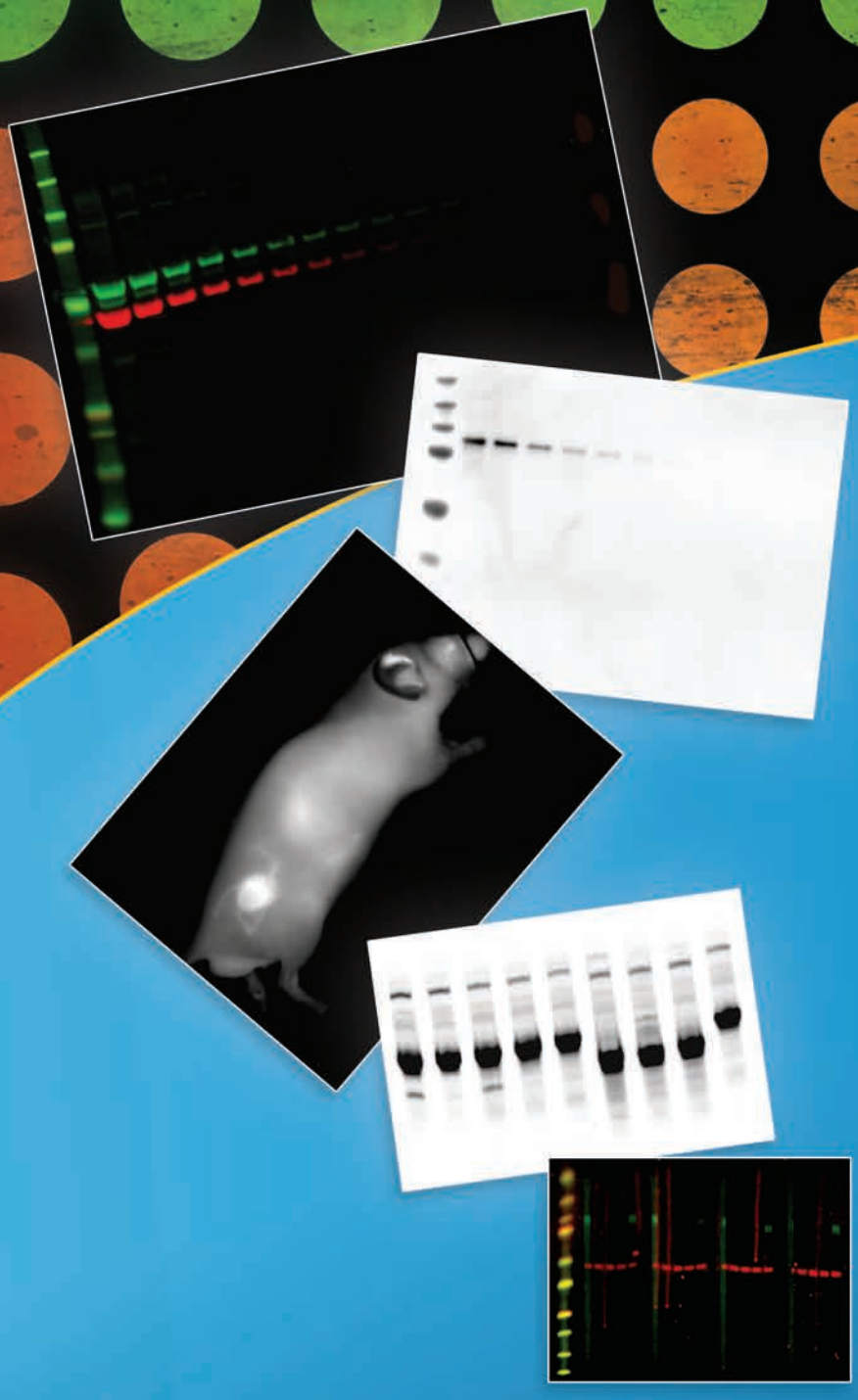
2012

Catalog and Applications Handbook



Featuring Products and Information for:

Western Blotting
Quantitative IR
Chemiluminescent

Small Animal Imaging

Gel Documentation
Protein Gels
Nucleic Acid Gels

Plate-based Assays

EMSA/Gel Shift Assays

Protein Arrays

RNAi Studies

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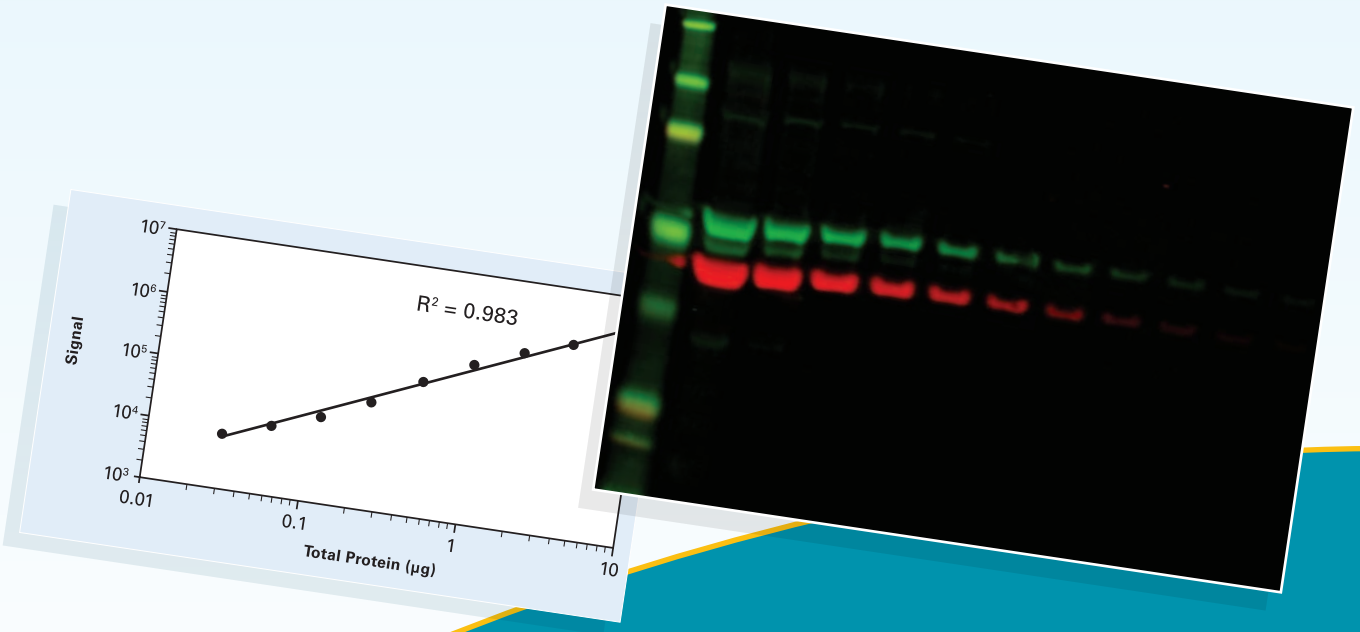

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Look at all the *NEW!* Products We Added in 2012!

Products added in 2012 are in the online version of this catalog only.

IRDye® BoneTag™ Optical Probes

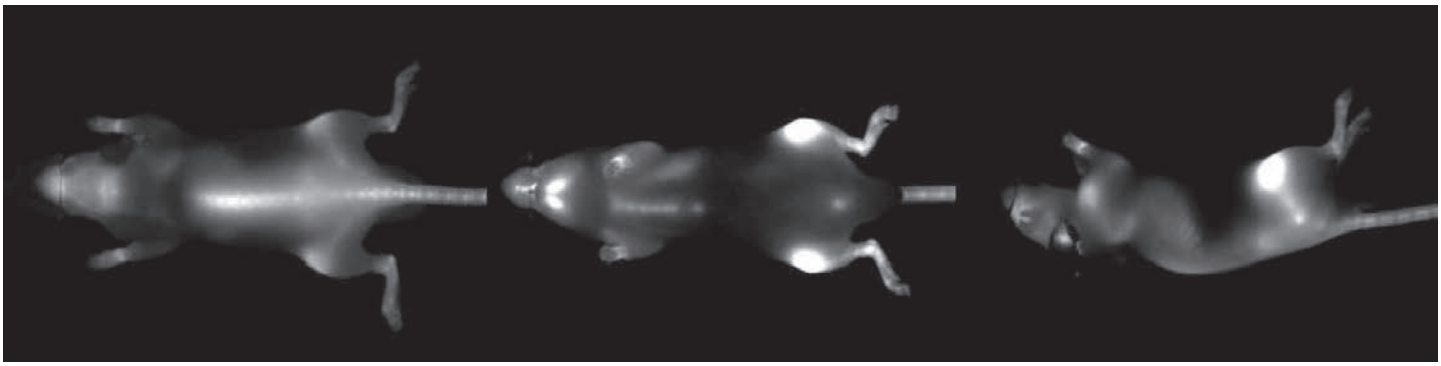


Figure 1. IRDye 680 BoneTag accumulation was imaged with the LI-COR® Pearl® Impulse (50 h after IV injection).

Calcium-chelating compounds are used for the detection of bone mineralization, growth, and morphological changes. BrightSite™ IRDye 680 BoneTag and IRDye 800CW BoneTag are calcium-chelating compounds labeled with IRDye 680RD or IRDye 800CW NIR fluorescent dyes. These probes are used to image bone structure and mineralization.

With IRDye BoneTag probes, bone anatomy and structures can be visualized over an extended period of weeks or months. This facilitates dual imaging with a second, spectrally distinct, optical probe that binds a disease-specific target. IRDye 800CW BoneTag may also be useful for bone metastasis and bone remodeling studies.

Cell-based assays and animal imaging were used to confirm specific targeting of these BoneTag probes.

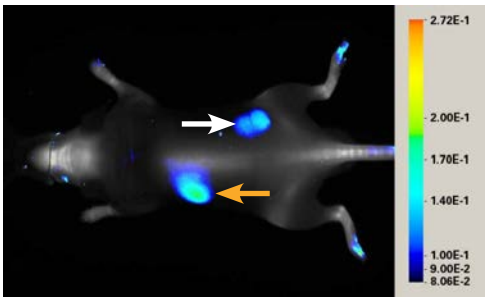


Figure 2. Cell-based assays and animal imaging were used to confirm specific targeting of these BoneTag probes.

926-09375 **IRDye 800CW BoneTag (4 x 10 nmol; approximately 20 injections) $325.00**
IRDye 800CW BoneTag is a calcium-chelating compound conjugated to IRDye 800CW NIR dye.

926-09374 **IRDye 680RD BoneTag (4 x 10 nmol; approximately 20 injections) $325.00**
IRDye 680RD BoneTag is a calcium-chelating compound conjugated to IRDye 680RD NIR dye.

Storage: -20°C prior to reconstitution/4°C after reconstitution

Look at all the *NEW!* Products We Added in 2011!

Imaging Systems, Software, and Accessories

Odyssey® CLx Infrared Imaging System 140

The Odyssey CLx is the next generation of Odyssey Classic, the most trusted and established standard in the quantitative Western blot industry for more than 10 years. Odyssey CLx is the most flexible and multifunctional platform of the Odyssey imaging systems.

AutoScan Function

- Wide dynamic range captures the entire range of data without saturation in a single, time-saving scan – no need for multiple scans to optimize intensity settings

Multiple Blot and Plate Scanning

- Simultaneously scan multiple samples of varied intensities in one scan for increased convenience

Odyssey® Sa with Barcode Function 141

The Odyssey Sa Infrared Imaging System offers the advantages of infrared imaging and the flexibility to handle both plate-based assays and quantitative Western blots, especially for high-throughput labs. The Odyssey Sa Barcode Reader is a factory-installed accessory that provides for the automated reading of barcodes on the sides of microplates.

- The Barcode Reader accessory is fully compatible with the Odyssey Sa and Sa Express Application Software and can read most common barcode symbologies
- This accessory may be added to ANY Odyssey Sa configuration

Odyssey® Fc with 600 nm Channel 142

In addition to its superior infrared fluorescence and chemiluminescence imaging performance, the Odyssey Fc Imaging System now features versatile nucleic acid detection. Image ethidium bromide and SYBR® family of DNA stains for agarose gel digital imaging with no harmful UV excitation light.

- SYTO® 60 near-infrared fluorescent DNA stain can also be used and imaged in the 700 nm channel of the Odyssey Fc
- Disposable imaging trays avoid instrument contamination

Image Studio 2.1 Imaging Software 146

Image Studio is a simple and easy-to-use imaging software package. It is compatible with Odyssey® CLx, Odyssey Classic, and Odyssey Fc Imaging Systems.

- Supports nine different types of analyses, including Western blots, DNA gel documentation, and small animal imaging (for the Odyssey CLx and Classic only)
- Provides an intuitive, application-driven ribbon interface that allows for easy user adoption while maintaining high quality data
- Compatible with PCs and Macs

Pearl® Impulse Small Animal Imaging Accessories

Compound Injection Clip 152

The Compound Injection Clip is a disposable accessory used to hold a syringe or catheter system in place while administering optical agents during small animal imaging. It is intended for use with the Pearl Impulse Small Animal Imaging System.

- Simplifies the administration of optical agents for researchers who need to monitor fast molecular events and to inject an animal while simultaneously imaging that animal

Organ Tray Base and Trays 152

The Organ Tray Base provides a way to image organs and tissue without compromising them due to heated imaging beds.

- Field-of-view markings on the trays ensure the organs will be placed within the actual imaging window
- Specially optimized polystyrene material provides very low background noise, ensuring high signal-to-noise ratios in images

IRDye® Secondary Antibodies

IRDye® 680RD Secondary Antibodies 48

IRDye 680RD secondary antibodies are the antibodies of choice for In-Cell Western™ assays, Western blot applications, and small animal imaging in the 700 nm channel. Two-color detection can be achieved by multiplexing IRDye 680RD secondary antibodies with IRDye 800CW secondary antibodies.

- Suitable for immunofluorescent microscopy and other fluorescent imaging applications

Anti-Human Secondary Antibodies 48

The new IRDye anti-human secondary antibodies are highly cross-adsorbed. These secondary antibodies are ideal for multiplexing in Western blot applications, including the In-Cell Western™ Assay.

Small pack sizes of IRDye® 800CW, IRDye 680RD, and IRDye® 680LT Goat anti-Mouse and Goat anti-Rabbit 47

The following six small scale antibodies [25 µL, (1 mg/mL)] are now available for sale:

- IRDye 800CW Goat anti-Mouse IgG (H+L)
- IRDye 680RD Goat anti-Mouse IgG (H+L)
- IRDye 680LT Goat anti-Mouse IgG (H+L)
- IRDye 800CW Goat anti-Rabbit IgG (H+L)
- IRDye 680RD Goat anti-Rabbit IgG (H+L)
- IRDye 680LT Goat anti-Rabbit IgG (H+L)

Subclass Specific Antibodies 47

IRDye® Goat anti-Mouse IgG_1, Goat anti-Mouse IgG_{2a} and Goat anti-Mouse IgG_{2b}, allow for two-color infrared detection using primary antibodies derived from the same species (mouse). IRDye Subclass Specific antibodies react with the heavy (gamma) chain only of the primary antibody. All other LI-COR IRDye secondary antibodies are raised against whole IgG (H + L) and react with the heavy (gamma) and light (kappa or lambda) chains of the primary antibody.

For more information about IRDye Subclass Specific antibodies, please see page 51 for the technical note: *Western Blot and In-Cell Western™ Assay Detection Using IRDye® Subclass Specific Antibodies.*

Small Animal Imaging Agents

IRDye® 680RD Optical Probes 97 98 99

IRDye 680RD is optimized for use in small animal imaging in the 680 nm range. It is ideal for labeling proteins, peptides, and antibodies and has excellent clearance, which allows for high signal-to-noise ratios for *in vivo* applications.

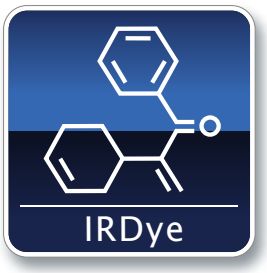
- IRDye 680RD RGD is designed to target integrins associated with tumor growth, tumor invasiveness, metastasis, tumor-induced angiogenesis, inflammation, osteoporosis, and rheumatoid arthritis
- IRDye 680RD EGF allows for labeling EGF receptors that are overexpressed in many types of cancer cells. It can be used for *in vivo* and *in vitro* analysis, as well as whole organ and tissue section analysis
- IRDye 680RD HA is a specific targeting agent for the lymphatic system and tumor cells that overexpress CD44 receptors

PSVue® 794 Reagent Kit 102

PSVue 794 is a near-infrared fluorescent probe for *in vivo* applications requiring the detection of bacteria, apoptotic and necrotic cells, or other anionic membranes.

- PSVue 794 binds to the phosphatidylserine (PS) residues exposed on the cell surface of apoptotic cells, as well as to a variety of cell types that have negatively charged phospholipids exposed on their membranes

Dyes



IRDye® 680RD Dyes 19

IRDye 680RD is spectrally matched to LI-COR® imaging systems and is the near-infrared fluorescent dye of choice for small animal imaging applications in the 700 nm channel. It has the lowest background compared to LI-COR's other 700 nm dyes. This infrared dye is ideal for labeling proteins, peptides, and antibodies and is suitable for Western blot and In-Cell Western™ (ICW) applications, available as NHS ester, malemide, and carboxylate.

IRDye® 650 and 750 Dyes 17 21

New IRDye dye choices in the far-red and near-infrared spectrum for your application needs. Available as NHS ester or maleimide reactive groups.

- IRDye 650 with peak excitation at 756 nm and emission at 776 nm
- IRDye 650 with maximum absorbance at 648 nm and emission at 665 nm

Kits

Quick Western Kit – IRDye® 680RD 57

The Quick Western Kit – IRDye 680RD provides a universal detection reagent that can be combined with the primary antibody incubation step, eliminating the need for a secondary antibody incubation step. The kit can be used to detect primary antibodies from a variety of hosts and has been shown to recognize recombinant tagged proteins (e.g. 6X His, Myc, DDK).

Odyssey® Western Blotting Kits 57 58

Odyssey Western Blotting Kits contain all reagents and membranes necessary to perform highly quantitative, infrared Western blots. All products in the Odyssey Western Blotting Kits have been through rigorous quality control testing

Labeling Kits 106

IRDye® 680RD labeling kits are suitable for labeling antibodies and other proteins for applications such as Western blots, In-Cell Western™ assays, *in vivo* imaging, and whole organ or tissue section assays.

Reagents

ELISA Substrates 79

LI-COR's proprietary peroxidase and phosphatase substrates are optimized for use in the near-infrared fluorescent region (700 nm) on Odyssey® CLx, Odyssey Classic, and Odyssey Sa Infrared Imaging Systems.

- Equal or better sensitivity compared to commercially-available chromogenic and chemiluminescent substrates and are ideal for endpoint assays
- Both the ELISA HRP substrate and the ELISA AP substrate provide excellent signal-to-noise ratios, consistency, and linearity

Introduction to **Near-infrared Fluorescence** *Technology*

Near-Infrared Fluorescence Imaging: Seeing Beyond the Visible With IRDye® Infrared Dyes

Harry L. Osterman, Ph.D. and Amy Schutz-Geschwender, Ph.D.

Introduction

Fluorescent dyes are important labeling tools for life science research. The major scientific milestone of the last decade, the sequencing of the human genome, was made possible by fluorescent detection. The first automated sequencing system, commercialized by Applied Biosystems, was based on fluorescein- and rhodamine-related visible dyes.[1] A different approach was taken in the development of a near-infrared (NIR) fluorescence detection system using NIR dyes (now called IRDye® infrared dyes).[2] This system, first commercialized in 1993 by LI-COR Biosciences, offered the key advantages of IRDye technology. Reduced autofluorescent background, high sensitivity, and wide dynamic range enabled the LI-COR® system to set new standards for read length and accuracy. As automated DNA sequencing technology evolves into massively parallel methods and single molecule detection, fluorescence continues to play an important role.

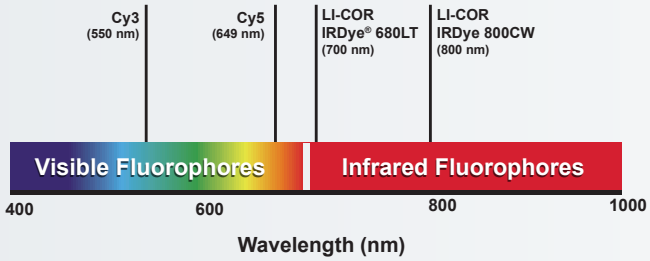


Figure 1. Emission wavelengths of various fluorophores.

Visible fluorescent dyes, which emit light in the visible spectrum (~400-650 nm), are used in a wide variety of biological assays (Figure 1). Commonly used dyes include the CyDye® family of carbocyanine dyes[3] developed in the late 1980's, and the Alexa Fluor® dye family. Although widely used, visible fluorophores do not offer optimal performance for all applications. Cells, animal tissue, plasticware, blotting membranes, and chemical compound libraries all possess intrinsic autofluorescence that can interfere with detection.[4] However, in the near-infrared (NIR) spectral region (700-900 nm), autofluorescent background is dramatically reduced. NIR fluorophores, such as IRDye infrared dyes (Table 1), exploit this property – enhancing detection sensitivity, signal-to-noise ratios, and dynamic range in applications where autofluorescence had been limiting. This extends the benefits of fluorescent detection to applications such as Western blotting and *in vivo* imaging, and can provide improved performance for cell-based assays, protein microarrays, microscopy, and screening of small molecule libraries.

Western Blotting

For decades, researchers have relied on Western blotting to confirm the presence or absence of target proteins in complex samples. After electrophoretic separation, proteins are transferred to a membrane support and interrogated with antibodies.[5] Western blot detection typically uses a primary antibody directed against a target protein and a secondary antibody conjugated with an enzyme reporter (horseradish peroxidase or alkaline phosphatase). Chemiluminescent or colorimetric detection of the enzyme conjugate confirms the presence of the target protein (Figure 2A).

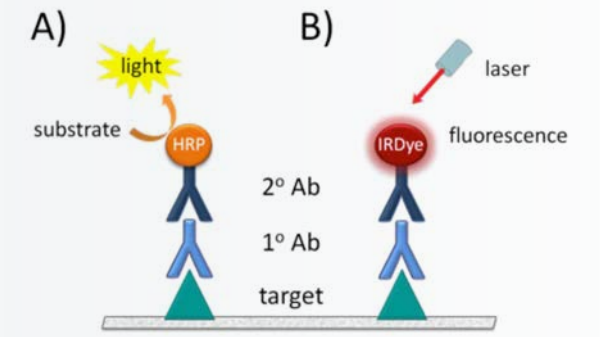


Figure 2. Western blot detection methods.
A) Chemiluminescent detection with horseradish peroxidase (HRP) conjugate. **B)** NIR fluorescent detection with IRDye® fluorescent conjugate.

In some cases, researchers also require quantitative information about how much protein is present. Fluorescent Western blot detection meets this need.[6,7] Secondary

Table 1. IRDye® fluorophore and characteristics

Dye	Exmax (nm)	Emmax (nm)	Reactive Group	Recommended for labeling	LI-COR channel
IRDye 800CW	778	794	NHS ester Maleimide	Proteins Peptides	800 nm
IRDye 800RS	770	786	NHS ester	Nucleic acids	800 nm
IRDye 680RD	680	694	NHS ester Maleimide	Proteins Peptides	700 nm
IRDye 680LT	680	694	NHS ester Maleimide	Proteins Peptides	700 nm
IRDye 700DX	680	687	NHS ester	Proteins Peptides	700 nm
IRDye 750	766	776	NHS ester Maleimide	Proteins Peptides	–
IRDye 650	651	668	NHS ester Maleimide	Proteins Peptides	–
IRDye 700	685	705	Phosphoramidite	Oligos	700 nm
IRDye 800	795	819	Phosphoramidite	Oligos	800 nm

antibodies are labeled with NIR fluorescent dyes (such as IRDye 800CW, IRDye 680RD, or IRDye® 680LT) for direct and non-enzymatic detection (Figure 2B). Blots are then documented with an NIR imager such as the Odyssey® CLx, Odyssey Classic, Odyssey Fc, or Odyssey Sa Imagers from LI-COR Biosciences. With this ratiometric approach, fluorescent signal is directly proportional to the amount of target protein present. NIR fluorescent detection dramatically improves quantitative accuracy and reproducibility – with excellent sensitivity for detection of endogenous protein levels.[7] Fluorescent signals are stable indefinitely, so blots can be stored and re-imaged later. Multiplexing of NIR dyes (Figure 3) allows two targets to be detected simultaneously, making normalization against an internal control easier and more accurate than comparison of two different blots (Figure 4).

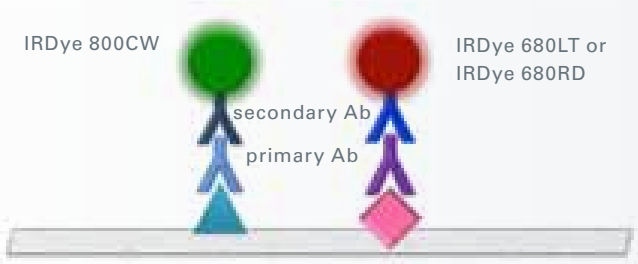


Figure 3. Multiplexing of spectrally-distinct IRDye® fluorophores.

Quantitative analysis can be very important in life science research, and IRDye fluorescence outperforms other Western blot detection methods. Studies comparing chemiluminescent and IRDye detection methods have demonstrated that IRDye detection is quantitative over a much broader linear dynamic range than ECL™ substrate (Figure 5).[6,7,8] Fluorescent conjugates avoid the enzyme kinetics and substrate availability limitations of chemiluminescence. This produces more consistent, straightforward, and accurate quantification.[7,8]

In carefully controlled experiments, absolute quantification can be performed. Wang et al[8] examined the stoichiometric relationships between p53 and its negative regulators. Cell numbers in each sample were carefully counted before lysis. Hdm2, HdmX, and p53 were then detected on NIR fluorescent Western blots. Results were normalized against an internal control protein. Cell extracts were compared against dilution curves of purified target proteins of known concentration, allowing the authors to estimate actual numbers of protein molecules per cell. Their results suggest that the stoichiometric balance between Hdm2, HdmX, and p53 is important for regulation of p53 activity.

Biological samples can be challenging for quantification. Because endogenous protein levels span an extremely wide dynamic range (an estimated 4-10 orders of magnitude),[9,10,11] the ability to image both low-abundance and high-abundance targets is critical. IRDye detection with the Odyssey family of imagers provides the widest linear dynamic range reported for any Western blot method.

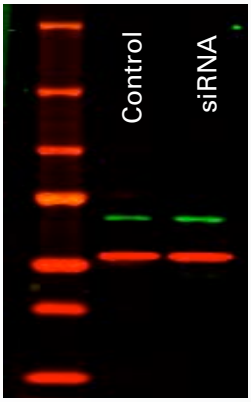


Figure 4. Multiplex detection for normalization against an internal control. Western blotting was used to validate siRNA knockdown of Akt1 (green). Tubulin (red) was used as a loading control. Normalization allowed precise quantification of Akt1 protein levels. Although Akt1 level may visually appear unchanged, normalized data reveal 32% knockdown of Akt1 relative to the scrambled control. Image captured with Odyssey Classic Imager.

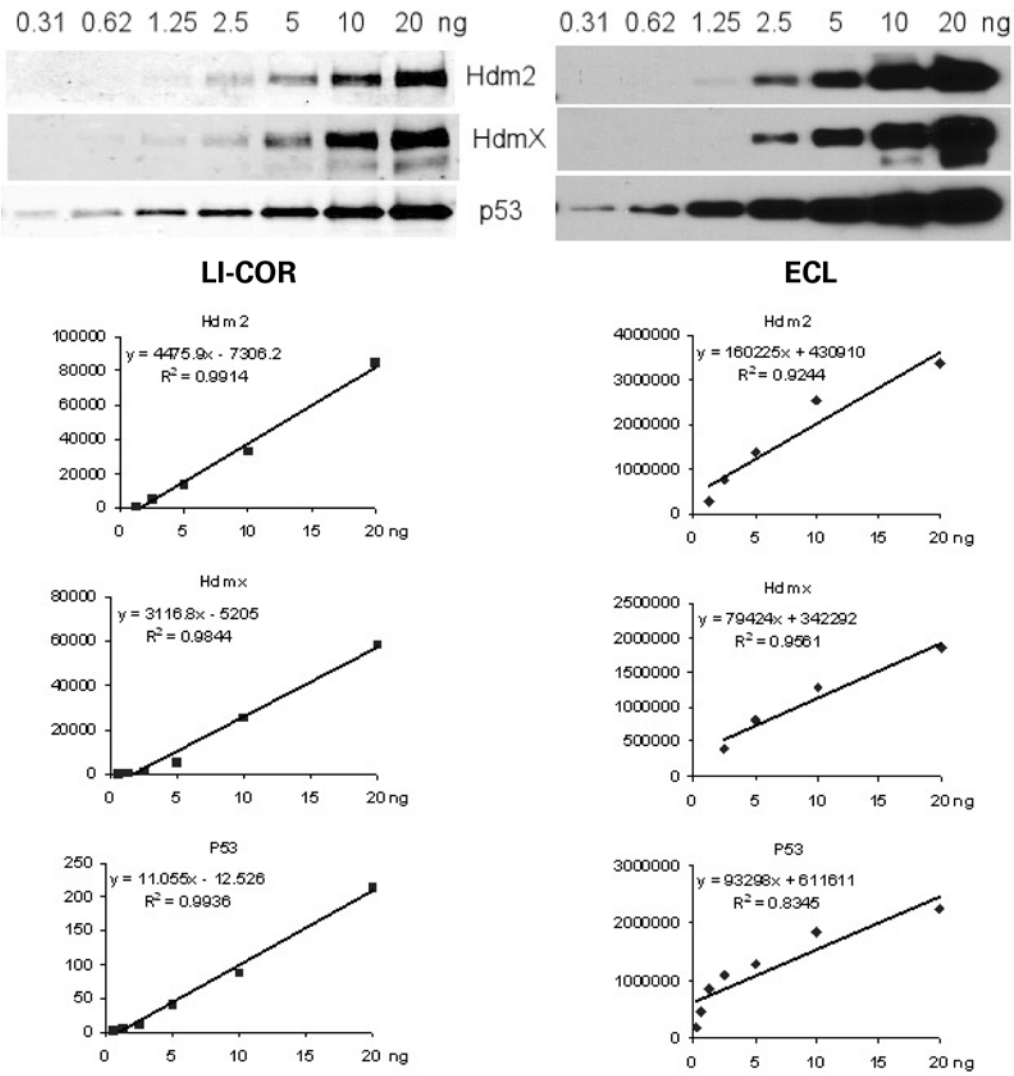


Figure 5. Comparison of detection methods for Western blot analysis. Pure recombinant p53, Hdm2, and Hdmx protein samples of known concentration were serially diluted with 100 ng/mL BSA and run in duplicate on two gels, followed by Western blot analysis. Proteins were detected by NIR fluorescence or standard ECL™ substrate. Signal intensities from each Western blot were quantified by using the LI-COR® Odyssey® Classic system or, for ECL, scanning of the developed films, followed by analysis with Un-Scan-It software. Signal intensities were plotted against corresponding protein concentrations. Reprinted from Wang, YV et al. *Proc Natl Acad Sci USA*. 104(30): 12365-70 (2007). Copyright (2007) National Academy of Sciences, U.S.A.

Detection of Hsp70 was reported to be linear over 4.3 orders of magnitude (up to 20,000 fold), from 5 pg to 100 ng[12] (Figure 5). Wide linear range allows all necessary information to be extracted from a single blot or image, without multiple exposures.

Multiplex Phosphorylation Analysis

A major application for two-color fluorescent Western blots is quantitative analysis of signal transduction pathways. The second fluorescent channel is used for normalization in order to correct for variation between samples and make quantification more accurate. One approach is to multiplex a phospho-specific antibody with a pan-antibody, which recognizes the target protein regardless of its phosphorylation state.[13,14,15] Two spectrally-distinct NIR fluorophores, attached to unique secondary antibodies, are used for detection. Phospho-signal is then normalized against the total level of that protein, using the target protein as its own internal control (Figure 6). Alternatively, the second color can be used to detect a different protein of interest or to normalize against a housekeeping protein. Multiplex phospho-analysis requires high sensitivity in both detection channels.

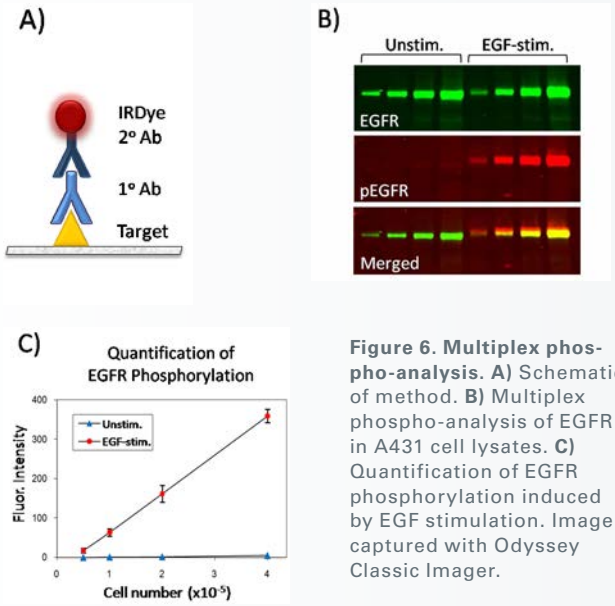


Figure 6. Multiplex phospho-analysis. A) Schematic of method. **B)** Multiplex phospho-analysis of EGFR in A431 cell lysates. **C)** Quantification of EGFR phosphorylation induced by EGF stimulation. Image captured with Odyssey Classic Imager.

Western blotting is also essential for validation of other assays. In mass spectrometry proteomics analysis, Western blots provide confirmation of the observed changes in protein abundance.[16,17] Western blots are also used to validate newly-developed ELISA methods.[18] Validation is particularly important for RNAi studies, because mRNA levels do not always accurately reflect protein levels. Quantitative Western analysis considers the impact of protein stability, yielding a more complete picture of RNA silencing.

Protein Microarrays

Protein arrays are a high-throughput way to generate information about protein abundance and/or modification state. Arrays allow a large number of samples to be interrogated simultaneously, with many replicates, and arrays help to conserve precious samples. Because arrays do not typically provide molecular weight information, proper controls and thorough antibody validation are important.[21,22]

Several types of protein arrays are commonly used: [23]

- Reverse phase (lysate) arrays (Figure 7A) contain complex samples, such as cell or tissue lysates, that are printed on an array surface and interrogated with antibodies. Lysate arrays are more quantitative and reproducible than Western blots (intra-chip variation ~0.1%[24]), with a wide dynamic range. They can provide very high sensitivity and are reported to detect femtogram or single-cell protein levels when NIR fluorescence is used.[24] Recombinant protein standards can be spotted for absolute quantification of target proteins.[24]
- Analytical arrays (Figure 7B) use affinity reagents such as antibodies or peptides to profile analytes in complex mixtures of proteins. These arrays can be spotted on chips or slides, or spotted into microplate wells. Antibody capture arrays are the most common form.[25,26]
- Functional protein arrays are spotted with many different purified proteins and used to assay biochemical functions such as protein-protein, protein-DNA, protein-small molecule interactions and enzyme activity.[27,28,29]

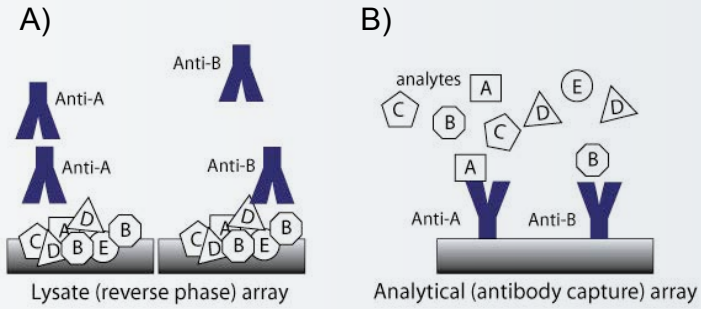


Figure 7. Common types of protein arrays.

Many types of protein microarrays can be detected with NIR fluorescence, and NIR detection is especially powerful for arrays spotted on membrane surfaces. Nitrocellulose-coated glass slides are widely used for protein arrays due to their high protein-binding capacity and ease of use, but their high autofluorescent background limits the sensitivity of visible fluorophores.[30] At NIR wavelengths, autofluorescence is dramatically reduced. Background levels and signal-to-noise ratios were compared for Cy3, Cy5, and IRDye® 800CW[31] (Figure 8). Because of the NIR advantage, IRDye 800CW delivers the highest signal-to-noise ratios (SNR) and sensitivity across the broadest dynamic range. Compared to Cy5, IRDye 800CW displayed more than an order of magnitude increase in SNR and a 4-fold increase in limit of detection. Compared to Cy3, signal-to-noise ratio increased by 3 orders of magnitude with a 125-fold increase in limit of protein target detection.

Reverse phase protein arrays on nitrocellulose slides are often used to determine relative protein levels in cell lysates, tissue lysates, and/or clinical samples. Chemiluminescent or colorimetric methods are often used for detection, but they are laborious, have a limited linear range, and can detect only a single target protein. This array format has been adapted for NIR detection.[32] Very small volumes of tumor biopsy lysates from breast cancer patients were arrayed on slides (Figure 9A). Antibodies against total and phosphorylated ERK were then applied, followed by multiplex

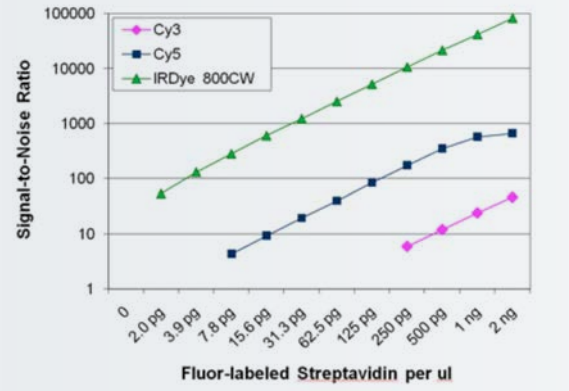


Fig 8. Comparison of visible and NIR dyes on nitrocellulose-coated slides.[31] Serial dilutions of streptavidin-dye conjugates (Cy3, Cy5, and IRDye 800CW) were spotted onto nitrocellulose-coated slides (5 replicates per slide; 3 slides per dye). Slides were imaged at the appropriate wavelengths. Background and signal-to-noise ratios (SNR) were calculated. SNR = (fluorescence intensity – mean background) / std. dev. of background; SNR cutoff=3 for limit of detection determination.

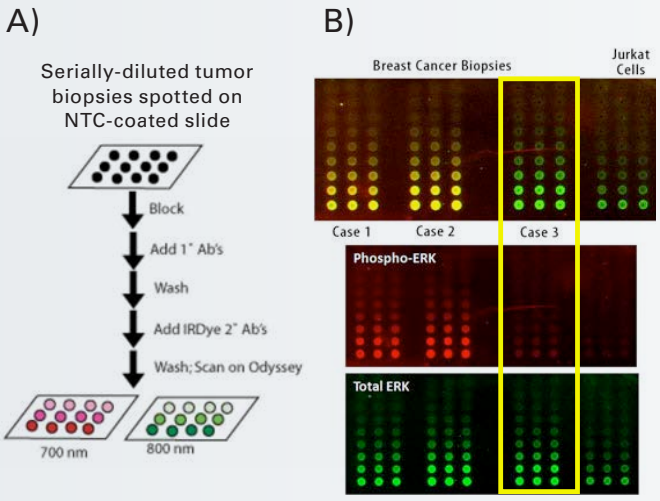


Figure 9. Multiplex detection of phosphorylated and total ERK protein levels on reverse phase protein arrays. A) Tissue samples were obtained from three human breast cancer patients; lysates were prepared from these and from a Jurkat T-cell control. **B)** The array was probed simultaneously with antibodies against phospho-ERK (mouse) and total ERK (rabbit), and detected with IRDye secondary antibodies (red, green). Overlaid image of total and phospho-ERK levels is shown at the top (red + green = yellow). A dramatic reduction in phospho-ERK was observed in Case #3. Image captured with Odyssey Classic Imager. Adapted from Calvert, VS et al. *Clin Prot J.* 1(1): 81-89 (2004).

detection with IRDye conjugates (Figure 9B). Quantitative analysis showed clear differences in ERK phosphorylation levels among the patient samples tested, although the levels of total ERK were nearly equivalent. Reproducibility and linear range were enhanced compared to the colorimetric method normally used to detect similar arrays.[32]

IRDye 800CW detection has been applied to antibody arrays in microplate wells (available commercially from Quansys Biotech and Thermo Fisher Scientific). Initially, microtiter plate arrays were developed for use with chemiluminescence. These arrays had several disadvantages, including limited linear range and signal blooming (light emission from a very bright spots spreading and obscuring the signal from adjacent spots). The method was converted to NIR detection using IRDye 800CW streptavidin. IRDye 800CW offered a much larger dynamic range (3 orders of magnitude) with sensitivity similar to chemiluminescent detection. In addition, the fluorescent method yielded sharp, distinct spots with no blooming of signal, permitting increased array density. IRDye 800CW streptavidin is also used for detection of R&D Systems Proteome Profiler Antibody Arrays. These nitrocellulose antibody arrays are designed for profiling of cytokines or angiogenesis-related proteins.

In-Cell Western™ Immunofluorescent Assay

IRDye fluorescence can also be applied to cell-based assays. The In-Cell Western™ Assay (ICW) is a microplate assay that uses immunofluorescent staining for fast, accurate measurement of protein levels in fixed cultured cells.[33,34] ICW assays are also called quantitative immunofluorescence assays, cytoblots, cell-based ELISA, In-Cell ELISA, or cLISA. R&D Systems' Cell-Based Infrared ELISA kits use IRDye secondary antibodies for detection.

The moderate-throughput ICW assay is based on standard immunofluorescent techniques. Cells are grown in 96- or 384-well microplates, exposed to the desired treatments or conditions, then fixed and permeabilized for immunostaining (Figure 10). Total fluorescent signal for each well is imaged and ratiometric analysis is performed. Two fluorescent channels can be used to simultaneously quantify two different protein targets, normalize against a housekeeping protein, or normalize for cell number using a DNA stain.[33,35,36] Normalization increases accuracy by correcting for unavoidable well-to-well variation in cell number. The ICW assay provides a "snapshot" of the protein expression or signal transduction status of the cell population in each well. This assay is a convenient alternative to flow cytometry for analysis of adherent cells and is much simpler than high content screening.[35]

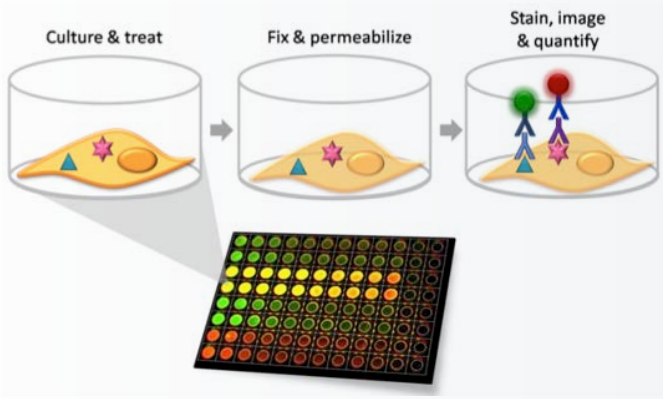


Figure 10. Schematic of In-Cell Western™ assay method, performed in a microwell plate.

ICW assays are a useful alternative to Western blotting. Because cell lysates, gels, and blotting are eliminated, many samples or replicates can easily and quickly be processed in parallel. Variability is greatly reduced, enabling enhanced reproducibility and more accurate analysis of changes in protein levels. This is especially useful for detailed signal transduction studies that require precise timing and sampling over an extended period.[36]

Several studies have shown that ICW results correlate very well with Western blot results.[37,38,39] In general, ICW assays are more reproducible than Western blotting and display significantly smaller standard deviations.[37,39] A G-protein-coupled receptor (GPCR) functional assay based on ICW detection of CREB phosphorylation demonstrated excellent agreement with radioligand binding and cAMP competitive immunoassays.[40]

The ICW assay is well-suited for analysis of protein phosphorylation (Figure 11) and the effects of drug compounds on signaling pathways, including IC_{50} determination.[33-34,36,38] The assay has also been used for a variety of other applications, including monitoring of GPCR functional activity;[34,40] screening for inhibitors of Tau protein;[41,42] tracking changes in gene expression;[43] viral quantification;[44] caspase-3 activation assays;[45] RNAi library screening;[35] and proliferation assays.[46,47,48] Modified ICW assays referred to as On-Cell Western Assays are used to analyze and quantify cell surface proteins, receptor trafficking, and receptor internalization.[49,50,51,52,53]

IRDye® 800CW and IRDye 680RD conjugates yield superior signal-to-noise ratios in ICW assays, allowing quantification of endogenous protein levels and phosphorylation status.

Cy5.5-labeled secondary antibodies did not exhibit sufficient signal-to-noise, and further experiments showed that Cy5.5 displayed strong, non-specific binding to cells (H. Chen, unpublished). Performance in ICW assays is generally best with 800 nm fluorophores, due to the lower autofluorescence and light scatter properties of plastic microplates at this wavelength. Below 680 nm, increasing autofluorescence adversely affects sensitivity and dynamic range.

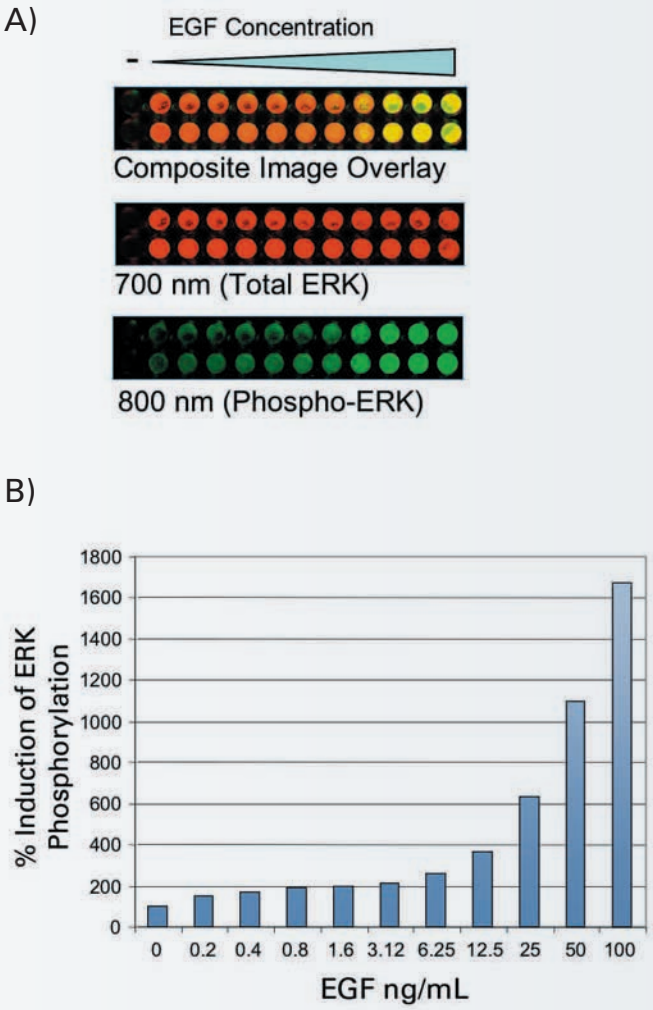


Figure 11. Phosphorylation of ERK in response to pathway stimulation. A) Detection of ERK phosphorylation. A portion of a 96-well plate is shown. Top: composite image showing fluorescence in both detection channels (duplicate wells are shown). Middle: detection of total ERK protein, regardless of phosphorylation status (red; 700 nm). Bottom: detection of increasing amounts of phospho-ERK, with increasing EGF stimulation (green; 800 nm). **B)** Quantification of fluorescence. Phospho-ERK signal was normalized against total ERK signal, to correct for well-to-well variation in cell number. Compared to the resting state, EGF stimulated cells displayed >16-fold increase in ERK phosphorylation. Image captured with Odyssey Classic Imager. Adapted from Chen, H et al. *Anal Biochem*. 338: 136 (2005).

Protein/Nucleic Acid Binding: Electrophoretic Mobility Shift Assays (EMSA)

The EMSA (electrophoretic mobility shift assay) method is used to study protein:DNA complexes and interactions. When electrophoresed on a non-denaturing gel, protein:DNA complexes will migrate more slowly than unbound linear DNA. Because DNA migration is "shifted" or “retarded” when bound to protein, this method is also called a gel shift or gel retardation assay.

Binding reaction

Short linear DNA fragments that contain consensus binding sequences are typically used as probes. Radiolabeling of probes with ^{32}P is commonly performed. Haptens such as biotin or digoxigenin are sometimes used for non-radioactive detection, but require secondary detection steps. Existing mobility shift assay protocols can be easily converted to NIR fluorescent assays by replacing the existing DNA oligonucleotides with IRDye® end-labeled oligonucleotides. Binding and electrophoresis conditions are very similar to other EMSA detection methods. After electrophoresis, fluorescent protein:DNA complexes can be imaged immediately while still in the gel.[28, 54, 55, 56]

With radioactive EMSA methods, gel drying and film exposure cause significant delays. Results are typically not available until the next day. Direct fluorescent detection provides immediate results, so a series of EMSA experiments can easily be performed in a single day.

Competitor DNAs are important to the assay. Non-specific competitors [irrelevant, unlabeled nucleic acids such as poly(dI-dC) or salmon sperm DNA] are used as blocking agents, to decrease binding of non-specific proteins to the labeled DNA probe. Specific competitors are very important controls used to confirm specificity of protein:DNA binding. Typically, the specific competitor contains exactly the same consensus sequence as the labeled probe. The competitor is unlabeled and is added to the binding reaction in a large molar excess (~200-fold). Unlabeled competitor out-competes the labeled probe for binding to the protein, eliminating or reducing the mobility shift (Figure 12). Mutant competitors can also be used. They contain mutated or low-affinity binding sites that will not compete with specific interactions and do not reduce the observed mobility shift.

Detection of bound probe

After electrophoresis of the protein:DNA complexes, detection is performed. If a radioactive probe is used, the gel is dried and exposed to X-ray film to document the binding results. If an IRDye fluorescent probe is used, binding results are imaged immediately by scanning the wet gel. Acrylamide gels can be scanned with the glass plates still in place, and gels can be returned to the electrophoresis unit and run longer if desired.

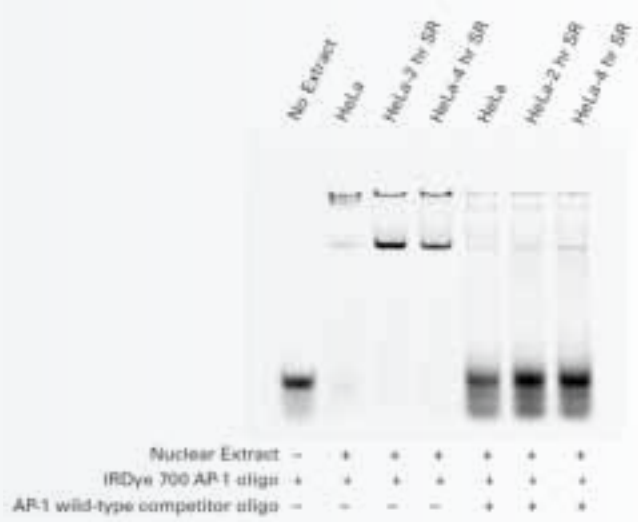


Figure 12. AP-1 EMSA using IRDye® 700 end-labeled oligonucleotide duplex. Competition reactions contained 100-fold molar excess of wild-type oligonucleotide duplex. Nuclear extracts of HeLa, HeLa 2-hour serum response (SR), and HeLa 4-hour serum response, were used to visualize an increase in AP-1 binding as a result of the serum treatment. Image captured with Odyssey Classic Imager.

Coomassie Gel Imaging

Coomassie Brilliant Blue is a dye that non-specifically stains proteins a dark blue color and is used to visualize proteins after gel electrophoresis. It was initially developed for use in the textile industry in the late 19th century. Coomassie-stained protein gels are typically detected visually with white-light imaging or densitometry. However, Coomassie is an excellent 700 nm fluorophore and can also be used for fluorescence imaging.[57] Fluorescence imaging of Coomassie with the Odyssey® family of imagers yields high sensitivity and quantitative protein detection with a reported linear range from ~10 ng to 20 mg.[57]

Fluorescence imaging is more sensitive than white-light imaging of Coomassie-stained gels (Figure 13).[57,58] It yields detection sensitivity comparable to Sypro Ruby (Figure 13),[58] with improved SNR (Figure 13G) and substantially reduced cost. IRDye® Blue Protein Stain, a Coomassie-based stain, is a convenient, safe alternative for gel staining. Unlike traditional Coomassie stains, which require methanol and acetic acid for staining and destaining, IRDye Blue Protein Stain is water-based and requires no hazardous solvents. This stain offers excellent detection sensitivity in the 700 nm channel of the Odyssey® family of imagers (5 ng of BSA can be reliably detected).

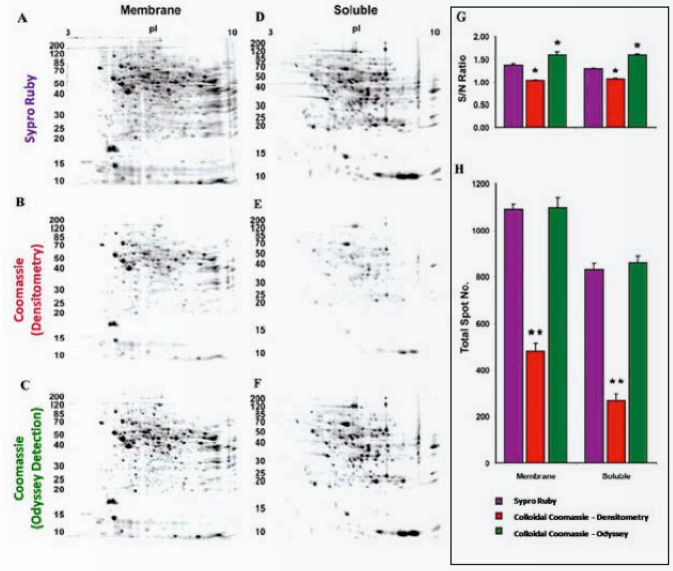


Figure 13. Comparison of Sypro Ruby and Coomassie Blue detection. Mouse liver membrane and soluble proteins were separated on 2D gels, then stained with Sypro Ruby followed by Coomassie. Image analysis indicates differences in average SNR (panel G) and total spot number (panel H); * and ** denote significant differences compared to Sypro Ruby ($P<0.05$ and $P<0.001$; n=3). Reprinted with permission from Harris, LR et al. *J Proteome Res*. 6(4): 1418-25 (2007). Copyright 2007 American Chemical Society.

IRDye FRET Assays for Protease Activity

Fluorescent dyes play an important role in screening chemical libraries for inhibitors of various enzyme targets, such as kinase and proteases. Background fluorescence from biological components, library compounds, and other external sources can produce false positives and reduce sensitivity. The use of IRDye 800CW can substantially reduce or eliminate background fluorescence by shifting the assay to higher wavelengths, thus reducing false positives.[59]

Many FRET (fluorescence resonance energy transfer) protease assays are based on non-fluorescent peptide substrates that become fluorescent upon cleavage. These quenched substrates have a fluorescent dye at one terminus and a quencher on the opposite side of the cleavage site. Cleavage by the protease physically separates the dye from the quencher, allowing the dye to fluoresce. These homogeneous assays do not require further processing or purification.

A caspase-3 protease assay has been developed using IRDye 800CW in combination with IRDye QC-1, a proprietary NIR quencher.[60, 61] IRDye 800CW/QC-1 Caspase-3 (CSP-3) Substrate is a sensitive reagent used to detect caspase-3 activity, measure potency of inhibitors, and screen compound collections for discovery of drug candidates. The substrate for this assay is a highly-quenched NIR-FRET peptide, IRDye 800CW-GDEVDGAK(IRDye QC-1)-COOH. Cleavage of the substrate peptide separates the donor-quencher pair (IRDye 800CW / IRDye QC-1) and restores the fluorescence signal of the donor (Figure 14). The CSP-3 substrate is optimized for use with the Odyssey family of imaging systems.

This caspase-3 substrate was used by the Kornbluth laboratory to monitor caspase activation in intact, living *Xenopus* ooctyes and early embryos.[62] The quenched substrate was microinjected into oocytes. Cleavage and activation of the substrate was detected by fluorescence imaging of the oocytes in microplates. This method enabled analysis of the kinetics of caspase activation and the role of inhibitory proteins, and revealed subtle details of apoptotic regulation.[62]

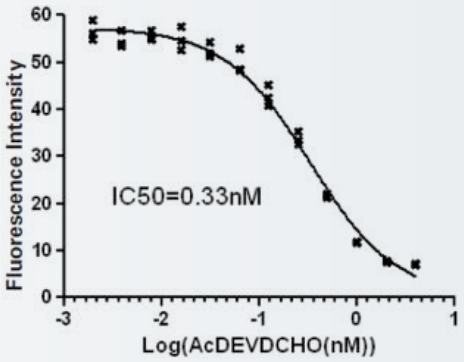


Figure 14. IC_{50} measurement for a known Caspase-3 inhibitor, Ac-DEVD-CHO, in a 96-well microplate. The serially diluted inhibitor solutions (15 µL/well; final conc. 0.00195 - 4 nM) were mixed with Caspase-3 (15 µL/well; final conc. 60 U/mL) for 30 minutes before adding substrate (30 µL/well; final conc. 200 nM). After incubating at room temperature for 90 minutes, the reactions were stopped with stop solution. Fluorescence intensity was measured with Aerius Infrared Imaging System.

Microscopy

Fluorescence detection is widely used in microscopy and has recently expanded into high content screening. IRDye® infrared dyes can provide additional colors for multicolor immunofluorescence imaging at NIR wavelengths, where cells and tissue have low autofluorescence (Figures 15-17). These additional colors offer wide spectral separation from commonly-used visible fluorophores. To image NIR wavelengths, microscopes must be equipped with appropriate filter sets, a CCD camera responsive in the NIR, and a light source with sufficient excitation energy at NIR wavelengths (such as a xenon lamp; for details, visit *www.licor.com/microscopy*).

NIR fluorophores have also been used for *in vivo* immunofluorescence microscopy for direct visualization of mouse vascular endothelial cells.[64] This study employed a two color approach using antibodies labeled with Cy5.5 and IRDye 38 (closely related to IRDye 800CW) to follow the dynamic expression of both E- and P-selectin. Interference from tissue autofluorescence was lower for IRDye 38 than for Cy5.5 and fluorescein. Two-photon excitation of fluorescein has been compared to single-photon excitation of IRDye 38 for *in vivo* imaging of microcapillaries labeled with anti-CD31 (JM Runnels, unpublished results). Single photon excitation of IRDye 38 produced lower background and clearer images than two photon excitation of fluorescein. The lower energy of long-wavelength NIR excitation light reduces cell and tissue damage from prolonged light exposure, making NIR excitation a better option for *in vivo* microscopy than shorter-wavelength visible light.

For imaging applications that require extended photostability, the phthalocyanine dye IRDye 700DX provides photostability combined with excellent water solubility.[65]

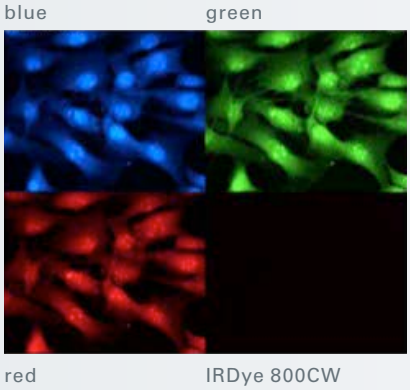


Figure 15. Autofluorescence of glutaraldehyde-fixed cells. NIH3T3 cells were fixed in glutaraldehyde. Unstained cells were viewed using standard blue, green, and red filters, and an IRDye 800CW custom filter set (Chroma Technology). No autofluorescent background could be detected at the IRDye 800CW wavelength. Exposure times: AMCA (blue) 5592 msec; FITC (green) 4977 msec; Cy3 (red) 6007 msec; IRDye 800CW 17,578 msec. Data courtesy of H. Fisk, University of Colorado, Boulder, CO.

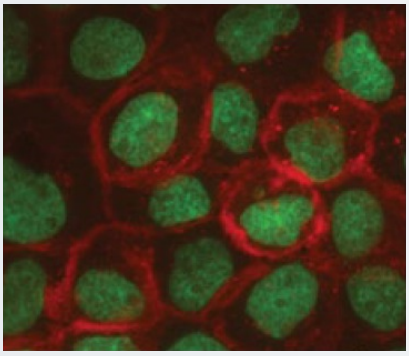


Figure 16. Binding and uptake of IRDye® 800CW EGF by A431 cells. EGF probe (shown in red) localizes to the cell membrane. Because the probe is internalized, punctate fluorescence is also seen in the cytoplasm. SYTOX Green DNA stain is shown in green. Image was captured with Leica epifluorescent microscope.

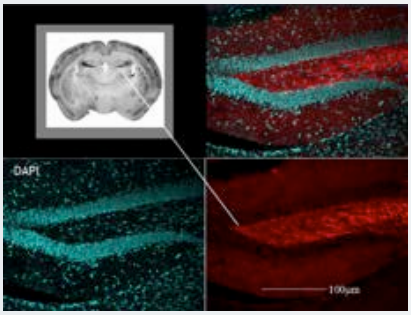


Figure 17. Imaging of stained brain tissue sections. 40 micron mouse brain sections treated with Rabbit anti-Orphan receptor antibody followed by Goat anti-Rabbit IRDye 680 secondary antibody. Image was taken on a Zeiss LSM-510 using 633 laser line at 100%. 640 bandpass filter. Pinhole=1 AU. DAPI stain (blue) used for focusing. Data courtesy of C. Kearn, University of Washington, Seattle, WA.

In vivo Optical Imaging[66]

Optical imaging enables non-invasive study of molecular targets inside the body of the living animal. This technology can be used to follow the progression of disease, the effects of drug candidates on the target pathology, the pharmacokinetic behavior of drug candidates, and the development of biomarkers indicative of disease and treatment outcomes. Currently, the three major types of labels used in optical imaging are bioluminescence, fluorescent proteins, and fluorescent dyes or nanoparticles. Bioluminescence and fluorescent proteins require engineering of cell lines or transgenic animals that carry the appropriate gene. Because fluorescent dyes do not have this requirement, they have the potential to translate to clinical applications.[67] For example, the carbocyanine dye indocyanine green (ICG; also known as Cardiogreen®), has been used in the clinic for many years as a dilution indicator for studies involving the heart, liver, lungs, and circulation.[68]

Near-infrared (NIR) fluorophores minimize the optical challenges of non-invasively detecting photons in tissues. A fundamental consideration in optical imaging is maximizing the depth of tissue penetration, which is limited by absorption and scattering of light. Light is absorbed by hemoglobin, melanin, lipids, and other compounds present in living tissue.[69] Because absorption and scattering decrease as wavelength increases, fluorescent dyes and proteins absorbing below 700 nm are difficult to detect in small amounts at depths below a few millimeters.[70]

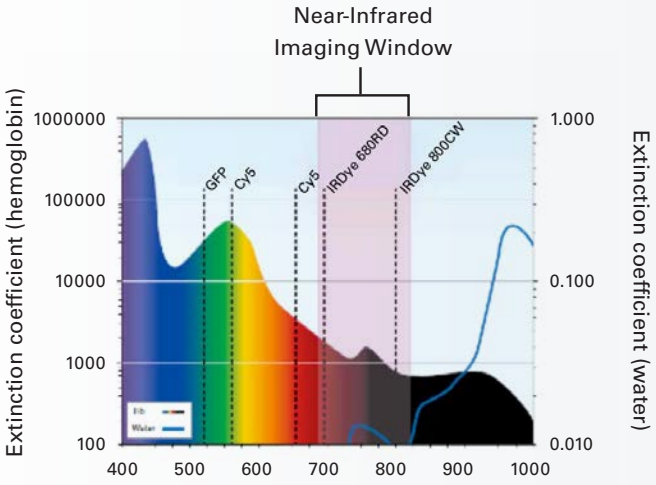


Figure 18. Animal tissue absorbs visible light. Hemoglobin (Hb) and other tissue components strongly absorb visible light. In the NIR region, where IRDye agents are detected, tissue absorbance is dramatically reduced. Above 820 nm, light absorbance by water increases and can affect performance.

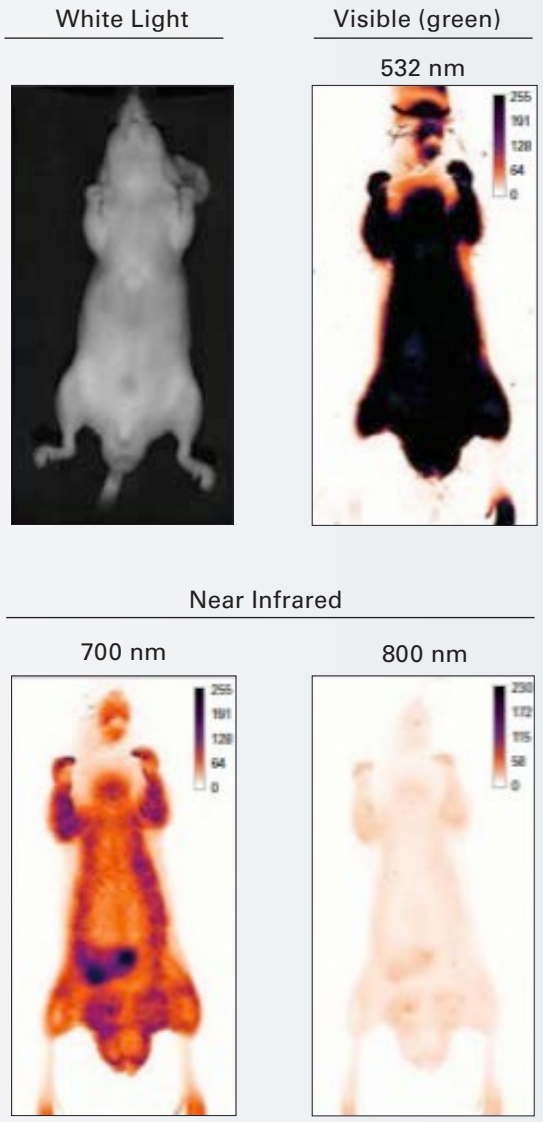


Figure 19. Tissue autofluorescence is much lower at NIR wavelengths. Untreated nude mouse was imaged with visible (532 nm) and NIR (700 nm or 800 nm) light. Autofluorescence at 532 nm (Cy3 channel) was very high.

In the NIR region (700-900 nm), the absorption coefficient of tissue is at its lowest and light can penetrate much more deeply.[71] Above 900 nm, light absorption by water begins to cause interference (Figure 18).

Autofluorescence is also an important consideration. Naturally-occurring compounds in animal tissue cause considerable autofluorescence throughout the visible spectral range, up to ~700 nm (Figure 19). Tissue autofluorescence can mask the desired signal and severely limit target-to-background ratios (Figure 20).[72]

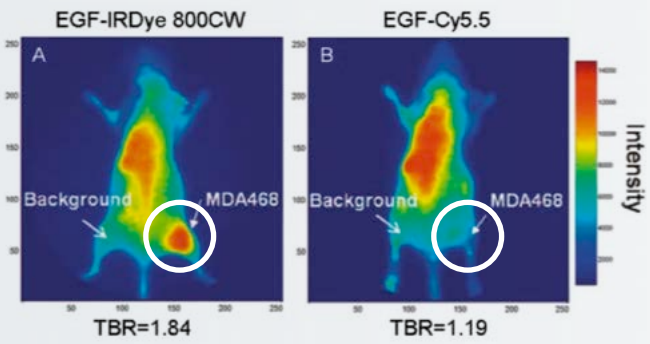


Figure 20. Comparison of target-to-background ratio (TBR) with IRDye® 800CW and Cy5.5 fluorescent conjugates. Mice bearing MDA-MB-468 tumors (white circles) were injected with 1 nmol of A) IRDye 800CW – EGF or B) Cy5.5 – EGF conjugates. Imaging was performed 24 h after injection. IRDye 800CW conjugate (A) yielded significantly higher TBR ratio than Cy5.5. Adapted from Adams, KE et al. *J Biomed Optics* 12: 024017 (2007).

A number of NIR dyes have been used for *in vivo* imaging, but IRDye 800CW is quickly becoming the dye of choice. Cy5.5 has been used historically, primarily due to the lack of other suitable dyes for imaging. The excitation/emission maxima for Cy5.5 (675 nm/694 nm) fall in the range affected by increased tissue autofluorescence, impacting its overall performance (Figure 29)[72], and Cy5.5 has been shown to cause higher background in cellular assays due to non-specific binding. In contrast, IRDye® 800CW has excitation/emission maxima at 785 nm/810 nm, precisely centered in the region known to give optimal signal-to-background ratio for optical imaging.[71] Quantum dots, with their photostability and bright emissions, have generated a great deal of interest; however, their size precludes efficient clearance from the circulatory and renal systems, and there are questions about their long-term toxicity.[73]

The Molecular Imaging Workflow

The basic steps for making, validating, and using an NIR fluorescent probe are summarized below, and in Figure 21. A more comprehensive discussion of approaches for the development of fluorescent contrast agents has also been published.[74] This article is reprinted on pages 82-94.

Probe Preparation

In vivo imaging projects typically begin with identification of a possible targeting agent or probe, such as a receptor ligand, peptide, small molecule, or antibody. Ready-to-use BrightSite™ fluorescent optical imaging agents make it possible to get started right away. These rigorously validated IRDye agents target a variety of biological characteristics, including cell surface protein expression, glucose metabolism and hypoxia, tumor angiogenesis, and vasculature/lymphatics. With IRDye reactive dyes (NHS ester or maleimide), custom probes can be developed to suit specific experimental needs.

Ready-to-use BrightSite optical agents make it easy to begin animal studies immediately. These bright fluorescent agents are labeled with IRDye fluorophores for NIR fluorescence optical imaging, and they target a variety of disease characteristics. Extensive probe development work (including probe labeling, purification, and quality control assays for binding and specificity) has been performed by LI-COR scientists. The agent can be administered and then imaged with any small animal imaging equipment with appropriate 680 nm or 800 nm filter sets. BrightSite optical agents and IRDye infrared dyes are compatible with many pre-clinical NIR fluorescence imagers, including the LI-COR Pearl

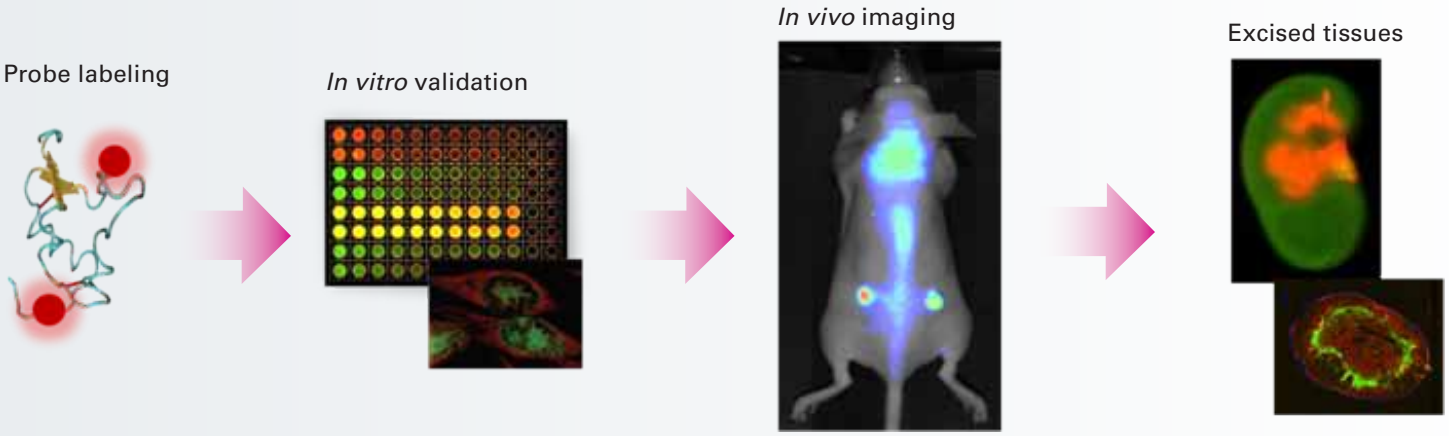


Figure 21. Validation and use of an IRDye® fluorescent probe. After probe labeling, *in vitro* cellular assays and microscopy are used to confirm specificity. The desired target is then imaged in animals. Excised organs and tissues can be examined for more detailed localization of the probe. Animal image captured with Pearl® Impulse.

Table 2. BrightSite™ Imaging Agents

Application	IRDye EGF	IRDye RGD	IRDye 2-DG	IRDye PEG	IRDye HA	CellVue	PSVue
Tumor Imaging	✓	✓	✓	✓	✓		
Metabolic Imaging			✓				
Inflammation/Arthritis		✓	✓				
Vasculature (Contrast)				✓			
Lymphatic Imaging				✓	✓		
Lymph Node Imaging				✓	✓		
Cell Trafficking						✓	
Apoptosis							✓

Impulse, LI-COR Odyssey CLx or Classic, Perkin Elmer (CRi Maestro, VisEn Medical FMT, Xenogen IVIS), Carestream, and Fluoptics (Fluobeam™ 700, Fluobeam 800).

For detailed information about BrightSite™ Imaging Agents and their use, see pages 95-100.

In vitro Validation

Cell-based assays can often be used to evaluate binding and specificity *in vitro* before animal studies begin. A variety of approaches have been used for *in vitro* testing, including the In-Cell Western™ format.[72, 74-77] Cultured tumor cells in microplates are incubated with the labeled targeting agent to assess binding. Specificity is evaluated by methods such as blocking access to the target with an antibody, or competition with an excess of unlabeled agent. Fluorescence emission from each microplate well is then quantified. Fluorescence microscopy is also used to validate targeting and localization of probes.[76]

In vivo Clearance

Clearance studies (with both the unconjugated dye, and the labeled probe) are important for accurate interpretation of imaging data.[74] Signal may be non-specifically retained in regions of the body that block or mimic the intended target (such as the liver, kidneys, or bladder), and could result in misinterpretation of data if these controls are not performed. Time courses of probe clearance also help to establish the optimal time for imaging in subsequent experiments.

Imaging

The probe can then be used to image the desired target in animal studies. If possible, specificity should also be confirmed *in vivo*. One approach is to pre-inject the animal with an excess of unlabeled agent or other compound that blocks or competes with binding of the targeting agent.[75,77]

Tissues and Organs

At the end of the imaging study, animals can be sacrificed, and organs or tissues can be excised and imaged to confirm the presence of the probe in the desired location.[74,77] Imaging of whole organs provides a quick and semi-quantitative estimate of signal intensity and can be used to evaluate biodistribution of the probe. For more detailed analysis, sections can be prepared from frozen or paraffin-embedded tissue and imaged at higher resolution.[74-76]

Summary

IRDye® infrared dyes are excellent fluorescent labels for protein and cellular assays, biochemical assays, microscopy, and *in vivo* molecular imaging. These dyes are bright, have excellent water solubility, and exhibit low non-specific binding. Background fluorescence from membranes, plastics, tissues, biological samples, and chemical compounds is substantially reduced at NIR wavelengths, enabling a variety of fluorescent applications that were previously impractical. In addition, the ability of NIR light to penetrate deep into animal tissue opens new windows of opportunity for *in vivo* imaging of small animals and possible clinical translation.[67]

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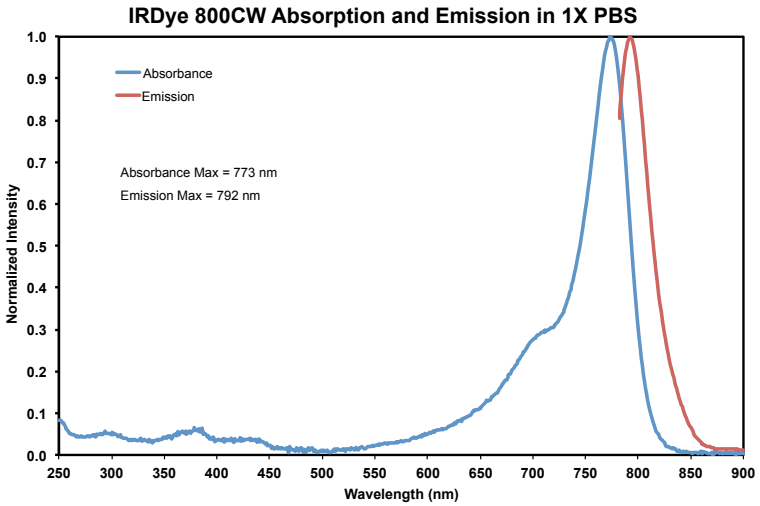
IRDye® Infrared Dyes

IRDye 800CW

IRDye 800CW is spectrally matched to LI-COR® imaging systems and is ideal for protein and antibody labeling, or nucleic acid applications with high labeling density. The dye offers optimal performance in Western blotting and immunocytochemical assays and superior performance for *in vivo* imaging. Dye structure may be found online at *www.licor.com/bio.*



Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	300,000	778	794
Water	240,000	774	789
1X PBS	240,000	774	789
PBS: Methanol	270,000	777	791

NHS Ester

The NHS ester reactive group provides the functionality for labeling primary and secondary amino groups (e.g. lysine residues).

929-70020 IRDye 800CW NHS Ester, 0.5 mg $130.00
929-70021 IRDye 800CW NHS Ester, 5.0 mg $750.00
929-70022 IRDye 800CW NHS Ester, 50 mg $4,990.00

Storage: -20°C

Maleimide

Maleimide reactive group provides the functionality for labeling molecules that contain free sulfhydryl (—SH) groups.

929-80020 IRDye 800CW Maleimide, 0.5 mg $130.00
929-80021 IRDye 800CW Maleimide, 5.0 mg $750.00

Storage: -20°C

Carboxylate

The carboxylate form of IRDye 800CW is in a non-reactive state, so it serves as a control for potential effects or retention of the dye itself. It provides a necessary control for both *in vitro* cell-based assays and *in vivo* animal imaging, when IRDye 800CW is used as the detection agent. It can also be used as a reference for HPLC purification of labeling reactions.

Note: *The carboxylate dye has no reactive group and cannot be used for labeling other molecules.*

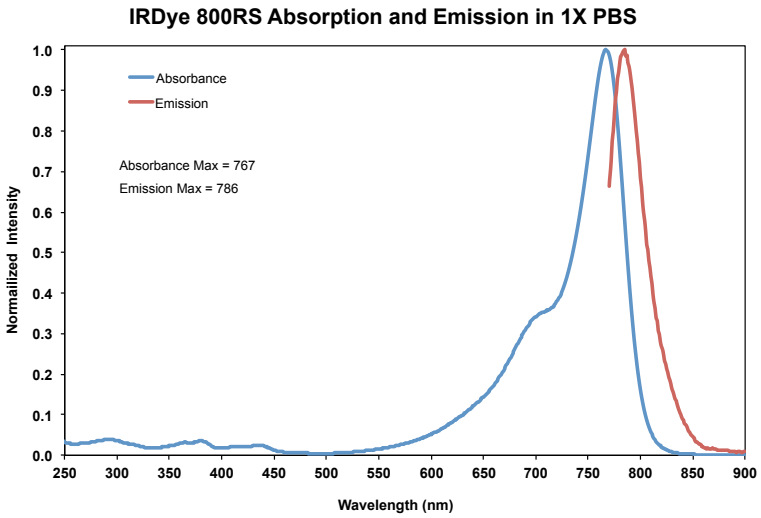
929-08972 IRDye 800CW Carboxylate, 100 nmol $130.00
929-09406 IRDye 800CW Carboxylate, 5.0 mg $525.00

Storage: -20°C

IRDye® 800RS

IRDye 800RS is the dye of choice for nucleic acid applications. The NHS ester reactive group provides the functionality for labeling primary and secondary amino groups. The dye has good water solubility but low salt tolerance. IRDye 800RS nucleic acids are easily purified by reverse-phase chromatography. Dye structure may be found online at *www.licor.com/bio.*

Note: *This dye is* ***not*** *recommended for most protein applications.*



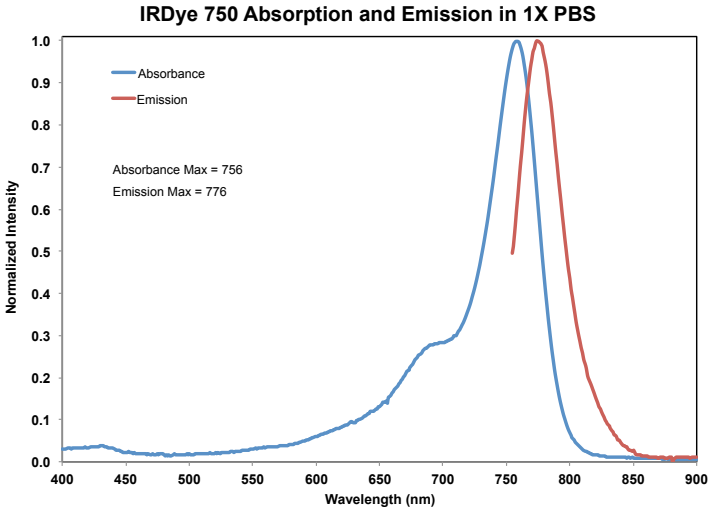
Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	300,000	770	786
Water	200,000	767	786
1X PBS	200,000	767	786
PBS: Methanol	200,000	770	786

929-72020 IRDye 800RS NHS Ester, 0.5 mg $130.00
929-72021 IRDye 800RS NHS Ester, 5.0 mg.....$750.00

Storage: -20°C

IRDye® 750

IRDye 750 has a maximum excitation at 766 nm and a maximum emission at 776 nm. It is spectrally similar to other 750 dyes that are used for microscopy and other applications. This dye is optimized for use with instruments that detect those wavelengths. Dye structure may be found online at *www.licor.com/bio.*



Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	330,000	766	776
Water	260,000	756	776

NHS Ester

IRDye 750 NHS Ester is an infrared dye with detection near 750 nm. The NHS ester reactive group provides the functionality for labeling primary and secondary amino groups.

929-70040 IRDye 750 NHS Ester, 0.5 mg $130.00
929-70041 IRDye 750 NHS Ester, 5.0 mg $750.00

Storage: -20°C

Maleimide

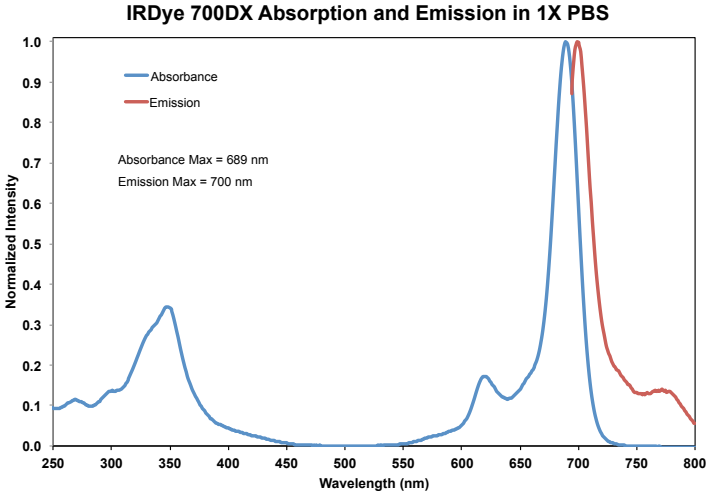
IRDye 750 Maleimide is an infrared dye with detection near 750 nm. Maleimide reactive group provides the functionality for labeling molecules that contain free sulfhydryl (—SH) groups. This reactive group allows conjugation reactions to be performed at physiological pH.

929-71040 IRDye 750 Maleimide, 0.5 mg $130.00
929-71041 IRDye 750 Maleimide, 5.0 mg $750.00

Storage: -20°C

IRDye® 700DX

IRDye 700DX is spectrally matched to LI-COR® imaging systems and is the most photostable 700 nm dye offered by LI-COR. It is an excellent dye for protein, antibody, and nucleic acid labeling and other applications where high water solubility is essential. This dye has excellent photostability, water solubility, and salt tolerance, but is sensitive to acids.



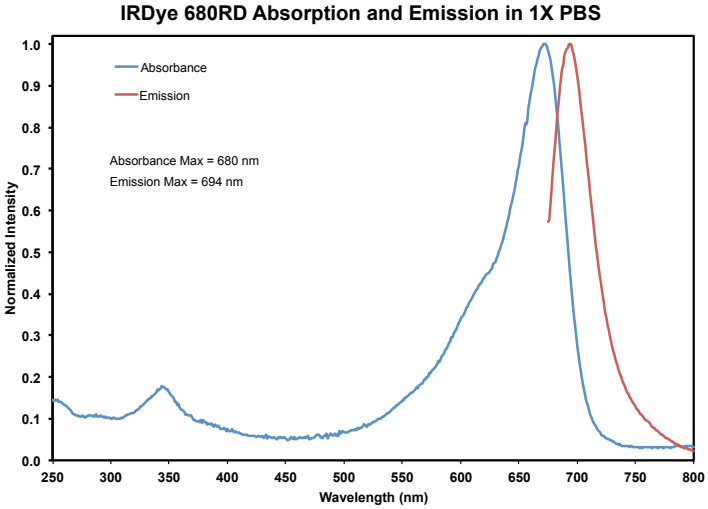
Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	210,000	680	687
Water	165,000	689	700
1X PBS	165,000	689	700

929-70010 IRDye 700DX NHS Ester, 0.5 mg $130.00
929-70011 IRDye 700DX NHS Ester, 5.0 mg $750.00

Storage: -20°C

IRDye® 680RD NEW!

IRDye 680RD is spectrally matched to LI-COR® imaging systems and is the near-infrared fluorescent dye of choice for small animal imaging applications. It has the lowest background compared to LI-COR's other 700 nm dyes. **This infrared dye is the dye of choice for labeling proteins, peptides, and antibodies and is suitable for Western blot and In-Cell Western™ (ICW) Assay applications.**



Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	170,000	680	694
1X PBS	165,000	672	694
PBS: Methanol	165,000	672	694

NHS Ester

The NHS ester reactive group provides the functionality for labeling primary and secondary amino groups (e.g. lysine residues).

929-70050 IRDye 680RD NHS Ester, 0.5 mg $130.00
929-70051 IRDye 680RD NHS Ester, 5.0 mg $750.00

Storage: -20°C

Maleimide

The maleimide reactive group provides the functionality for labeling molecules that contain free sulfhydryl (—SH) groups.

929-71050 IRDye 680RD Maleimide, 0.5 mg $130.00
929-71051 IRDye 680RD Maleimide, 5.0 mg $750.00

Storage: -20°C

Carboxylate

The carboxylate form of IRDye 680RD is in a non-reactive state, so it serves as a control for potential effects or retention of the dye itself. It can be used as a control for both *in vitro* cell-based assays and *in vivo* animal imaging when IRDye 680RD is used as the detection agent. It can also be used as a reference for HPLC purification of labeling reactions.

Note: *The carboxylate dye has no reactive group and cannot be used for labeling other molecules.*

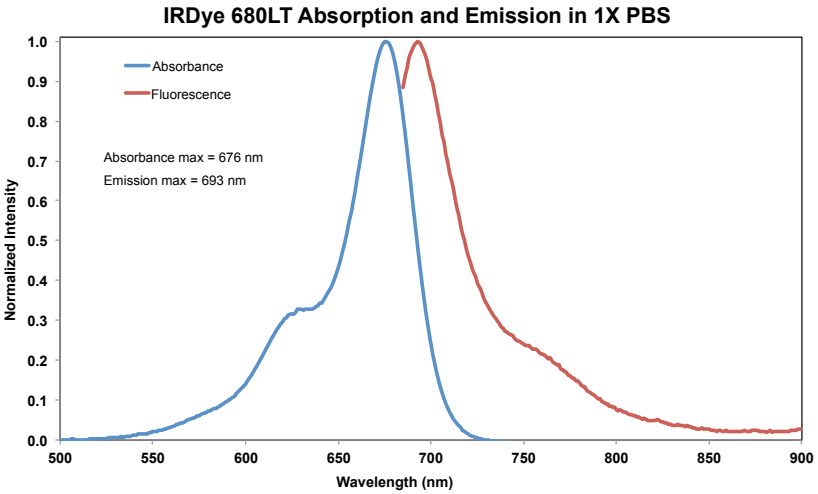
929-08980 IRDye 680RD Carboxylate, 100 nmol $130.00

Storage: -20°C

IRDye® 680LT

IRDye 680LT is spectrally matched to LI-COR® imaging systems and is significantly brighter and more photostable than many other 700 nm near-infrared dyes. This infrared dye is ideal for labeling proteins, peptides, and antibodies. It is brighter than Alexa Fluor® 680 for protein detection applications, including quantitative Western blots and fluorescence microscopy. Dye structure may be found online at *www.licor.com/bio.*

NOTE : *DO NOT USE FOR SMALL ANIMAL IMAGING.*



Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	250,000	680	694
1X PBS	250,000	676	693

NHS Ester

The NHS ester reactive group provides the functionality for labeling primary and secondary amino groups. This dye has the highest water solubility of the 700 series IRDye Infrared Dyes.

929-71010 IRDye 680LT NHS Ester, 0.5 mg $130.00
929-71011 IRDye 680LT NHS Ester, 5.0 mg $750.00

Storage: -20°C

Maleimide

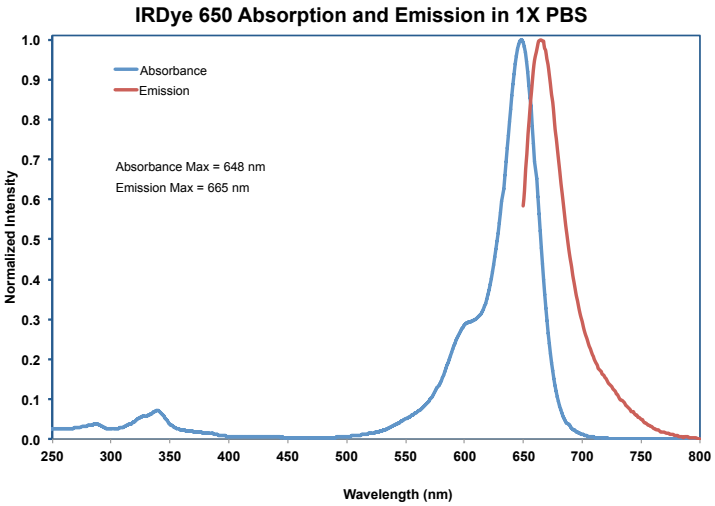
IRDye 680LT Maleimide is a functional derivative of infrared dye IRDye 680LT and is reactive with free sulfhydryl (—SH) groups such as cysteine residues.

929-71008 IRDye 680LT Maleimide, 0.5 mg $130.00
929-71009 IRDye 680LT Maleimide, 5.0 mg $750.00

Storage: -20°C

IRDye® 650

IRDye 650 has a maximum excitation at 651 nm and maximum emission at 668 nm and is spectrally similar to other 650 dyes that are used for flow cytometry, microscopy, and other applications. This dye is optimized for use with instruments that detect those wavelengths. Dye structure may be found online at *www.licor.com/bio.*



Solvent	Ext. Coeff. (M^{-1} cm^{-1})	Abs Max (nm)	Em Max (nm)
Methanol	240,000	651	668
Water	230,000	648	665
1X PBS	230,000	648	665

NHS Ester

IRDye 650 NHS Ester is an infrared dye with detection near 650 nm. The NHS ester reactive group provides the functionality for labeling primary and secondary amino groups.

929-71012 IRDye 650 NHS Ester, 0.5 mg $130.00
929-71013 IRDye 650 NHS Ester, 5.0 mg $750.00
Storage: -20°C

Maleimide

IRDye 650 Maleimide is an infrared dye with detection near 650 nm. Maleimide reactive group provides the functionality for labeling molecules that contain free sulfhydryl (—SH) groups. This reactive group allows conjugation reactions to be performed at physiological pH.

929-71014 IRDye 650 Maleimide, 0.5 mg $130.00
929-71015 IRDye 650 Maleimide, 5.0 mg $750.00
Storage: -20°C

Quencher

IRDye® QC-1 NHS Ester

IRDye QC-1 is a non-fluorescent dye that efficiently quenches fluorescence from a wide range of fluorophores from the visible to near-infrared regions (500-800 nm). It is a water-soluble, monoreactive NHS ester dye, which allows it to be used to label peptides, proteins, and nucleic acids, etc. through the amine groups on such molecules. Protease substrates using IRDye QC-1 do not require an organic co-solvent in assay buffer systems. IRDye QC-1 is suitable for FRET-based applications such as protease assays (see page 76 to learn about IRDye NIR-FRET Caspase Substrate). Dye structure may be found online at *www.licor.com/bio.*

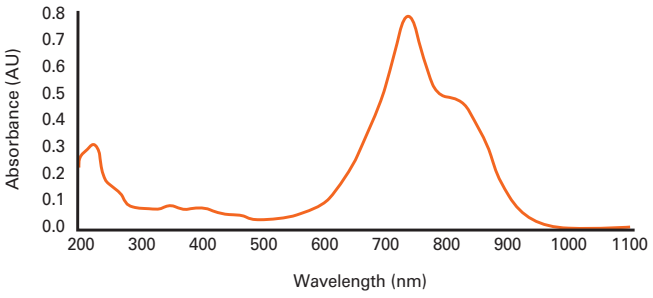
929-70030 IRDye QC-1 NHS Ester, 0.5 mg $130.00

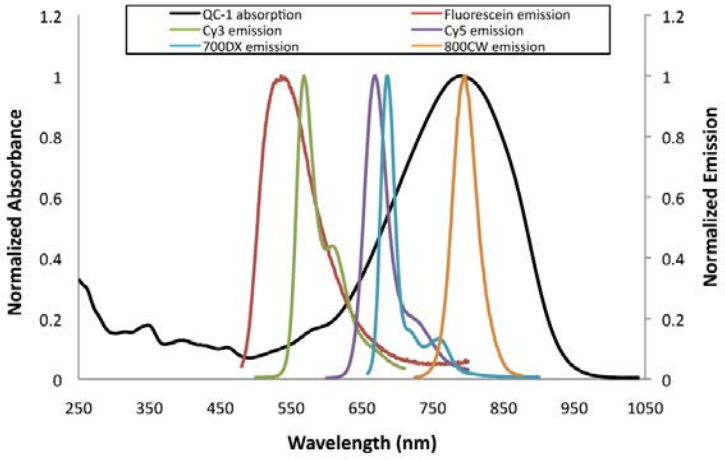
929-70031 IRDye QC-1 NHS Ester, 5.0 mg $750.00

Storage: -20°C

Solvent	Ext. Coeff. M^{-1} cm^{-1}	Absorption Max (nm)	MW g/mole
Methanol	98,000	788	1243.7
1X PBS	96,000	737	

IRDye QC-1 Absorption Spectrum in 1X PBS



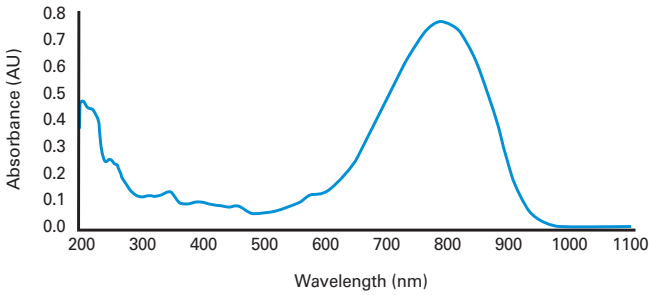


Spectral overlap between the absorption spectrum of IRDye QC-1 and the fluorescence spectra of fluorescein, Cy3, Cy5, IRDye 700DX, and IRDye 800CW

Fluorescence Quenching Efficiency of IRDye QC-1 Against Various Fluorophores in FRET-Based Octapeptide Caspase-3 Substrate Systems

Donor (Fluorophore)	Acceptor (IRDye QC-1)
Fluorescein	97.5%
Cy3	98.1%
Cy5	97.9%
IRDye 700DX	98.8%
IRDye 800CW	98.7%

IRDye QC-1 Absorption Spectrum in Methanol



Application *Workflows*

Applications Overview

LI-COR® Biosciences' imaging systems support many applications and techniques that today's life science researcher needs. Each system has a unique suite of applications that it supports, so you can choose the right system for the needs of your lab – both now and in the future. Below is a list of applications supported by each of the LI-COR Imaging Systems. This section contains application workflows that highlight the versatility and superior data quality of LI-COR imaging systems.

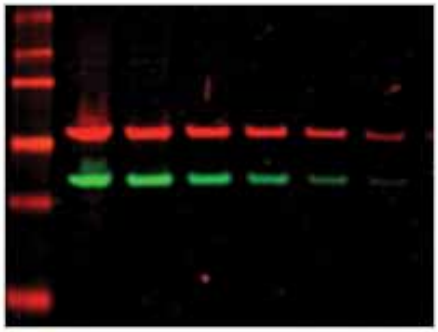
Also included is the technical note: *Immunofluorescent Mapping of Cannabinoid CB1 and Dopamine D2 Receptors in the Mouse Brain* by Dr. Christopher Kearn that highlights the use of the Odyssey® imager for tissue section imaging.

Application	Odyssey® CLx	Odyssey® Classic	Odyssey® Sa	Odyssey® Fc	Pearl® Impulse
Coomassie Protein Gel Imaging	✓	✓	✓	✓	
DNA Gel Imaging	✓	✓	✓	✓	
• SYTO®	✓	✓	✓	✓	
• Ethidium bromide				✓	
• SYBR® Safe Stains				✓	
ELISA	✓	✓	✓		
EMSA/Gel Shift Assay	✓	✓			
Glycoprotein Detection	✓	✓	✓	✓	
In-Cell Western™ Assay	✓	✓	✓		
Microwestern Array	✓	✓	✓		
Northern Blot	✓	✓	✓		
On-Cell Western Assay	✓	✓	✓		
Organ Imaging	✓*	✓*			✓
Protease Assay	✓	✓	✓		
Protein Array	✓	✓	✓		
Quantitative Western Blots	✓	✓	✓		
• Quantitative Infrared				✓	
• Chemiluminescent				✓	
Reporter Gene Assay	✓	✓			
Reverse Phase (Lysate) Array	✓	✓			
RNAi Analysis	✓	✓	✓		
Small Animal Imaging	✓	✓			✓
Southern Blot	✓	✓			
Tissue Section Imaging	✓	✓	✓		
Transcription Factor Assay	✓	✓			
Zymography	✓	✓	✓	✓	

* With MousePOD® *in vivo* Imaging Accessory

Near-Infrared Fluorescent Western Blots

Workflow: **Materials Used:**



Sample

- Protein Sample Loading Buffer (page 67)

Electro-phoresis

- Power Supply
- Electrophoresis Chamber
- Electrophoresis Buffer (page 67)
- Protein MW Markers (page 68)

Blotting and Transfer

- Blotting Module
- Transfer Buffer (page 67)
- Nitrocellulose or PVDF Membrane (page 59)
- Filter Paper

Near-Infrared Detection

- Odyssey® Blocking Buffer, Casein Blocking Buffer, Blocking Buffer Sample Pack (page 66)
- Primary Antibodies for Normalization (page 56)
- IRDye® 800CW, IRDye 680RD, and IRDye 680LT Secondary Antibodies (pages 47-48)
- Western Blot Incubation Boxes (page 149)
- PBS Wash Buffer (page 67)
- MPX™ Blotting System (page 60)
- Stripping Buffer (page 67)
- Western Blotting Kits (pages 57-58)

Imaging

- Odyssey CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Infrared Imaging System, (page 141)
- Odyssey Fc Imaging System (page 142)

Chemiluminescent Western Blots

Workflow: | **Materials Used:**

Sample

- Protein Sample Loading Buffer (page 67)

Electrophoresis

- Power Supply
- Electrophoresis Chamber
- Electrophoresis Buffer (page 67)
- Protein MW Markers (page 68)

Blotting and Transfer

- Blotting Module
- Transfer Buffer (page 67)
- Nitrocellulose or PVDF Membrane (page 59)
- Filter Paper

Antibody Incubation

- Blocking Buffer
- Primary Antibodies
- HRP Secondary Antibodies
- Western Blot Incubation Boxes (page 149)
- PBS Wash Buffer (page 67)
- MPX™ Blotting System (page 60)
- Stripping Buffer (page 67)

Detection

- Chemiluminescent Substrate
- PBS Wash Buffer (page 67)

Imaging

- Odyssey® Fc Dual-Mode Imaging System (page 142)

Purple Colored Text indicates LI-COR® Biosciences Products

Western Blot Cost Comparison: Odyssey® Systems

Save money with near-infrared fluorescence detection compared to chemiluminescence detection.

Cost Savings			
Reagents	IR Detection (2 Targets)	Chemiluminescence (1 Target)	Chemiluminescence (strip and reprobe for second target) 2-target total
Secondary Antibody (15 mL) Recommended Dilutions: (1:15,000 for IR#; 1:2,500 for Chemi)	$0.68	$0.33	$0.66
Chemiluminescent Substrate (2 mL)	---	$5.70 (2 mL)	$11.40 (2 mL)
Film (2-4 pieces of film/blot)	---	$7.68	$15.36
Protein Markers Two-color Protein Marker for IR (2 µL) Standard Protein Marker for Chemi (10 µL)	$1.16	$4.68	No charge to reuse marker
Cost **Extra Cost Per Blot Compared to IR***	**$1.84**	**$18.39 (2 mL)** **$16.55**	**$27.42 (2 mL)** **$25.58**

#IRDye 800CW and IRDye 680RD were used for IR calculations.
*Based on GE Healthcare pricing. September 20, 2011. Assumes 10 x 10 cm blot.

Easy Transition From Chemiluminescence

Perform electrophoresis and transfer as usual

↓

Block in Odyssey Blocking Buffer (page 66) 30 minutes to 1 hour

↓

Dilute primary antibody and incubate as usual

↓

Wash

↓

Dilute IRDye® Secondary Antibody (pages 47-48) and incubate 1 hour

↓

Wash

↓

Blot is ready for imaging

In-Cell Western™ Assays and On-Cell Western Assays

Workflow: **Materials Used:**

Culture Cells

- Typical Cell Culture Reagents
- 96 or 384-well Optically Clear-bottomed Microwell Plates

Stimulate/ Inhibit Cells

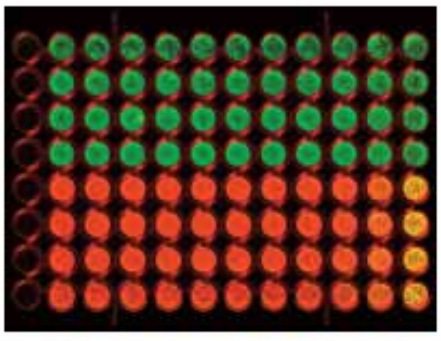
- Stimulants and Inhibitors
- PBS and Formaldehyde Fixing Solution
- PBS and 10% Triton® X-100 Washing Solution (for In-Cell Western Assays only)

Detection

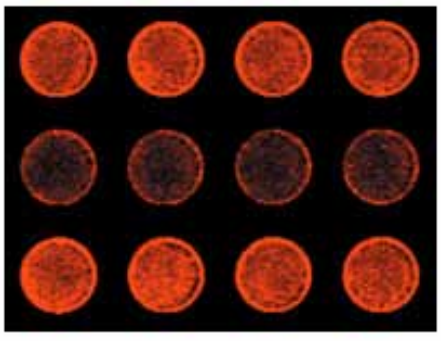
- Odyssey® Blocking Buffer (page 66)
- Primary Antibodies
- PBS Wash Buffer (page 67)
- IRDye® 800CW and IRDye 680RD Secondary Antibodies (pages 47-48)
- DRAQ5™ and Sapphire700™ (page 72) Normalization Stains
- In-Cell Western™ Assay Kits (page 72)
- Actin and Tublin Primary Antibodies for Normalization (page 56)
- NHS Ester Dyes (pages 15-21)

Imaging

- Odyssey CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Infrared Imaging System with optional Express Automation upgrade (page 141)



Multiplexed In-Cell Western™ Assay



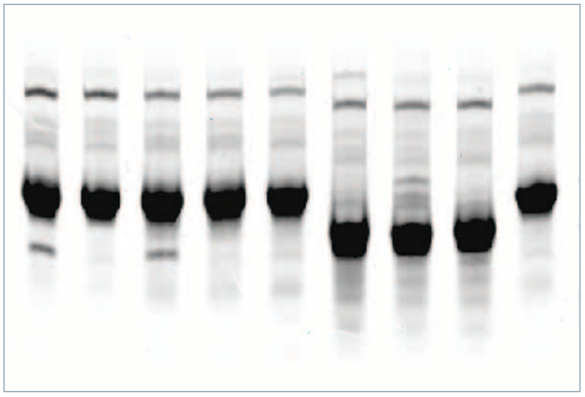
On-Cell Western Assay

Purple Colored Text indicates LI-COR® Biosciences Products

Coomassie Staining and Imaging of Protein Gels

Workflow:

Materials Used:



Sample

- Protein Sample Loading Buffer (page 67)
- Protein MW Markers for Westerns (page 68)

Electro-phoresis

- Power Supply
- Electrophoresis Chamber
- Electrophoresis Buffer (page 67)

Protein Staining

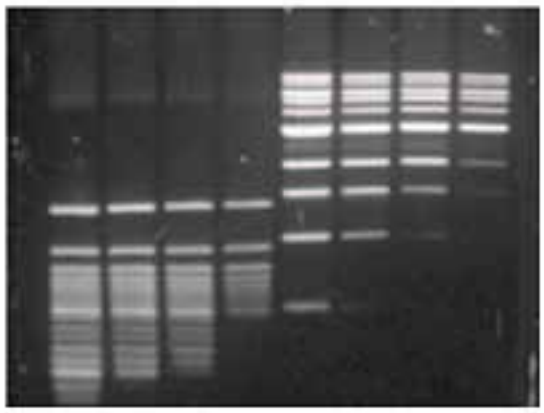
- IRDye® Blue Protein Stain (page 67)

Imaging in 700 nm channel

- Odyssey® CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Imaging System (page 141)
- Odyssey Fc Imaging System (page 142)

DNA Gel Staining and Imaging

Workflow: **Materials Used:**



Sample

- Orange Loading Dye (page 77)

Electrophoresis

- Power Supply
- Electrophoresis Chamber
- Agarose Gel
- DNA Ladder
- Electrophoresis Buffer

OR

Gel Staining

- SYTO® 60

Imaging

- Odyssey® CLx (700 nm) (page 140)
- Odyssey Classic (700 nm)
- Odyssey Sa (700 nm) (page 141)
- Odyssey Fc (700 nm) (page 142)

Gel Staining

- Ethidium Bromide
- SYBR® Safe Stains
- Other 600 nm Stains

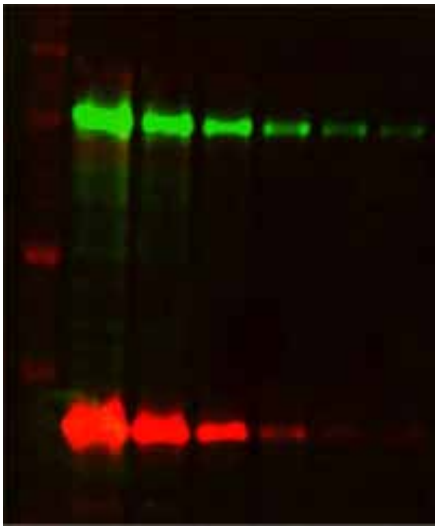
Imaging

- Odyssey Fc (600 nm) (page 142)

Purple Colored Text indicates LI-COR® Biosciences Products

In-Gel Western Assays

Workflow: **Materials Used:**



Sample

- Protein Sample Loading Buffer (page 67)

Electro-phoresis

- Power Supply
- Electrophoresis Chamber
- Precast Gels
- Electrophoresis Buffer (page 67)
- Protein MW Markers for Westerns (page 68)

Gel Fixation

- 50% Isopropanol + 5% Acetic Acid
- Ultrapure Water

Antibody Incubation

- Odyssey® Blocking Buffer (page 66)
- Primary Antibodies
- IRDye® 800CW, IRDye 680RD, and IRDye 680LT Secondary Antibodies (pages 47-48)
- Western Blot Incubation Boxes (page 149)
- PBS Wash Buffer (page 67)

Imaging

- Odyssey CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Infrared Imaging System (page 141)
- Odyssey Fc Dual-Mode Imaging System (page 142)

EMSA/Gel Shift Assays

Workflow:

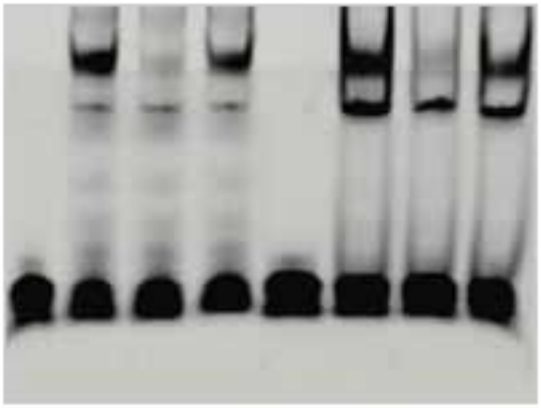
DNA-Protein Complex Formation

Electro-phoresis

Imaging

Materials Used:

- EMSA Buffer Kit (page 78)
- IRDye® 700 Consensus Oligonucleotides (pages 77-78)
- Extract or Protein of Interest
- Power Supply
- Electrophoresis Chamber
- Precast Gels
- Orange Loading Dye (page 77)
- Odyssey® CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System



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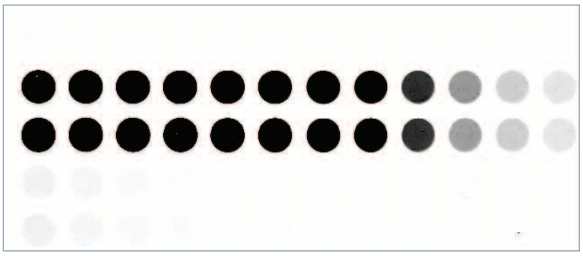
ELISA, Indirect Assay

Workflow: **Materials Used:**



Coat Plates and Incubate

- Antigen/Sample
- Microplate

Wash

- Odyssey® Blocking Buffer (page 66)
- Casein Blocking Buffer (page 66)
- Blocker Set (page 66)

Secondary Antibody Incubation

- HRP-Conjugated or AP-Conjugated Secondary Antibodies

Wash and Substrate Addition

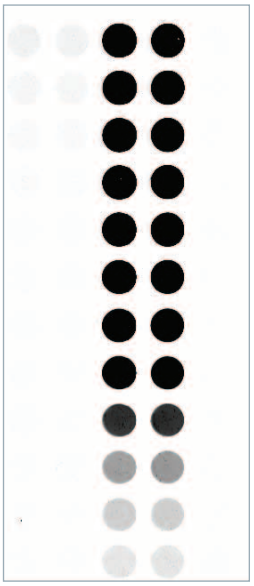
- PBS Wash Buffer (page 67)
- ELISA HRP Substrate (680) Pack (page 79)
- ELISA AP Substrate (680) Pack (page 79)

Imaging

- Odyssey CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Infrared Imaging System (page 141)

ELISA, Sandwich Assay

Workflow:	Materials Used
Coat Plates and Incubate	• Capture Antibody • Microplate
Wash and Sample	• PBS Wash Buffer (page 67) • Antigen/Sample
Primary Antibody Incubation	• Primary Antibodies
Wash	• PBS Wash Buffer (page 67)
Secondary Antibody Incubation	• HRP-Conjugated or AP-Conjugated Secondary Antibodies
Wash and Substrate Addition	• ELISA HRP Substrate (680) Pack (page 79) • ELISA AP Substrate (680) Pack (page 79) • PBS Wash Buffer (page 67)
Imaging	• Odyssey® CLx Infrared Imaging System (page 140) • Odyssey Classic Infrared Imaging System • Odyssey Sa Infrared Imaging System (page 141)



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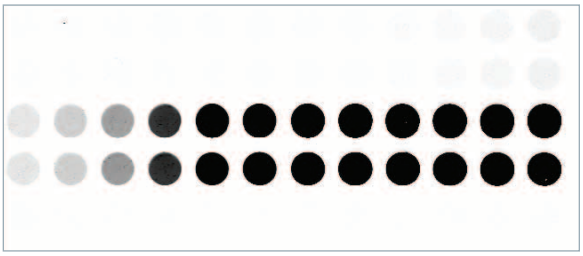
ELISA, Competitive Assay

Workflow: **Materials Used:**



Incubate and Plate

- Primary Antibody
- Antigen/Sample
- Microplates

Wash

- PBS Wash Buffer (page 67)

Secondary Antibody Incubation

- HRP-Conjugated or AP-Conjugated Secondary Antibodies

Wash

- PBS Wash Buffer (page 67)

Substrate Addition

- ELISA HRP Substrate (680) Pack (page 79)
- ELISA AP Substrate (680) Pack (page 79)

Imaging

- Odyssey® CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Infrared Imaging System (page 141)

Reverse Phase (Lysate) Protein Arrays

Workflow: **Materials Used:**

Spot Sample

- **Protein (lysate/tissue) spotted on Nitrocellulose-coated Slides**

Block

- **Odyssey® Blocking Buffer (page 66)**
- **Casein Blocking Buffer (page 66)**
- **Blocking Buffer Sample Pack (page 66)**

Primary Antibody Incubation

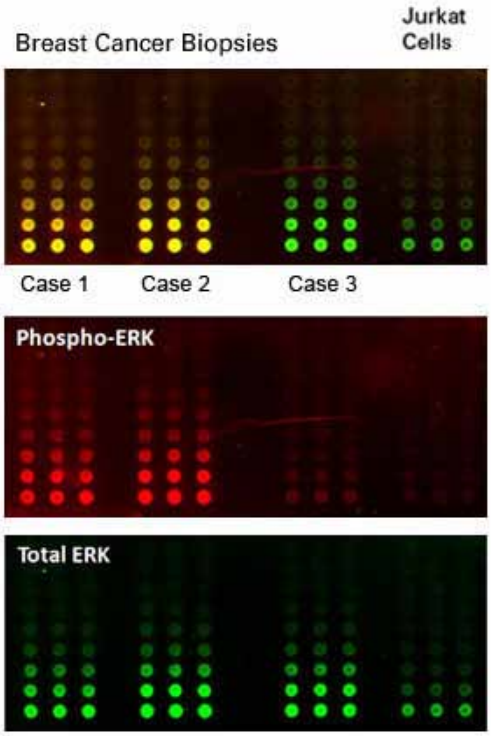
- **Primary Antibodies**
- **PBS Wash Buffer (page 67)**

Near-infrared Detection

- **IRDye® 800CW, IRDye 680RD, or IRDye 680LT Secondary Antibodies (pages 47-48)**
- **PBS Wash Buffer (page 67)**

Imaging

- **Odyssey CLx Infrared Imaging System (page 140)**
- **Odyssey Classic Infrared Imaging System**
- **Odyssey Sa Infrared Imaging System (page 141)**



Purple Colored Text indicates LI-COR® Biosciences Products

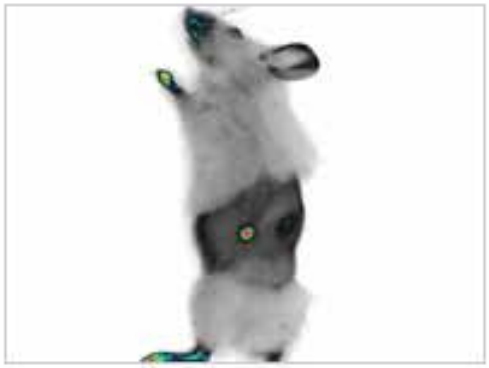
Glycoprotein Detection Methods

The Odyssey® Infrared Imaging Systems and IRDye® conjugates provide a single optimized solution for the detection of a variety of glycoproteins.

Detection Method	Materials Used:
Glycoprotein and Total Protein	• Glycoprotein Stain • Coomassie Stains such as IRDye® Blue Protein Stain (page 67) • Coomassie Stains can be imaged on the appropriate Odyssey® Infrared Imagers (pages 140-142) in the 700 nm channel
Indirect Lectin Detection	• Use biotinylated lectins in combination with IRDye Streptavidin (page 50) • Image on the appropriate Odyssey Infrared Imagers (pages 140-142)
Direct Lectin Detection	• Label lectins with IRDye infrared dyes using one of the IRDye Protein Labeling Kits (pages 104-107) • Image on the appropriate Odyssey Infrared Imagers (pages 140-142)
Glycoprotein Antibody Detection	• O-GlcNAc modifications can be detected using a specific antibody and IRDye Secondary Antibodies (pages 47-48) • Image on the appropriate Odyssey Infrared Imagers (pages 140-142)
Enzymatic Detection	• Use IRDye Streptavidin (page 50) with Click-iT™ Biotin Glycoprotein Detection Kit (Molecular Probes) • Image on the appropriate Odyssey Infrared Imagers (pages 140-142)
Arrays	• IRDye Labeling Kits (pages 104-107), IRDye Secondary Antibodies (pages 47-48), IRDye Streptavidin (page 50) used with Qproteome™ GlycoArray Kit • Image on the appropriate Odyssey Infrared Imagers (pages 140-142)

Molecular Imaging (*In Vivo* or Small Animal Imaging)

Workflow: **Materials Used:**



Probe Development

Target Identification

- **Application-specific Probe Development**

Label Conjugation

- IRDye® Protein Labeling Kits (pages 104-107)
- IRDye Infrared Dyes (pages 15-21)
- BrightSite™ Small Animal Imaging Agents (pages 95-100)
- CellVue® Stains (page 101)
- PSVue® 794 Reagent Kit (page 102)

In Vivo Imaging

***In vitro* Assays**

- **In-Cell Western™ Assays can be used to confirm binding and specificity *in vitro* on the Odyssey® CLx (page 140), Odyssey Classic, or Odyssey Sa Infrared Imaging Systems (page 141)**

***In Vivo* Clearance Studies**

- Pearl® Impulse Small Animal Imaging System (page 150)
- Odyssey CLx (page 140) or Odyssey Classic Infrared Imaging Systems with MousePOD® *in vivo* Small Animal Imaging accessory (page 148)
- SmartFlow Anesthesia Suite (page 151)
- SmartFlow Surgical Suite (page 151)

***In Vivo* Studies**

- Pearl® Impulse Small Animal Imaging System (page 150)
- Odyssey CLx or Odyssey Classic Infrared Imaging System with MousePOD® *in vivo* Small Animal Imaging accessory (page 148)
- SmartFlow Anesthesia Suite (page 151)
- SmartFlow Surgical Suite (page 151)

Post-Imaging Analysis

Imaging with Excised Organs/ Tissues

- Pearl Impulse Small Animal Imaging System (page 150)
- Odyssey CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System

Tissue Sections

- Odyssey CLx Infrared Imaging System (page 140)
- Odyssey Classic Infrared Imaging System
- Odyssey Sa Infrared Imaging System (page 141)

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TECHNICAL NOTE:
Immunofluorescent Mapping of Cannabinoid CB1 and Dopamine D2 Receptors in the Mouse Brain

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Identification of receptor expression patterns is an important initial element in delineating interactions between cellular signaling systems. In addition to understanding normal physiology, changes in receptor populations can be used to track disease progression. Here, we demonstrate the utility of infrared immunostaining for rapid and detailed evaluation of expression patterns for two G protein coupled receptors, the cannabinoid CB1 and dopamine D2 receptor in mouse brain.

Introduction

G protein coupled receptors (GPCR) represent the largest protein family with over 1000 distinct members and is the therapeutic target for the majority of current pharmaceuticals.[1] Alterations in GPCR expression have been associated with several disease processes. For example, decreased amounts of two GPCR's, the cannabinoid CB1 and dopamine D2 receptors in the basal ganglia are associated with Huntington's disease.[2]

The cannabinergic and dopaminergic systems exert diverse functions throughout the central nervous system. The CB1 receptor was identified first as the receptor for Δ9-tetrahydrocannabinol,[3,4] the major psychoactive component found in marijuana.[5] More recent studies indicate that CB1 receptor signaling helps tune motor coordination[6] and modulates satiety.[7] The D2 receptor also regulates movement through complex circuitry in the basal ganglia.[8] Additionally, dopamine signaling has been suggested as a common mediator for reinforcing and pleasurable stimuli.[9]

The neurodegenerative changes associated with Huntington's disease are well characterized and are likely the result of a genetic instability causing a polyglutamine expansion in the Huntington protein.[10] The best known of these neurological changes in Huntington's disease is a decrease in dopamine receptors in the basal ganglia.[11,12] However, the first known anatomical alteration is actually a decrease in CB1 receptors in the same area.[2,13,14] It is not known if this change in CB1 receptor density is causative or coincident with Huntington's disease progression. Interestingly, in a mouse model of Huntington's disease, environmental enrichment slows the progression of the disease[15,16] and slows the loss of CB1 receptors in the basal ganglia.[17] These findings suggest that CB1 receptor expression is a sensitive marker for Huntington's disease, which precedes cell death and loss of D2 receptors. We have employed the Odyssey® Imaging System to determine basal expression patterns and co-localization of CB1 and D2 receptors in normal mouse brain to validate this method to monitor changes in GPCR levels.

Materials and Methods

Slice Preparations

All protocols with mice were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult C57Bl/6 mice were anesthetized with pentobarbital and perfused intracardially with a solution of 4% paraformaldehye in 100 mM phosphate buffer, pH 7.4 (PB). The brains were excised and postfixed in the same fixative for 2 hours. The tissue was permeated with 30% w/v sucrose in PB at 4-8°C for no less than 48 hours. The brains were cut into 40 µm coronal or sagittal sections on a vibratome and placed in phosphate buffer until processed for immunohistochemistry.

Immunohistochemistry

Mouse brain sections were rinsed in 100 mM Tris buffered saline, pH 7.4 (TBS) and blocked in TBS containing 0.1% Triton® X-100 and 5% donkey serum (DS) for 1 to 2 hours at room temperature. The tissue sections were incubated with affinity purified antibodies in TBS containing 0.1% Triton X-100 and 2.5% DS overnight at 4-8°C. The specific antibodies employed in this study were anti-CB1-CT raised in goat (1:2500 dilution), and an anti-D2 antibody generated in rabbit (1:1500 dilution). The D2 antibody was raised against a fusion protein containing amino acids 216 to 311 of the human D2 long receptor isoform. The epitope displays approximately 90% identity with the mouse D2 receptor. Following four washes of 15 min. each with TBS containing 0.05% Tween® 20 (TBST), the tissue sections were incubated for 2 hours at room temperature with Alexa Fluor® 680 conjugated donkey antigoat IgG (H+L)

(Molecular Probes, Eugene OR) at a 1:5000 dilution and IRDye 800CW conjugated donkey anti-Rabbit IgG (H+L) (Rockland Immunochemicals, Gilbertsville, PA) at a 1:1500 dilution in TBS containing 0.1% Triton X-100 and 2.5% DS. The tissue sections were washed four times in TBST over two hours, placed in 100 mM Tris (pH 7.4) and mounted on microscope slides. Following a brief rinse with water, the sections were allowed to air dry for at least 1 hour. The fluorescent immunocomplexes were detected with the LI-COR Odyssey Classic (21 µm resolution, 1 mm offset with highest quality). Channel sensitivity was optimized for each set of stained sections then maintained for that group of samples. Typical sensitivity settings ranged from 1.5 to 3.0. The relative location of the slice and identification of brain regions were determined by comparison to images in (18). Regions of interest were defined and integrated intensities were determined with the associated Odyssey software and data were analyzed with the Prism® (GraphPad Software, San Diego, CA) program.

IRDye secondary antibodies for detection in the 700 nm and 800 nm channel are available from LI-COR and can be used for tissue section imaging as outlined in this Technical Note. Please see pages 46-49 for the full product listing.

Results

Free floating fixed mouse brain sections were incubated with primary antibody and fluorophore-conjugated secondary antibody then allowed to dry on glass slides. Unless noted, no additional tissue processing (i.e. fluorescent mounting medium) was used. Samples were imaged immediately after drying. For the stability studies, samples were stored at 4-8°C in the dark.

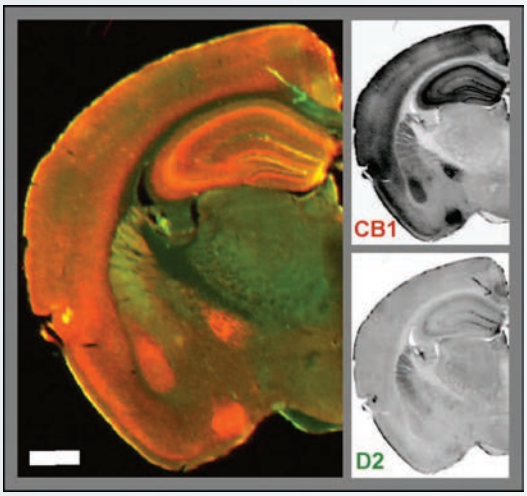

Figure 1. Coronal section of mouse brain immunostained for CB1 and D2 receptors. Fixed brain sections were incubated with goat anti-CB1 and rabbit anti-D2 antibodies followed by the secondary antibodies (see Methods). Right panels are grayscale images of the individual antibodies. The left images is a pseudocolored overlay of the two staining patterns with CB1 I.R. in red and D2 I.R. colored green. Scale bar = 1 mm.

Coronal sections

The coronal brain slice analyzed (Figure 1) is at the caudal extent of the cortical ventricles at approximately-1.34 mm relative to the bregma. The pseudo colored image depicts CB1 (red) and D2 (green) receptor immunoreactivity. The grayscale images are single channel fluorescence intensity images of CB1 (upper) and D2 (lower) receptor immunoreactivity. There is distinct CB1 receptor staining in the basolateral and basomedial amygdala. Intense staining is also observed in the medial globus pallidus and to a lesser extent, in the lateral globus pallidus. The hippocampus displays discrete intense staining for the CB1 receptor in regions corresponding to the pyramidal layer and hillus. CB1 receptors are expressed in discrete cortical lamina. The piriform cortex also exhibits strong CB1 receptor immunoreactivity.

The D2 receptor is found throughout the striatum and in a segregated portion of the hippocampal formation with marked expression in the hillus. Additionally, diffuse D2 receptor staining is observed in the cortex.

Sagittal Sections

Immunoreactivity for CB1 and D2 receptors is also readily apparent in sagittal sections of mouse brain (Figure 2).

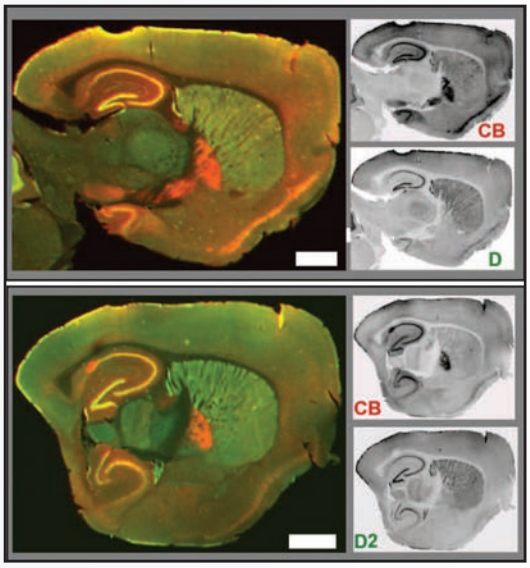

Figure 2. Sagittal sections of mouse brain immunostained for CB1 and D2 receptors. Fixed brain sections complexed with anti-CB1 and anti-D2 antibodies followed by fluorophore conjugated secondary antibodies. Right panels are grayscale images of the individual antibodies. The left images is a pseudocolored overlay of the two staining patterns with CB1 I.R. in red and D2 I.R. colored green. Scale bar = 1 mm.

The upper panels depict a brain slice approximately 1.7 mm lateral of the midline and the lower panels are of a slice approximately 2.5 mm from the midline. In addition to expression in the piriform layer, hippocampus and amygdala, CB1 receptors are clearly evident in the nigral-pallidal pathway with intense immunoreactivity in the substantia nigra reticulata, medial and lateral globus pallidus.

Similar high expression of CB1 receptors is found on the olfactory tubercle. In the cerebellum, there is discrete localization of CB1 receptor protein in the molecular layer and base of the Purkinje cell layer (Figure 3).

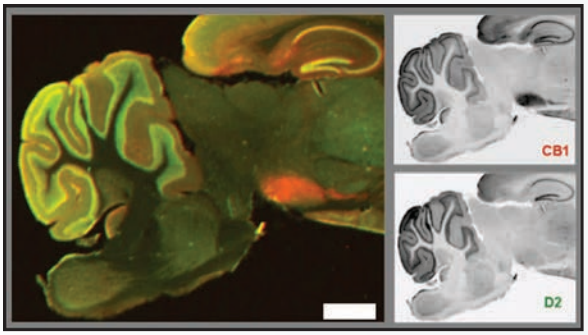

Figure 3. Sagittal section of mouse cerebellum and hippocampus immunostained for CB1(red) and D2 (green) receptors. Scale bar = 1 mm.

The caudate/putamen displays notable D2 receptor expression. In addition, the thalamus, medial and lateral geniculate are positive for D2 receptors. Discrete immunoreactivity for D2 receptors is also observed in the pyramidal layer and hillus of the hippocampus. In the cerebellum, the Purkinje and molecular layers are immunoreactive for D2 receptors.

Immunocomplex stability

The mounted tissue sections were stored in the dark at 4-8°C for six weeks and imaged under the same parameters used for freshly mounted samples. Four regions of interest in coronal sections were identified; the medial globus pallidus, basomedial amygdala, basolateral amygdala and a circular region in the thalamus. The intensity of CB1 receptor immunoreactivity was determined in these regions in freshly mounted tissue, and after six weeks of storage. Prolonged storage resulted in approximately a 20% decrease in signal intensity for all regions except the thalamus which has near basal staining intensity (Table 1).

Discussion

In this study, we have used the LI-COR Odyssey Classic to concurrently determine CB1 and D2 receptor expression in mouse brain. Global imaging of the entire brain slice permitted rapid and sensitive identification of receptor expression. It also allowed for discrimination of the laminar expression pattern of CB1 receptors in the cortex and both CB1 and D2 receptors in the hippocampus and cerebellum. This approach required less primary antibody, shorter incubation times and provided an extended window for analysis relative to standard immunohistochemical staining approaches used in our laboratory.

The distribution of CB1 receptors and D2 receptors in the rodent brain has been characterized previously.[19-24] Identification of the receptor protein with the LI-COR Odyssey described here recapitulates the expected distribution.

The CB1 receptor protein is expressed at high levels in the amygdala and basal ganglia including the globus pallidus. Expression of CB1 receptors in these regions which are known to regulate mood and motivation are likely responsible for cannabinoid modulation of affect and perception.[25,26] Although it is not possible to determine specific cellular localization of the CB1 receptor protein, the intense laminar staining for CB1 receptor protein in the hippocampus is consistent with CB1 receptors present on axons surrounding hippocampal pyramidal neurons.[22,23] These CB1 receptors are believed to be responsible for cannabinoid inhibition of memory consolidation.[27,28] Detection of D2 receptors with a polyclonal antibody reveals a widespread distribution throughout the mouse brain. Piriform cortex and thalamus express discernible levels of D2 receptors. A high level of D2 receptors are found in the hippocampus and especially in the hillus. In humans, decreases in D2 receptor levels in the hippocampus are associated with the memory loss characteristic of Alzheimer's disease.[29] The caudate putamen together

Table 1

Region	Integrated Intensity(Initial) counts mm^2	Integrated Intensity (6 week) counts mm^2	Percent Decrease
Basomedial amygdala	187.8	147.2	21.6
Basolateral amygdala	249.8	206.9	17.2
Medial globus pallidus	155.9	121.2	22.2
Thalamus	29.3	27.4	6.3

with the Purkinje and molecular layers of the cerebellum have marked expression of D2 receptors underlying the regulation of movement by this receptor.[8] While loss of D2 receptor in the cerebellum occurs as a function of aging,[30,31] receptors in the basal ganglia are specifically lost in Huntington's disease.[2] Additionally, a factor contributing to Alzheimer's dementia is the loss of D2 receptors in the striatum.[32]

The immunostaining conditions used for imaging on the Odyssey can be compared to a similar study using the same goat anti-CB1 antibody. Purification of the antibodies in our laboratory allows for similar lot to lot consistency in working antibody dilutions. Compared to our standard fluorescent immunostaining methods using FITC or Texas Red® conjugated secondary antibodies,[33] a more dilute primary antibody solution could be used (on the order of 5-8 fold more dilute). Signal amplification, such as an avidin-biotin complex, was not required. Impressively, imaging of the tissue sections was performed through the microscope slide on which the tissue was mounted, but not permeated with mounting medium. Tissue sections prepared in this manner could be stored for over a month with only minor decreases in signal intensity. Permeating the tissue sections in Gel/Mount™ (Biomedia Corp., Foster City, CA) or Vectashield® (Vector Laboratories Inc., Burlingame, CA) increased the overall fluorescent intensity but did not change the relative signal ratio among the regions of interest. The additional signal detection with mounting medium may be useful for immunostaining of rare proteins, however it was unnecessary for the parameters described here.

Fluorescent immunodetection of receptor proteins is a valuable tool for understanding physiology and disease processes. In this study, we have characterized expression of two GPCR's, the CB1 and D2 receptors concurrently in mouse brain slices. Expression of these receptors is known to decrease as a function of Huntington's disease progression.

Detection of the immunocomplex with the LI-COR Odyssey provided a rapid, sensitive platform for imaging of whole mouse brain slices. Few changes were required to optimize our standard fluorescence immunostaining protocol for use on the Odyssey. Indeed, the most notable change in protocol was a decrease in the amount of antibody required to obtain a sufficient signal.

As noted above, changes in GPCR levels have been detected in a number of neurological disorders. Alzheimer's disease, Huntington's chorea, schizophrenia, and Parkinson's disease all have identified changes in a specific population of GPCR's. Undoubtedly, additional correlations between changes in receptor density and disease progression will be found. The high throughput imaging opportunities provided by the LI-COR Odyssey provides a versatile platform for screening diverse receptor populations allowing for more rapid identification of the neurochemical alterations underlying neurological disorders.

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BrightSite™ Small Animal Imaging Agents Brochure

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Products and Technical Notes for Applications

Selecting the Right Secondary Antibody for Your Application

LI-COR® Biosciences offers a variety of IRDye® secondary antibodies for Western blotting, In-Cell Western™ Assays, immunohistochemistry, and many other applications. IRDye secondary antibodies are highly cross-adsorbed, making them suitable for two-color detection. They have been optimized for use with the Odyssey® family of imaging systems and can be used on instruments with similar excitation and emission filters.

IRDye® 800CW Antibodies

IRDye 800CW secondary antibodies are the antibodies of choice for a wide variety of applications in the 800 nm channel. IRDye 800CW secondary antibodies can be used for two-color detection when multiplexed with IRDye 680RD or IRDye 680LT secondary antibodies.

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- Microscopy
- 2D Gel Detection
- Small Animal Imaging
- Virus Titration Assay
- FRET-based Assays

IRDye® 680RD Secondary Antibodies

IRDye 680RD secondary antibodies are the antibodies of choice for In-Cell Western Assay and Western blot applications in the 700 nm channel. These antibodies can be used for two-color detection when multiplexed with IRDye 800CW secondary antibodies. These antibodies are our most universal use 700 nm channel antibodies.

Highly Recommended for:

- Western Blot
- In-Cell Western Assay
- On-Cell Western Assay
- Protein Array
- Immunohistochemistry
- Microscopy
- 2D Gel Detection
- Small Animal Imaging

IRDye® 680LT Antibodies

IRDye 680LT secondary antibodies provide the brightest signal for Western blot detection in the 700 nm channel and are comparable to Alexa Fluor 680 secondary antibodies. They are an excellent choice for low abundance targets and can be used for two-color detection when multiplexed with IRDye 800CW secondary antibodies.

Recommended for:

- Western Blot
- Protein Array
- Immunohistochemistry
- Microscopy
- 2D Gel Detection

Not Recommended for:

- In-Cell Western Assay
- On-Cell Western Assay
- Small Animal Imaging

IRDye® Secondary Antibodies

All IRDye® secondary antibodies are highly cross-adsorbed. Provided as IRDye conjugated purified immunoglobulin, lyophilized or liquid form in phosphate buffered saline, pH 7.4. Contains 10 mg/mL BSA (IgG and protease free) as a stabilizer and 0.01% sodium azide as a preservative. All IRDye secondary antibodies are stored at 4°C.

IRDye 800CW Secondary Antibodies

827-08364	**IRDye 800CW Goat anti-Mouse IgG (H + L), 25 µL, liquid** NEW!	**$59.00**
827-08365	**IRDye 800CW Goat anti-Rabbit IgG (H + L), 25 µL, liquid** NEW!	**$59.00**
926-32210	**IRDye 800CW Goat anti-Mouse IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32350	**IRDye 800CW Goat anti-Mouse IgG_1-Specific, 0.5 mg, lyophilized** NEW!	**$260.00**
926-32351	**IRDye 800CW Goat anti-Mouse IgG_{2a}-Specific, 0.5 mg, lyophilized** NEW!	**$260.00**
926-32352	**IRDye 800CW Goat anti-Mouse IgG_{2b}-Specific, 0.5 mg, lyophilized** NEW!	**$260.00**
926-32211	**IRDye 800CW Goat anti-Rabbit IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32219	**IRDye 800CW Goat anti-Rat IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32232	**IRDye 800CW Goat anti-Human IgG (H + L), 0.5 mg, lyophilized** NEW!	**$169.00**
926-32212	**IRDye 800CW Donkey anti-Mouse IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32213	**IRDye 800CW Donkey anti-Rabbit IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32214	**IRDye 800CW Donkey anti-Goat IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32218	**IRDye 800CW Donkey anti-Chicken IgG (H + L), 0.5 mg, lyophilized**	**$169.00**
926-32411	**IRDye 800CW Donkey anti-Guinea Pig IgG (H + L), 0.5 mg, lyophilized**	**$169.00**

IRDye® 680RD Secondary Antibodies NEW!

926-68170	IRDye 680RD Goat anti-Mouse IgG (H + L), 25 µL, liquid	$59.00
926-68171	IRDye 680RD Goat anti-Rabbit IgG (H + L), 25 µL, liquid	$59.00
926-68070	IRDye 680RD Goat anti-Mouse IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68071	IRDye 680RD Goat anti-Rabbit IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68076	IRDye 680RD Goat anti-Rat IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68078	IRDye 680RD Goat anti-Human IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68072	IRDye 680RD Donkey anti-Mouse IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68073	IRDye 680RD Donkey anti-Rabbit IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68074	IRDye 680RD Donkey anti-Goat IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68075	IRDye 680RD Donkey anti-Chicken IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68077	IRDye 680RD Donkey anti-Guinea Pig IgG (H + L), 0.5 mg, lyophilized	$169.00

IRDye® 680LT Secondary Antibodies

827-11080	IRDye 680LT Goat anti-Mouse IgG (H + L), 25 µL, liquid NEW!	$59.00
827-11081	IRDye 680LT Goat anti-Rabbit IgG (H + L), 25 µL, liquid NEW!	$59.00
926-68020	IRDye 680LT Goat anti-Mouse IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68050	IRDye 680LT Goat anti-Mouse IgG_1-Specific, 0.5 mg, lyophilized NEW!	$260.00
926-68051	IRDye 680LT Goat anti-Mouse IgG_{2a}-Specific, 0.5 mg, lyophilized NEW!	$260.00
926-68052	IRDye 680LT Goat anti-Mouse IgG_{2b}-Specific, 0.5 mg, lyophilized NEW!	$260.00
926-68021	IRDye 680LT Goat anti-Rabbit IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68029	IRDye 680LT Goat anti-Rat IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68032	IRDye 680LT Goat anti-Human IgG (H + L), 0.5 mg, lyophilized NEW!	$169.00
926-68022	IRDye 680LT Donkey anti-Mouse IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68023	IRDye 680LT Donkey anti-Rabbit IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68024	IRDye 680LT Donkey anti-Goat IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68028	IRDye 680LT Donkey anti-Chicken IgG (H + L), 0.5 mg, lyophilized	$169.00
926-68030	IRDye 680LT Donkey anti-Guinea Pig IgG (H + L), 0.5 mg, lyophilized	$169.00

LI-COR® IRDye® secondary antibodies are optimized for two-color Western blot detection. They are highly cross-adsorbed with a dye-to-protein ratio maximized for optimal signal-to-noise ratio in both Western blot and In-Cell Western™ Assay detection. Figures 1 and 2 show a comparison of LI-COR highly cross-adsorbed IRDye Goat anti-Mouse secondary antibody to a non-cross-adsorbed Goat anti-Mouse secondary antibody from another source.

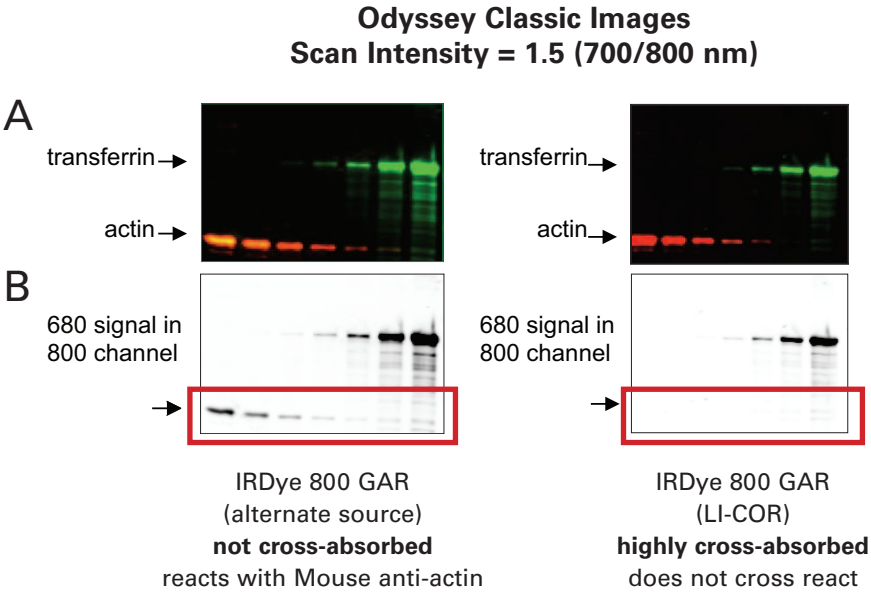

Figure 1. Example of a secondary antibody, not cross-adsorbed, cross-reacting with the actin primary antibody in a two-color Western blot. The highly cross-adsorbed secondary antibody from LI-COR does not show cross-reaction with the actin primary (Panel B). GAR = Goat anti-Rabbit

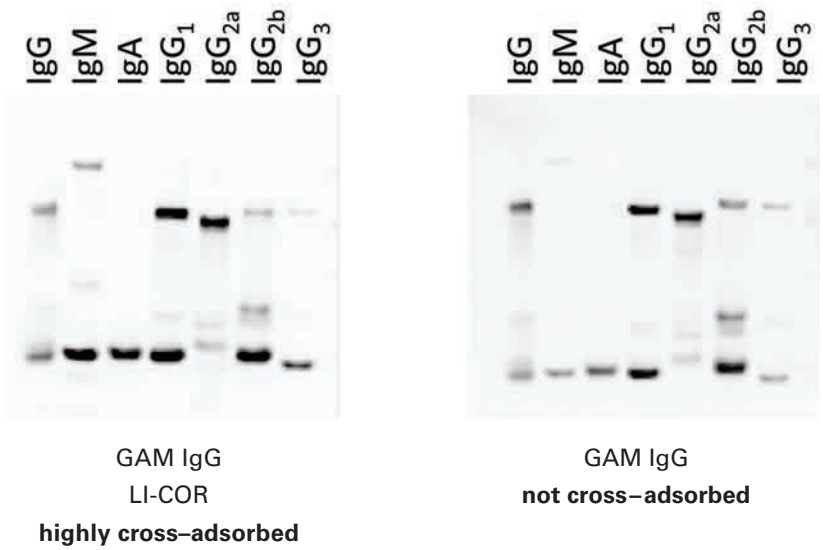

Figure 2. Mouse IgG subclass detection comparing LI-COR® IRDye® Goat anti-Mouse antibody to a similar antibody that was not cross-adsorbed. GAM = Goat anti-Mouse

IRDye® Streptavidin Conjugates

926-32230 **IRDye® 800CW Streptavidin, 0.5 mg** **$135.00**

IRDye 800CW Streptavidin can be used as a secondary detection reagent for protein arrays, microscopy, In-Gel Western Assays, and Western blotting.

Storage: 4°C

926-68079 **IRDye 680RD Streptavidin, 0.5 mg** NEW! **$135.00**

IRDye 680RD Streptavidin can be used as a secondary detection reagent for protein arrays, microscopy, In-Gel Western Assays, and Western blotting.

Storage: 4°C

926-68031 **IRDye 680LT Streptavidin, 0.5 mg** **$135.00**

IRDye 680LT Streptavidin can be used as a secondary detection reagent for Western blotting and other applications using biotinylated conjugates.

Storage: 4°C

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TECHNICAL NOTE:
Western Blot and In-Cell Western™ Assay Detection Using IRDye® Subclass Specific Antibodies

Introduction

This Technical Note is a guide to using IRDye® Subclass Specific antibodies for Western blotting and In-Cell Western™ Assay (ICW) applications. For more detailed descriptions of Western blotting and/or ICW techniques, refer to Western Blot Analysis and In-Cell Western Kits I and II on the LI-COR® Biosciences website (*www.licor.com*).

IRDye Goat anti-Mouse IgG_1, Goat anti-Mouse IgG_{2a} and Goat anti-Mouse IgG_{2b} allow for two-color detection using primary antibodies derived from the same species (mouse). IRDye Subclass Specific antibodies react with the heavy (gamma) chain only of the primary antibody. In mice, there are five unique subclasses of IgG: IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgG_3. Each subclass is based on small differences in amino acid sequences in the constant region of the heavy chains, so antibodies directed against a particular subclass will not recognize antibodies directed against other subclasses. For example, IRDye goat anti-Mouse IgG_1 recognizes mouse gamma 1; it will not recognize mouse gamma 2a, 2b, 2c or gamma 3. All other LI-COR IRDye secondary antibodies are raised against whole IgG (H + L) and react with the heavy (gamma) and light (kappa or lambda) chains of the primary antibody. Figure 1 demonstrates the differences in detection between the IRDye antibodies.

Antibody subclasses may also be designated by their light chains. There are two types of light chains, kappa (κ) or lambda (λ). In mice, 95% of light chains are kappa and 5% are lambda. These subclasses still contain the heavy (gamma) portion of the antibody, so IRDye Subclass Specific antibodies still recognize them. If the subclass of the primary antibody is unknown, LI-COR® whole IgG secondary antibodies may be used since they recognize most mouse IgG subclasses.

Suggested Materials

This section is intended as a guideline; other materials may be substituted, if desired.

- Proteins transferred to a nitrocellulose or PVDF membrane (for Western blot only)
- Cells that have been fixed and permeabilized in a 96 well plate (for ICW only)
- Odyssey® Blocking Buffer
- 10X PBS
- 20% Tween® 20
- SDS (if using PVDF membrane)

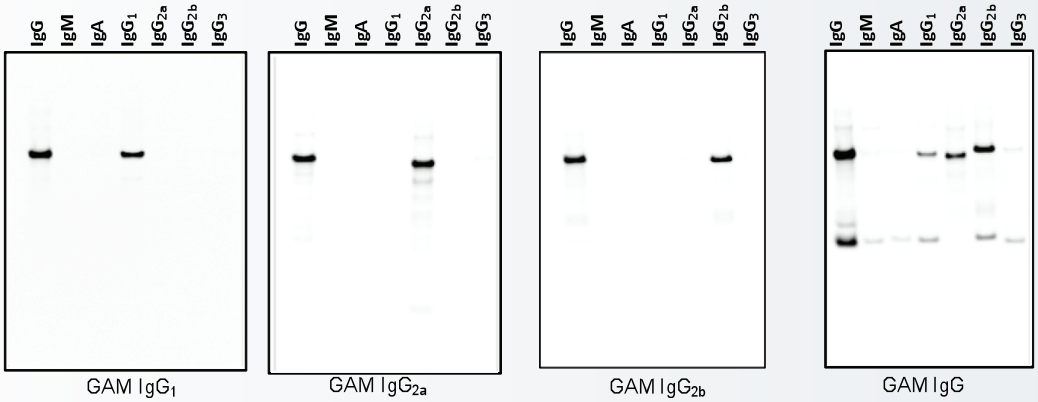


Figure 1. Western blot detection of various purified IgG subclasses. Each lane was loaded with 50 ng of antibody. Blots were detected with IRDye Subclass Specific antibodies or IRDye whole IgG.

Suggested mouse primary antibodies for normalization:

- Beta-Actin Mouse mAb IgG_{2b} (LI-COR P/N 926-42212)
- Alpha-Tubulin Mouse mAb IgG_1 (LI-COR P/N 926-42213)

One or two of the following IRDye® secondary antibodies:

Description	LI-COR P/N
IRDye 800CW Goat anti-Mouse IgG_1 Specific	926-32350
IRDye 800CW Goat anti-Mouse IgG_{2a} Specific	926-32351
IRDye 800CW Goat anti-Mouse IgG_{2b} Specific	926-32352
IRDye 680LT Goat anti-Mouse IgG_1 Specific	926-68050
IRDye 680LT Goat anti-Mouse IgG_{2a} Specific	926-68051
IRDye 680LT Goat anti-Mouse IgG_{2b} Specific	926-68052

Western Blot Detection

IRDye Subclass Specific antibodies are easily incorporated into the detection step of any Western blot protocol. The sample protocol provided below, optimized for LI-COR reagents, is recommended. After protein transfer to the membrane is complete, perform the following steps for one or two-color detection:

1. Wet the membrane in PBS for several minutes. If using a PVDF membrane that has been allowed to dry, pre-wet briefly in 100% methanol and rinse with ultrapure water before incubating in PBS.
2. Block the membrane in Odyssey® Blocking Buffer for 1 hour. Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 mL/cm² is suggested).
3. Dilute primary antibody in Odyssey Blocking Buffer. Optimum dilution depends on the antibody and should be determined empirically. A suggested starting range can usually be found in the product information from the vendor. To lower the background, add Tween® 20 to the diluted antibody at a final concentration of 0.1-0.2% prior to incubation.

   Note: If performing two-color detection, dilute primary antibodies together in the same buffer.
4. Incubate blot in primary antibody solution for a minimum of 60 minutes at room temperature, with gentle shaking. Optimum incubation times vary for different primary antibodies. Use enough antibody solution to completely cover the membrane.
5. Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1%Tween® 20 with gentle shaking, using a generous amount of buffer.
6. Dilute the IRDye Subclass Specific antibody in Odyssey Blocking Buffer. Avoid prolonged exposure of the antibody vial to light. Recommended dilution can be found in the pack insert for the IRDye conjugate. Add the same amount of Tween 20 to the diluted secondary antibody as was added to the primary antibody.

   Note: If performing two-color detection, dilute secondary antibodies simultaneously in the same buffer. Adding SDS to the diluted secondary antibody at a final concentration of 0.01%-0.02% will substantially reduce membrane background when using PVDF membrane.
7. Incubate blot in secondary antibody solution for 30-60 minutes at room temperature with gentle shaking. Protect from light during incubation.
8. Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween 20, with gentle shaking. Protect from light.
9. Rinse membrane with PBS (no detergent) to remove residual Tween 20. The membrane is now ready to image.

Two-Color Western Blot Considerations

Two different antigens can be detected simultaneously on the same blot using IRDye Subclass Specific or IRDye whole IgG antibodies that are visualized in different fluorescence channels (700 and 800 nm). Two-color detection requires careful selection of primary and secondary antibodies. The following guidelines will help with the design of two-color experiments:

- If the two primary antibodies are monoclonals (mouse) and are IgG_1, IgG_{2a}, or IgG_{2b}, IRDye Subclass Specific secondary antibodies must be used. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG_1 primary antibodies).
- If the two primary antibodies are derived from different host species (for example, primary antibodies from mouse and chicken), IRDye whole IgG secondary antibodies derived from the same host and labeled with different IRDye fluorophores must be used (for example, IRDye 800CW Donkey anti-Mouse and IRDye 680LT Donkey anti-Chicken).
- Before combining primary antibodies in a two-color experiment, always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible non-specific background bands.

Figures 2 (A-C) and 3 (A-C) demonstrate two-color Western blot detection using (A) IRDye Subclass Specific antibodies and (B) IRDye whole IgG antibodies, respectively. IRDye Subclass Specific secondary antibodies should NOT be used in

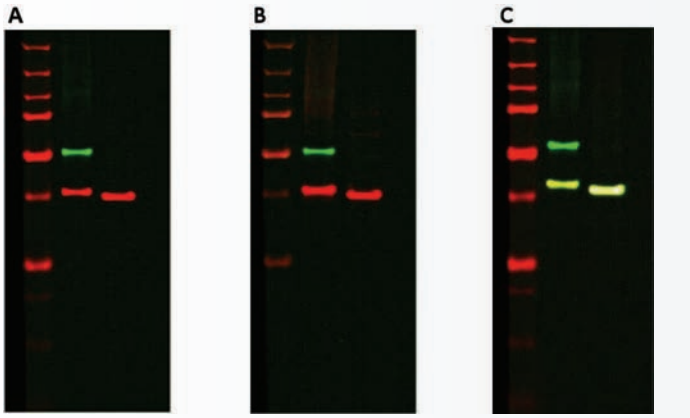


Figure 2. Western blot analysis of PTEN expression in mouse PTEN transfected 293T whole cell lysate (Lane 2) and non-transfected 293T lysate (Lane 3). Both lysates were loaded with 2 µg total protein per lane. Odyssey® Protein Molecular Weight Marker is loaded in Lane 1 (P/N 928-40000).

A. Blot was probed with mouse anti-PTEN (IgG_{2b}) and mouse anti-GAPDH (IgG_1) for normalization.The blot was detected with IRDye 800CW Goat anti-Mouse IgG_{2b} (GAM; P/N 926-32352) and IRDye 680LT GAM IgG_1; P/N 926-68050).

B. Blot was probed with mouse anti-PTEN (IgG_{2b}) and chicken anti-GAPDH for normalization.The blot was detected with IRDye 800CW Donkey anti-Mouse (DAM; P/N 926-32212) and IRDye 680LT Donkey anti-Chicken (DAC; P/N 926-68028).

C. Blot was probed with mouse anti-PTEN (IgG_{2b}) and mouse anti-GAPDH (IgG_1) for normalization. The blot was detected with IRDye 800CW GAM (P/N 926-32210) and IRDye 680LT GAM IgG_1 (P/N 926-68050).

Note: Apparent MW differences in GAPDH between lanes 2 and 3 could be due to post-translational differences (e.g., glycosylation, nitrosylation, glutathionylation) between cell lines. Colell, A., et.al., *Cell Death and Differentiation* (2009) 16, 1573-1581.

combination with IRDye whole anti-Mouse IgG secondary antibodies for two-color detection. IRDye whole anti-Mouse IgG secondary antibodies and IRDye Subclass Specific secondary antibodies both recognize the gamma chain of the primary antibody, causing detection in both channels (C). IRDye Subclass Specific antibodies can be used in combination with IRDye whole goat anti-Rabbit secondary antibodies.

Two-color Western blot detection can be achieved by multiplexing LI-COR® mouse primary antibodies and IRDye Subclass Specific antibodies. Figure 4 demonstrates two-color detection utilizing the LI-COR mouse primaries and IRDye Subclass Specific secondaries.

ICW Detection and Considerations

In-Cell Western™ assays commonly use primary and secondary antibodies for normalization in the 700 channel. For example, if phospho-ERK is the target of interest, an antibody against total ERK (or against a housekeeping protein) can be used to normalize for variations in cell number. IRDye Subclass Specific antibodies can be incorporated into the detection step of any ICW protocol. A recommended protocol is provided below. After cells have been fixed and permeabilized, perform the following steps:

1. Using a multi-channel pipettor, block cells by adding 150 µL of Odyssey® Blocking Buffer to each well. Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.
2. Allow blocking for 1.5 hours at room temperature with gentle shaking on a plate shaker. Dilute desired primary antibodies in Odyssey Blocking Buffer or other appropriate blocker. As a general guideline, 1:50 to 1:200 dilutions are recommended, depending on the primary antibody. If the antibody supplier provides dilution guidelines for immunofluorescent staining, start with that recommended range.
    a. It is important to include control wells that **DO NOT** contain primary antibody. These wells will be treated with secondary antibody only, and should be used to correct for background staining in the data analysis.
    b. Remove blocking buffer from step 1.
    c. Add 50 µL of Odyssey Blocking Buffer to the control wells and 50 µL of the desired diluted primary antibodies in Odyssey Blocking Buffer to the rest of the wells.
4. Incubate with primary antibody solution for 2 hours at room temperature with gentle shaking, or overnight at 4°C with no shaking.
5. Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer:
    a. Prepare Tween Washing Solution by adding 5 mL of 20% Tween 20 to 995 mL of 1X PBS.
    b. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.
    c. Allow wash to shake gently on a plate shaker for 5 minutes.
    d. Repeat washing steps 4 more times.

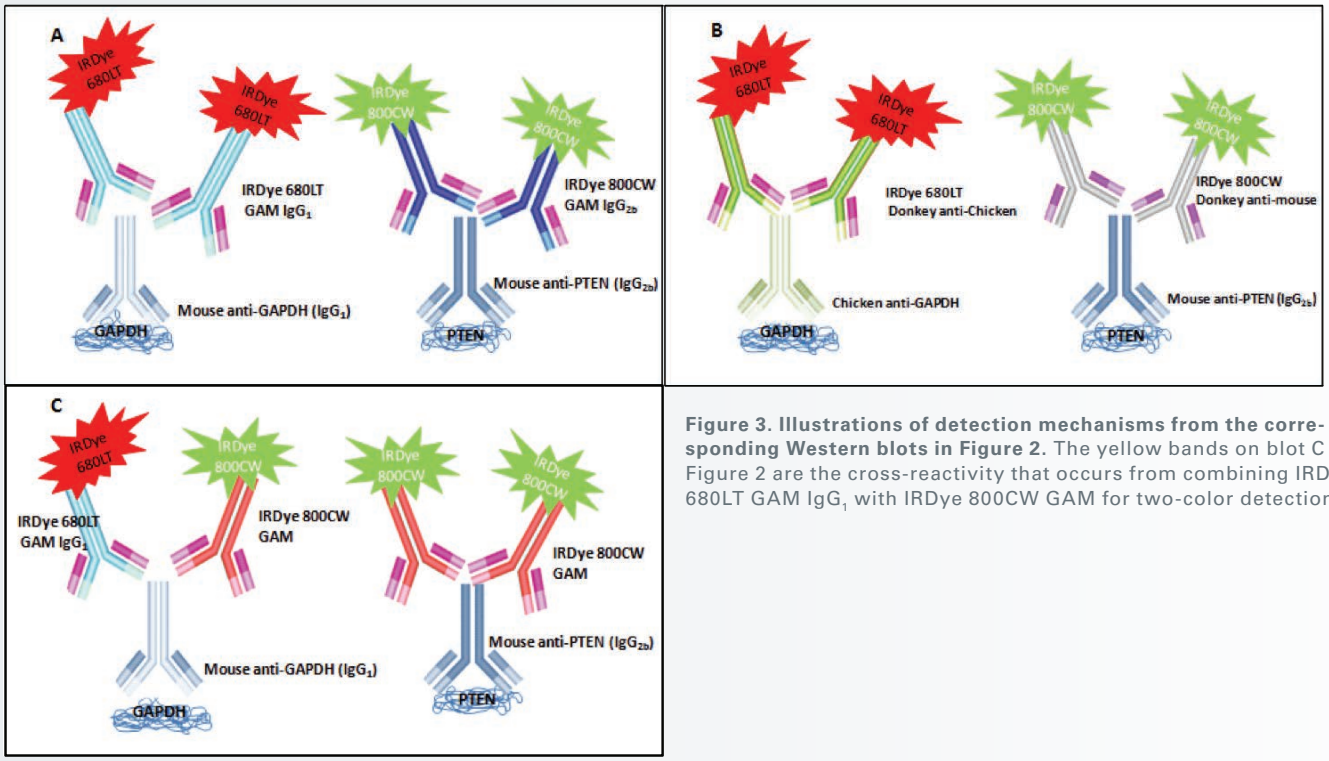


Figure 3. Illustrations of detection mechanisms from the corresponding Western blots in Figure 2. The yellow bands on blot C in Figure 2 are the cross-reactivity that occurs from combining IRDye 680LT GAM IgG_1 with IRDye 800CW GAM for two-color detection.

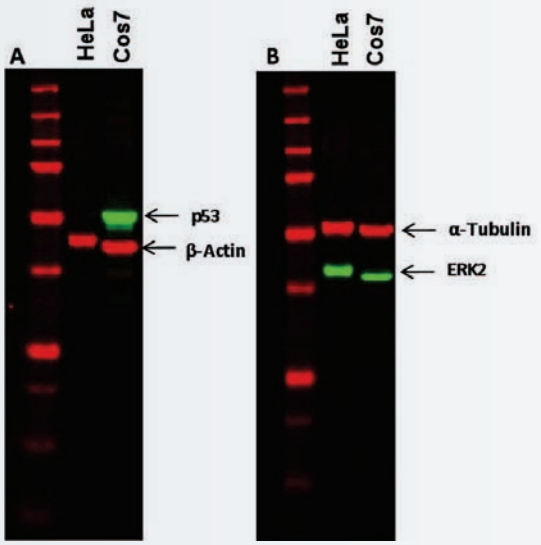


Figure 4. Two-color Western blot detection using LI-COR mouse primary antibodies. Two different blots were prepared using HeLa and COS7 whole cell lysates. Both lysates were loaded at 5 µg total protein per lane.

A. The blot was probed with Beta-Actin Mouse mAb IgG_{2b} (LI-COR P/N 926-42212) and p53 mouse mAb IgG_{2a}. The blot was detected with IRDye 800CW GAM IgG_{2a} and IRDye 680LT GAM IgG_{2b}.

B. The blot was probed with Alpha-Tubulin Mouse mAb IgG_1 (LI-COR P/N 926-42213) and ERK2 mouse mAb IgG_{2b}. The blot was detected with IRDye 680LT GAM IgG_1 and IRDye 800CW GAM IgG_{2b}.

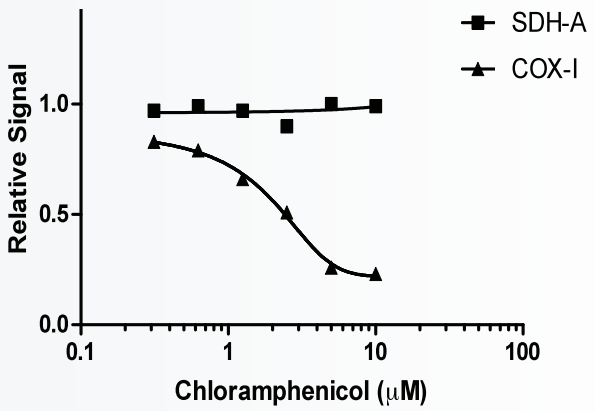


Figure 5. Inhibition of mitochondrial biogenesis by Chloramphenicol. The IC_{50} of a drug's effect on mitochondrial protein translation was determined using the MitoBiogenesis In-Cell ELISA Kit (IR). In this example, HepG2 cells were seeded at 3000 cells/well and allowed to grow for 3 cell doublings in a drug dilution series. Cells were fixed in a 96-well plate and targets of interest (COX-I and SDH-A) were detected with highly specific, well-characterized monoclonal antibodies supplied in the kit. The plate was scanned using an Odyssey imaging system. Average intensity values for each set of replicates were determined for COX-I and SDH-A and background subtracted (no primary antibody). Relative signal values were determined by normalizing the COX-I average intensity values to the SDH-A average intensity values. Chloramphenicol inhibits mtDNA-encoded COX-I protein synthesis relative to nuclear DNA-encoded SDH-A protein synthesis by 50% at 1.8 µM.

5. Dilute the IRDye Subclass Specific antibodies in Odyssey® Blocking Buffer or other appropriate blocker. The recommended dilution range is 1:200 to 1:1,200. The optimal dilution for your assay should be determined empirically. To lower background, add Tween® 20 at a final concentration of 0.2% to the diluted antibody. Secondary antibody staining is carried out simultaneously. Avoid prolonged exposure of the antibody vials to light.
6. Add 50 µL of secondary antibody solution into all wells. Incubate for 1 hour at room temperature, with gentle shaking. Protect plate from light during incubation.
7. Wash the plate five times with 1X PBS + 0.1%Tween 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer:
    a. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.
    b. Allow wash to shake gently on a plate shaker for 5 minutes.
    c. Repeat washing steps 4 more times. Protect plate from light during washing. The plate is now ready to image.

The same considerations for two-color Western blot detection apply to two-color In-Cell Western™ detection with the following addition:

- Choose primary antibodies that have been recommended for other immunofluorescence techniques such as IF-IC and IHC.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imaging system. To achieve the most consistent results, use the same blocking buffer for validation experiments and In-Cell Western assays. If significant non-specific binding is detected on a Western blot, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

Figures 5 and 6 demonstrate In-Cell Western Assay data generated using MitoSciences' MitoBiogenesis™ In-Cell ELISA Kit (IR). The kit utilizes IRDye Subclass Specific antibodies for detection.

Figure 7 compares IRDye whole IgG vs. IRDye Subclass Specific antibody detection by ICW. Extracellular-signal related kinase (ERK) phosphorylation was measured following the LI-COR protocol entitled, *In-Cell Western Assay For Assessing Response of A431 Cells to Stimulation with Epidermal Growth Factor.* This document can be found on the LI-COR website (*www.licor.com/bio*). All primary antibodies were qualified by Western blot prior to ICW (data not shown).

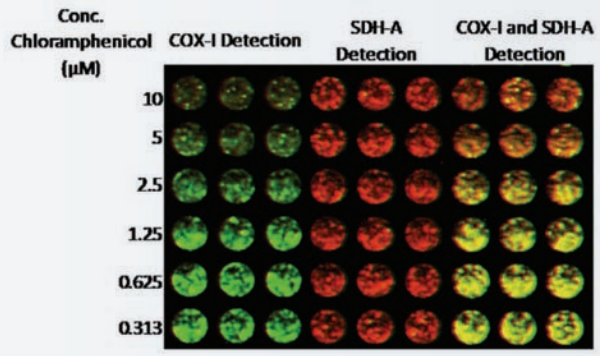


Figure 6. Odyssey® image of In-Cell ELISA (In-Cell Western™). COX-I detection is shown in green (800 channel) and SDH-A detection is shown in red (700 channel). COX-1 protein synthesis decreases with increasing amounts of Chloramphenicol.

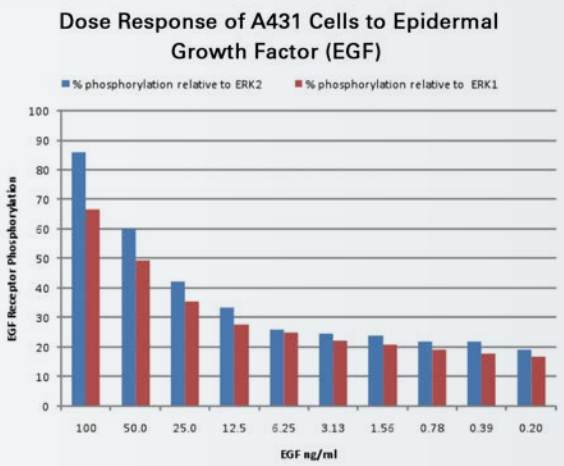


Figure 7. Dose response data. ERK phosphorylation is graphed relative to ERK2 (blue) or ERK1 (red). The ERK2 data was generated using mouse anti-pERK (IgG_{2a}) and mouse anti-ERK2 (IgG_{2b}) followed by detection with IRDye 800CW GAM IgG_{2a} (LI-COR P/N 926-32351) and IRDye 680LT GAM IgG_{2b} (LI-COR P/N 926-68052). ERK1 data was generated using mouse anti-pERK (IgG_{2a}) and rabbit anti-ERK1 followed by detection with IRDye 800CW GAM (LI-COR P/N 926-32210) and IRDye 680LT Goat anti-Rabbit (GAR).

Primary Antibodies for Normalization

926-42212 β-Actin Mouse mAb, 100 µL $239.00

This β-actin primary antibody can be used as a normalization antibody when performing two-color detection for Western blotting and In-Cell Western™ Assay applications. Detection of this primary antibody can be achieved with IRDye® Goat anti-Mouse, IRDye Donkey anti-Mouse, or IRDye Goat anti-Mouse IgG_{2b}–Specific secondary antibodies. The target molecular weight is 45 kDa.

926-42213 α-Tubulin Mouse mAb, 100 µL $239.00

This α-tubulin primary antibody can be used as a normalization antibody when performing two-color detection for Western blotting and In-Cell Western Assay applications. Detection of this primary antibody can be achieved with IRDye Goat anti-Mouse, IRDye Donkey anti-Mouse, or IRDye Goat anti-Mouse IgG_1–Specific secondary antibodies. The target molecular weight is 52 kDa.

926-42210 β-Actin Rabbit mAb, 100 µL $239.00

This β-actin primary antibody can be used as a normalization antibody when performing two-color detection for Western blotting and In-Cell Western Assay applications. Detection of this primary can be achieved with IRDye Goat anti-Rabbit, or IRDye Donkey anti-Rabbit secondary antibodies. The target molecular weight is 45 kDa.

926-42211 β-Tubulin Rabbit Antibody Polyclonal, 100 µL $239.00

This β-Tubulin primary antibody can be used as a normalization antibody when performing two-color detection for Western blotting and In-Cell Western Assay applications. Detection of this primary can be achieved with IRDye Goat anti-Rabbit or IRDye Donkey anti-Rabbit secondary antibodies. The target molecular weight is 55 kDa.

Storage for all antibodies: -20°C

Chemi-IR™ (Anti-HRP) Detection Kits

926-32234 Chemi-IR Detection Kit, 20 blot, 0.1 mg $89.00

Kit contains:

- Chemi-IR Conjugate (IRDye 800CW Rabbit anti-HRP, 0.1 mg, lyophilized)
- Chemi-IR Diluent (100 mL)
- Western Incubation Box, Large (11.5 x 8.8 x 2.8 cm)

Storage: 4°C

926-32236 Chemi-IR Detection Kit, 100 blot, 0.5 mg $249.00

Kit contains:

- Chemi-IR Conjugate (IRDye 800CW Rabbit anti-HRP, 0.5 mg, lyophilized)
- Chemi-IR Diluent (500 mL)
- Western Incubation Box, Large (11.5 x 8.8 x 2.8 cm)

Storage: 4°C

Odyssey® Western Blotting Kits

Quick Western Kit – IRDye® 680RD NEW!

The Quick Western Kit – IRDye 680RD provides a universal antibody detection reagent that can be combined with the primary antibody incubation step, eliminating the need for a secondary antibody. The overall time to complete a Western blot is reduced while providing the advantages of near infrared detection. The kit can be used to detect primary antibodies from a variety of hosts and has been shown to recognize primary antibodies to recombinant tagged proteins (e.g. 6X His, Myc, FLAG, etc.).

The kit also serves as a detection solution for post-immunoprecipitation samples by Western blot because it does not bind to denatured mouse monoclonal or rabbit monoclonal antibodies. The key benefit is the ability to use the same antibody for immunoprecipitation and postimmunoprecipitation detection by Western blot.

926-68100 **Quick Western Kit - IRDye® 680RD** **$230.00**

Kit contains:

- IRDye® 680RD Detection Reagent (1 vial)
- Odyssey Blocking Buffer (2 x 125 mL)
- Quick Reference Card

Odyssey Western Blotting Kits with PVDF Membrane

The Odyssey Western blotting kits give researchers the opportunity to sample infrared reagents and corresponding membranes and perform quantitative Western blots without committing to a large quantity of each. Additionally, all products in the Odyssey Western blot kits are rigorously tested to ensure that every product in each kit performs to the highest standards.

Each kit is optimized for 10 (7 cm x 8.5 cm) Western blots.

926-31062 **Odyssey Western Blotting Kit I LT** **$297.00**

Kit contains:

- IRDye® 800CW Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680LT Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Immobilon®-FL PVDF Membranes (0.45 µm, 10 cm x 10 cm)

926-31064 **Odyssey Western Blotting Kit II LT** **$297.00**

Kit contains:

- IRDye 800CW Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680LT Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Immobilon-FL PVDF Membranes (0.45 µm, 10 cm x 10 cm)

926-31081 **Odyssey Western Blotting Kit I RD** NEW! **$297.00**

Kit contains:

- IRDye 800CW Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680RD Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Immobilon–FL PVDF Membranes (0.45 µm, 10 cm x 10cm)

Odyssey® Western Blotting Kits with PVDF Membrane (Cont'd)

926-31082 **Odyssey Western Blotting Kit II RD** NEW! **$297.00**

Kit contains:

- IRDye® 800CW Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Immobilon–FL PVDF Membranes (0.45 µm, 10 cm x 10 cm)

Odyssey Western Blotting Kits with Nitrocellulose Membranes

Each kit is optimized for 10 (7 cm x 8.5 cm) Western blots.

926-31066 **Odyssey Western Blotting Kit III LT** **$297.00**

Kit contains:

- IRDye 800CW Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680LT Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Odyssey Nitrocellulose Membranes (0.22 µm, 7 cm x 8.5 cm)

926-31068 **Odyssey Western Blotting Kit IV LT** **$297.00**

Kit contains:

- IRDye 800CW Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680LT Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Odyssey Nitrocellulose Membranes (0.22 µm, 7 cm x 8.5 cm)

926-31083 **Odyssey Western Blotting Kit III RD** NEW! **$297.00**

Kit contains:

- IRDye 800CW Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680RD Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Odyssey Nitrocellulose Membranes (0.22 µm, 7 cm x 8.5 cm)

926-31084 **Odyssey Western Blotting Kit IV RD** NEW! **$297.00**

Kit contains:

- IRDye 800CW Goat anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
- 500 mL Odyssey Blocking Buffer
- 10 Odyssey Nitrocellulose Membranes (0.22 µm, 7 cm x 8.5 cm)

Storage for all kits: 4°C/Room Temperature depending on component

Membranes and Membrane Kits

926-31090 **1 Package of Ten 7 cm x 8.5 cm Odyssey® Nitrocellulose Membranes (0.22 µm) $54.00**

Storage: Room Temperature **Note:** Contains hazardous material. Additional shipping charges apply.

926-31092 **1 Roll of Odyssey Nitrocellulose Membrane (0.22 µm, 30 cm x 3 m) $200.00**

Storage: Room Temperature. **Note:** Contains hazardous material. Additional shipping charges apply.

926-31098 **Blocking Buffer and Membrane Kit..... $400.00**

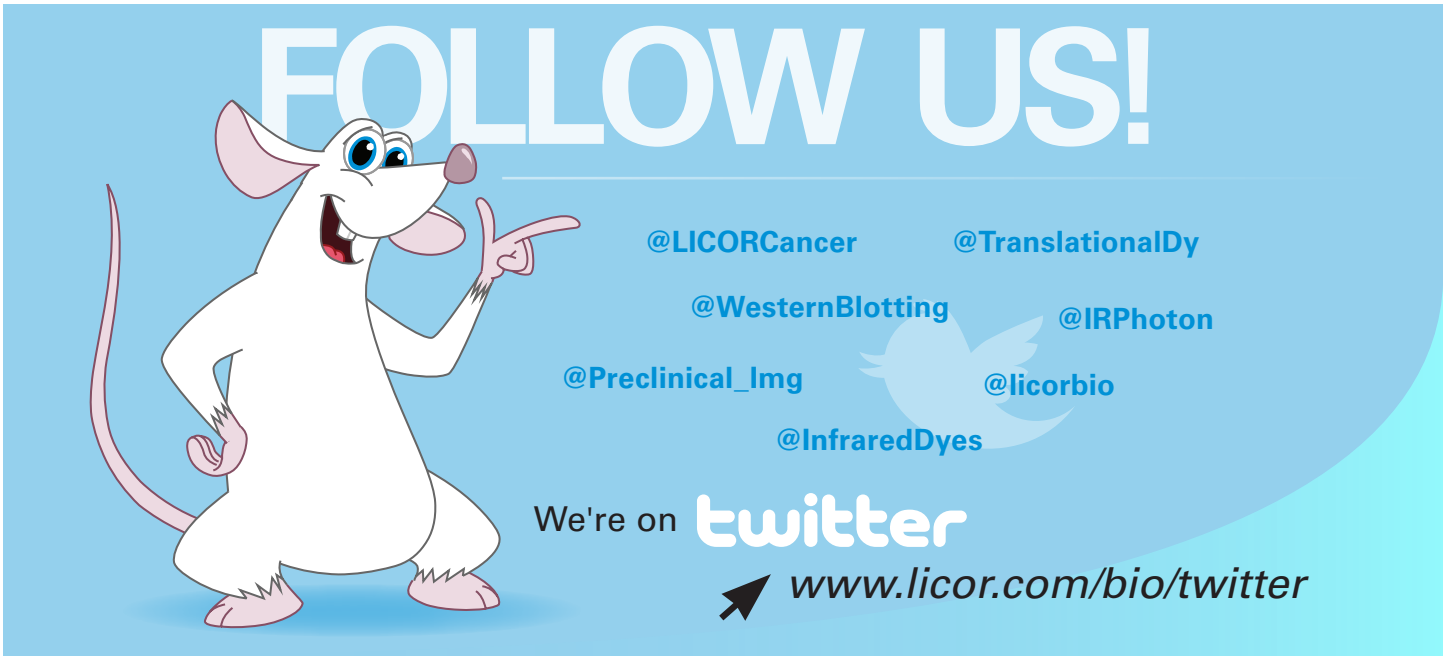
Kit contains:
- 1 bottle (500 mL) of Odyssey Blocking Buffer
- 1 roll of Immobilon®-FL PVDF Membrane (0.45 µm, 26.5 cm x 3.75 m)

Storage: 4°C/Room Temperature depending on component

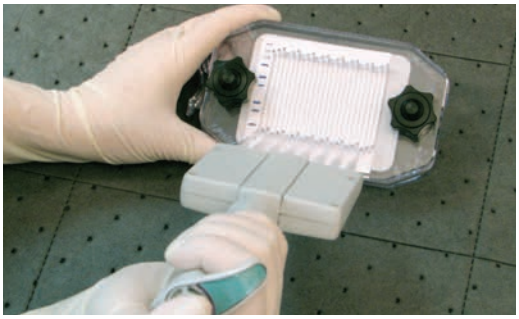
829-31080 **Blocking Buffer and Membrane Kit.....$1,350.00**

Kit contains:
- 10 bottles (500 mL) of Odyssey Blocking Buffer
- 1 roll of Immobilon-FL PVDF Membrane (0.45 µm, 26.5 cm x 3.75 m)

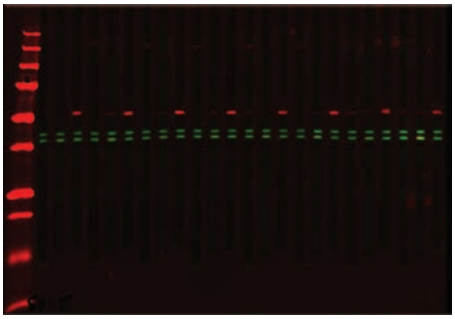
Storage: 4°C/Room Temperature depending on component



MPX™ (Multiplex) Blotting System



MPX Blotting System



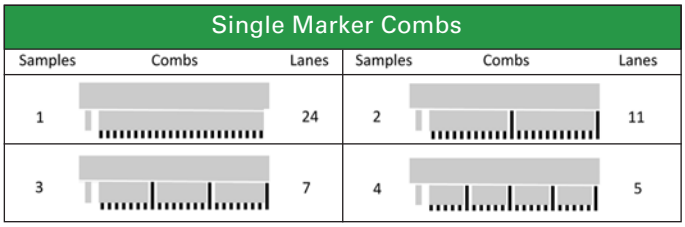
Representative MPX Western blot with one sample and multiple targets

- Maximize efficiency by screening multiple targets on the same blot
- Increase throughput by imaging on any of the Odyssey® Imaging Systems
- Conserve antibody and reduce cost with lower required reagent volumes (up to 160 µL)
- Accommodate most common Western blotting procedures and detection protocols

The MPX™ design creates a simple and convenient Western blotting workflow. The nesting dovetail joint on the base of the apparatus allows for convenient multiple-blot processing by the continuous linkage of units. Channel ports are spaced for use with both standard and multi-channel pipettes. The ports are also beveled and staggered for ease of use. The range of usable channels per sample is relative to comb size — from 24 on a single channel to four on a four-sample blot. Using LI-COR® Biosciences' selection of IRDye® Infrared Dyes on one of the Odyssey imagers provides a maximum of 48 targets on a single membrane.

Compatibility

The MPX Blotting System is ideal for any multiple-target Western blot application and can be adapted to nearly all common Western blotting procedures that utilize a PVDF or nitrocellulose membrane (7 cm x 8.5 cm). The MPX blotting system can be used with standard infrared, chemiluminescent, or colorimetric protocols. Quantitative analysis is possible with utilization of an Odyssey System and reagents optimized for use on any of the Odyssey Imagers.



921-00000 MPX Blotting System $395.00

Includes:

- MPX Unit, Top and Bottom
- Single and Two Lane Combs
- MPX Clamp Nuts (2 packs; Qty 2/pack), P/N 921-00110
- Membrane Cushions (10), P/N 921-00120
- MPX User Guide

921-00110 MPX Clamp Nut (2 ea.) $40.00

921-00120 MPX Membrane Cushion (pack of 10) $25.00

921-00200 Single Lane Comb with Marker Lane $45.00

921-00202 Two Lane Comb with Marker Lane $45.00

921-00204 Three Lane Comb with Marker Lane $45.00

921-00206 Four Lane Comb with Marker Lane $45.00

TECHNICAL NOTE:
One Blot Western Optimization Using the MPX™ Blotting System

Introduction

The independent channels of the LI-COR® MPX™ (Multiplex) Blotter facilitate the ability to optimize blocking buffer, primary antibody dilution, and secondary antibody dilution in a single Western blot. Western blotting procedures that generate a blot of 7.0 cm × 8.5 cm are easily adaptable to the MPX format. The process fits into any laboratory's standard Western blot workflow. Both self-poured and pre-cast gels can be used to generate blots. Run electrophoresis and transfer to standard nitrocellulose or PVDF membrane under standard conditions. Clamp the blot into the MPX Blotter, which creates up to 24 independent channels. The range of usable channels per sample is relative to comb size. For Western blot optimization, a single-well gel is all that is needed. For this application, any detection method can be used, including near-infrared fluorescence and chemiluminescence. The following is a general guideline for use with any of the Odyssey® Imaging Systems.

Suggested Materials

	Reagent/Supply	LI-COR P/N
Sample Preparation	4X Protein Sample Loading Buffer	928-40004
Electrophoresis	Odyssey Protein Molecular Weight Marker	928-40000
Blotting and Transfer	10X Tris Glycine (liquid or powder)	928-40010 or 928-40012
	Odyssey Nitrocellulose (7 cm x 8.5 cm or roll)	926-31090 or 926-31092
MPX Detection	LI-COR Blocking Buffer Sample Pack	927-40050
	Odyssey Blocking Buffer	927-40000 + Multiple P/N's
	Casein Blocking Buffer	927-40300 or 927-40200
	Commercial Milk Blocking Buffer	—
	IRDye Secondary Antibodies	Multiple P/Ns
	10X PBS (liquid or powder)	928-40018 or 928-40020
	MPX Membrane Cushion	921-00120
Imaging	Any of the Odyssey Imaging Systems	See pages 140-142

Table 1. Single sample pre-cast gel options for use with the MPX™ Blotter.

Vendor	Well Designation	Sample #	MW Marker Well	Usable Ports
Invitrogen	2D	1	Yes	19
Bio-Rad	2D/Prep	1	Yes	21
C.B.S. Scientific	1 Well	1	No	23

Gel Electrophoresis and Transfer

Gel Preparation

A wide variety of gel matrices are compatible with the MPX™ Blotter detection system. LI-COR® provides a solution for pouring gels with the NEXT GEL System (Amresco). The gel matrix can be used in your gel casting system with a single-well comb such as Bio-Rad (mini PROTEAN Comb, prep/2-D well, P/N 165-3361 1.0 mm, or P/N 165-3367, 1.5 mm) or from a vendor of the user's choice. Alternatively, pre-cast gels can be used. See Table 1 for a list of pre-cast gels available and the number of usable ports compatible with the MPX Blotter.

Sample Preparation

When using a single-well gel, a larger volume of sample is required. Prepare your protein sample so that the sample volume and concentration is equivalent to running all the lanes on a standard 10-well gel. Example: 5 µg of lysate per lane = 50 µg in a total volume of 100-150 µL, including loading buffer.

The following procedure can be used:

Dilute the sample 1:4 in 4X Protein Sample Loading Buffer (LI-COR P/N 928-40004) with β-Mercaptoethanol. See the pack insert at *biosupport.licor.com* for detailed instructions. Heat the sample at 95°C for 5 minutes.

Molecular Weight Marker

It is important to have a molecular weight marker that is visible to the eye because the marker is the primary tool used to align the blot in the MPX Blotter. Odyssey Protein Molecular Weight Marker (P/N 928-40000) is the recommended marker choice. This marker is easily visualized and is detected in the 700 nm channel of the Odyssey Imager.

Electrophoresis

Important: The maximum length of the separating gel should not exceed 50 mm—the length of the channels on the MPX Blotter.

Transfer

Always use clean forceps when handling membranes. Once electrophoresis is complete, transfer proteins to Odyssey® Nitrocellulose Membrane (LI-COR P/N 926-31092 or 926-31090) using standard transfer procedures. Mark the outside corners of the gel and sample wells with a pencil before separating the transferred gel from the membrane as in Figure 1. The marks help align the membrane once it is placed on the MPX Blotter. Allow the membrane to dry a minimum of one hour before proceeding with detection. **Important:** Ink from most pens will fluoresce on Odyssey Imagers.

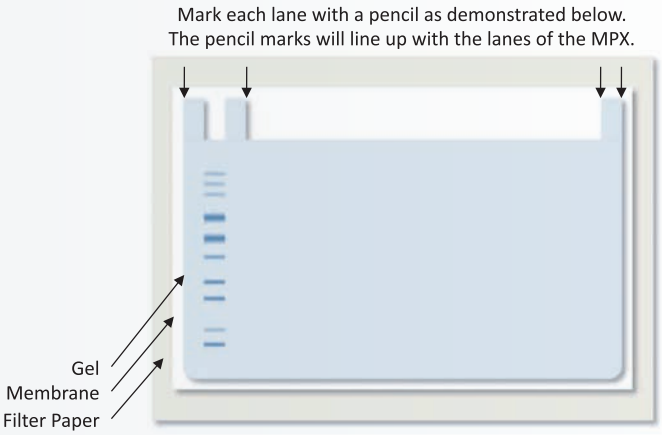


Figure 1. Diagram showing how to effectively mark the membrane for alignment into the MPX™ Blotter.

Membrane Blocking

Membrane Preparation

Cut the membrane into three individual blots as shown in Figure 2. Pre-wet membranes in PBS before proceeding with blocking.

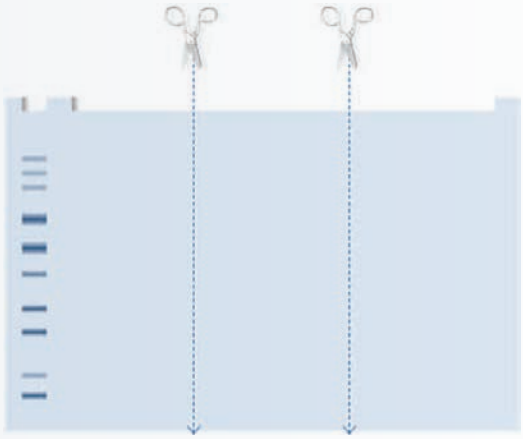


Figure 2. Diagram showing how to cut the membrane into three individual blots for blocking buffer optimization.

Blocking

Place the membranes into 3 different incubation boxes. In each box, cover the entire membrane with blocking buffer (approximately 0.4 mL/cm^2), using a different blocking buffer for each membrane. Block the membrane for 1 hour at room temperature.

Membrane	Blocker
1	Odyssey Blocking Buffer
2	Casein Blocking Buffer
3	Commercial Milk Blocking Buffer

Alignment in MPX™ Blotter

For detailed instruction on use of the MPX Blotter, see MPX Blotter Multiplex Western Blotting Accessory User Guide at *www.licor.com/mpxuserguide.* Place the three blocked membranes into the MPX Blotter so that there are at least 4 channels available for use on each membrane. See Figure 3.

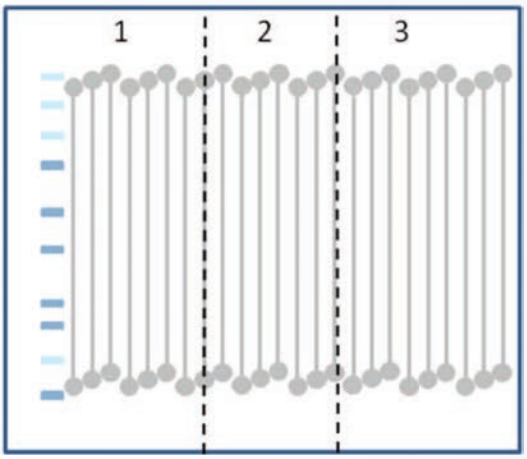


Figure 3. How to place three individual blots into the MPX™ Blotter.

Primary and Secondary Antibody Application

Primary Antibody Preparation

Two dilutions of primary antibody need to be made for each blocking buffer being evaluated. ***Dilutions should be chosen based on vendor recommendations.*** 500 µL of each dilution will be needed.

Membrane	Blocker	Primary Antibody Dilution	
1	Odyssey Blocking Buffer	1:500	1:1,000
2	Casein Blocking Buffer	1:500	1:1,000
3	Commercial Milk Blocking Buffer	1:500	1:1,000

Primary Antibody Application

The primary antibody/blocker dilutions should be loaded into the MPX Blotter to correspond with the same blocked membrane. Apply 2 replicates of each primary antibody dilution; see Figure 4. Fill the unused channels with appropriate corresponding blocking buffer. Incubate for 1-4 hours at room temperature. Wash primary antibody from the channels thoroughly according to MPX Blotter manual instructions, using 1X PBS-T.

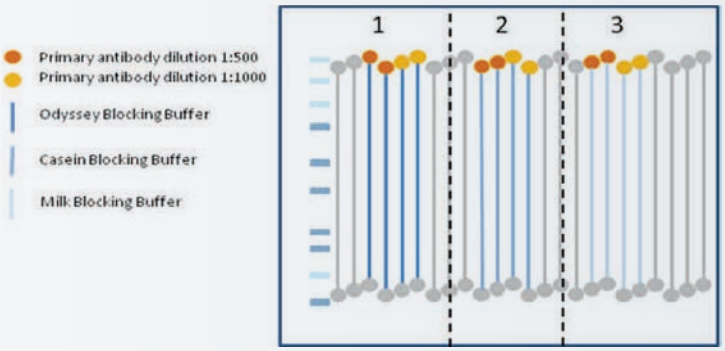


Figure 4. How to place primary antibodies into the MPX Blotter.

Secondary Antibody Preparation

Two dilutions of secondary antibody need to be made for each blocking buffer being evaluated. ***Dilutions should be chosen based on vendor recommendations.*** For IRDye® 800CW and IRDye 680RD secondary antibodies, we recommend 1:5,000 and 1:10,000 as a starting point. 500 µL of each antibody will be needed.

Membrane	Blocker	Secondary Antibody Dilution	
1	Odyssey Blocking Buffer	1:5,000	1:10,000
2	Casein Blocking Buffer	1:5,000	1:10,000
3	Commercial Milk Blocking Buffer	1:5,000	1:10,000

Secondary Antibody Application

The secondary antibody/blocker dilutions should be loaded into the MPX™ Blotter to correspond with the same blocked membrane. Add the secondary antibody dilutions to the primary antibody channels; see Figure 5. Fill the unused channels with the appropriate corresponding blocking buffer. Incubate 1 hour at room temperature. Wash secondary antibody from the channels thoroughly using 1X PBS-T, according to MPX Blotter manual instructions.

Blots can be removed from the MPX Blotter and washed in 1X PBS-T for 5 minutes, followed by a 1X PBS rinse.

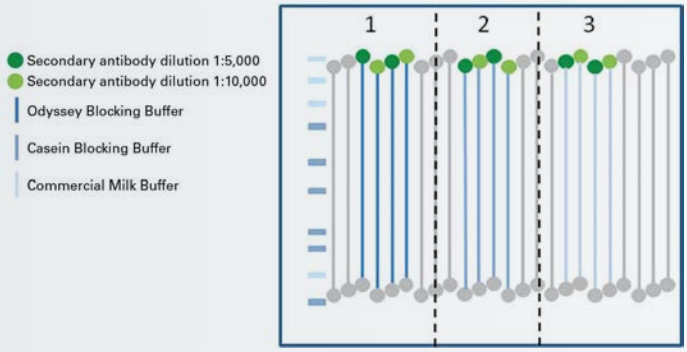


Figure 5. How to place secondary antibodies into the MPX™ Blotter.

Imaging

Membranes can be imaged immediately. Use standard Western blot imaging settings on the Odyssey® Imaging System. Example data is shown in Figure 6.

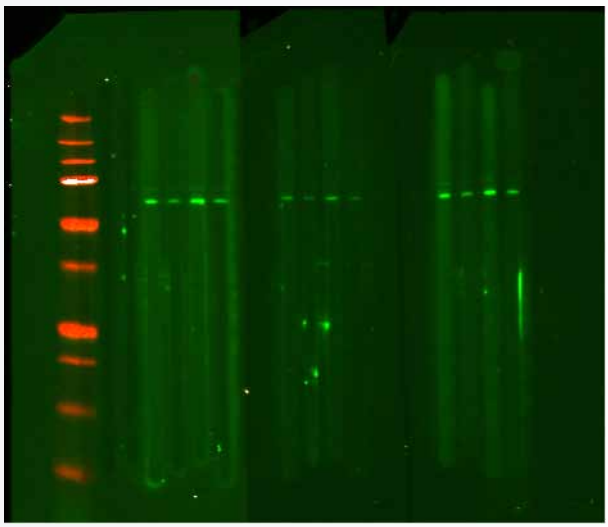


Figure 6. Akt antibody optimization using the MPX™ Blotter procedure.

NucleoCounter® NC-100™ Mammalian Cell Counter

Accurate and Automated Cell Counting of Mammalian Cells

The NucleoCounter NC-100 (P/N 930-00001) is a compact instrument that fits perfectly in any mammalian cell laboratory performing research, quality control, or monitoring of production. The NucleoCounter NC-100 is very simple to operate and requires minimal training.

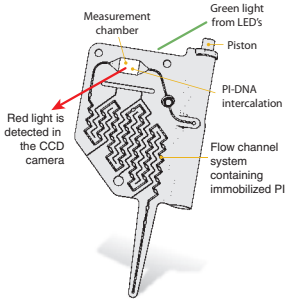
The NucleoCounter is an integrated fluorescence microscope designed to detect signals from the fluorescent dye, propidium iodide (PI), bound to DNA. Results from the NucleoCounter represent either total or nonviable cell concentration, depending on the sample preparation.

As Simple as 1-2-3

1. Sample Preparation
Mix a representative cell sample with equal volumes of Reagent A100 (lysis/disaggregation buffer) and Reagent B (stabilizing buffer).

2. Sampling
To load the NucleoCassette with the lysate, immerse the tip of the cassette in the solution and press the piston.

3. Analysis
Place the NucleoCassette in the NucleoCounter and press the "Run" key. Cell count is displayed in 30 seconds. Optionally, data may be transferred to an external PC using a USB connection or may be printed on an external printer.



NucleoCassette™ Cartridge

Propidium iodide is immobilized in the interior of the disposable NucleoCassette. When the cassette has been loaded with the cell lysate, the PI is dissolved, and the cellular DNA is stained.

After placement in the NucleoCounter, the stained mixture is automatically transferred to the measurement chamber. Green light excites the PI-DNA intercalation, and the red light emitted is registered by the CCD camera for correlation into a cell count.

Because the NucleoCassette contains the entire flow system, as well as the measurement chamber, there is no exposure to hazardous materials. No cleaning, calibration, or maintenance of the NucleoCounter instrument is needed.

930-00001	**NucleoCounter NC-100**	**$13,680.00**
930-41009	**NucleoCassette, 100 each** (Inquire for volume discounts)	**$266.00**
930-00003	**Reagent A100, 500 mL**	**$62.00**
930-00002	**Reagent B, 500 mL**	**$57.00**
930-02000	**Reagent C, 500 mL** (blood applications)	**$149.00**
930-50003	**NucleoView Software**	**$1,108.00**
930-10013	**NucleoCounter Cleaning Kit**	**$71.00**
930-20006	**NucleoCounter Test Kit**	**$213.00**
930-29001	**Bottle Stand**	**$51.00**
930-11001	**Dispenser 0.05-0.5 mL**	**$398.00**
930-29009	**Short Lid**	**$54.00**

Buffers and Reagents

Odyssey® Blocking Buffer

LI-COR® Odyssey Blocking Buffer is optimized for infrared assays including Western blots, In-Cell Western™ Assays, and On-Cell Western Assays. It is a non-mammalian blocking reagent with PBS (phosphate-buffered saline) containing 0.1% sodium azide.

WARNING: Contains 0.1% sodium azide; do not use as diluent for HRP conjugates.

927-40100 **Odyssey Blocking Buffer, 125 mL** **$40.00**

927-40000 **Odyssey Blocking Buffer, 500 mL** **$130.00**

927-40003 **Odyssey Blocking Buffer, 500 mL, 3 Pack** **$350.00**

927-40010 **Odyssey Blocking Buffer, 500 mL, 10 Pack** **$995.00**

927-40125 **Odyssey Blocking Buffer Pack, 25 × 500 mL** NEW! **$2,225.00**

927-40150 **Odyssey Blocking Buffer Pack, 50 × 500 mL** NEW! **$4,000.00**

Storage: 4°C

Casein Blocking Buffers

Casein Blocking Buffer is a blocking solution designed for Western blot applications. It also serves as a diluent for antigens, antibodies, and other proteins. Contains 0.5% Casein (Hammarsten Grade) in 1X PBS.

WARNING: Contains 0.1% sodium azide; do not use as diluent for HRP conjugates.

927-40300 **Casein Blocking Buffer, 100 mL** **$35.00**

927-40200 **Casein Blocking Buffer, 500 mL** **$110.00**

Storage: 4°C

Western Blocking Buffer Sample Pack

Western Blocking Buffer Sample Pack is designed to provide a blocking buffer solution for Western blot applications. These blockers also serve as diluents for antigens, antibodies, and other proteins.

927-40050 **Blocking Buffer Sample Pack** **$125.00**

Sample Pack Includes:

- 1 x 125 mL of Odyssey Blocking Buffer (927-40100)
- 1 x 100 mL of Casein Blocking Buffer (927-40300)

Storage: 4°C

Standing orders are offered for some products. Contact LI-COR for details.

See pages 69-71 for the Technical Note "Odyssey Western Blot Blocker Optimization"

Electrophoresis and Transfer Buffers

928-40010 **10X Tris-Glycine Liquid, 4 L** **$129.00**

928-40012 **10X Tris-Glycine Dry Pack, 2 packs; makes 2 L each** **$53.00**

928-40014 **10X Tris-Glycine-SDS Liquid, 4 L** **$129.00**

928-40016 **10X Tris-Glycine-SDS Dry Pack, 2 packs; makes 2 L each** **$53.00**

Storage: Room Temperature

Wash Buffers

928-40018 **10X PBS (Phosphate-buffered Saline) Liquid, 4 L** **$150.00**

928-40020 **10X PBS Buffer Dry Pack, 2 packs; makes 2 L each** **$65.00**

Storage: Room Temperature

Sample Loading Buffer

928-40004 **4X Protein Sample Loading Buffer, 15 mL** **$32.00**
Contains Orange Dye and is not visible on the Odyssey family of Imagers

Storage: Room Temperature

NewBlot™ Stripping Buffers

Note: Contains Hazardous Materials. Additional shipping charges apply.

928-40030 **5X NewBlot Nitro Stripping Buffer, 100 mL** **$130.00**
5X buffer optimized for use with IRDye® infrared dye-labeled antibody conjugates and Odyssey® nitrocellulose membranes. Other nitrocellulose membranes may be used. Sufficient for fifty 7 cm x 8.5 cm membranes.

928-40032 **5X NewBlot PVDF Stripping Buffer, 100 mL** **$105.00**
5X buffer optimized for use with IRDye infrared dye-labeled antibody conjugates and Millipore Immobilon®-FL PVDF membranes. Other PVDF membranes may be used. Sufficient for fifty 7 cm x 8.5 cm membranes.

Storage: Room Temperature

IRDye® Blue Protein Stain

IRDye® Blue Protein Stain is a convenient, safe alternative for protein gel staining. Unlike traditional Coomassie Blue stains, which require methanol and acetic acid for staining and destaining, IRDye Blue Protein Stain is water-based and requires no hazardous solvents. This stain offers excellent detection sensitivity in the 700 nm channel of any of the Odyssey family of Imagers. IRDye Blue Protein Stain is Coomassie-based and is provided as a ready-to-use 1X solution. Prewashing and de-staining steps are performed in water.

928-40002 **IRDye Blue Protein Stain, 1 L** **$95.00**
Colloidal Coomassie stain for visualizing protein gels in 700 nm channel.

Storage: Room Temperature

Protein MW Markers

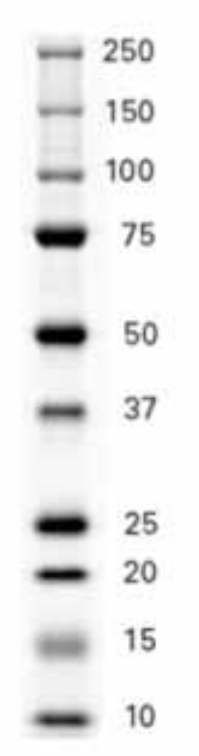
928-40000 **Odyssey® Protein Molecular Weight Marker, 0.5 mL (10-250 kDa)** **$185.00**

Odyssey Protein Molecular Weight Marker provides a ladder of convenient, consistent sizes. The markers include ten protein bands from 10 to 250 kDa, with molecular weight shifts of 5% and minimal band broadening. Three reference bands at 25, 50, and 75 kDa are three times as intense as other bands. The molecular weight markers are confirmed by migration in a Laemmli SDS-PAGE system.

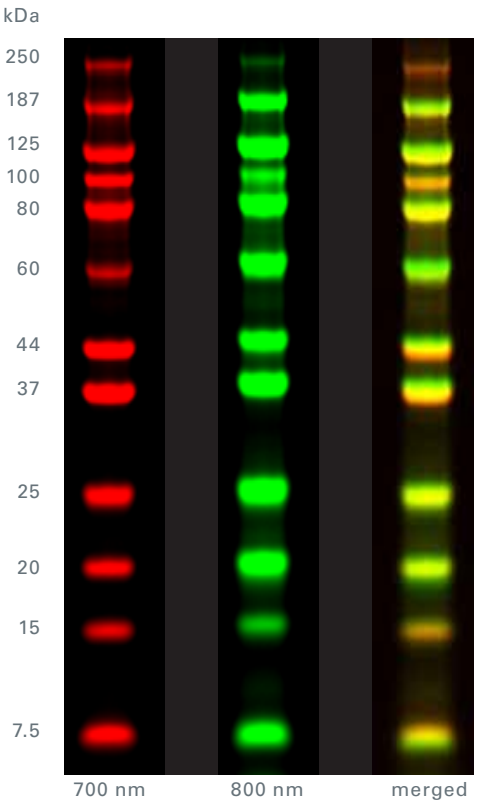
Storage: -20°C

928-40006 **IRDye® (680/800) Protein Marker** NEW! **$145.00**

The IRDye (680/800) Protein Marker is designed to provide a ladder of convenient and consistent protein sizes (7.5-250 kDa) for use with polyacrylamide gels and Western membranes.



Odyssey® Protein Molecular Weight Marker



IRDye (680/800) Protein Marker

TECHNICAL NOTE:
Odyssey® Western Blot Blocker Optimization

Blocker choice is important for immunoassay success.

Blocking buffers enhance sensitivity by reducing background interference, increasing signal-to-noise ratio, and promoting specific binding of the primary antibody while minimizing non-specific interactions.

- Insufficient blocking yields high background and reduced signal-to-noise ratios.
- Excessive blocking may cause loss of blotted proteins[1] or mask antibody:antigen interactions.
- Detection reagents may cross-react with certain blocking buffers.

Empirical testing is critical!

The Blocking Buffer Sample Pack provides smaller quantities of two LI-COR blockers, to help you identify the best choice for your antigen.

No single blocking buffer selection is suitable for ALL antigen-antibody pairs. Blocker choice may affect antibody specificity and non-specific binding.[2]

1. *J Immunol Methods* 1989, 122 (1), 129-35
2. *Proteomics* 2008, 8, 2379–2383

Required Reagents

- Protein MW Marker, see page 68 for products
- IRDye® Secondary Antibodies, see pages 47-48 for products
- Blocking Buffer, see page 66 for products
- Membrane and membrane kits, see pages 57-59 for products
- Primary antibodies (primary antibodies must be from host species compatible to the secondary antibodies being used-if using Subclass Specific antibodies, please refer to Technical Note: *Western Blot and In-Cell Western™ Assay Detection Using IRDye® Subclass Specific Antibodies pages 51-55)*
- Tween® 20
- PBS Buffer, see page 67 for products
- Methanol (when using Immobilon®-FL PVDF membrane)
- SDS (when using Immobilon-FL PVDF membrane)
- Western Blot Incubation Box, see page 149 for products

Gel Preparation for Blocker Optimization

Standard protein electrophoresis conditions and reagents can be used for gel and sample preparation. The following is a suggested template for sample electrophoresis to maximize blocker optimization and efficiently choose the best blocking conditions for a given primary antibody.

Using a 15-well gel, load the following samples in the order indicated:

Lane	Sample	Amount
1	Protein Marker	2-10 µL
2	Primary Antibody	5 µL of a 1:1000* dilution in PBS
3	Sample Lysate	10 µg
4	Sample Lysate	5 µg
5	Sample Lysate	2.5 µg
6	Protein Marker	2-10 µL
7	Primary Antibody	5 µL of a 1:1000* dilution in PBS
8	Sample Lysate	10 µg
9	Sample Lysate	5 µg
10	Sample Lysate	2.5 µg
11	Protein Marker	2-10 µL
12	Primary Antibody	5 µL of a 1:1000* dilution in PBS
13	Sample Lysate	10 µg
14	Sample Lysate	5 µg
15	Sample Lysate	2.5 µg

* Suggested starting point; may need to be adjusted for your primary antibody

Western Blocker Optimization Method

Western blot should be prepared using standard blotting procedures and Millipore Immobilon®-FL PVDF or Odyssey® Nitrocellulose Membrane. Allow blot to dry for at least 1 hour before proceeding with detection. Dry blots can be stored between filter paper overnight at room temperature, protected from light.

Note: Membranes should be handled only by their edges, with clean forceps. Take great care to never touch the membrane with bare or gloved hands.

Note: Do not write on membrane with an ink pen or marker, as the ink will fluoresce on the Odyssey Imager. Mark with pencil or Odyssey Pen (P/N 926-71804) to avoid this problem. Use pencil only for PVDF membrane, as wetting in methanol will cause ink to run.

If using the gel configuration outlined in the Gel Preparation for Blocker Optimization section above, cut the membrane, being careful not to touch the membrane along protein marker lanes 6 and 11 as shown in Figure 1. Label appropriately with pencil.

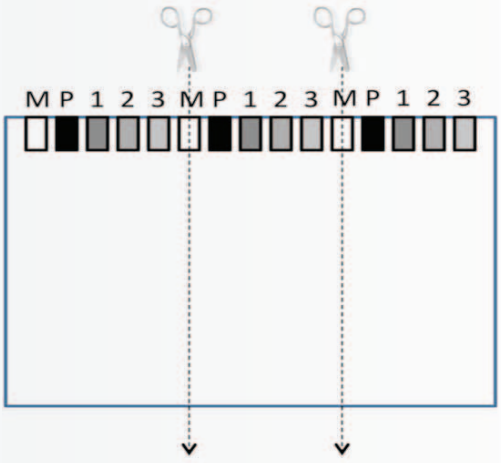


Figure 1. Cut Western blot along the Marker lanes into three individual optimization blots.

After cutting membrane, perform the following steps:

1. For Immobilon-FL PVDF membranes:
   a. Pre-wet 1 minute in 100% methanol
   b. Rinse with ultra pure water
   c. Wet in 1X PBS for 2 minutes

   For Odyssey Nitrocellulose Membranes:
   a. Wet in 1X PBS for 2 minutes
2. Place membranes into 3 different Western Blot Incubation Boxes and block the membrane in 10 mL Blocking Buffer for 1 hour while gently shaking.
   a. Box 1 – Odyssey Blocking Buffer
   b. Box 2 – Casein Blocking Buffer
   c. Box 3 – Blocking Buffer of your choice
3. Dilute primary antibody** in 10 mL of appropriate diluent listed below:
   a. Box 1 – Odyssey Blocking Buffer + 0.2% Tween® 20 + Primary Antibody
   b. Box 2 – Casein Blocking Buffer + 0.2% Tween 20 + Primary Antibody
   c. Box 3 – Blocking Buffer of your choice + Primary Anitbody

**The correct working range for antibody dilution depends on the characteristics of your primary antibody. Start with the dilution recommended by the primary antibody vendor for Western blot applications.

4. Incubate blots in diluted primary antibody for 1 to 4 hours at room temperature, or overnight at 4°C while gently shaking. **Note:** Incubation times vary for different primary antibodies.

5. Wash membranes:
   a. Pour off primary antibody solution.
   b. Rinse membrane with 1X PBS-T (0.1% Tween® 20).
   c. Cover blot with 1X PBS-T (0.1% Tween 20).
   d. Shake vigorously on platform shaker at room temperature for 5 minutes.
   e. Pour off wash solution.
   f. Repeat 3 additional times.

6. Dilute secondary antibody# in 10 mL of appropriate diluent listed below:

   Secondary antibody diluent for Immobilon®-FL PVDF membrane
   a. Box 1 – Odyssey® Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   b. Box 2 – Casein Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   c. Box 3 – Blocking Buffer of your choice + Secondary Antibody

   Secondary antibody diluent for Odyssey Nitrocellulose Membrane
   a. Box 1 – Odyssey Blocking Buffer + 0.2% Tween 20 + Secondary Antibody
   b. Box 2 – Casein Blocking Buffer + 0.2% Tween 20 + Secondary Antibody
   c. Box 3 – Blocking Buffer of your choice + Secondary Antibody

#IRDye conjugates have each been optimized for Western blot detection. Please see pack inserts for appropriate Western blot dilutions.

7. Incubate blot in diluted secondary antibody for 30-60 minutes at room temperature with gentle shaking. **Protect membrane from light during incubation.**

8. Protect from light during washes.
   Wash membranes:
   a. Pour off secondary antibody solution.
   b. Rinse membrane with 1X PBS-T (0.1% Tween 20).
   c. Cover blot with 1X PBS-T (0.1% Tween 20) using same volumes indicated above for Western blot incubation boxes.
   d. Shake vigorously on platform shaker at room temperature for 5 minutes.
   e. Pour off wash solution.
   f. Repeat 3 additional times.

9. Rinse membrane with 1X PBS to remove residual Tween 20. The membrane can be imaged wet or dry.

10. Image all three blots side-by-side.

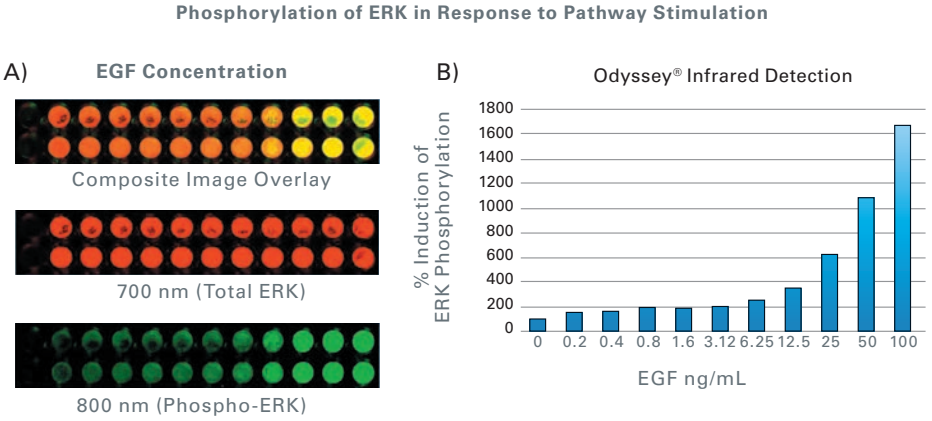
11. Visual inspection or data analysis with Odyssey application or Image Studio software can be used to determine which blocking buffer works best with the evaluated primary.

Tips

- Follow the protocol carefully.
- For additional Odyssey Western detection tips, see www.licor.com/bio > Applications > Quantitative Western Blots

In-Cell Western™ Assay Kits

The In-Cell Western™ Assay is an immunofluorescent assay that uses NIR fluorescence to detect and quantify proteins in fixed cells. Detecting proteins in their cellular context increases quantification precision. Proteins in fixed, cultured cells are detected directly in microplates, which yields higher throughput compared to Western blotting and eliminates error-prone Western blotting steps, such as cell lysate preparation, electrophoresis, and membrane transfer.



A. Detection of ERK phosphorylation. These images show a portion of a 96-well plate. Duplicate rows of microplate wells are shown. The top panel is a composite image showing the fluorescence in both the 700 nm and 800 nm detection channels. The middle panel shows detection of total ERK protein regardless of phosphorylation status. The bottom panel shows detection of increasing amounts of phospho-ERK as a function of increasing EGF stimulation. **B. Quantification of fluorescence.** Phospho-ERK signal has been normalized using the total ERK signal from each well, to correct for well-to-well variation in cell number. This experiment shows a greater than 16-fold increase in ERK phosphorylation compared to the resting state.

926-31070 **In-Cell Western Kit I** **$640.00**

Kit contains: IRDye® 800CW Goat anti-Mouse Secondary Antibody (0.5 mg)

- Odyssey® Blocking Buffer, (4 x 500 mL)
- DRAQ5™ Stain (100 µL) (Biostatus, Ltd.)
- Sapphire700™ Stain (100 µL)
  (Reagents for up to twenty 96-well plates or ten 384-well plates)

Storage: 4°C/Room Temperature depending on component

926-31072 **In-Cell Western Kit II** **$640.00**

Kit contains:

- IRDye 800CW Goat anti-Rabbit Secondary Antibody (0.5 mg)
- Odyssey Blocking Buffer, (4 x 500 mL)
- DRAQ5 Stain (100 µL)
- Sapphire700 Stain (100 µL)
  (Reagents for up to twenty 96-well plates or ten 384-well plates)

Storage: 4°C/Room Temperature depending on component

928-40022 **Sapphire700 Stain (100 µL)** **$95.00**

Cell stain may be used in combination with DRAQ5* Stain for normalization in In-Cell Western assays.

Sapphire700 is a non-specific cell stain that accumulates in both the nucleus and cytoplasm of fixed or dead cells, but not live cells. When used to stain serial dilutions of A431 cells in 96-well plates, Sapphire700 displays linearity of fluorescent signal for higher cell densities, from ~50,000 to ~250,000 cells/well. Simultaneous staining of cells with Sapphire700 and DRAQ5 (Biostatus, Ltd.)* Stain expands the linear range, allowing more accurate normalization of cell number across both low and high cell densities.

Storage: Room Temperature

*Can be purchased from *www.biostatus.com/product/draq5/*

TECHNICAL NOTE:
In-Cell Western™ Assay Quality Assessment Using Z'-Factor

Introduction

During In-Cell Western™ (ICW) assay optimization, it is important to assess the overall quality and reliability of the assay. The Z'-factor statistic provides a way to evaluate whether or not assay conditions (reagents, protocols, instrumentation, kinetics, and other conditions not directly related to the test compounds) are optimized. Z'-factor, introduced by Zhang et al., is a dimensionless value that represents both the variability and the dynamic range between two sets of sample control data.

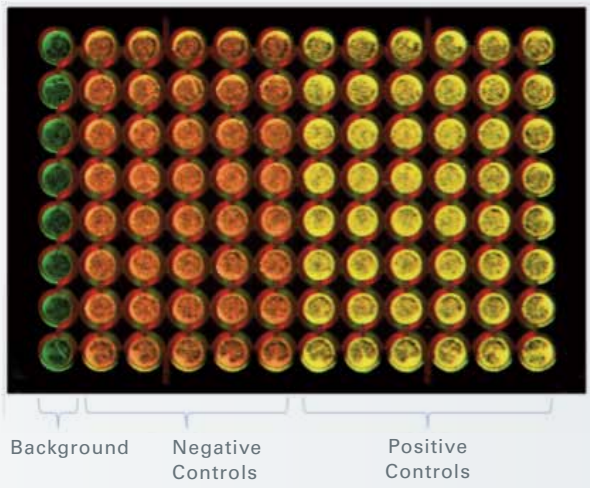
Z'-factor experiments are performed on one or more ICW assay plates containing replicate wells designated for background subtraction, negative control samples, and positive control samples. Typically, negative control wells are those in which the cells receive an appropriate treatment so as to elicit the lowest desired percent response (usually untreated cells); positive control wells are those in which the cells receive an appropriate treatment so as to elicit the maximum desired percent response; background wells are treated the same as the negative control wells, except primary antibody incubation is excluded.

Here we will briefly describe how to set up, analyze, and interpret an ICW Z'-factor experiment. For further information on Z'-factor analysis and cell-based assay validation in general, refer to the following publications:

- J.H. Zhang, T.D.Y. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, *J. Biomol. Screen.* 4 (1999) 67-73.
- V. Boveia, K. Ambroz, D.M. Olive, Using the Z'-Factor coefficient to monitor quality of near-infrared fluorescent cell-based assays, LI-COR Application Note (2009).
- P.W. Iversen, B.J. Eastwood, G.S. Sittampalam, K.L.Co, A comparison of assay performance measures in screening assays: signal window, Z' factor, and assay variability ratio, *J. Biomol. Screen.* 11 (2006) 247-252.
- B.J. Eastwood, M.W. Farmen, P.W. Iversen, T.J. Craft, J.K. Smallwood, K.E. Garbison, N.W. Delapp, G.F. Smith, The minimum significant ratio: a statistical parameter to characterize the reproducibility of potency estimates from concentration-response assays and estimation by replicate-experiment studies, *J. Biomol. Screen.*11 (2006) 253-261.

Experimental Design

As with any statistical parameter, the accuracy of the Z'-factor value improves with larger data sets used for the calculation. We recommend running at least one full 96-well ICW plate with background wells and replicate negative and positive control wells in order to obtain a meaningful Z'-factor value. For a better assay quality assessment, 3-4 plates should be run on different days. Wells can be arranged as desired, but a typical arrangement may look like this:



Follow these guidelines when you set up the ICW Z'-factor experiment:

- All reagents (apart from test compounds and reagents), antibodies, materials, and assay conditions should be the same as those that you intend to use for the final ICW assay.
- Background wells must be included on the assay plate to account for non-specific secondary antibody binding and plate autofluorescence. Background wells are treated the same as the negative control wells, except that primary antibody is excluded; only antibody diluent (i.e. blocking buffer) should be applied to background wells during this step.

Important: If DNA staining or NHS ester protein labeling is used for normalization, these reagents should also be excluded from the background wells.

- Be sure to correct for well-to-well variation by

normalizing against a second primary antibody, a DNA stain, or by total protein labeling. For more information on ICW assay normalization, see LI-COR technical note: *Labeling of Fixed Cells with IRDye NHS Ester Reactive Dyes for In-Cell Western Assay Normalization,* pages 108-110.

- When choosing treatment compounds to be used for Z'-factor analysis (i.e. stimulants, inhibitors, drugs, etc.), select well-characterized agents which give the best known response in an appropriate cell line.

Analysis

The Z'-factor value can be calculated manually using normalized intensity values, or by enabling Z'-factor calculations within the Odyssey® application software (v. 3.0 or later) or Image Studio Software.

Manual Calculation

1. Export the 700 and 800 channel intensity values Grid Sheet for each well of the microplate to a spreadsheet software application (e.g. Microsoft Excel). Values should not be background-subtracted. Refer to the Instrument User Guide or the Tutorial Manual for detailed instructions.
2. Calculate the mean background intensity in each channel for the background wells.
3. In each channel, subtract the appropriate mean background intensity value from each sample well (negative and positive controls).
4. In the channel used for normalization only, determine the maximum intensity value from the sample wells (negative and positive controls). Divide each intensity value by this maximum value. The resulting relative intensity values will normally be between 0.0 and 1.0, though negative numbers are possible.
5. In the channel used to analyze response, divide all values from the sample wells (negative and positive controls) by the corresponding relative intensity value calculated in step 4. This is the normalized intensity value that will be used to calculate the Z'-factor.
6. Calculate the mean and the standard deviation for each sample set (negative and positive controls) using the normalized intensity values for each of the sample sets.
7. Plug in the mean and standard deviation values calculated in step 6 into the formula below to obtain the unitless Z'-factor value:

$$Z' = 1 - \frac{(3\sigma_{c+} + \sigma_{c-})}{|\mu_{c+} + \mu_{c-}|}$$

Where:

- σ_{c+} = Standard deviation of positive controls
- c_{-} = Standard deviation of negative controls
- μ_{c+} = Mean of positive controls
- μ_{c-} = Mean of negative controls

Odyssey® Software Calculation

1. Z'-factor calculations are enabled in the Odyssey application software (v.3.0 or later) by choosing In-Cell Western > Change ICW Parameters and switching to the Calculations tab. At the bottom of the window select Calculations are for Z-Factor.

   Note: When **Calculations are for Z-Factor** is selected, In-Cell Western calculations are not performed, and any well links are ignored if links are defined on the Links tab of the Change ICW Parameters window.
2. Make sure **Subtract Background on All Channels** is selected.
3. Make sure **Calculate Relative Intensity in Channel** is selected and the correct channel for normalization is chosen.
4. Switch to the **Well Types** tab in the **Change ICW Parameters** window. Each well should be designated as a positive control, negative control, background well, or not used. To change well designations, begin by selecting the type of well you want to mark. Move the cursor over a well on the well assignment grid and click to assign the selected well type to that well. Multiple well assignments can be made at once by clicking and dragging through a range of wells. When the mouse button is released, all wells within the selection rectangle change to the color assigned to the chosen well type. If **Assign Remaining** is clicked, any wells that are Not Used will be assigned the currently selected well type. If you make a mistake and need to start over, click **Clear Rows** to change all wells to unused.
5. View the calculated Z'-factor value by choosing In-Cell Western > View ICW Analysis. The Z'-factor is listed in red above the data table in the View ICW Analysis window.

Image Studio Software Calculation

Refer to the online help system for Image Studio.

Interpretation

The Z′-factor formula can also be written as follows:

$$Z' = \frac{|\mu_{c+} + \mu_{c-}| - (3\sigma_{c+} + \sigma_{c-})}{|\mu_{c+} + \mu_{c-}|}$$

In this formula,

- $|\mu_{c+} - \mu_{c-}|$ Represents the assay dynamic range, and
- $|\mu_{c+} - \mu_{c-}|(3\sigma_{c+} + 3\sigma_{c-})$ Is the separation band between positive and negative control signals.
- These variables are represented in the illustration below.

Dimensionless Z′-factor values are always 1 or less as described below:

Z′ = 1 indicates an ideal assay. As standard deviations become very small or the difference between signals for positive and negative controls approaches infinity, Z′-factor approaches 1.

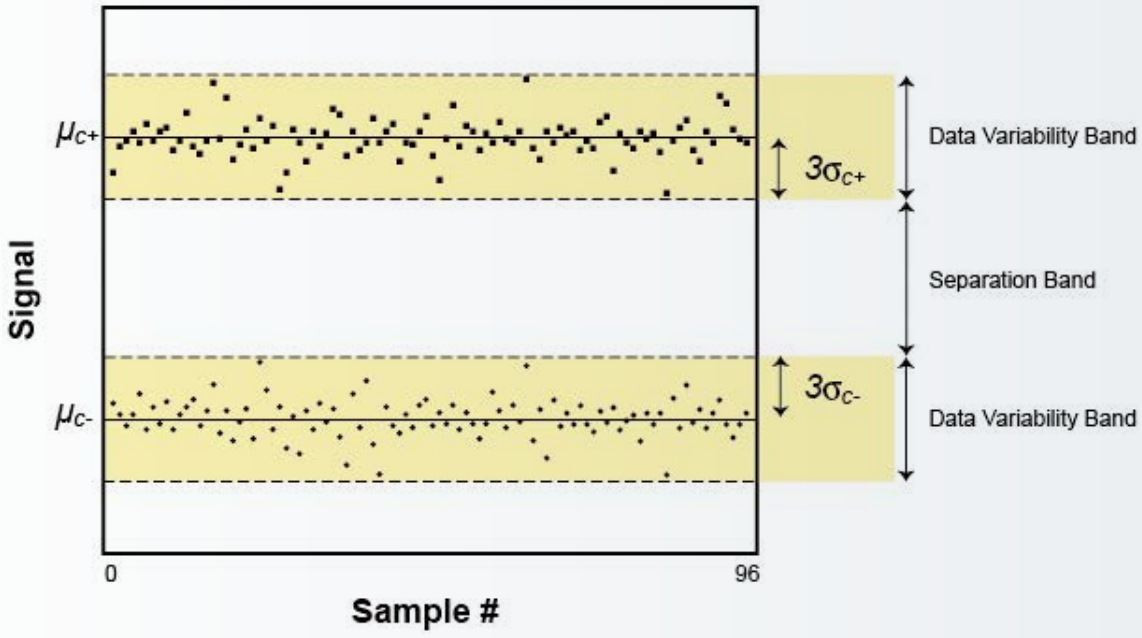
1 > Z′ ≥ 0.5 indicates a high quality assay exhibiting a wide separation between signal and background, and low data variability.

0.5 > Z′ > 0 indicates a poor quality assay with marginal distinction between signal and background and higher data variability.

Z′ ≤ 0 indicates unreliable data. Assay conditions are not optimized or the assay is not capable of generating meaningful data.

Z′ = -1 indicates there is no distinguishable difference between background signal and sample signal. If lower limit of detection (LLD) is expressed as the mean of the background signal plus three SD of the background signal, then the measured signal is equal to the LLD of the instrument.

The Z′-factor experimental guidelines described above can be utilized to evaluate ICW assay quality at any time throughout assay development, or near completion of assay optimization. Z′-factor measurement is also useful for monitoring assay quality over time, from user-to-user, or when conditions or reagents within the assay must be changed.



NIR-FRET Caspase Substrate

IRDye® Near-infrared Fluorescence Resonance Energy Transfer (NIR-FRET) Caspase Substrate

The IRDye Near-Infrared Fluorescence Resonance Energy Transfer (NIR-FRET) Caspase Substrate can be used for the detection of protease activity in NIR-FRET assays. The substrate has low intrinsic fluorescence due to self-quenching of the conjugated dye by IRDye QC-1 quencher (P/N 929-70030 and 929-70031, see page 22). Upon proteolytic cleavage of the substrate, fluorescence increases can be detected on an Odyssey® CLx, Classic, or Sa Infrared Imaging System or other imaging systems with near-infrared fluorescent detection capabilities.

LI-COR® offers NIR-FRET Caspase Substrate IRDye 800CW/QC-1 CSP-3 (P/N 926-08590). The substrate provides a sensitive method to detect protease activity, measure potency of inhibitors, and screen compound collections for discovery of drug candidates.

Caspases are a family of intracellular cysteine proteases that are vital in the process of apoptosis (programmed cell death). Caspase-3 functions as the most downstream executioner in the caspase-apoptosis signaling cascade.[1] Activation of Caspase-3 by upstream initiator caspases (Caspases 2, 8, and 9) results in the final execution of cell death.[2] Insufficient or accelerated apoptosis is implicated in a variety of diseases such as cancer, immune system diseases, and nervous system disease.[3]

References:

1. Zhang, J.-H.; Chung, T.D.Y.; Oldenburg, K.R. *J. Biomol. Screening* 4(2), 67-73 (1999)
2. Thornberry, N.A.; Lazebnik, Y. *Science*, 281, 1312-1316, 1998.
3. Slee, E.A.; Adrain, C.; Martin, S. *J. Cell Death Differ*, 6, 1067-1074, 1999.

926-08590 **IRDye 800CW/QC-1 CSP-3 Substrate** **$149.00**
(2.4 nmoles; 96 assays-one microplate)

Advantages:

- Subnanomolar enzyme detection
- NIR fluorescence detection dramatically reduces background, scattering, and interference from other compounds
- Suitable for high throughput screening of enzyme activity
- Up to 61-fold fluorescence intensity enhancement upon digestion
- Inhibitor and drug candidate IC_{50} measurement
- Excellent water solubility

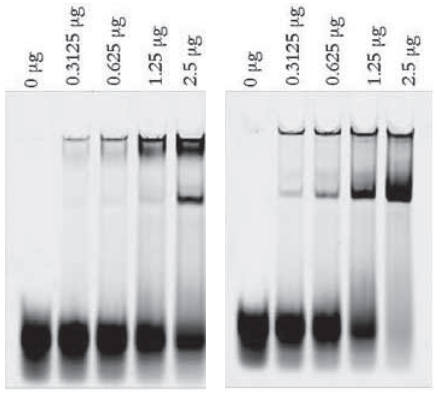
Storage: -20°C

EMSA/Gel Shift Assay Reagents

Existing electrophoretic mobility shift assay (EMSA) protocols are easily adapted to infrared assays by replacing radiolabeled oligonucleotides with IRDye® end-labeled oligonucleotides and imaging on the Odyssey® CLx or Odyssey Classic Infrared Imaging System. Binding conditions and electrophoresis conditions remain the same as other EMSA detection methods. Each IRDye 700 Consensus Oligonucleotide is sufficient for up to 25 reactions.

Advantages of IRDye® Mobility Shifts Assays

- Linear, 50-fold dilution range from 9.1 fmol to 0.18 fmol
- No hazardous radioisotope, no chemiluminescent substrate reagents, no film exposure or phosphoimager
- Gels are imaged in the glass plates (no gel transfer or drying)
- Multiple experiments can be done in one day due to the time savings



Infrared AP-1 EMSA was used to visualize changes in AP-1 binding following three different treatments of HeLa cells. HeLa, HeLa 2 h serum response (not shown), and HeLa 4 h serum response nuclear extracts were serially diluted and assayed under the same conditions using IRDye 700 end-labeled oligonucleotide duplex containing AP-1 binding sequence.

927-10100 **10X Orange Loading Dye (0.5 mg)..... $54.00**
Orange Loading Dye 10X is used as a loading buffer for DNA gel electrophoresis. This loading dye is not visualized when imaged on any of the Odyssey Infrared Imagers. When run on a 5% native acrylamide 1X TBE gel, the orange dye migrates with the 20-28 bp DNA fragment.

Storage: 4°C

829-07925 **IRDye 700 AP-1 Consensus Oligonucleotide (25 µL of 50 nM double-stranded DNA) $60.00**
5′—CGC TTG A TG ACT CA G CCG GAA—3′
3′—GCG AAC T ACTGA GT C GGC CTT—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07923 **IRDye 700 CREB Consensus Oligonucleotide* (25 µL of 50 nM double-stranded DNA)..... $60.00**
5′—AGA GAT TGC CT G ACG TCA GAG AGC TAG—3′
3′—TCT CTA ACG GA C TGC AGT CTC TCG ATC—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07929 **IRDye 700 HIF-1 Consensus Oligonucleotide (25 µL of 50 nM double-stranded DNA) $60.00**
5′—AGC TTG CCC T AC GTG CTG TCT CAG A—3′
3′—TCG AAC GGG A TG CAC GAC AGA GTC T—5′

Storage: -20°C Underlined nucleotides are the binding site.

*Shipping time for some EMSA Oligos is up to 2 weeks

829-07924 **IRDye 700 NFκB Consensus Oligonucleotide (25 µL of 50 nM double-stranded DNA) $60.00**
5′—AGTTGA GGG GACTTT CCC AGG C—3′
3′—TCA ACT CCC CTG AAA GGG TCC G—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07921 **IRDye 700 p53 Consensus Oligonucleotide* (25 µL of 50 nM double-stranded DNA) $60.00**
5′-TAC A GA ACA TGT CTA AGC ATG CTG GGG ACT—3′
3′-ATG T CT TGT ACA GAT TCG TAC GAC CCC TGA—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07926 **IRDye 700 Sp-1 Consensus Oligonucleotide (25 µL of 50 nM double-stranded DNA) $60.00**
5′—ATT CGA TC G GGG CGG GGC GAG C—3′
3′—TAA GCT AG C CCC GCC CCG CTC G—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07922 **IRDye STAT3 Consensus Oligonucleotide* (25 µL of 50 nM double-stranded DNA) $60.00**
5′-GAT CC TTCT GGG AATT CC TAG ATC—3′
3′-CTA GG A AGA CCC TTA A GG ATC TAG—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07933 **IRDye 700 ARE (Androgen Receptor) Consensus Oligonucleotide* (25 µL of 50 nM double-stranded DNA) $60.00**
5′—GTCT GG TAC A GG G TG TTCT TTTT—3′
3′—CAG A CC ATG T CC C AC AAG A AA AA—5′

Storage: -20°C Underlined nucleotides are the binding site.

829-07910 **EMSA Buffer Kit (100 reactions) $210.00**

Kit contains:

- 10X Binding Buffer, 100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5 (500 µL)
- 25 mM DTT, 2.5% Tween® 20 (500 µL)
- Poly (dI.dC), 1 µg/µL in 10 mM Tris, 1 mM EDTA; pH 7.5 (125 µL)
- Sheared Salmon Sperm DNA, 0.5 µg/µL in 10 mM Tris, 1 mM EDTA; pH 7.5 (125 µL)
- 50% Glycerol (500 µL)
- 1% NP-40 (500 µL)
- 1 M KCl (500 µL)
- 100 mM $MgCl_2$ (500 µL)
- 200 mM EDTA, pH 8.0 (500 µL)
- 10X Orange Loading Dye (500 µL)

Storage: -20°C

*Shipping time for some EMSA Oligos is up to 2 weeks

ELISA Substrates NEW!

LI-COR's proprietary peroxidase and phosphatase substrates are optimized for use in the near-infrared (NIR) fluorescent region (700 nm) on Odyssey CLx, Classic, or Sa Infrared Imaging Systems. This brings the additional versatility of another common laboratory application to the Odyssey platforms.

NIR fluorescence-based detection will also help overcome the limitation of chromogenic substrate detection, which does not allow for quantification of greater than four optical density units. NIR-based detection allows for a wider linear range.

Both substrates provide excellent signal-to-noise, consistency, and linearity, making them ideal for use in most ELISA applications. These substrates offer equal or better sensitivity as compared to commercially-available chromogenic and chemiluminescent substrates and are ideal for endpoint assays.

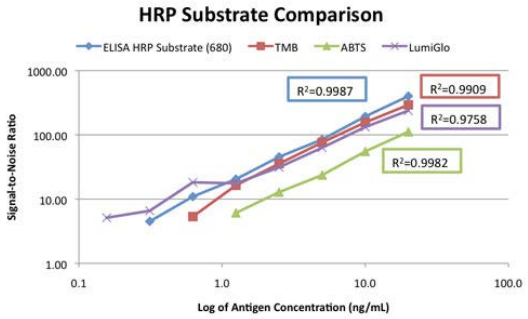


Figure 1. Substrate comparison of LI-COR® ELISA HRP Substrate (680) (P/N 926-34300) to various HRP substrates. Substrates were compared in a mouse IgG ELISA for linearity, signal-to-noise ratios (SNR) and limit of detection (LOD). SNR ratios were determined by dividing the background subtracted average value by the standard deviation of the average background. LOD was determined as any SNR value above 3. The LI-COR ELISA HRP Substrate (680) demonstrated superior SNR values and linearity compared to other substrates. The LOD values were 0.313, 0.625, 1.25, and 0.156 ng/mL for ELISA HRP Substrate (680), TMB, ABTS, and LumiGLo.

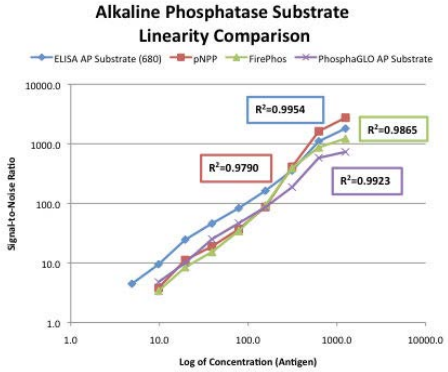


Figure 2. Substrate Linearity Comparison of Alkaline Phosphatase Substrates to LI-COR® ELISA AP Substrate (680) (P/N 926-34301). Signal-to-noise ratios were determined by dividing the background subtracted average value by the standard deviation of the average background. Limit of detection (LOD) was determined as any SNR value above 3. LOD was 4.9 ng/mL with the ELISA AP Substrate (680) and 9.8 ng/mL with all of the others.

Note: *These products cannot be used for Western blotting applications.*

926-34300 ELISA HRP Substrate (680) Pack $90.00

The peroxidase (HRP) substrate results in a colorless to light blue product in the presence of horseradish peroxidase. The reaction is stopped with a colored peroxidase stop solution, so the wells appear pink after the addition of the stop solution.

Kit contains:

- Horseradish Peroxidase (HRP) Substrate (1 vial)
- Assay Buffer (110 mL)
- Stop Solution (30 mL; adequate for 10 x 96-well plates)

926-34301 ELISA AP Substrate (680) Pack.....$90.00

The phosphatase (AP) substrate results in a colorless to light blue product in the presence of alkaline phosphatase. The reaction is stopped with a colored phosphatase stop solution, so the wells appear purple after the addition of the stop solution.

Kit contains:

- Alkaline Phosphatase (AP) Substrate (1 vial)
- Assay Buffer (110 mL)
- Stop Solution (30 mL; adequate for 10 x 96-well plates)

Storage: Refer to pack insert for individual storage recommendations.

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Small Animal *Imaging*

TECHNICAL NOTE:
A systematic approach to the development of fluorescent contrast agents for optical imaging of mouse cancer models

Joy L. Kovar, Melanie A. Simpson, Amy Schutz-Geschwender, and Dr. Michael Olive
Reprinted with permission from: Kovar, et al. *Anal. Biochem* 367(2007)1-12.

Abstract

Optical imaging is a rapidly developing field of research aimed at non-invasively interrogating animals for disease progression, determining the effects of a drug on a particular pathology, assessing the pharmacokinetic behavior of a drug, or identifying molecular biomarkers of disease. One of the key components of molecular imaging is the development of specific, targeted imaging contrast agents to assess these biological processes. The development of robust fluorochrome-labeled optical agents is a process that is often underestimated in terms of its complexity. Although many studies describe the use of these agents, guidelines for their development and testing are not readily available. This review outlines some of the general principles that are important when developing and using fluorochrome-labeled optical contrast agents for oncology investigations in animals.

In the last decade our increased understanding of the molecular basis of cancer has led to the development of novel targeted strategies for specific inhibition of cancer signaling pathways that control growth, proliferation, apoptosis, and angiogenesis. Several monoclonal antibody-based therapeutics and small molecule drugs have received clearance for use as human therapeutics.[1] However, among these successes are many candidate drugs that have failed in clinical trials despite promising pre-clinical results.[2]

The development of targeted therapeutics is expensive and time consuming. In their Critical Path Initiative, the United States Food and Drug Administration emphasized the need for more effective tools to facilitate the rapid development of improved cancer therapeutics. One such tool is the use of targeted molecular optical imaging probes or contrast agents to visualize the underlying processes in cancer.

Optical imaging, also known as molecular imaging, is a rapidly developing field of research aimed at non-invasively interrogating animals for disease progression, evaluating the effects of a drug, assessing the pharmacokinetic behavior of a drug, or identifying molecular biomarkers of disease. A prerequisite of molecular imaging is the development of specific, targeted imaging contrast agents to assess these biological processes. Several optical aids have shown great utility in animal studies, including bioluminescence, fluorescent proteins, and fluorochrome-labeled agents. However, only the latter have the advantage of being potentially relevant to human clinical applications.

The complexity of developing robust fluorochrome-labeled optical agents is often underestimated. Many studies describe the use of these agents, but guidelines for their development and testing are not readily available. The purpose of this review is to outline some of the considerations for developing and using fluorochrome-labeled optical contrast agents in animals. For simplicity, we have focused on the use of organic fluorochromes as labeling agents. These types of probes are generally the most straightforward to develop and have the greatest potential for translation to human clinical use. Nanoparticles such as quantum dots, while useful for some animal studies, are hampered by clearance issues and toxicity and will not be specifically discussed. However, the principles described here are generally applicable to any fluorescent optical imaging agent.

Principles of Fluorescence Imaging

The use of fluorochrome-labeled optical agents such as labeled antibodies, receptor-binding ligands, small molecules, peptides, and activatable probes offers a flexible and direct imaging methodology. The fluorescent labels can be visualized by excitation with an appropriate light source and capture of the emitted photons with a CCD camera or other optical detector. Several commercially-available imaging systems enable visualization of these probes in mice. These include systems from LI-COR® Biosciences, Perkin Elmer, Carestream, and Fluoptix. In fluorescent imaging there are generally three parameters used to characterize the interaction of photons with tissues: light absorption, light scattering, and fluorescent emission. One of the most important

considerations in optical imaging is maximizing the depth of tissue penetration. Absorption and scattering of light are largely a function of the wavelength of the excitation source.[3] Light is absorbed by endogenous chromophores found in living tissue, including hemoglobin, melanin, and lipid.[3-7] In general, light absorption and scattering decrease with increasing wavelength. Below ~700 nm these effects result in shallow penetration depths of only a few millimeters.[3] Thus, in the visible region of the spectrum, only superficial assessment of tissue features is possible.

Above 900 nm, water absorption can interfere with signal-to-background ratio. Because the absorption coefficient of tissue is considerably lower in the near-infrared (NIR) region (700-900 nm), light can penetrate more deeply, to depths of several centimeters.[3-6] Fluorochromes with emissions in the NIR are not hindered by interfering autofluorescence, so they tend to yield the highest signal-to-background. The combination of increased depth of penetration and reduced autofluorescence makes NIR fluorochromes ideally suited for optical imaging in small animals, and potentially in humans as well (Figure 1).

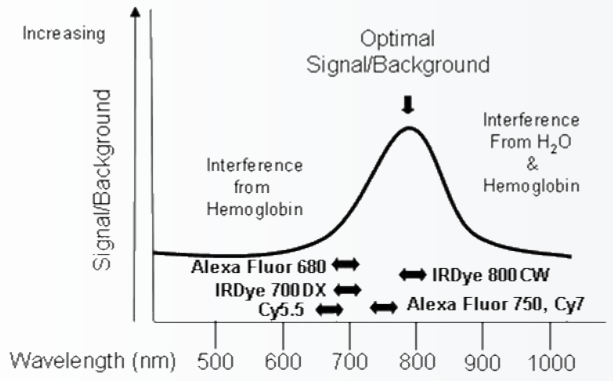


Figure 1. Schematic representation of the region of optimal signal-to-background ratio in tissue. Hemoglobin can interfere below 700 nm, while water interferes above 900 nm. The excitation and emission regions for several dyes commonly used in optical imaging are also indicated.

Near-infrared Fluorochromes

A key to enabling optical imaging has been the development of suitable NIR fluorochromes. Important criteria for effective optical imaging fluorochromes include: excitation and emission maxima in the NIR between 700-900 nm; high quantum yield;[3, 5-7] chemical and optical stability; and suitable pharmacological properties including aqueous solubility, low non-specific binding, rapid clearance of the free dye, and low toxicity. The dyes most commonly used for fluorescent optical imaging are listed in Table 1.

Table 1. Excitation and emission maxima of fluorescent dyes commonly used for optical imaging

Fluorophore	Excitation Max (nm)	Emission Max (nm)
Cy5.5	675	695
Alexa Fluor 680	679	702
IRDye 680RD	680	709
Alexa Fluor 700	702	723
IRDye 700DX	689	700
Alexa Fluor 750	749	775
Cy7	749	775
IRDye 800CW	774	805

The cyanine dye Cy5.5 has been used frequently for *in vivo* imaging. The excitation/emission maxima (675 nm/695 nm) are close to the NIR region, yielding acceptable signal-to-background. Other dyes with excitation and emission maxima in this region include IRDye® 700DX, IRDye 680RD, Alexa Fluor® 700, and Alexa Fluor 680. Although excitation and emission wavelengths of these dyes are maximal in the more favorable red region, they do not give the optimal performance that can be achieved by moving farther into the NIR. Thus, they are best used in situations when the highest sensitivity is not required.

However, the phthalocyanine dye IRDye 700DX has properties that may make it attractive for imaging. IRDye 700DX is considerably less sensitive to photobleaching than many organic fluorochromes; Peng et al.[8] have shown that IRDye 700DX is 100 times more photostable than Alexa Fluor 680 and Cy5.5. In addition, members of the phthalocyanine dye class have been used for photodynamic therapy in the treatment of several types of cancer.[9] The photodynamic characteristics relevant to tumor therapy require prolonged exposure to the light source and therefore would not interfere with tumor biology during the short exposures used for imaging. Therefore, phthalocyanines have the potential to serve not only as imaging agents but also therapeutics.

The most widely used dyes with true NIR character include IRDye 800CW, Cy7, and Alexa Fluor 750. The excitation/emission ranges for these dyes are shown schematically in Figure 1. In particular, IRDye 800CW has excitation/emission maxima of 774 nm/789 nm, which are centered at the optimal wavelength for *in vivo* imaging. This dye has been shown to be superior in performance to Cy5.5 in terms of signal-to-background.[3, 7, 10]

Targets and Ligands

Many targeted optical probes have been described in the literature. Targets include cell surface receptors, metabolic pathways, hormone receptors, apoptotic markers, and enzymatic activities.[11] High affinity probes may be developed by rational investigation, combinatorial chemical synthesis, or phage display. An effective agent reaches the target at a sufficient concentration and/or is retained there for a sufficient length of time to be visible at the time of imaging. Obstacles such as rapid excretion, metabolism, non-specific binding, and physical barriers to the agent reaching the target must be overcome in order for a targeted optical agent to function robustly.

Delivery barriers present the greatest obstacle but can be circumvented. One approach is to take advantage of normal cellular transport and endocytic processes by targeting surface receptors or transport pathways that internalize the optical agent. Growth factor receptors are an example in which the binding of a fluorochrome-labeled agent stimulates internalization via endocytosis. This has the added benefit of amplifying the fluorescent signal, since the fluorochrome will accumulate in the target cells. A second approach is to incorporate a peptide membrane translocation signal into the optical agent such that active transport of the imaging agent across the cell membrane results. Signal peptides such as the HIV tat peptide have been successfully used to transport nanoparticles into cells.[12]

Non-specific binding is another critical issue for noninvasive tumor imaging. *In vivo,* the inability to eliminate unbound ligand can cause low signal-to-background. In addition, non-specific binding or retention of the optical probe will yield false positive results. Careful assessment of the optical agent clearance pattern and verification of specific signal by competition experiments can address this issue.

Antibody Conjugates

Many of the first fluorochrome-conjugated targeted imaging agents were antibodies. For example, indocyanine-conjugated monoclonal antibodies against cells derived from a squamous cell carcinoma have been used to image A431 cell xenografts in nude mice.[13] Detection of the xenografts was sensitive and specific. Cy3, Cy 5, and Cy5.5-conjugated monoclonal antibodies have been used to direct SSEA-1 for detection of MH-15 teratocarcinoma xenografts.[14] In this study fluorescence did concentrate in the tumor, but significant background from the conjugate was observed in the kidneys and bladder. The NIR dye, Cy5.5, appeared to yield the best signal-to-background. Lastly, minibodies directed against the extra-domain B of fibronectin and conjugated to Cy7 have been used to successfully image atherosclerotic plaques in a mouse model.[15]

Although antibody conjugates have been successfully used, they have several undesirable features, primarily due to the size of the antibody. Larger molecules, such as antibodies, can elicit an adverse immune response from the host animal, and their long half-life in the blood results in high background fluorescence and long clearance times.[16,17] In addition large biomolecules are often taken up preferentially by the liver, precluding imaging of liver-proximal organs.[18] Finally, in order for the contrast agent to effectively penetrate to the target site, it must diffuse from the vasculature to the site of the pathology; larger molecules show very poor diffusion characteristics which may prevent them from reaching the target site.[19]

Tumor Surface Proteins

Tumor surface proteins offer diverse possibilities for targeting of optical probes. An excellent example is a receptor-binding ligand. Growth factors are a popular choice for optical imaging agents because, in addition to high affinity targeting, the ligand and its fluorescent label are often internalized by the normal endocytic pathway, amplifying the signal in the tumor cells. Fluorochrome-labeled epidermal growth factor (EGF) has been a versatile tumor imaging agent because the epidermal growth factor receptor (EGFR) is overexpressed on the surface of many types of tumor cells.[20-24] In a study measuring the diffusion of small molecules across the extracellular space in rat brains, Thorne et al.[25] showed that EGF labeled with Oregon Green 514 could be used as a reporter. Ke et al.[26] used Cy5.5-labeled EGF to target human breast tumor cells implanted in mice. The EGF-Cy5.5 accumulated specifically in the tumors and uptake was blocked by pretreatment of animals with C225 anti-EGFR monoclonal antibody (cetuximab).

Similarly we have demonstrated the utility of EGF labeled with IRDye® 800CW for analysis of orthotopic prostate tumors in mice.[27, 28] IRDye 800CW EGF accumulated specifically in the tumors, and metastatic spread of the primary orthotopic tumor to the para-aortic lymph nodes was detected. EGF is known to stimulate tumor growth, and this is an important concern for its use as an imaging agent. Comparison of tumors excised from animals injected with labeled EGF only at the study endpoint to tumors excised from mice that

had been injected at weekly intervals over a six week period demonstrated no stimulation of tumor growth by the fluorochrome-conjugated ligand used in this longitudinal study.[27]

Endostatin, a 20 kDa fragment of collagen XVIII, is a potent inhibitor of angiogenesis. Using Cy5.5-labeled endostatin, Citrin et al.[29] were able to demonstrate that the labeled optical agent bound specifically to tumor xenografts in C57BL mice, suggesting that the anti-angiogenic effect of endostatin is due to its action directly on the tumor cells rather than a general anti-angiogenic effect. The Cy5.5-endostatin bound specifically to the tumor and the signal persisted for up to seven days post-intraperitoneal injection.[29]

Apoptosis plays an important role in a number of disease pathologies, particularly cancer. One of the earliest markers of apoptosis is the externalization of phosphatidylserine. Annexin V, a 36 kDa protein, exhibits high affinity for phosphatidylserine and has been used to detect apoptosis *in vivo*. Petrovsky et al.[30] and Ntziachristos et al.[31] demonstrated the utility of Cy5.5-labeled annexin V for detection of apoptosis in a mouse tumor model. This marker may be useful in studying the anti-proliferative effects of chemotherapeutic agents on a variety of cancers.

Somatostatin receptors and their ligands have been used as a targeting system for tumor imaging and radiotherapy of cancer for over 15 years. Radiolabeled synthetic analogues of somatostatin have been used to successfully image gastric or pancreatic tumors as well as small cell lung cancer (SCLC).[32, 33] SCLC is a major cause of death in western countries. The substitution of fluorochrome-tagged somatostatin and several analogues has enabled imaging of human H69 SCLC tumor xenografts in mice using fiber-optic spectrofluorimetry. Thus, near-infrared conjugated peptides may have significant clinical impact on tumor detection by endoscopy, mammography, and intraoperative imaging.

Peptides and Small Molecules

Because of their small size, convenience of handling and attractive pharmacokinetic properties, peptides are useful agents for *in vivo* imaging. Peptides targeting integrins, a family of cell surface receptors that broadly regulate tumor growth, metastasis, and angiogenesis, have been successful agents for imaging neovascular density.[34-41] Chen et al.[37] and Achilefu et al.[41] used an NIR peptide conjugate, arginine-glycine-aspartic acid (RGD), targeting αvβ3 integrin, to detect and image tumor xenografts and to monitor angiogenesis. Tumor uptake of the Cy-5.5 RGD peptide was specific and could be blocked by pre-injection with unlabeled RGD. Houston et al. [42] used an RGD peptide doubly labeled with ^{111}indium and IRDye 800CW to directly compare NIR optical imaging with scintigraphy. The authors found the signal-to-background ratio significantly higher for IRDye 800CW than for the radioactive label. In general, small molecule and peptide imaging agents clear the system quickly, translating to a reduction in fluorescent background when imaging. In all cases described above, the agents cleared through the kidneys without pooling in the liver. The small size also greatly reduces the possibility of an adverse immune response. Finally, fluorochrome-labeled small molecules and peptides penetrate to the target efficiently because of their increased ability to diffuse from the vasculature.

Activatable Probes

The last category of fluorescent agents, activatable probes, or "molecular beacons,"[43, 44] specifically yield a fluorescent signal only when activated by an enzyme target. Most activatable probes are protease substrates. Protease levels are elevated in the extracellular space of many tumors, where they play a role in invasion and metastasis, [45-49] and present a physically accessible target. Typically these probes contain multiple NIR fluorochromes coupled to peptide sequences that can be cleaved by the protease. The proximity of the fluorochromes to each other results in quenching that is relieved, upon cleavage by the target protease, to generate a fluorescent signal. Use of fluorochrome/quencher pairs separated by the peptide target sequence has also been reported.

Proteases that have been targeted by activatable probes include cathepsins, matrix metalloproteinases (MMPs), prostate-specific antigen, thrombin, caspase-3, and interleukin-1β converting enzyme.[50] An example of the signal sensitivity that can be achieved using these agents is the visualization of MMP-2 activity.[50-52] *In vitro*, the authors observed an 850% increase in NIR fluorescence intensity when the probe was cleaved and specific activation could be blocked by MMP-2 inhibitors. MMP-2 positive human fibrosarcomas were visualized and differentiated from MMP-2 negative mammary adenocarcinomas using this probe.

Developing an Optical Imaging Agent

Below, we will discuss some of the critical parameters involved in developing, validating, and using an optical imaging agent. We present an overview of the process as it applies to tumor imaging, using as an example the IRDye

800CW EGF imaging agent we recently developed.[27, 28] Again, although we use this agent as an example, the principles described will be applicable to any dye-conjugate optical agent.

Conjugating Probes with NIR Dyes

Development of an optical targeting agent begins with covalent attachment of an NIR dye to the targeting compound. Many dyes are available with an N-hydroxysuccinimidyl (NHS) ester, which is activated for simple one-step coupling to free amines. NHS esterified NIR dyes may be purchased in bulk or in pre-packaged labeling kits. Methods for removal of unreacted dye may include HPLC, FPLC, or dialysis. Purification will be dictated by the molecular weight and chemical properties of the conjugates.

For *in vivo* imaging applications, the dye/protein ratio of the conjugate may affect biological or biochemical activity of the protein, signal-to-background ratio, clearance, and biodistribution.[52] The optimal ratio of coupling is unique to each targeting agent. For example, Ntziachristos et al.[31] found that annexin V labeled with Cy5.5 at a dye/protein ratio of 1 retained its ability to bind phosphatidylserine on the surface of cells induced for apoptosis, while the same molecule labeled at a dye/protein ratio of 2.4 had lost its binding capability.

If the optical imaging agent is based on a commercially available reagent, such as an antibody or receptor ligand, differences in preparation and purification may impact performance. We conjugated IRDye® 800CW to human recombinant EGF from five different commercial sources, at equivalent dye/protein ratios, and evaluated relative signal intensity by In-Cell Western™ Assay.[28] As shown in Figure 2, there were considerable differences in the amount of EGF bound to the cells. Variations in signal strength measured in this fashion have the potential to predict probe performance *in vivo* as we have demonstrated.[28]

Testing the Performance and Specificity of an Optical Imaging Agent *in vitro*

Validation of targeting agent efficacy and specificity *in vitro* is an important prelude to animal studies. Specificity can be demonstrated on cells in culture or in suspension by blocking the target with an antibody or by competition with the unlabeled agent. In targeting somatostatin receptors, Becker et al.[53] used a flow cytometric assay to quantify binding of the agent. As mentioned previously, Ntziachristos et al.[34] used whole cell competition assays to demonstrate both probe specificity and binding affinity. Another group used a

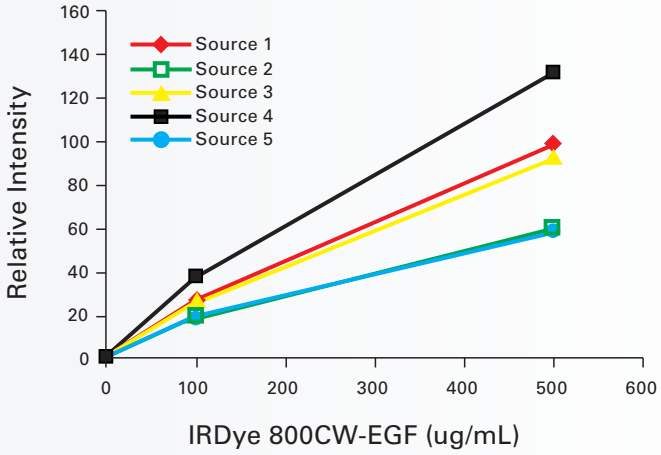


Figure 2. Binding activity of recombinant EGF from different commercial sources. EGF from five sources was conjugated to IRDye 800CW at similar dye/protein ratios and each was evaluated for its ability to bind confluent A431 monolayers by In-Cell Western as we have previously described.[27]

radioactive displacement assay.[54] ^{125}I-labeled echistatin, an integrin-binding ligand, was added to integrin-expressing cell cultures and allowed to bind. The degree of displacement of the radioactive echistatin by Cy7-labeled RGD peptide was used as a measure of specificity and binding affinity.

We have used the In-Cell Western™ (ICW) or cytoblot to evaluate IRDye® 800CW EGF for binding and specificity prior to actual testing in mice.[27, 55] In this assay, PC3M-LN4 and 22Rv1 human prostate adenocarcinoma cells were cultured in microtiter plates and treated with serial dilutions of labeled EGF (Figure 3A) to verify high affinity binding of EGFR-targeted dye relative to the low binding of free dye alone (Figure 3B). Specificity was then established in two ways: by blocking access of EGF to its receptor with the anti-EGFR monoclonal antibody C225 (also known as cetuximab or Erbitux, Figure 3C); and by competition with unlabeled EGF (Figure 3D). Fluorescence of the microplate was quantified by NIR imaging, and a DNA stain was used to normalize variations in cell number.

Characterization of the targeting agent in a cell-based assay can simplify probe development. While success in a cell-based assay format does not guarantee performance *in vivo*, failure in this assay is generally predictive of failure in the animal. In addition the competition assays developed may subsequently be useful for demonstrating specificity in animal experiments.

Animal Care and Use

All research animals must be handled according to protocols that comply with the animal care and use

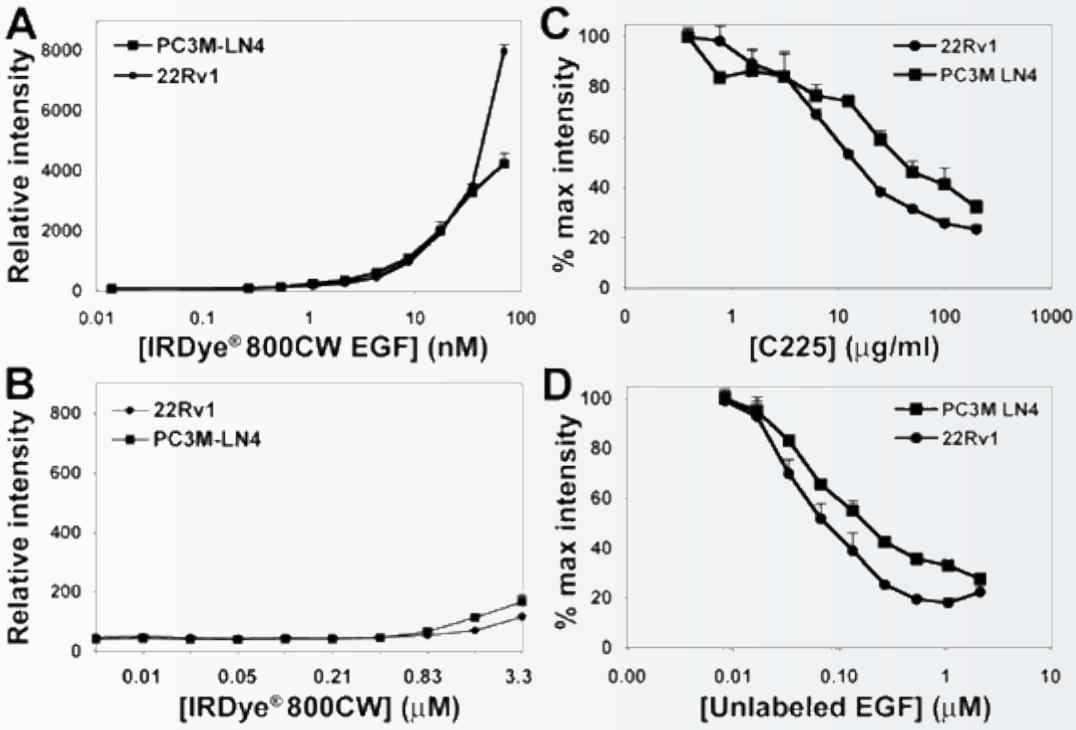


Figure 3. DNA binding specificity of IRDye® 800CW EGF for cultured PC3M-LN4 and 22Rv1 cells. Monolayer cells in 96-well microplates were incubated with increasing concentrations of IRDye 800CW EGF **(A)** or unconjugated, non-reactive IRDye 800CW **(B)** and normalized with TO-PRO-3 DNA staining. To demonstrate EGF receptor-targeting specificity, cells were incubated with 70 nM IRDye 800CW EGF in the presence of increasing concentrations of either C225 blocking antibody **(C)**, or unlabeled EGF **(D)**. The 800 nm signal, normalized to the 700 nm control, is plotted as the mean ± SD of three replicate wells.[27] This Figure reproduced with permission from the American Journal of Pathology.

regulations of the country and institution where the research will be performed. In the United States these regulations are described in a document compiled by the National Institutes of Health Organization for Lab Animal Welfare, available at *http://grants.nih.gov/grants/olaw/GuideBook.pdf.*

Considerations for Tumor Model Selection

An ideal tumor model system exhibits minimal background interference. Although much of the autofluorescence in the animal is ameliorated by imaging at NIR wavelengths, some anatomic regions inherently maintain higher fluorescent signals. For example, natural fluorescence from compounds in the animal's diet accumulates, and can be visualized, in the abdominal cavity. Organs involved in clearance of the dye, such as the liver and kidney, may also accumulate signal. Tumors arising in areas remote from these organs are detected with less ambiguity and higher sensitivity. In the case of subcutaneous xenografts, the placement of transplanted tissue in the flank, shoulder, or leg of the animal can minimize these interfering factors.

The model system selected will depend on the aims of the study. Transgenic, chemically induced, and spontaneously arising mouse models are available that recapitulate many aspects of the genesis, progression, and clinical course of human cancers. The National Cancer Institute of the United States has organized a Cancer Models Database (caMOD) to facilitate identification of appropriate models for cancer experimental design (*https://cancermodels.nci.nih.gov/camod/login.do*).

Three strains of immunodeficient mice are commonly used in tumorigenesis and metastasis research with human cell lines: nude, SCID and Rag1. The nude mouse (athymic; nu/nu) has a disruption in the Foxn1 gene, resulting in an absent or deteriorated thymus gland, diminished T cell numbers, and a severely impaired cellular immune system.[56] The resultant hairless phenotype also makes the nude mouse ideal for optical imaging, since animal hair blocks and scatters light. Over time these animals may regain partial cellular immune function, so nude mice may not be the best host for longer-term studies of tumor biology. SCID (severe combined immunodeficiency) mice lack both mature

T and B lymphocytes [57] and are an effective alternative to nude mice in cases where the partial immune system of the nude mouse presents a problem. The Rag1 mouse also lacks T and B lymphocytes and is not able to undergo V(D)J recombination. Thus, it fails to produce T-cell receptors and immunoglobulin molecules for antigen identification.[58-60] All three mouse models bear phenotypic and background strain characteristics that may impact a research project.

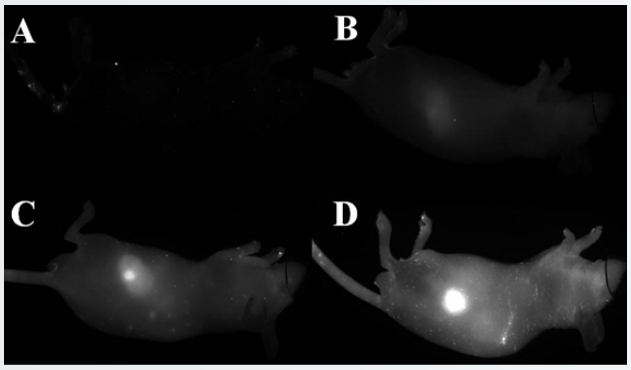


Figure 4. Dose response for an IRDye® 800CW conjugated optical imaging agent targeting tumors in nude mice. Animals were injected with 1x PBS as a negative control (A), or with 2.5 nmol (B), 5.0 nmol (C), and 10 nmol (D) of a tumor targeted IRDye 800CW conjugate. Images were acquired 24 hr post-injection.

Immunocompromised mice require Specific Pathogen Free (SPF) handling to avoid introducing infections. Institutional training is obligatory and includes instruction in the use of infection barriers, sterilized food, water and bedding, disinfected imaging surfaces, gloved handling, and aseptic technique.

Reducing Optical Interference for Tumor Imaging

Chlorophyll, which is often present in animal chow, absorbs at 655 nm and 411 nm, and fluoresces at 673 nm, producing strong signal in the abdominal cavity. For optimal fluorescent imaging performance, purified food formulations that do not contain plant products may be used. Fasting of the animal prior to imaging has been used in some studies and requires prior approval from the institutional committee governing animal care.

The hairless phenotype of the nude mouse makes it an ideal choice for NIR optical imaging, but this model may not always be appropriate for the research objectives. Thus, hair removal in the imaging region may be important for optimal signal detection. Animal hair interferes with imaging by blocking, absorbing, and scattering light. We evaluated this by implanting a tube containing IRDye® 800CW in the chest cavity of a deceased SCID mouse and imaging the animal before and after shaving. Following shaving, fluorescent signal increased by >50% (data not shown). An additional 12% signal was detected when the animal was treated with a depilatory cream (Nair, Church and Dwight Co., Inc.) presumably due to the removal of hair stubble.

Establishing Tumors in the Animal

For assays of tumorigenic and metastatic potential using cultured human cells or tissue may be implanted in animals subcutaneously, intravenously, intraperitonally, or orthotopically (i.e.; prostate cells implanted in mouse prostate). These assays are called xenografts, since they involve transplantation of cells, tissue, or organs from one species to another.

Depending on the aggressiveness of the cell line, we have established subcutaneous xenografts in mice by injection of 0.5-1 x 10^6 cells in ≈100 µL cell suspension. Tumors become palpable within 7-10 days. Orthotopic injections require fewer cells (we have used 1 x 10^5 cells) and reduced injection volume (10 µL), as the anatomical structure of the prostate is small. Tumors form in 2-3 weeks and metastasize within ≈6 weeks.[27, 28]

Probe Dosage and Administration

Tumor type may dictate the optical imaging agent selected and its optimal parameters for use. For example, A431 epidermoid carcinoma cells express EGFR at a much higher level than normal cells. However, HeLa ovarian carcinoma cells have low expression of EGFR. If IRDye 800CW EGF is used as an optical probe for both of these cell types, binding of the labeled ligand would be expected to vary dramatically. An optimal dosage of the imaging agent will afford the best signal-to-background, clearance, and imaging results. Excessively high doses will clear poorly, while a low dose may not saturate tumor uptake. Figure 4 illustrates a dose response to increasing concentrations of an optical agent.

Although background increases with the amount of probe administered, the signal is also greater at the highest dose. The quantified signal within the tumor, normalized to the mean background in several irrelevant surrounding regions, allowed us to determine that the optimal specific signal occurred at the two highest doses.

The route of targeting agent administration can affect its specific uptake and non-specific clearance. Intravenous injection via the tail vein or supraorbital cavity leads to rapid systemic dispersion (Figure 5). This method is appropriate for targeted contrast agents that bind a ubiquitous surface receptor present at a greater concentration on tumor cells. Uptake by the tumor cells is within the time window of agent clearance, and potential background from prolonged exposure to the probe is minimized. Performance of an agent that functions by incorporation into bone, however, may be enhanced by intraperitoneal injections, which prolong exposure through slower dispersion (Figure 5).

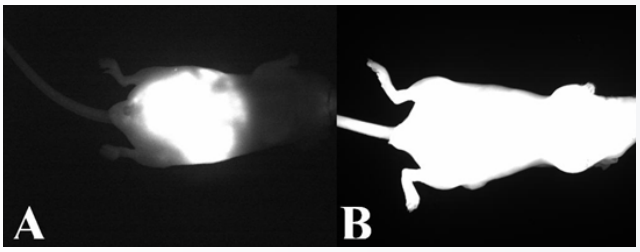


Figure 5. Impact of administration site on optical imaging agent dispersion in mice. Images were acquired approximately 15 minutes following intraperitoneal (A) or intravenous (B) injection of equivalent amounts of an optical imaging agent into a nude mouse.

Evaluation of Dye and Optical Agent Clearance

Performing initial imaging time courses following injection of the chosen targeting agent will establish the optimal time point for sensitive tumor analysis. The time course analysis begins with the unconjugated fluorochrome chosen for optical imaging, which should not be appreciably retained in the animal. An example of measurement of the clearance of IRDye® 800CW is shown in Figure 6. Other dyes may have different clearance characteristics. Secondly, a time course of agent clearance from non-tumor-bearing control animals, using the intended dose for tumor imaging, will yield a blueprint for whole-body non-specific background to assist interpretation of tumor images. Figure 7 shows the results of a clearance experiment with IRDye 800CW EGF in a tumor-negative mouse.

Finally, once it has been determined that the probe does not accumulate non-specifically, the time course is repeated with tumor-bearing animals to determine the imaging time at which the signal-to-background is greatest in the tumor. For IRDye 800CW EGF, mice bearing PC3M-LN4 subcutaneous xenografts were imaged over a three-day period. By 24 h, most of the initial fluorescence was gone from the non-tumor areas (Figure 8). However, signal-to-background continued to increase beyond this time point. Optimizing the time of imaging maximizes sensitivity for challenging applications such as detecting metastatic spread of a tumor.

Confirming probe specificity in *in vivo* clearance studies and optimization of timing will minimize non-specific fluorescence, but imaging artifacts may be misinterpreted nonetheless. For example, an apparent tumor detected in the liver upon targeting with an antibody probe that pooled in the liver should be confirmed by a competition test. One method is to pre-inject tumor-bearing animals with an excess (for example, 100-fold) of the unlabeled form of the optical agent. The labeled agent is injected shortly thereafter. Probe specificity is reflected as a decrease in the total fluorescence signal in animals that were pre-injected with the unlabeled agent. Specificity of Cy5.5-labeled EGF was demonstrated in this way by Ke et al.[26] Even if differential signal is not readily detected in intact animals, imaging analysis of excised whole and sectioned tumors may reveal the competition.

We used this approach to assess specificity of IRDye 800CW EGF imaging in prostate tumors.[27] Mice bearing either PC3M-LN4 subcutaneous or orthotopic tumors were injected with IRDye 800CW EGF; some animals were preinjected with C225 anti-EGFR monoclonal antibody. When we injected tumor-bearing animals with the probe and subsequently performed fluorescence imaging of tumor sections, IRDye 800CW EGF was clearly visible not only in the primary tumors, but also in lymph nodes extracted from the animals (Figure 9). Tumor-bearing animals that were preinjected with C225 exhibited a 33-49% decrease in fluorescent signal, indicating that binding of the labeled ligand was specific for EGFR.

An alternative approach to demonstrating specific signal is to quantify targeting agent uptake by both tumor and non-tumor tissue. For examining integrin-binding agents, Becker et al.[53] used an RGD peptide doubly labeled with ^{125}I and an indocarbocyanine dye. Radioactive content of the tumor, heart, liver, kidneys, and brain was quantified following imaging of the tumor. Thus, the authors were able to express the uptake of the probe as the percentage of injected dose per gram of tissue. This approach may also be useful for characterization of metabolic probes, for which high doses of unlabeled competitor may be toxic.

Summary and Conclusions

Fluorochrome-labeled molecular probes are valuable tools for non-invasive longitudinal study of tumorigenesis and

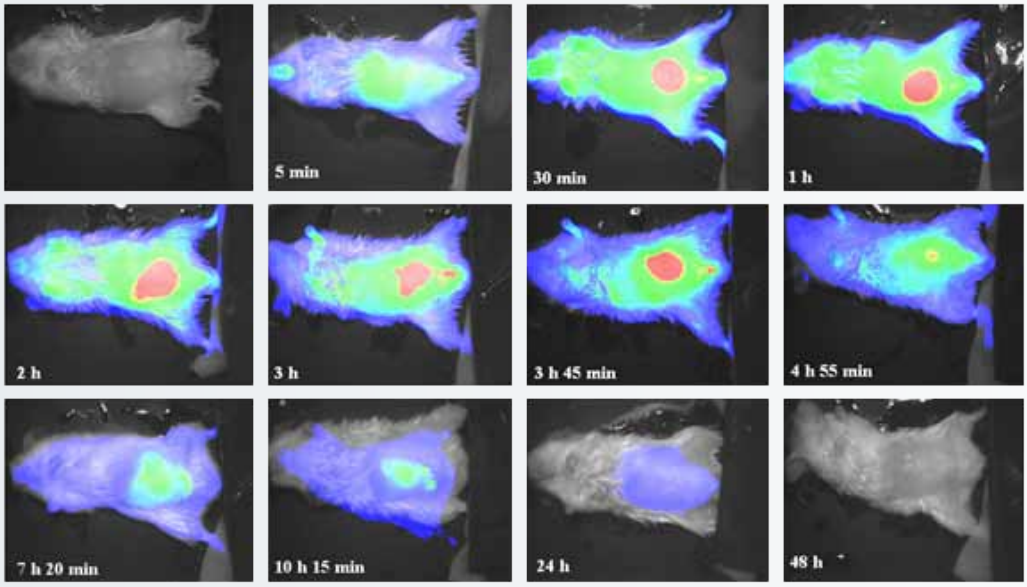


Figure 6. Clearance kinetics of unconjugated IRDye® 800CW. A SCID mouse was injected with 1 nmol of IRDye 800CW intravenously and monitored over time for complete clearance of the dye. Imaging times post injection are indicated on each individual image. Pseudocolored fluorescence is superimposed on the white light image of the mouse to illustrate rapid dispersion of the dye followed by complete clearance.

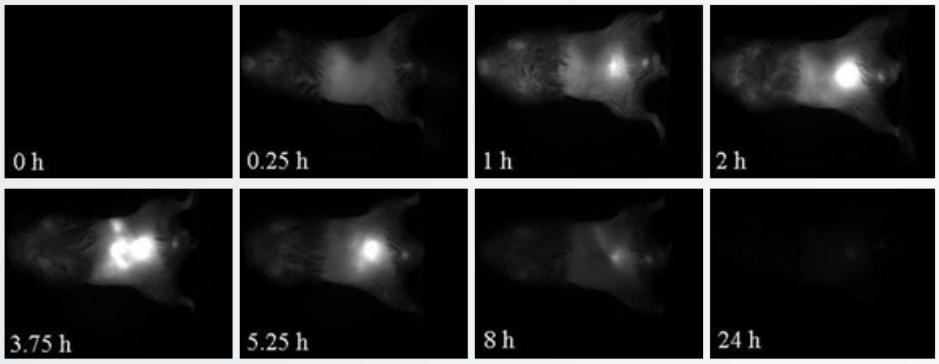


Figure 7. Time course for clearance of IRDye® 800CW EGF from a non-tumor-bearing mouse. A SCID mouse was injected with 1 nmol of the EGF-conjugated optical agent intravenously and imaged at the indicated time points over a 24 h period. Analysis of the abdominal region showed that >90% of the fluorescence disappeared in 24 h, and by 48 h (not shown) the probe had cleared completely.

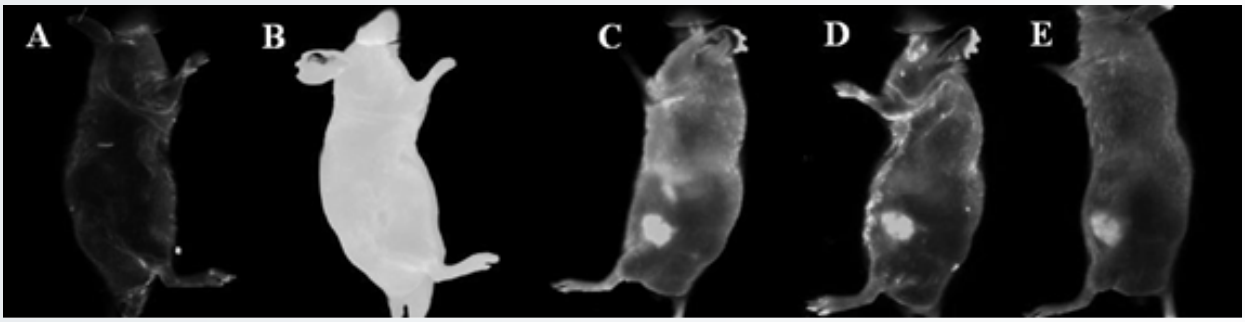
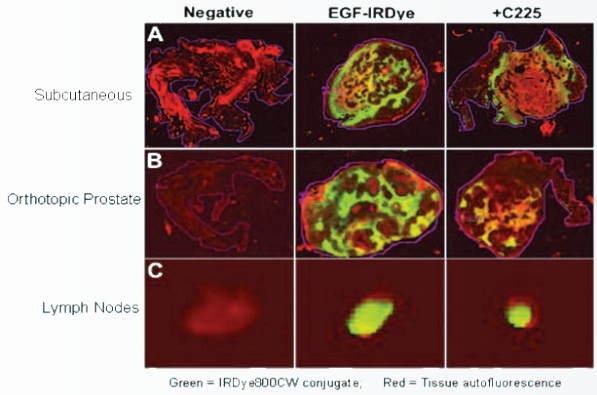


Figure 8. Time course for accumulation of IRDye® 800CW EGF in a subcutaneous tumor. Images of a nude mouse were collected prior to injection (A), or at 20 min (B), 24 h (C), 48 h (D), and 72 h (E) following intravenous injection of the animal with 1 nmol of IRDye 800CW EGF.



Fluorescence signal*/mm² by tissue

Treatment	Subcutaneous	Orthotopic	Nodes
Vehicle Only	0.01	0.14	0.01
IRDye 800CW EGF	0.69	1	1.15
C225 + IRDye 800CW EGF	0.38	0.52	0.76
Percentage Signal Blocked	45.7	49.0	33.7

***Integrated intensity in the 800 nm channel normalized to unit area of the section or tissue sample.**

Figure 9. Demonstration of specific tumor targeting *in vivo* for the IRDye 800CW EGF optical imaging agent. Tumors and lymph nodes were excised from animals bearing either subcutaneous (A) or orthotopic (B) prostate tumors, as indicated, injected intravenously with IRDye 800CW EGF or pre-injected with C225 anti-EGFR monoclonal antibody prior to dosing with IRDye 800CW EGF. The corresponding table presents IRDye 800CW EGF fluorescence signal per area tissue for vehicle control, optical agent only, and C225 competition for each tumor type (subcutaneous and orthotopic) and lymph nodes from the orthotopic tumor model (images in panel C).

metastasis, preclinical studies of the effects of therapeutic agents, and pharmacokinetic and pharmacodynamic studies of drug-target interactions. Because of this, these probes have significant potential for translation to human clinical use.

Several applications may expand the clinical utility of fluorochrome-labeled probes. For example, accurate definition of tumor margins is crucial to the therapeutic outcome of many surgical oncology procedures. A multimodal imaging agent, consisting of a magnetic iron oxide nanoparticle and Cy5.5, has been used as a preoperative nuclear magnetic resonance contrast agent and intraoperative optical probe to define the tumor margins in a rat gliosarcoma model.[61] Similar intraoperative imaging technology is being developed in the laboratory of Frangioni.[62] These procedures would allow a surgeon to identify and locate the tumor mass by MRI and subsequently remove the tumor accurately with visual guidance from an intraoperative near-infrared fluorescent imager.

Photodynamic therapy (PDT) is an application that has been used in oncology for over two decades.[9] A photosensitizing agent delivered to malignant tissue is exposed to light, generating cytotoxic singlet oxygen. The result is cell death through the induction of apoptosis, microvascular damage, and antitumor immune response. The major class of dyes used for this approach is phthalocyanines such as IRDye® 700DX.[8] NIR dyes conjugated to anti-tumor therapeutics such as Erbitux (anti-EGFR monoclonal antibody) may have similar clinical appeal for simultaneous treatment and monitoring of anti-cancer therapy.[63]

Imaging based on expression of luciferase or a fluorescent protein such as GFP has facilitated examination of intracellular signaling events *in vivo*. Hybrid gene constructs in which either the luciferase or GFP gene is placed under control of an inducible promoter, responsive to a signaling pathway of interest, have been used to directly assess the effects of anti-tumor agents on the gene regulation *in vivo*. A similar reporter system that directly images β-galactosidase activity on a far red substrate has been recently reported.[64] This fluorescent reporter system could provide a means for creating hybrid expression units to examine *in vivo* gene expression and regulation of a number of important pathways in cancer.

NIR-based imaging instrument technologies designed for human clinical use are in various stages of development.[62, 65, 66] Combined with new NIR-labeled biomarkers, these will expand the clinical options available for cancer management in the near future. Several excellent reviews describe the use of targeted markers in animal studies.[7, 67]

In this review we have discussed the basic validation of fluorochrome-labeled molecular probes. Although near-infrared optical excitation sources provide deeper tissue penetration, they are not great enough to provide unlimited application for human clinical use. Near-infrared imaging agents may be limited to accessible tissues such as breast tumors, or to intraoperative applications, endoscopy, and photodynamic therapy. However, these applications represent significant benefits for research, diagnosis, and treatment. Continued technological innovation in imaging instrumentation, biomarker discovery, and labeling chemistries will foster the clinical use of fluorescent optical imaging.

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BrightSite™ Imaging Agents Chart

BrightSite™ Small Animal Imaging Agents

Mechanism of Probe Retention

P/N	Product	Description	Amt (nmol)	MW (kDa)	Dose (IV)nmol	Ex_{max}	Em_{max}
Cell Surface Protein Binding							
926-08446	IRDye® 800CW EGF	Recombinant human EGF probe; targets overexpression of EGF receptor	20	7.3	1	779	795
926-08946	IRDye 800CW 2-DG	Glucose analog; targets GLUT receptor overexpression, increased glucose metabolism	100	1.3	10-20	774	791
926-09889	IRDye 800CW RGD	RGD (Arg-Gly-Asp); targets overexpression of integrins by tumor cells or during angiogenesis	15	1.7	1	776	792
926-50001	IRDYE 800CW HA	Hyaluronan probe; targets CD44 and other receptors in lymphatic system and lymph nodes	20	8-40	2	775	794
NEW! 926-08445	IRDye 680RD EGF	Recombinant human EGF probe; targets overexpression of EGF receptor	25	7.3	2	674	698
NEW! 926-09888	IRDye 680RD RGD	RGD (Arg-Gly-Asp); targets overexpression of integrins by tumor cells or during angiogenesis	25	1.7	2	673	695
NEW! 926-50000	IRDye 680RD HA	Hyaluronan probe; targets CD44 and other receptors in lymphatic system and lymph nodes	50	8-40	4	671	695
NEW! 929-90030	PSVue® 794	A near-infrared fluorescent probe for detection of apoptotic cells, necrotic cells, bacteria, and other anionic membranes	-	-	-	794	810
Calcium Chelating							
926-09374	IRDye 680RD BoneTag™	Calcium-binding probe; images bone structure and remodeling	40	-	2	675	697
926-09375	IRDye 800CW BoneTag	Calcium-binding probe; images bone structure and remodeling	40	-	2	780	795
Vascular/Lymphatic Contrast							
926-50401	IRDye 800CW PEG	Polyethylene glycol probe; general tumor contrast agent, also used to image surface vasculature and lymphatics	15	30-60	1	778	806
Cell Membrane							
929-90010	CellVue® Burgundy	Lipophilic fluorescent dye; stably integrates into cell membrane	-	-	-	683	707
929-90020	CellVue NIR815	Lipophilic fluorescent dye; stably integrates into cell membrane	-	-	-	786	814
Near-Infrared Fluorophores and Labeling Kits							
929-70021	IRDye 800CW NHS Ester	Labels primary and secondary amines, such as lysine residues in proteins	-	-	-	778	794
929-80021	IRDye 800CW Maleimide	Labels free sulfhydryl (-SH) groups, such as cysteine residues in proteins	-	-	-	778	794
929-09406	IRDye 800CW Carboxylate	Non-reactive form of dye; used as control for dye retention	-	-	-	778	794
NEW! 929-70051	IRDye 680RD NHS Ester	Labels primary and secondary amines, such as lysine residues in proteins	-	-	-	680	694
NEW! 929-71051	IRDye 680RD Maleimide	Labels free sulfhydryl (-SH) groups, such as cysteine residues in proteins	-	-	-	680	694
NEW! 929-08980	IRDye 680RD Carboxylate	Non-reactive form of dye; used as control for dye retention	-	-	-	680	694
928-38040	IRDye 800CW Labeling Kit, High MW	NHS ester reactive dye; for labeling of 3 X 1 mg protein (45-200 kDa)	-	-	-	778	794
928-38042	IRDye 800CW Labeling Kit, Low MW	NHS ester reactive dye; for labeling of 3 X 1 mg protein (14-45 kDa)	-	-	-	778	794
928-38044	IRDye 800CW Labeling Kit, Microscale	NHS ester reactive dye; for labeling of 3 X 100 µg protein (14-200 kDa)	-	-	-	778	794
NEW! 928-38072	IRDye 680RD Labeling Kit, High MW	NHS ester reactive dye; for labeling of 3 X 1 mg protein (45-200 kDa)	-	-	-	680	694
NEW! 928-38074	IRDye 680RD Labeling Kit, Low MW	NHS ester reactive dye; for labeling of 3 X 1 mg protein (14-45 kDa)	-	-	-	680	694
NEW! 928-38076	IRDye 680RD Labeling Kit, Microscale	NHS ester reactive dye; for labeling of 3 X 100 µg protein (14-200 kDa)	-	-	-	680	694

Application	IRDye EGF	IRDye RGD	IRDye 2-DG	IRDye PEG	IRDye HA	IRDye BoneTag	CellVue	PSVue
Tumor Imaging	●	●	●	●	●			
Metabolic Imaging			●					
Inflammation/Arthritis		●	●					
Vasculature (Contrast)				●				
Lymphatic Imaging				●	●			
Lymph Node Imaging				●	●			
Structural Imaging						●		
Cell Trafficking							●	
Apoptosis								●

IRDye® 2-DG Optical Probe

IRDye® 800CW 2-DG is a BrightSite™ fluorescent optical imaging agent that targets cells with elevated rates of glycolysis.[1] Cancer cells often exhibit increased glucose metabolism. Glucose analogues such as ^{18}F-2-deoxy-D-glucose (^{18}FDG) target this characteristic for PET imaging of primary tumors and metastasis. Fluorophore-labeled 2-deoxy-D-glucose (2-DG) is used for cancer imaging in small animals.

IRDye 800CW 2-DG exhibits the expected dose response in both cell-based and animal studies (cell lines tested include A431, SW620, 3T3-LI, and PC3LMN4). Uptake of the agent, which can be blocked by unlabeled 2-DG, enters cells and is localized to the cytoplasm. Although the exact mechanism of uptake is not known, experimental evidence implicates the GLUT transporter system.

1. Kovar, J et al. *Anal Biochem* 384: 254-262 (2009)

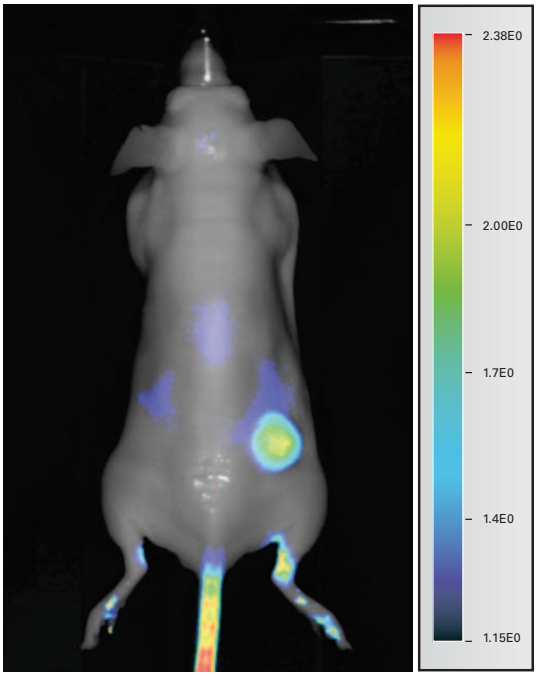

Figure 1. Increased glucose metabolism imaged with IRDye® 800CW 2-DG. Subcutaneous A431 tumor was detected. Image acquired with Pearl® Impulse.

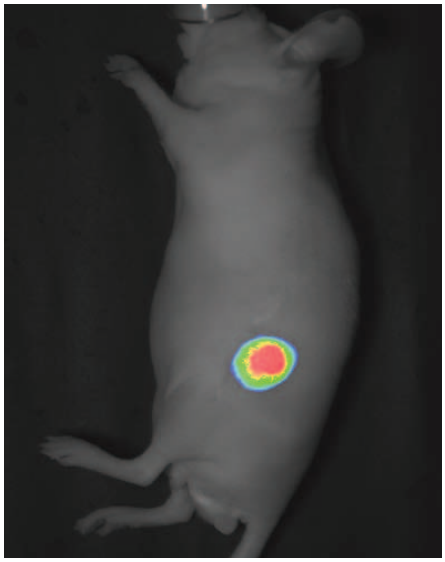

Figure 2. Tumor imaging with IRDye® 800CW 2-DG. PC3M-LN4 prostate carcinoma was detected on the left hip of a male athymic nu/nu mouse. Mouse was imaged 24 h after injection of probe. Image acquired with Pearl® Impulse.

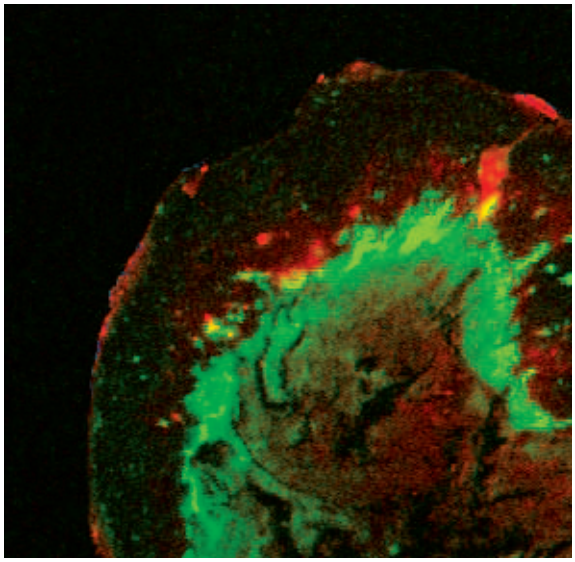

Figure 3. Uptake of IRDye® 800CW 2-DG probe by hypoxic tissue surrounding the necrotic center of a tumor. A431 tumor was excised, fixed, and embedded. Tissue sections were then imaged at 21 µm resolution with Odyssey® Classic Imager. Green indicates probe fluorescence (800 nm) and red indicates tissue autofluorescence.[1]

926-08946 IRDye 800CW 2-DG Optical Probe (100 nmol; approximately 10 injections).....$325.00

Storage: -20°C prior to reconstitution/4°C after reconstitution

IRDye® EGF Optical Probes

IRDye® 800CW and 680RD EGF Optical Probes are near-infrared (NIR) labeled recombinant human epidermal growth factor (EGF). Epidermal growth factor receptor (EGFR) is one of a family of receptor tyrosine kinases, found on the surface of epithelial cells, to which EGF binds. Many types of cancer cells have abnormally high EGFR levels on the cell surface (Table 1). *In vivo* assays of EGF binding have proven effective in monitoring specific solid tumor models that exhibit overexpression of EGFR. EGF, conjugated to LI-COR's IRDye 800CW or IRDye 680RD NIR fluorophores, provides a versatile probe that can be used for *in vitro* and *in vivo* assays as well as whole organ and tissue section analysis.

1. Kovar et al. *Amer J Pathol* 169 (4):1415-1426 (2006)
2. Kovar et al. *Anal Biochem* 361:47-54 (2007)

Table 1. Frequency of elevated EGFR expression by different types of epithelial tumors.

Tumor Type	% of Tumors Over–Expressing EGFR
Head and Neck	80-100
Renal Cell	50-90
Non-small-cell Lung	40-80
Glioma	40-50
Ovarian	35-70
Bladder	31-48
Pancreatic	30-50
Colon	25-77
Breast	14-91

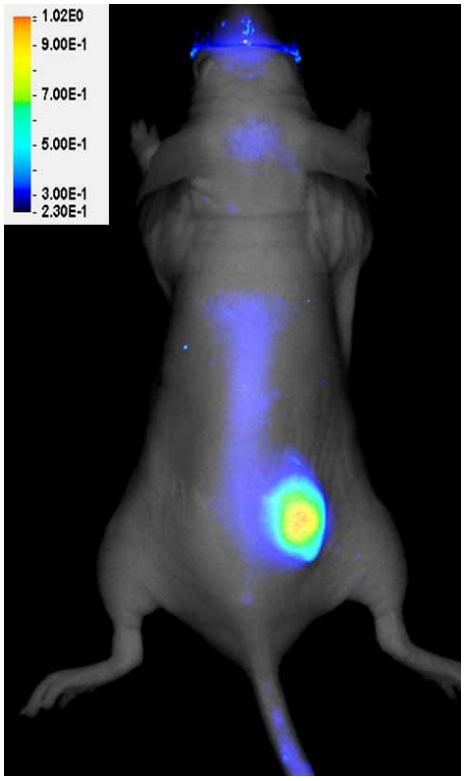

Figure 2. Intravenous administration of IRDye® 680RD EGF (2 nmol) binds to the A431 tumor located on the right hip. Image captured 24 h post injection on the Pearl® Impulse Imaging System.

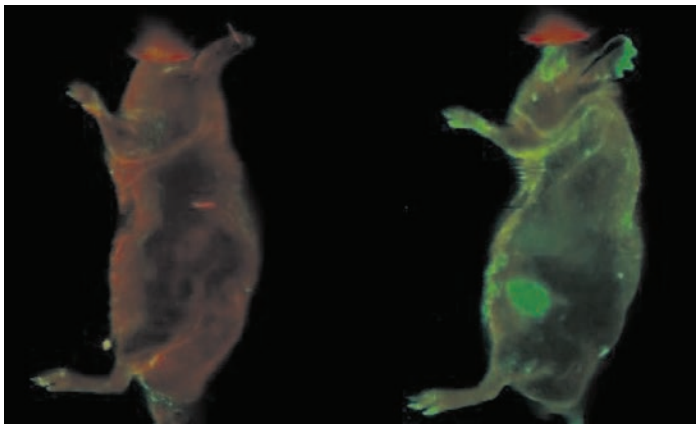

Figure 1. Athymic nu/nu tumor-bearing mice were injected with either 0.9% saline (left) or 1 nmol IRDye® 800CW EGF (right) 72 hr prior to imaging. The Odyssey® Classic Infrared Imaging System and MousePOD® accessory were used for imaging.

926-08446 **IRDye 800CW EGF Optical Probe (20 nmol; approximately 10 injections) $325.00**

Storage: -20°C prior to reconstitution/4°C after reconstitution

926-08445 **IRDye 680RD EGF Optical Probe (25 nmol; approximately 10 injections)** NEW! **.....$325.00**

Storage: -20°C prior to reconstitution/4°C after reconstitution

IRDye® RGD Optical Probes

Integrin receptors mediate interactions with the extracellular environment. The tripeptide recognition motif, RGD (Arg-Gly-Asp), targets integrin receptors such as $\alpha_v\beta_3$. Fluorescent RGD peptide ligands can be used to image and monitor disease *in vivo*.[1] This probe targets overexpression of integrins by tumor cells, or during tumor angiogenesis.

IRDye® 800CW and 680RD RGD were characterized by *in vitro* and *in vivo* binding assays with various tumor cell lines (U87, A431, PC3M-Ln4, and 22Rv1). *In vivo* tumor imaging is shown in Figures 1 and 2. To confirm specificity, binding of the agent was blocked with unlabeled RGD peptide, or with a non-specific RAD peptide.

1. Houston et al. *J Biomedical Optics* 105:054010 (2005)

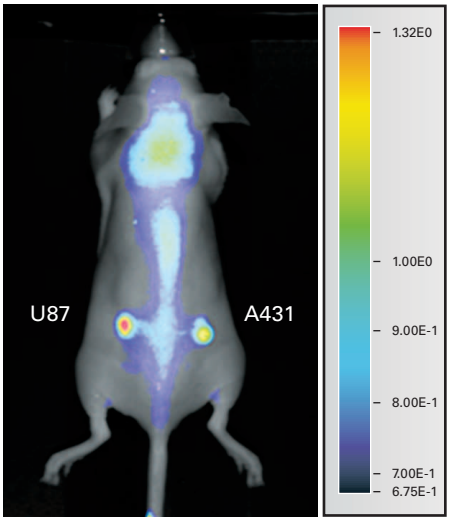

Figure 1. Integrin overexpression imaged with IRDye® 800CW RGD. Subcutaneous tumors were detected in a nude mouse (left hip, U87; right hip, A431). Image acquired with Pearl® Impulse.

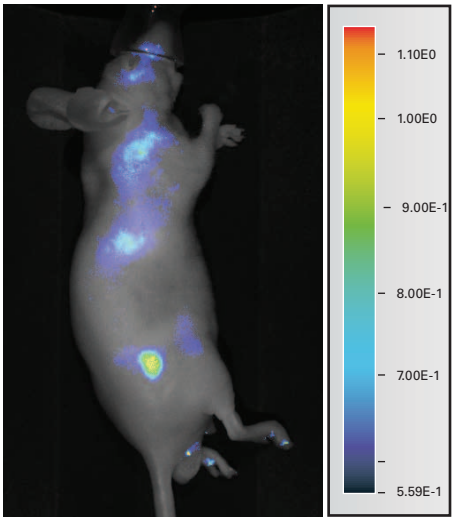

Figure 2. Elevated integrin expression in a U87 tumor, imaged with IRDye® 680RD RGD. Subcutaneous tumor is detected on the animal's hip. Image acquired with Pearl® Impulse.

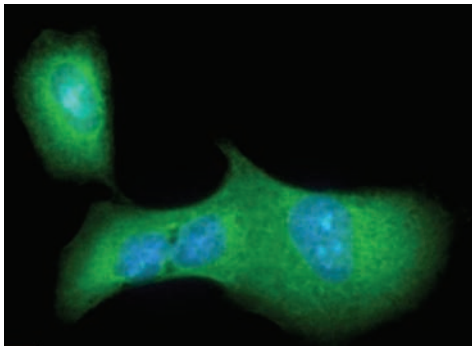

Figure 3. Binding and internalization of IRDye® 800CW RGD. Lung carcinoma cells were incubated with IRDye 800CW RGD (shown in green) and Sytox Green DNA stain (shown in blue). RGD probe localizes to the cytoplasm. Image was captured with Olympus epifluorescent microscope
(for details, visit *www.licor.com/microscopy*).

926-09889 **IRDye 800CW RGD Optical Probe (15 nmol; approximately 10 injections) $325.00**

Storage: -20°C prior to reconstitution/4°C after reconstitution

926-09888 **IRDye 680RD RGD Optical Probe (25 nmol; approximately 10 injections)** NEW! **..... $325.00**

Storage: -20°C prior to reconstitution/4°C after reconstitution

IRDye® HA Optical Probes

Hyaluronan (hyaluronic acid; HA) is an extracellular matrix glycosaminoglycan formed from disaccharide units containing N-acetylglucosamine and glucuronic acid. HA interacts with several cell surface receptors, namely CD44, RHAMM (receptor for hyaluronan mediated motility; CD168), LyVe-1 (lymphatic vessel endothelial HA receptor-1), HARE (hyaluronan receptor for endocytosis), layilin, and Toll-4. It binds to proteoglycans in cartilage and other tissues and fills an important structural role in the organization of the extracellular matrix. The agent is catabolized by receptor-mediated endocytosis and lysosomal degradation after transport, via the lymph, to lymph nodes.

IRDye® 800CW and IRDye 680RD HA may be used to target the lymphatic system and those tumors overexpressing CD44 receptors.

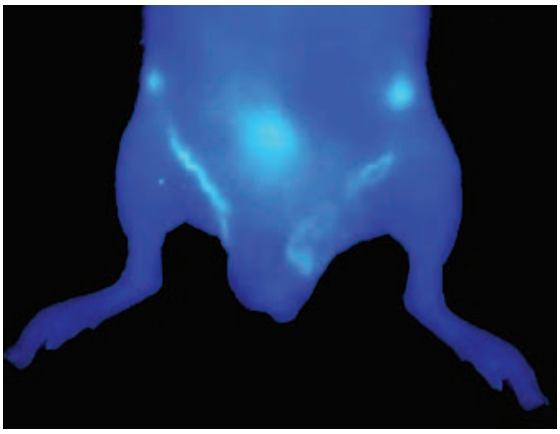

Figure 1. Imaging of lymph vessels leading to subiliac lymph nodes after intradermal injection of IRDye® 800CW HA. Image acquired with Pearl® Impulse.

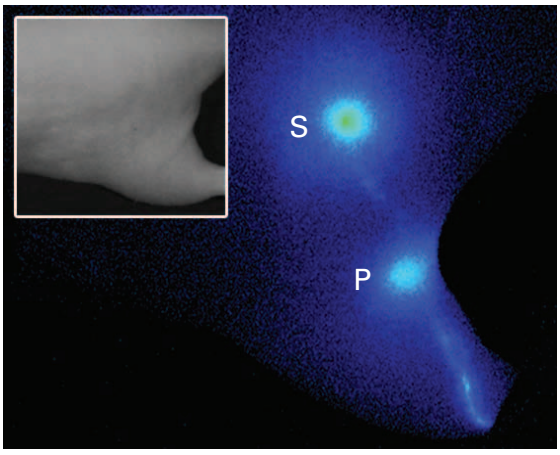

Figure 2. Lymph node imaging with IRDye® 680RD HA. After probe was injected intradermally in the footpad, it pulsed to the popliteal (P) and then sciatic (S) lymph nodes. Lymph tracks between the injection site and these nodes were clearly visualized. Inset shows white light image for orientation. Images acquired with Pearl® Impulse.

926-50001 **IRDye 800CW HA Optical Probe (20 nmol; approximately 10 injections).....$325.00**

Storage: -20°C prior to reconstitution/4°C after reconstitution

926-50000 **IRDye 680RD HA Optical Probe (2 x 25 nmol; approximately 10 injections)** NEW! **.....$325.00**

Storage: -20°C prior to reconstitution/4°C after reconstitution

See page III for details.

IRDye® PEG Fluorescent Contrast Agent

Enhanced permeability and retention (EPR) is a common characteristic of tumor vasculature. The vascular endothelium in the tumor microenvironment is often discontinuous, allowing molecules to diffuse into the surrounding tumor tissue. Lymphatic drainage for these regions is also poor.

IRDye® 800CW PEG Contrast Agent is a non-specific imaging agent that exploits EPR in tumor biology. In appropriate mouse models, the agent highlights surface vasculature for approximately 0.5 h post injection (Figure 1). Approximately 4 h post injection, retention of the labeled macromolecule is visible in the tumor (Figure 2). At 9 h post intravenous injection, the tumor is well defined (Figure 3).

IRDye 800CW PEG Contrast Agent is also used as a lymph tracking agent when given intradermally (Figure 4). All images captured with the Pearl® Impulse Small Animal Imaging System.

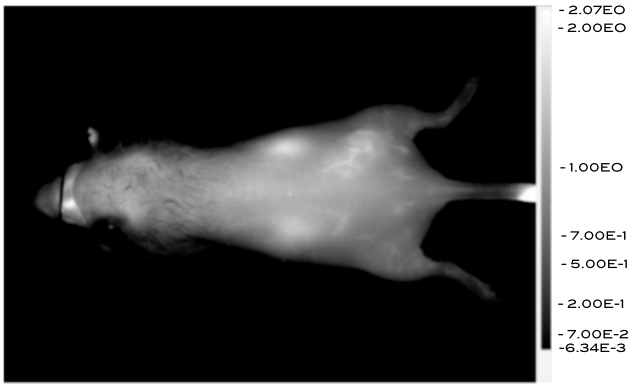

Figure 1. Athymic male nu/nu mouse, 0.5 h after receiving IRDye® 800CW PEG (1 nmol) intravenously. Surface blood vessels are visible.

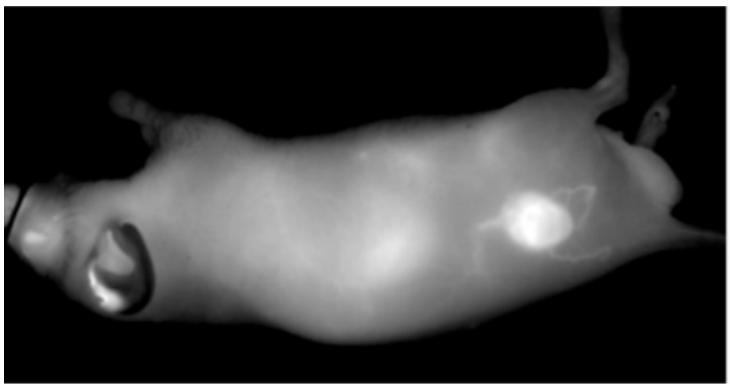

Figure 2. Athymic male nu/nu mouse, ~4 h after receiving IRDye® 800CW PEG (1 nmol) intravenously. Large blood vessels and tumor are visible.

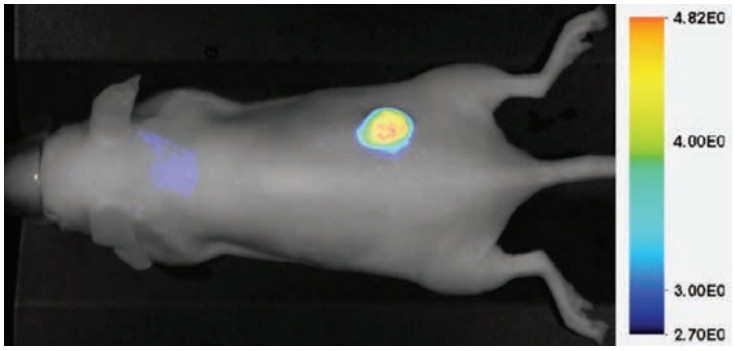

Figure 3. Athymic male nu/nu mouse, ~9 h after receiving IRDye® 800CW PEG (1 nmol) intravenously. Tumor is clearly defined.

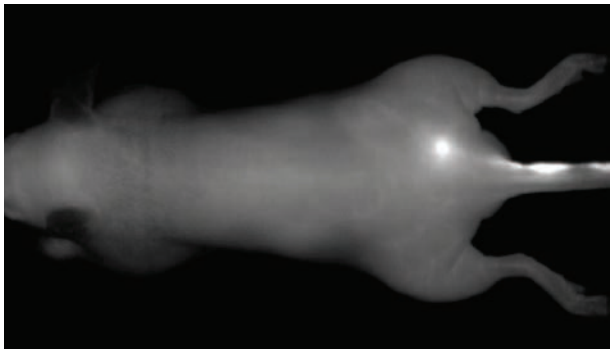

Figure 4. Athymic male nu/nu mouse, minutes after receiving IRDye® 800CW PEG (~0.1 nmol) intradermally on the tail (right side). Drainage of the agent to the sciatic lymph node is visualized.

926-50401 IRDye 800CW PEG Contrast Agent (15 nmol; approximately 10 injections) $325.00

Storage: -20°C prior to reconstitution/4°C after reconstitution

CellVue® Fluorescent Cell Labeling Kits

CellVue® Labeling Kits incorporate fluorescent dyes with long aliphatic hydrocarbon tails into lipid membranes. These extremely lipophilic fluorescent dyes rapidly and stably integrate into the phospholipid membrane of cells or membrane-containing bioparticles by non-covalent interactions. They are stably maintained within the lipid bilayer through strong hydrophobic interactions and do not transfer into the unstained membranes of adjacent cells. The pattern of fluorescence staining within the membrane may vary depending on the cell type.

CellVue dyes are provided in an easy-to-use kit format. The labeling Diluent C provided with the kit is an iso-osmotic aqueous solution that contains no physiologic salts or buffers, detergents, or organic solvents. It is designed to maintain cell viability while maximizing dye solubility and staining efficiency.

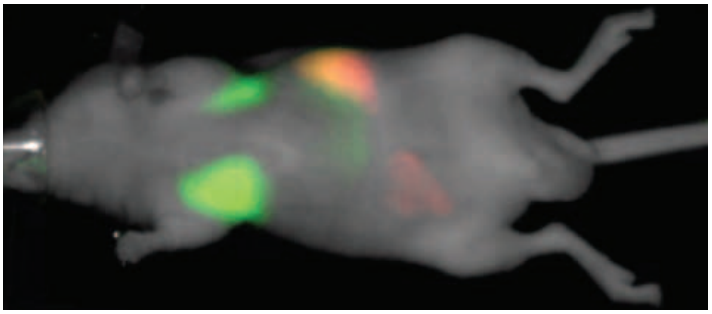

Figure 1. Imaging of CellVue® NIR815 in the capillary region of the lungs of a nude mouse. Image captured with Pearl® Impulse immediately following intravenous injection into tail vein. Green: CellVue NIR815 cell stain. Red: autofluorescence in animal's gut detected at 680 nm (caused by chlorophyll in the animal's diet). Both fluorescent signals are overlaid on a white light image of the mouse.

929-90010 CellVue Burgundy Fluorescent Cell Labeling Kit $195.00

Kit contains:

- 1 x 0.1 mL of 1 mM dye stock in ethanol and 1 x 10 mL of diluent
- Dye fluorescence properties: Ex max = 683 nm; Em max = 707 nm
- Provides stable labeling of the lipid regions of cell membranes

929-90020 CellVue NIR815 Fluorescent Cell Labeling Kit $195.00

Kit contains:

- 1 x 0.1 mL of 1 mM dye stock in ethanol and 1 x 10 mL of diluent
- Dye fluorescence properties: Ex max = 786 nm; Em max = 814 nm
- Provides stable labeling of the lipid regions of cell membranes

929-90001 Diluent C $175.00

Kit contains:

- Six vials containing 10 mL of Diluent C for membrane labeling with CellVue dyes

Storage: 4°C

PSVue® 794 Reagent Kit NEW!

PSVue® 794 is a near-infrared fluorescent probe for detection of apoptotic cells, necrotic cells, bacteria, and other anionic membranes. The PSVue 794 (formerly PSS 794) reagent kit contains components to provide a 1 mM solution of PSVue 794 in aqueous solution. The compound exhibits absorbance and fluorescence excitation maxima at 794 nm and emission maximum at 810 nm.

Through its zinc(II)-dipicolylamine (Zn-DPA) functionality, PSVue 794 has been found to bind strongly to negatively charged bacterial cell walls (e.g. *S. aureus, E. coli*) and necrotic regions present in various tumors (e.g. mammary, prostate, glioma) *in vitro* and *in vivo*. In particular, it has also been found to bind to the phosphatidylserine (PS) residues exposed on the cell surface of apoptotic cells, making it a more cost-effective alternative to fluorescently-labeled Annexin V in various cell death assays. The labeling vehicle provided with the kit (Diluent X) is designed to maximize dye solubility and is suitable for *in vitro* and *in vivo* use.

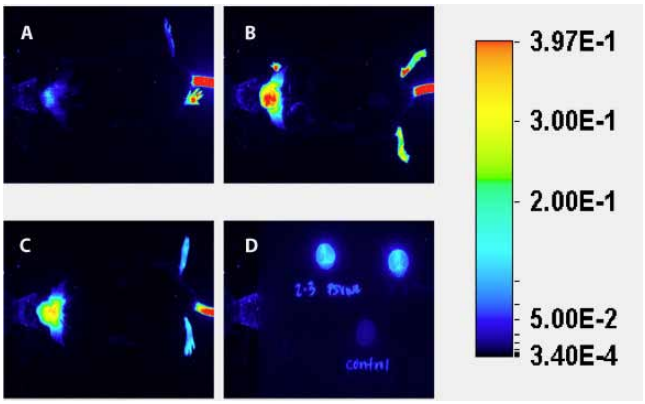

Figure 1. MPTP was used to induce cell death in mouse brains as a model for Parkinson's Disease. C57Bl/6 mice were treated with MPTP to selectively destroy dopaminergic neurons. Mice were then injected with PSVue® dye or control dye and imaged on the Pearl® Imager 68 hrs post injection. **A.** control (i.e. non-targeting) dye; **B.** and **C.** PSVue dye; **D.** excised brains from the three animals.

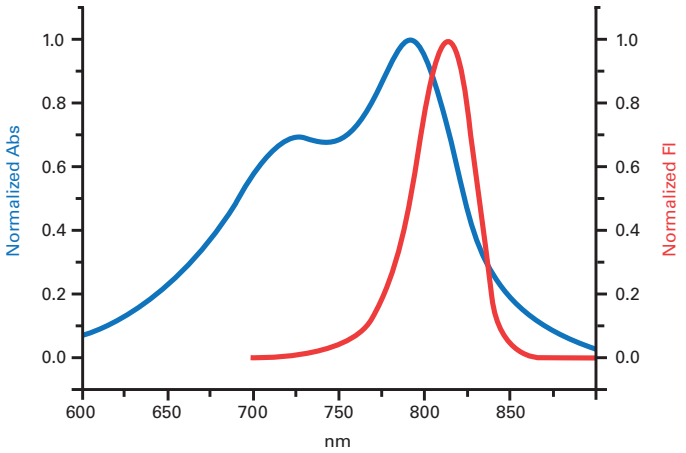

Figure 2. PSVue® 794 absorption and fluorescence emission spectra (5 µM solution: abs. max = 794 nm; fl. em max = 810 nm).

929-90030 **PSVue 794 Reagent Kit.....$325.00**

Kit contains:

- 1 vial containing pre-weighed amount of apo-PSS 794 solid dye (at least 1 mg)
- 1 vial of Diluent X (1 mL)
- 1 vial of 4.2 mM zinc nitrate solution in Diluent X (1 mL)

Storage: 4°C

Infrared Dye Labeling Overview

IRDye® Protein Labeling Kits label antibodies and other proteins for applications such as Western blots, In-Cell Western™ assays, *in vivo* imaging, and whole organ or tissue section assays. IRDye labeling kits are cost-effective alternatives to expensive, time-consuming, custom antibody labeling services.

Labeling Reactions

IRDye reactive dyes bear an N-hydroxysuccinimide (NHS) reactive group that couples to free amino groups and forms a stable conjugate. Proteins other than IgG antibodies can be labeled, but protocol adjustment and optimization may be necessary.

IRDye labeling kits are based on a simple conjugation protocol that uses fast, easy, spin column cleanup with resulting purity equivalent to purification by dialysis. Simply dissolve the protein in the provided buffer, combine it with the appropriate amount of water-soluble, amine-reactive IRDye, and separate the labeled conjugate from the free dye using the included spin columns. Labeling and purification are complete in approximately 2 hours.

Some IRDye infrared dyes are available with a maleimide reactive group. Maleimide dyes can be used to label molecules that contain free sulfhydryl (—SH) groups, such as cysteine residues. This reactive group allows conjugation reactions to be performed at physiological pH.

Table 1. Recommended IRDye® Infrared Dyes for Labeling Proteins, Peptides, or Nucleic Acids

IRDye	PROTEINS	PEPTIDES	NUCLEIC ACIDS
IRDye 800CW NHS Ester	✓	✓	
IRDye 800RS NHS Ester			✓
IRDye 750 NHS Ester	✓	✓	
IRDye 650 NHS Ester	✓	✓	
IRDye 680RD NHS Ester	✓	✓	
IRDye 680LT NHS Ester	✓	✓	
IRDye 650 NHS Ester	✓	✓	
IRDye 800CW Maleimide	✓	✓	
IRDye 750 Maleimide	✓	✓	
IRDye 680RD Maleimide	✓	✓	
IRDye 680LT Maleimide	✓	✓	
IRDye 650 Maleimide	✓	✓	
IRDye 800 Phosphoramidite			✓
IRDye 700 Phosphoramidite			✓

IRDye® Labeling Kits

IRDye® 800CW Labeling Kits

IRDye 800CW is functionalized with an NHS ester reactive group that will couple to free amino groups and form a stable conjugate. Fluorescent conjugates labeled with IRDye 800CW display an absorption maximum of 774 nm and an emission maximum of 789 nm in 1X PBS. These spectral characteristics match the 800 nm channel on the Odyssey® CLx, Odyssey Classic, Odyssey Fc, Odyssey Sa, and Pearl® systems. Labeled proteins may be used for Western blots, In-Cell Western™ Assays (ICW), *in vivo* imaging, and other applications.

928-38040 IRDye 800CW Protein Labeling Kit - High Molecular Weight $375.00
For labeling 3 x 1 mg of protein with molecular weight 45-200 kDa

Kit contains:

- 3 x IRDye 800CW Reactive Dye vials (0.1 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce® Zeba™ Desalting Spin Columns, P/N 89891

928-38042 IRDye 800CW Protein Labeling Kit - Low Molecular Weight $425.00
For labeling 3 x 1 mg of protein with molecular weight 15-45 kDa

Kit contains:

- 3 x IRDye 800CW Reactive Dye vials (0.5 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89891

928-38044 IRDye 800CW Protein Labeling Kit - Microscale $325.00
For labeling 3 x 100 µg of protein with molecular weight 14-200 kDa

Kit contains:

- 3 x IRDye 800CW Reactive Dye vials (0.1 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89882

Storage for all kits: -20/4°C depending on component

IRDye® 700DX Labeling Kits

The IRDye 700DX is functionalized with an NHS ester reactive group that will couple to free amino groups and form a stable conjugate. Fluorescent conjugates labeled with IRDye 700DX display an absorption maximum of 689 nm and an emission maximum of 700 nm in 1X PBS. These spectral characteristics match the 700 nm channel on the Odyssey® CLx, Odyssey Classic, Odyssey Fc, Odyssey Sa, and Pearl® systems. Labeled proteins may be used for Western blots, In-Cell Western™ Assays (ICW), *in vivo* imaging, and other applications.

928-38046 IRDye 700DX Protein Labeling Kit - High Molecular Weight $375.00
For labeling 3 x 1 mg of protein with molecular weight 45-200 kDa

Kit contains:
- 3 x IRDye 700DX Reactive Dye vials (0.175 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce® Zeba™ Desalting Spin Columns, P/N 89891

928-38048 IRDye 700DX Protein Labeling Kit - Low Molecular Weight $425.00
For labeling 3 x 1 mg of protein with molecular weight 15-45 kDa

Kit contains:
- 3 x IRDye 700DX Reactive Dye vials (0.5 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89891

928-38050 IRDye 700DX Protein Labeling Kit - Microscale $325.00
For labeling 3 x 100 µg of protein with molecular weight 14-200 kDa

Kit contains:
- 3 x IRDye 700DX Reactive Dye vials (0.175 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89882

Storage for all kits: -20/4°C depending on component

IRDye® 680RD Labeling Kits NEW!

The IRDye 680RD dye bears an NHS ester reactive group that will couple to free amino groups and form a stable conjugate. Fluorescent conjugates labeled with IRDye 680RD display an absorption maximum of 672 nm and an emission maximum of 694 nm in 1X PBS. These spectral characteristics match the 700 nm channel on the Odyssey® CLx, Odyssey Classic, Odyssey Fc, Odyssey Sa, and Pearl® systems. IRDye 680RD is the dye of choice for labeling proteins for use in Western Blotting, In-Cell Western™ Assays, and small animal imaging applications in the 700 nm wavelength.

928-38072 IRDye 680RD Protein Labeling Kit - High Molecular Weight $375.00
For labeling 3 x 1 mg of protein with molecular weight 45-200 kDa

Kit contains:
- 3 x IRDye 680RD Reactive Dye vials (0.1 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce® Zeba Desalting Spin Columns, P/N 89891

928-38074 IRDye 680RD Protein Labeling Kit - Low Molecular Weight.....$425.00
For labeling 3 x 1 mg of protein with molecular weight 15-450 kDa

Kit contains:
- 3 x IRDye 680RD Reactive Dye vials (0.5 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89891

928-38076 IRDye 680RD Protein Labeling Kit – Microscale.....$325.00
For labeling 3 x 100 µg of protein with molecular weight 14-200 kDa

Kit contains:
- 3 x IRDye 680RD Reactive Dye vials (0.1 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89892

Storage for all kits: -20/4°C depending on component

IRDye® 680LT Labeling Kits

IRDye 680LT is functionalized with an NHS ester reactive group that will couple to free amino groups and form a stable conjugate. Fluorescent conjugates labeled with IRDye 680LT display an absorption maximum of 676 nm and an emission maximum of 693 nm in 1X PBS. These spectral characteristics match the 700 nm channel on the Odyssey® CLx, Odyssey Classic, Odyssey Fc, and Odyssey Sa. Labeled proteins may be used for Western blots.

***Note**: Do Not Use For Small Animal Imaging*

928-38066 IRDye 680LT Protein Labeling Kit – High Molecular Weight.....$375.00
For labeling 3 x 1 mg of protein with molecular weight 45-200 kDa

Kit contains:

- 3 x IRDye 680LT Reactive Dye vials (0.5 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce® Zeba™ Desalting Spin Columns, P/N 89891

928-38068 IRDye 680LT Protein Labeling Kit – Low Molecular Weight.....$425.00
For labeling 3 x 1 mg of protein with molecular weight 15-45 kDa

Kit contains:

- 3 x IRDye 680LT Reactive Dye vials (0.5 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89891

928-38070 IRDye 680LT Protein Labeling Kit – Microscale.....$325.00
For labeling 3 x 100 µg of protein with molecular weight 14-200 kDa

Kit contains:

- 3 x IRDye 680LT Reactive Dye vials (0.125 mg each)
- 1 x 0.5 mL 1M Potassium Phosphate (K_2HPO_4), pH 9
- 1 x 25 mL 1X PBS
- 1 x 0.5 mL ultrapure water
- 3 x Pierce Zeba Desalting Spin Columns, P/N 89882

Storage for all kits: -20/4°C depending on component

TECHNICAL NOTE:
Labeling of Fixed Cells with IRDye® NHS Ester Reactive Dyes for In-Cell Western™ Assay Normalization

Introduction

The In-Cell Western™ assay is a popular immunoassay for the study of signal transduction, protein expression and function. A key feature in this assay is its ability to simultaneously measure two targets of interest or normalize the data for well-to-well variation in cell number. LI-COR® has developed three types of protocols for normalization.

The first method uses primary and secondary antibodies to detect two distinct targets. For example, phospho-ERK can be detected using a specific primary antibody and a secondary antibody labeled with IRDye® 800CW infrared dye. In a multiplex assay, a primary antibody against total ERK (or against a housekeeping protein or other target) can be detected with a secondary antibody labeled with a spectrally distinct IRDye fluorophore[1] such as IRDye 680RD. The protocol has been widely used in the scientific literature.[1-5]

In some cases, the cost of additional primary and secondary antibodies may preclude this approach, so more cost-effective alternative for normalization has also been developed. The method uses of two fluorescent cell stains (DRAQ5™ and Sapphire700™, which are detected in 700 nm channel of the Odyssey® and Aerius Imagers) to correct for cell number variation. DRAQ5 is a DNA stain, and Sapphire700 is a cell volume stain. When used together, these stains can quantify cell number across a wide linear range. However, quantitative accuracy may be affected by cell treatments that alter the status of nuclear DNA (such as treatments to induce apoptosis), so this method is not always the best choice.

The new protocol described here is very cost effective, and provides quantification over a wide linear range in a manner that does not use DNA staining and is not affected by changes in nuclear DNA. It was first described by Hoffman and colleagues.[6,7] This method uses IRDye reactive dyes to covalently label cellular proteins on lysine residues. IRDye 800CW or IRDye 700DX N-hydroxysuccinimidyl ester (NHS) reactive dyes can couple to free amine groups on lysine residues and form a stable conjugate. Because the cells are fixed, the reactive dye has access to both cell surface and internal lysine residues, which greatly increases the extent of labeling. These dyes are available in several formats:

As a component of IRDye Labeling Kits (such as P/N 928-38040 or 928-38046). These kits contain other components typically used to label antibodies and other proteins, which are not used in this cell labeling protocol.

- IRDye 700DX Protein Labeling Kit, High Molecular Weight, P/N 928-38046
- IRDye 800CW Protein Labeling Kit, High Molecular Weight, P/N 928-38040

As individual vials of reactive dye

- IRDye 700DX NHS ester, 0.5 mg, P/N 929-70010
- IRDye 800CW NHS ester, 0.5 mg, P/N 929-70020

This method adds only two brief steps to the protocol, and provides several advantages over the previous methods:

- **Extreme sensitivity**
  The lower limit detection is approximately 200 cells per well.
- **Quantitative accuracy**
  Wide linear range of signal extends from 200 to 200,000 cells in our experiments (Figure 1).
- **Cost effectiveness**
  Because highly dilute dye solutions are used, a 0.5 mg vial of IRDye 700DX reactive dye can label 50 plates of cells, and a 0.5 mg vial of IRDye 800CW is sufficient to label 500 plates.

Suggested Materials

- IRDye 800CW or IRDye 700DX NHS ester reactive dye
  - IRDye 800CW NHS ester, 0.5 mg (P/N 929-70020) or 5 mg (929-70021)
  - IRDye 700DX NHS ester, 0.5mg (P/N 929-70010) or 5 mg (929-70011)
- Dry (anhydrous grade) dimethyl sulfoxide (DMSO)
- Odyssey® Blocking Buffer (P/N 927-40000)
- 1X PBS wash buffer (LI-COR, P/N 928-40018 or 928-40020)
- Tissue culture reagents (serum, DMEM, trypsin, etc.)
- Clear or black well 96-well microplate, with clear bottom

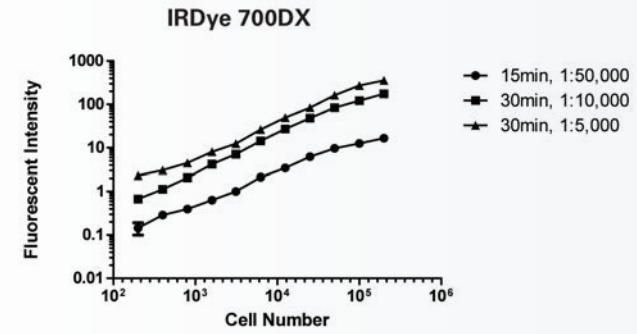

Figure 1a. Linear relationship between fluorescent intensity and number of cells, using IRDye® 700DX for labeling. Two-fold serial dilutions of HeLa cells were plated in clear, flat bottom 96-well plates, then fixed and permeabilized. Cells were labeled with IRDye 700DX with the dilutions and incubation times indicated.

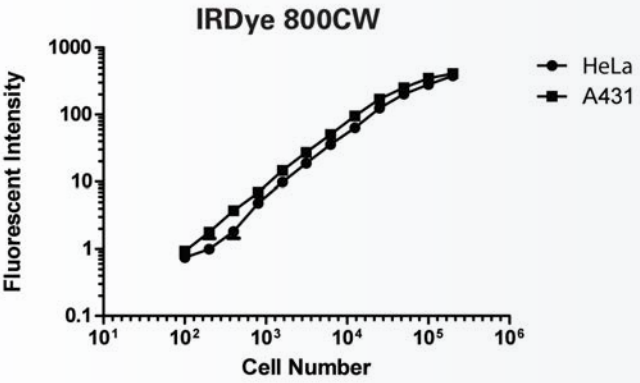

Figure 1b. Linear relationship between fluorescent intensity and number of cells, using IRDye® 800CW for labeling. Two-fold serial dilution of HeLa or A431 cells were plated in clear, flat bottom 96-well plates, then fixed and permeabilized. Cells were labeled with IRDye 800CW at 1:50,000 dilution for 20 minutes.

- 37% formaldehyde
- 20% Tween® 20
- 10% Triton X-100

Protocol

A. Cell Preparation and Fixation

Treat cells as desired with drug, stimulant, etc. Detailed In-Cell Western™ protocols for certain cell lines and target proteins may be downloaded at *www.licor.com/icwprotocols.*

Important Note: If optimal fixation and permeabilization conditions for immunofluorescent staining of your cell line and/or target protein are already known, these conditions may be more appropriate than the fixation protocol described here and would be an excellent starting point for In-Cell Western assay development. Most fixatives and fixation protocols for immunofluorescent staining may be adapted to the In-Cell Western format.

Note: If cells are loosely attached to plate, centrifuge plate at ~350 x g for 5-10 minutes during the last 10-15 minutes of this incubation. This will help to prevent cell loss.

1. Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

2. Remove media from microtiter plate manually or by aspiration.
3. Using a multi-channel pipette, immediately add 150 µL of fresh, room temperature Fixing Solution.
4. Add the Fixing Solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.
5. Allow incubation on bench top for 20 minutes at room temperature with no shaking.

B. Permeabilization

1. Wash five times with 1X PBS containing 0.1%Triton X-100 for 5 minutes per wash.
2. Prepare as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

3. Remove Fixing Solution (contains formaldehyde) to an appropriate waste container and dispose of properly.
4. Using a multi-channel pipette, add 200 µL of PBS+Triton solution to each well. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.
5. Allow wash to shake on a rotator for 5 minutes.

Note: If cells are loosely attached to plate, do not shake plate during washes! Instead, place the plate into a centrifuge and spin at ~350 X g for 5 minutes during each wash.

6. Repeat washing steps 4 more times, removing wash manually each time. Do not allow cells/wells to become dry during washing. Immediately add the next wash after each manual disposal.

C. Cell Number Staining

1. First, prepare a 1 mg/mL solution of the dye. LI-COR® supplies vials of reactive dye in lyophilized form. You must resuspend the dye in organic solvent (anhydrous DMSO) before use.

WARNING: DO NOT resuspend the contents of the dye vial in aqueous solution or buffer! The NHS ester reactive group is quickly hydrolyzed and inactivated by water. If you resuspend in aqueous buffer, then you must use the entire dye vial immediately and must discard ALL remaining dye after first use, because it will quickly hydrolyze during storage and become nonreactive.

- To preserve dye reactivity, resuspend the contents of the vial in dry (anhydrous grade) DMSO at a concentration of 1 mg/mL.
- After the contents of the dye vial are resuspended in DMSO, protect the vial from light and store at –20°C.

2. Dilute a small amount of IRDye 800CW or IRDye 700DX in aqueous solution (PBS) **FOR IMMEDIATE USE ONLY.** As a general guideline, a 1:50,000 dilution is recommended for IRDye 800CW NHS ester, and 1:5,000 for IRDye 700DX NHS ester.

1X PBS	25 mL
1 mg/mL IRDye 700DX in DMSO	5 µL
For one 96-well plate	

OR

1X PBS	25 mL
1 mg/mL IRDye 800CW in DMSO	0.5 µL
For one 96-well plate	

3. Remove PBS +Triton solution from each well of the plate manually or by aspiration. Add 200 µL diluted dye solution to each well.

- Incubate for 20 minutes for IRDye 800CW.
- Incubate for 30 minutes for IRDye 700DX.

D. Wash Out Unbound Dye

1. Wash each well 3 times with PBS + 0.1% Tween® 20 (PBS-T), 5 minutes per wash.

Note: If cells are loosely attached to plate, do not shake plate during washes! Instead, place the plate into a centrifuge and spin at ~350 X g for 5 minutes during each wash.

2. Block with Odyssey® Blocking Buffer and proceed with primary antibody staining as for a standard In-Cell Western™ Assay protocol.

References

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For Frequently Asked Questions about using Cells in Suspension for In-Cell Western™ Assays, see pages 134-135

Troubleshooting for some Protein Detection Applications

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Good Westerns Gone Bad: Tips to Make Your Near-infrared Western Blot Great

Introduction to Western Blotting

Western blotting is used to positively identify a protein from a complex mixture. It was first introduced by Towbin, et al. in 1979, as a simple method of electrophoretic blotting of proteins to nitrocellulose sheets. Since then, Western blotting methods for immobilizing proteins onto a membrane have become a common laboratory technique. Although many alterations to the original protocol have also been made, the general premise still exists. Macromolecules are separated using gel electrophoresis and transferred to a membrane, typically nitrocellulose or polyvinylidene fluoride (PVDF). The membrane is blocked to prevent non-specific binding of antibodies and probed with some form of detection antibody or conjugate.

Infrared fluorescence detection on the Odyssey® CLx, Odyssey Classic, Odyssey Fc, or Odyssey Sa Imaging Systems provides a quantitative two-color detection method for Western blots. This document will discuss some of the factors that may alter the performance of a near-infrared (IR) Western blot, resulting in "good Westerns, gone bad."

Factors That Alter the Performance of a Western Blot

Membrane

A low-background membrane is essential for IR Western blot success. Background can be attributed to membrane autofluorescence or to detection of antibody non-specifically binding to the membrane. Polyvinylidene fluoride (PVDF) and nitrocellulose are typically used for Western blotting applications. There are many brands and vendors for both types of membrane. Before any Western blot is performed on an Odyssey System, the membrane of choice should be imaged "out of the box" on an Odyssey System to determine the level of autofluorescence. LI-COR has evaluated many different membranes for Western blotting; examples of membrane performance can be seen in Figure 1. There is typically more variability in PVDF performance than nitrocellulose.

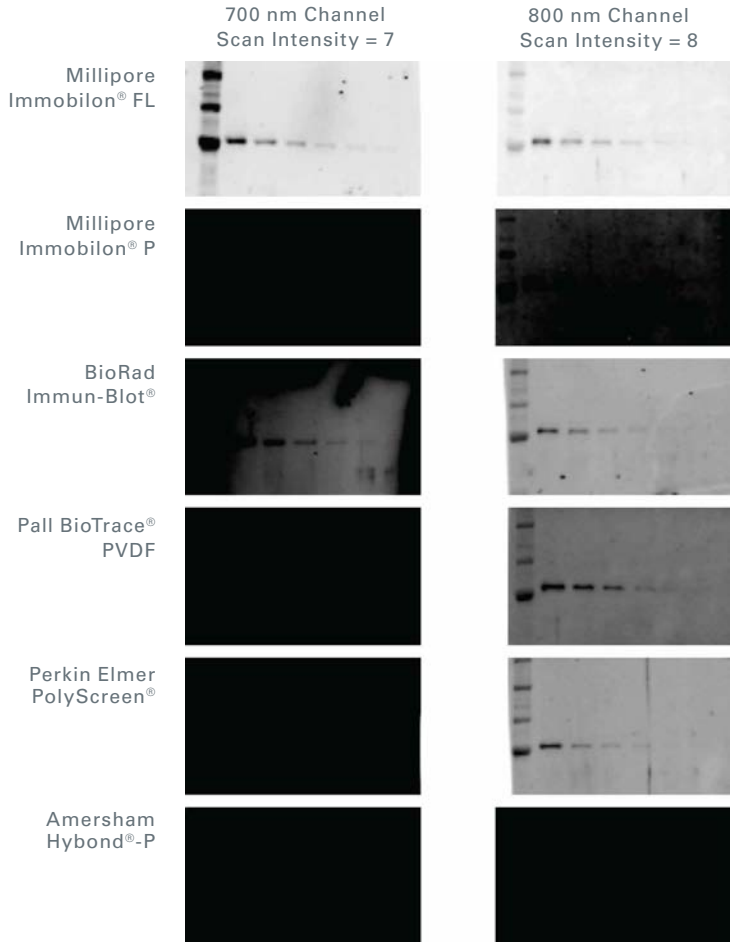


Figure 1. Western blot detection of transferrin using various vendors and brands of PVDF membrane on the Odyssey® Classic Infrared Imaging System in both 700 and 800 nm channels.

NOTE: Not all sources of PVDF and nitrocellulose have been evaluated by LI-COR; therefore, it is important to evaluate the membrane before use. Membranes can be quickly evaluated by imaging them both wet and dry on any Odyssey System.

Blocking Reagent

There are many different sources and types of blocking reagents sold for Western blot applications. Antibody performance can sometimes be compromised by the blocking reagent chosen. Milk-based blockers may contain IgG that can cross-react with anti-Goat antibodies. This can significantly increase background and reduce sensitivity.

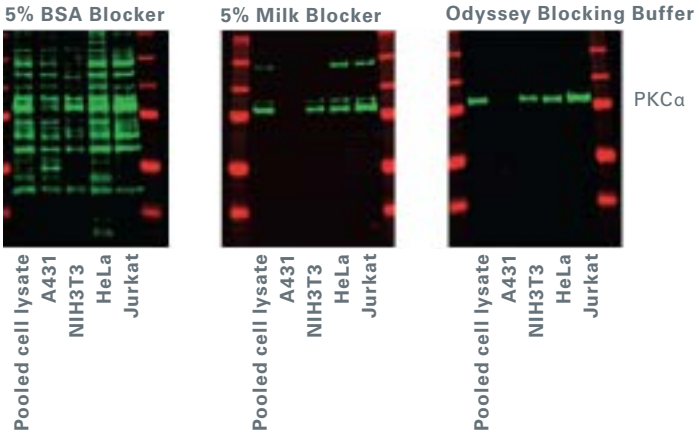


Figure 2. Western blots detected with anti-PKCα and IRDye® 800CW Goat anti-Mouse. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Classic Infrared Imager with scan intensity setting of 5, sensitivity of 5.

Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.

If an antibody fails with one blocking condition, it may be advantageous to try another. Figure 2 is an example of the behavior of the anti-PKCα antibody in 5% BSA, 5% Milk, and Odyssey® Blocking Buffer on a nitrocellulose membrane. Figure 3 is a similar example using Odyssey Blocking Buffer, I-Block™, and 5% BSA for detection of anti-pAkt and β-tubulin in 293T Cells stimulated withTGF-β.

We tested the PathScan® PDGFR Activity Assay: Phospho-PDGFR, Phospho-SHP2, Phospho-Akt, and Phospho-p44/42 MAPK (Erk1/2) Multiplex Western Detection Kit (P/N 7180), using five different blocking/diluent solutions. Figure 4 shows results from this experiment.The five phosphoproteins could

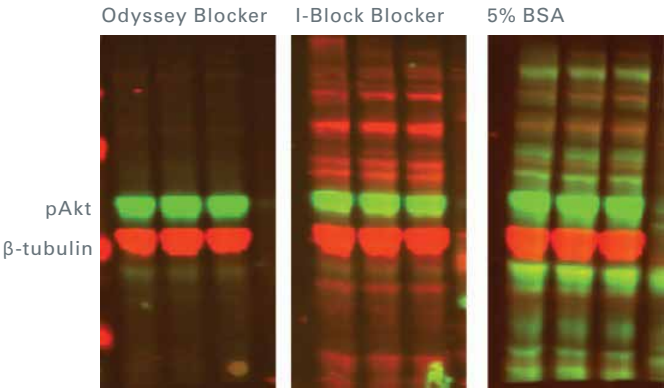


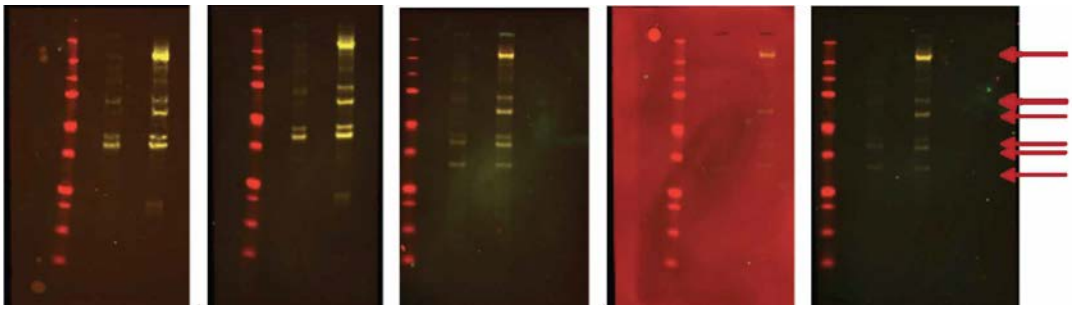
Figure 3. Western blots of 293T Cells stimulated withTGF-β (0, 2.5, and 5 minutes) detected with anti-pAkt and β-tubulin. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Classic Infrared Imager with scan intensity setting of 3.5/5 (700/800 nm), sensitivity of 5.

be clearly visualized with each of the blocking solutions, with the exception of 5% Milk, which had very high background. The S6 Ribosomal protein (total protein loading control) was almost completely absent in blots where Odyssey® Blocking Buffer (see page 66) was used. This data clearly suggests that there is not a universal blocker that is best for all antibodies.

Detergents

Addition of detergents to diluted antibodies can significantly reduce background on the blot. Optimal detergent concentration will vary, depending on the antibodies, membrane type, and blocker used. Keep in mind that some primaries do not bind as tightly as others and may be washed away by too much detergent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

1. **Tween® 20**
   a. Blocker: Do not put Tween 20 into the blocking reagent during blocking.
   b. Primary and secondary antibody diluents should have a final concentration of 0.1-0.2% Tween 20 for nitrocellulose membranes, and a final concentration of 0.1% for PVDF membranes. A higher concentration of Tween 20 may increase background on PVDF.
   c. Wash solutions should contain 0.1% Tween 20.
2. **SDS**
   a. Blocker: Do not put SDS into the blocking reagent during blocking.
   b. When using PVDF membrane, secondary antibody diluents should have a final concentration of 0.01-0.02% SDS. SDS can be added to the antibody diluents when using nitrocellulose to dramatically reduce overall membrane background and also reduce or eliminate non-specific binding. It is critical to use only a very small amount. SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process. When working with IRDye® 680LT conjugates on PVDF membranes, SDS (final concentration of 0.01-0.02%) and Tween 20 (final concentration of 0.1-0.2%) must be added during the detection incubation step.
   c. Wash solutions should not contain SDS.



a. b. c. d. e.

Figure 4. Above: Western blots utilizing PathScan® Multiplex primary antibody and both IRDye® 680 and IRDye® 800CW Goat anti-Rabbit for detection. Five different solutions were used for blocking and antibody dilution (antibody dilutions included 0.2% Tween® 20):

a. Odyssey® Blocking Buffer

b. Odyssey + PBS (1:1)

c. 5% BSA

d. 5% Skim Milk

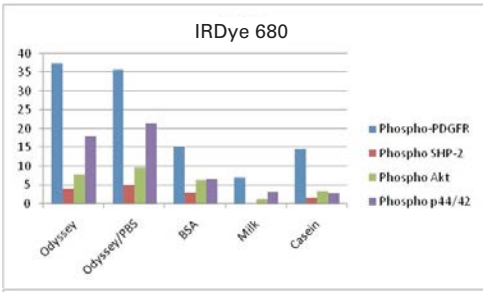
e. 0.5% Casein

In each image, arrows indicate band positions for each of the detected proteins. Starting from top: phospho-PDGFR, phospho-SHP2, phospho-Akt, phospho-p44/ p42, and S6.

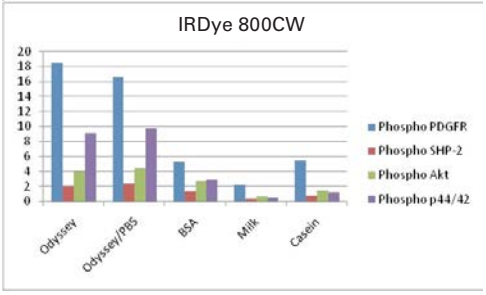
f. Quantification of 700 nm signal in each blocking solution.

g. Quantification of 800 nm signal in each blocking solution.

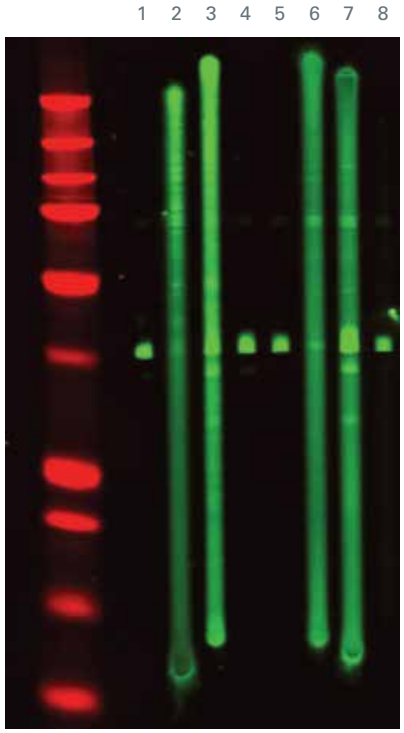
f.



g.



A.



B.

Antibody	Host	Manufacturer	P/N
1. α-GAPDH	Mouse	Ambion	4300
2. GAPDH	Sheep	AbCam	ab35348
3. GAPDH	Rabbit	Rockland	600-401-A33
4. GAPDH	Mouse	AbCam	ab8245
5. GAPDH	Chicken	ProSci Inc.	XW-7214
6. GAPDH (N-14)	Goat	Santa Cruz Bio	sc-20356
7. GAPDH (V-18)	Goat	Santa Cruz Bio	sc-20357
8. α-GAPDH	Mouse	Sigma	G8795

Figure 5. MPX™ screening of eight different GAPDH primary antibodies on a HeLa cell lysate sample. Primary antibodies were diluted in Odyssey Blocking Buffer according to manufacturer's recommendations.

Primary Antibody

An antibody produced to detect a specific antigen is called the primary antibody, and it binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species, such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the same antigen can perform very differently. It may be necessary to test multiple primary antibodies for the best performance in your Western blot system. Figure 5 is an example of how different primary antibodies may react.

Secondary Antibody Quality

One of the primary benefits of using an Odyssey® System for Western blot detection is the ability to detect two targets simultaneously. Two-color detection requires careful selection of primary and secondary antibodies. The two primary antibodies must be derived from different host species so they can be discriminated by secondary antibodies of different specificities (for example, primaries from rabbit and mouse will be discriminated by anti-Rabbit and anti-Mouse secondary antibodies). One secondary antibody must be labeled with IRDye® 680LT or IRDye 680RD, and the other with IRDye 800CW.

The exception to this is when using IRDye Subclass Specific antibodies. IRDye Goat anti-Mouse IgG_1, Goat anti-Mouse IgG_{2a}, and Goat anti-Mouse IgG_{2b} allow for two-color detection using primary antibodies derived from the same species (mouse). IRDye Subclass Specific antibodies react only with the heavy (gamma) chain of the primary antibody. In mice, there are five unique subclasses of IgG: IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgG_3. Each subclass is based on small differences in amino acid sequences in the constant region of the heavy chains, so antibodies directed against a particular subclass will not recognize antibodies directed against other subclasses. For example, IRDye Goat anti-Mouse IgG_1 recognizes mouse gamma 1, but will not recognize mouse gamma 2a, 2b, 2c, or gamma 3. For details and a complete description, refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies, pages 51-55*.

Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity as shown in Figure 6. LI-COR® IRDye®-conjugated secondary antibodies are optimized for two-color Western blot detection. They are highly cross-adsorbed with a dye-to-protein ratio maximized for optimal signal-to-noise ratio in both Western blot and In-Cell

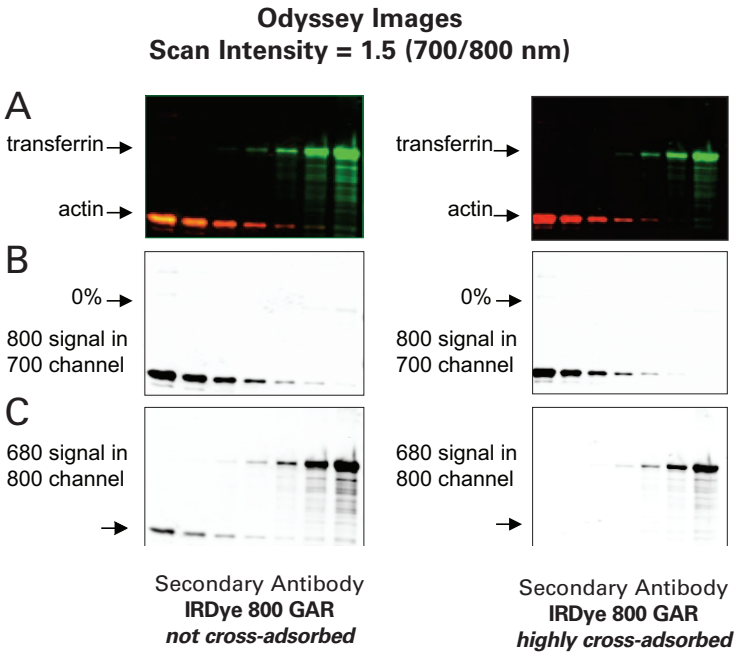


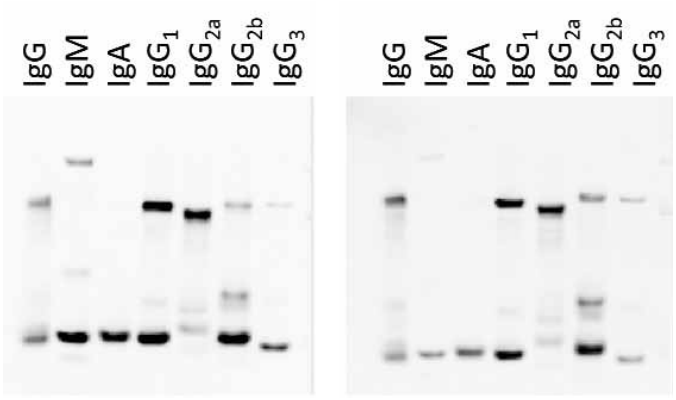
Figure 6. Example of a secondary antibody, not cross-adsorbed, cross-reacting with the actin primary antibody in a two-color Western blot.

Western™ assay detection. Figure 7 shows a comparison of LI-COR highly cross-adsorbed IRDye goat anti-mouse to a non-cross-adsorbed Goat anti-Mouse secondary antibody and their reactivity to the different mouse IgG subclasses.

There are many choices in secondary antibodies for Western blot detection. LI-COR offers IRDye whole IgG (H + L) secondary antibodies and IRDye Subclass Specific secondary antibodies. Figure 8 demonstrates the performance of LI-COR IRDye Goat anti-Mouse compared to various other secondary antibody options for detection of a mouse IgG primary antibody. Figure 9 demonstrates the differences between IRDye Subclass Specific detection and IRDye whole anti-Mouse IgG detection.

Secondary Antibody Dilution

The amount of secondary antibody that is used for IR Western blots can vary a great deal. When using LI-COR IRDye 800CW and IRDye 680RD conjugated secondary antibodies, the recommended dilution range is 1:5,000 to 1:25,000. When using LI-COR IRDye 680LT secondary antibodies, the recommended dilution range is 1:20,000 to 1:50,000. The dilution should be optimized for the primary antibody being used and the preferred appearance of the Western blot. The Odyssey imaging software can be used to maximize the appearance of the image using a wide range of secondary antibody dilutions (Figure 10).



GAM IgG LI-COR® highly cross-adsorbed

GAM IgG not cross-adsorbed

Figure 7. Mouse IgG subclass detection comparing LI-COR IRDye® Goat anti-Mouse antibody to a similar antibody that was not cross-adsorbed.

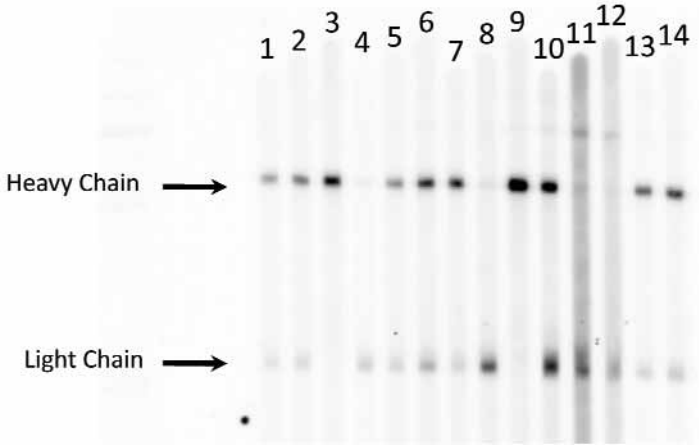


Figure 8. IRDye® 800CW anti-Mouse antibodies against purified mouse IgG.

Secondary antibodies used at a 1:5,000 dilution unless otherwise indicated.

1) Goat anti-Mouse IgA, IgG, IgM
2) Rabbit anti-Mouse IgG
3) Goat anti-Mouse IgG Fcy (heavy chain specific)
4) Goat anti-Mouse IgG F(ab)2
5) Goat anti-Mouse IgG, IgM
6) F(ab)2 Goat anti-Mouse IgG
7) F(ab)2 Goat anti-Mouse IgG, IgM
8) F(ab)2 Goat anti-Mouse IgG Fab
9) F(ab)2 Goat anti-Mouse IgG Fcy (heavy chain specific)
10) Donkey anti-Mouse (LI-COR)
11) Goat anti-Mouse IgM 1:5000
12) Goat anti-Mouse IgM 1:7500
13) Goat anti-Mouse IgG (LI-COR) 1:2500
14) Goat anti-Mouse IgG (LI-COR) 1:5000

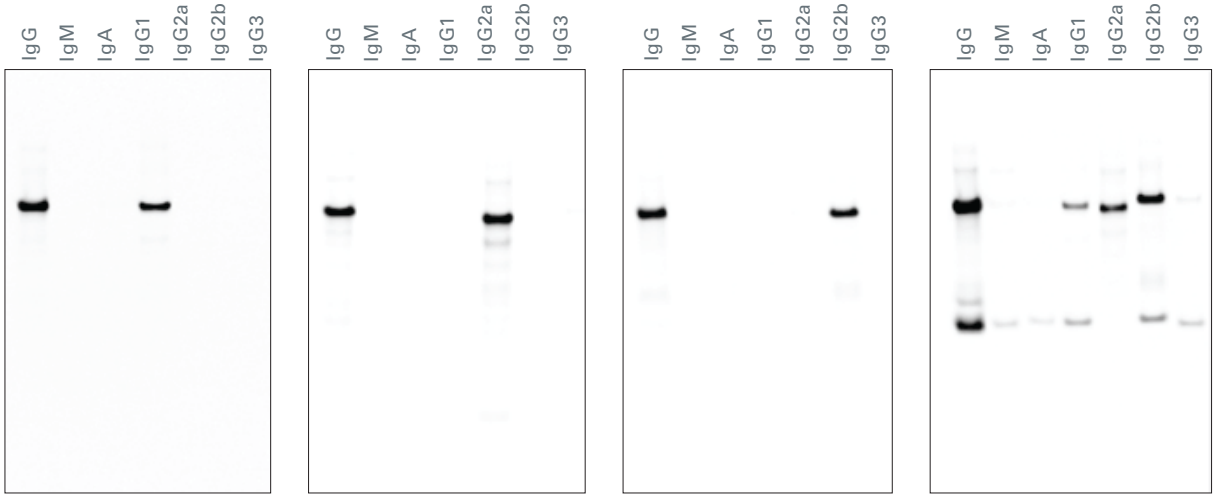


Figure 9. Western blot detection of various purified subclasses. Each lane was loaded with 50 ng of antibody. Blots were detected with IRDye labeled Subclass Specific antibodies or IRDye labeled whole IgG.

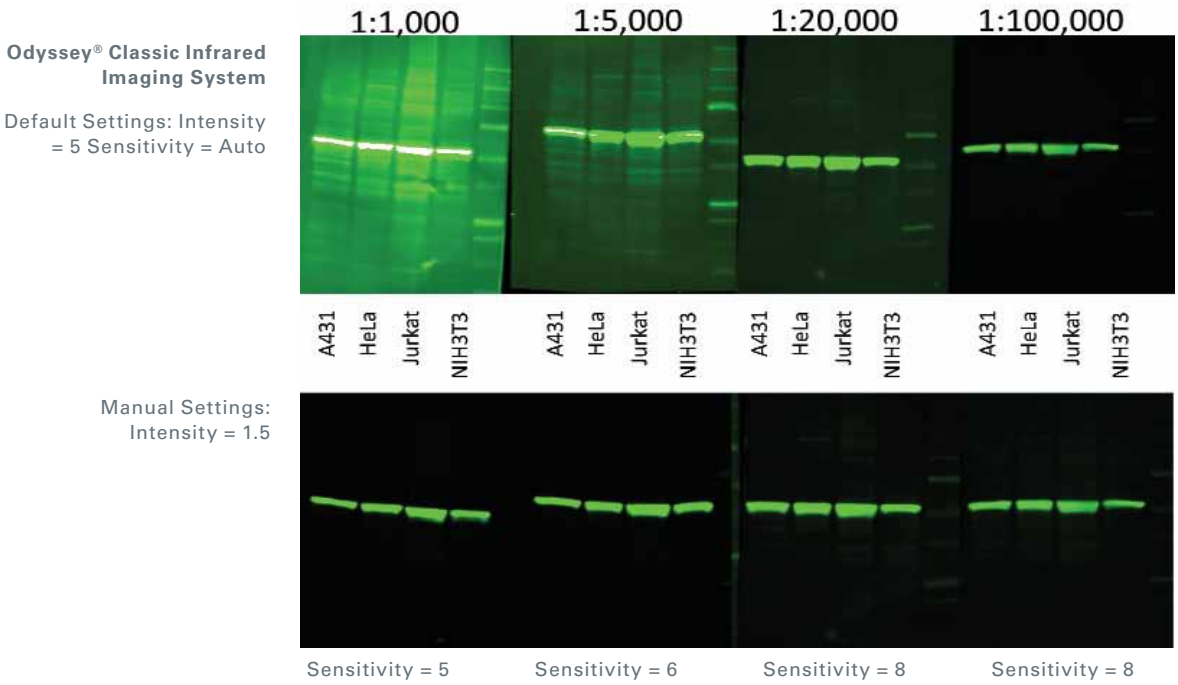


Figure 10. Secondary antibody concentration of IRDye® 800CW Goat anti-Mouse with maximized Odyssey® Classic imaging capabilities.

G. Miscellaneous Contamination

There are many things that can cause contamination of an infrared Western blot. Contamination can appear as a global increase in background, large smears of signal, or speckled blots. Common sources of contamination are listed in Table 1. Some example images are shown in Figure 11.

Imaging Issues That Can Alter the Performance of a Western Blot

There are adjustments that can be made during the process of imaging a Western on any Odyssey® Imaging System that can greatly influence data acquired from the instrument.

1. Starting with a clean scan bed or imaging tray is critical. If you acquire an image and the area that doesn't have a membrane appears to have signal in either channel, the scan bed or imaging tray is contaminated. The contamination source may be as simple as dust or as complex as dye.
2. Air bubbles can result in reduced signal detection during imaging. Flatten the membrane with a roller (P/N 926-71000) to remove bubbles and excess liquid. See Figure 12.
3. A Western blot can be imaged either wet or dry on any Odyssey Imaging System. Typically, the signal is higher when a dry blot is imaged; however, background will increase.

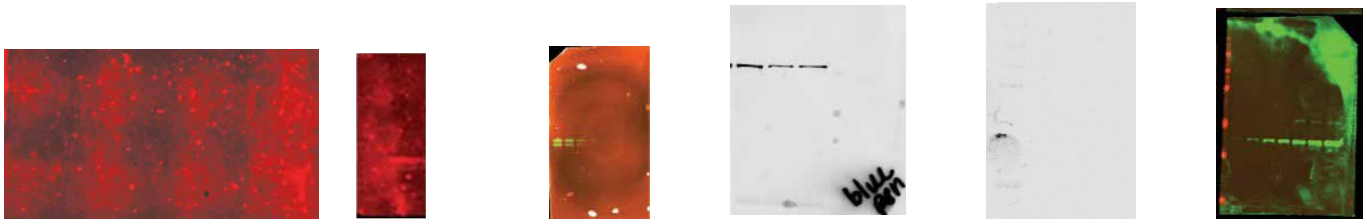


Figure 11. Examples of contamination events that may cause background on a Western blot.

NOTE: Once a blot is dry, or partially dried, stripping of the membrane for reuse is ineffective. See Figure 13.

Odyssey® Classic, CLx, and Sa

Focus Offset – Improper adjustment of the Focus Offset can result in reduced signal collection from the imager. The Focus Offset should be set at 0 mm for scanning a Western blot. For details, see the instrument's User Guide.

Scan Intensity – Improper optimization of the Scan Intensity can result in saturation of signal and reduced linear dynamic range. Figure 14 shows the quantification variation that can occur by changing intensity settings in which the image is acquired on the Odyssey Classic. Figure 15 illustrates AutoScan imaging functionality on the Odyssey CLx. Multiple scans, at four intensity settings, are required to reduce saturation, compared to a non-saturated image from a single Auto Intensity setting. For details, see the Image Studio Help System.

It is important to note that saturated pixels (pixels that appear white in the image) cannot be accurately quantified. Signal saturation can also result in signal transfer to the alternate channel. For example, saturated signal in the 800 nm channel can be seen as 700 nm signal in the 700 channel scan (see Figure 16). This can be easily eliminated by scanning at a lower intensity.

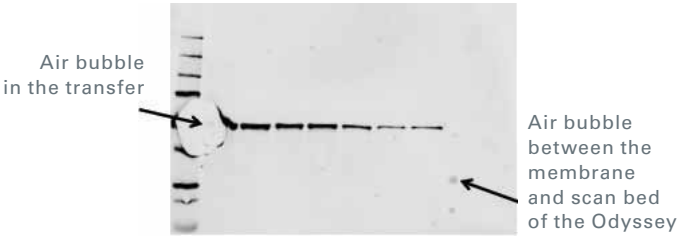


Figure 12. Examples of air bubbles in the transfer and on the Odyssey Classic Infrared Imager scan bed

Odyssey Fc

The Odyssey Fc Imaging System is optimized for acquiring Western blot images without saturated pixels or further adjustments by the operator.

Contamination Source	Appearance	Solution
Blue loading buffer used during gel electrophoresis	Smeared signal in the 700 nm channel	Use LI-COR 4X Protein Sample Loading Buffer (P/N 928-40004, see page 67)
Dirty transfer pads	Blotches can be seen on the blot that align with the transfer cassette holes	Replace transfer pads
Acrylamide residue on the membrane after transfer	Speckles and blotches can be seen in 700/800 nm channel	Carefully rinse off membrane in 1X PBS before it dries
Dirty processing containers: • Coomassie stain/gel stain/anything blue • Bacterial growth • Acrylamide residue	• In the 700 nm channel, entire membrane dark, smeared signal, or speckles depending on the amount of stain residue in container • Speckles and blotches can be seen in 700/800 nm channel • Speckles and blotches can be seen in 700/800 nm channel	• Use different containers for gel staining and Western blot detection • Wash containers with detergent, rinse thoroughly with distilled water, and a final rinse with methanol • Wash containers as indicated above
Fingerprints	Blotches can be seen in 700/800 nm channel where gloved/ungloved hands have touched the membrane	Handle Western membrane with clean forceps only
Dirty forceps	Blotches can be seen in 700/800 nm channel where gloved/ungloved hands have touched the membrane	Do not use rusty forceps. Forceps can be washed with detergent, rinsed with water, and a final rinse with methanol
Bacterial growth in antibodies (primary or secondary)	Speckles and blotches can be seen in 700/800 nm channel	Replace antibodies

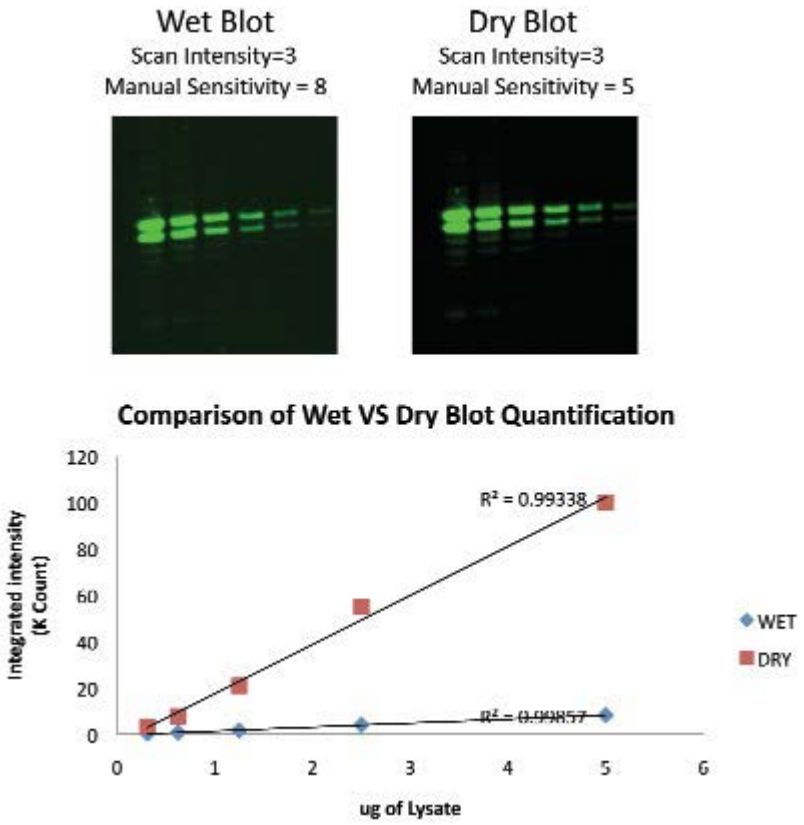


Figure 13. The same Western blot scanned wet and dry on an Odyssey® Classic. The images are represented using the optimal display settings. Quantification is shown in the chart below the images.

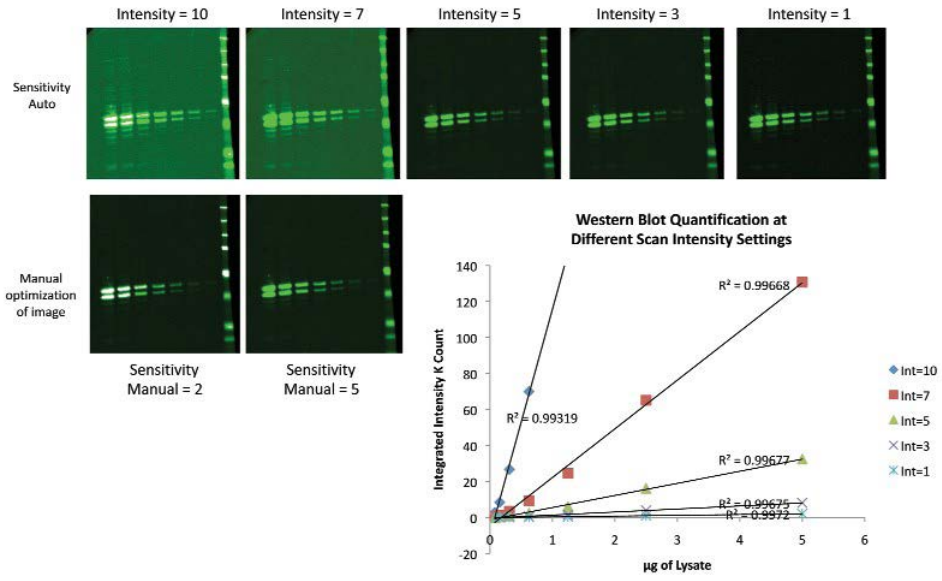


Figure 14. The same Western blot scanned at 5 different intensity settings on the Odyssey® Classic Infrared Imaging System. The top row of images are displayed using the Auto Sensitivity setting in the Odyssey Software. The bottom images were optimized using the Manual Sensitivity option for display. Quantification is shown in the chart. Note that the saturated signal at Intensity setting of 10 cannot be quantified.

IV. Software Adjustments for Image Optimization

There are two common problems that can be corrected with a few adjustments of the software.

- Blots that exhibit No Fluorescence
- Blots with Dim Bands

These software enhancements will only work on blots that are not experiencing binding chemistry problems.

Odyssey® Classic (ver. 1.X – 3.X application software) and Odyssey Sa (ver. 1.X application software)

No Fluorescence – Blots that unexpectedly exhibit no fluorescence can be enhanced by changing the sensitivity setting of the image from **Linear Auto** to **Linear Manual**. These settings can be changed from the **View** menu, then **Alter Image Display** menu. To enhance the image, simply click the **Linear Manual** radio button and adjust the slider. By manually adjusting the sensitivity settings, the most desirable image can be chosen. For details, see the User Guide.

Dim Bands – Improving the appearance of dim bands is as simple as adjusting the **Brightness** and **Contrast** of the image. The default software setting is 5. Adjust **Brightness** and **Contrast** sliders until the image is optimal. Each channel can be adjusted independently. Image adjustments can also be made in grayscale. Very faint bands are visualized well when black bands are displayed on a white background. For details, see the User Guide.

Odyssey Classic, Odyssey CLx, and Odyssey Fc (Image Studio Software, ver. 1.X - 2.X)

No Fluorescence – Click on the Auto Adjust button in the Image LookUp-Tables (LUTs) Tab. For details, see the Help System.

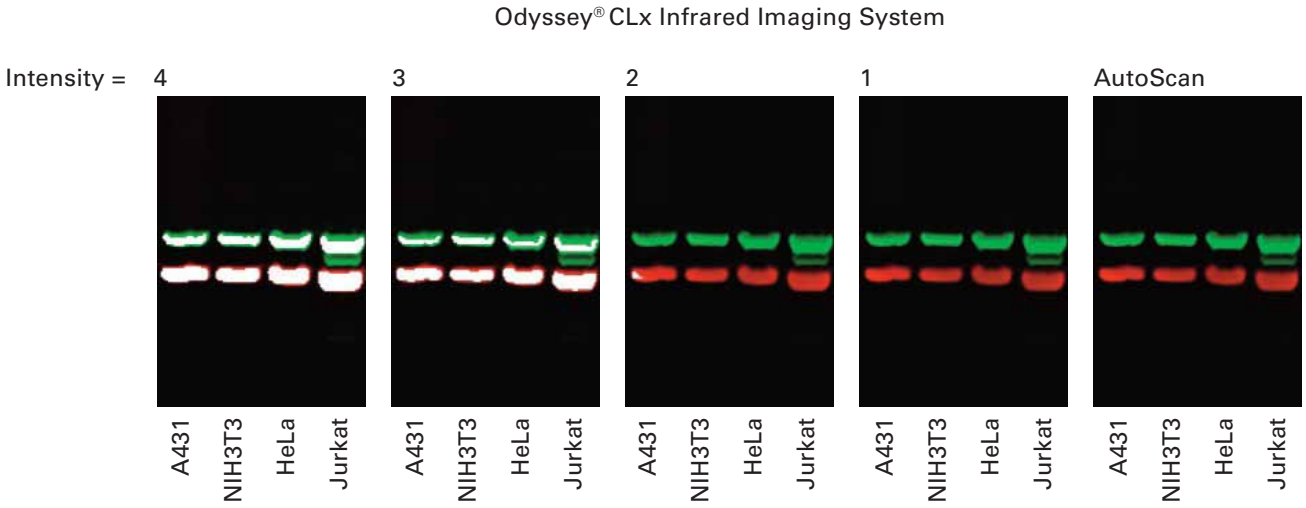


Figure 15. A single Western blot scanned on Odyssey® CLx at decreasing Scan Intensity settings, and finally using AutoScan function. Pixel saturation appears in white. The antigen targets for each lysate sample are displayed in green (rabbit anti-Tubulin detected with IRDye® 800CW Goat anti-Rabbit) and red (Mouse anti-Actin detected with IRDye 680LT Goat anti-Mouse).

Dim Bands – Click and drag the **min**, **max**, and **K value** dots (Figure 16) on the histogram (**Image LUT**s tab) to adjust the intensity of the image. Each channel can be adjusted independently. Image adjustments can be shown in grayscale and pseudocolor. Very faint bands are visualized well when black bands are displayed on a white background. For details, see the Help System.

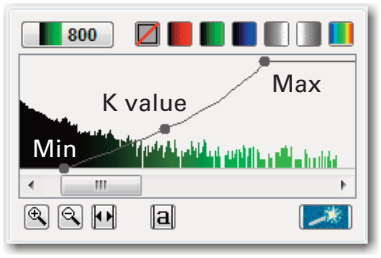


Figure 16. Histogram example.

V. Data Analysis Using the Odyssey® Classic

Odyssey Classic (Ver. 3.X application software)

Background - For accurate Western blot quantification, the Background setting must be applied effectively. The Background method sets the background calculation method for use in quantification, by measuring the intensity of the pixels selected as the background region. There are several methods for background subtraction, each unique to a specific need.

- **No Background** selection uses zero for the background calculations. This is the best choice for assays with their own background calculation methods, such as concentration standards used with In-Cell Western™ Assays. The No Background method is rarely used for Western blotting purposes.

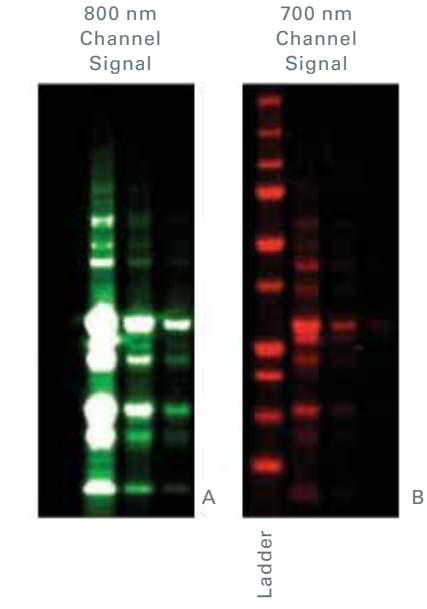
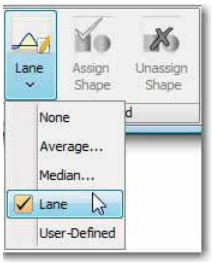


Figure 17. Saturated signal in the 800 nm channel (A) of the Odyssey® Classic Infrared Imaging System can be visualized in the 700 nm channel (B). The only detection that should be seen in the 700 nm channel is the ladder on the far left of the image. Optimizing scan intensity can eliminate this.

- **Average Background** takes the average value of pixels on all four sides of the feature. The sides (**All**, **Top/Bottom**, or **Right/Left**) of the feature can be selected to optimize quantification. It is possible to choose the number of pixels to include in the calculation by changing the **Border Width**.
- **Median** function sets the background level to the median value of the pixels outside the feature.
- **User–Defined** background selection averages the intensity of pixels enclosed by a selected feature. To implement this method, display both image channels, draw a feature over an area of typical background (be sure not to include any saturated pixels), select the feature, choose the **Background** icon from the toolbar, and change the background method to **User–Defined**. Click **Save**, and **OK** to the message. Notice that the feature has now changed to a Background feature. Multiple features can be selected for **User Defined Background**. This method is not preferred over Average or Median due to possible inconsistencies in noise across the image.

Image Studio (ver. 1.X – 2.X)

Background settings can be found in the **Background** group on the **Analyze** ribbon. To implement **User-Defined Background** selection in the Image Studio software, draw one or more shapes over an area of typical background. Select the shape(s) and click **Assign Shape** in the Background group in the Analyze ribbon. The background setting will change to **User-Defined**.



With the **Western Key**, the Background group on the Western and MPX™ Western Analysis ribbons includes the option of Lane background subtraction. This setting subtracts the background of the **Lane** from each Band. The same background settings used in Odyssey Classic 3.0 software can also be used in the Western and MPX™ Analysis ribbons.

Data analysis using the Odyssey® CLx or Odyssey Fc Image Studio (ver. 1.X – 2.X)

Background considerations, using Image Studio, are identical to those described in Section V. for the Odyssey Classic Infrared Imager.

Data analysis using the Odyssey Sa application software (ver. 1.X)

Background considerations, using the application software, are identical to those described in Section V. for the Odyssey Infrared Imager.

Summary

There are many ways to maximize the performance of a Western blot. A fully-optimized Western blot is the best place to start. LI-COR provides high-quality reagents for optimal Western blot detection. For a detailed protocol on how to do a Western blot with an Odyssey Family Imager, see the Odyssey Western Blot Analysis protocol.

X. Reference

1. Towbin, et al., (1979) *Proc. Natl. Acad. Sci. USA* 76; 4350-54

Troubleshooting Guide for Infrared Western Blots

This chart lists some commonly-occuring problems that may occur with near-infrared fluorescent Western blots and offers possible ways to either solve or prevent the issue.

Problem	Possible Cause	Solution / Prevention
High background, uniformly distributed	BSA used for blocking	Blocking solutions containing BSA may cause high membrane background. Try adding SDS to reduce background, or switch to a different blocker.
	Not using optimal blocking reagent	Compare different blocking buffers to find the most effective; try blocking longer.
	Background on nitrocellulose	Add Tween® 20 to the diluted antibodies to reduce background. Try adding SDS to diluted secondary antibody.
	Background on PVDF	Use low-fluorescent PVDF membrane. With IRDye® 680LT conjugates, always use SDS (0.01-0.02% final concentration) and Tween 20 (0.1-0.2% final) during the detection incubation step.
	Antibody concentrations too high	Optimize primary and secondary antibody dilutions using MPX™ blotting system. For details, see *One Blot Western Optimization Using the MPX Blotting System* at *http://biosupport.licor.com* or on pages 61-64.
	Insufficient washing	Increase number of washes and buffer volume.
		Make sure that 0.1% Tween 20 is present in buffer and increase if needed. Note that excess Tween 20 (0.5-1%) may decrease signal.
	Cross-reactivity of antibody with contaminants in blocking buffer	Use Odyssey® Blocking Buffer instead of milk. Milk is usually contaminated with IgG and will cross-react with anti-Goat secondary antibodies.
	Inadequate antibody volume used	Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times (use heat-seal bags if volume is limiting). Do not allow any area of membrane to dry out.
		Use agitation for all antibody incubations.
	Membrane contamination	Always handle membranes carefully and with clean forceps. Do not allow membrane to dry. Use clean dishes, bags, or trays for incubations.
Uneven, blotchy, or speckled background	Blocking multiple membranes together in small volume	If multiple membranes are being blocked in the same dish, ensure that blocker volume is adequate for all membranes to move freely and make contact with liquid.
	Membrane not fully wetted or allowed to partially dry	Keep membrane completely wet at all times. This is particularly crucial if blot will be stripped and re-used.
		If using PVDF, remember to first pre-wet in 100% methanol.
	Contaminated forceps or dishes	Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away.
		Use clean dishes, bags, or trays for incubations.
	Dirty scanning surface, silicone mat, or imaging tray	Clean scanning surface, mat, or tray carefully before each use. Dust, lint, and residue will cause speckles.
	Incompatible marker or pen used to mark membrane	Use only pencil or Odyssey pen (nitrocellulose only) to mark membranes.

Troubleshooting Guide for Infrared Western Blots (Cont'd)

Problem	Possible Cause	Solution / Prevention
Weak or no signal	Not using optimal blocking reagent	Primary antibody may perform substantially better with a different blocker.
	Insufficient antibody used	Primary antibody may be of low affinity. Increase amount of antibody or try a different source.
		Extend primary antibody incubation time (try 4-8 hr at room temperature, or overnight at 4°C).
		Increase amount of primary or secondary antibody, optimizing for best performance.
		Try substituting a different dye-labeled secondary antibody.
		Primary or secondary antibody may have lost reactivity due to age or storage conditions.
	Too much detergent present; signal being washed away	Decrease Tween® 20 and/or SDS in diluted antibodies. Recommended SDS concentration is 0.01-0.02%, but some antibodies may require an even lower concentration.
	Insufficient antigen loaded	Load more protein on the gel. Try using the narrowest possible well size to concentrate antigen.
	Protein did not transfer well	Check transfer buffer choice and blotting procedure.
		Use pre-stained molecular weight marker to monitor transfer, and stain gel after transfer to make sure proteins are not retained in gel.
	Protein lost from membrane during detection	Extended blocking times or high concentrations of detergent in diluted antibodies may cause loss of antigen from the blotted membrane.
	Proteins not retained on membrane during transfer	Allow membrane to air dry completely (1-2 hr) after transfer. This helps make the binding irreversible.
		Addition of 20% methanol to transfer buffer may improve antigen binding. Note: Methanol decreases pore size of gel and can hamper transfer of large proteins.
		SDS in transfer buffer may interfere with binding of transferred proteins, especially for low molecular weight proteins. Try reducing or eliminating SDS. **Note**: Presence of up to 0.05% SDS does improve transfer efficiency of some proteins.
		Small proteins may pass through membrane during transfer ("blowthrough"). Use membrane with smaller pore size or reduce transfer time.

Troubleshooting Guide for Infrared Western Blots (Cont'd)

Problem	Possible Cause	Solution/Prevention
Non-specific or unexpected bands	Antibody concentrations too high	Reduce the amount of antibody used.
		Reduce antibody incubation times.
		Increase Tween® 20 in diluted antibodies.
		Add or increase SDS in diluted secondary antibodies.
	Not using optimal blocking reagent	Choice of blocker may affect background bands. Try a different blocker.
	Cross-reactivity between antibodies in a two-color experiment	Double-check the sources and specificities of the primary and secondary antibodies used.
		Use only highly cross-adsorbed secondary antibodies.
		There is always potential for cross-reactivity in two-color experiments. Use less secondary antibody to minimize this.
		Always test the two colors on separate blots first so you know what bands to expect and where.
		Avoid using mouse and rat antibodies together, if possible. Because the species are so closely related, anti-Mouse will react with rat IgG to some extent, and anti-Rat with mouse IgG. Sheep and goat antibodies may exhibit the same behavior.
	Bleed through of signal from one channel into other channel	Check the fluorescent dye used. Fluorophores such as IRDye 750 or Alexa Fluor® 750 may appear in both channels and are not recommended for use with the Odyssey® Imaging Systems.
		If signal in one channel is very strong (near or at saturation), it may generate a small amount of bleed through signal in the other channel. Minimize this by using a lower scan intensity setting in the problem channel.
		Reduce signal by reducing the amount of protein loaded or antibody used.

Troubleshooting Guide for Chemiluminescent Western Blots

Chemiluminescent Western blots are probed with a primary antibody against the target protein, followed by a secondary antibody labeled with HRP (horseradish peroxidase) enzyme. A chemiluminescent substrate for the HRP enzyme is carefully applied to the blot, and light is emitted when the HRP enzyme modifies the substrate. Photographic film or an imaging system with a digital CCD camera captures the emitted light as an image.

The Odyssey® Fc Imaging System is a CCD-based imager that detects chemiluminescent signal, visible signal at 600 nm, and near-infrared fluorescent signals at 700 nm and 800 nm wavelengths. This versatile, multichannel system can image both chemiluminescent and near-infrared Western blots. It can detect near-infrared fluorescent markers on a chemiluminescent blot as well, providing a less expensive alternative to HRP-labeled markers.

There are several factors that affect the outcome of a chemiluminescent Western blot. The following section addresses some of the commonly-asked questions as well as some of the challenges you may face in optimizing your chemiluminescent Western blot.

Blocking Buffer

1. **Can I dilute the HRP-conjugated secondary antibodies in the Odyssey Blocking Buffer?**

   No. Odyssey Blocking Buffer contains sodium azide as a preservative. Sodium azide binds irreversibly to the HRP enzyme, inhibiting the binding of the substrate and slowing the chemiluminescent reaction. This results in less light production that may affect the appearance of less intense bands or even the entire blot. For optimal results, do not use any solutions containing sodium azide for chemiluminescent Western blotting.

2. **Can I use the Odyssey Blocking Buffer to block my blot?**

   Yes. Use only for the blocking step and be aware that the sodium azide from the Odyssey Blocking Buffer may still be present on the membrane at the detection step and will bind to the HRP enzyme, resulting in reduced light production and less intense bands.

3. **Can I use milk-based blockers?**

   Yes. Milk-based blockers can be used for chemiluminescent detection but should be avoided when detecting phosphoproteins or glycoproteins. Milk-based blockers may contain endogenous biotin and glycoproteins, resulting in higher background on the membrane.

4. **What is the best blocker for chemiluminescent Western blots?**

   It is best to try several blockers to find the one that gives the most satisfying data for each antigen and antibody pair. There is not a best blocker for all conditions.

Primary and Secondary Antibodies

5. **Why is the signal missing in the middle of the bands?**

   Too much secondary antibody on the membrane results in consumption of all the substrate in that area. Without substrate, there is no chemiluminescent signal and a white spot appears in the center of the band. Try different dilutions of the primary and secondary antibodies to find what gives the best results, or try changing the substrate.

6. **Does it matter where I purchased the HRP-conjugated secondary antibodies?**

   The reactivity of secondary antibodies ranges widely between vendors. Additionally, the ratio of HRP enzyme to antibody varies, and may affect the detection of the target. If the secondary antibodies from one vendor are not working, trying antibodies from other vendors may be helpful.

7. **Should the HRP-conjugated secondary antibodies be highly cross-adsorbed?**

   Although highly cross-adsorbed antibodies are essential for two-channel, multiplex detection, it is not always necessary with chemiluminescent blotting for a single target.

Washing Buffer

8. **Does it matter how I wash the membranes after antibody incubation?**

   Yes. Adequately washing the membranes will greatly improve the appearance of the chemiluminescent Western blot. Wash the membranes with a saline-buffered solution containing 0.05 to 0.1% of a non-ionic detergent such as Tween® 20. Wash four times for five minutes each time with ample wash solution on a shaker or rotator.

Substrate

9. Which substrate do I use?

There is a wide variety of chemiluminescent substrates for HRP detection, and some are better suited for digital imaging than others. Generally, choose a substrate with a faster rate of reaction for use with the Odyssey® Fc Imaging system. (Some substrates that are designed for optimal performance on film may not be suitable for detection on an imaging system.)

10. How do I apply the substrate?

Make sure the substrate is at room temperature before use. Apply carefully and avoid pooling to prevent splotches and areas of high background (Figure 1A). Carefully wick off any pools of substrate before imaging.

11. The membrane dried during imaging. Can I apply more substrate and image again?

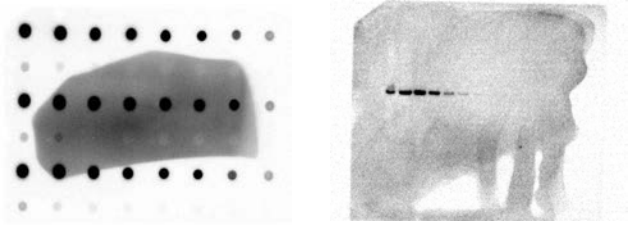
No. Applying more substrate to a dried membrane will likely result in high background (Figure 1B).

12. How do I keep the membrane from drying out?

Place a clear, flat plastic covering on the chemiluminescent Western blot to keep the substrate in contact with the HRP enzyme and to prevent the membrane from drying out. Image the plastic covering by itself first to determine if there is autofluorescence that will cause high background. You may need to try several types of plastic coverings before finding the best one.

High Background Due to Substrate

Pools of excess substrate on the membrane can lead to areas of high background, as can adding more substrate to a membrane that has dried (Figure 1). Apply the substrate carefully and wick off any pools of substrate before imaging. Do not allow the membrane to dry.



A. Pooled substrate B. Substrate addition to dried blot

Figure 1. High background due to excess substrate (A) and addition of substrate to a dried blot (B).

Imaging

13. Can I use the Odyssey Fc Imaging Tray multiple times?

It is important to image with a clean tray to prevent unwanted background, so you may want to use a new tray. You can clean a previously-used tray with ultrapure water or methanol to remove any traces of substrate or dye. If you have cleaned a used tray, image the tray by itself first to see if there is any contamination left. If there is still signal detected, clean the tray again with ultrapure water or methanol and re-image. If necessary, dispose of the contaminated tray and use a new tray (Figure 2).

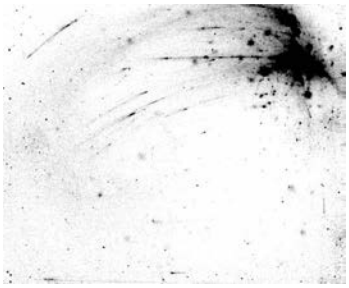


Figure 2. Contamination of the Odyssey® Fc Imaging Tray.

14. Can I wrap the blot in plastic wrap before imaging?

Wrapping the blot in plastic wrap may cause unwanted background, especially if it is folded or handled roughly (Figure 3). If using plastic wrap, it is important to avoid wrinkles as they scatter light, resulting in high background. You can also image the plastic wrap alone first to determine if the plastic itself scatters light. If it does, try different brands of plastic wrap to find the best one.

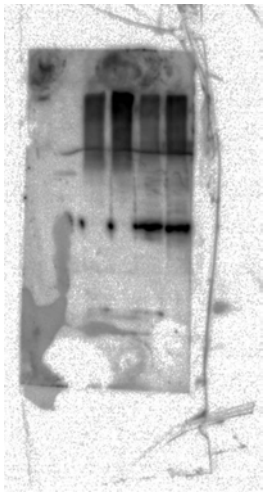


Figure 3. Unwanted background caused by plastic wrap.

Optimizing the Image Display

Use the LookUpTable (LUT) in Image Studio to adjust how the data are mapped to the display pixels of your computer screen. Overlaying the LookUpTable histogram is a curve with three adjustable points. Move the Max Point to the left to map more of the higher intensity data to the brighter display pixels and make the bands appear darker. Move the Min Point to the right to map the lower intensity data to the background color, creating a visually cleaner background. The middle point (K value) smoothly adjusts the mapping from linear to logarithmic. Changing to a more logarithmic mapping reduces the contrast between the lower and higher intensity data, so the appearance of less intense bands is improved while avoiding overly dark bands.

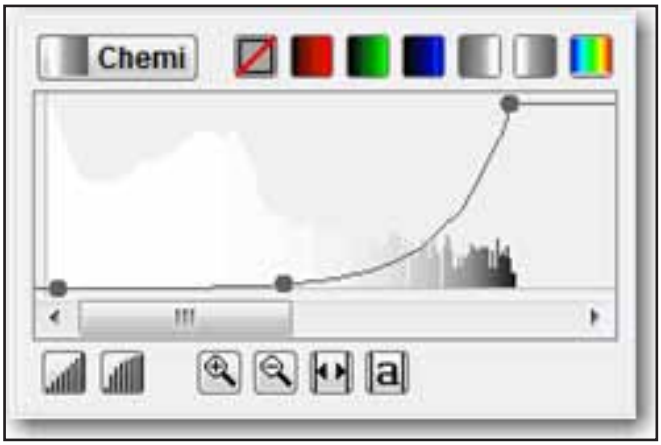


Figure 4. Use the three adjustable points to optimize the displayed image.

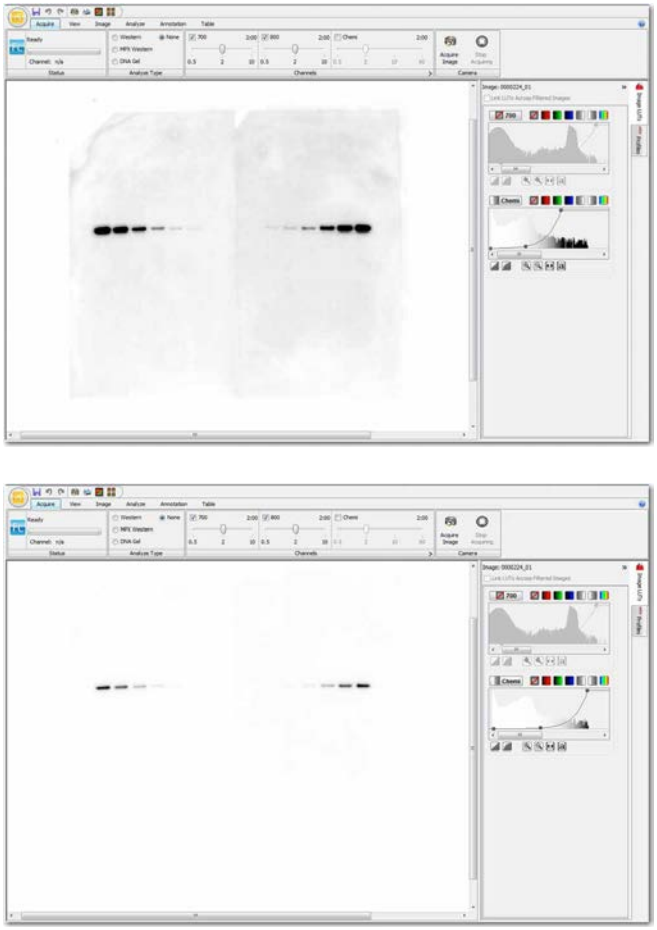


Figure 5. The curve overlaying the histogram in the LookUp Table was adjusted by moving the Max point to the left to make the bands appear darker in the second image.

Troubleshooting Guide for EMSA/Gel Shift Assays

IRDye® end-labeled oligonucleotides and the Odyssey® CLx or Odyssey Classic Infrared Imaging System provide a safe and sensitive alternative to radioactive EMSA detection. You can easily adapt existing mobility shift assay protocols by replacing the radiolabeled oligonucleotides with IRDye end-labeled oligonucleotides. You can also perform direct fluorescent detection in the wet gel, without waiting for gel drying or film exposures. Go from binding reactions to results in less than two hours.

Here are some helpful tips for optimizing your gel shift assay in order to obtain superior results.

Problem	Possible Cause	Solution / Prevention
Weak or no signal	Did not add DTT/Tween® 20 to binding reaction	Add 1 µL of 25 mM DTT/ 2.5% Tween 20 to binding reaction.
	Not enough IRDye labeled DNA used	Increase amount of IRDye labeled DNA added to the reaction.
	Target DNA degraded	Verify integrity of DNA.
	Imaged in the wrong channel of the Odyssey	When using IRDye 700-labeled DNA, turn on the 700 nm laser.
No shift bands detected or weak signal	Auto Sensitivity selected in Odyssey Software	Change Sensitivity setting to manual and adjust Sensitivity manually.
	Scanned gel with Intensity too low	Increase Intensity parameter in Odyssey Software to 8 and scan again.
	Incorrect focus offset	Adjust the Focus Offset in Odyssey software to equal the thickness of the glass plate plus half the thickness of the gel, and scan again.
	DNA/Protein complex disrupted due to heat or vortexing	Run gel with cooled buffer.
		Do not vortex binding reaction.
	Not enough extract	Add more extract to reaction.
	Degraded extract	Minimize freeze/thaw cycles.
		Use protease inhibitors.
	System not fully optimized	Use additives in the kit to determine their effects on binding efficiency.
Spots or speckling	Contamination on glass surfaces	Clean glass gel plates and the Odyssey scanning surface with isopropanol.

Troubleshooting Guide for In-Gel Westerns

Infrared fluorescence In-Gel Western detection avoids transfer problems by directly detecting target proteins within the polyacrylamide gel matrix, using the Odyssey® CLx or Odyssey Classic Infrared Imaging Systems. It saves time, reduces cost, and eliminates the variables introduced by the transfer step or subsequent blocking of the membrane. In-Gel Western detection can be performed with standard Odyssey reagents – no special kit is required. In addition, two-color Western detection of two different protein targets can be performed within the gel. In-gel detection can enable faster results and eliminate inconsistencies due to transfer.

If your target proteins don't transfer well (for example, large proteins or glycoproteins that are retained in the gel, or small proteins that may pass through the membrane during transfer), In-Gel detection bypasses this problem. Near-infrared In-Gel Westerns also offer unparalleled sensitivity in the low picogram range with the Odyssey Infrared Imaging systems. This technique provides a very useful tool for protein detection and research. However, it is important to note that In-Gel Western detection may not be quantitative.

Here are some helpful troubleshooting hints and tips for you to optimize your In-Gel Western experiment.

Problem	Possible Cause	Solution / Prevention
High background	Stacking gel is still present	Cut the stacking gel away after electrophoresis.
	Too much antibody	Reduce concentration of secondary antibody.
	Uneven gel background may result from insufficient solution volumes for incubations	Use enough solution at each step (fixation, washes, and antibody incubations) to completely immerse the gel.
	Pressing or squeezing gel during fixation and staining can cause splotchy background	Handle the gel gently, with gloved hands, and by the edges whenever possible.
	Gel was not thoroughly washed	Use plenty of wash buffer to allow gel to move freely. Do not allow the gel to stick to bottom of container.
		Extend wash times or increase number of washes. Background may decrease if the gel is allowed to soak in PBS overnight at room temperature (protect from light).
	Contaminated scanning surface	Before each use, clean scanning surface with methanol or ethanol followed by ultrapure water and wipe with lint-free tissues to remove residual dye. Remove any visible smears with isopropanol. Use canned air to remove any lint or dust.

Troubleshooting Guide for In-Gel Westerns (Cont'd)

Problem	Possible Cause	Solution / Prevention
Weak or no signal	Not enough antibody	Increase amount of primary and/or secondary antibody. Extend primary antibody incubation to overnight at 4°C to increase signal.
		Remember that In-Gel detection is not as sensitive as blot detection; adjust sample loading and antibody concentrations accordingly.
	Antibody dilution buffer is not optimal for your primary antibody	Try a different dilution buffer; this can significantly affect performance of some primary antibodies.
		Suggested buffers include 3-5% BSA, Odyssey® Blocking Buffer, and PBS or TBS (all with 0.1% Tween® 20). Other blockers (milk, casein, commercial blockers) and Tween 20 concentrations can also be tested.
	Gel type is not optimal	Amresco NEXT gels or Invitrogen NuPAGE® BisTris pre-cast gels are recommended for In-Gel detection. Other commercial gel sources and home-made gels can be used, but may show reduced sensitivity and require further optimization.
	Antibody did not penetrate gel sufficiently or evenly	Acrylamide percentage was too high. Try a lower percentage or a gradient gel.
		Increase volume for antibody incubations so that gel is completely immersed in antibody solution.
		Make sure gel is adequately fixed. Some monoclonal antibodies may be sensitive to residual acid in the gel; in this situation, eliminate acetic acid from the fix or extend the water wash step.
	Gel was left in isopropanol/acetic acid too long	This may cause protein to be lost from the gel. Fix for 15 minutes only.

Troubleshooting Guide for In-Gel Westerns (Cont'd)

Problem	Possible Cause	Solution / Prevention
Fuzzy or irregularly shaped bands	Gel type is not optimal	We recommend Amresco NEXT gels or Nu-PAGE® Bis-Tris pre-cast gels for In-Gel detection. Other commercial gel sources and homemade gels can be used, but may show reduced sensitivity and require further optimization.
	Gel is overloaded	Try loading less protein; bands can appear "blobby" if the amount of target protein in the band is too high.
	Inadequate fixation of gel	If problem persists when gel is fixed according to the protocol, try adjusting isopropanol or acetic acid concentrations. Fixing in isopropanol alone (no acetic acid) can cause irregularly shaped bands.
Non-specific or unexpected bands	Antibody concentration too high	Reduce amount of antibody used or reduce incubation times.
	Cross-reactivity between antibodies in a two-color experiment	Antibodies must be chosen carefully. Read *Guidelines for Two-Color Western Detection* (below).
	Antibody dilution buffer is not optimal for primary antibody	Try a different dilution buffer; this can significantly affect performance of some primary antibodies.
		Suggested buffers include 3-5% BSA, Odyssey® Blocking Buffer, and PBS or TBS (all with 0.1% Tween® 20).
	Bleed through between 700 nm and 800 nm channels	If signal is extremely strong (saturated) in one channel, it may appear faintly in the other channel. Re-scan gel at a lower intensity or repeat using less antibody or protein.

Guidelines for Two-Color Western Detection

It is absolutely critical that primary and secondary antibodies be carefully selected for two-color detection or cross-reactivity will result. The following guidelines should be used when selecting primary and secondary antibodies for two color detection:

1. All secondary antibodies must be highly cross-adsorbed to eliminate cross-reactivity.
2. The two primary antibodies used must be derived from different host species so they can be discriminated by secondary antibodies of different specificities. Example: Rabbit anti-Protein X + Mouse anti-Protein Y primary antibodies.
3. The two secondary antibodies used must be derived from the same host species so they will not react against one another. The secondary antibodies should not recognize immunoglobulins from other species that may be present in the sample Example: Goat anti-Rabbit IgG + Goat anti-Mouse IgG.
4. One secondary antibody should be labeled with IRDye® 800CW and the other with IRDye 680LT or IRDye 680RD, or other commercially available near-infrared dyes.
5. Always perform preliminary blots with each antibody alone to determine the expected banding pattern for each, before combining them in a two-color experiment. Slight cross-reactivity may occur, particularly if the antigen is very abundant, and can complicate interpretation of your blot. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.
6. For best results, avoid using primary antibodies from mouse and rat together for a two-color experiment. Because the species are so closely related, it is not possible to completely adsorb away cross-reactivity. Substantial cross-reactivity between bands may occur. If using mouse and rat together, it is crucial to run single-color blots first with each individual antibody to be certain of expected band sizes.

Troubleshooting Guide for Labeling with IRDye® Infrared Dyes

Problem	Possible Cause	Solution/Prevention
Protein is over-labeled (D/P ratio is too high)	Free dye present (i.e. the real amount of labeling may be less than it appears)	Process conjugates through a second spin column to remove free dye.
	Reactive dye and/or protein concentration in labeling reaction is not optimal	Use less dye or more protein in the labeling reaction.
	Labeling reaction carried out too long	Incubate reaction for 2 hours. Purify by spin column immediately.
Protein is under-labeled (D/P ratio is too low)	Reactive dye and/or protein concentration in the labeling reaction not optimal	Re-label the conjugate to increase the D/P ratio.
	Protein with inherently low labeling efficiency	Use more dye.
	Reactive contaminant(s) or preservatives present in original protein solution	Dialyze or desalt unlabeled protein prior to labeling reaction.
	NHS ester content too low	Use a fresh vial of dye for labeling reaction. Do not allow dye solution to stand for more than a few minutes before use.
	Protein concentration too low	Concentrate protein before labeling reaction.
High background in Westerns	Excessive free dye	Process conjugates through a second spin column to remove free dye.
	Primary antibody detects non-specific bands	Test alternative primary antibodies with one labeled secondary antibody.
	Over-labeling of protein	Re-check D/P ratio and possibly repeat labeling reaction using a lower amount of dye. For secondary antibodies, try more dilute solutions.
	Poor blocking	Try a different blocking buffer.
		Try a different membrane.
Low signal in Westerns	Protein under-labeled	Re-label conjugate to increase D/P ratio.
	Inappropriate blocker used	Try a different blocking buffer. Primary antibody performance is highly dependent on choice of blocker.
High background in In-Cell Western™ assays	Excessive free dye	In-Cell Western assays are very sensitive to free dye; process conjugates through a second spin column to remove free dye.
	Over-labeling	D/P ratios higher than 2:1 will cause excess background in this assay. Label new antibody with a lower D/P ratio.

Frequently Asked Questions for Using Cells in Suspension for In-Cell Western™ Assays

Handling Suspension Cells

1. **How do you culture suspension cells?**

   For instructions on culturing suspension cells, look up your cell line at *http://www.atcc.org/* and follow the guidelines.

2. **How do you make non-adherent cells (suspension cells) attach to plates?**

   A simple trick is to replace your complete media containing 10% serum (usually fetal bovine serum) with the same media minus the serum. Then allow the cells to sediment, forming a monolayer of cells within 10 minutes. **Caution**: Although cells appear attached to the plates, they are relatively loosely attached and therefore, extreme caution is required during solution-changing steps.

3. **How do I know that I have a monolayer?**

   Method #1 - Examine cells in the round bottom 96-well plates under a light microscope. The center of the wells should all have a small flat circular surface area where all the cells in that field are "in focus". Moving the plane of focus, up or down, will cause cells to be "off focus".

   Method #2 - Hold the round bottom 96-well plate under a light source. The monolayer should look opaque rather than transparent. Cells will not attach on top of the cell monolayer, so the opaqueness is due only to the monolayer.

4. **I cannot get a monolayer of cells. I get lots of spaces between cells. Is seeding 200,000 cells/well enough?**

   Seeding 200,000 cells/well is more than enough to form a complete cell monolayer. It is necessary to allow the cells in serum-free media to sediment in the T75 flask (or other tissue culture plates) for approximately 30 minutes before counting cells using a hemacytometer. When cells in serum-free media are placed, for example, in a T7 tissue culture flask, a monolayer of cells will immediately begin to form on the bottom of the flask. This will dramatically decrease the number of cells in suspension that are available for plating. **Note**: Once a complete monolayer has formed on the plate, the rest of the cells will remain in suspension. Count these cells in suspension and the cells attached to the T75 flask can be discarded later.

5. **During my washing steps, cells are coming off the plates.**

   a. **Are you using the recommended round bottom 96-well plate (BD Bioscience, P/N 353077)?**

      If **no**, cells will more easily detach from the flat bottom plates than the round bottom plates. The multi-channel pipettors will generate enough pressure when expelling liquid from the pipet to cause cell detachment when using flat bottom plates. Cells will detach even when pipetting down the sides of the wells.

      If **yes**, make sure you pipet down the sides of the wells and not directly onto the cells. If this doesn't help, you may need to change your multi-channel pipettor because different brands of pipettors have different amount of pressure required to expel the liquid from the pipet. The recommended multi-channel pipettor is the 12-channel Finnpipette (Thermo Fisher Corp, P/N 4610050).

   b. **Are you shaking or rotating the plates at a moderate to high speed?**

      If **yes**, gentler shaking/rotating is needed to prevent cells from detaching. Cells will detach. Set shaking or rotating speed to very low speed.

      If **no**, are you dumping the solutions straight from the plates? Dumping causes cells to detach. Either aspirate very slowly or manually pipet using the sides of the wells.

Round vs. Flat Bottom 96-well Plates

6. **Why can't I use the flat bottom 96-well plates?**

   LI-COR® Biosciences recommends using the round bottom 96-well plates.

7. When I scan an empty round bottom 96-well plate, I get lots of background noise.

The round bottom plate shows some background auto-fluorescence. The background fluorescence is relatively small compared to signal (about 200-fold difference, depending on the intensity of the signal) and can be subtracted from the signal. It is necessary to include background wells containing cells and only the secondary antibodies in order to completely subtract away the background noise originating from the plate as well as from the non-specific binding of the secondary antibodies.

Scan Settings

8. Why does my scanned image look so weak?

Assuming that you followed the protocol correctly and your antibodies work, did you set the focus offset to 3.0 to 3.5 mm for the BD Bioscience round bottom plates (P/N 353077)? If using the Nunc® round bottom plates (P/N 16332), the default setting for the flat bottom 96-well plates (3.0 mm) will not produce much signal. The focus offset for the Nunc round bottom plates should be set to 3.5 to 3.95 mm. For maximum signal strength with the Odyssey® Infrared Imagers, BD Bioscience round bottom plates are recommended. Both BD Bioscience and Nunc round bottom plates work well with the Odyssey Sa instrument.

Other Suspension Cell Lines and Different Pathways

9. Have you tested other suspension cell lines?

Yes. Suspension cell lines tested include Jurkat, K-562, and THP-1. A sample protocol can be downloaded from *http://biosupport.licor.com*.

10. Have you tested other pathways?

Yes. Pathways tested include ERK activation and apoptosis using cleaved caspase-3 as a marker (Figure 1). A sample protocol can be downloaded from *http://biosupport.licor.com*.

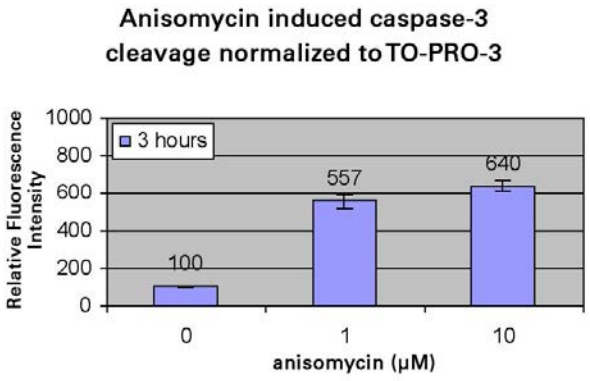
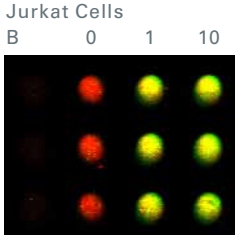
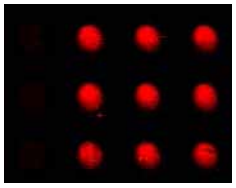


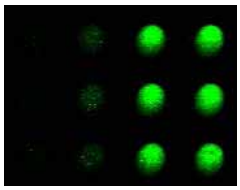
Figure 1. Anisomycin-induced apoptosis in Jurkat cells. The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 nm channels detecting TO-PRO®-3 DNA staining and cleaved caspase-3 (Asp175), respectively. The image was scanned using the Odyssey® Sa Infrared Imaging system with scan setting of 200 µm resolution, focus offset of 3.5, and intensity of 3.5 (700 channel) and 4 (800 channel). Background (B) wells were incubated with a secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the increase in caspase-3 cleavage in response to anisomycin treatment for 3 hours in Jurkat cells.



Overlay: Two-color In-Cell Western detection of cleaved capase-3



700 nm channel: TO-PRO-3 for normalization



800 nm channel: cleaved caspase-3

In-Cell Western™ Brochure

Quantitative Cell-based Assays

Download a Free Copy at
www.licor.com/icwbrochure

Imaging Systems

Choosing the Right Imaging System

LI-COR® Biosciences offers a variety of imaging systems capable of being used for many different applications. Pick your applications and then choose the right system for your lab.

Application	Odyssey® CLx Infrared Imaging System	Odyssey® Sa Infrared Imaging System	Odyssey® Fc Dual-Mode Imaging System	Pearl® Impulse Small Animal Imaging System
Coomassie Protein Gel Imaging	✓	✓	✓	
DNA Gel Staining - SYTO® 60 IR Fluorescent Stain	✓	✓	✓	
DNA Gel Staining – EtBr, SYBR® Safe Stains			✓	
EMSA/Gel Shift Assay	✓			
Gel Documentation	✓	✓	✓	
Glycoprotein Detection	✓	✓	✓	
High Throughput Plate-based Assay		✓**		
In-Cell ELISA	✓	✓		
In-Cell Western™ Assay	✓	✓		
In-Gel Western Assay	✓			
Microwestern Array	✓			
Multiplex Infrared Western Blot	✓	✓	✓	
Northern Blot	✓	✓		
On-Cell Western Assay	✓	✓		
Organ Imaging	✓			✓
Protease Assay	✓	✓		
Protein Array	✓	✓		
Protein Gel Staining	✓	✓	✓	
Reporter Gene Assay	✓	✓		

Choosing the Right Imaging System (Cont'd)

Application	Odyssey® CLx Infrared Imaging System	Odyssey® Sa Infrared Imaging System	Odyssey® Fc Dual-Mode Imaging System	Pearl® Impulse Small Animal Imaging System
Reverse Phase (Lysate) Protein Array	✓	✓		
RNAi Analysis	✓	✓		
Small Animal Imaging	✓*			✓
Southern Blot	✓	✓		
Tissue Section Imaging	✓	✓		
Transcription Factor Assay	✓	✓		
Western Blot				
• Chemiluminescent			✓	
• Quantitative Infrared	✓	✓	✓	
Zymography	✓	✓		

✓* With the MousePOD® *in vivo* Imaging Accessory for the Odyssey CLx Infrared Imaging System.

✓** With the optional Odyssey Sa Express Software, the BioTek® BioStack™ microplate reader, and Barcode reader.

MousePOD® *in vivo* Imaging Accessory for the Odyssey CLx Infrared Imaging System.

Odyssey® CLx Infrared Imaging System *NEW!*

The Odyssey CLx is the next generation of the Odyssey Classic, the most trusted and established standard in quantitative Western blot technology.

- The most flexible and multifunctional platform of the Odyssey imaging systems
- Accommodates a wide variety of applications
- Largest imaging surface of all Odyssey imaging systems (25 cm x 25 cm)

Now Featuring:

AutoScan Function

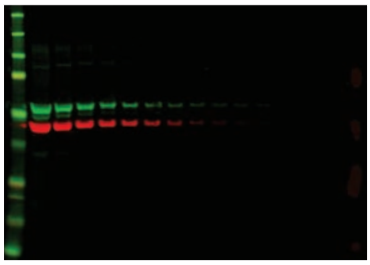
- Wide dynamic range captures the entire range of data without saturation in a single, time-saving scan — no need for multiple scans to optimize intensity settings
- An even wider dynamic range is available when detecting high-abundance proteins in a single image

Multiple Blot and Plate Scanning

- Simultaneously scan multiple samples of varied intensities in one scan for increased convenience

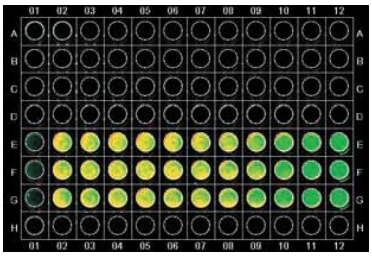
Easy-to-Use Image Studio Software (see pages 146-147)

- One-button image acquisition
- Quick user adoption
- Saves time needed to acquire and analyze data
- Compatible with PCs and Macs



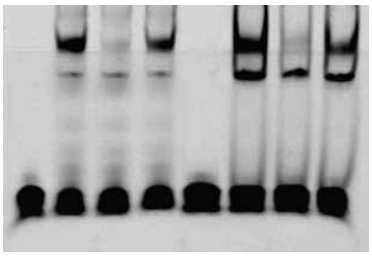
Wide Linear Dynamic Range

Accurately detect strong and weak signals over a broad, linear dynamic range (4 logs with manual scan feature; more than six logs with AutoScan feature).



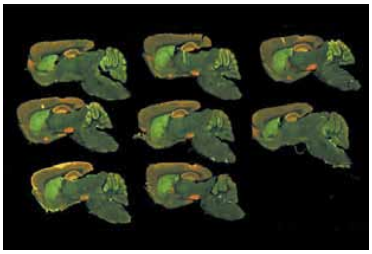
Two-Color Detection and Quantification

Detecting two targets simultaneously on the same membrane increases the accuracy of quantification and comparison.



High Sensitivity

Near-infrared fluorescence provides low background, high signal-to-noise and sensitivity equal to or better than chemiluminescence.



Direct Detection for Better Data

No film, darkroom, plastic wrap, or substrates. IRDye® signals on membranes are stable indefinitely if stored properly.

Applications:

- Coomassie Gel Imaging
- DNA Gel Imaging
- ELISA
- EMSA/Gel Shift Assay
- Glycoprotein Detection
- In-Cell Western™ Assay
- In-Gel Western Assay
- Microwestern Array
- Northern Blot
- On-Cell Western Assay
- Protease Assay
- Protein Array
- Quantitative Western Blot
- Reporter Gene Assay
- Reverse Phase (Lysate) Array
- RNAi Studies
- Small Animal Imaging*
- Southern Blot
- Tissue Section Imaging
- Transcription Factor Assay
- Zymography

*with the MousePOD® *in vivo* Imaging Accessory (page 148)

Odyssey® Sa Infrared Imaging System

The Odyssey Sa Infrared Imaging System offers the advantages of infrared imaging and the flexibility to handle both plate-based assays and quantitative Western blots.

Affordable Price

- Less expensive alternative to the more versatile Odyssey CLx Infrared Imaging System
- Excellent choice for smaller budgets while still offering membrane, gel, and plate imaging capabilities

Quantitative IR Western Blot Analysis

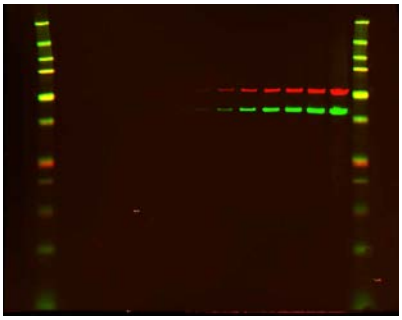
- Two-color infrared fluorescent detection for true quantitative analysis
- Detection down to picogram levels, comparable to the industry-leading Odyssey CLx Infrared Imaging System
- Wide linear dynamic range (4-5 logs in a single scan) comparable to the industry-leading Odyssey CLx Infrared Imaging System

Convenient and Flexible Software

- Sample format accommodates microplates, membranes, or gels
- In-Cell Western™ Assay analysis templates and easy-to-use analysis software

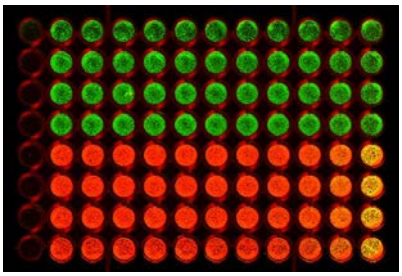
Walk-away Automation

- Upgrade to Odyssey Sa Express Automation Software for convenient multiple plate scanning
- Integrate with BioTek® BioStack™ Microplate Stacker for easy automation of up to 30 or 50 plates per run



Barcode Reader Accessory (see page 148)

The Odyssey Sa Barcode Reader is a factory-installed accessory that offers automated reading of barcodes on the sides of microplates. This accessory is fully compatible with the Odyssey Sa and Sa Express Application Software and can read most common barcode symbologies. This accessory may be added to ANY Odyssey Sa Configuration.



Multiple Packages

Tailor the Odyssey Sa Infrared Imaging System and Software to your lab's specific needs. Ask your local sales representative for more information.

Odyssey® Sa Applications

- Quantitative Western Blots
- In-Cell Western™ Assays
- On-Cell Western Assays
- RNAi Screens
- ELISAs and other Plate-based Assays
- Protein Gel Documentation
- Nucleic Acid Gel Documentation

Odyssey® Fc Dual-Mode Imaging System

Eliminate Film and Darkroom

Infrared Fluorescence plus Chemiluminescence Detection

The Odyssey Fc Imaging System provides superior infrared (IR) fluorescence and chemiluminescence imaging performance in a single system. Using the proprietary FieldBrite™ XT Optical System, the Odyssey Fc is specifically designed to detect a wide range of protein levels (see pages 143-145 for a White Paper on this technology).

Two-color Multiplex Detection

Near-infrared solid-state laser diodes at 685 and 785 nm provide two-channel IR direct detection with this advanced CCD-based imaging technology.

Streamlined Chemiluminescence Detection

Chemiluminescence detection can be done more quickly and more easily. Eliminate multiple exposures due to "blow out" of bands or saturation. Film and darkroom steps are eliminated.

Nucleic Acid Detection

Image ethidium bromide and SYBR® family of DNA stains for agarose gel digital imaging with no harmful UV excitation light

- Disposable imaging trays avoid instrument contamination
- SYTO® 60 near-infrared fluorescent DNA stain can also be used and imaged in the 700 nm channel of the Odyssey Fc

Easy-to-use, CCD-based Technology

Simple, elegant design with no manual filter wheels, manual focusing, or multiple light sources to contend with.

FieldBrite™ XT Optical System Offers: (see page 143)

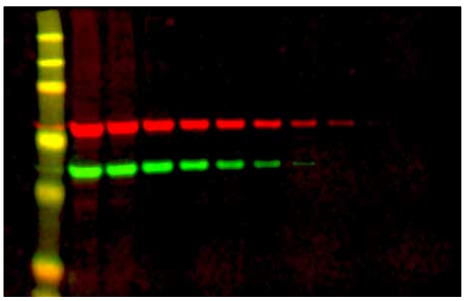
- No need for any post-image acquistion data manipulation by the user, such as flat fielding, binning, or image "stacking"
- Uniform imaging over entire field of view
- Improved signal to noise with increased image acquisition time

Image Studio Software Features:

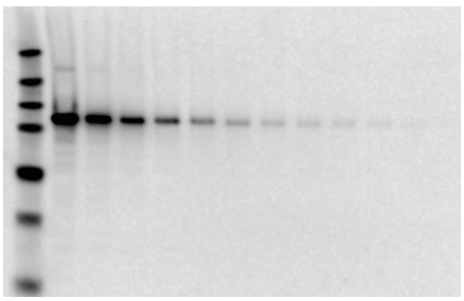
- Compatible with PCs and Macs
- Straight-forward project file structure
- Simple, one-button acquisition for optimal image with little or no user intervention
- Intuitive, application-based ribbon format leads you through from image acquisition to analysis
- Lock multiple image look-up tables together for accurate visual comparison between images
- Easily export data
- Quickly create electronic lab notebooks with customizable layout, headers, and print to paper or PDF

See pages 146-147 for more information on Image Studio software.

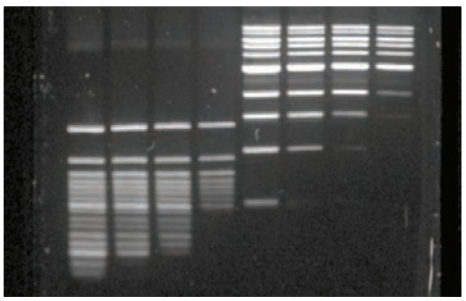
Odyssey® Fc Applications



Quantitative IR Western blots



Chemiluminescent Western blots



Coomassie, Ethidium Bromide (shown), and other Gel Stains

White Paper:
FieldBrite™ XT Optical System Within the Odyssey® Fc Imaging System

Introduction

Digital imaging with a CCD can often pose problems that require data manipulation for optimal image correction and quality. Methods that are commonly used for data manipulation in CCD systems include:

- Image Stacking
- Flat Fielding
- Binning

From a research perspective, it would be advantageous to have a system that provides highly accurate quantitation, without the need for post-imaging data correction.

Image Saturation and Stacking

The signal level a CCD generates is the rate of light that hits the detector (photon flux) multiplied by the exposure length (Figure 1). With a fixed photon flux, changing the exposure time can control the signal level a CCD generates.

Selecting the appropriate exposure length is critical for obtaining a quality image with quantifiable data. In order for the generated signal to be reliably measured, it needs to be strong enough to overcome the noise, but not stronger than the saturation level of the CCD. However, without knowing the level of light being emitted from a given sample, it can be difficult for the user to initially select the correct exposure time. With many CCD systems on the market, the user will generally need to acquire more than one image, at different exposure lengths, to finally obtain a high quality image. Some systems need to have the option to "stack" multiple images at a set of exposure times. This method will select the best image from the "stack" of images, or it will combine the frames in the "stack" into a single image through software manipulation.

Some imaging systems have image "auto exposure" features, an iterative approach to finding an exposure length that achieves a predefined maximum intensity level without saturation. In this case, image saturation can be avoided but the exposure still may not be optimal for the signal of interest.

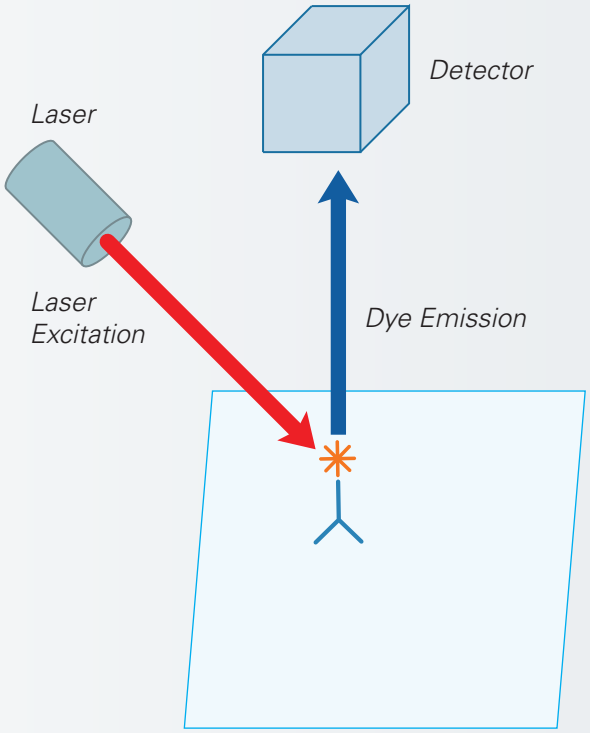


Figure 1. The amount of light reaching a detector (photon flux) is a critical component of imaging sensitivity.

The optimal method for digital imaging with CCD detection would include a system to allow for a single, accurate exposure.

Flat Fielding

Some CCD-based imaging systems offer a flat fielding technology that corrects for image nonuniformity within the initial image capture. This nonuniformity may be due to the lens, and for fluorescence imaging, the illumination, or the filters. Ideally, an instrument would be designed to give a reproducible imaging experience with low coefficients of variation (CV) without data manipulation (Figure 2).

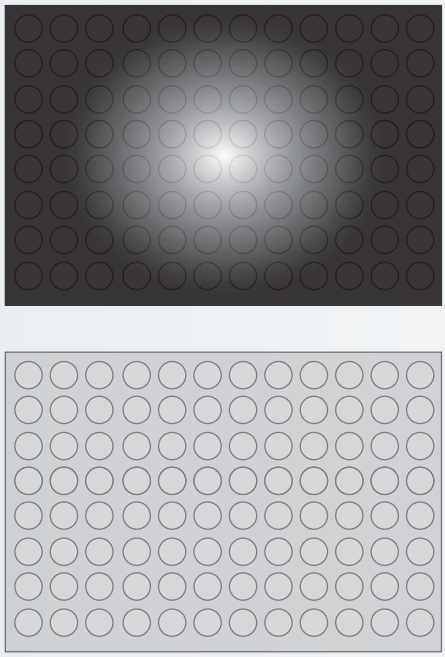


Figure 2. The top image shows data that were imaged with CCD detection and resulted in image nonuniformity. The bottom image shows the same data after being altered with flat fielding technology. With proper optical design, the bottom image should be possible without image manipulation.

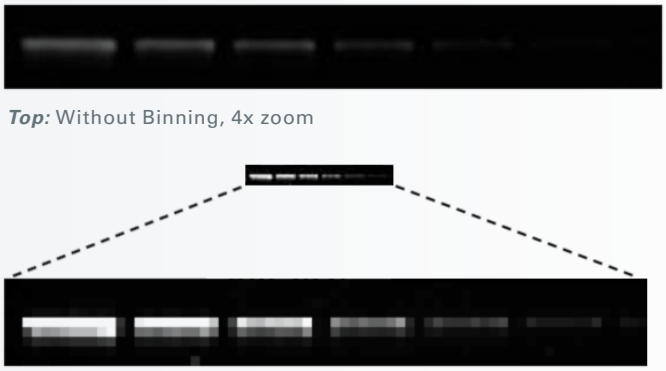
Noise Reduction Techniques and Binning

Camera manufacturers and CCD users have found many techniques to reduce the noise of the camera relative to the signal the CCD generates. These techniques enable reliable detection of samples emitting low amounts of signal. As mentioned above, one technique is image "stacking", which includes the acquisition of multiple frames of the same data and combining them into one image.

Another common technique used to reduce noise is binning. Binning consists of the addition of signal from a group of pixels together as they are read out of the CCD to form a larger pixel. This can produce higher signal, relative to noise, and results in improved sensitivity. This technique of combining pixels, however, results in lower image resolution (Figure 3).

FieldBrite™ XT Technology

The FieldBrite™ XT optical system within the Odyssey® Fc System has a highly advanced design that eliminates the need for post-image capture data correction. The FieldBrite XT system also provides images with an exceptionally wide dynamic range without the need for the user to take multiple



Top: Without Binning, 4x zoom

Bottom: With Binning, 4x zoom

Figure 3. An example of the effect of binning on a sample – it will produce a brighter image, but with a reduced resolution quality.

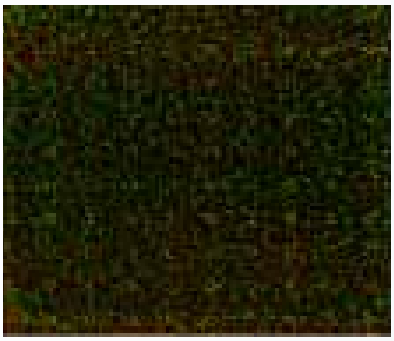
exposures or perform post-imaging data manipulation, such as flat fielding, noise reduction, or binning.

The FieldBrite XT optical system eliminates the need for image "stacking" and the worry of image saturation. The user selects only the total acquisition time before taking the one, and only, image necessary. This system provides images with a six-log dynamic range that produces a single image showing both strong and weak bands, without saturation. Because there is no need for software manipulation post-image capture, the FieldBrite XT optical system consistently produces quantifiable data.

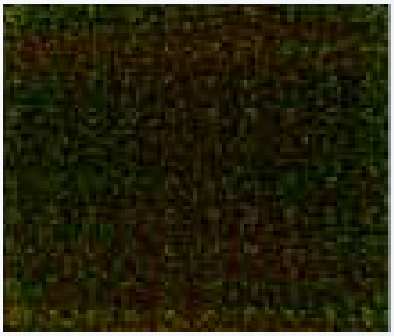
The FieldBrite XT optical system provides uniformity across the entire field of view with a coefficient of variation of less than 3% (Figure 4). Even laser illumination is superior to flat fielding technology, which is simply a post-image capture software manipulation, because it offers an image that is already flat — no additional software corrections are required by the user.

The even laser illumination, along with a dynamic range of greater than six logs, allows a FieldBrite XT user to obtain a high quality image the first time, every time, without worrying about what exposure time to use in order to avoid saturation, or where to place the sample for best results. Because this technology does not require data manipulation, it is presented in its most accurate, quantifiable form. This feature also allows for superior data reproducibility.

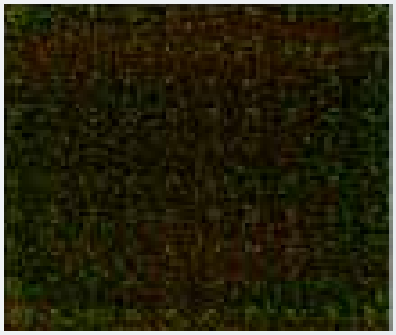
In FieldBrite XT technology, binning is not needed to increase sensitivity because of the low background resulting from its patented design. Because binning sacrifices resolution, this optical system has optimized resolution and sensitivity for high quality performance and image quality.



30 s
700 nm – 1.88% CV
800 nm – 1.36% CV



2 min
700 nm – 1.83% CV
800 nm – 1.42% CV



10 min
700 nm – 1.86% CV
800 nm – 1.41% CV

Figure 4. The three images above were acquired using the Odyssey® Fc Imaging System, with 100 spots analyzed per image across the entire field of view showing acquisition times with %CV per channel.

The noise reduction techniques employed in the FieldBrite XT optical system enable the detection of low light levels at the full resolution of the CCD. The only limitations in imaging with this technology almost always come from non-specific binding or optical background from the sample, not from the system itself.

Conclusions

Because FieldBrite™ XT technology offers an optimized optical system, data images only need to be captured once, without the need for post-image data manipulation. The patented design includes a wide dynamic range that eliminates the need for image stacking and taking multiple exposures at different times. An even field of illumination means that there is no need for flat fielding manipulation – the user can be confident that, without software adjustments, the original image is already flat. The FieldBrite XT optical system is sensitive with low background – there is no need to sacrifice resolution to gain sensitivity with this system.

Image Studio Imaging Software

Image Studio imaging software is now available for use with the Odyssey® CLx, Odyssey Classic, and Odyssey Fc Imaging Systems. The software is also compatible with PCs or Macs. This newly re-designed software is popular with LI-COR customers because it provides an extremely simple, intuitive interface that allows for easy user adoption while maintaining high quality data.

Image Studio simplifies the workflow for many applications with the following benefits:

Image Acquisition

Application-driven Ribbon Interface (Figure 1)

- Each analysis type has a corresponding ribbon with tools for analysis and formatting that are only relevant for that application

Ability to scan multiple areas in a single scan

- With an enhanced acquisition interface, the user can divide a single scan into multiple scan areas

Data Analysis

Definition of Lanes and Bands

- Simply define the sample area and the total number of lanes

Placement of Shapes by Simple Point-and-Click Method

- Click on a band to see a rectangle placed around it automatically

Rotation of Objects

- If a band or blot are not in the desired orientation, objects can be rotated as a group or individually

Data Analysis Tools

- The user has full control of the data display and can rearrange each column through sorting and filtering

Annotation Controls

- Simple to change fonts and controls, including rotational control of text and arrows

Linking of LookUp Tables

- View different scans under identical display conditions

Supports Many Types of Analysis, including:

- Western Blots
- In-Cell Western™ Assays
- Multi-Well Plates
- Coomassie Gel Documentation
- MPX™ Western Blots
- EMSA/Gel Shift Assays
- Tissue Imaging
- Grid Arrays
- DNA Gel Documentation
- Small Animal Imaging

Image & Data Export and Printing

Image Export Tools

- Image can be exported in multiple file formats for better end-use flexibility
- Image and accompanying information can be easily transferred from one computer to another for data analysis
- New sizing and resolution options are now available, as well as expanded file formats

Data Export Tools

- Launch an external spreadsheet program and automatically import selected rows of data into the spreadsheet program

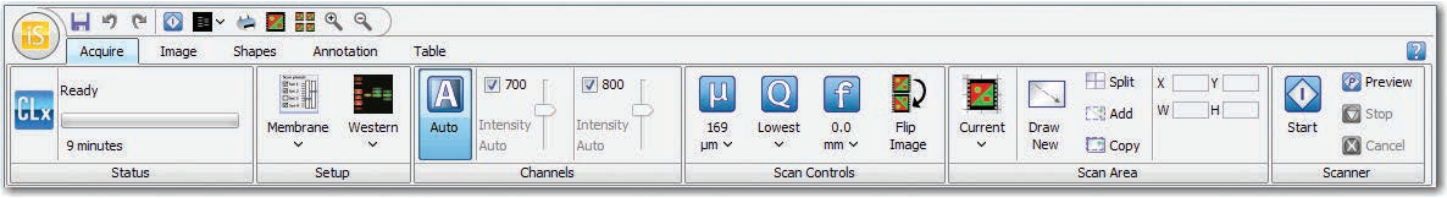


Figure 1. Image Studio is a ribbon-based application that displays analysis and formatting tools for user selection and implementation. The ribbon shown is for the Odyssey® CLx.

- Export experimental images and tables for further analysis in several downstream software packages, such as Adobe® Photoshop, Microsoft® PowerPoint, and GraphPad Prism® Software

Customizable Lab Notebooks (Figure 2)

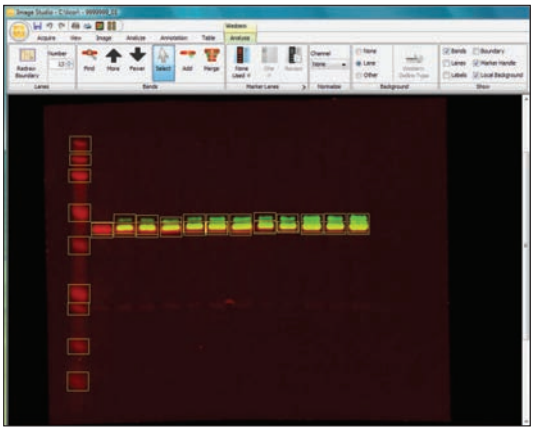
- Ability to create a direct-to-PDF export of data with separate templates for each analysis type. This PDF format is fully customizable to meet different documentation requirements

Data Storage

Simplified File Access

- Easier, more convenient access to user data

Figure 2. Create electronic or hard-copy lab reports customized to meet specific needs and documentation requirements, such as GLP or ISO.



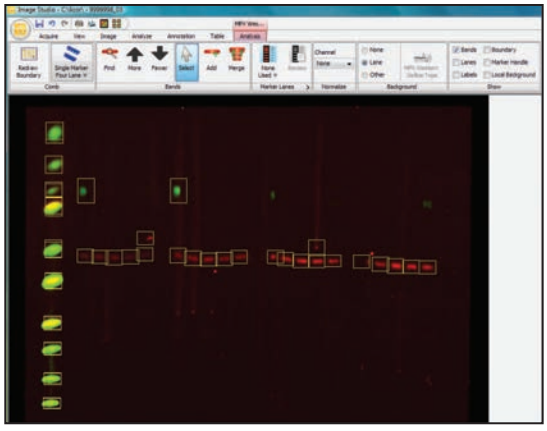


Figure 3. Dedicated Western Analysis and MPX™ Western ribbons offer tools for quick placement of lane and band markers on the image to speed up data analysis.

Image Studio software available in Windows or Macintosh versions.

www.licor.com/imagestudiosoftware

Odyssey® Family Accessories

926-40020 **Odyssey Fc Imaging Tray** **$45.00**
Imaging trays for use with the Odyssey Fc Imaging System. Five (5) trays per pack.

926-70000 **Silicone 13 x 13 cm Mat** **$30.00**
Used for flattening membranes against the glass surface of the Odyssey CLx or Classic Infrared Imaging Systems.

926-70001 **Silicone 25 x 25 cm Mat** **$72.00**
Used for flattening membranes against the glass surface of the Odyssey CLx or Classic Infrared Imaging Systems.

926-71000 **4" Soft Roller** **$38.50**
Used for eliminating air bubbles from membranes on the glass surface of the Odyssey CLx or Classic Infrared Imaging Systems or in the Odyssey Fc Imaging System imaging tray.

926-71804 **Odyssey Pens (Package of 4)** **$20.00**
Used for writing on nitrocellulose membranes to be scanned on any of the Odyssey Imaging Systems. Does not fluoresce in the 700 or 800 nm channel.

926-72000 **1 MP Alignment Guide** **$35.00**
Suitable for aligning 1 multi-well plate on the glass surface of the Odyssey CLx or Classic Infrared Imaging Systems. Multi-well plates purchased separately.

926-72200 **6 MP Alignment Guide** **$35.00**
Suitable for aligning up to 6 multi-well plates on the glass surface of the Odyssey CLx or Classic Infrared Imaging Systems. Multi-well plates purchased separately.

926-73000 **Membrane Carrier** **$99.00**
The glass membrane carrier included with the Odyssey Sa Imaging System. Used for Western blotting methods and other analyses requiring scans of membranes. Carrier has the same dimensions as a microplate, but with a 3 mm ridge around the edges to prevent liquid from spilling into the Imager.

9201-550 **Odyssey Classic ICW Software Application Module** **$2,175.00**
For *in vitro* assays in microplate format for the Odyssey Software (P/N 9201-500). Automatically finds wells for 96- and 384-well microplates, and performs ratiometric calculations using the two imaging channels.

9201-570 **Odyssey Classic SAI Software Application Module** **$2,175.00**
Includes Auto Shape tool for Odyssey Software (P/N 9201-500) to quickly mark tumors, organs, and other regions of interest. Pseudocolor display style helps to quickly isolate regions of interest.

2000-501 **Image Studio In-Cell Western™ Analysis Key** ..... NEW! **$2,175.00**
For *in vitro* assays in microplate format for the Image Studio Software (P/N 9140-500). Automatically finds wells for 96- and 384-well microplates, and performs ratiometric calculations using the two imaging channels of the Odyssey CLx or Classic Infrared Imaging Systems.

2000-502 **Image Studio Small Animal Imaging Analysis Key** ..... NEW! **$2,175.00**
Includes Auto Shape tool for Image Studio Software (P/N 9140-500) to quickly mark tumors, organs, and other regions of interest. Pseudocolor display style helps to quickly isolate regions of interest.

9201-MP **MousePOD® *In vivo* Imaging Accessory** **$10,000.00**
Fits on the Odyssey Classic or CLx Imagers scanning surface and accommodates up to 3 mice at one time. It regulates air temperature to maintain animal's temperature during scanning and facilitates gas anesthesia to animals via nose cones (SmartFlow Anesthesia Suite not included; see page 151). Includes Small Animal Imaging Module (P/N 9201-570) for Odyssey Software with Auto Shape tool and Image Studio Small Animal Analysis Key.

929-97902 **Rat Nose Cone for MousePOD Accessory** **$60.00**

9260-61 **Barcode Reader for Odyssey Sa Infrared Imaging System** ..... NEW! **$2,500.00**
A factory-installed accessory that provides for the automated reading of barcodes on the sides of microplates. Fully compatible with the Odyssey Sa and Sa Express Application Software and can read most common barcode symbologies. May be added to ANY Odyssey Sa Configuration.

Western Blot Incubation Boxes

A convenient way to protect your near-infrared fluorescent Western blots during incubations and washes.

Small

929-97101 Western Incubation Box, Small, 1 box, 2 3/16" x 1 7/8" x 1 3/16" (7.2 x 4.9 x 3.0 cm) $11.00

929-97105 Western Incubation Box, Small, 5 pack $38.50

929-97110 Western Incubation Box, Small, 10 pack $61.50

Medium

929-97201 Western Incubation Box, Medium, 1 box, 3 1/2" x 2 7/16" x 1 1/8" (8.9 x 6.3 x 2.8 cm) $12.50

929-97205 Western Incubation Box, Medium, 5 pack $42.50

929-97210 Western Incubation Box, Medium, 10 pack $72.50

Large

929-97301 Western Incubation Box, Large, 1 box, 4 1/2" x 3 7/16" x 1 1/8" (11.5 x 8.8 x 2.8 cm) $15.40

929-97305 Western Incubation Box, Large, 5 pack $55.00

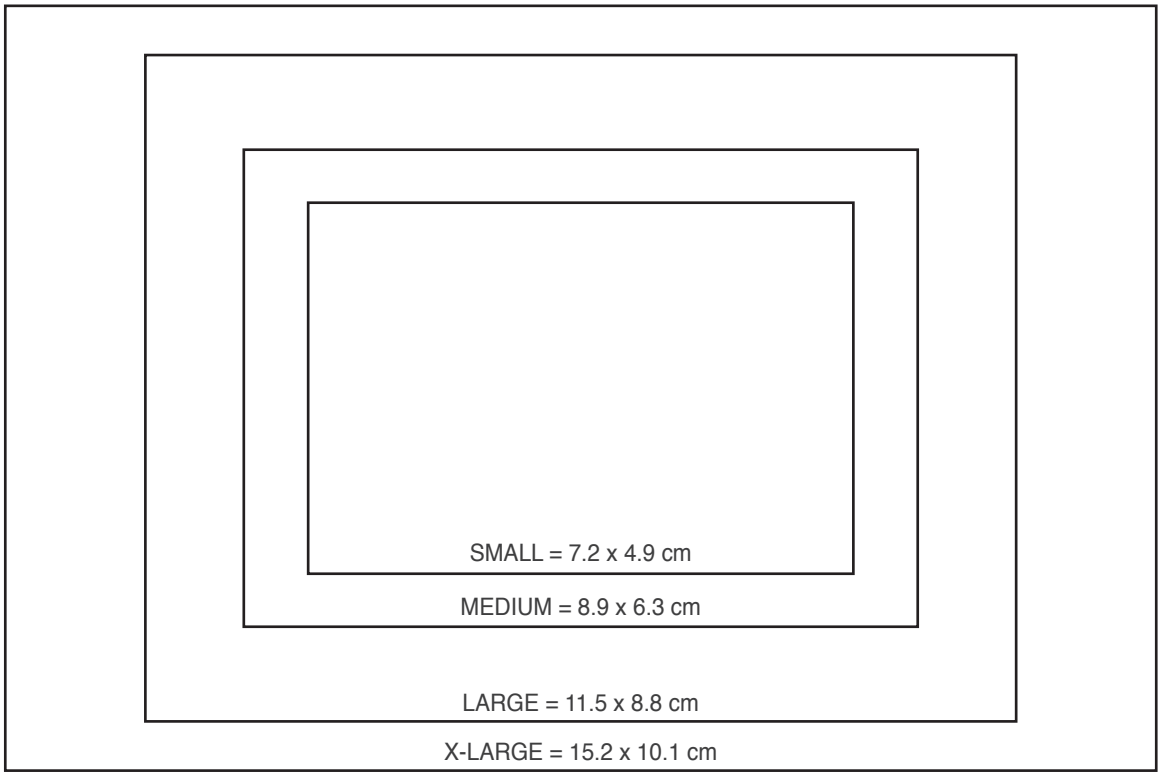
929-97310 Western Incubation Box, Large, 10 pack $99.00

Extra Large

929-97401 Western Incubation Box, XL, 1 box, 5 15/16" x 3 15/16" x 1 1/4" (15.2 x 10.1 x 3.1 cm) $16.50

929-97405 Western Incubation Box, XL, 5 pack $66.00

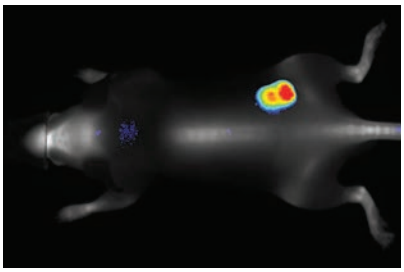
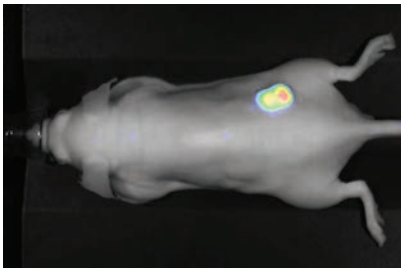
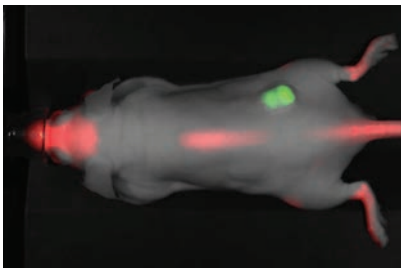
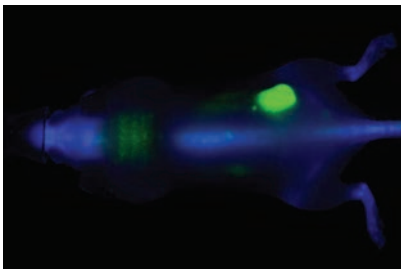
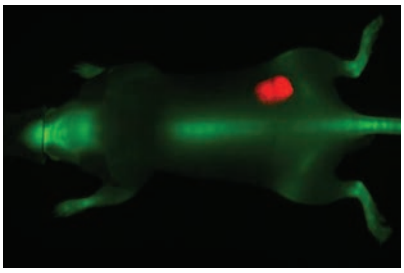
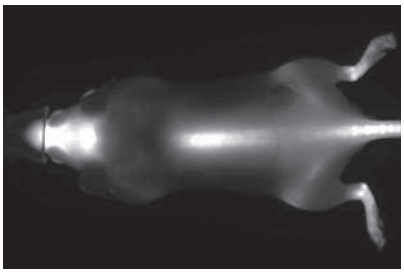
929-97410 Western Incubation Box, XL, 10 pack $115.50



Pearl® Impulse Small Animal Imaging System

Near-Infrared Detection for the Highest Sensitivity

- The Pearl Impulse uses a FieldBrite™ Xi CCD-based optical system that is specifically designed for *in vivo* imaging in the near-infrared (NIR) fluorescence spectral region. This near-infrared region is ideal for *in vivo* imaging because it offers extremely low tissue autofluorescence and light scattering.
- Patented optical design dramatically reduces noise.
- Signal detection is optimized for industry-leading IRDye® near-infrared dyes and BrightSite™ optical probes.
- Near-infrared laser illumination offers deeper tissue penetration than white light.
- Smaller targets can be visualized at greater depths, allowing for earlier tumor detection.
- Acquires single-channel fluorescent images as often as every 500 milliseconds. Acquires optimized multi-channel images (700 nm, 800 nm, and white light) in about 30 seconds. Speed minimizes animal stress and increases productivity.
- The Pearl Impulse is portable and does not take up much room. This small footprint simplifies mobility and accommodates placement into most labs.
- Six logs (22 bits) of dynamic range available for each image: Up to 4 logs of usable dynamic range when imaging mice in the 800 nm channel, or up to 6 logs of dynamic range when imaging excised tumors or organs.

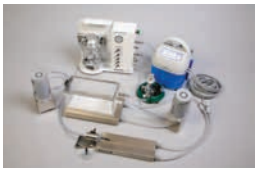
Multiple Packages

Tailor the Pearl Impulse and Software to your lab's specific needs. Ask your local sales representative for more information.

Check out our video on connecting the SmartFlow Anesthesia System to the Pearl Impulse Small Animal Imaging System.

www.licor.com/smartflowsetup

Pearl® Accessories



SmartFlow Anesthesia Suite

9000-101 SmartFlow Anesthesia Suite (120V) $12,900.00
9000-102 SmartFlow Anesthesia Suite (230V) $12,900.00

LI-COR® Biosciences SmartFlow Anesthesia Suite is the most advanced inhalation anesthesia design available. The innovative design ensures accurate gas flow for up to five different breathing devices with the simple flip of a switch, eliminating the complex and error-prone calculations required on manual systems associated with adjusting the flow rates for multiple breathing systems. Unlike traditional systems, the SmartFlow system accurately controls the flow rate of oxygen for each breathing device, which reduces overall costs by more efficient use of oxygen and Isoflurane.

The system accommodates an induction chamber plus up to four other breathing units (e.g. Pearl® Impulse, Docking Station, Surgical Suite, plus the MousePOD® *in vivo* Imaging accessory for the Odyssey® Classic or CLx).

Benefits:

- Preset flow rates reduce the usage of Isoflurane and eliminate the need for flow meters and manual adjustments
- Easy-to-use on/off switches reduce the chances for operator errors
- Flow rate is not impacted by the number or type of accessories attached to the unit
- Consistent flow rates are obtained over distances greater than 100 feet
- Broad worldwide compatibility with either a 120V mode or 230V mode

Includes:

- SmartFlow Vaporizer Unit
- Charcoal Filters (8)
- Induction Chamber
- Induction Chamber Heater
- Water Pump (120V or 230V)
- Oxygen Regulator (H Tank)
- Filter Stand
- Gas Inflow Hose, Outflow Hose, and Oxygen Hose
- Anesthesia Accessories
- Manual and QuickStart Guide

SmartFlow Surgical System

9000-120 SmartFlow Surgical Suite $4,500.00

The SmartFlow Surgical Suite is an important accessory to the SmartFlow Anesthesia Suite, allowing mice to be safely and easily kept warm (water pump not included; water pump is included with SmartFlow Anesthesia Suite) and anesthetized during preparation and surgical procedures. For use with animals up to 500 grams.

Includes:

- SmartFlow Surgical Bed
- SmartFlow Microflex Non-Rebreather Breathing Device
- Filter Stand
- Charcoal Filter
- Two Nose Cones and Plugs
- Surgical Suite Quickstart Guide

Pearl® Accessories

9000-140 Pearl Imaging Cart $6,900.00
This heavy duty stainless steel Pearl Imaging Cart turns your optical imaging lab into a mobile workstation. Sturdy design with plenty of room to accommodate a wide variety of accessories including: SmartFlow Anesthesia Suite, SmartFlow Surgical Suite, Pearl Docking Station, computer, oxygen tanks, and more.

9300-20 Pearl Docking Station $6,900.00
The Docking Station provides heat and HEPA-filtered anesthesia to either the Pearl Imaging Bed or the Pearl Clean Box (Anesthesia System, Imaging Bed, and Clean Box all sold separately). The Docking Station is composed of the Docking Station and Flowmeter and has a small footprint that allows it to fit into traditional laminar flow hoods.

9300-21 Pearl Imaging Bed $3,900.00
The Pearl Imaging Bed is compatible with both the Pearl Impulse and the Pearl Docking Station. Once inserted into either the Pearl Docking Station or Pearl Impulse, the Imaging Bed will be supplied with both heat and anesthesia.

9300-22 Pearl Clean Box $6,900.00
The Clean Box is compatible with both the Pearl Impulse and the Pearl Docking Station. Once inserted into either the Pearl Docking Station or Pearl Impulse, the Clean Box will be supplied with both heat and anesthesia. This unique imaging accessory allows immunocompromised mice to be transported from the docking station clean area to the Pearl Impulse in a closed system, to avoid exposure to outside elements.

9300-23 Organ Tray Base *NEW!* **..... $1,900.00**
9300-836 Disposable Organ Trays (5 pack) $100.00
9300-837 Disposable Organ Trays (25 pack) $400.00
The Organ Tray Base provides a way to image organs and tissue without compromising them due to heated imaging beds. Field-of-view markings on the trays ensure that organs will be placed within the actual imaging window, and the specially optimized polystyrene material provides very low background noise ensure high signal-to-noise ratios in organs. Includes 5 disposable organ trays.

Consumables

9000-802 Mouse Nose Cone Large (3) $195.00
9000-803 Mouse Nose Cone Small (3) $195.00
9000-804 Nose Cone Plug Large (6) $125.00
9300-800 Nose Cone Plug Small (6) $125.00
9300-801 Rubber Cord for Imaging Bed: 10' .. $100.00
9300-820 Charcoal Canisters (8 canisters) $160.00
9300-821 HEPA Filter Assembly $200.00
9300-834 Clean Box Cover Slip $100.00
9300-830 Clean Box Cleaning Kit $200.00
9300-831 Heavy Duty Optical Wipes (300) $100.00
9300-832 Optical Tissue (150 tissues) $100.00
9300-810 Imaging Blankets (25 blankets) $125.00
9300-833 Optical Cleaner (3 pack) $100.00
9300-835 Glass Replacement Kit $750.00
9300-840 Dye Decontamination Kit $125.00
9300-841 Povidone Iodine Refills (400 pads) ... $80.00
9300-842 Alcohol Prep Pad Refills (400 pads) .. $80.00
9300-850 Compound Injection Clip (25) *NEW!* **..... $100.00**
9300-851 Compound Injection Clip (100) *NEW!* **..... $300.00**
9957-046 Pearl Desiccant Kit $195.00

Create a Complete Workstation

Combining the Odyssey® CLx Infrared Imaging System with the Pearl® Impulse Small Animal Imaging System creates a versatile workstation for *in vivo* and *in vitro* imaging. Probes developed using IRDye® infrared dyes can be used for evaluation of binding capacity for *in vivo*, *in vitro*, and tissue imaging. This technology offers researchers the ability to take research from the cell to the animal, all within one lab.

Odyssey® CLx Infrared Imaging System Capabilities:

- Cell-based assays (binding capacity, specificity, competition, etc.) for optical agent development
- Histology and whole organ imaging for studying clearance and specificity
- Simultaneous two-color detection for two targets or one target with sample normalization

Pearl® Impulse Small Animal Imaging Capabilities:

- Visualize biological processes in living animals with IRDye® optical agents
- Monitor multiple biological targets simultaneously
- Small, easy-to-use platform that is compatible with ready-to-use imaging agents – no transfections required

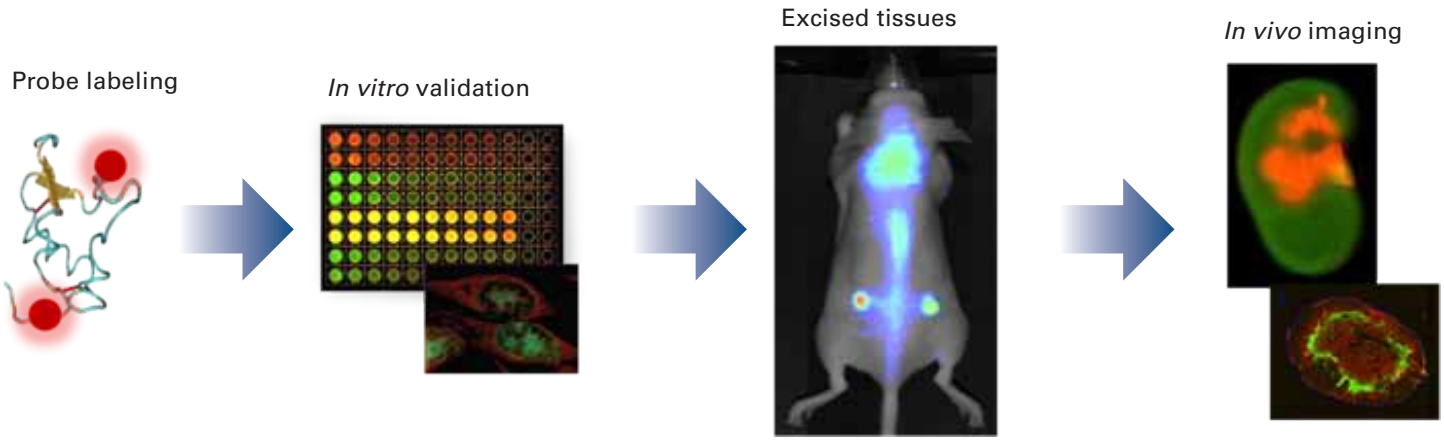


Figure 1. Validation and Use of an IRDye® Fluorescent Probe. After probe labeling, *in vitro* cellular assays and microscopy are used to confirm specificity. The desired target is then imaged in animals. Excised organs and tissues can be examined for more detailed localization of the probe. Animal image captured with Pearl® Impulse. A more comprehensive discussion of approaches for the development of fluorescent contrast agents has also been published. (see pages 82-94) Reference: Kovar, et al. *Anal Biochem* 367(2007) 1-12.

Pearl® Impulse Small Animal Imaging System Brochure

Download a Free Copy at
www.licor.com/pearlbrochure

Translational *Research*

Translational Research

Translating IRDye® Technology into the Clinic

LI-COR Translational Research is working to facilitate the use of IRDye® 800CW-labeled imaging agents in clinical studies for fluorescence-assisted cancer surgery. In preclinical studies, IRDye 800CW has been conjugated to a variety of targeted agents by a number of leading molecular imaging laboratories with the intent to translate these to clinical use.

Molecular Imaging has the promise to improve surgical procedures by increasing the visual information available to the surgeon. Near-infrared (NIR) fluorescent probes are being developed for several procedures, including:

- Angiography
- Location and mapping of lymphatics
- Identification of positive lymph nodes
- Precise resection of a tumor margin
- Identification of normal vital structures (i.e. ducts, nerves, lymphatics)

Toxicity Study

In 2010, LI-COR Biosciences announced the successful completion of animal toxicity studies for IRDye® 800CW infrared dye carboxylic acid using a protocol reviewed by the Food and Drug Administration (FDA). Currently LI-COR is exploring options for additional studies as next steps in the FDA approval process leading toward clinical use. In expectation of clinical applications, LI-COR has established cGMP manufacturing of IRDye 800CW along with an FDA drug master file. We continue to work with collaborators to translate optical imaging agents to the clinic.

IRDye® 800CW Scale up and GMP Manufacture

In 2010, LI-COR® Biosciences announces it has completed manufacturing of IRDye® 800CW near-infrared dye under a GMP process. This is considered a key step toward translating near-infrared contrast agents into clinical use.

IRDye® 800CW Active Substance Master File and Drug Master File Submitted

In 2011, LI-COR Biosciences announced an Active Substance Master File (ASMF) for IRDye 800CW N-succinimidyl ester is on record with European regulatory authorities in support of a clinical trial testing a targeted agent for optical imaging-assisted surgery. In addition, a Drug Master File has been registered with the United States Food and Drug Administration in anticipation of similar clinical trials in the U.S. The dye is manufactured under GMP and is the subject of a published toxicity study.

Contact Info:

If you would like to learn more about IRDye 800CW or have an interest licensing the dye for a clinical application, e-mail: **TranslationalResearch@licor.com** or call **1-402-467-0904.**

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Worldwide Distributors

For pricing information and ordering information outside the United States: contact your local distributor. Information on LI-COR distributors available at *www.licor.com/distributors.*

Prices subject to change without notice. See *www.licor.com/terms* for terms and conditions of sale. Ground shipping available for selected items.

"Trust in the LORD with all your heart and do not lean on your own understanding. In all your ways acknowledge Him, and He will make your paths straight." —Proverbs 3:5,6

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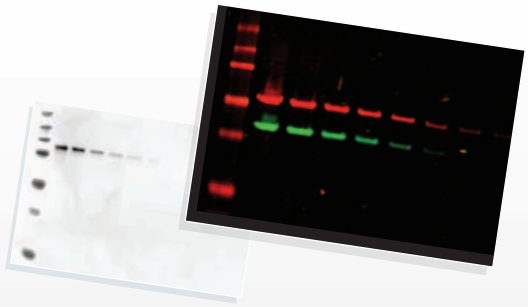

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