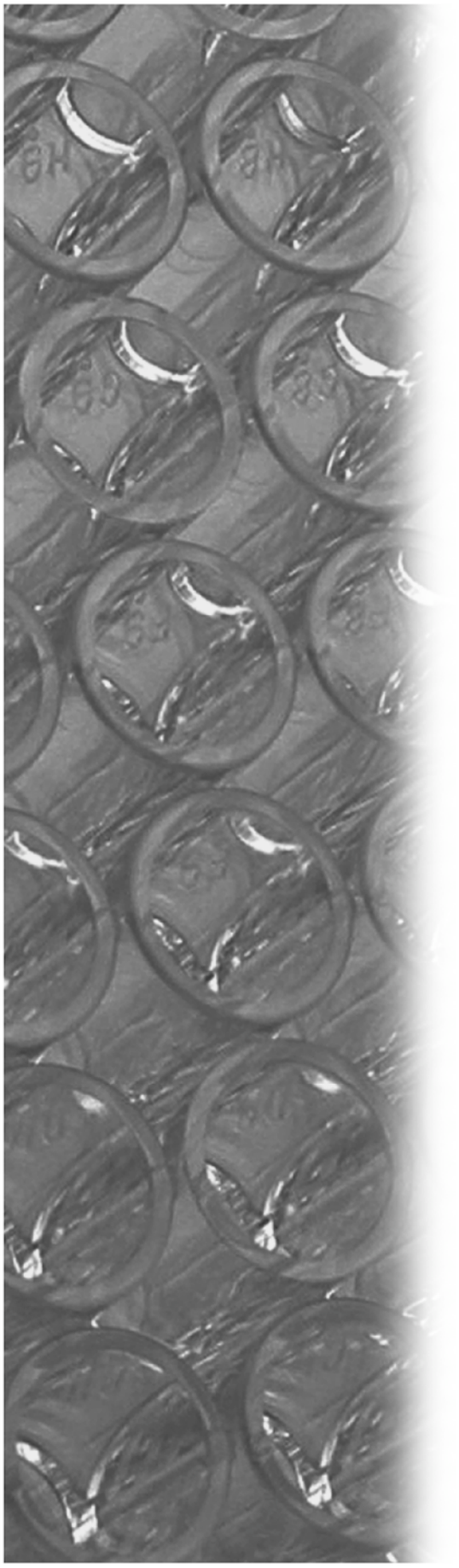

ODYSSEY

INFRARED IMAGING SYSTEM

Application Protocols

LI-COR®
Biosciences

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Additional Protocols at http://biosupport.licor.com

988-12030 4/11

Western Blot Analysis

Developed for:

Odyssey Family of Imagers

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your model of Odyssey Imager.

LI-COR®
Biosciences

Published 2003. Revised February 2011.
The most recent version of this protocol
is posted at: http://biosupport.licor.com

Contents

I. Required Reagents

- Blotted nitrocellulose (LI-COR, P/N 926-31090)
  or low-fluorescent PVDF membrane (LI-COR, P/N 926-31098)
- Odyssey Blocking Buffer (LI-COR, P/N 927-40000)
- Primary antibodies
- Infrared IRDye® secondary antibodies (LI-COR)
- Tween® 20
- PBS wash buffer (LI-COR, P/N 928-40018 or 928-40020)
- Ultrapure water
- Methanol for wetting of PVDF
- SDS (if desired)
- Other blocking buffers (if desired)
- NewBlot™ Stripping Buffer, if desired, for nitrocellulose (LI-COR, P/N 928-40030)
  or PVDF (LI-COR, P/N 928-40032) membranes

Fluorescent Dyes Appropriate for Use with the Odyssey System

Dye	Sensitivity	Odyssey Channel
IRDye 800CW	+ + +	800
IRDye 680LT	+ + +	700
IRDye 680	+ + +	700
IRDye 700DX	+ +	700
Alexa Fluor® 680	+ + +	700
Alexa Fluor 700	+ +	700
Alexa Fluor 750	+ +	700/800 (not recommended; signal appears in both channels)
Alexa Fluor 647	+	700
Cy®5.5	+ +	700
Cy5	+	700

The most current information on dye compatibility can be found on the LI-COR website (www.licor.com).

II. Western Detection Methods

Nitrocellulose or PVDF membranes may be used for protein blotting. Pure cast nitrocellulose is generally preferable to supported nitrocellulose. Protein should be transferred from gel to membrane by standard procedures. Membranes should be handled only by their edges, with clean forceps.

After transfer, perform the following steps:

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. **Notes:** • Ink from most pens and markers will fluoresce on the Odyssey® Imagers. The ink may wash off and re-deposit elsewhere on the membrane, creating blotches and streaks. Pencil should be used to mark membranes. (The Odyssey pen doesn't fluoresce and can be used with nitrocellulose membranes, since the membrane will not be soaked in methanol causing the ink to run.)
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/cm^2 is suggested). **Notes:** • Membranes can be blocked overnight at 4°C if desired. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution, but be aware that milk may cause higher background on PVDF membranes. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required). • Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.
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 background, add 0.1 - 0.2% Tween® 20 to the diluted antibody before incubation. The optimum Tween 20 concentration will depend on the antibody. **Notes:** • Two-color detection requires careful selection of primary and secondary antibodies. For details, see *III. Guidelines for Two Color Western Detection*. • The MPX™ Blotting System can be used to efficiently determine the optimum antibody concentration. For details, see *One Blot Western Optimization Using the MPX Blotting System (979-10184)* at http://biosupport.licor.com.
4.	Incubate blot in primary antibody for 60 minutes or longer 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 fluorescently-labeled secondary 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. **Notes:** • For detection of small amounts of protein, try using more secondary antibody (1:5000-1:10,000 dilution). • Be careful not to introduce contamination into the antibody vial. • Diluted secondary antibody can be saved and re-used. Store at 4°C and protect from light. However, for best sensitivity and performance, use freshly diluted antibody solution. • Adding 0.01% - 0.02% SDS to the diluted secondary antibody (in addition to Tween 20) will substantially reduce membrane background, particularly when using PVDF. However, DO NOT add SDS during blocking or to the diluted primary antibody. See *V. Adapting Western Blotting Protocols for Odyssey Detection* for more information about how and why to use SDS in the secondary antibody incubation. • The MPX™ Blotting System can be used to efficiently determine the optimum antibody concentration. For details, see *One Blot Western Optimization Using the MPX Blotting System (979-10184)* at http://biosupport.licor.com.
7.	Incubate blot in secondary antibody for 30-60 minutes at room temperature with gentle shaking. Protect from light during incubation. **Notes:** • Incubating more than 60 minutes may increase background.
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 to remove residual Tween 20. The membrane is now ready to scan. **Notes:** • Scan in the appropriate channels (see *I. Required Reagents* for details). • Protect the membrane from light until it has been scanned. • Keep the membrane wet to strip and re-use it. Once a membrane has dried, stripping is ineffective. • Blots can be allowed to dry before scanning if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wetted for scanning. • The fluorescent signal on the membrane will remain stable for several months, or longer, if protected from light. Membranes may be stored dry or in PBS buffer at 4°C. • If signal on membrane is too strong or too weak, re-scan the membrane at a lower or higher scan intensity setting, respectively.

Molecular Weight Marker

If you loaded the Odyssey Prestained Molecular Weight Marker (LI-COR, P/N 928-40000), it will be visible in the 700 nm channel and also faintly visible in the 800 nm channel. If the marker is subjected to numerous freeze/thaw cycles, it may degrade. This is observed as multiple, high-molecular weight bands appearing in the 800 nm channel. If this occurs, discard the aliquot and use a fresh one.

Prestained blue molecular weight markers from other sources can also be used. Load 1/3 to 1/5 of the amount you would normally use for Western transfer. Too much marker can result in very strong marker bands that may interfere with visualization of sample lanes. If using multicolored markers, some bands may not be visualized.

Optimization Tips

- Follow the protocol carefully.
- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If it is difficult to detect the target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for infrared detection.
- Addition of detergent such as Tween® 20 can reduce membrane background and non-specific binding. Refer to *V. Adapting Western Blotting Protocols for Odyssey® Detection* for details.
- To avoid background speckles on blots, use ultrapure water for buffers and rinse plastic dishes well before and after use. Never perform Western incubations or washes in dishes that have been used for Coomassie staining.
- Membranes should be handled only by their edges, with clean forceps.
- After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with distilled water and/or ethanol. If forceps are not cleaned after being dipped in antibody solutions, they can cause spots or streaks of fluorescence on the membrane that are difficult to wash away.
- Do not wrap the membrane in plastic when scanning.
- If a Western blot will be stripped, do not allow the membrane to dry. Stripping is ineffective once a membrane has dried, or even partially dried.

III. Guidelines for Two-Color Detection

Two different antigens can be detected simultaneously on the same blot using antibodies labeled with IR dyes 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 design two-color experiments:

- 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).
- 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). For details refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies*).
- 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 background bands. Slight cross-reactivity may occur and can complicate interpretation of a blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.

- One secondary antibody must be labeled with a 700 channel dye, and the other with an 800 channel dye. For a list of fluorescent dyes and the channels where they can be visualized, see *I. Required Reagents*.
- Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use highly cross-adsorbed antibodies may result in cross-reactivity.
- For best results, avoid using primary antibodies from mouse and rat together for a two-color experiment. It is not possible to completely adsorb away cross-reactivity because the species are so closely related. 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.

When performing a two-color blot, use the standard Western blot protocol with the following modifications:

- Combine the two primary antibodies in the diluted antibody solution in step 3. Incubate simultaneously with the membrane (step 4).
- Combine the two dye-labeled secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with the membrane (step 7).

IV. Stripping the Membrane

Typically, both PVDF and nitrocellulose membranes can be stripped up to three times. LI-COR® NewBlot™ Stripping Buffer is available under P/N 928-40030 for nitrocellulose or 928-40032 for PVDF. If a blot is to be stripped, DO NOT allow it to dry before, during, or after imaging (keep the blot as wet as possible). Complete usage instructions are given in the NewBlot Stripping Buffer pack insert that is shipped with the product. Before proceeding, read the instructions in the pack insert, including the frequently asked questions.

V. Adapting Western Blotting Protocols for Odyssey Detection

When adapting Western blotting protocols for Odyssey® detection or using a new primary antibody, it is important to determine the optimal antibody concentrations. Optimization will help achieve maximum sensitivity and consistency. Three parameters should be optimized: primary antibody concentration, dye-labeled secondary antibody concentration, and detergent concentration in the diluted antibodies.

Primary Antibody Concentration

Primary antibodies vary widely in quality, affinity, and concentration. The correct working range for antibody dilution depends on the characteristics of the primary antibody and the amount of target antigen to detect. Suggested dilutions are 1:500, 1:1500, 1:5000, and 1:10,000 (start with the dilution factor normally used for chemiluminescent detection, and also refer to the product information from the vendor). Use the MPX™ Blotting System to optimize the primary dilution to achieve maximum performance and conserve antibody (refer to *One Blot Western Optimization Using the MPX Blotting System* at http://biosupport.licor.com).

Secondary Antibody Concentration

Optimal dilutions of dye-conjugated secondary antibodies should also be determined. Suggested starting dilutions to test are 1:5000, 1:10,000, and 1:20,000 (refer to the IRDye® conjugate pack insert for recommendations). The amount of secondary required varies depending on how much antigen is being detected – abundant proteins with strong signals require less secondary antibody. Use the MPX™ Blotting system to optimize (refer to *One Blot Western Optimization Using the MPX Blotting System* at http://biosupport.licor.com).

Detergent Concentration

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.

Tween® 20:

- Add Tween 20 to both the primary antibody and secondary antibody solutions when the antibodies are diluted in blocking buffer. A final concentration of 0.1 - 0.2% is recommended for nitrocellulose membranes, and a final concentration of 0.1% is recommended for PVDF membranes (higher concentrations of Tween 20 may actually cause increased background on PVDF).
- Wash solutions should contain 0.1% Tween 20.

SDS:

- Adding 0.01 - 0.02% SDS to the diluted secondary antibody can dramatically reduce overall membrane background and also reduce or eliminate non-specific binding. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process.
- Addition of SDS is particularly helpful for reducing the higher overall background that is seen with PVDF membrane. 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 detection incubation step to avoid non-specific background staining.
- *DO NOT add SDS during the blocking step or to the diluted primary antibody.* Presence of SDS during binding of the primary antibody to its antigen may greatly reduce signal. *Add SDS only to the diluted secondary antibody.*
- When diluting the dye-labeled secondary antibody in blocking buffer, add both 0.1 - 0.2% Tween 20 and 0.01 - 0.02% SDS to the antibody solution.
- Wash solutions should contain 0.1% Tween 20, but no SDS.
- Some antibody-antigen pairs may be more sensitive to the presence of SDS and may require even lower concentrations of this detergent (less than 0.01%) for best performance. Titrate the amount of SDS to find the best level for the antibodies used.
- If high background is seen when using BSA-containing blocking buffers, adding SDS to the secondary antibody may alleviate the problem.

VI. General Tips

- Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. Milk may also contain endogenous biotin or phospho-epitopes that can cause higher background.
- Store the IRDye® secondary antibody vial at 4°C in the dark. Do not thaw and refreeze the vial, as this will affect antibody performance. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.
- Protect membrane from light during secondary antibody incubations and washes.
- Use the narrowest well size possible for the loading volume to concentrate the target protein.
- The best transfer conditions, membrane, and blocking agent for experiments will vary, depending on the antigen and antibody. If there is high background or low signal level, a good first step is to try a different blocking solution.
- Small amounts of purified protein may not transfer well. Adding non-specific proteins of similar molecular weight can have a "carrier" effect and substantially increase transfer efficiency.
- For proteins <100 kDa, try blotting in standard Tris-glycine buffer with 20% methanol and no SDS. Addition of SDS to the transfer buffer can greatly reduce binding of transferred proteins to the membrane (for both PVDF and nitrocellulose).
- Soak the gel in transfer buffer for 10-20 minutes before setting up the transfer. Soaking equilibrates the gel and removes buffer salts that will be carried over into the transfer tank.
- To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (approximately 1-2 hours).
- Do not over-block. Long blocking incubations, particularly with nonfat dry milk at 2% or higher, can cause loss of target protein from the membrane (*J. Immunol. Meth.* 122:129-135, 1989).
- To enhance signal, try extended primary antibody incubation at room temperature or overnight incubation at 4°C. Avoid extended incubations in secondary antibody.

VII. Imaging of Coomassie-Stained Protein Gels

IRDye® Blue Protein Stain is a convenient, safe alternative for gel staining to provide confirmation of protein transfer to the membrane. 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 the Odyssey® imaging systems (< 5 ng of BSA can be detected). IRDye Blue Protein Stain is Coomassie-based and is provided as a ready-to-use 1X solution. Prewashing and destaining steps are performed in water.

1.	Wash gels with ultrapure water for 15 minutes.
2.	Submerge gel in IRDye Blue Stain for 1 hour.
3.	Destain with ultrapure water for 30 minutes or overnight if needed.
4.	Scan on an Odyssey imaging system in the 700 nm channel only. If using the Odyssey software, select the Protein Gel scan preset. If using the Odyssey Sa software, set the focus offset to 3.0 plus one-half the thickness of the gel. In Image Studio, select Western.

VIII. Troubleshooting Guide

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.
	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.

Problem	Possible Cause	Solution / Prevention
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 or silicone mat.	Clean scanning surface and mat 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.
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.

Problem	Possible Cause	Solution / Prevention
Weak or no signal (continued)	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 ("blow-through"). Use membrane with smaller pore size or reduce transfer time.

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 (see *III. Guidelines for Two-Color Detection*).
		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.
	Bleedthrough of signal from one channel into other channel.	Check the fluorescent dye used. Fluorophores such as 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 bleedthrough 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.

LI-COR® Biosciences
4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe, Middle East, and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
All other countries, contact LI-COR Biosciences or a local LI-COR distributor: http://www.licor.com/distributors
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988-11873 02/11

Good Westerns Gone Bad:

Tips to Make Your NIR Western Blot Great

Developed for:

Odyssey Family of Imagers

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your model of Odyssey Imager.

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Contents

I. 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® Imaging System 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."

II. Factors That Alter the Performance of a Western Blot

A. 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 and examples of membrane performance can be seen in Figure 1. There is typically more variability in PVDF performance than nitrocellulose.

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 the Odyssey.*

B. 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. 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 reagents on a nitrocellulose membrane. Figure 3 is a similar example using Odyssey blocking reagent, I-Block™, and 5% BSA for detection of anti-pAkt and β-tubulin in 293T Cells stimulated with TGF-β.

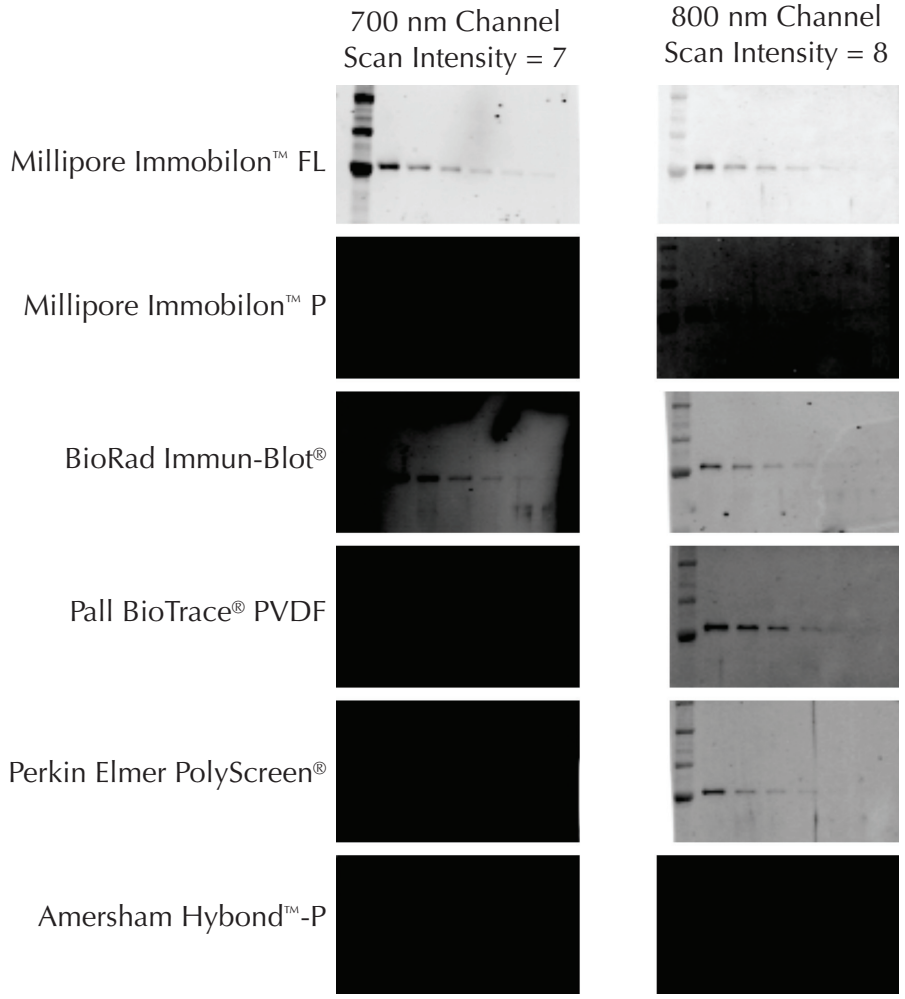


Figure 1. Western blot detection of transferrin using various vendors and brands of PVDF membrane on the Odyssey Infrared Imaging System in both 700 and 800 nm channels.

We tested the PathScan PDGFR Tyrosine Kinase Activity Assay (Cell Signaling Technology, P/N 7180), using five different blocking/diluent solutions. Figure 4 shows results from this experiment. The five phosphoproteins could 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 (P/N 927-40010, 927-40003, 927-40000, 927-40100) was used. This data clearly suggests that there is not a universal blocker that is best for all antibodies.

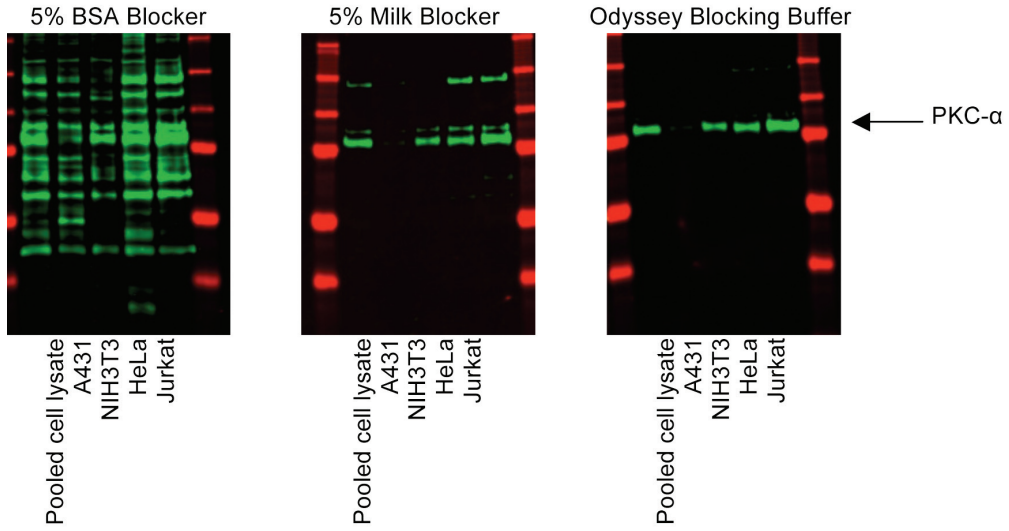


Figure 2. Western blots detected with anti-PKCα and IRDye® 800CW Goat anti-mouse. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey® Infrared Imager with scan intensity setting of 5, sensitivity of 5.

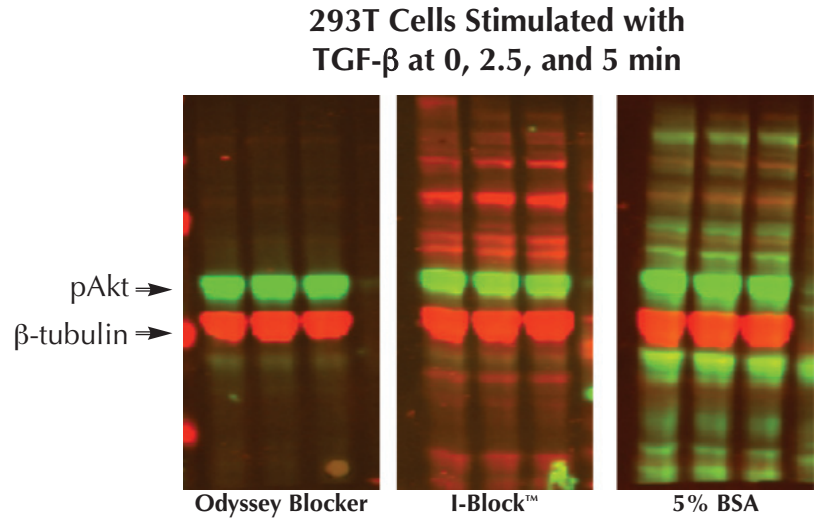


Figure 3. Western blots of 293T Cells stimulated with TGF-β (0, 2.5, and 5 minutes) detected with anti-pAkt and β-tubulin. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Infrared Imager with scan intensity setting of 3.5/5 (700/800 nm),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

C. 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 deter gent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

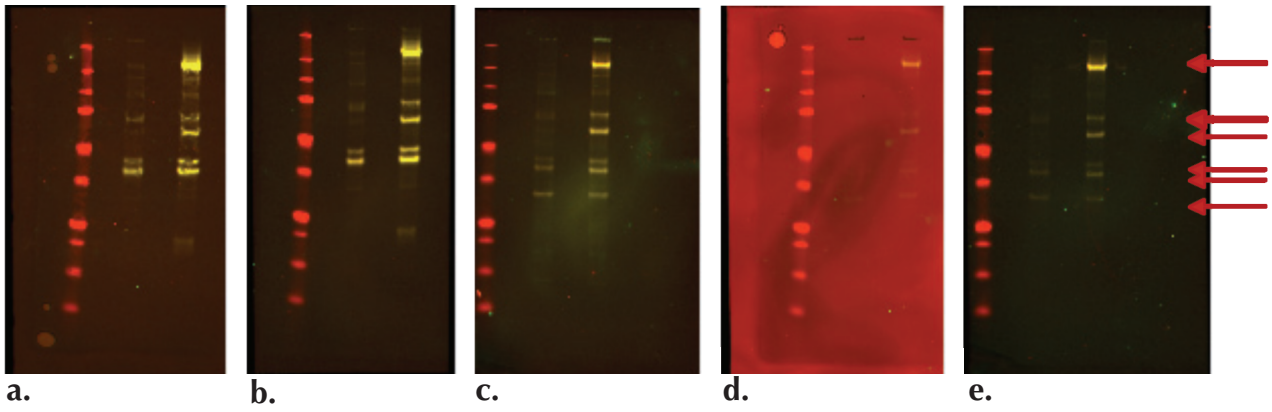
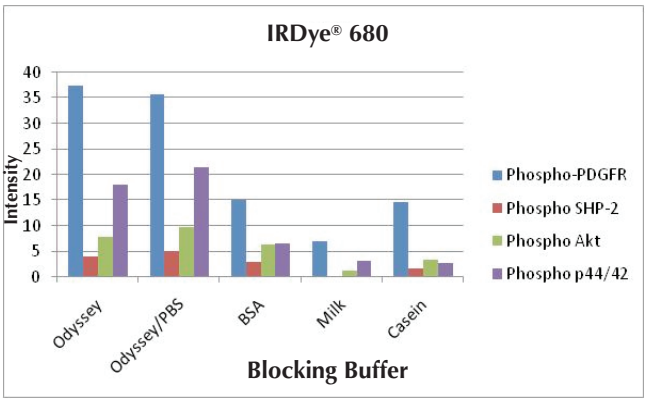
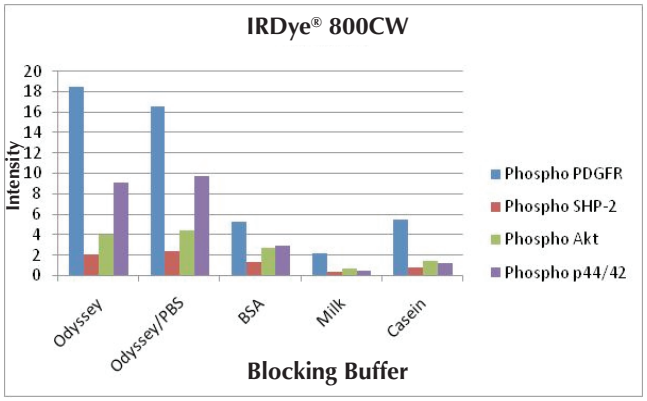


Figure 4. Above: Western blots utilizing PathScan Multiplex primary antibody and both IRDye® 680 and IRDye 800CW goat anti-rabbit for detection. Five different solutions were used for blocking and antibody dilution (antibody dilutions included 0.2% Tween® 20):
a. Odyssey® Blocking Buffer;
b. Odyssey + PBS (1:1);
c. 5% BSA;
d. 5% Skim Milk;
e. 0.5% Casein.
In each image, the arrows indicate the band positions for each of the detected proteins. Starting from the 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.

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.
   c. Wash solutions should not contain SDS.

D. Primary Antibody

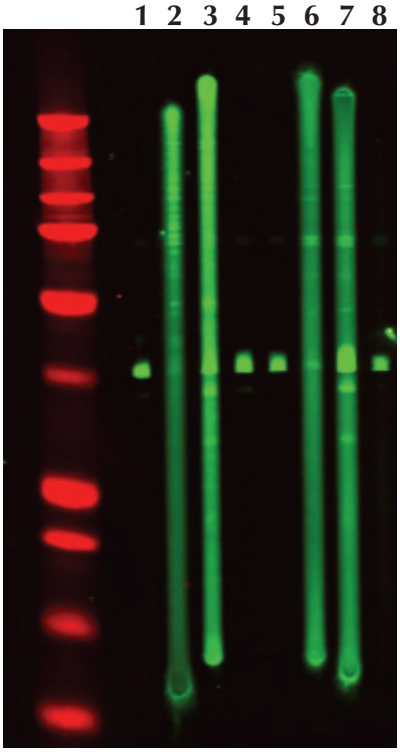
An antibody produced to detect a specific antigen is called the primary antibody. It binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the same 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.

E. 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 680 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 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, but will not recognize mouse gamma 2a, 2b, 2c or gamma 3. For details, refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies*, for a complete description.

A.



B.

	Antibody	Host	Manufacturer	Part #
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.

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 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 sub-classes.

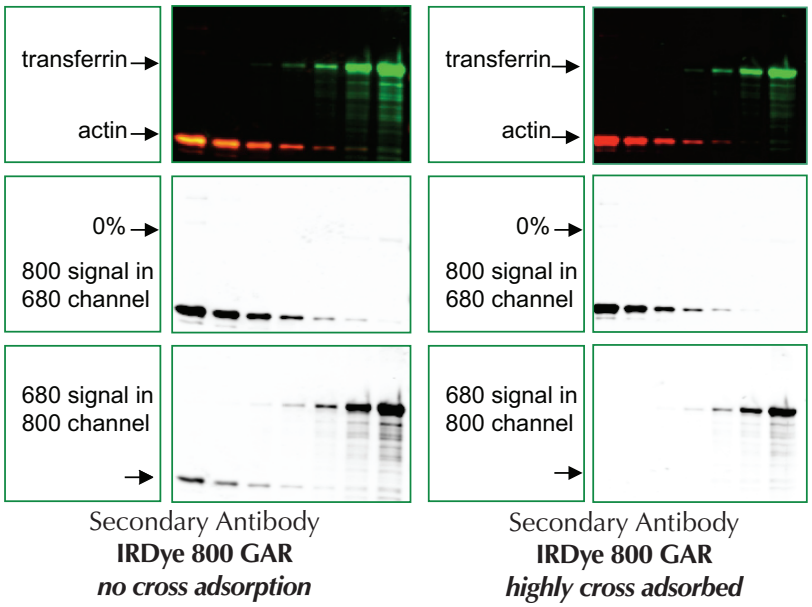


Figure 6. Example of a secondary antibody not cross adsorbed, cross-reacting with the second antibody pair in a two-color Western blot.

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.

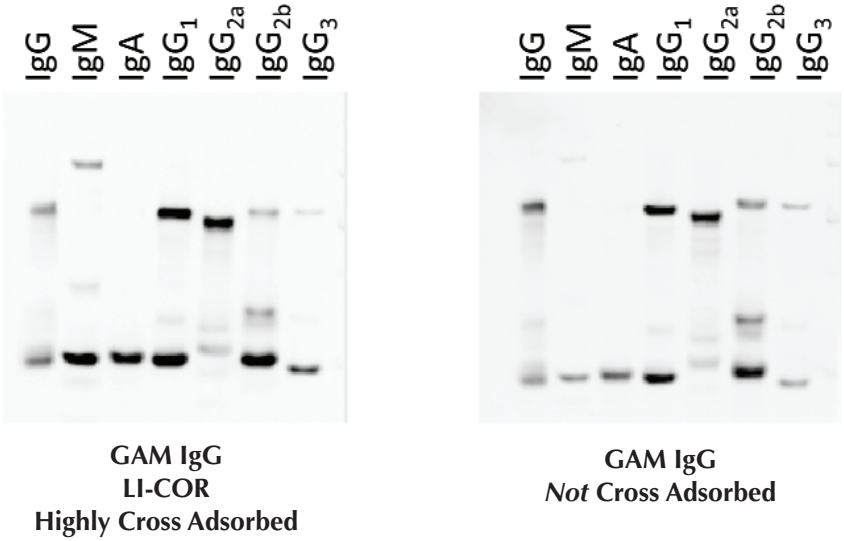


Figure 7. Mouse IgG Subclass detection comparing LI-COR IRDye goat anti-mouse antibody to a similar antibody that was not cross-adsorbed.

F. 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 680 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 1:10,000 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).

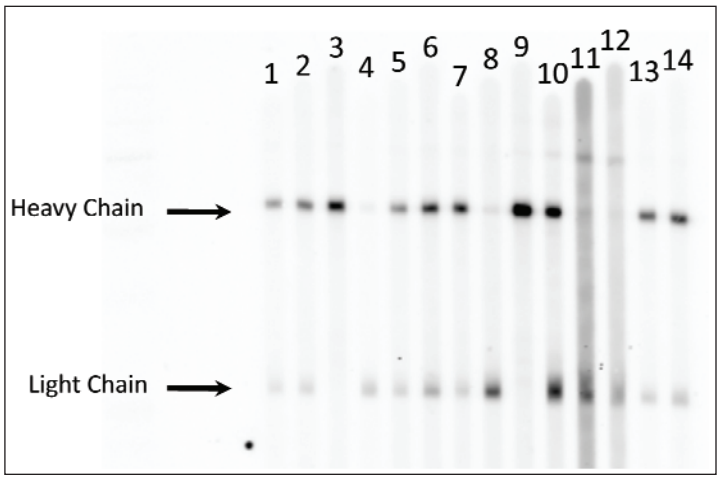
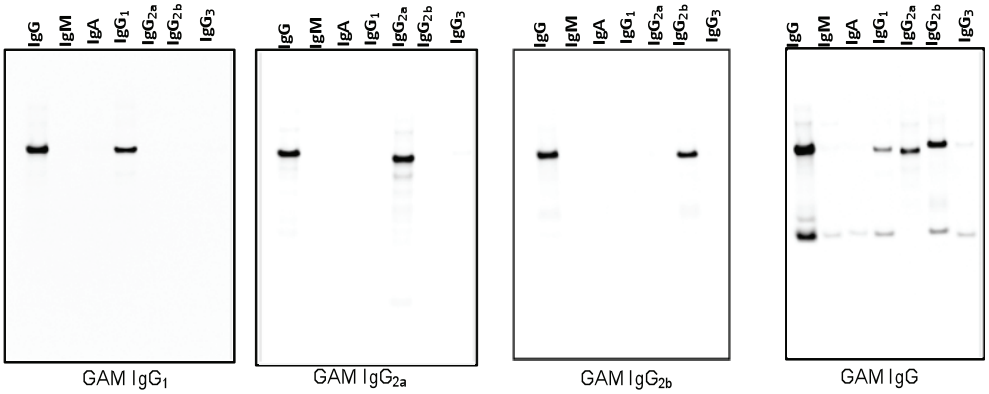


Figure 8. IRDye® 800CW labeled anti-mouse antibodies against purified mouse IgG.

Secondary Antibodies used at a 1:5000 dilution unless otherwise indicated

1) Goat anti-Mouse IgA, IgG, IgM
2) Rabbit anti-Mouse IgG
3) Goat anti-Mouse IgG Fcγ (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 Fcγ (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.



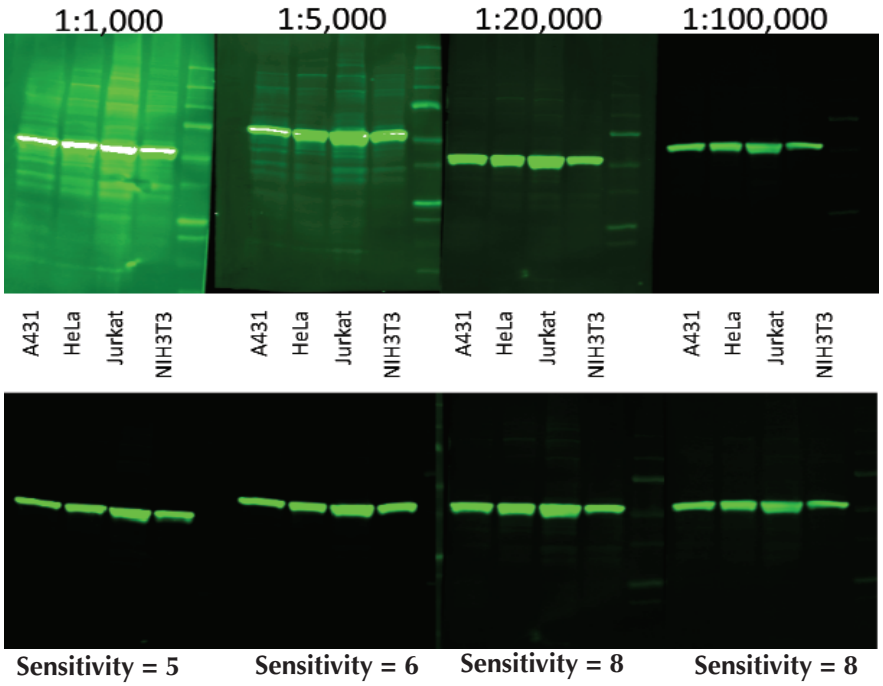


Figure 10. Secondary Antibody Concentration of IRDye 800CW goat anti-mouse with maximized Odyssey imaging capabilities.

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 on the following page.

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 (Part #928-40004).
Dirty transfer pads	Blotches can be seen on the blot that align with the transfer cassette holes	Replace transfer pads.
Acrylamide residue on membrane after transfer	Speckles and blotches can be seen in 700/800 nm channel	Carefully rinse off membrane in 1X PBS before it dries.
Blue pen used on membrane	Smeared signal in the 700 nm channel	Use pencil to mark blots.
Dirty processing containers: 1. Coomassie Stain/gel stain/ anything blue	1. In the 700 nm channel, entire membrane dark, smeared signal, or speckles, depending on the amount of stain residue in container.	1. Use different containers for gel staining and Western blot detection.
2. Bacterial Growth	2. Speckles and blotches can be seen in 700/800 nm channel.	2. Wash containers with detergent, rinse thoroughly with distilled water and a final rinse with methanol.
3. Acrylamide Residue	3. Speckles and blotches can be seen in 700/800 nm channel.	3. 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 forceps 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.

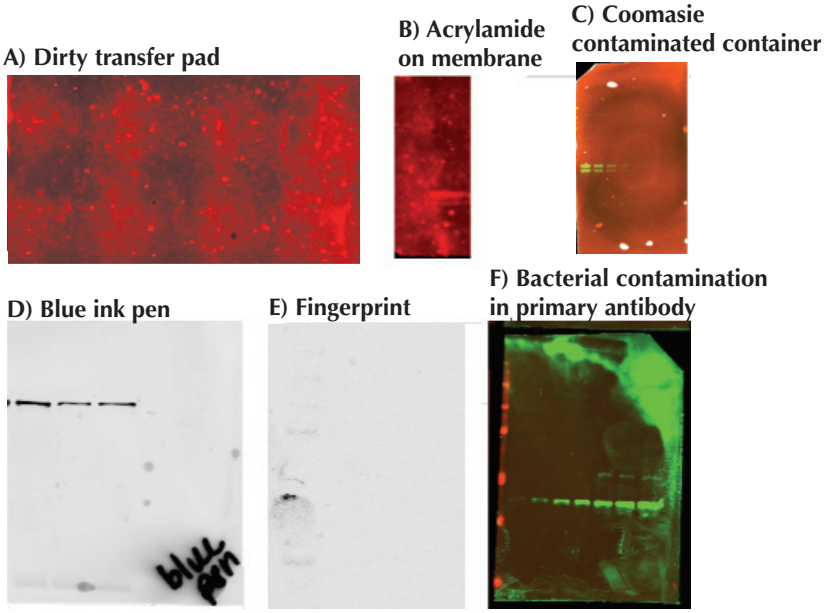


Figure 11. Examples of contamination events that may cause background on a Western blot.

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

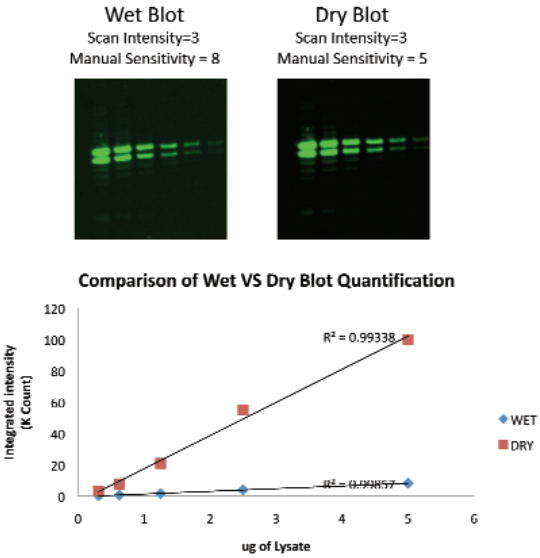


Figure 13. The same Western blot scanned on an Odyssey system wet and dry. The images are represented using the optimal display settings. Quantification is shown in the chart below the images.

III. 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 an Odyssey System that can greatly influence the data acquired from the instrument.

A. 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.

B. Air bubbles can result in reduced signal detection during imaging. Flatten the membrane with a roller to remove bubbles and excess liquid. See Figure 12.

C. A Western blot can be imaged either wet or dry on an Odyssey System. Typically, the signal is higher when a dry blot is imaged; however, the background also will increase. ***Note:*** *Once a blot is dry, or partially dried, stripping of the membrane for reuse is ineffective.* See Figure 13.

The Odyssey Fc Imaging System is optimized for acquiring Western blot images without saturated pixels or further adjustment by the operator. The following two items apply only to the Odyssey Infrared Imaging System.

D. Improper adjustment of the Odyssey Focus Offset can result in reduced signal collection from the Odyssey Infrared Imaging System. The focus offset should be set at 0 mm for scanning a Western blot. This can be done in the "Scan Console" Window of the Odyssey software. For more details see Chapter 2: Starting Scans, in the Odyssey User Guide.

E. Improper optimization of the Odyssey Scan Intensity can result in saturation of signal and reduced linear dynamic range. Figure 14 shows the quantification variation that can occur by changing the intensity settings in which the image is acquired. Intensity optimization can be done in the Scan Console Window of the Odyssey software. For more details, see Chapter 2: Starting Scans, in the Odyssey®

User Guide. 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 in the Odyssey® Infrared Imaging System. For example, saturated signal in the 800 nm channel of the Odyssey can be seen as 700 nm signal in the 700 channel scan (see Figure 15). This can easily be eliminated by scanning at a lower intensity.

There are two common problems that can be corrected with a few adjustments of the Odyssey Infrared Imaging System software or the Image Studio software on the Odyssey Fc Imaging System. These include blots that exhibit: • No Fluorescence • Dim Bands. Keep in mind that these software enhancements will only work on blots that are not experiencing binding chemistry problems.

For the Odyssey Infrared Imaging System – 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 Alter Image Display menu. To enhance the image, simply click the Linear Manual radio button and adjust the slider. By manually managing the sensitivity settings, the most desirable image can be chosen. For more details, see Chapter 11: Changing the Appearance of Scanned Images, in the Odyssey 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 50. Adjust the Brightness and Contrast sliders to brighten and darken the pixels until the image is optimal. Each channel can be adjusted independently. Image adjustments can also be made in grayscale; very faint bands can be visualized better in gray. For more details, see Chapter 11: Changing the Appearance of Scanned Images, in the Odyssey User Guide. Additional enhancement of images can also be done using "Adjust Image Display Curves".

For the Odyssey Fc Imaging System – No Fluorescence

Click on the Auto Adjust button in the Image LUTs tab. For more details, see Chapter 5: Manipulating an Image in the Odyssey Fc Tutorial (Doc #984-11074).

Dim Bands

Click and drag the min, max, and K value dots on the histogram in the Image LUTs tab to adjust the intensity of the image. For more details, see Chapter 5: Manipulating an Image in the Odyssey Fc Tutorial.

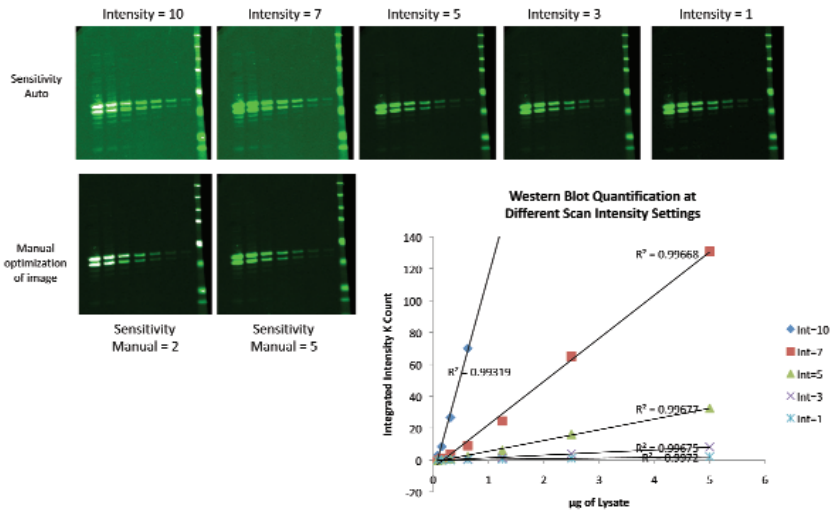


Figure 14. The same Western blot scanned on the Odyssey Infrared Imaging System at 5 different intensity settings. The top row of images are displayed using the auto sensitivity setting in the Odyssey Software. The bottom images were optimized using the manual sensitivity option for display. Quantification is shown in the chart. Note that the saturated signal at the Intensity setting of 10 cannot be quantified.

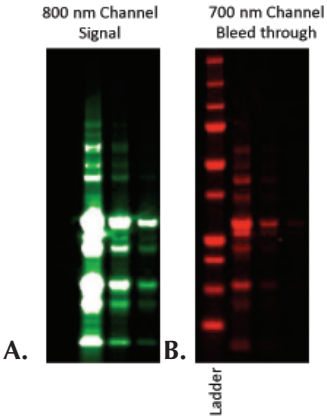


Figure 15. Saturated signal in the 800 nm channel (A) of the Odyssey Infrared Imaging System can be visualized in the 700 nm channel (B). The only detection that should be seen in the 700 nm channel is the ladder on the far left of the image. Optimizing scan intensity can eliminate this.

IV. Data Analysis Using the Odyssey® Infrared Imaging System

Background

For accurate Western blot quantification, the background setting in the Odyssey software must be applied effectively. The Background method sets the background calculation method for use in quantification, by averaging the intensity of the pixels selected as the background region. There are several different methods for background subtraction, each unique to a specific need.

i. ***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.

ii. ***Average Background*** takes the average value of the pixels on all four sides of the feature. It is possible to choose the number of pixels to include in the calculation by changing the Border Width.

iii. ***Median*** function sets the background level to the median value of the pixels outside the feature. The sides (All, Top/Bottom, or Right/Left) of the feature can be selected to optimize quantification. This feature is also available with the Average Background method.

iv. ***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 hot 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 'regular 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.

V. Data Analysis using the Odyssey Fc Imaging System

Background

The same background settings used in the Odyssey software are available in the Image Studio software on the Odyssey Fc Imaging System. They can be found by clicking on 'Define Type' in the Background group on the Analyze ribbon. To implement the 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 on '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 also includes the option of Lane background subtraction. This setting subtracts the background of the Lane from each Band. The same background settings as above can also be used in the Western and MPX Western Analysis ribbons by clicking on 'Other' and 'Western Define Type'.

VI. 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 more detailed protocol on how to do an Odyssey Western blot, see the Odyssey Western Blot Analysis protocol).

VII. References

Towbin, *et al.*, (1979) *Proc. Natl. Acad. Sci USA* 76; 4350-4

LI-COR® Biosciences
4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
North America: 800-645-4267 • International: 402-467-0700 • FAX: 402-467-0819
LI-COR GmbH Germany, Serving Europe, Middle East, and Africa: +49 (0) 6172 17 17 771
LI-COR UK Ltd., Serving UK, Ireland, Scandinavia: +44 (0) 1223 422104
All other countries, contact LI-COR Biosciences or a local LI-COR distributor:
http://www.licor.com/distributors
www.licor.com

Doc #988-11874
0311

In-Gel Western Detection Using Near-Infrared Fluorescence

Developed for:

Odyssey® Infrared Imaging System

LI-COR® Biosciences

Published January 2006, revised December 2010.
The most recent version of this protocol is posted at: *http://biosupport.licor.com*

Contents

I. Reagents

Required Reagents:

- IRDye® secondary antibodies (LI-COR)*
- Smart™ Gels (LI-COR, P/N 928-40041, 928-40043, and 928-40045), Bis-Tris acrylamide gels, Tris-Glycine gels, or equivalent for electrophoresis
- 50% isopropanol + 5% acetic acid in ultrapure water
- Blocking buffer (5% BSA)
- Primary antibodies
- Tween® 20 detergent
- PBS buffer
- Ultrapure water

Optional Reagents:

- Odyssey Blocking Buffer (LI-COR, P/N 927-40000)

*Go to www.licor.com for the current list of the LI-COR® IRDye Conjugates

II. Description

Western blot detection of proteins requires separation of protein mixtures by electrophoresis, followed by transfer of the separated proteins to nitrocellulose or PVDF membranes for detection. The Odyssey® Infrared Imaging system allows you to detect target proteins while still embedded in the gel, without transfer to a membrane using near-infrared secondary antibodies, such as the LI-COR IRDye Conjugates.

Using near-infrared fluorescence detection methods for In-Gel Westerns makes this a powerful technique. 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. After electrophoresis, the gel is fixed briefly in a solution of isopropanol and acetic acid. Following a wash step to remove the alcohol,

the gel is incubated in diluted antibodies and washed in a manner similar to an ordinary Western blot. The wet gel is ready to scan on the Odyssey® Imager after washing. There is no substrate to apply, no plastic wrap, and no film exposures. 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 system. This technique provides a very useful tool for protein detection and research. However, it is important to note that In-Gel detection may not be quantitative.

III. Electrophoresis

1.	Separate the proteins of interest by electrophoresis. **Notes:** • Gel type will affect the success and sensitivity of In-Gel Western detection. Best results can be obtained with LI-COR® Smart™ Gels (for self-poured gels) or NuPAGE® Bis-Tris pre-cast gels. The performance of different pre-cast gels may vary. Other gel types can be used but may require optimization. • Gel thickness and acrylamide percentage affect the ability of antibody molecules to penetrate the gel. We generally recommend that gel percentage be 12% or less, with a gel thickness of 1 - 1.5 mm.

IV. In-Gel Western Detection Protocol

2.	Following electrophoresis, separate the two plates and cut away any stacking gel present at the top of the gel using a scalpel or razor blade. ***Note:*** *The stacking gel will exhibit high background when the gel is imaged.* Notch one corner of the gel for orientation, if desired.
3.	Incubate the gel in 50% isopropanol + 5% acetic acid (prepared with ultrapure water) for 15 minutes. Use enough solution that the gel is completely covered and can move freely. Shake gently. ⚠ **Important:** Always use clean gloves and incubation trays when handling the gel to avoid high background. Handle the gel gently. Squeezing or pressing can cause splotches or fingerprints to appear in the image.

Continued

4.	Remove isopropanol/acetic acid and wash the gel in ultrapure water for 15 minutes with gentle shaking. Use enough water that the gel is completely submerged and can move freely. The gel may curl and/or float to the surface. Gently flatten the gel or turn it over, making sure that it is completely covered. Residual alcohol on the gel surface can cause diffuse bands. **Tip**: If desired, you may stop here and store the gel overnight in water at 4°C.
5.	No blocking step is required before antibody incubations. • Dilute primary antibody to desired concentration in 5% BSA, Odyssey Blocking Buffer, or PBS (5% BSA is recommended). • Include 0.1% Tween® 20 in the diluted antibody solution. • Since In-Gel detection is not as sensitive as a standard Western blot, more primary antibody than usual may be needed. Make sure the gel is completely covered with antibody solution. • Incubate gel for 1 hour with gentle shaking.
6.	Primary antibody incubation can be extended to several hours, or carried out overnight at 4°C. Extended incubation will increase signal.
7.	Wash the gel 3 times for 10 minutes in PBS + 0.1% Tween® 20 with gentle shaking, using a generous amount of wash buffer.
8.	• Dilute secondary antibody at 1:1000 – 1:5000 in the appropriate diluent with 0.1% Tween 20. • Incubate gel in secondary antibody for 1 hour with gentle shaking, and protect from light. • Use enough antibody solution to completely cover the gel.
9.	Wash the gel 3 times for 10 minutes in PBS + 0.1% Tween 20 with gentle shaking, using a generous amount of wash buffer.
10.	Wash the gel for 5 minutes in PBS.
11.	Lay the wet gel on the scanning surface of the Odyssey instrument. The gel can be scanned uncovered, or covered with plastic wrap to prevent drying. Set the focus offset to 1/2 the gel thickness (e.g., for a 1 mm gel, set the focus offset to 0.5 mm). If the stacking gel was not removed from the top of the gel, it can be cropped from the image when creating a new analysis in the Odyssey software.
12.	If image background is high, the background may be reduced by soaking the gel several hours or overnight in PBS and re-scanning. Store the gel at 4°C and protect from light. Gels can be kept in PBS at 4°C for several days, if desired.

V. 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:

a. **All secondary antibodies must be highly cross-adsorbed** to eliminate cross-reactivity.

b. **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

c. **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

d. One secondary antibody should be labeled with IRDye® 800 and the other with IRDye 680LT, or other commercially available near-infrared dyes.

e. 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.

f. 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.

Protocol Modifications for Two-Color Detection

For two-color detection, follow **IV. In-Gel Western Detection Protocol** with the following modifications:

1. Use two labeled secondary antibodies that are labeled with dyes that are detected in two different channels.
   Example: IRDye 680LT (700 nm) and IRDye 800CW (800 nm)
2. Make sure that antibody specificities and hosts are appropriate and will not cross-react.
3. Combine the two primary antibodies in antibody diluent in step 5, and incubate simultaneously with the gel.
4. Combine the two IRDye secondary antibodies in the antibody diluent in step 8. Incubate simultaneously with the gel.

VI. Optimization

The In-Gel detection protocol may require optimization for each target protein or gel type. Sensitivity of In-Gel Westerns may be lower than standard Western blots. (Transfer to a membrane concentrates the target protein, whereas in gels, protein is dispersed through the thickness of the gel.)

Use the following guidelines for optimization:

- Optimization of primary and secondary antibody dilutions, as well as amounts of Tween® 20 in diluted antibodies, may be needed to achieve maximum signal and minimum background. Recommended Tween 20 concentration is 0.1%.
- Try different buffers for dilution of the antibodies, including PBST alone, Odyssey Blocking Buffer (LI-COR, P/N 927-40000), or milk. Changing the buffer solution may dramatically improve performance.
- To avoid background issues, use high-quality ultrapure water. Rinsing previously used incubation boxes or trays with methanol can reduce background contamination on gels.
- For experiments utilizing streptavidin labeled with IRDye® infrared dyes, add 0.01% SDS in addition to Tween 20 in the antibody diluents and wash buffer.

VII. Troubleshooting Guide

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 buffers 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, apply 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.

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.	LI-COR® Smart™ gels or NuPAGE® Bis-Tris pre-cast gels are recommended for In-Gel detection. Other commercial gel sources and homemade 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.

Problem	Possible Cause	Solution / Prevention
Fuzzy or irregularly shaped bands	Gel type is not optimal.	We recommend LI-COR® Smart™ 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 V. Guidelines for Two-Color Western Detection.
	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).
	Bleedthrough 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.

LI-COR Biosciences • 4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 or 402-467-0819
LI-COR GmbH, Germany (Serving Europe, Africa, and the Middle East): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
In other countries, contact LI-COR or a local LI-COR distributor.
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In-Cell Western™ Assay

For Assessing Response of A431 Cells to Stimulation with Epidermal Growth Factor

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com

Contents

I. Required Reagents

LI-COR® Reagents

- IRDye® 800CW goat anti-mouse secondary antibodies (P/N 926-32210)
- IRDye 680 goat anti-rabbit secondary antibodies (P/N 926-32221)

  Note: IRDye 680LT goat anti-rabbit secondary antibodies (P/N 926-68021) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (P/N 927-40000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 20% Tween® 20
- Epidermal Growth Factor (Upstate Group Inc., P/N 01-107)
- 37% formaldehyde
- 10% Triton® X-100
- Nunc® 96 Microwell™ Plate (Nunc, P/N 167008)
- Primary antibodies as described below

Special Note: Phosphorylated-EGFR and phosphorylated-ERK are purchased from Cell Signaling Technonolgy and Santa Cruz Biotechnology, respectively. **Serum starvation of the cells is required to obtain maximal response.**

II. Seeding, Stimulation, and Detection of the A431 Cellular Response to Epidermal Growth Factor

1.	Allow A431cell growth in a T75 flask in DMEM and 10% fetal calf serum (FCS; Gibco®) using standard tissue culture procedures until cells reach 80%-90% confluency (~1.5 x 10^7 cells).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells.
3.	Neutralize displaced cells with culture media and pellet by centrifugation.
4.	Remove supernatant and resuspend cell pellet in remaining media by manually tapping the collection tube. Avoid vigorous pipetting or vortexing to resuspend cells in order to maintain cell integrity.
5.	Dilute cells to 20 mL in complete media and count cells using a hemacytometer.
6.	Dilute cells with complete media to concentration of 200,000 cells/mL.
7.	Gently mix the cell suspension thoroughly.
8.	Under sterile conditions dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ plate (40,000 cells plated per well).
9.	Incubate cells and monitor cell density until cells are consistently confluent in each well. This should take approximately three days.
10.	Warm serum free media (DMEM; Gibco) to 37°C.
11.	Remove complete media from the microwell plate by aspiration.
12.	Replace media with 200 µL of pre-warmed serum-free media per well and incubate 4 to 16 hours.
13.	In a separate 96-well Microwell plate, dispense 100 µL of DMEM per well.
14.	Leave the first and second wells without EGF (resting cells controls). In the remaining wells, add aliquots of a solution of EGF to make serial dilutions ranging 0.2 to 100 ng/mL in the microplate. The experimental layout should look like that shown in **Figure 1**.
15.	Remove starvation media from plate wells by aspiration.
16.	Transfer EGF dilutions from the dilution plate into the cell-containing plate.
17.	Incubate at 37°C for 7.5 minutes.

18.	Prepare fresh *Fixing Solution* as follows: 1X PBS 45.0 mL 37% Formaldehyde 5.0 mL 3.7% Formaldehyde 50.0 mL
19.	Remove EGF-containing media by aspiration. Immediately fix cells by addition of 150 µl of fresh *Fixing Solution* and incubate at room temperature (RT) for 20 minutes with no shaking. **Add the solution carefully by pipetting down the side of the wells to avoid detaching the cells.**
20.	Prepare *Triton® Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL
21.	Remove the *Fixing Solution* by aspiration.
22.	Wash four times with 200 µL of *Triton Washing Solution* for 5 minutes per wash to permeabilize the cells. **Notes:** • Allow each wash to shake on a plate shaker for 5 minutes at RT. • Do not allow cells/wells to become dry during washing. Add washes immediately after each other.
23.	Remove the *Triton Washing Solution* by aspiration.
24.	To each well, carefully add 150 µL of LI-COR® Odyssey® Blocking Buffer (P/N 927-40000) down the side of the wells and incubate for 1.5 hours at RT with moderate shaking on a rotating platform. **Notes:** • No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for infrared fluorescent detection. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required). • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.

25.	Dilute the antibodies in LI-COR® Odyssey® Blocking Buffer to give the concentrations specified below. Primary antibodies can be added in a variety of combinations. Generally one antibody will be directed against the phosphorylated form of the target protein and the second antibody will be directed against the target protein regardless of phosphorylation status. The following are suggested combinations of primary antibodies depending upon the target to be detected: a. Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237) Total EGFR (Mouse; 1:500 dilution; Biosource International, P/N AHR5062) b. Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237) Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology, P/N SC-1647) c. Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383) Total ERK1 (Rabbit; 1:200 dilution; Santa Cruz Biotechnology, P/N SC-94) d. Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237) Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383)
26.	Add 50 µL of LI-COR Odyssey Blocking Buffer to one set of wells. These wells will serve as a control for any potential background due to the dye-labeled secondary antibody. See **Figure 1** for an example of the desired plate layout.
27.	Mix the primary antibody solution thoroughly before addition to wells.
28.	Remove the blocking buffer by aspiration and add 50 µL of the desired primary antibody combination to the remaining wells. The antibody solution should cover the bottom of each well.
29.	Incubate with primary antibody for 2 hours with gentle shaking at RT. **Notes:** • For greatest sensitivity, continue incubation overnight at 4°C with no shaking. • To avoid the cells drying out, cover the plates if left overnight.
30.	Prepare *Tween® Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL
31.	Wash the plate with *Tween Washing Solution* by gently adding buffer down the side of the wells to avoid detaching the cells. Use a generous amount of buffer (200-500 µL). Allow wash to shake gently on a rotator for 5 minutes at RT.
32.	Repeat wash 4 more times.

33.	Calculate the amount of secondary antibody required for the experiment. Dilute the fluorescently-labeled secondary antibodies in Odyssey® blocking buffer according to the dilution factors specified below. To lower background, add Tween® 20 to the diluted antibody to a final concentration of 0.2%. a. Goat anti-rabbit IRDye® 680 (1:200 dilution; LI-COR®, P/N 926-32221) b. Goat anti-mouse IRDye 800CW (1:800 dilution; LI-COR, P/N 926-32210) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
34.	Mix the antibody solutions thoroughly, add 50 µL of the secondary antibody solution to each well and incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.
35.	Wash the plate with *Tween Washing Solution* (step 30) by gently adding buffer down the side of the wells to avoid detaching the cells. Use a generous amount of buffer (200-500 µL). Allow wash to shake gently on a rotator for 5 minutes at RT. ⚠ **Protect plate from light during washing.**
36.	Repeat wash 4 more times.
37.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
38.	Before scanning, clean the bottom plate surface and the Odyssey Infrared Imager scanning bed (if applicable) with moist, lint-free paper to avoid obstructions during scanning.
39.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey System. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**

- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

- All Odyssey® Imaging systems require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments. (The actual minimum and maximum focus offset will vary with each instrument.)
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality, with 169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument provides satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening plate-like lysates through Western blotting and detection on an Odyssey instrument. If significant non-specific binding is present, choose alternative primary antibodies to avoid results with non-specific signal detection.

IV. Experimental Results

Color images can be seen at http://biosupport.licor.com.

Quantitative and simultaneous measurements of EGFR and phosphorylation of EGFR in response to EGF stimulation.

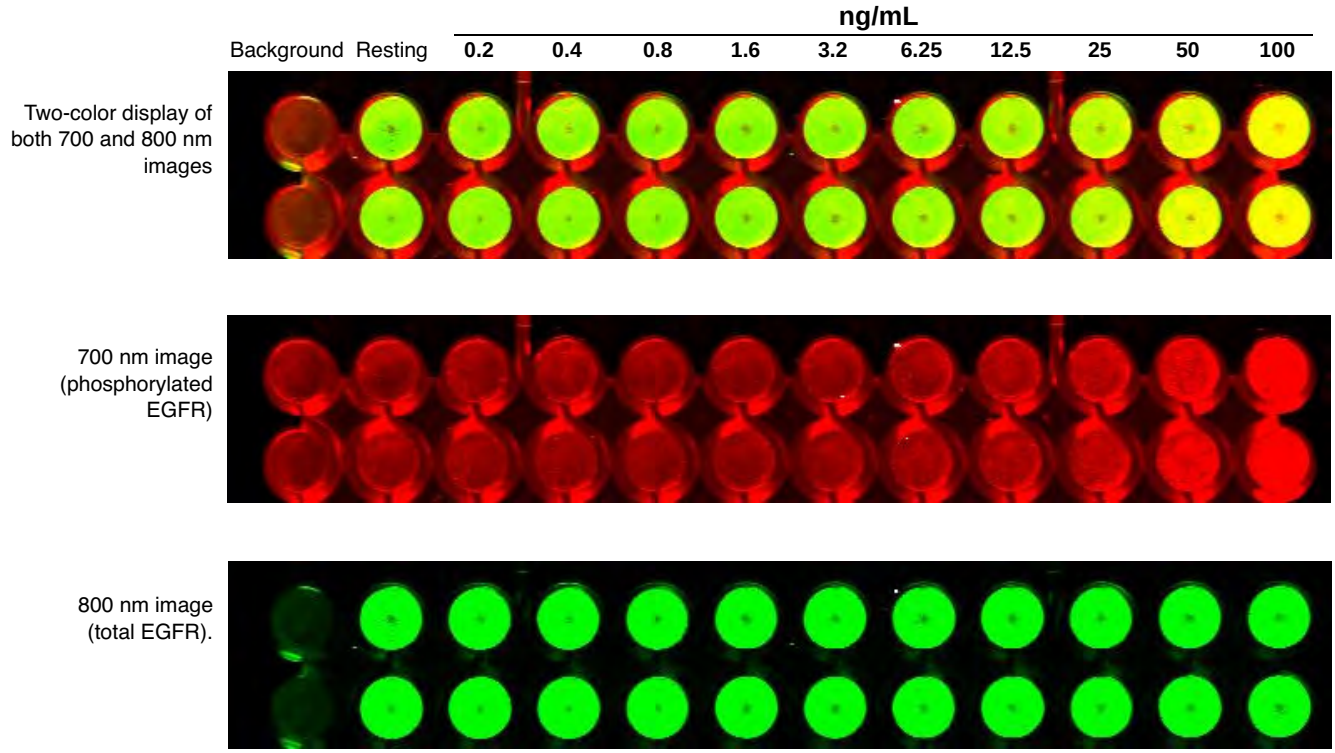
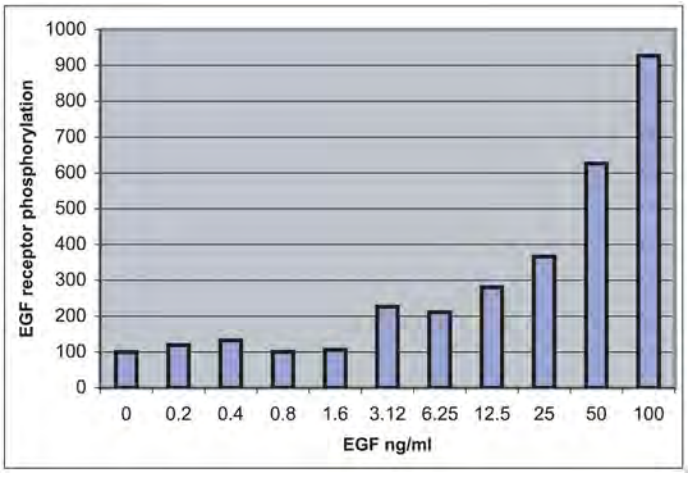


Figure 1. Dose response of A431 cells to Epithelial growth factor (EGF) as measured by specific antibody detecting phosphorylated EGF receptor (Tyr1045). The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting total EGF receptor (as normalization) and phosphorylated EGF receptor, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of EGF receptor.



Quantitative and simultaneous measurements of ERK and phosphorylation of EGF receptor in response to EGF stimulation.

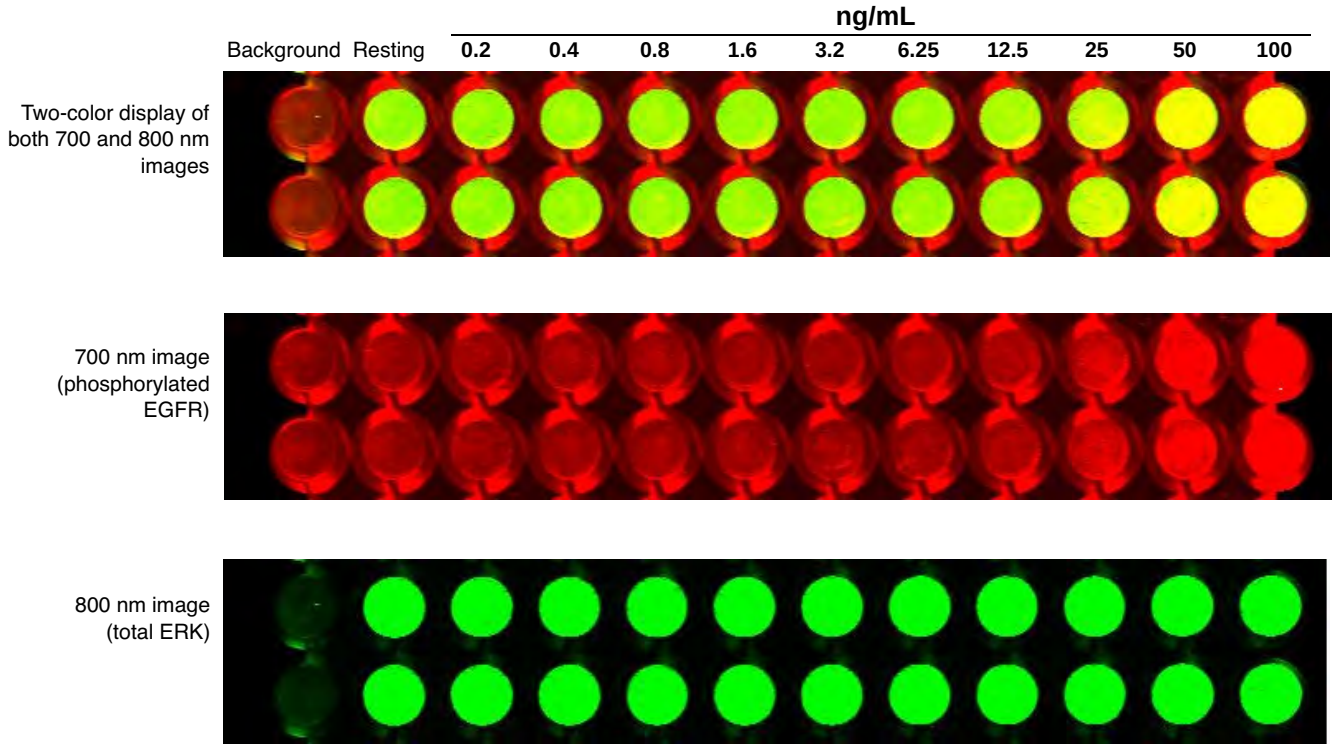
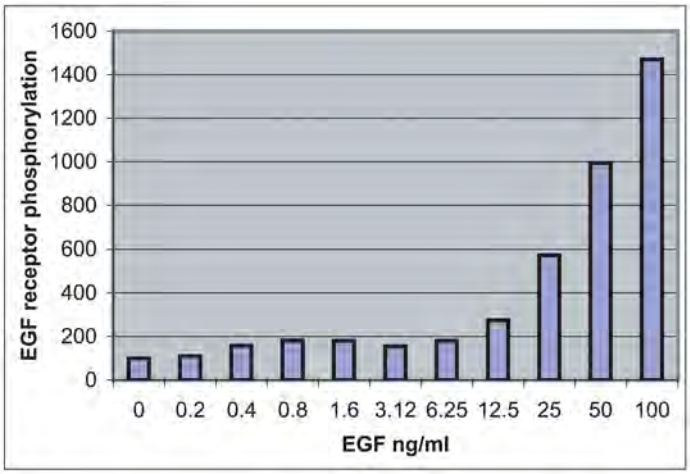


Figure 2. Dose response of A431 cells to Epithelial growth factor (EGF) as measured by specific antibody detecting phosphorylated EGF receptor (Tyr1045). The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting total ERK (as normalization) and phosphorylated EGF receptor, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of EGF receptor.



Quantitative and simultaneous measurements of total ERK and phosphorylation of ERK in response to EGF stimulation.

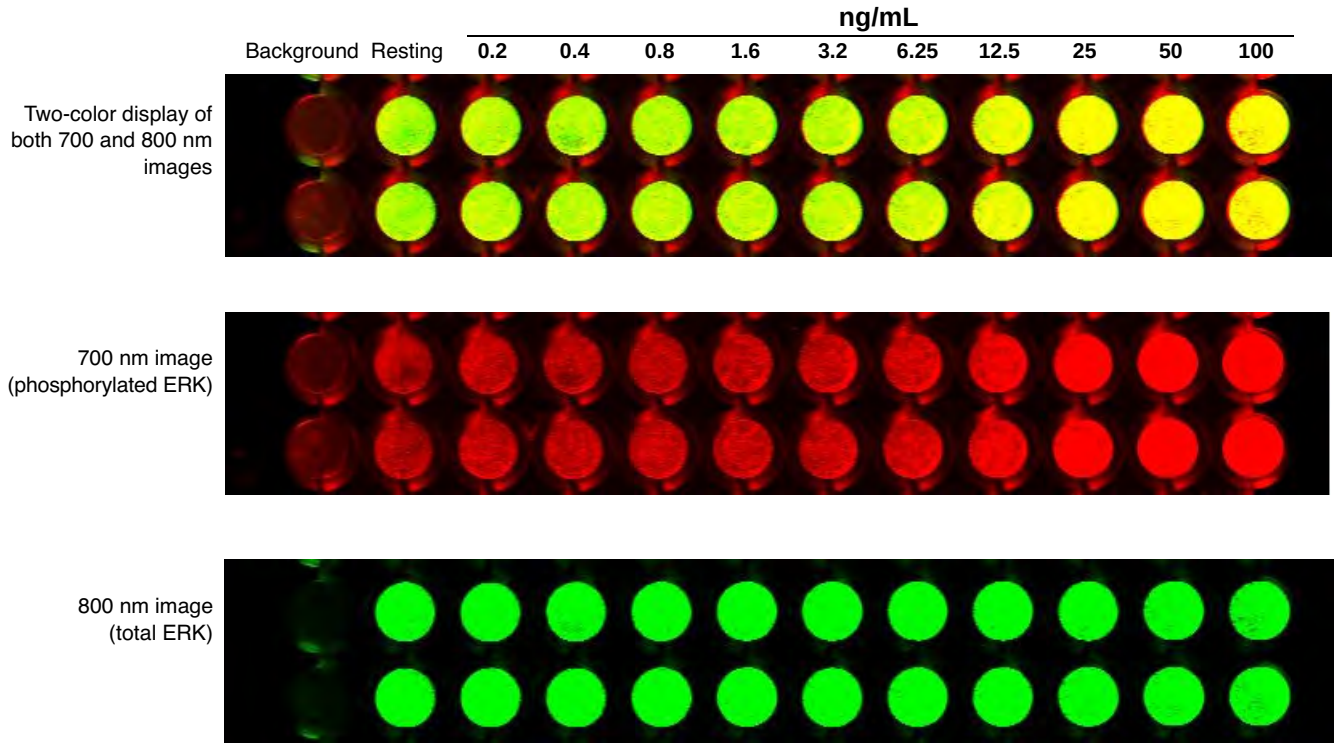
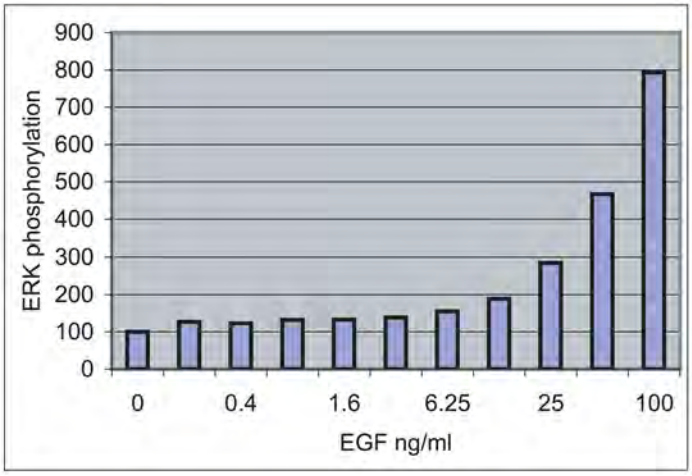


Figure 3. Dose response of A431 cells to Epithelial growth factor (EGF) as measured by specific antibody detecting phosphorylated ERK (Tyr204). The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting total and phosphorylated ERK, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of ERK.



Quantitative and simultaneous measurements of EGFR and ERK phosphorylation in response to EGF stimulation.

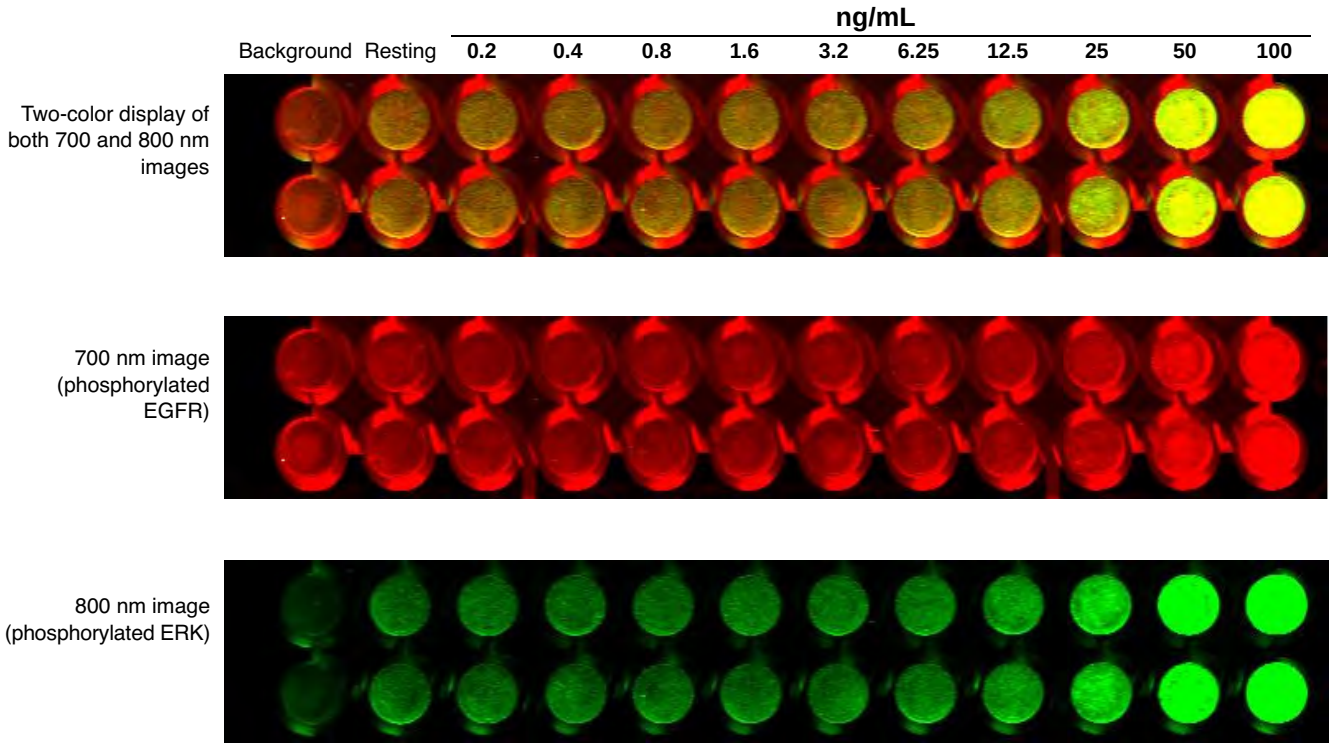
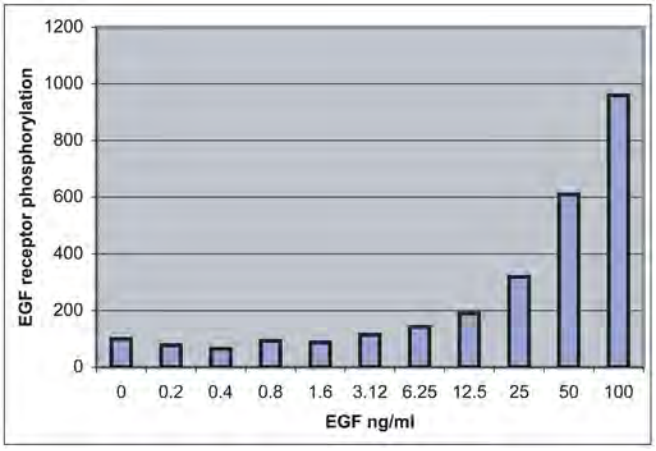
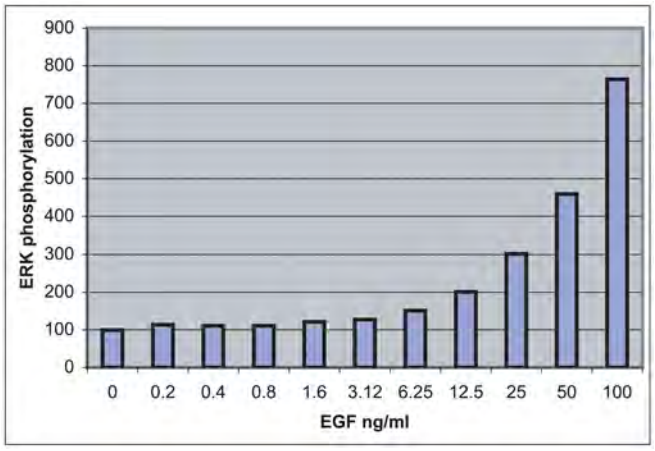


Figure 4. Dose response of A431 cells to Epithelial growth factor (EGF) as measured by specific antibody detecting phosphorylated EGF receptor (Tyr1045) and phosphorylated ERK (Tyr204) simultaneously. The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting phosphorylated ERK (Tyr204) and phosphorylated EGF receptor, respectively. Background wells were incubated with secondary antibody but no primary antibody.





Signal specificity confirmation of In-Cell Western Assay using conventional Western blots.

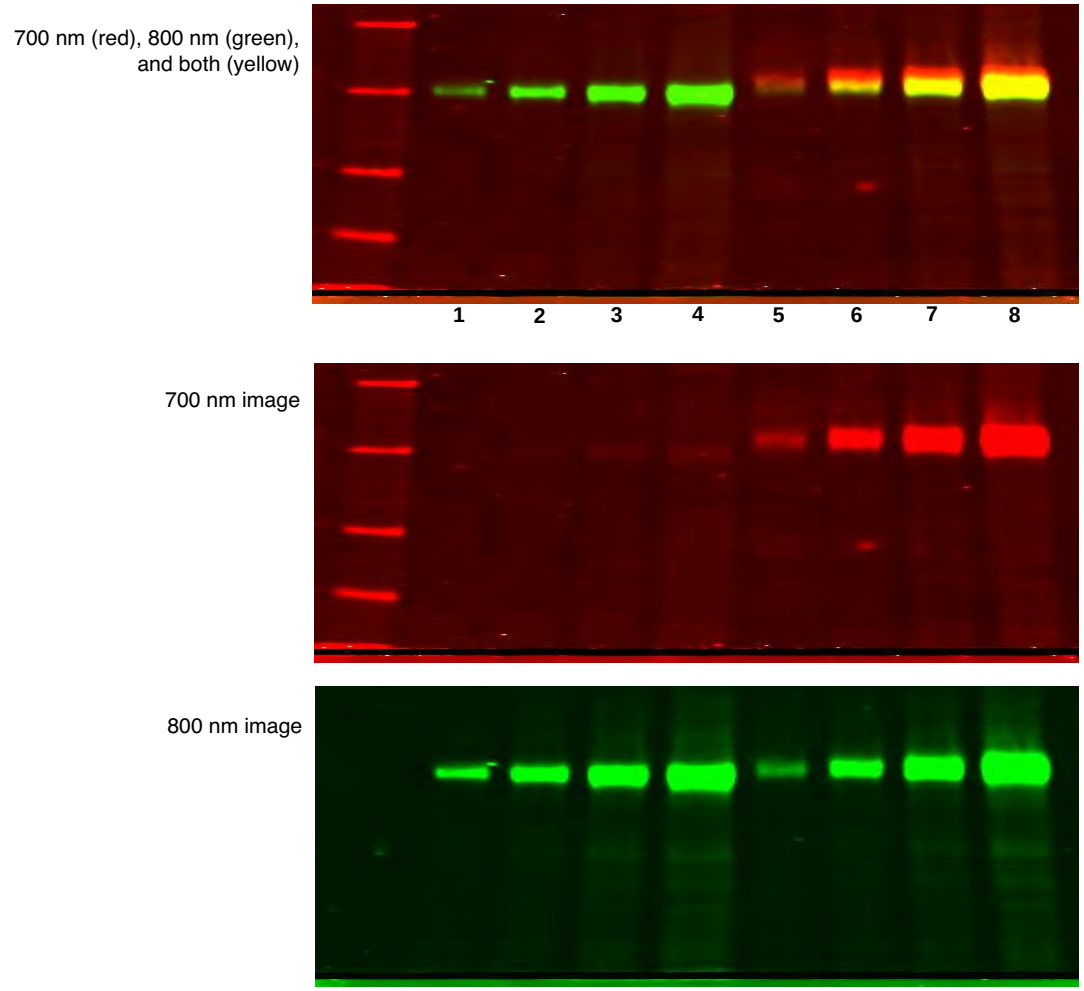
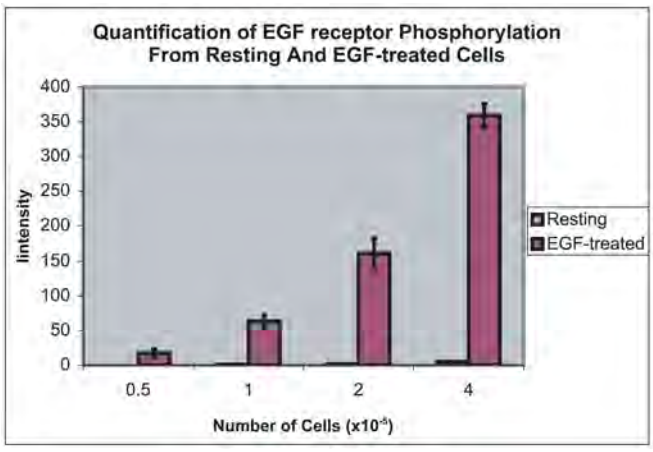
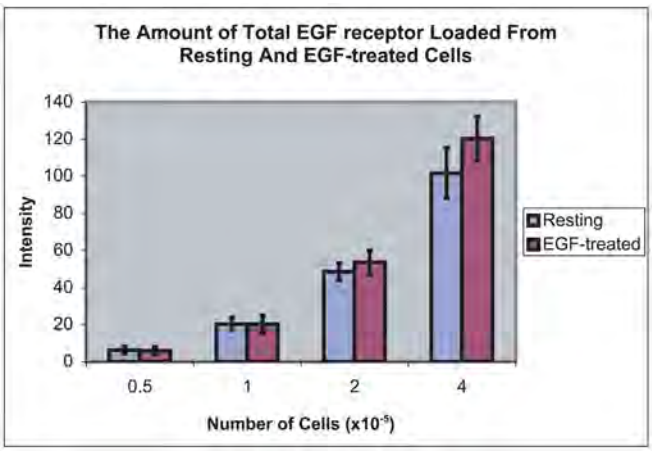


Figure 5. Simultaneous measurement of total and phosphorylated EGF receptor in resting and EGF-treated A431 cell lysates. Two-fold serials of dilutions of resting (lane 1 to 4) and EGF treated (lane 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated EGFR (700 nm, red) and total EGFR (800 nm, green) in these lysates were simultaneously assessed.





Simultaneous measurement of phosphorylated EGF receptor and total ERK in resting and EGF-treated A431 cell lysates.

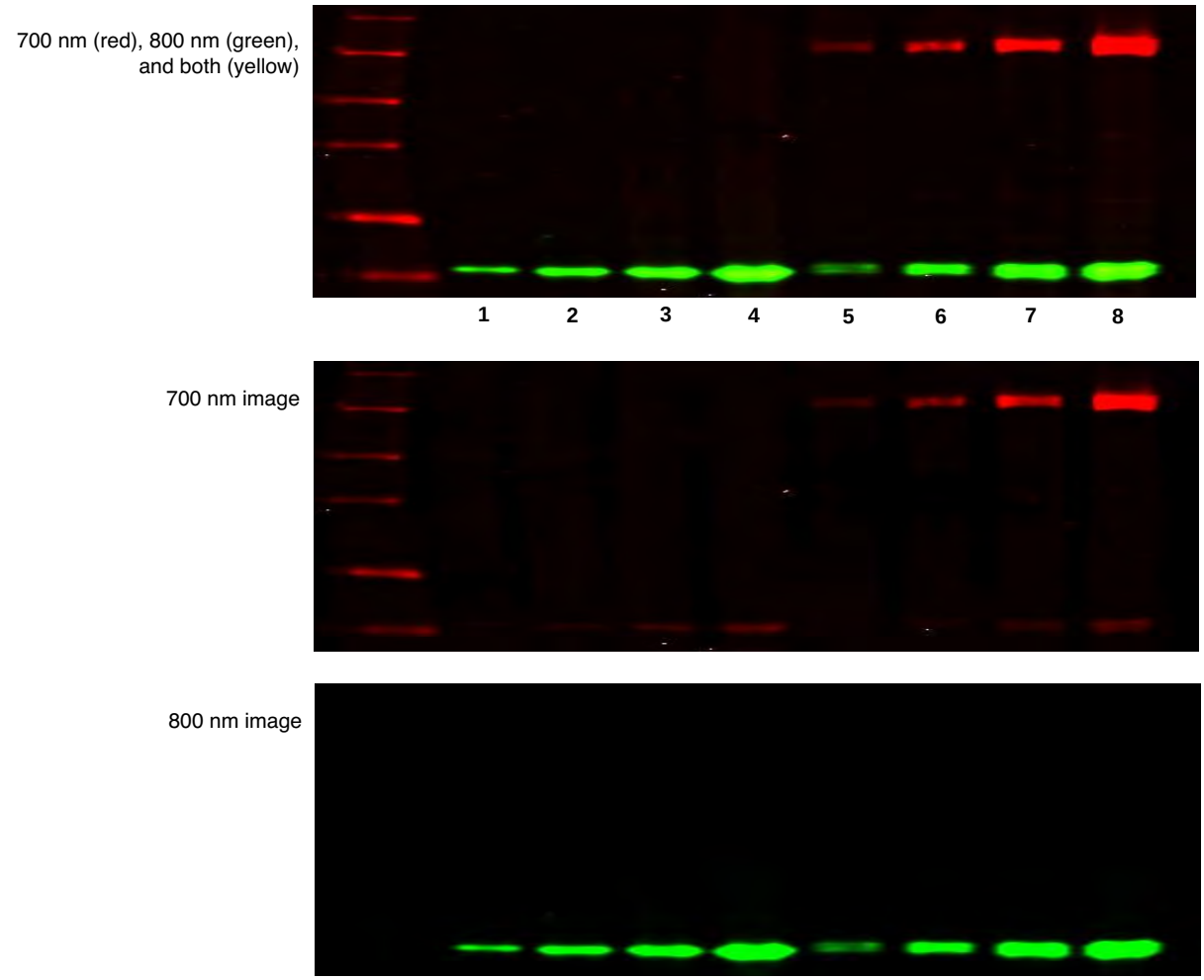
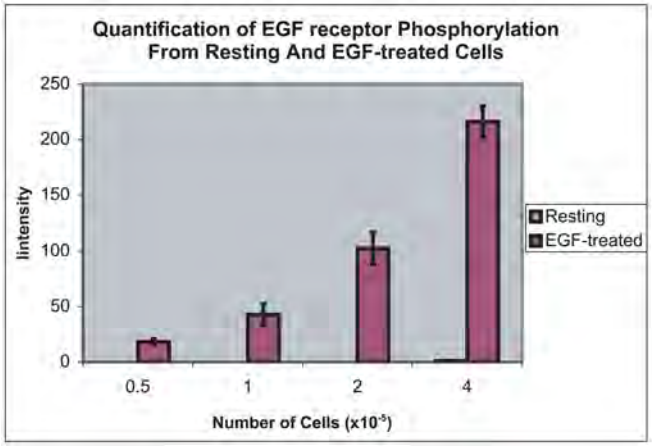
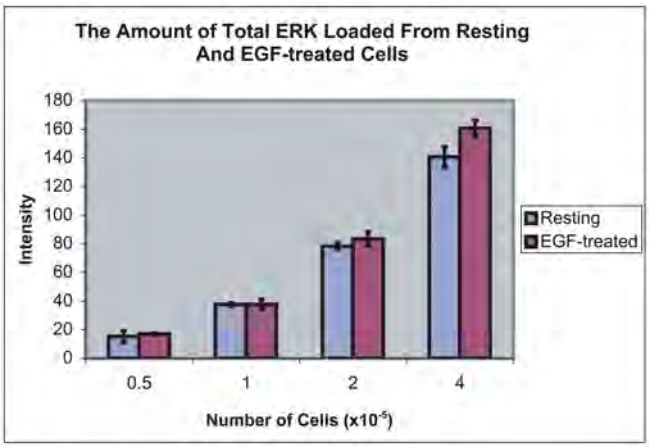


Figure 6. Two-fold serials of dilutions of resting (lane 1 to 4) and EGF-treated (lane 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated EGFR (700 nm, red) and total ERK (800 nm, green) in these lysates were simultaneously assessed.





Simultaneous measurement of total and phosphorylated ERK in resting and EGF-treated A431 cell lysates.

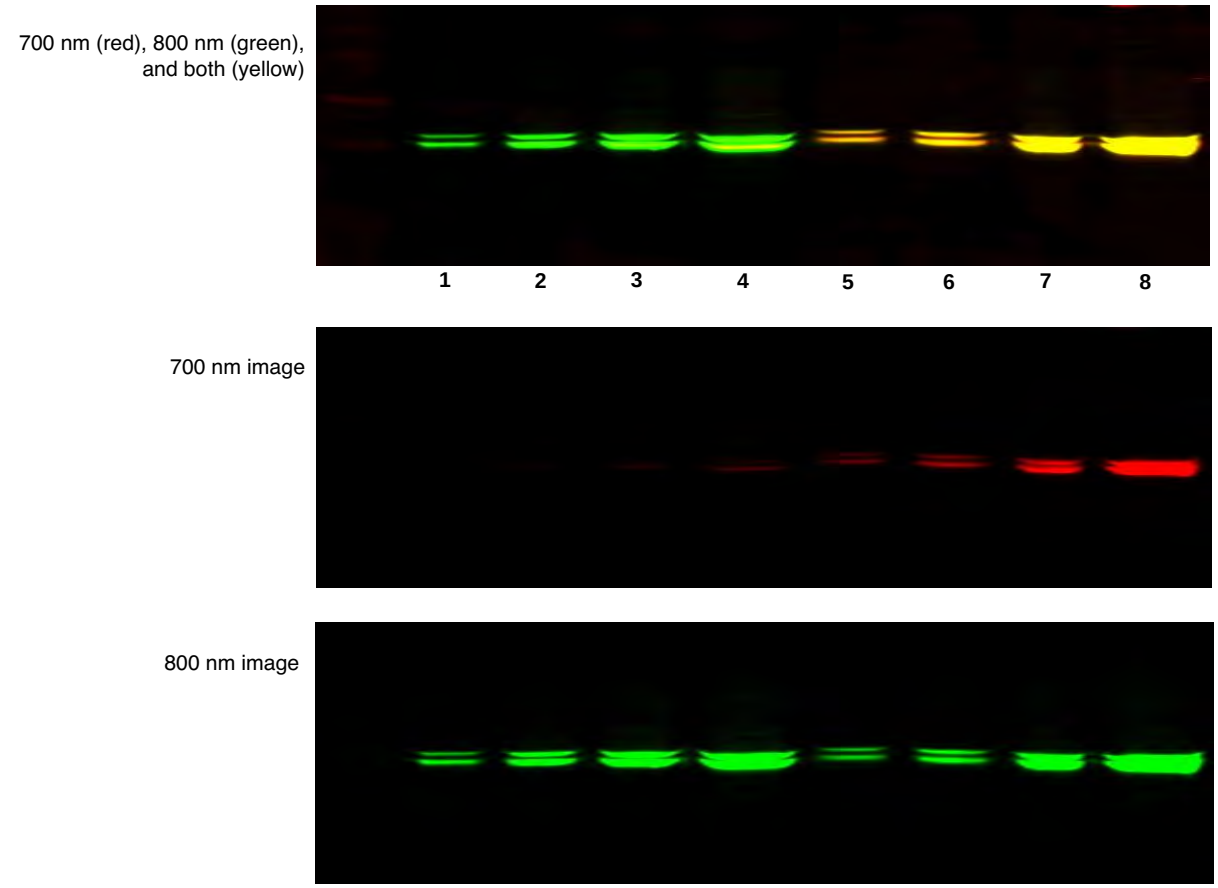
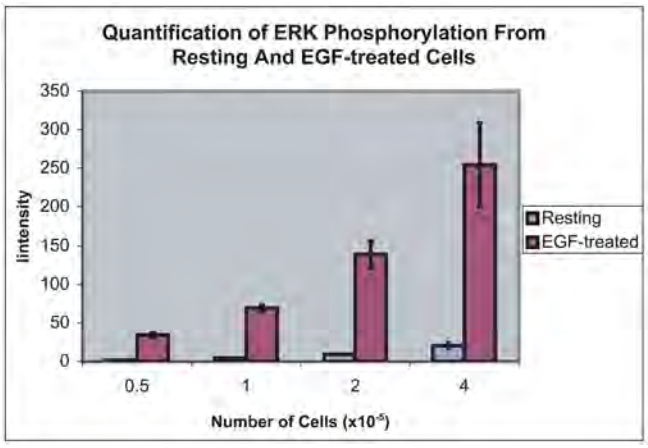
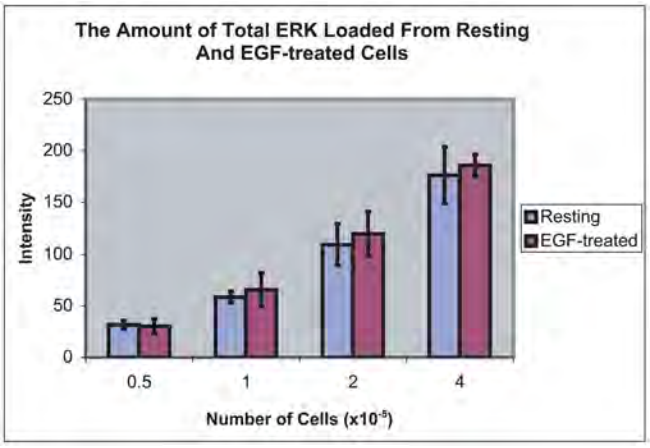


Figure 7. Two-fold serials of dilutions of resting (lane 1 to 4) and EGF treated (lane 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated ERK (700 nm, red) and total ERK (800 nm, green) in these lysates were simultaneously assessed.





Simultaneous measurement of phosphorylated EGF receptor and ERK in resting and EGF-treated A431 cell lysates.

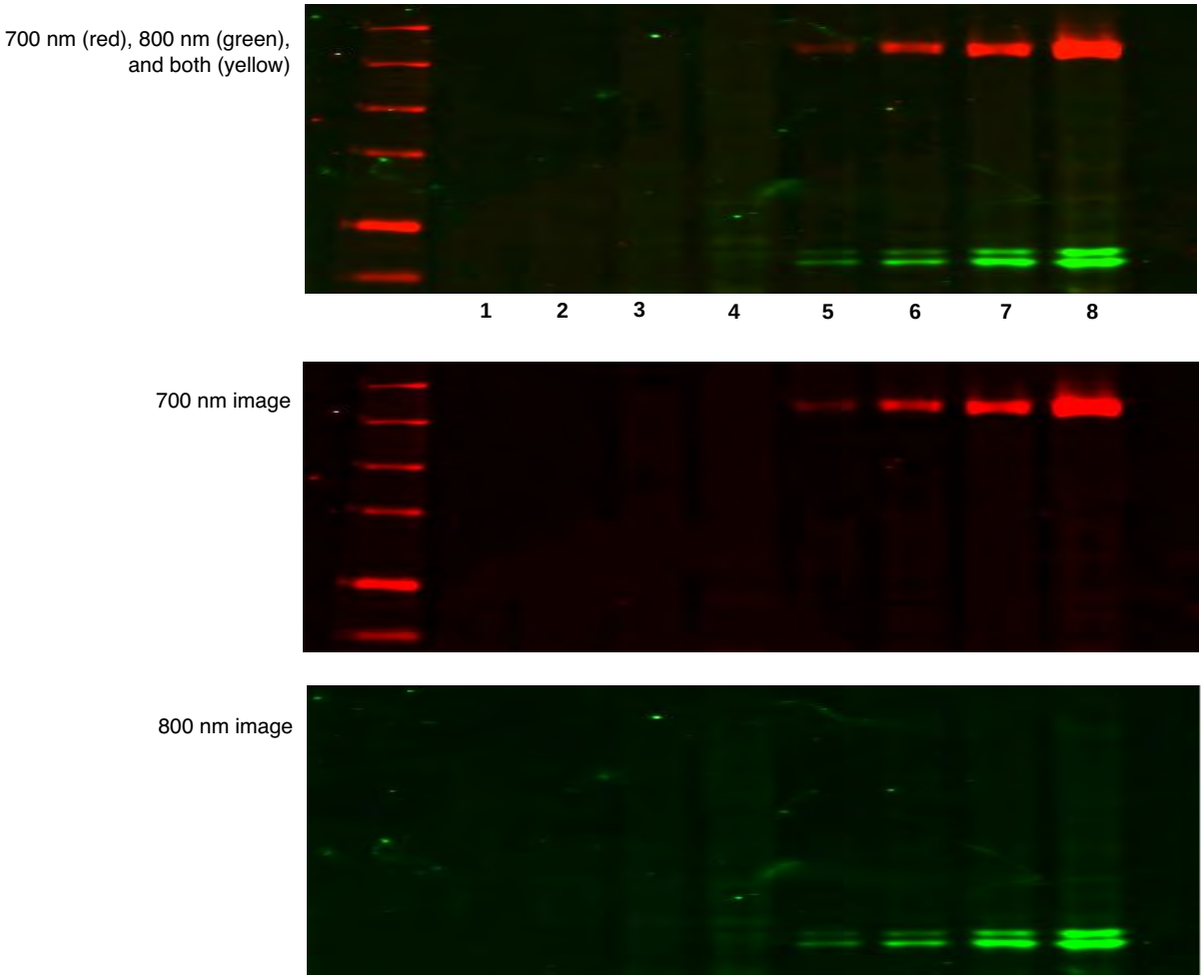
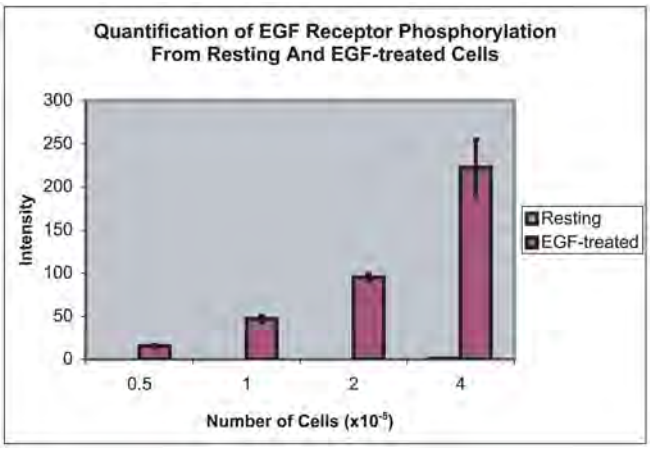
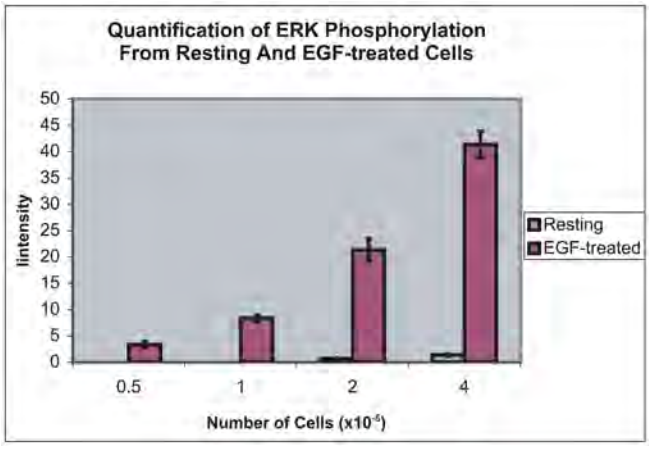


Figure 8. Two-fold serials of dilutions of resting (lane 1 to 4) and EGF-treated (lane 5 to 8) A431 cellular lysates were loaded, then the levels of phosphorylated EGFR (700 nm, red) and phosphorylated ERK (800 nm, green) in these lysates were simultaneously assessed.





4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
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988-11453 09/10

In-Cell Western™ Assay

IRDye® 800CW EGF Competition and Binding Assay Using A431 Cells

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

LI-COR® Reagents

- IRDye 800CW EGF Optical Probe (P/N 926-08446)
- Odyssey® Blocking Buffer (P/N 927-40000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- TO-PRO®-3 was purchased from Molecular Probes
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100
- Nunc® 96 Microwell™ Plate (Nunc, P/N 167008)
- Primary antibodies as described below

Special Note: Serum starvation of the cells is required to obtain maximal response.

II. Seeding, Stimulation, and Detection with IRDye® 800CW EGF

The following protocol is intended to illustrate the process for testing a particular cell type with IRDye 800CW EGF Optical Probe for eventual use *in vivo*. A431 (epithelial carcinoma) cells are used in this example, because of its over-expression of EGFR. Media considerations and certain cell characteristics will naturally alter this existing protocol.

1.	Allow A431 cell growth in a T75 flask in DMEM and 10% fetal calf serum (FCS; Gibco®) using standard tissue culture procedures until cells reach 80%-90% confluency (~1.5 x 10^7 cells).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells.
3.	Neutralize displaced cells with culture media and pellet by centrifugation.
4.	Remove supernatant and resuspend cell pellet in remaining media by manually tapping the collection tube. **Avoid vigorous pipetting or vortexing to resuspend cells in order to maintain cell integrity.**
5.	Dilute cells to 20 mL in complete media and count cells using a hemacytometer.
6.	Dilute cells with complete media to concentration of 200,000 cells/mL.
7.	Gently mix the cell suspension thoroughly.
8.	Under sterile conditions dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ plate (40,000 cells plated per well).
9.	Incubate cells and monitor cell density until cells are consistently confluent in each well. This should take approximately three days.
10.	Warm serum-free media (DMEM; Gibco) to 37°C.
11.	Remove complete media from the microwell plate by aspiration or inversion of the plate and tap excess media on tissue.
12.	Replace media with 200 µL of pre-warmed serum-free media per well and incubate 4 to 16 hours.
13.	In a separate 96-well Microwell plate devoid of cells, prepare a dilution series of reagents for the binding and competition assays discussed in step 14. Dispense 50 µL of DMEM per well (exclude Wells 1, 2, and 12).

14.	Continue preparation of the dilutions for both assays in the cell-less microtiter plate discussed above prior to adding to A431 cells. *Binding Assay*: Prepare 1:2 serial dilutions of IRDye® 800CW EGF ranging from 0.2 to 100 ng/mL. Dilute in DMEM. Add 100 µL of IRDye 800CW EGF (100 ng/mL) to Well 12 in triplicate rows. Transfer 50 µL from Well 12 to Well 11 and mix by pipetting up and down. Repeat this process through to Well 3. Leave the first and second wells without EGF (background controls) and add 100 µL DMEM only. An example image of the experimental layout should look like that shown in Figure 1A. *Competition Assay*: Prepare 1:2 serial dilutions of unlabeled EGF (15 µg/mL; Dilute in DMEM.) ranging from 15 to 0.03 µg/mL. Add 100 µL of 15 µg/mL unlabeled EGF to Well 12 in triplicate rows. Then transfer 50 µL from Well 12 to Well 11 and mix by pipetting up and down. Repeat this process to Well 3. Leave the first and second wells without EGF (background controls) and add 100 µL DMEM only. Remove and discard 50 µL from Well 3 so all wells now contain 50 µL except Wells 1 and 2. Add 50 µL of 200 ng/mL IRDye 800CW EGF to Wells 3-12 for triplicate *Competition Assay* rows giving a final concentration of labeled EGF of 100 ng/mL. An example image of the experimental layout should look like that shown in Figure 1B. ⚠ **Do not store.** Move directly to cell-containing plate and begin.
15.	Remove starvation media from cells by aspiration or inversion of the plate and tap excess media on tissue.
16.	Transfer 50 µL EGF dilutions for *Binding Assay* and 100 µL EGF mixtures for *Competition Assay* from the dilutions prepared in Step 14 into the cell-containing plate. Transfer these mixtures quickly (~20 sec) as the cellular responses are quick.
17.	Incubate at room temperature for 2 minutes.
18.	Prepare fresh *Fixing Solution* as follows: 1X PBS 45.0 mL 37% Formaldehyde 5.0 mL 3.7% Formaldehyde 50.0 mL
19.	Remove EGF-containing media by aspiration or inversion. Immediately fix cells by addition of 150 µL of fresh *Fixing Solution* and incubate at room temperature (RT) for 20 minutes with no shaking. **Add the solution carefully by pipetting down the side of the wells to avoid detaching the cells.** **Note:** • Cover plate with foil to protect from light.
20.	Prepare *Triton® Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL

21.	Remove the *Fixing Solution* by aspiration.
22.	Wash four times with 200 µL of *Triton® Washing Solution* for 5 minutes per wash to permeabilize the cells. **Notes:** • Allow each wash to shake on a plate shaker for 5 minutes at RT. • Do not allow cells/wells to become dry during washing. Add washes immediately after each other. • Cover plate to protect from light during procedure washings.
23.	Remove the *Triton Washing Solution* by aspiration or inversion.
24.	To each well, carefully add 150 µL of LI-COR® Odyssey® Blocking Buffer (P/N 927-40000) + 0.1% Tween® 20 down the side of the wells and incubate for 1.5 hours at RT with moderate shaking on a plate shaker.
26.	Add 50 µL of LI-COR Odyssey Blocking Buffer + 0.1% Tween 20 to Wells 1 and 2. These wells will serve as controls for any potential background due to the stain.
27.	Dilute TO-PRO®-3 (1:5,000 dilution; Molecular Probes, P/N T-3605) in Odyssey blocking buffer. Add 50 µL to each well (except Well 1) and incubate for 1 hour with gentle shaking. **Notes:** • IRDye® 800CW EGF will be detected in the 800 nm channel. • TO-PRO-3 is a cell stain that will be detected in the 700 nm channel. It can be used to normalize the IRDye 800CW EGF signal, to correct for variations in cell number from well to well. ⚠ **Avoid prolonged light exposure of the EGF and stain vials.**
28.	Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL
29.	Wash the plate with *Tween Washing Solution* (step 28) by gently adding buffer down the side of the wells to avoid detaching the cells. Use a generous amount of buffer (200 µL). Allow wash to shake gently on a plate shaker for 5 minutes at RT. ⚠ **Protect plate from light during washing.**
30.	Repeat wash 3 more times.
31.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
32.	Before scanning, clean the bottom plate surface and the Odyssey scanning bed with moist, lint-free paper to avoid obstructions during scanning.

33.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey® Imaging System. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

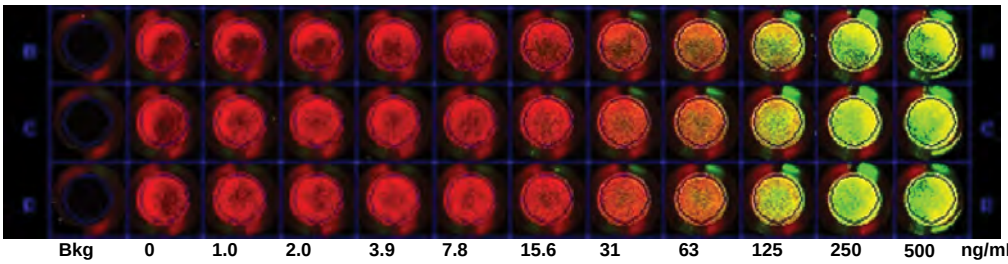
- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

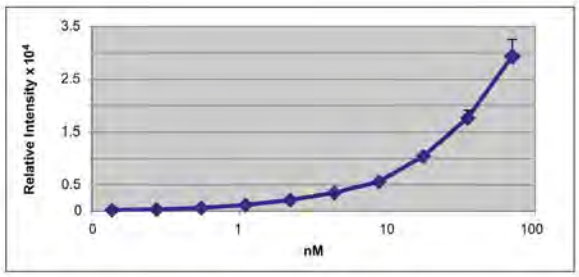
- All Odyssey Imaging systems require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments. (The actual minimum and maximum focus offset will vary with each instrument.)
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.

IV. Experimental Results

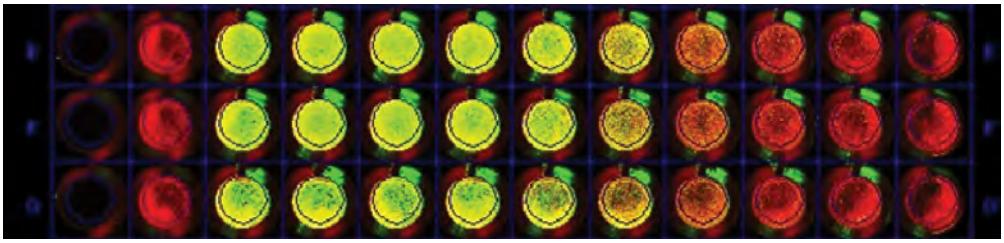
(A)



Bkg	0	1.0	2.0	3.9	7.8	15.6	31	63	125	250	500	ng/mL IRDye® 800CW EGF



(B)



Bkg	0	0.03	0.05	0.1	0.2	0.5	0.9	1.9	3.8	7.5	15	µg/mL Unlabeled EGF
Bkg	0	100	100	100	100	100	100	100	100	100	100	ng/mL IRDye 800CW EGF

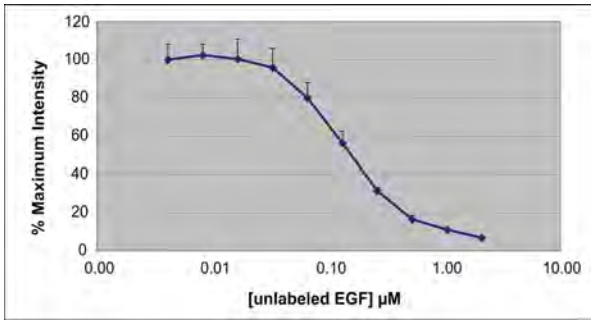


Figure 1. Plate set up for binding (A) and competition (B) assays. Subsequent analyses are shown below. Normalization using TO-PRO®-3 is detected in the 700 nm channel for both experiments. IRDye 800CW EGF binding is depicted in (A). IRDye 800CW EGF blocking with increasing concentrations of unlabeled EGF is depicted in (B).

Note: The level of blocking achieved on any given cell line will be a helpful indicator of how effective the probe will be *in vivo*.

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
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988-11464 09/10

In-Cell Western™ Assay

Complete Sample Protocol for Measuring IC_{50} of Inhibitor PD168393 in A431 Cells Responding to Epidermal Growth Factor

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

- IRDye® 800CW and IRDye 680 secondary antibodies (LI-COR® Biosciences)

  Note: IRDye 680LT secondary antibodies are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR, P/N 927-40000)
- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 20% Tween® 20
- Epidermal Growth Factor (Upstate Group Inc., P/N 01-107)
- Protein Tyrosine Kinase Inhibitor PD168393 (CALBIOCHEM®, P/N 513033)
- 37% formaldehyde
- 10% Triton® X-100
- Falcon™ 384-well microplate (P/N 353961)
- Primary antibodies

Special Note: Anti-phosphorylated-EGFR and anti-phosphorylated-ERK antibodies are purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Cell starvation is needed to obtain maximal response when these two phospho-antibodies are used. This is in contrast to use of anti-phospho-ERK from BD Pharmingen and from Cell Signaling Technology.

II. Sample Protocol

1.	Allow A431cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation.
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.
6.	Dilute cells with complete media such that 200,000 cells/mL is achieved.
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions dispense 50 µL of the cell suspension per well in Falcon 384-well microplate (10,000 cells plated per well).
9.	Incubate cells and monitor cell density until confluency is achieved with well-to-well consistency; approximately three days.
10.	Warm serum-free media (DMEM; Gibco) to 37°C.
11.	Remove complete media from plate wells by aspiration or manual displacement.
12.	Replace media with 50 µl of pre-warmed serum-free media per well and incubate 4 to 16 hours.
13.	Warm serum-free media (DMEM; Gibco) to 37°C.
14.	Dissolve PD168393 in DMEM to make 3 µM stock. Make two fold serial dilutions of inhibitor using DMEM so that the final concentration of inhibitor range from 3 µM to 90 pM, as shown in section *IV. Experimental Results*.
15.	Remove media in A431 cell plate.
16.	Add 50 µL of serial diluted inhibitor into cells and incubate 1 to 2 hours.
17.	Remove inhibitor from plate wells by aspiration or manual displacement.
18.	Add either serum-free media for resting cells (mock) or serum-free media with 100 ng/mL EGF. Use 50 µL of resting/activation media per well.
19.	Allow incubation at 37°C for 7.5 minutes.

20. Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature.

a. Prepare fresh *Fixing Solution* as follows:

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

b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.**

c. Allow incubation on bench top for 20 minutes at RT with no shaking.

21. Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash.

a. Prepare *Triton Washing Solution* as follows:

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

b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde).

c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.**

d. Allow wash to shake on a plate shaker for 5 minutes at RT.

e. Repeat washing steps 4 more times after removing wash manually.

⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**

22. Using a multi-channel pipettor, block cells/wells by adding 50 µL of LI-COR® Odyssey® Blocking Buffer to each well. Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.

Notes:

- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different nonspecific banding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for Odyssey detection.
- Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).
- Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.

23.	Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.
24.	Add the two primary antibodies into a tube containing Odyssey® Blocking Buffer. Choose one of the following primary antibody pairs: • Phospho-ERK; mouse (1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383) Total ERK1; rabbit (1:200 dilution; Santa Cruz Biotechnology, P/N SC-94) • Phospho-EGFR Tyr1045; rabbit (1:100 dilution; Cell Signaling Technology, P/N 2237) Total ERK2; mouse (1:75 dilution; Santa Cruz Biotechnology, P/N SC-1647) • Phospho-EGFR Tyr1045; rabbit (1:100 dilution; Cell Signaling Technology, P/N 2237) Phospho-ERK; mouse (1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383) • Phospho-EGFR Tyr1045; rabbit (1:100 dilution; Cell Signaling Technology, P/N 2237) Total EGFR; mouse (1:500 dilution; Biosource International, P/N AHR5062) a. Mix the primary antibody solution thoroughly before addition to wells. b. Remove blocking buffer from the blocking step and add 20 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well. c. Make sure to include control wells without primary antibody to serve as a source for background well intensity. Add 50 µL of Odyssey Blocking Buffer only to control wells.
25.	Incubate with primary antibody overnight with gentle shaking at RT.
26.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor add 200 µL of *Tween Washing Solution* (RT). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow wash to shake on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.

27.	Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer as specified below. To lower background, add Tween® 20 to the diluted antibody for a final concentration of 0.2%. IRDye® 680 goat anti-rabbit (1:200 dilution; LI-COR®, P/N 926-32221) IRDye 800CW goat anti-mouse (1:800 dilution; LI-COR, P/N 926-32210) Or IRDye 680 goat anti-mouse (1:200 dilution; LI-COR, P/N 926-32220) IRDye 800CW goat anti-rabbit (1:800 dilution; LI-COR, P/N 926-32211) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the dye-labeled secondary antibody vials to light.** **Note:** • Use IRDye 800CW secondary antibody to detect phosphorylation and IRDye 680 secondary antibody to detect total protein.
28.	Mix the antibody solutions thoroughly and add 20 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.
29.	Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 26). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** b. Allow wash to shake on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ **Protect plate from light during washing.**
30.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
31.	Before plate scanning, clean the bottom plate surface and the Odyssey scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
32.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey Imaging System. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

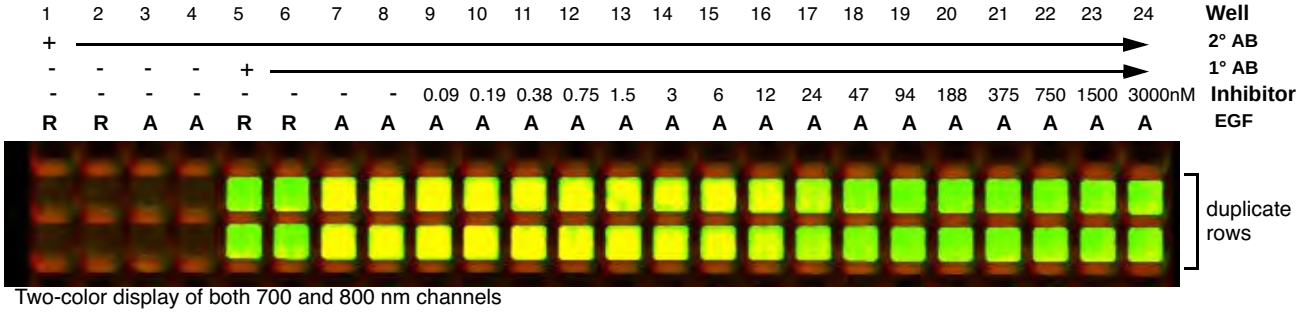
- All Odyssey® Imaging systems require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments. (The actual minimum and maximum focus offset will vary with each instrument.)
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imaging system. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results

IV. Experimental Results

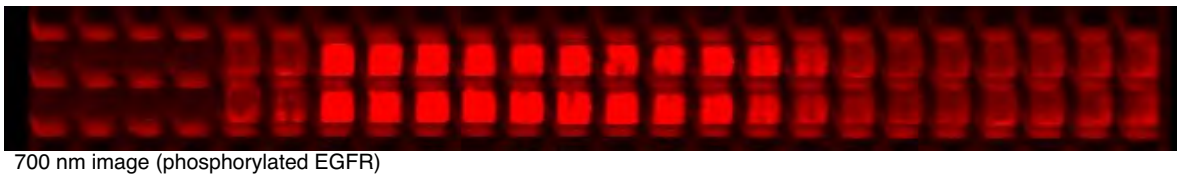
Simultaneous measurement of the effect of PD168393 on the phosphorylation of EGFR and downstream ERK

Four different experiments were conducted on the same microplate. (Color images can be seen at http://biosupport.licor.com.)

Experiment 1. Effect on the phosphorylation of EGFR (normalized against total EGFR)



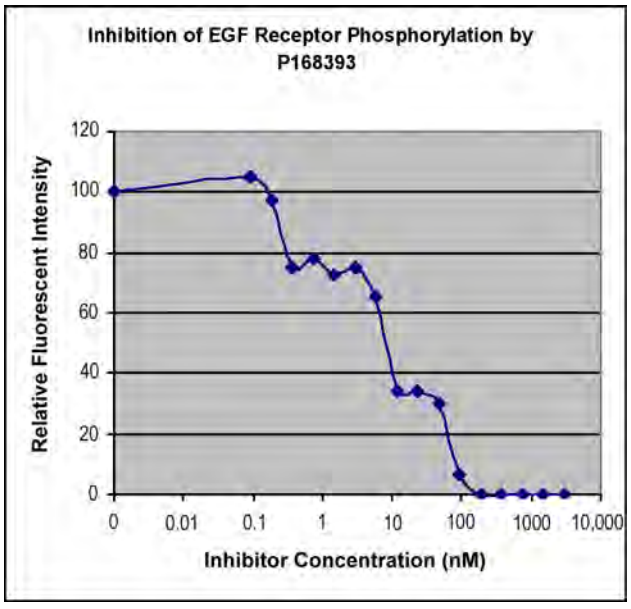
Two-color display of both 700 and 800 nm channels



700 nm image (phosphorylated EGFR)

800 nm image (total EGFR)

Figure 1. The level of EGFR phosphorylation was assessed in the 700 nm channel. A dramatic increase of EGFR phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of EGFR phosphorylation by PD168393 was observed in the 9th to 24th wells. Similar amounts of EGFR are present in all wells, as indicated by the 800 nm channel. The 1st to 4th wells, reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



Experiment 2. Effect on the phosphorylation of EGFR (normalized against total ERK)

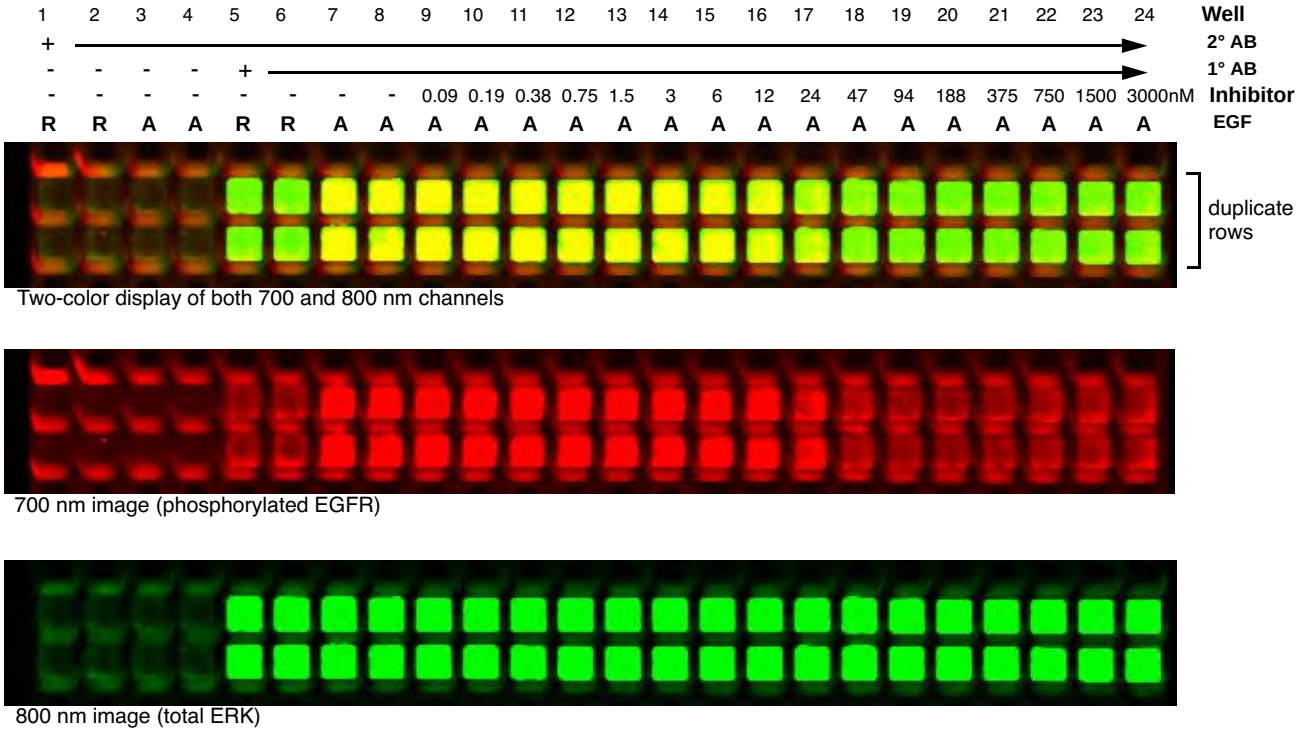
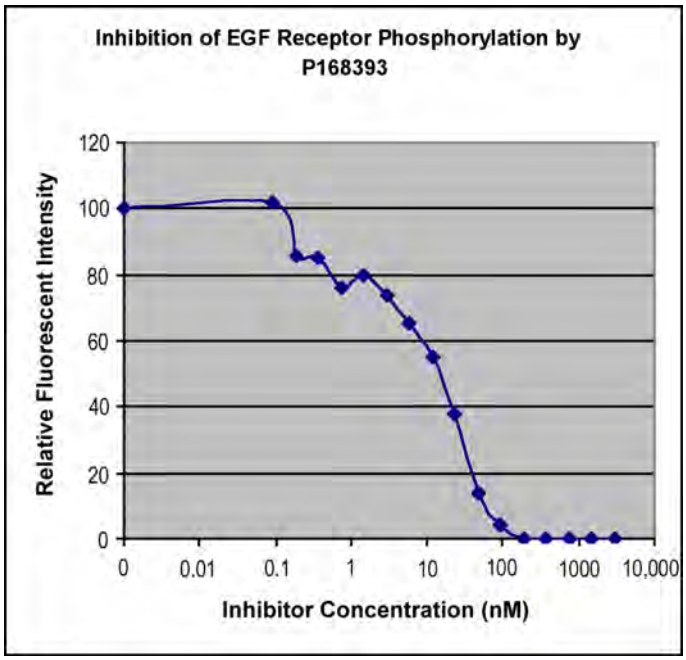


Figure 2. The level of EGFR phosphorylation was assessed in the 700 nm channel. A dramatic increase of EGFR phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of EGFR phosphorylation by PD168393 was observed in the 9th to 24th wells. Similar amounts of ERK are present in all wells, as indicated by the 800 nm channel. The 1st to 4th wells, reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



Experiment 3. Effect on the phosphorylation of ERK (normalized against total ERK)

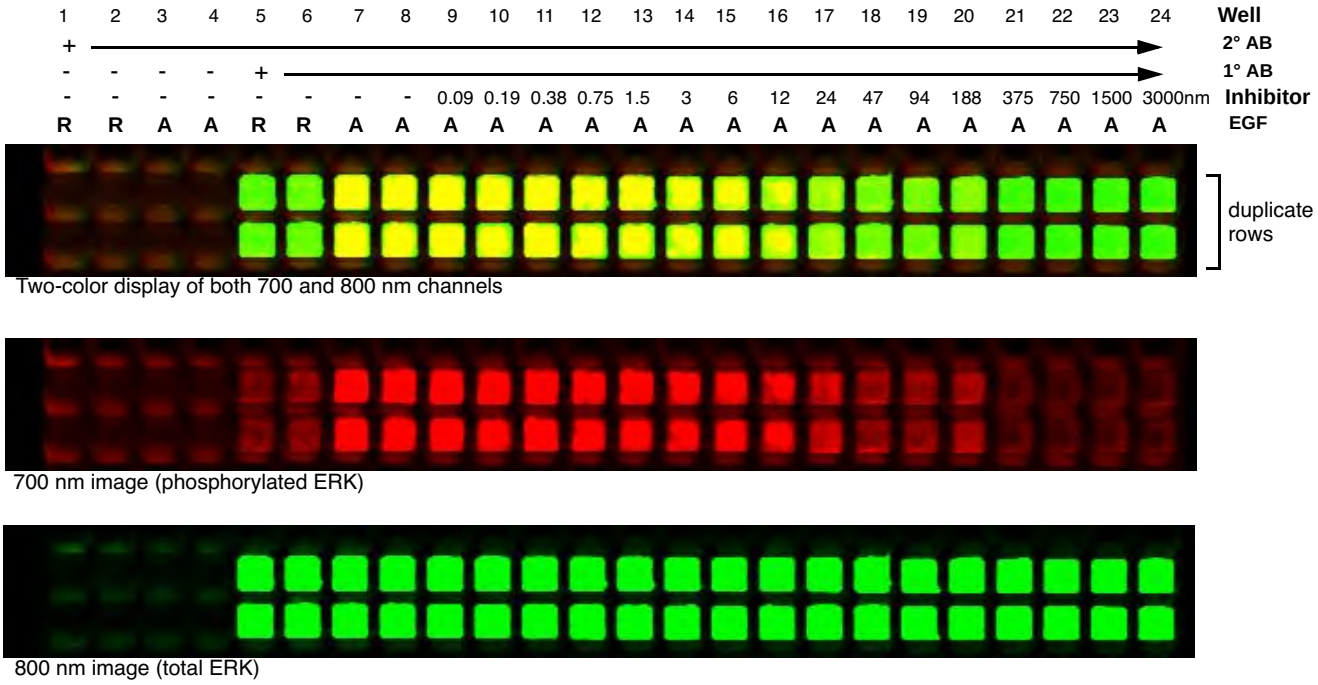
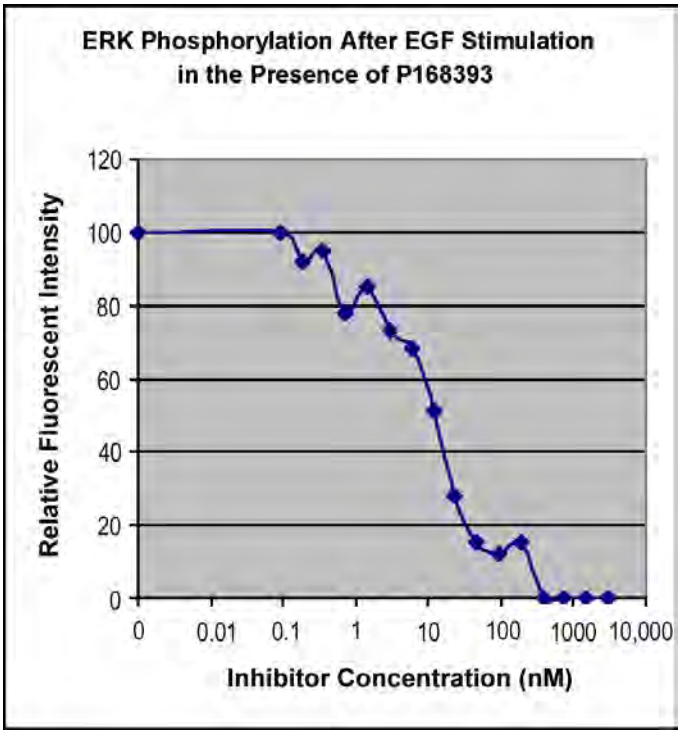


Figure 3. The level of ERK phosphorylation was assessed in the 700 nm channel. A dramatic increase of ERK phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of ERK phosphorylation by PD168393 was observed in the 9th to 24th wells. Similar amounts of ERK are present in all wells, as indicated by the 800 nm channel. The 1st to 4th wells, reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



Experiment 4. Effect on the phosphorylation of EGFR and ERK

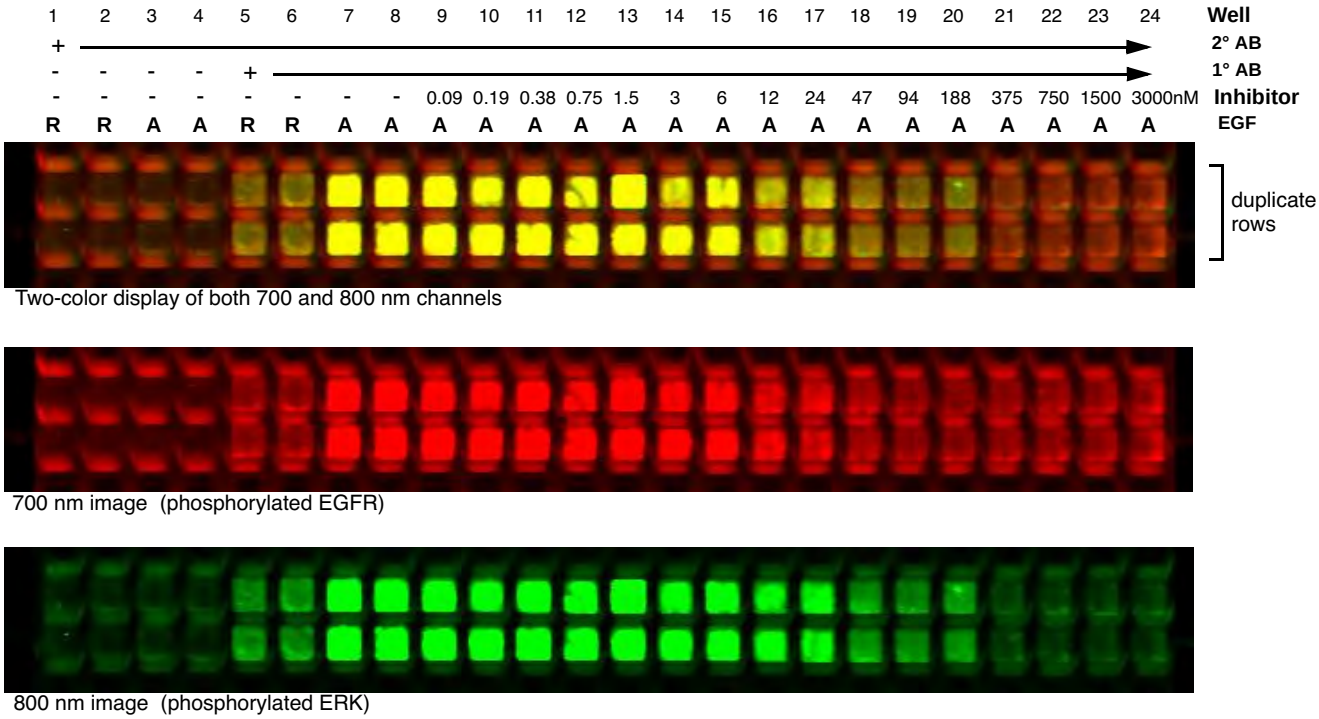
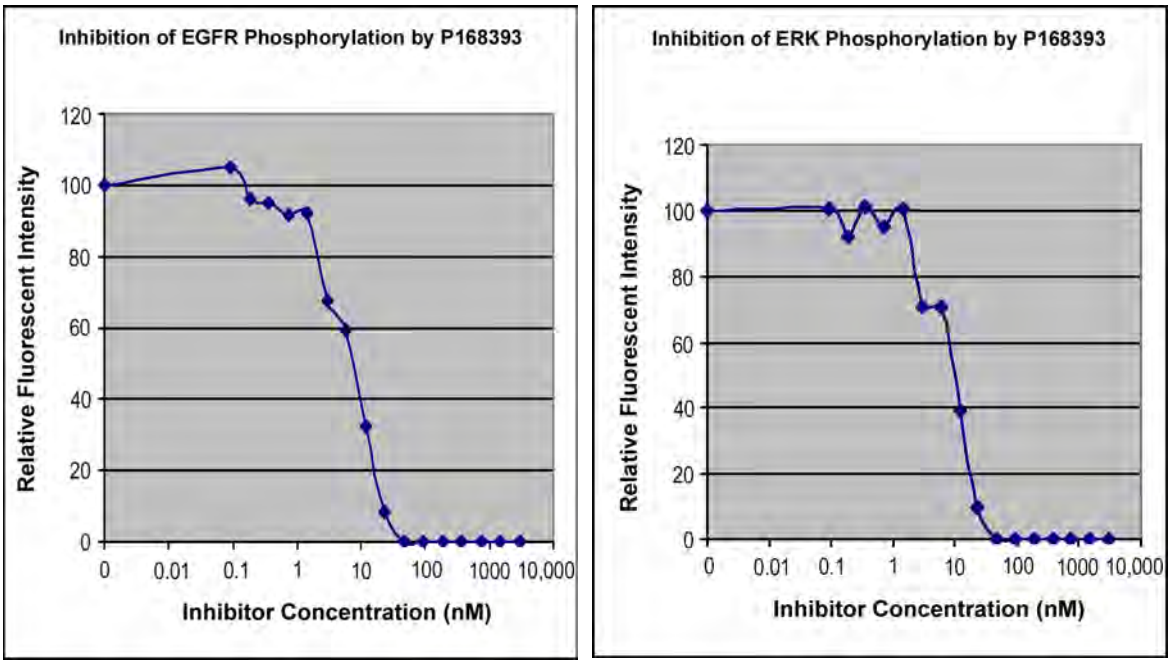


Figure 4. The degree of phosphorylation of EGFR and ERK was assessed in the 700 nm (red) and 800 nm (green) channels, respectively. A dramatic increase of phosphorylation of EGFR and ERK in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of phosphorylation of EGFR and ERK by PD168393 was observed in 9th to 24th wells. The 1st to 4th wells, reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
In other countries, contact LI-COR Biosciences or a local LI-COR distributor.
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988-11462 09/10

In-Cell Western™ Assay

Complete Sample Protocol for Measuring IC_{50} of Inhibitor U0126 in NIH3T3 Responding to Acidic Fibroblast Growth Factor (aFGF-1)

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September 2010. The most recent version of this protocol is posted at http://biosupport.licor.com

Contents

I. Required Reagents

LI-COR® Reagents

- IRDye® 800CW goat anti-mouse secondary antibodies (P/N 926-32210)
- IRDye 680 goat anti-rabbit secondary antibodies (P/N 926-32221)

  Note: IRDye 680LT goat anti-rabbit secondary antibodies (P/N 926-68021) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (P/N 927-40000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- SigmaScreen™ Poly-D-Lysine-coated 96-well microplate (Sigma®, P/N Z38249-3)
- Heparin (CALBIOCHEM®, P/N 375097)
- Acidic Fibroblast Growth Factor (Upstate Group Inc., P/N 01-116)
- MEK inhibitor U0126 (Promega®, P/N V1121)
- Primary antibodies
- 20% Tween® 20
- 37% Formaldehyde
- 10% Triton® X-100

Special Note: NIH3T3 cells do not adhere strongly to TC-treated plates resulting in the need for poly-D-lysine-coated plates in this assay. However, even with lysine-coated plates the adherence of cells remains relatively weak compared to other cell lines.

⚠ **Be very cautious and delicate with plate handling and pipetting when washing, removing, and adding solutions to avoid detaching the cells.**

II. Sample Protocol

1.	Allow NIH3T3 (ATCC; CRL-1555) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.
6.	Reconstitute cells and dilute in 40 mL of complete media such that 75,000 cells/mL is achieved (2 plates x 96 wells x 200 µL/well = ~ 40 mL).
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions, dispense 200 µL of the cell suspension per well into a SigmaScreen™ Poly-D-Lysine 96-well microplate (15,000 cells plated per well).
9.	Incubate cells and monitor cell density until 70% confluency is achieved (about 24 hours). ⚠ **70% confluency is very important. 90 to 100% confluent cells are certain to detach during washing.**
10.	Warm serum-free media (DMEM; Gibco) to 37°C.
11.	Dissolve U0126 in DMSO to make 10 mM stock. Make two fold serial dilutions of inhibitor using DMEM. Add 10 µL of serial diluted inhibitor into cells so that the final concentration of inhibitor range from 1 to 125 µM (see Figure 1, section *IV. Experimental Results*). Incubate 1 to 2 hours.
12.	Remove media and inhibitor from plate wells by aspiration or manual displacement.
13.	Add either serum-free media for resting cells (mock) or serum-free media with 100 ng/mL aFGF combined with 10 µg/mL heparin for activated cells. Use 100 µL of resting/activation media per well.
14.	Allow incubation at 37°C for 7.5 minutes.

15.	Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature. a. Prepare fresh *Fixing Solution* as follows: 1X PBS 45 mL 37% Formaldehyde 5 mL 3.7% Formaldehyde 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on bench top for 20 minutes at RT with no shaking.
16.	Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.** d. Allow wash to shake on a plate shaker for 5 minutes at RT. e. Repeat washing steps 4 more times after removing wash manually. ⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**

17.	Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.** **Notes:** • No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required). • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.
18.	Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.
19.	Add the two primary antibodies to a tube containing Odyssey Blocking Buffer. Combine the solutions defined below for ERK target analysis: • Phospho-ERK (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 9101) Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology, P/N SC-1647) a. Mix the primary antibody solution thoroughly before adding it to the wells. b. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well. c. **Make sure to include control wells without primary antibody to serve as a source for background well intensity.** Add 50 µL of Odyssey Blocking Buffer only to control wells.
20.	Incubate with primary antibody overnight with gentle shaking at RT.
21.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **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 on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.

22.	Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer as specified below. To lower background, add Tween® 20 to the diluted antibody to a final concentration of 0.2%. Goat anti-rabbit IRDye® 680 (1:200 dilution; LI-COR® Biosciences) Goat anti-mouse IRDye 800CW (1:800 dilution; LI-COR Biosciences) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
23.	Mix the antibody solutions well and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.
24.	Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 21). **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 on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ **Protect plate from light during washing.**
25.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
26.	Before plate scanning, clean the bottom plate surface and the Odyssey Imager scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
27.	Scan the plate with detection in both 700 and 800 nm channels. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

III. Experimental Considerations

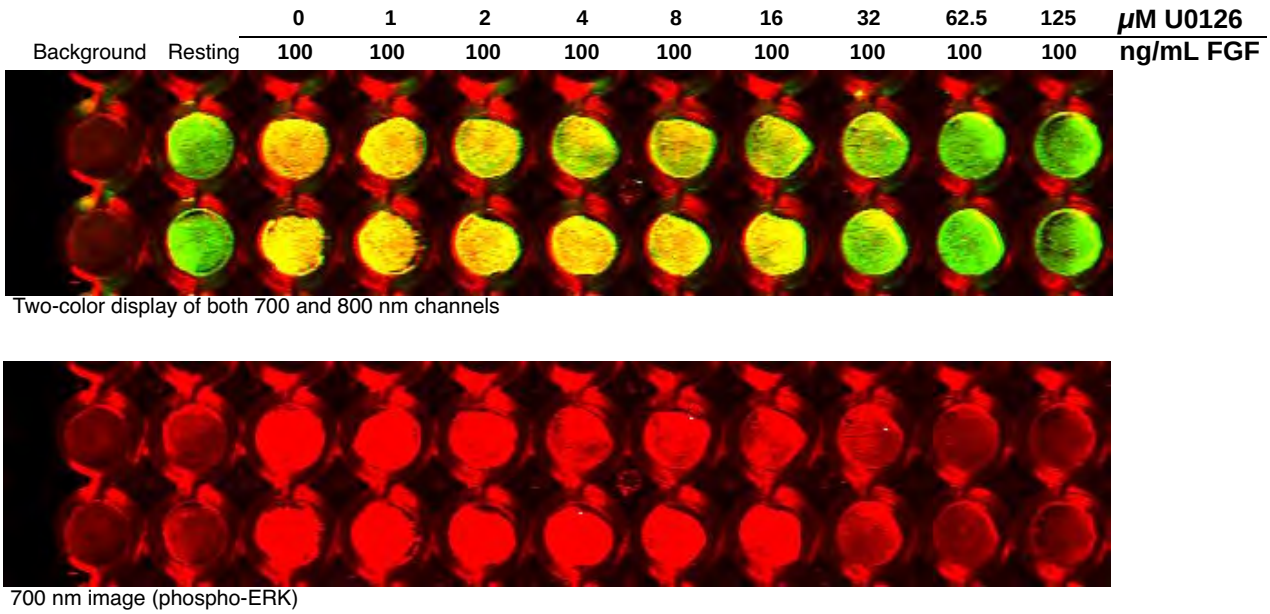
- All Odyssey Imaging systems require that microplates have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). The recommended focus offset is 3.0 mm for the SigmaScreen™ microplates specified for this assay.
- If plates other than the recommended SigmaScreen microplates are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets

for the Odyssey® Sa instrument (actual maximum and minimum values vary with each instrument). Use the same intensity settings for each scan. After reviewing the collected scans, use the focus offset with the highest signal-to-noise as the focus offset for experiments.

- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager and 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality, with 169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey instrument. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

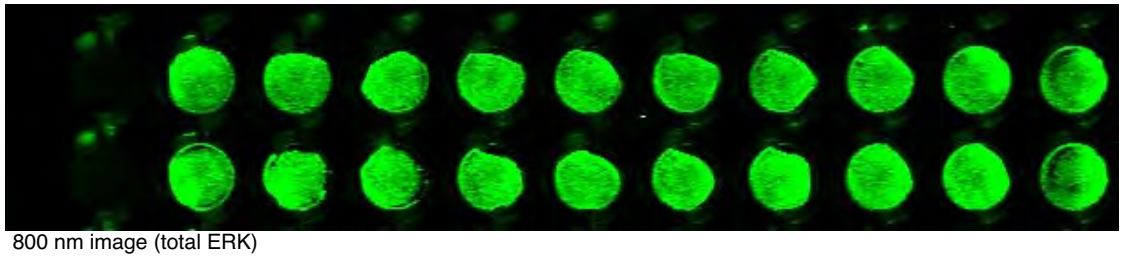
IV. Experimental Results

Color images can be seen at http://biosupport.licor.com.



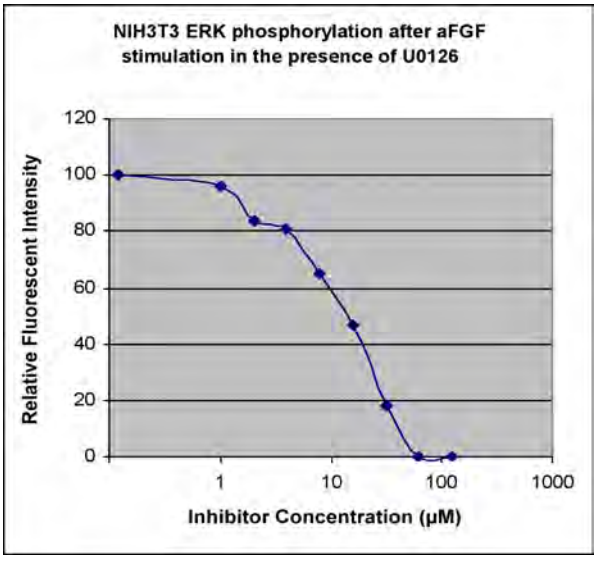
Two-color display of both 700 and 800 nm channels

700 nm image (phospho-ERK)



800 nm image (total ERK)

Figure 1. U0126 inhibition of ERK phosphorylation in NIH3T3 cells stimulated with FGF. The graph demonstrates the inhibitory effect of the MEK inhibitor U0126 as determined through the detection of ERK phosphorylation (Thr202/Tyr204) within an In Cell Western assay. Resulting data were plotted and the IC_{50} of U0126 was determined to be ~15μM, correlating well with the IC_{50} reported in literature (1) for *in vitro* and *in vivo* assays.



1. Ahn, N.G. *et al* (1999). U0126: An Inhibitor of MKK/ERK Signal Transduction in Mammalian Cells. Promega Notes 71, p. 4.

LI-COR® Biosciences
4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
www.licor.com

988-11454 09/10

In-Cell Western™ Assay

Complete Sample Protocol Detailing the Seeding, Stimulation, and Detection of the HeLa Cellular Response to Epidermal Growth Factor

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com

Contents

I. Required Reagents

LI-COR® Reagents

- IRDye® 800CW goat anti-mouse secondary antibodies (P/N 926-32210)
- IRDye 680 goat anti-rabbit secondary antibodies (P/N 926-32221)

  Note: IRDye 680LT goat anti-rabbit secondary antibodies (P/N 926-68021) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (P/N 927-40000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 20% Tween® 20
- Epidermal Growth Factor (Upstate Group Inc., P/N 01-107)
- 37% formaldehyde
- 10% Triton® X-100
- Nunc® 96 Microwell™ Plate (Nunc, P/N 167008)
- Primary antibodies

Special Note: (1) Long passage HeLa cells are not suitable for the assay because of high basal level phosphorylation of ERK. As a result, ordering a new sample of this cell line from ATCC is highly recommended. (2) No starvation is needed or recommended.

II. Sample Protocol

1.	Allow HeLa (ATCC; CCL-2) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation.
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.
6.	Dilute cells with complete media such that 75,000 cells/mL is achieved.
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions, dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ Plate (15,000 cells plated per well).
9.	Incubate cells and monitor cell density until confluency is achieved with well-to-well consistency; approximately two to three days.
10.	Warm serum-free media (DMEM; Gibco) to 37°C. Dispense 100 µL of DMEM per well in the Nunc 96-well microplate.
11.	Make serial dilutions of EGF in the microplate ranging from 0.2 to 100 ng/mL. Leave the first and second wells without EGF (resting cells as control), as shown in section *IV. Experimental Results*.
12.	Remove complete media from plate wells by aspiration or manual displacement.
13.	Transfer media from the dilution plate into the experimental plate.
14.	Allow incubation at 37°C for 7.5 minutes.
15.	Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature. a. Prepare fresh *Fixing Solution* as follows: 1X PBS — 45 mL 37% Formaldehyde — 5 mL 3.7% Formaldehyde — 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on bench top for 20 minutes at RT with no shaking.

16.	Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.** d. Allow wash to shake on a plate shaker for 5 minutes at RT. e. Repeat washing steps 4 more times after removing wash manually. ⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**
17.	Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer to each well. Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells. **Notes:** • No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required). • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.
18.	Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.

19.	Add the two primary antibodies to a tube containing Odyssey® Blocking Buffer. Combine the following solutions as defined below for ERK target analysis: • Phospho-ERK (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 9101) • Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology, P/N SC-1647) a. Mix the primary antibody solution thoroughly before addition to wells. b. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well. c. Make sure to include control wells without primary antibody to serve as a source for background well intensity (see first well in **Figure 1**). Add 50 µL of Odyssey Blocking Buffer only to control wells.
20.	Incubate with primary antibody for 2 hours with gentle shaking at RT. **Note:** • For greatest sensitivity, continue incubation overnight at 4°C with no shaking.
21.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor add 200 µL of *Tween Washing Solution* (RT). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow wash to shake on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.
22.	Dilute the fluorescently-labeled secondary antibody in Odyssey Blocking Buffer as specified below. To lower background, add Tween 20 to the diluted antibody for a final concentration of 0.2%. Goat anti-rabbit IRDye® 680 (1:200 dilution; LI-COR® Biosciences) Goat anti-mouse IRDye 800CW (1:800 dilution; LI-COR Biosciences) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
23.	Mix the antibody solutions well and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

24.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 21). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** b. Allow wash to shake on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ **Protect plate from light during washing.**
25.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
26.	Before plate scanning, clean the bottom plate surface and the Odyssey® scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
27.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey Imaging System. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

- All Odyssey Imaging systems require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.

- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey® Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for the experiments. (The actual minimum and maximum focus offset will vary with each instrument.)
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imaging system. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

IV. Experimental Results

Color images can be seen at http://biosupport.licor.com.

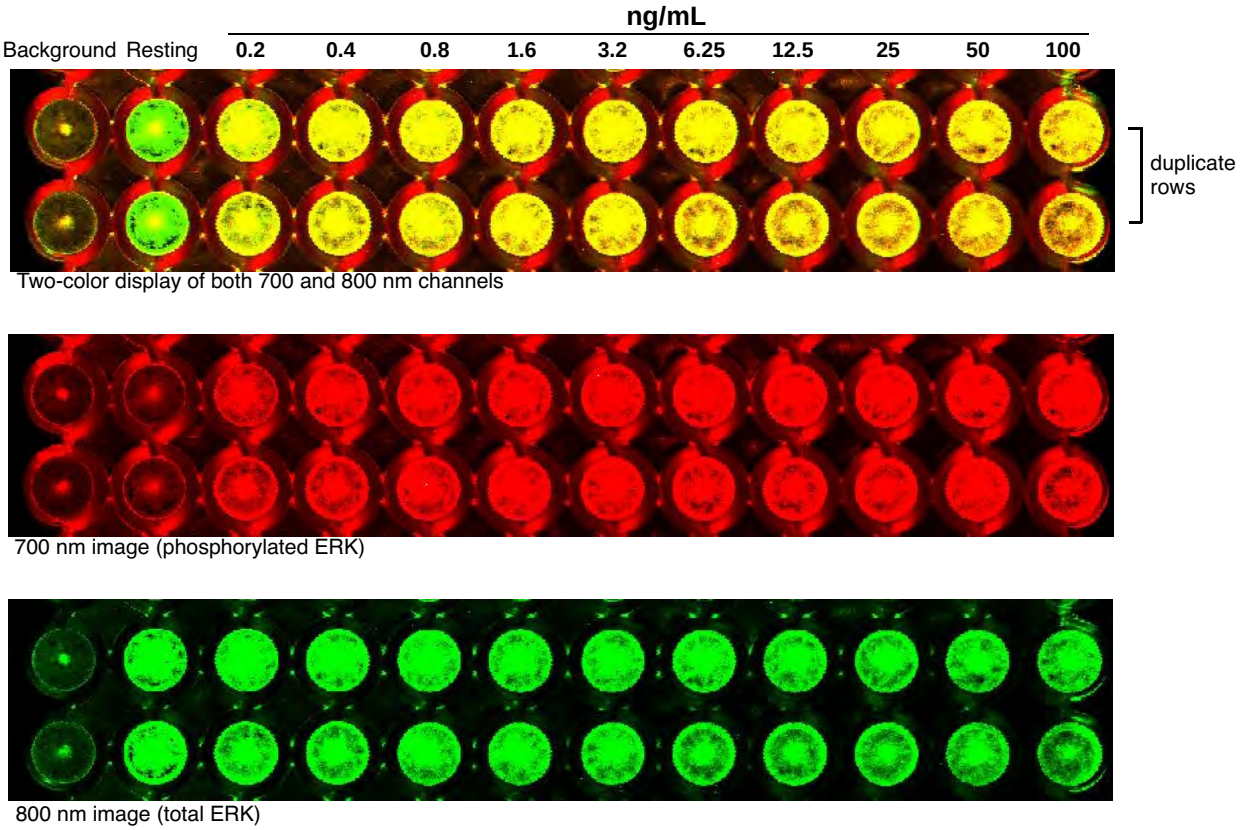
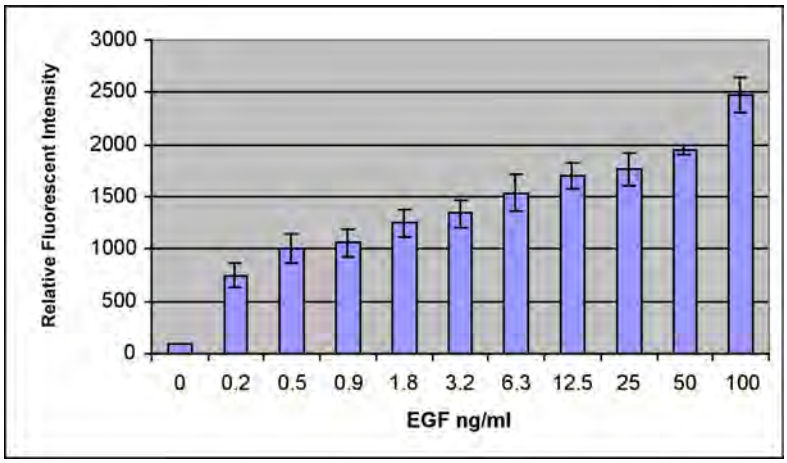


Figure 1. Dose response of HeLa cells to epidermal growth factor (EGF) as measured by specific antibody detecting dual-phosphorylated ERK (Thr202/Tyr204). The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting total and phosphorylated ERK, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of ERK.



LI-COR® Biosciences
4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
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988-11455 09/10

In-Cell Western™ Assay

Complete Sample Protocol Detailing the Seeding, Stimulation, and Detection of the NIH3T3 Cellular Response to Platelet Derived Growth Factor BB (PDGF-BB)

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

- IRDye® 800CW goat anti-rabbit secondary antibodies (P/N 926-32211)
- IRDye 680 goat anti-mouse secondary antibodies (P/N 926-32220)

  Note: IRDye 680LT goat anti-mouse secondary antibodies (P/N 926-68020) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR, P/N 927-40000)
- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- SigmaScreen™ Poly-D-Lysine-coated 96-well microplate (Sigma®, P/N Z38249-3)
- Platelet Derived Growth Factor BB (PDGF-BB) (Upstate Group Inc., P/N 01-305)
- Primary antibodies
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100

Special Note: NIH3T3 cells do not adhere strongly to TC-treated plates resulting in the need for poly-D-lysine-coated plates in this assay. However, even with lysine-coated plates the adherence of cells remains relatively weak compared to other cell lines.

⚠ **Be very cautious and delicate with plate handling and pipetting when washing, removing, and adding solutions to avoid detaching the cells.**

II. Sample Protocol

1.	Allow NIH3T3 (ATCC; CRL-1555) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).
4.	Remove supernatant and disrupt the cell pellet manually by hand tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.
6.	Reconstitute cells and dilute in 40 mL of complete media such that 75,000 cells/mL is achieved (2 plates x 96 wells x 200 µL/well = ~ 40 mL).
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions, dispense 200 µL of the cell suspension per well into a SigmaScreen™ Poly-D-Lysine 96-well microplate (15,000 cells plated per well).
9.	Incubate cells and monitor cell density until 70% confluency is achieved (it takes about 24 hours). ⚠ **70% confluency is very important. 90 to 100% confluent cells are certain to detach during washing.**
10.	Warm serum-free media (DMEM; Gibco) to 37°C.
11.	Remove media and inhibitor from plate wells by aspiration or manual displacement.
12.	Add either serum-free media for resting cells (mock) or serum-free media with serial concentrations of PDGF-BB ranging from 0.4 to 200 ng/mL for activated cells. Use 100 µL of resting/activation media per well.
13.	Allow incubation at 37°C for 7.5 minutes.
14.	Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature. a. Prepare fresh *Fixing Solution* as follows: 1X PBS — 45 mL 37% Formaldehyde — 5 mL 3.7% Formaldehyde — 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on bench top for 20 minutes at RT with no shaking.

15.	Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.** d. Allow wash to shake on a rotator for 5 minutes at RT. e. Repeat washing steps 4 more times after removing wash manually. ⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**
16.	Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.** **Notes:** • No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required). • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.
17.	Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.

18.	Add the two primary antibodies into a tube containing Odyssey® Blocking Buffer. Combine the solutions defined below for phospho-Akt target analysis, using total ERK2 for normalization: • Phospho-Akt (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 9271 or 4058) • Total ERK2 (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-1647) a. Mix the primary antibody solution thoroughly before adding it to the wells. b. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well. c. **Make sure to include control wells without primary antibody to serve as a source for background well intensity.** Add 50 µL of Odyssey Blocking Buffer only to control wells.
19.	Incubate with primary antibody overnight with gentle shaking at RT.
20.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **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 on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.
22.	Dilute the fluorescently-labeled secondary antibody in Odyssey Blocking Buffer as specified below. To lower the background, add Tween 20 to the diluted antibody to a final concentration of 0.2%. IRDye® 800CW goat anti-rabbit (1:800 dilution; LI-COR, P/N 926-32211) IRDye 680 goat anti-mouse (1:200 dilution; LI-COR, P/N 926-32220) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
23.	Mix the antibody solutions thoroughly and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

24.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 20). **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 on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ **Protect plate from light during washing.**
25.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
26.	Before scanning, clean the bottom plate surface and the Odyssey® Imager scanning surface (if applicable) with moist, lint-free paper to avoid any obstructions during scanning.
27.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey Imaging system. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

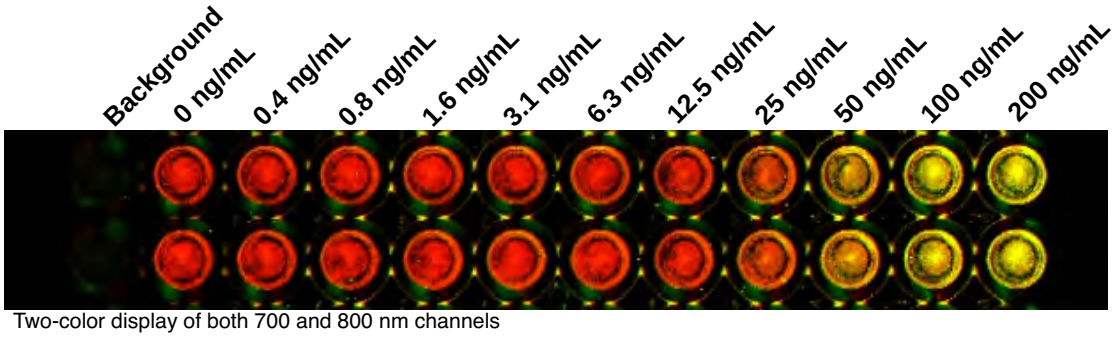
III. Experimental Considerations

- All Odyssey Imaging systems require microplates that have a maximum of 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). The recommended focus offset is 3.0 mm for the SigmaScreen™ microplates specified for this assay.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. (The actual minimum and maximum focus offset will vary with each instrument.) Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).

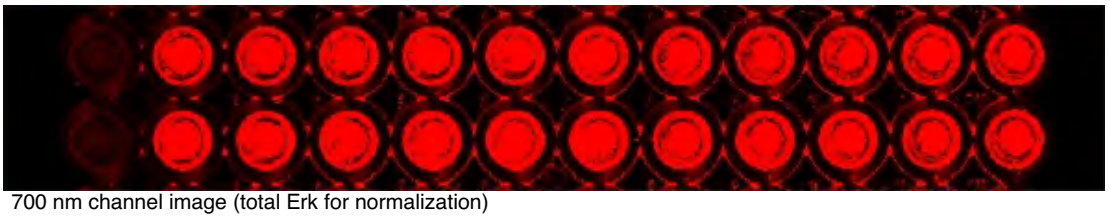
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey® Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imager. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

IV. Experimental Results

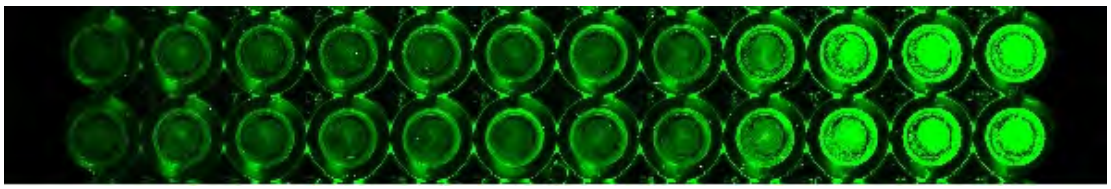
Color images can be seen at http://biosupport.licor.com.



Two-color display of both 700 and 800 nm channels



700 nm channel image (total Erk for normalization)



800 nm channel image (phospho-Akt)

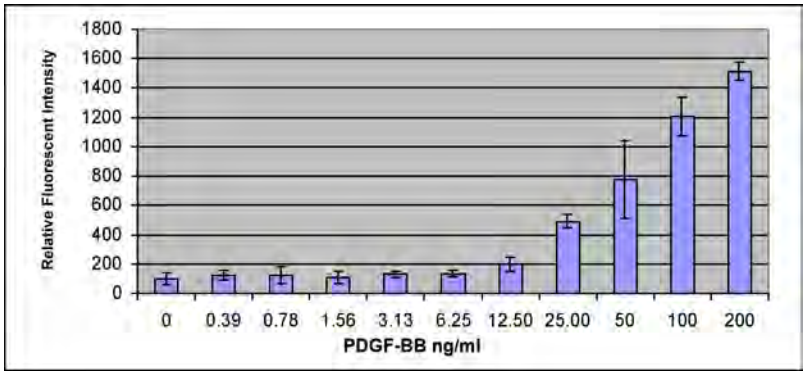


Figure 1. Dose response of NIH3T3 cells to Platelet Derived Growth Factor (PDGF-BB) as measured by specific antibody detecting phosphorylated Akt (Ser473) using total ERK2 for normalization. The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 channels detecting total ERK2 and phosphorylated Akt (Ser473), respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents average of four sets of quantitative data demonstrating the percent induction of phosphorylated Akt (Ser473).

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
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988-11456 09/10

In-Cell Western™ Assay

Complete Sample Protocol Detailing the Seeding, Stimulation, and Detection of the NIH3T3 Cellular Response to Acidic Fibroblast Growth Factor (aFGF-1)

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

- IRDye® 800CW goat anti-mouse secondary antibodies (P/N 926-32210)
- IRDye 680 goat anti-rabbit secondary antibodies (P/N 926-32221)

  Note: IRDye 680LT goat anti-rabbit secondary antibodies (P/N 926-68021) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR, P/N 927-40000)
- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- SigmaScreen™ Poly-D-Lysine-coated 96-well microplate (Sigma®, P/N Z38249-3)
- Heparin (CALBIOCHEM®, P/N 375097)
- Acidic Fibroblast Growth Factor (Upstate Group Inc., P/N 01-116)
- Primary antibodies
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100

Special Note: NIH3T3 cells do not adhere strongly to TC-treated plates resulting in the need for poly-D-lysine-coated plates in this assay. However, even with lysine-coated plates the adherence of cells remains relatively weak compared to other cell lines.

⚠ **Be very cautious and delicate with plate handling and pipetting when washing, removing, and adding solutions to avoid detaching the cells.**

II. Sample Protocol

1.	Allow NIH3T3 (ATCC; CRL-1555) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.
6.	Reconstitute cells and dilute in 40 mL of complete media such that 75,000 cells/mL is achieved (2 plates x 96 wells x 200 µL/well = ~ 40 mL).
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions, dispense 200 µL of the cell suspension per well into a SigmaScreen™ Poly-D-Lysine 96-well microplate (15,000 cells plated per well).
9.	Incubate cells and monitor cell density until 70% confluency is achieved (it takes about 24 hours). ⚠ **70% confluency is very important. 90 to 100% confluent cells are certain to detach during washing.**
10.	Warm serum-free media (DMEM; Gibco) to 37°C.
11.	Remove media and inhibitor from plate wells by aspiration or manual displacement.
12.	Add either serum-free media for resting cells (mock) or serum-free media with serial concentrations of aFGF-1 ranging from 0.2 to 100 ng/mL, combined with 10 µg/mL heparin for activated cells. Use 100 µL of resting/activation media per well.
13.	Allow incubation at 37°C for 7.5 minutes.
14.	Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature. a. Prepare fresh *Fixing Solution* as follows: 1X PBS — 45 mL 37% Formaldehyde — 5 mL 3.7% Formaldehyde — 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on bench top for 20 minutes at RT with no shaking.

15.	Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.** d. Allow wash to shake on a plate shaker for 5 minutes at RT. e. Repeat washing steps 4 more times after removing wash manually. ⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**
16.	Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.** **Notes:** • No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required). • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.
17.	Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.

18.	Add the two primary antibodies to a tube containing Odyssey® Blocking Buffer. Combine the solutions defined below for phospho-ERK target analysis, using total ERK2 for normalization: • Phospho-ERK (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 9101) • Total ERK2 (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-1647) a. Mix the primary antibody solution well before adding it to the wells. b. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well. c. Make sure to include control wells without primary antibody to serve as a source for background well intensity. Add 50 µL of Odyssey Blocking Buffer only to control wells.
19.	Incubate with primary antibody overnight with gentle shaking at RT.
20.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **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 on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.
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22.	Mix the antibody solutions thoroughly and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

23.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 20). **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 on a rotator for 5 minutes at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ **Protect plate from light during washing.**
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25.	Before plate scanning, clean the bottom plate surface and the Odyssey® Imager scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
26.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey Imaging system. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

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- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imaging system or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. (The actual minimum and maximum focus offset will vary with each instrument.) Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
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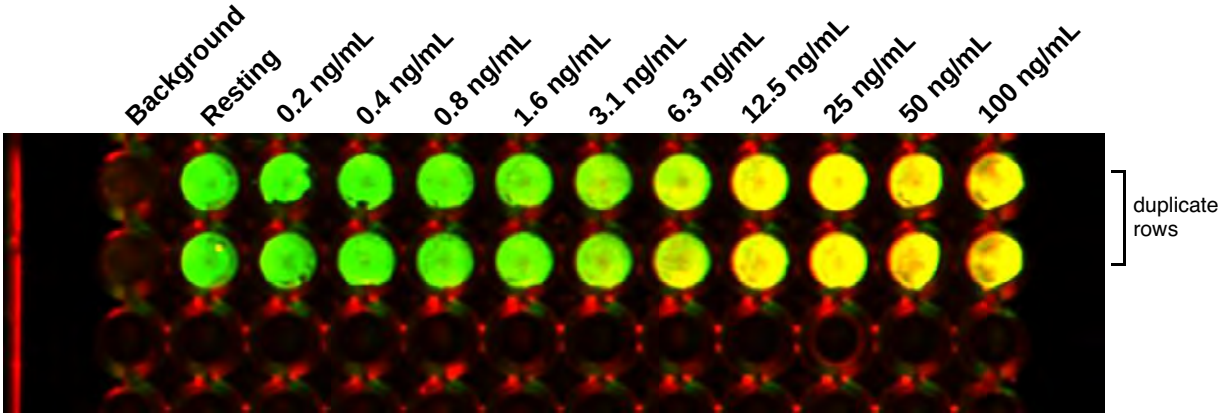
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imaging system. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

IV. Experimental Results

Color images can be seen at http://biosupport.licor.com.



Two-color display of both 700 and 800 nm channels

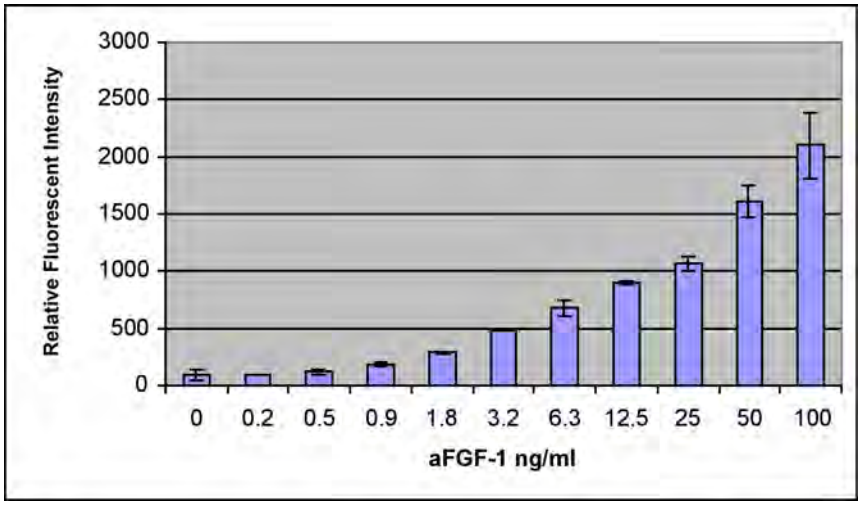


Figure 1. Dose response of NIH3T3 cells to Fibroblast growth factor (aFGF-1) as measured by specific antibody detecting dual-phosphorylated ERK (Thr202/Tyr204). The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting total and phosphorylated ERK respectively. Background wells were incubated with secondary antibody, but no primary antibody. The graph represents normalized quantitative data demonstrating the percent phosphorylation of ERK.

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Technical Support: 800-645-4260
North America: 800-645-4267
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988-11457 09/10

In-Cell Western™ Assay

Complete Apoptosis Assay Example Detailing the Seeding, Induction, and Detection of the HeLa Cellular Response to Anisomycin Treatment

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 20% Tween® 20
- Anisomycin (Sigma®, P/N A9789)
- Trypsin-EDTA Solution (1X) (Sigma, P/N T-3924)
- 37% formaldehyde
- 10% Triton® X-100
- Nunc® Nalgene 96 Microwell™ Plate (VWR, P/N 167008) or Nunc Optical Bottom Plates 96-well Black-walled/clear bottom with lid (VWR, P/N 37000-558)
- Secondary antibodies:

  IRDye® 800CW goat anti-mouse (LI-COR®, P/N 926-32210)
  IRDye 800CW goat anti-rabbit (LI-COR, P/N 926-32211)
  IRDye 680 goat anti-mouse (LI-COR, P/N 926-32220)
  IRDye 680 goat anti-rabbit (LI-COR, P/N 926-32221)

  Note: IRDye 680LT goat anti-rabbit (P/N 926-68021) and IRDye 680LT goat anti-mouse (P/N 926-68020) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR, P/N 927-40000) or StartingBlock™ (PBS) blocking buffer (Pierce, P/N 37538). **Note:** When using primary antibodies discussed in this protocol, either blocking buffer will provide low background. This may not hold true for other primary antibodies.
- Primary antibodies:

  Cleaved Caspase-3 (Cell Signaling Technologies, P/N 9661)
  ß-Tubulin (D-10), [Santa Cruz, P/N SC-5274] or Anti-ß-Tubulin, clone AA2, [Upstate Group Inc., P/N 05-661]

II. Apoptosis Assay Example

1.	Allow HeLa (ATCC; CCL-2) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and displace cells with 5 mL Trypsin-EDTA (Sigma®).
3.	Neutralize displaced cells with culture media and clarify by centrifugation.
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Reconstitute cells in complete media such that 50,000 cells/mL is achieved.
6.	Manually mix the cell suspension thoroughly.
7.	Under sterile conditions, dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ Plate (10,000 cells plated per well).
8.	Incubate cells and monitor cell density until ~ 80% confluency is achieved.
9.	Warm serum-free media (DMEM; Gibco) to 37°C.
10.	Add either serum-free media for resting cells (mock) or serum-free media containing dilution series (1:2) of Anisomycin ranging in concentration from 0.07-40 µM. Add 100 µL of resting or activation media per well.
11.	Transfer media from the dilution plate into the experimental plate.
12.	Allow incubation at 37°C for 4 hours.
13.	When incubation period is complete, remove activation media manually or by aspiration. Immediately fix cells with *Fixing Solution* (4% formaldehyde in 1X PBS) for 20 minutes at room temperature (RT). a. Prepare fresh *Fixing Solution* as follows: 1X PBS — 45 mL 37% Formaldehyde — 5 mL 3.7% Formaldehyde — 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (RT). **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on bench top for 20 minutes at RT with no shaking.

14. To permeabilize cells, wash five times with 1X PBS containing 0.1% Triton® X-100 for 5 minutes per wash.

a. Prepare *Triton Washing Solution* as follows:

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

b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde).

c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.**

d. Allow plate to shake on a plate shaker for 5 minutes at RT.

e. 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 manual disposal.**

15. Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer or Pierce StartingBlock blocking buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.**

Notes:

- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution.
- Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).
- Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.

16. Allow blocking for 1.5 hours at RT with moderate shaking on a plate shaker.

17.	Dilute the two primary antibodies in Odyssey® or StartingBlock™ blocking buffer. Combine the following antibodies for Cleaved Caspase-3 target analysis: • Cleaved Caspase-3; rabbit (1:100 dilution; Cell Signaling Technology, P/N 9661) • **Normalizing antibody:** Anti-ß-Tubulin, clone AA2; mouse (1:100 dilution; Upstate, P/N 05-661) or ß-Tubulin (D-10); mouse (1:100 dilution; Santa Cruz Biotechnology, P/N SC-5274) a. Mix the primary antibody solution thouroughly before adding it to the wells. b. Remove blocking buffer and add 50 µL of the desired primary antibody or antibodies in Odyssey or StartingBlock blocking buffer to cover the bottom of each well. c. **Make sure to include control wells without primary antibody to serve as a source for background well intensity.** To control wells, add 50 µL of Odyssey or StartingBlock blocking buffer only.
18.	Incubate with primary antibody for 2 hours with gentle shaking at RT. **Note:** • For greatest sensitivity, continue incubation overnight at 4°C with no shaking.
19.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor add 200 µL of *Tween Washing Solution* (RT). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow plate to shake on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.
20.	Dilute the fluorescently-labeled secondary antibody in Odyssey or StartingBlock blocking buffer and add 0.5% Tween 20 to the diluted antibody to lower background as specified below. IRDye® 680 goat anti-rabbit (1:200 dilution; LI-COR®, P/N 926-32221) IRDye 800CW goat anti-mouse (1:800 dilution; LI-COR, P/N 926-32210) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
21.	Mix the antibody solutions thoroughly and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

22.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 19). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** b. Allow plate to shake on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times, removing wash manually each time. ⚠ **Protect plate from light during washing.**
23.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for several weeks (protected from light).
24.	Before plate scanning, clean the bottom plate surface and the Odyssey® Imager scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
25.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey System. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics, including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

- All Odyssey Imagers require microplates that have a maximum of 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). The recommended focus offset is 3.0 mm for the microplates specified above for this assay.

- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey® Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. (The actual minimum and maximum focus offset will vary with each instrument.) Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imager. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

IV. Experimental Results

Color images can be seen at http://biosupport.licor.com.

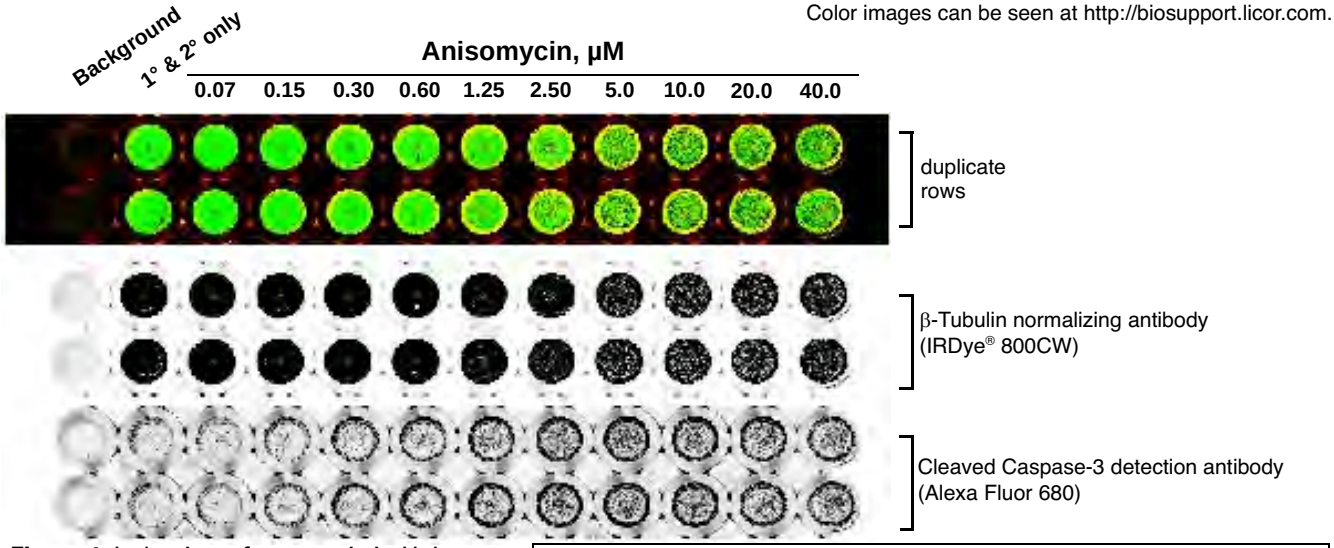
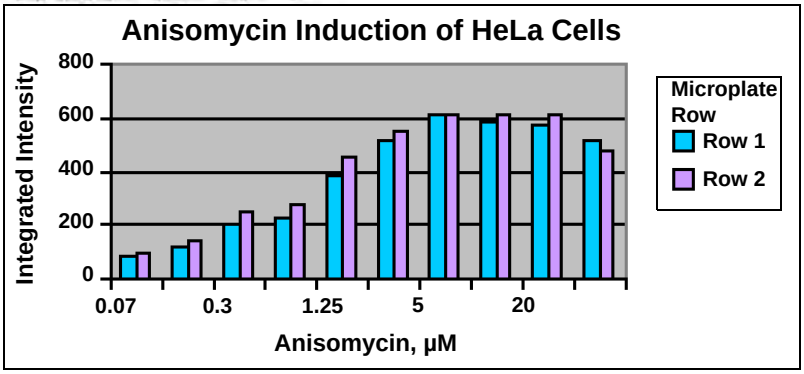


Figure 1. Induction of apoptosis in HeLa cells was achieved with increasing concentrations of anisomycin. An increase in Cleaved Caspase-3, a cleaved by-product indicative of apoptosis, is illustrated in the graph. The ultimate result of apoptosis induction is cell death. This can clearly be seen at high concentrations of anisomycin (5-40 µM) in this example. The reduction in cell number per well is taken into account when normalizing with another antibody or a DNA stain. In an assay such as this, normalization is very important.



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Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
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988-11458 09/10

In-Cell Western™ Assay

Phospho-p53 Detection in COS Cells in Response to Hydroxyurea

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

- IRDye® 800CW and IRDye 680 secondary antibodies (LI-COR® Biosciences)

  Note: IRDye 680LT secondary antibodies are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR, P/N 927-40000)
- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- Clear or black 96-well microplate (see *III. Experimental Considerations*)
- Hydroxyurea (Sigma®, P/N H8672)
- Anti-phospho-p53 (Cell Signaling Technology, P/N 9286)
- Normalization antibody: Anti-total ERK1 (Santa Cruz Biotechnology, P/N SC-94)
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100

II. Assay Example

1.	Allow Cos-7 (ATCC; CRL-1651) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. **Do not pipet or vortex during pellet disruption to maintain cell integrity.**
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.

6.	Reconstitute cells and dilute in 40 mL of complete media such that 100,000 cells/mL is achieved (2 plates x 96 wells x 200 µL/well = ~ 40 mL).
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions, dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ Plate (20,000 cells plated per well).
9.	Incubate cells and monitor cell density until 80 - 90% confluency is achieved (approximately 72 hours).
10.	Warm serum-free media (DMEM; Gibco®) to 37°C.
11.	Remove complete media from plate wells by aspiration or manual displacement.
12.	Add either serum-free media for resting cells (mock) or serum-free media with serial concentrations of Hydroxyurea ranging 0.04 - 20 mM for activated cells. Add 100 µL of resting/activation media per well.
13.	Allow incubation at 37°C overnight (16-24 hours).
14.	Remove activation or stimulation media manually or by aspiration. Immediately fix cells with *Fixing Solution* (4% formaldehyde in 1X PBS) for 20 minutes at room temperature. a. Prepare fresh *Fixing Solution* as follows: 1X PBS 45 mL 37% Formaldehyde 5 mL 3.7% Formaldehyde 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom**. c. Allow incubation on bench top for 20 minutes at RT with no shaking.
15.	To permeabilize, wash five times with 1X PBS containing 0.1% Triton® X-100 for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.** d. Allow plate to shake on a rotator for 5 minutes at RT. e. 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 manual disposal.**

16. Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.**

Notes:

- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution.
- Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).
- Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. *BSA-containing blockers are not generally recommended* and should be used only when the primary antibody requires BSA as blocker.

17. Allow blocking for 1.5 hours at RT with moderate shaking on a plate shaker.

18. Dilute the two primary antibodies in Odyssey Blocking Buffer. Combine the following antibodies for phospho-p53 target analysis, using total ERK1 for normalization:

- Phospho-p53; mouse (1:400 dilution; Cell Signaling Technology, P/N 9286)
- Total ERK1; rabbit (1:100 dilution; Santa Cruz Biotechnology, P/N SC-94)

a. Mix the primary antibody solution thoroughly before addition to wells.

b. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well.

c. **Make sure to include control wells without primary antibody to serve as a source for background well intensity.** Add 50 µL of Odyssey Blocking Buffer only to control wells.

19. Incubate with primary antibody overnight with gentle shaking at 4°C.

20. Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

a. Prepare *Tween Washing Solution* as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS with 0.1% Tween 20	1000 mL

b. Using a multi-channel pipettor add 200 µL of *Tween Washing Solution* (RT). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.**

c. Allow wash to shake on a rotator for 5 minutes at RT.

d. Repeat washing steps 4 more times.

21.	Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer as specified below. To lower background, add Tween® 20 at a final concentration of 0.2% to the diluted antibody. IRDye® 800CW goat anti-rabbit (1:800 dilution; LI-COR®, P/N 926-32211) IRDye 680 goat anti-mouse (1:200 dilution; LI-COR, P/N 926-32220) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
22.	Mix the antibody solutions well and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.
23.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 20). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** b. Allow wash to shake on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times. ⚠ **Protect plate from light during washing.**
24.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for several weeks (protected from light).
25.	Before plate scanning, clean the bottom plate surface and the Odyssey Imager scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
26.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey Imaging system. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels (optimization may be required.)

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

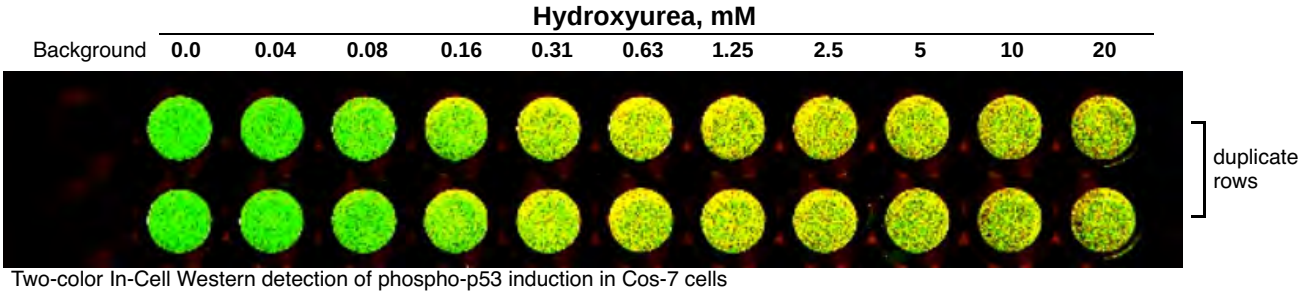
- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well, clear	Nunc® (P/N 167008, 161093)
96-well, clear	Falcon™ (P/N 353075, 353948)
96-well, black with clear bottom	Nunc (P/N 165305)
384-well, clear	Nunc (P/N 164688, 164730)
384-well, clear	Falcon (P/N 353229, 353289)
384-well, black with clear bottom	Nunc (P/N 142761)

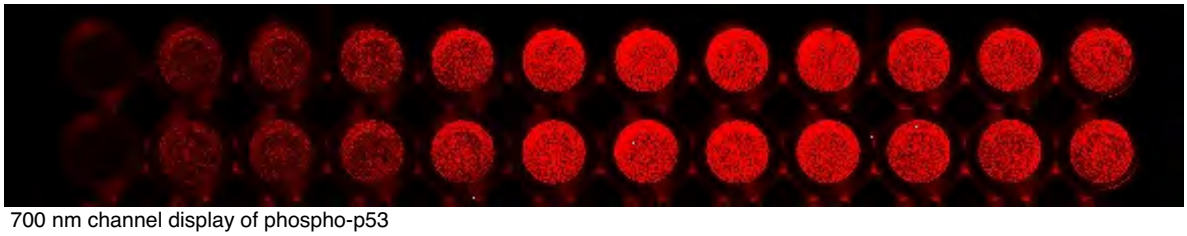
- All Odyssey® Imaging systems require that microplates have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments. (The actual minimum and maximum focus offset will vary with each instrument.)
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager and 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality, with 169 µm resolution for the Odyssey Infrared Imaging system or 200 µm resolution for the Odyssey Sa instrument provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.

IV. Experimental Results

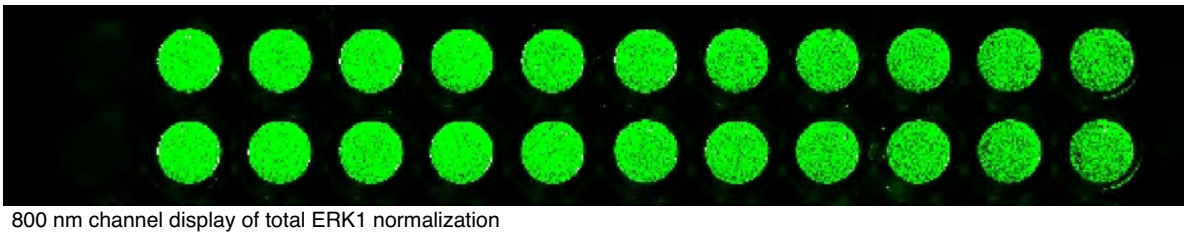
Color images can be seen at http://biosupport.licor.com/support.



Two-color In-Cell Western detection of phospho-p53 induction in Cos-7 cells



700 nm channel display of phospho-p53



800 nm channel display of total ERK1 normalization

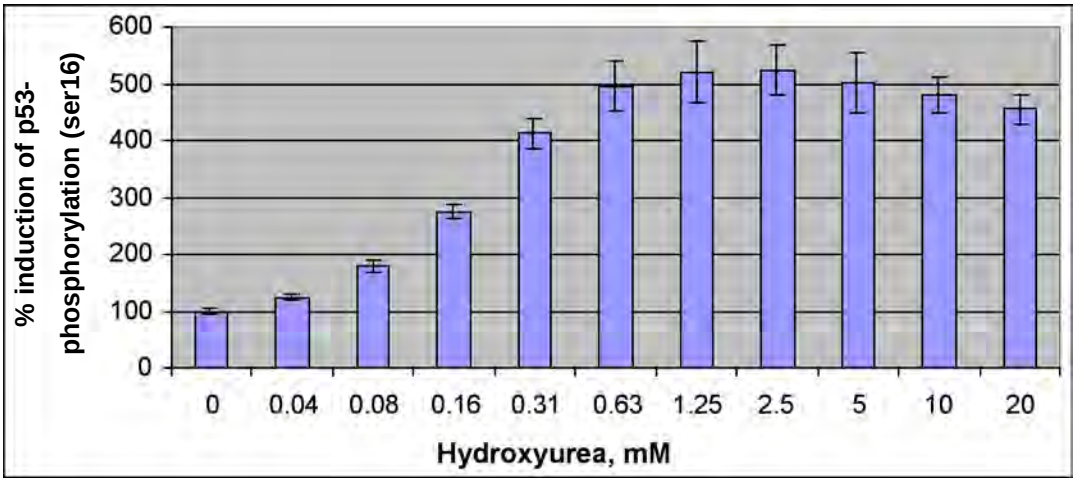


Figure 1. Dose response of Cos-7 cells to Hydroxyurea as measured by specific antibody detecting phosphorylated-p53 (Ser16) using total ERK1 for normalization. The image represents a 96-well two-color In-Cell Western with the 700 and 800 nm channels detecting phosphorylated-p53 (Ser16) and total ERK1, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents the average of four sets of quantitative data, demonstrating the percent induction of phosphorylated-p53 (Ser16).

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
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988-11459 09/10

In-Cell Western™ Assay

Phospho-p38 Detection in HeLa Cells in Response to Anisomycin

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com

Contents

I. Required Reagents

- IRDye® 800CW and IRDye 680 secondary antibodies (LI-COR® Biosciences)

  Note: IRDye 680LT secondary antibodies are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR Biosciences, P/N 927-40000)
- 1X PBS wash buffer (LI-COR Biosciences, P/N 928-40018 or 928-40020)
- Standard tissue culture reagents (serum, DMEM media, Trypsin-EDTA, etc.)
- HeLa cells (ATCC, P/N CCL-2)
- Nunc® 96 Microwell™ Plate (Nunc, P/N 167008)
- Anisomycin (Sigma-Aldrich, P/N A9789)
- Anti-phospho-p38 antibody (Cell Signaling Technology, P/N 9211)
- Normalization antibody (e.g., anti-total ERK2 (Santa Cruz Biotechnology, P/N SC-1647)
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100

II. Assay Example

1.	Allow HeLa cell growth in a T75 flask, using standard tissue culture procedures, until cells reach near confluency (1.5×10^7 cells).
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.
3.	Neutralize displaced cells with culture media and clarify by centrifugation.
4.	Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube. Avoid use of pipet or vortex during pellet disruption to maintain cell integrity.
5.	Resuspend cells in 20 mL of complete media and count cells using a hemacytometer.
6.	Dilute cells with complete media such that 75,000 cells/mL is achieved.

7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions, dispense 200 µL of the cell suspension per well in a Nunc® 96 Microwell™ Plate (15,000 cells plated per well).
9.	Incubate cells at 37°C with 5% CO_2 in air atmosphere; monitor cell density until confluency is achieved with well-to-well consistency (two to three days).
10.	Warm serum-free media (DMEM; Gibco®) to 37°C. In a fresh 96-well microplate, prepare two-fold serial dilutions of anisomycin, ranging from 2 to 1000 nM (0.5 to 265 ng/mL). Leave the first and second wells without anisomycin (resting cells as control), as shown in section *IV. Experimental Results*.
11.	Remove complete media from plate wells by aspiration or manual displacement.
12.	Transfer media and anisomycin dilutions from the dilution plate into the experimental plate.
13.	Incubate plate at 37°C for 30 minutes.
14.	Remove stimulation media manually or by aspiration. Immediately fix cells with 3.7% formaldehyde for 20 minutes at room temperature. a. Prepare fresh *Fixing Solution* as follows: 1X PBS 45 mL 37% Formaldehyde 5 mL 3.7% Formaldehyde 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature solution, RT). **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on bench top for 20 minutes at RT with no shaking.
15.	Wash five times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.** d. Allow wash to shake on a plate shaker for 5 minutes at RT. e. Repeat washing steps 4 more times after removing wash manually. ⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**

16. Using a multi-channel pipettor, block cells/wells by adding 150 µL of LI-COR® Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.**

    Notes:
    - No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution.
    - Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).
    - Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. BSA-containing blockers are not generally recommended and should be used only when the primary antibody requires BSA as blocker.

17. Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.

18. Add the two primary antibodies to a tube containing Odyssey Blocking Buffer. Combine the following solutions as defined below for phospho-p38 target analysis, using total ERK2 for normalization:
    - Phospho-p38 (rabbit; 1:100 dilution; Cell Signaling Technology, P/N 9211)
    - Total ERK2 (mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N sc-1647)

    a. Mix the primary antibody solution thoroughly before adding it to the wells.

    b. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well.

    c. Make sure to include control wells without primary antibody to serve as a source for background well intensity. Add 50 µL of Odyssey Blocking Buffer only to control wells.

19. Incubate with primary antibody for 2 hours with gentle shaking at RT.

    Note:
    - For greatest sensitivity, continue incubation overnight at 4°C with no shaking.

20.	Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Using a multi-channel pipettor add 200 µL of *Tween Washing Solution* (RT). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow wash to shake on a plate shaker for 5 minutes at RT. d. Repeat washing steps 4 more times.
21.	Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer as specified below. To lower background, add Tween 20 to the diluted antibody for a final concentration of 0.2%. IRDye® 800CW goat anti-rabbit (1:800 dilution; LI-COR® , P/N 926-32211) IRDye 680 goat anti-mouse (1:200 dilution; LI-COR, P/N 926-32220) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.**
22.	Mix the antibody solutions well and add 50 µL of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.
23.	Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (see step 20). **Make sure to carefully add solution down the sides of the wells to avoid detaching the cells from the well bottom.** b. Allow wash to shake on a plate shaker for 5 minutes at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ **Protect plate from light during washing.**
24.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
25.	Before plate scanning, clean the bottom plate surface and the Odyssey scanning bed (if applicable) with moist lint-free paper to avoid any obstructions during scanning.
26.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey Imaging system. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels (optimization may be required.)

III. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR® Biosciences:

96-well, clear	Nunc® (P/N 167008, 161093)
96-well, clear	Falcon™ (P/N 353075, 353948)
96-well, black with clear bottom	Nunc (P/N 165305)
384-well, clear	Nunc (P/N 164688, 164730)
384-well, clear	Falcon (P/N 353229, 353289)
384-well, black with clear bottom	Nunc (P/N 142761)

- All Odyssey® Imaging systems require that microplates have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa Software and then clicking Scanner Information). When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for the experiments. (The actual minimum and maximum focus offset will vary with each instrument.)
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager and 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality, with 169 µm resolution for the Odyssey Infrared Imaging system or 200 µm resolution for the Odyssey Sa instrument provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.

IV. Experimental Results

Color images can be seen at http://biosupport.licor.com.

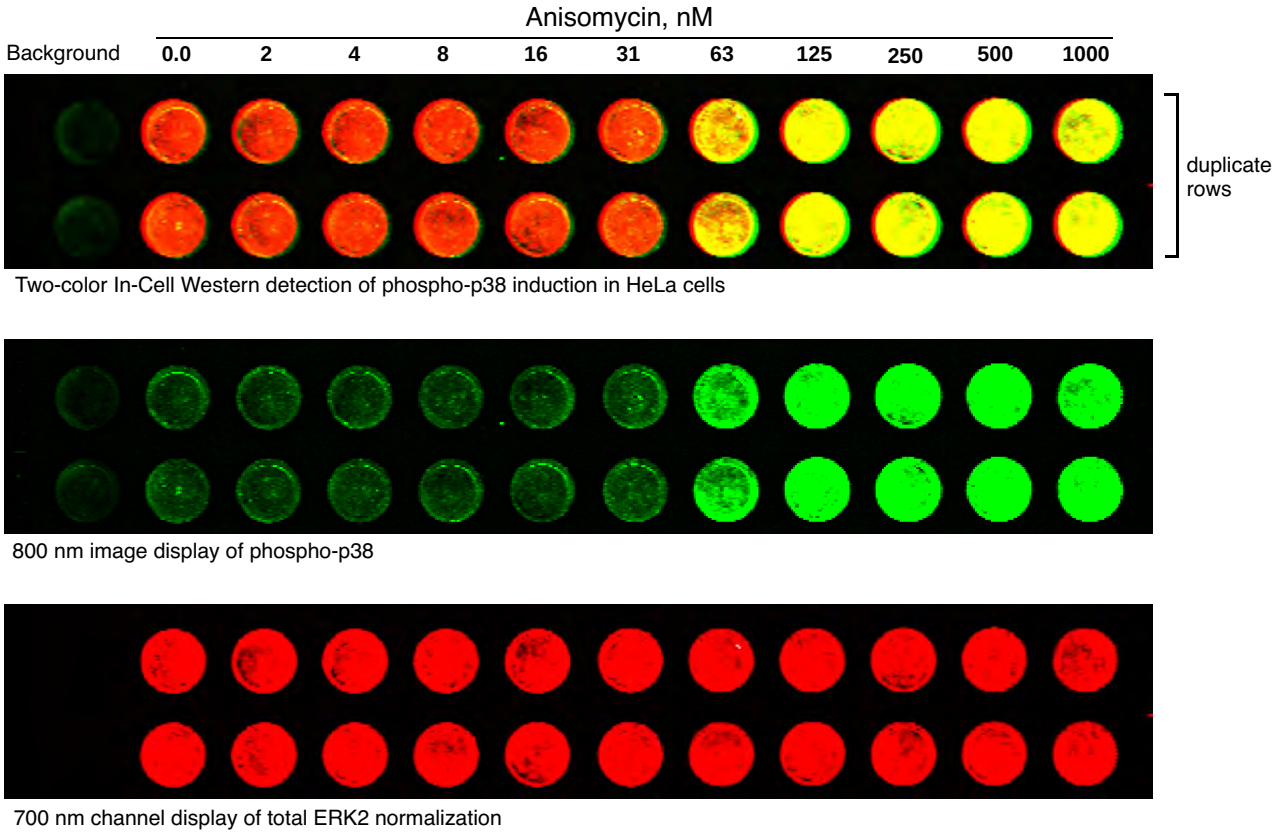
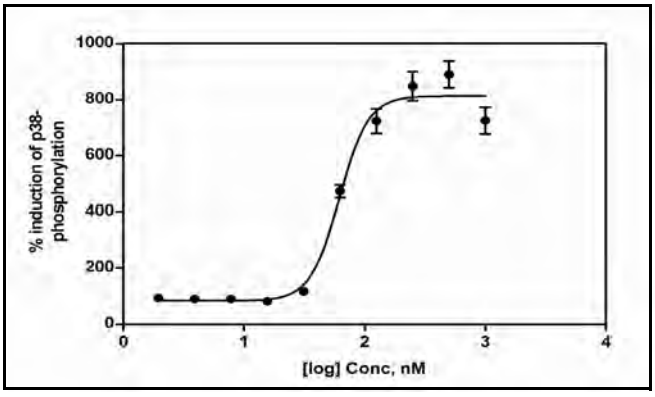


Figure 1. Dose response of HeLa cells to Anisomycin as measured by specific antibody detection of phosphorylated-p38 (Thr180/Tyr182), using total ERK2 for normalization. The image represents a 96-well two-color In-Cell Western assay with the 800 and 700 channels detecting phosphorylated-p38 (Thr180/Tyr182) and total ERK2, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents the average of eight sets of quantitative data, demonstrating the percent induction of phosphorylated-p38 (Thr180/Tyr182).



4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
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988-11467 09/10

In-Cell Western™ Assay

Complete Sample Protocol for PMA-induced ERK Activation in Suspension Cell Lines

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

Contents

I. Required Reagents

LI-COR® Reagents

- IRDye® 800CW goat anti-rabbit secondary antibody: (LI-COR, P/N 926-32211)
- IRDye 680 goat anti-mouse secondary antibody: (LI-COR, P/N 926-32220)

  Note: IRDye 680LT goat anti-mouse secondary antibodies (LI-COR, P/N 926-68020) are also available. This protocol may require optimization if IRDye 680LT secondary antibodies are used.
- Odyssey® Blocking Buffer (LI-COR, P/N 927-40000)
- Large Western Incubation Box: (LI-COR, P/N 929-97803)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (RPMI 1640, fetal bovine serum, etc.)
- Round-bottom 96-well plates: (BD Bioscience, P/N 353077)
- Round-bottom 96-well plates: (Nunc®, P/N 167008)
- Jurkat cells: (ATCC®, P/N TIB-152™)
- THP-1 monocytes: (ATCC, P/N TIB-202™)
- K-562 lymphocytes: (ATCC, P/N CCL-243™)
- Concentrated Prefer (5X): (Anatech LTD, P/N 411)
- TO-PRO®-3: (Molecular Probes, P/N T-3605)
- PMA (phorbol 12-myristate 13-acetate): (Sigma®, P/N P1585)
- DMSO (dimethyl sulfoxide): (Sigma, P/N D8418)
- ERK rabbit antibody: (Santa Cruz, P/N SC-94)
- pERK mouse antibody: (Cell Signaling Technology, P/N 9106)

II. Experiment Design

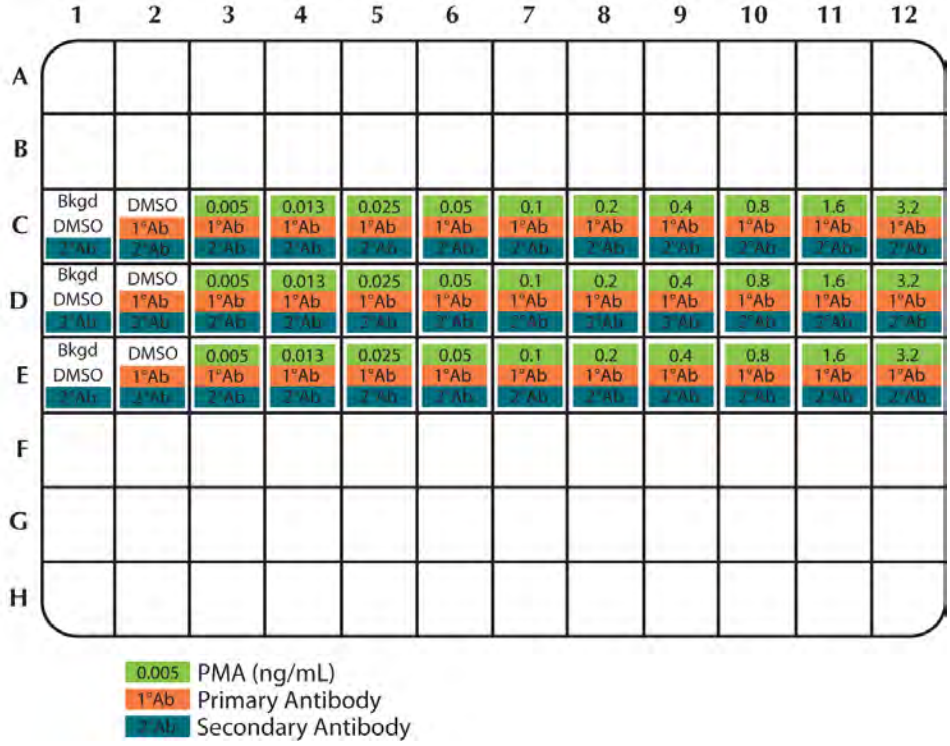


Figure 1. Experimental design for Figure 2. Round bottom 96-well plate: ~200,000 Jurkat cells per well (C1-E12) in triplicate samples

III. Assay Example

1.	Allow Jurkat (ATCC®, P/N TIB-152™) cells to grow in a T75 flask using standard tissue culture procedures. Avoid growing cells to density greater than 2 x 10^6 cells.
2.	Transfer cells in growth media to 50 mL conical tubes and centrifuge at 500 x g for 5 minutes.
3.	Remove media and resuspend cell pellet in 10 mL of serum-free media (pre-warmed to 37°C). **Pipet very slowly in order to maintain cell integrity while disrupting the cell pellet.** Transfer resuspended cells into T75 flask and place in an incubator (37°C and 5% CO_2). ⚠ **Important:** It is the serum withdrawal from the complete media that allows suspension cells to attach to plates (i.e., T75 flask). Gravity will cause cells to form a monolayer over time (10 to 15 minutes). Once a monolayer is formed, the rest of the cells in the serum-free media will remain in suspension and will not further attach to the plates. Only the cells in suspension in the T75 flask will be used in the following steps.

4.	Allow cells to settle down for 30 minutes before taking a 50 µL aliquot of cells for cell counting using a hemacytometer.
5.	Add appropriate volume of serum-free media such that 1 x 10^6 cells/mL is achieved (1 plate x 96 wells x 200 µL of cells/well = ~20 mL/plate).
6.	Serum deprive cells by replacing cells suspended in serum-free media back in the incubator for an additional 3.5 hours or overnight.
7.	Add 2 µL of DMSO for both the background samples (serves as non-specific background fluorescence) and resting cells (serves as basal control) in triplicate wells. Add 2 µL of 1:1 serial dilutions of PMA ranging from 0.005 to 3.2 ng/mL in triplicate wells. ⚠ **Important note about choosing round bottom plates for suspension cell assays:** If imaging with the Odyssey® Sa Imager, LI-COR® Biosciences recommends clear, round bottom 96-well plates from BD Bioscience (P/N 353077) or from Nunc® (P/N 167008). If imaging with the Odyssey Infrared Imager, use the plates from BD Bioscience (P/N 353077). Imaging Nunc round bottom plates with the Odyssey Infrared Imager will yield quantitatively accurate results, but the images will not be as visually satisfying and over all signal intensity will be lowered. This is due to differences in the optical properties of the round bottom wells.
8.	Using a multi-channel pipettor, transfer 200 µL of suspended cells (~200,000 cells) per well into the wells containing 2 µL of DMSO or PMA from step 7.
9.	Allow incubation at 37°C for 15 minutes. ⚠ **Important:** Be careful not to disrupt the cells during this PMA-induced activation step. During this critical step, cells will sediment to the bottom of the wells by gravity forming a monolayer. This monolayer can be easily viewed under a light source. The monolayer will appear opaque rather than transparent. Clumping of cells will lead to detachment from plates during incubation and washing steps. Be careful of handling the plate at this stage because the cells will be very loosely attached to the bottom of the wells.
10.	Directly add to the cell suspension, 50 µL of concentrated (5X) Prefer (or 25 µL of 37% formaldehyde; final concentration is 4%) into each well. ⚠ **Important:** Gently add Prefer into wells using side of the wells to avoid detaching the cells from the well bottom. During fixation, the cell monolayer will attach more firmly to the wells. However, the strength of the attachment is never as strong as adherent cells grown on plates. Thus, a degree of caution is needed during every step of this procedure.
11.	Allow cells to fix for 20 minutes at room temperature with very gentle rotation (set at speed 2 on The Belly Dancer®; Stovall).
12.	Centrifuge at 1500 rpm (332 rcf) for 10 minutes.

13.	To permeabilize cells, wash three times with 100 µL of 1X PBS containing 0.1% Triton® X-100 for 5 minutes each by centrifugation at 1500 rpm (332 rcf). a. Prepare *Triton Permeabilization Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove fixing solution (if using formaldehyde, collect in an appropriate waste container). c. Using a multi-channel pipettor, add 100 µL of fresh *Triton Permeabilization Solution*. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.** d. Centrifuge at 1500 rpm (332 rcf) for 5 minutes. e. Gently remove *Triton Permeabilization Solution* by manually pipetting. f. Repeat washing step two more times. ⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.** **Note:** If detecting cell surface proteins with exofacial antigens, you do not need to permeabilize cells. After fixing cells in step 11, wash cells (see step 18) before proceeding to blocking (step 14).
14.	Using a multi-channel pipettor, block cells/wells by adding 100 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.**
15.	Allow blocking for 1 hour at room temperature with very gentle shaking on a plate shaker.
16.	Dilute the two primary antibodies in Odyssey Blocking Buffer. Combine the following antibodies for ERK target analysis: • Rabbit anti-ERK antibody (1:200 dilution; Santa Cruz) • Mouse anti-phospho-ERK antibody (1:100 dilution; Cell Signaling Technology) a. Mix the primary antibody solution thoroughly before addition to wells. b. Remove blocking buffer from the blocking step. c. Add 50 µL of Odyssey Blocking Buffer only to the background control wells (serves as non-specific background fluorescence). d. Add 50 µL of the primary antibody solution into rest of wells.
17.	Incubate with primary antibody for 2 hours at room temperature or overnight at 4°C with very gentle shaking on a plate shaker.

18.	Wash the plates five times with 200 µL of 1X PBS + 0.1% Tween® 20 for 5 minutes by centrifugation at 1500 rpm (332 rcf). a. Prepare *Tween Washing Solution* as follows: 1X PBS 995 mL 20% Tween 20 5 mL 1X PBS with 0.1% Tween 20 1000 mL b. Remove primary antibody solution. c. 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.** d. Centrifuge at 1500 rpm (332 rcf) for 5 minutes. e. Gently remove *Tween Washing Solution* by manually pipetting. f. Repeat washing steps 4 more times.
19.	Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer with 0.2% Tween 20 in order to lower background as specified below. IRDye® 680 goat anti-mouse (1:200 dilution; LI-COR®, P/N 926-32220) IRDye 800CW goat anti-rabbit (1:800 dilution; LI-COR, P/N 926-32211) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the secondary antibody vials to light.**
20.	Mix the antibody solutions thoroughly and add 50 µL of the secondary antibody solution to each well. Incubate for one hour with very gentle shaking on a plate shaker at room temperature. Protect plate from light during incubation. Use a large black Western Incubation Box (LI-COR, P/N 929-97803) to protect plate from light during subsequent steps.
21.	Wash the plates five times with 200 µL of 1X PBS + 0.1% Tween 20 at room temperature for 5 minutes by centrifugation at 1500 rpm (332 rcf). a. Remove secondary antibody solution. 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.** c. Centrifuge at 1500 rpm (332 rcf) for 5 minutes. d. Gently remove *Tween Washing Solution* by manually pipetting. e. Repeat washing step four more times. ⚠ **Protect plate from light during washing.**
22.	After final wash, remove wash solution completely from wells. For best results, scan plate immediately; plates may also be stored at 4°C for several weeks (protected from light).

23.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey System. For the Odyssey Infrared Imager, use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels. For the Odyssey Sa instrument, use 200 µm resolution, 3.0 mm focus offset, and an intensity setting of 7 for both 700 and 800 nm channels.

IV. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- Clear round bottom plates are recommended for In-Cell Western analysis of non-adherent cells:

  Odyssey® Infrared Imaging System

  96-well format BD Bioscience (P/N 353077)

  Note: Clear round bottom plates from Nunc® (P/N 163320) can also be used and will yield quantitatively accurate data. However, the images may be less visually satisfying when imaged on the Odyssey Infrared Imager due to differences in the optical properties of the round bottom wells. The Nunc plates work well on the Odyssey Sa instrument.

  Odyssey Sa Infrared Imaging System

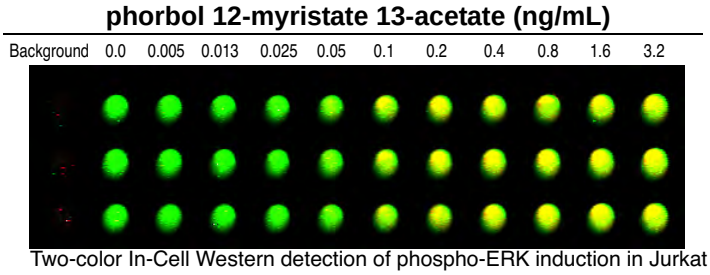
  96-well format BD Bioscience (P/N 353077)
  Nunc (P/N 167008)

- Both the flat and round bottom plates show some plate autofluorescence. However, the plate autofluorescence is relatively small compared to the actual signal. When using the recommended BD Bioscience round bottom plate, the recommended focus offset is 3.0 to 3.5 mm. The recommended focus offset for the Nunc round bottom plate is 3.5 to 3.95.
- If plates other than those recommended above are used, the focus offset can be determined by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey® Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. (The actual minimum and maximum focus offset will vary with each instrument.) Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Intensity for both 700 and 800 nm channels should be set to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.

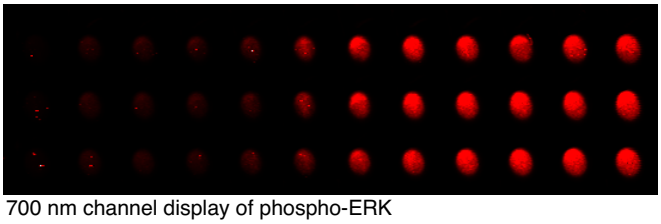
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imager. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

V. Experimental Results

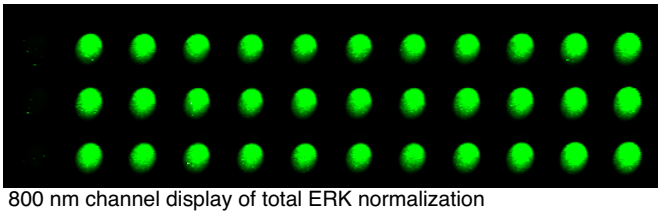
Color images can be seen at http://biosupport.licor.com.



Two-color In-Cell Western detection of phospho-ERK induction in Jurkat cells



700 nm channel display of phospho-ERK



800 nm channel display of total ERK normalization

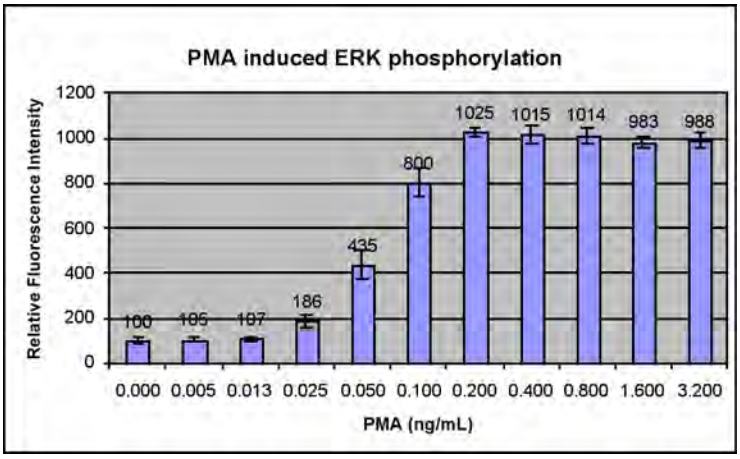
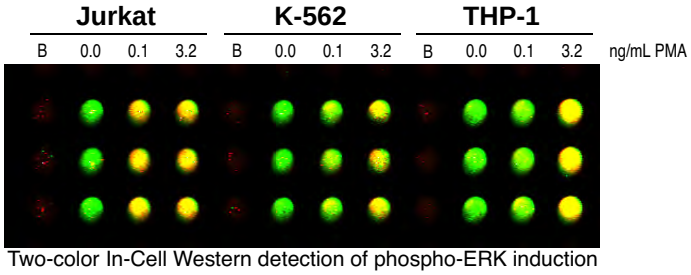
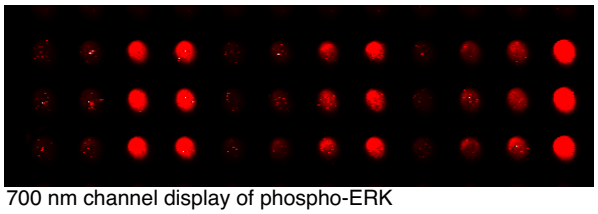


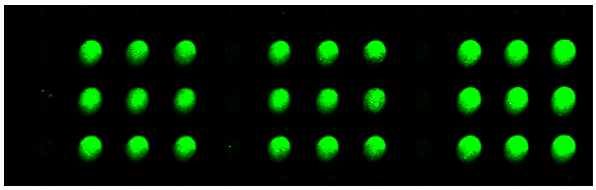
Figure 2. Dose response of Jurkat cells to phorbol 12-myristate 13-acetate (PMA) as measured by specific antibody detecting dual-phosphorylated ERK (Thr202/Tyr204). The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 nm channels detecting phosphorylated and total ERK, 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 2 (700 nm channel) and 4 (800 nm channel). Background wells were incubated with secondary antibodies but no primary antibodies. The graph represents normalized quantitative data demonstrating the increase in ERK phosphorylation in response to PMA stimulation.



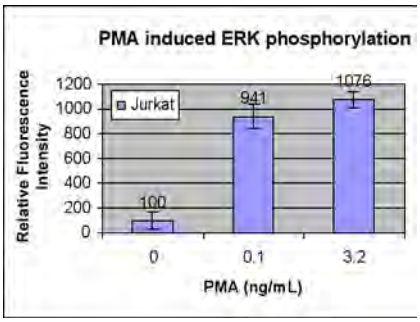
Two-color In-Cell Western detection of phospho-ERK induction

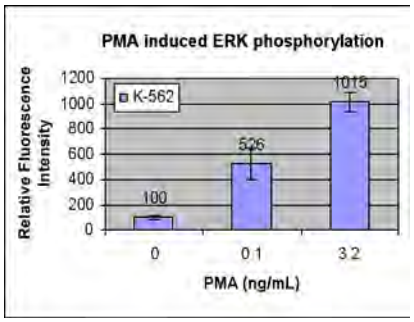


700 nm channel display of phospho-ERK



800 nm channel display of total ERK normalization





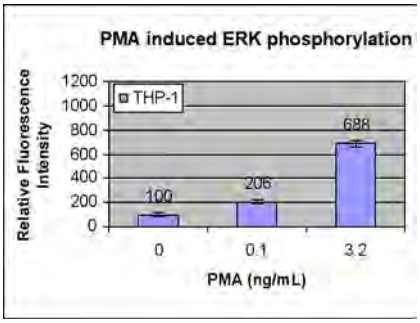


Figure 3. ERK activation in Jurkat, K-562 and THP-1 non-adherent cells in response to PMA as measured by specific antibody detecting dual-phosphorylated ERK (Thr202/Tyr204). The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 nm channels detecting phosphorylated and total ERK, 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 nm channel) and 5 (800 nm channel). Background (B) wells were incubated with secondary antibodies but no primary antibodies. The graph represents normalized quantitative data demonstrating the increase in ERK phosphorylation in response to PMA stimulation.

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
In other countries, contact LI-COR Biosciences or a local LI-COR distributor.
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988-11460 09/10

In-Cell Western™ Assay

FAQs for Suspension Cells

Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

LI-COR® Biosciences

Revised September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com.

1. Handling Suspension Cells

1.1 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.

1.2 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.

1.3 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.

1.4 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.

1.5 During my washing steps, cells are coming off the plates.

1.5.1 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 Electron Corp, P/N 4610050).

1.5.2 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.

2. Round vs. Flat Bottom 96-well Plates

2.1 Why can't I use the flat bottom 96-well plates?

LI-COR® Biosciences recommends using the round bottom 96-well plates. For an explanation, see 1.5 above.

2.2 When I scan an empty round bottom 96-well plate, I get lots of background noise.

The round bottom plate shows some background autofluorescence. 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.

3. Scan Settings

3.1 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 3.95mm. For maximum signal strength with the Odyssey® Infrared Imager, BD Bioscience round bottom plates are recommended. Both BD Bioscience and Nunc round bottom plates work well with the Odyssey Sa instrument.

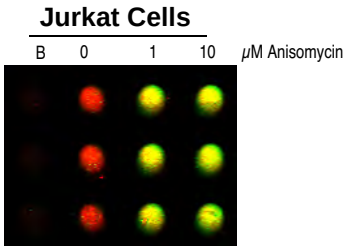
4. Other Suspension Cell Lines and Different Pathways

4.1 Have you tested other suspension cell lines?

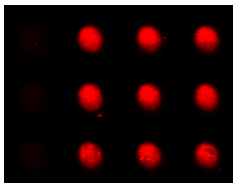
Yes. Suspension cell lines tested include Jurkat, K-562 and THP-1. A sample protocol can be downloaded from http://biosupport.licor.com.

4.2 Have you tested other pathways?

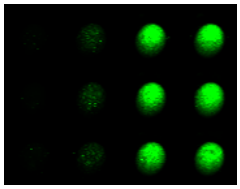
Yes. Pathways tested include ERK activation and apoptosis using cleaved caspase3 as a marker (Figure 1). A sample protocol can be downloaded from http://biosupport.licor.com.



Two-color In-Cell Western™ detection of cleaved caspase-3



700 nm channel display of TO-PRO-3 for normalization



800 nm channel display of cleaved caspase-3

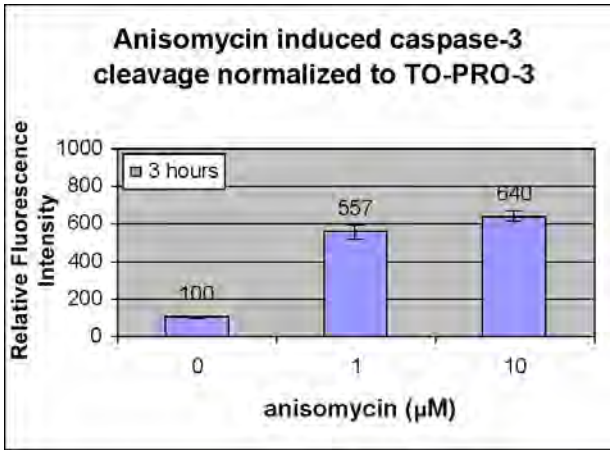


Figure 1. Anisomycin-induced apoptosis in Jurkat cells. The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 nm channels detecting TO-PRO®-3 DNA staining and cleaved caspase-3 (Asp175), respectively. The image was scanned using the Odyssey® Sa Infrared Imaging system with 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.

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4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
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988-11461 09/10

In-Cell Western™ Assay Kits I and II

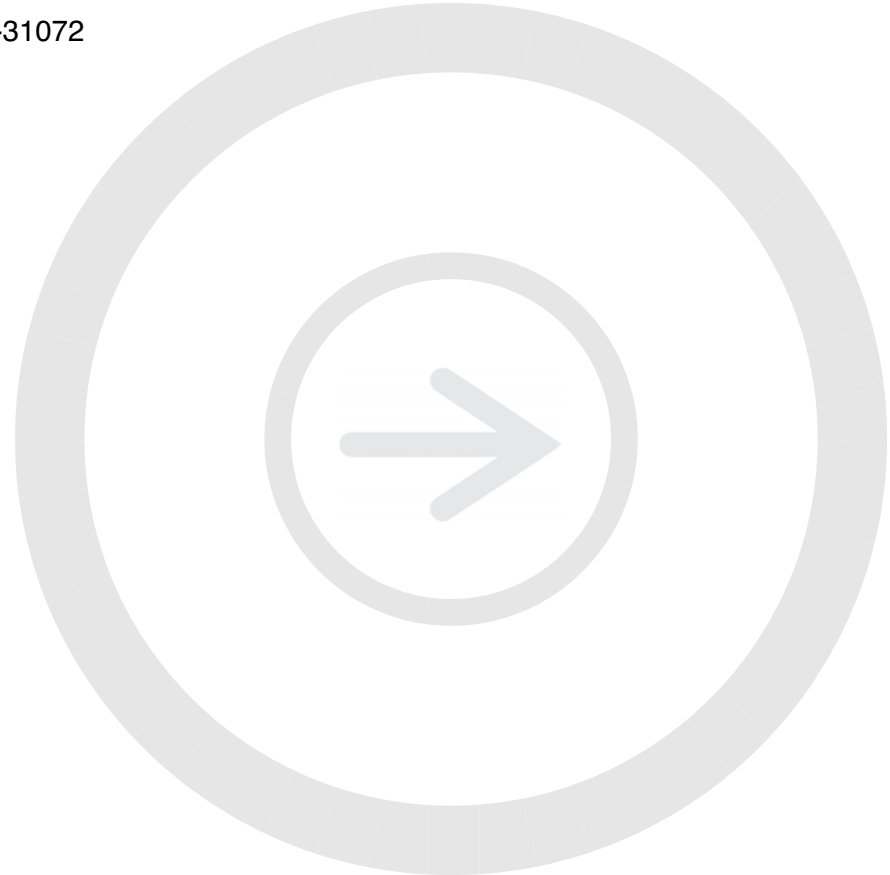
Developed for:

Odyssey® Infrared Imaging System

Odyssey Sa Infrared Imaging System

Part Numbers: 926-31070 and 926-31072

Storage: 4°C



LI-COR® Biosciences

Published November, 2006. Revised October 2010. The most recent version of this protocol is posted at: *http://biosupport.licor.com*

The In-Cell Western Kits provide detection reagents for cell-based In-Cell Western Assays. Each kit includes blocking buffer, IRDye® 800CW secondary antibody for detection of a specific protein target in the 800 nm channel, and two fluorescent cell stains that are used in combination in the 700 nm channel to normalize well-to-well variations in cell number. This approach allows the target of interest to be detected accurately and cost-effectively.

If the application detects two protein targets with two different primary antibodies, use two different secondary antibodies (one labeled with IRDye 800CW and the other with either IRDye 680LT or IRDye 680) in a multiplex assay. IRDye secondary antibodies for multiplex detection can be purchased at: **www.licor.com** Dilution factors and blocking conditions should be optimized for target and primary antibody combinations.

Using DRAQ5™ and Sapphire700™ for Cell Number Normalization

The cell stains included in this kit are designed to be used in combination to provide accurate normalization over a broad range of cell densities. DRAQ5 is a cell-permeable DNA-interactive agent that can be used for stoichiometric staining of DNA in live or fixed cells. DRAQ5 is part of this kit, but is otherwise sold separately by Biostatus Limited (visit: **http://www.biostatus.com/product/draq5/**). When serial dilutions of A431 human epithelial carcinoma cells are plated in 96-well plates, DRAQ5 demonstrates linearity of fluorescent signal for lower cell densities, up to ~50,000 cells/well (Figure 1A).

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 (Figure 1B). Simultaneous staining of cells with DRAQ5 and Sapphire700 expands the linear range, allowing more accurate normalization of cell number across both low and high cell densities (Figure 1A and B). Sapphire700 can be purchased separately from LI-COR Biosciences (P/N 928-40022).

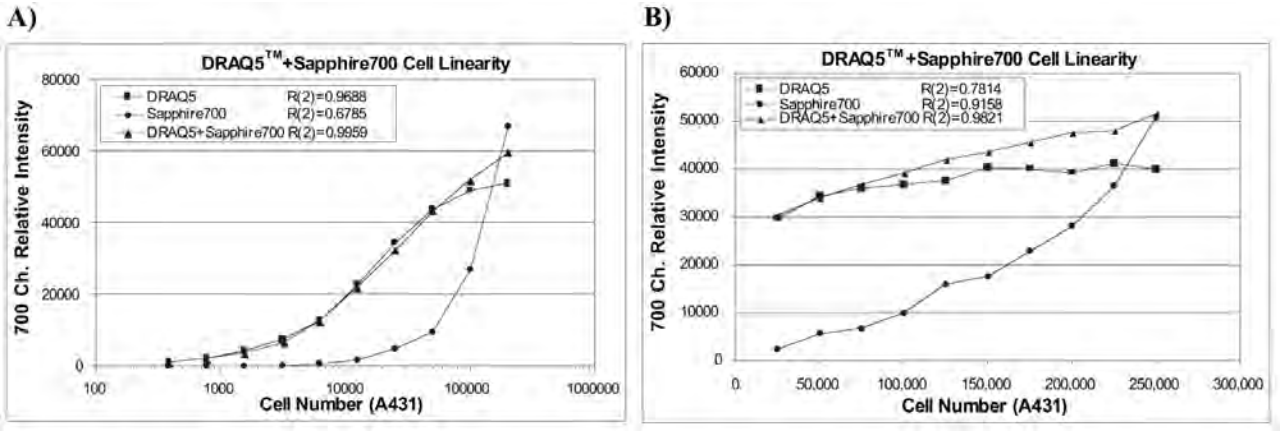


Figure 1. DRAQ5 and Sapphire700 as normalizing agents for In-Cell Western assays. Dilutions of A431 cells were plated on clear, flat bottom 96-well plates, then fixed and permeabilized. Cells were stained with DRAQ5 alone, Sapphire700 alone, or both stains combined. A) Two-fold dilutions of cells, over a wide range of cell densities (0 - 200,000 cells/well). B) Closer examination of linearity of signal over the range of 25,000 - 250,000 cells/well, in dilution increments of 25,000 cells.

In-Cell Western assays commonly use primary and secondary antibodies for normalization in the 700 nm 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. Staining with DRAQ5™ and Sapphire700™ eliminates the need for these additional primary and secondary antibodies, and yields the same quantitative measurement of ERK phosphorylation (Figure 2).

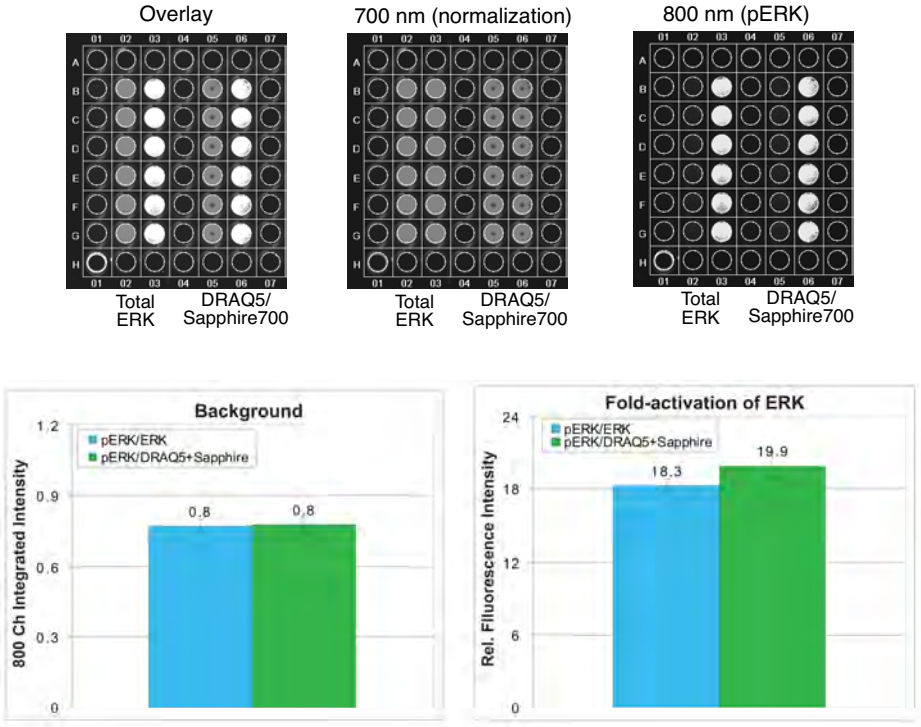


Figure 2. Comparison of normalization methods. ERK activation was induced in A431 cells by stimulation with epidermal growth factor (EGF). Phospho-ERK was detected in the 800 nm channel with anti-phospho-ERK primary antibody and IRDye 800CW secondary antibody. Normalization was performed in two ways: anti-total-ERK primary antibody and IRDye 680 secondary antibody; or DRAQ5 and Sapphire700 staining. The EGF-induced ERK activation measured by the two methods was indistinguishable.

In-Cell Western Kit: Protocol for Use

Kit Components (store kit at 4°C)

- IRDye® 800CW secondary antibody, 0.5 mg (lyophilized)
- Odyssey® Blocking Buffer, 4 x 500 mL (LI-COR, P/N 927-40000)
- DRAQ5, 100 µL
- Sapphire700, 100 µL (LI-COR, P/N 928-40022)

Additional Reagents (required but not included)

- Primary antibody
- 1X PBS wash buffer (LI-COR, P/N 928-40018, 10X PBS)
- Tissue culture reagents (serum DMEM, trypsin, etc.)

- Clear or black 96-well microplate (see *V. Experimental Considerations*)
- 37% formaldehyde
- 20% Tween® 20
- 10% Triton® X-100

I. Reconstitution of Antibody

1.	Protect from light. Store IRDye® 800CW secondary antibody at 4°C prior to reconstitution.
2.	Reconstitute contents of antibody vial with 0.5 mL sterile distilled water. Mix gently by inverting, and allow to rehydrate for at least 30 minutes before use. Centrifuge product if solution is not completely clear after standing at room temperature.
3.	Dilute only immediately prior to use. Reconstituted antibody is stable for 3 months at 4°C when stored undiluted as directed. For extended storage, aliquot and freeze at -20°C or below; avoid repeated freeze-thaw cycles.

II. Cell Preparation and Fixation

1.	Treat cells as desired with drug, stimulant, etc. Detailed In-Cell Western protocols for certain cell lines and target proteins may be downloaded at: **http://biosupport.licor.com**
2.	Remove media manually or by aspiration. Immediately fix cells with *Fixing Solution* (3.7% formaldehyde in 1X PBS) for 20 minutes at room temperature (RT). a. Prepare fresh *Fixing Solution* as follows: 1X PBS 45 mL 37% Formaldehyde 5 mL 3.7% Formaldehyde 50 mL b. Using a multi-channel pipettor, add 150 µL of fresh, room temperature *Fixing Solution* to each well. **Add carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.** c. Allow incubation on the bench top for 20 minutes at RT with no shaking. **Note:** • If optimal fixation 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.

3.	To permeabilize, wash five times with 1X PBS containing 0.1% Triton® X-100 for 5 minutes per wash. a. Prepare *Triton Washing Solution* as follows: 1X PBS 495 mL 10% Triton X-100 5 mL 1X PBS + 0.1% Triton X-100 500 mL b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde). c. Using a multi-channel pipettor, add 200 µL of room temperature *Triton Washing Solution* to each well. **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.** d. Allow wash to shake on a plate shaker for 5 minutes. e. 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. **Note:** • If an alternative permeabilization method (for example, ice-cold methanol) is known to work well for immunofluorescent staining of your protein target, you may prefer to use that permeabilization method rather than the Triton method described here.

III. Cell Staining

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.** **Notes:** • No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If you have used the primary antibody successfully for immunofluorescent staining, consider trying the same blocking buffer for In-Cell Western detection. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS, or commercial blocking buffers, can also be used for blocking and antibody dilution. When using anti-goat antibodies, milk-based reagents may be contaminated with endogenous IgG, biotin, or phospho-epitopes that can interfere with detection.
2.	Allow blocking for 1.5 hours at room temperature with moderate shaking on a plate shaker.

3. Dilute desired primary antibody 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.

   Note:
   - If using DRAQ5™ and Sapphire700™ for normalization, only one primary antibody will be used. Alternatively, you may choose to normalize with a second primary antibody in your assay. The second primary antibody MUST be from a different host, and an appropriate IRDye® 680LT or IRDye 680 secondary antibody (not provided in the kit) will be required for detection.

   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 2.

   c. Add 50 µL of Odyssey Blocking Buffer to the control wells and 50 µL of the desired primary antibody in Odyssey Blocking Buffer to the rest of the wells.

4. Incubate with primary antibody for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.

5. Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

   a. Prepare *Tween Washing Solution* as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS with 0.1% Tween 20	1000 mL

   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 on a plate shaker for 5 minutes.

   d. Repeat washing steps 4 more times.

6. Dilute the fluorescently-labeled secondary antibody in Odyssey Blocking Buffer or other appropriate blocker. The recommended dilution range is 1:200 to 1:1,200, with a suggested starting dilution of 1:800. 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. **Avoid prolonged exposure of the antibody vials to light.**

   a. Secondary antibody staining and normalization staining are carried out simultaneously. To stain for normalization, add DRAQ5 and Sapphire700 to the diluted secondary antibody solution and apply this mixture to the cells. Suggested dilutions for normalization stains:
      Sapphire700: 1:1000
      DRAQ5 from LI-COR® ICW kit (1 mM): 1:2000
      DRAQ5 from Biostatus (5 mM): 1:10,000

   b. For control wells (used to calculate background), do not add DRAQ5 and Sapphire700. Add only diluted secondary antibody to these wells.

7.	Add 50 µL of secondary antibody solution *without* DRAQ5™ and Sapphire700™ into each of the control wells and 50 µL of secondary antibody solution *with* DRAQ5 and Sapphire700 into rest of wells. Incubate for 1 hour at room temperature with gentle shaking. **Protect plate from light during incubation.**
8.	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 on a plate shaker for 5 minutes. c. Repeat washing steps 4 more times. **Protect plate from light during washing.**

IV. Imaging

1.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
2.	Before plate scanning, clean the bottom plate surface and the scanning bed (if applicable) with moist lint-free paper.
3.	Scan the plate with detection in both 700 and 800 nm channels using an Odyssey® Imaging System (700 nm detection for normalization stains, and 800 nm detection for IRDye® 800CW antibody). Generally, a scan resolution of 169 µm (Odyssey Imager) or 200 µm (Odyssey Sa Imager) is appropriate. An initial intensity setting of 5 (Odyssey Imager) or 7 (Odyssey Sa Imager) is suggested. Focus offset position is critical to proper plate imaging; for more information about choosing the correct focus offset, see Section V.

V. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics including well depth, plate autofluorescence, and well-to-well signal crossover. Use the general considerations for microplate selection provided below.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. **Do not use plates with white walls, since the autofluorescence from the white surface will create significant noise.**
- All Odyssey Imaging systems require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa instrument and is found by choosing Settings > System Administration in the Odyssey Sa software and then clicking Scanner Information). Optimal signal will be achieved when the focus offset position is set as accurately as possible, and the best offset may need to be determined empirically (see below).

- In-Cell Western assays require sterile plates for tissue culture growth. The following plates and focus offset settings are recommended.

Well Number	Well Bottom	Manufacturer	Part Number	Offset - Odyssey® Imager	Offset - Odyssey Sa Imager
96	Flat	Nunc®	161093, 165305	3.0 mm	3.0 mm
96	Flat	BD Falcon™	353075, 353948	3.0 mm	3.0 mm
96	Round*	BD Falcon	353077	3.0 mm	*not recommended*
96	Round*	Nunc	163320	*not recommended*	3.9 mm
384	Flat	Nunc	164688, 164730	3.0 mm	3.0 mm
384	Flat	BD Falcon	353961, 353962	3.0 mm	3.0 mm

* For use with suspension cells. For more information, please refer to the suspension cell protocols available in the Application Protocols Manual or on the LI-COR® website (**http://biosupport.licor.com**).

- Find the optimal focus offset by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Infrared Imager or 1.7, 2.0, 3.0, and 4.0 mm focus offsets for the Odyssey Sa instrument. (The actual minimum and maximum focus offset will vary with each instrument.) Use the same intensity settings for each scan. After reviewing the collected scans, use the focus offset with the highest signal-to-noise for experiments. Focus offset can be further fine-tuned in 0.1-0.5 mm increments if desired, and this may result in additional improvement in signal strength.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at room temperature or 4°C.
- Set the intensity settings for both 700 and 800 nm channels to 5 for the Odyssey Infrared Imager or 7 for the Odyssey Sa instrument for initial scanning. If the image signal is saturated or too high, re-scan using a lower intensity setting (i.e., 2.5 for the Odyssey Infrared Imager or 4 for the Odyssey Sa instrument). If the image signal is too low, re-scan using a higher intensity setting (i.e., 7.5 for the Odyssey Infrared Imager or 8 for the Odyssey Sa instrument).
- Scan settings of medium to lowest quality (169 µm resolution for the Odyssey Infrared Imager or 200 µm resolution for the Odyssey Sa instrument) provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- 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.

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4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH, Germany (Serving Europe, Africa, and the Middle East): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104
In other countries, contact LI-COR Biosciences or a local LI-COR distributor.
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988-11466 10/10

Electrophoretic Mobility Shift Assay (EMSA) Using IRDye® Oligonucleotides

Designed for:
Odyssey Infrared Imaging System

LI-COR® Biosciences

Published May 2004. Revised February 2011.
The most recent version of this protocol, with color figures, is posted at http://biosupport.licor.com

I. Introduction

Gel shift assays or electrophoretic mobility shift assays (EMSA) provide a simple method to study DNA-protein interactions. This assay is based on the principle that a DNA-protein complex will have different mobility during electrophoresis than non-bound DNA. These shifts can be visualized on a native acrylamide gel using labeled DNA to form the DNA-protein binding complex. To date, protocols require labeling DNA by radioisotope (1), digoxygenin (2), or biotin (3). The Odyssey® Infrared Imaging System (LI-COR® Biosciences) offers a quick and easily-adapted alternative method to radioisotopic and chemiluminescent detection methods for EMSA analysis and visualization.

A DNA oligonucleotide end-labeled with a LI-COR IRDye infrared dye is a good substrate for protein binding. LI-COR offers pre-annealed oligonucleotides specific to eight unique binding proteins. Oligonucleotides end-labeled with IRDye 700 and IRDye 800 can also be ordered from Integrated DNA Technologies (IDT) to study the competitive binding of a protein to two DNA fragments. DNA detection using IRDye reagents is linear within a 50-fold dilution range, from 9.1 fmol to 0.18 fmol. Additional benefits include no hazardous radioisotope, no gel transfer to membrane or gel drying, no chemiluminescent substrate reagents, and no film exposure. Following electrophoresis, the gel can be imaged on the Odyssey Infrared Imaging System while remaining in the glass plates. If necessary, the gel can be placed back in the electrophoresis unit and run longer.

Existing mobility shift assay protocols can be easily transformed into infrared assays by replacing the existing DNA oligonucleotides with oligonucleotides end-labeled with IRDye reagents. The binding conditions and electrophoresis conditions will remain the same as with any other EMSA detection method.

II. General Methodology

EMSA Oligonucleotides Labeled with IRDye 700

	Part Number
p53 IRDye 700 Labeled Oligo	829-07921
STAT3 IRDye 700 Labeled Oligo	829-07922
CREB IRDye 700 Labeled Oligo	829-07923
NFkB IRDye 700 Labeled Oligo	829-07924
AP-1 IRDye 700 Labeled Oligo	829-07925
Sp-1 IRDye 700 Labeled Oligo	829-07926
HIF-1 IRDye 700 Labeled Oligo	829-07929
ARE (Androgen Receptor) IRDye 700 Labeled Oligo	829-07933
EMSA Buffer Kit for the Odyssey	829-07910

Labeling DNA Fragments with IRDye Infrared Dyes

To obtain DNA fragments end-labeled with IRDye infrared dyes, oligos labeled with IRDye infrared dyes are used. It is critical that the DNA fragment is end-labeled rather than having dye incorporated into the DNA, which interferes with the formation of the DNA-Protein complex.

Synthetic oligonucleotides 5′ end-labeled with IRDye 700 phosphoramidite or IRDye 800 phosphoramidite are available from Integrated DNA Technologies (IDT) (www.idtdna.com). Oligonucleotides are manufactured in single strand form; therefore, both forward and reverse DNA oligonucleotides must be purchased. Once oligonucleotides are obtained, they need to be annealed to form a double-stranded DNA fragment.

Oligonucleotides are annealed by placing the oligonucleotide set in a 100°C heat block for 5 minutes and then leaving the oligonucleotides in the heat block and turning it off to slowly cool to room temperature.

Important: *Both oligonucleotide sequences should be end-labeled with the same IRDye infrared dye. There is a significant decline (~70%) in signal intensity when using only one end-labeled oligonucleotide.*

III. Mobility Shift Sample Protocol (NFkB)

Each oligo labeled with IRDye 700 provided by LI-COR® Biosciences for EMSA reactions will have an optimized protocol to measure the protein-DNA interaction. See the specific EMSA oligo pack insert for more information. As an example, the p53 protein-DNA interaction will be described in this document.

Gel Preparation: Native pre-cast polyacrylamide gels such as 5% TBE (BioRad) or 4-12% TBE (Invitrogen) are recommended. Alternatively, the recipe below can be used to prepare a 4% native gel.
Note: *The protein shift detected on each gel type (i.e., 5% vs 4-12%) will be unique.*

Prepare 4% native polyacrylamide gel containing 50 mM Tris, pH 7.5; 0.38 M glycine; and 2 mM EDTA:

For 40 mL mix:

5 mL 40% polyacrylamide stock (Polyacrylamide-BIS ratio = 29:1)
2 mL 1 M Tris, pH 7.5
7.6 mL 1 M Glycine
160 µL 0.5 M EDTA
26 mL H_2O
200 µL 10% APS
30 µL TEMED
Pour the gel between glass plates and wait about 1-2 hours to polymerize.

Oligo Preparation: EMSA oligonucleotides from LI-COR Biosciences are pre-annealed; however, if IRDye oligonucleotides from IDT are used, the following protocol can be used as a guideline:

1. Dilute oligos in 1X TE for final concentration of 20 pmol/µL.
2. Place 5 µL of forward IRDye 700 oligo into a new tube and add 5 µL of reverse IRDye 700 oligo.
3. Place 5 µL of forward IRDye 800 oligo into a new tube and add 5 µL of reverse IRDye 800 oligo.
4. Anneal oligos by placing the oligo set in a 100°C heat block for 3 minutes. Leave the oligos in the heat block and turn it off to slowly cool to room temperature.
5. Dilute annealed oligos 1 µL in 199 µL water. This is your working DNA stock. Oligos can be stored at -20°C for up to a year if protected from light.

Binding Reaction: For NFkB IRDye 700 oligonucleotide, the following binding reaction is a good starting point.

Reaction	µL
10 X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5)	2
Poly(dI•dC) 1 µg/µL in 10 mM Tris, 1 mM EDTA; pH 7.5	1
25 mM DTT/2.5% Tween® 20	2
Water	13
NFkB IRDye 700 Oligonucleotide	1
Raji nuclear extract (Positive control) (5 µg/µL)	1
TOTAL	**20**

After the addition of the DNA to the protein-buffer mix, reactions are incubated to allow protein binding to DNA. A typical incubation condition is 20-30 minutes at room temperature. Since IRDye 700 infrared dye is sensitive to light, it is best to keep binding reactions in the dark during incubation periods (e.g., put tubes into a drawer or cover the tube rack with aluminum foil).

Electrophoresis:

1. Add 1 µL of 10X Orange loading dye (LI-COR, P/N 927-10100), mix, and load on a gel.
2. Run the gel at 10 V/cm for about 30 minutes in non-denaturing buffer (i.e., 1X TGE or TBE buffer).

Note: For best results, electrophoresis should be performed in the dark (simply put a cardboard box over the electrophoresis apparatus).

Imaging: Gels can be imaged either inside the glass plates or removed from the glass plate. When removing gel from the glass plates, take care not to deform or tear the gel.

In glass plate:

- Scan the gel inside the glass plates using 1.5 mm focus offset (assuming 1 mm thick gel and glass plates are 1 mm thick). If glass plates and gel are thicker/thinner, use larger/smaller offset (so that plane of focus is in the middle of the gel).
- Start with Scan Intensity setting of 8.

Removed from glass plate:

- Adjust the focus offset to 0.5 (assuming 1 mm thick gel). Start with a scan intensity of 8.

Figure 1. NFkB IRDye 700 oligonucleotides were separated on a native polyacrylamide gel (4-12% TBE, Invitrogen EC62352BOX) and imaged on the Odyssey® Infrared Imaging System. Lane 1) no nuclear extract; Lanes 2 and 5) 10 µg Raji nuclear extract; Lanes 3 and 6) 5 µg Raji nuclear extract; Lanes 4 and 7) 2.5 µg Raji nuclear extract.

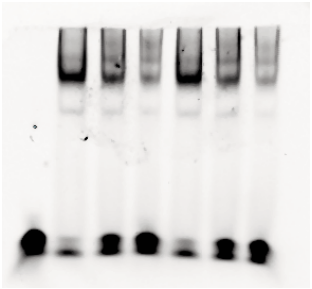
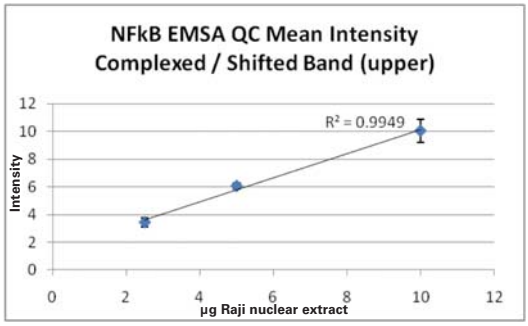


Figure 2. The uppermost shifted band in Lanes 2-7 of Figure 1 was analyzed to determine the level of NFkB binding to the NFkB IRDye Oligonucleotides.



One of the benefits of using the Odyssey® Infrared Imaging System for EMSA analysis is that it provides an easy method for quantification. However, there are issues to consider when using the Odyssey Imager to quantify EMSA results. The primary issue is that the free DNA fragment has much less signal than the DNA fragment when bound to a protein, making quantification of the unbound DNA inaccurate. The addition of DTT/Tween® 20 to the binding reaction stabilizes the dye and reduces this phenomenon. In addition, it is unrealistic to perform quantification analyses under the assumption that the free DNA band in the control, containing DNA only (no extract), should equal the sum of the signals of the free and bound DNA in the samples where the protein-DNA binding reaction occurs. Using end-labeled oligonucleotide duplexes as the DNA source and nuclear extract as a protein source renders this assumption impractical, due to the non-specific binding that occurs from using a nuclear extract. Oligonucleotides can also complicate quantification because the free oligonucleotides form a smear rather than a tight band. This makes it more difficult to assign an intensity value to bands.

Optimization

Binding Reaction

A universal binding condition that applies to every protein-DNA interaction cannot be recommended, since binding conditions are specific for each protein-DNA interaction. Thus, the user should establish binding reaction conditions for each protein-DNA pair. Binding buffer should be the same for this method as with any other mobility shift detection method used.

After the addition of DNA to the protein-buffer mix, reactions are incubated to allow protein to bind to DNA. Time required for binding is the same as when radioactively-labeled DNA fragments are used; a typical incubation condition is 20-30 minutes at room temperature. Since IRDye reagents are sensitive to light, it is best to keep binding reactions in darkness during incubation periods (e.g., put tubes into a drawer or simply cover the tube rack with aluminum foil). After the incubation period, native loading dye is added to the binding reaction.

> ***Note:*** In some cases, it was observed that DNA control reactions (no protein) have lower signal than reactions containing protein. This may be due to lower stability of the dye in certain buffer conditions. The addition of 5 mM DTT and 0.5% Tween 20 to all reactions reduces this phenomenon.

> ***Important:*** It is critical not to use any blue loading dye (e.g., bromophenol blue), as this will be visible on the Odyssey image. Use 10X Orange loading dye instead (LI-COR, Part #927-10100).

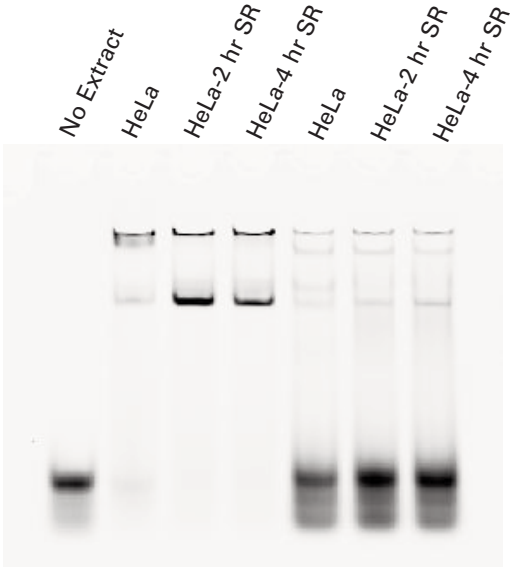


Figure 3. AP-1 EMSA using IRDye 700 end-labeled oligonucleotide duplex.
It is common to use unlabeled DNA duplex to determine binding specificity. Excess unlabeled DNA is added to the binding reaction; therefore, it competes with the labeled DNA for binding sites. If competition eliminates labeled DNA binding, no shift is observed (see last three lanes in gel), indicating that the binding reaction is specific.

Competition reactions contained 100-fold molar excess of wild-type oligonucleotide duplex. Nuclear extracts of HeLa, HeLa 2-hour serum response, and HeLa 4-hour serum response, were used to visualize an increase in AP-1 binding as a result of the serum response treatment to the HeLa cells.

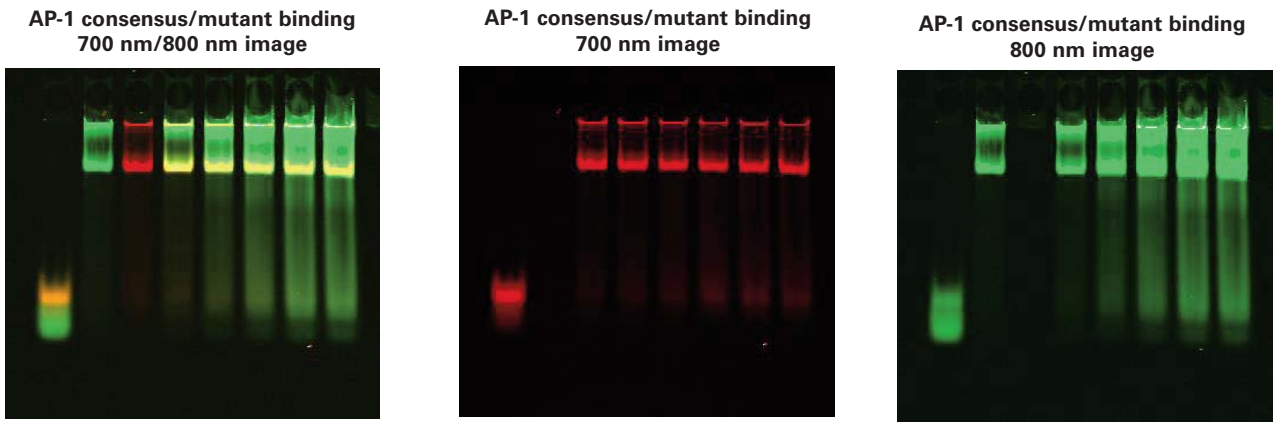


Figure 4. AP-1 EMSA using 2.5 µg Hela 4-hour serum response nuclear extract to demonstrate binding specificity of AP-1 consensus DNA duplex. Binding specificity determination using Odyssey® two-color imaging. (A copy of this document with color figures can be downloaded from http://biosupport.licor.com.)

Competition using mutant DNA duplexes is another common method to determine binding specificity. A mutant DNA sequence is used to compete with the wild-type binding sequence. Specific binding is observed when mutant DNA (unlabeled) does not reduce the binding of labeled wild-type DNA. Two-color analysis of mutant vs. wild-type binding is done using the Odyssey Infrared Imaging System. The wild-type oligos are labeled with IRDye 700 phosphoramidite and mutant oligos with IRDye 800 phosphoramidite. In the figure above, the mutant non-specific binding is very intense (800 nm image); however, there is no decrease in wild-type binding (700 nm image).

Lane 1 – Free AP-1 consensus oligonucleotide IRDye 700 end-labeled and AP-1 mutant oligonucleotide IRDye 800 end-labeled with no nuclear extract;
Lane 2 – Nuclear extract with 0:1 ratio of AP-1 consensus oligonucleotide IRDye 700 end-labeled to AP-1 mutant oligonucleotide IRDye 800 end-labeled;
Lane 3 – Nuclear extract with 1:0 ratio of AP-1 consensus to mutant oligonucleotide;
Lane 4 – Nuclear extract with 1:1 ratio of AP-1 consensus to mutant oligonucleotide;
Lane 5 – Nuclear extract with 1:2 ratio of AP-1 consensus to mutant oligonucleotide;
Lane 6 – Nuclear extract with 1:3 ratio of AP-1 consensus to mutant oligonucleotide;
Lane 7 – Nuclear extract with 1:4 ratio of AP-1 consensus to mutant oligonucleotide.
Lane 8 – Nuclear extract with 1:5 ratio of AP-1 consensus to mutant oligonucleotide.

References

1. Wolf, S. S., Hopley, J. G., and Schweizer, M. (1994) The Application of ^{33}P-Labeling in the Electrophoretic Mobility Shift Assay. *Biotechniques* 16, 590-592.
2. Suske, G., Gross, B., and Beato, M. (1989) Non-radioactive method to visualize specific DNA-protein interactions in the band shift assay. *Nucleic Acids Research*, 17, 4405.
3. Ludwig, L. B., Hughes, B. J., and Schwartz, S. A. (1995) Biotinylated probes in the electrophoretic mobility shift assay to examine specific dsDNA, ssDNA or RNA-protein interactions. *Nucleic Acids Research*, 23, 3792-3793

LI-COR® Biosciences

4647 Superior St. • P.O. Box 4000 • Lincoln, Nebraska 68504
LI-COR Biosciences North America: 800-645-4267 / 402-467-0700
FAX: 402-467-0819 • Technical Support: 800-645-4260

LI-COR GmbH, Germany: Serving Europe, Africa, and the Middle East: +49 (0) 6172 17 17 771

LI-COR Ltd, UK: Serving UK, Ireland and Scandinavia: +44 (0) 1223 422104

In other countries, contact LI-COR Biosciences or a local LI-COR distributor:
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Doc # 988-11801
0211

Odyssey®

Imaging Systems

Syto® 60 Staining of Nucleic Acids in Gels

Published June 2010. The most recent version of this pack insert is posted at http://biosupport.licor.com/support

LI-COR®

Biosciences

The Syto® 60 stain is a red fluorescent nucleic acid stain supplied as a 5 mM solution in DMSO by Invitrogen, P/N S-11342. Any questions regarding the Syto® 60 stain should be directed to Invitrogen (www.invitrogen.com).

I. Introduction

Invitrogen's patented Syto® dyes are cell-permeant cyanine dyes that bind to nucleic acids. Several Syto dyes are available with varying cell permeability, fluorescence enhancement upon binding to nucleic acids, excitation and emission spectra, and nucleic acid selectivity and binding affinity. The Syto 60 stain has absorption and fluorescence emission maxima of 652/678 nm. Nucleic acids stained with the Syto 60 stain can be detected and quantified on the Odyssey® Infrared and Odyssey Fc Imaging Systems using the 700 nm channel.

In the procedures outlined, the Syto 60 dye was used to stain serial dilutions of a 1 kb DNA ladder and a 50 bp DNA ladder (New England Biolabs, P/N N3232 and N3236, respectively). Three methods are presented for staining of DNA in this technical note. The Syto 60 stain can be included in the DNA sample for detection using an Odyssey Imaging system. The Syto 60 stain can also be combined with ethidium bromide (EtBr) and included in the DNA sample for visualization on an Odyssey Imaging System and on a UV transilluminator; or the Syto 60 stain can be diluted and used alone as a post-electrophoresis gel stain.

II. Methods

Method I. Electrophoretic Staining

Purpose: To obtain an archivable, digital image of a DNA agarose gel using an Odyssey Imaging System.

Method:

1. Dilute the Syto 60 stain 1:1000 in TE buffer, mix well.
   Note: Syto 60 stain is stable for up to 1 week at 4°C when diluted.
2. Prepare DNA samples in loading dye and reserve an additional 1 µl in the final volume to accommodate the 1 µl of Syto 60 stain for loading.
3. To each sample, add 1 µl of the diluted Syto 60 stain and mix well with a pipettor.
4. Incubate at room temperature for 5 minutes.
5. Load the samples on the gel.
6. Run the gel at ~5-10 V/cm for ~1 hour or less.
7. Use the Odyssey Infrared or Odyssey Fc Imaging Systems to obtain a digital image of the Syto 60 stained DNA.
   Odyssey Infrared Imaging System Settings:
   - Gel face down on scan bed
   - 700 nm channel intensity: 5-8
   - Focus offset: 0.5 mm

   Odyssey Fc System Settings:
   - Gel face up on imaging tray
   - Acquisition time: 2 min.

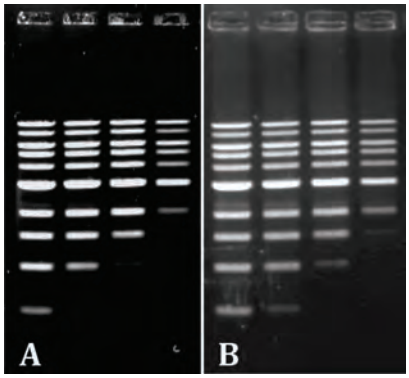


Figure 1. Two-fold dilutions of 1 kb ladder, from 1 µg to 0.125 µg, separated on a 1.2% agarose gel at 8V/cm in 1X TAE buffer for 1 hour. Panel A is the image of the gel obtained from the Odyssey Infrared Imaging System using an intensity of 5.0, gel face down. Panel B is the image of the gel acquired for 2 minutes using the Odyssey Fc Imaging System 700 nm channel, gel face up.

Method II. Dual Electrophoretic Staining

Purpose: To obtain a digital image using an Odyssey® Imaging System and then visualize DNA bands on a UV transilluminator for excision.

1. Dilute the Syto 60 stain 1:1000 in TE buffer, mix well.
   Note: *The Syto 60 stain is stable for up to 1 week at 4°C when diluted.*
2. Dilute EtBr (10 mg/ml solution) 1:500 in TE buffer, mix well (made fresh).
3. Prepare DNA samples in loading dye and reserve an additional volume of 2 µl to accommodate the volume of Syto 60 stain and EtBr for loading.
4. To each sample, add 1 µl of the diluted EtBr and mix with a pipettor.
5. To each sample, add 1 µl of the diluted Syto 60 stain and mix with pipettor.
6. Incubate at room temperature for 5 minutes.
7. Load the samples.
8. Run the gel at ~5-10 V/cm for ~1 hour or less.
   Note: *Longer run times result in fading of the Syto 60 intensity.*
9. Image on an Odyssey Imaging System in the 700 nm channel to obtain a digital image of Syto 60-stained DNA.
   Odyssey Infrared Imaging System Settings:
   - Gel face down on scan bed
   - 700 nm channel intensity: 5-8
   - Focus offset: 0.5 mm

   Odyssey Fc System Settings:
   - Gel face up on imaging tray
   - Acquisition time: 2 min.

   UV Transilluminator:
   - Place gel on UV transilluminator to identify bands for excision. If the band(s) to be excised are not bright enough, the gel can be soaked for a short time in a 2 mg/ml solution of EtBr in TAE or TBE buffer after imaging on an Odyssey System.

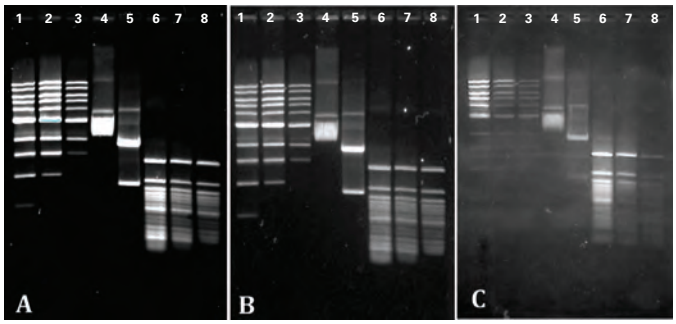


Figure 2. A 1.2% agarose gel was imaged using the Odyssey Infrared Imaging System (panel A), Odyssey Fc Imaging System (panel B) or a UV transilluminator and the image captured using Polaroid 667 film (panel C). Lane 1) 1 µg 1 kb ladder; Lane 2) 0.5 µg 1 kb ladder; Lane 3) 0.25 µg 1 kb ladder; Lane 4) 0.5 µg pUC 19; Lane 5) 0.5 µg pUC19/HindIII / XmnI; Lane 6) 1 µg 50 bp ladder; Lane 7) 0.5 µg 50 bp ladder; Lane 8) 0.25 µg 50 bp ladder. The gel was electrophoresed for 8 V/cm in 1X TAE buffer for 1 hr. The Odyssey intensity setting for the 700 nm channel was 8 and focus offset was 0.5 with the gel face down. The Odyssey Fc acquisition was 2 minutes, gel face up.

Hints and Tips for Method I and II

1. The range of dilution for the Syto 60 stain is 1:500 to 1:20,000. The dilution to use is dependent on the DNA size, concentration, and whether the Syto 60 stain will be used in combination with EtBr. ***Warning:*** *Smaller bands may not be visualized (<100 bp).*
2. The Syto 60 stain, diluted within the recommended range in TE buffer, is stable for 1 week at 4°C.
3. EtBr is not stable in TE and should be diluted fresh each time.
4. The grade of agarose is important. High grade or Molecular Biology grade agarose is less likely to cause "speckling" on Odyssey images.
5. When using the Odyssey® Infrared Imaging System to image DNA gels stained with Syto 60 stain, it may be necessary to scan the gel with the front side on the glass and/or adjust the focus offset, depending on the gel thickness. A 5 mm-7 mm thick gel is optimum.
6. Addition of EtBr to the gel and running buffer with the Syto 60 stain added in the sample is not recommended.

Method III. Post-electrophoretic staining

Purpose: To obtain an archivable, digital image of a DNA agarose gel using an Odyssey Imaging System.

Method:

1. Two parallel 1.3% agarose/TBE gels were loaded with serial two fold dilutions of 100 bp DNA ladder (New England Biolabs) from 1 µg to 0.3 µg per lane.
2. The gels were electrophoresed in 1X TBE running buffer at approximately 5 V/cm.
3. One gel was stained with Syto 60 dye diluted 1:2500 in water for 45 minutes at room temperature, rinsed briefly with double distilled water and then imaged in the 700nm channel using an Odyssey Imaging system.
4. The other gel was stained in 0.5 µg/ml ethidium bromide for 20 minutes at room temperature, rinsed briefly in water and imaged using a UV transilluminator and a standard CCD camera.

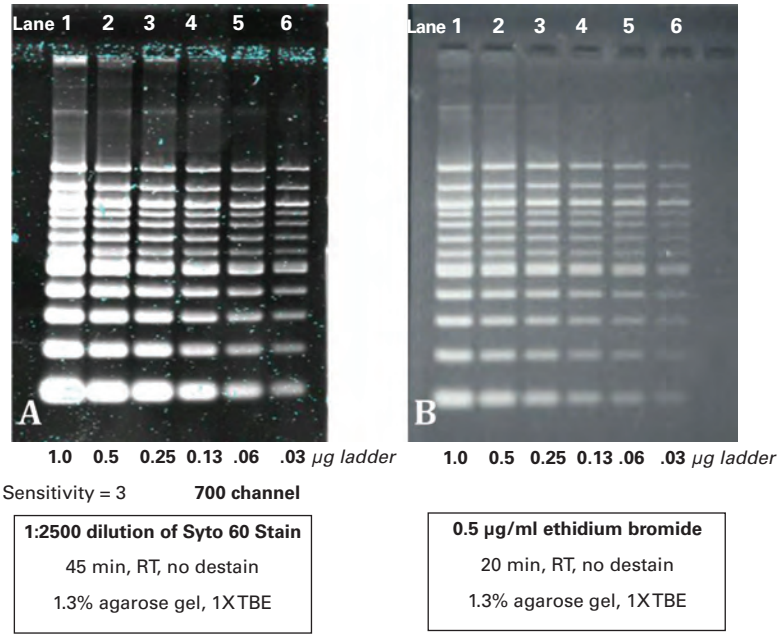


Figure 3. Panel A: Image of a Syto 60 stained gel using the Odyssey Infrared Imaging System.
Panel B: Image of ethidium bromide-stained gel using a CCD camera.

Recommended Dilutions and Time Requirements for Method III.

Gel Conditions: A 10 x 10 cm agarose gel, 5-8 mm thick, made with high grade or molecular biology grade agarose in 1X TAE or TBE buffer

Syto® 60 Nucleic Acid Stain Dilution	Minimum Staining Time
1:2000	5-15 min
1:2500	15-30 min
1:5000	30-45 min
1:10000	45 min
1:15000	45 min
1:20000	45 min

The quickest staining time was 5 minutes using 1:2000 dilution of the Syto 60 stain in water. Gels were stained sufficiently in 15 minutes using a 1:2500 dilution. A 1:5000 dilution of Syto 60 stain requires at least 30-45 minutes of staining. The most dilute solution tested was 1:20,000 and the gel was stained sufficiently after 45 minutes. There was no significant improvement in sensitivity from 60 to 120 minutes using 1:10,000, 1:15,000 and 1:20,000 dilutions.

Speckle Reduction

The appearance of speckles on the gel may be present after post-electrophoretic staining. The Odyssey® Infrared Imaging System software's "FILTER" then "Noise Removal" function and the Odyssey Fc Image Studio's "NOISE REDUCTION" function can be used to improve the appearance of the images (see Figure 4). To reduce the appearance of speckles on the gel, cut off the wells before post-electrophoretic staining and rinse the gel in water.

Note: *The type and concentration of agarose will affect the degree of speckling. For example, low melting-point agarose tends to be highly prone to speckling.*

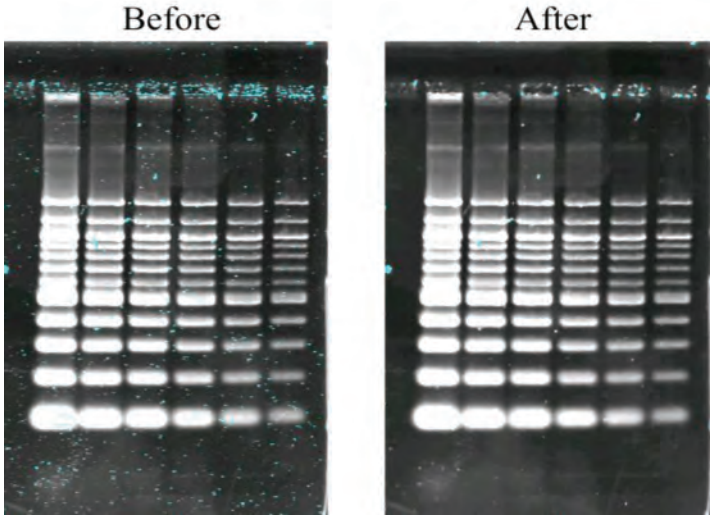


Figure 4. Image of agarose gel showing before and after using Odyssey Infrared Imaging System software's "FILTER" then "Noise Removal" function.

Conclusions

A table of cost comparisons for the Syto 60 stain and the ethidium bromide staining reagents used for each method is provided below. The recommended dilution of the Syto 60 staining reagent makes it more competitive with ethidium bromide on a cost basis, and the inclusion of a small amount of the Syto 60 stain in the sample is environmentally friendly.

Cost Comparisons

	Dilution	Staining Method	Cost
Syto 60 stain	1:1000	Method I or II	$0.006 (1 µl/well, 8 wells)
Syto 60 stain	1:20000	Method I	$0.0003 (1 µl/well, 8 wells)
Syto 60 stain	1:2500	Method III	$7.44 (25 ml)
EtBr	1:500	Method II	$0.00007 (1 µl/well, 8 wells)
EtBr	1:2000	Method III	$0.05 (25ml)

LI-COR is an ISO 9001 registered company.

LI-COR® Biosciences
4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH Germany, Serving Europe and Africa: +49 (0) 6172 17 17 771
LI-COR UK Ltd. UK, Serving UK, Ireland, Scandinavia: +44 (0) 1223 422104
www.licor.com

Doc# 988-11204

Odyssey®

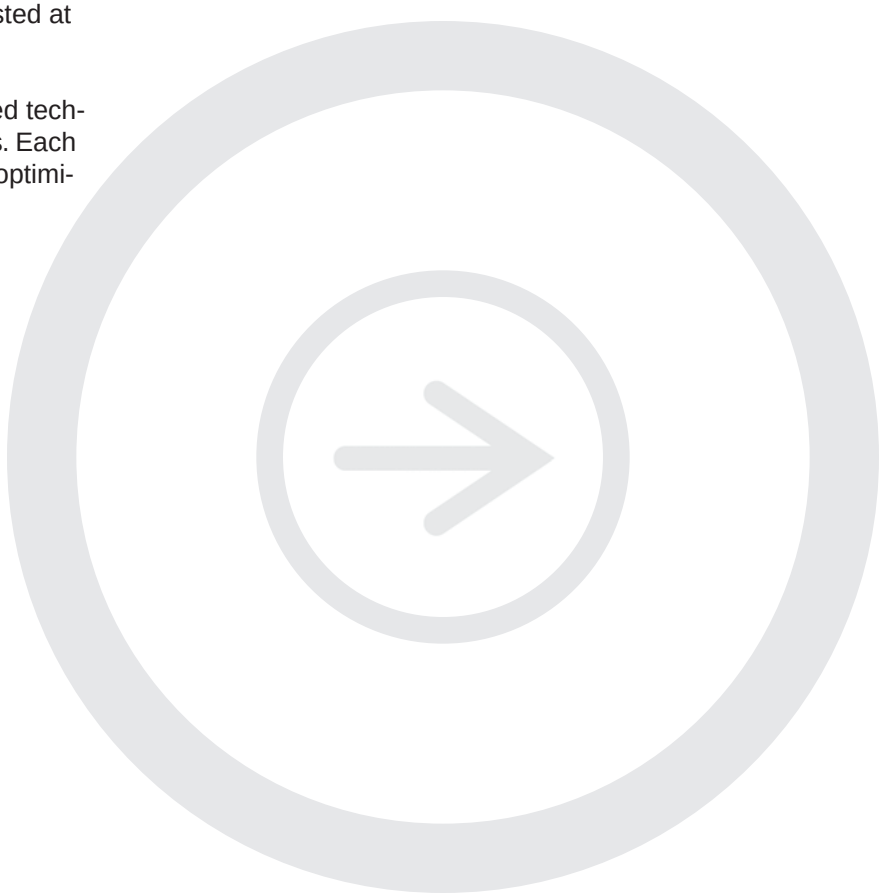
Infrared Imaging System

Technical Note

Fluorophore-Linked Immunosorbent Assay (FLISA) Recommendations

Published January, 2006. The most recent version of this Technical Note is posted at http://biosupport.licor.com/support.

Notice: LI-COR provides only limited technical support for FLISA applications. Each FLISA assay will require individual optimization and performance may vary.



LI-COR® Biosciences

Doc# 988-08352

The following should be used only as a guideline for adapting a Fluorophore-Linked Immunosorbent Assay (FLISA) to the Odyssey® Infrared Imaging System. LI-COR provides only limited technical support for FLISA (ELISA) application.

I. Assay Components

Microplates

Many commercially available microplates and strip wells designated for ELISA or EIA/RIA use are compatible with Odyssey. A few recommended examples are given below. Consider the following characteristics when deciding to use a particular microplate: whether to use clear or opaque microplates, the diameter at the base of each well, and the distance from the bottom of the wells to the bottom of the supporting edge of the plate.

Clear vs. Opaque: LI-COR recommends black microplates with optically clear bottoms to facilitate quantification. Clear microplates perform well, but produce significant laser light scatter around the well edges; extra care must be taken to exclude these edges during quantification.

Well Diameter: It is necessary to know the well diameter for a particular microplate or 8-well strip; this diameter is used by the Odyssey application software for grid placement and determining well area for quantification. Well diameter can be determined by physically measuring the inside diameter of a well, searching the microplate manufacturer's specifications, or using the selection drawing tool within the application software (dimensions of a selected area are listed at the bottom of the window).

Focus Offset: For microplates, the focus offset required in Odyssey application software is the distance from the inner surface at the base of the wells to the plate base that contacts the Odyssey scanning surface. Focus offset can be determined either physically, by measuring the distance (in millimeters); or empirically, by incrementing the focus offset over multiple scans of the same plate containing experimental or control samples. Quantify the same well, or wells, on each scan and determining the focus offset with the highest mean integrated intensity.

Microplate Examples:

- Greiner Bio-One 96-well, polystyrene microplate; high binding capacity
  Black with flat, optically clear well bottoms
  Manufacturer's part number: 65509
- Greiner Bio-One 384-well, polystyrene microplate; high binding capacity
  Black with flat, optically clear well bottoms
  Manufacturer's part number: 781097
- Greiner Bio-One 96-well, polystyrene strip plate; 12 x 8-well removable strips; high binding capacity
  Flat, clear strips/wells in a microplate frame
  Manufacturer's part number: 762071
- Corning Costar 96-well, polystyrene microplate; high binding capacity
  Black with flat, optically clear well bottoms
  Manufacturer's part number: 3601
- Corning Costar 96-well, polystyrene StripWell™ strip plate; 12 x 8-well removable strips
  Flat, clear strips/wells in a microplate frame; high binding capacity
  Manufacturer's part number: 2592
- Corning Costar 96-well, clear polystyrene microplate; high binding capacity
  Manufacturer's part number: 9018

Suggested Reagents

Wash Buffers

- 1X PBS: This is commonly made from a 10X solution (LI-COR Biosciences Cat# 928-40018 or 928-40020) containing 1 M sodium phosphate and 1.5 M sodium chloride, but can also include potassium phosphate and/or potassium chloride depending on your preference. Check pH prior to use and adjust to 7.2 - 7.4 as necessary.
- Alternate: 1x PBS + 0.05% Tween®-20 may be used when greater stringency is required for washing. A final rinse with 1X PBS helps alleviate some of the frothing effect of the Tween-20. Take care when adding detergents to the wash buffer (e.g. SDS) as they can adversely affect other reagent components in the assay.

Blocking Buffer

- 1% BSA
- 5% Sucrose
- 0.05% Sodium Azide
- Dillute in 1x PBS
- Store at 4 °C

Alternate #1
0.1% Casein
0.1% Tween-20 (optional)
Dilute in 1X PBS
Store at 4 °C

Alternate #2
Odyssey Blocking Buffer (LI-COR Biosciences Cat# 927-40000)
0.1% Tween-20 (optional)

Note: There are many other commonly used blocking reagents used for ELISA, each with its own advantages and disadvantages. If the aforementioned blocking buffers do not perform well in your system (i.e. the assay produces an exorbitant amount of nonspecific background signal), then you may want to experiment with other blocking agents and detergents.

Reagent Diluent

- 0.1% BSA
- 0.05% Tween-20
- Dilute in 1X TBS (0.02M Tris base, 0.15M Sodium Chloride)
- Adjust to pH 7.2 - 7.4 with HCl
- 0.2 µm filtered
- Store at 4 °C

Alternatively, dilute with an appropriate blocking buffer or simply 1X PBS. Reagent diluent should only be used to dilute protein sample and detection antibody; dilute dye-labeled streptavidin and dye-labeled secondary antibody with 1X PBS.

Capture Buffer

- 15 mM sodium carbonate
- 35 mM sodium bicarbonate
- 0.02% sodium azide
- Dilute in water
- Store at room temperature

Detection Reagents

Streptavidin Conjugates

- IRDye® 800CW labeled Streptavidin (LI-COR Biosciences Cat# 926-32230)

Secondary Antibody Conjugates

- IRDye 680 and 800CW labeled secondary antibodies are available from LI-COR Biosciences.

Protein Labeling Kits

- IRDye 800CW Protein Labeling Kit (LI-COR Biosciences Cat# 928-38040, 928-38042, or 928-38044)
- EZ-Link® Sulfo-NHS-LC Biotinylation Kit (Pierce Biotechnology Cat# 21430)

II. Optimization Considerations

Reagent Titration

Ideally, each reagent involved in the assay should be titrated in pairs; start by titrating the capture antibody and antigen, followed by titrating the detection antibody and secondary antibody or streptavidin conjugate. Realistically, however, the amount of antibody and/or antigen available may be a limiting factor in such an extensive titration. An example of a more efficient, though less thorough, titration of capture antibody, antigen, and detection antibody, is outlined in Figure 1. In addition, incubation temperatures and times can have significant effects on assay performance, and should be considered as part of the experimental design. In general, the temperature of incubation will determine the length of incubation time for each step in the assay. For example, standard/sample incubation performed at 4 °C will usually require an overnight incubation time, while incubation at 37°C will require a much shorter incubation time to obtain an equivalent fluorescent signal.

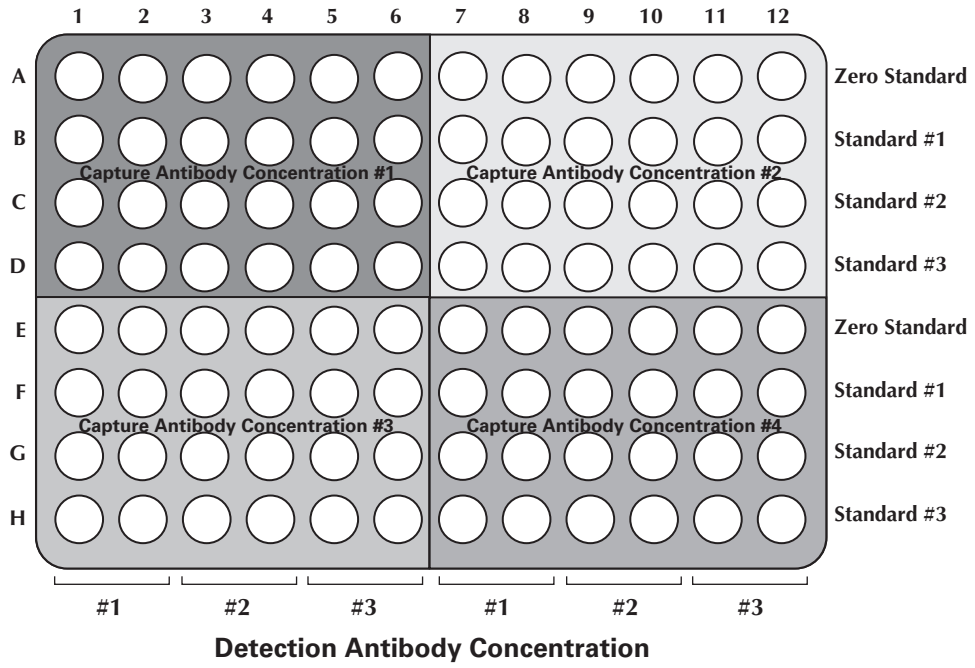


Figure 1. Example of a plate outline for reagent titration. Each quadrant of this particular plate tests three different detection antibody concentrations, each with four standard concentrations in duplicate (including a zero standard). These titrations are repeated in each quadrant for four di stinct capture antibody concentrations. Antibody/streptavidin conjugate is maintained at a fixed concentration.

Analyte

Standards: A calibration curve, run in duplicate, should be generated for each experiment. Serial dilution range can be determined after an initial standard titration (see above) and should include a zero standard.

Samples: If possible, samples should be filtered, column purified, dialyzed, or otherwise purified for best results. Dilute samples in Reagent Diluent (recommended), blocking buffer, or 1X PBS.

Fluorescent Detection

The recommended method of detection for FLISA assays is biotin/streptavidin because many commercially available ELISA kits use biotinylated detection antibodies along with streptavidin-conjugated horseradish peroxidase or alkaline phosphatase. This method allows you to purchase an ELISA kit and substitute the HRP/AP-streptavidin with a fluorescent IRDye-labeled streptavidin.

For cases in which a biotin conjugated detection antibody is either unavailable or undesirable, an alternative detection option would be to utilize a dye-labeled secondary antibody against an unlabeled detection antibody; however, this format generally does not provide the level of sensitivity of biotin/streptavidin. Another detection alternative is to employ a dye-labeled antibody conjugate directly; this option has the advantage of requiring one less reagent, but may introduce additional problems with non-specific fluorescent signal.

Wash Procedure

The wash steps in the FLISA assay are critical to obtaining good results. Use the following guidelines for best results:

- Standard laboratory wash bottles work well for washing. Automatic plate washers may be used when higher throughput is necessary, as long as cross-contamination between wells can be prevented and the guidelines below can be adhered to. Wells can be overfilled and sprayed vigorously, but not harshly. Make sure each well is filled completely with buffer for each wash (exception: for the last set of washes, use a pipet to dispense and aspirate wash buffers. This will prevent extraneous signal caused by dye conjugate spilling over between wells.).
- For more stringent washing, first wash 2-3 times with 1X PBS-T, then rinse with 1X PBS. After the final wash, briefly centrifuge the plate upside-down on a clean paper towel at low speed (~600 x g); this will ensure all liquid is removed and allow for better reagent contact in the microplate wells for the successive steps. If a centrifuge is not available, rigorously (but not harshly) blot the plate on paper towels until all liquid is removed from the wells.
- Optionally for each wash, allow the wash buffer to sit in the wells for 1-2 minutes with gentle rocking/shaking.

III. Odyssey Settings

Scan

Start by defining a Scan Preset to use with the FLISA assay. Choose **Settings > Scan Presets** in Odyssey application software and edit the default "MicroPlate2" scan preset. Change the focus offset to match the distance measured from the well bottom to the base of the plate. Click **Save As** and save the Scan Preset using a new name. Consult the Odyssey User Guide for complete information on using the Scan Preset settings.

Sample Quantification

Quantification in Odyssey application software is performed by applying a grid to the microplate image (choose **Analyze > Add Grid**). If one of the default Grid Templates does not fit your microplate, use the Grid Template settings (choose **Settings > Grid Templates**) adjust the well diameter and other parameters to correspond with the microplate you are using. Quantification data can be viewed in the grid sheet (choose **Analyze > Grid Sheet**). Consult the Odyssey User Guide for more detailed microplate quantification procedures.

IV. General FLISA Protocol

The following is an example protocol for performing a 96-well microplate sandwich FLISA with the Odyssey Infrared Imaging System. Reagent concentrations, incubation times and temperatures, and method of fluorescent detection are given only as a typical FLISA example; these characteristics may vary considerably with the samples and antibodies in your assay system.

1.	Bring all reagents to room temperature before use.
2.	Prepare an appropriate amount of 2 µg/ml capture antibody. Pipet 100 µl into each desired well. Incubate microplate at 37 °C for 30 minutes, room temperature for 2 hours, or 4 °C overnight (16-18 hours). Gently agitate plate on a shaker or rocker during incubation.
3.	Remove capture antibody solution from wells. Wash 3 times with 1X PBS-T and once with 1X PBS; use enough wash buffer to fill each well. Gently agitate wash buffer for 1-2 minutes before removing. Ensure wash buffer is removed completely before proceeding to the next step.
4.	Pipet 300 µl of blocking solution into each well. Incubate at room temperature with gentle agitation for at least one hour. Prepare samples and standards while waiting for blocking to proceed.
5.	Remove blocking buffer completely.
6.	Add 100 µl of the appropriate samples/standards dilutions to each well. Incubate at 37 °C for 30 minutes, room temperature for two hours, or 4 °C for 4-6 hours. Gently agitate plate during incubation.
7.	Remove samples/standards from wells. Wash 3 times with 1X PBS-T and once with 1X PBS as in step (3).
8.	Prepare an appropriate amount of 200 ng/ml biotinylated detection antibody. Pipet 100 µl into each well. Incubate at 37 °C for 30 minutes, room temperature for 1 hour, or 4 °C for 2-3 hours.
9.	Remove detection antibody solution from wells. Wash 3 times with 1X PBS-T and once with 1X PBS as in step (3).
10.	Prepare an appropriate amount of 1 µg/ml streptavidin conjugate. Add 100 µl to each well. Protect the plate from light and incubate at room temperature with gentle agitation for 30 minutes.
11.	Carefully decant the streptavidin solution with a pipet. Wash 4 times with 1X PBS-T and once with 1X PBS; take extra care during this set of wash steps so as not to allow wash buffer to spill between wells (and thereby introduce a potential source for extraneous fluorescent signal).
12.	Place the microplate on the Odyssey scanning surface with well A1 in upper left orientation. Use the microplate scanning guide as described in the Odyssey Operator's Manual. Scan the microplate following the aforementioned guidelines and instructions in the Odyssey User Guide.

4308 Progressive Avenue • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH (Germany, Austria, Switzerland, Czech Republic, Hungary, Slovakia): +49 (0) 6172 17 17 771
LI-COR UK Ltd.: +44 (0) 1223 422104
www.licor.com

In Vivo Imaging

Scanning a Mouse on the Odyssey® System: Hints and Tips

Developed for:

Odyssey Infrared Imaging System

LI-COR® Biosciences

Published September, 2010. The most recent version of this protocol is posted at http://biosupport.licor.com

I. Diet Considerations

Mouse chows generally have high fluorescent signal in the 700 and 800 nm channels due to plant based ingredients that contain chlorophyll. If imaging in the abdominal region where intestinal fluorescence will be an issue, feed a purified diet containing no plant based ingredients. Figure 1 illustrates the issues with regular mouse chows (signal is saturated) as compared to two purified diets provided by the same company.

Figure 1. Three mouse chows from Harlan Teklab imaged on the Odyssey system.

An example of the level of interference that can be seen when imaging a mouse on the Odyssey system is shown in Figure 2. The circle on the 700 nm channel image indicates the intestinal signal due to the mouse diet while the circle on the 800 nm channel image pin-points the abdominal tumor.

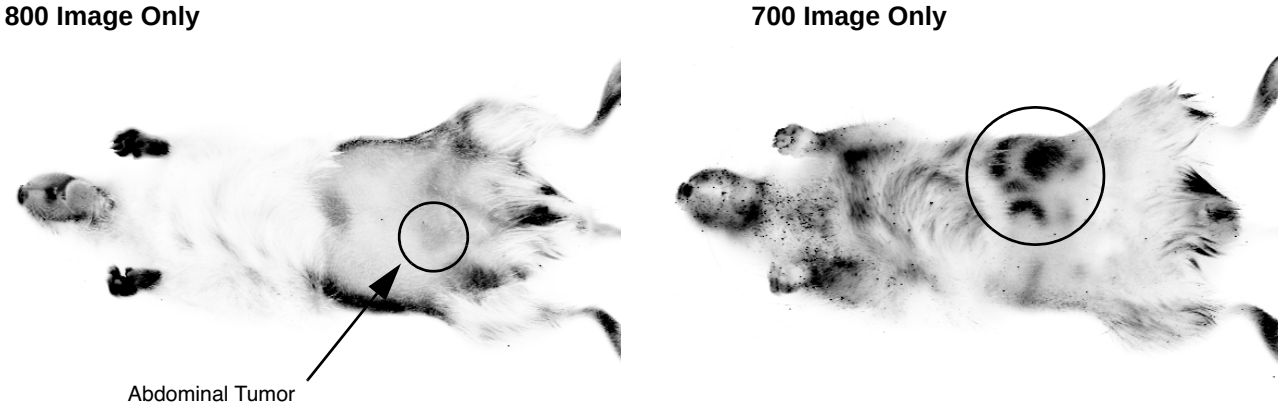


Figure 2. A typical mouse scan on the Odyssey Imager where an abdominal tumor was present. Scan parameters include resolution = 169 µm; quality = medium; focus offset = 2.0; intensity values = L1 for 700 channel and 3 for 800 channel.

II. Pre-Scan

It is always beneficial to scan the mouse prior to probe injection to document the amount of background/autofluorescence the mouse emits. Start with intensity setting of L1 and 3 for the 700 and 800 nm channel, respectively, until the level of expected signal is known.

III. Resolution

The first image should be at the lowest resolution (i.e., 337 µm), which gives a good preview of what to expect from the particular mouse model being evaluated. If the mouse is optimally positioned, this short preview scan will provide a more accurate estimate of the correct intensity settings. Generally, images scanned at 169 µm are a good compromise between resolution and scan time. Scanning quickly with live mice is important to minimize image anomalies caused by mice moving during a scan and to minimize stress to the animal.

IV. Hair vs. Shaved Animal

If working with a haired mouse model (i.e., something other than nude mice), shave the animal in the region of interest prior to imaging. Up to 50% of the signal is blocked when imaging through hair. Hair removal can be accomplished by shaving (i.e., mustache shavers work well) or by the use of Nair®. To demonstrate, a mouse was implanted with a tube containing IRDye® 800CW in the thoracic cavity and imaged before and after shaving. Figure 3 shows the effect of hair with the difference between Panel A (before) and Panel B (after) being 53%.

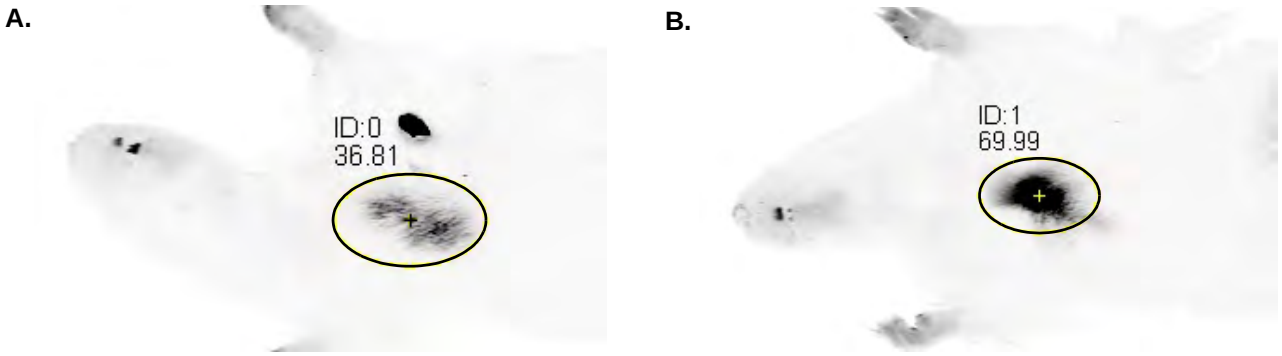


Figure 3. Signal from IRDye 800CW in the thoracic cavity when imaged on the Odyssey® system before (Panel A) and after (Panel B) shaving.

V. Reflections

Any areas that may cause a reflection will be an issue. For example, when shaving, avoid nicking the skin as the open nick will cause reflection and signal.

VI. Focus Offset for Surface and Abdominal Tumors

A focus offset of 1.0 mm is a good starting point for a surface (subcutaneous) or intra-abdominal tumor.

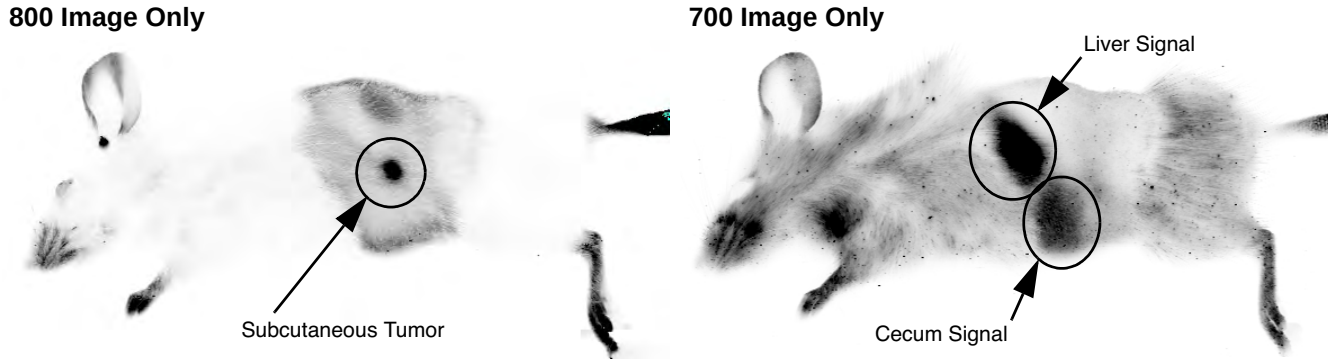


Figure 4. Subcutaneous tumor (800 nm channel) and liver and cecum signal present in the 700 nm channel.

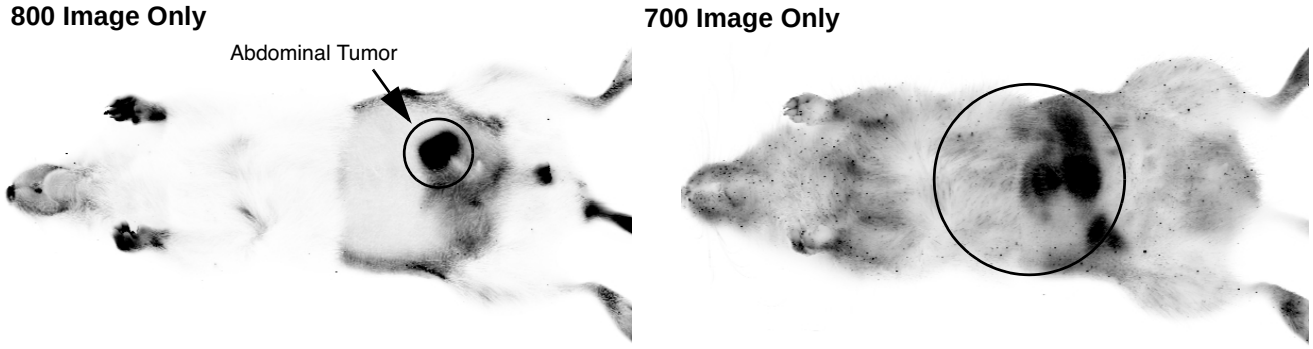


Figure 5. Abdominal tumor in the 800 nm channel and intestinal signal in the 700 nm channel.

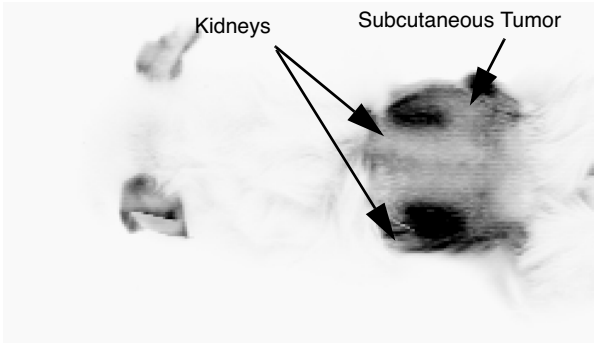


Figure 6. The 800 nm channel image illustrates clearance of the particular IRDye® 800CW labeled probe from the kidneys. A subcutaneous side tumor is also visible.

LI-COR Biosciences • 4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 or 402-467-0819
LI-COR GmbH, Germany (Serving Europe and Africa): +49 (0) 6172 17 17 771
LI-COR UK Ltd., UK (Serving UK, Ireland, and Scandinavia): +44 (0) 1223 422104

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