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I. Starting a Therapeutics Research Project

Developing a therapeutic agent that has the power to improve human health relies on both basic and translational research. Significant advancement in these fields depends on both proper development and implementation of multiple biological assays that are robust and reliable.

The type of reagents, reaction conditions, signal measured (fluorescence, luminescence, absorbance, etc.), instrumentation, and statistical models used for analysis all impact the development of a quality assay. LI-COR Biosciences provides imagers, dyes, reagents, software, protocols, and support that will enable you to confidently develop reliable assays for your therapeutic research projects.

The aim of this handbook is to describe assays you can use to answer vital questions related to therapeutics research. Creative uses of these assays are included to spark ideas.

II. Assays

The following assays are frequently used in therapeutics research and can be conducted using LI-COR solutions. Remember that you can always use your creativity to adapt any of these assays to fit your research needs. More information is available from a number of sources, including:

- Detailed protocols and information about other assays are available at licor.com/applications.
- On-demand Western blot education is available on the Lambda U® Education Portal (lambdau.net).

Western Blot

Researchers rely on Western blotting to detect target proteins and compare target protein levels in complex samples. This type of protein detection is essential to early phases of therapeutics research for qualitative target analysis and quantitative comparison of relative target abundance.
Near-infrared (NIR) Western blot technology enables detection and comparison of multiple proteins in a sample using secondary antibodies conjugated to NIR fluorophores. NIR Western blot detection enables more reliable target analysis in a variety of ways, including the following two:

- Detection of stable NIR signal eliminates problems caused by chemiluminescent signal changing over time.
- Multiplex protein detection in each sample enables accurate normalization that minimizes the effect that intrinsic variability can have on your results.

Getting Started with NIR Western Blots

Although a Western blot protocol has many steps, publisher guidelines provide a valuable template for designing and conducting reliable Western blot experiments for therapeutics research. (A select list of these guidelines is provided in the "References" on page 33.) These guidelines emphasize the importance of experimental design validation and choosing a normalization method that will produce accurate results.

Drawing from these valuable ideas, LI-COR has created protocols and application guides that provide detailed information your lab can use to achieve reliable Western blot results. (A select list of these resources is provided in the "References" on page 33.)

Validation

Because Western blots and Cell-based Westerns are fundamentally similar, similar validation steps are required for both.

Antibody Characterization

Verify that your primary antibody is both specific and selective to the target antigen in your experimental conditions.

- To quickly get started choosing an antibody, you can ask LI-COR for published information relevant to the target you are trying to detect (licor.com/antibodyrequest).
- Watching the Antibody Learning Path on the Lambda U® Western Blot Education Portal (lambdua.net) will quickly get everyone in your lab up to speed on antibody characterization.

Linear Range Validation

Quantitative Western blot analysis is accurate only if the target protein and internal loading control (ILC) can both be detected within the same linear range – a range that must be determined experimentally for each target and loading control. The combined linear range is
then used to determine how much sample should be loaded to produce a linear signal response for both the target protein and the ILC.

The Linear Range Determination protocol (licor.com/LinearRange) explains how to determine an appropriate amount of sample to load for quantitative Western blot analysis.

**Housekeeping Protein Validation**

If you use a housekeeping protein (HKP) for normalization, you must ensure that the HKP (or other internal reference protein) that you use is stably expressed under the conditions in your experiment.

The *Housekeeping Protein Validation* protocol (licor.com/HKP-Validation) explains how to validate an HKP for use as an ILC, by demonstrating that HKP expression is stable in the relevant experimental samples.

**Normalization**

Normalization minimizes the effect that small, intrinsic variation in your Western blot protocol will have on your data – thereby improving the accuracy and reliability of your Western blot results. Accurate normalization requires comparison of target data to an ILC that is known to be stable under the conditions in your experiment and can be detected within the same combined linear range as your target (1).

The *Normalization Handbook* (licor.com/handbook) describes how to choose and validate an appropriate ILC.

Empiria Studio® Software provides step-by-step workflows so that everyone in your lab can perform Western blot analysis using normalization methods recommended by publishers – for accuracy and to minimize user-to-user variation. Normalized signal intensities for target proteins are automatically calculated based on the type of ILC used in the experiment. The following describes some ILC options.

**Total Protein Stain**

This approach is used to detect the total amount of sample protein in each lane to correct for variation in sample loading and transfer. Total protein staining has emerged as a reliable and widely applicable normalization method for quantitative immunoblotting (15, 16). Revert™ 700 Total Protein Stain (licor.com/revert) provides linear signal over a broad range of sample concentrations and is compatible with subsequent Western blot immunodetection methods.

**Housekeeping Protein**

A housekeeping protein is a single endogenous protein that is sometimes used as a readout of sample loading for normalization. To use a housekeeping protein for normalization, you must validate that its expression is stable in your experiment. Housekeeping proteins such as
The Housekeeping Normalization protocol (licor.com/HKP-Normalization) describes how to use a housekeeping protein for Western blot normalization and quantitative analysis.

**Post-Translational Modification**

If you are studying post-translational modification, publishers recommend using the total form of the target (regardless of modification) as an ILC for normalization (1). For example, phospho-AKT could be normalized to pan-AKT.

The Pan/Phospho Normalization protocol (licor.com/PanProteinNormalization) describes how to use pan-specific antibodies as an ILC for normalization.
Protocol Overview

1. Prepare Samples
2. Perform Electrophoresis
3. Transfer to Membrane
4. Block
5. Incubate with Primary Antibodies
6. Wash
7. Incubate with Near-Infrared Fluorescent Secondary Antibodies
8. Wash
9. Detect with an Odyssey® Imager
10. Analyze with Empiria Studio® Software
Protocol

The following generalized protocol shows the milestones in a reproducible Western blot experiment.

- The detailed *NIR Western Blot Detection Protocol* is available here licor.com/nirwesternprotocol.
- *Good Westerns Gone Bad* provides troubleshooting tips and examples licor.com/gwgb.

**Step 1. Prepare Samples**

For normalization to be effective, a protein concentration assay must be used to estimate the protein concentration of your samples, allowing you to adjust sample concentration to make sample loading as consistent as possible.

The Protein Concentration Learning Path on Lambda U® (lambdau.net) explains how to use protein concentration assays effectively.

**Step 2. Perform SDS-PAGE Electrophoresis**

Be sure to load the same sample volume and concentration in each well.

Chameleon® Pre-Stained Protein Ladders (licor.com/chameleon) can be detected in the NIR region, and can be visually inspected to ensure that protein is migrating in the gel as expected.

**Step 3. Transfer to Membrane**

Determine the best membrane type to use for your experiment. Both nitrocellulose and PVDF are available (licor.com/membranes).

- Nitrocellulose membranes generally offer the lowest background.
- PVDF membranes typically display higher levels of autofluorescence, but they can be an appropriate choice when higher binding capacity or better tensile strength are required.

Odyssey® Loading Indicator (licor.com/oli) provides a simple, convenient method to evaluate the consistency of sample loading volume across gel lanes, as well as the uniformity of Western blot transfer.

**Step 4. Dry Membrane**

Membranes should be dried immediately after transfer to allow proteins to firmly bind the membrane, reducing protein loss from the membrane throughout the protocol and ensuring stronger target signal.
Step 5. Block the Membrane

Blocking the membrane helps prevent non-specific binding of antibodies to the membrane, which would lead to high background signal that can interfere with interpretation of your results. Because no single blocking buffer is ideal for every experiment, several varieties of Intercept® Blocking Buffer are available.

- Intercept Blocking Buffer ([licor.com/intercept]) is formulated to provide highly efficient blocking and low background for reliable quantitative Western blots and other immunoassays.

- Protein-based and protein-free Intercept Blocking Buffers are available in TBS and PBS formulations to fit the needs of a wide variety of experiments. In general, TBS blocking reagents are used to detect phosphorylated protein targets. The phosphate in PBS blocking reagents may competitively bind with antibodies to the phosphorylated target.

Step 6. Incubate with Primary Antibodies

When performing multiplex Western blot detection on targets of similar molecular weight, the primary antibodies must be from different host species or subclasses.

Dilute primary antibodies according to the vendor’s recommended dilution in a solution of 0.2% Tween 20 in the blocking buffer you used for the blocking step. Antibody concentration is typically 1 µg/mL, but it’s important to use enough antibody solution to cover the entire blot.

Alternatively, Intercept T20 Antibody Diluent formulations are available to pair with the type of Intercept Blocking Buffer formulation used in your protocol. With pre-made antibody diluent, your lab can focus on developing therapeutics instead of spending time on routine tasks like making antibody diluent.

Step 7. Wash

Be sure to use the same buffer system throughout your Western blot protocol. If you use a PBS-based buffer for blocking, then use PBS-based buffer for all other steps in the protocol.

Step 8. Incubate with Secondary Antibodies

Performance of secondary antibodies is vital for Western blotting and many other applications. IRDye® Near-Infrared Fluorescent Secondary Antibodies ([licor.com/secondaries]) are highly cross-adsorbed, so they do not cause cross-reactivity and are good for multiplex detection.

- Choose IRDye 800CW secondary antibodies for lowest background and maximum sensitivity in the 800nm channel. Use IRDye 800CW for detecting low abundance targets that require the highest sensitivity.
• Choose IRDye 680RD secondary antibodies for the lowest background and easiest optimization when detecting in the 700nm channel.

• Choose IRDye 680LT secondary antibodies for applications that require greater light stability (such as microscopy) and when optimal Western blot conditions for specific targets have already been well-established. IRDye 680LT secondary antibodies can typically be used at lower concentrations, but can require more optimization to ensure low background.

**Step 9. Wash**

Be sure to use the same buffer system throughout your Western blot protocol. If you use PBS-based buffer for blocking, then use a PBS-based buffer for all other steps in the protocol.

**Step 10. Image**

Relative abundance of different protein targets varies widely based on the sample and treatment. Some bands on a Western blot may be barely perceptible by eye, while others may be very bright. For an imager to accurately detect biologically relevant differences in band intensity, it must have a wide enough linear dynamic range to capture both faint bands from less abundant proteins and bright bands from highly abundant proteins. An imager with a wide linear dynamic range increases the likelihood that a target and ILC can be detected in the same linear range for accurate quantification, normalization, and results.

The Odyssey® CLx Imager is the gold standard for quantitative Western blot imaging, and Odyssey Imagers have been cited in over 11,000 peer-reviewed publications.

• The Odyssey CLx is a laser-point scanner that provides over 6 logs of linear dynamic range, so you can accurately image faint and strong bands together on the same Western blot in a single acquisition. The Odyssey CLx can also image a variety of other assays ([licor.com/clx](licor.com/clx)).

• The Odyssey Fc is a CCD-based imaging system that provides over 6 logs of linear dynamic range and can image NIR and chemiluminescent Western blots ([licor.com/fc](licor.com/fc)).

When using an Odyssey Imager, blots can be imaged either wet or dry. Imaging a dry membrane can provide more signal intensity than imaging the membrane wet, but a dry blot may also have increased background.

**Step 11. Analysis**

Journals have published clear guidelines ([1](#)) for validation and normalization steps that researchers can follow to gather reliable data from Western blot experiments. Whether your intent is to publish groundbreaking research or to bring a therapeutic agent to market, these guidelines provide the basis for procedures your lab can follow to gather Western blot data that can advance your therapeutics research.
Traditional Western blot analysis software does not provide features that allow you to quantify blots for these recommended steps. Empiria Studio® Software (licor.com/empiria) was developed with specific analysis workflows based on publisher recommendations for validation and normalization. Empiria Studio also uses a patented background subtraction algorithm to automatically subtract background, eliminating variability caused by subjective background subtraction choices (licor.com/empiria-studio-background).

Figure 1. Empiria Studio Software provides analysis workflows for publisher-recommended steps in a reliable Western blot project. Publisher guidelines recommend validating key aspects of your Western blot experimental design (Antibody Validation, Linear Range Determination, and Housekeeping Protein Validation) and strategies for normalizing target data. Empiria Studio provides analysis options for each of these steps.

DigiWest® High-Content Protein Profiling Services

The novel and proprietary DigiWest® protein-profiling technology enables simultaneous detection of up to 800 targets from 20 – 60 µg of protein sample. This assay is a versatile way to perform pathway profiling, mode-of-action studies, compound characterization, and biomarker discovery with a large number of targets and limited sample.

LI-COR Biosciences and NMI TT Pharmaservices have partnered to bring you DigiWest® as a contract research service. Visit licor.com/digiwest to get more information and to request the service.
Description

DigiWest® combines a traditional Western blot protocol with multiplex detection on a Luminex® imager to achieve high-content protein profiling with small samples of protein.

Sample lanes on a Western blot membrane are cut horizontally to separate blotted proteins into molecular weight fractions. Proteins are then eluted into solution in micro-well plates, where they are bound to color-coded beads and probed with a wide selection of primary antibodies and phycoerythrin-labeled secondary antibodies for signal generation.
Protocol

1. Proteins are size separated via SDS-PAGE.

2. Proteins are transferred to a blotting membrane and biotinylated.

3. Sample lanes are cut into 96 strips to generate 96 molecular weight fractions immobilized on membrane.

4. Biotinylated proteins are eluted into 96-well plates.

5. Biotinylated proteins are immobilized on the surface of Neutravidin-coated Luminex beads. One distinct bead set is added to each of the 96 wells, which results in a collection of distinguishable protein-loaded bead sets from a single sample.

6. Beads are pooled to reconstruct the initial sample lane. A defined color code correlates with each molecular weight.

7. A small aliquot of the bead pool is incubated with antibodies before phycoerythrin-labeled secondary antibodies are added for signal generation.

8. The sample is detected on a Luminex FLEXMAP 3D® instrument.

9. You will be provided with the data needed to do further analysis (such as cluster analysis or pathway analysis), and a record of the protocol used to generate the data.

Request the DigiWest® Contract Research Service

Visit licor.com/digiwest to get more information and to request the service.
Quantitative Cell-Based Westerns

The In-Cell Western™ Assay and On-Cell Western Assay are quantitative NIR fluorescence assays that allow you to detect multiple proteins and quantify target protein abundance in many replicate samples in multi-well plates (commonly with 96 or 384 wells).

The In-Cell Western Assay uses primary antibodies and NIR fluorescent secondary antibodies to detect protein targets inside fixed and permeabilized cells. The On-Cell Western assay is similar, but cells are not permeabilized and cell surface proteins are detected. In this handbook, Cell-based Western will be used to reference both In-Cell Western Assays and On-Cell Western Assays.

Cell-based Westerns are similar to traditional Western blots, but have fewer steps that can contribute to variability. Low variability and higher throughput make Cell-based Westerns suitable for many experiments that are commonly used for the development of therapeutics.

The following is a limited set of examples. For a more complete list of how quantitative Cell-based Westerns have been used, visit licor.com/icw.

- Dose response IC50 assays (2, 3)
- Tau protein accumulation assays (4)
- Viral titration assays (5, 6, 7, 8)
- Apoptosis assays (9)
- RNAi library screening assays (10)
- Cell proliferation assays (11, 12, 13)
- Binding affinity assays (14)

Comparison to Traditional Western Blotting

Cell-based Western assays and traditional Western blots are fundamentally similar.

Immunodetection with a specific primary antibody and secondary antibody labeled with an IRDye near-infrared fluorescent dye are employed in both, although the detection occurs in a lysate for the Western blot and within the context of the cell for Cell-based Westerns.

Purpose

Although similar in principle, Western blots and Cell-based Westerns allow you to gather different types of data.
- Western blots allow you to confirm the molecular weight of your target and detect multiple targets simultaneously.

- Cell-based Westerns allow you to rapidly screen many experimental conditions, compound libraries, or dose-response treatments in replicate.

Validation

Both traditional Western blots and Cell-Based Westerns require antibody validation, linear range determination, and housekeeping protein validation (if you’re using a housekeeping protein for normalization).

The following shows examples of assays that can be used to validate each part of the validation process for Western blots and Cell-Based Westerns.

<table>
<thead>
<tr>
<th>Antibody Validation</th>
<th>Linear Range Determination</th>
<th>Housekeeping Protein Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blot</td>
<td>Western blot</td>
<td>Western blot</td>
</tr>
<tr>
<td>Cell-Based Western</td>
<td>Western blot, immunofluorescence microscopy, immunohistochemistry</td>
<td>Cell-Based Western</td>
</tr>
</tbody>
</table>

Normalization

Both traditional Western blots and Cell-Based Westerns require normalization.

- In Western blots, normalization accounts for small, intrinsic variation in the protocol, including the transfer step.

- In Cell-Based Westerns, normalization can account for variation, such as a change in cell number caused by treatment or plating density (see the example experiment on page 30).

The following table shows normalization options for Western blots and Cell-Based Westerns.

<table>
<thead>
<tr>
<th>Validated Housekeeping Protein</th>
<th>Pan-Protein</th>
<th>Protein or Cell Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blot</td>
<td>Yes</td>
<td>Yes Revert™ 700 Total Protein Stain</td>
</tr>
<tr>
<td>Cell-based Western</td>
<td>Yes</td>
<td>Yes CellTag™ 700 Stain</td>
</tr>
</tbody>
</table>

Getting Started with Cell-Based Western Assays

Superior performance and robustness of any assay is a product of good design, construction, execution, and analysis. The development process is often cyclic with multiple validation steps.

Key components to the design, validation, and implementation of a reliable Cell-based Western assay are described below.
Watch the webinar: Rethinking the Traditional Western Blot provides guidance for getting started with In-Cell Western Assays.

**Plate Type**

Using the correct type of plate for your experiment is critical.

Among other considerations, the color of the plate is important. Plates with a clear bottom and either clear or black wells are recommended. Do not use white plates, because autofluorescence from white plates leads to high noise in your images.

**Fixation and Permeabilization**

Fixation and permeabilization are critical steps in the process to preserve cell morphology and retain antigenicity of target molecules. No single fixation and permeabilization method will work optimally for every cell line, antibody, and epitope combination. Optimal conditions should be determined empirically. Established conditions for detecting your antigen in immunofluorescent microscopy or other cell-based assays are a good starting point.

The *Fixation and Permeabilization* guide describes methods you can evaluate for your assay (licor.com/ICWFixative).
Validation

Because Western blots and Cell-based Westerns are fundamentally similar, similar validation steps are required for both.

The validation phase is also a good opportunity to determine the optimal time point for cellular response. Without finding the optimal time, it is possible to underestimate the response or miss the response altogether.

Antibody Characterization

In a Cell-based Western assay, proteins are not separated into molecular weight fractions via electrophoresis. Western blots can be used as part of the antibody characterization process to determine if the antibody recognizes a target at the correct molecular weight.

**Note:** The specificity of a given antibody for a given target may be different in a Western blot than a Cell-based Western.

Immunofluorescent microscopy can also be used to confirm localization of the antibody to the area of the cell where your target is located. For example, if your target is in the nucleus but you find signal spread throughout the cytoplasm, you may need try a different antibody. Positive and negative controls are important in evaluating specificity and selectivity of your antibody.

**More Info:** More information about antibody validation is available in the In-Cell Western™ Assay Antibody Validation Tech Note (licor.com/icw_antibody_guide).

Linear Range Validation

For Cell-based Westerns, it is important to find the range in experimental conditions that produces a linear response in signal intensity for both the target and ILC.

Housekeeping Protein Validation

If you use a housekeeping protein for normalization, you must ensure that the HKP you use is stably expressed under the conditions in your experiment.
Normalization

Because Western blots and Cell-based Westerns are fundamentally similar, similar methods of normalization can be used for each.

Cell Stain

Similar to how Revert™ 700 Total Protein Stain is used to detect all the protein in a Western blot lane for normalization, accurate normalization for Cell-based Westerns can be achieved by staining cells.

- CellTag™ 700 Stain: This cell stain accumulates in the nucleus and cytoplasm of permeabilized cells, providing an accurate method for normalization to cell number (licor.com/CellTag).

- Total protein staining: First developed by Hoffman and colleagues (10), this method uses reactive infrared dyes to covalently label cellular proteins on lysine residues. IRDye® 800CW or IRDye 700DX N-hydroxysuccinimidyl ester (NHS) can couple to free amine groups on lysine residues to form a stable conjugate. Cells in an In-Cell Western assay are fixed and permeabilized, so the reactive dye has access to both cell surface and internal lysine residues, which greatly increases the amount of proteins that can be labeled. The full protocol is available (licor.com/FixCellNormalization).

Housekeeping Protein

As with a Western blot, you must validate that a housekeeping protein is stable in the conditions in your experiment before using the housekeeping protein for normalization in a Cell-based Western.

Post-Translational Modification

If you are studying a post-translational modification, you can normalize to the total form of the protein (regardless of modification). For example, phospho-ERK detected with a specific primary antibody and an IRDye 800CW secondary antibody can be normalized to total ERK detected with a specific primary antibody and a spectrally-distinct IRDye 680RD secondary antibody.
Well Types

- Background wells are not incubated with primary antibody or normalization reagent. These wells are used to identify nonspecific secondary antibody binding.

- Negative controls are incubated normally with primary and secondary antibodies, and, for example, do not receive treatment.

- Positive controls are incubated normally with primary and secondary antibodies, and receive the appropriate treatment to elicit the maximum desired response.

The Microplate Setup Guide (licor.com/MicroplateSetup) will help you set up and organize your plates with all the necessary well types.

Z’-Factor Analysis

The Z’-Factor statistic is a dimensionless value that represents the variability and dynamic ranges between two sets of sample control data. The Z’-Factor provides a way to evaluate if assay conditions are optimized and that the assay is reliable.

Before using a Cell-based Western to characterize targeting agent activity, it is best to determine the overall quality and reliability of the assay using a Z’-Factor assay. A Z’-Factor Assay consists of a plate with background, negative control, and positive control wells.

You can read about the Z’-Factor analysis in the Z’-Factor Tech Note (licor.com/zfactor) or the Z’-Factor White Paper (licor.com/zfactor_app_note).
Protocol Overview

1. Culture cells

2. Stimulate or inhibit cells

3. Fix and permeabilize cells

4. Add blocking buffer

5. Incubate cells with primary antibody

6. Incubate cells with IRDye® secondary antibody

7. Image with an Odyssey® CLx Imager
Protocol (In-Cell Western Assay)

This protocol highlights the main steps of an In-Cell Western™ Assay.

The On-Cell Western Assay protocol is very similar. The main difference is that cells are not permeabilized in an On-Cell Western Assay.

Be sure to characterize primary antibody specificity and conduct a Z’-Factor Assay before performing an In-Cell Western Assay.

**Step 1. Prepare Cell Culture**

Prepare cell culture in a micro-well plate.

**Step 2. Apply Treatment**

Apply treatment for your experiment, e.g. inhibitor, stimulant, or other potential therapeutic agent.

**Step 3. Remove Media**

Remove media by aspiration. Aspiration can lead to cell loss and should be done carefully, but it is still recommended over inverting the plate.

Proceed immediately to the next step.

**Step 4. Fix Cells**

Immediately fix cells with Fixing Solution appropriate for your experiment. If optimal fixing conditions are known for your cell line and target, those conditions may be an appropriate starting point.

Using a multichannel pipettor, gently pipette Fixing Solution down the side of each well. Be careful not to dislodge cells.

**Step 5. Permeabilize Cells**

**Note:** For On-Cell Western Assays, cells are not permeabilized.

1. Remove fixing solution to an appropriate waste container.
2. Add Wash Solution to each well. Be careful to avoid detaching the cells.
3. Repeat wash steps 4 more times. Do not allow wells to become dry.
Step 6. Block Cells

No single blocking reagent will be optimal for every antigen-antibody pair, so several varieties of Intercept® Blocking Buffer are available.

Intercept Blocking Buffer can be used for Cell-based Western assays. It is available in protein-based and protein-free formulations in TBS and PBS to provide a wide variety of options for many different types of experiments.

Using a multi-channel pipettor, add Intercept Blocking Buffer to each well. Be careful not to dislodge cells.

Step 7. Dilute Primary Antibody

The primary antibody should be diluted in the same solution used for blocking.

Incubate with Primary Antibody

1. Remove blocking buffer.
2. Add Intercept Blocking Buffer to the control wells and diluted primary antibody to the rest of the wells.
3. Incubate with primary antibody in conditions appropriate for your experiment.

Step 8. Wash Plate

1. Using a multichannel pipettor, add Washing Solution to each well. Carefully avoid detaching cells while adding Washing Solution and shaking on a plate shaker.
2. Repeat washing steps 4 more times.

Step 9. Dilute Secondary Antibody

The secondary antibody should be diluted in the same solution used for blocking, but with Tween 20 added.

Intercept T20 Antibody Diluent consists of Intercept Blocking Buffer with high-quality Tween® 20 already diluted. To work with all varieties of Intercept Blocking Buffer, Intercept T20 Antibody Diluent is available in protein-based and protein-free formulations in TBS and PBS.

Note: Protect the antibody vials and plates from light.
Step 10. Incubate with Secondary Antibody

Secondary antibody staining and normalization are carried out simultaneously. For background wells, add only diluted secondary antibody without the normalization agent, such as CellTag™ 700 Stain (licor.com/CellTag).

1. Add CellTag™ 700 Stain to the diluted secondary antibody solution.
2. To the control wells, add secondary antibody solution without CellTag 700 Stain.
3. To the remaining wells, add secondary antibody solution with CellTag 700 Stain.
4. Incubate in conditions appropriate for your experiment. Protect from light during incubation.

Step 11. Wash Plate

**Note:** Protect plate from light during wash.

1. Using a multichannel pipettor, add Washing Solution to each well. Carefully avoid detaching cells while adding Washing Solution and shaking on a plate shaker.
2. Repeat wash procedure 4 more times.

Step 12. Remove Wash Solution

Remove wash solution completely from the wells.

Turn the plates upside down, and tap or blot gently on paper towels to remove traces of wash buffer.

Step 13. Scan with an Odyssey® CLx Imager

For best results, scan the plate immediately. However, plates can be stored for up to several weeks at 4 °C.

1. Clean the bottom of the plate with a lint-free wipe.
2. Scan the plate in the 700 and 800 nm channels using an Odyssey CLx Imager.

Step 14. Analysis

You can analyze the plate image using Image Studio Software with the In-Cell Western™ Assay Analysis Key (licor.com/AnalysisKeys).
Figure 2. The Image Studio Software In-Cell Western™ Assay Analysis key provides options for normalization, percent response, and Z’-Factor analysis.

**Tissue Section Imaging and Microscopy**

Tissue section imaging and microscopy are effective for granular identification of targeting agent locations within an organ.

If you are developing a therapeutic agent for clinical use, the Odyssey® CLx Imager can be used to image tissue sections macroscopically to measure uptake and localization of your probe in tissues and organs ex vivo. The sections can be labeled with standard immunohistochemical protocols or in vivo as part of an animal imaging study.
Tissue sections are stained with IRDye® labeled antibodies, and imaged at 21 μM resolution for macro-level analysis. Thirty slides can be imaged in a single scan for screening, so valuable microscope time can be used efficiently.

With appropriate microscopy equipment, you can assess NIR fluorescent-labeled targeting agent location (e.g. membrane bound, intracellular, or interstitial) in more detail.

The Tissue Section Imaging protocol (licor.com/tissueimaging) describes how to prepare tissue sections for imaging on an Odyssey® CLx Imager.

**In Vivo and Ex Vivo Imaging**

Studying the specificity of your potential therapeutic agent in vivo allows you to confirm localization to a specific tissue or organ, and it allows you to identify any non-specific interactions. Cell surface receptors and transporters are the most common targets for in vivo specificity studies.

Low autofluorescence from tissue components and deeper penetration of light in the NIR spectrum allows sensitive detection and semi-quantitative assessment of your potential therapeutic agent’s affinity to the intended target. Uniform NIR laser illumination across the entire imaging area in the Pearl® Trilogy Imager (licor.com/pearl) enables reproducible in vivo measurement of specificity. Along with uniform illumination and detection, the Pearl Trilogy also provides a uniquely wide dynamic range and high sensitivity.

For more information about ex vivo imaging, see the Ex Vivo Imaging protocol (licor.com/ex_vivo_guide).

**III. Example Experiments**

Creative uses of these assays are included below to spark ideas.

**Target Identification**

Identifying which targets make sense for your therapeutic can be a daunting task. But with DigiWest® high-content profiling, you can rapidly assess hundreds of potential targets.

**Discover Biomarkers DigiWest® Protein-Profiling**

This study used DigiWest® for analysis of mechanisms and biomarkers of platinum resistance in ovarian cancer patients.

- A set of 24 fresh frozen tumor specimens from relapsed vs cured platinum-treated ovarian cancer patients was analyzed by DigiWest®, using 466 antibodies (total and phospho
proteins) covering various cell signaling pathways.

- DigiWest® protein signatures distinguished platinum resistant vs sensitive patients, and revealed 8 promising biomarker candidates.

Figure 3. Image shows a heat map of potential biomarkers (targeted with antibodies shown on the y-axis) in each tumor sample (x-axis). Red squares indicate an increase in biomarker expression compared to control, black indicates no change, and green squares indicate a decrease. (digiwest.de)

**Gene Therapy**

Knocking down genes that code for signaling proteins can be useful for pathway characterization and for therapeutic purposes.

Studying Adenoviral CRISPR/Cas9 Effects on Fibrosis Development Using Near-Infrared Western Blots Imaged on an Odyssey® CLx Imager

Example Study: “Highly efficient gene inactivation by adenoviral CRISPR/Cas9 in human primary cells.”
Research conducted by Voets, O., et al, Galapagos BV, PLoS One.23

Efficient gene knockdown techniques enable therapeutics researchers to better understand disease processes and advance drug discovery by identifying and validating biological targets. An example from respiratory disease research is the use of Adenoviral (AdV) CRISPR/Cas9 to reduce the expression of SMAD3, a protein involved in triggering fibrosis.
Figure 4. AdV CRISPR/Cas9 exhibited significant SMAD3 knockdown in human primary cells. SMAD3 (red) and Cas9 (red) were detected in normal human lung fibroblasts (NHLF) and human bronchial epithelial cells (HBEC) and normalized to β-actin (green) using IRDye® Secondary Antibodies on NIR Western blots. A control guide RNA (gRNA C1) provided additional verification of knockdown specificity and efficiency. Images were acquired on an Odyssey® CLx Imager. Adapted from Voets, O., et al.

**Binding Specificity**

Whether you are characterizing ligand binding or therapeutic binding to targets, binding specificity or competition assays will be useful.

**Assessing Binding Specificity Using an On-Cell Western Assay Imaged on an Odyssey Imager**

Example Study: “On-Cell Western Plate-Based Assay for Targeted Near-Infrared-Labeled Optical Imaging Agent Development: Receptor-Based Binding and Competition Assay.” Research conducted by LI-COR Biosciences. 14

It is critical to establish binding specificity of a potential therapeutic agent to its purported target early in the therapeutics research process. The On-Cell Western format can be used to study specificity of an unlabeled ligand by comparison to a form of the ligand labeled with an NIR fluorescent dye.
Figure 5. The binding specificity of EGF to EGFR is observed using a competition assay with unlabeled EGF and EGF labeled with IRDye® 800CW (IRDye 800CW-EGF). IRDye 800CW-EGF was detected in the 800 nm channel (green) and normalized to cell number measured using CellTag™ 700 Stain detected in the 700 nm channel (red). A) Wells were treated with increasing concentrations of IRDye 800CW-EGF and a corresponding increase in normalized 800 nm signal indicated an increase in binding. B) IRDye 800CW-EGF binding is blocked with increasing concentrations of unlabeled EGF, demonstrating specific binding of unlabeled EGF.

**Dose Response**

Understanding how much ligand or therapeutic is needed to stimulate a response is essential to therapeutics research projects.

**Determining IC_{50} by In-Cell Western™ Assay Imaged on an Odyssey® CLx Imager**

Example Study: “Small molecule inhibitors reveal PTK6 kinase is not an oncogenic driver in breast cancers.” Research conducted by Qiu, L., et al, Pfizer, PLoS One.³

PTK6 is abnormally expressed in breast cancers, and researchers have described kinase-dependent functions of PTK6 in driving tumor growth. As with any therapeutic approach, using small molecule inhibitors to target PTK6 activity requires validation of specificity and efficacy.
Figure 6. Inhibition of tumor cell growth by PTK6 inhibitors is independent of PTK6 kinase activity inhibition, providing evidence against using PTK6 kinase inhibition as a therapeutic strategy for breast cancer treatment. In-Cell Western™ Assay imaged on an Odyssey® CLx Imager were used to assess cellular levels of PTK6 auto-phosphorylation normalized to cell number (measured by CellTag™ 700 Stain). Adapted from Qiu, L., et al.

Therapeutic Characterization

Therapeutics will be characterized for their mode of action, off target effects, and contrasted to other lead candidates or current drugs on the market. High content profiling can make this part of the research project efficient.

Characterizing Lead Compounds with DigiWest® Protein-Profiling

In this study, DigiWest® was used to perform a mechanism-of-action study of compounds in comparison to a reference therapeutic.

- Calu1 cells were treated with 1 MEK inhibitor vs 1PI3K inhibitor vs 2 experimental lead compounds (data not shown) vs DMSO. Cell samples were analyzed by DigiWest® using 156 selected antibodies (total and phospho proteins) covering different signal transduction pathways.
- DigiWest® yielded distinct signatures for each compound and allowed for in-depth characterization of lead compounds as compared to reference therapeutic.
Assessing Phenotypic Effects

Understanding the cellular response to a potential therapeutic agent is critical information to gather in early phases of therapeutics research.

Detecting Apoptosis Using an In-Cell Western™ Assay

Example Study: “Detecting Apoptosis in HeLa Cells Following Anisomycin Treatment” Research conducted by LI-COR Biosciences.9

Understanding the processes that lead to programmed cell death (apoptosis) is of utmost importance in the study of many diseases, including autoimmune diseases, neurological diseases, and cancer (25).

The following example illustrates how the In-Cell Western Assay format is useful for quantifying apoptosis in response to drug treatment. Anisomycin, a protein synthesis inhibitor, has been shown to trigger apoptosis in several cancer cell lines (26 - 32). The Caspase-3 protein has been implicated as an effector caspase associated with the initiation of apoptosis (33). In this study, the effect of anisomycin on HeLa cells was measured by Caspase-3 activation.
Figure 8. 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. Error bars represent the standard deviation from four technical replicates. The ultimate result of apoptosis induction is cell death. The reduction in cell number per well is taken into account when normalizing with CellTag™ 700 Stain. In an assay such as this, normalization is very important. See www.licor.com/ICWnormalization for more information on normalization options for an In-Cell Western™ Assay.

Delivery and In Vivo Characterization

As your therapeutics research advances, it will become important to assess delivery methods of your therapeutic agent and localization of the therapeutic agent in vivo.

Assessing Biodistribution of an siRNA Delivery System for a Promising Cancer Treatment Using the Pearl® Trilogy Imager


Ovarian and uterine cancers are often diagnosed at advanced stages, underscoring the need to identify therapeutic targets and means of delivering therapies to tumors. One promising therapeutic target is the AXL protein, which is involved in ovarian and uterine cancer metastasis. One potential treatment option is to silence AXL expression with siRNA, but a mechanism is needed to deliver the siRNA to the tumor.
**Figure 9.** p5RHH-siRNA nanoparticles were found to localize to tumor cells in vivo. To determine localization of p5RHH nanoparticles and to identify the best delivery method, mice bearing either uterine or ovarian cancer tumors were injected either intraperitoneally (IP) or intravenously (IV) with p5RHH-siControl nanoparticles labeled with a fluorescent probe. Excised tumors from IP-injected mice showed more probe localization than those from IV-injected mice. Live mouse and whole organ images were acquired on a Pearl® Trilogy Imager.
IV. References


