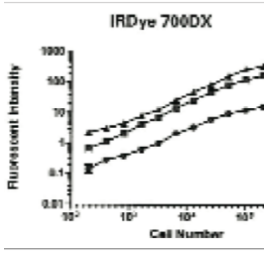
Sep 28, 2018

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



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LI-COR Biosciences[1]

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Manuscript citation:

1. Chen, H et al. A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy. Analyt Biochem 338: 136-142 (2005).

2. Wong, SKF. A 384-well cell-based phospho-ERK assay for dopamine D2 and D3 receptors. Analyt Biochem 333: 265-272 (2004).

3. Selkirk, JV et al. A novel cell-based assay for G-protein-coupled receptor-mediated cyclic adenosine monophosphate response element binding protein phosphorylation. Biomolecular Screening 11: 351-358 (2006).

4. Kumar, N et al. Multipathway model enables prediction of kinase inhibitor cross-talk effects on migration of Her2-overexpressing mammary epithelial cells. Mol Pharmacol 73:1668-1678 (2008).

5. Hannoush, RN. Kinetics of Wnt-driven b-catenin stabilization revealed by quantitative and temporal imaging. PLoS ONE 3(10):e3498 (2008).

6. Hoffman, G et al. A functional siRNA screen for novel regulators of mTORC1 signaling. Poster presentation, American Society for Cell Biology Annual Meeting (2008).

7. Hoffman, GR et al. A high-throughput, cell-based screening method for siRNA and small molecule inhibitors of mTORC1 signaling using the In-Cell Western technique. Assay Drug Dev Technol. doi:10.1089/adt.2009.0213 (2010).

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We use this protocol and it's working

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Abstract

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

Attachments

AppNote_Reagents_IRD.
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142KB

Guidelines

I. Introduction

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

The first method uses primary and secondary antibodies to detect two distinct targets. For example, phospho-ERK can be detected using a specific primary antibody and a secondary antibody labeled with IRDye 800CW infrared dye. In a multiplex assay, a primary antibody against total ERK (or against a housekeeping protein or other target) can be detected with a secondary antibody labeled with a spectrally-distinct IRDye fluorophore1, such as IRDye 680RD. The protocol has been widely used in scientific literature[1-5].

LI-COR also offers a one-step solution for In-Cell Western assay normalization using CellTag™ 700 Stain (LI-COR P/N 926-41090). CellTag 700 Stain allows for normalization across the same range of cell densities as Sapphire700TM and DRAQ5® Stains combined and enables accurate measurement of target protein levels when combined with an IRDye 800CW secondary antibody. Please visit http://www.licor.com/bio for more detailed information regarding CellTag 700 Stain.

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

As a component of IRDye Infrared Dye labeling kits. These kits contain other components typically used to label antibodies and other proteins, which are not used in this cell labeling protocol.

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

This method adds only two brief steps to the protocol and provides several advantages over previous methods:
Extreme sensitivity. The lower-limit detection is approximately 200 cells per well.

Quantitative accuracy. Wide linear range of signal extends from 200 to 200,000 cells in our experiments (Fig. 1).

Cost-effectiveness. Because highly-diluted dye solutions are used, a 0.5 mg vial of IRDye 700DX reactive dye can label 50 plates of cells; a 0.5 mg vial of IRDye 800CW is sufficient to label 500 plates (Figure 2).

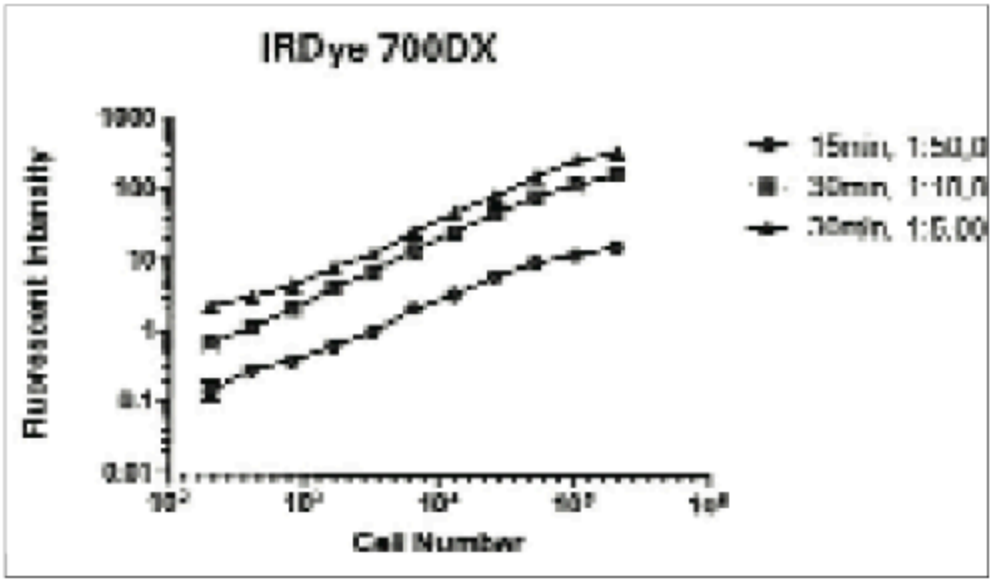

Figure 1. Linear relationship between fluorescent intensity and number of cells, using IRDye 700DX for labeling. Two-fold serial dilutions of HeLa cells were plated in clear, flat-bottomed 96-well plates, then fixed and permeabilized. Cells were labeled with IRDye 700DX with the dilutions and incubation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

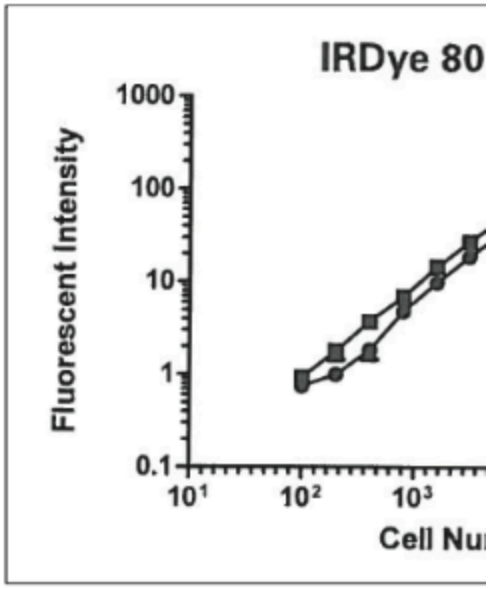

Figure 2. Linear relationshi
tensity and number of cells
labeling. Two-fold serial dil
were plated in clear, flat-bo
then fixed and permeabiliz
IRDye 800CW at 1:50,000

II. Suggested Materials

IRDye 800CW OR IRDye 700DX NHS ester reactive dye

- IRDye 800CW NHS ester, 0.5 mg (P/N 929-70020) or 5 mg (929-70021)
- IRDye 700DX NHS ester, 0.5 mg (P/N 929-70010) or 5 mg (929-70011)

Dry (anhydrous grade) dimethyl sulfoxide (DMSO)

Odyssey® Blocking Buffer (PBS) (P/N 927-40000)

1X PBS wash buffer

Tissue culture reagents (serum, DMEM, trypsin, etc.)

Clear or black 96-well microplate, with clear bottom

37% formaldehyde

20% Tween® 20

10% Triton® X-100

III. Protocol

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

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

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

See 'STEPS'

References

Chen, H et al. A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy. Analyt Biochem 338: 136-142 (2005).

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Materials

MATERIALS

- Odyssey® Blocking Buffer (PBS) **LI-COR Catalog #**927-40000 927-40100
- IRDye 700DX Protein Labeling Kit, High Molecular Weight **LI-COR Catalog #**928-38046
- IRDye 800CW Protein Labeling Kit, High Molecular Weight **LI-COR Catalog #**928-38040
- IRDye 700DX NHS ester, 0.5 mg **LI-COR Catalog #**929-70010
- IRDye 800CW NHS ester, 0.5 mg **LI-COR Catalog #**929-70020

Safety warnings

See SDS (Safety Data Sheet) for hazards and safety warnings.

Before start

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

Cell Preparation and Fixation

1 Prepare fresh ***Fixing Solution*** as follows:

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

45 mL 1x PBS

5 mL 37% Formaldehyde

50 mL 3.7% Formaldehyde

2 Remove media from microtiter plate manually or by aspiration.

3 Using a multi-channel pipette, immediately add 150 µL of fresh, room-temperature ***Fixing Solution.***

Note

Add the ***Fixing Solution*** carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.

150 µL Fixing Solution

4 Allow incubation on bench top for 20 minutes at room temperature with no shaking.

00:20:00 Incubation

Note

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

Permeabilization

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

Prepare PBS + Triton X-100 as follows:

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

Note

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

6 Remove ***Fixing Solution*** (contains formaldehyde) to an appropriate waste container and dispose of properly.(wash 1/5)

7 Using a multi-channel pipette, add 200 µL of PBS + Triton X-100 to each well. (wash 1/5)

Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.

200 µL PBS + Triton X-100

8 Allow wash to shake on a rotator for 5 minutes. (wash 1/5)

00:05:00

Note

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

9 Remove wash manually. (wash 1/5)

10 Using a multi-channel pipette, add 200 µL of PBS + Triton X-100 to each well. Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells. (wash 2/5)

200 µL PBS + Triton X-100

11 Allow wash to shake on a rotator for 5 minutes. (wash 2/5)

00:05:00

12 Remove wash manually. (wash 2/5)

13 Using a multi-channel pipette, add 200 µL of PBS + Triton X-100 to each well. Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells. (wash 3/5)

200 µL PBS + Triton X-100

14 Allow wash to shake on a rotator for 5 minutes. (wash 3/5)

00:05:00

15 Remove wash manually. (wash 3/5)

16 Using a multi-channel pipette, add 200 µL of PBS + Triton X-100 to each well. Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells. (wash 4/5)

200 µL PBS + Triton X-100

17 Allow wash to shake on a rotator for 5 minutes. (wash 4/5)

00:05:00

18 Remove wash manually. (wash 4/5)

19 Using a multi-channel pipette, add 200 µL of PBS + Triton X-100 to each well. Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells. (wash 5/5)

200 µL PBS + Triton X-100

20 Allow wash to shake on a rotator for 5 minutes. (wash 5/5)

00:05:00

21 Remove wash manually. (wash 5/5)

Cell Number Staining

22 Prepare a 1 mg/mL solution of the dye.

LI-COR supplies vials of reactive dye in lyophilized form. You must resuspend the dye in an organic solvent (anhydrous DMSO) before use.

Note

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

23 To preserve dye reactivity, resuspend the contents of the vial in dry(anhydrous grade) DMSO at a concentration of 1 mg/mL.

24 After the content of the dye vial is resuspended in DMSO, protect the vial from light and store at -20 °C.

-20 °C Storage

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

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

or

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

26 Remove PBS + Triton® X-100 from each well of the plate manually or by aspiration.

27 Add 200 µL diluted dye solution to each well.

200 µL diluted dye solution

28 Incubate for 20 minutes for IRDye 800CW.

00:20:00

29 Incubate for 30 minutes for IRDye 700DX.

00:30:00

Wash Out Unbound Dye

30 Wash each well with PBS + 0.1% Tween® 20, for 5 minutes.

00:05:00

Note

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

31 Wash each well with PBS + 0.1% Tween® 20, for 5 minutes.
00:05:00

32 Wash each well with PBS + 0.1% Tween® 20, for 5 minutes.
00:05:00

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