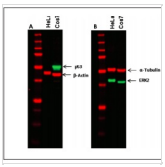

Working

Sep 28, 2018

ICW Detection and Considerations

Forked from a private protocol

LI-COR Biosciences[1]

[1]LI-COR Biosciences

dx.doi.org/10.17504/protocols.io.guqbwvw

LI-COR Biosciences

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ABSTRACT

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

Developed for: Odyssey® Family of Imagers

EXTERNAL LINK

https://www.licor.com/documents/86xilzixljcaz6rreqy9fx8dwz4rps55

TechNote_IRWesternBlot_ICW_SubclassSpecAb_0311_11784.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

I. Introduction

IRDye Goat anti-Mouse IgG1, Goat anti-Mouse IgG2a and Goat anti-Mouse IgG2b, 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}, IgG2b, IgG_{2c} and IgG_3. Each subclass is based on small differences in amino acid sequences in the constant region of the heavy chains so antibodies directed against a particular subclass will not recognize antibodies directed against other subclasses. For example, IRDye goat anti-mouse IgG_1 recognizes mouse gamma 1; it will not recognize mouse gamma 2a, 2b, 2c or gamma 3. All other LI-COR IRDye secondary antibodies are whole IgG (H+ L) and react with the heavy (gamma) and light (kappa or lambda) chains of the primary antibody. Figure I demonstrates the differences in detection between the IRDye antibodies.

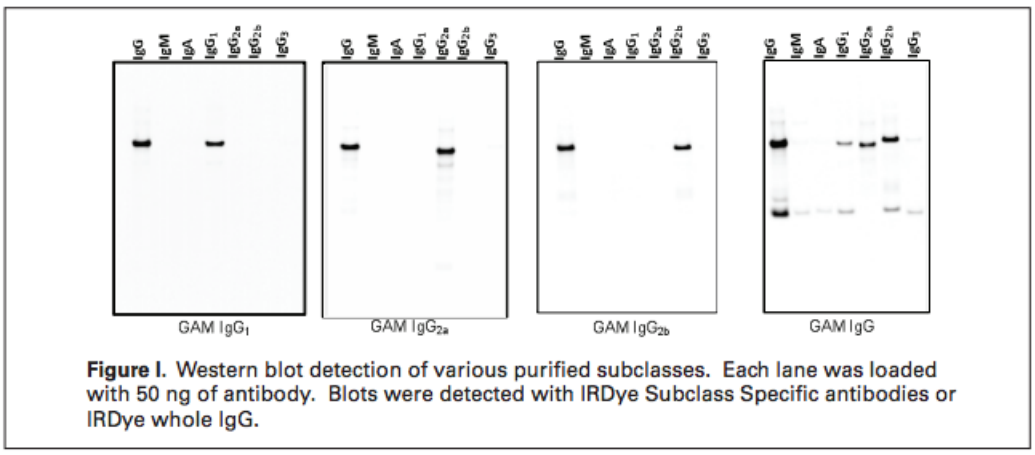

Figure I. Western blot detection of various purified subclasses. Each lane was loaded with 50 ng of antibody. Blots were detected with IRDye Subclass Specific antibodies or IRDye whole IgG.

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

II. Suggested Materials

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

- Proteins transferred to a nitrocellulose or PVDF membrane (for Western blot only)
- Cells that have been fixed and permeabilized on a 96 well plate (for ICW only)
- Odyssey® Blocking Buffer
- 10X PBS
- 20% Tween® 20
- SDS (if using PVDF membrane)
- Suggested mouse primary antibodies for normalization:
  - Beta-Actin Mouse mAb IgG_{2b} (LI-COR P/N 926-42212)
  - Alpha-Tubulin Mouse mAb IgG_1 (LI-COR P/N 926-42213)
- One or two of the following IRDye secondary antibodies

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

III. Western Blot Detection

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

IV. Two-Color Western Blot Considerations

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

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

Figures II (A-C) and III (A-C) demonstrate two-color Western blot detection using (A) IRDye Subclass Specific antibodies and (B) IRDye whole IgG antibodies, respectively. IRDye Subclass Specific secondary antibodies should NOT be used in combination with IRDye whole anti-mouse IgG secondary antibodies for two-color detection. IRDye whole anti-mouse IgG secondary antibodies and IRDye Subclass Specific secondary antibodies both recognize the gamma chain of the primary antibody, causing detection in both channels (C). IRDye Subclass Specific antibodies can be used in combination with IRDye whole goat anti-rabbit secondary antibodies.

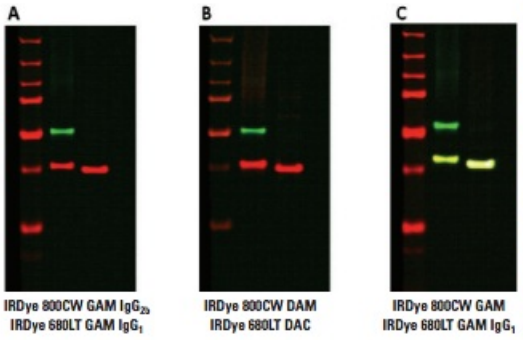

Figure II. Western blot analysis of PTEN expression in mouse PTEN transfected 293T whole cell lysate (Lane 2) and non-transfected 293T lysate (Lane 3). Both lysates were loaded with 2 µg total protein per lane. LI-COR® Molecular Weight Marker is loaded in Lane 1 (LI-COR P/N 928-40000).
A. Blot was probed with mouse anti-PTEN (IgG_{2b}) and mouse anti-GAPDH (IgG_1) for normalization. The blot was detected with IRDye 800CW GAM IgG_{2b} (LI-COR P/N 926-32352) and IRDye 680LT GAM IgG_1 (LI-COR P/N 926-68050).
B. Blot was probed with mouse anti-PTEN (IgG_{2b}) and chicken anti-GAPDH for normalization. The blot was detected with IRDye 800CW DAM (LI-COR P/N 926-32212) and IRDye 680LT DAC (LI-COR P/N 926-68028).
C. Blot was probed with mouse anti-PTEN (IgG_{2b}) and mouse anti-GAPDH (IgG_1) for normalization. The blot was detected with IRDye 800CW GAM (LI-COR P/N 926-32210) and IRDye 680LT GAM IgG_1 (LI-COR P/N 926-68050).

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

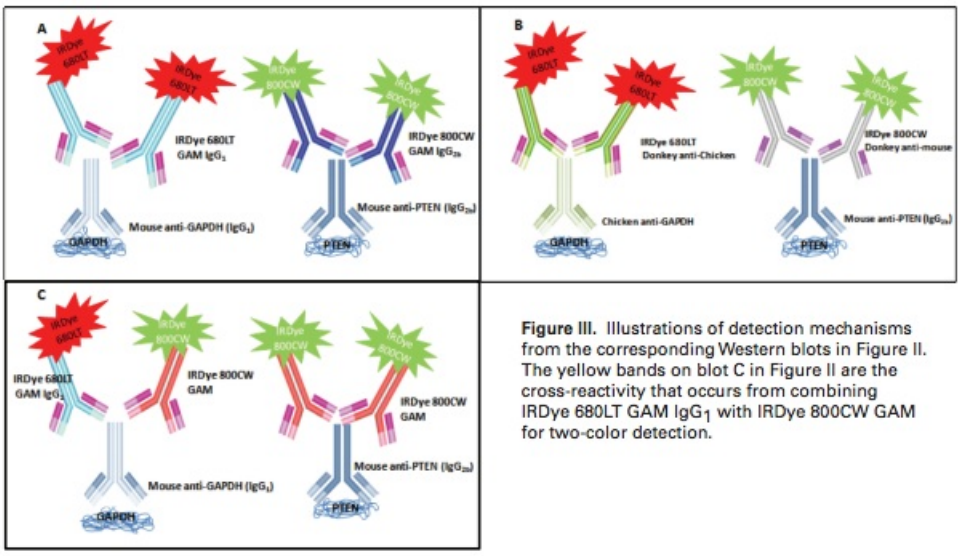

Figure III. Illustrations of detection mechanisms from the corresponding Western blots in Figure II. The yellow bands on blot C in Figure II are the cross-reactivity that occurs from combining IRDye 680LT GAM IgG_1 with IRDye 800CW GAM for two-color detection.

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

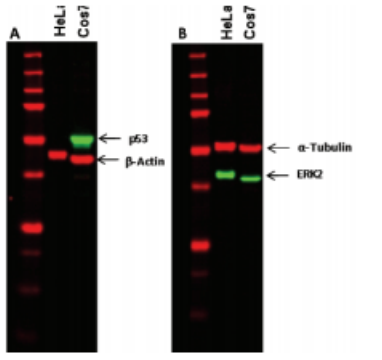

Figure IV. Two-color Western blot detection using LI-COR® mouse primary antibodies. Two different blots were prepared using HeLa and COS7 whole cell lysates. Both lysates were loaded at 5 µg total protein per lane.
A. The blot was probed with Beta-Actin Mouse mAb IgG_{2b} (LI-COR P/N 926-42212) and p53 mouse mAb IgG_{2a}. The blot was detected with IRDye 800CW GAM IgG_{2a} and IRDye 680LT GAM IgG_{2b}.
B. The blot was probed with Alpha-Tubulin Mouse mAb IgG_1 (LI-COR P/N 926-42213) and ERK2 mouse mAb IgG_{2b}. The blot was detected with IRDye 680LT GAM IgG_1 and IRDye 800CW GAM IgG_{2b}.

V. ICW Detection and Considerations

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

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

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

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

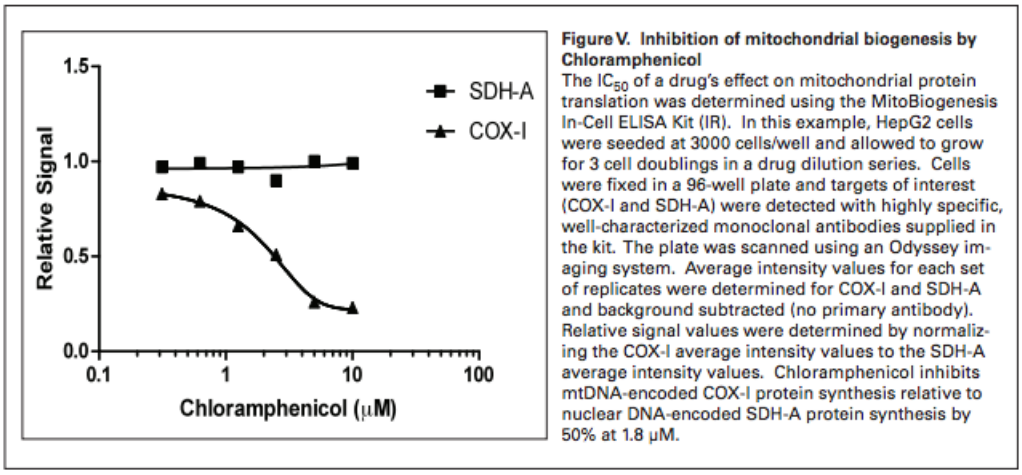

Figure V. Inhibition of mitochondrial biogenesis by Chloramphenicol
The IC_{50} of a drug's effect on mitochondrial protein translation was determined using the MitoBiogenesis In-Cell ELISA Kit (IR). In this example, HepG2 cells were seeded at 3000 cells/well and allowed to grow for 3 cell doublings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

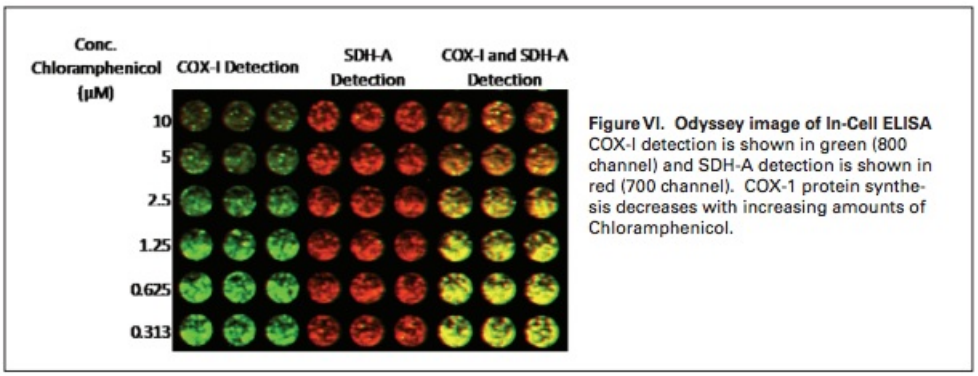

Figure VI. Odyssey image of In-Cell ELISA
COX-I detection is shown in green (800 channel) and SDH-A detection is shown in red (700 channel). COX-1 protein synthesis decreases with increasing amounts of Chloramphenicol.

Figure VII compares IRDye whole IgG vs. IRDye Subclass Specific antibody detection by ICW. Extracellular-signal related kinase (ERK) phosphorylation was measured following the LI-COR protocol entitled, In-Cell Western Assay For Assessing Response of A431 Cells to Stimulation with Epidermal Growth Factor. This document can be found on the LI-COR website (Doc# 988-11453). All primary antibodies were qualified by Western blot prior to ICW (data not shown).

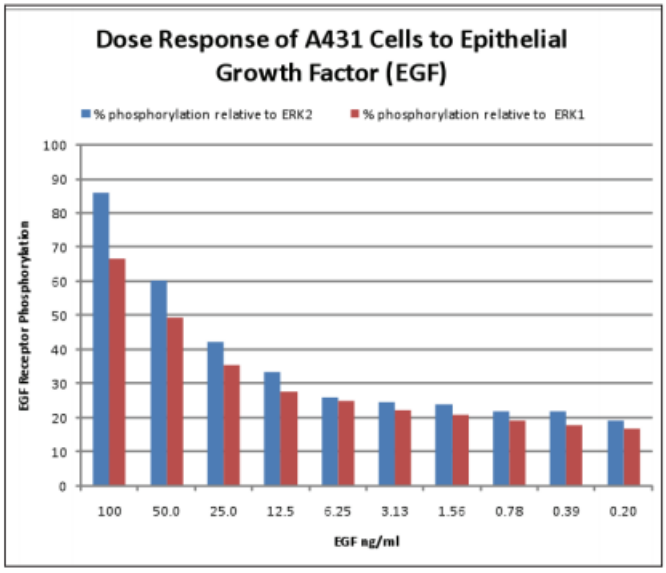

Figure VII. Dose response data
ERK phosphorylation is graphed relative to ERK2 (blue) or ERK1 (red). The ERK2 data was generated using mouse anti-pERK (IgG_{2a}) and mouse anti-ERK2 (IgG_{2b}) followed by detection with IRDye 800CW GAM IgG_{2a} (LI-COR P/N 926-32351) and IRDye 680LT GAM IgG_{2b} (LI-COR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

MATERIALS

NAME	CATALOG #	VENDOR
Beta-Actin Mouse mAb IgG2b	926-42212	LI-COR
Alpha-Tubulin Mouse mAb IgG1	926-42213	LI-COR
IRDye 800CW Secondary Antibodies	926-32350 926-32351 926-32352	LI-COR
IRDye 680LT Secondary Antibodies	926-68050 926-68051 926-68052	LI-COR

SAFETY WARNINGS

See SDS (Safety Data Sheets) for warnings and hazards.

1 Using a multi-channel pipettor, block cells by adding 150 µL of Odyssey® Blocking Buffer to each well.

NOTE
Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.

150 µl Odyssey® Blocking Buffer

2 Allow blocking for 1.5 hours at room temperature with gentle shaking on a plate shaker.

01:30:00

3 Dilute desired primary antibodies in Odyssey Blocking Buffer or other appropriate blocker.

NOTE
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.

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.

4 Remove blocking buffer from step 1.

5 Add 50 µL of Odyssey Blocking Buffer to the control wells and 50 µL of the desired diluted primary antibodies in Odyssey Blocking Buffer to the rest of the wells.

50 µl Odyssey Blocking Buffer

50 µl desired primary antibodies

6 Incubate with primary antibody solution for 2 hours at room temperature with gentle shaking, or overnight at 4°C with no shaking.

02:00:00

7 For washing the plate five times with 1X PBS + 0.1% Tween® 20, prepare Tween Washing Solution by adding 5 mL of 20% Tween 20 to 995 mL of 1X PBS.

5 ml 20% Tween 20

995 ml 1X PBS

8 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.** (wash 1/5)

200 µl Tween Washing Solution

9 Allow wash to shake gently on a plate shaker for 5 minutes. (wash 1/5)

00:05:00

10 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.** (wash 2/5)

200 µl Tween Washing Solution

11 Allow wash to shake gently on a plate shaker for 5 minutes. (2/5)

00:05:00

12 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.** (wash 3/5)

200 µl Tween Washing Solution

13 Allow wash to shake gently on a plate shaker for 5 minutes. (3/5)

00:05:00

14 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.** (wash 4/5)

200 µl **Tween Washing Solution**

15 Allow wash to shake gently on a plate shaker for 5 minutes. (4/5)

00:05:00

16 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.** (wash 5/5)

200 µl **Tween Washing Solution**

17 Allow wash to shake gently on a plate shaker for 5 minutes. (5/5)

00:05:00

18 Dilute the IRDye labeled Subclass Specific antibodies in Odyssey® Blocking Buffer or another appropriate blocker.**Avoid prolonged exposure of the antibody vials to light.**

NOTE

The recommended dilution range is 1:200 to 1:1,200. The optimal dilution for your assay should be determined empirically. To lower background, add Tween® 20 at a final concentration of 0.2% to the diluted antibody. Secondary antibody staining is carried out simultaneously.

19 Add 50 µL of secondary antibody solution into all wells. Incubate for 1 hour at room temperature, with gentle shaking. **Protect plate from light during incubation.**

50 µl **secondary antibody**

01:00:00 **incubation**

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

21 Using a multi-channel pipettor, add 200 µL of Tween Washing Solution (from STEP 8). **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom. Protect plate from light during washing.** (wash 1/5)

200 µl **Tween Washing Solution**

22 Allow wash to shake gently on a plate shaker for 5 minutes. (wash 1/5)

00:05:00

23 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. Protect plate from light during washing.** (wash 2/5)

200 µl **Tween Washing Solution**

24 Allow wash to shake gently on a plate shaker for 5 minutes. (wash 2/5)

00:05:00

25 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. Protect plate from light during**

washing. (wash 3/5)

200 µl **Tween Washing Solution**

26 Allow wash to shake gently on a plate shaker for 5 minutes. (wash 3/5)

00:05:00

27 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. Protect plate from light during washing.** (wash 4/5)

200 µl **Tween Washing Solution**

28 Allow wash to shake gently on a plate shaker for 5 minutes. (wash 4/5)

00:05:00

29 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. Protect plate from light during washing.** (wash 5/5)

200 µl **Tween Washing Solution**

30 Allow wash to shake gently on a plate shaker for 5 minutes. (wash 5/5)

00:05:00

31 The plate is now ready to image.