On-Cell Western Plate-Based Assay for Targeted Near-Infrared-Labeled Optical Imaging Agent Development: Receptor-Based Binding and Competition Assays

LI-COR Biosciences

Abstract

This protocol is intended to illustrate the process for testing a particular cell type with the IRDye 800CW EGF for eventual use in vivo. A431 (epithelial carcinoma) cells are used in this example because of their over-expression of EGFR. Media considerations and certain cell characteristics will naturally alter this protocol if a different cell line is used.

Developed for:

Aerius,

Odyssey® Classic,

Odyssey CLx, and

Odyssey Sa

Infrared Imaging Systems

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Guidelines

I. Required Reagents

LI-COR Reagents

- IRDye 800CW EGF (P/N 926-08446)
- CellTagTM 700 Stain (LI-COR, P/N 926-41090)
- Odyssey® Blocking Buffer (PBS) (LI-COR, P/N 927-40000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- 100% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100
- Costar™ 96-well Black Clear-Bottom Plate (Corning)

SPECIAL NOTE: Serum starvation of the cells is required to obtain maximal response.

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

"See STEPS"

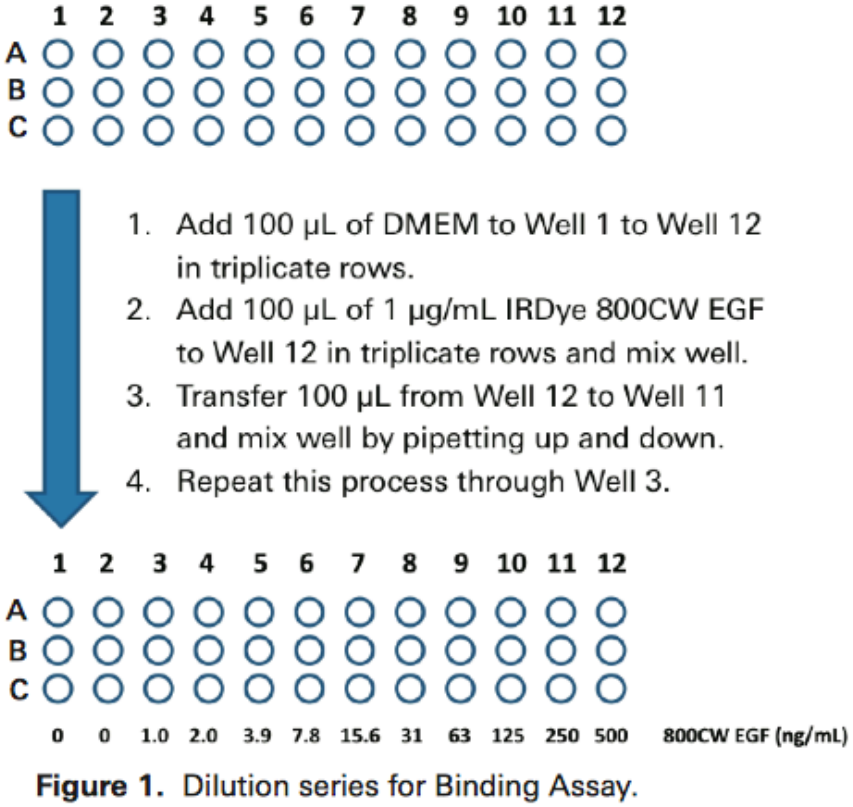
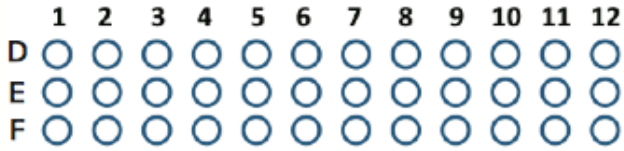


Figure 1. Dilution series for Binding Assay.



1. Add 100 µL of DMEM to Well 1 to Well 11 in triplicate rows.
2. Add 200 µL of 32 µg/mL unlabeled EGF to Well 12 in triplicate rows.
3. Transfer 100 µL from Well 12 to Well 11 and mix well by pipetting up and down.
4. Repeat this process through Well 3.
5. Add 100 µL of DMEM only to Wells 1 and 2. These are background controls and contain no EGF.
6. Prepare 10 mL of 50 ng/mL IRDye® 800CW EGF.
7. Add 100 µL of IRDye 800CW EGF stock solution to Wells 3-12 for triplicate Competition Assay rows for final concentration of labeled EGF of 25 ng/mL.

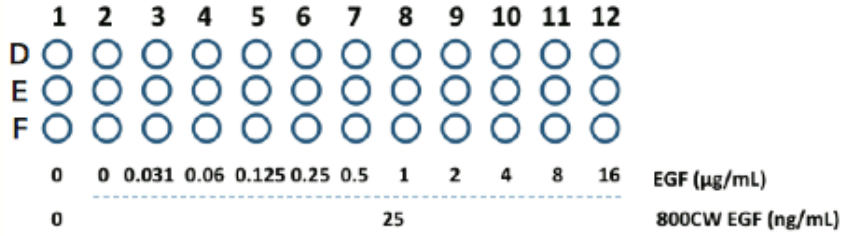


Figure 2. Plate setup for Competition Assay.

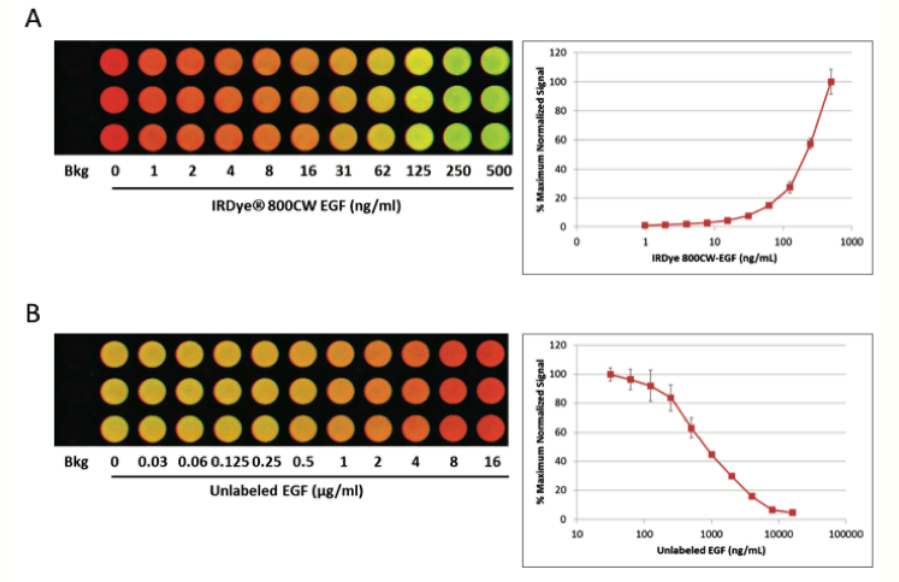


Figure 3. Plate setup for binding (A) and competition (B) assays. Subsequent analyses are shown on the right. Normalization using CellTag™ 700 Stain is detected in the 700 nm channel (red) while IRDye® 800CW EGF is detected in the 800 nm channel (green). Binding of IRDye 800CW EGF to EGF receptor is shown in Figure 1A. In Figure 1B, binding of IRDye 800CW EGF (25 ng/mL) is blocked with increasing concentrations of unlabeled EGF, demonstrating that the observed binding is specific.

III. Experimental Considerations

Proper selection of microplates can significantly affect the results of an analysis, 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.

- On-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 and black-sided plates with clear bottoms. Do not use plates with white wells, since the autofluorescence from the white surface will create significant noise.
- On-Cell Western assays require sterile plates for tissue culture growth. The following plates and focus offset settings are recommended by LI-COR Biosciences. Please be aware that manufacturers' specifications for culture plates are subject to change.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.

Well Number	Well Bottom	Manufacturer	Part Numbers	Odyssey & Odyssey CLx Offset	Aerius & Odyssey Sa Offset
96	Flat	Nunc	161093, 165305	3.0 mm	3.0 mm
96	Flat	BD Falcon	353075, 353948	3.0 mm	3.0 mm
96	Flat	Corning	3603	3.0 mm	3.0 mm

384	Flat	Nunc	164688, 164730	3.0 mm	3.0 mm
384	Flat	BD Falcon	353961, 353962	3.0 mm	3.0 mm

Focus Offset Optimization - Plates deviating from LI-COR recommendations may require lower or higher focus offsets for optimal resolution and detection.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	0.5, 1.0, 2.0, 3.0 & 4.0
Odyssey Sa & Aerius	1.7, 2.0, 3.0 & 4.0

Alternatively, consult the plate manufacturer to obtain the measured distance from the skirt to the bottom of the plate. Microplates are required to have a distance of no more than 4.0 mm from the instrument surface to the target detection area of the plate.

Intensity Setting Optimization – Intensity settings may need to be optimized depending on results. If signal is weak or saturated, try the suggested intensity settings in the table below to achieve optimal results.

Instrument	Initial Intensity Setting (700/800 nm)	Intensity Settings: Weak Signal (700/800 nm)	Intensity Settings: Saturated Signal (700/800 nm)
Odyssey® Classic	5 / 5	7.5 / 7.5	2.5 / 2.5
Odyssey CLx	5 / 5	7.5 / 7.5	2.5 / 2.5
	AutoScan*	-	-
Odyssey Sa	7 / 7	8 / 8	4 / 4
Aerius	7 / 7	8 / 8	4 / 4

*The Image Studio AutoScan function for the Odyssey CLx alleviates the need to scan the plate at multiple intensity settings.

Materials

Odyssey® Blocking Buffer (PBS) 927-40000 927-40100 by LI-COR

IRDye 800CW EGF 926-08446 by LI-COR

CellTag™ 700 Stain 926-41090 by LI-COR

Protocol

Step 1.
Allow A431 cell growth in a 100-mm dish in DMEM containing 10% fetal bovine serum (FBS; ATCC 30-2020) and 1% PEN/STREP (Gibco®) using standard tissue culture proce- dures until cells reach 80% - 90% confluency (1.5 x 10^7 cells).

Step 2.
Remove growth medium, wash cells with sterile 1X PBS, and trypsinize cells.

Step 3.
Neutralize displaced cells with culture medium and pellet by centrifugation.

Step 4.
Remove supernatant and resuspend cell pellet in remaining medium by manually tapping the collection tube. To maintain cell integrity, avoid vigorous pipetting or vortexing to re- suspend cells.

Step 5.
Dilute cells to 20 mL in complete medium and count cells using a hemocytometer.

Step 6.
Dilute cells with complete medium to a concentration of 200,000 cells/mL.

Step 7.
Gently mix the cell suspension thoroughly.

Step 8.
Under sterile conditions, dispense 200 µL of the cell suspension per well in a 96-well plate (40,000 cells plated per well).

Step 9.
Incubate cells and monitor cell density until cells are 80 - 90% confluent in each well.

Step 10.
Warm serum-free medium (DMEM; Gibco®) to 37 °C.

Step 11.
Remove complete medium from the 96-well plate by aspiration or inversion of the plate and blot excess medium by tapping the inverted plate gently on a tissue.

Step 12.
Replace medium with 200 µL of pre-warmed, serum-free medium per well and incubate 4 hours at 37 °C.

DURATION
04:00:00

Step 13.
Prepare a dilution series of reagents for the binding and competition assays in a separate 96-well deep well plate as described in Figures 1 and 2.

Binding Assay

Step 14.

Prepare 2-fold serial dilutions of IRDye® 800CW EGF ranging from 1.0 ng/mL to 500 ng/mL according to the experiment layout shown in Figure 1.

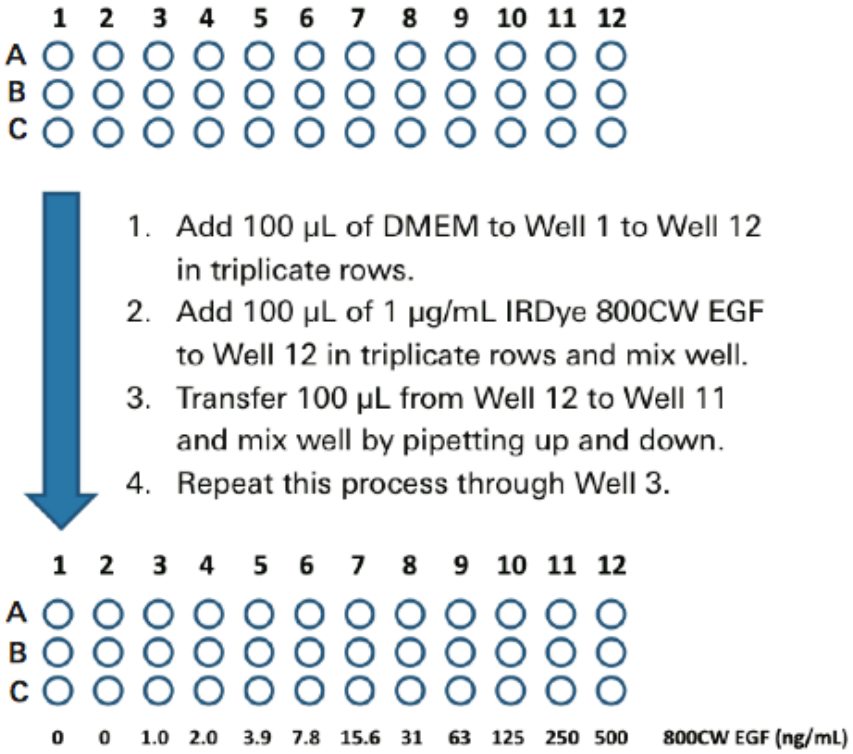


Figure 1. Dilution series for Binding Assay.

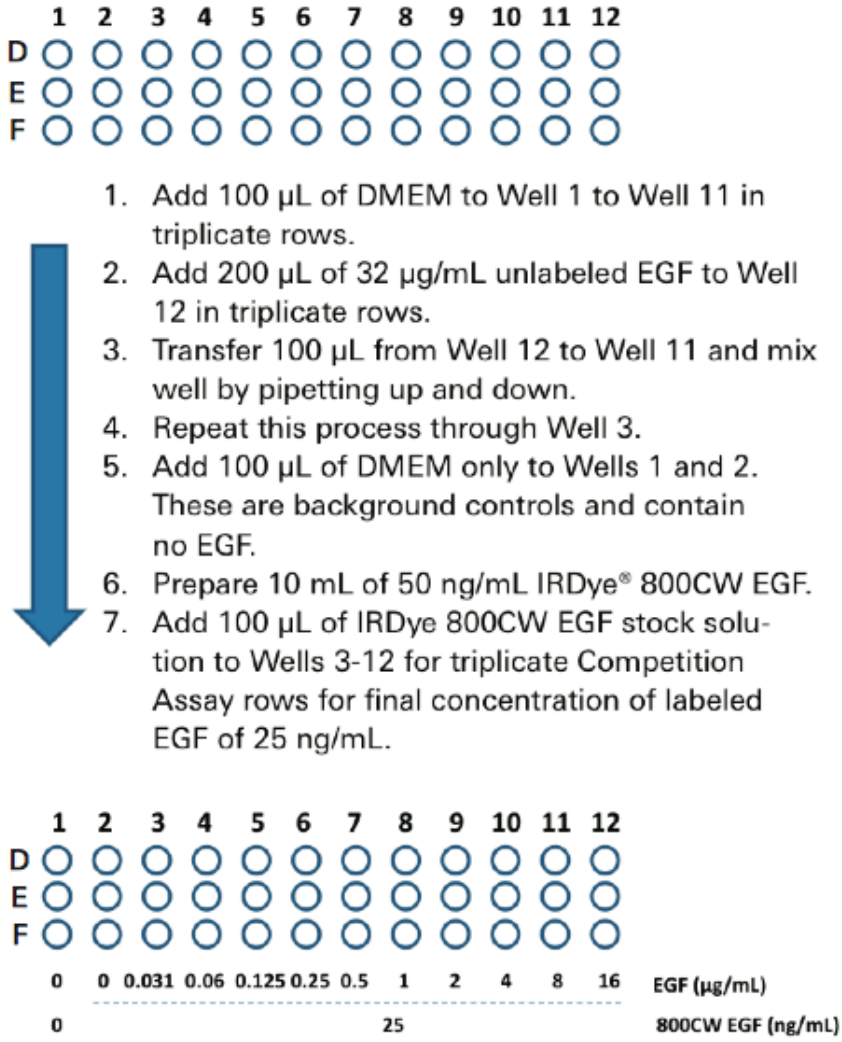


Figure 2. Plate setup for Competition Assay.

Binding Assay

Step 15.

Prepare 1 mL of 1 µg/mL IRDye 800CW EGF in DMEM.

Binding Assay

Step 16.

Wells 1 and 2 are background controls and contain no IRDye 800CW EGF.

Binding Assay

Step 17.

Do not store; proceed immediately to step 21.

Competition Assay

Step 18.

Prepare 2-fold serial dilutions of unlabeled EGF diluted in DMEM ranging from 0.06 to 16 µg/mL according to the experiment layout shown in Figure 2.

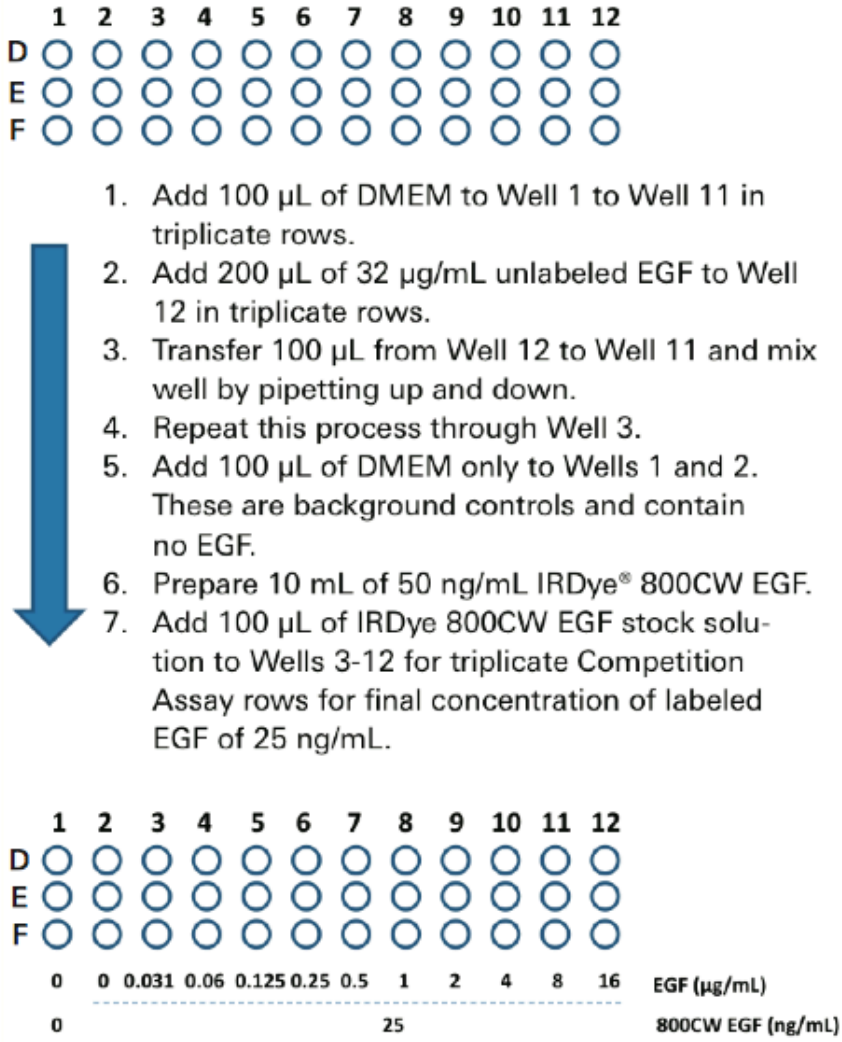


Figure 2. Plate setup for Competition Assay.

Competition Assay

Step 19.

Prepare 1 mL of 32 µg/mL unlabeled EGF in DMEM.

Competition Assay

Step 20.

Do not store; proceed immediately to step 21.

Step 21.
Retrieve cell-containing plate from 37 °C incubator. Remove starvation medium from cells by aspiration or inversion of the plate and blot excess medium by tapping plate gently on tissue.

Step 22.
Transfer 100 µL Binding Assay mixtures to rows A-C of the plate and 100 µL Competition Assay mixtures to rows D-F into the cell-containing wells. Use a multi-channel pipette and transfer these mixtures quickly (20 sec), as cellular responses are quick.

Step 23.
Incubate at 37 °C for 2 minutes.

DURATION
00:02:00

Step 24.
Prepare fresh Fixing Solution as follows:

1X PBS	45.0 mL
37% Formaldehyde	5.0 mL
3.7% Formaldehyde	50.0 mL

Step 25.

Remove EGF-containing medium by aspiration or inversion.

Step 26.

Immediately fix cells with the addition of 150 µL of fresh Fixing Solution and incubate at room temperature (RT) for 20 minutes with no shaking. **Add the Fixing Solution carefully by pipetting down the side of the wells to avoid detaching the cells.**

DURATION
00:20:00

Step 27.
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

Step 28.
Remove the Fixing Solution by aspiration.

Step 29.
Wash with 200 µL of Triton Washing Solution for 5 minutes per wash to permeabilize the cells. (wash 1/4)

DURATION
00:05:00

NOTES
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NOTES:

- Allow each wash to shake on a rotator for 5 minutes at RT.
- Do not allow cells/wells to become dry during washing. Add each wash immediately after the preceding wash is removed.

Step 30.

Wash with 200 µL of Triton Washing Solution for 5 minutes per wash to permeabilize the cells. (wash 2/4)

DURATION
00:05:00

Step 31.

Wash with 200 µL of Triton Washing Solution for 5 minutes per wash to permeabilize the cells. (wash 3/4)

DURATION
00:05:00

Step 32.

Wash with 200 µL of Triton Washing Solution for 5 minutes per wash to permeabilize the cells. (wash 4/4)

DURATION
00:05:00

Step 33.
Remove the Triton Washing Solution by aspiration or inversion.

Step 34.
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 hour at RT with moderate shaking on a rotating platform.

DURATION
01:00:00

Step 35.
IRDye® 800CW EGF will be detected in the 800 nm channel. CellTag™ 700 Stain is a cell stain that can be used to normalize the signal from binding of the labeled EGF, to correct for variations in cell number from well to well. CellTag 700 Stain will be detected in the 700 nm channel.

Step 36.
Add 50 µL of LI-COR Odyssey Blocking Buffer + 0.1% Tween 20 to Well 1. This will serve as a control for any potential background.

Step 37.
Dilute 0.1 mM CellTag 700 Stain 1:500 in Odyssey Blocking Buffer. Add 50 µL to each well (except Well 1) and incubate for 1 hour with gentle shaking.

DURATION
01:00:00

NOTES
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NOTE: Protect from light.

Step 38.
Prepare Tween Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL

1X PBS with 0.1% Tween 20 1000 mL

Step 39.
Remove Odyssey Blocking Buffer and CellTag 700 Stain solution by aspiration or inversion.

Step 40.
Wash the plate with Tween Washing Solution by gently adding solution down the side of the wells to avoid detaching the cells. Use a generous amount of solution (200 µL/well). (wash 1/5)

Step 41.
Allow wash to shake gently on a rotator for 5 minutes at RT.

DURATION
00:05:00

NOTES
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NOTE: Protect plate from light during washing.

Step 42.
Wash the plate with Tween Washing Solution by gently adding solution down the side of the wells to avoid detaching the cells. Use a generous amount of solution (200 µL/well). (wash 2/5)

Step 43.

Allow wash to shake gently on a rotator for 5 minutes at RT. (wash 2/5)

Step 44.

Wash the plate with Tween Washing Solution by gently adding solution down the side of the wells to avoid detaching the cells. Use a generous amount of solution (200 µL/well). (wash 3/5)

Step 45.

Allow wash to shake gently on a rotator for 5 minutes at RT. (wash 3/5)

Step 46.

Wash the plate with Tween Washing Solution by gently adding solution down the side of the wells to avoid detaching the cells. Use a generous amount of solution (200 µL/well). (wash 4/5)

Step 47.

Allow wash to shake gently on a rotator for 5 minutes at RT. (wash 4/5)

Step 48.

Wash the plate with Tween Washing Solution by gently adding solution down the side of the wells to avoid detaching the cells. Use a generous amount of solution (200 µL/well). (wash 5/5)

Step 49.

Allow wash to shake gently on a rotator for 5 minutes at RT. (wash 5/5)

Step 50.
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 solution. For best results, scan plate immediately; plates may also be stored at 4 °C for up to several weeks (protected from light).