In-Cell Western™ Assay (COS Cells Phospho-p53 Detection in Response to Hydroxyurea)

LI-COR Biosciences

Abstract

This is an In-Cell Western™ Assay protocol for the detection of Phospho-p53 in COS cells in response to Hydroxyurea.

Developed for:

Aerius,
Odyssey® Classic,
Odyssey CLx, and
Odyssey Sa
Infrared Imaging Systems

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

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Guidelines

Required Reagents

LI-COR Reagents

- IRDye® 800CW Goat anti-Rabbit Secondary Antibody (LI-COR P/N 925-32211 or 926-32211)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (LI-COR P/N 925-68070 or 926-68070)
- Odyssey® Blocking Buffer (LI-COR, P/Ns 927-40000 or 927-50000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- Clear or black 96-well or 384-well microplates (See 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

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 following general considerations for microplate selection.

- In-Cell Western analyses use detection at the well surface with minimal 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 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, Eppendorf (P/Ns 0030730119 or 0030741030)
  96-well, clear, Nunc® (P/Ns 167008, 161093)
  96-well, clear, FalconTM (P/Ns 353075, 353948)
  96-well, black with clear bottom, Nunc (P/N 165305)
  96-well format, Corning (P/N 3603)

  384-well, clear, Nunc (P/Ns 164688, 164730)
  384-well, clear, Falcon (P/Ns 353229, 353289)
  384-well, black with clear bottom, Nunc (P/N 142761)
  384-well format, Corning (P/N 3712)

- **Focus Offset Optimization** – If plates other than those recommended are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	1.0, 1.5, 2.0, 2.5, 3.0, 3.5, & 4.0
Odyssey Sa & Aerius	1.5, 2.0, 2.5, 3.0, & 3.95

Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise ratio for experiments. The actual minimum and maximum focus offset will vary with each instrument. Alternatively, the plate manufacturer can be consulted to obtain the measured distance from the skirt bottom to the bottom of the plate.

- All Aerius and Odyssey® Imaging systems (excluding Odyssey Fc) 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 Aerius and Odyssey Sa instrument and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified for In-Cell Western assays, the recommended focus offset is 3.5 mm or higher.
- **Intensity Setting Optimization -**

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

- 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.

Experimental Results

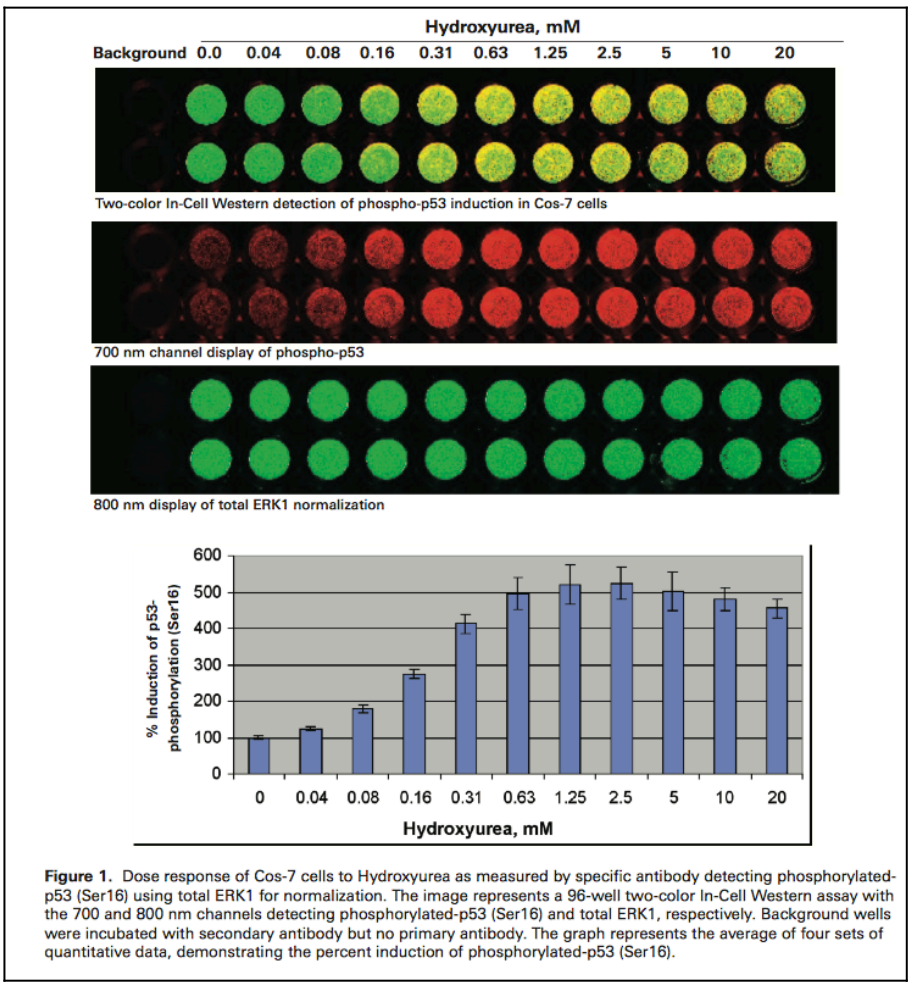

Figure 1. Dose response of Cos-7 cells to Hydroxyurea as measured by specific antibody detecting phosphorylated-p53 (Ser16) using total ERK1 for normalization. The image represents a 96-well two-color In-Cell Western assay with the 700 and 800 nm channels detecting phosphorylated-p53 (Ser16) and total 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).

Materials

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

Odyssey Blocking Buffer (TBS) 927-50000 927-50100 by LI-COR

IRDye® 800CW Goat anti-Mouse Secondary Antibody 925-32210 926-32210 by LI-COR

IRDye 680RD Goat anti-Rabbit Secondary Antibody 925-668071 926-68071 by LI-COR

Normalization antibody: Anti-total ERK1 SC-94 by Santa Cruz Biotechnology

Anti-phospho-p53 9286 by Cell Signaling Technology

Hydroxyurea H8627 by Sigma Aldrich

Protocol

Cell Preparation

Step 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®).

Cell Preparation

Step 2.

Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.

Cell Preparation

Step 3.

Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).

Cell Preparation

Step 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.**

Cell Preparation

Step 5.

Resuspend cells in 20 mL complete media and count cells using a hemocytometer.

AMOUNT

20 ml Additional info:
complete media

Cell Preparation

Step 6.

Reconstitute cells and dilute in 40 mL of complete media so that 100,000 cells/mL is achieved (2 plates x 96 wells x 200 µL/well = 40 mL).

AMOUNT

40 ml Additional info:
complete media

Cell Preparation

Step 7.

Manually mix the cell suspension thoroughly.

Cell Preparation

Step 8.

Under sterile conditions, dispense 200 µL of the cell suspension per well in a 96-well plate (20,000 cells plated per well).

AMOUNT

200 µl Additional info: Cell
suspension

Cell Preparation

Step 9.

Incubate cells and monitor cell density until 80 - 90% confluency is achieved (approximately 72 hours).

DURATION

12:00:00

Cell Treatment

Step 10.

Warm serum-free media (DMEM, Gibco®) to 37 °C.

TEMPERATURE

37 °C Additional info:

Cell Treatment

Step 11.

Carefully remove complete media from plate wells by aspiration or manual displacement.

Cell Treatment

Step 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.

AMOUNT

100 µl Additional info:
resting/activation media
per well

Cell Treatment

Step 13.

Allow incubation at 37 °C overnight (16 - 24 hours).

TEMPERATURE

37 °C Additional info:
Incubation

DURATION

24:00:00

NOTES

James Thornton Jr 10 May 2017

Towards the end of the incubation, prepare the Fixing Solution, as described in Step14.

Fixing/Permeabilizing Cells

Step 14.

Prepare fresh Fixing Solution as follows:

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

Fixing/Permeabilizing Cells

Step 15.

When the Cell Treatment incubation period is complete, remove activation or stimulation media manually or by aspiration.

NOTES

James Thornton Jr 10 May 2017

Immediately fix cells with Fixing Solution (4% formaldehyde in 1X PBS) for 20 minutes at room temperature, as detailed in steps 16-17.

Fixing/Permeabilizing Cells

Step 16.

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

AMOUNT

150 µl Additional info:
Fresh fixing solution

Fixing/Permeabilizing Cells

Step 17.

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

DURATION

00:20:00

Fixing/Permeabilizing Cells

Step 18.

To permeabilize, wash 5 times with 1X PBS containing 0.1% Triton® X-100 for 5 minutes per wash. Detailed in steps 19-35.

NOTES

James Thornton Jr 05 Jan 2017

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

Fixing/Permeabilizing Cells

Step 19.

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

Fixing/Permeabilizing Cells

Step 20.

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

Fixing/Permeabilizing Cells

Step 21.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 1/5)

AMOUNT

200 µl Additional info:
Triton Washing solution

Fixing/Permeabilizing Cells

Step 22.

Allow plate to shake on a rotator for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 23.

Remove wash manually. (wash 1/5)

Fixing/Permeabilizing Cells

Step 24.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 2/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 25.

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

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 26.

Remove wash manually. (wash 2/5)

Fixing/Permeabilizing Cells

Step 27.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 3/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 28.

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

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 29.

Remove wash manually. (wash 3/5)

Fixing/Permeabilizing Cells

Step 30.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 4/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 31.

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

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 32.

Remove wash manually. (wash 4/5)

Fixing/Permeabilizing Cells

Step 33.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 5/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 34.

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

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 35.

Remove wash manually. (wash 5/5)

Blocking Cells

Step 36.

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

AMOUNT

150 µl Additional info:
Odyssey Blocking Buffer

NOTES

James Thornton Jr 14 Dec 2016

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 reused for morethan a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammarsten- grade casein is not required).

James Thornton Jr 14 Dec 2016

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.

Ashley Humphrey 31 May 2018

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. Non-fat 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 reused for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammarsten- 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.

Blocking Cells

Step 37.

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

DURATION

01:30:00

Primary Antibodies

Step 38.

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)

Primary Antibodies

Step 39.

Mix the primary antibody solution thoroughly before adding to wells.

Primary Antibodies

Step 40.

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

AMOUNT

50 µl Additional info:
primary antibody or
antibodies

Primary Antibodies

Step 41.

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.

AMOUNT

50 µl Additional info:
Odyssey Blocking Buffer
only to control wells

Primary Antibodies

Step 42.

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

TEMPERATURE

4 °C Additional info:
Incubation

Primary Antibodies

Step 43.

Wash the plate 5 times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. Detailed in steps 44 - 58

Primary Antibodies

Step 44.

Prepare Tween Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS + 0.1% Tween 20	1,000 mL

Primary Antibodies

Step 45.

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

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 46.

Allow wash to shake on rotator for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Primary Antibodies

Step 47.

Remove wash manually.(wash 1/5)

Primary Antibodies

Step 48.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution**

down the sides of the wells carefully to avoid detaching the cells from the well bottom. (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 49.

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

DURATION

00:05:00

Primary Antibodies

Step 50.

Remove wash manually.(wash 2/5)

Primary Antibodies

Step 51.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 52.

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

DURATION

00:05:00

Primary Antibodies

Step 53.

Remove wash manually.(wash 3/5)

Primary Antibodies

Step 54.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 55.

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

DURATION

00:05:00

Primary Antibodies

Step 56.

Remove wash manually.(wash 4/5)

Primary Antibodies

Step 57.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 58.

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

Remove wash manually when done. (wash 5/5)

DURATION

00:05:00

Secondary Antibodies

Step 59.

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)
- IRDye 680RD Goat anti-Mouse (1:800 dilution)

NOTES

James Thornton Jr 14 Dec 2016

Recommended dilution range is 1:200 to 1:1,200.

James Thornton Jr 14 Dec 2016

Avoid prolonged exposure of the antibody vials to light.

Secondary Antibodies

Step 60.

Mix the antibody solutions well and add 50 µL of the secondary antibody solution to each well.

AMOUNT

50 µl Additional info:
secondary antibody
solution

Secondary Antibodies

Step 61.

Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

DURATION

01:00:00

Secondary Antibodies

Step 62.

Wash the plate 5 times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. Detailed in steps 63-77.

NOTES

James Thornton Jr 05 Jan 2017

Protect plate from light during washing.

James Thornton Jr 05 Jan 2017

NOTE: If using an Eppendorf glass-bottom plate (P/N 0030741030), rinse briefly with PBS, then wash once with PBS on a rotator for 5 min at room temperature.

Secondary Antibodies

Step 63.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 1/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 64.

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

Secondary Antibodies

Step 65.

Remove wash manually.(wash 1/5)

Secondary Antibodies

Step 66.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 67.

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

DURATION

00:05:00

Secondary Antibodies

Step 68.

Remove wash manually.(wash 2/5)

Secondary Antibodies

Step 69.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 70.

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

DURATION

00:05:00

Secondary Antibodies

Step 71.

Remove wash manually.(wash 3/5)

Secondary Antibodies

Step 72.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 73.

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

DURATION

00:05:00

Secondary Antibodies

Step 74.

Remove wash manually.(wash 4/5)

Secondary Antibodies

Step 75.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 76.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Imaging

Step 77.
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

Imaging

Step 78.
For best results, scan plate immediately; plates may also be stored at 4 °C for several weeks (protected from light).

TEMPERATURE

4 °C Additional info:
Storage temperature

Imaging

Step 79.
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.

Imaging

Step 80.
Scan plate with detection in both 700 and 800 nm channels using an Odyssey or Aerius System described following:

Instrument	Resolution*	Focus Offset	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey Classic	169 µm	3.5	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µm	3.5	medium-lowest	5 / 5	7 min
	169 µm	3.5	medium-lowest	AutoScan	16 min
Odyssey Sa	200 µm	3.5	medium-lowest	7 / 7	3 min
Aerius	200 µm	3.5	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

NOTES

Ashley Humphrey 31 May 2018
All settings may require adjustment for optimal data quality

Warnings

See SDS for safety and hazards.