

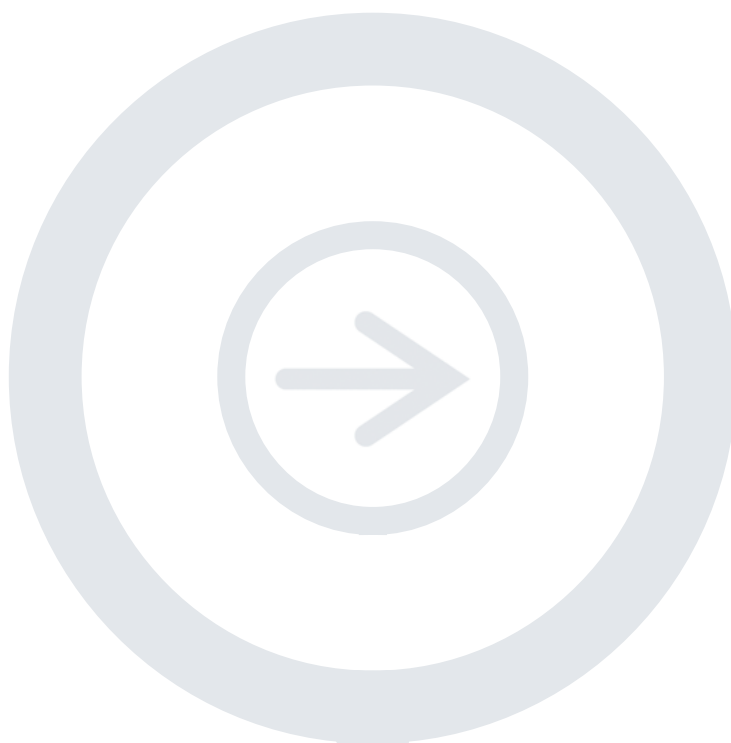
Application Note

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

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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I. Required Reagents

LI-COR Reagents

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

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- TO-PRO®-3 (Invitrogen, P/NT3605)
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100
- Nunc™ 96 Microwell™ Plate (Nunc, P/N 167008)
- Primary antibodies as described below

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

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

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.

1. Allow A431 cell growth in a T75 flask in DMEM and 10% fetal calf serum (FCS; Gibco®) using standard tissue culture procedures until cells reach 80% - 90% confluency (~1.5 x 10⁷ cells).
2. Remove growth medium, wash cells with sterile 1X PBS, and trypsinize cells.
3. Neutralize displaced cells with culture medium and pellet by centrifugation.
4. Remove supernatant and resuspend cell pellet in remaining medium by manually tapping the collection tube. Avoid vigorous pipetting or vortexing to resuspend cells in order to maintain cell integrity.
5. Dilute cells to 20 mL in complete medium and count cells using a hemocytometer.
6. Dilute cells with complete medium to a concentration of 200,000 cells/mL.
7. Gently mix the cell suspension thoroughly.
8. Under sterile conditions, dispense 200 µL of the cell suspension per well in a Nunc 96 Micro-well plate (40,000 cells plated per well).

9. Incubate cells and monitor cell density until cells are ~80 - 90% confluent in each well. This should take approximately three days.
10. Warm serum-free medium (DMEM; Gibco®) to 37°C.
11. Remove complete medium from the microwell plate by aspiration or inversion of the plate and tap excess medium on tissue.
12. Replace medium with 200 µL of pre-warmed, serum-free medium per well and incubate 4 to 16 hours.
13. In a separate 96-well Microwell™ plate lacking cells, prepare a dilution series of reagents for the binding and competition assays. Begin by dispensing 50 µL of DMEM per well (exclude Well 12). Continue preparation of the dilutions for both assays in this plate.
14. **Binding Assay:** Prepare 2-fold serial dilutions of IRDye® 800CW EGF ranging from 1.0 to 500 ng/mL.
 - a. Prepare stock solution (1 µg/mL) in DMEM.
 - b. Add 100 µL to Well 12 in triplicate rows.
 - c. Transfer 50 µL from Well 12 to Well 11 and mix by pipetting up and down.
 - d. Repeat this process through Well 3.
 - e. Add 100 µL DMEM only to Wells 1 and 2. These are background controls and contain no EGF. An image of the experimental layout should look like that shown in Figure 1A (Page 5).

Competition Assay: Prepare 2-fold serial dilutions of unlabeled EGF diluted in DMEM ranging from 0.3 to 15 µg/mL.

- a. Prepare stock solution (30 µg/mL) in DMEM.
 - b. Add 100 µL to Well 12 in triplicate rows.
 - c. Transfer 50 µL from Well 12 to Well 11 and mix by pipetting up and down.
 - d. Repeat this process through Well 3.
 - e. Add 100 µL DMEM only to Wells 1 and 2. These are background controls and contain no EGF.
 - f. Remove and discard 50 µL from Well 3 so the final volume equals 50 µL in all wells except Wells 1 and 2.
 - g. Prepare a stock solution of IRDye 800CW EGF (200 ng/mL).
 - h. Add 50 µL of IRDye 800CW EGF stock solution to Wells 3-12 for triplicate Competition Assay rows for final concentration of labeled EGF of 100 ng/mL.
 - i. An example image of the experimental layout should look like that shown in Figure 1B.
 - j. **Do not store;** move directly to cell-containing plate and begin.
15. Retrieve cell-containing plate from 37°C incubator. Remove starvation medium from cells by aspiration or inversion of the plate and tap excess medium on tissue.
 16. Transfer 50 µL Binding Assay mixtures and 100 µL Competition Assay mixtures from the dilutions prepared in Step 14 into the cell-containing wells. Use a multi-channel pipettor and transfer these mixtures quickly (~20 sec), as cellular responses are quick.
 17. Incubate at 37°C for 2 minutes.

18. Prepare fresh *Fixing Solution* as follows:

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

19. Remove EGF-containing medium by aspiration or inversion. Immediately fix cells with the addition of 150 μ L 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.**

20. Prepare *Triton[®] Washing Solution* as follows:

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

21. Remove the *Fixing Solution* by aspiration.

22. Wash four times with 200 μ L of *Triton Washing Solution* for 5 minutes per wash to permeabilize the cells.

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.

23. Remove the *Triton Washing Solution* by aspiration or inversion.

24. To each well, carefully add 150 μ L of LI-COR[®] Odyssey[®] Blocking Buffer (P/N 927-40000) + 0.1% Tween[®] 20 down the side of the wells and incubate for 1.5 hours at RT with moderate shaking on a rotating platform.

25. IRDye[®] 800CW EGF will be detected in the 800 nm channel. TO-PRO[®]-3 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. TO-PRO-3 will be detected in the 700 nm channel.

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

27. Dilute TO-PRO-3 1:5,000 in Odyssey Blocking Buffer. Add 50 μ L to each well (except Well 1) and incubate for 1 hour with gentle shaking.
NOTE: Protect from light.

28. Prepare *Tween Washing Solution* as follows:

1X PBS	995 mL
20% Tween 20	5 mL
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1X PBS with 0.1% Tween 20	1000 mL

29. Remove Odyssey Blocking Buffer and TO-PRO-3 solution by aspiration or inversion.

30. 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 μ L/well). Allow wash to shake gently on a rotator for 5 minutes at RT.
NOTE: Protect plate from light during washing.

31. Repeat wash 4 more times.

32. 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).

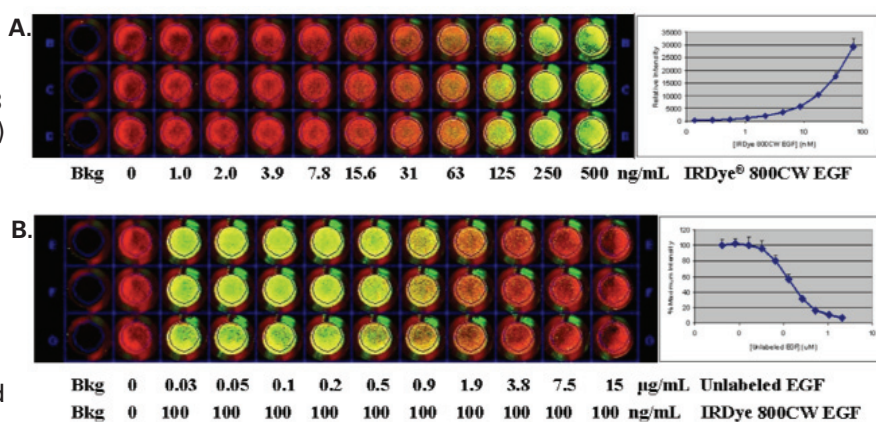
33. Before scanning, clean the bottom plate surface and the Odyssey® Imager scanning bed with moist, lint-free paper to avoid obstructions during scanning.

34. Scanning parameters by instrument:

Instrument	Resolution	Focus Offset	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey Classic	169 μ m	3.0	medium-lowest	5/7	7 min
Odyssey CLx	169 μ m	3.0	medium-lowest	5/7	7 min
	169 μ m	3.0	medium-lowest	AutoScan	16 min
Odyssey Sa	200 μ m	3.0	medium-lowest	5/7	3 min
Aerius	200 μ m	3.0	medium-lowest	5/7	3 min

Figure 1. Plate setup for binding (A) and competition (B) assays. Subsequent analyses are shown on the right. Normalization using TO-PRO®-3 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 the dye-labeled ligand 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
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

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, 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 –

	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.

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