In-Cell ELISA protocol

In-Cell ELISA (also known as cell based ELISA, in cell western or cytoblot) is an immunocytochemistry method used to quantify target protein or post-translational modifications of the target protein, in cultured cells. Cells are cultured (or treated if required) and seeded into a coated 96-well microplate. After fixation and permeabilization, primary antibody is added to the well and is incubated followed by addition of a labeled secondary antibody. Detection can be colorimetric or fluorescent for a single target using our In-Cell ELISA kits or primary antibodies characterized for In-Cell ELISA. Dual targets can be quantified using IR-conjugated secondary antibodies in our In-Cell ELISA kits. Kits include highly-specific, well-characterized primary antibodies generated from mouse or rabbit, and appropriately conjugated secondary antibodies for colorimetric, fluorescent or infra-red detection.

Below, we provide a general protocol for a 96-well microplate that can be used for all our In-Cell ELISA kits as well as with our antibodies characterized for In-Cell ELISA. Reagent preparation and hints and tips for a successful assay are included in the appendices.

Quick protocol

1. Adherent cell seeding

- Seed cells into 96-well microplate at desired density. (For 384-well microplate, seed at ¼ of the density).
- Allow cells to adhere
- Treat cells as desired in total volume of 100 µL media for 96-well microplate (or ¼ of volume of 384-well microplate).

2. Fix cells to microplate

- Add an equal volume (100 µL) of 8% paraformaldehyde solution to fix and crosslink the cells to the microplate.
- Incubate for 15 minutes.
- Wash wells with PBS (microplate may be stored at 4°C at this point).

3. Permeabilize cells

- Add 200 µL of 1X permeabilization solution to wells for 30 min.
- Add 200 µL of 2X blocking solution to each well. Incubate for 2 hr.
- Wash thoroughly.

4. Incubation with primary antibody

- Dilute the primary antibody stock to the specified In-Cell ELISA concentration in 1X incubation buffer and add to appropriate wells.
- Cover and incubate overnight at 4°C.
- Wash thoroughly.

5. Incubation with secondary antibody

- Dilute the secondary antibody stock to the required concentration (as defined in kit protocol or as determined by user) in 1X incubation buffer and add 100 µL diluted secondary antibodies to appropriate wells.
- Incubate for 2 hr at room temperature.
- Wash thoroughly.

6. Measure signal

- As appropriate, image the microplate with an IR scanner or develop the HRP labeled microplate and read it with a spectrophotometer. Export data.

7. Whole cell staining with Janus Green

- If desired, stain with Janus Green and measure relative cell seeding density in an IR imager or micromicroplate spectrophotometer. Export data.
- Determine background corrected signal and then ratio signal to Janus Green if desired.

Materials

- In-Cell ELISA characterized primary antibody
- Appropriate IRDye® or HRP-labeled secondary antibody
- For HRP detection, HRP solution substrate
- 20% paraformaldehyde
- Distilled or deionized water
- 10X Phosphate Buffered Saline (PBS) (80 mM Na_2HPO_4, 14 mM KH_2PO_4, 1.4 M NaCl, 27 mM KCl, adjust pH to 7.4 with NaOH)
- 100X Triton X-100 (10% solution in H_2O)
- 400X Tween-20 (20% solution in H_2O)
- 10X blocking buffer
- 0.3% solution Janus Green Stain (ab111622) in water
- 0.5 M HCl

Equipment

- 96-well microplate. Clear bottom (black wall necessary for IRDye® only), preferably coated (amine/collagen) for optimal cell culture
- Appropriate microplate reader: For HRP detection, use a microplate reader capable of measuring absorbance at 650 nm (preferably in a kinetic mode) or 450 nm. For IRDye® use a LI-COR® Odyssey® or Aerius® near-infrared imaging system.
- Multichannel pipette (recommended)

Please read through the protocol before starting the experiments. For best results, please do not deviate from the protocol.

Prepare cells:

1.1. Adherent cells can be grown in the recommended assay microplates or seeded directly into the assay microplate and allowed to attach for several hours or overnight. The optimal cell seeding density is cell type dependent and should be determined for each experiment (see Appendix 1). For example, HeLa cells should be seeded between 25,000 and 50,000 cells per well for a 96-well microplate.

1.2. Treat the attached cells as desired in 100 µL media (up to 10% serum is acceptable). (Duration of cell treatment should be determined by user).

When treating cells with drug of interest, we recommend including wells with untreated cells and cells treated with the drug solvent.

2. Fix cells to microplate

2.1. Immediately add an equal volume (100 µL) of 8% paraformaldehyde solution to the wells containing culture media.
2.2. Incubate for 15 minutes.
2.3. Gently aspirate the paraformaldehyde solution from the microplate and wash the microplate 3 times with 300 µL 1X PBS per well.
2.4. Add 100 µL of 1X PBS to the wells. The microplate can now be stored at 4°C for several days. Cover the microplate with provided seal. For prolonged storage, supplement PBS with 0.02% sodium azide.

The microplate should not be allowed to dry at any point during or before the assay. Both paraformaldehyde and sodium azide are toxic, handle with care and dispose of according to local regulations.

3. Permeabilize cells

It is recommended to use a plate shaker (~300 rpm) during incubation steps. Any step involving removal of buffer or solution should be followed by gently tapping the microplate on paper towel to remove all solutions from the wells.

3.1. Remove PBS and add 200 µL of freshly prepared 1X permeabilization buffer to each well. Incubate for 30 min.
3.2. Remove 1X permeabilization buffer and add 200 µL of 2X blocking solution to each well. Incubate for 2 hr.

4. Incubation with primary antibody

4.1. Prepare 1X primary antibody solution by diluting provided stock antibody in 1X incubation buffer. See Appendix 2 for more information.
4.2. Remove 2X blocking solution and add 100 µL diluted primary antibody solution to each well. Incubate overnight at 4°C.

To determine the background signal it is essential to omit primary antibody from at least one well containing cells for each experimental condition and detector antibody used.

5. Incubation with secondary antibody

5.1. Remove primary antibody solution and wash the microplate 3 times with 250 µL 1X wash buffer per well. Do not remove the last wash until step 5.3.
5.2. Prepare 1X secondary antibody solution by diluting as directed in the kit protocol (or antibody datasheet) in 1X incubation buffer.

Protect fluorescently labeled antibodies from light.

5.3. Remove the 1X wash buffer and add 100 µL 1X secondary antibody solution to each well. Incubate for 2 hr.
5.4. Remove secondary antibody solution and wash 4 times with 250 µL 1X wash buffer per well. Do not remove last wash.

6. Measure signal

6.1. When using dual detection kits (with IRDye® conjugated secondary antibodies), wipe the bottom of the microplate and the scanner surface with 70% ethanol and scan the microplate on the LI-COR® Odyssey® system using both 700 nm and 800 nm channels according to manufacturer's instructions (suggested intensity range is 5-7).
6.2. For HRP conjugated secondary antibodies, remove the last wash and tap the microplate face down on paper towels to remove excess liquid. Add 100 µL HRP development solution. Pop any bubbles and immediately

record the blue color development in the microplate reader set up as follows:

Mode:	Kinetic
Wavelength:	650 nm
Time:	30 min
Interval:	20 sec – 1 min
Shaking:	Shake between reading
Alternative:	In place of kinetic reading, at a user-defined time record endpoint OD data at 640 nm or stop the reaction by adding 100 µL 0.5 M HCl and record OD data at 450 nm.

6.3. Save data and export raw data to Excel.

Analyze data using LI-COR® ICW software, or other suitable data analysis software, such as Microsoft Excel or GraphPad Prism.

7. Whole cell staining with Janus Green (optional)

The (IR or HRP) signal of antibody-specific complexes can be normalized to the Janus Green staining intensity to account for differences in cell seeding density. It is recommended to use a plate shaker (~300 rpm) during all incubation steps.

7.1. Empty microplate wells and add 50 µL of 1X Janus Green Stain per well. Incubate microplate for 5 min at room temperature.
7.2. Remove dye, wash microplate 5 times in deionized water or until excess dye is removed.
7.3. Remove last water wash, blot to dry, add 200 µL of 0.5 M HCl and incubate for 10 min.
7.4. Measure using a LI-COR® Odyssey® scanner in the 700 nm channel or measure OD at 595 nm in a standard microplate spectrophotometer.

8. Data Analysis

8.1. Correct the raw In-Cell ELISA signal for the background signal by subtracting the mean signal of well(s) incubated in the absence of the primary antibody from all other readings.
8.2. Correct the Janus Green signal of wells that do not contain cells from all other Janus Green readings.
8.3. Normalize the In-Cell ELISA signal by dividing the background-corrected In-Cell ELISA signal by the "background-corrected" Janus Green signal.

Appendix 1 – Hints and tips for a successful In-Cell ELISA

Cells

Cell seeding density, culture medium and other growth conditions are key parameters for a successful and reproducible experiment. Cell-type specific parameters must be defined by the user.

Cell attachment and fixation

Adherent cells can be grown and treated directly in the assay plate. Cell attachment can be checked with a microscope. When the cells are fully attached the media can be removed and replaced with media containing the treatment reagent. Culture media containing up to 10% fetal serum does not interfere with the cell fixation and cross-linking to the plate but may interfere with the treatment reagent.

Cell Treatment

When using a drug (inhibitor or activator), untreated cells and cells treated with only the drug solvent should be included in the experiment.

Cell seeding density

The cell seeding density of the assay microplate is dependent on the cell type. It depends on the cell size (diameter, in case of the adherent cells) and the abundance of the target protein. As a general guideline, a monolayer is recommended for robust signal. The cell seeding can be determined experimentally by microscopic observation of cell density of serially diluted cells. For adherent cells, prepare a serial dilution of the cells in a microplate (of similar well dimensions) and observe the cell density under a microscope. Working on the high end of this range will generate stronger overall signal and allow detection of small signal increases. *E.g.,* HeLa and HepG2 cells should be seeded from 25,000 to 50,000 cells per well, human fibroblasts (HdFN) from 15,000 to 25,000 cells per well.

Controls

It is essential to omit primary antibody in at least one well to provide a background signal for the experiment which can be subtracted from all measured data. This should be done for each experimental condition and with each detector antibody.

Abcam primary antibodies are supplied with a recommended final concentration for In-Cell ELISA; this is found on each product data sheet.

Scalability

This assay can also be performed in 384-well microplate format by using ¼ of the volume of reagents and cells, as specified in the above protocol.

Appendix 2 – Buffer and reagent preparation

Reagent	Reagent preparation instructions	When to prepare?
1 X PBS	Dilute 45 mL 10X PBS in 405 mL distilled or deionized water. Mix well. Store at room temperature.	At start of experiment
1X Wash Buffer	Dilute 625 µL 400X Tween-20 in 250 mL of 1X PBS. Mix well. Store at room temperature	At start of experiment
8% paraformaldehyde solution	Mix 6.25 mL distilled or deionized water with 1.25 mL10X PBS and 5.0 mL 10% paraformaldehyde. Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.	Prepare immediately before use
1 x Permeabilization Buffer	Dilute 250 µL Triton X-100 in 24.75 mL 1X PBS. Mix well.	Prepare immediately before use
2X Blocking Solution	Dilute 5 mL 10X blocking solution in 20 mL 1X PBS.	Prepare immediately before use
1X Incubation Buffer	Dilute 2.5 mL 10X blocking solution in 22.5 mL 1X PBS	Prepare immediately before use

Related products

Reagents required for you to design your own In-Cell ELISA experiment are available for purchase in our In-Cell ELISA Support Pack (ab111542), which can be used with adherent and suspension cells. In-Cell ELISA Support Pack (without plates) (ab111541) is also available for purchase.

We also offer a range of In-Cell ELISA characterized primary antibodies, as well as Janus Green cell normalization stain.