MitoSciences
1850 Millrace Drive, Suite 3A
Eugene, Oregon 97403
www.mitosciences.com

In-Cell ELISA Protocol

Rev.0

DESCRIPTION

This In-Cell ELISA (ICE) protocol is for use with adherent cells. The protocol is optimized for use with MitoSciences' ICE-validated antibodies.

For any ICE assay it is necessary to purchase both primary antibody(ies) and labeled secondary antibody(ies). Antibodies are sold separately, allowing customization of the target(s) of interest, method of detection and multiplexing. For IR detection a LI-COR ® system is necessary and for HRP detection an HRP substrate solution and a standard microplate reader are required.

For non adherent cells or cells likely to detach we recommend MitoSciences' Suspension Cell Support pack MS922.

PRINCIPLE

In-Cell ELISA is a quantitative immunocytochemistry method to measure protein levels or post-translational modifications of cultured adherent cells. The cells are grown in and fixed to the bottom of a coated 96-well plate. Targets of interest are detected by primary antibodies, which are in turn are quantified with labeled secondary antibody(ies). MitoSciences offers highly-specific, well-characterized primary antibodies generated from mouse or rabbit host species. Available secondary antibodies include IRDye®- or HRP-labeled anti-mouse and anti-rabbit antibodies, as well as IRDye®-labeled isotype specific anti-mouse antibodies. By combining primary antibodies of different species or isotype and appropriate IR-labeled secondary antibodies, two color multiplexing can be achieved in the 800/700 channels using a LI-COR® Odyssey® or Aerius® system. While assays using HRP labeled secondary antibodies are quantified using a standard spectrophotometer but cannot be multiplexed.

BACKGROUND

This protocol is a general protocol for ICE analysis and can be used with any of MitoSciences' ICE-validated antibodies. Antibodies are available in single quantities or are available in smaller amounts as part of a **sample ICE pack**. Specific scientific information, background and working concentration for each antibody are detailed in each antibody's corresponding Technical Data Sheet.

MATERIALS REQUIRED

- MitoSciences ICE-validated primary antibody(ies)
- Appropriate 2500X IRDye®- or HRP- labeled secondary antibody(ies) which can be obtained from MitoSciences (MS923-929)
- For HRP detection, HRP solution substrate is necessary such as KPL Sureblue TMB solution.
- Adequate instrumentation. For IRDye® use a LI-COR® Odyssey® or Aerius® near-infrared imaging system. For HRP detection use a spectrophotometer plate reader (Molecular Dynamics

SpectraMax recommended) capable of measuring absorbance at 650 (preferably in a kinetic mode) or 450 nm.

- 20% paraformaldehyde (Electron Microscopy Sciences #15713)
- Deionized water
- Multichannel pipette (recommended)
- 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 Sigma blocking buffer (Sigma B6429)
- 0.3% solution Janus Green Stain (Sigma J1000 in H_2O)
- Clear bottom, (black wall necessary for IRDye only), 96-Well Assay Microplate, preferably coated (amine/collagen) for optimal cell culture
- 0.5 M HCl

ASSAY PROTOCOL

Note: Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

A. Preparation of sufficient buffers and working solutions to analyze a single microplate

1. Prepare 1X PBS by diluting 45 mL of 10X PBS in 405 mL Nanopure water or equivalent. Mix well. Store at room temperature.

2. Prepare 1X Wash Buffer by diluting 0.625 mL of 400X Tween-20 in 250 mL of 1X PBS. Mix well. Store at room temperature.

3. Immediately prior to use prepare 8% Paraformaldehyde Solution by mixing 6.25 mL Nanopure water, 1.25 mL10X PBS and 5.0 mL 20% paraformaldehyde.

   Note – Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.

4. Immediately prior to use prepare 1X Permeabilization Buffer by diluting 0.25 mL 100X Triton X-100 in 24.75 mL 1X PBS. Mix well.

5. Immediately prior to use prepare 2X Blocking Solution by diluting 5 mL 10X Blocking Solution in 20 mL 1X PBS.

6. Immediately prior to use prepare 1X Incubation Buffer by diluting 2.5 mL 10X Blocking Solution in 22.5 mL 1X PBS.

B. Cell Seeding

Note – A blank plate map for notation of treatments and cells seeding is provided on page 8.

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

1. Adherent cells can be grown in the recommended assay plates or seeded directly into the assay plate and allowed to attach for several hours to overnight. The optimal cell seeding density is cell type dependent. For suggestions regarding the cell seeding see Appendix. As an example, HeLa cells should be seeded between 25,000 and 50,000 cells per well.

2. The attached cells can be treated as desired in 100 µL media. For suggestions regarding the treatment to induce apoptosis, positive and negative controls see Appendix.

   Note – When treatment with drug of interest is performed, it is recommended to include wells with untreated cells and cells treated with the vehicle only.

3. After treatment proceed to Step C1.

C. Cell Fixation

1. Immediately add an equal volume (100 µL) of 8 % Paraformaldehyde Solution to the wells of the plate containing culture media.

2. Incubate for additional 15 minutes.

3. Gently aspirate the Paraformaldehyde Solution from the plate and wash the plate 3 times briefly with 1X PBS. For each wash, rinse each well of the plate with 300 µL of 1X PBS. Finally, add 100 µL of 1X PBS to the wells of the plate. The plate can now be stored at 4℃ for several days. Cover the plate with provided seal. For prolonged storage supplement PBS with 0.02% sodium azide.

   Note – The plate 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.

D. Assay Procedure

Note – 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 blotting the plate gently upside down on a paper towel.

1. Remove PBS and add 200 µL of freshly prepared 1X Permeabilization Buffer to each well of the plate. Incubate 30 minutes.

2. Remove 1X Permeabilization Buffer and add 200 µL of 2X Blocking Solution to each well of the plate. Incubate 2 hours.

3. Prepare 1X Primary Antibody Solution by diluting MitoSciences stock antibody(ies) into 1X Incubation Buffer. *See Appendix for more information.*

4. Remove 2X Blocking Solution and add 100 µL diluted Primary Antibody Solution to each well of the plate. Incubate overnight at 4℃.

   Note – 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. Remove Primary Antibody Solution and wash the plate 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 250 µL of 1X Wash Buffer. **Do not remove the last wash until step 8.**

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

6. Prepare 1X Secondary Antibody Solution by diluting 4.8 µL of 2500X labeled-secondary antibody(ies) into 12 mL 1X Incubation Buffer.

   Note – Protect fluorescently labeled antibodies from light.

7. Remove the 1X Wash Buffer and add 100 µL 1X Secondary Antibody Solution to each well of the plate. Incubate 2 hours.

8. Remove Secondary Antibody Solution and wash 4 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 250 µL of 1X Wash Buffer. **Do not remove the last wash.**

9. For IRDye conjugated secondary antibodies, wipe the bottom of the plate and the scanner surface with 70% ethanol and scan the plate on the LI-COR® Odyssey® system using both 700 and 800 channels according to manufacturer's instructions (Suggested intensity range 5-7).

   For HRP conjugated secondary antibodies, remove the last wash and blot the plate face down to remove excess liquid. Add 100 µL HRP Development Solution. Pop any bubbles and immediately record the blue color development in the microplate reader prepared as follows:

Mode:	Kinetic
Wavelength:	650 nm
Time:	30 min
Interval:	20 sec – 1 min
Shaking:	Shake between readings

   Alternative – In place of a kinetic reading, at a ***user-defined*** time record the endpoint OD data at 650 nm or stop the reaction by adding 100 µL 0.5M HCl and record OD data at 450 nm.

10. Save data and export raw data to Excel. Next perform the in-well Janus Green staining step if desired.

E. Whole Cell Staining with Janus Green (Optional)

Note – 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 incubation steps.

11. Empty microplate wells and add 50 µL of 1X Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.

12. Remove dye, wash plate 5 times in deionized water or until excess dye is removed.

13. Remove last water wash, blot to dry, add 200 µL of 0.5 M HCl and incubate for 10 minutes.

14. Measure using a LI-COR® Odyssey® scanner in the 700 nm channel or measure OD at 595 nm in a standard microplate spectrophotometer.

F. Data Analysis

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

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

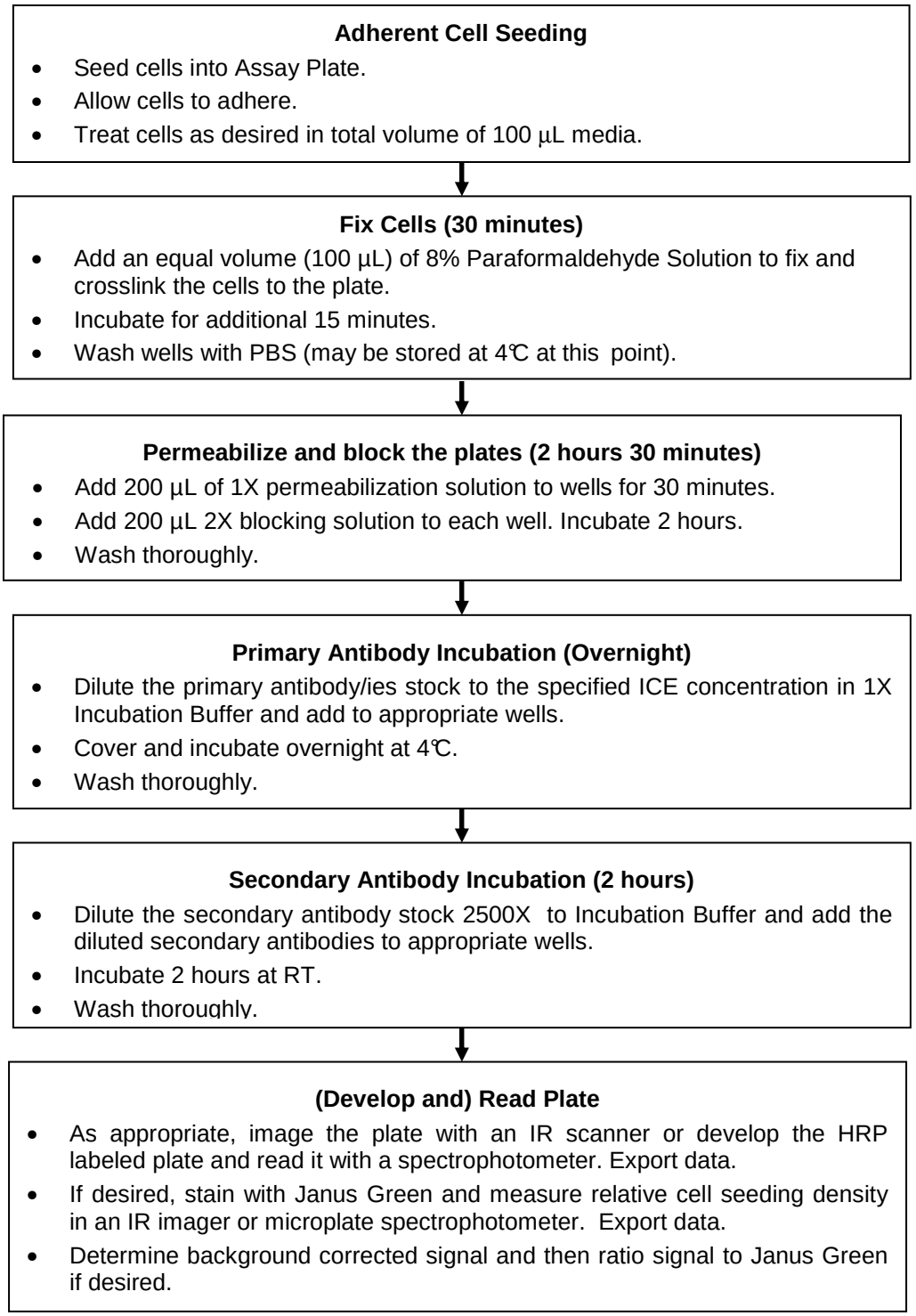
1. Correct the raw ICE signal for the background signal by subtracting the mean signal of well(s) incubated in the absence of the Primary Antibody from all other readings.

2. This step is optional. Correct the Janus Green for the background signal by subtracting the mean Janus Green signal of well that do not contain cells from all other Janus Green readings.

3. Normalize the ICE signal. Divide the background-corrected ICE signal by the (background-corrected) Janus Green signal.

FLOW CHART

For quick reference only. Be completely familiar with previous details of this document before performing the assay.



APPENDIX

Cell seeding density, culture medium and other growth conditions are important and cell-type specific parameters should be defined by the user.

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 treatment of interest. Culture media containing up to 10% fetal serum does not interfere with the cell fixation and crosslinking to the plate.

The cell seeding density of the Assay Plate is cell type dependent. It depends on the cell size (diameter, in case of the adherent cells) and the abundance of the target protein. As a general guideline, if final fixed cells form a monolayer, ICE assays using Mitosciences ICE-validated antibodies give robust signals. 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 plate (of similar well dimensions) and observe the cell density in a microscope. Working on the high end of cell densities will generate stronger signals and allow small signal increases to be measured accurately. As an example, 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, Working on the high end of this range will generate stronger signals and allow greater reductions to be measured accurately, this is important in particular for less abundant targets or lower affinity antibodies.

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 detector antibody used.

Primary antibodies are supplied by MitoSciences with a recommended final concentration for ICE which can be found in the Technical Data Sheet for each antibody.

The assay can be also performed in 384-well plate format. Contact MitoSciences representative to inquire about 384 well Assay Plates.

COMPLEMENTARY PRODUCTS

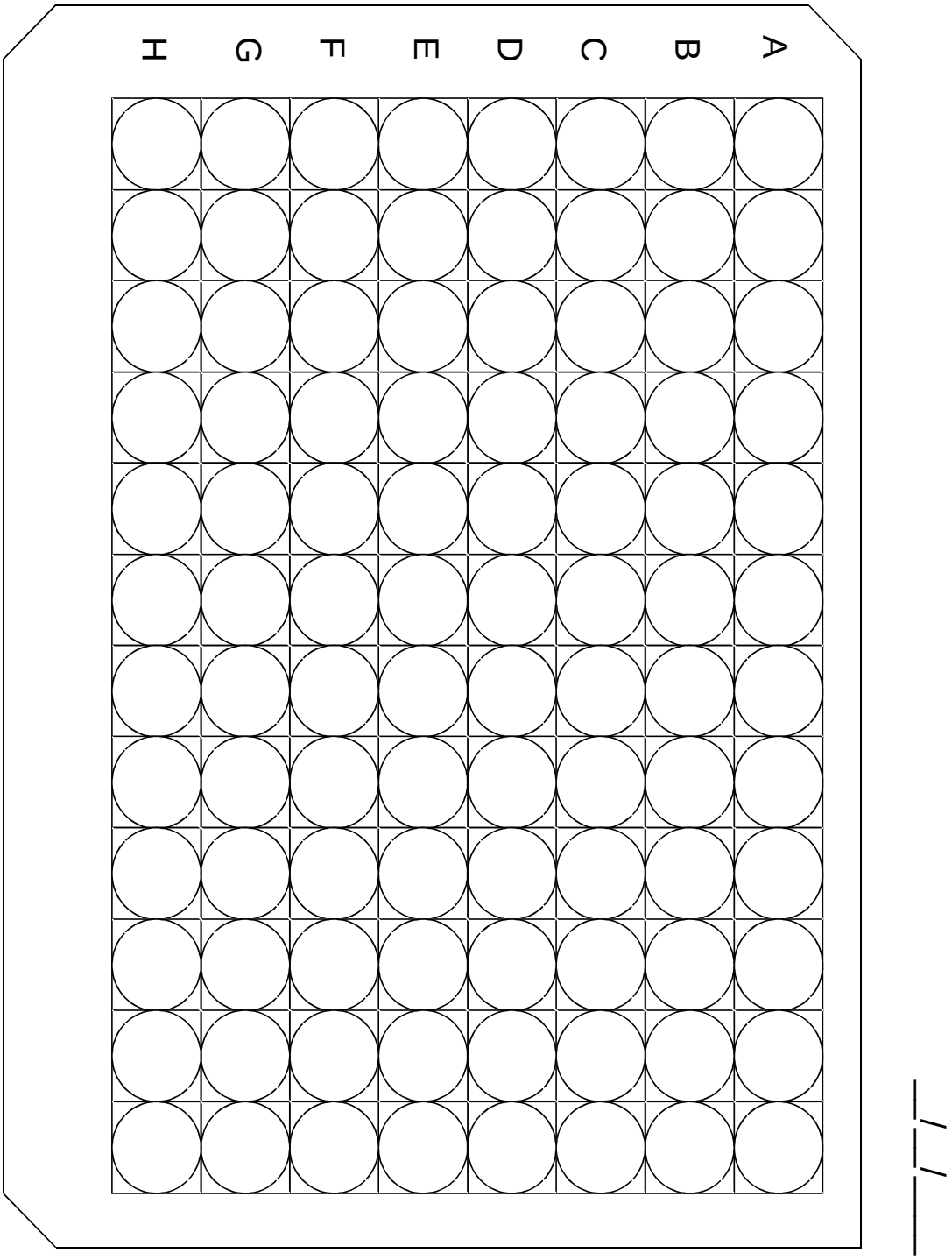
Mitosciences offers this product in kit form (MS922 Support pack). This support pack can be used not only with adherent cells but also with suspension cells.

Mitosciences also offers a range of ICE-validated primary antibodies.
http://www.mitosciences.com/in-cell-elisa.html

Mitosciences offers 2500X IRDye conjugated secondary antibodies for detection of mouse antibodies and rabbit antibodies as well as isotype specific anti-mouse antibodies in the 700 and 800 IR channels (MS923-927). By combining antibodies of different species or isotype and appropriate IR labeled secondary antibodies, two color multiplexing can be achieved in the 800/700 channels. Alternatively Mitosciences also offers HRP-conjugated anti-rabbit (MS929) and anti-mouse (MS928) secondary antibodies for colorimetric detection. HRP development solution (TMB) is required to complete the assay.

OTHER RESOURCES

MitoSciences has published an application guide which provides an overview of the ICE platform and ICE compatible antibodies with experimental data from various cell treatments which can be found at:
http://www.mitosciences.com/in-cell-elisa.html



LI-COR®, Odyssey®, Aerius® and IRDye® are registered trademarks or trademarks of LI-COR Biosciences Inc.

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com