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ab111541 –

ICE (In-Cell ELISA) Support Pack w/o plates

Instructions for Use

For use with suspension, apoptotic/detaching cells and adherent cell lines.

This product is for research use only and is not intended for diagnostic use.

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Product Overview

ab111541 (MS921) is for use with suspension, apoptotic/detaching cells and adherent cell lines. The pack contains buffers and a protocol to perform ICE with Abcam ICE-validated antibodies. For an ICE assay, it is necessary to purchase both primary antibody (ies) and labeled secondary antibody (ies). Antibodies are sold separately, allowing customizing the target(s) of interest, method of detection and multiplexing.

In-Cell ELISA uses quantitative immunocytochemistry to measure protein levels or post-translational modifications within cells. The cells are fixed to the bottom of a coated 96-well plate (not provided). Targets of interest are detected by primary antibodies, which are in turn quantified with labeled secondary antibody (ies). Abcam offers highly-specific, well-characterized primary antibodies, IRDye®- or HRP-labeled anti-mouse and anti-rabbit secondary antibodies, as well as IRDye®-labeled isotype specific anti-mouse antibodies. 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. IR imaging and quantification is performed using a LI-COR® Odyssey® or Aerius® system. HRP-labeled complexes are developed and quantified colorimerically using spectrophotometer.

Two protocols are available when using this kit. Protocol (1) suspension cells and cells likely to detach under experimental conditions (for example, adherent cells undergoing apoptosis readily detach from a culture plate). Protocol (2) - A second protocol is available for normal adherent cells. These protocols can be used with any of Abcam's ICE-validated antibodies. Specific scientific information, background and working concentration for each antibody are detailed in each antibody's corresponding datasheet.

Note - This kit does not include cell culture plates. 96 well or 384 well plates must be purchased separately and which are appropriate for the requirements of your cell line. However an alternative support pack ab111542 (MS922) is available which contains 5x high quality 96-well microplates for ICE.

1. Introduction - suspension or apoptotic /detaching cells

ab111541 In-Cell ELISA (ICE) Support Pack is for use with suspension or apoptotic/detaching cells. The pack contains enough buffers and a protocol to perform ICE with Abcam ICE-validated antibodies. A cell culture microplate is not provided in this pack, as an alternative Abcam offers a support pack containing cell culture microplates (ab111542/MS922).

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

In-Cell ELISA – Suspension Cell Protocol uses quantitative immunocytochemistry to measure protein levels or post-translational modifications of cultured suspension or apoptotic/detaching cells. The cells are fixed to the bottom of a coated 96-well plate (not provided). Targets of interest are detected by primary antibodies, which are in turn quantified with labeled secondary antibody(ies). Abcam offers highly-specific, well-characterized primary antibodies, IRDye®- or HRP-labeled anti-mouse and anti-rabbit secondary antibodies, as well as IRDye®-labeled isotype specific anti-mouse

antibodies. 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. IR imaging and quantification is performed using a LI-COR® Odyssey® or Aerius® system. HRP-labeled complexes are developed and quantified colorimerically using spectrophotometer.

To perform an ICE assay with cultured suspension cells, the cells must be physically attached to the assay plate. This protocol relies on combination of a coating of the assay plate and simple centrifugation steps. The suspension cells, treated as desired, are transferred into the assay plate, sedimented by centrifugation and, after the addition of fixative, sedimented again. When this protocol is used, nearly all cells are crosslinked to the assay plate (see Figure 1) and the crosslinked cells remain attached on the plate within the course of ICE assay (see Figure 2). In addition, it is advantageous that no serum-washing step is required; the suspension cells can be fixed onto the assay plate directly in culture medium containing serum (see Figures 1, 2, 3 and 4).

This protocol is also applicable for adherent cells that may detach under experimental conditions. For example, adherent cells undergoing apoptosis readily detach from a culture plate. The detachment of apoptotic cells often leads to their loss and thus

underestimation the proportion of apoptotic cells. This protocol eliminates the loss of the apoptotic/detaching cells and thus it is recommended for ICE assay on adherent cells undergoing apoptosis (see Figure 3). Materials and separate protocol sections are provided to achieve efficient attachment of suspension as well as apoptotic/detaching adherent cells. This protocol is a general protocol for ICE analysis and can be used with any of Abcam's ICE validated antibodies. Specific scientific information, background and working concentration for each antibody are detailed in each antibody's corresponding datasheet.

2. Assay Summary - suspension or apoptotic /detaching cells

Suspension Cell Seeding

- Seed cells into a plate or dish
- Treat cells as desired.
- (Concentrate) & transfer the cells into the Assay Plate in 100 µl.

Adherent Cell Seeding

- Seed cells into Assay Plate.
- Allow cells to adhere.
- Treat cells as desired in total volume of 100 µl.

Cell Fixation (30 minutes)

- Centrifuge the plate at 500x g for 8 minutes at room temperature.
- Immediately add an equal volume (100 µl) of 8% Paraformaldehyde
- Solution to fix and crosslink the cells to the plate.
- Immediately centrifuge the plate at 500x g for 8 minutes at room temperature.
- Incubate for additional 15 minutes.
- Wash wells with PBS (may be stored at 4°C at this point).

Permeabilization and Blocking (2.5 hours)

- Dilute the 100X Triton X-100 stock one hundred times in 1X PBS and add the 1X Permeabilization Buffer. Incubate 30 minutes at RT.
- Dilute the 10X Blocking Solution five times in 1X PBS and add the 2X Blocking Solution. Incubate 2 hours at RT.

Primary Antibody Incubation (Overnight)

- Dilute the primary antibody stock 500X in 1X Incubation Buffer and add the diluted primary antibodies.
- Incubate overnight at 4°C.
- Wash thoroughly.

Secondary Antibody Incubation (2 hours)

- Dilute the secondary antibody stock 1000X in 1X Incubation Buffer and add the diluted secondary antibodies.
- Incubate 2 hours at RT.
- Wash thoroughly.

Develop and Read Plate

- As appropriate, image the plate with an IR scanner or develop the HRP labelled plate and read it with a spectrophotometer. Export data.
- If desired, stain with Janus Green and measure relative cell seeding density in an IR imager or microplate spectrophotometer. Export data.
- Determine background corrected signal and then ratio signal to Janus Green.

3. Kit Contents - suspension or apoptotic /detaching cells

Part Number	Item	Quantity
8209725	10X Phosphate Buffered Saline (PBS)	250 ml
8209726	100X Triton X-100 (10% solution)	1.25 ml
8209727	400X Tween-20 (20% solution)	4 ml
8209728	10X Blocking Solution	40 ml
8209729	1X Janus Green Stain	30 ml

4. Storage and Handling - suspension or apoptotic /detaching cells

Store all components upright at 4°C.

5. Additional Materials Required - suspension or apoptotic /detaching cells

- Appropriate cell culture microplates with a surface coating to allow cell attachment. Cell culture treated plates must have clear bottom wells and black walls are recommended.
- Abcam ICE-validated primary antibody(ies)
- Appropriate Abcam 1000X IRDye®- or HRP- labeled secondary antibody (ies).
- For HRP detection HRP solution substrate is necessary.
- Adequate instrumentation. For IRDye® use a LI-COR® Odyssey® or Aerius® near-infrared imaging system. For HRP detection use a spectrophotometer plate reader capable of measuring absorbance at 650 (preferably in a kinetic mode) or 450 nm.

- Centrifuge equipped with standard microplates holders.
- 20% paraformaldehyde.
- Deionized water.
- Multichannel pipette (recommended).
- 0.5 M HCl (optional for Janus Green cell staining procedure).

6. Preparation of Reagents - suspension or apoptotic /detaching cells

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.

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 deionized water. 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 deionized 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.

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.

7. Assay Method - suspension or apoptotic /detaching cells

A. Cell Seeding- Suspension Cells:

1. To ensure efficient crosslinking of the suspension cells to Assay Plate, cells must be grown and treated (as desired) in a different plate or dish of choice (not provided).

2. The treated suspension cells are then transferred to the provided Assay Plate in 100 µl of media per well. The cell seeding density of the Assay Plate is cell type-dependent. For suggestions regarding the cell culture and seeding, see Appendix. If necessary, cells can be concentrated by centrifugation and re-suspended in PBS (preferred) or in media to desired concentration. As an example, HL-60 should be seeded between 150,000 and 300,000 cells per well, Jurkat cells between 100,000 and 200,000 cells per well in 100 ml of PBS (preferred) or media.

   Note – The media should contain no more than 10 % fetal serum otherwise efficiency of the cell crosslinking to the plate may be compromised

3. After treatment proceed to Step B1.

B. Cell Fixation

1. Centrifuge the Assay Plate at 500x g for 8 minutes at room temperature.

2. Immediately add 100 µl of 8 % Paraformaldehyde Solution to the wells of the plate.

3. Immediately centrifuge the plate at 500x g for 8 minutes at room temperature.

4. Incubate for additional 15 minutes.

5. 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°C 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.

C. 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 2X Blocking Solution to each well of the plate. Incubate 2 hours.

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

4. Remove 2X Blocking Solution and add 100 µl 1X Primary Antibody Solution to each well of the plate. Incubate overnight at 4°C.

   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.**

6. Prepare 1X Secondary Antibody Solution by diluting 12 µl 1000X labeled Secondary Antibody 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. Based on the chosen Abcam labelled secondary antibody (ies), use appropriate detection/imaging and instrumentation. Refer to the protocols accompanying the secondary antibodies.

D. Whole Cell Staining with Janus Green (Optional)

Note – The (IR or HRP) signal 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.

1. Remove last 1X Wash and add 50 µl of 1X Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.

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

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

4. Measure using a measure LI-COR® Odyssey® scanner in the 700 nm channel or OD595 nm using a standard microplate spectrophotometer.

8. Data Analysis - suspension or apoptotic /detaching cells

Note – Analyze data using 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 signal 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.

9. Supporting Data - suspension or apoptotic /detaching cells

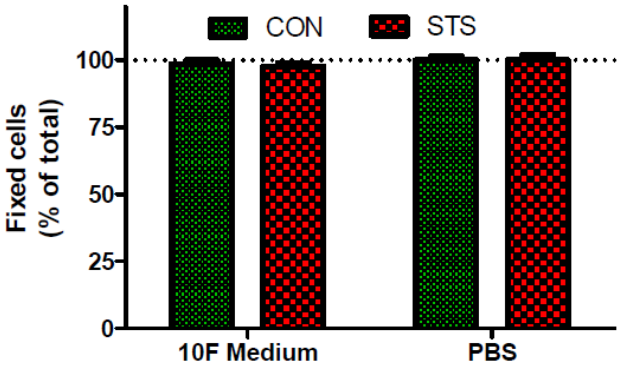

Figure 1. Suspension cells are efficiently crosslinked to the assay plate. Untreated (CON) or 4 hour Staurosporine-treated (STS, 1 mM) Jurkat cells were seeded at 200,000 cells per well into the assay plate in media containing 10% bovine fetal serum (10F Medium) or in PBS, and fixed as described in the ab111541(MS921) Protocol. After the fixation the number of cells attached (fixed) to the plate was determined and expressed as percentage of total seeded cells. Mean and standard error of the mean (n=2) is shown. Note that virtually all cells (untreated or STS-treated) attached to the plate whether the fixative was added to cells in 10F media or PBS.

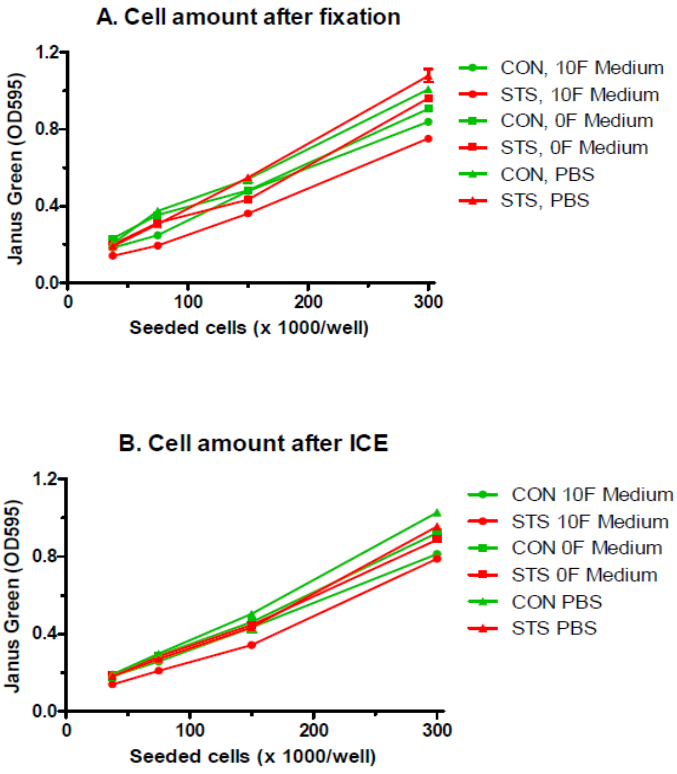

Figure 2. Suspension cells remain firmly attached to the assay plate within the ICE assay. Untreated (CON) or 4 hour Staurosporine-treated (STS, 1 mM) HL-60 cells were seeded in the indicated amounts into the assay plate in media containing 10% bovine fetal serum (10F Medium), media without serum (0F Medium) or PBS, and fixed as described in the ab111541(MS921) Protocol. The cell amount attached to the assay plate was determined by Janus green staining either just after the fixation (Panel A) or at the end of ICE assay (Panel B). Mean and standard error of the

mean (n=2) is shown. Note: (1) that virtually all fixed cells remained attached to the plate within the duration of the ICE assay (compare the cell amounts in Panel A to the cell amounts in Panel B), (2) that the STS-treated cells attached nearly as efficiently as the untreated cells and (3) that the cells attach efficiently even in media containing 10% serum.

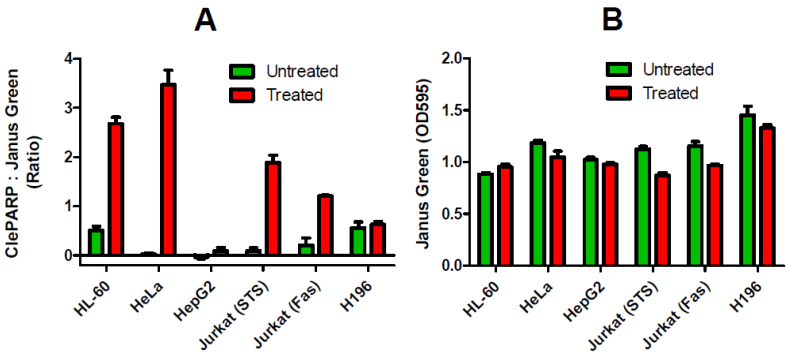

Figure 3. Apoptotic adherent and suspension cells are efficiently crosslinked to the assay plate. Adherent cell lines were seeded (HeLa and HepG2 at 50,000 per well, H196 at 150,000 per well) directly in assay plate, allowed to attach overnight and treated with 1 mM Staurosporine (STS) as indicated. Suspension cells were treated with 1 mM STS or 50 ng/ml Fas antibody as indicated, concentrated by centrifugation, re-suspended in media containing 10% serum and transferred (HL-60 at 300,000 per well, Jurkat at 200,000 per well) to the assay plate. Cells were fixed as described in the ab111541 (MS921) Protocol and the plate was analyzed by ICE to measure the cleaved PARP using ab110215 (MSA43). Mean and standard error of the mean (n=3) is shown. (A) Relative levels of apoptosis measured as PARP cleavage normalized to cell amount measured by Janus Green whole cell stain. Note HepG2 cells are resistant to undergoing apoptosis

under these conditions, consistent with all of our previous observations. (B) Cell amounts measured by Janus Green. Note no or very small differences between Janus Green staining of treated and untreated cells indicating that the treated cells undergoing apoptosis are efficiently crosslinked to the assay plate.

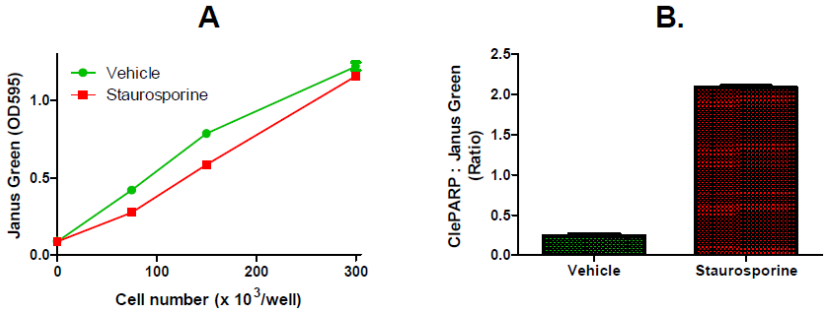

Figure 4. Apoptotic suspension cells are efficiently crosslinked to the assay plate. HL-60 cells were seeded as indicated into a 96-well plate and treated with 1 mM Staurosporine or vehicle for 4 hours. The treated cells were directly transferred to the 96 well assay plate. Cells were fixed as described in the ab111541 (MS921) Protocol and the plate was analyzed by ICE to measure the cleaved PARP using ab110215 (MSA43). (A) Cell amounts measured by Janus Green. Mean and standard error of the mean (n=6, coefficient of variation 0.05 or less) is shown. Note no or very small differences of Janus Green staining between vehicle-and Staurosporine-treated cells indicating that the Staurosporine-treated cells undergoing apoptosis are efficiently crosslinked to the assay plate. (B) Relative levels of apoptosis measured as PARP cleavage normalized to cell amount measured by Janus Green whole cell stain. Mean and standard error of the mean (n=3) for cells seeded at 300,000 per well is shown.

10. Appendix - suspension or apoptotic /detaching cells

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

The suspension cells should be grown and treated as desired in a separate dish or plate of choice. We recommend simple (not treated) polystyrene vessels to minimize cell attachment during the cell culture and/or drug treatment. Do not use the coated Assay Plates for cell culturing (including drug treatment) or the efficiency of cell crosslinking to the Assay Plate may be compromised. At the end of the treatment or other experimental condition, the cells are transferred into the Assay Plate in PBS (preferred) or in media containing up to 10% fetal serum. No major difference in the efficiency of crosslinking of HL-60 cells to the Assay Plate was observed whether the cells were in RPMI1640 media containing 10% fetal serum or in 1X PBS. However, media containing more than 10% serum may compromise the cell crosslinking and may lead to significant cell loss.

Adherent cells can be seeded and treated directly in the Assay Plate. The coating of the Assay Plate does not interfere with the cell growth and enhances the cell attachment. Cell attachment can be checked with a microscope. When the cells are fully attached the media

can be removed and replaced with media containing drug 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 suspension cells) and the abundance of the target protein. As a general guideline, if final fixed cells form a monolayer, ICE assays using Abcam ICE-validated antibodies give robust signals. The cell seeding can be determined experimentally by microscopic observation of cell density of serially diluted cells. For suspension cells, prepare a serial dilution of the cells in a plate (of similar well dimensions) and observe the cell density in a microscope after a simple centrifugation step (8 min at 500x g). For adherent cells, prepare serial dilution of the cells in a plate (of similar well dimensions) and allow them to attach prior to observation. The Assay Plate has flat bottom wells of bottom surface 29.8 mm^2. Working on the high end of cell densities will generate stronger signals and allow small signal increases to be measured accurately, this is important in particular for less abundant targets or lower affinity antibodies. 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, Jurkat cells from 100,000 to 200,000 cells per well and HL-60 from 150,000 to 300,000 cells per well.

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 Abcam with a recommended final concentration for ICE which can be found in datasheet for each antibody. The assay can be also performed in 384-well plate format. Contact Abcam to inquire about 384 well Assay Plates.

Abcam offers a range of ICE-validated primary antibodies.

Abcam offers IR dye 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. By combining antibodies of different species or isotype and appropriate IR labelled secondary antibodies, two colour multiplexing can be achieved in the 800/700 channels.

Abcam also offers HRP-conjugated secondary anti-rabbit and anti-mouse secondary antibodies for colorimetric detection. These products also include the development solution to complete the assay.

11. Introduction – adherent cells

(ab111541/MS921) protocol is for use with adherent cells. It contains enough materials to perform five 96 well microplate ICE experiments with Abcam's ICE-validated antibodies. A cell culture microplate is not provided in this pack, as an alternative Abcam offers a support pack containing cell culture microplates (ab111542/MS922).

For an 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. For HRP detection HRP substrate solution and a standard microplate reader are required. For non adherent cells or cells likely to detach see our suspension cell protocol ab111541 (MS921).

In-Cell ELISA – Adherent Cells uses quantitative immunocytochemistry to measure protein levels or posttranslational 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 quantified with labeled secondary antibody(ies). Abcam offers highly-specific, well-characterized primary antibodies, IRDye®- or HRP-labeled anti-mouse and anti-rabbit secondary antibodies, as well as IRDye®-

labeled isotype specific anti-mouse antibodies. 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. IR imaging and quantification is performed using a LI-COR® Odyssey® or Aerius® system. HRP-labeled complexes are developed and quantified colorimerically using spectrophotometer.

This protocol is a general protocol for ICE analysis and can be used with any of Abcam's ICE validated antibodies. Specific scientific information, background and working concentration for each antibody are detailed in each antibody's corresponding datasheet.

12. Assay Summary – adherent cells

Adherent Cell Seeding

- Seed cells into Assay Plate.
- Allow cells to adhere.
- Treat cells as desired in total volume of 100 µl.

Cell Fixation (30 minutes)

- Add an equal volume (100 µl) of 8% Paraformaldehyde Solution to fix and crosslink the cells to the plate.
- Incubate for additional 15 minutes.
- Wash wells with PBS (may be stored at 4°C at this point).

Primary Antibody Incubation (Overnight)

- Dilute the primary antibody stock 200X in 1X Incubation Buffer and add the diluted primary antibodies.
- Incubate overnight at 4°C.
- Wash thoroughly.

Secondary Antibody Incubation (2 hours)

- Dilute the secondary antibody stock 1000X in 1X Incubation Buffer and add the diluted secondary antibodies.
- Incubate 2 hours at RT.
- Wash thoroughly.

Develop and Read Plate

- As appropriate, image the plate with an IR scanner or develop the HRP labelled plate and read it with a spectrophotometer. Export data.
- If desired, stain with Janus Green and measure relative cell seeding density in an IR imager or microplate spectrophotometer. Export data.
- Determine background corrected signal and then ratio signal to Janus Green if desired.

13. Kit Contents – adherent cells

Part Number	Item	Quantity
8209725	10X Phosphate Buffered Saline (PBS)	250 ml
8209726	100X Triton X-100 (10% solution)	1.25 ml
8209727	400X Tween-20 (20% solution)	4 ml
8209728	10X Blocking Solution	40 ml
8209729	1X Janus Green Stain	30ml

14. Storage and Handling – adherent cells

Store all components upright at 4°C.

15. Additional Materials Required – adherent cells

- Appropriate cell culture microplates with a surface coating to allow cell attachment. Cell culture treated plates must have clear bottom wells and black walls are recommended (consult Abcam for advice).
- Abcam ICE-validated primary antibody (ies).
- Appropriate Abcam 1000X IRDye®- or HRP- labelled secondary antibody (ies).
- For HRP detection HRP solution substrate is necessary.
- Adequate instrumentation. For IRDye® use a LI-COR® Odyssey® or Aerius® near-infrared imaging system. For HRP detection use a spectrophotometer plate reader capable of measuring absorbance at 650 (preferably in a kinetic mode) or 450 nm.
- 20% paraformaldehyde.
- Deionized water.
- Multichannel pipette (recommended)

- 0.5 M HCl (optional for Janus Green cell staining procedure).

16. Preparation of Reagents – adherent cells

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.

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 deionized 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 deionized water, 1.25 ml 10X 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.

17. Assay Method – adherent cells

A. Cell Seeding- Adherent Cells

1. Adherent cells can be 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 with a drug of interest to induce apoptosis. 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 B1.

B. 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°C 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.

C. 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 2X Blocking Solution to each well of the plate. Incubate 2 hours.

3. Prepare 1X Primary Antibody Solution by diluting Abcam 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°C.

   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.**

6. Prepare 1X Secondary Antibody Solution by diluting 12 µl of 1000X labelled-secondary antibody (ies) into 12 ml 1X Incubation Buffer.

   Note – Protect fluorescently labelled 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. Based on the chosen Abcam labelled secondary antibody (ies), use appropriate detection/imaging and

instrumentation. Refer to the protocols accompanying the secondary antibodies.

D. Whole Cell Staining with Janus Green (Optional)

Note – The (IR or HRP) signal 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.

1. Remove last 1X Wash Buffer and add 50 µl of 1X Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.

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

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

4. Measure using a LI-COR® Odyssey® scanner in the 700 nm channel or measure OD595 nm in a standard microplate spectrophotometer.

18. Data Analysis – adherent cells

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 signal 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.

19. Appendix – adherent cells

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. The coating of the Assay Plate does not interfere with the cell growth and enhances the cell attachment. Cell attachment can be checked with a microscope. When the cells are fully attached the media can be removed and replaced with media containing drug 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 Abcam 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 Abcam with a recommended final concentration for ICE which can be found in the datasheet for each antibody.

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

Abcam offers an equivalent In Cell ELISA support pack for Suspension cells.

Abcam also offers a range of ICE-validated primary antibodies.

Abcam offers IR dye 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. By combining antibodies of different species or isotype and appropriate IR labelled secondary antibodies, two colour multiplexing can be achieved in the 800/700 channels. Alternatively Abcam also offers HRP-

conjugated secondary anti-rabbit and anti-mouse secondary antibodies for colorimetric detection. These products also include the development solution to complete the assay.

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