

ab111542

In-cell ELISA Support Pack for Suspension Cells

Instructions for use:

For use with suspension or apoptotic/detaching cells

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

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1. BACKGROUND

ab111542 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.

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.

This 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 Aeries® system. HRP-labeled complexes are developed and quantified colorimetrically using spectrophotometer.

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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

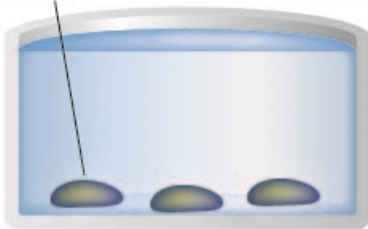
INTRODUCTION

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).

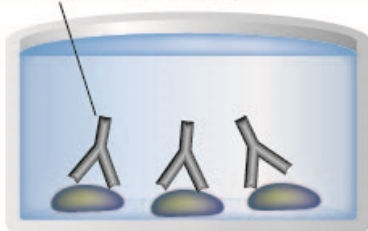
This protocol is also applicable for adherent cells that may detach under experimental conditions. For example, adherent cells undergoing apoptosis readily detach from a culture plate. The detachment of apoptotic cells often leads to their loss and thus underestimation the proportion of apoptotic cells.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

Sample



Primary Antibody



Labeled HRP-Conjugate



Substrate

Colored Product



Seed cells and incubate overnight. Apply treatment activators or inhibitors. Fix cells with Fixing Solution. Centrifuge at 500g for 8 minutes at room temperature add permeabilization and blocking buffers..

Add prepared primary antibody to each well used. Incubate at room temperature.

Empty and wash each well. Add prepared secondary antibody. Incubate at room temperature.

Image plate and analyze data. If desired, stain with Janus Green and measure relative cell seeding density in a microplate spectrophotometer or IR imager. Calculate ratios and perform data analysis.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

GENERAL INFORMATION

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Phosphate Buffered Saline (PBS)	250 mL	4°C
100X Triton X-100 (10% solution)	1.25 mL	4°C
400X Tween-20 (20% solution)	4 mL	4°C
10X Blocking Solution	40 mL	4°C
1X Janus Green Stain	30 mL	4°C
Sterile 96-Well Assay Microplate	5 EA	4°C
Plate seals	5 EA	4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

- 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 Aeries® 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)

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. 1X PBS

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

9.2. 1X Wash Buffer

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

9.3. 8% Paraformaldehyde Solution

Immediately prior to use prepare 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.

9.4. 1X Permeabilization Buffer

Immediately prior to use prepare by diluting 0.25 mL 100X Triton X-100 in 24.75 mL 1X PBS.

9.5. 2X Blocking Solution

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

9.6. 1X incubation Buffer

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

10. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- Prepare all reagents, working, and samples as directed in the previous sections.

Cell Seeding- Suspension Cells:

- 10.1. 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).
- 10.2. The treated suspension cells are then transferred to the provided Assay Plate in 100 μ l 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*

- 10.3. After treatment proceed to Step 10.4.

Cell Fixation

- 10.4. Centrifuge the Assay Plate at 500x g for 8 minutes at room temperature.
- 10.5. Immediately add an equal volume (100 μ L) of 8 % Paraformaldehyde Solution to the wells of the plate.
- 10.6. Immediately centrifuge the plate at 500x g for 8 minutes at room temperature.
- 10.7. Incubate for additional 15 minutes.

ASSAY PROCEDURE

- 10.8. 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 μ L of 1X PBS. Finally, add 100 μ L 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.*

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.*

- 10.9. Remove PBS and add 200 μ L of freshly prepared 1X Permeabilization Buffer to each well of the plate. Incubate 30 minutes.
- 10.10. Remove 1X Permeabilization Buffer and add 200 μ L 2X Blocking Solution to each well of the plate. Incubate 2 hours.
- 10.11. Prepare 1X Primary Antibody Solution by diluting Abcam stock antibody(ies) into 1X Incubation Buffer. *See Appendix for more information.*
- 10.12. Remove 2X Blocking Solution and add 100 μ L 1X Primary Antibody Solution to each well of the plate. Incubate overnight at 4°C.
- 10.13. *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.*
- 10.14. 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 μ L of 1X Wash Buffer. **Do not remove the last wash until step 10.16.**

ASSAY PROCEDURE

10.15. Prepare 1X Secondary Antibody Solution by diluting 12 μ L 1000X labelled Secondary Antibody(ies) into 12 mL 1X Incubation Buffer.

NOTE – *Protect fluorescently labeled antibodies from light.*

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

10.17. Remove Secondary Antibody Solution and wash 4 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 250 μ L of 1X Wash Buffer. **Do not remove the last wash until step 10.20.**

10.18. Based on the chosen Abcam labeled secondary antibody(ies), use appropriate detection/imaging and instrumentation. Refer to the protocols accompanying the secondary antibodies.

10.19. **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.*

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

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

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

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

11. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

NOTE – *Analyze data using suitable data analysis software, such as Microsoft Excel or GraphPad Prism.*

- 11.1. 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.
- 11.2. 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.
- 11.3. Normalize the ICE signal. Divide the background-corrected ICE signal by the (background-corrected) Janus Green signal.

12. TYPICAL DATA

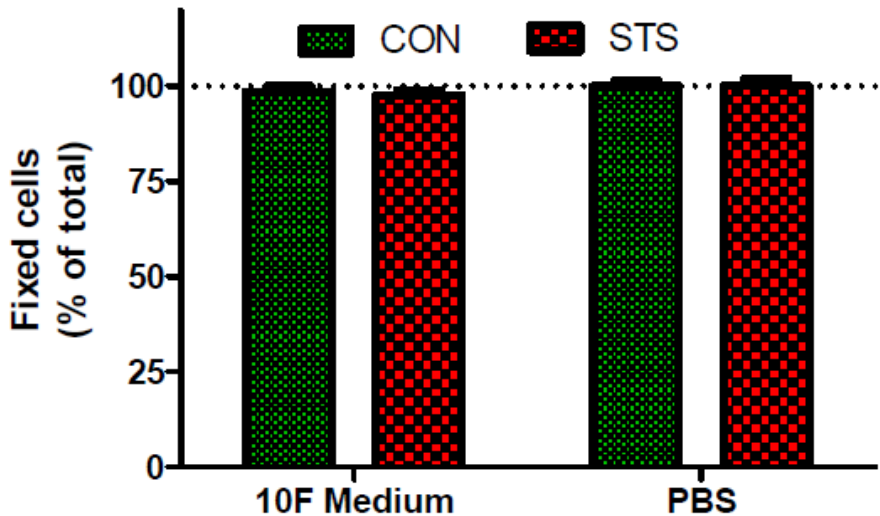


Figure 1. Suspension cells are efficiently crosslinked to the assay plate. Untreated (CON) or 4 hour Staurosporine-treated (STS, 1 mM) Jurkat cells were seeded at 200,000 cells per well into the assay plate in media containing 10% bovine fetal serum (10F Medium) or in PBS, and fixed as described in the ab111542 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.

DATA ANALYSIS

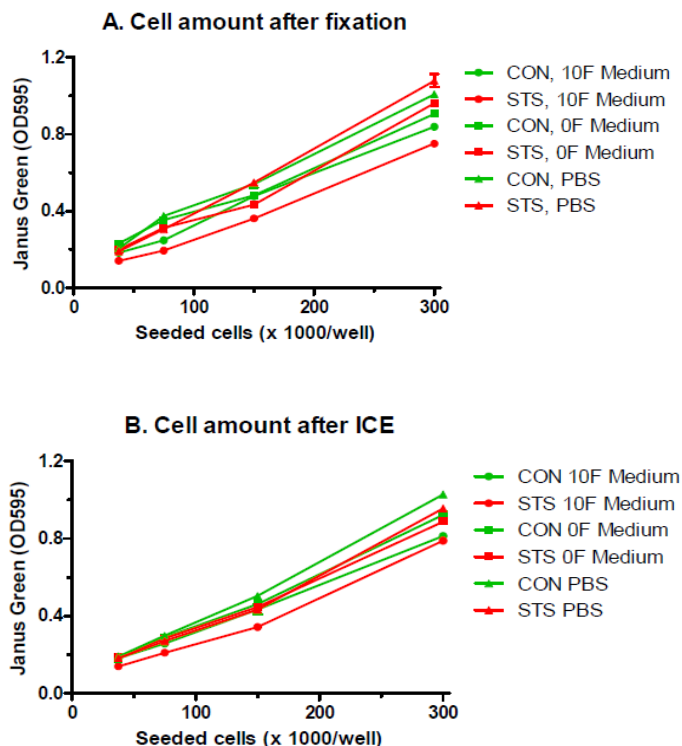


Figure 2. Suspension cells remain firmly attached to the assay plate within the ICE assay. Untreated (CON) or 4 hour Staurosporine-treated (STS, 1 mM) HL-60 cells were seeded in the indicated amounts into the assay plate in media containing 10% bovine fetal serum (10F Medium), media without serum (0F Medium) or PBS, and fixed as described in the ab111542 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.

DATA ANALYSIS

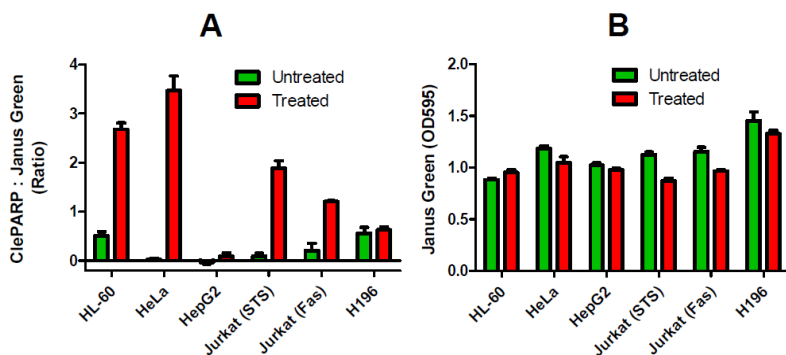


Figure 3. Apoptotic adherent and suspension cells are efficiently crosslinked to the assay plate. Adherent cell lines were seeded (HeLa and HepG2 at 50,000 per well, H196 at 150,000 per well) directly in assay plate, allowed to attach overnight and treated with 1 mM Staurosporine (STS) as indicated. 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 ab111542 Protocol and the plate was analyzed by ICE to measure the cleaved PARP using ab110215. 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.

DATA ANALYSIS

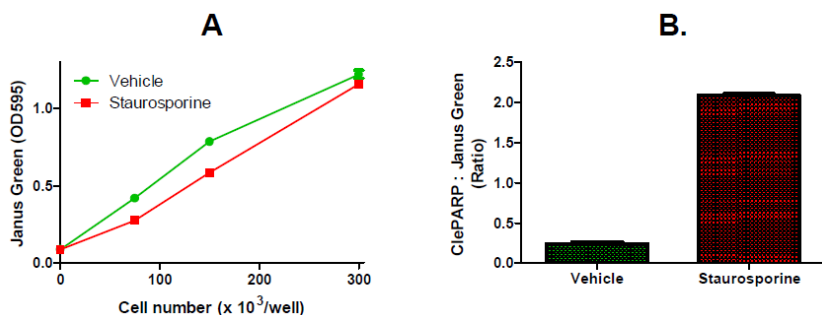


Figure 4. Apoptotic suspension cells are efficiently crosslinked to the assay plate. HL-60 cells were seeded as indicated into a 96-well plate and treated with 1 mM Staurosporine or vehicle for 4 hours. The treated cells were directly transferred to the 96 well assay plate. Cells were fixed as described in the ab111542 Protocol and the plate was analyzed by ICE to measure the cleaved PARP using ab110215. (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.

13. APPENDIX

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 mm². Working on the high end of cell densities will generate stronger signals and allow small signal increases to be measured

RESOURCES

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 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 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 color 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.

14. NOTES

RESOURCES

RESOURCES

RESOURCES

UK, EU and ROW

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