

ab151280

AMP alpha1 (Total and phosphor T172) In-Cell Elisa Kit (IR)

Instructions for use:

For measuring in high throughput levels of AMPK alpha1 total protein and phosphorylated at pT172 in human cell lines.

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

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1. BACKGROUND

ab151280 is an In-Cell ELISA (ICE) assay kit that uses quantitative immunocytochemistry to measure levels of AMPK α 1 total protein and phosphorylated at threonine 172 in cultured cells. Cells are fixed in a microplate and targets of interest are detected with highly specific, well-characterized antibodies. Relative target levels are quantified using an IRDye®-labeled Secondary Antibody Cocktail and IR imaging using a LI-COR® Odyssey® or Aeries® system. Optionally, antibody signal intensity can be normalized to the total cell stain Janus Green.

In-Cell ELISA (ICE) technology is used to perform quantitative immunocytochemistry of cultured cells with a near-infrared fluorescent dye-labeled detector antibody. The technique generates quantitative data with specificity similar to Western blotting, but with much greater quantitative precision and higher throughput due to the greater dynamic range and linearity of direct fluorescence detection and the ability to run up to 96 samples in parallel. This method rapidly fixes the cells in situ, stabilizing the in vivo levels of proteins and their post-translational modifications, and thus essentially eliminates changes during sample handling, such as preparation of protein extracts. Finally, the signal can be normalized to cell amount, measured by the provided Janus Green whole cell stain, to further increase the assay precision.

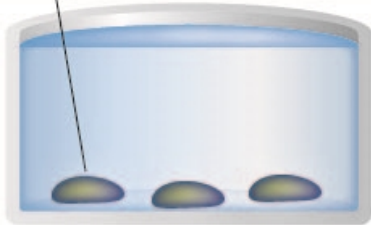
AMP-activated protein kinase (AMPK) is an energy sensor protein kinase that plays a key role in regulating cellular energy homeostasis. Mammalian AMPK is a heterotrimer kinase, containing a catalytic subunit (α) and two regulatory subunits (β and γ). Each subunit has different isoforms (α 1, α 2, β 1, β 2, γ 1, γ 2, γ 3) with differential tissue expression, cellular localization and functionality. It has been hypothesized that when ADP or AMP are present at high levels, these nucleotides bind directly to the γ subunit, leading to a conformational change that allows phosphorylation of Thr172 at the α subunit. Phosphorylation of AMPK α activates the kinase which leads to downstream effects concerted to increase catabolic and suppress anabolic pathways in order to restore levels of cellular ATP and ultimately cell fate.

INTRODUCTION

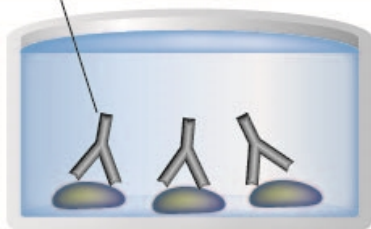
AMPK is activated physiologically due to stresses such as low nutrients and prolonged exercise. Furthermore AMPK may be activated pharmacologically by metformin (the most widely prescribed Type 2 diabetes drug), phenformin, AICAR (acadesine/AICA riboside) and resveratrol. When active due to low nutrients, AMPK coordinates the control of cell growth and autophagy via suppression of the mammalian target of rapamycin complex 1 (mTORC1) pathway. Furthermore, AMPK also controls metabolism via direct phosphorylation of metabolic enzymes such as acetyl-CoA carboxylase (ACC1 and ACC2) HMG-CoA reductase, hormone sensitive lipase (HSL), adipocyte triglyceride lipase (ATGL), insulin receptor substrate 1 (IRS1), phosphofructo-kinase (PFKFB). Control of metabolism also occurs long term through control of transcription and chromatin structure via phosphorylation of transcription factors (SREBP1, PPAR γ), coactivators (CRTC family, PGC1 α), acetyltransferase p300, histone H2B and histone deacetylases (HDACs class IIa). Activation of AMPK has also been linked to circadian clock regulation via phosphorylation of Cry1, coupling daily light and dark cycles to the metabolic control of fed and fasting cycles. In addition, it has been suggested that AMPK may control cell polarity and cytoskeletal dynamics in some settings.

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. Incubate at room temperature. Add Quenching Buffer. Incubate at room temperature. Add Blocking Buffer. Incubate at 37°C.

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 at least 6 months 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.

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)	100 mL	4°C
33X Triton X-100	1.5 mL	4°C
400X Tween – 20	2 mL	4°C
10X Blocking Buffer	15 mL	4°C
Antigen Retrieval Buffer	25 mL	4°C
100X AMPK α (Total and pT172/T183) Primary Antibody Cocktail	120 μ L	4°C
500X IRDye®-Labeled Secondary Antibody Cocktail (anti-Mouse IRDye800® and anti-Rabbit IRDye680®)	30 μ L	4°C
Janus Green Stain	17 mL	4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

- A LI-COR® Odyssey® or Aeries® infrared imaging system.
- Heated Water bath
- Plate heater
- 96 or 384-well amine coated plate(s).
- 20% paraformaldehyde.
- Nanopure water or equivalent.
- Multi and single channel pipettes.
- 0.5 M HCl (optional for Janus Green cell staining procedure).
- Optional: Humid box for overnight incubation step.
- Optional: Plate shaker for all incubation steps.

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 or control 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 you have the right type of plate for your detection method of choice.
- 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
- Equilibrate all reagents to room temperature.

9.1. 1X PBS

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

9.2. 1X Wash Buffer

Prepare by diluting 750 μ L of 400X Tween-20 in 300 mL of 1X PBS. Mix well. Store at room temperature.

9.3. 8% Paraformaldehyde Solution

Immediately prior to use prepare in PBS. To make 8% Paraformaldehyde combine 6 mL of nanopure water or equivalent, 1.2 mL of 10X PBS and 4.8 mL of 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 / 2X Blocking Solution

Immediately prior to use prepare by diluting 0.66 mL of 33X Triton X-100, 4.4 mL of 10X blocking buffer in 17 mL of 1X PBS. Mix well.

9.5. 1X incubation solution

Immediately prior to use prepare by diluting 0.72 mL of 33X Triton X-100, 2.4 mL of 10X blocking buffer in 21 mL of 1X PBS. Mix well.

ASSAY PROCEDURE

10. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- It is recommended to assay all controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- Make certain that the microplate does not dry out at any time before or during the assay procedure

Seeding

10.1. Seed adherent cells directly into an amine coated plate and allow them to attach for at least 6 hours or overnight. It is advised to seed in a 100 μ L volume of the same media used to maintain the cells in bulk culture. Optimal seeding density is cell type dependent and the goal is to seed cells such that they are just reaching confluence (but not over-confluent) at the time of fixation.

Table 1: Optimal Seeding Ranges

Typical working ranges	
Sample Type	Seeding Range [cells/well]
Hek293T	12,000 – 100,000
HepG2	20,000 – 70,000
C2C12 treated with 0.5mM AICAR for 3 hours	20,000 – 80,000
H4IIE treated with 0.5mM AICAR for 3 hours	25,000 – 200,000

For optimization of seeding in other cell lines see section 14.2 below. The attached cells can be treated if desired with a drug of interest.

10.2. Fix cells by adding a final concentration of 4% Paraformaldehyde Solution. Either by: (1) Add an equal volume of 8% Paraformaldehyde Solution to the culture volume (e.g. add 100 μ L 8% Paraformaldehyde to a well with 100 μ L media) or (2) gently remove culture media from the wells and replace with 100 μ L 4% Paraformaldehyde Solution.

ASSAY PROCEDURE

- 10.3. Incubate for 10 minutes at room temperature.
- 10.4. Remove 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 200 μ L of 1X PBS. Finally, add 100 μ L of 1X PBS to the wells of the plate. If desired, the plate can now be stored at 4°C for several days. Cover the plate with a lid or seal while stored. 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

- It is recommended to use a plate shaker (~200 rpm) during all incubation steps. Any step involving removal of buffer or solution should be followed by blotting the plate gently upside down on a paper towel before refilling wells. Unless otherwise noted, incubate at room temperature.
 - During development of this assay we have not observed problems with edge effects. However if edge effects are of concern, the perimeter wells of the plate can be used as control wells (primary antibody omitted). Regardless, it is required to leave at minimum one well from which the primary antibodies are excluded to determine background signals of the assay.
- 10.5. Decant the Antigen retrieval into a glass – heat resistant container and equilibrate to 90°C in water bath.
- 10.6. Preheat the plate heater to 80°C.
- 10.7. Remove the 1X PBS from the microplate wells and add 200 μ L of the heated antigen retrieval buffer to each well of the plate. Incubate for 15 min on plate heater.
- 10.8. Remove the antigen retrieval buffer and wash the plate three times. For each wash, rinse each well of the plate with 200 μ L of 1X PBS.

ASSAY PROCEDURE

- 10.9. Remove the 1X PBS and add 200 μ L of the Permeabilization & Blocking Solution to each well of the plate. Incubate for 1 hour at room temperature.
- 10.10. Prepare 1X Primary Antibody Cocktail Solution by diluting the cocktail stock 100X into appropriate volume of 1X incubation solution (i.e. 12 mL of Incubation Solution + 120 μ L of the 100X Anti-AMPK α mouse mAb + Anti-AMPK α pT172 rabbit mAb).
- 10.11. Remove Permeabilization & Blocking Solution and add 100 μ L of 1X Primary Antibody Solution to each well of the plate. Incubate for 2 hours at room temperature or 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.*
- 10.12. Remove the Primary Antibody Solution and wash the plate three times. For each wash, rinse each well of the plate with 200 μ L of 1X Wash Buffer. **Do not remove the last wash until step 10.13.**
- 10.13. Prepare 1X Secondary Antibody Solution by diluting 24 μ L of 500X IRDye®-Labeled Secondary Antibody Cocktail into of 12 mL 1X incubation solution. Protect labeled antibodies from light. *Note – The secondary antibody cocktail is 1:1 a mixture of IRDye680®-labelled anti-rabbit antibody and IRDye800®-labelled anti-mouse antibody.*
- 10.14. Remove the 1X Wash Buffer and add 100 μ L of the 1X Secondary Antibody Solution to each well of the plate. Incubate 2 hours at room temperature in the dark.
- 10.15. Remove 1X Secondary Antibody Cocktail Solution and wash 3 times. For each wash, rinse each well of the plate with 200 μ L of 1X Wash Buffer.
- 10.16. Wash 2 times with 1XPBS, using 200 μ L of 1X PBS for each well. **Do not remove the last wash.**

ASSAY PROCEDURE

- 10.17. Wipe the bottom of the plate and the scanner surface with a damp lint-free cloth before scanning the plate on the LI-COR® Odyssey® system. Collect data in the 700 and 800 channels according to manufacturer's instructions. The optimal focus off-set for typical amine plates is 3.9. The Total AMPK α protein signal corresponds to the 800 channel (IRDye800®) and the AMPK α (pT172) protein signal corresponds to the 700 channel (IRDye680®). *Note – The absolute value of the IR signal is dependent on the intensity settings. Value 8.5 is recommended for initial scanning. Adjust as needed so that the signal is not saturated in any well.*
- 10.18. After collecting IR data remove the last PBS wash and add 100 μ L of Janus Green Stain to each well of the plate. Incubate plate for 5 minutes at room temperature.

NOTE – *The IR data should be normalized to the Janus Green staining intensity to account for differences in cell seeding density.*
- 10.19. Remove the dye and wash the plate 5 times in deionized water or until excess dye is removed.
- 10.20. Remove last water wash, blot to dry, add 100 μ L of 0.5 M HCl to each well of the plate and incubate for 10 minutes in a plate shaker.
- 10.21. Measure OD595 nm using a standard microplate spectrophotometer or measure a signal in the 800 nm channel using a LI-COR® Odyssey® scanner.

11. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- 11.1. Background subtraction. Determine the raw signal intensity (Integrated Intensity) values for the IR680 and IR800 channels for the wells that lacked primary antibody. Subtract the mean background values from all other IR680 or IR800 experimental values respectively.
 - 11.2. Janus Green normalization of both targets. Divide the background subtracted IR intensities (from 11.1) by the Janus Green value of the corresponding well. The result is the “normalized intensity”.
 - 11.3. Normalization of Phospho signal from total protein. Divide the phospho AMPK α normalized intensity by the total AMPK α normalized intensity.

12. TYPICAL DATA

Assay performance was tested using various treated and untreated cell lines containing high levels of endogenous AMPK α total protein and AMPK α phosphorylation at T172. Figure 1 shows dynamic range of the assay on amine coated plates.

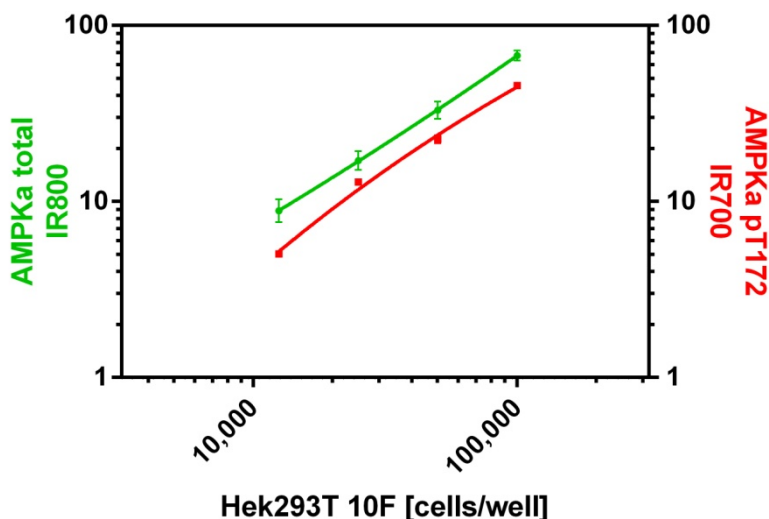


Figure 1. Dynamic range of AMPK α total and pT172. Cells were seeded the day before fixation at the specified cell density and allowed to adhere overnight. Cells were then fixed and signal was obtained using this kit as described. In this experiment, the Hek293T cells were permeabilized with methanol at -20°C due to their sensitivity to antigen retrieval. Total AMPK α and AMPK α pT172 are shown after background subtraction.

Antibody Specificity - Because confidence in antibody specificity is critical to ICE data interpretation, the primary antibodies in this kit were also validated on: (1) the ICE platform using lambda phosphatase and models known to upregulate the phosphorylation levels, (2) the Western blot platform targeting the correct band size as well as recombinant AMPK α .
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DATA ANALYSIS

protein and (3) immunocytochemistry to show cytoplasmic co-localization of AMPK α total and AMPK α pT172 signal. The amino acids surrounding the phosphorylation site at threonine 172 from AMPK α 2 has 100% sequence similarity to the phosphorylation site at threonine 183 from AMPK α 1. Hence, if isoform α 1 is expressed in the sample of interest, the antibody included in this kit will measure both phosphorylation sites (T172 and T183). In Figure 2A specificity of the signal on the ICE platform is shown with the use of 0.5 mM AICAR (ab120358) and 1 μ M oligomycin for 3 hours. Both AICAR and oligomycin are known activators of AMPK activity in live cells. Figure 2B, on the other hand, shows specificity of AMPK pT172 with lambda phosphatase (LP) which artificially dephosphorylates AMPK α pT172 after fixation. LP crosses the plasma membrane minimally after permeabilization with 0.3% Triton (used in this kit). Methanol is required instead to allow complete entry of the enzyme.

In Figure 3, AMPK α total and pT172 show specific co-localization in the cytoplasm. The cytoplasmic signal for the pT172 target is absent on serum starved H4IIE cells treated with DMSO only, whereas it is significantly induced with the use of 1 μ M oligomycin.

In Figure 4 a single band is found at about 64kDa for both AMPK α total and phosphorylated. The phosphorylated band is completely removed in lysates treated with 1/100 dilution of LP at 34°C for 45 minutes and it is also upregulated in serum starved mouse C2C12 myoblasts.

Reproducibility - ICE results provide accurate quantitative measurements of antibody binding and hence cellular antigen concentrations. The coefficient of the intra-assay variation for this assay kit on Hek293T cells is typically 4.7% for total and 8.7% for pT172. The assay was also found to be highly robust with a mean Z factor from multiple cell densities of 0.81 for AMPK α total (3k – 100k/well) and 0.64 for AMPK α pT172 (12 – 100k/well).

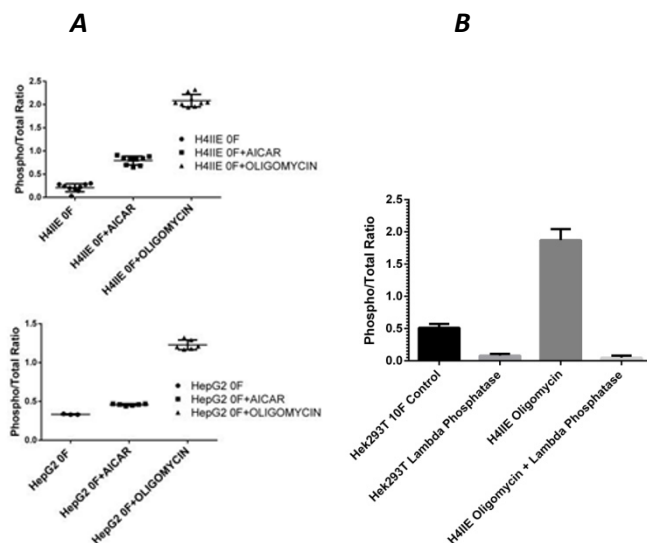


Figure 2. Specificity of Signal by In Cell ELISA. (A) H4IIE and HepG2 cells were seeded on amine coated plates within the working range of the assay the day before fixation. Levels of total AMPK α and phosphorylated protein at T172/183 were measured after serum starvation and treatment with AICAR (ab120358) or oligomycin. Normalized signal intensities were ratio to show the effect of treatment on the phosphorylation status of AMPK. (B) H4IIE cells treated with oligomycin and untreated Hek293T were fixed on a 96 well plates at densities within the working range of the assay. After fixation, cells were permeabilized with methanol at -20°C for 30 minutes and treated with and without Lambda Phosphatase at 40°C for 45 minutes on a plate heater. Blocking and antibody incubations were carried out according to this protocol (without the use of Triton X-100). Normalized signal intensities were ratio to show the effect of treatment on the phosphorylation status of AMPK.

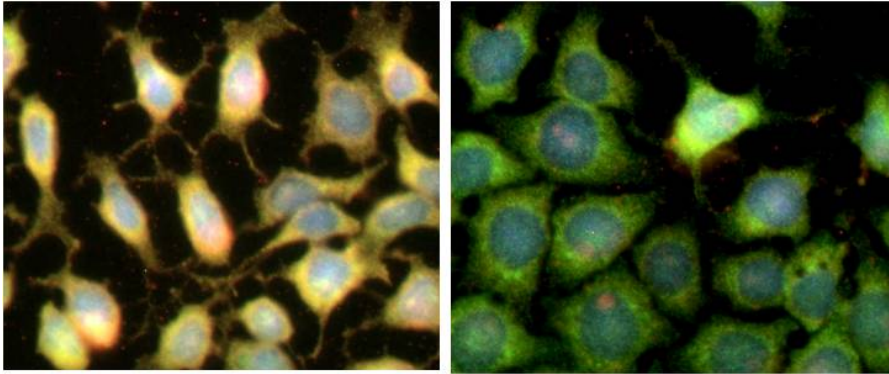


Figure 3. Specificity of Signal by Immunocytochemistry. H4IIE cells were seeded on glass coverslips and allowed to adhere for a few hours. Cells were then serum starved overnight and treated the next day with 1 μ M oligomycin (left) or DMSO (right). Levels of AMPK α total and phosphorylated protein at T172 were measured following this protocol. The total AMPK α signal is shown in green and AMPK α p172 in red. The left panel shows up-regulation of phosphorylation levels due to oligomycin treatment.

DATA ANALYSIS

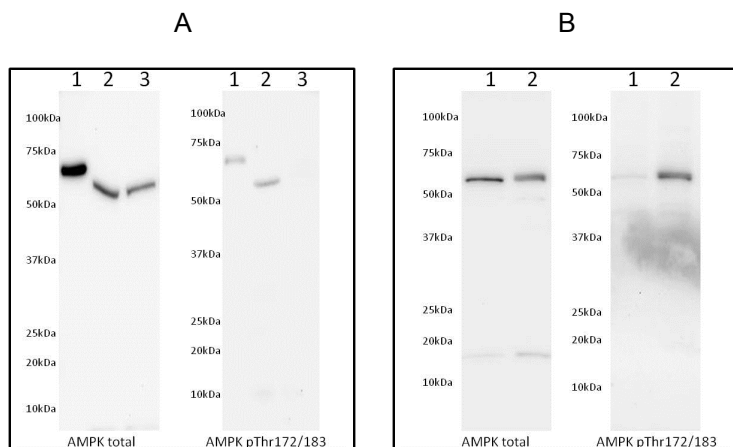


Figure 4. Specificity of signal by Western Blot. Western Blot was run on a 4-20% gradient acrylamide gel. (A) Samples were loaded as follows: (1) 40ng of AMPK α human recombinant protein, (2) 40 μ g of C2C12 myoblasts serum starved and (3) 40 μ g of C2C12 myoblasts in 10% FCS. (B) Samples were loaded as follows: (1) 40 μ g Hek293T in 10% FCS treated with 1/100 dilution of LP and (2) 40 μ g Hek293T in 10% FCS. AMPK α total membrane was blocked with 5% Milk in TBST, AMPK α pT172 was blocked with 1X Blocking buffer (ab126587) in TBST.

RESOURCES

13. TROUBLESHOOTING

Problem	Cause	Solution
Low Signal	Too brief incubation times	Ensure sufficient incubation times
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Insufficient cells	Increase seeding density of cells; goal is nearly confluent cells at time of fixation.
	Cell detachment	Refer to section 14.4
High CV	Plate is insufficiently washed	Review the manual for proper washing. If using a plate washer, check that all ports are free from obstruction
	Contaminated wash buffer	Prepare fresh wash buffer
	Artifacts creating increased signal on IR	Troughs used for multichannel pipetting should be cleaned or replaced.
	Edge effects	Do not use the edges of the plate. Incubate in a humid box
	Variable cell seeding	Plate cells with care and normalize with Janus Green

14.F.A.Q

- 14.1. I do not have a plate heater or a water bath to perform the antigen retrieval step. How can I do this assay without antigen retrieval?

If a plate heater and a water bath are not available, we suggest to permeabilize the cells with 100% ice cold methanol for at least 30 minutes at -20°C. After permeabilization, wash the plate three times with PBS and then proceed with the blocking and antibody incubation steps as specified in this protocol, but without the addition of Triton X-100.

- 14.2. How many cells do I seed per well?

The cell seeding density varies by cell type and depends both on the cell size and the abundance of the target protein. The cell seeding will likely need to be determined experimentally (see Table 1 for some guidelines). For adherent cells, prepare serial dilution of the cells in a plate and allow them to attach prior to observation. The goal is to have cells that are just confluent at the time of fixation. Overly confluent cells may have compromised viability and tend to not adhere as well to the plate. Under-seeded cells may yield too low a signal, depending on the analyte. Keep in mind that drug treatments or culture conditions may affect cell density/growth.

- 14.3. Do I have to use an amine-coated microplate?

We have tested black wall amine and cell culture treated microplates and found that amine coated plates improve reproducibility and specificity in comparison to standard plates. In addition, multiple cell types appear to have favorable growth and also more even seeding on amine coated plates. The assay performance is only guaranteed with amine plates.

RESOURCES

- 14.4. A treatment causes cells detachment. Is there a way to prevent the lost of detaching cells?

Loss of floating cells can be easily prevented by inserting two centrifugation steps into the protocol: (1) Immediately prior the addition of Paraformaldehyde Solution (step 10.3) centrifuge the microtiter plate at 500x g for 5-10 minutes, (2) Immediately after the addition of Paraformaldehyde Solution centrifuge the microtiter plate again at 500x g for 5-10 minutes. Continue in the fixation for a total of 15 - 20 minutes.

- 14.5. Can I use suspension cells for ICE?

The In-Cell ELISA can be easily adapted for use with suspension cells. In this case an amine plate must be used. To ensure efficient cross-linking of the suspension cells to the amine plate, cells must be grown and treated in a different plate or dish of choice. The treated suspension cells are then transferred to the amine plate in 100 μ L of media per well. The cell seeding density of the amine plate is cell type-dependent. 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 and Jurkat cells should be seeded, respectively, at 300,000 and 200,000 cells per well in 100 μ L of PBS (preferred) or media. After the cells are transferred to the amine plate follow immediately the fixation procedure as described in section 14.4.

Note – With suspended cells, the media should contain no more than 10% fetal serum, otherwise efficiency of the suspension cell cross-linking to the plate may be compromised.

- 14.6. I grow my cells in 15% FBS, will this interfere with the cell fixation?

Culture media containing up to 15% fetal serum does not interfere with the cell fixation and cross-linking to the plate on adherent cells.

RESOURCES

14.7. How do I measure the assay background?

It is essential to omit primary antibody in at least one well (3 wells recommended) to provide a background signal for the experiment which can be subtracted from all measured data. This should be done for each experimental condition. It is also recommended to include at least one well without cells to provide primary antibody background.

14.8. Is Janus Green normalization necessary?

Janus Green is a whole-cell stain that is useful to determine if a decrease in antibody signal intensity in a well is due to a relevant down-regulation or degradation of the target analyte or if it is a function of decreased cell number (e.g. due to cytotoxic effect of a treatment). As such it is not a required readout, but it is useful in the analysis to determine a normalized intensity value (section 11.2).

14.9. I am testing my experimental condition at more than one seeding density and after normalizing to Janus green I get different values at the different densities. How can I interpret the results?

Phosphorylation of AMPK α is known to occur during stress conditions. Confluence and depletion of media may result in stress which can phosphorylate more readily AMPK α under certain conditions/treatments. Therefore the induction of phosphorylation may not follow a linear pattern during a cell titration in the same way Janus green staining does. When this is the case, interpretation of results can be aided with the generation of a phospho/total AMPK protein ratio. This ratio will not change regardless of the density in which the cells are at.

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