

# AMPK $\alpha$ [pT172] ELISA Kit

Catalog Number KH00651 (96 tests)

Pub. No. MAN0014681 Rev. 3.0 (30)

**CAUTION!** This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

**Note:** For safety and biohazard guidelines, see the “Safety” appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

## Product description

The Invitrogen™ AMPK $\alpha$  [pT172] ELISA Kit is a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA). This assay is designed to detect and quantify the level of AMPK $\alpha$  protein that is phosphorylated at threonine residue 172 in lysates from human, mouse, and rat cells. The assay recognizes both natural and recombinant AMPK $\alpha$  [pT172].

AMPK (5'-prime-AMP-activated protein kinase) is a metabolite and stress-sensing kinase that regulates homeostasis, which serves as an energy sensor in all eukaryotic cell types. AMPK functions as a serine/threonine protein kinase, and exists as a heterotrimeric complex comprised of a catalytic alpha (alpha 1 or alpha 2) subunit and non-catalytic, regulatory beta and gamma (beta 1 or beta 2 and gamma 1, gamma 2, or gamma 3) subunits that differ in their subcellular localization and AMP-dependence.

## Contents and storage

Upon receipt, store the kit at 2°C to 8°C.

| Contents  | Cat. No. KH00651 (96 tests) |
|---|-----------------------------|
| AMPK $\alpha$ [pT172] Standard; contains 0.1% sodium azide.                                   | 2 vials                     |
| Standard Diluent Buffer; contains 0.1% sodium azide; red dye <sup>[1]</sup>                   | 25 mL                       |
| Antibody Coated Wells, 96-well plate  | 1 plate                     |
| AMPK $\alpha$ [pT172] Detection Antibody; contains 0.1% sodium azide; blue dye <sup>[1]</sup> | 11 mL                       |
| Anti-Rabbit IgG HRP (100X)  | 0.125 mL                    |
| HRP Diluent. Contains 3.3 mM thymol; yellow dye <sup>[1]</sup>                                | 25 mL                       |
| Wash Buffer Concentrate (25X)   | 100 mL                      |
| Stabilized Chromogen, Tetramethylbenzidine (TMB)  | 25 mL                       |
| Stop Solution   | 25 mL                       |
| Plate Covers, adhesive strips   | 3                           |

<sup>[1]</sup> In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored Standard Diluent Buffer, Detection Antibody, and HRP Diluent to help monitor the addition of solutions to the reaction wells. This does not in any way interfere with the test results.

## Required materials not supplied

- Distilled or deionized water
- Microtiter plate reader with software capable of measurement at or near 450 nm
- Plate washer—automated or manual (squirt bottle, manifold dispenser, or equivalent)
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solutions; beakers, flask and cylinders for preparation of reagents
- Cell Extraction Buffer (Cat. No. FNN0011, or see “Prepare Cell Extraction Buffer”)

## Before you begin

**IMPORTANT!** Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

- Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at [thermofisher.com](http://thermofisher.com).
- Allow reagents to reach room temperature before use. Mix to redissolve any precipitated salts.

## Prepare 1X Wash Buffer

1. Dilute 16 mL of Wash Buffer Concentrate (25X) with 384 mL of deionized or distilled water. Label as 1X Wash Buffer.
2. Store the concentrate and 1X Wash Buffer in the refrigerator. Use the diluted buffer within 14 days.

## Prepare Cell Extraction Buffer

**Note:** See the *ELISA Technical Guide* for detailed information on preparing Cell Extraction Buffer.

1. Prepare Cell Extraction Buffer.  
Cell Extraction Buffer consists of 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton™ X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate.
2. Immediately before use, add PMSF (0.3 M stock in DMSO) to 1 mM and 50  $\mu$ L protease inhibitor cocktail (e.g., Sigma Cat. No. P-2714) for each 1 mL of Cell Extraction Buffer.

## Prepare cell lysate

1. Collect cells by centrifugation (non-adherent cells) or scraping from culture flasks (adherent cells), then wash cells twice with cold PBS.
2. Remove and discard the supernatant and collect the cell pellet. The pellet can be stored at  $-80^{\circ}\text{C}$  and lysed at a later date if desired.
3. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes, on ice. Vortex at 10-minute intervals.

**Note:** The volume of Cell Extraction Buffer used depends on the number of cells in the cell pellet, and expression levels of AMPK $\alpha$  [pT172]. [Researchers must optimize the extraction procedures for their own applications.]

4. Transfer the lysate into microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at  $4^{\circ}\text{C}$ .
5. Transfer the supernatant into clean microcentrifuge tubes. Samples can be stored at  $-80^{\circ}\text{C}$  (avoid multiple freeze-thaw cycles).

## Pre-dilute samples

Sample concentrations should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application.

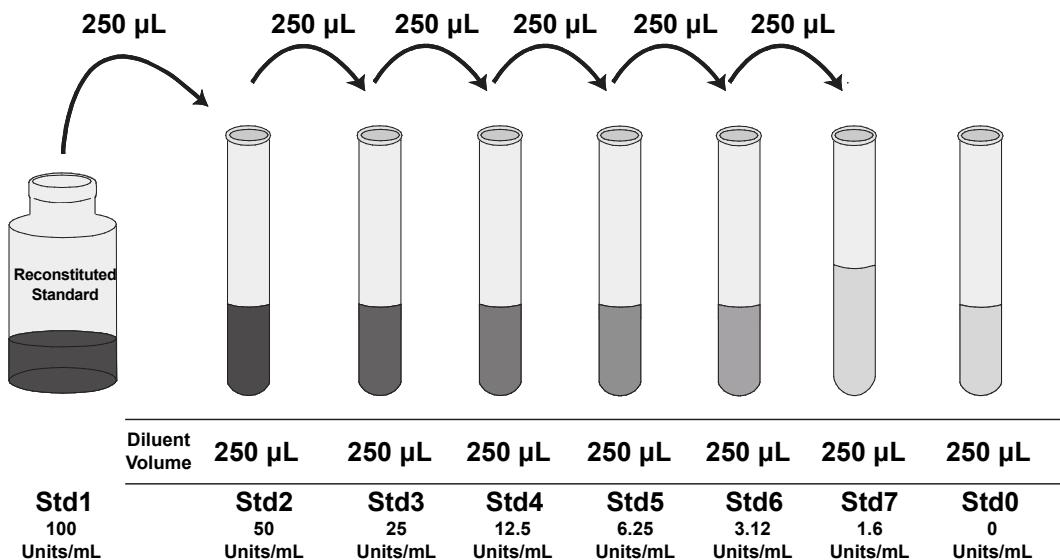
- Perform sample dilutions with Standard Diluent Buffer.
- Pre-dilute samples prepared in Cell Extraction Buffer 1:10 or greater in Standard Diluent Buffer (e.g., 10  $\mu\text{L}$  sample into 90  $\mu\text{L}$  buffer). This dilution is necessary to reduce the matrix effect of the Cell Extraction Buffer. SDS concentration should be less than 0.01% before adding to the plate.

## Dilute standards

**Note:** Use glass or plastic tubes for diluting standards.

**Note:** This AMPK $\alpha$  [pT172] standard is prepared from phosphorylated recombinant AMPK $\alpha$ . One unit of standard is equivalent to the amount of AMPK $\alpha$  [pT172] in 0.4  $\mu\text{g}$  of GTL16 cell lysate treated with 500  $\mu\text{M}$  oligomycin for 1 hour.

1. Reconstitute AMPK $\alpha$  [pT172] Standard to 100 Units/mL with Standard Diluent Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow the contents to sit for 10 minutes to ensure complete reconstitution. Label as 100 Units/mL AMPK $\alpha$  [pT172]. **Use the standard within 1 hour of reconstitution.**
2. Add 250  $\mu\text{L}$  Standard Diluent Buffer to each of 7 tubes labeled as follows: 50, 25, 12.5, 6.25, 3.12, 1.6, and 0 Units/mL AMPK $\alpha$  [pT172].
3. Make serial dilutions of the standard as shown in the following dilution diagram. Mix thoroughly between steps.
4. Remaining reconstituted standard should be discarded or frozen in aliquots at  $-80^{\circ}\text{C}$  for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.



## Prepare 1X Anti-Rabbit IgG HRP solution

**Note:** Prepare 1X Anti-Rabbit IgG HRP solution within 15 minutes of usage.

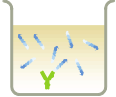




1. For each 8-well strip used in the assay, pipet 10  $\mu\text{L}$  Anti-Rabbit IgG HRP (100X) solution, and dispense the solution into a tube containing 1 mL of HRP Diluent. Mix thoroughly.
2. Return the unused Anti-Rabbit IgG HRP (100X) solution to the refrigerator.

## Perform ELISA (Total assay time: 4 hours)

**IMPORTANT!** Perform a standard curve with each assay.

- Allow all components to reach room temperature before use. Mix all liquid reagents prior to use.
- Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2°C to 8°C for future use.



|          |  |   |
|----------|--|---|
| <b>1</b> | <b>Bind antigen</b><br>             | <ol style="list-style-type: none"> <li>Add 100 <math>\mu</math>L of standards, controls, or samples (see "Pre-dilute samples" on page 2) to the appropriate wells. Leave the wells for chromogen blanks empty.</li> <li>Cover the plate with a plate cover and incubate 2 hours at room temperature.</li> <li>Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.</li> </ol> |
| <b>2</b> | <b>Add detector antibody</b><br>    | <ol style="list-style-type: none"> <li>Add 100 <math>\mu</math>L of AMPK<math>\alpha</math> [pT172] Detection Antibody solution into each well except the chromogen blanks.</li> <li>Cover the plate with a plate cover and incubate 1 hour at room temperature.</li> <li>Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.</li> </ol>                                     |
| <b>3</b> | <b>Add IgG HRP</b><br>              | <ol style="list-style-type: none"> <li>Add 100 <math>\mu</math>L 1X Anti-Rabbit IgG HRP solution into each well except the chromogen blanks.</li> <li>Cover the plate with plate cover and incubate for 30 minutes at room temperature.</li> <li>Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.</li> </ol>  |
| <b>4</b> | <b>Add Stabilized Chromogen</b><br> | <ol style="list-style-type: none"> <li>Add 100 <math>\mu</math>L Stabilized Chromogen to each well. The substrate solution begins to turn blue.</li> <li>Incubate for 30 minutes at room temperature in the dark.</li> </ol> <p><b>Note:</b> TMB should not touch aluminum foil or other metals.</p>  |
| <b>5</b> | <b>Add Stop Solution</b><br>       | Add 100 $\mu$ L Stop Solution to each well. Tap the side of the plate to mix. The solution in the wells changes from blue to yellow.  |

## Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 2 hours after adding the Stop Solution.
2. Use curve-fitting software to generate the standard curve. A 4 parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

**Note:** Dilute samples producing signals greater than the upper limit of the standard curve in Standard Diluent Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

## Performance characteristics

### Standard curve example

The following data were obtained for the various standards over the range of 0 to 100 Units/mL AMPK $\alpha$  [pT172].

| Standard AMPK $\alpha$ [pT172] (Units/mL) | Optical Density (450 nm) |
|---|--------------------------|
| 100                                       | 3.18                     |
| 50  | 1.96                     |
| 25  | 1.07                     |
| 12.5                                      | 0.61                     |
| 6.25                                      | 0.36                     |
| 3.12                                      | 0.23                     |
| 1.6                                       | 0.17                     |
| 0   | 0.09                     |

### Recovery

To evaluate recovery, AMPK $\alpha$  [pT172] ELISA Kit Standard was spiked at 3 different concentrations into 10% Cell Extraction Buffer. The average recovery was 90%.

### Inter-assay precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

| Parameters                 | Sample 1 | Sample 2 | Sample 3 |
|----------------------------|----------|----------|----------|
| Mean (Units/mL)            | 46.4     | 14.9     | 6.1      |
| Standard Deviation         | 4.1      | 1.1      | 0.4      |
| % Coefficient of Variation | 8.9      | 7.3      | 6.6      |

### Intra-assay precision

Samples of known AMPK $\alpha$  [pT172] concentrations were assayed in replicates of 16 to determine precision within an assay.

| Parameters                 | Sample 1 | Sample 2 | Sample 3 |
|----------------------------|----------|----------|----------|
| Mean (Units/mL)            | 45.3     | 14.8     | 6.2      |
| Standard Deviation         | 2.6      | 0.69     | 0.24     |
| % Coefficient of Variation | 5.8      | 4.6      | 3.9      |

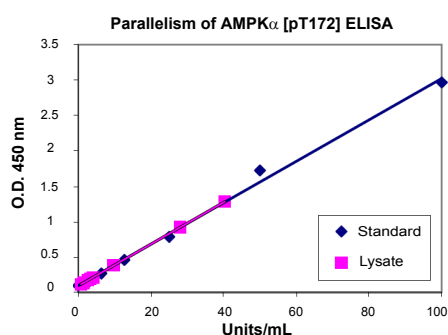
## Linearity of dilution

GTL16 cells were grown in tissue culture medium with 10% fetal bovine serum, treated with 500  $\mu$ M oligomycin for 1 hour, and lysed with Cell Extraction Buffer. This lysate was diluted in Standard Diluent Buffer over the range of the assay and measured for AMPK $\alpha$  [pT172]. Linear regression analysis of sample values versus the expected concentrations yielded a correlation coefficient of 0.99.

| Dilution | Measured (Units/mL) | Expected   |     |
|----------|---------------------|------------|-----|
|          |                     | (Units/mL) | %   |
| Neat     | 40.33               | 40.28      | 100 |
| 1/2      | 27.89               | 20.16      | 138 |
| 1/4      | 9.59                | 10.08      | 95  |
| 1/8      | 4.03                | 5.04       | 80  |
| 1/16     | 3.25                | 2.52       | 128 |

## Parallelism

Natural AMPK $\alpha$  [pT172] from oligomycin treated GTL16 cells lysates were serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the AMPK $\alpha$  [pT172] standard curve. The standard accurately reflects AMPK $\alpha$  [pT172] content in samples.



## Sensitivity

The analytical sensitivity of the assay is <1 Unit/mL AMPK $\alpha$  [pT172]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

In addition, the sensitivity of the ELISA is ~2-fold greater than that of western blot when tested against known quantities of lysates from GTL16 cells treated with oligomycin.

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### Product label explanation of symbols and warnings

|            |                |            |            |  |                        |  |        |  |              |  |                              |  |   |
|------------|----------------|------------|------------|--|------------------------|--|--------|--|--------------|--|------------------------------|--|---|
| <b>REF</b> | Catalog Number | <b>LOT</b> | Batch code |  | Temperature limitation |  | Use by |  | Manufacturer |  | Consult instructions for use |  | Caution, consult accompanying documents |
|------------|----------------|------------|------------|--|------------------------|--|--------|--|--------------|--|------------------------------|--|---|

**Manufacturer's address:** Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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Western blot (63 kDa)



ELISA: O.D. 450 nm

|      |      |      |      |      |      |      |     |
|------|------|------|------|------|------|------|-----|
| 0.11 | 0.13 | 0.17 | 0.19 | 0.22 | 0.38 | 0.91 | 1.3 |
|------|------|------|------|------|------|------|-----|

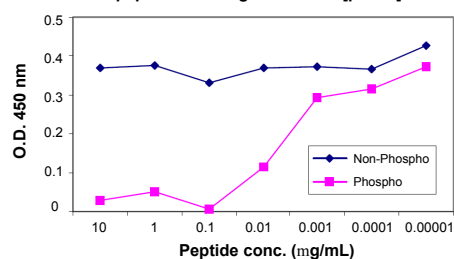
GTL16 lysate ( $\mu$ g/test)

|   |       |       |      |     |   |    |    |
|---|-------|-------|------|-----|---|----|----|
| 0 | 0.313 | 0.625 | 1.25 | 2.5 | 5 | 10 | 20 |
|---|-------|-------|------|-----|---|----|----|

## Specificity

AMPK $\alpha$  [pT172] specificity of the AMPK $\alpha$  [pT172] ELISA Kit was confirmed by peptide competition. The data shows that only the phosphopeptide containing the phosphorylated threonine blocks the ELISA signal. The same sequence with a non-phosphorylated threonine at position 172 was not blocked.

Effect of peptide blocking on AMPK $\alpha$  [pT172] ELISA



Lysates (400  $\mu$ g/mL) from GTL16 cells treated with 500  $\mu$ M oligomycin for 1 hour were assayed with the AMPK $\alpha$  [pT172] ELISA Kit. The results show that oligomycin induces phosphorylation of AMPK $\alpha$ , and that the assay corresponds well to western blot analysis of the same samples (inset).

Effect of oligomycin on levels of AMPK $\alpha$  [pT172]

