AMPKα [pT172] ELISA Kit

Catalog Number KH00651 (96 tests)

Pub. No. MAN0014681 **Rev.** 2.0

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

AMPK (5'-prime-AMP-activated protein kinase) is a metabolite and stress-sensing kinase that regulates homeostatis, 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)
AMPKα [pT172] Standard; contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume	2 vials
Standard Diluent Buffer; contains 0.1% sodium azide; red dye [1]	25 mL
Antibody Coated Wells, 96-well strip-well plate	1 plate
AMPKα [pT172] Detection Antibody; contains 0.1% sodium azide; blue dye[1]	11 mL
Anti-Rabbit IgG HRP (100X); contains 3.3 mM thymol	0.125 mL
HRP Diluent. Contains 3.3 mM thymol; yellow dye[1]	25 mL
Wash Buffer Concentrate (25X)	100 mL
Stabilized Chromogen, Tetramethylbenzidine (TMB)	25 mL
Stop Solution	25 mL
Plate Covers, adhesive strips	3

[1] 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
- 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**.
- 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 5 mL of 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 $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton™ X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate.
2. Immediately before use, add 1 mM PMSF (0.3 M stock in DMSO) and 250 µL protease inhibitor cocktail (e.g., Sigma Cat. No. P-2714).

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°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 AMPKα [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°C.
5. Transfer the supernatant into clean microcentrifuge tubes. Samples can be stored at −80°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.

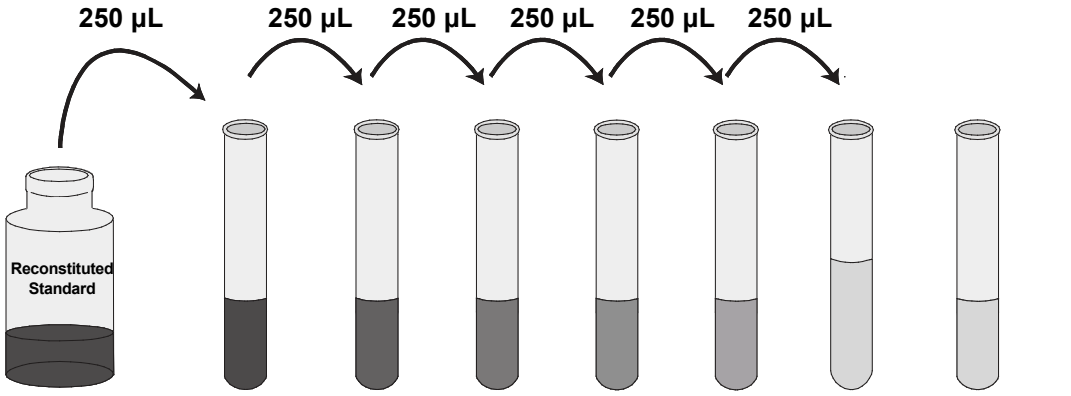
- Perform sample dilutions with Standard Diluent Buffer.
- Pre-dilute samples prepared in Cell Extraction Buffer 1:10 or greater in Standard Diluent Buffer (e.g., 10 µL sample into 90 µL buffer). This dilution is necessary to reduce the matrix effect of the Cell Extraction Buffer. SDS concentration should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

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

Note: This AMPKα [pT172] standard is prepared from phosphorylated recombinant AMPKα. One unit of standard is equivalent to the amount of AMPKα [pT172] in 0.4 µg of GTL16 cell lysate treated with 500 µM oligomycin for 1 hour.

1. Reconstitute AMPKα [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 AMPKα [pT172]. **Use the standard within 1 hour of reconstitution.**
2. Add 250 µL 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 AMPKα [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°C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.



	Diluent Volume	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL
Std1		Std2	Std3	Std4	Std5	Std6	Std7	Blank
100 Units/mL		50 Units/mL	25 Units/mL	12.5 Units/mL	6.25 Units/mL	3.12 Units/mL	1.6 Units/mL	0 Units/mL

Prepare 1X Anti-Rabbit IgG HRP solution

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

The Anti-Rabbit IgG HRP (100X) is in 50% glycerol, which is viscous. To ensure accurate dilution:

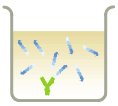
1. For each 8-well strip used in the assay, pipet 10 µL Anti-Rabbit IgG HRP (100X) solution, wipe the pipette tip with clean absorbent paper to remove any excess solution, and dispense the solution into a tube containing 1 mL of HRP Diluent. Mix thoroughly.
2. Return the unused Anti-Rabbit IgG HRP (100X) 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.

1 Bind antigen



a. Add 100 µL of standards, controls, or samples (see "Pre-dilute samples"on page 2) to the appropriate wells. Leave the wells for chromogen blanks empty.
b. Cover the plate with a plate cover and incubate 2 hours at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

2 Add detector antibody

a. Add 100 µL of AMPKα [pT172] Detection Antibody solution into each well except the chromogen blanks.
b. Cover the plate with a plate cover and incubate 1 hour at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

3 Add IgG HRP

a. Add 100 µL 1X Anti-Rabbit IgG HRP solution into each well except the chromogen blanks.
b. Cover the plate with plate cover and incubate for 30 minutes at room temperature.
c. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.

4 Add Stabilized Chromogen

a. Add 100 µL Stabilized Chromogen to each well. The substrate solution begins to turn blue.
b. Incubate for 30 minutes at room temperature in the dark.

Note: TMB should not touch aluminum foil or other metals.

5 Add Stop Solution

Add 100 µL 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 AMPKα [pT172].

Standard AMPKα [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, AMPKα [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 AMPKα [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

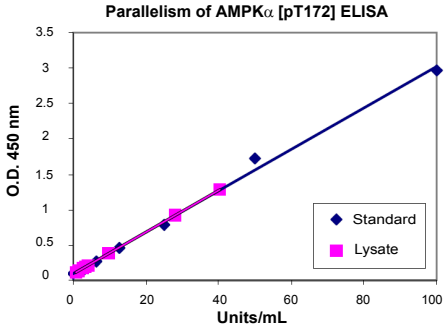
Linearity of dilution

GTL16 cells were grown in tissue culture medium with 10% fetal bovine serum, treated with 500 µM 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 AMPKα [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

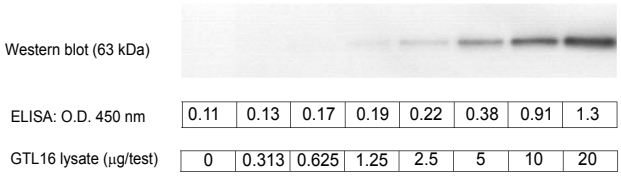
Natural AMPKα [pT172] from oligomycin treated GTL16 cells lysates were serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the AMPKα [pT172] standard curve. The standard accurately reflects AMPKα [pT172] content in samples.



Sensitivity

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

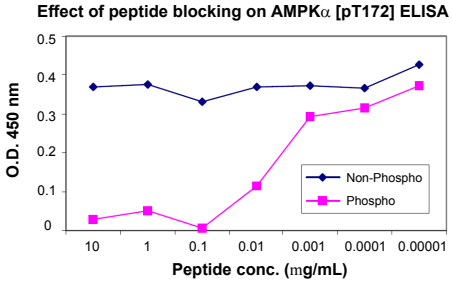
In addition, the sensitivity of the ELISA is ~2-fold greater than that of western blot when tested against known quantities of lysates from GTL16 cells treated with oligomycin.



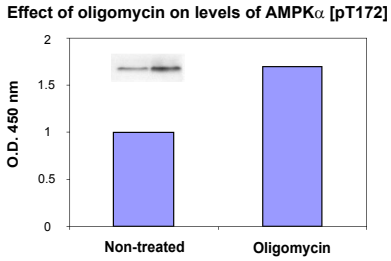
ELISA: O.D. 450 nm	0.11	0.13	0.17	0.19	0.22	0.38	0.91	1.3
GTL16 lysate (µg/test)	0	0.313	0.625	1.25	2.5	5	10	20

Specificity

AMPKα [pT172] specificity of the AMPKα [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.



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



Limited product warranty

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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

Manufacturer's address: Life Technologies Corporation | 7335 Executive Way | Frederick, MD 21704 | USA