Product Manual

[pThr^{202}/Tyr^{204}]Erk1/2 ELISA kit

Catalog #: ADI-900- 098A

96 Well Kit

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Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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DESCRIPTION

The [pThr^{202}/Tyr^{204}]Erk1/2 ELISA kit is a complete kit for the quantitative determination of pERK in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to ERK immobilized on a microtiter plate to bind the pERK in the standards or sample. A recombinant pERK Standard is provided in the kit. After a short incubation the excess sample or standard is washed away and a rabbit polyclonal antibody to pERK is added. This antibody binds to the pERK captured on the plate. After a short incubation the excess antibody is washed away and goat anti-rabbit IgG conjugated to horseradish peroxidase is added, which binds to the polyclonal pERK antibody. Excess conjugate is washed away and substrate is added. After a short incubation, the enzyme reaction is stopped and the generated color is read at 450nm. The measured optical density is directly proportional to the concentration of pERK in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway exists in all eukaryotes. It consists of several subgroups including ERK 1/2 (extracellular signal-regulated kinase), JNK and p38 kinases. These kinases regulate and control fundamental cellular processes including proliferation, apoptosis, survival and differentiation. They are activated by diverse stimuli including cytokines, growth factors, irradiation, changes in osmolarity, heat and shear stress. MAP kinases are characterized by their requirement for dual phosphorylation for full activation. ERK1 (44kDa) and ERK2 (42kDa) kinases are characterized by their requirement for dual phosphorylation at a conserved T-E-Y motif. The literature contains numerous and extensive reviews on MAP kinase.

PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Handle with care

1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of buffers, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The pERK Standard provided, Catalog No. 80-0942, should be handled with care because of the known and unknown effects of pERK.
5. The pERK Standard should be stored at or below -20°C. Do not repeatedly freeze-thaw.

Avoid freeze / thaw cycles

MATERIALS SUPPLIED

1. **ERK Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0936:**

   A plate using break-apart strips coated with a mouse monoclonal antibody specific to ERK

2. **pERK Antibody, 10ml, Catalog No. 80-0937:**

   A yellow solution of a rabbit polyclonal antibody to pERK.

3. **Assay Buffer 21, 100ml, Catalog No. 80-1519:**

   Tris buffered saline containing proteins, detergents and phosphatase inhibitor.

4. **pERK Conjugate, 10ml, Catalog No. 80-1319:**

   A blue solution of goat anti-rabbit IgG conjugated to Horseradish peroxidase.

5. **Wash Buffer Concentrate, 100ml, Catalog No. 80-1287:**

   Tris buffered saline containing detergents.

6. **pERK Standard, 2 vials, Catalog No. 80-0942:**

   Two vials containing 1,000pg each of lyophilized recombinant pERK..

7. **TMB Substrate, 10ml, Catalog No. 80-0350:**

   A solution of 3,3’,5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide. Protect from prolonged exposure to light.

8. **Stop Solution 2, 10ml, Catalog No. 80-0377:**

   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic**.

9. **RIPA Cell Lysis Buffer 2, 100ml, Catalog No. 80-1284:**

   50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS.

10. **pERK Assay Layout Sheet, 1 each, Catalog No. 30-0163.**

11. **Plate Sealer, 3 each**

Reagants require separate storage conditions.

STORAGE

All components of this kit, except the pERK Standard, are stable at 4°C until the kit's expiration date. The pERK Standard must be stored at or below -20°C.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.
3. Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent.
4. Sodium orthovanadate, Sigma #S6508 or equivalent.
5. Sodium pyrophosphate, Sigma #S6422 or equivalent.
6. Precision pipets for volumes between 100µl and 1,000µl.
7. Repeater pipet for dispensing 100µl.
8. Disposable beakers for diluting buffer concentrates.
9. Graduated cylinders.
10. A microplate shaker.
11. Adsorbent paper for blotting.
12. Microplate reader capable of reading at 450nm, preferably with correction between 570nm and 590nm.

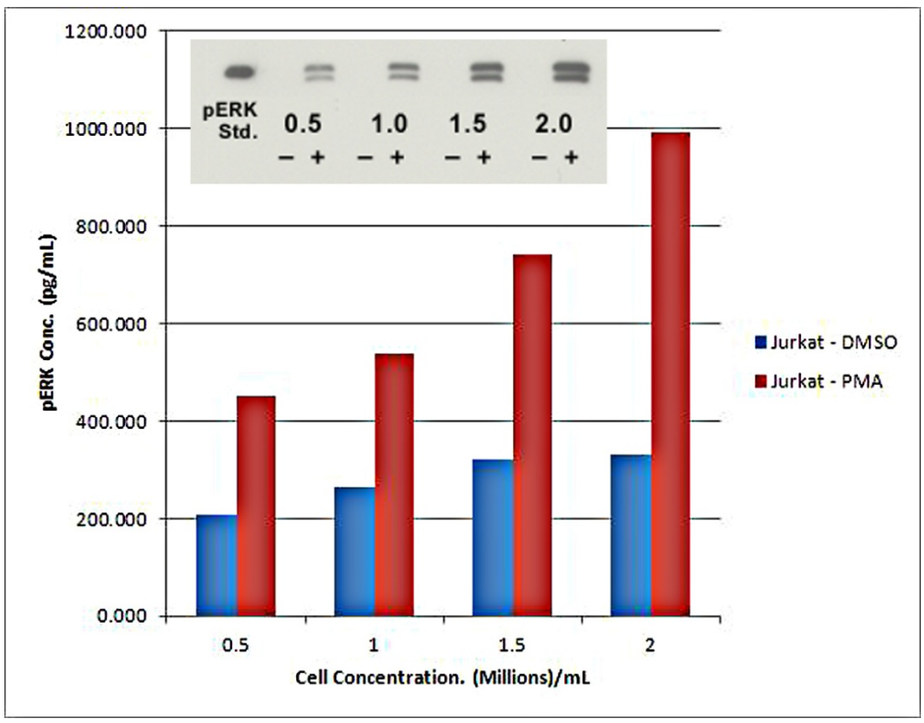
SAMPLE HANDLING

The pERK ELISA is compatible with pERK samples in a wide range of cell lysates and buffers. Samples diluted sufficiently into Assay Buffer 21 plus Inhibitors (see Reagent Preparation) can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 13 for details of suggested dilutions. It is recommended that all samples be lysed with the provided RIPA Cell Lysis Buffer 2 modified by the addition of PMSF and PIC (see Reagent Preparation) immediately prior to use. Samples lysed in RIPA Cell Lysis Buffer 2 plus Inhibitors must be diluted at least 1:8 with Assay Buffer 21 plus Inhibitors prior to assaying.

If the end user chooses to use another lysis buffer, a greater number of cells, or varies from the stimulation procedure noted below, it is up to the end user to determine the appropriate dilution of samples and assay validation. Samples must be stored frozen at or below -70°C to avoid loss of bioactive pERK. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4°C slowly and gently mixed.

JURKAT CELL STIMULATION EXPERIMENT

This experiment was adapted from a protocol outlined in reference #12. The number of Jurkat cells used in this experiment were: 2.0, 1.5, 1.0 and 0.5 million per ml. They were stimulated with 50nM Phorbol 12-myristate 13-acetate (PMA) or with DMSO (for a negative control) for 5 minutes at 37°C. Cells were centrifuged at 1,500rpm for 5 minutes and the supernatant discarded. The cell pellets were re-suspended and washed with PBS. Cells were pelleted at 1,500rpm for 5 minutes and the supernatant discarded. The cell pellets were re-suspended with RIPA Cell Lysis Buffer 2, vortexed and placed on ice for 5 minutes. The lysates were vortexed and centrifuged at 14,000rpm for 10 minutes. The supernantants were split for the Western blot and ELISA to generate the data illustrated. Note that 20µl of sample was used per lane in the Western blot. The exposure time for development was 20 seconds.



PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards must be made up in polypropylene tubes.
4. Pre-rinse the pipet tip with reagent. Use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

REAGENT PREPARATION

1. Wash Buffer

Prepare the Wash Buffer by diluting 50ml of the supplied concentrate with 950ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Assay Buffer 21 plus Inhibitors

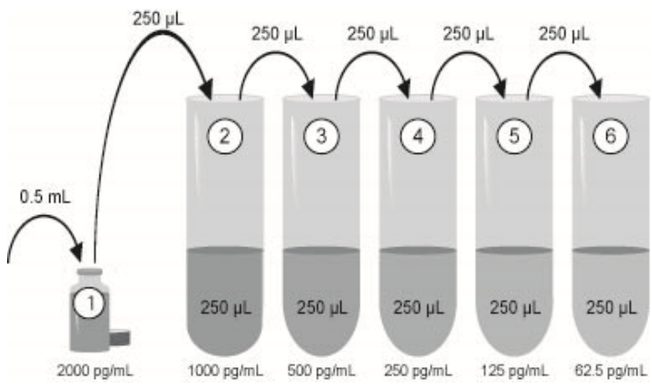
Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5µl/ml PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1mM.

This modified Assay Buffer 21 must be used for standard reconstitution and all sample and standard dilutions to ensure optimal integrity of pERK. Fresh Assay Buffer 21 plus Inhibitors must be made for each assay.

3. pERK Standards

Allow the lyophilized pERK standard to warm to room temperature. Add 0.5ml of Assay Buffer 21 plus Inhibitors to the lyophilized pERK vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard #1. Label five 12x75mm polypropylene tubes #2 through #6. Pipet 250µl of Assay Buffer 21 plus Inhibitors into tubes #2 though #6. Add 250µl of reconstituted standard #1 into tube #2 and vortex. Add 250µl of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

The concentration of pERK in standard vial #1 and tubes #2 through #6 will be 2,000, 1,000, 500, 250, 125 and 62.5pg/ml respectively. See pERK Assay Layout Sheet for dilution details.



Reconstituted and diluted standards should be used within 60 minutes of preparation.

Discard any unused reconstituted standard and subsequent dilutions.

4. RIPA Cell Lysis Buffer 2 plus Inhibitors

Allow to come to room temperature. Ensure buffer is completely in solution prior to use. Immediately prior to use in cell lysis, protease inhibitors (PIC and PMSF) as well as phosphatase inhibitors (Sodium orthovanadate and Sodium pyrophosphate) must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5µl/ml PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1mM. Add Sodium orthovanadate, such as Sigma #S6508, to a final concentration of 2mM and Sodium pyrophosphate, such as Sigma #S6422, to a final concentration of 20mM.

Fresh RIPA Cell Lysis Buffer 2 plus Inhibitors must be made each time the cells are lysed.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100µl of Assay Buffer 21 plus Inhibitors into the S0 (0pg/ml standard) wells.
3. Pipet 100µl of Standards #1 through #6 into the appropriate wells.
4. Pipet 100µl of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500rpm*.
7. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100µl of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500rpm*.
10. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100µl of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500rpm*.
13. Empty the contents of the wells and wash by adding 400µl of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100µl of Substrate Solution into each well.

15. Incubate for 30 minutes at room temperature on a plate shaker at ~500rpm*.
16. Pipet 100µl Stop Solution to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 450nm, preferably with correction between 570 and 590nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

    ***** The plate shaker speed was based on a BellCo Mini Orbital Shaker (mod no. 7744-08096). The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of pERK in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program (such as AssayBlaster™, catalog number ADI-28-0002). Such software is often supplied by plate reader manufacturers. If data reduction software is not readily available, the concentration of pERK can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus pERK concentration in each standard. Approximate a straight line through the points. The concentration of pERK in the unknowns can be determined by interpolation.

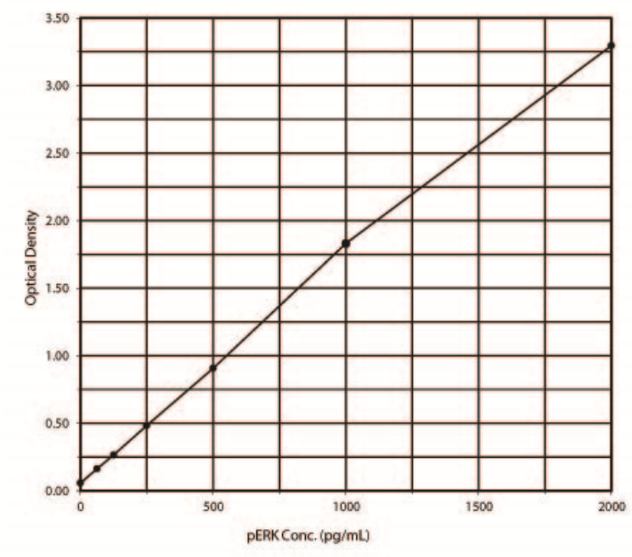
TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results.

Sample	Average Net OD	pERK (pg/ml)
Blank(mean)	(0.046)	
S0	0.057	**0**
S1	3.295	**2,000**
S2	1.831	**1,000**
S3	0.907	**500**
S4	0.483	**250**
S5	0.265	**125**
S6	0.163	**62.5**

TYPICAL STANDARD CURVES

A typical standard curve is shown below. This curve must not be used to calculate pERK concentrations; each user must run a standard curve for each assay.



Units of Measure

Samples measured in the phospho-ERK ELISA can be expressed in terms of concentration by weight or activity. When reconstituted according to direction, the standard stock concentration is 2000pg/ml. To convert this value to Units/ml, the weight concentration is multiplied by the specific activity of the standard. The specific activity of the standard is ~10,000,000 Units/mg where one Unit of pERK activity is equal to 1 pmole phosphate incorporated into 100µM myelin basic protein per minute at 30°C in a total reaction volume of 30µl.

PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[13].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for twenty four (24) wells run with 0pg/ml Standard, and comparing to the average optical density for twenty four (24) wells run with Standard #6. The detection limit was determined as the concentration of pERK measured at two (2) standard deviations from the 0pg/ml Standard along the standard curve. The sensitivity of the assay was determined to be 2.67pg/ml.

Linearity

A sample containing 1400pg/ml pERK was serially diluted 5 times 1:2 in the Assay Buffer 21 supplied in the kit and measured in the assay. The data was plotted graphically as actual pERK concentration versus measured pERK concentration.

The line obtained had a slope of 1.014 with a correlation coefficient of 0.998.

Precision

Intra-assay precision was determined by assaying 20 replicates of 3 buffer controls containing pERK in a single assay.

pg/ml	%CV
1099.7	4.4
384.3	2.9
190.9	3.7

Inter-assay precision was determined by measuring buffer controls of varying pERK concentrations in multiple assays over several days.

pg/ml	%CV
1121.6	5.5
367.2	5.6
187.0	7.8

Cross Reactivities

The pERK EIA kit is specific for bioactive pERK. There is less than 0.01% cross-reactivity with non-phosphorylated ERK, phospho p38, non-phosphorylated p38, phospho-JNK, non-phosphorylated JNK, phospho-AKT, non-phosphorylated AKT.

SAMPLE RECOVERIES

Please refer to pages 5-8 for Sample Handling recommendations and Reagent Preparation.

pERK concentrations were measured in RIPA Cell Lysis Buffer 2. pERK was spiked into the undiluted samples of these matrices which were then diluted with the kit assay buffer and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
RIPA Cell Lysis Buffer 2	103.6%	1:8

WARNING: If the end user chooses to not use the provided RIPA Cell Lysis Buffer 2, it is up to the end user to determine the appropriate dilution of samples and assay validation for their chosen cell lysis buffer.

* See Sample Handling instructions on page 5 for details.

REFERENCES

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