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Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA

ProductInformation

Product Number **PE0100**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative detection of Erk1&2 double phosphorylated at threonine 185 and tyrosine 187 in cell lysates. It does not detect non-phosphorylated Erk1&2 protein or the Erk1&2 phosphorylated at other residues. A monoclonal antibody specific for Erk1&2 (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate provided. Erk standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the Erk1&2 antigen binds to the immobilized (capture) antibody. After incubation, excess reagents are washed away and an antibody, specific for Erk1&2 double phosphorylated at threonine 185 and tyrosine 187 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized Erk1&2. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. It binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess Anti-Rabbit IgG-HRP, substrate solution is added, which is acted upon by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of Erk1&2 (pThr^{185}/pTyr^{187}) present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of Erk1&2 (pThr^{185}/pTyr^{187}).

Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA is designed to detect and quantify the level of both dual-phosphorylated Erk2 at threonine 185 and tyrosine 187 (Erk2 (pThr^{185}/pTyr^{187}) and Erk1 at threonine 202 and tyrosine 204 (Erk1 (pThr^{202}/pTyr^{204}). The level of the phosphorylation of Erk1&2 can be an indirect indication of the activity of upstream kinases of Erk1&2 or the activity of Erk1&2 themselves. Although performance characterization of this ELISA kit is done primarily on human cell lines, it kit can be used for detection of Erk1&2 in mouse and rat cells. This assay is intended for detection of Erk1&2 dual phosphorylation from cell lysates. For normalization of Erk1&2 content in the samples an Erk1&2 ELISA kit, which is independent of phosphorylation status, is available from Sigma (Product No. EK0100).

Erk (Extracellular Signal Regulated Kinase), also known as MAPK (Mitogen-Activated Protein Kinase), has two closely related isoforms. Erk1, also known as MAP kinase 1 or p44 MAP kinase, is a 44 kDa protein and Erk2, also known as MAP kinase 2 or p42 MAP kinase, is a 42 kDa protein. These kinases belong to a family of serine/threonine kinases that are activated upon treatment of cells with a large variety of stimuli including mitogens, hormones, growth factors, cytokines, and bioactive peptides. Cell stimulation induces the activation of a signaling cascade, the downstream effects of which have been linked to the regulation of cell growth and differentiation as well as regulation of the cytoskeleton. Erk1 and Erk2 are serine/threonine kinases expressed broadly in normal tissues and various cell lines. They are activated through the phosphorylation of a threonine and a tyrosine residue (within a Thr-Glu-Tyr motif) within the activation loop by MEKs (MAPK/ERK kinases), including MEK1 (MAPK/Erk kinase 1) or MEK2. The phosphorylation occurs on threonine 202 and tyrosine 204 of human Erk1, and on threonine 185 and tyrosine 187 of human Erk2 and the dual phosphorylation is required for enzyme activity of Erk1 and Erk2. Once activated, Erk1 and Erk2 can phosphorylate PXS/TP motifs in many different proteins including cytoskeletal proteins, translation regulators, the Rsk family of protein kinases and transcription factors, such as Elk-1.

Reagents

- **Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) Standard, 2 vials, Product No. E 2905** – lyophilized from full length human recombinant Erk2.
- **Standard Diluent Buffer**, **25 mL**, **Product No. S 3943** - containing BSA and sodium azide as a preservative.

- **Monoclonal Anti-Erk1&2-coated 96-well plate, 1 plate, Product No. E 2655** - A plate using break-apart strips coated with monoclonal antibody specific for Erk1&2 (regardless of phosphorylation state).
- **Phospho-Anti-Erk1&2 (pThr^{185}/pTyr^{187}) 11 mL, Product No. E 4155** – A detection antibody, specific for ERK phosphorylated at threonine 185 and tyrosine 187. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate, 100X**, **Product No. I 4908 -** contains 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 8912** – contains thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25X, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions.*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**.

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL
- Cell extraction buffer (see recommended extraction procedure)
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard
- Absorbent paper towels to blot the plate
- Calibrated beakers and graduated cylinders in various sizes
- Vortex mixer

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
- Cell Extraction Buffer
  10 mM Tris, pH 7.4
  100 mM NaCl
  1 mM EDTA
  1 mM EGTA
  1 mM NaF
  20 mM Na4P2O7
  2 mM Na3VO4
  1% Triton X-100
  10% Glycerol
  0.1% SDS
  0.5% Deoxycholate
  1 mM PMSF (stock is 0.3 M in DMSO).
  PMSF is very unstable and must be added prior to use, even if added previously.
  Protease inhibitor cocktail (Product. No. P 2714).
  Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 ºC or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 ºC.

Thaw on ice. Add the protease inhibitors just before use.

Procedure for Extraction of Proteins from Cells

The recommended Cell Extraction Buffer and procedure are optimized to achieve effective protein phosphorylation. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 ºC and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute

intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of Erk1&2 (pThr^{185}/pTyr^{187}). For example, 10^8 Jurkat cells grown in RPMI plus 10% FBS and treated with 50 ng/mL PMA can be extracted in 1 mL of Extraction Buffer.

5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
6. Aliquot the clear lysate to clean microcentrifuge tubes.

Pretreatment of Cell Extracts before assay

This ELISA efficiently detects denatured Erk1&2 proteins in the cell extracts. Both, increased SDS concentration in cell extracts and heating of samples are acceptable pretreatment alternatives intended to maximize Erk protein recognition and detection by the antibodies. The choice method depends on the total protein concentration in the sample.

- We recommend the heating option for samples containing <2.5 mg/mL total protein.
- Samples containing >2.5 mg/mL, should be pretreated with an elevated concentration of SDS.
- The treatment chosen should be optimized for each experimental method. Figure 1 and 2 show the examples of both treatments:

SDS Treatment of cell extracts

1) Extracts containing 10, 5, 2.5 and 1 mg/mL total protein from PMA-treated Jurkat cells were treated by addition of 1, 0.5, 0.25 and 0.1% of SDS respectively.
2) The cell extracts were then diluted 1:100, 1:50, 1:25 and 1:10 in *Standard Diluent Buffer* to maintain a final concentration of 0.01% SDS
3) Upon dilution, equal aliquots of 10 µg total protein were assayed by the Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA.

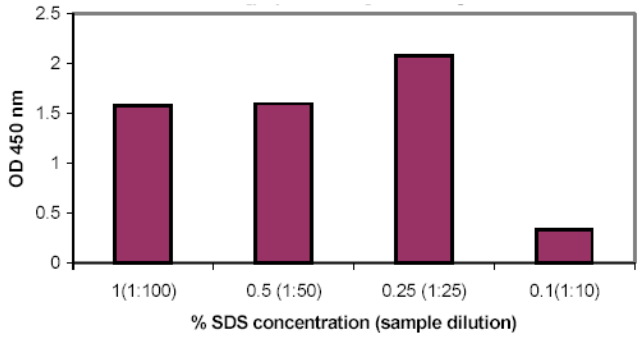

Fig.1 Effect of SDS concentration in cell extracts on Erk (pThr^{185}/pTyr^{187}) signal

4) The data in Figure 1 show that cell extracts with the addition of 0.25-1% SDS generate higher signals than the extract with 0.1% SDS.

Boiling of cell extracts

1. Cell extracts with total protein concentrations of 1, 2, 5 and 8 mg/mL were boiled for 5 minutes.
2. Upon cooling, the extracts were centrifuged.
3. Equal aliquots of 10 µg total protein were assayed by the Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA.

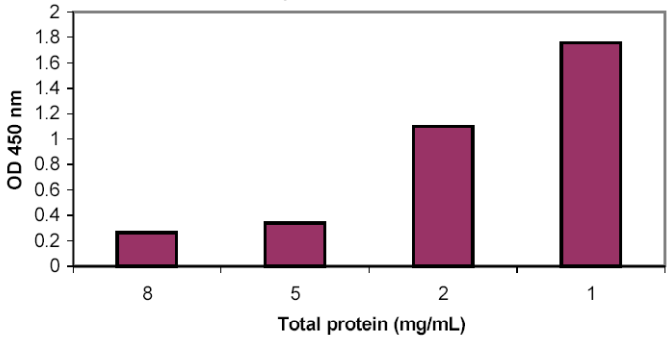

Fig. 2 Effect of boiling on Erk1&2 (pThr^{185}/pTyr^{187}) recovery

4. The data in Figure 2 show that protein concentrations above 2.5 mg/mL negatively affected the ELISA signal. perhaps due to protein aggregation or precipitation after boiling.
5. The signal remained strong in the protein concentration range 1-2.5 mg/mL.

Reagent Preparation

Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) Standard

Erk1&2 (pThr^{185}/pTyr^{187}) standard is prepared using purified full length human recombinant active Erk2.

1. Reconstitute one vial of Standard with Standard Diluent Buffer according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. Label as **100 Units/mL Erk1&2 (pThr^{185}/pTyr^{187})**
4. Prepare serial standard dilutions as follows:

Tube#	Standard Diluent Buffer	Standard from tube #:	Final Erk1&2 Units/mL
1	Reconstitute according to label instructions		100 U/mL
2	0.25 mL	0.25 mL (1)	50
3	0.25 mL	0.25 mL (2)	25
4	0.25 mL	0.25 mL (3)	12.5
5	0.25 mL	0.25 mL (4)	6.25
6	0.25 mL	0.25 mL (5)	3.12
7	0.25 mL	0.25 mL (6)	1.6
8	0.5 mL	-	0

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution

1 unit of standard is equivalent to the amount of Erk1&2 ($pThr^{185}/pTyr^{187}$) derived from 40 pg of Erk1&2 that was phosphorylated by MEK1. Subsequent lots of standard will be normalized to this lot of material to allow consistency of Erk1&2 ($pThr^{185}/pTyr^{187}$) quantitation.

Anti-Rabbit IgG-HRP concentrate (100X), contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 µL IgG-HRP Concentrate +1 mL HRP Diluent
   (sufficient for one 8-well strip, prepare more as needed)
4. Label as **Anti-Rabbit IgG-HRP Working Solution.**
5. Return the unused Anti-IgG-HRP concentrate to the refrigerator.

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as **Working Wash Buffer.**
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2 to 8 ºC. Any unused reconstituted standard should be discarded or frozen at -70 ºC. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 ºC).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4^{th} wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.

- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

Assay Summary
Phospho- Erk1&2 (pThr^{185}/pTyr^{187}) ELISA

1) Incubate 100 µL of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)

aspirate and wash 4x

2) Incubate 100 µL of Detection Antibody for 1 hour at RT.

aspirate and wash 4x

3) Incubate 100 µL of HRP Anti-Rabbit IgG for 30 minutes at RT.

aspirate and wash 5x

4) Incubate 100 µL of stabilized Chromogen for 30 minutes at RT.

5) Add 100 µL of Stop Solution and read at 450nm.

Total Time 4 hours

1st incubation
a Add 100 µL Standard Diluent to zero wells.
b Add 100 µL standards, samples or controls to the appropriate wells. *Samples in Cell Extraction Buffer must be diluted at least 1:10 (1:25 or 1:50 were found to be optimal) in Standard Diluent Buffer. The dilutions should be optimized for each assay.*
c Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2 to 8 °C.*
d Wash wells 4 times following washing instructions.
e After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

2nd incubation
a Add 100 µL Anti- Erk1&2 (pThr^{185}/pTyr^{187}) detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation
a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation
a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction
a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading
a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).
c Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results

1. The results may be calculated using any immunoassay software package.

2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of Erk may be calculated manually.
4. Calculate the Average Net OD (nm) (average reading of 2 wells) for each standard dilution and samples as follows:
   Average Net OD (nm) =
   Average Bound OD (nm) – Average Chromogen Blank OD (nm)
5. On graph paper plot the Average Net OD (nm) of standard dilutions (nm) against the standard concentration (Units/mL) of Erk1&2 ($pThr^{185}/pTyr^{187}$). Draw the best curve through these points to construct the standard curve.
6. The Erk1&2 ($pThr^{185}/pTyr^{187}$) concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
7. Multiply the values obtained for the samples by dilution factor of each sample.
8. Samples producing signals higher than the 100 units/mL standard should be further diluted and assayed again.

Product Profile

Typical Results
The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Phospho- Erk1&2 ($pThr^{185}/pTyr^{187}$) Standard (U/mL)	Optical Density 450 nm
0	0.181
1.6	0.212
3.12	0.237
6.25	0.308
12.5	0.482
25	0.798
50	1.423
100	2.771

Limitations:
- Do not extrapolate the standard curve beyond the 100 Units/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- Although Erk1&2 degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity
The specificity of this assay for dually phosphorylated Erk1&2 was confirmed by peptide competition. The data presented in Figure 3 show that only phospho-peptide containing the phosphorylated threonine and tyrosine could block the ELISA signal. The same sequence containing non-phosphorylated threonine and tyrosine at position 185/187 or mono-phospho peptides did not block the signal by more then 10%.

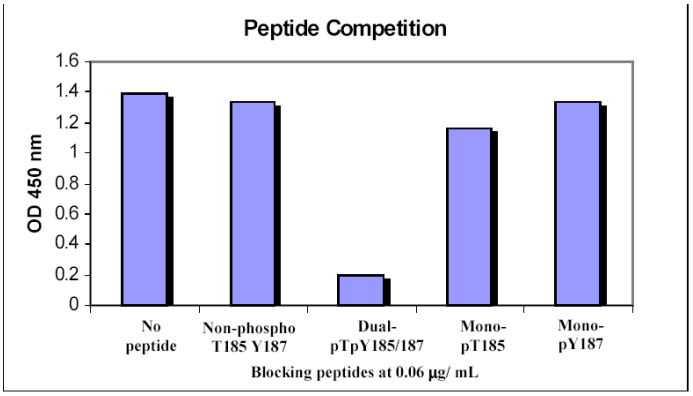

Figure 3

Jurkat cells were treated with 50 ng/mL PMA for 10 minutes. Untreated Jurkat cells were used as control. Cell extracts were prepared in 0.1% SDS, boiled, and analyzed in the Phospho-Erk1&2 ($pThr^{185}/pTyr^{187}$) ELISA and Erk1&2 (Total) ELISA (Prod. No. EK0100). The Phospho-Erk1&2 ($pThr^{185}/pTyr^{187}$) ELISA kit detected phosphorylated Erk1&2 in PMA-treated Jurkat cells, but not the non-phosphorylated Erk1&2 in untreated Jurkat cells. In contrast Erk1&2 ELISA detected both phosphorylated and non-phosphorylated Erk1&2 in PMA-treated cells and untreated control (Figure 4).

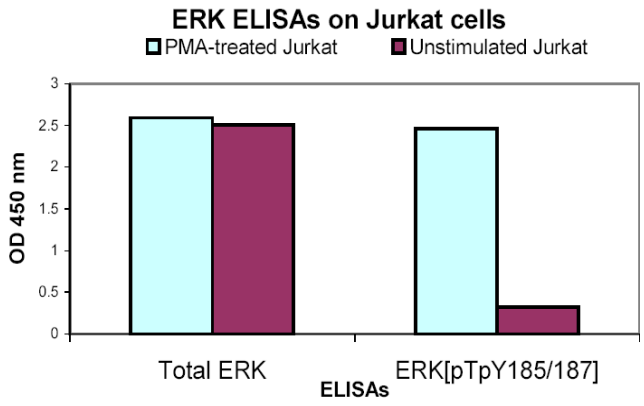

Figure 4

Sensitivity

Sensitivity of this assay is <0.8 Units/mL**.** Sensitivity was calculated by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to immunoblotting using known quantities of Erk1&2 &2 (pThr^{185}/pTyr^{187}).

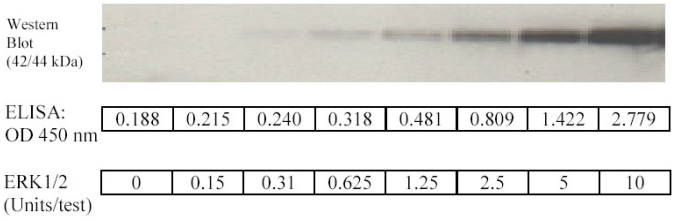

Figure 5 Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA vs. immunoblotting

The results in Figure 5 show that ELISA is approximately 4 times more sensitive in detecting Erk1&2 (pThr^{185}/pTyr^{187}) than immunoblotting. The bands shown in the immunoblot were developed using rabbit anti-Erk1&2 (pThr^{185}/pTyr^{187}); an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography

Precision

1. Intra-Assay Precision

Samples of known Erk1&2 (pThr^{185}/pTyr^{187}) concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	56.8	29.3	7.0
Standard Deviation (SD)	2.5	1.6	0.7
% Coefficient of Variation	4.5	5.5	9.2

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	56.8	29.3	7.0
Standard Deviation (SD)	2.5	1.6	0.7
% Coefficient of Variation	4.5	5.5	9.2

Sample Recovery

To evaluate recovery Erk1&2 standard at 3 different concentrations was spiked into10% cell extract buffer and assayed in this ELISA. The recovery averaged 95%.

Parallelism

Natural Erk1&2 (pThr^{185}/pTyr^{187}) from PMA-treated Jurkat cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against Erk1&2 (pThr^{185}/pTyr^{187}) standard curve. Parallelism demonstrated by the figure 6 indicated that the standard accurately reflects natural Erk1&2 (pThr^{185}/pTyr^{187}) content in samples.

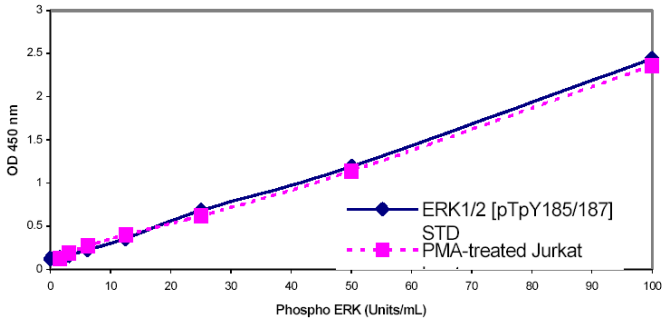

Fig. 6 Parallelism: Natural and standard Erk1&2 (pThr^{185}/pTyr^{187}) in ELISA

Linearity of Dilution

Jurkat cells were PMA-treated and lysed with cell extraction buffer. The lysate was diluted in *Standard Diluent Buffer* over the range of the assay and analyzed in Phospho-Erk1&2 (pThr^{185}/pTyr^{187}) ELISA. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Cell Lysate			
Dilution	**Measured (Units/mL)**	**Expected (Units/mL)**	**% Expected**
Neat	97.0	97.0	100%
1:2	47.3	48.5	97%
1:4	24.8	23.7	104%
1:8	14.7	12.4	118%
1:16	8.6	7.3	116%
1:32	3.4	3.3	113%

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