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Phospho-Akt/PKB (pThr^{308}) ELISA

Product Information

Product Number **CS0110**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

Phospho-Akt/PKB (pThr^{308}) ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of Akt/PKB protein phosphorylated on threonine 308 in cell lysates. A monoclonal antibody specific for Akt/PKB regardless of phosphorylation state has been coated onto the wells of the multiwell plate provided. Phospho-Akt/PKB (pThr^{308}) standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the Akt/PKB antigen binds to the immobilized (capture) antibody. After incubation, excess reagents are washed away and a rabbit antibody specific for Phospho-Akt/PKB (pThr^{308}) is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized Akt/PKB protein captured during the first incubation. 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 all the excess Anti-Rabbit IgG-HRP, substrate solution is added, which is acted upon by the bound enzyme to produce a yellow color. The intensity of this colored product is directly proportional to the concentration of Phospho-Akt/PKB (pThr^{308}) present in the original specimen. The optical density measured at 450 nm in the multiwell reader is used to calculate the concentration of Phospho-Akt/PKB (pThr^{308}) The ELISA detects Phospho-Akt/PKB (pThr308) in human, mouse and rat cell lysates. The researcher may normalize his results using an Akt/PKB ELISA (Product No.CS0160), which measures Akt/PKB independent of phosphorylation status.

Akt, also known as the protein kinase B-a?(PKB-a) or RAC-PKa, was initially identified as one of the downstream targets of PI-3 Kinase (PI3-K). Akt is now known to consist of three highly conserved isoforms, which are designated in humans as Akt1, Akt2, and Akt3. Each isoform consists of an N-terminus pleckstrin homology (PH) domain, which mediates lipid-protein or protein-protein interactions, and a C-terminus kinase catalytic domain. Although each kinase is expressed differentially in a tissue-specific manner, they respond in a similar fashion to various stimuli. Akt can be activated by a diverse array of growth factors and physiologic stimuli in a PI3-K-dependent manner. Activation of Akt kinase is a multi-step process involving both membrane translocation and phosphorylation. Activated PI3-K generates 3' phosphoinositide products, 3,4,5-triphosphates (PI-3,4,5-P3) and PI-3,4-P2.[1-3]

Akt is recruited from the cytosol to the plasma membrane through the interaction of its PH domain with these phosphoinositides. Upon membrane localization, Akt undergoes a conformational change, which makes it accessible to phosphorylation at two kinase regulatory sites, threonine-308 of the kinase activation segment becomes activated by PDK-1 and serine-473 of the hydrophobic module by putative serine 473 kinase. Carboxyl-terminal modulator protein (CTMP) binds to Akt/PKB a and reduces the activity by inhibiting phosphorylation at both threonine 308 and serine 473. These findings identify CTMP as a negative regulatory component of the pathway controlling PKB activity. Activated Akt/PKB acts as a key mediator of signals for cell survival, proliferation, angiogenesis, and a number of metabolic effects of insulin. The effects of Akt activation may be mediated by modulation of expression or activity of various molecules including Bcl-2, BAD, caspase-9, endothelial nitric oxide synthase (eNOS), glycogen synthase, and transcription factors (NF-kB, Forkhead, CREB, Mdm2).[4]

Because of its growth-promoting effects, Akt is also emerging as a central player in tumorigenesis. A number of oncogenes and tumor suppressor genes act upstream of Akt to influence cancer progression. Deletion of PTEN, a tumor suppressor gene that encodes a phosphatase, correlates with increased Akt

activity in several cancers. Similarly, over expression of active Ras, Her/Neu, or Akt genes causes hyperactivation of Akt in many cancers including pancreatic, gastric, breast, ovarian and prostate adenocarcinomas. Small-molecule therapeutics that block PI3K signaling might inhibit cancer cells by blocking many aspects of the tumor-cell phenotype.[5-7]

Reagents

- **Phospho-Akt/PKB (pThr^{308}) Standard, Human, 2 vials, Product No. P 6370** – lyophilized, full-length recombinant Akt/PKB protein, expressed in *Sf21* cells and phosphorylated by MAPKAP2 and PDK1. *Refer to vial label for quantity and reconstitution volume.*
- **Standard Diluent Buffer**, **25 mL**, **Product No. S 3068** - containing BSA and sodium azide as a preservative.
- **Monoclonal Anti-Akt/PKB Antibody-coated 96 well plate, 1 plate, Product No. H 9287**- A plate using break-apart strips coated with monoclonal antibody specific for full length Akt/PKB.
- **Anti-phospho-Akt/PKB (pThr^{308}), 11 mL, Product No. R 9527** – A detection antibody, produced in rabbit, which recognizes Akt/PKB phosphorylated on threonine 308. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate (100x), 1 vial, 0.125 mL, Product No. R 9652** – contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Catalog No H 8912** – contains 3.3 mM 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 (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 Handling

- 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.

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. In such cases, they should assay their lysates for the satisfactory extraction and/or phosphorylation.

- 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)
  Protease inhibitor cocktail (Product No. P 2714)
  PMSF is very unstable and must be added prior to use, even if added previously.
  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.*

- Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent). Wash twice with cold PBS.
2. 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).
3. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
4. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of Akt/PKB. For example, $4X10^7$ Jurkat cells grown in RPMI plus 10% FBS 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.

Before assay: extracted cell lysate samples containing Phospho-Akt/PKB ($pThr^{308}$) protein should be diluted with Standard Diluent Buffer at least 1:10 (dilutions 1:20 to 1:50 may be required). This dilution is necessary to reduce the matrix effect of the cell lysate buffer. Example: 0.1-0.5 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of Phospho-Akt/PKB ($pThr^{308}$).

Reagent Preparation

Phospho-Akt/PKB (pThr308) Standard

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 U/mL Phospho-Akt/PKB ($pThr^{308}$)**.
4. Prepare serial standard dilutions as follows:

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

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution.

One Unit of standard = the amount of Phospho-Akt/PKB (pThr308) derived from 500 pg of Akt/PKB which was phosphorylated by MAPKAP2 and PDK1.

Subsequent lots of standard will be normalized to this lot of material to allow consistency of Phospho-Akt/PKB ($pThr^{308}$) quantitation.

Anti-Rabbit IgG Horseradish Peroxidase (HRP), 100x Concentrate

Solution 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 with 1 mL HRP Diluent (sufficient for one 8-well strip, prepare more if needed)
4. Label as **Anti-Rabbit IgG-HRP Working Solution.**
5. Return the unused Anti-Rabbit IgG-HR concentrate to the refrigerator.

Wash Buffer

1. Equilibrate Wash Buffer Concentrate 25x to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25x with 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 for 6 months from the date of purchase.
Any unused reconstituted standard should be discarded or frozen at –80 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. to ensure 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.
- Stabilized Chromogen (TMB) is light sensitive. Avoid prolonged exposure to light. Avoid contact with metal, it may cause color development.

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 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.

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.

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 1 hours at RT.**

   aspirate and wash 4x

3) **Incubate 100 µL of Anti-Rabbit IgG-HRP 30 min. at RT.**

   aspirate and wash 4x

4) **Incubate 100 µL of stabilized Chromogen for 30 minutes at RT *(in the dark).***

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

Total Time 4 hours

- **Summary of Phospho-Akt/PKB (pThr^{308}) Assay**

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 (1:50 or 1:100 may be necessary) 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. 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-phospho-Akt/PKB ($pThr^{308}$) (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 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 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 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

The results may be calculated using any immunoassay software package. The four-parameter algorithm provides the best curve fit. If the software program is not readily available, the concentrations of Phospho-Akt/PKB ($pThr^{308}$) may be calculated as follows:

1. 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)
2. On graph paper plot the Average Net OD (nm) of standard dilution against the concentration (U/mL) of Phospho-Akt/PKB ($pThr^{308}$) for the standards.
3. Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
4. The Phospho-Akt/PKB ($pThr^{308}$) concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply value(s) obtained for sample(s) by dilution factor to correct for the dilution in step I.b.
6. Samples producing signals higher than the highest standard (100 Units/mL) should be further diluted in *Standard Diluent Buffer* and re-analyzed, multiplying the concentration by the appropriate dilution factor.
7. Values of Phospho-Akt/PKB ($pThr^{308}$) should be normalized for total Akt/PKB content. Use Akt/PKB ELISA (Product No. CS0160) to quantitate total Akt/PKB concentration in samples and controls.

Product Profile

Typical Results

The standard curve below is for illustration purposes only and **should not be used** to calculate results in your assay. Run standard curve in each assay.
The following data were obtained for the various standards over the range of 0 to 100 Units/m Phospho-Akt/PKB ($pThr^{308}$):

OD 450 nm	Phospho-Akt/PKB (pThr^{308}) Standard U/mL
0.181	0
0.214	1.6
0.259	3.12
0.339	6.25
0.511	12.5
0.874	25
1.388	50
2.513	100

Limitations:

- Do not extrapolate the standard curve beyond the 100 U/mL standard point.
- The dose response is non-linear in this region and accuracy is compromised.
- Dilute samples >100 Units/mL with *Standard Diluent Buffer;* re-analyze these and multiply results by the appropriate dilution factor.
- Other buffers and matrices have not been investigated.
- Although Akt/PKB degradation or dephosphorylation of Phospho-Akt/PKB (pThr^{308}) 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

This Phospho-Akt/PKB (pThr^{308}) ELISA is specific for measurement of human, mouse and rat Akt/PKB phosphorylated at threonine 308. The specificity of this assay for AKT phosphorylated at threonine 308 was confirmed by peptide competition. The data presented in Figure 1 show that only the phospho-peptide containing the phosphorylated threonine could block the ELISA signal. The same sequence containing non-phosphorylated threonine at position 308 did not block the signal.

The assay was found to have no cross-reactivity with the following recombinant phosphoproteins tested at 100 ng/mL: p38 MAPK, p44 ERK1, p42 ERK2, JNK1, human insulin receptor, rat insulin receptor, human HGFR (c-met).

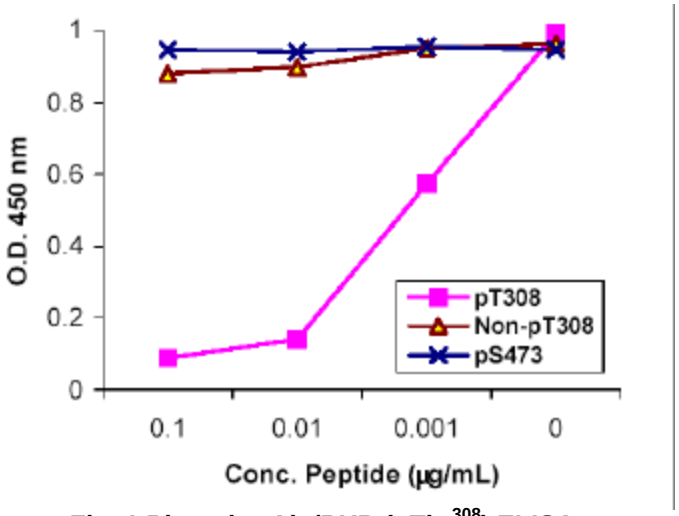

Fig. 1 Phospho-Akt/PKB (pThr^{308}) ELISA: Peptide Blocking

Sensitivity

Sensitivity of this assay is **<0.8 U/mL** of Akt/PKB(pThr^{308}). 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 Akt/PKB(pThr^{308}).

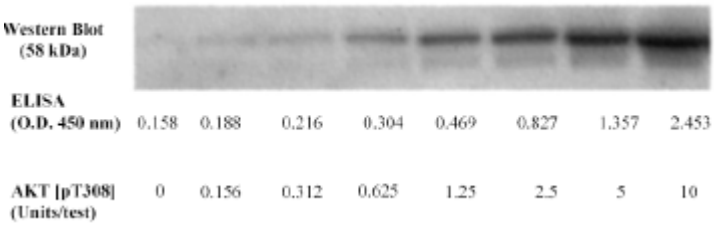

Fig. 2 Detection of Phospho-Akt/PKB (pThr^{308}) by ELISA vs. immunoblot

The data presented in Figure 1 show that the sensitivity of the ELISA is approximately the same as that of immunoblotting. The bands shown in the immunoblotting data were developed using rabbit anti-Akt/PKB (pThr^{308}); an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Precision

1. Intra-Assay Precision
Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (U/mL)	42.6	9.0	2.0
Standard Deviation (SD)	1.7	0.3	0.1
% Coefficient of Variation	4.1	3.0	3.0

2. Inter-Assay Precision
Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (U/mL)	39.0	9.0	2.0
Standard Deviation (SD)	2.1	0.5	0.1
% Coefficient of Variation	5.4	5.2	5.9

Sample Recovery

To evaluate recovery, extract buffer was diluted 1:10 with *Standard Diluent Buffer* to bring the SDS concentration to <0.01%. Recombinant Akt/PKB (pThr^{308}) at 3 levels was spiked into the cell extract and percent recovery calculated over endogenous levels. On average, 102% recovery was observed.

Parallelism

Natural Phospho-Akt/PKB (pThr^{308}) from extracts of Jurkat cells cultured in RPMI with 10% FCS were serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the Phospho-Akt/PKB (pThr^{308}) standard curve. Parallelism demonstrated in figure 3 indicated that the standard accurately reflects full length Phospho-Akt/PKB (pThr^{308}) content in samples.

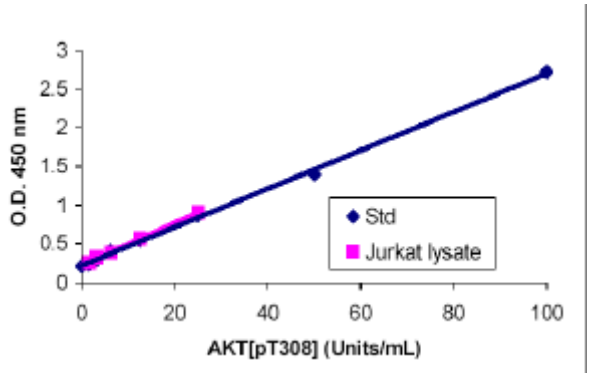

Fig. 3 Phospho-Akt/PKB (pThr^{308}) ELISA: Parallelism

Linearity of Dilution

Extract Buffer was spiked with Akt/PKB (pThr^{308}) and serially diluted in Standard Diluent Buffer over the range of the assay.

Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Cell Lysate			
Dilution	**Measured (U/mL)**	**Expected (U/mL)**	**% Expected**
Neat	75	75	100
1:2	38.5	37.5	103
1:4	21.7	18.8	116
1:8	11.9	9.4	126

References

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