Product Manual

[pThr180/Tyr182)p38 ELISA kit

Catalog #: ADI-900-101

96 Well Kit

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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 [pThr180/Tyr182) p38 ELISA kit is a complete kit for the quantitative determination of phospho-p38 in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to p38 immobilized on a microtiter plate to bind the phospho-p38 in the standards or sample. A recombinant phospho-p38 Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a rabbit polyclonal antibody to phospho-p38 is added.

This antibody binds to the phospho-p38 captured on the plate. After a short incubation the excess antibody is washed out and goat anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the p38 (phospho-Thr180/Tyr182) polyclonal antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450nm. The measured optical density is directly proportional to the concentration of phospho-p38 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

p38 MAP kinase (MAPK) is also known as Mitogen-Activated Protein Kinase 14, MAP Kinase p38, p38 alpha, Stress Activated Protein Kinase 2A (SAPK2A), RK, MX12, CSBP1, CSBP2 included, and Cytokine Suppressive Anti-Inflammatory Drug Binding Protein (CSAID binding protein). There are many recent reviews in the literature detailing the function and activity of p38[3-7]. It is the mammalian counterpart of the yeast HOG kinase (High Osmolarity Glycerol response kinase). p38 is involved in a signaling system that controls cellular responses to cytokines and stress[8-11]. Similar to the SAPK/JNK pathway, p38 MAP Kinase is activated by a range of cellular stimuli including osmotic shock, lipopolysaccharides (LPS), inflammatory cytokines, UV light and growth factors[4-8]. MKK3 and SEK activate p38 MAP Kinase by phosphorylation at Thr180 and Tyr182. Activated p38 MAP Kinase has been shown to phosphorylate MAPKAP Kinase-2[10] and to phosphorylate the transcription factors ATF-2[12] and Max[13].

SAFETY WARNINGS & PRECAUTIONS

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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 phospho-p38 Standard provided, Catalog No. 80-0965, should be handled with care because of the known and unknown effects of phospho-p38.
5. The phospho-p38 Standard should be stored at or below -20°C. Do not repeatedly freeze-thaw.

MATERIALS SUPPLIED

1. **p38 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0964**

   A plate using break-apart strips coated with a mouse monoclonal antibody specific to p38.

2. **phospho-p38 Antibody, 11 ml, Catalog No. 80-0963**

   A yellow solution of rabbit polyclonal antibody to phospho-p38.

3. **Assay Buffer 4 Concentrate, 5x, 100 ml, Catalog No. 80-0935**

   MOPSO buffered saline containing proteins and detergents**.**

4. **phospho-p38 Conjugate, 11 ml, Catalog No. 80-1164**

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

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

   Tris buffered saline containing detergents.

6. **phospho-p38 Standards, 2 each, Catalog No. 80-0965**

   Two vials containing 5,000 pg each of lyophilized recombinant phospho-p38.

7. **TMB Substrate, 11 ml, 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, 11 ml, Catalog No. 80-0377**

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

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

   M Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS

10. **phospho-p38 Assay Layout Sheet, 1 each, Catalog No. 30-0171**

11. **Plate Sealer, 3 each, Catalog No. 30-0012**

Reagents require separate storage conditions.

STORAGE

All components of this kit, **except the Standard**, are stable at 4°C until the kit's expiration date. The 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.
13. Graph paper for plotting the standard curve.

SAMPLE HANDLING

The (pThr180/Tyr182)p38 ELISA kit is compatible with phospho-p38 samples in a wide range of cell lysates and buffers. Samples diluted sufficiently into Assay Buffer 4 plus Inhibitors (see Reagent Preparation, s 8, #2) can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 15 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 immediately prior to use (see Reagent Preparation page 9, #4). Samples lysed in RIPA Cell Lysis Buffer 2 plus Inhibitors must be diluted at least 1:80 with Assay Buffer 4 plus Inhibitors prior to running in the assay.

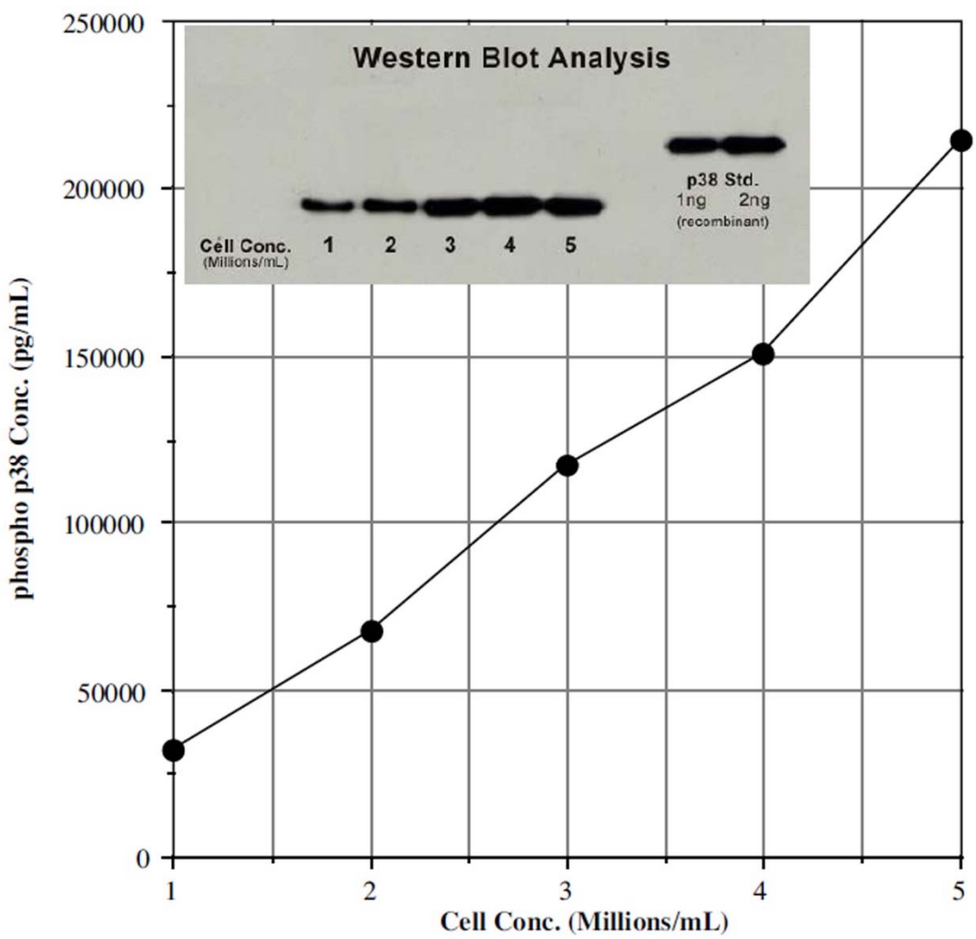
Note that this dilution is based on the lysis of 4 million Jurkat cells per ml. The 1:80 dilution contained 50,000 cells per ml with a calculated recovery of 96.8%.

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.

Only standard curves generated in Assay Buffer 4 plus Inhibitors should be used to calculate the concentration of phospho-p38. Samples must be stored frozen at or below -70°C to avoid loss of bioactive phospho-p38. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4°C slowly and gently mixed.

JURKAT CELL LYSIS EXPERIMENT

The number of Jurkat cells used in this experiment were: 5.0,4.0, 3.0, 2.0 and 1.0 million per ml. Cells were centrifuged at 1,500rpm for 5 minutes and the supernatant discarded. The cell pellets were resuspended and washed with PBS. Cells were pelleted at 1,500rpm for 5 minutes and the supernatant dicarded. The cell pellets were resuspended with modified 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 90 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 can be made up in either glass or plastic 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 50 ml of the supplied concentrate with 950 ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **Assay Buffer 4 plus Inhibitors**

   Ensure that the Assay Buffer 4 Concentrate is completely in solution prior to use. Prepare the Assay Buffer 4 by diluting 100 ml of the supplied concentrate with 400 ml of deionized water. This can be stored at 4°C until the kit expiration, or for 3 months, whichever is earlier. 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 4 must be used for standard reconstitution and all sample and standard dilutions to ensure optimal integrity of phospho-38. Fresh Assay Buffer 4 plus Inhibitors must be made for each assay.

3. **phospho-p38 Standards**

   Allow the lyophilized phospho-p38 standard to warm to room temperature. Add 1 ml of Assay Buffer 4 plus Inhibitors to the lyophilized phospho-p38 vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard #1. Label five 12x75 mm glass tubes #2 through #6. Pipet 500µl of Assay Buffer 4 plus Inhibitors into tubes #2 though #6. Add 500µl of reconstituted standard #1 to tube #2 and vortex. Add 500µl of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

   The concentration of phospho-p38 in standard vial #1 and tubes #2 through #6 will be 5,000, 2,500, 1,250, 625, 312.5 and 156.25 pg/ml respectively. See phospho-p38 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 4 plus Inhibitors into the S0 (0 pg/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. 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 2 to each well. This stops the reaction and the plate should be read immediately.
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 phospho-p38 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 this sort of data reduction software is not readily available, the concentration of phospho-p38 can be calculated as follows:

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

   Average Net OD = Average Bound OD - Average NSB OD

2. Plot the Average Net OD for each standard versus phospho-p38 concentration in each standard. Approximate a straight line through the points. The concentration of phospho-p38 in the unknowns can be determined by interpolation.

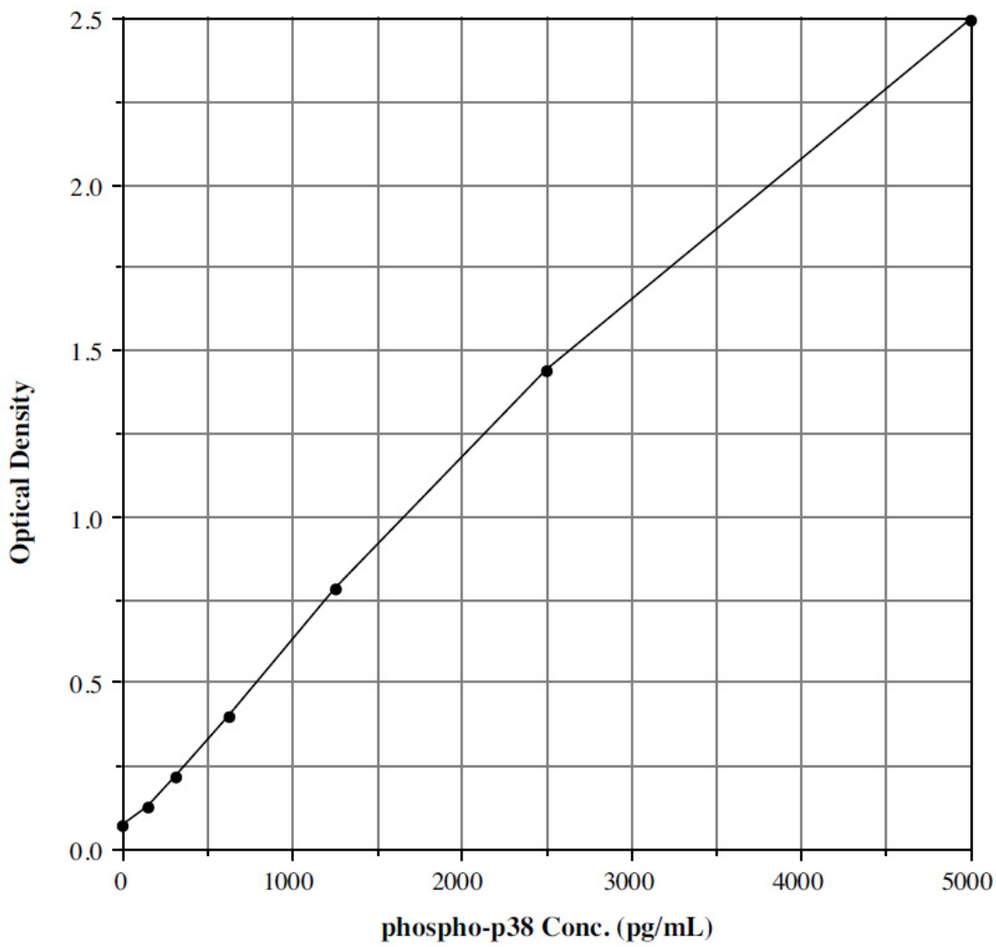
TYPICAL RESULTS

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

Sample	Average OD	Net OD	phospho-p38 (pg/ml)
Blank	0.129		
S0	0.195	0.067	0
S1	2.619	2.490	5,000
S2	1.566	1.438	2,500
S3	0.912	0.783	1,250
S4	0.525	0.397	625
S5	0.345	0.216	313
S6	0.252	0.124	156
Unknown 1	0.752	0.623	810
Unknown 2	0.471	0.342	240

TYPICAL STANDARD CURVES

A typical standard curve is shown below. This curve must not be used to calculate phospho-p38 concentrations; each user must run a standard curve for each assay.



Units of Measure

Samples measured in the (pThr180/Tyr182)p38 ELISA for can be expressed in terms of concentration by weight or activity. When reconstituted according to direction, the standard stock concentration is 5,000 pg/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 76 Units/mg where one Unit of phospho-p38 activity is equal to 1nmol phosphate incorporated into 0.33 mg/ml myelin basic protein per minute at 30°C in a total reaction volume of 50µl.

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 sixteen (16) wells run with 0 pg/ml Standard, and comparing to the average optical density for sixteen (16) wells run with Standard #6. The detection limit was determined as the concentration of phospho-p38 measured at two (2) standard deviations from the 0 pg/ml Standard along the standard curve.

Mean OD for S0 = 0.051 ± 0.009 (18.4%)

Mean OD for Standard #6 = 0.105 ± 0.010 (9.5%)

Delta Optical Density (156.25 - 0 pg/ml) = 0.105 - 0.051 = 0.054

2 SD's of 0 pg/ml Standard = 2 x 0.009= 0.018

$$\text{Sensitivity} = \frac{0.018}{0.054} \times 156.25 \text{ pg/ml} = 52.1 \text{ pg/ml}$$

Linearity

A sample containing 2,850 pg/ml phospho-p38 was serially diluted 4 times 1:2 in the Assay Buffer 4 plus Inhibitors supplied in the kit and measured in the assay. The data was plotted graphically as actual phospho-p38 concentration versus measured phospho-p38 concentration.

The line obtained had a slope of 0.9432 with a correlation coefficient of 0.9997.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of phospho-p38 and running these samples multiple times (n=18) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of phospho-p38 in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of phospho-p38 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Phospho-p38 (pg/ml)	Intra-assay %CV	Inter-assay %CV
Low	534	6.8	
Medium	1069	13.8	
High	2022	7.0	
Low	500		6.9
Medium	936		9.3
High	1893		12.1

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactants in the kit assay buffer at a concentration of 50,000 pg/ml. The samples were then measured in the phospho-p38 assay.

Compound	Cross Reactivity
phospho-p38	100%
non-phosphorylated p38	<0.01%
phospho-JNK	<0.01%
non-phosphorylated JNK	<0.01%
AKT	<0.01%
non-phosphorylated ERK	<0.01%
phospho-ERK	<0.01%

SAMPLE RECOVERIES

Phospho-p38 concentrations were measured in modified RIPA Cell Lysis Buffer 2. Phospho-p38 was spiked into the undiluted sample of this matrix which was then diluted with the kit assay buffer and assayed in the kit.

The following result was obtained:

Sample	% Recovery*	Recommended Dilution*
RIPA Cell Lysis Buffer	90.0	1:40

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NOTES

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