



TiterZyme[®] CLIA

phospho-ERK 1/2

Chemiluminescence Enzyme Immunometric Assay Kit

Catalog No. 910-098

96 Well Kit

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Description

Assay Designs' phospho-Extracellular signal-Regulated Kinase (pERK)1/2 TiterZyme® CLIA 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 out 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 out and goat anti-rabbit IgG conjugated to alkaline phosphatase is added, which binds to the polyclonal pERK antibody. Excess conjugate is washed out and chemiluminescent substrate is added. The substrate reacts with the bound alkaline phosphatase conjugate to produce light emission at approximately 530 nm. The intensity of the emitted light 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 Chard¹ or Tijssen².

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.

1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by high concentrations of chelators (>10 mM) such as EDTA and EGTA.
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.

Materials Supplied

1. **ERK Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1151**
A white plate using break-apart strips coated with a mouse monoclonal antibody specific to ERK.
2. **pERK CLIA Antibody, 10 mL, Catalog No. 80-1152**
A yellow solution of rabbit polyclonal antibody to pERK.
3. **Assay Buffer 21, 100 mL, Catalog No. 80-1519**
Tris buffered saline containing proteins, detergents and phosphatase inhibitor.
4. **pERK CLIA Conjugate, 10 mL, Catalog No. 80-1153**
A blue solution of goat anti-rabbit IgG conjugated to alkaline phosphatase.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
Tris buffered saline containing detergents and sodium azide as preservative.
6. **pERK Standard, 2 vials, Catalog No. 80-0942**
Two vials containing 1,000 pg each of lyophilized recombinant pERK.
7. **Lumiphos 530™ CLIA Substrate*, 20 mL, Catalog No. 80-0134**
Alkaline phosphatase substrate in diethanolamine buffer at pH 9.5, containing fluorescent enhancers.
8. **RIPA Cell Lysis Buffer 2, 100 mL, Catalog No. 80-1284**
50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS.
9. **pERK CLIA Assay Layout Sheet, 1 each, Catalog No. 30-0198**
10. **Plate Sealer, 3 each, Catalog No. 30-0012**

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.

Materials Needed but Not Supplied

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 100 µL and 1,000 µL.
7. Repeater pipet for dispensing 100 µL.
8. Disposable beakers for diluting buffer concentrates.
9. Graduated cylinders.
10. A microplate shaker.
11. Adsorbent paper for blotting.
12. Plate luminometer capable of reading glow chemiluminescence. Some radiation counters may be suitable. Please refer to the counter instruction manual for recommendations on suitability for chemiluminescence measurements.
13. Graph paper for plotting the standard curve.

*Lumiphos 530 is the trademark of Lumigen Inc., Southfield, MI, USA and supplied under US patents 4,857,652; 4,983,779; 4,959,182; 5,004,565; 4,962,192, & 5,386,017; European patents 254051B1 & 352713B1; Japanese patent 5-45590; Australian patent 603,736; Korean patent 69,259 and Taiwanese patent 46,563.

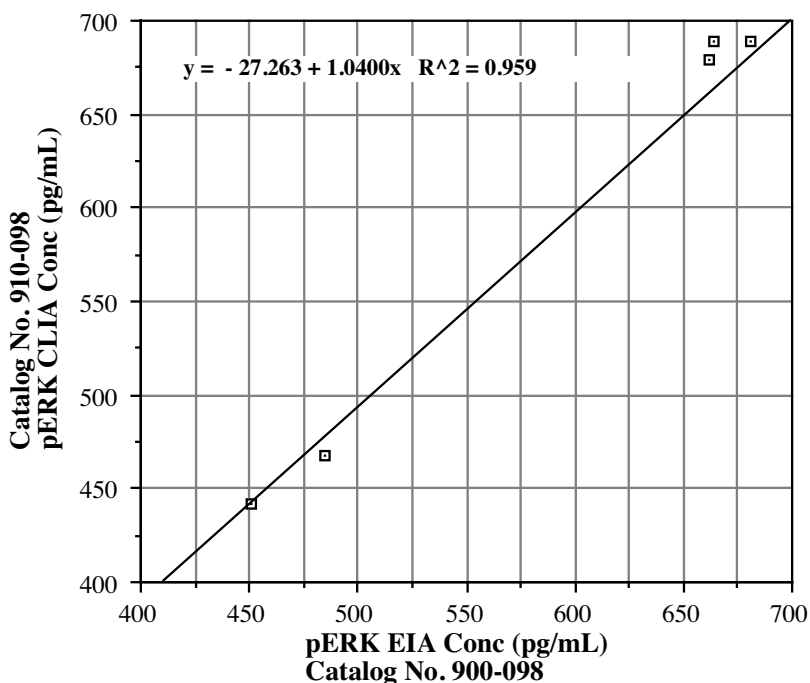
Sample Handling

Assay Designs' TiterZyme® CLIA 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, page 5, #2) can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 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, page 6, #4) immediately prior to use. Samples lysed in RIPA Cell Lysis Buffer 2 plus Inhibitors must be diluted at least 1:4 with Assay Buffer 21 plus Inhibitors prior to assaying. Note that this dilution is based on the lysis of 1.5 million Jurkat cells per mL. The 1:4 dilution contained 375,000 cells per mL with a calculated recovery of 100.3%.

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 21 plus Inhibitors should be used to calculate the concentration of pERK. 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: 1.5, 1.0 and 0.5 million per mL. They were stimulated with 50 nM Phorbol 12-myristate 13-acetate (PMA) or with DMSO (for a negative control) for 5 minutes at 37 °C. Cells were centrifuged at 1,500 rpm for 5 minutes and the supernatant discarded. The cell pellets were resuspended and washed with PBS. Cells were pelleted at 1,500 rpm for 5 minutes and the supernatant discarded. The cell pellets were resuspended with RIPA Cell Lysis Buffer 2, vortexed and placed on ice for 5 minutes. The lysates were vortexed and centrifuged at 14,000 rpm for 10 minutes. The supernatants were split for the CLIA and EIA (Assay Designs' Catalog No. 900-098) to generate the data illustrated. Note that correlation between EIA and CLIA for same samples was used because the lysates measured were not detectable by Western blot analysis due to its limit of detection.



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. Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. **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.**
10. **The chemiluminescent signal generated is read after 30 minutes. The signal is still being generated so the wells must be read in the order in which the substrate was added. If you do not have a luminometer that will precisely time the substrate incubation the following protocol must be followed. We suggest adding the substrate at 10 second intervals between wells and reading the generated chemiluminescence for 2 seconds at 10 second intervals for consistency. If luminometer injection is not used, we suggest using a repeater type syringe, such as an Eppendorf™ Repeater™ Pipette, Catalog Number 2226000-6 and a 5 mL repeater Combitip™ set for delivery of 200 µL. Use the repeater to add substrate to the wells in the order in which they will be read. Please note the order that plate luminometers read wells and ensure substrate addition follows this sequence.**

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 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 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

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 0.5 mL of Assay Buffer 21 plus Inhibitors to the lyophilized pERK vial for a stock solution of 2,000 pg/mL and vortex. Wait 5 minutes and vortex again prior to use. Label five 12x75 mm polypropylene tubes #1 through #5. Pipet 350 μ L of Assay Buffer 21 plus Inhibitors into tube #1. Pipet 250 μ L of Assay Buffer 21 plus Inhibitors into tubes #2 through #5. Add 150 μ L of reconstituted standard stock into tube #1 and vortex. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5.

The concentration of pERK in tubes #1 through #5 will be 600, 300, 150, 75 and 37.5 pg/mL respectively. See pERK Assay Layout Sheet for dilution details.

Reconstituted and diluted standards should be used within 30 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 0.5 μ L/mL PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM. Add Sodium orthovanadate, such as Sigma #S6508, to a final concentration of 2 mM and Sodium pyrophosphate, such as Sigma #S6422, to a final concentration of 20 mM.

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, controls 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 100 μ L of Assay Buffer 21 plus Inhibitors into the S0 (0 pg/mL standard) wells.
3. Pipet 100 μ L of Standards #1 through #5 into the appropriate wells.
4. Pipet 100 μ L 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 ~500 rpm.
7. Empty the contents of the wells and wash by adding 400 μ L 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 100 μ L 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 ~500 rpm.
10. Empty the contents of the wells and wash by adding 400 μ L 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 100 µL 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 ~500 rpm.
13. Empty the contents of the wells and wash by adding 400 µL 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 200 µL of the CLIA Substrate Solution into each well. **Note: Refer to substrate addition timing and sequence on page 5.**
15. Incubate for 30 minutes at room temperature .
16. Read each well for 2 seconds each on a suitable luminometer.

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 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of pERK can be calculated as follows:

1. Calculate the average net Relative Light Units (RLU) bound for each standard and sample by subtracting the average Blank RLU from the average RLU for each standard and sample.

$$\text{Average Net RLU} = \text{Average RLU} - \text{Average Blank RLU}$$

2. Using linear graph paper, plot the Average Net RLU 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.

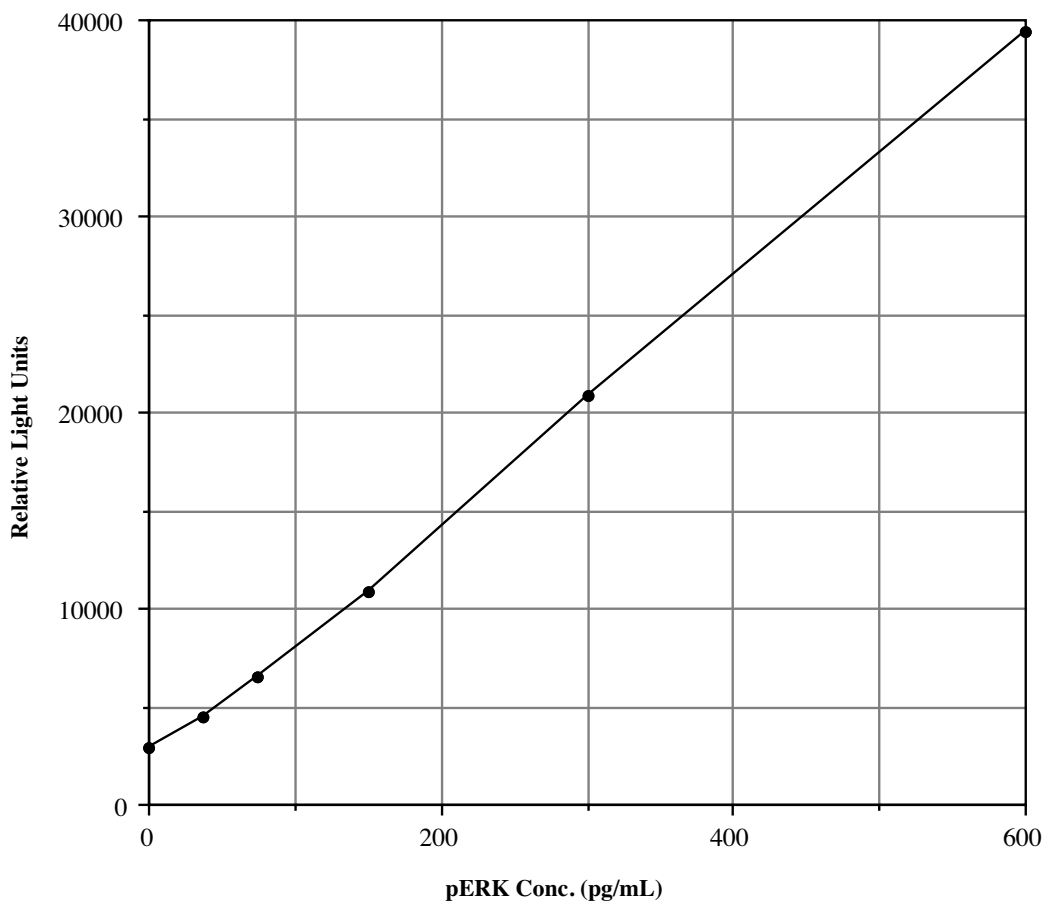
Typical Results

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

<u>Sample</u>	<u>Average RLU</u>	<u>Net RLU</u>	<u>pERK (pg/mL)</u>
Blank	(262)		
S0	3095	2833	0
S1	39604	39342	600
S2	21108	20846	300
S3	11123	10861	150
S4	6811	6549	75
S5	4706	4444	37.5
Unknown1	31522	31260	462.5
Unknown2	5019	4757	42.8

Typical Standard Curve

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 Assay Designs' TiterZyme EIA[®] for phospho-ERK can be expressed in terms of concentration by weight or activity. When reconstituted according to direction, the standard stock concentration is 2000 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 ~10,000,000 Units/mg where one Unit of pERK activity is equal to 1 pmole phosphate incorporated into 100 μ M myelin basic protein per minute at 30 °C in a total reaction volume of 30 μ L.

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹³.

Sensitivity

Sensitivity was calculated by determining the average RLU signal bound for sixteen (16) wells run with 0 pg/mL Standard, and comparing to the average RLU signal for sixteen (16) wells run with Standard #6. The detection limit was determined as the concentration of pERK measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean RLU for S0 = 4,428 ± 594 (13.4%)

Mean RLU for Standard #5 = 5,723 ± 879 (15.4%)

Delta RLU's (37.5 - 0 pg/mL) = 5,723 - 4,428 = 1295.125

2 SD's of 0 pg/mL Standard = 2 x 594.215 = 1188.429

Sensitivity = $\frac{1188.429}{1295.125} \times 37.5 \text{ pg/mL} = \mathbf{34.4 \text{ pg/mL}}$

Linearity

A sample containing 543.6 pg/mL pERK was serially diluted 3 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 0.964 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of pERK and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring two samples with low and high concentrations of pERK in multiple assays (n=8).

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

	pERK (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	127.8	11.3	
Medium	184.5	7.7	
High	568.7	5.2	
Low	92.5		14.2
High	520.1		11.4

Cross Reactivities

The pERK TiterZyme® CLIA 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 as determined in the pERK TiterZyme® EIA Colorimetric assay.

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard 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 pERK EIA kit. The following results were obtained:

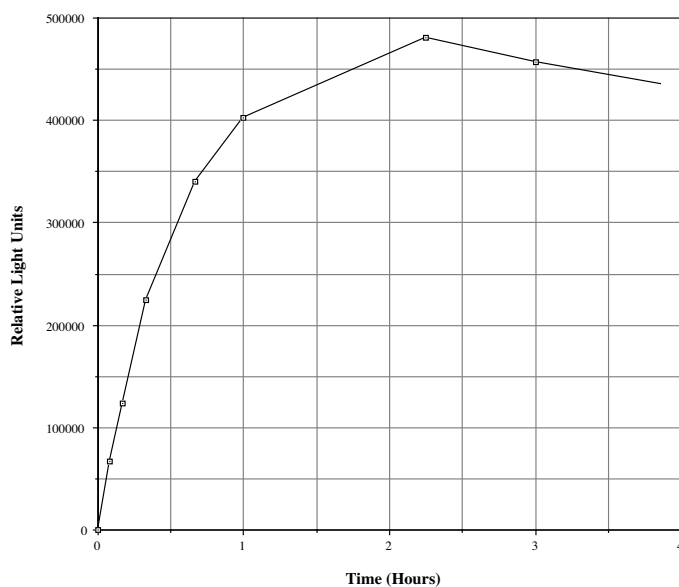
Sample	% Recovery*	Recommended Dilution*
RIPA Cell Lysis Buffer 2	105.5%	≥1:4

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 4 for details.

Time Course of Chemiluminescent Emission

The chemiluminescent signal generated from the reaction of the alkaline phosphatase conjugate and the CLIA substrate is a kinetic reaction that reaches a maximum light output after approximately 4 hours. The chemiluminescent emission will last for several hours. The data is presented below.



References

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LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

**Assay Designs, Inc.
5777 Hines Drive
Ann Arbor, MI 48108
U.S.A.**

**Telephone: (734) 668-6113
(800) 833-8651 (USA & Canada only)
Fax: (734) 668-2793
e-mail: info@assaydesigns.com
website: www.assaydesigns.com**

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