Cayman Practice ELISA Kit

Item No. 10009658

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Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Number	Item	96 wells Quantity/Size
1	Practice ELISA Antiserum	1 vial/100 dtn*
2	Practice AChE Tracer	1 vial/100 dtn*
3	Practice ELISA Standard	1 vial
4	ELISA Buffer Concentrate	2 vials/10 ml
5	Wash Buffer Concentrate	1 vial/5 ml
5a	Polysorbate 20	1 vial/3 ml
6	Mouse Anti-rabbit IgG Coated Plate	1 plate
7	96-Well Cover Sheet	1 cover
8	Ellman's Reagent	3 vials/100 dtn*
14	ELISA Tracer Dye	1 vial
15	ELISA Antiserum Dye	1 vial

*dtn = determinations

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Cayman Practice ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of "UltraPure" water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ("UltraPure"). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. *NOTE: UltraPure water is available for purchase (Item No. 400000).*

INTRODUCTION

About This Assay

This assay has been developed for researchers that do not have experience performing enzyme immunoassays (ELISAs). It can be used as a practice tool allowing the user to become comfortable with running Cayman ELISAs. Practicing these assays can help decrease errors when samples, time, and costs are at risk.

This kit contains enough reagents to run at least four complete standard curves, including blank wells, NSB wells, and maximum binding (B_0) wells. For more information regarding the development and science behind the manufacturing of ELISA's please contact our technical service department.

Description of AChE Competitive ELISAs[1-3]

Competitive enzyme immunoassays are based on the competition between an unlabeled analyte and an enzyme-labeled analyte. In this particular assay, the enzyme labeled to the analyte is acetylcholinesterase (AChE tracer). These analytes compete for a limited number of analyte-specific rabbit antiserum binding sites. Because the concentration of the tracer is held constant while the concentration of free analyte varies, the amount of tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free analyte in the well. This rabbit antiserum-analyte (free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate for AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount of free analyte present in the well during the incubation; or

$$\text{Absorbance} \propto \text{[Bound Analyte Tracer]} \propto 1/\text{[Analyte]}$$

A schematic of this process is shown in Figure 1 on page 7.

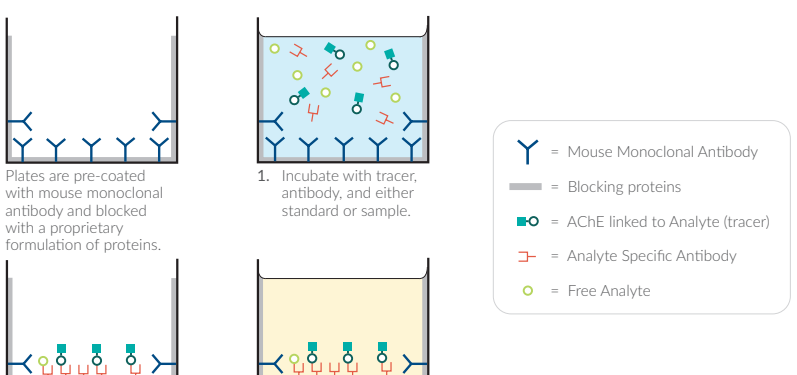


Figure 1. Schematic of the AChE ELISA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *E. electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in AChE enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, on page 9). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a "stop" reagent, and the plate may be read whenever it is convenient.

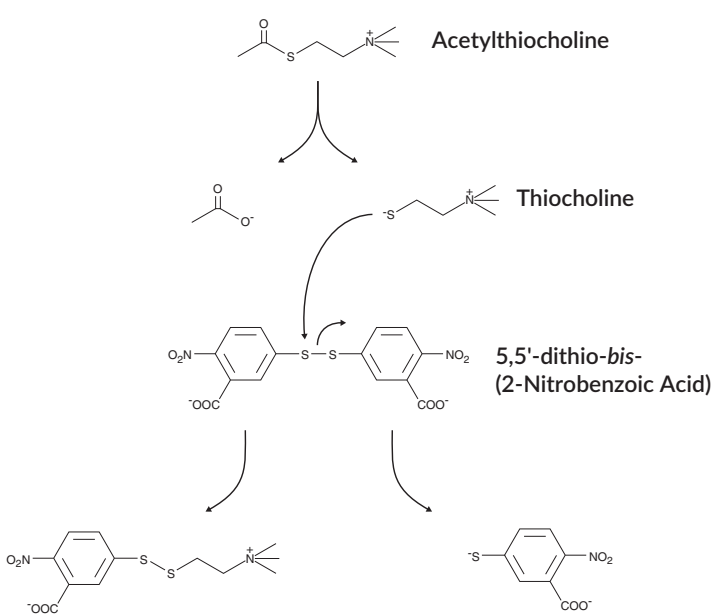


Figure 2. Reaction catalyzed by acetylcholinesterase

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the NSB wells.

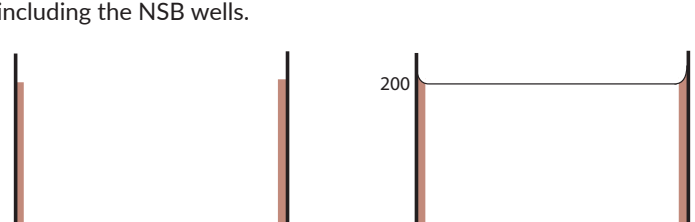


Figure 3. Blank Well

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

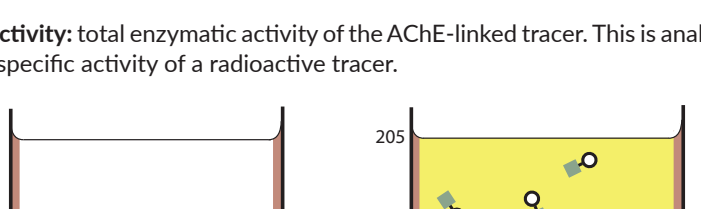


Figure 4. Total Activity Well

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding. Do not forget to subtract the Blank absorbance values.

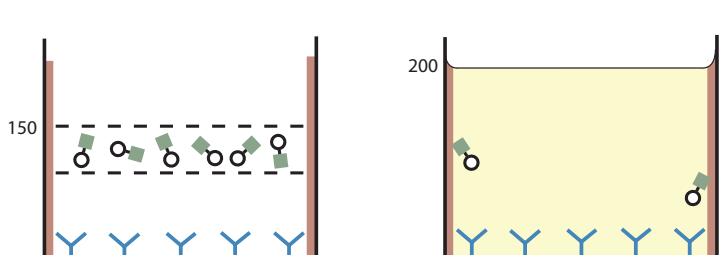


Figure 5. Non-Specific Binding Well

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

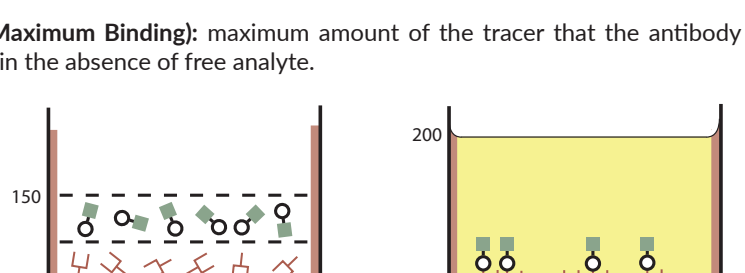


Figure 6. Maximum Binding (B_0) Well

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

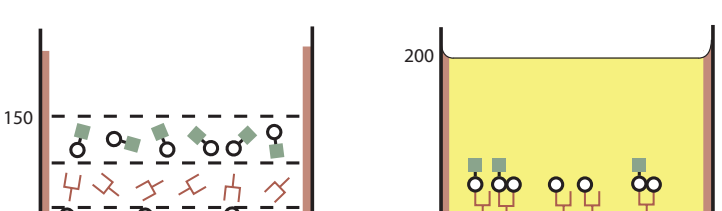


Figure 7. Standard Curve Well

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ("UltraPure"). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **ELISA Buffer Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (vial #4) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit; vial #5): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (vial #5a).

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Practice ELISA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the Practice ELISA Standard (vial #3) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 10 ng/ml. It will be stable for at least six weeks.

NOTE: If assaying culture medium samples that have not been diluted with ELISA Buffer, culture medium should be used in place of ELISA Buffer for dilution of the standard curve.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 500 µl ELISA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. The diluted standards may be stored at 4°C for no more than 24 hours.

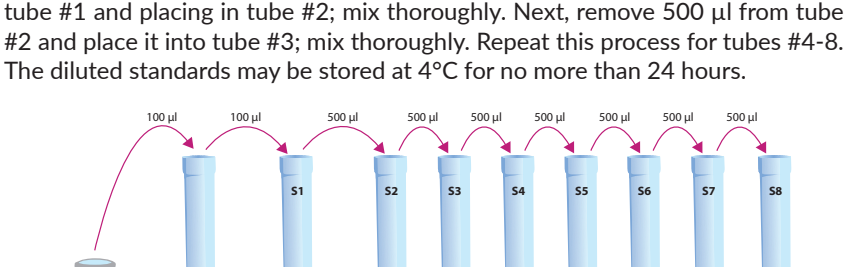


Figure 8. Preparation of the ELISA standards

Practice AChE Tracer

Reconstitute the Practice AChE Tracer as follows:

100 dtn Practice AChE Tracer (96-well kit; vial #2): Reconstitute with 6 ml ELISA Buffer.

Store the reconstituted Practice AChE Tracer at 4°C *(do not freeze!)* and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

Practice ELISA Antiserum

Reconstitute the Practice ELISA Antiserum as follows:

100 dtn Practice ELISA Antiserum (96-well kit; vial #1): Reconstitute with 6 ml ELISA Buffer.

Store the reconstituted Practice ELISA Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of antiserum has been included to account for any incidental losses.

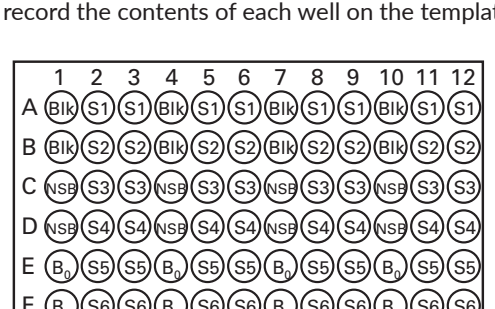
Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum).

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

A suggested plate format is shown in Figure 9, below. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 20, for more details). We suggest you record the contents of each well on the template sheet provided (see page 26).



Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8

Figure 9. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **ELISA Buffer**

   Add 100 µl ELISA Buffer to NSB wells. Add 50 µl ELISA Buffer to B_0 wells.

2. **Practice ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Practice AChE Tracer**

   Add 50 µl to each well *except* the TA and the Blk wells.

4. **Practice ELISA Antiserum**

   Add 50 µl to each well *except* the TA, the NSB, and the Blk wells.

Well	ELISA Buffer	Practice Standard	AChE Practice Tracer	Practice Antiserum
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with 96-well cover sheet (item #7) and incubate for two hours at room temperature.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; vial #8): Reconstitute with 20 ml of UltraPure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays be run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well
4. Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's reagent from splashing on the cover. *NOTE: Any loss of Ellman's reagent will affect the absorbance readings. If Ellman's reagent is present on the cover, use a pipette to transfer the Ellman's reagent into the well. If too much Ellman's reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's reagent.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either %B/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

$$\%\ B/B_0 = \left[\frac{(\text{Standard Absorbance - NSB})}{(\text{Average } B_0 \text{ Absorbance}) - \text{NSB}}\right] \times 100$$

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B_0 divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the ***Sample Data*** *(see page 22). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 24 for* ***Troubleshooting****).*

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* analyte concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\textbf{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Performance Characteristics

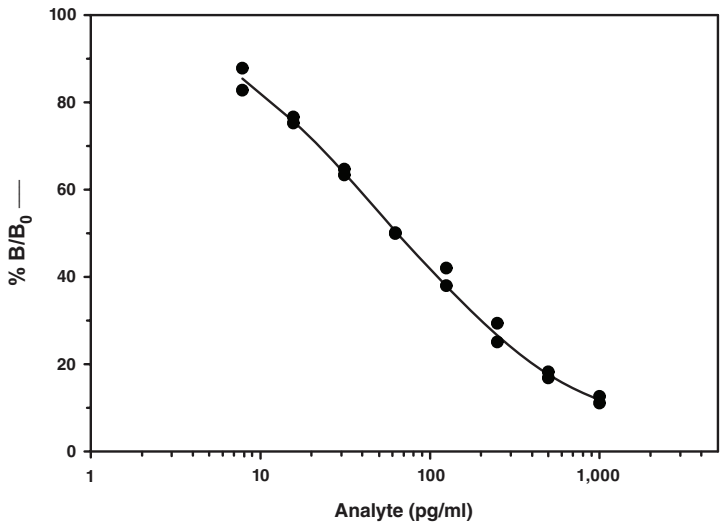
Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the value of your samples. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	1.009	1.174	1.091	
NSB	0	0	0	
B_0	0.514	0.532		
	0.498	0.536	0.520	0.520

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
1,000	0.065	0.057	0.065	0.057	12.5	11.0
500	0.094	0.087	0.094	0.087	18.1	16.7
250	0.152	0.130	0.152	0.130	29.2	25.0
125	0.197	0.189	0.197	0.189	38.0	36.3
62.5	0.260	0.259	0.260	0.259	50.0	49.8
31.3	0.329	0.336	0.329	0.336	63.3	65.0
15.6	0.391	0.398	0.391	0.398	75.2	76.5
7.8	0.430	0.456	0.430	0.456	82.7	87.7

Table 2. Typical results



Assay Range = 7.8-1,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 11 pg/ml
Mid-point (defined as 50% B/B_0) = 50-120 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer.

Figure 10. Typical standard curve

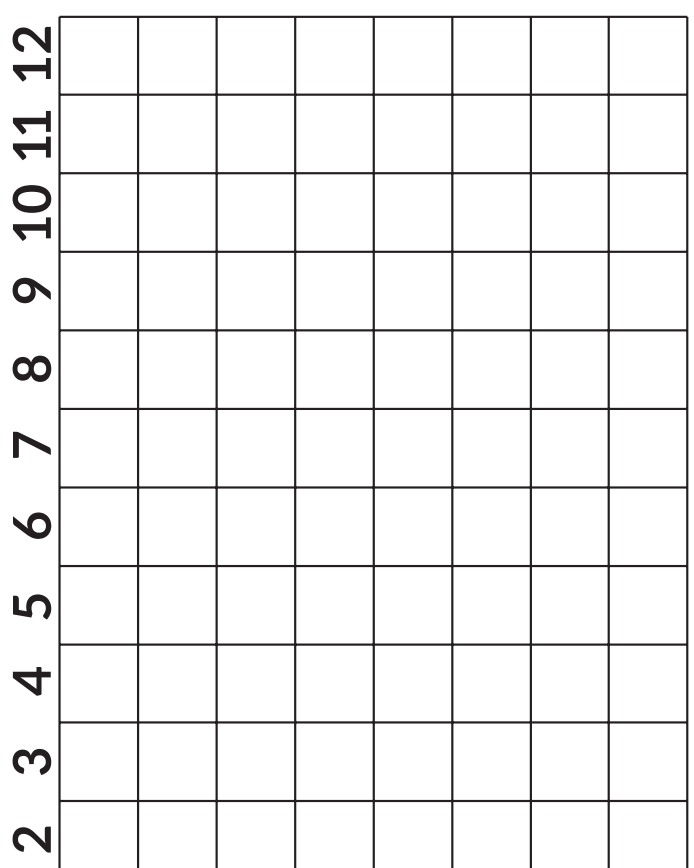
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Rewash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[1]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

References

1. Maxey, K.M., Maddipati, K.R., and Birkmeier, J. Interference in enzyme immunoassays. *J. Clin. Immunoassay* **15**, 116-120 (1992).
2. Pradelles, P., Grassi, J. and Maclouf, J. Enzyme immunoassays of eicosanoids using acetylcholinesterase as label: An alternative to radioimmunoassay. *Anal. Chem.* **57**, 1170-1173 (1985).
3. Maclouf, J., Grassi, J., and Pradelles, P. Development of enzyme-immunoassay techniques for the measurement of eicosanoids, Chapter 5, *in* Prostaglandin and Lipid Metabolism in Radiation Injury. Walden, T.L., Jr. and Hughes, H.N., editors, Plenum Press, Rockville, 355-364 (1987).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.