DNA Methylation ELISA Kit

Item No. 589324

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage Temperature
489332	5-Methyl-2'-deoxycytidine ELISA Monoclonal Antibody	1 vial/100 dtn	-20°C
489330	5-Methyl-2'-deoxycytidine AChE Tracer	1 vial/100 dtn	-20°C
489334	5-Methyl-2'-deoxycytidine ELISA Standard	1 vial	-20°C
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml	RT
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400008/400009	Goat Anti-Mouse IgG Coated Plate	1 plate	4°C
400012	96-Well Cover Sheet	1 cover	RT
400050	Ellman's Reagent	3 vials/100 dtn	-20°C
400040	ELISA Tracer Dye	1 vial	RT
400042	ELISA Antiserum Dye	1 vial	RT

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 DNA Methylation 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, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 14).

INTRODUCTION

Background

DNA methylation is an important epigenetic process regulating gene expression. Methylation occurs on carbon 5 of 2'-deoxycytidine yielding the modified base 5-methyl-2'-deoxycytidine. The methylation pattern of cells is tightly regulated during development with the methylation profile being transmitted from parent to daughter cells during cell division.[1] Methylation results in long-term silencing of genes, while unmethylated regions of DNA can be actively transcribed.[1,2]

One region of the genome of particular interest in regard to methylation is CpG islands. CpG islands are regions approximately 200 bp to several thousand bp in length which often span the promoter and first few exons of many housekeeping and tumor suppressor genes. These regions remain essentially unmethylated throughout development.[1] As the name implies, the CpG dinucleotide is overrepresented in CpG islands, with the frequency being approximately five times greater in CpG islands than in the remainder of the genome.[1]

It is well established that alterations in DNA methylation are a common feature of cancer.[3] In addition to global genomic hypomethylation, there are also discrete areas of dense hypermethylation particularly in the normally unmethylated CpG islands.[3] Because many tumor suppressor genes contain CpG islands, these genes are among those silenced by hypermethylation. The first report of methylation of a tumor suppressor CpG island was Retinoblastoma (Rb) which was discovered in 1989.[4] Interestingly, the pattern and level of gene hypermethylation for a given cancer is specific to that malignancy, with cancer of the GI tract tending to show a greater level of hypermethylation than do other cancers. In the digestive tract, methylation of genes, including the estrogen receptor, occurs as a normal part of the aging process.[5-7] During carcinogenesis, this age-related methylation may progress to hypermethylation.[5,6]

Global changes in methylation can be quantified by measuring plasma or urinary levels of 5-methyl-2'-deoxycytidine.[8-10] These changes in methylation can provide valuable information about cancer status of an individual. For example, patients with leukemia excrete significantly elevated levels of 5-methyl-2'-deoxycytidine compared to healthy individuals.[8] Global methylation within tissues can be measured in a similar manner, allowing study of tissue-specific changes that occur as a result of differentiation, aging, or carcinogenesis.[11]

DNA methylation is regulated by a family of DNA methyltransferases (DNMTs), with DNMT1, DNMT3a, and DNMT3b all implicated in carcinogenesis.[12] Three cytosine nucleoside analogs (azacitidine, decitabine, and zebularine) which incorporate into DNA during synthesis are being actively investigated as anti-cancer drugs.[13] DNMTs bind irreversibly to these cytidine analogs, resulting in suppression of methylation and the possibility that genes which had been inappropriately methylated may resume their normal function.[13]

About This Assay

Cayman's DNA Methylation ELISA Kit is a competitive assay that can be used for the quantification of 5-methyl-2'-deoxycytidine in urine, culture supernatants, plasma, and other sample matrices. The assay has a range from 1.17-150 ng/ml and a sensitivity (80% B/B_0) of approximately 3 ng/ml.

Description of AChE Competitive ELISAs[14,15]

This assay is based on the competition between 5-methyl-2'-deoxycytidine and a 5-methyl-2'-deoxycytidine-acetylcholinesterase (AChE) conjugate (5-Methyl-2'-deoxycytidine Tracer) for a limited amount of 5-Methyl-2'-deoxycytidine ELISA Monoclonal Antibody. Because the concentration of the 5-Methyl-2'-deoxycytidine Tracer is held constant while the concentration of 5-methyl-2'-deoxycytidine varies, the amount of 5-Methyl-2'-deoxycytidine Tracer that is able to bind to the 5-Methyl-2'-deoxycytidine ELISA Monoclonal Antibody will be inversely proportional to the concentration of 5-methyl-2'-deoxycytidine in the well. This antibody-5-methyl-2'-deoxycytidine complex binds to goat polyclonal anti-mouse 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 to 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 5-Methyl-2'-deoxycytidine Tracer bound to the well, which is inversely proportional to the amount of free 5-methyl-2'-deoxycytidine present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound 5-Methyl-2'-deoxycytidine Tracer}] \propto 1/[\text{5-Methyl-2'-deoxycytidine}]$$

A schematic of this process is shown in Figure 1, on page 9.

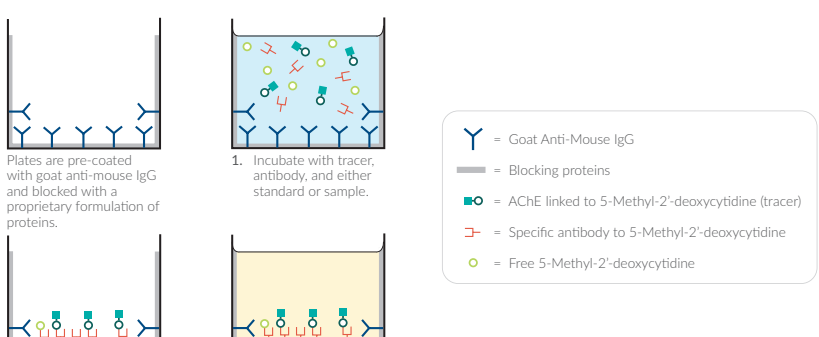

Figure 1. Schematic of the AChE ELISA

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 11). 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.

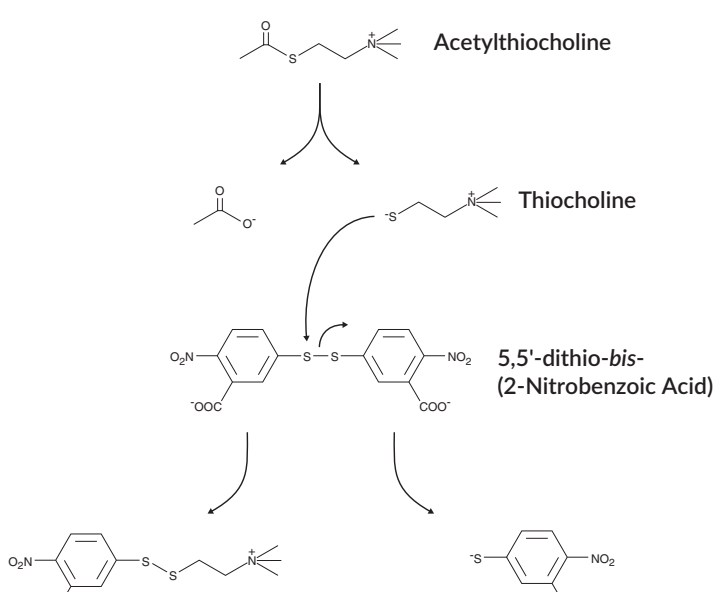

Figure 2. Reaction catalyzed by acetylcholinesterase

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 NSB wells.

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

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.

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

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the 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.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% B/B}_0 \text{ value for the primary analyte}}{\text{50\% B/B}_0 \text{ value for the potential cross reactant}} \right] \times 100\%$$

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 dilluted 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) (Item No. 400060) 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**

   Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035).

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.

Sample Preparation

Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples that cannot be assayed immediately should be stored as indicated below.
- Samples of mouse origin may contain antibodies which interfere with the assay by binding to the goat anti-mouse plate. We recommend that all mouse samples be purified prior to use in this assay.

Urine

Urine samples should be stored at -20°C immediately after collection. Interference in urine is infrequent; dilutions appropriate for this assay (*i.e.*, dilutions falling between 20-80% B_0 (approximately 1:100-1:1,000)) show a direct linear correlation between 5-methyl-2'-deoxycytidine immunoreactivity and 5-methyl-2'-deoxycytidine concentration (see figure 3, on page 17). Urinary concentrations of 5-methyl-2'-deoxycytidine vary considerably. It is recommended that the values obtained from urine samples be standardized to creatinine levels using Cayman's Creatinine ELISA Kit (Item No. 502330), Creatinine (urinary) Colorimetric Assay Kit (Item No. 500701), or a similar assay.

Plasma/Serum

Collect plasma using established methods and store at -80°C. The concentration of free 5-methyl-2'-deoxycytidine in plasma is very low relative to the level of DNA-incorporated 5-methyl-2'-deoxycytidine. Glomerular filtration results in excretion of 5-methyl-2'-deoxycytidine into the urine, while the DNA-incorporated 5-methyl-2'-deoxycytidine remains in the blood. The differing fates of free *versus* DNA-incorporated 5-methyl-2'-deoxycytidine should be considered in experimental design. If you choose to measure DNA-incorporated 5-methyl-2'-deoxycytidine in plasma, it is possible to purify DNA using a commercially available kit and treat the DNA with a combination of nuclease and alkaline phosphatase to liberate the individual bases. Due to the complexities of measuring 5-methyl-2'-deoxycytidine in plasma, urine is often a more appropriate matrix.

Culture Medium Samples

Collect culture medium samples and store at -80°C. Fetal bovine serum contains 5-methyl-2'-deoxycytidine; therefore assays should either be performed in serum-free medium or PBS. If the 5-methyl-2'-deoxycytidine concentration is high enough to dilute the sample 10-fold with ELISA Buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted 1:10 with ELISA Buffer), dilute the standard curve in the same culture medium as that used for the experiment. This will ensure that the matrix for the standards is comparable to the samples. We recommend that a standard curve be run first to ensure that the assay will perform in a particular culture medium.

Cell Lysates

Collect lysates using established methods and store at -80°C until use. Purify DNA using a commercially available extraction kit. Digest DNA using nuclease P1 (Sigma N8630 or equivalent) following the manufacturer's instructions. Adjust pH to 7.5-8.5 using 1M Tris. Add 1 unit of alkaline phosphatase per 100 µg of DNA and incubate at 37°C for 30 minutes. Boil for 10 minutes and place on ice until use.

Tissue Samples

Snap-freeze tissue samples in liquid nitrogen immediately after collection. Store at -80°C until use. When ready to use the samples, thaw and add 5 ml of homogenization buffer (0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA) per gram of tissue. Homogenize the sample using either a Polytron-type homogenizer or a sonicator. Centrifuge at 1,000 x g for 10 minutes and purify the supernatant using a commercially available DNA extraction kit. Digest DNA using nuclease P1 (Sigma N8630 or equivalent) following the manufacturer's instructions. Adjust the pH to 7.5-8.5 using 1 M Tris. Add 1 unit of alkaline phosphatase per 100 µg of DNA and incubate at 37°C for 30 minutes. Boil for 10 minutes and place on ice until use.

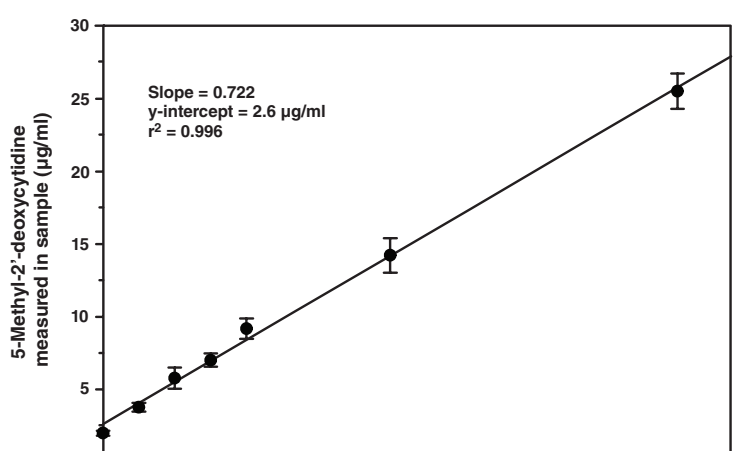

Figure 3. Recovery of 5-methyl-2'-deoxy-cytidine from urine
Urine samples were spiked with 5-methyl-2'-deoxycytidine, diluted as described in the **Sample Preparation** section and analyzed using the DNA Methylation ELISA Kit. The y-intercept corresponds to the amount of 5-methyl-2'-deoxycytidine in unspiked urine. Error bars represent standard deviations obtained from multiple dilutions of each sample.

Preparation of Assay-Specific Reagents

5-Methyl-2'-deoxycytidine ELISA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the 5-Methyl-2'-deoxycytidine ELISA Standard (Item No. 489334) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 1.5 µg/ml. Store this solution at 4°C; it will be stable for approximately 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 (1.5 µg/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.

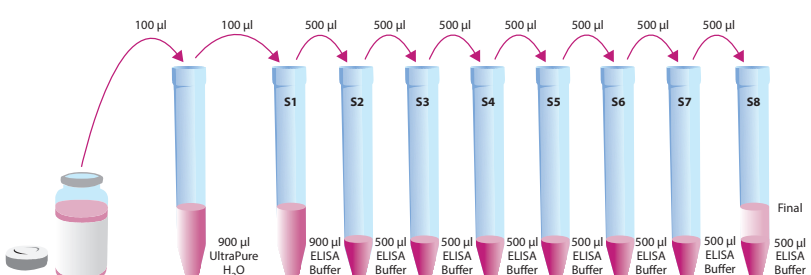

Figure 4. Preparation of the 5-Methyl-2'-deoxycytidine standards

5-Methyl-2'-deoxycytidine AChE Tracer

Reconstitute with 6 ml ELISA Buffer. Store the reconstituted 5-Methyl-2'-deoxycytidine 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). *NOTE: Do not store tracer with dye for more than 24 hours.*

5-Methyl-2'-deoxycytidine ELISA Monoclonal Antibody

Reconstitute with 6 ml ELISA Buffer. Store the reconstituted 5-Methyl-2'-deoxycytidine ELISA Monoclonal Antibody at 4°C. It will be stable for at least four weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). *NOTE: Do not store antibody with dye for more than 24 hours.*

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *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.*

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 26, for more details). We suggest you record the contents of each well on the template sheet provided (see page 34).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 5. 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. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl ELISA Buffer to NSB wells).

2. **5-Methyl-2'-deoxycytidine 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. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **5-Methyl-2'-deoxycytidine AChE Tracer**

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

5. **5-Methyl-2'-deoxycytidine ELISA Monoclonal Antibody**

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

Well	ELISA Buffer	Standard/Sample	Tracer	Antibody
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 plastic film (Item No. 400012) and incubate 18 hours at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent (Item No. 400050) immediately before use 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 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 well.
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 90-120 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 are in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, wash the plate, add fresh Ellman's Reagent and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plot 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.)

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 28). 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 32 for* ***Troubleshooting****).*

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* 5-methyl-2'-deoxy-cytidine 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.*

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

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

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.

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 values of your samples. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	1.459	1.568	1.514	
NSB	0.000	0.000	0.000	
B_0	1.048	1.128		
	1.062	0.991	1.057	1.057

Dose (ng/ml)	Raw Data		Corrected		%B/B_0	
150	0.057	0.053	0.057	0.053	5.4	5.0
75	0.100	0.109	0.100	0.109	9.4	10.3
37.5	0.195	0.193	0.195	0.193	18.4	18.3
18.75	0.320	0.322	0.320	0.322	30.3	30.5
9.38	0.496	0.483	0.496	0.483	46.9	45.7
4.69	0.659	0.696	0.659	0.696	62.4	65.8
2.34	0.762	0.785	0.762	0.785	71.1	74.2
1.17	0.878	0.901	0.878	0.901	83.1	85.2

Table 2. Typical results

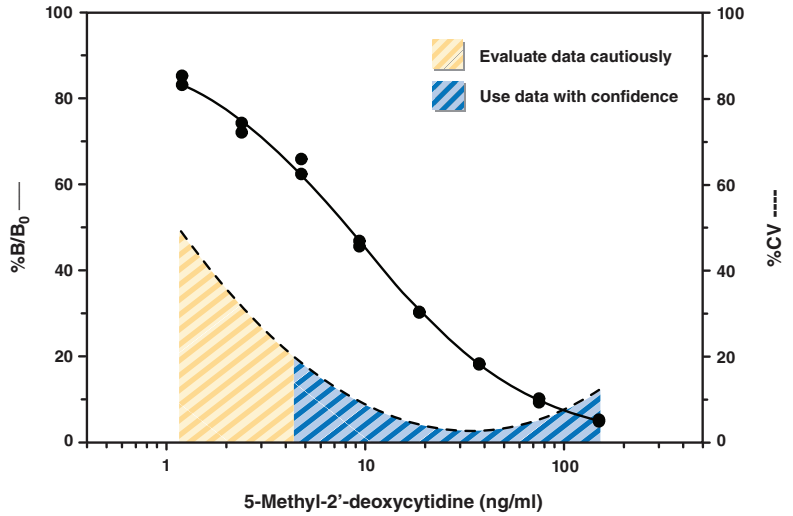

Figure 7. Typical standard curve

Precision:

The intra- and inter-assay CVs have been determined at multiple points on the standard curve. These data are summarized in the graph on page 29 and in the table below.

Dose (ng/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
150	9.1	13.8
75	7.6	5.0
37.5	5.2	4.2
18.75	6.6	7.5
9.38	8.2	14.7
4.69	13.2	21.8
2.34	†	†
1.17	†	†

Table 3. Intra- and inter-assay variation

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

†Outside of the recommended usable range of the assay.

Cross Reactivity:

Compound	Cross Reactivity
5-Methyl-2'-deoxycytidine	100%
5-Methylcytidine	20%
2'-Deoxycytidine	0.1%
Cytidine	0.1%
Thymidine	<0.01%

Table 4. Cross Reactivity of the 5-Methyl-2'-deoxycytidine ELISA

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[16]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

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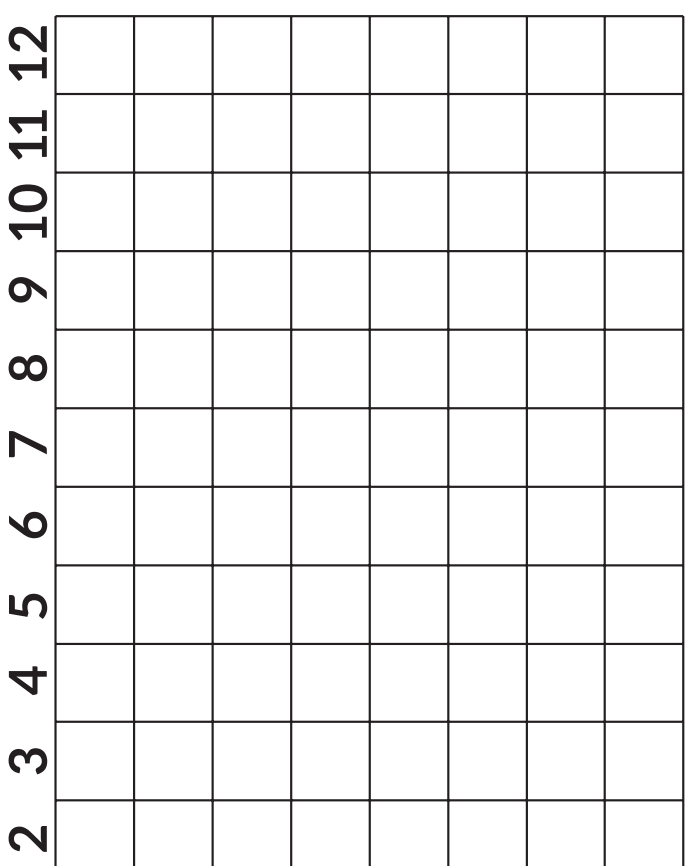

NOTES

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