DNA Methylation EIA Kit

Cat. No.:DEIA5038
Pkg.Size:96T

**Intended use**

CD’s DNA Methylation EIA Kit is a competitive assay that can be used for the quantification of 5-methyl-2-deoxy cytidine in urine, culture supernatants, plasma, and other sample matrices. The EIA typically displays IC50 (50% B/B0) and IC80 (80% B/B0) values of approximately 12 and 3 ng/ml, respectively.

**General Description**

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

It is well established that alterations in DNA methylation are a common feature of cancer. In addition to global genomic hypomethylation, there are also discrete areas of dense hypermethylation particularly in the normally unmethylated CpG islands. 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. 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. During carcinogenesis, this age-related methylation may progress to hypermethylation.

Global changes in methylation can be quantified by measuring plasma or urinary levels of 5-methyl-2-deoxy cytidine. 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-deoxy cytidine compared to healthy individuals. 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.

DNA methylation is regulated by a family of DNA methyltransferases (DNMTs), with DNMT1, DNMT3a, and DNMT3b all implicated in carcinogenesis. Three cytosine nucleoside analogs (azacitidine, decitabine, and zebularine) which incorporate into DNA during synthesis are being actively investigated as anti-cancer drugs. 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.

**Principle Of The Test**

This assay is based on the competition between 5-methyl-2-deoxy cytidine and a 5-methyl-2-deoxy cytidine-acetylcholinesterase (AChE) conjugate (5-methyl-2-deoxy Cytidine Tracer) for a limited amount of 5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody. Because the concentration of the 5-methyl-2-deoxy Cytidine Tracer is held constant while the concentration of 5-methyl-2-deoxy cytidine varies, the amount of 5-methyl-2-deoxy Cytidine Tracer that is able to bind to the 5-methyl-2-deoxy
Cytidine EIA Monoclonal Antibody will be inversely proportional to the concentration of 5-methyl-2-deoxy cytidine in the well. This antibody-5-methyl-2-deoxy cytidine 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-deoxy Cytidine Tracer bound to the well, which is inversely proportional to the amount of free 5-methyl-2-deoxy cytidine present in the well during the incubation; or

\[ \text{Absorbance} \propto \frac{[\text{Bound 5-methyl-2-deoxy Cytidine Tracer}]}{[5-\text{methyl-2-deoxy Cytidine}]} \]

### Reagents And Materials Provided

1. 5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody, 1 vial/100 dtn
2. 5-methyl-2-deoxy Cytidine AchETracer, 1 vial/100 dtn
3. 5-methyl-2-deoxy Cytidine EIA Standard, 1 vial
4. EIA Buffer Concentrate (10X), 2 vials/10 ml
5. Wash Buffer Concentrate (400X), 1 vial/5 ml
6. Tween 20, 1 vial/3 ml
7. Goat Anti-Mouse IgG Coated Plate, 1 plate
8. 96-Well Cover Sheet, 1 cover
9. Ellman’s Reagent, 3 vials/100 dtn
10. EIATracer Dye, 1 vial
11. EIA Antiserum Dye, 1 vial

### Materials Required But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeat pipettor.
3. A source of 'UltraPure' water. Water used to prepare all EIA 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 EIA.
4. Materials used for Sample Preparation

### Storage

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.

### Specimen Collection And Handling

#### 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

1. All samples must be free of organic solvents prior to assay.
2. Samples that cannot be assayed immediately should be stored as indicated below.
3. 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% B0 (approximately 1:100-1:1,000)) show a direct linear correlation between 5-methyl-2-deoxy cytidine immunoreactivity and 5-methyl-2-deoxy cytidine concentration. Urinary concentrations of 5-methyl-2-deoxy cytidine vary considerably and, as with any urinary marker, we recommend standardizing the values obtained by EIA to creatinine levels. CD sells a Creatinine Assay Kit which may be used for this purpose.

**Plasma/Serum**
Collect plasma using established methods and store at -80°C. The concentration of free 5-methyl-2-deoxy cytidine in plasma is very low relative to the level of DNA-incorporated 5-methyl-2-deoxy cytidine. Glomerular filtration results in excretion of 5-methyl-2-deoxy cytidine into the urine, while the DNA-incorporated 5-methyl-2-deoxy cytidine remains in the blood. The differing fates of free versus DNA-incorporated 5-methyl-2-deoxy cytidine should be considered in experimental design. If you choose to measure DNA-incorporated 5-methyl-2-deoxy cytidine 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-deoxy cytidine 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-deoxy cytidine; therefore assays should either be performed in serum-free medium or PBS. If the 5-methyl-2-deoxy cytidine concentration is high enough to dilute the sample 10-fold with EIA Buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted 1:10 with EIA 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.

**Plate Preparation**
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 (B0), 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.

**Reagent Preparation**
NOTE: Water used to prepare all EIA 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 EIA. UltraPure water may be purchased from CD.

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

1. **EIA Buffer Preparation**

   Dilute the contents of one vial of EIA Buffer Concentrate (10X) 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): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Tween 20. Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Tween 20 (0.5 ml/liter of Wash Buffer).

   NOTE: Tween 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.

### Preparation of Assay-Specific Reagents

#### 5-methyl-2-deoxy Cytidine EIA 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-deoxy Cytidine EIA Standard 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 EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve.

   To prepare the standard for use in EIA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl EIA Buffer to tube #1 and 500 µl EIA 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.

#### 5-methyl-2-deoxy Cytidine AChE Tracer

   Reconstitute the 5-methyl-2-deoxy Cytidine AChE Tracer as follows: 100 dtn 5-methyl-2-deoxy Cytidine AChE Tracer: Reconstitute with 6 ml EIA Buffer.

   Store the reconstituted 5-methyl-2-deoxy Cytidine 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).

   NOTE: Do not store tracer with dye for more than 24 hours.

#### 5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody

   Reconstitute the 5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody as follows: 100 dtn 5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody: Reconstitute with 6 ml EIA Buffer.

   Store the reconstituted 5-methyl-2-deoxy Cytidine EIA 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 or add 300 µl of dye to 30 ml of antibody).

   NOTE: Do not store antibody with dye for more than 24 hours.

### Assay Steps

#### 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.
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.

B0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B0) well.

Standard Curve: a plot of the %B/B0 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.

**Pipetting Hints**
1. Use different tips to pipette each reagent.
2. 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).
3. Do not expose the pipette tip to the reagent(s) already in the well.

**Addition of the Reagents**
1. **EIA Buffer**
   Add 100 µl EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl EIA Buffer to Maximum Binding (B0) wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for EIA Buffer in the NSB and B0 wells (i.e., add 50 µl culture medium to NSB and B0 wells and 50 µl EIA Buffer to NSB wells).
2. **5-methyl-2-deoxy Cytidine EIA 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-deoxy Cytidine AChE Tracer**
   Add 50 µl to each well except the Total Activity (TA) and the Blank (Blk) wells.
5. **5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody**
   Add 50 µl to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

**Incubation of the Plate**
Cover each plate with plastic film and incubate 18 hours at 4°C.

**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: 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 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 Total Activity 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., B0 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 B0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B0 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.

**Calculation**

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/B0 versus log concentration using a four-parameter logistic fit or as logit B/B0 versus log concentration using a linear fit.

**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 B0 wells.
3. Subtract the NSB average from the B0 average. This is the corrected B0 or corrected maximum binding.
4. Calculate the B/B0 (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 B0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B0 for a logistic four-parameter fit, multiply these values by 100.)

**NOTE:** The total activity (TA) values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B0 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. Erratic absorbance values and a low (or no)% Bound could indicate the presence of organic solvents in the buffer or other technical problems.

**Plot the Standard Curve**

Plot %B/B0 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 linearized using a logit transformation. The equation for this conversion is shown below. **NOTE:** Do not use %B/B0 in this calculation.

\[
\text{logit} \left( \frac{B}{B_0} \right) = \ln \left[ \frac{B}{B_0} \left( 1 - \frac{B}{B_0} \right) \right]
\]

Plot the data as logit (B/B0) versus log concentrations and perform a linear regression fit.

**Determine the Sample Concentration**

Calculate the B/B0 (or %B/B0) 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/B0 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.

**Typical Standard Curve**

**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.

<table>
<thead>
<tr>
<th>Dose (ng/ml)</th>
<th>Raw Data</th>
<th>Corrected</th>
<th>%B/B₀</th>
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<tbody>
<tr>
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<td>0.053</td>
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<td>37.5</td>
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<td>0.483</td>
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<td>1.17</td>
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Typical results
Specificity

For Specificity data see table below.
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<th>Compound</th>
<th>Cross-reactivity</th>
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<tr>
<td>5-methyl-2-deoxy Cytidine</td>
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<td>20%</td>
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<tr>
<td>2-deoxy Cytidine</td>
<td>0.1%</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.1%</td>
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<tr>
<td>Thymidine</td>
<td>&lt;0.01%</td>
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**Specificity of the 5-methyl-2-deoxy Cytidine EIA Monoclonal Antibody**

**Reproducibility**

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

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

†Outside of the recommended usable range of the assay.

<table>
<thead>
<tr>
<th>Dose (ng/ml)</th>
<th>%CV* Intra-assay variation</th>
<th>%CV* Inter-assay variation</th>
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**Intra- and inter-assay variation**

**REFERENCES**
