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ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

5-mC DNA ELISA Kit

Catalog Nos. **D5325** & **D5326**

Highlights

- For high-throughput, detection of global 5-methylcytosine (5-mC) in DNA.
- The streamlined workflow can be completed in less than 3 hours.

Contents

Product Contents 1
Specifications.....1
Product Description.....2
Experimental Considerations 3
Buffer Storage.....3
Protocol.....4
Appendix.....5
Ordering Information 6
Related Products 7

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Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888-882-9682.

Product Contents

5-mC DNA ELISA Kit (Kit Size)	D5325 (1 x 96 wells)	D5326 (2 x 96 wells)	Storage Temperature
5-mC Coating Buffer	15 ml	30 ml	4 °C
5-mC ELISA Buffer	250 ml	250 ml x 2	4 °C
Anti-5-Methylcytosine (0.5 µg/µl)	15 µl	30 µl	-20 °C
Secondary Antibody (1 µg/µl)	15 µl	30 µl	-20 °C
HRP Developer	15 ml	30 ml	4 °C
Negative Control (100 ng/µl)	50 µl	50 µl	- 20 °C
Positive Control (100 ng/µl)	50 µl	50 µl	- 20 °C
96-well plate (12 x 8-well Strips)	1 plate	2 plates	Room Temp.
Protocol	1	1	-

Note - Integrity of kit components is guaranteed for up to up to six (6) months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

Specifications

Sample Sources – Purified genomic DNA, plasmid DNA, PCR amplification products, or DNA fragments in water, Tris-EDTA, or similar.

DNA Quantity – This protocol is optimized for 100 ng input DNA/well. Compatible with DNA in the range of 10-200 ng.

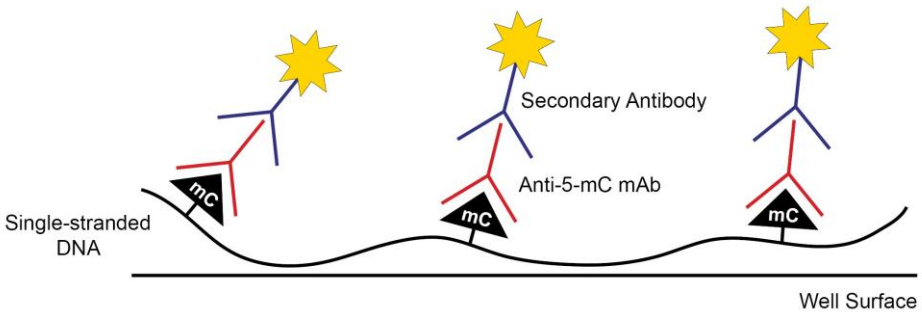
Detection – ≥ 0.5% 5-methylcytosine (5-mC) per 100 ng single-stranded DNA.

Equipment Required – Incubator and ELISA plate reader. A multi-channel pipettor is recommended. An automated plate washer may be used for blocking and wash steps.

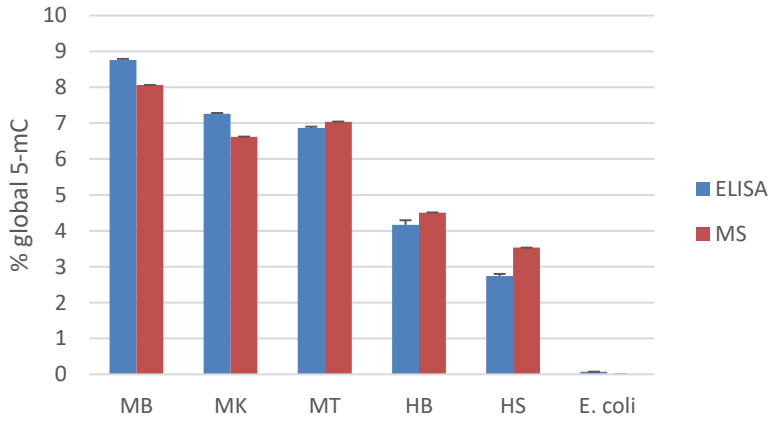
Product Description

The ability to efficiently detect and quantify DNA methylation (i.e., 5-methylcytosine) has become essential for epigenetic-based research. To date, a number of methods have been developed for this purpose including high-performance capillary electrophoresis, bisulfite sequencing, and methylated DNA immunoprecipitation.

The **5-mC DNA ELISA Kit** is a convenient and powerful tool that allows the researcher to accurately quantitate 5-mC in *any* DNA sample in less than 3 hours. The kit features a unique **Anti-5-Methylcytosine** monoclonal antibody that is both sensitive and specific for 5-mC. The assay is compatible with a wide range of input DNA from vertebrate, plant, and microbial sources as well as PCR amplicons and fragmented DNA. Percent 5-mC in a DNA sample can be accurately quantified from a standard curve generated with specially designed controls included with the kit. Also, the fast, streamlined workflow is ideal for high-throughput analyses.



The 5-mC DNA ELISA Kit utilizes the indirect ELISA technique in its workflow. Denatured, single-stranded DNA samples are coated on the well surfaces in **5-mC Coating Buffer**. **Anti-5-Methylcytosine** monoclonal antibody (Anti-5-mC mAb) and the HRP-conjugated **Secondary Antibody** are prepared in **5-mC ELISA Buffer** and added to the wells. Detection of 5-mC occurs after addition of the **HRP Developer**.



The 5-mC DNA ELISA Kit can quantify 5-mC in numerous DNA samples with close correlation to LC-MS. 100 ng of genomic DNA from mouse brain (MB), mouse kidney (MK), mouse thymus (MT), human brain (HB), human spleen (HS), and E. coli ER2925 were used to coat wells, in triplicate. Percent 5-mC was calculated using the logarithmic equation of the line from the standard curve that was constructed with the **Negative Control** and the **Positive Control** (see Appendix, page 5). The percent 5-mC calculated in DNA samples using the 5-mC DNA ELISA Kit (ELISA) strongly correlates to mass spectrometry (MS) data of 5-mC found in the respective gDNA sample

For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

Experimental Considerations

- All DNA *must* be denatured (single-stranded) for use with the kit (refer to DNA Coating steps on page 4). The protocol is optimized for the detection of 5-mC in 100 ng of double stranded DNA per well that has been denatured. All samples should be assayed in duplicate (meaning a total of 200 ng DNA/sample will be used with this assay). However, depending on your experimental design, 10 to 200 ng of sample DNA can be used in the assay.
  Note: When using inputs other than 100 ng per well, the amount of control DNA used must be adjusted to equal the amount of sample used. This will ensure accurate % 5-mC quantification.

- The **Negative** and **Positive Controls** consist of double stranded DNA at a concentration of 100 ng/µl and can used for the detection/quantification of 5-mC in DNA. For 5-mC detection, both controls should be assayed. For 5-mC quantification, the **Negative Control** should be mixed with the **Positive Control** at different ratios to construct a standard curve (see Appendix, page 5). All standards should be assayed in duplicate.

- **Secondary Antibody** is a horseradish peroxidase (HRP) conjugate and supplied at a concentration of 1 µg/µl. Avoid freeze/thaw cycles; if necessary make aliquots of the antibody and keep at -20°C for long term. Store thawed antibody at 4°C for short periods of time (~1 week).

Buffer Storage

- ✓ **5-mC Coating Buffer** is stable at room temperature or 4 °C for extended periods of time.

- ✓ **5-mC ELISA Buffer** should be storage at 4°C and used within 6 months. Alternatively, the buffer may be dispensed into multiple aliquots and kept at -20°C for long term storage. Avoid repeated freeze/thaw cycles.

- ✓ **HRP Developer** must be stored at 4°C and used within 6 months. Do not freeze. For more rapid color development, bring to room temperature before adding to the wells.

Protocol

This protocol is optimized for 100 ng of DNA per well.
***Duplicate samples** are recommended for accurate 5-mC detection and quantification.*

DNA Coating:

1. Remove the necessary number of well strips[1] to assay DNA samples and controls[2].
2. Add 100 ng of each DNA[3] to a PCR tube and bring the final volume to 100 µl with **5-mC Coating Buffer**.

   Example: If the DNA concentration is 20 ng/µl, add 5 µl of DNA to 95 µl of **5-mC Coating Buffer** for a final volume of 100 µl.
3. Denature the DNA at 98°C for 5 minutes in a thermal cycler. After denaturation, transfer immediately to ice for 10 minutes.
4. Add the entire volume (100 µl) denatured DNAs to the wells of the plate, cover with foil, and incubate at 37 °C for 1 hour.

Blocking:

1. Discard the buffer from the wells[4].
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer**. *Discard the buffer after each wash.*
3. Add 200 µl of **5-mC ELISA Buffer** to each well. Cover the plate with foil and incubate at 37 °C for 30 minutes.

Antibody Addition:

1. Discard buffer from the wells.
2. Prepare an antibody mix[5] consisting of **Anti-5-Methylcytosine** and **Secondary Antibody** in **5-mC ELISA Buffer** according to the following table:

	Dilution	Volume (µl)	Example (18 wells)
5-mC ELISA Buffer	N/A	(# wells + 2) 100	2,000 µl
Anti-5-Methylcytosine	1:2,000	Buffer Vol. / 2,000	1 µl
Secondary Antibody	1:1,000	Buffer Vol. / 1,000	2 µl

3. Add 100 µl of this antibody mix to each well. Cover the plate with foil and incubate at 37°C for 1 hour.

Color Development:

1. Discard the antibody mix from the wells.
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer**.
3. Add 100 µl of **HRP Developer** to each well. Allow color to develop for 10-60 minutes[6] at room temperature.
4. Measure absorbance at 405-450 nm using an ELISA plate reader.

Notes:

[1] The well strips should be stored in a clean, dry, dark place for later use.

[2] For more information regarding 5-mC detection and quantification using the **Negative** and **Positive Controls**, refer to the Appendix, page 5.

[3] Make sure that the volume of the DNA added to the **5-mC Coating Buffer** does not exceed 20% of the final volume.

[4] Tap out any remaining buffer onto a paper towel after emptying a well.

[5] The antibody mix can be prepared during the blocking step and kept on ice until it is needed.

[6] The development time will depend on the temperature of the **HRP Developer** (see p. 3). Development time may vary according to experimental design as well.

Notes:

[1] The **Negative** and **Positive Controls** must be included on the same plate as the DNA samples for each assay.

[2] A new standard curve should be generated for each assay.

[3] The number of standard curve mixtures for 5-mC quantification can vary. In the example given in the table, seven mixtures were prepared. Leftover mixtures can be frozen at or below -20 ºC for future use.

Appendix - Analysis with Negative and Positive Control DNAs

For 5-mC Detection:

The presence or absence of 5-mC can be determined by comparing the absorbance of samples to **Negative** (0% methylation) and **Positive** (100% methylation) **Controls**[1].

For 5-mC Quantification:

To quantify the percentage of 5-mC in a DNA sample, a standard curve[2] must be generated. This is done by preparing mixtures[3] of the **Negative Control (100 ng/µl)** and **Positive Control (100 ng/µl)** to generate standards of known 5-mC percentage (see table below). These must be prepared *prior to denaturation* and assayed in parallel with the samples. Add 1 µl (i.e., 100 ng) of each mixture to a PCR tube and bring the final volume to 100 µl with **5-mC Coating Buffer**. Proceed with Coating Step 3 of the protocol (p. 4).

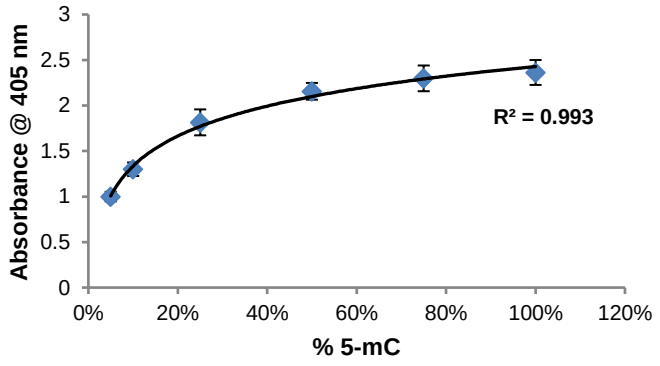
% 5-mC	Negative Control (100 ng/µl)	Positive Control (100 ng/µl)
0%	10.0 µl	0 µl
5%	9.5 µl	0.5 µl
10%	9.0 µl	1.0 µl
25%	7.5 µl	2.5 µl
50%	5.0 µl	5.0 µl
75%	2.5 µl	7.5 µl
100%	0 µl	10.0 µl

Table highlights the preparation of seven mixtures using the **Negative Control** and **Positive Control** to be used to generate a standard curve. Total volume of each is 10 µl at a concentration of 100 ng/µl.

The absorbance for each mixture must be plotted as a function of Absorbance @ 405 nm (Y-axis) vs. % 5-mC (X-axis). Using the equation below, derived from the logarithmic second-order regression, determine the 5-mC percentage for DNA samples (unknowns) based on their absorbance.

Note: The **Positive** and **Negative Control DNAs** consist of *Escherichia coli* gDNA. The **Positive Control DNA** has been treated with CpG Methylase (Catalog # E2010/11). The density of CpG dinucleotides varies between species and to quantitate the %5-mC simply multiply the calculated %5-mC by the fold difference in CpG density from *E. coli* and the sample species. For example, *E.coli* CpG sites/genome length is 0.075 and mouse CpG sites/genome length is 0.0081, therefore, the fold difference between *E. coli* and mouse CpG density is 9.22.

$$\%\ 5\text{-mC} = e^{\left\{\frac{(\text{Absorbance} - \text{y-intercept})}{\text{Slope}}\right\}}$$



Standard curve generated with DNA mixtures. The curve was generated using the absorbance values of the mixtures indicated in the table above. A logarithmic relationship was observed with a correlation of 0.99.

Ordering Information

Product Description	Catalog No.	Kit Size
5-mC DNA ELISA Kit	D5325 D5326	1 x 96 wells 2 x 96 wells

For Individual Sale	Catalog No.	Amount
5-mC Coating Buffer	D5325-1-15 D5325-1-30	15 ml 30 ml
5-mC ELISA Buffer	D5325-2-250	250 ml
Anti-5-Methylcytosine (0.5 µg/µl)	A3002-15 A3002-30	15 µl 30 µl
Secondary Antibody (1 µg/µl)	D5325-3-15 D5325-3-30	15 µl 30 µl
HRP Developer	D5425-4-15 D5425-4-30	15 ml 30 ml
Negative Control (100 ng/µl)	D5325-5-1	50 µl
Positive Control (100 ng/µl)	D5325-5-2	50 µl
96-well ELISA plate (12 x 8-well Strips)	C2020	1 plate

Related Products for 5-mC Analysis:

Product Name	Size	Catalog No.
Methylated-DNA IP Kit	10 Rxns.	D5101
***OneStep* qMethyl™ Kit**	1 x 96	D5310
***OneStep* qMethyl™-Lite**	1 x 96	D5311
Zymo*Taq*™ DNA Polymerase	50 Rxns. 200 Rxns.	E2001 E2002
Zymo*Taq*™ PreMix	50 Rxns. 200 Rxns.	E2003 E2004
EZ DNA Methylation™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5001 D5002 D5003 D5004
EZ DNA Methylation-Gold™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5005 D5006 D5007 D5008
EZ DNA Methylation-Direct™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5020 D5021 D5022 D5023
EZ DNA Methylation-Startup™ Kit	50 Rxns.	D5024
EZ Bisulfite DNA Clean-up Kit™	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5025 D5026 D5027 D5028
Universal Methylated DNA Standard	1 set	D5010
Universal Methylated Human DNA Standard	1 set	D5011
Universal Methylated Mouse DNA Standard	1 set	D5012
Human HCT116 DKO Methylation Standards	1 set	D5014
Human HCT116 DKO Non-methylated DNA Standard	5 µg	D5014-1
Human HCT116 DKO Methylated DNA Standard	5 µg	D5014-2
Bisulfite Converted Universal Methylated Human DNA Standard	1 set	D5015
***E. coli* Non-methylated Genomic DNA**	5 µg	D5016
ChIP DNA Clean & Concentrator™	50 50	D5201 D5205
Anti-5-Methylcytosine Monoclonal Antibody (clone 10G4)	50 µg 200 µg	A3001-50 A3001-200
CpG Methylase (M.SssI)	200 units 400 units	E2010 E2011
5-Methyl dCTP [10 mM]	1 µmol	D1035
5-Methylcytosine dNTP Mix [10 mM]	2.5 µmol	D1030

Additional Products for Epigenetics Research:

Product Name	Size	Catalog No.
Quest 5-hmC™ DNA ELISA Kit	1x96	D5425
	2x96	D5426
Anti-5-Hydroxymethylcytosine Polyclonal Antibody	50 µg	A4001-50
	200 µg	A4001-200
Quest 5-hmC™ DNA Enrichment Kit	25 Preps.	D5420
	50 Preps.	D5421
Quest 5-hmC Detection Kit™	25 Preps.	D5410
	50 Preps.	D5411
Quest 5-hmC Detection Kit™-Lite	25 Preps.	D5415
	50 Preps.	D5416
Quest*Taq*™ PreMix	50 Rxns.	E2050
	200 Rxns.	E2051
Human Matched DNA Set	2 x 5 µg	D5018
Mouse ^{5hm}C & ^{5m}C DNA Set	4 x 5 µg	D5019
5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set	3 x 2 µg	D5405
DNA Degradase™	500 units	E2016
	2,000 units	E2017
DNA Degradase Plus™	250 units	E2020
	1,000 units	E2021
5-hmC Glucosyltransferase	100 units	E2026
	200 units	E2027
5-Hydroxymethyl dCTP [100 mM]	10 µmol	D1045
5-Hydroxymethylcytosine dNTP Mix [10 mM]	2.5 µmol	D1040

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