Global DNA Methylation LINE-1 Kit

Catalog No. 55017

(version A2)

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Revision History

Revision	Date	Description of Change
A2	Dec 2018	Clarified recommendations for colorimetric reaction development time

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Overview

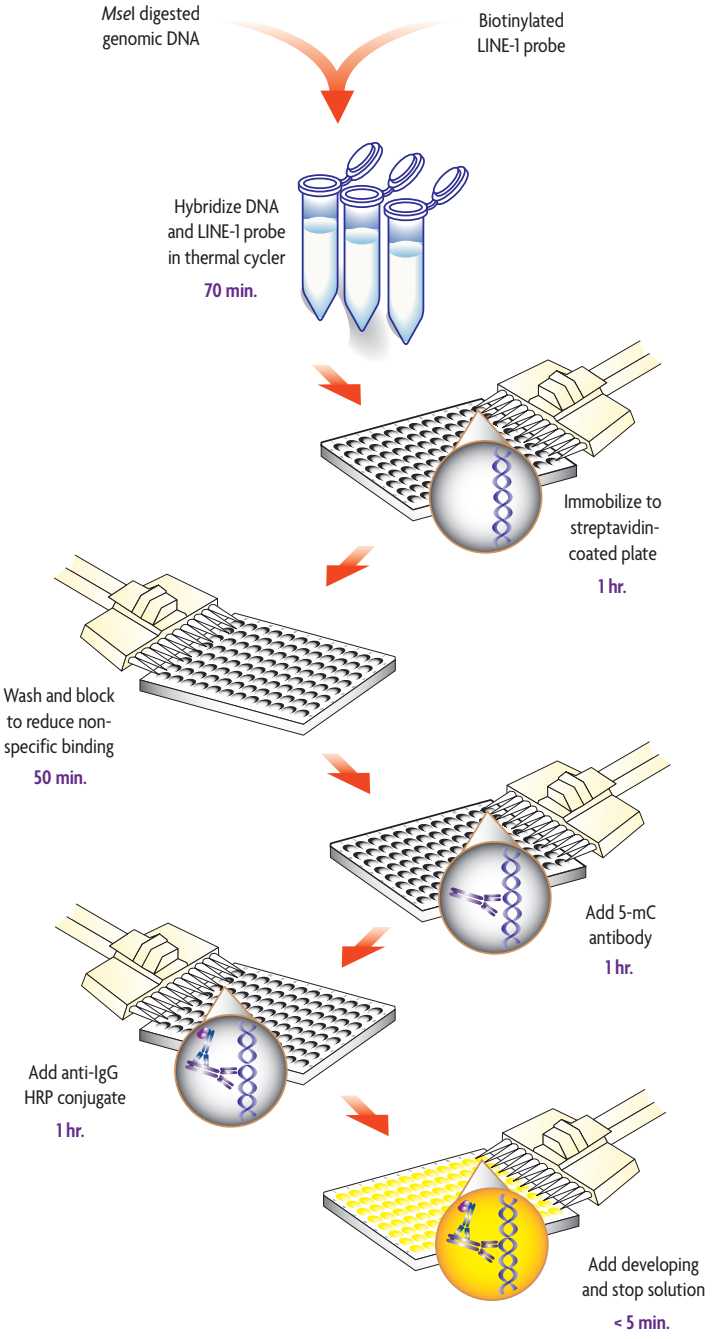
The Global DNA Methylation – LINE-1 Kit is designed to detect methylation in Long Interspersed Nucleotide Element 1 (LINE-1) repeat elements of human genomic DNA. LINE-1 methylation serves as a surrogate for global DNA methylation levels and can be utilized to compare relative changes in 5-methylcytosine levels across different sample types, treatment conditions, clinical outcomes, dietary history or environmental backgrounds.

In the Global DNA Methylation – LINE-1 Kit, genomic DNA of interest is fragmented by enzymatic digestion and hybridized to a biotinylated human LINE-1 consensus probe. Hybridized DNA is immobilized onto a streptavidin-coated plate, while unbound DNA fragments are washed away. A 5-Methylcytosine antibody and a secondary antibody conjugated to horseradish peroxidase (HRP) are used for detection of methylated fragments. The colorimetric readout is easily quantified by spectrophotometry using a microplate reader at 450nm.

The assay contains an optimized protocol and reagents necessary to perform DNA fragmentation, hybridization, capture, and colorimetric detection of 5-methylcytosine. For added convenience, methylated and non-methylated DNA standards containing known levels of LINE-1 methylation are included in the kit. By generating a standard curve, the relative 5-methylcytosine levels in each DNA sample can be determined.

product	format	catalog no.
Global DNA Methylation – LINE-1 Kit	1 x 96 rxns	55017

Flow Chart of Process



Introduction

DNA Methylation and Long Interspersed Nuclear Element 1 (LINE-1)

DNA methylation, which usually occurs at the fifth position of the cytosine pyrimidine ring of CpG dinucleotides (5-mC) in mammals and other vertebrates, is an important epigenetic mechanism regulating gene expression. Aberrant changes in DNA methylation have been linked to the development and progression of disease, including cardiovascular disease, lupus, cancer, birth defects and neuropsychiatric disorders[1-9]. Consequently, DNA methylation patterns and/or levels represent disease biomarkers with potential in both diagnostic and prognostic applications.

Repetitive elements comprise approximately 45% of the human genome and consist of either tandem repeats of simple (satellite DNA) or complex sequences, or interspersed repeats derived from non-autonomous or autonomous transposable elements[10-14]. It is estimated that more than one-third of DNA methylation occurs in repetitive elements[15-17]. Literature indicates that analysis of the methylation status of high copy number repeat elements such as the long interspersed nuclear element (LINE-1) can serve as a surrogate marker for global genomic DNA methylation[18-20]. A comparison of the methylation status of the LINE-1 repeat elements correlates well with total 5-methylctyosine content when compared to high performance liquid chromatography (HPLC) or combined bisulfite restriction analysis (COBRA) and pyrosequencing[18,20]. LINE-1 repeats represent approximately 17-18% of the human genome, with a copy number estimated at roughly half a million[21]. LINE-1 hypomethylation is associated with elevated risk of several disease states including cancer, stroke and heart disease and is considered a prognostic factor in gliomas[22-28].

Using Active Motif's Global DNA Methylation – LINE-1 assay, genomic DNA from different sample types (e.g. normal and tumor), treatment conditions, clinical outcomes, dietary history or environmental backgrounds can be analyzed for differences in global DNA methylation levels. First, genomic DNA of interest is enzymatically digested using the provided *Mse*I enzyme to generate the appropriate fragmentation to hybridize to a biotinylated consensus sequence corresponding to human LINE-1 transposon. The LINE-1 probe is designed to assay a 290 bp region of the LINE-1 repeat element containing 88 cytosine residues, of which 12 are in a CpG context. Hybridized samples are immobilized to a 96-well plate and methylated cytosines are identified using a 5-methylcytosine antibody, HRP-conjugated secondary antibody and colorimetric detection reagents. Generating a standard curve using the included DNA standards with known LINE-1 methylation levels provides the relative level of 5-methylcytosine in each DNA sample.

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Traditional Methods to Study DNA Methylation

To date, there are several methods used for DNA methylation analysis:

1. **High performance liquid chromatography (HPLC):** DNA can be digested into single nucleotides which are separated according to size. Both cytosine and methylated cytosine are quantified. Variants of this approach include tandem mas spectrometry (LC-MS/MS) as well as two dimensional thin layer chromatography and high performance capillary electrophoresis. While these methods are highly quantitative, they require large amounts of DNA and are not easily amenable to high throughput.
2. **Bisulfite conversion:** Bisulfite conversion consists of the treatment of genomic DNA with sodium bisulfite, leading to deamination of unmethylated cytosines into uracil. Converted DNA is then PCR amplified and sequenced. Bisulfite-based techniques can be cumbersome, involving time- and labor-intensive chemical treatments that damage DNA and limit throughput. It is most readily used to analyze gene-specific sequences instead of global methylation.
3. **Methylated DNA Immunoprecipitation (MeDIP):** Methylated DNA Immunoprecipitation is an immunocapture technique in which an antibody specific for methylated cytosines is used to immunoprecipitate methylated genomic DNA fragments. Enriched DNA can be used for analysis of the methylation status of a particular gene by PCR, or in combination with NGS. MeDIP is prone to antibody bias and the technique does not consider total cytosine content.

Kit Performance and Benefits

The Global DNA Methylation – LINE-1 Kit is used to quantitate 5-methylcytosine levels in human genomic DNA when comparing the samples to the provided DNA standards.

Range of detection: The dynamic range of the suggested standard curve can detect methylation between 1% to 13%. The assay has been shown to detect as little as 0.5% methylation.

Input DNA can be used in the range of 10 ng - 200 ng per well. However, if the OD 450nm readings fall outside the limits of detection for the assay (as determined by the standard curve), we suggest performing a sample titration of the DNA to determine the appropriate quantity to use within the assay. Then perform the rest of the assay at the determined concentration. We recommend a starting concentration of 100 ng/well.

Cross-reactivity: The assay is specific to human LINE-1 repeat elements.

Assay time: 5 hours

Global DNA Methylation – LINE-1 Kit

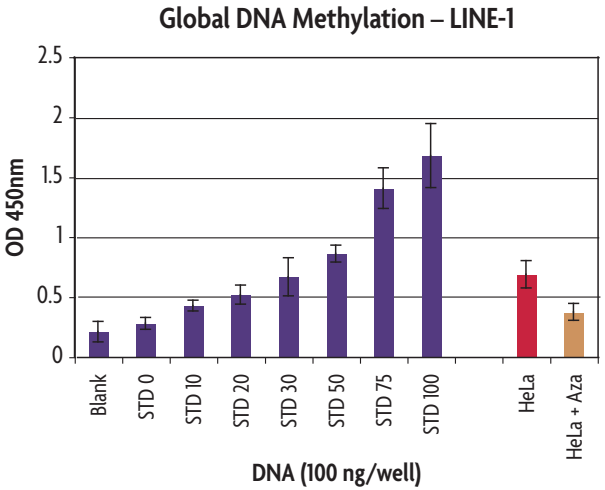

Figure 1: The Global DNA Methylation – LINE-1 assay compares 5-mC levels across treatment conditions.
The Global DNA Methylation – LINE-1 kit was used to assay 100 ng per well of *MseI* digested genomic DNA from HeLa cells or HeLa cells treated with 0.05 µM 5-azacytidine. Treatment of cells with 5-azacytidine, a known inhibitor of DNA methyltransferases (DNMTs), results in a decrease in global methylation as compared to untreated HeLa cells as expected. Using the provided standard standards and curve fitting software, the percent 5-methylcytosine for each DNA sample can be determined.

Global DNA Methylation – LINE-1 Kit Components and Storage

Global DNA Methylation Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
LINE-1 Probe (2.5 µM)	20 µl	-20°C
*Mse*I enzyme (10 U/µl)	16 µl	-20°C
10X *Mse*I Reaction Buffer	65 µl	-20°C
Methylated DNA Standard (10 ng/µl)	185 µl	-20°C
Non-methylated DNA Standard (10 ng/µl)	270 µl	-20°C
5-Methylcytosine mAb	110 µl	-20°C
Anti-mouse HRP-conj. secondary Ab	450 µl	4°C
Assay Buffer AM3	40 ml	4°C
20X Wash Buffer	25 ml	4°C
96-well Streptavidin plate	1 ea	4°C
Developing Solution	11 ml	4°C
Stop Solution	11 ml	RT
5X Hybridization Buffer	1 ml	RT
10X Buffer W	10 ml	RT
Plate sealer	2 ea	RT
0.2 ml PCR stripwell tubes	1 pk	RT

Additional materials required

- Sample DNA
- Orbital shaker (*e.g.* Multi-microplate Genie from Scientific Industries)
- 1.5 or 2 ml microcentrifuge tubes
- Microcentrifuge
- Vortex
- DNase-free sterile water
- Nanodrop, Qubit or equivalent method to determine DNA concentration
- (Optional) Graphing program with curve fitting capabilities

Buffer Preparation and Recommendations

***Mse*I enzyme**

The *Mse*I enzyme is provided ready to use at 10 U/µl. Enough enzyme is provided to perform 32 DNA digestions as described in Step A of the protocol. Digested DNA is assayed in triplicate.

10X *Mse*I Reaction Buffer

The reaction buffer is provided ready to use at a 10X concentration. Please follow the instructions in the protocol for use.

LINE-1 Probe (See the Quick Chart for Preparing Buffers)

The LINE-1 Probe is provided at 2.5 µM. Prepare a master mix of probe diluted in Hybridization Buffer for use with all assay samples, standards and blanks. Follow the recommendations in the Quick Chart for Buffer Preparation to prepare only the amount of diluted probe needed for each experiment. Use 25 µl per well (0.5 pmol/well).

5X Hybridization Buffer (See the Quick Chart for Preparing Buffers)

The 5X Hybridization Buffer is provided ready to use. Please follow the recommendations in the Quick Chart for Buffer Preparation for use.

1X Buffer W (See the Quick Chart for Preparing Buffers)

Prepare the amount of 1X Buffer W required for the assay as follows: For every 10 ml of 1X Buffer W required, dilute 1 ml 10X Buffer W with 9 ml sterile water.

5-Methylcytosine mAb (See the Quick Chart for Preparing Buffers)

Prepare a 1:100 dilution of 5-methylcytosine monoclonal antibody into Assay Buffer AM3. Follow the recommendations in the Quick Chart for Buffer Preparation to prepare only the amount of antibody needed for each experiment. Use 100 µl per well.

Anti-mouse HRP-conj. secondary antibody (See the Quick Chart for Preparing Buffers)

Prepare a 1:25 dilution of anti-mouse HRP-conj. secondary antibody into Assay Buffer AM3. Follow the recommendations in the Quick Chart for Buffer Preparation to prepare only the amount of antibody needed for each experiment. Use 100 µl per well.

1X Wash Buffer (See the Quick Chart for Preparing Buffers)

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 5 ml 20X Wash Buffer with 95 ml sterile water. Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 20X Wash Buffer may form visible aggregates, therefore, homogenize the buffer by incubating at 50°C for 2 minutes and mixing prior to use.

Developing Solution (See the Quick Chart for Preparing Buffers)

The Developing Solution should be warmed to room temperature before use. The Developing

Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. However, a blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting into the wells. After use, discard remaining Developing Solution.

Stop Solution (See the Quick Chart for Preparing Buffers)
Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container. After use, discard remaining Stop Solution.

WARNING: The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* safety glasses, gloves and labcoat.

Methylated and Non-methylated DNA Standards
Methylated and Non-methylated DNA Standards are provided ready to use at a concentration of 10 ng/µl. Both standards are derived from human Jurkat genomic DNA. The methylated standard has been *in vitro* methylated with *M.Sss*I, then digested with *Mse*I to produce the appropriate fragmentation. The Non-methylated DNA Standard was whole genome amplified to remove endogenous methylation. DNA Standards can be used as assay controls, or can be mixed in varying proportions to create a standard curve for quantitation of percent methylated cytosine (see Section B of the protocol). Enough DNA is provided to perform two standard curves in triplicate. Please follow the instructions in the protocol for use.

The provided DNA standards are suitable for relative quantitation, but do not account for copy number variances that may exist in different sample types. If copy number variation is a concern, please follow the recommendations in Appendix Section J to prepare Methylated and Non-methylated DNA standards for your specific DNA sample.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	For 1 well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 12 strips (96 wells)
LINE-1 Probe Solution	LINE-1 Probe	0.2 µl	1.8 µl	10.8 µl	21.6 µl
	5X Hybridization Buffer	10 µl	90 µl	540 µl	1.08 ml
	Distilled water	14.8 µl	133.2 µl	800 µl	1.6 ml
	TOTAL REQUIRED	**25 µl**	**225 µl**	**1.35 ml**	**2.7 ml**
1X Buffer W	Distilled water	720 µl	7.2 ml	40.5 ml	81 ml
	10X Buffer W	80 µl	0.8 ml	4.5 ml	9 ml
	TOTAL REQUIRED	**800 µl**	**8 ml**	**45 ml**	**90 ml**
5-Methylcytosine Ab	5-Methylcytosine Ab	1 µl	9 µl	54 µl	108 µl
	Assay Buffer AM3	99 µl	891 µl	5.35 ml	10.7 ml
	TOTAL REQUIRED	**100 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
HRP-conjugated anti-mouse Ab	HRP-conjugated anti-mouse IgG	4 µl	36 µl	216 µl	432 µl
	Assay Buffer AM3	96 µl	864 µl	5.2 ml	10.4 ml
	TOTAL REQUIRED	**100 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
1X Wash Buffer	Distilled water	1.9 ml	17.1 ml	95 ml	190 ml
	20X Wash Buffer	100 µl	0.9 ml	5 ml	10 ml
	TOTAL REQUIRED	**2 ml**	**18 ml**	**100 ml**	**200 ml**
Developing Solution	**TOTAL REQUIRED**	**100 µl**	**900 µl**	**5.4 ml**	**10.8 ml**
Stop Solution	**TOTAL REQUIRED**	**100 µl**	**900 µl**	**5.4 ml**	**10.8 ml**

Assay Protocol

Read the entire protocol before use.

Prior to starting the Global DNA Methylation – LINE-1 Kit, genomic DNA is fragmented using restriction digestion with the provided *Mse*I enzyme. This enzymatic digestion will generate the appropriate fragments in the LINE-1 repeat elements of the genomic DNA to enable hybridization to the assay's LINE-1 probe.

Section A: *Mse*I Digestion of Genomic DNA

We recommend preparing high-quality genomic DNA using a commercially available kit or a standard established protocol and determining DNA concentration by UV spectrophotometry or fluorescence-based quantitation. The LINE-1 Global DNA Methylation assay can be performed on approximately 100 ng *Mse*I fragmented genomic DNA per well. We suggest using 1 µg genomic DNA per sample type for digestion and then performing the assay in triplicate.

1. In microcentrifuge tubes, set up a restriction digest for each DNA sample to be tested. Also, prepare a mock digestion reaction that will be used as a blank for DNA quantification following the digestion reaction. A small loss of DNA is anticipated as part of the digestion process, therefore quantification MUST be performed following *Mse*I digestion to accurately determine the DNA quantity for use in the assay. A single mock digestion reaction can be used as a blank for all the DNA digestions performed on the same day.

Reagents	**DNA Sample**	**Mock Digestion**
Genomic DNA (1 µg)	_____ µl	0 µl
10X Reaction Buffer	2 µl	2 µl
*Mse*I enzyme (10 U/µl)	0.5 µl	0.5 µl
Sterile water	Up to 20 µl	17.5 µl
Total Volume	**20 µl**	**20 µl**

2. Mix well by pipetting and incubate at 37°C for 4 hours or overnight.
3. Heat-inactivate *Mse*I by incubating the reaction mixture at 65°C for 20 minutes. Quick spin tubes to collect any condensate.
4. Measure the DNA concentration of each sample using the mock digestion as a blank. We suggest using 1 µl of the digestion reaction for quantification. The Global DNA Methylation assay is very sensitive to DNA concentration so DNA MUST be quantified following *Mse*I digestion. Failure to properly quantify the DNA following digestion will lead to variability in the assay results. Make sure your samples fall within the range of detection for your spectrophotometer or use a more sensitive fluorescence-based quantitation.
5. If the assay will not be performed immediately, the digested DNA can be stored at -20°C.

Section B: Standard Curve Preparation (Optional)

The provided Methylated and Non-methylated DNA Standards can be used as assay controls for non-quantitative analysis, or can be mixed in varying proportions to create a standard curve for quantitation of percent methylated cytosine in each sample. For non-quantitative assay controls we recommend using: Blank, STD 0 and STD 100 as shown in the table below. To create a quantitative standard curve, prepare the entire range of standards.

1. In microcentrifuge tubes, prepare a mixture of Methylated and Non-methylated DNA standards according to the following table. Volumes listed below are sufficient to prepare one standard curve in triplicate.

Standard Name	Methylated Standard (ng)	Non-Meth. Standard (ng)	Volume Methylated Standard	Volume Non-Meth. Standard	Volume Water
BLANK	0	0	0 µl	0 µl	80 µl
STD 0	0	100	0 µl	32 µl	48 µl
STD 10	10	90	3.2 µl	28.8 µl	48 µl
STD 20	20	80	6.4 µl	25.6 µl	48 µl
STD 30	30	70	9.6 µl	22.4 µl	48 µl
STD 50	50	50	16 µl	16 µl	48 µl
STD 75	75	25	24 µl	8 µl	48 µl
STD 100	100	0	32 µl	0 µl	48 µl

2. Vortex to mix thoroughly. Quick spin to collect liquid.
3. Use 25 µl of each Standard per well.

Section C: DNA Sample Hybridization

1. Determine the appropriate number of 0.2 ml stripwell PCR tubes required for testing samples, standards and blanks in triplicate. Label each tube.
2. Add 25 µl LINE-1 Probe Solution prepared on page 9 to all PCR tubes being used.
3. Dilute the *Mse*I digested DNA samples to a concentration of 4 ng/µl in sterile water. Mix well by vortexing. (*e.g.* To prepare triplicate reactions, dilute 320 ng of DNA in sterile water for a final volume of 80 µl. Use 25 µl per reaction.)
4. Add DNA to the appropriately labeled PCR tube. The final volume is 50 µl per tube.

   Blanks: Add 25 µl Blank per well (no DNA wells)

   DNA Standards: Add 25 µl of each DNA Standard per well (100 ng DNA/well)

   ***Mse*I-digested Samples**: Add 25 µl of each DNA Sample per well (100 ng DNA/well)

5. Cap tubes and briefly vortex to mix. Collect liquid to bottom of tubes.
6. Place samples in a thermal cycler and incubate at 98°C for 10 minutes, then quickly ramp to 68°C for 1 hour followed by a quick ramp to 25°C.

Section D: Binding to Streptavidin-coated Plate

Remove the appropriate number of microwell strips required for testing. Store the unused strips in the aluminum pouch at 4°C. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the plate sealer while you perform the assay. The unused wells are stable at room temperature for the duration of the assay if kept dry. Once the assay is finished, unused strips should be returned to the aluminum pouch and stored at 4°C for a separate assay. Use the strip holder while performing the assay. An example plate layout is shown below.

Prepare buffers as described in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the Buffer W, 1X Wash Buffer, Developing Solution and Stop Solution into the wells being used.

	DNA Standards			*Mse*I-digested Samples		
	1	2	3	4	5	6
A	Blank	Blank	Blank	Sample 1	Sample 1	Sample 1
B	STD 0	STD 0	STD 0	Sample 2	Sample 2	Sample 2
C	STD 10	STD 10	STD 10	Sample 3	Sample 3	Sample 3
D	STD 20	STD 20	STD 20	Sample 4	Sample 4	Sample 4
E	STD 30	STD 30	STD 30	Sample 5	Sample 5	Sample 5
F	STD 50	STD 50	STD 50	Sample 6	Sample 6	Sample 6
G	STD 75	STD 75	STD 75	Sample 7	Sample 7	Sample 7
H	STD 100	STD 100	STD 100	Sample 8	Sample 8	Sample 8

1. Collect any condensate to the bottom of the PCR tubes. Transfer the contents of each PCR tube to a separate well of the streptavidin-coated assay plate. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at room temperature with mild agitation (100-300 rpm on an orbital plate shaker). Keep any unused wells covered during the remaining steps in order to preserve those wells for future assays.
2. Remove the plate sealer and discard the unbound DNA by quickly inverting the plate over a liquid waste receptacle. Then, tap the inverted plate 3 times on absorbent paper towels.
3. Add 200 µl 1X Buffer W to each well for a quick rinse and immediately discard solution as described in Step 2. Repeat for a second rinse.
4. Wash each well 2 times with 200 µl 1X Buffer W and incubate each wash for 10 minutes with mild agitation.
5. Add 200 µl Assay Buffer AM3 to each well. Cover the plate with the adhesive sealer and

incubate at room temperature for 30 minutes with mild agitation.

6. During this incubation, prepare the 5-methylcytosine antibody dilution as described in the section Buffer Preparation and Recommendations.

Section E: Binding of Primary Antibody

1. Remove the plate sealer and discard the blocking buffer by quickly inverting the plate over a liquid waste receptacle, then tap the inverted plate 3 times on absorbent paper towels.
2. Add 100 µl of diluted 5-Methylcytosine antibody to each well being used, including blank wells. Cover the plate and incubate for 1 hour at room temperature with mild agitation.
3. During this incubation, prepare the anti-mouse HRP antibody dilution as described in the section Buffer Preparation and Recommendations.
4. After the incubation, rinse the wells 3 times with 200 µl 1X Wash Buffer.

Section F: Binding of Secondary Antibody

1. Add 100 µl of diluted HRP-conjugated anti-mouse antibody to each well being used, including blank wells. Cover the plate and incubate for 1 hour at room temperature with mild agitation.
2. During this incubation, place the Developing and Stop Solutions at room temperature.
3. After the incubation, rinse the wells 3 times with 200 µl of 1X Wash Buffer.

Section G: Colorimetric Reaction

1. Remove as much of the final wash as possible by blotting the plate on paper towels.
2. Add 100 µl of room temperature Developing Solution to all wells being used.
3. Incubate for the amount of developing time specified on the Certificate of Analysis at room temperature, protected from direct light. Please read the Certificate of Analysis supplied with this kit for optimal development time associated with this lot number. Monitor the blue color development in the standard curve wells containing the highest concentrations of methylated DNA (STD 100) until they turn medium to dark blue. The Blank wells should be a faint blue color. Do not overdevelop.
4. Add 100 µl of Stop Solution to all wells. In presence of the acid, the blue color turns yellow.
5. Read absorbance on a spectrophotometer within 5 minutes at 450nm with an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

   Reading the reference wavelength is optional. Most microtiter plate readers are equipped to perform dual wavelength analysis and with the appropriate software, will automatically subtract the reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, you may read the plate twice, once at 450 nm and

once at 655 nm then manually subtract the 655 nm OD from the 450 nm OD values.

Section H: Analysis of Results

1. Average the triplicate readings for all Blank, Standard and Sample wells. Then, subtract the averaged Blank OD 450nm values from the average Standard and the averaged Sample values. These are the Net readings.
2. For non-quantitative analysis, OD 450nm values for samples can be compared to the assay controls to determine relative levels of LINE-1 DNA methylation. The Blank wells represent the baseline signal from the assay reagents in the absence of DNA. The STD 0 (non-methylated DNA only) value displays the background signal of the assay in the presence of unmethylated DNA samples, while the STD 100 (methylated DNA only) represents a sample with a high degree of DNA methylation.
3. For quantitative analysis, the % 5-mC associated with each sample can be calculated two different ways. The first method determines % 5-mC based on the CpG content. The second method determines the % 5-mC of CpG residues as it relates to total cytosine content. Determine the method of quantitation that is most appropriate for your experimental analysis.

Method 1: Determines the % 5-mC associated with the detectable CpG residues.	**Standard Name**	**% 5-mC**
The 290 bp LINE-1 *Mse*I fragments that hybridize to the LINE-1 probe contain 10 detectable CpG residues. The provided DNA standards are either 100% methylated or 0% methylated for all 10 CpGs. Therefore, the standard curve generated by mixing the DNA standards together quantitates methylation in the range of 0% to 100% 5-mC for the CpG content of the samples. See the values to the right for the specific % 5-mC for each DNA Standard.	STD 0	0%
	STD 10	10%
	STD 20	20%
	STD 30	30%
	STD 50	50%
	STD 75	75%
	STD 100	100%

Method 2: Determines the % 5-mC measured relative to the total cytosine content.	**Standard Name**	**% 5-mC**
The 290 bp LINE-1 *Mse*I fragments that hybridize to the LINE-1 probe contains 10 detectable CpG residues out of a total of 76 cytosines. The provided DNA standards are either 100% methylated or 0% methylated for all 10 CpGs. By dividing the methylated CpG content used in each DNA standard by the total number of cytosines, the % 5-mC of the sample as it relates to total cytosine content can be determined in the range of 1% to 13% methylation. See the values to the right for the specific % 5-mC for each DNA Standard.	STD 0	0%
	STD 10	1.3%
	STD 20	2.6%
	STD 30	3.9%
	STD 50	6.6%
	STD 75	9.9%
	STD 100	13.1%

4. After selecting a method for % 5-mC determination, use a graphing program containing curve fitting tools to plot the % 5-mC associated with each DNA Standard along the x-axis and the Net readings along the y-axis. Since the OD values will vary with color development time, the best fit curve needs to be determined for each experimental run. Extrapolate the % 5-mC methylation for each DNA sample. Readerfit (https://readerfit.com) is a freely available curve fitting program that may be used for analysis.

   Note: If the amount of sample DNA used within the assay was a value other than the suggested 100 ng DNA/well, the extrapolated value of the % 5-mC should be divided by the mass of the input sample to achieve a corrected % 5-mC for that DNA sample. For example, if the 100 ng Standard Curve was used to extrapolate the % 5-mC for a DNA sample that only contained 50 ng/well, then the extrapolated % 5-mC values are divided by 50 and multiplied by 100% to predict the relative level of methylation in the 50 ng sample.

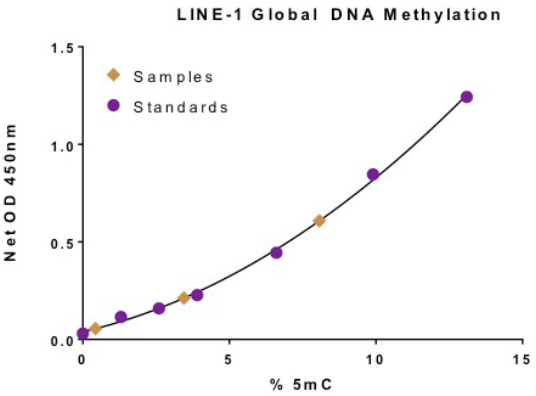

Figure 2: Graphical representation of the best curve fit as determined by analysis software.

The % 5 methylcytosine represented by each DNA standard was plotted on the x-axis and the Net OD 450nm readings for both the standards (triangles) and samples (circles) were plotted on the y-axis. The best curve fit was determined and the % 5-mC values for the DNA samples were extrapolated from the curve.

Appendix

Section I: Troubleshooting Guide

Problem/question	Possible cause	Recommendation
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in all wells in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity by mixing a small aliquot of HRP and Developing Solution together
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction. Follow our recommendations to prepare buffers
	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader
	Incorrect assay temperature	Bring Developing Solution and Stop Solution to room temperature before using
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
High background in all wells	Developing time too long	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue or the blank wells turn a faint blue color
	Concentration of antibodies is too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations. Make sure to perform the 10 minute incubations in Buffer W during Section D, step 4. Failure to incubate the washes may increase the background signal.
Uneven color development between replicates	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations. Ensure no residual wash buffer remains prior to addition of developing solution
	Well cross-contamination	Follow washing recommendations
	Inaccurate pipetting	Verify that the same amount of hybridized sample, primary antibody, secondary antibody, developing and stop solutions are added to each well
Signal in sample well beyond the detectable range for the microplate reader	Too much sample per well	Decrease amount of sample per well. Titrate the amount of genomic DNA to use per well such that it falls within the range of the DNA standards. We suggest a range of 10-200 ng/well
	Concentration of antibodies is too high	Perform antibody titration to determine optimal working concentration. Start using 1:50 for primary antibody and 1:10 for the secondary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough sample per well	Increase amount of sample per well. Titrate the amount of genomic DNA to use per well such that it falls within the range of the DNA standards. We suggest a range of 10-200 ng/well
No signal or weak signal in standard curve wells	Omission of key reagent	Ensure that all reagents have been added to the wells in the correct order. Verify that DNA standards were prepared as instructed in Step B of the manual.

Section J. Sample-specific DNA standards

The provided Methylated and Non-methylated DNA standards included in the assay can be used to generate a standard curve for relative quantitation. These DNA standards are prepared from human Jurkat genomic DNA to provide researchers known quantities of LINE-1 methylation that can be used to approximate the level of 5-mC methylation in their DNA samples. However, the copy number of the LINE-1 repeat element may vary between sample types. To improve the accuracy of the methylation quantitation based on copy number variances, customers could prepare a standard curve mixture using genomic DNA from their specific cell line or tissue of interest.

i. Isolate genomic DNA of interest. Reserve 10 ng of DNA for use in preparing the non-methylated DNA standard and 1 µg to perform the assay. Use 4 µg of the remaining DNA to prepare the methylated DNA standard.

ii. Fully methylated one fraction of the genomic DNA using M.SssI enzyme following the manufacturer's instructions.

Note: We have found the following conditions improve the methylation reaction: Use 2 units M.SssI enzyme (4 U/µl) per µg DNA and 640 µM final concentration of the methyl donor SAM. Incubate the reaction at 37°C for 2 hours and then spike in additional SAM (the same amount used initially) and continue to incubate at 37°C for another 2 hours.

iii. Fragment the fully methylated genomic DNA using *Mse*I enzyme.

iv. Quantify the fragmented fully methylated genomic DNA. Dilute to 10 ng/µl in sterile water. This DNA can now be used as the Methylated DNA standard to generate the sample-specific standard curve.

v. Prepare non-methylated DNA standards by performing whole-genome amplification of the 10 ng fraction reserved of the isolated genomic DNA. Amplification will remove any endogenous methylation to generate a non-methylated pool of DNA. Since the DNA is generated by PCR amplification it will already be present in fragments. The DNA prepared by whole genome amplification does not require additional *Mse*I digestion.

vi. Quantify the whole-genome amplified genomic DNA. Dilute to 10 ng/µl in sterile water. This DNA can now be used as the Non-Methylated DNA standard to generate the sample-specific standard curve.

Follow the recommendations for standard curve preparation in the manual using the sample-specific standards. Analysis of DNA samples from the same cell type as the standard curve will normalize for any copy number variances during quantitation of 5-mC levels.