MLH1 Methylation Detection Kit

Real-Time PCR assay

- Detects promoter methylation in regions C and D of the MLH1 gene
- No bisulfite conversion neeed
- 24 tests per kit
- Available as RUO (US) / IVD (EU)

INTENDED USE:

The MLH1 Methylation Detection Kit combines methylation-sensitive restriction enzymes (MSER) with qPCR for methylation detection of the mismatch repair mutL homolog 1 (MLH1) promoter. This assay contains primer/probe mixes for methylation detection in two MLH1 promoter regions.

FOR USE WITH:

- Bio-Rad - CFX96
- Applied Biosystems® - 7500/7500 Fast
- Thermo Fisher - QS5

PACKAGE CONTENTS

This product contains the following materials sufficient for 24 reactions, including samples and controls.

Description	Tubes
2x Reaction Mix	2
MLH1 Primer/Probe Mix	2
Restriction Enzyme	1
MLH1 Positive Control Mix	1

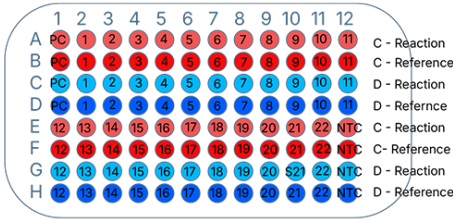
HANDLING AND STORAGE

This product is shipped on frozen ice packs. The contents of the shipment should be stored at -25°C to -15°C in a non-frost-free freezer immediately upon receipt.

- Store all unopened components in original containers.
- Primer/probe mixes should be protected from light at all times to prevent photobleaching of the fluorescent dyes.
- Centrifuge the tubes before opening.
- This product will maintain its performance through the expiration date printed on the box label. Its performance is not guaranteed after the expiration date.

PLATE SETUP

Suggested plate layout for a single experiment analyzing 22 unknown samples, PC and NTC; RE- restriction enzyme reaction. Reference – uncut DNA reaction.



PRINCIPLE OF THE ASSAY

The MLH1 Promoter Methylation Detection Kit is a single-tube Methylation Sensitive Restriction Enzyme (MSRE)-based qPCR assay. Each reaction contains specific primers and probes for the amplification of regions C or D in MLH1 promoter (labeled by FAM), and a B2M internal control gene (labeled by CFO560/VIC).
Each DNA sample is tested in two (paired) reactions. DNA sample in one reaction tube is treated with methylation-sensitive restriction enzymes (RE) prior to the qPCR reaction, while the other is amplified without the addition of restriction enzymes. The latter is used as reference for methylation ratio calculations. Restriction enzymes cut only the non-methylated sequences, making them unavailable for amplification. Differences in qPCR fluorescence signals of target MLH1 promoter regions C or D between the RE and the reference reaction are indicative of the levels of methylation in those regions. In addition, amplification of an internal input control (IC) gene is used to ensure that the same amount of sample is loaded. Using this approach, extracted DNA can be tested directly without the need for pre-treatment, such as bisulfite conversion.

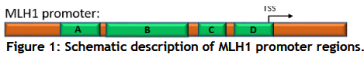
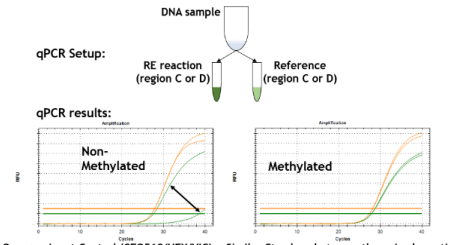


Figure 1: Schematic description of MLH1 promoter regions.



Orange - Input Control (CFO560/HEX/VIC) - Similar Ct values between the paired reactions.
Green - MLH1 promoter (FAM) - Signal depends on restriction enzyme activity.

Figure 2: Schematic description of MLH1 MSRE-qPCR assay.

ASSAY PERFORMANCE

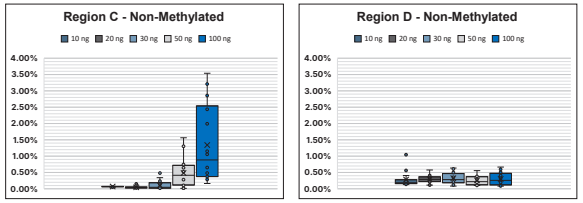
Limit of Blank

No-Template Reactions

Limit of Blank was assessed on the CFX96 instrument with 38 reactions per promoter region, using molecular grade water. Results demonstrate no non-specific amplification in blank reactions (no signal under Ct of 40 was detected in FAM or CFO560).

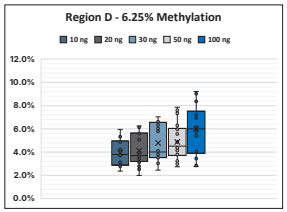
Non-Methylated DNA

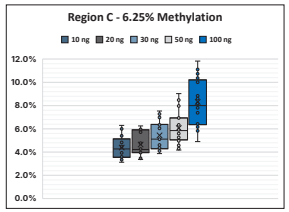
The MLH1 Methylation Detection Assay was tested with non-methylated gDNA at an input of 10 ng, 20 ng, 30 ng, 50 ng and 100 ng. The restriction step was done by a 60 minute incubation at 37°C with 0.5 µl RE. All runs were performed on the CFX96 instrument. Methylation result distribution for each input is shown below using box and whisker plots.



Limit of Detection

Limit of detection was assessed with 6.25% gDNA methylation standards, at 10 ng, 20 ng, 30 ng, 50 ng and 100 ng inputs, tested in at least 18 replicates. Restriction step was done by 60 minutes incubation at 37°C with 0.5 µl RE. All runs were performed on the CFX96 instrument. Methylation results distribution for each input is shown below using box and whisker plots.





Precision

Precision of the MLH1 Methylation Detection Kit was assessed using four kit lots and multiple gDNA samples for a total of 21 replicates per region. Samples were tested over a six-month period at an input of 30 ng/reaction. The restriction step was done by a 60 minute incubation at 37°C with 0.5 µl RE. All runs were performed on the CFX96 instrument. The resulting averages for both promoter regions are tabled below.

Precision Results in Region C:

Tested Sample	FAM Restriction Reaction		FAM Reference (Uncut) Reaction		CFO560 Restriction Reaction		CFO560 Reference (Uncut) Reaction		Methylation Results			
	Average CT	SD	Average CT	SD	Average CT	SD	Average CT	SD	Average % Meth	SD	Min	Max
100% Methylation	27.92	0.21	27.89	0.22	28.10	0.33	28.13	0.26	**96.2%**	7.5%	80.1%	109.4%
50% Methylation	28.88	0.19	27.76	0.21	28.03	0.24	28.06	0.21	**45.1%**	2.1%	42.0%	49.3%
25% Methylation	29.92	0.24	27.77	0.22	28.12	0.27	28.11	0.20	**22.8%**	2.1%	20.0%	27.7%
12.5% Methylation	30.92	0.27	27.76	0.26	28.09	0.24	28.09	0.25	**11.2%**	1.8%	8.8%	14.5%
6.3% Methylation	31.91	0.27	27.69	0.28	28.01	0.24	28.03	0.27	**5.4%**	1.2%	3.9%	7.5%
Non-Methylation	38.56	1.54	27.81	0.29	28.13	0.24	28.13	0.27	**0.1%**	0.1%	0.0%	0.5%

Precision Results in Region D:

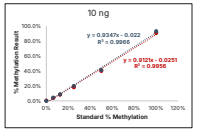
Tested Sample	FAM Restriction Reaction		FAM Reference (Uncut) Reaction		CFO560 Restriction Reaction		CFO560 Reference (Uncut) Reaction		Methylation Results			
	Average CT	SD	Average CT	SD	Average CT	SD	Average CT	SD	Average % Meth	SD	Min	Max
100% Methylation	28.26	0.32	28.21	0.34	28.18	0.26	28.20	0.28	**94.6%**	4.2%	84.7%	101.4%
50% Methylation	29.25	0.31	28.08	0.27	28.11	0.22	28.12	0.21	**44.4%**	3.7%	39.8%	51.4%
25% Methylation	30.24	0.35	28.04	0.27	28.08	0.25	28.10	0.24	**21.6%**	3.1%	17.3%	27.9%
12.5% Methylation	31.36	0.45	28.02	0.26	28.08	0.27	28.07	0.25	**10.2%**	2.5%	7.1%	15.6%
6.3% Methylation	32.24	0.57	28.00	0.26	28.08	0.26	28.06	0.25	**4.8%**	1.6%	2.5%	7.0%
Non-Methylation	36.56	0.92	28.03	0.24	28.16	0.26	28.11	0.27	**0.3%**	0.2%	0.1%	0.6%

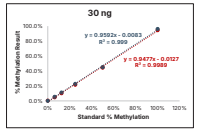
Linearity

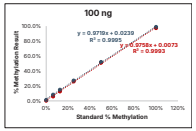
The Linearity study was conducted on the CFX96 instrument using intact gDNA methylation standards (by 60 minutes incubation at 37°C with 0.5 µl RE), and serial dilutions of FFPE samples (120 minutes incubation at 37°C with 2 µl RE).

Linearity Intact DNA

Linearity of the MLH1 Methylation Detection Assay for intact gDNA samples was assessed using a series of methylated standards at 0%, 6.25%, 12.5%, 25%, 50% and 100% methylation. These intact gDNA samples were tested at an input of 10 ng, 30 ng and 100 ng, with each data point being comprised of at least 18 replicates. Linearity was calculated by plotting the experimental results against the rates of the methylated standards. The results for intact gDNA sample inputs are shown in the graphs below, with region C graphed in blue and region D graphed in red.







Accuracy

Accuracy was assessed by first determining the methylation status of FFPE samples with the MLH1 Methylation Detection Kit (the restriction step was done by a 120 minute incubation at 37°C with 2 µl RE). In parallel, samples were treated with a bisulfite conversion kit (Entrogen, cat. # BCK-RT50), which facilitates the deamination of unmethylated cytosines into uracils, thus allowing the discrimination between methylated and non-methylated cytosines. Sanger Sequencing of the bisulfite treated samples was performed on MLH1 promoter regions C and D using region-specific primers. Sanger Sequencing is not a quantitative method, therefore, the results were qualitatively assessed as "methylated" or "non-methylated" based on observed chromatogram peaks. Samples that were outside the input range of the MLH1 assay, or failed Sanger Sequencing, were excluded from this study. A total of 84 samples were evaluated for methylation in region C and 64 were evaluated for methylation in region D. Results are summarized in the table below.

		Sanger Sequencing					
		Region C			Region D		
		Methylated	Non-Methylated	Total	Methylated	Non-Methylated	Total
MLH1 MSRE Assay	Methylated*	3**	2***	**5**	2**	0	**2**
	Non-Methylated*	0	79	**79**	0	62	**62**
Total		**3**	**81**	**84**	**2**	**62**	**64**

Region C			Region D		
PPA	NPA	OPA	PPA	NPA	OPA
3/3 (100%)	79/81 (98%)	82/84 (98%)	2/2 (100%)	62/62 (100%)	64/64 (100%)

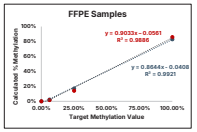
* Samples with MLH1 assay result of ≥4% methylation were considered methylated. Samples with lower values were considered non-methylated.

** These samples were identified as methylated by both MLH1 assay and Sanger sequencing.
Methylation results for positive samples in region C according to the MLH1 assay: 21.0%, 19.9% and 39.0%.
Methylation results for positive samples in region D according to the MLH1 assay: 4.0% and 29.5%.

*** These samples were identified with low methylation levels in region C by the MLH1 assay (7.6% and 4.5%), but were called as non-methylated by Sanger Sequencing.

Linearity FFPE Samples

Linearity of the MLH1 Methylation Detection Assay for FFPE samples was assessed using 5 artificially methylated FFPE samples. Samples were methylated using a methyltransferase enzyme, then serially diluted in the background of their non-methylated matched control. The graph below shows the average experimental methylation results for each serial dilution of the 5 FFPE samples plotted against the expected percent methylation rates; with region C shown in blue and Region D shown in red.



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For in vitro diagnostic use (IVD) in the European Union