Validation of EntroGen Methylation Sensitive Restriction Enzyme Based qPCR assay To Determine MLH1 Promoter Methylation Status in Colorectal and Endometrial Tumors

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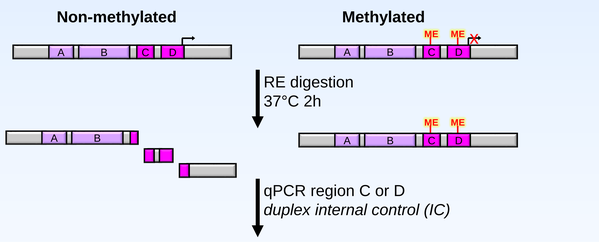
Introduction

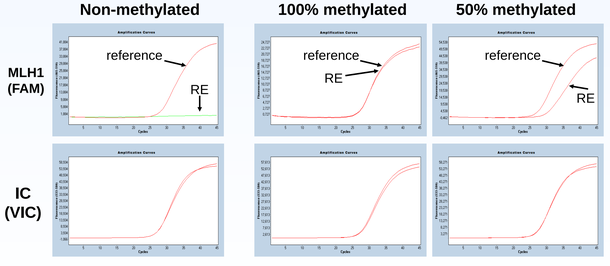
- MLH1 promotor methylation is found in colorectal and endometrial cancers of somatic origin, and result in loss of expression of mismatch repair (MMR) proteins MLH1 and PMS2.
- Lynch syndrome is an autosomal dominant genetic syndrome that is also characterized by the loss of expression of MMR proteins, but is caused predominantly by a germline mutation of MLH1 or MSH2.
- MLH1 methylation analysis is a cost and time efficient pre-screening tool prior to genetic analysis for the diagnostic of Lynch Syndrome.

Objective:
Entrogen has developed MLH1 promotor methylation detection kit that is validated for Bio-Rad CFX96 and Applied Biosystems® 7500/7500 Fast, QS5 qPCR instruments. Our objective was to test and validate the Entrogen kit on our two qPCR instruments: Roche LightCycler 480 II and Applied Biosystems® QuantStudio3.

Methods

EntroGen MLH1 Methylation Detection Kit is a Methylation Sensitive Restriction Enzyme (MSRE)-based qPCR assay. Restriction enzymes (RE) cut only the non-methylated sequences, making them unavailable for amplification. Differences in qPCR amplification of target MLH1 promoter regions C or D between the RE reaction and a reference reaction without RE are indicative of the levels of methylation in those regions. As mix contains restriction enzymes and primers/probes for qPCR, it is a single-step experiment.

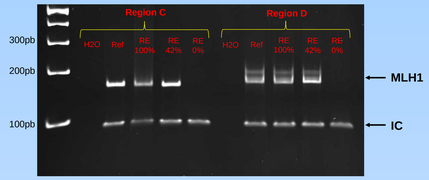




$$\% \text{ Methylation} = 2^{[(CP_{MLH1\_Ref} - CP_{MLH1\_RE}) - (CP_{IC\_Ref} - CP_{IC\_RE})]}$$

Results

Amplification specificity
Acrylamide gel electrophoresis of qPCR products for regions C and D shows amplification of the expected amplicons and the absence of non-specific amplification.



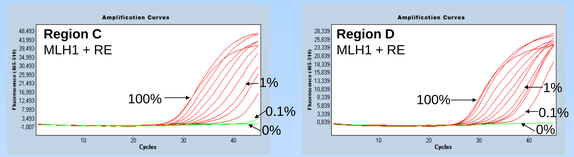
Effect of DNA concentration for FFPE tissues
DNA concentrations under and over the recommended range of 5-15ng/µl were tested, using Formalin-Fixed Paraffin-Embedded (FFPE) tissues up to two years old. CP values for the MLH1 reference reaction were within the acceptable 26-34 range for all but one 1ng/µl sample. Δ CP for IC between RE and reference reactions were all smaller than 0.5. Methylation % were similar within a range of 1-30ng/µl, suggesting that low quality FFPE tissues could still generate good results, but that highly concentrated DNA should be diluted for optimal amplification.

			Region C				Region D			
	Sample	Concentration	MLH1 CP	IC CP	% methylation	Δ CP IC	MLH1 CP	IC CP	% methylation	Δ CP IC
RE reaction	FFPE-1	148 ng/µl		27.3	none	0.03		27.2	none	0.06
		30 ng/µl	40	28.3	0.06%	0.2		28.3	none	0.13
		10 ng/µl		29.2	none	0.16		29.2	none	0.11
		5 ng/µl		31.0	none	0.19		31.5	none	0.76
		2.5 ng/µl		32.1	none	0.1		32.2	none	0.1
		1 ng/µl		33.8	none	0.48		34.6	none	1.23
	FFPE-2	148 ng/µl	38.0	26.3	0.22%	0.28	36.5	26.2	0.20%	-0.09
		30 ng/µl	30.3	26.5	25%	0.13	30.2	26.5	9.3%	0.14
		10 ng/µl	31.5	28.0	25%	0.28	31.6	27.9	9.2%	0.13
		5 ng/µl	32.4	29.0	29%	0.02	33.0	29.1	8.6%	0.09
		2.5 ng/µl	33.4	30.1	34%	0.08	34.1	30.2	8.8%	0.09
		1 ng/µl	35.8	31.7	18%	0.19	35.6	31.5	8.5%	0.17
	FFPE-3	5 ng/µl	31.2	28.9	61%	0.02	31.6	29.0	20%	0.08
		2.5 ng/µl	32.2	29.9	54%	0.04	32.4	30.0	23%	0.15
		1 ng/µl	33.7	31.6	67%	0.24	33.9	31.6	27%	0.15
	FFPE-4	5 ng/µl	34.3	29.5	11%	0.18	33.3	29.5	10%	0.18
		2.5 ng/µl	36.2	30.8	7%	0.15	34.6	30.8	10%	0.17
		1 ng/µl	37.9	32.3	6%	0.07	36.5	32.5	9%	0.32
	Sample	Concentration	MLH1 CP	IC CP		MLH1 CP 26-34	MLH1 CP	IC CP		MLH1 CP 26-34
Reference reaction	FFPE-1	148 ng/µl	31,0	27,3			28,0	27,2		
		30 ng/µl	29.2	28.1		ok	28.1	28.2		ok
		10 ng/µl	30.2	29.0		ok	29.0	29.1		ok
		5 ng/µl	31.9	30.8		ok	30.9	30.8		ok
		2.5 ng/µl	33.0	32.0		ok	32.0	32.1		ok
		1 ng/µl	34.5	33.3		out of range	33.6	33.4		ok
	FFPE-2	148 ng/µl	28.9	26.0		ok	27.6	26.3		ok
		30 ng/µl	28.2	26.3		ok	26.7	26.3		ok
		10 ng/µl	29.3	27.8		ok	28.1	27.8		ok
		5 ng/µl	30.6	29.0		ok	29.4	29.0		ok
		2.5 ng/µl	31.8	30.0		ok	30.6	30.1		ok
		1 ng/µl	33.1	31.5		ok	31.9	31.4		ok
	FFPE-3	5 ng/µl	30.5	28.9		ok	29.2	29.0		ok
		2.5 ng/µl	31.3	29.9		ok	30.1	29.9		ok
		1 ng/µl	32.9	31.3		ok	31.9	31.5		ok
	FFPE-4	5 ng/µl	31.0	29.3		ok	29.8	29.4		ok
		2.5 ng/µl	32.1	30.6		ok	31.1	30.6		ok
		1 ng/µl	33.8	32.2		ok	32.8	32.2		ok

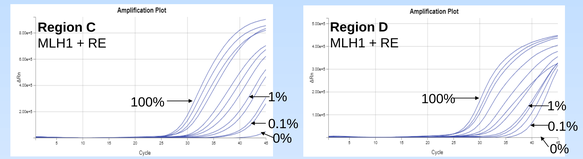
Results

Limit of detection
Dilutions of commercial methylated and non-methylated DNA controls were tested on both LC480 and QuantStudio3. We estimate that methylation was detected down to 1%, as shown with amplification curves distinct from background level of amplification for both regions C and D. The kit instruction recommends using 4% methylation as a cutoff.

LC480

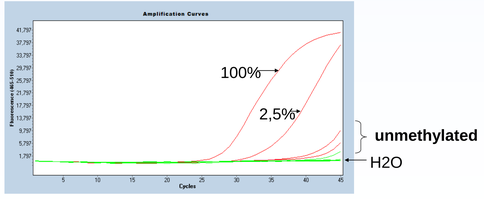


QuantStudio3



	Region C				Region D			
	LC480		QuantStudio3		LC480		QuantStudio3	
Dilution	MLH1+RE CP	% methylation	MLH1+RE Cq	% methylation	MLH1+RE CP	% methylation	MLH1+RE Cq	% methylation
100%	28.1	90%	26,4	70%	27.0	91%	25,9	101%
75%	28.6	62%	26,8	40%	27.3	71%	26,3	99%
50%	29.3	35%	27,5	31%	27.9	43%	26,5	52%
25%	30.2	18%	27,4	20%	28.8	24%	28,1	23%
10%	31.7	6%	30,2	5%	29.9	10%	29,9	5%
5%	34,0	1%	30,8	2%	31,3	4%	31,0	3%
2,5%	36.6	0.18%	31,7	1%	33.3	0.91%	31,8	2%
1%	39.0	0.04%	33,6	0,28%	35.7	0.19%	34,5	0,19%
0,5%	40	0,02%	34,9	0,10%	36.8	0.09%	35,3	0,13%
0,25%	40	0.02%	37,9	0,01%	37.1	0.07%	36,7	0,05%
0,1%	-	none	38,7	0,01%	40	0.01%	38,3	0,02%
0%	-	none	43,6	0%	-	none	-	none

Background level of amplification
Small amplification curves can often be measured in non-methylated samples and in the positive control of the kit. Amplification is caused either by incomplete RE digestion of non-methylated DNA, or by background level of methylation in DNA. Calculated methylation level in non-methylated samples is usually under 0.1%, well under the recommended cutoff of 4%.



Results

Correlation for patients
We tested 31 patients whose methylation status had been measured in a reference lab using bisulfite conversion followed by qPCR using TaqMan probes. Correlation was 94% (29/31). For endometrial cancer tissues, we used the remaining of the FFPE blocks, which may explain the negative result for Endometrial-12. For Colorectal-6, methylation was detected, but was under the cutoff of 4%.

Sample	Known methylation status	Region C		Region D	
		LC480	QuantStudio3	LC480	QuantStudio3
Endometrial-1	Methylated	11%	12%	16%	7%
Endometrial-2	Methylated	32%	13%	32%	18%
Endometrial-3	Methylated	17%	7%	2%	2%
Endometrial-4	Methylated	49%	16%	25%	19%
Endometrial-5	Methylated	9%	9%	8%	8%
Endometrial-6	Methylated	14%	13%	7%	5%
Endometrial-7	Methylated	23%	18%	16%	9%
Endometrial-8	Methylated	43%	39%	43%	38%
Endometrial-9	Methylated	15%	28%	19%	21%
Endometrial-10	Methylated	46%		32%	
Endometrial-11	Methylated	47%		50%	
Endometrial-12	Methylated	0,08%		0,03%	
Endometrial-13	Methylated	11%		11%	
Colorectal-1	Methylated	40%	58%	15%	8%
Colorectal-2	Methylated	54%	49%	10%	8%
Colorectal-3	Methylated	48%	12%	26%	37%
Colorectal-4	Methylated	28%	20%	12%	7%
Colorectal-5	Methylated	59%	86%	4%	4%
Colorectal-6	Methylated	2%	4%	0,70%	1%
Colorectal-7	Methylated	54%	47%	1%	2%
Colorectal-8	Methylated	18%		15%	
Colorectal-9	Non-methylated	0,14%	0,01%	0%	none
Colorectal-10	Non-methylated	0,06%	none	0,03%	none
Colorectal-11	Non-methylated	none	none	none	none
Colorectal-12	Non-methylated	0,07%	none	none	none
Colorectal-13	Non-methylated	0,05%		none	
Colorectal-14	Non-methylated	0,33%		none	
Colorectal-15	Non-methylated	0,07%		0,03%	
Colorectal-16	Non-methylated	none		none	
Colorectal-17	Non-methylated	0,06%		none	
Colorectal-18	Non-methylated	0,24%		none	

Conclusion

- The MSRE-based qPCR assay of the EntroGen kit, is an easy, fast and robust method to measure MLH1 promotor methylation in tumor tissues, with hand-on time of 20-30 minutes and no bisulfite modification step.
- Lower DNA concentrations can generate good results, including DNA extracted from two years old FFPE tissues.
- Methylation results obtained with the EntroGen kit correlate with those obtained with a bisulfite method in a reference lab.
- Small pipetting variations can result in a larger methylation % difference. This test is not quantitative, but % accuracy is important around the cutoff to determine if methylated or non-methylated.
- EntroGen MLH1 Methylation Detection Kit was validated on our two qPCR instruments, Roche LightCycler 480 and Applied Biosystems® QuantStudio3, and is now routinely used in our lab to determine MLH1 methylation status in colorectal and endometrial tumors, allowing to differentiate between sporadic (MLH1 methylation) and hereditary (MLH1 germline mutation) cancers.