Quantitative Methylation Specific PCR (qMSP)

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[Abstract] Detection of low copies of methylated DNA targets in clinical specimens is challenging. The quantitative Methylation-Specific PCR (qMSP) assays were designed to specifically amplify bisulphite-converted methylated DNA target sequences in the presence of an excess of unmethylated counterpart sequences. These qMSP assays are real-time PCR assays utilizing, sequence-specific primers and an intervening, also sequence specific, Taqman probe to cover an amplicon of approximately 100 bp in length. The use of Taqman probes bearing a minor groove binding (MGB) allow for the use of shorter probes and therefore facilitate design and significantly increases the analytical specificity of the reaction. In the context of the biomarker discovery program of the Liverpool Lung Project (LLP), ten gene promoters were selected. qMSP assays were developed, validated and used to screen 655 bronchial washings from patients with lung cancer and age/sex matched controls with non malignant lung disease (Nikolaidis *et al*., 2012).

Materials and Reagents

1. TaqMan® Universal Master Mix II no UNG (Life Technologies, catalog number: 4440039)
2. TaqMan MGB probes (custom synthesis) (Life Technologies)
3. EZ DNA Methylation-Gold™ kit (ZymoResearch, catalog number: D5006)
4. The methylation-specific primer and probe sequences (see Recipes)

Equipment

1. PCR cabinet
2. PCR plates
3. Centrifuge (Sigma-Aldrich)
4. PCR thermal cycler (Life Technologies/Applied Biosystems, model: 9700)
5. Real time PCR machine (Life Technologies/Applied Biosystems, model: 7500 FAST)

Procedure

1. Primer/probe design

Note: Primer/probe design is of major importance for the specificity of the reaction, i.e. to amplify only methylated bisulphite-converted target in the presence of excess unmethylated target. The primers should include 3-5 CG dinucleotides. The inclusion of at least two such CGs within the six 3' end of the primer significantly increases specificity. This is in addition to the usual rules on primer stability, GC content and secondary structure avoidance apply in real time PCR assay design.

2. One µg DNA was converted by sodium bisulphite using the EZ DNA Methylation-Gold™ kit and following the supplier's protocol but eluting in 50 µl elution buffer (instead of the recommended 10 µl).
3. The qMSP reactions contained 1x TaqMan® Universal Master Mix II non-UNG, 250 nM probe, 300-900 nM primers (Table 1) and 2 µl eluate from the bisulphite treated DNA sample.

   Note: The primer concentration is an important determinant of analytical sensitivity/specificity and has to be ascertained experimentally In other words the analytical sensitivity threshold is set to the dilution that has an overlapping 95% Confidence Interval with the unmethylated control reaction. In practical terms, the highest sensitivity one can use is the one that is always at least 2 ΔCt. lower than the unmethylated control.

Table 1. Primer-probe concentrations for oligo mixes

Primer/probe mix	Final concentration (nM)		
	Fwd primer	Rev Primer	Probe
p16	700	700	250
TERT	250	250	250
RASSF1	700	700	250
TMEFF2	900	900	250
CYGB	300	300	250
RARb	500	500	250
DAPK1	250	250	250
p73	250	250	250
WT1	750	750	250
CDH13	250	250	250
ACTB	900	900	250

4. PCR plates were sealed and span at 4,000 *x g* for 1 min prior to be placed in the thermal cycler in order to bring all the reaction volume to the bottom and ensure removal of bubbles from the reaction mix.

5. The reactions were performed in duplicate on a 7500 FAST real time cycler under the following thermal profile: 95 °C for 10 min activation step followed by 50 cycles consisted of denaturation at 95 °C for 15 sec, annealing and extension at 58 °C-65 °C (Table 2, depending on the assay) for 1 min.

Table 2. Annealing information for qMSP optimised conditions

Genes	Annealing temp (°C)	Time (sec)
p16	60	60
RASSF1	60	60
CYGB	64	5
	61	55
RARB	65	5
	62	55
TERT	65	5
	62.5	55
WT1	62	60
ACTB	58	20
	60	40
CDH13	64	5
	61	55
DAPK	65	5
	62.5	55
P73	65	5
	62.5	55
TMEFF	58	20
	60	40

Recipes

1. The methylation-specific primer and probe sequences are listed in Table 3. In the initial steps of assay development it became apparent that probes bearing minor groove binding moiety (Taqman MGB probes) provided significantly higher assay specificity. In addition, due to their smaller size, they allow for a more flexible assay design.

Table 3. Nucleotide sequences of methylation specific primers and probes for the qMSP assays utilised in the BW screening. The ACTB assay is methylation-independent acting as DNA input control.

Primer/probe name	Sequence 5' →3'	Modification
p16meth_F	GGAGGGGGTTTTTTCGTTAGTATC	
p16meth_R	CTACCTACTCTCCCCCTCTCCG	
p16meth_P	AACGCACGCGATCC	FAM-MGB
RASSF1meth_F	GTGGTGTTTTGCGGTCGTC	
RASSF1meth_R	AACTAAACGCGCTCTCGCA	
RASSF1_P	CGTTGTGGTCGTTCG	FAM-MGB
TMEFF2meth_F	GGAGAGTTAAGGCGTTTCGTAGTTC	
TMEFF2meth_R	CGTGGGAAGAGGTAGTCGGG	
TMEFF2meth_P	GTTTTTAGTTCGTTCG	FAM-MGB
TERTmeth_F	TTGGGAGTTCGGTTTGGTTTC	
TERTmeth_R	CACCCTAAAAACGCGAACGA	
TERTmeth_P	AGCGTAGTTGTTTCGG	FAM-MGB
CYGBmeth_F	GTGTAATTTCGTCGTGGTTTGC	
CYGBmeth_R	CCGACAAAATAAAAACTACGCG	
CYGBmeth_P	TGGGCGGGCGGTAG	FAM-MGB
RARbmeth_F	GATTGGGATGTCGAGAACGC	
RARbmeth_R	ACTTACAAAAAACCTTCCGAATACG	
RARbmeth_P	AGCGATTCGAGTAGGGT	FAM-MGB
DAPK1meth_F	CGAGCGTCGCGTAGAATTC	
DAPK1meth_R	ACCCTACAAACGAACTAACGACG	
DAPK1meth_P	AGCGTCGGTTTGGTAG	FAM-MGB
p73meth_F	TTGTTTTTTGGATTTTAAGCGTTTC	
p73meth_R	CACCCGAATCTCTCCTAACCG	
p73meth_P	TAACGCTAAACTCCTCG	FAM-MGB
WT1meth_F	GAGGAGTTAGGAGGTTCGGTC	
WT1meth_R	CACCCCAACTACGAAAACG	
WT1meth_P	AGTTCGGTTAGGTAGC	FAM-MGB

CDH13meth_F	CGTGTATGAATGAAAACGTCGTC	
CDH13meth_R	CACAAAACGAACGAAATTCTCG	
CDH13meth_P	CGTTTTTAGTCGGATAAAA	FAM-MGB
ACTBmgb_F	GGGTGGTGATGGAGGAGGTT	
ACTBmgb_R	TAACCACCACCCAACACACAAT	
ACTBmgb_P	TGGATTGTGAATTTGTGTTTG	VIC-MGB

Cycle threshold (Ct) values for each target were normalized for DNA input by calculating the $\Delta Ct=Ct_{(Target)}-Ct_{(ACTB)}$. The values for al samples were transformed to relative quantity (RQ) compare to the *calibrator* (0.5% standard methylated DNA dilution) included in all experiments using the following type:

$RQ_{sample} = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$.

Note: qMSP is a challenging version of real-time PCR and one needs to gain a very good understanding *of the latter prior to engaging in qMSP experiments. The main additional challenge is the use of bisulphite DNA which is of lower quality but most importantly of lower complexity. This significantly affects the thermodynamic behavior of this template in the reaction. The authors are very happy to provide assistance to colleagues if needed; please email Dr. T Liloglou (tliloglo@liv.ac.uk).*

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This protocol is adapted from Nikolaidis *et al*. (2012).

References

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