Supplemental Data

MNAzyme qPCR with superior multiplexing capacity

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Contents

1. Target information and oligonucleotides

1.1. Genes or transcripts amplified in manuscript

Targets amplified and quantified in this study included the genes *KRAS* (NT_009714.16), *TFRC* (NT_029928.13), *B2M* (NT_010194.8), *HMBS* (NT_ 033899), *HFE* (NT_007592.15), *HPRT1* (NT_011786.16), *GSTP1* (NT_167190.1), *RARB* (NT_022517.18), *APC* (NT_034772.6) and *ACTB* (NT_007819.17) and the transcripts *KRAS* (NM_033360), *RPLP0* (NM_001002), *HMBS* (NM_000190), and *ACTB* (NM_001101), *BCR* (NM_004327.3), *TP53* (NM_000546.5), *PGK1* (NM_000291), *PMM1* (NM_002676), *PSMB6* (NM_002798.1), *TFRC* (NM_003234), *B2M* (NM_004048) *PPIA* (NM_021130) and *HPRT1* (NM_000194.2)

1.2. Oligonucleotides used in manuscript

The sequences of all oligonucleotides used in this paper are listed in Table 1. In the partzyme sequences; the bases in underlined type form at least part of the active catalytic core of the assembled MNAzyme, bases in bold type hybridise with the target, and bases in italics type hybridise to the substrate. The “-P” at the end of a partzyme name indicates that the oligonucleotide was 3’ phosphorylated.

Unless stated otherwise the PCR primer amplifies both DNA and RNA transcript and partzymes detect both DNA and cDNA. When designated (RNA) or “r” the oligo is specific for RNA and when designate (DNA) or “d” the oligo is specific for DNA otherwise the oligonucleotide can be used for both DNA and RNA. “For” represents Forward primer and “Rev” represents Reverse primer. Primer names that are followed with a (U) denote a universal tag sequence has been added to the primer to increase its Tm (see section 1.3 for more details).

Table 1. PCR primers and partzyme A and B sequences

Type	Name	Sequence (5’-3’)
For primer (RNA)	5rTRFC(U)	CTAAGGTCATCAGGATTGCCTAATA
For primer (DNA)	5dTRFC(U)	CTAAAACAATAACTCAGAACTTACG
Rev primer	3TFRC(U)	CTAACTTTCTGAGGTTACCATCCTA
Partzyme A	TFRCA/3-P	**GGAATATGGAAGGAGACTGTC**ACAACGA*GGTTGTGCTG*
Partzyme B	TFRCB/3-P	*CGGTTGGTGA*GGCTAGCT**CCTCTGACTGGAAAACAGACT**
Partzyme A	TFRCA/2-P	**GGAATATGGAAGGAGACTGTC**ACAACGA*GAGGAAACCTT*
Partzyme B	TFRCB/2-P	*TGCCCAGGGA*GGCTAGCT**CCTCTGACTGGAAAACAGACT**

For primer (RNA)	5rB2M(U)	CTAAGGCTATCCAGCGTACTCCAAAG
For primer (DNA)	5dB2M(U)	CTAATCTTTTCCCGATATTCCTCAG
Rev primer	3B2M(U)	CTAAAGCAATTCAGGAAATTTGAC
Partzyme A	B2MA4/6-P	**ATTCAGGTTTACTCACGTCATC**ACAACGA*GAGGCGTGAT*
Partzyme B	B2MB5/6-P	*CTGGGAGGAA*GGCTAGCT**CAGCAGAGAATGGAAAGTCAAA**
For primer (RNA)	5rKRAS	GCCTGCTGAAAATGACTGAATA
For primer (DNA)	5dKRAS	TAGTGTATTAACCTTATGTGTGAC
Rev primer	3KRAS	AATGATTCTGAATTAGCTGTATC
Partzyme A	KRASA/2-P	**TAAACTTGTGGTAGTTGGAG**ACAACGA*GAGGAAACCTT*
Partzyme B	KRASB/2-P	*TGCCCAGGGA*GGCTAGCT**CTGGTGGCGTAGGCAAGAGTGCC**
Partzyme A	KRASA/4-P	**TAAACTTGTGGTAGTTGGA**GACAACGA*GTGCGCCATG*
Partzyme B	KRASB/4-P	*TACTTCTCCCAA*GGCTAGCT**CTGGTGGCGTAGGCAAGAGTGC**C
Rev primer (RNA)	5rRPLP0	CATTCTATCATCAACGGGTA
Rev primer (RNA)	3rRPLP0	CAAAGGCAGATGGATCAG
Partzyme A (RNA)	rRPLP0 A/2-P	**CAAACGAGTCCTGGCCTTGTCT**ACAACGA*GAGGAAACCTT*
Partzyme B (RNA)	rRPLP0 B/2-P	*TGCCCAGGGA*GGCTAGCT**GTGGAGACGGATTACACCTTC**
For primer (RNA)	5rACTB_1	CATTGCCGACAGGATGCAGA
For primer (RNA)	5rACTB_1(U)	CTAACATTGCCGACAGGATGCAGA
Rev primer (RNA)	3rACTB_1	GAGCCGCCGATCCACACG
Partzyme A (RNA)	rACTB_1A/3-P	**TGAAGATCAAGATCATTGCTCC**ACAACGA*GGTTGTGCTG*
Partzyme B (RNA)	rACTB_1B/3-P	*CGGTTGGTGA*GGCTAGCT**TCCTGAGCGCAAGTACTCCG**
Partzyme A (RNA)	rACTB_1A/2-P	**TGAAGATCAAGATCATTGCTCC**ACAACGA*GAGGAAACCTT*
Partzyme B (RNA)	rACTB_1B/2-P	*TGCCCAGGGA*GGCTAGCT**TCCTGAGCGCAAGTACTCCG**
For primer	5HPRT1(U)	CTAACTTTGCTGACCTGCTGGATTA
For primer	5HPRT1	CTTTGCTGACCTGCTGGATTA
Rev primer (RNA)	3rHPRT1	CCTGTTGACTGGTCATTACAA
Rev primer (DNA)	3dHPRT1	CAATAGCTCTTCAGTCTGATAA
Partzyme A	HPRT1A/2-P	**CTGAATAGAAATAGTGATAGATC**ACAACGA*GAGGAAACCTT*
Partzyme B	HPRT1B/2-P	*TGCCCAGGGA*GGCTAGCT**CATTCCTATGACTGTAGATTTTA**
Partzyme A	HPRT1A/7-P	**ACTGAATAGAAATAGTGATAGAT**ACAACGA*GTGCCATGTTAA*
Partzyme B	HPRT1B/7-P	*TATCACAGCCAA*GGCTAGCT**CCATTCCTATGACTGTAGATT**
For primer	5HMBS	ACCCACACACAGCCTACTTTC
For primer	5HMBS(U)	CTAAACCCACACACAGCCTACTTTC
Rev primer (RNA)	3rHMBS	TACCCACGCGAATCACTCTCA
Rev primer (DNA)	3dHMBS	AGCCCAAAGTGTGCTGGTCA
Partzyme A (RNA)	rHMBSA/6-P	**GCCATGTCTGGTAACGGCAA**ACAACGA*GAGGCGTGAT*
Partzyme B (RNA)	rHMBSB/6-P	*CTGGGAGGAA*GGCTAGCT**TGCGGCTGCAACGGCGGAA**
Partzyme A (RNA)	rHMBSA/2-P	**GCCATGTCTGGTAACGGCAA**ACAACGA*GAGGAAACCTT*
Partzyme B (RNA)	rHMBSB/2-P	*TGCCCAGGGA*GGCTAGCT**TGCGGCTGCAACGGCGGAA**

Partzyme A (DNA)	dHMBSA/6-P	**GCCATGTCTGGTAACGGCAA**ACAACGA*GAGGCGTGAT*
Partzyme B (DNA)	dHMBSB/6-P	*CTGGGAGGAA*GGCTAGCT**TGCGGCTGCAACGGCGGTG**
For primer (DNA)	3dHFE	TCCAGGCAAAAGAACAATTG
Rev primer (DNA)	5dHFE	GGGTGGAGGTCTCTAGG
Partzyme A (DNA)	dHFEA/3-P	**GGCTCCTGAGAGAGGCCTA**ACAACGA*GGTTGTGCTG*
Partzyme B (DNA)	dHFEB/3-P	*CGGTTGGTGA*GGCTAGCT**CCTCGGGCCTTTCCCCACTC**
For primer (RNA)	5rBCR(U)	CTAACACTCAGCCACTGGATTTAAG
Rev primer	3BCR	GCGCGTCTTTGCTTTATTCA
Partzyme A (RNA)	rBCRA/2-P	**GTTCAAATCTGTACTGCACC**ACAACGA*GAGGAAACCTT*
Partzyme B (RNA)	rBCRB/2-P	*TGCCCAGGGA*GGCTAGCT**CTGGAGGTGGATTCCTTTGG**
For primer (RNA)	5rPGK1(U)	CTAATTGGAGAGCCCAGAGCGAC
Rev primer	3PGK1	CATTCCACCACCAATAATCATC
Partzyme A	PGK1A/2-P	**AGCTAAAGTTGCAGACAAGATC**ACAACGA*GAGGAAACCTT*
Partzyme B	PGK1B/2-P	*TGCCCAGGGA*GGCTAGCT**CAGCTCATCAATAATATGCTGG**
For primer	5PMM1	CGGAATGGCATGCTGAACATC
Rev primer (RNA)	3rPMM1	CTGTTTTCAGGGCTTCCACGA
Partzyme A	PMM1A/2-P	**CCGGAGCTGCACCCTGGAG**ACAACGA*GAGGAAACCTT*
Partzyme B	PMM1B/2-P	*TGCCCAGGGA*GGCTAGCT**GAGAGGATCGAGTTCTCCGA**
For primer (RNA)	5rPMSB6	GGACTCCAGAACAACCACTG
Rev primer	3PSMB6	CAGCTGAGCCTGAGCGACA
Partzyme A	PSMB6A/2-P	**GAGTGACTGACAAGCTGACAC**ACAACGA*GAGGAAACCTT*
Partzyme B	PSMB6B/2-P	*TGCCCAGGGA*GGCTAGCT**CTATTCACGACCGCATTTTCT**
For primer (RNA)	5TP53_1	CATCATCACACTGGAAGACTC
Rev primer	3TP53	CTCTGTGCGCCGGTCTCTC
Partzyme A	TP53A/2-P	**GACGGAACAGCTTTGAGGTG**ACAACGA*GAGGAAACCTT*
Partzyme B	TP53B/2-P	*TGCCCAGGGA*GGCTAGCT**CGTGTTTGTGCCTGTCCTGG**
For primer (RNA)	5PPIA_1	ATCTGCACTGCCAAGACTGAG
Rev primer	3PPIA	GCGCTCCATGGCCTCCAC
Partzyme A	PPIAA/3-P	**TGGTTGGATGGCAAGCATGTG**ACAACGA*GGTTGTGCTG*
Partzyme B	PPIAB/3-P	*CGGTTGGTGA*GGCTAGCT**GTGTTTGGCAAAGTGAAAGAAG**

Table 2. PCR primers and partzyme A and B sequences for use with MSP quadruplex MNAzyme assay

Name	Sequence (5' to 3')	Source
Outer primers		
O5GP1	CGGTTAGTTGCGCGGCGATTTCG	(B)
O3GSTP1	CGAAAACCTCGCGACCTCCGAAC	(B)
O5APC	CACTACGAAATACGAATCGAAAAACGA	(B)
O3APC	AGTGATATTTTGGCGGGTTGTATTAATA	(B)
O5RAR	CGAGTTGTTTGAGGATTGGGATGTC	(B)

O3RAR	TTACTCGACCAATCCAACCGAAACG	(B)
Inner Primers		
I5GP1	TGTAGCGGTCGTCGGGGTTG	(A)
I3GP1	GCCCCAATACTAAATCACGACG	(A)
I5APC	CGCTAAATACGAACCAAAACGC	(B)
I3APC	TAGTTATATGTCGGTTACGTGCG	(B)
I5RAR	AACGCGAGCGATTCGAGTAG	(A)
I3RAR	CTTACAAAAAACCTTCCGAATACG	(A)
I5ACT	GGAGTATATAGGTTGGGGAAGTTTG	(A)
I3ACT	AACACACAATAACAAACACAAATTCAC	(A)
Partzymes		
GSTP1A/2-P	CGACCGCTCTTCTAAAAAATCCCTACAACGAGAGGAAACCTT	(B)
GSTP1B/2-P	TGCCCAGGGAGGCTAGCGCGAACTCCCGCCGACCC	(B)
APCA/7-P	CGTTTATATTTAGTTAATCGGACAACGAGTGCCATGTTAA	(B)
APCB/7-P	TATCACAGCCAAGGCTAGCTCGGGTTTTCGACGGGAA	(B)
RARA/6-P	TTCCGAATCCTACCCCGTACAACGAGAGGCGTGAT	(B)
RARB/6-P	CTGGGAGGAAGGCTAGCACGATACCCAAACAAACC	(B)
ACTA/3-P	CAAAAAACTTACTAAACCTCCTACAACGAGGTTGTGCTG	(B)
ACTB/3-P	CGGTTGGTGAGGCTAGCTCCATCACCACCCCACACA	(B)

Sourced from (A) Vener *et al* (2008) or (B) designed in house.

1.3. Universal method to improve efficiency of MNAzyme qPCR assays

MNAzyme qPCR utilises asymmetric primer ratios to ensure an excess of the strand that contains the target sequence the MNAzyme binds. Limiting the concentration of one of the primers changes its thermodynamics in the reaction. In most cases there is no negative impact to the amplification efficiency however there are occasions where the efficiency is affected seen by either increased Cqs or lower reaction efficiencies (Sanchez et al, 2004). To compensate for this we have added a short generic tag of ~10°C to the 5' end of the limiting primer, when this has been used we place a (U) after the primer name. The generic tag has also been used on the primer in excess which further improved the amplification efficiency in some cases. The use of a generic tag instead of gene specific sequence to increase the Tm of the primer, maintains the specificity of the primer for its intended target while attaining a primer with a higher Tm after the first round of amplification.

2. Further experimental details

2.1. Standard MNAzyme qPCR; singleplex vs triplex; Primers, partzymes, substrates and their respective concentrations

The comparison of a single verse multiplex MNAzyme qPCR reaction was performed using the genes *TFRC*, *B2M* and *KRAS.* The oligonucleotides used for each gene are displayed in Table 3. The sequences of each oligonucleotide are available in Table 1. The concentration of the oligonucleotides used for each target whether singleplexed or multiplexed was 40 nM forward primer, 200 nM of each reverse primer, partzyme A, partzyme B and probe. Primer names that are followed with a (U) denote that a universal tag sequence has been added to the primer to increase its Tm (see section 1.3).

Table 3. Oligonucleotides used in the singleplex and triplex reactions

Gene	Forward Primer	Reverse Primer	Partzyme A	Partzyme B	Reporter Probe
TFRC	5dTFRC(U)	3TFRC(U)	TFRCA/3-P	TFRCB/3-P	Sub3-JB
B2M	5dB2M(U)	3B2M(U)	B2MA/6-P	B2MB/6-P	Sub6-TRB2
KRAS	5dKRAS	3KRAS	KRASA/2-P	KRASB/2-P	Sub2-FB

2.2. Quantification of a gene of interest (GOI) with two reference genes (RG); Primers, partzymes, substrates and their respective concentrations

MNAzyme single and triplex RT-qPCR amplification and quantification was performed on a series of gene transcripts of interest (GOI) *ACTB*, *BCR*, *HPRT1*, *KRAS*, *TP53*, *HMBS*, *PGK1*, *PMM1*, *PSMB6*, *RPLP0*, *TFRC*, and two gene transcripts *B2M* and *PPIA* to be used as reference gene transcripts (RG). The oligonucleotides used for each gene are displayed in Table 4. The sequences of each oligonucleotide are available in Table 1. The concentration of the oligonucleotides used for each target whether singleplexed or multiplexed was 40 nM forward primer, 200 nM of each reverse primer, partzyme A, partzyme B and probe. Primer names that are followed with a (U) denote a universal tag sequence has been added to the primer to increase its Tm (see section 1.3).

Table 4. Oligonucleotides used for each GOI and RG

Gene	Forward Primer	Reverse Primer	Partzyme A	Partzyme B	Reporter Probe
TFRC	5rTRFC(U)	3TFRC	TFRCA/2-P	TFRCB/2-P	Sub2-FB
KRAS	5rKRAS	3KRAS	KRASA/2-P	KRASB/2-P	Sub2-FB
RPLP0	5rRPLP0	3rRPLP0	rRPLP0 A/2-P	rRPLP0 B/2-P	Sub2-FB
ACTB_1	5rACTB_1(U)	3ACTB_1	rACTB_1A/2-P	rACTB_1B/2-P	Sub2-FB
HPRT1	5HPRT1(U)	3rHPRT1	HPRT1A/2-P	HPRT1B/2-P	Sub2-FB
HMBS	5HMBS(U)	3rHMBS	rHMBSA/2-P	rHMBSB/2-P	Sub2-FB
BCR	5rBCR(U)	3BCR	rBCRA/2-P	rBCRB/2-P	Sub2-FB
PGK1	5rPGK1(U)	3PGK1	PGK1A/2-P	PGK1B/2-P	Sub2-FB
PMM1	5PMM1	3rPMM1	PMM1A/2-P	PMM1B/2-P	Sub2-FB
PSMB6	5rPMSB6	3PSMB6	PSMB6A/2-P	PSMB6B/2-P	Sub2-FB
TP53	5rTP53	3TP53	TP53A/2-P	TP53B/2-P	Sub2-FB
B2M	5rB2M(U)	3B2M	B2MA/6-P	B2MB/6-P	Sub6-TRB2
PPIA	5rPPIA	3PPIA	PPIAA/3-P	PPIAB/3-P	Sub3-JB

2.3. Quintuplex MNAzyme RT-qPCR; Primers, partzymes, substrates and their respective concentrations

Single-tube quintuplex MNAzyme RT-qPCR amplification and quantification was performed on the genes *KRAS*, *RPLP0, HMBS, HPRT1* and *ACTB.* The oligonucleotides used for each gene are displayed in Table 5. The sequences of each oligonucleotide are available in Table 1. The concentrations of each oligonucleotide used in the quintuplex reaction are displayed in Table 5. Some minor optimisation was required for the efficient and robust amplification of all targets in the quintuplex MNAzyme RT-qPCR. The primer concentration of the lower expressing genes, like *HPRT1*, was increased to improve amplification efficiency. The reporter probes that were labelled with a weaker dye, like Alexa 350 or were known to be less catalytically active than the others, like Sub4, were also increased (optimisation data not shown).

Table 5. Oligonucleotides and their concentration used in the quintuplex MNAzyme RT-qPCR reaction

Gene	Forward Primer	Reverse Primer	Partzyme A	Partzyme B	Reporter Probe
KRAS	5rKRAS 40 nM	3KRAS 200 nM	KRASA/4-P 200 nM	KRASB/4-P 200 nM	Sub4-TRB2 400 nM[+]
RPLP0	5rRPLP0 40 nM	3rRPLP0 200 nM	rRPLP0 A/2-P 200 nM	rRPLP0 B/2-P 200 nM	Sub2-A3B 600 nM[+]
ACTB_1	5rACTB_1 60 nM[+]	3rACTB_1 300 nM[+]	rACTB_1A/3-P 200 nM	rACTB_1B/3-P 200 nM	Sub3-Q6B2 200 nM
HPRT1	5HPRT1 80 nM[+]	3rHPRT1 400 nM[+]	HPRT1A/7-P 200 nM	HPRT1B/7-P 200 nM	Sub7-JB 200 nM
HMBS	5HMBS 60 nM[+]	3rHMBS 300 nM[+]	rHMBSA/6-P 200 nM	rHMBSB/6-P 200 nM	Sub6-FB 200 nM

[+] Oligonucleotide concentration was increased to improve the amplification and detection

2.4. MSP quadruplex MNAzyme qPCR on methylated and unmethylated gDNA; Primers, partzymes, substrates and their respective concentrations

The real-time PCR amplification and MNAzyme quantification of *GSTP1, RARB, APC and ACTB* was performed in a quadruplex reaction. The oligonucleotides used for each gene are displayed in Table 6. MNAzymes were designed to the regions identified by Vener and colleagues (2008) and some primer sequences were sourced from their paper. The sequences of each oligonucleotide are available in Table 2. The concentrations of each oligonucleotide used in the quadruplex reaction are also displayed in Table 6. Some minor primer concentration optimisation was required for the efficient and robust amplification of all targets in the MSP quadruplex MNAzyme qPCR as indicated in Table 6.

Table 6. Oligonucleotides used in MNAzyme MSP assay

Gene	5'Outer Primer	3'Outer Primer	5'Inner Primer[#]	3'Inner Primer[#]	Partzyme A[^]	Partzyme B[^]	Reporter Probe
GSTP1	O5GP1 20 nM	O3GP1 20 nM	I5GP1 200 nM	I3GP1 40 nM	GP1A/2-P 200 nM	GP1B/2-P 200 nM	Sub2-FB 200 nM
RARB	O5RAR 20 nM	O3RAR 20 nM	I5RAR 200 nM	I3RAR 40 nM	RARA/6-P 200 nM	RAR/6-P 200 nM	Sub6-Q5B2 200 nM
APC	O5APC 20 nM	O3APC 20 nM	I5APC 300 nM[+]	I3APC 60 nM[+]	APCA/7-P 200 nM	RO5B/7-P 200 nM	Sub7-TRB2 200 nM
ACTB	O5ACT 20 nM	O3ACT 20 nM	I5ACT 200 nM	I3ACT 40 nM	ACTA/3-P 200 nM	ACTB/3-P 200 nM	Sub3-Q6B2 200 nM

[+] Oligonucleotide concentration was increased to improve amplification and detection.
[#] Sequences from Vener et al (2008)

2.5. MNAzyme qPCR; standard vs rapid thermocycling; Primers, partzymes, substrates and their respective concentrations

PCR amplification and MNAzyme quantification of *HMBS*, *HFE* and *HPRT1* was performed by comparing standard and rapid thermocycling parameters. The oligonucleotides used for each gene are displayed in Table 7. The sequences of each oligonucleotide are available in Table 1. The concentration of the oligonucleotides used for each target was 40 nM forward primer, 200 nM of each reverse primer, partzyme A, partzyme B and probe.

Table 7. Oligonucleotides used in the standard and rapid MNAzyme qPCR reaction

Gene	Forward Primer	Reverse Primer	Partzyme A	Partzyme B	Reporter Probe
HPRT1	5HPRT1	3dHPRT1	HPRT1A/2-P	HPRT1B/2-P	Sub2-Q7B2
HMBS	5HMBS	3dHMBS	dHMBSA/6-P	dHMBSB/6-P	Sub6-FB
HFE	5dHFE	3dHFE	dHFEA/3-P	dHFEB/3-P	Sub3-JB

3. Further result details

3.1. Standard MNAzyme qPCR

A calibration curve was generated for each gene when single and triplexed and the reaction efficiency and correlation coefficient are shown in Table 8. For each gene the multiplex assay was compared to a singleplex and the delta Cq was calculated from the difference in Cq between single and triplex reactions for each of the six standards and then averaged.

Table 8. Comparison of singleplex vs triplex data

Target	-plex	Efficiency	R^2	Average ΔCq	SD
KRAS	single	100.2%	0.999	0.12	0.08
	tri	103.0%	0.999		
TFRC	single	99.7%	0.999	0.12	0.10
	tri	99.3%	0.998		
B2M	single	103.8%	0.998	0.11	0.06
	tri	103.0%	0.999		

3.2. Quantification of a gene of interest (GOI) with two reference genes (RG)

To determine the effect of multiplexing a GOI with 2 RGs, the Cqs of the singleplex and multiplex reaction were compared (Table 9). Multiplexing the assays showed very little impact on Cq values when compared to singleplex, the delta Cq ranged from 0.03 to 0.21.

Table 9. Cq values compared between GOI singleplex reaction and triplex reaction with two different RGs.

	Genes	Singleplex	Triplex	ΔCq
GOI	***TFRC***	21.2	21.2	0.03
	PMM1	25.0	25.1	0.12
	PSMB6	24.8	24.7	0.14
	RPLP0	18.3	18.4	0.07
	KRAS	24.1	24.1	0.06
	TP53	25.1	24.7	0.16
	ACTB_1	20.6	20.5	0.13
	BCR	24.2	24.0	0.05
	HPRT1	22.3	22.1	0.21
	HMBS	24.4	24.0	0.13
	PGK1	22.3	22.4	0.20
RG	***PPIA***	19.6	19.5 +/-0.14	0.07
	B2M	20.5	20.6 +/-0.15	0.01

All GOI and RG singleplex data represents the average of a duplicate reaction. The RG triplex represents the average of 11 duplicate reactions.

3.3. MSP quadruplex MNAzyme qPCR on methylated and unmethylated gDNA

Each reaction was run in duplicate and the Cq values for each duplicate were averaged and are displayed in Table 10. The three methylation markers all produced duplicate Cq values for detection of 1 methylated allele amplified in a background of 1000 unmethylated. *GSTP1* also produced duplicate Cq values when 1 methylated allele was amplified in a background of 10000, however for *RARB* and *APC* only one of the duplicate reactions produced a Cq value. The averaged Cq value for *ACTB* was 12.8 +/- 0.17.

Table 10. MNAzyme methylation quadruplex qPCR assay

Sample	Average Cq^			
	GSTP1	***RARB***	***APC***	***ACTB***
methylated (LNCap)	10.4	12.6	13.5	13.1
1 in 10	13.4	16.2	17.1	12.6
1 in 100	16.6	19.2	20.0	12.8
1 in 1000	19.9	24.1	22.4	12.7
1 in 10000	23.4	25.7 (1 of 2)	26.9 (1 of 2)	12.9
unmethylated (K562)	n/a	n/a	n/a	12.8
No Template	n/a	n/a	n/a	n/a

^ Cq duplicates averaged except for where only one replicate worked

3.4. MNAzyme qPCR; standard vs rapid thermocycling; Development of rapid cycling protocol

The demand for high throughput assays has seen the implementation of rapid cycling protocols. There are many facets of a qPCR that can be modified for rapid cycling; a summary is available in the BioRad Fast PCR Bulletin[2]. To develop rapid cycling conditions for MNAzyme qPCR, the first step was to switch to MyTaqHS™ polymerase (Bioline) that reduced the upfront denaturation to 2 minutes, as opposed to 10 minutes with Immolase. To reduce overall cycling times, the time held at each temperature was investigated. The original denaturation time of 15 seconds was compared to 5, 3 and 1 seconds. The annealing and extension times were combined for a two temperature cycle format and run at 53°C. The original annealing time of 60 seconds was reduced to 30, 20, 15, 10 and 5 seconds (not all data shown). It was found that the optimal denaturation time was 5 seconds and optimal annealing/extension time was 15 seconds.

For each gene the time to reach the Cq value was calculated for each concentration (Table 11). Calibration curves for all genes produced the amplification efficiencies and correlation coefficients as seen in Table 12.

Table 11. Cq values for standard and rapid thermocycling of MNAzyme qPCR and time to reach Cq for the gDNA concentrations of 125, 8 and 0.5 ng

Gene	gDNA (ng)	Standard qPCR		Rapid qPCR	
		Cq	Minutes	Cq	Minutes
HMBS	125	25.3	52	26.4	30
	7.8	29.3	60	30.4	35
	0.5	33.6	69	34.2	39
HFE	125	23.2	48	24.1	28
	7.8	27.1	56	28.1	32
	0.5	31.4	64	32.2	37
HPRT1	125	25.1	52	26.2	30
	7.8	29.1	60	30.5	35
	0.5	31.4	68	34.7	40

Table 12. Comparison of correlation coefficients and PCR efficiencies for standard and rapid thermocycling

Thermocycling	***HMBS***	***HFE***	***HPRT1***
Standard	R^2 = 0.998 Eff = 97%	R^2 = 0.997 Eff = 98%	R^2 = 0.997 Eff = 93%
Rapid	R^2 = 0.999 Eff =104%	R^2 = 0.999 Eff = 100%	R^2 = 0.998 Eff = 93%

4. Optimising an MNAzyme qPCR reaction

The concentration of magnesium in MNAzyme qPCR is generally higher than for other qPCR protocols since magnesium is required for the MNAzymes' catalytic activity. However, this additional magnesium does not impact the reaction specificity as four binding events are needed for signal in MNAzyme qPCR. While concentrations in the range of 6 to 10 mM are suitable for most qPCR reactions; 8 mM has been found to be universally robust for singleplex reactions. This may be increased to 10 mM in multiplex reactions detecting three or more targets. When using commercial mixes it is advisable to add additional magnesium to the mix to give a final concentration of 8 mM.

Other components in a MNAzyme qPCR reaction such as polymerase, dNTPs and reverse transcriptase are used following the manufacturer's instructions. When three or more targets are multiplexed, the addition of more units of polymerase and/or reverse transcriptase can improve efficiency in some assays. Certain additives such as betaine or single stranded binding protein have been found to have a negative effect on MNAzyme activity.

5. References

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(3) Sanchez, J.A., Pierce, K.E., Rice, J.E. and Wangh, L.J. (2004) Linear-after-the-exponential (LATE)-PCR: an advanced method of asymmetric PCR and its uses in quantitative real-time analysis. *Proc Natl Acad Sci USA*. 101(7):1933-8.