

Standardisation of real-time PCR

Dr P. Lewis White Public Health Wales



The MIQE Guidelines

- Publications reporting real-time PCR are ubiquitous
- "Lack of sufficient experimental detail"
- Limits the potential to evaluate results or repeat experiments
- Published to highlight the inadequacies in manuscripts reporting realtime PCR assays
- Aim of providing specifications for the minimum information required for publication qPCR manuscripts.



Table 1. MIQE checklist for authors, reviewers, and editors."

I	Item to check	Importance	Item to check	Importance
ł	Experimental design		qPCR oligonucleotides	
l	Definition of experimental and control groups	ε	Primer sequences	Ε
ł	Number within each group	£	RTPrimerD8 identification number	D
	Assay carried out by the core or investigator's laboratory?	D	Probe sequences	Dd
	Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
	Sample		Manufacturer of oligonucleotides	D
	Description	E	Purification method	D
	Volume/mass of sample processed	D	qPCR protocol	
ł	Microdissection or macrodissection	E ·	Complete reaction conditions	E
l	Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
I	If frozen, how and how quickly?	E	Primer, (probe), Mg ²⁺ , and dNTP concentrations	E
l	If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
l	Sample storage conditions and duration (especially for FFPE ^b samples)	E	Buffer/kit identity and manufacturer	E
l	Nucleic acid extraction		Exact chemical composition of the buffer	D
	Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
l	Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Ì	Source of additional reagents used	Ð	Complete thermocycling parameters	E
۱	Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
l	Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
l	Nucleic acid quantification	E	qPCR validation	
l	Instrument and method	E	Evidence of optimization (from gradients)	D
l	Purity (A ₂₆₀ /A ₂₈₀)	D	Specificity (gel, sequence, melt, or digest)	£
l	Yield	D	For SYBR Green I, C _q of the NTC	E
	RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
l	RIN/RQI or C _q of 3' and 5' transcripts	E	PCR efficiency calculated from slope	E
l	Electrophoresis traces	D	Cis for PCR efficiency or SE	D
l	Inhibition testing (C _q cilutions, spike, or other)	E	r ² of calibration curve	E
l	Reverse transcription		Linear dynamic range	E
l	Complete reaction conditions	E	C _{th} variation at LOD	£
l	Amount of RNA and reaction volume	E	Cis throughout range	D
ļ	Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
l	Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
ļ	Temperature and time	E	Data analysis	
l	Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
I	C _e s with and without reverse transcription	D*	Method of C _q determination	E
l	Storage conditions of CDNA	D	Outlier identification and disposition	E
	Grock target intomation		Nesults for NILS	
I	Security accession number		Justification of number and choice of reference genes	ь
I	Sequence accession name		Description of normalization metrico	
I	Amelian least		Number and concordance of biological replicates	U
I	Autprecis rengen		Number and stage (reverse transcription or gPCA) of technical replicates	
I	Providences activity screen (boost, and so bit)		Repeatability (intraassay variation)	с
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ł	Secondary structure analysis of amplican	0	runner energins Cestistical analysis	υ -
I	inclusion of each primer by even or intensities of applicable)	¢	sonation memory for results significance	ь ,
l	What relice variants are tweeted)	-	C or row data submission with PDMI	1 D
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^a All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.

*FPE, formalin-fixed, parafilm-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deconnucleoside triphosphate. Assessing the absence of DNA with a no-reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of an o-versent transcription compt of desirable but no longer essential.

^d Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

86 criteria 57 essential

Apply the MIQE guidelines to Fungal PCR – combine with EAPCRI recommendations



Sample acquisition and processing

- Sample Choice "1st Potential Source of experimental variability" Bustin 4:1
 - Disease manifestation origin and progression
 - Incidence of disease
 - Underlying condition of patient
 - Frequency and volume required
- Potential target within sample
 - The fungus
 - Free DNA
 - Phagocytosed
- Sample storage and period

• Influence choice of extraction protocol and efficiency

• Interpretation of results



Options for Fungal PCR





EAPCRI Extraction recommendations

	Serum Testing	Whole Blood testing				
Sample type	N/A	EDTA only				
Sample volume	≥0.5ml	≥3.0ml				
Potential target	Free circulating DNA	Phagocytosed fungal fragments				
Extraction requirements	Commercial extraction kits	Red and White cell lysis, Bead- beating, commercial extraction kits.				
Elution volume	Elu	ıte in <100μl,				
Special requirements	Screen all reagents for contamination with fungal DNA. Positive and negative extraction controls recommended					
"Nucleic Acid extraction is a second critical step" Bustin 4:2						

Bustin *et al* 2009; White *et al* 2010, 2011



Monitoring for Inhibition

- "The potential for inhibition must be addressed in a publication.....and it is important to ensure no inhibitors distort results" Bustin 5:2
 - Overall detection
 - Quantification
- Different PCR reactions will not be equally susceptible to inhibition.
 - Higher burdens with earlier Cq values may be less affected
 - Significant in Fungal PCR where Cq values are typically >35 cycles



EAPCRI IC recommendations

• Internal control Cq values should be representative of typical *Aspergillus* PCR positive results (≥35 cycles)

 Human DNA targets avoided due to the possibility of variable target amounts within individual specimens

 When using serum IC should be incorporated at the beginning of the extraction process to also monitor for extraction efficiency

White et al 2010, 2011



qPCR amplification

- Optimisation is critical (Bustin 7)
 - Cycling conditions (temp/time/instrument)
 - Mastermix composition
 - Mg²⁺, oligonucleotide and polymerase concentrations
 - DNA template and final reaction volume
- Oligonucleotide design is critical (Bustin 7:1)
 - Avoid Oligonucleotide dimers and hairpin loops
 - Software is readily available
- Oligonucleotide Specificity (Bustin 7:2)
 - Blast search
 - Similarity to homologous or unexpected genes



300 300 300

450 450 450

500 500

600 600

650 650 650

700 700 700

850 850 850

1150 1150 1150

Problems - Oligonucleotide design

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IUMAN1 18ALB4 7UM18. IUMAN1 18ALB4 7UM18.

FUM18.

UM18.

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TUM18.

BALB4

UM18.

RALEA

UMI8.

FUM18.

UMBNI

UMAN1 SALB4 UM18.

UMAN1 BALB4

IOMAN1 8ALB4 FOM18. IOMAN1 8ALB4 FOM18.

UM18.

UMAN10

• rRNA operon

- 18S rRNA gene
- Panfungal primers
- Genus sp. Probe White *et al. 2006*
- Block-based/Sybr Green
 False positives
- Probe based assay
 - False negatives

S.SEQ SEQ				50 50 50 50 50	HUMAN18S.SEQ CDIFF16S.SEQ MRSA16S.SEQ	1 1 1	TAC NINNNN	TCCTGCCAG	TA CANTG		AGATTAAGC	
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S.SEQ SEQ	160 151 0000000000000000000000000000000000	170 AAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAA	Leo 190 C CCCGGC S T Free T - CCCGGC S T Free	200 CCCCTC 200 GTC 200	HUMAN183.SEQ CDIFF165.SEQ MRSA165.SEQ	151 151 151	160 AC TO		180 TAC TO	190 GACCING T TGTCCAA		
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S.SEQ SEQ	260 251 c c c c 251	270 CGGGGG GCGGGCG	280 290 CCG GCGG 290	300 TAG 300 300	HUMAN185.SEQ CDIFF165.SEQ	251 251	260 CGGCCC T	270 CGGCCGGGGG	280 GC GGC C G TA	290 GCGGCTTTGG	300 TGACTCT	
S.SEQ SEQ	310 301			350 350 350 350 350	HUMAN185.SEQ CDIFF165.SEQ	301 301	310 A C C GG	320 C C C C C C - C C C	330 GCCCCCCC	340 CGGC AC	350 ATTCGAA	
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S.SEQ SEQ	401 C 410			450 450 450 450	HUMAN185.SEQ CDIFF165.SEQ MPS8165 SEQ	401	410 CCGGGGA	420 GGGGGAATCA	430 GGGTT	440	450 AGCC GAA	/
S.SEQ SEQ	451 460 451 460	470 4		500 500 500 500	HUMAN185.SEQ CDIFF165.SEQ MPS8165_SEQ	451	460 ACTA	470 CCAA		490 CADATT	ACT CT C	
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S.SEQ SEQ	551 C C	570 C C T	80 590 T A	600 600 600 600	HUMAN18S.SEQ CDIFF16S.SEQ MRSA16S.SEQ	551 551	560 TAAT	570 ATC GT	580 A T T AAT	590 CCTT A C	600 G A CA T	
S.SEQ SEQ	601 610 601 601	620		650 650 Attente 650	HUMAN185.SEQ CDIFF165.SEQ MRSA165.SEQ	601 601 601	610 GG C GT	620 GG C G G	630 CCGC T	640 AATTCCAGCT	650 cc 1 c c	
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S.SEQ SEQ	760 751 cm cm cgc 751			800 C GAA 800 800	HUMAN185.SEQ CDIFF165.SEQ	751 751 751	760 C CT C CC	770 CCTCGAT	780 cereces	790 And 1 CC	800 GGGC	
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S, SEQ SEQ	901 910 901 TCGA	920 920 R Ag Ce 2	30 940	900 950 950 950 950	HUMAN185.SEQ CDIFF165.SEQ	901 901	910 TTTTC	920 CTGA G CA	930 T AAGA	940 Geococce	950 Geographics	
S.SEQ SEQ *	960 951 100 GC C T 951 100 GC C T	970 5	80 990 C CC CC CC	1000 CCGA 1000 1000	HUMAN185.SEQ CDIFF165.SEQ	951 951	960 TCGCCGC	970 A	980 1111 G C C	990 CGGC C A	1000 CGG -A	
S.SEQ SEQ	1010 1001 1001	1020 10 A	30 1040	1050 CG A 1050 1050	HUMAN188.SEQ CDIFF168.SEQ	1001 1001	1010 A AT	1020 T GC 744 74	1030 TTTCATT	1040 AATCA -A	1050 TCG	
EQ S.SEQ SEQ	1001 1060 1051 107 1051 107	1070 10	80 1090 TCCG	1050 1100 1100 1100	HUMAN185.SEQ CDIFF165.SEQ	1051 1051	1060 A 7 6 6	1070	1080	1090 TCGA	1100 CC	
EQ SEQ	11051 1101 CG C C C C		30 ,1140 CG S	1100 1150 1150 1150	HUMAN18S.SEQ	1101 1101	1110 cc cc cca	1120 CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	1130 TACCATG 	1140 A C C G	1150 A TT CGC C CC	
EQ S.SEQ SEQ	1101 1160 1151 100 1160		80 1190 A 1190	1150 1200 1200 1200	HUMAN18S.SEQ CDIFF16S.SEQ M853165.SEQ	1151 1151 1151	1160 GAALCCLAG	1170				
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PCR Controls - Bustin 7:3

- No template controls
 - Contamination or non-specific amplification
- Positive PCR controls
 - Monitor for temporal assay variation
- Calibrators
 - Used to generate standard curves for quantification
- AsTeC EAPCRI collaboration developing an international *Aspergillus* PCR calibrator



Assay performance (Bustin 7.

- PCR efficiency
 - Calibration curves
 - E=10-1/slope-1: Achieve >90%
- Cq >40 cycles should not be reported
 - Care with Arbitrary thresholds
 - False positive/negative results
 - IA Cq >40 cycles have been deemed significant
- Analytical sensitivity
 - LoD (95% probability of detection)
 - Optimal Theoretical LoD: 3 copies/reaction

Bustin et al. 2009; Millon et al. 2006



Assay performance

- Analytical specificity
 - Assay detecting the correct target sequence
 - Detection range or cross reactivity
- Accuracy: Comparison of the measured and the actual value
- Precision: Comparison of values generated when testing the same sample
- Linear dynamic range
 - The range of copy numbers established by a calibration curve



Summary

- Know your disease
- Know your optimal sample
- Know your potential NA source
- Evaluate and optimize NA extraction
- PCR amplification is only as efficient as the NA extraction technique will allow.
- Design, Optimize and evaluate PCR amplification prior to clinical evaluation