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 real-time PCR assays
- Aim of providing specifications for the minimum information required for publication qPCR manuscripts.

Bustin *et al.* 2009

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		**qPCR oligonucleotides**	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
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**	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	**qPCR validation**	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
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
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	**Data analysis**	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

[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.
[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.
[c] 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 a no–reverse transcription control is 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

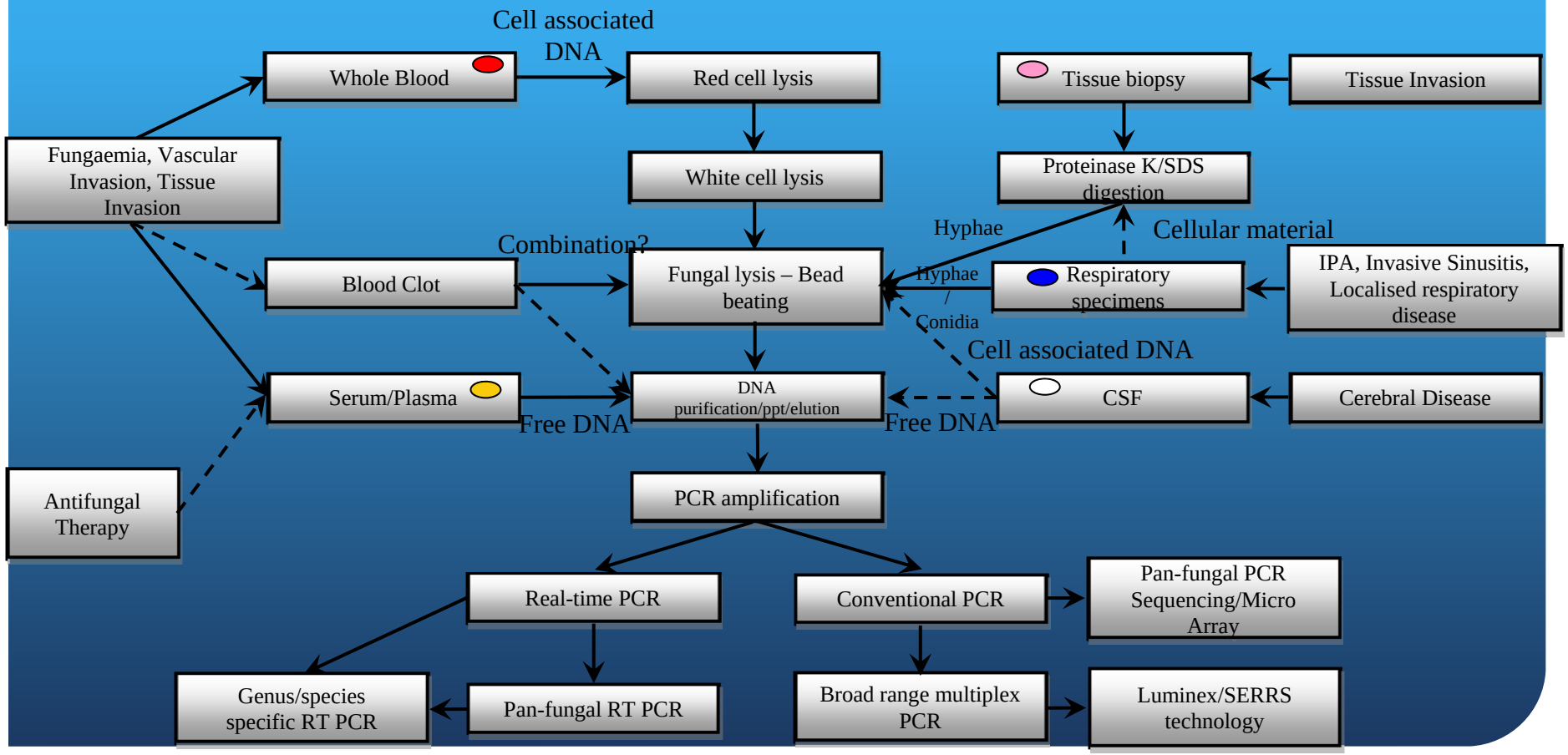
Apply the MIQE guidelines to Fungal PCR – combine with EAPCRI recommendations

Bustin *et al.* 2009

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	Elute 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
    - Mg^{2+}, 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

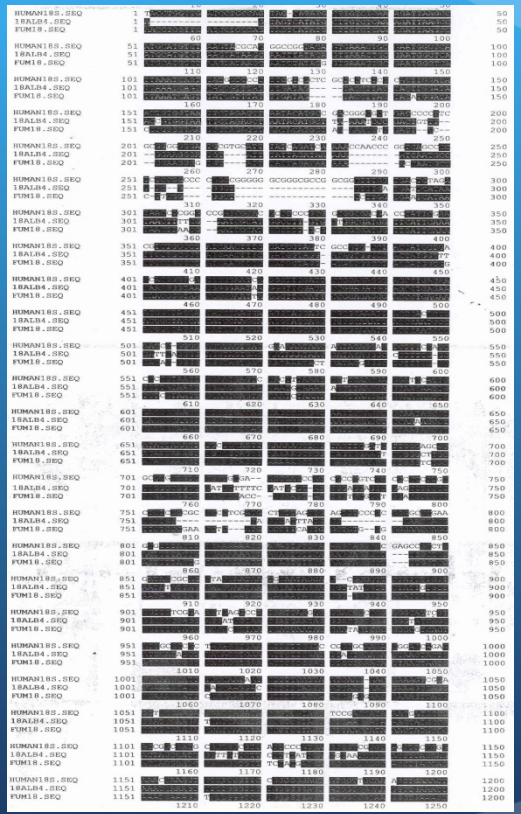
Bustin *et al.* 2009

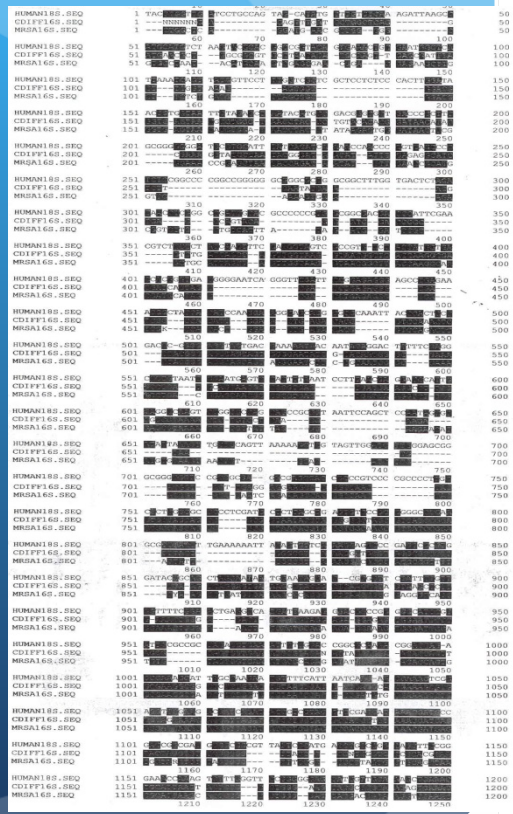
Problems – Oligonucleotide design

- 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





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

Bustin *et al.* 2009

Assay performance (Bustin 7.4)

- 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

Bustin *et al.* 2009

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