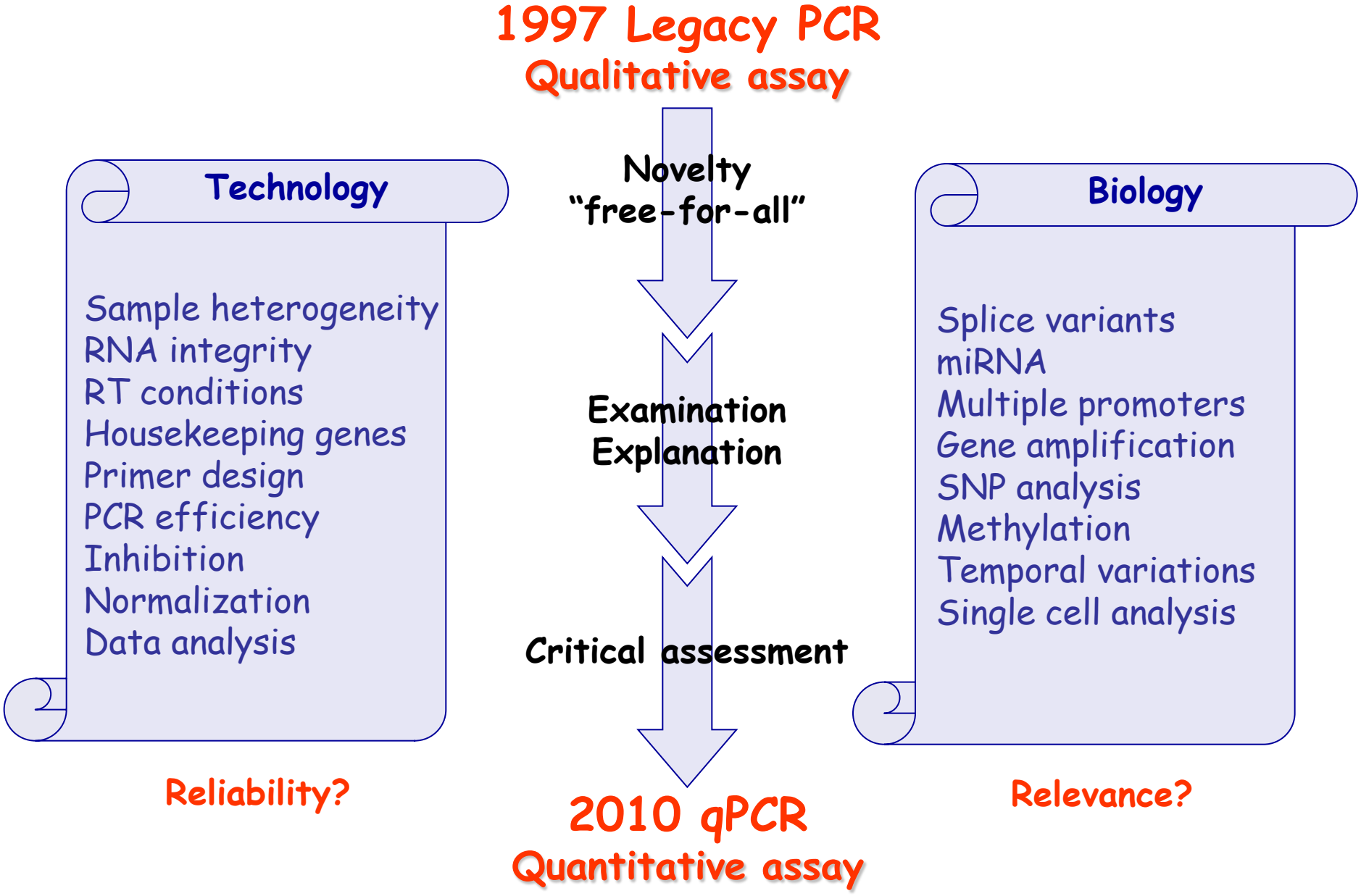
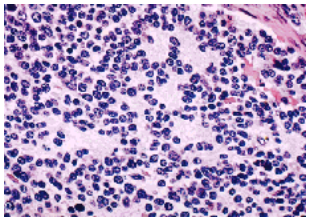
Le linee guida MIQE

Paola Scaruffi

S.S. Oncologia Traslazionale Pediatrica
Istituto Nazionale per la Ricerca sul Cancro (IST)
Genova

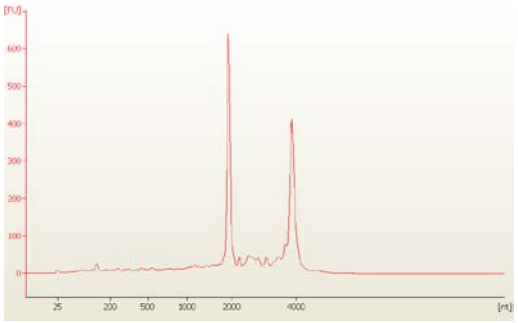

1997 Legacy PCR
Qualitative assay
Technology
Sample heterogeneity
RNA integrity
RT conditions
Housekeeping genes
Primer design
PCR efficiency
Inhibition
Normalization
Data analysis
Novelty
"free-for-all"
Examination
Explanation
Critical assessment
Biology
Splice variants
miRNA
Multiple promoters
Gene amplification
SNP analysis
Methylation
Temporal variations
Single cell analysis
Reliability?
Relevance?
2010 qPCR
Quantitative assay



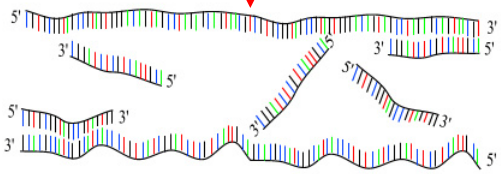
Sample selection and handling



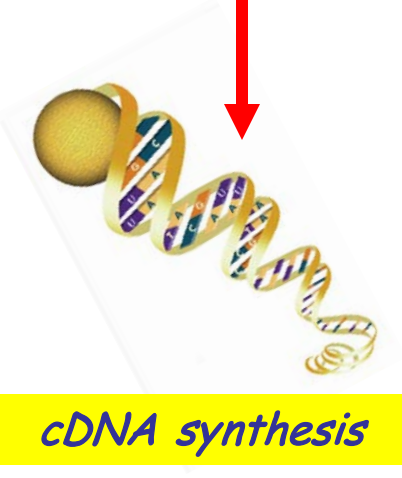
Sample extraction



RNA quality assessment



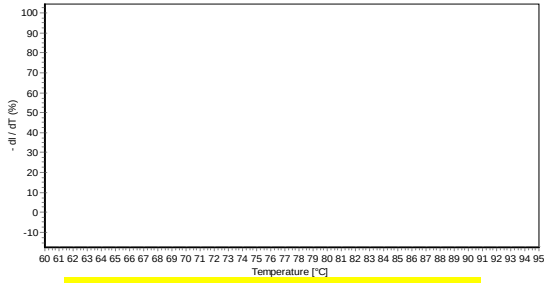
PCR primer design



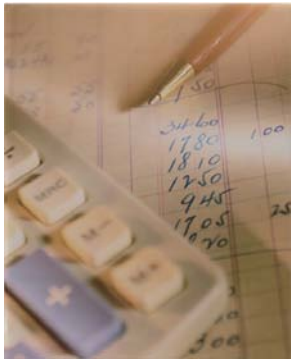
cDNA synthesis



Choice of qPCR chemistry



Assay validation



Data analysis & reporting



Real time cycler

THE PROBLEM

A lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments.

The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

- SAMPLE: quality assessment
- EXPERIMENTAL DESIGN: primers, probes
- PROTOCOL: reaction conditions
- ANALYSIS: normalization
- INTERPRETATION: significant of fold-changes

MIQE

Minimum Information for Publication of Quantitative Real-time PCR Experiments

= is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments

Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: *M*inimum *I*nformation for Publication of Quantitative Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

The MIQE guidelines

AIM:

to provide authors, reviewers, and editors specifications for the minimum information, that must be reported for a qPCR experiment to ensure its relevance, accuracy, correct interpretation, and repeatability



they target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency

The MIBBI Project
Minimum Information for Biological and Biomedical Investigations

History of the MIBBI Project

The MIBBI Project was initially conceived during the HUPO Proteomics Standards Initiative's 2006 spring meeting in San Francisco.

VOLUME 26 NUMBER 8 AUGUST 2008 NATURE BIOTECHNOLOGY

Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project

Chris F Taylor[*1,2], Dawn Field[2,3], Susanna-Assunta Sansone[1,2], Jan Aerts[4], Rolf Apweiler[1], Michael Ashburner[5], Catherine A Ball[6], Pierre-Alain Binz[7,8], Molly Bogue[9], Tim Booth[2], Alvis Brazma[1], Ryan R Brinkman[10], Adam Michael Clark[11], Eric W Deutsch[12], Oliver Fiehn[13], Jennifer Fostel[14], Peter Ghazal[15], Frank Gibson[16], Tanya Gray[2,3], Graeme Grimes[15], John M Hancock[17], Nigel W Hardy[18], Henning Hermjakob[1], Randall K Julian Jr[19], Matthew Kane[20], Carsten Kettner[21], Christopher Kinsinger[22], Eugene Kolker[23,24], Martin Kuiper[25], Nicolas Le Novère[1], Jim Leebens-Mack[26], Suzanna E Lewis[27], Phillip Lord[16], Ann-Marie Mallon[17], Nishanth Marthandan[28], Hiroshi Masuya[29], Ruth McNally[30], Alexander Mehrle[31], Norman Morrison[2,32], Sandra Orchard[1], John Quackenbush[33], James M Reecy[34], Donald G Robertson[35], Philippe Rocca-Serra[1,36], Henry Rodriguez[22], Heiko Rosenfelder[31], Javier Santoyo-Lopez[15], Richard H Scheuermann[28], Daniel Schober[1], Barry Smith[37], Jason Snape[38], Christian J Stoeckert Jr[39], Keith Tipton[40], Peter Sterk[1], Andreas Untergasser[41], Jo Vandesompele[42] & Stefan Wiemann[31]

The Minimum Information for Biological and Biomedical Investigations (MIBBI) project provides a resource for those exploring the range of extant minimum information checklists and fosters coordinated development of such checklists.

MI projects registered with MIBBI

CIMR	Core Information for Metabolomics Reporting

Projects/MIqPCR

Minimum Information about a quantitative Polymerase Chain Reaction Experiment

1	*General features*	
1.1	**Domain**	quantitative Polymerase Chain Reaction
1.2	**Document Type**	Checklist
1.3	**Group**	RDML-Consortium
1.4	**Main Website**	http://www.rdml.org/
1.5	**MI Checklist's Name**	Minimum Information about a quantitative Polymerase Chain Reaction experiment
1.6	**MI Checklist's Acronym**	MIqPCR

2	*Contact Person*	
2.1	**Full Name**	Andreas Untergasser
2.2	**Email Address**	andreas[@]untergasser.de

2	*Contact Person*	
2.1	**Full Name**	Jo Vandesompele
2.2	**Email Address**	joke.vandesompele[@]ugent.be

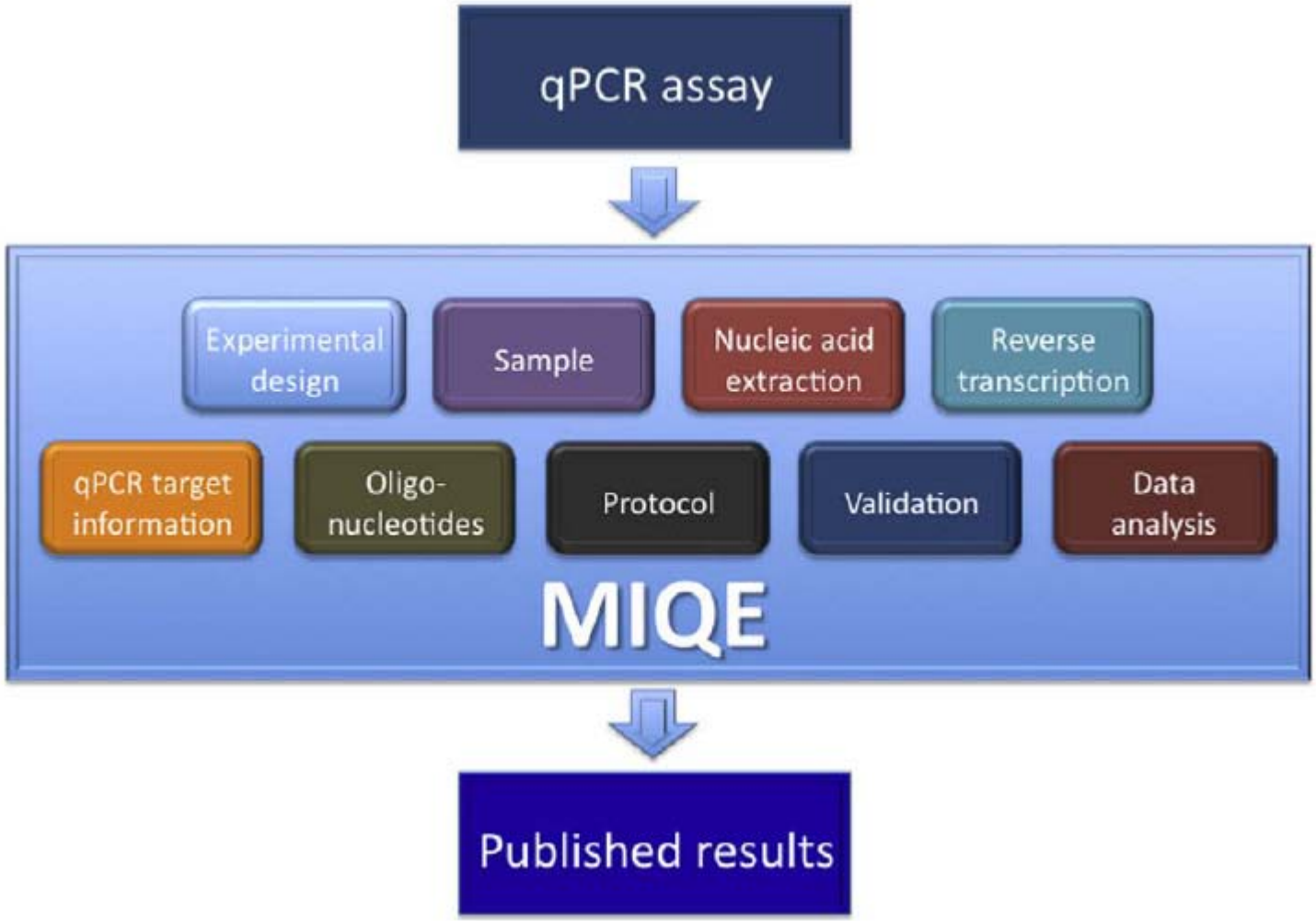
2	*Contact Person*	
2.1	**Full Name**	Jan Hellemans
2.2	**Email Address**	jan.hellemans[@]ugent.be

MIRIAM	Minimal Information Required In the Annotation of biochemical Models
MISFISHIE	Minimum Information Specification For In Situ Hybridization and Immunohistochemistry Experiments
STRENDA	Standards for Reporting Enzymology Data
TBC	Tox Biology Checklist

NOMENCLATURE

RDML (Real-Time PCR Data Markup Language) data standard
http://www.rdml.org

- ✓ The abbreviation **qPCR** for **quantitative real-time PCR** and **RT-qPCR** for **reverse transcription-qPCR**
- ✓ **Quantification** not **quantitation**
- ✓ **Hydrolysis probes** not **TaqMan**
- ✓ **LightCycler-type probes** should be referred to as **dual hybridization probes**
- ✓ **Quantification cycle (Cq)** replaces threshold cycle (**Ct**), crossing point (**Cp**), take-off point (**TOP**)
- ✓ **Reference** gene not **housekeeping** gene



CHECKLIST

Bustin SA et al. Methods, 2009

CHECKLIST

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.

Bustin SA et al. Clin Chem, 2009

EXPERIMENTAL DESIGN & SAMPLE

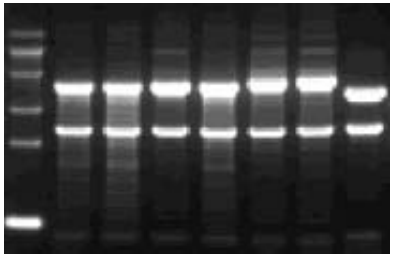
Item to check	Importance
Experimental design	
Definition of experimental and control groups	E
Number within each group	E
Assay carried out by the core or investigator's laboratory?	D
Acknowledgment of authors' contributions	D
Sample	
Description	E
Volume/mass of sample processed	D
Microdissection or macrodissection	E
Processing procedure	E
If frozen, how and how quickly?	E
If fixed, with what and how quickly?	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E

2.

NUCLEIC ACID EXTRACTION

Nucleic acid extraction	
Procedure and/or instrumentation	E
Name of kit and details of any modifications	E
Source of additional reagents used	D
Details of DNase or RNase treatment	E
Contamination assessment (DNA or RNA)	E
Nucleic acid quantification	E
Instrument and method	E
Purity (A_{260}/A_{280})	D
Yield	D
RNA integrity: method/instrument	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E
Electrophoresis traces	D
Inhibition testing (C_q dilutions, spike, or other)	E

RNA QUALITY CONTROL

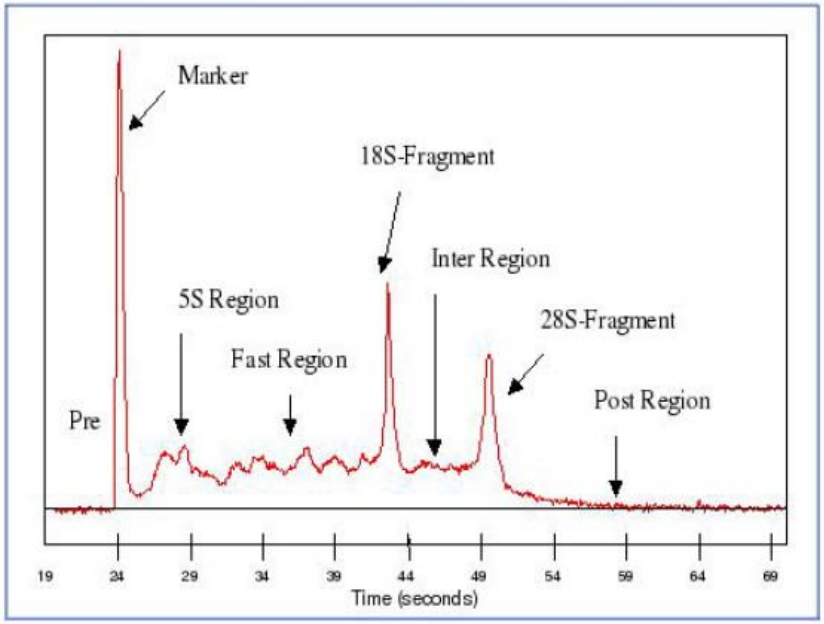


ratio of 28S:18S

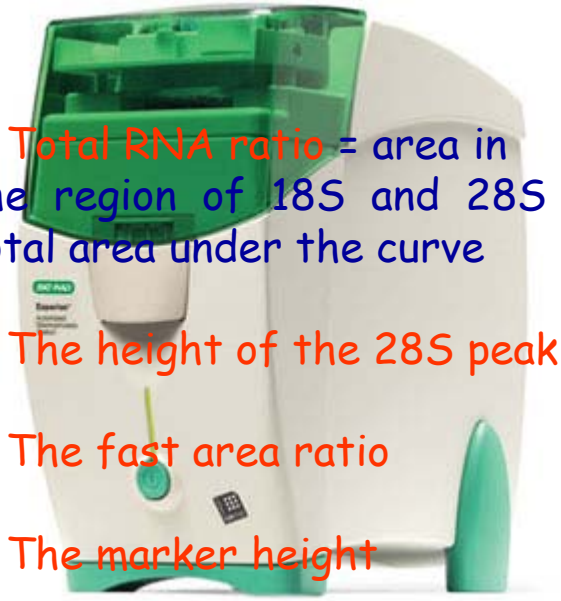
microcapillary electrophoretic RNA separation

RNA Integrity Number algorithm (RIN)
Range 1-10





2100 BioAnalyzer (Agilent Technologies)



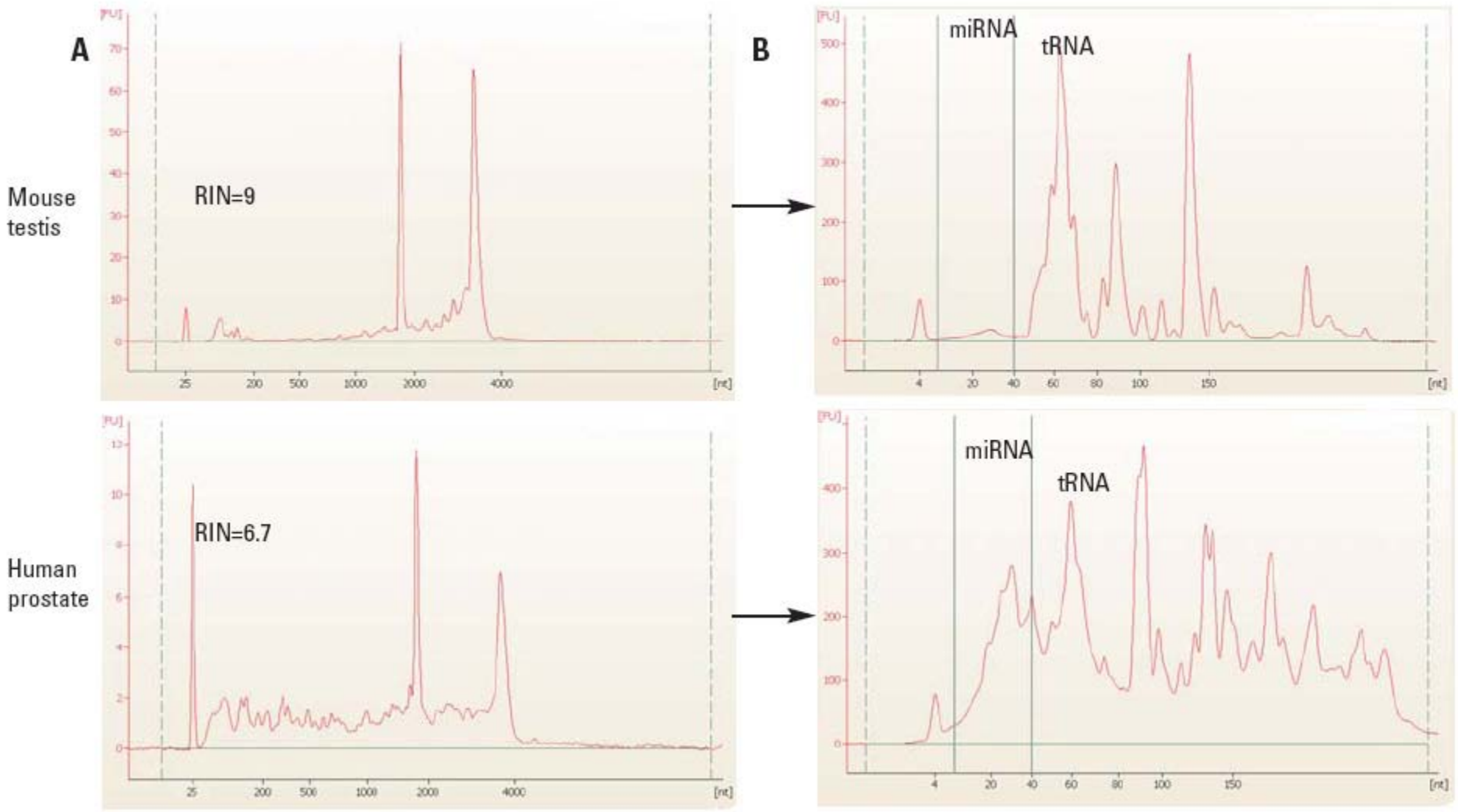
- ✓ Total RNA ratio = area in the region of 18S and 28S / total area under the curve
- ✓ The height of the 28S peak
- ✓ The fast area ratio
- ✓ The marker height

Experion (Bio-Rad)

Schroeder A. et al. BMC Molecular Biology, 2006

mRNA and microRNA QUALITY CONTROL

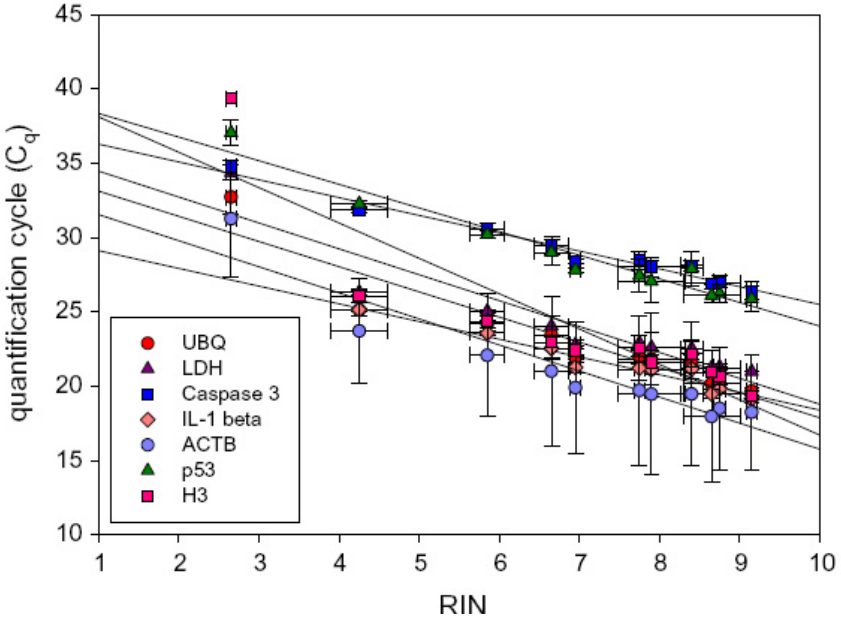
RNA 6000 Nano assay | **Small RNA assay**



↑ RNA degradation → ↑ miRNA amount

RNA INTEGRITY and qPCR performance

mRNA

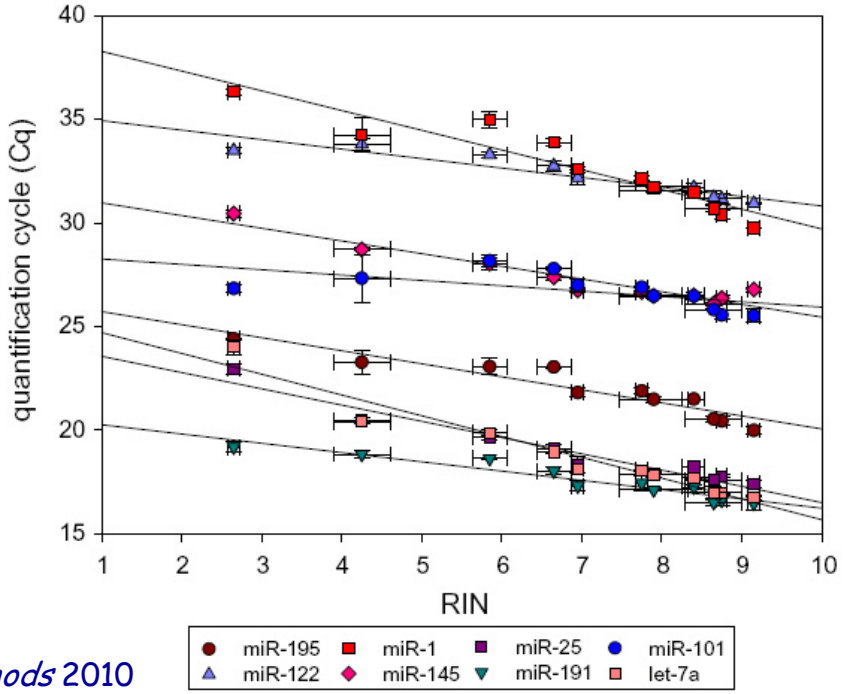


r^2_{mean} = 0.837

P < 0.001

mean slope of the regression line = 1.578

microRNA



r^2_{mean} = 0.835

P < 0.001

mean slope of the regression line = 0.784

Threshold for reliable PCR results: RIN > 6

Becker C et al. *Methods* 2010

REVERSE TRANSCRIPTION

Reverse transcription	
Complete reaction conditions	E
Amount of RNA and reaction volume	E
Priming oligonucleotide (if using GSP) and concentration	E
Reverse transcriptase and concentration	E
Temperature and time	E
Manufacturer of reagents and catalogue numbers	D
C_qs with and without reverse transcription	D^c
Storage conditions of cDNA	D

TARGET INFORMATION

qPCR target information	
Gene symbol	E
Sequence accession number	E
Location of amplicon	D
Amplicon length	E
In silico specificity screen (BLAST, and so on)	E
Pseudogenes, retropseudogenes, or other homologs?	D
Sequence alignment	D
Secondary structure analysis of amplicon	D
Location of each primer by exon or intron (if applicable)	E
What splice variants are targeted?	E

5.

qPCR OLIGONUCLEOTIDES

qPCR oligonucleotides	
Primer sequences	E
RTPrimerDB identification number	D
Probe sequences	D[d]
Location and identity of any modifications	E
Manufacturer of oligonucleotides	D
Purification method	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."

(Bustin SA et al. Clin Chem, 2009)

http://www.primerdesign.co.uk/research_with_integrity.html



Primer sequences supplied

...research with integrity

A scientific understanding of real-time PCR data requires more than just the interpretation of PCR amplification plots. What precisely do your plots relate to and which sequences or splice variants are excluded from the analysis? This information is contained within the primer sequences used in the PCR reaction. We believe that real-time PCR research can only be performed with integrity when researchers have this information, and are able to share it within the scientific community. This information, critical to interpreting the data, is too important to be left in the hands of commercial enterprises. Editors are correct to insist that primer sequences be made available so that data can be accurately replicated, and be challenged at the level of sequence specificity.

Purchase a custom kit from us and the primer sequences are made available to researchers as standard with no questions asked. Of course, restrictions on their use apply, but the sequences can be published in academic journals. If you also require the probe sequence for publication, we will supply this too - just ask.

We want to build trusting relationships with our customers and empower you to fully understand the results that you obtain from our kits. We are scientists first, and improving the quality of published real-time PCR data is an integral part of our mission. We hope that you will support us in this aim by sourcing your real-time PCR kits from PrimerDesign Ltd.

qPCR PROTOCOL

qPCR protocol	
Complete reaction conditions	E
Reaction volume and amount of cDNA/DNA	E
Primer, (probe), Mg^{2+}, and dNTP concentrations	E
Polymerase identity and concentration	E
Buffer/kit identity and manufacturer	E
Exact chemical composition of the buffer	D
Additives (SYBR Green I, DMSO, and so forth)	E
Manufacturer of plates/tubes and catalog number	D
Complete thermocycling parameters	E
Reaction setup (manual/robotic)	D
Manufacturer of qPCR instrument	E

qPCR VALIDATION

qPCR validation	
Evidence of optimization (from gradients)	D
Specificity (gel, sequence, melt, or digest)	E
For SYBR Green I, C_q of the NTC	E
Calibration curves with slope and *y* intercept	E
PCR efficiency calculated from slope	E
CIs for PCR efficiency or SE	D
r^2 of calibration curve	E
Linear dynamic range	E
C_q variation at LOD	E
CIs throughout range	D
Evidence for LOD	E
If multiplex, efficiency and LOD of each assay	E

8.

DATA ANALYSIS

Data analysis	
qPCR analysis program (source, version)	E
Method of C_q determination	E
Outlier identification and disposition	E
Results for NTCs	E
Justification of number and choice of reference genes	E
Description of normalization method	E
Number and concordance of biological replicates	D
Number and stage (reverse transcription or qPCR) of technical replicates	E
Repeatability (intraassay variation)	E
Reproducibility (interassay variation, CV)	D
Power analysis	D
Statistical methods for results significance	E
Software (source, version)	E
C_q or raw data submission with RDML	D

REFERENCE GENE SELECTION

"Normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described.
The optimal number and choice of reference genes must be experimentally determined and the method reported."

(Bustin SA et al. Clin Chem, 2009)





geNorm: algorithm to determine the most stable reference genes from a set of tested candidate genes in a given sample panel, and to calculate a gene expression normalization factor for each sample based on the geometric mean of a user-defined number of reference genes

http://medgen.ugent.be/%7Ejvdesomp/genorm/
http://www.primerdesign.co.uk/

DATA ANALYSIS

Data analysis	
qPCR analysis program (source, version)	E
Method of C_q determination	E
Outlier identification and disposition	E
Results for NTCs	E
Justification of number and choice of reference genes	E
Description of normalization method	E
Number and concordance of biological replicates	D
Number and stage (reverse transcription or qPCR) of technical replicates	E
Repeatability (intraassay variation)	E
Reproducibility (interassay variation, CV)	D
Power analysis	D
Statistical methods for results significance	E
Software (source, version)	E
C_q or raw data submission with RDML	D

RDML

Real-Time PCR Data Markup Language

Published online 17 February 2009

Nucleic Acids Research, 2009, Vol. 37, No. 7 **2065–2069**
doi:10.1093/nar/gkp056

SURVEY AND SUMMARY

RDML: structured language and reporting guidelines for real-time quantitative PCR data

Steve Lefever[1], Jan Hellemans[1,*], Filip Pattyn[1], Daniel R. Przybylski[2], Chris Taylor[3], René Geurts[4], Andreas Untergasser[4] and Jo Vandesompele[1], on behalf of the RDML consortium

[1]Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium, [2]Bio-Rad Laboratories, Inc., Hercules, CA, USA, [3]European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK and [4]Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, The Netherlands

RDML
http://www.rdml.org

Real-time PCR Data Markup Language

RDML

Home | News | Data Standard | Development | MIQE | Tools | Consortium

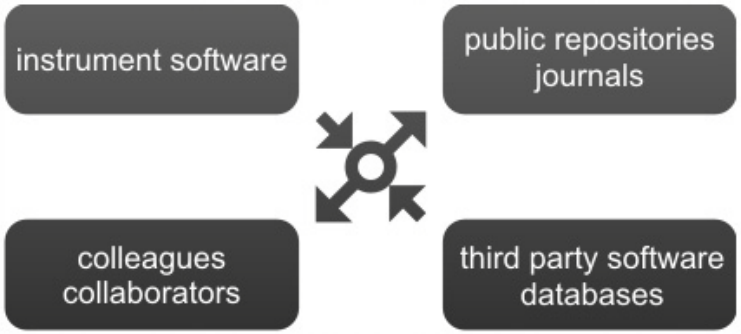
The RDML standard is based on XML (eXtensible Markup Language)

Tools

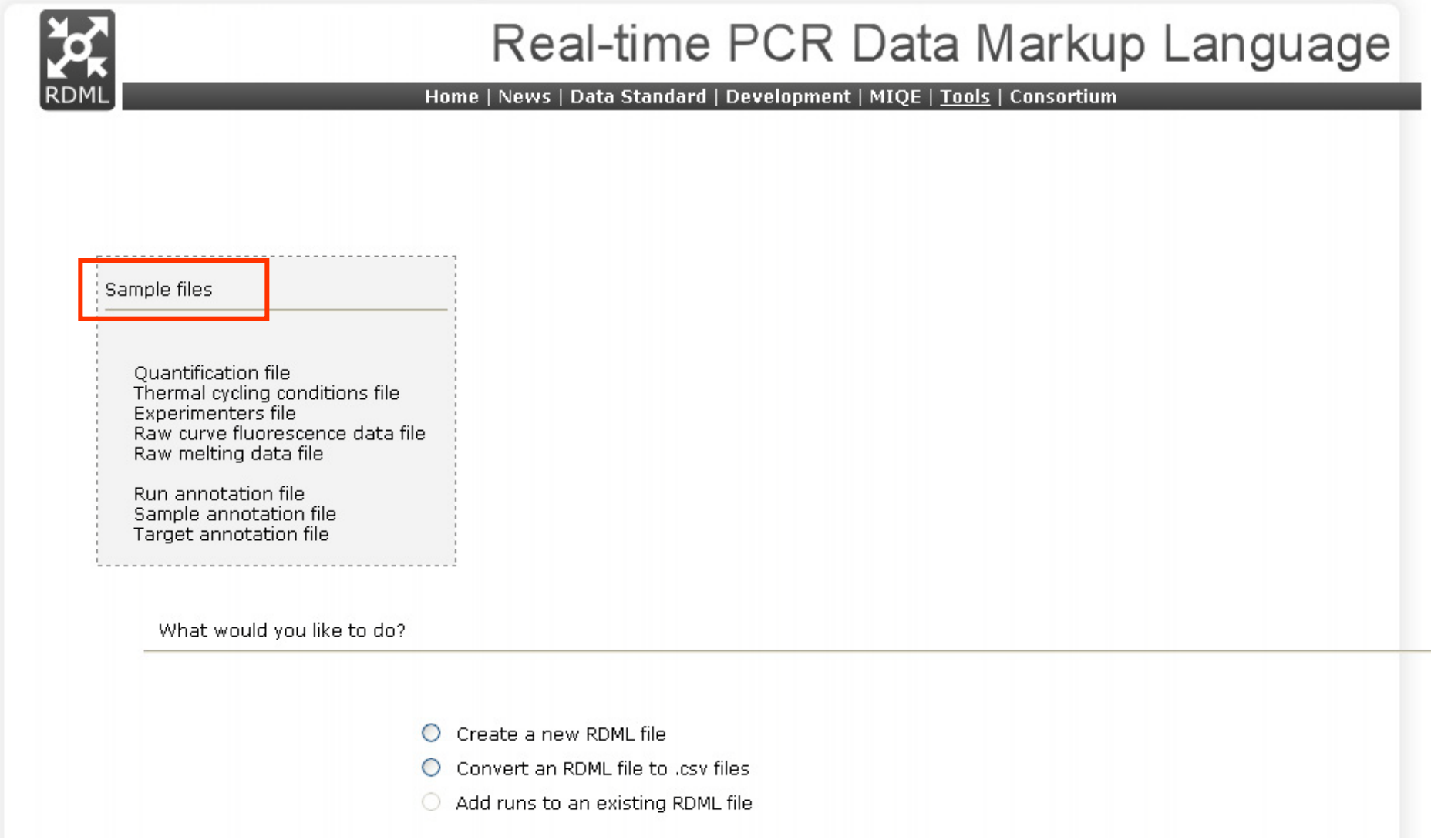
Create RDML file
Validate RDML file
Browse libraries

Join or support

The Real-time PCR Data Markup Language (RDML) is a structured and universal data standard for exchanging quantitative PCR (qPCR) data. Together with the accompanying RDML guidelines, the data standard should contain sufficient information to understand the experimental setup, re-analyse the data and interpret the results. The data standard is a flat text file in Extensible Markup Language (XML) and enables transparent exchange of annotated qPCR data between instrument software and third-party data analysis packages, between colleagues and collaborators, and between authors, peer reviewers, journals and readers. To support the public acceptance of this standard, both an on-line RDML file generator is available for end users, as well as RDML software libraries to be used by software developers, enabling import and export of RDML data files.



RDML GENERATOR



Sample files

- ✓ Quantification file
- ✓ Thermal cycling conditions file
- ✓ Experimenters file
- ✓ Raw curve fluorescence data file
- ✓ Raw melting data file

- ✓ Run annotation file
- ✓ Sample annotation file
- ✓ Target annotation file

Target Annotation file

Quantification file

reactionId	cq
A4	40
A3	40

Id	type	dye
18S	ref	VIC
TUCR51	toi	SYBRgreen
TUCR58	toi	SYBRgreen
TUCR59	toi	SYBRgreen
TUCR64	toi	SYBRgreen
TUCR70	toi	SYBRgreen
TUCR81	toi	SYBRgreen
TUCR84	toi	SYBRgreen
TUCR100	toi	SYBRgreen
TUCR121	toi	SYBRgreen
TUCR122	toi	SYBRgreen
TUCR129	toi	SYBRgreen
TUCR165	toi	SYBRgreen
TUCR167	toi	SYBRgreen
TUCR185	toi	SYBRgreen
TUCR189	toi	SYBRgreen

Run annotation file

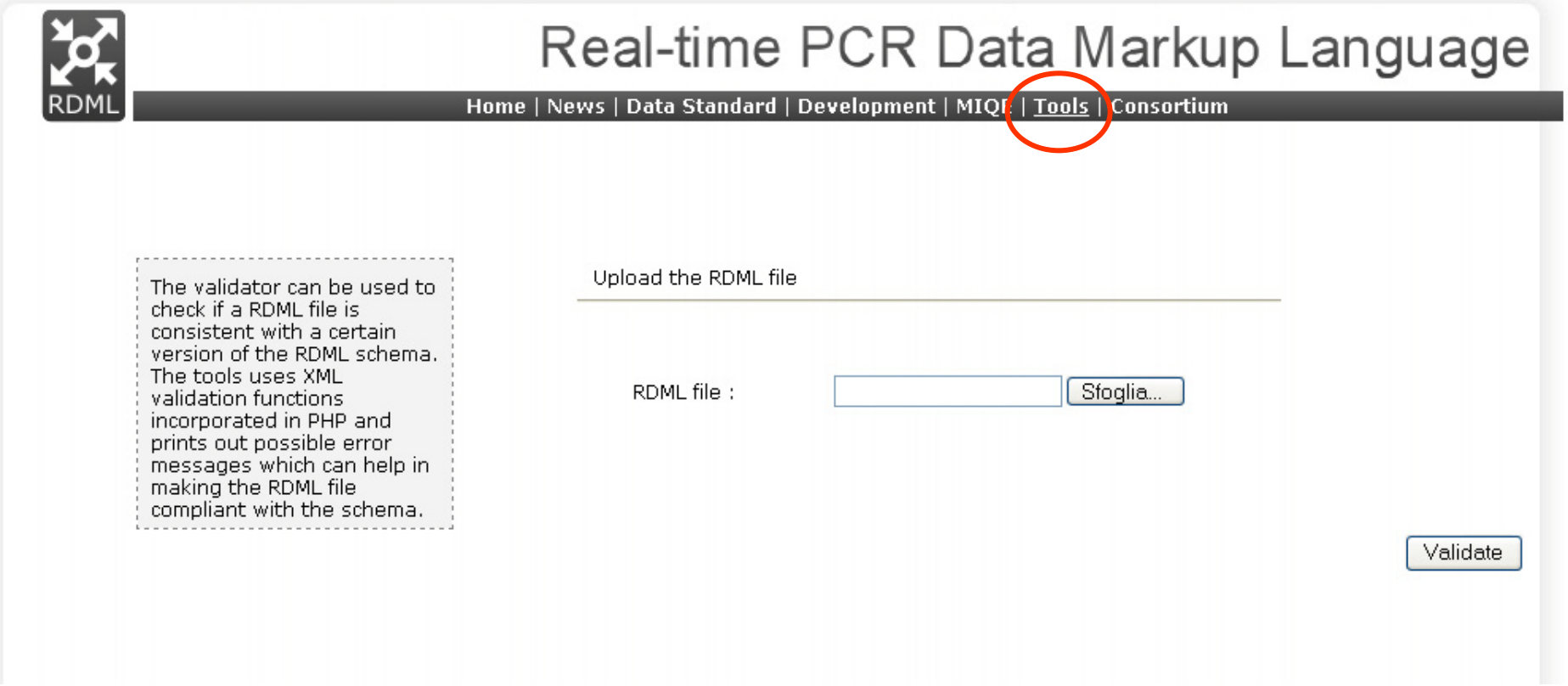
id	instrument	pcrFormat	software		thermConditions
run001	RealPlex4 system (Eppendorf, Hamburg, Germany	96-well plate 8x12	RealPlex	ne settings	TUCR
run002	RealPlex4 system (Eppendorf, Hamburg, Germany	96-well plate 8x12	RealPlex	baseline settings	TUCR
run003	RealPlex4 system (Eppendorf, Hamburg, Germany	96-well plate 8x12	RealPlex	d baseline settings	TUCR
run004	RealPlex4 system (Eppendorf, Hamburg, Germany	96-well plate 8x12	RealPlex	d baseline settings	TUCR
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Experimenters file

firstName	lastName	email		labAddress
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RDML VALIDATOR

http://www.rdml.org/validate.php



THE OUTPUT:
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MIQE & PUBLICATIONS

MIQE details should be published either in abbreviated form or as an online supplement

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25th March 2009

Dear Editor

Re: MIQE: Information for Publication of Quantitative Real-Time PCR Experiments

Many research publications employ quantitative real time PCR (qPCR) for nucleic acid analysis. However, most provide inadequate experimental detail and many use unsuitable data analysis to reach inappropriate conclusions. As a result, the quality of qPCR data is variable, manuscripts can be difficult to evaluate by reviewers, and the studies may be impossible to repeat. Consequently, there is a clear need for a common publication standard that comprehensively describes qPCR experiments.

The recently published "MIQE" guidelines provide a uniform standard for reporting qPCR data and delineate the level of detail needed to enable appropriate interpretation of qPCR experiments. These guidelines are modeled after similar recommendations for microarrays, proteinomics, and others under the umbrella of MIBBI (Minimum Information for Biological and Biomedical Investigations, http://www.mibbi.org). MIQE was developed by an international consortium of academic scientists with a conscious focus to avoid any commercial bias.

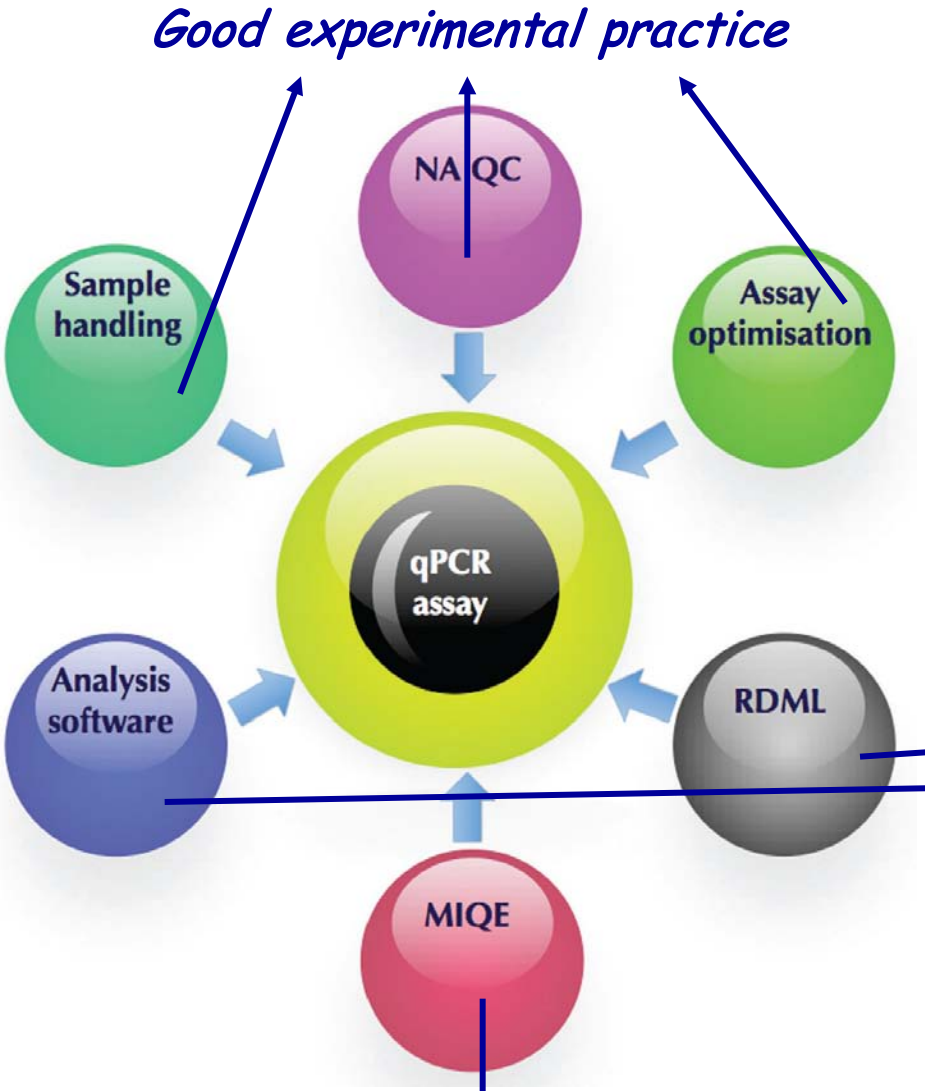
For manuscripts that use qPCR, please consider adopting these MIQE standards. We suggest that authors include the accompanying checklist upon initial submission, enabling even non-expert reviewers to assess the validity of the qPCR data and any conclusion drawn. Attached please find an expanded justification for the need of these guidelines, the published article and the qPCR checklist that can also be found at http://www.RDML.org/MIQE.

Yours sincerely,

Prof Stephen A Bustin on behalf of all authors:

Dr Vladimir Benes, EMBL Heidelberg, Germany
Dr Jeremy A. Garson University College London, UK
Dr Jan Hellemans Ghent University Hospital, Belgium
Dr Jim Huggett University College London UK
Prof Mikael Kubista TATAA Biocenter, Sweden and Institute of Biotechnology, Czech Republic
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Dr Gregory L. Shipley The University of Texas Health Science Center, USA
Prof Jo Vandesompele Ghent University Hospital, Belgium
Prof Carl T. Wittwer, University of Utah and ARUP Institute for Clinical and Experimental Pathology, USA

SUMMARY: COMPONENTS OF A RELIABLE qPCR ASSAY



Bustin SA, Eur Pharm Rev 2008

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