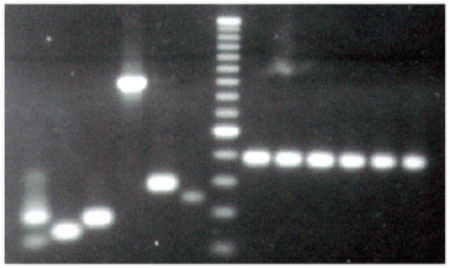
RT-qPCR Guidelines:

From designing to publishing your data

Emir Hodzic D.V.M., Ph.D.

Real-Time PCR Molecular & Diagnostic Core Facility

UC Davis, January 31, 2011



1997

Technology

PCR efficiency
Normalization
RT condition
Primer design
RNA integrity
Inhibition
Sample heterogeneity
Reporting standards

Reliability?

Novelty
"Free-for-all"

Reflection
Examination
Explanation

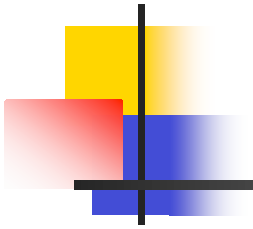
Critical assessment

qPCR 2011

Biology

Allele specific expression
Splice variants
miRNA
mRNA localization
Multiple promoters
Temporal variation
Single cell expression

Relevance?



Many publications using RT-qPCR report seriously misleading results

Publishe
Science
Vol. 309
DOI: 10.

RESEAR

The
Apex

Tao Hu

Auth

* To wh

Note ad
shoot a

ABST

Day
but h
Arab
gene
flowe
activ
signa

Retraction

WE WISH TO RETRACT OUR RESEARCH ARTICLE "THE MRNA OF THE *ARABIDOPSIS* GENE *FT* MOVES from leaf to shoot apex and induces flowering" (*1*). After the first author (T.H.) left the Umeå Plant Science Centre for another position, analysis of his original data revealed several anomalies. It is apparent from these files that data from the real-time RT-PCR were analyzed incorrectly. Certain data points were removed, while other data points were given increased weight in the statistical analysis. When all the primary real-time RT-PCR data are subjected to correct statistical analysis, most of the reported significant differences between time points disappear. Because of this, we are retracting the paper in its entirety.

In new experiments, we have reproduced the floral induction caused by a heat-shock induction of *FT* in a single leaf, but we have failed to detect movement of the transgenic *FT* mRNA from leaf to shoot apex. We therefore retract the conclusion that *FT* mRNA is part of the florigenic inductive signal moving from leaf to shoot apex.

We deeply regret any scientific misconceptions that have resulted from the publication of these data.

The first author of the paper (T.H.) strongly objects to the retraction of the paper and has therefore declined to be an author of the retraction.

Our related *Science* Report on the *CO*/*FT* regulatory module in trees (*2*) is not affected by this Retraction. In this paper, T.H. was involved in the construction and analysis of the *PtCEN1* experiments reported in the Supporting Online Material. These data have been reevaluated and found to be correctly reported.

HENRIK BÖHLENIUS,[1] SVEN ERIKSSON,[1] FRANÇOIS PARCY,[2] OVE NILSSON[1]

[1]Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-90183 Umeå, Sweden. [2]Laboratoire de Physiologie Cellulaire Végétale, Département Réponse et Dynamique Cellulaires (DRDC/PCV), Unité Mixte de Recherche 5168 [(UMR) Joint Research Unit], Centre National de la Recherche Scientifique (CNRS), Commissariat à l'Energie Atomique (CEA), Institut National de la Recherche Agronomique (INRA), Université Joseph Fourier, 17 rue des Martyrs, batiment C2-38054, Grenoble Cedex 9, France.

References

1. T. Huang, H. Böhlenius, S.Eriksson, F. Parcy, O. Nilsson, *Science* **309**, 1694 (2005).
2. H. Böhlenius *et al.*, *Science* **312**, 1040 (2006).

✗ incorrect analysis

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 22, Number 12, 2006, pp. 1253–1259
© Mary Ann Liebert, Inc.

Quantitative Analysis of Human Endogenous Retrovirus-W *env* in Neuroinflammatory Diseases

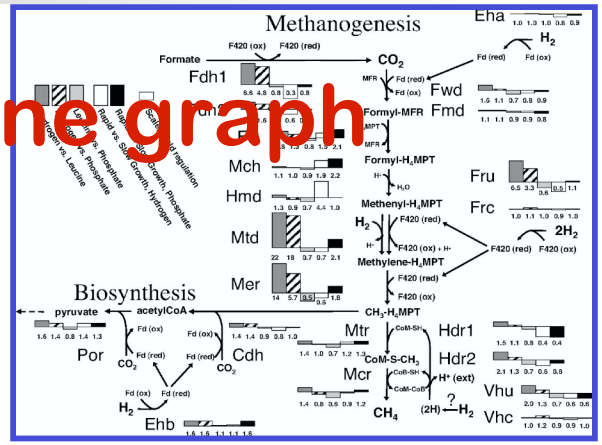
JOSEPH M. ANTONY,[1] MARYAM IZAD,[1,2] AMIT BAR-OR,[3] KENNETH G. WARREN,[4] MOHAMMED VODJGANI,[2] FRANCOIS MALLET,[5] and C. POWER[1,4]

previously from our group.[14] To quantify syncytin-1 DNA or RNA levels, a standard curve was generated with serial 10-fold dilutions (10^{11} copies/8 μg to 1 copy/8 μg) of the DNA plasmid (pBS-syncytin-1) or cDNA derived from the *in vitro* transcribed RNA, respectively.

FIG. 1. Syncytin-1 DNA copy numbers are increased in brain of MS patients. A standard curve for DNA copy number (correlation coefficient: 0.979; slope: −1.365; intercept: 29.435; $y = -1.365x + 29.435$) was derived (**A**). The number of viral DNA copies indicated a significant increase in brain of MS patients (9.8 $\log_{10}$) relative to non-MS controls (7.9 $\log_{10}$) (**B**). A standard curve for GAPDH (correlation coefficient: 0.965; slope: −2.276; intercept: 29.371; $y = -2.276x + 29.371$) was derived (**C**).

Slope: -1.365
Slope: -2.276

The problems



All theories proven with one graph

Insufficient information

- ***sample:*** quality assessment
- ***experimental design:*** primers, probe, and much more
- ***protocol:*** reaction condition
- ***analysis:*** normalization
- ***interpretation:*** significance of fold change methods

Clinical Chemistry 55:4
611–622 (2009)

The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative 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]

Aims

- Maximize confidence in technical merit of publications using real-time PCR
- Reliable and unequivocal interpretation of results
- Full disclosure of reagents and analytical methods
- Published results easier to reproduce
- Focus on their biological relevance

Nomenclature

- ✓qPCR and RT-qPCR
- ✓Reference gene(s) ***not*** housekeeping genes
- ✓Quantification ***not*** quantitation
- ✓Hydrolysis probes ***not*** TaqMan
- ✓Quantification cycles (Cq) replaces Ct, Cp, TOP*

**Ct=cycle threshold-AB; Cp=crossing point-Roche; TOP=take of point-Corbett*

Are You MIQE* Compliant?

SIGMA-ALDRICH

SIGMA Life Science Where bio begins™

PREMIER Biosoft International

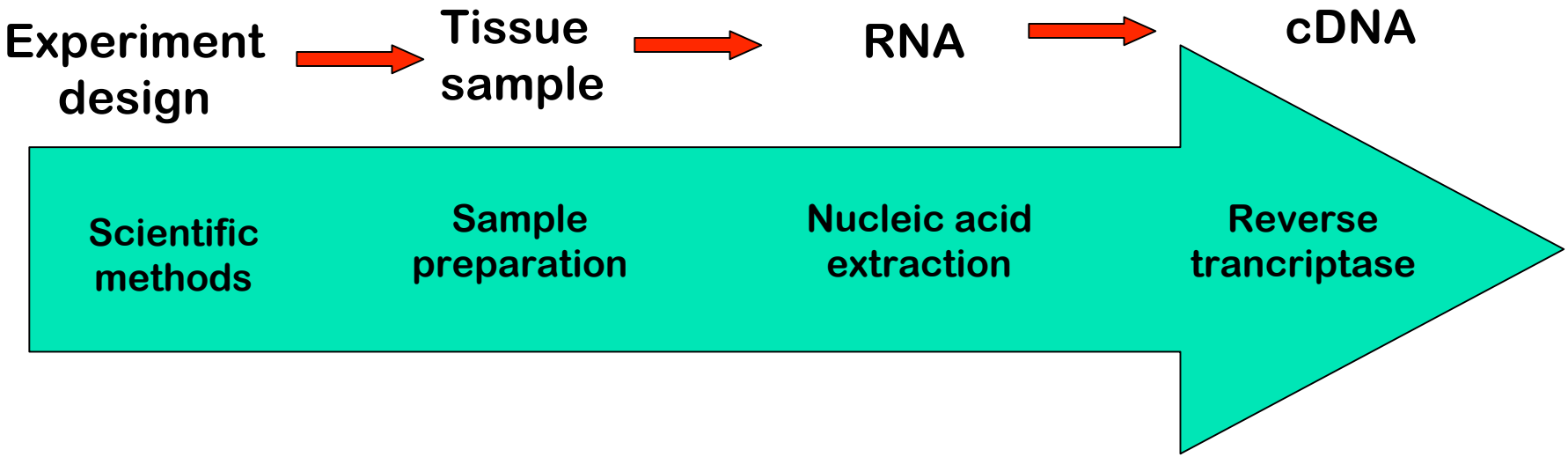
Are You MIQE* Compliant?

*Minimum Information for Publication of Quantitative Real-Time PCR Experiments

The MIQE guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Primerdesign

RT-qPCR: steps and variables



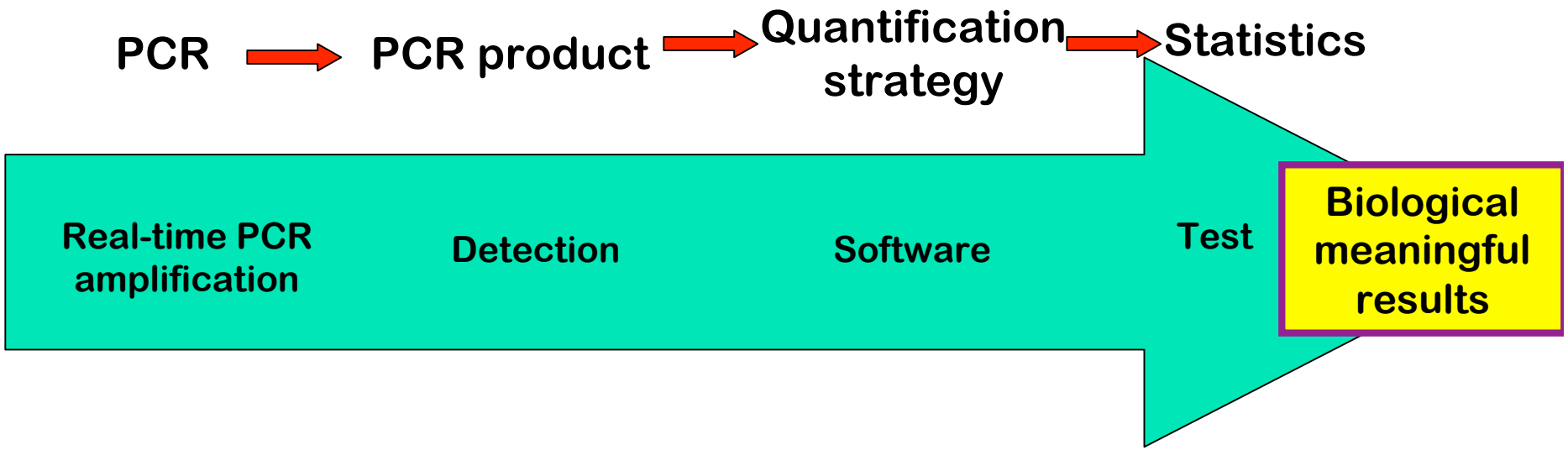
Experimental procedure:
-Observation
-Define problem
-Hypothesis
-Experimental and control groups
-Experimental replicates

Sampling method:
-Biopsy
-Stability of DNA/RNA
-Tissue storage
-Liquid Nitrogen
-RNA later

Extraction method:
-Total RNA
-mRNA
-Columns
-Robot
-OD
-Bioanalyzer 2100
-Storage

Efficiency of RT:
-RT enzyme type
-RT temperature
-Poly-T primer
-Random hexamers
-Specific primer
-One step
-Two step

RT-qPCR: steps and variables



Efficiency and specificity:
-Primer design
-Primer specificity
-gDNA/cDNA input
-Enzyme types & mixture
-Cycler

Detection method:
-Sybr Green I
-Probes; TaqMan, Beacons, etc
-Background correction
-Fit point method
-Other models (Log)
-Singleplex, multiplex

Quantification strategy:
-Absolute quantification
  * Using calibration curve
-Relative quantification
  * Normalization with RG
  * Normalization with more (>3) RGs

Test method:
-SAS, SPSS, Excel
-Normality of data
-t-Test
-ANOVA
-Randomization test

Guidelines

Experimental design
Sample handling
Nucleic acid extraction
Reverse transcription
Target
Primers & probes
Assay details
Validation
Data analysis

The MIQE guidelines
-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

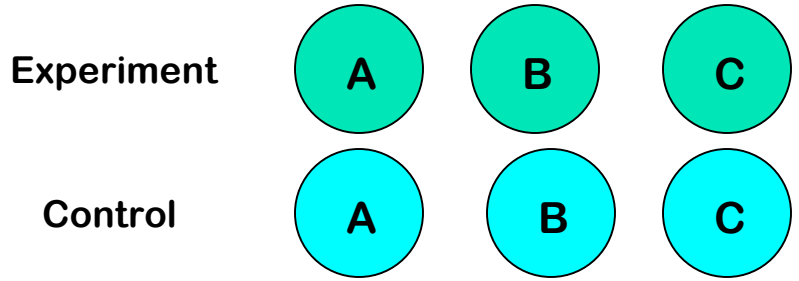
E = essential, D = desirable

Experimental design

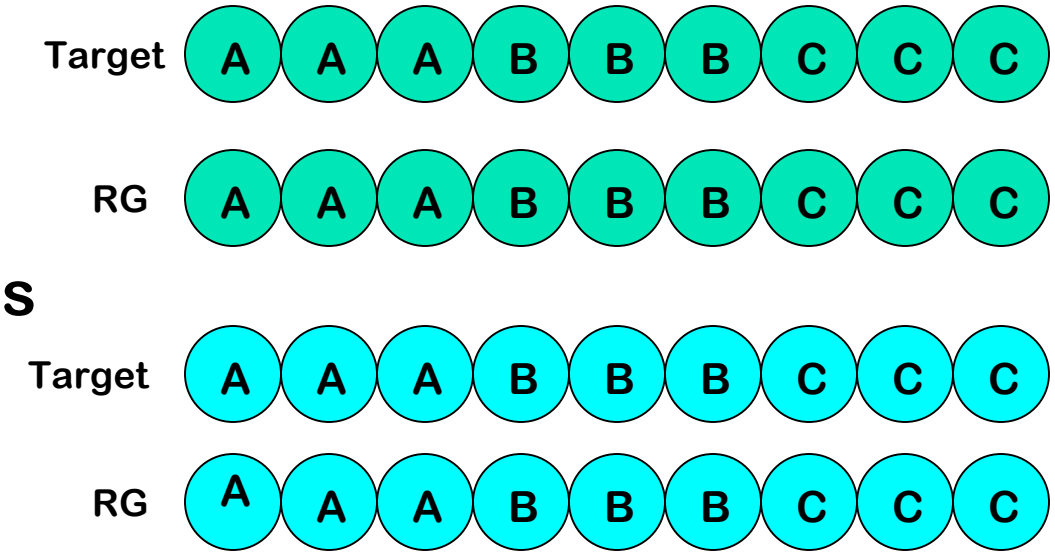
Exp't procedure	Control groups	Replicates	Exp't conditions
Disease or treatment groups	Time course of study	Biological samples	Growth conditions
Target genes	Normal vs. disease	Technical samples	Days of development
Reference genes	Untreated vs. treated		Amount of drug/mass
These steps define the: experimental parameters; the goals and the samples based on literature or previous experimental data.			Phenotype
			Incubation time

Experimental replicates

Biological replicates



RT-qPCR samples
Technical replicates



The MIQE guidelines
-Sample handling-

➢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 samples)	E

E = essential, D = desirable, FFPE = formalin-fixed paraffin embedded

Sample handling

- Source
- Method of preservation

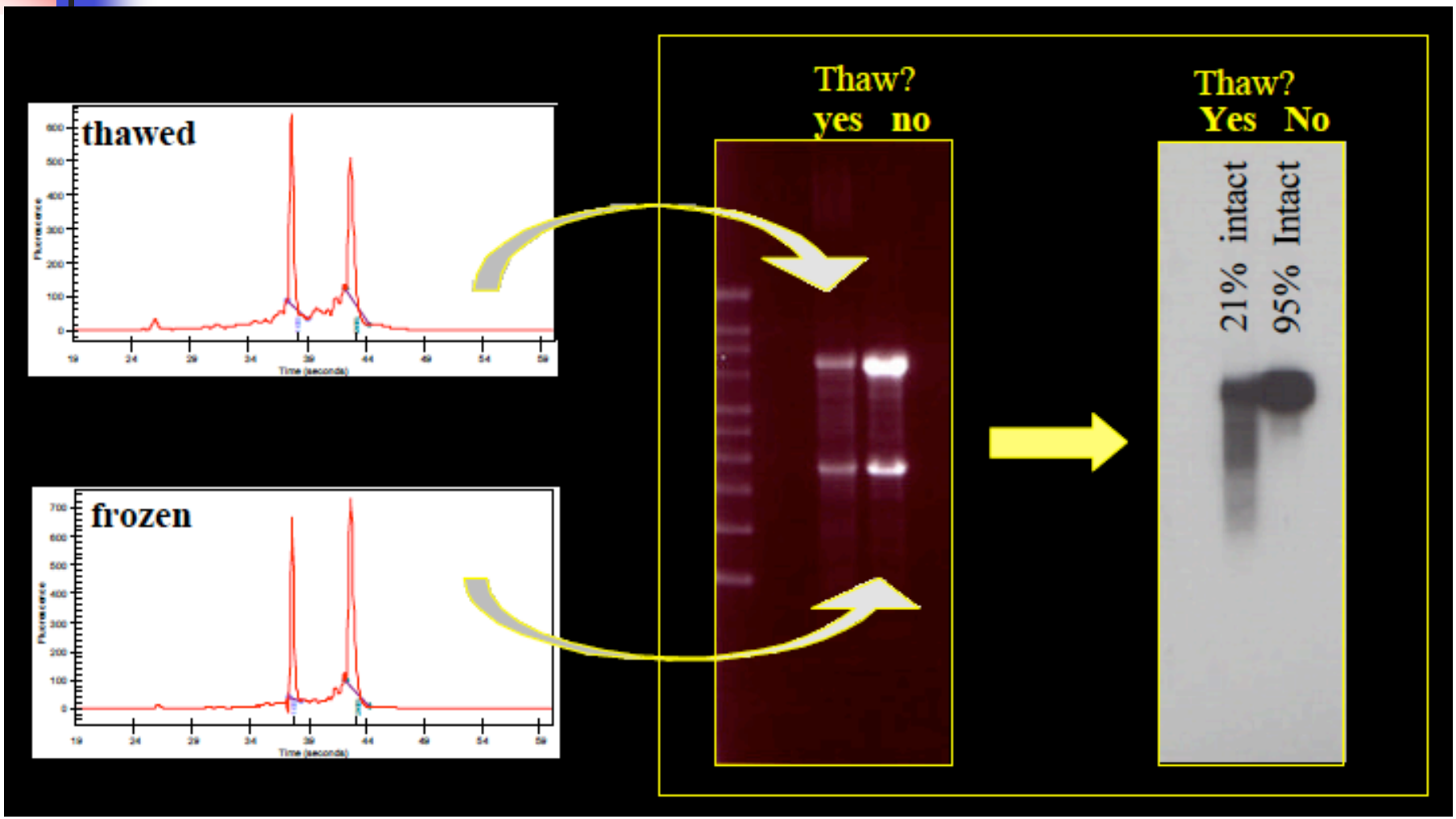
  Snap freeze

  RNAlater®
- Storage time

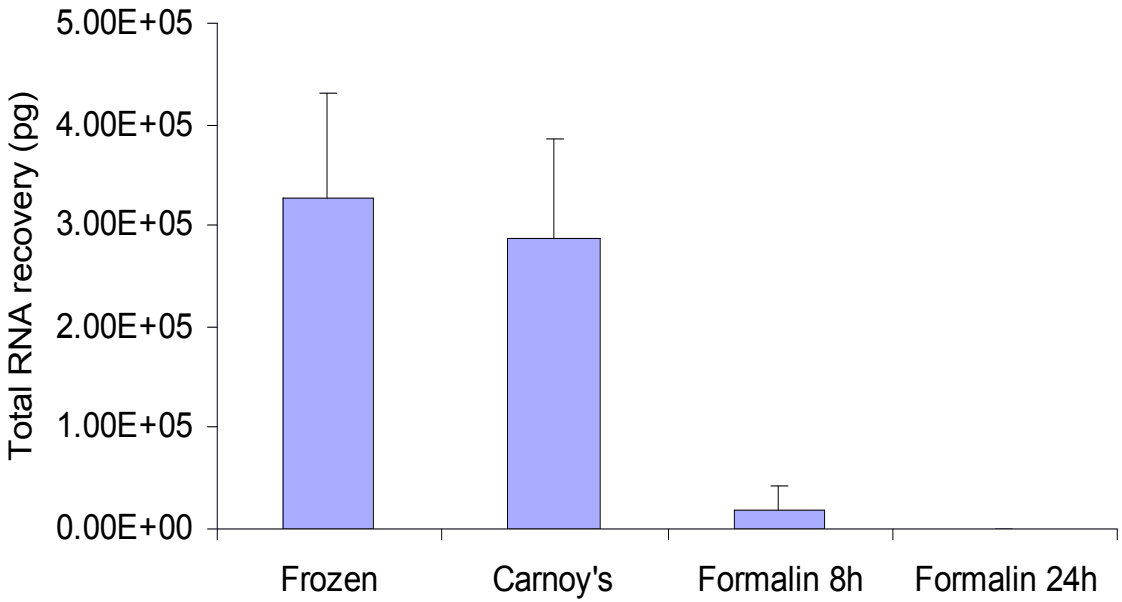
  RNAlater®: at 37^{0}C 24 h, at 25^{0}C 1-2 week, at 4^{0}C for months
- Handling

  Tissue grinding, freeze/thaw
- Disruption

Freeze/thaw effect



mRNA recovery

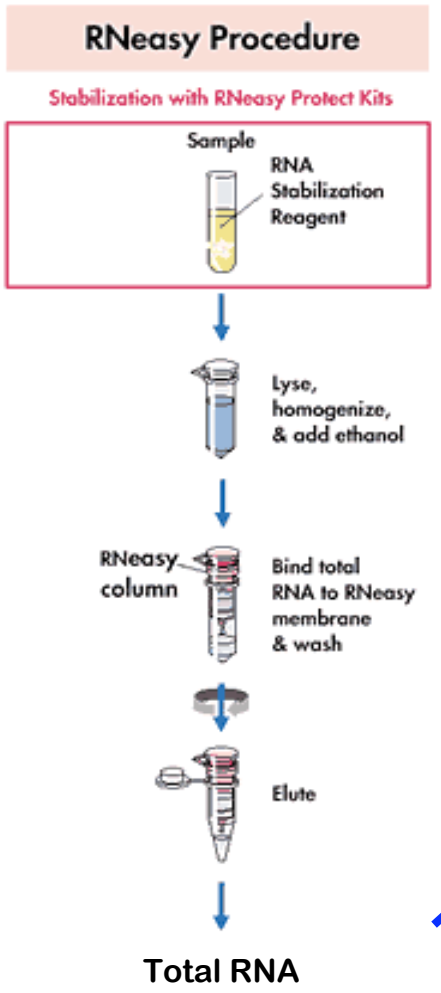


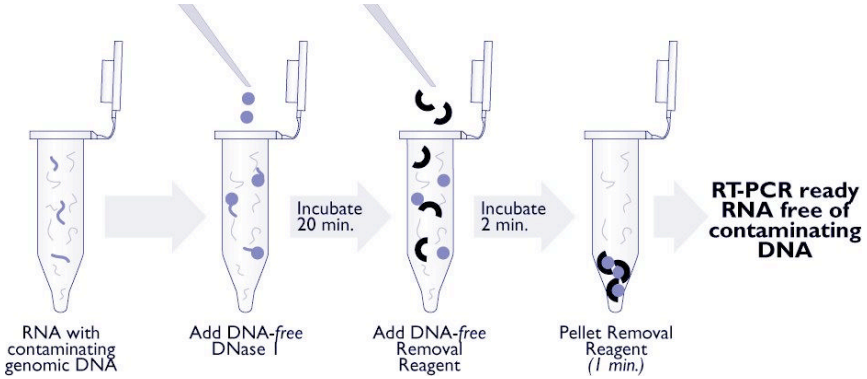
The MIQE guidelines
-Nucleic acid extraction-

➢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 (A260/A280)	D
✓Yield	D
➢RNA integrity: method/instrument	E
✓RIN/RQI or Cq of 3 and 5 transcripts	E
✓Electrophoresis traces	D
➢Inhibition testing (Cq dilutions, spike, or other)	E

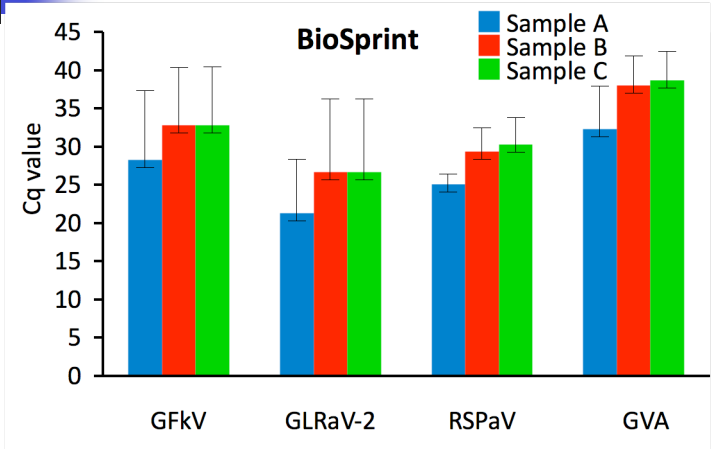
E = essential, D = desirable

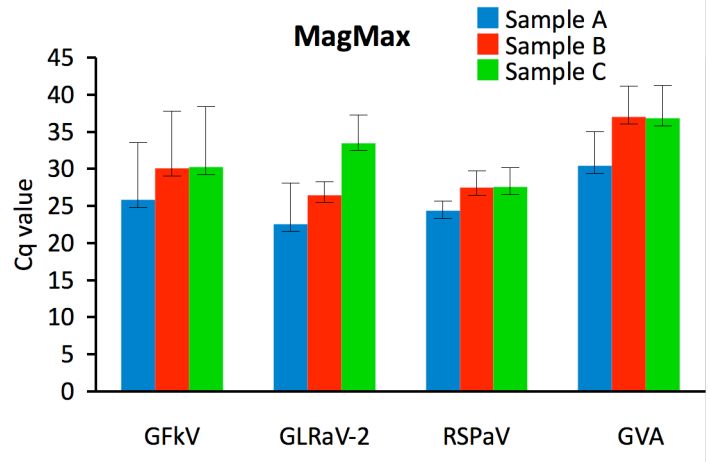
Nucleic acid extraction

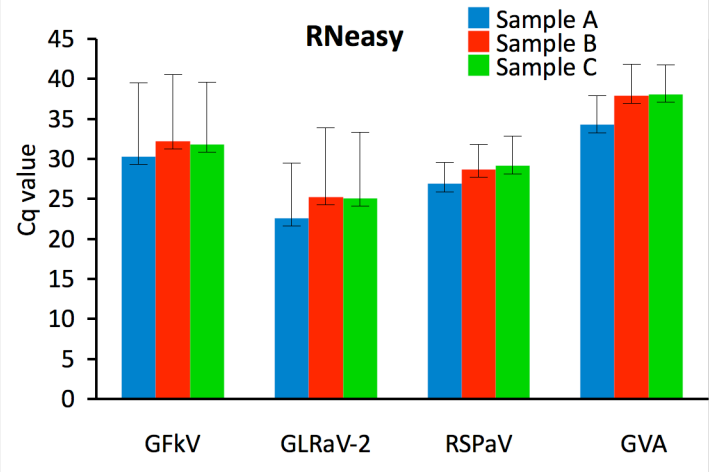


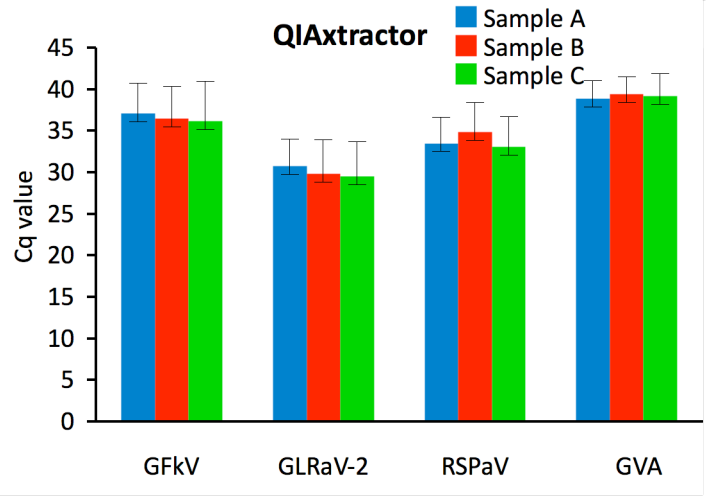


Extraction method





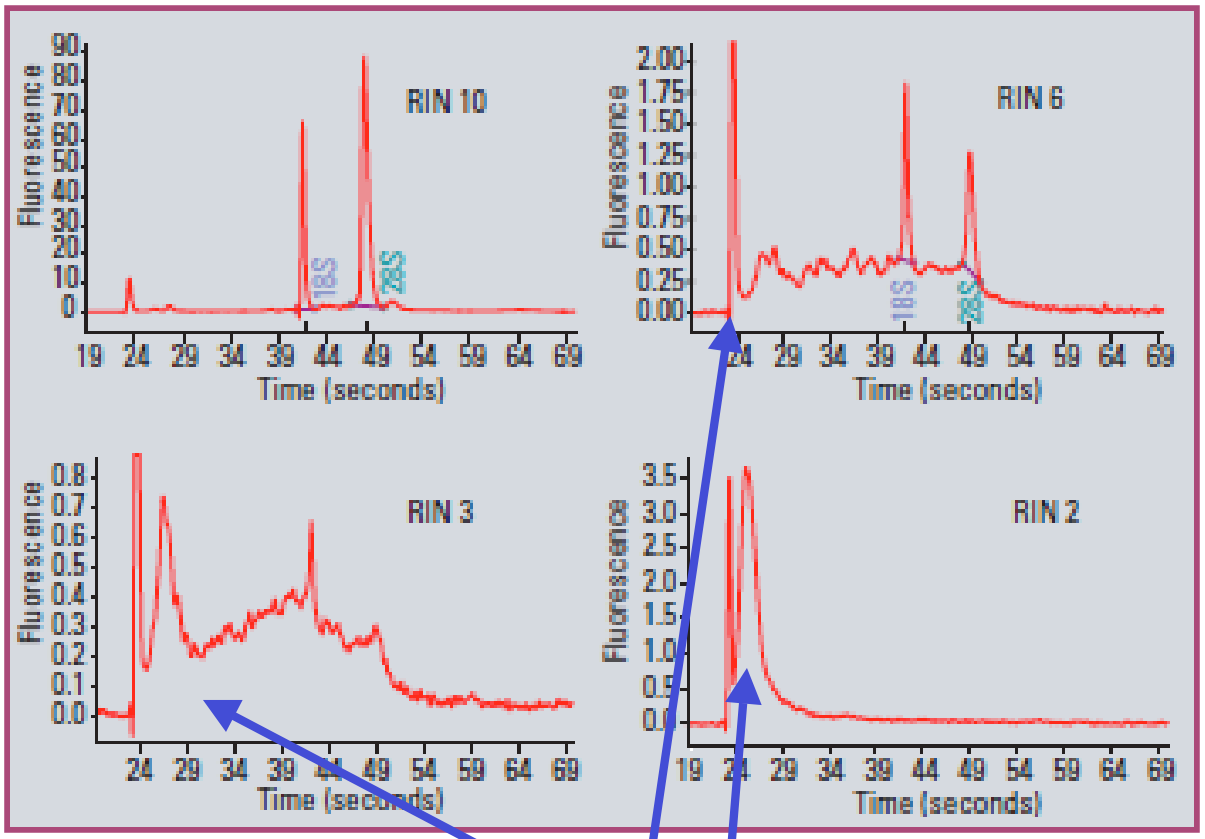




Total RNA quality

- Should be free of protein (absorbance 260nm/280nm - >1.8)
- Undegraded (28S/18S - 1.8 to 2.0)
- Free of gDNA
- Free of any substance which complex with reaction co-factors, like Mg2+ or Mn2+
- Free of nucleases
- Free of PCR inhibitors
  - Purification methods
  - Clean-up methods

Total RNA analysis



RNA degradation

RIN classification:

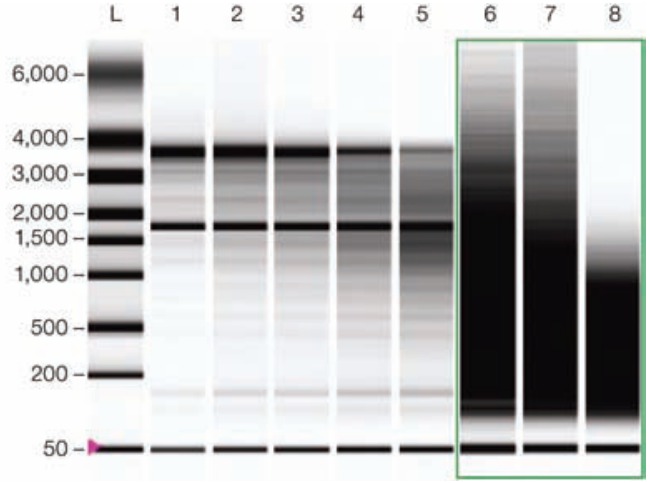
High: RIN ≥ 7

Medium: 2.5 ≥ RIN < 7

Low: RIN < 2.5

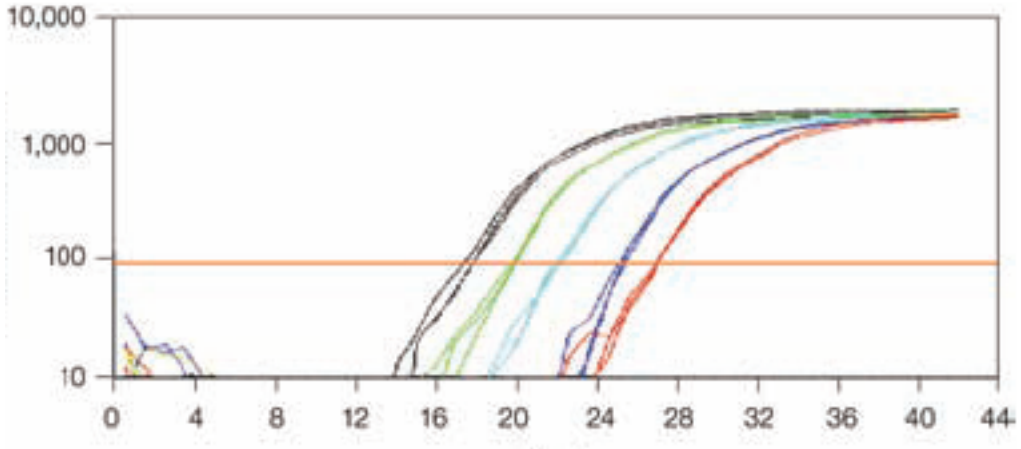


Analysis of RNA purity and integrity

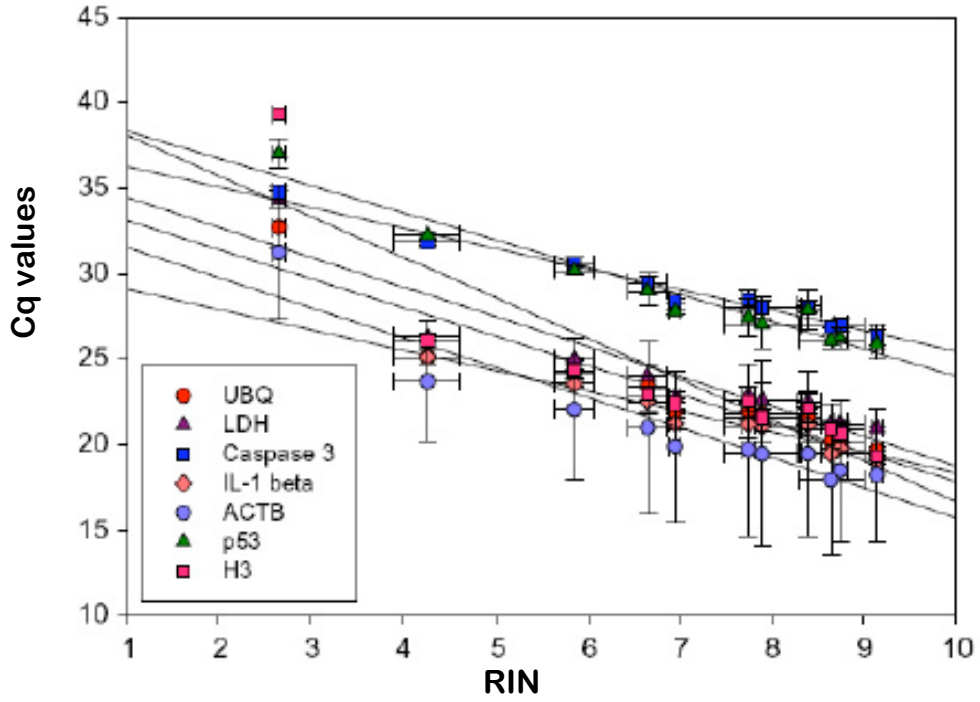


Sample		$A_{260/280}$*	$A_{260/230}$*
1	Control—no heat	1.90	2.44
2	3 min @ 90°C	1.93	2.40
3	5 min @ 90°C	2.06	2.37
4	10 min @ 90°C	2.03	2.37
5	15 min @ 90°C	2.02	2.31
6	1 hr @ 90°C	1.99	2.18
7	2 hr @ 90°C	2.00	2.32
8	4 hr @ 90°C	1.89	2.23

Accepted ratios for good quality RNA are >1.8



Influence of RIN on Cq



r^2_{mean}=0.837
P<0.001
Mean slope of the regression line=1.578

Threshold for reliable qPCR results: RIN > 6

Becker C et al. Methods 2010

PCR inhibition: in what samples?

Input material:

- fecal sample
- cartilage, liver, cornea, etc
- environmental sample: soil, water, air, stones, wood, swabs:
- humic acid
- plants: …
- whole blood: heme
- anticoagulant: heparin

Caused by extraction system / wrong protocol: total RNA or gDNA

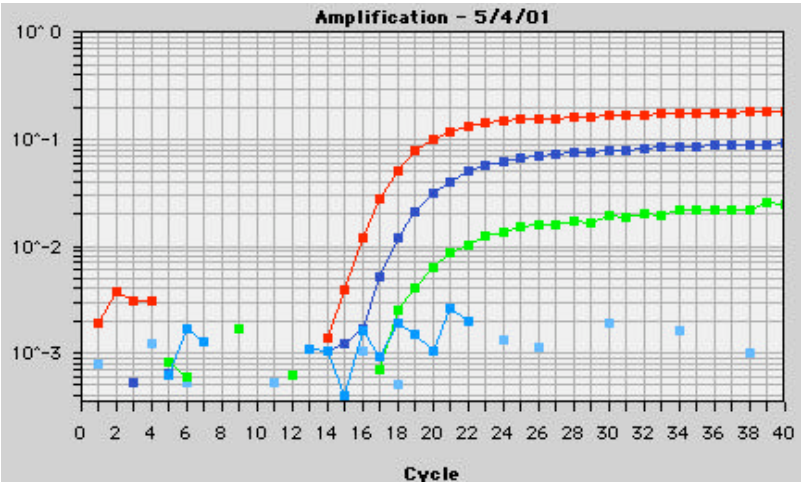
- insufficient centrifugation with second wash: ethanol in elute inhibits RT step and PCR step
- RNA precipitation with isopropanol; ethanol wash steps: ethanol leftover

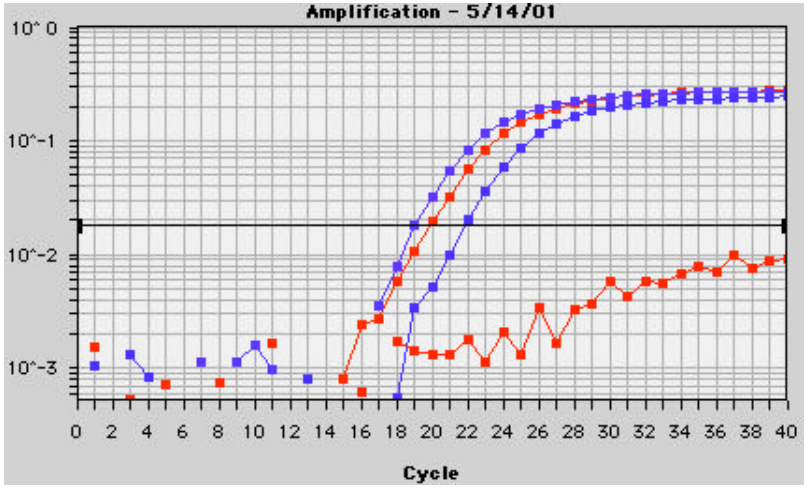
cDNA or gDNA too concentrated:

- more than 10% of cDNA into PCR: inhibition
- too much gDNA in PCR; inhibition

PCR inhibition

Can be relieved by heat-denaturation





Removal of PCR inhibition

Stool sample

	Dilution of NA					
	1:10	1:5	1:1			
	Volume used for PCR					
E-No	0.5	1 ul	5 ul		oCT	@
15	23.17	22.72	30.22		22.72	1:5
16	21.85	22.35	-		21.85	1:10
17	27.08	31.24	-		27.08	1:10
18	20.71	20.8	-		20.71	1:10
19	20.01	20.02	30.02		20.01	1:10
20	21.3	24.7	-		21.3	1:10
21	19.35	19.04	34.58		19.04	1:5
22	33.88	22.99	-		22.99	1:5
23	22.04	30.79	-		22.04	1:10
24	-	-	-		19.99	-
25	19.99	21.43	-		17.62	1:10
26	17.62	18.3	-		17.62	1:10
27	35	-	-		35	1:10

The MIQE guidelines
-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
➢Cqs with and without RT	D
➢Storage conditions of cDNA	D

E = essential, D = desirable

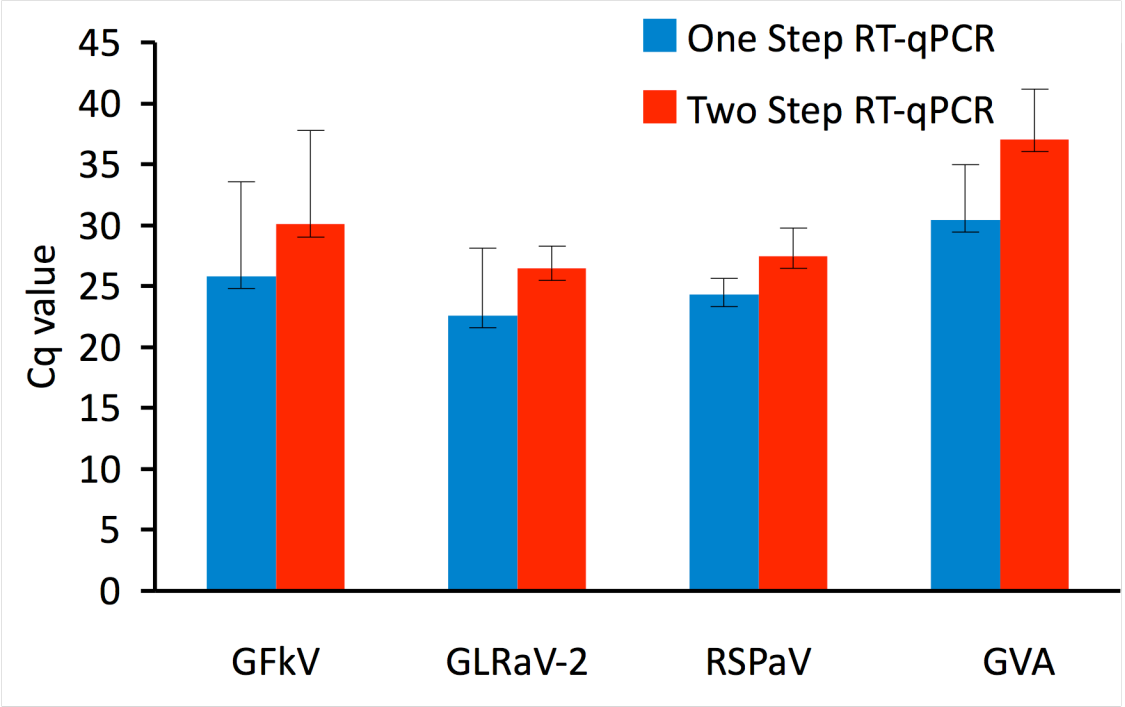
Reverse transcription

The conversion of mRNA to cDNA is a major obstacle and arguably is the single most variable step in the whole quantification procedure.

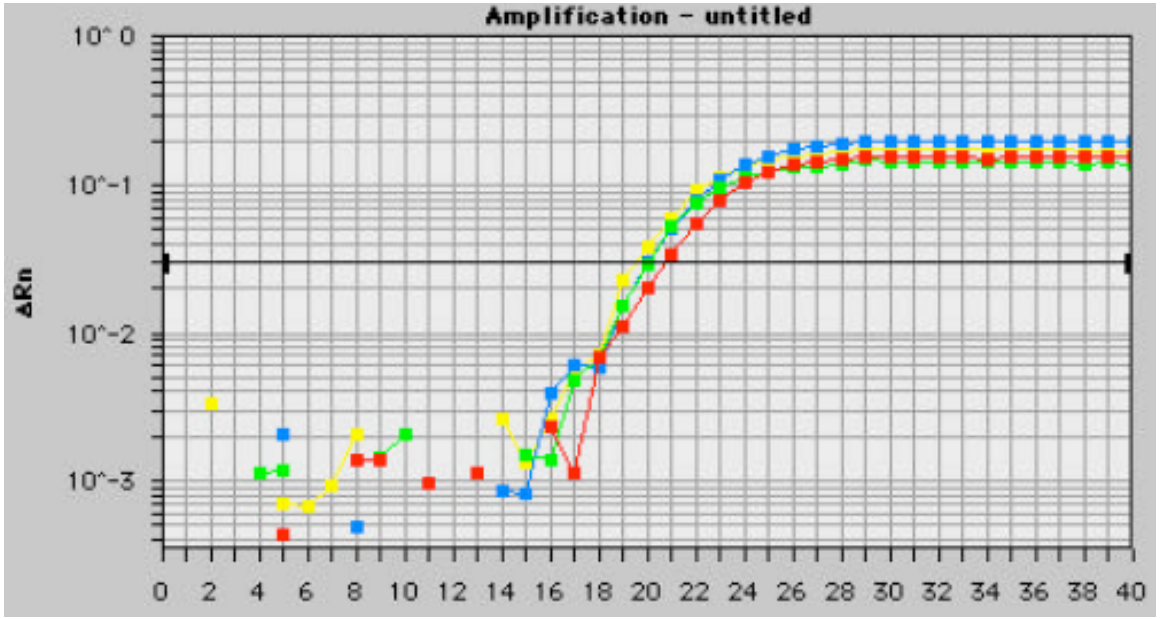
One step vs. two step

	One-Step qRT-PCR	Two-step qRT-PCR
Description	RT and qPCR reactions performed in the same tube Optimized working buffer for both the RT and PCR enzymes	RT and qPCR reactions performed in separate tubes
Pros	Save pipetting steps No contamination between RT and qPCR steps Lower background in SYBR® assays Best option for High-Throughput Screening (less time consuming than 2-step reactions)	More efficient because random primers and oligo d(T) can be used Possibility to stock cDNA to quantify several targets More flexible (separate optimization possible for the two reactions)
Cons	No possibility to use UNG carry-over prevention Usually less sensitve than a Two-Steps qRT-PCR assay	RNase inhibitors that can influence the PCR reaction after the RT Higher background when performing a SYBR® assay

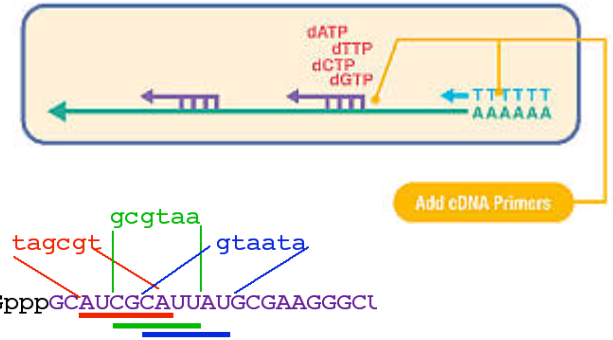
One step vs. two step



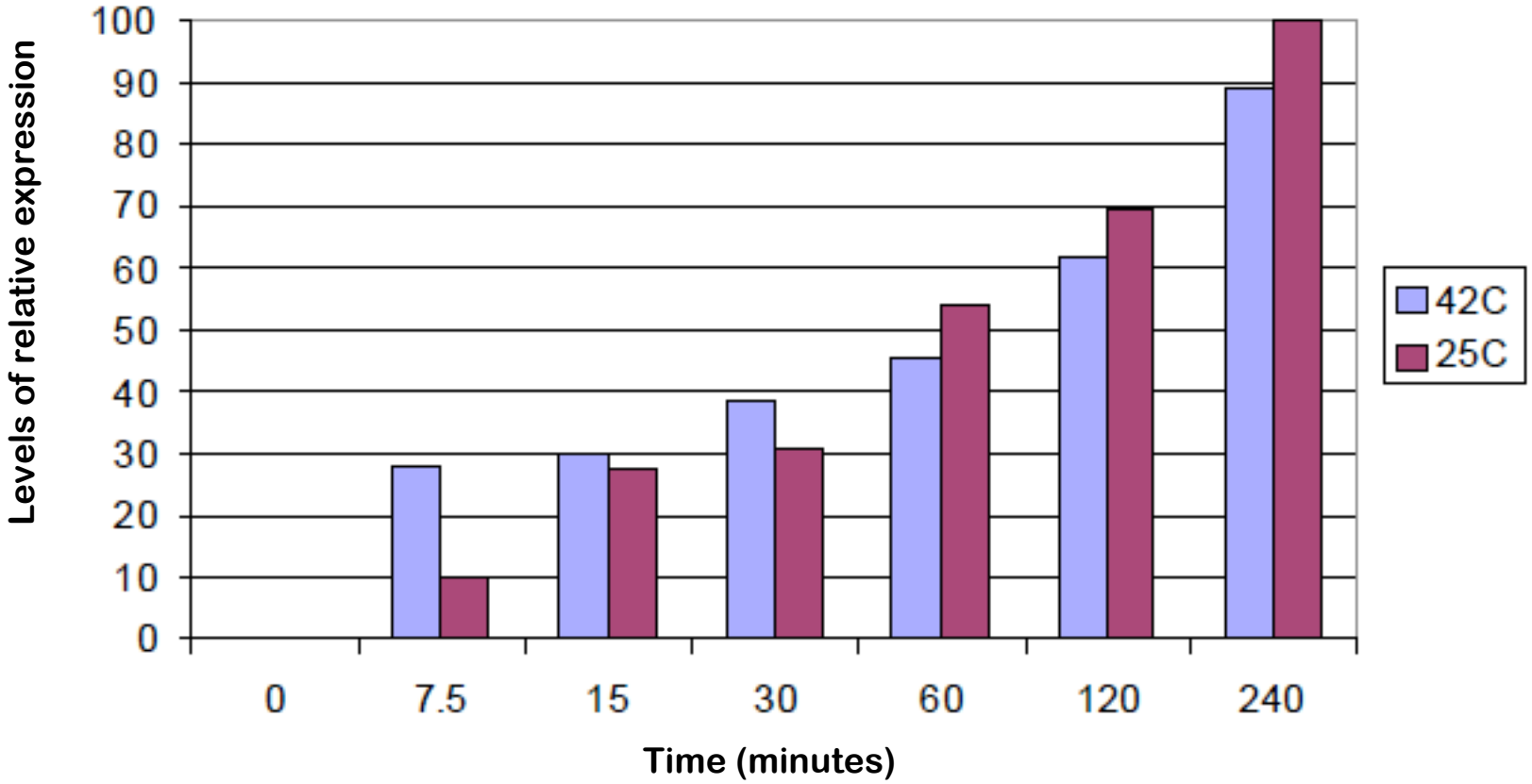
Efficiency of RT primers



- random primer
- oligo dT
- specific primers
  (more sensitive?)
- control



cDNA synthesis: 25^0C vs. 42^0C

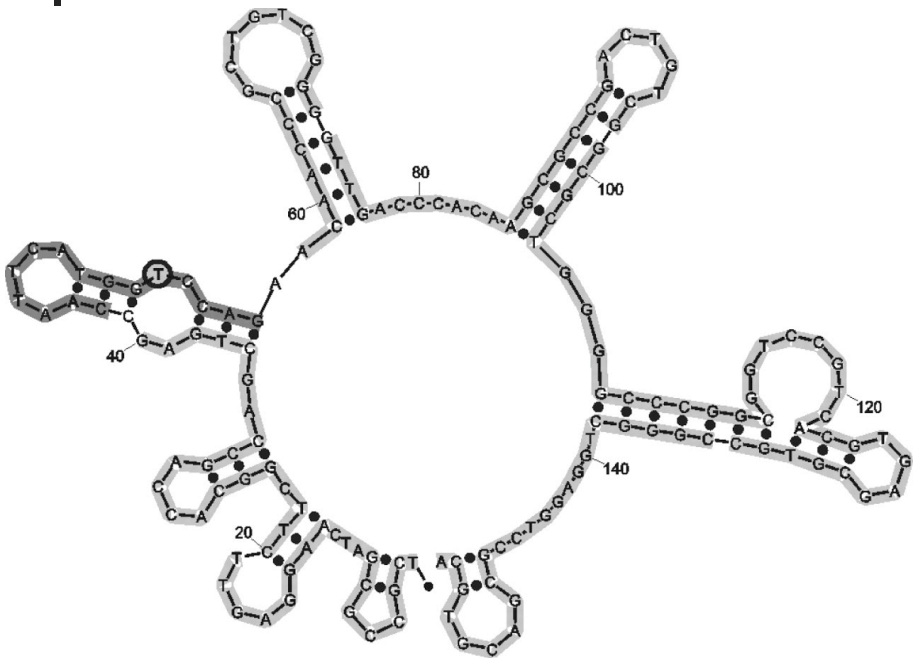


The MIQE guidelines
-qPCR target information-

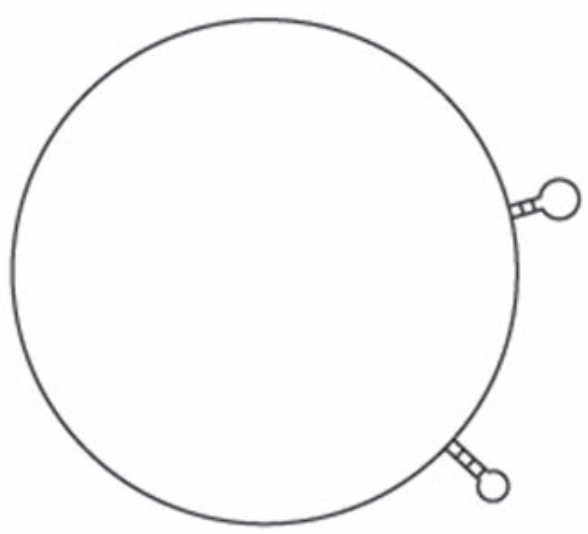
➢Gene symbol	E
➢Sequence accession number	E
➢Location of amplicon	D
➢Amplicon length	E
➢In silico specificity screen (BLAST, and so on)	E
➢Pseudogenes, retropseudogenes, or other homologs?	D
➢Sequence alignment	D
➢Secondary structure analysis of amplicon	D
➢Location of each primer by exon or intron (if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

E = essential, D = desirable

Amplicon secondary structure



60^{0}C



65^{0}C

http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form

The MIQE guidelines -qPCR oligonucleotides-

➢Primer sequences	E
➢RTPrimerDB identification number	D
➢Probe sequences	D
➢Location and identity of any modifications	E
➢Manufacturer of oligonucleotides	D
➢Purification method	D

E = essential, D = desirable

"Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it can not be an essential requirement. Use of such assays is discouraged."
(Bustin SA et al.Clin Chem, 2009)

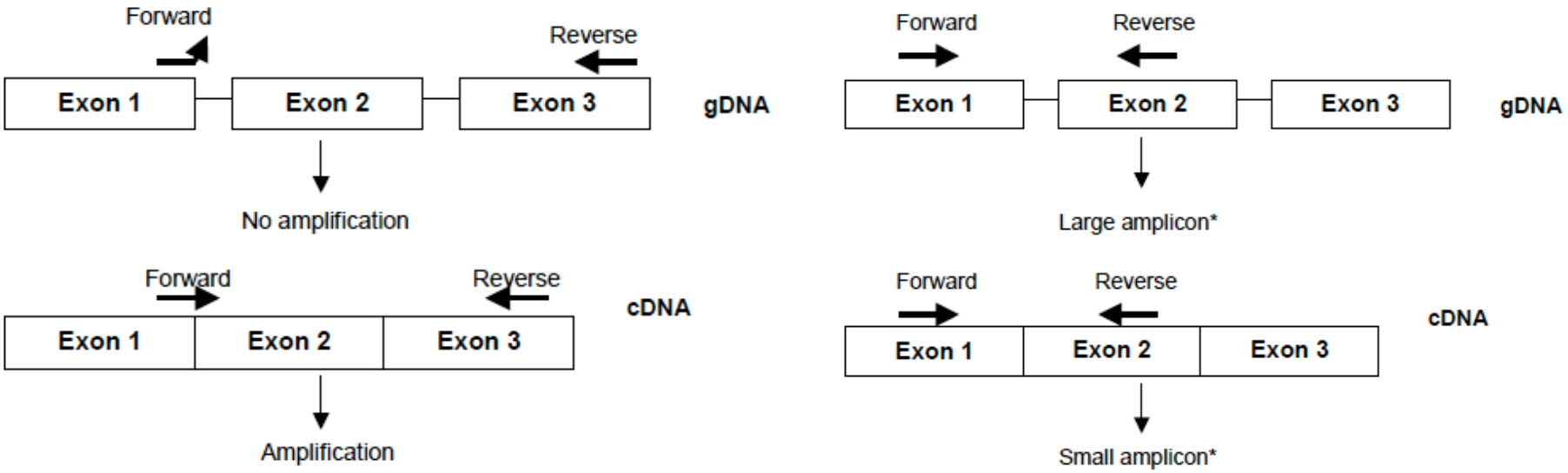
qPCR oligonucleotides

- Primer-BLAST your sequence (NCBI) to find unique primers and amplicon sequences.
- Enter sequence in “M-fold” to assure minimal secondary structure.
- Choose at least two sets of primer pairs and validate with thermal gradient and standard curve.

qPCR oligonucleotides

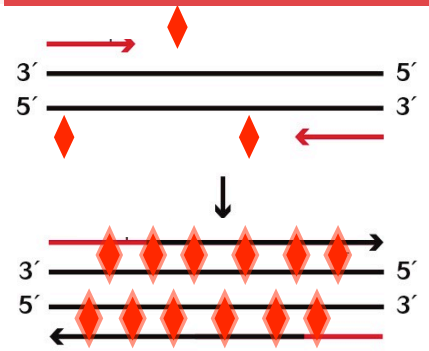
- Gene accession number
- Amplicon size
- Primers/probe sequence
- Specificity
- Efficiency
  - No primer-dimers
  - Ideally should not give a DNA signal
    - cross exon/exon boundary

Intron spanning/flanking primers



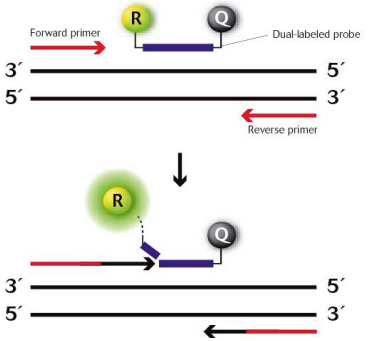
Detection Systems

Intercalating dye

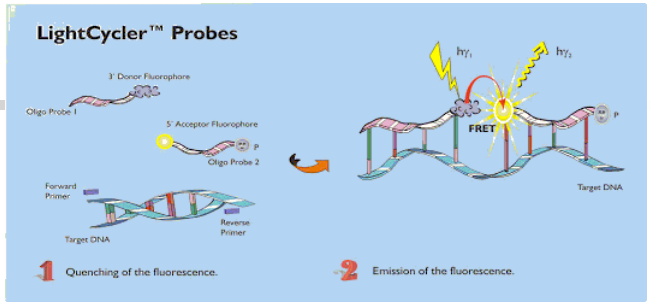


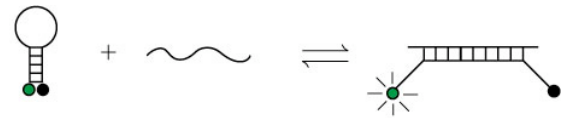
SYBR Green I®

Probe systems

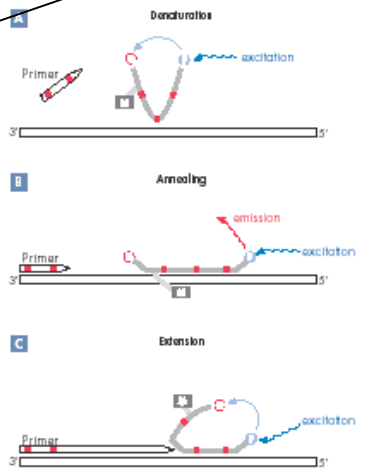


TaqMan®



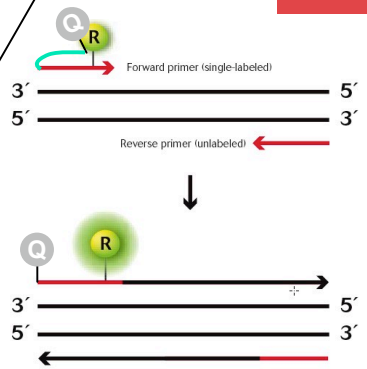


Molecular Beacon | Target | Hybrid

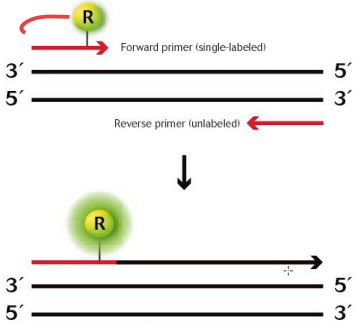


Quantiprobe®

Primer systems



Amplifluor™



LUX™

The MIQE guidelines
-qPCR protocol-

➢Complete reaction conditions	E
➢Reaction volume and amount of cDNA/DNA	E
➢Primer, (probe), Mg2 , 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

E = essential, D = desirable

RT-qPCR assay characteristics

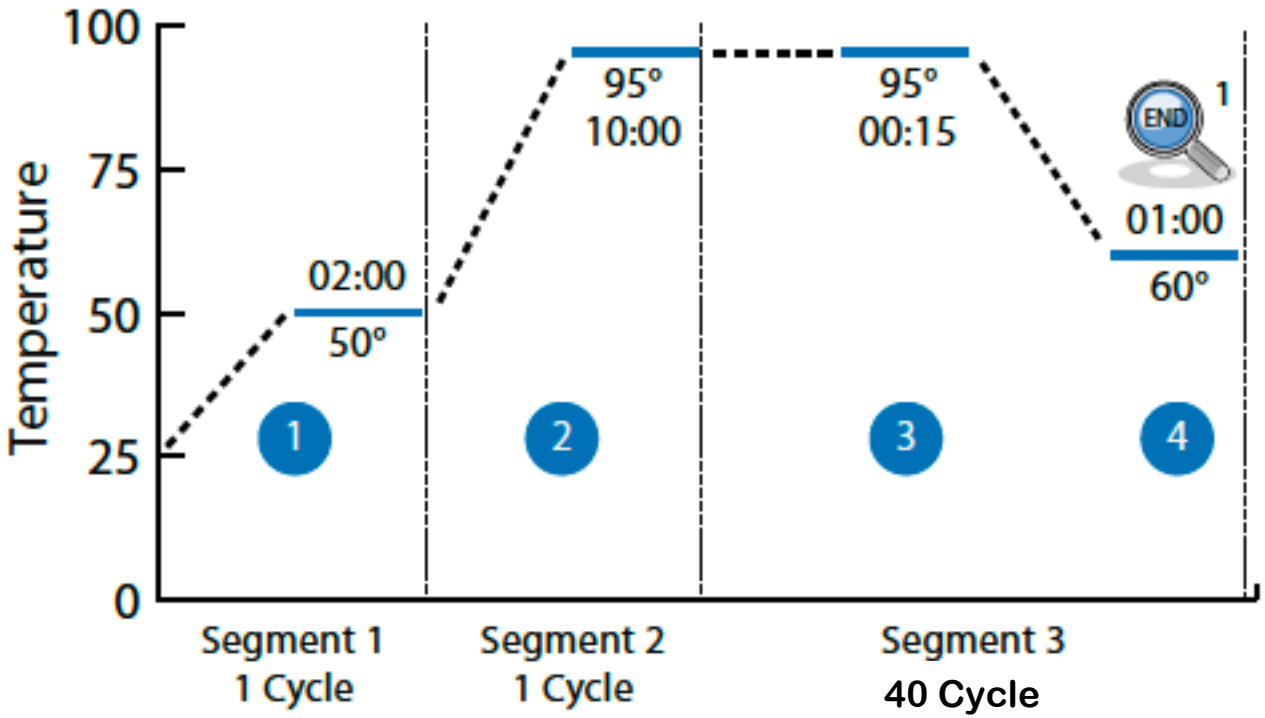
- **Analytical Sensitivity:** reproducible threshold at 5 molecules
- **Precision:** CV

RNA transcripts:	50	500	5000
Within run	9.29	11.72	6.43
Between runs	13.56	11.63	10.22

Core kits or MasterMixes?

- Taq Polymerase
  - ✓antibody-blocked *Taq*
  - ✓chemically-blocked *Taq*
- dNTPs / dUTPs
  - ✓dNTPs increases the sensitivity
  - ✓dUTPs brings security to the assay
- UNG (Uracil-N-Glycosylase)
  - ✓hydrolyses all single-stranded and double-stranded
  - ✓DNA containing dUTPs
- ROX
- MgCl2
- Inert colored dye

Thermal profile



Thermocycler	Supplier	Format	Number of channels	Optical Excitation system	Notes
GeneAmp® SDS 5700	Applied Biosystems	96-well plate	1	Halogen lamp	Sales discontinued
ABI Prism® SDS 7000	Applied Biosystems	96-well plate	4	Tungsten-halogen lamp	Sales discontinued
ABI Prism® SDS 7700	Applied Biosystems	96-well plate	4	Laser	Sales discontinued
ABI Prism® SDS 7900 HT	Applied Biosystems	96-well plate or 384-well plate	4	Laser	Robotic plate loading system for high throughput use Normal or FAST block
ABI Prism® SDS 7300	Applied Biosystems	96-well plate	4	Tungsten-halogen lamp	
ABI Prism® SDS 7500	Applied Biosystems	96-well plate	5	Tungsten-halogen lamp	Normal or FAST block
StepOne® / StepOnePlus®	Applied Biosystems	48-well /96-well	3/4	LEDs	
iCycler iQ®	BioRad	96-well plate	4	Halogen lamp	Sales discontinued
My iQ®	BioRad	96-well plate	1	Tungsten- halogen lamp	Singleplex FAM/SYBR
iQ5®	BioRad	96-well plate	5	Tungsten-halogen lamp	
CFX 96®	BioRad	96-well plate	5	LEDs	PCR cycler converted into Real-Time cycler
MiniOpticon®	BioRad	48-well plate	2	LEDs	
DNA Engine Opticon® 1	BioRad	96-well plate	1	LEDs	Sales discontinued
DNA Engine Opticon® 2	BioRad	96-well plate	2	LEDs	
Chromo 4®	BioRad	96-well plate	4	LEDs	
Mx3000P®	Stratagene	96-well plate	4	Quartz tungsten-halogen lamp	Customizable filters
Mx3005P®	Stratagene	96-well plate	5	Quartz tungsten-halogen lamp	Customizable filters
Mx4000®	Stratagene	96-well plate	4	Quartz tungsten-halogen lamp	Sales discontinued
Mastercycler ®ep realplex2	Eppendorf	96-well plate	2	LEDs	
Mastercycler ®ep realplex4	Eppendorf	96-well plate	4	LEDs	
LightCycler® 1.5	Roche	32 glass capillaries	3	LEDs	
LightCycler® 2	Roche	32 glass capillaries	6	LEDs	
LightCycler 480®	Roche	96-well or 384-well plate	6	Xenon lamp	Optional HRM module
Smartcycler®	Cepheid Innovation	Up to 96 plastic tubes.	4	LEDs	Independent block of 16 wells. Up to 6 blocks can be combined.
Rotor-Gene™ 2000 / 3000	Corbett Life Science	16 / 32 or 72 plastics tubes	2/4	LEDs	Sales discontinued
Rotor-Gene™ 6000	Corbett Life Science		6	LEDs	Includes HRM module

The MIQE guidelines
-qPCR validation-

➢Evidence of optimization (from gradients)	D
➢Specificity (gel, sequence, melt, or digest)	E
➢For SYBR Green I, Cq 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
➢r2 of calibration curve	E
➢Linear dynamic range	E
➢Cq variation at LOD	E
➢CIs throughout range	D
➢Evidence for LOD	E
➢If multiplex, efficiency and LOD of each assay	E

E = essential, D = desirable, LOD = Limit of detected

Reaction validation

- Start with a gradient RT-qPCR to optimize annealing temperature.
- Create standard curve from a serial dilution of template to test primers/probe across broad dynamic range.
- Evaluate:
  - Specificity
  - Efficiency
  - Reproducibility
  - Dynamic range
  - Intra assay variation

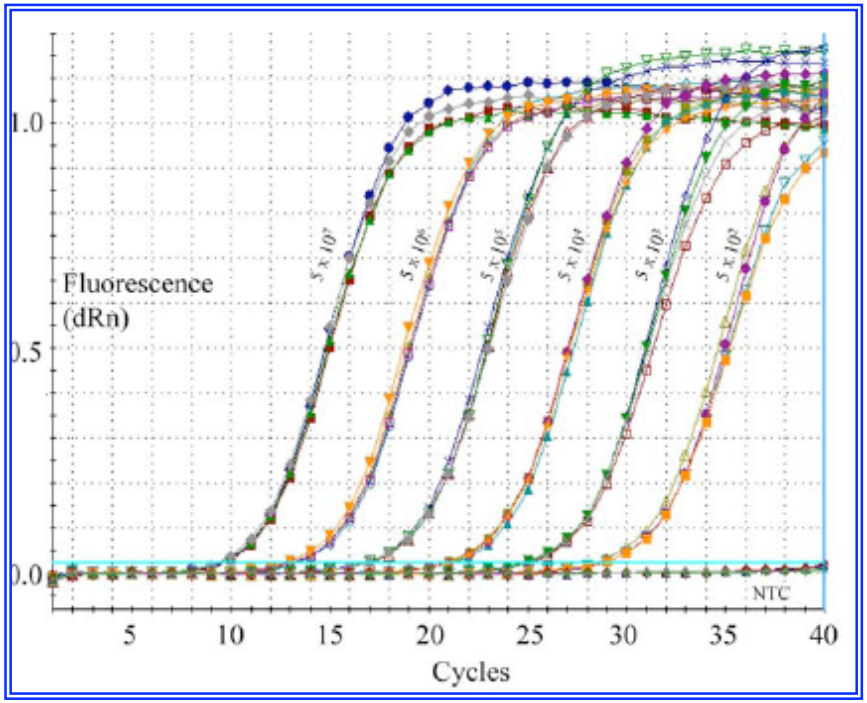
Conceptual considerations

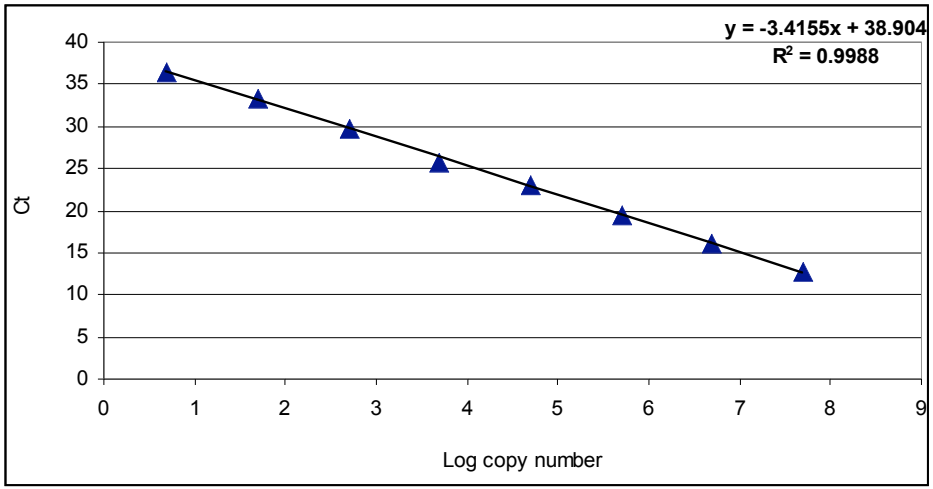
- Analytical sensitivity, clinical sensitivity, limit of detection
- Analytical specificity
- Accuracy
- Repeatability
- Reproducibility

The hallmarks of an optimized RT-qPCR

- **Linear standard curve (R^2 > 0.985)**
- **High amplification efficiency**
- **Consistency across replicate reactions**

Standard curve





Amplification efficiency

Difference in PCR efficiency generate falsely calculated differences in gene expression

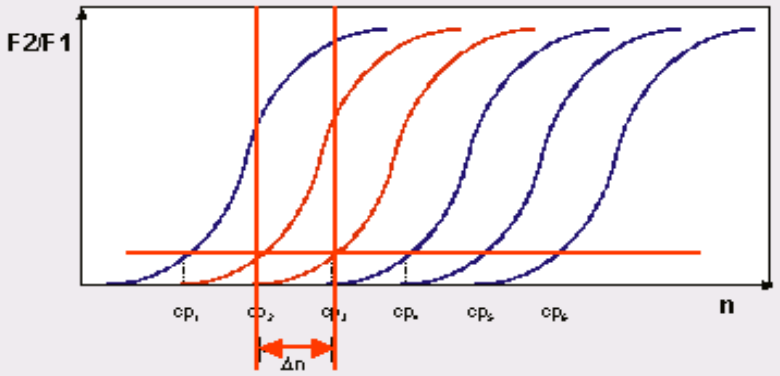
The efficiency of the PCR should be 95-100% (ideal slope = -3.32)

Several methods are described to calculate real-time PCR efficiency

Amplification efficiency

Calculation of real-time PCR efficiency

$E = 10^{-1/slope}$ => $E = 10^{-1/-3.337}$ => $E = 10^{0.299}$ => $E = 1.99$



$\Delta CP = \Delta n$

$N_2 = N_{0,2} \times E^{n2}$ $N_3 = N_{0,3} \times E^{n3}$

For $N_2 = N_3$:

$N_{0,2} / N_{0,3} = E^{(n_3 - n_2)} = E^{\Delta n}$

For $N_{0,2} = 10 \times N_{0,3}$ (10-fold dilutions):

$\Delta n = 1 / \log E$ $E = 10^{-1/slope}$

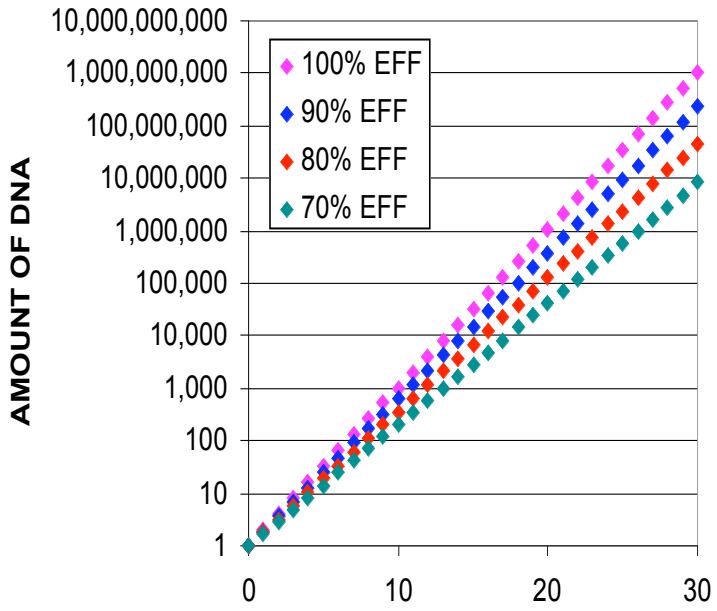
e.g. E = 2.0 Δn = 3.32
E = 1.9 Δn = 3.58
E = 1.8 Δn = 3.91

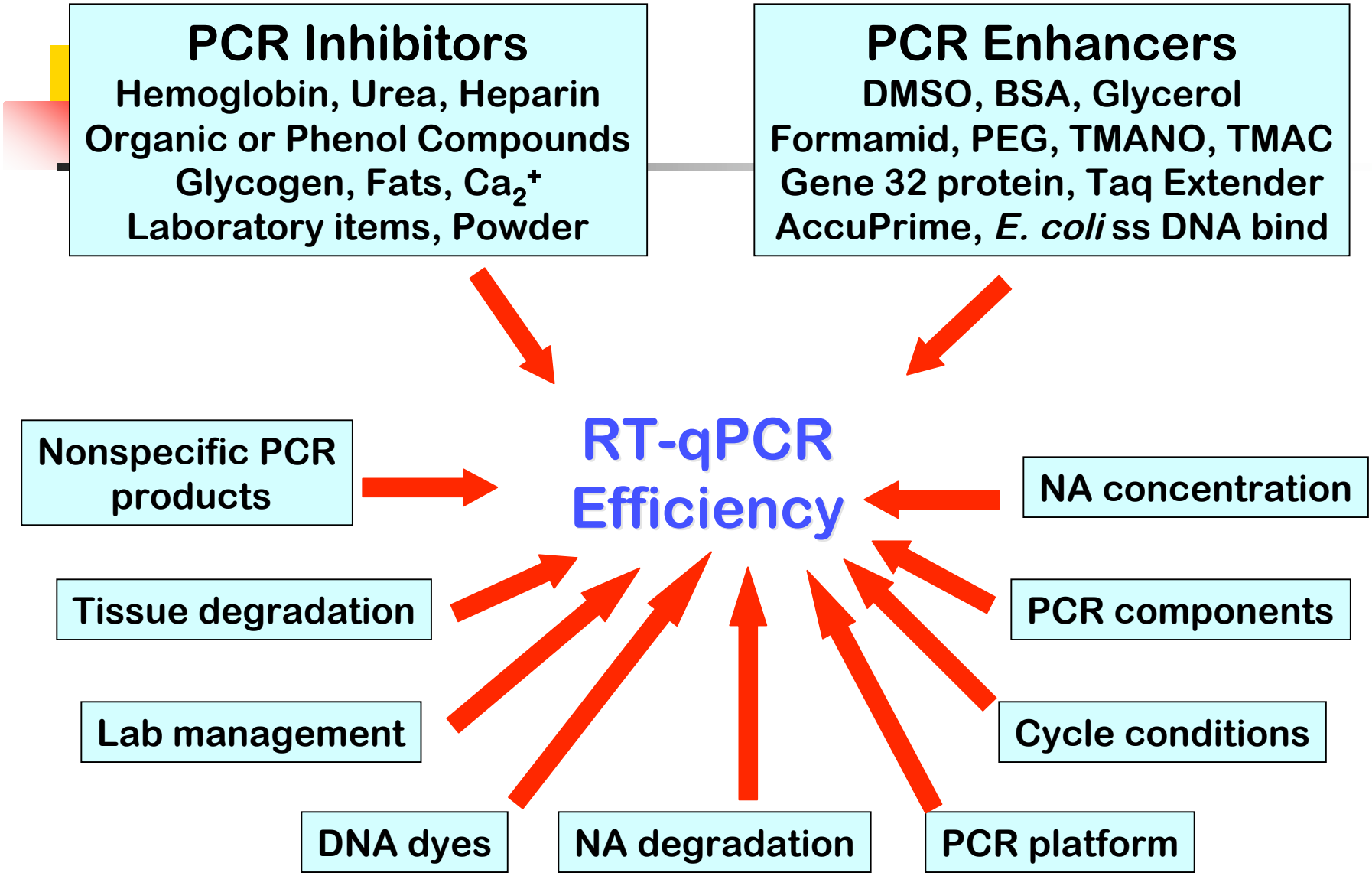
Roche Diagnostics, LC rel. Quantification software, March 2001

Rasmussen, R (2001) *Quantification on the LightCycler. In: Meuer, S, Wittwer, C, Nakagawara, K, eds. Rapid Cycle Real-time PCR, Methods and Applications Springer Press, Heidelberg; page 21-34*

Amplification efficiency

CYCLE	AMOUNT OF DNA 100% EFFICIENCY	AMOUNT OF DNA 90% EFFICIENCY	AMOUNT OF DNA 80% EFFICIENCY	AMOUNT OF DNA 70% EFFICIENCY
0	1	1	1	1
1	2	2	2	2
2	4	4	3	3
3	8	7	6	5
4	16	13	10	8
5	32	25	19	14
6	64	47	34	24
7	128	89	61	41
8	256	170	110	70
9	512	323	198	119
10	1,024	613	357	202
11	2,048	1,165	643	343
12	4,096	2,213	1,157	583
13	8,192	4,205	2,082	990
14	16,384	7,990	3,748	1,684
15	32,768	15,181	6,747	2,862
16	65,536	28,844	12,144	4,866
17	131,072	54,804	21,859	8,272
18	262,144	104,127	39,346	14,063
19	524,288	197,842	70,824	23,907
20	1,048,576	375,900	127,482	40,642
21	2,097,152	714,209	229,468	69,092
22	4,194,304	1,356,998	413,043	117,456
23	8,388,608	2,578,296	743,477	199,676
24	16,777,216	4,898,763	1,338,259	339,449
25	33,554,432	9,307,650	2,408,866	577,063
26	67,108,864	17,684,534	4,335,959	981,007
27	134,217,728	33,600,615	7,804,726	1,667,711
28	268,435,456	63,841,168	14,048,506	2,835,109
29	536,870,912	121,298,220	25,287,311	4,819,686
30	1,073,741,824	230,466,618	45,517,160	8,193,466




PCR Inhibitors
Hemoglobin, Urea, Heparin
Organic or Phenol Compounds
Glycogen, Fats, Ca_2^+
Laboratory items, Powder
PCR Enhancers
DMSO, BSA, Glycerol
Formamid, PEG, TMANO, TMAC
Gene 32 protein, Taq Extender
AccuPrime, *E. coli* ss DNA bind
RT-qPCR Efficiency
Nonspecific PCR products
Tissue degradation
Lab management
DNA dyes
NA degradation
PCR platform
NA concentration
PCR components
Cycle conditions

The MIQE guidelines
- Data analysis-

➢qPCR analysis program (source, version)	E
➢Method of Cq 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
➢Cq or raw data submission with RDML	D

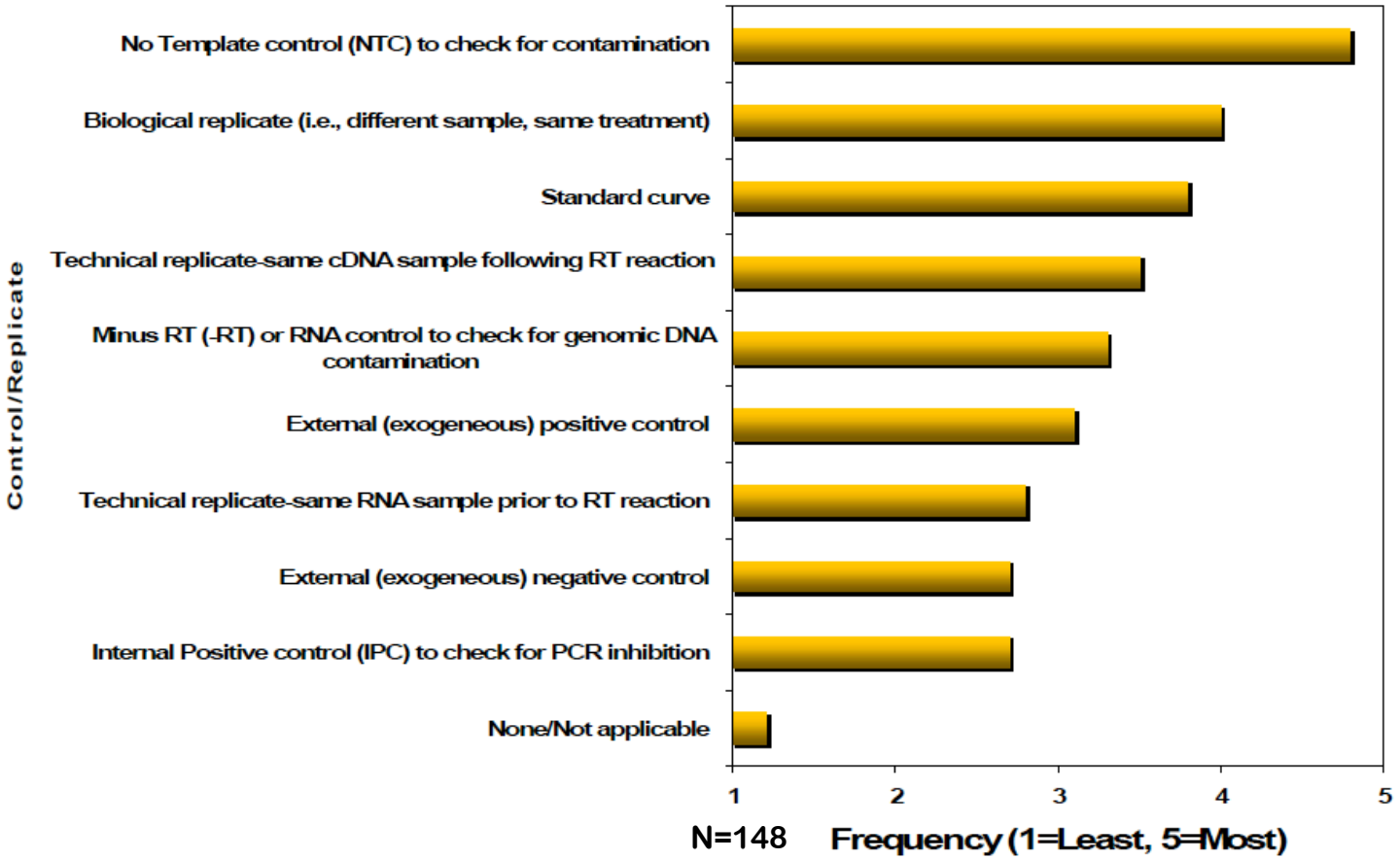
E = essential, D = desirable,

Importance of controls

NEGATIVE	AIM
• No Template Control	Detection of primers dimers and contamination
• No Amplification Control	Detection of probe's degradation
• No RT Control	Detection of genomic DNA contamination

POSITIVE	AIM
• Endogenous Control (same sample, different target)	Check quality of reagents. Also used for normalization.
• Exogenous Control (same target, different sample)	Check quality of reagents.
• Spiking Control (additional DNA spiked into the sample, different target)	Detect inhibitors presence Reject false negative in diagnostic assays

Frequency of use of controls



From Nucleic Acid Research Group, NARG survey 2007, http://www.abrf.org/NARG/

Normalization of RT-qPCR

- Real-time PCR can be very accurate over a large dynamic range
- Measuring RNA by RT-qPCR can be affected by error:
  - Inherent variability of RNA
  - Multi-step process
- Normalization required to control for error

Reference genes

- Using a reference gene is a simple and effective method
- Must be validated for the specific experiment using the actual samples being measured
- Failure to do so may generate incorrect results

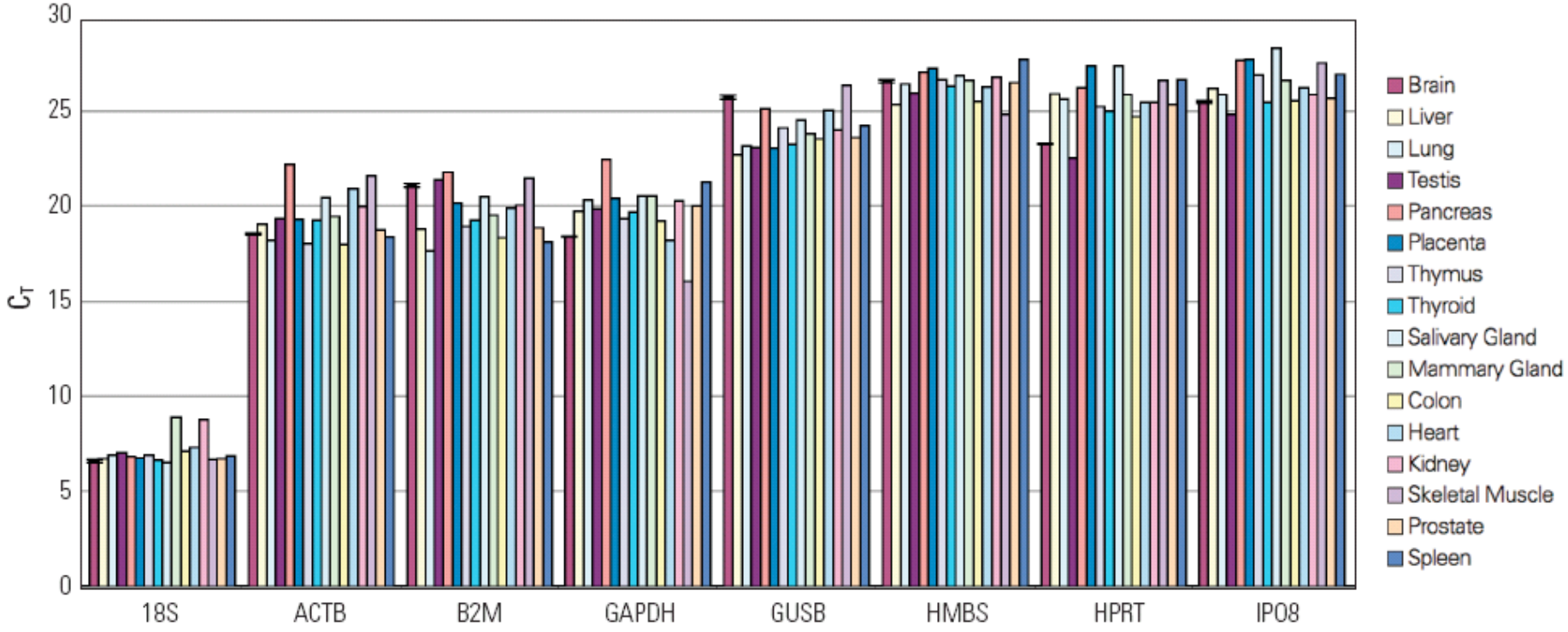
Reference genes

- Valid reference gene should correct differences in RNA sampling.
- Reference gene should have:
  - constant RNA transcription
  - transcribed at different experimental conditions
  - sufficiently abundant across different tissues and cell types
- The "perfect" RG does NOT exist.

Reference genes

Name	Pseudogene	Relative expression level
18S rRNA0		Very high
28S rRNA0		Very high
Beta actin	+	High
Glyceraldehyde-3-phosphate dehydrogenase	+	High
Ubiquitin C	-	High
Beta-2-microglobulin	-	High
Tyrosine 3-monooxygenase activation protein, Zeta polypeptide (Phospholipase A2)	+	Medium
Ribosomal protein L13a	+	Medium
Succinate dehydrogenae complex, subunit A	+	Medium
Hypoxanthine photshoribosyl-transferase I	+	Medium
TAT box binding protein	-	Low
Hydroxymethyl-bilane synthase		Low

Reference genes



Genes that show little variation from the calibrator should be chosen as reliable reference genes.

There is a lot of published data that has used invalidated reference gene(s) for normalization

Guideline to reference gene selection for quantitative real-time PCR

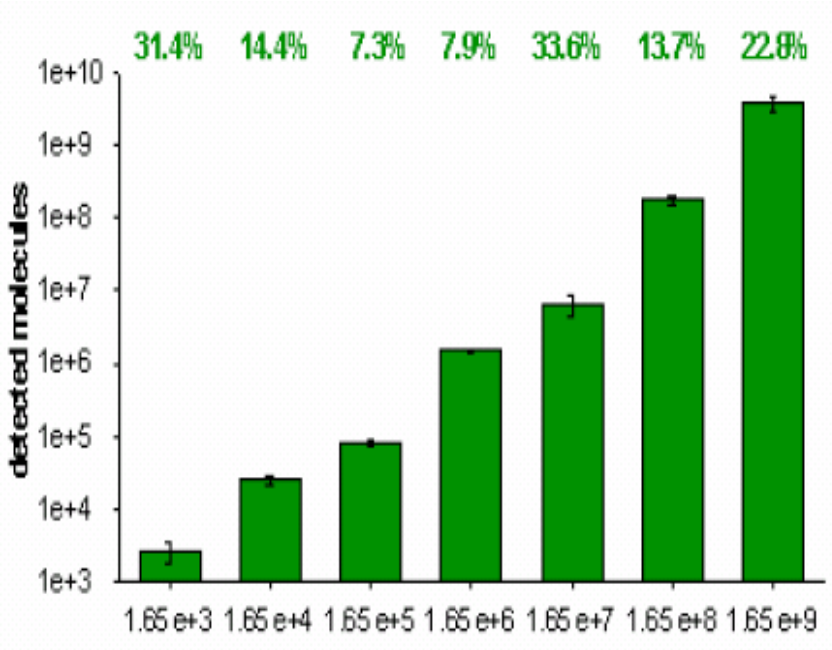
Aleksandar Radonić,[a] Stefanie Thulke,[a] Ian M. Mackay,[b] Olfert Landt,[d] Wolfgang Siegert,[a] and Andreas Nitsche[a,c,d,*]

Biochemical and Biophysical Research Communications 313 (2004) 856–862

Software: geNorm
BestKeeper
General pattern recognition

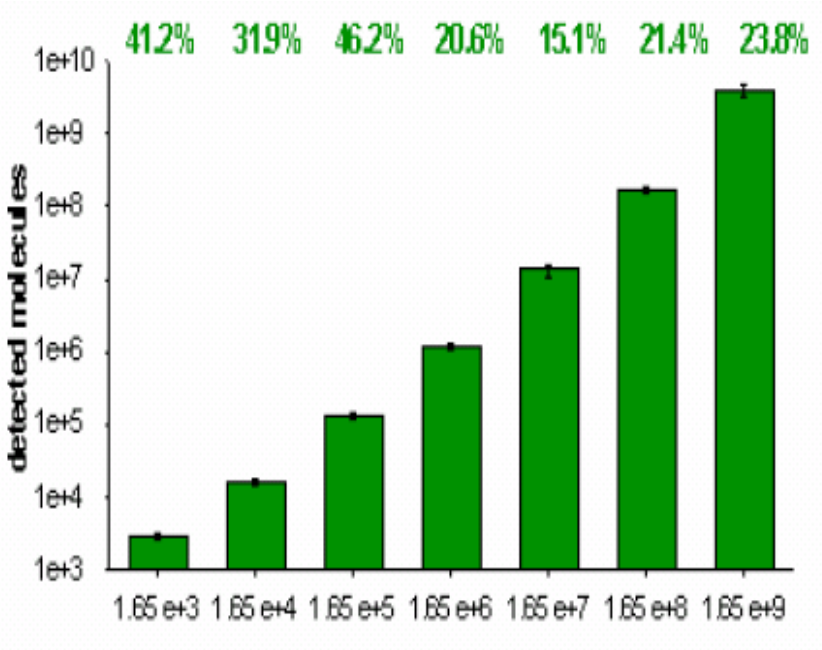
Intra/inter-assay variation

Intra-assay variation
CV=18.7%



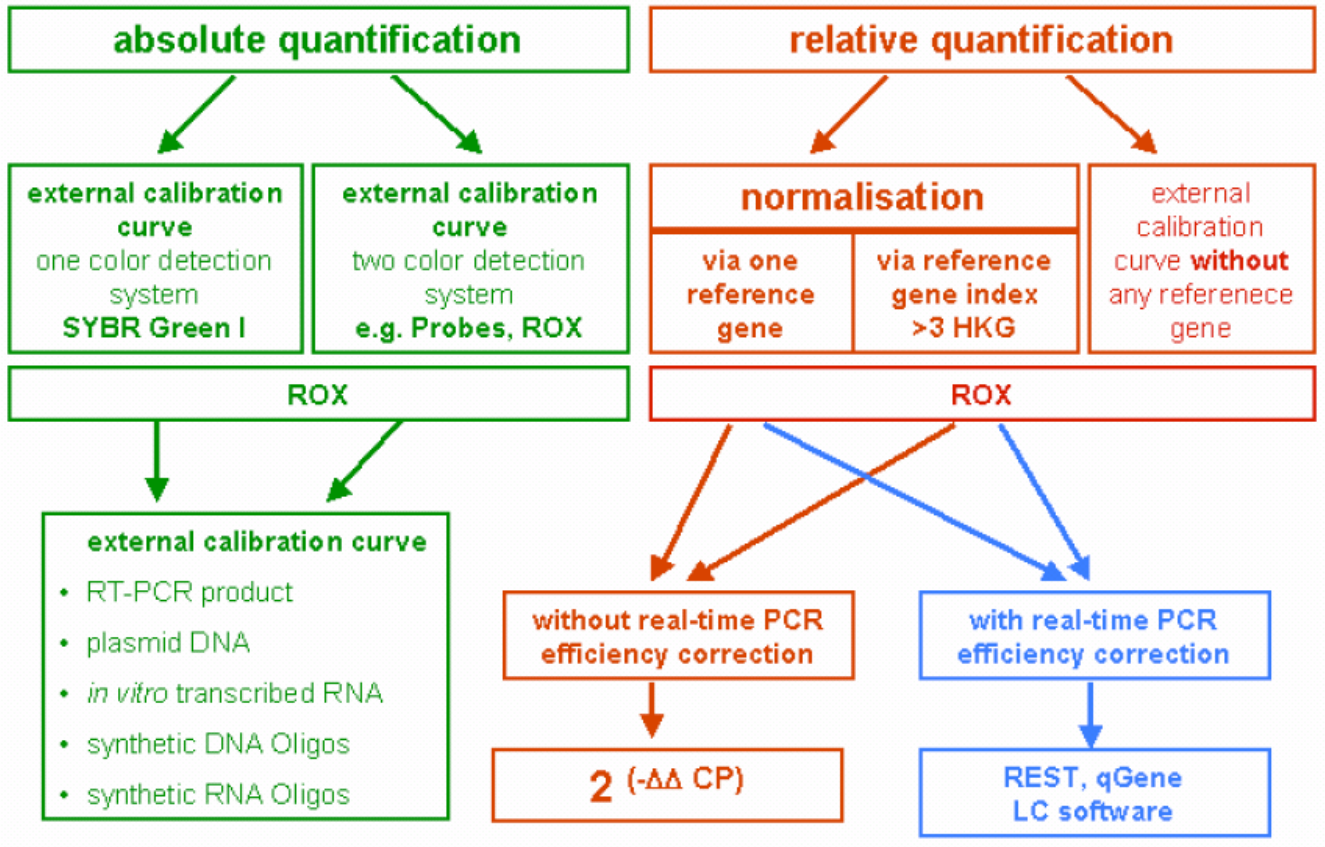
Input RNA

Inter-assay variation
CV=28.6%



Input RNA

Quantification strategies in RT-qPCR



Pfaffl, M.W., BioSpectrum 2004

Relative quantification

The mRNA expression is relative to:

- non treated control
- time point zero
- another gene of interest
- mean expression of a target gene
- universal calibration curve
- constant expressed RG
- RG index of more RGs (>3)
- etc ?????

Relative quantification

Three general procedures of calculation:

1. ΔC_q ($R = 2^{[Cq\ sample - Cq\ control]}$, $R = 2^{\Delta Cq}$), $\Delta\Delta C_q$ method ($R = 2^{-[\Delta Cq\ sample - \Delta Cq\ control]}$, $R = 2^{-\Delta\Delta Cq}$) without efficiency correction.
2. Calculation models, based on ONE sample and MULTIPLE samples

$$ratio = \frac{(E_{target})^{\Delta C_P\ target\ (control - sample)}}{(E_{Ref})^{\Delta C_P\ Ref\ (control - sample)}}$$

$$ratio = \frac{(E_{Ref})^{C_P\ sample}}{(E_{target})^{C_P\ sample}} \div \frac{(E_{Ref})^{C_P\ calibrator}}{(E_{target})^{C_P\ calibrator}}$$

$$ratio = \frac{(E_{target})^{\Delta C_P\ target\ (MEAN\ control - MEAN\ sample)}}{(E_{Ref})^{\Delta C_P\ Ref\ (MEAN\ control - MEAN\ sample)}}$$

3. Calculation models, based on MULTIPLE sample and on MULTIPLE reference genes, consisting at least of three reference genes

$$R = \frac{(E_{target})^{\Delta C_P\ target\ (MEAN\ control - MEAN\ sample)}}{(E_{Ref\ index})^{\Delta C_P\ Ref\ index\ (MEAN\ control - MEAN\ sample)}}$$

Data analysis methods

- Standard curve
- ΔΔCt
- REST software (free)
- RealTime Statminer® software (AB, $3,000/1 year)
- GenEx software
- LinRegPCR software
- qBase software
- DART-PCR software
- Q-Gene software
- CAamER software
- Gene expression Macro software (Bio-Rad)
- qPCR-DAMS software
- LinRegPCR software
- Other

The key steps for most RT-qPCR experiments include:

- Sample procurement which requires following strict experimental protocols for acquisition, processing, and storage to assure biological reproducibility and minimize SD between replicates.
- Appropriate number of biological ($n \geq 3$) and technical ($n \geq 2$) replicates with SD between technical replicates of less than 0.3 Cq.
- Use an RNA isolation procedure that produces high-quality total RNA and high yield.
- Digest purified RNA with DNase I.
- Perform RT reactions with a robust reverse transcriptase.
- Test cDNA yield and quality.

The key steps for most RT-qPCR experiments include:

- Design gene-specific PCR primers.
- Reduce technical errors in PCR reaction setup.
- For relative quantification test gene-specific primers for at least four potential reference genes.
- Perform real-time PCR on test and reference genes in parallel for each sample (≤0.5 Cq between all experimental conditions or time points).
- Determine which reference gene(s) is best for normalization.
- Calculate relative transcript abundance for each gene in each sample.
- RT-qPCR experiments with appropriate positive and no template controls.

RDML
Real-Time PCR Data Markup Language

Nucleic Acids Research, 2009, 1–5
doi:10.1093/nar/gkp056

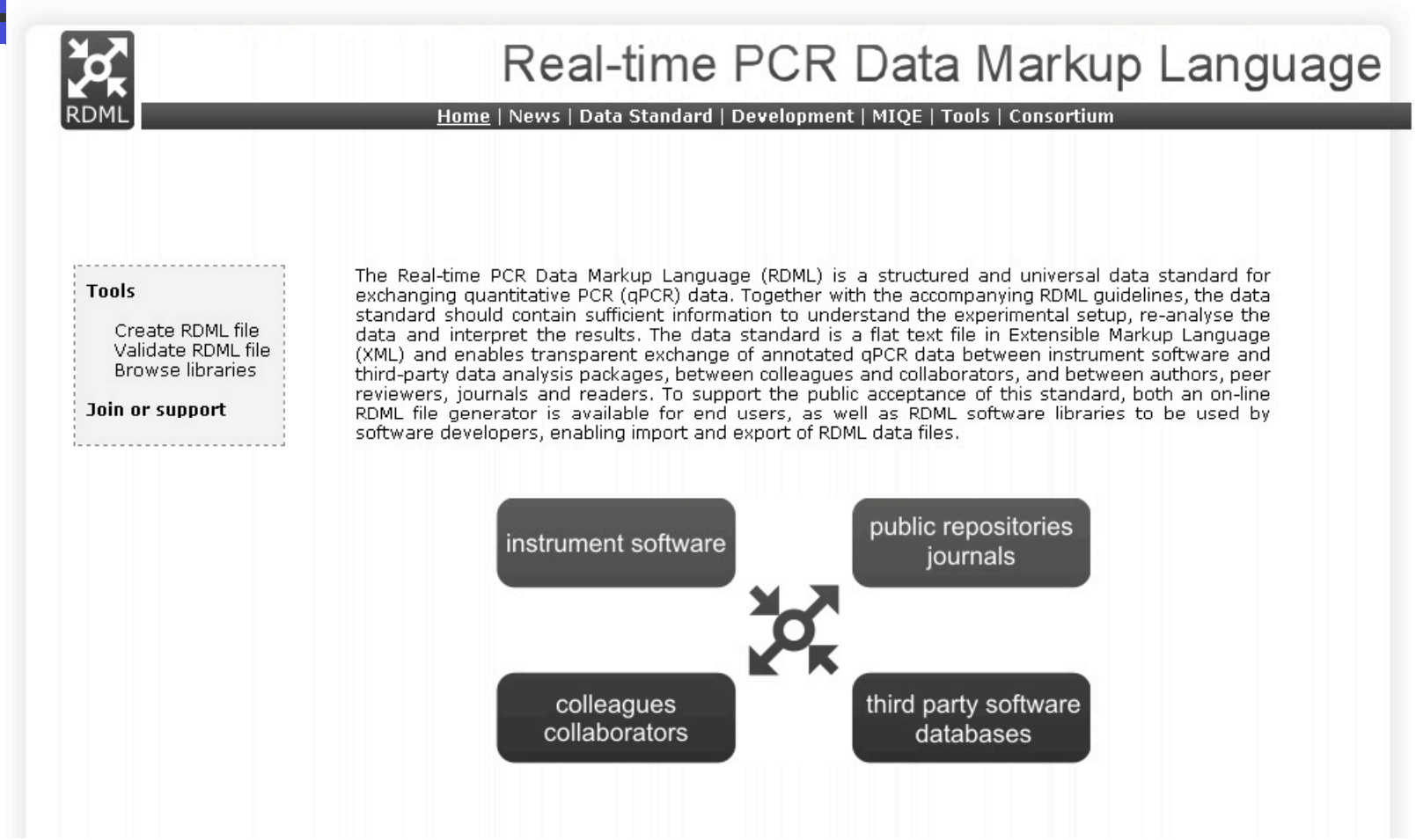
SURVEY AND SUMMARY

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

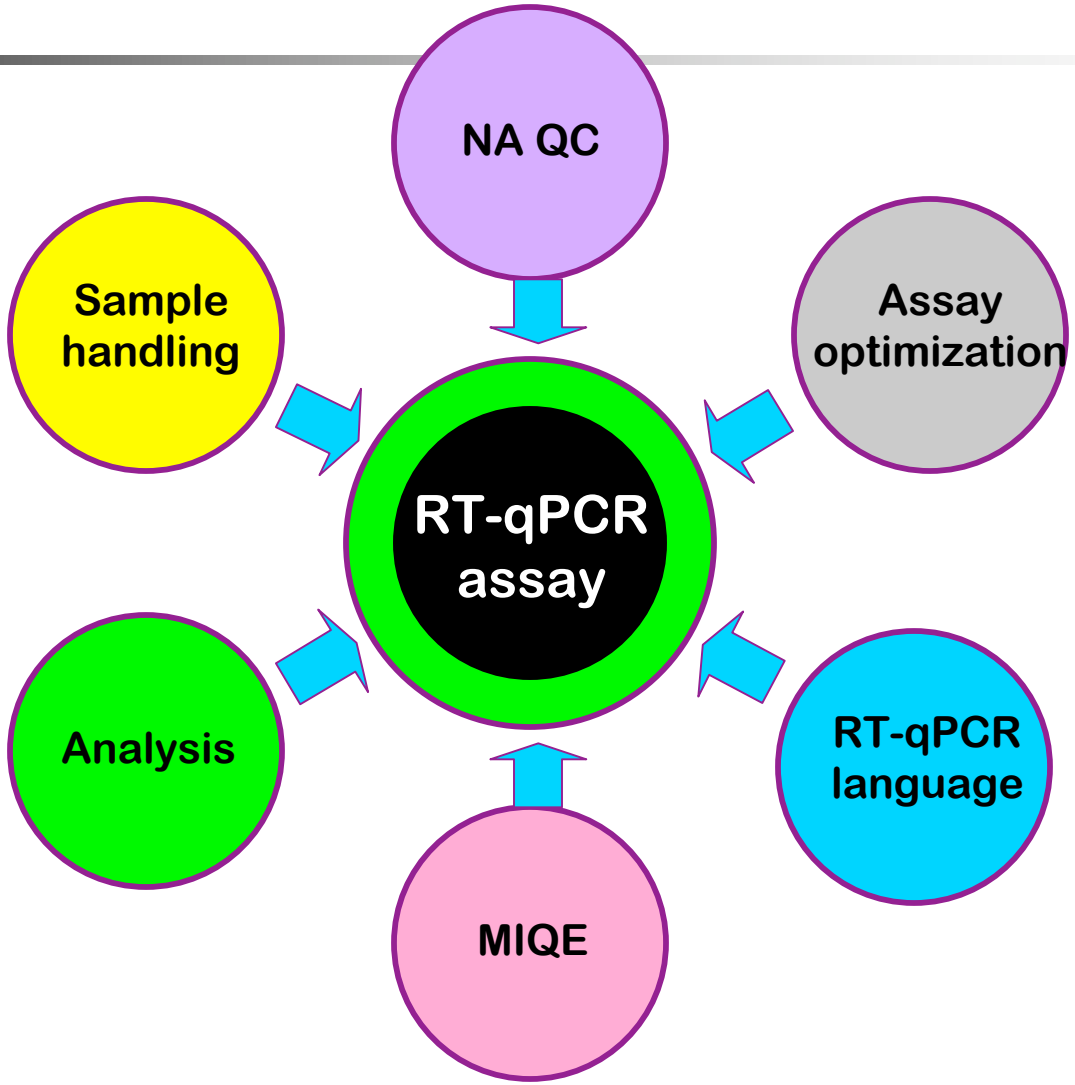
Steve Lefever[1], Jan Hellemans[1,*], Filip Pattyn[1], Daniel R. Przybylski[2], Chris Taylor[3], René Geurts[4], Andreas Untergasser[4] and Jo Vandesompele[1], on behalf of the RDML consortium

[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



Components for publishing a reliable RT-qPCR assay



Acknowledgments

qPCR Core Facility

Alicja Omanska
Cara Wademan
Cathy Inta
Greg Simonek
Tammi Olineka

Dr. Nicola Pusterela
Samantha Mapes

Dr. Adib Rowhani
Dr. Fatima Osman

Dr. Stephen W. Barthold
Dr. Sunlian Feng
Edlin Escobar
Kimberly Olsen

Dr. Jane Sykes