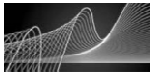


BIO-RAD

MIQE Guidelines

Minimum Information for Publication of Quantitative Real-Time PCR Experiments

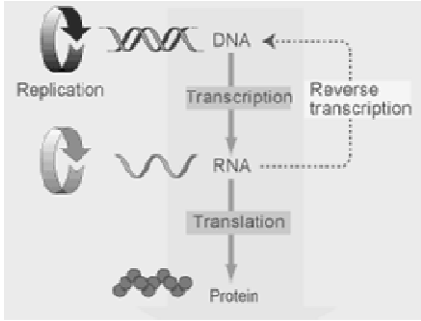
產品專員 / 陳立德

正茂生物科技有限公司
Genmall Biotechnology Co.,Ltd.
TEL：(02)2731-3369 FAX：(02)2731-9590
免費諮詢服務及訂貨專線：0800-213-029

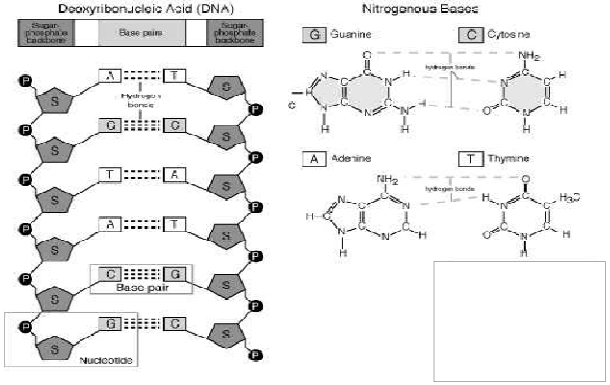
Central Dogma

Central dogma describes information flow from

DNA→RNA→protein



DNA Structure

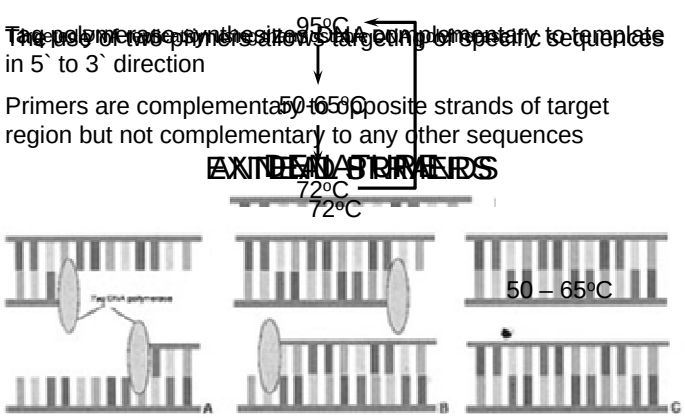


Polymerase Chain Reaction

The use of two primers allows targeting of specific sequences

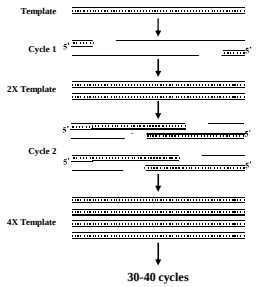
Taq polymerase synthesizes DNA complementary to template in 5` to 3` direction

Primers are complementary to opposite strands of target region but not complementary to any other sequences

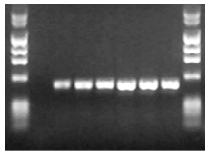


PCR – 1st Generation

- Amplify target DNA with end point analysis to distinguish products
  - No relationship between end point and starting target copies



End point analysis



What is Real-Time PCR?

Simply - **fluorescent molecules** are used to monitor the reaction while amplification is taking place.

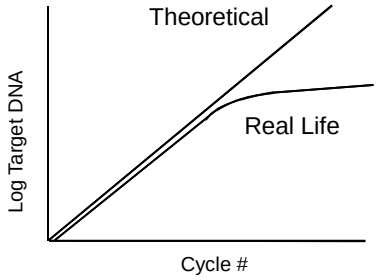
You are able to view this occurring in **real-time** on your instrument.

Reality vs. Theory

Amplification is exponential, but the exponential increase is limited:

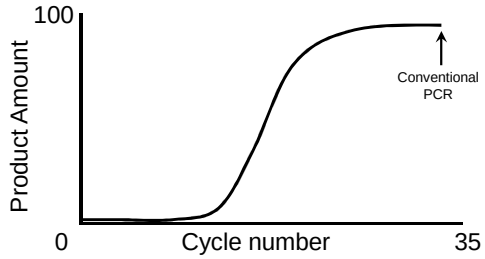
1) **A linear increase follows exponential**
2) **Eventually plateaus**

Real-Time PCR allows us to 'see' the exponential phase so we can calculate how much we started with.



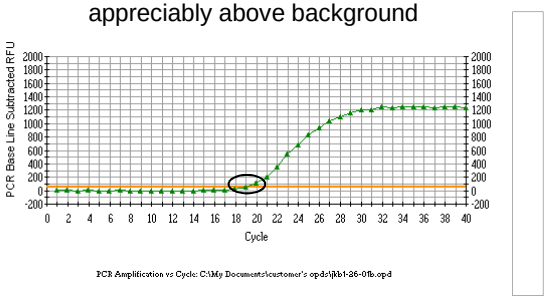
Quantitative PCR (qPCR)

Real-time qPCR enables assessment of the reaction after each cycle



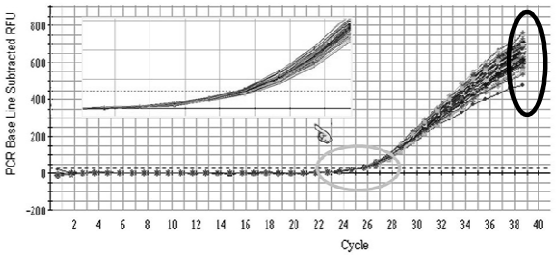
Quantification cycle, Cq

The point at which the fluorescence rises appreciably above background



End Point Measurements

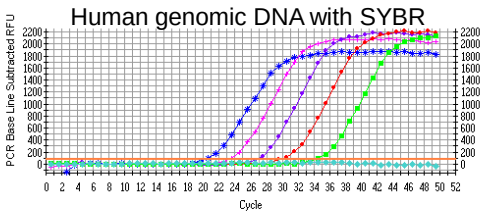
96 replicates of an identical reaction can have very different final amounts of fluorescence



Quantification cycle, Cq

- Correlates strongly with the starting copy number
  - If you have twice the template, you get to C_q one cycle earlier
  - If you have half the template, you reach C_q one cycle later
- Is linear with the log of starting copy number over six or more orders

Human genomic DNA with SYBR



50-0.005 ng of template- FV Leiden primers

PCR Amplification vs Cycle: 15-Aug-01LUFV5YBRMC.opd

C_q value v.s. concentration

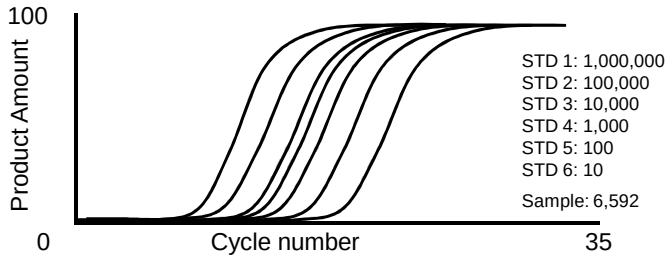
- 1 cycle = 2 fold difference
- 3.32 cycles ≅ 10 fold difference
- Assumes 100% efficiency

$Y = N_0\, 2^n \quad \Longrightarrow \quad \mathbf{Y = N_0\,(1+E)^n}$

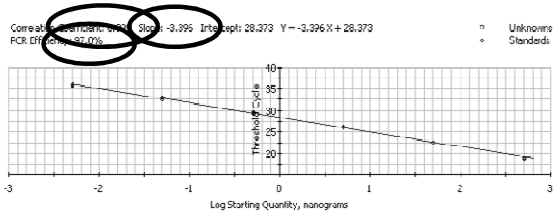
Quantitative PCR (qPCR)

Create a **standard curve** with 10-fold serial dilutions of PCR product – assign arbitrary values

Compare values from standards with values for unknown sample



C_q is linear with the log of starting copy number (standard curve)



r = is a measure of how well the actual data fit to the standard curve.
= (explained variation/total variation)

Aim for R value (Correlation Coefficient) of ≥0.98

The slope of the standard curve can be directly correlated to the efficiency of the reactions:

Efficiency (E) = $[10^{(-1/slope)}]$ - 1

When slope = -3.32, Efficiency = 100%

Slope = - [1/log (1+E)]

log (1+E) = - (1/slope)

E = $10^{[-1/slope]}$ - 1

E	Slope
0.5	-5.679
0.6	-4.899
0.7	-4.339
0.8	-3.917
0.9	-3.587
1	-3.322
1.1	-3.103
1.2	-2.920
1.3	-2.765

Aim for Efficiency Values:

Good = 90 – 110%

Fantastic = 95 – 105%

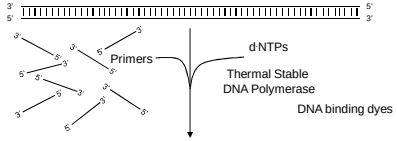
What are the most common detection strategies used for Real-Time PCR?

Real-Time PCR

- These fluorescent molecules can be used
  - Non-specific DNA binding dyes
    - SYBR® Green I
    - Ethidium Bromide
  - Specific Hybridization Probes/Primers
    - TaqMan™
    - molecular beacons
    - dual-oligo FRET pairs
    - Scorpions™/Amplifluor™/LUX™

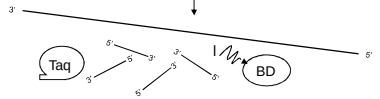
DNA binding dyes

Add Master Mix & Sample



Reaction Tube

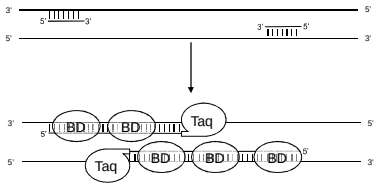
Denaturation



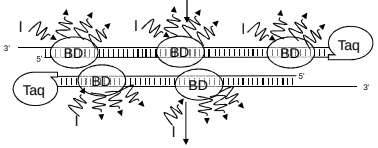
Annealing

DNA binding dyes

Extension



Extension Continued
Apply Excitation Wavelength

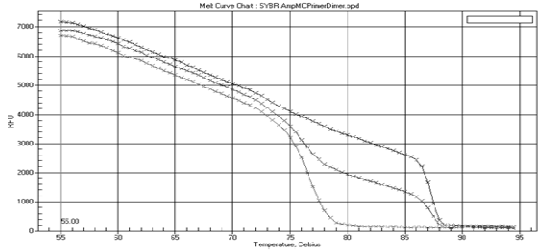


Repeat

Melt Curve Analysis

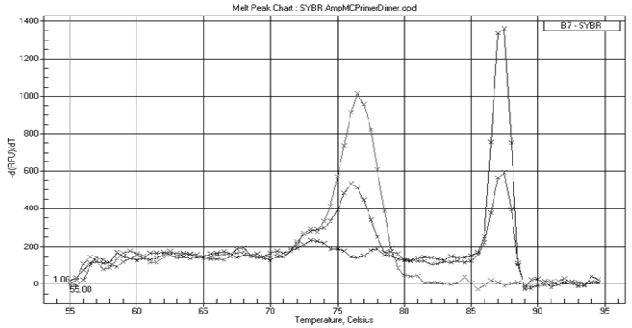
Fluorescence decrease as the temperature increase:

1. DNA strands start to separate
2. SYBR green looses its binding to the DNA
3. Fluorescence rapidly decreases



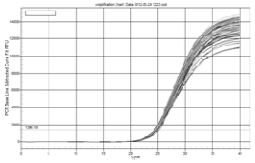
Melt Curve Analysis

-The melting temperature of the amplicon can easily be detected.
-Contaminating DNA, primer dimer or false priming is seen as an additional peak.

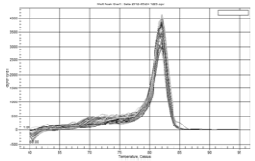


DNA binding dyes

- Easy to start qPCR Exp.
- Inexpensive
- Accurate
- Large dynamic range

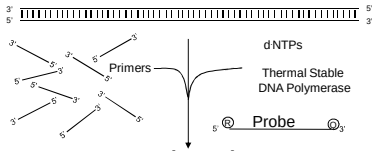


- Primer specificity
- Single target



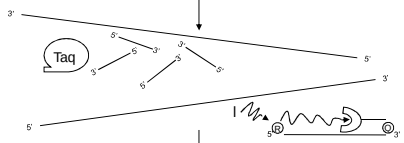
Cleavage-based assay: TaqMan Probe

Add Master Mix & Sample



Reaction Tube

Denaturation

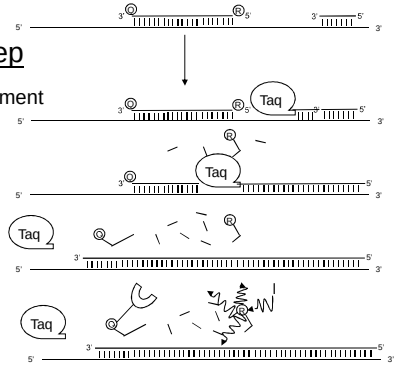


Annealing

Cleavage-based assay: TaqMan Probe

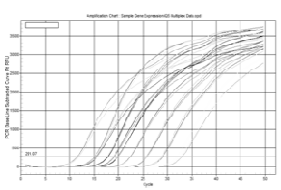
Extension Step

1. Strand Displacement
2. Cleavage
3. Polymerization Complete
4. Detection



Probe-based assay

- More specific than dye base method
- Multiplex able using different reporter dye
- SNP genotyping application
- More high cost than dye base method



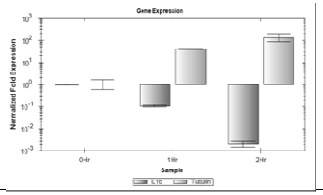
Historically… before qPCR

- Laborious techniques were used to examine specific [illegible]ls;

10 fold! I don't see this as 10 fold. It looks more like 3 fold to me!

- These methods were Simi-quantitative at best.

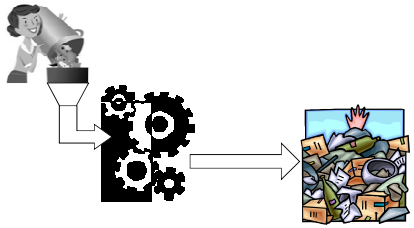
After the arrival of qPCR



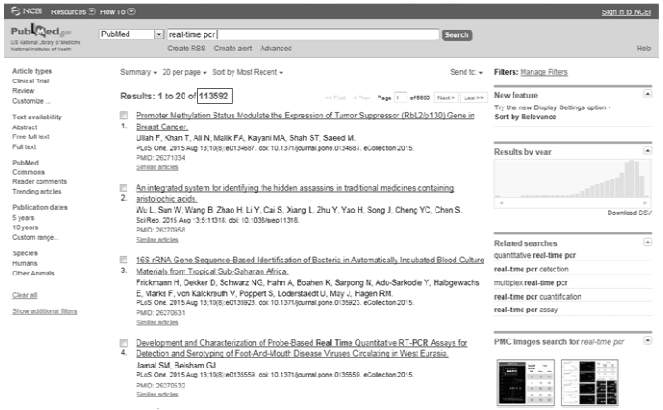
- Assay development was much easier.
- Sample required was much smaller.
- Results were achieved much faster.
- Data sets grew larger.
- Instruments generated numerical outputs.

The Problem

- Perceived simplicity of running qPCR experiments has resulted in many publications containing seriously misleading results.



QPCR papers



Questionable Data

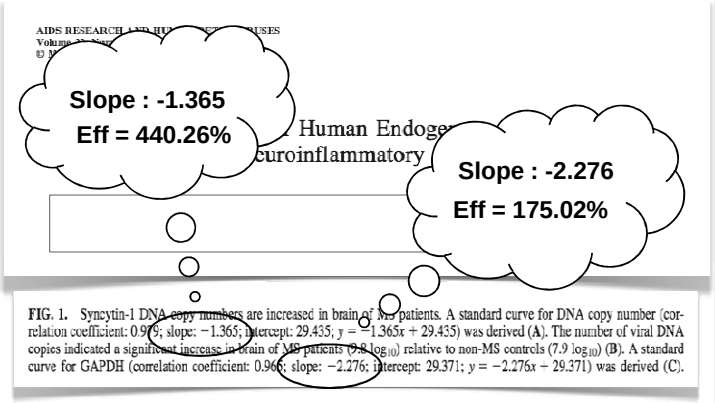
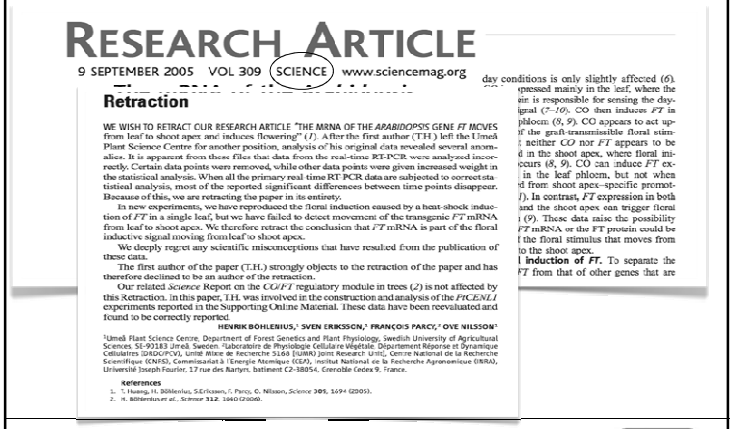


FIG. 1. Syncytin-1 DNA copy numbers are increased in brain of MS patients. A standard curve for DNA copy number (correlation coefficient: 0.979; slope: −1.365; intercept: 29.435; $y = -1.365x + 29.435$) was derived (**A**). The number of viral DNA copies indicated a significant increase in brain of MS patients (9.8 $\log_{10}$) relative to non-MS controls (7.9 $\log_{10}$) (**B**). A standard curve for GAPDH (correlation coefficient: 0.966; slope: −2.276; intercept: 29.371; $y = -2.276x + 29.371$) was derived (**C**).

Retracted Paper



RESEARCH ARTICLE

9 SEPTEMBER 2005 VOL 309 SCIENCE www.sciencemag.org

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 (TH) 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 floral 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 *CGFT* 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 *PtCENL1* experiments reported in the Supporting Online Material. These data have been reevaluated and found to be correctly reported.

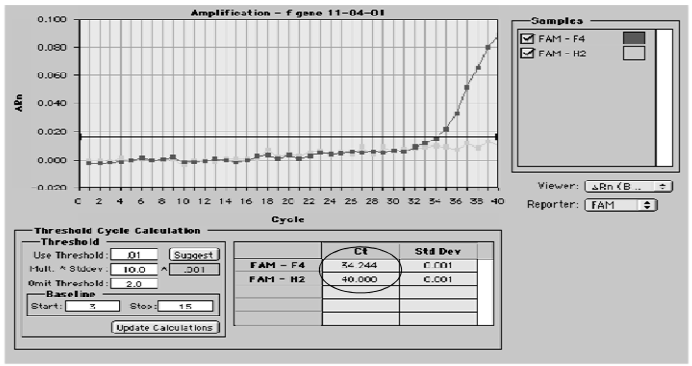
HENRIK BÖHLENIUS,[1] SVEN ERIKSSON,[2] FRANÇOIS PARCY,[3] OVE NILSSON[1]

[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, bâtiment C2-38054, Grenoble Cedex 9, France.

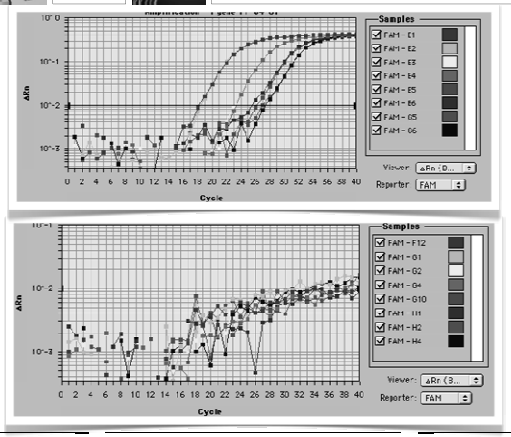
References

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

Autism and MMR Vaccine



Autism and MMR Vaccine



Lingering Effects of Bad Data

The NEW ENGLAND JOURNAL of MEDICINE

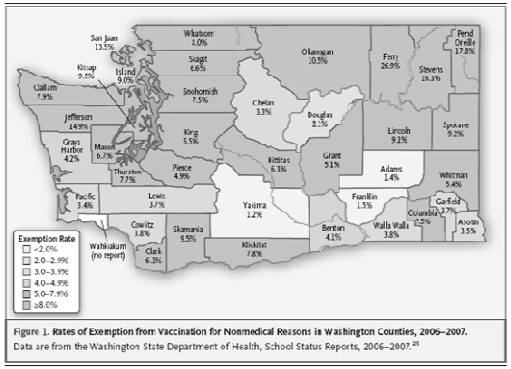


Figure 1. Rates of Exemption from Vaccination for Nonmedical Reasons in Washington Counties, 2005–2007.
Data are from the Washington State Department of Health, School Status Reports, 2006–2007.[25]

What can be done?

MIBBI, RDML and MIQE

- **Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nature biotechnology***, volume 26, number 8, August 2008, 889-896.
- **RDML: structured language and reporting guidelines for real-time quantitative PCR data.** Lefever S, Hellemans J, Pattyn F, Przybylski DR, Taylor C, Geurts R, Untergasser A, Vandesompele J; on behalf of the RDML consortium. ***Nucleic Acids Res.*** 2009 Feb 17.
- **The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.** Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. ***Clin Chem.*** 2009 Feb 26.

A new beginning

Clinical Chemistry 55:4
611–622 (2009)

Special Report

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]

57 Essential information
(E) must be submitted with the manuscript

28 Desirable information
(D) should be submitted if available

What is MIQE? It's a Checklist

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

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	Commercial identification number	D
Assay carried out by the core or investigator's laboratory?	D	Manufacturer	D*
Acknowledgment of authors' contributions	D	Amplicon length	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D*
Volume/mass of sample processed	E	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	Oligonucleotide identity and concentration	E
Sample storage conditions and duration (especially for FFPE samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact thermal composition of the buffer	D
Procedure and/or instrumentation	E	Additives (DMSO, betaine, BSA)	E
Name of kit and method of any modifications	E	Manufacturer of polymerase and cycling conditions	D
Source of additional reagents used	E	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 pilots)	D
Purity (A_{260}/A_{280})	D	Agarose gel, capillary electrophoresis, or other	E
Yield	D	For SYBR Green I, T_m of the PPC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y-intercept	E
RIN/RQI or T_m of "melt" transition	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	Or the PCR efficiency and	E
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	Or throughout range	E
Priming oligonucleotide (if using GSP) and concentration	E	Evidence that 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_q with and without reverse transcription	E	Method of C_q determination	E
Amount of cDNA or DNA	E	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	E	Number and concordance of biological replicates	E
Amplicon length	E	Number and range/variance from pilots of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intra-assay variation)	E
Pseudogenes, retrogenes, or other homology?	D	Reproducibility (inter-assay variation, LOD	D
Sequence alignment	E	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
Whether probe is dual-labeled	E	C_q or raw data submitted with RDML	D

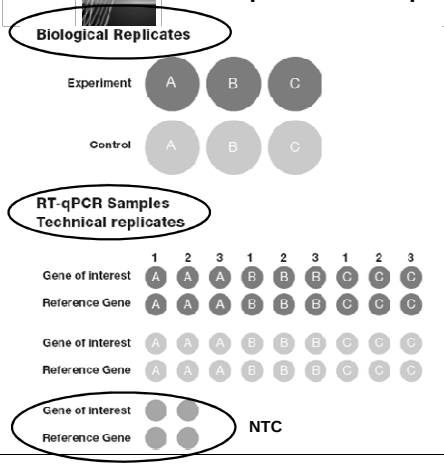
* All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from PrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.
† RIN, Agilent 2100 bioanalyzer; RQI, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; RNT, deoxynucleoside 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 RNA free, inclusion of a no-reverse transcription control is not necessary for every sample analyzed.
§ Disclosure of the probe sequence is highly desirable and strongly encouraged; however, assessment of vendor-of-commercial-predesigned assays provided this information is somewhat an essential requirement. Use of such assays is discouraged.

- qPCR community driven guidelines for essential and desired information in litterature;
  - Experimental Design
  - Sample Information
  - Nucleic Acid Extraction
  - Reverse Transcription
  - qPCR Target Information
  - qPCR Oligonucleotides
  - qPCR Protocol
  - qPCR Validation
  - Data Analysis

Experimental Design

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

Experimental Replicates



Sample Information

Item to check	Importance
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

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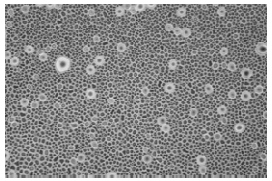
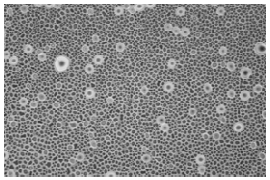
Sample Information – Acquisition

- Source (soil, blood, urine, plant tissues …)
- Sampling techniques (biopsy materials, single-cell sampling, laser microdissection…)
- Description (percentage of the biopsy made up of tumor cells)
- Mass/Volume
- Homogeneity



Sample Information – Processing and Storage

- Processing procedure
- Storage (-80C vs -20C; duration…)
- Prep time to extraction (-80C)
- Sample degradation



Nucleic Acid Extraction

Item to check	Importance
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

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Sample Extraction

- DNA / RNA
  - Aquapure™ Genomic DNA Isolation Kit
  - Aurum™ Total RNA Kits
- Contaminants
  - Starches
  - Lipids
  - Metals
- Extraction contaminants
  - Phenol chloroform
  - Salts
- Sample degradation

Inhibitors copurified

BMC Research Notes

BioMed Central

Short Report

Open Access

Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon

Jim F Huggett*[1], Tarya Novak[1], Jeremy A Garson[2], Clare Green[1], Stephen D Morris-Jones[3], Robert F Miller[4] and Alimuddin Zumla[1]

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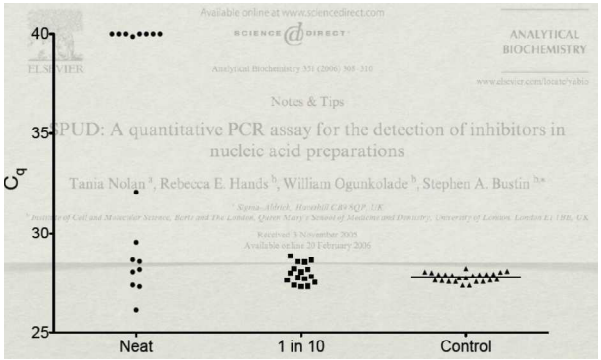
BMC Research Notes 2008, 1:70 doi:10.1186/1756-0500-1-70

Received: 8 August 2008

Accepted: 28 August 2008

Results: When copurified inhibitors were assessed using two different PCR reactions one reaction appeared to be inhibited whilst the other was not. Further experiments using various concentrations of unextracted urine to inhibit six different PCR reactions revealed that susceptibility to inhibition was highly variable between reactions. Similar results were obtained using EDTA as the PCR inhibitor. We could find no obvious explanation why one reaction should be more susceptible to inhibition than another, although a possible association with amplicon GC content was noted.

Effects of EDTA



Template DNA Preparation

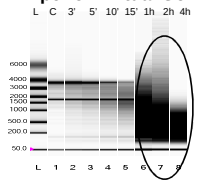
- Genomic DNA
  - Cut with restriction enzyme that does not cut within amplicon
  - Boil DNA for 10 min and then onto ice
- Plasmid DNA
  - If it doesn't work, linearize plasmid with restriction enzyme that does not cut within amplicon
- cDNA
  - Prior to RT: Treat RNA with RNase-free DNase
  - After RT: use enzyme that has RNaseH activity to digest away RNA from RNA:DNA hybrid

Sample and Template Preparation

- Extract and analyze RNA
- Careful quantification is necessary
  - RiboGreen Assay - Quantification
  - NanoDrop – Quantification and purity
  - Experion™ - Quality and quantification

Analysis of RNA purity and integrity

Experion Virtual Gel



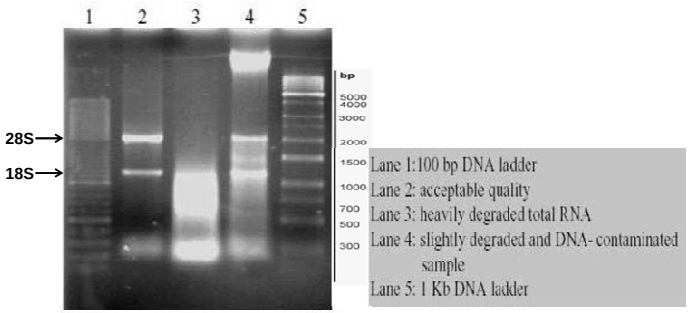
Samples 6,7,8 are highly degraded.

Corresponding Nanodrop Readings

Sample	Conc (ng/ul)	A260/280*	A260/230*
Control-no heat	115	1.90	2.44
3 min @ 90C	114	1.93	2.40
5 min @ 90C	115	2.06	2.37
10 min @ 90C	115	2.03	2.37
15 min @ 90C	116	2.02	2.31
1 hr @ 90C	109	1.99	2.18
2hr @ 90C	117	2.00	2.32
4 hr @ 90C	118	1.89	2.23

*Generally accepted ratios (A260/280 and A260/230) for good quality RNA are > 1.8.

Total RNA analysis

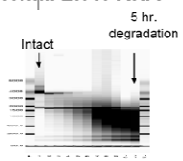


1% Agarose gel electrophoresis

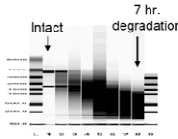
AMPLIFICATION

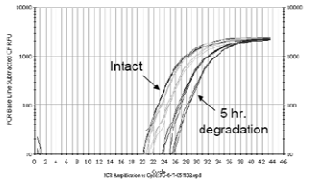
Total RNA analysis

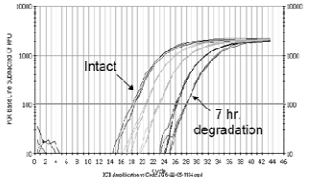
Normal Liver RNA



Carcinoma Liver RNA



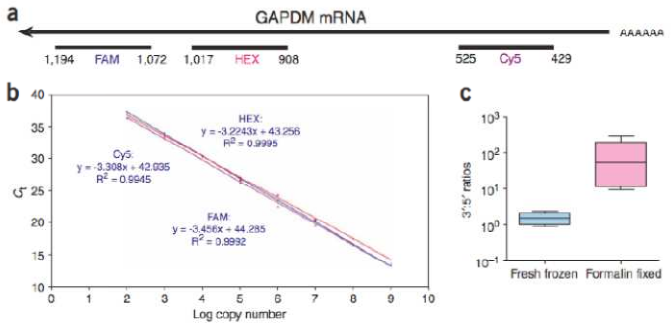




qPCR: GAPDH gene

AMPLIFICATION

Cq of 5' and 3' transcripts



AMPLIFICATION

Reverse Transcription

Item to check	Importance
Reverse transcription	
Complete reaction conditions	F
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

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AMPLIFICATION

RT protocols:

RNA was reversed transcribed,

Papers in Press. Published October 31, 2014 as doi:10.1373/clinchem.2014.230615
The latest version is at http://hwmaint.clinchem.org/cgi/doi/10.1373/clinchem.2014.230615

Clinical Chemistry 61:1
000–000 (2015)

Molecular Diagnostics and Genetics

Variability of the Reverse Transcription Step: Practical Implications

Stephen Bustin,[1]* Harvinder S. Dhillon,[1] Sara Kirvell,[1] Christina Greenwood,[1] Michael Parker,[1] Gregory L. Shipley,[2] and Tania Nolan[3]

BACKGROUND: The reverse transcription (RT) of RNA to cDNA is a necessary first step for numerous research and molecular diagnostic applications. Although RT efficiency is known to be variable, little attention has been paid to the practical implications of that variability.

RESULTS: RT efficiency is enzyme, sample, RNA concentration, and assay dependent and can lead to variable correlation between mRNAs from the same sample. This translates into relative mRNA expression levels that generally vary between 2- and 3-fold, although higher levels are also observed.

Variability of the Reverse Transcription Step: Practical Implications

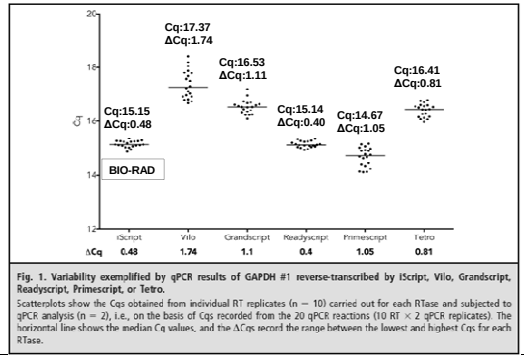


Fig. 1. Variability exemplified by qPCR results of GAPDH #1 reverse-transcribed by iScript, Vilo, Grandscript, Readyscript, Primescript, or Tetro.

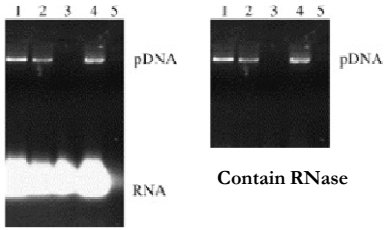
Scatterplots show the Cqs obtained from individual RT replicates (n = 10) carried out for each RTase and subjected to qPCR analysis (n = 2), i.e., on the basis of Cqs recorded from the 20 qPCR reactions (10 RT × 2 qPCR replicates). The horizontal line shows the median Cq values, and the ΔCqs record the range between the lowest and highest Cqs for each RTase.

Reverse transcription Strategies

- Oligo dT
- Random primers
- Oligo dT and Random primers
- Gene specific primers
- RNase H
- RNase inhibitors

RNase is STRONG

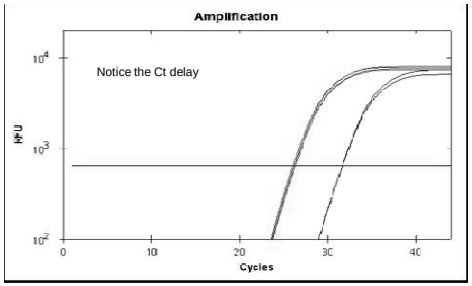
Plasmid extracted with traditional 3-steps and eluted with sterile ddH_2O.



NO RNase

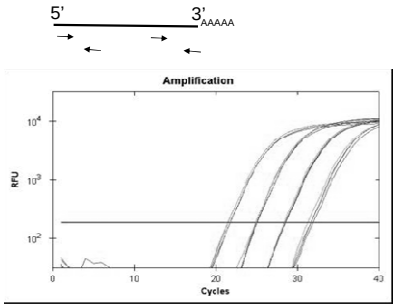
Contain RNase

Obtain Accurate Results – **potent blend of RNase inhibitor**



RT with iScript (green trace)
RT with iScript + RNaseA (red trace)
RT with iScript without RNase inhibitor + RNase (black trace)

Increase primer design flexibility & prevent 5' 3' bias – **optimum blend of oligo(dT) & random primers**



RT 100, 10, 1, and 0.1 ng input RNA
Primer pairs for 5' end (green trace - ~60 bp)
Primer pairs for 3' end (orange trace - ~70 bp)

iScript works with a broad range of RNA

- Range of iScript is100fg – 1µg of input RNA
- Competitors cannot reliably achieve this range, and sometimes require two RTs for low and high amounts of input RNA

iScript

Competitors

Bio-Rad's
iScript cDNA Synthesis supermix

~45 min Completed

qPCR Target Information

Item to check	Importance
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

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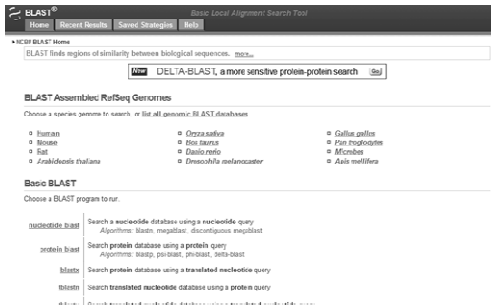
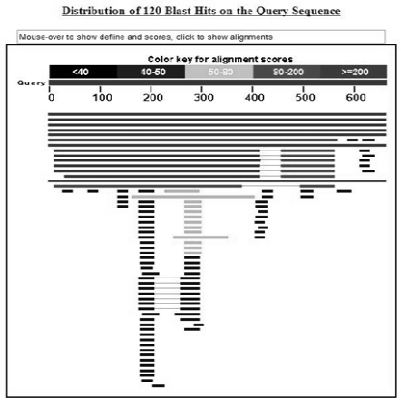
NCBI



- Free resource
- Blast sequence
- Stack sequence

Blast analysis



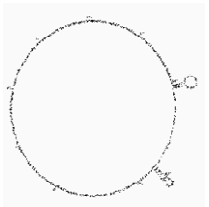
http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi

Amplicon Secondary Structures

http://mfold.rna.albany.edu/?q=mfold

- Bad location for primers

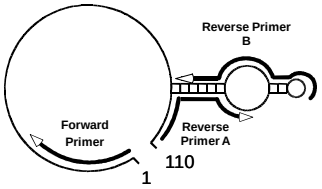
- Good location for primers

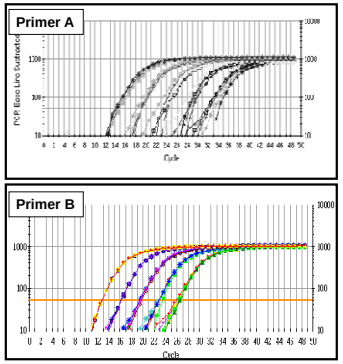


Effect of primer location

Primer A: η = 66.3 %

Primer B: η = 95.8 %





qPCR Oligonucleotides

Item to check	Importance
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

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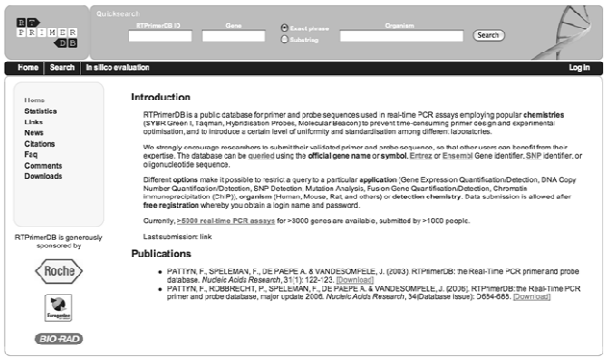
Primer Design

- Designs Primers
- Designs Internal Oligos
- Provides multiple outputs
- Free

Web software provided by *Steve Rozen* and *Whitehead Institute for Biomedical Research.*
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

RTPrimerDB



qPCR Protocol

qPCR protocol	Item to check	Importance
	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

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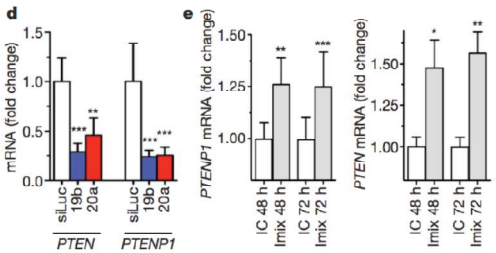
Vol 465|24 June 2010|doi:10.1038/nature09144 nature

ARTICLES

A coding-independent function of gene and pseudogene mRNAs regulates tumour biology

Laura Poliseno[1*†], Leonardo Salmena[1*], Jiangwen Zhang[2], Brett Carver[3], William J. Haveman[1] & Pier Paolo Pandolfi[1]

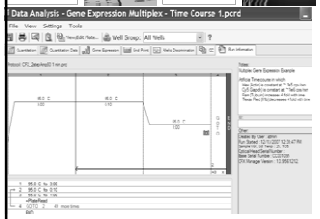
The canonical role of messenger RNA (mRNA) is to deliver protein-coding information to sites of protein synthesis. However, given that microRNAs bind to RNAs, we hypothesized that RNAs could possess a regulatory role that relies on their ability to compete for microRNA binding, independently of their protein-coding function. As a model for the protein-coding-independent role of RNAs, we describe the functional relationship between the mRNAs produced by the *PTEN* tumour suppressor gene and its pseudogene *PTENP1* and the critical consequences of this interaction. We find that *PTENP1* is biologically active as it can regulate cellular levels of PTEN and exert a growth-suppressive role. We also show that the *PTENP1* locus is selectively lost in human cancer. We extended our analysis to other cancer-related genes that possess pseudogenes, such as oncogenic *KRAS*. We also demonstrate that the transcripts of protein-coding genes such as *PTEN* are biologically active. These findings attribute a novel biological roie to expressed pseudogenes, as they can regulate coding gene expression, and reveal a non-coding function for mRNAs.



METHODS SUMMARY

Cell lines were cultured under standard conditions. miRNA overexpression was obtained by transient transfection (si-miRNAs). *PTENP1/PTEN/KRAS1P 3'* UTR overexpression was achieved by transient transfection (pCMV expression vectors) or stable infection with MSCV-PIG retroviral constructs. miRNA–target interaction was measured by luciferase reporter assay. *PTENP1*, *PTEN*, *KRAS*, *KRAS1P* and mRNA expression level was detected by real-time PCR. Proliferation, foci and soft agar assays were performed according to standard protocols.

Recipe, Protocol, Consumable, and Instrument



	1 RXN Number	# RXNs of RXNs	
	1	90	
TE (1X)	2.8	252.0	
2X iQ PowerMix (-->1X)	10	900.0	
10uM IL1β5101F (-->0.3 uM)	0.6	54.0	
10uM IL1β246R (-->0.3 uM)	0.6	54.0	
10uM FAM-IL1β BHline (--> 0.2uM)	0.4	36.0	
10uM GAPDH 169F (-->0.3 uM)	0.6	54.0	
10uM GAPDH 290R (-->0.3 uM)	0.6	54.0	
100uM HEX-GAPDHBHLine (--> 0.2uM)	0.4	36.0	
		1440.0	Total
Template	4		

The DNA binding dye reagents of Bio-Rad

Reagents

- **iQ SYBR Green Supermix**
- **Ssofast EvaGreen Supermix**
- **iTaq™ Universal SYBR® Green One-Step Kit**

Key Features

- Sensitive SYBR Green or EvaGreen
- Simplified reaction set up with ready-to-use 2x reaction mix
- iTaq hot-start DNA polymerase, hot-start in 30 sec or 3 min at 95℃
- Linear results over 7 orders of magnitude

The probes based reagents of Bio-Rad

Reagents

- **iTaq universal supermix for Probe**
- **iTaq™ Universal probe One-Step Kit**
- **iQ Multiplex Powermix**

Key Features

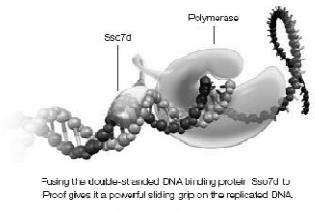
- Simplified reaction set up with ready-to-use 2x reaction mix
- hot-start DNA polymerase, hot-start in 30 sec or 3 min at 95℃
- Linear results over 7 orders of magnitude
- Detection of up to 2 or 5 targets, where one target can be as much as 10^6-fold different in input quantity

3rd Generation Reagents:

SsoFast™ Series Supermix

- High Speed
- High Dynamic Range
- High Tolerance

3rd Generation Reagents



Fusing the double-stranded DNA binding protein Sso7d to Proof gives it a powerful sliding grip on the replicated DNA.

SsoFast EvaGreen Supermix

Sso7d from *Sulfolobus solfataricus*

- 7kD, 63 aa.
- Thermostable (Tm >90° C)
- No sequence preference
- Binds to dsDNA (3-6 bp/protein molecule)
- Monomeric

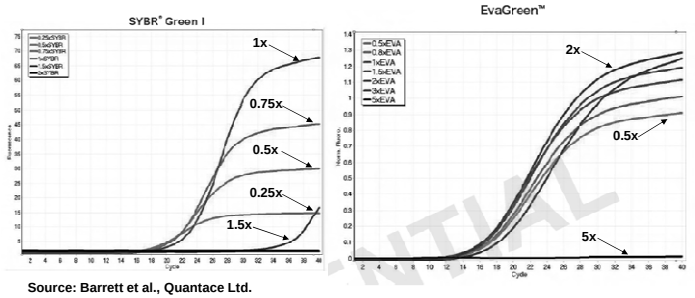
- Minimal inhibition of PCR by use of EvaGreen
- Higher activity
- Tolerant to PCR inhibitors

Sso7d-fusion enzymes
Enhanced processivity

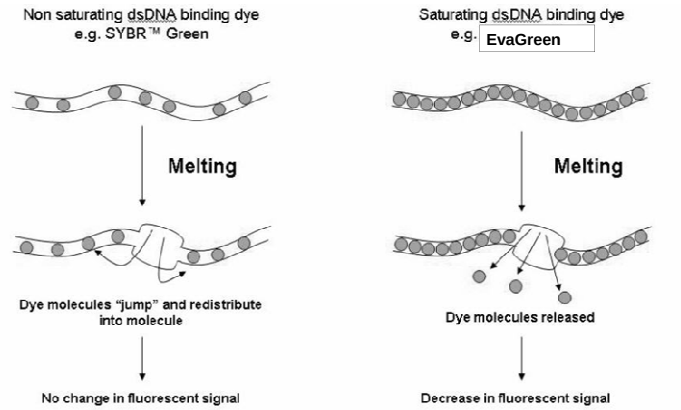
Enzyme	Processivity
Taq	15-19nt
Sso7d-Taq	**96-109nt**
Pfu	2-3nt
Pfu-Sso7d	**35-39nt**
ΔTaq	2-4nt
Sso7d- ΔTaq	**26-39nt**

Processivity is enhanced 5 to 10-fold

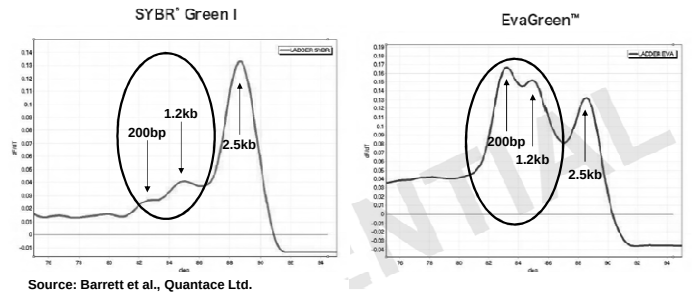
PCR Inhibition caused by Dye



Source: Barrett et al., Quantace Ltd.



Even Dye Redistribution



Source: Barrett et al., Quantace Ltd.

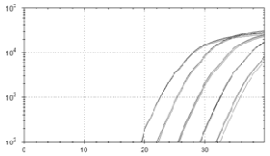
The even dye redistribution gives equal signal intensities from different sized DNA fragments

AMPLIFICATION

High Dynamic Range

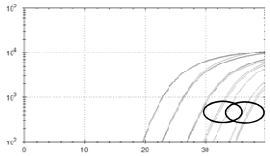
Fast Protocol 36 min

SsoFast EvaGreen Supermix



Efficiency = 104.0%, r = 0.992

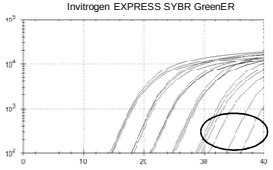
ABI Fast SYBR Green Mastermix



Efficiency = 110.4%, r = 0.966

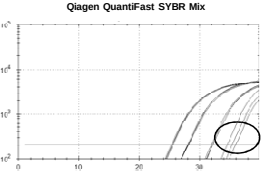
Fast Protocol 36 min

Invitrogen EXPRESS SYBR GreenER



Efficiency = 89.3%, r = 0.986

Qiagen QuantiFast SYBR Mix

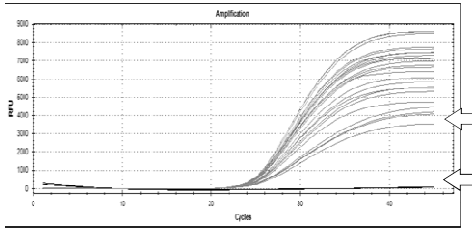


Efficiency = 98.5%, r = 0.988

Bio-Rad Mix in Green
Competitor Mix in Blue

AMPLIFICATION

Phenol Chloroform (extraction mix)



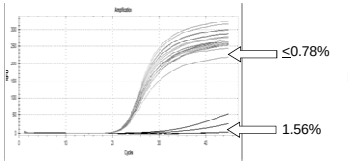
≤1.56%

3.125%

CCI26 amplified using Bio-Rad SsoFast EVAGreen Supermix: 5ul Assay
98ºC 30sec / 50x95ºC 1 sec 60ºC 5 sec / melt analysis

AMPLIFICATION

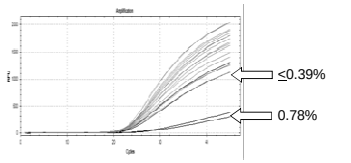
Phenol Chloroform (extraction mix)



≤0.78%

1.56%

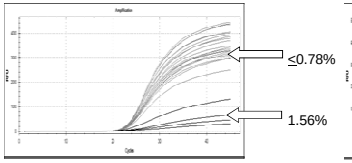
CCI26 amplified using Bio-Rad iQ SYBR Green Supermix:
5ul Assay95ºC 3 min / 50x 95ºC 10 sec 60ºC 60 sec / melt



≤0.39%

0.78%

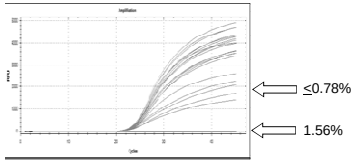
CCI26 amplified using Other Reagent A: 5ul Assay
95ºC 5min / 50x 95ºC 15 sec 60ºC 60 sec / melt analysis



≤0.78%

1.56%

CCI26 amplified using Other Reagent B: 5ul Assay
95ºC 20sec / 50x 95ºC 3 sec 60ºC 30 sec / melt analysis



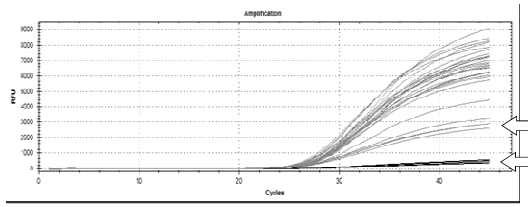
≤0.78%

1.56%

CCI26 amplified using Other Reagent C: 5ul Assay
95ºC 20sec / 50x 95ºC 3 sec 60ºC 30 sec / melt analysis

AMPLIFICATION

Blood Serum



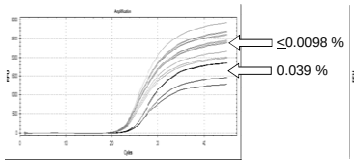
≤2.5 %

10 %

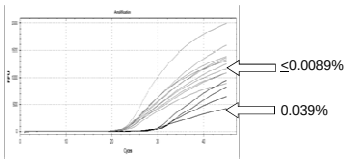
CCI26 amplified using Bio-Rad SsoFast EVAGreen Supermix: 5ul Assay
98ºC 30sec / 50x95ºC 1 sec 60ºC 5 sec / melt analysis

AMPLIFICATION

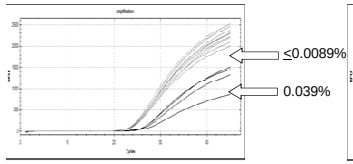
Blood Serum



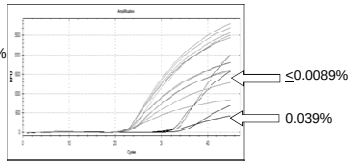
CCI26 amplified using Bio-Rad iQ SYBR Green Supermix
5ul Assay 95oC 3 min / 50x 95oC 10 sec 60oC 60 sec / melt



CCI26 amplified using Other Reagent A: 5ul Assay
95ºC 5min / 50x 95ºC 15 sec 60ºC 60 sec / melt analysis



CCI26 amplified using Other Reagent B: 5ul Assay
95ºC 20sec / 50x 95ºC 3 sec 60ºC 30 sec / melt analysis



CCI26 amplified using Other Reagent C: 5ul Assay
95ºC 20sec / 50x 95ºC 3 sec 60ºC 30 sec / melt analysis

AMPLIFICATION

Inhibitor Concentration Not Having Any Significant Effect on qPCR Reaction

Inhibitor	Max Tested	DilutionSeries	EVA	iQ SYBR	ABI	INV	Euro
Ethanol	25 %	2 fold	12.5 %	**6.25 %**	3.125 %	3.125 %	3.125 %
Isopropanol	17.5 %	2 fold	8.75 %	**4.375 %**	2.18 %	**4.375 %**	**4.375 %**
KCl	500 mM	2 fold	**62.5 mM**	31.25 mM	125 mM	**62.5 mM**	31.25 mM
NaAc	250 mM	2 fold	62.5 mM	62.5 mM	62.5 mM	62.5 mM	15.63 mM
Phenol/Chloroform	6.25 %	2 fold	1.53 %	**0.781 %**	**0.781 %**	**0.781 %**	0.391 %
Blood Serum	10 %	4 fold	2.5 %	0.0098 %	0.0098 %	0.0098 %	0.0098 %
Total Blood (+hep)	10 %	4 fold	0.156 %	0.0024 %	**0.0098 %**	0.0024 %	0.0024 %
Heparin	0.0188 U	2 fold	0.0188 U	**0.00469 U**	**0.00059 U**	**0.00023 U**	0.00012 U
Soil	25 %	4 fold	6.25 %	**1.56 %**	**1.56 %**	6.25 %	0.39 %
Coffee	25 %	4 fold	6.25 %	1.56 %	1.56 %	1.56 %	1.56 %

AMPLIFICATION

qPCR Validation

qPCR validation: Item to check	Importance
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

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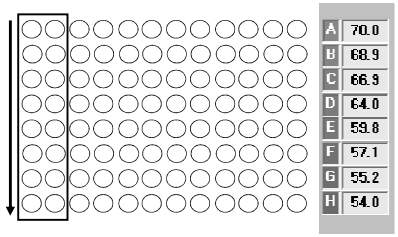
AMPLIFICATION

Optimization

How to choose the annealing temperature?

Evidence of optimization - thermal gradient

dynamic thermal gradient



10°C Above

6°C Below

Gradient optimization

Single Plate Optimisation of Annealing Temperature and PCR Efficiency via Slope of Calibration Curve

Dilution series →

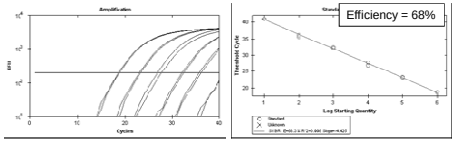
Thermal gradient ↓

		10^7		10^6		10^5		10^4		10^3		10^2	
		1	2	3	4	5	6	7	8	9	10	11	12
A	68.0	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
B	67.6	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
C	66.9	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
D	65.5	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
E	63.8	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
F	62.4	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
G	61.5	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
H	61.0	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6

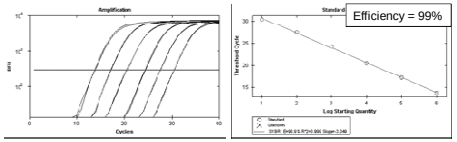
Gradient Optimization of Annealing Temperature

- Serial dilutions at 8 temps from 55°C to 68°C
- Reactions at 62°C annealing have low Cts and highest reaction efficiency

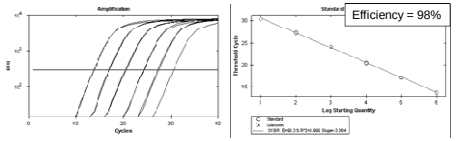
67°C



62°C

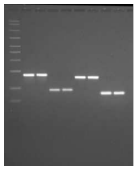


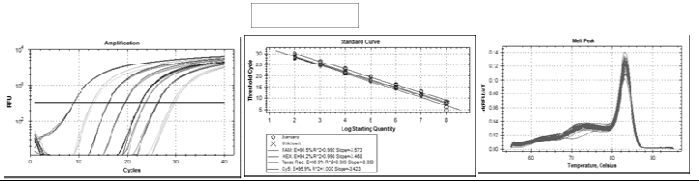
56°C



QPCR Validation

- Use a serial dilution of template to test primers across a broad dynamic range.
- Include representative unknown samples.
- Evaluate specificity, efficiency, reproducibility and dynamic range.
- Gel checking.





Data Analysis

Item to check	Importance
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

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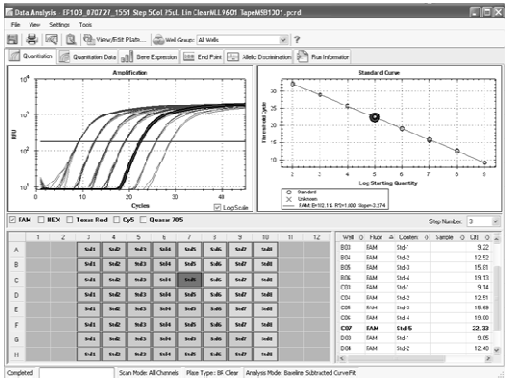
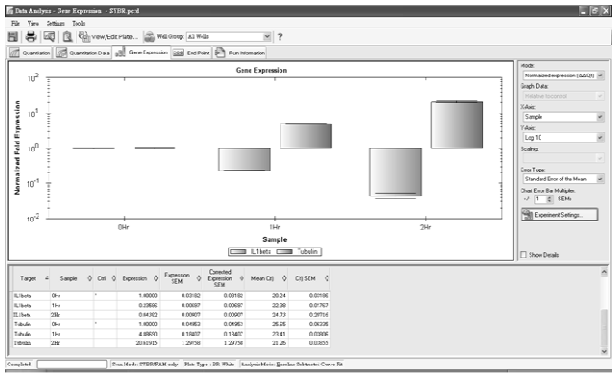
qPCR Program/Software

CFX Manager

Absolute Quantization



Relative Quantization



Gene Expression Normalization - Comparative C_q

- Relative Quantity (ΔCq)
  - Not normalized
  - Normalization accomplished via equal loading of samples
  - Post analysis normalization
- Normalized Expression (ΔΔCq)
  - Accounts for loading differences
  - Usually normalize to reference gene
  - Relative quantity of Cq is normalized by the relative quantity of the reference genes

Relative Quantification - ΔCq

	Cq
Sample :	**22**
Control :	**24**
Delta Ct:	**22 - 24 = - 2**

Fold induction = $2^{-(-2)} = 4$

Comparative Cq Method ($2^{-\Delta\Delta Cq}$)

- **Normalize Cq of target gene to that of reference gene**
- **Normalize ΔCq of sample to ΔCq of control sample**

		Reference	Cq
	Tissue #1: (sample)	**21**	**22**
	Tissue #2: (control)	**20**	**24**
1st Delta	**Delta Cq #1:**		**22-21 = 1**
	Delta Cq #2:		**24-20 = 4**
2nd Delta	**Delta Cq:**		**1 - 4 = - 3**

Fold induction = $2^{-(-3)} = 8$

Relative Quantification –Pfaffl Modification

		Reference	Target Cq
	Tissue #1: (sample)	**21**	**22**
	Tissue #2: (control)	**20**	**24**
(From Standard curve)	**Efficiency:**	**90% = 1.9**	**100% = 2**
	Delta Cq:	**20-21 = -1**	**24-22 = 2**

$$\text{Fold induction} = \frac{2_{\text{target Cq}}^{\text{deltaCq}_{\text{target GOI}}\ (24-22=2)}}{1.9_{\text{reference}}^{\text{deltaCq}_{\text{reference}}\ (20-21=-1)}} = \frac{4}{0.53} = 7.5$$

AMPLIFICATION

Gene Expression Normalization - Normalized Expression

- ΔΔCq
  - Assume 100% efficiency
  - Only one Ref Gene
- Pfaffl Modification
  - Accounts for efficiency differences
  - Only one Ref Gene
- Vandesompele Method
  - Accounts for efficiency differences
  - Allows multiple reference genes for normalization

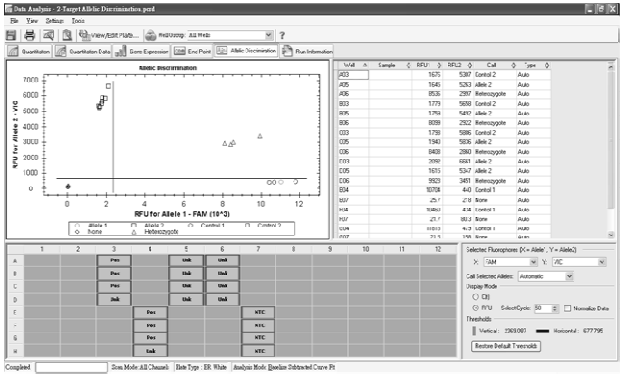
Simple

↓

Complex

AMPLIFICATION

Allelic Discrimination



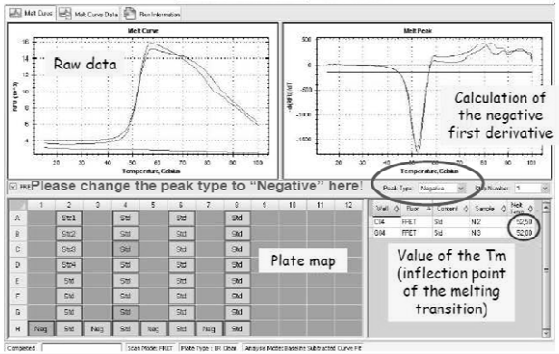
AMPLIFICATION

Protein Folding analysis

Sample : 2.5 µg of purified protein in 23 µl tested with 2 µl of Sypro Orange (25µl / dupli

Protocol: melting curve ranging from 15°C to 100°C with increment of 0.5°C

Estimated run time : 1h 35min



AMPLIFICATION

HRM mutation analysis

Lung cancer screen in *KRAS* mutation on CFX

SsoFast EvaGreen Supermix Protocol

98ºC – 2 mins

(40 cycles)

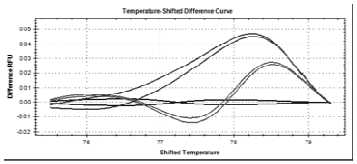
98ºC – 5 sec

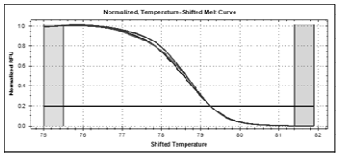
61ºC – 10 sec

(Melt)

95ºC – 30 sec

75ºC – 5 sec (+0.2ºC/ cycle to 95ºC)

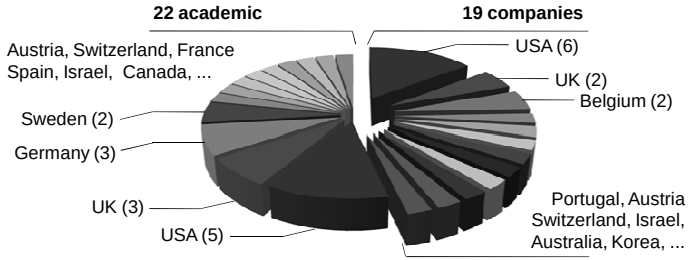




Well	Content	Sample	Cluster	Percent Confidence
A06	Unkn-1	A549-92bp	Cluster 1	99.7
B06	Unkn-1	A549-92bp	Cluster 1	99.7
C06	Unkn-2	HCT116_92bp	Cluster 2	96.8
D06	Unkn-2	HCT116_92bp	Cluster 2	97.2
E06	Unkn-3	48T-92bp	Cluster 3	96.5
F06	Unkn-3	48T-92bp	Cluster 3	97.3
G06	NTC	NTC	<Excluded>	N/A
H06	NTC	NTC	<Excluded>	N/A

The RDML International Consortium

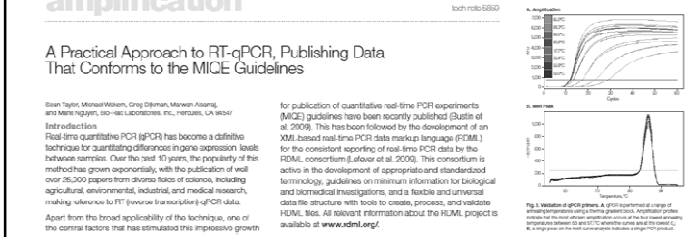
- Key developer group, a member community and supporters
- **41 supporters and members from 20 different countries**



Goal: develop and maintain the RDML data exchange format

Conforming to MIQE: an example

- The Bio-Rad technote 5859 provides an excellent summary of a MIQE compliant experiment in practice, from start to finish


amplification

tech note 5859

A Practical Approach to RT-qPCR, Publishing Data That Conforms to the MIQE Guidelines

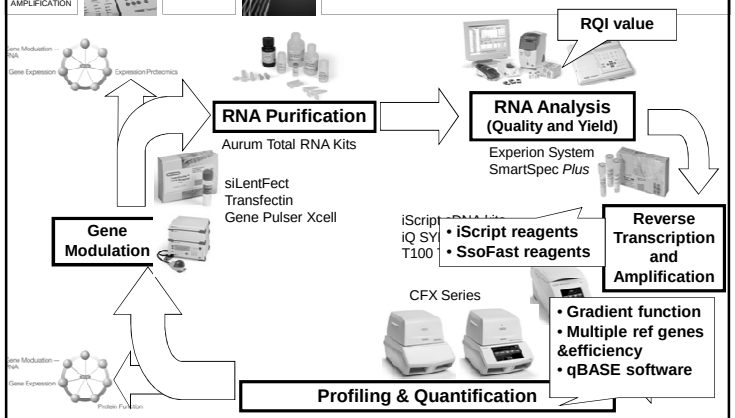
Sean Taylor, Michael Wakem, Greg Dijkman, Marwan Alsarraj, and Marie Nguyen, Bio-Rad Laboratories Inc., Hercules, CA 94547

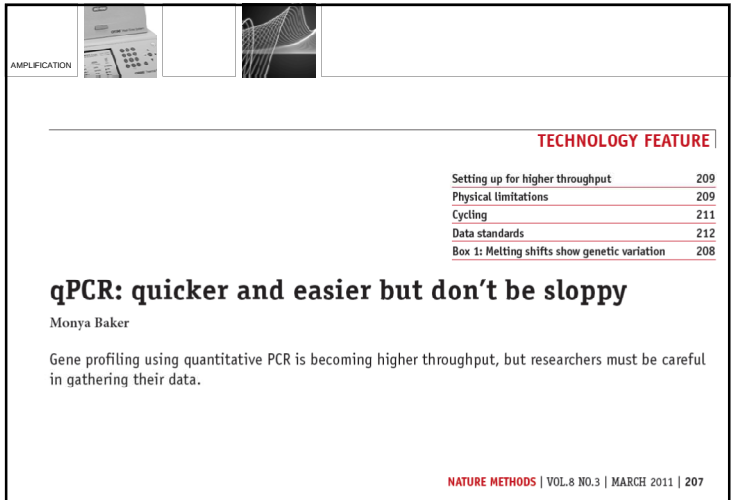
Introduction

Real-time quantitative PCR (qPCR) has become a definitive technique for quantifying differences in gene expression levels between samples. Over the past 10 years, the popularity of this method has grown exponentially, with the publication of well over 35,000 papers from diverse fields of science, including agricultural, environmental, industrial, and medical research, making reference to RT (reverse transcription)-qPCR data.

Apart from the broad applicability of the technique, one of the central factors that has stimulated this impressive growth for publication of quantitative real-time PCR experiments (MIQE) guidelines have been recently published (Bustin et al. 2009). This has been followed by the development of an XML-based real-time PCR data markup language (RDML) for the consistent reporting of real-time PCR data by the RDML consortium (Lefever et al. 2009). This consortium is active in the development of appropriate and standardized terminology, guidelines on minimum information for biological and biomedical investigations, and a flexible and universal data file structure with tools to create, process, and validate RDML files. All relevant information about the RDML project is available at **www.rdml.org/.**

Complete solutions




TECHNOLOGY FEATURE

qPCR: quicker and easier but don't be sloppy

Monya Baker

Gene profiling using quantitative PCR is becoming higher throughput, but researchers must be careful in gathering their data.

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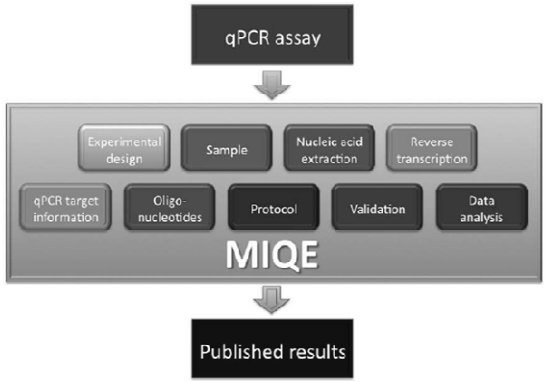
AMPLIFICATION

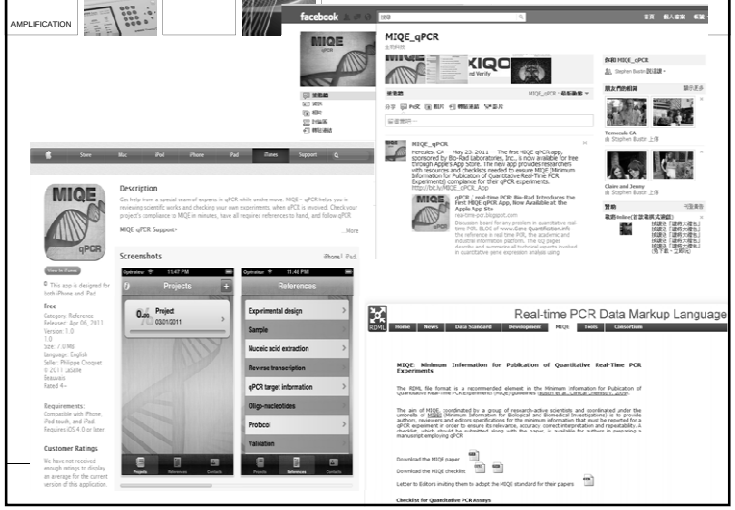
Questions?



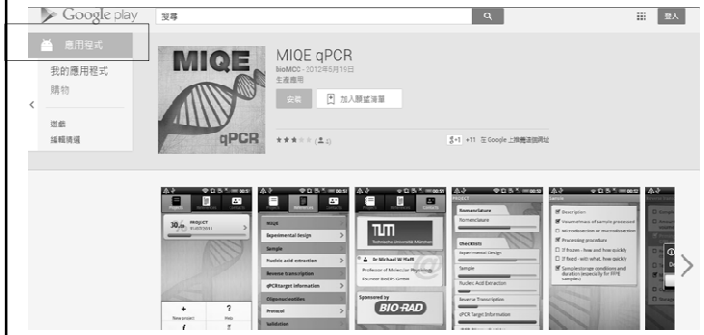
www.bio-rad.com/pcr

BIO-RAD

AMPLIFICATION



AMPLIFICATION



www.bio-rad.com/pcr

BIO-RAD