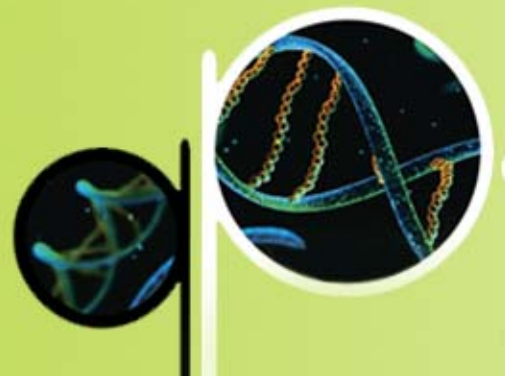


Science Webinar Series

The Future of qPCR

Best practices, Standardization, and the MIQE Guidelines

Science AAAS

30 September, 2010

Brought to you by the *Science*/AAAS Business Office

Participating Experts:

Dr. Stephen A. Bustin
Barts and the London School of Medicine and Dentistry
London, UK

Dr. Gregory L. Shipley
University of Texas Health Science Center at Houston
Houston, TX

Manju R. Sethi
ThermoFisher Scientific
Wilmington, DE

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Why MIQE?

Dr. Stephen A. Bustin
Barts and The London
School of Medicine and Dentistry

Real-time PCR

Barts and The London
School of Medicine and Dentistry

- Simple
- Mature
- “Gold standard”

Barts and The London
School of Medicine and Dentistry



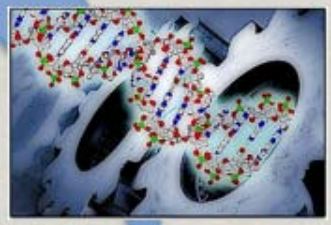
Primer design



RT



Chemistry

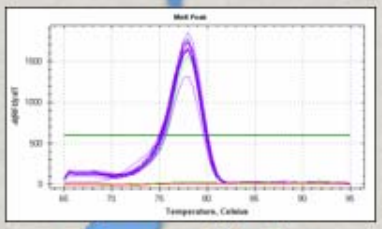


cDNA synthesis



RNA QA





Assay validation



Sample extraction

qbase PLUS

Data analysis


Diagnosis of the Small Round Blue Cell Tumors Using Multiplex Polymerase Chain Reaction

Data reporting



Sample selection and handling

Real-time PCR

- Simple
- Mature
- “Gold standard”

- Complex
- Evolving
- Variable

Journal of Clinical Virology 43 (2008) 367–371

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv



Development of working reference materials for clinical virology

Jacqueline F. Fryer[a,*,1], Sally A. Baylis[a,1,2], Anna L. Gottlieb[a,1], Morag Ferguson[a], Giuseppe A. Vincini[b], Valerie M. Bevan[b], William F. Carman[c], Philip D. Minor[a]

[a] Division of Virology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK
[b] Evaluations and Standards Laboratory, Centre for Infections, Health Protection Agency, 61 Colindale Avenue, London NW9 5EQ, UK
[c] West of Scotland Specialist Virology Centre, Gartnaval General Hospital, Great Western Road, Glasgow G12 0YN, UK

6. Conclusions

The results presented here show that there is considerable variation in the performance of clinical real-time PCR assays for these target viruses. For the influenza and norovirus candidate run controls a number of assays failed to detect these targets in one or more runs, although in some cases this was reported to be due to inhibition of the PCR. For all run controls, a high level of both inter- and intra-assay performance variation was observed. The highest

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Table 1
Summary of candidate run controls undergoing phase II evaluation

Run control	Strain	No. of responders/participants (%)	No. of datasets[a]	Overall mean C_T value ± 2S.D.[b]	Overall C_T value range[b]
Influenza A (H1)[c]	A/Christchurch/1/2003 (H1N1)	11/13 (85)	15	31.29 ± 5.24	25.30–40.66
Influenza A (H3)[c]	A/Wyoming/3/2003 (H3N2)	11/13 (85)	15	30.33 ± 5.40	24.30–37.88
Influenza B[c]	B/Jiangsu/10/2003	12/13 (92)	16	28.14 ± 4.55	22.46–36.75
Norovirus	Genogroup II	15/17 (88)	20	33.14 ± 9.00	23.00–44.03
HSV-1	17	14/23 (61)	15	28.91 ± 7.72	18.73–39.23
HSV-2	HG52	14/23 (61)	15	29.43 ± 6.27	20.83–39.00
HCMV	AD169	15/23 (65)	16	29.28 ± 5.51	24.87–37.40
EBV	Raji	10/18 (56)	10	28.03 ± 8.82	17.28–36.56

[a] More than one dataset was returned by some laboratories.
[b] Excluding results that were negative or outside the cut-off for the assay.
[c] Excluding results for one laboratory performing nested real-time PCR.

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ΔC_q=15 (3.2x10^4-fold)
ΔC_q=13 (0.8x10^4-fold)
ΔC_q=14 (1.6x10^4-fold)
ΔC_q=21 (2.1x10^6-fold)
ΔC_q=21 (2.1x10^6-fold)
ΔC_q=19 (5.2x10^5-fold)
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Key Issues

- Sample quality
- Reverse transcription
- Assay validation/optimisation
- PCR efficiency
- Normalisation
  - >1 RG
  - validation

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 22, Number 12, 2006, pp. 1253–1259
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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]

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**).

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slope: -1.365

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

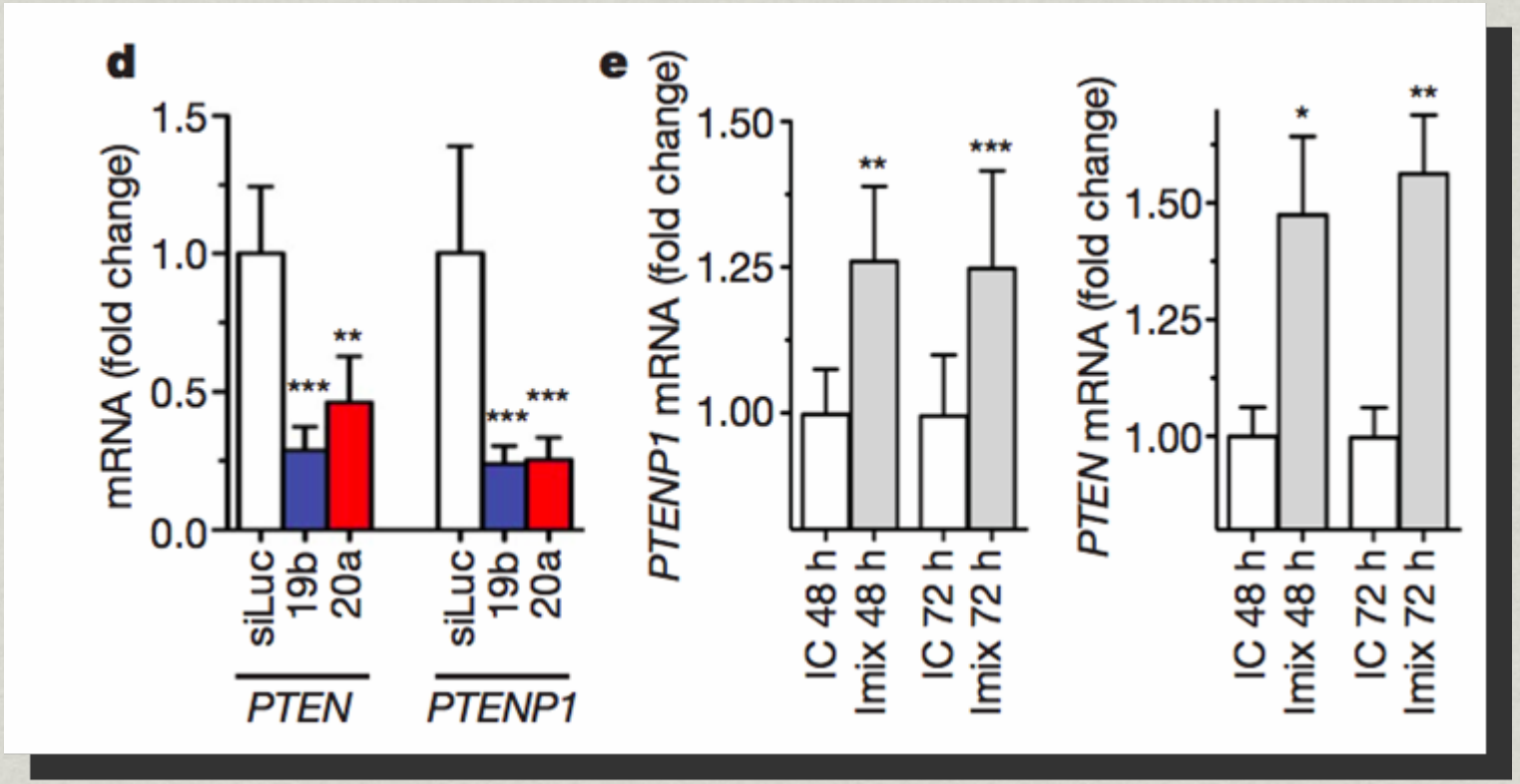
ARTICLES

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 role to expressed pseudogenes, as they can regulate coding gene expression, and reveal a non-coding function for mRNAs.

competitive endogenous RNA (ceRNA)


d
mRNA (fold change)
1.5
1.0
0.5
0.0
***
**
***
***
siLuc
19b
20a
siLuc
19b
20a
PTEN
PTENP1
e
PTENP1 mRNA (fold change)
1.50
1.25
1.00
**
***
IC 48 h
Imix 48 h
IC 72 h
Imix 72 h
PTEN mRNA (fold change)
1.50
1.25
1.00
*
**
IC 48 h
Imix 48 h
IC 72 h
Imix 72 h

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 miRNA expression level was detected by real-time PCR. Proliferation, foci and soft agar assays were performed according to standard protocols.

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***PTEN* forward**	**CCTTCTCCATCTCCTGTGTAATCAA**
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***PTEN* FAM probe**	**CCAGTGCTAAAATTCA**

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Supplementary Figure 1. Alignment between *PTEN* and *PTENP1* 3'UTR. *PTEN* (NM_000314) and *PTENP1* (NM_023917) 3'UTR are shown. Matched nucleotides are in black, unmatched are in white. The

Homo sapiens taste receptor, type 2, member 9 (TAS2R9), mRNA

NCBI Reference Sequence: NM_023917.2

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NCBI Reference Sequence: NM_023917.2

Homo sapiens phosphatase and tensin homolog pseudogene 1 (PTENP1), non-coding RNA

NCBI Reference Sequence: NR_023917.1

RESEARCH ARTICLE

9 SEPTEMBER 2005 VOL 309 SCIENCE www.sciencemag.org

The mRNA of the *Arabidopsis* Gene *FT* Moves from Leaf to Shoot Apex and Induces Flowering

Tao Huang,[1] Henrik Böhlenius,[1] Sven Eriksson,[1] François Parcy,[2] Ove Nilsson[1]*

Day length controls flowering time in many plants. The day-length signal is perceived in the leaf, but how this signal is transduced to the shoot apex, where floral initiation occurs, is not known. In *Arabidopsis*, the day-length response depends on the induction of the *FLOWERING LOCUS T* (*FT*) gene. We show here that local induction of *FT* in a single *Arabidopsis* leaf is sufficient to trigger flowering. The *FT* messenger RNA is transported to the shoot apex, where downstream genes are activated. These data suggest that the *FT* mRNA is an important component of the elusive "florigen" signal that moves from leaf to shoot apex.

day conditions is only slightly affected (*6*). *CO* is expressed mainly in the leaf, where the CO protein is responsible for sensing the day-length signal (*7*–*10*). CO then induces *FT* in the leaf phloem (*8*, *9*). CO appears to act upstream of the graft-transmissible floral stimulus, but neither *CO* nor *FT* appears to be expressed in the shoot apex, where floral initiation occurs (*8*, *9*). CO can induce *FT* expression in the leaf phloem, but not when expressed from shoot apex–specific promoters (*9*, *11*). In contrast, *FT* expression in both the leaf and the shoot apex can trigger floral initiation (*9*). These data raise the possibility that the *FT* mRNA or the FT protein could be a part of the floral stimulus that moves from the leaf to the shoot apex.

Local induction of *FT*. To separate the role of *FT* from that of other genes that are

Barts and The London
School of Medicine and Dentistry

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 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 *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 *PtCENL1* 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

ORIGINAL ARTICLE

Potential viral pathogenic mechanism for new variant inflammatory bowel disease

V Uhlmann*, C M Martin*, O Sheils, L Pilkington, I Silva, A Killalea, S B Murch, J Walker-Smith, M Thomson, A J Wakefield, J J O'Leary

J Clin Pathol: Mol Pathol 2002;**55**:84–90

See end of article for authors' affiliations

Correspondence to: Professor J J O'Leary, Department of Pathology, Coombe Women's Hospital, Dublin 8, Ireland; joleary@coombe.ie

Accepted for publication 8 November 2001

Aims: A new form of inflammatory bowel disease (ileocolonic lymphonodular hyperplasia) has been described in a cohort of children with developmental disorder. This study investigates the presence of persistent measles virus in the intestinal tissue of these patients (new variant inflammatory bowel disease) and a series of controls by molecular analysis.

Methods: Formalin fixed, paraffin wax embedded and fresh frozen biopsies from the terminal ileum were examined from affected children and histological normal controls. The measles virus Fusion (F) and Haemagglutinin (H) genes were detected by TaqMan reverse transcription polymerase chain reaction (RT-PCR) and the Nucleocapsid (N) gene by RT in situ PCR. Localisation of the mRNA signal was performed using a specific follicular dendritic cell antibody.

Results: Seventy five of 91 patients with a histologically confirmed diagnosis of ileal lymphonodular hyperplasia and enterocolitis were positive for measles virus in their intestinal tissue compared with five of 70 control patients. Measles virus was identified within the follicular dendritic cells and some lymphocytes in foci of reactive follicular hyperplasia. The copy number of measles virus ranged from one to 300 000 copies/ng total RNA.

Conclusions: The data confirm an association between the presence of measles virus and gut pathology in children with developmental disorder.

ORIGINAL ARTICLE

Potential viral pathogenic mechanism for new variant inflammatory bowel disease

V Uhlmann*, C M Martin*, O Sheils, L Pilkington, I Silva, A Killalea, S B Murch,
J Walker-S

News

MEASLES ONCE AGAIN ENDEMIC IN THE UNITED KINGDOM

Editorial team (eurosurveillance@ecdc.europa.eu)[1]
1. European Centre for Disease Prevention and Control, Stockholm, Sweden

See end of article
authors' affiliation

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Department of Pa
Coombe Women'
Hospital, Dublin 8
joleary@coombe.

Accepted for publ
8 November 200

Fourteen years after the local transmission of measles was halted in the United Kingdom (UK), the disease has once again become endemic, according to the Health Protection Agency (HPA), the public health body of England and Wales. In an update on measles cases in its weekly bulletin last week, the agency stated that, as a result of almost a decade of low mumps-measles-rubella (MMR) vaccination coverage across the UK, 'the number of children susceptible to measles is now sufficient to support the continuous spread of measles' [1].

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1. Health Protection Agency. Confirmed measles cases in England and Wales – an update to end of May 2008. Health Protection Report (serial online). 20 June 2008. 2;25. Available from: http://www.hpa.org.uk/hpr/news/default.htm#meas0805
2. Health Protection Agency. Confirmed cases of measles in England and Wales – an update. Health Protection Report (serial online). 23 May 2008. 2;(21). Available from: http://www.hpa.org.uk/hpr/archives/2008/news2108.htm#measls
3. Vaccine uptake, measles, mumps, rubella, whooping cough. HPS Weekly Report. Health Protection Scotland. 25 June 2008. 42;2008/26. Available from: http://www.documents.hps.scot.nhs.uk/ewr/pdf2008/0826.pdf

Barts and The London School of Medicine and Dentistry

ORIGINAL ARTICL

Potential viral p
inflammatory b

V Uhlmann*, C M Mart
J Walker-S

News

MEASLES

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1. European Centre

See end of article
authors' affiliation

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joleary@coombe.

Accepted for publ
8 November 200

Fourteen years af
in the United Kingd
endemic, according
public health body
cases in its weekly
a result of almost a
vaccination covera
susceptible to meas
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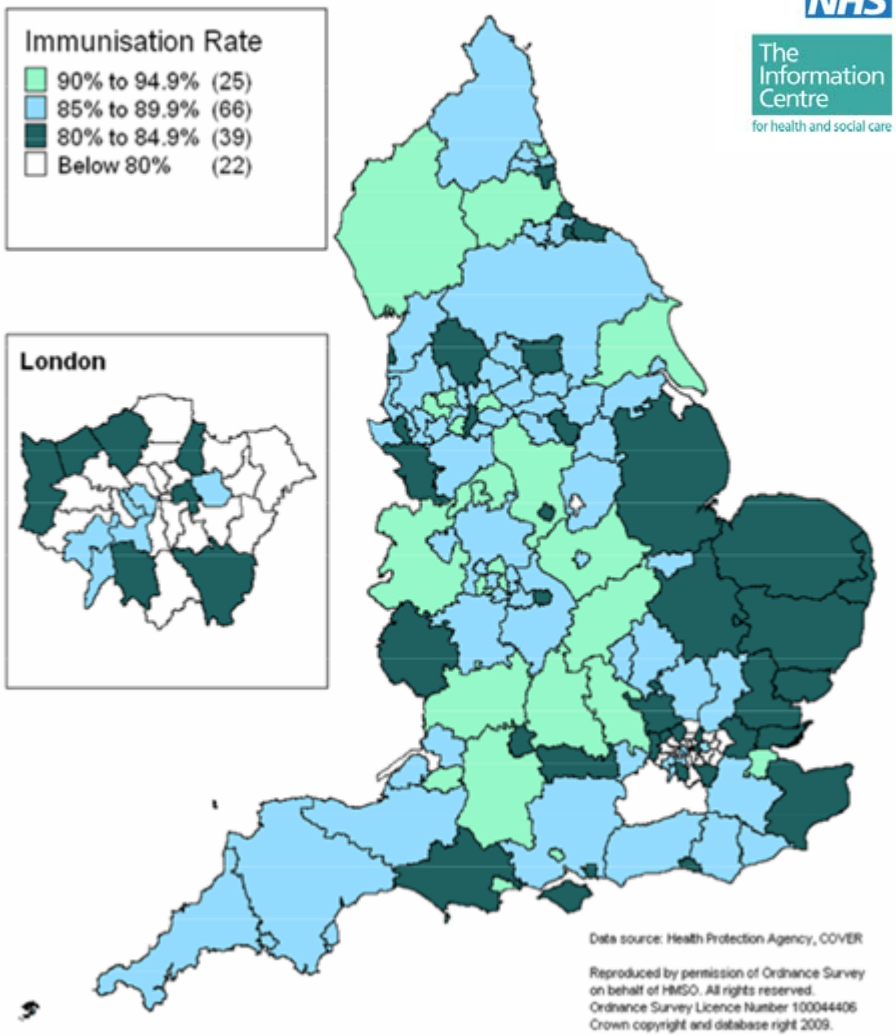
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t (serial online).
pr/news/default.

in England and
ne). 23 May 2008.
/2008/news2108.

PS Weekly Report.
ble from: http://

Figure 2: Percentage of children immunised against MMR at 24 months, by Primary Care Trust, 2008-09



Key Issues

	Science (n=17)	Cell (n=15)	BMC (n=16)
Sample Quality	0	0	6
RT	5	1	8
Assay Optimisation	0	2	2
PCR efficiency	1	0	6
Normalisation >1 RG	0	1	4
RG validation	0	0	6

Science Webinar Series

The Future of qPCR

Best practices, Standardization, and the MIQE Guidelines

30 September, 2010

Brought to you by the *Science*/AAAS Business Office

Participating Experts:

Dr. Stephen A. Bustin
Queen Mary, University of London
London, UK

Dr. Gregory L. Shipley
University of Texas Health Science Center at Houston
Houston, TX

Manju R. Sethi
ThermoFisher Scientific
Wilmington, DE

Sponsored by: Thermo SCIENTIFIC

MIQE Guidelines- Tips and Tricks

Gregory L. Shipley, Ph.D.



UTHealth
The University of Texas
Health Science Center at Houston

Outline

1. What is real-time qPCR?

2. MIQE PCR Target information

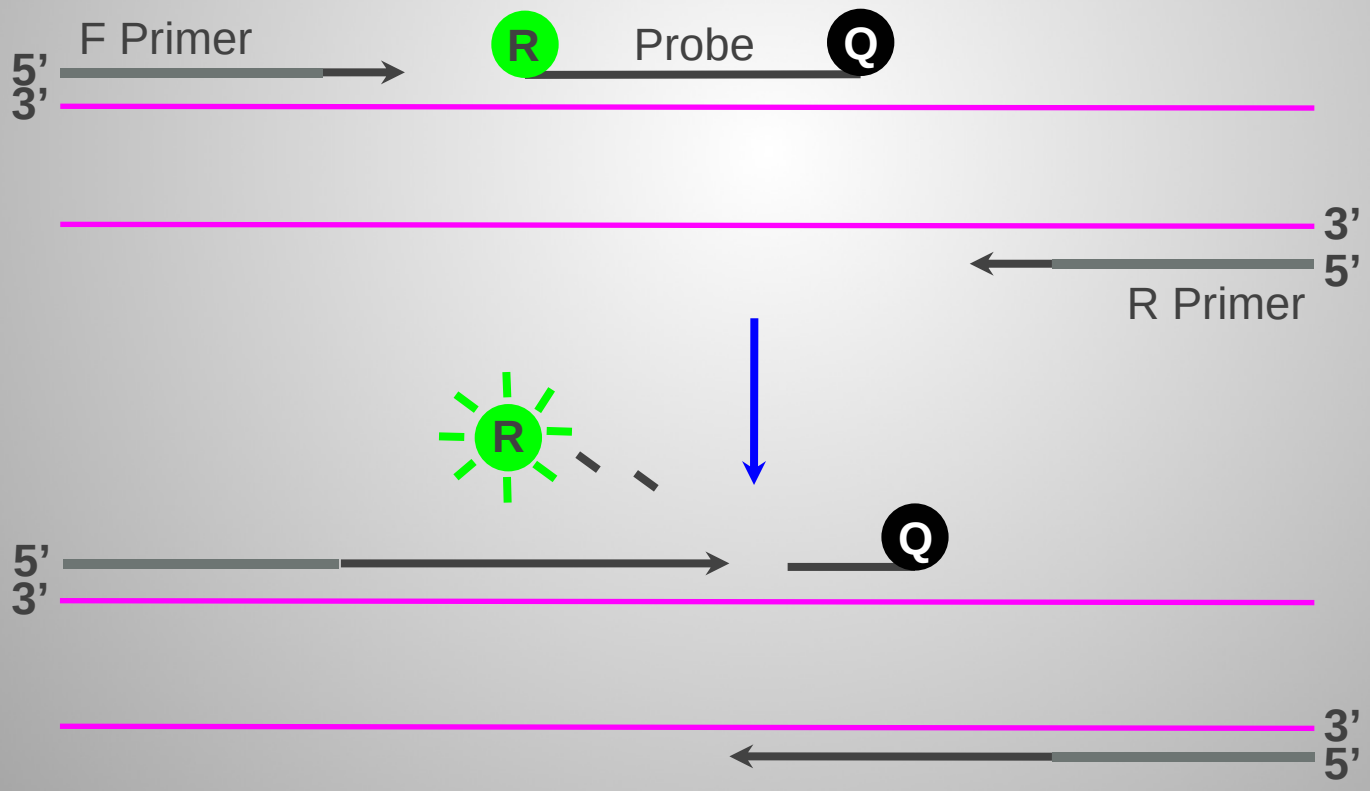
3. MIQE Assay Validation - how to

UTHealth
The University of Texas
Health Science Center at Houston

What is Real-Time qPCR?

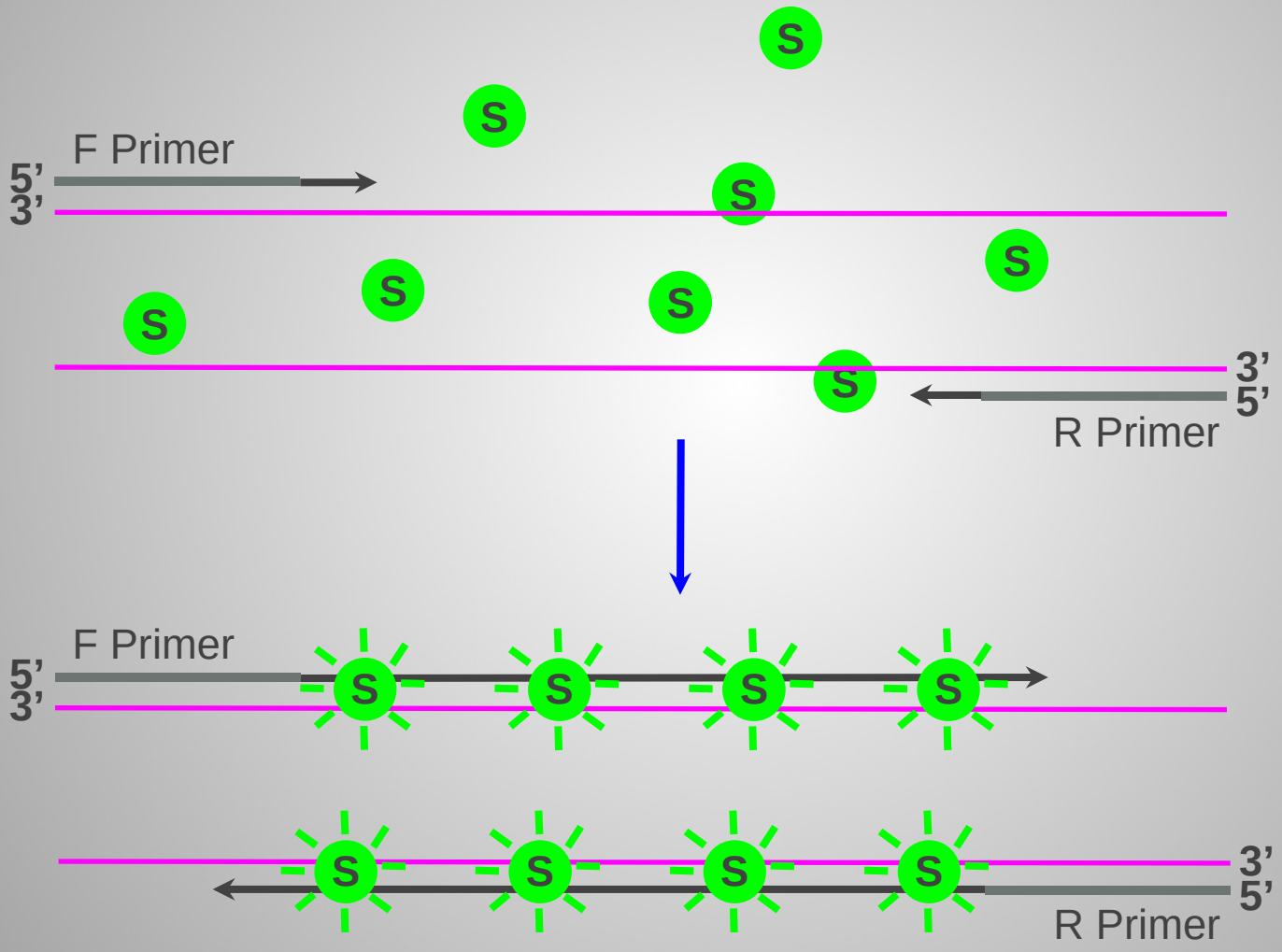
Collecting an increasing fluorescent signal for one or more gene targets in real time during a Polymerase Chain Reaction (PCR) leading to a quantitative value for each target.

1- Hydrolysis probe-based detection - FRET



What is Real-Time qPCR?

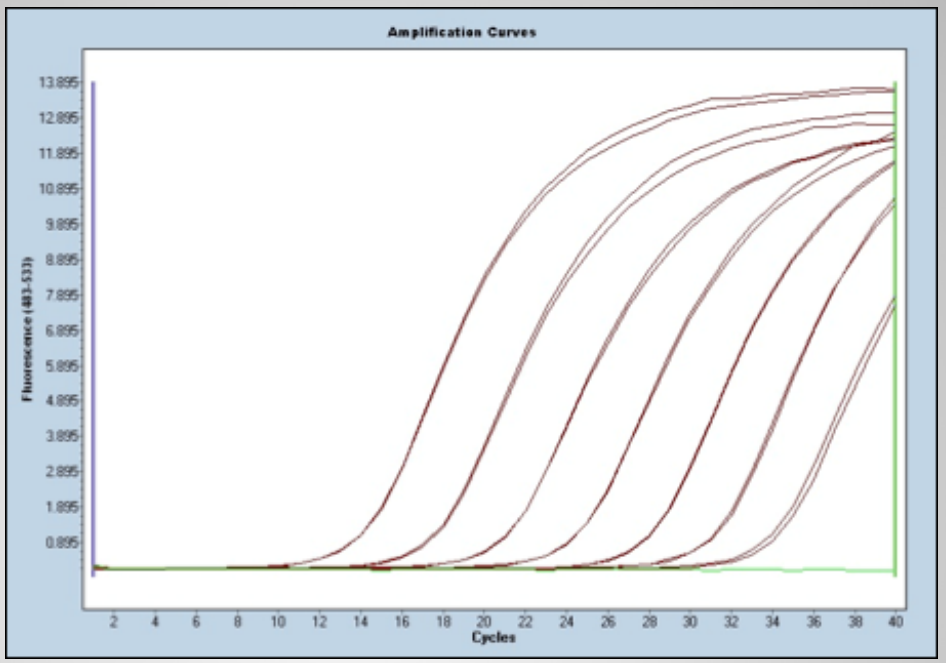
2- Dye-based Detection - based on SYBR Green I



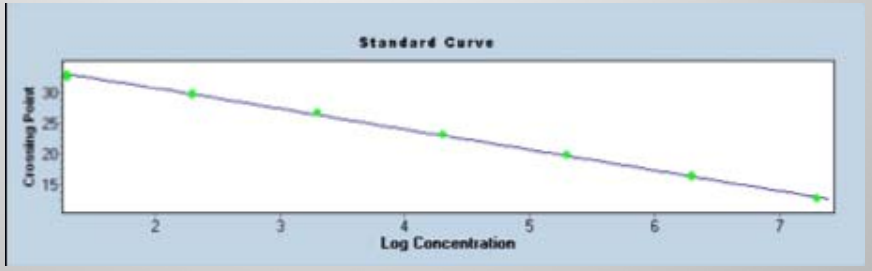
What is Real-Time qPCR?

7-log Std Curve using oligo DNA standard

Name	Cq
Std #1	12.65
Std #1	12.58
Std #2	16.43
Std #2	16.20
Std #3	19.68
Std #3	19.74
Std #4	23.15
Std #4	23.20
Std #5	26.68
Std #5	26.76
Std #6	29.69



Human PTEN Assay



Error: 0.0803
Efficiency: 1.978
Slope: -3.375
YIntercept: 37.53

Roche LC480 Instrument

PCR Target Information



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

m-fold server: http://mfold.bioinfo.rpi.edu/

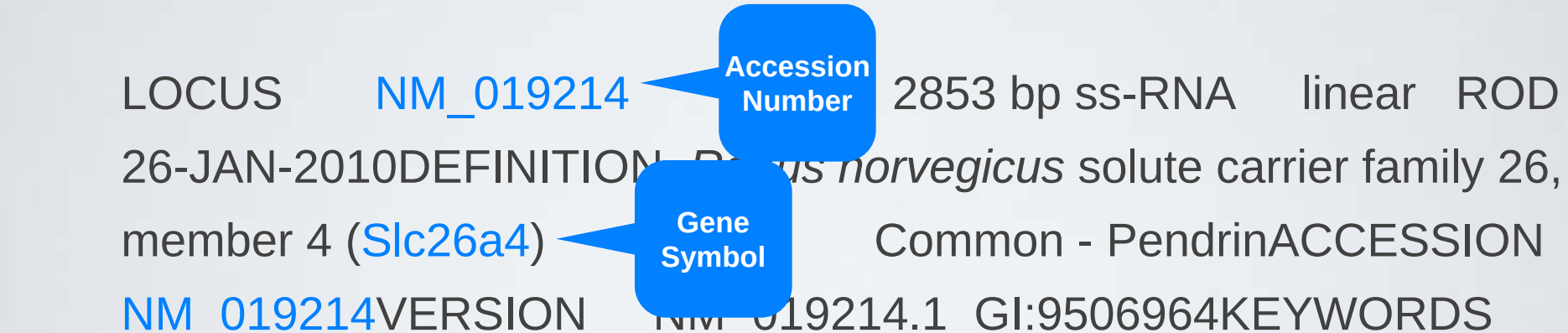
PCR Target Information

Useful Websites:

http://www.ncbi.nlm.nih.gov/

http://genome.ucsc.edu/

http://www.embl.de/services/bioinformatics/index.php

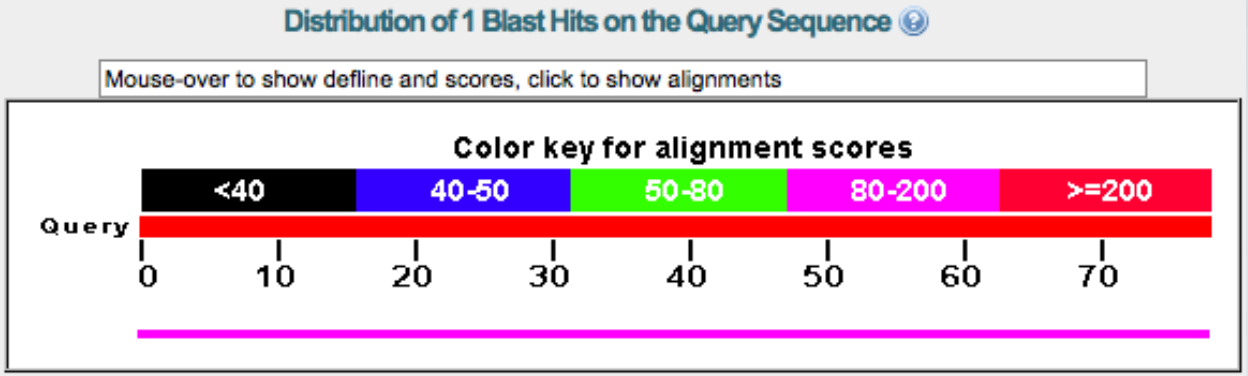


LOCUS NM_019214 2853 bp ss-RNA linear ROD 26-JAN-2010DEFINITION *Rattus norvegicus* solute carrier family 26, member 4 (Slc26a4) Common - PendrinACCESSION NM_019214VERSION NM_019214.1 GI:9506964KEYWORDS .SOURCE *Rattus norvegicus* (Norway rat)

PCR Target Information

BLAST search result for rSlc26a4 (Pendrin) PCR amplicon
Use BLASTN - more sensitive than MegaBLAST

Graphic Summary



Descriptions

Sequences producing significant alignments:	Score (Bits)	E Value
ref|NM_019214.1| Rattus norvegicus solute carrier family 26, ...	141	7e-34

PCR Target Information

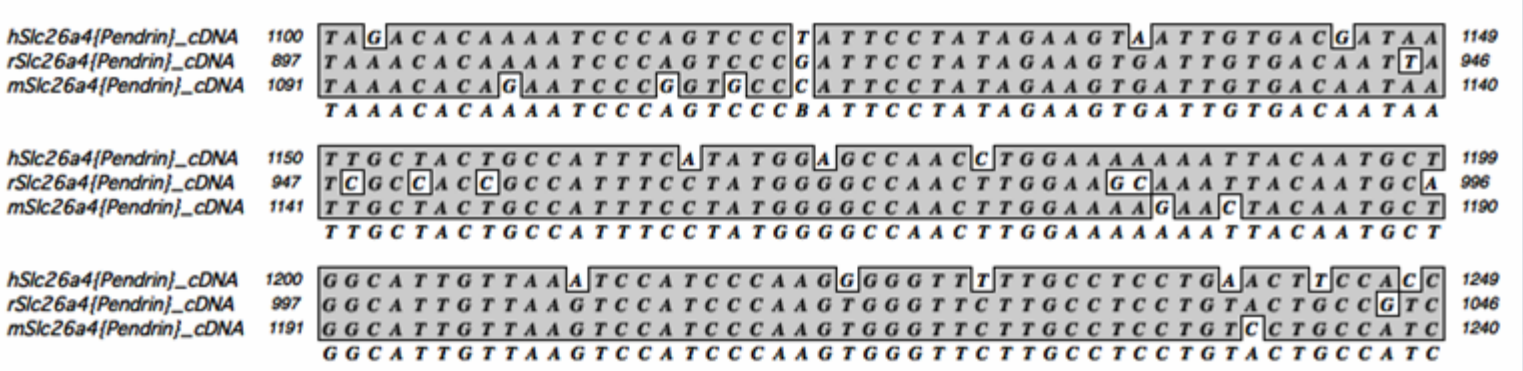
rSLC26a4

911(+) CAGTCCCGATTCCTATAG = 1114 in the human sequence

988(-) AATTTGCTTCCAAGTTGG = 1191 in the human sequence

940(+) FAM-ACAATTATCGCCACCGCCA-BHQ1

78 base PCR amplicon length; crosses the exon 7/8 boundary



exon	990	1142	/gene=SLC26A4 /gene_synonym=DFNB4 /inference=alignment:Splign /number=7 /note=EVA; PDS; TDH2B
exon	1143	1225	/gene=SLC26A4 /gene_synonym=DFNB4 /inference=alignment:Splign /number=8 /note=EVA; PDS; TDH2B

Can determine splice junctions in the rat using the human sequence information plus an alignment

PCR Target Information

Final Table Information for Publication

rat Slc26A4 assay (syn: Pendrin, PDS, DFNB4)
NM_019214
911(+) CAGTCCCGATTCCTATAG
988(-) AATTTGCTTCCAAGTTGG
940(+) FAM-ACAATTATCGCCACCGCCA-BHQ1
78 base PCR amplicon length; crosses the exon 7/8 boundary
PCR efficiency = 97%; LOD = 23 copies (or Cq value)
No known splice variants; single target by BLAST search
No folding issues following m-fold analysis w/in the PCR amplicon

qPCR Assay Validation





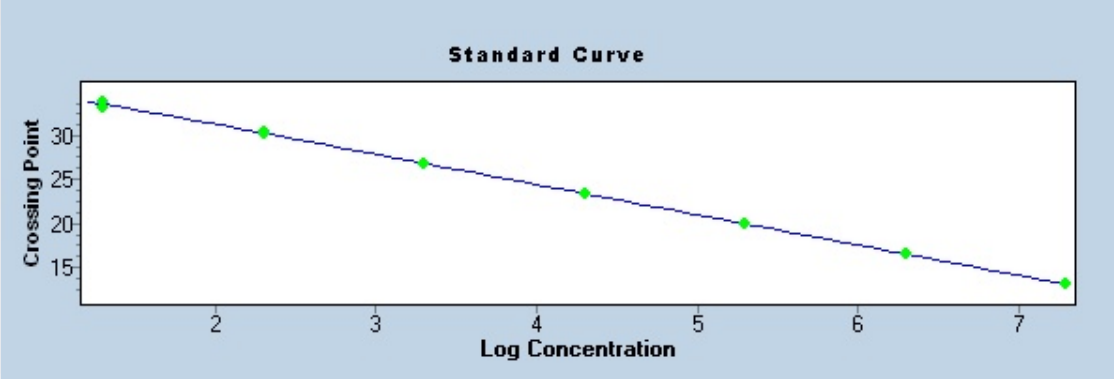


qPCR validation	
Evidence of optimization (from gradients)	D
Specificity (gel, sequence, melt, or digest)	E
For SYBR Green I, C_q of the NTC **(NTC = no-template control)**	E
Calibration curves with slope and *y* intercept	E
PCR efficiency calculated from slope	E
CIs for PCR efficiency or SE **(CI = confidence interval)**	D
r^2 of calibration curve	E
Linear dynamic range **(LOD = limit of detection)**	E
C_q variation at LOD	E
CIs throughout range	D
Evidence for LOD	E
If multiplex, efficiency and LOD of each assay	E

qPCR Validation

Assay Validation- Hydrolysis Probes:

1. Probe-based assays (all) depend upon 3 oligonucleotides working in concert to get a fluorescent signal
2. Greatly increases template specificity
3. A standard curve over a wide range of template concentrations will give assay specifications



hIAPP (islet amyloid polypeptide) assay using ssDNA oligo template over 7-logs
Cq values from 13 to 33, copy numbers from $1x10^7$ to $1x10^1$ copies (calculated) = LOD

Assay stats:
Slope = -3.435; $r^2 = 0.999$; y-intercept = 38.06 cycles; PCR efficiency $(10^{-1/slope})-1)*100 = 95.5\%$
Variation at the LOD is not significant
Minimum assay QC requirements (QGCL)- PCR efficiency ≥93%; LOD <30 copies (PCR)

qPCR Validation

Assay Validation- SYBR Green or other primer-based assay:

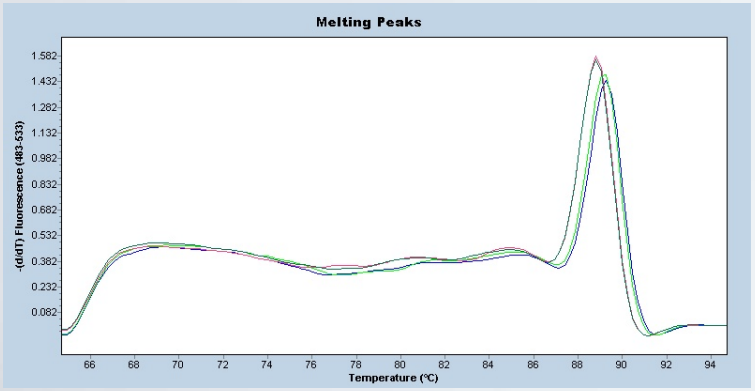
1. Must show template specificity:

asymmetric restriction digest on DNA acrylamide gel or sequencing of PCR product

2. Discriminating among sequences from large sequence-related families can be difficult

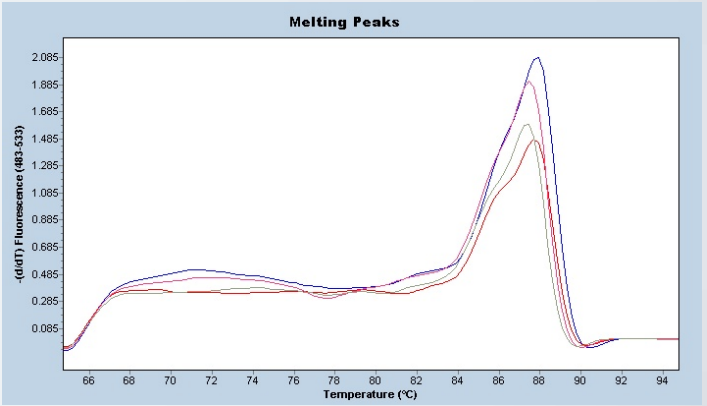
3. A melt curve alone is not sufficient to show template specificity but is useful information

4. A standard curve over a wide range of template concentrations will give all remaining information



rCDC42ep1

Melt Analyses



rTnk2

qPCR Validation

SYBR Assay Validation made painless

1- Generate RT-PCR or PCR product (1st run), monitor with real-time qPCR

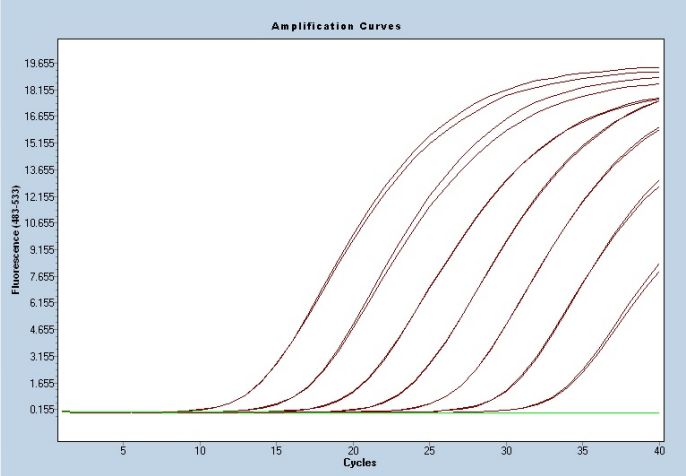
2- Depending on the Cq value, dilute PCR product 1/100 up to 1/10,000 in *E. coli* tRNA at10-100 ng/µl

3- Make a 7-8 log standard curve in 10-fold decrements in carrier *E. coli* tRNA

4- Run dilutions in duplicate PCRs

5- Collect data as below for assay stats and publication

6- Record lowest Cq value for each assay that is still linear with the lower dilutions



Macaca Collagen 1A1: 7-log dilution series

macCol1A1 SYBR Assay stats:

Slope = -3.312

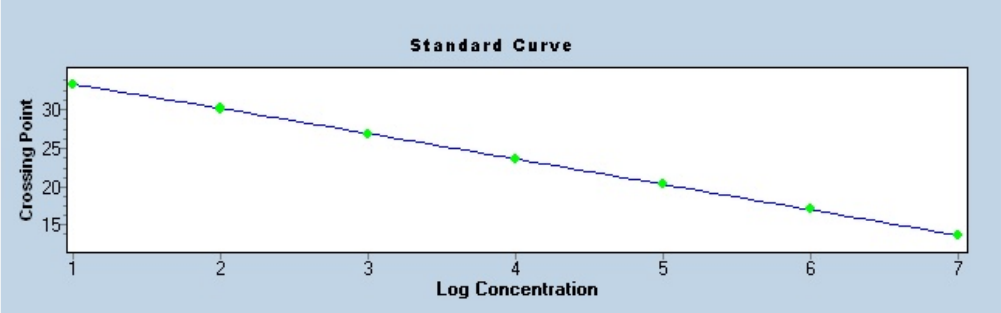
r^2 = 0.999

y-int = 36.70 cycles

PCR efficiency = 100%

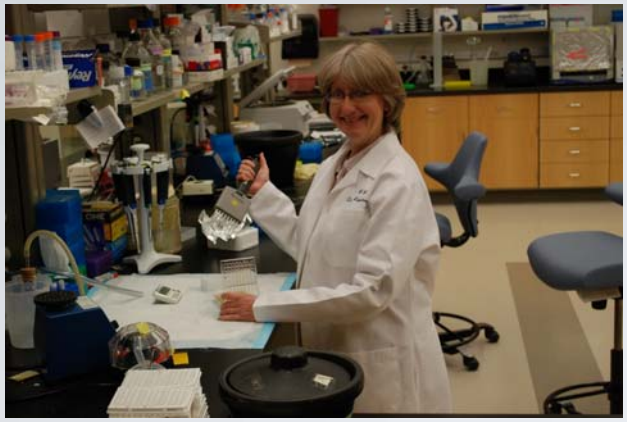
LOD = 33 cycles

Cq variation at LOD - not significant



Macaca Collagen 1A1

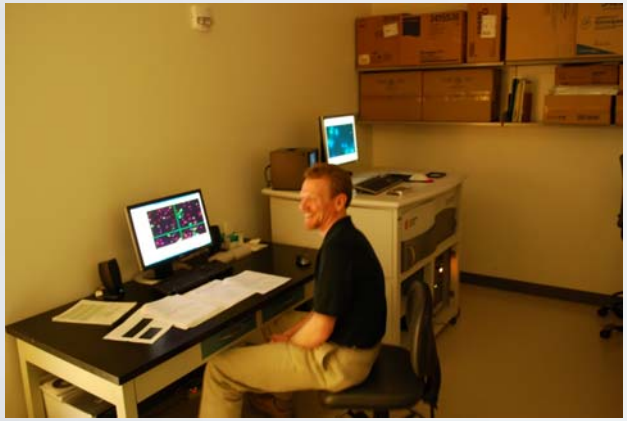
The Important Folks



Ms. Mary Sobieski



Ms. Xiaoying Wang



Dr. Cliff Stephan



Ms. Nancy Shipley

Science Webinar Series

The Future of qPCR

Best practices, Standardization, and the MIQE Guidelines

30 September, 2010

Brought to you by the *Science*/AAAS Business Office

Participating Experts:

Dr. Stephen A. Bustin
Queen Mary, University of London
London, UK

Dr. Gregory L. Shipley
University of Texas Health Science Center at Houston
Houston, TX

Manju R. Sethi
ThermoFisher Scientific
Wilmington, DE

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Making the MIQE Guidelines work for you: practical applications

Manju Sethi, B.Tech, M.S. (ChemE)
Senior Product Manager, NanoDrop Products
September 30, 2010

MIQE Guidelines Checklist

Nucleic Acid QC

qPCR assays

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

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

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MIQE Guidelines Checklist – QC of Nucleic Acids

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

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

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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Why these QC parameters?

- Why is quantity so important?
  - For absolute quantification, samples must lie within the standard curve
  - For relative quantification, large differences in template quantity increase the potential error in calculated expression ratio, low quantities increase error
  - In methods using relative quantification for genotyping, signal strength in unknown samples should be similar to that for standards
- Why is purity so important?
  - Residual chemical contamination from extraction procedures can drastically influence downstream analysis
- Why is integrity so important?
  - Reliable results depend on establishment of threshold criteria for RNA quality

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What tools are available to do this?

- Quantification
  - UV-Vis spectrophotometry (A_{260}) is the accepted method for nucleic acid quantification, providing accuracy and reliability

    e.g. Thermo Scientific NanoDrop spectrophotometers
  - Fluorescent RNA-binding dyes

    e.g. RiboGreen

- Purity
  - A_{260}/ A_{280} PLUS Spectral data

    e.g. Thermo Scientific NanoDrop spectrophotometers

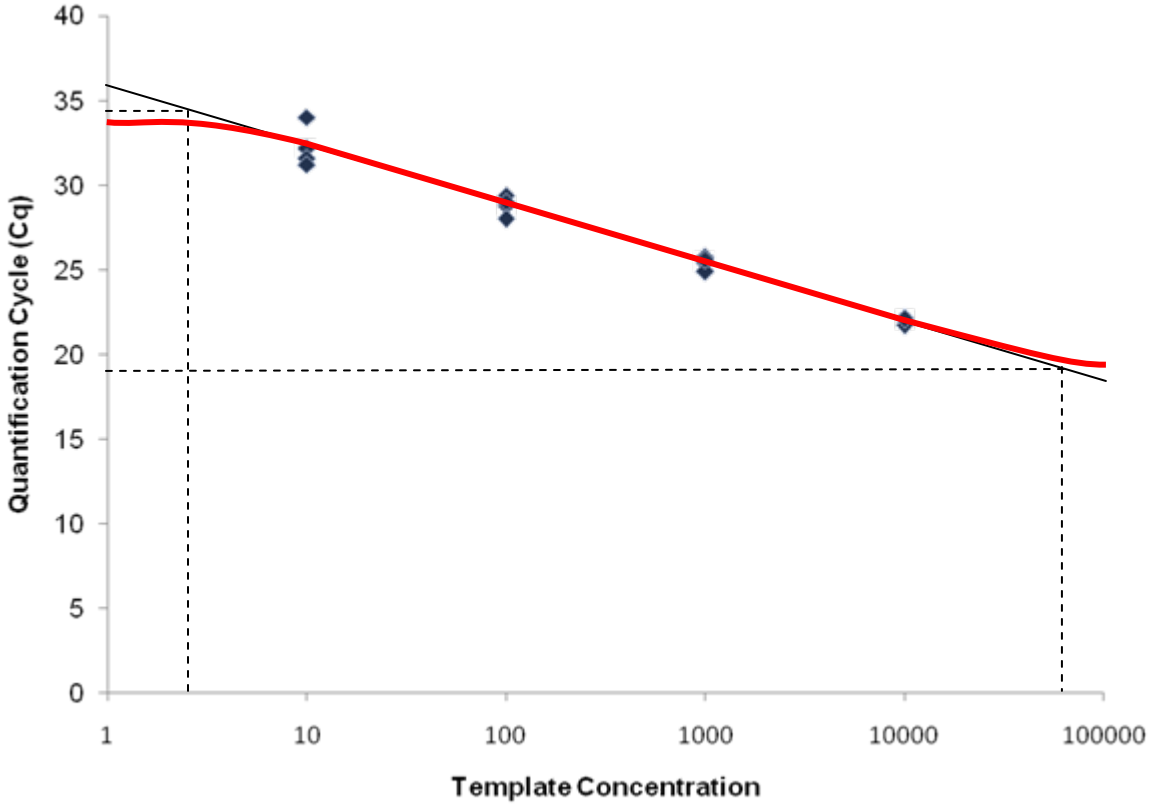
- Integrity
  - Gel analysis, RNA integrity number, RNA quality indicator are not perfect, but the best that's available today

    e.g. standard agarose gel analysis, Agilent 2100 Bioanalyzer (RIN), Bio-Rad Experion (RQI)

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Absolute Quantification

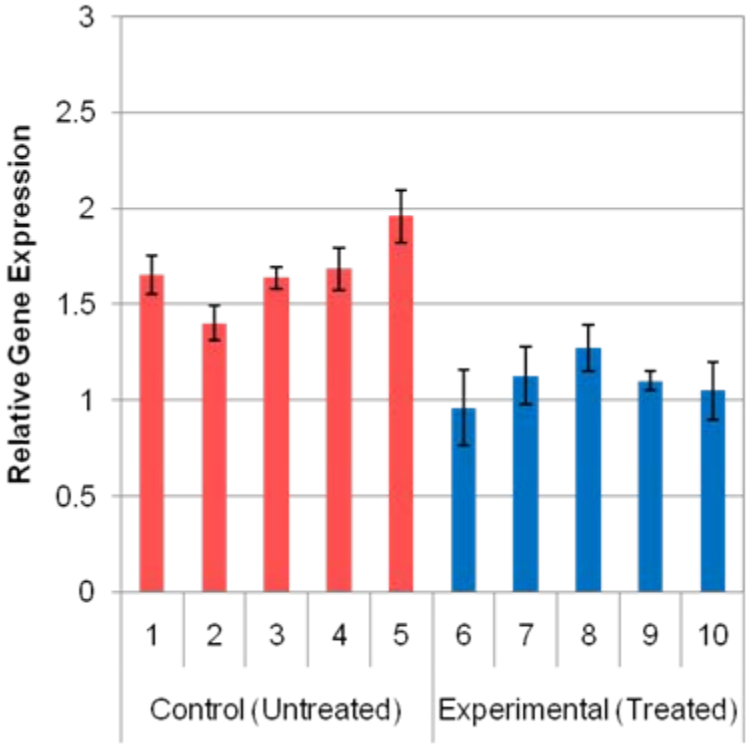
- 4 point std curve
- 2 outlying samples
  - Cq = 34
  - Cq = 19
- Cq = 34
  - Background?
  - Contamination?
  - Variability?
- Cq = 19
  - Reduced efficiency?



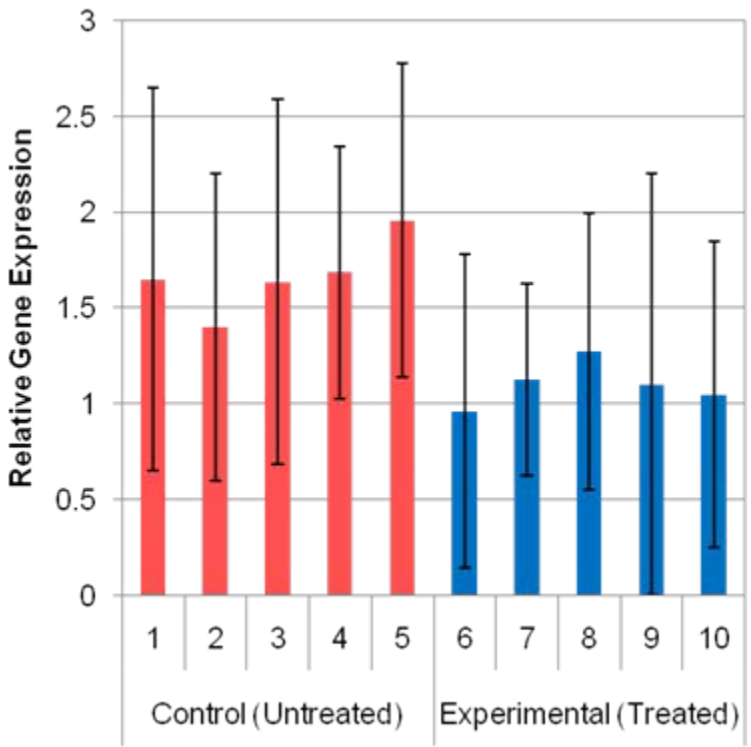
Extrapolate at your own peril; better to ensure before qPCR that your sample concentrations are within a suitable range

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Relative Quantification: Gene Expression



Normal template quantity

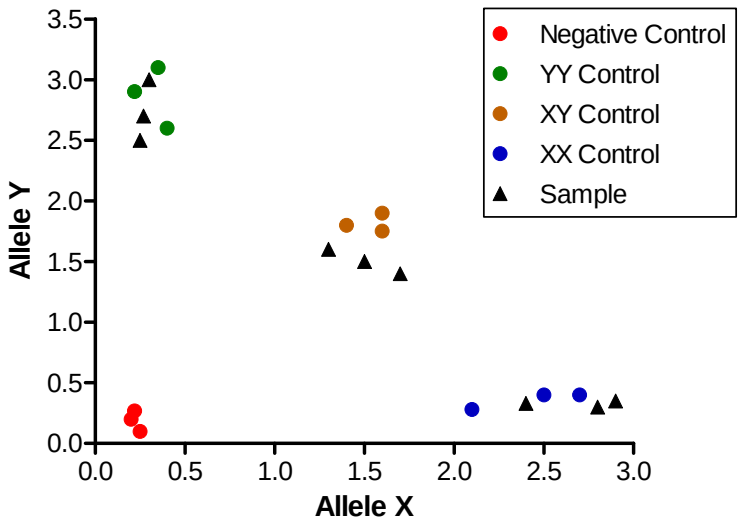


Low template quantity

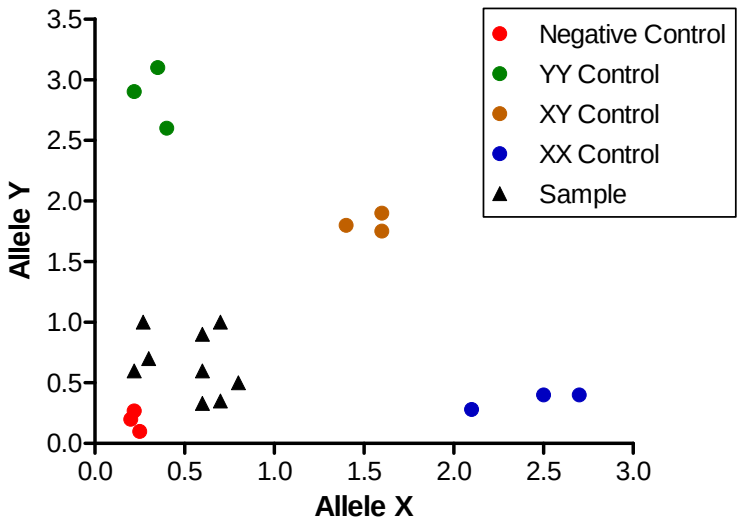
Low template quantities result in data variability

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Relative Quantification: SNP Genotyping



Optimal template quantity



Low template quantity

Low template quantities make allele calls unreliable or impossible

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When exact quantification is critical…

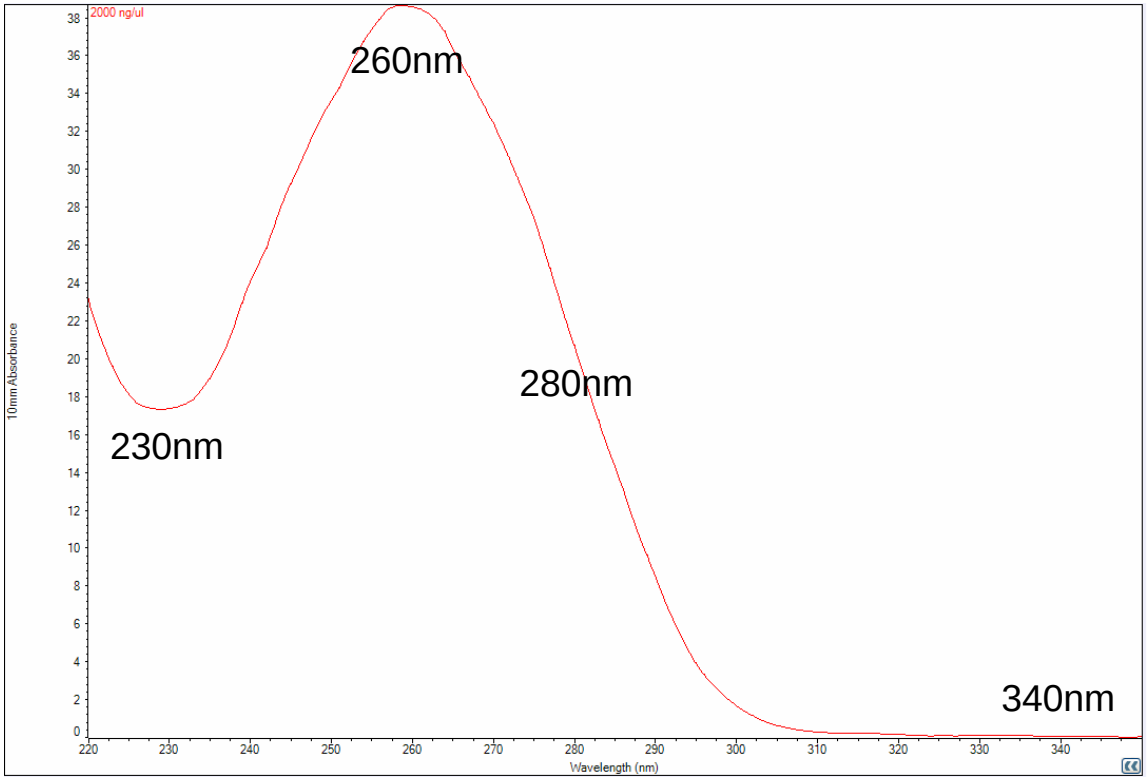
Sometimes equal loading of different templates is an integral part of the experimental design

- Gene expression studies normalized to total RNA input
  - Use of a normalization gene is more popular, but not always possible
- Validation of new normalization genes
  - Extract RNA from multiple samples, equalize template loading, look for equal quantities of candidate gene

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What about the nucleic acid purity?

- Historically, A_{260}/A_{280} has been the primary measure of purity
- Users are now gathering more information by examining the entire absorbance spectra, including A_{260}/A_{230}



Pure DNA

A_{260}/A_{280} ~ 1.8

A_{260}/A_{230} ~ 1.8 – 2.2

Pure RNA

A_{260}/A_{280} ~ 2.0

A_{260}/A_{230} ~ 1.8 – 2.2

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Using spectra to diagnose problems

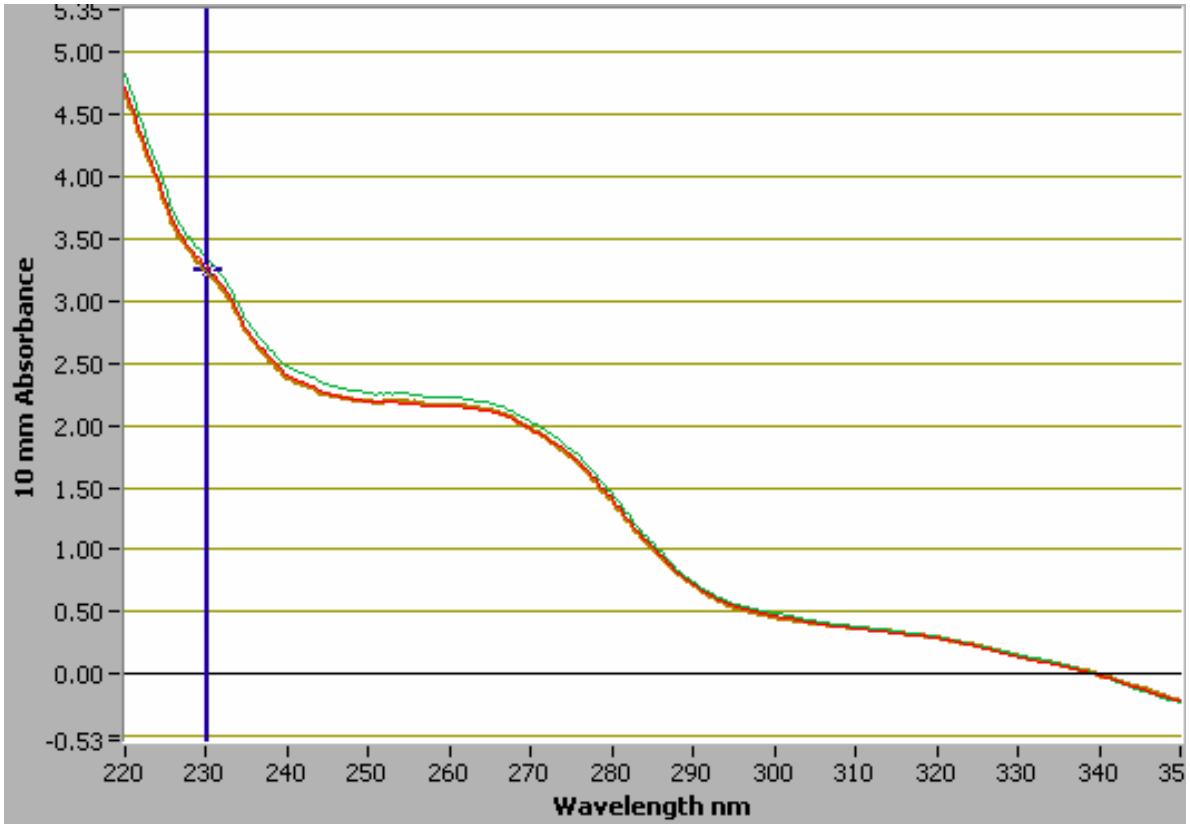
- Contaminants can create problems with downstream applications – so it's important to know when they are present
- They can affect the sample's absorbance spectra – so look for clues in the spectral data
- Consider your starting material *and* purification method when troubleshooting

  For example: if you have a high A_{230} (poor A_{260}/A_{230} ratio):

  - Did you purify using phenol?
  - Did you use a kit containing guanidine?
  - Did you precipitate using glycogen?
  - Could this be carbohydrate (often a problem with plants)?

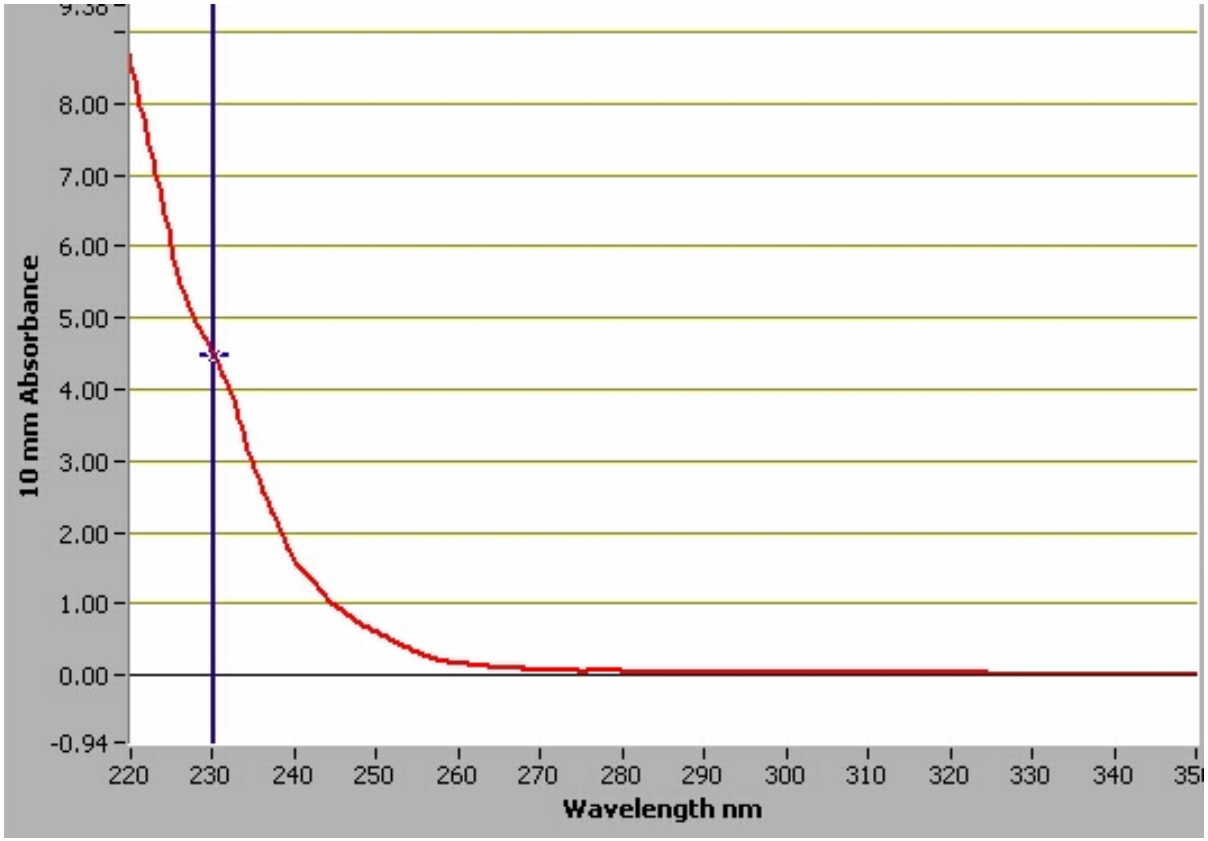
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Glycogen



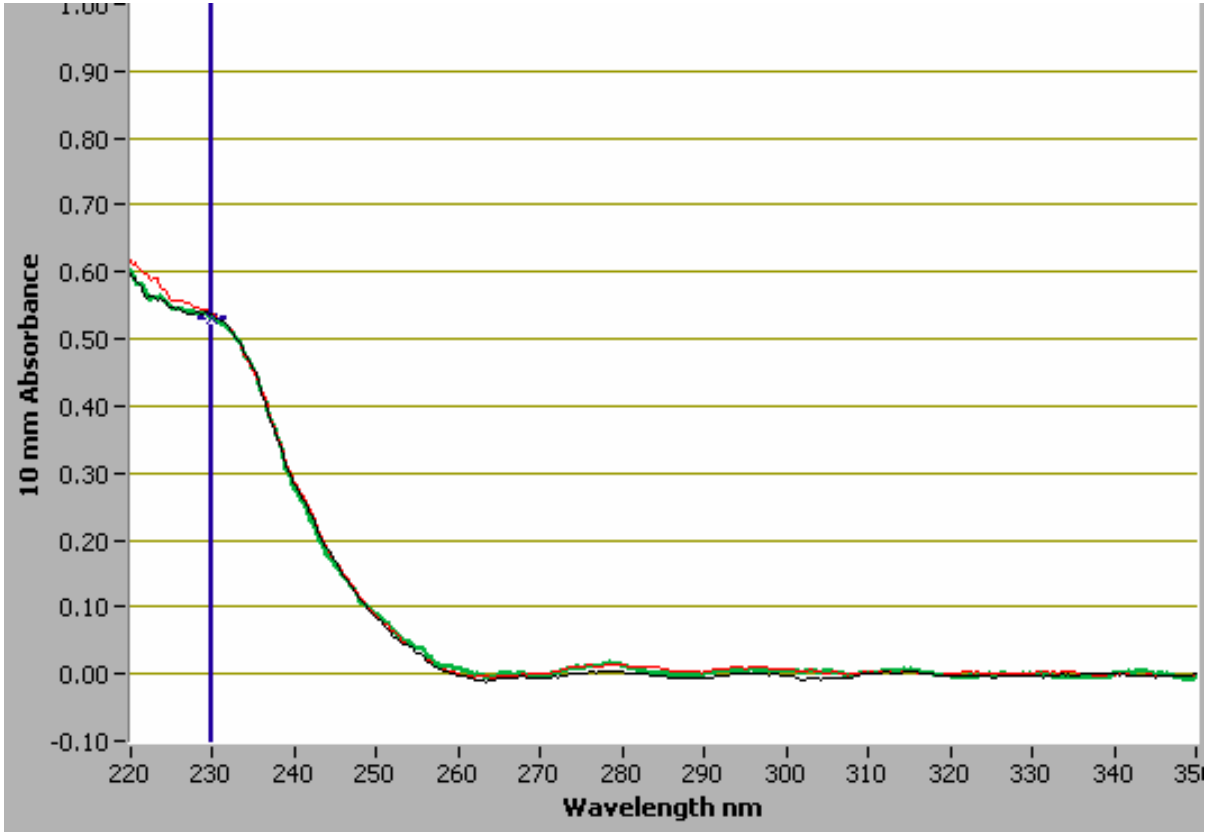
ThermoFisher SCIENTIFIC

EDTA



ThermoFisher SCIENTIFIC

TE



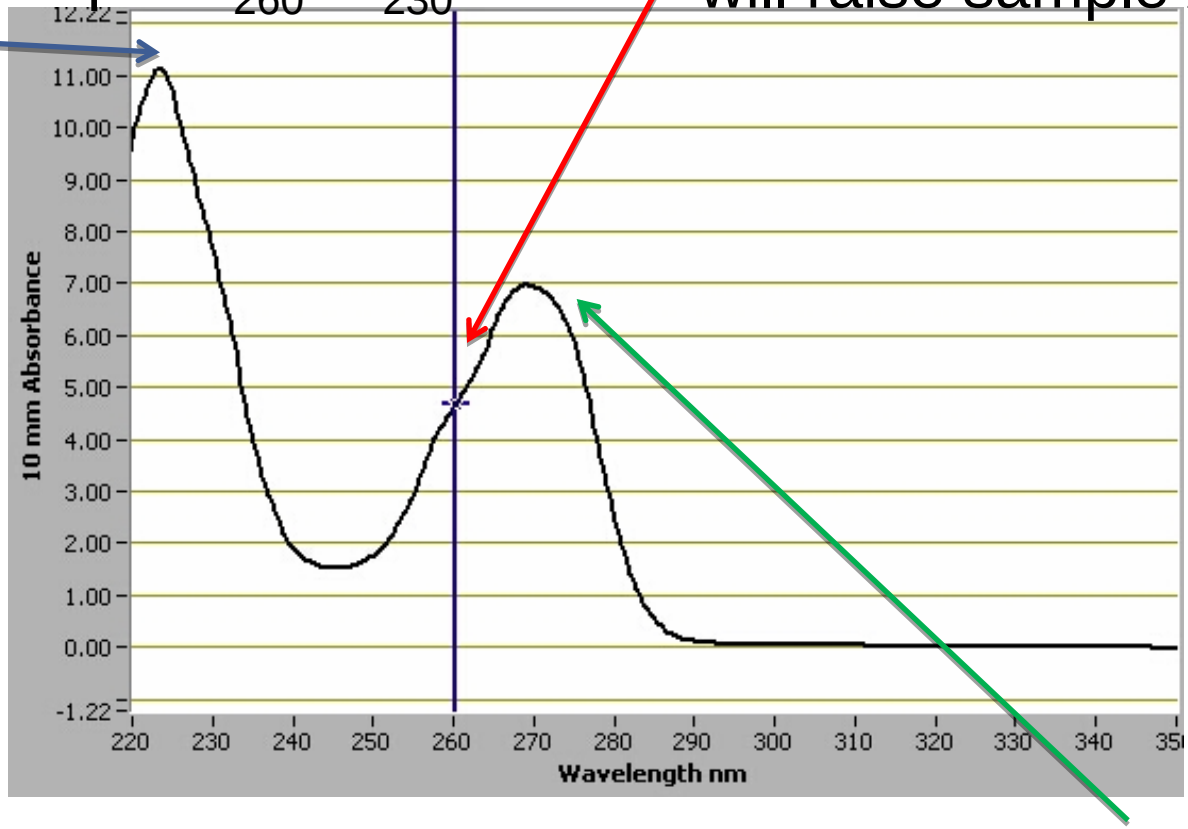
(Using the same buffer for both blank and sample measurements eliminates this problem)

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Phenol

230 nm Absorbance –
will lower sample A_{260}/ A_{230}

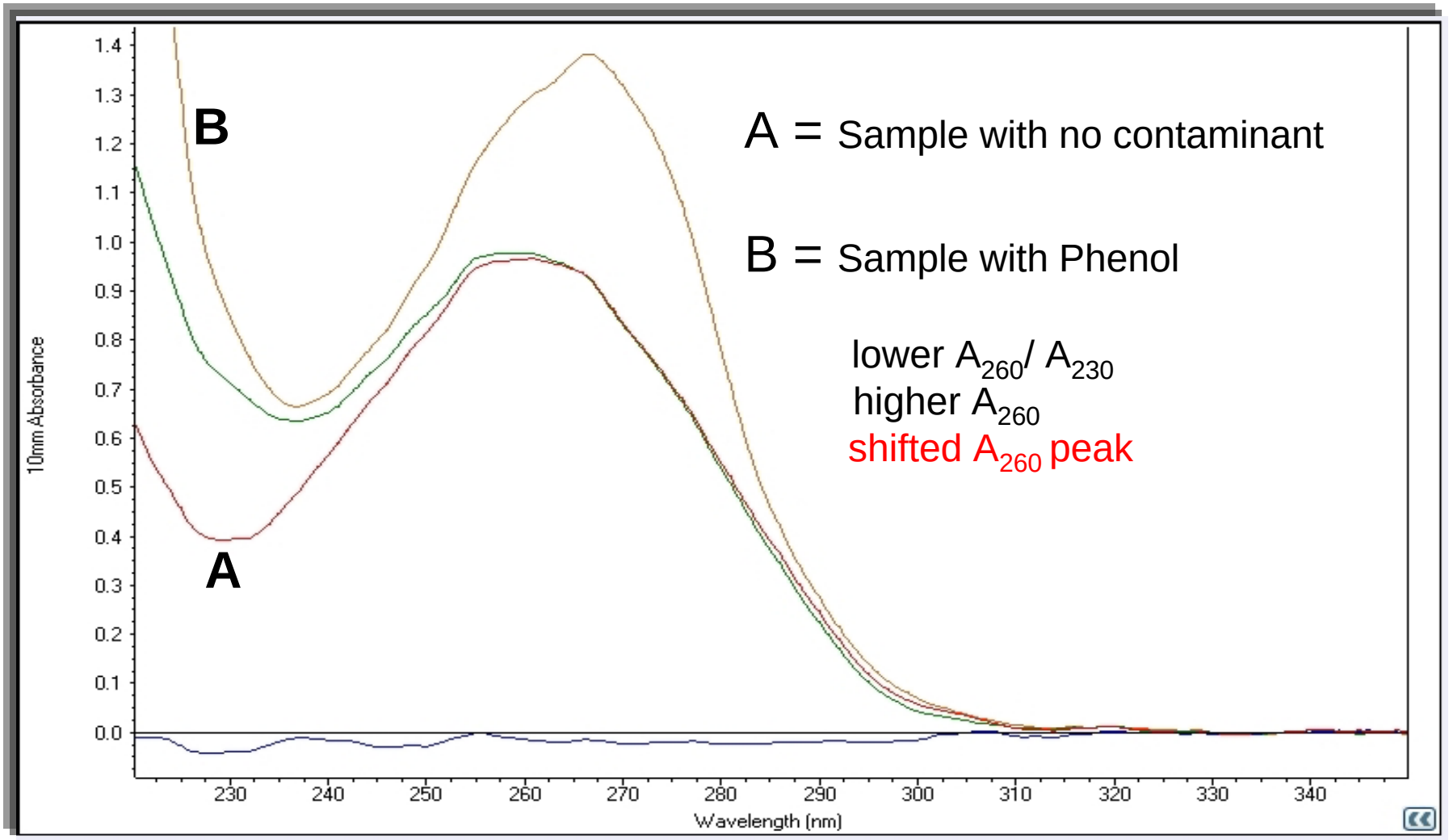
260 nm Absorbance –
will raise sample A_{260}



270+ nm peak –
will shift sample A_{260} peak

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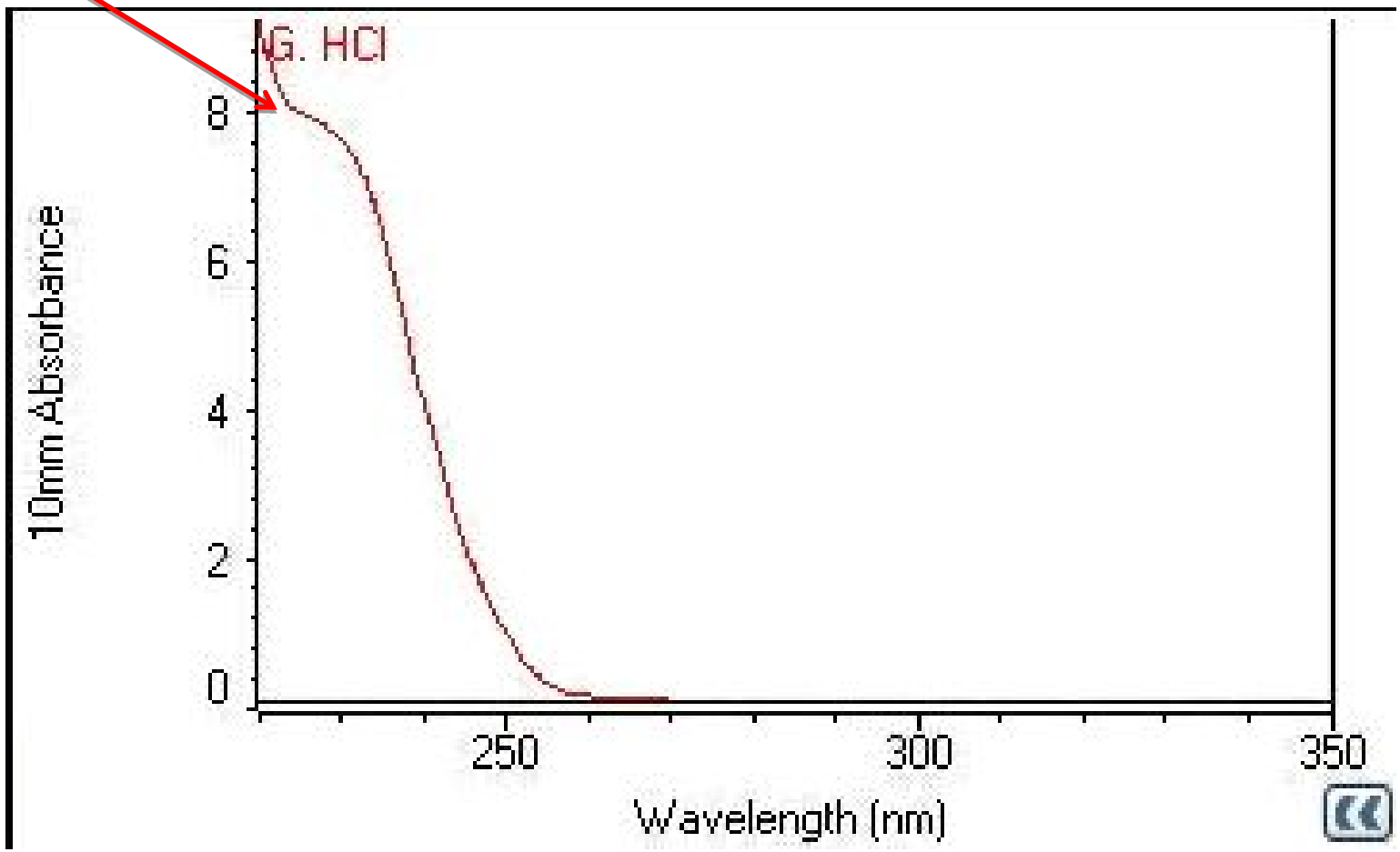
How does Phenol affect my sample?



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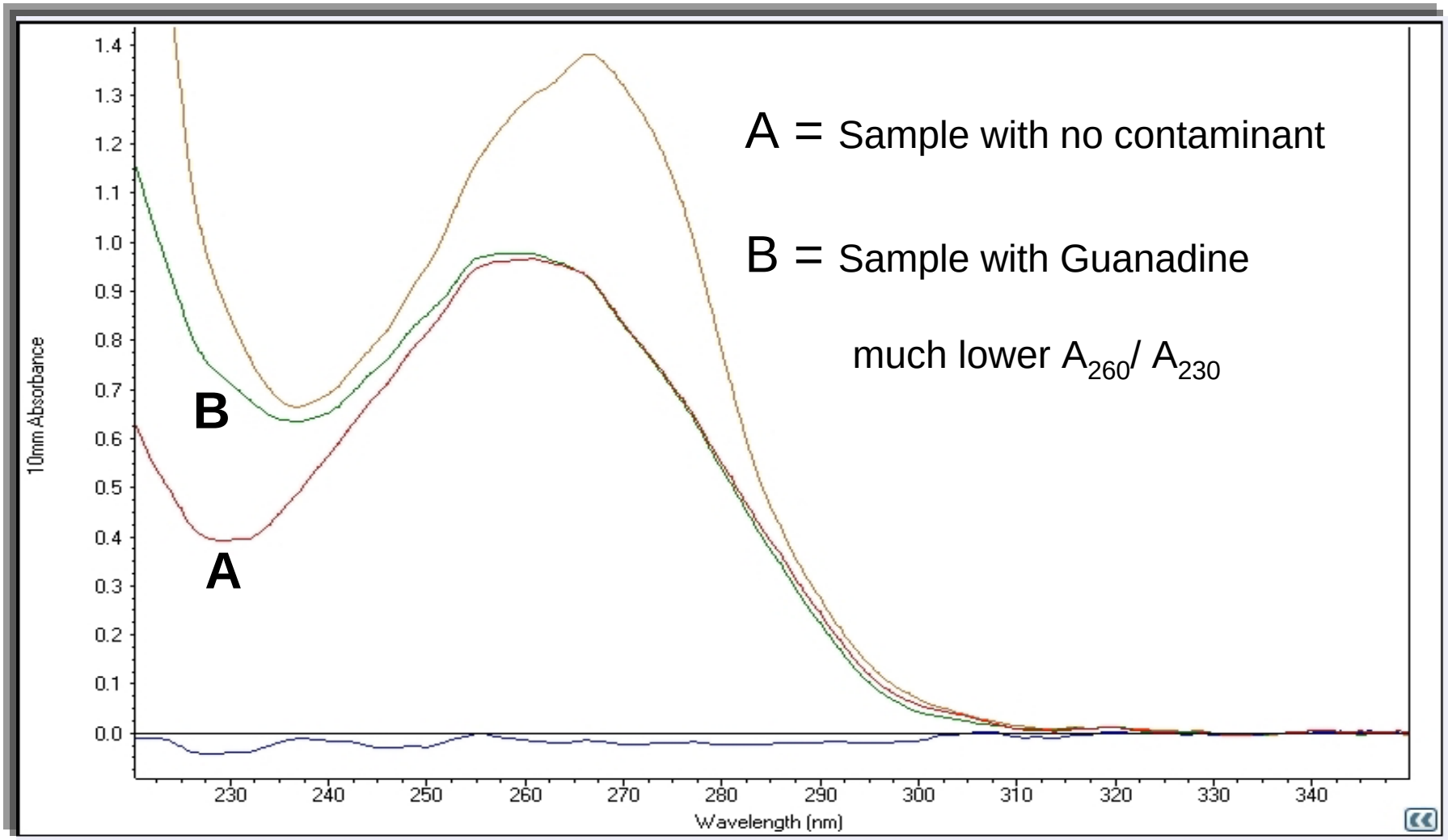
Guanidine HCl

230 nm Absorbance –
will lower sample A_{260}/ A_{230}



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How does Guanadine affect my sample?



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QC of Nucleic Acids summary

Use complementary technologies:

For Quantification
UV-Vis spectrophotometer
(e.g. NanoDrop)

For Purity UV-Vis
spectrophotometer
(e.g. NanoDrop)

For Integrity
Gel Electrophoresis
(e.g. Standard gel analysis,
Bioanalyzer 2100 or Experion)

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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MIQE Guidelines – qPCR assay

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

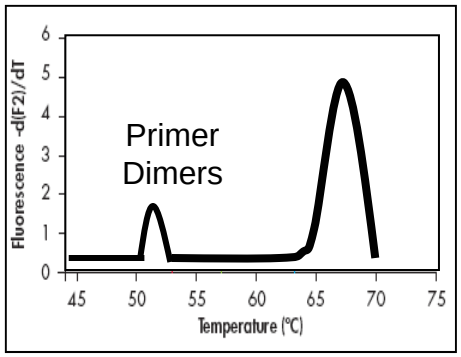
Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences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

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

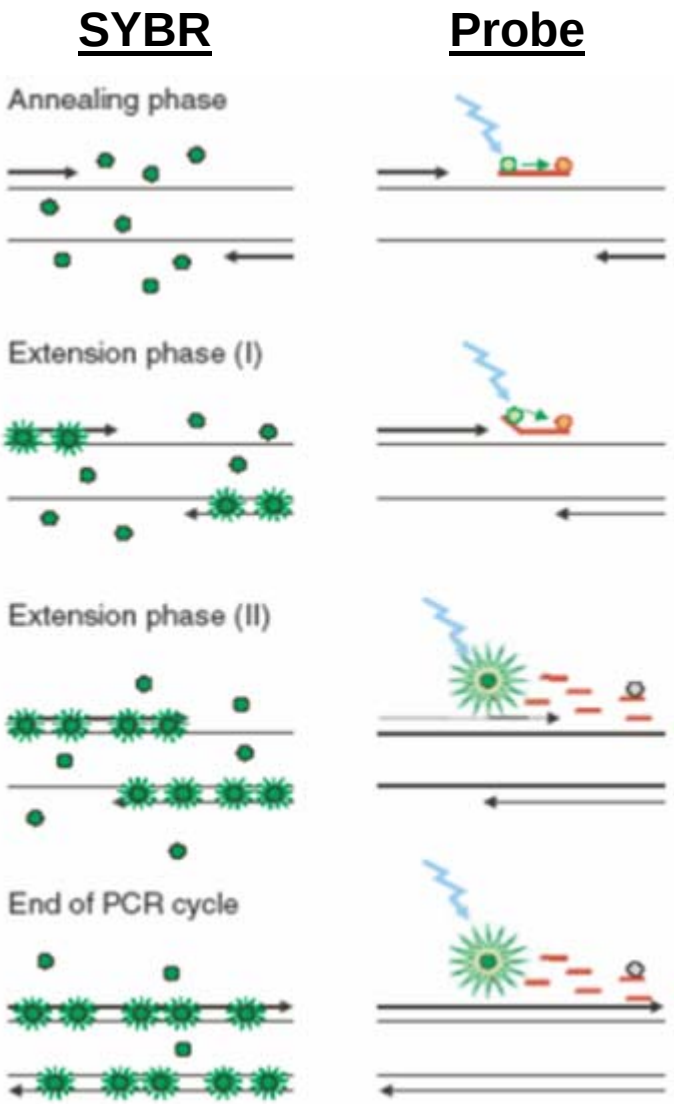
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Probe vs. SYBR qPCR Detection

- SYBR-based detection
  - high throughput- screening approach
  - cannot multiplex
  - binds to **ANY** dsDNA formed
  - Melt curve analysis
  - Validation of primers



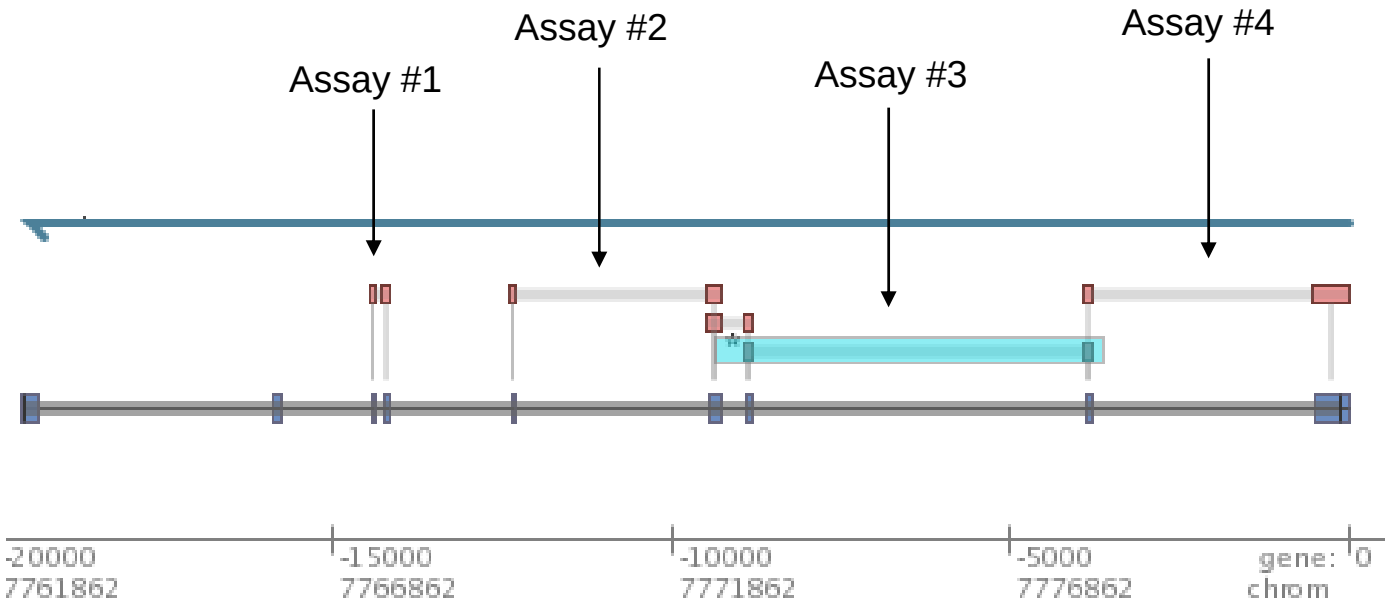
- Probe-based detection
  - more focused approach
  - binds **selectively** to target
  - can perform multiplexing
  - few probes allow melt curve analysis



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Difficulties with Assay Selection

- Encountering multiple assays where it is unclear which assay will perform best?



Which of these assays is the best one to choose?

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How does MIQE guide which assay to select?

Primer sequences	E
Probe sequences	D[1]

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

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Performance criteria

- Efficiency of amplification
  - Is there a doubling of amplicon with every cycle (2^n)?
- Dynamic range of assay
  - How many log dilutions fall within the line?
- Sensitivity / Lower Limit of Detection
  - How many copies of target can be reliably detected?
- Splice variant coverage
  - How many assays would you need to run to get wide splice variant coverage for GOI?
- Specificity
  - No unexpected amplification of gDNA
  - No off-targeting, primer dimer formation
    - Single band on agarose gel

Select assays that will give high quality data - confidence in publishing
e.g. Thermo Scientific Solaris qPCR Gene Expression Assays

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In summary

- Use the MIQE checklist as you design and conduct your experiments

- Selecting the right tools/assays can make a significant impact on the quality of your results, and ensure confidence in published material.

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Science Webinar Series

The Future of qPCR

Best practices, Standardization, and the MIQE Guidelines

30 September, 2010

Brought to you by the *Science*/AAAS Business Office

Participating Experts:

Dr. Stephen A. Bustin
Queen Mary, University of London
London, UK

Dr. Gregory L. Shipley
University of Texas Health Science Center at Houston
Houston, TX

Manju R. Sethi
ThermoFisher Scientific
Wilmington, DE

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JoVE – what is it?

Journal of Visualized Experiments (JoVE)
is a peer reviewed, PubMed indexed journal
devoted to the publication of biological research
in a video format.

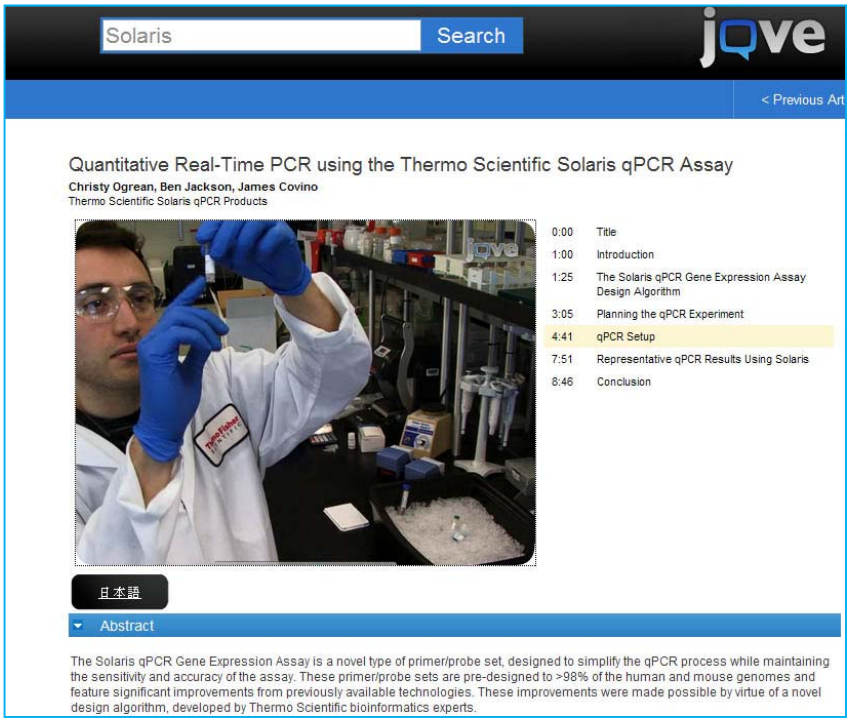
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Thermo Scientific Protocols Available on JoVE

Quantitative Real-Time PCR using the Thermo Scientific Solaris qPCR Assay

- Ogrean, Jackson, and Covino

In this video real time PCR is demonstrated using Solaris qPCR assays – these are gene-specific probe and primer pairs designed to detect all known splice variants of a given gene under universal thermal cycling conditions.

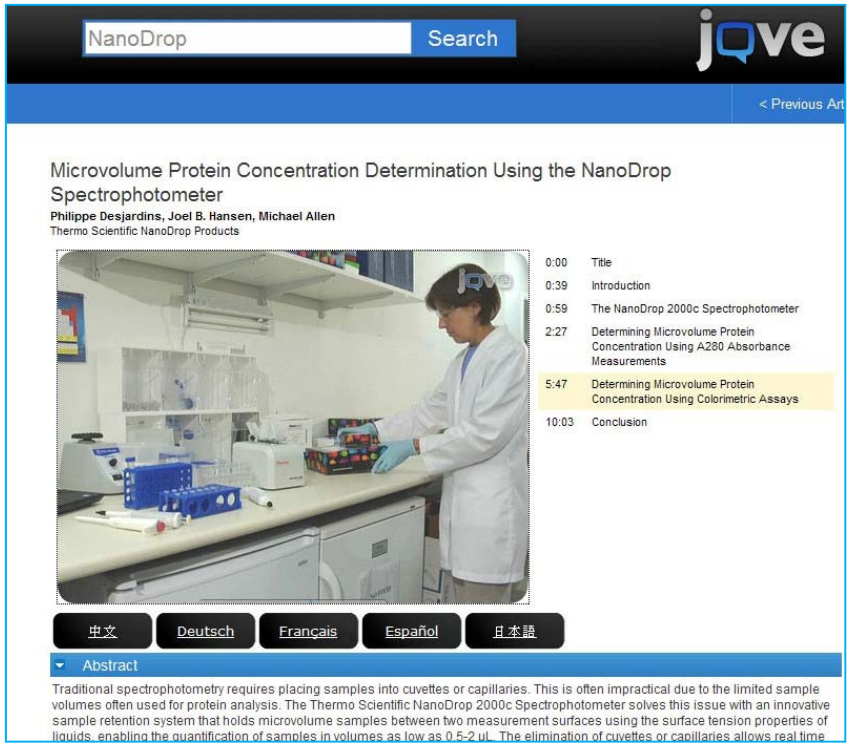


http://www.jove.com/index/details.stp?ID=1700

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Thermo Scientific Protocols Available on Journal of Visual Experiments (www.JoVE.com)

Microvolume Protein Concentration Determination Using the NanoDrop Spectrophotometer
- Desjardins, Hansen, and Allen



This video article demonstrates the use of the NanoDrop 2000c
UV-Vis spectrophotometer, with its patented retention technology for microvolume samples.

In this example, protein quantification is performed using two common methods – A_{280} absorbance and the BCA assay.
A JoVE video for nucleic acid quantification and purity assessment will be released soon.

http://www.jove.com/index/details.stp?ID=1610

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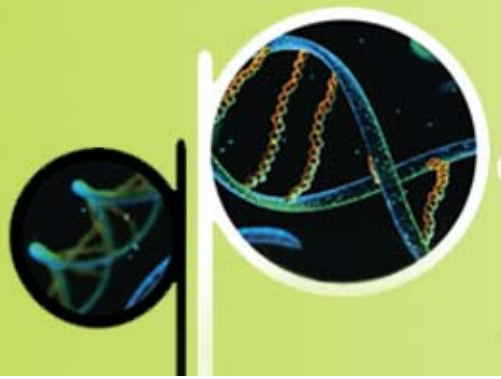
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