How NOT to mess up your qPCR

Promega GmbH

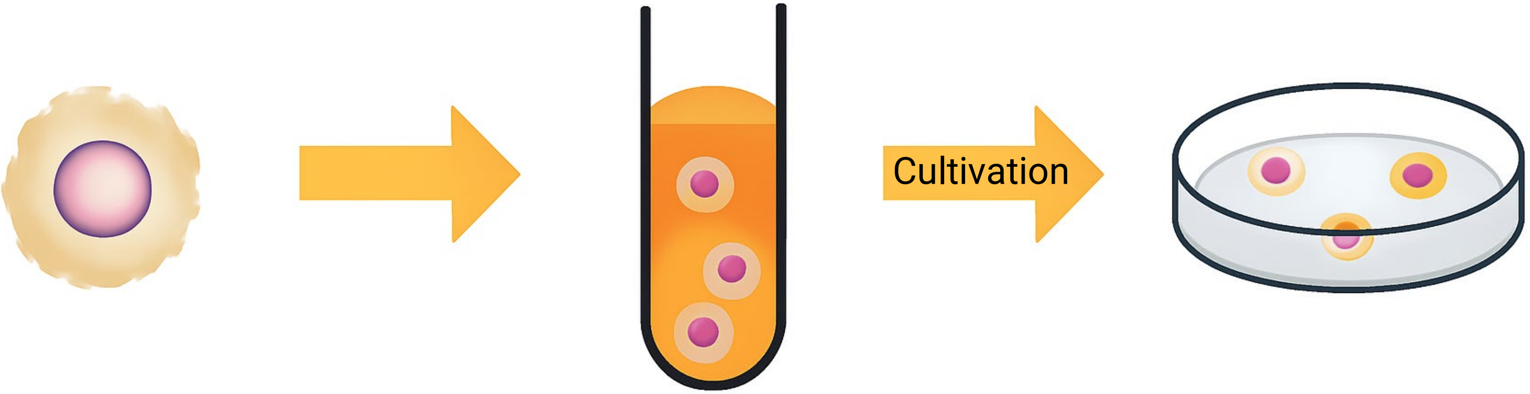
Stimulus-Triggered Acquisition of Pluripotency (STAP)

ARTICLE

doi:10.1038/nature12968

Stimulus-triggered fate conversion of somatic cells into pluripotency

Haruko Obokata[1,2,3], Teruhiko Wakayama[3]†, Yoshiki Sasai[4], Koji Kojima[1], Martin P. Vacanti[1,5], Hitoshi Niwa[6], Masayuki Yamato[7] & Charles A. Vacanti[1]



Cultivation

Lymphocytes

Mild acid bath

Pluripotent cells

„STAP Cell Scandal“

News | Published: 18 March 2014

Stem-cell method faces

The STAP case scandal: Researcher Har ta

resigns after failing to

News | Published: 01 April 2014

Stem-cell scientist found guilty of misconduct

P Cell Scandal: Japan's Biggest Science Fraud

The Final Word on STAP

Researchers fail to replicate STAP study; computational analysis reveals genomic inconsistency

Promega

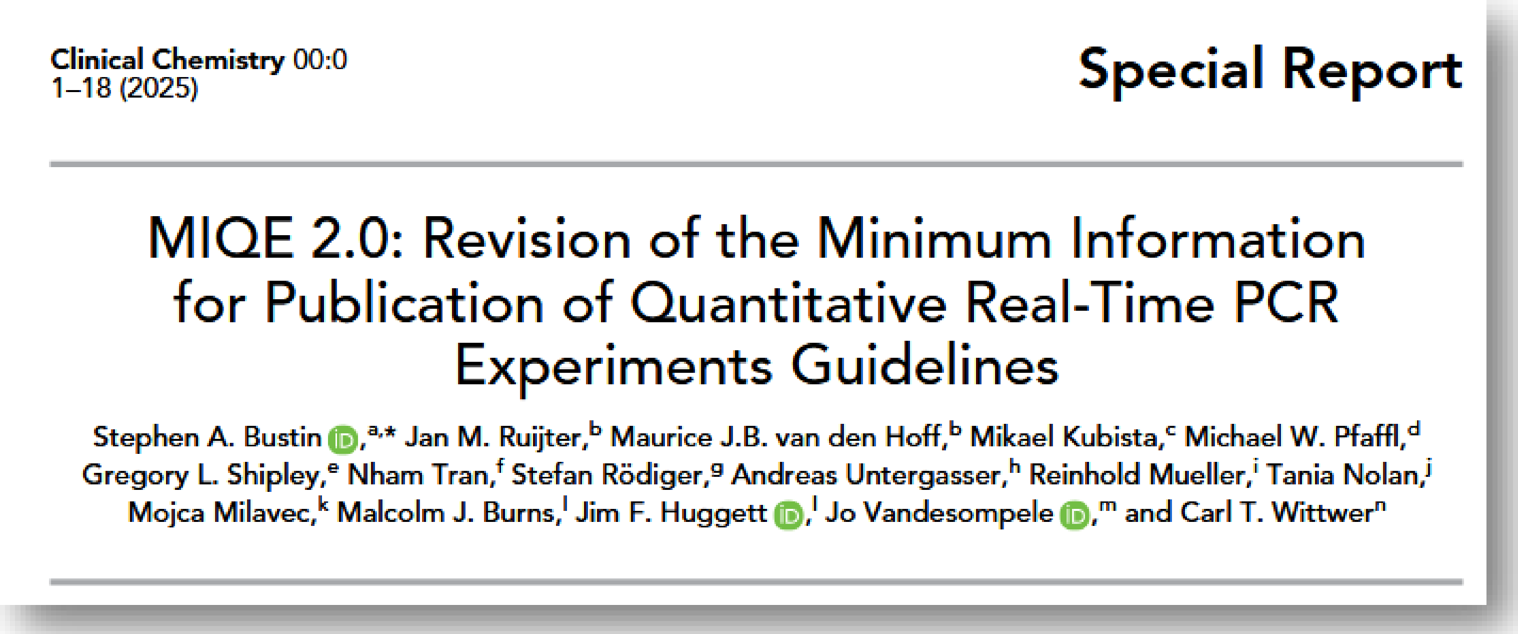
Lessons Learned: Why Standards Are Crucial

MIQE 2.0 Guidelines

Standardized set of recommendations for

- Designing
- Optimizing
- Validating
- Analysing
- Reporting

of **qPCR** results



Clinical Chemistry 00:0
1–18 (2025)

Special Report

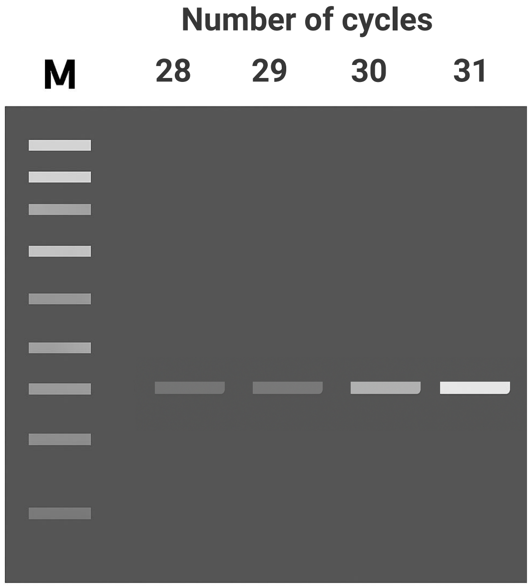
MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines

Stephen A. Bustin,[a,*] Jan M. Ruijter,[b] Maurice J.B. van den Hoff,[b] Mikael Kubista,[c] Michael W. Pfaffl,[d] Gregory L. Shipley,[e] Nham Tran,[f] Stefan Rödiger,[g] Andreas Untergasser,[h] Reinhold Mueller,[i] Tania Nolan,[j] Mojca Milavec,[k] Malcolm J. Burns,[l] Jim F. Huggett,[l] Jo Vandesompele,[m] and Carl T. Wittwer[n]

What is Real-Time PCR?

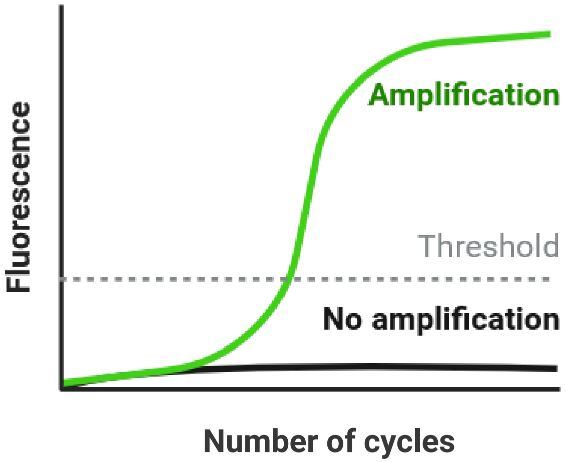
End-point PCR
Product formation measured

after reaction is complete



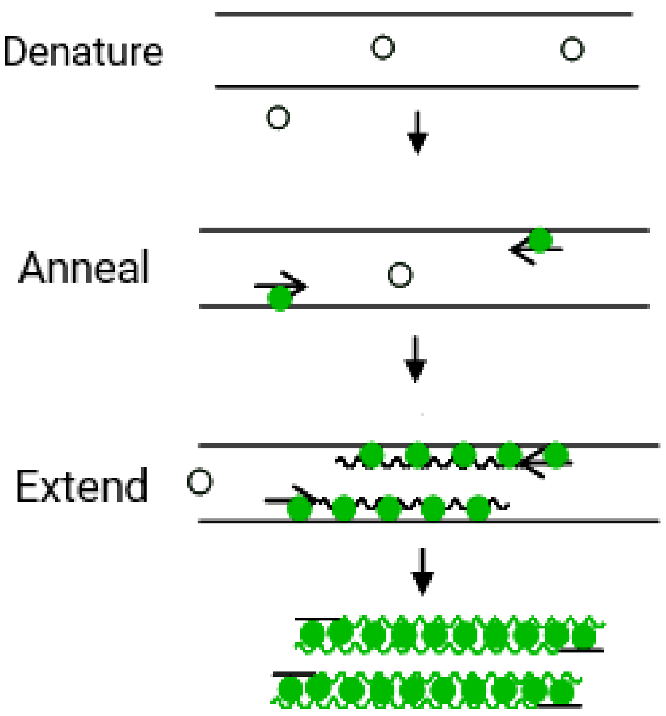
Real-Time PCR
Product formation measured

at each cycle, during the reaction

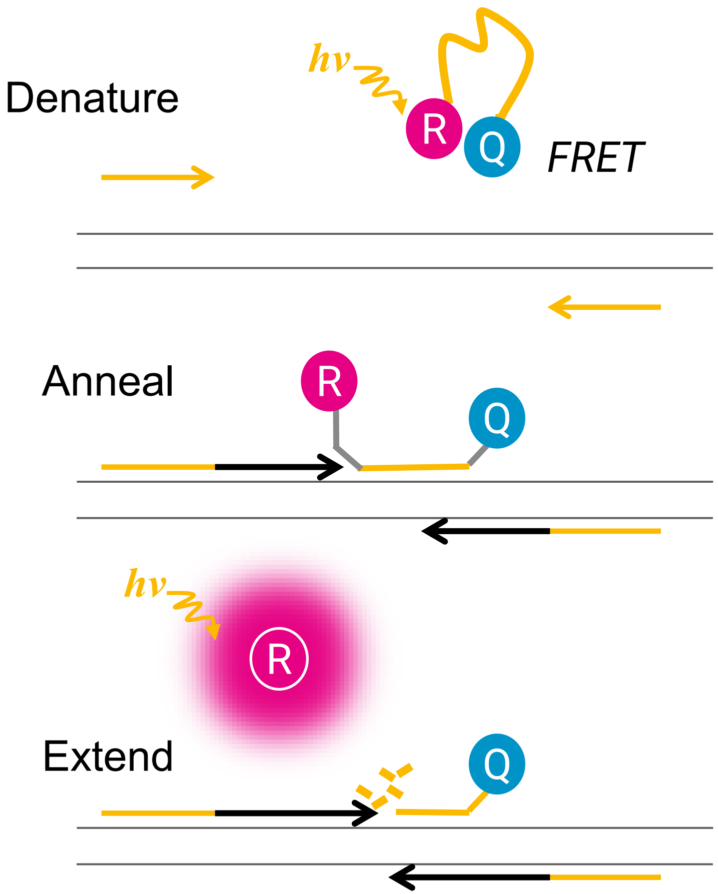


Real-time PCR Chemistries: Dye-based vs. Probe-based qPCR

Dye-based

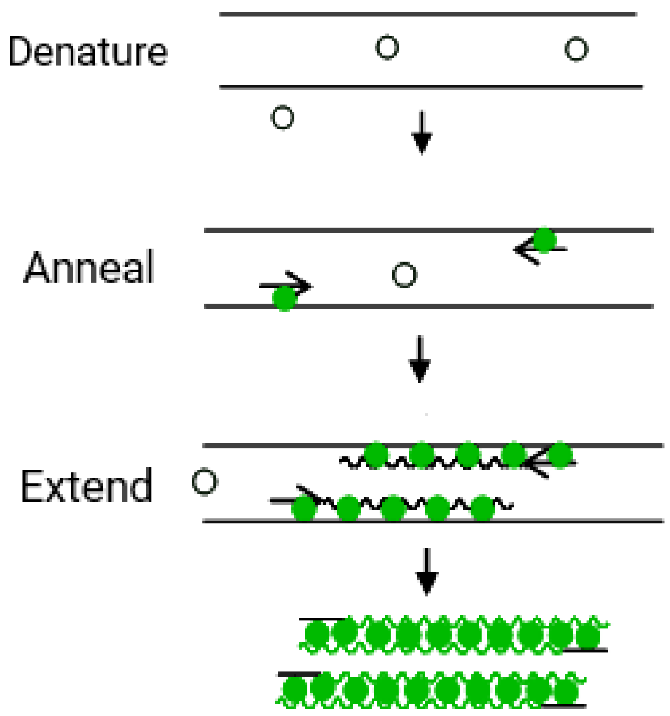


Probe-based



Real-time PCR Chemistries: Dye-based

Dye-based



As more PCR product is produced, more dye is bound

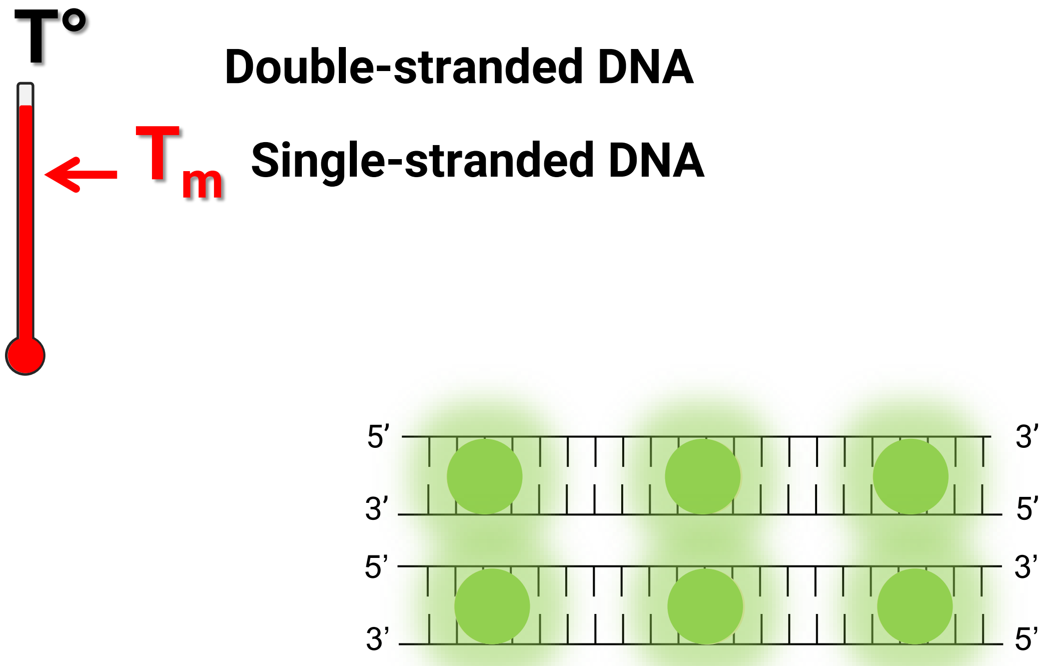
- **dsDNA-binding dye is included in PCR master mix**
- Standard primers used
- Dye associates with PCR product

○ **Free Dye → low fluorescence**
● **Bound Dye → high fluorescence**

Fluorescence is proportional to the amount of PCR product

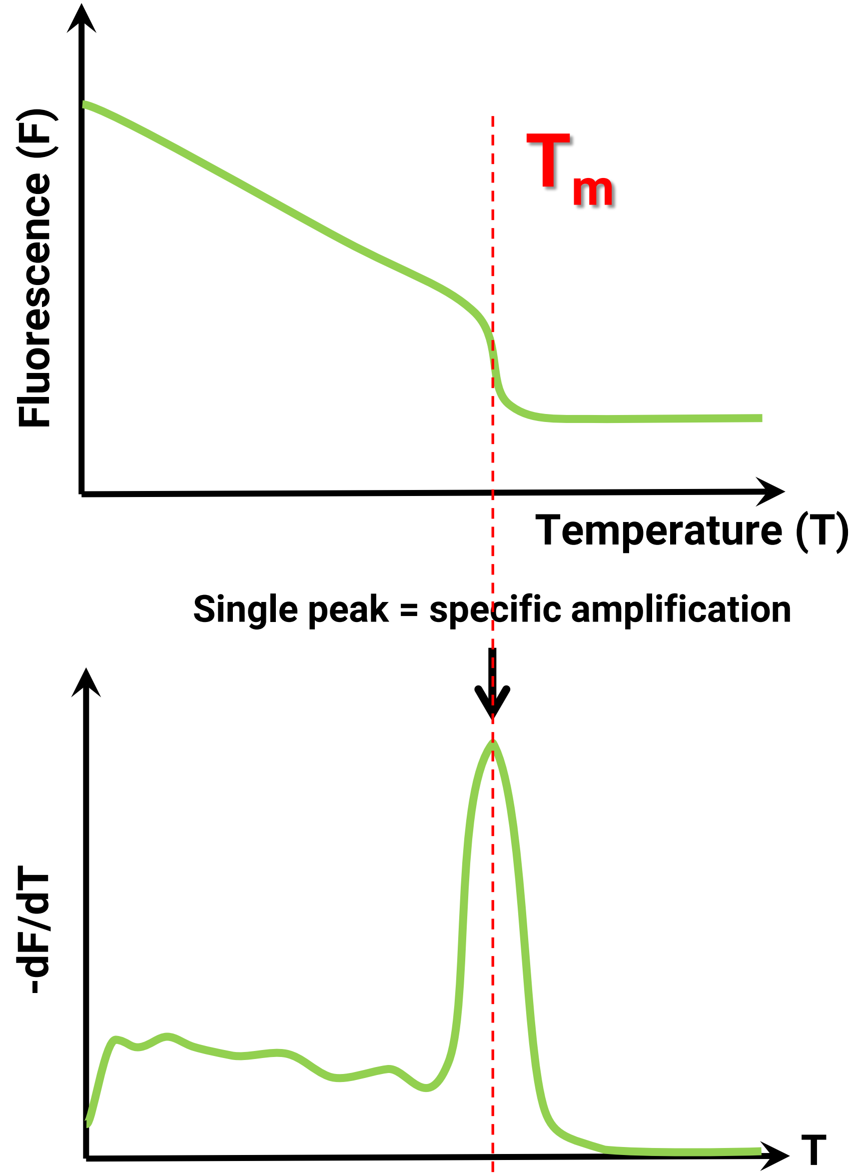
Melting Curve

- Produced in a second, linked thermal profile performed after amplification
- Product is heated slowly, signal is continually measured



Double-stranded DNA

Single-stranded DNA



Single peak = specific amplification

Provides qualitative information about PCR products – primarily, number & size

Real-time PCR Chemistries: Probe-based qPCR

TaqMan® is the most familiar type:

- 2 PCR primers + 1 probe
- Probe labeled with reporter & quencher

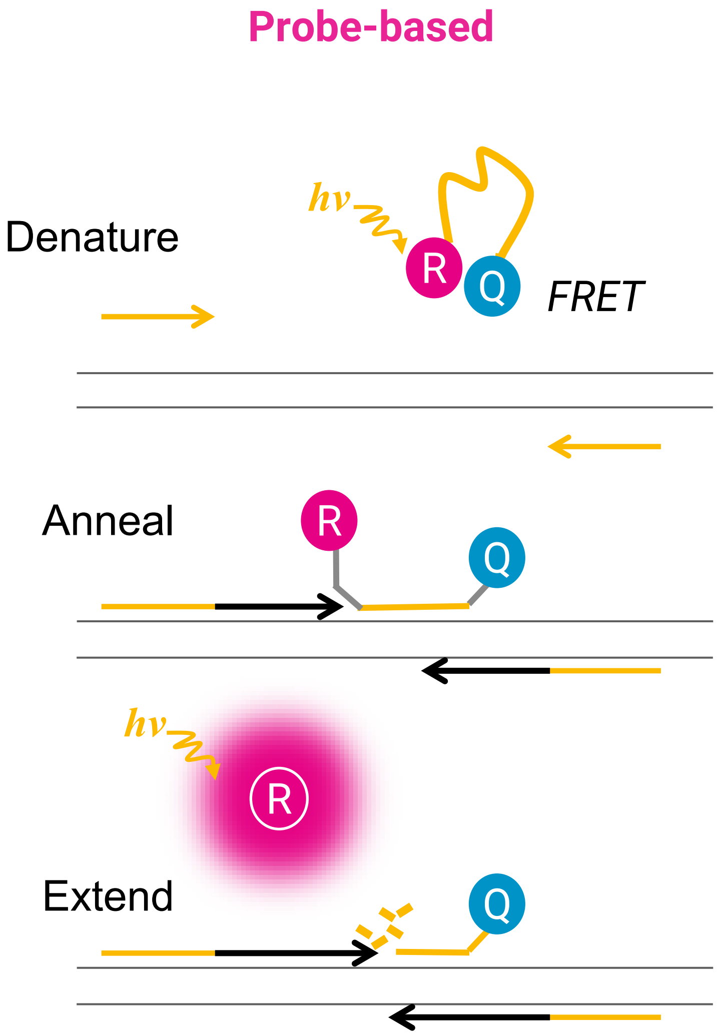
- Primers & probe anneal to target

- During extension, 5' nuclease activity of Taq degrades probe

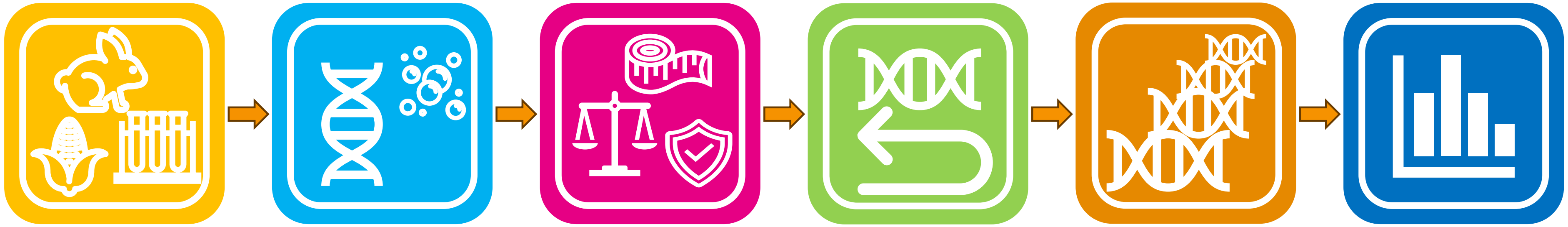
Free probe → FRET occurs

Degraded probe → reporter unquenched

Fluorescence is proportional to the amount of proper PCR product



Experimental Workflows



RT-qPCR	**Sample collection & Processing**	**RNA Extraction**	**RNA QC & Protection**	**Reverse Transcription**	**qPCR**	**Data Analysis**
qPCR	**Sample collection & Processing**	**DNA Extraction**	**DNA QC**		**qPCR**	**Data Analysis**

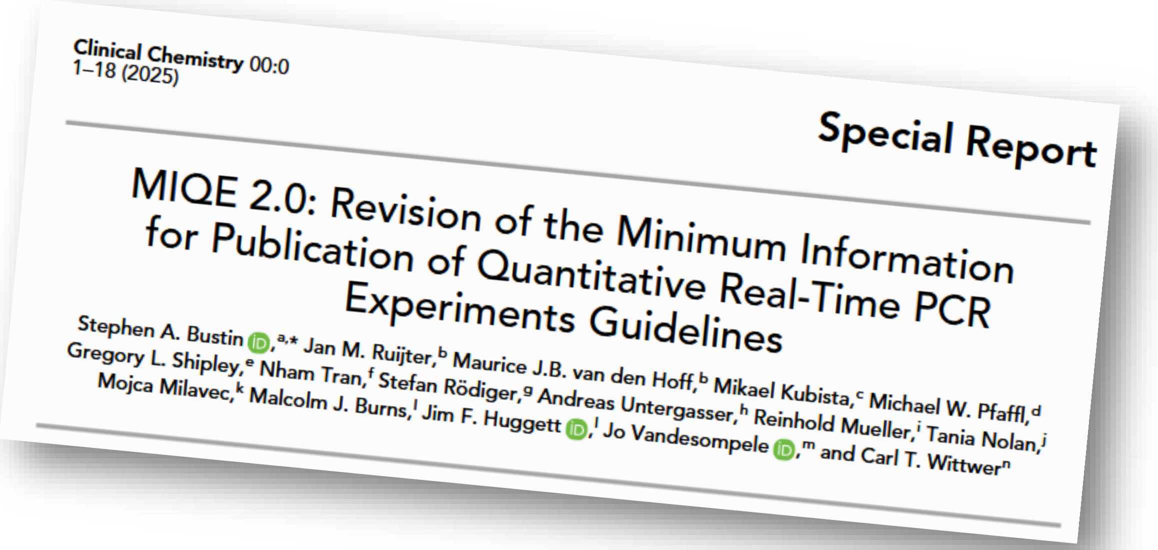
What general precautions should be taken when working with nucleic acids?

- Change **gloves** regularly
- **RNase-** and **DNase-free environment**

- Avoid **cross-contamination**:
  - Use sterile, certified RNase- and DNase-free **pipette tips**
  - **Separate work areas** pre- & post-amplification
  - Uracil N-glycosylase (**UNG**), **dUTP** instead of dTTP
- Avoid gDNA contamination:
  - Extended incubation with **DNase I**
  - **gDNA shearing** with syringe

Ask Yourself 5 Questions Before You Publish Your qPCR Results

1. Can I Trust This Sample As A Valid Starting Point For My Quantification?
2. Did I Normalize The Right Way?
3. What Is My Result And What Does It Mean? How Do I Interpret Cq Values?
4. What Controls Have My Back?
5. Can Someone Else Re-Run My Analysis?



Clinical Chemistry 00:0
1–18 (2025)

Special Report

MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines

Stephen A. Bustin,[a,*] Jan M. Ruijter,[b] Maurice J.B. van den Hoff,[b] Mikael Kubista,[c] Michael W. Pfaffl,[d] Gregory L. Shipley,[e] Nham Tran,[f] Stefan Rödiger,[g] Andreas Untergasser,[h] Reinhold Mueller,[i] Tania Nolan,[j] Mojca Milavec,[k] Malcolm J. Burns,[l] Jim F. Huggett,[l] Jo Vandesompele,[m] and Carl T. Wittwer[n]

Keep in mind: **MIQE isn't paperwork, it's guardrails.**

It ensures that you work cleanly, remain confident, and don't lose valuable time.

1. Can I Trust This Sample As A Valid Starting Point For My Quantification?

How Do I Prepare My Sample Correctly?

Maxwell® RSC Instruments

→ Research Use Only

Isolation of high-quality DNA, RNA or TNA

Maxwell® CSC Instruments

→ CE-IVD certified

Efficient

Up to 16 or 48 samples simultaneously

Flexible

Wide range of samples types
From Aspergillus to Zebrafish

Time-saving

On average < 45 minutes per run

1. Can I Trust This Sample As A Valid Starting Point For My Quantification?

How do I store my sample correctly?

- Extracted RNA:

-20°C ➔ days,

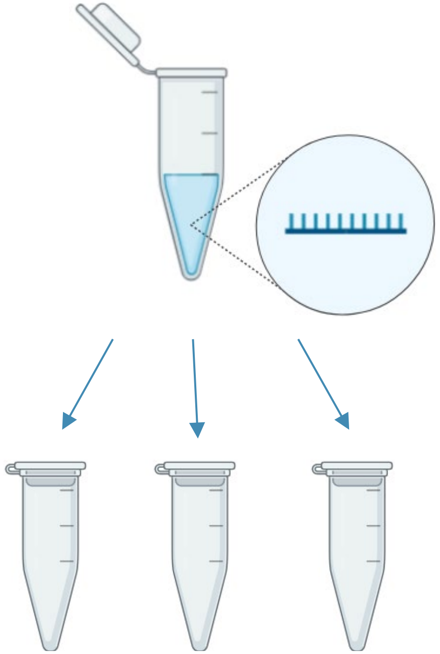
-80°C ➔ months,

-180°C (liquid N_2) ➔ 5 years

- Store DNA/RNA in small aliquots

- Avoid multiple freeze-thaw cycles

- Check nucleic acid concentration after each thawing



1. Can I trust this sample as a valid starting point for my quantification?

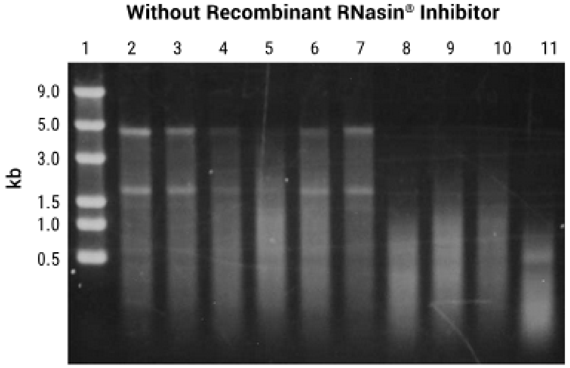
Is my sample intact or degraded?

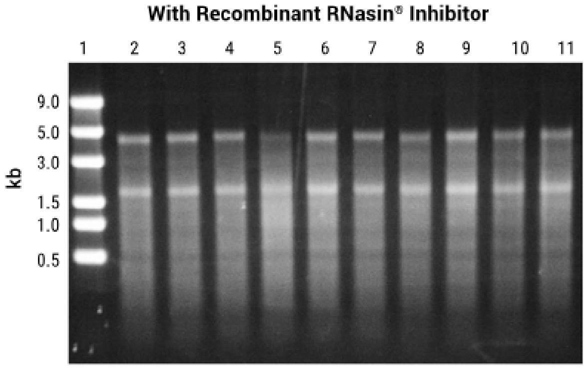
Electrophoresis

- Size
- Integrity
- Quantity

Dye Staining (QuantiFluor)

- Concentration (very sensitive)


Without Recombinant RNasin® Inhibitor

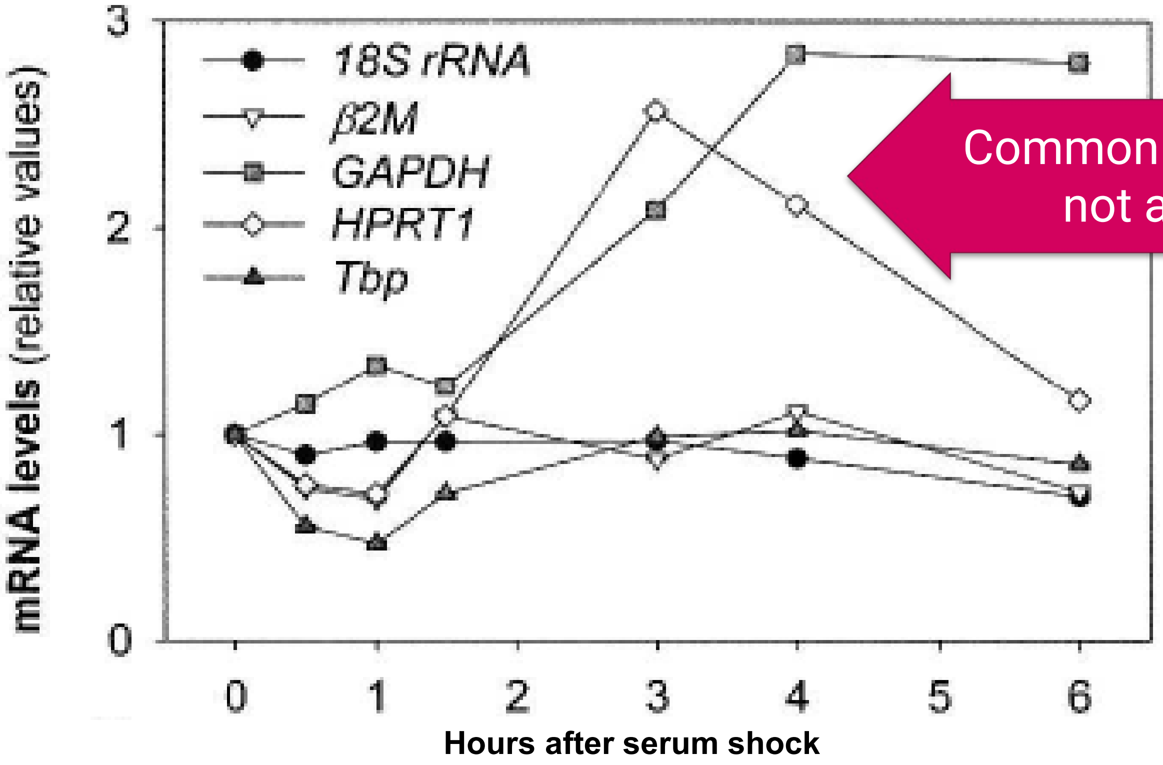

With Recombinant RNasin® Inhibitor

MIQE suggests **combination of electrophoresis & dye staining** to assess
size, quantity, concentration and integrity
(although this does not reveal contamination)

Quality is more important than yield!

2. Did I Normalize the Right Way?

Reference genes

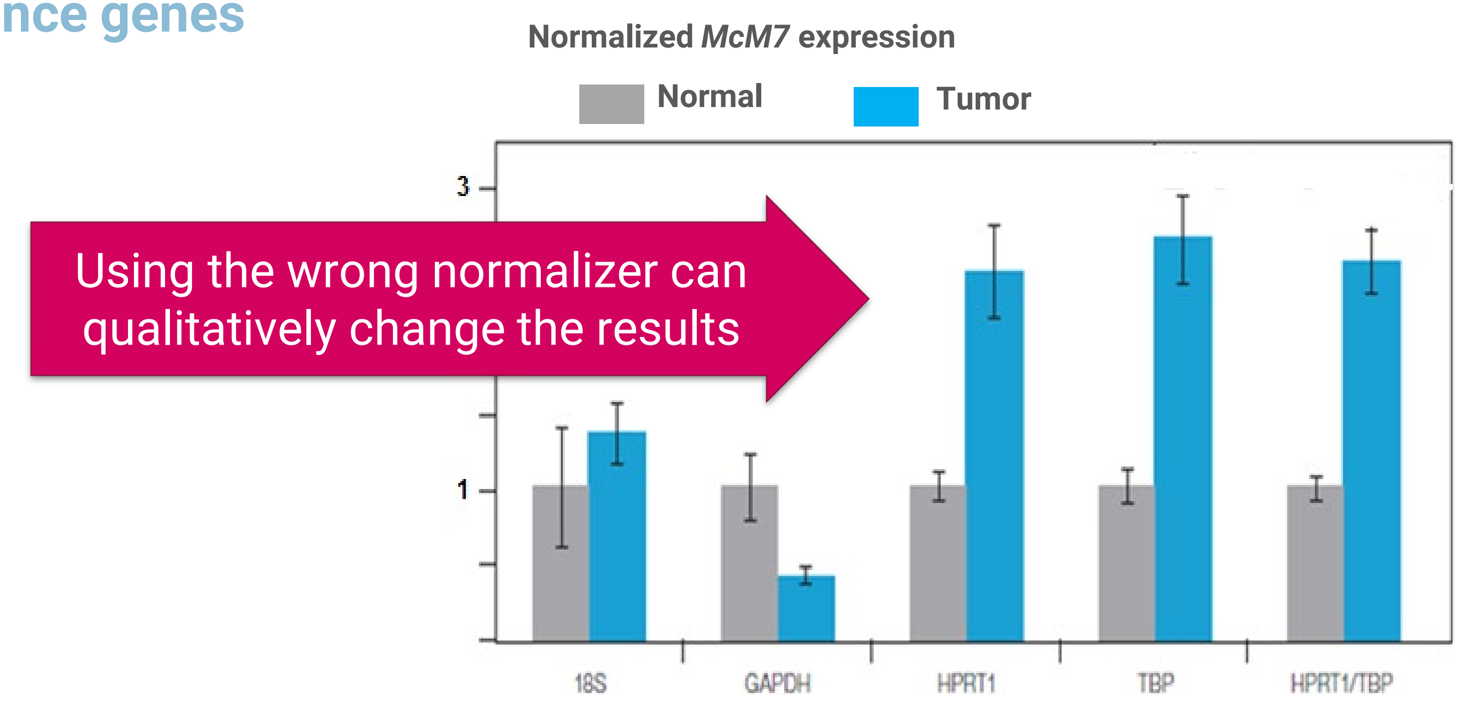


Commonly used normalizers are not always constitutive

Garabino-Pico, E. et al. (2007) RNA

2. Did I Normalize the Right Way?

Reference genes


Normalized *McM7* expression

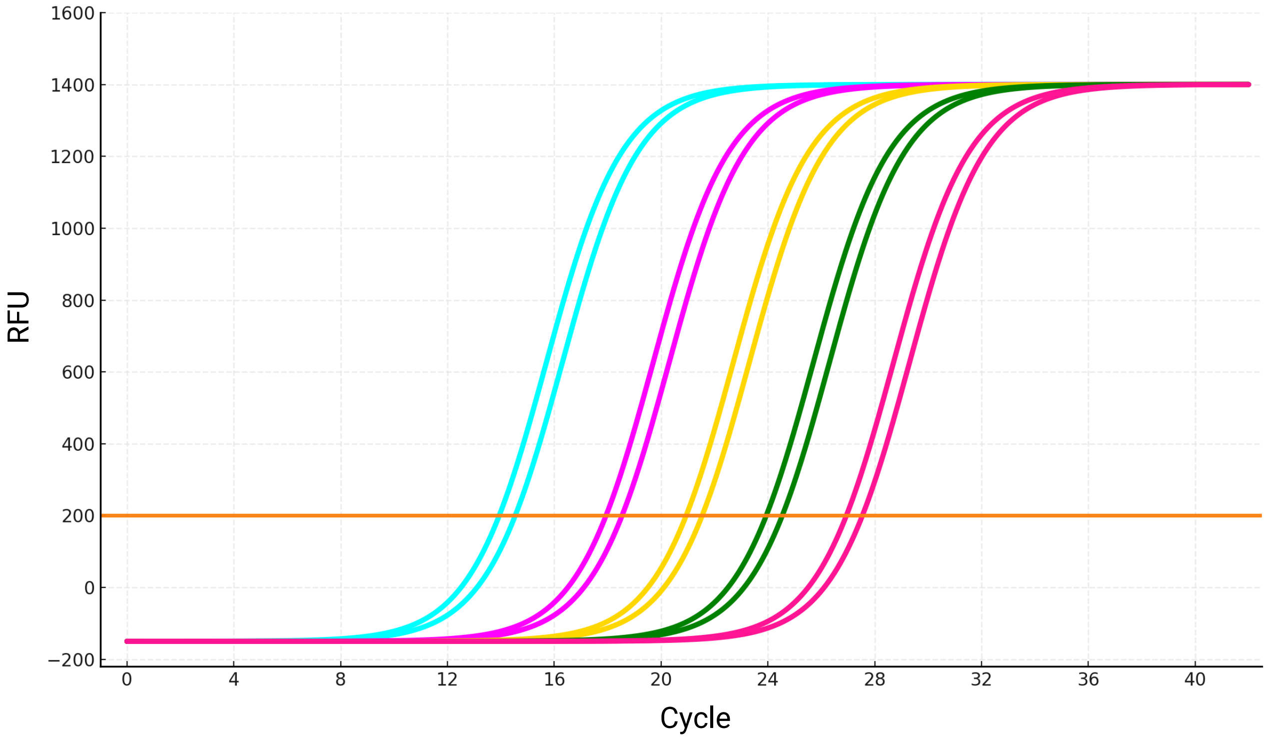
Using the wrong normalizer can qualitatively change the results

Adapted from Taylor, S. (2011) Bio-Rad tech note 6245

- MIQE suggests at least two normalizers
- Software to evaluate:

  geNORM (https://genorm.cmgg.be/)
- Expression of normalizers should be in the range of your GOI

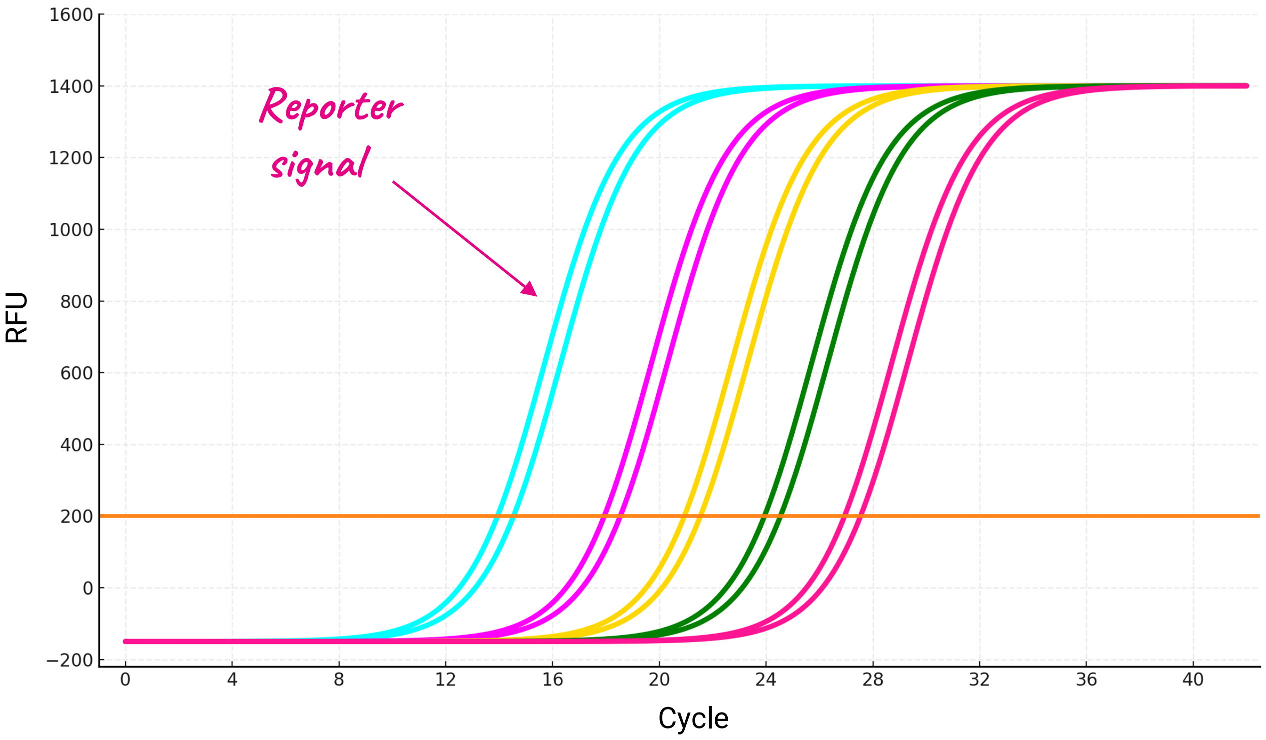
3. What Is My Result And What Does It Mean? How Do I Interpret Cq Values?



3. What Is My Result And What Does It Mean? How Do I Interpret Cq Values?

Amplification Curve – shows accumulation of product as PCR progresses

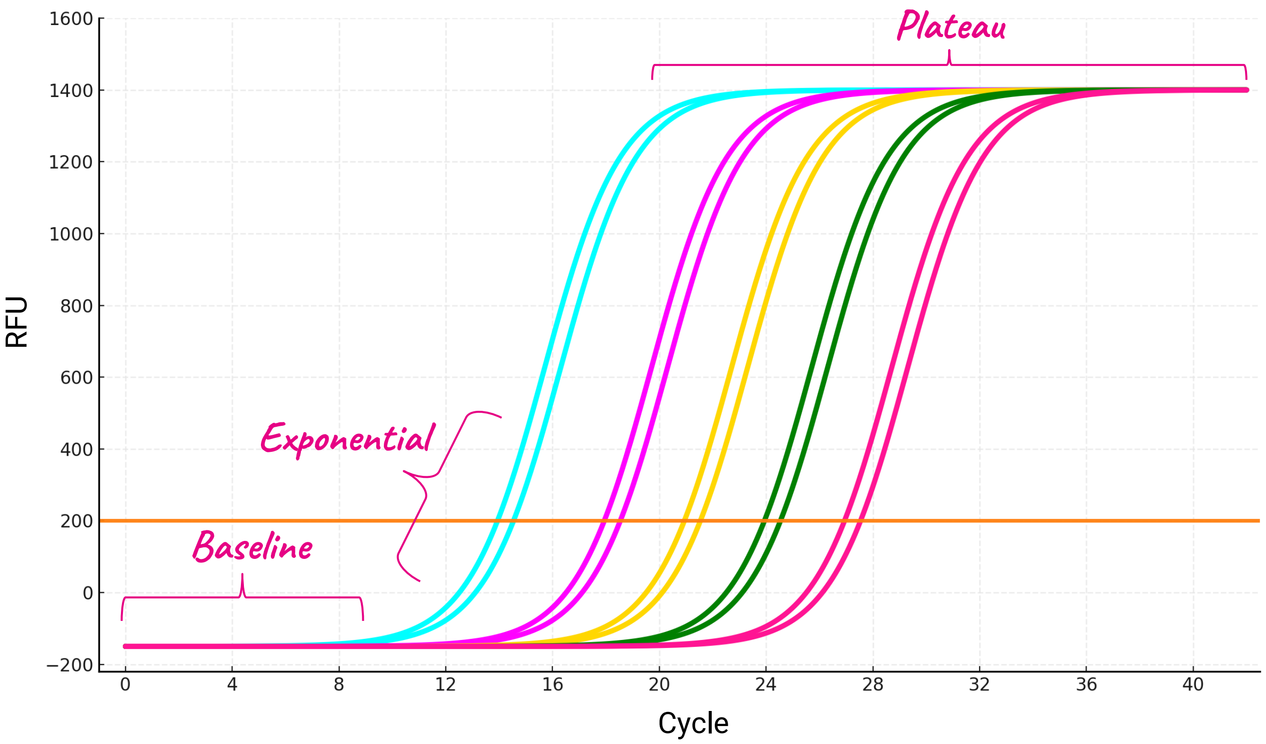
- **Reporter** – fluorescent dye or label used to monitor PCR product formation
- **RFU** – relative fluorescence unit



Primary output is the amplification curve

Amplification Curve – shows accumulation of product as PCR progresses

- **Baseline** – initial reporter fluorescence, before significant product formation occurs
- **Exponential phase** – stage of reaction when product is doubling with each cycle
- **Plateau phase** – stage of reaction when rate of product formation is diminishing

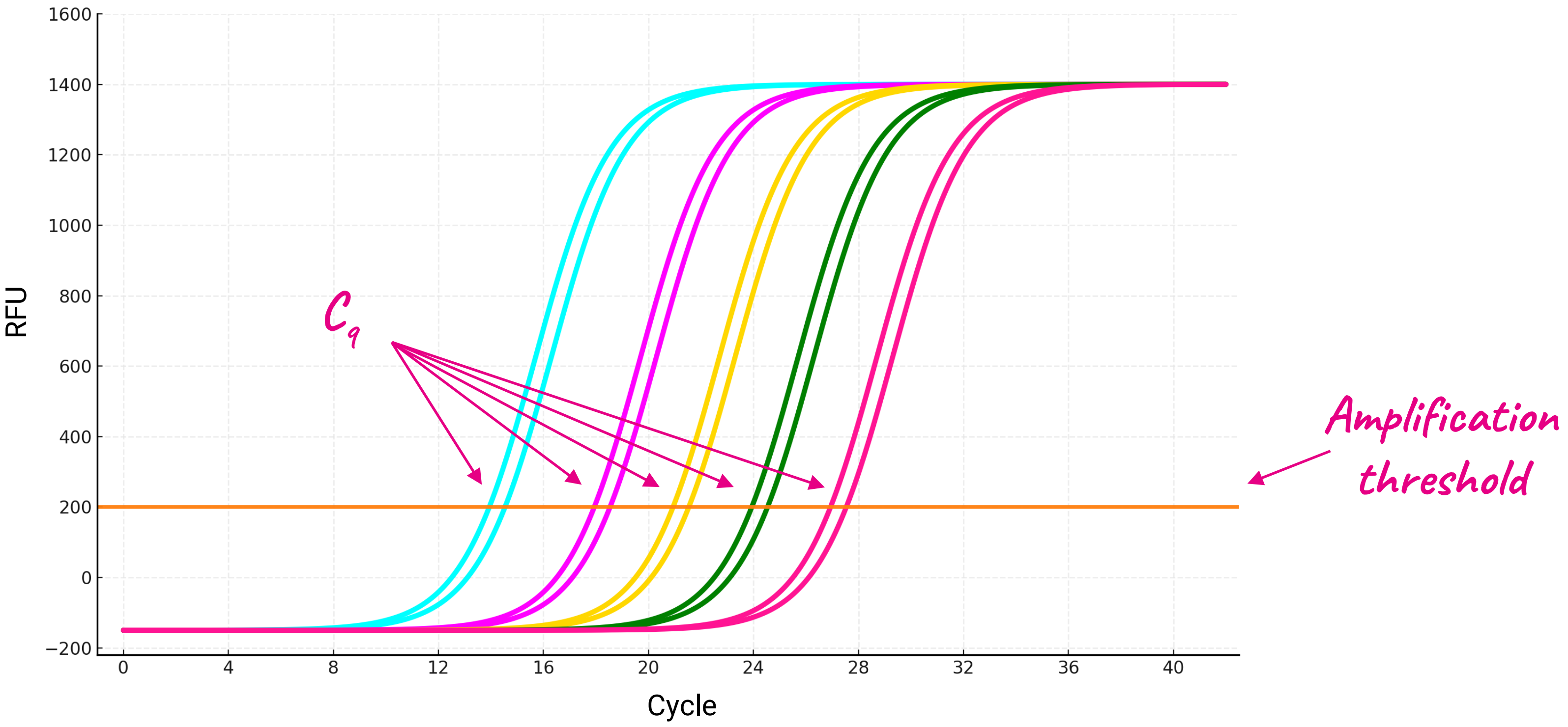


What is a C_q value?

C_q = quantification cycle

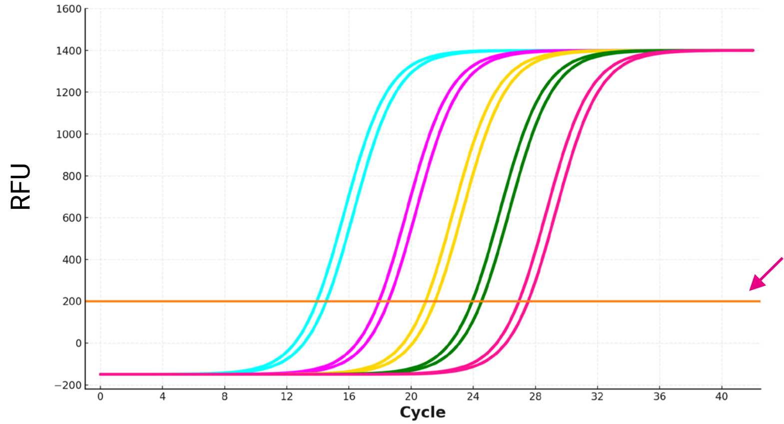
Cycle number at which amplification curve crosses amplification threshold – this is the “take-away” metric…

C_q value is inversely proportional to amount of starting template



What do I do with the Cq value?

Cq values depend on how the threshold is set and are therefore not directly comparable.

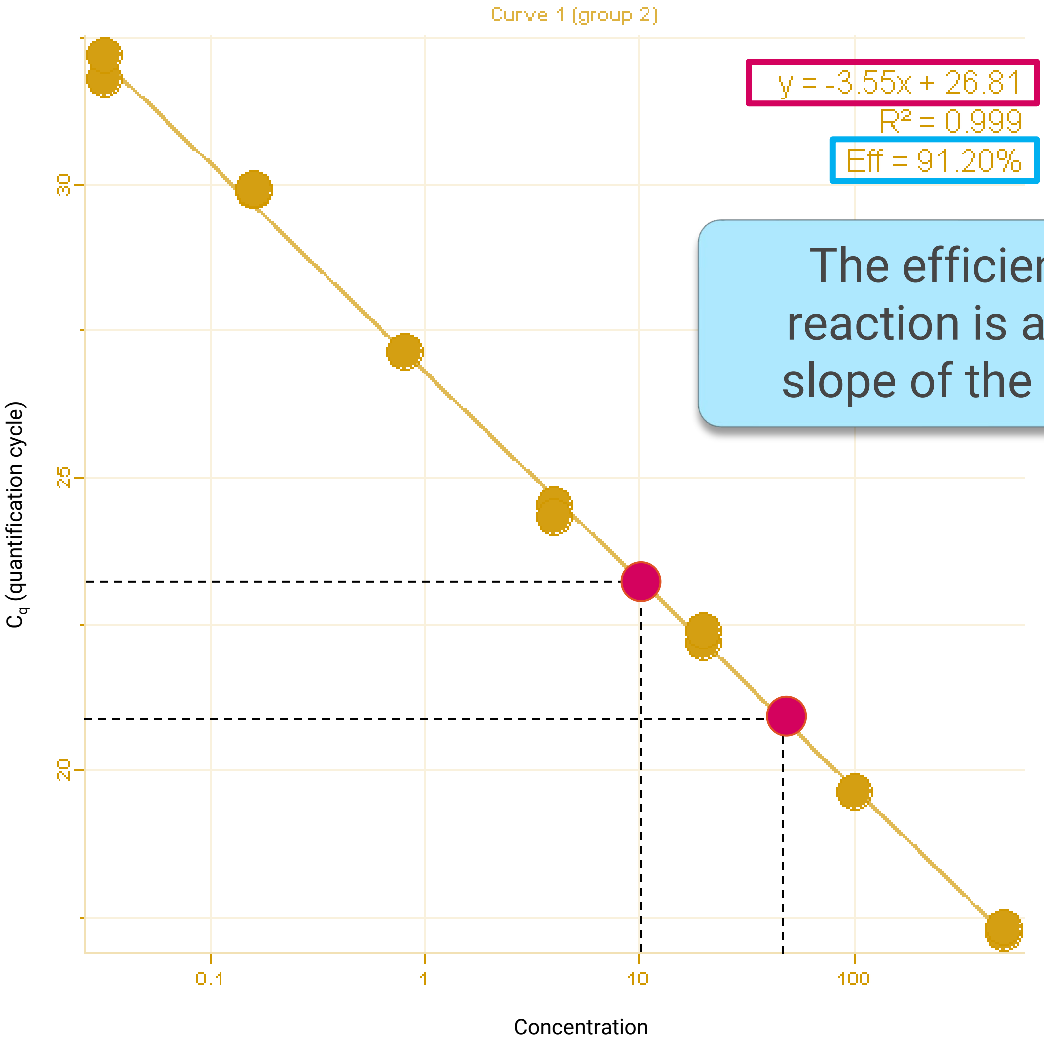


Amplification threshold

1. **Absolute quantification** = measurement of the expression of a target gene using a standard curve
2. **Relative quantification** = measurement of the expression of a target gene compared to references

Absolute Quantifizierung über die Standardkurve

The standard curve is created by plotting Cq against log (concentration).



The efficiency of the PCR reaction is a function of the slope of the standard curve.

The concentrations of unknown samples are derived from the Cq value using the standard curve.

Versch modelle

Absolut
relativ

(Normalized) Relative Quantitation

Livak and Schmittgen (2001)

- 100% PCR efficiency
- 1 reference gene

$$NRQ = 2^{\Delta\Delta Cq}$$

$$= \Delta C_{q,goi} - \Delta C_{q,ref}$$

$$= \frac{2^{\Delta C_{q,goi}}}{2^{\Delta C_{q,ref}}}$$

Pfaffl (2001)

- Experimentell bestimmte PCR efficiency
- 1 reference gene

$$NRQ = \frac{E_{goi}^{\Delta Cq_{,goi}}}{E_{ref}^{\Delta Cq_{,ref}}}$$

reminder

$$RQ = \overset{E}{\cancel{2}}{}^{\Delta C_q}$$

PCR efficiency

qBase model (2007)

- exp PCR efficiency
- Multiple reference genes

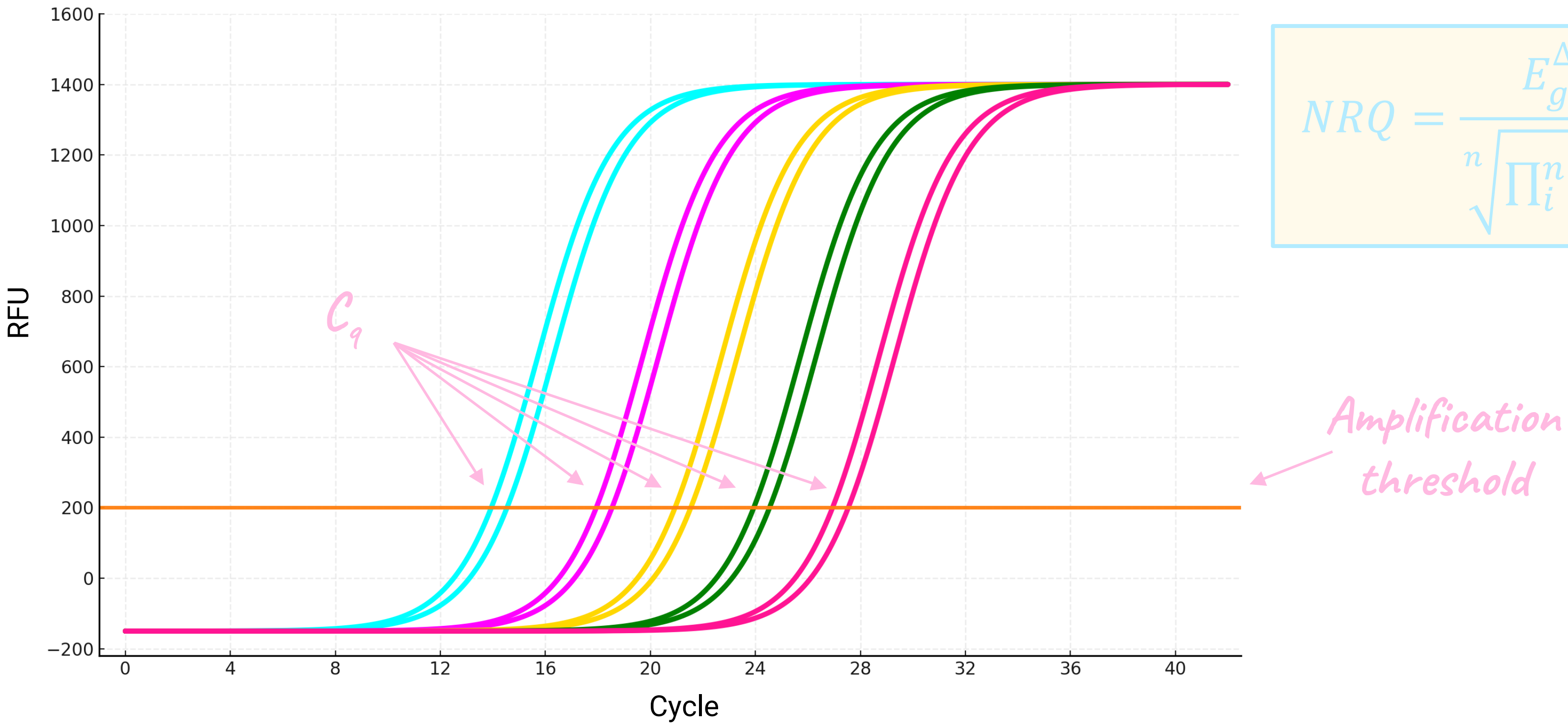
$$NRQ = \frac{E_{goi}^{\Delta Cq_{,goi}}}{\sqrt[n]{\prod_i^n E_{ref_i}^{\Delta Cq_{,ref i}}}}$$

geometric mean

Don‘t worry!

Any real-time software can perform these functions

- Automatically
- User-defined



$$NRQ = \frac{E_{goi}^{\Delta Cq,goi}}{\sqrt[n]{\prod_i^n E_{ref_i}^{\Delta Cq,ref_i}}}$$

4. What controls have my back?

- **Positive Controls**
  - **Nucleic acid target sequence**

    If positive:
    - helps determine LOD and LOQ,
    - establishes sensitivity and linear dynamic range of the assay
    - permits detection of potential inhibitors or suboptimal reaction conditions
    - aids in threshold setting

- **Negative Controls**
  - **Carrier nucleic acid (not target sequence)**

    If positive:
    - unknown sample should be considered positive only if its Cq precedes that of the negative control by at least 5 cycles

- **No RT Controls**
  - **Sample without reverse transcriptase enzyme (only RT-qPCR)**

    If positive:
    - gDNA contamination

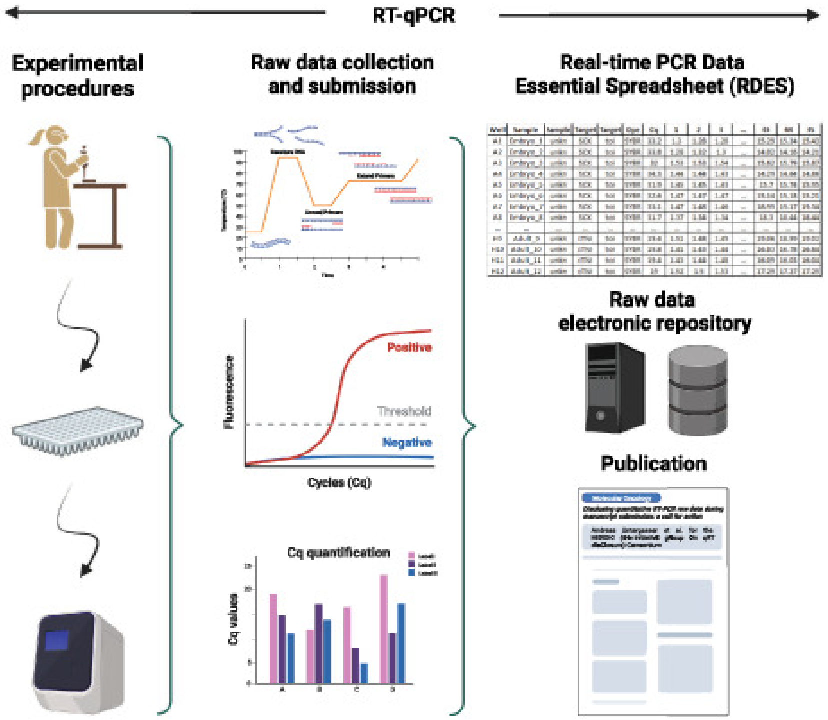
- **No Template Controls**
  - **Water or buffer instead of nucleic acid**

    If positive:
    - (Cross-) contamination = results of the entire run are invalid or caution should be taken when interpreting results

5. Can someone else re-run my analysis?

Save your raw data!

- Raw data export
- Thresholding method
- Baseline settings



Adapted from Untergasser *et al.*, 2023

MIQE suggests to publish raw data in the **supplementary material as RDES or RDML files**

Für jede Probe der richtige Mix!

SATISFACTION
PCR
GUARANTEE

Product	GoTaq® qPCR	GoTaq® Probe	GoTaq® Enviro	GoTaq® Endure
Method of detection	Dye-based	Probe-based	Probe-based	Probe-based
Suitable for	Almost all types of samples	Almost all types of samples	Environmental samples	Samples with a lot of inhibitors
Inhibitor resistance	✓	✓	✓ ✓ ✓	✓ ✓ ✓

Probe-based GoTaq® Endure Master Mix

Inhibitors like EDTA or humic acid can distort your qPCR results!

If you have samples with many inhibitors that are difficult to remove during sample preparation, use a master mix that is resistant to inhibitors

Request a free sample!

www.promega.com/testEndure

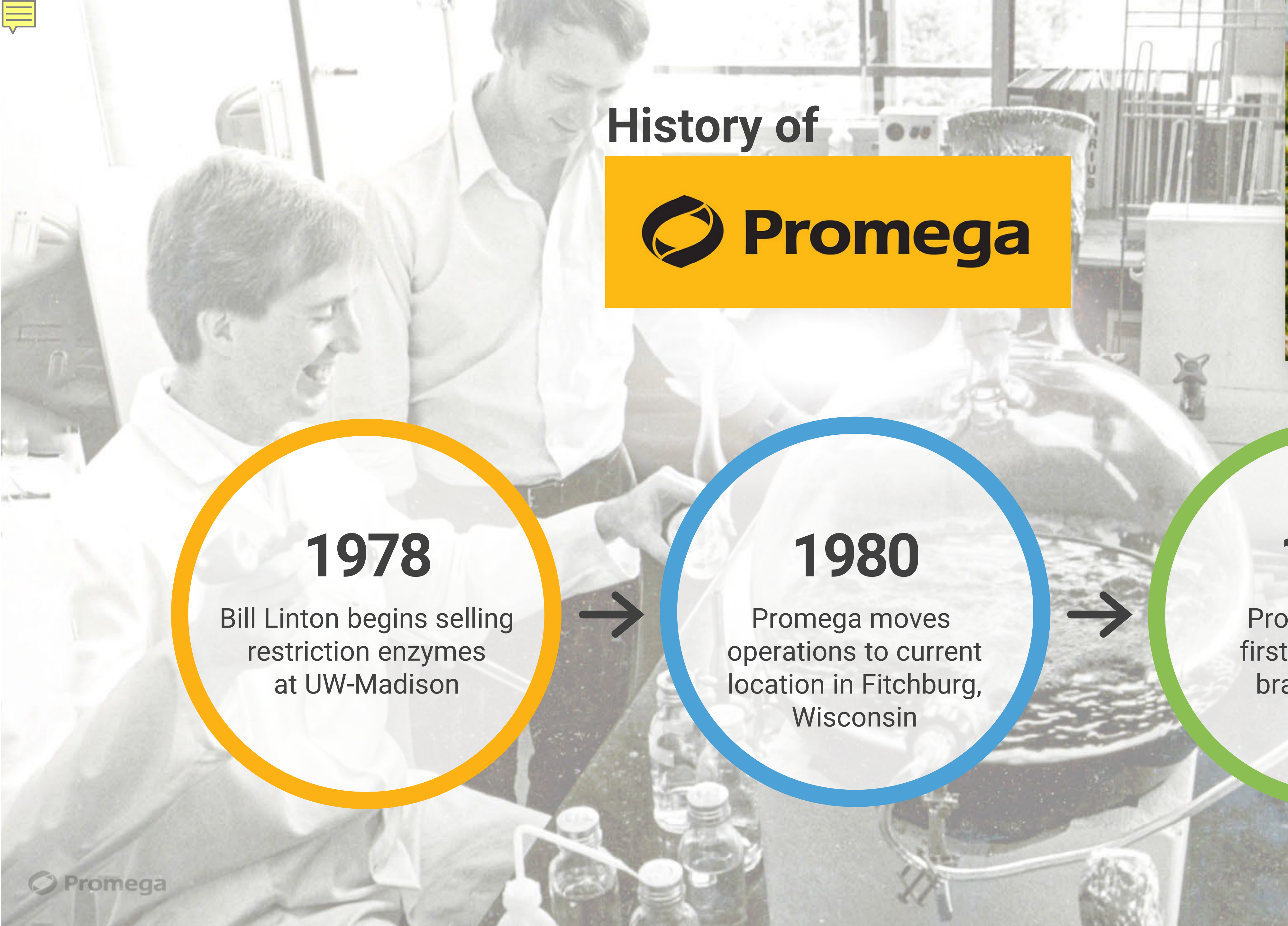
GoTaq® Endure
qPCR & RT-qPCR Master Mixes

Suitable for a variety of sample types:

- ✓ Blood
- ✓ Bacteria
- ✓ Virus
- ✓ Feces
- ✓ Soil
- ✓ Plant
- ✓ Food

Get your free sample now!

- Probe-based
- Extremely high inhibitor tolerance
- Fast Cycling
- Multiplexing



History of Promega

1978

Bill Linton begins selling restriction enzymes at UW-Madison

→

1980

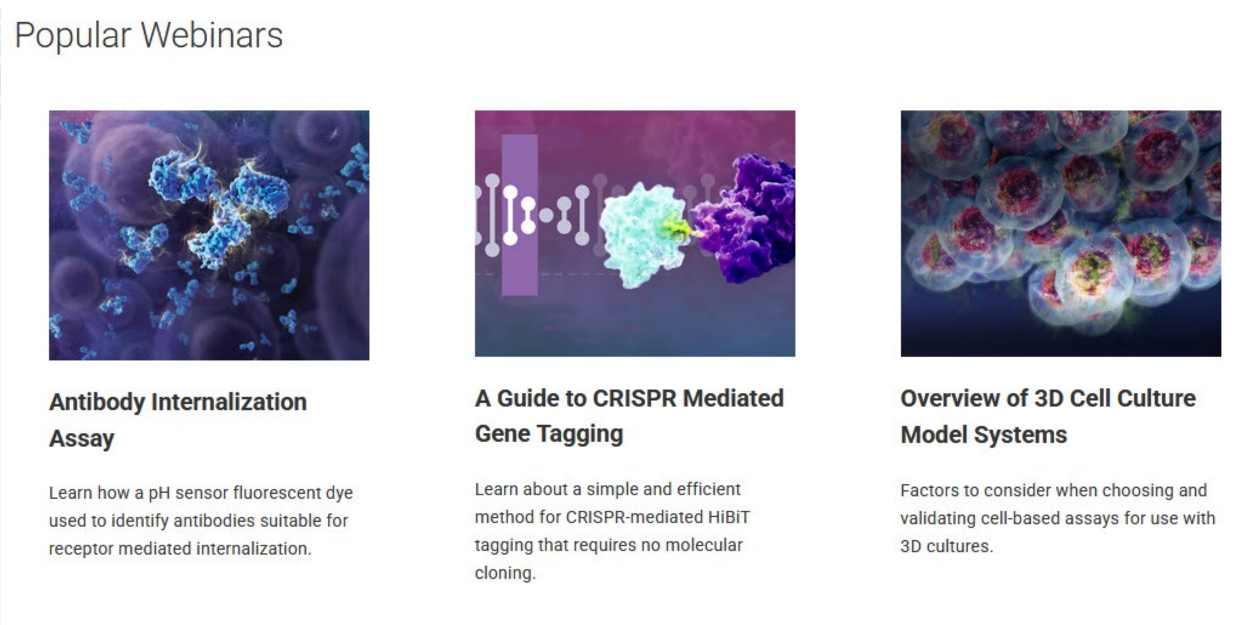
Promega moves operations to current location in Fitchburg, Wisconsin

→

1983

Promega opens first international branch offices

Learning @Promega



Popular Webinars

Antibody Internalization Assay

Learn how a pH sensor fluorescent dye used to identify antibodies suitable for receptor mediated internalization.

A Guide to CRISPR Mediated Gene Tagging

Learn about a simple and efficient method for CRISPR-mediated HiBiT tagging that requires no molecular cloning.

Overview of 3D Cell Culture Model Systems

Factors to consider when choosing and validating cell-based assays for use with 3D cultures.

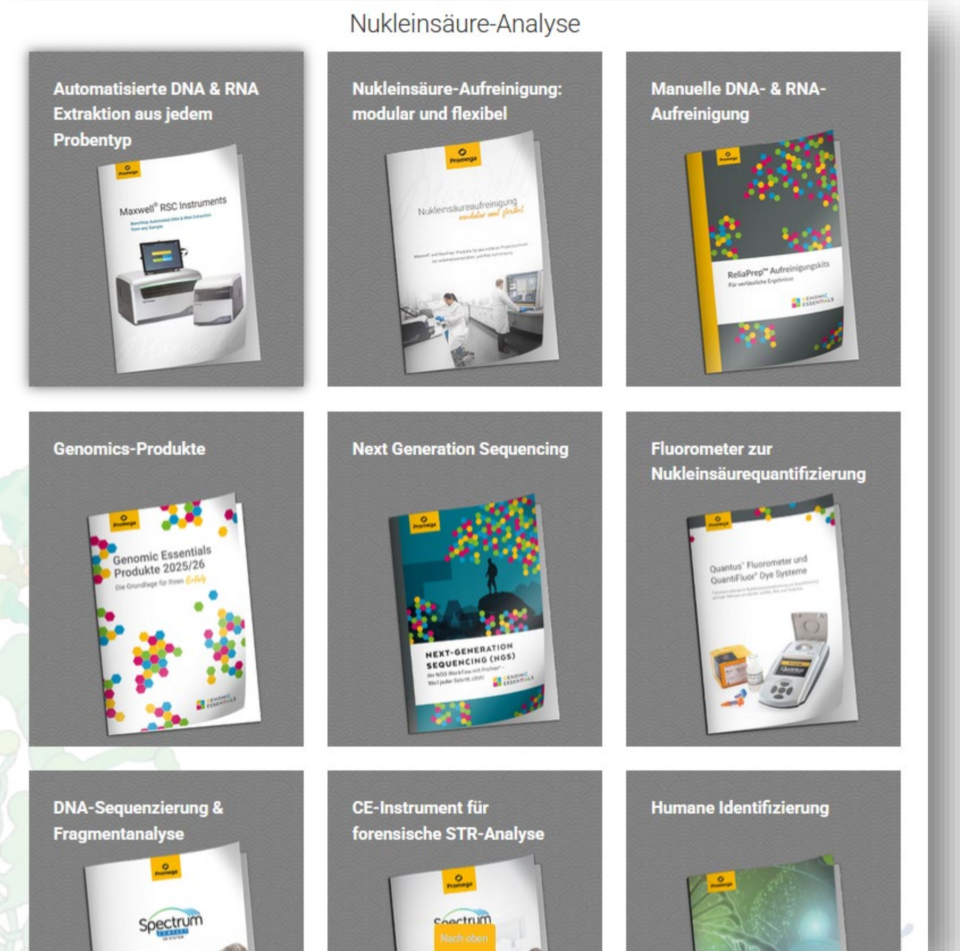
Promega-Academy

Wissenschaftliche Seminare: Zugeschnitten auf Ihre Bedürfnisse!

Promega Connections

Thoughts, tech tips and news about science

NEW » LAB
STARTUP
PROGRAM



Nukleinsäure-Analyse

- Automatisierte DNA & RNA Extraktion aus jedem Probentyp
- Nukleinsäure-Aufreinigung: modular und flexibel
- Manuelle DNA- & RNA-Aufreinigung
- Genomics-Produkte
- Next Generation Sequencing
- Fluorometer zur Nukleinsäurequantifizierung
- DNA-Sequenzierung & Fragmentanalyse
- CE-Instrument für forensische STR-Analyse
- Humane Identifizierung

Technical Service

Visit our

- **qPCR Seminar**
- **qPCR Workshop**

Or watch our **qPCR Webinar**!

If you need help with your (PCR) reactions, feel free to contact our technical service!

Dr. Hannah Buntz

Manager Training Lab
Tel. +49 151 580 421 05
hannah.buntz@promega.com

Technical Services Team


Christof
Brückmann


Sabine
Wawro


Petra
Numrich


Thorsten
Stehlik


Jan
Adam

Contact us in case of any questions or problems

de_techserv@promega.com
+49 6227 6906 290

Request your sample!

Thank you!

Feel free to ask questions!

in Let's connect!

Contact your local sales representative for products & sales relevant information: www.promega.com/c/local sales/sales contacts/

Most images created by biorender.com or are partly AI generated