Promega

Navigating qPCR: Experimental Design, Data Analysis, and Best Practices

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Area Manager, Central Eastern Europe
Promega Germany
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Our Mission

Provide innovative biological reagents and integrated systems used in research and applied technology worldwide.

Our Products Support

Government and Academic Research Laboratories

Forensic and Paternity Laboratories

Pharmaceutical and Biotechnology Industries

Clinical and Molecular Diagnostics Laboratories

Food and Water Safety Testing Facilities

qPCR Applications

- **Research tool**
  - Gene expression studies in disease
  - Drug discovery and development
- **Diagnostic tool**
  - Disease detection
  - Newborn screening
- **Pathogen detection (bacteria, viruses)**
  - Clinical samples
  - Environmental samples
    - Water quality
    - Soil contamination
    - Food safety

Image is generated using AI

How qPCR Analyzes Gene Expression

- **Size** – examine differential splicing
- **Sequence** – predict protein product
- **Abundance** – measure expression levels
- **Dynamics of expression** – temporal, developmental, tissue specificity

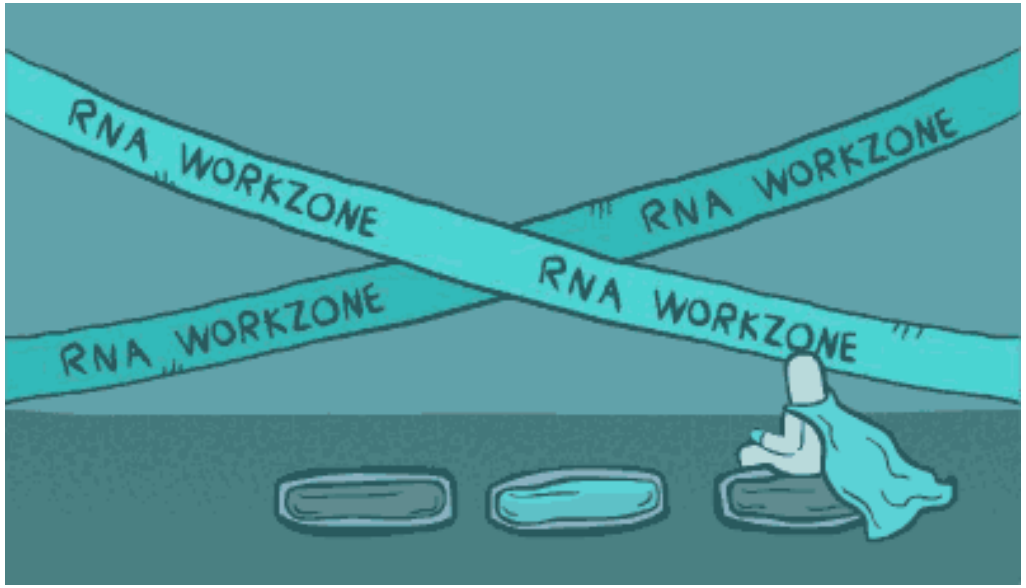
Workflow

MIQE Guidelines
Minimum Information for Publication of Quantitative Real-Time PCR Experiments
Stephen Bustin et al. (2009) Clinical Chemistry, 55:4

MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines Stephen Bustin et al. (2024) Clinical Chemistry, 71:6

Protecting RNA Starts at the Bench

- Temperature abuse of samples before/during /after collection
- Dissection takes too long
- Sample dimensions too large – takes too long to freeze & thaw
- Insufficient tissue disruption



Protecting RNA Starts at the Bench

- Change gloves regularly
- Use sterile, certified RNase- and DNase-free pipette tips
- Prevent gDNA contamination:
  - Extended incubation with DNase I
  - gDNA shearing" with syringe

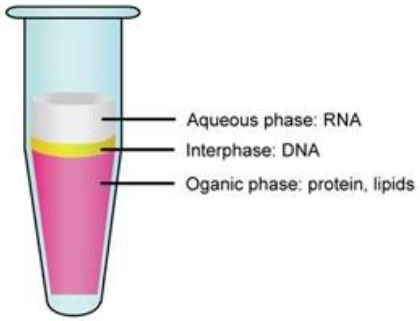
Cellular Total RNA

- Messenger RNA (mRNA): 1-5%
- Ribosomal RNA (rRNA): >80%
- Transfer RNA (tRNA): 10-15%
- MicroRNA (miRNA): <1%

The choice of purification method is an important consideration when isolating specific RNA molecules of interest.

Phenol/chloroform/Trizol extraction

- Advantages
  - Price
  - Yield (tRNA + mRNA)
- Disadvantages
  - Time-consuming: >2h of work; many steps
  - Toxic, organic waste 
  - Purity (gDNA contamination, inhibitor carryover)
  - Reproducibility



https://www.creative-diagnostics.com/images/Protocol-Total-Protein-Extraction-by-Trizol-1-phase-separation.jpg

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ReliaPrep™ RNA Miniprep Systems

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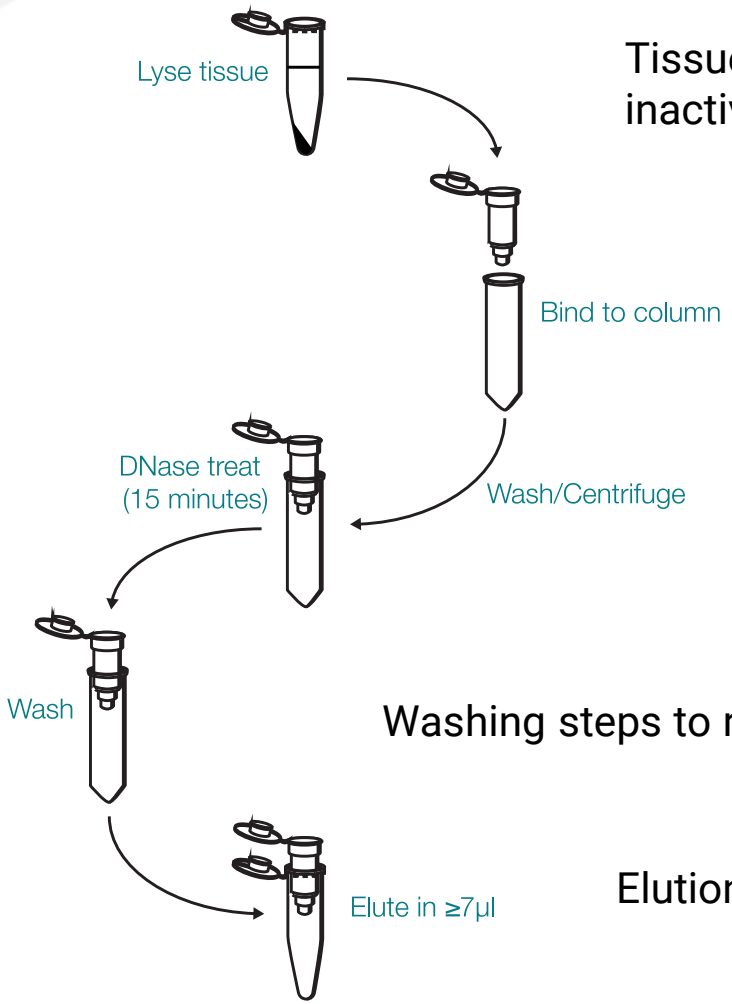


Tissue/Cell homogenization without organic reagents → release of RNA, inactivation of RNases

Dehydration → binding of RNA to membrane in the presence of chaotropic salts

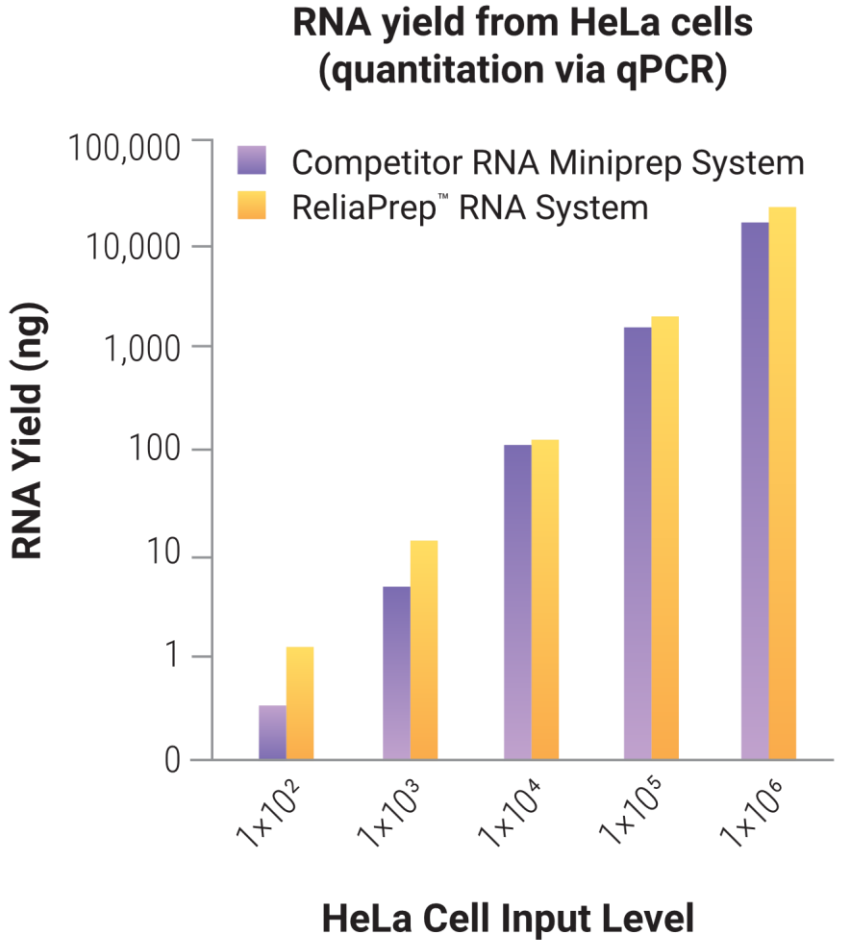
On column digestion of gDNA with DNase I

Washing steps to remove contaminants (salts, proteins, cellular components)

Elution of RNA in 7-50 µl (Water/TE-Buffer)

ReliaPrep™ RNA Miniprep Systems

- Advantages
  - **Working without a hood – no organic reagents**
  - **Save time**: 30-40 minutes including DNase digestion (already included in kit)
  - **High purity**: A260/280 ≥ 2.0 and A260/230 ≥ 2.0
  - **Flexibility**:
    - Adjustable elution volume: 7 µL - 50 µL
    - Input : 10 - 5*10^6 cells or 0.003 - 20 mg tissue
  - Keep in mind: **miRNA isolation** requires a purification system specifically designed for recovery of small RNAs



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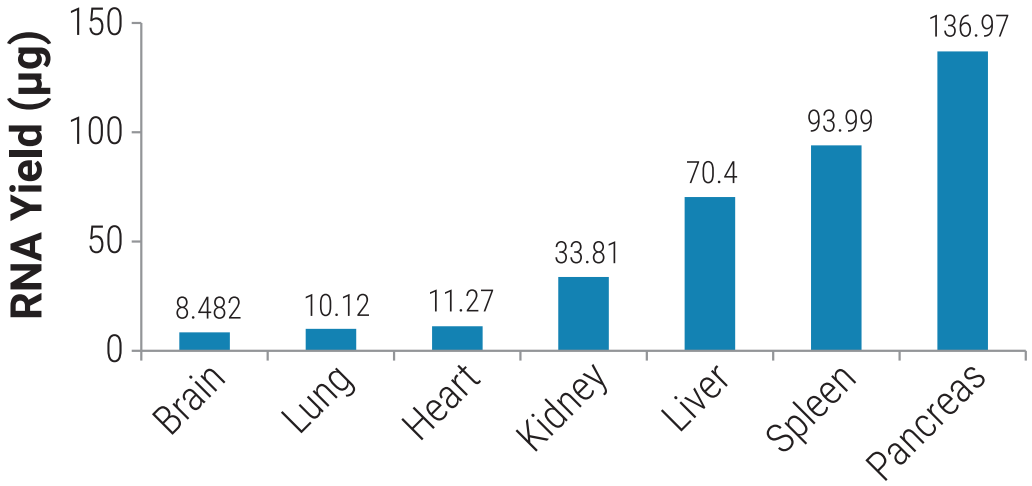
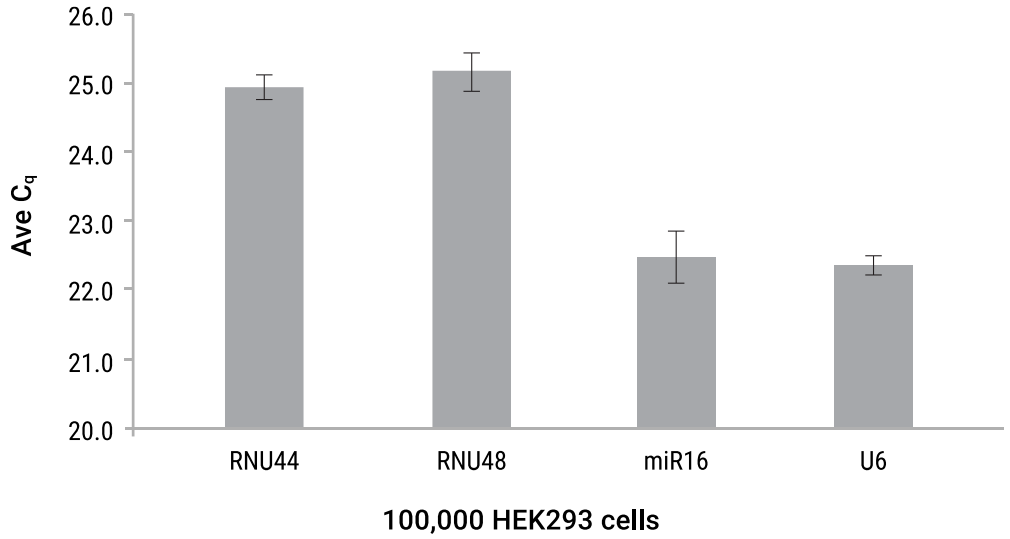
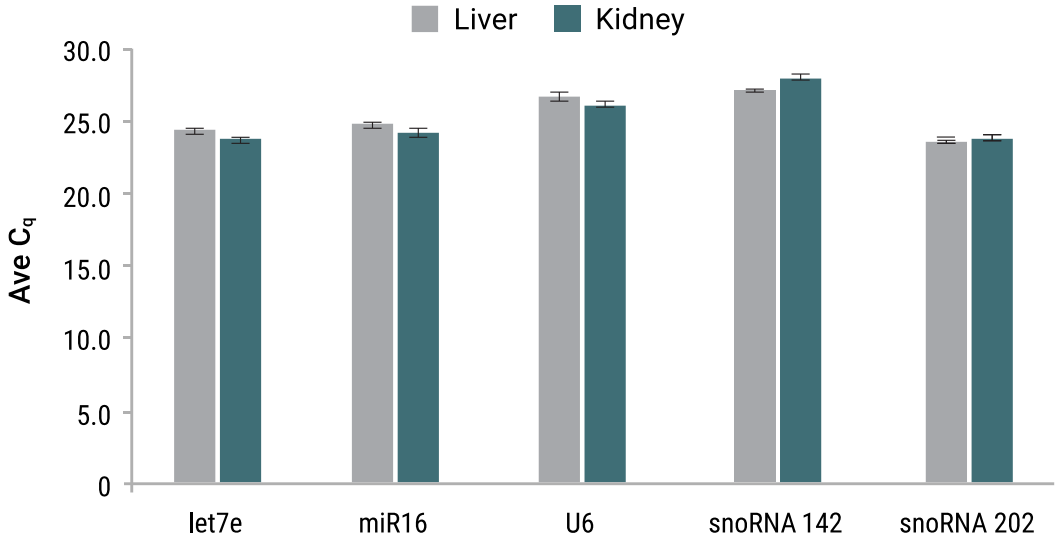


ReliaPrep™ miRNA Cell and Tissue Miniprep System

Isolates total RNA including microRNA (miRNA) and other small non-coding RNA (sncRNA) subspecies from a variety of cell and tissue types.

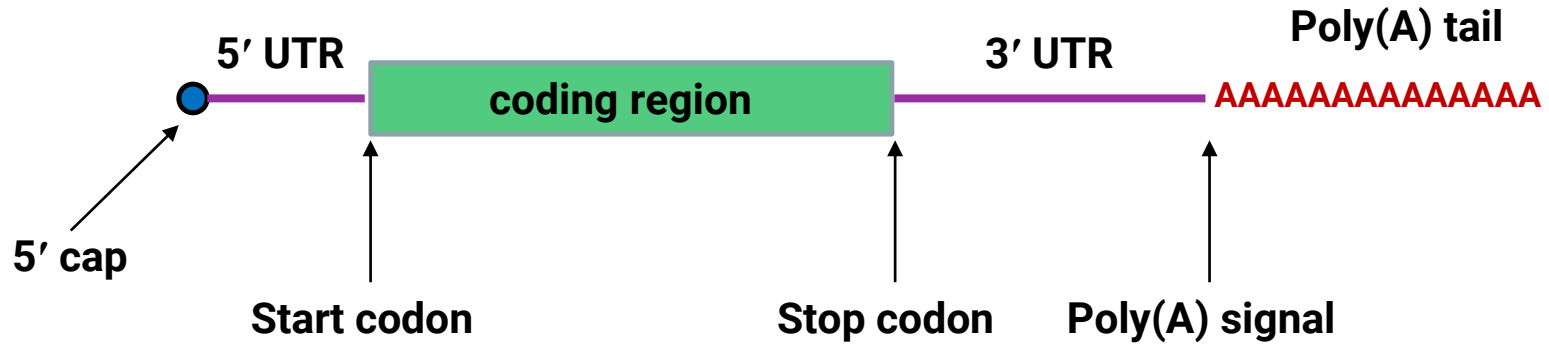




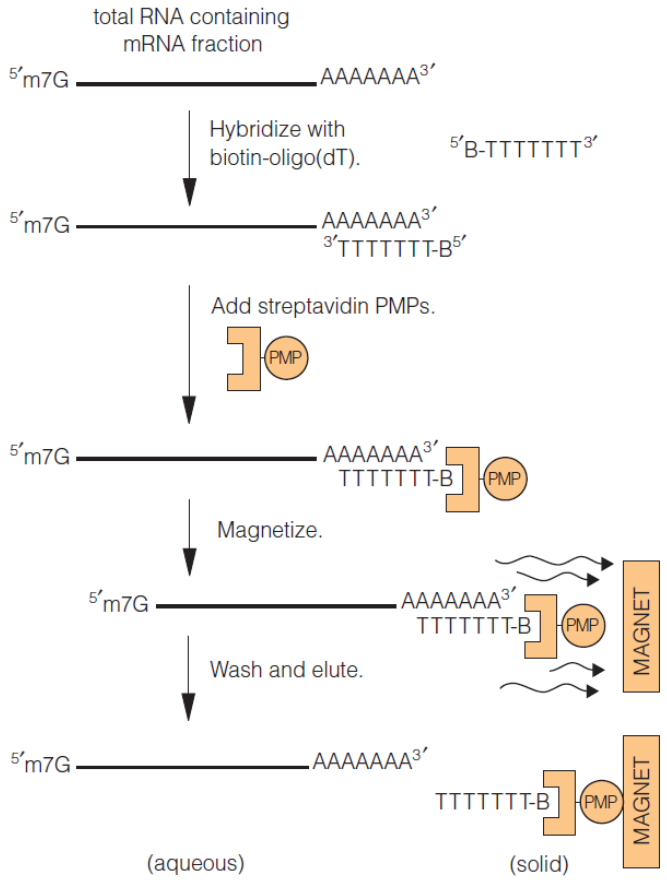
snoRNA: RNU44, RNU48, snoRNA 142, snoRNA 202 **miRNA**: miR-16, let7e **snRNA**: U6

Messenger RNA Extraction

- mRNA molecules have a tail of A's at the 3' end (poly-A tail)
- Oligo(dT) probes can be used to purify mRNA from other RNAs



PolyATract® mRNA Isolation Systems



The systems use a biotinylated oligo(dT) primer to hybridize to the 3´ poly(A)+ region present in most mature eukaryotic mRNAs.

The hybrids are bound to streptavidin coupled to paramagnetic particles.

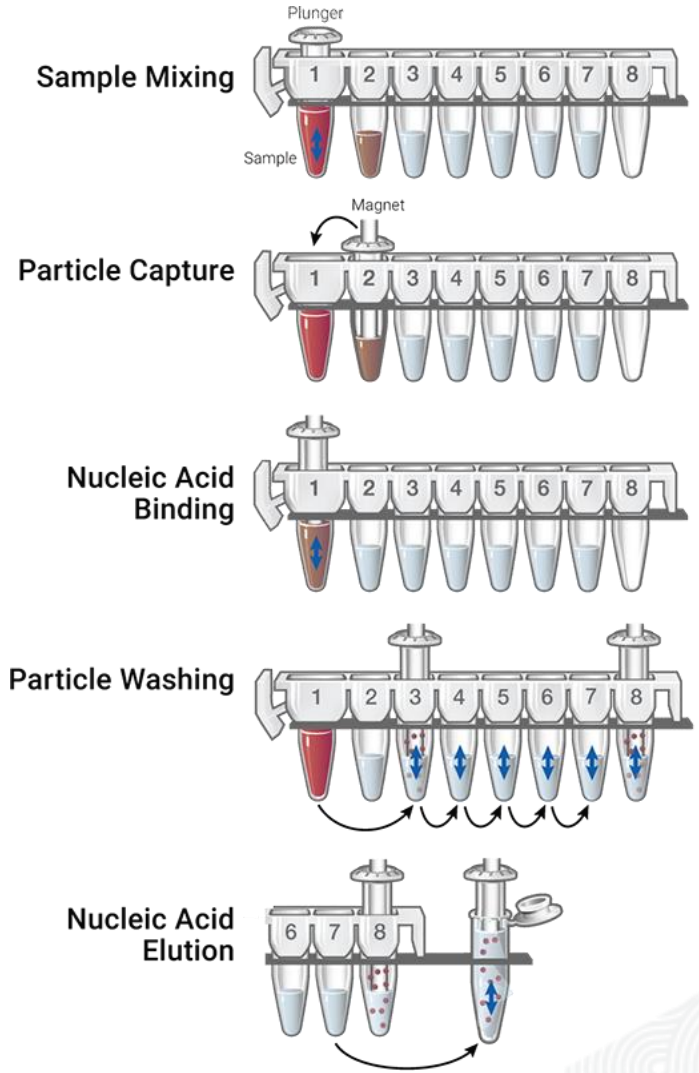
PMP' are captured using a magnetic separation stand and washed at high stringency.

Maxwell® RSC Systems – Automate Your Workflow

- Suitable for various downstream applications
- Purification from multiple sample types
- Prefilled cartridges and preinstalled methods
- Up to 48 samples per run in 25-60 minutes
- Integrated UV decontamination

Maxwell® Systems – Automate Your Workflow

- Maxwell® Instruments = Transport of paramagnetic particles
- **No drips**: No detectable cross-contamination.
- **No clogs and fewer breakdowns**: Fewer interruptions to your workflow and improved efficiency



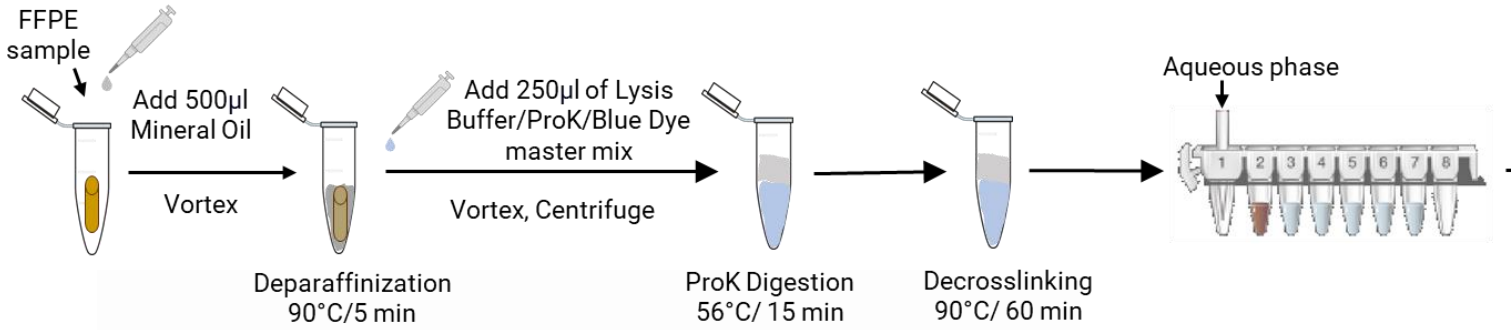
Maxwell® RSC Systems – Automate Your Workflow

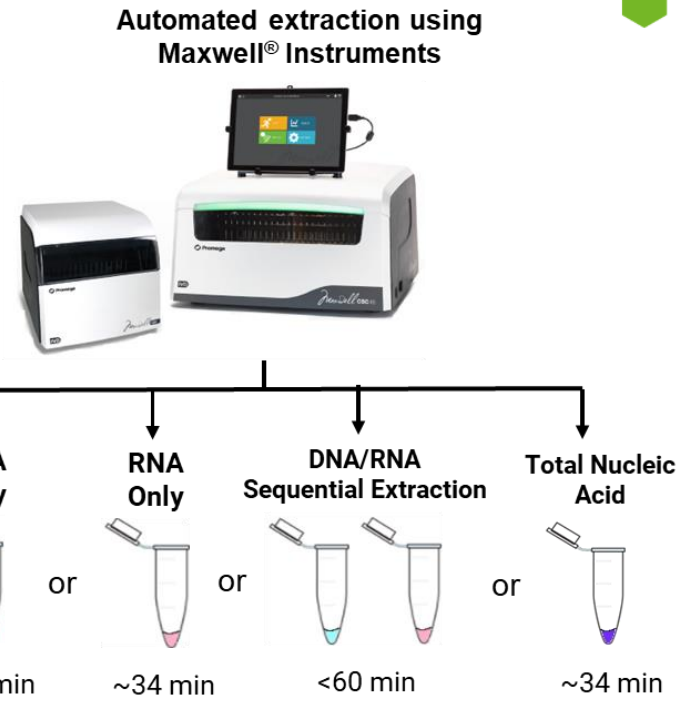
Biofluids	Cells/Tissues	Environmental	Microbes & Viruses
Blood	Mammalian Tissue	Feces	Bacteria
Saliva & Mucus	FFPE	Soil	Fungi
Plasma/Serum	Plant & Insect	Water	Archaea
Urine	Swabs	Wastewater	Protist

Over 130 application notes provide protocol details and purification data from numerous sample types.

Maxwell® RSC XtractAll FFPE DNA-RNA Kit (AS1570)

"A Versatile Kit for All FFPE Nucleic Acid Extraction Needs!"





Sequential gDNA and RNA extraction separately from the same FFPE tissue. Also, singular extraction of DNA or RNA, as well as total nucleic acid.

Maximized, comprehensive genomic and transcriptomic information with minimal sample consumption.

High-quality DNA and RNA extraction starting with FFPE sections in ~2.5 hours.

Less plastic waste: a single cartridge and plunger per workflow.

High-Throughput Automated DNA and RNA Extraction

Ready-to-Go Extraction Kits

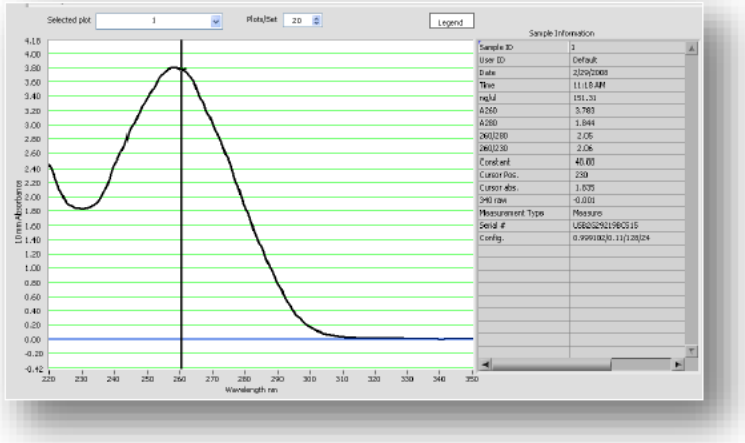
Assay Scale-Up and Custom Support

Expert Support for Any Liquid Handling System

RNA Purity Matters — Understanding Measurement Limitations

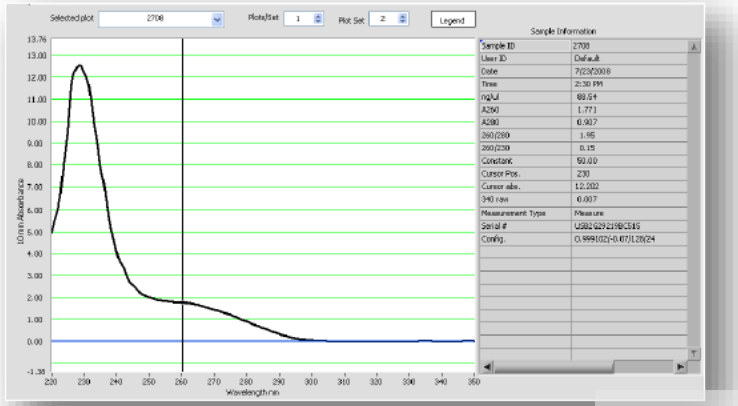
Spectrophotometry measures total absorbance: signals at 260nm can include RNA alongside other absorbing compounds

Optimal Spectra



A260 = 3,78
A_{260}/A_{280} = 2,06

Strong Peak ~230nm Contributes to 260nm Reading



A260 = 1,77
A_{260}/A_{280} = 1,95

Is there really DNA/RNA?

Absorbance-based measurements may overestimate RNA concentration in the presence of contaminants

Fluorescence-based RNA quantification — sensitive & specific

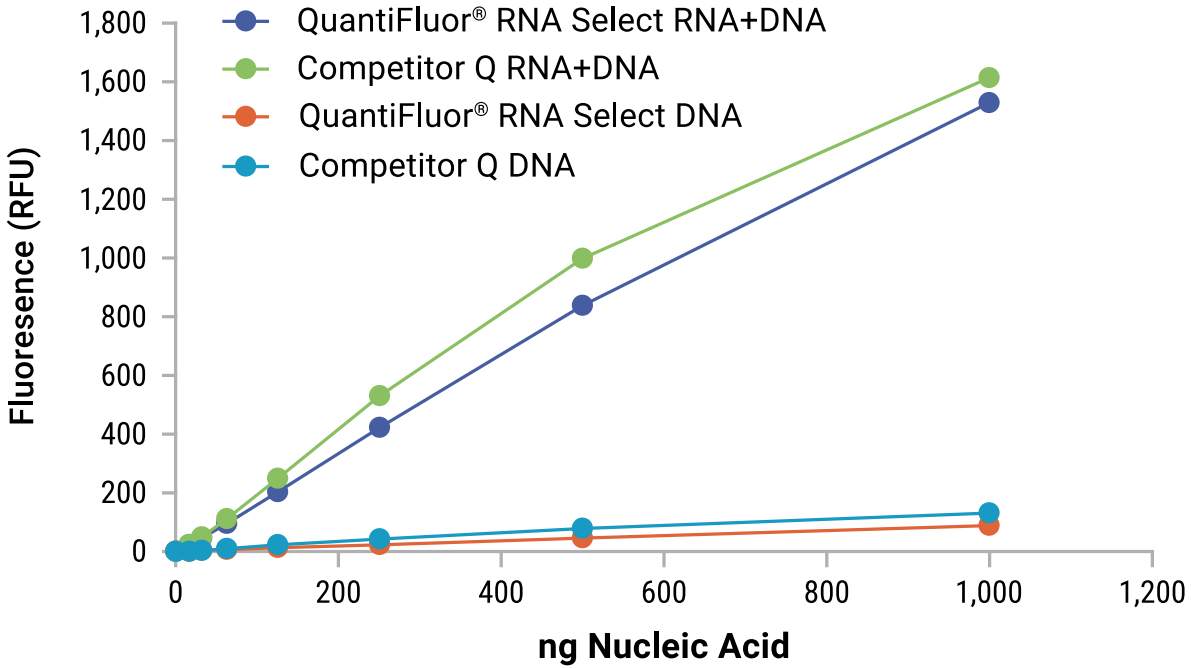
Principle

- Fluorescent dyes bind selectively to nucleic acids
- Signal generated only upon binding — minimal background
- Minimal interference from contaminants
- Measured signal reflects true nucleic acid concentration

QuantiFluor® RNA Select

- Standard dyes detect RNA and DNA together
- DNA presence may influence quantification results
- RNA Select enables RNA-specific detection
- Ideal for sequencing applications
- Improves accuracy for RNA-focused applications
- Ambient shipping-compatible, increased shelf life and reduced storage temperature requirements

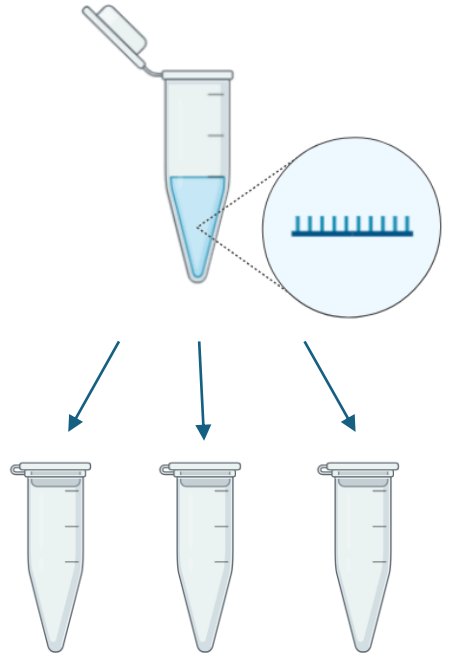
RNA Specificity Comparison



GREENER SCIENCE,
SMARTER SOLUTIONS

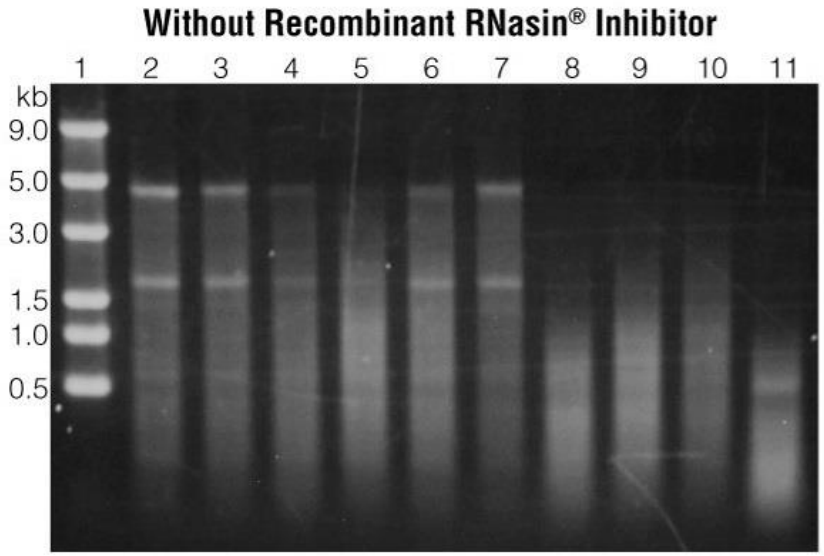
How do I store my sample correctly?

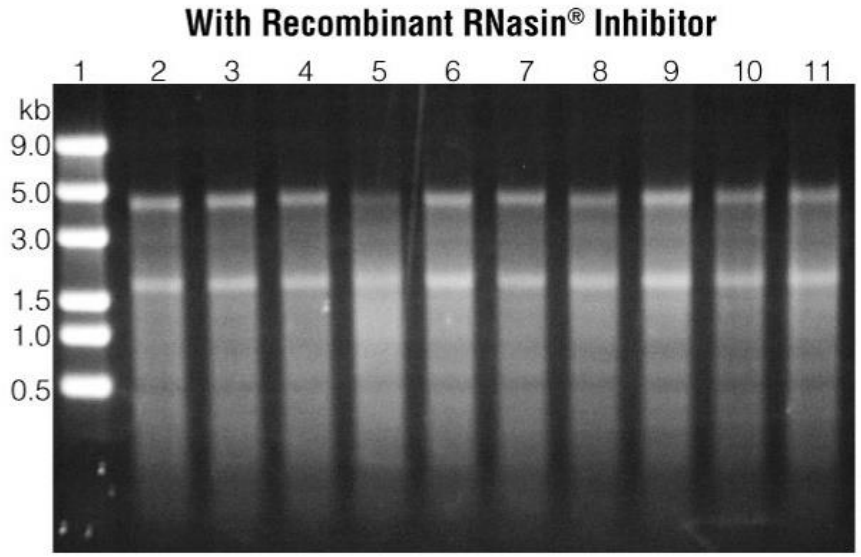
- Extracted RNA:
  - -20°C → days,
  - -80°C → months,
  - -180°C (liquid N_2) → 5 years
- Store DNA/RNA in small aliquots
- Avoid multiple freeze-thaw cycles
- Check nucleic acid concentration after each thawing



Protect RNA Integrity with RNasin®

- Protects RNA from RNase degradation
- Active across broad temperature & pH range
- Compatible with RT, PCR, and transcription workflows
- Available in standard and oxidation-resistant formats





Reverse Transcription – Tips & Tricks

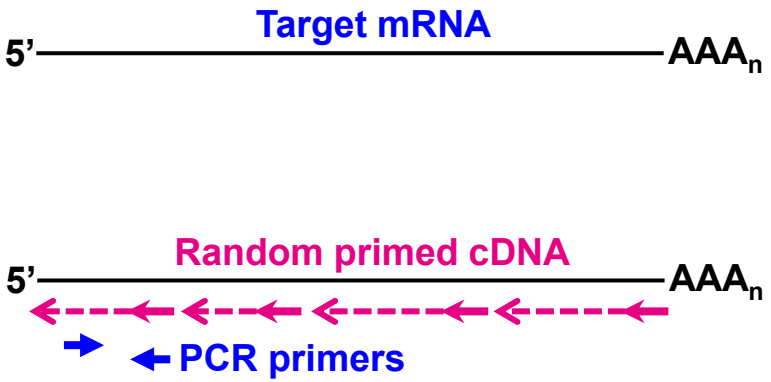
RNA input

- Depends on the abundance of the target in each sample
- Typical 1-2 µg RNA
  - High-copy-number transcript may be detected in as little as 1-10pg
  - Rare or long targets (>8 kb) may require 100 ng - 1 µg or even more

Reverse Transcription – Tips & Tricks

RT Primers

- Random Hexamers
  - cDNA Synthesis from all RNA molecules independent of poly(A)+ tail (including prokaryotic RNA)
- Oligo$(dT)_{15}$ primer
  - Annealing at the 3‘ end of any polyadenylated RNA molecule



Reverse Transcription – Tips & Tricks

Control Reactions

- No Template Control (NTC)
  - Check for contamination within the system independent of your RNA isolation
- -RT (No Reverse Transcription Control)
  - Test for the presence of contaminating genomic DNA or plasmid DNA in the RNA template
- Positive Control
  - Known positive sample

Reverse Transcription – Tips & Tricks

Choice of RT enzyme

- Depends on
  - Length
  - Secondary structures
  - GC content
- of transcripts

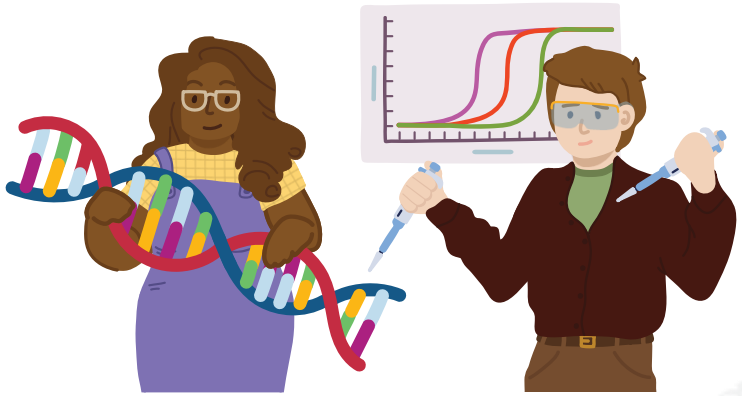
FEATURES	GoScript™ Reverse Transcriptase	AMV Reverse Transcriptase	M-MLV Reverse Transcriptase	M-MLV Reverse Transcriptase RNase H-, Point Mutant
Reaction temperature	37–55°C	37–58°C	37–42°C	40–55°C
cDNA length	Up to 9 kb	Up to 4 kb	Up to 5 kb	Up to 7.5 kb
Sensitivity	0.2 fg–5 µg total RNA	1 pg–1 µg total RNA 1 pg–100 ng poly(A)+ RNA	NA	100 fg–100 ng total RNA
RNase H-activity	low	yes	low	no
Suitable for RNAs with secondary structure	★★★	★★★	★	★★★
Error rate	NA	Approx. 5 errors in 10,000 bases	Approx. 1 error in 10,000 bases	Approx. 1 error in 10,000 bases
Main applications	› RT-PCR › Incorporation of marked nucleotides › Primer extension/RACE	› Reverse transcription › Primer extension/RACE	› Reverse transcription › Primer extension/RACE	› Reverse transcription › Primer extension/RACE
Advantage	› Low RNase H activity › For cDNA up to 9 kb › Optimized conditions for one-tube RT-PCR and RT-qPCR › Particularly resistant to inhibitors	› Especially suitable for RNA with secondary structures › For cDNA up to 4 kb › High processivity	› Low RNase H activity › For cDNA up to 5 kb	› No RNase H activity › For cDNA over 7.5 kb › Reaction temperature up to 55°C › Very stable › High selectivity

End-point PCR

Qualitative detection = "Is something there? Can I detect it?"

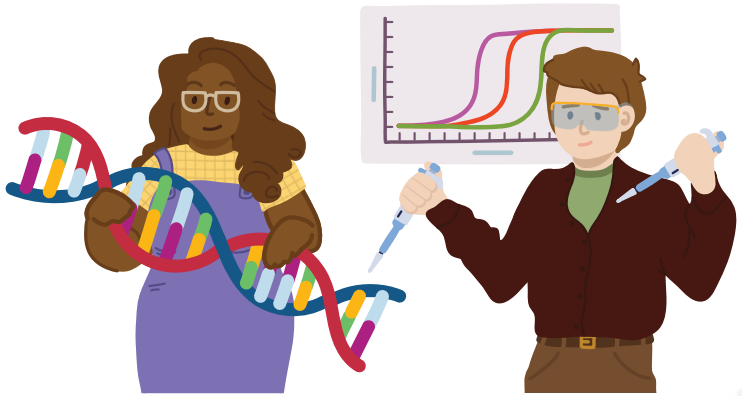
Real-Time PCR

Quantitative detection = "Exactly how much is there?"



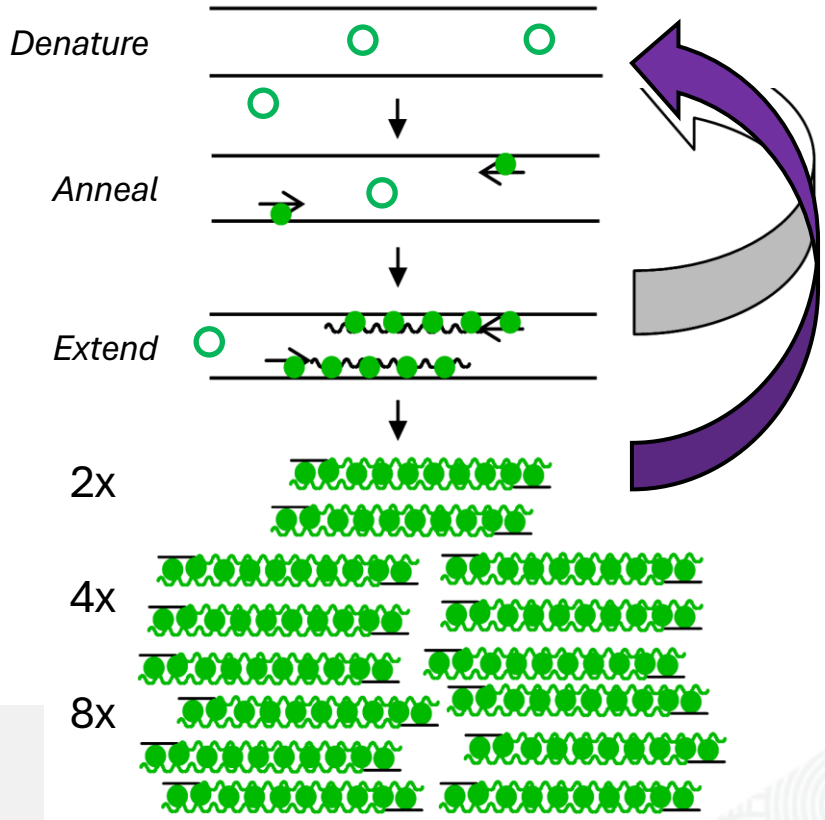
qPCR Chemistries

- A fluorescent reporter is used to detect product formation
  - Part of the reaction mix
  - Two general types
    - dsDNA binding dye
    - Labelled primer or probe



Dye-based approach

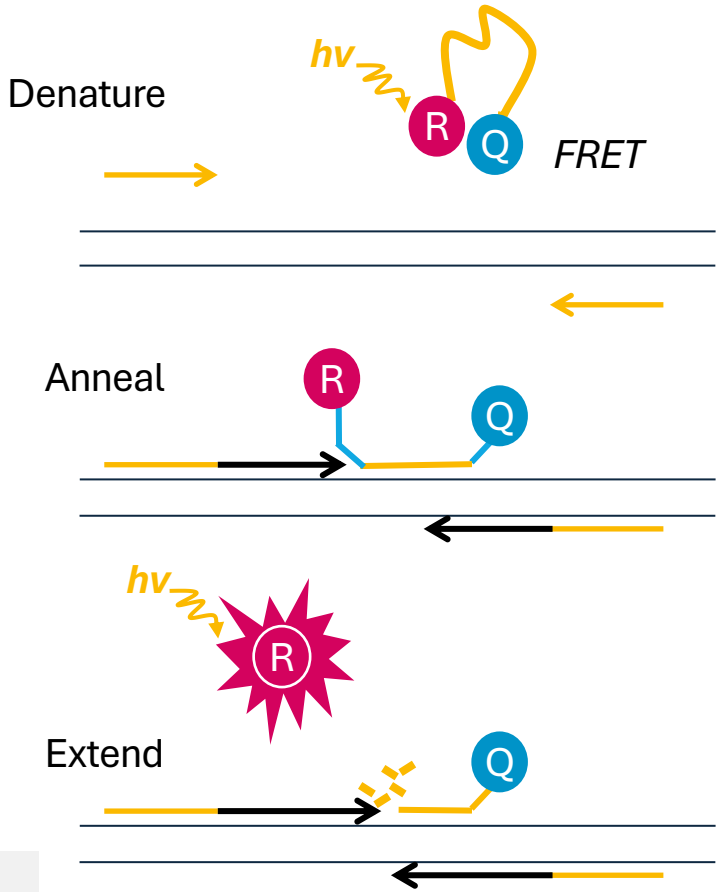
- dsDNA-binding dye is included in PCR master mix
- dye associates with PCR product
  - Free Dye → low fluorescence
  - Bound Dye → high fluorescence
- As more PCR product is produced, more dye is bound



Fluorescence is proportional to the amount of product

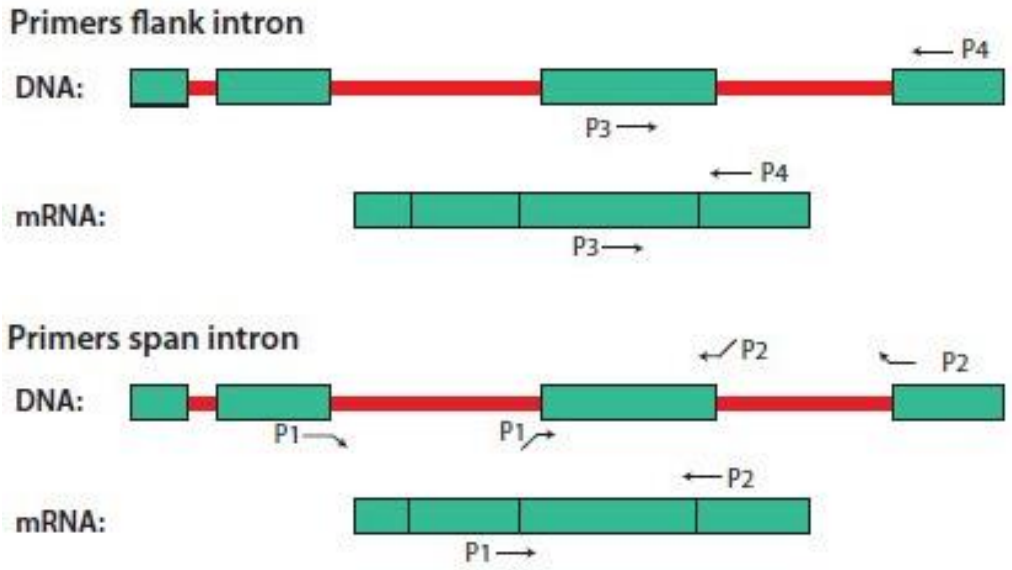
Probe-based approach

- 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 un-quenched



Fluorescence is proportional to the amount of product

Optimize your primer design



→ Possible gDNA amplification

→ no gDNA amplification even in presence of gDNA

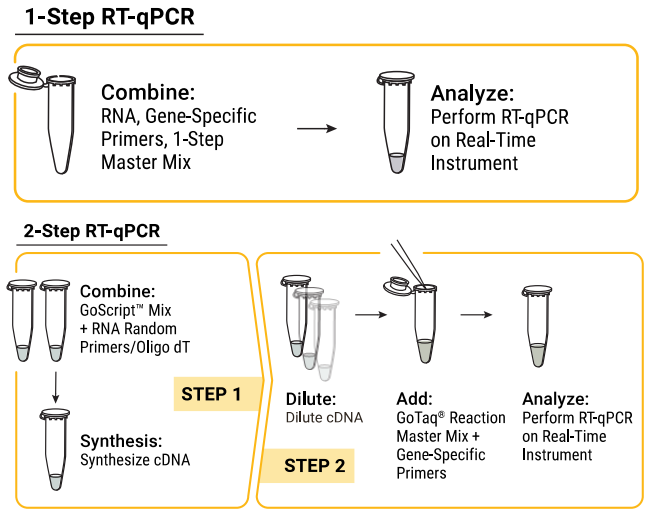
http://www.sigmaaldrich.com/technical-documents/articles/biology/pcr-qpcr-dpcr-assay-design.html

qPCR Chemistries

	Dye-based qPCR	Probe-based qPCR
PCR product labeling	dsDNA-binding dye	Fluorescently labeled probes
Cost	Lower cost	Higher cost
Instrumentation	All qPCR instruments	Must match probes to filters
Specificity	Measures all dsDNA	Measures amplicon with probe sequence
Multiplexing	No	Yes – different dyes/filters
Melt analysis (QC and genotyping)	Yes	No (TaqMan)
Throughput	High	Highest (multiplexed)
Sample required	Low	Lowest (multiplexed)
Requires validation	Yes	Yes

Promega's qPCR Chemistries

	Dye-based qPCR	Probe-based qPCR
Genomic DNA / cDNA	GoTaq® qPCR Master Mix	GoTaq® Probe qPCR Master Mix
RNA	GoTaq® 1-Step RT-qPCR	GoTaq® Probe 1-Step RT-qPCR
	GoTaq® 2-Step RT-qPCR	GoTaq® Probe2-Step RT-qPCR



- Low chances of cross contamination
- Faster results
- No need to store the cDNA

- Optimized performance of both RT and PCR steps
- cDNA available for other procedure
- Many targets per sample

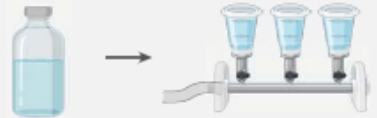
Sample collection & Processing | RNA Extraction | RNA QC & Protection | Reverse Transcription | qPCR

GoTaq® Probe	GoTaq® Enviro	GoTaq® Endure
Broad spectrum of applications	Specifically for environmental samples	Specially developed for very high inhibitor concentrations
		
Compatible with variety of samples.	Tested samples like water, soil and biological material	Tested for blood, bacteria, viruses, feces, soil, plants and food samples
Tested for inhibitors, but only up to a certain concentration (e.g. up to 50 µM hematin, see Endure: 500 µM hematin)	Tested for inhibitors such as humic and tannic acid.	Tested for inhibitors such as EDTA, EtOH, Humaic acid, Hematin (500 µM), Heparin, Sodium Citrate but not tested for tannic acid
For general use rather than extreme conditions		Fewer reaction failures and optimizations → Saves time and costs
Multiplexing capability ✓	Multiplexing capability ✓	Multiplexing capability ✓
Probe-based ✓	Probe-based ✓	Probe-based ✓
Fast-Cycling ✓	Fast-Cycling ✓	Fast-Cycling ✓

Legionella Testing from Water Samples using qPCR

Optional

Sample Filtration



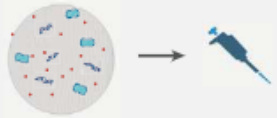
OR

Suspension Sample

15 min

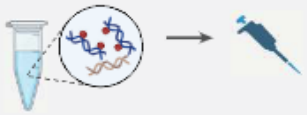
Concentrate bacteria through filtration or use an aliquot from a sample.

On-Filter Viability Reagent Treatment & Neutralization



OR

Suspension Viability Reagent Treatment & Neutralization



75 min

To eliminate false positive results, DNA of non-viable cells is blocked from amplification by treating the filter or suspended sample with the viability reagent (included in the vPCR kits).

DNA Purification

Automated

Maxwell® RSC PureWater (AS2110)

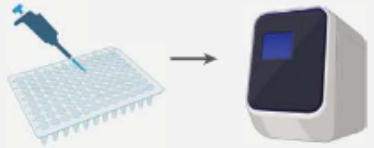
Manual

Wizard® PureWater (A3130)

40–60 min

DNA isolation and purification using either the automated Maxwell® RSC PureWater kit, or for manual methods, the Wizard® PureWater kit.

Real-time PCR



GoTaq® *Legionella pneumophila* qPCR Kit (AM2201/AM2205)

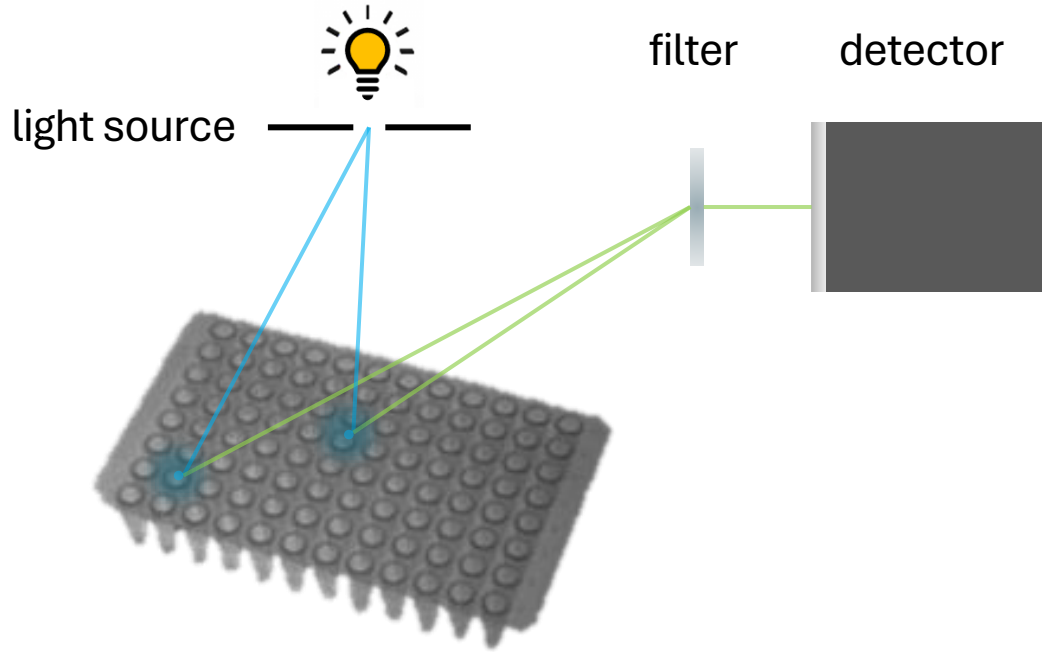
GoTaq® *Legionella spp/pneumophila/SG1* qPCR Kit (AM2202/AM2206)

120 min

PCR amplification of *Legionella spp., L. pneumophila,* and Serogroup 1 (SG1). 2 kit configurations are available either with or without viability treatment.

Viability Selection is a new feature **outside of** ISO 12869:2019

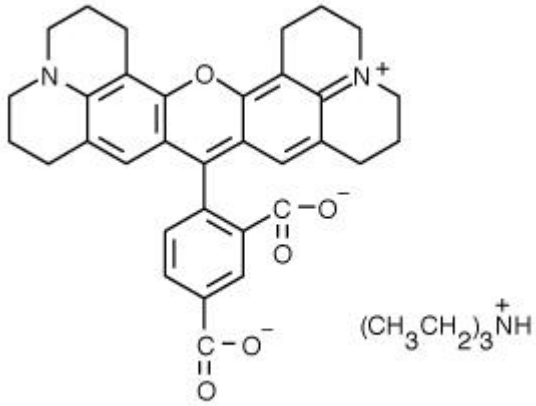
Passive Reference



second dye in solution
as passive reference for
signal normalization

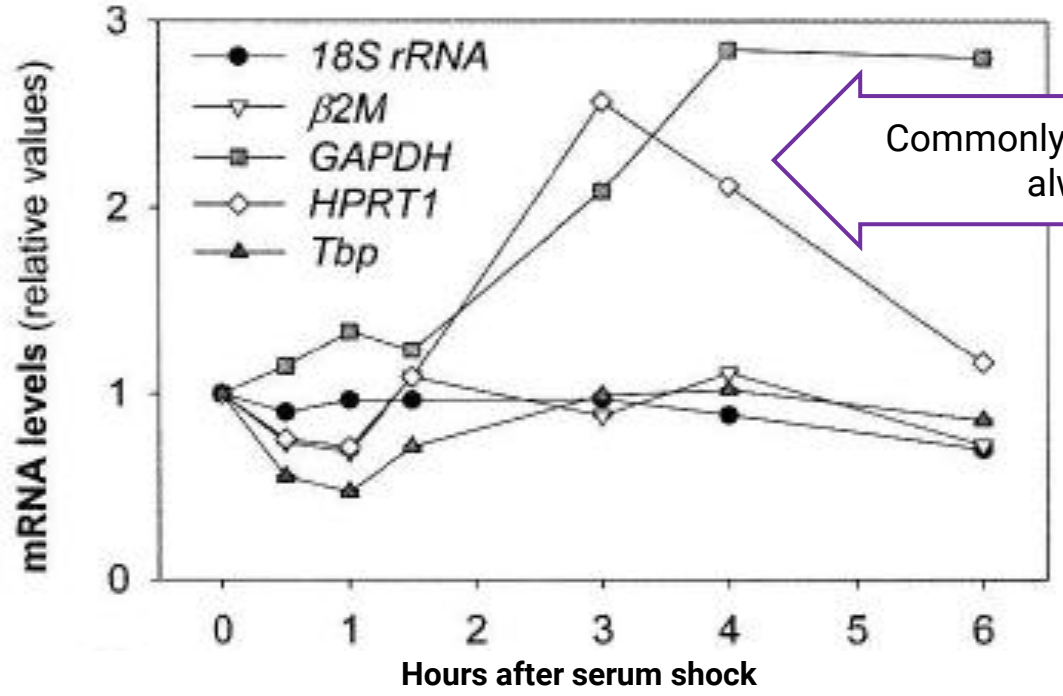
differences in intensity due to
- different beam path length
- variation in signal collection
- technical artifacts

→ **Almost all cyclers need additional CXR**



5-carboxy-X-rhodamine, triethylammonium salt
(CXR = ROX™)

Reference genes



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

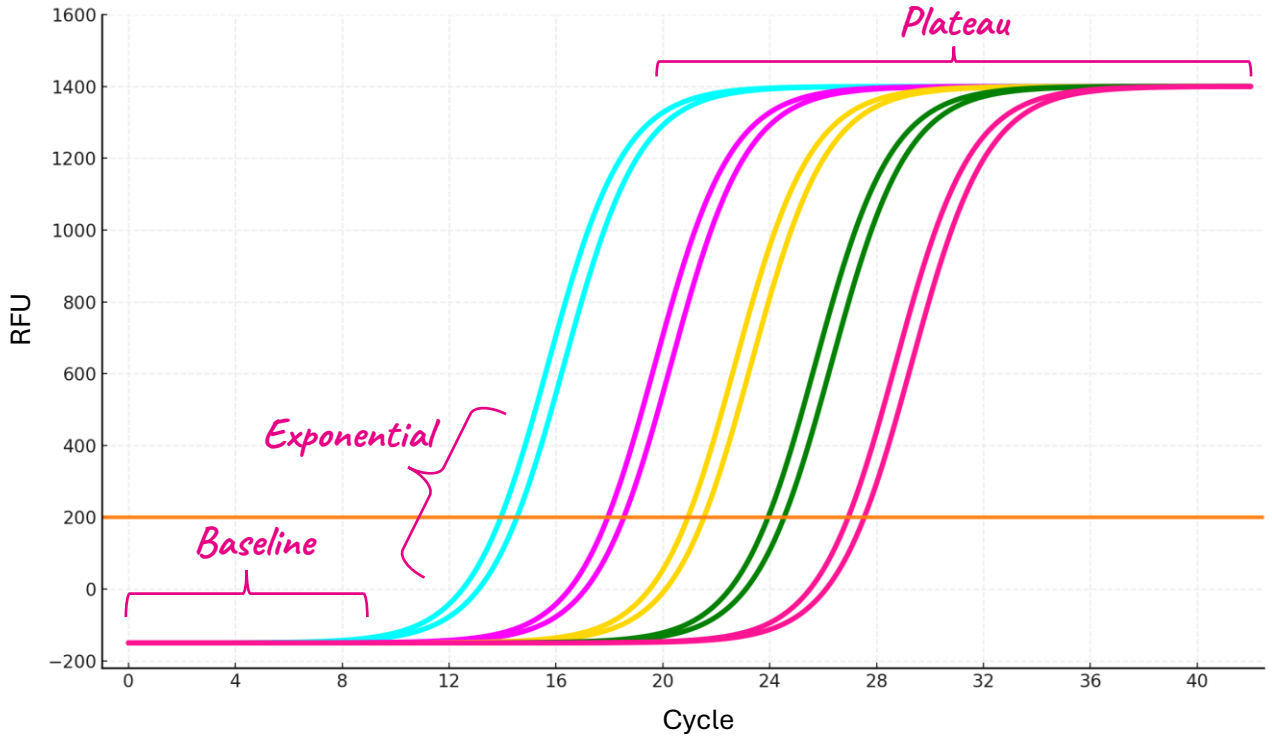
Commonly used normalizers are not always constitutive

one fits **NOT** all!!

Primary output: Amplification Curve

Amplification Curve – shows accumulation of product as PCR progresses

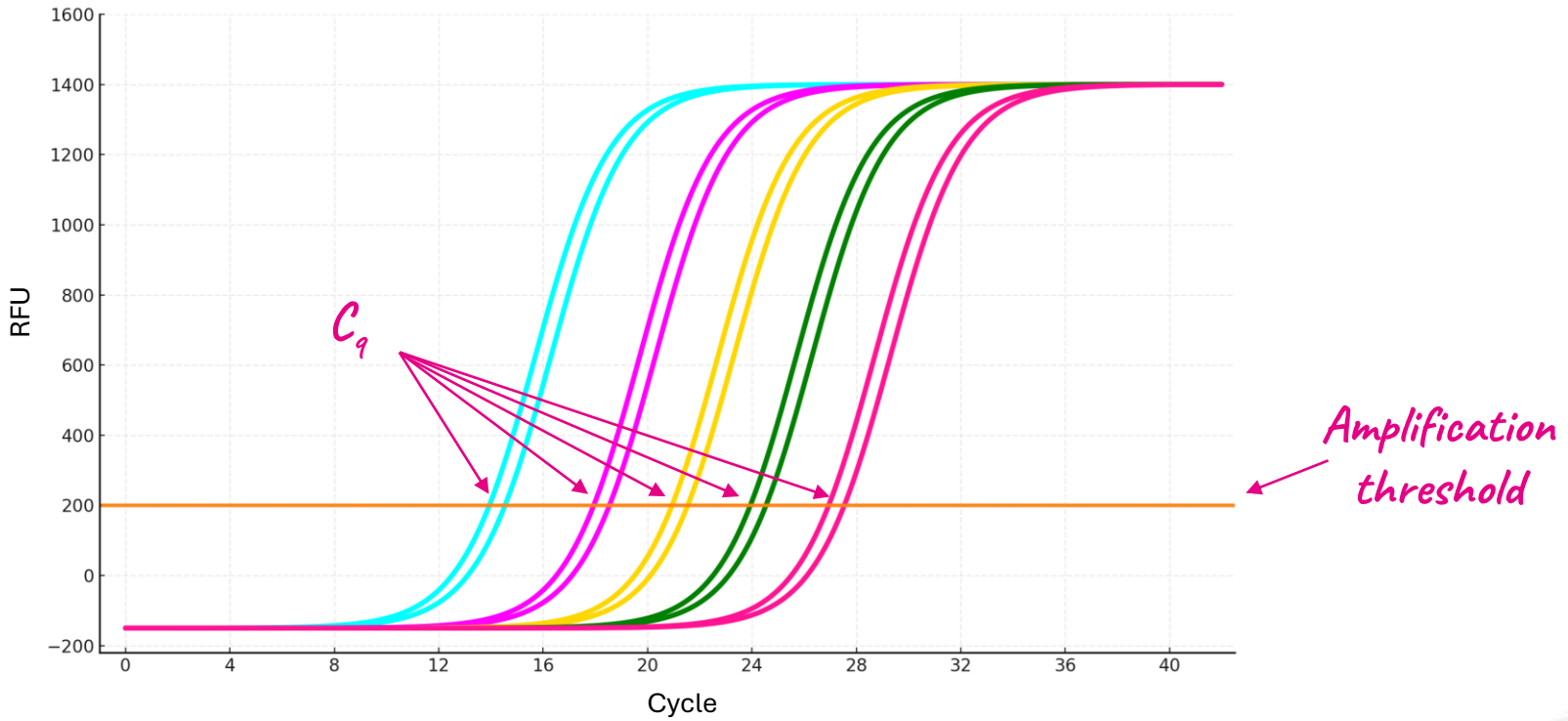
- **Baseline** – initial reporter fluorescence, before significant product formation occurs
- **Exponential phase** – stage of reaction when product is doubling with each cycle
- **Plateau phase** – stage of reaction when rate of product formation is diminishing



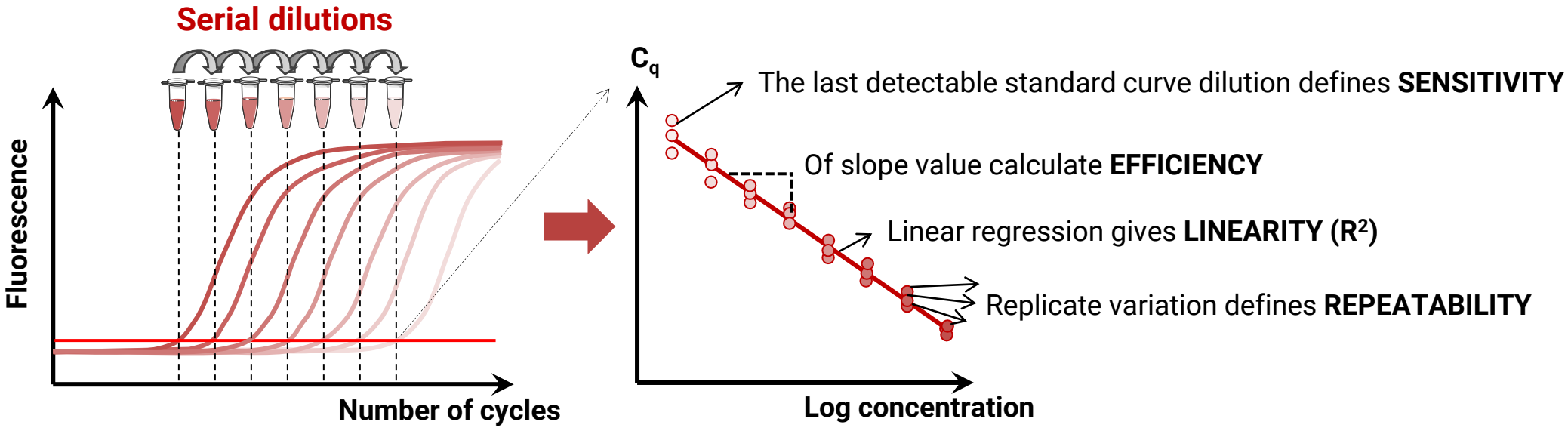
Analysis of the amplification curves gives Cq values

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



Validation of qPCR reactions

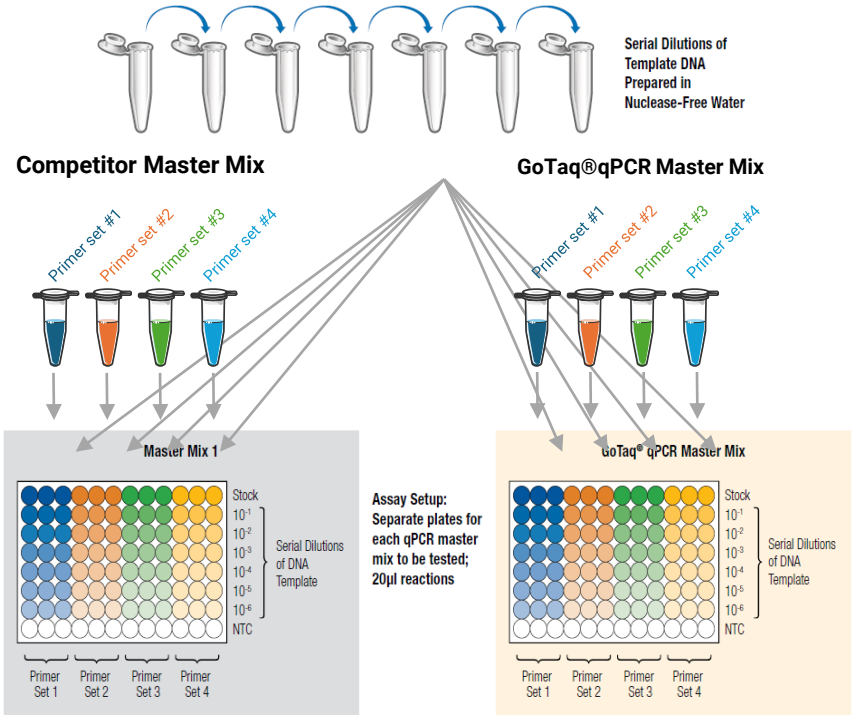


STANDARD CURVE should be:

- At least 5 log dilutions starting at 100 ng
- 1:5 or 1:10 serial dilution
- In triplicates

SENSITIVITY	Cover the sample concentration range	
EFFICIENCY	$E = 10^{\frac{-1}{slope}} - 1$	90% < E < 110%
LINEARITY	$0.98 < R^2 < 1$	
REPEATABILITY	St dev between tech replicates < 0.5	

Assay setup for a qPCR reagent comparison



		MM1	GoTaq®
Activation	1 cycle	5 min, 95°C	**2 min, 95°C**
Denaturation	40 cycle	15 sec, 95°C	
Annealing/Extension		40 sec, 60°C	
Melt (dye-based)	Instrument defined		

1) **Prepare serial dilutions**
- Use same standards dilutions with both reagents

2) **Make bulk reaction mixes of reagents**
- 2X qPCR Master Mix
- Primers
- Water
- CXR (if required)

3) **Use separate plates**
- 1 plate for each Master Mix
- 2 separate runs

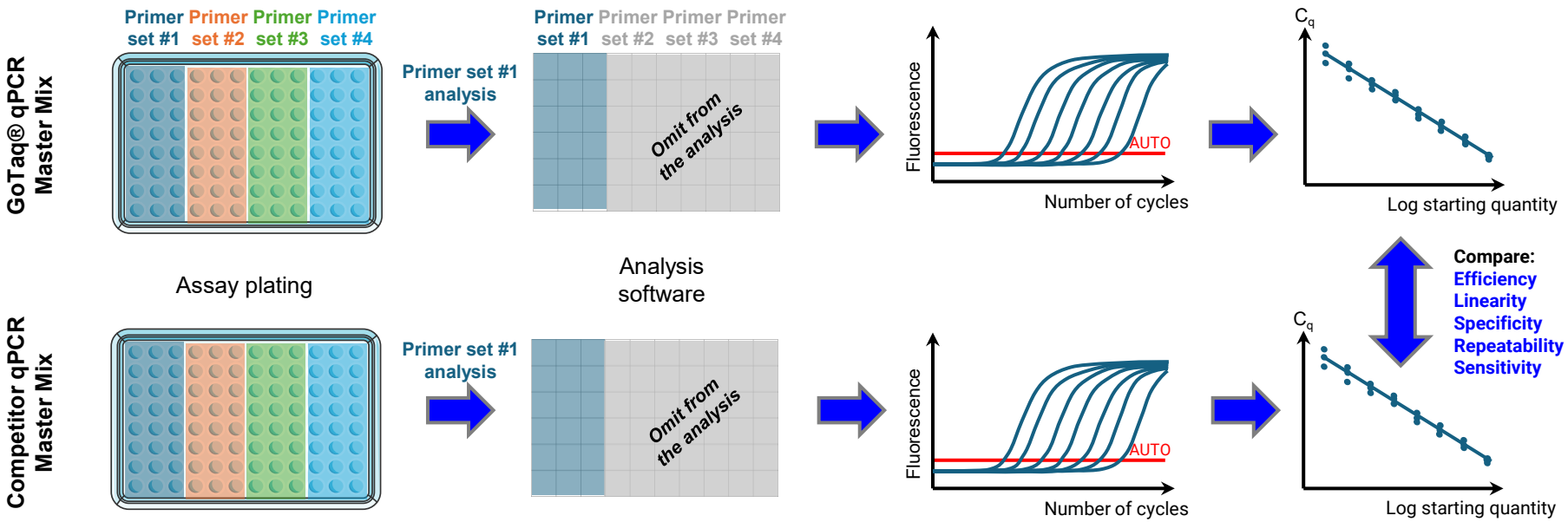
4) **Program thermal cycling conditions**
- Use optimized cycling conditions
- **But** change activation for GoTaq® to 2 min, 95°C

https://www.promega.de/resources/tools/biomath-calculators/

Data analysis of a qPCR reagent comparison

5) Analyze reactions for each assay separately

- Different reagent, primers, & fluorophores can influence settings



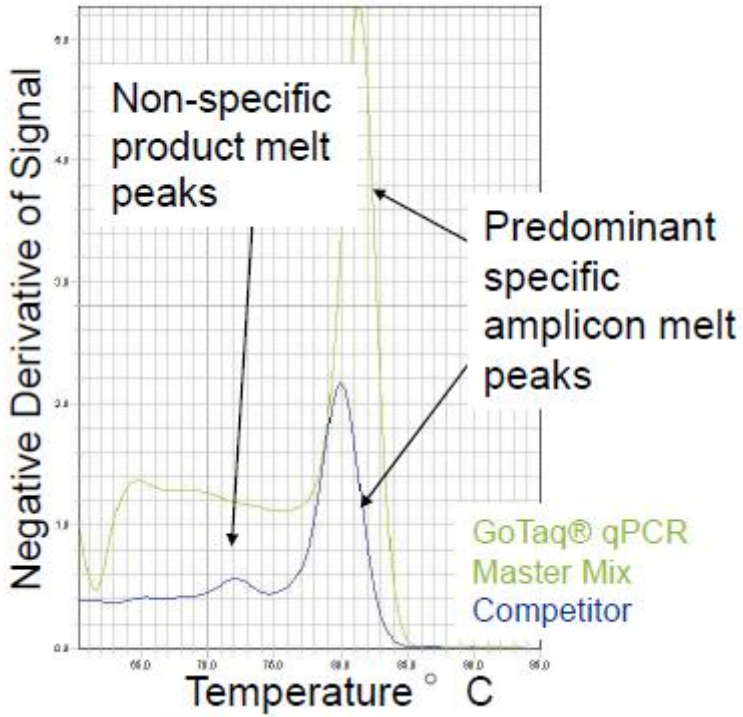
6) Use automatic baseline and threshold settings for each master mix separately

- Changes in threshold can alter C_q by >3 cycles!
- Use auto-threshold for best comparison even if you typically use a manual threshold

Data analysis of a qPCR reagent comparison

Reaction specificity by melt curve and/or gel analysis

- Specificity is a factor of primer design and can be influenced by the composition of the qPCR master mix (i.e., salts)
- To assess specificity, look for non-specific amplification products
  - Melt curve analysis with dye-based qPCR chemistries
    → no secondary peaks/shoulders
  - And/or electrophoresis gel with probe-based chemistries
    → no additional bands



Teter et al. (2016) Promega Poster

Summary

- Proper sampling handling is very important for RNA extraction
- Using ReliaPrepTM RNA Miniprep Systems for RNA extraction leads to high quality RNA
- Specialist kits for miRNA and mRNA extraction: ReliaPrep™ miRNA Cell and Tissue Miniprep System and PolyATtract® mRNA Isolation Systems
- RNasin® minimizes the risk of RNA degradation, improves long-term storage, prevents oxidation
- GoTaq® Dye-based Real Time PCR Systems and GoTaq® Probe qPCR System
- Factors to consider for protocol optimization: primer design, concentrations of RNA and primers as well as choice of house keeping genes
- Data quality & data validation are crucial for data interpretation

Questions?

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Thank You!
Děkuju