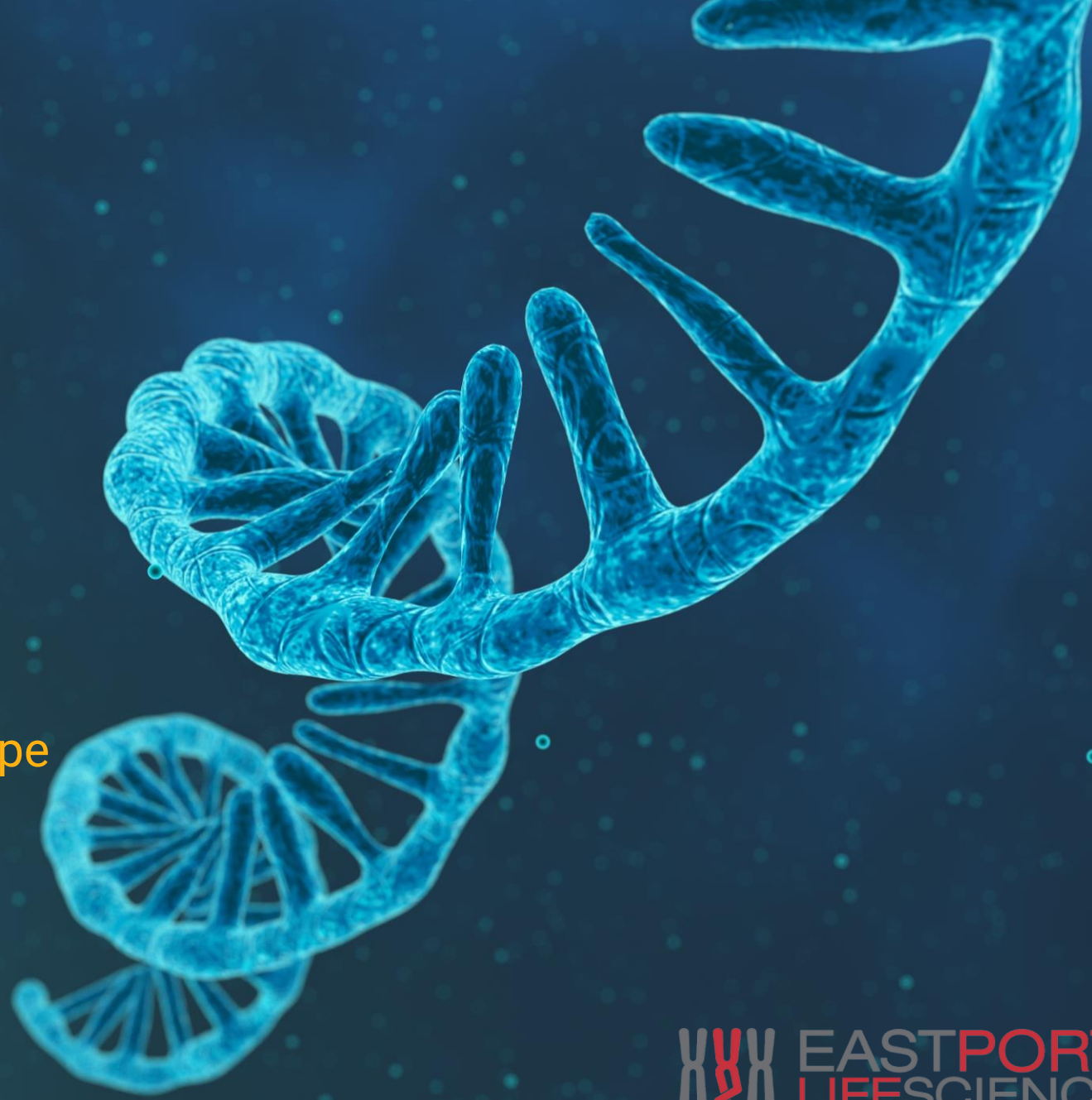
Promega

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



Dr. Kerem Yıldırım
Area Manager, Central Eastern Europe
Promega Germany
June 12, 2025

EASTPORT LIFESCIENCE

Our Mission

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

Supporting Science Around the World

100+
Countries

50+
Distributors

- Headquarters
- R&D and Manufacturing
- Branch Office

Our Products Support

Government and Academic Research Laboratories

Forensic and Paternity Laboratories

Pharmaceutical and Biotechnology Industries

Clinical and Molecular Diagnostics Laboratories

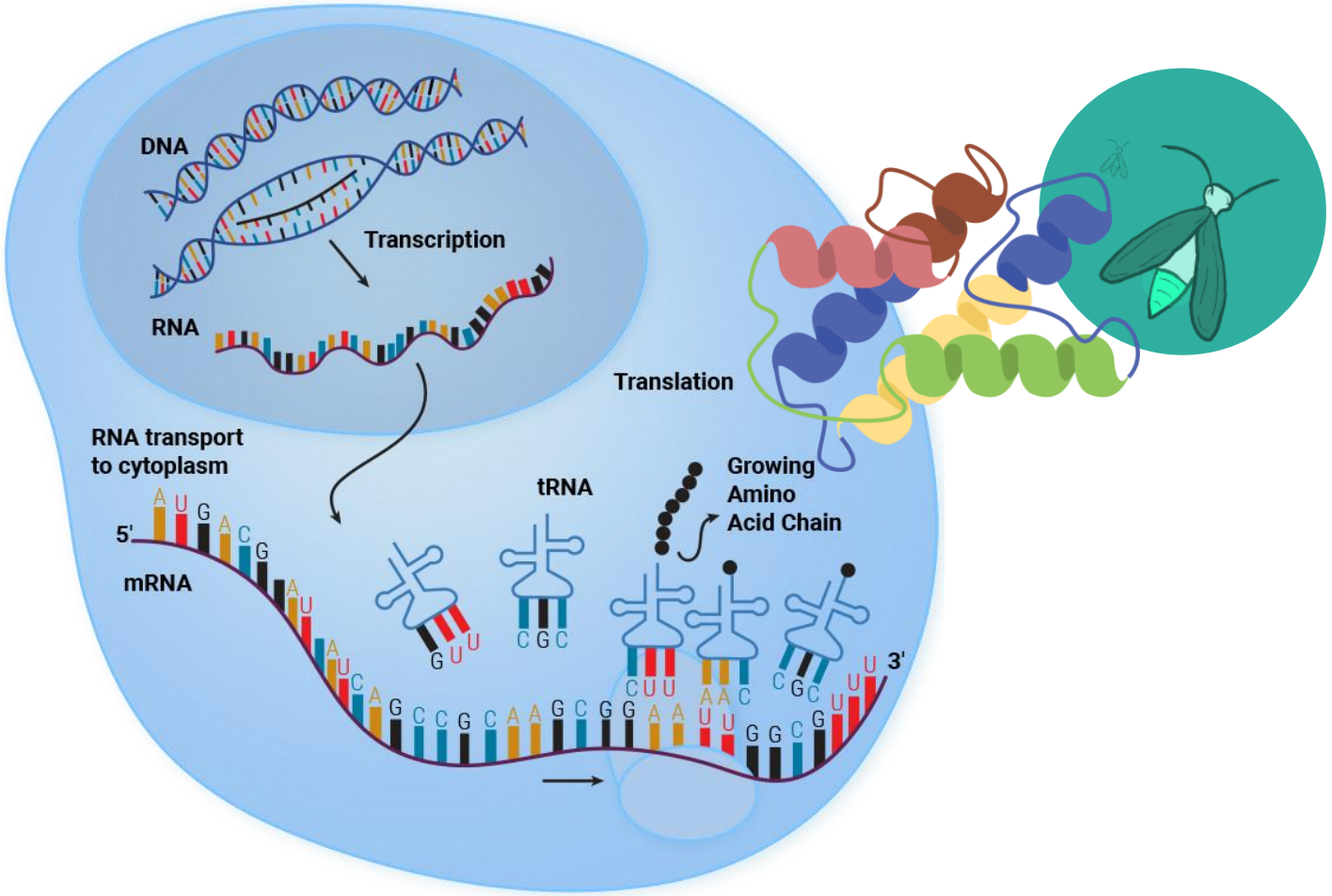
Food and Water Safety Testing Facilities

Product Portfolio

DNA & RNA Analysis	Cellular Analysis	Protein Analysis	Genetic Identity	Molecular Diagnostic	Drug Development
• DNA and RNA Purification • DNA Amplification PCR and qPCR • Reverse Transcription and RNA protection • Sequencing Sample Preparation • Cloning, Enzymes and DNA Markers • Transfection and Epigenetics	• Cell Health (viability, cytotoxicity, apoptosis) • Cellular Metabolism • Cell Signaling • Reporter Assays • Imaging	• Mass Spectrometry • Immunoassays • Protein Quantification • Protein Expression • Protein Purification • Protein Interaction	• Forensic and Paternity Testing • STR Typing	• cGMP Manufacturing • Gene Analysis and Mutation Determination	• Biologics • Small-Molecule Drug Discovery
Instrumentation					
• Instruments for DNA and RNA Extraction and Quantification	• Luminometer, Fluorometer and Bioluminescence Imager		• Capillary Electrophoresis Systems		

In 2020, PCR testing became a household name due to its role in detecting COVID-19. Almost overnight, the world witnessed the power of molecular diagnostics at an unprecedented scale.

The Central Dogma



Gene Expression is Analyzed via RNA

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

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
    - Biological weapons

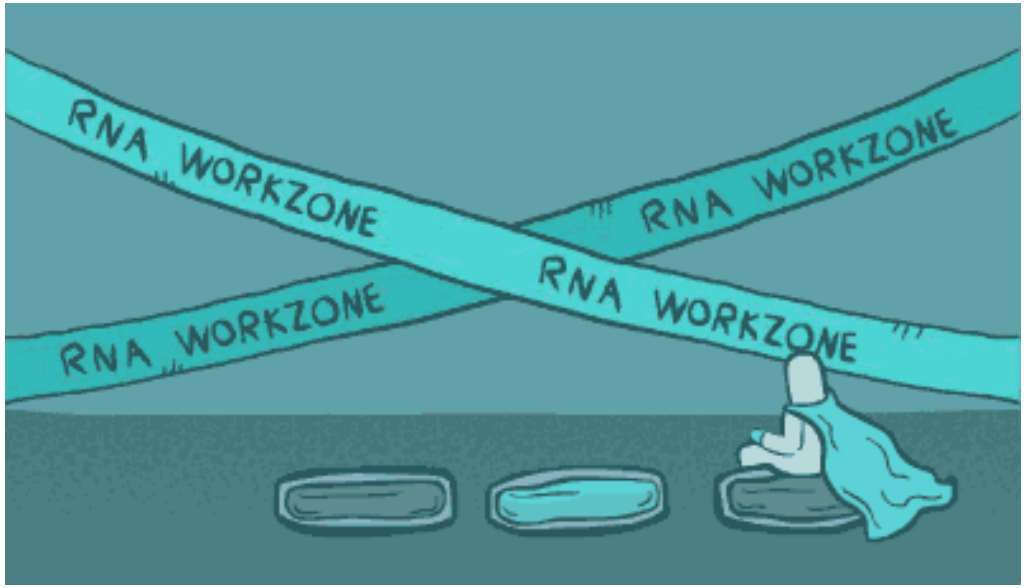
Image is generated using AI

Workflow

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

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



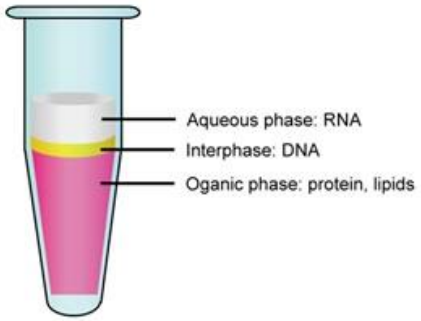
Cellular Total RNA

- 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



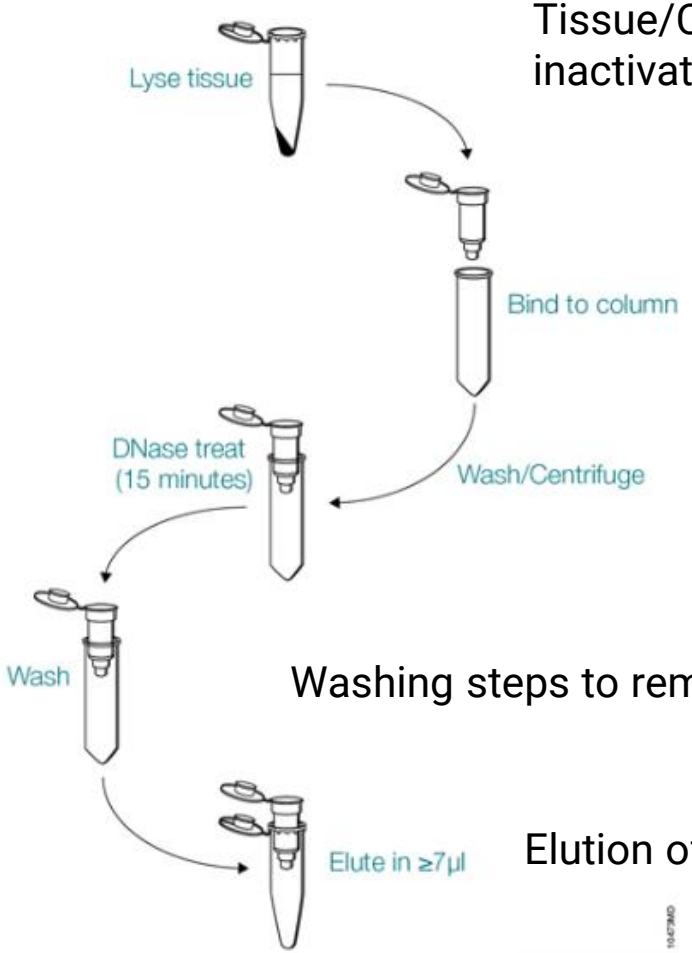
https://www.creative-diagnostics.com/images/Protocol-Total-Protein-Extraction-by-Trizol-1-phase-separation.jpg

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

ReliaPrep™ RNA Miniprep Systems

ReliaPrep™ RNA Miniprep Systems



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)

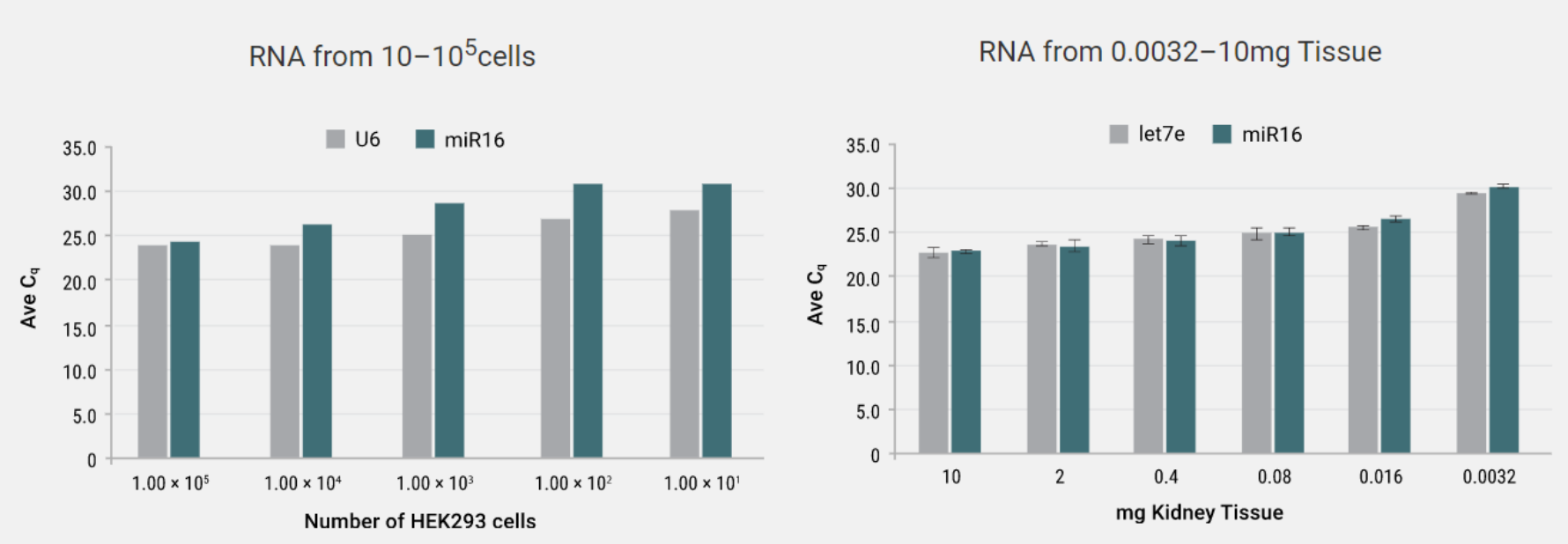
Elution of RNA in 7-50 µl (Water/TE-Buffer)

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

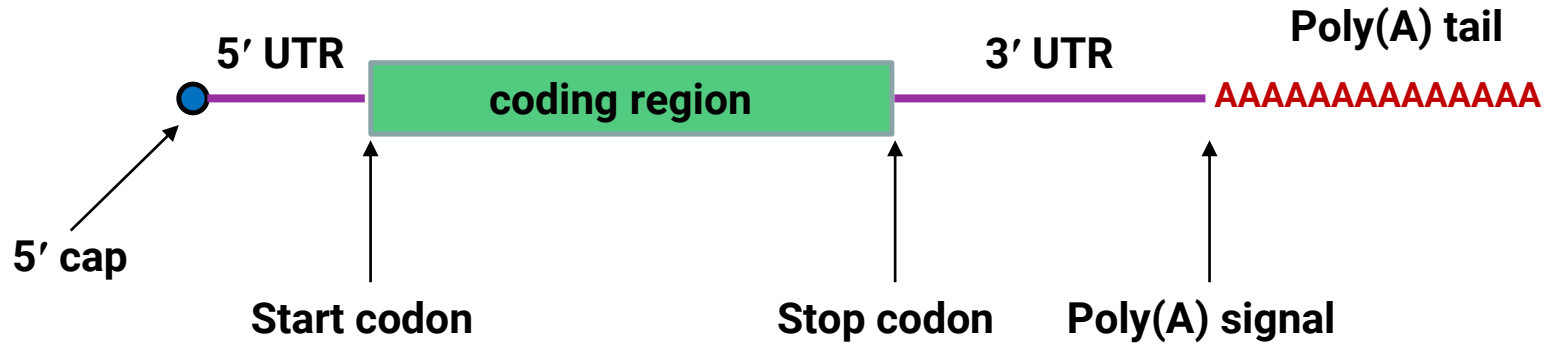
ReliaPrep™ miRNA Cell and Tissue Miniprep System

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

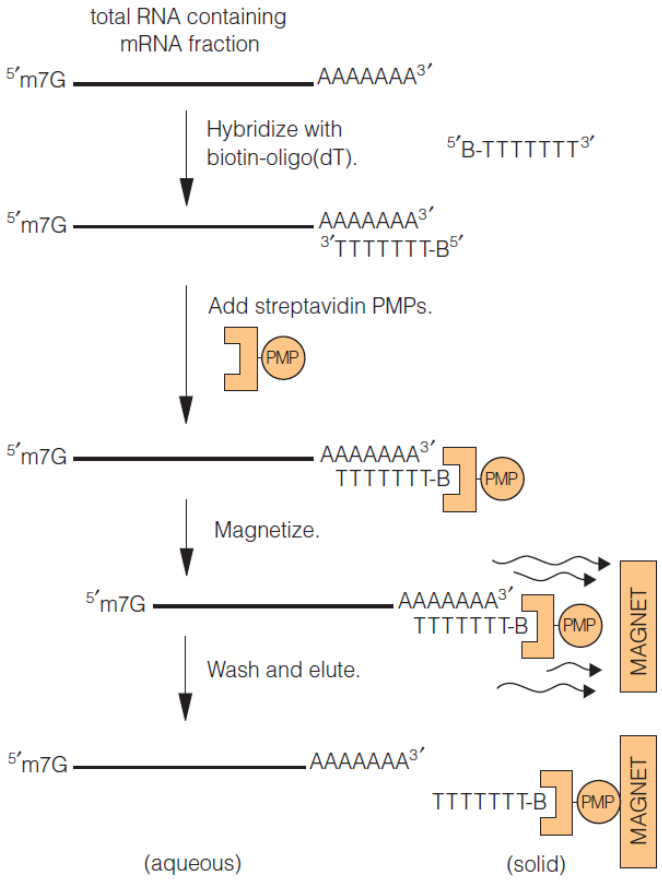


Messenger RNA Extraction

- 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



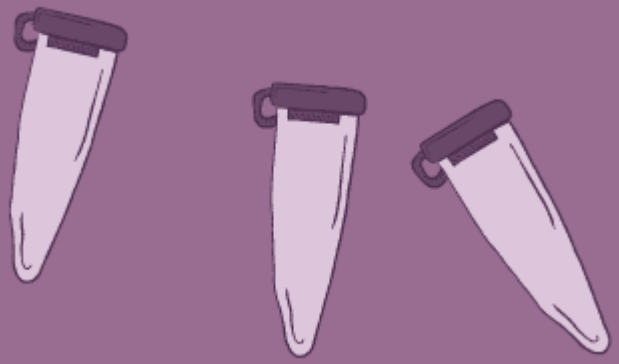
PolyATract® mRNA Isolation Systems



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.

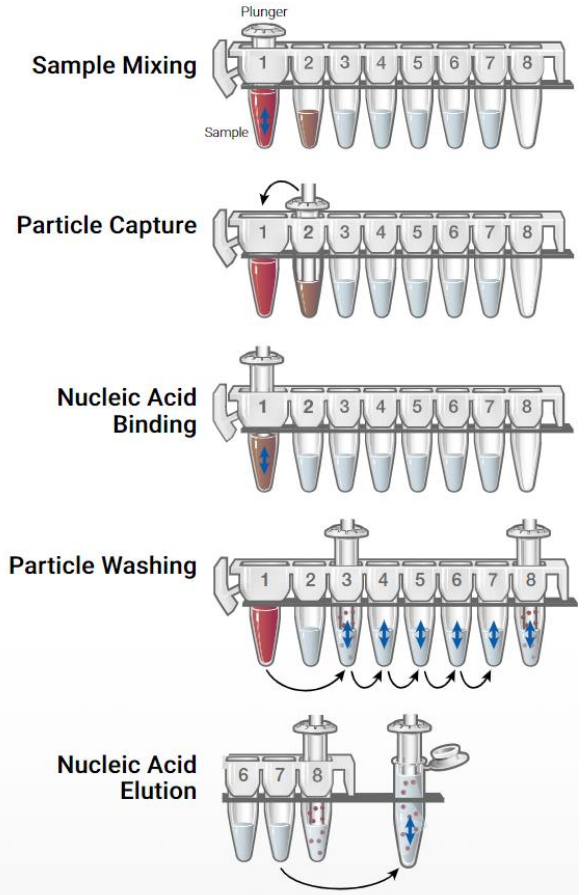


Maxwell® RSC Systems – Automate Your Workflow

- 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® RSC Systems – Automate Your Workflow

- A magnetic particle mover, not a liquid handler, it offers advantages over other automated systems
- Minimal risk of cross-contamination
- No clogging worries
- Increased sensitivity and reproducibility due to the higher binding capacity of paramagnetic particles
- No additional equipment, e.g., centrifuges for spin columns, is required



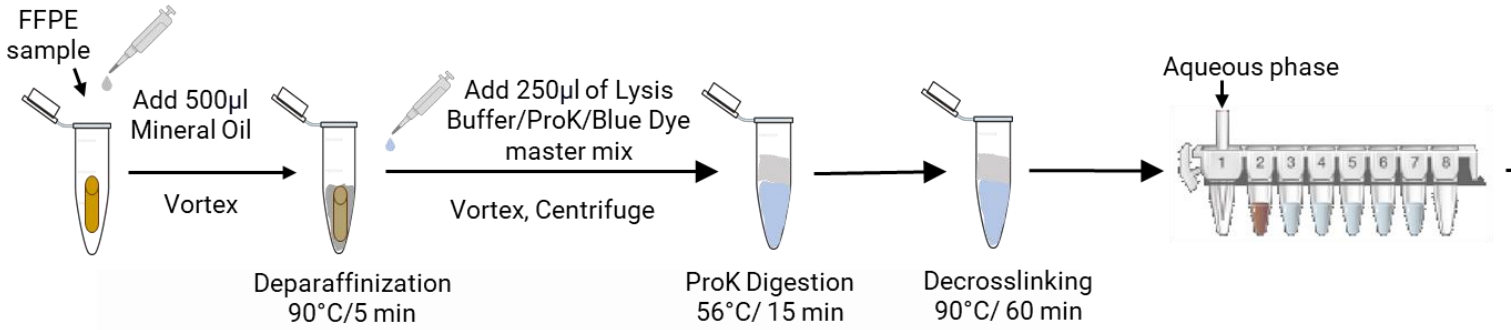
Maxwell® RSC Systems – Automate Your Workflow

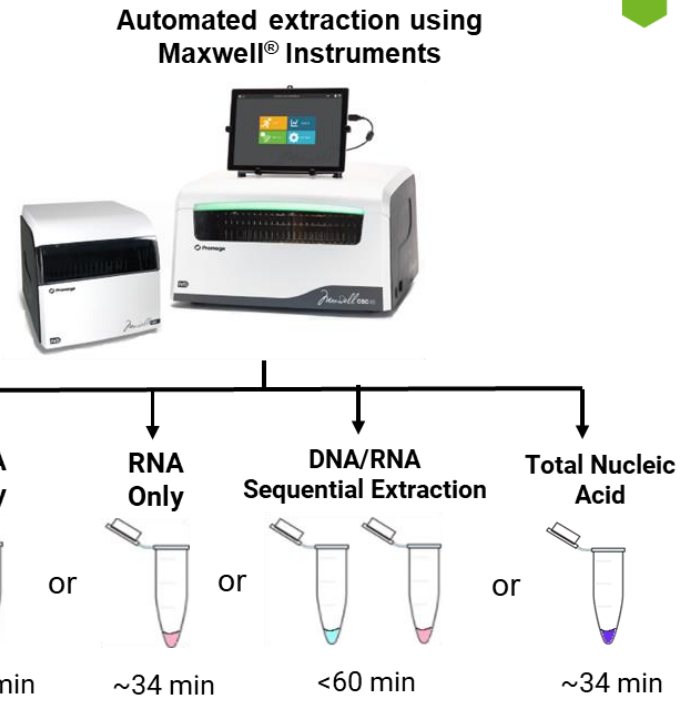
Biofluids	Cells/Tissues	Environmental	Microbes & Viruses
Blood	Mammalian Tissue	Feces	Bacteria
Saliva & Mucus	FFPE	Soil	Fungi
Plasma/Serum	Plant & Insect	Water	Archaea
Urine	Swabs	Wastewater	Protist

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

Analysis of RNA Purity

- RNA quality and purity are much more important than yield
- RNA sample absorbances are determined on the spectrophotometer at 260nm, 280nm, and 230nm
  - 260nm: Nucleic acid (DNA, RNA, nucleotides)
  - 280nm: Protein
  - 230nm: Some organic compounds and chaotropic salts

$$A_{260}/A_{280} \ \& \ A_{260} / A_{230}$$

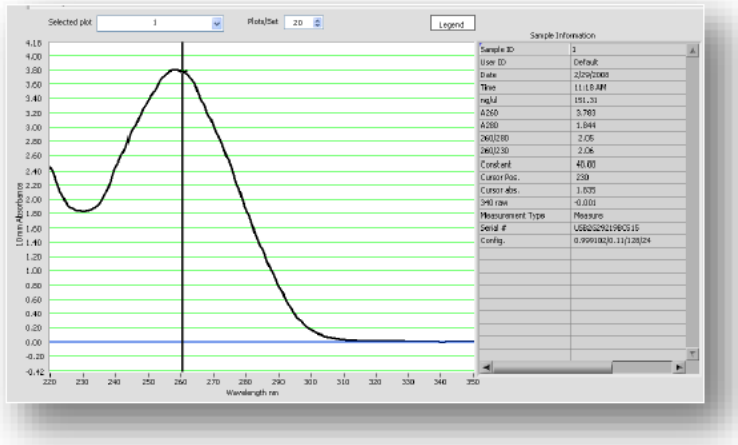
  - Properly purified RNA should exhibit ratios within the range of 1.8 - 2.0.
  - If the RNA exhibits a ratio lower than 1.7, this may indicate the presence of co-purified contaminants

RNA/DNA Quality Check with NanoDrop™

Spectrophotometer: Absorbance ratios

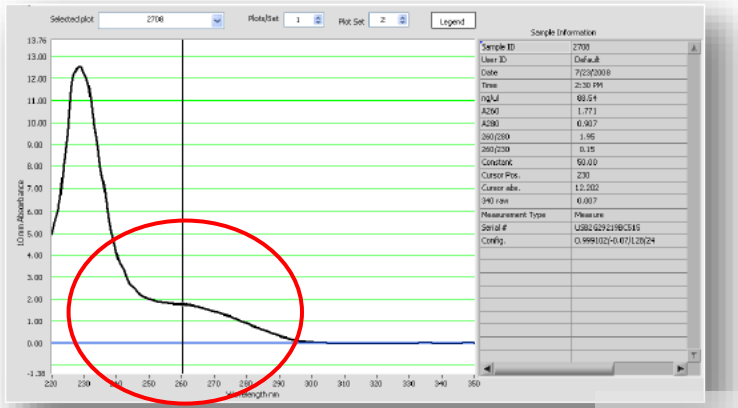
Large peaks at wavelengths lower than 260nm can influence the measured peak at 260nm, and low absorbance levels often yield unreliable concentrations

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?

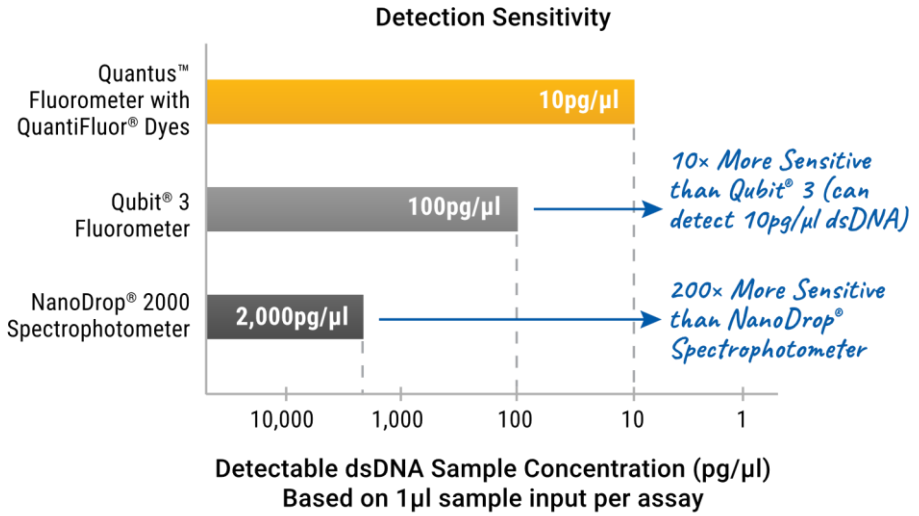
Possible overestimation of nucleic acid concentration due to contaminants

RNA/DNA Quantification

- *Bioanalyzer – RNA 6000 Nano total RNA-Kit:*
  - 5 – 500 ng/µl
- *Quantus™ Fluorometer*
  - Fluorescent dye offers greatest sensitivity & dynamic range
  - Detection limit: RNA 100 pg/µl // dsDNA 10 pg/µl,

Quantus Fluorometer & QuantifFluor® Dye Systems

- **Sensitive DNA and RNA Quantification**
- Highly sensitive fluorescent detection
- Ready to use with optimized QuantiFluor® Dyes
  - QuantiFluor® RNA System

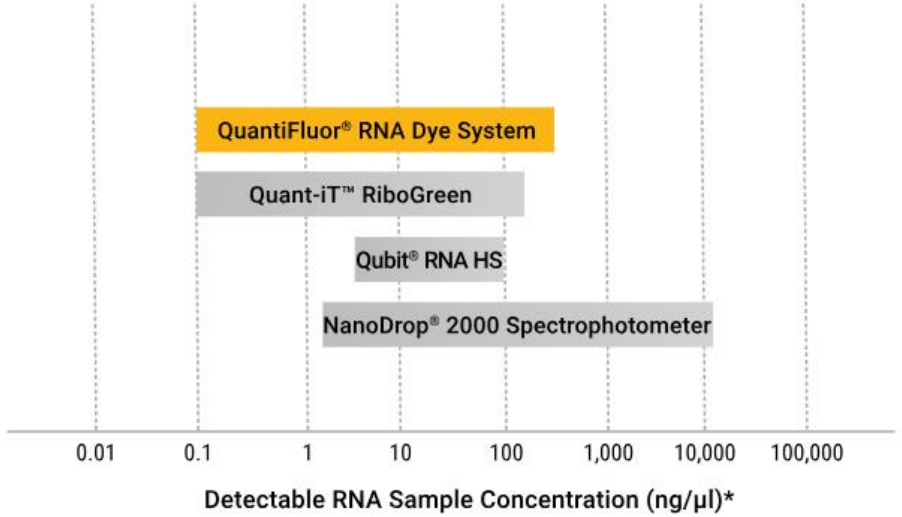


Quantus Fluorometer & QuantifFluor® Dye Systems

- **Sensitive DNA and RNA Quantification**
- Highly sensitive fluorescent detection
- Ready to use with optimized QuantiFluor® Dyes
  - QuantiFluor® RNA System

	Sample*	Assay
QuantiFluor® RNA System	0.1–500ng/µl	0.1–500ng
Quant-iT™ RiboGreen	0.1–200ng/µl	0.1–200ng
Qubit® RNA HS (5–100ng)	5–100ng/µl	5–100ng
NanoDrop® 2000 Spectrophotometer	2–12,000ng/µl	2–12,000ng

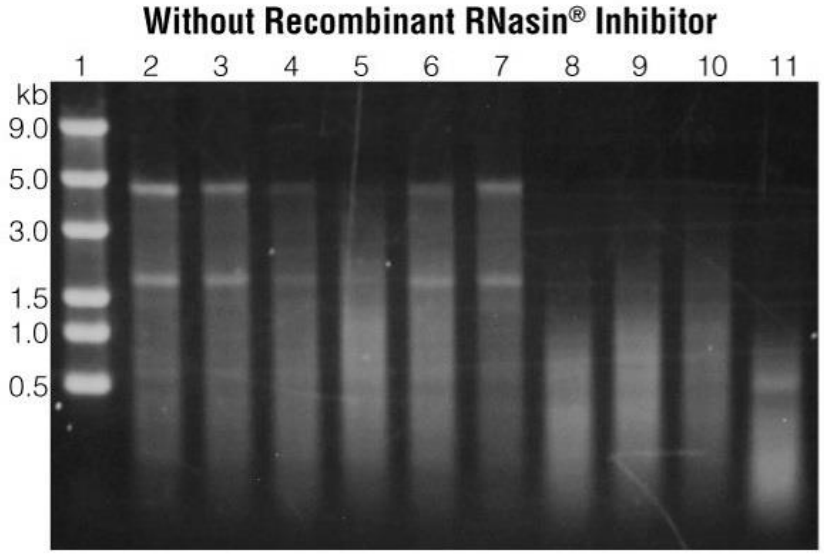
*Based on 1µl sample input per assay. Quantitation of more dilute samples is possible using more input RNA per assay.

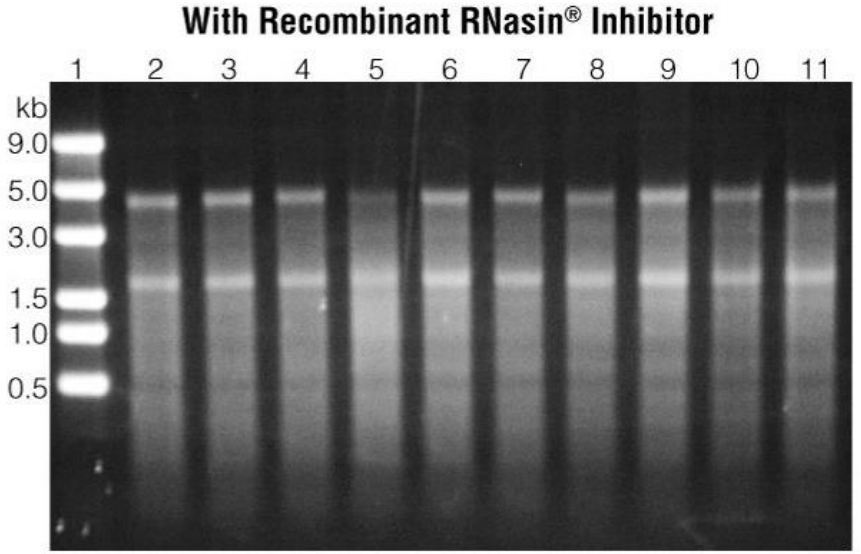


Protect RNA with the best - RNasin®

- Native, recombinant and oxidation-resistant forms available
- Maintains RNase inhibitory activity over a wide temperature range
- Inhibits a broad spectrum of eukaryotic RNases (RNase A, B and C and human placental RNases) over a wide pH range (pH 5–8).
- Does not inhibit SP6, T7 or T3 RNA Polymerase; GoScript™ Reverse Transcriptase, AMV or M-MLV Reverse Transcriptase; or Taq DNA polymerase
- If a long-term storage (e.g. for Bio-banking) is intended, RNasin Plus should be used due to the improved stability against oxidation and heat for 15 min at 70°C
- Cited in approx. 11.000 publications

Protect RNA with the best - RNasin®





Reverse Transcription – Tips & Tricks

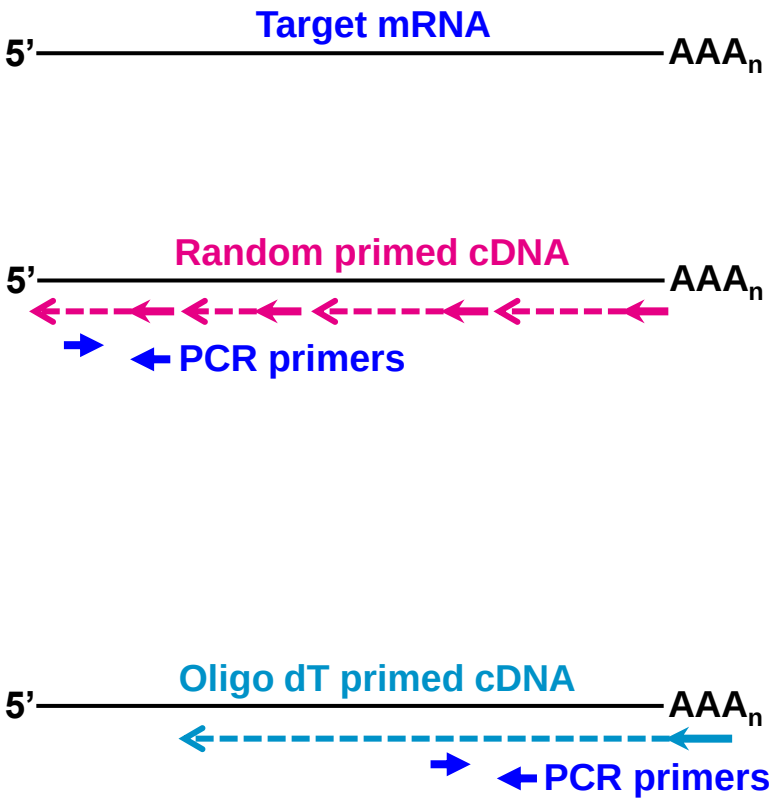
RNA input

- 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

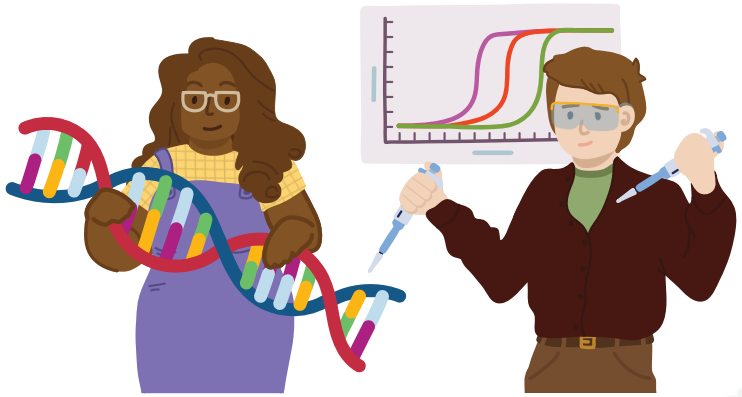
FEATURES	GoScript™ Reverse Transcriptase	AMV Reverse Transcriptase	M-MLV Reverse Transcriptase	M-MLV Reverse Transcriptase RNase H-, Point Mutant
Reaction temperature	37–55°C	37–58°C	37–42°C	40–55°C
cDNA length	Up to 9 kb	Up to 4 kb	Up to 5 kb	Up to 7.5 kb
Sensitivity	0.2 fg–5 µg total RNA	1 pg–1 µg 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?"

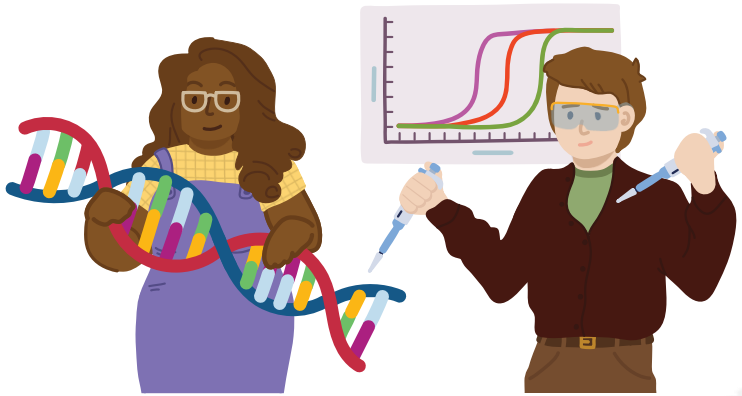
Real-Time PCR

Quantitative detection = "Exactly how much is there?"



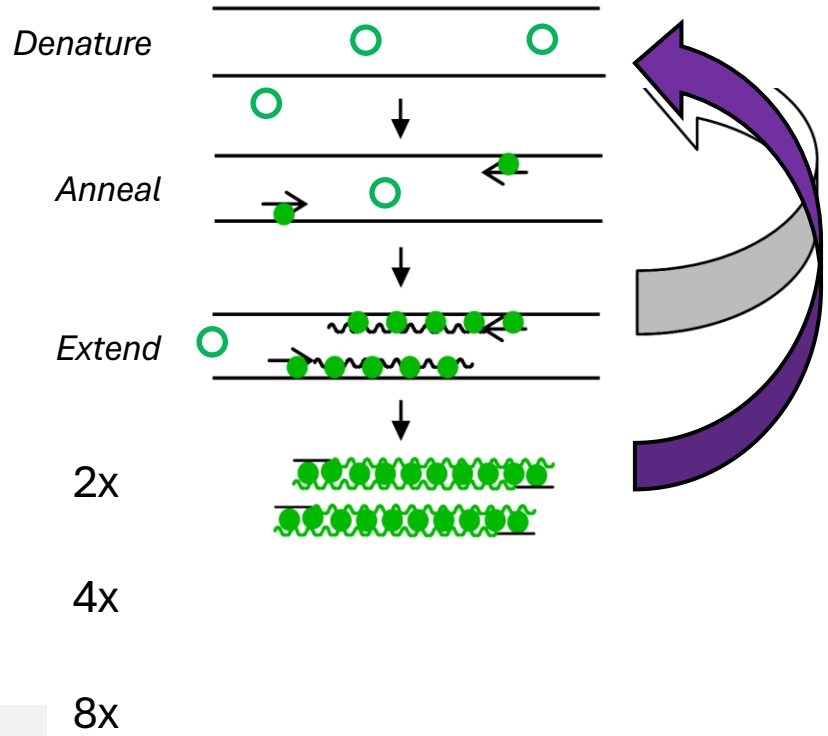
qPCR Chemistries

- 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

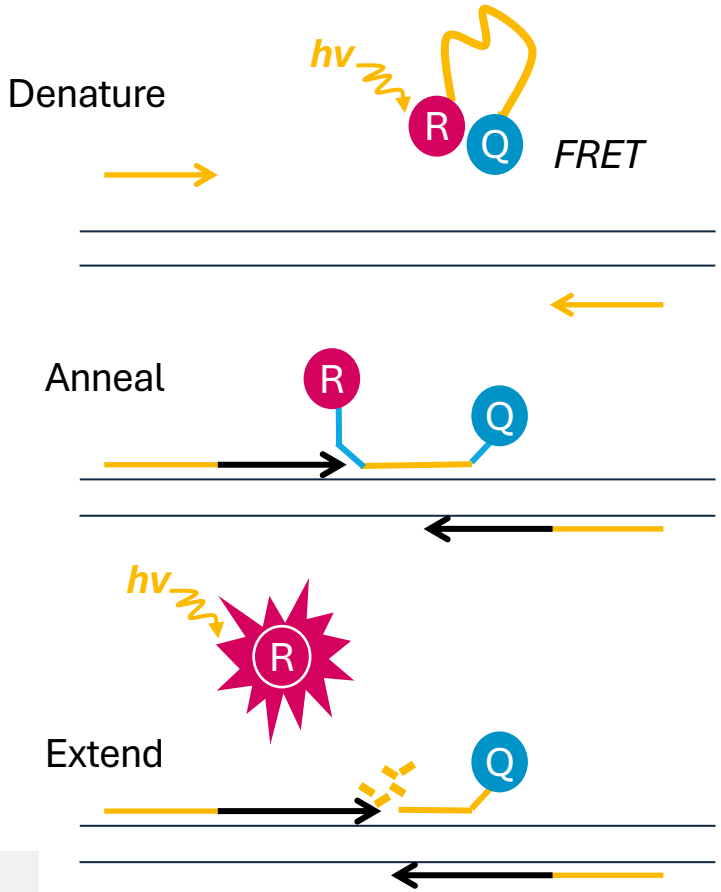
- dsDNA-binding dye is included in PCR master mix
- dye associates with PCR product
  - Free Dye → low fluorescence
  - Bound Dye → high fluorescence
- As more PCR product is produced, more dye is bound



Fluorescence is proportional to the amount of product

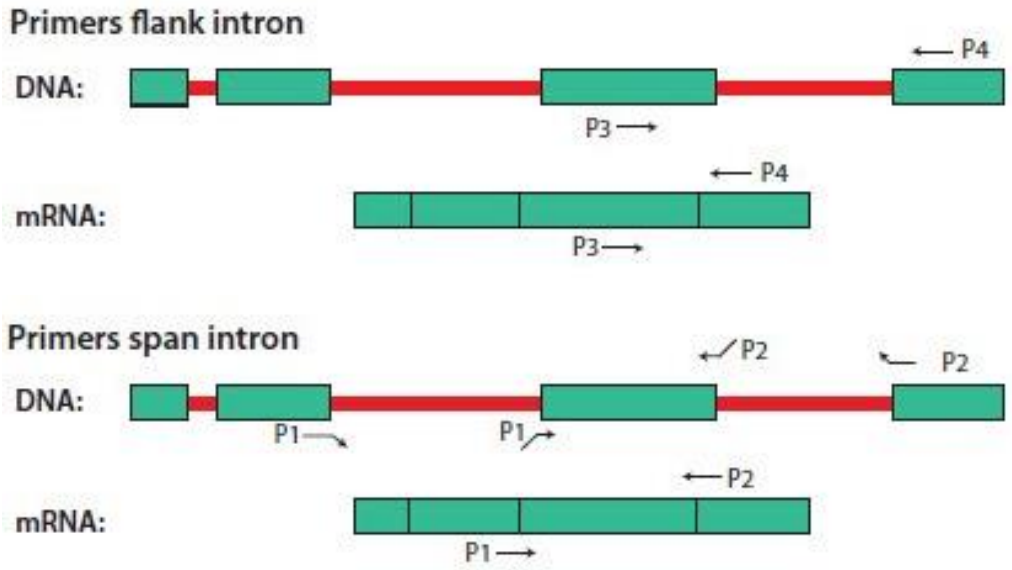
Probe-based approach

- 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

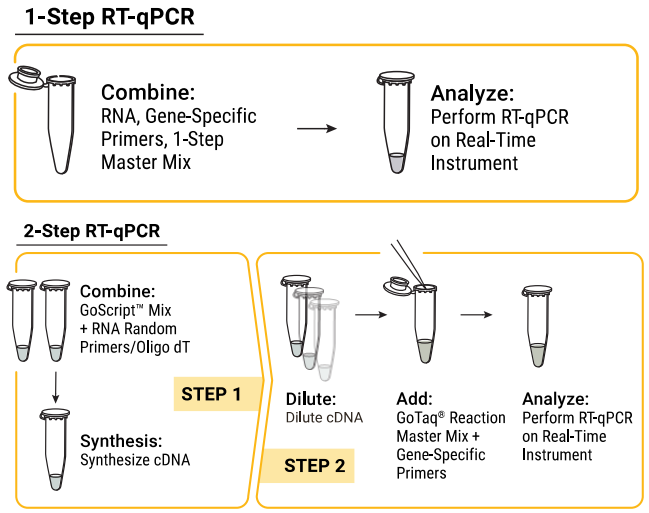
http://www.sigmaaldrich.com/technical-documents/articles/biology/pcr-qpcr-dpcr-assay-design.html

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



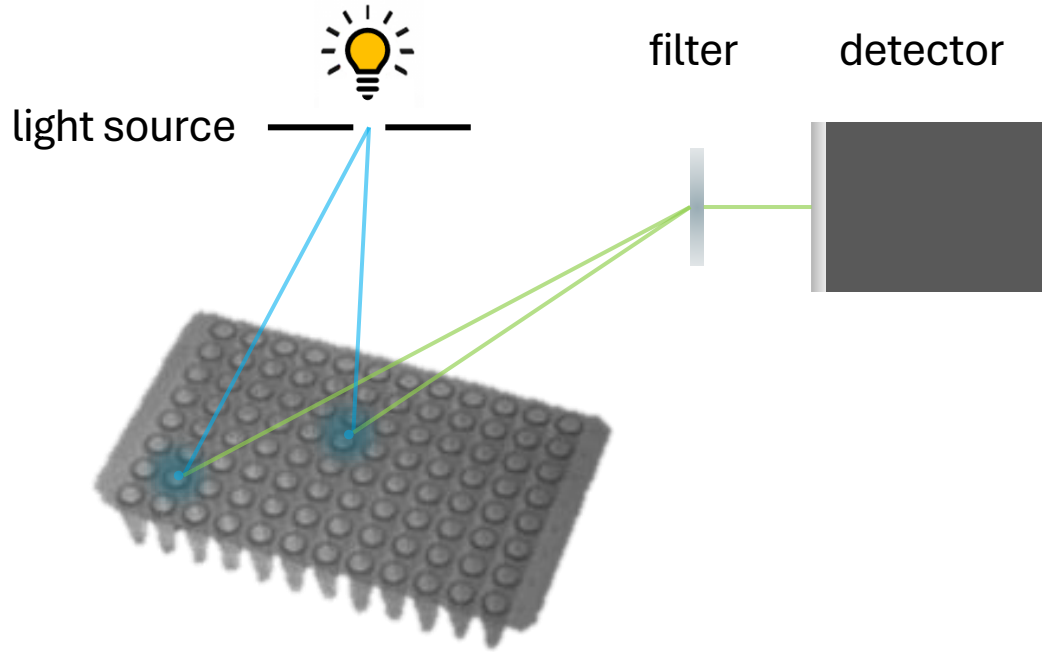
- Low chances of cross contamination
- Faster results
- No need to store the cDNA

- 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
contains RNasin Plus ✓	contains RNasin Plus ✓	contains RNasin Plus ✓
Multiplexing capability ✓	Multiplexing capability ✓	Multiplexing capability ✓
Probe-based ✓	Probe-based ✓	Probe-based ✓
Fast-Cycling ✓	Fast-Cycling ✓	Fast-Cycling ✓

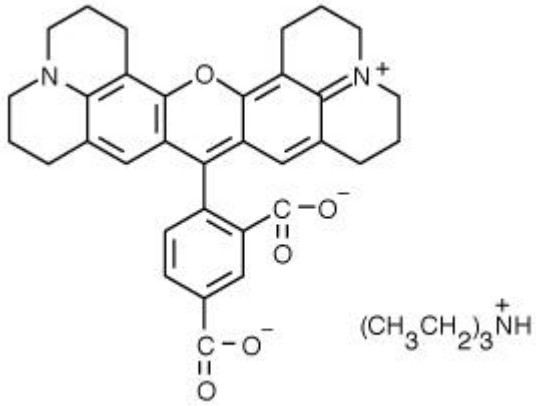
Passive Reference



second dye in solution
as passive reference for
signal normalization

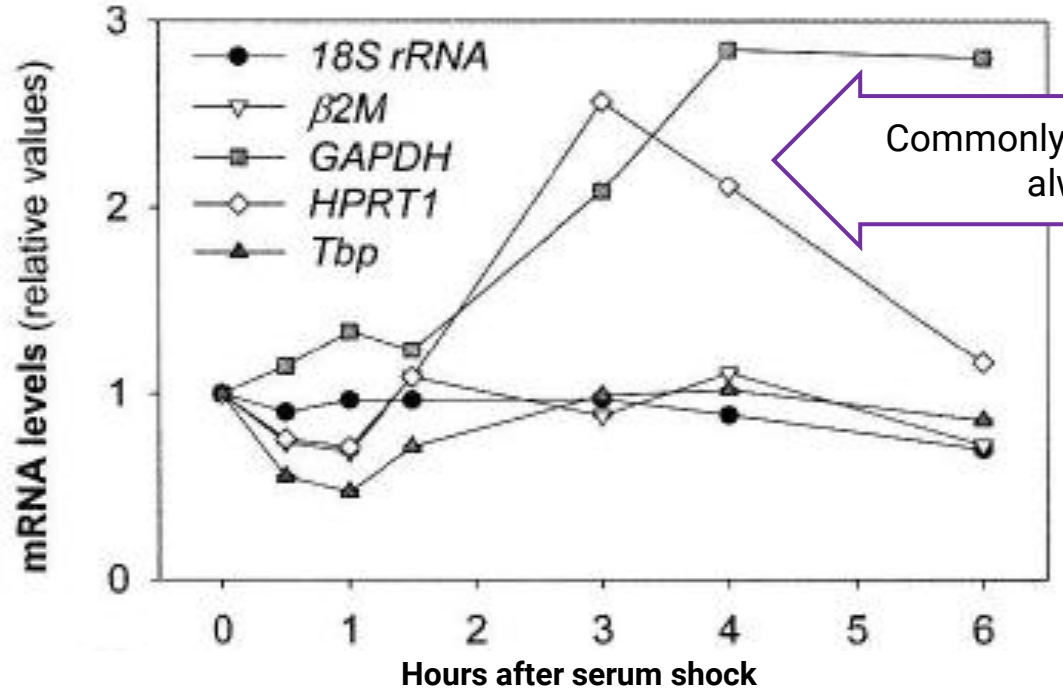
differences in intensity due to
- different beam path length
- variation in signal collection
- technical artifacts

→ **Almost all cyclers need additional CXR**



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

Reference genes



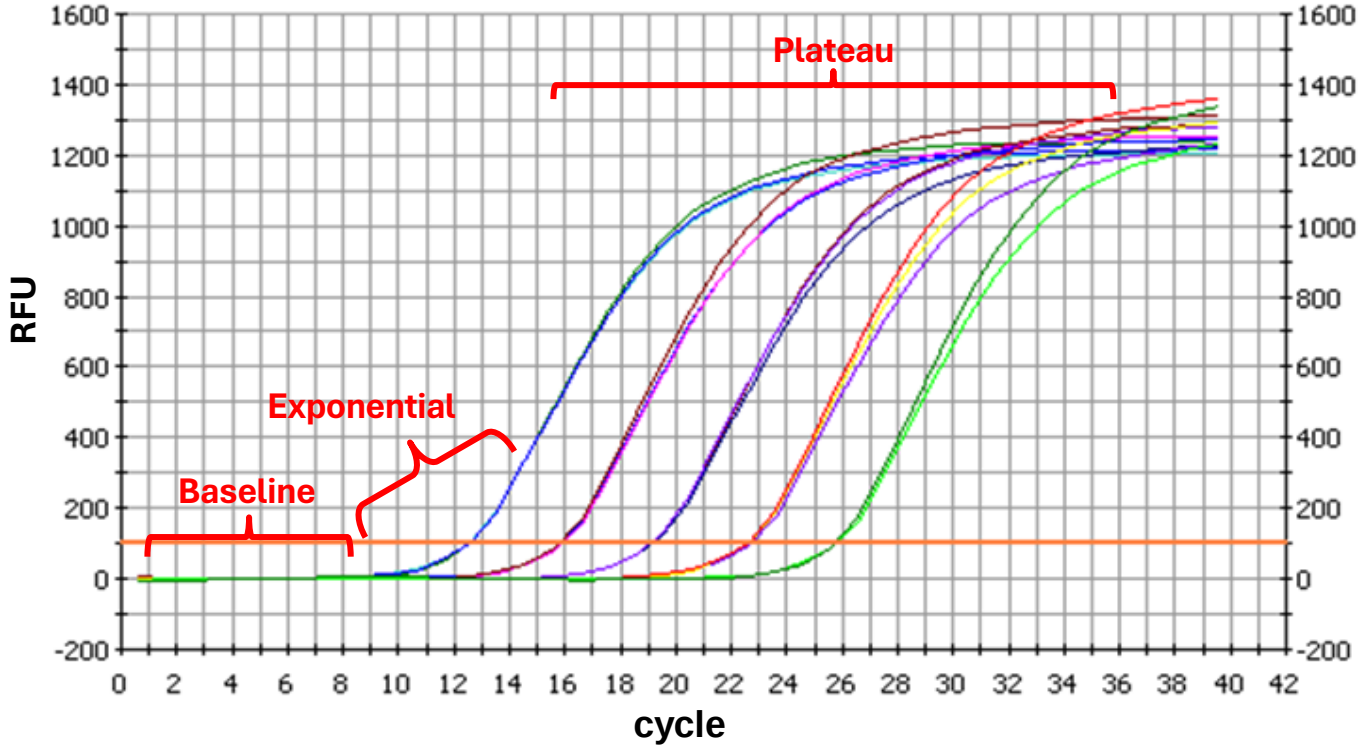
Garabino-Pico, E. et al. (2007) RNA

Commonly used normalizers are not always constitutive

one fits **NOT** all!!

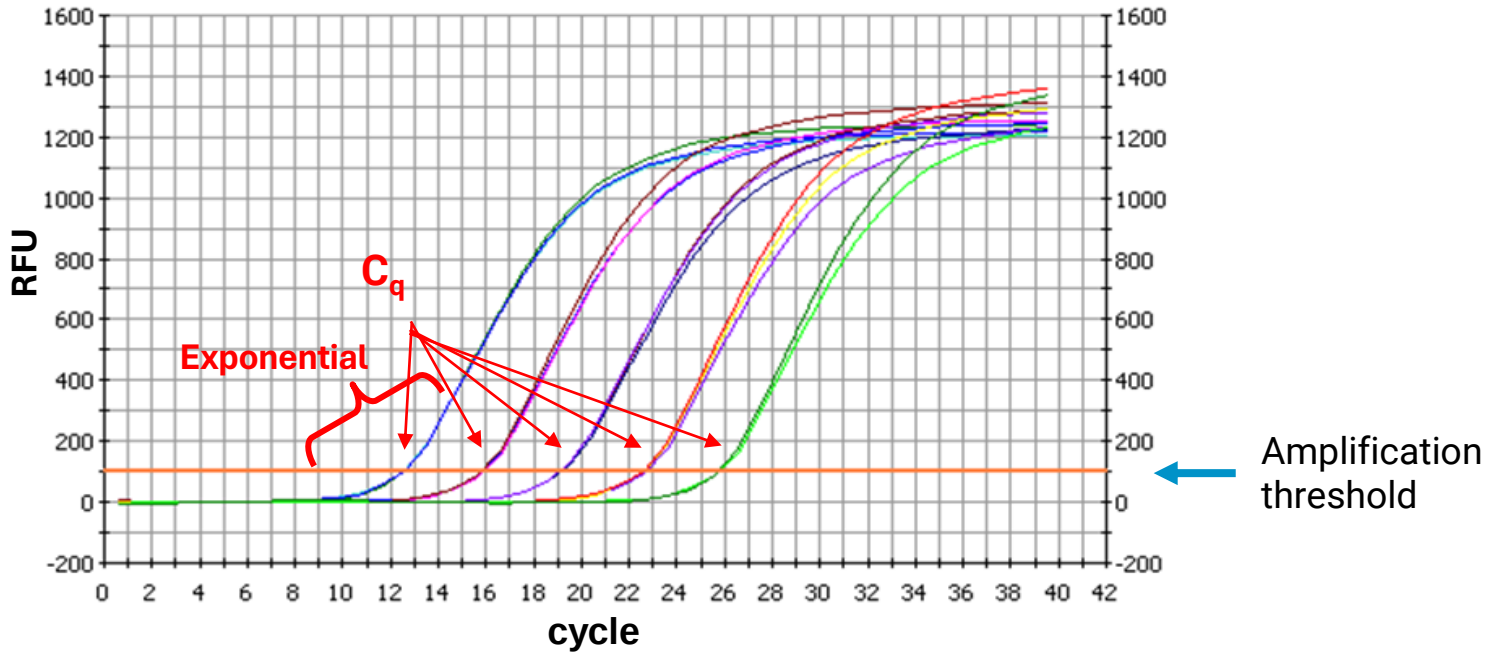
Primary output: Amplification Curve

This graph shows amplification curves for 15 different samples (wells) in an assay (3 replicates of 5 serial 10-fold dilutions of a positive control sample).



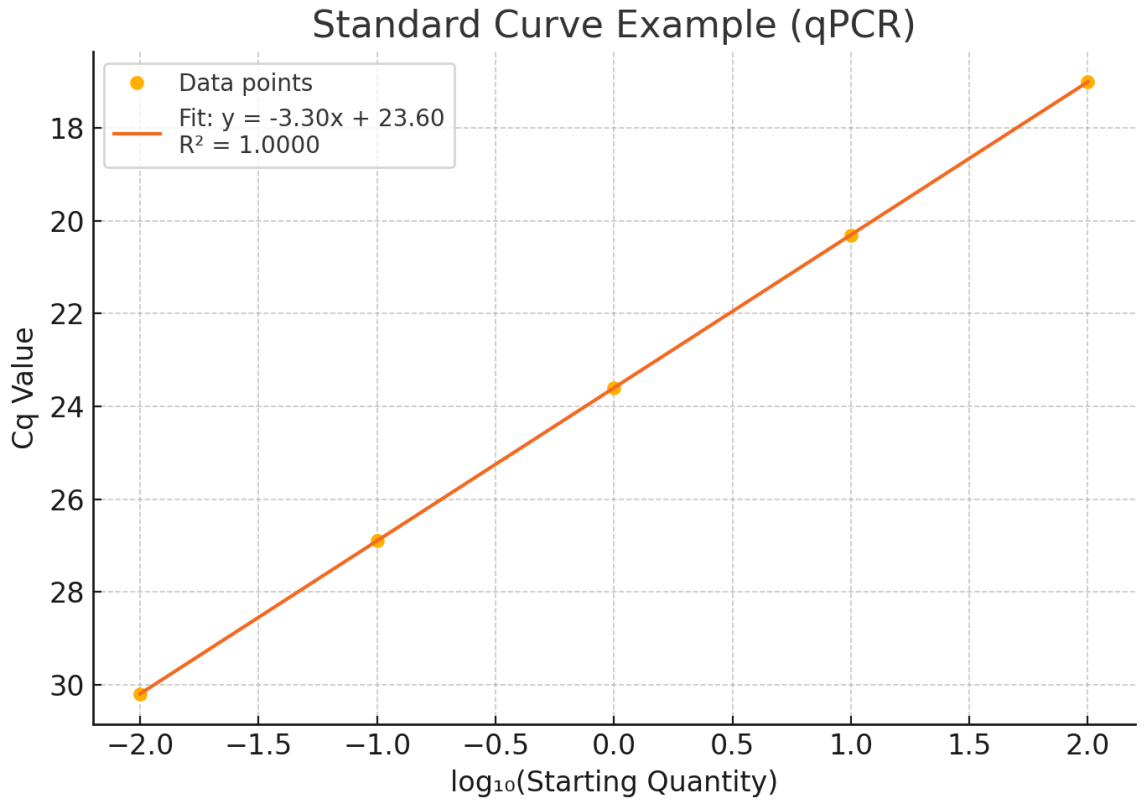
Primary output: Cq - value

- quantification cycle: cycle number at which amplification curve crosses amplification threshold - this is the “take-away“ metric…
- Cq - value is inversely proportional to amount of starting template



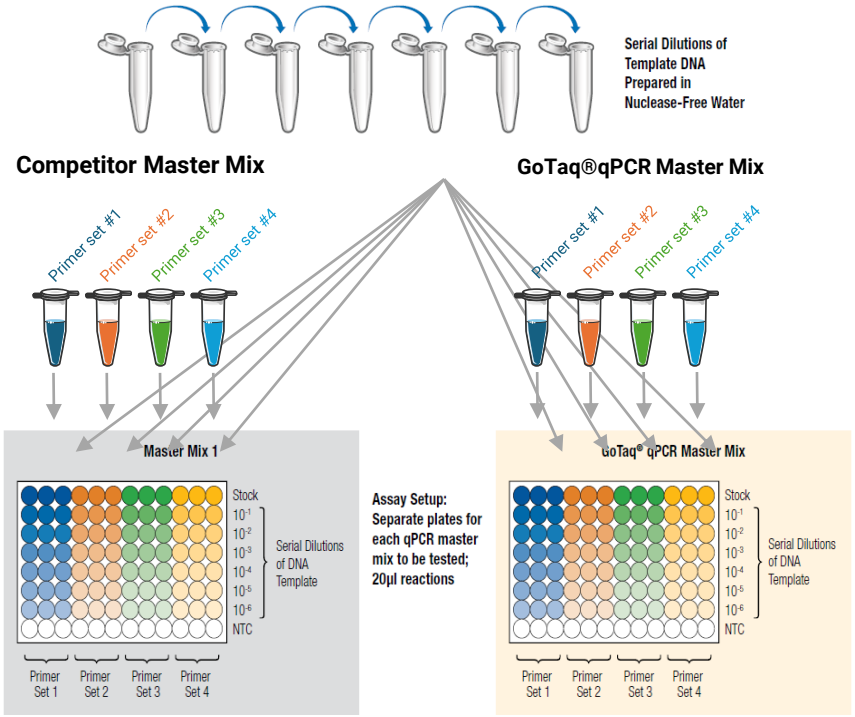
Validating Assay Performance with a Standard Curve

Consistent means each dilution gives the expected shift in Cq. Linear means the Cq values follow a straight-line relationship with concentration — and that's what makes our standard curve valid



E = (10^(-1/slope) - 1) × 100
= (10^(-1/3.3) - 1) × 100
= 100%

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

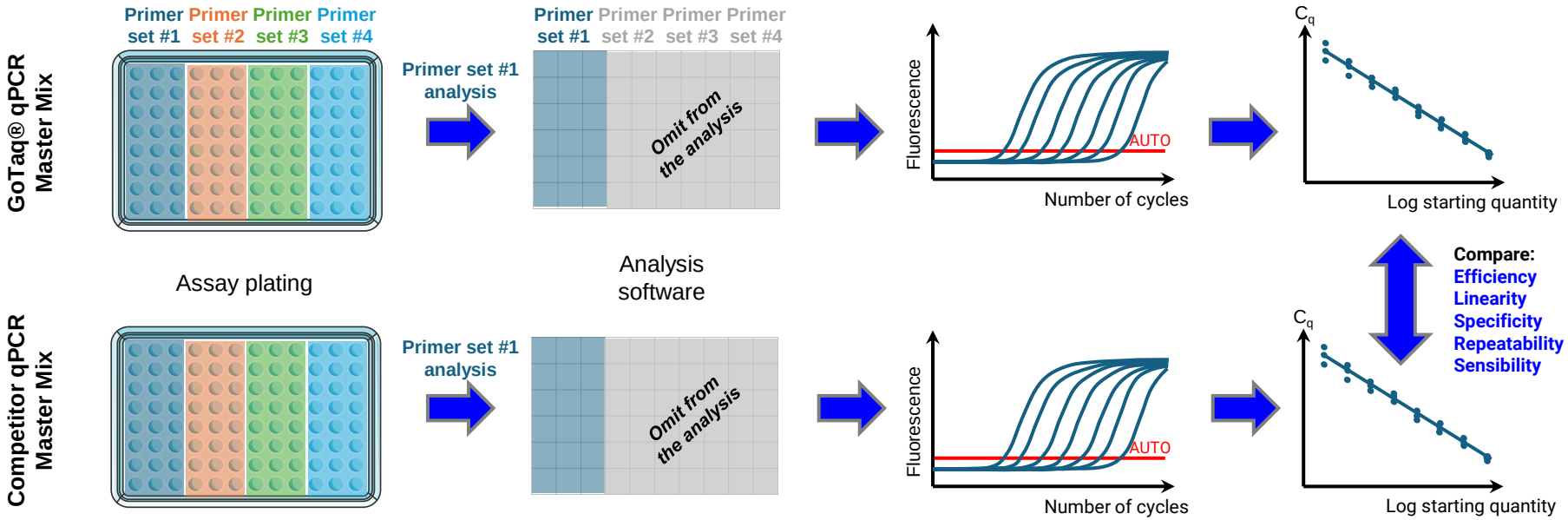
4) **Program thermal cycling conditions**
- Use optimized cycling conditions
- **But** change activation for GoTaq® to 2 min, 95°C

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

Data analysis of a qPCR reagent comparison

5) Analyze reactions for each assay separately

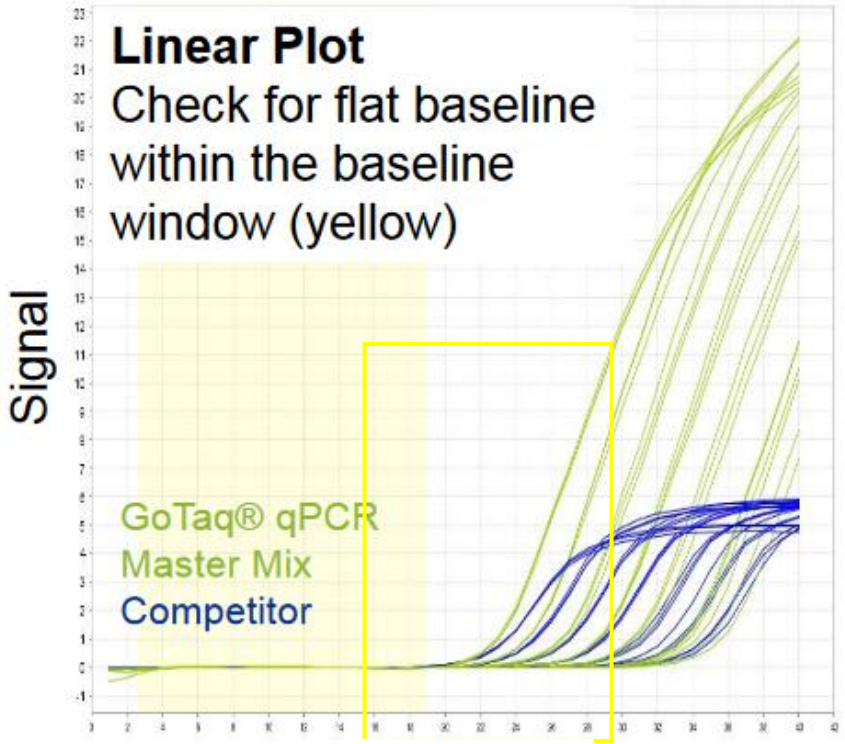
- Different reagent, primers, & fluorophores can influence settings



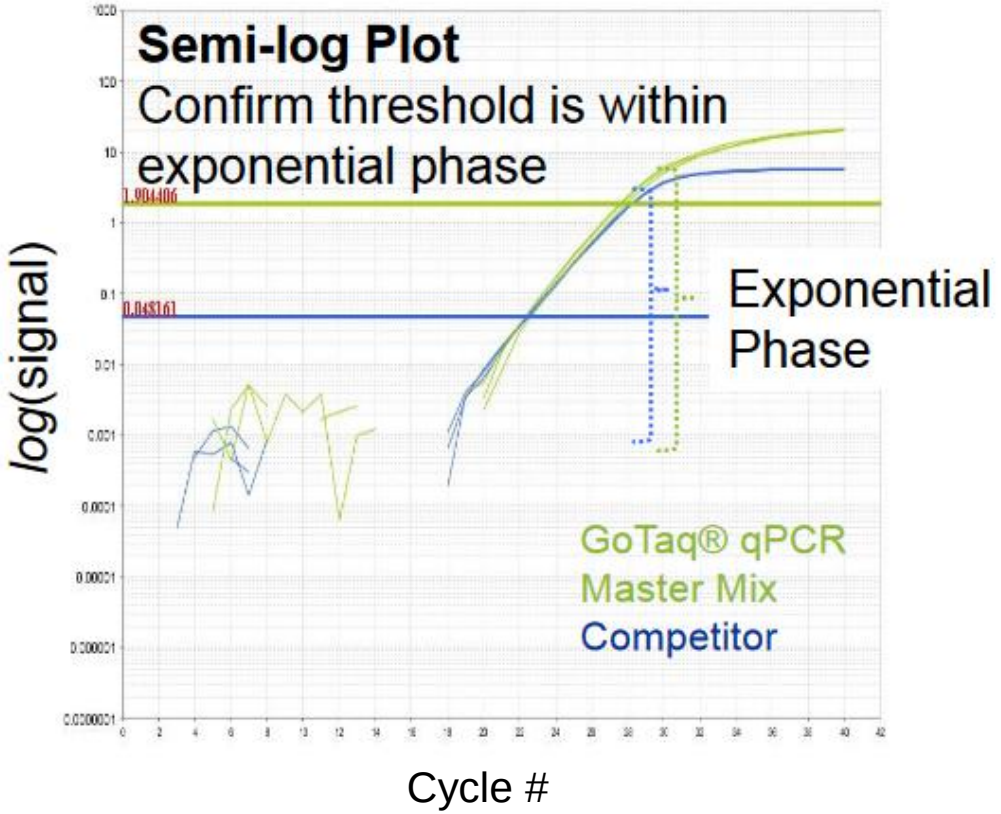
6) Use automatic baseline and threshold settings for each master mix separately

- 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



Is the threshold in your exponential phase?



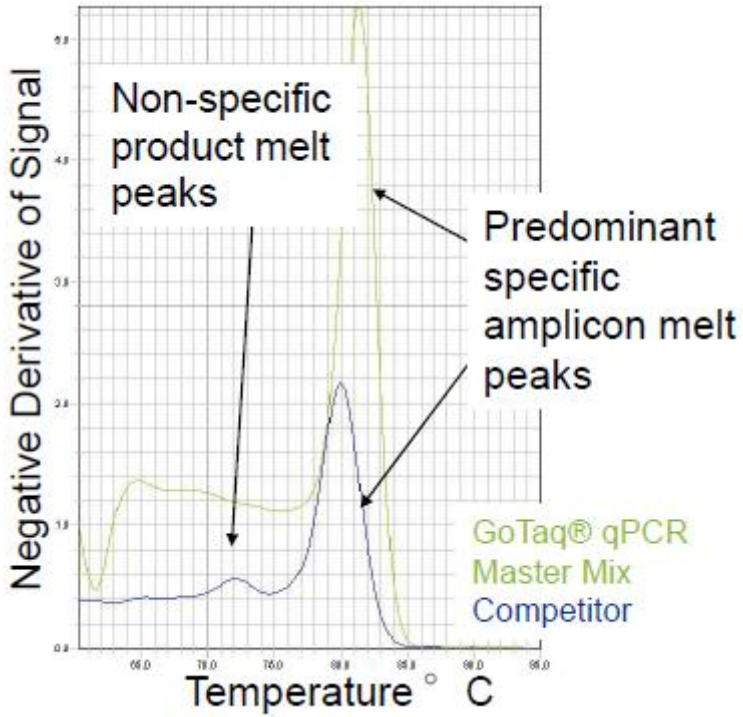
Expression at low C_q will influence your baseline calculation → dilute DNA 1:10 – 1:100

Teter et al. (2016) Promega Poster

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

qPCR resources

- Promega: qPCR Master mix comparison guideline - short version - https://www.promega.de/resources/pubhub/applications-notes/an298-real-time-qpcr-considerations-for-comparing-reagent-performance/

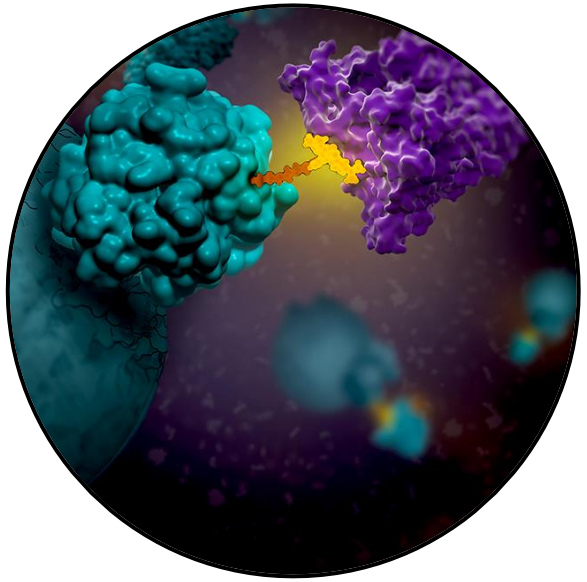
- Promega: Guidelines for a Comparison of Reagent Performance - https://www.promega.de/resources/pubhub/applications-notes/an299-real-time-qpcr-guidelines-for-a-comparison-of-reagent-performance/

- Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) - http://rdml.org/miqe.html

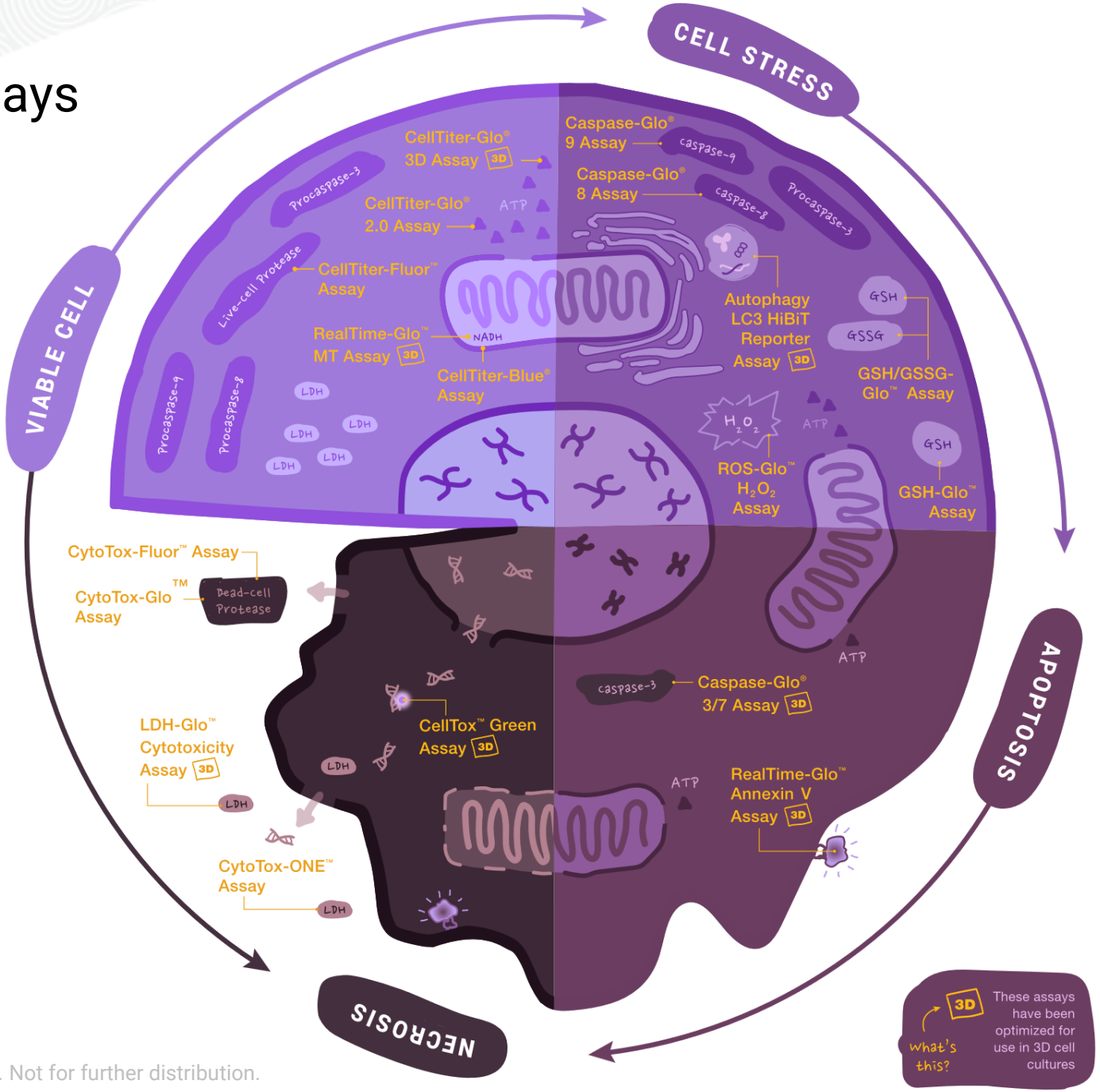
Summary

- 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

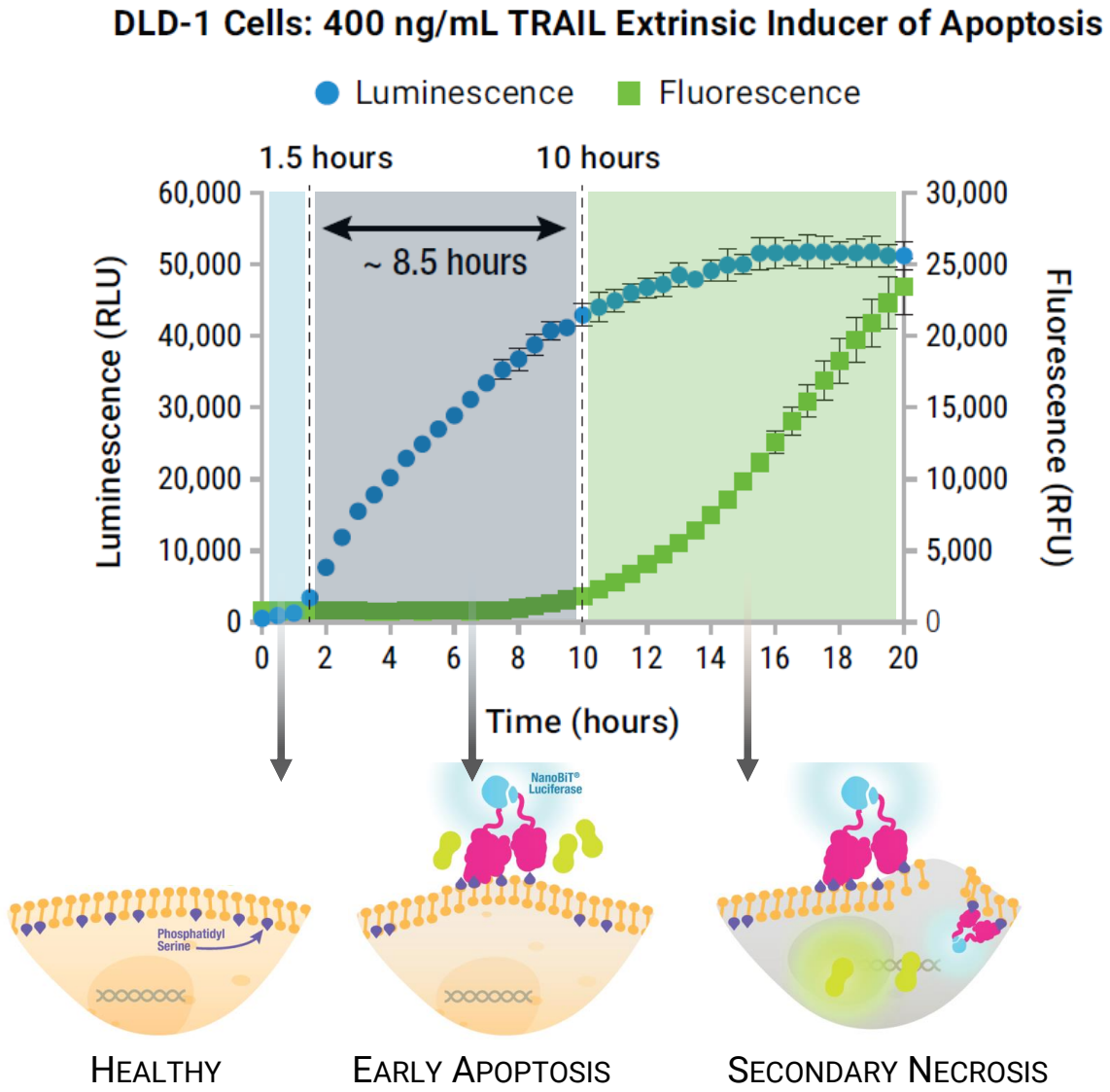
Nucleic acid isolation is a key step for many applications

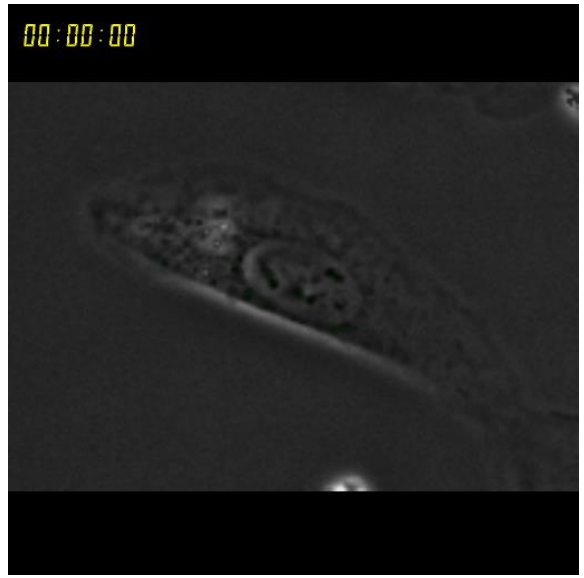


Cell Health Assays

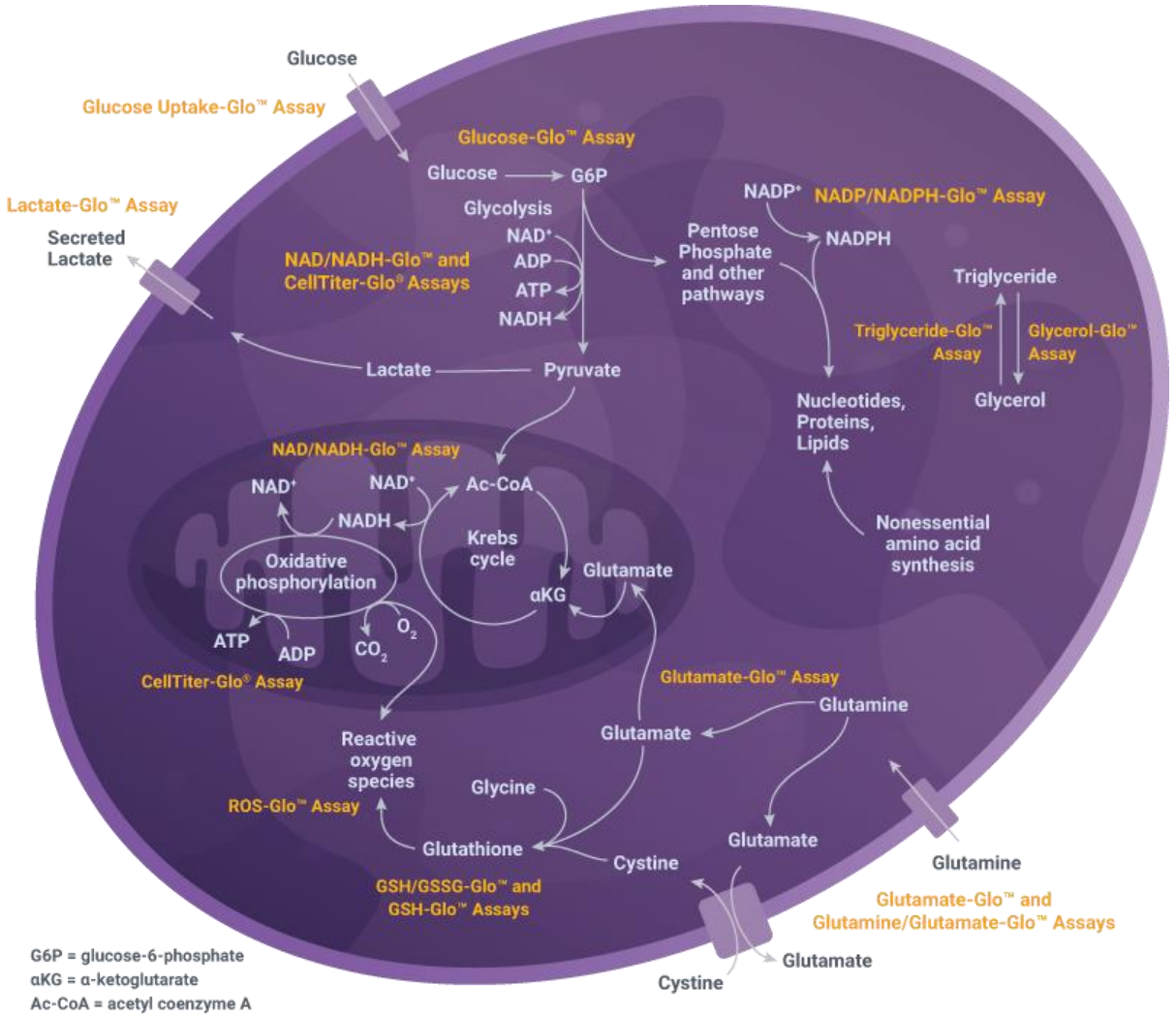


RealTime-Glo® Annexin V Apoptosis and Necrosis Assay

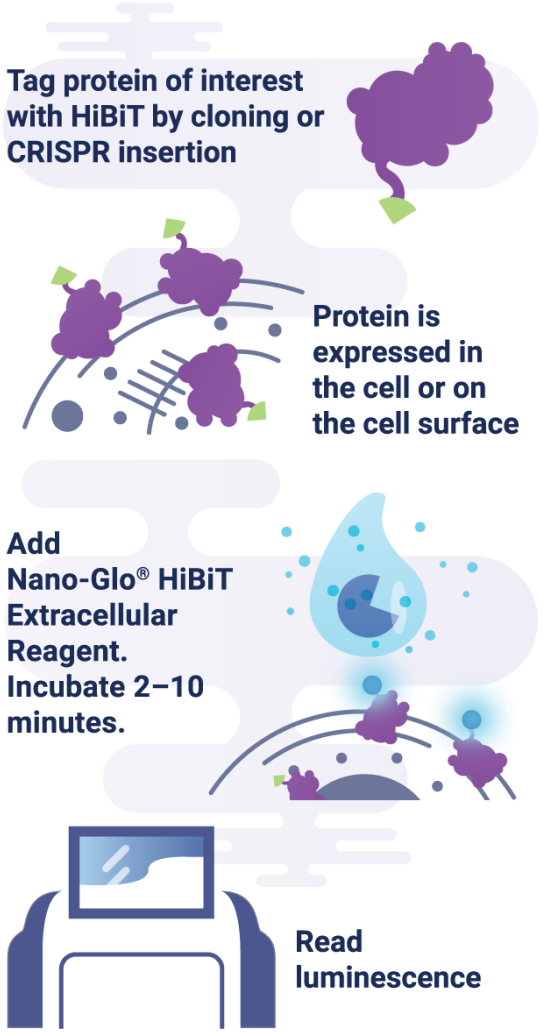


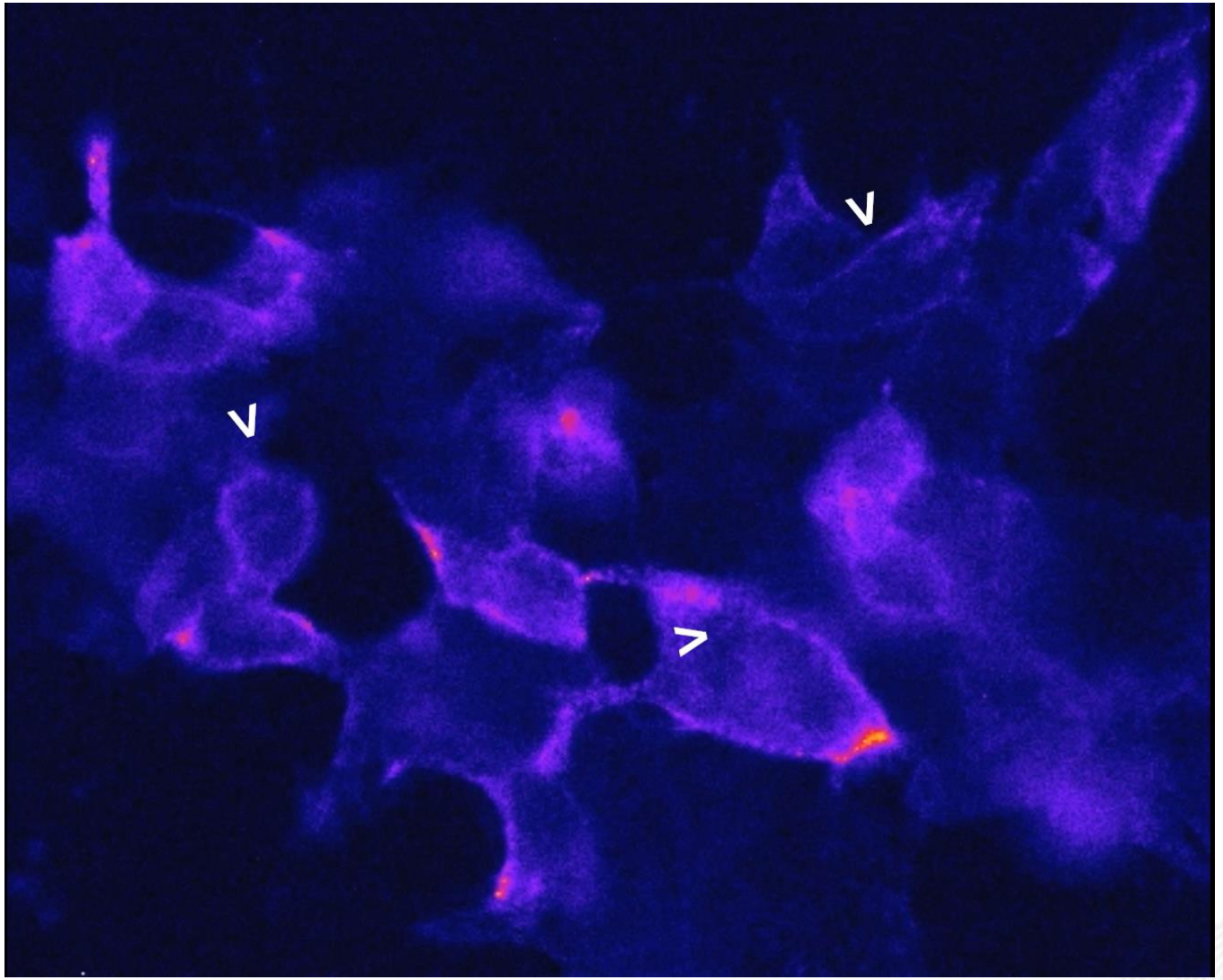


Cell Energy Metabolism

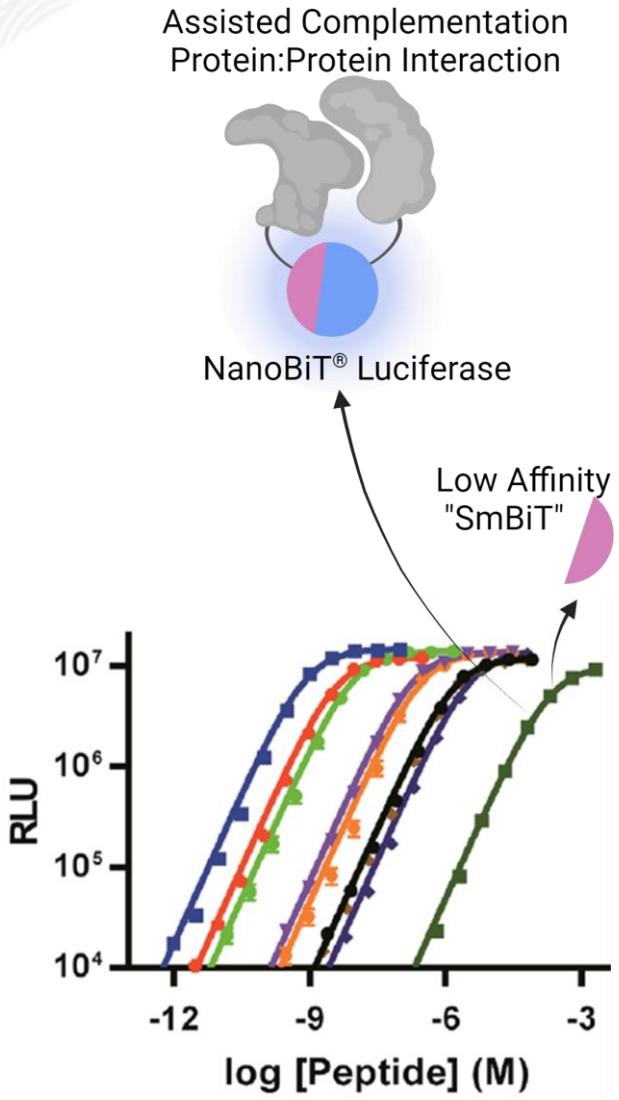


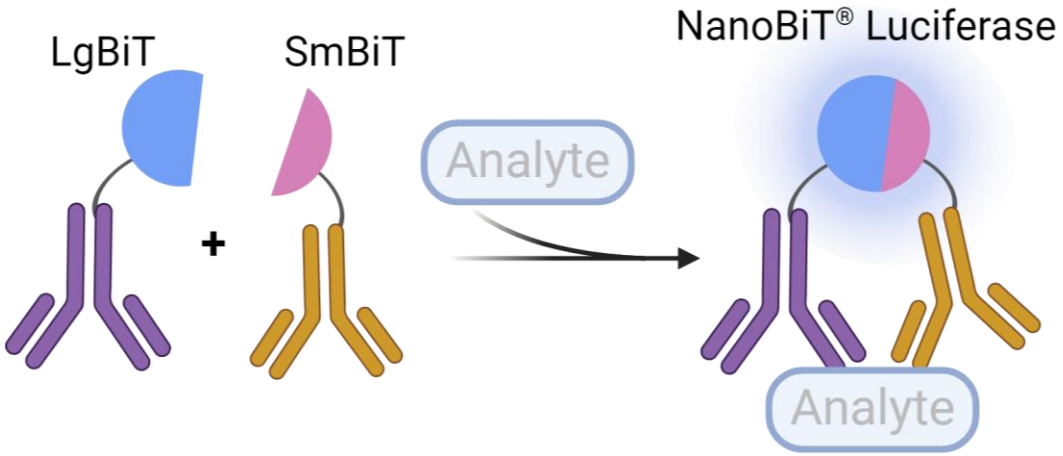
HiBiT Protein Tagging System

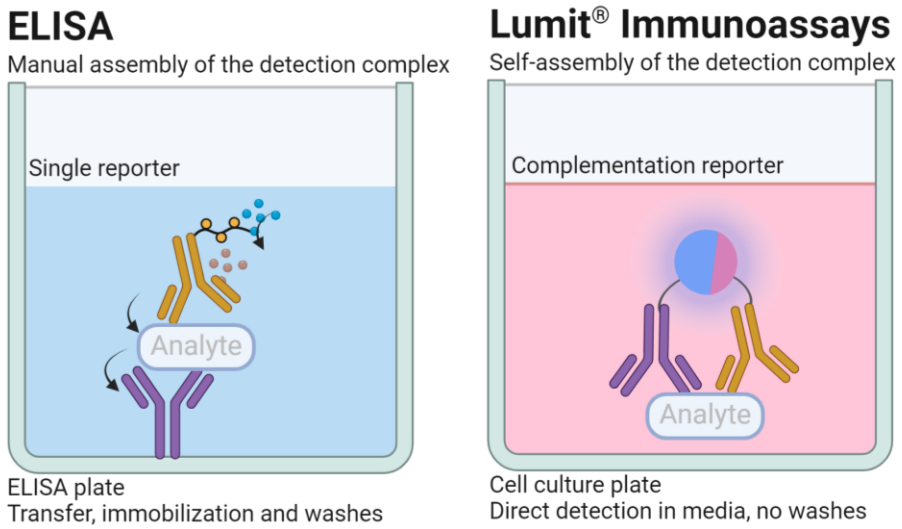




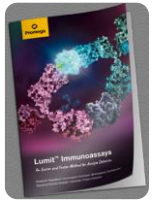
Lumit® Immunoassays: Detect Analytes and Molecular Interactions







Lumit™ is a platform technology.
Learn more in this eBook:



Questions?

For additional questions please contact:
kerem.yildirim@promega.com

Let's connect!

THANK YOU

Your main contact for products & sales relevant information:

vojtech.andrle@eastport.cz

vojtech.ledvina@eastport.cz

ondrej.ptacek@eastport.cz

EASTPORT LIFESCIENCE

Promega

Thank You!
Děkuju