Optimizing RNA Expression Analysis from Start to Finish

Doug Wieczorek, Ph.D. May 2012

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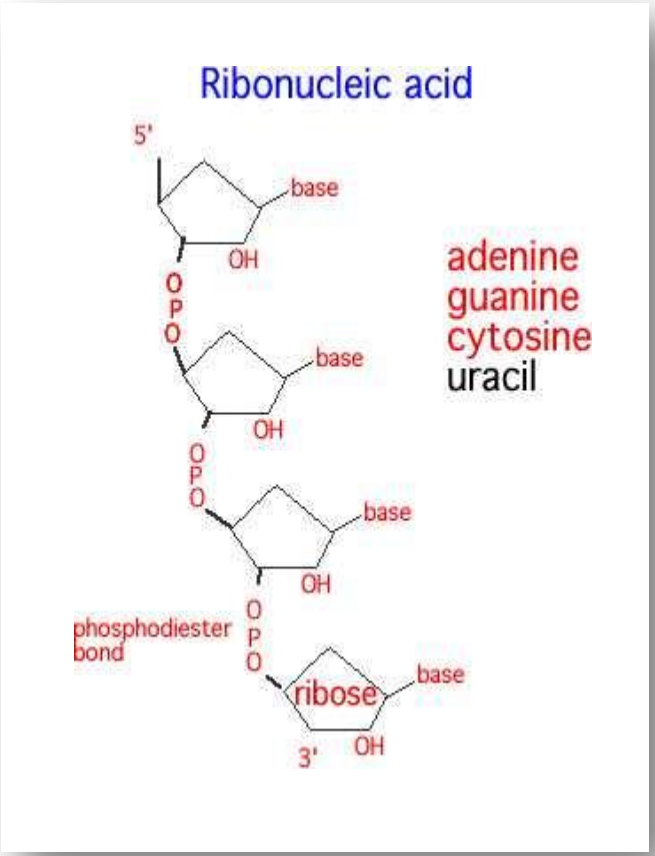
Presentation Outline

RNA Analysis Workflow

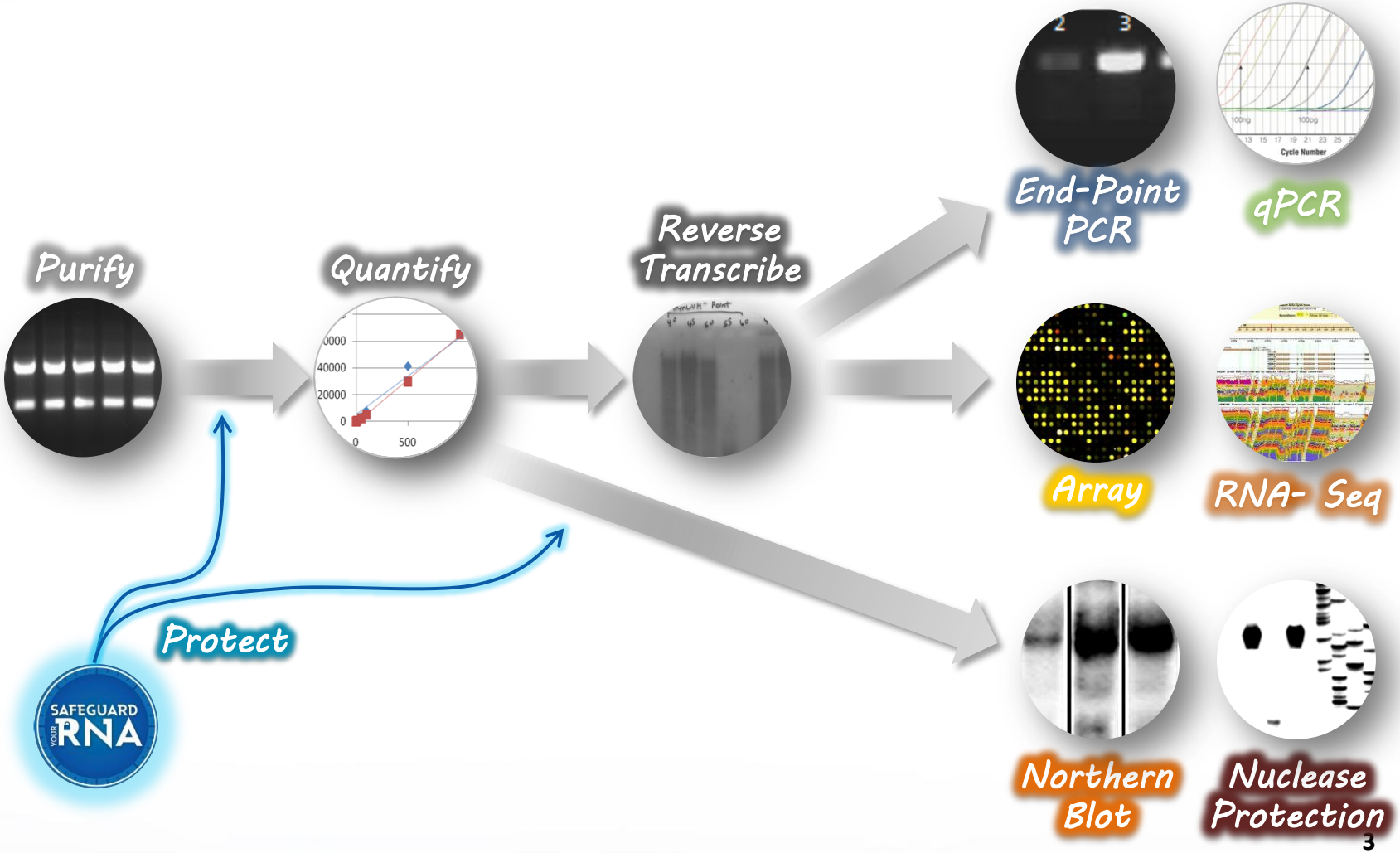
- Critical considerations at each step
  - Protecting RNA from degradation
  - Purification
  - Quantitation
  - Reverse Transcription
  - Analysis Methods

- ***Careful execution of each step is critical to successful results***



RNA Expression Analysis Workflow

Each Step Affects the Quality of the Final Results



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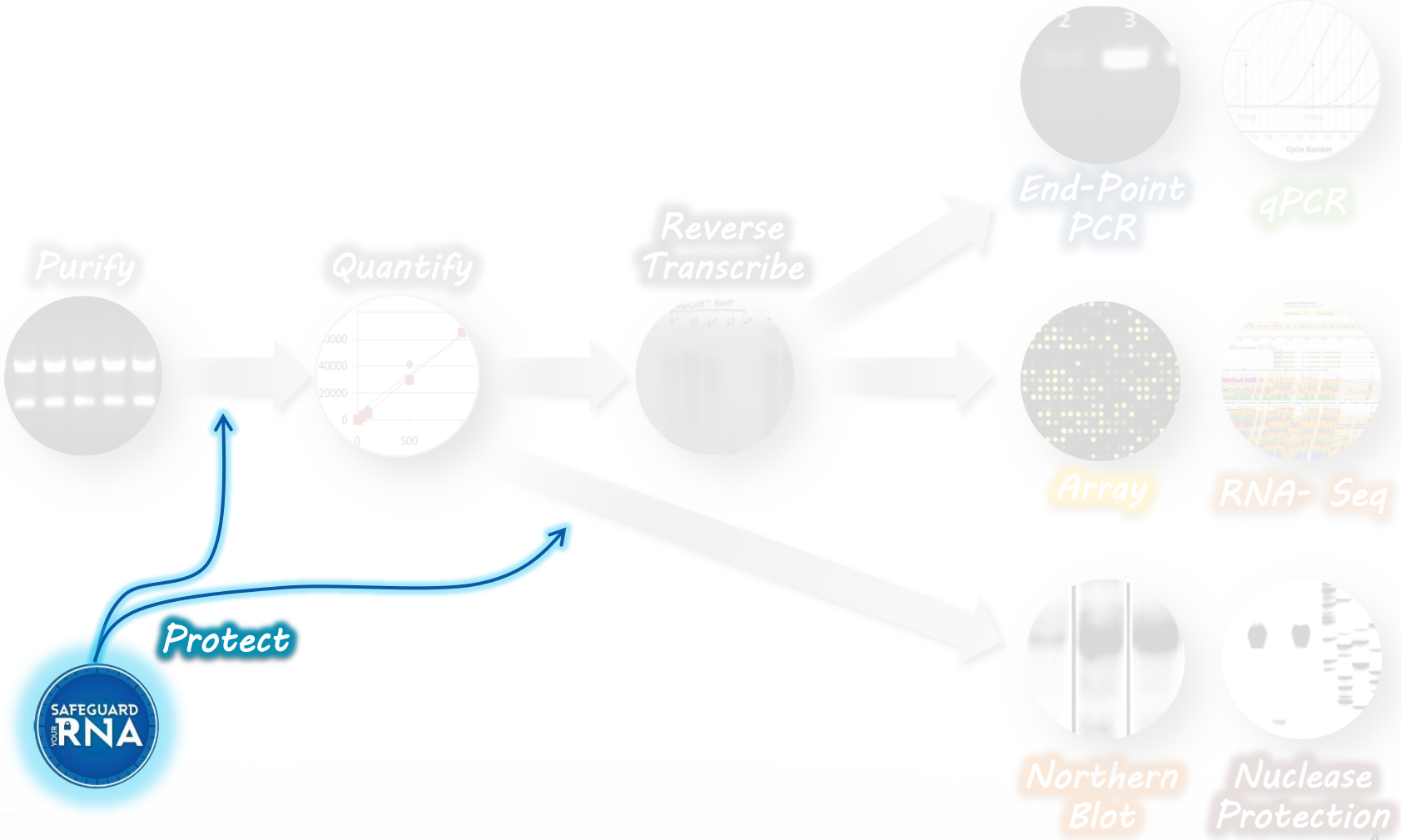

Protecting RNA from Degradation

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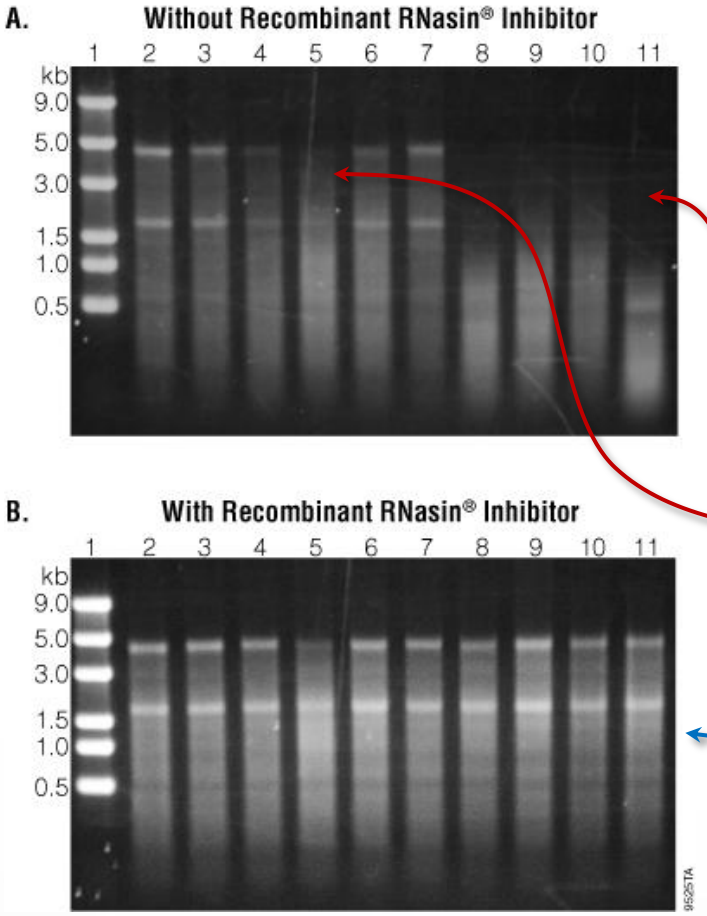
- **RNA is Fragile!**
  - Maintaining RNA integrity is essential for downstream analysis
  - RNases are ubiquitous and ***want to chew up your RNA***
  - Proper handling is essential
    - Gloves
    - Storage conditions
    - RNaseZAP®, ELIMINASE®
    - RNasin® Ribonuclease Inhibitors

RNase Contamination Happens; Recombinant RNasin® Inhibitor Can Safeguard Your Samples. Hendricksen A, Hook B, and Schagat T.

Protecting Your Valuable RNA with RNasin® Ribonuclease Inhibitors



1. RNA + lab solutions* were incubated overnight +/- RNasin® Ribonuclease Inhibitor
   a. Water
      - Autoclaved Milli-Q
      - Purchased DEPC-treated
   b. Other lab solutions used with RNA
   c. Tested both common and personal stocks

2. Analyzed by agarose gel electrophoresis

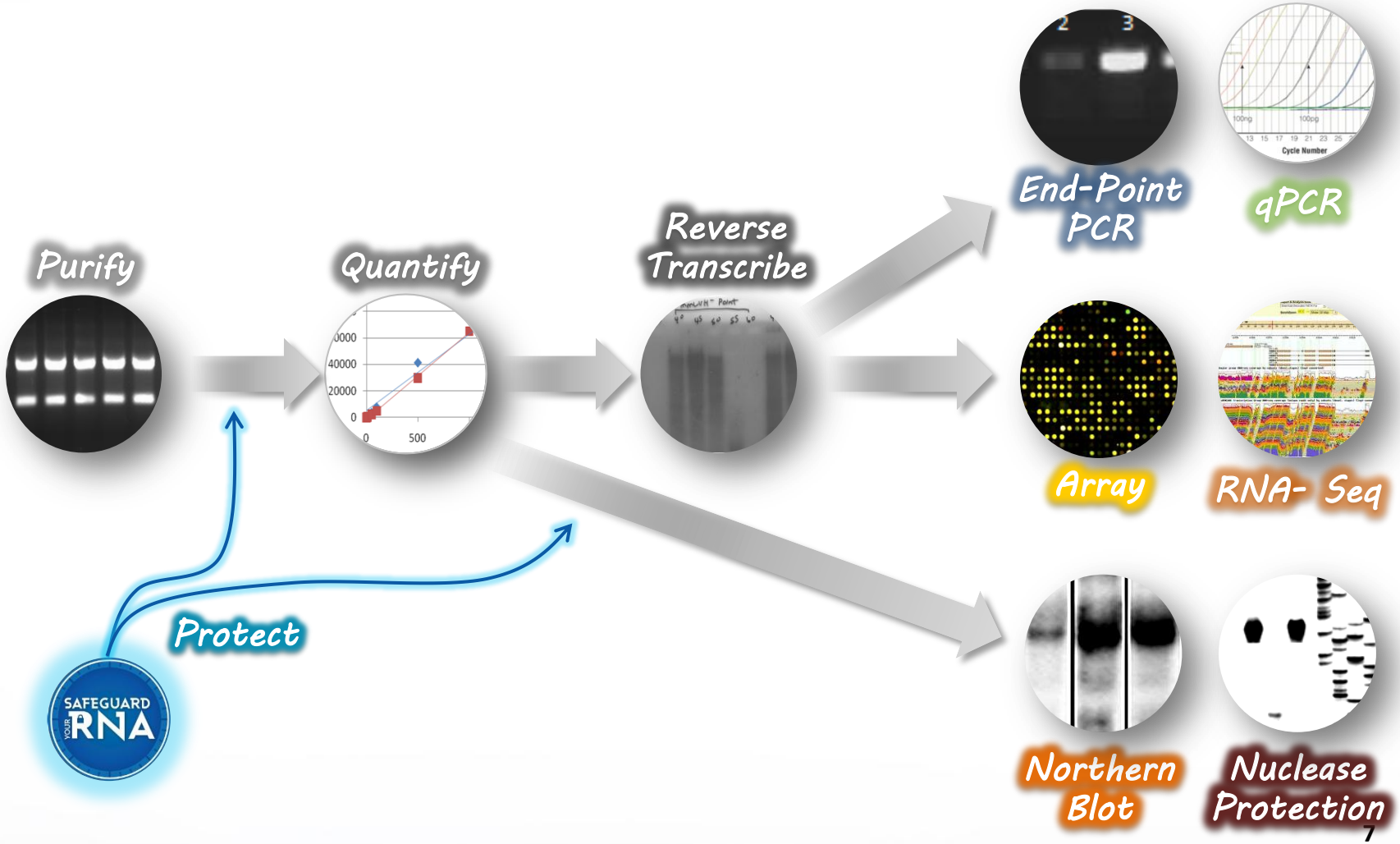
RNase contamination degraded samples in this RNA laboratory!

Despite "RNase-free" label!

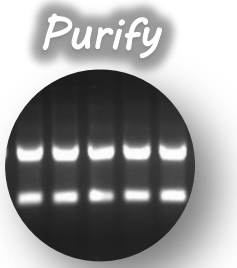
RNasin® protected

RNA Purification

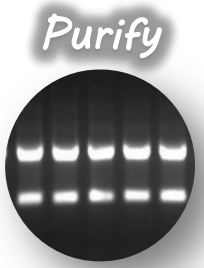
Purity and Yield are Critical to Downstream Success



RNA Purification
Purity and Yield are Critical to Downstream Success



RNA Purification
Purity and Yield are Critical to Downstream Success



Key Challenges

- Purifying sufficient RNA from small, precious samples
- Maintaining RNA integrity
- Isolating pure RNA
  - No gDNA contamination
  - No enzyme inhibitors to affect RT and PCR steps

RNA Purification
Choice of Purification Kit Impacts Purity and Yield

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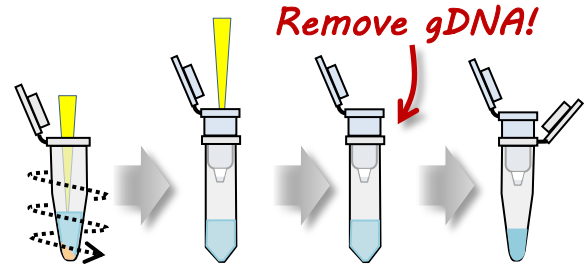
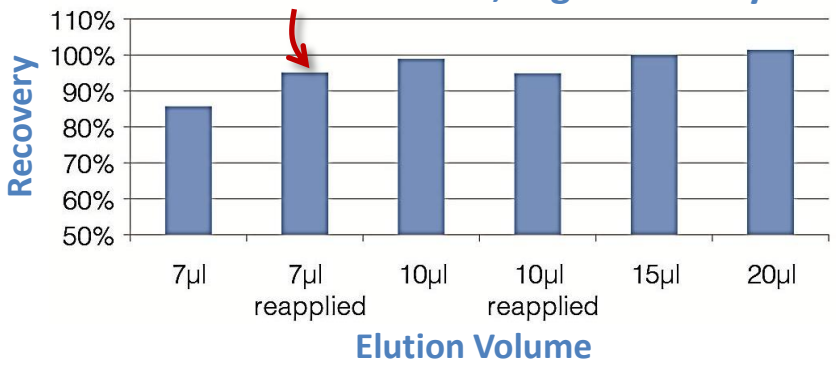
ReliaPrep™ RNA Cell Miniprep System

Quick, 20 Minute Protocol



Lyse cell pellet (vortexing or pipeting) | Transfer & Spin to Capture | Wash DNase Treat Wash 3X | Elute purified Total RNA

Low Elution Volumes, High Recovery



RT-qPCR Inhibitors in RNA Purified with Competitor Kit

	qPCR Input Volume		
	1µl	5µl	9.5µl
Competitor Kit (30µl Elution)	**100%**	**94%**	**85%**
ReliaPrep™ Cell (30µl Elution)	**100%**	**105%**	**109%**
ReliaPrep™ Cell (15µl Elution)	**100%**	**115%**	**117%**

RNA Purification
Contamination gDNA Affects RT-qPCR Quantitation

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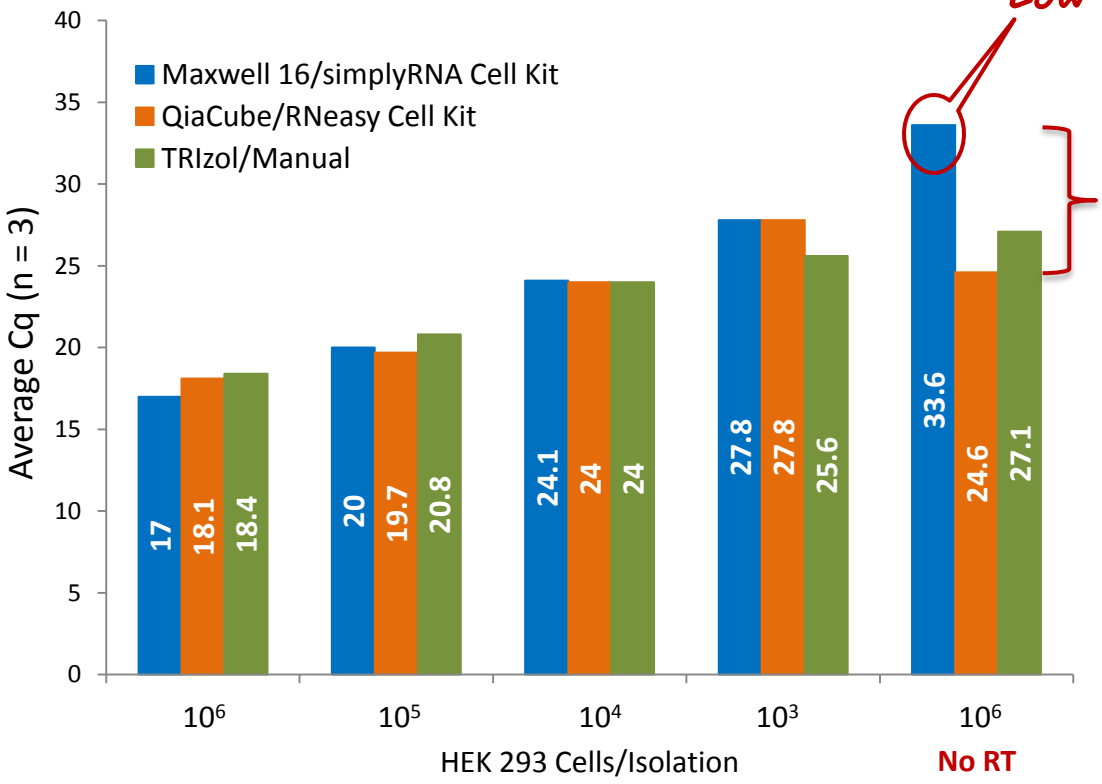
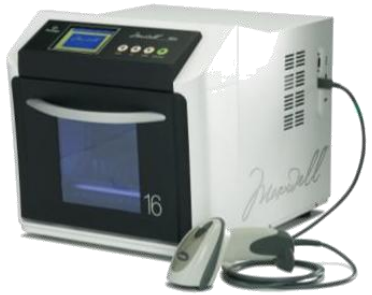
Amplification of Human β2-microglobulin from Equal Volumes of Eluate



Low gDNA content (High Cq)

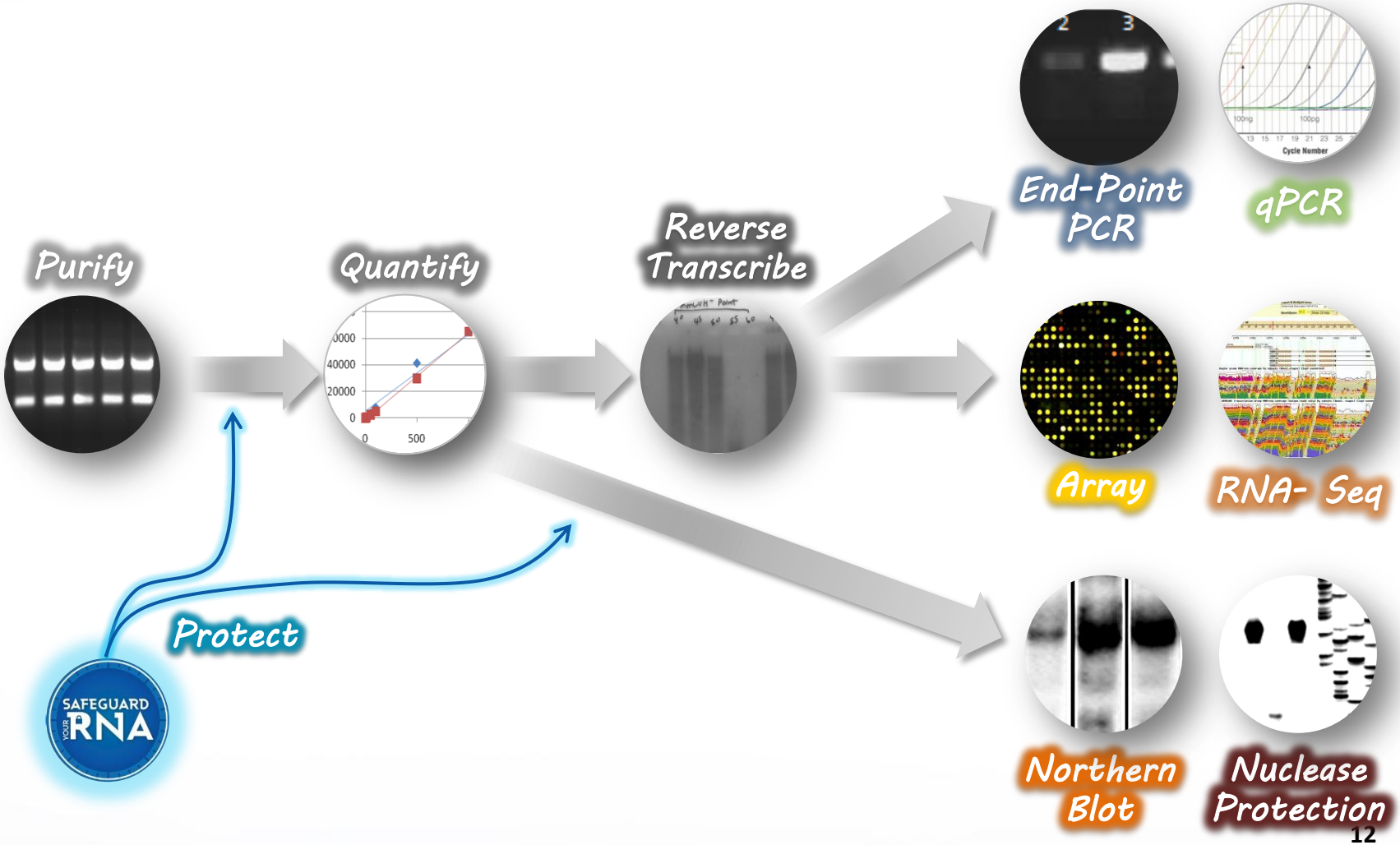
gDNA contamination

6.5-9 Cq difference means 90-500 fold more gDNA in these preps



RNA Quantitation

Accurate Measurement of RNA Yield and Purity Helps



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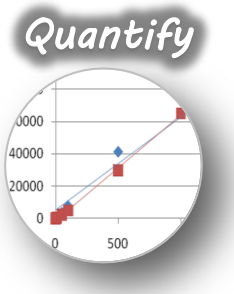


Key Challenges

- Effectively measuring small RNA amounts
- Assessing integrity
- Determining purity
  - Organics carryover
  - gDNA contamination

Total RNA Quantitation and Quality Assessment

- UV Absorbance
  - Spectrophotometer
  - NanoDrop®/NanoVue™
- Fluorescent Dye-based Quantitation
  - Plate Reader
  - Hand-held Instruments
- Gel Electrophoresis
  - Agarose
  - Acrylamide
- Agilent 2100 Bioanalyzer

What information does each method give us……

What information does each method not give us……

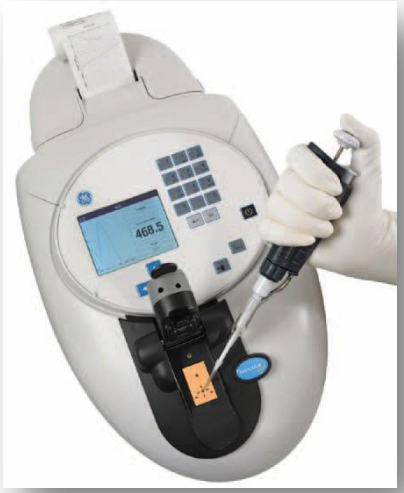
What are the advantages and disadvantages of each method…..

UV Absorbance
Measuring Concentration and Purity

Spectrophotometer
(various manufacturers)

NanoDrop®
(Thermo Scientific)

NanoVue™
(GE Healthcare)



Measure nucleic acid:

- ✓ Concentration
- ✓ Purity

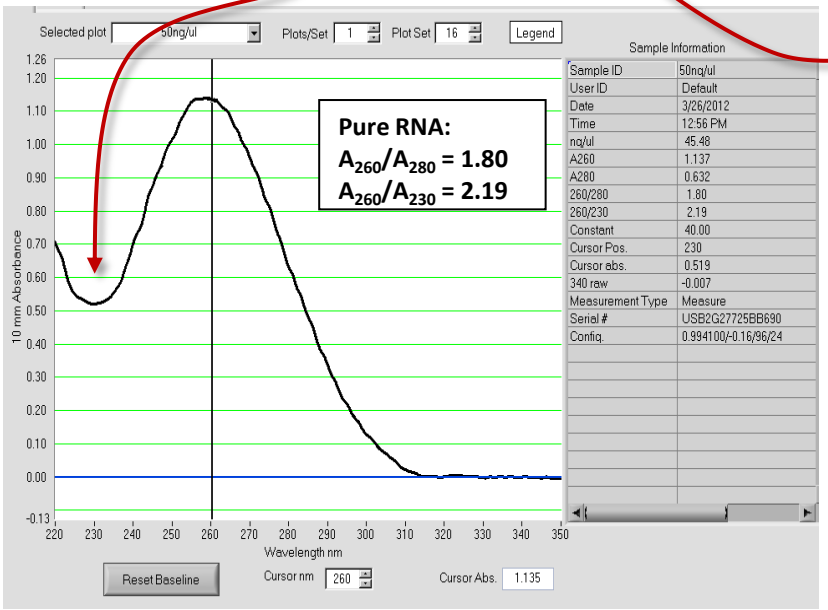
UV Absorbance – Wavelengths
Each Wavelength Measures Different Components

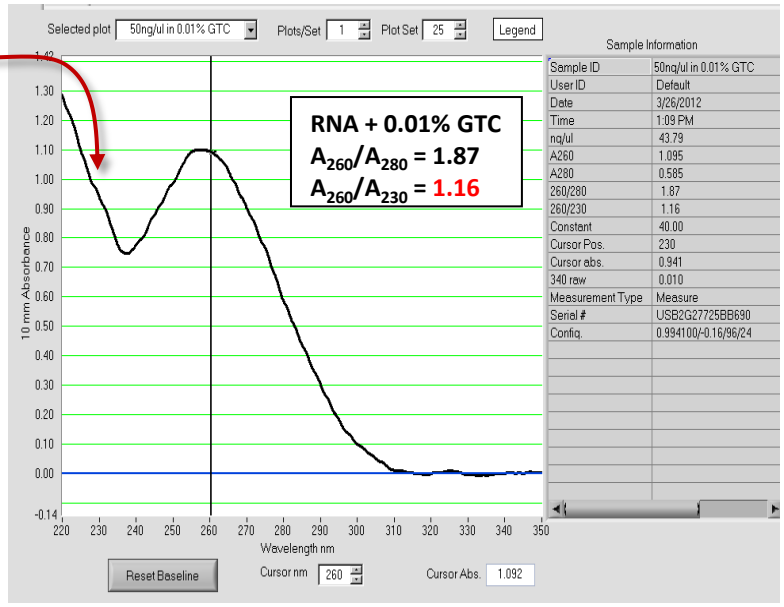
Wavelength	Measurement
260nm	Amount of nucleic acid present in a sample A_{260nm} of 1.0 = 50µg/ml for dsDNA 40µg/ml for RNA 33µg/ml for ssDNA
280nm	Amount of protein present in a sample
230nm	Amount of other contaminants present in a sample
320nm	Amount of light scattering components present in a sample; used for background subtraction

UV Absorbance – Purity
Guanidine Thiocyanate Lowers A_{260}/A_{230} Ratio

Purity Measurement	Acceptable Ratios
A_{260}/A_{280}	Generally 1.8 – 2.2
A_{260}/A_{230}	Generally >1.7

Guanidine thiocyanate affects A_{260}/A_{230} ratio

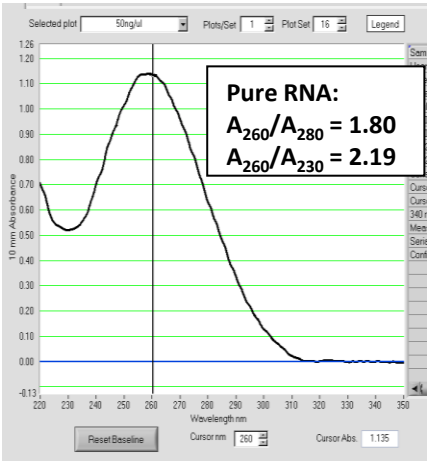




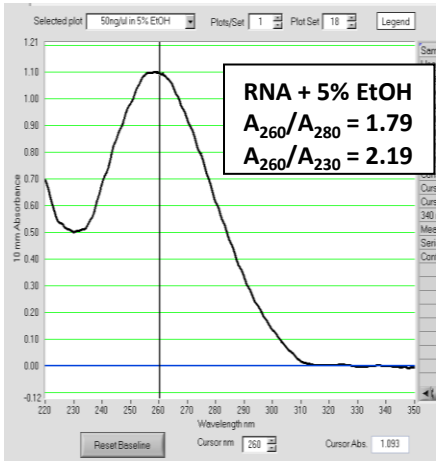
UV Absorbance – Purity
Alcohols Do Not Significantly Affect Ratios

Purity Measurement	Acceptable Ratios
A_{260}/A_{280}	Generally 1.8 – 2.2
A_{260}/A_{230}	Generally >1.7

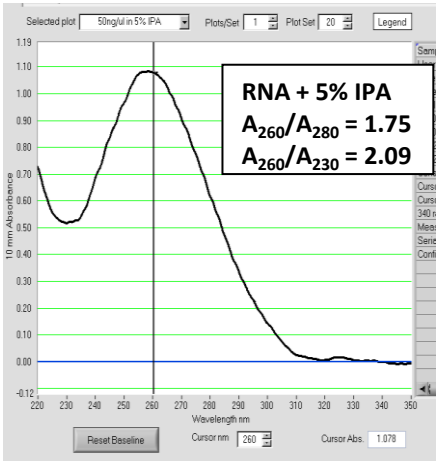
Ethanol and isopropanol have little effect on purity ratios



Pure RNA



RNA + 5% EtOH



RNA + 5% IPA

If you can't detect alcohols, you need clean RNA from your purification kits!

UV Absorbance - NanoDrop®
Measures Small Volumes with Good Sensitivity

Promega

Features:

- ✓ Measures the absorbance of small volume samples of nucleic acids
- ✓ 0.5 – 2µl of sample required
- ✓ 190nm – 840nm wavelength range
- ✓ Wide detection range
  - 2ng/µl minimum (RNA)
  - 12,000ng/µl maximum (RNA)
- ✓ Measurements in less than 30 seconds
- ✓ No other reagents or accessories required

UV Absorbance - NanoDrop®

Simple to Use, Easy to Interpret

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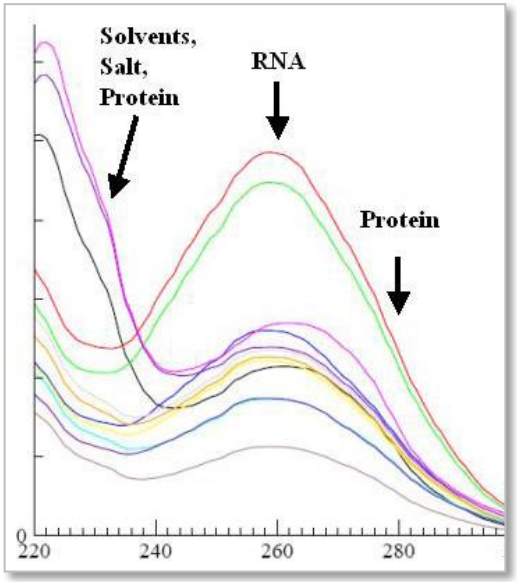
1. Select Read Type:
   Nucleic Acid, RNA

2. Read:
   - Water ✓
   - Blank ✓
   - Sample ✓

Output



Report | Test type: Nucleic Acid | 1/25/2012 1:33 PM | Exit

eport Name | Max Buffer Size: 200 | Buffer Mode: Save Report & Clear

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.
DNased RNA 4-12-11 #132	Default	1/25/2012	1:25 PM	15.06	0.377	0.223	1.69	0.28	40.00	230
DNased RNA 4-12-11 #134	Default	1/25/2012	1:26 PM	17.05	0.426	0.259	1.65	0.45	40.00	230
DNased RNA 4-12-11 #136	Default	1/25/2012	1:26 PM	12.27	0.307	0.186	1.65	0.06	40.00	230
DNased RNA 4-12-11 #138	Default	1/25/2012	1:27 PM	12.51	0.313	0.212	1.47	0.40	40.00	230
DNased RNA 4-12-11 #140	Default	1/25/2012	1:28 PM	13.54	0.339	0.224	1.51	0.51	40.00	230
DNased RNA 4-12-11 #142	Default	1/25/2012	1:28 PM	10.21	0.255	0.182	1.40	0.39	40.00	230
DNased RNA 4-12-11 #407	Default	1/25/2012	1:29 PM	12.95	0.324	0.208	1.55	0.47	40.00	230
DNased RNA 4-12-11 #409	Default	1/25/2012	1:30 PM	11.44	0.286	0.191	1.50	0.43	40.00	230
DNased RNA 4-12-11 #411	Default	1/25/2012	1:30 PM	12.07	0.302	0.197	1.53	0.46	40.00	230
DNased RNA 4-12-11 #413	Default	1/25/2012	1:31 PM	11.68	0.292	0.192	1.52	0.43	40.00	230
DNased RNA 4-12-11 #415	Default	1/25/2012	1:32 PM	12.93	0.323	0.209	1.54	0.46	40.00	230
DNased RNA 4-12-11 #417	Default	1/25/2012	1:33 PM	11.54	0.288	0.172	1.67	0.41	40.00	230

- Concentration (ng/µl)
- Purity Ratios:
  - A_{260}/A_{280}
  - A_{260}/A_{230}

UV Absorbance – Disadvantages
No Nucleic Acid Specificity or Info on Integrity

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- Lack of specificity – cannot distinguish between dsDNA, RNA, or ssDNA
- gDNA contamination cannot be determined
- Contaminants that absorb at 260nm – overestimation of nucleic acid
- No information on integrity – degraded RNA (nucleotides) will still contribute to 260nm reading

Total RNA Quantitation and Quality Assessment

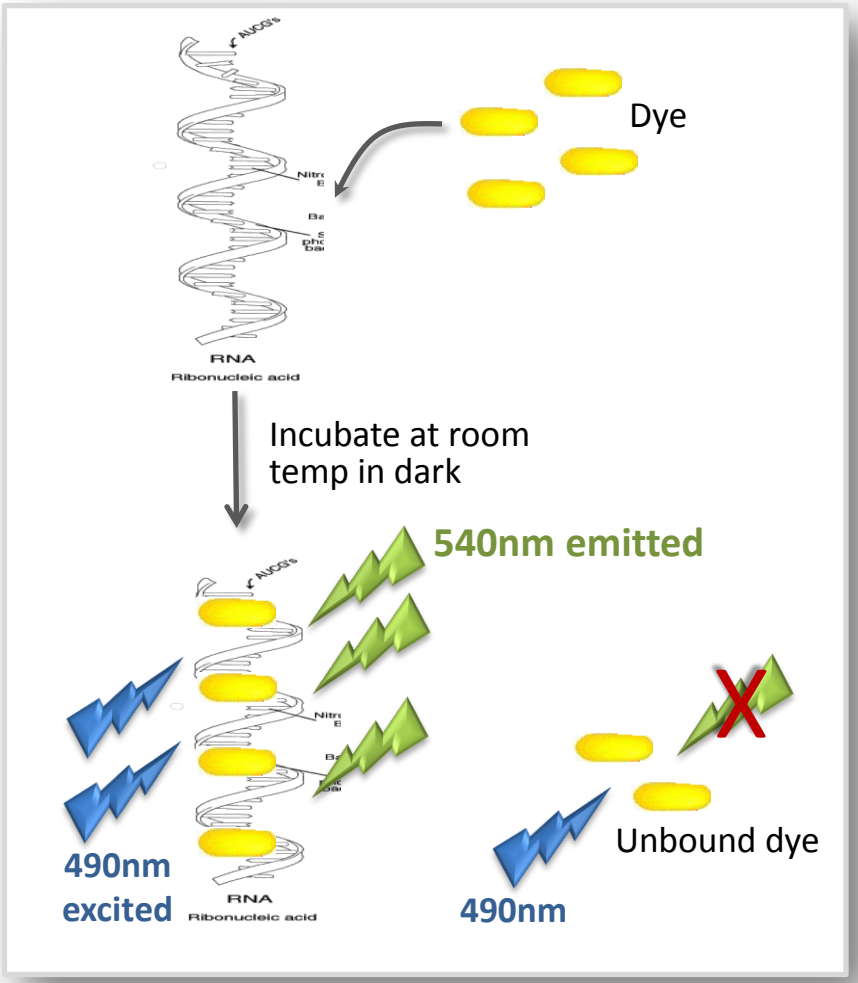
- UV Absorbance
  - Spectrophotometer
  - NanoDrop®/NanoVue™
- Fluorescent Dye-based Quantitation
  - Plate Reader
  - Hand-held Instruments
- Gel Electrophoresis
  - Agarose
  - Acrylamide
- Agilent 2100 Bioanalyzer

Total RNA Quantitation and Quality Assessment

- Fluorescent Dye-based Quantitation
  - Plate Reader
  - Hand-held Instruments

Fluorescent Dye-based Quantification
Lower Background for Increased Sensitivity

- Binding of dye to RNA causes conformation change in dye allowing it to fluoresce when excited
- Fluorescence output is directly proportional to amount of RNA in sample
  - More RNA = higher fluorescence
- Unbound dye molecules do not under go conformational change and therefore do not fluoresce
  - Lower background = improved sensitivity



Microplate & PCR Tube Formats
Flexible Formats for Multiple Detection Instruments

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1. Dilute RNA dye in 1xTE buffer to make dye working solution and protect from light.

2. Generate standards and unknown samples
   - Make standard curve and add sample to microtiter plate, or 0.5ml PCR tubes
   - Dilute unknown samples in 1xTE and add to microtiter plate, or 0.5ml PCR tubes

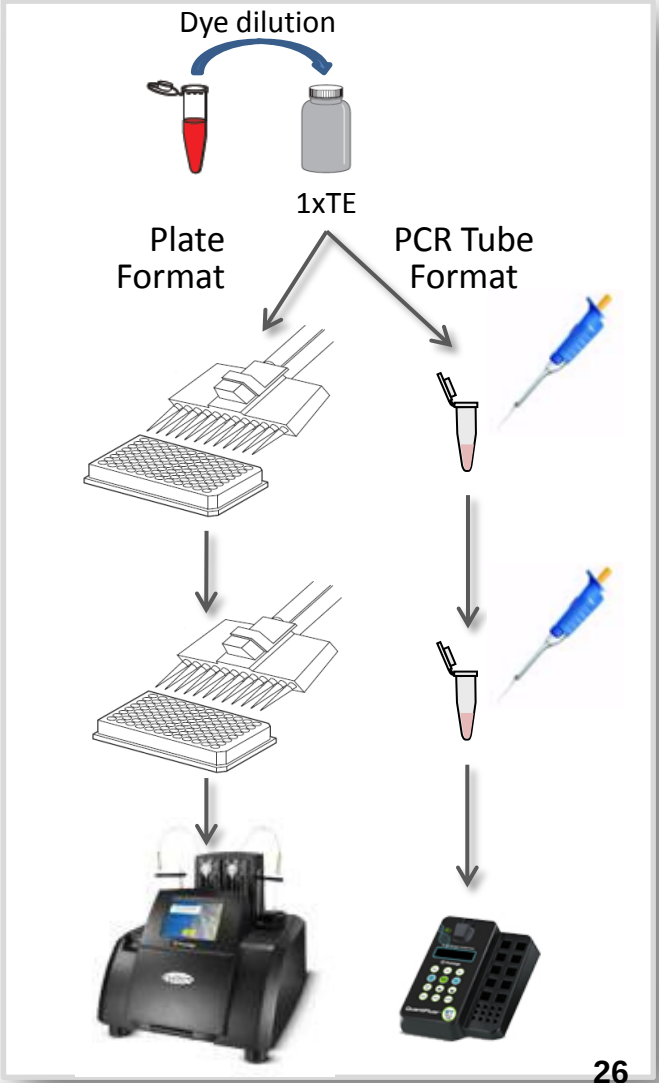
3. Add dye working solution to each sample well and incubate at RT protected from light.

4. Measure fluorescence
   - Microplate reader

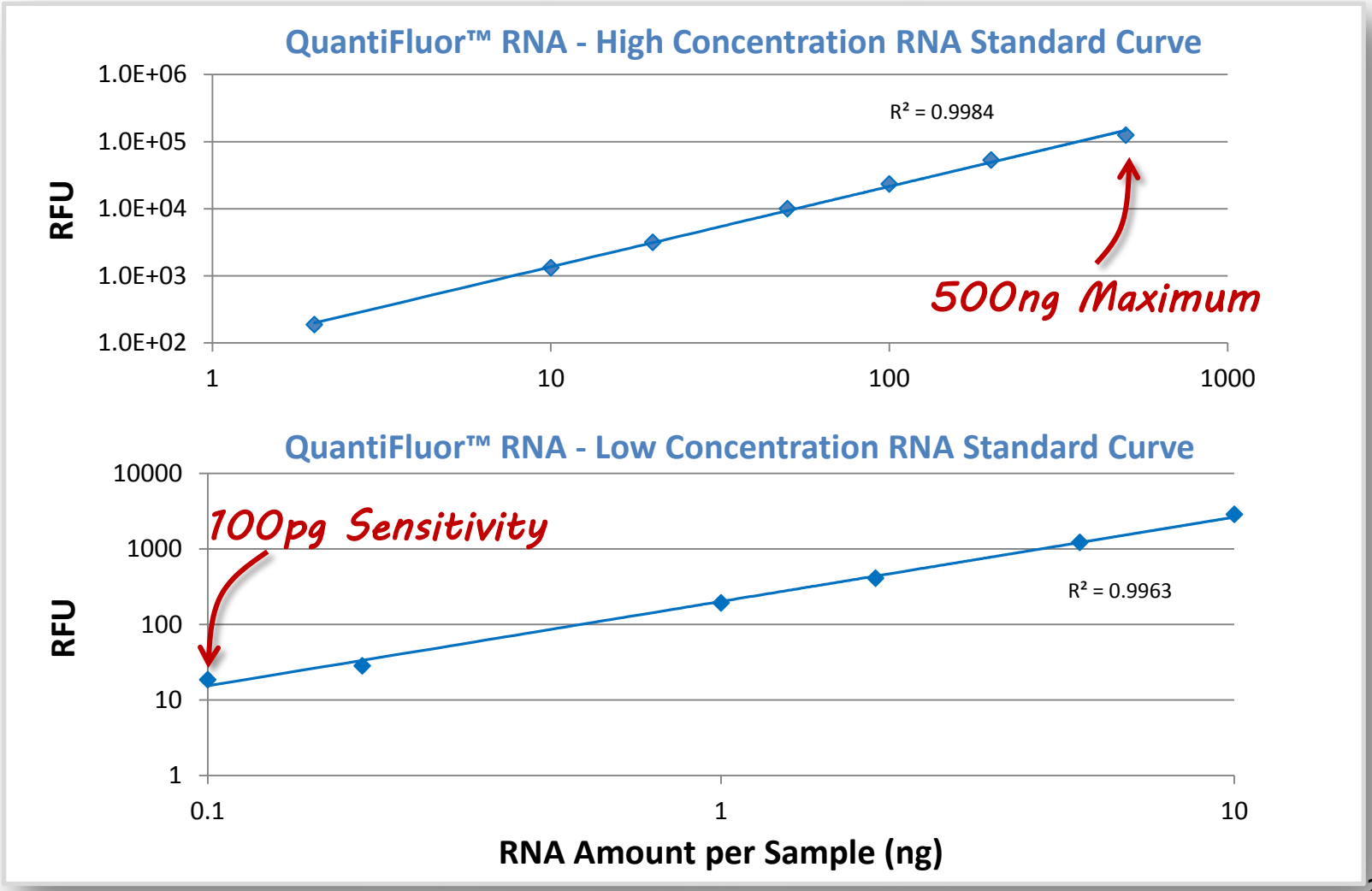
     GloMax®-Multi+ Microplate Multimode Reader

   - Single tube formats

     QuantiFluor™ Single-Tube Fluorometers



High and Low Concentration Standard Curves
High Sensitivity Down to 100pg per Sample



Fluorescent Dye-based Quantitation–Disadvantages
No Information on Purity or Integrity

- ✓ Lack of specificity
  - QuantiFluor™ and RiboGreen not RNA specific – may require DNase treatment
- ✓ Creation of standards – High and Low concentration
- ✓ No information on purity – separate dye based quantification systems are available for DNA and protein.
- ✓ No information on integrity
- ✓ Fluorescent dyes are potentially hazardous

Total RNA Quantitation and Quality Assessment

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  - Agarose
  - Acrylamide
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Total RNA Quantitation and Quality Assessment

- Gel Electrophoresis
  - Agarose
  - Acrylamide

Gel Electrophoresis
Most Common Analysis Method

- Nucleic acid fragments separated on the basis of size
- Agarose and acrylamide gel electrophoresis
- Fragments stained with a fluorescent dye that binds specifically to the nucleic acid and visualized by the excitation of the dye bound to the nucleic acid.
  - Ethidium bromide, SYBR® Green, and SYBR® Gold.
- RNA quantitation
  - Estimate the relative intensity of fluorescence compared to known amounts
  - Gel densitometry

Gel Electrophoresis
Visual Analysis of Integrity and gDNA Contamination

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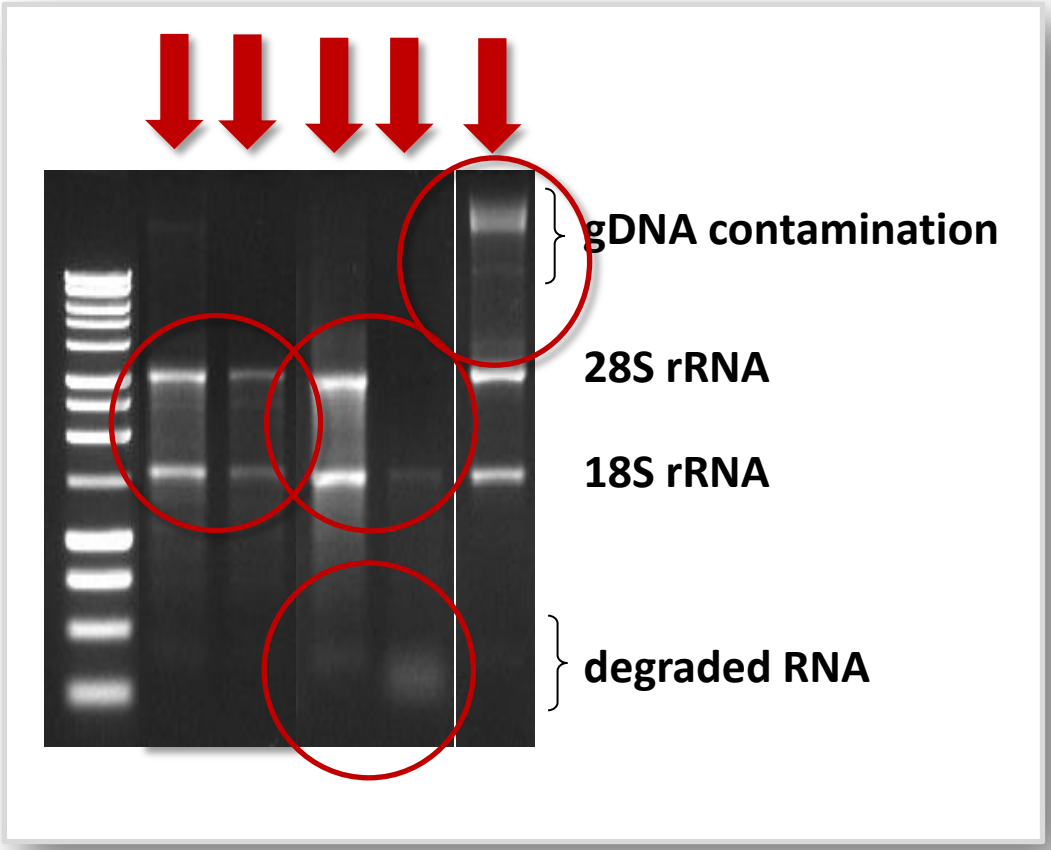
Qualitative analysis:

- RNA integrity

  For mammalian ribosomal RNA, a 28S:18S rRNA ratio of 2:1 is generally considered to be representative of good quality RNA.

- Genomic DNA contamination



Gel Electrophoresis
Drawbacks and Alternatives to Ethidium Bromide

- Low cost, but requires significant amount of handling and hands on time.
- Typically requires a few nanograms of RNA
  - Minimum detectable mass detectable varies by stain
- SYBR® Green II and SYBR® Gold dyes 2.4X and 7.9X more sensitive than ethidium bromide[1]
- Suspected carcinogens - SYBR® Green II and SYBR® Gold dyes can be viewed as safer alternatives to ethidium bromide[2]

[1] Tuma, R.S. et al. Characterization of SYBR Gold nucleic acid gel stain: a dye optimized for use with 300-nm ultraviolet transilluminators. Anal Biochem. 1999, 268(2), 278-88.

[2] Kirsanov, K.I. et al. SYBR Gold and SYBR Green II are not mutagenic in the Ames test. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2010, 1–2 (17), 1–4.

Total RNA Quantitation and Quality Assessment

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Total RNA Quantitation and Quality Assessment

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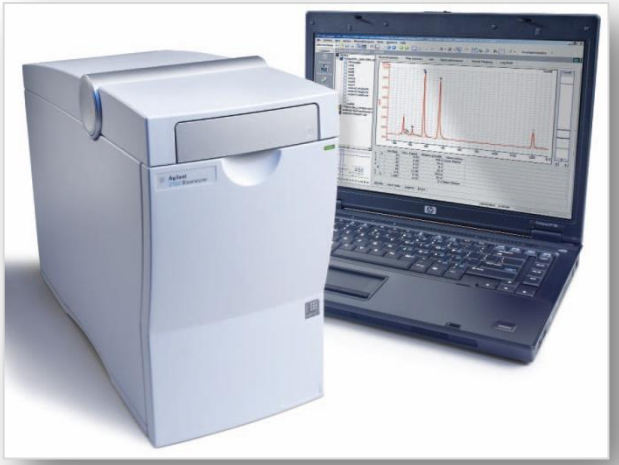
Agilent 2100 Bioanalyzer
Microfluidics to Analyze 1µl of Sample

Promega

- Uses microfluidics to analyze DNA, RNA, protein, and cells using sample specific chips
- Samples are combined with a fluorescent dye and added into wells in the chip.
- The samples are separated by electrophoresis.
- The samples are detected by fluorescence, and electropherograms and gel-like images are provided for sizing and quantification.
- 1µl of sample is required, 11-12 samples can be run on the same chip, and analysis is complete in 30-40 minutes.



Agilent 2100 Bioanalyzer – RNA Analysis
High Sensitivity, RNA Integrity Assessment

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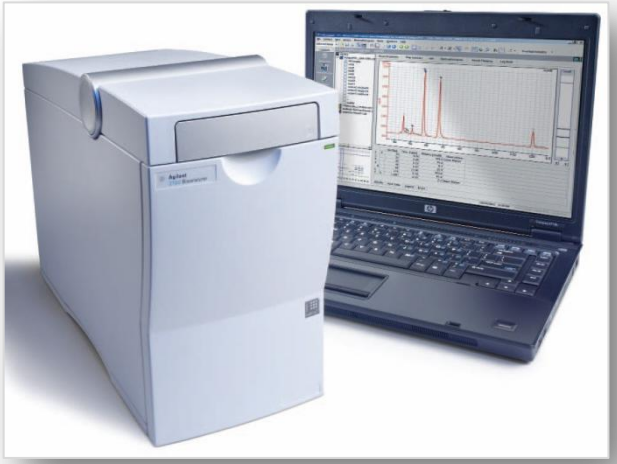
- RNA analysis kits
  - RNA 6000 Nano Kit - total RNA and mRNAs (5ng/µl up to 500ng/µl)
  - RNA 6000 Pico Kit - total RNA and mRNAs (50pg/µl up to 5000pg/µl)
  - Small RNA Kit - small and microRNAs
- Information provided:
  - RNA Integrity Number (RIN) ←
  - RNA concentration
  - 28S : 18S ratio
  - Gel-like image



Agilent 2100 Bioanalyzer – RIN=10.0

High Quality RNA with Minimal Degradation

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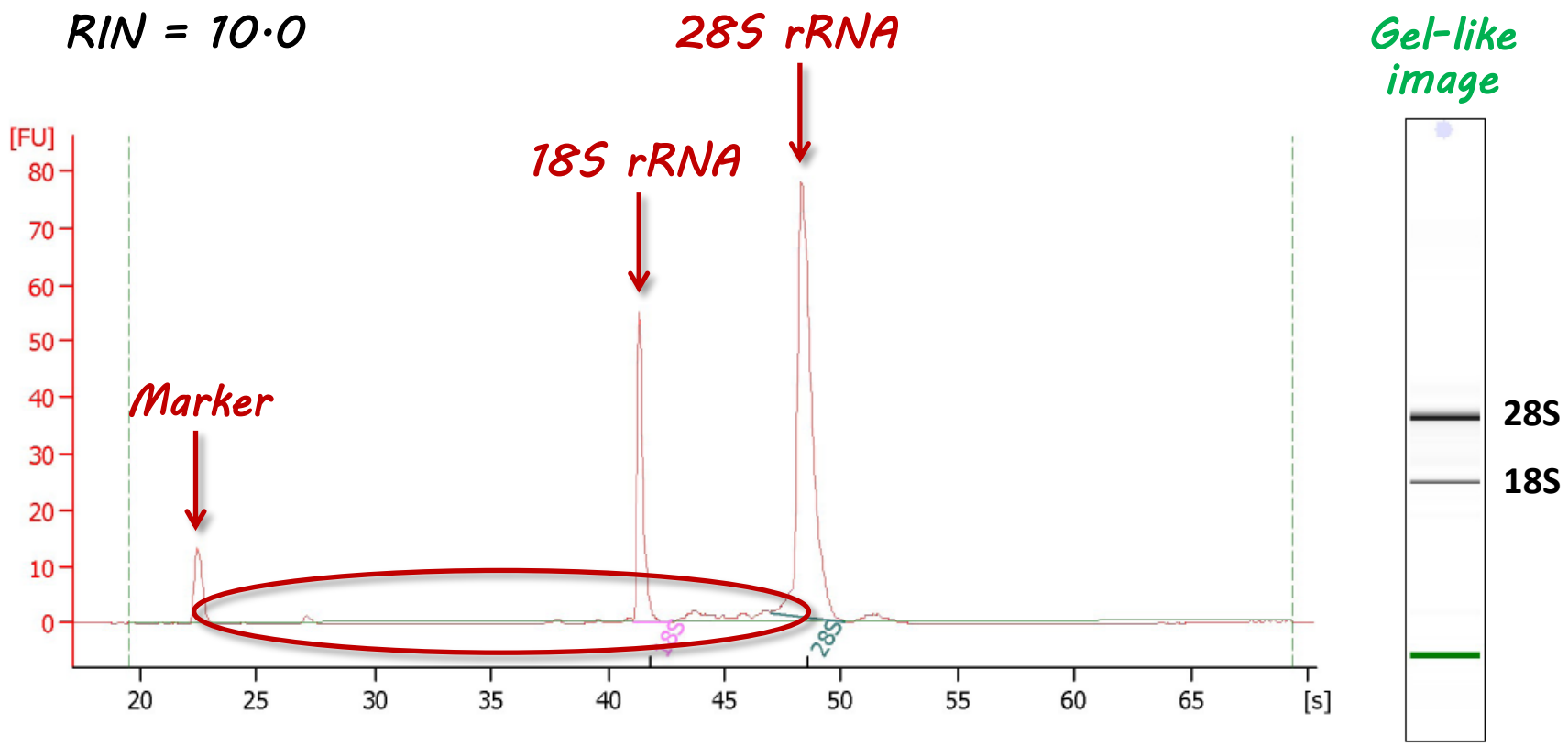
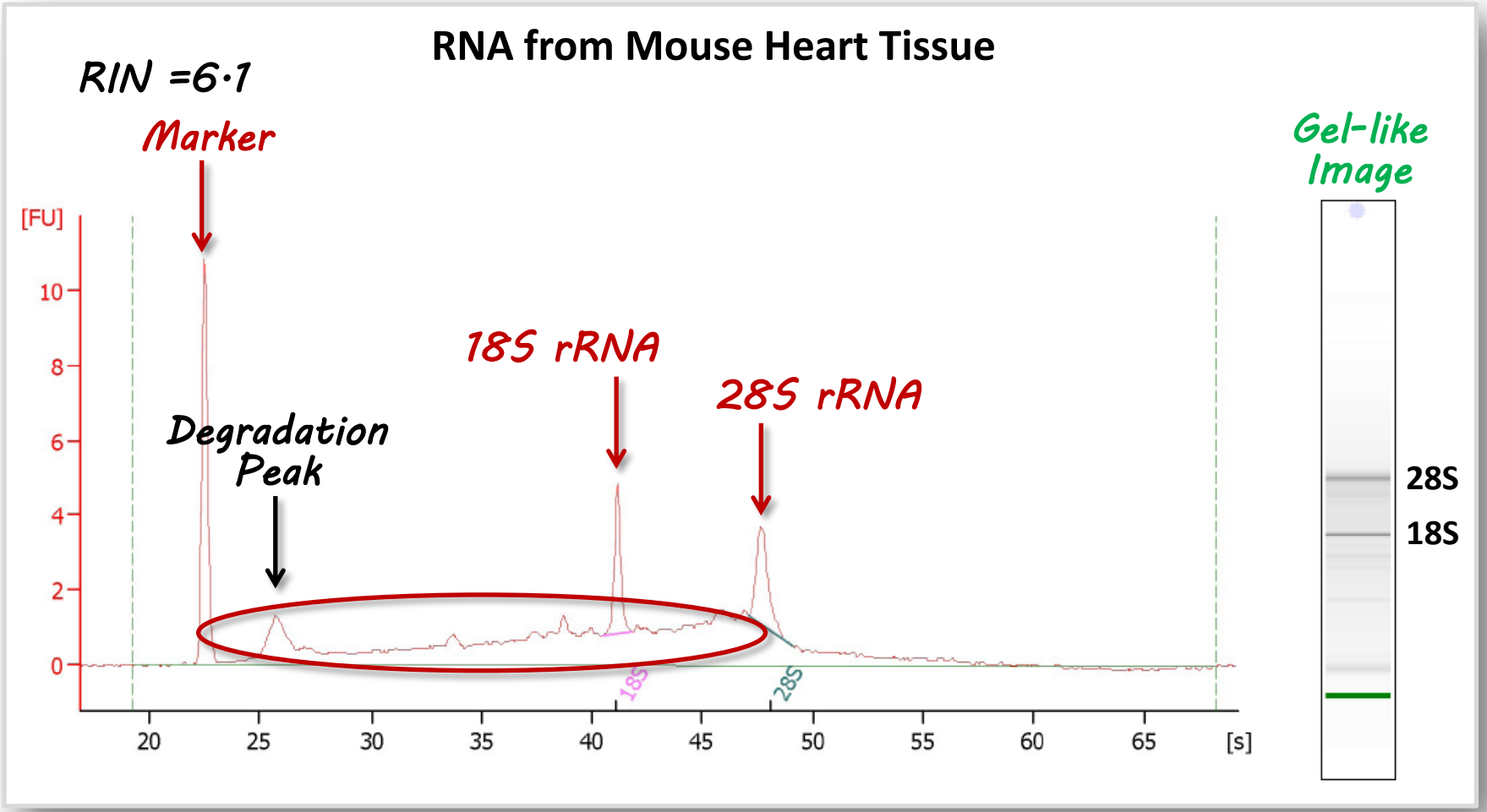
RNA from HEK293 cells



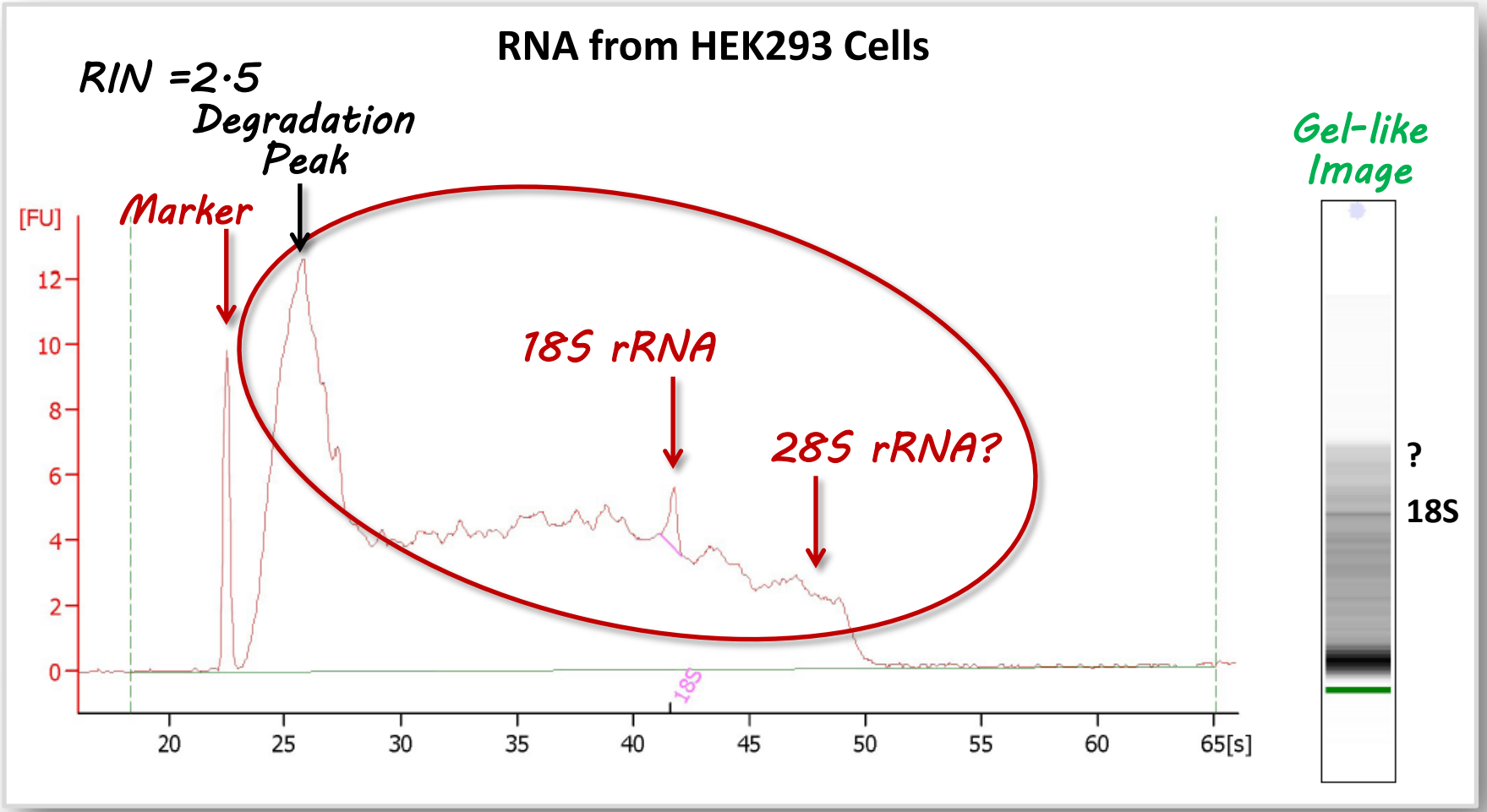
Agilent 2100 Bioanalyzer – RIN=6.1

RNA Degradation Becomes Apparent



Agilent 2100 Bioanalyzer – RIN=2.5

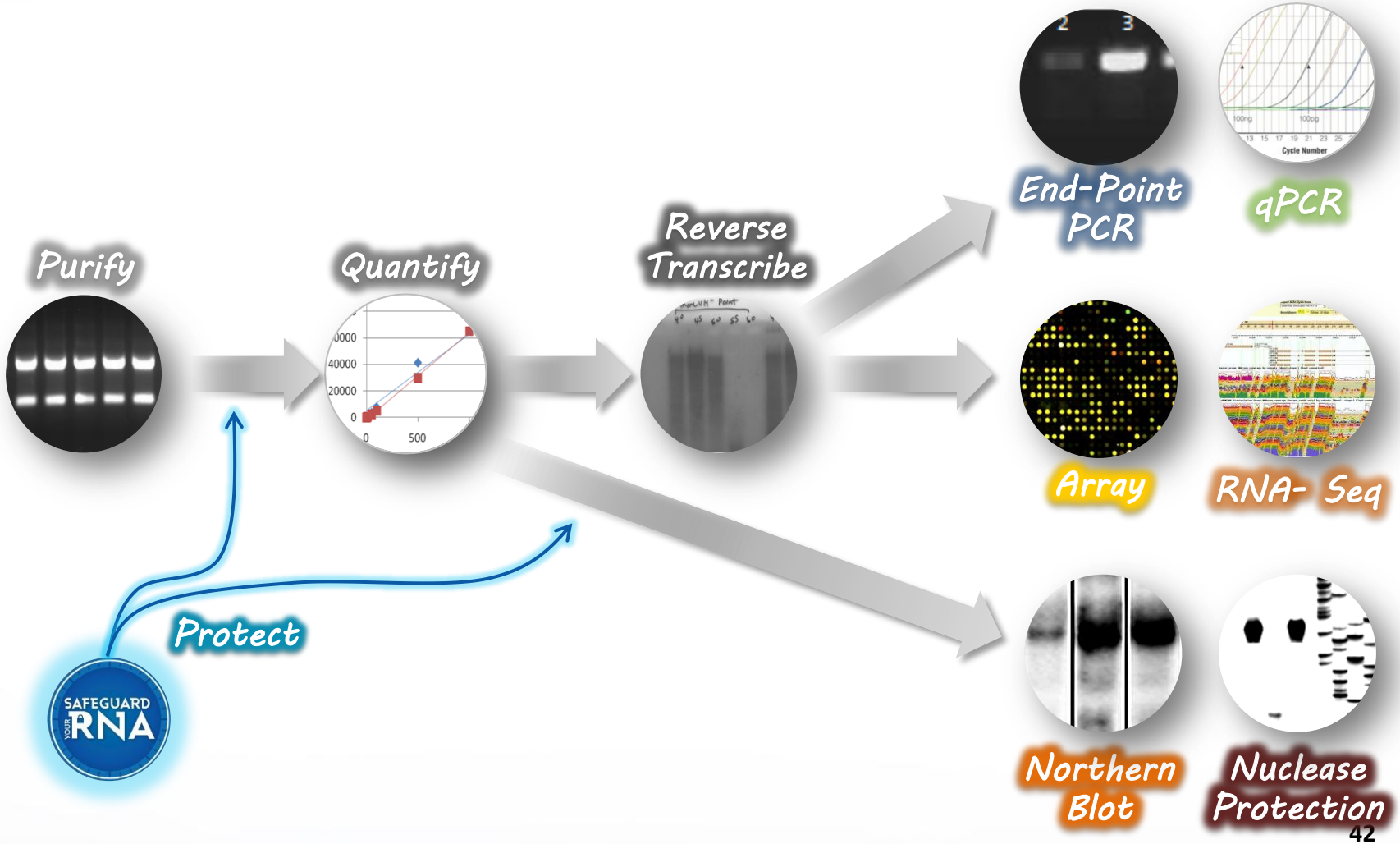
Low Quality RNA Shows Loss of rRNA Peaks



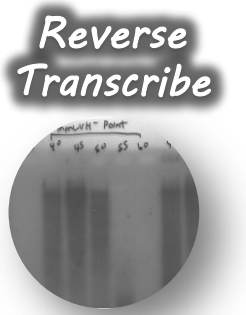
Agilent 2100 Bioanalyzer – Disadvantages
No Assessment of Purity

- No information on purity
  - gDNA too large to be analyzed
  - No detection of organic contaminants
- High costs of instrumentation, reagents, and chips

Reverse Transcription
Converting RNA into cDNA for Downstream Analysis



Reverse Transcription
Converting RNA into cDNA for Downstream Analysis

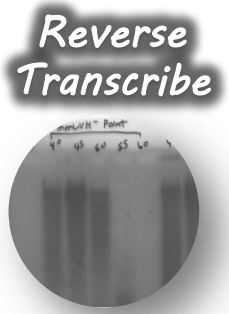


Reverse Transcription
Converting RNA into cDNA for Downstream Analysis

Promega

Key Challenges

- GC-rich mRNA targets
- Secondary structure in mRNA
- Presence of RT enzyme inhibitors in RNA preps

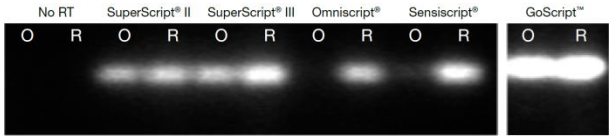


Reverse Transcription
The RT System You Choose Makes a Difference

- **GoScript® Reverse Transcriptase & Kits**
  - M-MLV reverse transcriptase
  - Proprietary & optimized buffers
- **Key Features:**
  - Efficiently transcribes long mRNAs
  - Improved synthesis through RNA secondary structure
  - Performs better in the presence of inhibitors such as ethanol

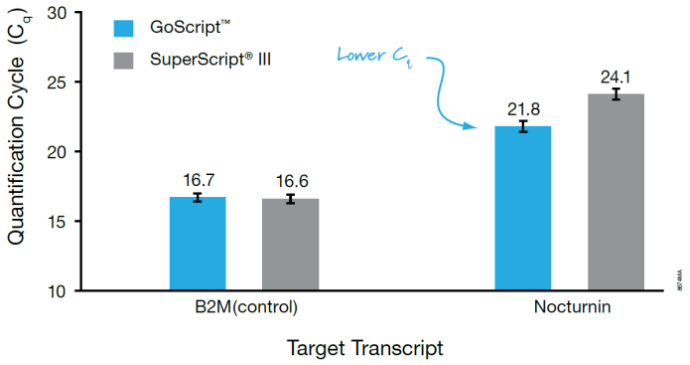
Lining Up the Scripts: Reverse Transcriptase Comparison Study. Ammerschläger, M. et al.

Better cDNA Length



O = Oligo dT primers R = Random primers

Improved RT Through RNA Secondary Structure

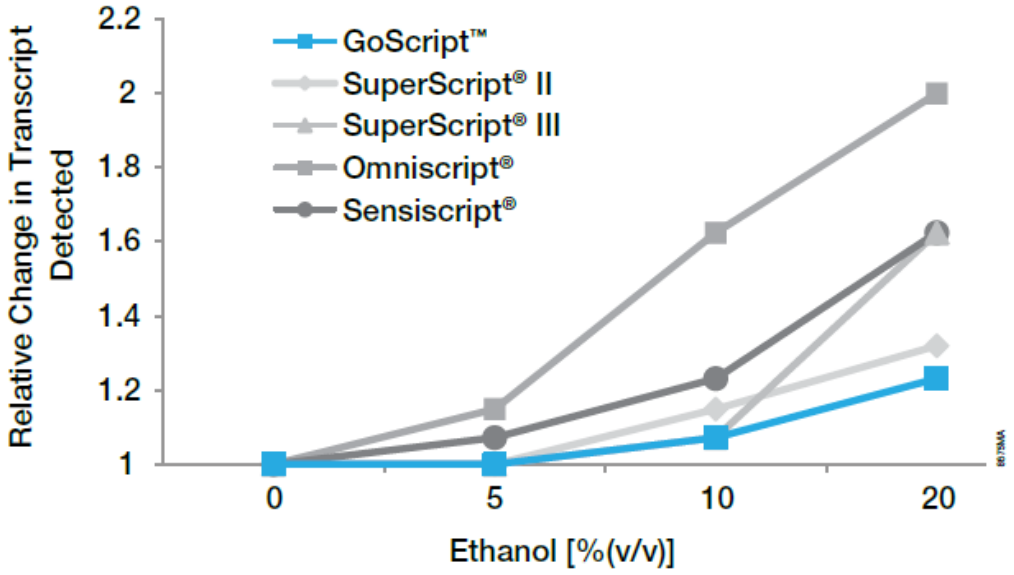


RT → GoTaq® qPCR Master Mix

Reverse Transcription
The RT System You Choose Makes a Difference

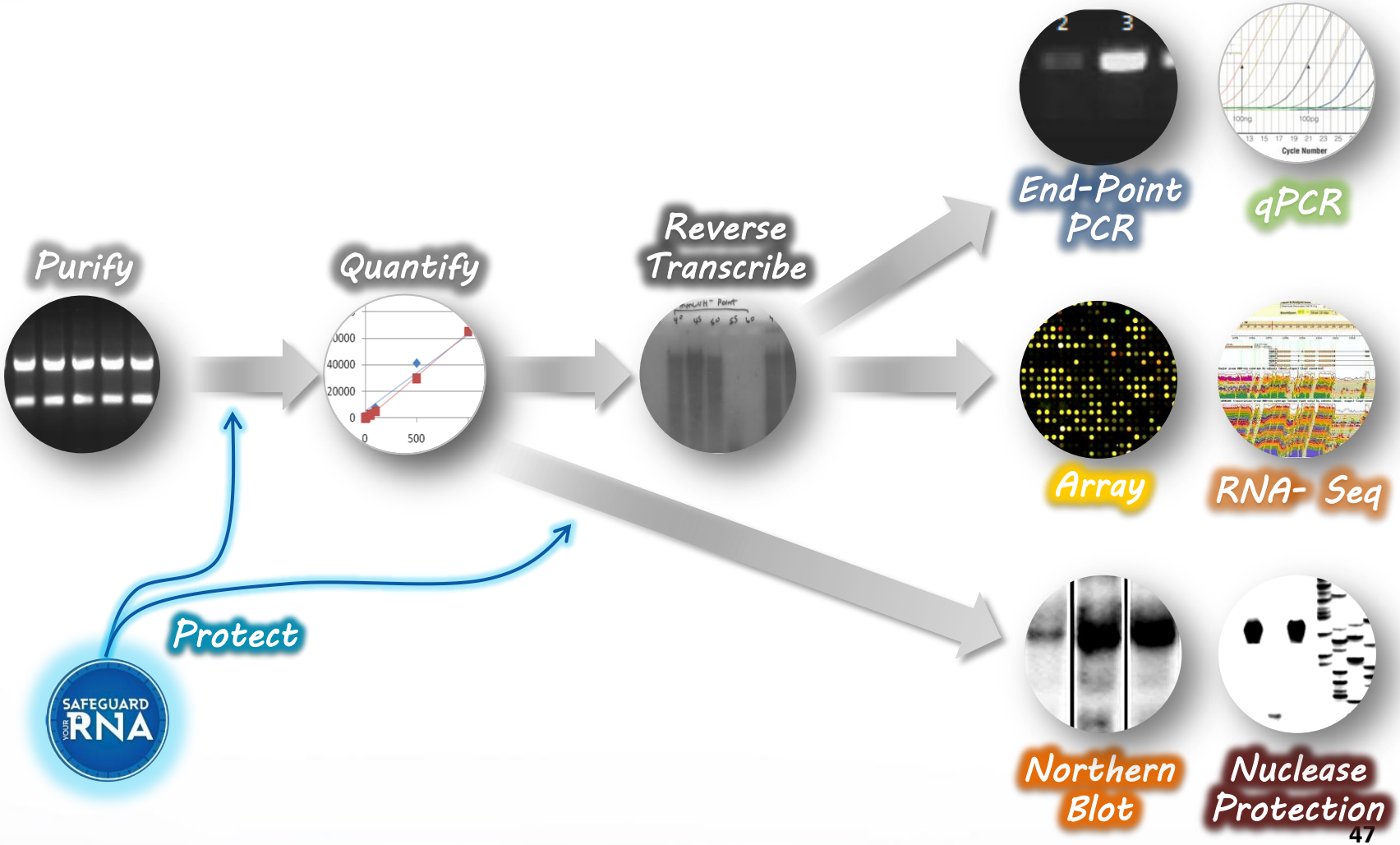
Promega

Better Resistance to Alcohols



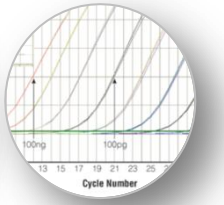
Better reverse transcription means better, more sensitive downstream analysis!

RNA Analysis Methods
Many Options Depending on Research Goal

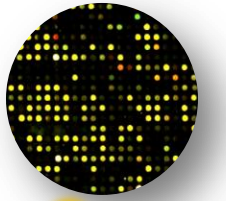


RNA Analysis Methods
Many Options Depending on Research Goal

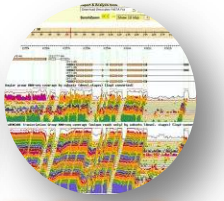
End-Point PCR



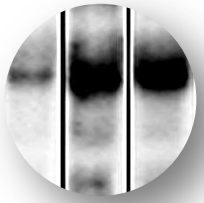
qPCR



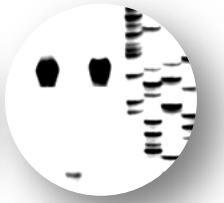
Array



RNA- Seq



Northern Blot



Nuclease Protection

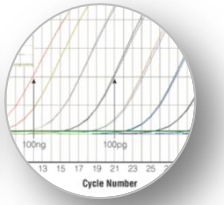
RNA Analysis Methods
Many Options Depending on Research Goal

Promega

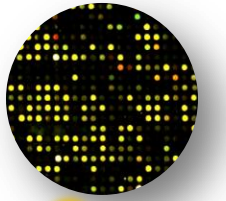
Key questions to ask yourself:

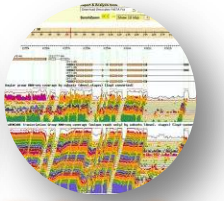
- How many target RNAs do I want to measure?
- Am I interested in whole transcriptome analysis?
- Am I interested in identifying splice variants?
- Do I want information on target size, integrity?
- Do I want highly quantitative data or is qualitative assessment OK?

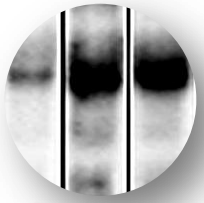
End-Point PCR

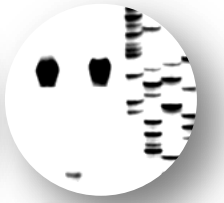


qPCR

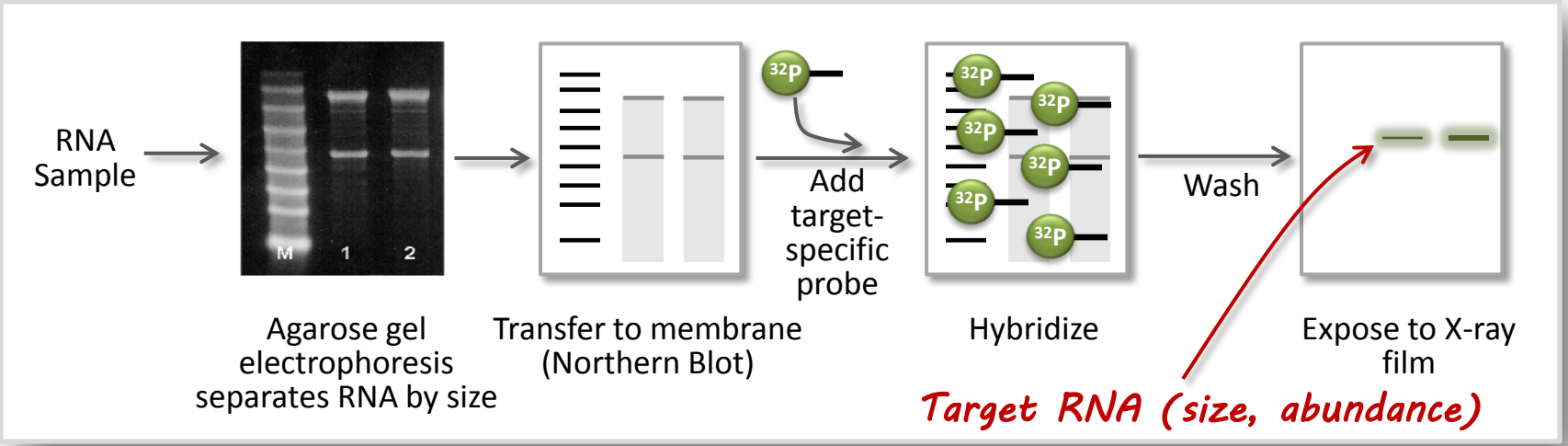

Array


RNA- Seq


Northern Blot


Nuclease Protection

Northern Blotting
Provides Info on Size and Expression Level



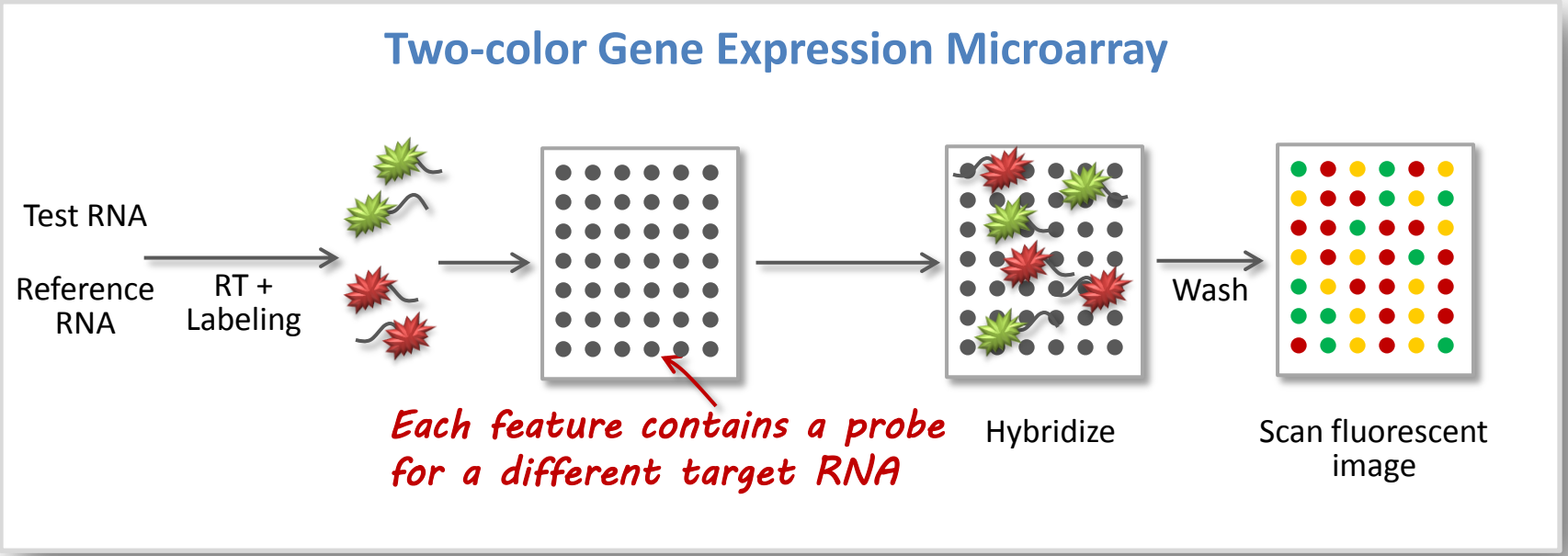
Advantages

- Provides target RNA size and information on alternative splice variants
- Illustrates RNA integrity (smears indicate degraded samples)
- Semi-quantitative measure of expression level

Disadvantages

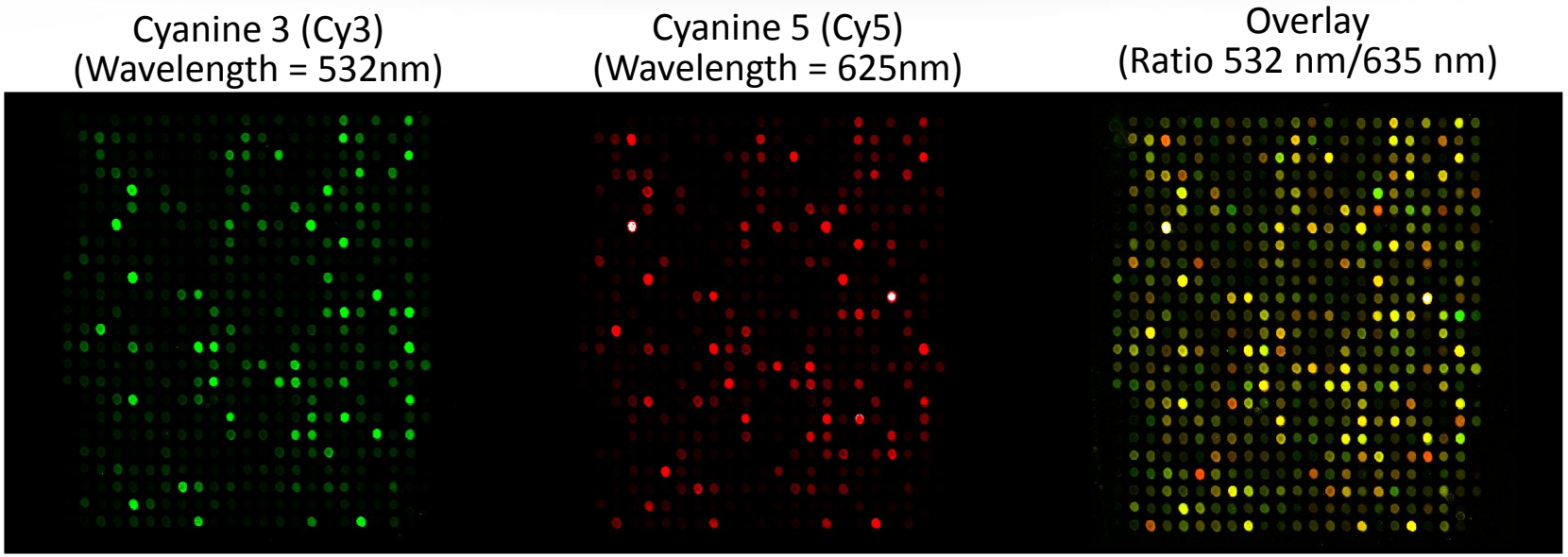
- Assays only 1 target RNA per hybridization (can do more than 1 if well separated by size and previously characterized)
- Semi-quantitative

Microarrays
"Multiplex Northern Blot"



- RNA samples are labeled, probes on arrays are unlabeled
- One-color microarrays are also performed (Affymetrix arrays)
- Compare one array to another to determine relative expression levels

Microarrays
"Multiplex Northern Blot"



Advantages

- Simultaneous measurement of all known mRNA
- Provides relative expression levels between two samples – great comparative, exploration tool

Disadvantages

- Difficult to detect and measure alternative splice variants
- Requires previous sequence knowledge of mRNAs to be analyzed

RNA-seq
The Sexy, New Kid on the Block

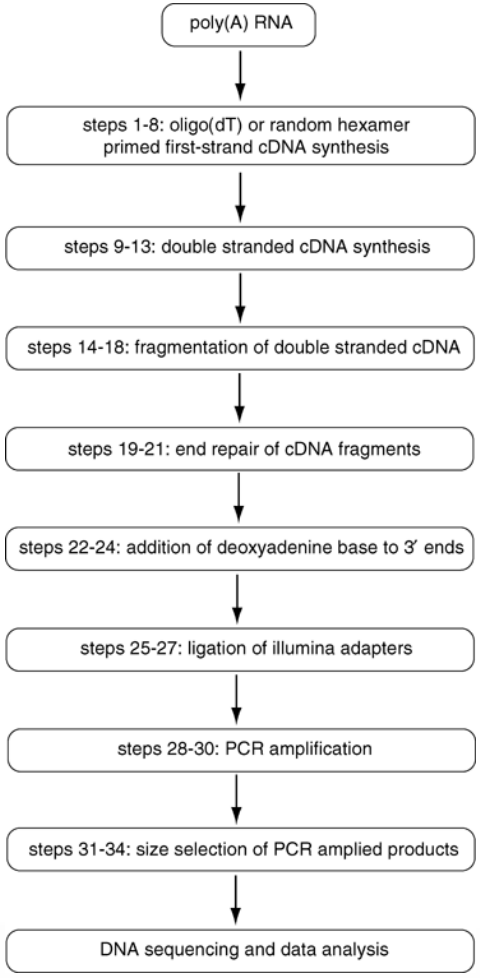
Advantages

- Provides the most information in a single experiment
  - Absolute quantitation of known mRNAs
  - Absolute quantitation of previously undiscovered mRNAs (genes)
  - Splice variants
  - Allelic expression patterns

Disadvantages

- Expensive
- Requires extensive bioinformatic support
- Also requires large data storage capabilities

Illumina Protocol



From: Curr. Protoc. Mol. Biol. **89**:4.11.1-4.11.13.

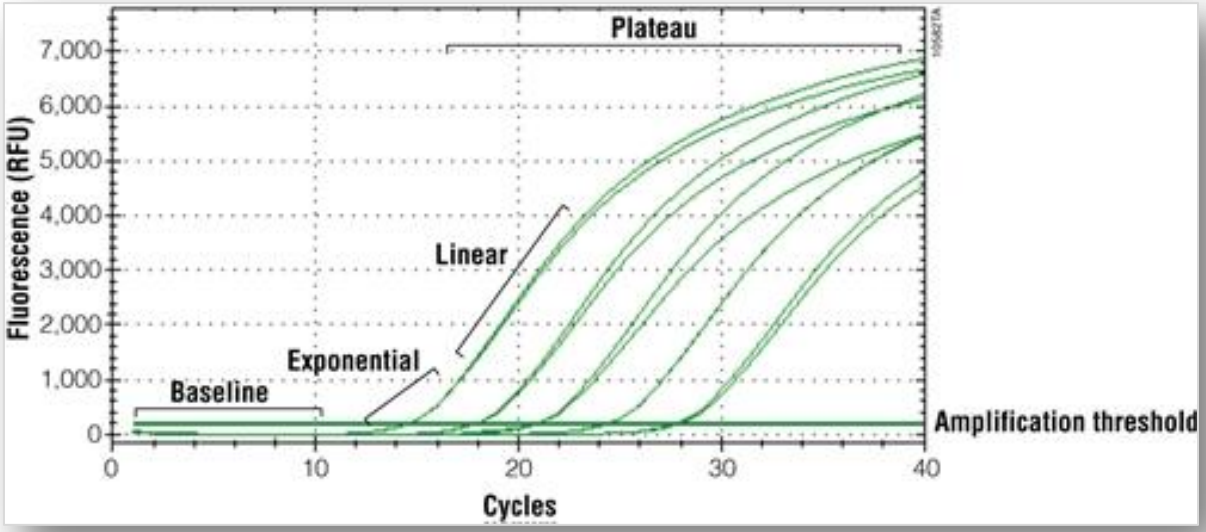
Real-Time PCR
Measuring Product After Each PCR Cycle

What is end-point PCR?

The amount of amplified product is typically determined only after a set number of amplification cycles is completed

What is Real-Time PCR?

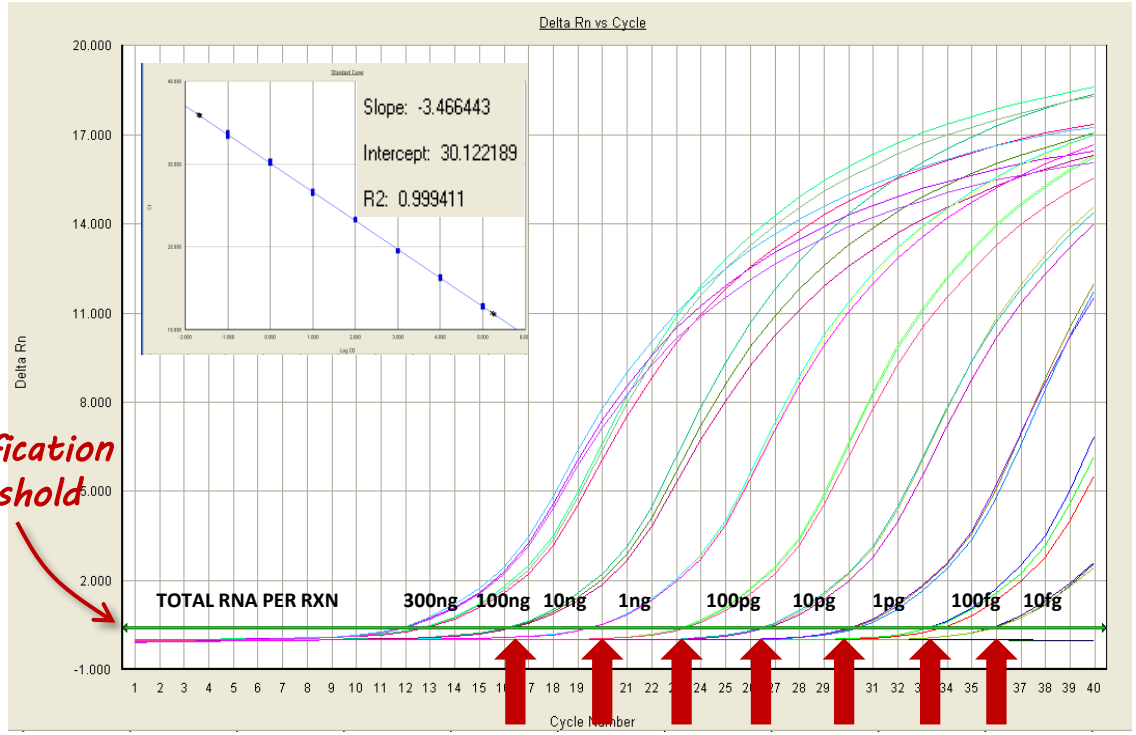
The amount of amplified product is measured after each PCR amplification cycle



Serial Dilution of Total RNA - GAPDH Transcript
C_q Used to Measure Starting Template Amount

C_q = quantification cycle = Cycle number at which amplification curve crosses amplification threshold

C_q value is inversely proportional to amount of starting template



Each cycle results in the doubling of the product:

- ✓ 1 Cq difference = 2-fold difference in starting material
- ✓ 3.3 Cq difference = 10-fold difference in starting material

Real-Time PCR
Learn More About the Ins & Outs of RT-qPCR

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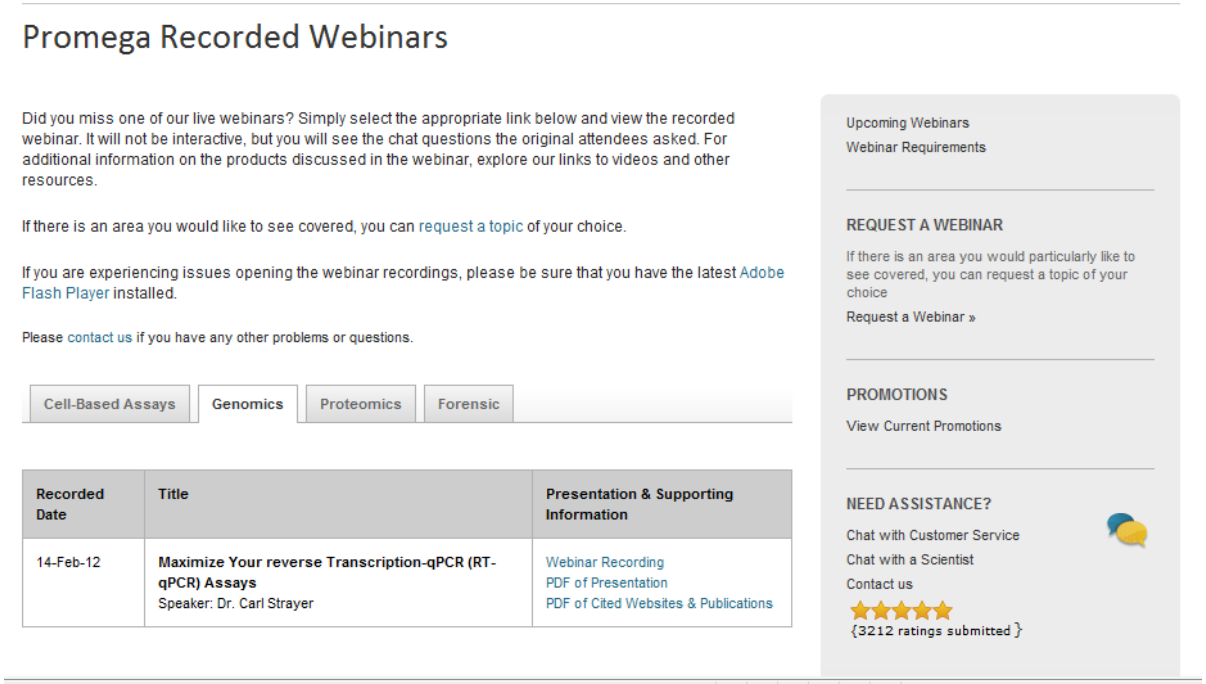
Maximize Your Reverse Transcription-qPCR (RT-qPCR) Assays

Speaker: Dr. Carl Strayer



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Recorded Date	Title	Presentation & Supporting Information
14-Feb-12	**Maximize Your reverse Transcription-qPCR (RT-qPCR) Assays** Speaker: Dr. Carl Strayer	Webinar Recording PDF of Presentation PDF of Cited Websites & Publications

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Getting the Best Results from Your Reverse Transcription Quantitative PCR Assay

RT-qPCR
Suitable for Relative or Absolute Quantitation

RT-qPCR can be used to measure the amount of a specific RNA target in a sample by incorporating a reverse transcription step (RT) followed by qPCR.

- **Relative quantification** – Sample C_q is compared to a reference gene C_q
- **Absolute quantification** - A standard of known target amounts is run in parallel with samples of interest to create a standard curve and enable comparison to the unknowns

RT-qPCR is often the downstream application for RNA analysis, but it can be used to qualify RNA for other downstream applications as well.

- Analyze samples for the presence of PCR inhibitors
- Delay in C_q generation may indicate the presence of PCR contaminants that could affect subsequent downstream applications
- Assay integrity (e.g. Is there amplifiable RNA from FFPE samples?)

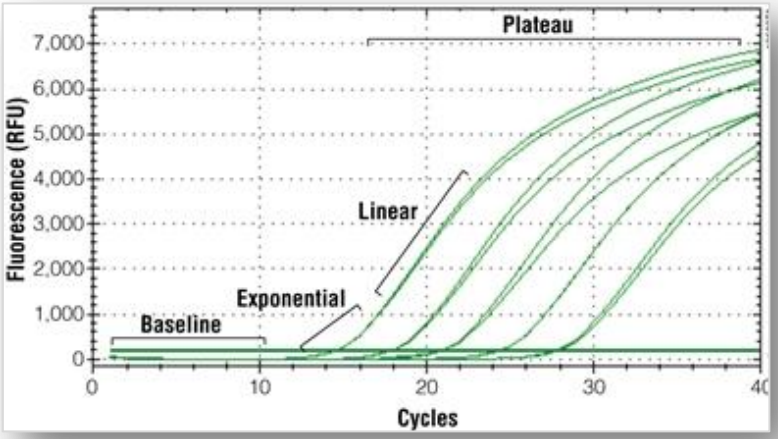
RT-qPCR
Highly Quantitative Measure of mRNA Levels

Advantages

- Highly quantitative measurements
- Limited multiplexing is possible (probe-based systems only)
- Wide dynamic range

Disadvantages

- Limited number of target mRNAs assessed per reaction

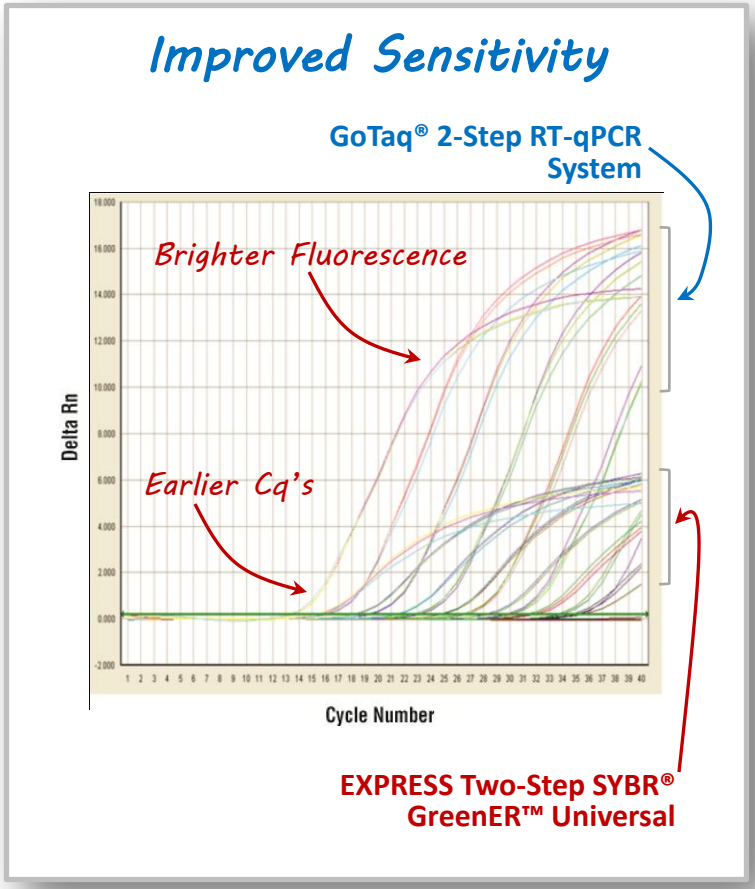


Key Challenges

- Wide variation in mRNA expression levels per sample
- RT-qPCR enzymes may be sensitive to inhibitors in starting RNA sample – e.g.:
  - RT and/or *Taq* DNA Pol in 1-step RT-qPCR systems
  - RT in 1st step of 2-step RT-qPCR experiments

RT-qPCR
Pick a System that Provides the Greatest Sensitivity

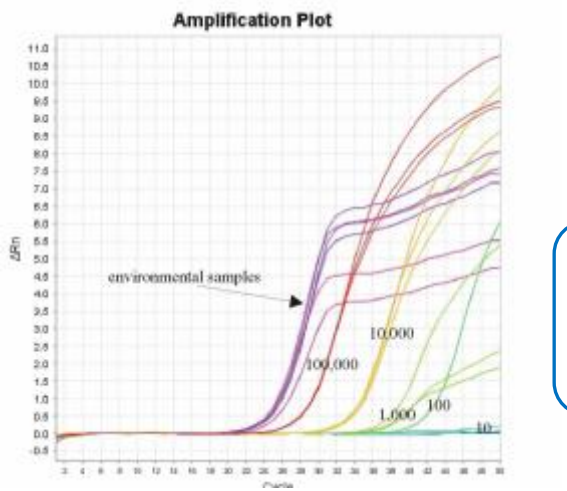
- **GoTaq® 2-Step RT-qPCR System**
  - Combination of GoScript® RT Kit and GoTaq® qPCR Master Mix
- **GoTaq® 1-Step RT-qPCR System**
  - Single tube rxn for both RT and qPCR
  - Built on strength of GoScript® RT and GoTaq® qPCR Master Mix
  - Sensitive, reproducible detection of low abundance transcripts in a single tube



RT-qPCR
Pick a System that Provides the Greatest Sensitivity

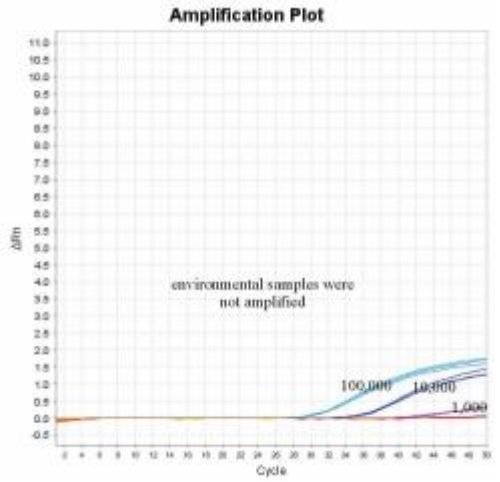
Promega

GoTaq® qPCR Master Mix



5 cycle difference in C_q's

Power SYBR® Green Master Mix



Customer data courtesy of **Andrei C., University of Louisiana Lafayette**

Amplification of target RNA in difficult samples with high levels of contaminants

Summary

- The quality of your final RNA expression analysis experiments depends on careful execution of each step in the workflow
  - Isolation of pure, intact RNA free of contaminants
  - Accurate quantitation and quality assessment of total RNA
  - Selection of the best target RNA-specific quantitation method

- Products are available that overcome some of the major challenges associated with each step of the RNA expression analysis workflow
  - Isolation of pure RNA free of gDNA or ethanol carryover with the ReliaPrep™ Cell RNA Miniprep System
  - Enzymes and kits that are resistant to inhibitors that may be present in your RNA samples (if you don't use a Promega RNA purification kit!)
    - GoScript® RT- and GoTaq®-based RT-kits and RT-qPCR kits

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