DNA and RNA Workflow

Marine Biological Laboratory

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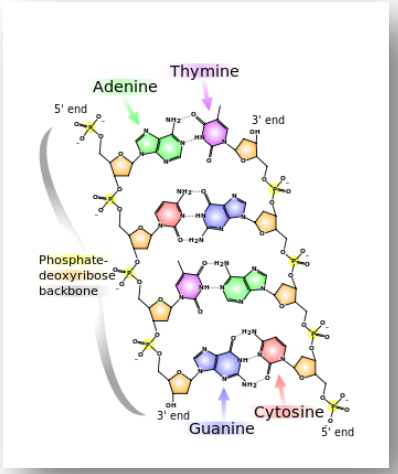
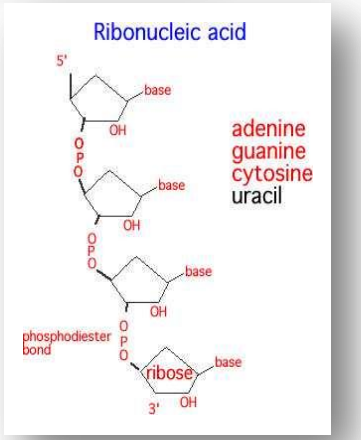
August 2014

Presentation Outline

DNA and RNA Workflow

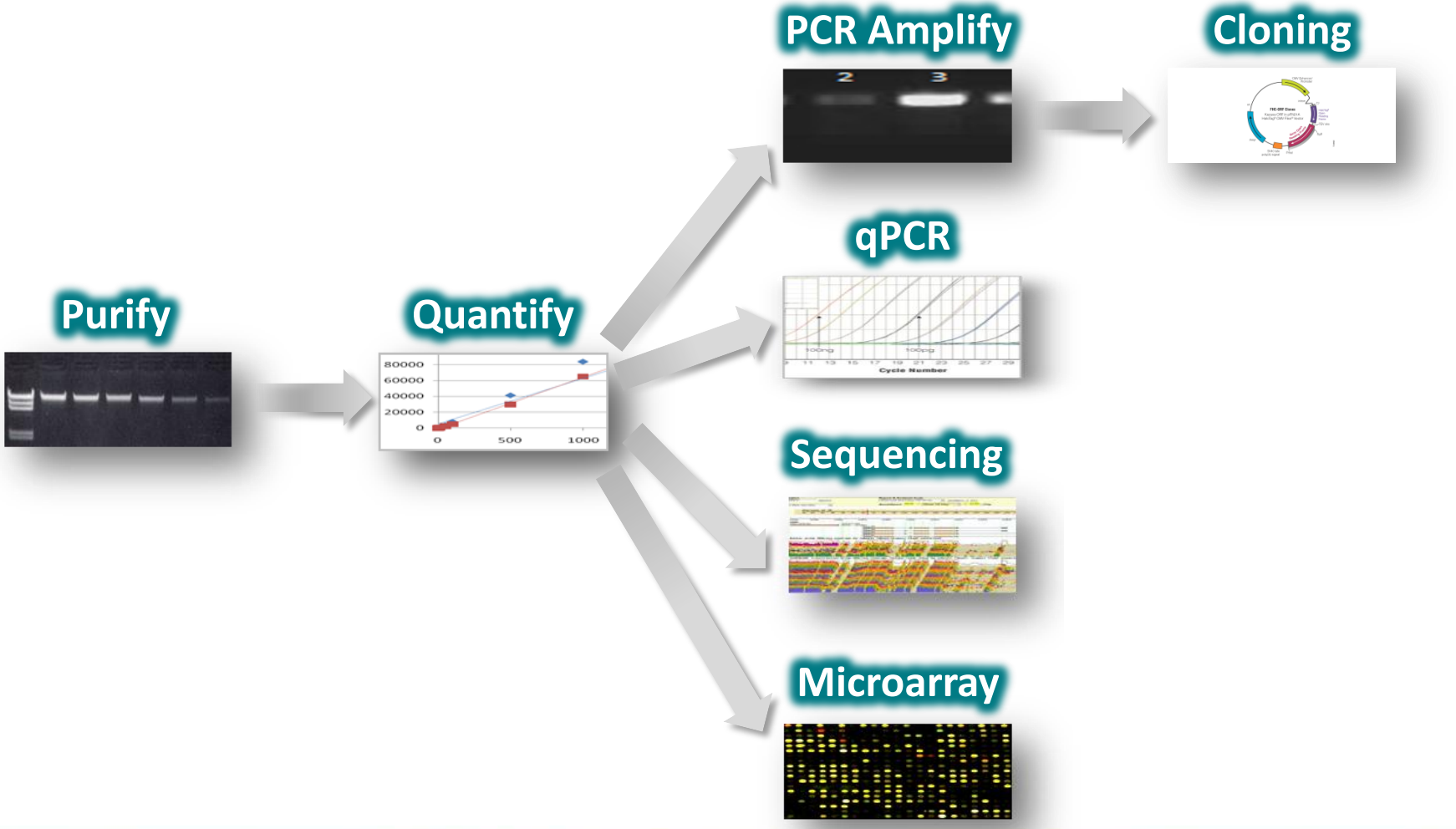
- Purification
- Quantitation
- Qualification
- Analysis Methods



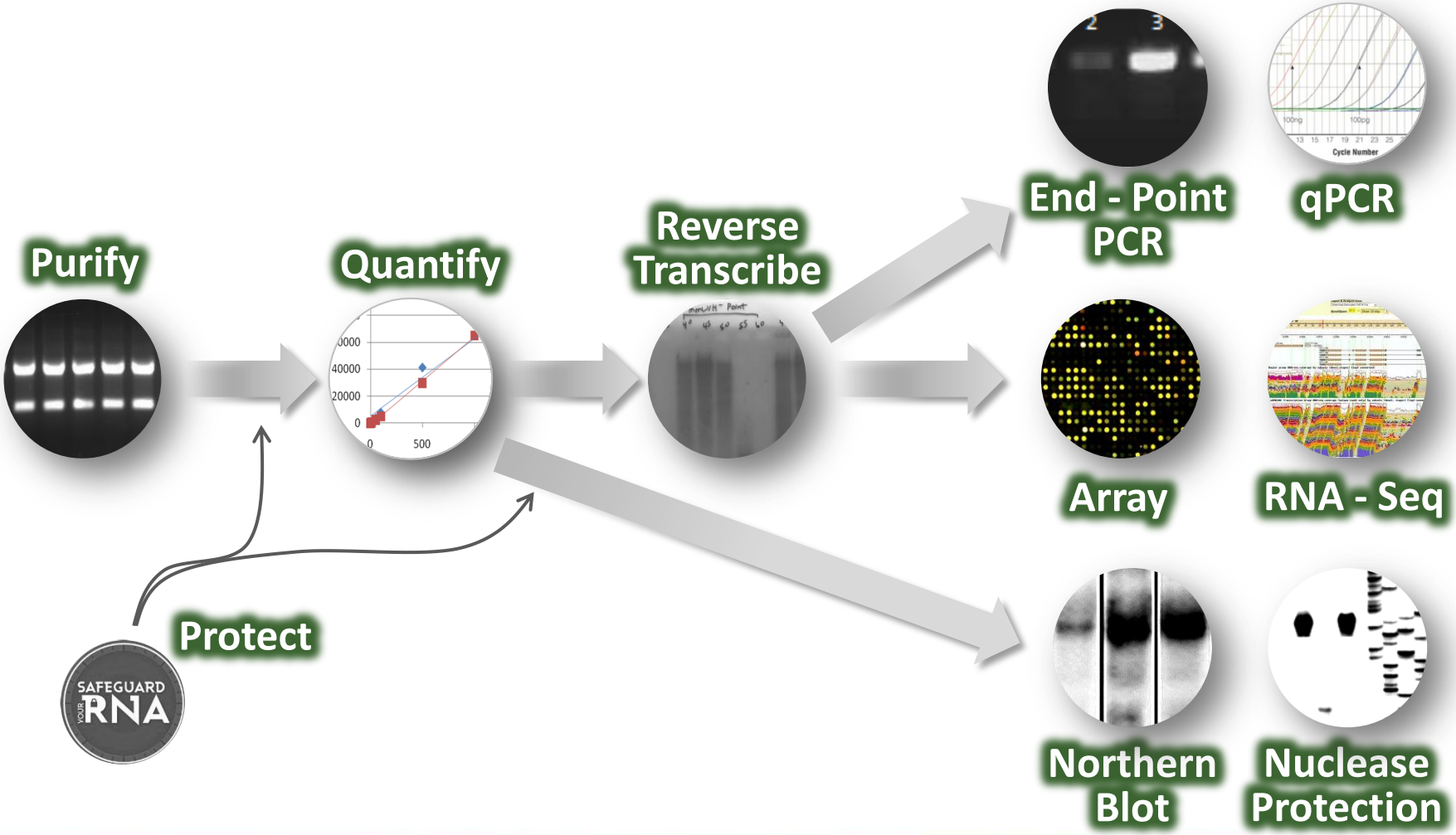


- ✓ Key considerations at each step
- ✓ Ways to overcome major challenges
- ✓ Examples of challenging samples

DNA Workflow
Each Step Affects the Quality of the Final Data



RNA Workflow
Each Step Affects the Quality of the Final Data



Downstream Assays
Different Tools Based on Experimental Needs

PCR	qPCR	Sequencing	Arrays
Cloning	SNP Genotyping	SNP detection	SNP Detection
Sanger Sequencing: • Confirmation • Identification • Targeted regions/application	Methylation Analysis	Mutation Discovery	Comparative Genomic Hybridization
STR Analysis (ID)	Mutation detection	De Novo Genome Sequencing	Loss of Heterozygosity
Methylation Analysis	Pathogen identification	Microbiome Research	Pathogen Identification
Genotype Confirmation (KOs)		Chromatin Immoprecipitation	
Chromatin Immoprecipitation		Methylation Analysis	

Downstream Applications
Importance of Input DNA Characteristics

	PCR	qPCR	Sequencing	Arrays
Quantity of DNA	+	+	++	+++
Integrity of DNA	+/- Depending on amplicon	- Typically small amplicons	+++ More important with longer read technologies, but many providers assume large fragments	++ Typically small fragments, but providers expect minimum fragment sizes
Lack of Inhibitors	++	++	+++	++
Accurate Quantitation	++	+++	+++	+++

Next-Gen Sequencing

Many methods, many applications, many considerations

Several technologies exist and additional technologies are in development for large scale sequencing of genomes with many applications:

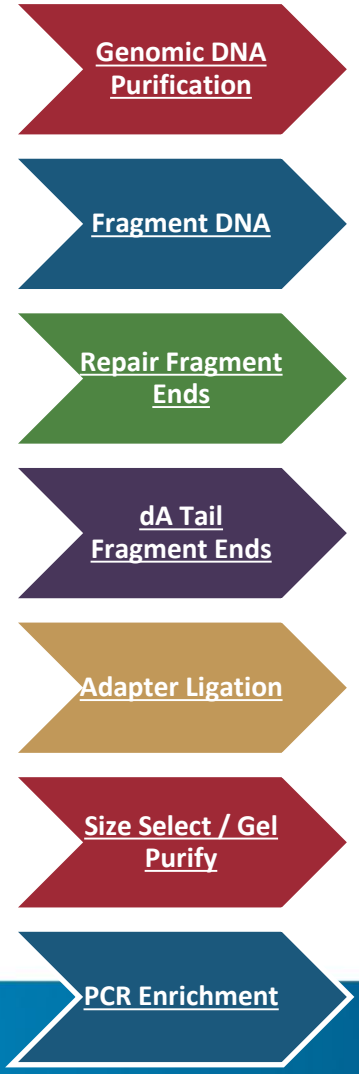
- SNP discovery
- Mutation discovery/detection
- Identity
- Methylation

Ultimately, sequencing data is dependent on the integrity and quality of the starting material

Sequencing Library Prep Considerations

While the DNA is fragmented in the first step in preparation, protocols assume certain degrees of intactness.

Inhibitors of downstream enzymes can effect the production of libraries – best to use a purification kit that provides pure, intact DNA



Purification:

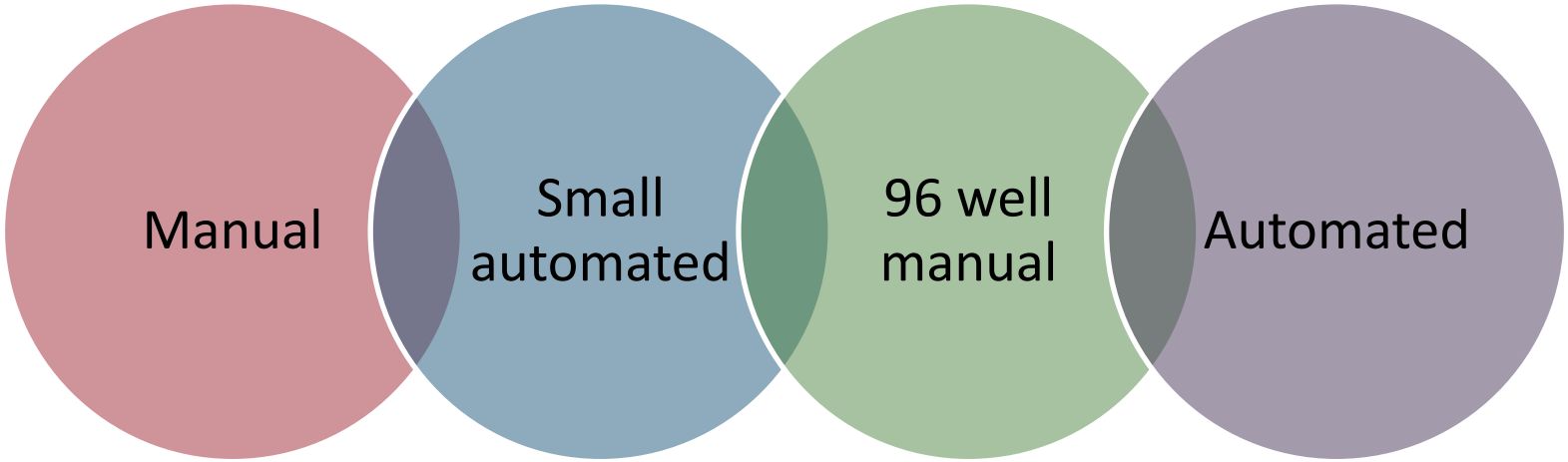
Setting the Stage for Downstream Success

Purification
Yield, Integrity & Purity are Critical to Success

Key Challenges

- Purifying sufficient DNA/RNA from:
  - Low biomass samples
  - Difficult samples
  - Degraded samples
- DNA/RNA integrity
- Isolating pure DNA/RNA
  - No enzyme inhibitors to affect downstream applications
  - No contaminating DNA/RNA

DNA Purification Technologies
All Provide Advantages Depending on Specific Needs

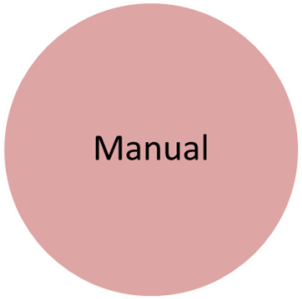


Purification, Manual
Low Investment and Scalability are Attractive

Manual columns and scalable solution-based purification are attractive low-throughput options for standard or difficult samples

Advantages	Reasons to Consider Other Options
Low initial investment vs. automation	Greater throughput desired
Flexibility in sample processing	Time constraints
Lower price per prep	Error reduction
Minimal set up time	

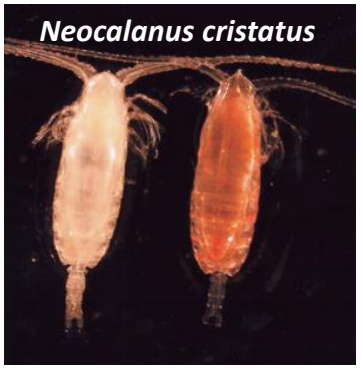
Many sample types supported: Blood, Tissue, FFPE, Plant…

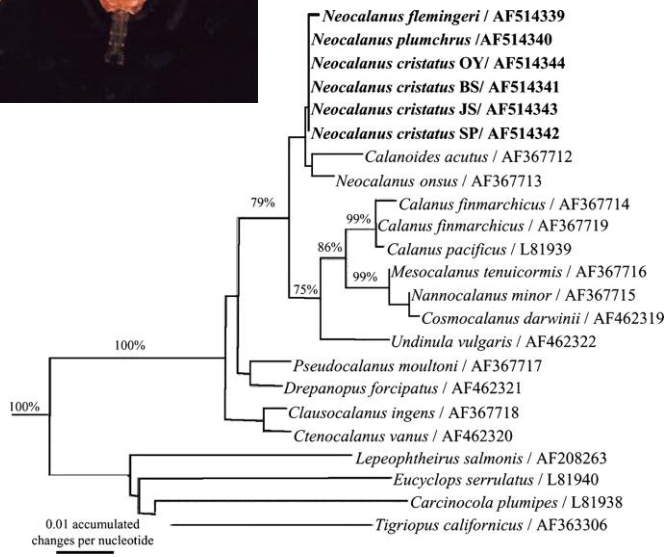


DNA from copepod crustaceans using the Wizard® Genomic DNA Purification Kit

Tomoo Sawabe and Tsutomu Ikeda
Research Faculty of Fisheries Sciences, Hokkaido University, Japan.

- *18S rRNA gene amplification and sequence analysis to study phylogeny of Neocalanus* copepods
- Used modified animal tissue protocol (mouse liver and brain)
- Compared with standard phenol/chloroform extraction
- Reproducible amplification with Wizard® Genomic – no amplification with phenol/chloroform





Taniguchi, M. *et al.* (2004) Molecular phylogeny of *Neocalanus* copepods in the subarctic Pacific Ocean, with notes on non-geographical genetic variations for *Neocalanus cristatus*. *J. Plankton Res.* **26**, 1249–55.

DNA Purification, Small Scale Automation
Small Automated Systems Offer Major Benefits

Small, dedicated purification instruments allow individuals to automate purification and increase productivity

Advantages	Reasons to Consider Other Options
Minimal initial investment	Not enough throughput to justify
Frees time for other activities	Even greater throughput desired
Fewer purification errors	Input sample volume incompatibility
Increases sample throughput	

Maxwell® 16:
5 minute setup – 30-45 minutes to extract 1-16 samples

Small automated

Maxwell® 16 DNA Purification Kits (SEV) Applications

Sample Type	Input	DNA Yield	DNA Conc.	A_{260}/A_{280}	A_{260}/A_{230}	Protocol Testing	Notes
Yeast: *S. cerevesiae*	1 large colony	721ng	n.d.	n.d.	n.d.	PT	
Zebrafish: *Danio rerio*	50mg	2.5µg	11ng/µl	n.d.	n.d.	PT	
Tick	1 or more	n.d.	n.d.	n.d.	n.d.	PT	Detected by PCR only.
Tomato Leaf: *solanum lycopersicum*	8mm punch	734ng	n.d.	n.d.	n.d.	PT	Suitable for real-time qPCR.
Drosophila	1, 5 flies	324ng, 1.5µg	n.d.	n.d.	n.d.	PT	
Mosquito	1 mosquito	3.6µg	16ng/µl	n.d.	n.d.	PT	
Blue-green Algae: *Spirulina*	~7mg, 13mg	n.d.	~50ng/µl, ~100ng/µl	n.d.	n.d.	CT	
Brine Shrimp: *Artemia fransciscana*	1 shrimp (3-4mm)	1.7µg	7.7ng/µl	n.d.	n.d.	PT	Suitable for real-time qPCR.
Canola Seed: *Brassica napus*	5 seeds	2.3µg	n.d.	n.d.	n.d.	PT	Suitable for real-time qPCR.
Celery	100mg ground	16.4µg	82ng/µl	1.8	n.d.	PT	
Feces, Mouse	1 pellet	48µg	218ng/µl	n.d.	n.d.	PT	May have to dilute or run eluate over PVPP column to remove more PCR inhibitors.

Maxwell® 16 LEV Blood DNA Applications

- Various organ tissues
- Bacteria
- Various plant types
- Bone marrow
- Yeast
- Stool
- Waste water
- Environmental filters

Stool

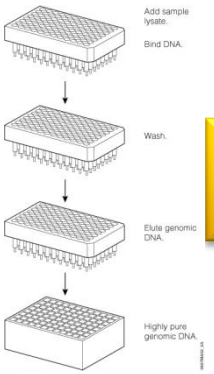
- Up to 250mg in 1ml of Lysis Buffer.
- Incubate at 99°C for 10 minutes.
- Centrifuge for 2 minutes to pellet the solid material and transfer 300ul to a new tube
- Add 30ul of ProK and incubate at 56°C for 20 minutes.
- Transfer entire volume to cartridge and run on LEV Blood.
- Yields are highly variable, but human and bacterial DNA are both detectable.

Environmental Filters

- Add 600µl of Tail Lysis Buffer to a 2ml screw cap tube.
- Add filter piece and vortex.
- Incubate at 99°C for 10 minutes.
- Let cool for 2 minutes and add 60µl of Proteinase K.
- Incubate at 56°C for 20 minutes.
- Add liquid to the LEV Blood cartridge.
- Elute in 50µl.
- Concentrations highly variable – we were able to get 23ng/ul and 7ng/ul from a quarter of a 5 inch filter (had two liters of water filtered over it).

DNA Purification, Manual 96 Well
Vacuum Purification Increases Sample Throughput

Advantages	Reasons to Consider Other Options
Low initial investment	96 well processing can be tedious
High sample throughput	Desire to reduce errors
Offers performance equal to spin columns	Staff time has become rate limiting
	Greater throughput desired
	Input sample volume incompatibility

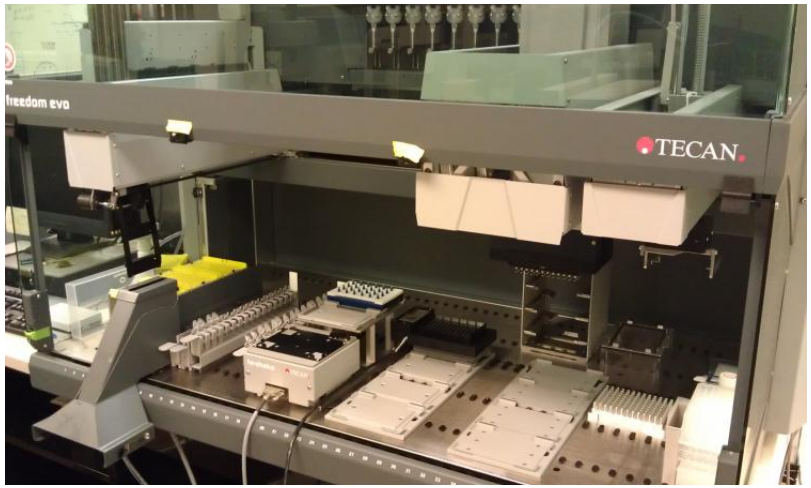


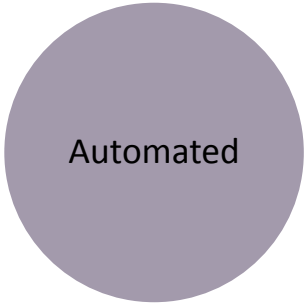
The Wizard® SV 96 Genomic system can isolate gDNA from many sample types in less than 60 minutes

96 well manual

DNA Purification, Automated 96 Well
Increases Laboratory Throughput and Lowers Costs

Advantages	Reasons to Consider Other Options
Increases laboratory productivity	High initial cost
Aids in sample tracking	Not enough throughput to justify
Increases consistency of results	
Can automate many activities	

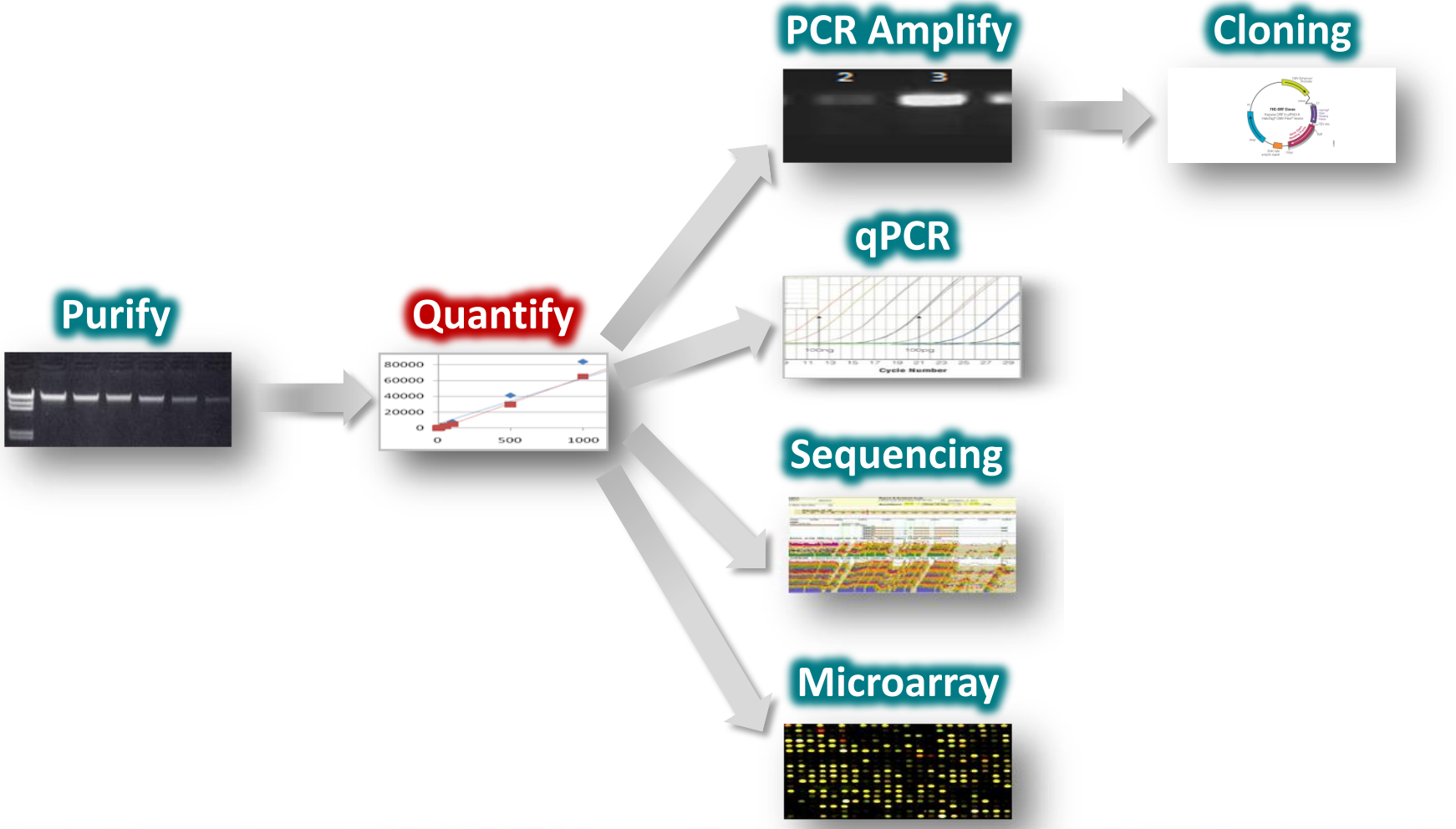




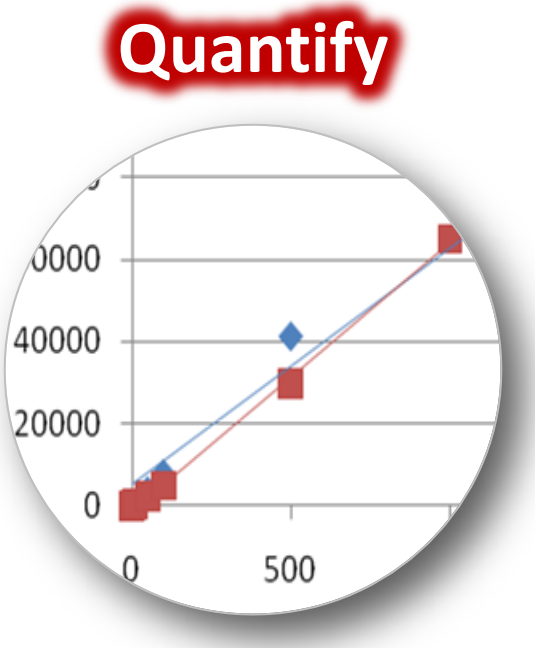
Quantitation:

A Simple But Critical Step in Analysis

Quantitation
A Simple But Critical Step in Analysis



Key Challenges Include Sensitivity, Accuracy, and Nucleic Acid Specificity



Challenges

- ✓ Sensitivity
  - effectively measuring small nucleic acid amounts
- ✓ Accuracy
  - affected by purity
  - detection range
- ✓ Specificity
  - dsDNA vs ssDNA vs RNA
  - human vs non-human

Three Common Methods of Quantitation Utilize UV Absorbance, Fluorescent Dyes and qPCR

- UV Absorbance
  - Spectrophotometer
  - NanoDrop®/NanoVue™
- Fluorescent Dye-based Quantitation
  - Plate Reader
  - Hand-held Instruments
- Real-Time PCR

UV Absorbance Spectroscopy Gives a Rapid Assessment of Concentration and Purity

Spectrophotometer
(various manufacturers)

NanoDrop®
(Thermo Scientific)

Measure nucleic acid:

- ✓ Concentration
- ✓ Purity

UV Absorbance Measures Different Components with Distinct Wavelengths

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

NanoDrop® Measures UV Absorbance of Small Volumes with Good Sensitivity

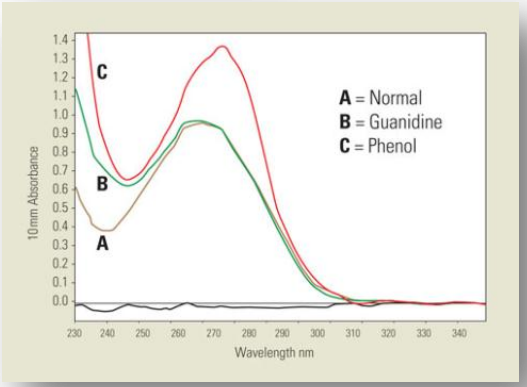
Features:

- ✓ Measures the absorbance of small volume samples
- ✓ 0.5 – 2µl of sample required
- ✓ 190nm – 840nm wavelength range
- ✓ Wide detection range
  - 2ng/µl minimum (DNA)
  - 15,000ng/µl maximum (DNA)
- ✓ Measurements in less than 30 seconds
- ✓ No other reagents or accessories required

NanoDrop® is an Easy-to-use and Popular System

Choose Read Type: dsDNA, ssDNA, or RNA

Read:
- Water
- Blank
- Sample

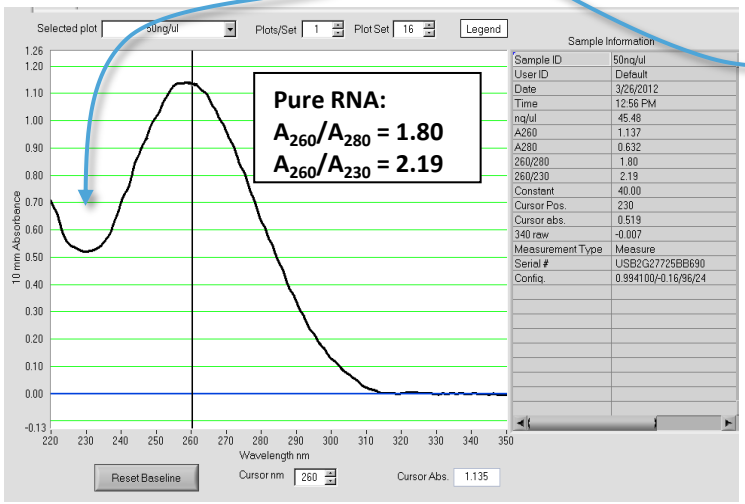


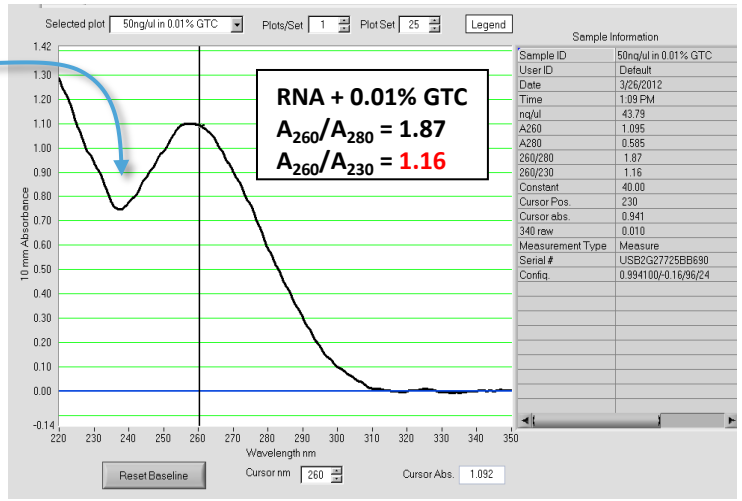
Output:
- Spectra
- Table
  - Concentrations
  - Purity ratios
  - Absorbance readings

Contaminants such as Guanidine Thiocyanate Lower the A_{260}/A_{230} Purity Ratio

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

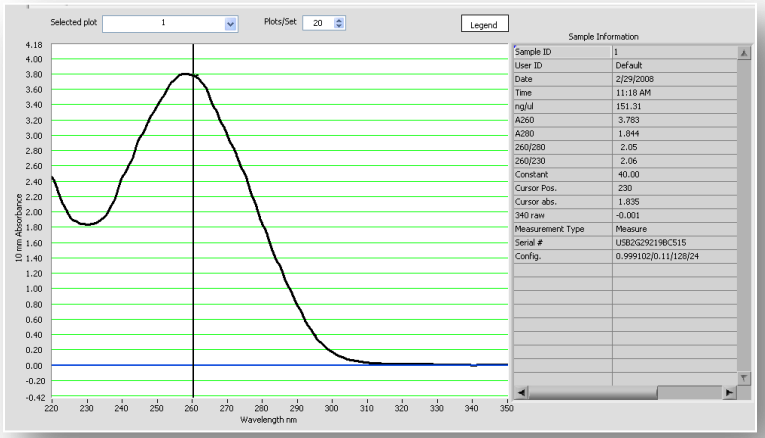
Guanidine Thiocyanate Affects A_{260}/A_{230} Ratio



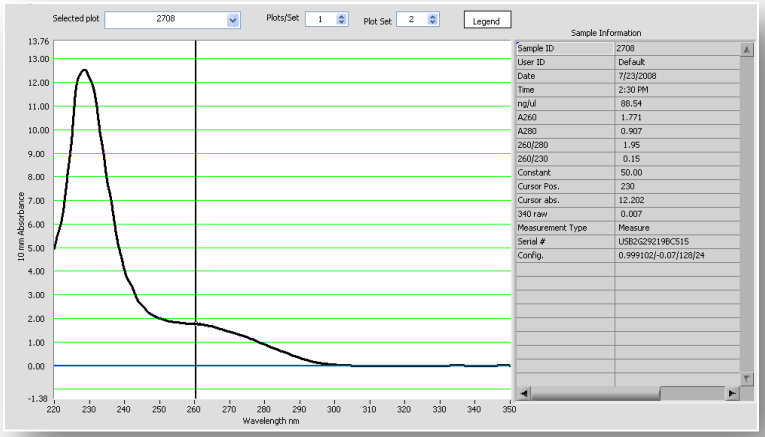


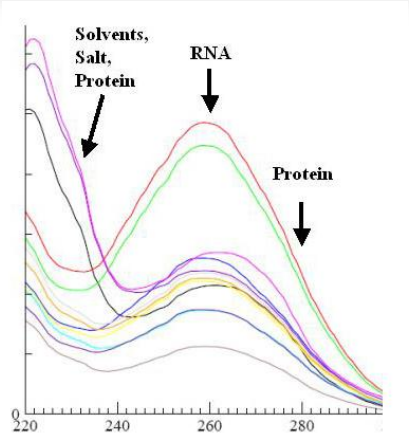
Large Peaks at Wavelengths Lower than 260nm Can Influence the Measured Peak at 260nm

Optimal Spectra



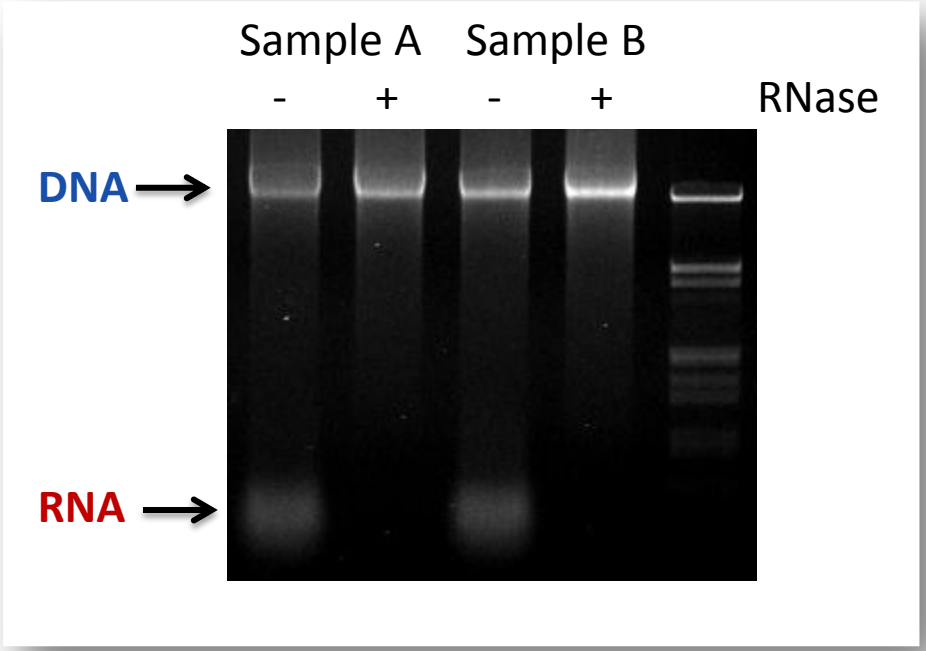
Strong Peak ~230nm Contributes to 260nm Reading





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

A_{260} Absorbance is Not Specific and Cannot Distinguish Between dsDNA, RNA, or ssDNA



	NanoDrop® (ng/µl)	QuantiFluor™ dsDNA (ng/µl)
Sample A	213.5	158.0
Sample B	87.5	66.0

260nm reading represents total amount of all nucleic acid present in sample

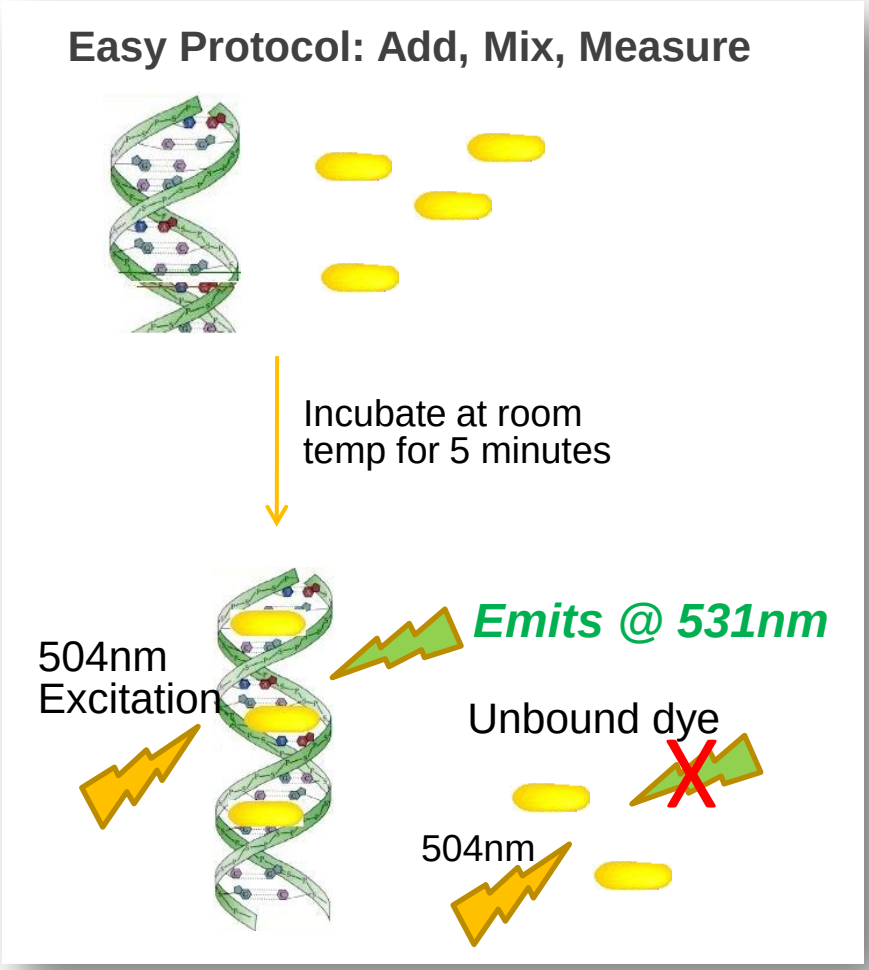
Cannot distinguish between dsDNA, ssDNA, or RNA

Disadvantages of Absorbance Include Lack of Specificity, Overestimation, and Lack of Integrity Information

- ✓ Lack of Specificity
  - Cannot distinguish between dsDNA, RNA or ssDNA
  - Nucleic acid contamination cannot be determined
- ✓ Overestimation of nucleic acid concentration due to contaminants
  - Many contaminants absorb at or around 260nm
- ✓ No information on integrity
  - Nucleotides and small nucleic acid fragments still contribute to the 260nm reading

Fluorescent Dye-based Quantitation is a More Sensitive Method

- Dye binds nucleic acid – the resulting conformation shift produces in fluorescence when excited
- Fluorescence is directly proportional to the amount of nucleic acid in the sample
- Higher signal = more nucleic acid present
- Unbound dye does not fluoresce
- Low background increases sensitivity



Two Formats to Match Your Workflow
Single Tube or Plate Assays

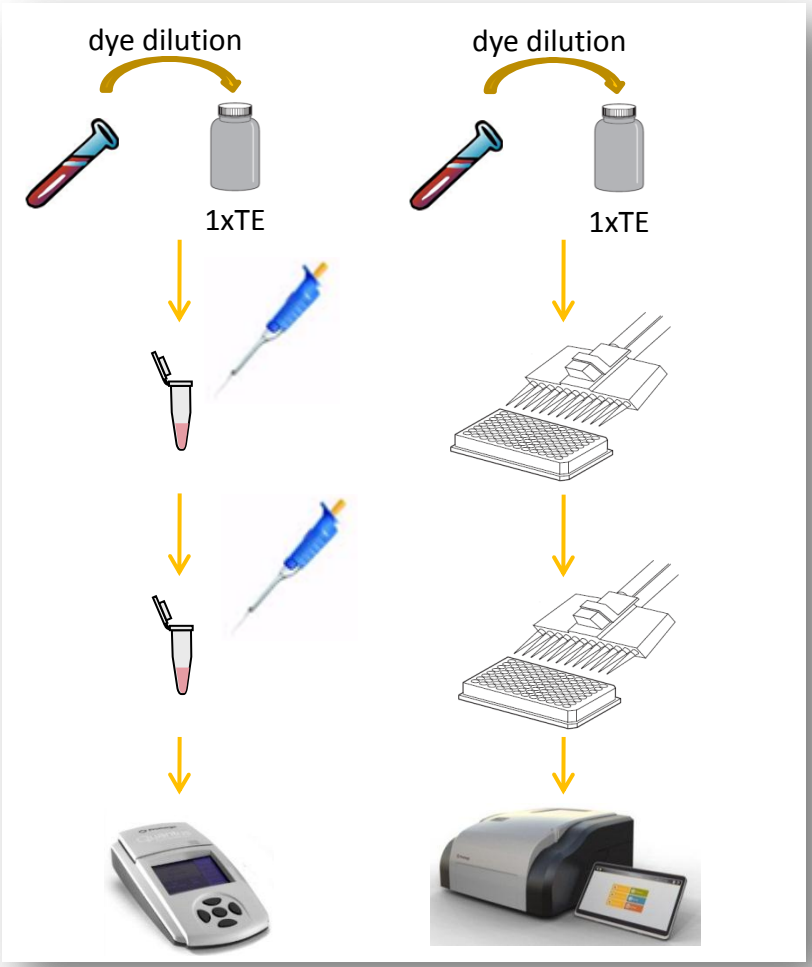
1. Dilute dye
2. Prepare standards and samples
3. Add dye and incubate
4. Measure fluorescence

Single tube format:

Quantus™ Fluorometer

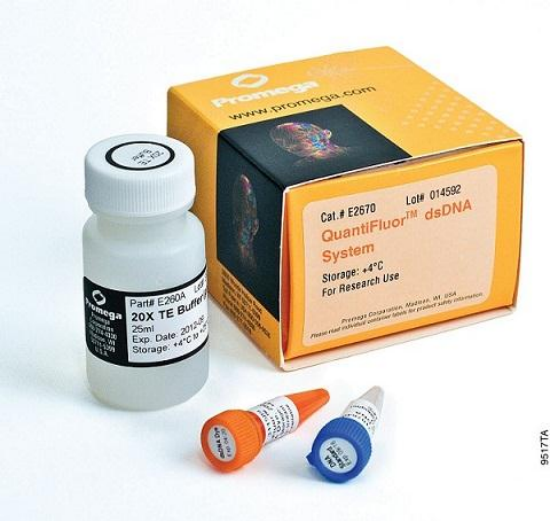
Microplate reader:

GloMax® Discover Detection System

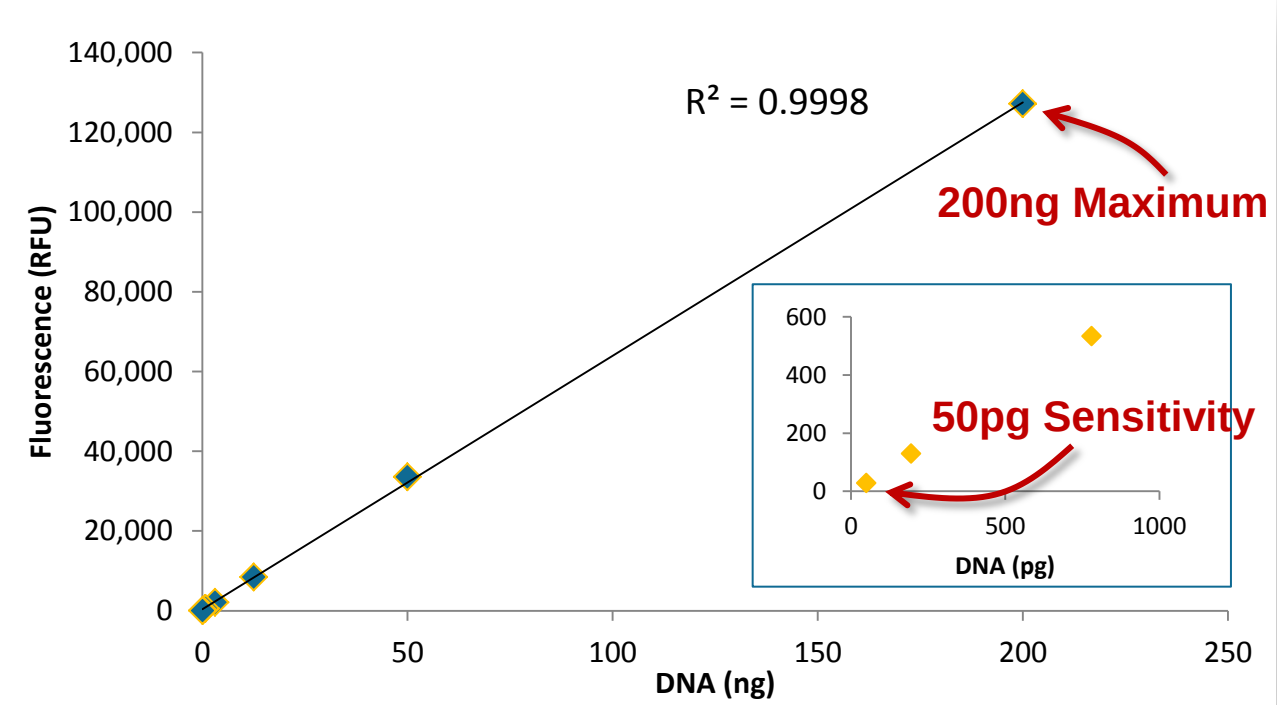


QuantiFluor® Dyes are Optimized for the Different Types of Nucleic Acids

- ✓ QuantiFluor® dsDNA System
  - Specific for dsDNA with minimal binding to ssDNA, RNA, protein and interfering compounds
  - Sensitivity down to 50 pg/ml in a microplate system (200µl volume)
- ✓ QuantiFluor® One dsDNA System*
- ✓ QuantiFluor® ssDNA System
- ✓ QuantiFluor® RNA System



QuantiFluor® dsDNA System Exhibits High Sensitivity Down to 50pg



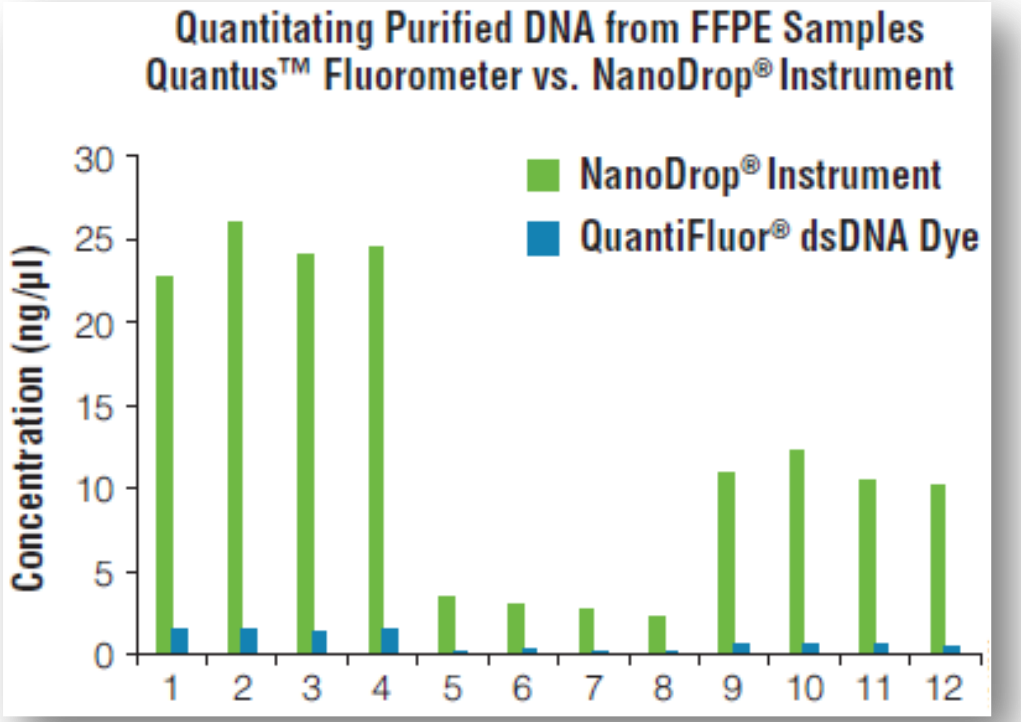
Low Concentration DNA Samples May Fall Outside of the Range of the NanoDrop®

Comparing Concentrations Obtained Using the NanoDrop® and the QuantiFluor™ dsDNA system

	NanoDrop®			QuantifFluor™ dsDNA
FFPE Sample	**ng/µl**	**A_{260}/A_{280}**	**A_{260}/A_{230}**	**ng/µl**
Kidney	7.02	1.77	1.69	5.96
Liver	8.65	1.76	1.77	6.09
Spleen	9.86	1.65	1.17	8.29
Heart	nd			1.32
Brain	4.26	1.72	1.07	3.86
Int. tumor 1	nd			0.617
Int. tumor 2	nd			1.02
Int. tumor 3	nd			0.544

The NanoDrop® Instrument Often Overestimates the Amount of DNA in Solution

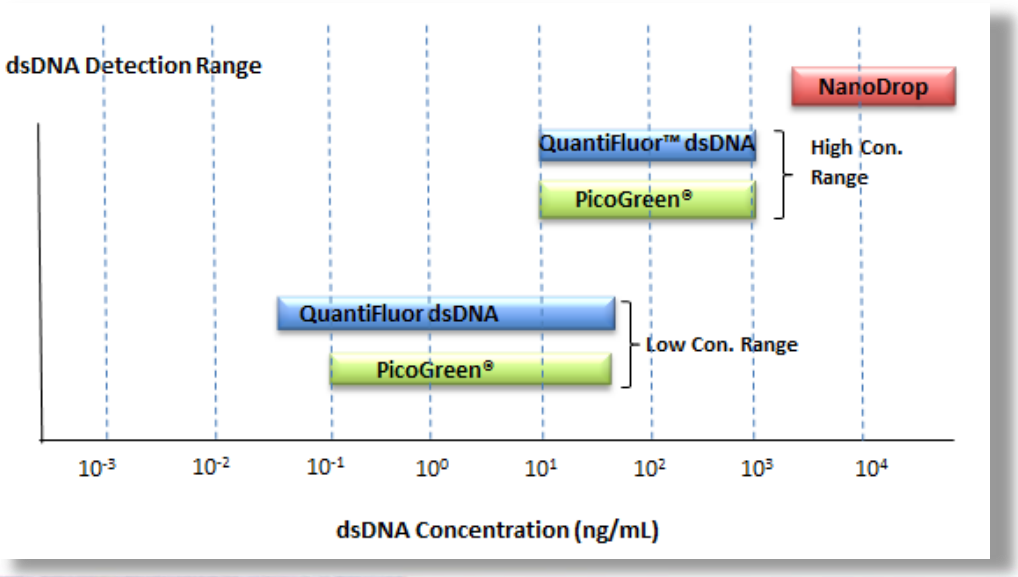
- Accurate quantitation is critical for many downstream applications
- Many FFPE tissue sections are small, and isolated DNA samples have concentrations well below the limit of detection of traditional spectrophotometric assays
- Even with highly purified DNA, the NanoDrop® consistently overestimates the amount of DNA in solution



The Key Advantage of Fluorescent Dye-based Quantitation is Sensitivity

- Some sample types such as FFPE contain low levels of nucleic acid
- Absorbance methods (NanoDrop®) lack detection *sensitivity* as well as *target-specificity*
- Proper nucleic acid quantitation improves success in a variety of downstream assays such as PCR, cloning, next gen sequencing (NGS), transfections

Fluorescence is 40,000X more sensitive than NanoDrop® UV



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

- ✓ Must create standards
- ✓ No information on purity
  - Separate dye-based quantification systems are available for ssDNA, RNA and protein
- ✓ No information on integrity
- ✓ Lack of specificity with dyes
- ✓ Fluorescent dyes are potentially hazardous

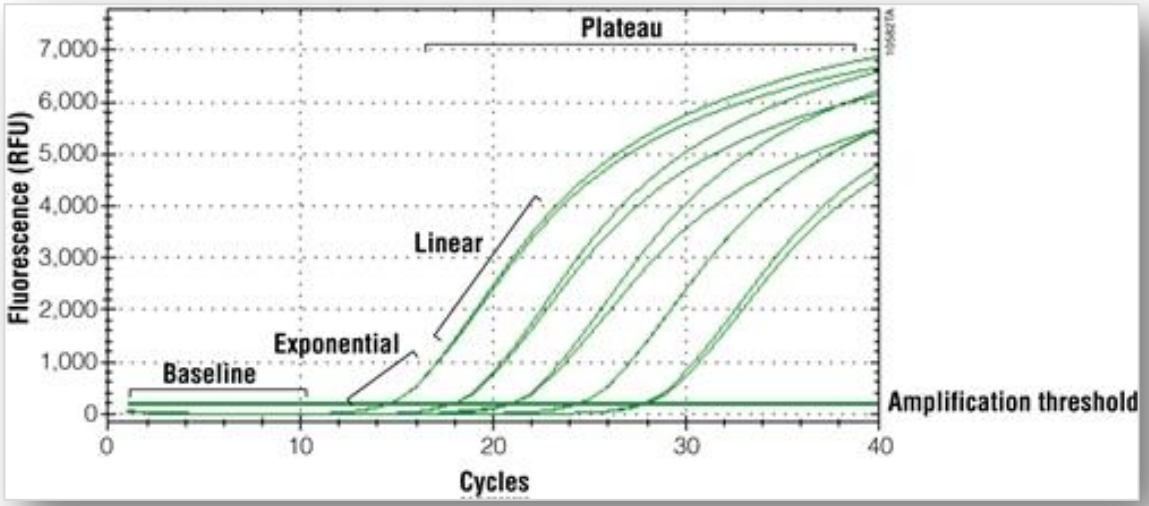
Real-Time PCR (quantitative PCR, qPCR) Quantitation Involves Detection of Product at Each Cycle

What is end-point PCR?

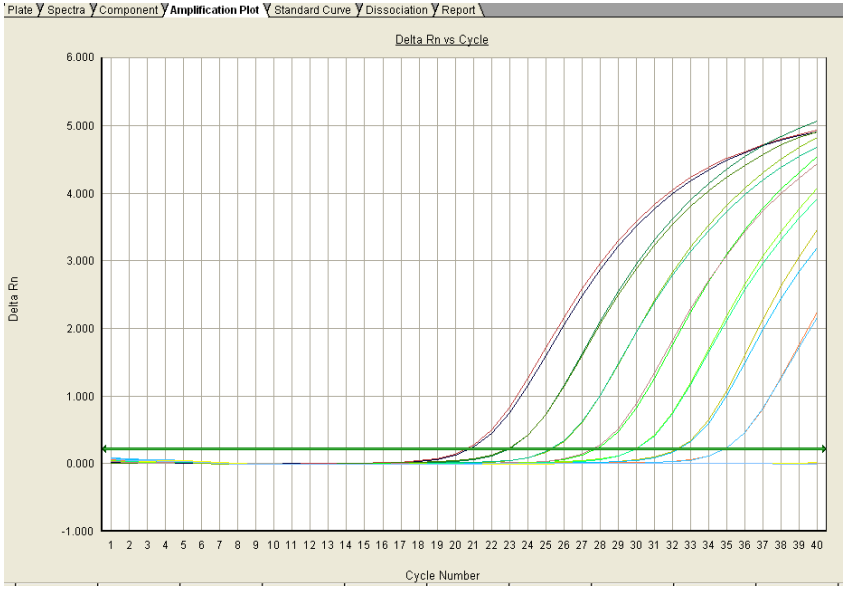
The amount of amplified product is typically determined only after a set number of amplification cycles is completed

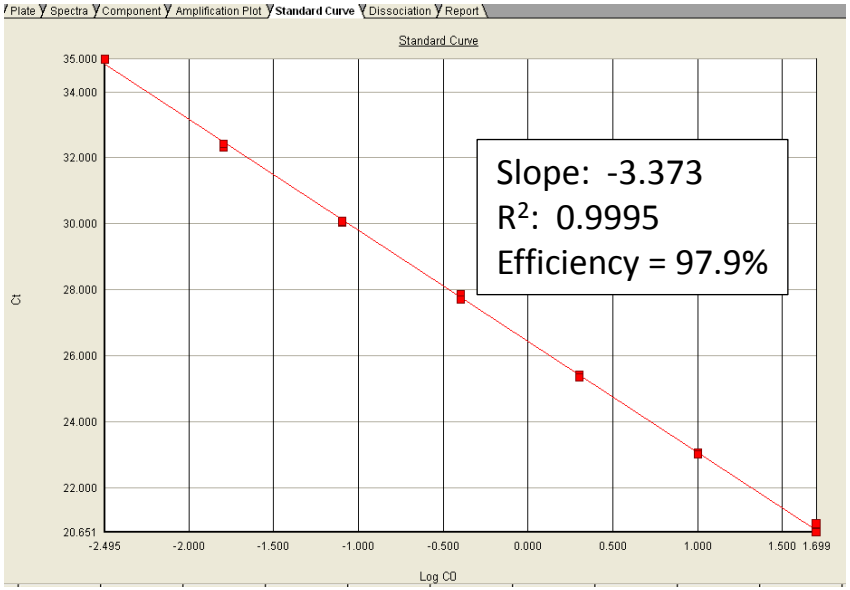
What is Real-Time PCR?

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



Absolute Quantitation can be Determined by Using a Standard Curve





- Standard curve is generated by plotting C_q vs (log) concentration for known sample
- Concentrations of unknown samples are extrapolated from the standard curve, based on C_q
- Amplification efficiency is also derived from the standard curve (function of slope)

RT-qPCR is the Detection of RNA Transcripts Using a Combination of Reverse Transcription & qPCR

- RT-qPCR: Reverse Transcription followed by qPCR amplification.
- Conversion of a specific RNA molecule into cDNA followed by DNA-based PCR amplification.
- RT-qPCR is a powerful method of gene expression analysis.
  - Determine the presence or absence of a transcript
  - Quantitate mRNA expression levels (transcript abundance)

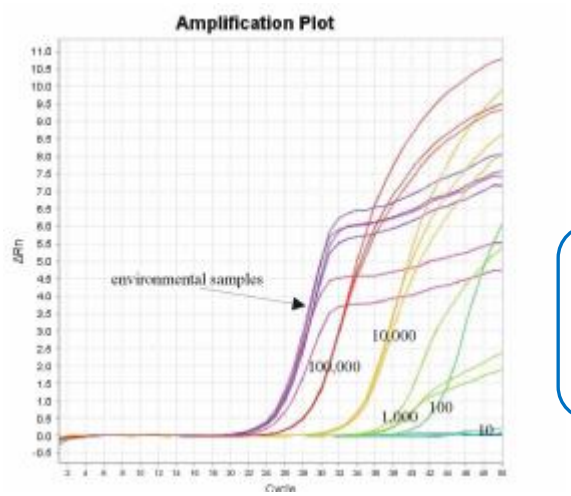
Template Purity Is Key to Amplification as Inhibitors May Be Present in Starting Samples

- Environmental samples can be challenging with qPCR analysis
  - Food samples, fecal samples, soil, wood, plant material, textiles
  - Inhibitors from these samples may be present in “purified” nucleic acid
- Inhibitors can also be introduced during nucleic acid purification
  - Part of purification protocol/kit
  - Chaotropes, alcohols, chelators, detergents, solvents

Some qPCR master mixes will perform better in the presence of inhibitors

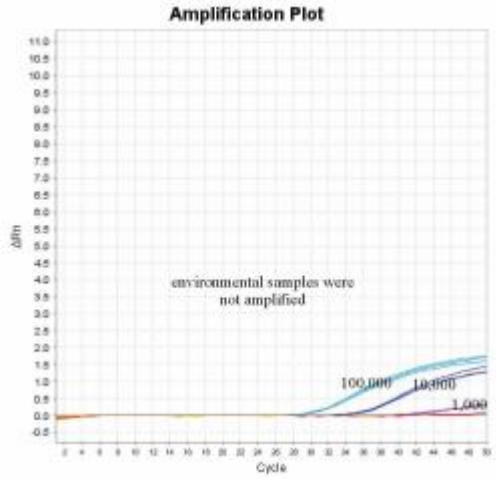
Higher Tolerance to Inhibitors Allows for Amplification of RNA from Difficult Samples

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**
(DNA from Desulfovibrio vulgaris bacteria isolated from seawater)

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

Template Integrity is Important as Fragmented Samples May Affect qPCR Results

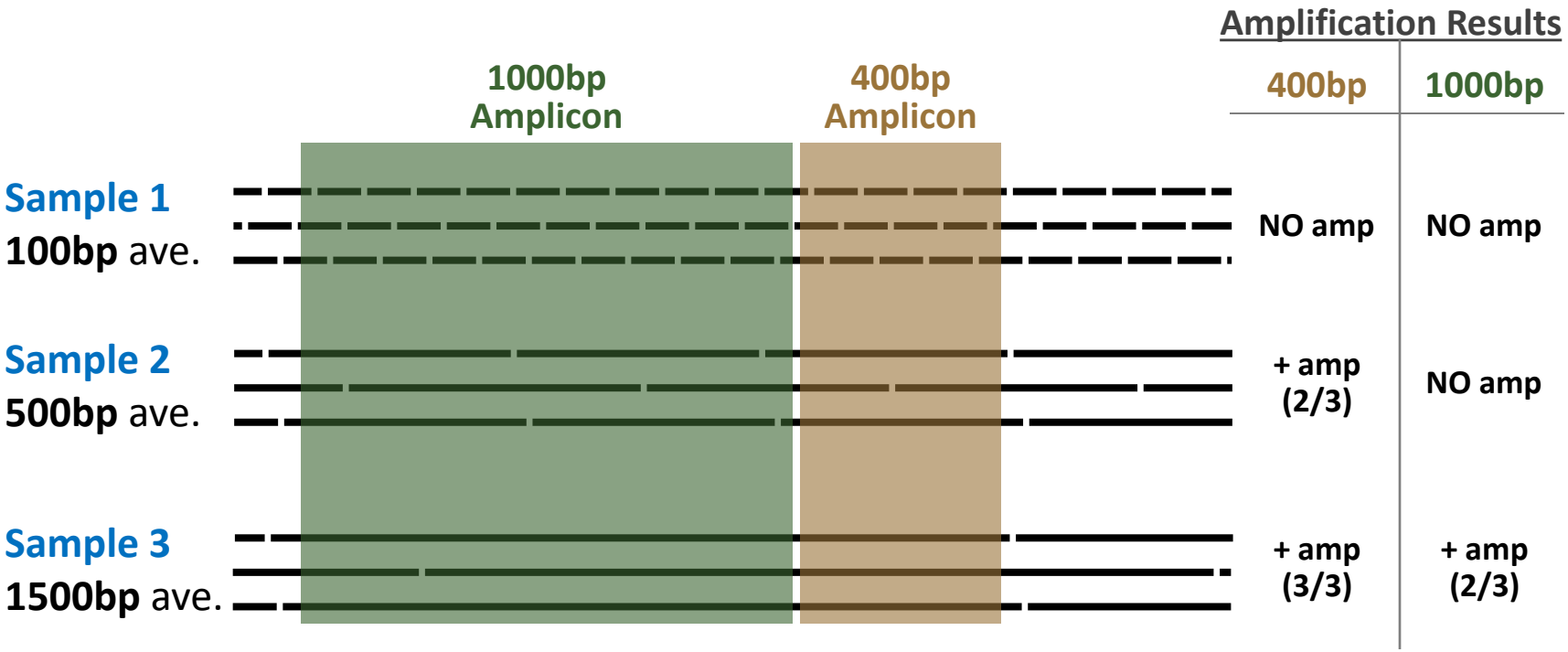
Some sample types are more likely to be degraded

- Samples exposed to nucleases, heat and UV light
- FFPE samples: cross-linking introduced by the fixation and embedding process results in nucleic acids that are characteristically partially degraded after extraction
- When designing downstream amplification assays, best results will be achieved when targeting regions of 200 nucleotides or less

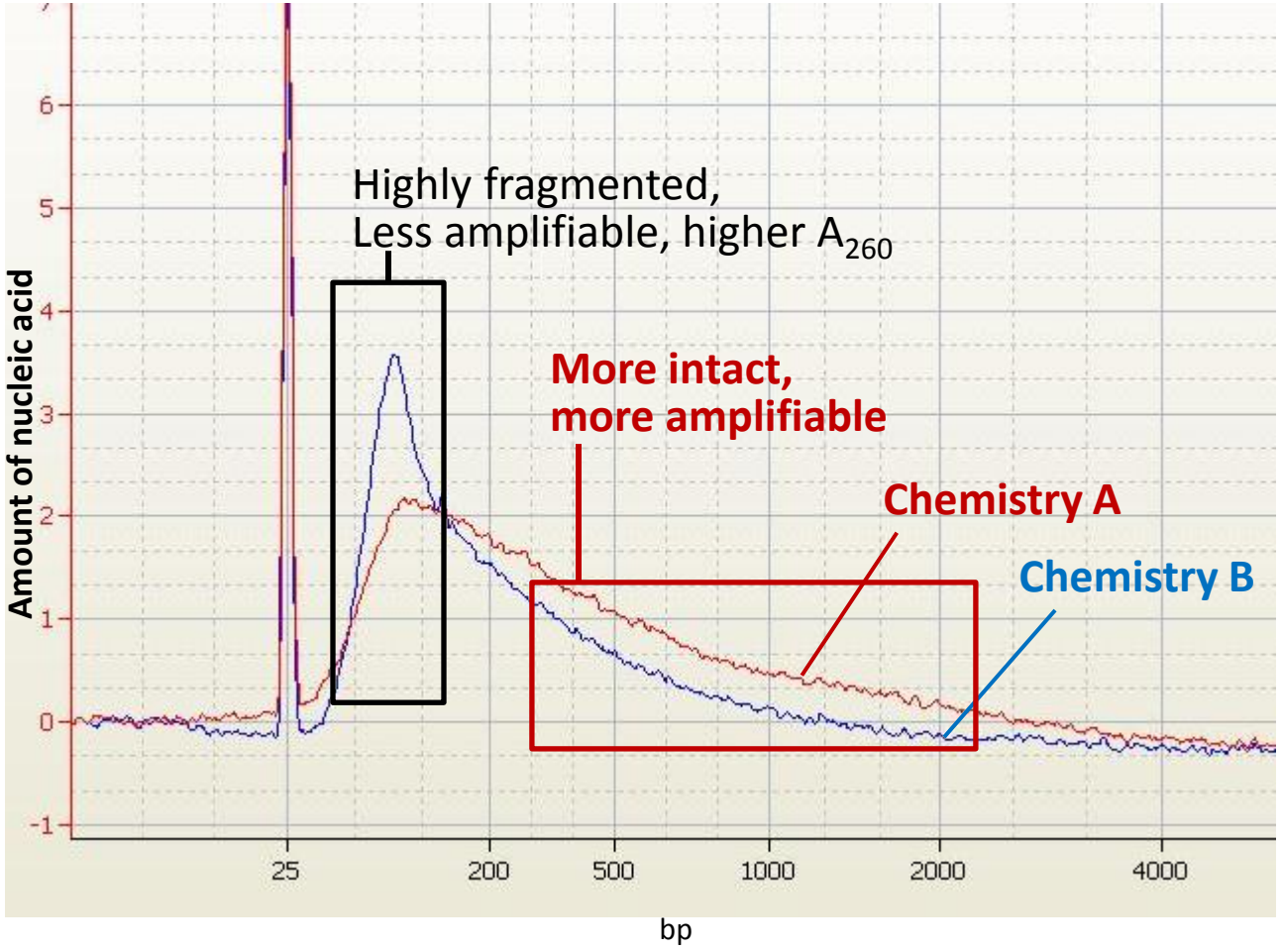
Options for assessing template integrity, include:

- Gel analysis
- Agilent Bioanalyzer (Agilent Technologies)
- Fragment Analyzer (Advanced Analytical)

DNA Fragmentation Directly Affects Amplifiability



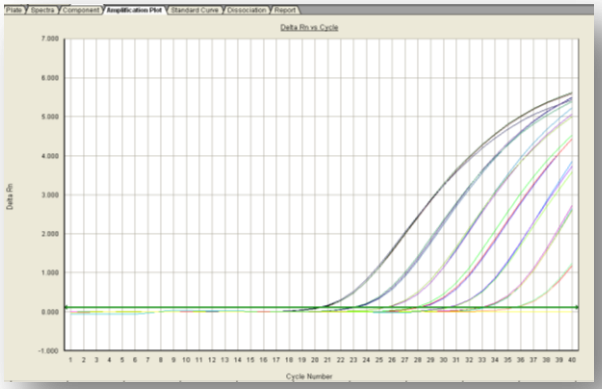
Amplifiability of the Sample Can Affect Quantitation



- Important to note that fragmentation occurs as part of fixation
- Highly fragmented gDNA is less amplifiable, as the peak is about 100bp
- The difference in area under the curve >300bp is the advantage of Chem A over Chem B

Advantages of Real-Time PCR Quantitation Include High Sensitivity and Wide Dynamic Range

- ✓ Amplification and detection occur together
- ✓ Highly sensitive
- ✓ Wide dynamic range
- ✓ High throughput capability
- ✓ Multiplex capability
- ✓ Requires minimal sample



Disadvantages of Real-Time PCR Quantitation Include Expensive Equipment and Sensitivity to Inhibitors

- ✓ Requires specialized instrumentation
- ✓ Higher cost compared to UV absorbance and fluorescent dye-based methods
- ✓ Sensitivity to inhibitors

ABI 7500
Real Time System

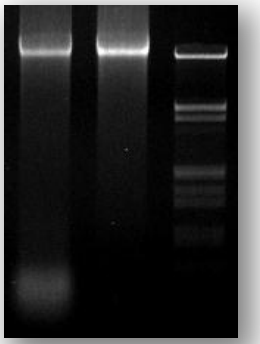
Bio-Rad MyiQ2

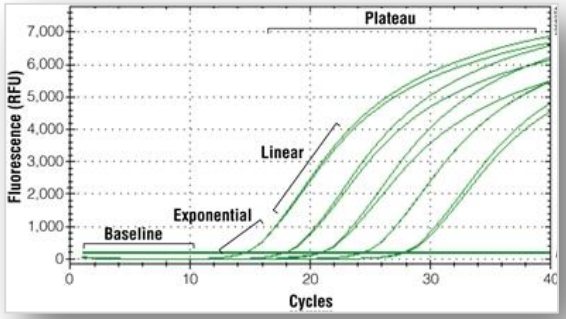
Bio-Rad QX100™ Droplet Digital™ PCR System

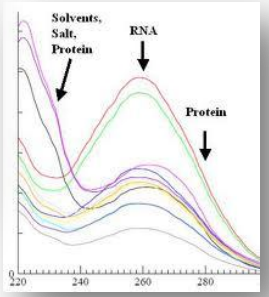
Qualification and Analysis

Several Different Metrics Can Be Used to Predict the Likelihood of Success in Downstream Assays

- **Quantitation by:**
  - Absorbance
  - Fluorescent dye
  - Amplification (qPCR)
- **Purity by:**
  - Absorbance ratio
- **Factors that impact quality:**
  - Purification chemistry contaminants
  - Sample specific inhibitors
  - Fragmentation





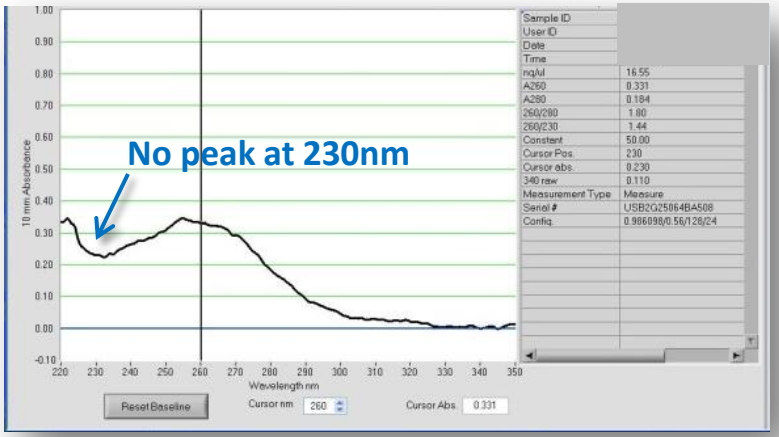


All are used to predict likelihood of success in downstream assays

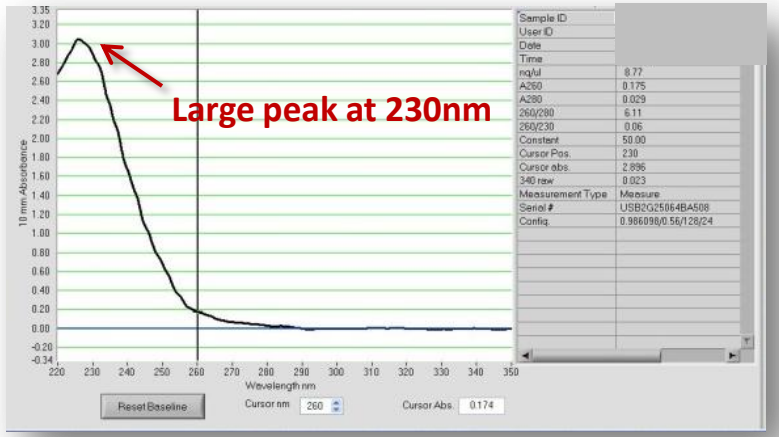
Quantitation of DNA by Absorbance can be Overestimated Due to Contaminants

gDNA Extraction from Matched Lung Tissue FFPE Slides

Chemistry A

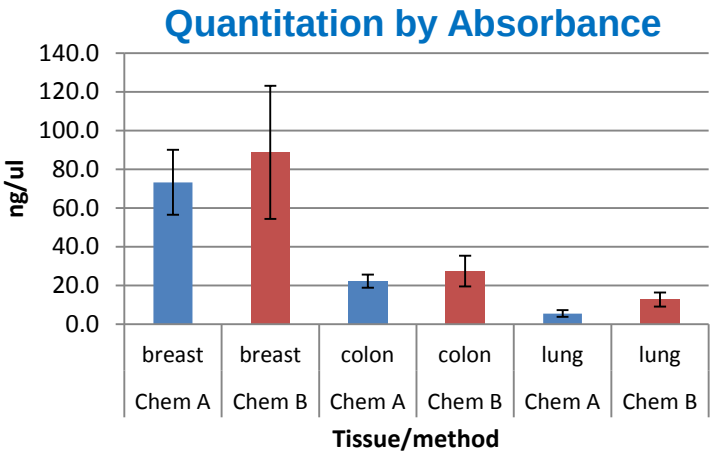


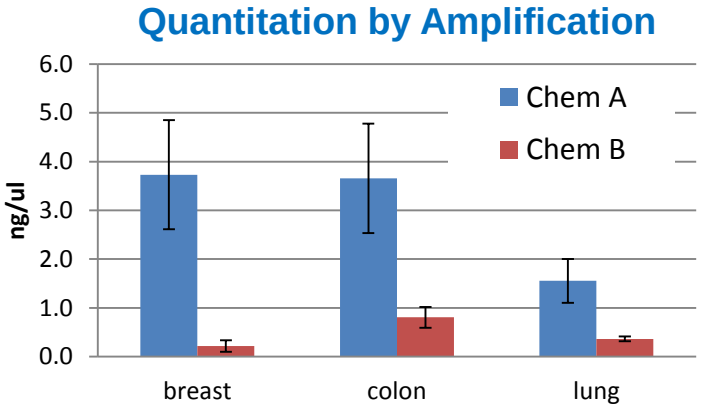
Chemistry B



Absorbance is unreliable for Chemistry B – is qPCR a better choice?

Absorbance at 260nm May Not Be an Accurate Measure of Amplifiable Yield



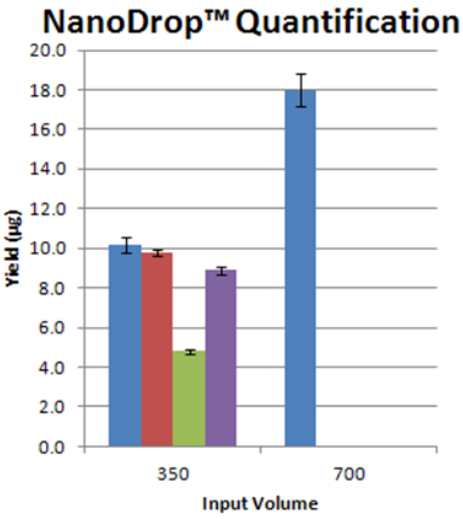


Large difference in quantitation

- Absorbance at 260nm may not be an accurate measure of amplifiable yield
- Absorbance and amplifiability may correlate, but several other factors play a role

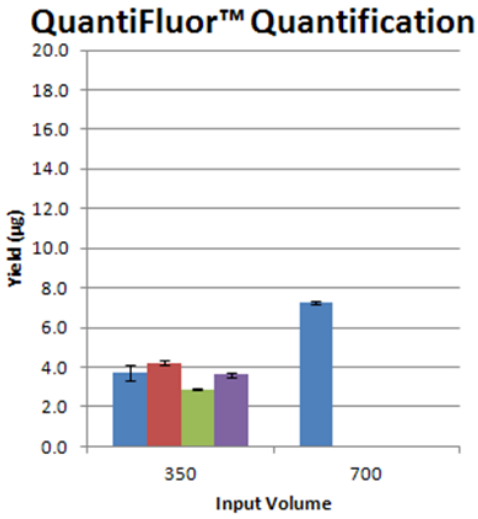
Sample of DNA Isolated from Saliva and Quantitated by the Three Methods Shows Different Results

Absorbance



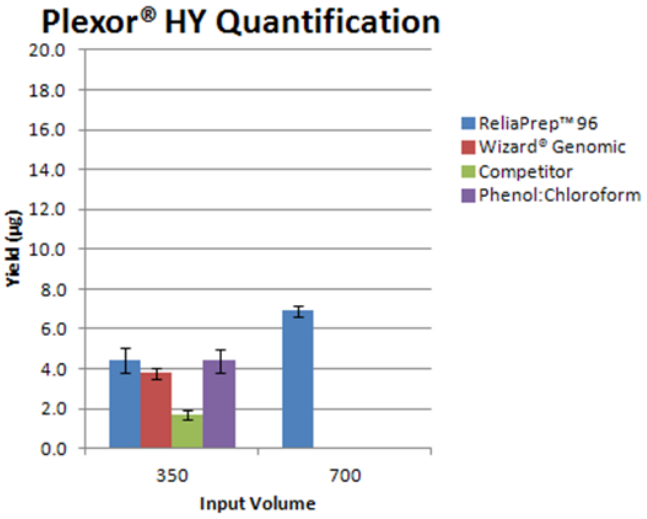
Absorbance shows all nucleic acid present: dsDNA, ssDNA and RNA

Fluorescent Dye



Fluorescent-dye quantitation measures only dsDNA present

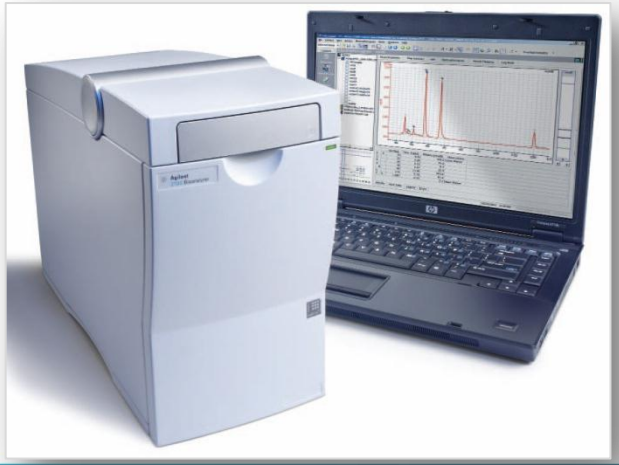
qPCR for Human Target



qPCR quantitates human DNA present

Agilent 2100 Bioanalyzer
Microfluidics to Analyze 1µl of Sample

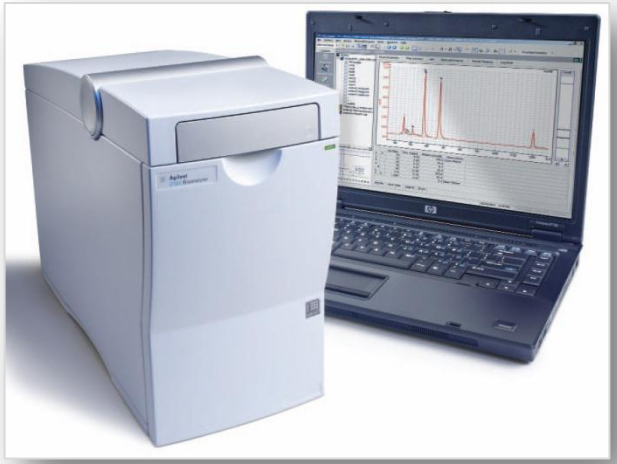
- Uses microfluidics to analyze DNA, RNA, protein, and cells using sample specific chips
- Samples are combined with a fluorescent dye and added into wells in the chip.
- The samples are separated by electrophoresis.
- The samples are detected by fluorescence, and electropherograms and gel-like images 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

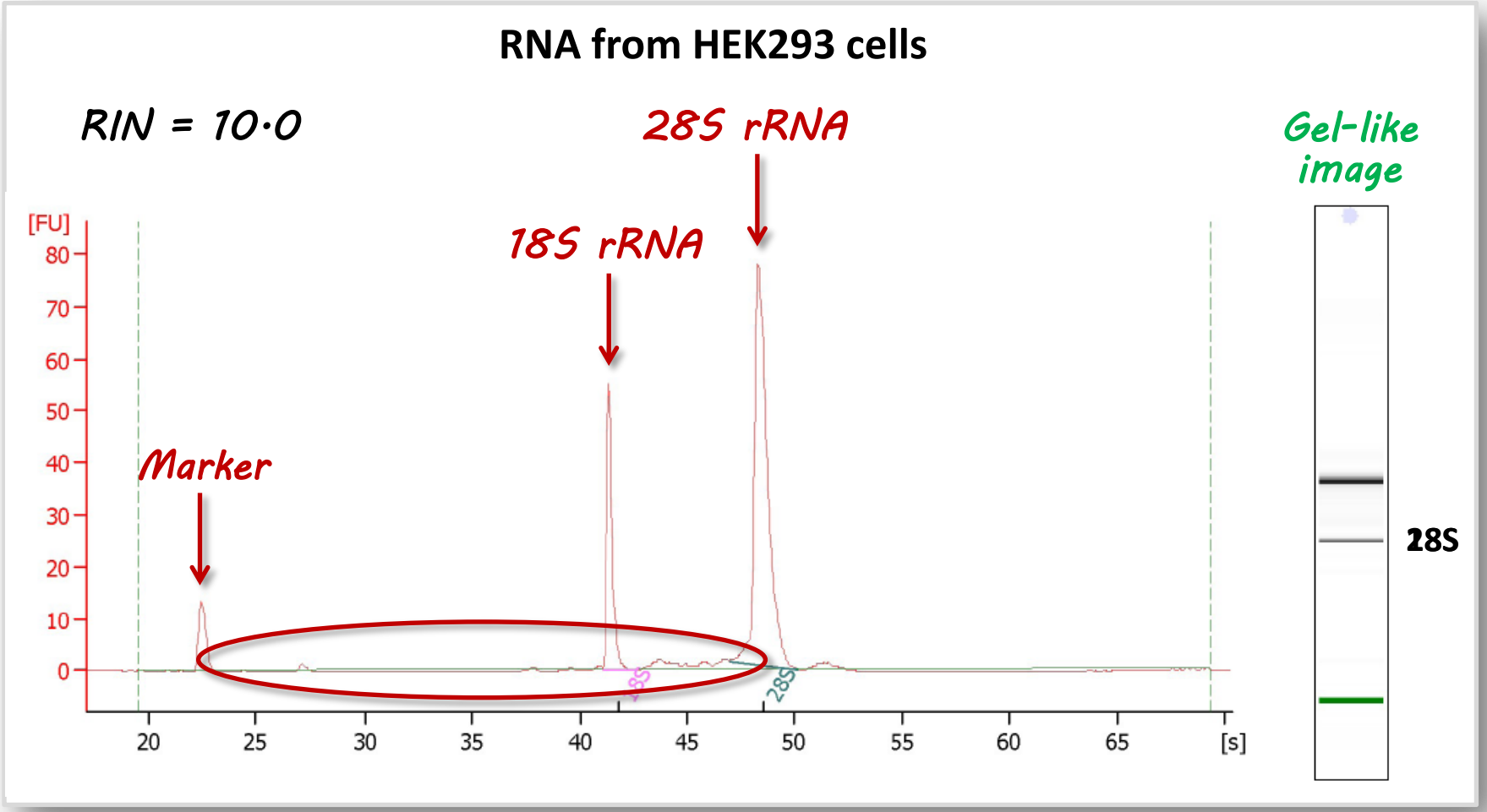
- RNA analysis kits
  - Total RNA and mRNAs
  - Small and microRNAs

- Information provided:
  - RNA Integrity Number (RIN)
  - RNA concentration
  - 28S : 18S ratio
  - Gel-like image

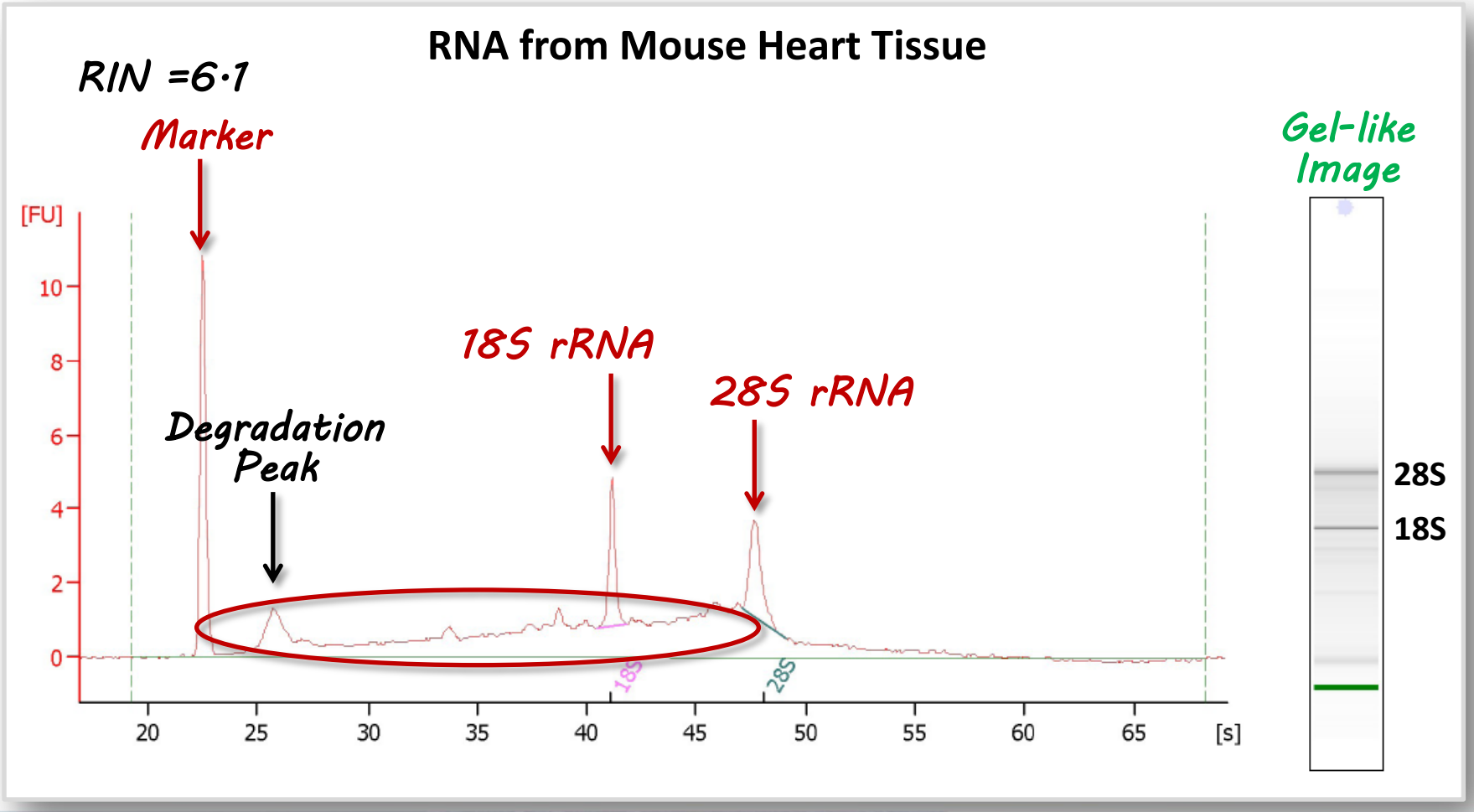


- DNA Analysis Kits
  - PCR fragments, restriction digests or fragmented DNA

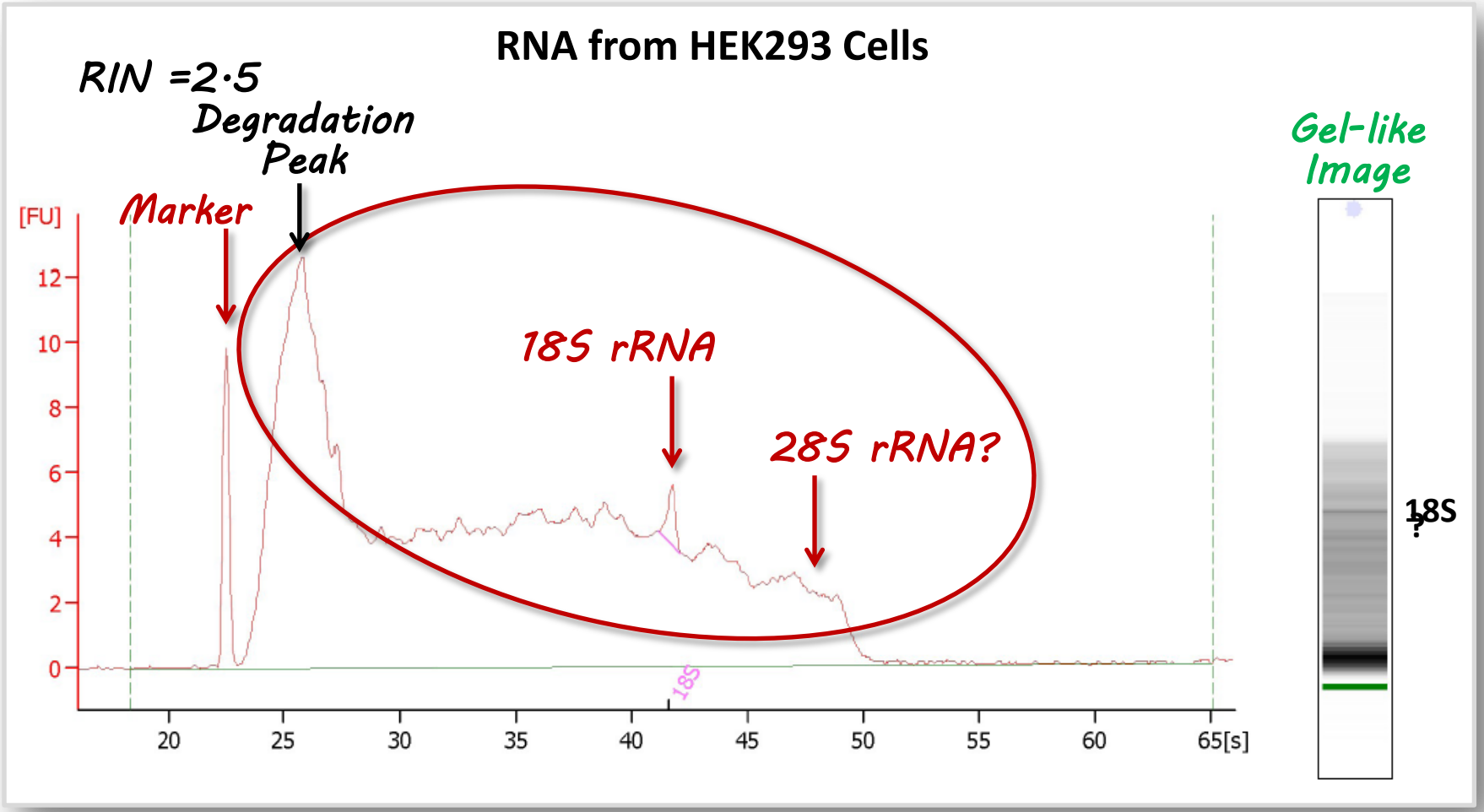
Agilent 2100 Bioanalyzer – RIN=10.0
High Quality RNA with Minimal Degradation



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

Summary

- Sequencing data is largely dependent on the integrity and quality of the starting material

- The quality of analysis depends on careful execution of each step in the workflow
  - Isolation of pure, intact nucleic acid free of contaminants
  - Accurate quantitation and quality assessment

- Reagents and instrumentation are available that overcome many of the major challenges associated with each step of the workflow
  - Isolation of nucleic acid from difficult, degraded or low biomass samples
  - Enzymes that are resistant to inhibitors