Successfully Overcoming the Challenges of Working with FFPE Samples

Trista Schagat, Ph.D.

November 2014

Promega Corporation

Webinar Outline

Overview of Formalin-Fixed, Paraffin-Embedded (FFPE) tissue

Molecular analysis workflow: challenges, key considerations and tips for success

- Sample preparation
- Purification
- Quantitation
- Downstream applications

Why Work with FFPE Samples?

- FFPE is the most common format for archiving solid tissue pathology specimens, especially tumor samples.
- Nucleic acid extraction from FFPE enables both prospective and retrospective opportunities to correlate disease state and tissue morphology with genotype (DNA) and/or gene expression (RNA).
- FFPE samples can be used for MDx and CDx applications in drug development and personalized medicine.

FFPE Samples are Highly Crosslinked and Difficult Starting Samples for Molecular Analyses

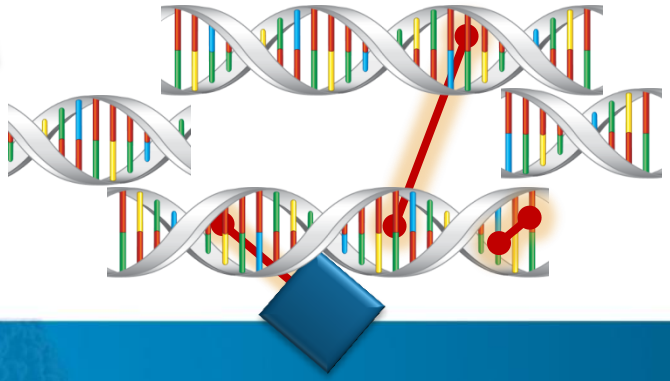
FFPE Sample

Normal Sample

1. Reverse crosslinks
2. Degrade/remove proteins

"Perfect" Extracted gDNA

"Real-life" FFPE Extracted gDNA

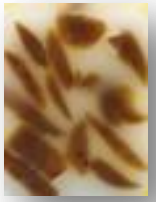


= Formalin crosslinks

Molecular Analysis Workflow

Molecular Analysis Workflow

FFPE Samples



Purify NA → Quantify → End - point PCR → qPCR → Microarray → Next - Gen Sequencing

Sample Challenges

- Degraded/fragmented DNA or RNA
- Crosslinked DNA/RNA & proteins
- Insufficient amount of sample

Preservation of Samples by Formalin Fixation and Paraffin Embedding (FFPE)

Fix tissue
(formalin, neutral pH)

↓

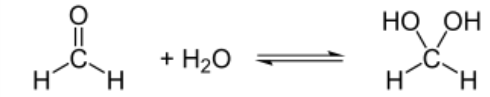
Dehydrate
1. Ethanol
2. Xylene (Toluene)

↓

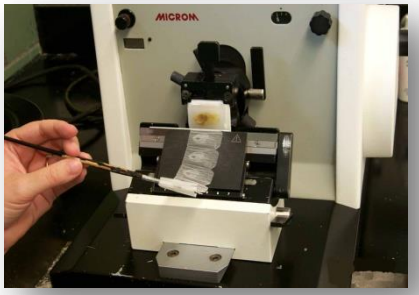
Paraffin embed
(hot wax)

↓

Sectioning and Storage
1. Microtome
2. Store at room temperature

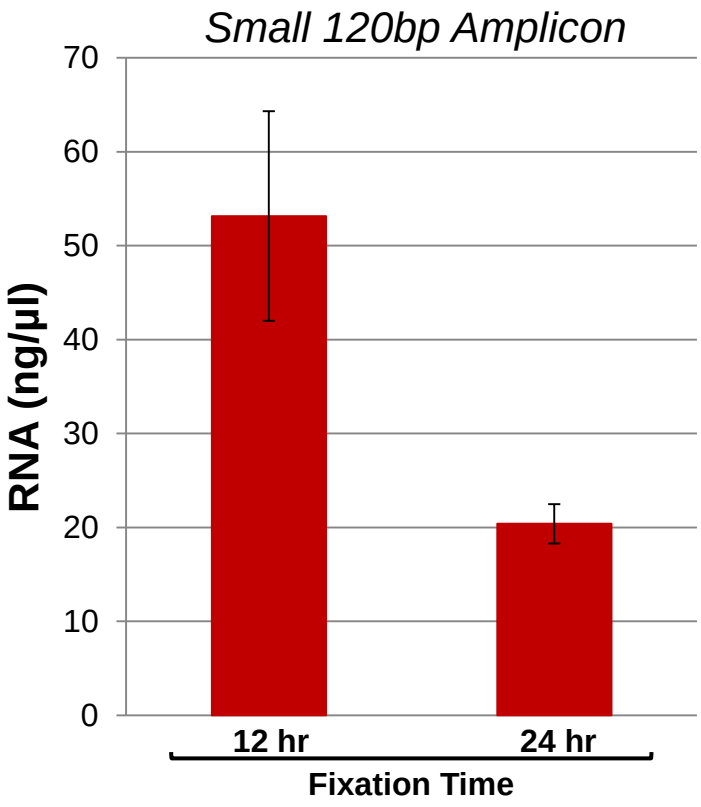


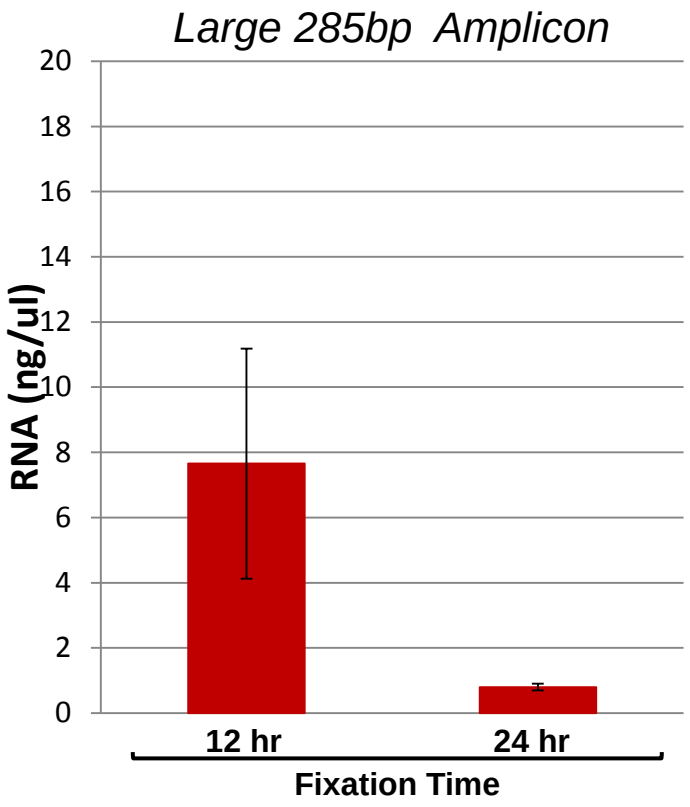
Denatures protein, crosslinks with DNA



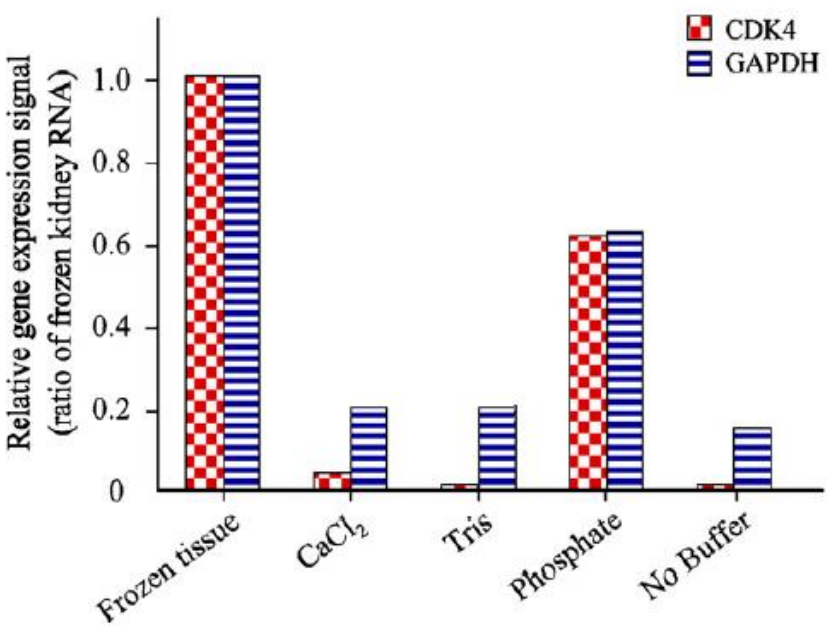
Fixation Time Impacts Nucleic Acid Recovery

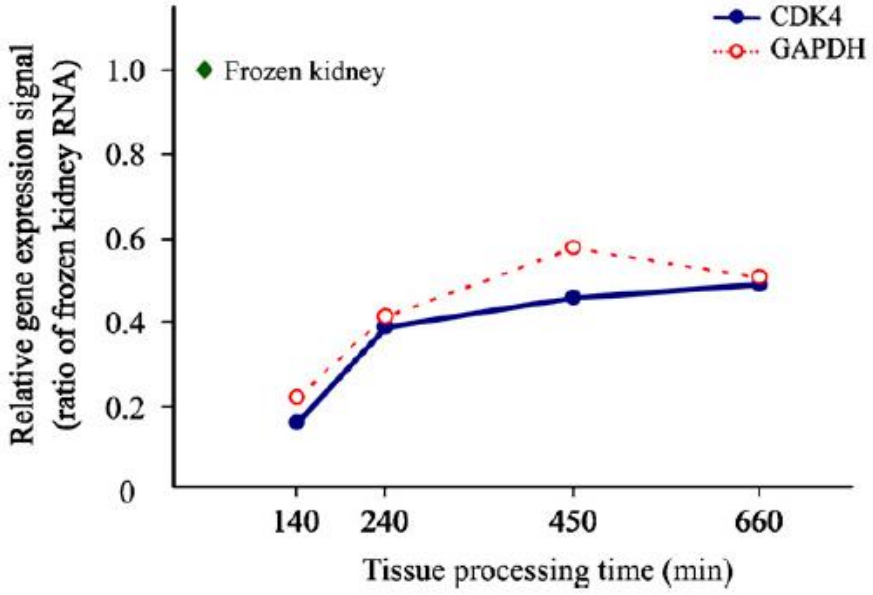
Longer Fixation Time Reduces Subsequent Nucleic Acid Recovery





Fixation Buffer and Processing Time Impact Recovery



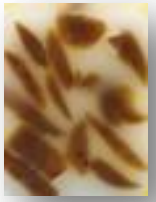


Phosphate-containing fixation buffer and longer processing (dehydration – impregnation) time helps with amplifiable RNA recovery

Chung, et al. J Histochem Cytochem. 2008; 56(11): 1033-42.

Molecular Analysis Workflow

FFPE Samples



Purify NA

Quantify

End - point PCR

qPCR

Microarray

Next - Gen Sequencing

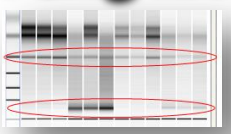
Sample Preparation

- Output will only be as good as input
- Follow methods maximized for quality nucleic acid recovery

Molecular Analysis Workflow

FFPE Samples

Purify NA



Quantify

End - point PCR

qPCR

Microarray

Next - Gen Sequencing

Purification Challenges

- Reversing crosslinks
- Removing enough protein
- Extracting intact, amplifiable gDNA or RNA
- Working with hazardous organics to deparafinize samples

Improvements to Nucleic Acid Purification from FFPE Tissue

Step	Traditional
De-paraffinize	Xylenes or other organics
Lyse/De-crosslink	Proteinase K + heat
Purify nucleic acid	Phase extraction (phenol chloroform)
Remove salts etc.	Precipitation & alcohol wash
Recover nucleic acid	Precipitation/Rehydration
Remove contaminating nucleic acids	DNase/RNase treatment
Time	2 days

Improved FFPE Nucleic Acid Purification Offerings are Available in Manual and Automated Formats

Manual Kits

- ReliaPrep™ FFPE gDNA Miniprep System
- ReliaPrep™ FFPE RNA Miniprep System

Automated System

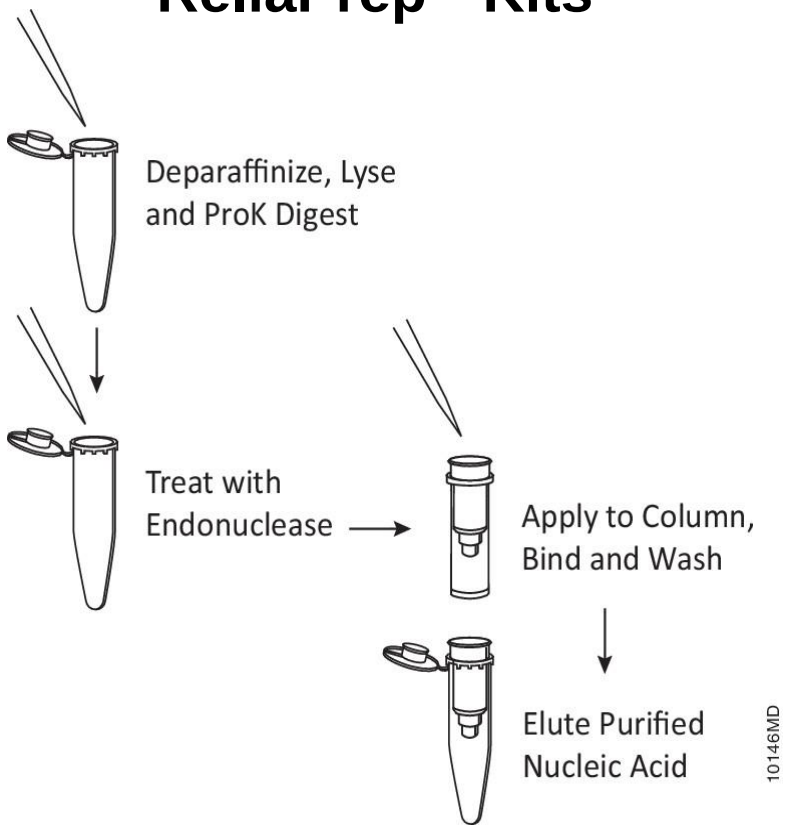
Maxwell® Instruments & Kits

- Maxwell® 16 FFPE Tissue LEV DNA Purification Kit*
- Maxwell® 16 FFPE Plus LEV DNA Purification Kit*
- Maxwell® 16 LEV RNA FFPE Kit

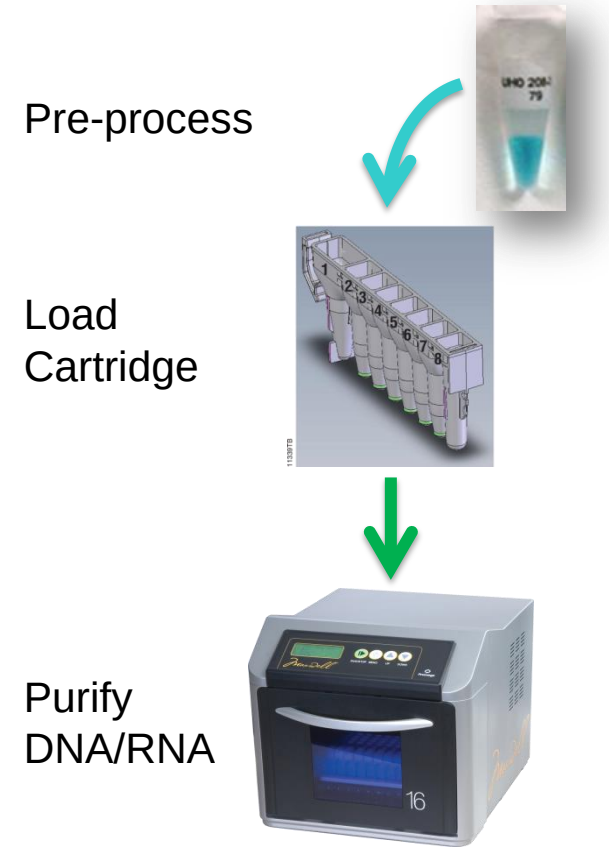
*For Laboratory Use Only

Both Manual and Automated Methods Include the Same Improvements

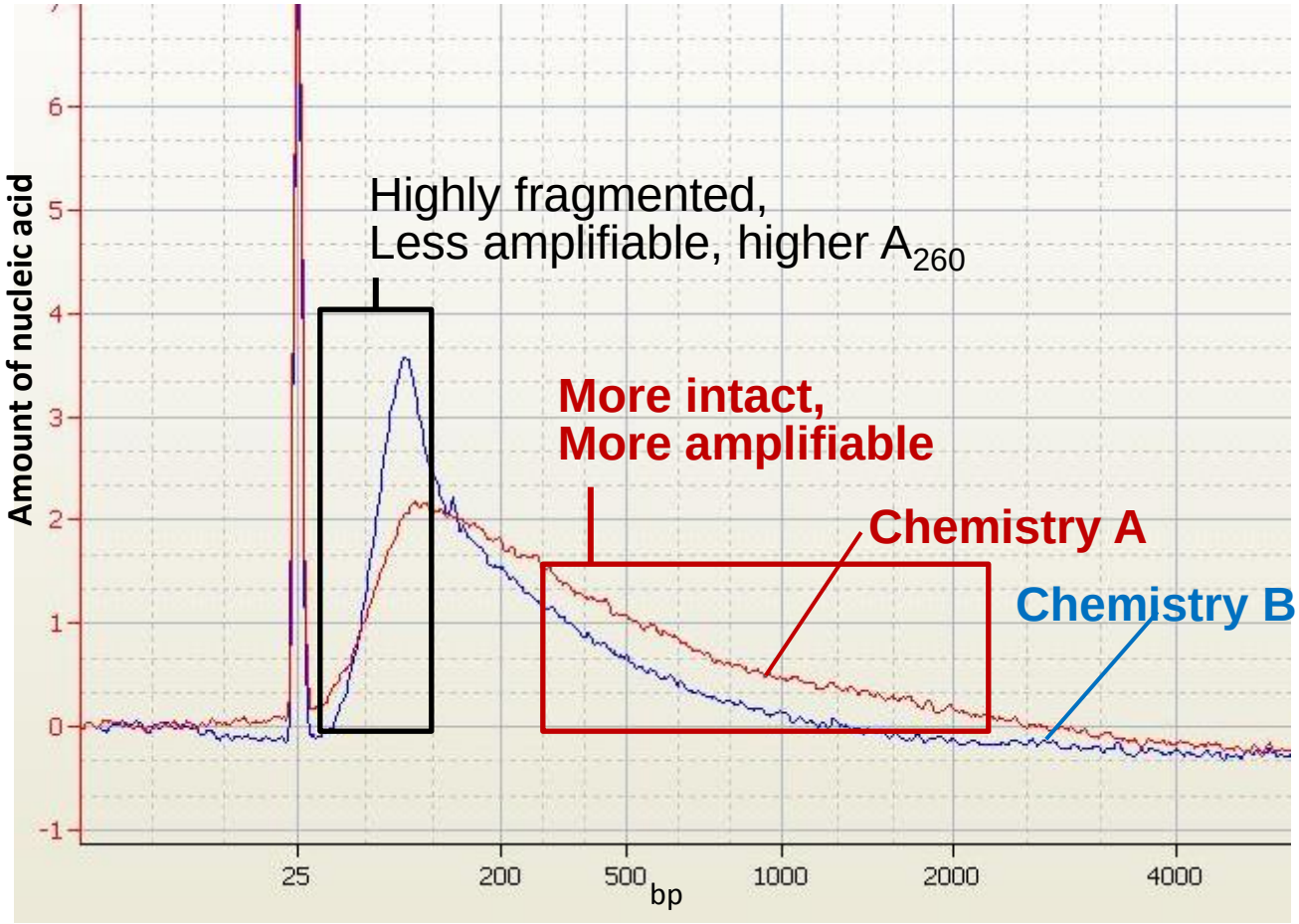
ReliaPrep™ Kits



Maxwell® System



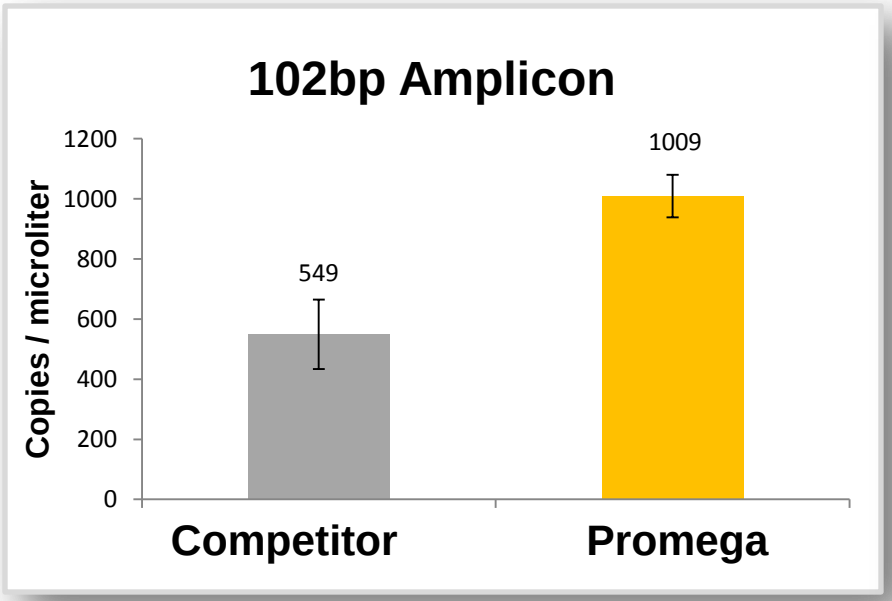
Purification Chemistry Affects Average gDNA Fragment Size Recovered

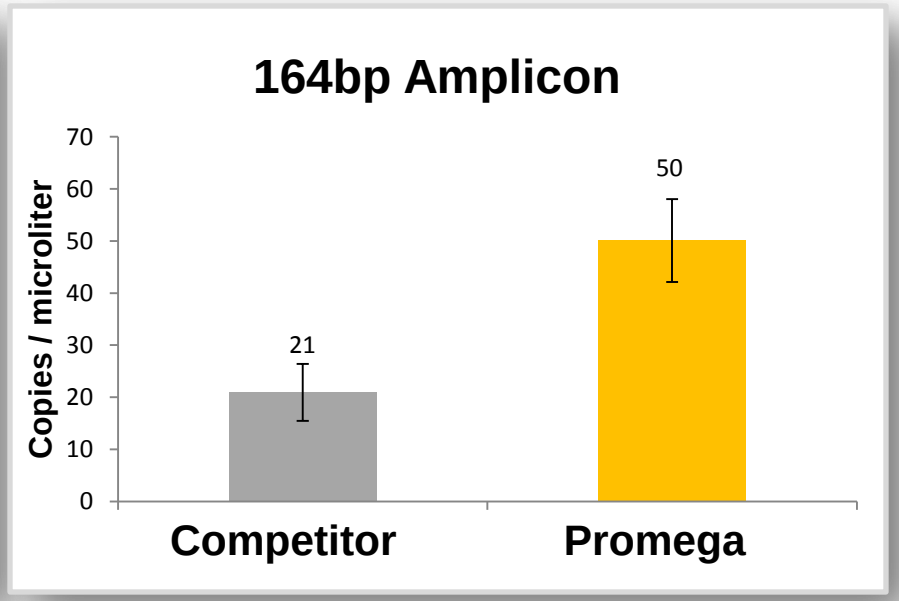


- Fragmentation occurs as part of fixation
- Highly fragmented gDNA is less amplifiable, as the peak is about 100bp
- The difference in area under the curve >300bp is the advantage of Chem A over Chem B

New Technologies Offer Better Performance

Promega vs Competitor FFPE DNA Extraction:
More amplifiable DNA extracted with Promega Kit



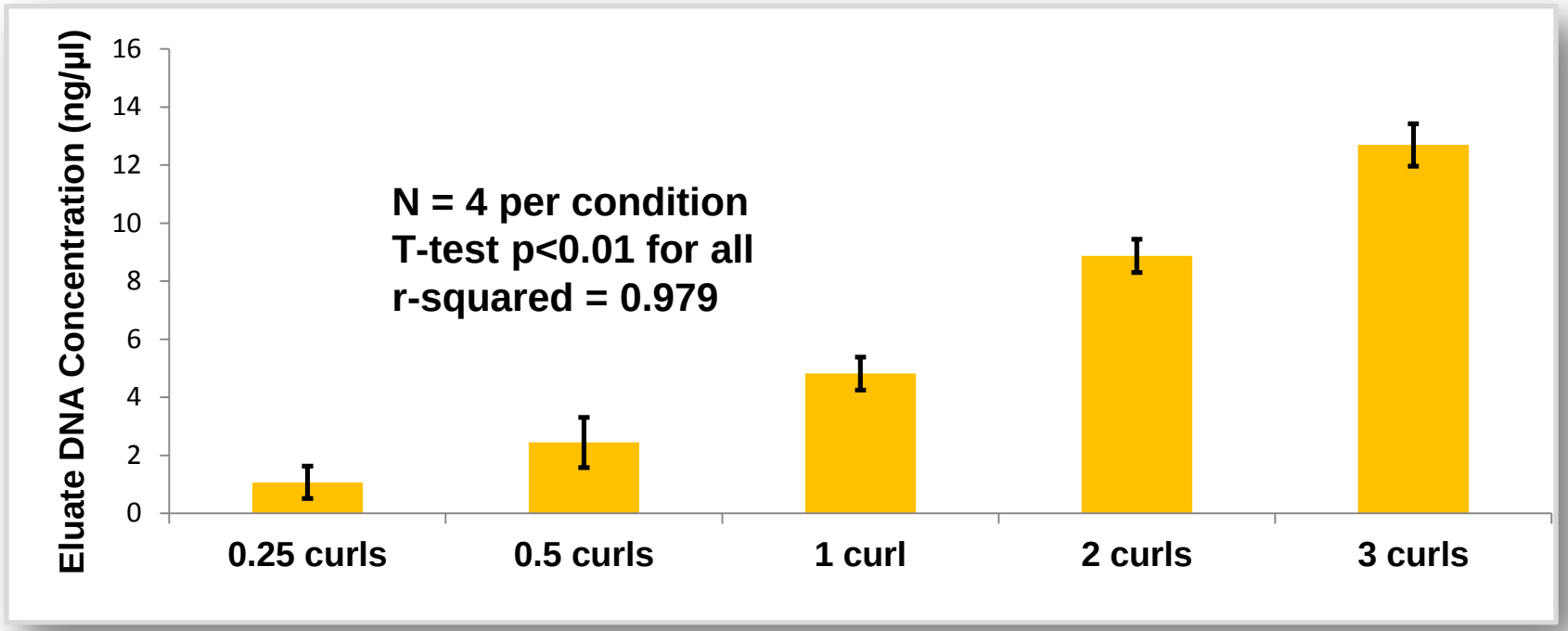


Mounted Slides (5 micon):
Breast Tumor Tissue, n=4

Parameters that Affect Nucleic Acid Purification from FFPE Samples

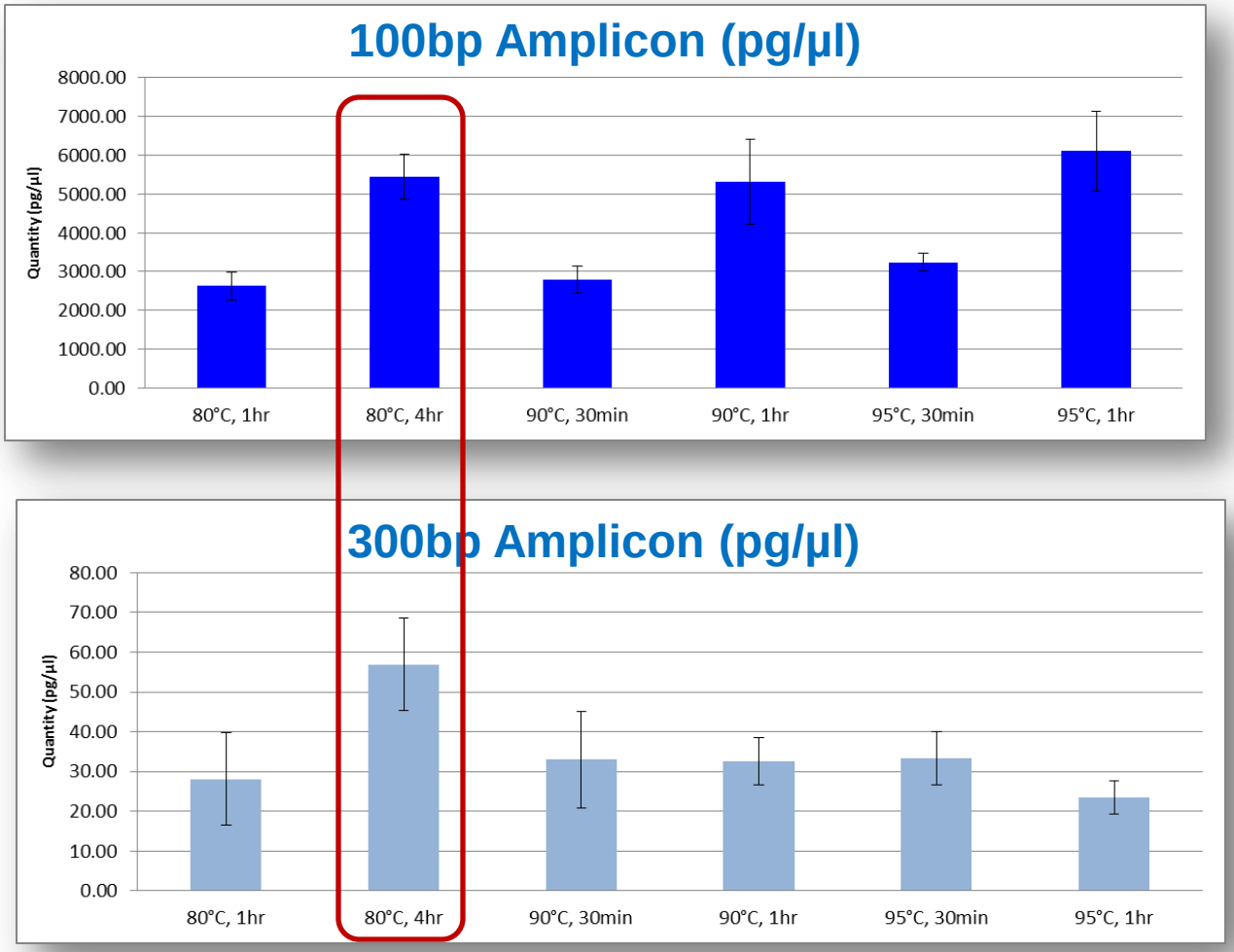
- Input tissue (amount, age, fixation time and process)
- De-paraffinization
- Proteinase K (amount, time, temperature)
- De-crosslinking (time, temperature)
- Lysis and binding reagent composition
- Solid support for nucleic acid capture
- Elution volume

Input Tissue: Number of Curls Scales with Recovery



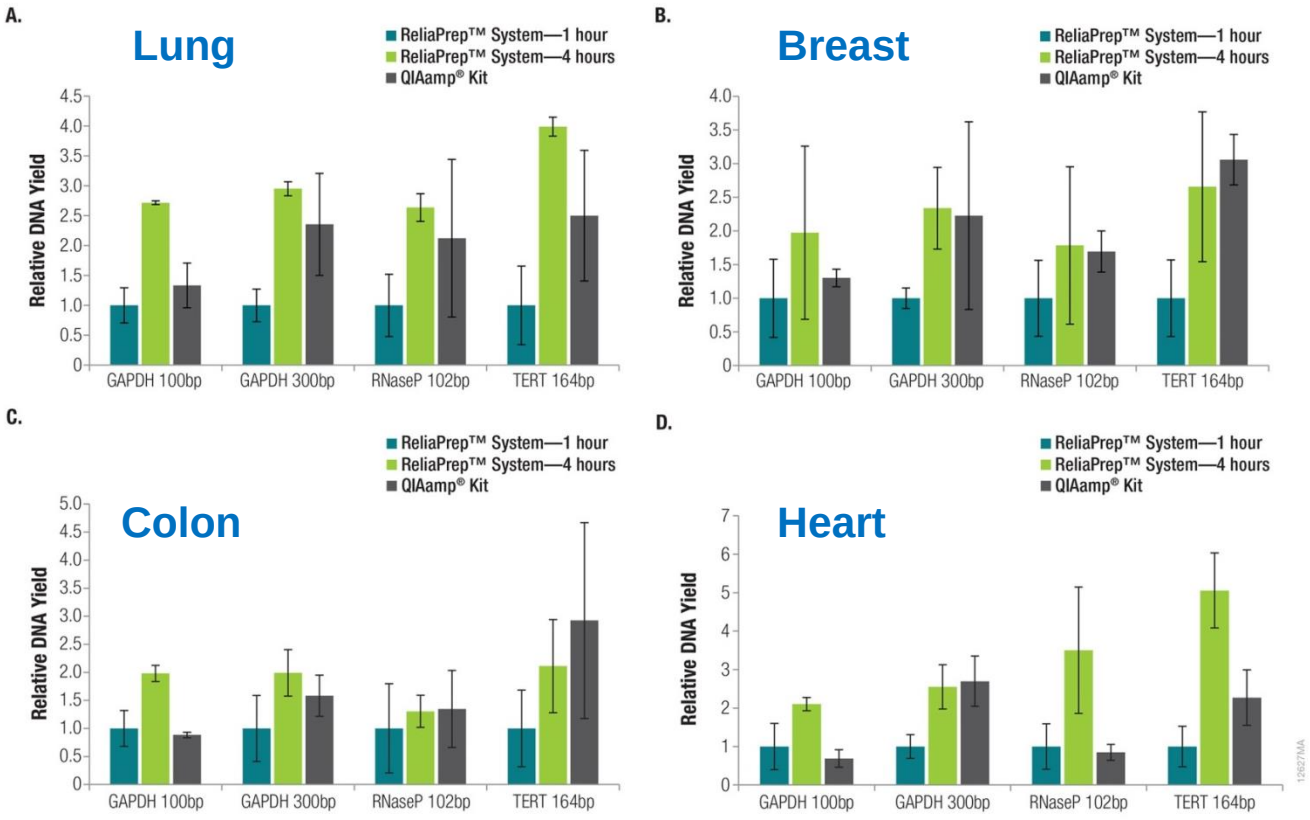
Direct correlation between the input amount of FFPE tissue and the amount of DNA recovered

De-crosslinking Time & Temperature Affect Yield



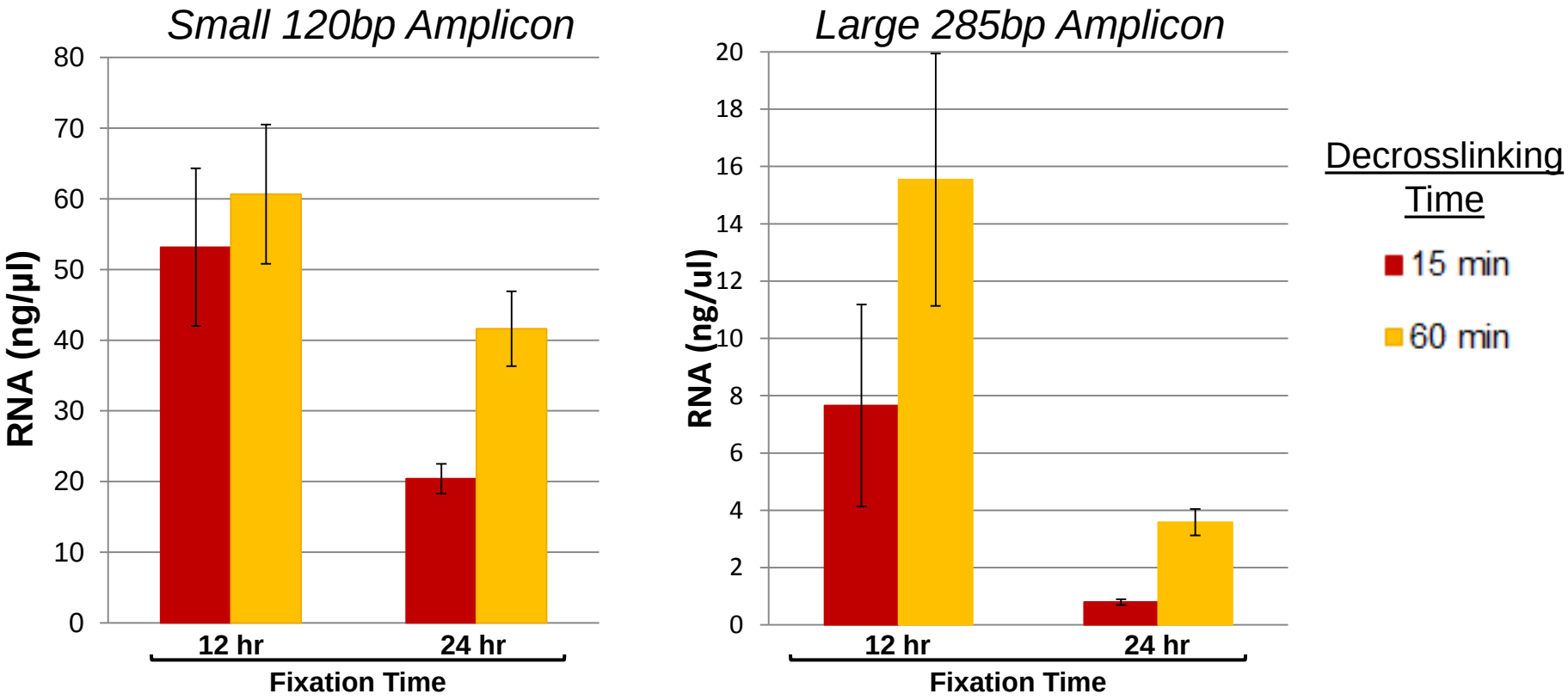
- Maxwell® DNA
- 4 breast sample slides each condition
- **80°C for 4 hours** produces most amplifiable DNA for both 100bp and 300bp amplicons

Tissue Type Influences Optimal De-crosslinking Time



ReliaPrep™ FFPE DNA System, 4 hour de-crosslinking improves yields in most, but not all conditions.

De-crosslinking Time Impacts RNA Recovery

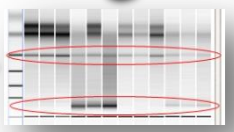


Maxwell® 16 FFPE RNA: Longer de-crosslinking increases recovery RNA

Molecular Analysis Workflow

FFPE Samples

Purify NA



Quantify

End - point PCR

qPCR

Microarray

Next - Gen Sequencing

Purification

- Omit use of organics
- Maximize recovery of functional nucleic acid
- Effectively remove contaminants

Molecular Analysis Workflow

Quantitation Challenges

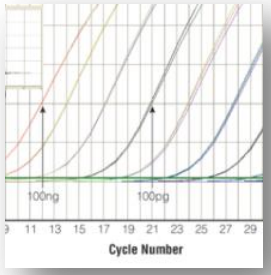
- Contaminants affect UV absorbance-based measurements
- Low NA yields often limit usefulness of quantitation by UV absorbance
- Residual crosslinking and fragmentation make predicting performance in downstream assays difficult based on initial quantitation

There are Many Methods for Measuring Nucleic Acid Quantity & Quality

UV Absorbance
NanoDrop® 2000
(Thermo Scientific)

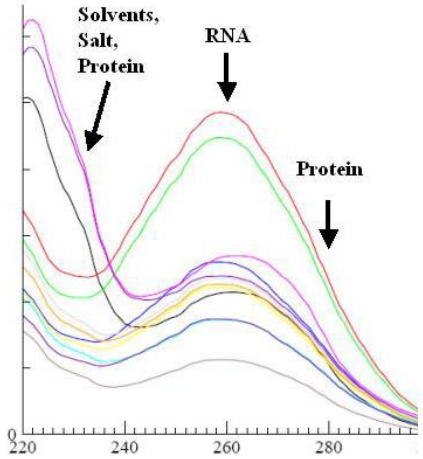
Fluorescence
Quantus™ Fluorometer &
QuantiFluor® ONE dsDNA Kit
(Promega)

Microfluidic Analysis
Agilent Bioanalyzer



qPCR
GoTaq® qPCR and RT-qPCR Systems
(BRYT Green® dye or probe-based)
(Promega)

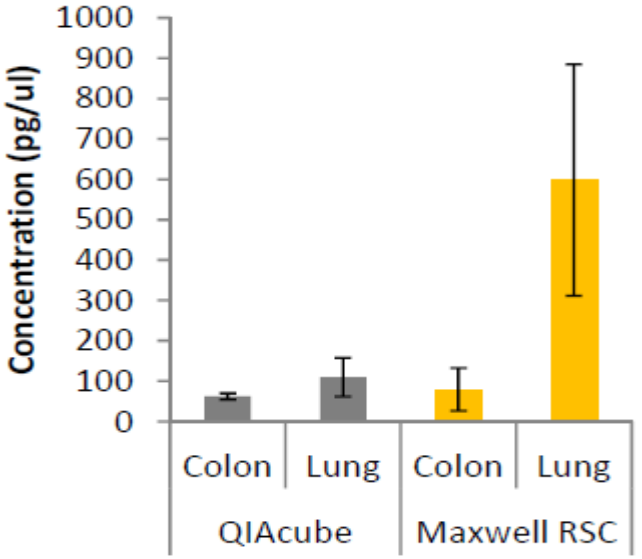
Absorbance Measures More than Just Nucleic Acid Quantity



- Molecules other than NA absorb UV
- FFPE NA concentrations are low
- The lower the NA concentration, the greater the impact of contaminants on absorbance readings

FFPE RNA Extracted and Assessed by NanoDrop® and RT-qPCR

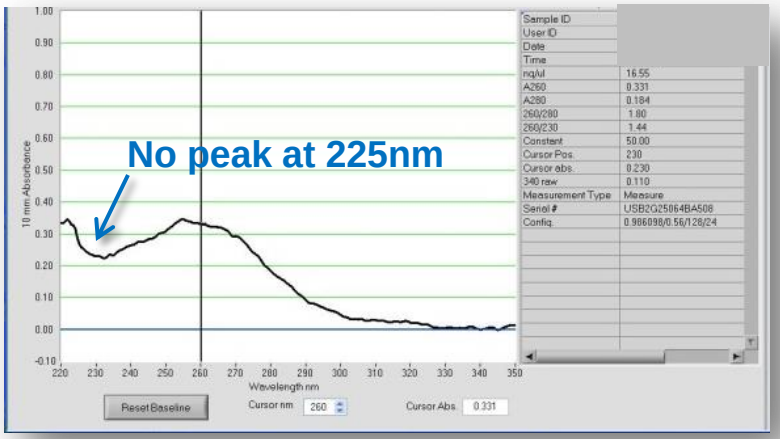
		ng/µl NanoDrop
QIAcube	Colon	16.45
	Lung	54.7
Maxwell® RSC	Colon	13.48
	Lung	28.59



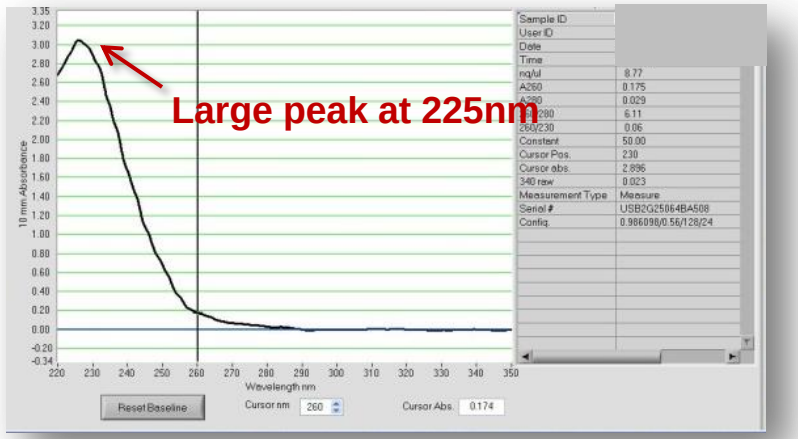
Absorbance is Not Reliable for FFPE Nucleic Acid Quantitation

gDNA extraction from matched lung tissue FFPE slides

Chemistry A

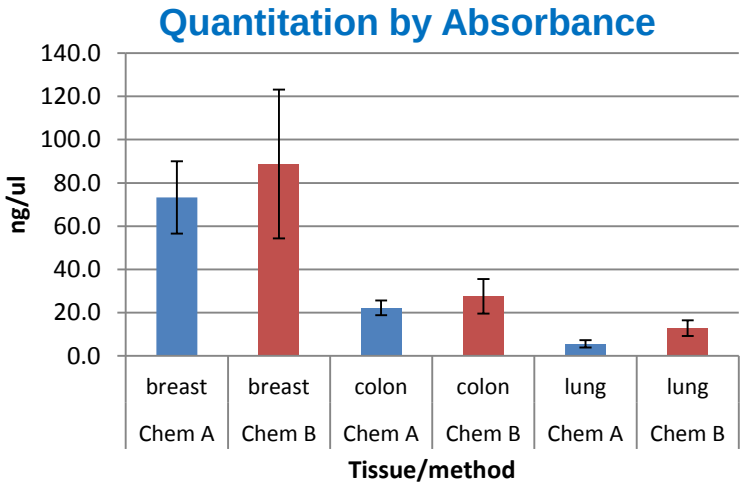


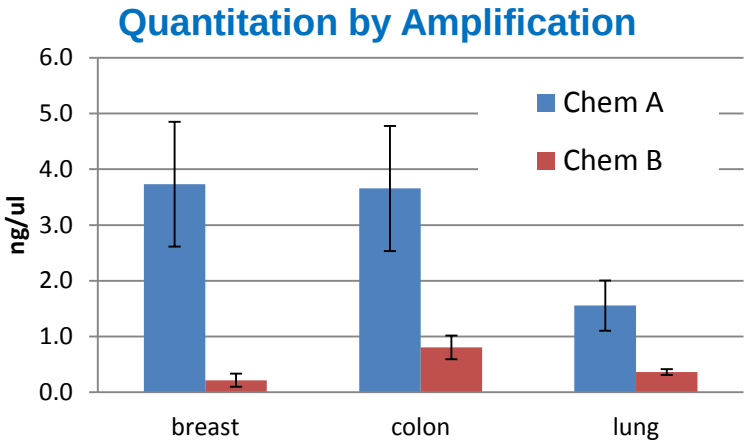
Chemistry B



- Large peaks at wavelengths lower than 260nm can influence the measured peak at 260nm
- ***Concentration and ratios are not reliable at low absorbance levels***

Absorbance-based Quantity Does Not Correlate with qPCR



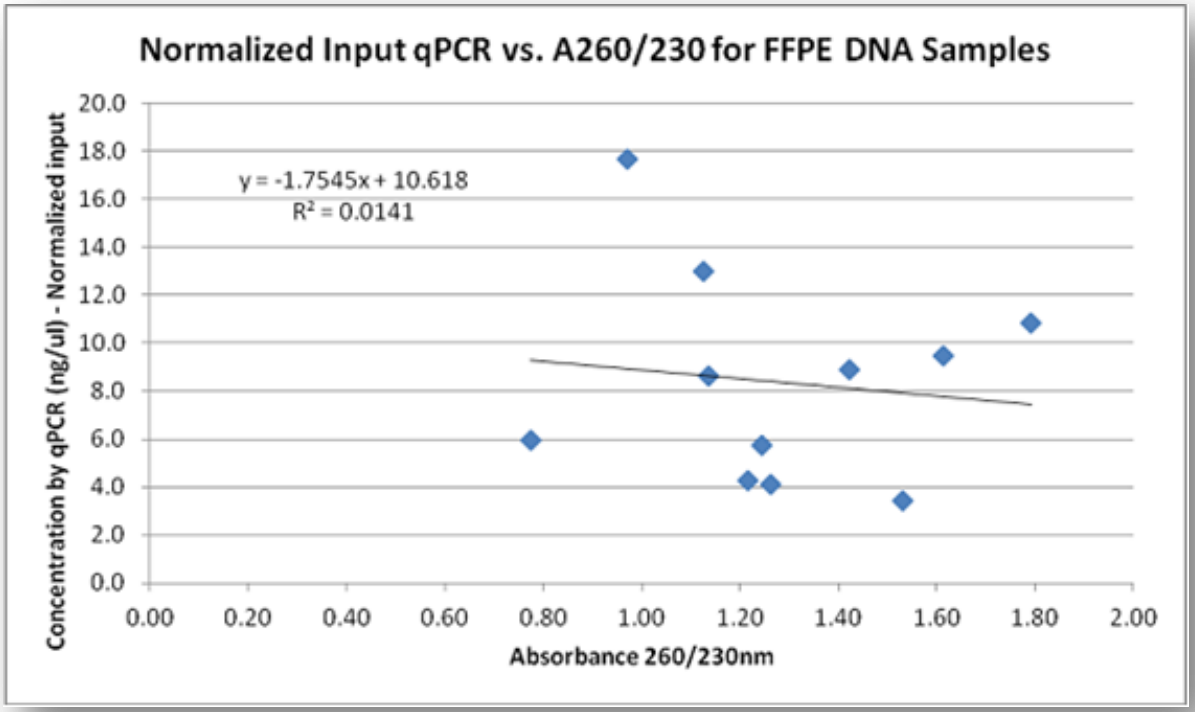


Large difference in quantitation

- Absorbance at 260nm is not always an accurate measure of NA
- Absorbance does not necessarily predict downstream performance

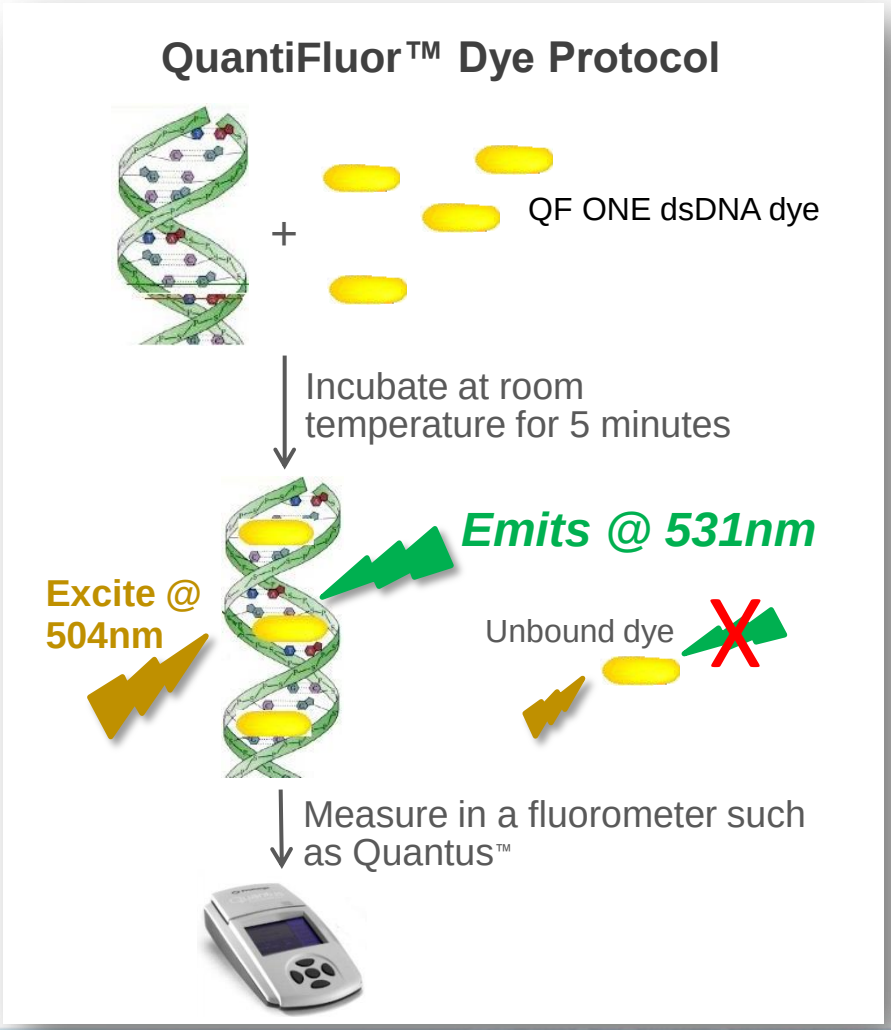
No Correlation Between A_{260}/A_{230} and Amplifiability

- Input normalized to A_{260}; Samples run using quantitative PCR
- Variety of chemistries tested across a single tissue type (breast) to include a range of A_{260}/A_{230} ratios



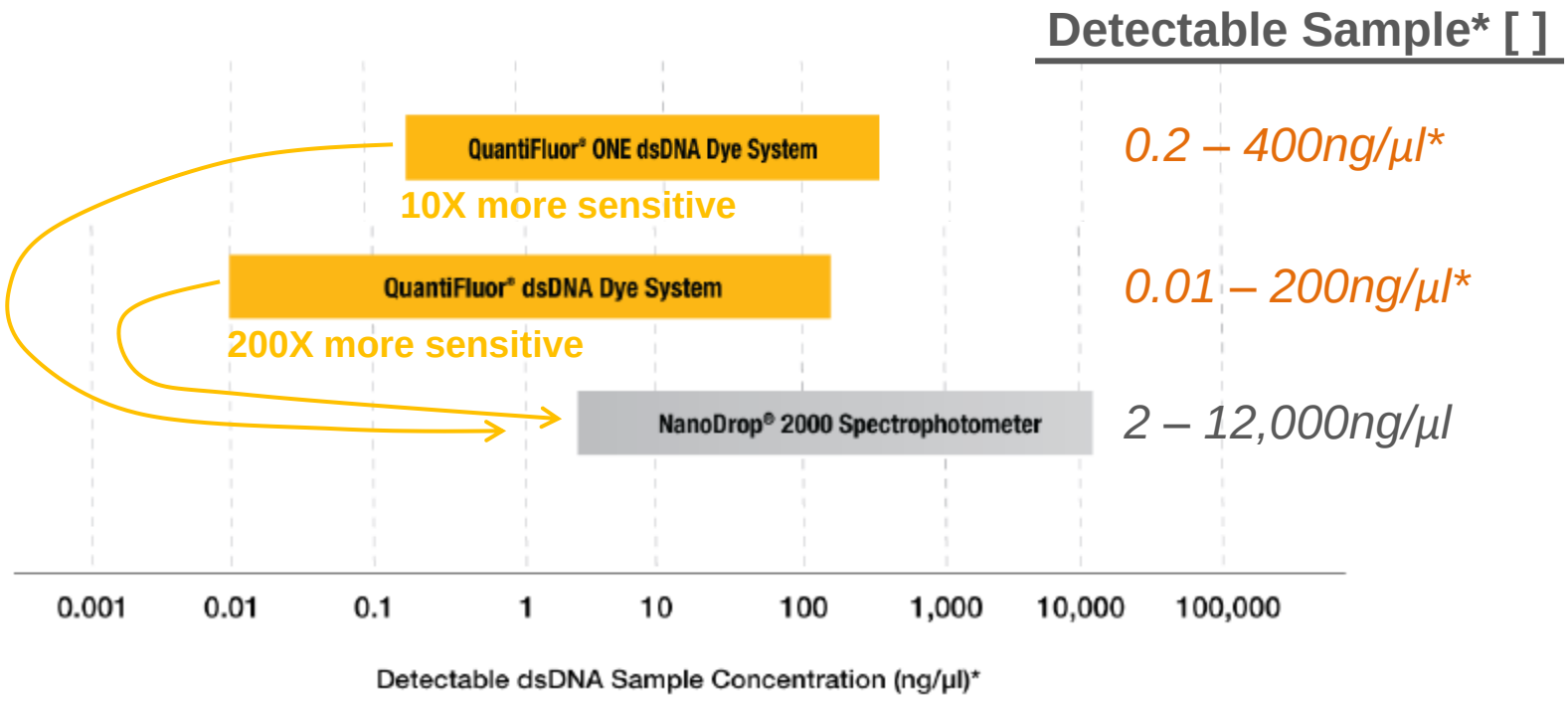
- ***No correlation between A_{260}/A_{230} and amplifiability***
- *For QC, a more predictive test is needed*

Fluorescent Nucleic Acid Quantitation Uses Nucleic Acid - Binding Dyes



- Easy protocol: add, mix, read
- Greater specificity than UV absorbance
  - Unaffected by contaminants in the samples unlike UV absorbance – especially at low NA concentrations like those obtained from FFPE tissues
- Greater sensitivity than UV absorbance

Fluorescence-based Quantitation is More Sensitive than UV Absorbance

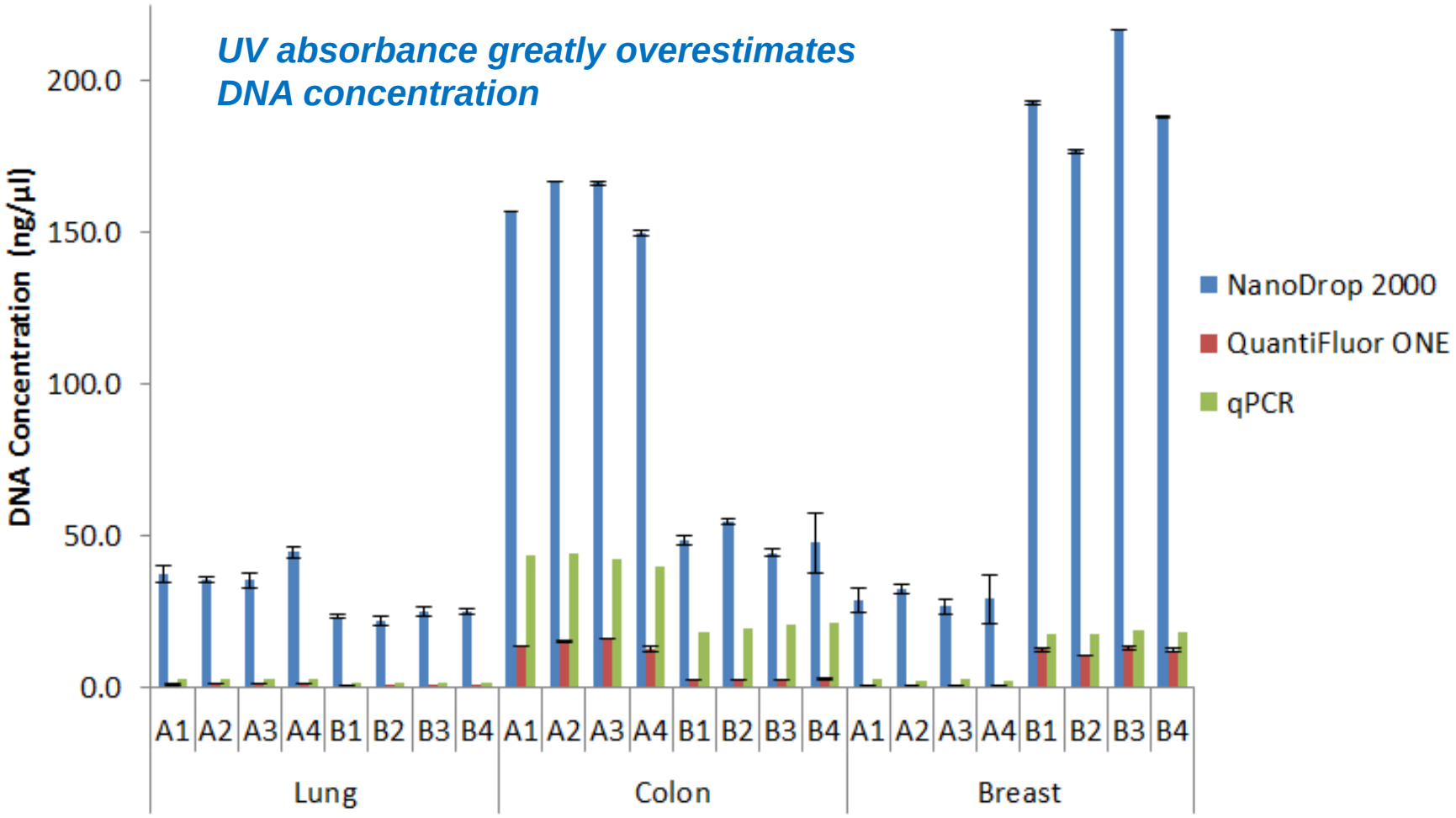


QuantiFluor® RNA Dye System:
0.1 – 500ng/µl Sensitivity

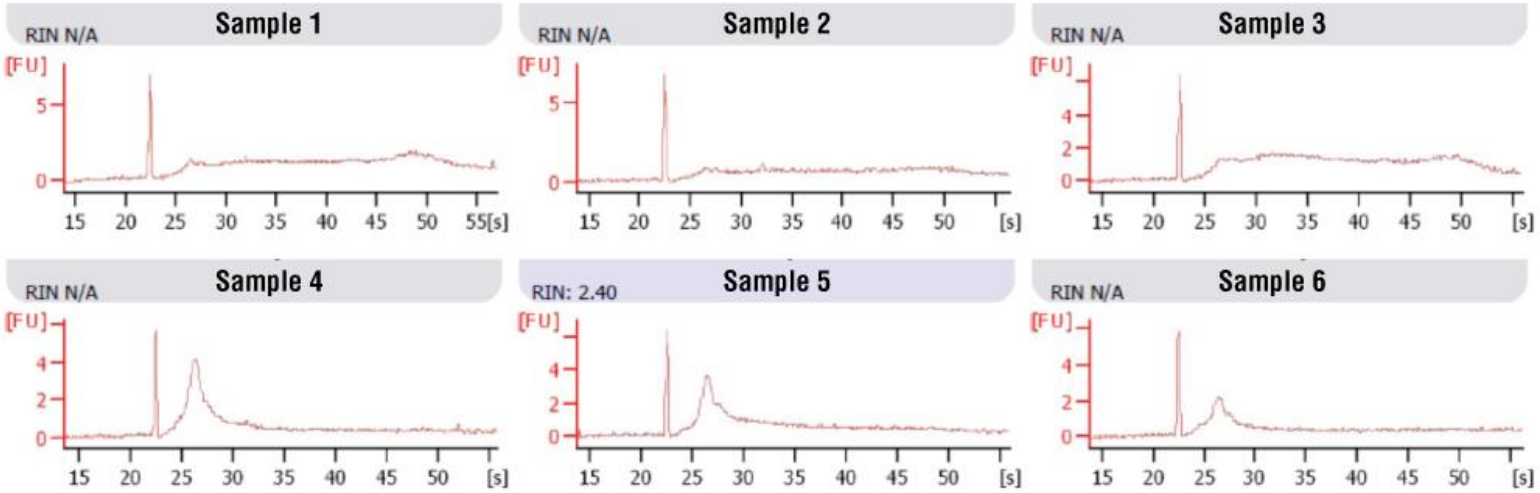
***** Based on using 1µl of sample per assay

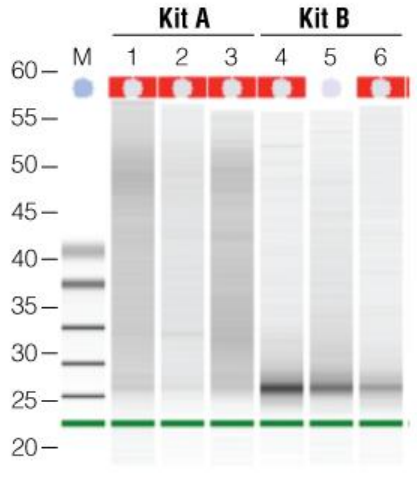
Note: Fluorescence sensitivity can be increased by assaying more sample volume

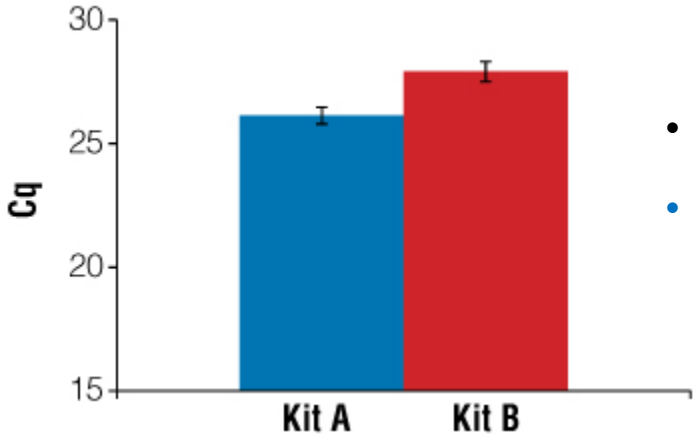
Fluorescence-based Quantitation Correlates Best with qPCR



Bioanalyzer Assesses Nucleic Acid Integrity





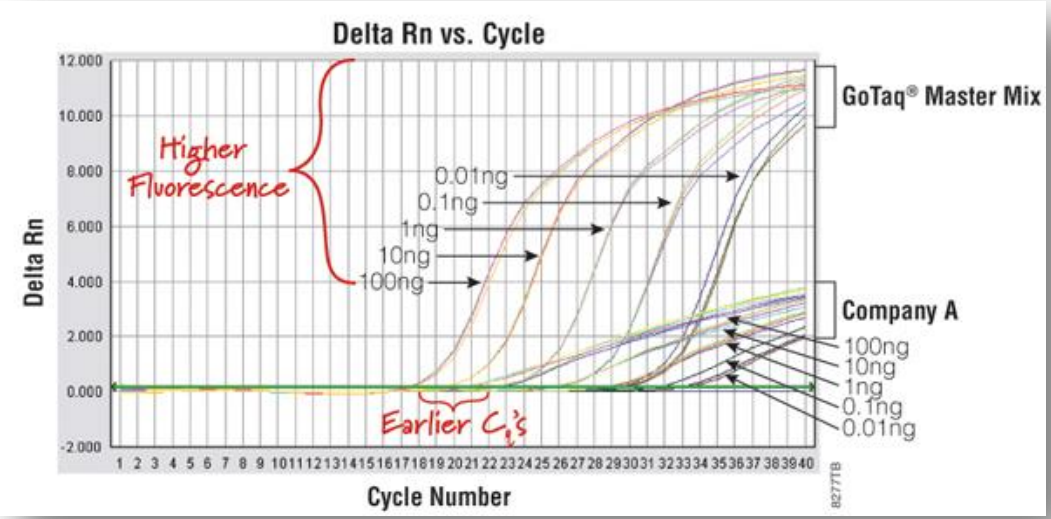


- *FFPE RNA has low RIN*
- ***Integrity does not predict downstream performance***

Real-Time qPCR is the Method of Choice for "Functional" Nucleic Acid Quantitation

Real-Time qPCR assays (e.g. GoTaq® qPCR Master Mixes)

- Measure NA quantities by comparison to standard
- Degraded, crosslinked NA will not amplify, so the qPCR result reflects amplifiable DNA
- GoTaq® BRYT Green® dye exhibits greater fluorescence enhancement upon binding to dsDNA than does SYBR® Green I.



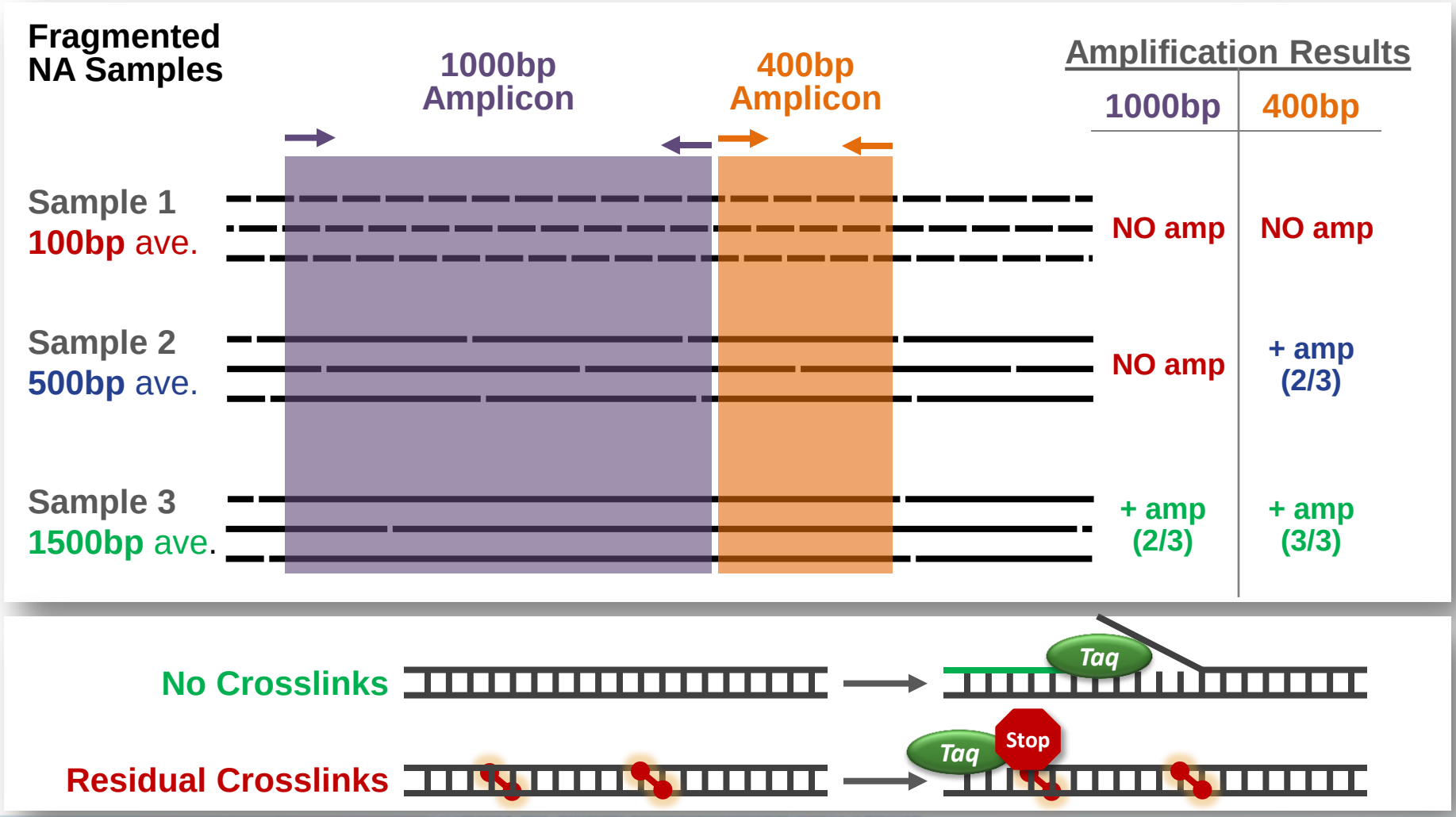
Pros

- Quantitates
- Indicates amplifiability of NA (both template amount & presence of inhibitors)

Cons

- Longer protocol than spectrophotometry or fluorometric methods.

Isolated Fragment Size and Residual Crosslinks are Key Determinants of Downstream Assays Success



qPCR Quantitation is Affected by Amplicon Length Due to Highly Fragmented Nature of FFPE NA

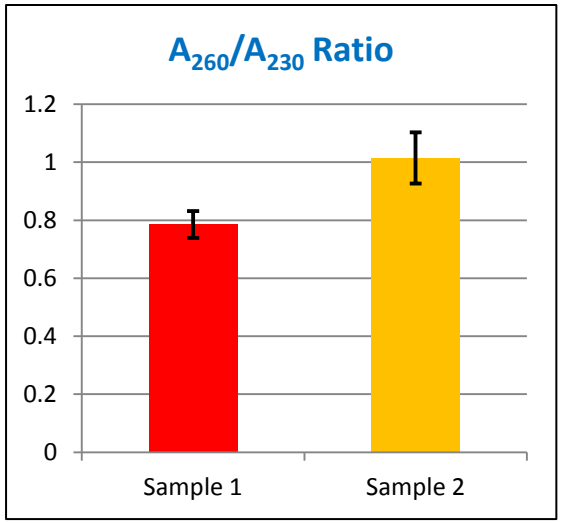
qPCR DNA Quantitation Using Different Amplicon Lengths				
FFPE Sample	100bp Amplicon (ng/µl)	200bp Amplicon (ng/µl)	300bp Amplicon (ng/µl)	400bp Amplicon (ng/µl)
1	39.6	5.35	1.24	1.61
2	54.7	5.85	1.37	1.47
3	53.6	2.26	1.34	1.7
4	50.9	1.95	0.96	1.12

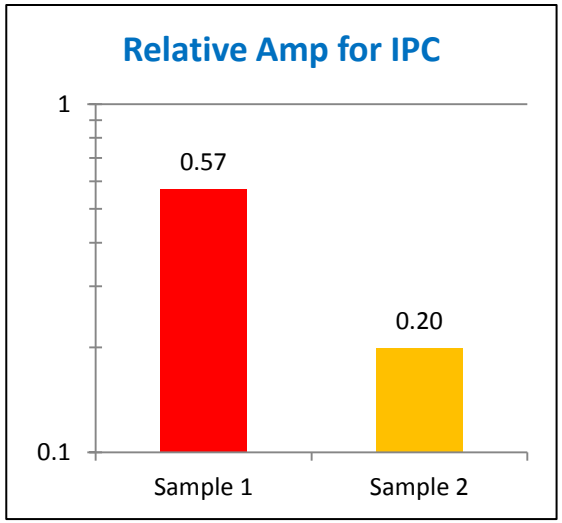
Compare

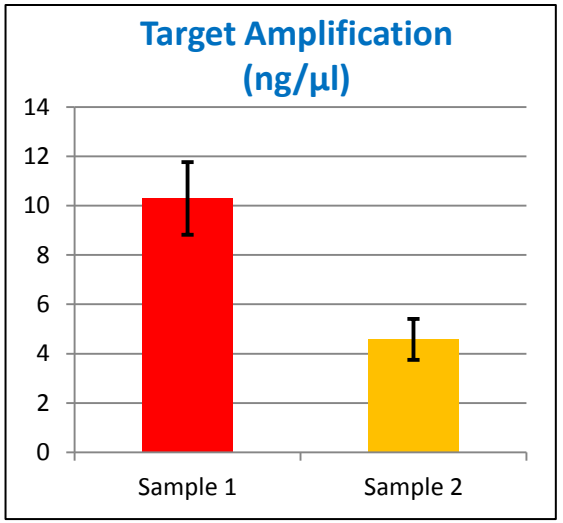
gDNA estimation is greater when using a shorter amplicon

Internal Positive Control (IPC) Assay Can Assess Inhibitor Carryover/Presence

Exogenous amplification target is added with master mix and the effect of adding NA eluates is monitored

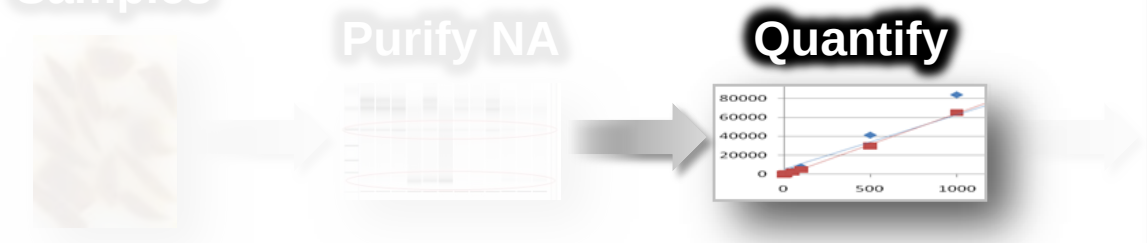






- The purity measure, A_{260}/A_{230} ratio, fails to correlate with amplification
- *IPC assay shows relative amplification that tracks with amplification of a target in the FFPE sample.*

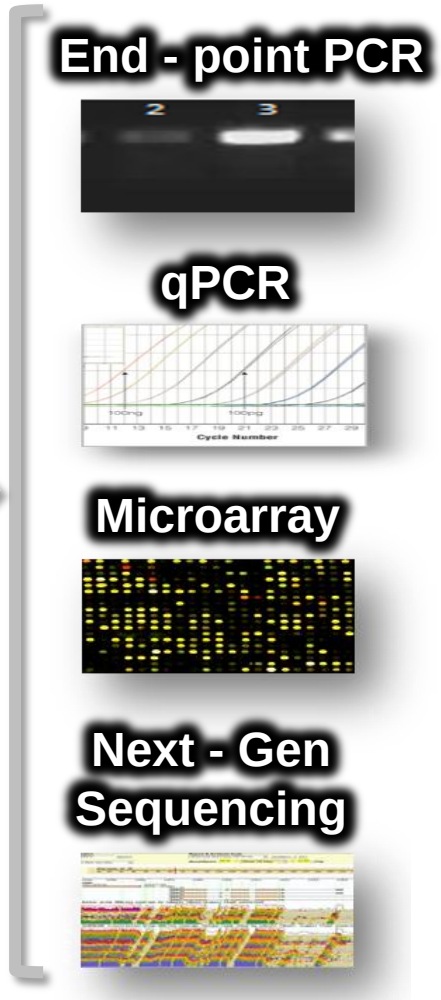
Molecular Analysis Workflow



Quantitation

- UV absorbance-based measurements tend to be inaccurate
- Fluorescent dyes are ideal for low NA samples
- qPCR provides a functional assessment

Molecular Analysis Workflow



Assay Challenges

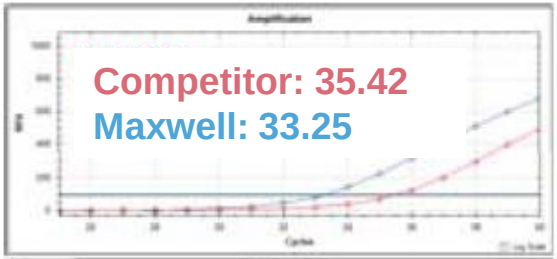
- Enzyme inhibitors in “purified” DNA may inhibit downstream assays
- Input DNA is highly fragmented and not good templates for many assays

Allele-specific qPCR Affected by FFPE DNA Purification Method

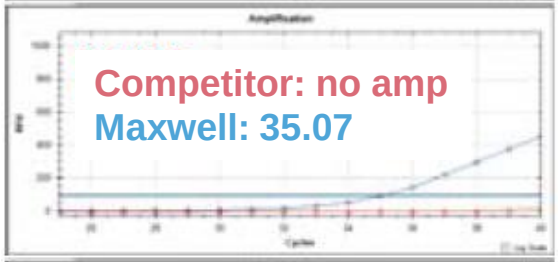
Sample	ΔC_q Value (Competitor – Maxwell)
01	1.11
02	**2.17**
03	0.37
04	0.66
05	0.73
06	1.94
07	**N/A**
08	-0.02
09	1.72
10	**2.60**
11	**2.55**
12	0.05

(Customer provided data)

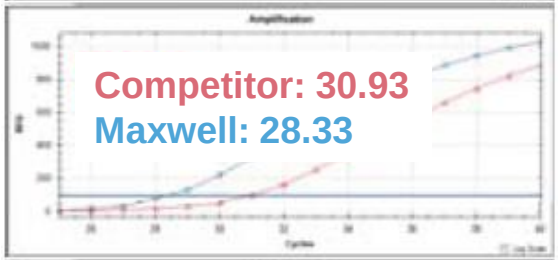
#2



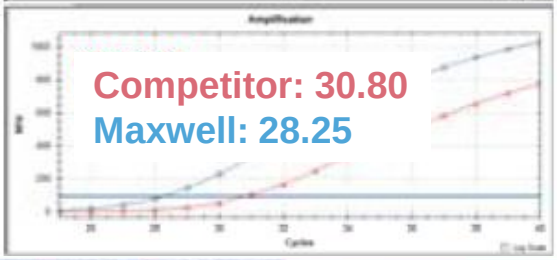
#7



#10



#11

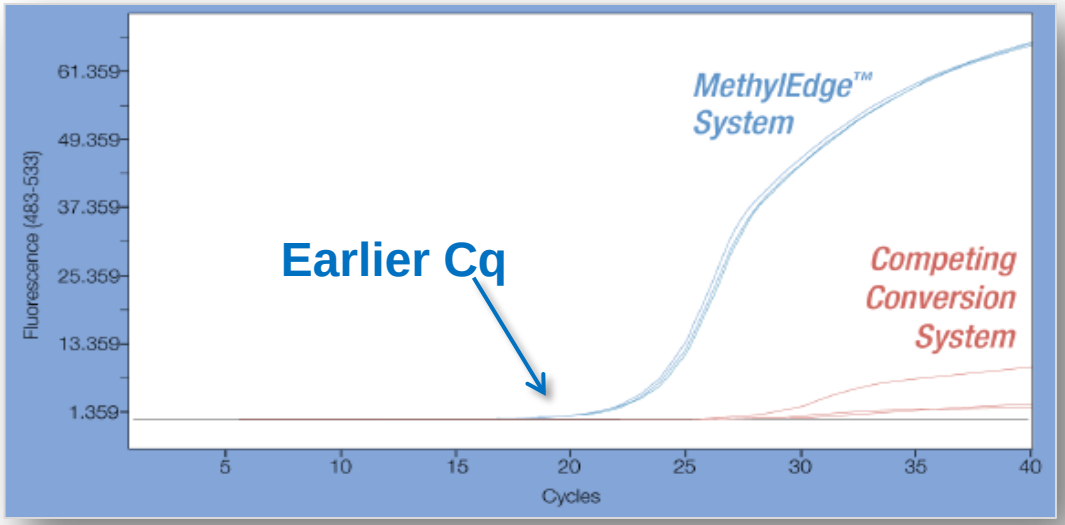


Maxwell FFPE DNA averaged 1.3 C_q earlier than competition (2.4x more DNA)

Bisulfite-specific PCR Chemistry Impacts FFPE DNA Performance

Bisulfite conversion (to study DNA methylation patterns) fragments gDNA – choose a chemistry that minimizes fragmentation of already challenged FFPE DNA samples.

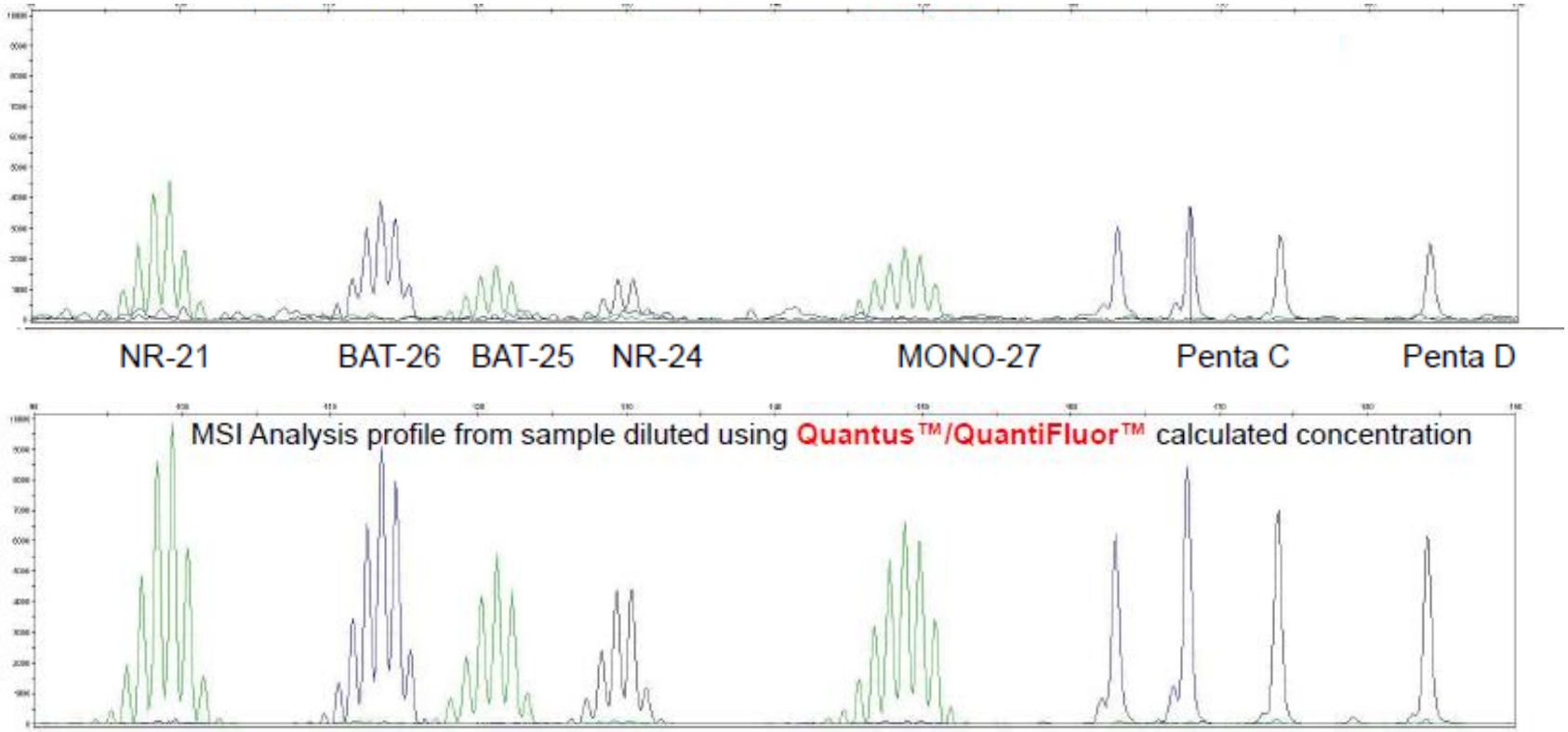
- FFPE samples prepped with ReliaPrep™ FFPE gDNA System
- Bisulfite converted with MethylEdge™ (<2 hr) and competitor kit
- Analyzed with GoTaq® qPCR Master Mix using bisulfite-specific PCR



MethylEdge™ bisulfite conversion results in less fragmented DNA & greater detection sensitivity.

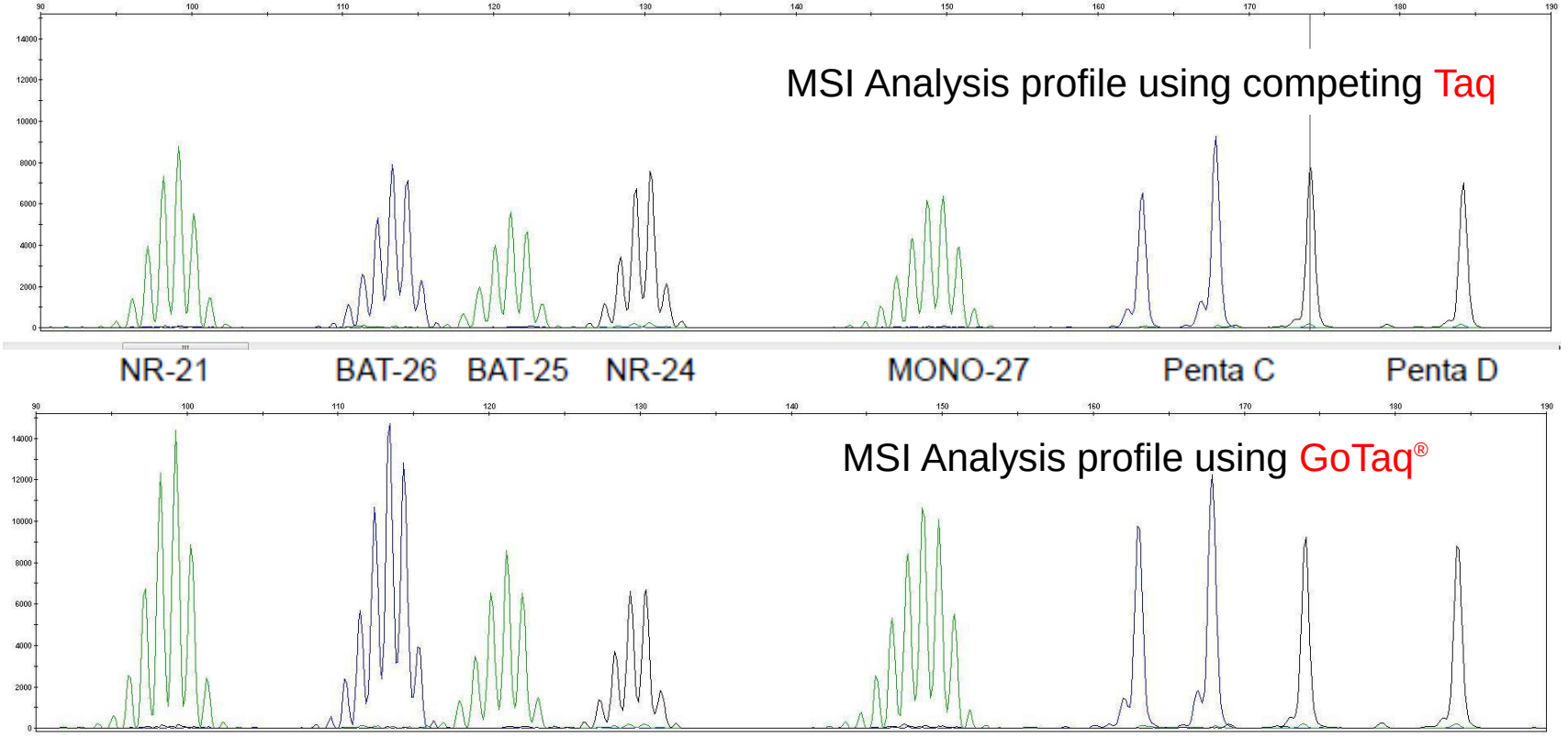
MSI (Microsatellite Instability) Analysis Influenced by Input Mass of FFPE DNA

MSI Analysis System (Promega) uses multiplex PCR followed by capillary electrophoresis for high resolution distinction of loci-specific repeats.



NanoDrop® overestimated [FFPE DNA]; less mass was added; lower peak heights achieved.

Maximize FFPE MSI Analysis with GoTaq® DNA Polymerase

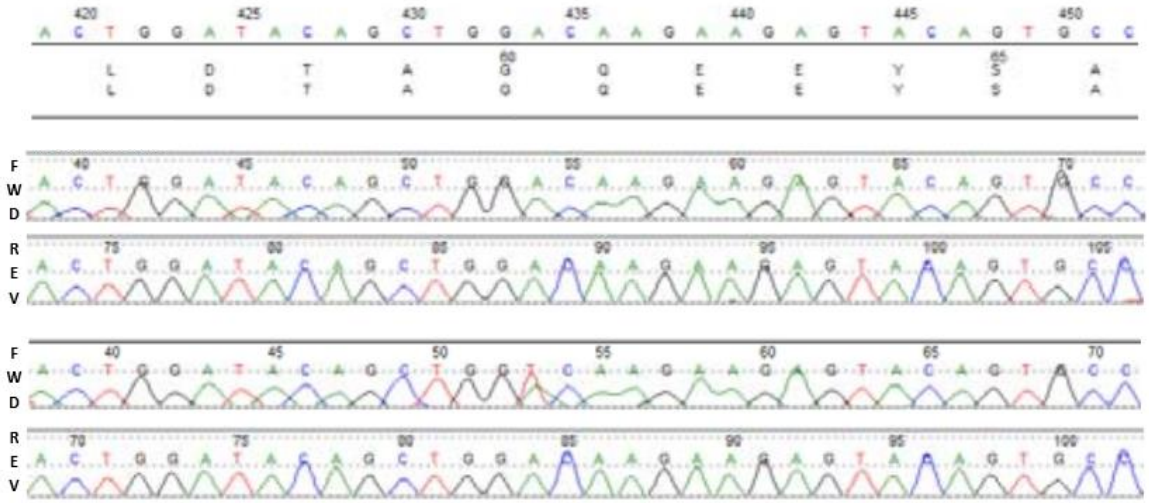


GoTaq® DNA Polymerase helps maximize signal intensity in multiplex qPCR

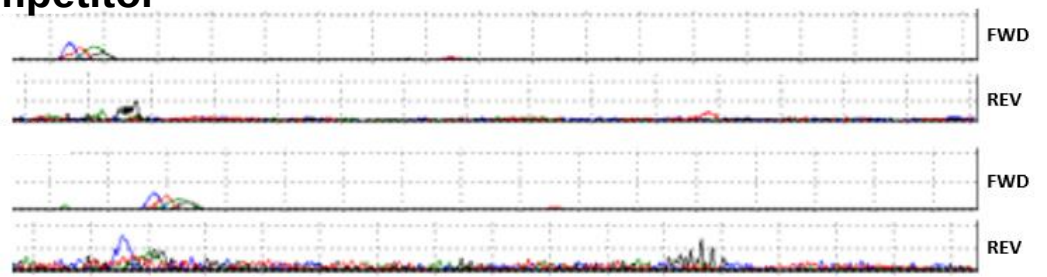
Purification Chemistry Impacts Sanger Sequencing

Sanger sequencing of a cancer target using two different FFPE tissue DNA purification kits

Promega



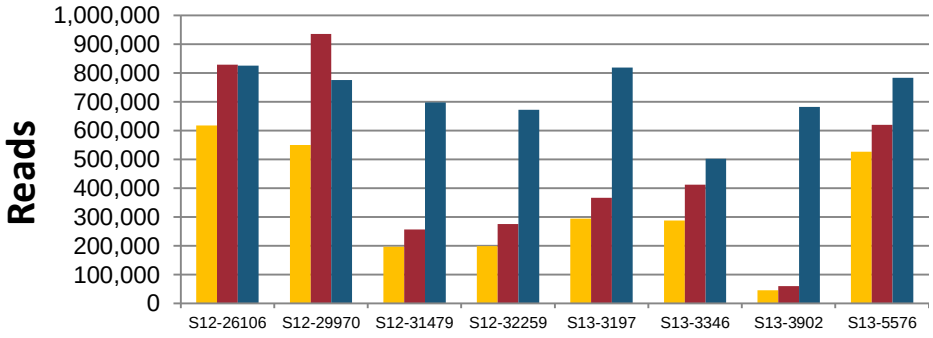
Competitor



(Customer provided data)

Next Gen Sequencing Affected by FFPE Nucleic Acid Input Mass

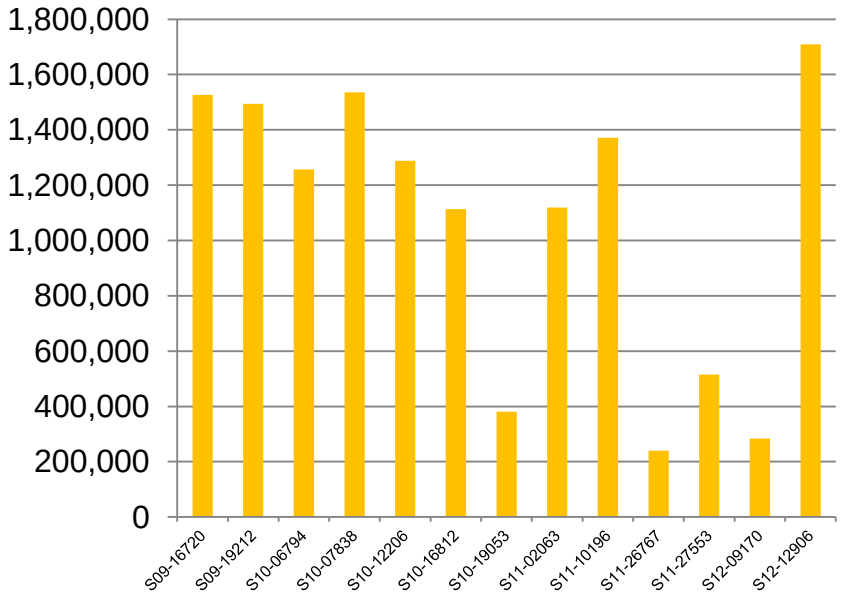
Input Amount Affects Success:
High Quality FFPE DNA Means Less may be Best



Maxwell (4µl)
Avg. 340,000

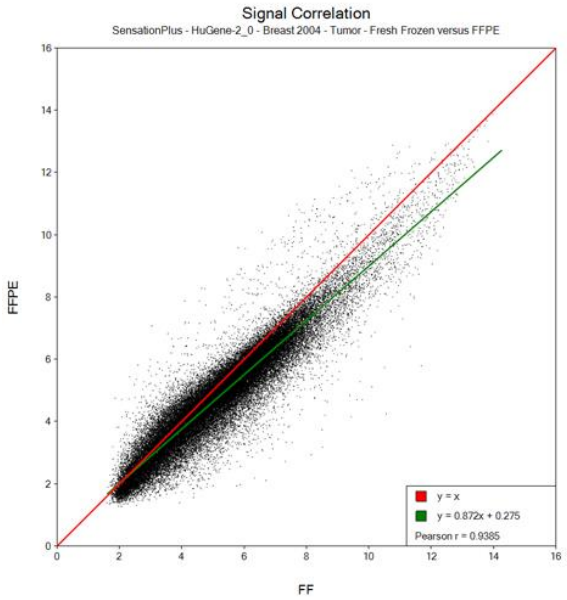
Maxwell (3µl)
Avg. 470,000

Competitor (4µl)
Avg. 719,000



Reduce Maxwell to 2µl Input
Avg. >1M reads

High Quality FFPE RNA Can Be Used for Gene Expression Profiling by Microarray



SensationPlus™ FFPE Amplification and WT Labeling Kit (Affymetrix) for whole-transcriptome analysis of FFPE samples with the GeneChip® human gene ST arrays

High correlation between fresh and FFPE tissue (breast tumor shown with GeneChip® 2.0 ST Array)

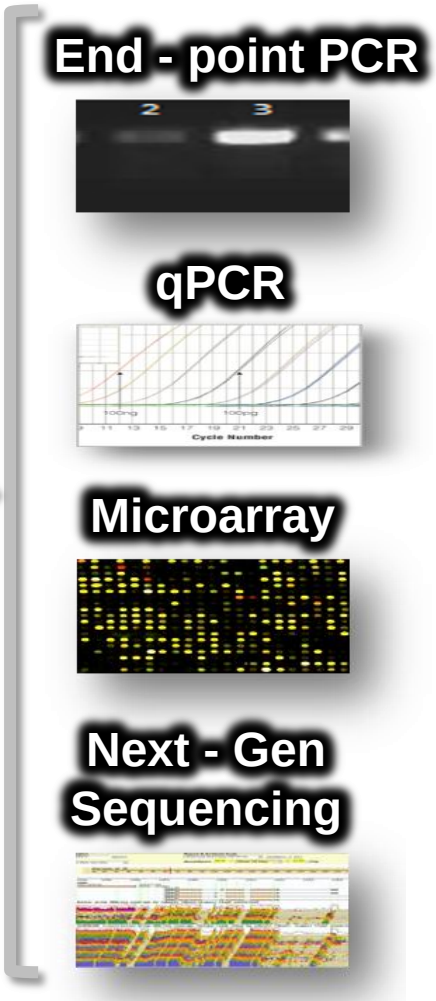
RNA isolation kits successfully validated with SensationPlus™ FFPE WT Reagent Kit	
Supplier	**Description**
Promega	ReliaPrep™ FFPE Total RNA Miniprep System

http://www.affymetrix.com/

Molecular Analysis Workflow

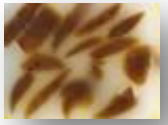
Assays

- Use robust enzymes and assays to maximize signal
- Optimize input considering quantitation method and quality of FFPE NA



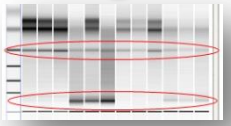
Key Tips for Successful Molecular Analysis of FFPE Samples

FFPE Samples



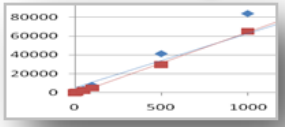
- Preserve samples immediately after collection
- Fixation (minimize time)

Purify NA



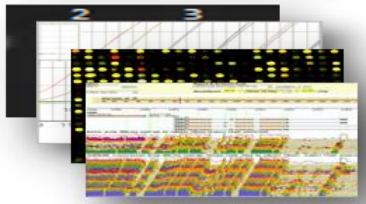
- Use NAP kits that maximize “functional” nucleic acid yield
- Optimized de-crosslinking time and temperature
- Effectively remove contaminants

Quantify



- Fluorescent dyes are ideal for low nucleic acid samples
- qPCR provides a functional assessment

Downstream Assays



- Use robust enzymes and assays to maximize signal
- Optimize input considering quantitation method and quality of FFPE nucleic acid

Promega Field Applications Specialists Are Your Local Promega Resource

Please Contact Us to Obtain Contact Information for Your Local FAS

United States: 800-356-9526

Canada: 608-274-4330

For Countries Outside North America, Please Visit:

http://www.promega.com/support/worldwide-contacts/

Technical Services Scientists Are Ready to Help

By phone: 800-356-9526

- Available 6am-5pm MST M-F

By Online Chat @ promega.com

- Available 6am-5pm MST M-F
- Global Chat with Branch office tech serv scientists, too, after hours (language dependent)

By email: techserv@promega.com

- Guaranteed answers within 24hr
- Most responses within 2hrs

North American Branch Technical Support Team