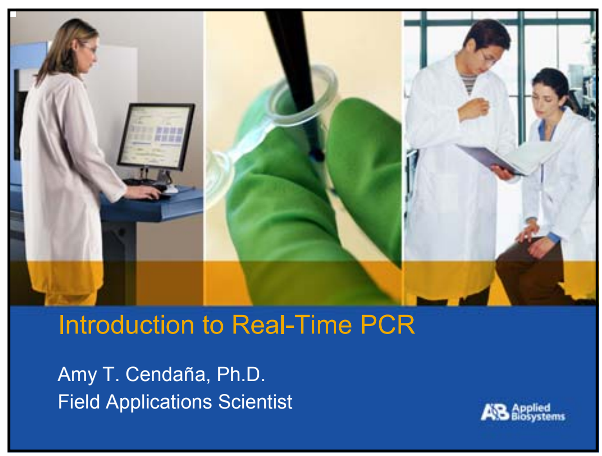
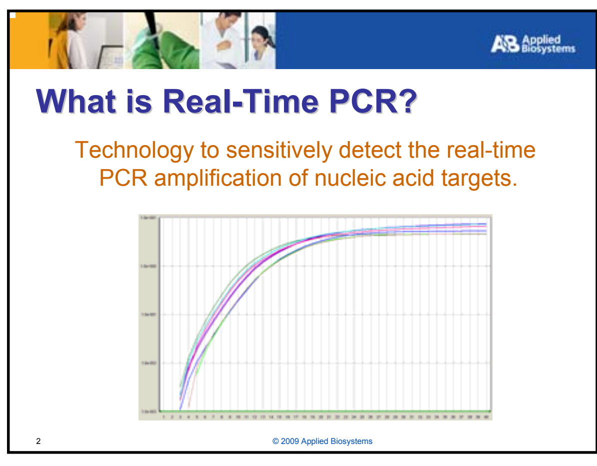
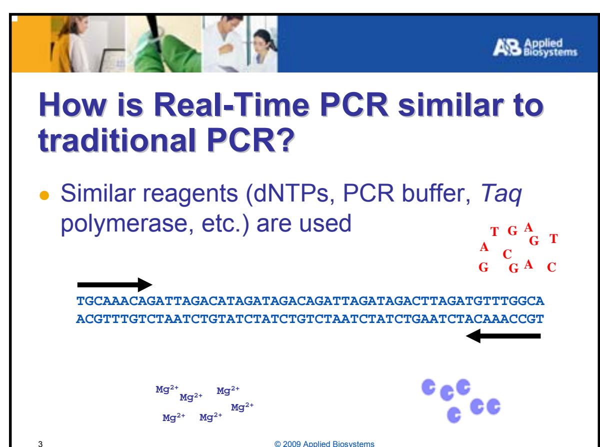


Introduction to Real-Time PCR
Amy T. Cendaña, Ph.D.
Field Applications Scientist
Applied Biosystems

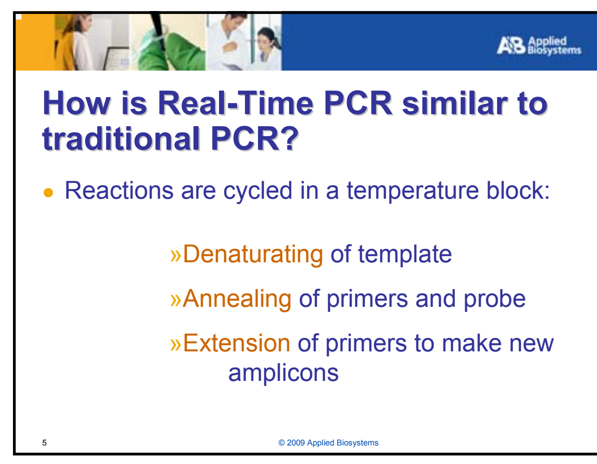


Applied Biosystems
What is Real-Time PCR?
Technology to sensitively detect the real-time PCR amplification of nucleic acid targets.
2
© 2009 Applied Biosystems

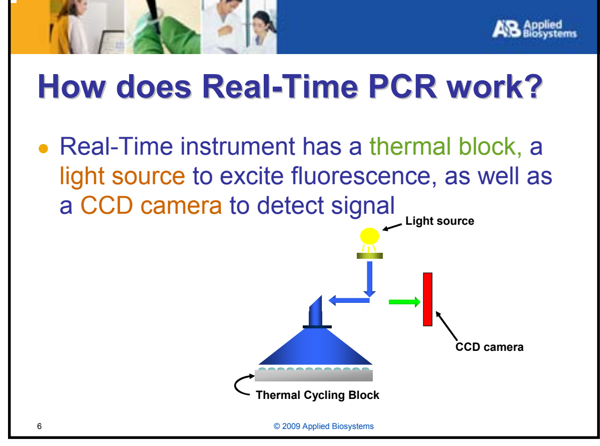


Applied Biosystems
How is Real-Time PCR similar to traditional PCR?
Similar reagents (dNTPs, PCR buffer, Taq polymerase, etc.) are used
TGCAAACAGATTAGACATAGATAGACAGATTAGATAGACTTAGATGTTTGGCA
ACGTTTGTCTAATCTGTATCTATCTGTCTAATCTATCTGAATCTACAAACCGT
3
© 2009 Applied Biosystems

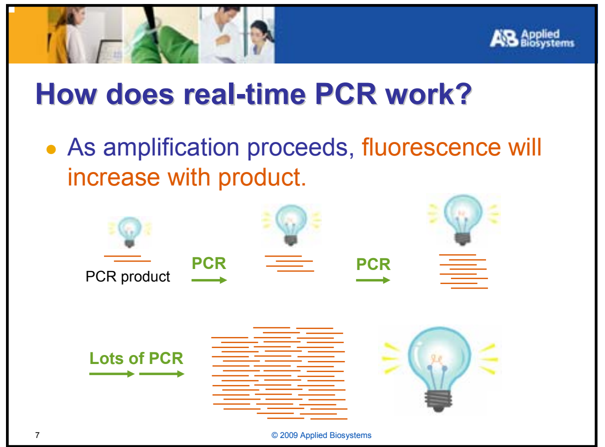
Applied Biosystems
How does Real-Time PCR work?
Some form of fluorescence is added to the PCR mix
4
© 2009 Applied Biosystems



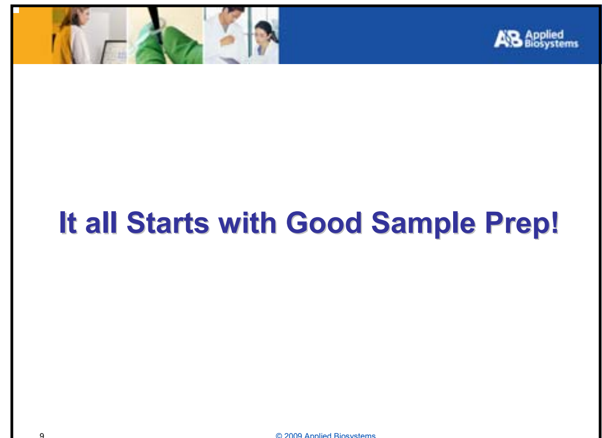
Applied Biosystems
How is Real-Time PCR similar to traditional PCR?
Reactions are cycled in a temperature block:
»Denaturating of template
»Annealing of primers and probe
»Extension of primers to make new amplicons
5
© 2009 Applied Biosystems

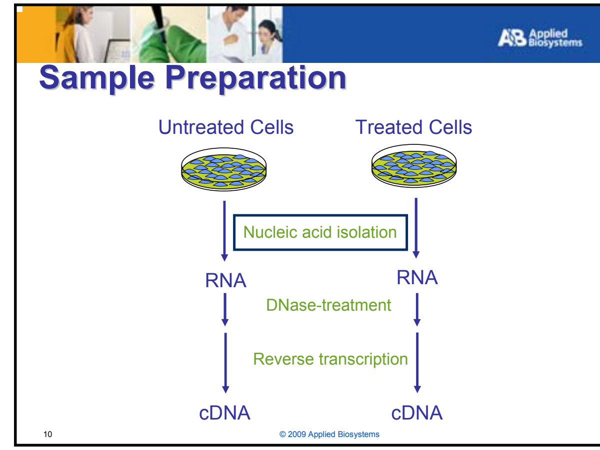


Applied Biosystems
How does Real-Time PCR work?
Real-Time instrument has a thermal block, a light source to excite fluorescence, as well as a CCD camera to detect signal
Light source
CCD camera
Thermal Cycling Block
6
© 2009 Applied Biosystems

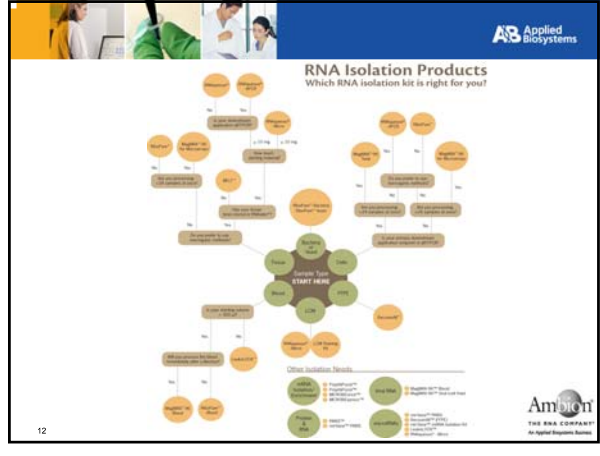

How does real-time PCR work?
As amplification proceeds, fluorescence will increase with product.
PCR product
PCR
PCR
Lots of PCR
7
© 2009 Applied Biosystems

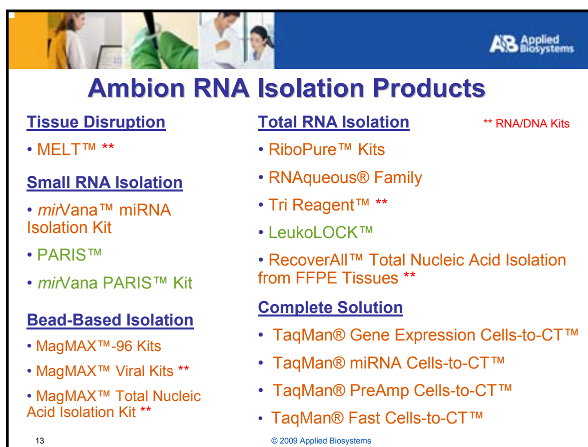
How does Real-Time PCR work?
Finally, data are presented graphically for easy analysis
8
© 2009 Applied Biosystems

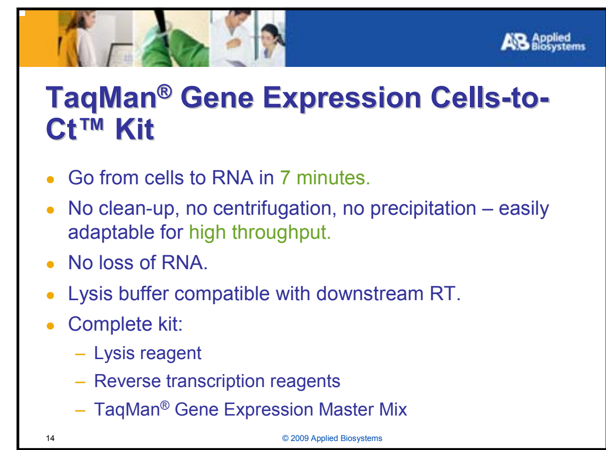

It all Starts with Good Sample Prep!
9
© 2009 Applied Biosystems

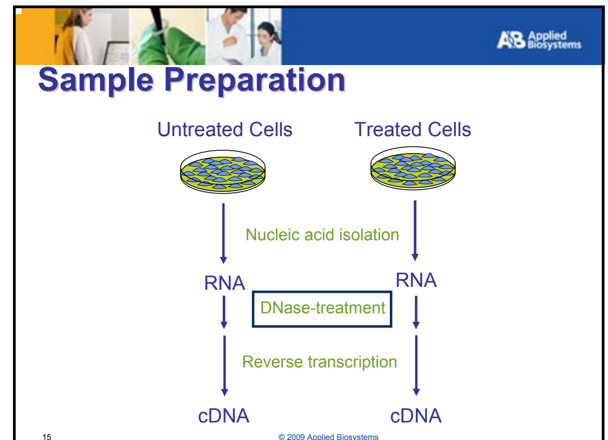

Sample Preparation
Untreated Cells
Treated Cells
Nucleic acid isolation
RNA
RNA
DNase-treatment
Reverse transcription
cDNA
cDNA
10
© 2009 Applied Biosystems

Ambion : The RNA Company
Ambion
Maximum results. Minimum effort.
11
© 2009 Applied Biosystems

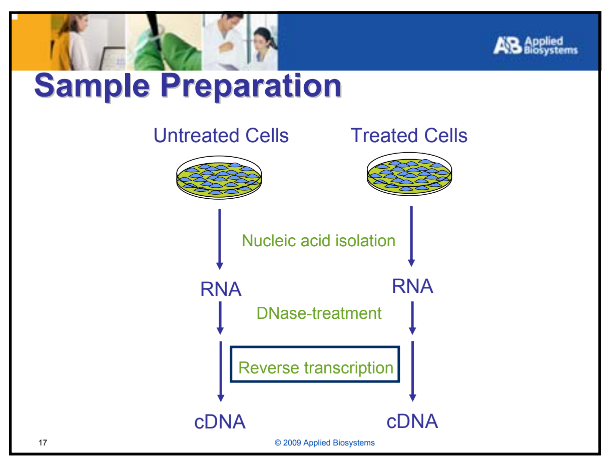

RNA Isolation Products
Which RNA isolation kit is right for you?
12


Applied Biosystems
Ambion RNA Isolation Products
Tissue Disruption
• MELT™ **
Small RNA Isolation
• mirVana™ miRNA Isolation Kit
• PARIS™
• mirVana PARIS™ Kit
Bead-Based Isolation
• MagMAX™-96 Kits
• MagMAX™ Viral Kits **
• MagMAX™ Total Nucleic Acid Isolation Kit **
Total RNA Isolation
** RNA/DNA Kits
• RiboPure™ Kits
• RNAqueous® Family
• Tri Reagent™ **
• LeukoLOCK™
• RecoverAll™ Total Nucleic Acid Isolation from FFPE Tissues **
Complete Solution
• TaqMan® Gene Expression Cells-to-CT™
• TaqMan® miRNA Cells-to-CT™
• TaqMan® PreAmp Cells-to-CT™
• TaqMan® Fast Cells-to-CT™
13
© 2009 Applied Biosystems

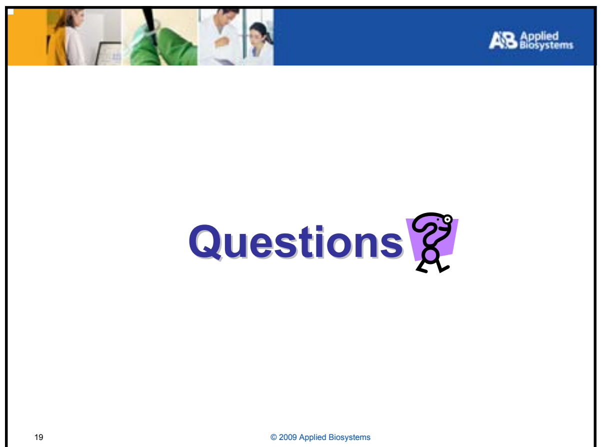

Applied Biosystems
TaqMan® Gene Expression Cells-to-Ct™ Kit
• Go from cells to RNA in 7 minutes.
• No clean-up, no centrifugation, no precipitation – easily adaptable for high throughput.
• No loss of RNA.
• Lysis buffer compatible with downstream RT.
• Complete kit:
– Lysis reagent
– Reverse transcription reagents
– TaqMan® Gene Expression Master Mix
14
© 2009 Applied Biosystems

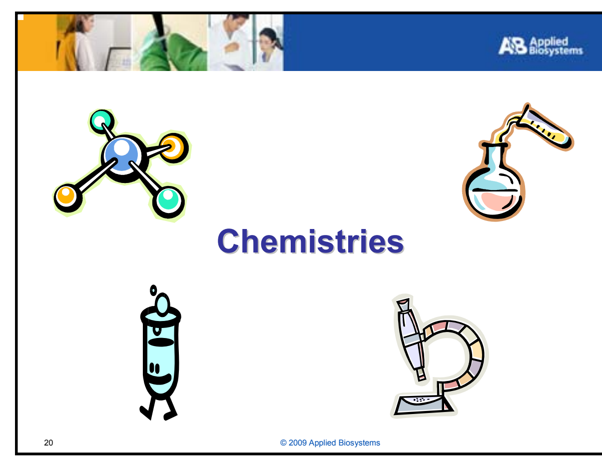

Applied Biosystems
Sample Preparation
Untreated Cells
Treated Cells
Nucleic acid isolation
RNA
RNA
DNase-treatment
Reverse transcription
cDNA
cDNA
15
© 2009 Applied Biosystems

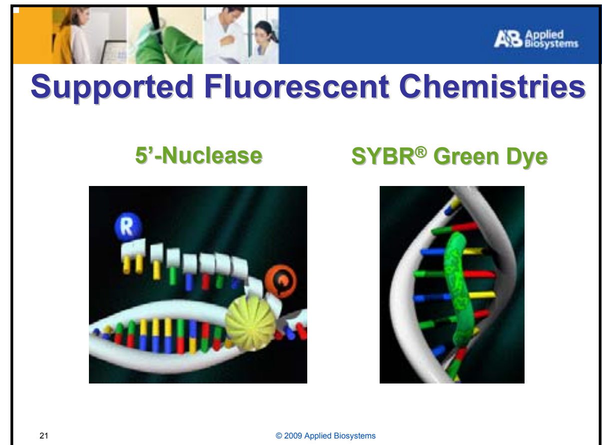
Applied Biosystems
TURBO DNA-free™: gentle on RNA
TURBO DNA-free™
16
© 2009 Applied Biosystems

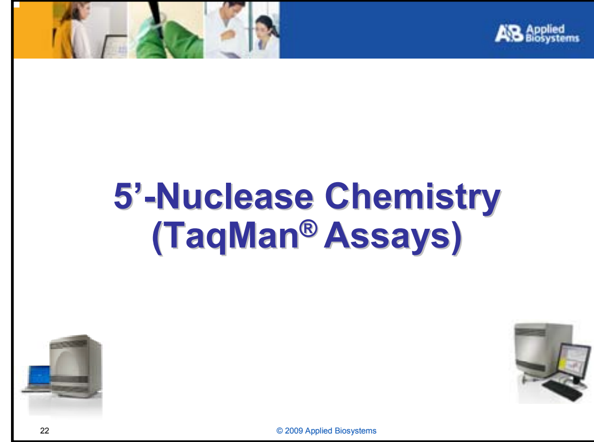

Applied Biosystems
Sample Preparation
Untreated Cells
Treated Cells
Nucleic acid isolation
RNA
RNA
DNase-treatment
Reverse transcription
cDNA
cDNA
17
© 2009 Applied Biosystems

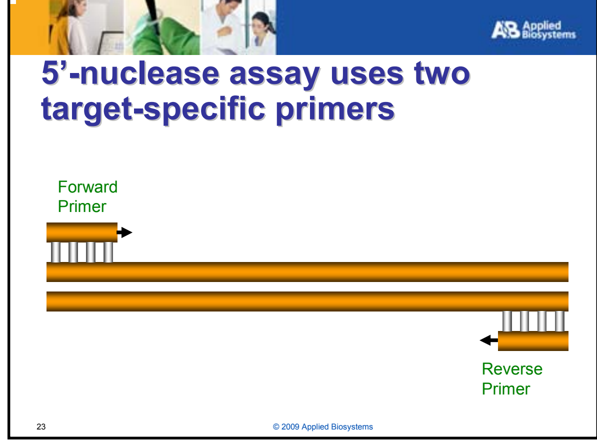
Applied Biosystems
High Capacity RNA-to-cDNA Master Mix
Single-tube first strand synthesis:
• Very high efficiency – excellent conversion rate.
• Broad dynamic range – reproducible results over a large concentration range.
• Convenient 5x formulation.
• Optional no-RT control formulation tube.
• Liquid master mix at -20° – eliminates freeze/thaw cycles.
18
© 2009 Applied Biosystems

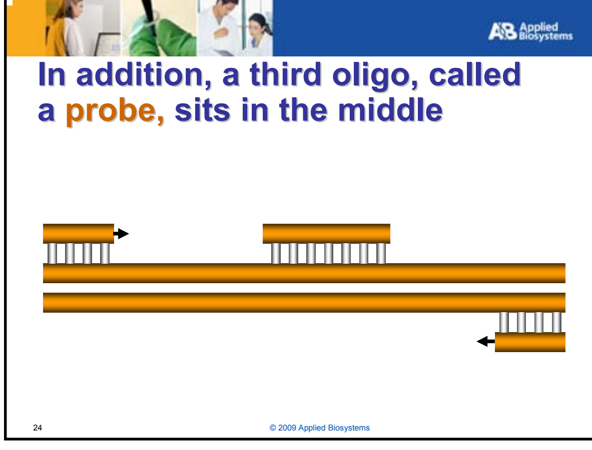

Applied Biosystems
Questions
19
© 2009 Applied Biosystems

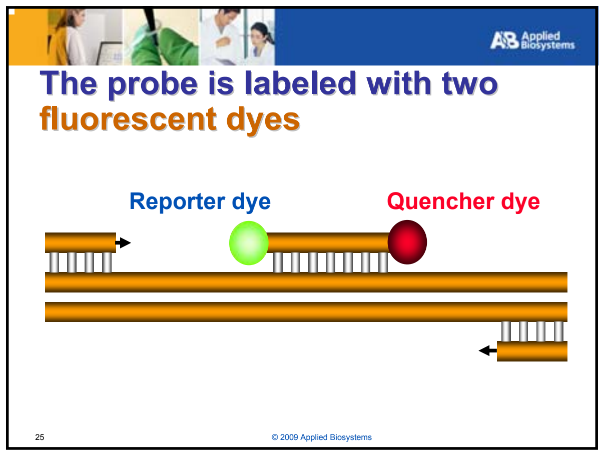

Applied Biosystems
Chemistries
20
© 2009 Applied Biosystems

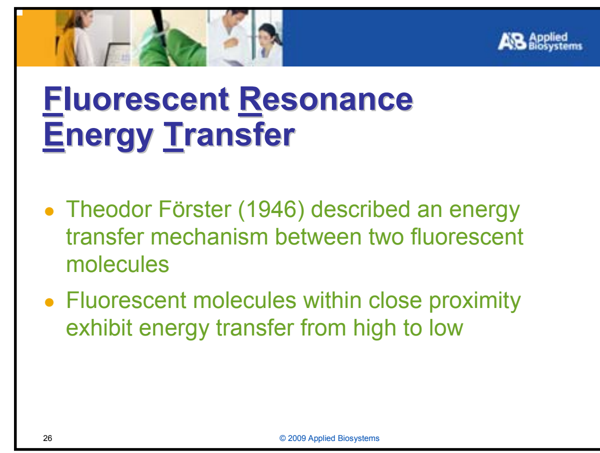

Applied Biosystems
Supported Fluorescent Chemistries
5'-Nuclease
SYBR® Green Dye
21
© 2009 Applied Biosystems

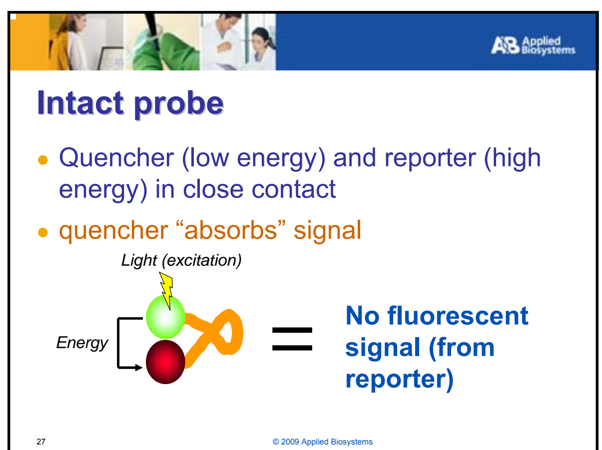

Applied Biosystems
5'-Nuclease Chemistry
(TaqMan® Assays)
22
© 2009 Applied Biosystems

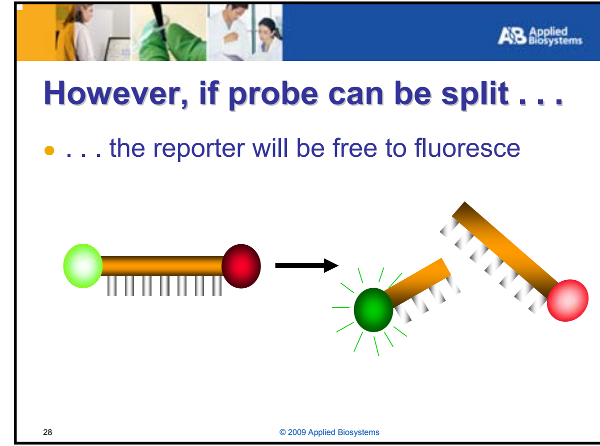

Applied Biosystems
5'-nuclease assay uses two target-specific primers
Forward Primer
Reverse Primer
23
© 2009 Applied Biosystems

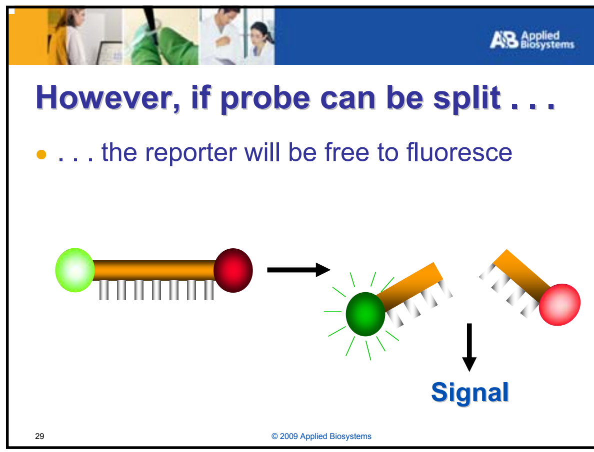

Applied Biosystems
In addition, a third oligo, called a probe, sits in the middle
24
© 2009 Applied Biosystems

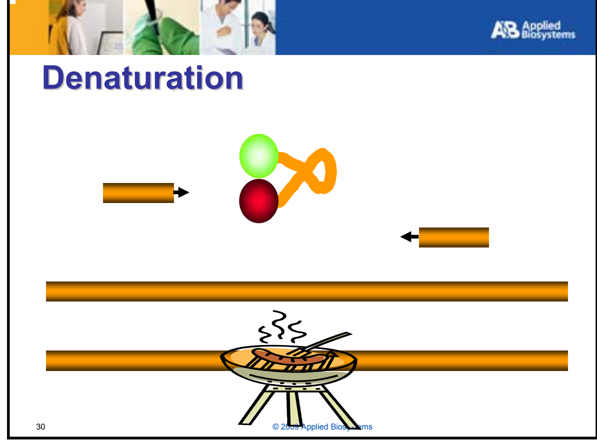

The probe is labeled with two fluorescent dyes
Reporter dye
Quencher dye
25
© 2009 Applied Biosystems

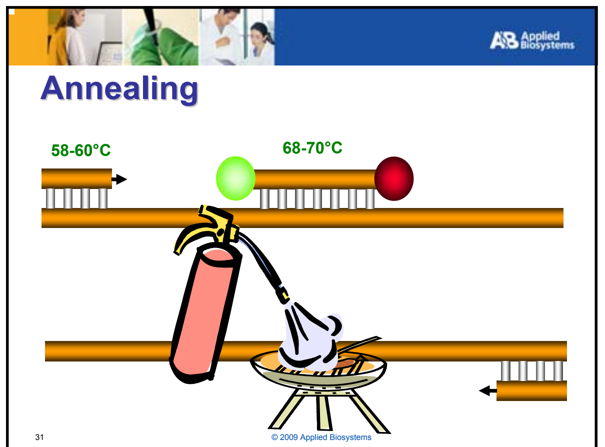

Fluorescent Resonance Energy Transfer
Theodor Förster (1946) described an energy transfer mechanism between two fluorescent molecules
Fluorescent molecules within close proximity exhibit energy transfer from high to low
26
© 2009 Applied Biosystems

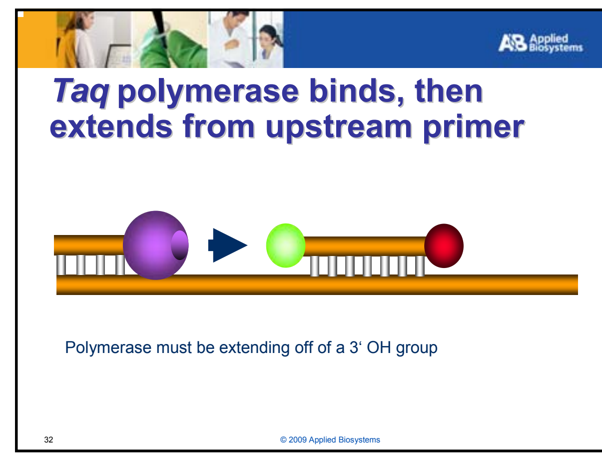

Intact probe
Quencher (low energy) and reporter (high energy) in close contact
quencher "absorbs" signal
Light (excitation)
Energy
No fluorescent signal (from reporter)
27
© 2009 Applied Biosystems

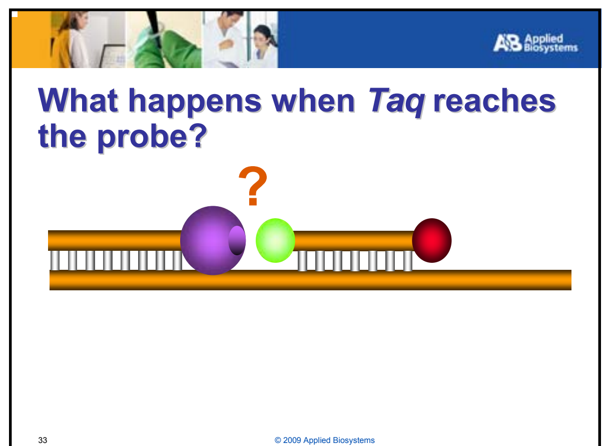

However, if probe can be split . . .
. . . the reporter will be free to fluoresce
28
© 2009 Applied Biosystems

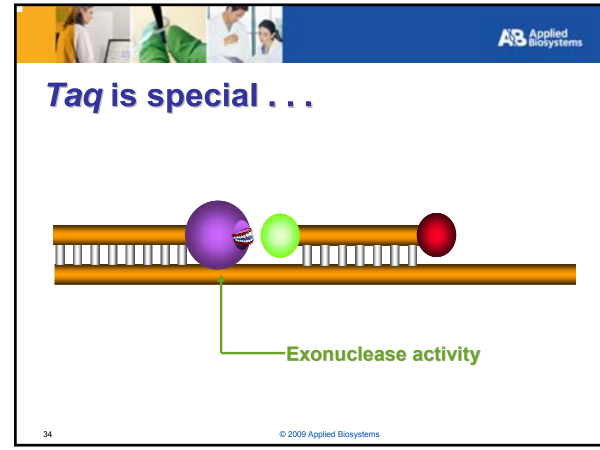

However, if probe can be split . . .
. . . the reporter will be free to fluoresce
Signal
29
© 2009 Applied Biosystems


Denaturation
30

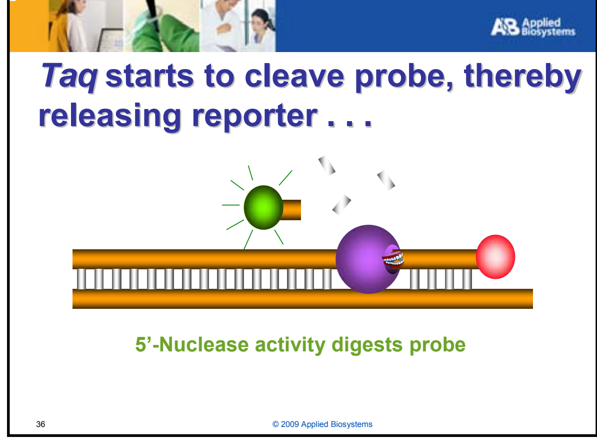

Applied Biosystems
Annealing
58-60°C
68-70°C
31
© 2009 Applied Biosystems

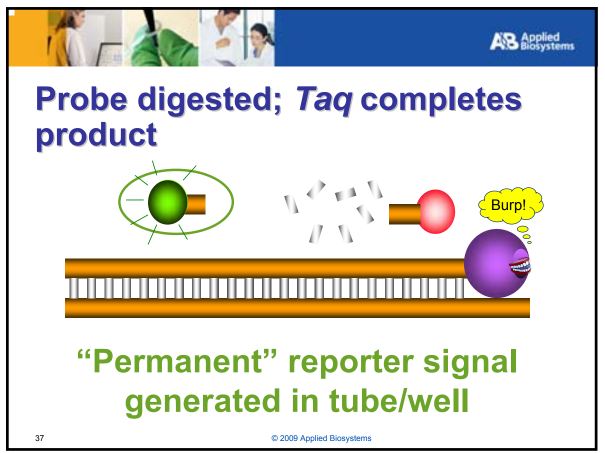

Applied Biosystems
Taq polymerase binds, then extends from upstream primer
Polymerase must be extending off of a 3' OH group
32
© 2009 Applied Biosystems

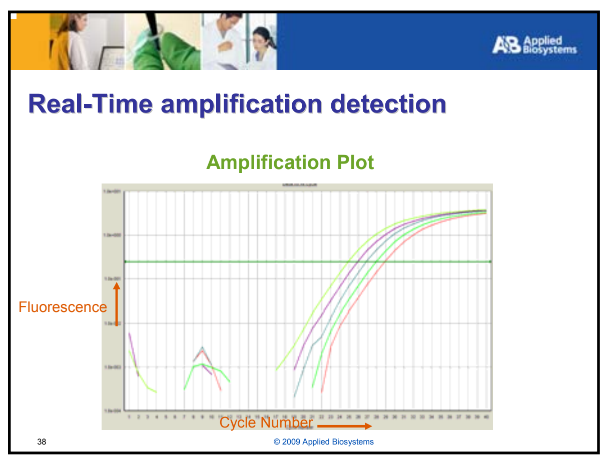

Applied Biosystems
What happens when Taq reaches the probe?
?
33
© 2009 Applied Biosystems

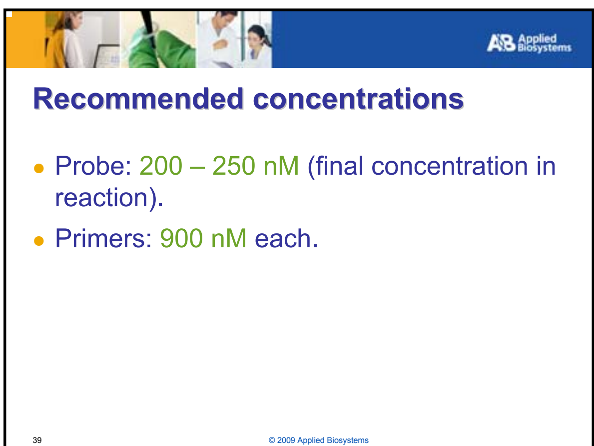

Applied Biosystems
Taq is special . . .
Exonuclease activity
34
© 2009 Applied Biosystems

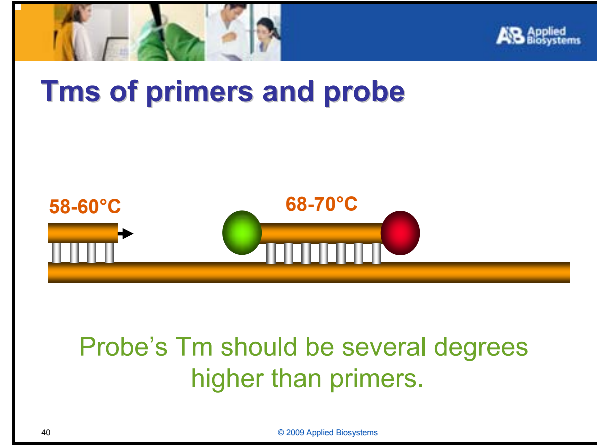
Applied Biosystems
What happens when Taq reaches the probe?
Probe will get digested*
*Must be associated with a 5' hydrogen bond
35
© 2009 Applied Biosystems

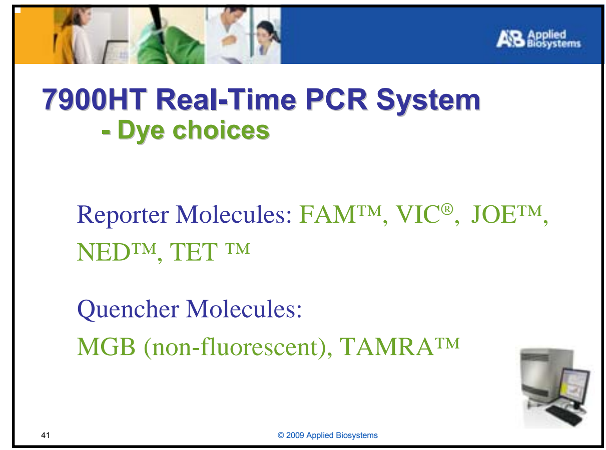

Applied Biosystems
Taq starts to cleave probe, thereby releasing reporter . . .
5'-Nuclease activity digests probe
36
© 2009 Applied Biosystems


Probe digested; Taq completes product
Burp!
"Permanent" reporter signal generated in tube/well
37
© 2009 Applied Biosystems

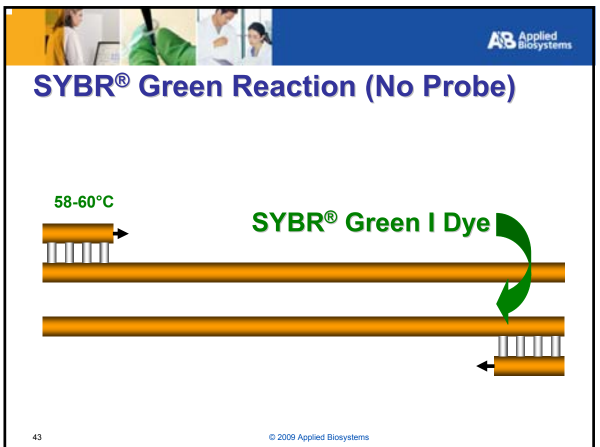

Real-Time amplification detection
Amplification Plot
Fluorescence
Cycle Number
38
© 2009 Applied Biosystems

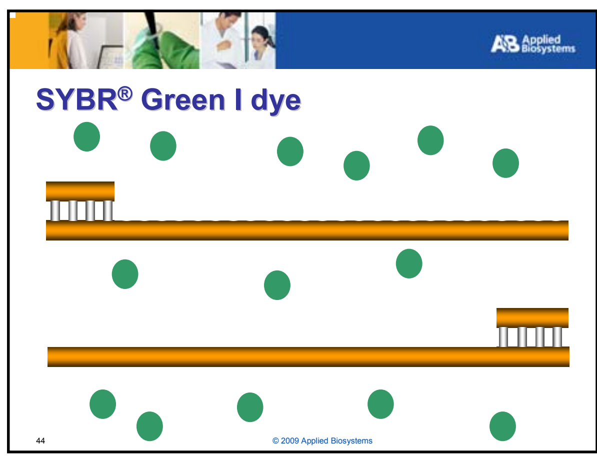

Recommended concentrations
Probe: 200 – 250 nM (final concentration in reaction).
Primers: 900 nM each.
39
© 2009 Applied Biosystems

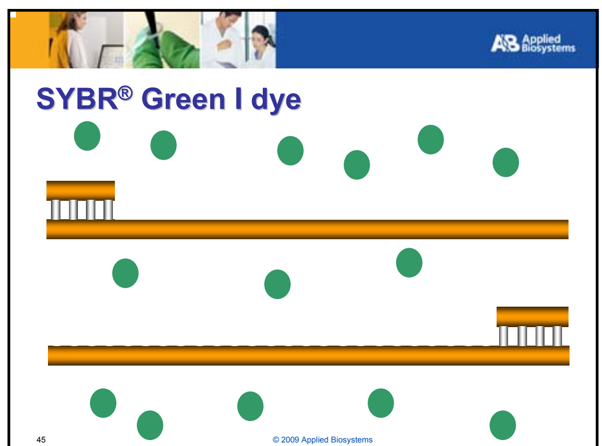

Tms of primers and probe
58-60°C
68-70°C
Probe's Tm should be several degrees higher than primers.
40
© 2009 Applied Biosystems

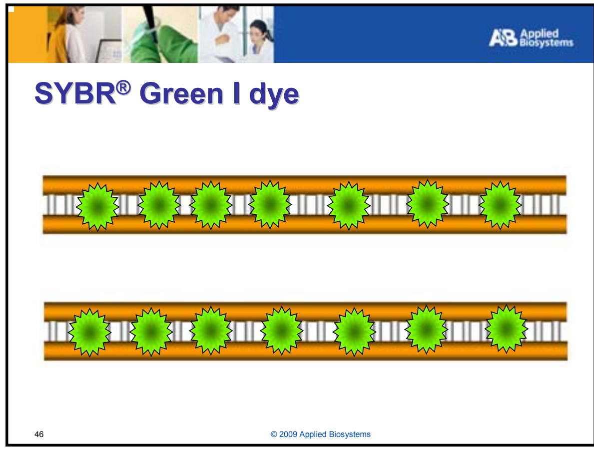

7900HT Real-Time PCR System
- Dye choices
Reporter Molecules: FAM™, VIC®, JOE™, NED™, TET ™
Quencher Molecules:
MGB (non-fluorescent), TAMRA™
41
© 2009 Applied Biosystems

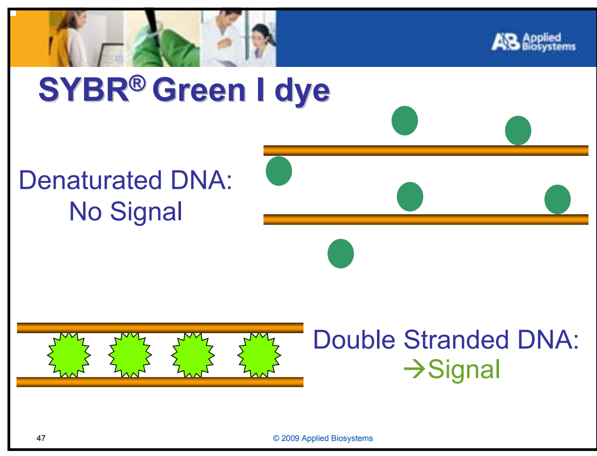
SYBR® Green I Reaction
42
© 2009 Applied Biosystems

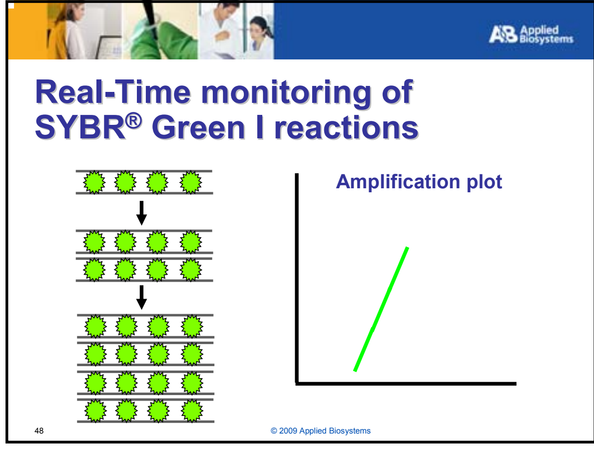

Applied Biosystems
SYBR® Green Reaction (No Probe)
58-60°C
SYBR® Green I Dye
43
© 2009 Applied Biosystems

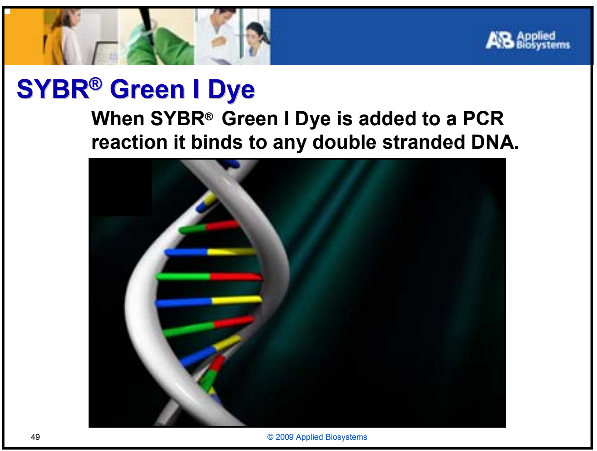

Applied Biosystems
SYBR® Green I dye
44
© 2009 Applied Biosystems


Applied Biosystems
SYBR® Green I dye
45
© 2009 Applied Biosystems


Applied Biosystems
SYBR® Green I dye
46
© 2009 Applied Biosystems


Applied Biosystems
SYBR® Green I dye
Denaturated DNA:
No Signal
Double Stranded DNA:
→Signal
47
© 2009 Applied Biosystems


Applied Biosystems
Real-Time monitoring of
SYBR® Green I reactions
Amplification plot
48
© 2009 Applied Biosystems


Applied Biosystems
SYBR® Green I Dye
When SYBR® Green I Dye is added to a PCR reaction it binds to any double stranded DNA.
49
© 2009 Applied Biosystems

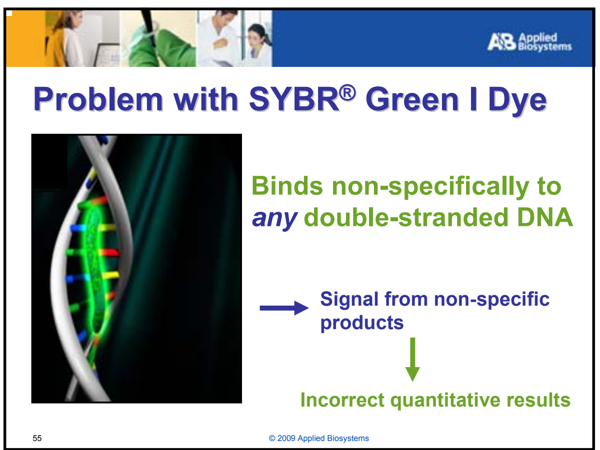
Applied Biosystems
SYBR® Green I Dye
50
© 2009 Applied Biosystems

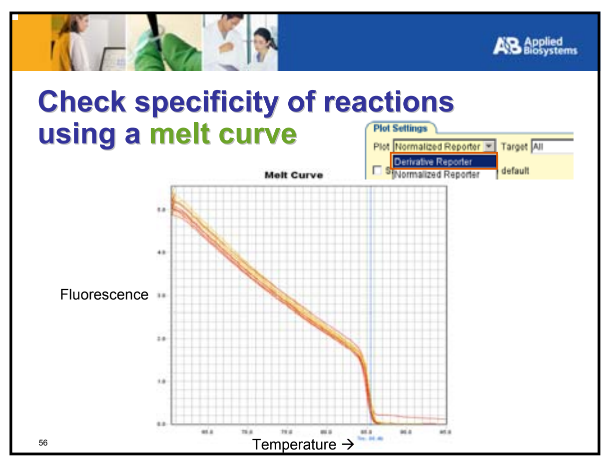
Applied Biosystems
SYBR® Green I Dye
51
© 2009 Applied Biosystems

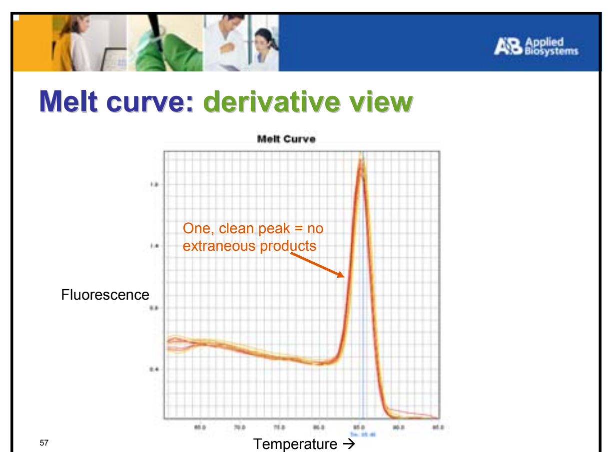
Applied Biosystems
SYBR® Green I Dye
52
© 2009 Applied Biosystems

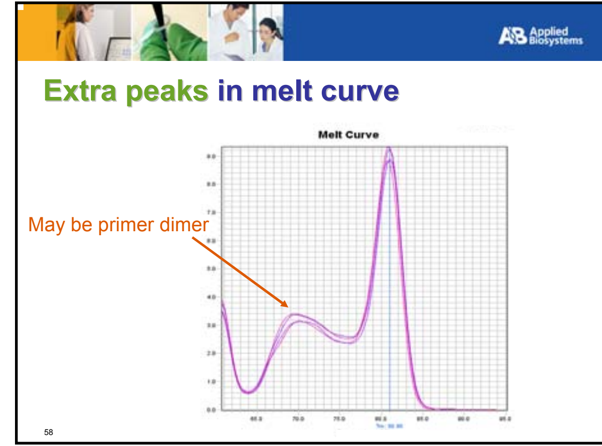
Applied Biosystems
SYBR® Green I Dye
53
© 2009 Applied Biosystems

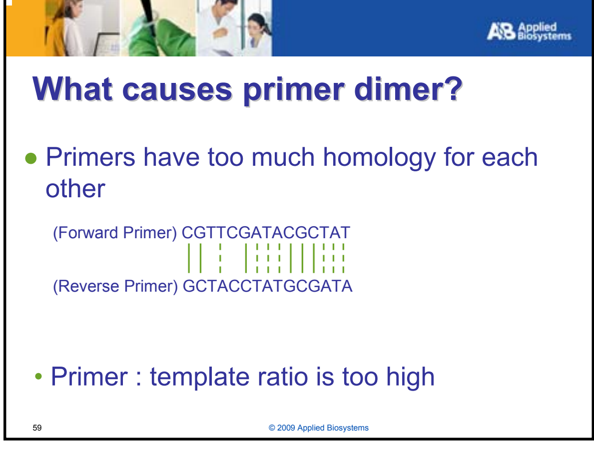
Applied Biosystems
SYBR® Green I Dye
54
© 2009 Applied Biosystems

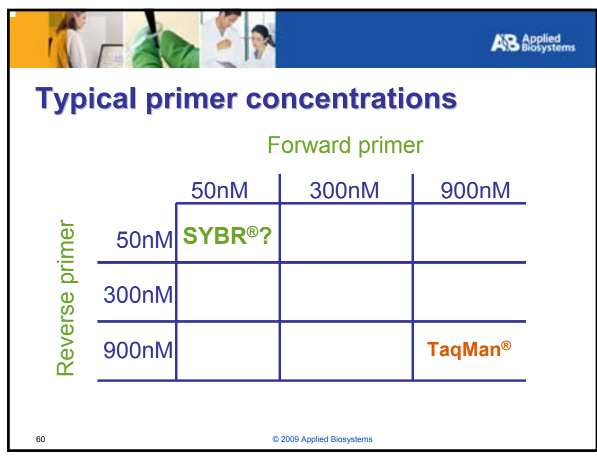

Applied Biosystems
Problem with SYBR® Green I Dye
Binds non-specifically to any double-stranded DNA
Signal from non-specific products
Incorrect quantitative results
55
© 2009 Applied Biosystems


Applied Biosystems
Check specificity of reactions using a melt curve
Plot Settings
Plot Normalized Reporter
Derivative Reporter
Normalized Reporter
Target All
default
Melt Curve
Fluorescence
Temperature →
56


Applied Biosystems
Melt curve: derivative view
Melt Curve
One, clean peak = no extraneous products
Fluorescence
Temperature →
57


Applied Biosystems
Extra peaks in melt curve
Melt Curve
May be primer dimer
58


Applied Biosystems
What causes primer dimer?
Primers have too much homology for each other
(Forward Primer) CGTTCGATACGCTAT
(Reverse Primer) GCTACCTATGCGATA
Primer : template ratio is too high
59
© 2009 Applied Biosystems


Applied Biosystems
Typical primer concentrations
Forward primer
Reverse primer
50nM
300nM
900nM
50nM
SYBR®?
300nM
900nM
TaqMan®
60
© 2009 Applied Biosystems

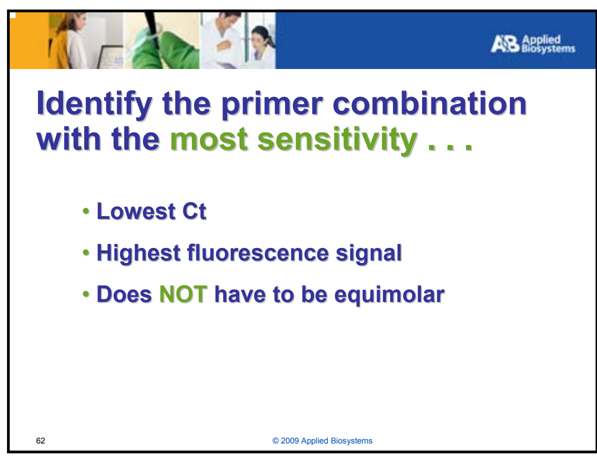
Applied Biosystems

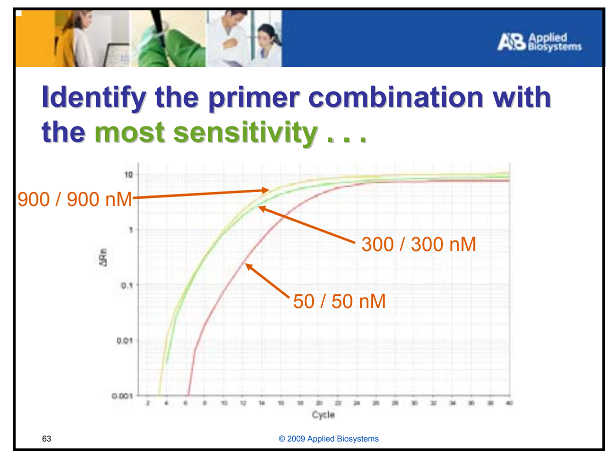
Run a primer matrix

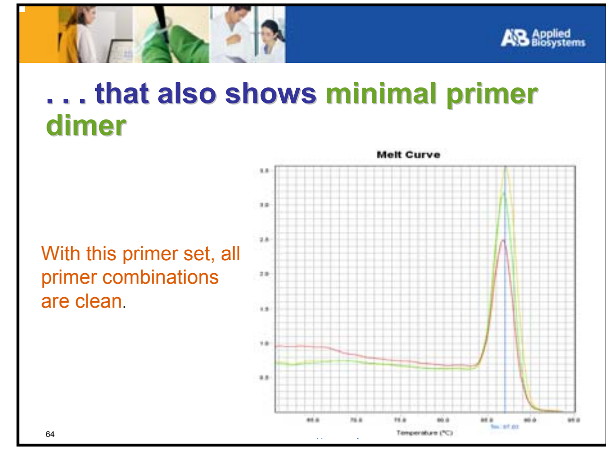
Forward primer (columns) / Reverse primer (rows)

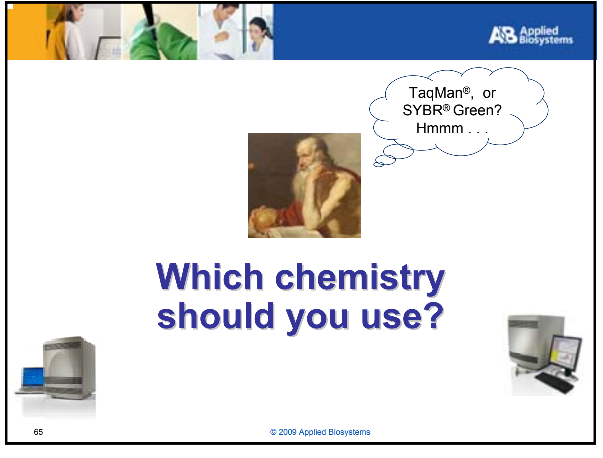
Reverse primer	50nM	300nM	900nM
50nM	X		
300nM		X	
900nM			X

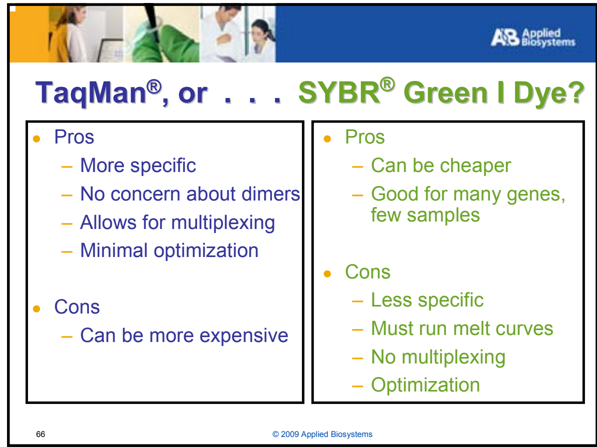
© 2009 Applied Biosystems

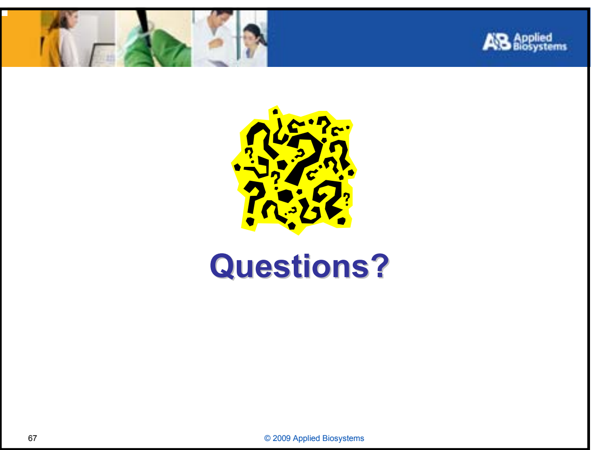


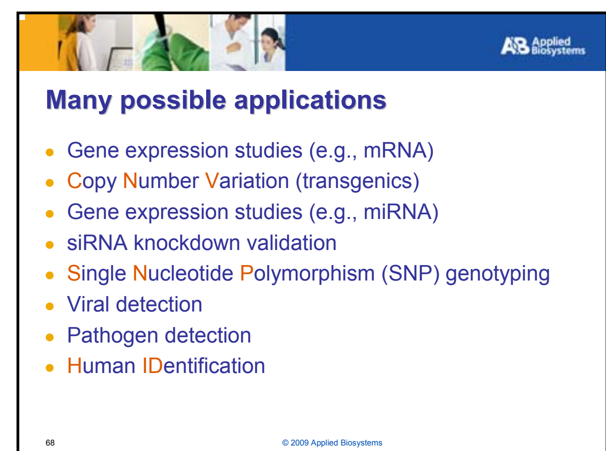


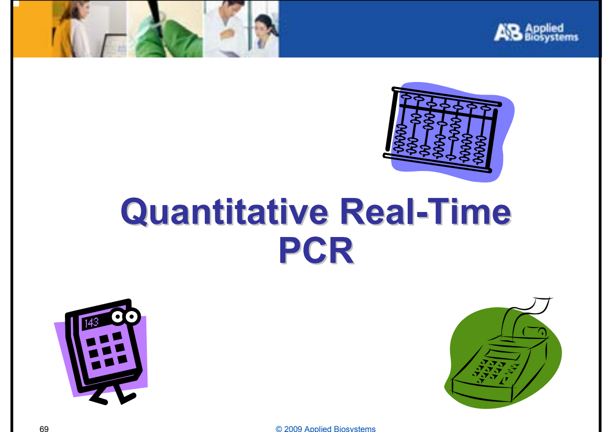


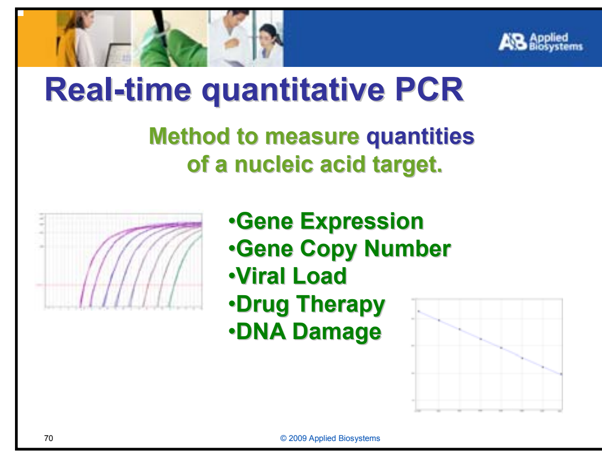


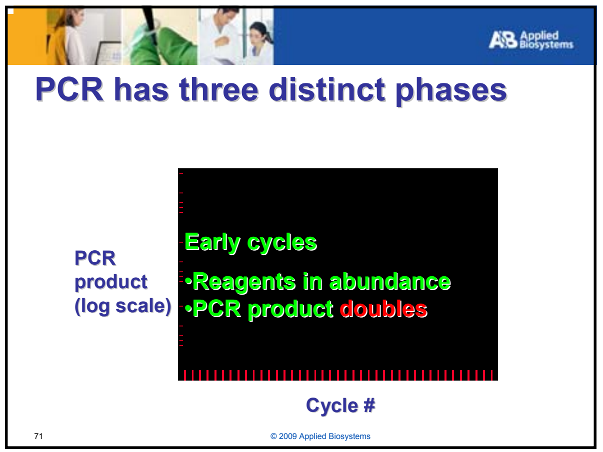


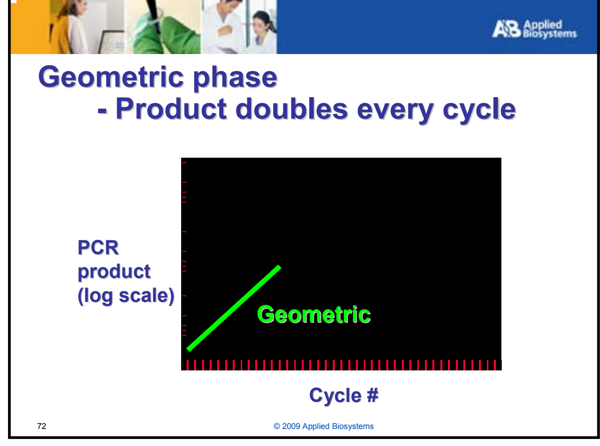

Applied Biosystems
Questions?
67
© 2009 Applied Biosystems

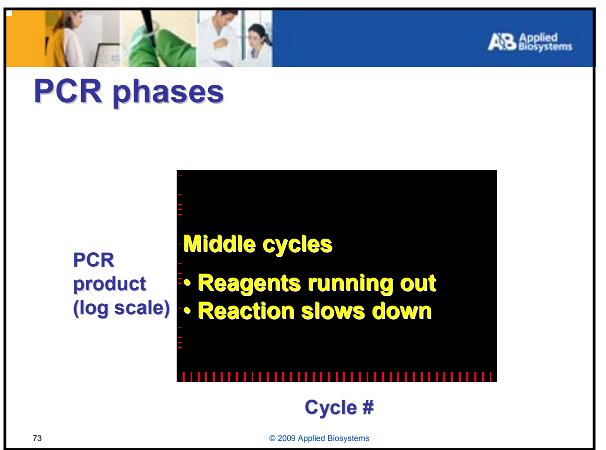

Applied Biosystems
Many possible applications
Gene expression studies (e.g., mRNA)
Copy Number Variation (transgenics)
Gene expression studies (e.g., miRNA)
siRNA knockdown validation
Single Nucleotide Polymorphism (SNP) genotyping
Viral detection
Pathogen detection
Human IDentification
68
© 2009 Applied Biosystems

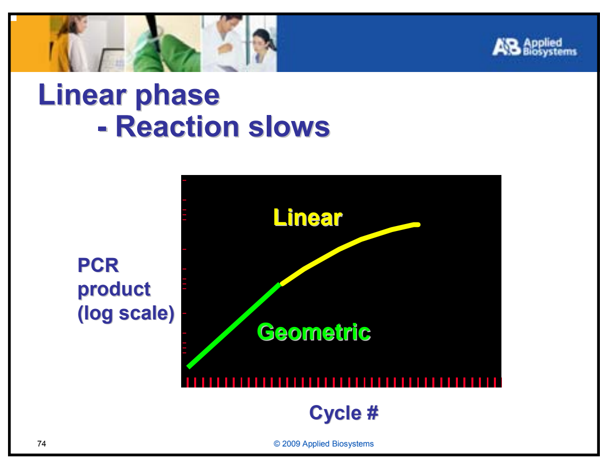

Applied Biosystems
Quantitative Real-Time PCR
69
© 2009 Applied Biosystems

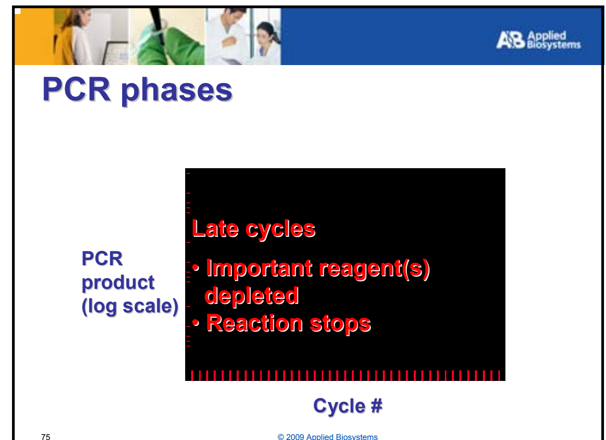

Applied Biosystems
Real-time quantitative PCR
Method to measure quantities of a nucleic acid target.
•Gene Expression
•Gene Copy Number
•Viral Load
•Drug Therapy
•DNA Damage
70
© 2009 Applied Biosystems

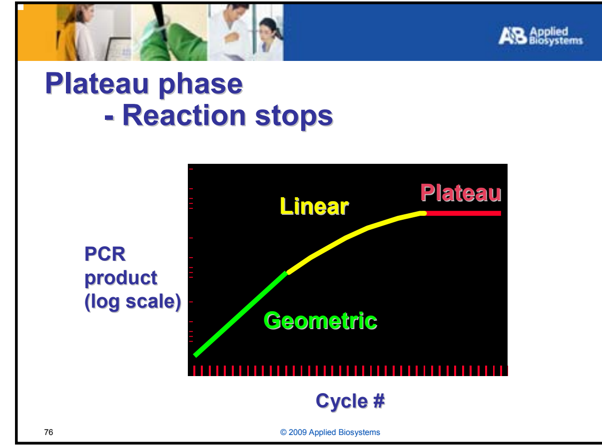

Applied Biosystems
PCR has three distinct phases
PCR product (log scale)
Early cycles
•Reagents in abundance
•PCR product doubles
Cycle #
71
© 2009 Applied Biosystems

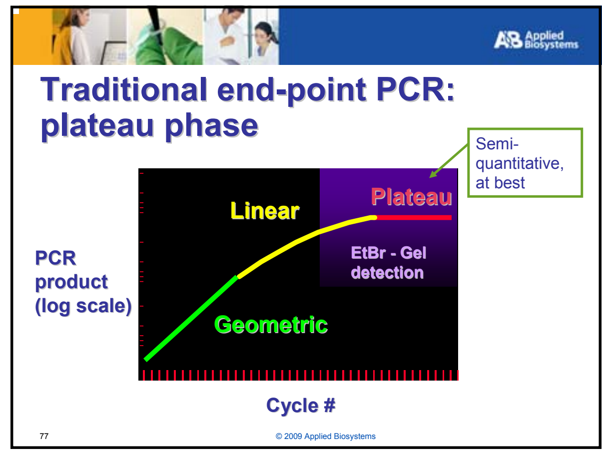

Applied Biosystems
Geometric phase
- Product doubles every cycle
PCR product (log scale)
Geometric
Cycle #
72
© 2009 Applied Biosystems

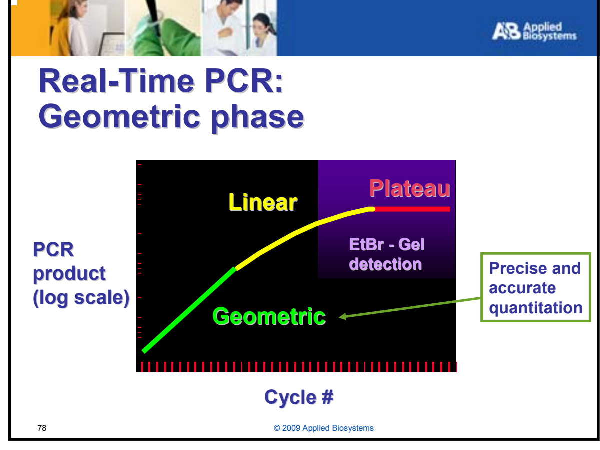

Applied Biosystems
PCR phases
PCR product (log scale)
Middle cycles
• Reagents running out
• Reaction slows down
Cycle #
73
© 2009 Applied Biosystems

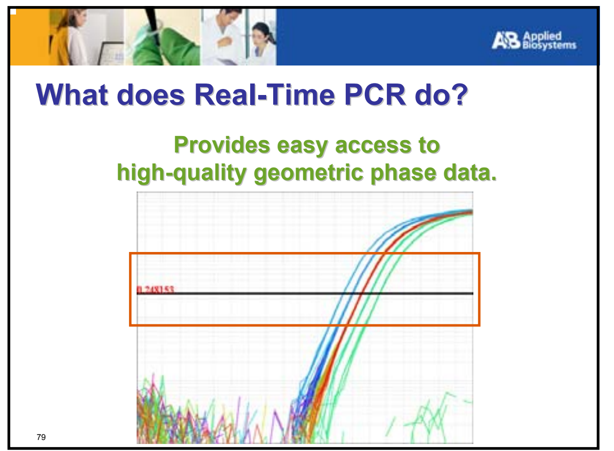

Applied Biosystems
Linear phase
- Reaction slows
PCR product (log scale)
Linear
Geometric
Cycle #
74
© 2009 Applied Biosystems


Applied Biosystems
PCR phases
PCR product (log scale)
Late cycles
• Important reagent(s) depleted
• Reaction stops
Cycle #
75
© 2009 Applied Biosystems

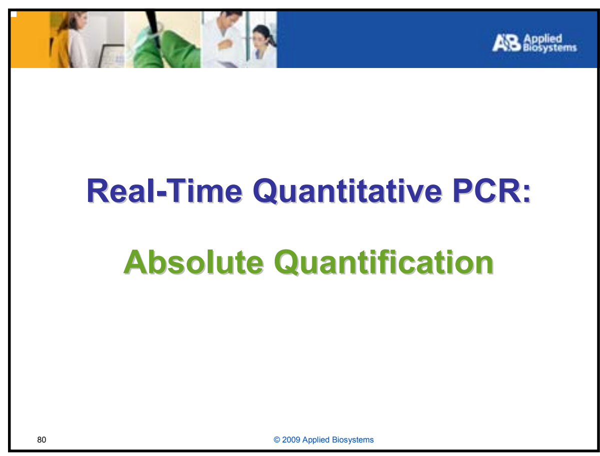

Applied Biosystems
Plateau phase
- Reaction stops
PCR product (log scale)
Linear
Plateau
Geometric
Cycle #
76
© 2009 Applied Biosystems


Applied Biosystems
Traditional end-point PCR: plateau phase
Semi-quantitative, at best
PCR product (log scale)
Linear
Plateau
EtBr - Gel detection
Geometric
Cycle #
77
© 2009 Applied Biosystems

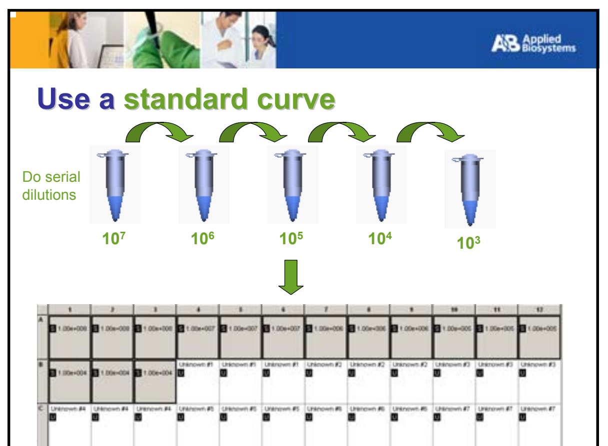

Applied Biosystems
Real-Time PCR: Geometric phase
PCR product (log scale)
Linear
Plateau
EtBr - Gel detection
Geometric
Precise and accurate quantitation
Cycle #
78
© 2009 Applied Biosystems



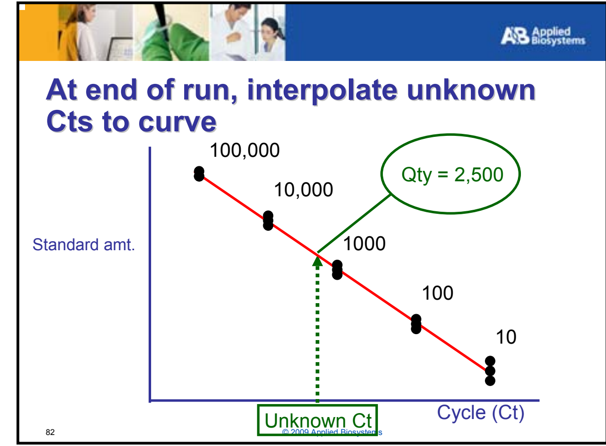
Applied Biosystems
What does Real-Time PCR do?
Provides easy access to
high-quality geometric phase data.
79



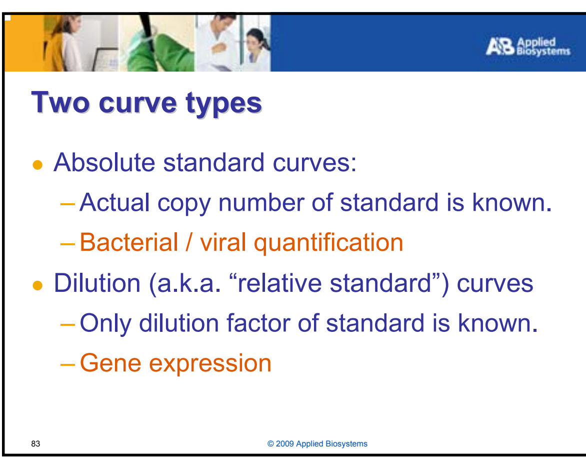
Applied Biosystems
Real-Time Quantitative PCR:
Absolute Quantification
80
© 2009 Applied Biosystems



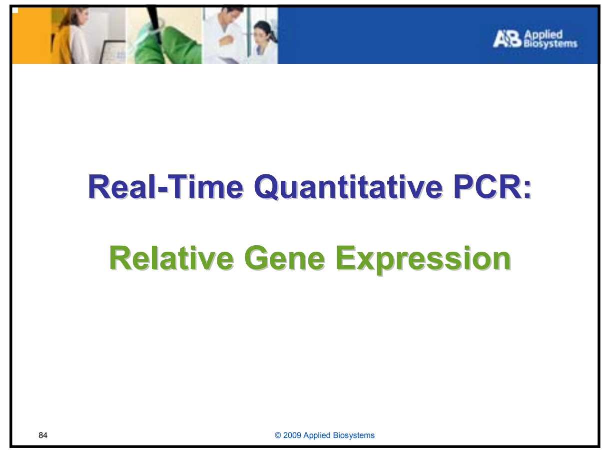
Applied Biosystems
Use a standard curve
Do serial dilutions
10⁷
10⁶
10⁵
10⁴
10³



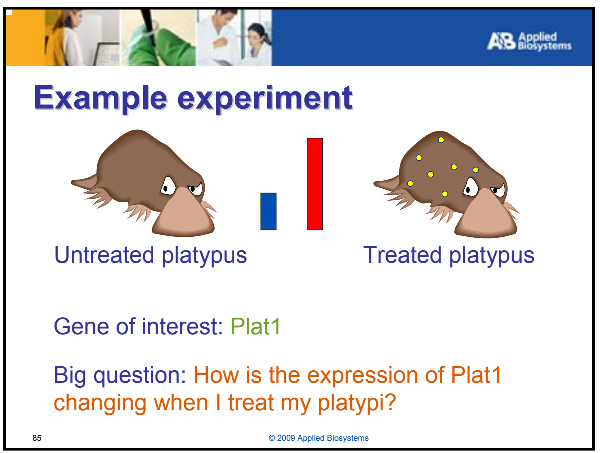
Applied Biosystems
At end of run, interpolate unknown Cts to curve
100,000
10,000
Qty = 2,500
Standard amt.
1000
100
10
Unknown Ct
Cycle (Ct)
82

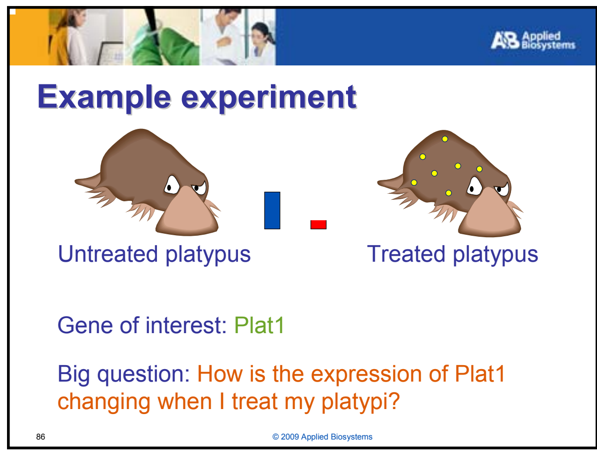


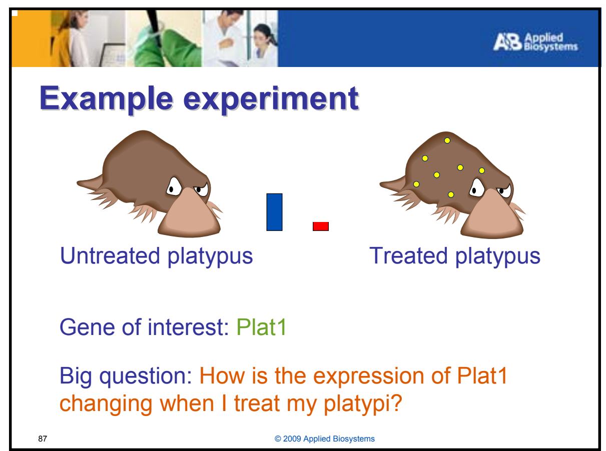
Applied Biosystems
Two curve types
• Absolute standard curves:
– Actual copy number of standard is known.
– Bacterial / viral quantification
• Dilution (a.k.a. “relative standard”) curves
– Only dilution factor of standard is known.
– Gene expression
83
© 2009 Applied Biosystems

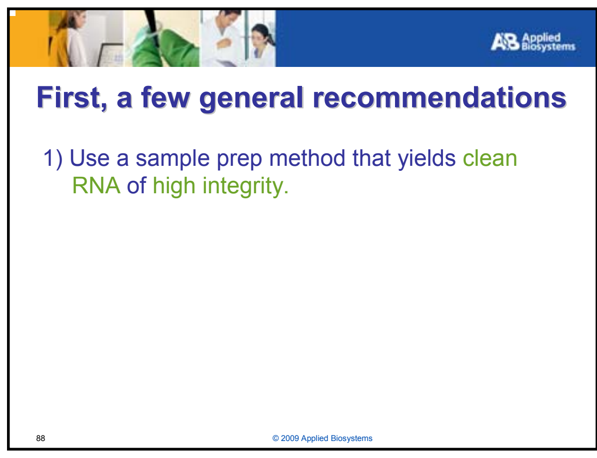


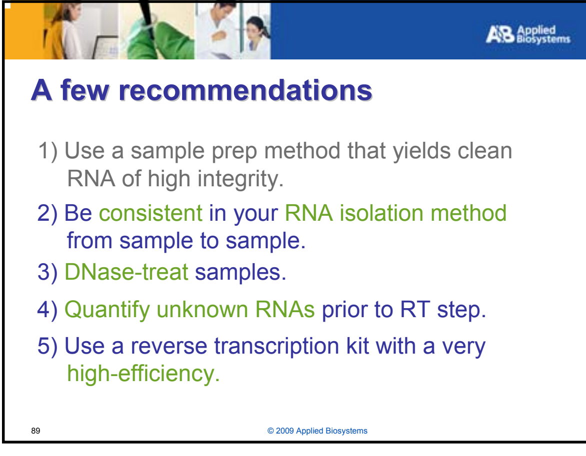
Applied Biosystems
Real-Time Quantitative PCR:
Relative Gene Expression
84
© 2009 Applied Biosystems


Applied Biosystems
Example experiment
Untreated platypus
Treated platypus
Gene of interest: Plat1
Big question: How is the expression of Plat1 changing when I treat my platypi?
85
© 2009 Applied Biosystems

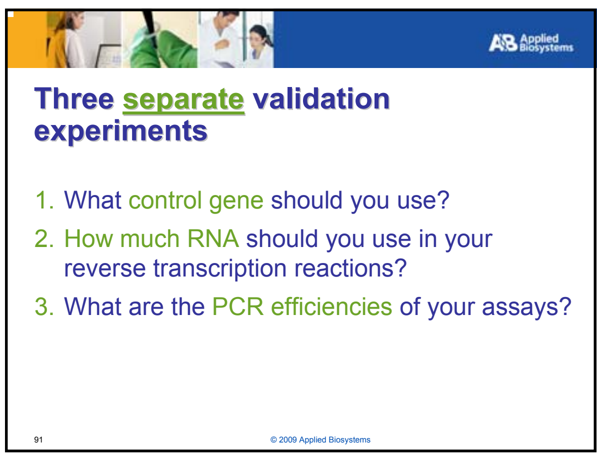

Applied Biosystems
Example experiment
Untreated platypus
Treated platypus
Gene of interest: Plat1
Big question: How is the expression of Plat1 changing when I treat my platypi?
86
© 2009 Applied Biosystems

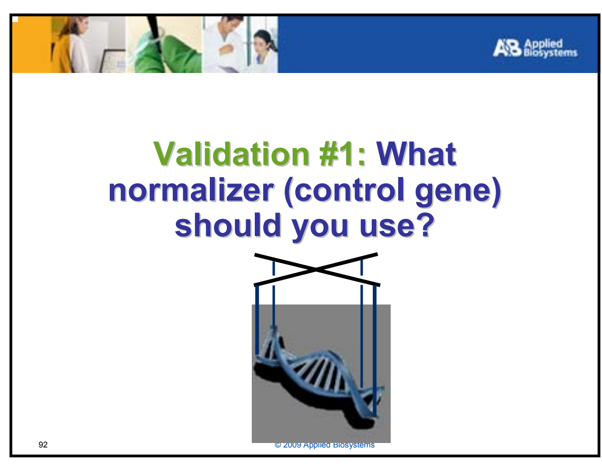

Applied Biosystems
Example experiment
Untreated platypus
Treated platypus
Gene of interest: Plat1
Big question: How is the expression of Plat1 changing when I treat my platypi?
87
© 2009 Applied Biosystems

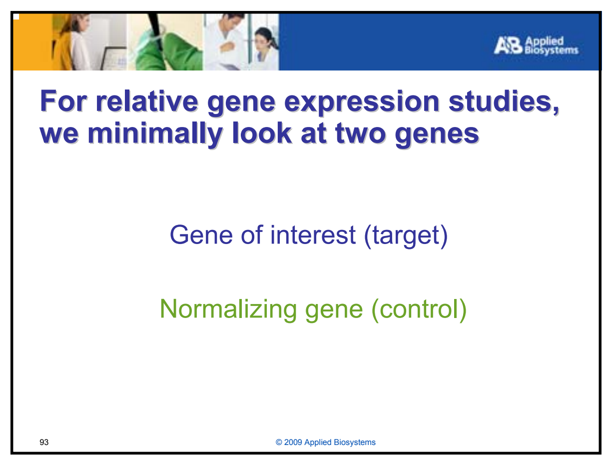

Applied Biosystems
First, a few general recommendations
1) Use a sample prep method that yields clean RNA of high integrity.
88
© 2009 Applied Biosystems

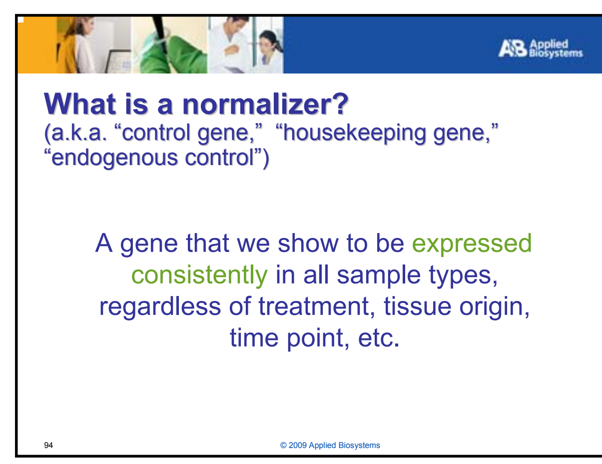

Applied Biosystems
A few recommendations
1) Use a sample prep method that yields clean RNA of high integrity.
2) Be consistent in your RNA isolation method from sample to sample.
3) DNase-treat samples.
4) Quantify unknown RNAs prior to RT step.
5) Use a reverse transcription kit with a very high-efficiency.
89
© 2009 Applied Biosystems

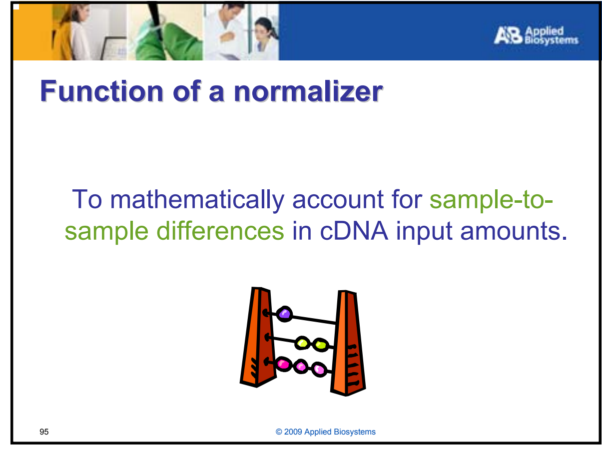
Applied Biosystems
Three validations for a successful relative gene expression experiment
90
© 2009 Applied Biosystems

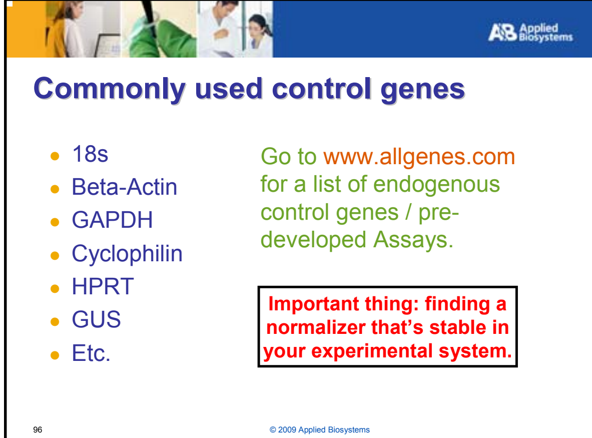

Applied Biosystems
Three separate validation experiments
1. What control gene should you use?
2. How much RNA should you use in your reverse transcription reactions?
3. What are the PCR efficiencies of your assays?
91
© 2009 Applied Biosystems

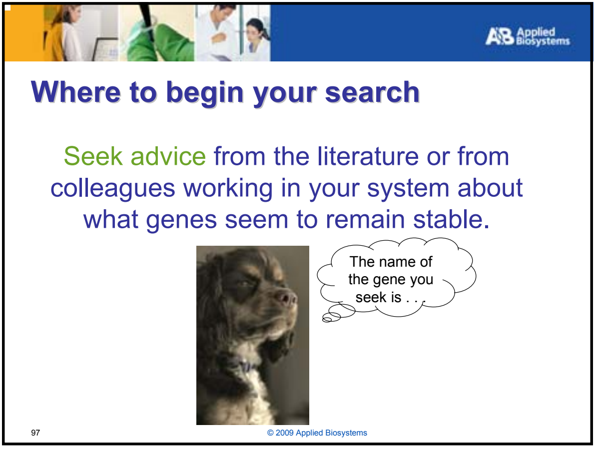

Applied Biosystems
Validation #1: What normalizer (control gene) should you use?
92

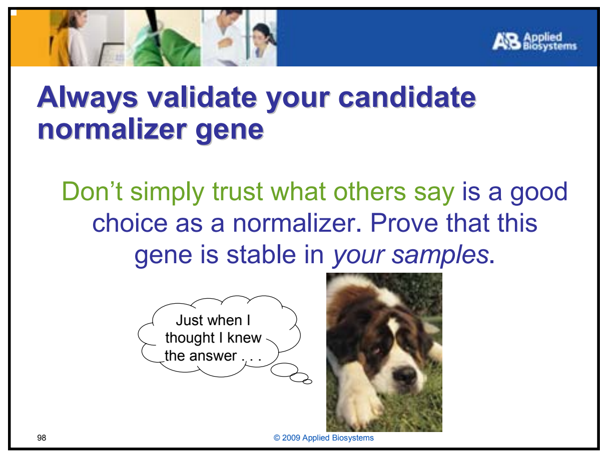

Applied Biosystems
For relative gene expression studies, we minimally look at two genes
Gene of interest (target)
Normalizing gene (control)
93
© 2009 Applied Biosystems

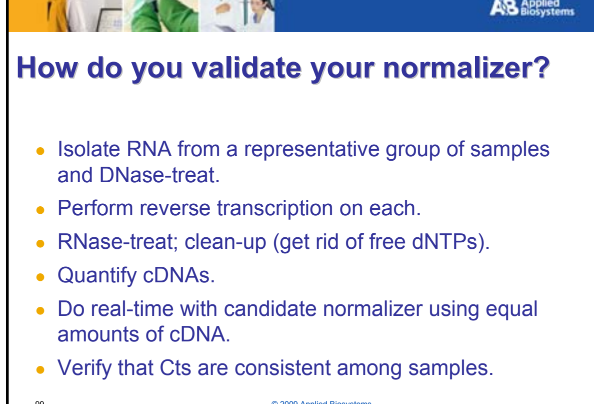

Applied Biosystems
What is a normalizer?
(a.k.a. "control gene," "housekeeping gene," "endogenous control")
A gene that we show to be expressed consistently in all sample types, regardless of treatment, tissue origin, time point, etc.
94
© 2009 Applied Biosystems

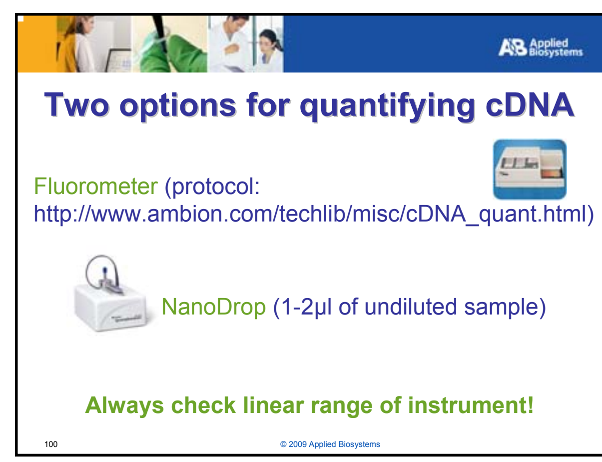

Applied Biosystems
Function of a normalizer
To mathematically account for sample-to-sample differences in cDNA input amounts.
95
© 2009 Applied Biosystems

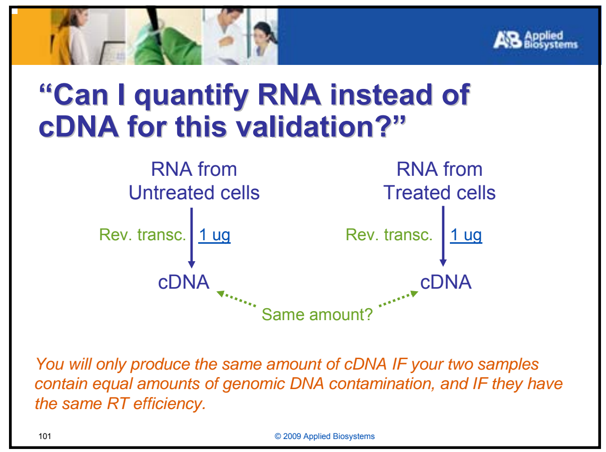

Applied Biosystems
Commonly used control genes
18s
Beta-Actin
GAPDH
Cyclophilin
HPRT
GUS
Etc.
Go to www.allgenes.com for a list of endogenous control genes / pre-developed Assays.
Important thing: finding a normalizer that's stable in your experimental system.
96
© 2009 Applied Biosystems

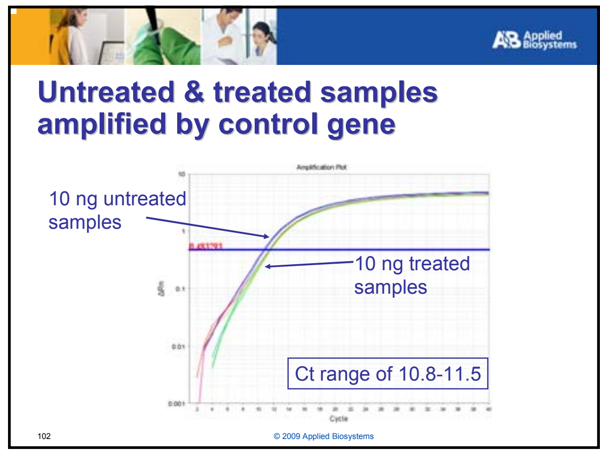

Where to begin your search
Seek advice from the literature or from colleagues working in your system about what genes seem to remain stable.
The name of the gene you seek is . . .
97
© 2009 Applied Biosystems

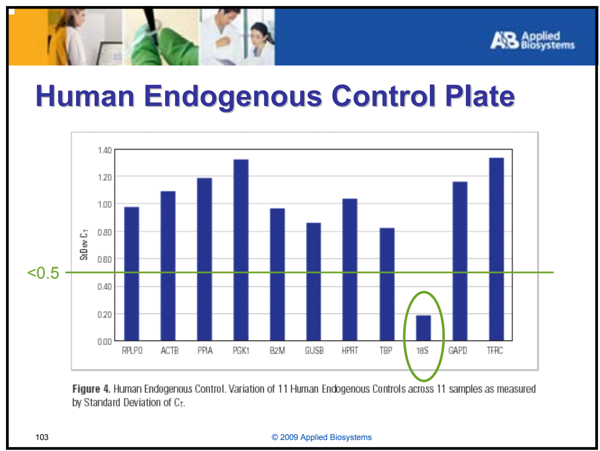

Always validate your candidate normalizer gene
Don't simply trust what others say is a good choice as a normalizer. Prove that this gene is stable in your samples.
Just when I thought I knew the answer . . .
98
© 2009 Applied Biosystems

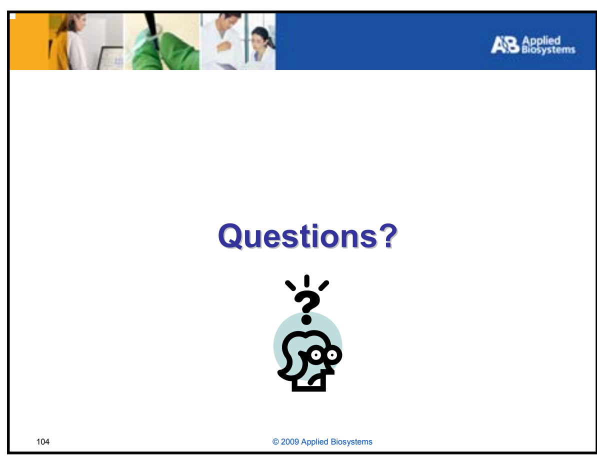

How do you validate your normalizer?
Isolate RNA from a representative group of samples and DNase-treat.
Perform reverse transcription on each.
RNase-treat; clean-up (get rid of free dNTPs).
Quantify cDNAs.
Do real-time with candidate normalizer using equal amounts of cDNA.
Verify that Cts are consistent among samples.
99
© 2009 Applied Biosystems

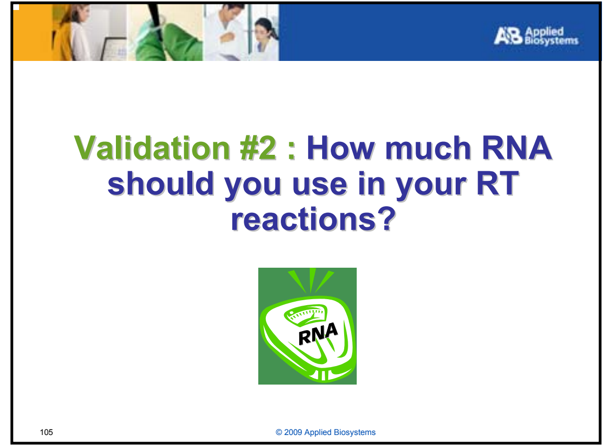

Two options for quantifying cDNA
Fluorometer (protocol: http://www.ambion.com/techlib/misc/cDNA_quant.html)
NanoDrop (1-2µl of undiluted sample)
Always check linear range of instrument!
100
© 2009 Applied Biosystems

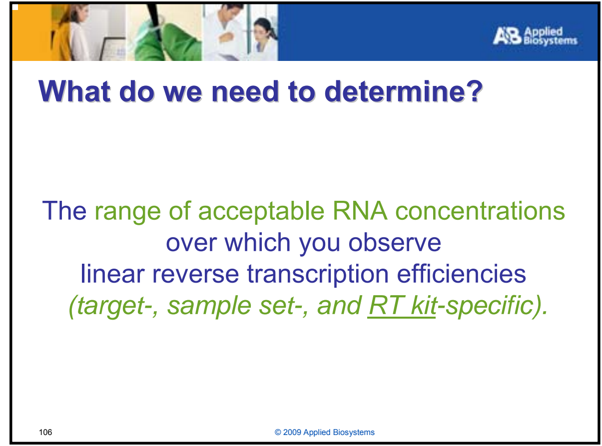

"Can I quantify RNA instead of cDNA for this validation?"
RNA from Untreated cells
RNA from Treated cells
Rev. transc.
1 ug
Rev. transc.
1 ug
cDNA
cDNA
Same amount?
You will only produce the same amount of cDNA IF your two samples contain equal amounts of genomic DNA contamination, and IF they have the same RT efficiency.
101
© 2009 Applied Biosystems

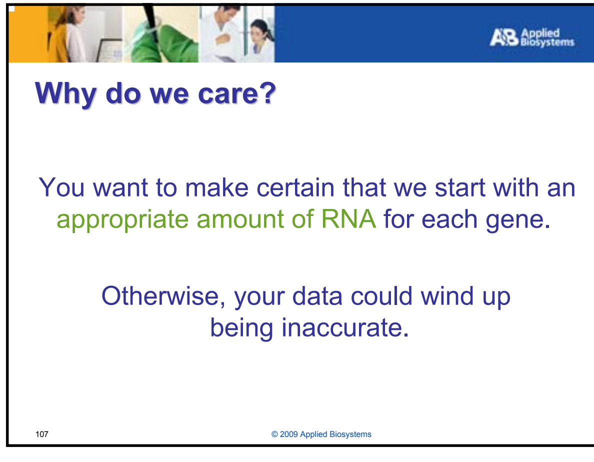

Untreated & treated samples amplified by control gene
10 ng untreated samples
10 ng treated samples
Ct range of 10.8-11.5
Cycle
102
© 2009 Applied Biosystems

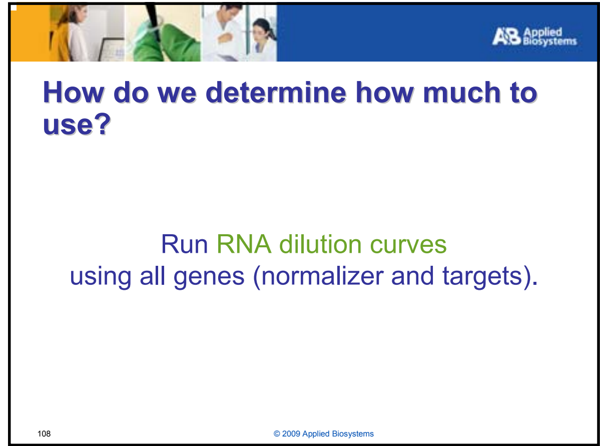

Human Endogenous Control Plate
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
<0.5
RPLP0
ACTB
PPIA
PGK1
B2M
GUSB
HPRT
TBP
18S
GAPD
TFRC
Figure 4. Human Endogenous Control. Variation of 11 Human Endogenous Controls across 11 samples as measured by Standard Deviation of CT.
103
© 2009 Applied Biosystems

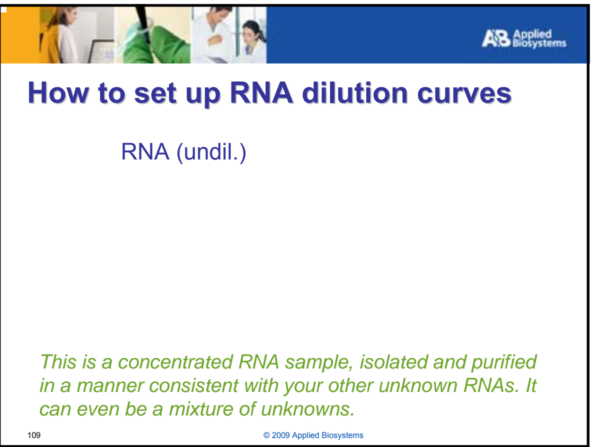

Questions?
104
© 2009 Applied Biosystems

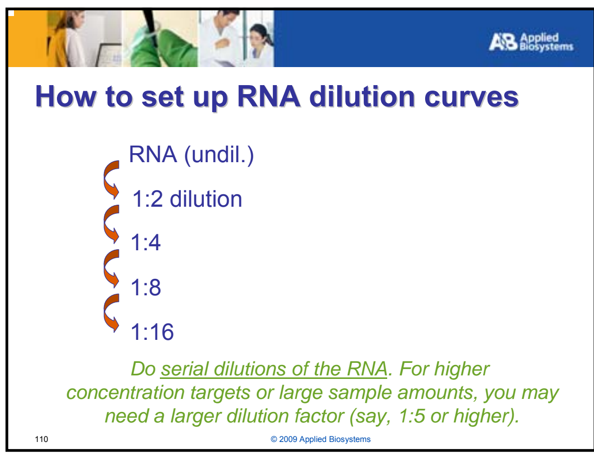

Validation #2 : How much RNA should you use in your RT reactions?
RNA
105
© 2009 Applied Biosystems

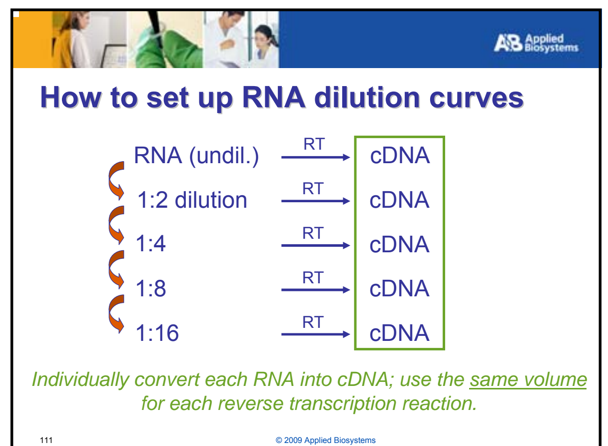

What do we need to determine?
The range of acceptable RNA concentrations over which you observe
linear reverse transcription efficiencies
(target-, sample set-, and RT kit-specific).
106
© 2009 Applied Biosystems

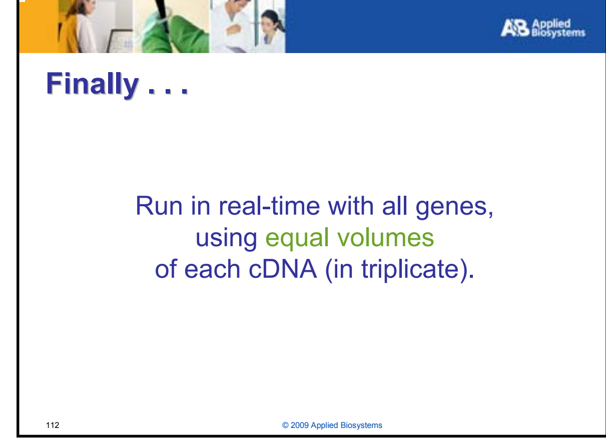

Why do we care?
You want to make certain that we start with an appropriate amount of RNA for each gene.
Otherwise, your data could wind up being inaccurate.
107
© 2009 Applied Biosystems

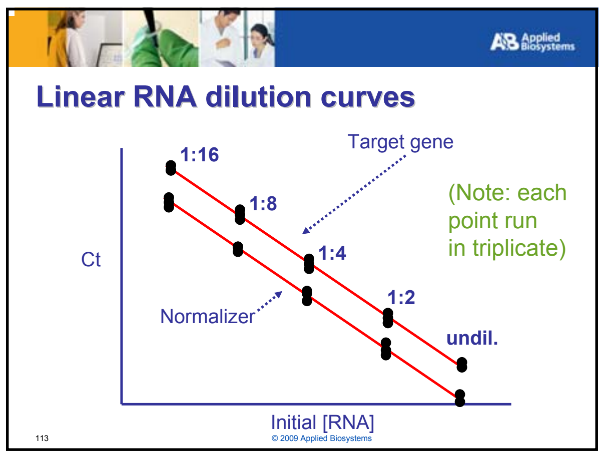

How do we determine how much to use?
Run RNA dilution curves
using all genes (normalizer and targets).
108
© 2009 Applied Biosystems

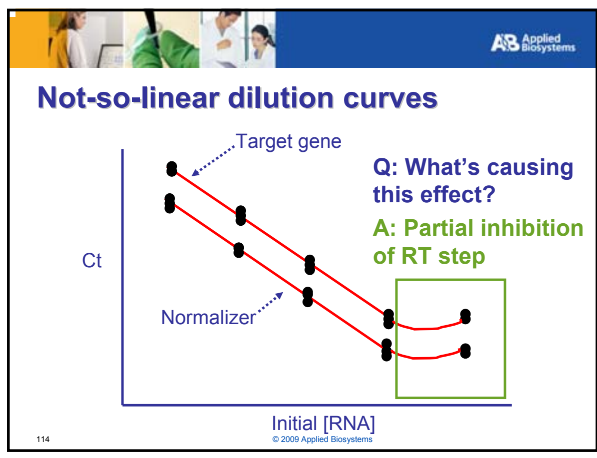

How to set up RNA dilution curves
RNA (undil.)
This is a concentrated RNA sample, isolated and purified in a manner consistent with your other unknown RNAs. It can even be a mixture of unknowns.
109
© 2009 Applied Biosystems

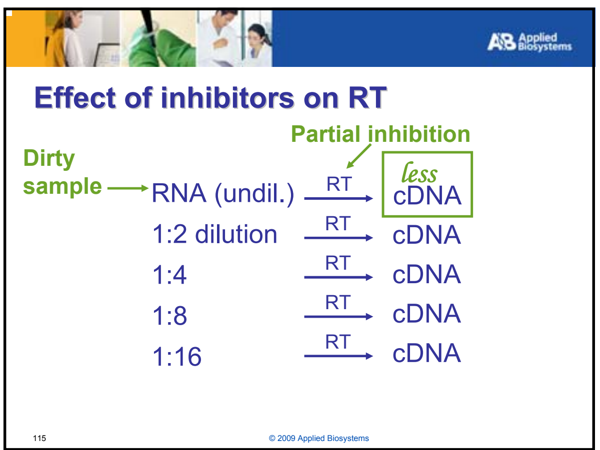

How to set up RNA dilution curves
RNA (undil.)
1:2 dilution
1:4
1:8
1:16
Do serial dilutions of the RNA. For higher concentration targets or large sample amounts, you may need a larger dilution factor (say, 1:5 or higher).
110
© 2009 Applied Biosystems

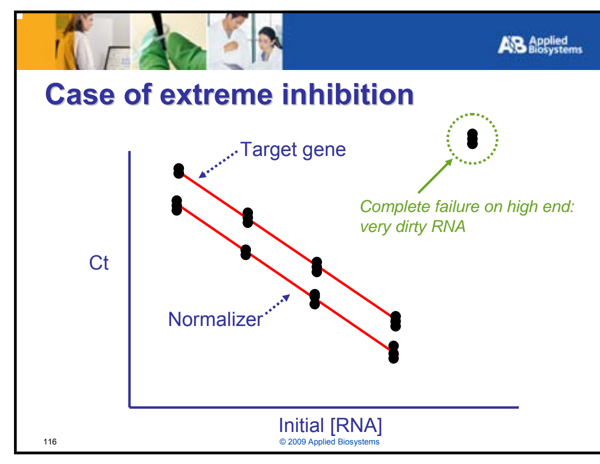

How to set up RNA dilution curves
RNA (undil.)
1:2 dilution
1:4
1:8
1:16
RT
cDNA
Individually convert each RNA into cDNA; use the same volume for each reverse transcription reaction.
111
© 2009 Applied Biosystems

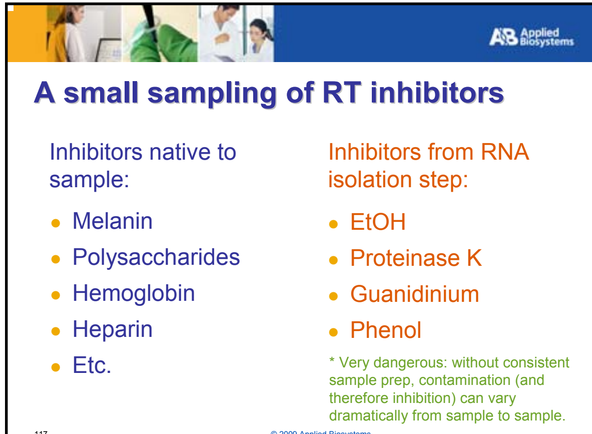

Finally . . .
Run in real-time with all genes, using equal volumes of each cDNA (in triplicate).
112
© 2009 Applied Biosystems

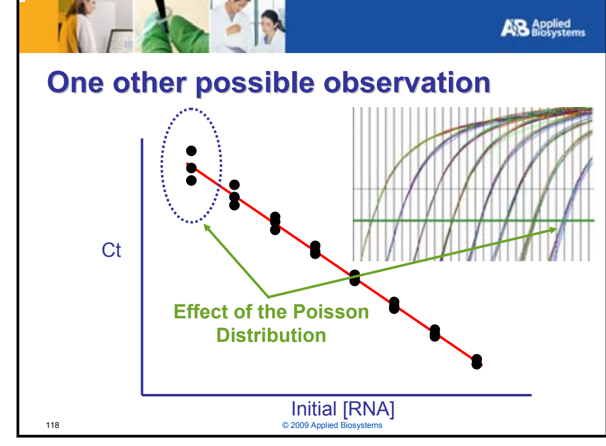

Linear RNA dilution curves
Target gene
(Note: each point run in triplicate)
1:16
1:8
1:4
1:2
undil.
Ct
Normalizer
Initial [RNA]
113
© 2009 Applied Biosystems

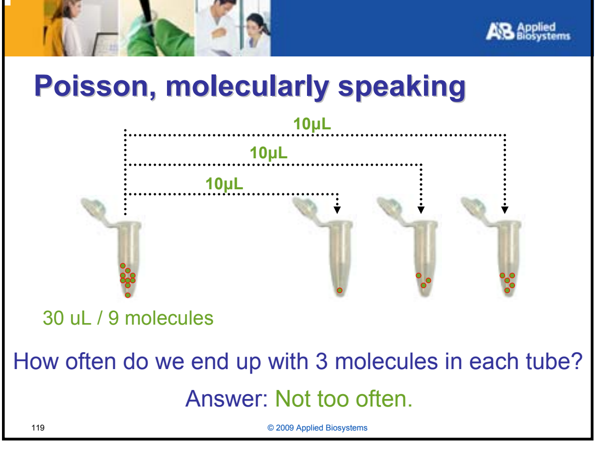

Not-so-linear dilution curves
Target gene
Q: What's causing this effect?
A: Partial inhibition of RT step
Ct
Normalizer
Initial [RNA]
114
© 2009 Applied Biosystems

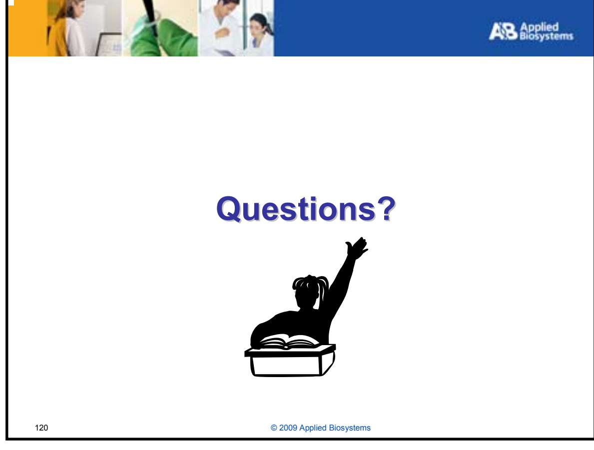

Effect of inhibitors on RT
Partial inhibition
Dirty sample
RNA (undil.)
RT
less cDNA
1:2 dilution
RT
cDNA
1:4
RT
cDNA
1:8
RT
cDNA
1:16
RT
cDNA
115
© 2009 Applied Biosystems

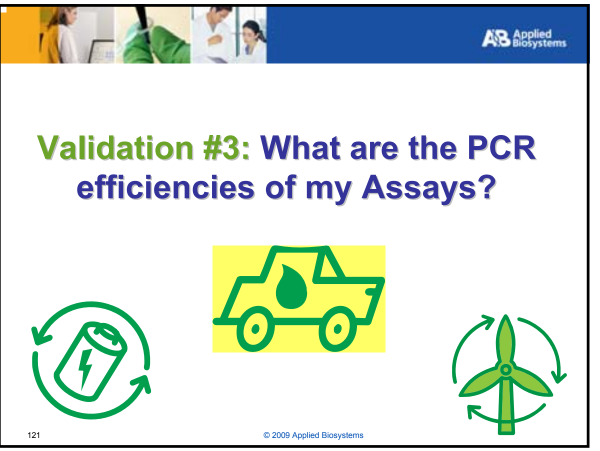

Case of extreme inhibition
Target gene
Complete failure on high end: very dirty RNA
Ct
Normalizer
Initial [RNA]
116
© 2009 Applied Biosystems

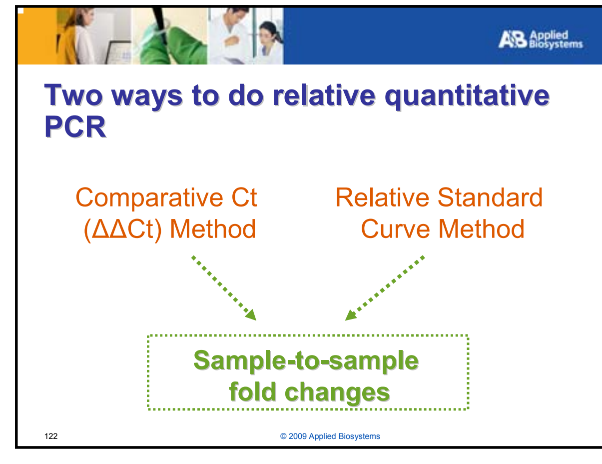

A small sampling of RT inhibitors
Inhibitors native to sample:
Melanin
Polysaccharides
Hemoglobin
Heparin
Etc.
Inhibitors from RNA isolation step:
EtOH
Proteinase K
Guanidinium
Phenol
* Very dangerous: without consistent sample prep, contamination (and therefore inhibition) can vary dramatically from sample to sample.
117
© 2009 Applied Biosystems

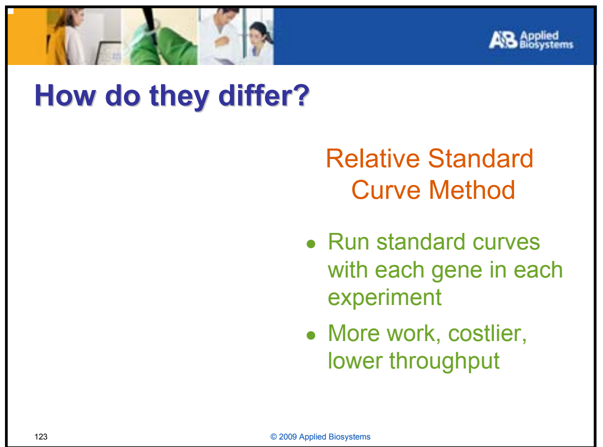

One other possible observation
Ct
Effect of the Poisson Distribution
Initial [RNA]
118
© 2009 Applied Biosystems

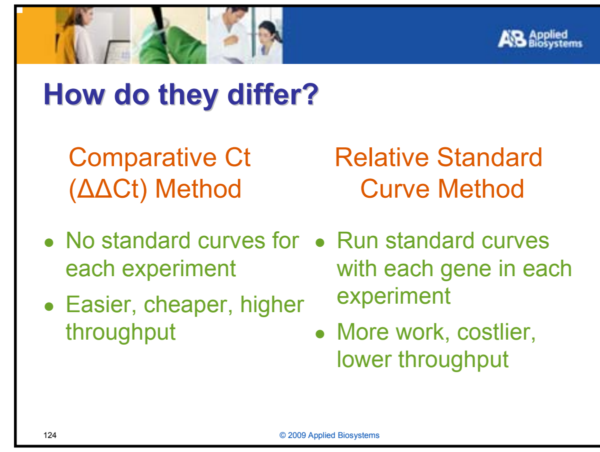

Poisson, molecularly speaking
10µL
10µL
10µL
30 uL / 9 molecules
How often do we end up with 3 molecules in each tube?
Answer: Not too often.
119
© 2009 Applied Biosystems

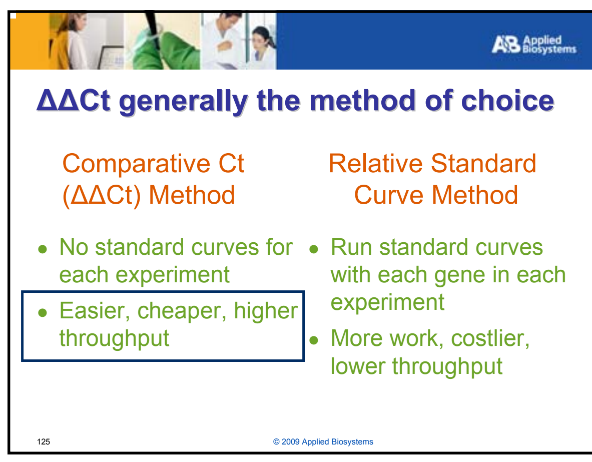

Questions?
120
© 2009 Applied Biosystems

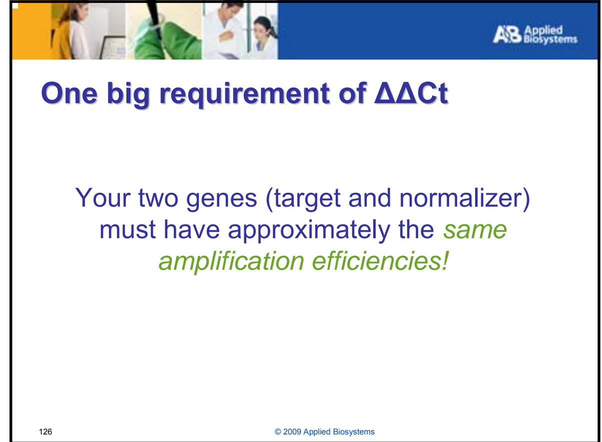

Applied Biosystems
Validation #3: What are the PCR efficiencies of my Assays?
121
© 2009 Applied Biosystems

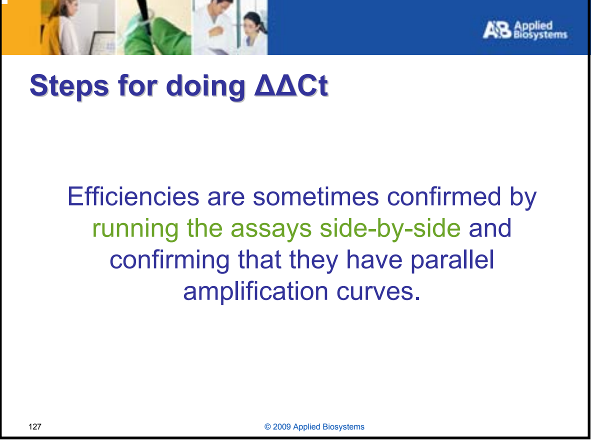

Applied Biosystems
Two ways to do relative quantitative PCR
Comparative Ct (ΔΔCt) Method
Relative Standard Curve Method
Sample-to-sample fold changes
122
© 2009 Applied Biosystems

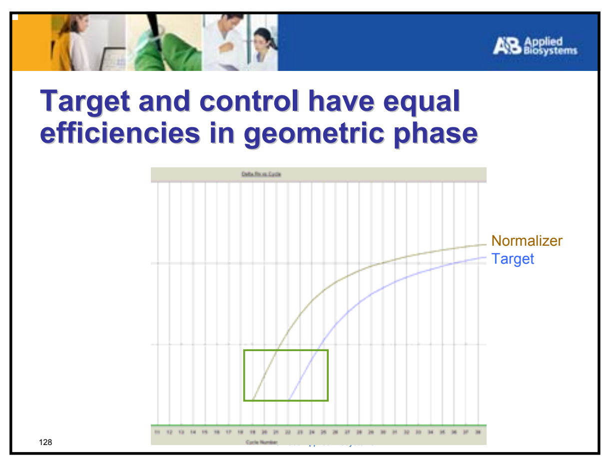

Applied Biosystems
How do they differ?
Relative Standard Curve Method
Run standard curves with each gene in each experiment
More work, costlier, lower throughput
123
© 2009 Applied Biosystems

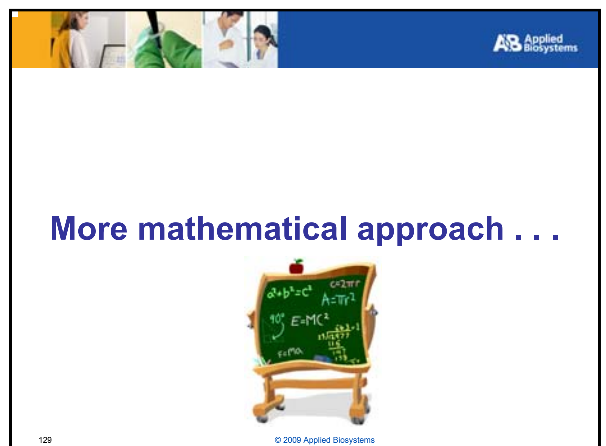

Applied Biosystems
How do they differ?
Comparative Ct (ΔΔCt) Method
Relative Standard Curve Method
No standard curves for each experiment
Easier, cheaper, higher throughput
Run standard curves with each gene in each experiment
More work, costlier, lower throughput
124
© 2009 Applied Biosystems

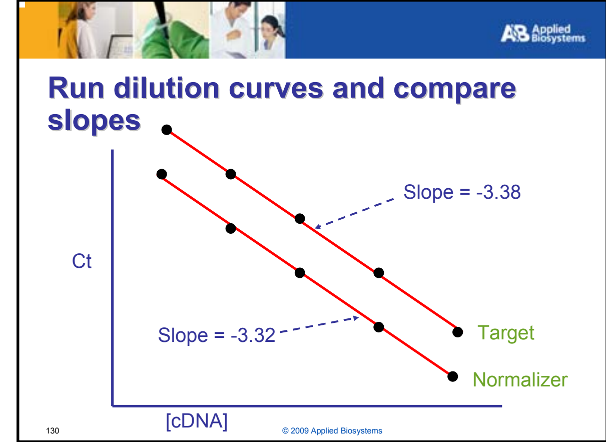

Applied Biosystems
ΔΔCt generally the method of choice
Comparative Ct (ΔΔCt) Method
Relative Standard Curve Method
No standard curves for each experiment
Easier, cheaper, higher throughput
Run standard curves with each gene in each experiment
More work, costlier, lower throughput
125
© 2009 Applied Biosystems

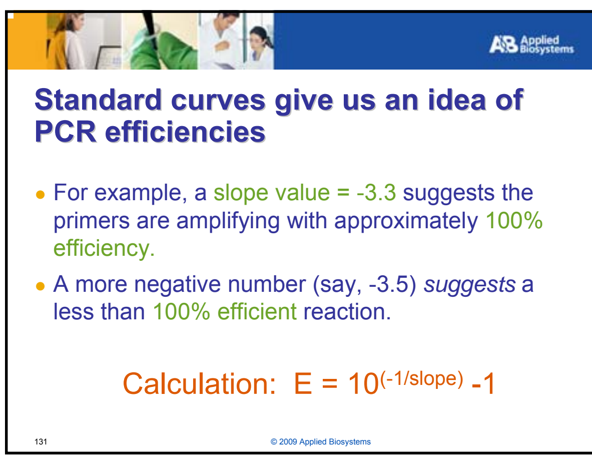

Applied Biosystems
One big requirement of ΔΔCt
Your two genes (target and normalizer) must have approximately the same amplification efficiencies!
126
© 2009 Applied Biosystems


Applied Biosystems
Steps for doing ΔΔCt
Efficiencies are sometimes confirmed by running the assays side-by-side and confirming that they have parallel amplification curves.
127
© 2009 Applied Biosystems

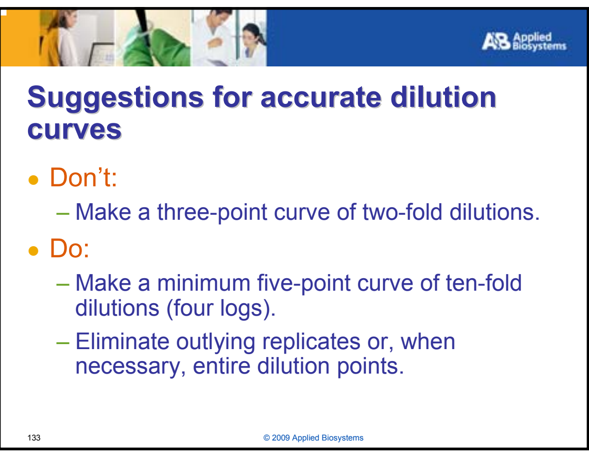

Applied Biosystems
Target and control have equal efficiencies in geometric phase
Normalizer
Target
128

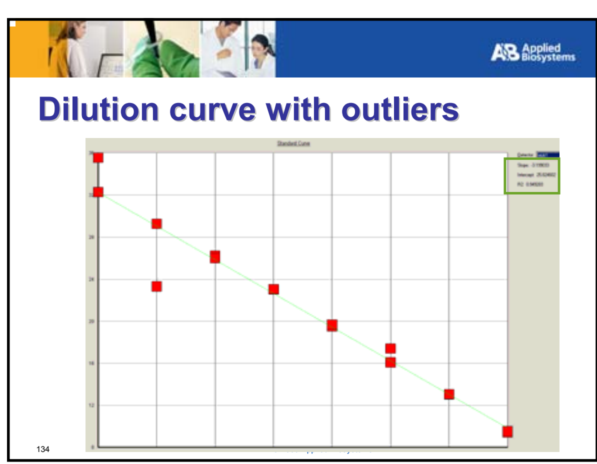

Applied Biosystems
More mathematical approach . . .
129
© 2009 Applied Biosystems

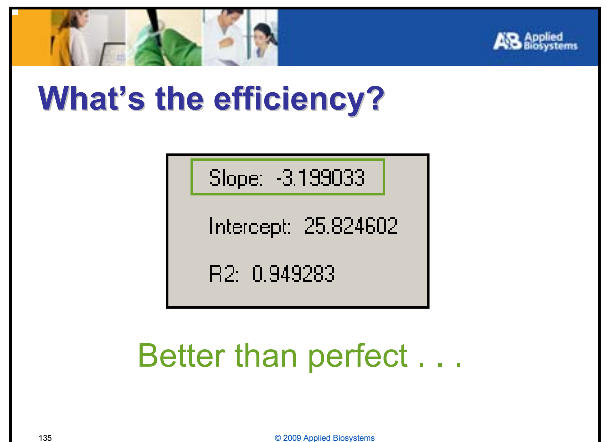

Applied Biosystems
Run dilution curves and compare slopes
Slope = -3.38
Ct
Slope = -3.32
Target
Normalizer
[cDNA]
130
© 2009 Applied Biosystems

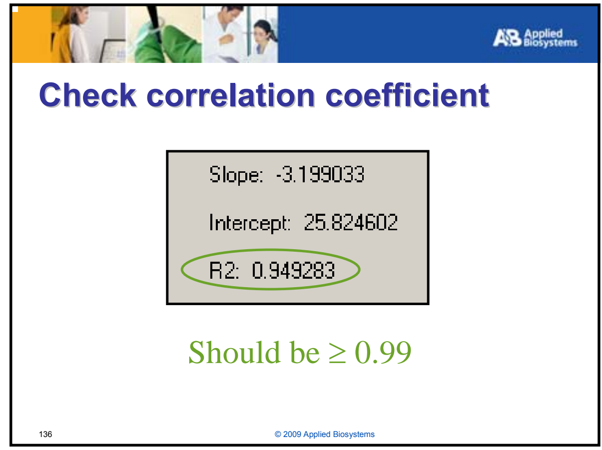

Applied Biosystems
Standard curves give us an idea of PCR efficiencies
For example, a slope value = -3.3 suggests the primers are amplifying with approximately 100% efficiency.
A more negative number (say, -3.5) suggests a less than 100% efficient reaction.
Calculation: E = 10(-1/slope) -1
131
© 2009 Applied Biosystems

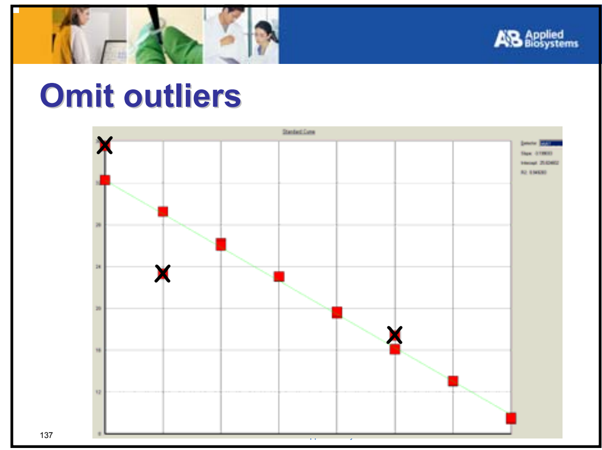
Applied Biosystems
#1 mistake among my real-time customers
Generating inaccurate dilution curves (and thus inaccurate efficiency calculations).
132
© 2009 Applied Biosystems

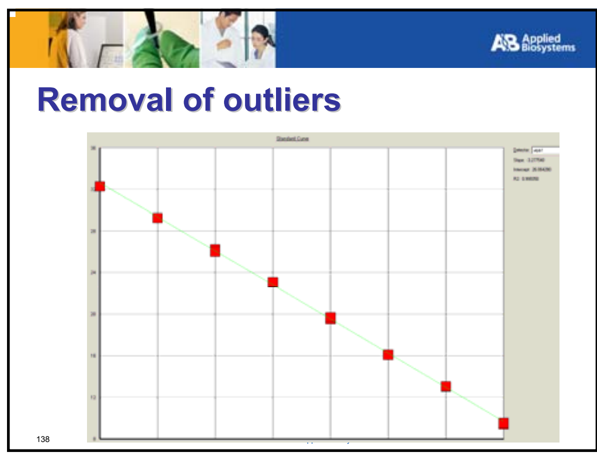

Applied Biosystems
Suggestions for accurate dilution curves
Don't:
– Make a three-point curve of two-fold dilutions.
Do:
– Make a minimum five-point curve of ten-fold dilutions (four logs).
– Eliminate outlying replicates or, when necessary, entire dilution points.
133
© 2009 Applied Biosystems

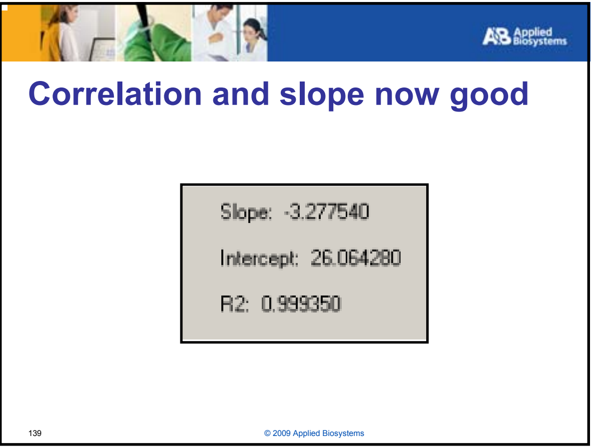

Applied Biosystems
Dilution curve with outliers
134

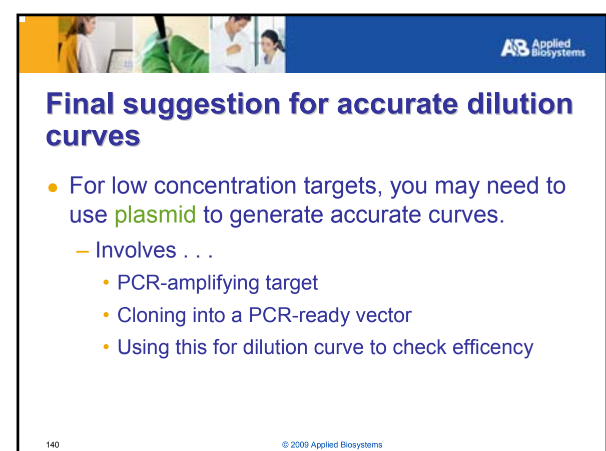

Applied Biosystems
What's the efficiency?
Slope: -3.199033
Intercept: 25.824602
R2: 0.949283
Better than perfect . . .
135
© 2009 Applied Biosystems

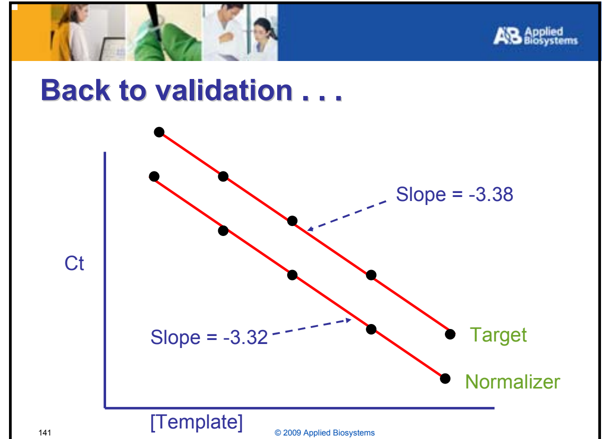

Applied Biosystems
Check correlation coefficient
Slope: -3.199033
Intercept: 25.824602
R2: 0.949283
Should be ≥ 0.99
136
© 2009 Applied Biosystems

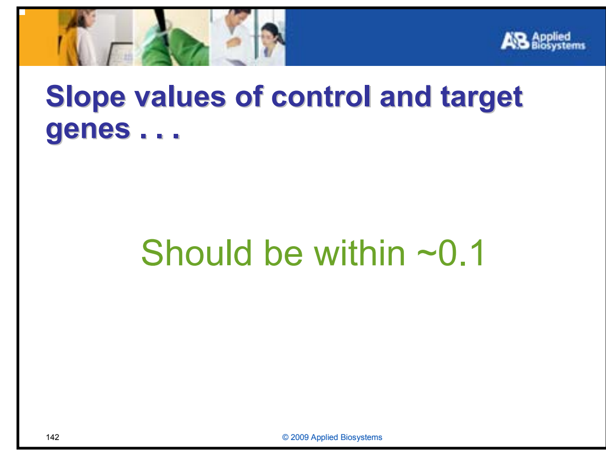

Applied Biosystems
Omit outliers
137

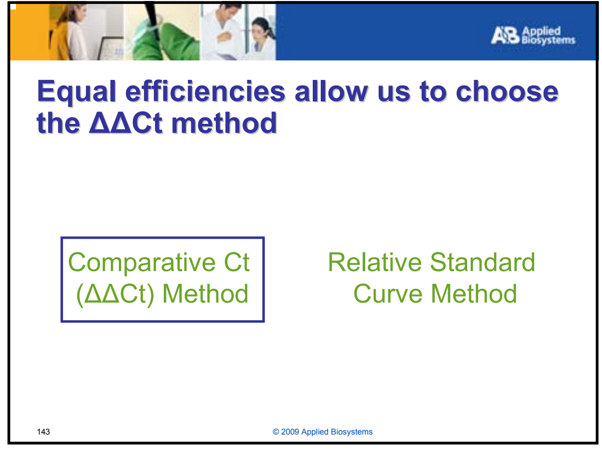

Applied Biosystems
Removal of outliers
138

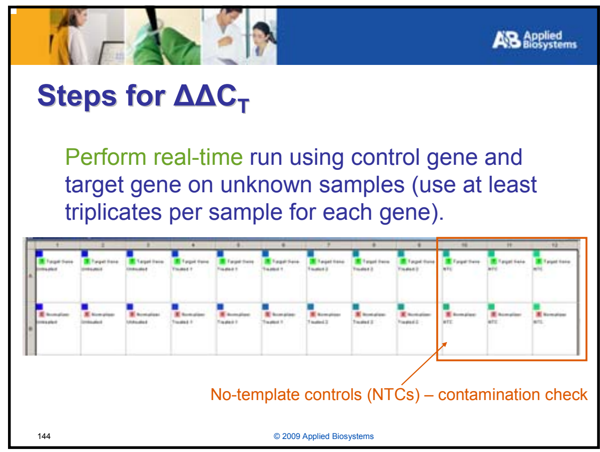

Applied Biosystems
Correlation and slope now good
Slope: -3.277540
Intercept: 26.064280
R2: 0.999350
139
© 2009 Applied Biosystems

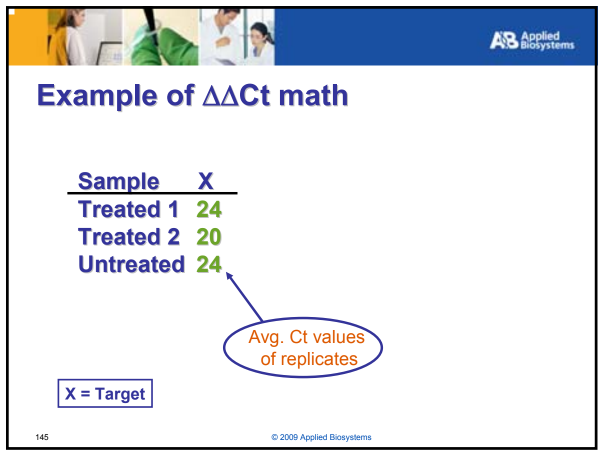

Applied Biosystems
Final suggestion for accurate dilution curves
For low concentration targets, you may need to use plasmid to generate accurate curves.
– Involves . . .
PCR-amplifying target
Cloning into a PCR-ready vector
Using this for dilution curve to check efficency
140
© 2009 Applied Biosystems

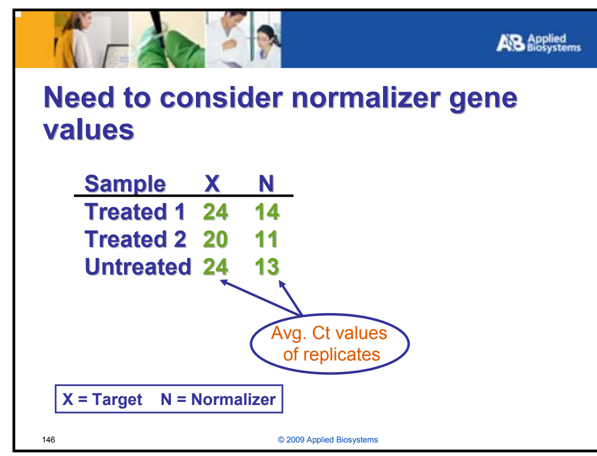

Applied Biosystems
Back to validation . . .
Slope = -3.38
Ct
Slope = -3.32
Target
Normalizer
[Template]
141
© 2009 Applied Biosystems

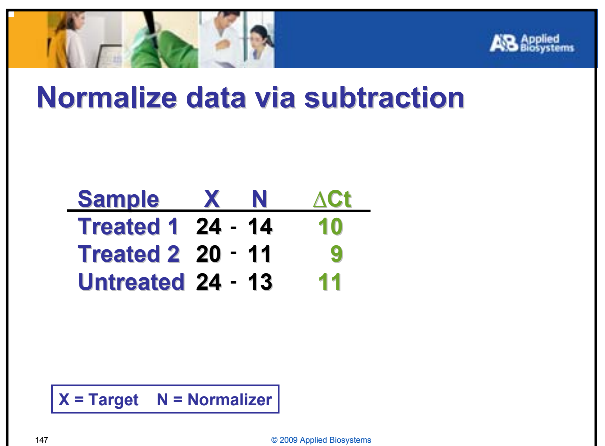

Applied Biosystems
Slope values of control and target genes . . .
Should be within ~0.1
142
© 2009 Applied Biosystems

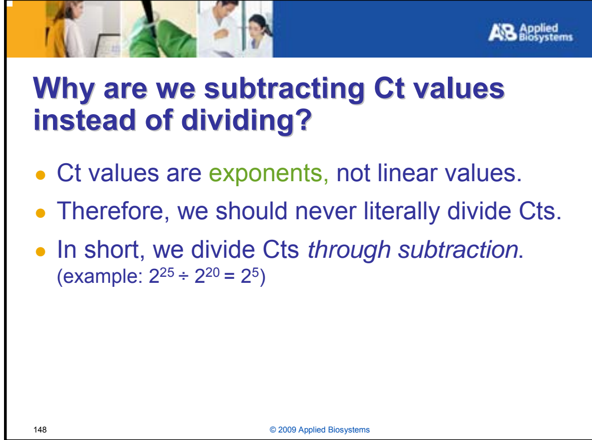

Applied Biosystems
Equal efficiencies allow us to choose the ΔΔCt method
Comparative Ct (ΔΔCt) Method
Relative Standard Curve Method
143
© 2009 Applied Biosystems

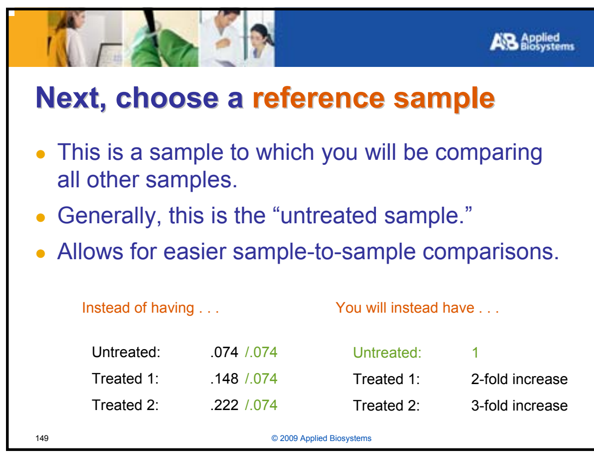

Applied Biosystems
Steps for ΔΔCT
Perform real-time run using control gene and target gene on unknown samples (use at least triplicates per sample for each gene).
No-template controls (NTCs) – contamination check
144
© 2009 Applied Biosystems

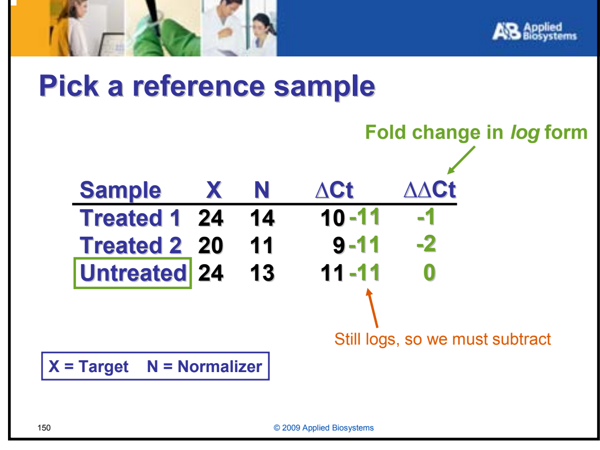

Applied Biosystems
Example of ΔΔCt math
Sample X
Treated 1 24
Treated 2 20
Untreated 24
Avg. Ct values of replicates
X = Target
145
© 2009 Applied Biosystems

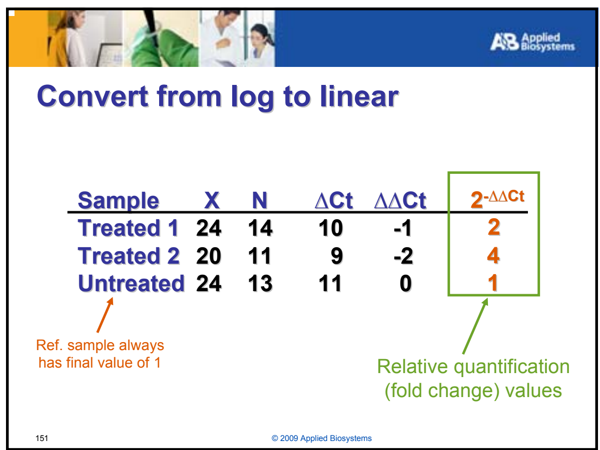

Applied Biosystems
Need to consider normalizer gene values
Sample X N
Treated 1 24 14
Treated 2 20 11
Untreated 24 13
Avg. Ct values of replicates
X = Target N = Normalizer
146
© 2009 Applied Biosystems


Applied Biosystems
Normalize data via subtraction
Sample X N ΔCt
Treated 1 24 - 14 10
Treated 2 20 - 11 9
Untreated 24 - 13 11
X = Target N = Normalizer
147
© 2009 Applied Biosystems

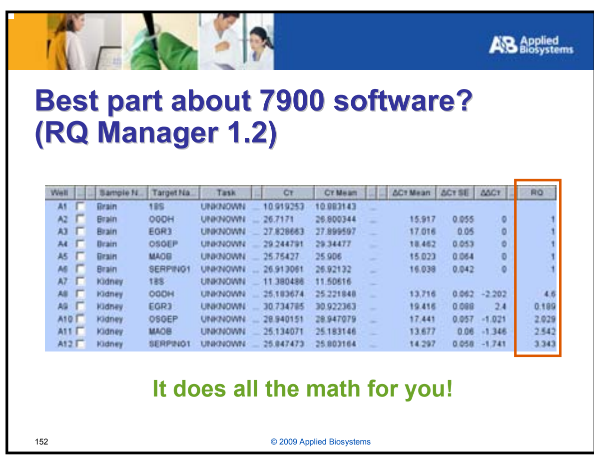

Applied Biosystems
Why are we subtracting Ct values instead of dividing?
Ct values are exponents, not linear values.
Therefore, we should never literally divide Cts.
In short, we divide Cts through subtraction.
(example: 2^25 ÷ 2^20 = 2^5)
148
© 2009 Applied Biosystems


Applied Biosystems
Next, choose a reference sample
This is a sample to which you will be comparing all other samples.
Generally, this is the “untreated sample.”
Allows for easier sample-to-sample comparisons.
Instead of having . . .
Untreated: .074 /.074
Treated 1: .148 /.074
Treated 2: .222 /.074
You will instead have . . .
Untreated: 1
Treated 1: 2-fold increase
Treated 2: 3-fold increase
149
© 2009 Applied Biosystems

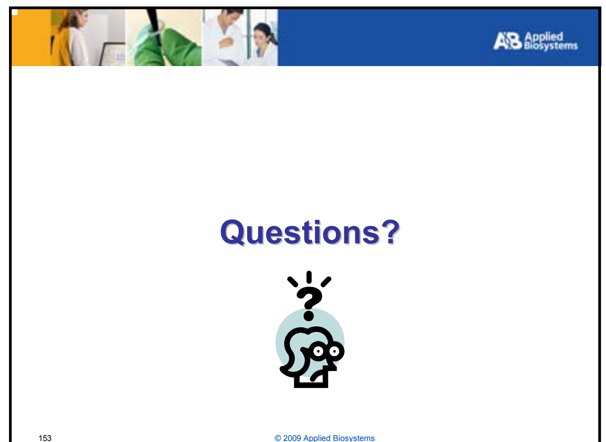

Applied Biosystems
Pick a reference sample
Fold change in log form
Sample X N ΔCt ΔΔCt
Treated 1 24 14 10 -11 -1
Treated 2 20 11 9 -11 -2
Untreated 24 13 11 -11 0
Still logs, so we must subtract
X = Target N = Normalizer
150
© 2009 Applied Biosystems

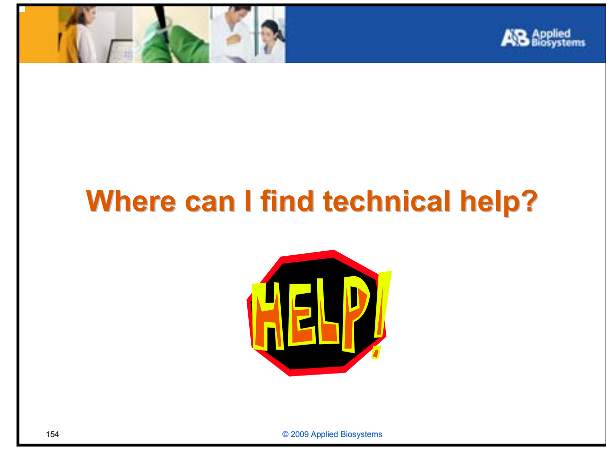


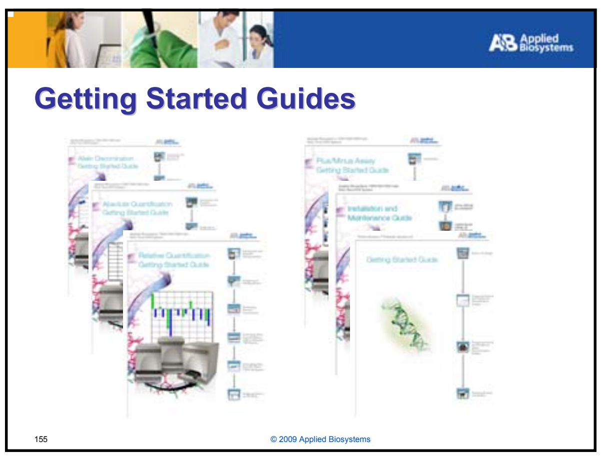
Sample	X	N	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
Treated 1	24	14	10	-1	2
Treated 2	20	11	9	-2	4
Untreated	24	13	11	0	1

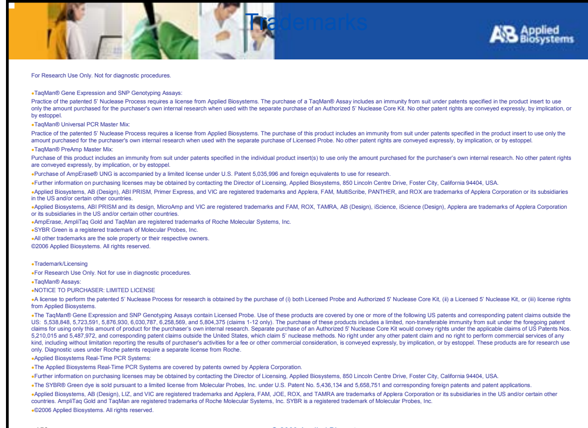


Well	Sample N...	Target Na...	Task	CT	CT Mean	ΔCT Mean	ΔCT SE	ΔΔCT	RQ
A1	Brain	18S	UNKNOWN	10.919253	10.883143				
A2	Brain	OGDH	UNKNOWN	26.7171	26.800344	15.917	0.055	0	1
A3	Brain	EGR3	UNKNOWN	27.828663	27.899597	17.016	0.05	0	1
A4	Brain	OSGEP	UNKNOWN	29.244791	29.34477	18.462	0.053	0	1
A5	Brain	MAOB	UNKNOWN	25.75427	25.906	15.023	0.064	0	1
A6	Brain	SERPING1	UNKNOWN	26.913061	26.92132	16.038	0.042	0	1
A7	Kidney	18S	UNKNOWN	11.380486	11.50616				
A8	Kidney	OGDH	UNKNOWN	25.183674	25.221848	13.716	0.062	-2.202	4.6
A9	Kidney	EGR3	UNKNOWN	30.734785	30.922363	19.416	0.088	2.4	0.189
A10	Kidney	OSGEP	UNKNOWN	28.940151	28.947079	17.441	0.057	-1.021	2.029
A11	Kidney	MAOB	UNKNOWN	25.134071	25.183146	13.677	0.06	-1.346	2.542
A12	Kidney	SERPING1	UNKNOWN	25.847473	25.803164	14.297	0.058	-1.741	3.343









Trademarks

For Research Use Only. Not for diagnostic procedures.

- TaqMan® Gene Expression and SNP Genotyping Assays:

Practice of the patented 5' Nuclease Process requires a license from Applied Biosystems. The purchase of a TaqMan® Assay includes an immunity from suit under patents specified in the product insert to use only the amount purchased for the purchaser's own internal research when used with the separate purchase of an Authorized 5' Nuclease Core Kit. No other patent rights are conveyed expressly, by implication, or by estoppel.

- TaqMan® Universal PCR Master Mix:

Practice of the patented 5' Nuclease Process requires a license from Applied Biosystems. The purchase of this product includes an immunity from suit under patents specified in the product insert to use only the amount purchased for the purchaser's own internal research when used with the separate purchase of Licensed Probe. No other patent rights are conveyed expressly, by implication, or by estoppel.

- TaqMan® PreAmp Master Mix:

Purchase of this product includes an immunity from suit under patents specified in the individual product insert(s) to use only the amount purchased for the purchaser's own internal research. No other patent rights are conveyed expressly, by implication, or by estoppel.

- Purchase of AmpErase® UNG is accompanied by a limited license under U.S. Patent 5,035,996 and foreign equivalents to use for research.
- Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.
- Applied Biosystems, AB (Design), ABI PRISM, Primer Express, and VIC are registered trademarks and Applera, FAM, MultiScribe, PANTHER, and ROX are trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries.
- Applied Biosystems, ABI PRISM and its design, MicroAmp and VIC are registered trademarks and FAM, ROX, TAMRA, AB (Design), iScience, iScience (Design), Applera are trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries.
- AmpErase, AmpliTaq Gold and TaqMan are registered trademarks of Roche Molecular Systems, Inc.
- SYBR Green is a registered trademark of Molecular Probes, Inc.
- All other trademarks are the sole property or their respective owners.

©2006 Applied Biosystems. All rights reserved.

- Trademark/Licensing
- For Research Use Only. Not for use in diagnostic procedures.
- TaqMan® Assays:
- NOTICE TO PURCHASER: LIMITED LICENSE
- A license to perform the patented 5' Nuclease Process for research is obtained by the purchase of (i) both Licensed Probe and Authorized 5' Nuclease Core Kit, (ii) a Licensed 5' Nuclease Kit, or (iii) license rights from Applied Biosystems.
- The TaqMan® Gene Expression and SNP Genotyping Assays contain Licensed Probe. Use of these products are covered by one or more of the following US patents and corresponding patent claims outside the US: 5,538,848, 5,723,591, 5,876,930, 6,030,787, 6,258,569, and 5,804,375 (claims 1-12 only). The purchase of these products includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. Separate purchase of an Authorized 5' Nuclease Core Kit would convey rights under the applicable claims of US Patents Nos. 5,210,015 and 5,487,972, and corresponding patent claims outside the United States, which claim 5' nuclease methods. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. These products are for research use only. Diagnostic uses under Roche patents require a separate license from Roche.
- Applied Biosystems Real-Time PCR Systems:
- The Applied Biosystems Real-Time PCR Systems are covered by patents owned by Applera Corporation.
- Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.
- The SYBR® Green dye is sold pursuant to a limited license from Molecular Probes, Inc. under U.S. Patent No. 5,436,134 and 5,658,751 and corresponding foreign patents and patent applications.
- Applied Biosystems, AB (Design), LIZ, and VIC are registered trademarks and Applera, FAM, JOE, ROX, and TAMRA are trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries. AmpliTaq Gold and TaqMan are registered trademarks of Roche Molecular Systems, Inc. SYBR is a registered trademark of Molecular Probes, Inc.

- ©2006 Applied Biosystems. All rights reserved.

© 2009 Applied Biosystems