

qPCR with stochastic profiling samples

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Lecture Objectives

qPCR detection method in Janes lab

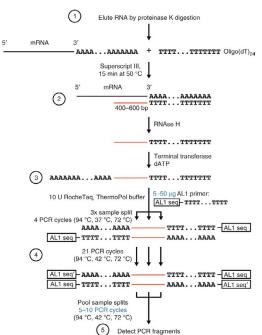
Primer design and validation

Troubleshooting primers and amplifications

What we are going to do in the lab today

Workflow for qPCR with stochastic profiling samples

Small-sample cDNA amplification



Extraction of RNA

Abbreviated RT

cDNA amplification

Primer Design and Validation

qPCR Setup

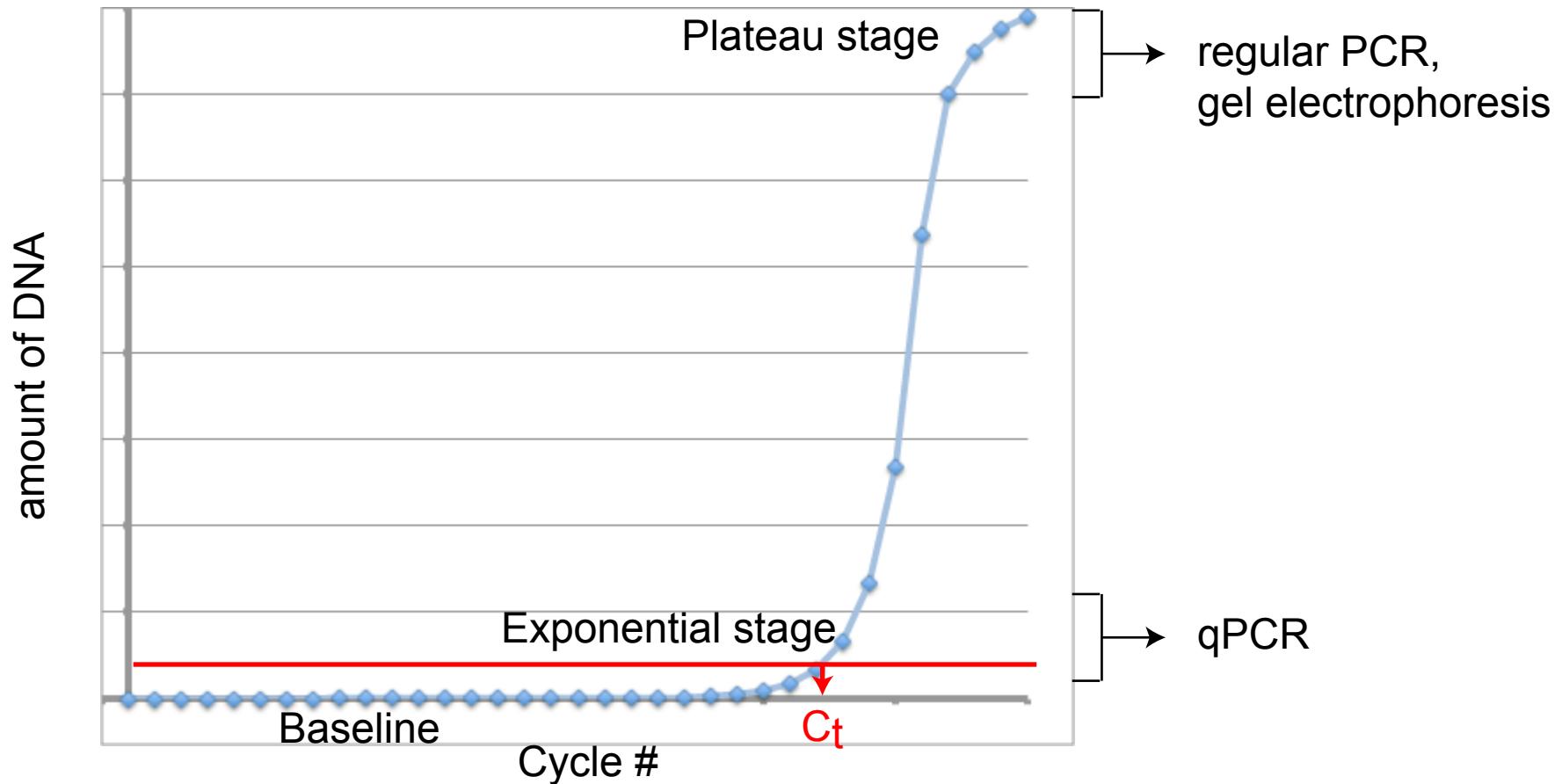
Data Output and Analysis

cDNA reamplification and purification

RNAseq and data analysis

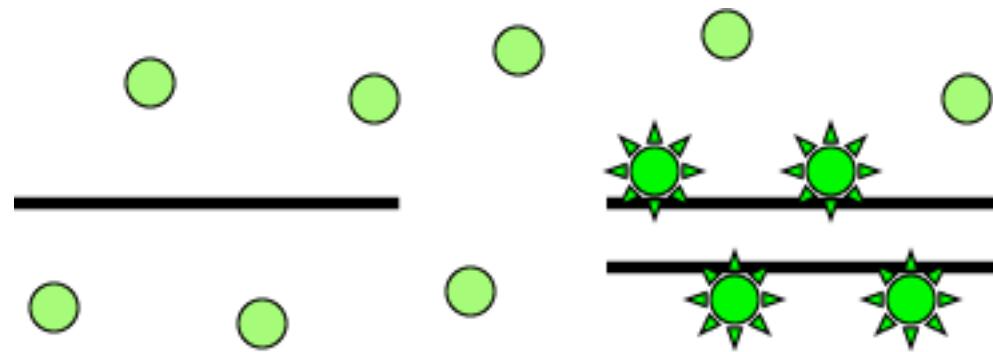
What is qPCR?

PCR amplification: Product (P) = Template (T) x $(2)^n$



Detection method in Janes lab

SYBR green



Fluorescence increases with concentration of dsDNA.
Detects double stranded products, including primer dimers.

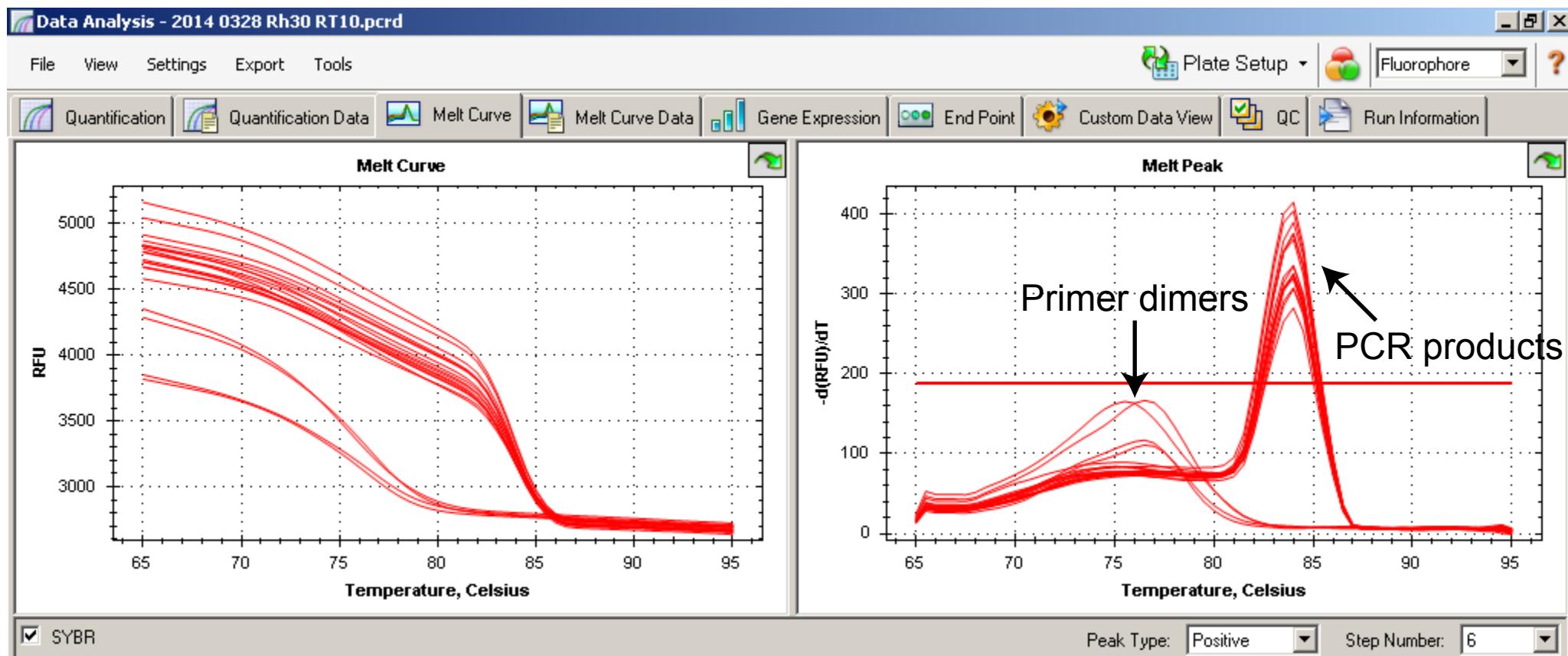
Important controls

blank sample, no template control (NTC):
to check primer dimers and contaminants

no RT sample:
to check genomic DNA

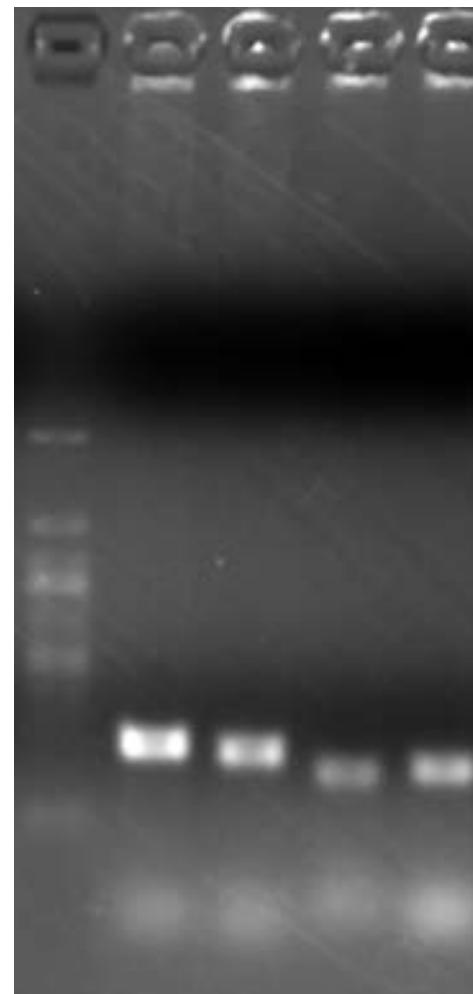
Whether qPCR reactions have produced single, specific products?

Melting Curves Analysis



Whether qPCR reactions have produced single, specific products?

Agarose gel analysis



← PCR products

← Primer dimers

Primer design

Gene of interest: mRNA RefSeq identification

Primer design

Primer3

Amplicon position:

within ~400 bp from the 3' end of the transcripts and do not typically span introns, because of the abbreviated RT.

Amplicon Length: 150-200bp

Primer length: opt. 20nt

T_m: opt 60°C

Primer3: WWW primer tool

pick primers from a DNA sequence

Paste source sequence below (5'->3', string of ACGTNacgn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a [Mispriming Library \(repeat library\)](#):

Pick left primer or use left primer below. Pick hybridization probe (internal oligo) or use oligo below. Pick right primer or use right primer below (5'->3' on opposite strand).

Sequence Id: A string to identify your output.

Targets: E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the [source sequence](#) with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.

Excluded Regions: E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the [source sequence](#) with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.

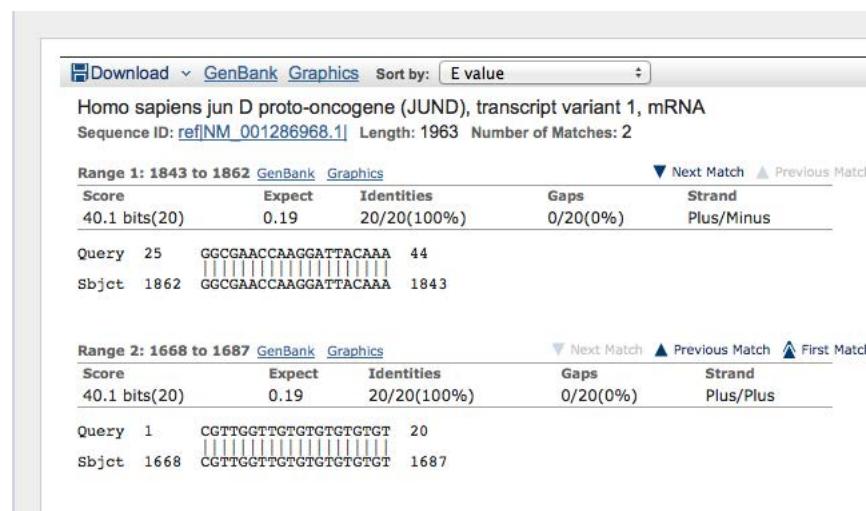
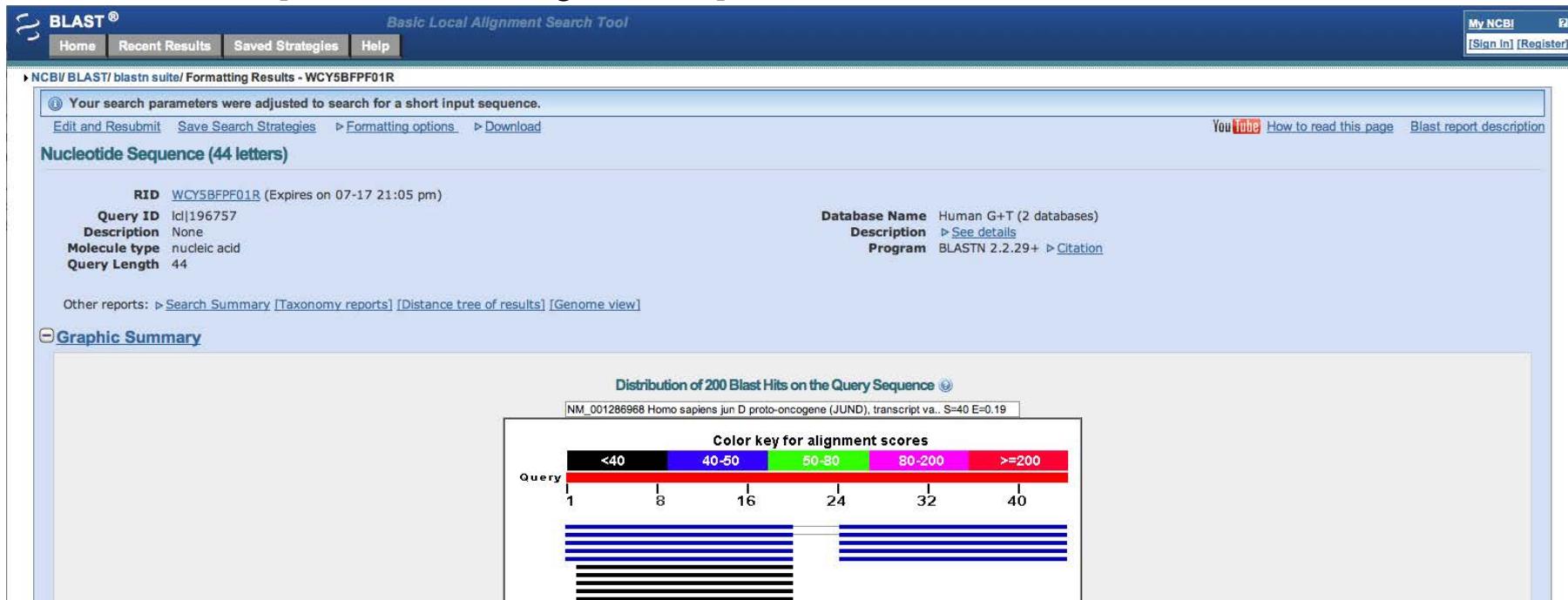
Product Size Min: Opt: Max:

Number To Return: Max 3' Stability:

Max Mispriming: Pair Max Mispriming:

Primer design

BLAST: Specificity of primers



Primer design

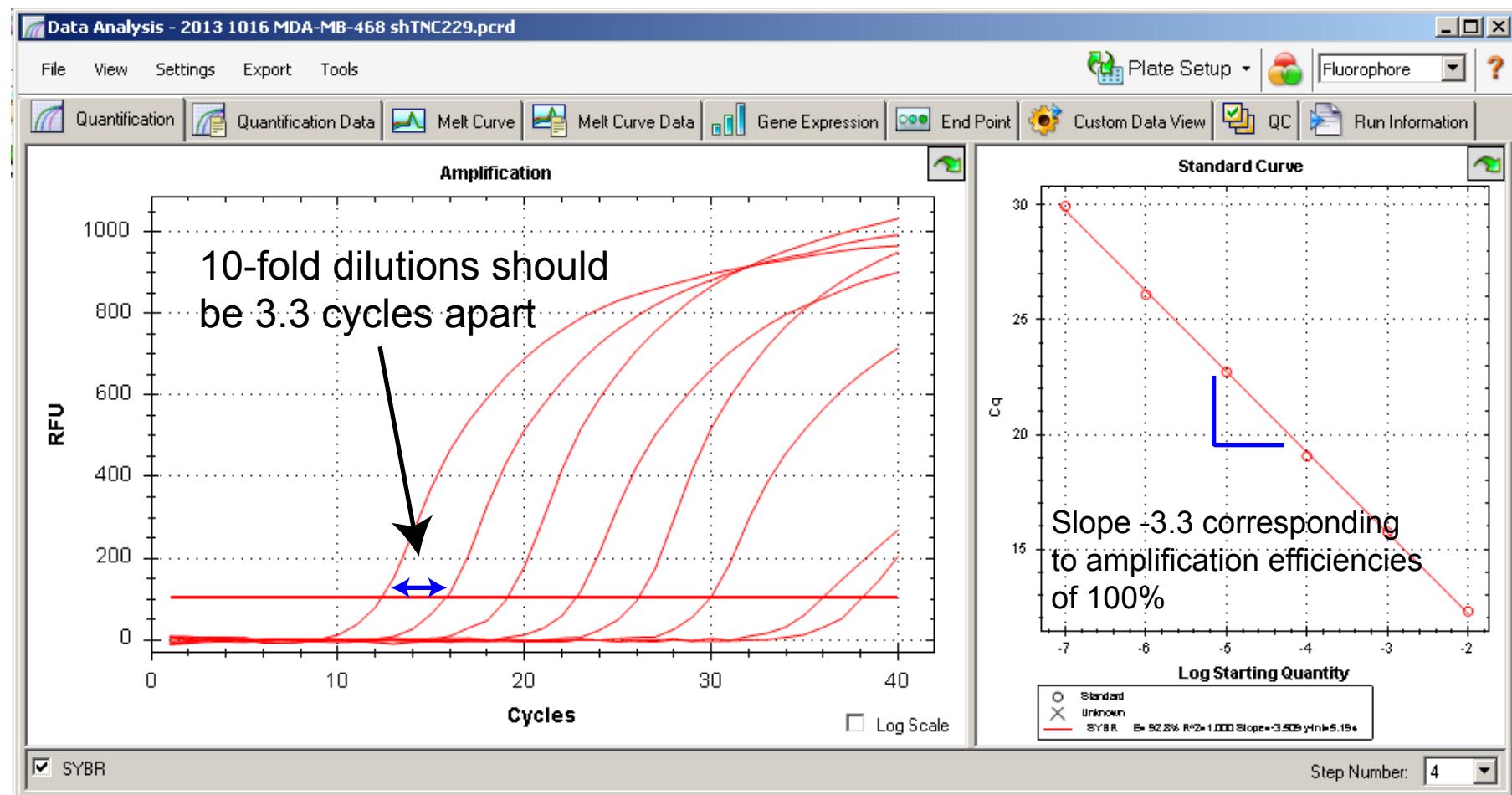
Order 2 sets of primer pairs
from a low-cost provider of custom synthesis

We order 25 nmole with standard desalting.
The cost is \$0.35 per base
in the scale of 15-60 bps.

Primer validation

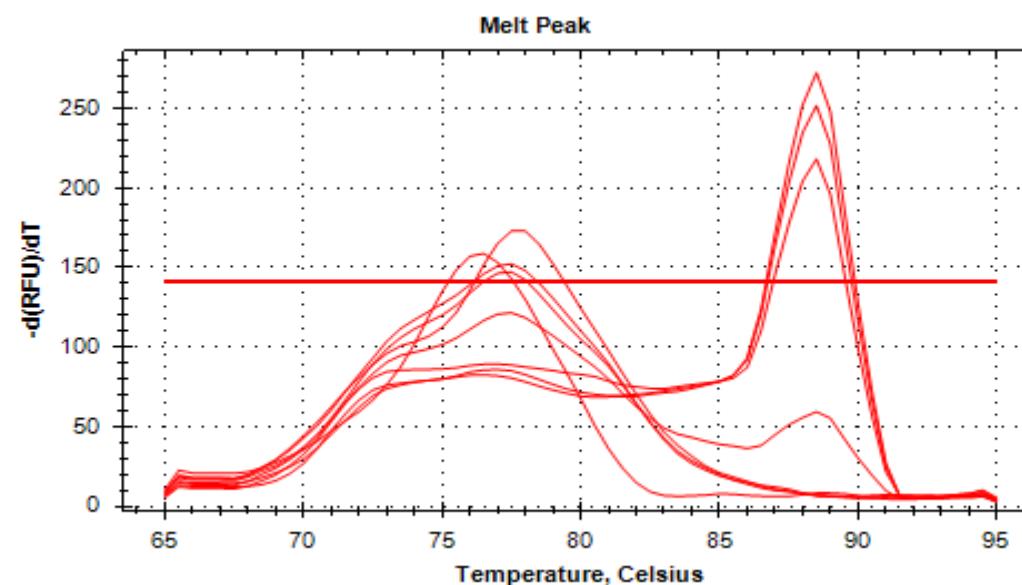
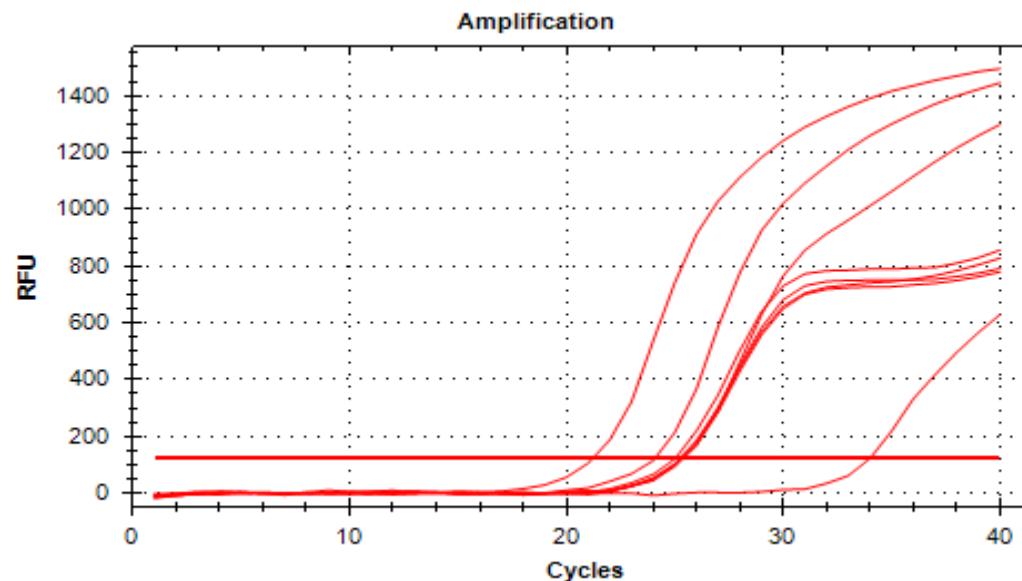
Verify that the single product is produced:
first-stand cDNA sample vs the blank sample
in melting curve or gel electrophoresis.

Quantitative accuracy and amplification efficiency:



Considerations for qPCR of stochastic samples

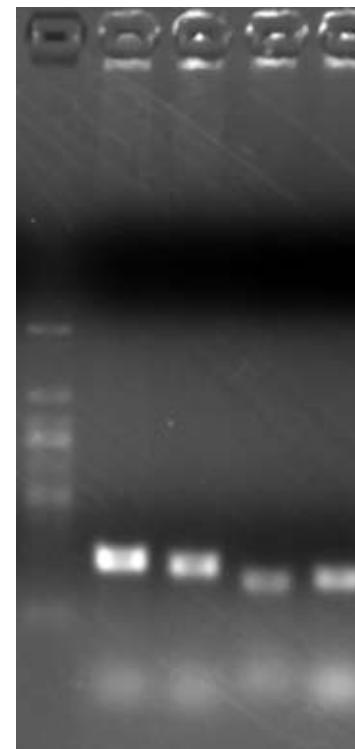
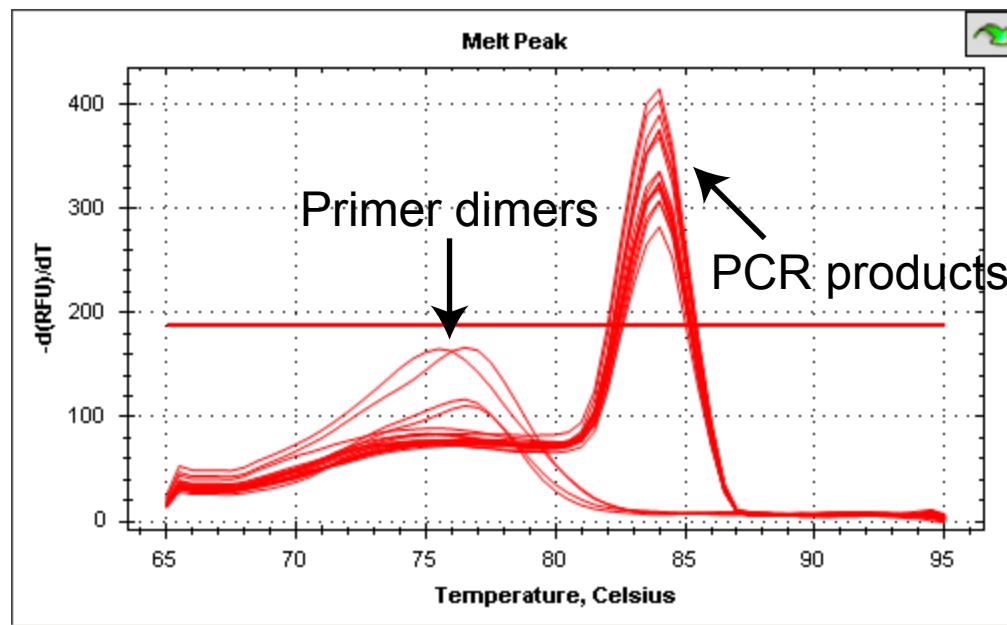
Large dilution in H₂O to reduce competition from AL1 primer



Troubleshooting primers

Primer dimers:

Detections: melting curve, gel electrophoresis



◀ PCR products

◀ Primer dimers

Troubleshooting primers

Primer dimers:

Detections: melting curve, gel electrophoresis

Solutions:

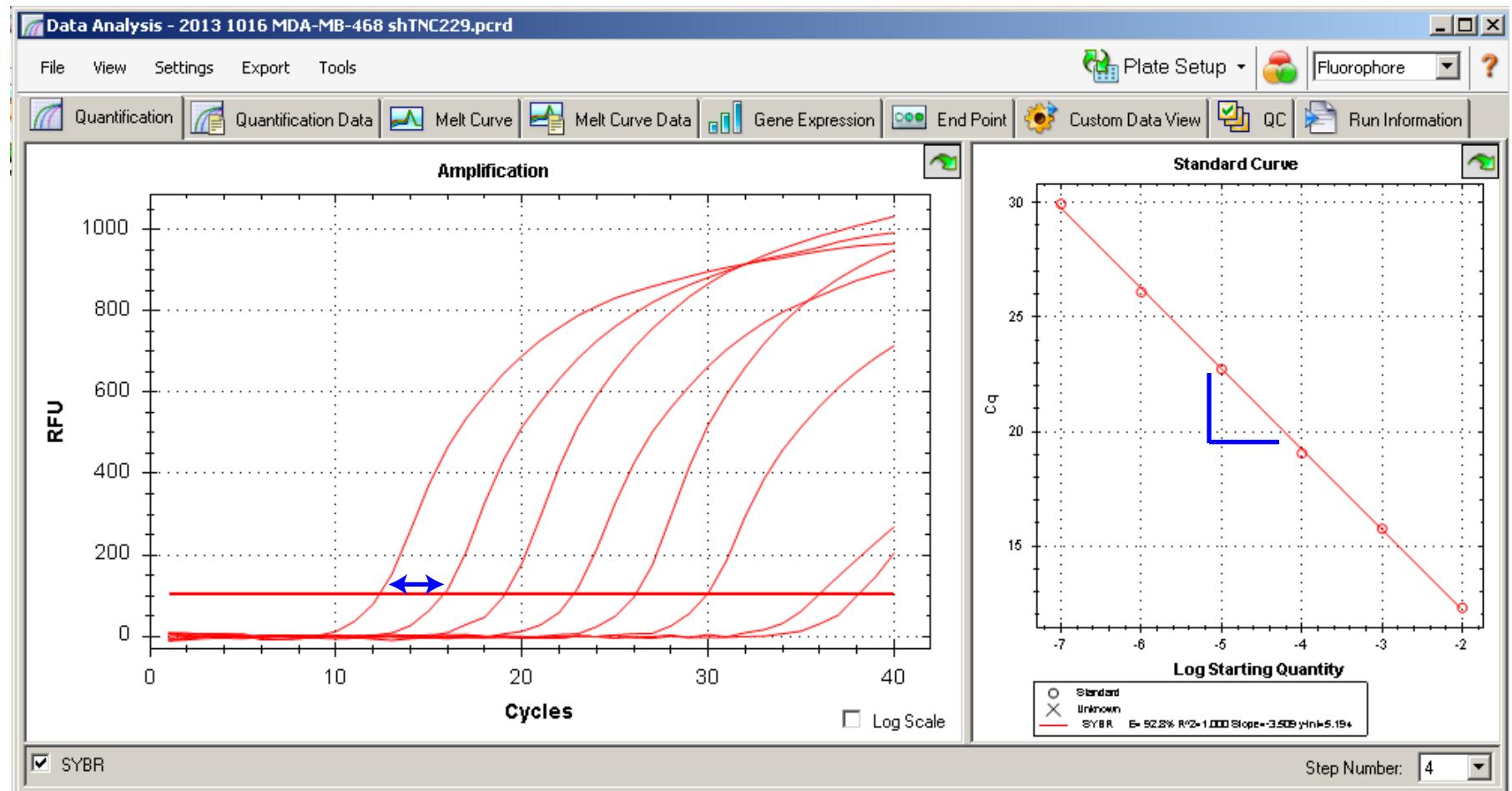
proper primer design that prevents the formation of hairpins, self dimers, and cross dimers.

decreasing primer concentration two-fold in the RT-qPCR reaction

Troubleshooting primers

Amplification efficiencies of qPCR primer:

Slope of standard curve in the range of -2.9 to -3.9,
corresponding to amplification efficiencies (E) of 80-120%



Troubleshooting primers

Amplification efficiencies of qPCR primer:

Low efficiencies:

Main reasons:

bad primer design (2nd structure,
not appropriate Tm);

not-optimal reagent concentration.

Solutions:

increasing the primer concentration two-fold
in the RT-qPCR reaction

Troubleshooting primers

Amplification efficiencies of qPCR primer:
exceedingly high efficiencies:

Reasons:

1. the presence of inhibitors of polymerase enzyme in cDNA samples.
Most concentrated samples should be omitted.

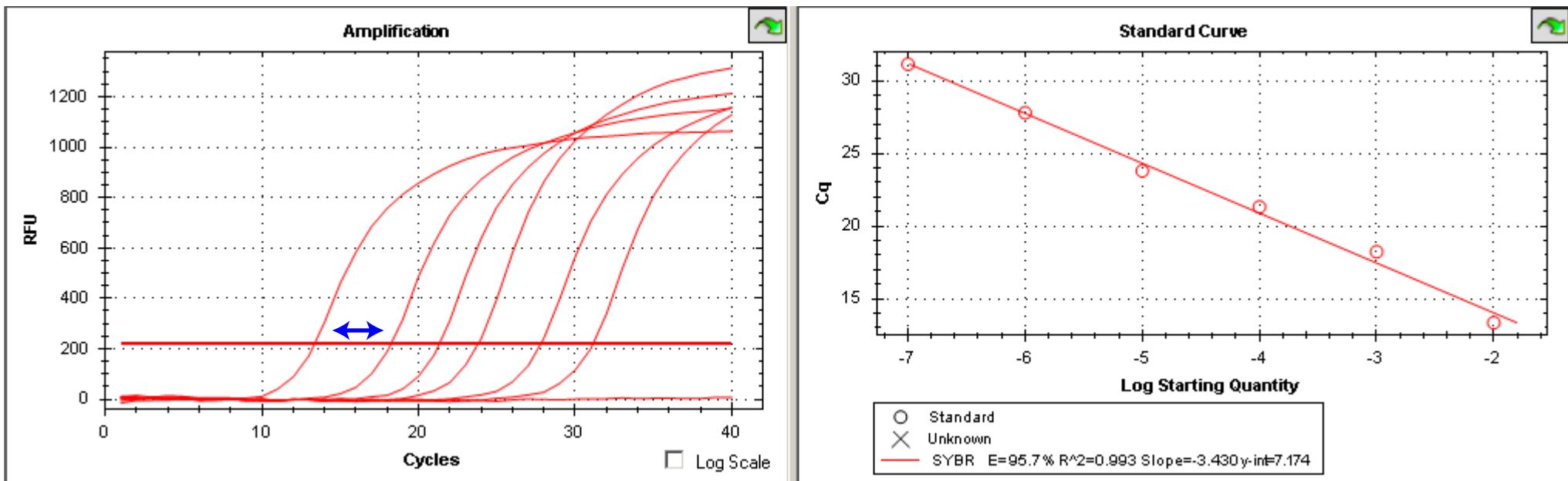
2. primer dimers:

Decreasing the primer concentration two-fold
in the RT-qPCR reaction

3. contamination, inappropriate dilution series

Troubleshooting primers

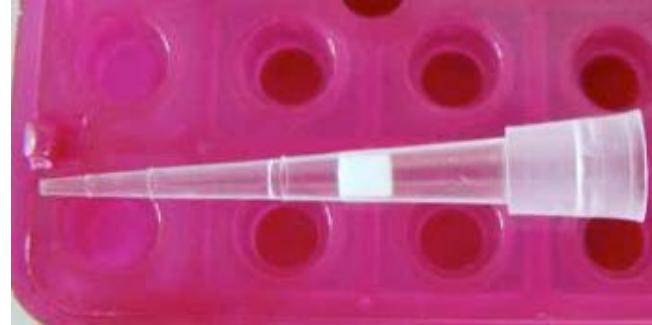
Pipetting issues:



Troubleshooting primers

Pipetting issues:

use barrier tips



In serial dilution for a standard curve:

1. change tips to prevent the carryover on the tips.
2. not to vortex the tubes so vigorously that the liquid hits the cap and produces contamination when opening it.

Troubleshooting amplifications

qPCR cycle thresholds are all very low (< 15)

Possible reason: cDNA is overamplified

Solution: Reduce AL1 primer amount or PCR cycle numbers

qPCR cycle thresholds are all very high (> 25)

Possible reason: RNA in tissue is degraded or amplification is defective

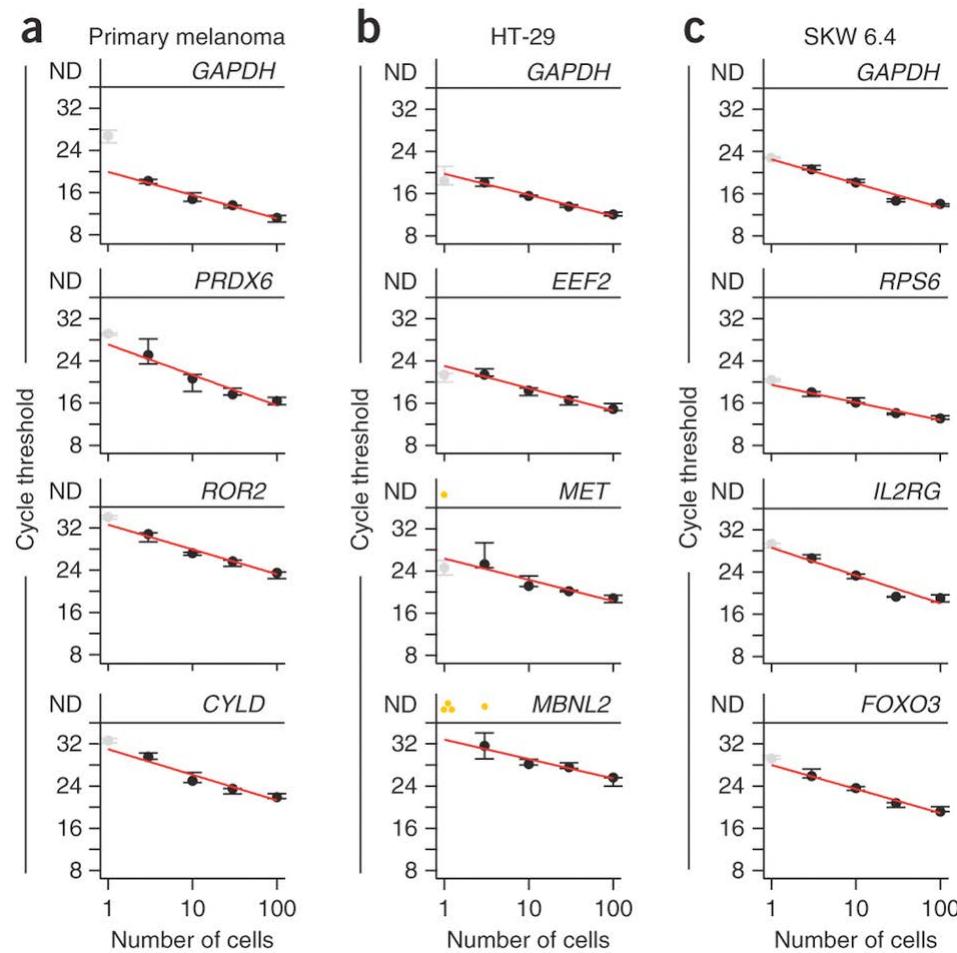
Solution: Perform an amplification with ~100 pg of purified RNA

Possible reason: cDNA is underamplified

Solution: Increase AL1 primer amount or PCR cycle numbers

Anticipated results

Optimized small-sample cDNA amplifications in three distinct biological contexts



What we are going to do in the lab today:

1. Standard curve with purified amplicon

high-abundant genes: GAPDH, HINT1

middle-abundant gene: MRPL33

low abundance gene: ANGPTL4

2. Optimization with Stochastic Profiling Samples

Standard curve with purified amplicon

	1	2	3	4
A	NTC	NTC	NTC	NTC
B	standard -2	standard -2	standard -2	standard -2
C	-3	-3	-3	-3
D	-4	-4	-4	-4
E	-5	-5	-5	-5
F	-6	-6	-6	-6
G	-7	-7	-7	-7
H				
initials, gene names				

Thermocycler #:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
initials, gene names												

Standard curve with purified amplicon

Optimization with Stochastic Profiling Samples

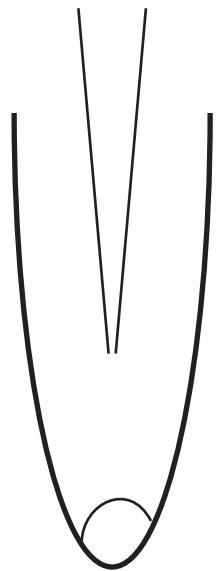
	1	2	3
A	NTC	AL1: 10 µg 25	AL1: 25 µg 25
B	NRT	30	30
C		35	35
D		40	40
E	AL1: 5 µg cycle 25	AL1: 15 µg 25	AL1: 50 µg 25
F	cycle 30	30	30
G	cycle 35	35	35
H	cycle 40	40	40
initial and gene name			

Thermocycler #:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
initials, gene names												

Optimization with Stochastic Profiling Samples

qPCR setup

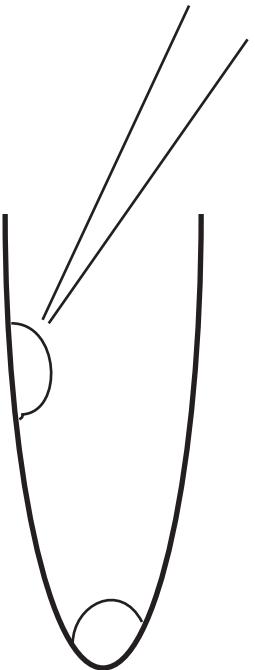


4.5 μ l of each samples

Standard Curve: dilute 1 μ l of amplicon of **449 μ l** of H₂O

Stochastic Profiling Samples: Step 50 in Nature Protocol paper
dilute 1 μ l of amplicon of **449 μ l** of H₂O

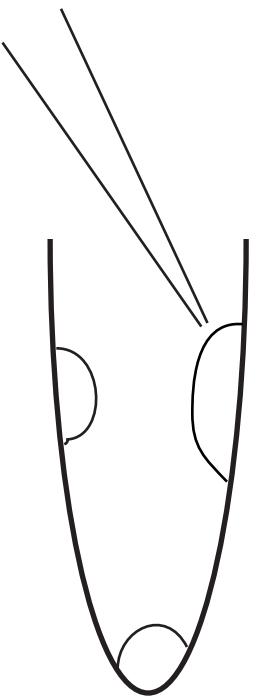
qPCR setup



3 µl of primer master mix

Fwd primer
Rev primer
H₂O

qPCR setup



7.5 µl of
2X RT-qPCR master mix

2 mm
Taq DNA polymerase
100x SYBR