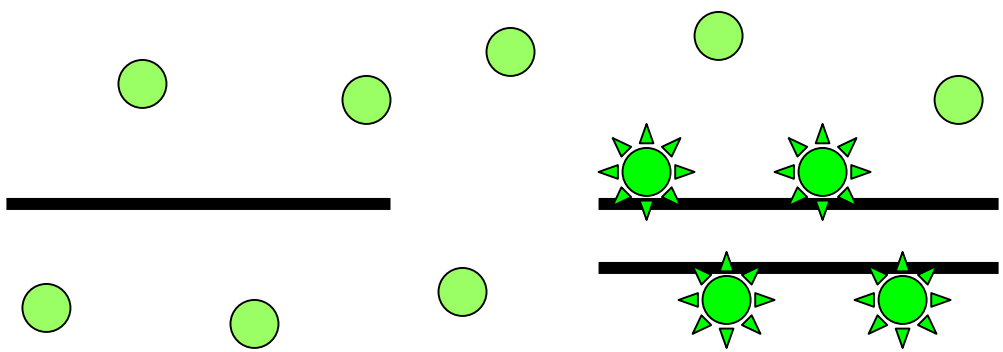
REAL TIME PCR

USING SYBR GREEN



THE PROBLEM

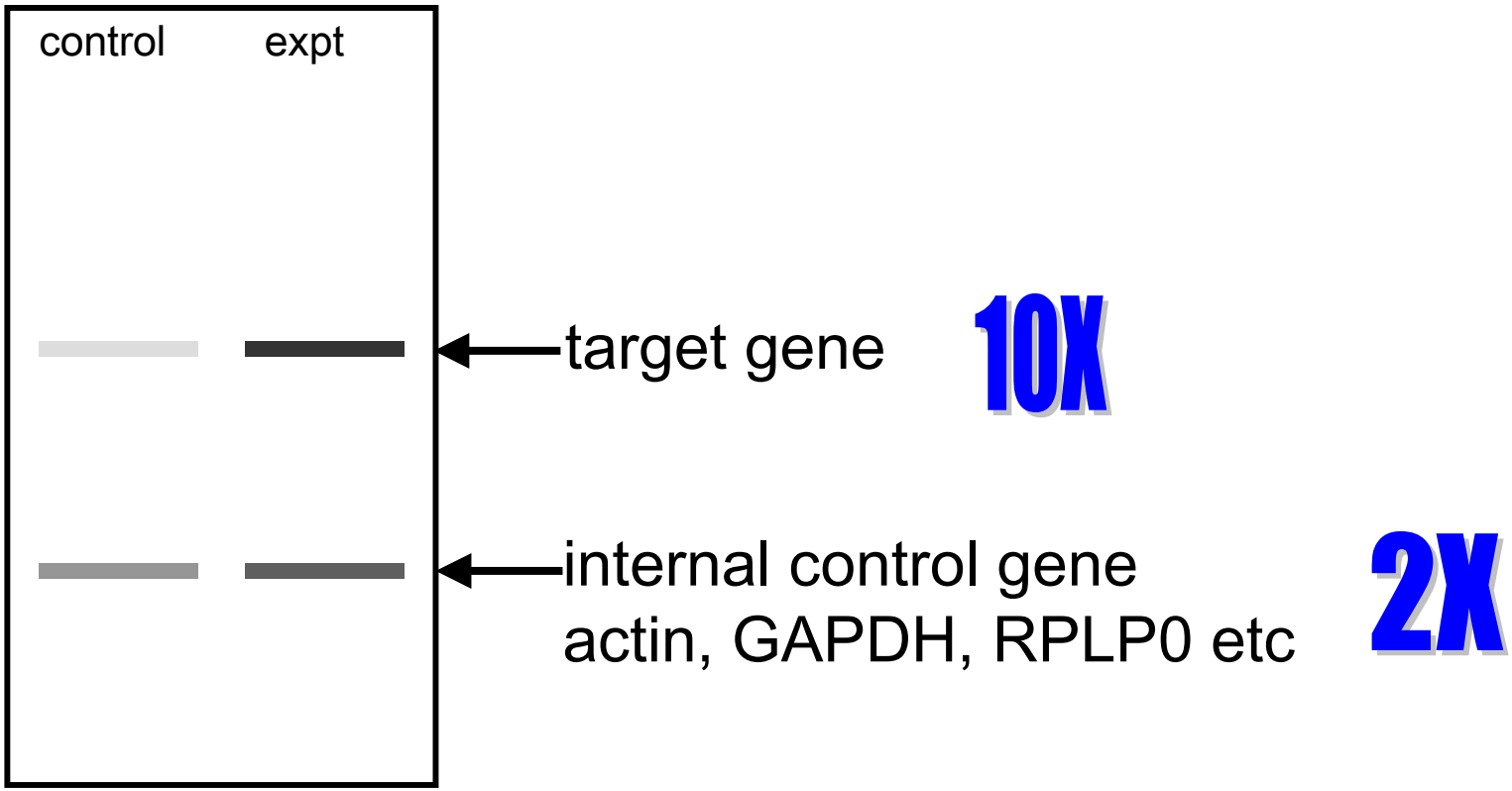
- NEED TO QUANTITATE DIFFERENCES IN mRNA EXPRESSION

- SMALL AMOUNTS OF mRNA
  - LASER CAPTURE
  - SMALL AMOUNTS OF TISSUE
  - PRIMARY CELLS
  - PRECIOUS REAGENTS

THE PROBLEM

- QUANTITATION OF mRNA
  - northern blotting
  - ribonuclease protection assay
  - in situ hybridization
  - PCR
    - most sensitive
    - can discriminate closely related mRNAs
    - technically simple
    - **but** difficult to get truly quantitative results using conventional PCR

NORTHERN



Corrected fold increase = 10/2 = 5

$$\text{Ratio target gene in experimental/control} = \frac{\text{fold change in target gene}}{\text{fold change in reference gene}}$$

Standards

- same copy number in all cells
- expressed in all cells
- medium copy number advantageous
  - correction more accurate

Standards

- **The perfect standard does not exist**

Standards

- Commonly used standards
  - Glyceraldehyde-3-phosphate dehydrogenase mRNA
  - Beta-actin mRNA
  - MHC I (major histocompatability complex I) mRNA
  - Cyclophilin mRNA
  - mRNAs for certain ribosomal proteins
    - **E.g. RPLP0 (ribosomal protein, large, P0; also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0)**
  - 28S or 18S rRNA

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
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10	1,024
11	2,048
12	4,096
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14	16,384
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18	262,144
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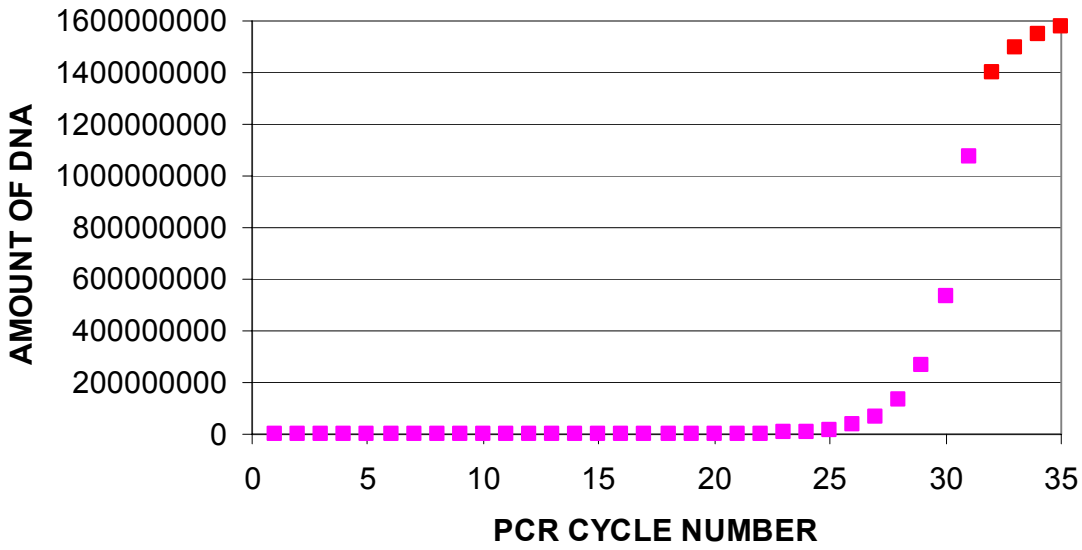
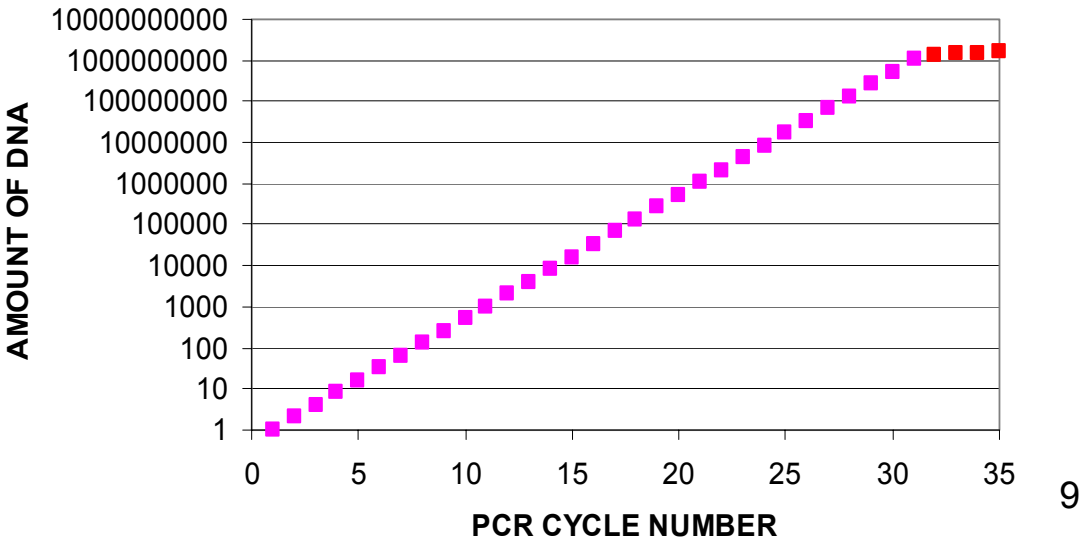
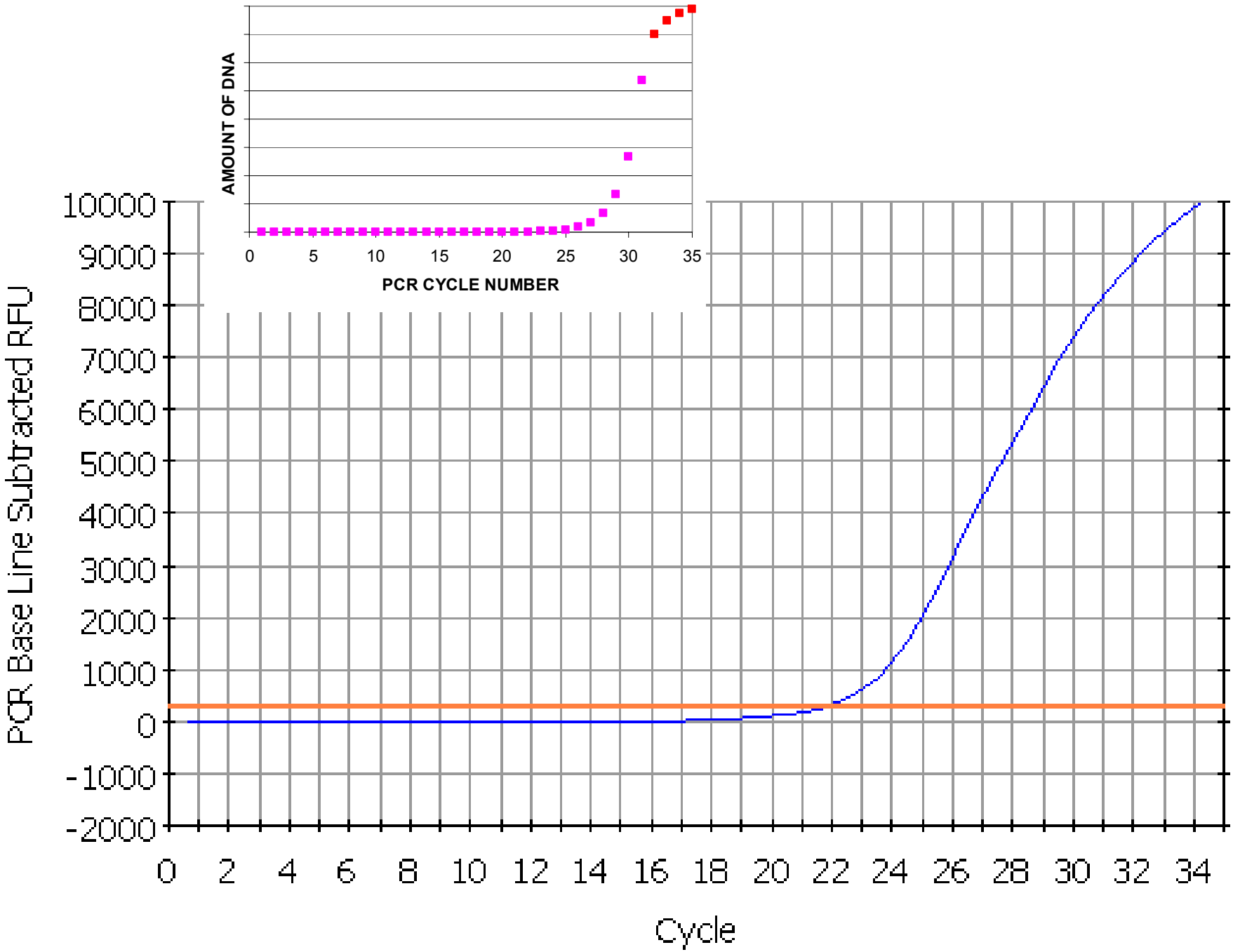
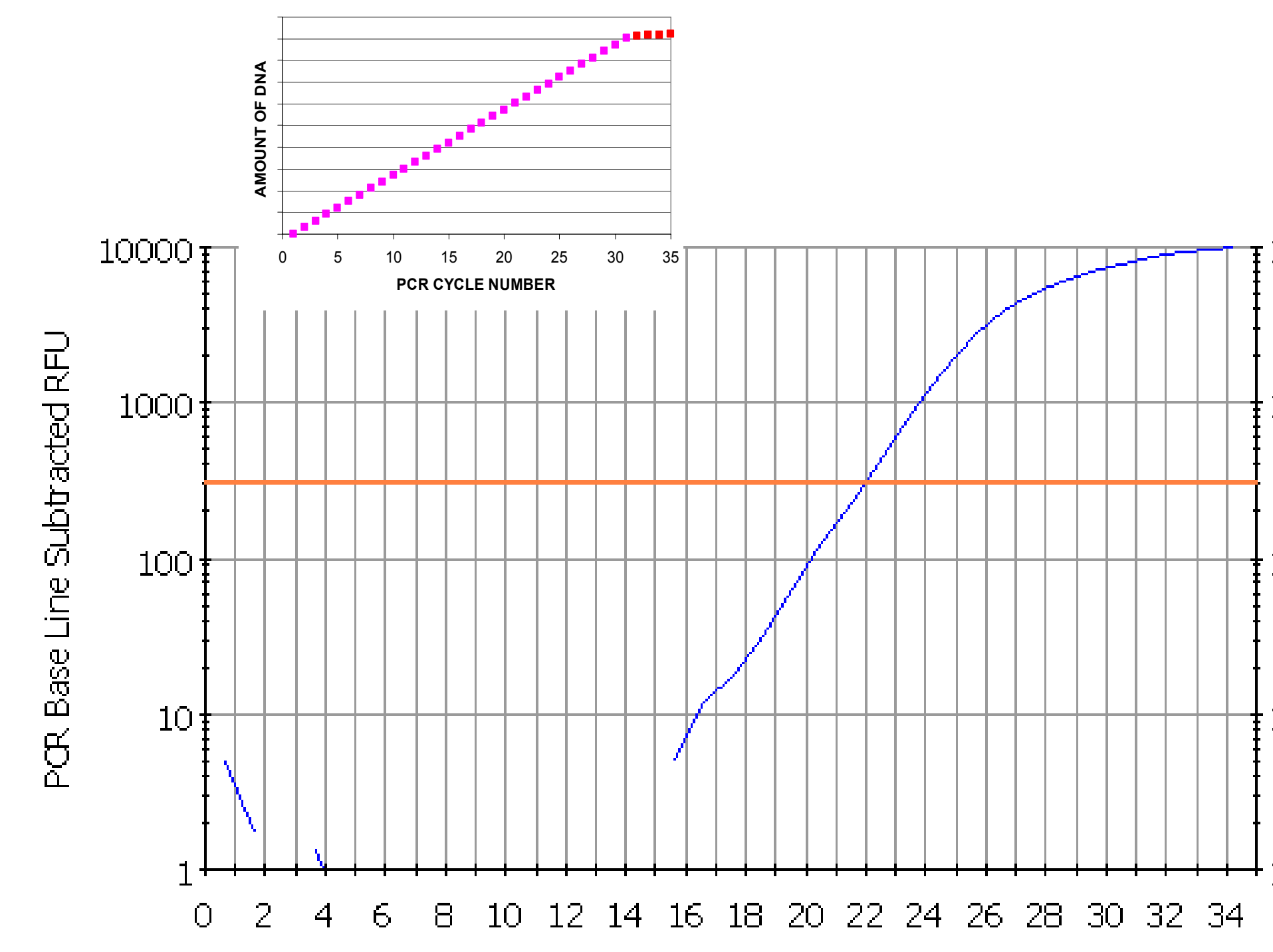
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

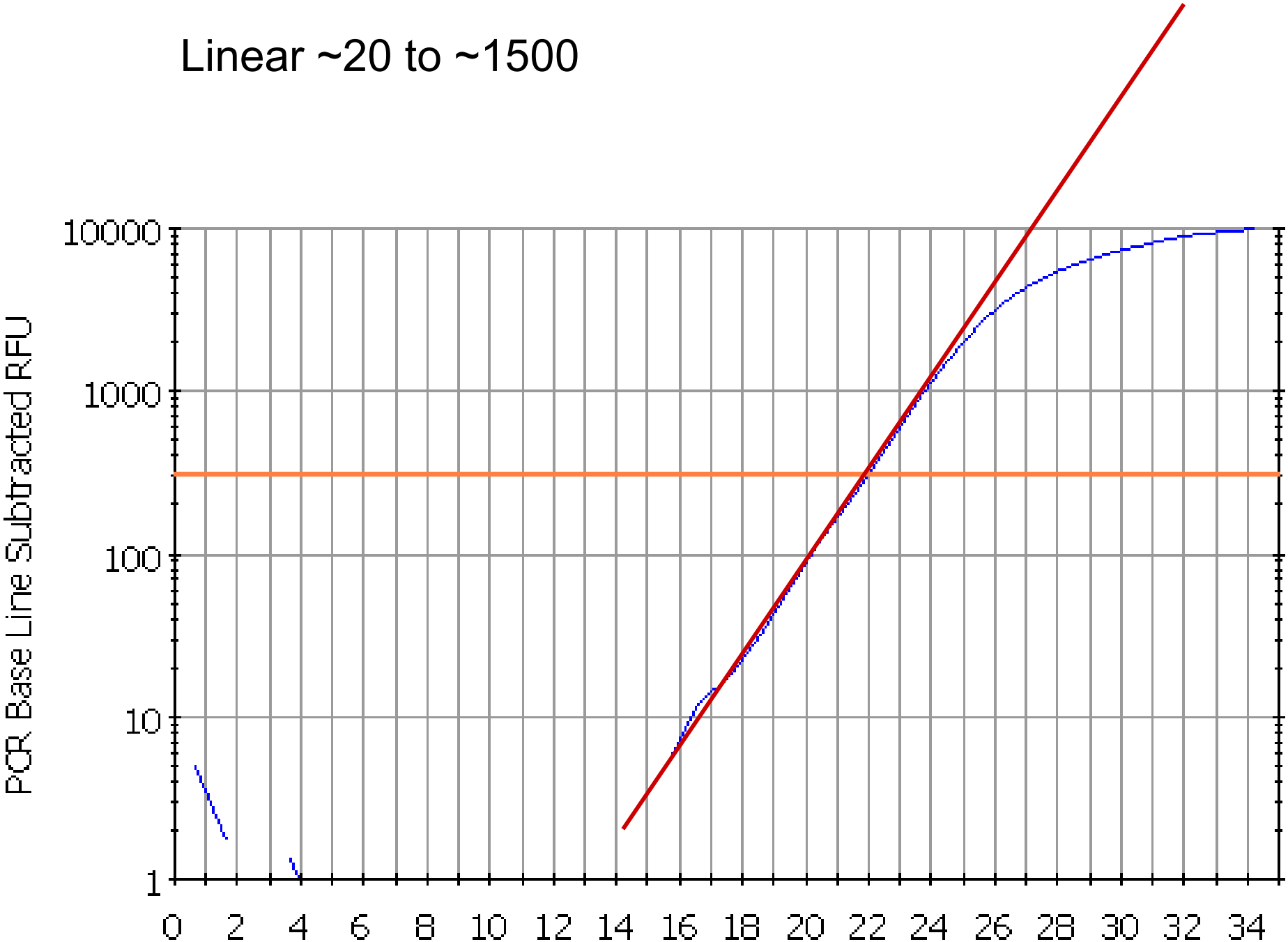




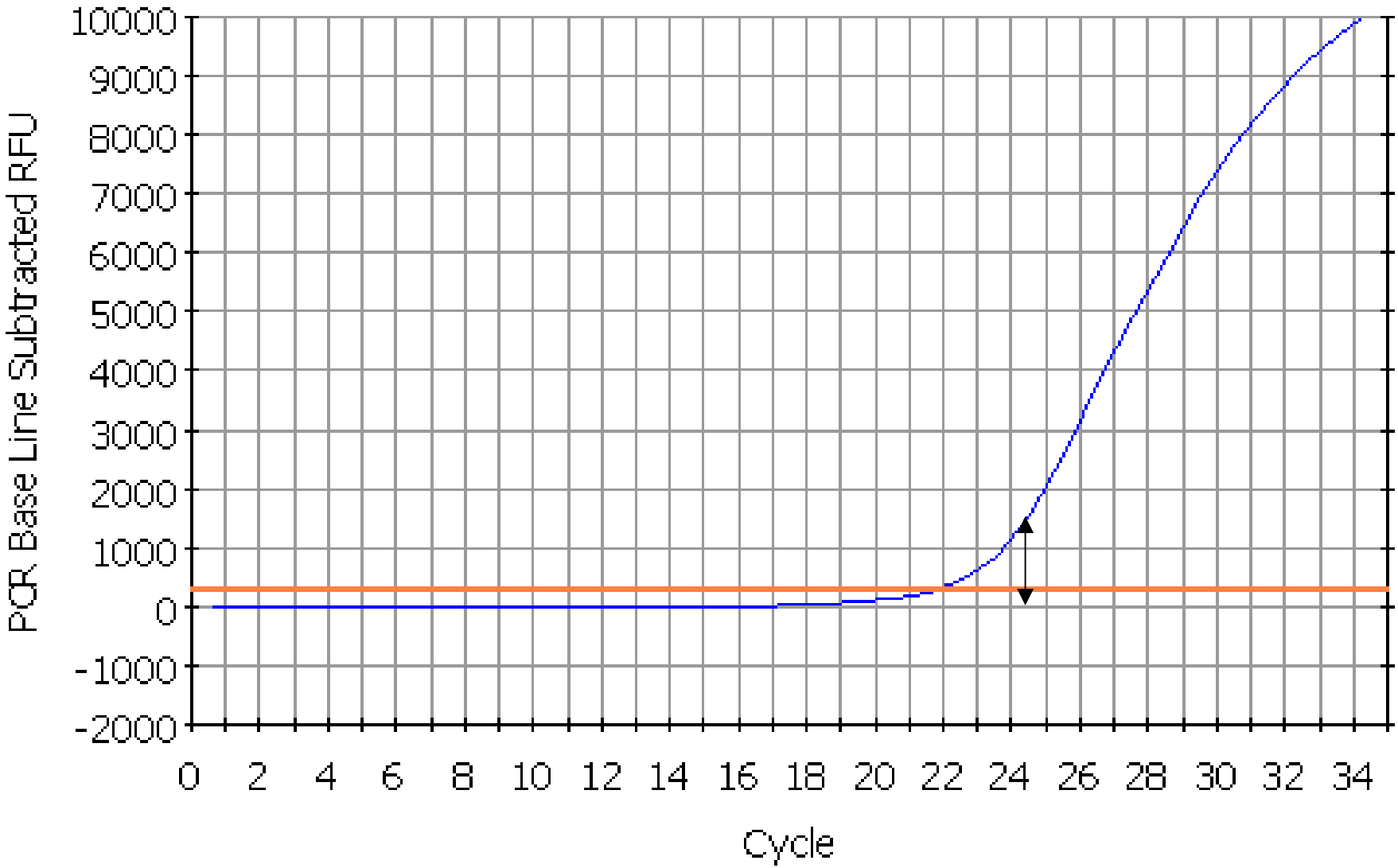

AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Cycle


AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

Linear ~20 to ~1500

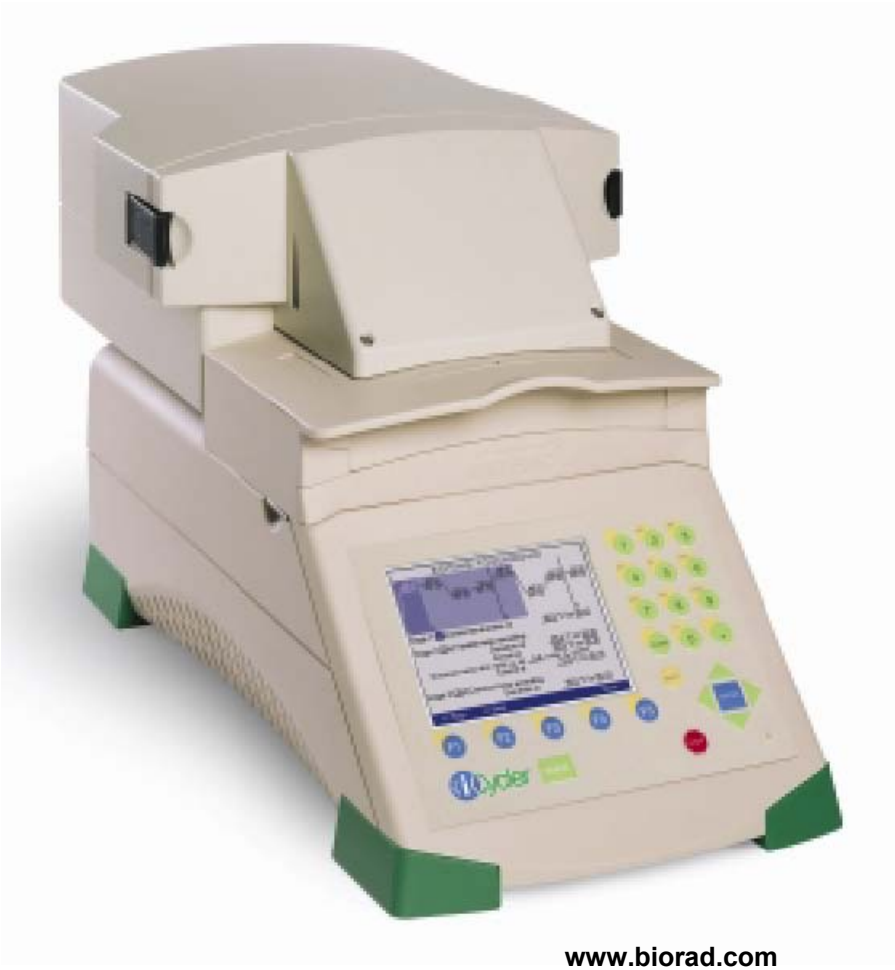


Linear ~20 to ~1500



REAL TIME PCR

- kinetic approach
- early stages
- while still linear



www.biorad.com

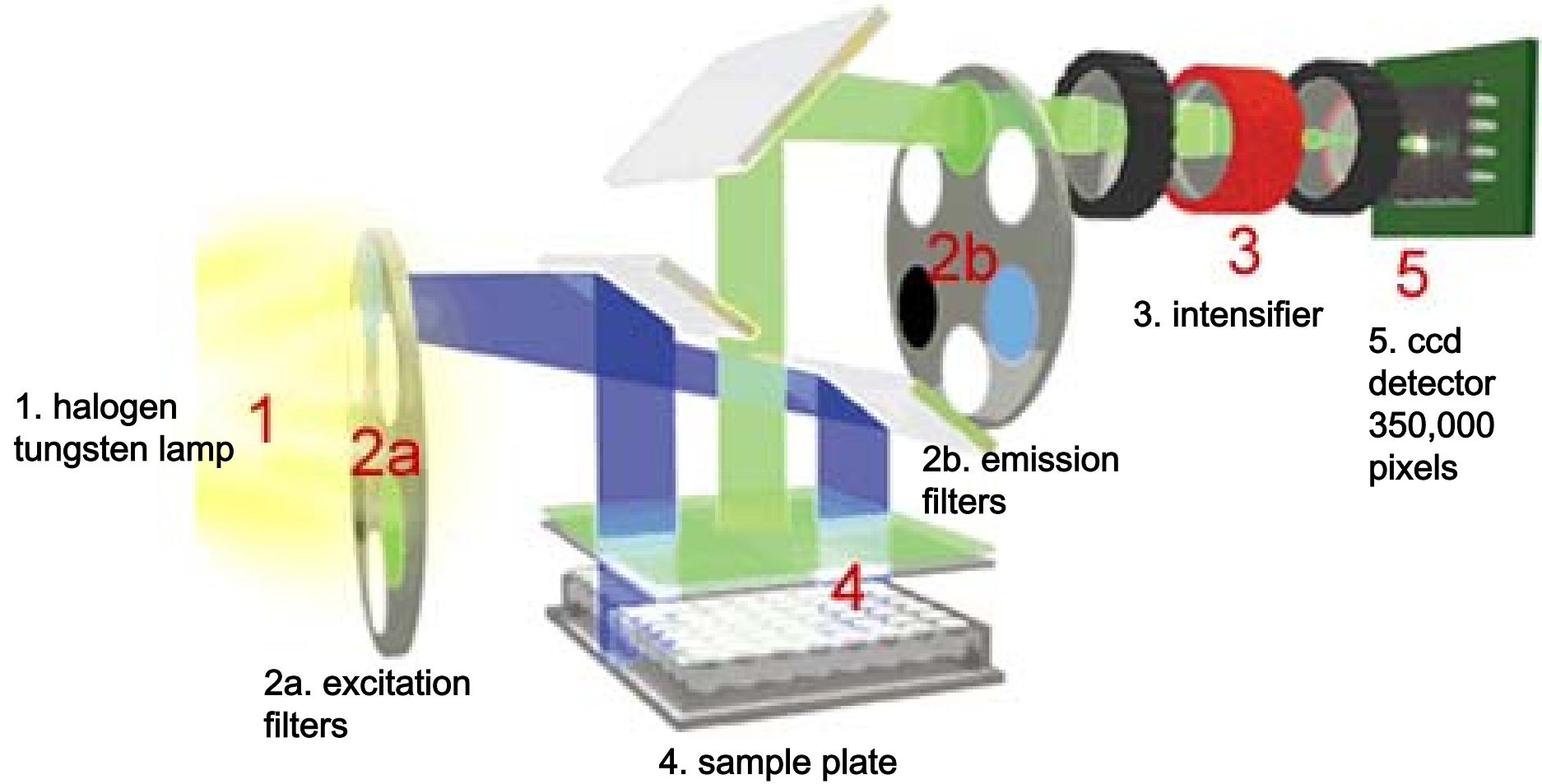


Fig. 1.2. Representation of Optical Detection System layout.

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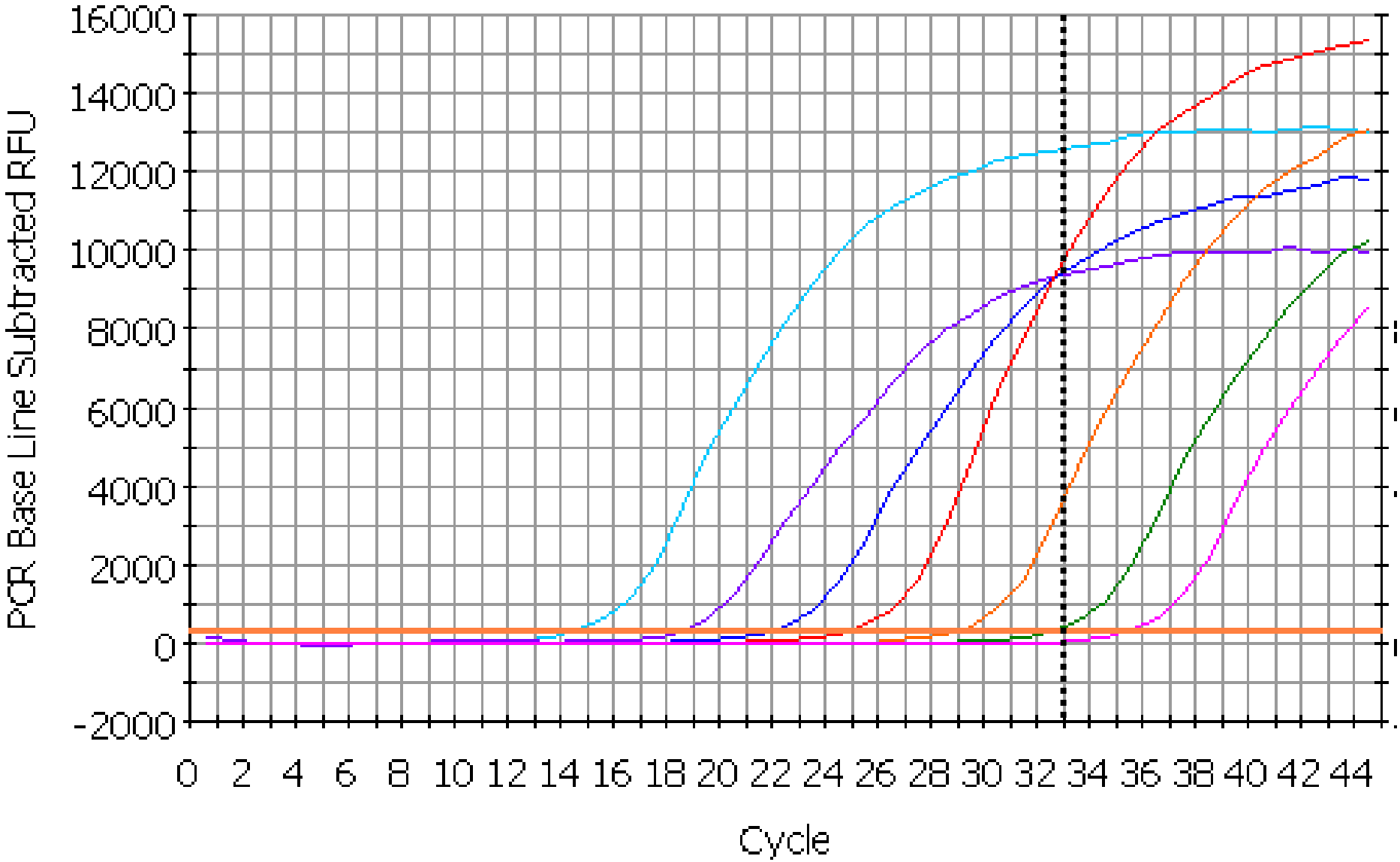
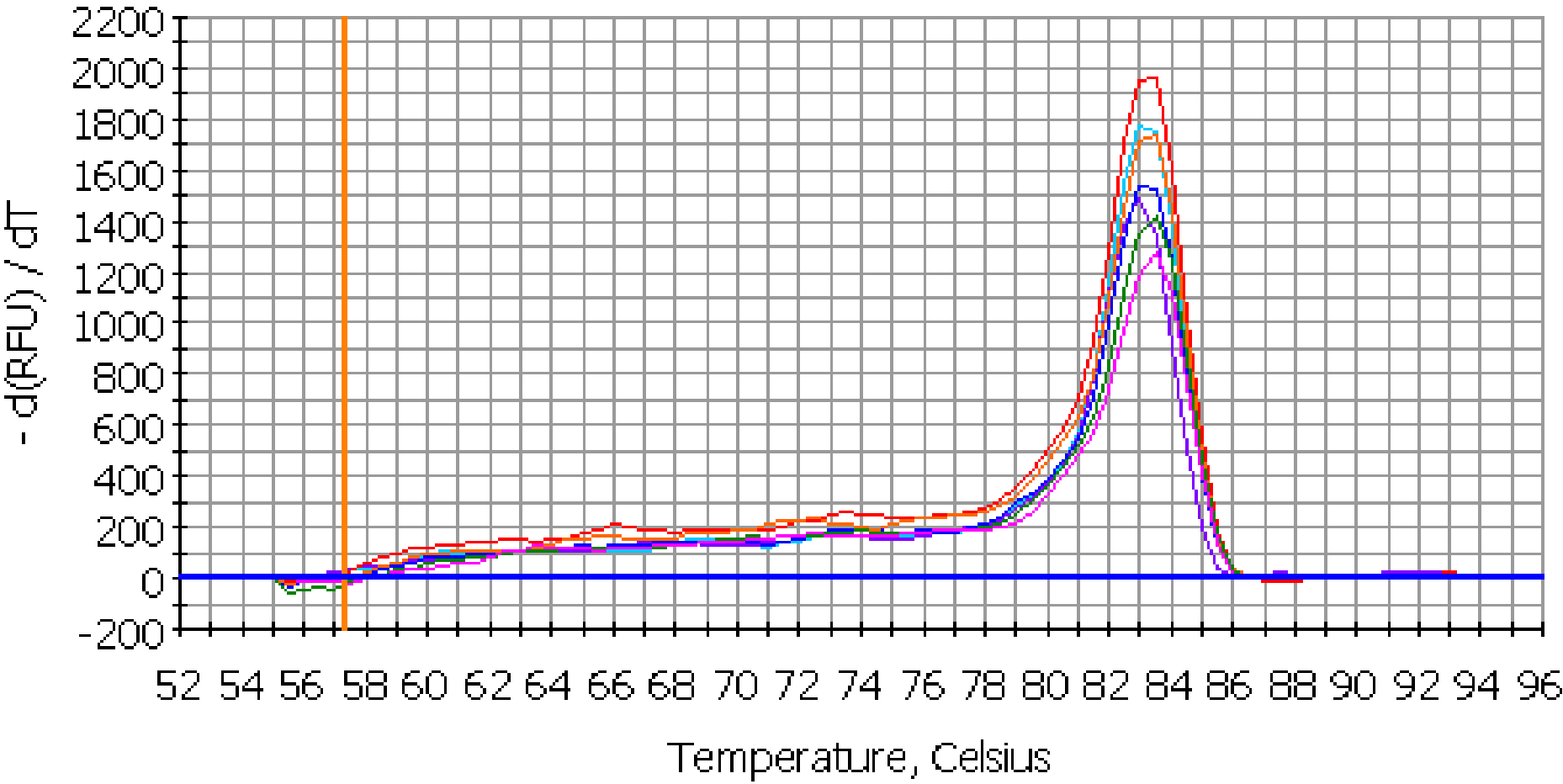
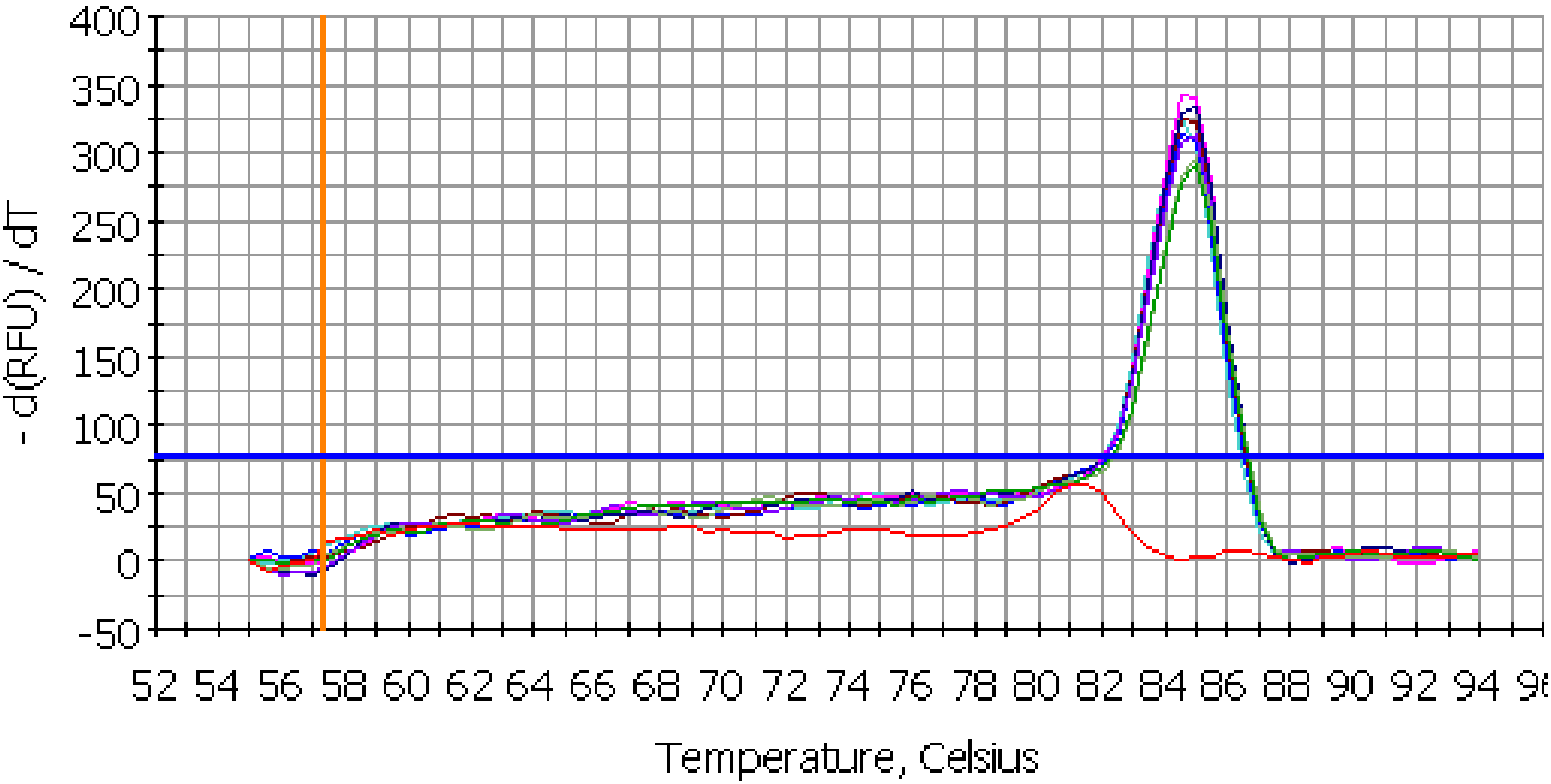
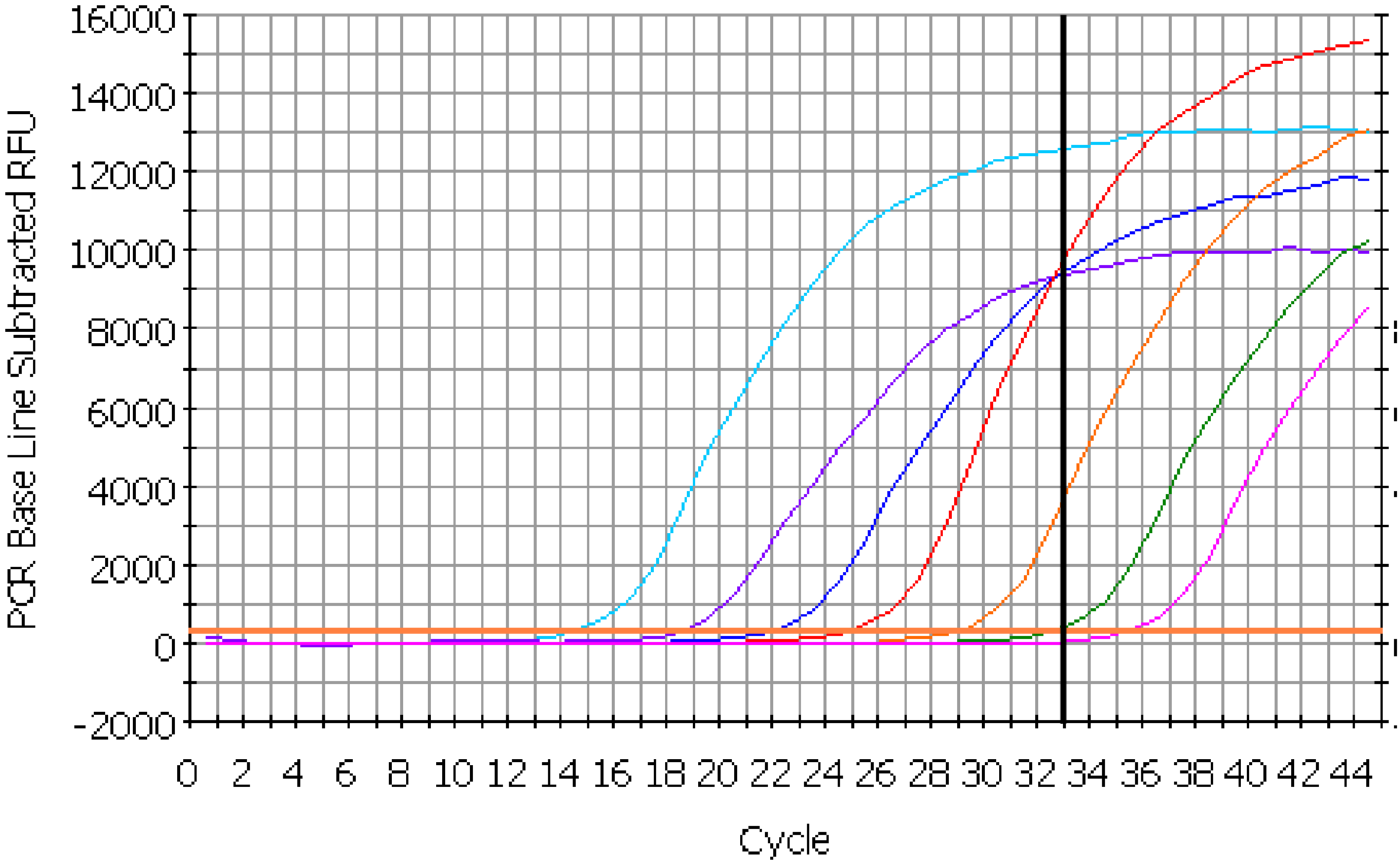


SERIES OF 10-FOLD DILUTIONS

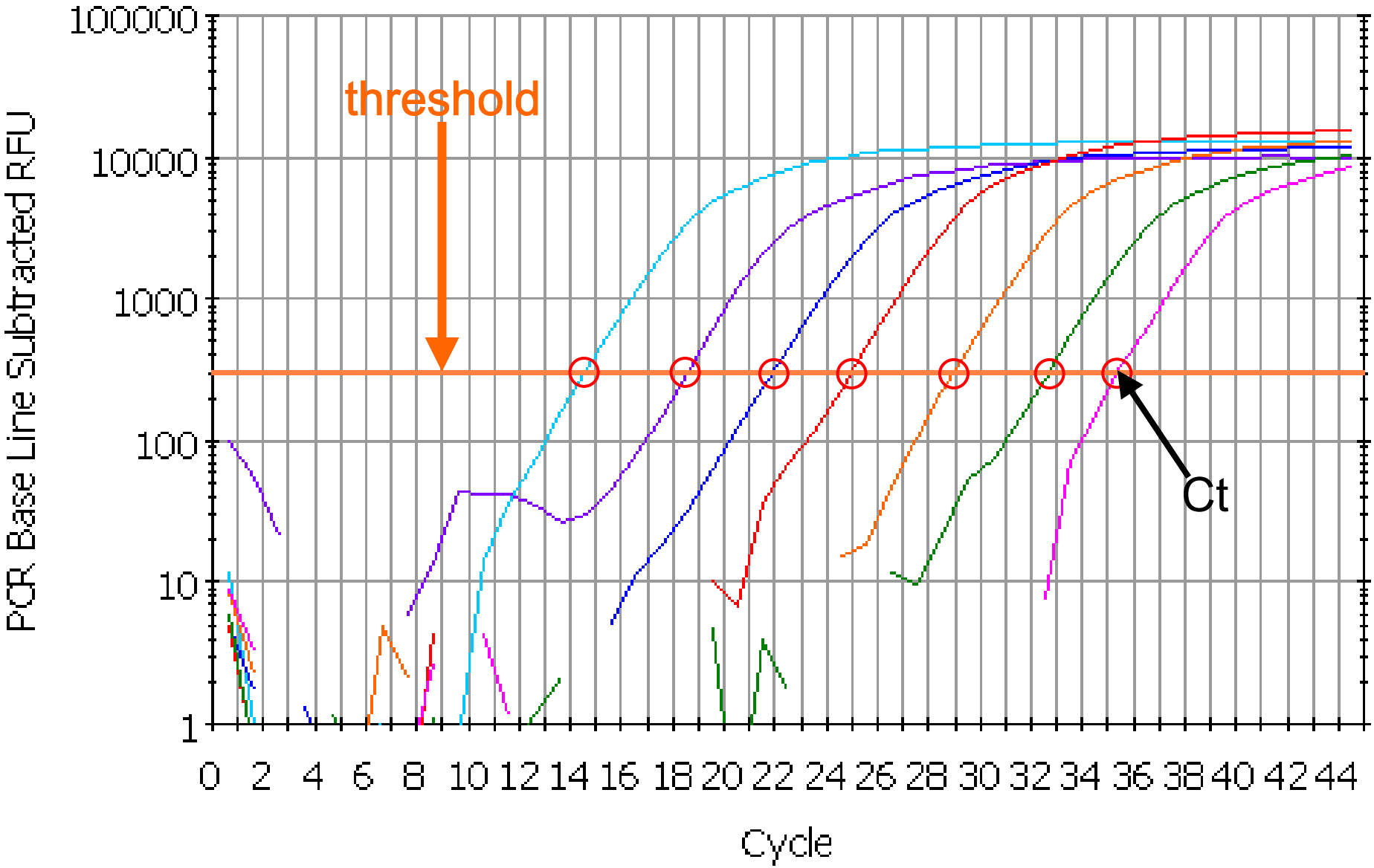

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
- d(RFU) / dT
52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96
Temperature, Celsius



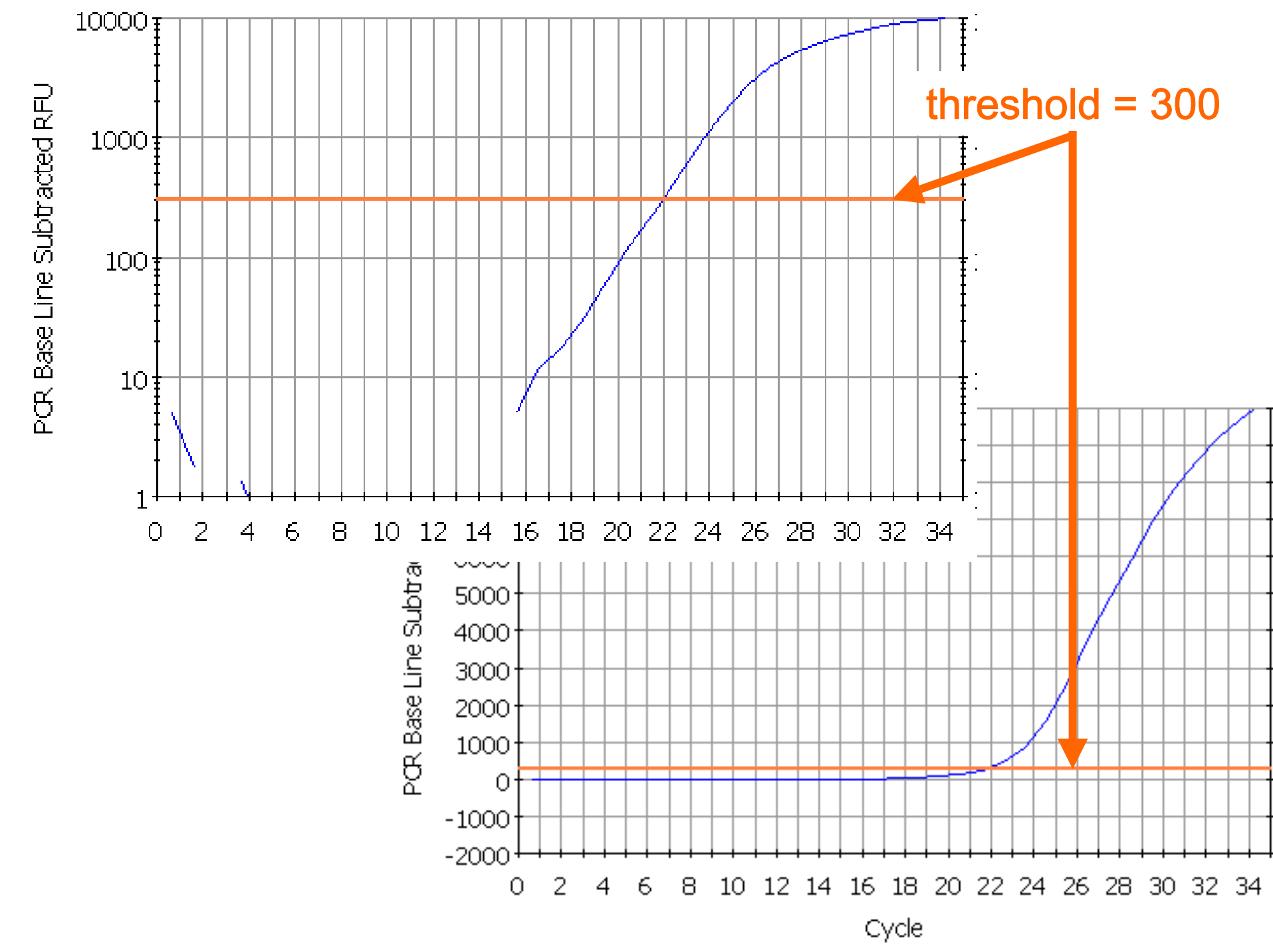
Melt Peak: Data 10-Mar-03 1259 ed.opd

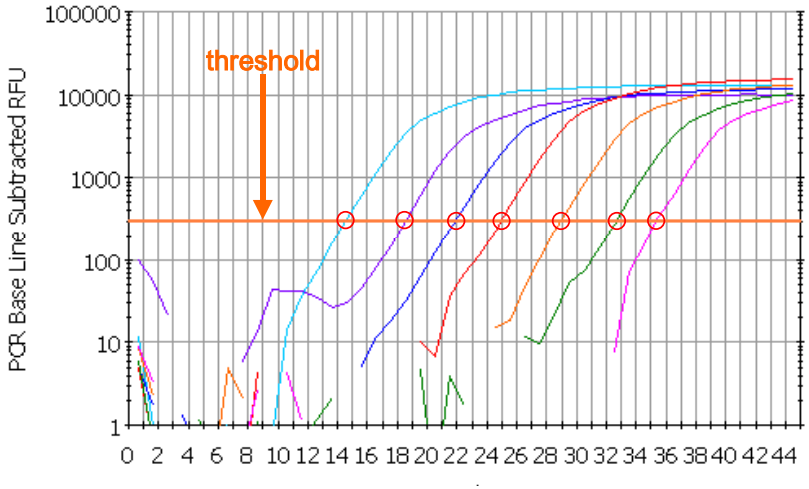


SERIES OF 10-FOLD DILUTIONS



SERIES OF 10-FOLD DILUTIONS

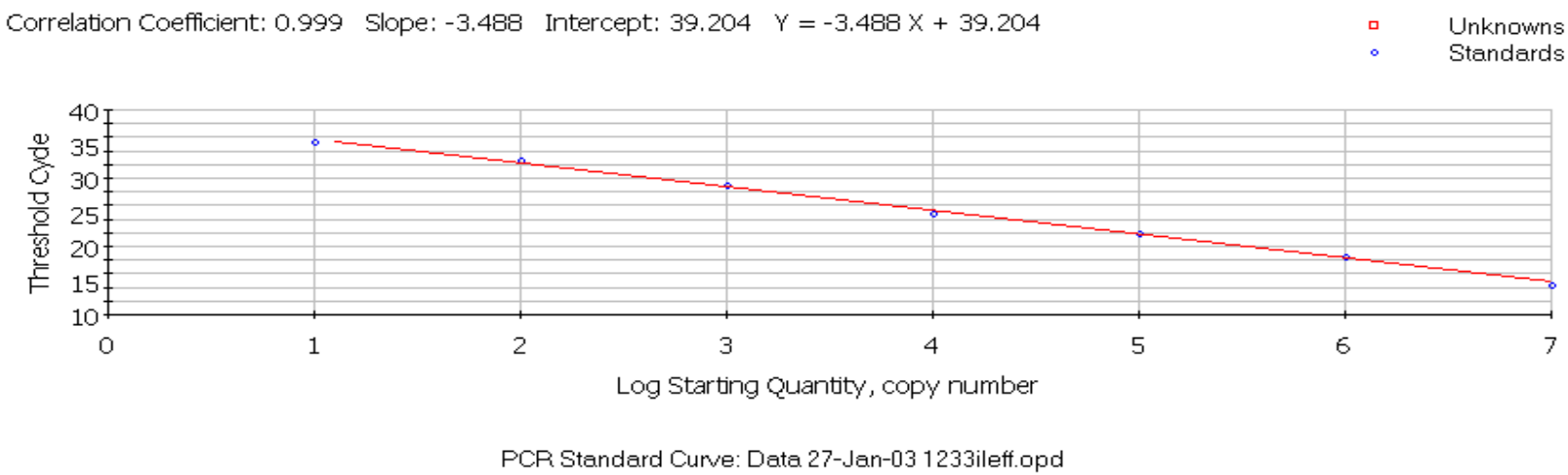

threshold = 300
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
PCR Base Line Subtra
5000
4000
3000
2000
1000
0
-1000
-2000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Cycle

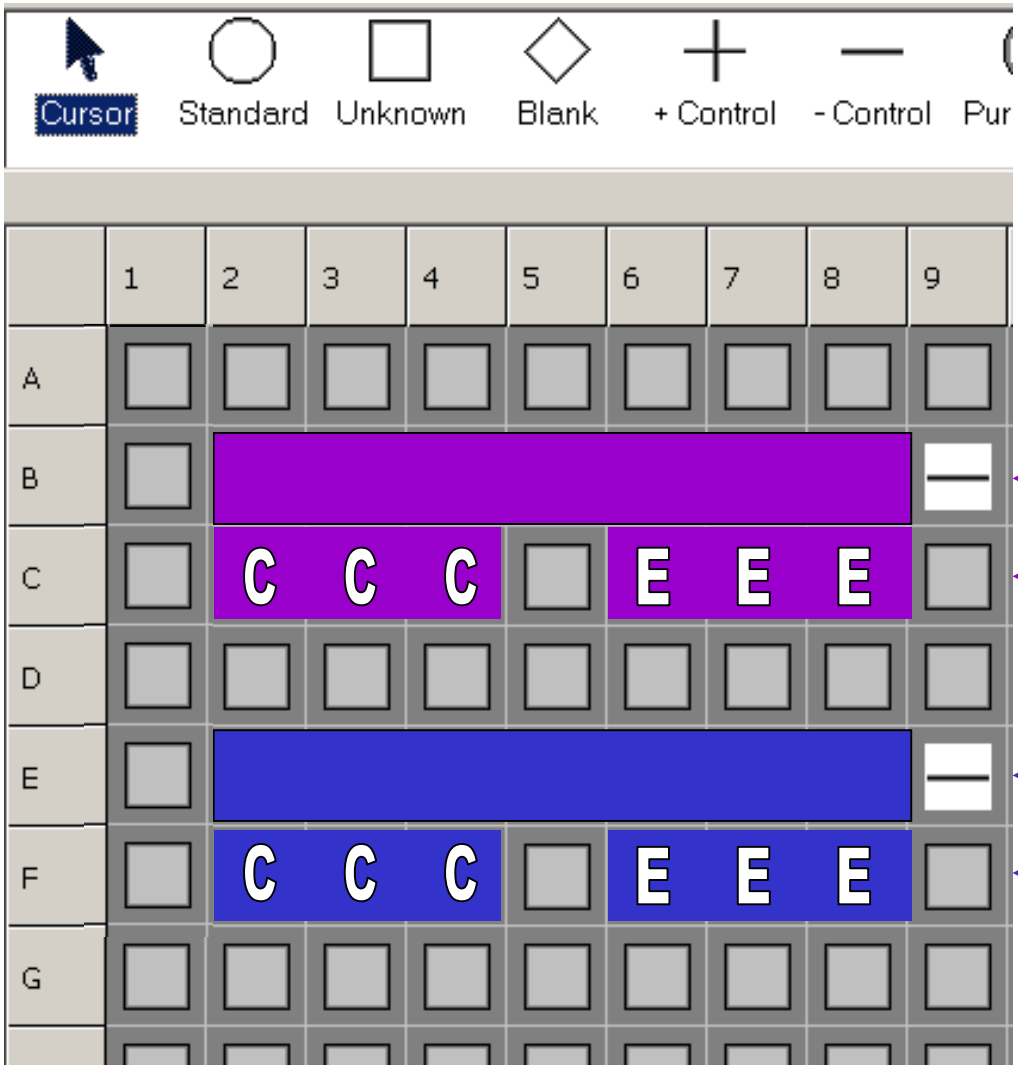


Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204

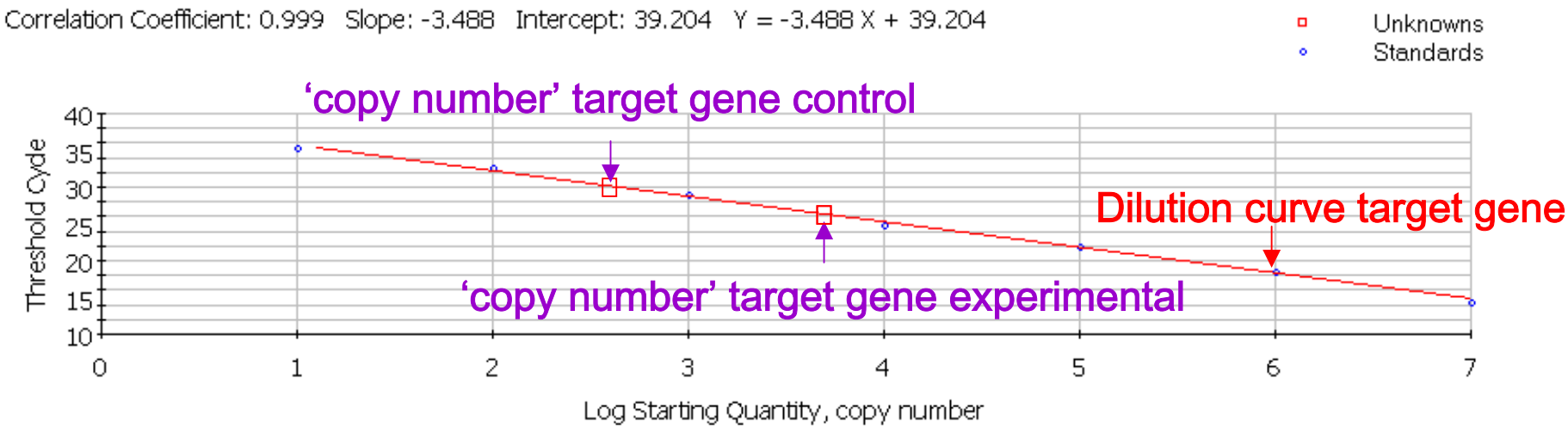
PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

STANDARD CURVE METHOD

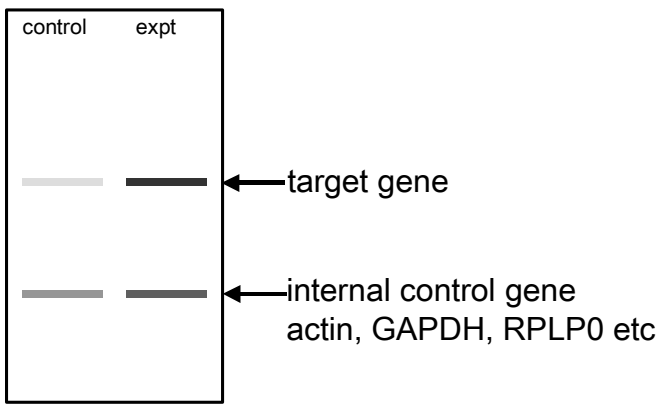




Standard curve method

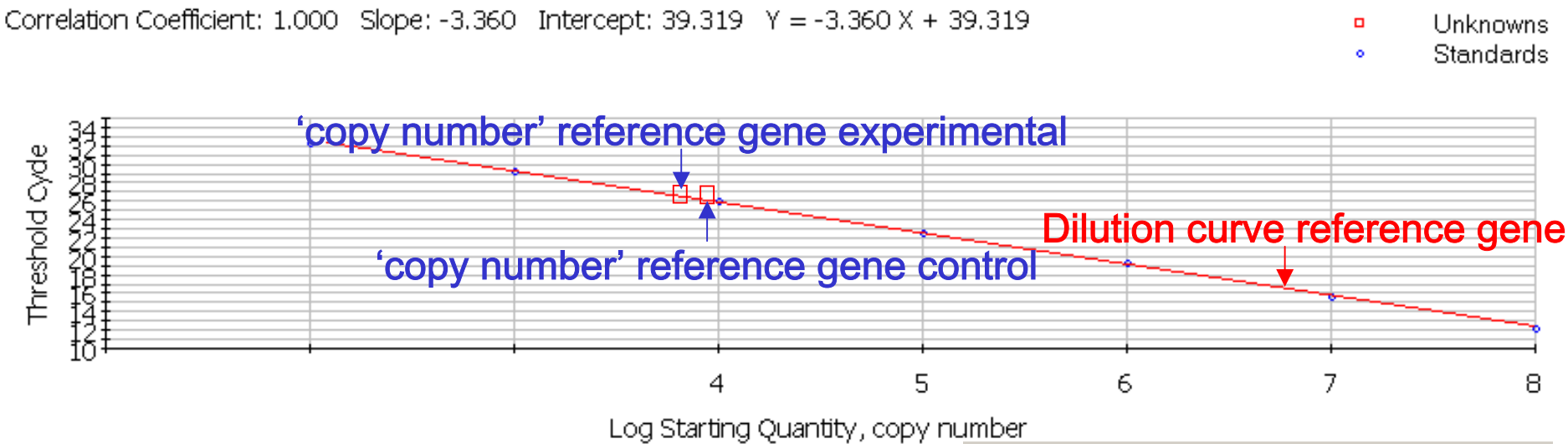


NORTHERN

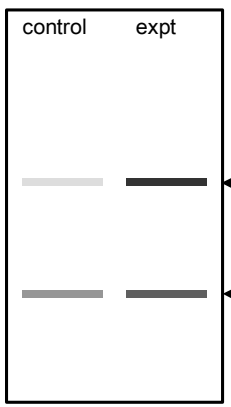


fold change in target gene= copy number experimental / copy number control

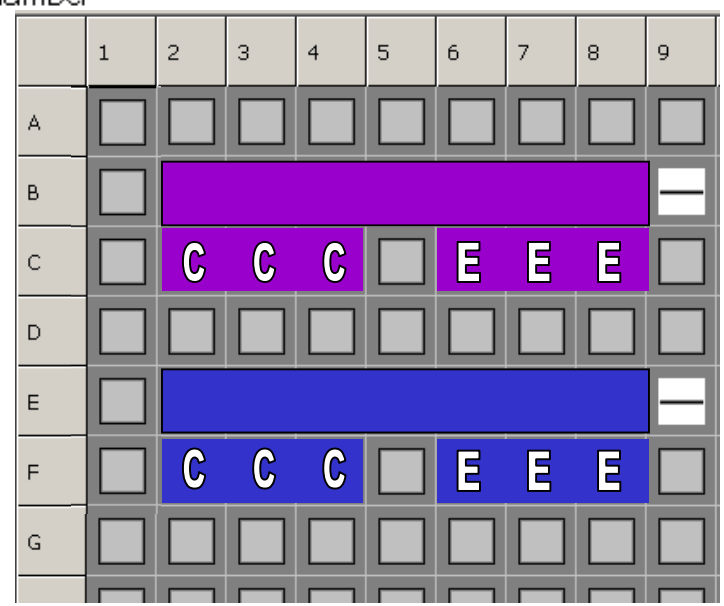
Ratio experimental/control = fold change in target gene / fold change in reference gene



NORTHERN



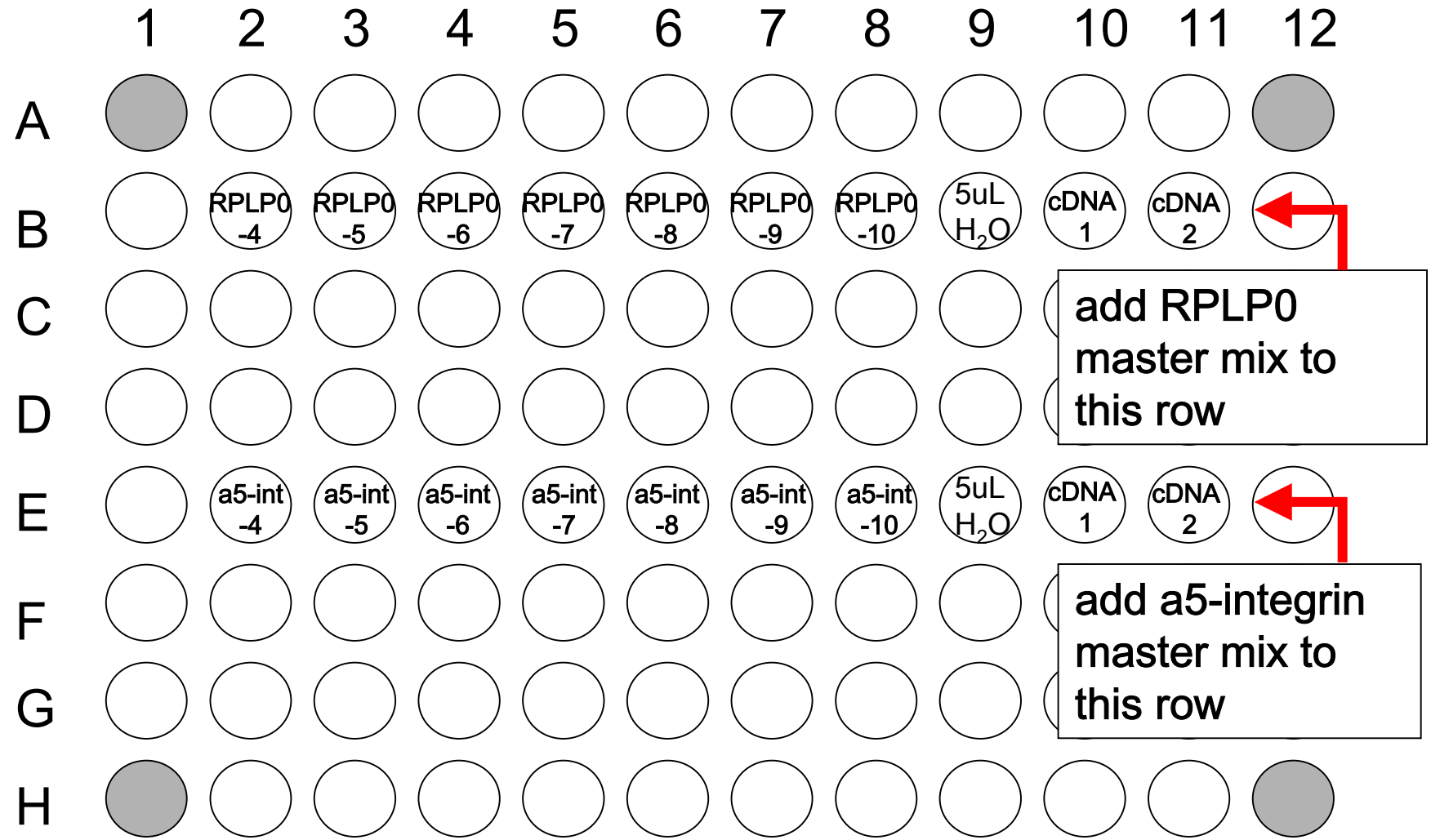
Ratio experimental/control = $\frac{\text{fold change in target gene}}{\text{fold change in reference gene}}$



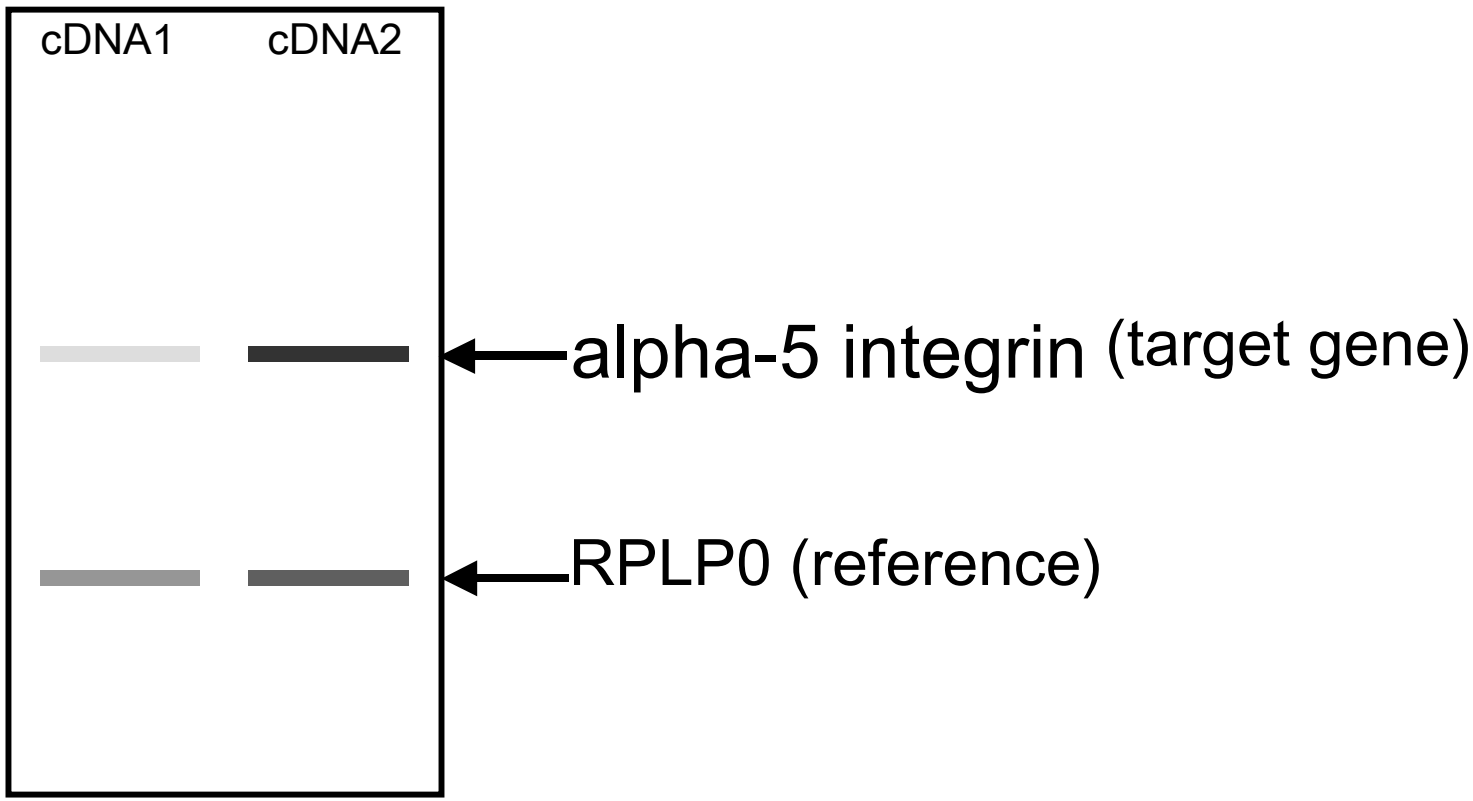
Real time pcr - week 1

- Two different series of diluted DNAs to do standard curve plus two unknowns
  - RPLPO (ribosomal protein, reference gene)
  - alpha-5 integrin
- Get standard curve and efficiency RPLP0 and alpha-5 integrin
- Determine ratio of RPLP0 and alpha-5 integrin in two unknowns (cDNA 1 and cDNA 2)
- Determine melting temperature RPLP0 and alpha-5 integrin
- Each person will do either RPLP0 or alpha-5 integrin

Date: protocol:



NORTHERN



Ratio alpha-5 integrin cDNA2 to cDNA1 = $\frac{\text{fold change in alpha-5 integrin}}{\text{fold change in RPLP0}}$

Importance of controls

- negative control
  - checks reagents for contamination

Importance of cleanliness in PCR

- Contamination is major problem
- Huge amplification contributes to this
- Bacterial vectors contribute to this
- Amplification of ds DNA is more sensitive than that of cDNA

PFAFFL METHOD

– M.W. Pfaffl, Nucleic Acids Research 2001 29:2002-2007

EFFECTS OF EFFICIENCY

CYCLE	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA
	100% EFFICIENCY	90% EFFICIENCY	80% EFFICIENCY	70% EFFICIENCY
0	1	1	1	1
1	2	2	2	2
2	4	4	3	3
3	8	7	6	5
4	16	13	10	8
5	32	25	19	14
6	64	47	34	24
7	128	89	61	41
8	256	170	110	70
9	512	323	198	119
10	1,024	613	357	202
11	2,048	1,165	643	343
12	4,096	2,213	1,157	583
13	8,192	4,205	2,082	990
14	16,384	7,990	3,748	1,684
15	32,768	15,181	6,747	2,862
16	65,536	28,844	12,144	4,866
17	131,072	54,804	21,859	8,272
18	262,144	104,127	39,346	14,063
19	524,288	197,842	70,824	23,907
20	1,048,576	375,900	127,482	40,642
21	2,097,152	714,209	229,468	69,092
22	4,194,304	1,356,998	413,043	117,456
23	8,388,608	2,578,296	743,477	199,676
24	16,777,216	4,898,763	1,338,259	339,449
25	33,554,432	9,307,650	2,408,866	577,063
26	67,108,864	17,684,534	4,335,959	981,007
27	134,217,728	33,600,615	7,804,726	1,667,711
28	268,435,456	63,841,168	14,048,506	2,835,109
29	536,870,912	121,298,220	25,287,311	4,819,686
30	1,073,741,824	230,466,618	45,517,160	8,193,466

AFTER 1 CYCLE

100% = 2.00x

90% = 1.90x

80% = 1.80x

70% = 1.70x

CYCLE	AMOUNT OF DNA 100% EFFICIENCY	AMOUNT OF DNA 90% EFFICIENCY	AMOUNT OF DNA 80% EFFICIENCY	AMOUNT OF DNA 70% EFFICIENCY
0	1	1	1	1
1	2	2	2	2
2	4	4	3	3
3	8	7	6	5
4	16	13	10	8
5	32	25	19	14
6	64	47	34	24
7	128	89	61	41
8	256	170	110	70
9	512	323	198	119
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AFTER 1 CYCLE

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80% = 1.80x

70% = 1.70x

AFTER N CYCLES:

fold increase = $(\text{efficiency})^n$

CYCLE	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA
	100% EFFICIENCY	90% EFFICIENCY	80% EFFICIENCY	70% EFFICIENCY
0	1	1	1	1
1	2	2	2	2
2	4	4	3	3
3	8	7	6	5
4	16	13	10	8
5	32	25	19	14
6	64	47	34	24
7	128	89	61	41
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9	512	323	198	119
10	1,024	613	357	202
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12	4,096	2,213	1,157	583
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15	32,768	15,181	6,747	2,862
16	65,536	28,844	12,144	4,866
17	131,072	54,804	21,859	8,272
18	262,144	104,127	39,346	14,063
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21	2,097,152	714,209	229,468	69,092
22	4,194,304	1,356,998	413,043	117,456
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25	33,554,432	9,307,650	2,408,866	577,063
26	67,108,864	17,684,534	4,335,959	981,007
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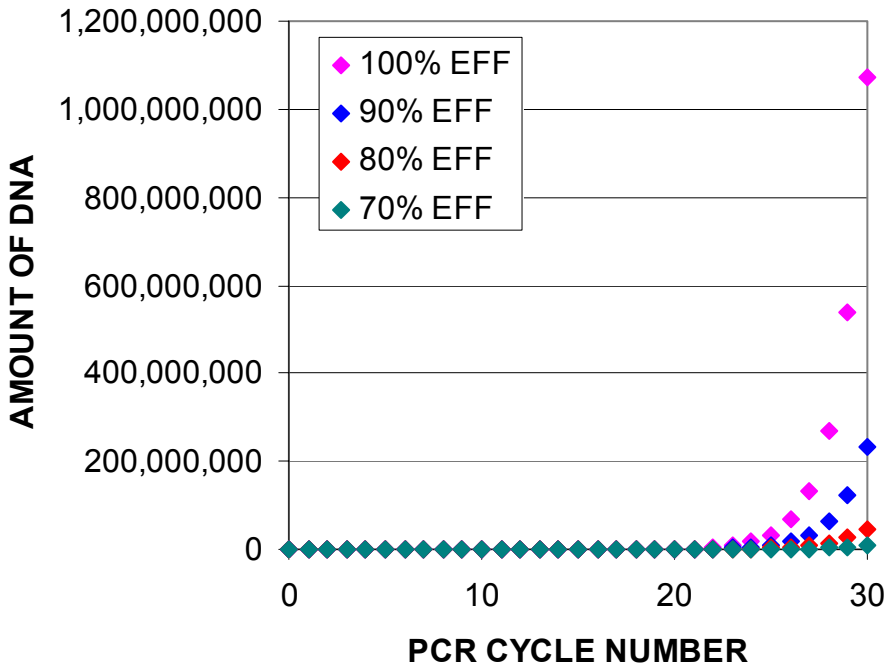
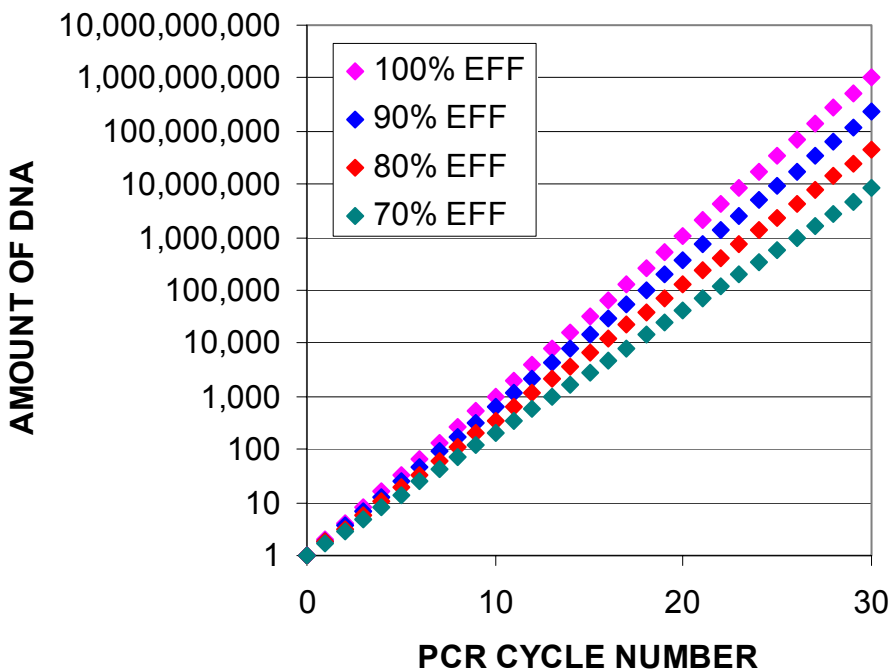
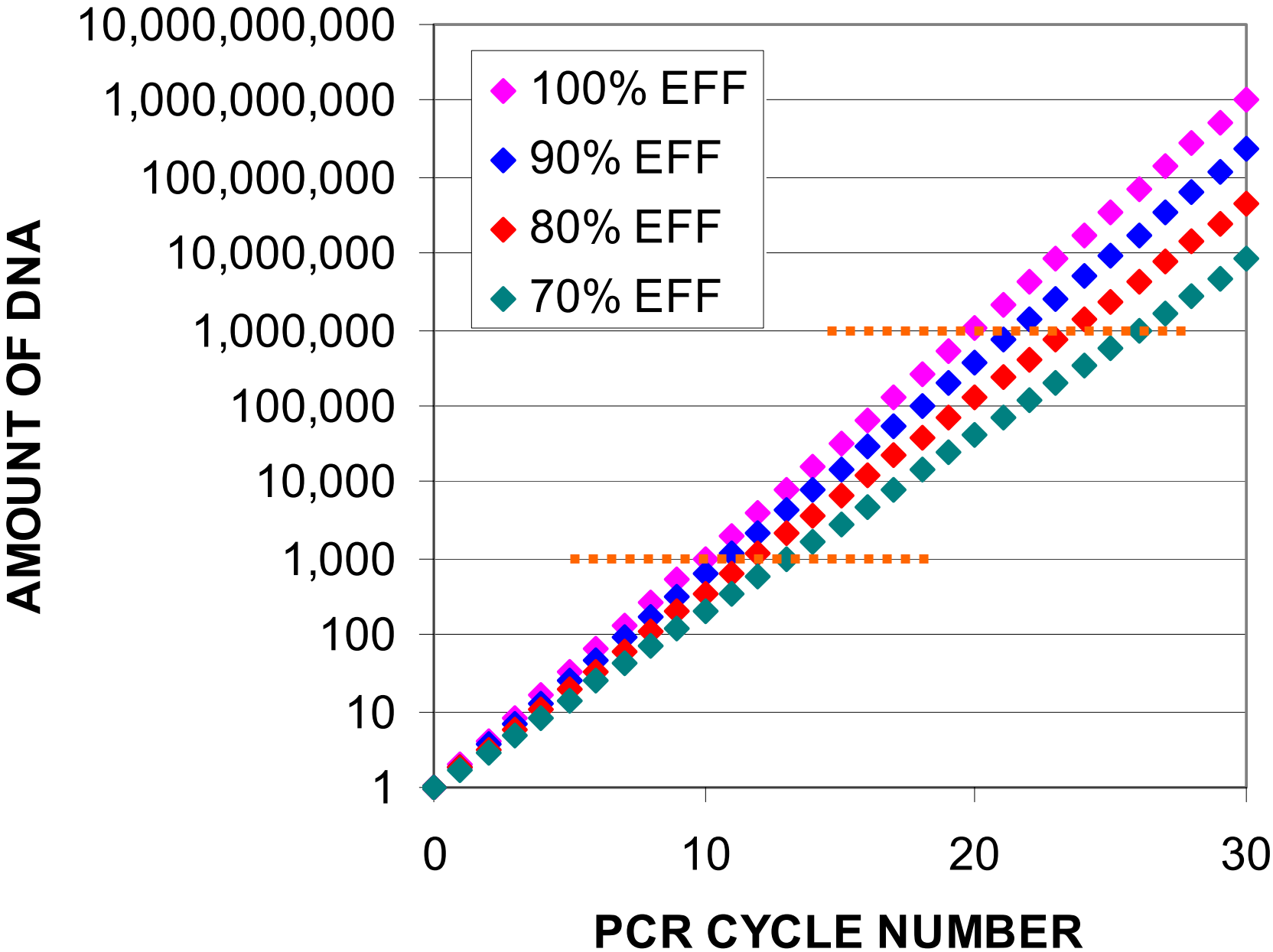
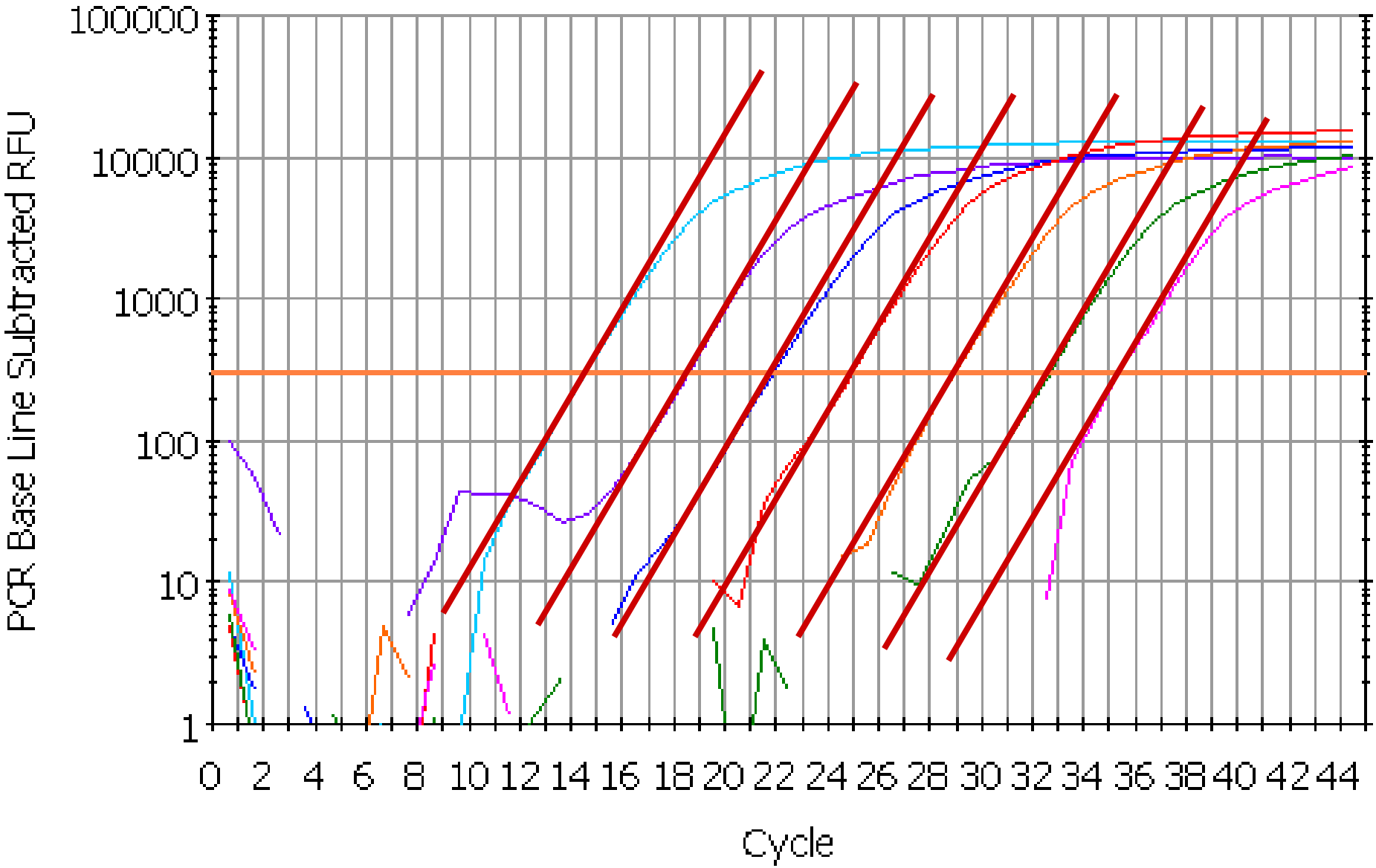
28	268,435,456	63,841,168	14,048,506	2,835,109
29	536,870,912	121,298,220	25,287,311	4,819,686
30	1,073,741,824	230,466,618	45,517,160	8,193,466

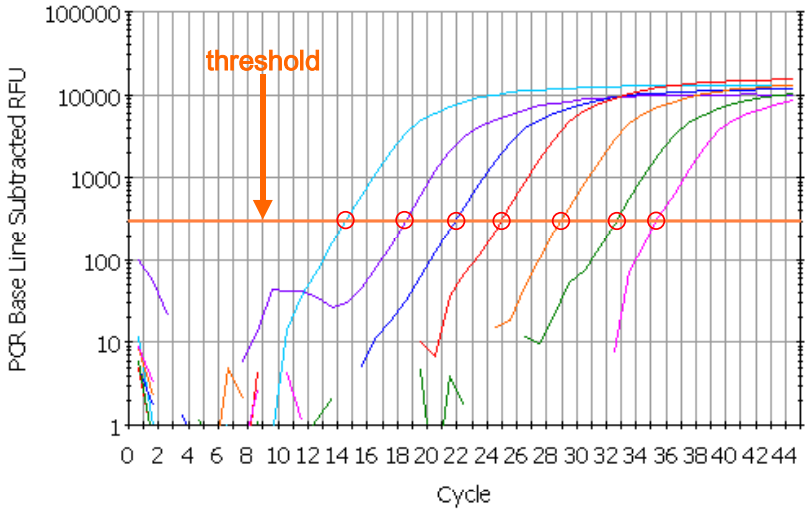





10,000,000,000
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
AMOUNT OF DNA
100% EFF
90% EFF
80% EFF
70% EFF
0
10
20
30
PCR CYCLE NUMBER



SERIES OF 10-FOLD DILUTIONS



Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204

PCR Efficiency: 93.5 %

Unknowns

Standards

PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

QUALITY CONTROL -EFFICIENCY CURVES

- use pcr baseline subtraction (not curve fitting default option) - see next slide
- set the threshold manually to lab standard
- check all melting curves are OK
- check slopes are parallel in log view
- delete samples if multiple dilutions cross line together (usually at dilute end of curve)
- delete samples if can detect amplification at cycle 10 or earlier
- make sure there are 5 or more points
- check correlation coefficient is more than 1.990

iCycler

Services Help

Library

Workshop

Run-Time Central

Data Analysis

View/Save Data | PCR Quantification | PCR Standard Curve | Melt Curve | Allelic Discrimination N/A

Data File: Data 10-Mar-03 1259 ed.opd

Select analysis mode: PCR Base Line Subtracted

- Background Subtracted
- PCR Base Line Subtracted
- PCR Base Line Subtracted Curve Fit

Threshold Cycle Cal

Baseline Cycles

- Auto Calculated
- User Defined

Threshold Position: 100.0

- Auto Calculated
- User Defined

Select Wells

Reports

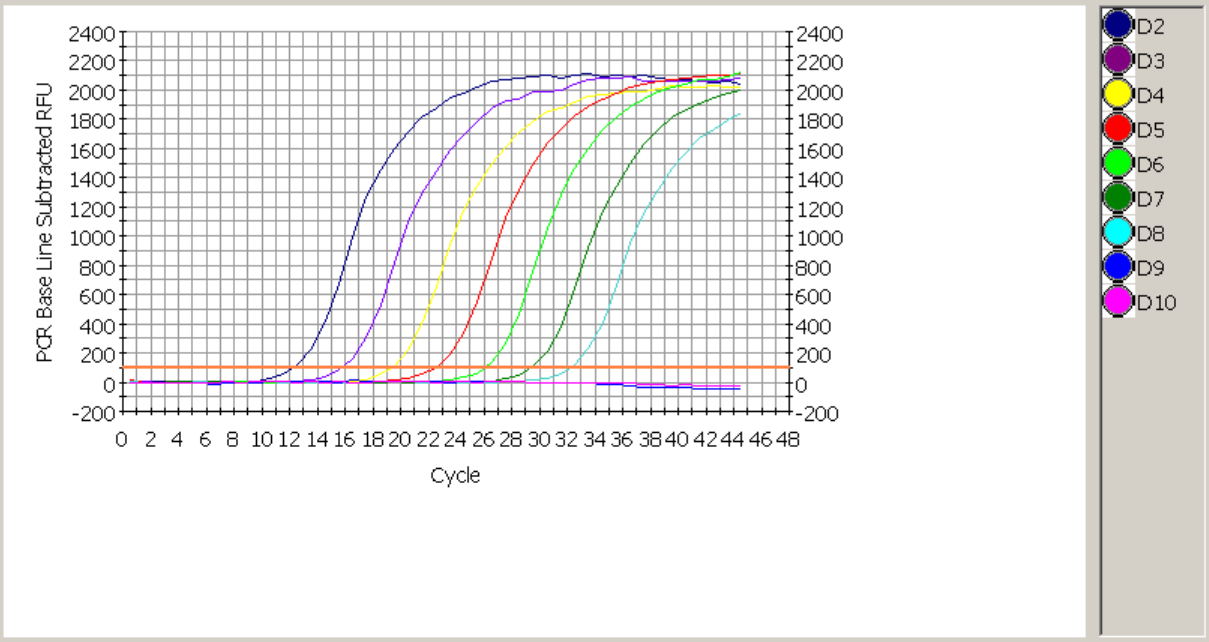
Recalculate Threshold Cycles

FAM-490

Select a Reporter: FAM-490

Save for X-axis Allelic Analysis

Save for Y-axis Allelic Analysis



	Threshold Cycle Ct	Identifier
D2	12.3	
D3	15.8	
D4	19.3	
D5	22.6	
D6	26.0	
D7	29.4	
D8	32.3	
D9	N/A	
D10	N/A	

ACTION: | Data Window: Last 95% | Filter: Weighted Mean | Analysis: Post-Run

QUALITY CONTROL -EFFICIENCY CURVES

- use pcr baseline subtraction (not curve fitting default option)
- set the threshold manually to lab standard
- check all melting curves are OK
- check slopes are parallel in log view
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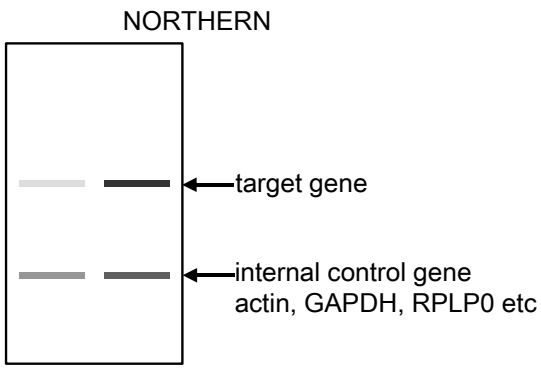
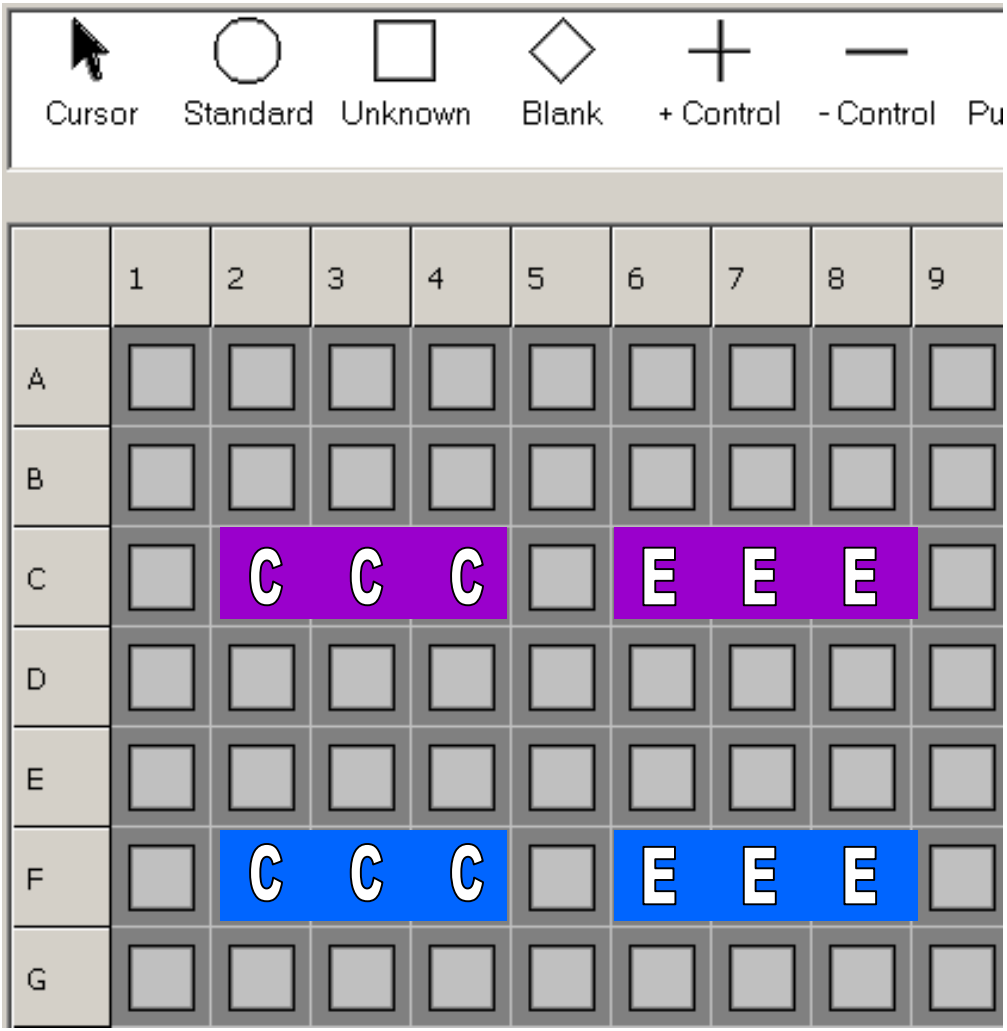
- delete samples if can detect amplification at cycle 10 or earlier
- make sure there are 5 or more points
- check correlation coefficient is more than 1.990

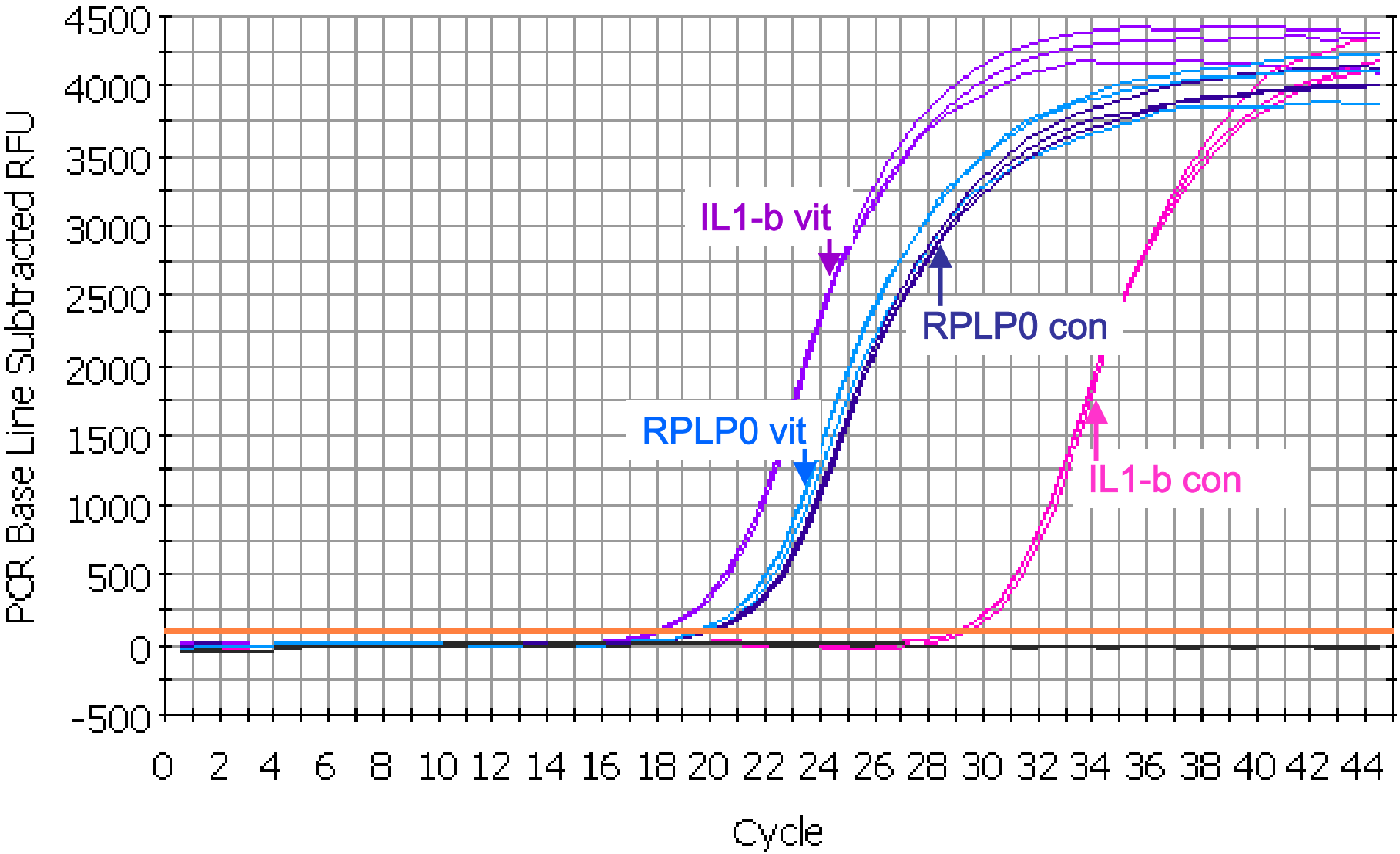
PFAFFL METHOD

M.W. Pfaffl, Nucleic Acids Research
2001 29:2002-2007

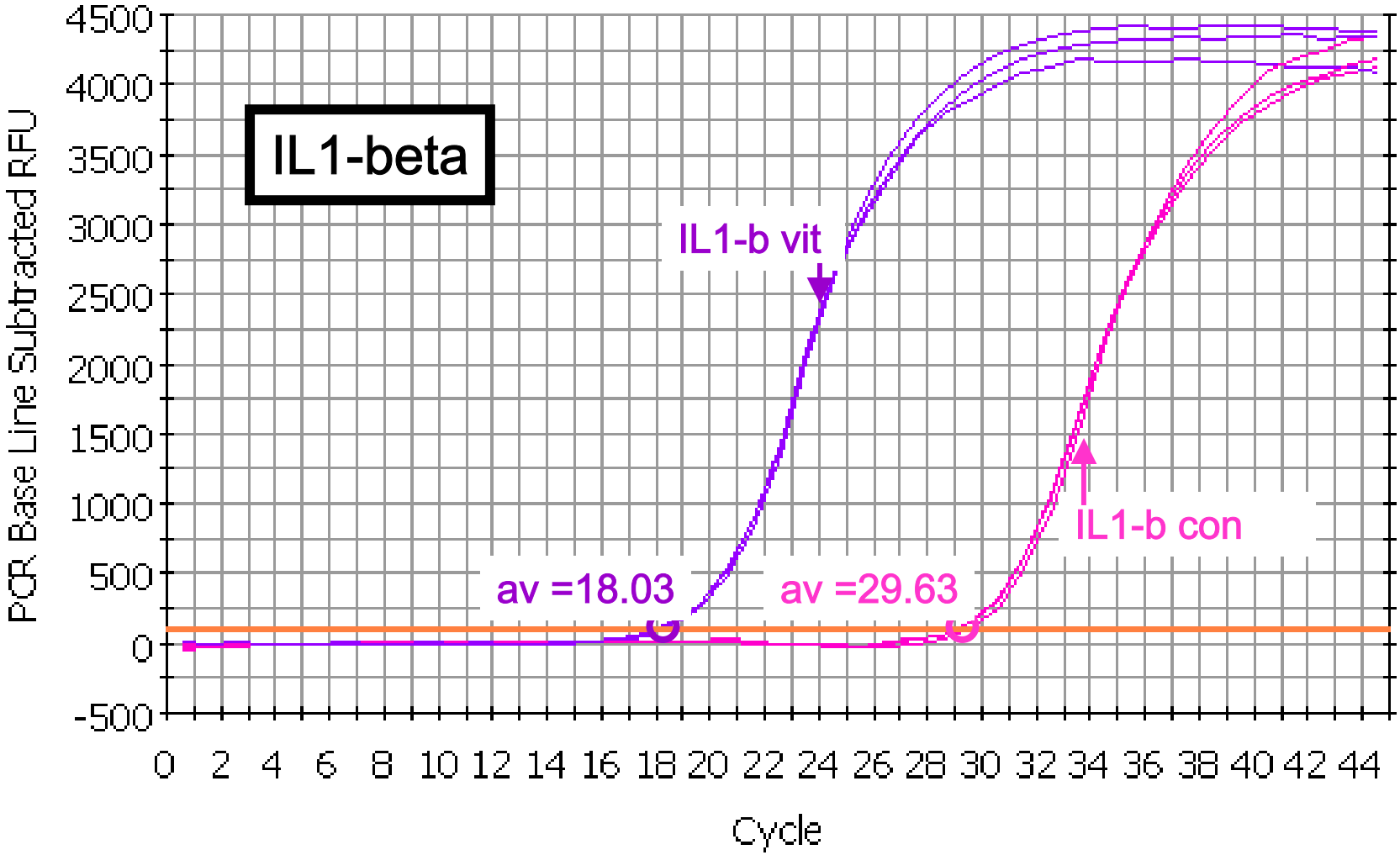


$$\text{ratio} = \frac{\text{fold increase in target gene}}{\text{fold increase in reference gene}}$$


Cursor
Standard
Unknown
Blank
+ Control
- Control
Pu
1
2
3
4
5
6
7
8
9
A
B
C
D
E
F
G
C C C
E E E
C C C
E E E
triplicates cDNA
target primers
triplicates cDNA
reference primers



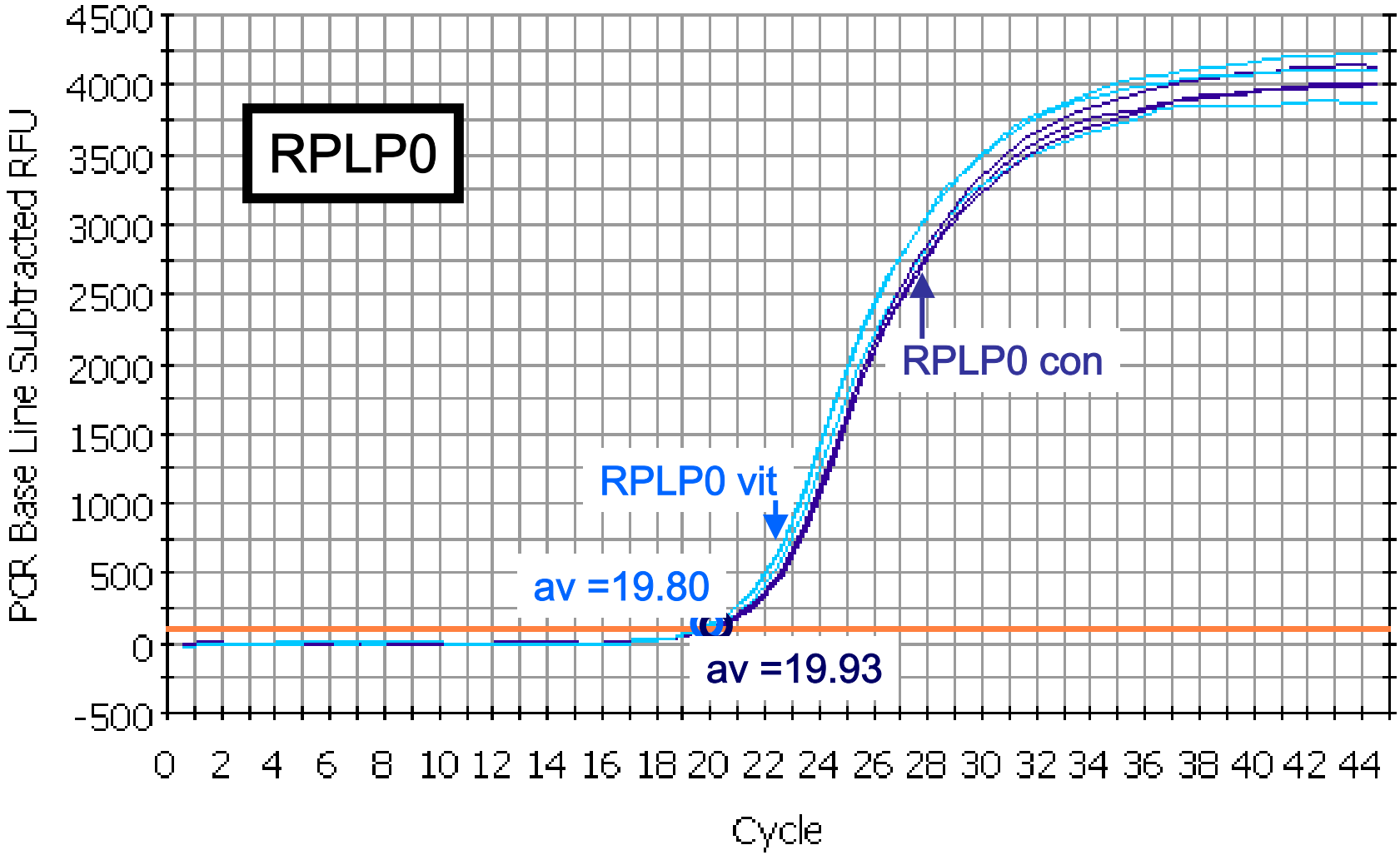
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



AFTER N CYCLES: change = $(\text{efficiency})^{n}$

AFTER N CYCLES: ratio vit/con = $(1.93)^{29.63-18.03} = 1.93^{11.60} = 2053$

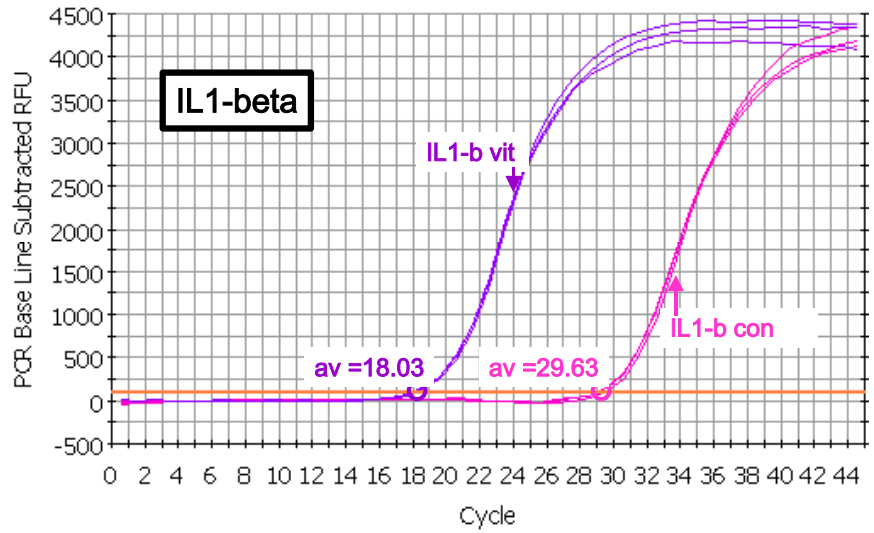
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



AFTER N CYCLES: change = $(\text{efficiency})^{n}$

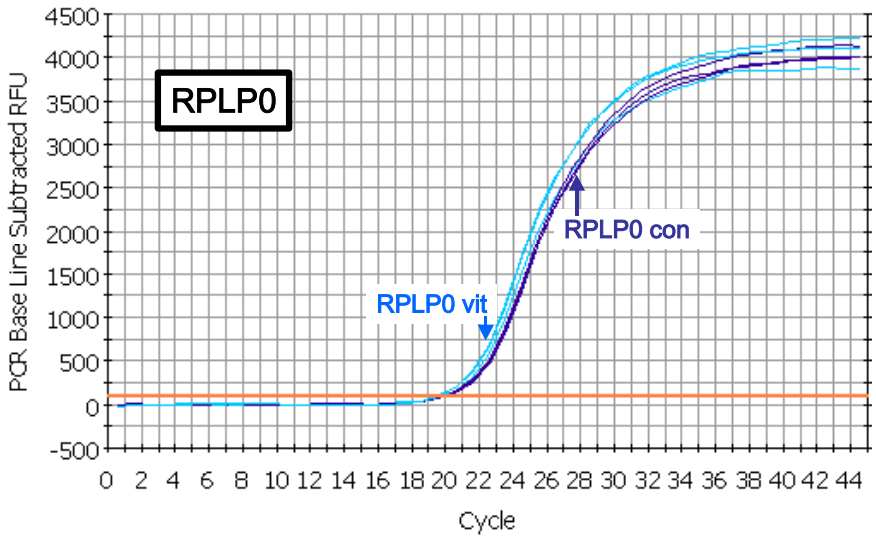
AFTER N CYCLES: ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



AFTER N CYCLES: increase = (efficiency)^n

Ratio vit/con = $(1.93)^{29.63-18.03} = 1.93^{11.60} = 2053$



AFTER N CYCLES: increase = (efficiency)^n

Ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

$$\text{ratio} = \frac{\text{change in IL1-B}}{\text{change in RPLP0}} = 2053/1.08 = 1901$$

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct \text{ target (control-treated)}}}{(E_{ref})^{\Delta Ct \text{ ref (control-treated)}}}$$

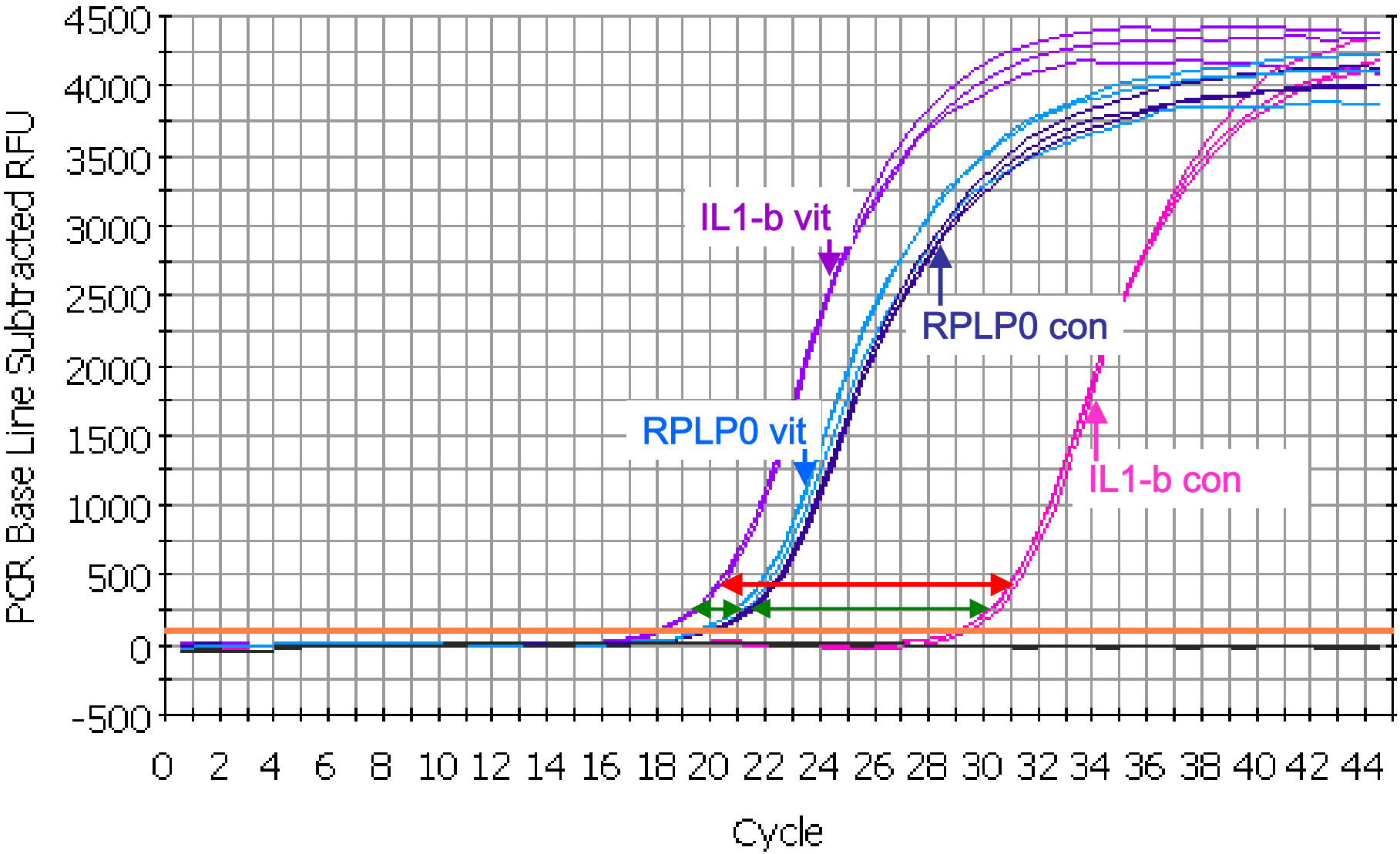
	A	B	C	D	E	F	G	H	J	K
1		CONTROL RPLP0	CONTROL TARGET GENE	TREATED RPLP0	TREATED TARGET GENE	Ct CONTROL- Ct TREATED FOR TARGET GENE	PFAFFL EQUATION TOP LINE	Ct CONTROL- Ct TREATED FOR RPLP0	PFAFFL EQUATION BOTTOM LINE	RATIO TARGET GENE IN TREATED/CONTROL
2		average Ct	average Ct	average Ct	average Ct		(fold change in target gene)		(fold change in reference gene)	(corrected for internal standard)
3		20.87	23.73	20.57	22.13	1.60	2.88	0.30	1.22	2.4
4	-> EXCEL formula used for the data in row 3					=C-E	=POWER(1.936,F)	= B-D	=POWER(1.943,H)	=G/J
5										

An example of a step-by-step way to set up the calculations for the Pfaffl method in EXCEL.

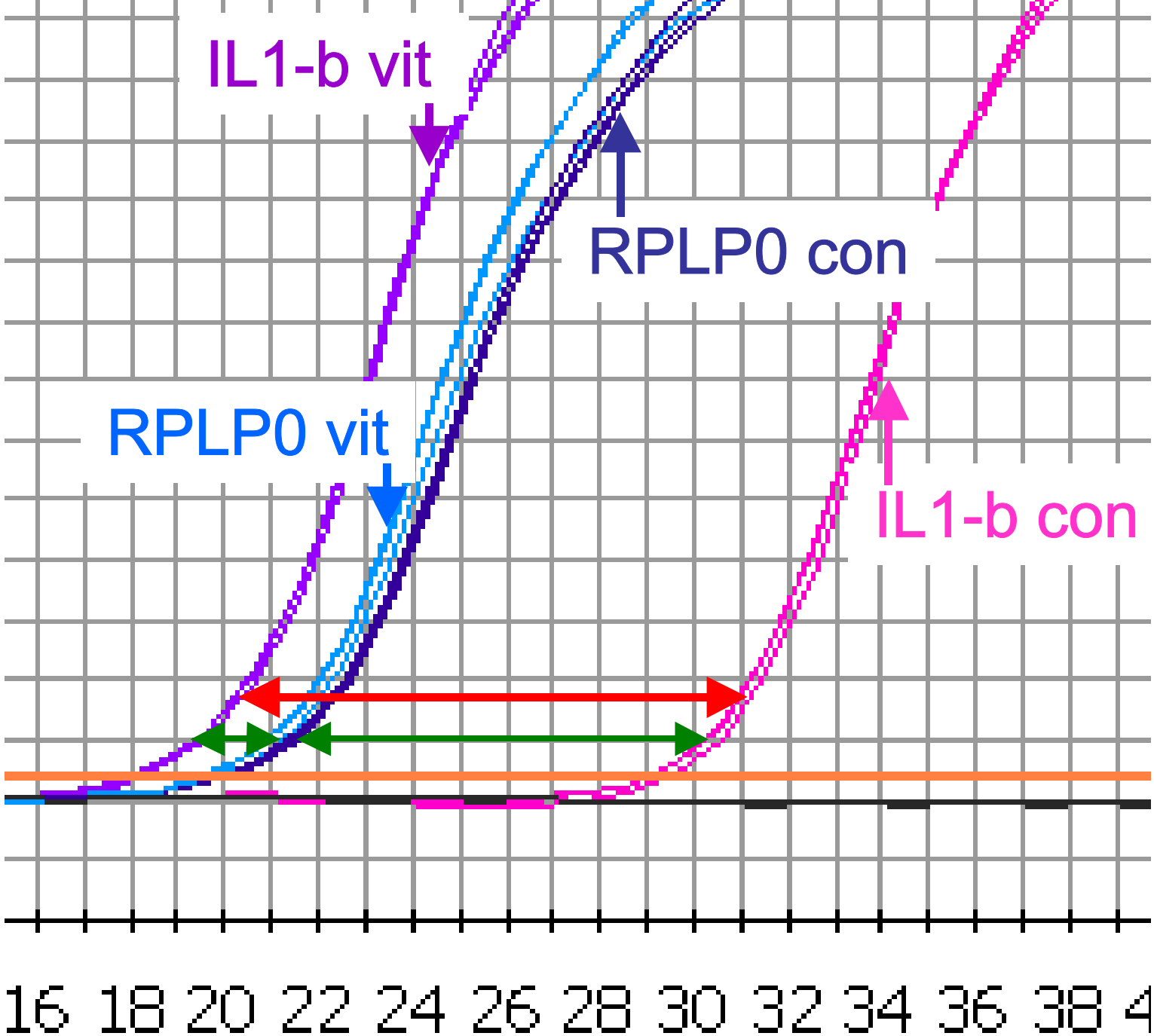
Row 3, columns B, C, D, and E are the average Ct values from real time. In separate experiments, the average efficiency for the target gene was determined to be 1.936 and for RPLP0 was 1.943

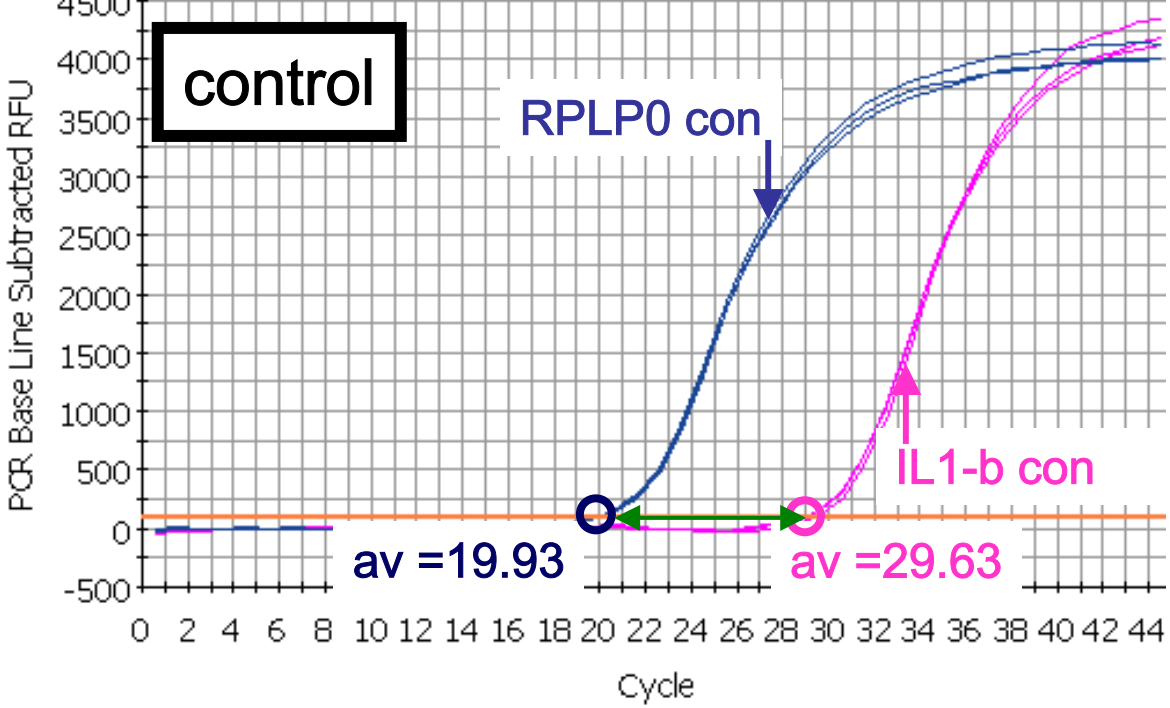
EFFICIENCY $^{\Delta\Delta Ct}$ METHOD

APPROXIMATION METHOD



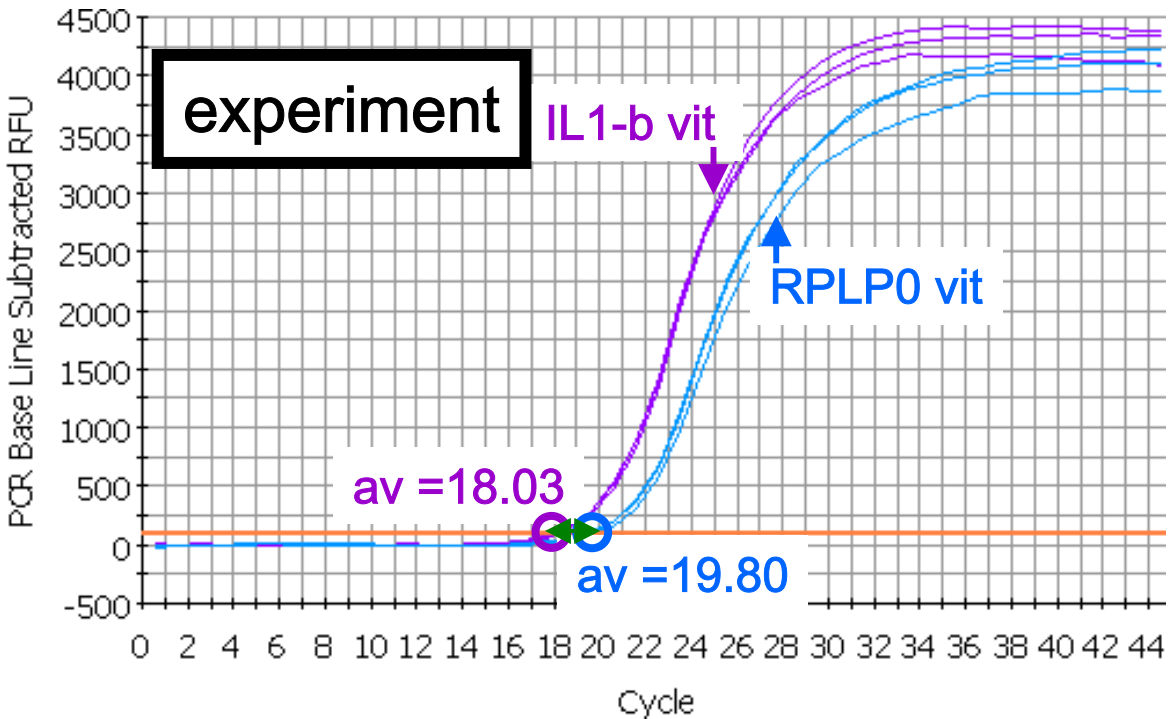
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd


IL1-b vit
RPLP0 con
RPLP0 vit
IL1-b con
16 18 20 22 24 26 28 30 32 34 36 38 4



Δ Ct = target - ref

Δ Ct = 9.70



Δ Ct = target - ref

Δ Ct = -1.7

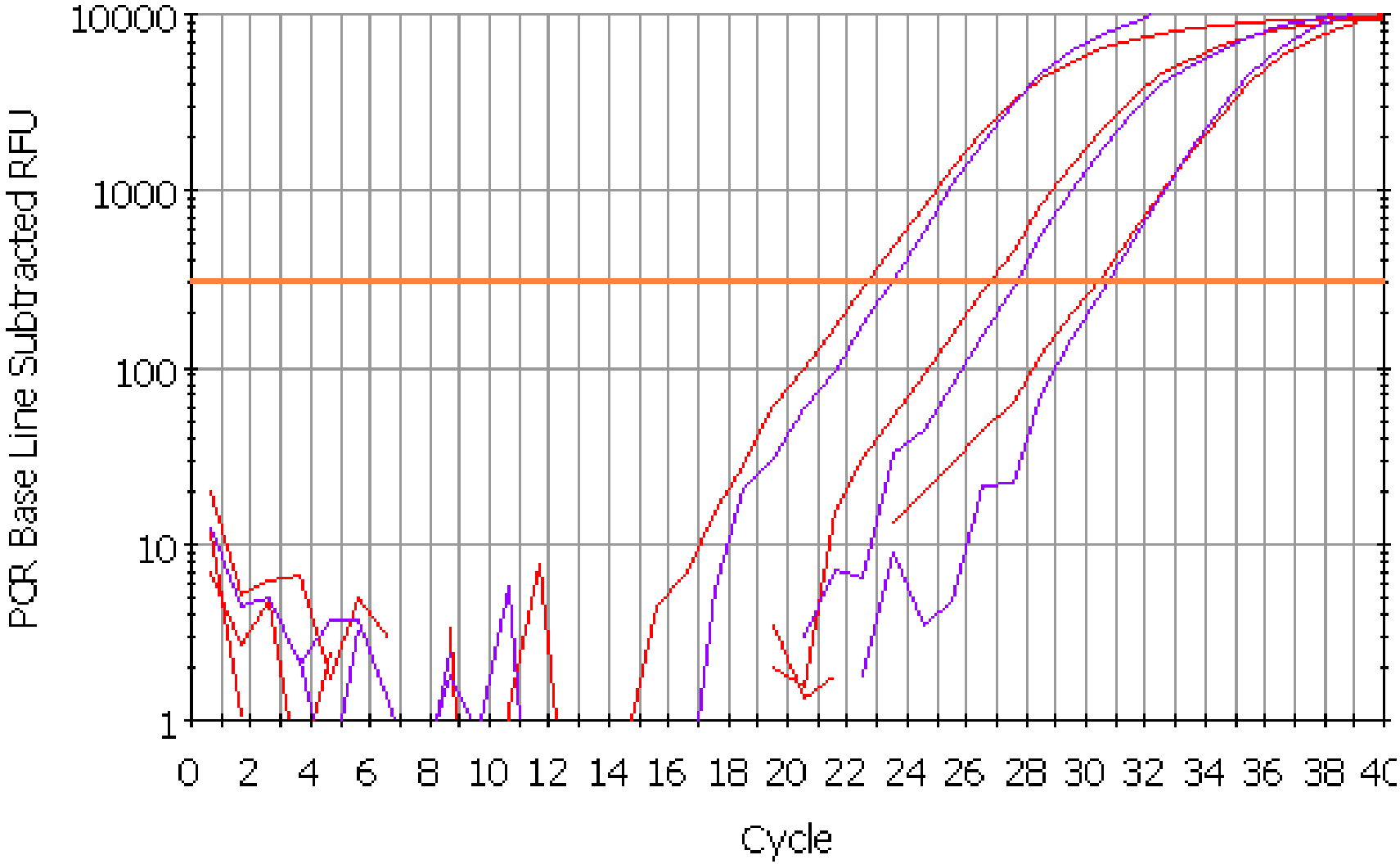
Difference = ΔCt-ΔCt
= ΔΔCt
= 9.70-(-1.7)
= 11.40

$\Delta\Delta$Ct = 11.40 for IL1-beta

- 2 $^{\Delta\Delta Ct}$ variant: assumes efficiency is 100%
  Fold change = $2^{11.40}$ = 2702

- But our efficiency for IL1-beta is 93%
  - Fold change = $1.93^{11.40}$ = 1800

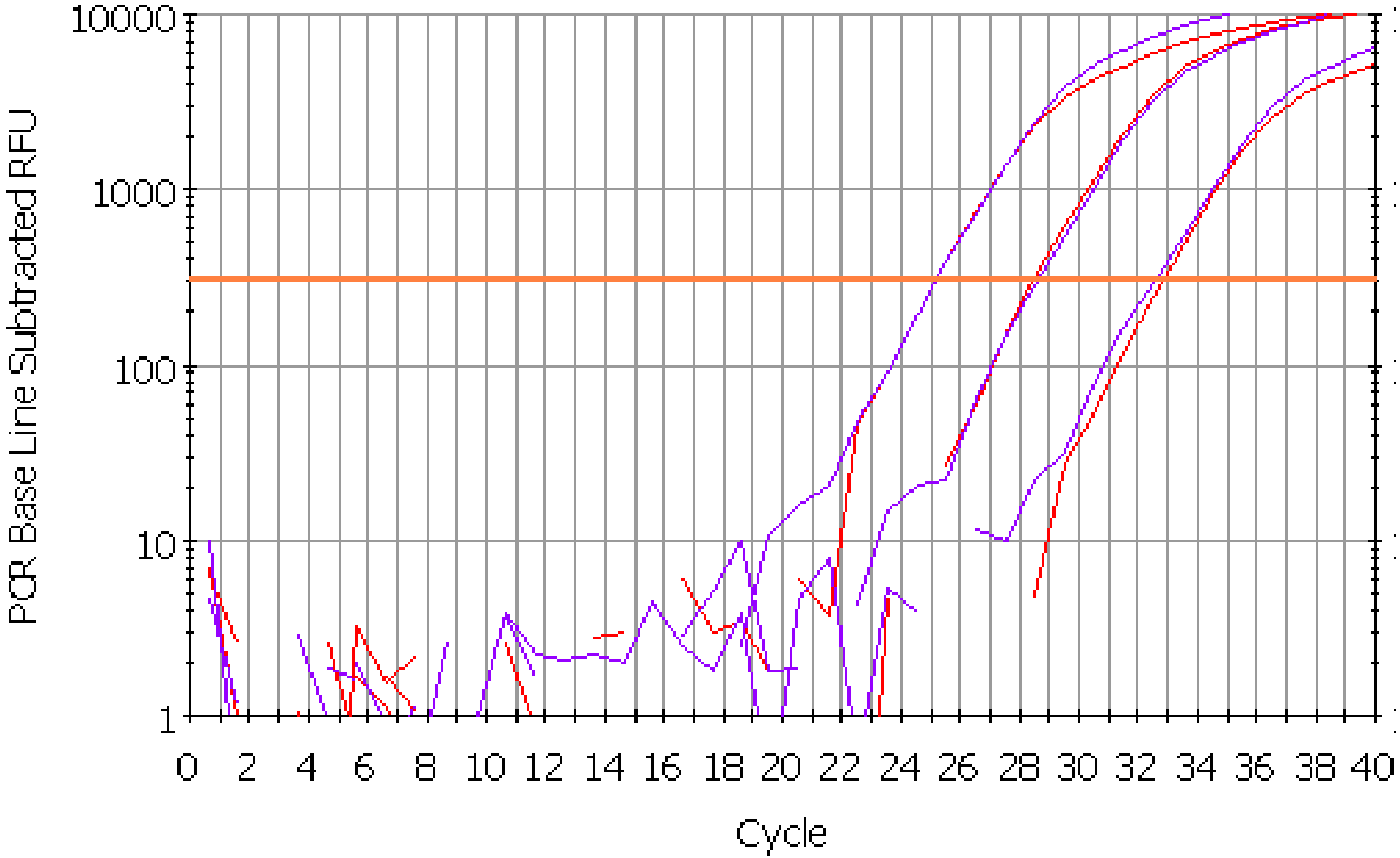
- Pfaffl equation corrected for RPLP0 efficiency
  - Fold change = 1901



SERIAL 10-FOLD DILUTIONS

RED: 83% efficiency
PURPLE: 93% efficiency

PCR Amplification vs Cycle: Data 28-Aug-02 1113.opd



SERIAL 10-FOLD DILUTIONS

RED: 94% efficiency
PURPLE: 94% efficiency

PCR Amplification vs Cycle: Data 28-Jan-03 1124ileff.opd

EFFICIENCY $^{\Delta\Delta Ct}$ METHOD

- assumes
  - minimal correction for the standard gene, **or**
  - that standard and target have similar efficiencies
    - 2 $^{\Delta\Delta Ct}$ variant assumes efficiencies are both 100%

- approximation method, but need to validate that assumptions are reasonably correct - do dilution curves to check ΔCts don't change

- The only extra information needed for the Pfaffl method is the reference gene efficiency, this is probably no more work than validating the approximation method

Real time pcr - week 2

- Two different cDNAs derived from cells which have undergone control or vitreous treatment
- Do levels of alpha-5 integrin change relative to RPLPO?
  - Calculate according to Pfaffl method

RNA from control RPE cells → cDNA from control RPE

RNA from TGF-b treated RPE cells → cDNA from TGF-b treated RPE cells

? Is there any change in a5-integrin expression ?

OVERVIEW

tissue

↓

extract RNA

↓

copy into cDNA
(reverse transciptase)

↓

do real-time PCR

↓

analyze results

OVERVIEW

tissue

↓

extract RNA

↓

copy into cDNA
(reverse transciptase)

↓

do real-time PCR

↓

analyze results

IMPORTANCE OF RNA QUALITY

- Should be free of protein (absorbance 260nm/280nm)
- Should be undegraded (28S/18S ~2:1)
- Should be free of DNA (DNAse treat)
- Should be free of PCR inhibitors
  - Purification methods
  - Clean-up methods

OVERVIEW

tissue

extract RNA

copy into cDNA
(reverse transciptase)

do real-time PCR

analyze results

Importance of reverse transcriptase primers

- Oligo (dt)
- Random hexamer (NNNNNN)
- Specific

REVERSE TRANSCRIPTION

- adds a bias to the results
- efficiency usually not known

OVERVIEW

tissue

↓

extract RNA

↓

copy into cDNA
(reverse transciptase)

↓

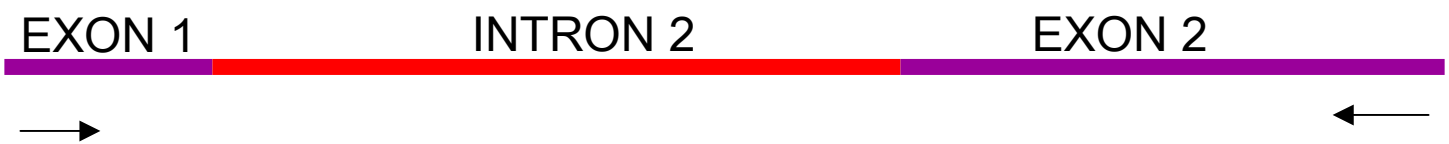
do real-time PCR

↓

analyze results

Importance of primers in PCR

- specific
- high efficiency
- no primer-dimers
- Ideally should not give a DNA signal
  - cross exon/exon boundary



DNA

RNA

How are you going to measure the PCR product

- Directly
  - Sybr green
  - Quality of primers critical
- Indirectly
  - In addition to primers, add a fluorescently labeled hybridization probe
  - Many different approaches to this, see Bustin J.Mol.Endocrinol. (2000) 25:169

Importance of controls

- negative control (no DNA)
  - checks reagents for contamination
- no reverse transcriptase control
  - detects if signal from contaminating DNA
- positive control
  - checks that reagents and primers work
  - especially importance if trying to show absence of expression of a gene

Standards

- same copy number in all cells
- expressed in all cells
- medium copy number advantageous
  - correction more accurate
- reasonably large intron
- no pseudogene
- no alternate splicing in region you want to PCR

RNA from control RPE cells → cDNA from control RPE

RNA from TGF-b treated RPE cells → cDNA from TGF-b treated RPE cells

? Is there any change in a5-integrin expression ?

RNA from control RPE cells → cDNA from control RPE

→ No RT for control RPE
(to see if any genomic DNA signal)

RNA from TGF-b treated RPE cells → cDNA from TGF-b treated RPE cells

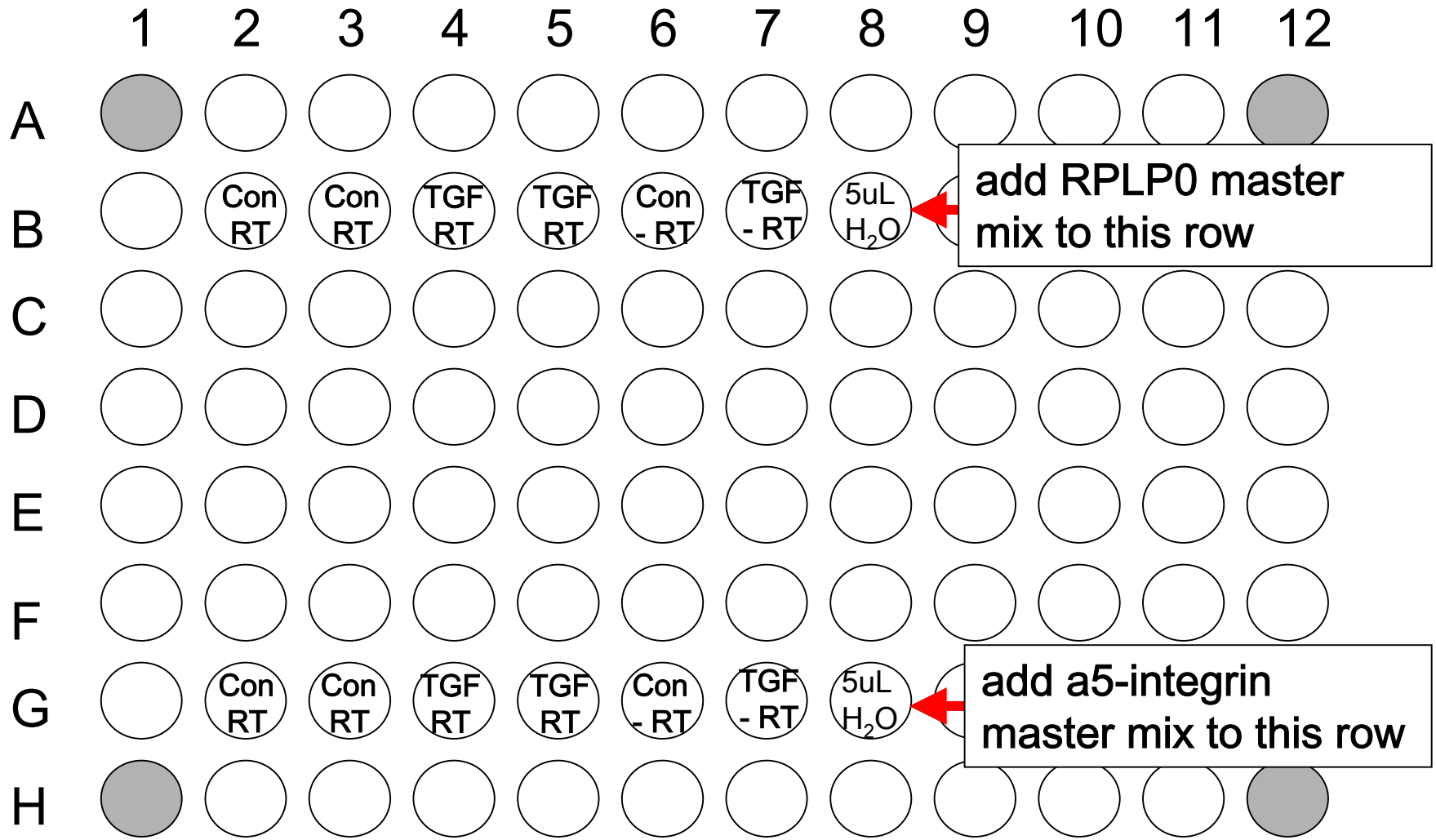
→ No RT for TGF-b treated RPE
(to see if any genomic DNA signal)

? Is there any change in a5-integrin expression ?

THE REVERSE TRANSCRIPTION REACTIONS HAVE BEEN DONE FOR YOU

- reactions done as 20ul reactions with oligo (dT) as primer and 1ug total RNA
- reactions done under oil
- reactions were incubated 1 hr 37C, then diluted to 150ul with water, and incubated in a boiling water bath for 10 mins
- You will use 5uL of this diluted cDNA in your reactions

Date: protocol:



SPECIAL THANKS TO

- Dr. Joyce Nair-Menon and Lei Li for the use of their real-time PCR results

- Anyone who has ever discussed their real-time PCR results with me

- NEI - EY12711 for the money