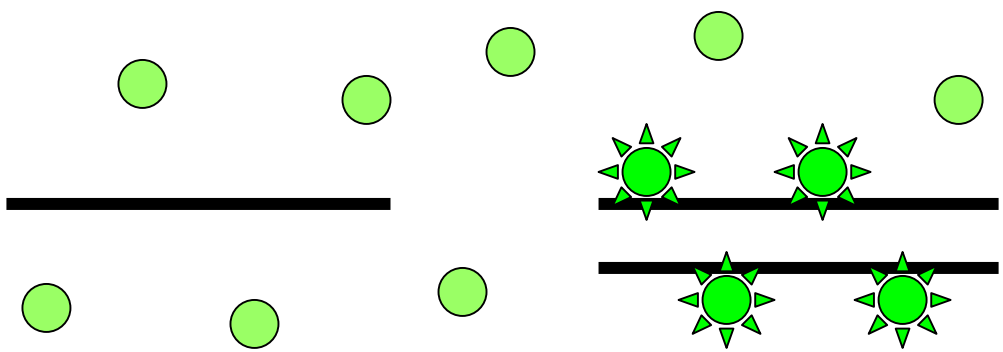
REAL TIME PCR

SYBR GREEN



THE PROBLEM

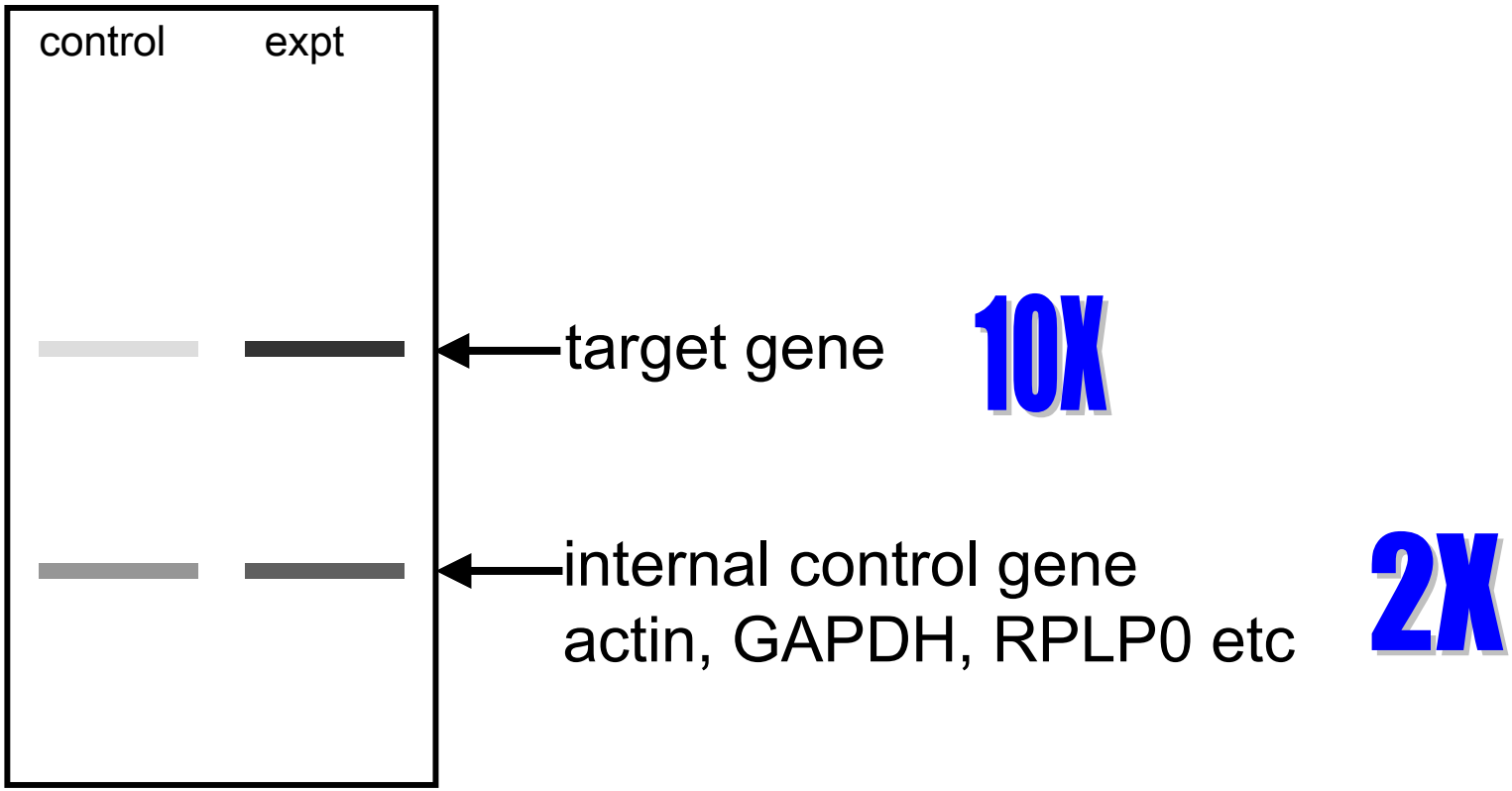
- NEED TO QUANTITATE DIFFERENCES IN mRNA EXPRESSION

- 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

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

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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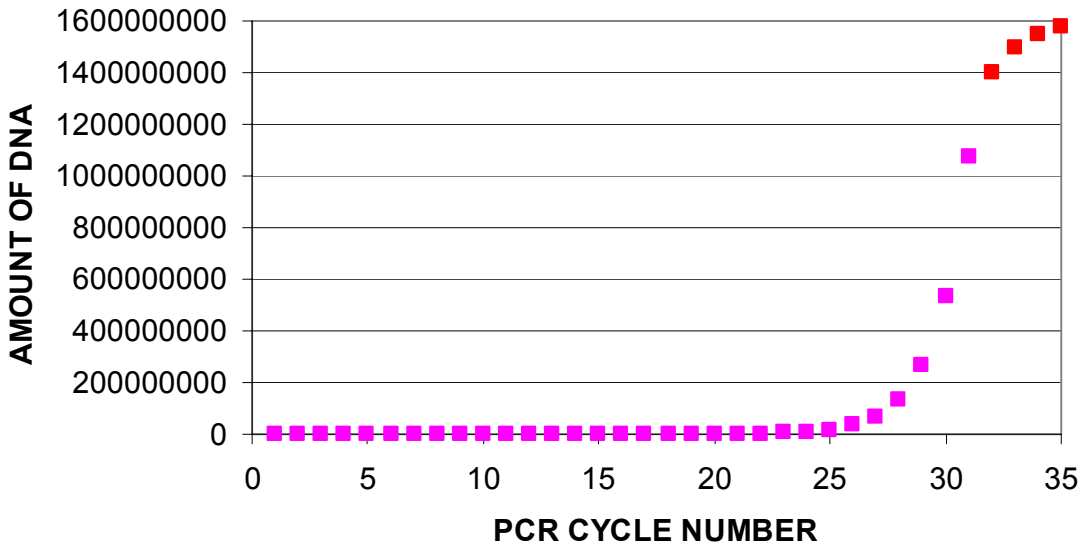
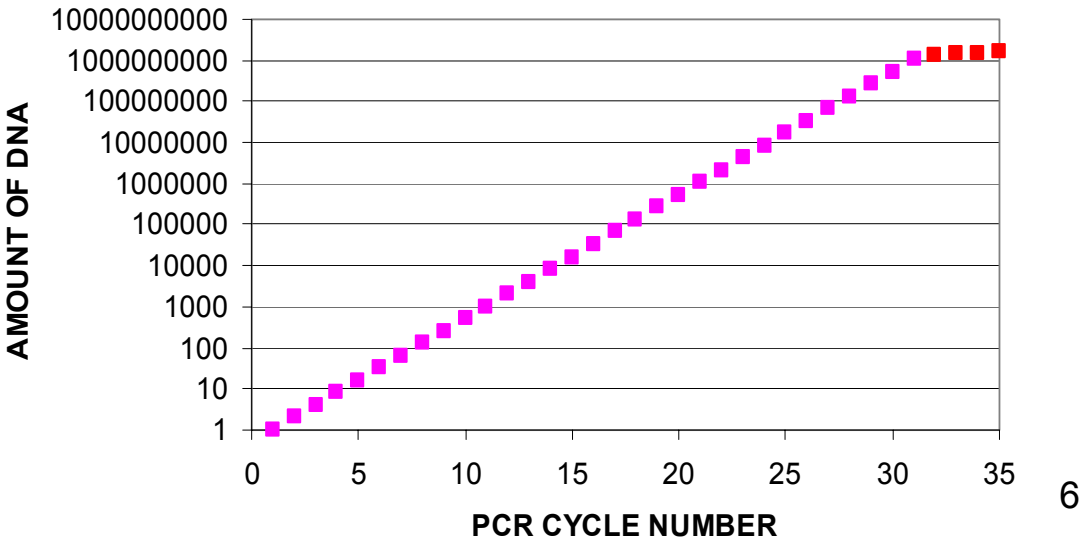
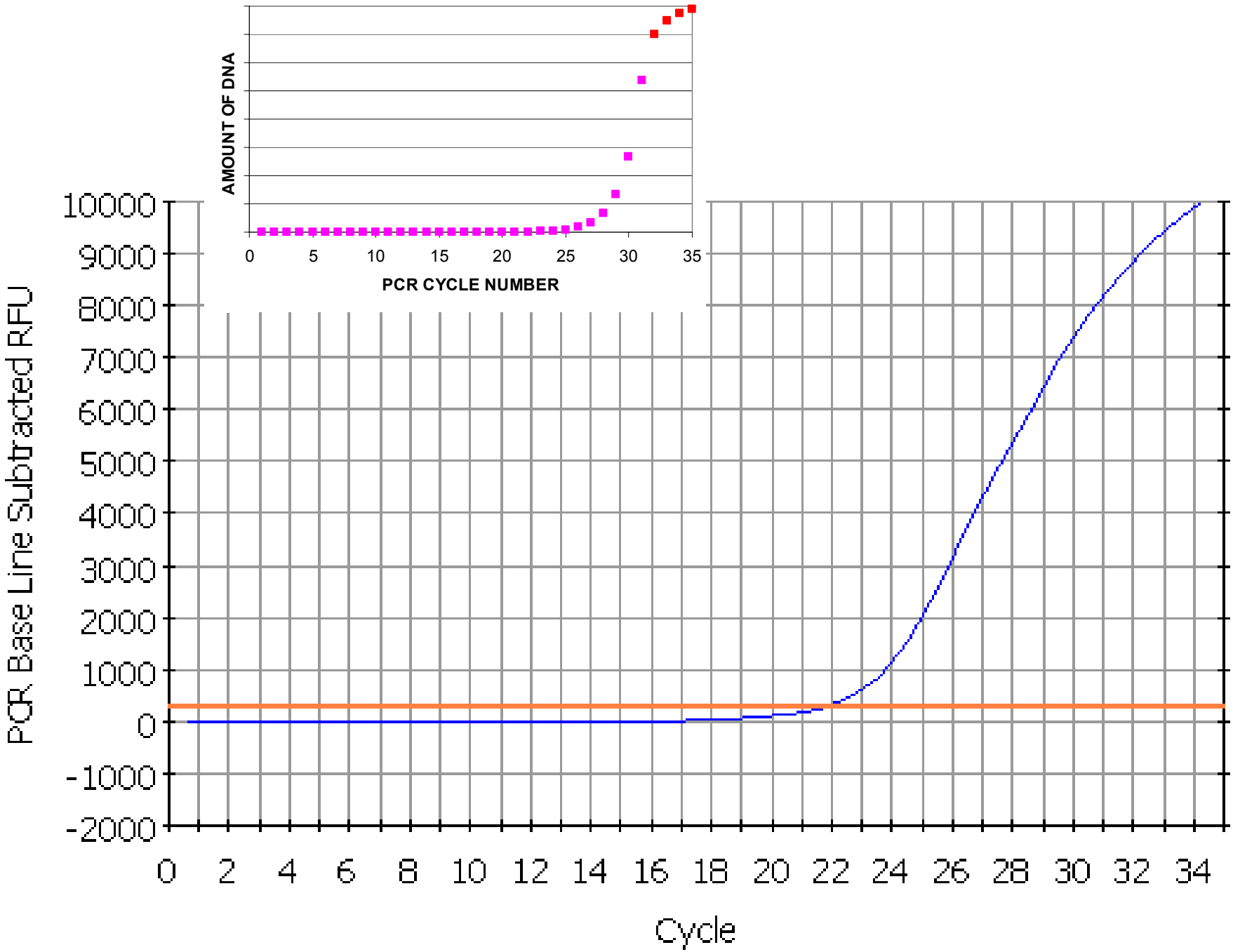
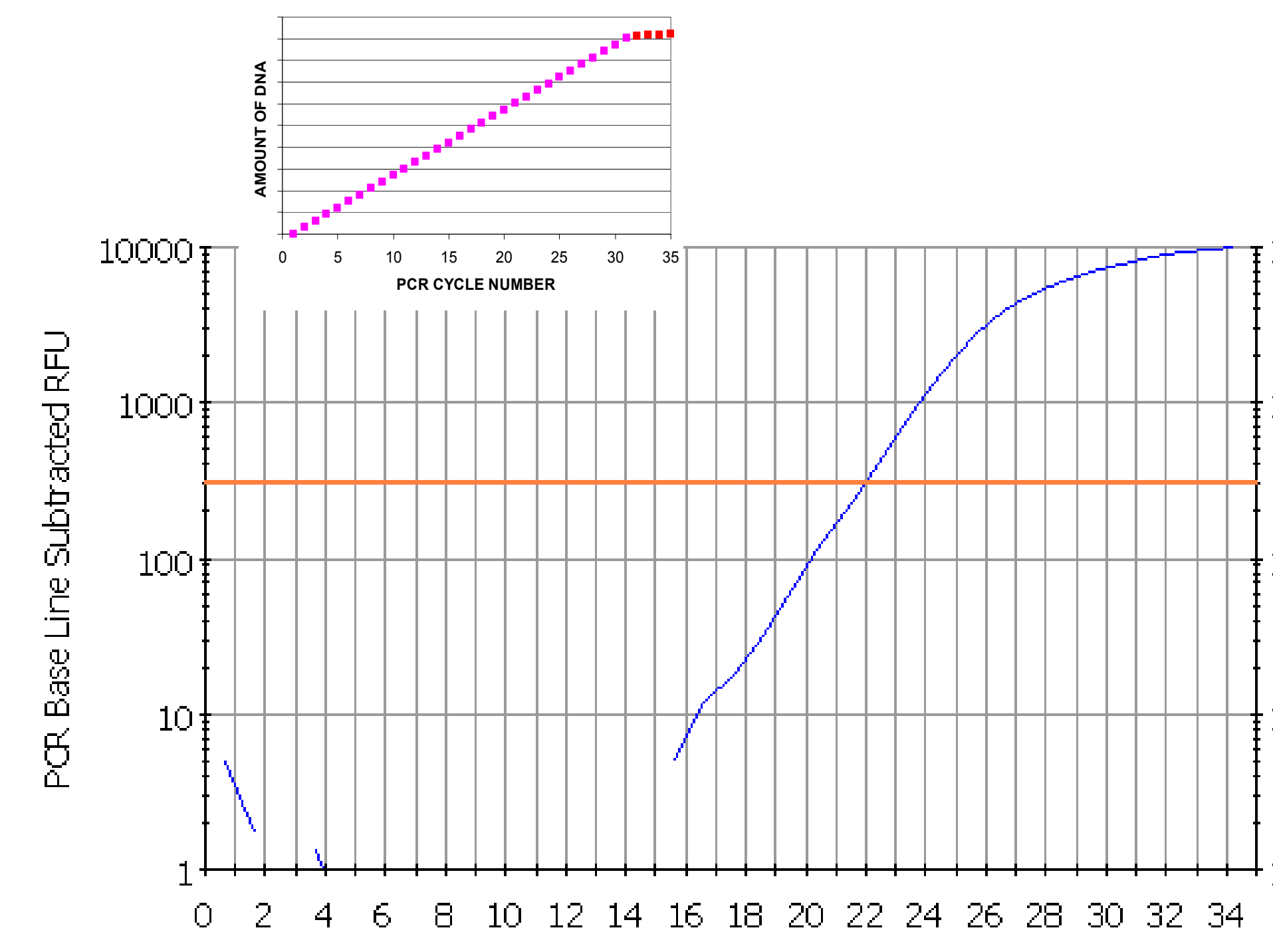
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

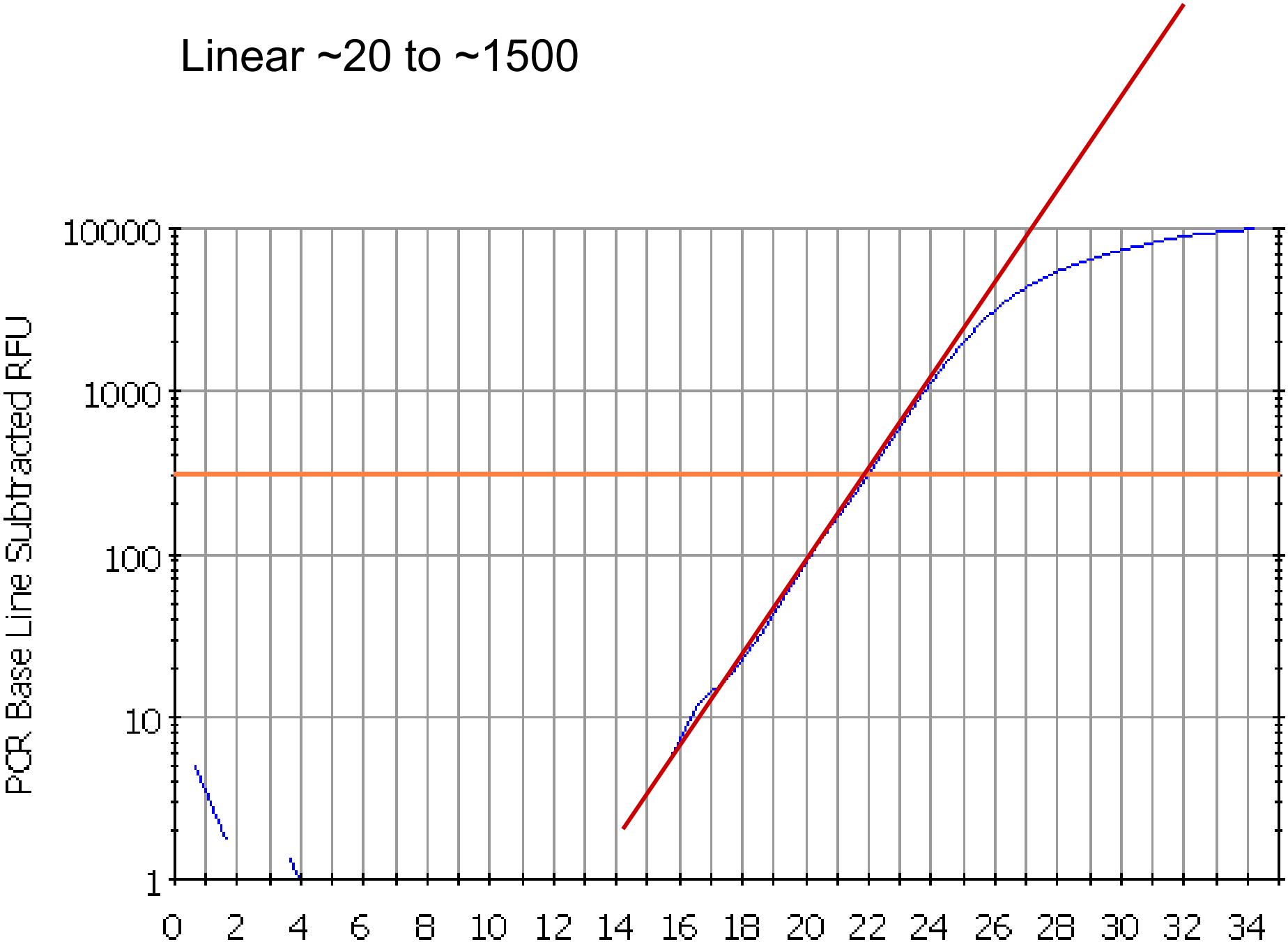




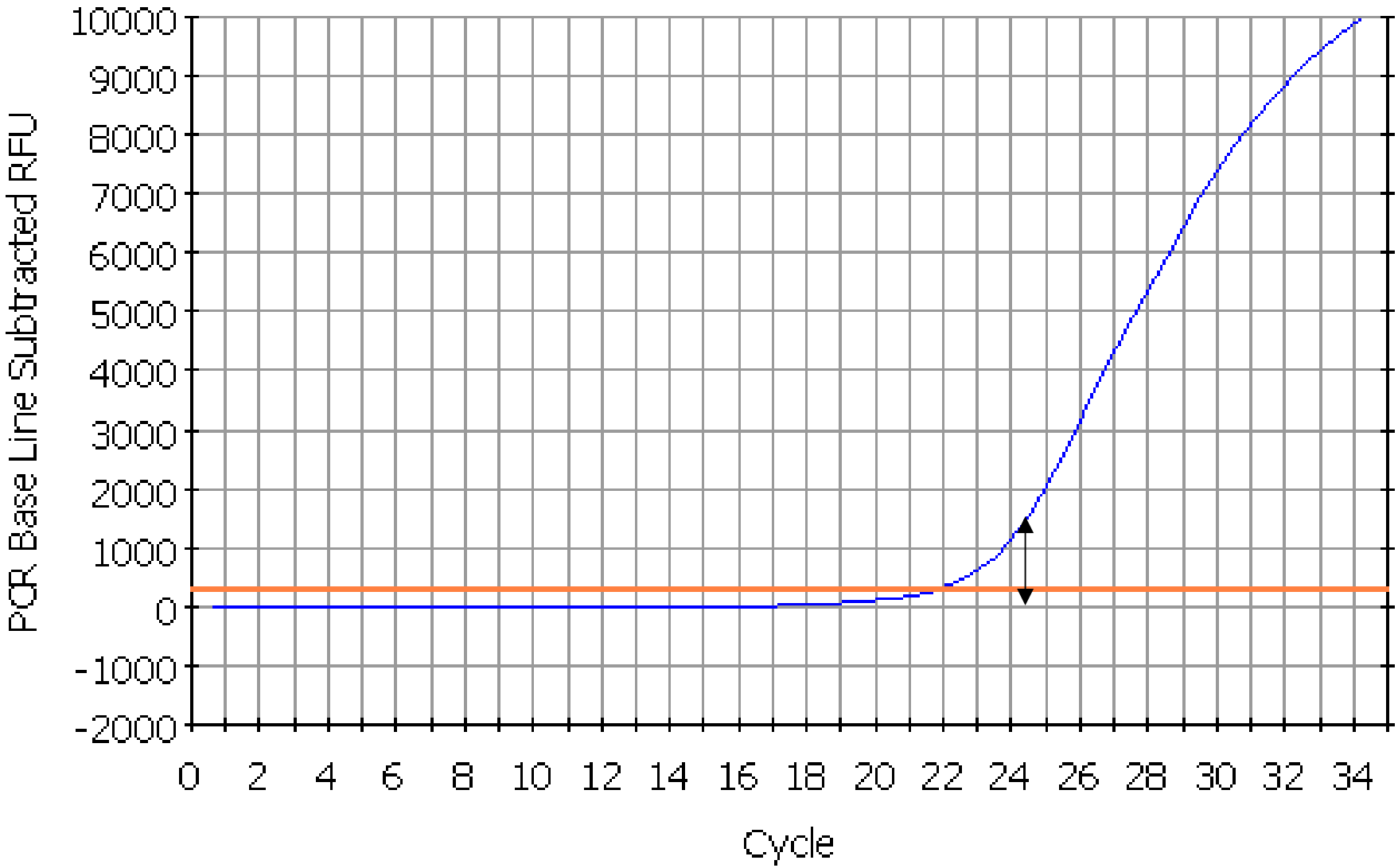

AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Cycle


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

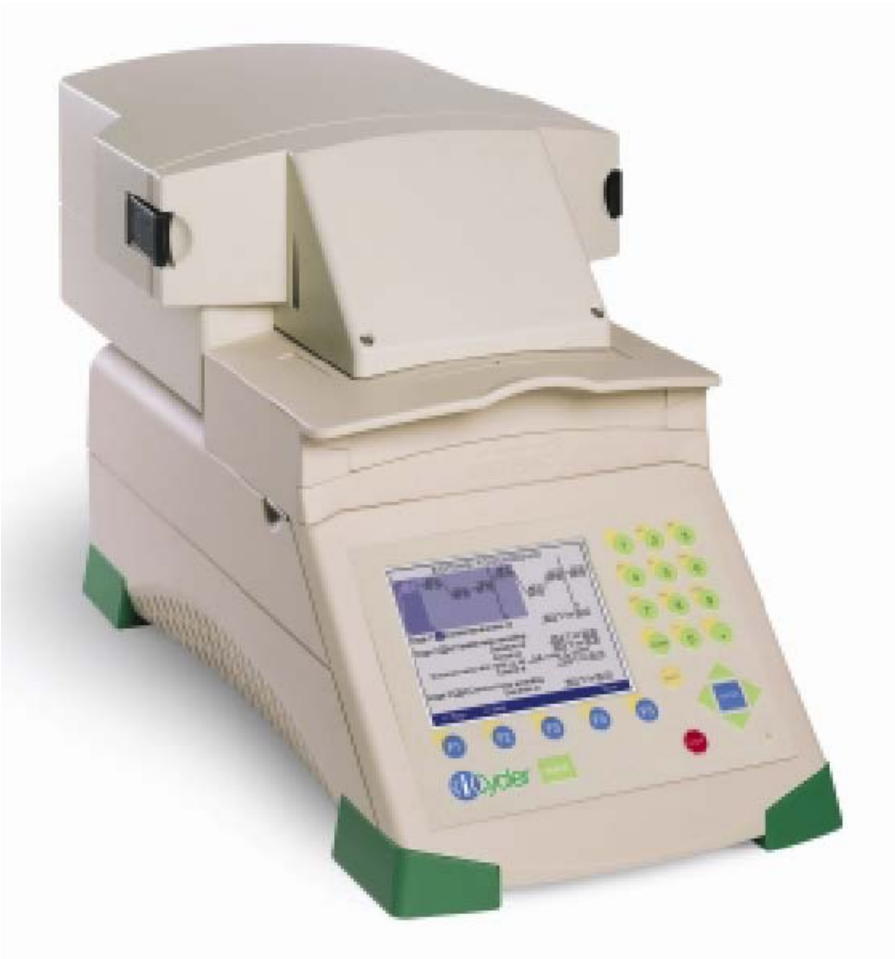


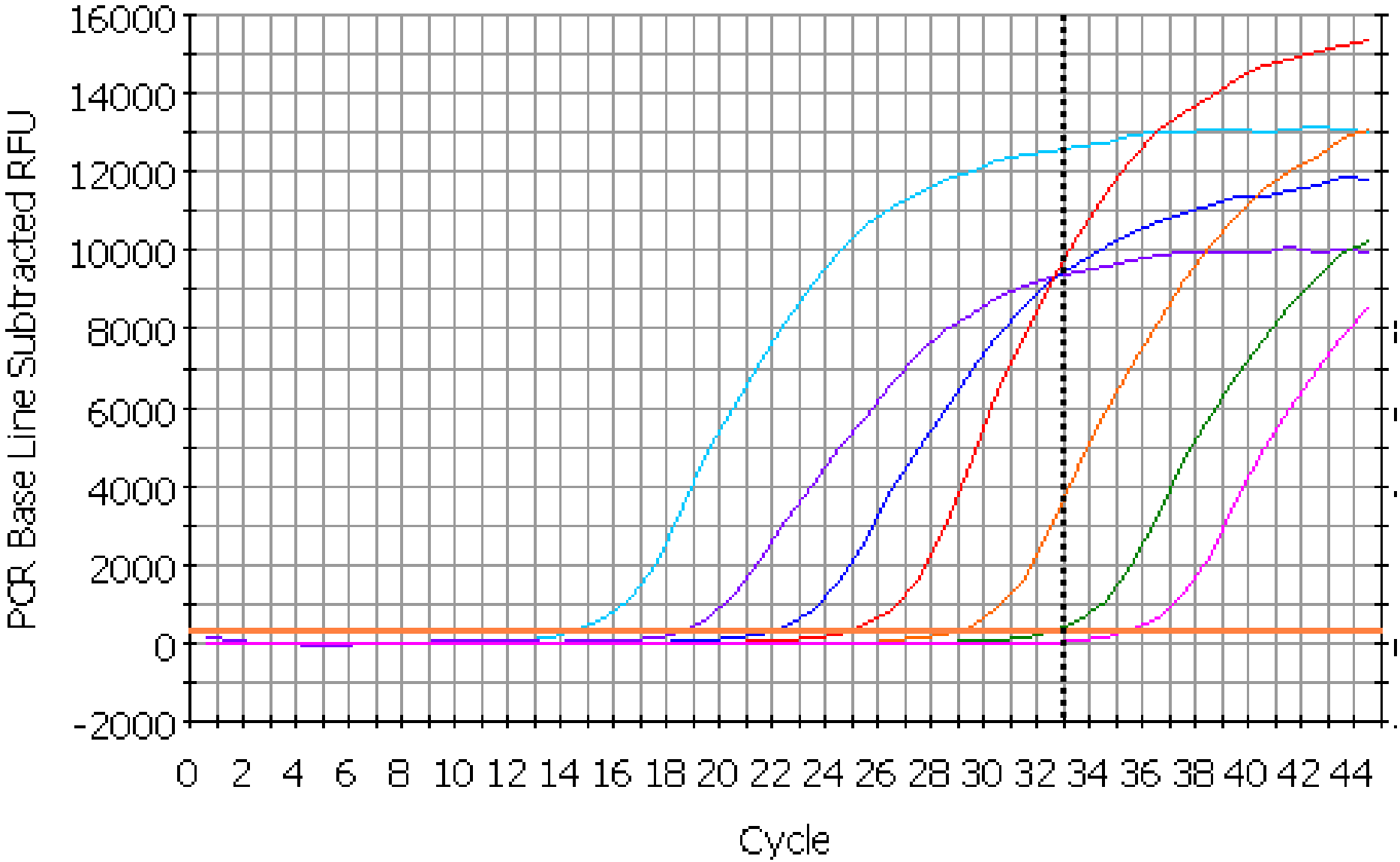
Linear ~20 to ~1500



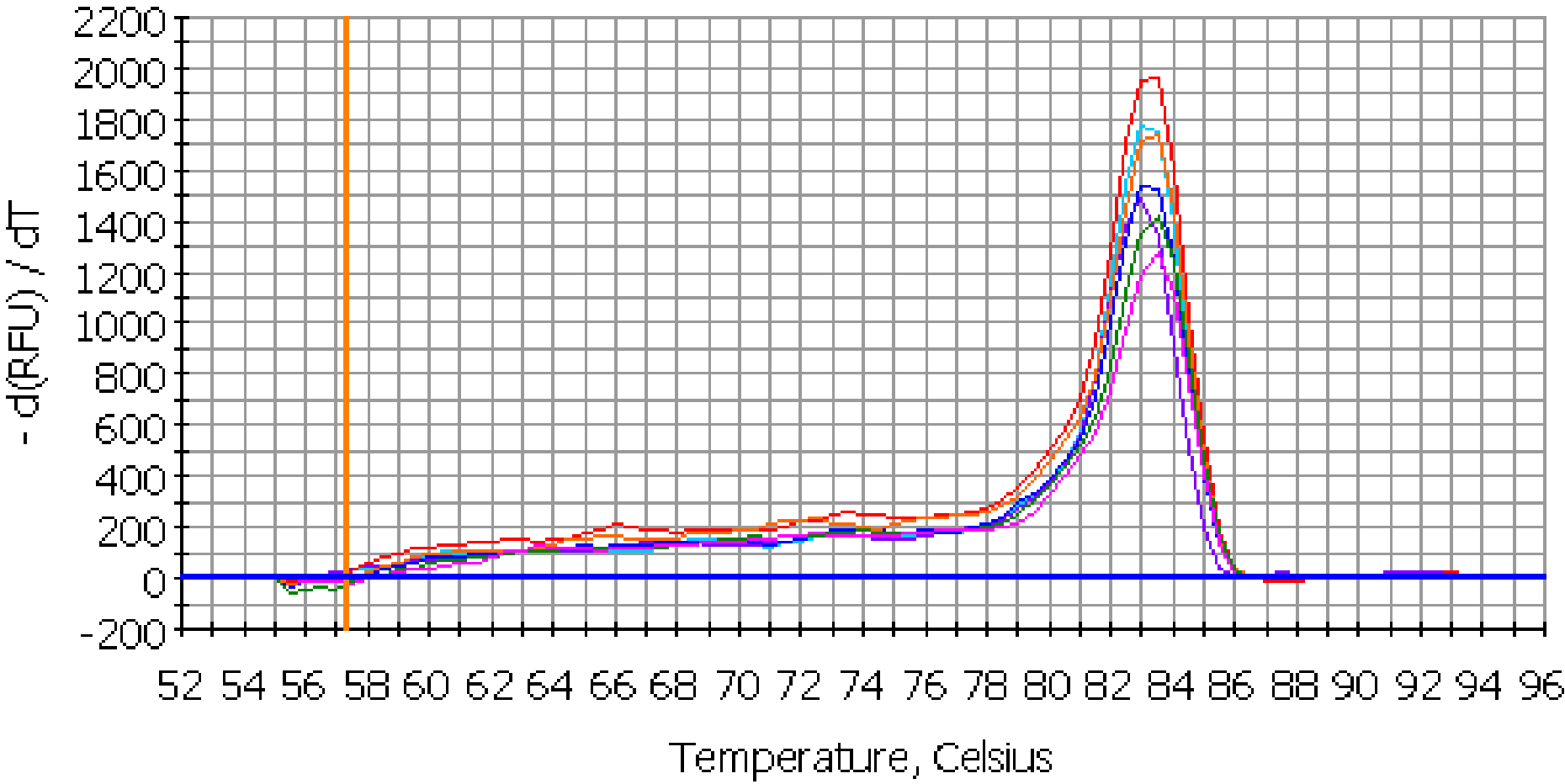
REAL TIME PCR

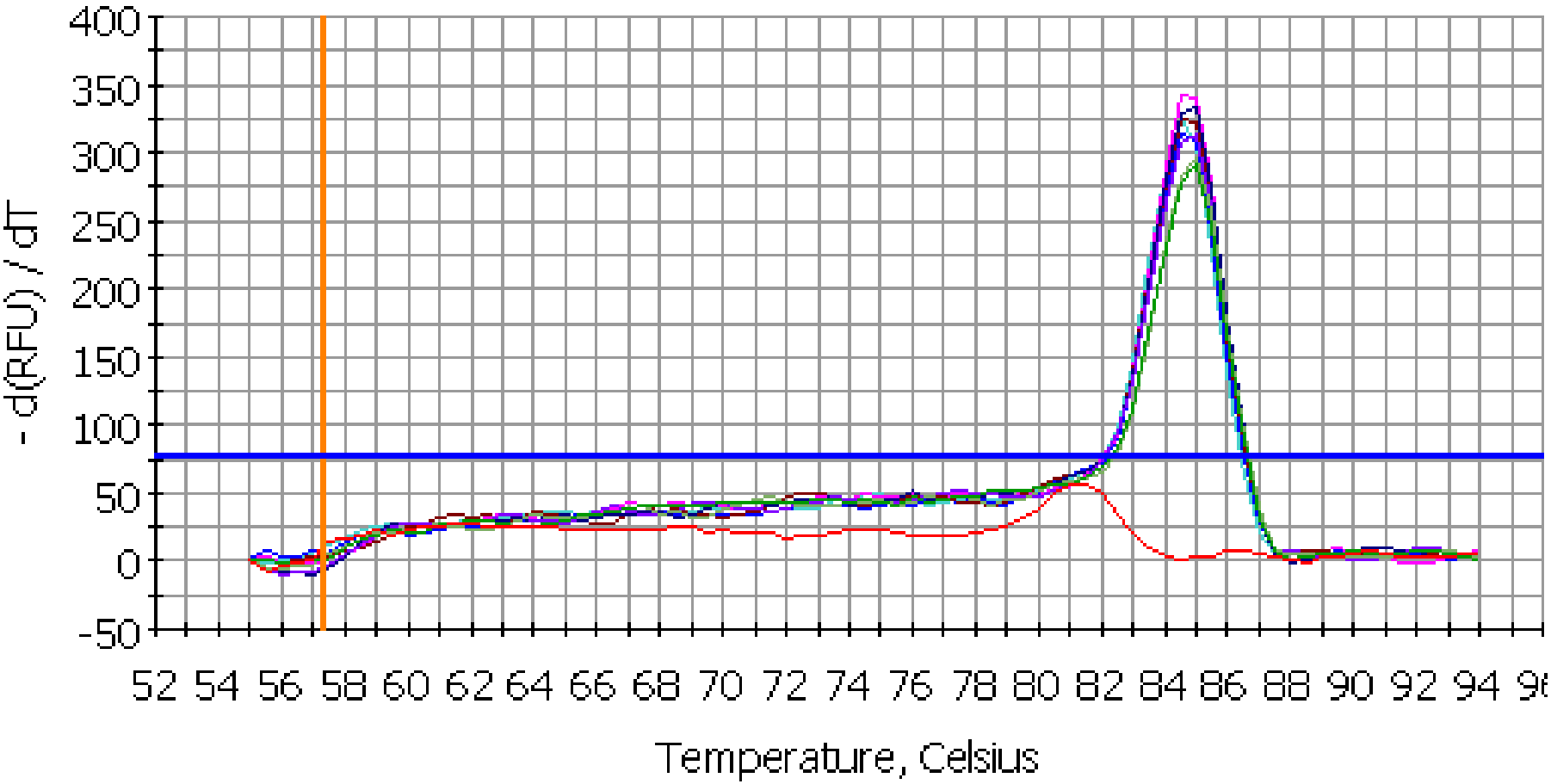
- kinetic approach
- early stages
- while still linear



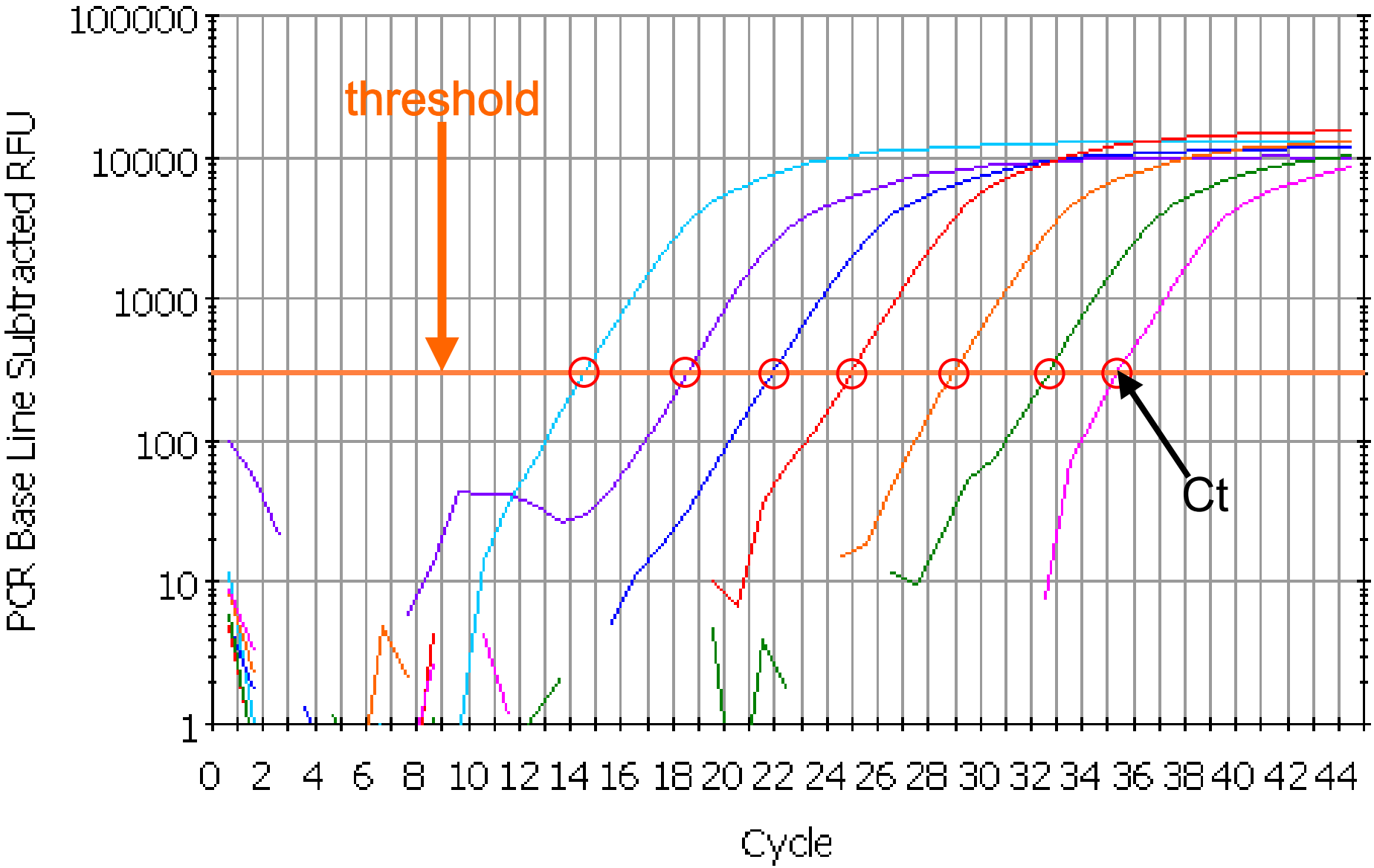


SERIES OF 10-FOLD DILUTIONS

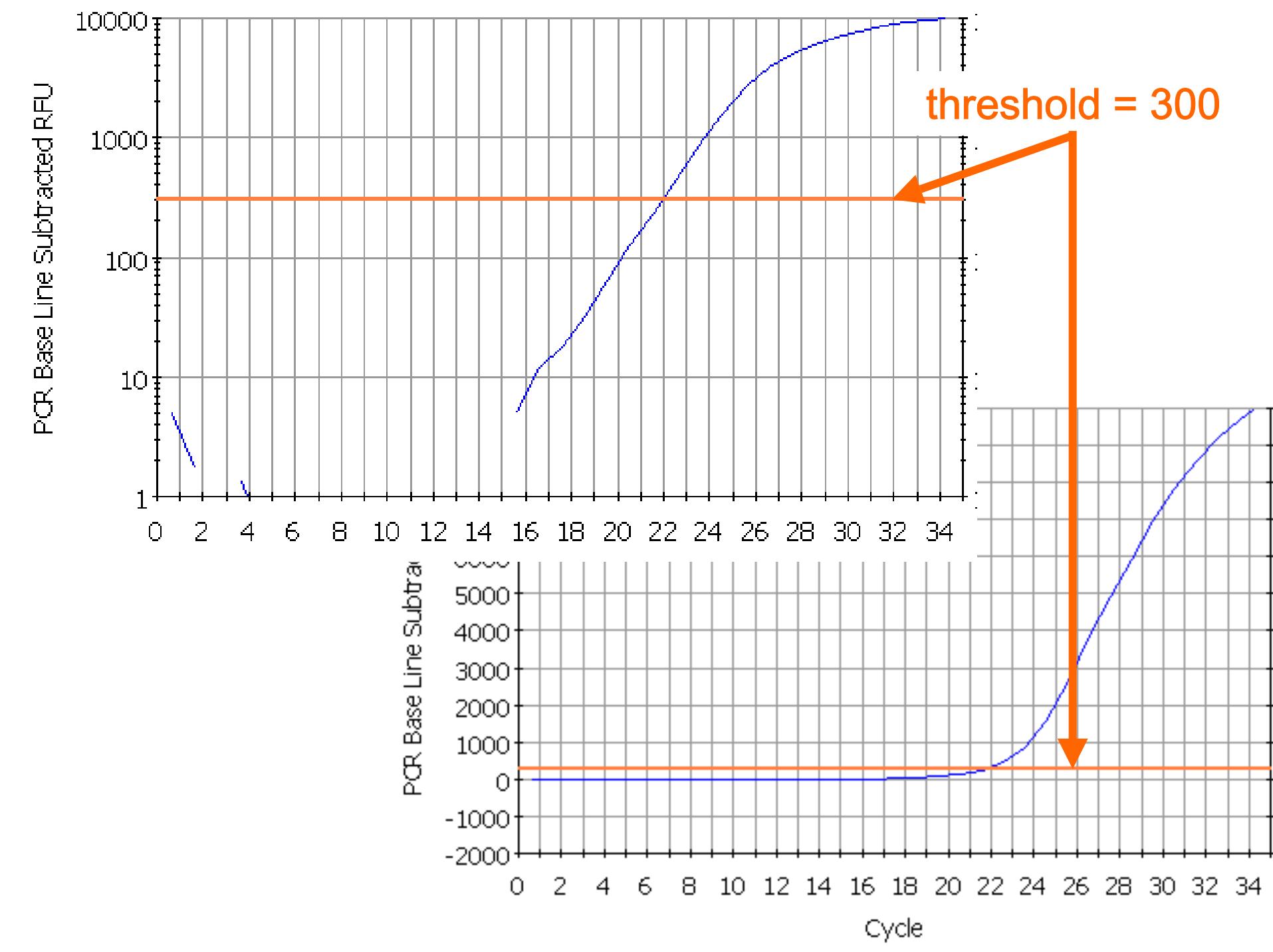

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
- d(RFU) / dT
52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96
Temperature, Celsius

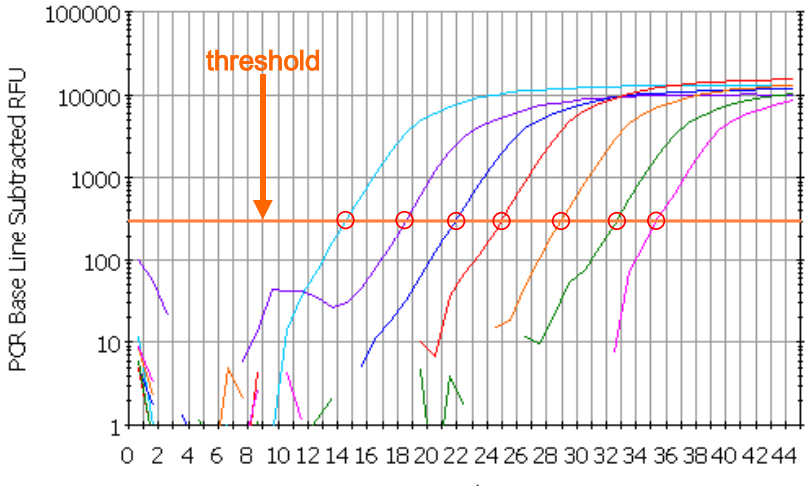


Melt Peak: Data 10-Mar-03 1259 ed.opd



SERIES OF 10-FOLD DILUTIONS

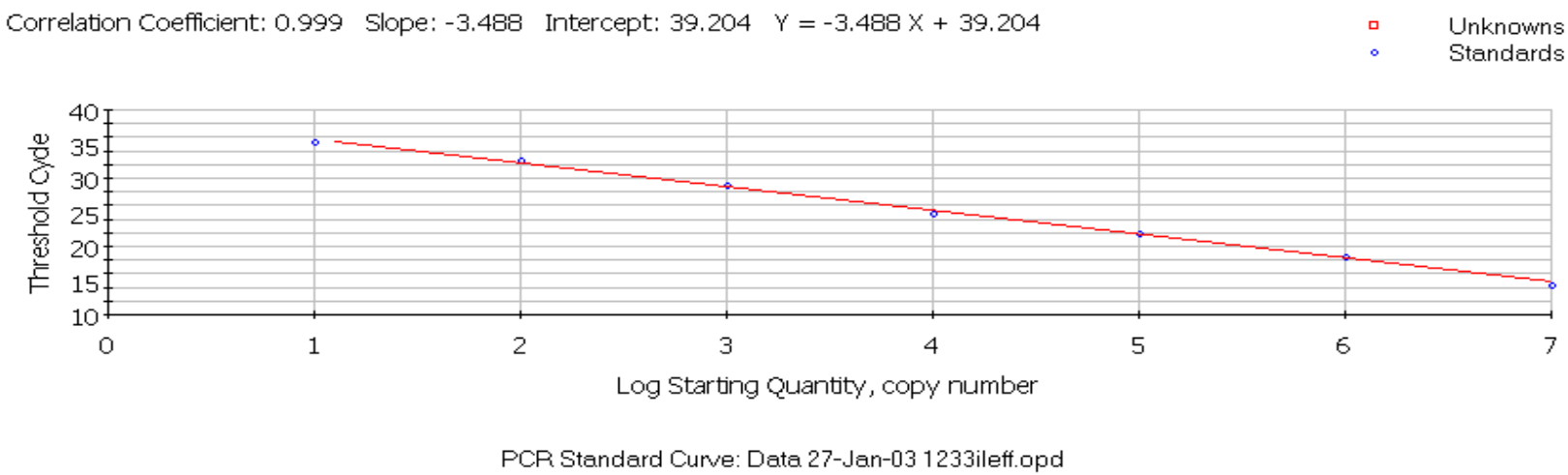

threshold = 300
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
PCR Base Line Subtra
5000
4000
3000
2000
1000
0
-1000
-2000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Cycle

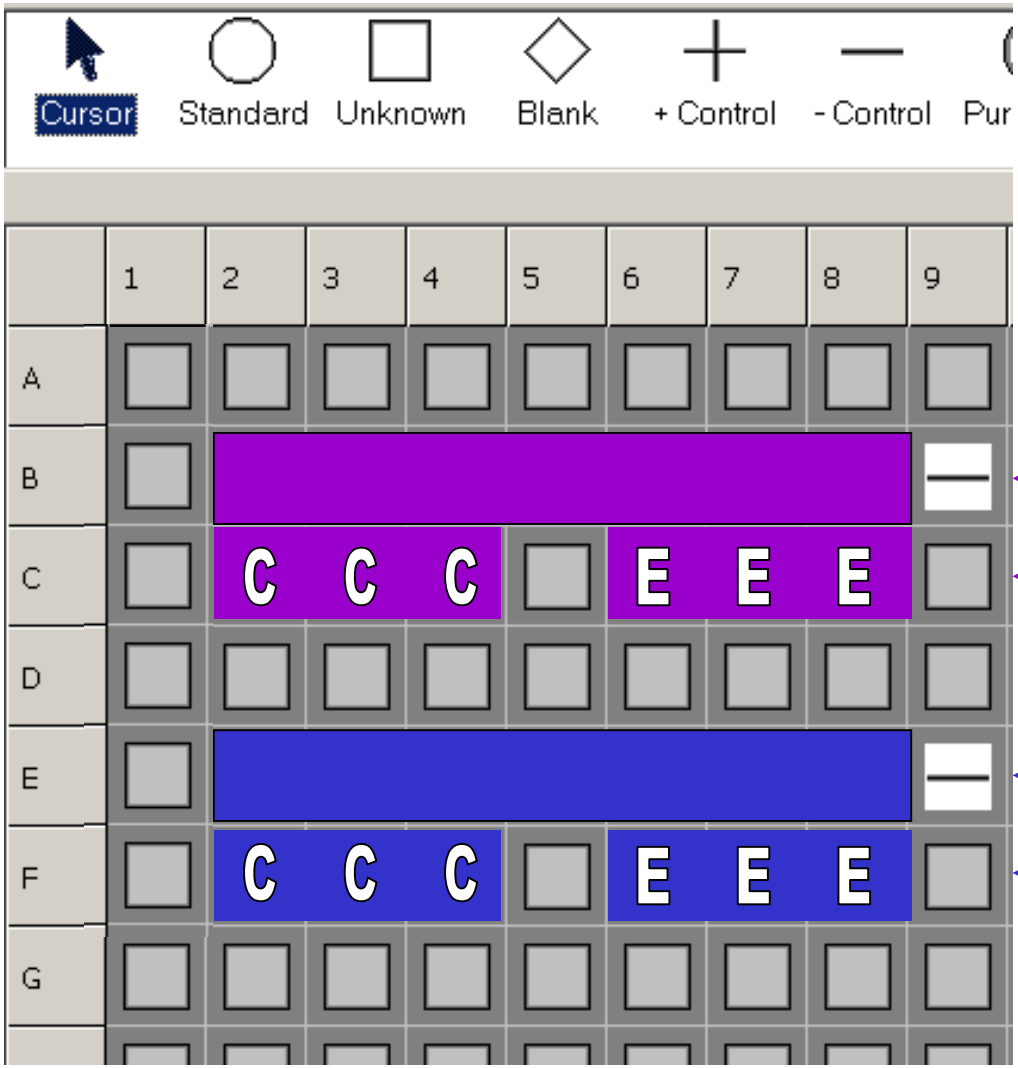


Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204

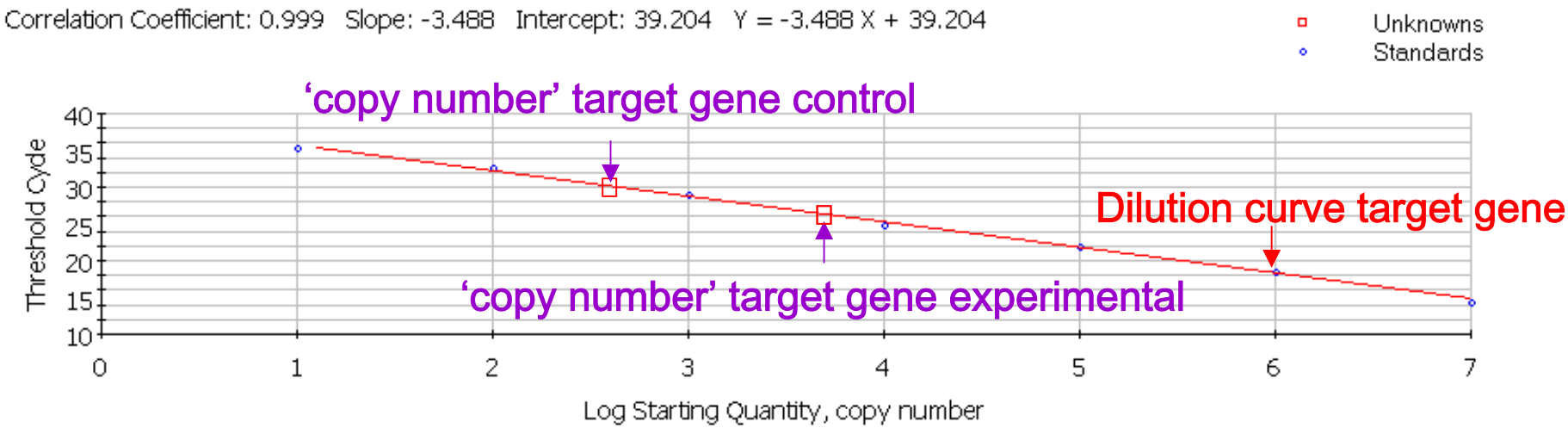
PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

STANDARD CURVE METHOD

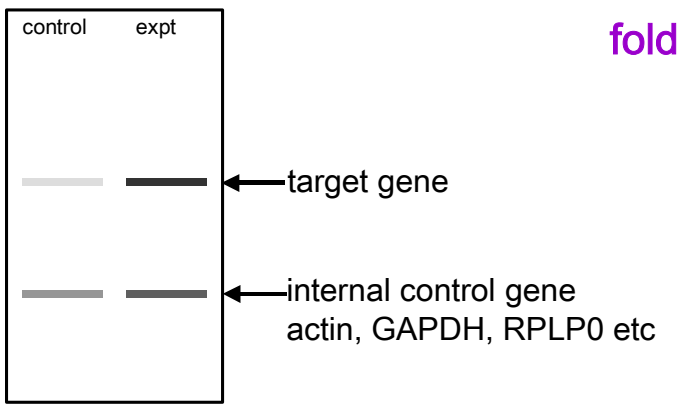




Standard curve method

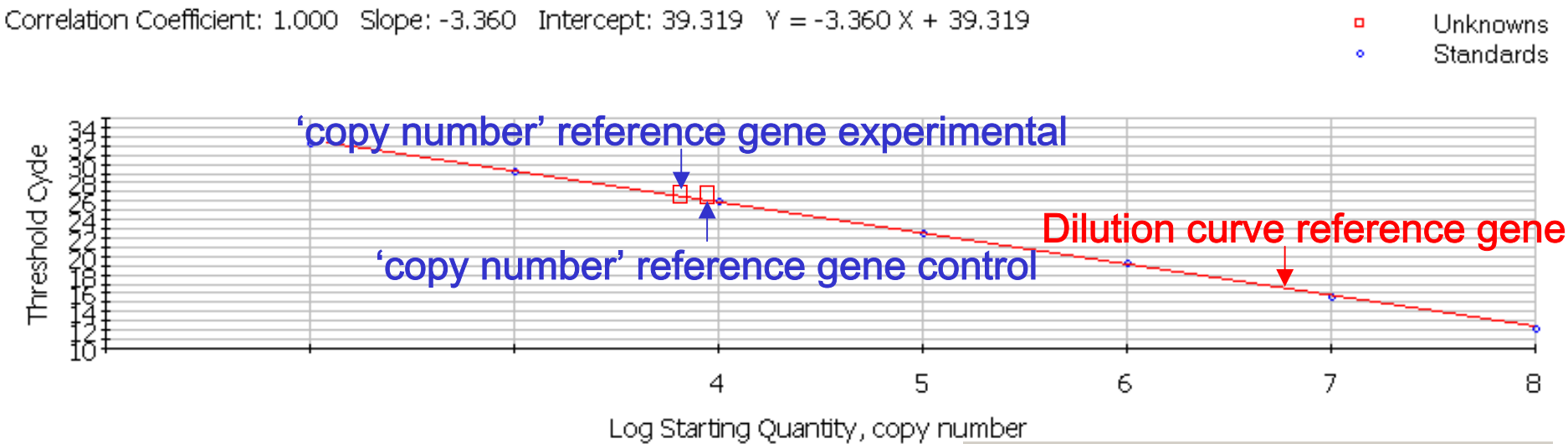


NORTHERN

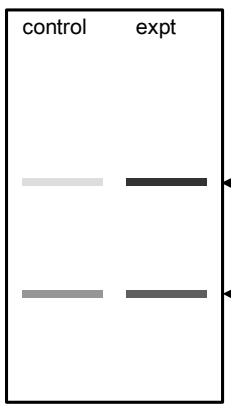


fold change in target gene= copy number experimental / copy number control

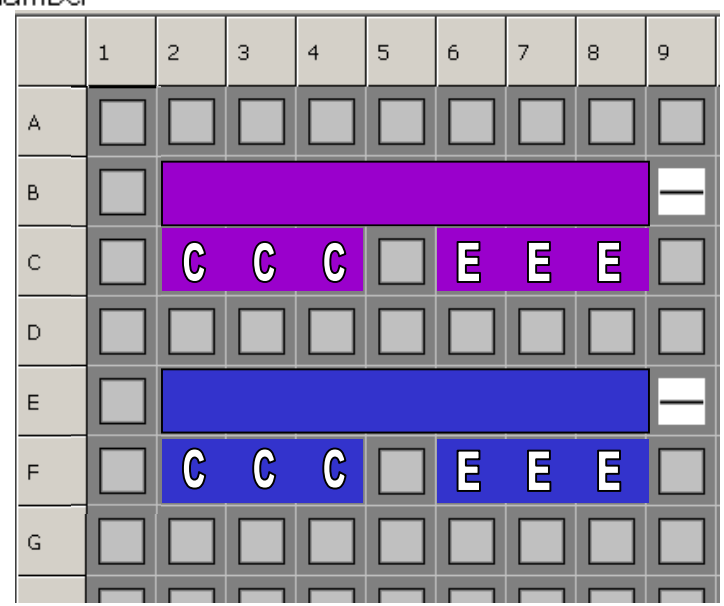
Ratio experimental/control = fold change in target gene / fold change in reference gene



NORTHERN



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



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
10	1,024	613	357	202
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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

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
8	256	170	110	70
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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
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
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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
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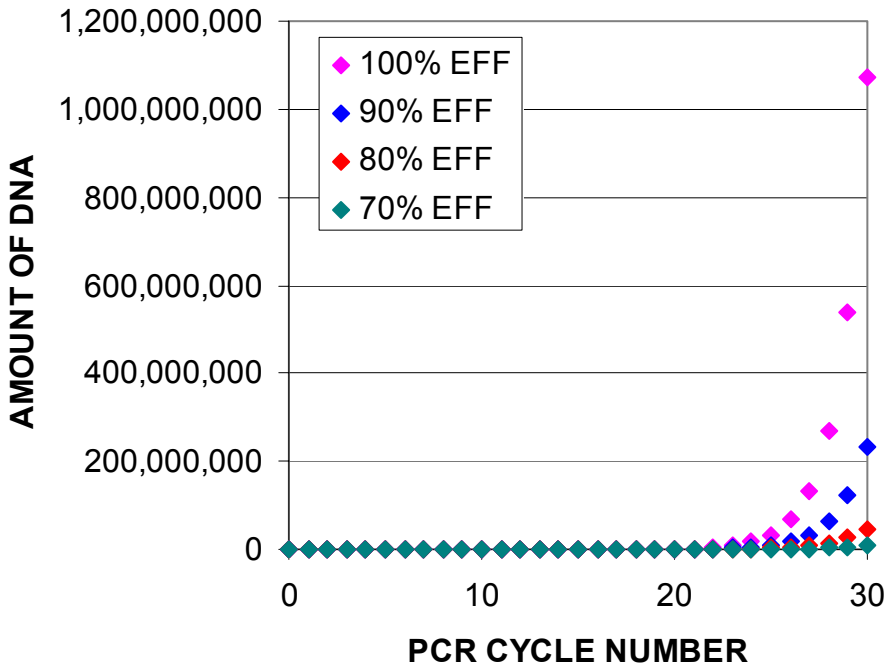
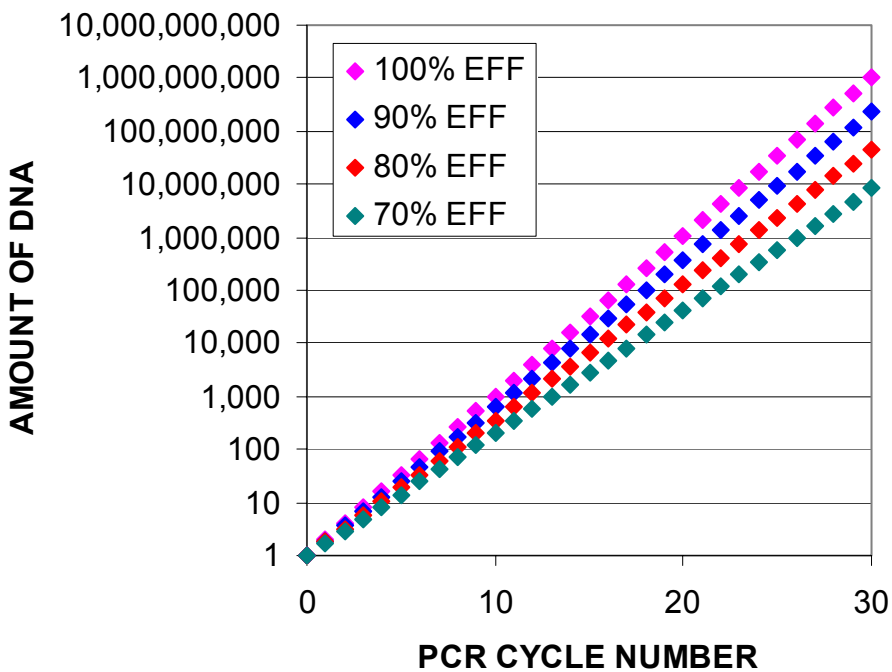
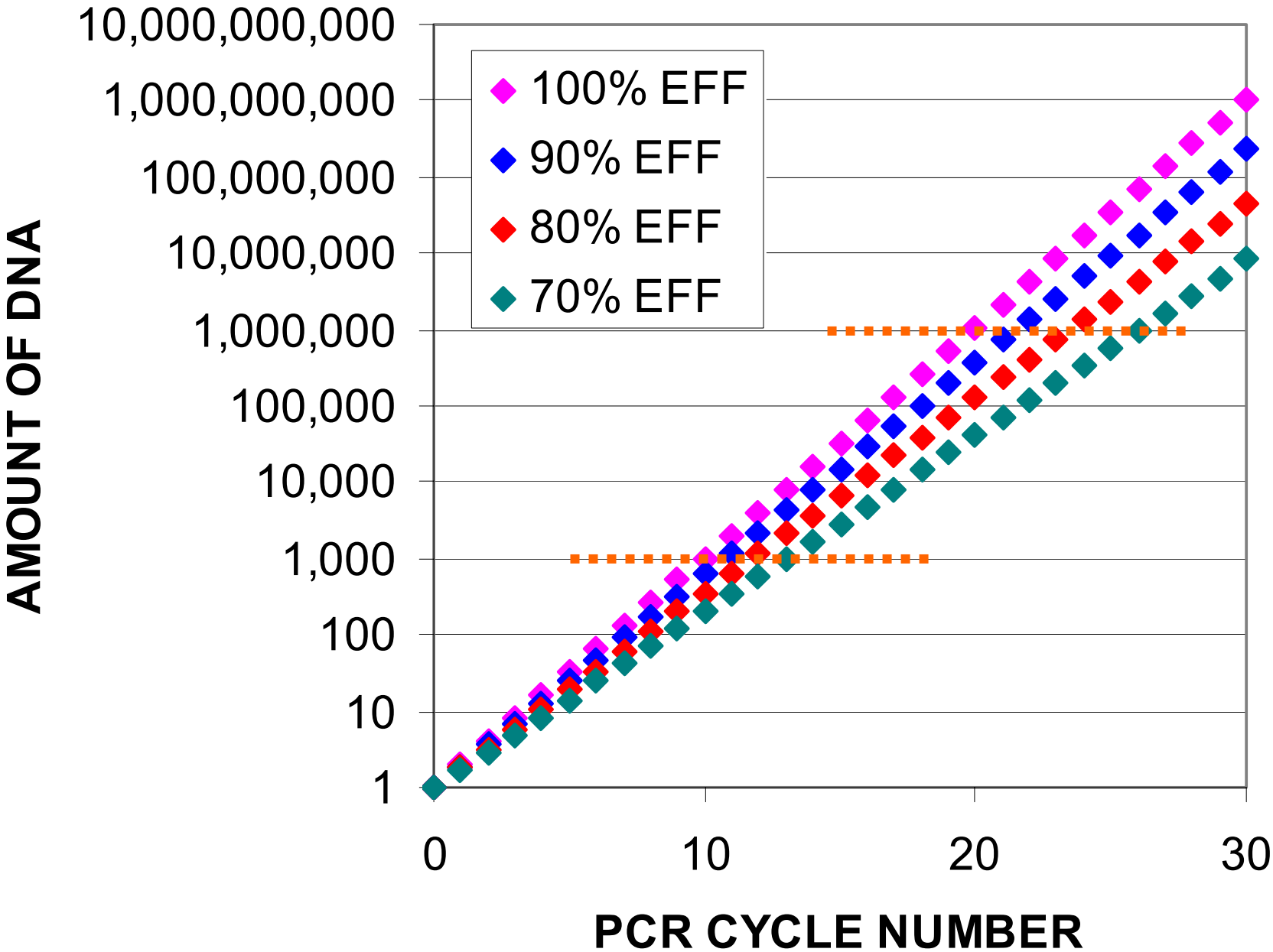
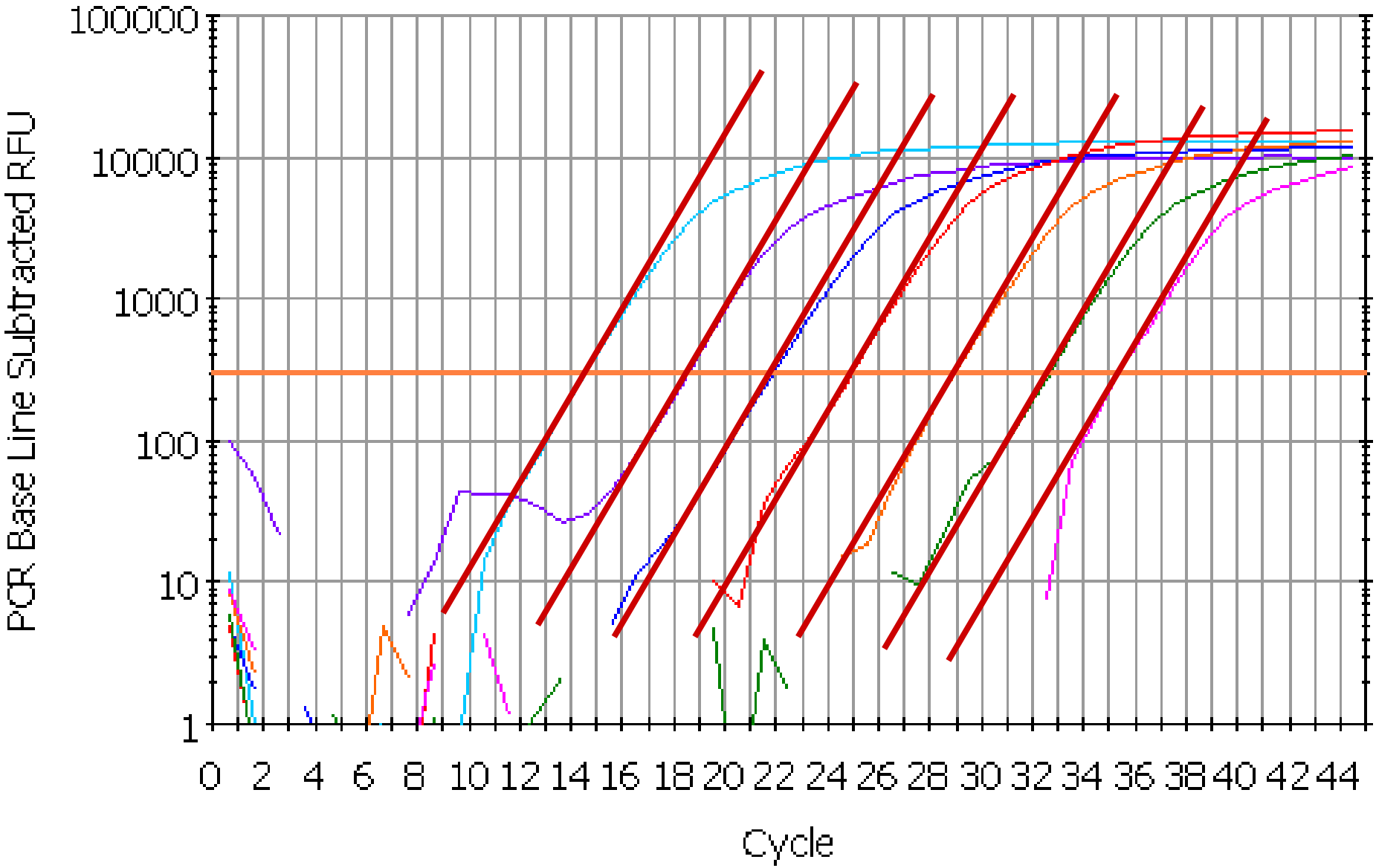
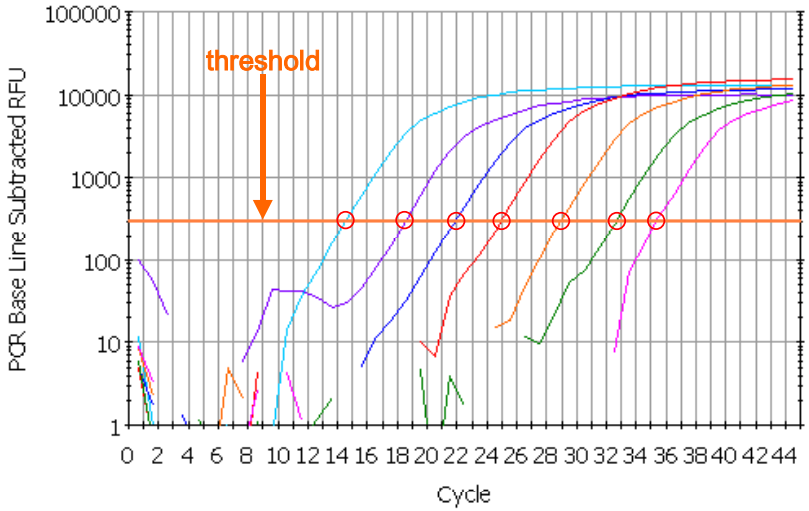





10,000,000,000
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
AMOUNT OF DNA
100% EFF
90% EFF
80% EFF
70% EFF
0
10
20
30
PCR CYCLE NUMBER



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)
- set the threshold manually to lab standard
- check all melting curves are ok
- check slopes 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 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.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

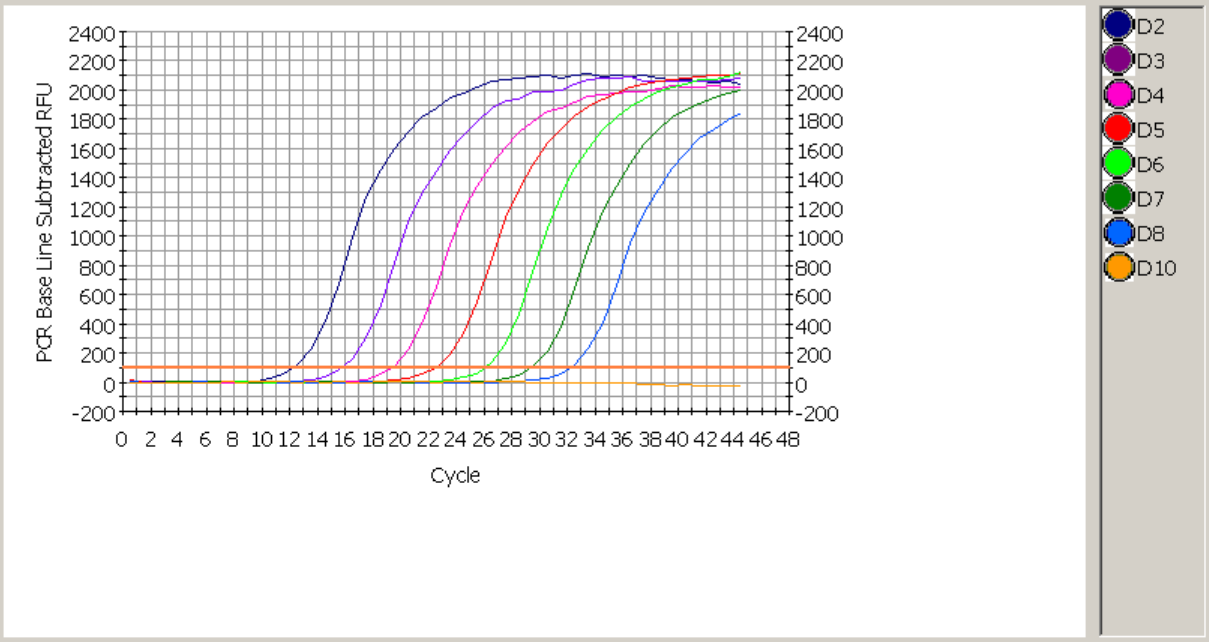
FAM-490

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	
D10	N/A	

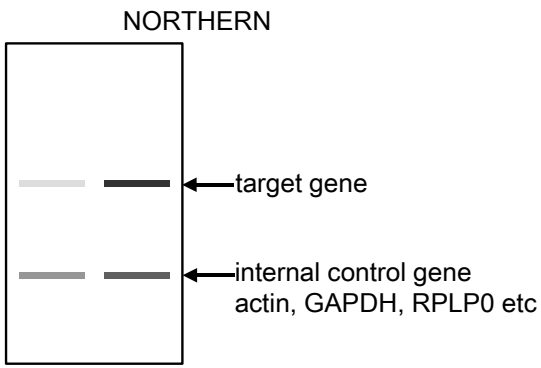
ACTION: | Data Window: Last 95% | Filter: Weighted Mean | Analysis: Post-Run

QUALITY CONTROL -EFFICIENCY CURVES

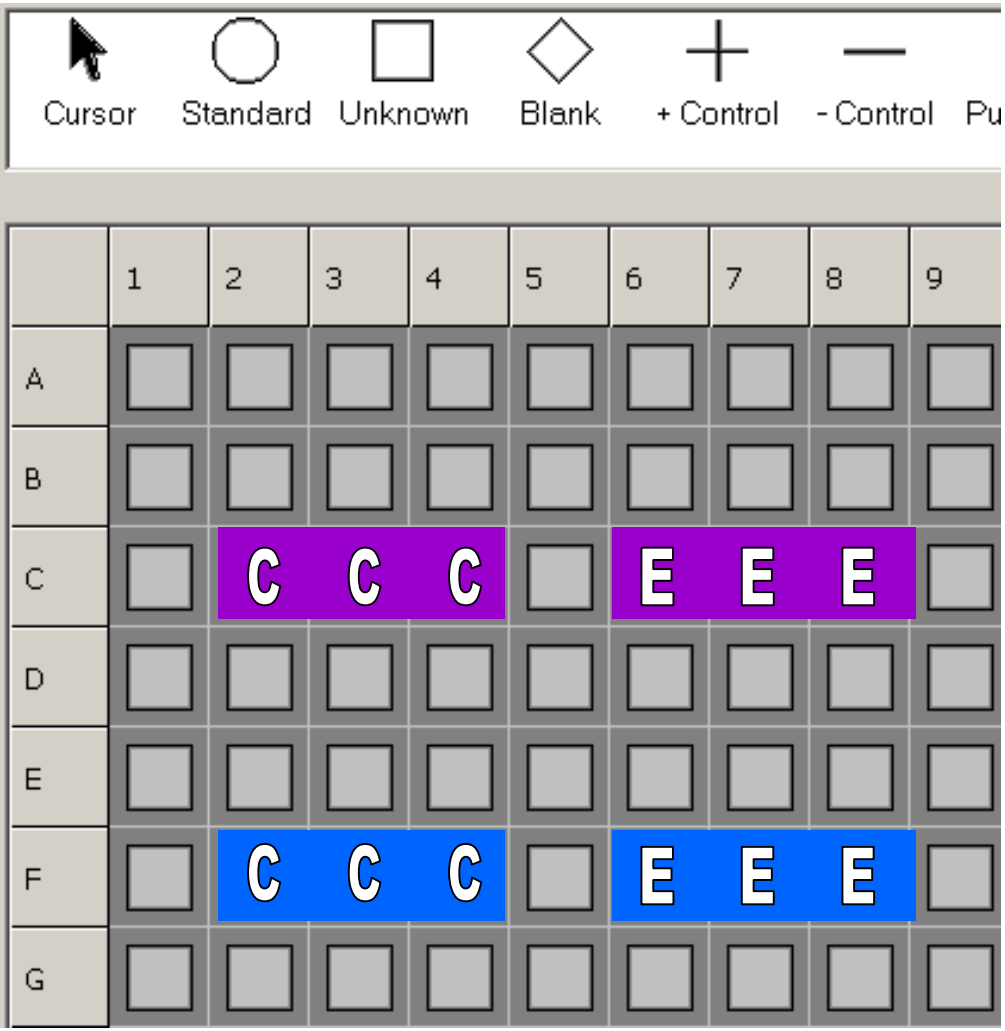
- use pcr baseline subtraction (not curve fitting default option)
- set the threshold manually to lab standard
- check all melting curves are ok
- check slopes parallel in log view
- delete samples if multiple dilutions cross line together (usually at dilute end of curve)
- delete samples if can detect amplification at cycle 10 or earlier
- make sure there are 5 or more points
- check correlation coefficient more than 0.990

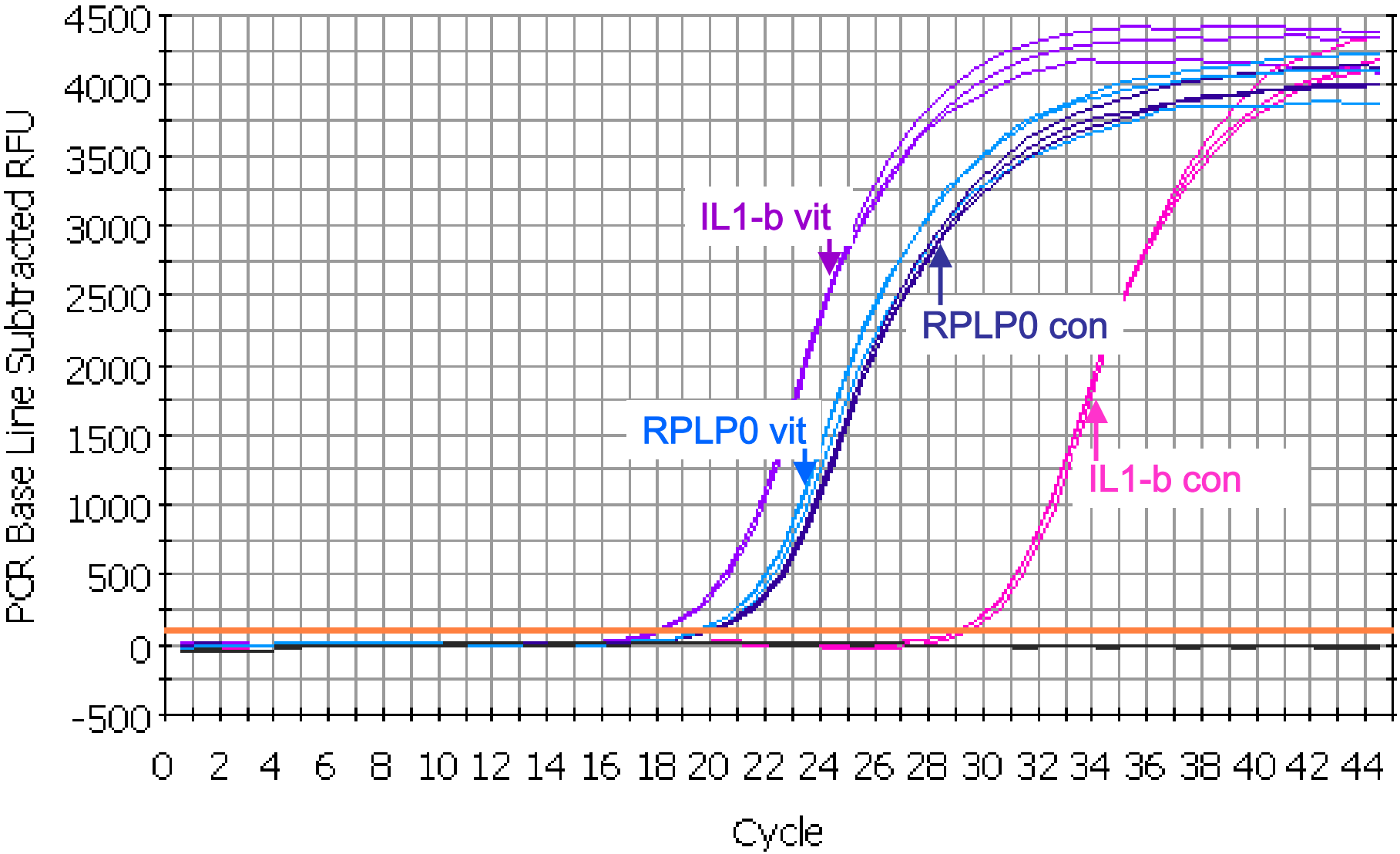
PFAFFL METHOD

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

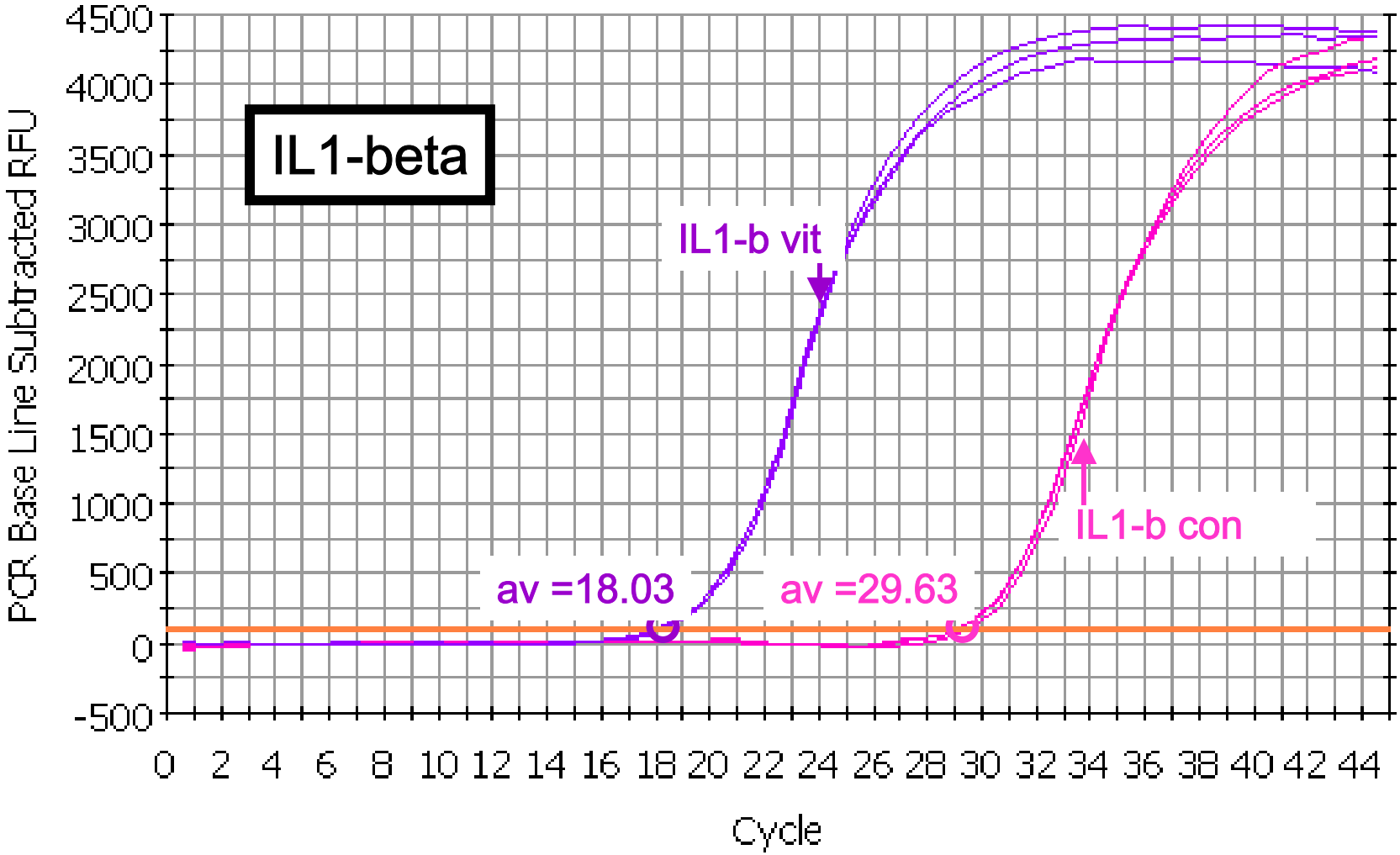


$$\text{ratio} = \frac{\text{fold increase in target gene}}{\text{fold increase in reference gene}}$$


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



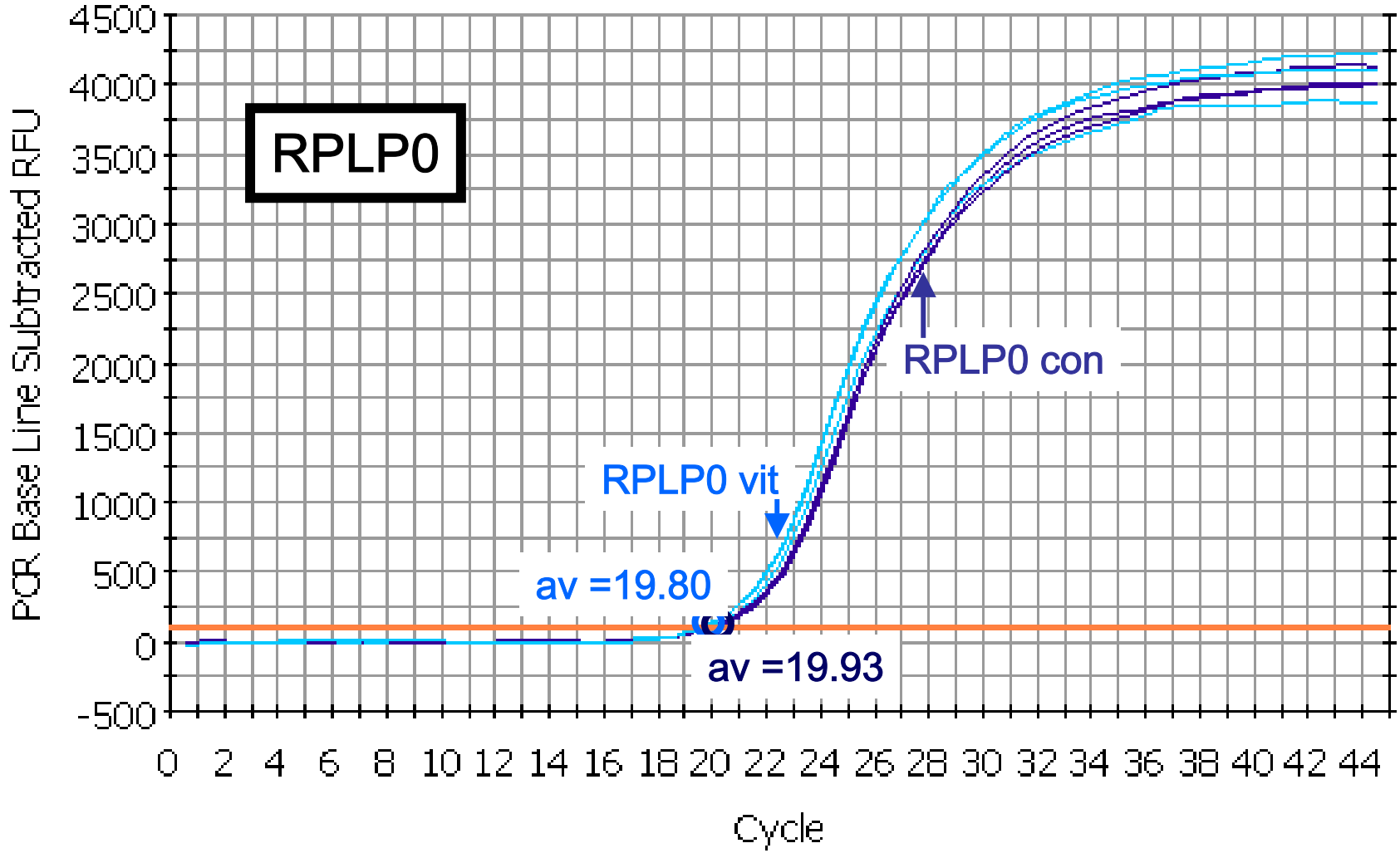
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



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

AFTER N CYCLES: increase = $(1.93)^{29.63-18.03} = 1.93^{11.60}$ = 2053 fold increase

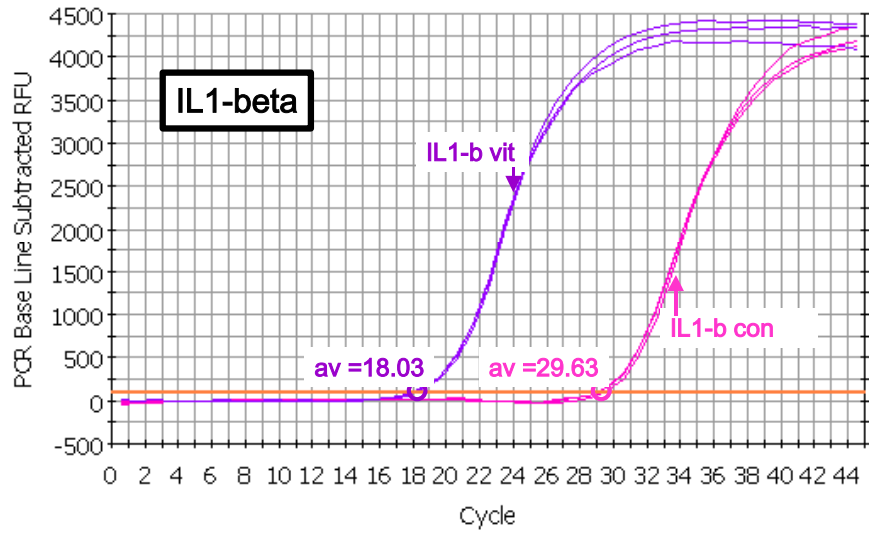
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



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

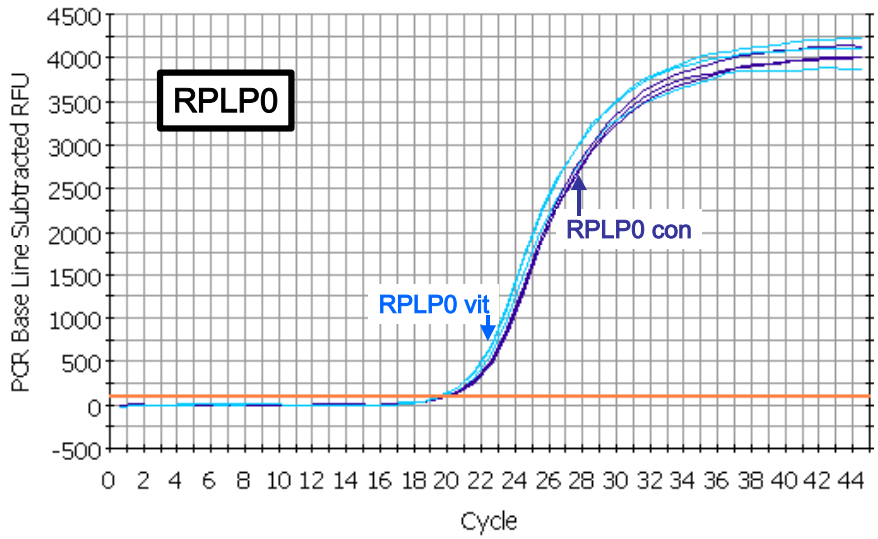
AFTER N CYCLES: increase = $(1.87)^{19.93-19.80} = 1.87^{0.13}$ = 1.08 fold increase

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



AFTER N CYCLES: increase = (efficiency)^n

increase = $(1.93)^{29.63-18.03} = 1.93^{11.60}$ = 2053 fold increase



AFTER N CYCLES: increase = (efficiency)^n

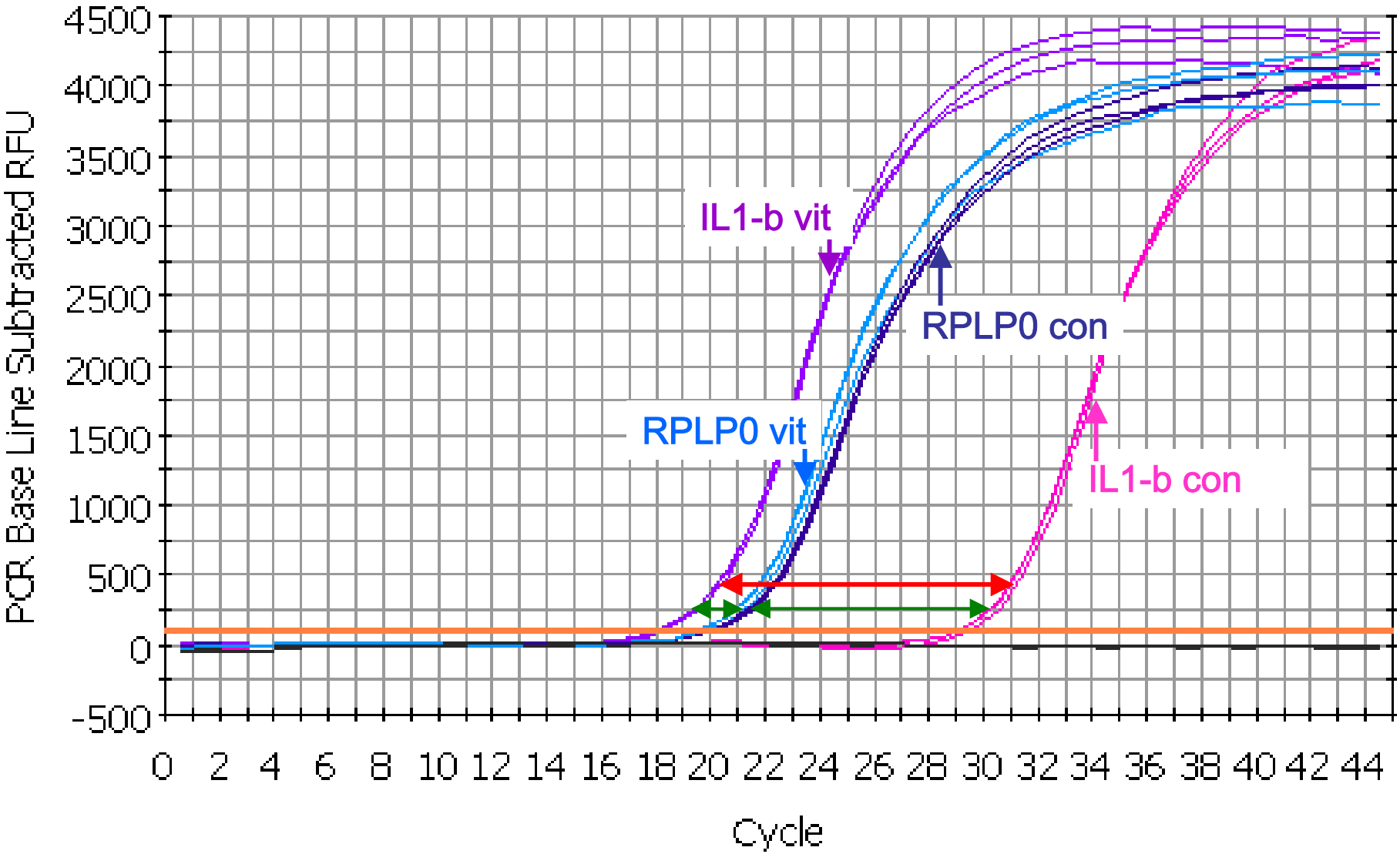
increase = $(1.87)^{19.93-19.80} = 1.87^{0.13}$ = 1.08 fold increase

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

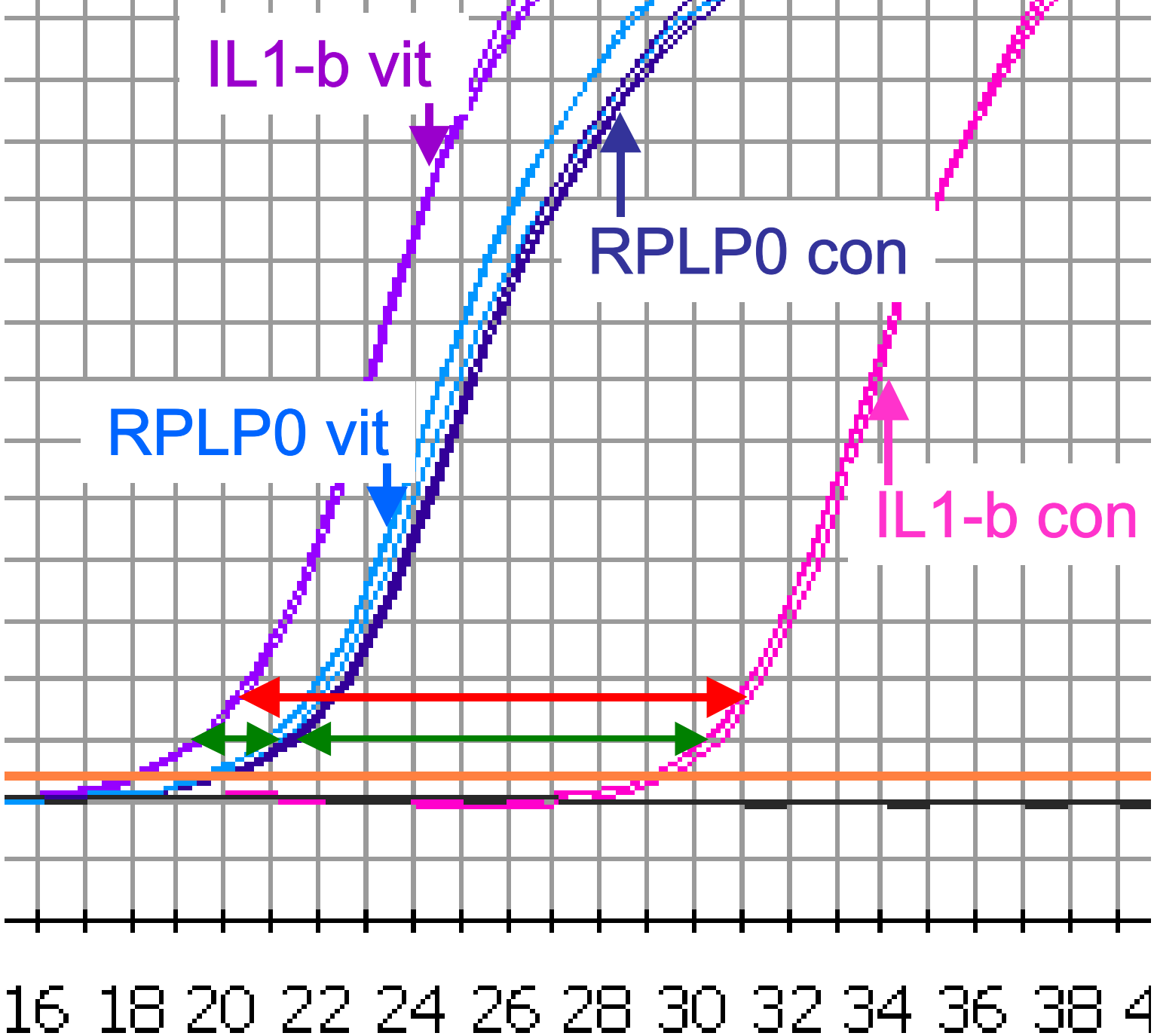
$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control\text{-}treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control\text{-}treated)}}$$

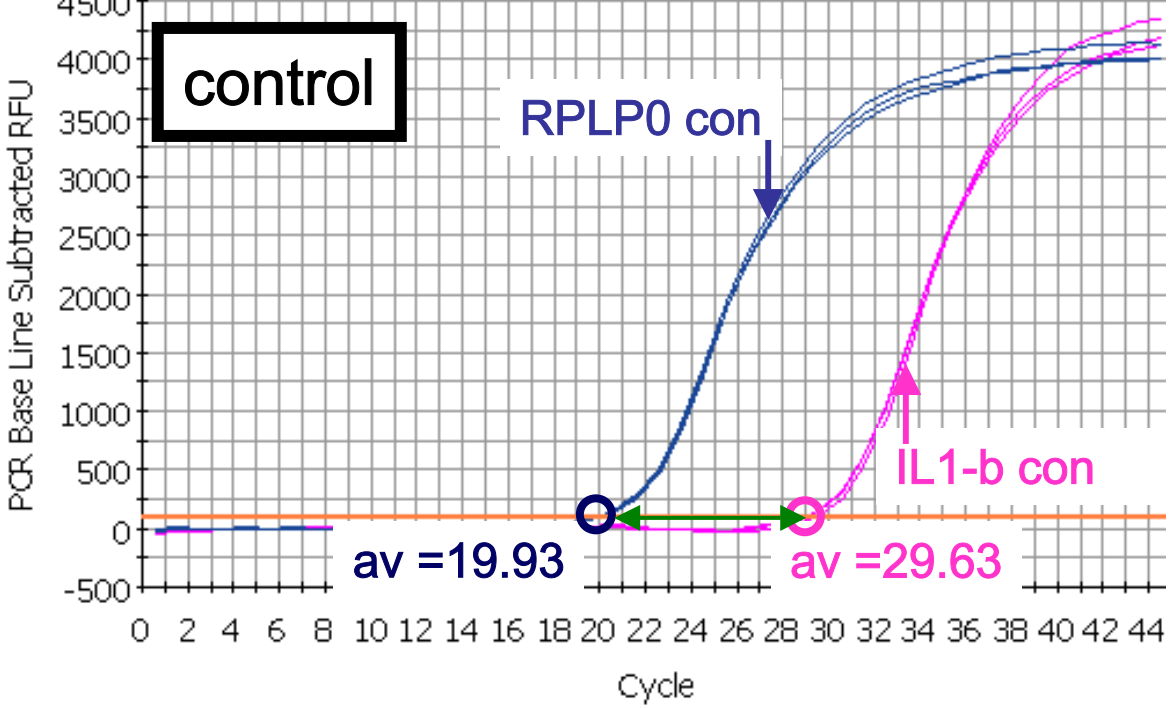
EFFICIENCY$^{\Delta\Delta Ct}$ METHOD

APPROXIMATION METHOD



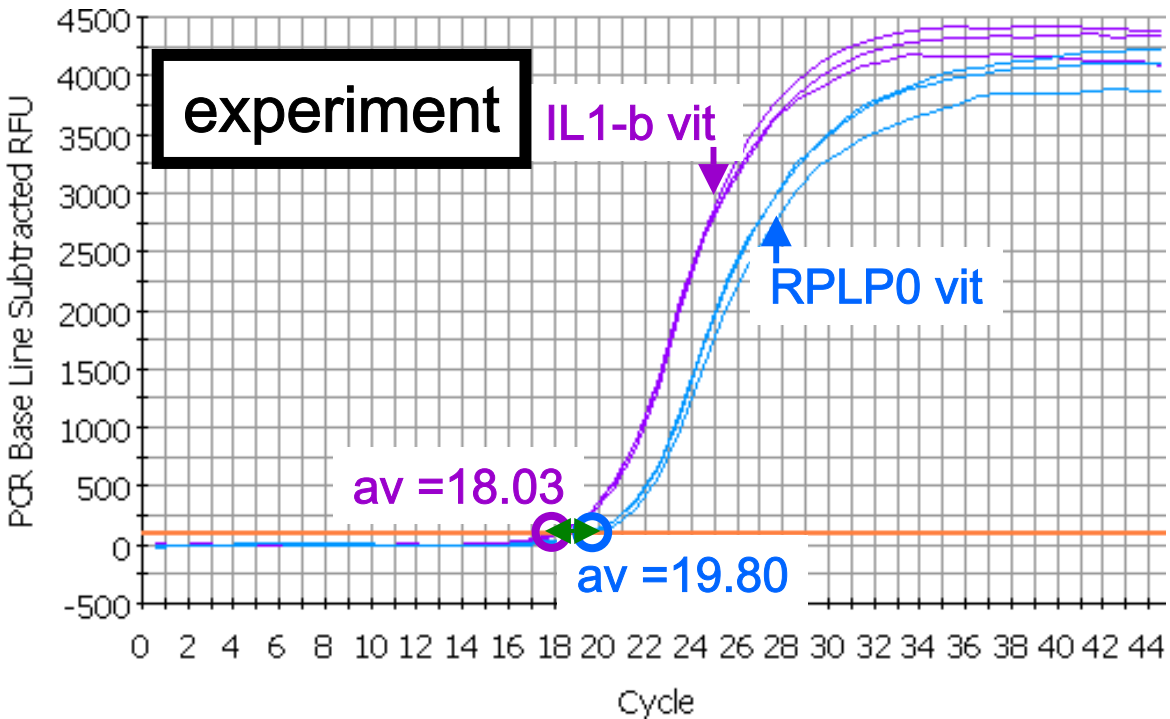
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd


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



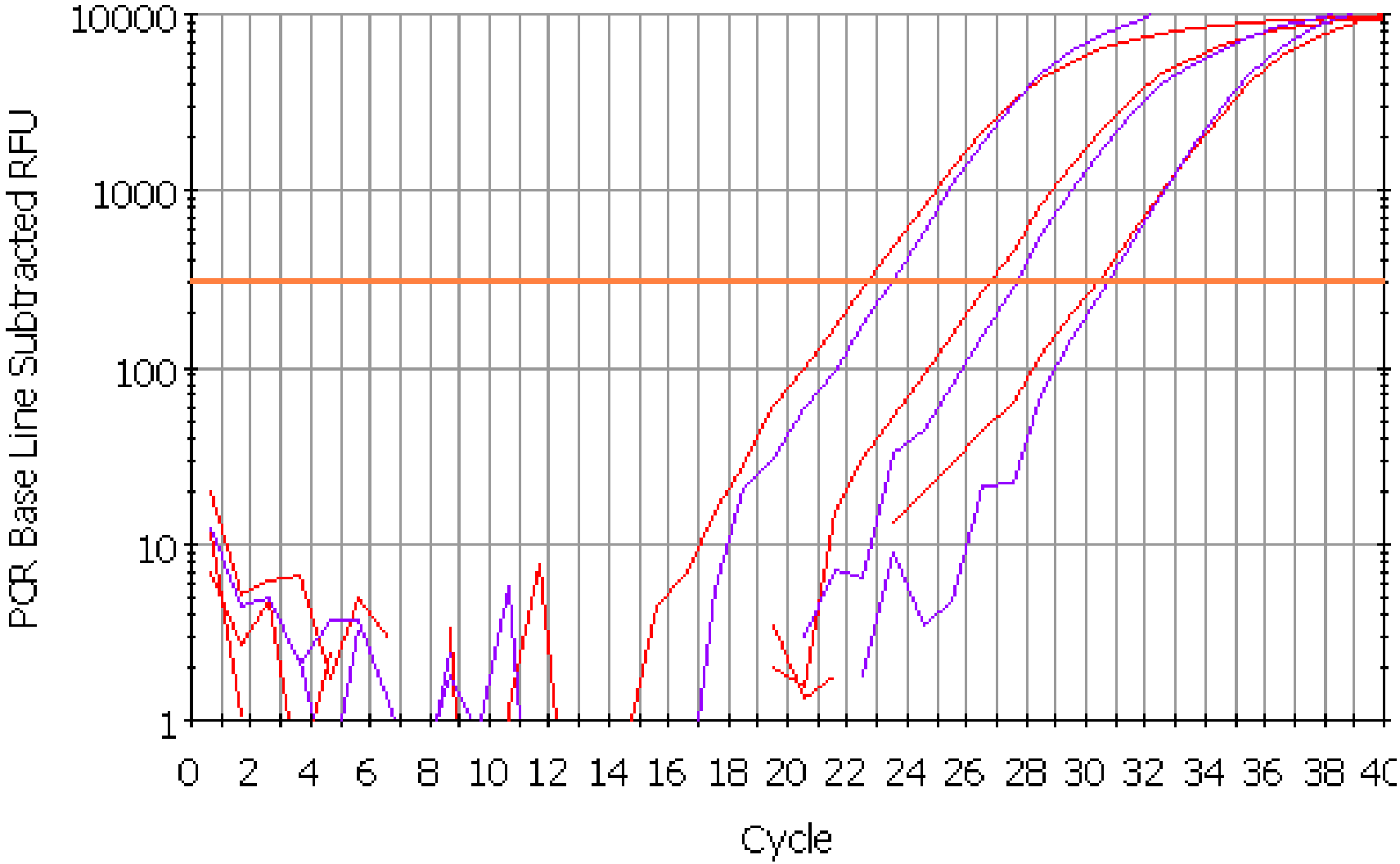
Δ Ct = target - ref

Δ Ct = -1.7

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

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

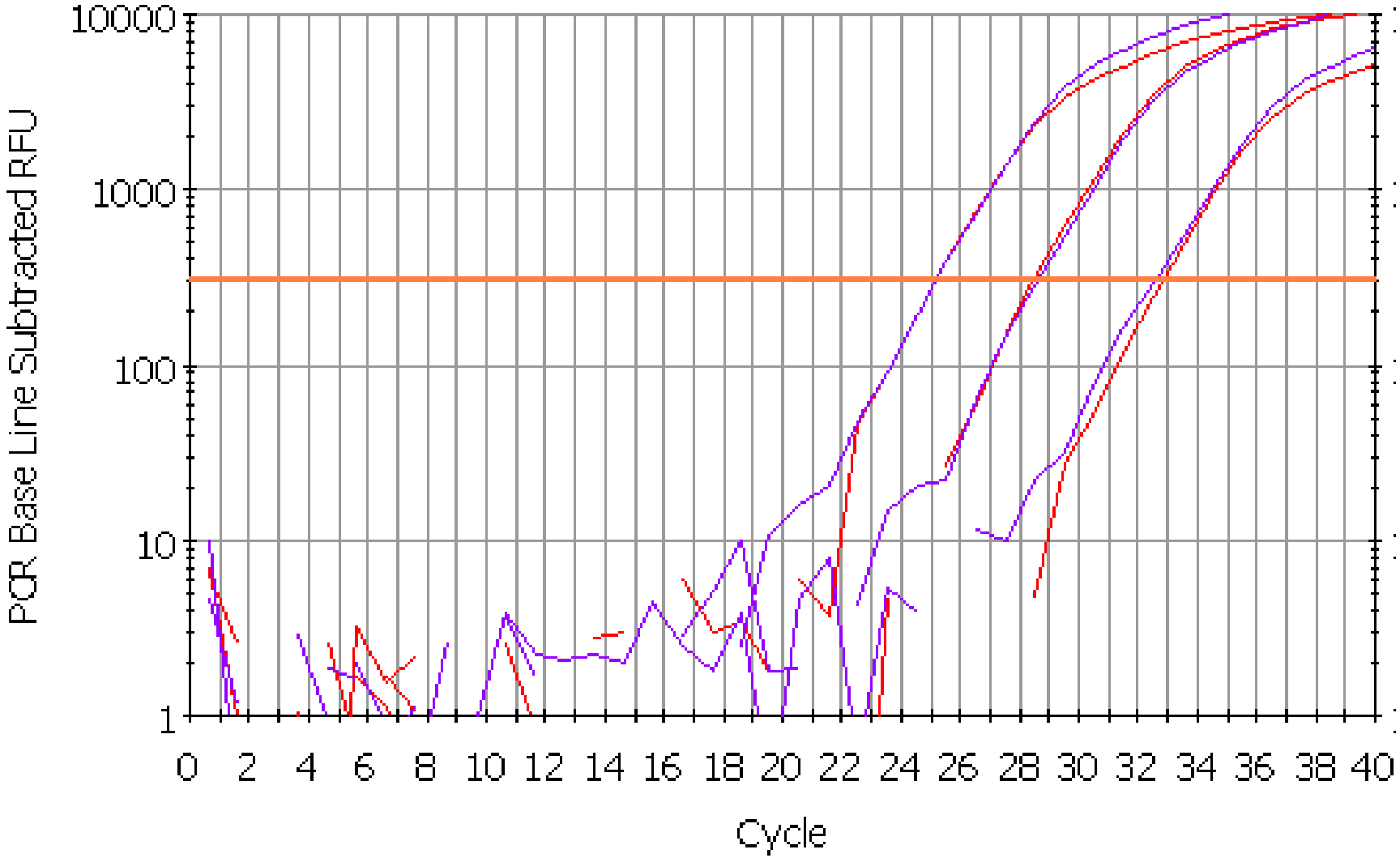
- If efficiency is 100% then
  - Fold change = $2^{11.40}$ = 2702
- Efficiency for IL1-beta is 93%
  - Fold change = $1.93^{11.40}$ = 1800
- Pfaffl equation corrected for RPLP0 efficiency
  - Fold change = 1901



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, little more work than validating the approximation method

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