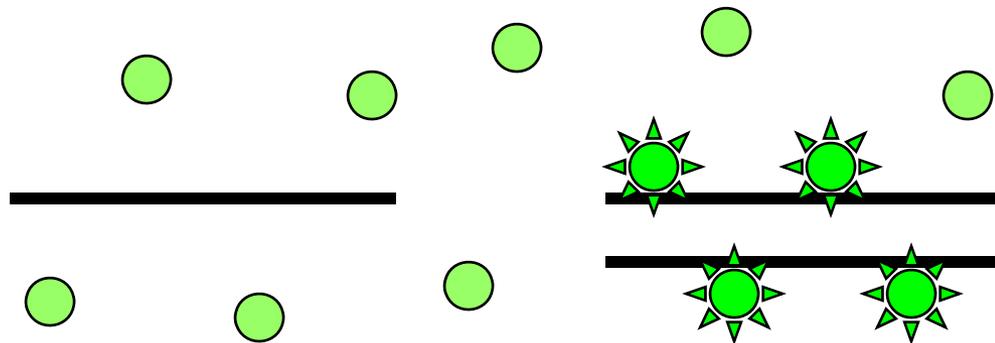# REAL TIME PCR

## USING SYBR GREEN

# THE PROBLEM

- NEED TO QUANTITATE DIFFERENCES IN mRNA EXPRESSION

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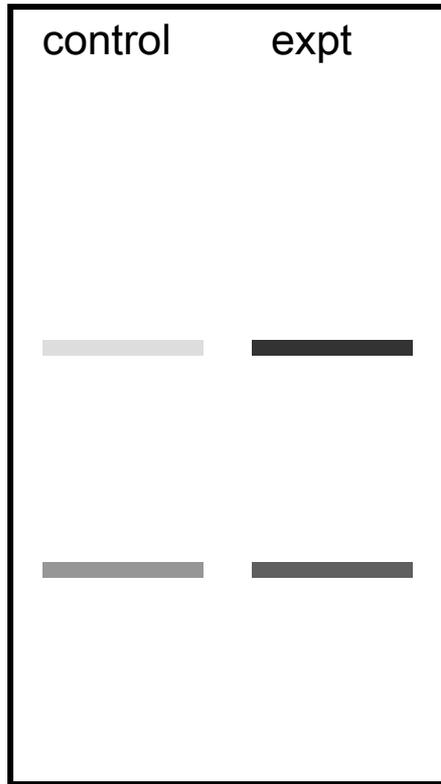  - PRIMARY CELLS
  - PRECIOUS REAGENTS

# 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



| control | expt |
|---------|------|

target gene — **10X**

internal control gene
actin, GAPDH, RPLP0 etc — **2X**

**Corrected fold increase = 10/2 = 5**

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

4

# Standards

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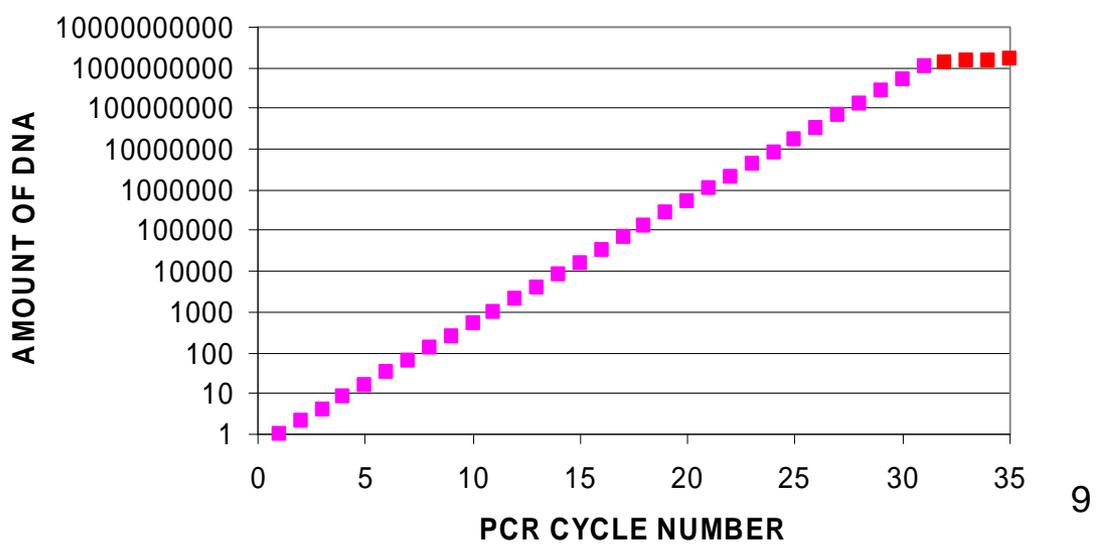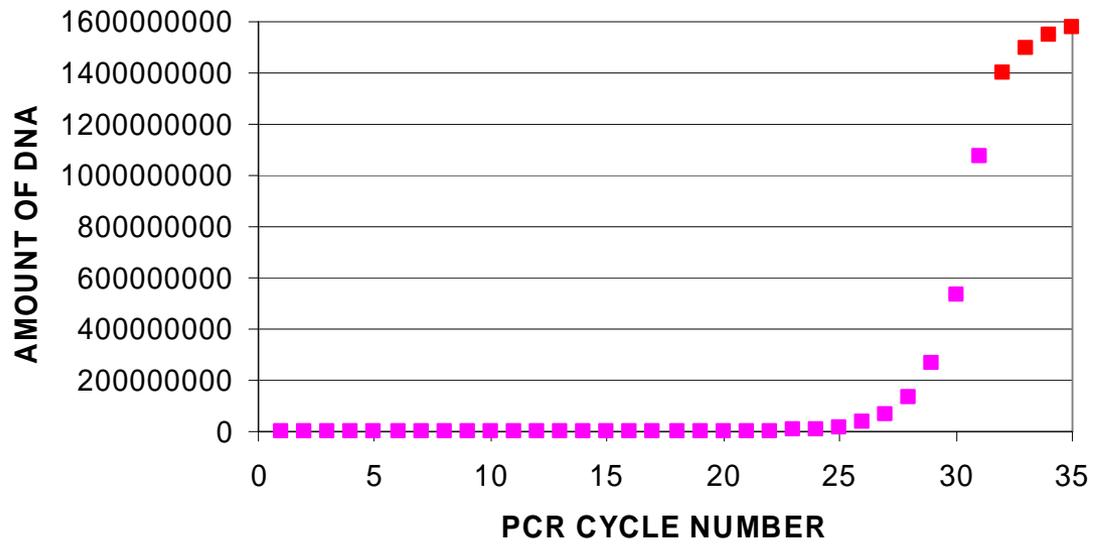- medium copy number advantageous
  - correction more accurate

# Standards

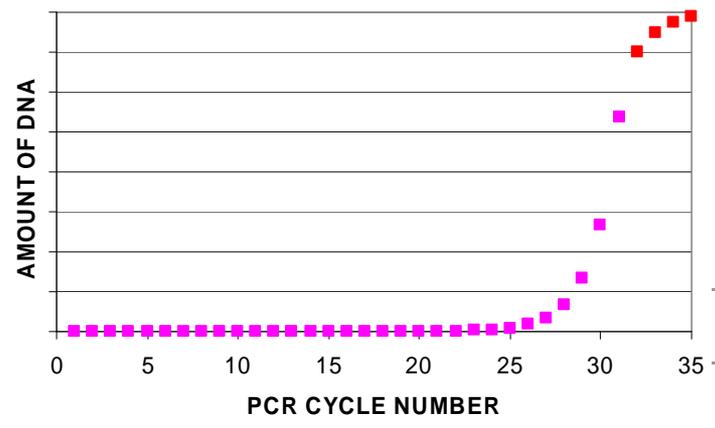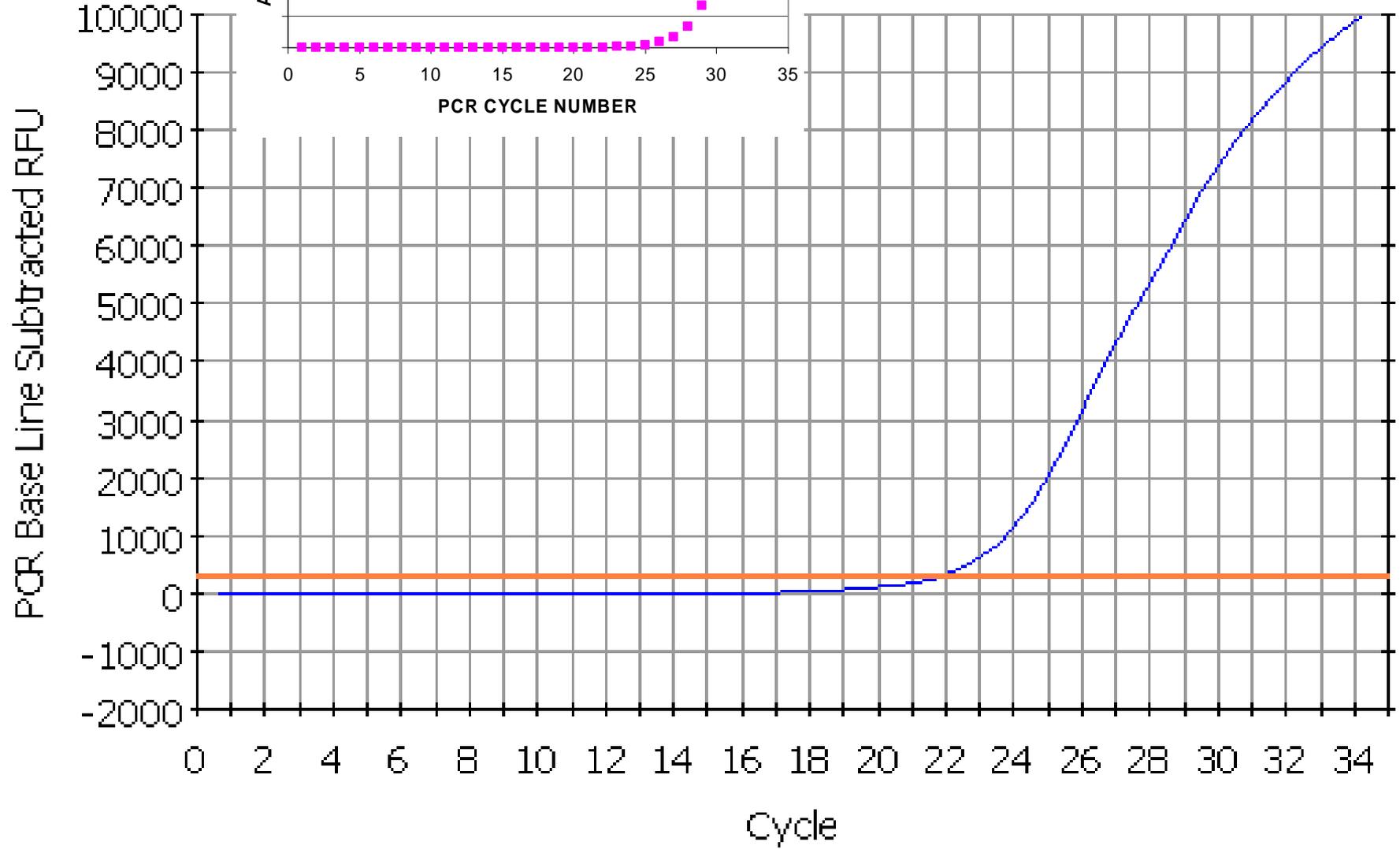- **The perfect standard does not exist**

# Standards

- Commonly used standards
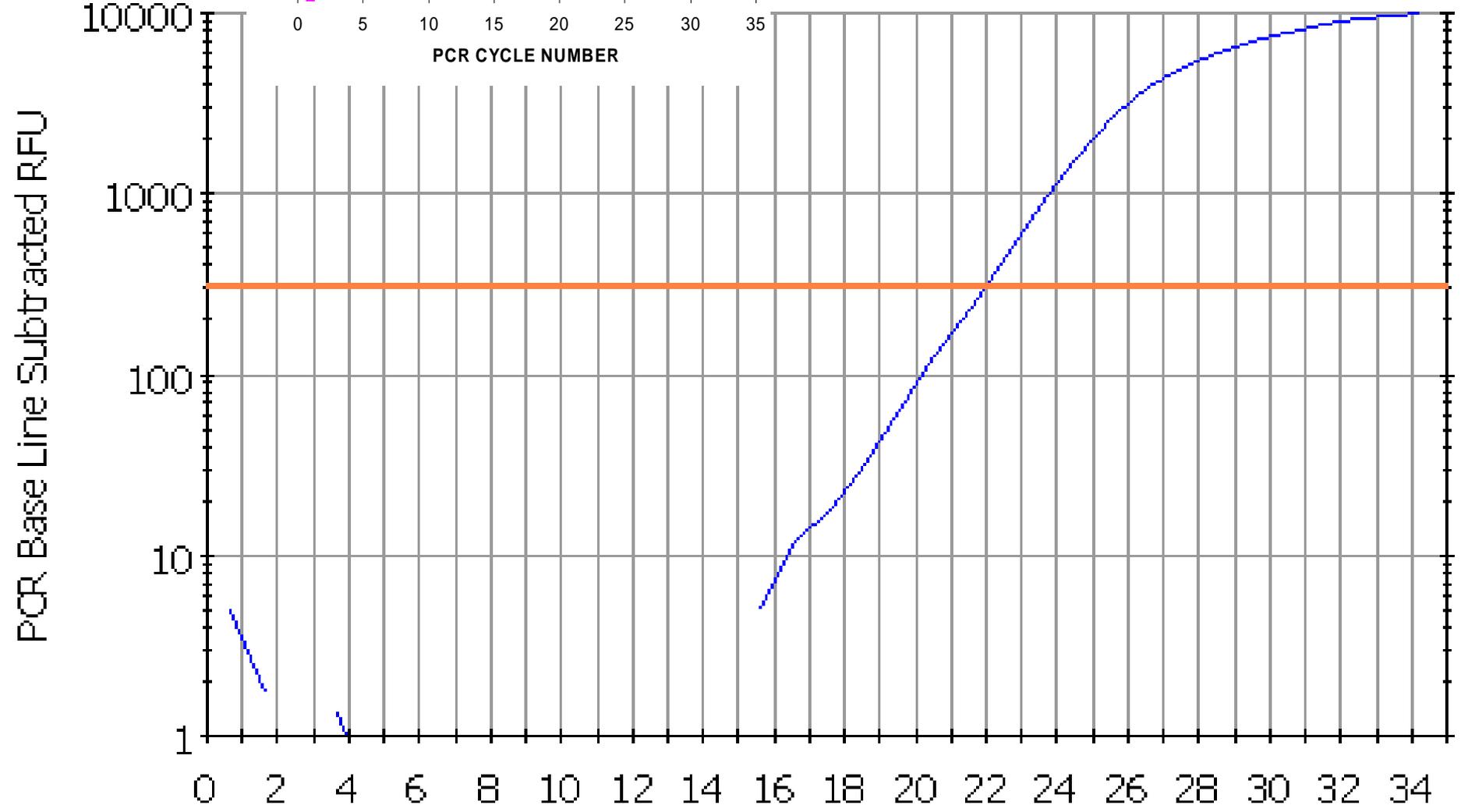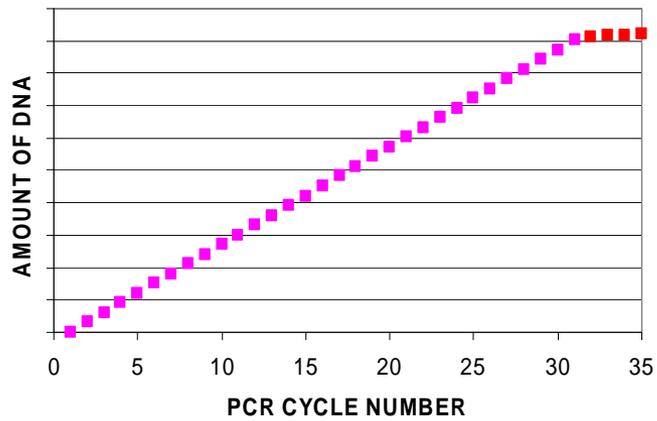  - Glyceraldehyde-3-phosphate dehydrogenase mRNA
  - Beta-actin mRNA
  - MHC I (major histocompatability complex I) mRNA
  - Cyclophilin mRNA
  - mRNAs for certain ribosomal proteins
    - **E.g. RPLP0 (ribosomal protein, large, P0; also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein 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 |
| 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 |
| 31 | 1,400,000,000 |
| 32 | 1,500,000,000 |
| 33 | 1,550,000,000 |
| 34 | 1,580,000,000 |





9

AMOUNT OF DNA

PCR CYCLE NUMBER

PCR Base Line Subtracted RFU

10000

1000

100

10

1

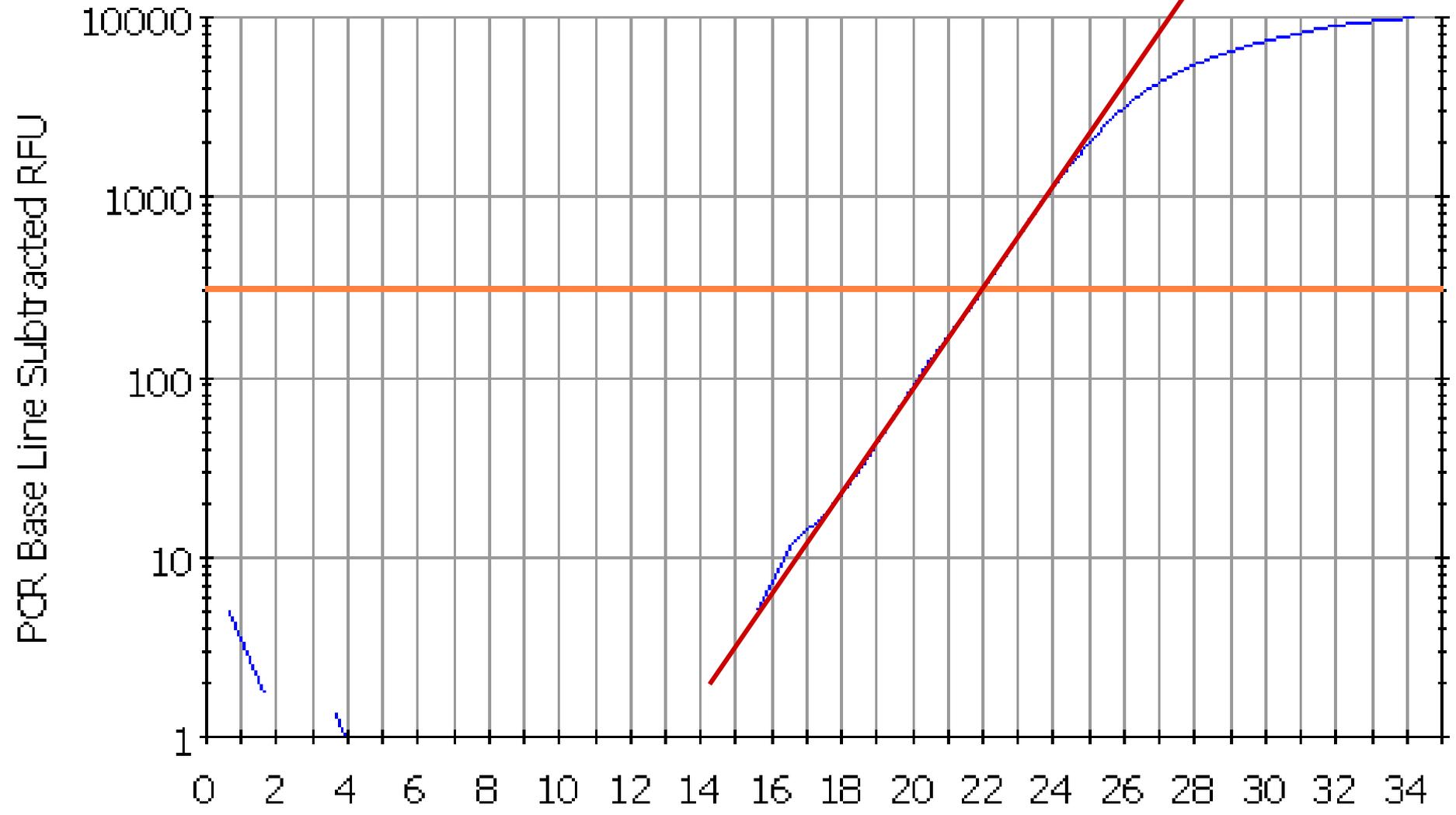0  2  4  6  8  10  12  14  16  18  20  22  24  26  28  30  32  34
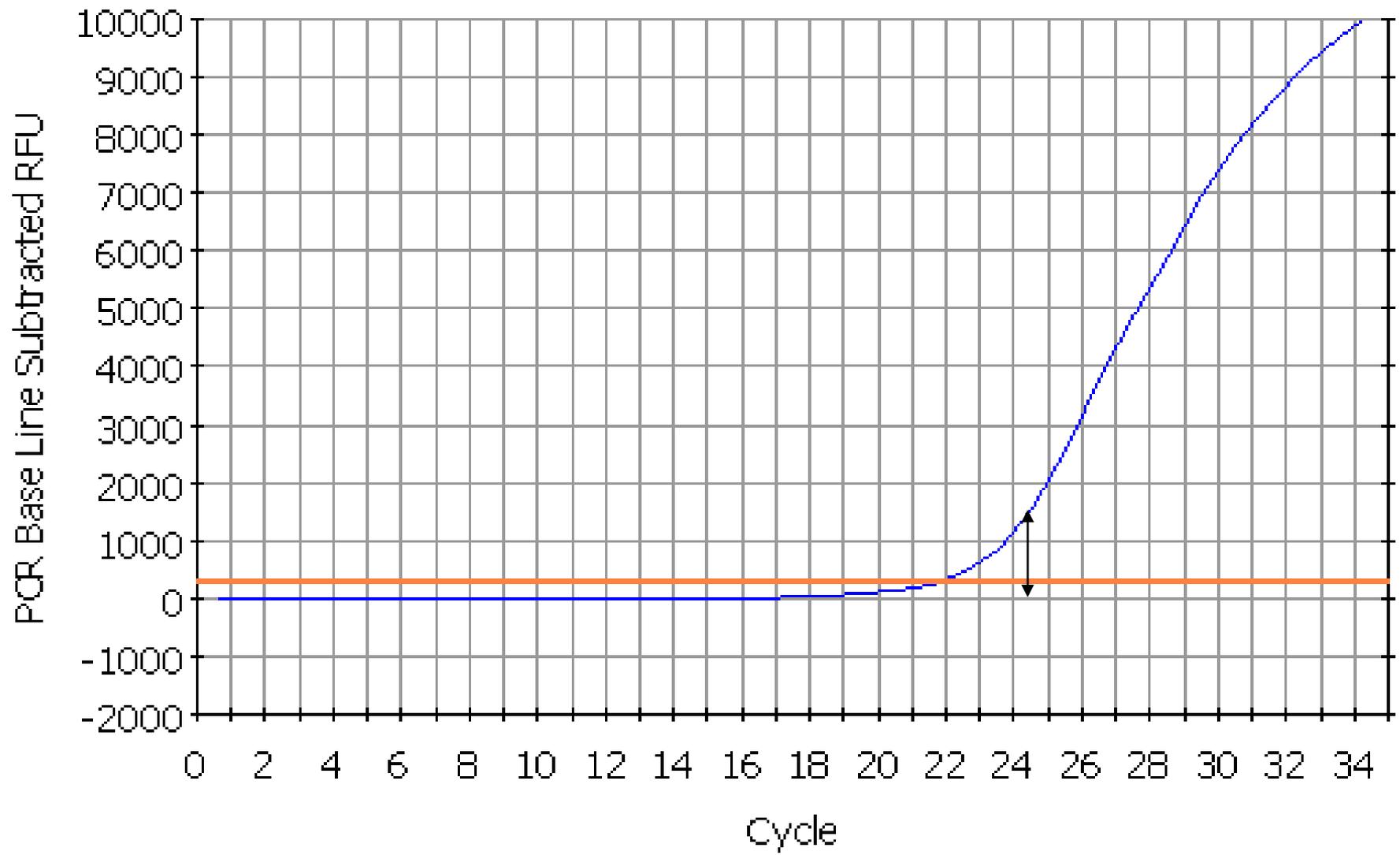
Linear ~20 to ~1500

Linear ~20 to ~1500

# REAL TIME PCR

- kinetic approach
- early stages
- while still linear

www.biorad.com

1. halogen tungsten lamp

2a. excitation filters

2b. emission filters
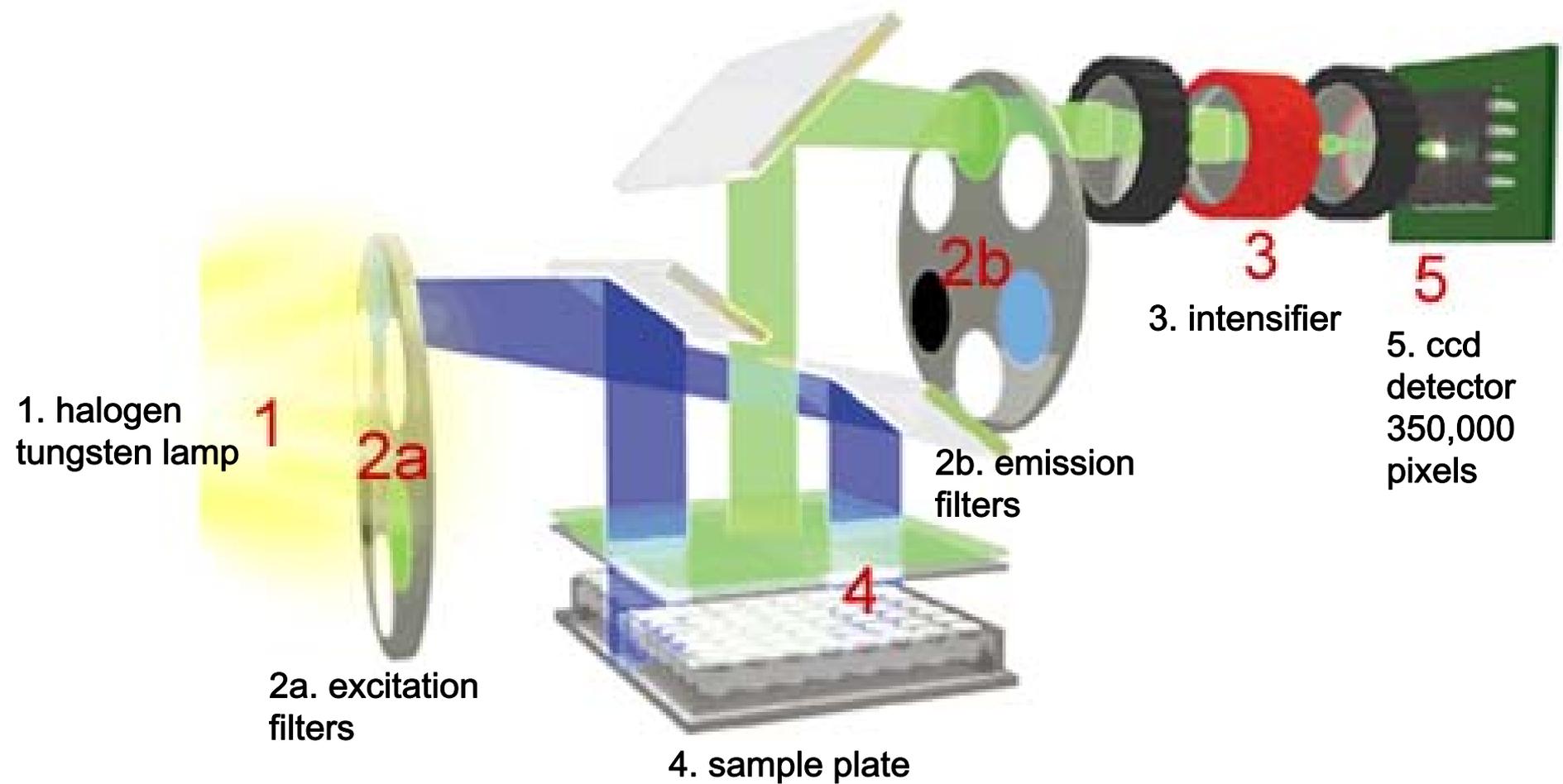
3. intensifier

4. sample plate

5. ccd detector 350,000 pixels
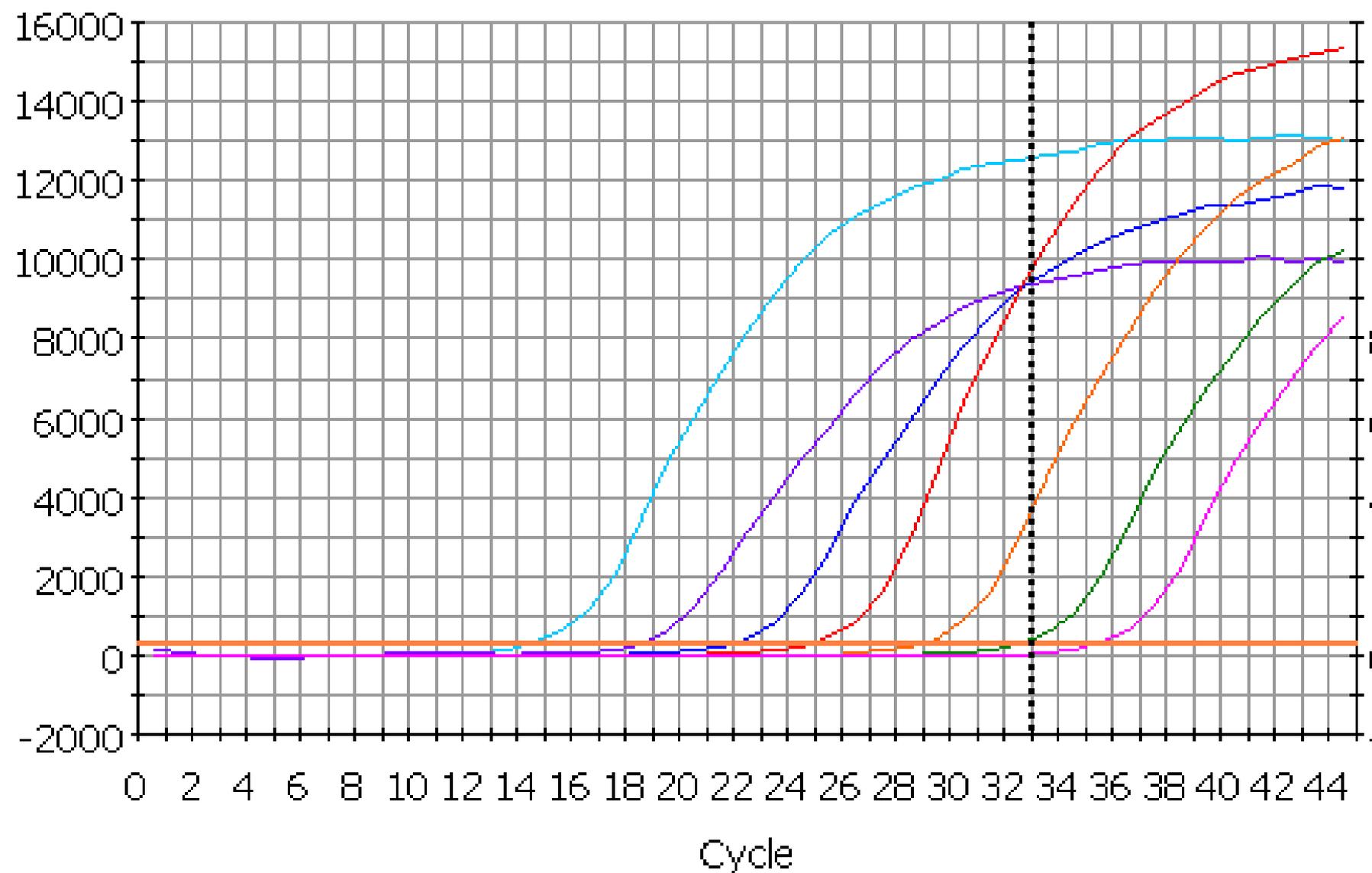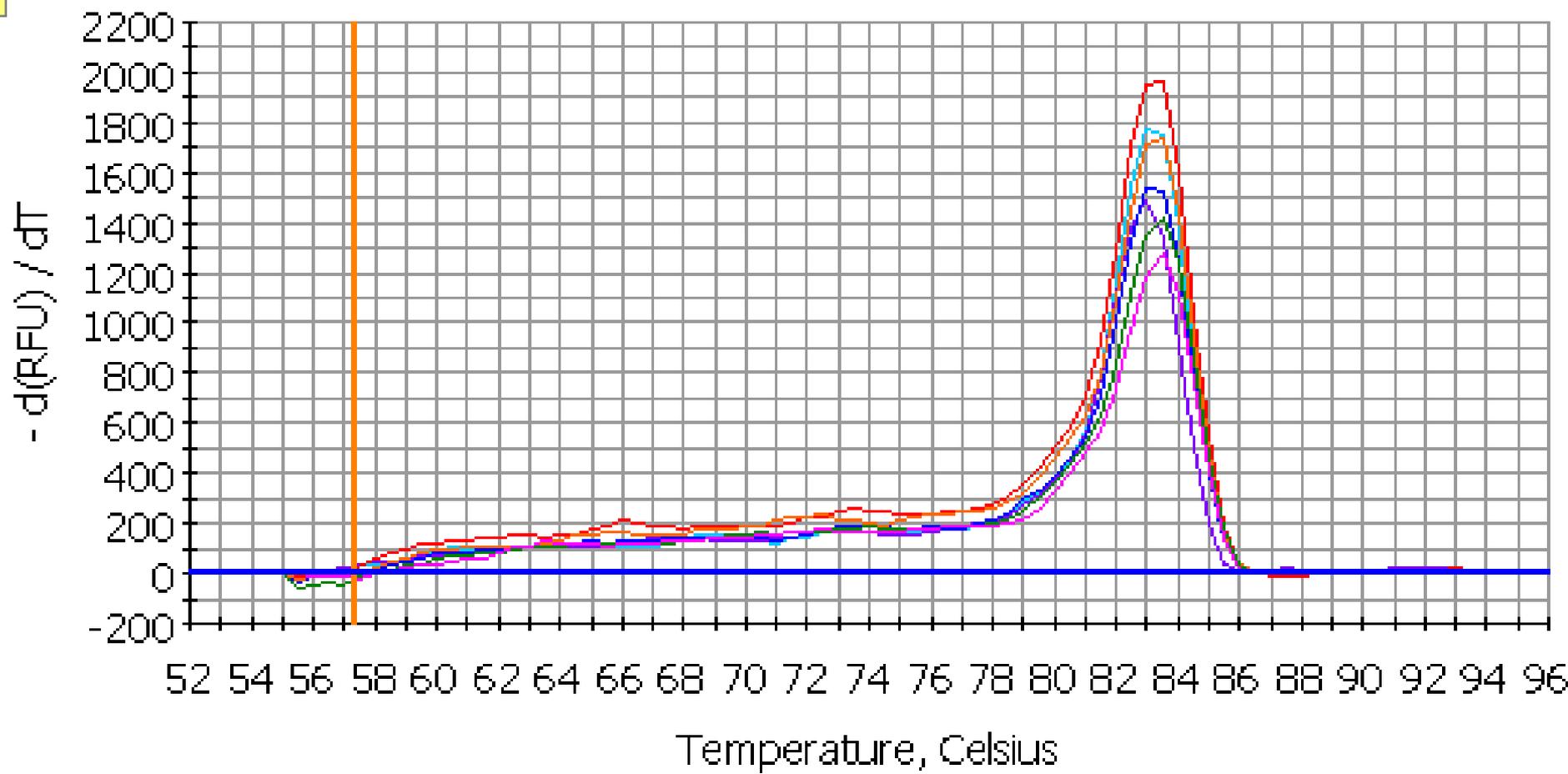
Fig. 1.2. Representation of Optical Detection System layout.

www.biorad.com

SERIES OF 10-FOLD DILUTIONS
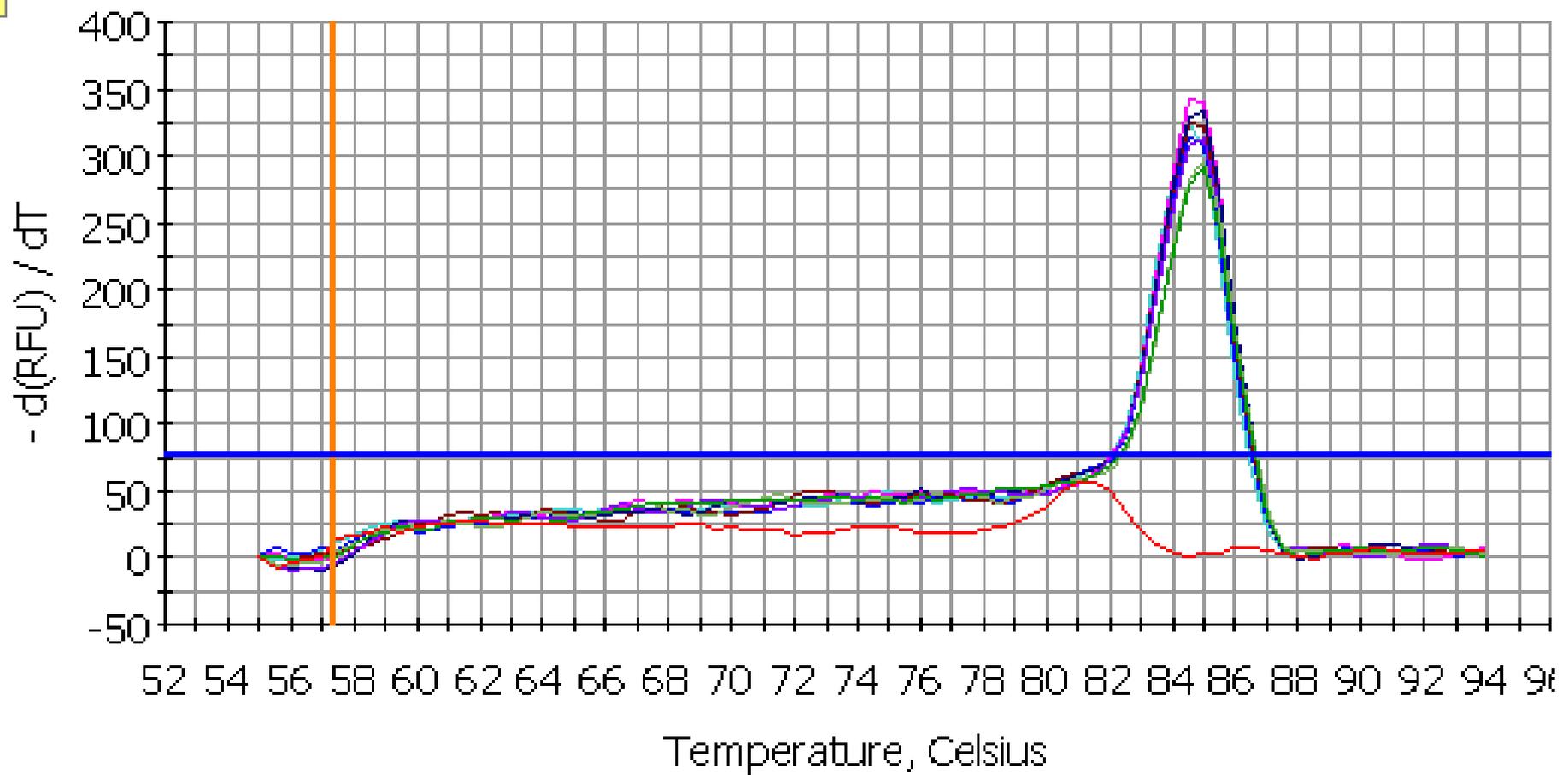
Melt Peak: Data 10-Mar-03 1259 ed.opd
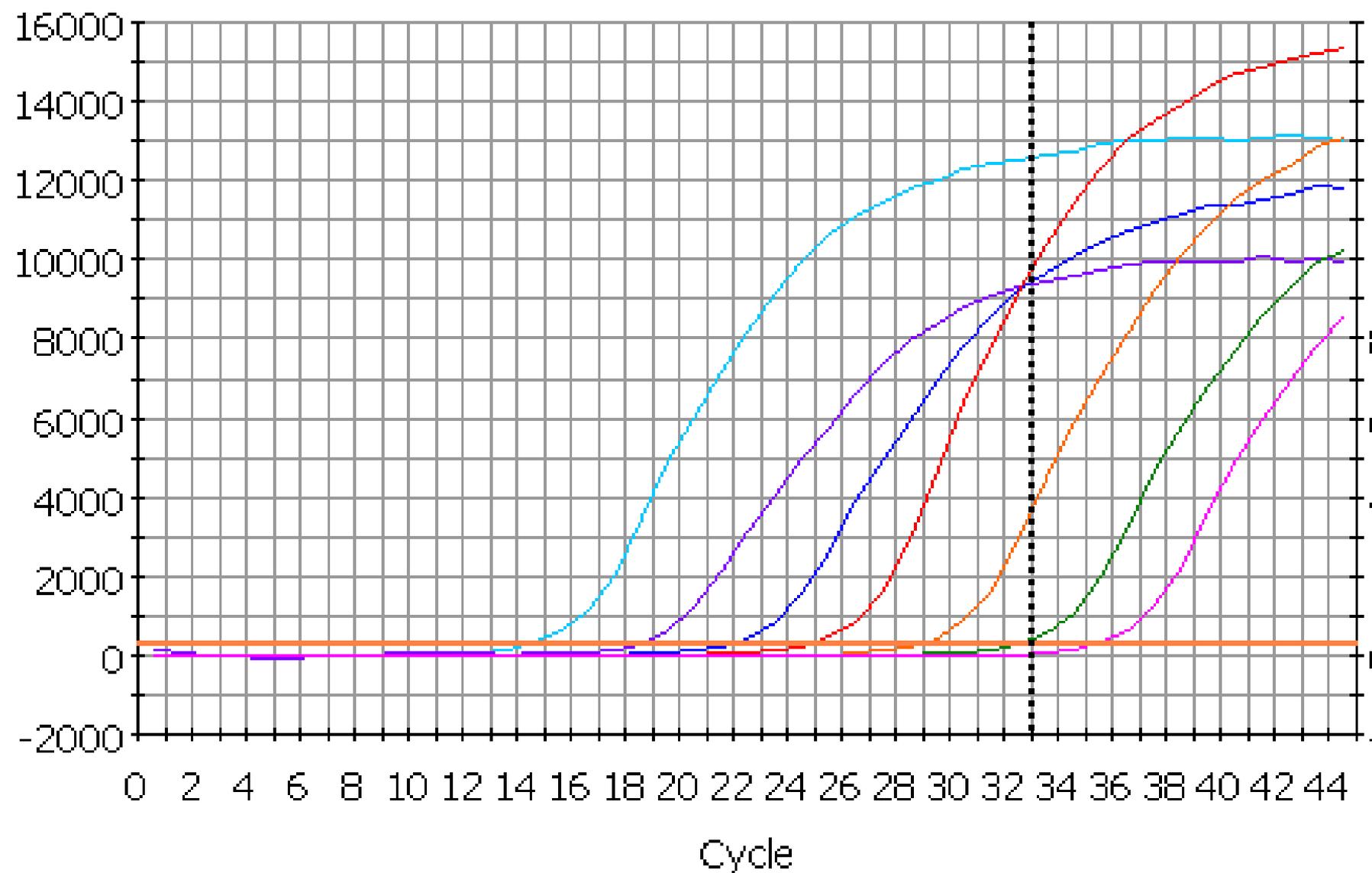
SERIES OF 10-FOLD DILUTIONS

SERIES OF 10-FOLD DILUTIONS

threshold = 300

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

□ Unknowns
○ Standards

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

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# STANDARD CURVE METHOD

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

Unknowns
Standards

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

dilutions target DNA
triplicates cDNA
target primers

dilutions reference DNA
triplicates cDNA
reference primers

# Standard curve method

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

□ Unknowns
○ Standards

'copy number' target gene control

Dilution curve target gene

'copy number' target gene experimental

Threshold Cycle (y-axis: 10, 15, 20, 25, 30, 35, 40)

Log Starting Quantity, copy number (x-axis: 0, 1, 2, 3, 4, 5, 6, 7)

NORTHERN

control    expt

← target gene

← internal control gene
   actin, GAPDH, RPLP0 etc

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

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

27

Correlation Coefficient: 1.000    Slope: -3.360    Intercept: 39.319    Y = -3.360 X + 39.319

□ Unknowns
∘ Standards

'copy number' reference gene experimental

Dilution curve reference gene

'copy number' reference gene control

Threshold Cycle

Log Starting Quantity, copy number

NORTHERN

control    expt

← target gene

← internal control gene
actin, GAPDH, RPLP0 etc

Ratio experimental/control = fold change in target gene
                             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:

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| A | ● | | | | | | | | | | | ● |
| B | | RPLP0 -4 | RPLP0 -5 | RPLP0 -6 | RPLP0 -7 | RPLP0 -8 | RPLP0 -9 | RPLP0 -10 | 5uL $H_2O$ | cDNA 1 | cDNA 2 | ← |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | a5-int -4 | a5-int -5 | a5-int -6 | a5-int -7 | a5-int -8 | a5-int -9 | a5-int -10 | 5uL $H_2O$ | cDNA 1 | cDNA 2 | ← |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | ● | | | | | | | | | | | ● |

add RPLP0 master mix to this row

add a5-integrin master mix to this row

30

NORTHERN

cDNA1    cDNA2

← alpha-5 integrin (target gene)

← RPLP0 (reference)
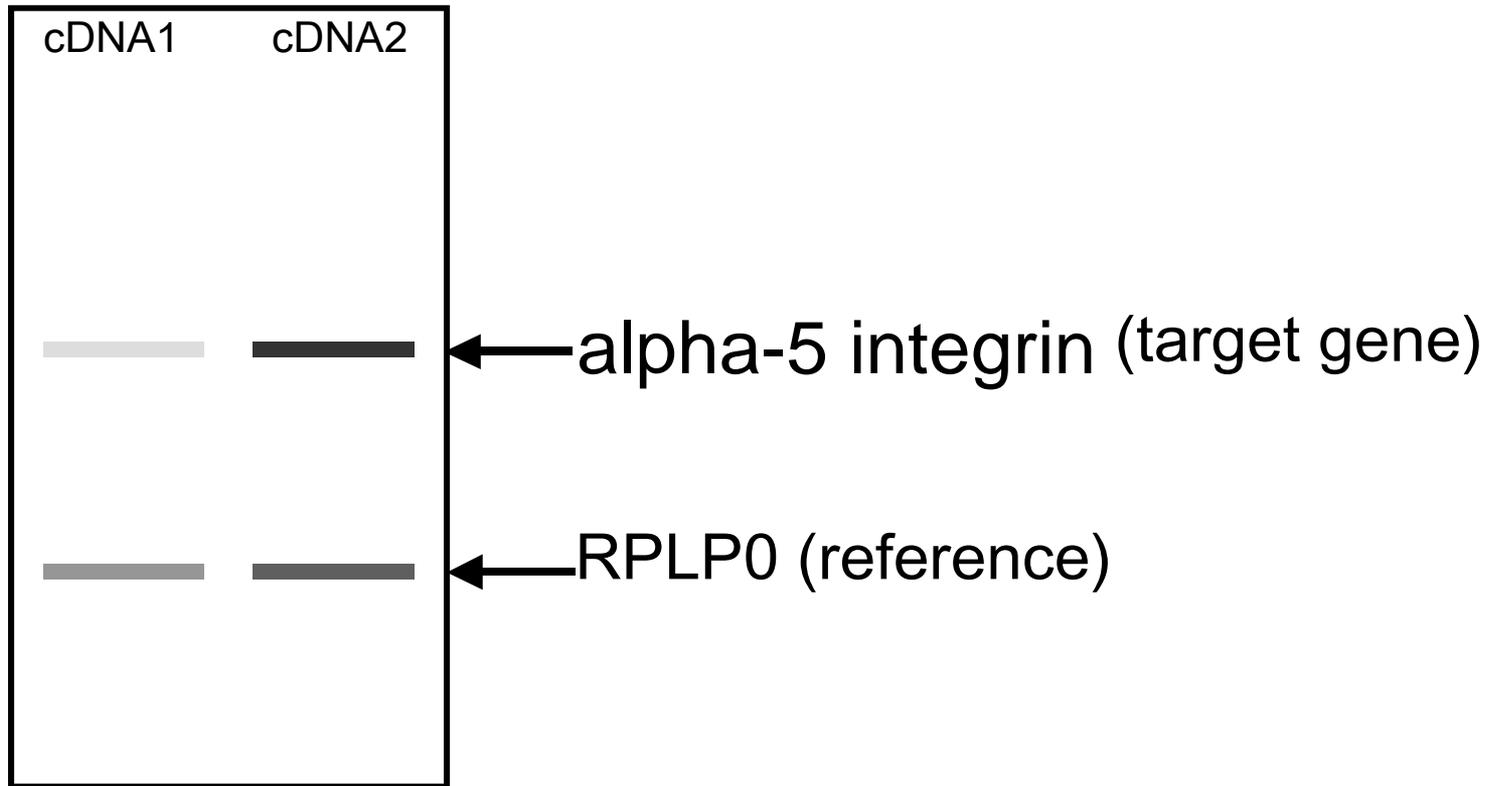
Ratio alpha-5 integrin cDNA2 to cDNA1 = $\dfrac{\text{fold change in alpha-5 integrin}}{\text{fold change in RPLP0}}$

# 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 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 |
| 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

36

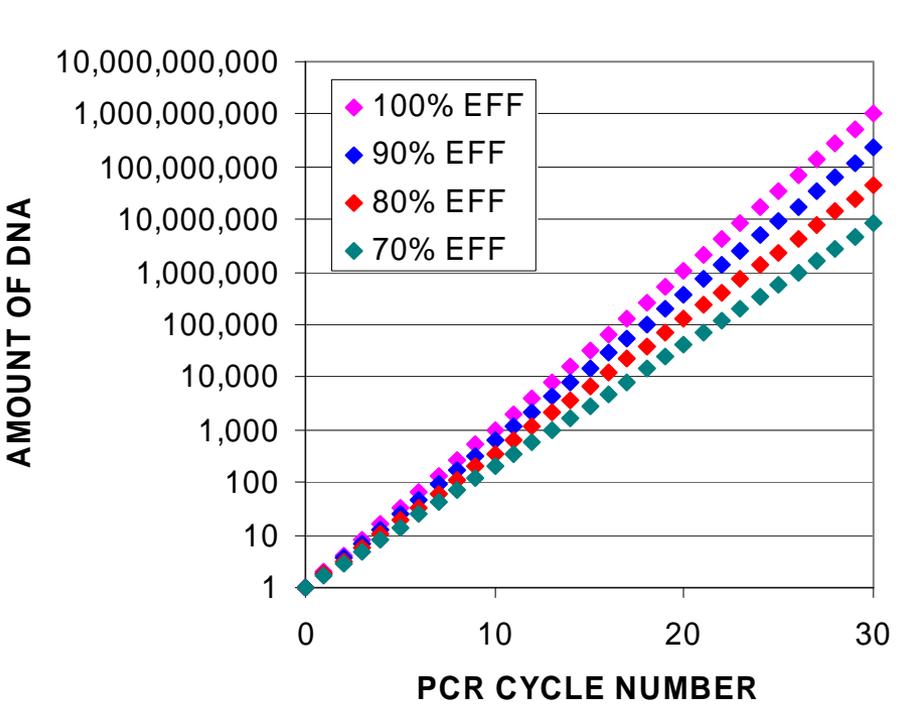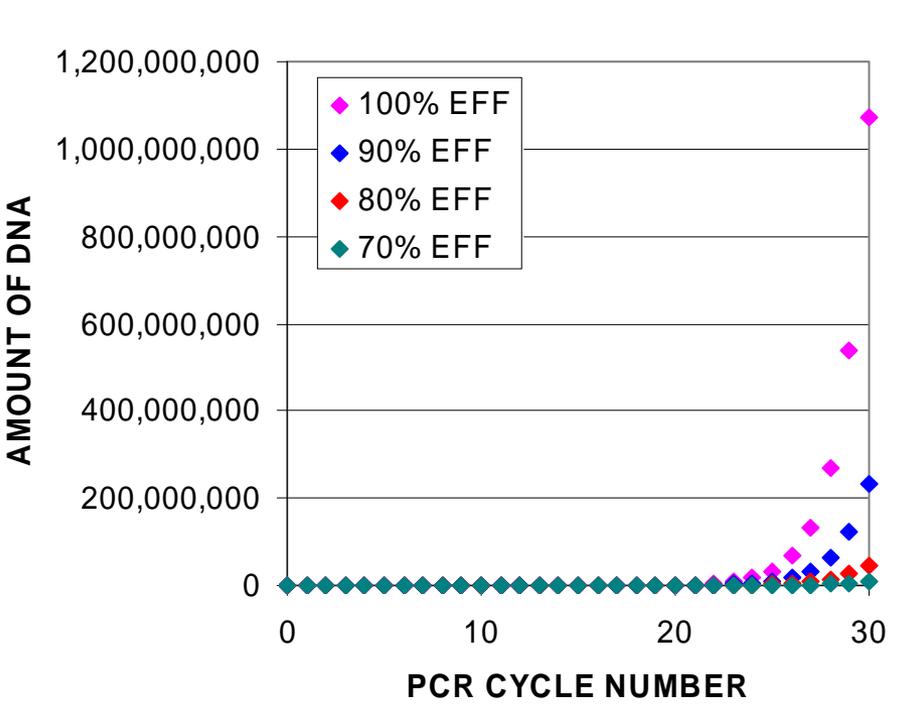| CYCLE | AMOUNT OF DNA 100% EFFICIENCY | AMOUNT OF DNA 90% EFFICIENCY | AMOUNT OF DNA 80% EFFICIENCY | AMOUNT OF DNA 70% EFFICIENCY |
|---|---|---|---|---|
| 0 | 1 | 1 | 1 | 1 |
| 1 | 2 | 2 | 2 | 2 |
| 2 | 4 | 4 | 3 | 3 |
| 3 | 8 | 7 | 6 | 5 |
| 4 | 16 | 13 | 10 | 8 |
| 5 | 32 | 25 | 19 | 14 |
| 6 | 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 |
| 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
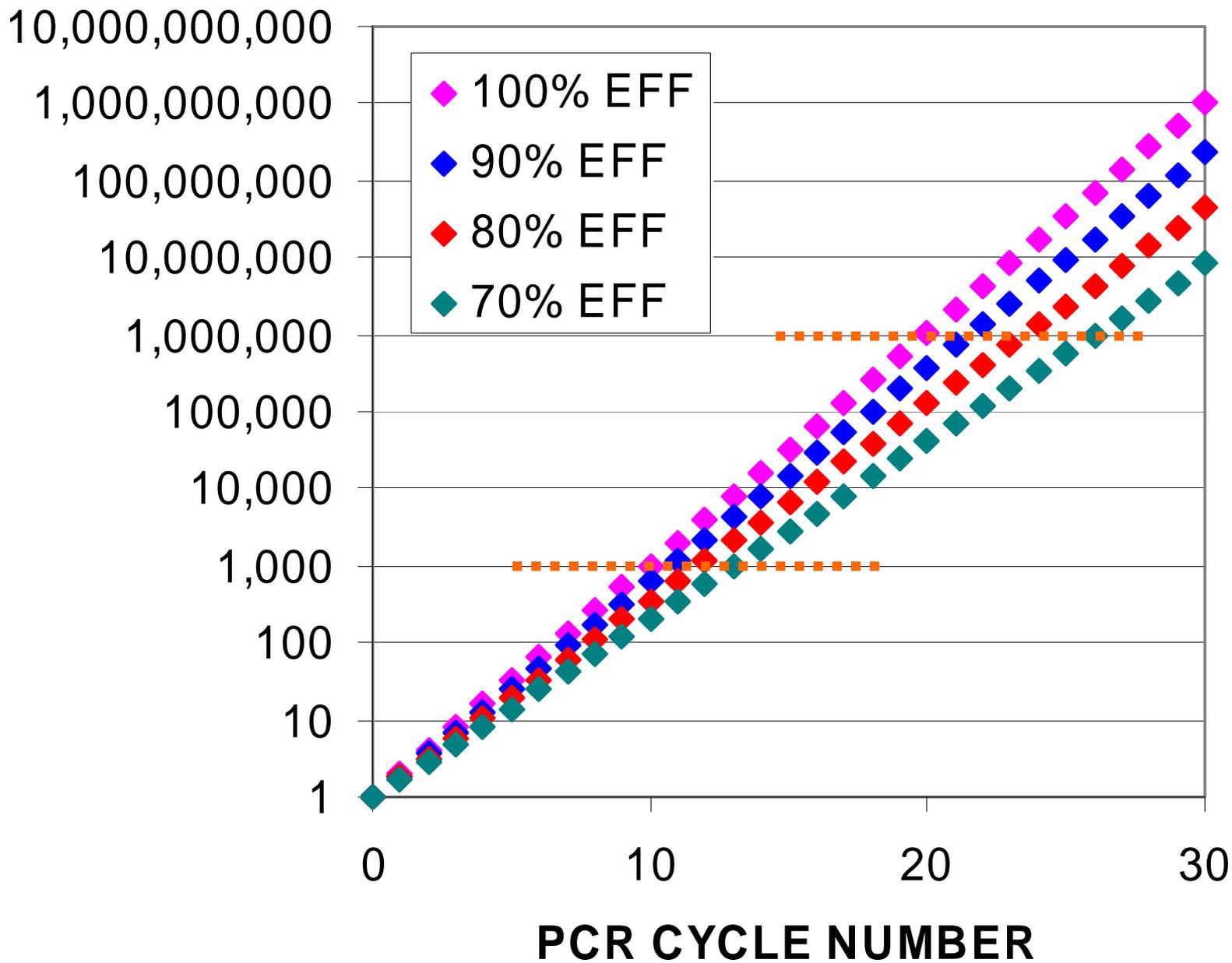90%  = 1.90x
80%  = 1.80x
70%  = 1.70x
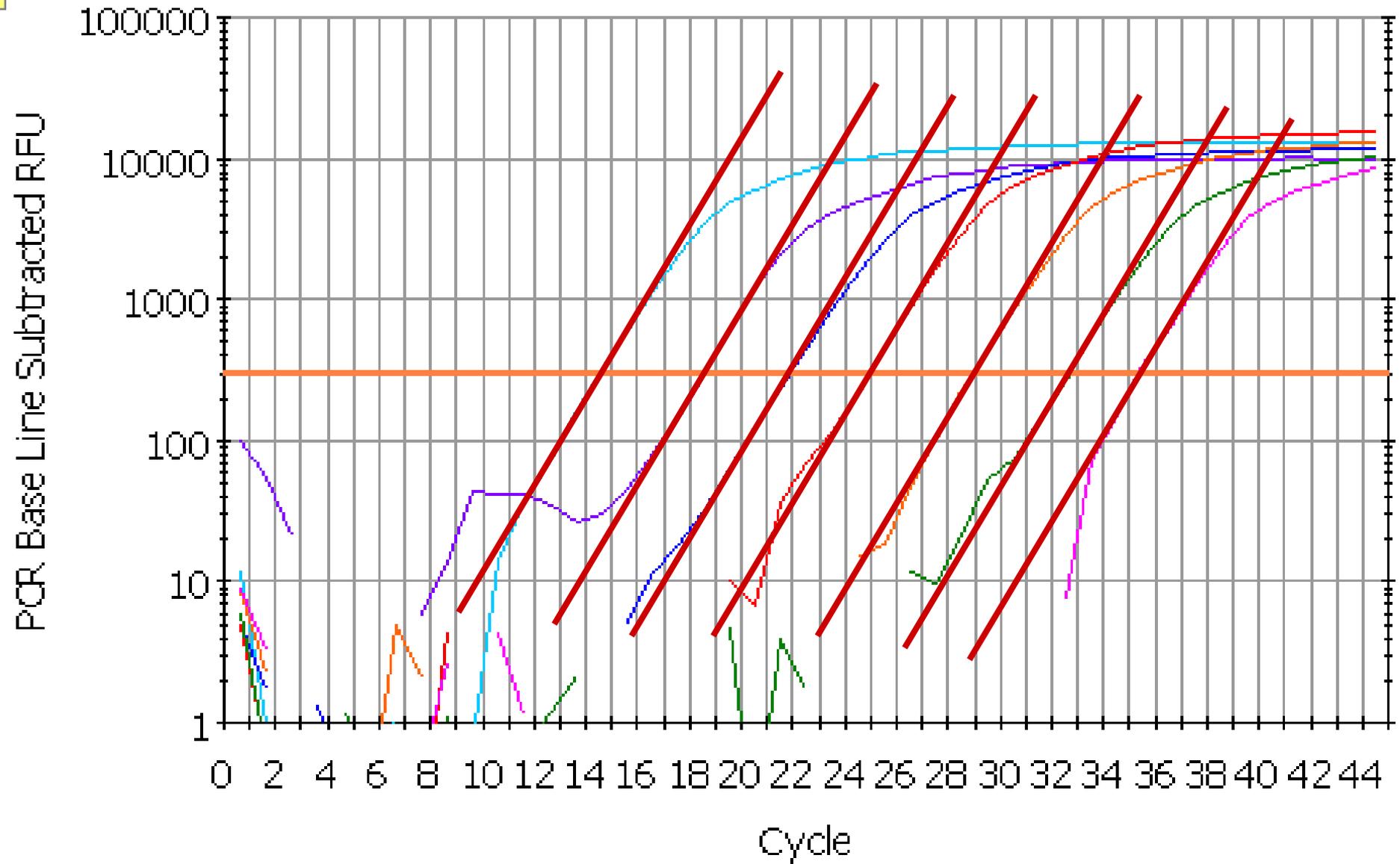
AFTER N CYCLES:
fold increase =
$(\text{efficiency})^n$

37

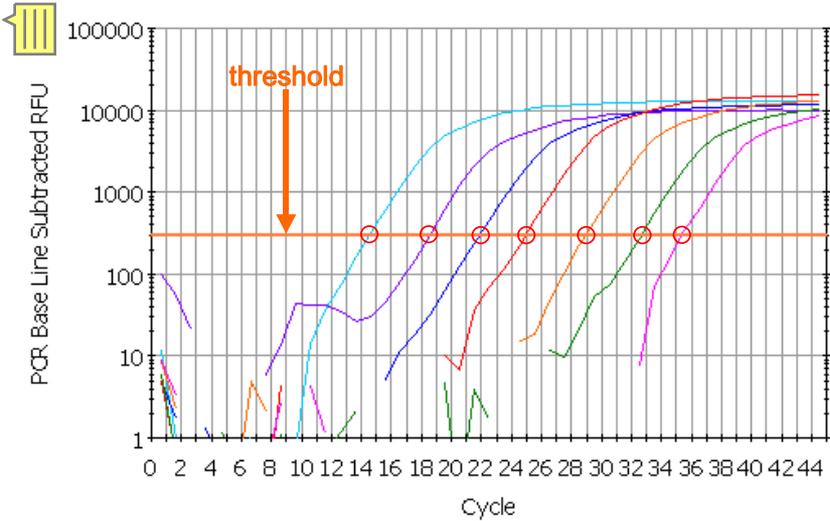| CYCLE | AMOUNT OF DNA 100% EFFICIENCY | AMOUNT OF DNA 90% EFFICIENCY | AMOUNT OF DNA 80% EFFICIENCY | AMOUNT OF DNA 70% EFFICIENCY |
|---|---|---|---|---|
| 0 | 1 | 1 | 1 | 1 |
| 1 | 2 | 2 | 2 | 2 |
| 2 | 4 | 4 | 3 | 3 |
| 3 | 8 | 7 | 6 | 5 |
| 4 | 16 | 13 | 10 | 8 |
| 5 | 32 | 25 | 19 | 14 |
| 6 | 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 |
| 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 |

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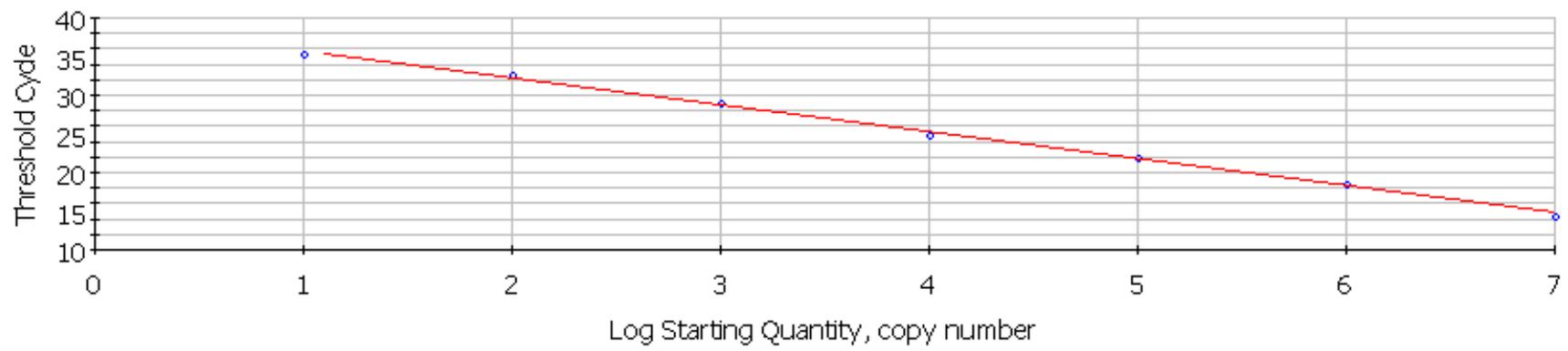
PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

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# 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

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

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

**FAM-490**

Select analysis mode: | PCR Base Line Subtracted ▼

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

☐ Select Wells

🔍📋 Reports

Select a Reporter

✏️ FAM-490

Threshold Cycle Cal

Baseline Cycles

○ Auto Calculated
○ User Defined

Recalculate Threshold Cycles

Threshold Position

100.0

○ Auto Calculated
● User Defined

Save for X-axis Allelic Analysis

Save for Y-axis Allelic Analysis
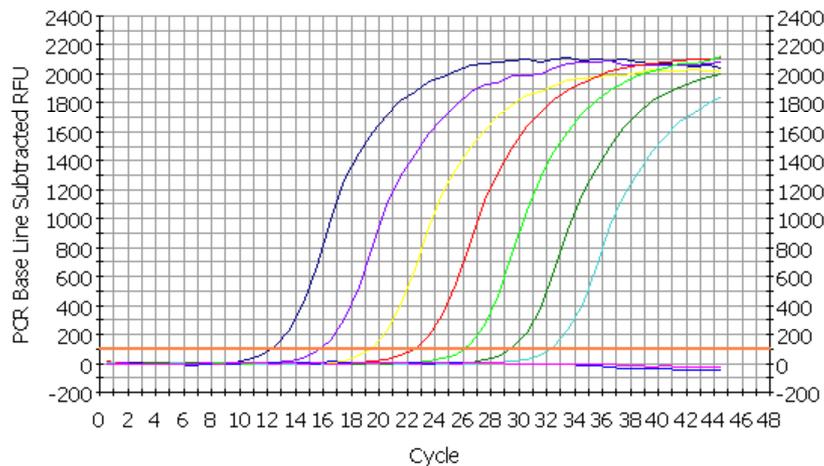
| | Threshold Cycle Ct | Identifier |
|---|---|---|
| D2 | 12.3 | |
| D3 | 15.8 | |
| D4 | 19.3 | |
| D5 | 22.6 | |
| D6 | 26.0 | |
| D7 | 29.4 | |
| D8 | 32.3 | |
| D9 | N/A | |
| D10 | N/A | |

D2
D3
D4
D5
D6
D7
D8
D9
D10

PCR Base Line Subtracted RFU

Cycle

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
- 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

# PFAFFL METHOD

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

NORTHERN

← target gene

← internal control gene
actin, GAPDH, RPLP0 etc
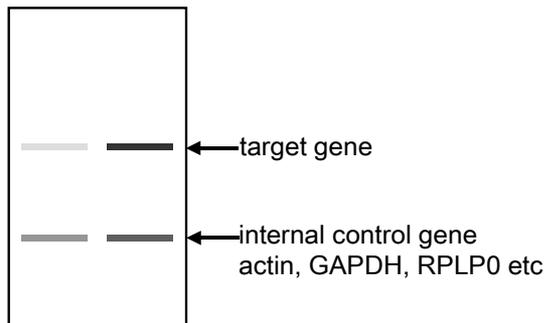
ratio = fold increase in target gene
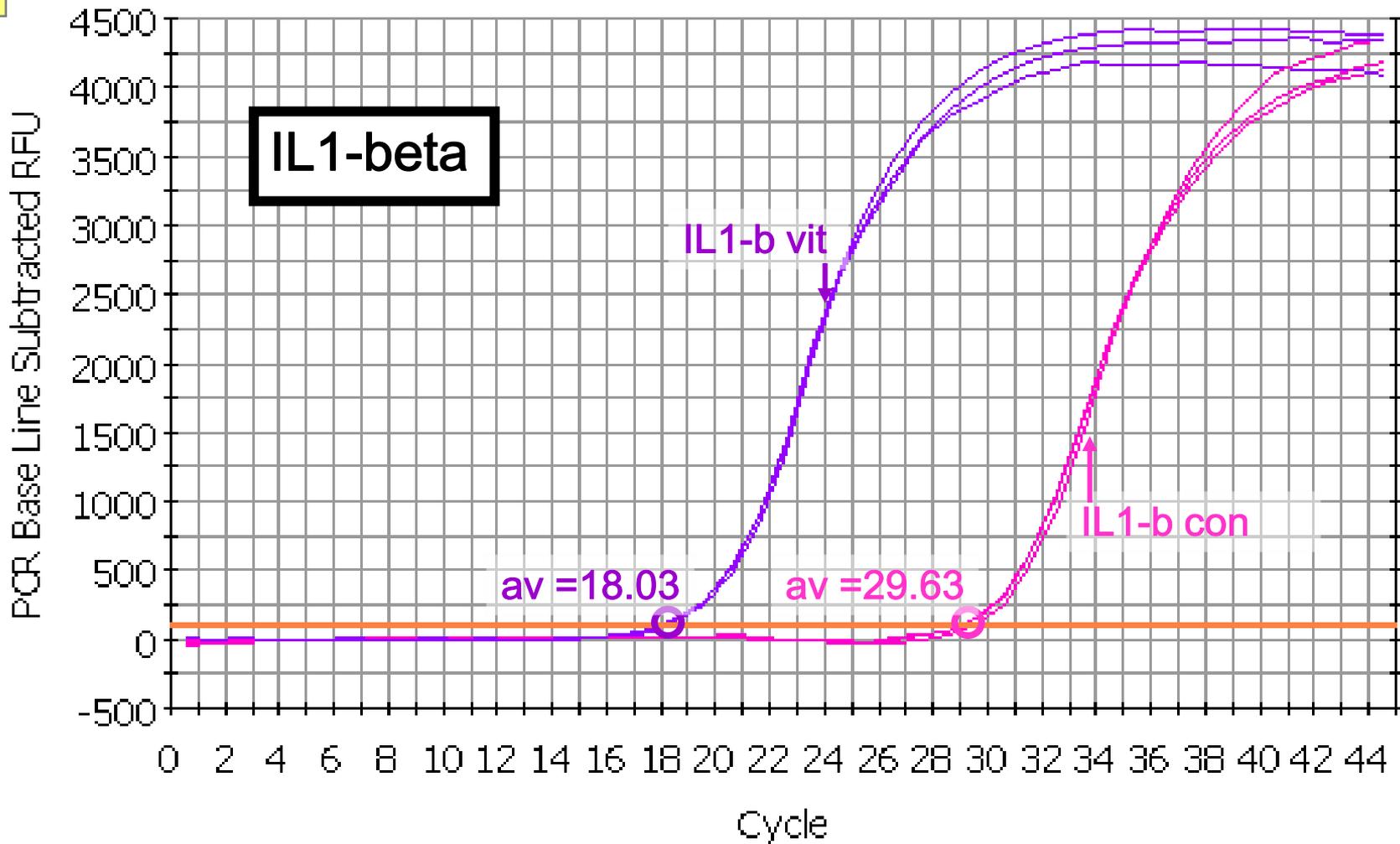fold increase in reference gene

triplicates cDNA ← }target primers

triplicates cDNA ← }reference primers

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PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd
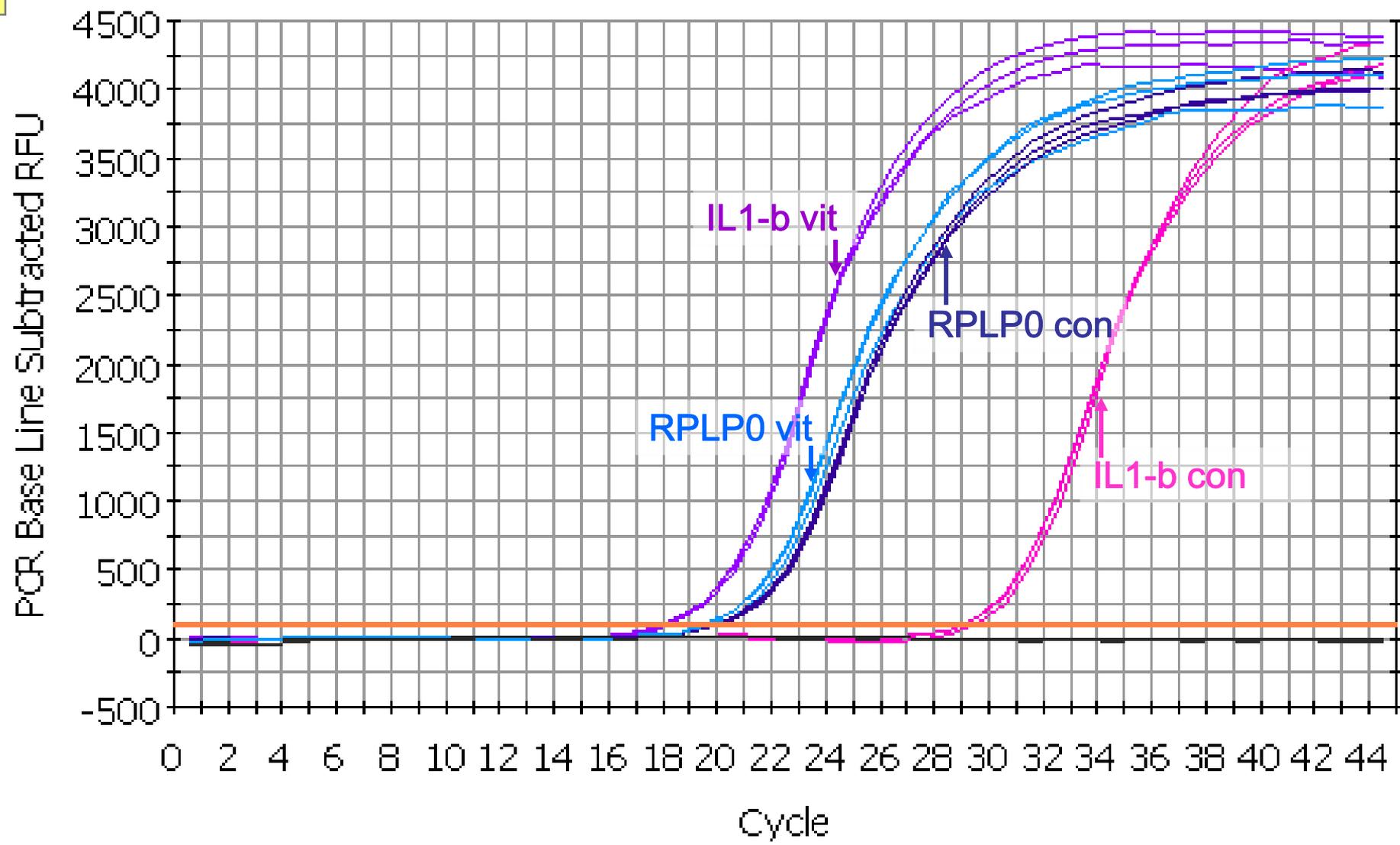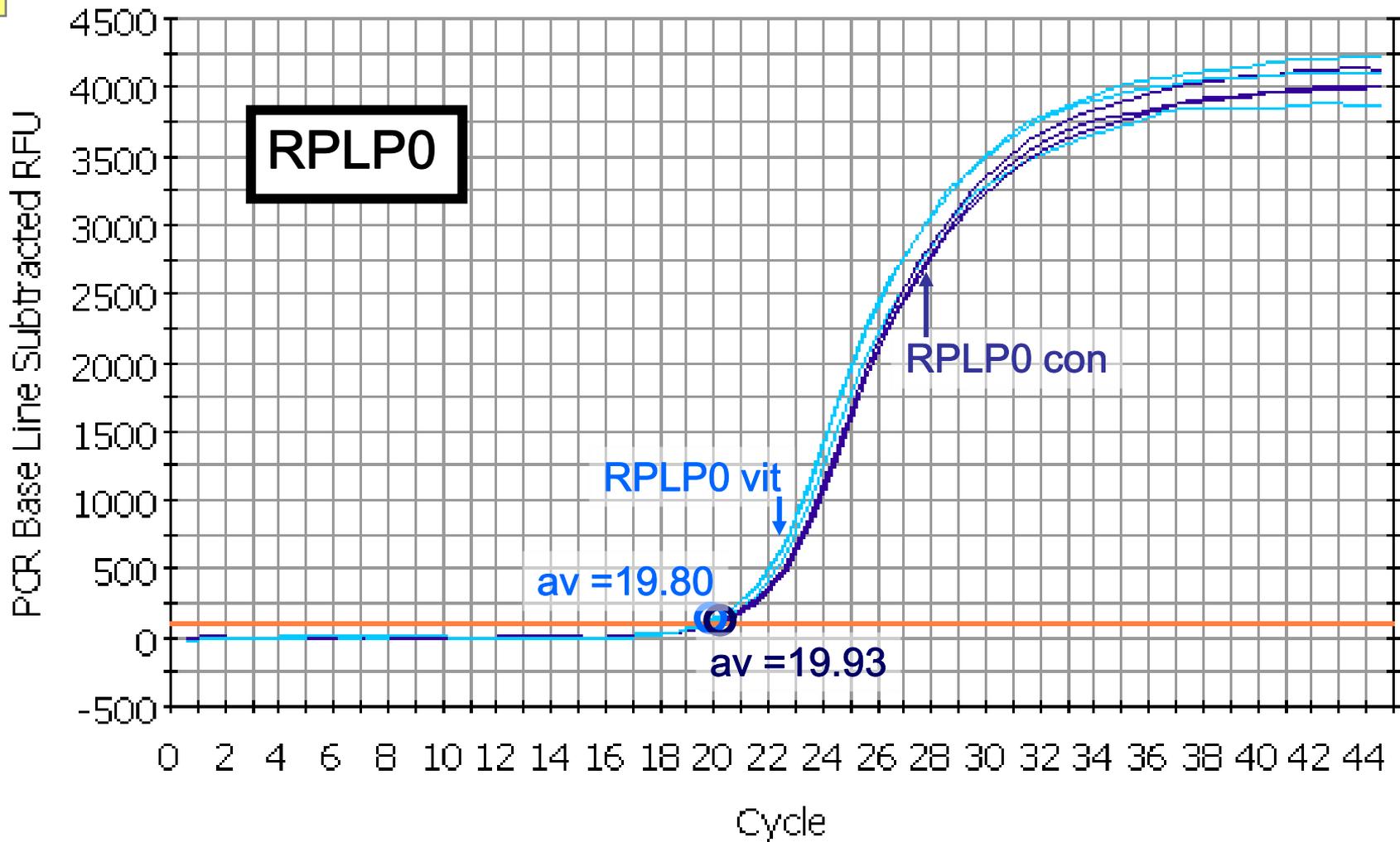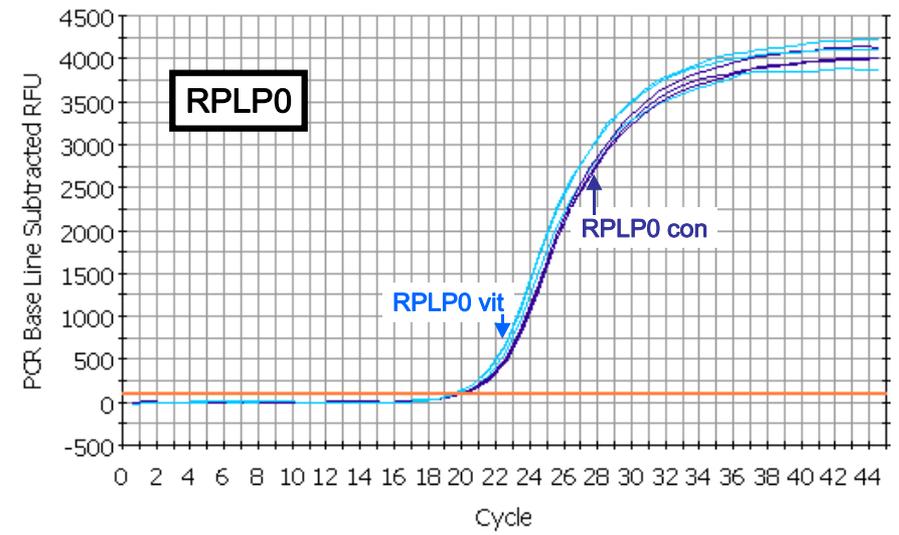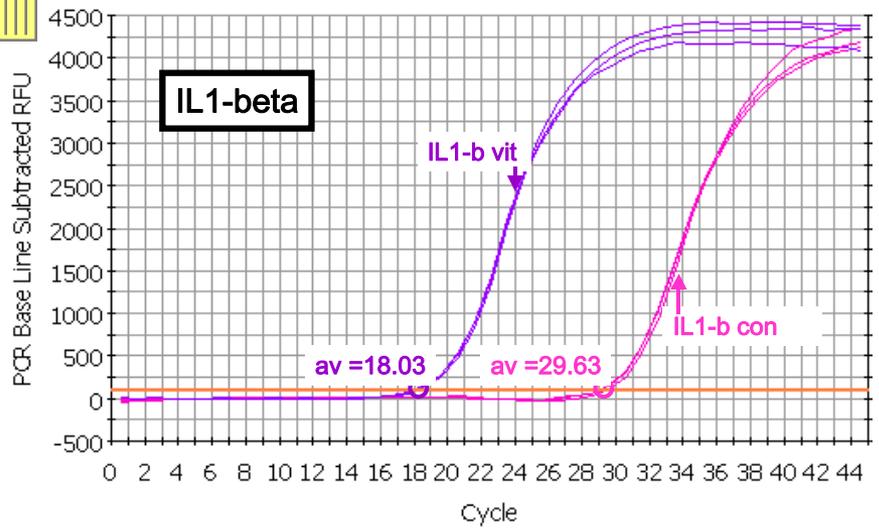
AFTER N CYCLES: change = (efficiency)$^n$

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

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

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

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IL1-beta

IL1-b vit

av =18.03    av =29.63    IL1-b con

AFTER N CYCLES: increase = $(efficiency)^n$

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

RPLP0

RPLP0 con

RPLP0 vit

AFTER N CYCLES: increase = $(efficiency)^n$

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

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

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

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| | 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 | | | | | | | | | | |

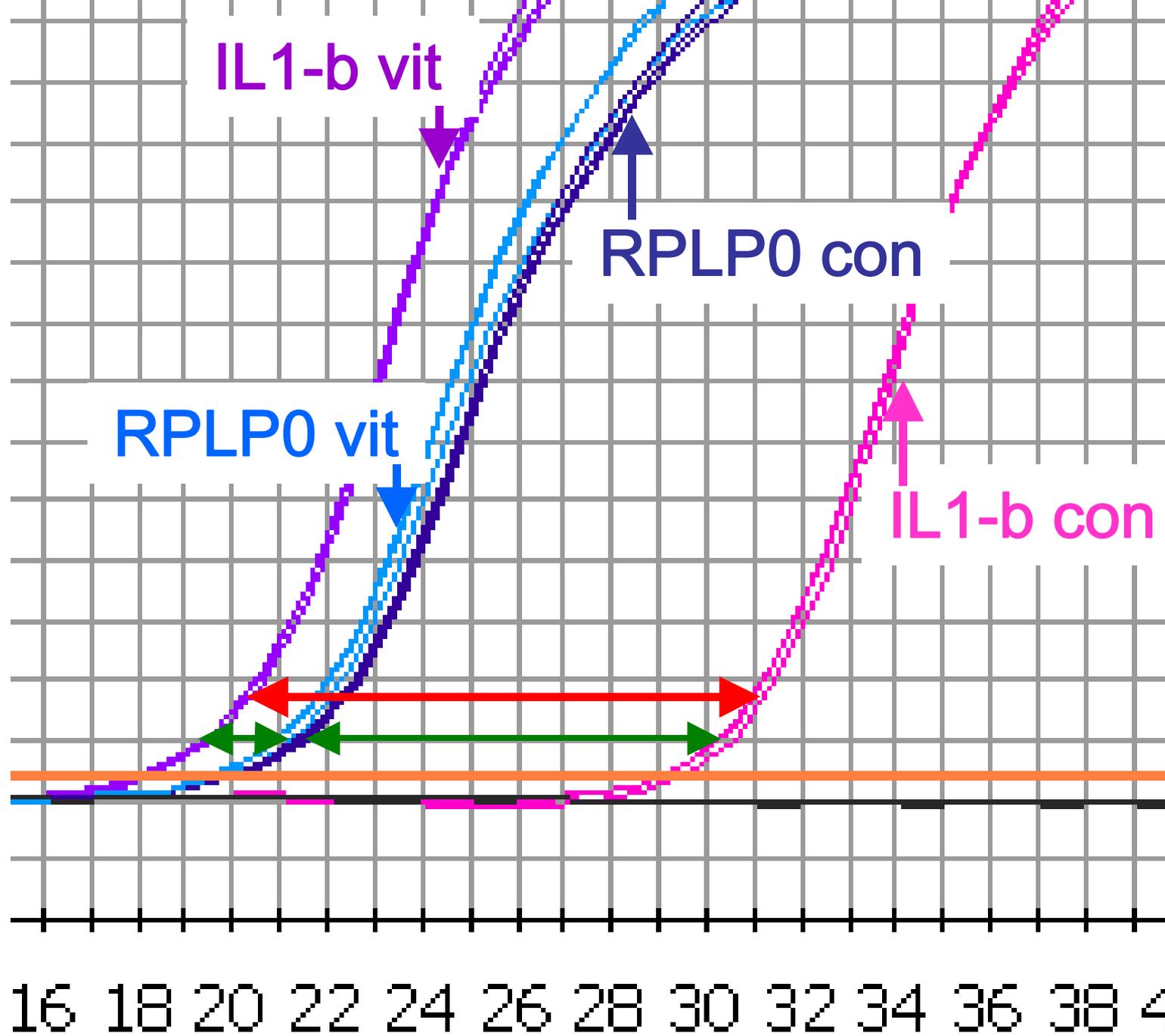An example of a step-by-step way to set up the calculations for the Pfaffl method in EXCEL.
Row 3, columns B, C, D, and E are the average Ct values from real time. In separate experiments, the average efficiency for the target gene was determined to be 1.936 and for RPLP0 was 1.943
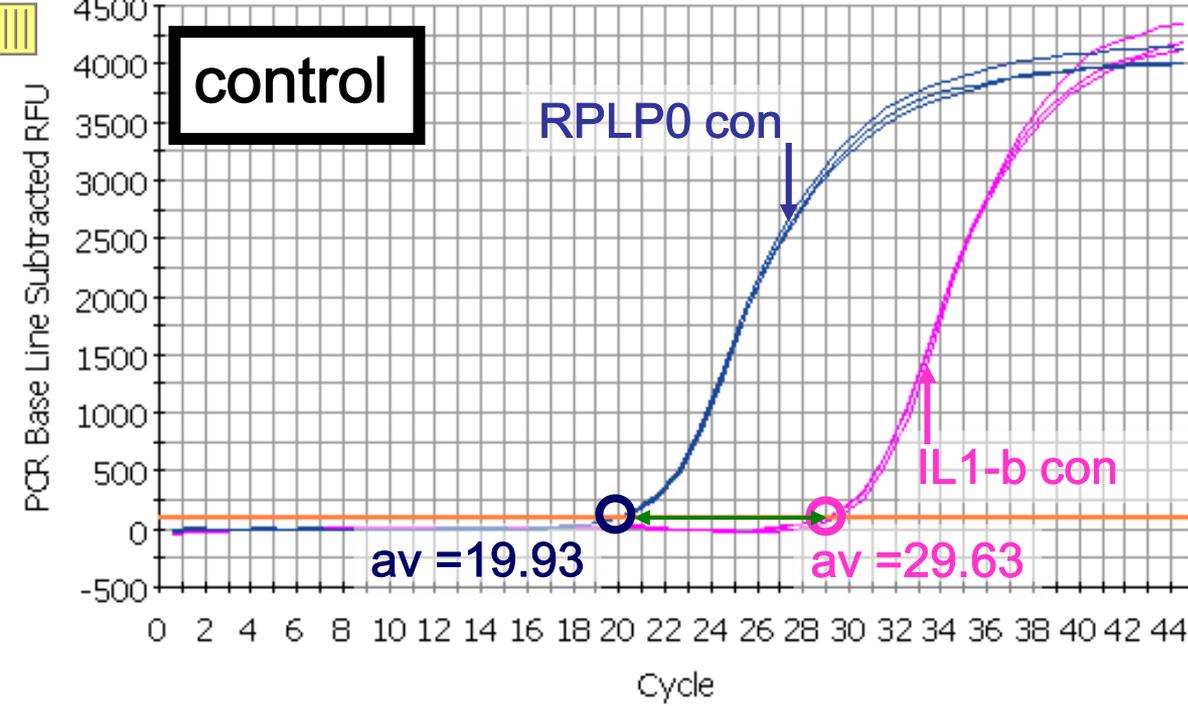
# EFFICIENCY$^{\Delta\Delta Ct}$ METHOD
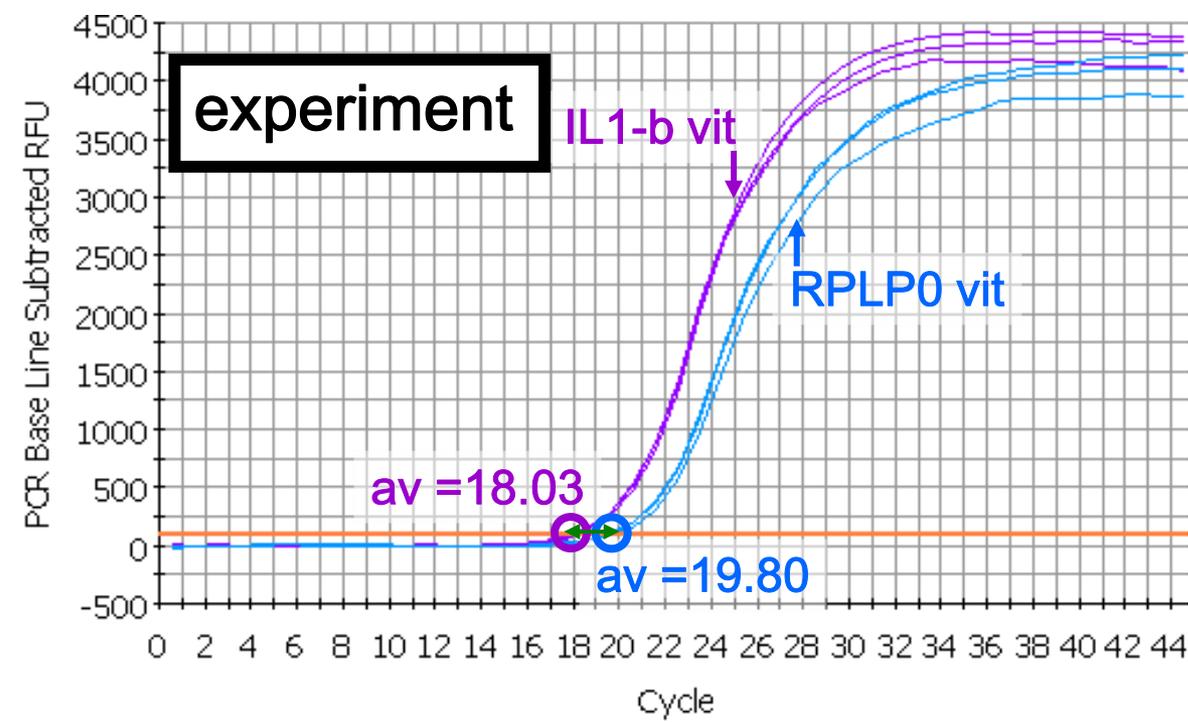
## APPROXIMATION METHOD

PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd
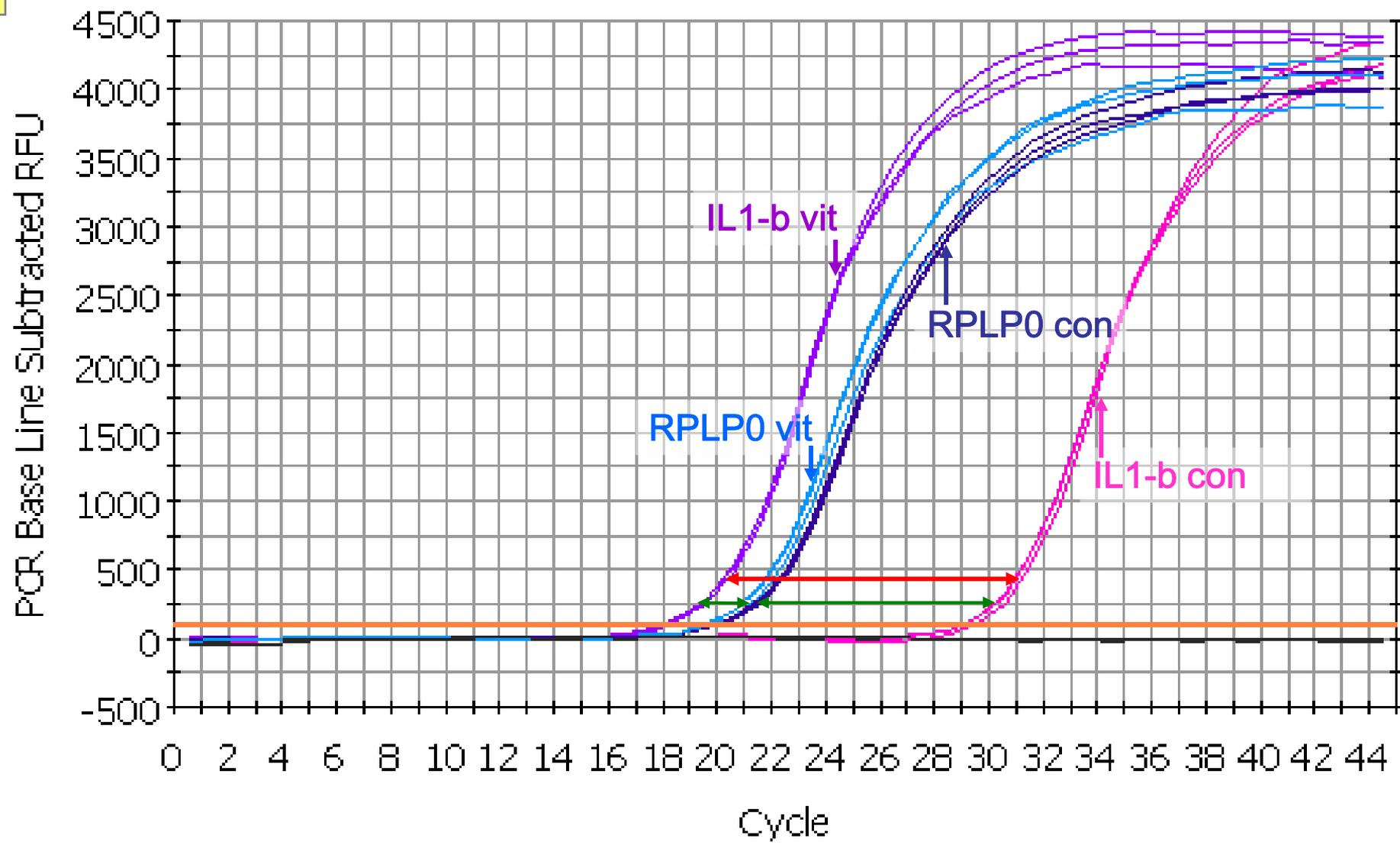
$\Delta$ Ct = target - ref

$\Delta$ Ct = 9.70

$\Delta$ Ct = target - ref

$\Delta$ Ct = -1.7
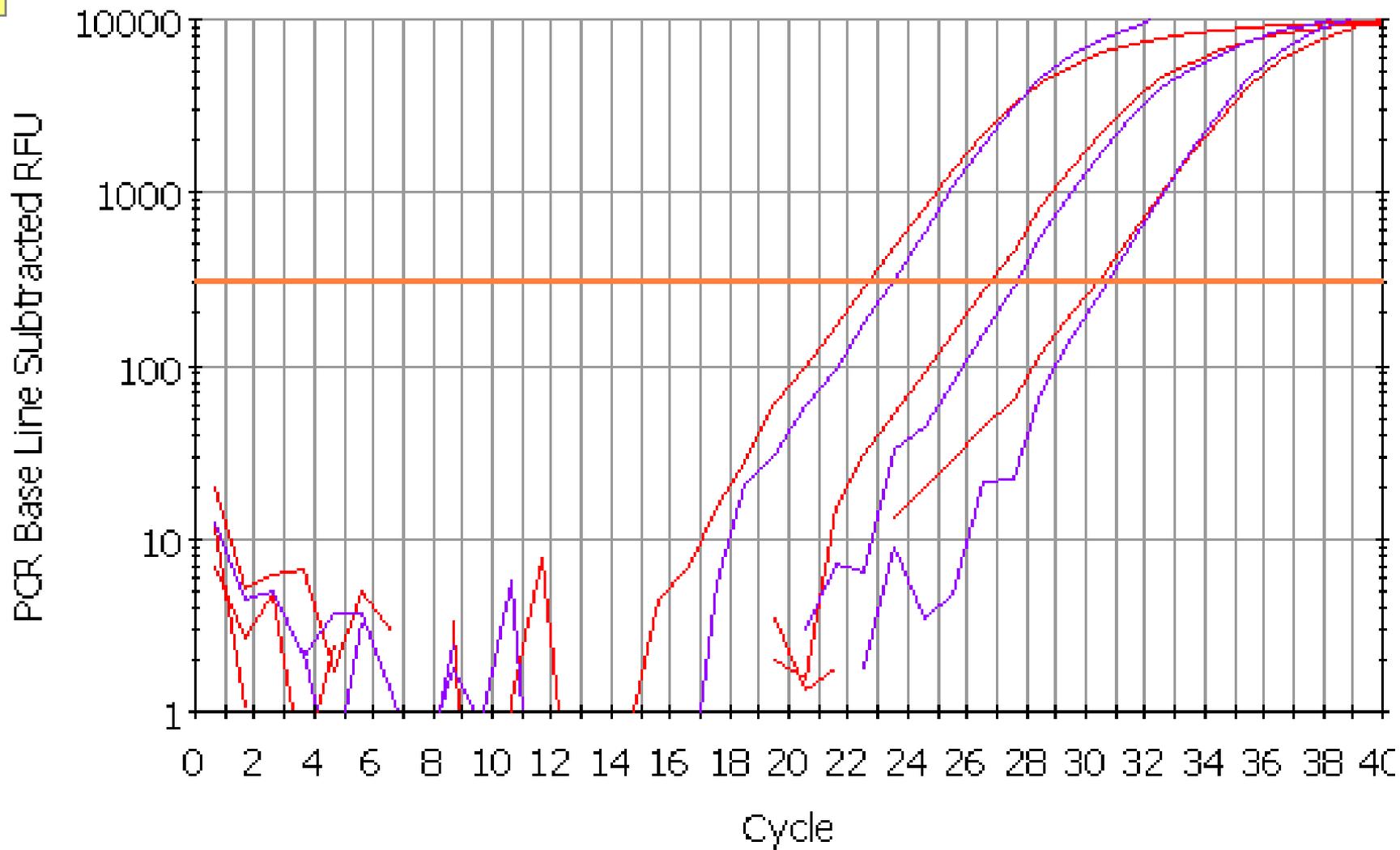
Difference = $\Delta$Ct-$\Delta$Ct
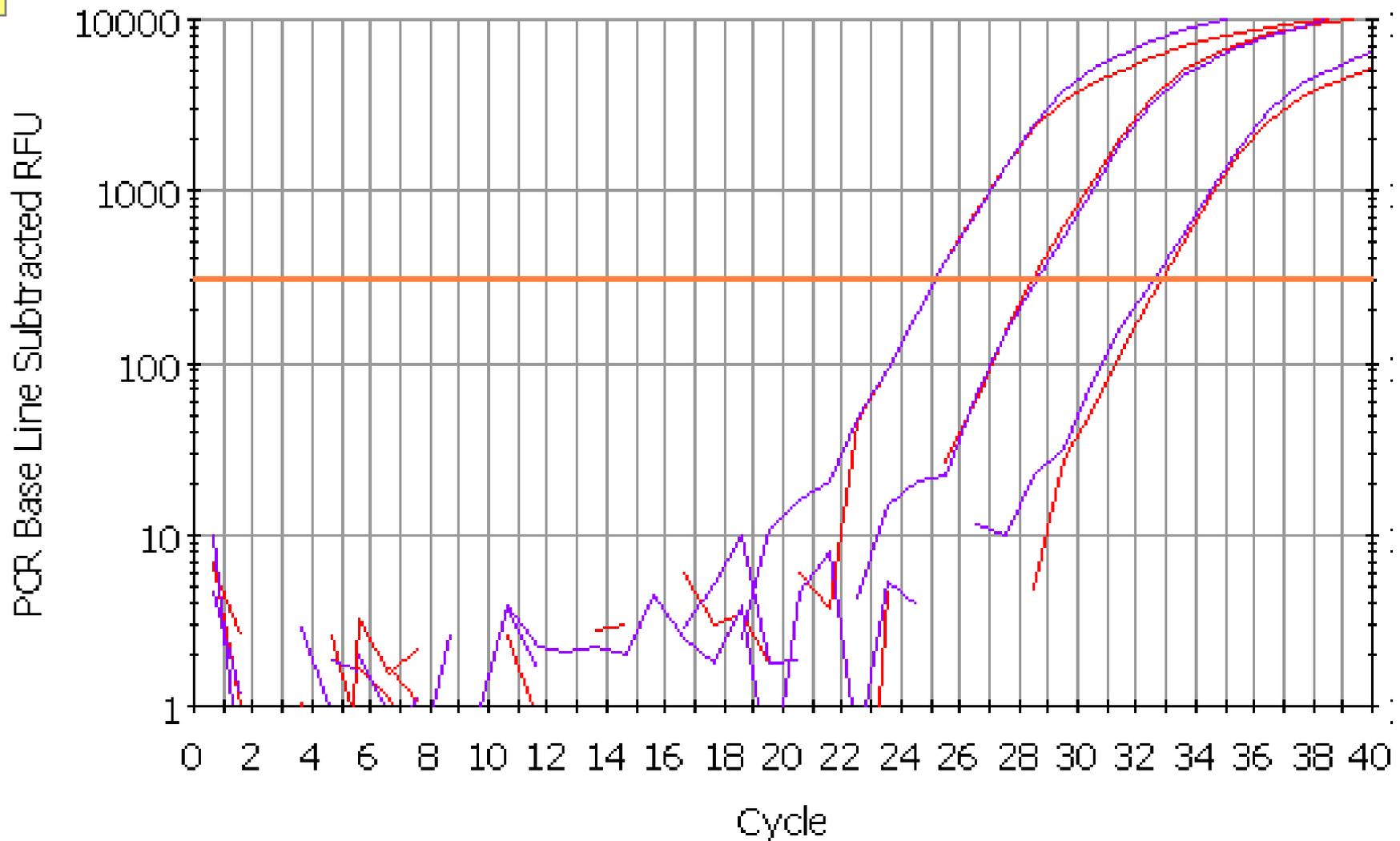
= $\Delta\Delta$Ct

= 9.70-(-1.7)

= 11.40

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# $\Delta\Delta$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

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 $\Delta$Cts 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?

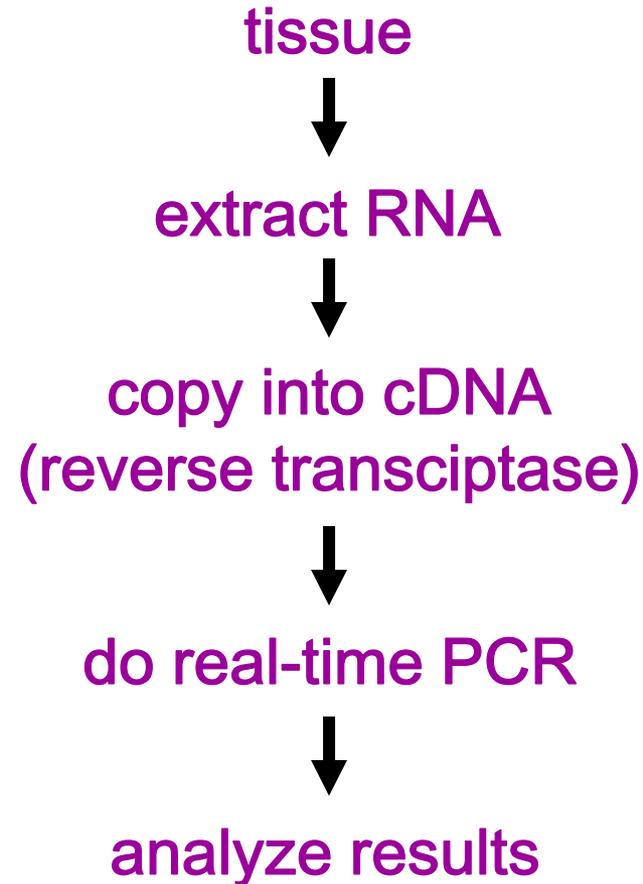  – Calculate according to Pfaffl method

RNA from control RPE cells $\longrightarrow$ cDNA from control RPE

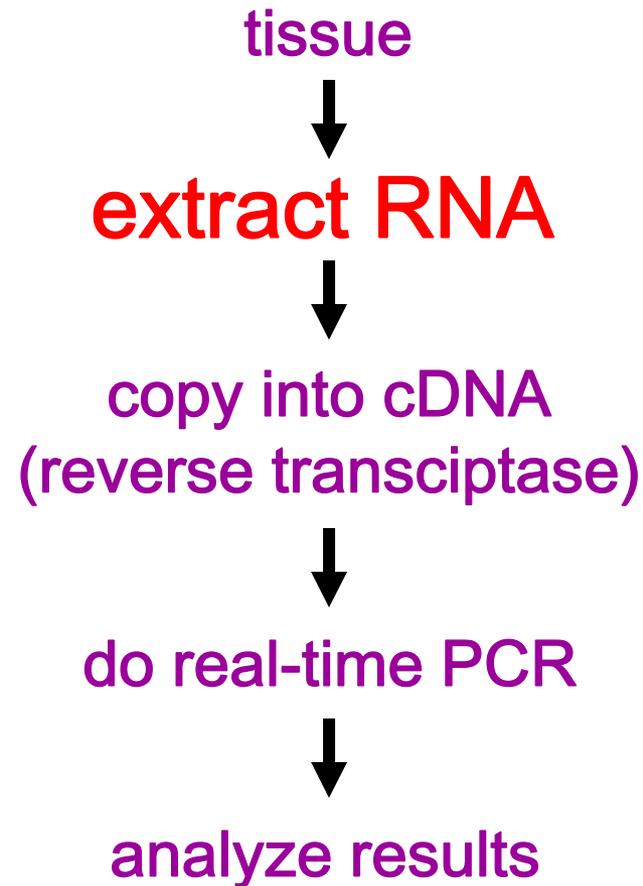RNA from TGF-b treated RPE cells $\longrightarrow$ cDNA from TGF-b treated RPE cells

?  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
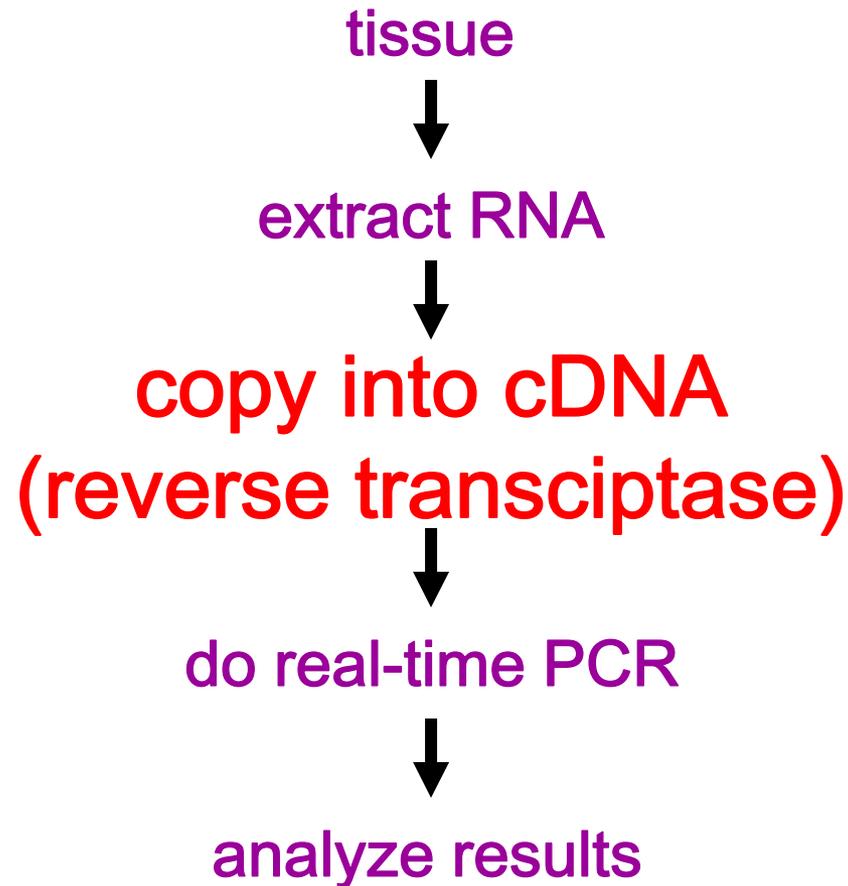(reverse transciptase)

↓

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
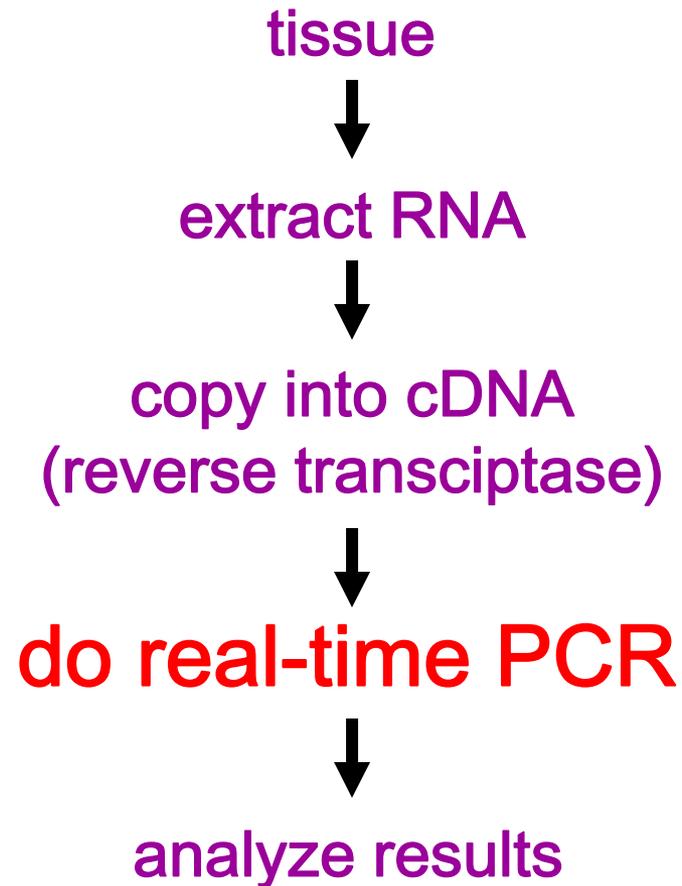
↓

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

# 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 $\longrightarrow$ cDNA from control RPE

RNA from TGF-b treated RPE cells $\longrightarrow$ 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

# 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