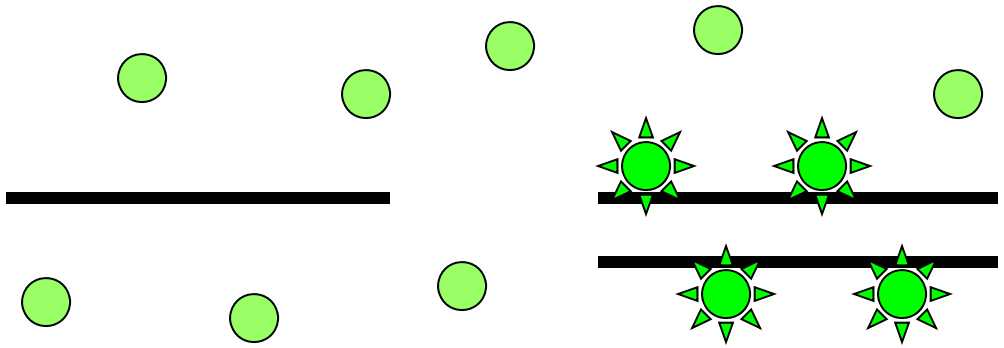
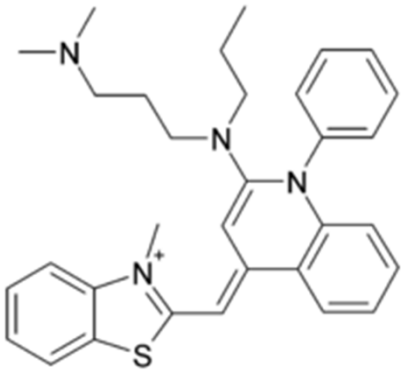
Quantitative Real Time PCR

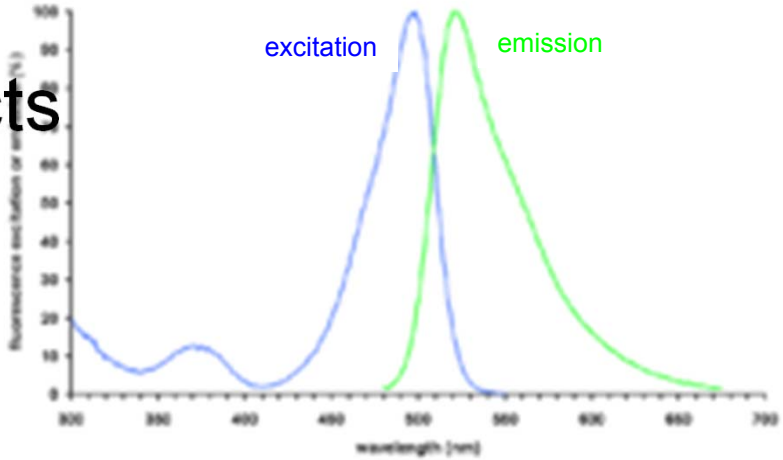
USING SYBR GREEN



SYBR Green



- SYBR Green is a cyanine dye that binds to double stranded DNA.
- When it is bound to D.S. DNA it has a much greater fluorescence than when bound to single stranded DNA.
- This can be used to follow the production of new PCR products



THE PROBLEM

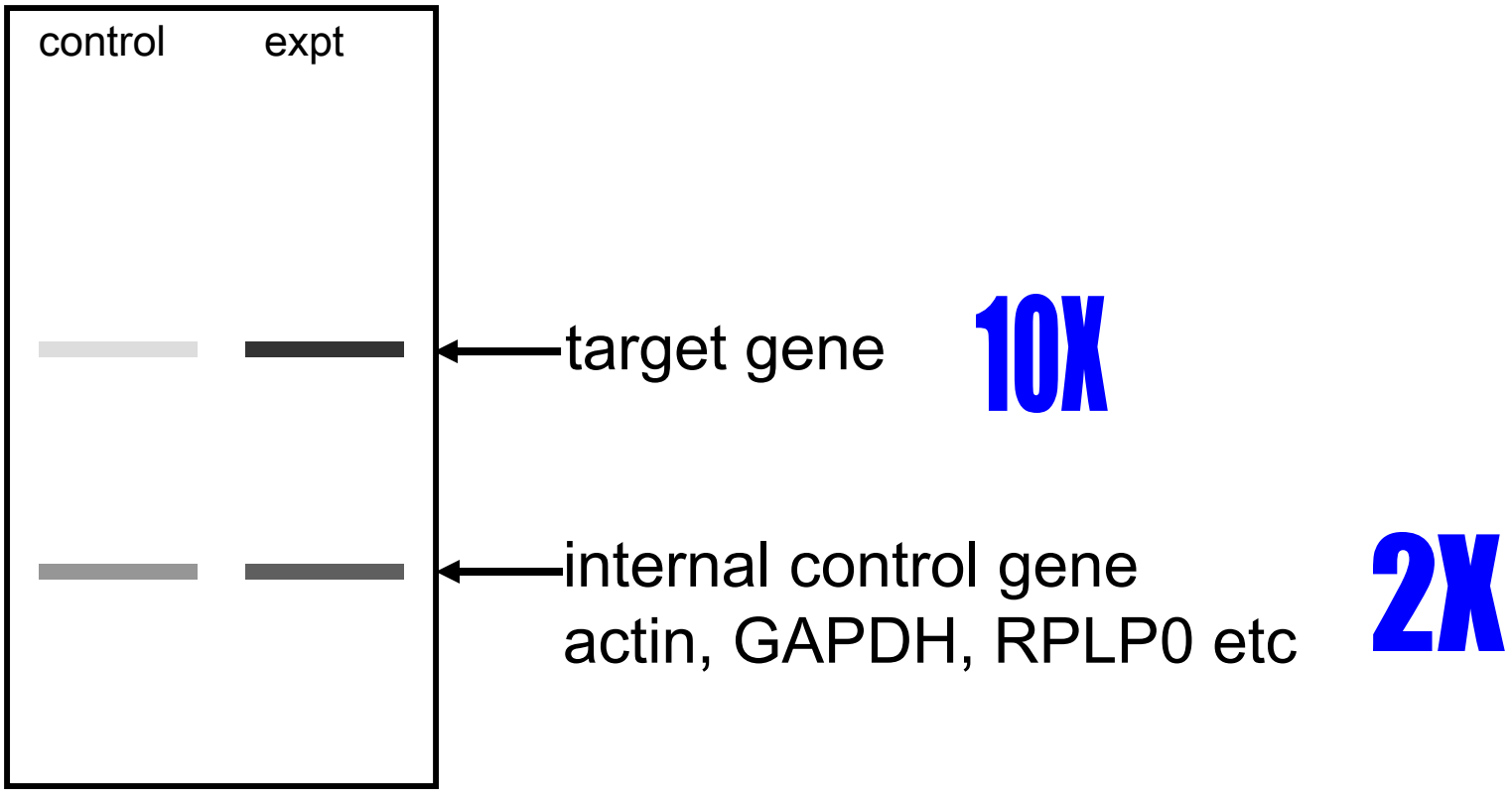
- NEED TO QUANTITATE DIFFERENCES IN GENE (mRNA) EXPRESSION

- SMALL AMOUNTS OF mRNA
  - LASER CAPTURE
  - SMALL AMOUNTS OF TISSUE
  - PRIMARY CELLS
  - mRNA FROM CHICKEN LIPS

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 BLOT



Corrected fold increase = 10/2 = 5

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

Normalization Standards

- corrects for loading errors

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

Normalization Standards

- **The perfect standard does not exist**
- **You have to determine which is best for your organism and questions**

Standards

- Commonly used standards
  - Glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH)
  - Beta-actin mRNA
  - MHC I (major histocompatability complex I) 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

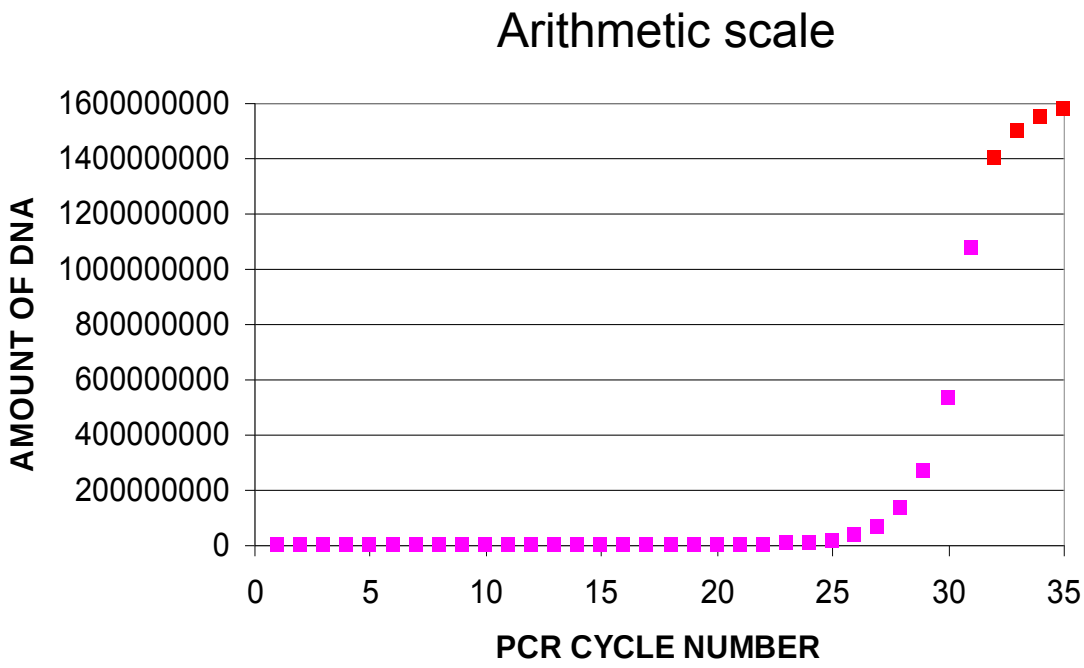
PCR

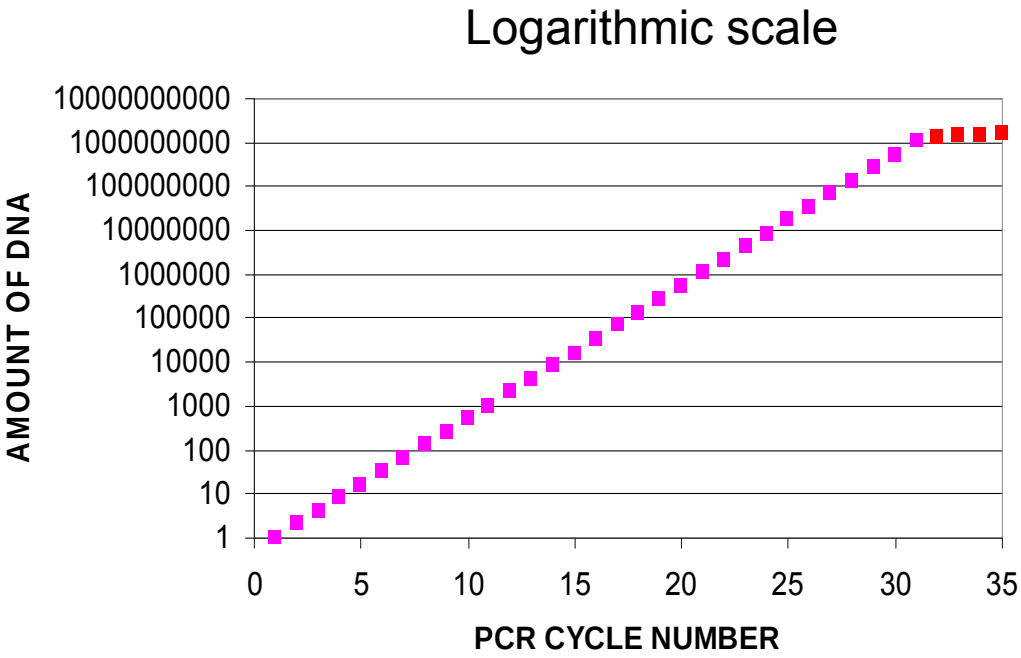
The amount of DNA doubles after each cycle

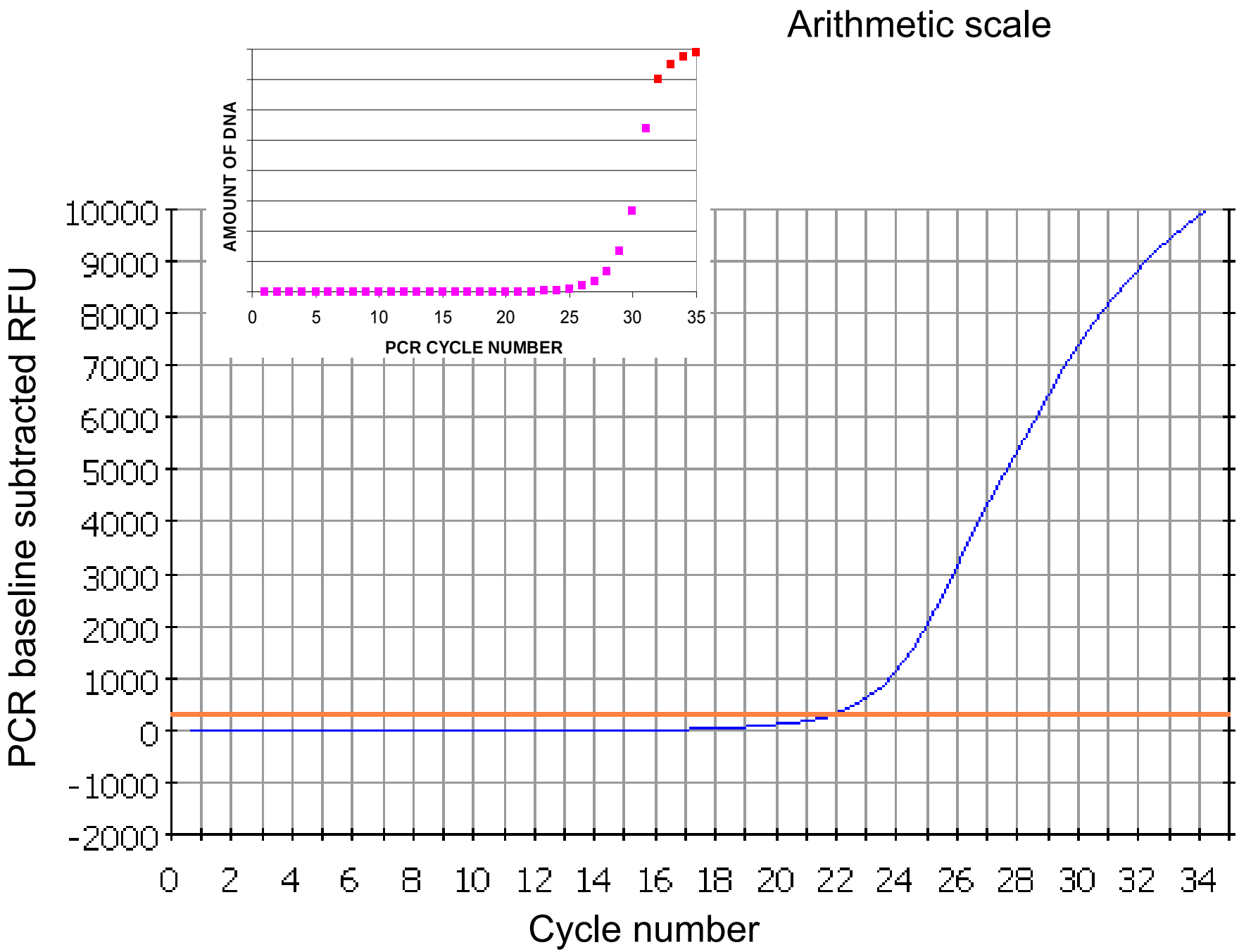
Assuming 100% efficient PCR reactions

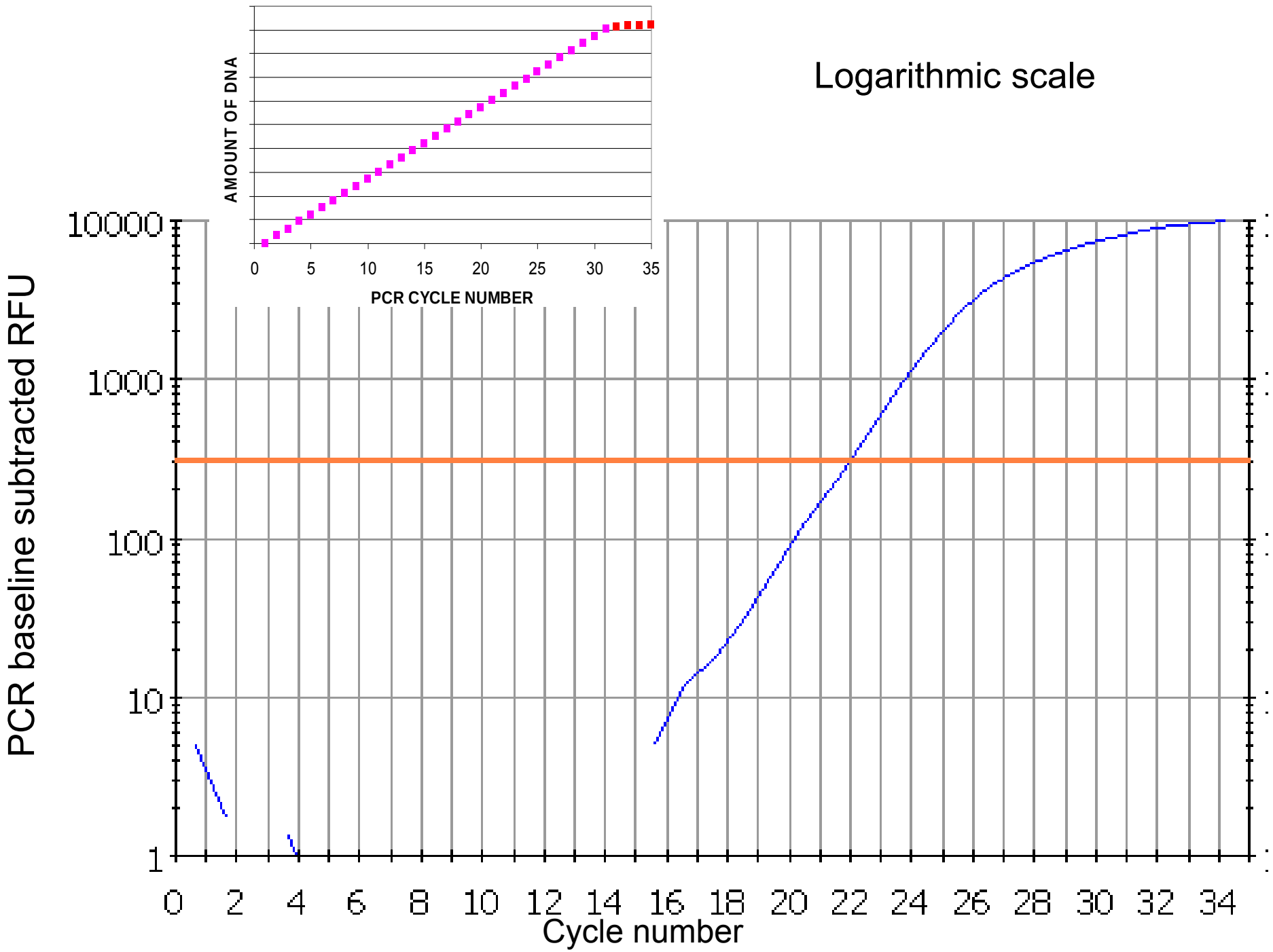
After n cycles there will be 2^n times as much DNA

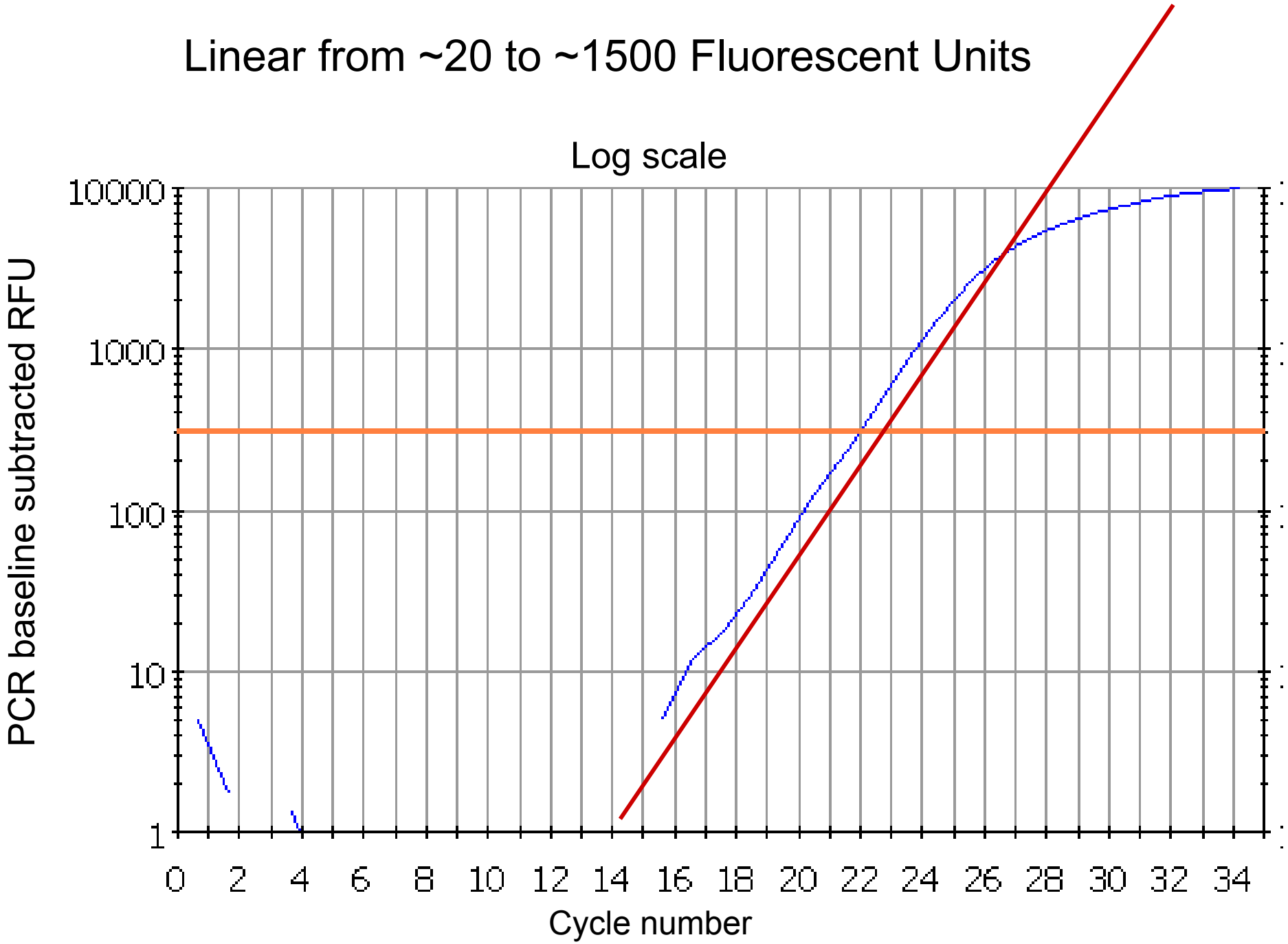
CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1.500.000.000



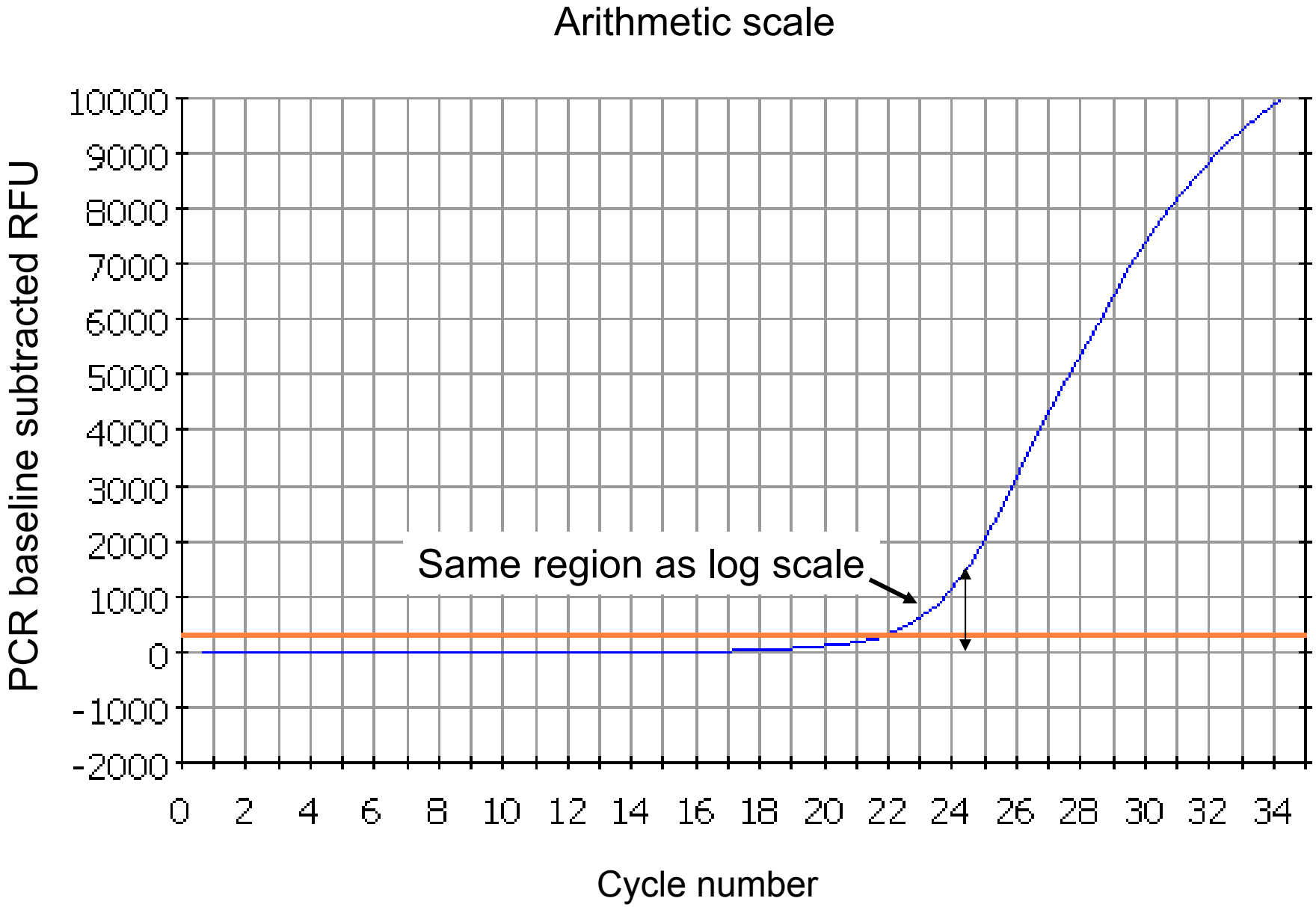


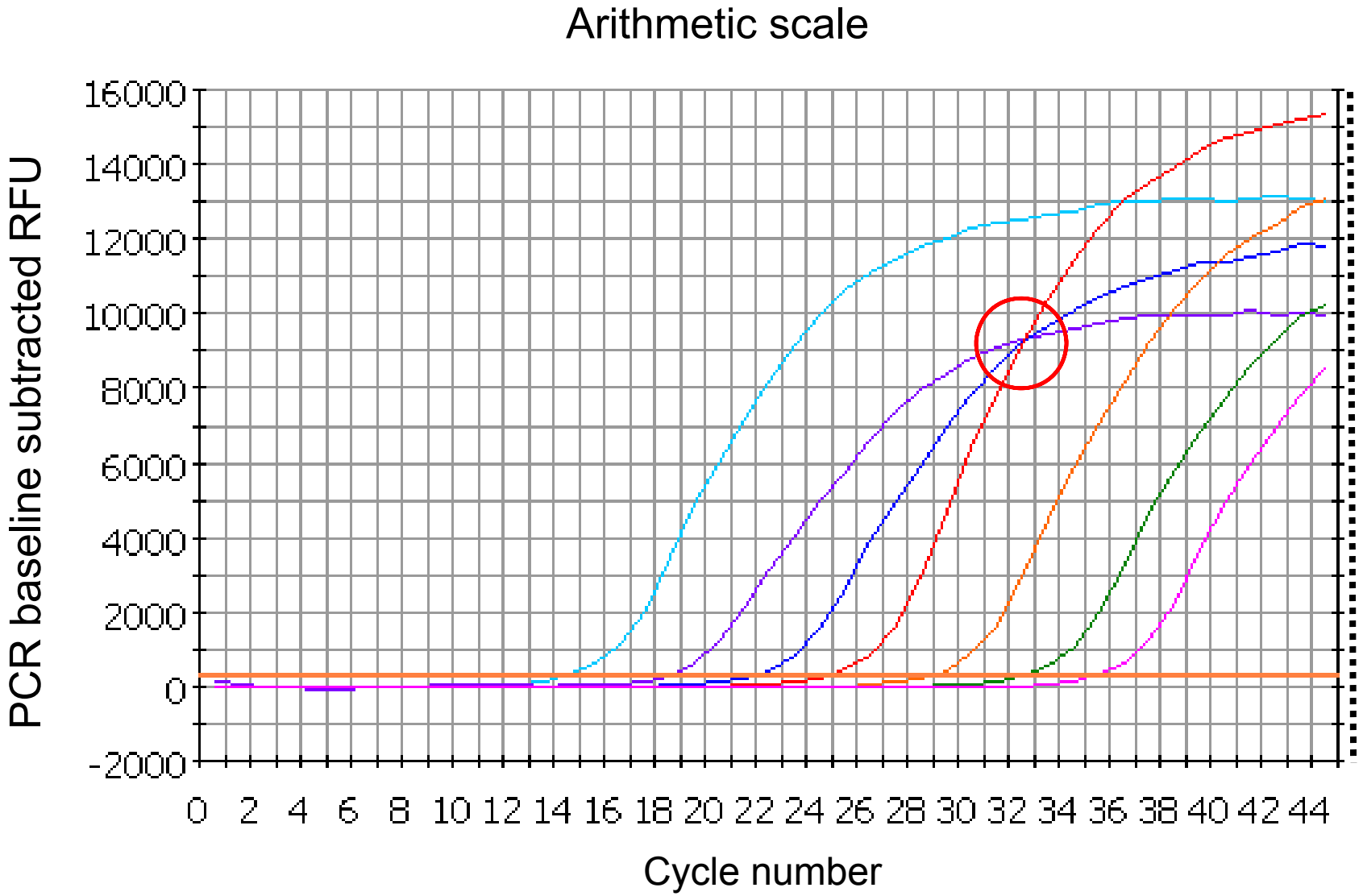

Arithmetic scale
AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR baseline subtracted RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Cycle number


AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
Logarithmic scale
PCR baseline 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
Cycle number

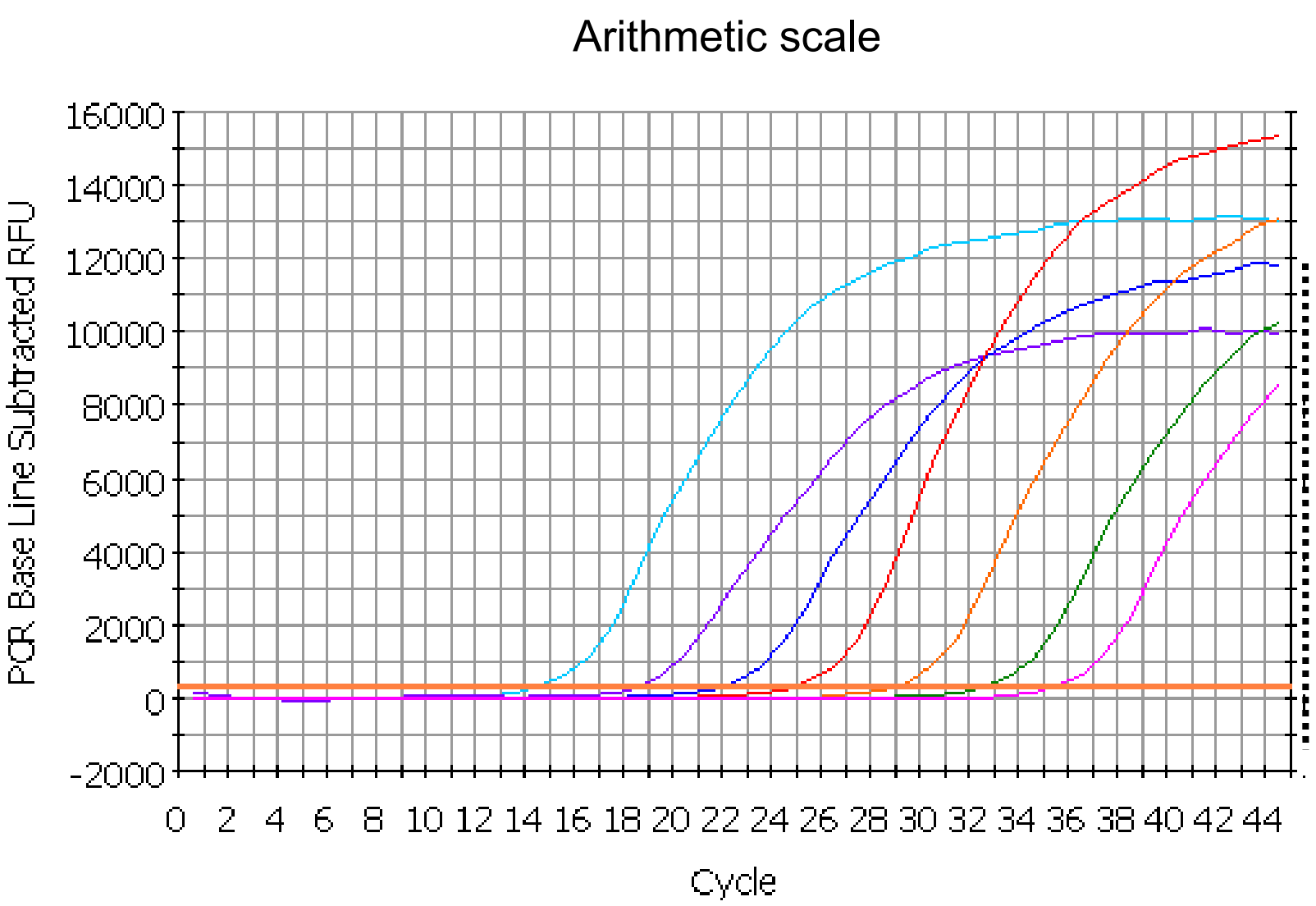

Linear from ~20 to ~1500 Fluorescent Units
Log scale
PCR baseline subtracted RFU
10000
1000
100
10
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Cycle number

Linear ~20 to ~1500 Fluorescent Units

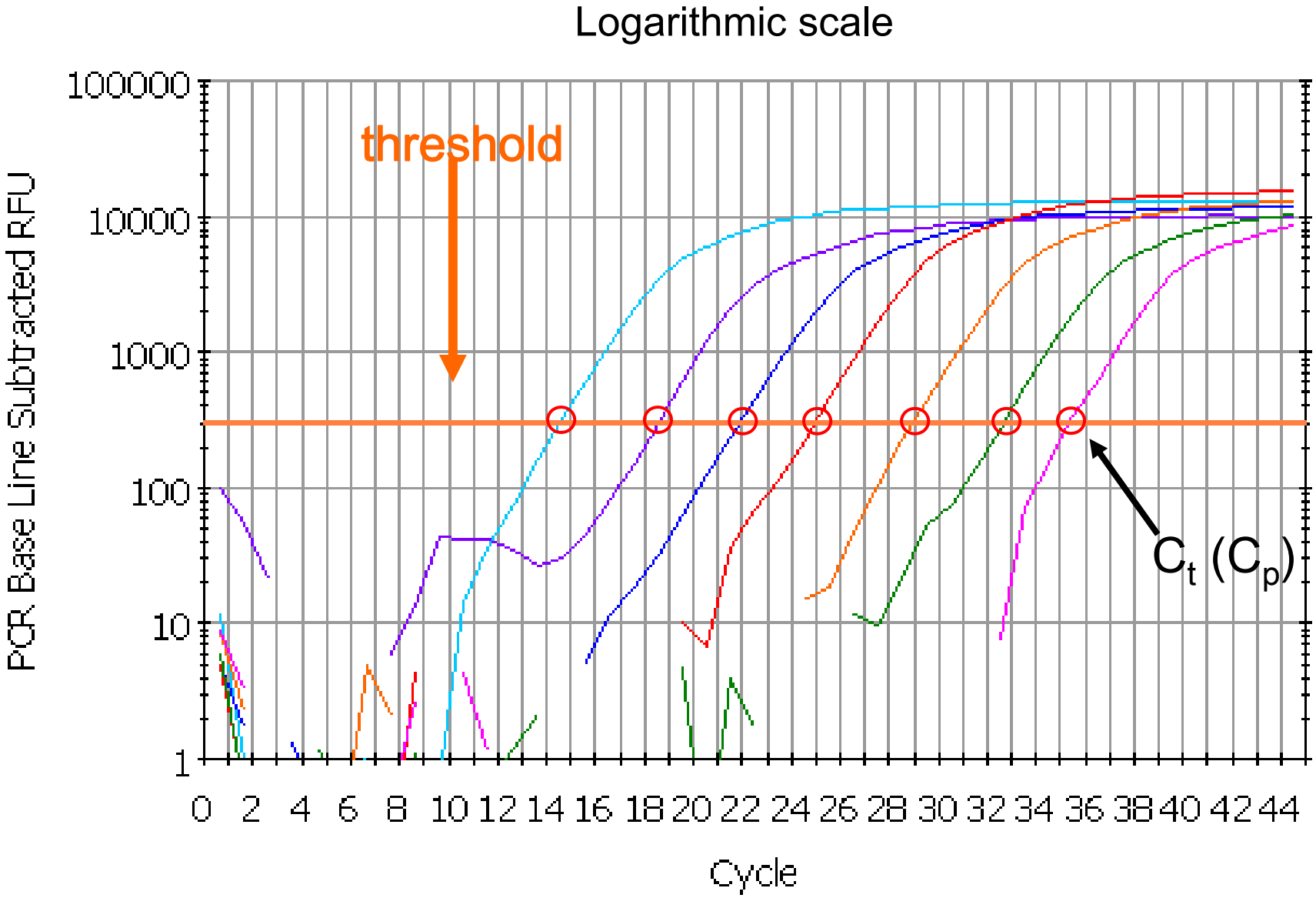




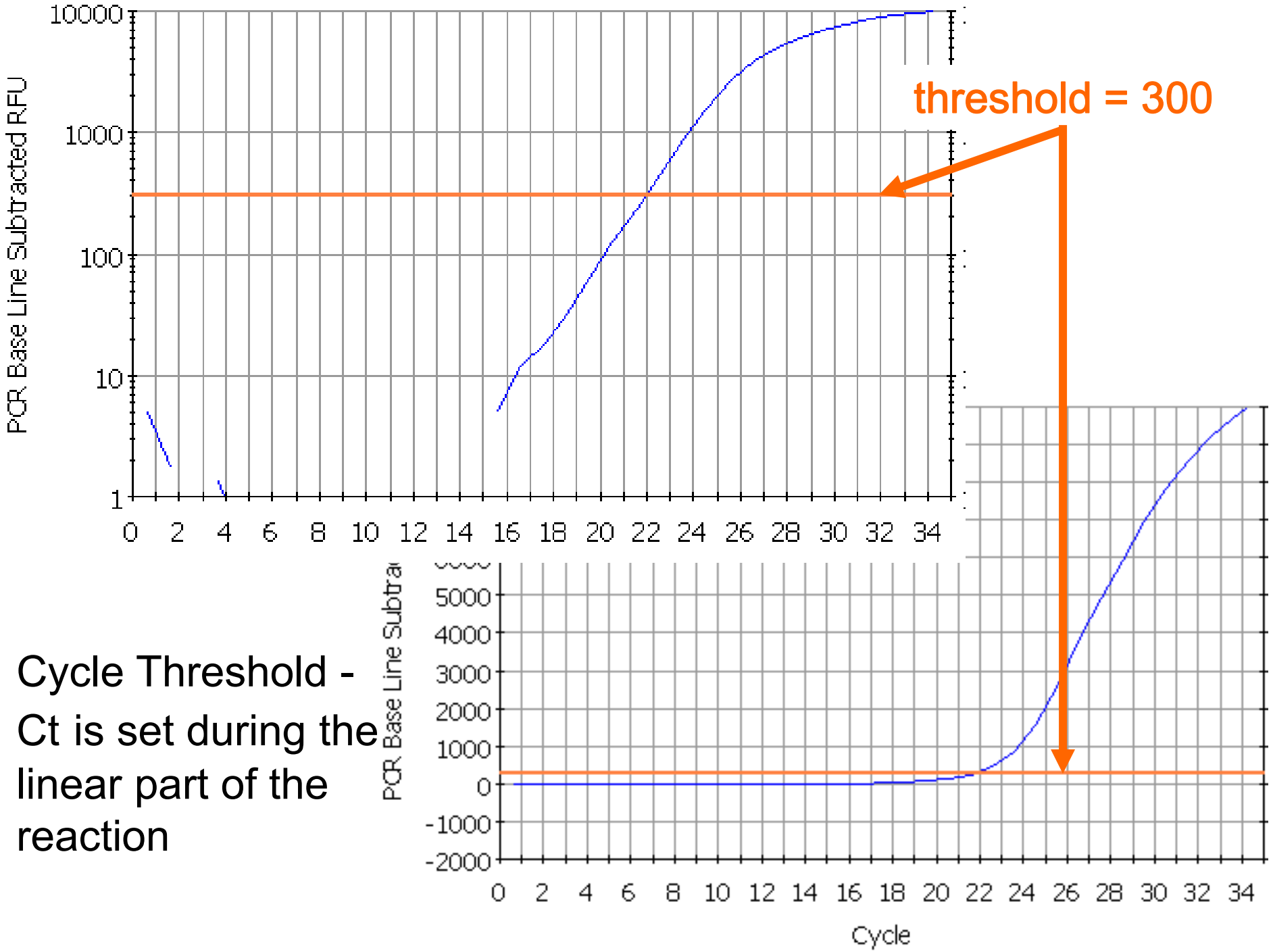
SERIES OF 10-FOLD DILUTIONS OF TEMPLATE



SERIES OF 10-FOLD DILUTIONS



SERIES OF 10-FOLD DILUTIONS



Cycle Threshold -

Ct is set during the linear part of the reaction

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

AFTER 1 CYCLE

100%= 2.00x

90% = 1.90x

80% = 1.80x

70% = 1.70x

Much different values depending on the 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
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AFTER 1 CYCLE

100%= 2.00x

90% = 1.90x

80% = 1.80x

70% = 1.70x

AFTER N CYCLES:
fold increase =
$(1 + \text{efficiency})^n$

Only 1% of 100% efficiency amount

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
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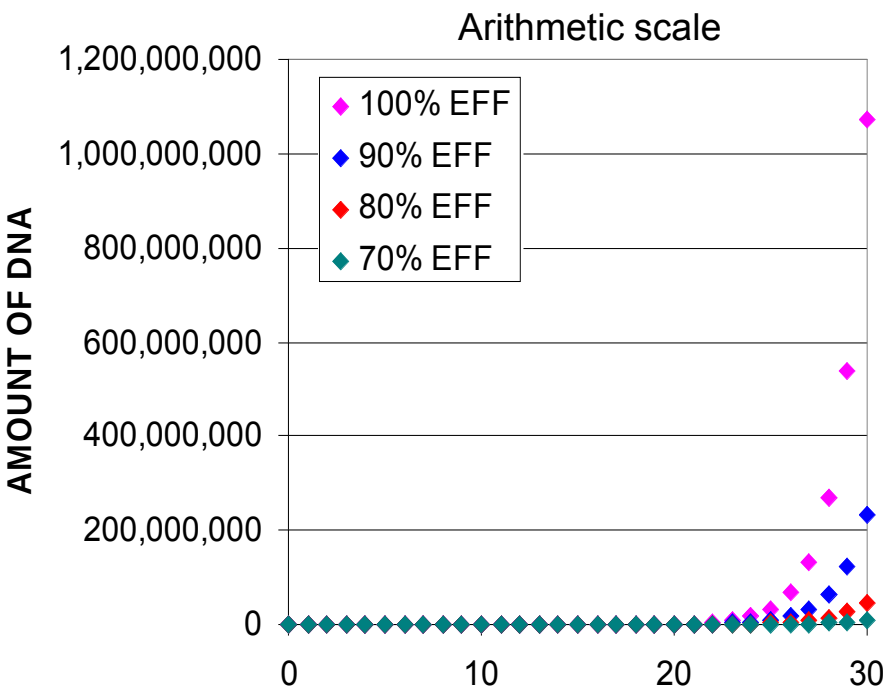
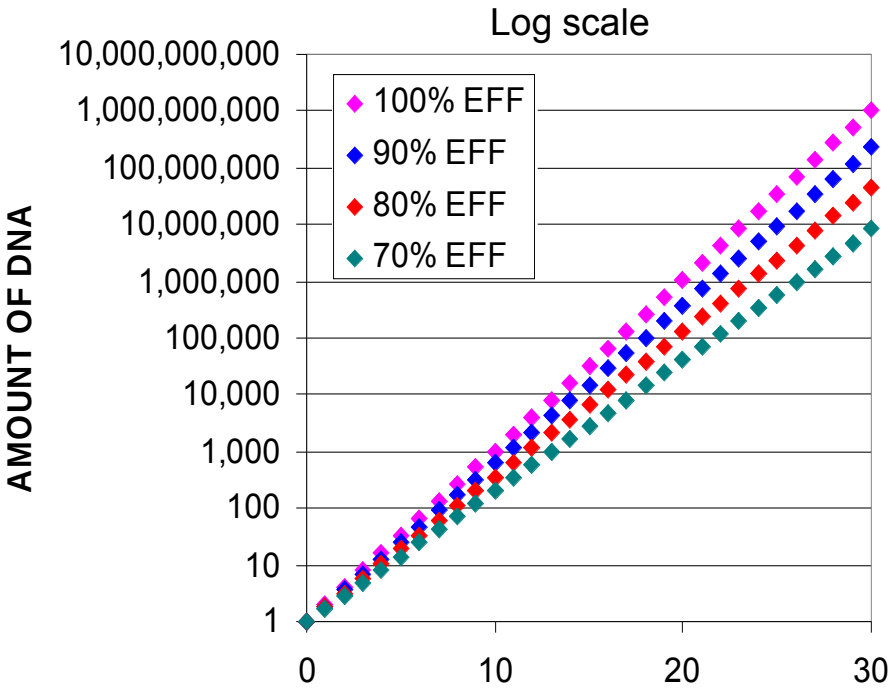
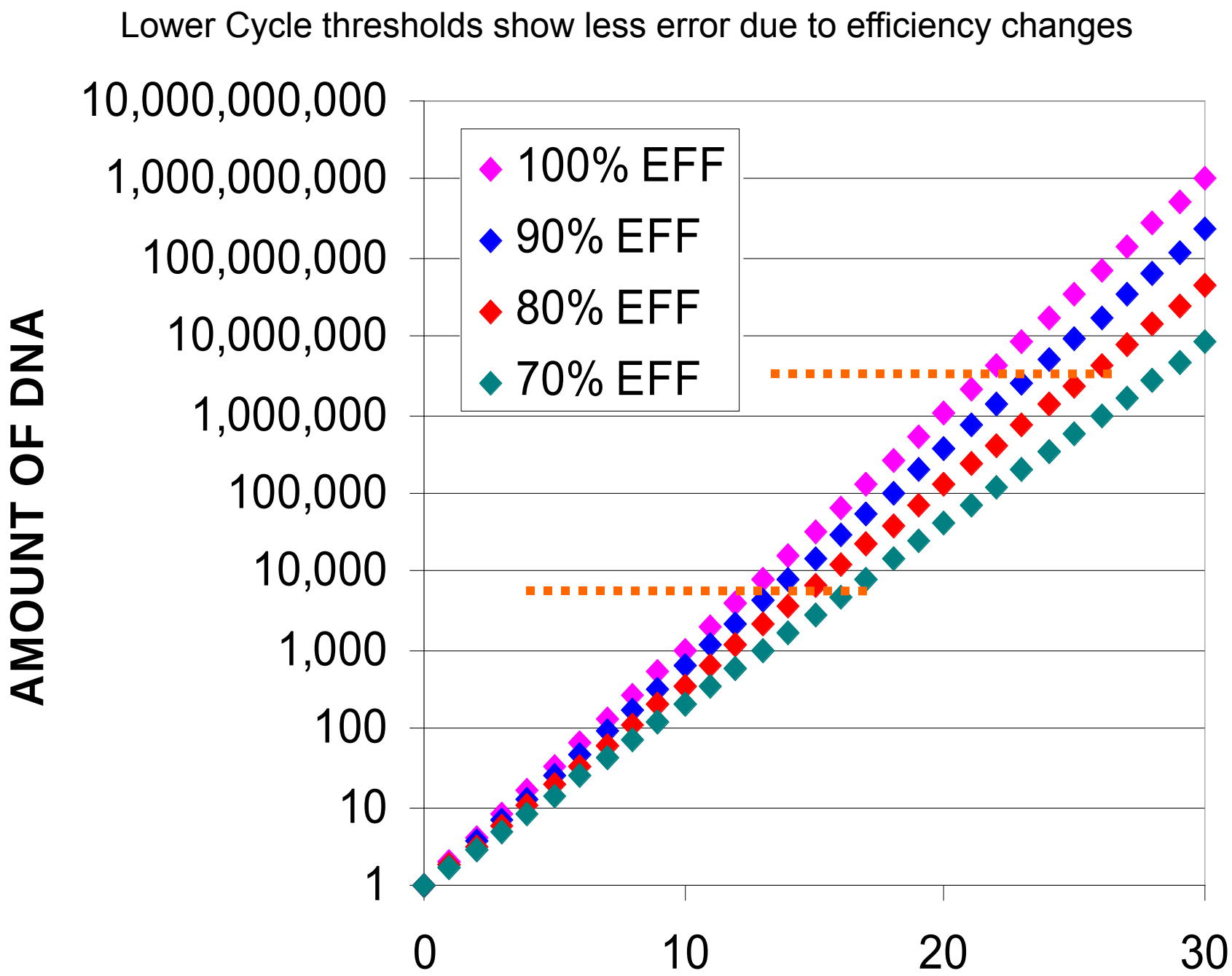
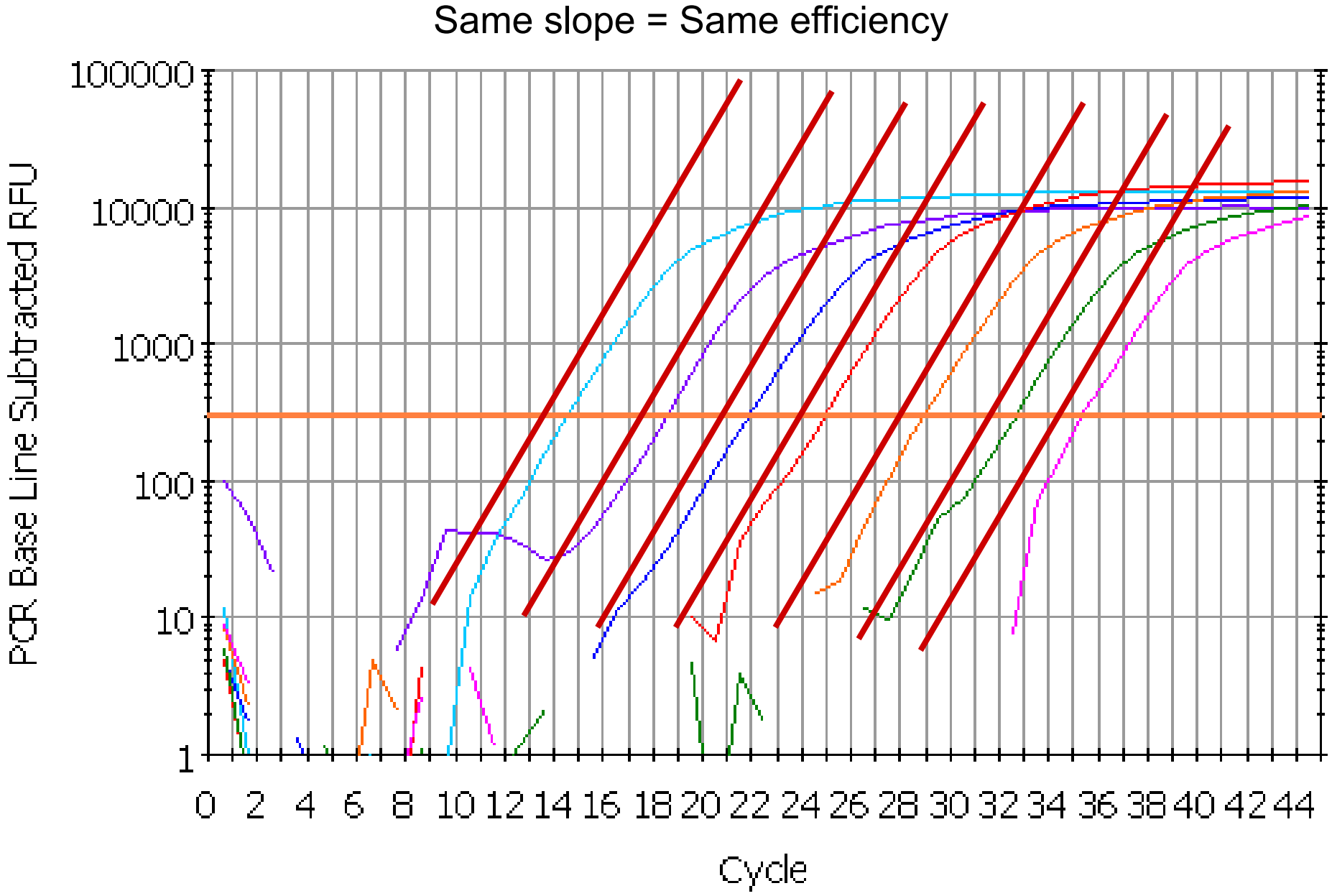
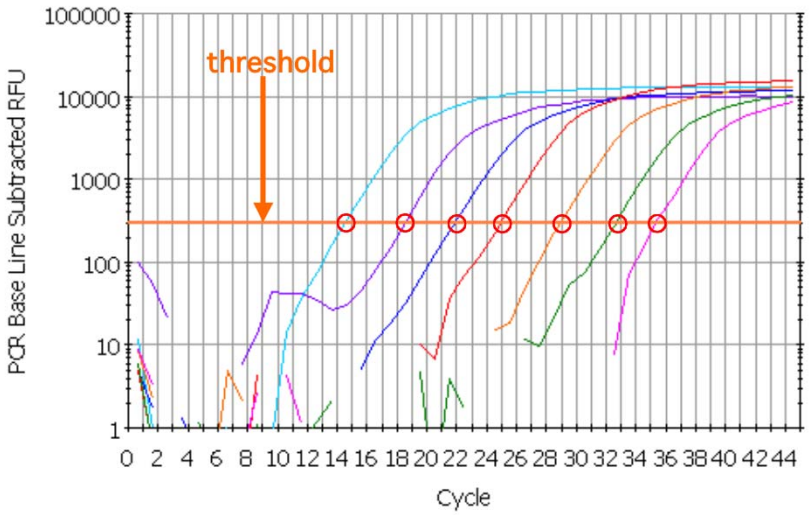




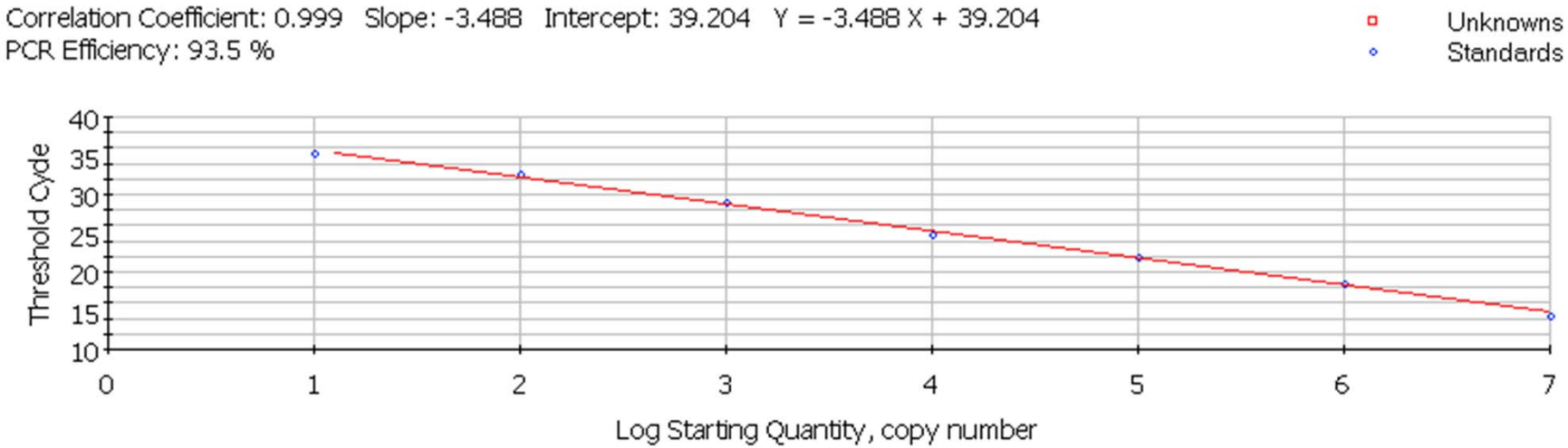

Lower Cycle thresholds show less error due to efficiency changes
AMOUNT OF DNA
10,000,000,000
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
0
10
20
30
100% EFF
90% EFF
80% EFF
70% EFF



SERIES OF 10-FOLD DILUTIONS



Plot the Ct values for the dilutions vs. concentration, the slope of the line can be used to calculate the PCR efficiency



Melt curve analysis

SYBR Green will bind to any double-stranded DNA.

Primer-dimers will contribute to the signal too.

How can you distinguish between amplification of the gene of interest and artifacts?

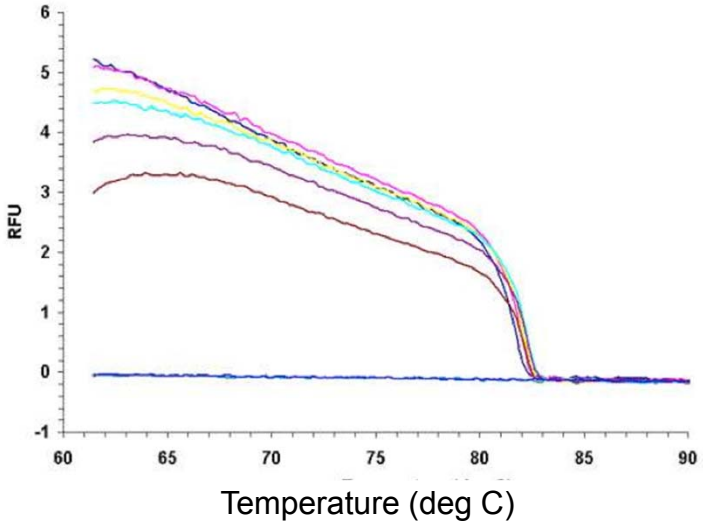
Remember SYBR Green binds to double-stranded DNA but not single stranded DNA.

You can ‘melt’ the newly created DNA and the SYBR Green will dissociate and the fluorescence decreases.

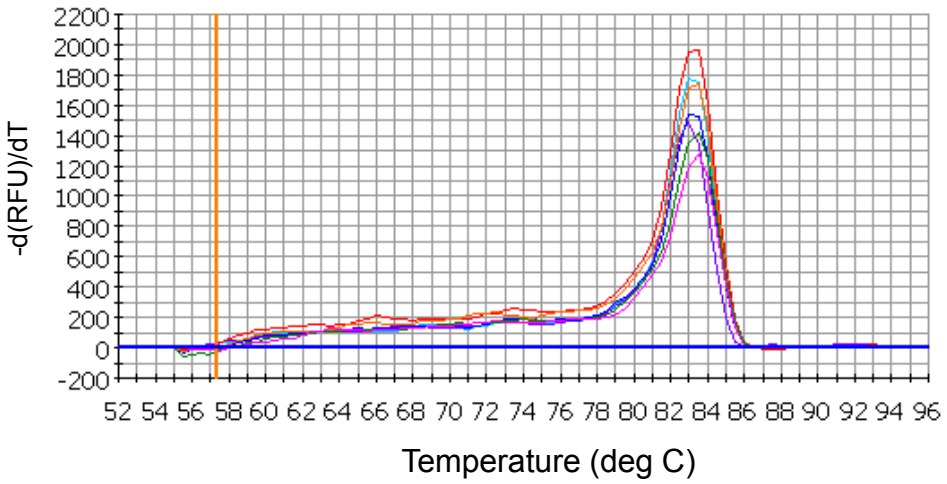
Melt curve analysis

The key is that DNA of different base composition and length will ‘melt’ at difference temperatures.

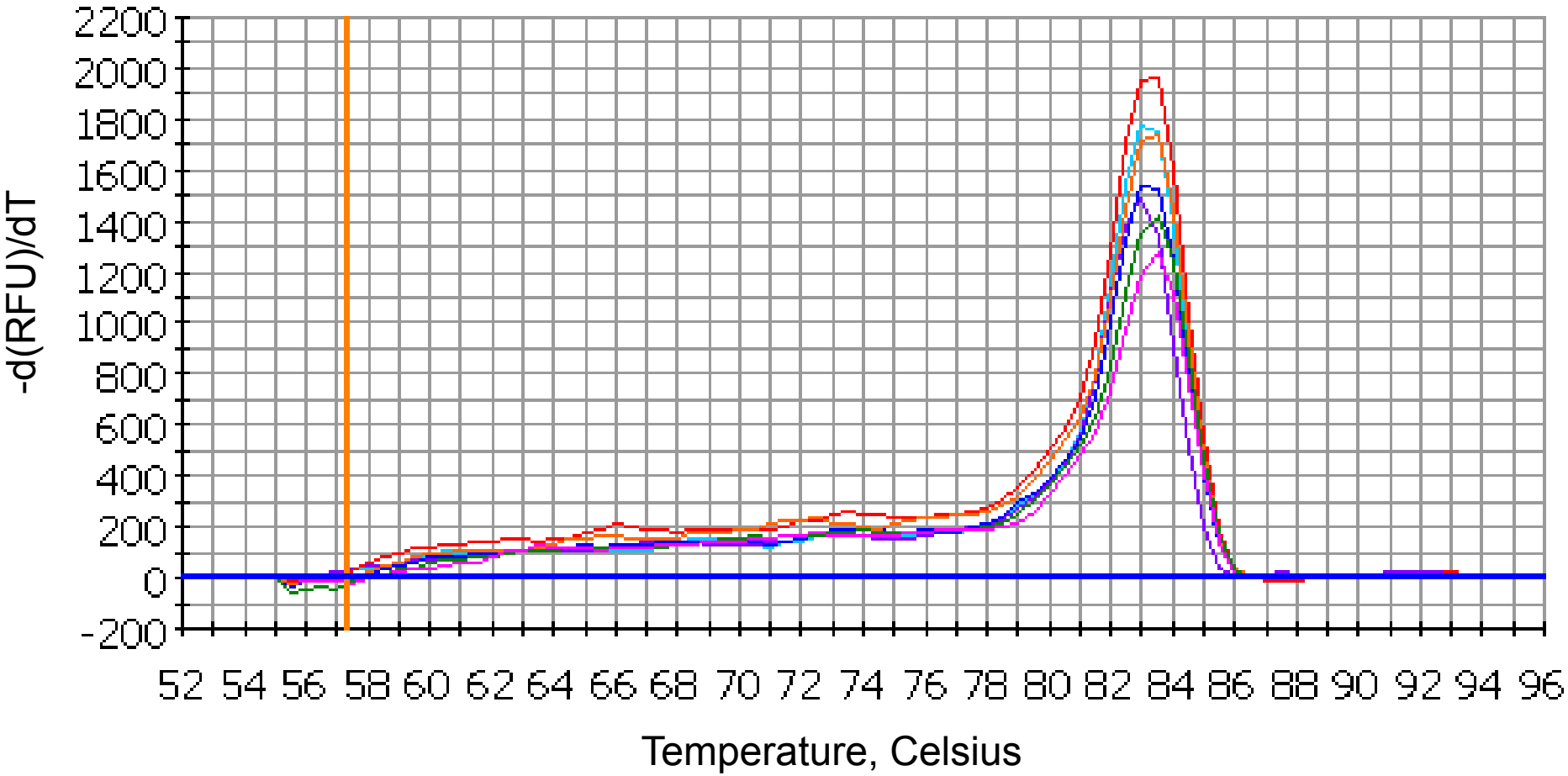
By slowly and accurately increasing the temperature there will be changes in the rate of the fluorescence decrease if there is more than one kind of DNA present.



Raw melt-curve

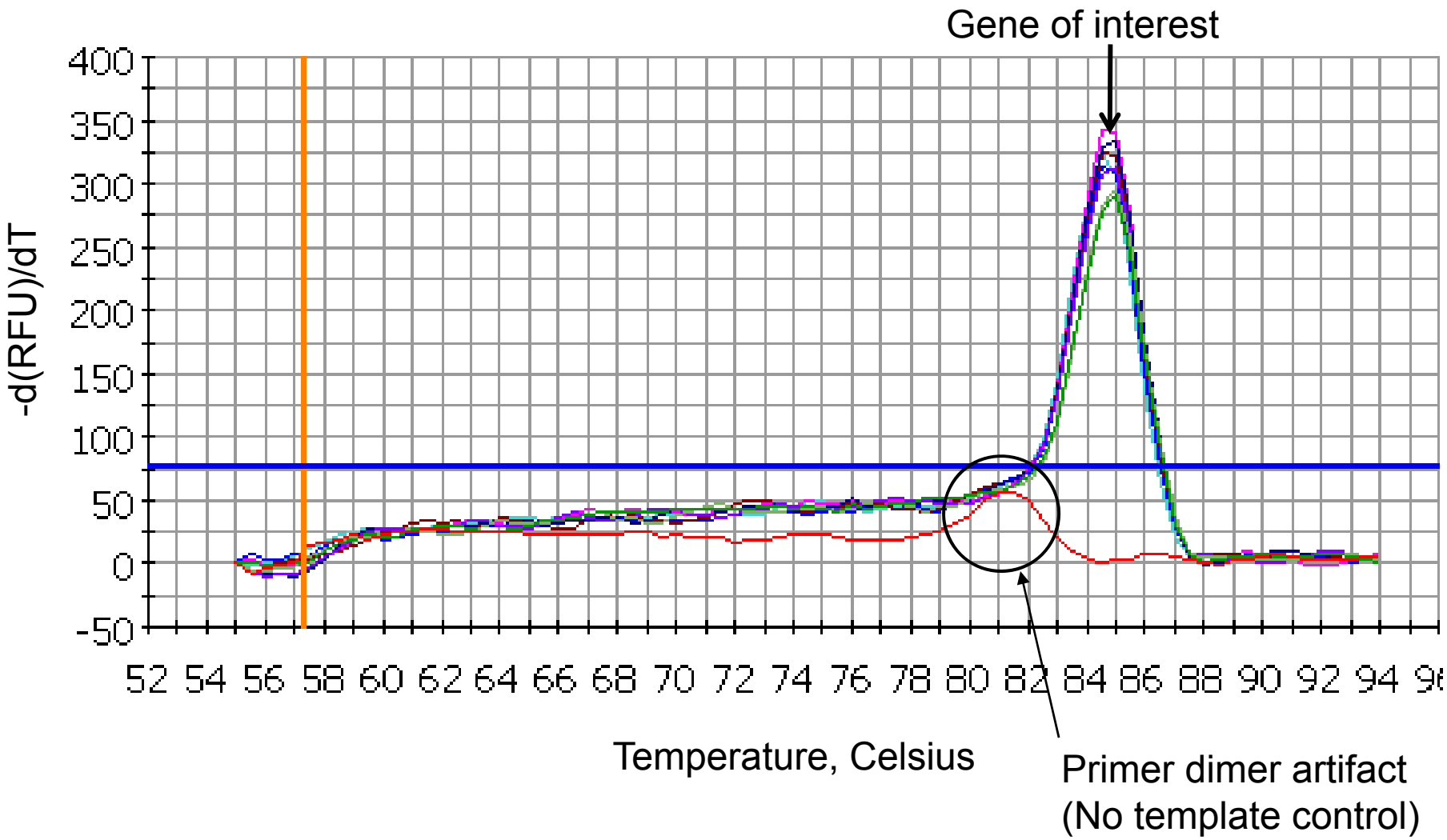


Derivative of melt-curve

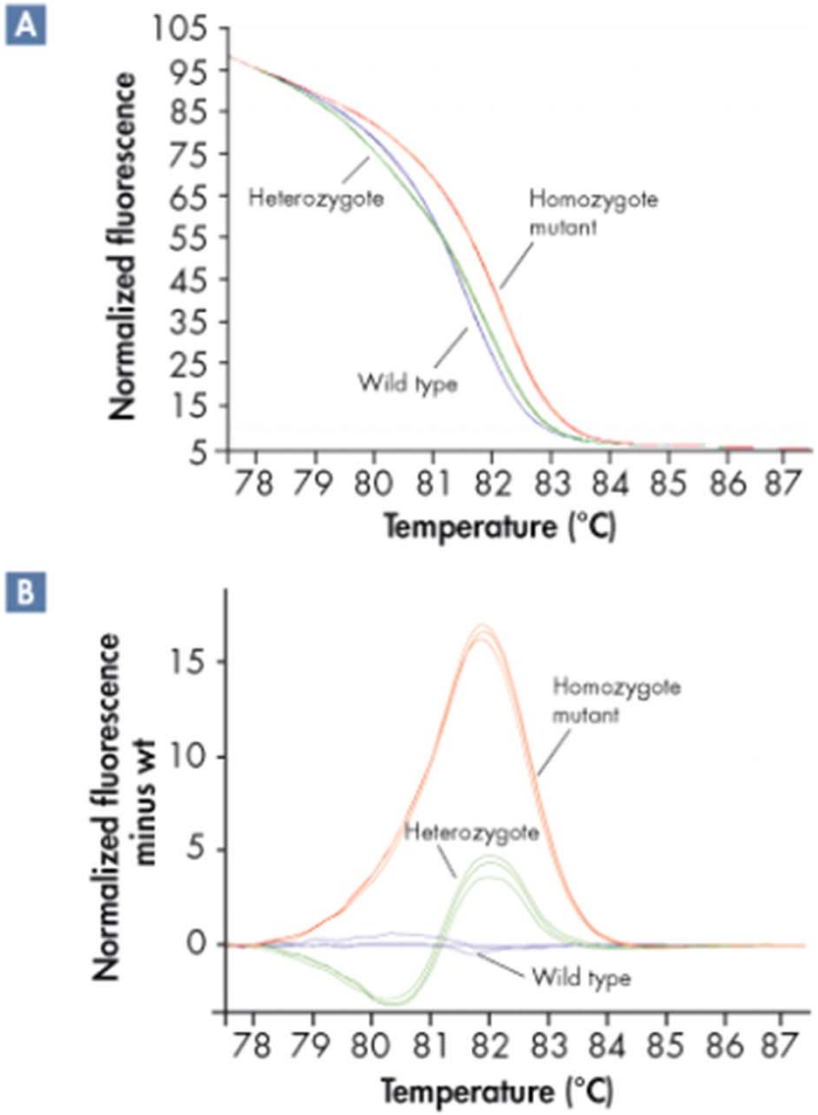


Melt curve analysis (derivative of fluorescence decrease as the DNA becomes single stranded)

The Melt-Curve shows the different types of DNA present



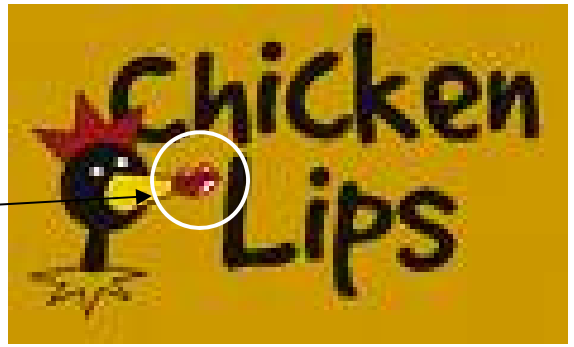
Melt curve analysis can also be used for allelic discrimination



Newer RT-PCR thermocyclers can perform High Resolution Melt Curve analyses

Used for allelic discrimination analyses in populations

GENE EXPRESSION ANALYSIS OVERVIEW



Obtain tissue

↓

extract RNA

↓

copy into cDNA
(reverse transcriptase)

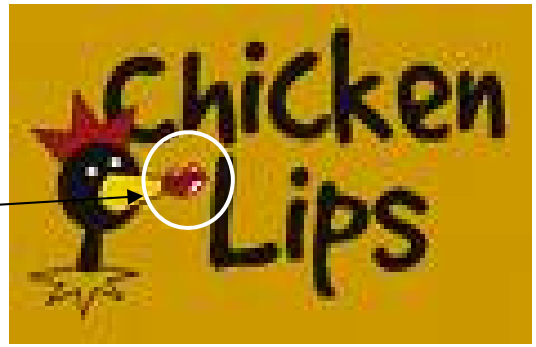
↓

real-time PCR

↓

analyze results

GENE EXPRESSION ANALYSIS OVERVIEW



Obtain tissue

↓

Extract RNA

↓

Copy into cDNA
(reverse transcriptase)

↓

Real-time PCR

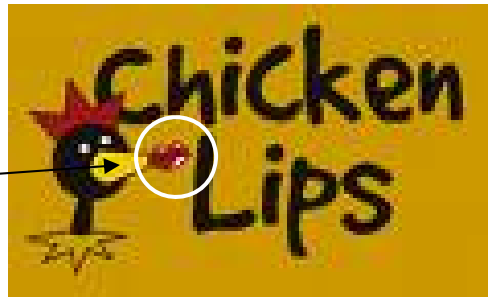
↓

Analyze results

IMPORTANCE OF RNA QUALITY

- Should be free of protein (absorbance 260nm/280nm > 1.8)
- Should be intact (28S/18S ~2:1)
- High RIN (use Agilent Bioanalyzer)
- Should be free of DNA (treat with DNAse)
- Should be free of PCR inhibitors
  - Purification methods
  - Clean-up methods

OVERVIEW



Obtain tissue

↓

Extract RNA

↓

Copy into cDNA
(reverse transcriptase)

↓

Real-time PCR

↓

Analyze results

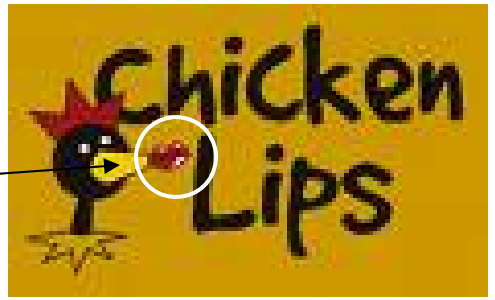
Importance of reverse transcriptase primers

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

REVERSE TRANSCRIPTION

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

OVERVIEW



Obtain tissue

↓

Extract RNA

↓

Copy into cDNA
(reverse transcriptase)

↓

Real-time PCR

↓

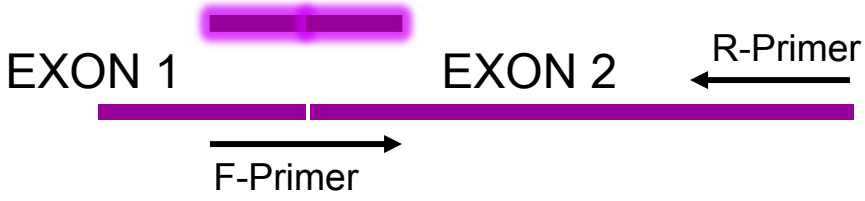
Analyze results

Importance of primers in qPCR

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

Primer will not bind to genomic DNA because the 3’ end is not complementary to the Intron

genomic DNA



cDNA

Primer will bind to the cDNA because the primer is complementary to the Exon-Exon boundary after the intron is cleaved out

How will you 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

RNA from control cells → cDNA from control cells

RNA from treated cells → cDNA from treated cells

Is there any change in your gene expression?

RNA from control cells → cDNA from control

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

RNA from treated cells → cDNA from treated cells

→ No RT for treated cells
(to see if any genomic DNA signal)

Is there any change in your gene expression?

*RT - Reverse Transcriptase

qPCR Data Analysis

Depends on the goal of the experiment—

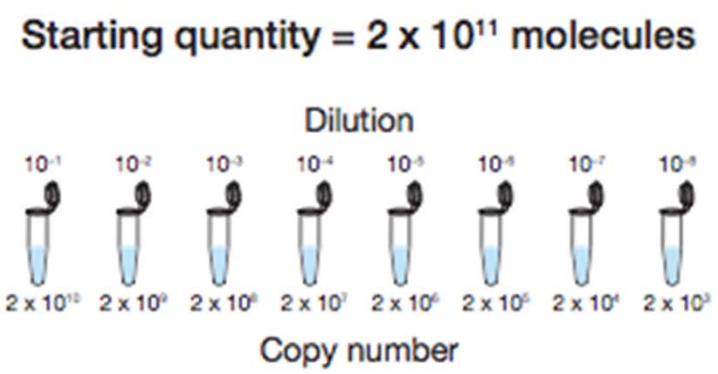
- Absolute quantification allows actual copy numbers to be determined but is labor intensive.

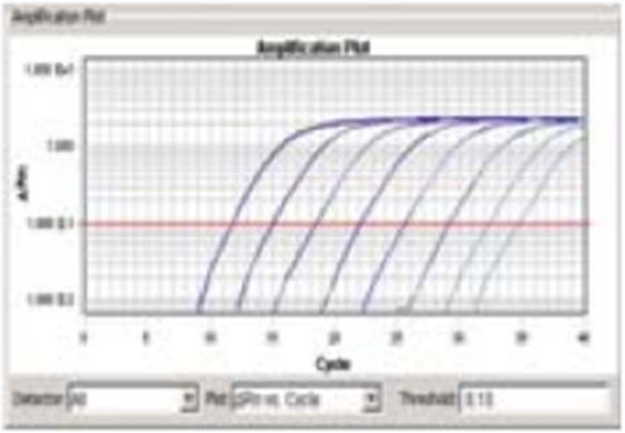
- Comparative quantification determines relative abundance rather than exact copy.

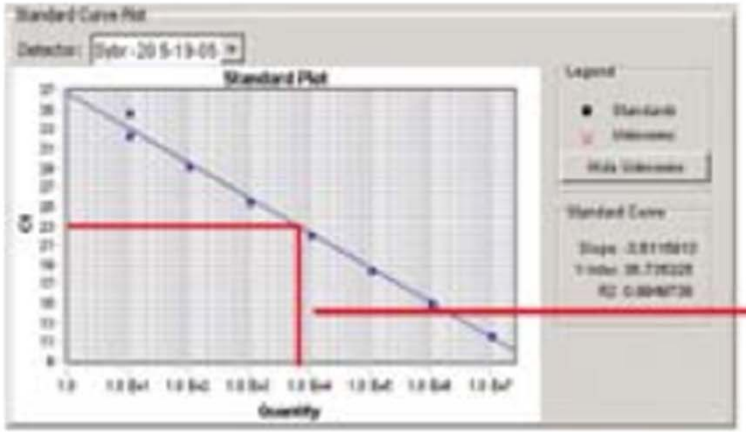
  Most often used for gene expression studies and has two main options for quantitation:
  $\Delta\Delta C_t$ and standard curve quantitation.

Absolute quantification

A standard curve is generated using a single template species that is diluted over several orders of magnitude. C_t (C_p) vs concentration is plotted.







$y = mx + b$

$y = C_t$

m = slope

b = y-intercept

x = copy number

Standard curve generation – template choice

DNA standards—PCR amplicon of the target of interest, or plasmid clone containing the target of interest

Pros: Easy to generate, quantify, and maintain stability with proper storage.

Cons: Avoids the reverse transcription phase of qRT-PCR, which can impact reaction efficiency significantly.

RNA standards—In *vitro–transcribed* RNA of the target of interest

Pros: Incorporates RT efficiency and mimics the target of interest most similarly.

Cons: Time-consuming to generate and difficult to maintain accuracy over time due to instability.

Comparative quantification – ΔC_t method

Most basic form is to obtain a C_t value for the gene of interest and a calibrator sample (such as time zero sample). The difference is the ΔC_t

$$\text{Fold difference} = 2^{\Delta C_t}$$

This basic method does not incorporate a normalizer or corrects for efficiency.
It assumes that the same amount of template was present and the amplification efficiency is the same.

Comparative quantification – $\Delta\Delta C_t$ method

An improvement over ΔC_t is the $\Delta\Delta C_t$ method

$$\text{Fold difference} = 2^{-\Delta\Delta Ct}$$

$$\Delta C_{t\ \text{sample}} - \Delta C_{t\ \text{calibrator}} = \Delta\Delta C_t$$

$$C_{t\ \text{GOI}^s} - C_{t\ \text{norm}^s} = \Delta C_{t\ \text{sample}}$$

$$C_{t\ \text{GOI}^c} - C_{t\ \text{norm}^c} = \Delta C_{t\ \text{calibrator}}$$ e.g. Time zero

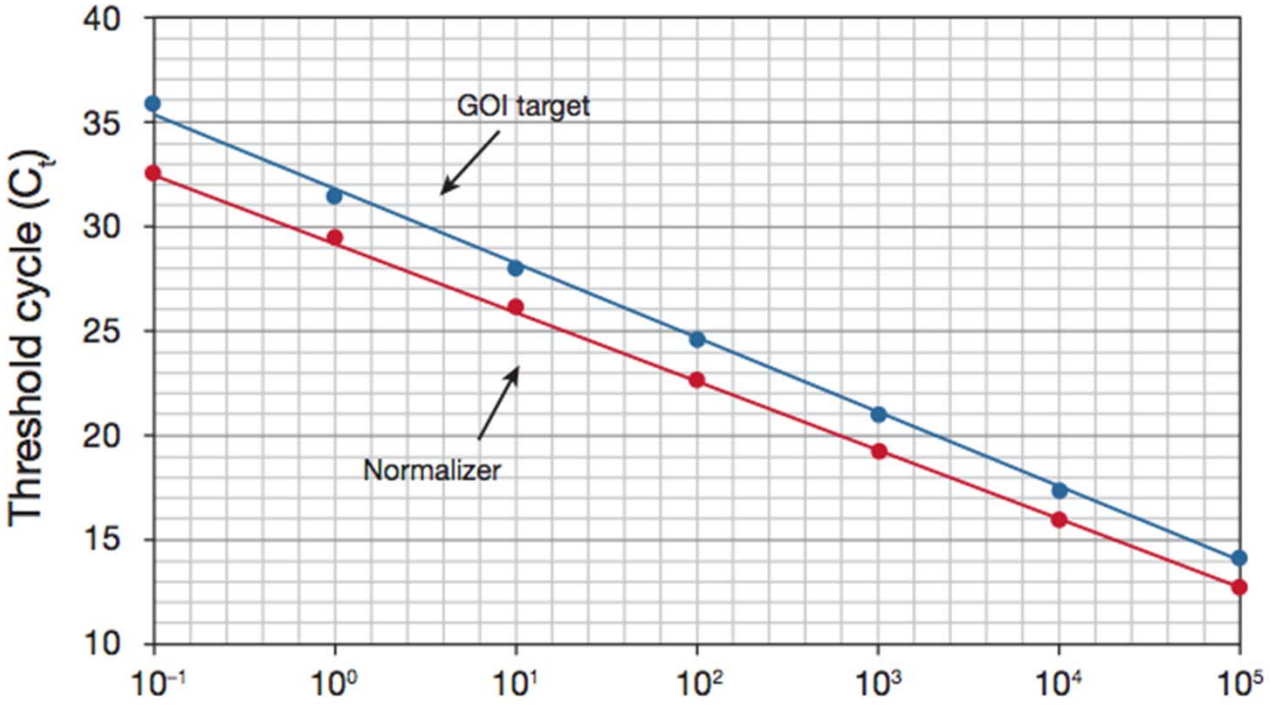
Comparative quantification – standard curve method

Fold difference = $(E_{target})^{\Delta Ct\ target} / (E_{normalizer})^{\Delta Ct\ normalizer}$

E = efficiency from standard curve $E = 10^{[-1\ /slope]}$

$\Delta Ct\ target = C_{t\ GOI\ c} - C_{t\ GOI\ s}$

$\Delta Ct\ normalizer = C_{t\ norm\ c} - C_{t\ norm\ s}$



References:

Several pdfs for this talk are available at:
http://botany.okstate.edu/resources/pcr_core.html

Another good website with loads of information:
http://www.gene-quantification.de/

Any Questions?