2nd Report due TOMORROW 5 PM

qPCR

Powerful technique to amplify + detect trace amounts mRNA

Gold standard used to be Northern Blot Hybridization

- Longer procedure
- Needs a lot of RNA

q PCR

Advantages:
- V. sensitive
- V. specific
- Reproducible
- Wide dynamic range
  - ↳ i.e can detect trace amts RNA to large amts RNA
- Easy to see both up-regulated + down-regulated expression

Major observation underpinning qPCR:

The amount of PCR product formed is directly related to the amount of target DNA (present in PCR) in a linear fashion

Amplification products are measured using a fluoresent label

We will use SYBRGreen

SYBRGreen intercalates ω dsDNA and fluoresces

∴ Fluorescence ∝ [DNA template]

qPCR unit detects fluoresence produced during a PCR run

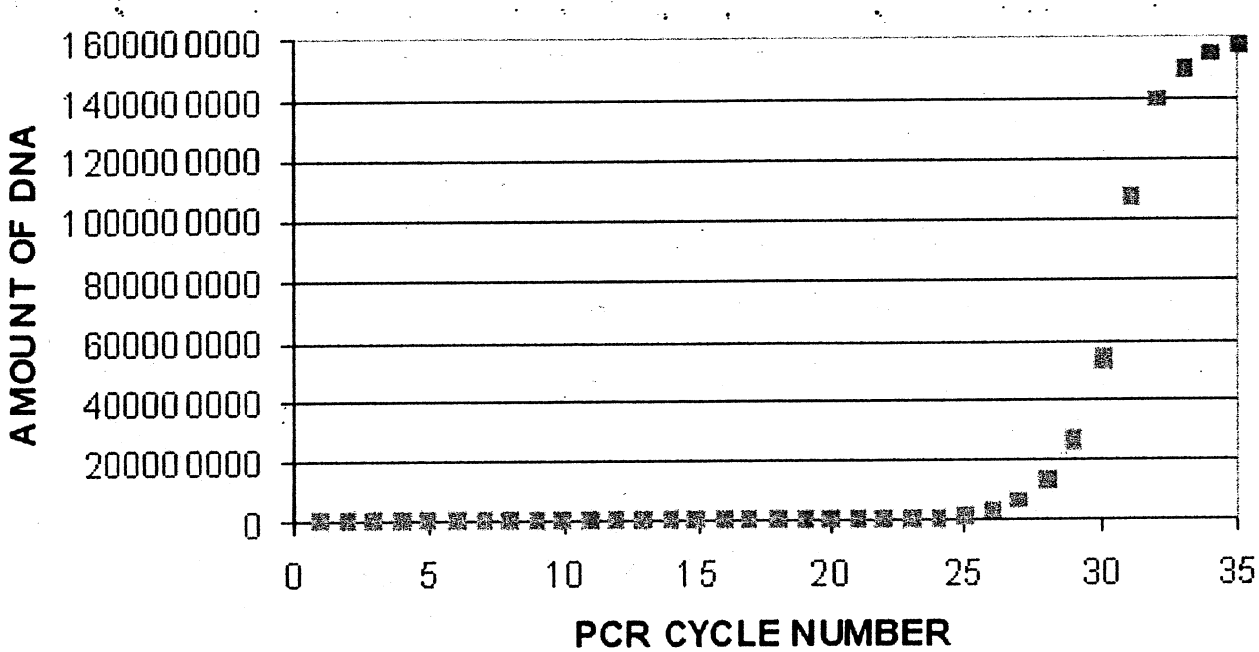
The point at which fluorescence is deteted as being significantly higher than background = Threshold cycle or C_T value

C_T valve is used to quantify the amount of target DNA

C_T is inversely correlated to log [target DNA]
i.e the higher the amount of target DNA in your sample the lower the C_T value

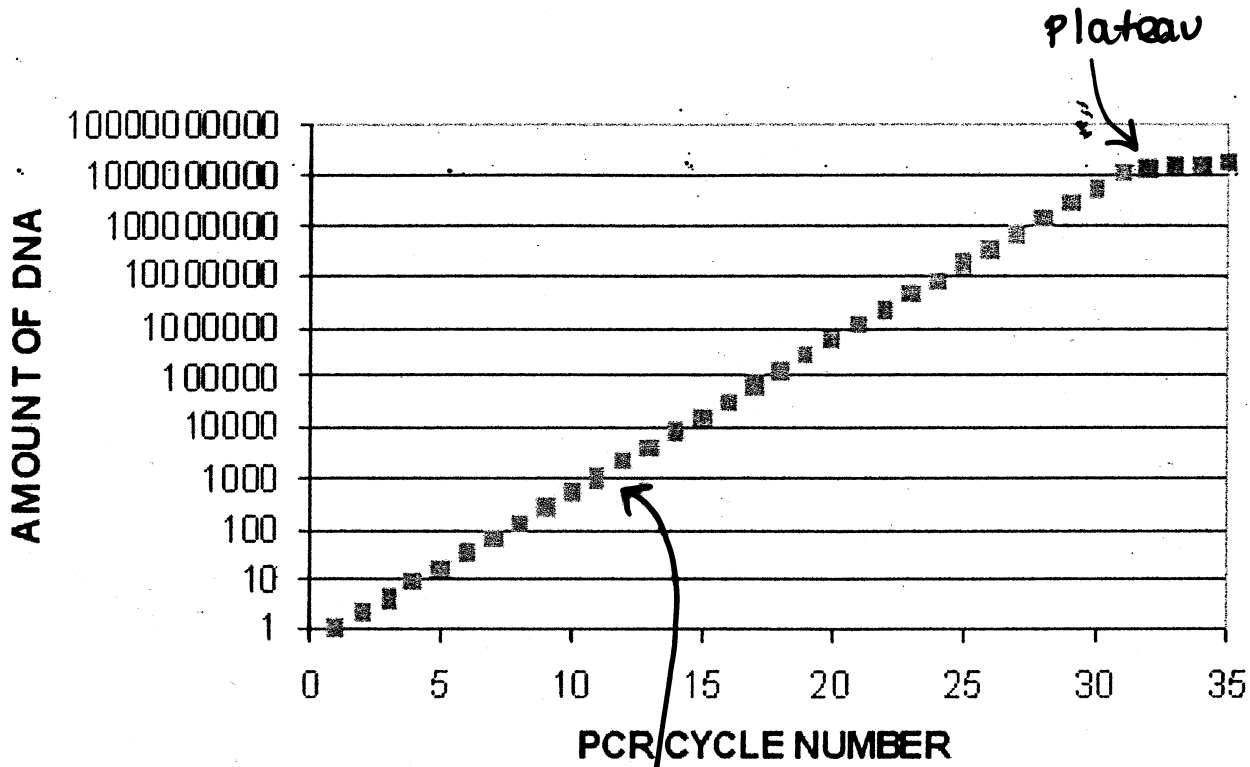
PCR amplification is exponential

Linear scale. Prod^c PCR amplification product



Images from http://pathmicro.med.sc.edu/pcr/realtime-home.htm

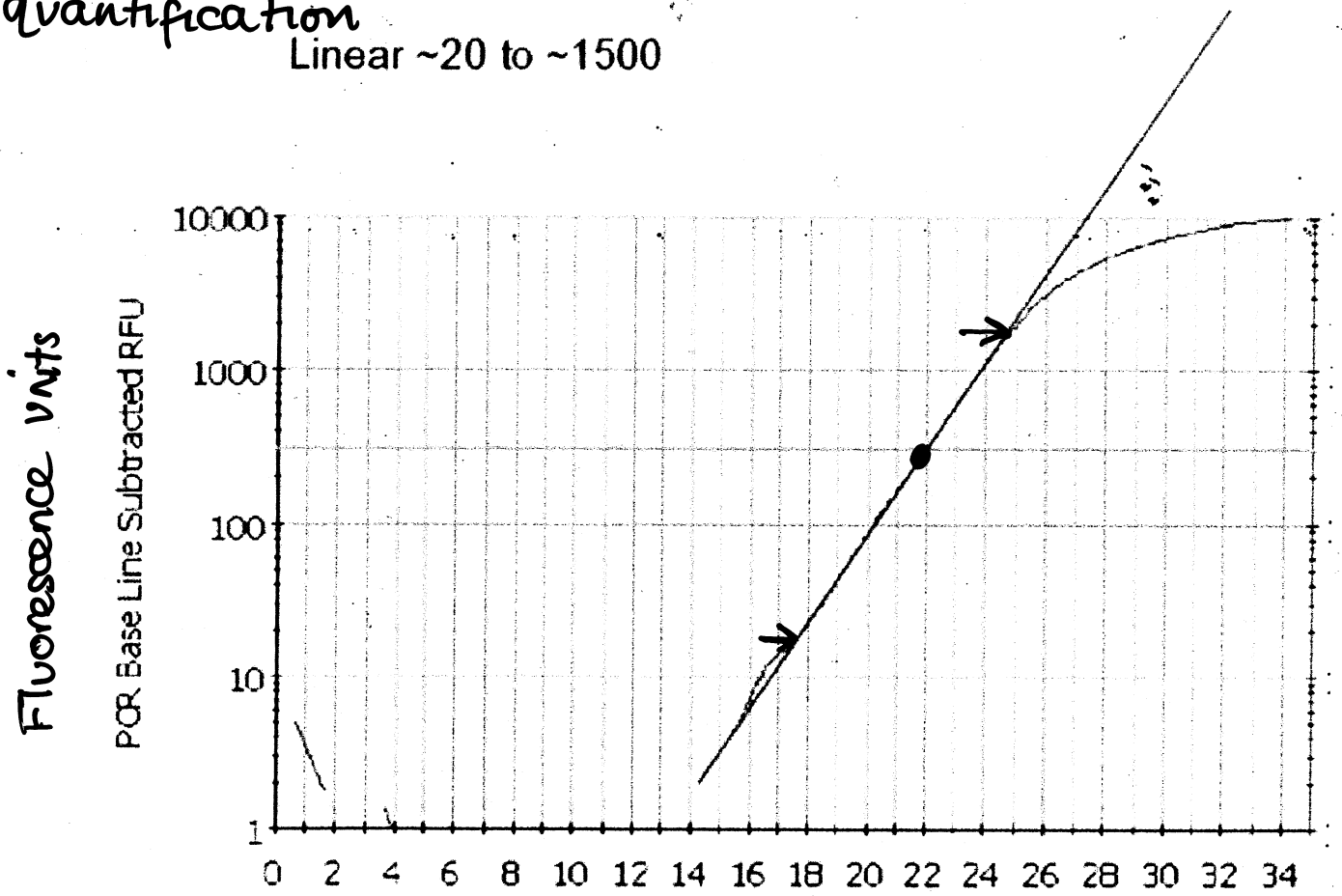
Log scale. Production amplification product



Linear relationship btwn amount target DNA and cycle #

Log scale shows that linear phase of PCR amplificat^{n} occurs between ~20 + ~1500 RFU

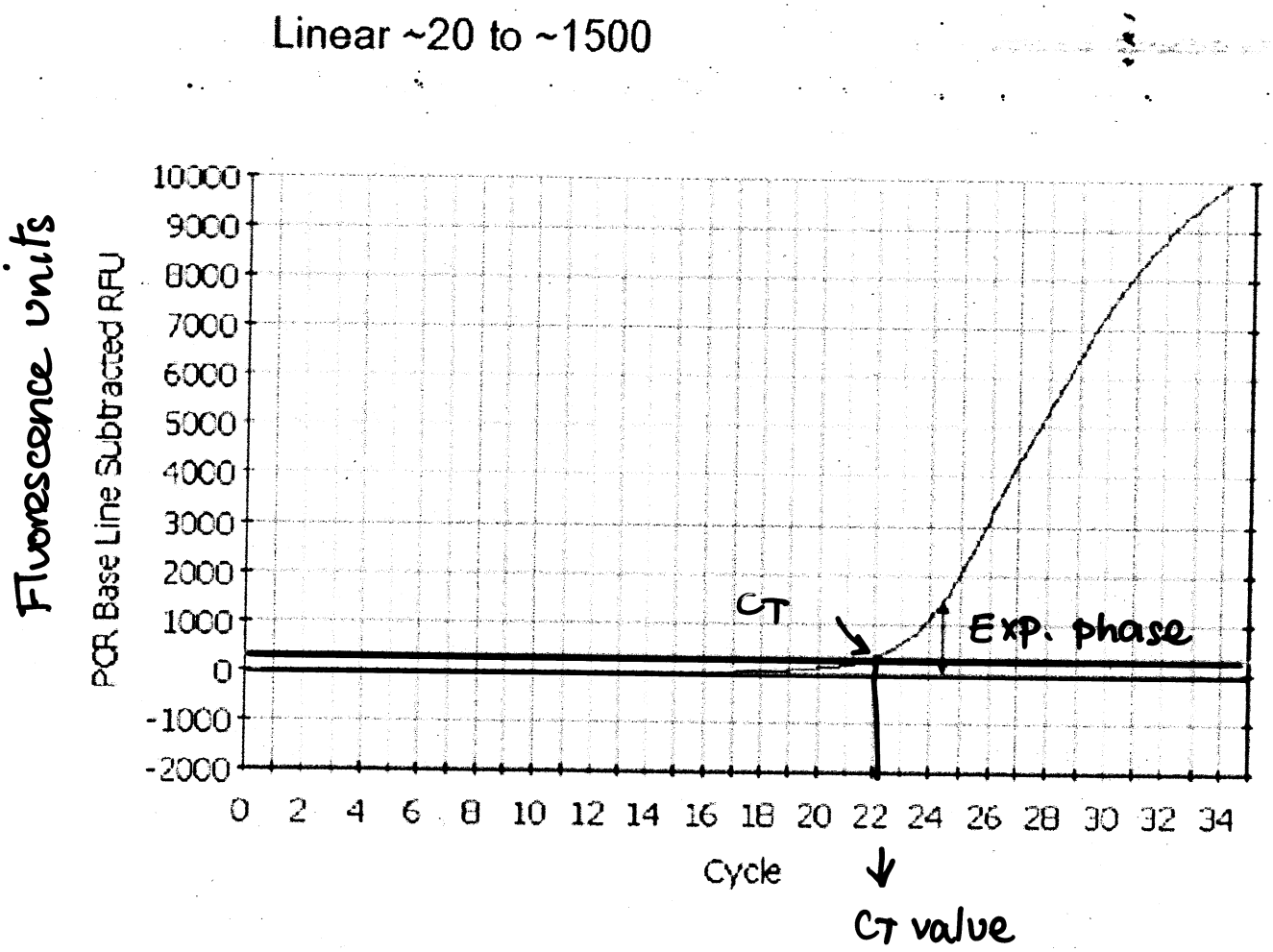
This corresponds to exponential phase + is used for quantification



Linear scale

[redacted] phase occurs at beginning of curve
Exponential

∴ C_T is set close to the bottom of the curve



C_T = cycle # at which the ↑ in fluoresence is exponential

QUANTIFICATION

C

Relative quantification determines the change in steady state mRNA levels of a gene across samples expressed relative to the level of a reference (housekeeping) gene

Based on comparison of differences in C_T

= ΔC_T methods

or

$\Delta\Delta C_T$ methods (used when comparing a treated to a non-treated sample)

Reference (housekeeping gene)

- Expression should not vary WRT treatment
- May be difficult to find
- We will use >1 but all show minimal fluctuations in expression btwn treatments

Quantification example

	Ubi (ref 1)	ef1a (ref 2)	ef1b (ref 3)	NCED3 (target)	
C_T values →	18.53	18.8	19.9	27.8	Control Roots
→	18.66	18.6	19.7	27.5	
	19.2	19.4	19.2	23.6	drought * Roots
	18.9	19.5	18.9	24.1	

Calc. geometric mean for reference genes!

Control 19.2

Drought 19

Relative gene expression $= 2^{*\,[\Delta C_T \text{ drought} - \Delta C_T \text{ control}]}$

$= 2^{\Delta\Delta C_T}$

ΔC_T target $- \Delta C_T$ ref $= \Delta C_T$ Control

$= 27.7 - 19.2$

$= 8.5$

* **Assumes 100% efficiency PCR**

ΔC_T target − ΔC_T ref = ΔC_T drought

= 23.4 − 19

= 4.4

ΔC_T control − ΔC_T drought = 8.2 − 4.4

$\Delta\Delta C_T$ = 3.8

$2^{-\Delta\Delta C_T}$ = $2^{-3.8}$ = 13.93

∴ NCED3 was up-regulated 13.93 fold in roots responding to drought stress.