Theoretical course: Basic biochemical methods and ischemic heart models

The polymerase chain reaction

Collected by Ernő Zádor

PhD.

2011

Supported by: HURO/0901/069/2.3.1
HU-RO-DOCS

Definition and developer

- The polymerase chain reaction (PCR) is a molecular biology technique to amplify a single or a few copies of a piece of DNA up to several orders of magnitude ($10^{11\text{-}12}$ copies) of a particular DNA sequence.
- This automated process bypasses the need to use bacteria for amplifying DNA.
- Nobel Prize in Chemistry (1993) to the developers, Kary Mullis along with Michael Smith

The procedure

- Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps (usually three)
- The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90), and followed by one hold at the end for final product extension (~72°C) or brief storage (~4°C).

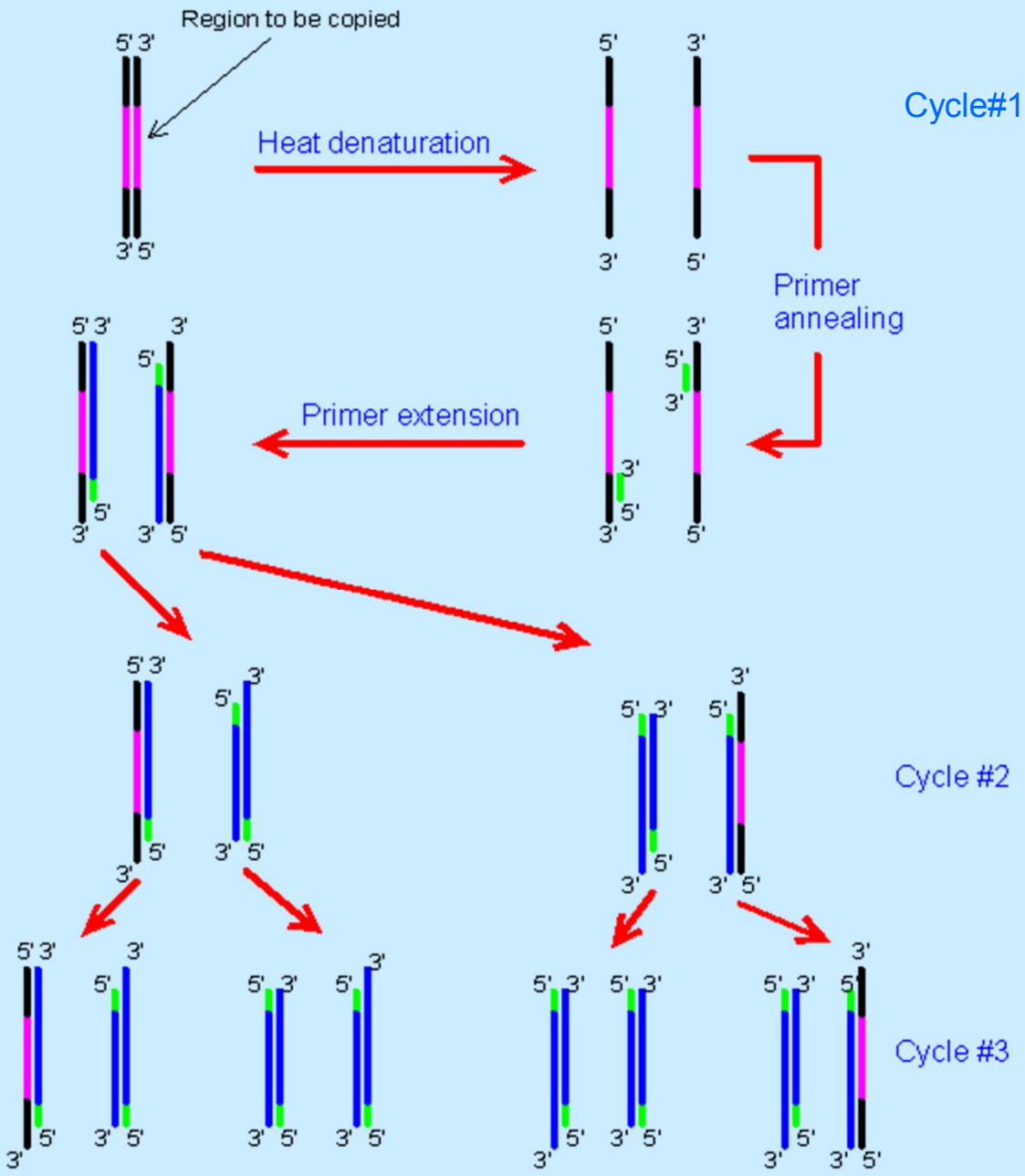
The scheme of a cycle and a 3-step-PCR

- One cycle - three temperature shifts:

1.) Denaturation at 94-96 °C

2.) Primer annealing at 45-60 °C (dependig on the primer)

3.) Primer extension (ussually) at 72 °C



The temperature

- The temperatures used and the length of time applied in each cycle depend on a variety of parameters, i.e. the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers
- The melting temperature (*Tm*) is defined as the temperature at which half of the DNA strands are in the double-helical state and half are in the random coil states
- Being related to hydrogene bounds, the melting temperature depends on both the length, and the specific nucleotide sequence composition of the primer.

Steps of the cycle

- *Initialization step*: consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR

Steps of the cycle (2)

- *Denaturation step*: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Steps of the cycle (3)

- *Annealing step*: The reaction temperature is lowered to 50–65 °C and held for 20–60 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 C^{o} below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Steps of the cycle (4)

- *Extension/elongation step*: The temperature depends on the DNA polymerase used; Taq polymerase has its optimum activity at 75–80 °C, and commonly a 72 °C is used with this enzyme. The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Steps of the cycles (5-6)

- *Final elongation*: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- *Final hold*: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Principles and procedure

- Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb) (some techniques up to 40 kb)
- A basic PCR set up requires several components and reagents in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes)

The components of a PCR reaction

1.) DNA template that contains the DNA region (target) to be amplified

2.)Two *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target

3.) Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C

The components of a PCR reaction (cont.)

4.) *Deoxynucleoside triphosphates* (the four nucleotides containing triphosphate groups; dNTPs), the building-blocks from which the DNA polymerase synthesizes a new DNA strand

5.) *Buffer solution*, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

6.) *Monovalent cation* potassium ions.

The components of a PCR reaction (cont.)

7.) *Divalent cations*, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis

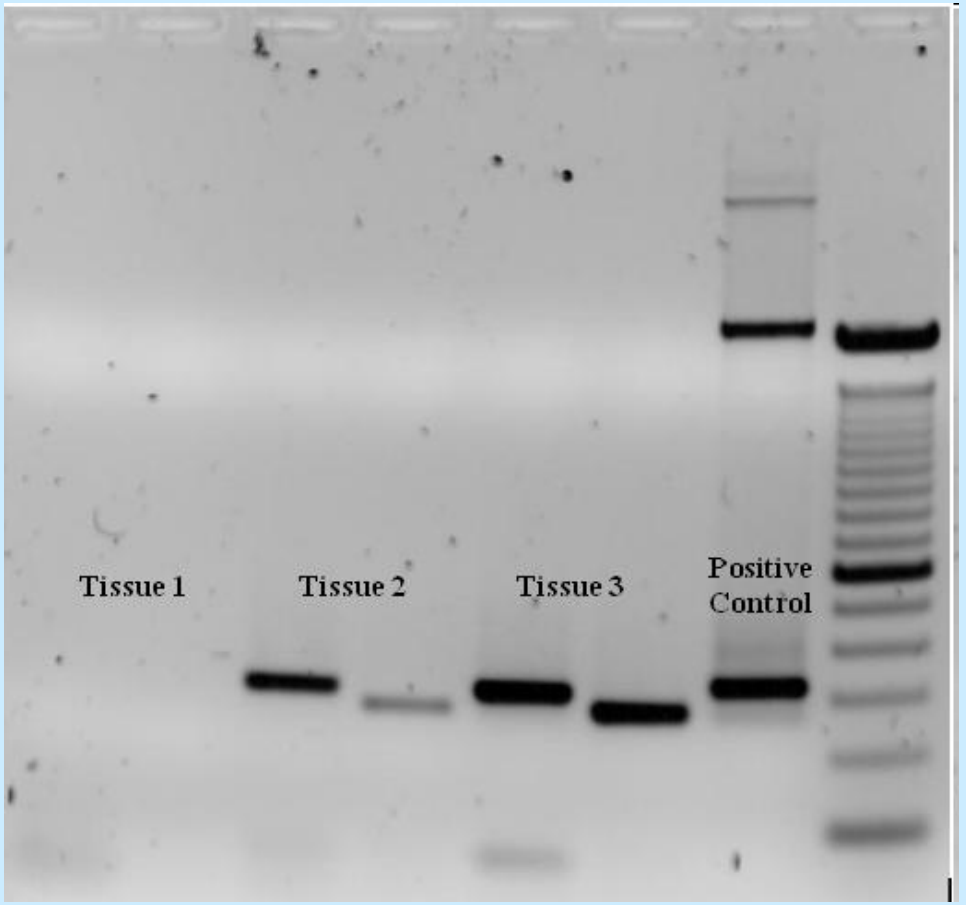
- The reaction is set up in a thin walled PCR tube permit favorable thermal conductivity to allow for rapid thermal equilibration in a thermal cycler

The thermal cycler

- The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction
- Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current.
- Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube
- Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

Checking fragment identity

- To control whether the PCR generated the expected DNA fragment, 1-2% agarose or 6% acrylamide gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a lenght marker, several DNA fragments of known size) run on the same gel alongside the PCR products



Expression of a specific gene in three different tissues was tested by two separate sets of primers. As the gel shows, Tissue1 lacks that gene, whereas Tissue2 and 3 expresses it. A positive control shows that the PCR conditions were adequate and a 1kb ladder refers to the size of the bands.

The magnitute of amplification with PCR

- An optimalised reaction in 50 μl volume might well produce 0.2 μg of 150 bp DNA from one hundred template molecules after 35-40 cycles (even with poor efficiency)
- The molar weight of this fragment (taking 330 daltons per nucleotide and 660 Da for an average base pair) is 99 000 Da
- There are $6x10^{23}$ molecules (Avogadro number) in 99 000 g of the fragment
- So 0.2 μg ($2x10^{-7}$ g) fragment has $9.9x10^{4}$ / $2x10^{-7}$ = $4.95x\ 10^{11}$ times less than $6x10^{23}$ molecules, which is identical with $1.21x\ 10^{12}$ molecules
- Therefore the amplification from 10^{2} to 10^{12} is $x10^{10}$ - 10 billion-fold

Extreme applications

- PCR can be used for forensic analysis, when only a trace amount of DNA is available as evidence.
- PCR-based techniques have been successfully used to analyse ancient DNA (tens of thousands of years old), such as a forty-thousand-year-old mammoth
- Also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of family tree of a Russian tsar.

Basic PCR techniques

- *Allele-specific PCR*: a diagnostic or cloning technique based on single-nucleotide polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer indicates presence of the specific SNP in a sequence.

Basic PCR techniques

- *Assembly PCR* or *Polymerase Cycling Assembly (PCA)*: artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product

Basic PCR techniques

- *Asymmetric PCR*: preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as *L*inear-*A*fter-*T*he-*E*xponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Basic PCR techniques

- *Helicase-dependent amplification*: similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

Basic PCR techniques

- *Hot start PCR*: a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

Basic PCR techniques

- *Intersequence-specific PCR* (ISSR): a PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths
- *Inverse PCR*: is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence.

Basic PCR techniques

- *Ligation-mediated PCR*: uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA footprinting
- *Methylation-specific PCR* (MSP): it detects methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

Basic PCR techniques

- *Miniprimer PCR*: uses a thermostable polymerase (S-Tbr) that can extend from short primers ("smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene
- *Multiplex Ligation-dependent Probe Amplification* (*MLPA*): permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR (see below).

Basic PCR techniques

- *Multiplex-PCR*: consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

Basic PCR techniques

- *Nested PCR*: increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

Basic PCR techniques

- *Overlap-extension PCR* or *Splicing by overlap extension (SOE)* : a genetic engineering technique that is used to splice together two or more DNA fragments that contain complementary sequences. It is used to join DNA pieces containing genes, regulatory sequences, or mutations; the technique enables creation of specific and long DNA constructs.

Basic PCR techniques

- *Quantitative PCR (Q-PCR)*: used to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. *Quantitative real-time PCR* has a very high degree of precision. QRT-PCR (or QF-PCR) methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (*R*eal *T*ime PCR) or RQ-PCR. QRT-PCR or RTQ-PCR are more appropriate contractions, since RT-PCR commonly refers to reverse transcription PCR (see below), often used in conjunction with Q-PCR.

Basic PCR techniques

- *R*everse *T*ranscription PCR (RT-PCR): for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (*Rapid Amplification of cDNA Ends*).

Basic PCR techniques

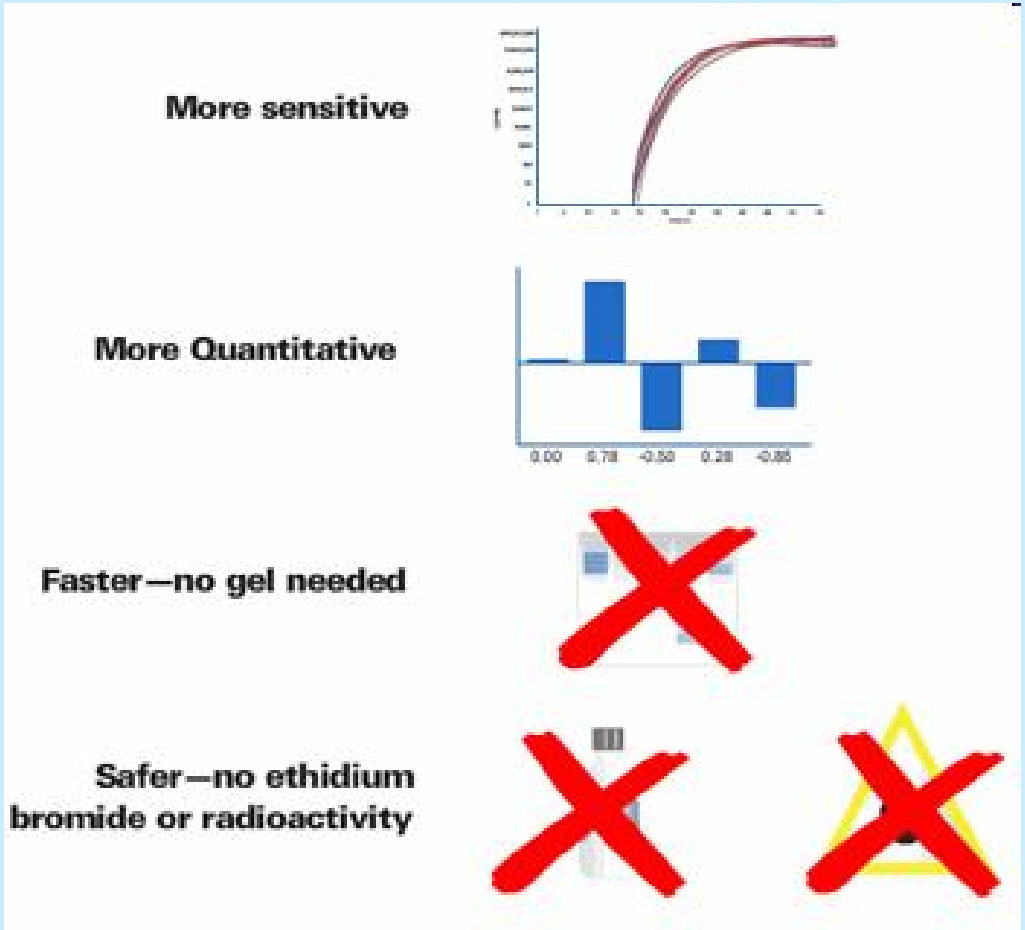
- *Solid Phase PCR*: encompasses multiple meanings, including Polony Amplification (where PCR colonies are derived in a gel matrix, for example), Bridge PCR (primers are covalently linked to a solid-support surface), conventional Solid Phase PCR (where Asymmetric PCR is applied in the presence of solid support bearing primer with sequence matching one of the aqueous primers) and Enhanced Solid Phase PCR (where conventional Solid Phase PCR can be improved by employing high Tm and nested solid support primer with optional application of a thermal 'step' to favour solid support priming).

- *Thermal asymmetric interlaced PCR (TAIL-PCR)*: for isolation of an unknown sequence flanking a known sequence. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.
- *Touchdown PCR* (*Step-down PCR*): a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the Tm of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer Tm. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.

Basic PCR techniques

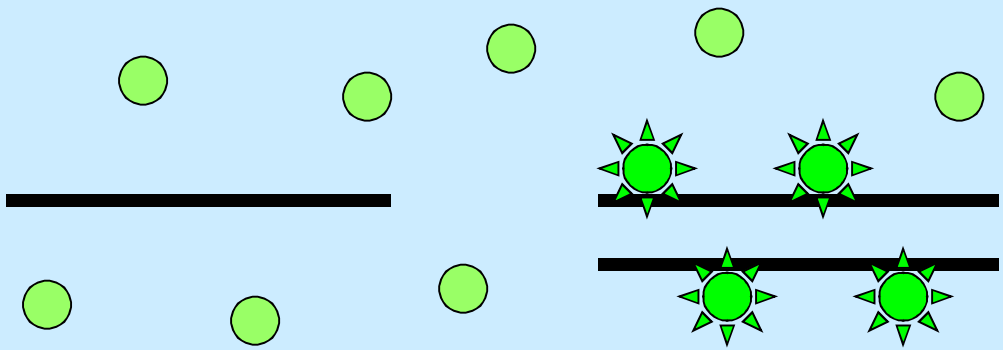
- *PAN-AC*: uses isothermal conditions for amplification, and may be used in living cells
- *Universal Fast Walking*: for genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional 'one-sided' approaches (using only one gene-specific primer and one general primer — which can lead to artefactual 'noise') by virtue of a mechanism involving lariat structure formation. Streamlined derivatives of UFW are LaNe RAGE (lariat-dependent nested PCR for rapid amplification of genomic DNA ends), 5'RACE LaNe and 3'RACE LaNe

Digital real time vs. Traditional PCR

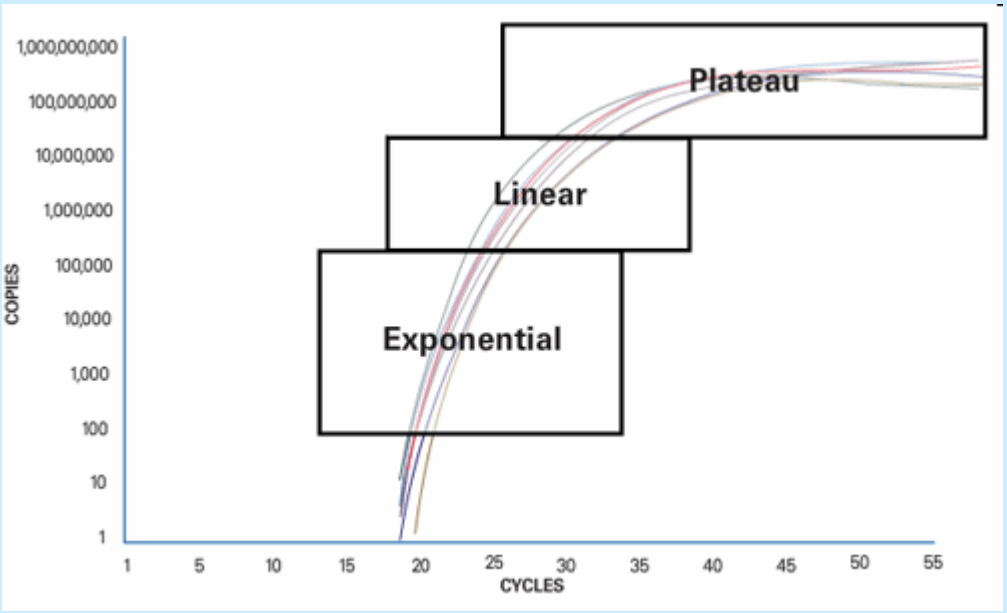


REAL TIME PCR

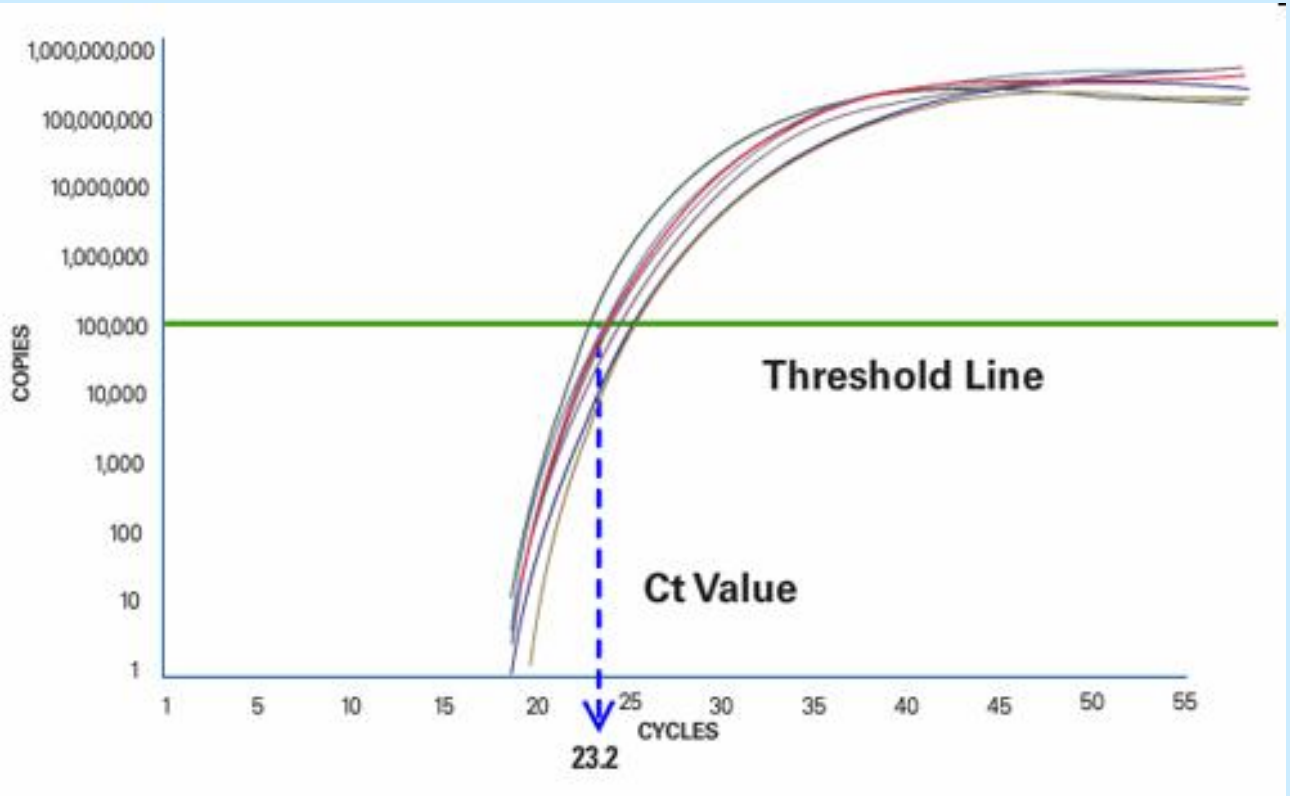
USING SYBR GREEN

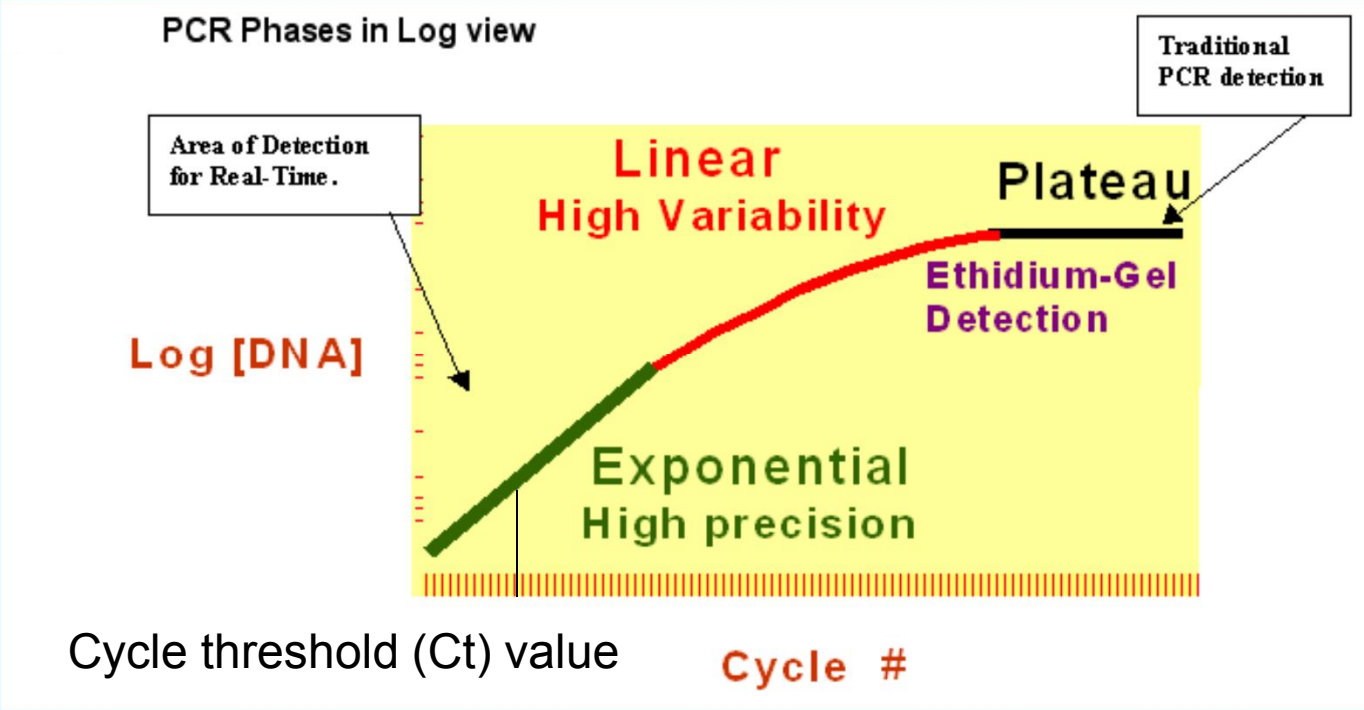


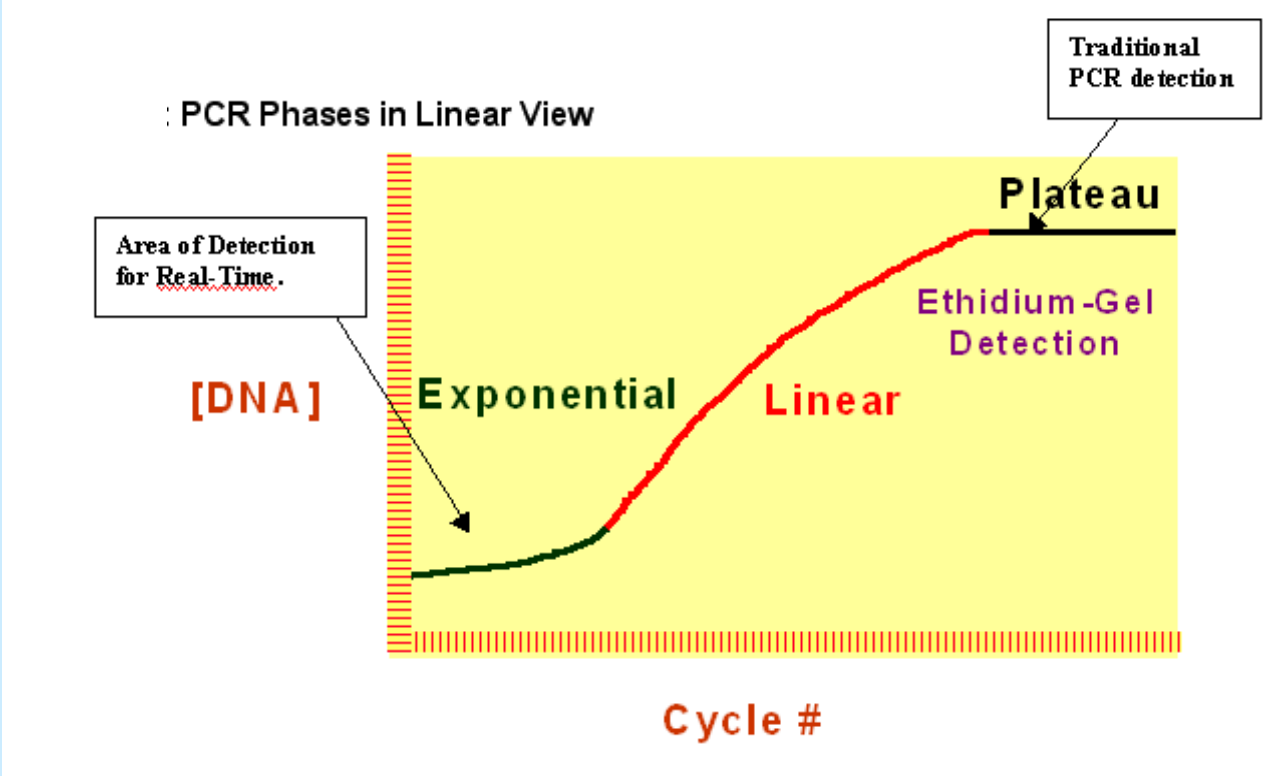
The different phases of amplification

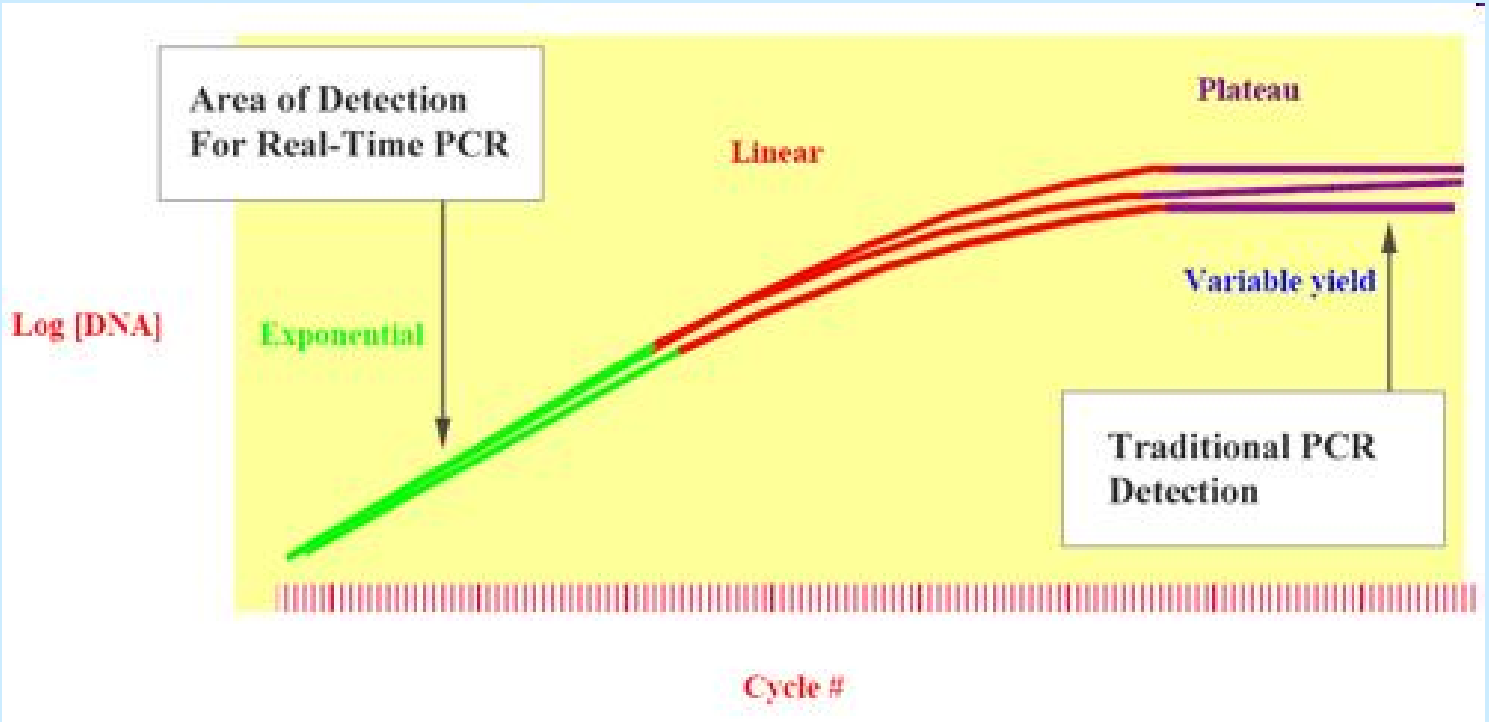


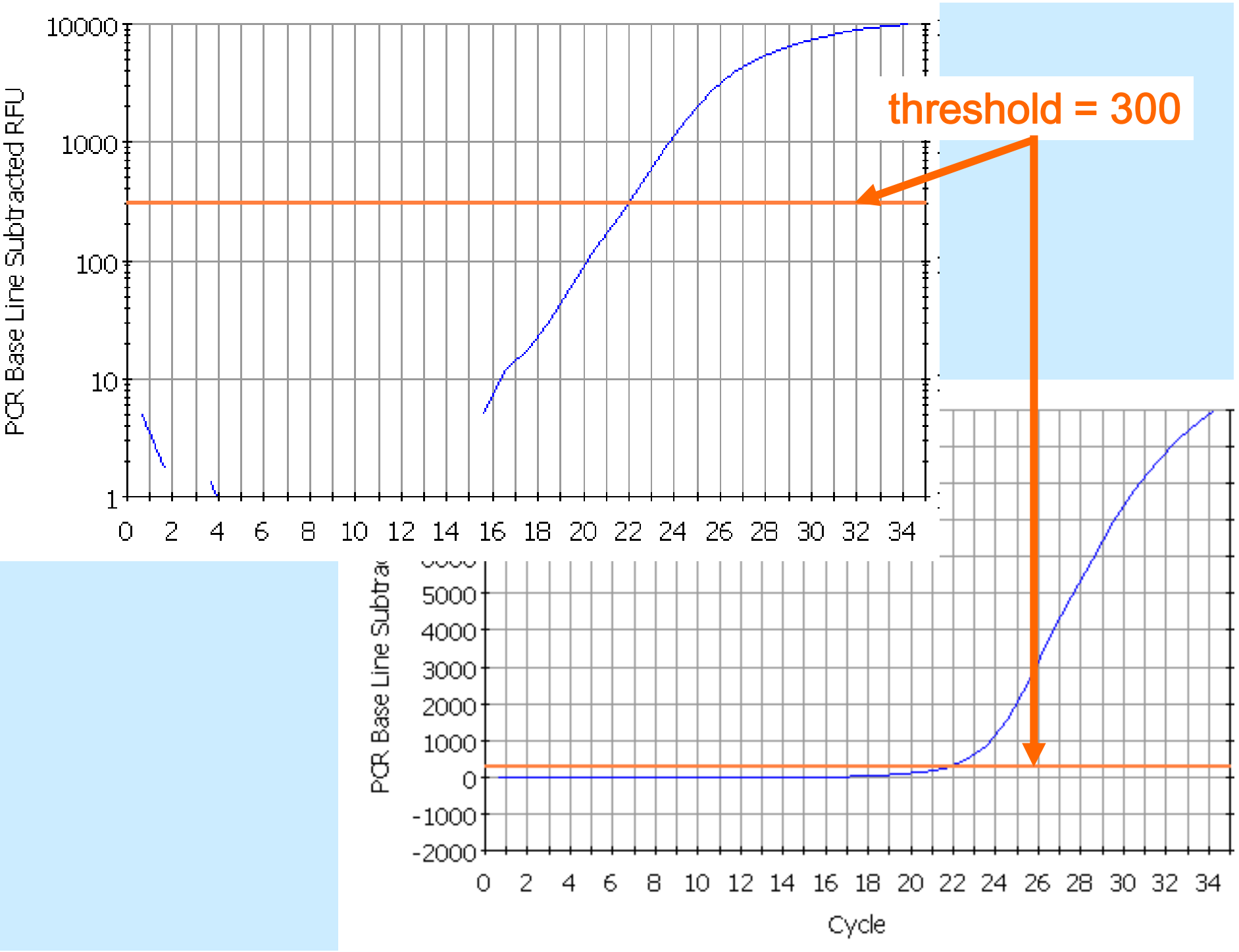
The level of detection



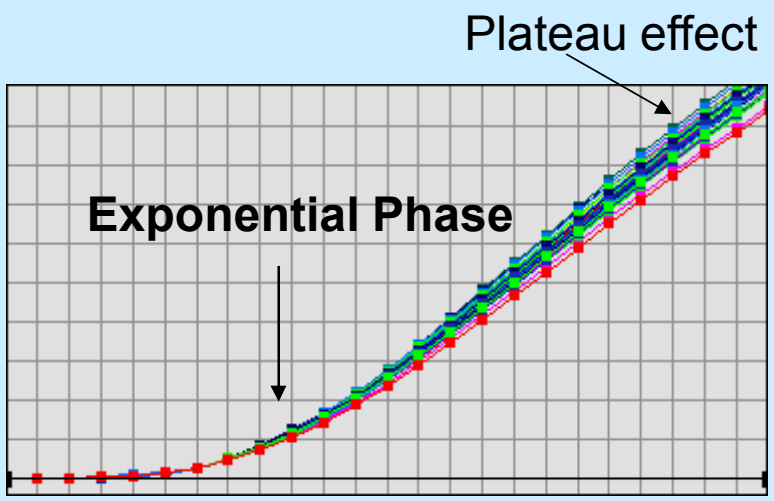

PCR Phases in Log view
Traditional PCR detection
Area of Detection for Real-Time.
Linear
High Variability
Plateau
Ethidium-Gel Detection
Log [DNA]
Exponential
High precision
Cycle threshold (Ct) value
Cycle #


PCR Phases in Linear View
Traditional PCR detection
Plateau
Area of Detection for Real-Time.
Ethidium-Gel Detection
[DNA]
Exponential
Linear
Cycle #

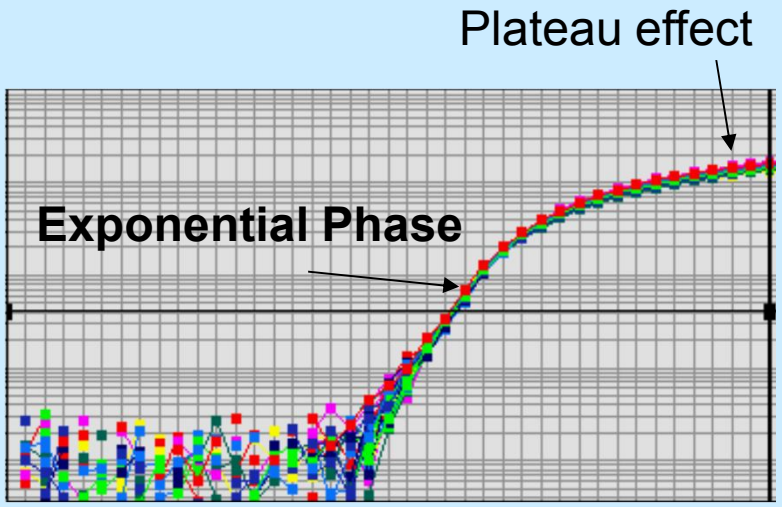

Area of Detection
For Real-Time PCR
Plateau
Linear
Variable yield
Log [DNA]
Exponential
Traditional PCR
Detection
Cycle #


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
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

Linear view 96 replicates

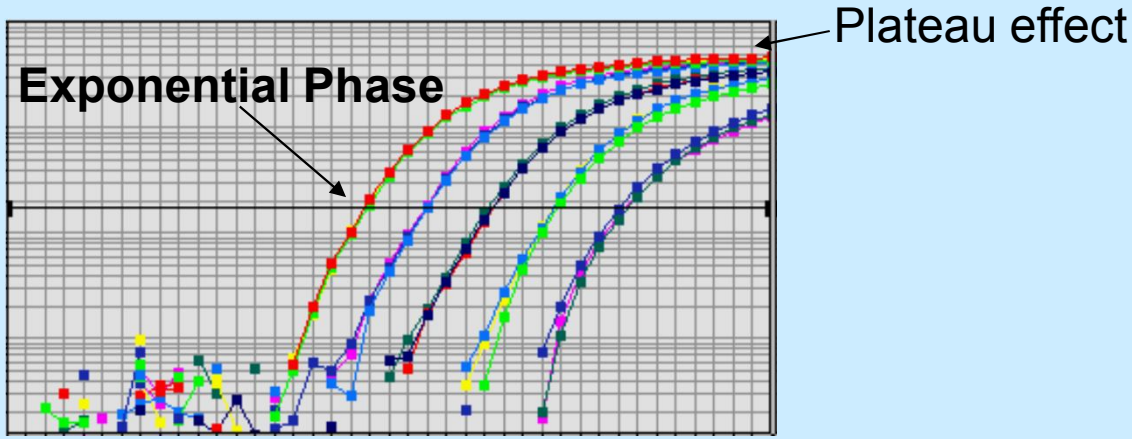


Log view 96 replicates

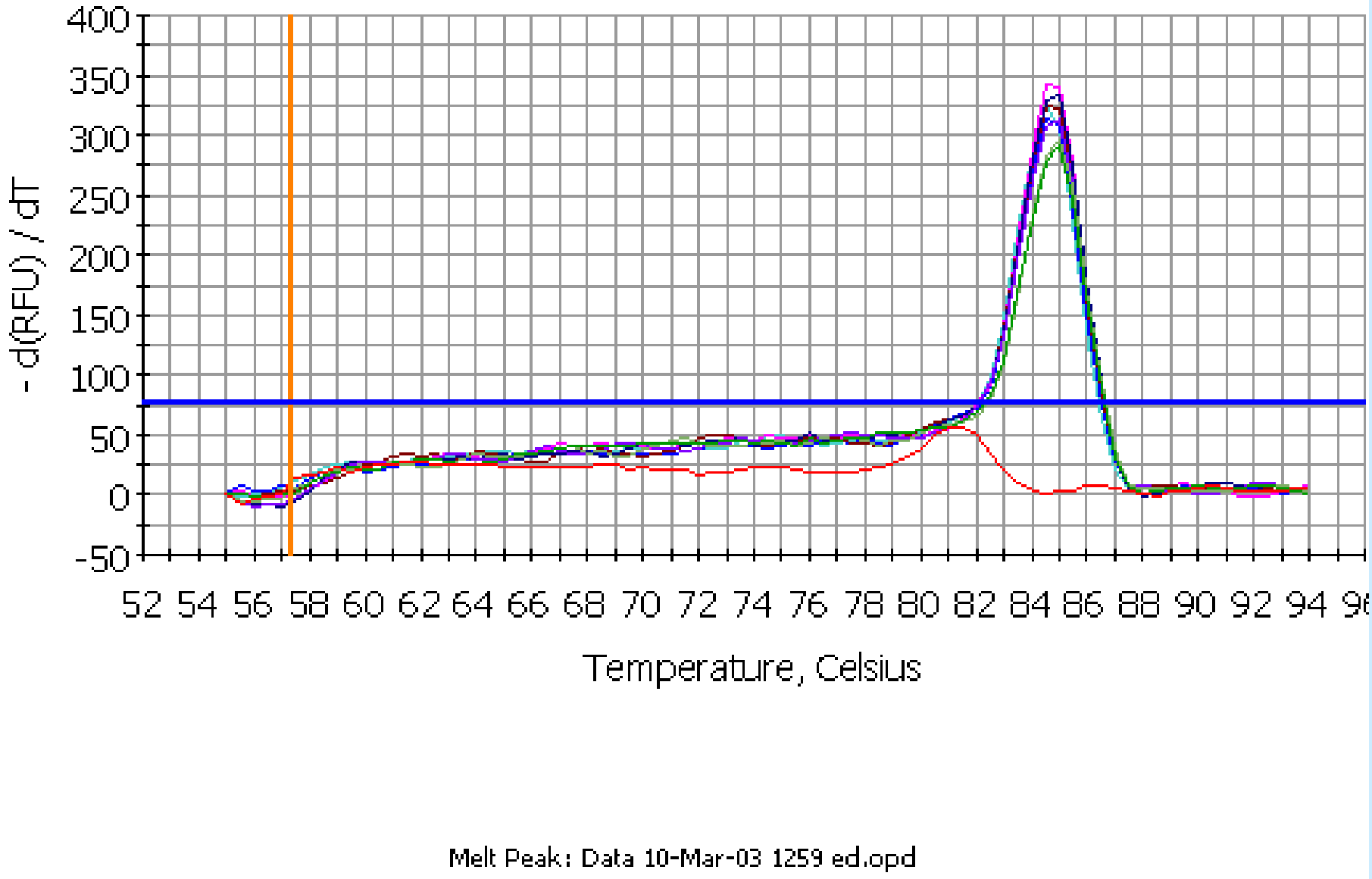


Difference between the exponential phase and the plateau phase can be seen clearly only in the log view. The horizontal line signs Ct.

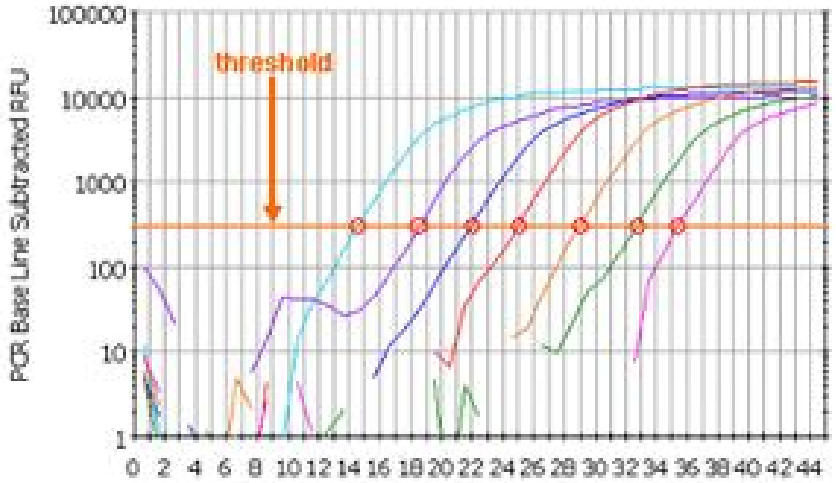
Log view 5-fold dilution series

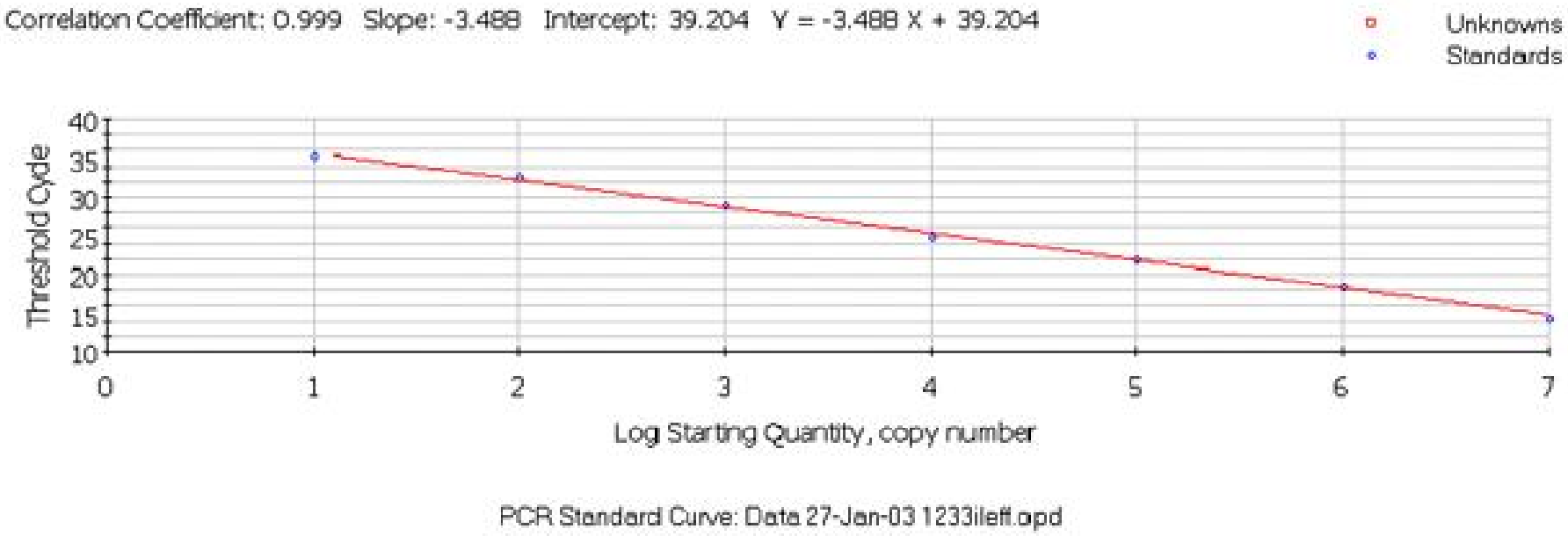


Difference between the dilution can be measured only in the exp. (log.) phase



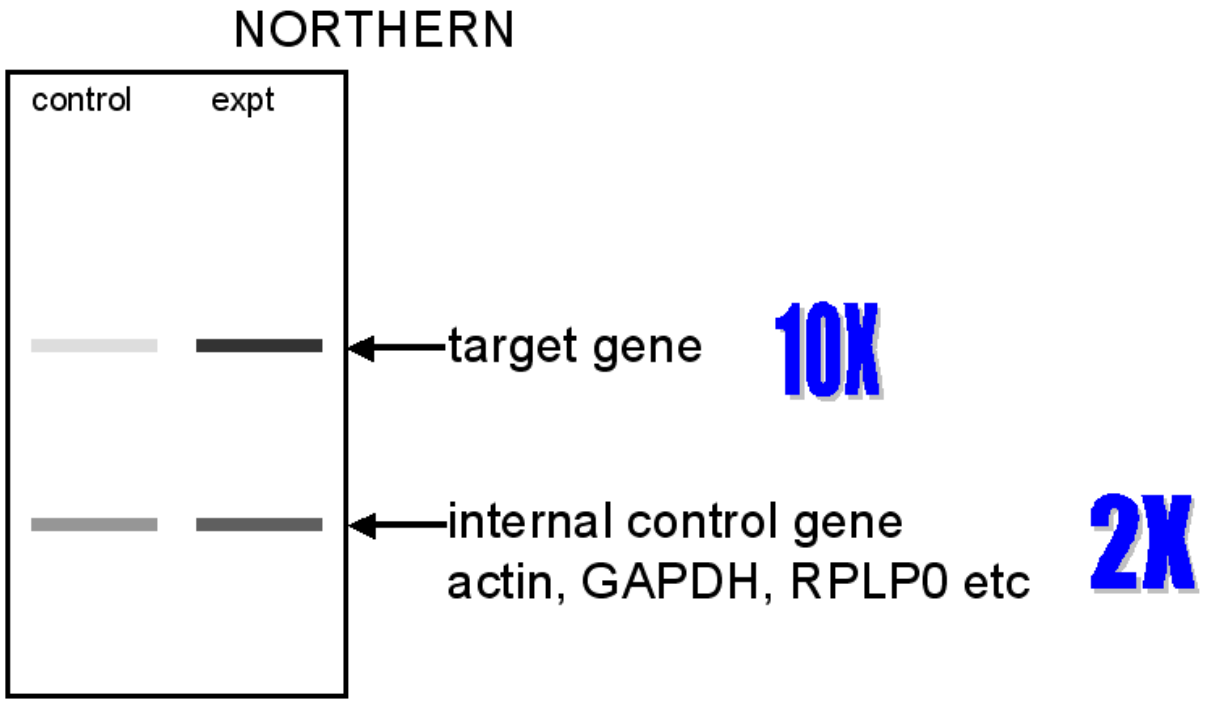
Melting curve analysis – the red line displays lower melting curve due to primer dimers because there was probably no DNA in the tube.





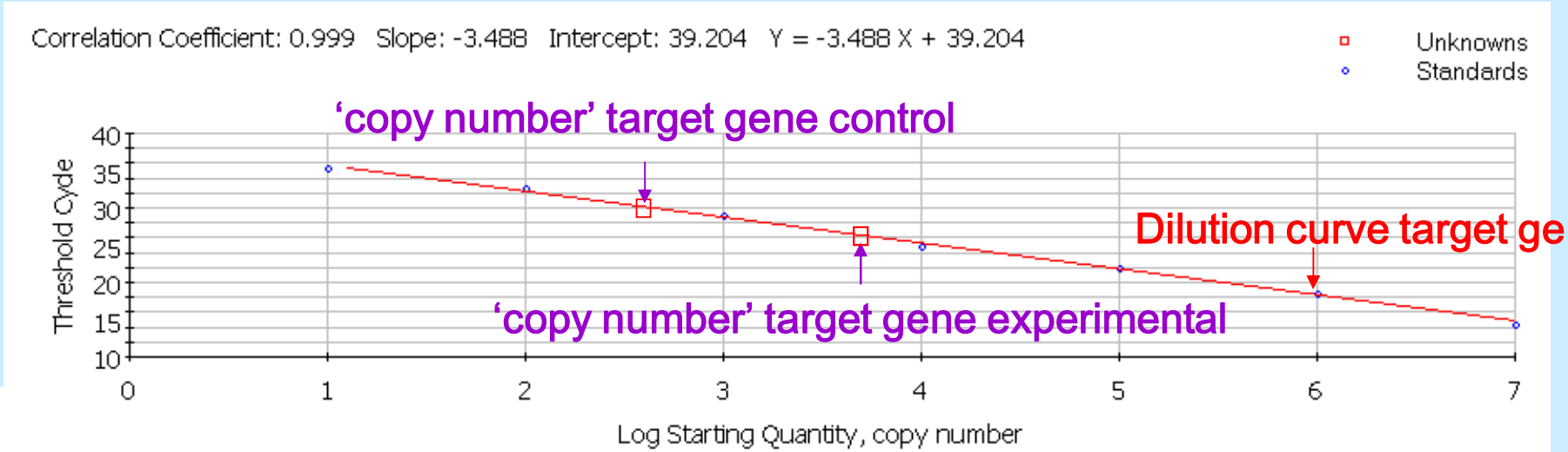
We can plot the Ct values for the dilutions against concentration - the result is a linear graph. It should have an excellent correlation coefficient (certainly more than 0.990). The slope of this graph is a measure of efficiency, and can be readily used to calculate efficiency – *source: Margaret Hunt: Real time PCR*

Normalisation to an internal control (the Northern equation)

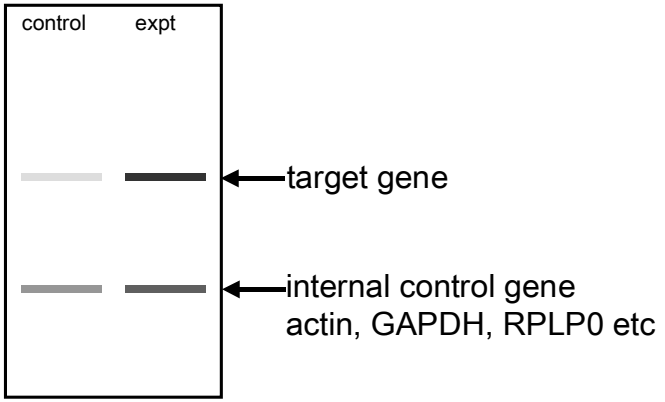


Corrected fold increase = 10/2 = 5

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

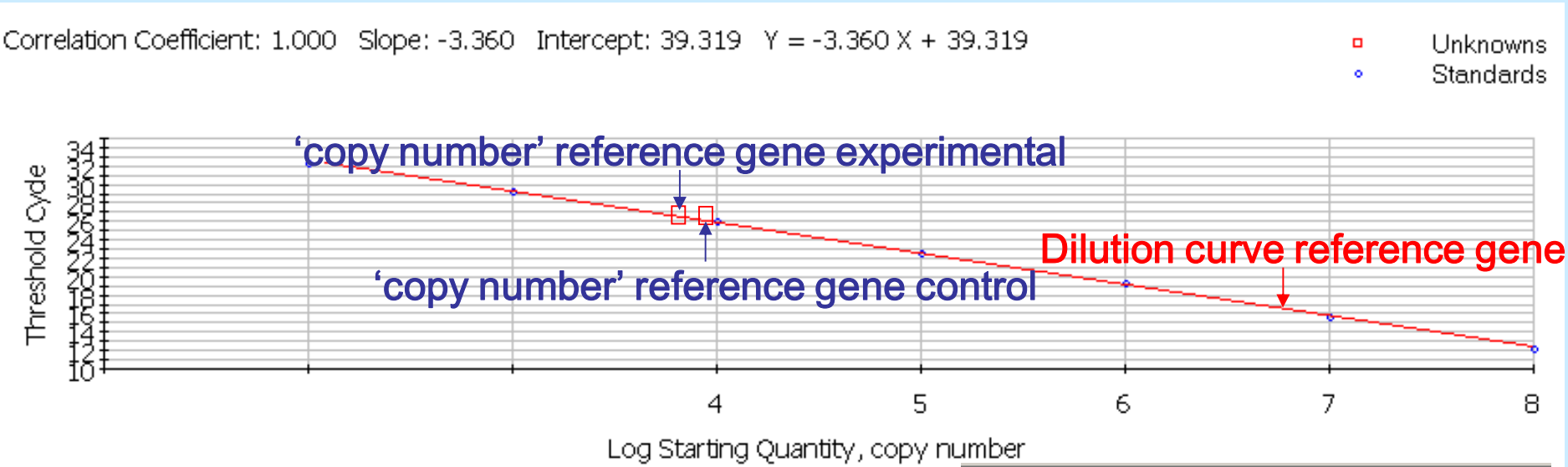


NORTHERN

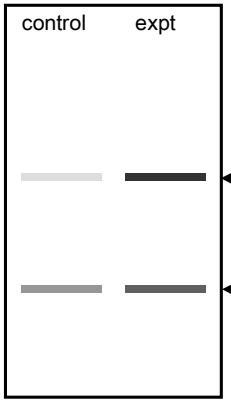


Ratio experimental/control = $\frac{\text{fold change in target gene}}{\text{fold change in reference gene}}$

fold change in target gene= $\frac{\text{copy number experimental}}{\text{copy number control}}$



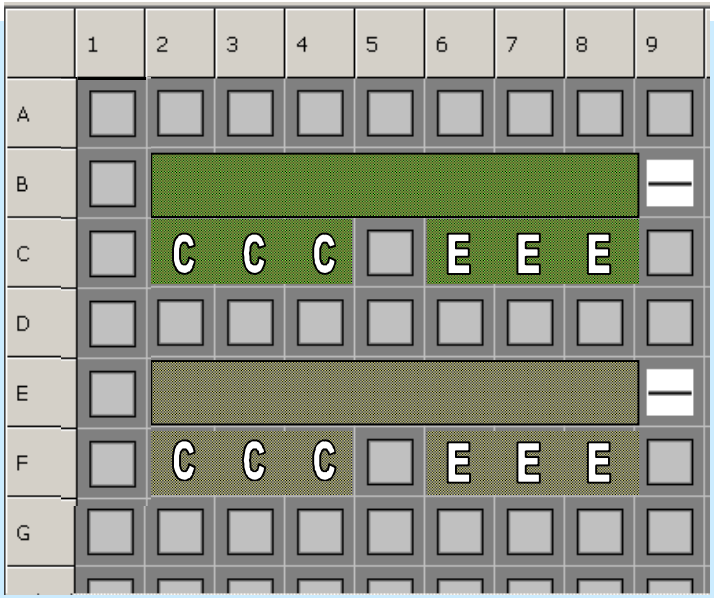
NORTHERN



target gene

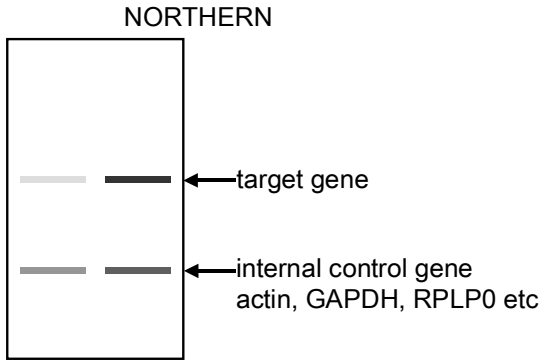
internal control gene
actin, GAPDH, RPLP0 etc

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

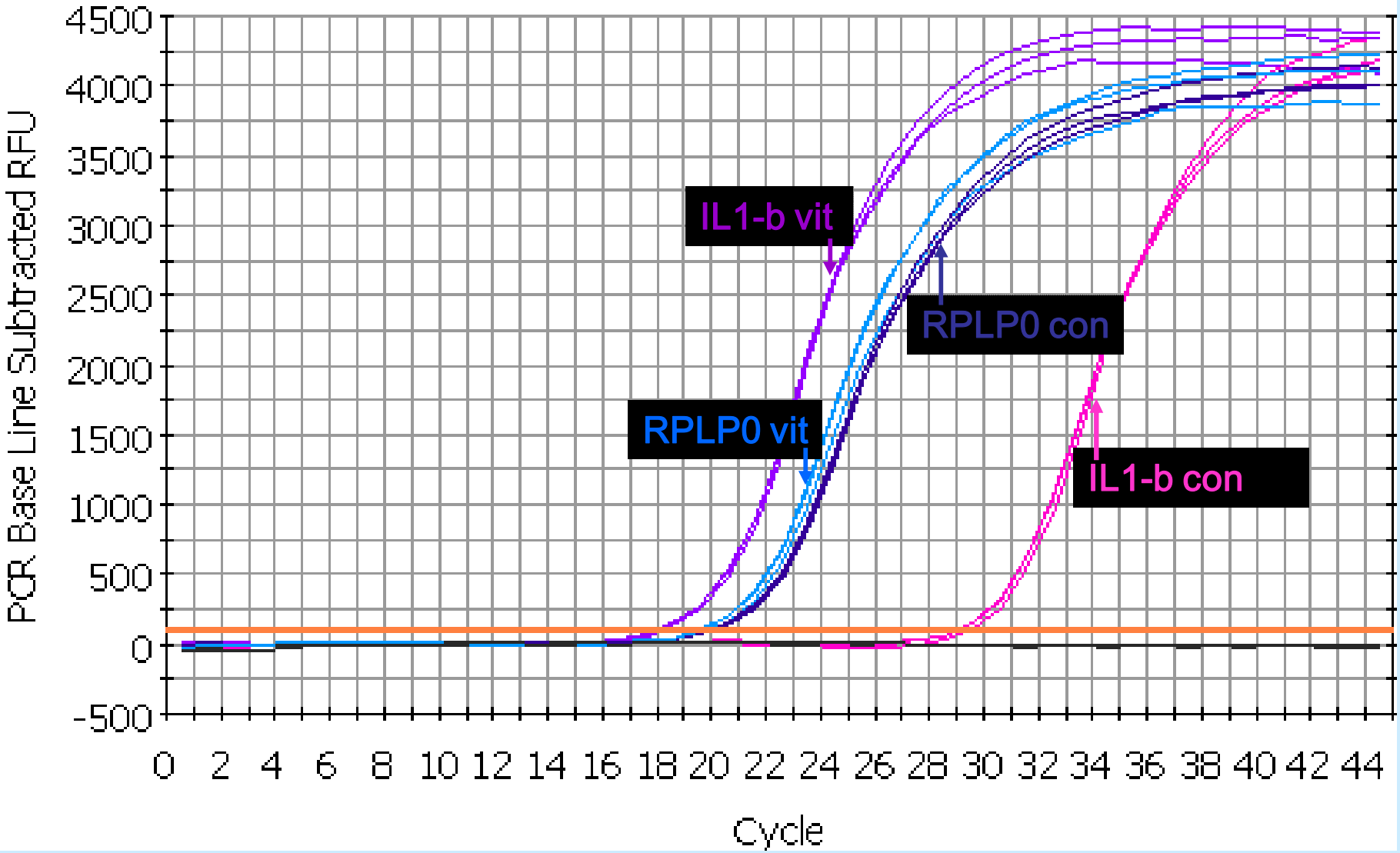


PFAFFL METHOD

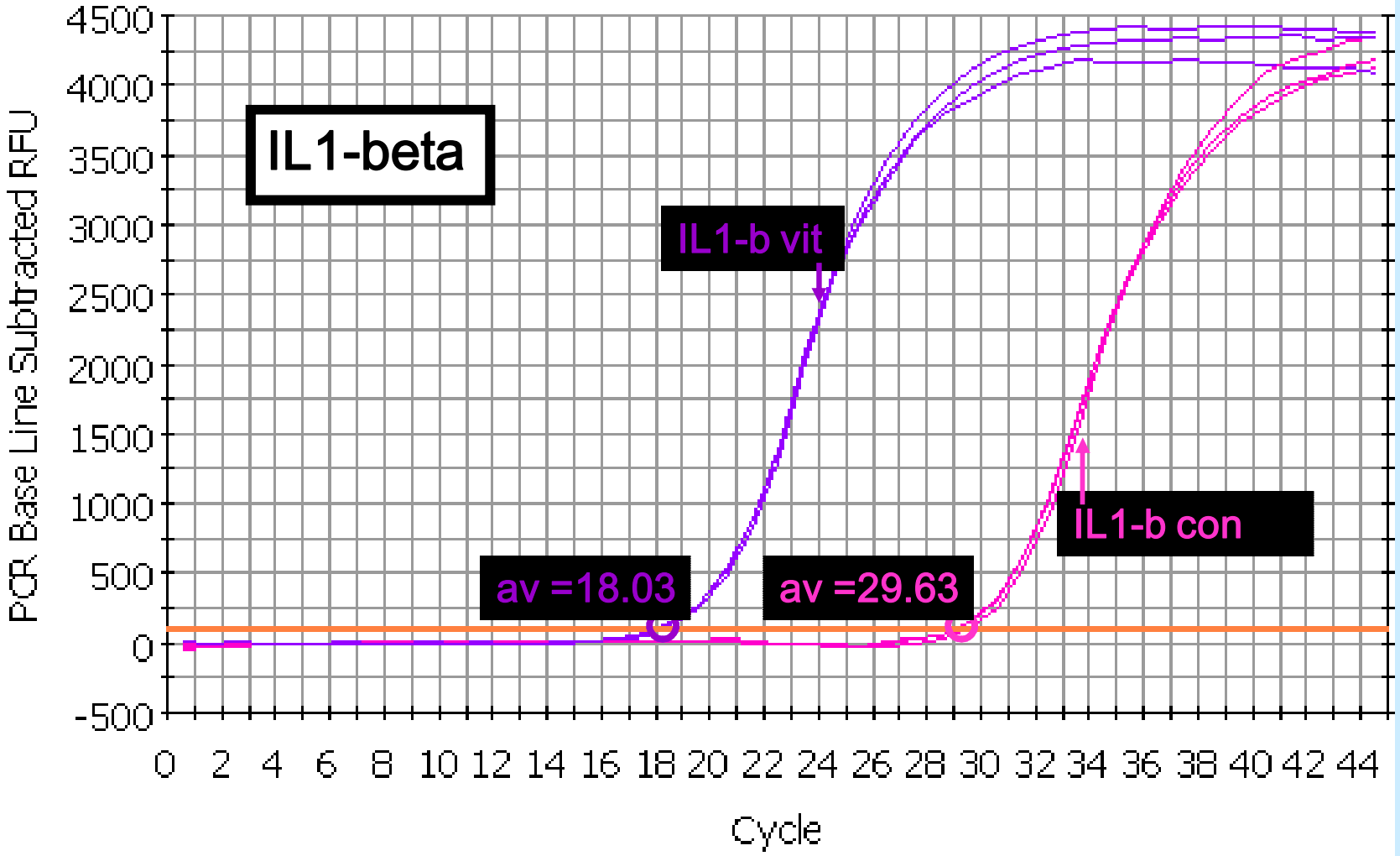
M.W. Pfaffl, Nucleic Acids Research
2001 29:2002-2007



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



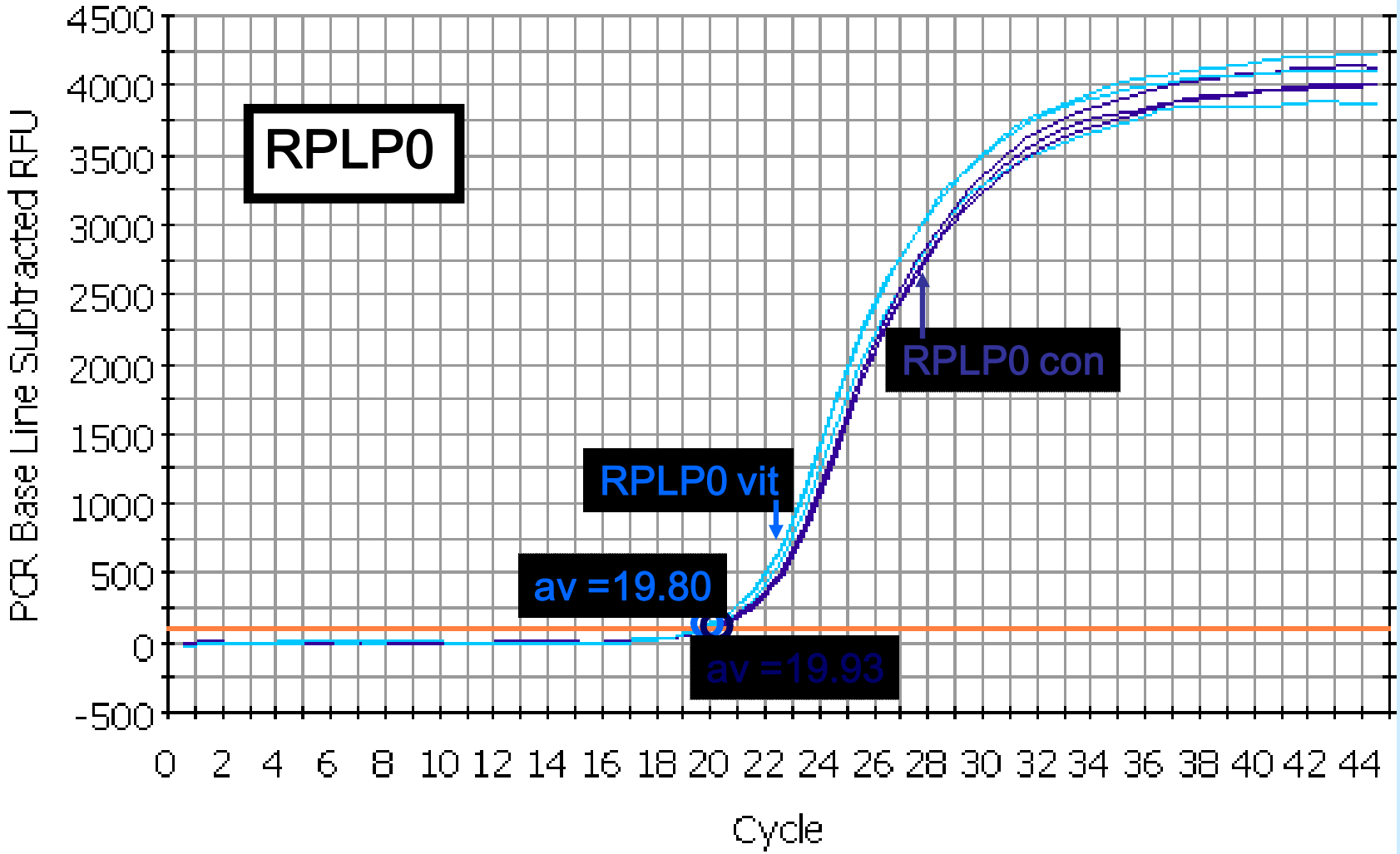
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



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

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

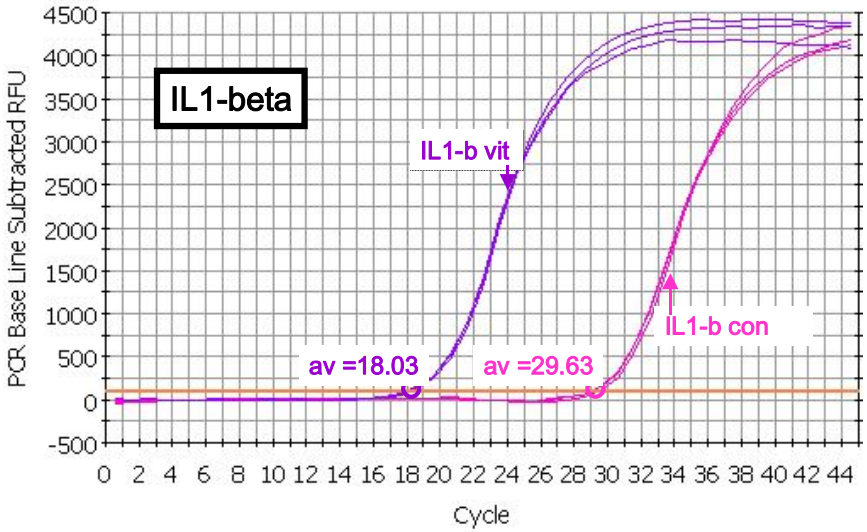
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



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

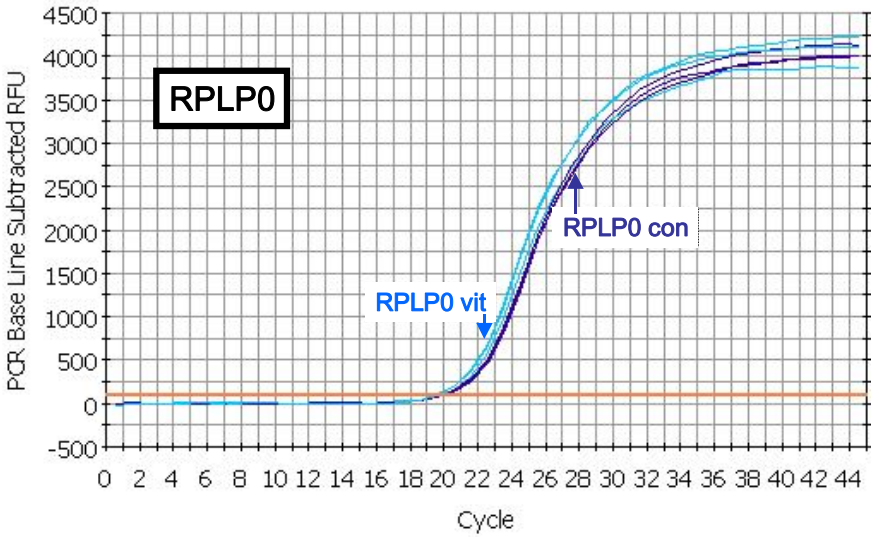
AFTER N CYCLES: ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

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



AFTER N CYCLES: increase = (efficiency)^n

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



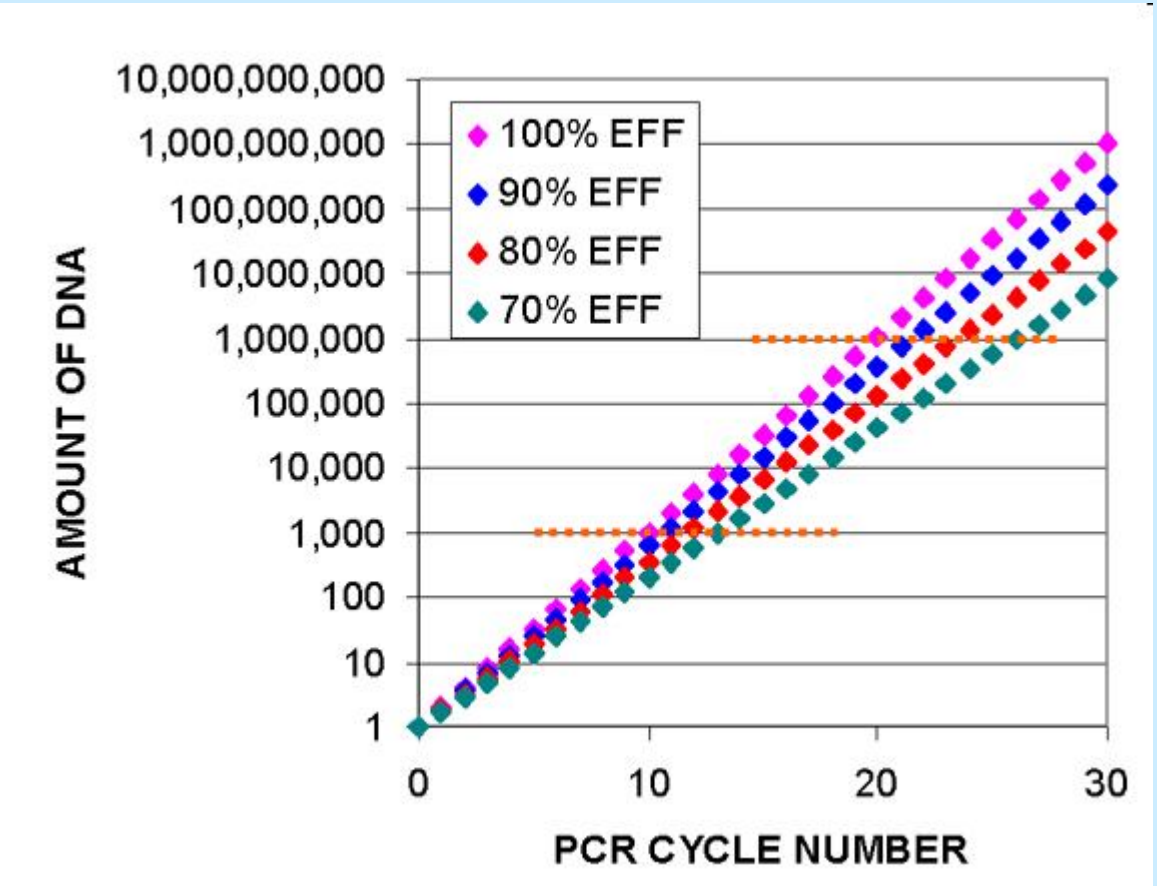
AFTER N CYCLES: increase = (efficiency)^n

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

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

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

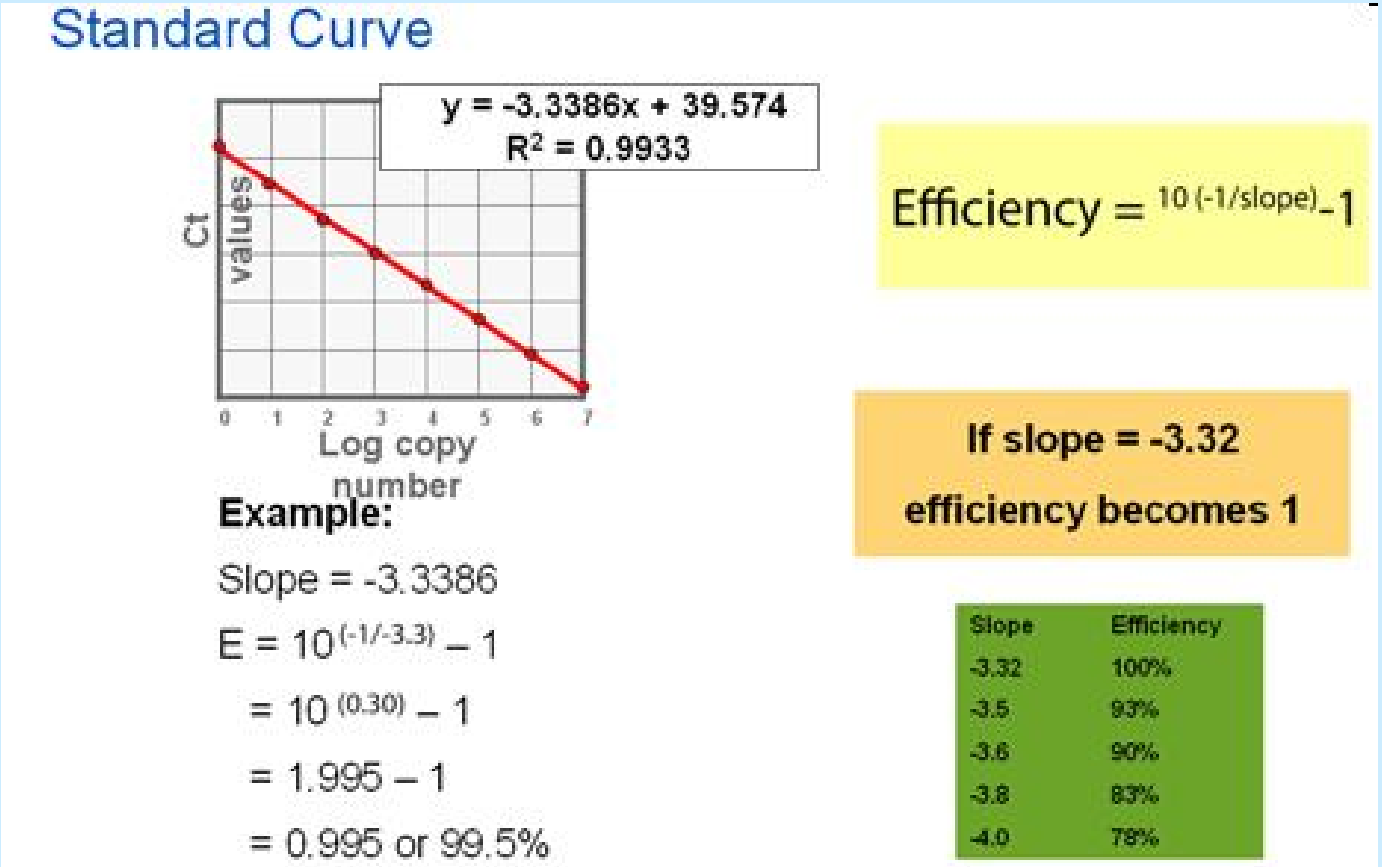
Amplification of different efficiency



Efficiency$^{\Delta\Delta Ct}$

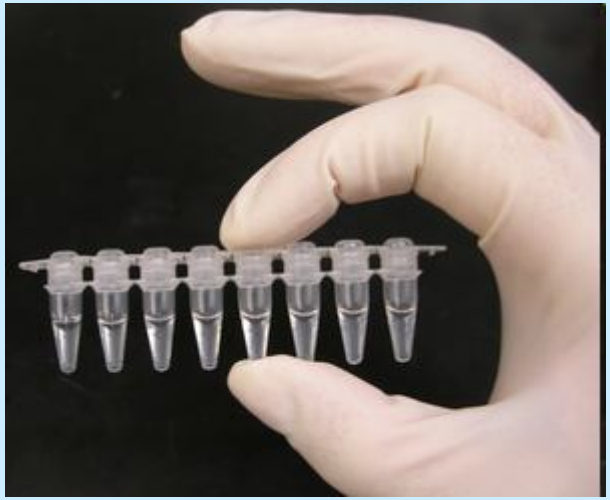
- The approximation can be improved by calculating the efficiency for both the target and the control amplification
- If the efficiency is 95% the difference (fold change) is $1.95^{11.5}$=2164.702, in case of ΔCt=11.5
- The efficiency can be determined from the the dilution curve

The efficiency of PCR can be calculated from a dilution series used for the target assay



- The efficiency of the PCR should be between 90–100% (−3.6 ≥ slope ≥ −3.3). If the efficiency is 100%, the CT values of the 10 fold dilution will be 3.3 cycles apart (there is a 2-fold change for each change in CT). If the slope is below –3.6, then the PCR has poor efficiency.

Thank you for your attention and have a good play with the art!



EDIROL PCR M1

Resources

- Wikipedia, The free encyclopedia - Polymerase chain reaction
- http://www.dnalc.org/resources/animations/pcr.html
- *Margaret Hunt: Real time PCR http://pathmicro.med.sc.edu/pcr/realtime-home.htm*
- appliedbiosystems.com