Cube

Computational Systems Biology

# Quantitative Methoden in der Molekularbiologie

## *8. Quantification of nucleotide sequences (RT-qPCR)*

*WS 2015/16*

# Exam question

The following experimental plan is proposed for the quantification of proteins in yeast mutants. Correct all errors in this plan.

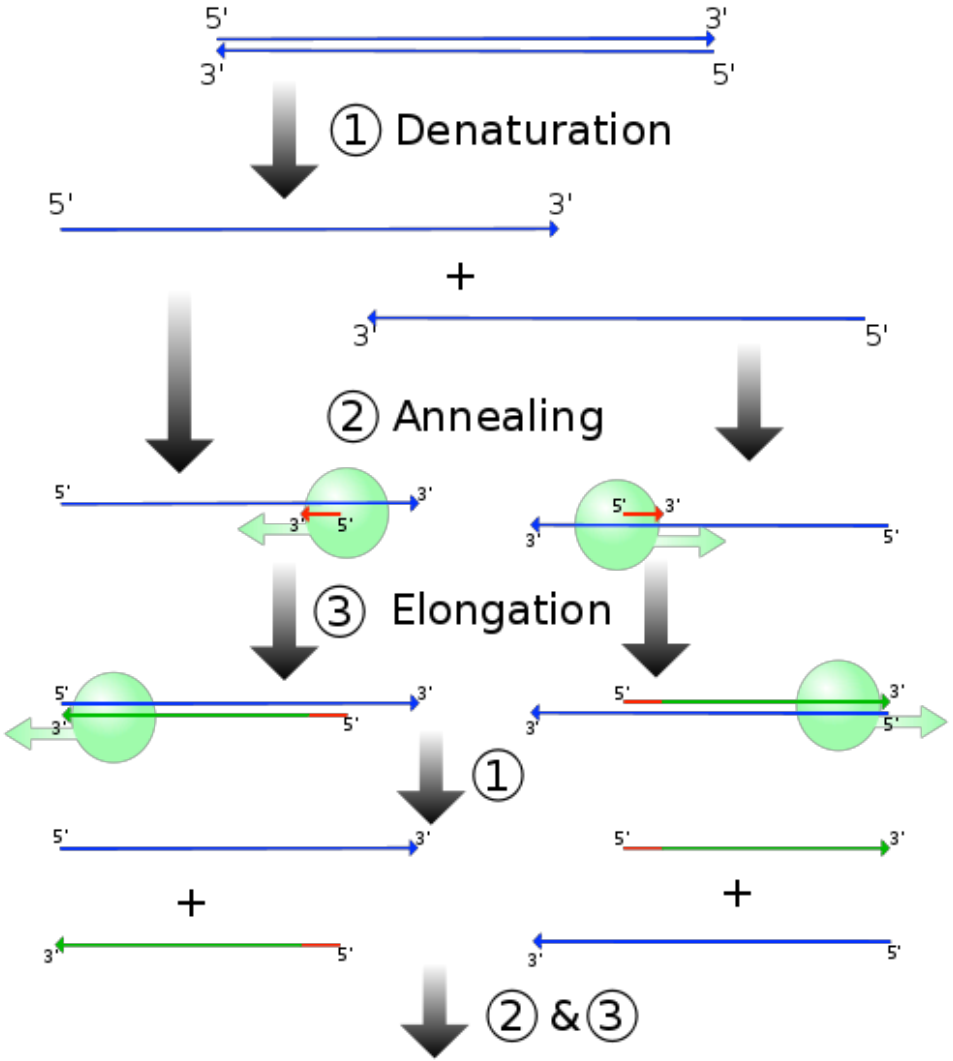
| Sample | Method | Technician |
|---|---|---|
| Wildtype | Mass spectrometer/ICAT | Person 1 |
| Mutant A | Mass spectrometer/ICAT | Person 1 |
| Mutant A | Mass spectrometer/ICAT | Person 1 |
| Mutant B | Protein isolation and Bradford assay | Person 2 |
| Mutant B | Protein isolation and Bradford assay | Person 2 |
| Negative control | Protein isolation and Bradford assay | Person 2 |

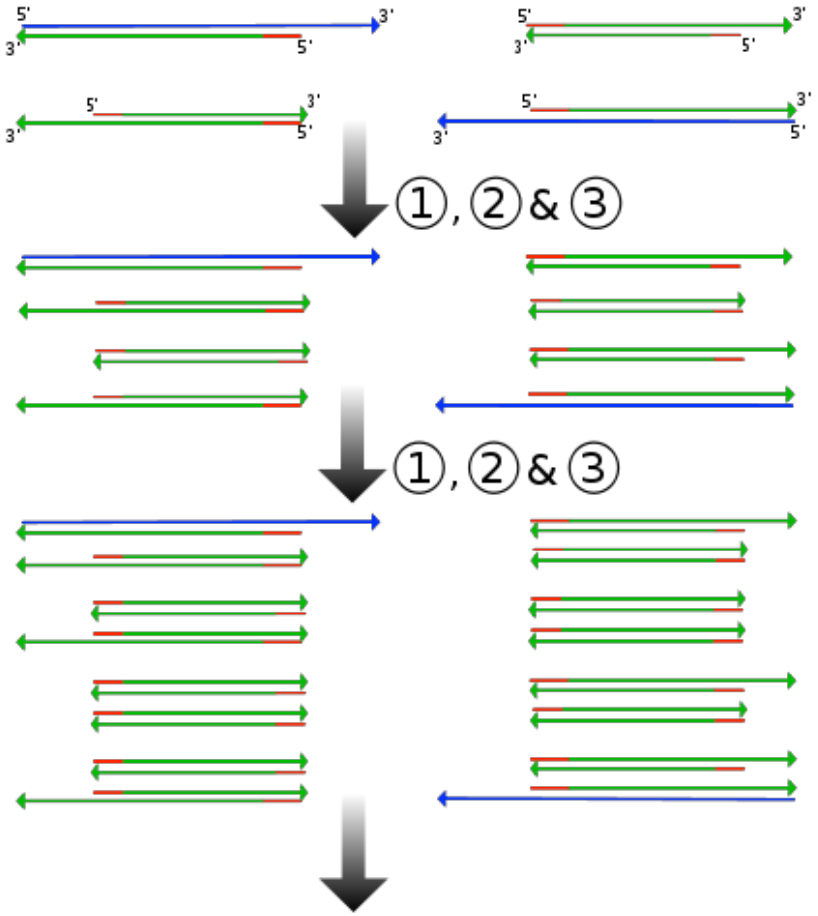
# Outline

1. PCR

2. RT-qPCR (basics)

# Polymerase Chain Reaction (PCR)




Exponential growth of short product

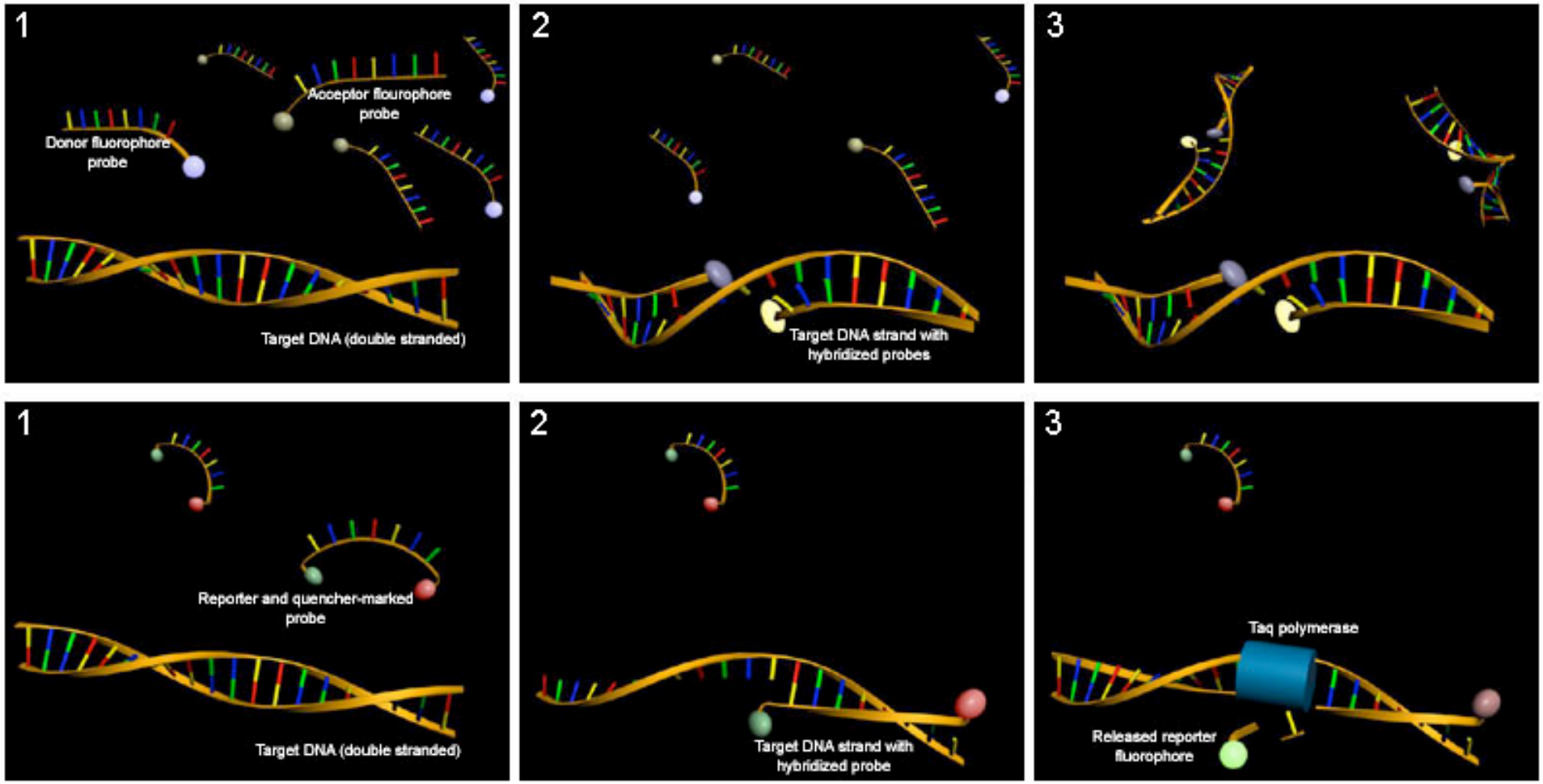
# Exponential growth

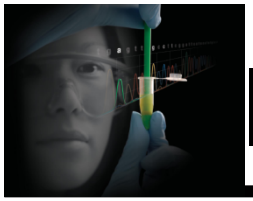
The legend goes that the tradition of serving Paal Paysam to visiting pilgrims started after a game of chess between the local king and the lord Krishna.

The king was a big chess enthusiast and had the habit of challenging wise visitors to a game of chess. One day a traveling sage was challenged by the king. To motivate his opponent the king offered any reward that the sage could name. The sage modestly asked just for a few grains of rice in the following manner: the king was to put a single grain of rice on the first chess square and double it on every consequent one.

Having lost the game and being a man of his word the king ordered a bag of rice to be brought to the chess board. Then he started placing rice grains according to the arrangement: 1 grain on the first square, 2 on the second, 4 on the third, 8 on the fourth and so on…

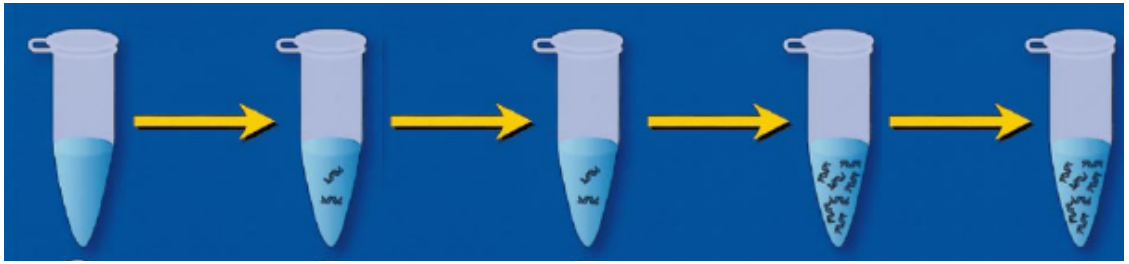
# Real-time (RT)-PCR Principle



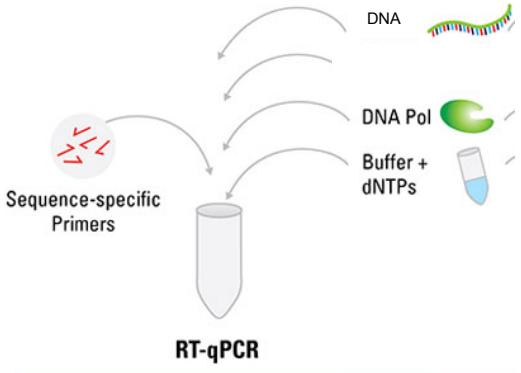


## Lab Experiment

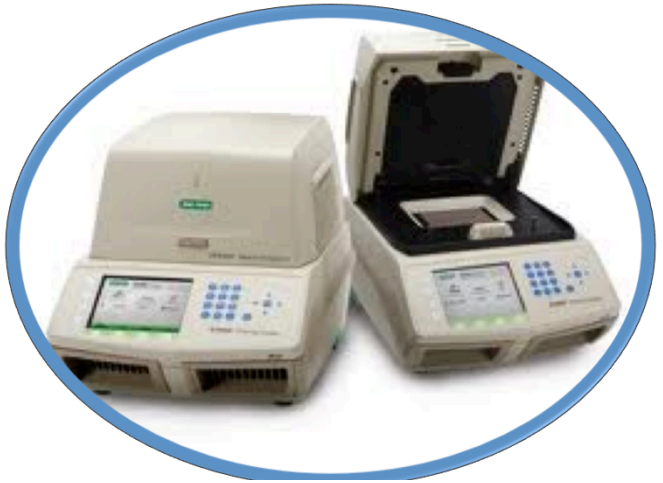
1: Dilution series of DNA with known concentration



2: Adding DNA samples with primers and buffer on the plate




3: Running on the RT-PCR machine



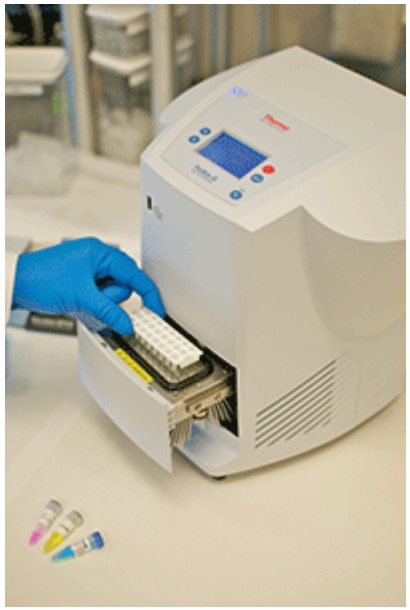
# Real-Time PCR Instruments

AB StepOnePlus
Fast Real-Time PCR System

Roche Light Cycler


Qiagen's Rotor-gene

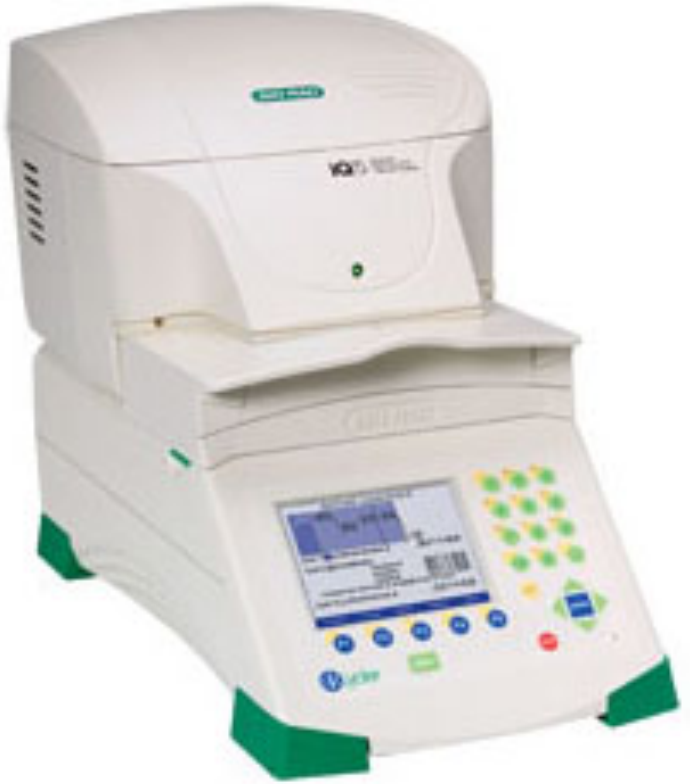
Bio-Rad CFX-96



Thermo PikoReal

# Real-Time PCR Instruments

- 96 or 384 well format
- Run time
- 10-100 µl reaction mix
- Dyes (FAM, SYBR green I, TET, HEX, JOE, VIC, Yakima Yellow, TAMARA, Cy3, Cy5, Texas Red, ROX, Alexa Fluor 350)

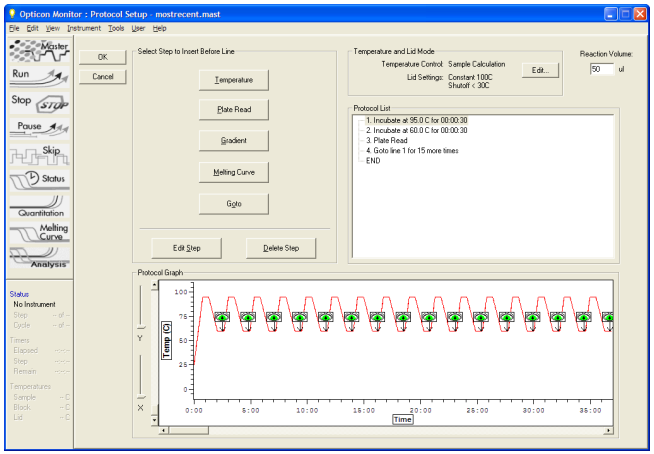


**Bio-Rad iQ5 real-time PCR instrument**

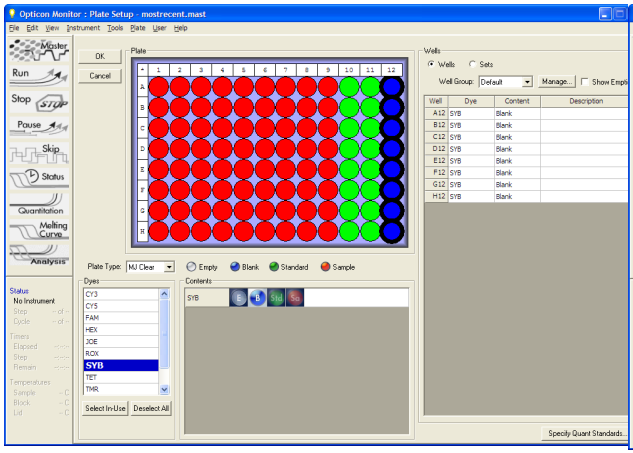
# Real-Time PCR Software

1. **Set up PCR protocol** (annealing, denaturation and hybridization)
2. **Set up plate layout.**
3. **Collect data.**
4. **Analyze data.**

1




2

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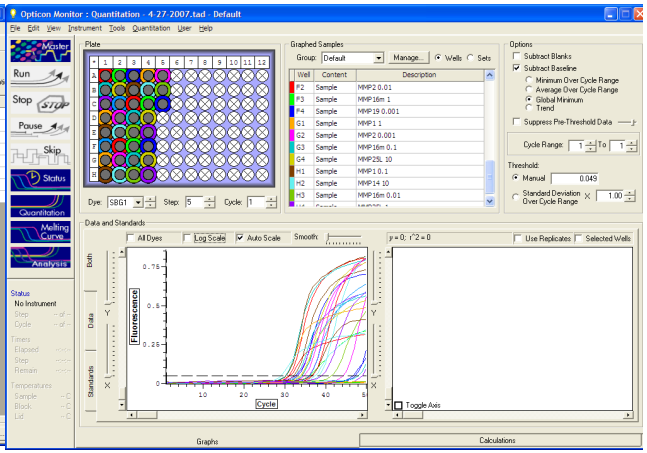
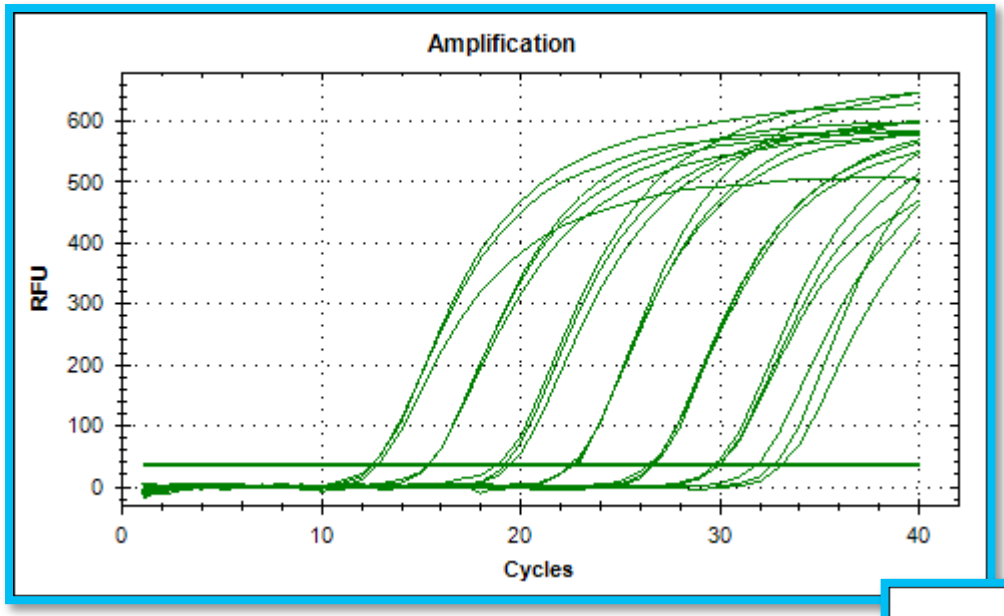
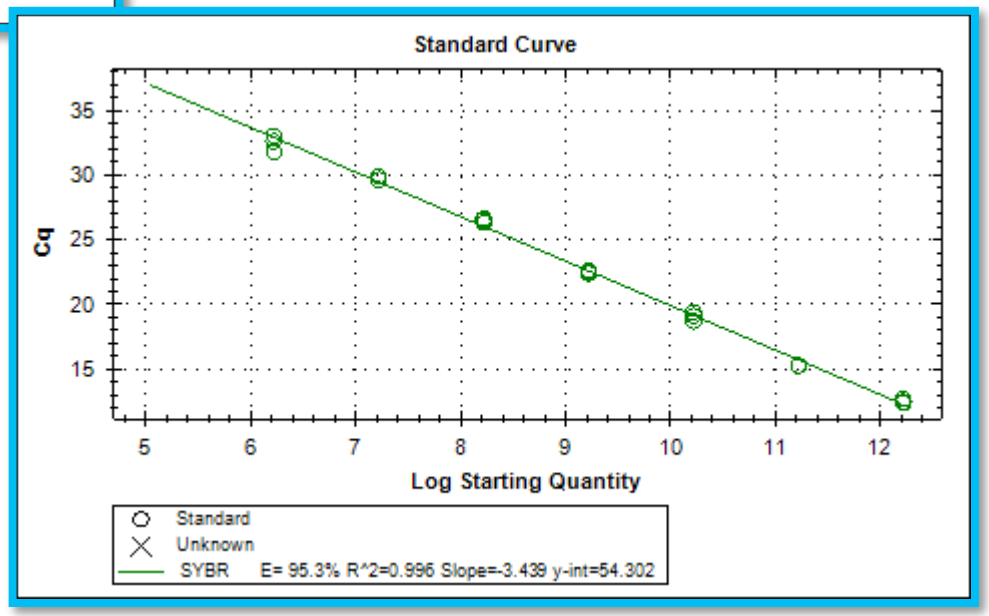


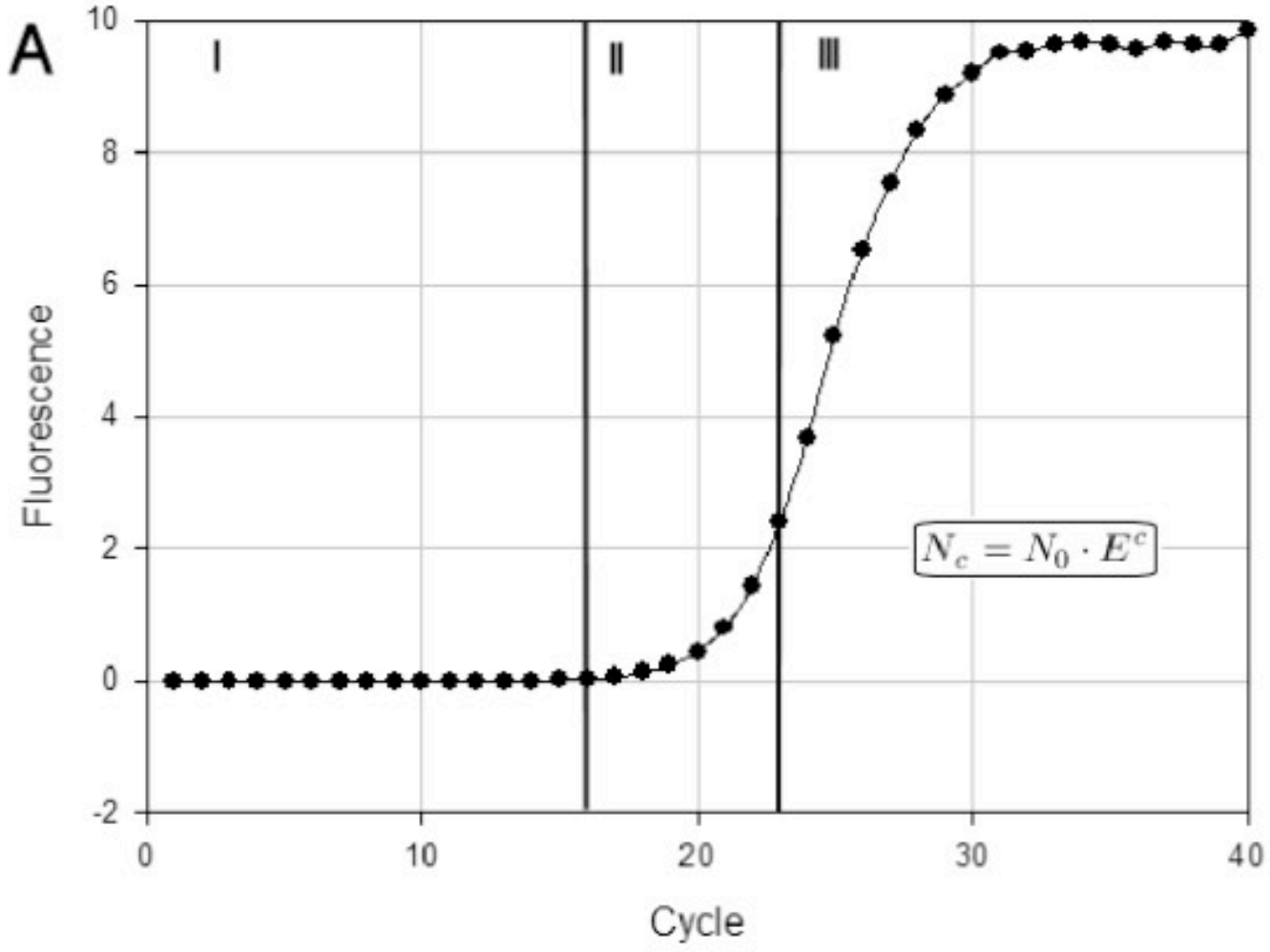



Dilution series of DNA with known concentration



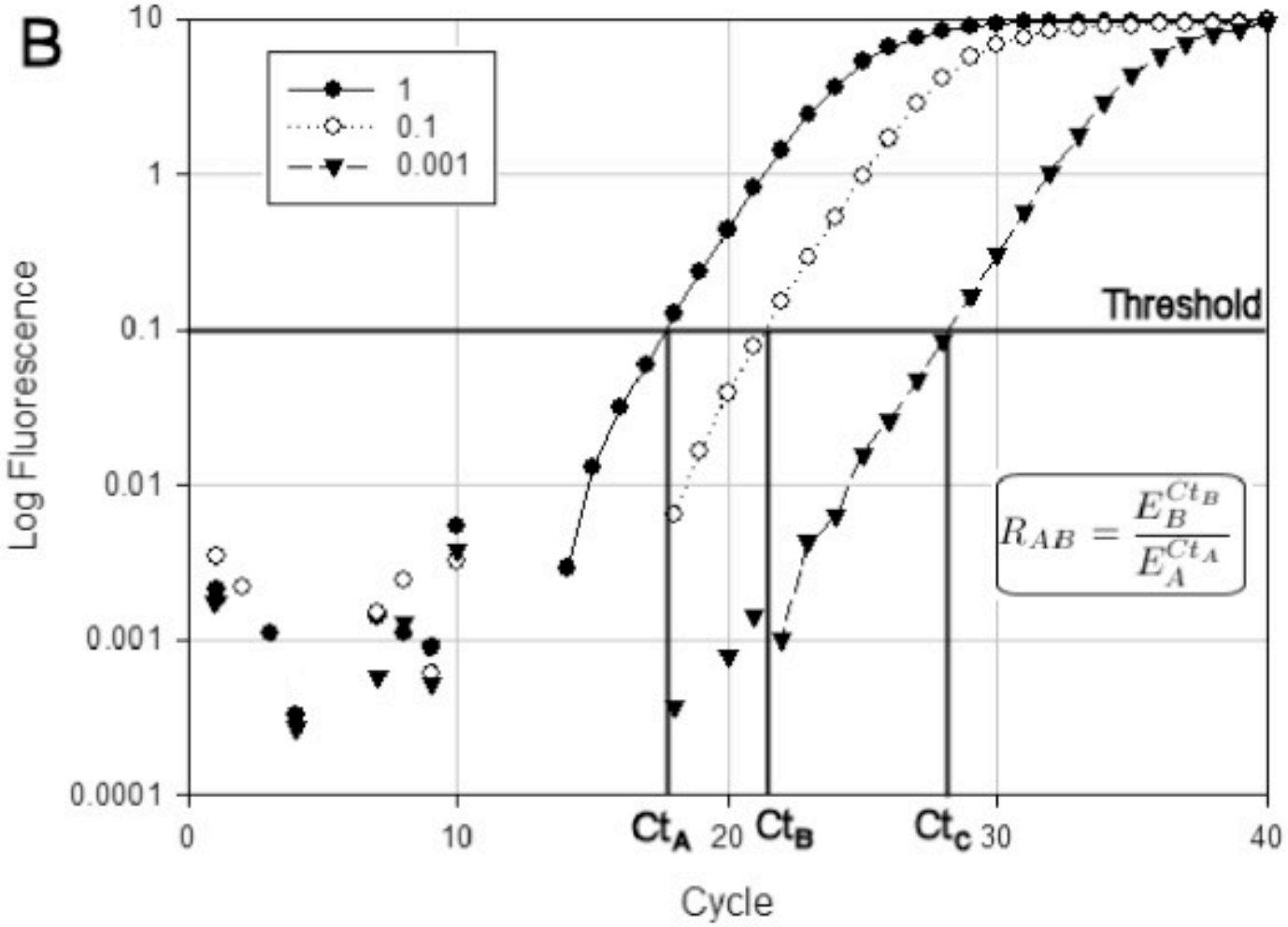

Efficiency of the reaction: 95%

# RT-PCR Phases




*Karlen et al. BMC Bioinformatics 2007*

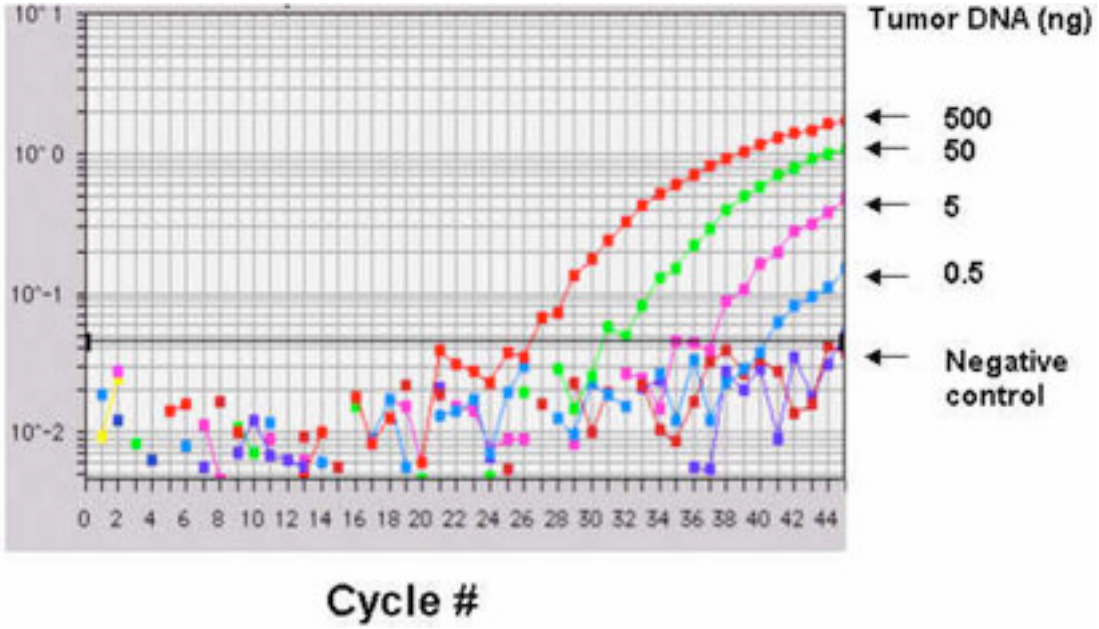
# RT-PCR Phases and $C_T$ values




*Karlen et al. BMC Bioinformatics 2007*

# Threshold T

$$T=\mu(noise)+10*\sigma(noise)$$



# Problem

Two DNA samples, A and B, are run in a real-time PCR experiment in which the β-Actin gene is assayed.

Sample A yields a $C_T$ value of 21.8.

Sample B yields a $C_T$ value of 23.2.

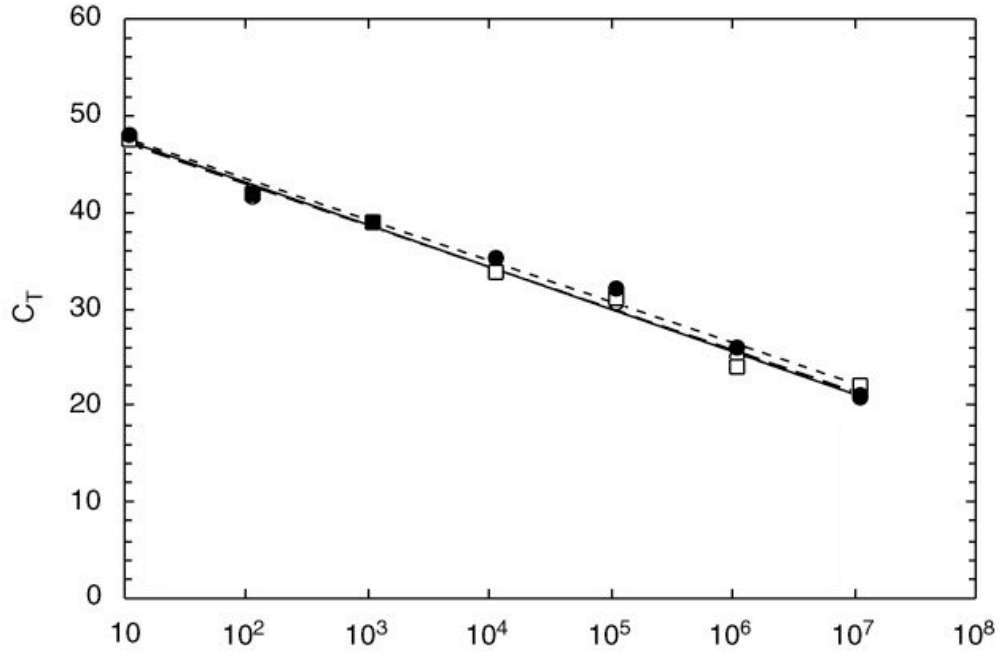
What is the fold increase in the amount of Sample A over Sample B?

# Solution

- A has higher amount compared to B (lower $C_T$)
- Fold change (concentration ratio) proportional to difference of $C_T$ values: $\Delta C_T = 23.2 - 21.8 = 1.4$
- Fold change = $2^{\Delta CT} = 2^{1.4} = 2.64$

# Absolute quantification

Requires a standard curve to be generated from a serially diluted DNA sample having a concentration determined by independent method:



# Efficiency

$$Y = X * (1+E)^n$$

- Y amount of PCR product
- X initial number of template molecules
- E Efficiency
- n cycle number

# Efficiency and slope

During exponential amplification, a 100% efficient reaction will double every cycle and will produce a 10-fold increase in PCR product every 3.32 cycles ($\log_2 10 = 3.3219$)

- Theoretical efficiency E=1 -> slope -3.32
- Slope < -3.32: efficiency <100%
- Slope > -3.32: experimental errors, template problems

Amplification $= 10^{(-1/\text{slope})}$

Efficiency $E = 10^{(-1/\text{slope})} - 1$

# Controls

- No template control (NTC): all reagents, primers, probes, but no template
- Exogenous control: known oligonucleotide of known concentration
- Passive reference: dye not participating in amplification and not inhibiting it