qPCR course

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What do you want to know?

Data Analysis and interpretation

Lecture 2

Application of qPCR

Lecture 2

Statistical analysis of qPCR

Basics

Lecture 1

How qPCR works

Lecture 2

Normalization

Lecture 2

Troubleshooting

Everything

More

Results of the quiz

1. Did you learn anything new from the lecture

A lot	yes	no
4	11	0

2. If yes, do you think you will remember it tomorrow?

No	some (not the equations)	yes, after revising it	yes
1	4	7	3

3. Would you prefer to have more information in the lecture, keep it the same, reduce it?

Reduce/split in 2 parts	Keep the same	More information
6	8	1

a requests for less calculations!

abbreviation of each symbol

4. Were the tasks too easy, ok, or too hard?

too hard	ok	too easy
9	6	0

will be simplified

Results of the quiz

6. was/would be the homework helpful

Nope	a bit	Yes
3	2	10

10. Would you suggest any changes?

it was a bit too fast/I need more time to think

more group work

Application part was too fast and not clear

I. Efficiency

II. Quantification

Absolute qPCR

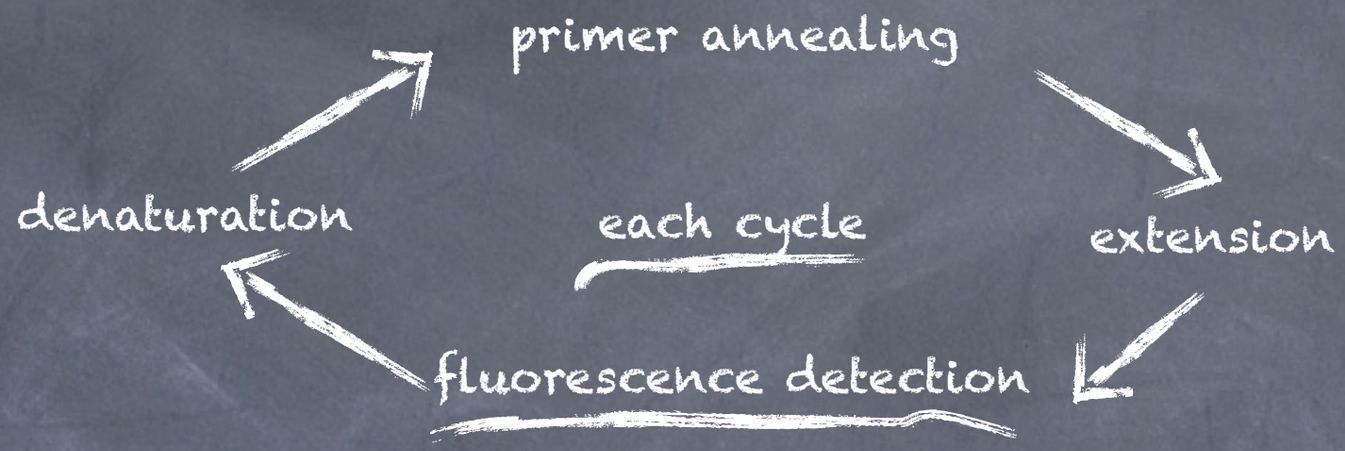
Relative qPCR

III. Primer design

IV. The practical part

Why a short product?

- the biggest issue seem to be the efficiency (see the 72°C for 5 minutes step in end point PCR)
- than shorter is the amplicon than less its amplification is susceptible to suboptimal conditions
- depending on the kit, there might be a risk of "running out"of components during the reaction
- time issue. you want your results asap



Please draw one cycle and write the temperatures for each one

Helicase-dependent isothermal DNA amplification. Vincent et al.,EMBO Reports. 2014

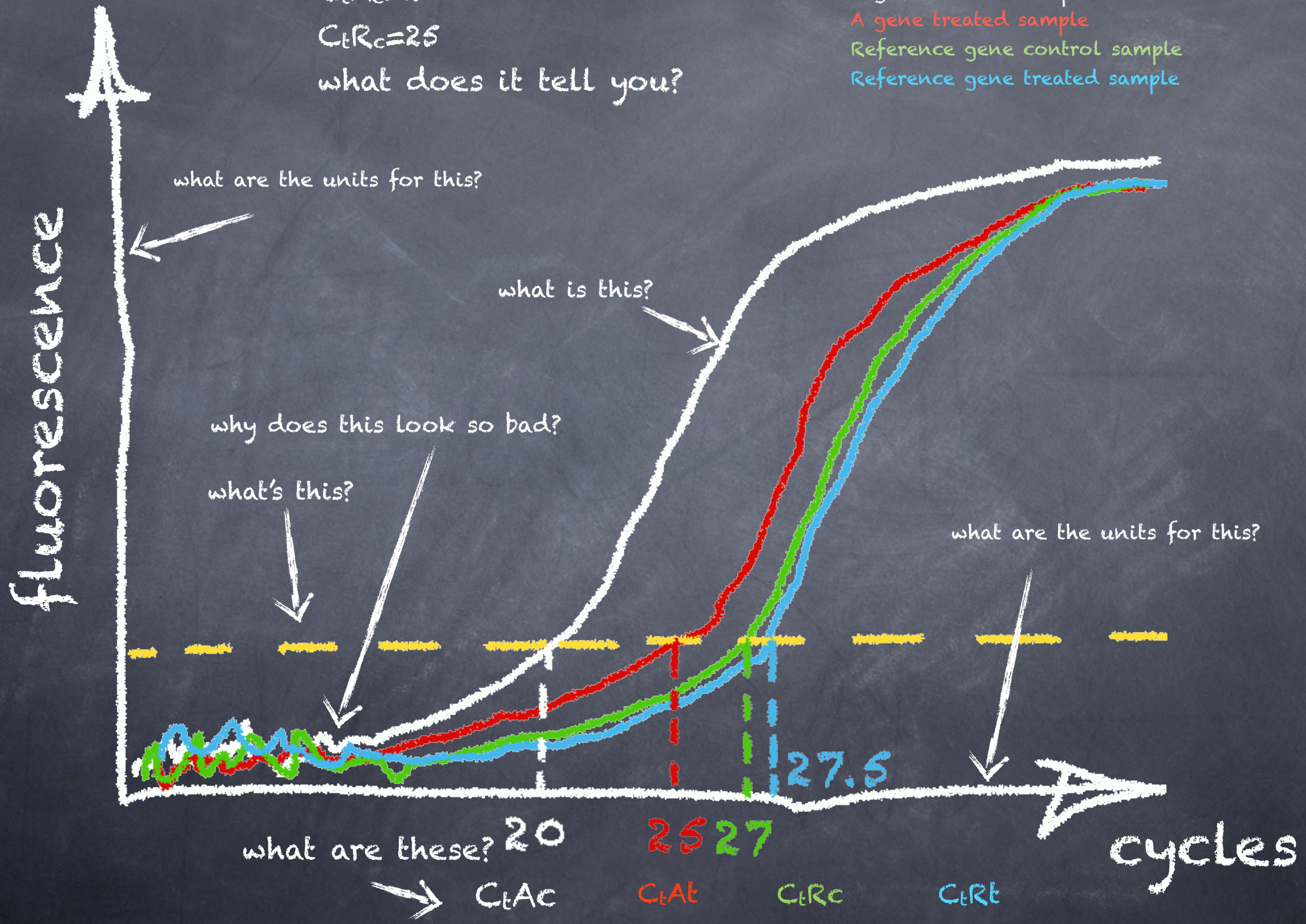
- Can you PCR RNA?
- What is the difference between end point and real-time PCR?
- What is absolute qPCR ?
- What is relative qPCR?
- Why do you need to normalise your PCR results?
- What are the ways to normalise them?

$C_tA_c=20$

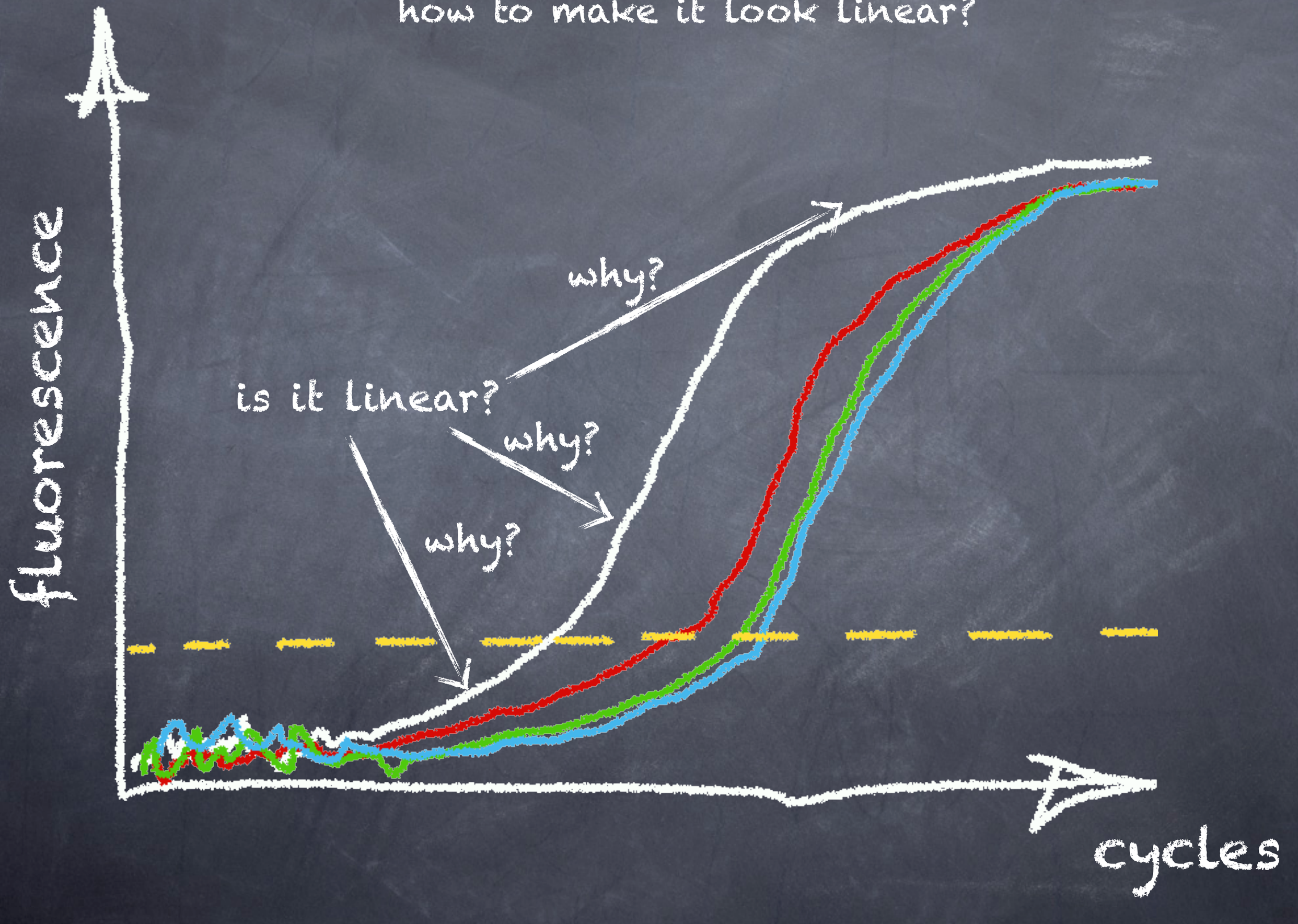
$C_tR_c=25$

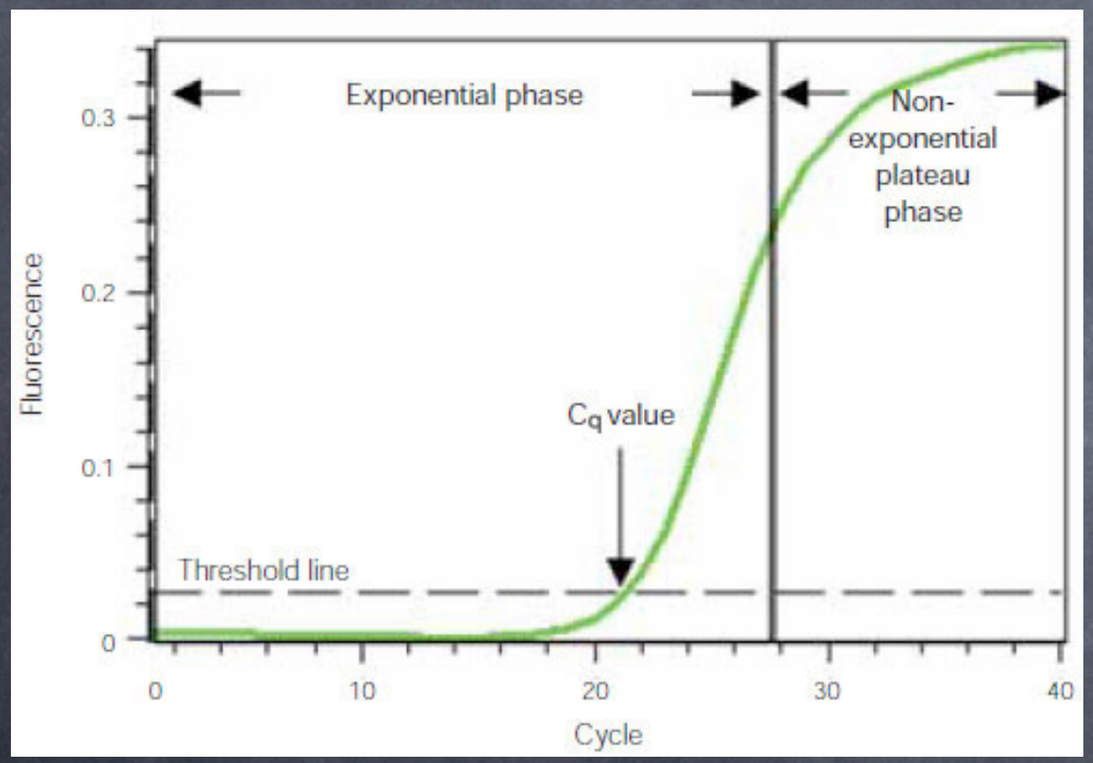
what does it tell you?

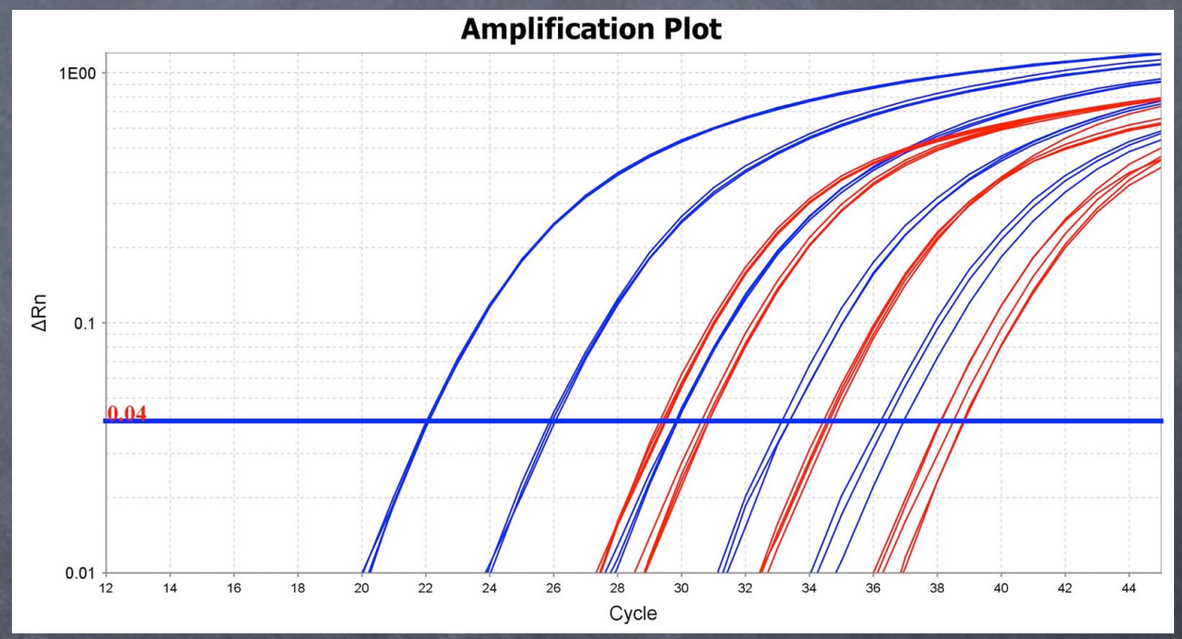
A gene control sample
A gene treated sample
Reference gene control sample
Reference gene treated sample



how to make it look linear?




Exponential phase
Non-exponential plateau phase
C_q value
Threshold line
Fluorescence
0.3
0.2
0.1
0
0
10
20
30
40
Cycle


Amplification Plot
1E00
0.1
0.01
ΔRn
0.04
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
Cycle

what is log?

$\log_a b = c \quad \Rightarrow \quad a^c = b$

$\log_3 9 = ?$

$\log_2 8 = ?$

$\log_{749} = ?$

$\log_5 1 = ?$

$\log_{10} 16 = ? \quad \Rightarrow \quad 10^{1.2} = 16$

$\log_{10} 8 = ? \quad \Rightarrow \quad 10^{0.9} = 8$

$\log_{10} 4 = ? \quad \Rightarrow \quad 10^{0.6} = 4$

$\log_{10} 2 = ? \quad \Rightarrow \quad 10^{0.3} = 2$

- What is primer Efficiency?
- What does it mean when Efficiency is 100%?
- What does it mean when E=2 ?
- How can it be more than 100% ?
- Why can it be less than 100% ?
- How to estimate efficiency?

E = 100%

DNA → C_t=20

is it important to know concentration of the initial sample?

↓ dilute twice

DNA → C_t=21

↓ dilute twice

DNA → C_t=22

↓ dilute twice

DNA → C_t=23

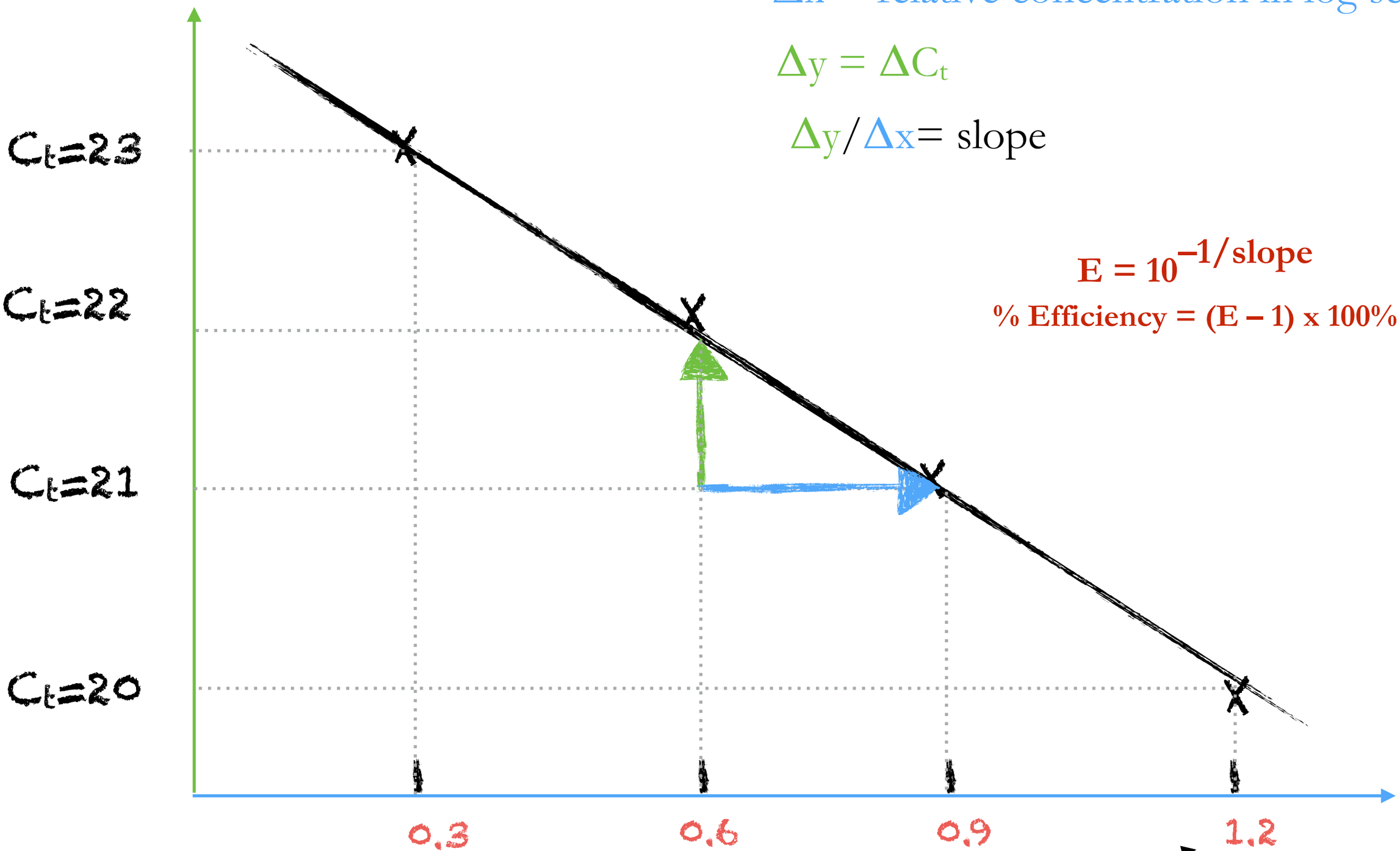
Δx = relative concentration in log scale

$\Delta y = \Delta C_t$

$\Delta y / \Delta x$ = slope

$$E = 10^{-1/slope}$$

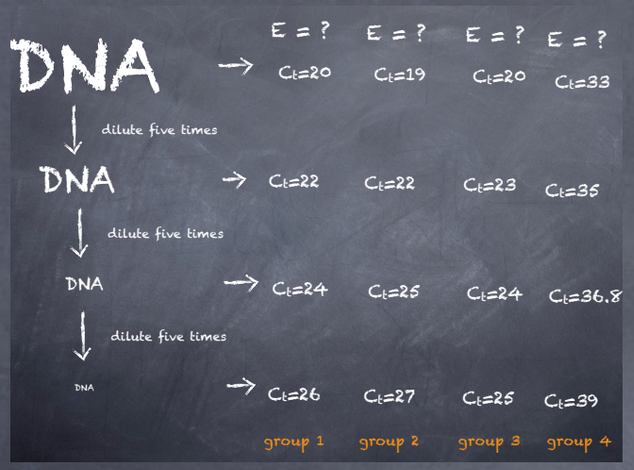
% Efficiency = (E – 1) x 100%



where do these numbers come from?

why in log scale again?

DNA	→	E = ? Ct=20	E = ? Ct=19	E = ? Ct=20	E = ? Ct=33
↓ dilute five times					
DNA	→	Ct=22	Ct=22	Ct=23	Ct=35
↓ dilute five times					
DNA	→	Ct=24	Ct=25	Ct=24	Ct=36.8
↓ dilute five times					
DNA	→	Ct=26	Ct=27	Ct=25	Ct=39
		group 1	group 2	group 3	group 4



why it is not important to know the true concentration, again?

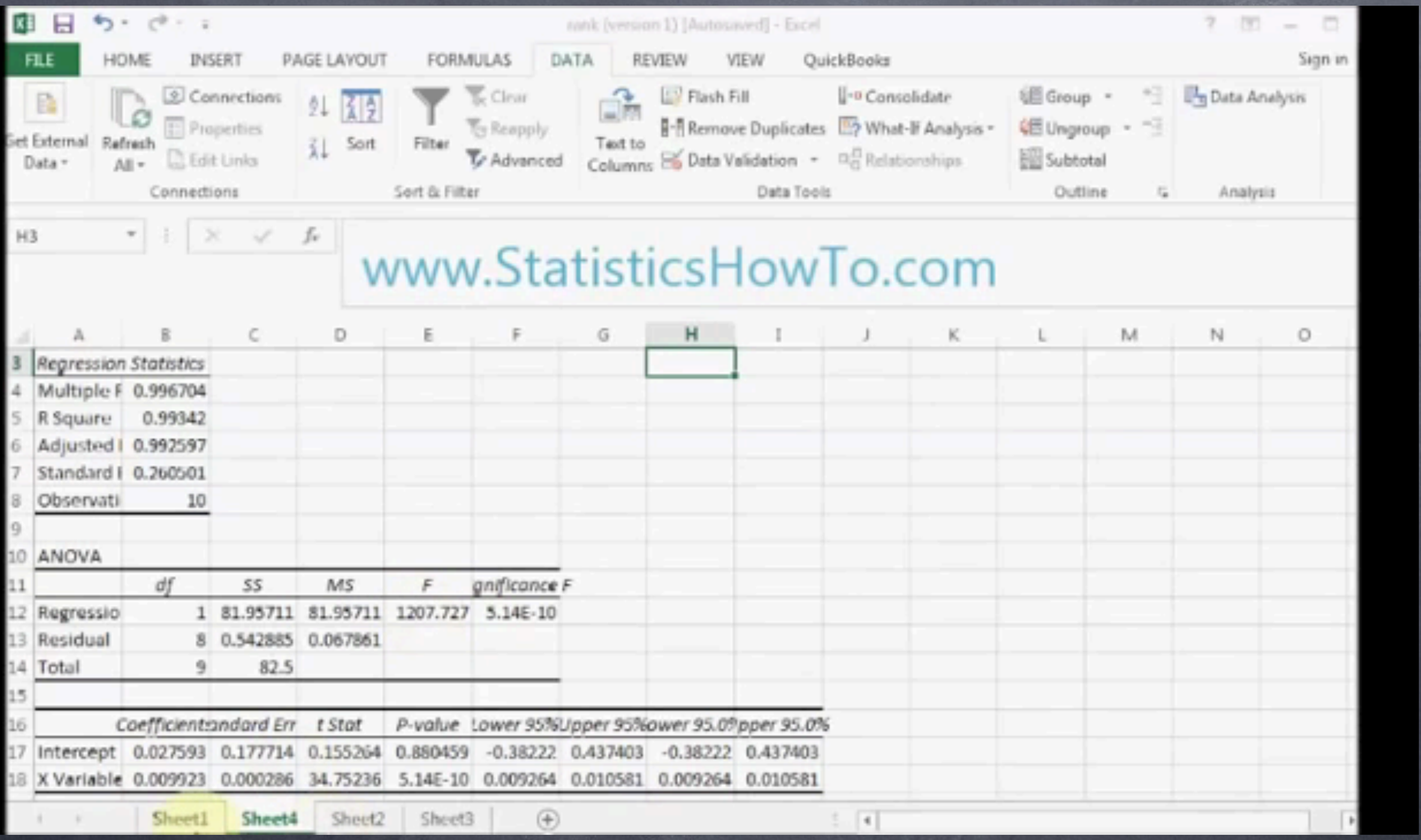
step 1 ? set amount of DNA in the undiluted sample to your favourite number

step 2 ? determine the $\log_{10}$ of your DNA concentration

step 3 ? calculate the slope=$\Delta y/\Delta x$ or? linear function $y = kx + m$

Manually: http://www.youtube.com/watch?v=CfrWexuiZyU slope

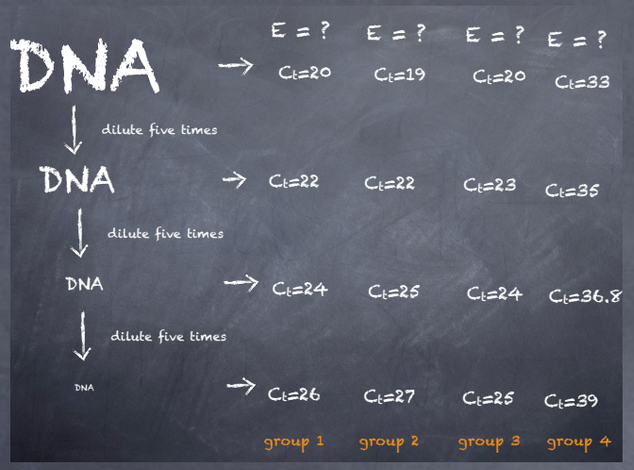
Linear regression calculation



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
3	*Regression Statistics*														
4	Multiple F	0.996704													
5	R Square	0.99342													
6	Adjusted I	0.992597													
7	Standard I	0.260501													
8	Observati	10													
9															
10	ANOVA														
11		*df*	*SS*	*MS*	*F*	*gnificance F*									
12	Regressio	1	81.95711	81.95711	1207.727	5.14E-10									
13	Residual	8	0.542885	0.067861											
14	Total	9	82.5												
15															
16		*Coefficient*	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*						
17	Intercept	0.027593	0.177714	0.155264	0.880459	-0.38222	0.437403	-0.38222	0.437403						
18	X Variable	0.009923	0.000286	34.75236	5.14E-10	0.009264	0.010581	0.009264	0.010581						

You can also find this video on the website of the course

http://www.youtube.com/watch?v=OIxiOJ26r_k



Calculation of Efficiency

why it is not important to know the true concentration, again?

step 1 ? set amount of DNA in the undiluted sample to your favourite number

step 2 ? determine the $\log_{10}$ of your DNA concentration

step 3 ? calculate the slope=$\Delta y/\Delta x$ or? linear function $y = kx + m$

Manually: http://www.youtube.com/watch?v=CfrWexuiZyU slope

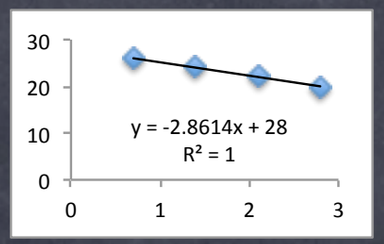
step 4 ? calculate $E = 10^{-1/slope}$

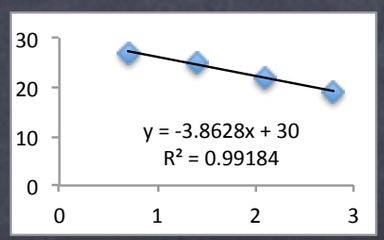
step 5 ? if you want % calculate (E-1)*100%

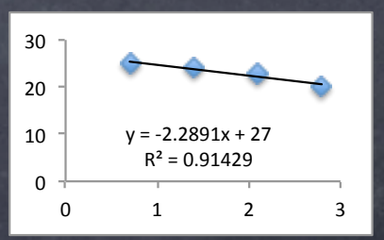
step 6 ? CHECK if your result MAKES SENSE! how would you do it?

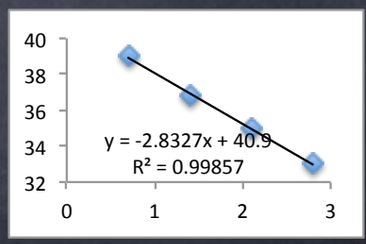
			group 1		group 2		group 3		group 4	
	amount of DNA	**log10 of the DNA amoount**	**x**	**y**	**x**	**y**	**x**	**y**	**x**	**y**
initial DNA sample	625	2,795880017	2,795880017	20	2,795880017	19	2,795880017	20	2,795880017	33
1st 5 fold dilution	125	2,096910013	2,096910013	22	2,096910013	22	2,096910013	23	2,096910013	35
2d 5 fold dilution	25	1,397940009	1,397940009	24	1,397940009	25	1,397940009	24	1,397940009	36,8
3d 5 fold dilution	5	0,698970004	0,698970004	26	0,698970004	27	0,698970004	25	0,698970004	39

slope	**k**	**-2,8614**	**-3,8628**	**-2,2891**	**-2,8327**
	R^2	**1**	**0,99184**	**0,91429**	**0,99857**
E=10^(-1/slope)	E	2,2360384	1,8150122	2,73434249	2,2543434
%E=(E-1)*100%	%E	123,60384	81,501228	173,434249	125,43434









Efficiency

http://www.gene-quantification.de/efficiency01.html

You can also find this link on the website of the course

Absolute qPCR

- what is absolute qPCR?
- how do you calculate the amount of DNA in your sample?
- what is the difference between the standard curve for absolute qPCR and for primer Efficiency?
- can you use the same steps for calculating?

Calculation of Efficiency vs Absolute qPCR standard curve

is it applicable for absolute qPCR standard curve?

step 1 set amount of DNA in the undiluted sample to your favourite number

step 2 determine the $\log_{10}$ of your DNA concentration

step 3 calculate the slope=$\Delta y/\Delta x$ or? linear function $y = kx + m$

slope

axis y is Ct

C_t=23

C_t=22

C_t=21

C_t=20

? ? ? ?

$y = kx + m$

$x = ?$

0.3 0.6 0.9 1.2

axis x is quantity in log scale

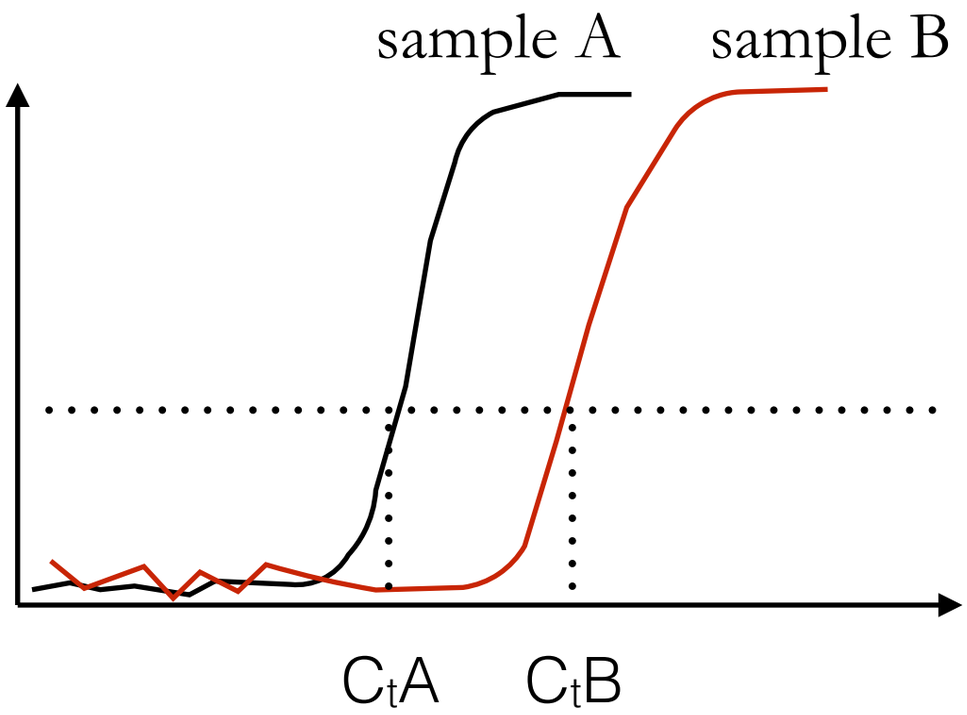
Relative qPCR

- what is relative qPCR?
- how do you make sure that your samples have the same amount of total DNA?

ΔCt method

what is delta?

For PCR exactly the same amount of DNA was used from sample A and from sample B
The gene of interest (GOI) was detected.
How much more/less of this gene is present in the sample A



what is the possible disadvantage of this approach?

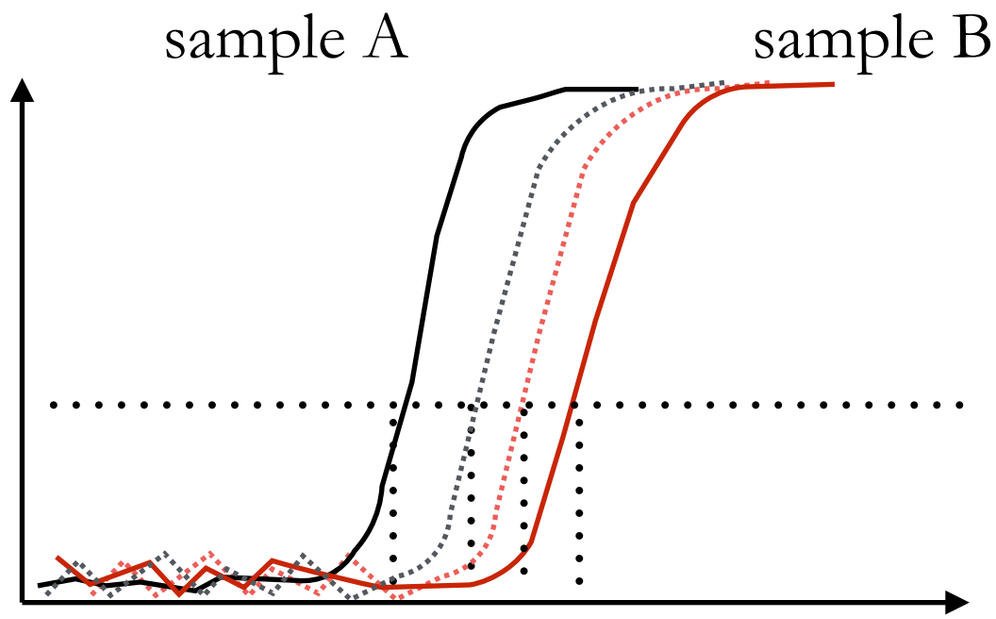
how would you try to solve it?

normalized to what?

Normalized expression ratio = $E^{\Delta Ct} = E^{(CtA - CtB)}$

$\Delta\Delta Ct$

why do you think there are two deltas here?



1. Normalize expression of gene of interest in A and B

$$E_{GOI}^{-\Delta C_t GOI} = E_{GOI}^{-(C_t A - C_t B)}$$

what will it tell you?

C_tA C_tAr C_tBr C_tB

2. Normalize expression of reference gene in A and B

$$E_{ref}^{-\Delta C_t ref} = E_{ref}^{-(C_t Ar - C_t Br)}$$ ← what will it tell you?

3. Calculate expression ratio of GOI and reference gene

$$E_{geneA}^{-\Delta C_t GOI} / E_{ref}^{-\Delta C_t ref} = ?$$

Primer design

do you have suggestion, what to take into consideration?

DyNAmo Flash SYBR Green qPCR Kit

- optimal amplicon size more than 50 bp, but less than 250 bp
- GC content as close to 50%
- length: not shorter than 18 ntp, preferably not longer than 30. 22-25 bp is usually good.
- both primers should have the same melting T (Tm)
- check for self annealing and primer dimer formation
- check for specificity, allow several mismatches
- you can eliminate or detect genomic DNA contamination
- make sure, that all your primers are written down as 5'->3' (including the reverse one)
- T/A on 3' end might decrease not specific amplification
- try Primer3 and BLAST-primer softwares (on the course site)

reference genes

- you want them to be equally expressed in control and test samples:
    - check available literature and ask people around
    - check available microarray data (GENEVESTIGATOR, the link to it is on the website of the course, you will need to create an account to use it)
    - figure out what genes are involved in key pathways in all cells present in your sample
    - avoid picking genes within the same pathway or cross-talking pathways
    - pick only single member family genes
    - choose the least conservative coding part of a gene you picked
- it is on your conscience to trust single reference gene data. just for you information, good people use more than one reference.

On the website of the course you can download the Excel file for primer order

Please fill it in and send to me not later than 4 pm on the 7th of October (next Tuesday!!)

The practical part

Experiment

Absolute qPCR

my sample and my primers

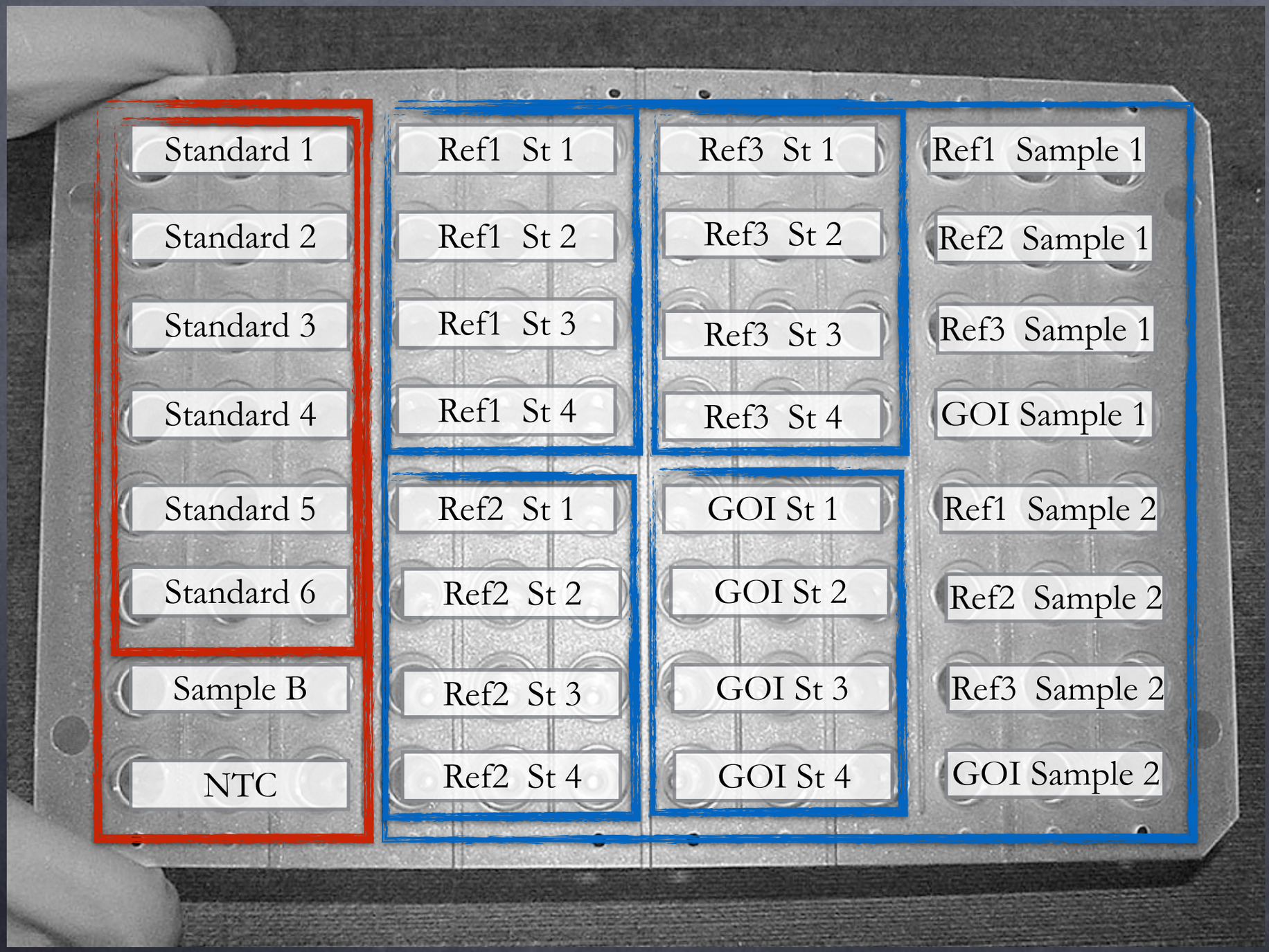
Relative RT-qPCR

your samples and your primers

Experiment layout

Absolute qPCR

Relative RT-qPCR



Absolute qPCR

You will get from me:

1. Sample A = an eppendorf with a plasmid of known concentration

2. Sample B = an eppendorf with the same plasmid with concentration known only to me

3. primers to detect the plasmid and data about their E

You will need to:

here 1. Run an absolute qPCR

home 2.find out the concentration of the plasmid in the sample B

home 3. write a report in a form of Results + Materials and Methods + Figure with Legend for an article in a given journal

home 4. make a short presentation of your results + stuff you want to share

Relative RT-qPCR

You will need to:

home 1. Have RNA from 2 samples you want to compare

home 2. Design 4 pairs of primers to detect 1 gene of your interest and 3 reference genes in your samples

here 3. Run RT

here 4. Run primer efficiency test for all 4 primer pairs

here 5. Run PCR on your samples using all 4 primer pairs

home 6. Calculate primer efficiency

home 7. Assess performance of your reference genes. Pick 2 best.

home 8. Use these 2 reference genes to normalize your data using ΔC_t and $\Delta\Delta C_t$ methods

home 9. Make up data to perform a statistical analysis

home 10. Write a report in a form of Results + Materials and Methods +Figure with Legend for an article in a given journal

home 11. include results into your short presentation