Advanced Cell Biology & Biotechnology

Biotechnology Project Lab

Giovanna Gambarotta
& Isabella Tarulli

The lecture of December 5th 2021 is about to begin….

Exercises (29-11-2021 & MTT)

I part

- MTT primers & ingredients
- Exercises about Ct in diluted or concentrated samples
- relative expression of soluble NRG1 & ErbB2
- absolute expression of ErbB4

Question 1 Not yet answered Marked out of 1.00

To amplify this entire sequence you have to prepare a sense and an antisense primer.

```
5′-ATGGAGGGCG CCGGCGGCGA GAACGAGAAG AAAAATAGGA TGAGTTCCGA GCACGTCGAA-3′
3′-TACCTCCCGC GGCCGCCGCT CTTGCTCTTC TTTTTATCCT ACTCAAGGCT CGTGCAGCTT-5′
```

Please, design sense and antisense primers 10 base long, writing only nucleotides, not numbers or symbols:

SENSE:

ANTISENSE:

Question 1

Not yet answered Marked out of 1.00

You have to prepare a reverse transcription reaction.

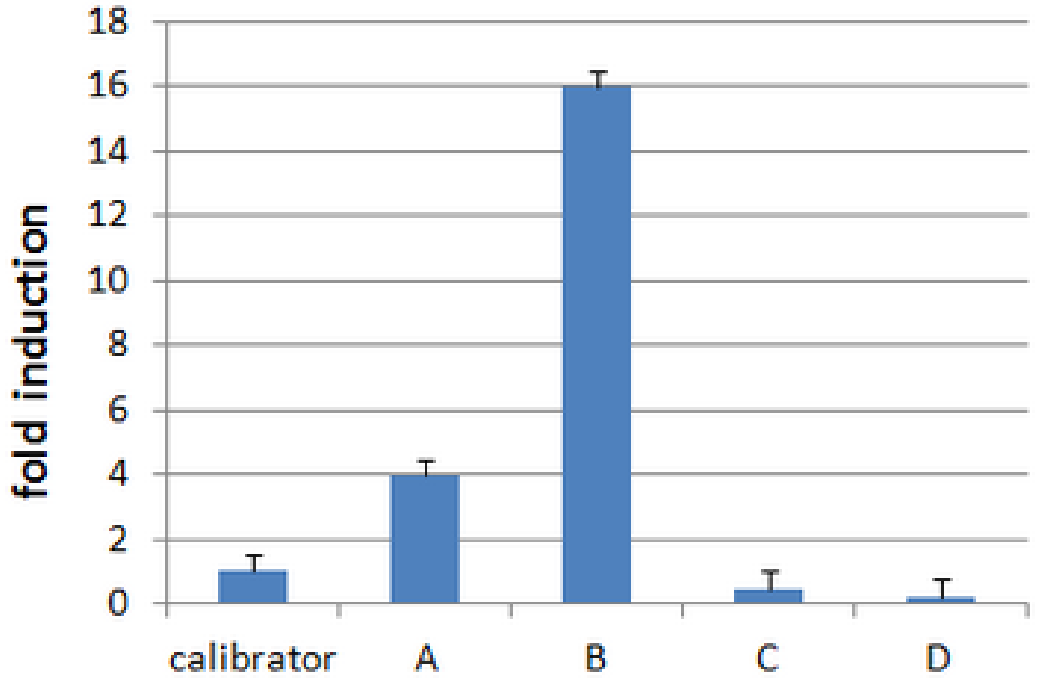
Please fill in the protocol writing the µl to be added for the missing ingredients (RNA, buffer, BSA).

Use the point (.) instead of a comma (,) for decimals; write only the number, not "µl"

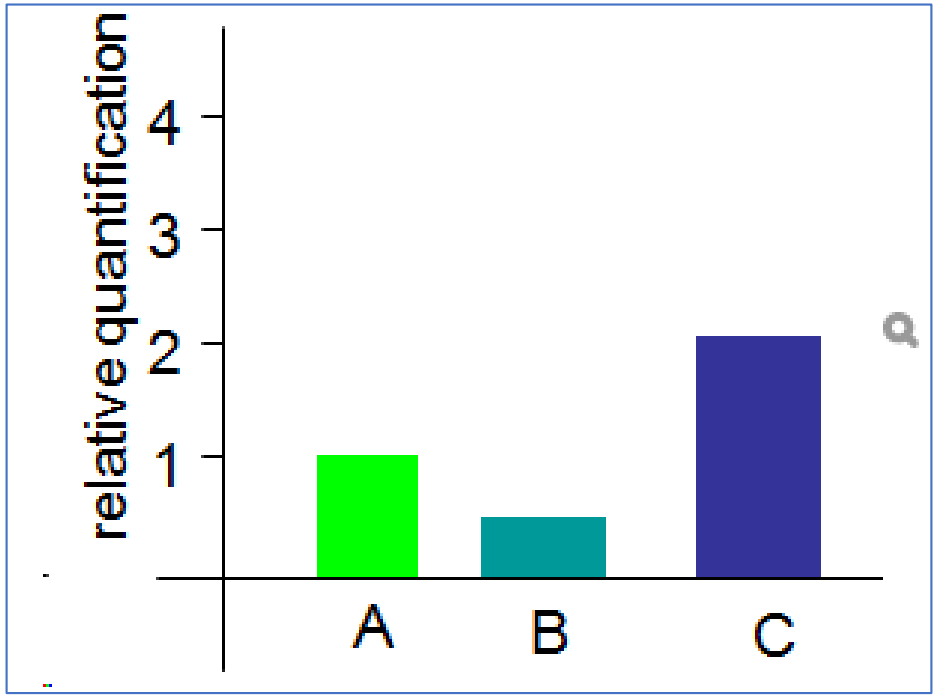
ingredients	stock concentration	amunt(in ul)	final concentration (or final amount)
RNA	0.20 µg/µl	µl	1 µg
Buffer	10x	µl	1x
BSA	1µg/µl	µl	0.01µg/µl
reverse transcriptase	50 units/µl	µl	200units/reaction
RNAse inhibitor	30 units/µl	µl	60 units/reaction
H_2O		enough to 100 µl	
total		100 µl	

The threshold cicle (CT) of your calibrator sample is 20.

Please, watch carefully this graph: which will be the CTs of samples A, B, C, D?



sample	fold induction	CT
calibrator	1	20
A	4	
B	16	
C	0.5	
D	0.25	



The threshold cycle (CT) of a sample is determined by the amount of template present in that sample at the beginning of the amplification reaction.

In your experiment, **A** is your calibrator sample with a **CT=22**, therefore:
- the relative expression of the calibrator is set = **1**.

- If the relative expression of sample B is 0.5 (see the figure), which is the **CT** of sample **B**?

- If the relative expression of sample C is 2 (see the figure), which is the **CT** of sample **C**?

- Which will be the relative expression of a sample with a **CT=25**?

- Which will be the relative expression of a sample with a **CT=20**?

(use dots and not commas for decimals)

Exercise 1

Real time PCR, relative quantification

	strain	age	sample number	CT soluble NRG1	CT ErbB2	CT HKG
5	WT	P3	59.55	31,393	22,62	24,33
6			59.57	30,725	22,33	24,23
7			59.59	30,416	22,33	24,09
8		P16	59.67	29,293	23,83	24,68
9			59.68	29,415	23,7	25,06
10			59.69	28,97	23,51	24,63
11		1 month	59.73	31,263	25,25	25,32
12			59.74	30,977	25,96	25,74
13			59.75	30,649	24,72	25,24
14		2 months	59.79	32,22	27,13	27,12
15			59.80	32,027	27,82	26,7
16			59.81	33,01	28,12	27,41
17	CMT1A +/-	P3	59.61	30,658	22,87	24,69
18			59.63	30,221	22,41	24,14
19			59.65	29,808	22,3	23,99
20		P16	59.70	25,753	22,6	24,65
21			59.71	25,477	23,75	24,59
22			59.72	25,852	23,02	24,79
23		1 month	59.76	25,096	23,86	24,65
24			59.77	25,851	24,08	24,74
25			59.78	25,588	24,83	25,05
26		2 months	59.82	26,295	25,57	26,2
27			59.83	27,057	26,04	26,19

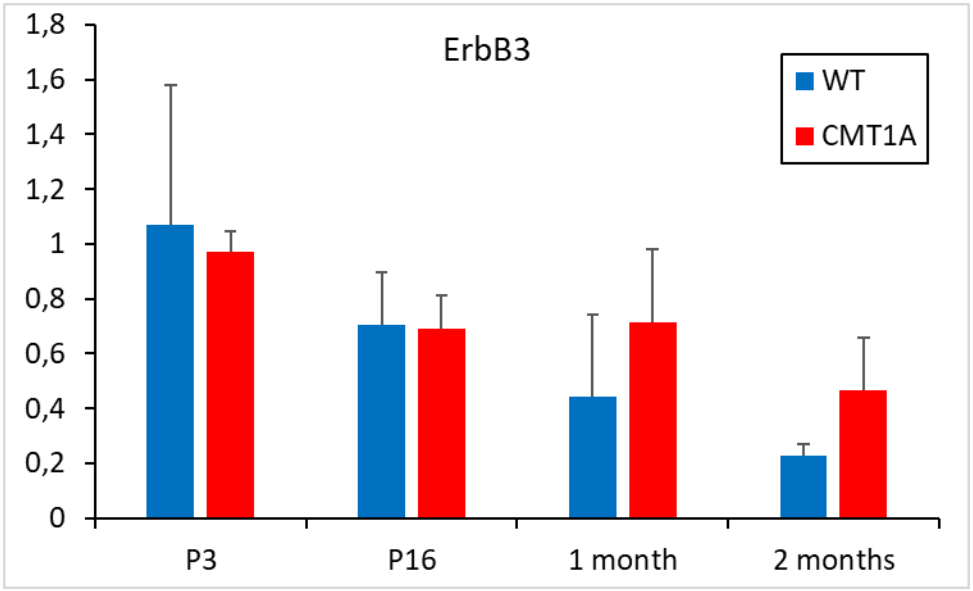
soluble NRG1 | ErbB2 | ErbB3 | HKG

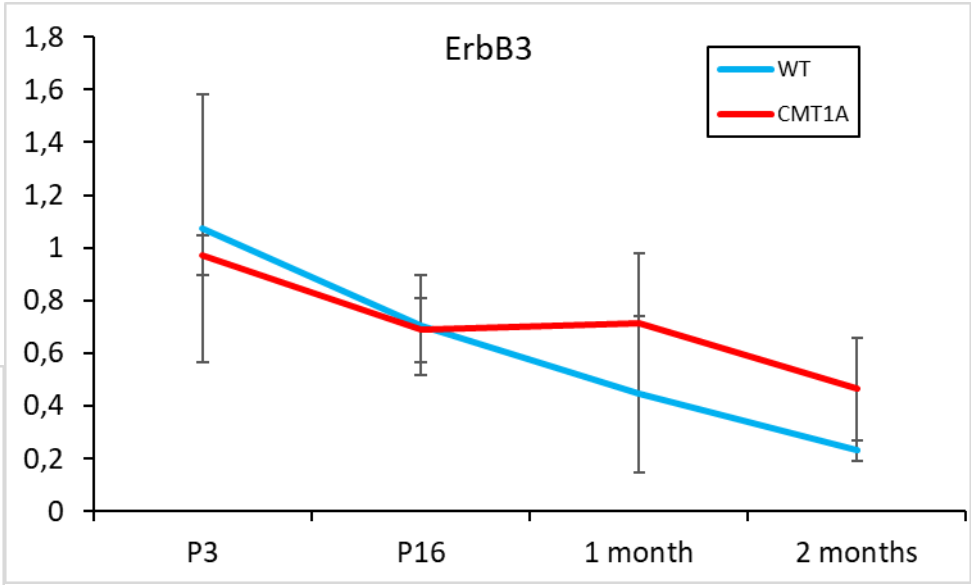
Exercise 1 Example

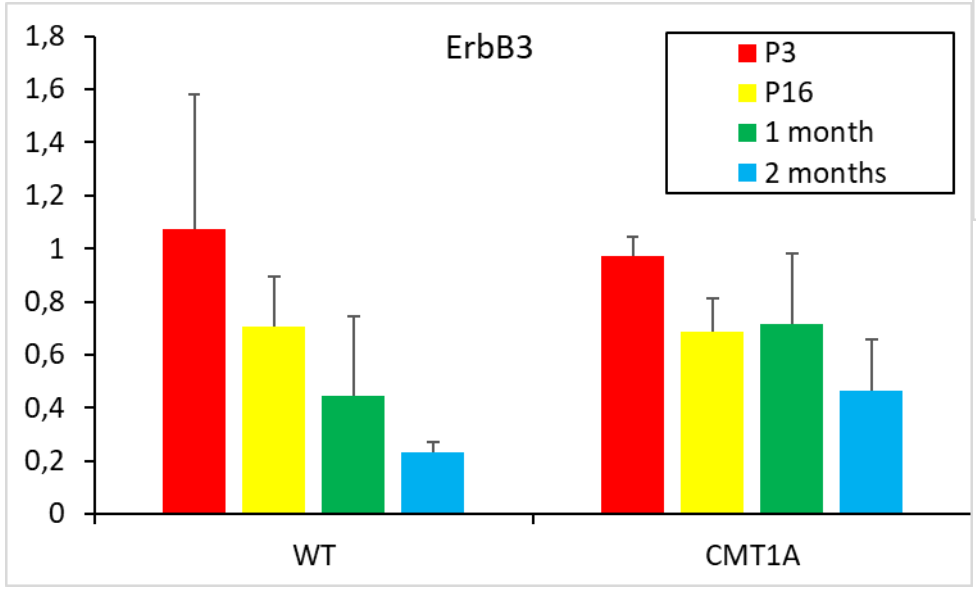
	B	C	D	E	F	G	H	I	J	K	L
4	strain	age	sample number	CT ErbB3	CT HKG	Δ CT	ΔΔCT	−ΔΔCT	2^-ΔΔCt	average	standard deviation
5	WT	P3	59.55	20,115	24,33	-4,219	0,218	-0,218	0,85975649	1,07189	0,5081638
6			59.57	19,072	24,23	-5,161	-0,724	0,724	1,65175533		
7			59.59	20,155	24,09	-3,931	0,506	-0,506	0,70417211		
8		P16	59.67	20,933	24,68	-3,748	0,689	-0,689	0,62028365	0,70646	0,1885933
9			59.68	21,418	25,06	-3,642	0,795	-0,795	0,57634317		
10			59.69	20,309	24,63	-4,321	0,116	-0,116	0,92274249		
11		1 month	59.73	22,639	25,32	-2,678	1,759	-1,759	0,29545289	0,44461	0,2981065
12			59.74	23,296	25,74	-2,44	1,997	-1,997	0,2505204		
13			59.75	21,15	25,24	-4,093	0,344	-0,344	0,78785389		
14		2 months	59.79	24,729	27,12	-2,392	2,045	-2,045	0,24232245	0,23037	0,0399401
15			59.80	24,694	26,7	-2,009	2,428	-2,428	0,18582287		
16			59.81	24,899	27,41	-2,51	1,927	-1,927	0,26297544		
17	CMT1A	P3	59.61	20,242	24,69	-4,44567	-0,009	0,00867	1,00602768	0,97079	0,0756747
18			59.63	19,881	24,14	-4,259	0,178	-0,178	0,88392753		
19			59.65	19,524	23,99	-4,469	-0,032	0,032	1,02242853		
20		P16	59.70	20,628	24,65	-4,025	0,412	-0,412	0,75158074	0,68878	0,1222355
21			59.71	20,54	24,59	-4,054	0,383	-0,383	0,76684133		
22			59.72	21,218	24,79	-3,569	0,868	-0,868	0,54790588		
23		1 month	59.76	20,988	24,65	-3,664	0,773	-0,773	0,58519932	0,71455	0,2651889
24			59.77	20,279	24,74	-4,465	-0,028	0,028	1,01959768		
25			59.78	21,502	25,05	-3,545	0,892	-0,892	0,53886657		
26		2 months	59.82	22,956	26,2	-3,243	1,194	-1,194	0,43708931	0,46516	0,1945434
27			59.83	22,323	26,19	-3,864	0,573	-0,573	0,6722175		
28			59.84	23,591	26,22	-2,632	1,805	-1,805	0,28618104		

soluble NRG1 | ErbB2 | **ErbB3** | HKG

Exercise 1
Example







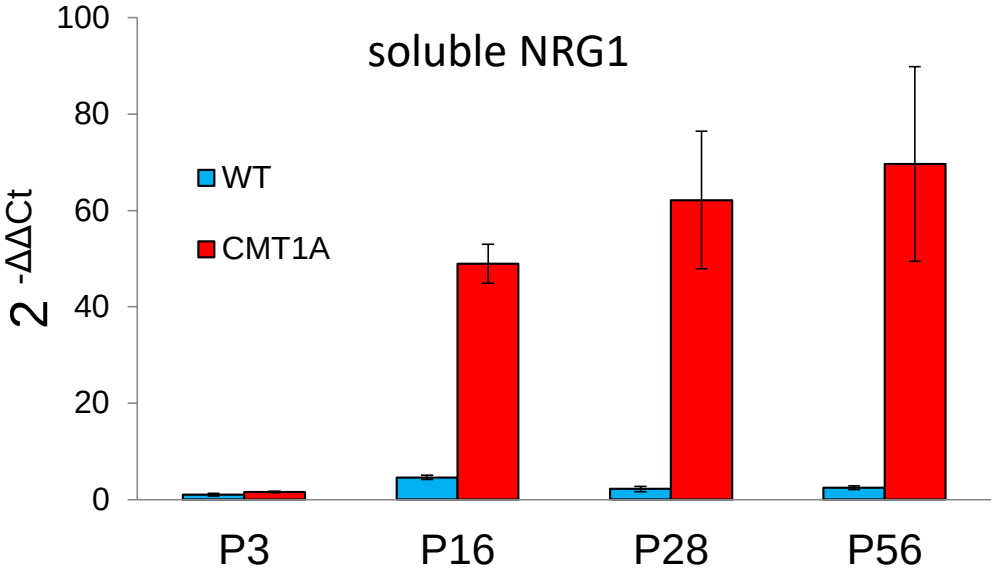
Exercise 1

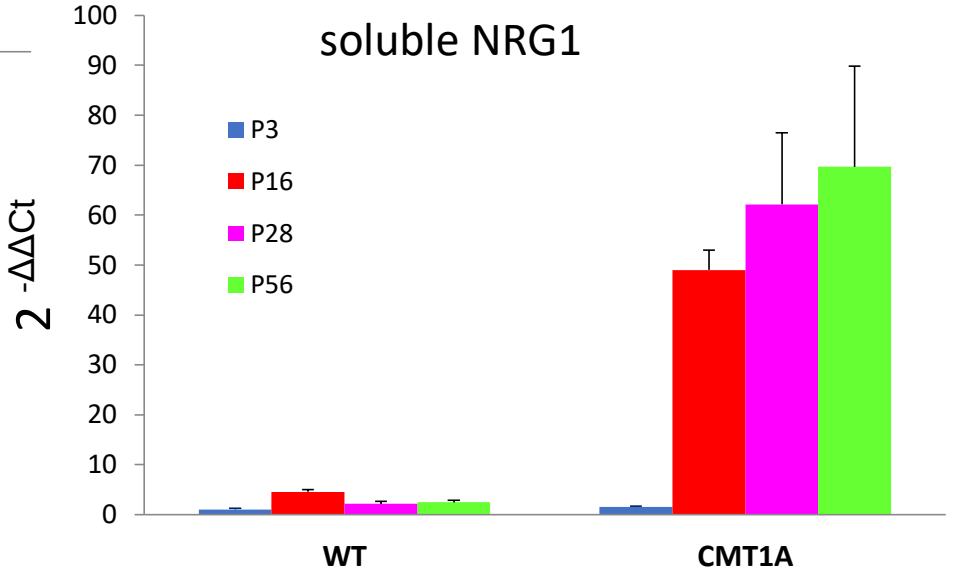
NRG1 real time PCR, relative quantification

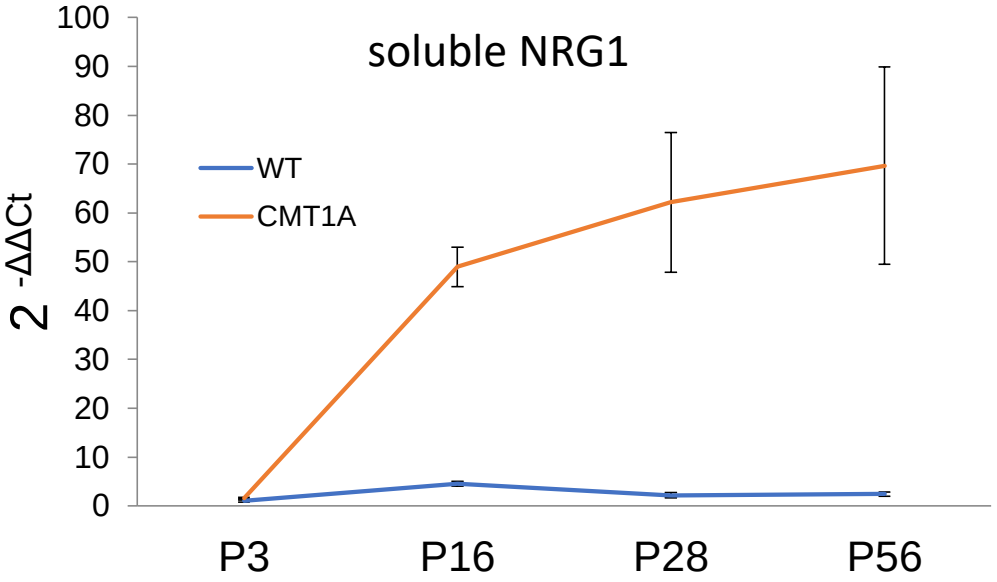
strain	age	#	CT NRG1 I/II	CT HKG	Δ CT	ΔΔCT	–ΔΔCT	2^-ΔΔCt	average	standard deviation
WT	P3	59.55	31,393	24,33	7,059	0,432	-0,432	0,741233505	1,02263777	0,252286446
		59.57	30,725	24,23	6,492	-0,135	0,135	1,098092814		
		59.59	30,416	24,09	6,33	-0,297	0,297	1,22858698		
	P16	59.67	29,293	24,68	4,612	-2,015	2,015	4,041805786	4,5840434	0,470269359
		59.68	29,415	25,06	4,355	-2,272	2,272	4,829922366		
		59.69	28,97	24,63	4,34	-2,287	2,287	4,880402041		
	1 month	59.73	31,263	25,32	5,946	-0,681	0,681	1,603250659	2,18262119	0,521245519
		59.74	30,977	25,74	5,241	-1,386	1,386	2,613530508		
		59.75	30,649	25,24	5,406	-1,221	1,221	2,331082396		
	2 months	59.79	32,22	27,12	5,099	-1,528	1,528	2,883857743	2,46254749	0,423764633
		59.80	32,027	26,7	5,324	-1,303	1,303	2,46741434		
		59.81	33,01	27,41	5,601	-1,026	1,026	2,0363704		
CMT1A +/-	P3	59.61	30,658	24,69	5,97033	-0,65667	0,65667	1,576439714	1,59737177	0,148913282
		59.63	30,221	24,14	6,081	-0,546	0,546	1,460032011		
		59.65	29,808	23,99	5,815	-0,812	0,812	1,755643595		
	P16	59.70	25,753	24,65	1,1	-5,527	5,527	46,10975196	48,9819427	4,034097221
		59.71	25,477	24,59	0,883	-5,744	5,744	53,5940152		
		59.72	25,852	24,79	1,065	-5,562	5,562	47,24206098		
	1 month	59.76	25,096	24,65	0,444	-6,183	6,183	72,65549454	62,1577159	14,28785161
		59.77	25,851	24,74	1,107	-5,52	5,52	45,88656794		
		59.78	25,588	25,05	0,541	-6,086	6,086	67,93108537		
	2 months	59.82	26,295	26,2	0,096	-6,531	6,531	92,47554547	69,6591412	20,19517839
		59.83	27,057	26,19	0,87	-5,757	5,757	54,0791286		
		59.84	26,886	26,22	0,663	-5,964	5,964	62,42274954		

Exercise 1

NRG1 real time PCR, relative quantification







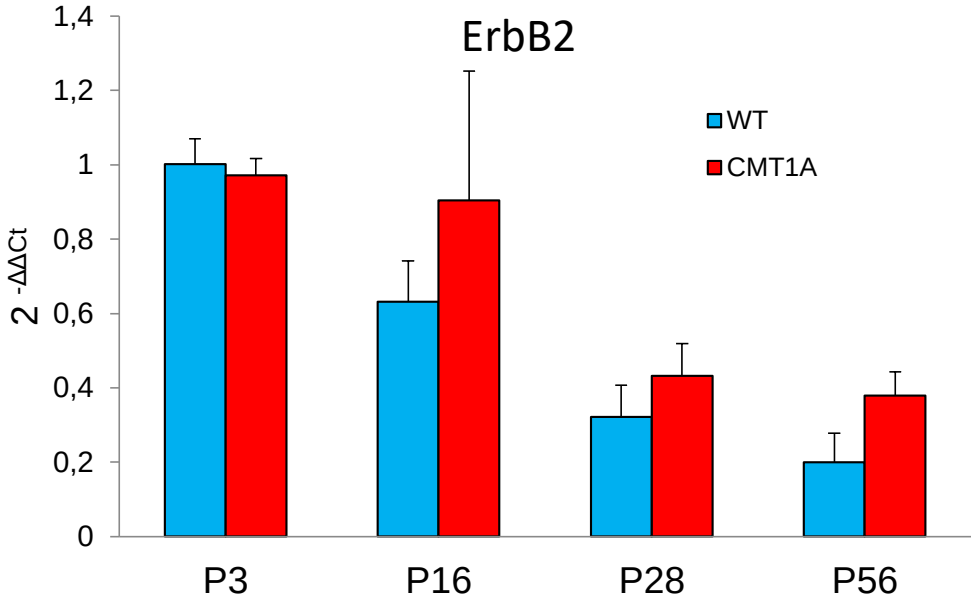
Exercise 1

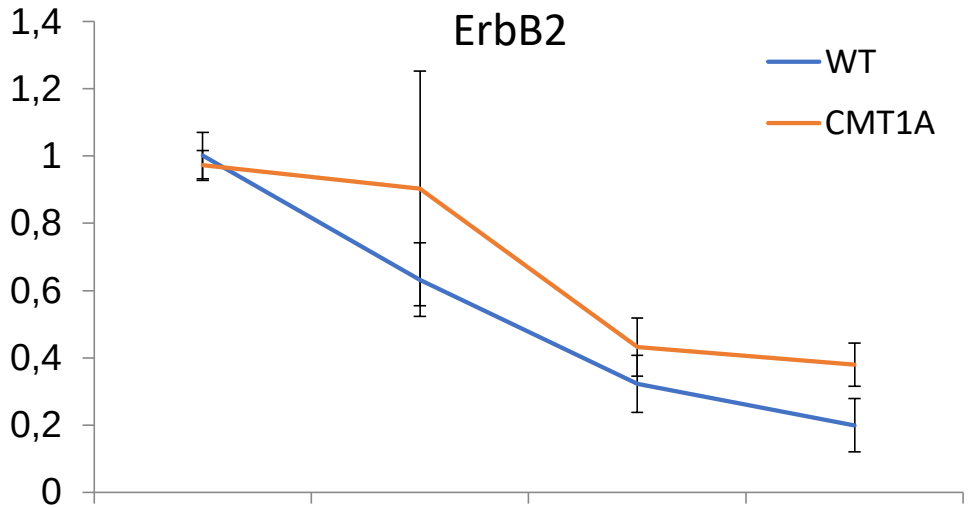
ErbB2 real time PCR, relative quantification

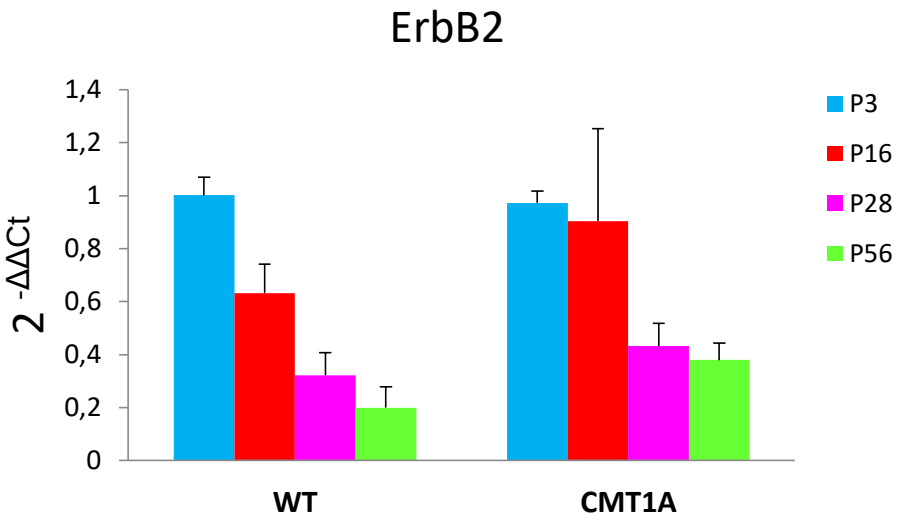
strain	age	#	CT ErbB2	CT HKG	Δ CT	ΔΔCT	–ΔΔCT	2^-ΔΔCt	average	standard deviation
WT	P3	59.55	22,619	24,33	-1,715	0,074	-0,074	0,950000383	1,00155219	0,06902936
		59.57	22,333	24,23	-1,9	-0,111	0,111	1,07997656		
		59.59	22,334	24,09	-1,752	0,037	-0,037	0,974679631		
	P16	59.67	23,827	24,68	-0,854	0,935	-0,935	0,52304247	0,63214342	0,109613199
		59.68	23,701	25,06	-1,359	0,43	-0,43	0,742261785		
		59.69	23,505	24,63	-1,125	0,664	-0,664	0,631126016		
	1 month	59.73	25,25	25,32	-0,067	1,722	-1,722	0,303128205	0,32232692	0,084951682
		59.74	25,955	25,74	0,219	2,008	-2,008	0,248617542		
		59.75	24,722	25,24	-0,521	1,268	-1,268	0,415235012		
	2 months	59.79	27,131	27,12	0,01	1,799	-1,799	0,287373712	0,19940392	0,079221312
		59.80	27,817	26,7	1,114	2,903	-2,903	0,133693386		
		59.81	28,117	27,41	0,708	2,497	-2,497	0,177144675		
CMT1A	P3	59.61	22,869	24,69	-1,81867	-0,02967	0,02967	1,020778608	0,97234447	0,044157001
		59.63	22,407	24,14	-1,733	0,056	-0,056	0,961927455		
		59.65	22,302	23,99	-1,691	0,098	-0,098	0,934327347		
	P16	59.70	22,596	24,65	-2,057	-0,268	0,268	1,204137381	0,90362696	0,349219283
		59.71	23,747	24,59	-0,847	0,942	-0,942	0,520510799		
		59.72	23,018	24,79	-1,769	0,02	-0,02	0,986232704		
	1 month	59.76	23,856	24,65	-0,796	0,993	-0,993	0,50243191	0,43226993	0,086321039
		59.77	24,08	24,74	-0,664	1,125	-1,125	0,458502022		
		59.78	24,832	25,05	-0,215	1,574	-1,574	0,335875856		
	2 months	59.82	25,574	26,2	-0,625	1,164	-1,164	0,446273486	0,37947975	0,063558343
		59.83	26,043	26,19	-0,144	1,645	-1,645	0,319746395		
		59.84	25,859	26,22	-0,364	1,425	-1,425	0,372419366		

Exercise 1

ErbB2 real time PCR, relative quantification



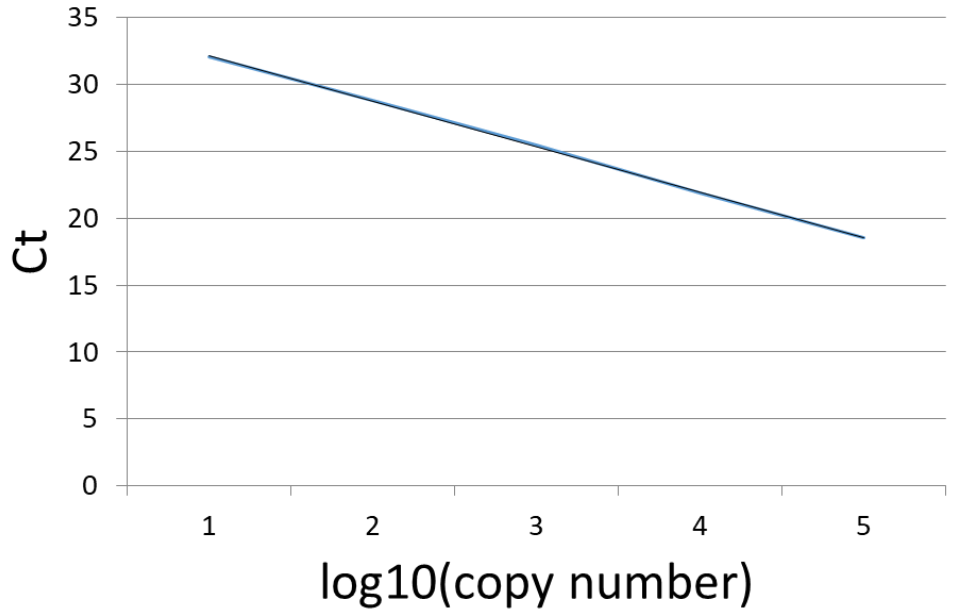




Exercise 2

ErbB4			
copy number	log_{10}(copy number)	Ct	Ct average
10	1	32,1	
		31,85	
		32,35	
100	2	28,5	
		28,6	
		28,95	
1000	3	25,36	
		25,58	
		25,94	
10000	4	21,87	
		21,98	
		21,84	
100000	5	18,69	
		18,55	
		18,49	

ErbB4, real time PCR, absolute quantification



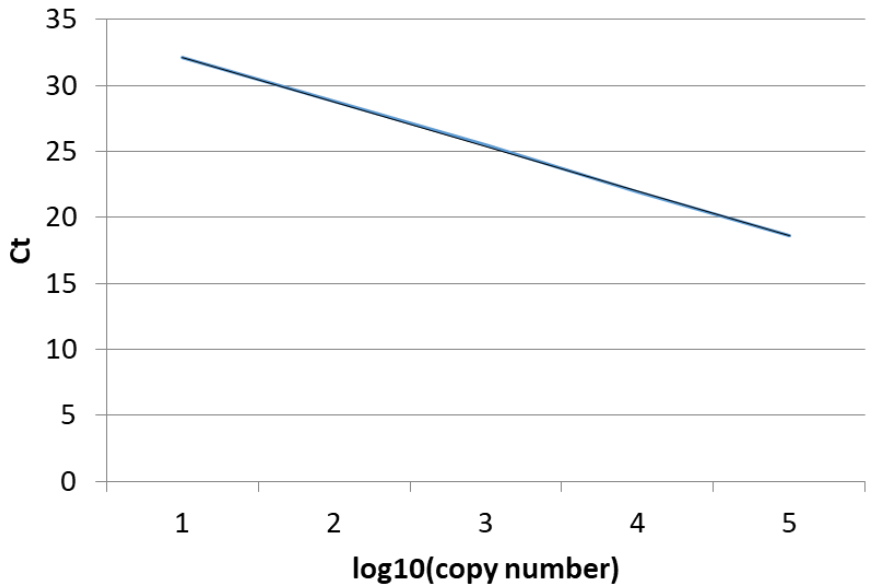
1-Calculate the Ct average

2-Prepare the regression line and display the regression equation (on X axis: log_{10} (copy number); on Y axis: Ct average)

Calculate the Ct average

Sample	Detector	Task	Ct	Avg Ct
1	ErbB4	Target	23,388	
1	ErbB4	Target	23,248	
2	ErbB4	Target	21,828	
2	ErbB4	Target	21,727	
3	ErbB4	Target	24,464	
3	ErbB4	Target	24,589	
4	ErbB4	Target	18,217	
4	ErbB4	Target	18,205	
5	ErbB4	Target	28,07	
5	ErbB4	Target	27,905	
6	ErbB4	Target	31,951	
6	ErbB4	Target	32,274	

Starting from the Ct average calculate the number of template copy using the regression equation



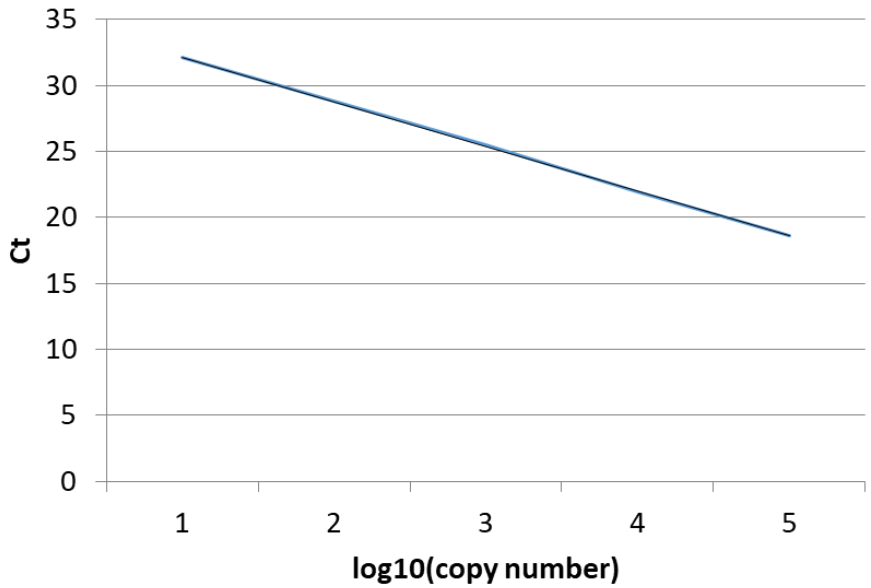
$y=mx + n$

$x=(y-n)/m$

$\log_{10}(\text{copy number})=(CT - n)/m$

Sample	Avg Ct	$\log_{10}$(copy number)	copy number
1			
2			
3			
4			
5			
6			

Starting from the Ct average calculate the number of template copy using the regression equation



$y=mx + n$

$x=(y-n)/m$

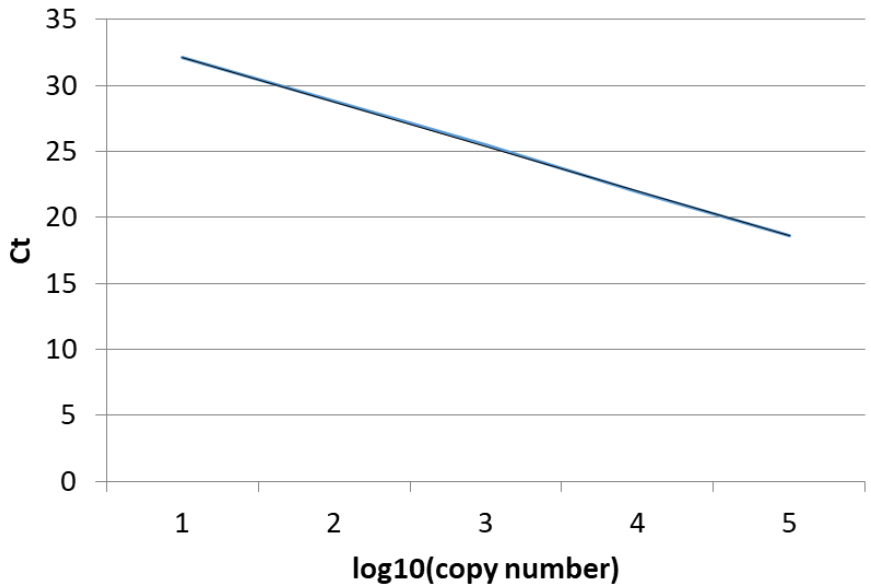
$\log_{10}(\text{copy number})=(CT - n)/m$

The logarithm of a number is the exponent to which another number, the base, must be raised to produce that number.

If $\mathbf{10^y = x}$, then the logarithm of x to base 10, denoted $\mathbf{\log_{10}(x) = y}$

Sample	Avg Ct	$\log_{10}$(copy number)	copy number
1			
2			
3			
4			
5			
6			

Starting from the Ct average calculate the number of template copy using the regression equation



$y=mx + n$

$x=(y-n)/m$

$\log_{10}(\text{copy number})=(CT - n)/m$

The logarithm of a number is the exponent to which another number, the base, must be raised to produce that number.

If $\mathbf{10^y = x}$, then the logarithm of x to base 10, denoted $\mathbf{\log_{10}(x) = y}$

$$\mathbf{10^{\log10(x)} = x}$$

$$\mathbf{10^{\log10\,(copy\ number)} = copy\ number}$$

Sample	Avg Ct	$\log_{10}$(copy number)	copy number
1			
2			
3			
4			

PROTEIN CONCENTRATION

Exercize: Prepare the regression line and display the regression equation. Starting from the OD average calculate the protein concentration and the µl necessary to load in gel 40 µg proteins.

standard curve

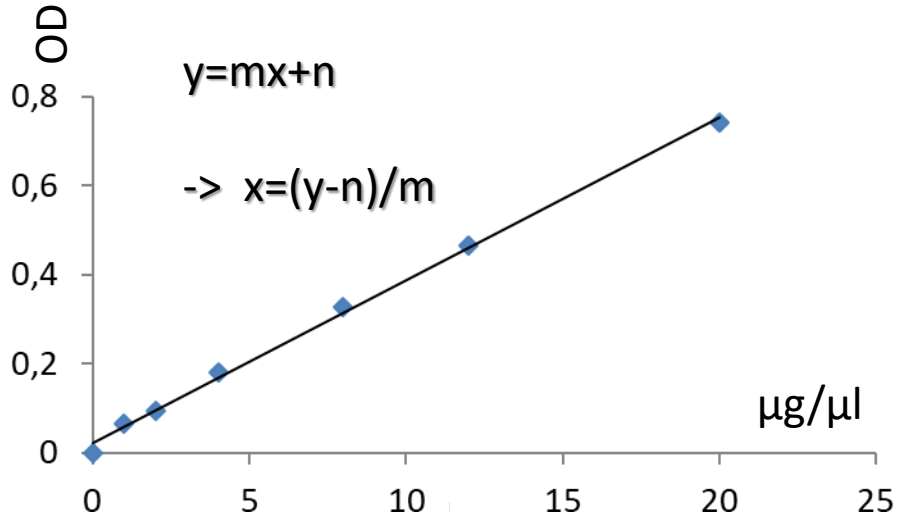
BSA (µg/µl)	OD 1	OD2	OD average	OD-blank
0	0,111	0,119		
1	0,179	0,183		
2	0,205	0,21		
4	0,283	0,306		
8	0,437	0,448		
12	0,572	0,589		
20	0,85	0,866		

PROTEIN CONCENTRATION

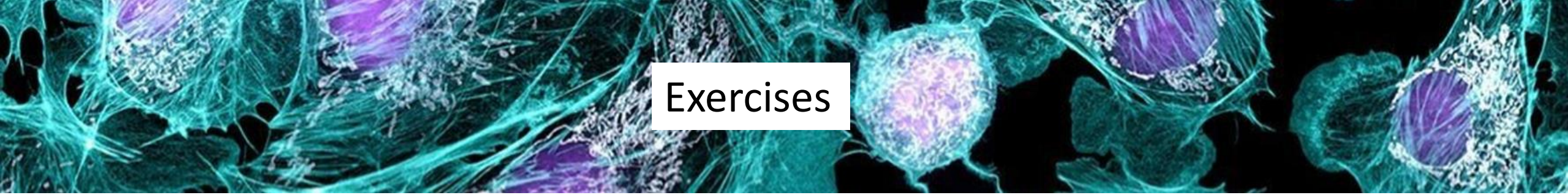
Exercize: Prepare the regression line and display the regression equation. Starting from the OD average calculate the protein concentration and the µl necessary to load in gel 40 µg proteins.

standard curve

BSA (µg/µl)	OD 1	OD2	OD average	OD-blank
0	0,111	0,119		
1	0,179	0,183		
2	0,205	0,21		
4	0,283	0,306		
8	0,437	0,448		
12	0,572	0,589		
20	0,85	0,866		



genotype	age	#	OD1	OD2	OD average	OD -blank	µg/µl	µl/40µg
WT	P3	**59.55**	0,201	0,22				
WT	P16	**59.67**	0,418	0,412				
WT	1m	**59.73**	0,435	0,451				
WT	2m	**59.79**	0,388	0,408				
CMT1A	P3	**59.61**	0,329	0,337				
CMT1A	P16	**59.70**	0,532	0,538				
CMT1A	1m	**59.76**	0,685	0,673				
CMT1A	2m	**59.82**	0,686	0,7				



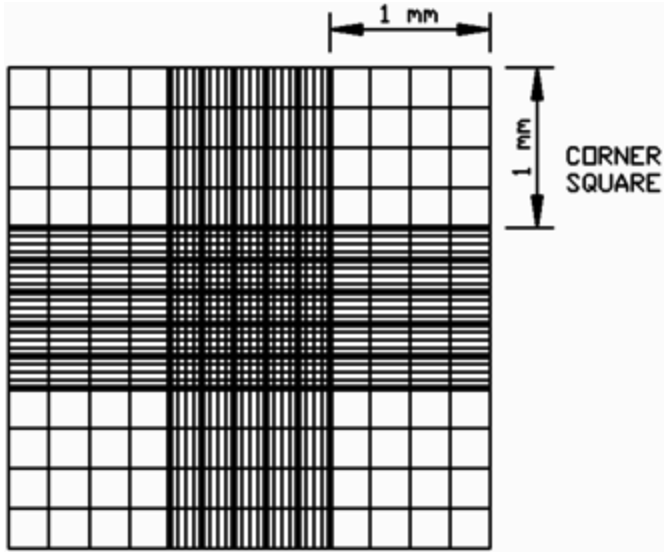
Exercises

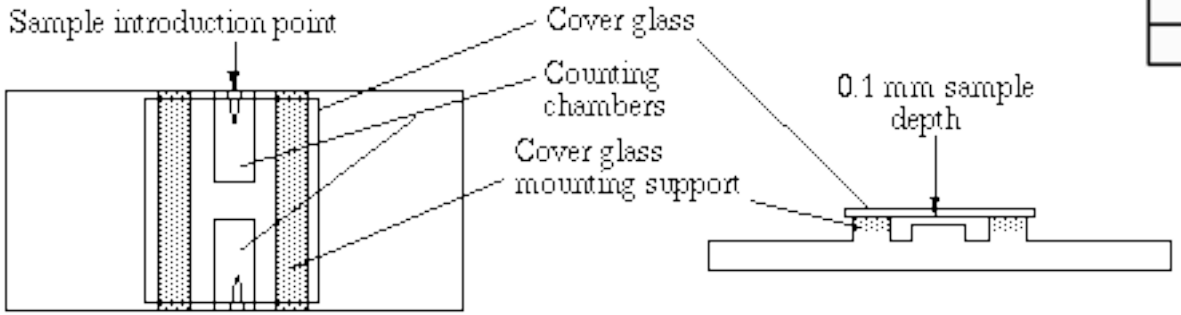
II part

- proliferation assay
- transwell assay
- reagents for western blot

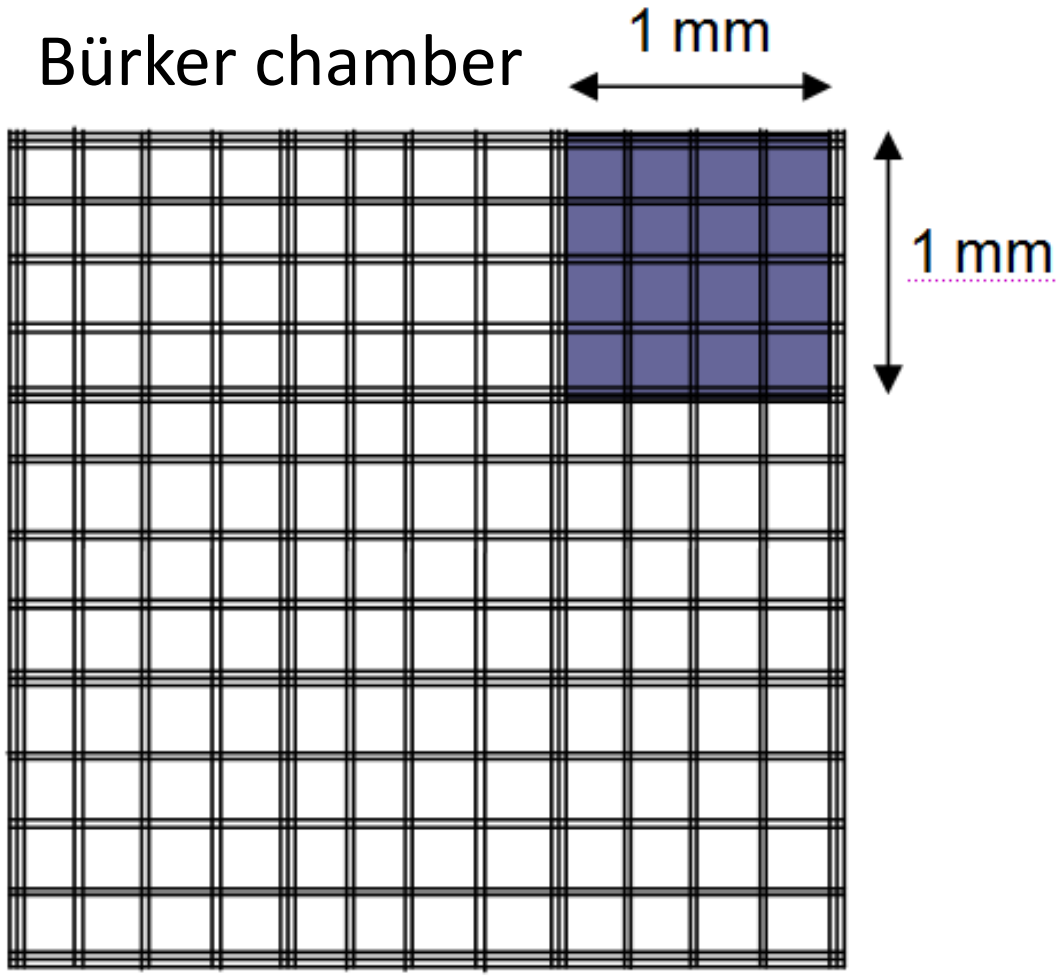
Cell count

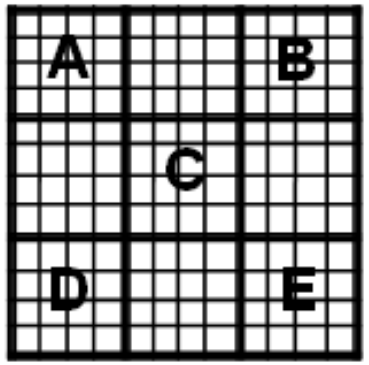

0.0025 mm²
0.04 mm²
MARIENFELD
LABORATORY GLASSWARE


1 mm
1 mm
CORNER SQUARE


Sample introduction point
Cover glass
Counting chambers
Cover glass mounting support
0.1 mm sample depth

Bürker chamber



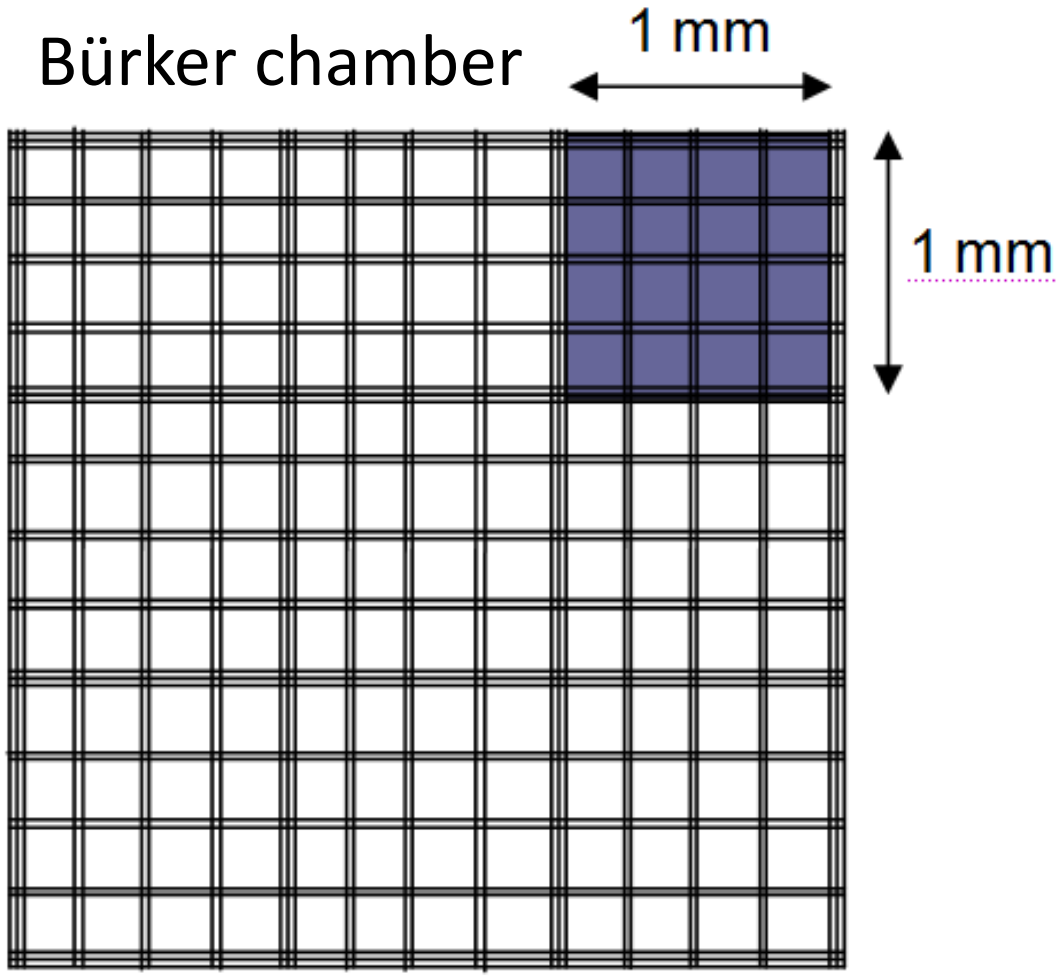


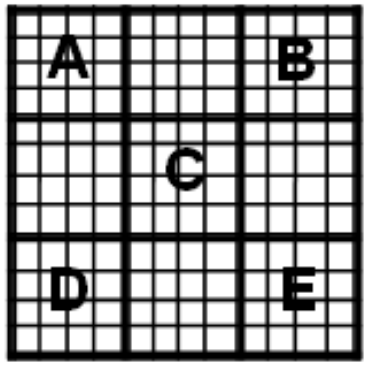
$$\frac{A+B+C+D+E}{5}$$

1mm X 1mm X 0,1mm = 0,1 mm^3

If you count the number of cells in 0,1 mm^3 , how can you calculate the number of cells in 1ml?

Bürker chamber





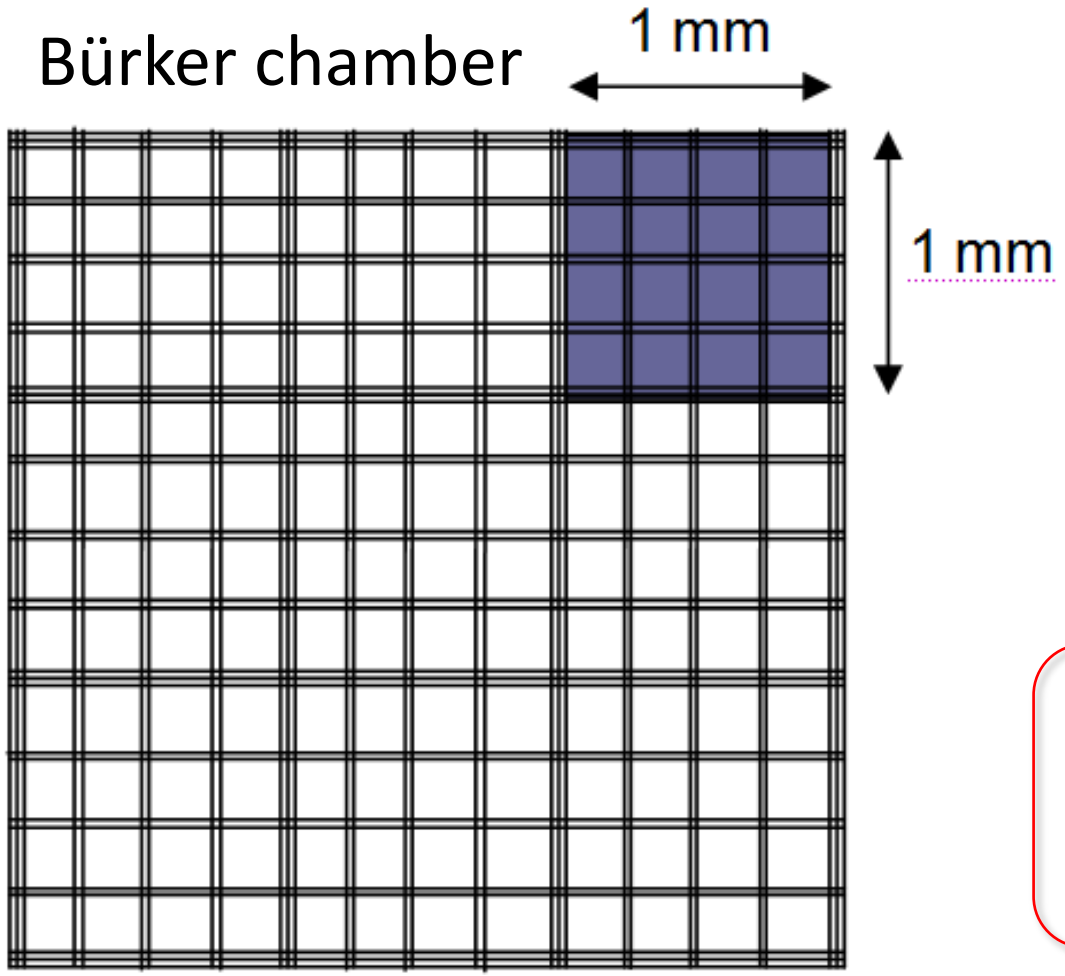
$$\frac{A+B+C+D+E}{5}$$

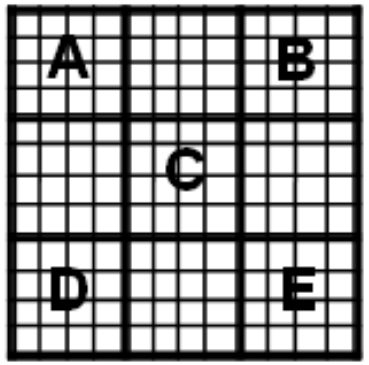
1mm X 1mm X 0,1mm = 0,1 mm^3

If you count the number of cells in 0,1 mm^3 , how can you calculate the number of cells in 1ml?

1l = 1000 ml = 1 dm^3 = 10cm X 10cm X 10cm = 1000 cm^3
1ml = 1cm^3 = 10mm X 10mm X 10mm = 10^3 mm^3

Bürker chamber





$$\frac{A+B+C+D+E}{5} \cdot 10^4 = \text{cells/ml}$$

$1mm \times 1mm \times 0{,}1mm = 0{,}1\ mm^3$

$1ml = 1cm^3 = 10mm \times 10mm \times 10mm = 10^3\ mm^3$

If you count the number of cells in $0{,}1\ mm^3$, how can you calculate the number of cells in 1ml?

$1ml = 0{,}1mm^3 \times 10^4$ ⟶ cells counted in $0{,}1mm^3$ must be multiplied X 10^4

Exercise #1 - Proliferation assay

- take a 10cm diameter plate containing confluent cells
- aspirate medium
- wash with 5 ml PBS
- aspirate PBS
- add 1,5 ml trypsin, incubate 2 min at 37°C
- add 6,5 ml medium containing 10% FBS (foetal bovine serum) to inactivate trypsin
- resuspend well the cells pipetting up and down
- take a drop to count the cell number with the Bürker chamber
- you count for example: 15 27 32 26 20

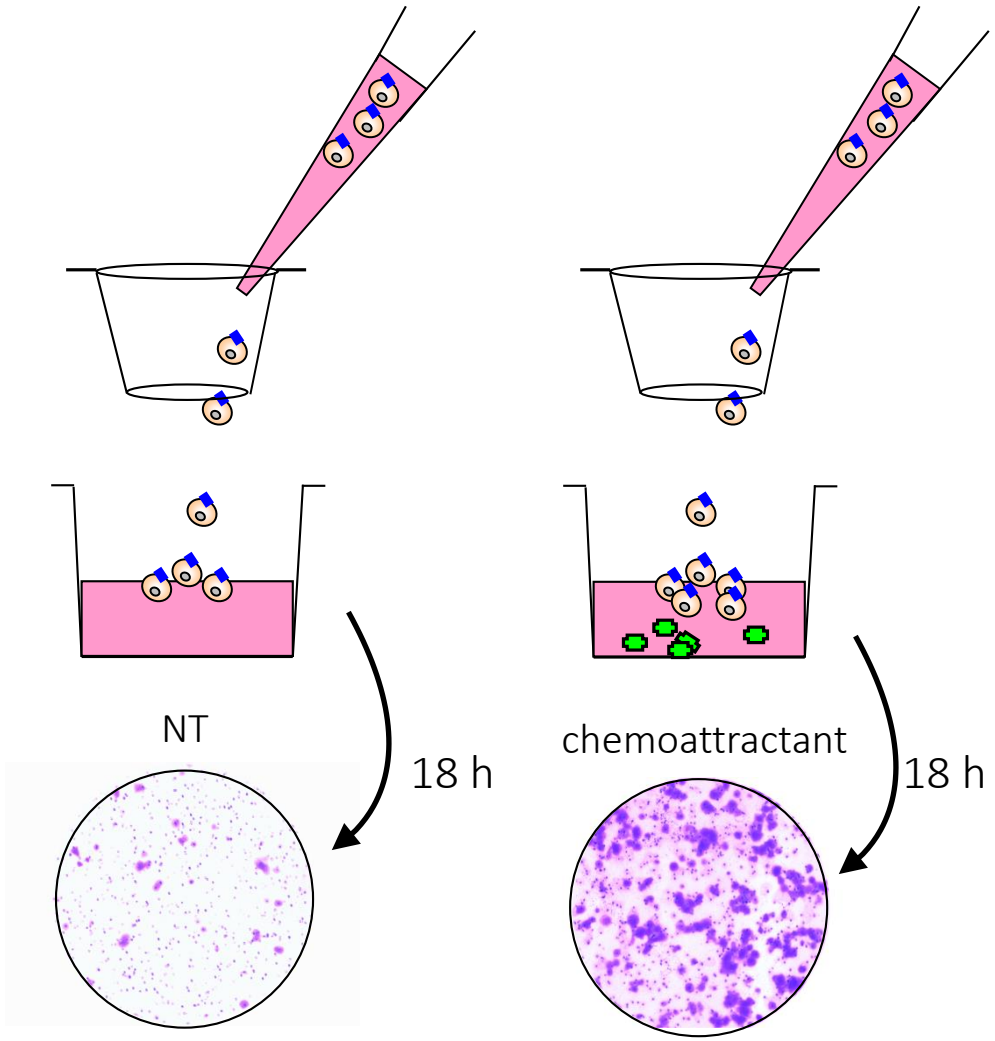
- how many cells do you have in 1 ml?
- how many µl do you have to use if you want to plate 10^5 cells?

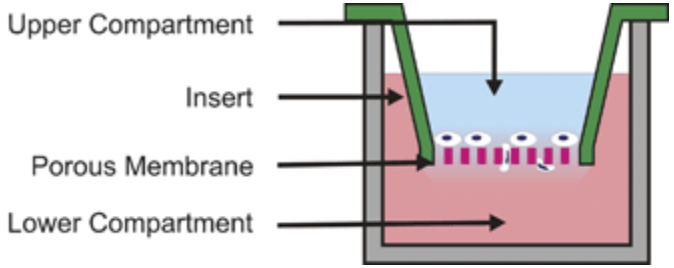
- if you want to do a proliferation assay in a 96 well plate, you have to use 10^3 cells/well
- how many µl of cells do you need if you want to plate 1000 cells in a well?
- if you prepare 4 plates 96 wells for a time course assay (control=time 0, 1 day, 3 days, 5 days) you can prepare a solution containing all the cells and all the medium necessary for the entire experiment.
- If you add 100µl/ well and 1000 cells/well, how do you prepare your cell mix solution?
- how many cells? Number and µl?
- how much medium? ml?

- in the lab usually we prepare a solution more abundant in order to be sure to have enough material, but now we plan to prepare the precise volume

TRANSWELL ASSAY

http://www.youtube.com/watch?v=6SON7VAA5-k





Exercise #2 – Transwell assay

Migration Assay

The Transwell migration assay was used to measure three-dimensional movement. Cells (**10^5**) resuspended in **200 µl** of DMEM containing 2% FBS were seeded in the upper chamber of a Transwell (cell culture insert, no. 353097, BD Biosciences) on a porous transparent polyethylene terephthalate membrane (8.0-µm pore size, 1 X 10^5 pores/cm^2). The lower chamber (a 24-well plate well) was filled with 800 µl DMEM containing 2% FBS with or without 5 nM recombinant NRG1β1. The 24-well plates containing cell culture inserts were incubated at 33 °C in a 5% CO_2 atmosphere saturated with H_2O. After 18 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed with 2% glutaraldehyde in PBS for 15 min at room temperature, washed five times with water, stained with 0.1% crystal violet and 20% methanol for 20 min at room temperature, washed five times with water, air-dried, and photographed using an Olympus IX50 inverted microscope equipped with a Cool SNAP-Pro CCD camera; images were edited with Image Pro-Plus software.

Exercise #2 – Transwell assay

- grow cells until confluence in a 10cm diameter dish
- aspirate medium
- wash with 5 ml PBS
- aspirate PBS
- add 1,5 ml trypsin, incubate 2 min at 37°C
- add 6,5 ml medium containing 10% FBS (foetal bovine serum)
- resuspend well the cells pipetting up and down
- take a drop to count the cell number with the Bürker chamber
- spin cells in the centrifuge 5 min, 800 rpm, room temperature
- discard supernatant
- resuspend the pellet in **XX** ml of 2% FBS DMEM in order to have a suitable concentration;

-> Indeed, you want to pipet **200 µl** containing **10^5** cells in different transwells

- Add 200 µl containing 10^5 cells to different transwell and put them in a multiwell containing 800 µl 2% FBS DEMEM with or without ligands.

Exercise #2 – Transwell assay

Question:

If you count **35 51 45 31 46** cells in five squares of the Bürker chamber (0,1mm^3 each)

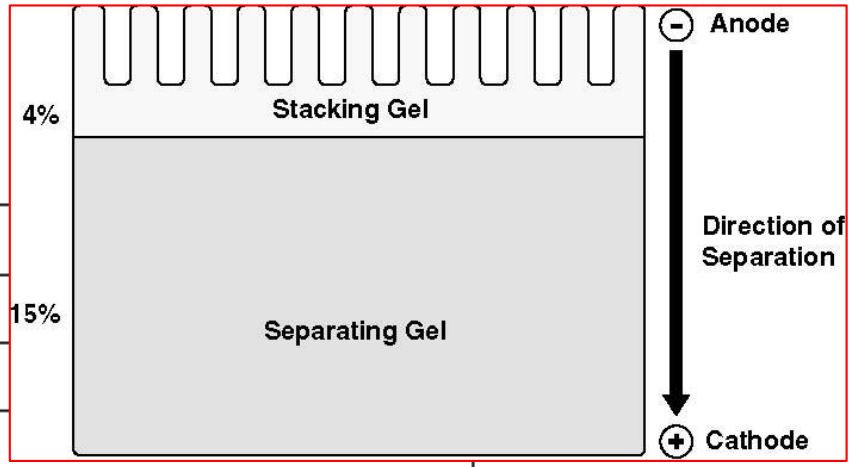
- How many cells do you have? …..
- in which volume of 2% FBS DMEM do you have to resuspend the cells to have 100000 cells in 200 µl? ….ml (use 2 decimal numbers)

To be more precise, you count again your cells to be sure that you really put 10^5 cells/transwell.

You count again and you find: **46 44 42 50 34**

How many microliters of cells do you have to put in the transwell to have 10^5 cells? (no decimal numbers)

Exercise # 3 - Western blot



- **For a 5 ml stacking gel:**

H_2O	2.975 ml
→ 0.5 M Tris-HCl, pH 6.8	1.25 ml
→ 10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

- **For a 10ml separating gel:**

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
→ 1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
→ 10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

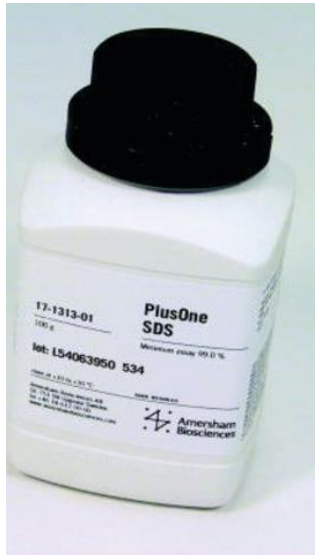
Exercise # 3 – Reagents for western blot

TRIS Base

Molecular Weight 121,14

1,5 M Tris pH 8.8 - How many grams for 250 ml?

0,5 M Tris pH 6,8 - How many grams for 250 ml?



Sodium dodecyl sulfate (SDS)

Molecular Weight 288.38

10% SDS - How many grams for 250 ml?