RT-qPCR

Results of the quiz

1. Did you learn anything new from the lecture

	A lot	yes	a bit	no
lecture 1 (PCR)	13%	62%	25%	0
lecture 2 (qPCR)	27%	73%	0	0
Seminar 1 (qPCR)	17%	75%	8%	0

← the best

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

	yes	yes, after revising it	I hope/some	no
lecture 1 (PCR)	56%	13%	31%	0
lecture 2 (qPCR)	20%	47%	26%	7%
Seminar 1 (qPCR)	58%	25%	17%	0

← the worst

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

	Less	the same	More
lecture 1 (PCR)	0	94%	6%
lecture 2 (qPCR)	40%	53%	7%
Seminar 1 (qPCR)	8%	92%	0

← the worst

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

	too hard	ok	too easy
lecture 1 (PCR)	19%	69%	12%
lecture 2 (qPCR)	60%	40%	0
Seminar 1 (qPCR)	0	100%	0

← the worst

6. was/would be the homework helpful

	Nope	a bit	yes
lecture 1 (PCR)		-	
lecture 2 (qPCR)	20%	13%	66.7%
Seminar 1 (qPCR)	0	0	100%

8. Would you suggest any changes?

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

more group work

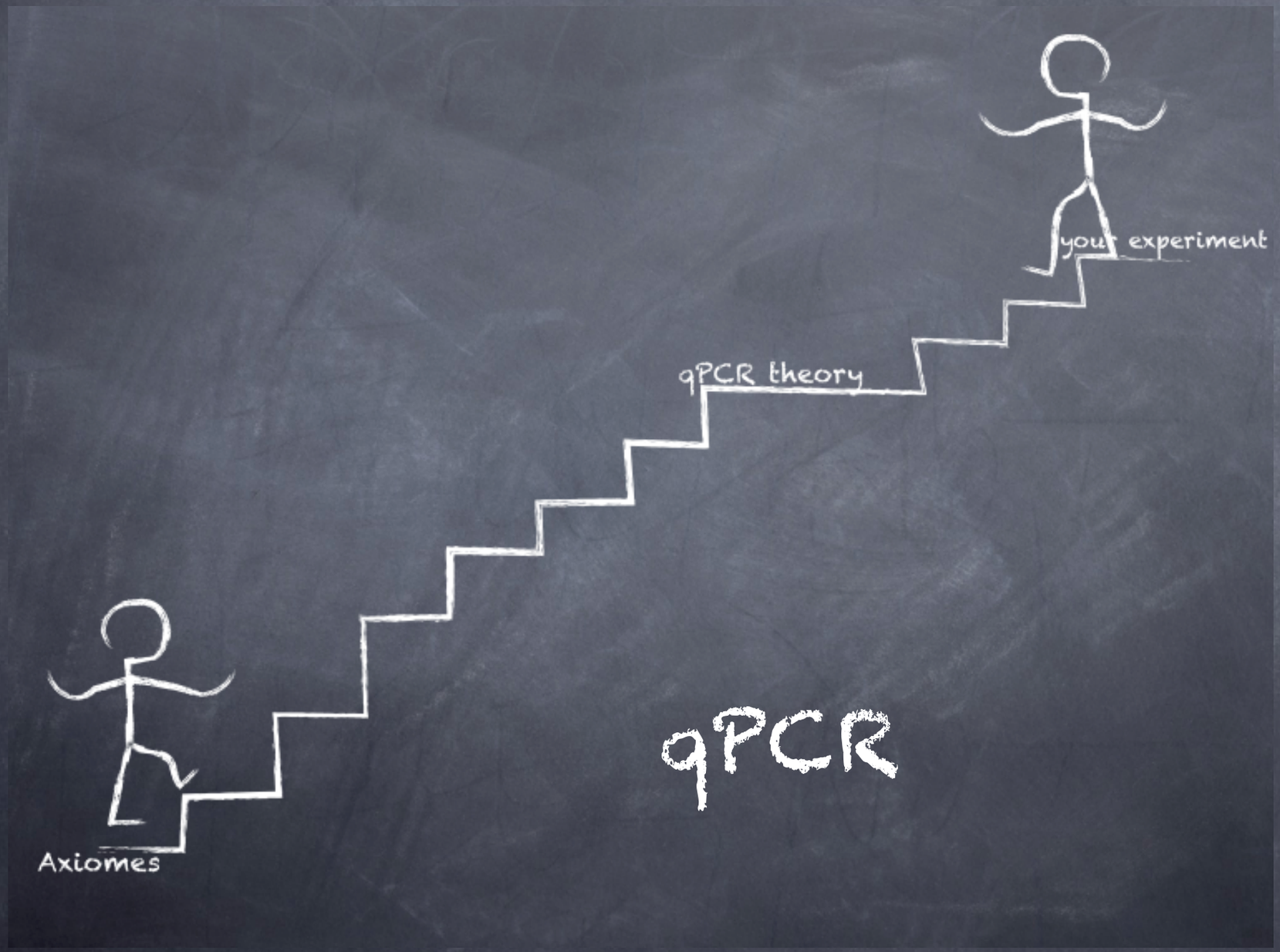
homework to repeat stuff from previous lectures

some repetitions are unnecessary


your experiment
qPCR theory
Maartje
Daniel
Alyona
Axiomes

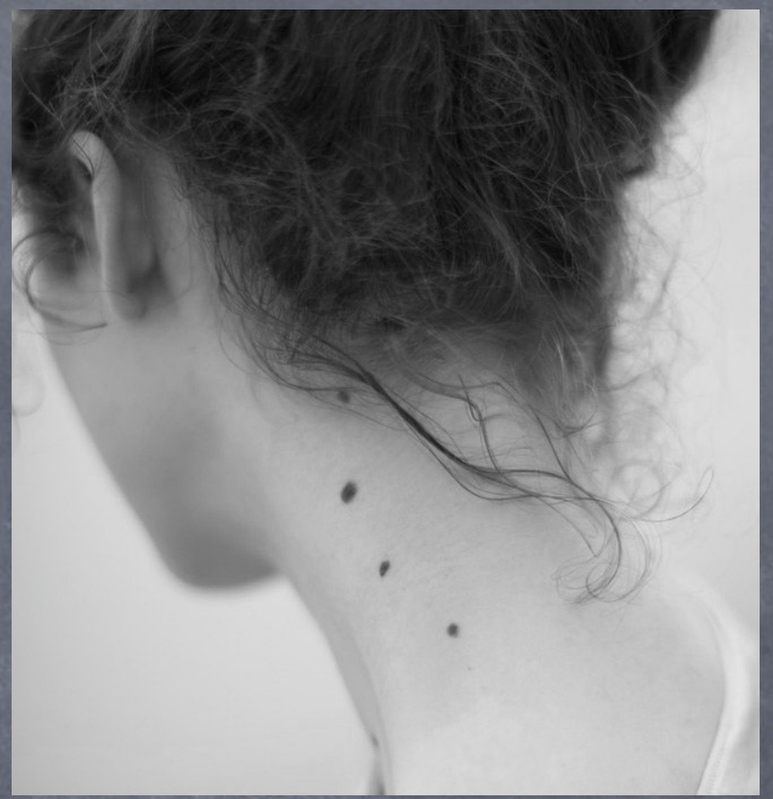
Next Generation Sequencing

Microarray



What is Mole?

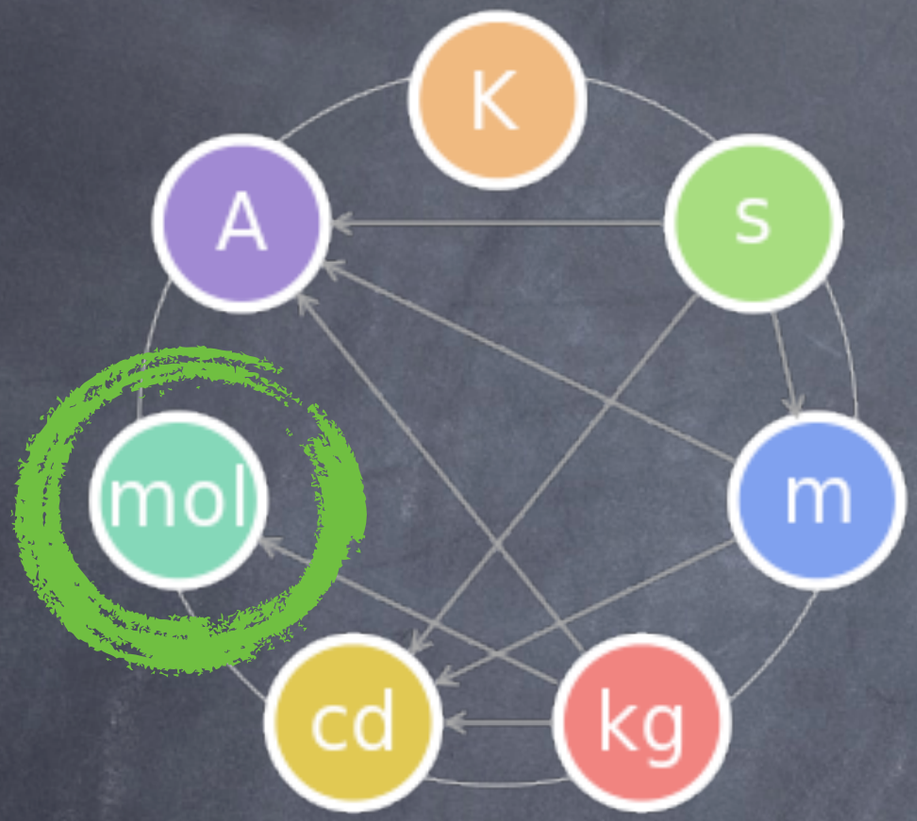
What is Mole?




Kim Philby


INGREDIENTS FOR
mexican mole sauce
(VEGAN & GLUTEN-FREE)
VEGETABLE BROTH
GARLIC
ONIONS
DARK CHOCOLATE
OREGANO
CINNAMON
CUMIN
SEA SALT
GF FLOUR MIX
BLACK PEPPER
OLIVE OIL
CHILI PEPPER
LIAGRIFFITH.COM

Mole is a unit of measurement



SI from French: Le Système International d'Unités

The seven SI base units:
K = Kelvin (temperature)
S = second (time)
m = metre (length)
kg = kilogram (mass)
cd = candela (luminous intensity)
mol = mole (amount of substance)
A = ampere (electric current)

it is used a lot in chemistry/biology

Mole is a unit of measurement

one mole of whatever = 6.022×10^{23} of this whatevers parts

Avogadro constant

= amount of atoms in 12 grams of pure carbon-12 (^{12}C)

why this many?

true chemist nerds celebrate the Mole Day on the October 23d between 6:02 AM and 6:02 PM

sucrose is composed of sucrose molecules
one mole of sucrose = 6.022×10^{23} of sucrose molecules

Lets say your primer stock is composed of 20bp long DNA molecules

one mole of primer = 6.022×10^{23} of 20bp long DNA molecules

what is molarity?

molarity of whatever = a value telling you how many moles of this whatever you have in a certain volume

mol/L

and what is molality?

molarity = mol/L

why to use molarity of primers and not ng?

mMol uMol nMol pMol fMol

	Mol	10^{1} Mol
milli	mMol	10^{-3} Mol
micro	uMol	10^{-6} Mol
nano	nMol	10^{-9} Mol
pico	pMol	10^{-12} Mol
femto	fMol	10^{-15} Mol

Homework part 1

You have:

1. sample A = cDNA made on a blood sample from a patient infected with Ebola virus
2. sample B = 200 ng of viral genomic cDNA (it is an RNA virus)
3. the length of the viral genomic cDNA is 19 kb.

You made:

1. four 10-fold dilutions of the sample B
2. ran qPCR with sample A and dilutions of the sample b

Your data are:

dilution 1 C_t= 20
dilution 2 C_t= 23
dilution 3 C_t= 26.8
dilution 4 C_t= 30.4
sample A C_t= 32.7

Please find out:

1. Is it possible to estimate the efficiency of primers from these data?
2. If yes, what is it in %? (If not, assume it is 100%)
3. Amount of viral cDNA in the patients blood
4. Is it possible to estimate the amount of viruses in the patient's blood?

Homework part 2

Experiment

You have:
1. sample A = cDNA isolated from 100 mg of Arabidopsis roots (grown under normal conditions)
2. sample B = cDNA isolated from 100 mg of Arabidopsis roots (grown under stress conditions)
3. primers to ABA-receptor gene (E=98%), primers to reference gene E2F (E=93%)

You made:
1. ran qPCR with sample A and sample B using both primer sets

Your data are:
sample A ABA-receptor gene C_t= 29.4
sample A E2F gene C_t= 27.3
sample B ABA-receptor gene C_t= 25.1
sample B E2F gene C_t= 29.6

Please find out how much more/less of the ABA-receptor mRNA there is in the sample A by using:
1. delta Ct
2. Livak method
3. Pfaffl method

Homework part 3

Group 1:
Reza
Konstantia
Enid
Martin
Mohammed

Group 2:
Jun
Zaenab
Jule
Kiran

Group 3:
Anna
Maite
Enrique
Shirin
Anders

your topic is

RT with oligodT

your topic is

RT with random hexamers

your topic is

RT with gene-specific primers

Please answer the questions:
1. what is it?
2. is it applicable for qPCR (if not, what is it for)
3. what are advantages/disadvantages of it

Group work 1

Group 1:
Reza
Konstantia
Enid
Martin
Mohammed

Group 2:
Jun
Zaenab
Jule
Kiran

Group 3:
Anna
Maite
Enrique
Shirin
Anders

your topic is

RT with oligodT

your topic is

RT with random hexamers

your topic is

RT with gene-specific primers

Please discuss your topic within your group (5 minutes)

1. what is it?
2. is it applicable for qPCR (if not, what is it for)
3. what are advantages/disadvantages of it

Group work 2

Group 1:
Reza
Konstantia
Zaenab
Jule
Enrique
Shirin

Group 2:
Jun
Enid
Martin
Anders

Group 3:
Anna
Maite
Kiran
Mohammed

Please present your topic to the new members of your group
(10 minutes)
1. what is it?
2. is it applicable for qPCR (if not, what is it for)
3. what are advantages/disadvantages of it

Group work 3

Group 1:
Reza
Konstantia
Zaenab
Jule
Enrique
Shirin

Group 2:
Jun
Enid
Martin
Anders

Group 3:
Anna
Maite
Kiran
Mohammed

Please discuss within your group:
(10 minutes)
1. can you combine these three RT-methods, or any two of them
2. what would be advantage/disadvantage of the mixed methods

So would you use any combination of these RT-methods?

Maxima First Strand cDNA Synthesis Kits for RT-qPCR (# K1641)

what does it contain?

why to mix it in this order?

Add on ice in the indicated order:

1. 5X Reaction Mix	4 µL
2. Maxima Enzyme Mix	2 µL
3. Template RNA	1 pg – 5 µg
4. Water, nuclease-free to	20 µL

→ Mix gently → centrifuge

how much should one take for RT?

what happens here?

10 min at 25°C →

what happens here?

15–30 min at 50–65°C →

what happens here?

85°C for 5 min

why to use longer time?

why to use higher T?

store at -20°C for up to one week
for longer storage use -80°C

Group work

Group 1:
Reza
Konstantia
Enid
Martin
Mohammed

sample 1: 10 ng/ul
sample 2: 57 ng/ul

Group 2:
Jun
Zaenab
Jule
Kiran

sample 1: 31 ng/ul
sample 2: 580 ng/ul

Group 3:
Anna
Maite
Enrique
Shirin
Anders

sample 1: 2000 ng/ul
sample 2: 1500 ng/ul

please calculate the composition of RT reaction for RT-qPCR experiment:
1. for sample 1 and sample 2 given to your group
2. for sample 1 and sample 2 the members of your group have

Add on ice in the indicated order:

1. 5X Reaction Mix	4 µL
2. Maxima Enzyme Mix	2 µL
3. Template RNA	1 pg - 5 µg
4. Water, nuclease-free to	20 µL

Maxima First Strand cDNA Synthesis Kits for RT-qPCR (# K1641)

Add on ice in the indicated order:

1. 5X Reaction Mix	4 µL
2. Maxima Enzyme Mix	2 µL
3. Template RNA	1 pg - 5 µg
4. Water, nuclease-free to 20 µL	

what if you have 40 RNA samples to synthesize cDNA?

2014. 09.24 qPCR o

125% Zoom | Function | Table | Chart | Text | Shape | Media | Comment

RT | plates | results

		RNA concentraion ng/ul	ul/RT		ul of MQ/RT	
1	Col-0	68	=1000÷D2		=5−E2	
2	qrt1-2	804	1,2	☑	3,8	☑
3	srt1-3	771	1,3	☑	3,7	☑
4	srt2-1	554	1,8	☑	3,2	☑
5	srt2-2	203	4,9	☑	0,1	☑

Enzyme mix (ThermoScientific, Maxima RT kit)			Program	
MQ	49,5		25 C	10'
5x buffer	=4×5,5		60 C	40'
Enzyme	11		85 C	5'
	15 ul/reaction		4 C	Hold

DyNAmo Flash SYBR Green qPCR Kit (# F-415L,)

- Perform the reaction setup in an area separate from nucleic acid preparation and ...uct analysis
- As the hot-start DNA polymerase is inactive during PCR setup, it is not nece... the setup on ice
- Pipette with sterile filter tips
- Minimize the exposure of the qPCR master mix to light.
- Minimize pipetting errors by using calibrated pipettes a... paring premixes to avoid pipetting very small volumes (recommended total V=20-50 ul, also ... sensitivity of your qPCR machine).
- Use optically clear caps or sealers to achieve ... signal.
- Use a cap sealing tool or firm finger ... close caps properly, or use a film sealer.
- Avoid touching the optical surfa... cap or sealing film without gloves, as fingerprints may interfere with fluorescence measuremen...
- Use powder-free glov...
- Plates or strips ... centrifuged before starting the cycling program to force the solution to the bottom of the ... to remove any bubbles.
- Use n... tology grade H2O.

Please read manual for YOUR kit BEFORE starting the experiment

DyNAmo Flash SYBR Green qPCR Kit (# F-415L,)

Can be mixed at room temperature, minimise exposure to light!

what does it contain?

should one ever change it?

1. 2X Master Mix	10 µL
2. Fw primer	125 nM
3. Re primer	125 nM
4. Template cDNA	5 ul (<100 ng)
5. Water, nuclease-free to	20 µL

how to decide how much to add?

the volume can be changed depending on your template concentration.
please be aware that adding tiny volumes increases pipetting error!

DyNAmo Flash SYBR Green qPCR Kit (# F-415L,)

Premix = the same pipetting error for all reactions?

	x1 reaction	xN reactions	
1. 2X Master Mix	10 µL	?	
2. Fw primer	125 nM	?	which components to
3. Re primer	125 nM	?	premix?
4. Template cDNA	5 ul (<100 ng)	?	
5. Water, nuclease-free to	20 µL	?	

can one pre-premix primers?

what would be the molarity of your primer stock?

eurofins | Genomics

Oligonucleotide Synthesis Report

Dr. Alyona Minina
Uppsala BioCenter, SLU

Order ID: 3404981
Customer ID: 943162
Your Order ID (PO#): 480MTR

Order Date: 07.10.2014
Lab No.: 2537
No. of Oligos: 84/84

Eurofins Genomics
Anzingerstraße 7a
D- 85560 Ebersberg

No.	Oligo Name	Sequence (5' -> 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [pmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode IDO	QC Report
1	SA_DAL19_F	CGGTTTTGACATCACCTG (18)	4.2	124	22.6	-	226	53.7	5465	50 %	0.01 µmol	HPSF	-	018656743	-
2	SA_DAL19_R	CGATAGGAAGGGGATCT TATAC (22)	6.2	164	24.0	-	240	58.4	6823	45.5 %	0.01 µmol	HPSF	-	018656744	-
3	SA_Polyubq_ F	TGGTCGTACTCTGGCCG ATTATA (23)	7.2	209	29.6	-	296	60.6	7045	47.8 %	0.01 µmol	HPSF	-	018656745	-
4	SA_Polyubq_ R	ACACCTAGCGGCACACA GTTAA (22)	7.3	195	29.2	-	292	60.3	6697	50 %	0.01 µmol	HPSF	-	018656746	-
5	SA_Actin_F	ATTGGGATGGAAGCTGC TG (19)	2.5	68	11.6	-	116	56.7	5923	52.6 %	0.01 µmol	HPSF	-	018656747	-
6	SA_Actin_R	CCCACCACTAAGCACAAT G (19)	3.5	95	16.7	-	167	56.7	5710	52.6 %	0.01 µmol	HPSF	-	018656748	-
7	SA_His2A_F	GGCTAAGCGAAACGATC AAC (20)	5.1	132	21.5	-	215	57.3	6144	50 %	0.01 µmol	HPSF	-	018656749	-
8	SA_His2A_	GATCTGGGCCAAATTTCT GA (20)	5.9	164	26.7	-	267	55.3	6132	45 %	0.01 µmol	HPSF	-	018656750	-
9	AJ_IL-17_Plus	CTGAGAACTTCATCCGTG TC (20)	5.3	154	25.4	-	254	57.3	6067	50 %	0.01 µmol	HPSF	-	018656751	-

DyNAmo Flash SYBR Green qPCR Kit (# F-415L,)

Now use your primer stock concentration and please write down how many microliters of each component you would add to a premix for 7 samples

why?

sic!! ALWAYS make premix for 0.5-2 more reaction than you are going to use

	x1 reaction	x? reactions
1. 2X Master Mix	10 µL	
2. Fw primer	125 nM	
3. Re primer	125 nM	
4. Template cDNA	5 µL	
5. Water, nuclease-free to	20 µL	

qPCR

2 experiments on one plate

Absolute

how much of GOI is in your sample

Relative

which of your samples has more of GOI

Absolute qPCR

sample A: 50 ul of plasmid, concentration 10 ng/ul

sample B: the same plasmid, concentration is unknown ← what is the concentration?

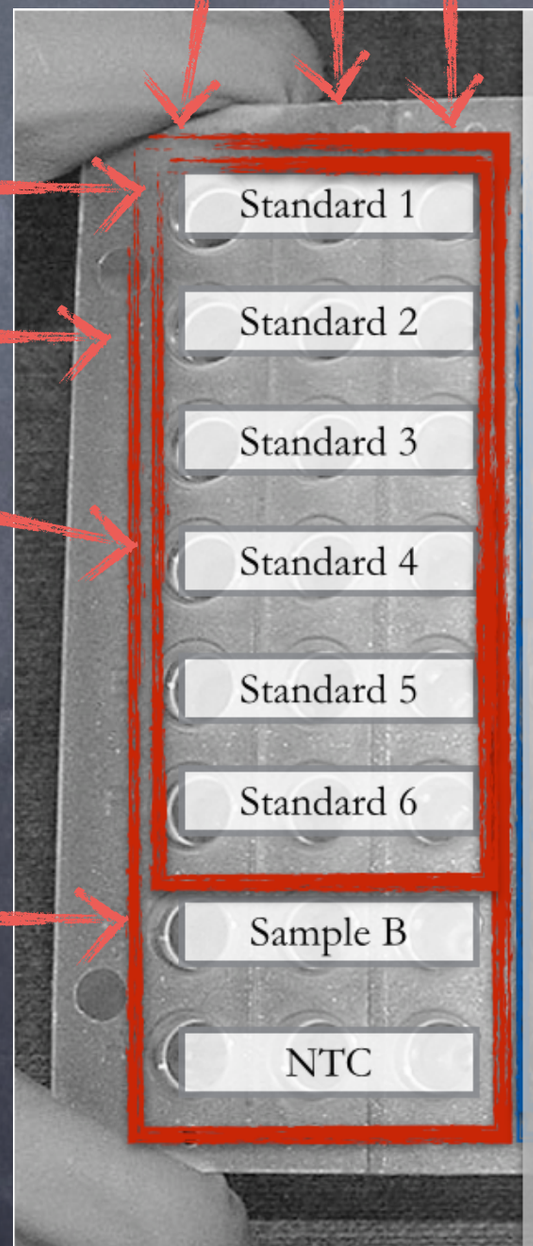
Fw and Re primers to detect a gene on the plasmid

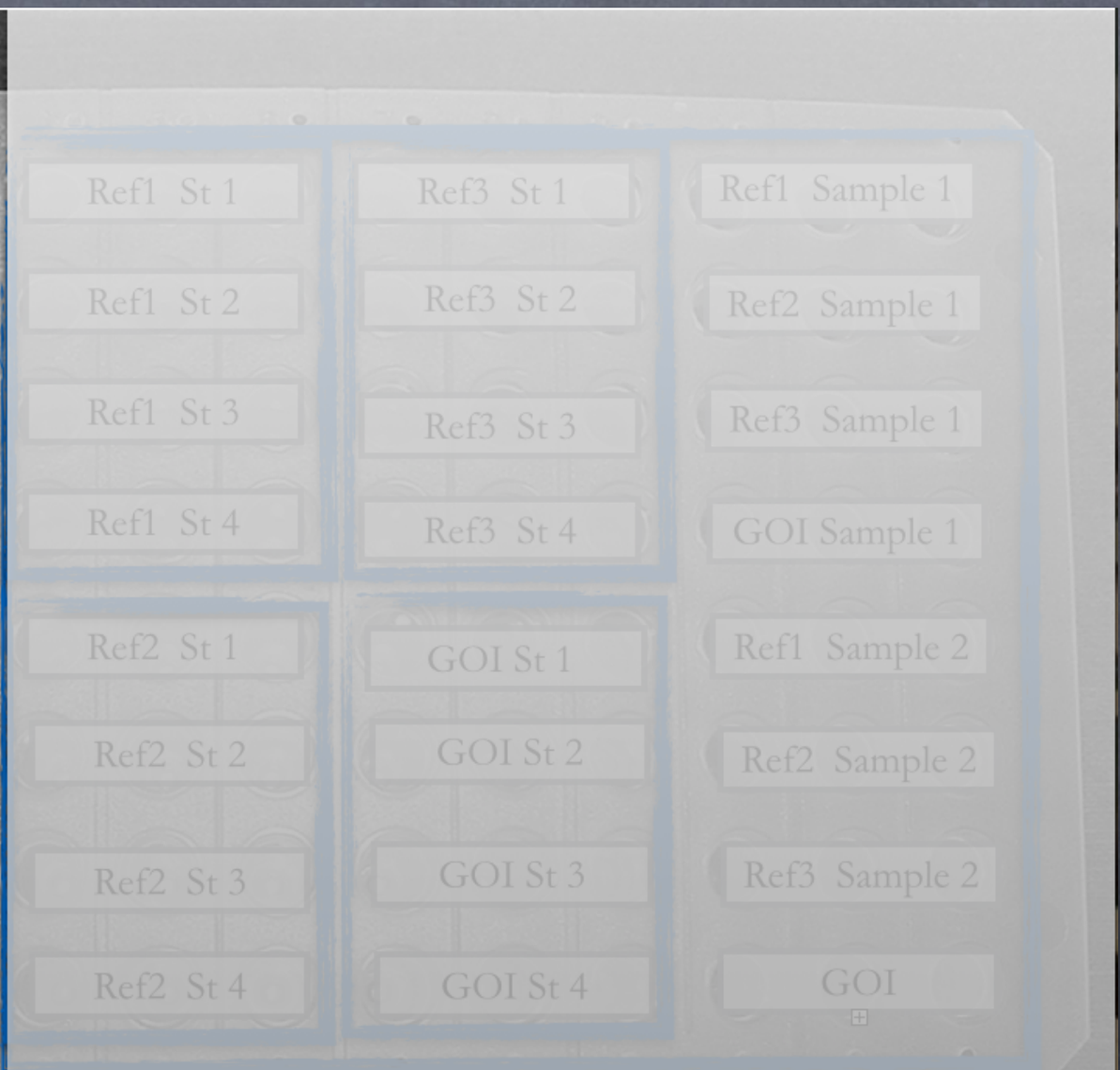
Technical triplicates for each PCR reaction

Dilution series of the sample with known concentration for the Standard curve

sample with unknown concentration

NTC= no template control= water control for contamination of PCR





$y=kx+m$

R^2 >?

C_t for standard 6

C_t for standard 5

C_t for standard 4

C_t=for sample B

C_t for standard 3

C_t for standard 2

C_t for standard 1

Standard 6 Standard 5 Standard 4 Standard 3 Standard 2 Standard 1

$\log_{10}$ [ng of plasmid]

ng of sample B =?

ng of sample B =10^x

$\log_{10}$ [ng of sample B]

Absolute qPCR

sample A: 50 ul of plasmid, concentration 10 ng/ul

sample B: the same plasmid, concentration is unknown

Fw and Re primers to detect a gene on the plasmid

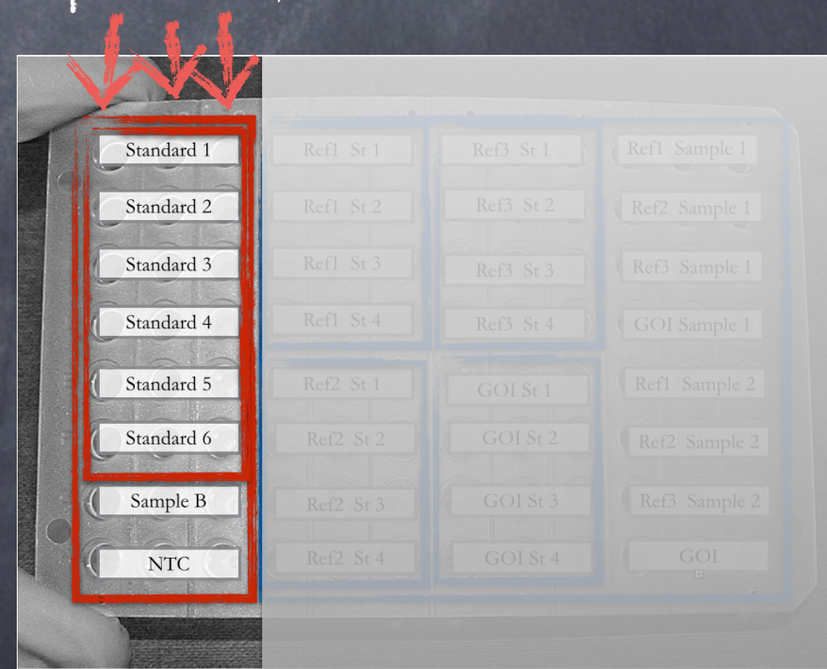
1. Please calculate the amount of reactions for this experiment

2. Use sample A to make 6 serial 10 fold dilutions. Please calculate the volume of dilutions you are going to make, and how you are going to mix them

3. Please calculate amount of components for the premix you will use for the absolute qPCR

	x1 reaction
1. 2X Master Mix	10 µL
2. Fw primer	125 nM
3. Re primer	125 nM
4. Template cDNA	5 µL
5. Water, nuclease-free to	20 µL

Technical triplicates for each PCR



sample B

NTC= no template control= water

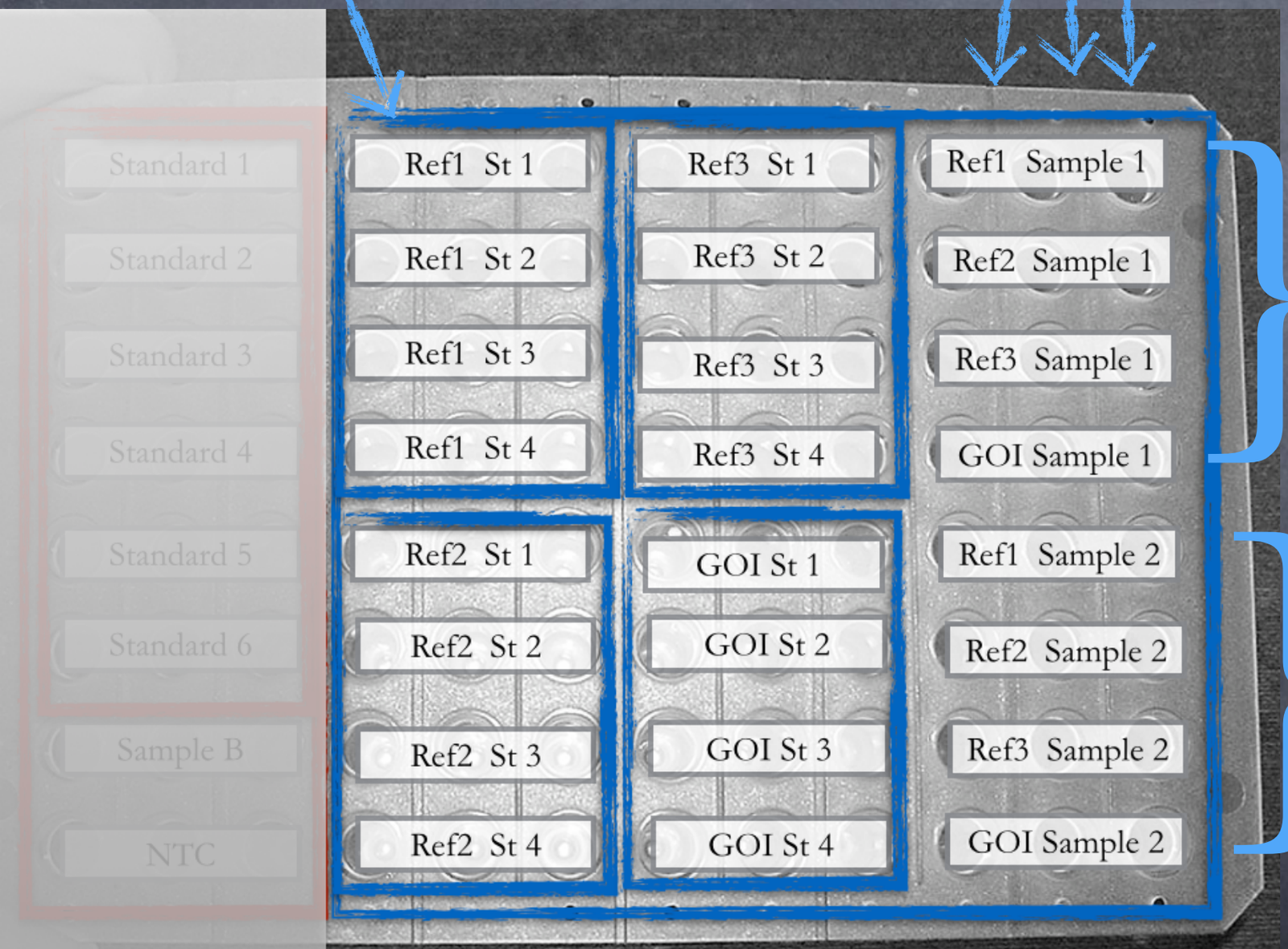
Relative qPCR

sample 1: cDNA of your choice
sample 2: another cDNA of your choice
one pair of primers to detect GOI
3 pairs of primers to detect 3 reference genes

which of your samples has more of the GOI?

Dilution series of the cDNA pool to estimate primer efficiency

Technical triplicates for each PCR reaction



detection of all four genes in the sample 1

detection of all four genes in the sample 2

Relative qPCR

sample 1: cDNA of your choice
sample 2: another cDNA of your choice
one pair of primers to detect GOI
3 pairs of primers to detect 3 reference genes

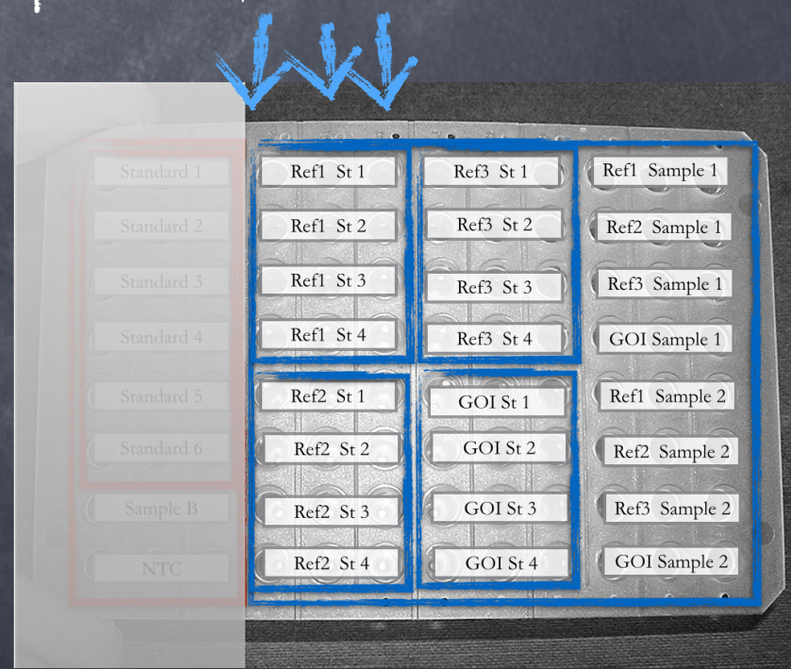
1. Please calculate the amount of reactions for each primer pair

2. Please calculate the volume cDNA pool and its dilutions you are going to make, and how you are going to mix them

3. Please calculate amount of components for the premixes you will use for the absolute qPCR

	x1 reaction
1. 2X Master Mix	10 µL
2. Fw primer	125 nM
3. Re primer	125 nM
4. Template cDNA	5 µL
5. Water, nuclease-free to	20 µL

Technical triplicates for each PCR reaction



RT | plates | results

2014.09.24 PLATE 1 PRIMER EFFICIENCY

1	2	3	4	5	6	7	8	9	10	11	12
cDNA pool 1:20			cDNA pool 1:20			cDNA pool 1:20			cDNA pool 1:20		
cDNA pool 1:40			cDNA pool 1:40			cDNA pool 1:40			cDNA pool 1:40		
cDNA pool 1:80			cDNA pool 1:80			cDNA pool 1:80			cDNA pool 1:80		
cDNA pool 1:160			cDNA pool 1:160			cDNA pool 1:160			cDNA pool 1:160		
cDNA pool 1:320			cDNA pool 1:320			cDNA pool 1:320			cDNA pool 1:320		
cDNA pool 1:20			cDNA pool 1:160			cDNA pool 1:20			cDNA pool 1:160		
cDNA pool 1:40			cDNA pool 1:320			cDNA pool 1:40			cDNA pool 1:320		
cDNA pool 1:80			RNA pool control (NTC)			cDNA pool 1:80			RNA pool control (NTC)		

srt1 flank	srt1-3 Fw (GCCCCAAAGGAATTTGGADGTTACA) / qPCR srt1 flanking Re (CACATCTCCATAAAAGAGTCTCCATG)
srt1 outsidehp	qPCR srt1 before hp (TGTTCTGTTGAAAAGTGTGGAGCT) / srt1-3 Re (ATTGCAAGCAGGGGTTATCTGCAAA)
srt2 flank	qPCR srt2 flanking Fw (GGAAGATATCCACAAGTTGTATDGAC) / qPCR srt2 flanking Re (CCTCCTCCATCCAGCGTAACTC)
srt2 outside hp	qPCR srt2 after hairpin (AGAGAAGAGCTTTGGCATGAAAC) / srt2-2 Re (TCTTTGGAGACAACATCCCGAAG)
PP2A	
HEL	

A	B	C	D	E	F	G	H

reaction mixes	x1	x18
2x master mix	10	180
5 uM primer mix	1	18
template	5	
MQ	4	72

program			
95C	7'		
95C	10"		
60C	30"	aquisition	40 cycles
95C			
60C-95C	0.5C step	aquisition for melt curve	

	cDNA	MQ
cDNA pool 1:20	4 ul from 5 samples	380 ul
cDNA pool 1:40	200 ul of 1:20	200 ul
cDNA pool 1:80	200 ul of 1:40	200 ul
cDNA pool 1:160	200 ul of 1:80	200 ul
cDNA pool 1:320	200 ul of 1:160	200 ul

	total RNA	MQ
RNA pool	3 ul from 5 samples	135 ul
	100 uM stock (100pmol/ul)	MQ
5 uM primer mix	5 ul of Fw+ 5 ul of Re	90 ul

2014.09.24 PLATE 2 SRT EXPRESSION

1	2	3	4	5	6	7	8	9	10	11	12
Col-0			Col-0			Col-0			Col-0		
qrt1-2			qrt1-2			qrt1-2			qrt1-2		
srt1-3			srt1-3			srt1-3			srt1-3		
srt2-1			srt2-1			srt2-1			srt2-1		
srt2-2			srt2-2			srt2-2			srt2-2		
Col-0			srt2-1			Col-0			srt2-1		
qrt1-2			srt2-2			qrt1-2			srt2-2		
srt1-3						srt1-3					

srt1 flank
srt2 flank
srt1 outsidehp
srt2 outside hp
PP2A
HEL

Each primer pair has its colour

DNA templates are named

tion mixes	x1	x18	x18 (srt2 outside hp)
master mix	10	180	180
primer mix	1	18	36
template	5		
MQ	4	72	54

program			
95C	7'		
95C	10"		
60C	30"	aquisition	40 cycles
95C			
60C-95C	0.5C step	aquisition for melt curve	

	cDNA	MQ
cDNA 1:30	4 ul	116

Homework

1. Please make your own Excel file for the Absolute and Relative qPCRs

2.Please make an Excel file for the experiment where you have:

- samples from 4 different conditions (control and 3 treatments)
- 3 biological replicates for each condition
- one genes of interest
- 2 reference genes