Esercitazione 8: ANALISI DATI

1. Real-Time PCR Chemistry
2. Amplification plot, melting curve
3. Basics on controls biological and technical replicates
4. Analysis of qPCR data generated in the laboratory course

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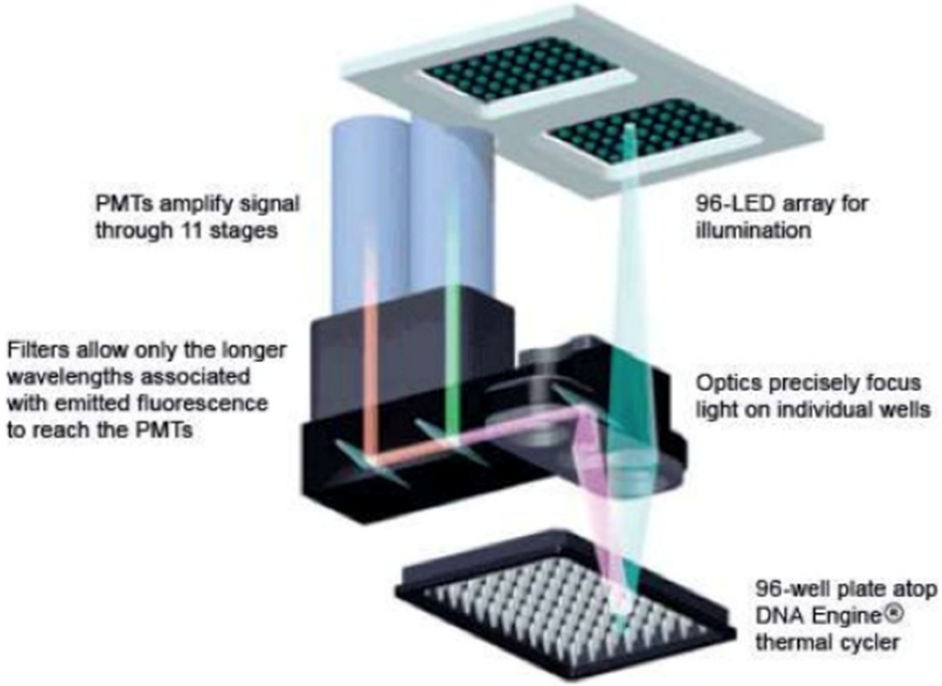
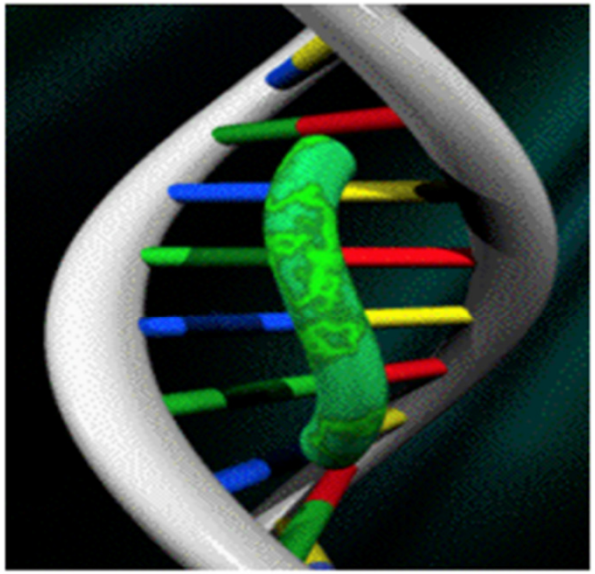
Real time PCR and Data analysis



…follow the accumulation of PCR products during increasing cycle numbers in "real-time" using a detection system (gel electrophoresis non necessary)

Real-Time PCR Chemistry

SYBR® Green I dye

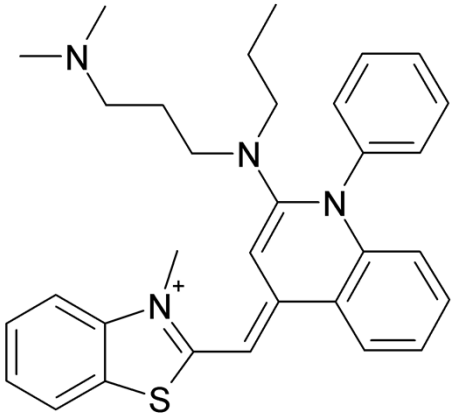


Binds double stranded DNA

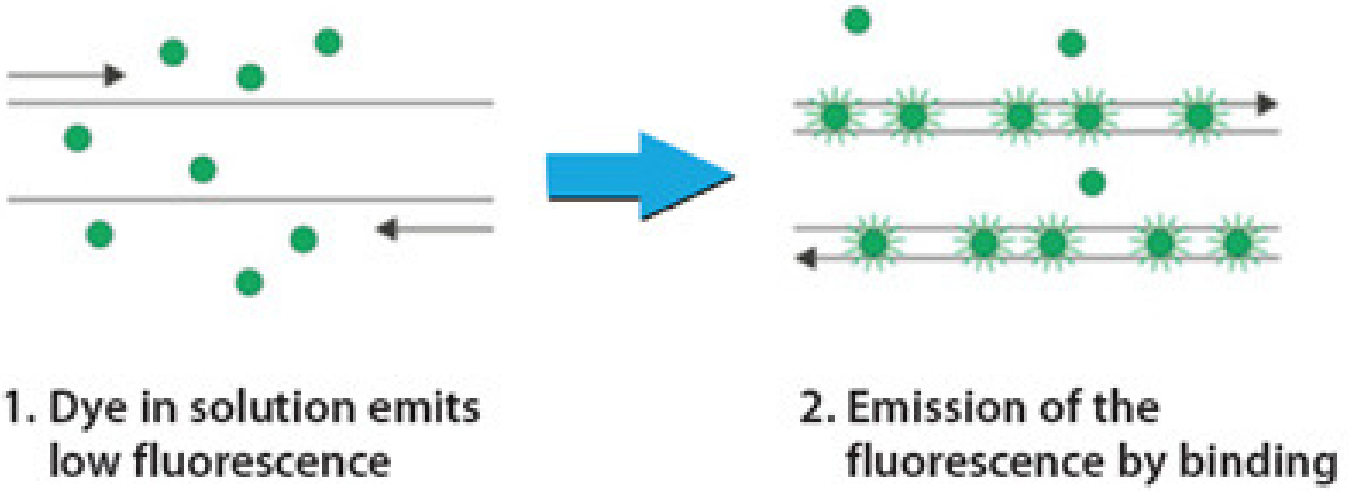
1. SYBR® Green I Dye Assay Chemistry

Classic PCR setup with addition of SYBR Green:

SYBR Green is a green fluorescent cyanine dye that has high affinity for double-stranded DNA. The mode of binding is believed to be a combination of DNA intercalation and external binding. When bound, SYBR absorbs at a wavelength around 497 nm and emits fluorescence around 520 nm.



Denaturation
Annealing



DNA synthesis
Detection of emission of fluorescence

Fluorescence emmission is increasing with increasing of PCR cycles

Basics of real-time PCR measurements

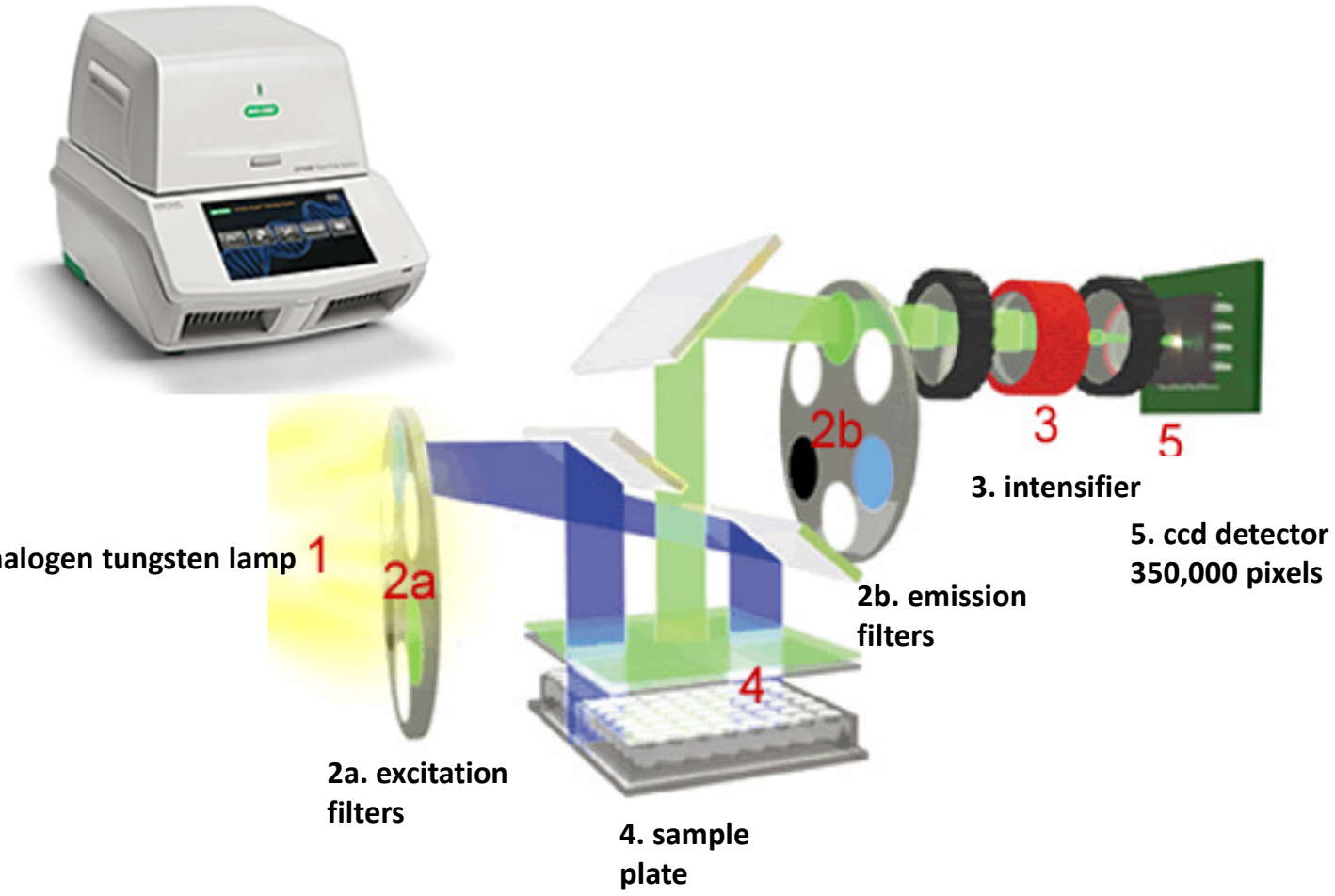


Fig. 1.2. Representation of Optical Detection System layout.

www.biorad.com

Every PCR cycle:

1. Exitation of SYBR green (497nm)
2. Measurment of emission from SYBR green (520nm)

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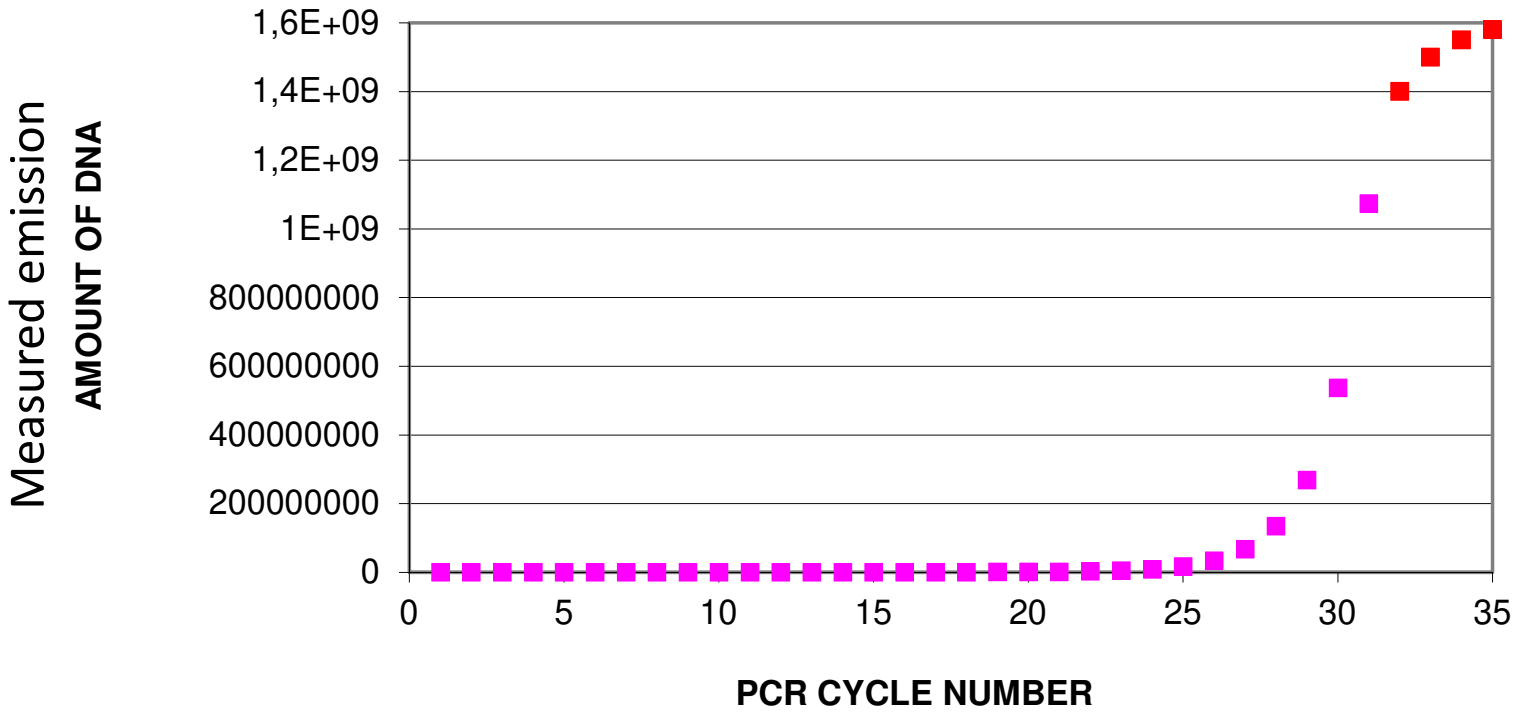
Basics of real-time PCR measurements

AMPLIFICATION BLOT

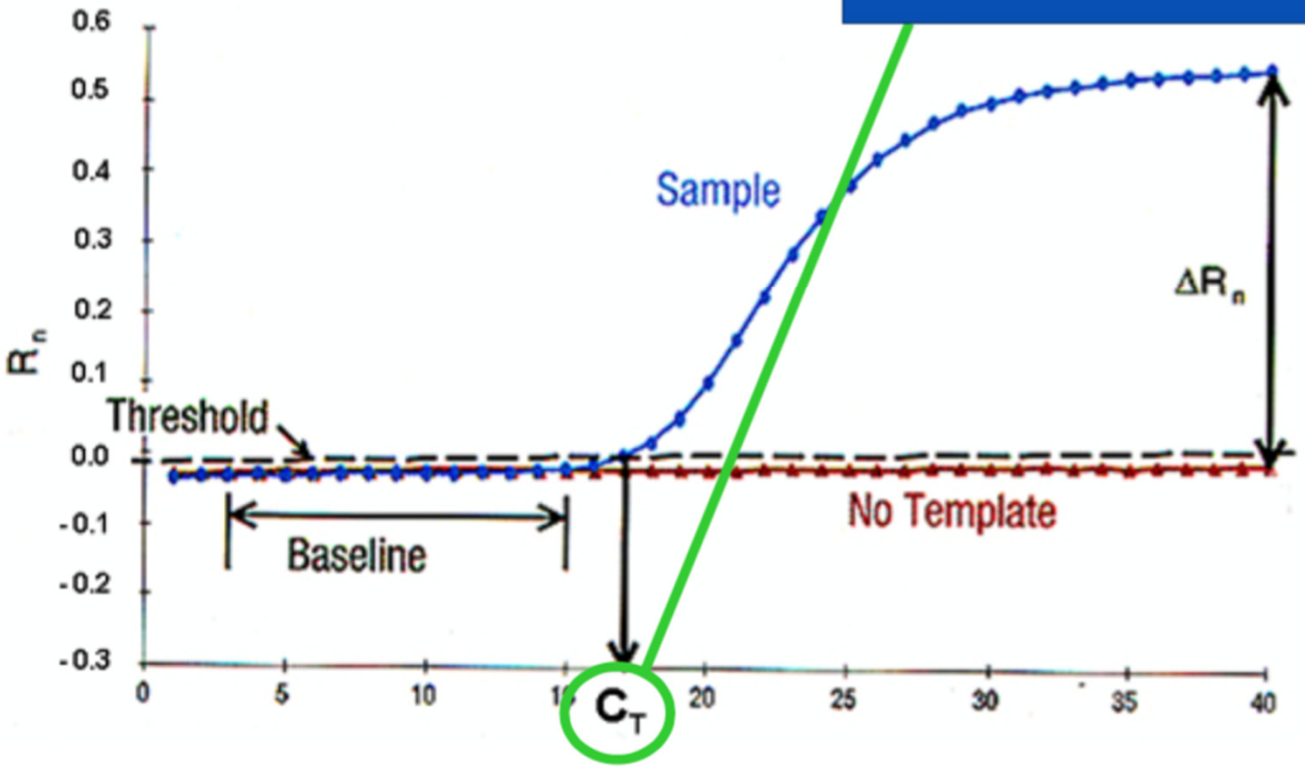


Quantitative information

Terminology of amplification blots

Cycle Threshold (C_T):

The cycle (point in time) at which the PCR product crosses the threshold of detection.

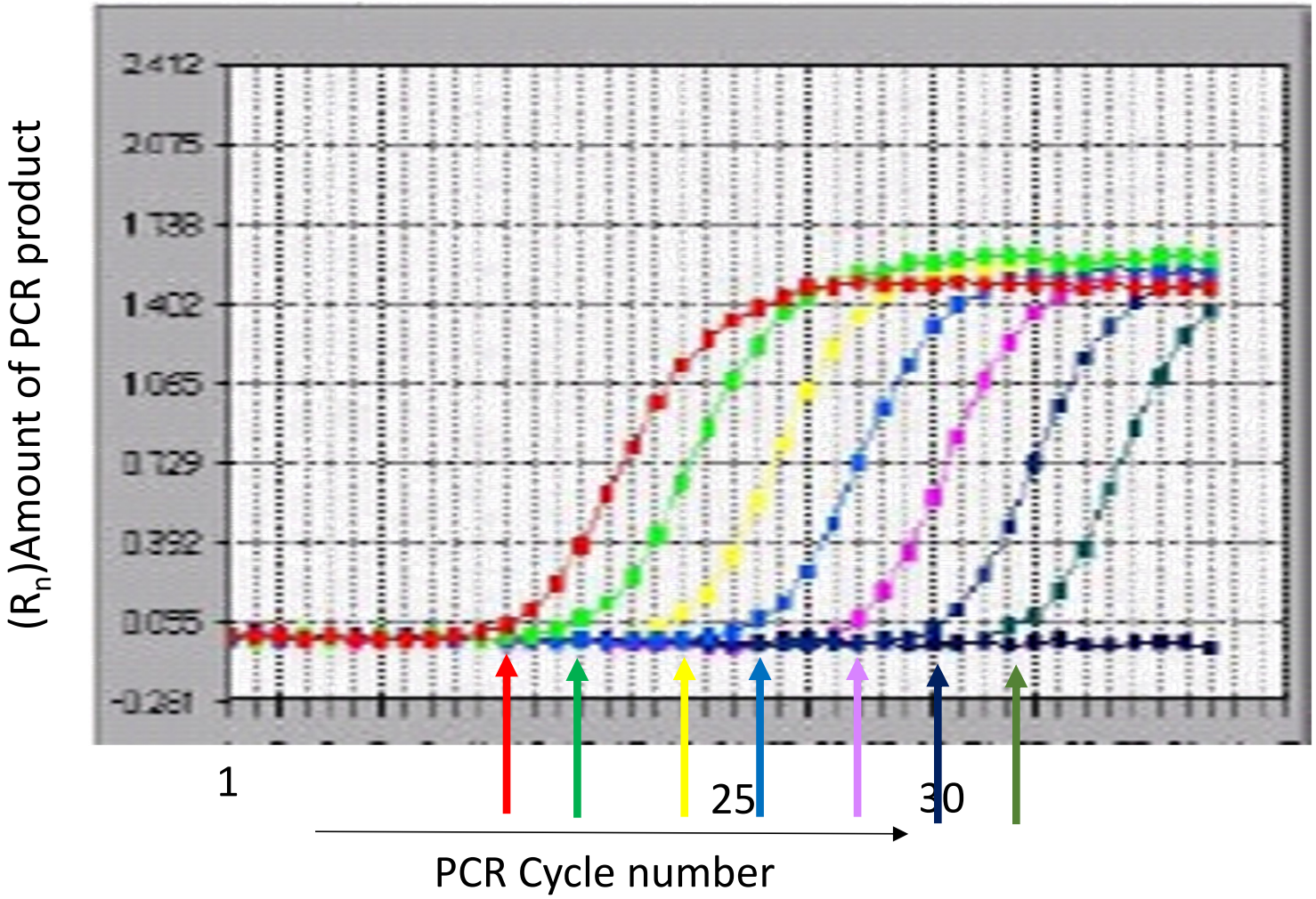


C_t VALUE: Most important value for the analysis of real-time PCR data

C_t = threshold cycle: è il ciclo della reazione di amplificazione in cui il segnale di fluorescenza del campione è maggiore rispetto a quello della threshold

Basics of real-time PCR measurements – Amplification blot

Follow PCR product amplification in real-time (RT-PCR)



Many samples are analyzed at same time (typically in 96 well format)

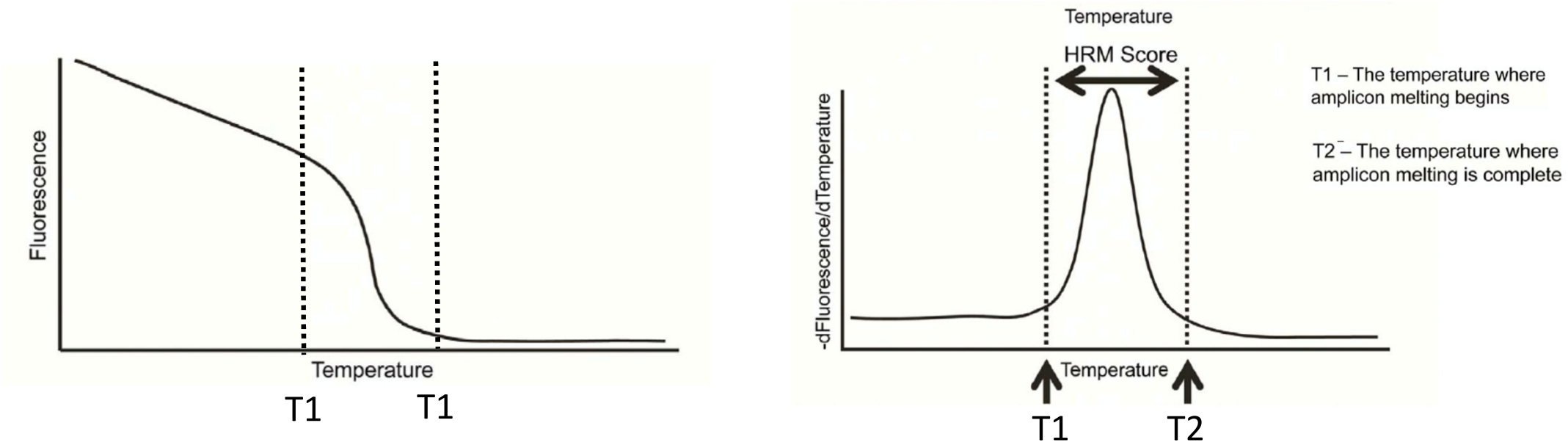
Follow the amplification of PCR amplicons in "REAL-TIME" in all biological samples analyzed = REAL TIME PCR

Basics of real-time PCR measurements – Melting curve

METLTING CURVE ANALYSIS (HRM = high resolution melting score)

The temperature-dependent dissociation between two DNA-strands can be measured using a DNA-intercalating fluorophore such as SYBR green,or fluorophore-labelled DNA probes. In the case of SYBR green (emmitting fluorescense 1000-fold more intensely while intercalated in the minor groove of two strands of DNA), the dissociation of the DNA during heating is measurable **by the large reduction in fluorescence that results.**

The temperature at which 50% of DNA is denatured is known as the melting temperature.



Generation of melting curves, melting peaks, and HRM scores. Melting curves (top panel) are generated by graphing Fluorescence against Temperature. Fluorescence declines as the DNA melts. DNA melting is visualized through the use of a saturating duplex-dependent DNA intercalating dye. As the DNA melts, the dye is released; unbound dye does not fluoresce. Melting peaks (bottom panel) are generated by taking the negative derivative of Fluorescence with respect to Temperature and graphing these values against Temperature (2 dF/dT vs T).

Melting curve is determined after the last cycle of PCR:

- → **PCR machine heats up PCR products from 0°C to 100°C**
- → **Dissociation of SYBR from dsDNA filaments is measured**
- → **IF PCR HAS AMPLIFIED SPECIFICALLY A SPECIFIC REGION → ALL DNA MOLECULES WILL MELT AT A SPECIFIC TEMPERATURE → melting temperature is determined by DNA sequence!!!**
- → **IF YOU RUN PCR PRODUCT ON AGAROSE GEL, ONLY ONE BAND WILL BE VISIBLE**

Basics of real-time PCR measurements – Melting curve

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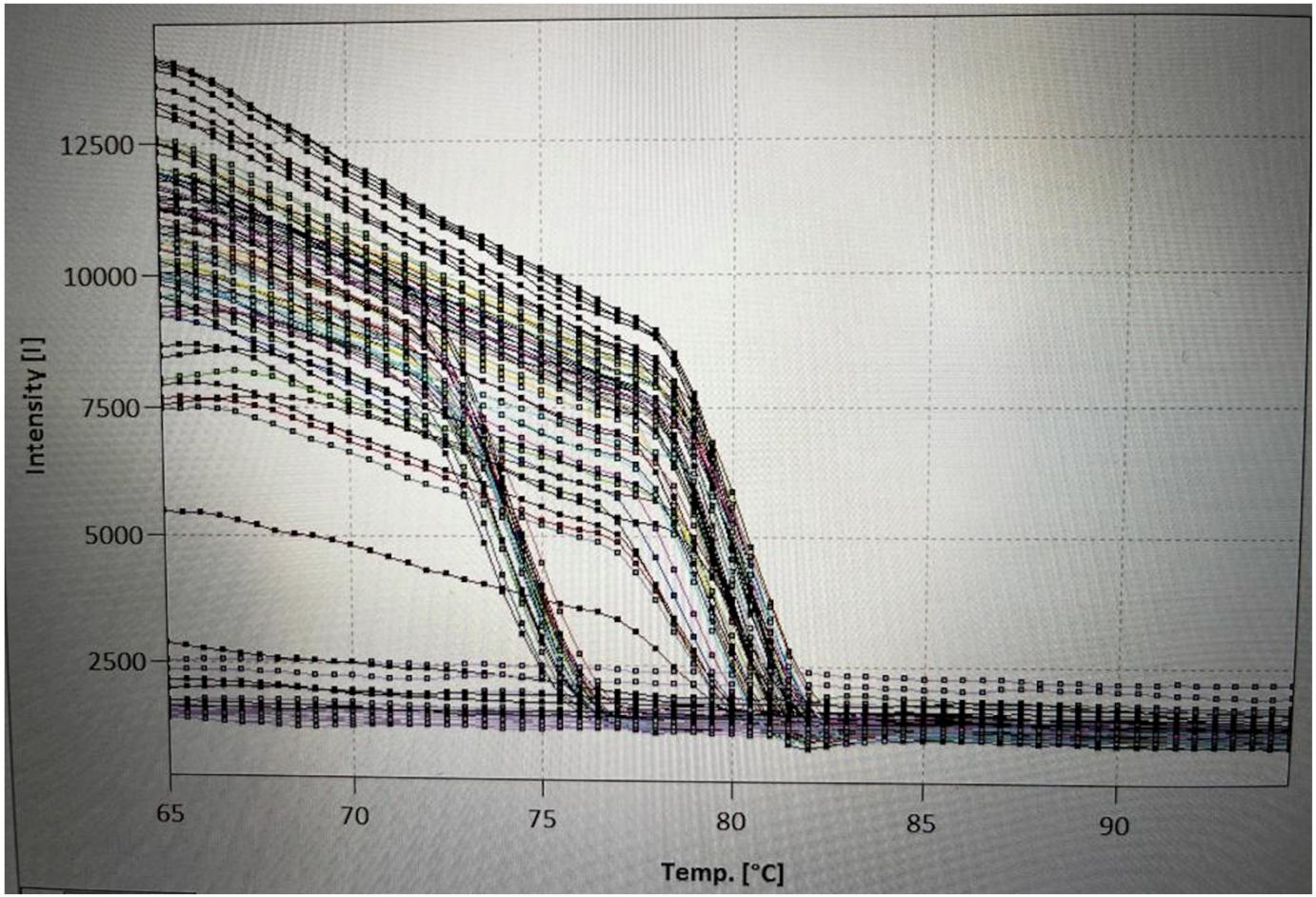

PCR amplicon SFPQ

PCR amplicon ACTIN

PCR amplicon IFI44/CCL5

Basics of real-time PCR measurements – Melting curve

METLTING CURVE ANALYSIS (HRM = high resolution melting score)

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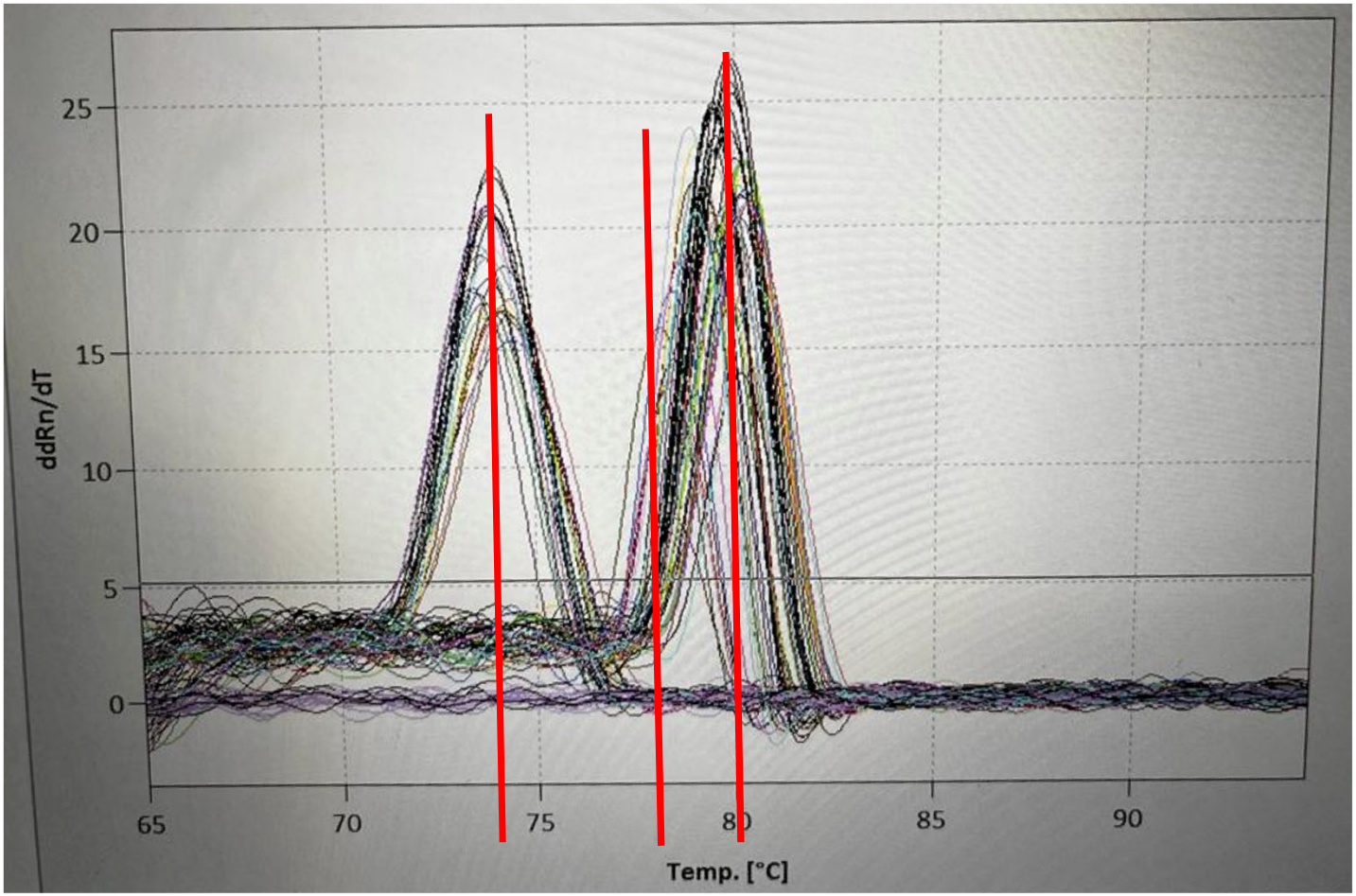


T_m: PCR amplicon XXX: 74°C

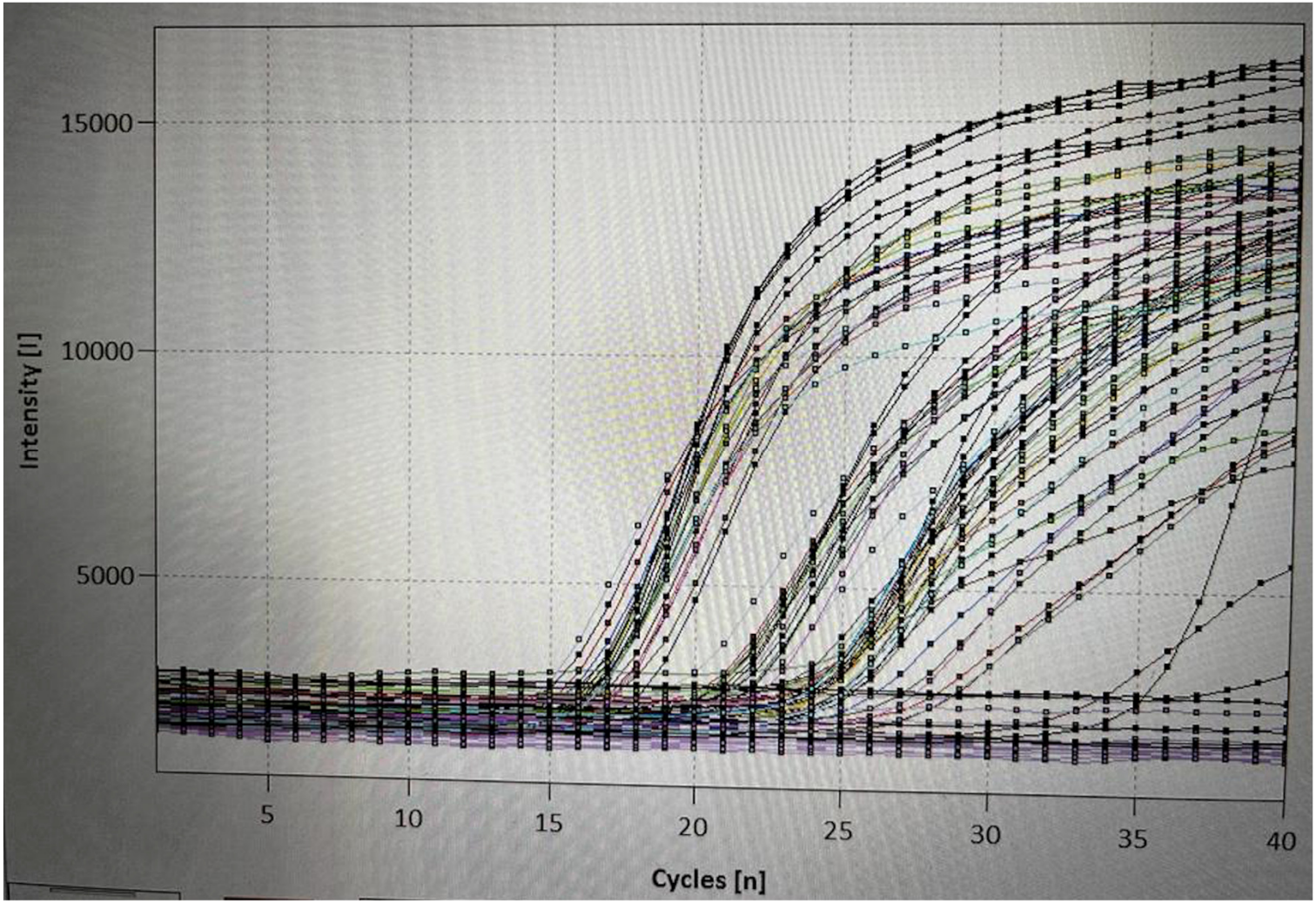
T_m: PCR amplicon XXX: 76°C

T_m: PCR amplicon XXX: 80°C

Tm: depends on length of PCR aplicon and seuquence context (proportion A-T and G-C);
Specific for each PCR amplification product

Generated by taking the negative derivative of Fluorescence with respect to Temperature and graphing these values against Temperature (2 dF/dT vs T).

Basics of real-time PCR measurements – Amplification blot



Follow the amplification of PCR amplicons in "REAL-TIME" in all biological samples analyzed
= REAL TIME PCR

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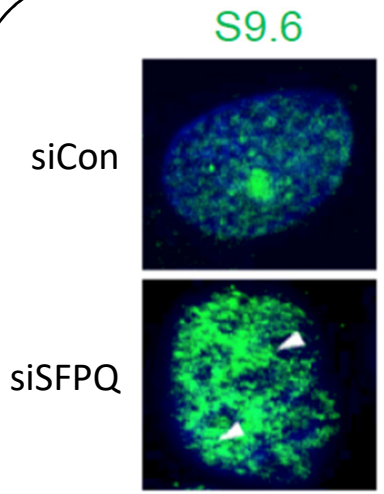
Analysing experimental data:

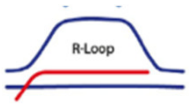
BIOLOGICAL BACKGROUND: LOSS OF SFPQ FUNCTION

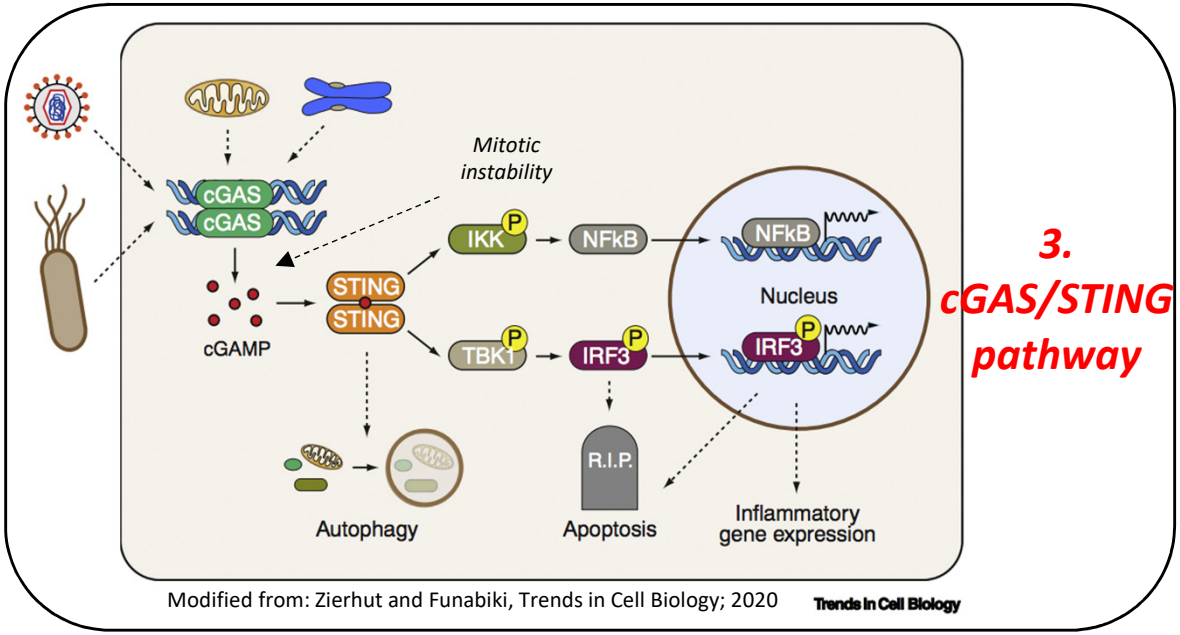


Blue: DNA stain (DAPI)

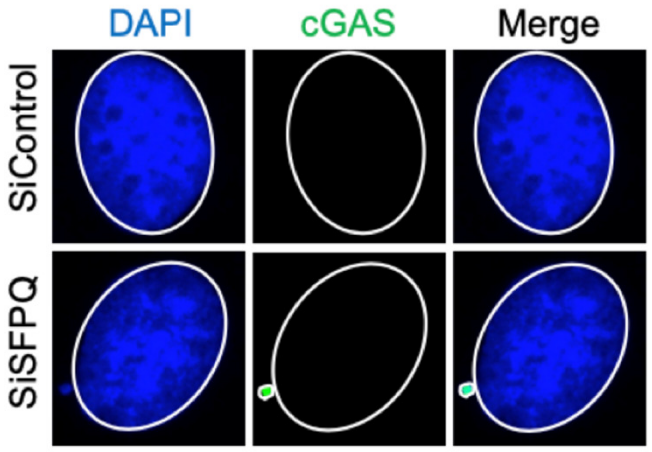
Green: R-loops (detected using the S9.6 monoclonal antibody)

1. More R-loops





3. cGAS/STING pathway



2. More cytoplasmatic DNA

READ OUT: *Expression of CCL5*
(central gene in interferon signalling – innate immunity)

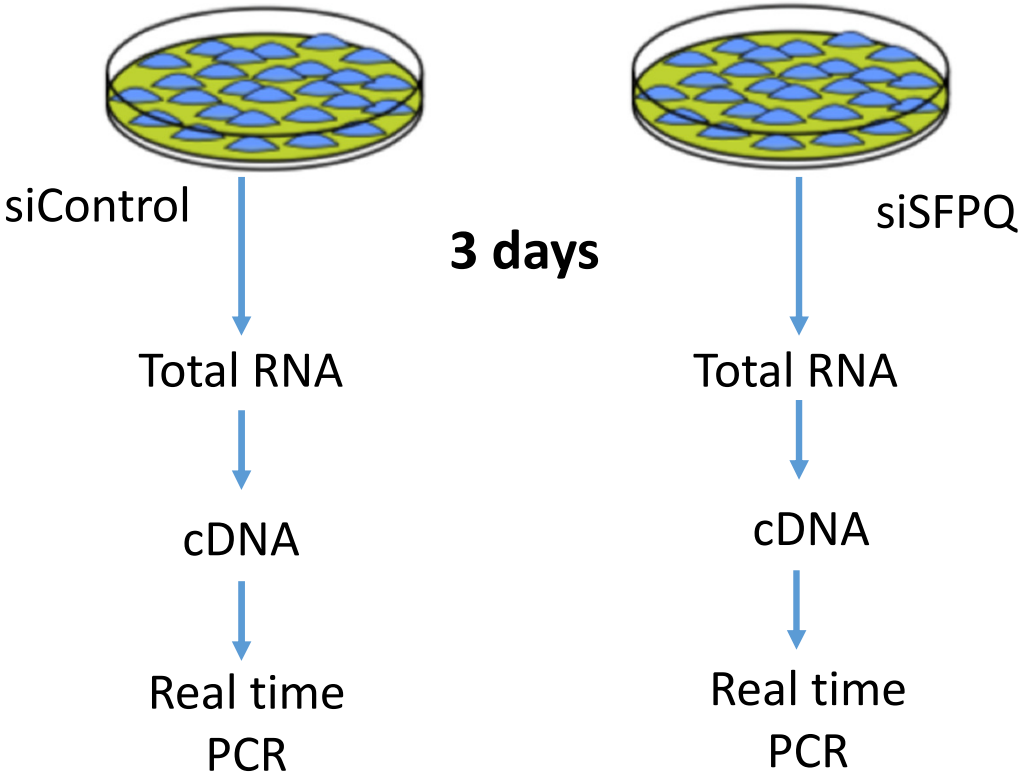
- Tumor-suppressive effect (Therapy)

- Tumor-promoting effect

Analysing experimental data:

1. All relevant biological samples are proessed at same time with same procedures

U2-OS cells transfected with siRNA (siControl, siSFPQ)



CONTROL EXPERIMENT

MANIPULATION OF BIOLOGICAL CONTEXT

!!!! Control experiments are essential!!!!

!!!! All relevant experimental samples have been processed (RNA prep; cDNA sysnthesis, qPCR) in parallel, at the same time by sme operator !!!!

!!! Only in this case experimental data can be compared with each other (Control and siSFPQ)!!!

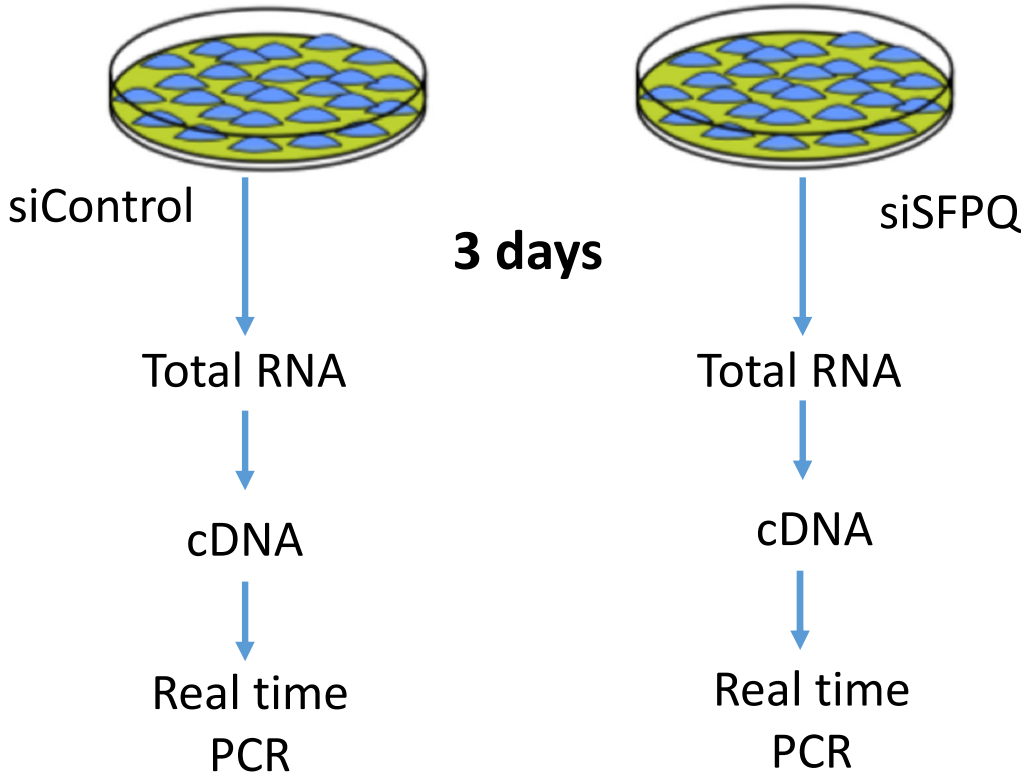
!!!Experiments are repeated to evidence eventual biological or technical variations !!!

Analysing experimental data:

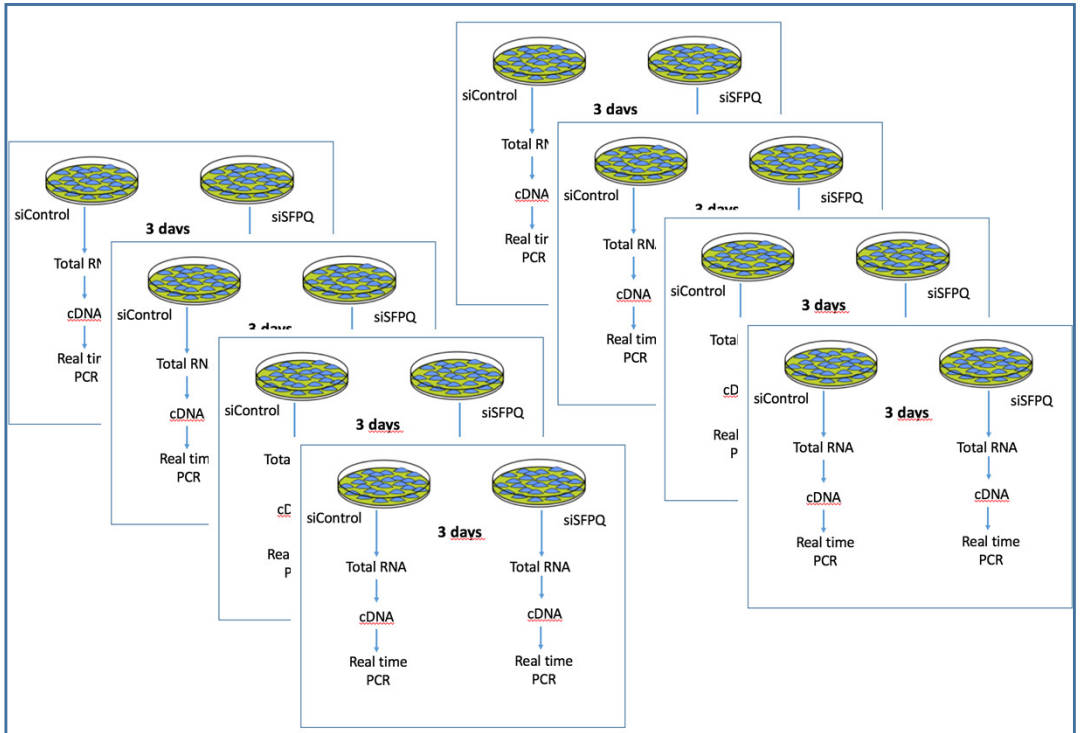
2. Replicates are necessary to produce relevant scientific data

BIOLOGICAL REPLICATES:

Le repliche biologiche sono misurazioni parallele di campioni biologicamente distinti che catturano variazioni biologiche casuali, che possono essere esse stesse oggetto di studio o fonte di rumore.



Student 1 --- biological replicate 1



Student 2 – Student 9: biological replicates 2-9

Variazioni biologiche possibili:

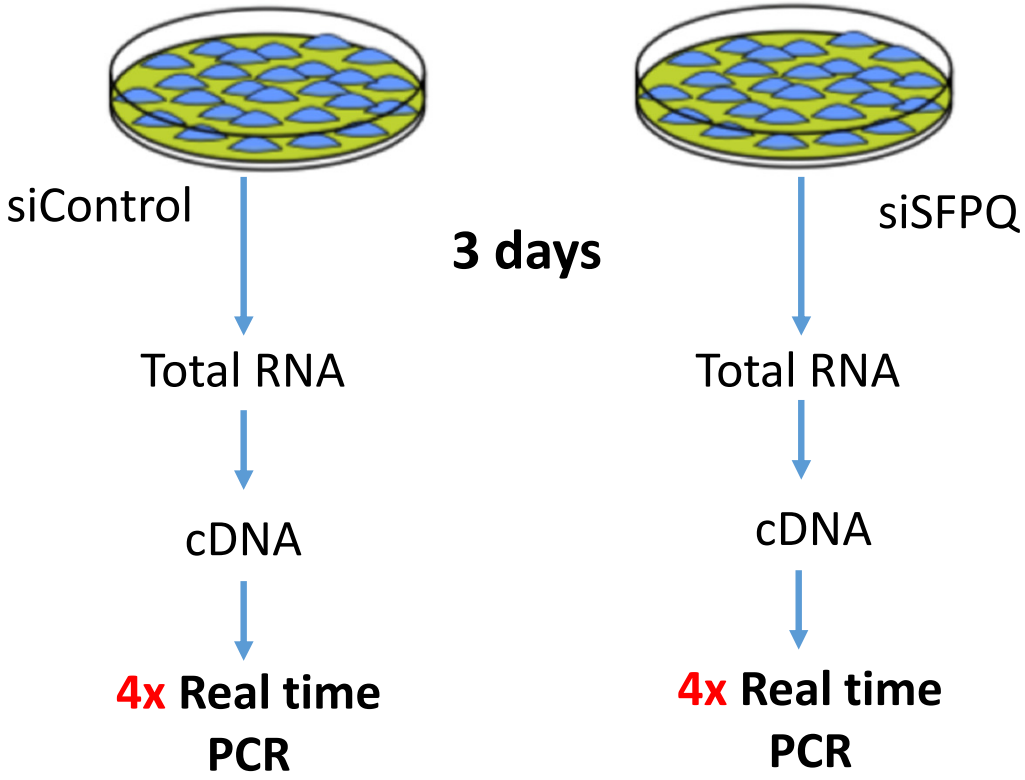
- **Confluenza cellulare**
- **Efficacia di trasfezione**
- **Durata del knock-down**
- **…..**

Analysing experimental data:

2. Replicates are necessary to produce relevant scientific data

TECHNICAL REPLICATES:

Le repliche tecniche sono misurazioni ripetute dello stesso campione che rappresentano misure indipendenti del rumore casuale associato a protocolli o apparecchiature.



I campioni sono ottenuti da un singolo esperimento. Il metodo specifico viene applicato più volte sullo stesso campione

Note: we did not do technical replicates in the laboratory course
(in normally at least 2 technological replicates need to be performed)

Analysing experimental data:

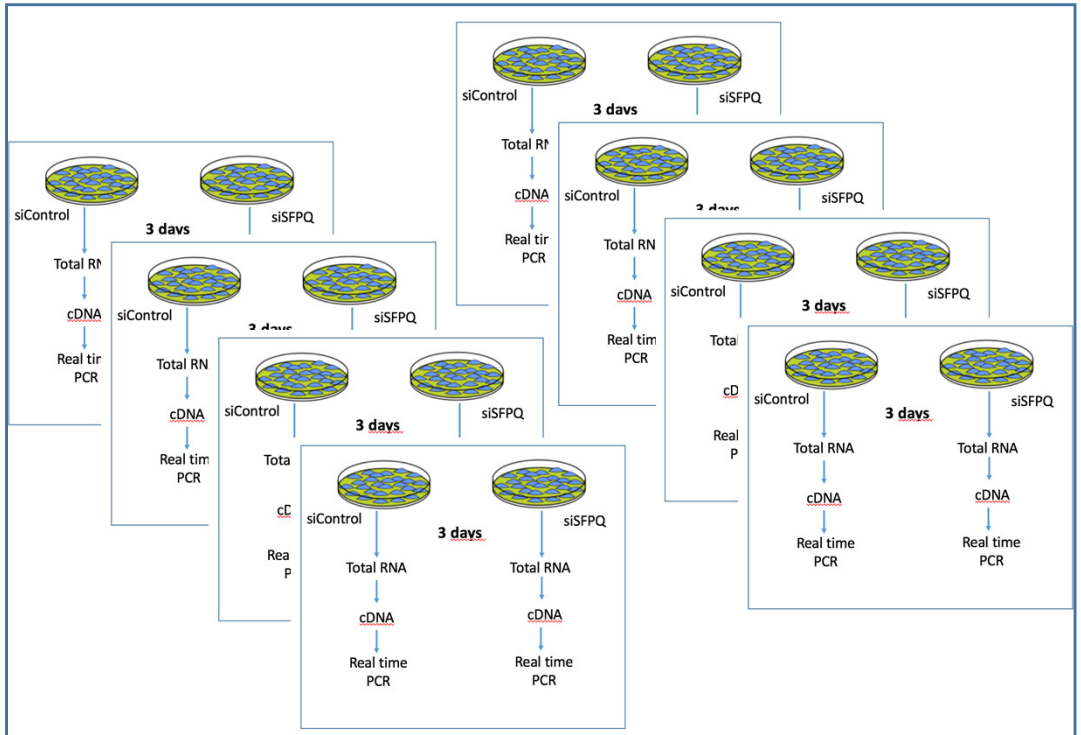
2. Tests to evaluate the robustness and significance of biological data

Step 1: Deviazione standard:

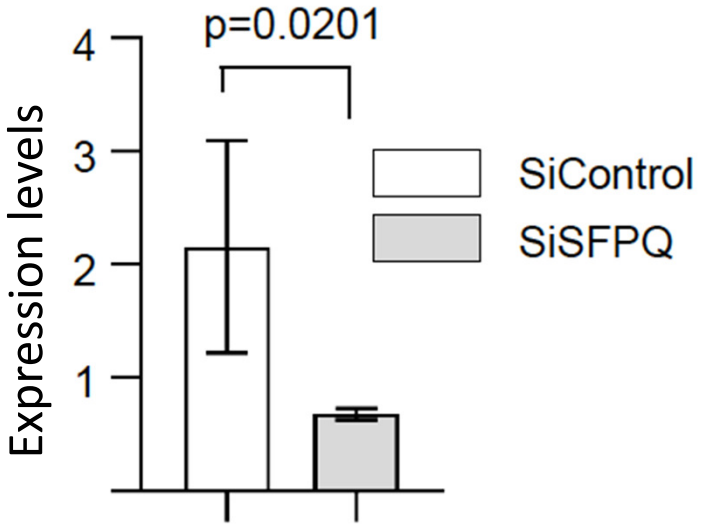
Una deviazione standard è una misura di quanto sono dispersi i dati rispetto alla media. Una deviazione standard bassa o piccola indica che i dati sono raggruppati strettamente attorno alla media, mentre una deviazione standard alta o grande indica che i dati sono più sparsi.

Step 2: Student's t-test:

Un t-test è un tipo di analisi statistica utilizzata per confrontare le medie di due gruppi e determinare se le differenze tra loro hanno maggiori probabilità di derivare da un caso casuale.



Biological replicates 1-9



p<0,05: statisticamente significante

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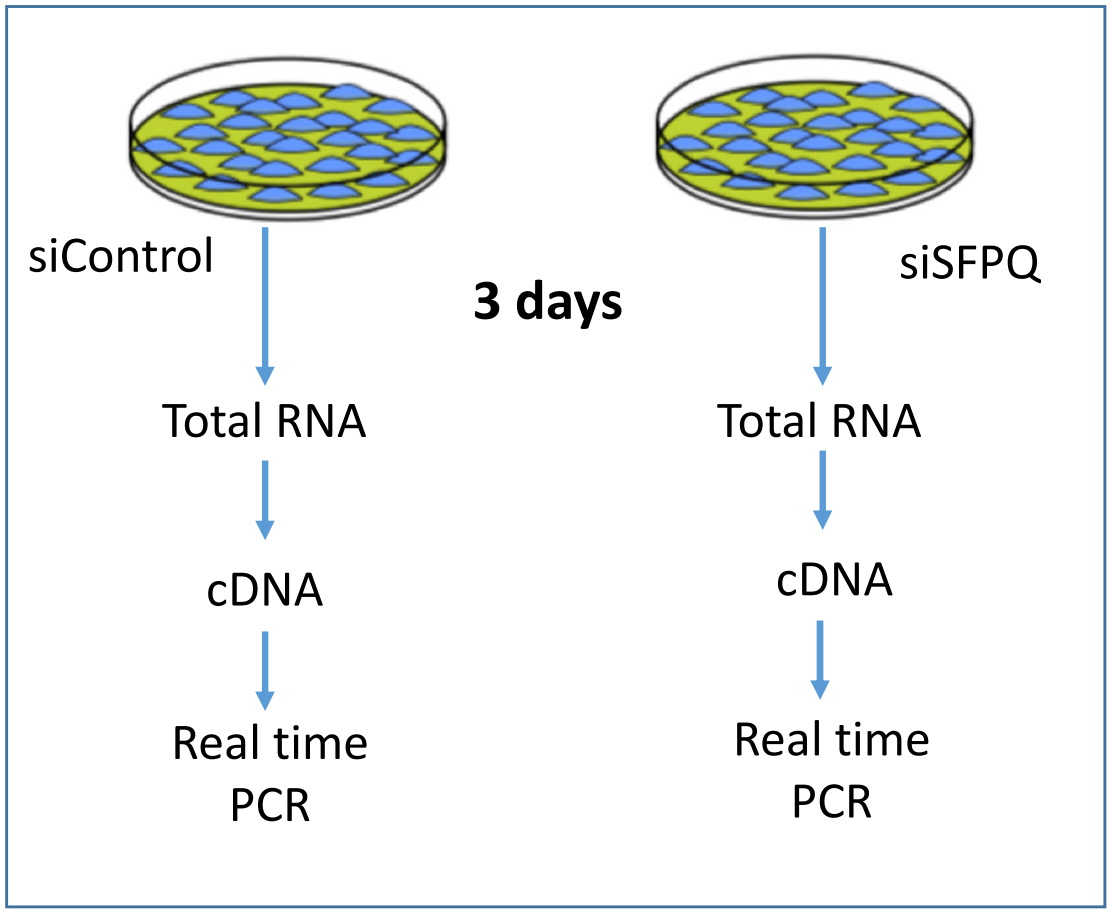


A. Obtaining Ct values for siCon; siSFPQ

B. Calculation of ΔCt value

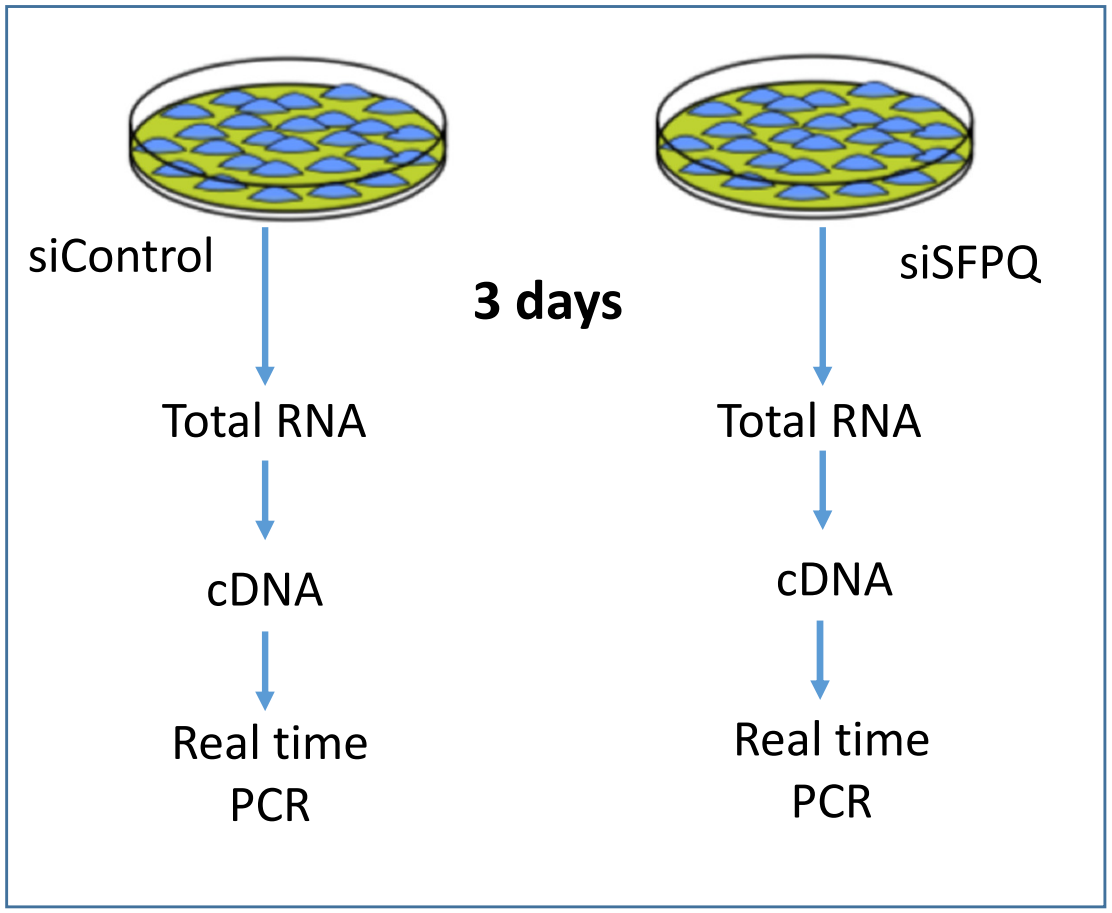
C. Calculation of $\Delta\Delta$Ct values

D. Calculation of fold changes

E. Calculation of StdDev; p-value

F. Generation of Barblot diagram with StdDev, p-values, labelling of axes

Analysis of qPCR data generated in the laboratory course



A. **Obtaining Ct values for siCon; siSFPQ**

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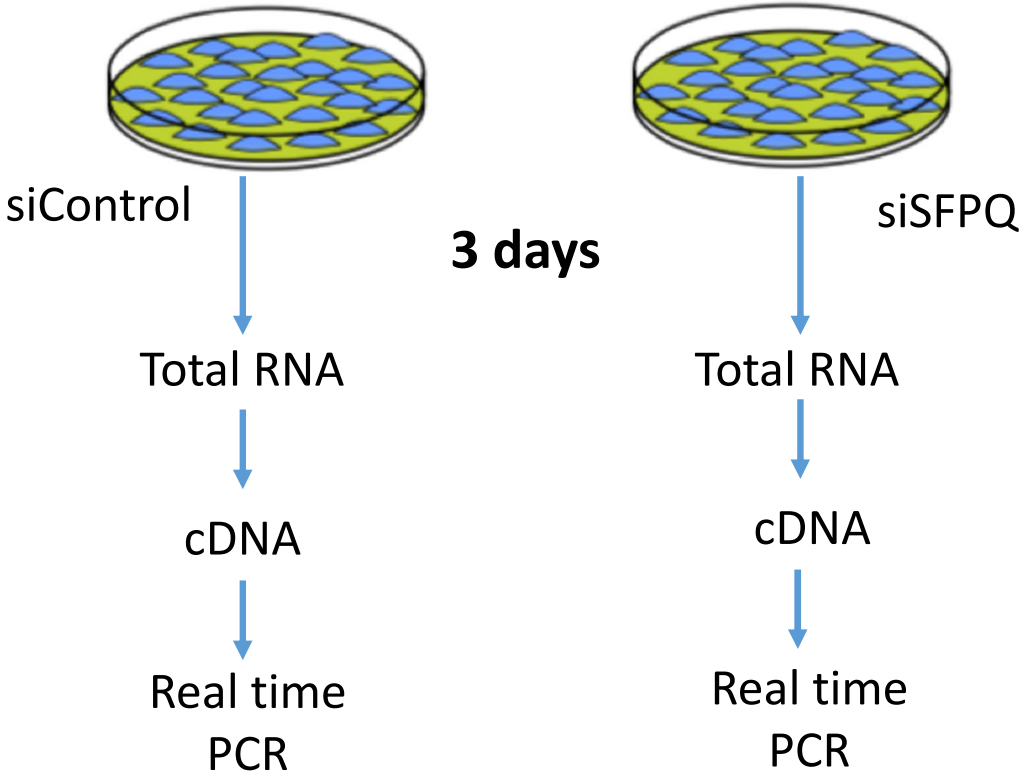
Basics for the analysis of real-time PCR data: relative quantitation

Obtaining Ct values for siCon; siSFPQ

U2-OS cells transfected with siRNA (siControl, siSFPQ)



All relevant experimental samples have been processed (RNA prep; cDNA sysnthesis, qPCR) in parallel, at the same time

A. Obtaining Ct values for siCon; siSFPQ

GENES OF INTEREST (SFPQ, CCL5):

- **altered expression in relevant biological context expected (siControl, siSFPQ)**
- **Biological interest is focussed on these genes**

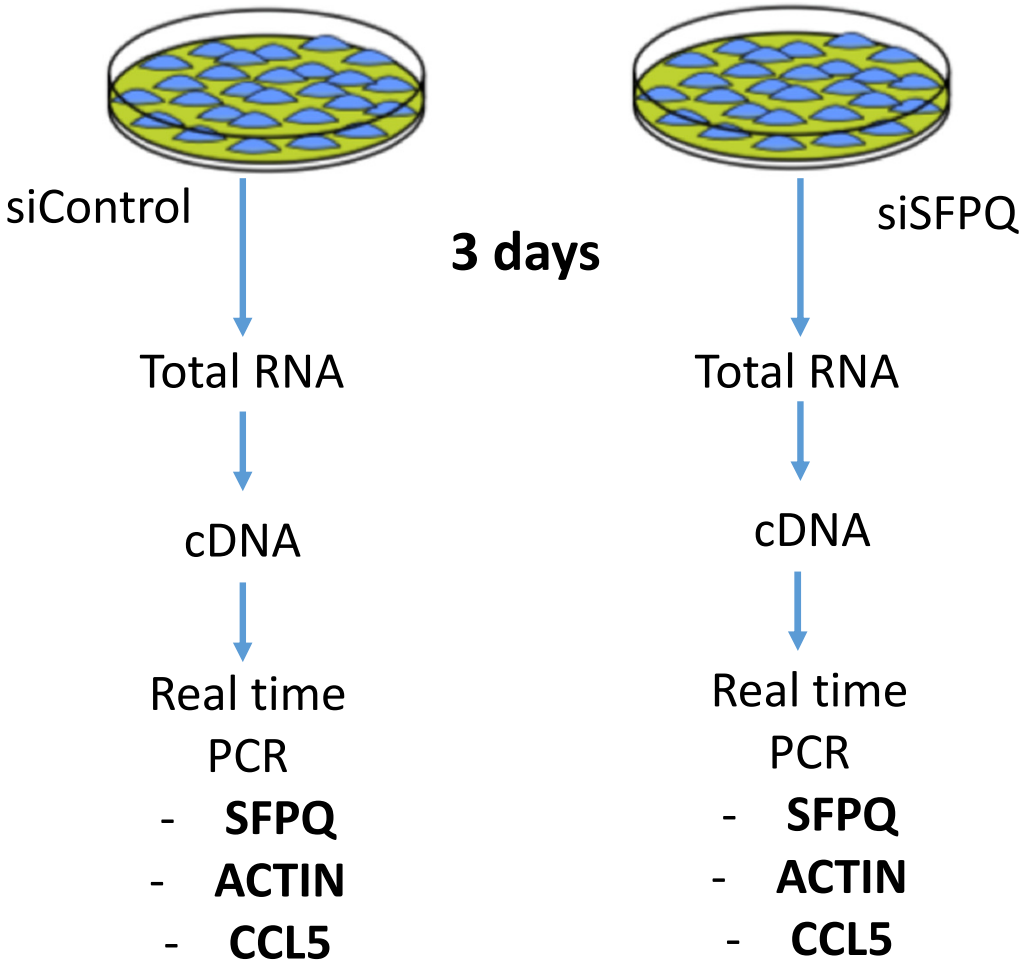
REFERENCE GENE (ACTIN):

- **no altered expression in relevant biological context (siControl, siSFPQ)**
- **preferentially expressed at high levels**
- **Serves to control of sample quantity**
- **Serves to control for pipetting errors**
- **Other examples:18S rRNA, GAPDH, β-actin, tubilin, RNA polymerase II, histone H3**

Basics for the analysis of real-time PCR data: relative quantitation

Obtaining Ct values for siCon; siSFPQ

U2-OS cells transfected with siRNA (siControl, siSFPQ)



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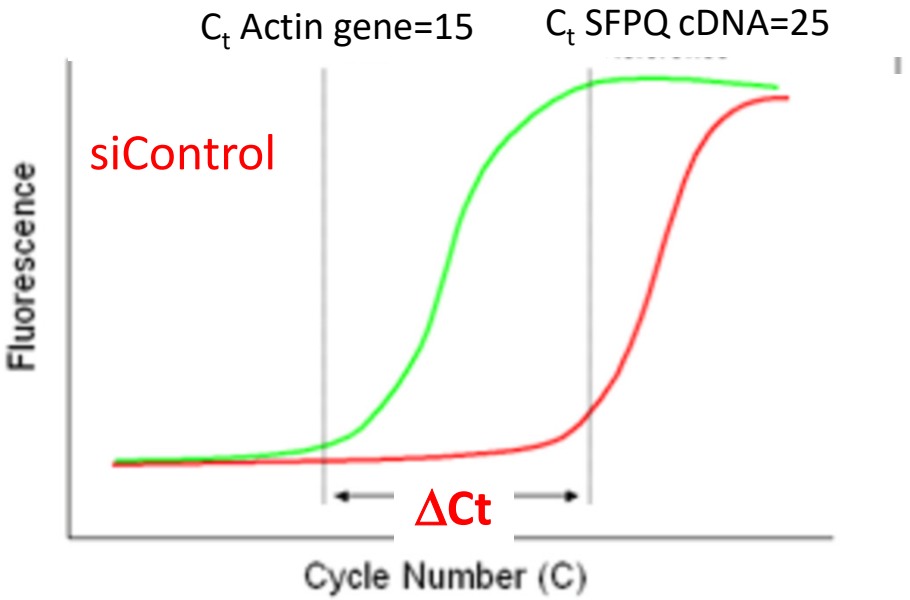
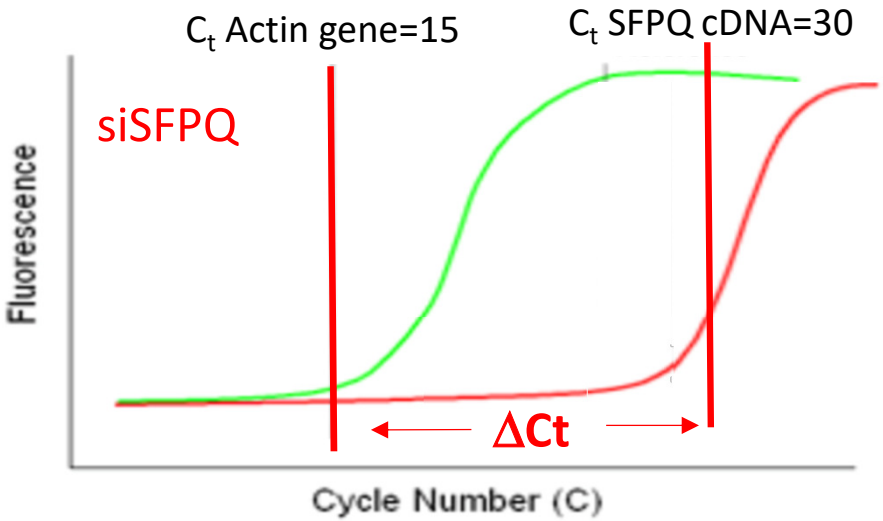
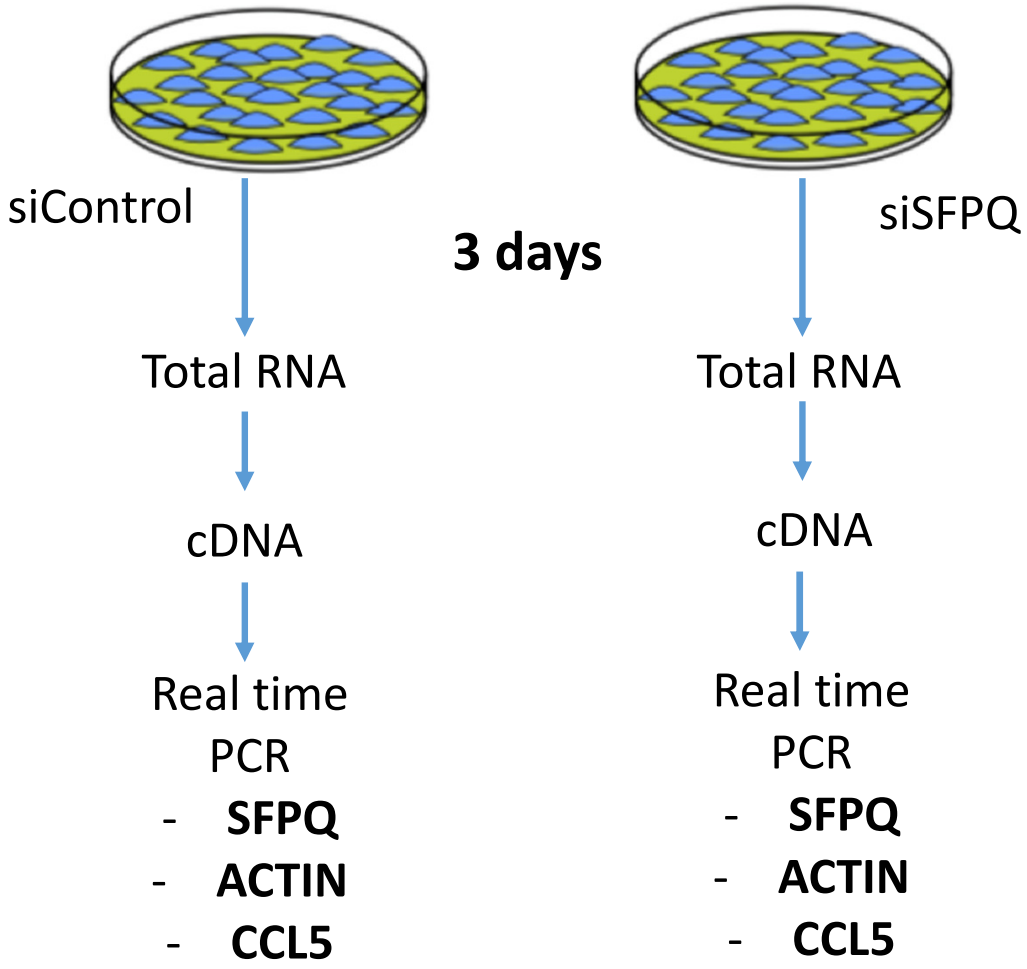




Basics for the analysis of real-time PCR data: relative quantitation

Reference gene compensates for evential errors by operator

U2-OS cells transfected with siRNA (siControl, siSFPQ)



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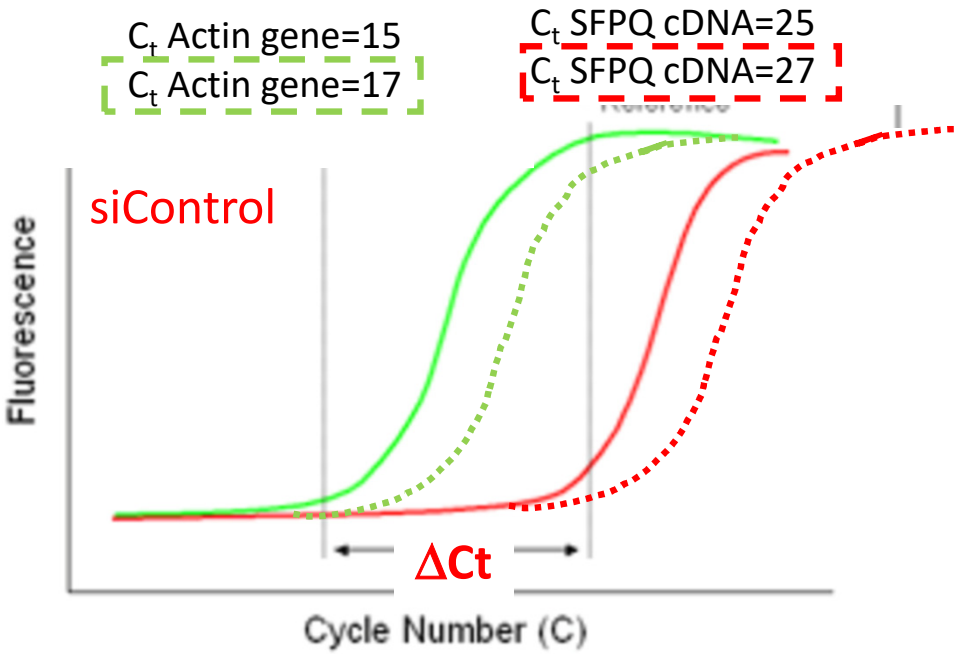
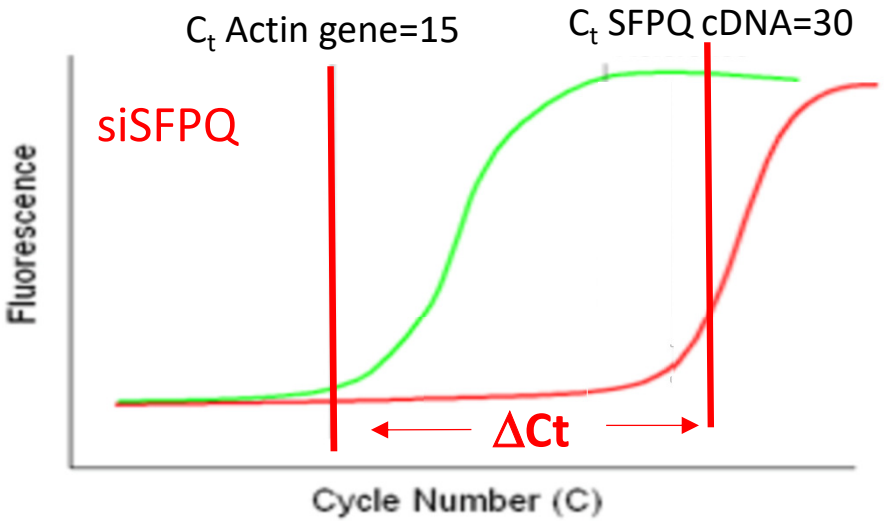
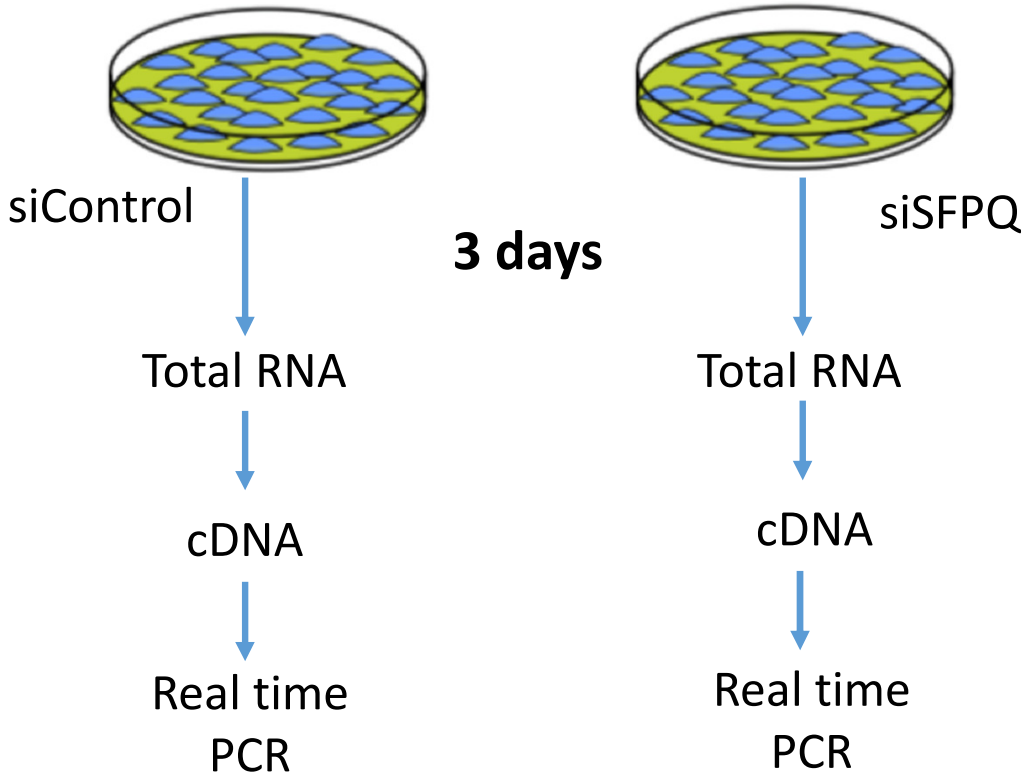




Basics for the analysis of real-time PCR data: relative quantitation

Obtaining Ct values for siCon; siSFPQ

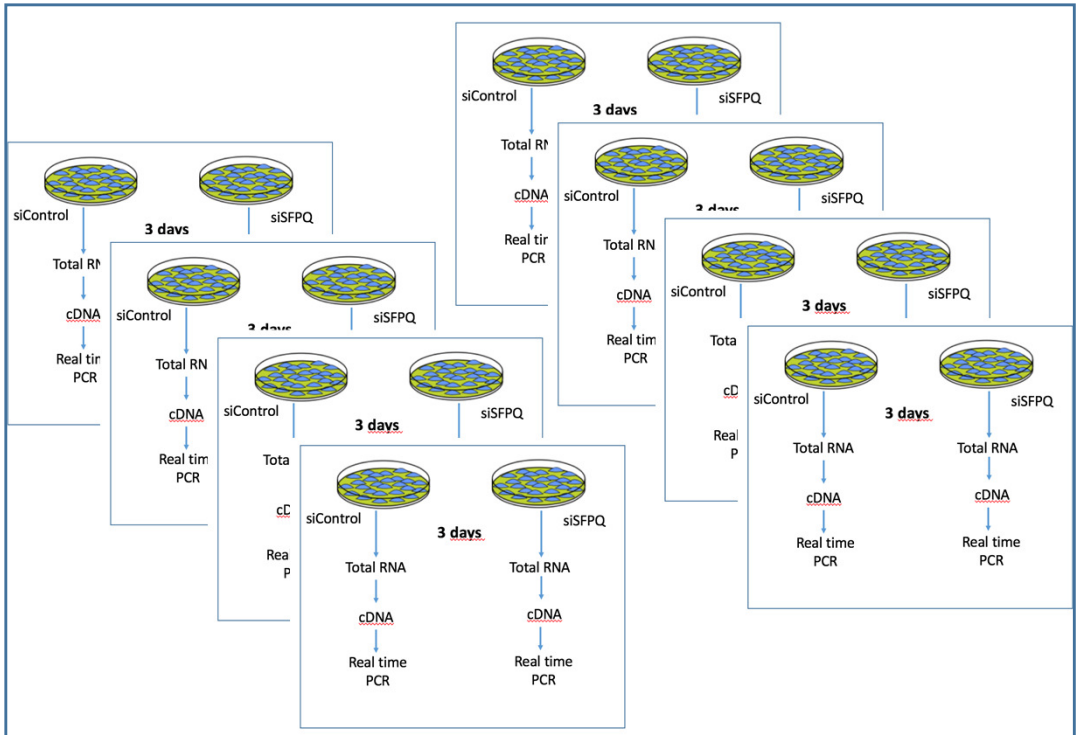
Step 1 Processing individual data



Student 1 --- biological replicate 1
Tutor --------- biolocical replicate 2
Tutor --------- biolocical replicate 3

A. Obtaining Ct values for siCon; siSFPQ

Step 1 Processing data of entire tuno



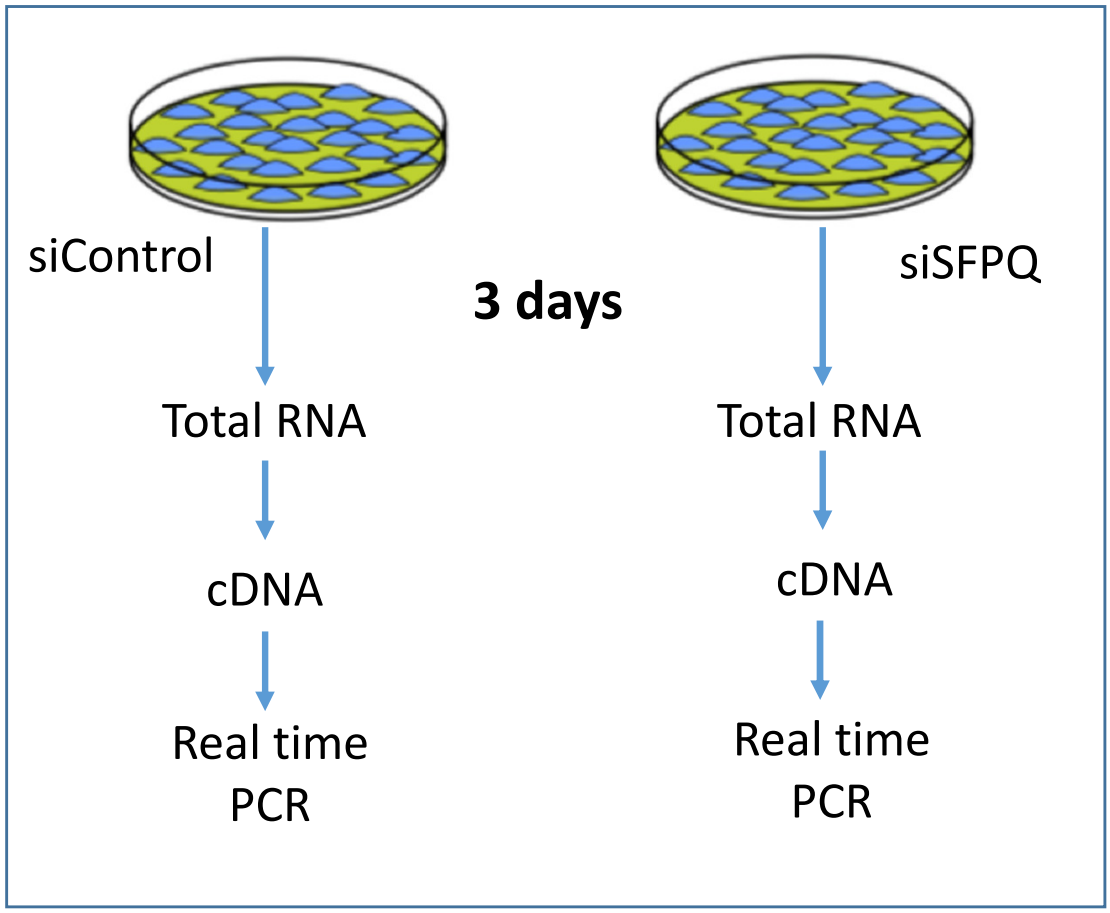
Student 1 – Student 9: biological replicates 1-9

ANALYSING DATA OF THE LABORATORY COURSE

Obtaining Ct values for siCon; siSFPQ

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1		**ACTIN**						**SFPQ**						**CCL5**			
2																	
3	**Replicate**		**siControl**	**siSFPQ**			**Replicate**		**siControl**	**siSFPQ**			**Replicate**		**siControl**	**siSFPQ**	
4	1	CT	12,43	13,15			1	CT	17,33	21,32			1	CT	20,51	19,96	
5	2	CT	12,13	13,28			2	CT	17,34	21,64			2	CT	23,8	29,85	
6	3	CT	13,29	12,79			3	CT	17,99	21,17			3	CT	21,82	20,02	
7	4	CT	12,68	12,37			4	CT	16,82	20,54			4	CT	23,13	21,7	
8	5	CT	11,15	13,59			5	CT	15,91	21,57			5	CT	18,17	24,58	
9	6	CT	11,94	14,61			6	CT	16,79	22,22			6	CT	19,6	21,08	
10	7	CT	12,88	14,5			7	CT	17,31	21,87			7	CT	21,03	21,17	
11	8	CT	13,01	13,53			8	CT	17,53	20,82			8	CT	20,76	20	
12	9	CT	13,69	13,12			9	CT	18,49	20,4			9	CT	23,43	20,26	
13																	
14																	

ANALYSING DATA OF THE LABORATORY COURSE



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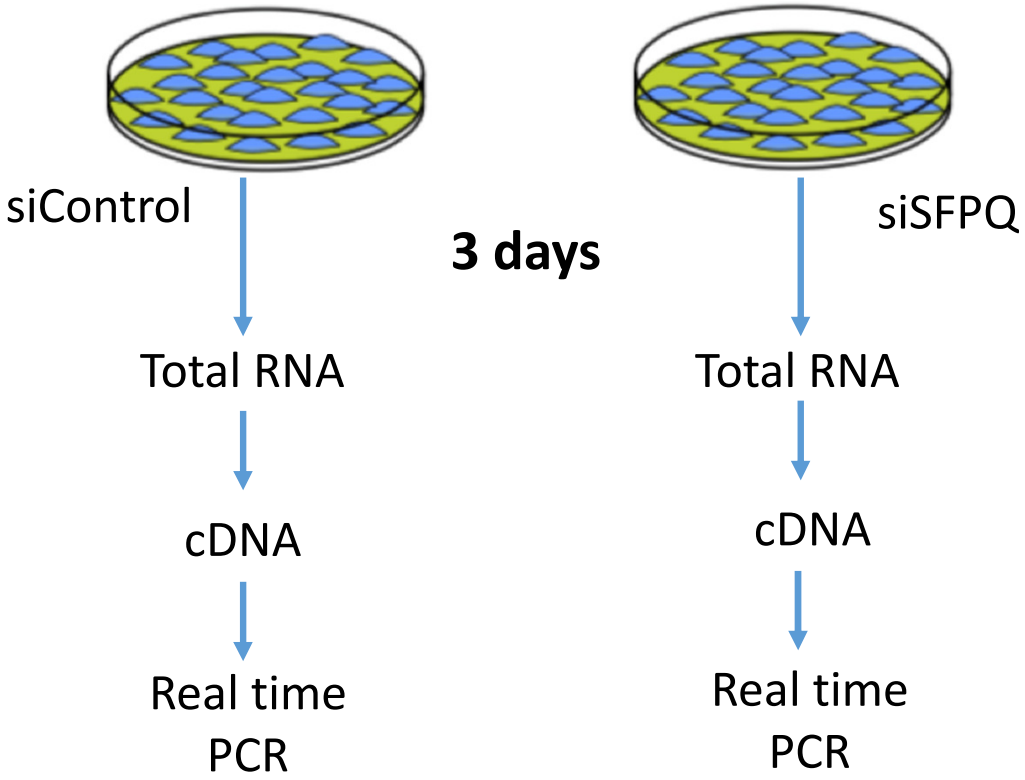
D. Calculation of fold changes

E. Calculation of StdDev; p-value

F. Generation of Barblot diagram with StdDev, p-values, labelling of axes

ANALYSING DATA OF THE LABORATORY COURSE

Calculation of ΔCt value, ΔΔCt values, fold changes



Student 1 --- biological replicate 1
Tutor --------- biolocical replicate 2
Tutor --------- biolocical replicate 3

B. Calculation of deltaCt value

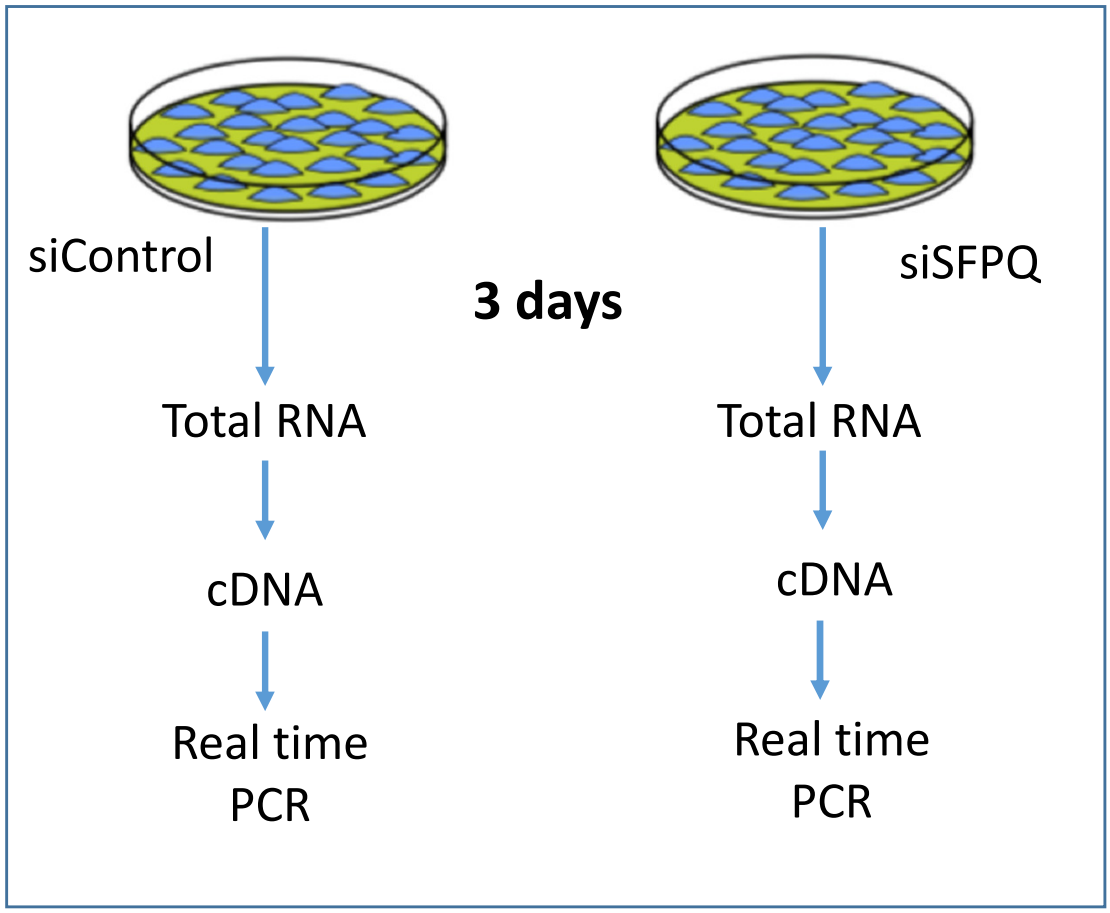
siControl

Replicate		ACTIN siControl	SFPQ siControl	siControl delta Ct
1	CT	12,43	17,33	4,9
2	CT	12,13	17,34	5,21
3	CT	13,29	17,99	4,7
4	CT	12,68	16,82	4,14
5	CT	11,15	15,91	4,76
6	CT	11,94	16,79	4,85
7	CT	12,88	17,31	4,43
8	CT	13,01	17,53	4,52
9	CT	13,69	18,49	4,8

siSFPQ

Replicate		ACTIN siSFPQ	SFPQ siSFPQ	siSFPQ delta Ct
1	CT	13,15	21,32	8,17
2	CT	13,28	21,64	8,36
3	CT	12,79	21,17	8,38
4	CT	12,37	20,54	8,17
5	CT	13,59	21,57	7,98
6	CT	14,61	22,22	7,61
7	CT	14,5	21,87	7,37
8	CT	13,53	20,82	7,29
9	CT	13,12	20,4	7,28

ANALYSING DATA OF THE LABORATORY COURSE



A. Obtaining Ct values for siCon; siSFPQ

B. Calculation of ΔCt value

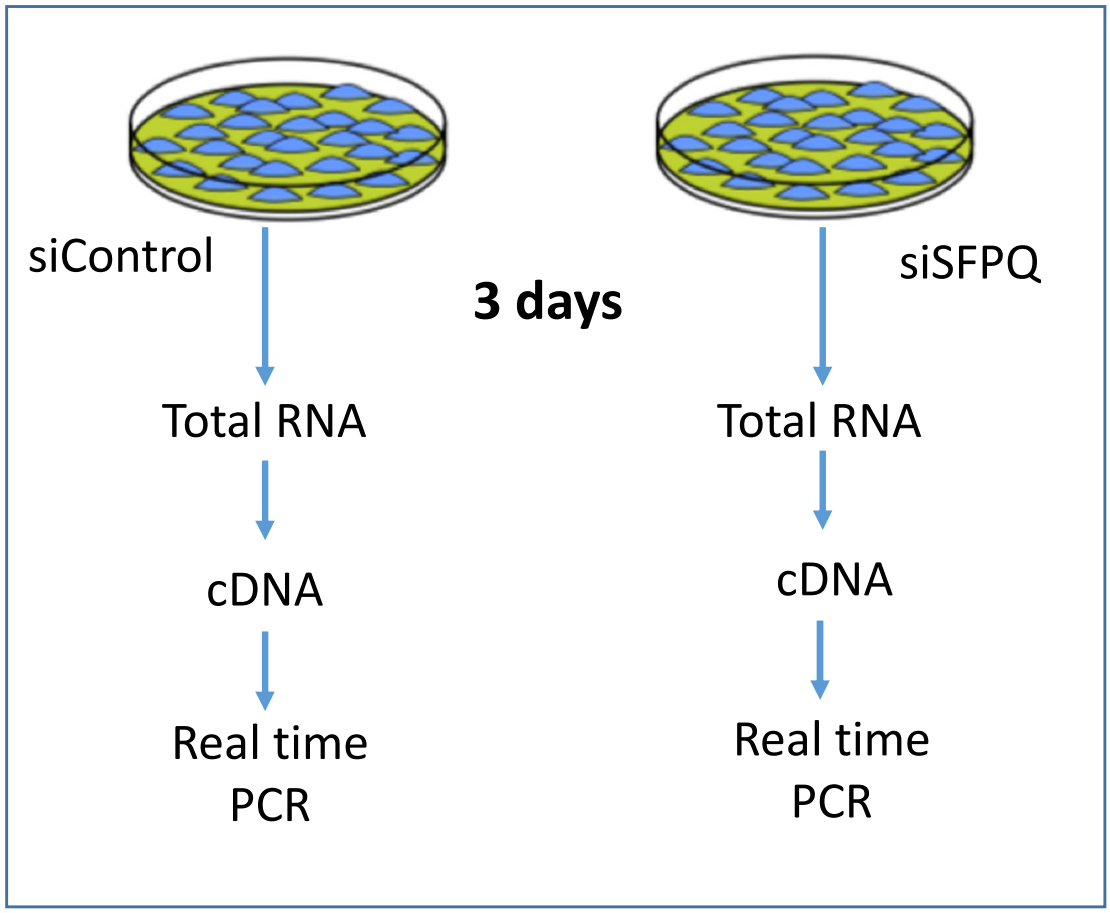
C. Calculation of ΔΔCt values

D. Calculation of fold changes

E. Calculation of StdDev; p-value

F. Generation of Barblot diagram with StdDev, p-values, labelling of axes

ANALYSING DATA OF THE LABORATORY COURSE



C. Calculation of ΔΔCt value

siControl

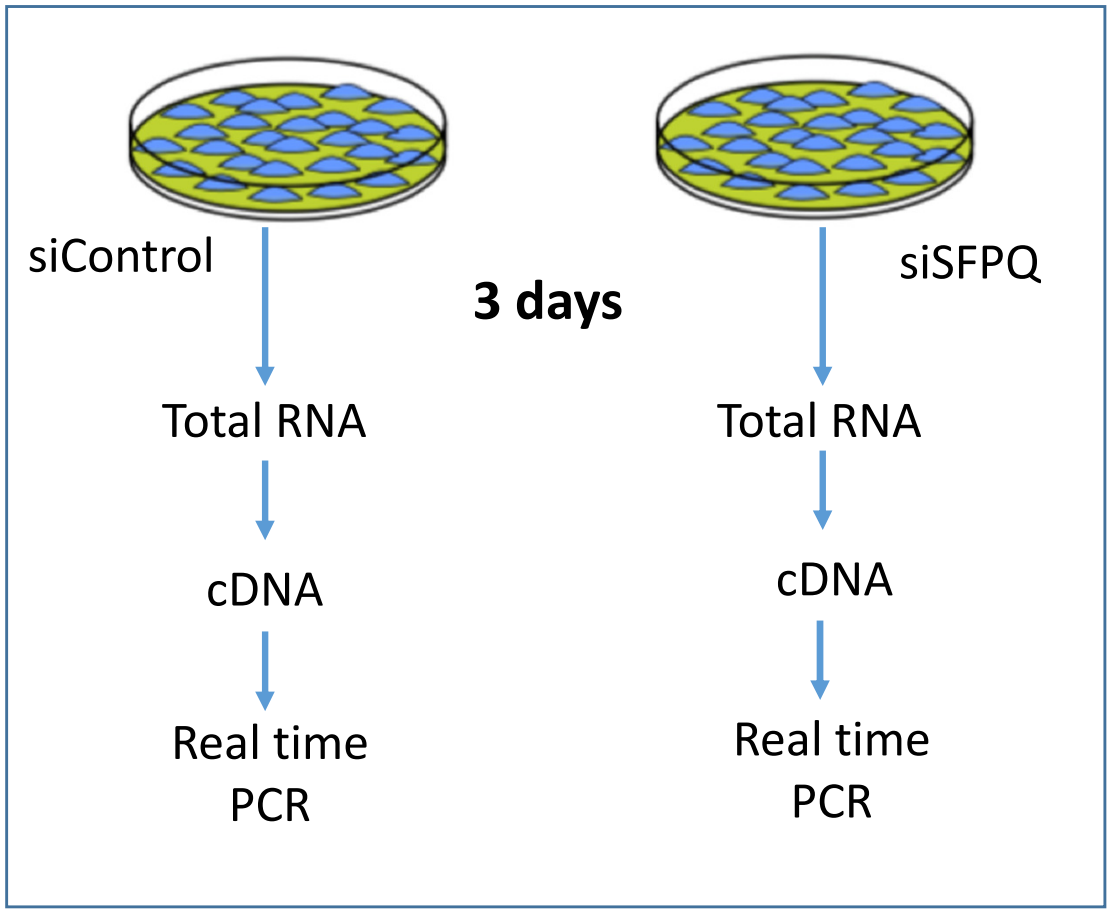
Replicate		ACTIN siControl	SFPQ siControl	siControl delta Ct
1	CT	12,43	17,33	4,9
2	CT	12,13	17,34	5,21

siSFPQ

Replicate		ACTIN siSFPQ	SFPQ siSFPQ	siSFPQ delta Ct
1	CT	13,15	21,32	8,17
2	CT	13,28	21,64	8,36

Replicate		siControl delta Ct	siSFPQ delta Ct	delta deltaCt
1	CT	4,9	8,17	-3,27
2	CT	5,21	8,36	-3,15
3	CT	4,7	8,38	-3,68
4	CT	4,14	8,17	-4,03
5	CT	4,76	7,98	-3,22
6	CT	4,85	7,61	-2,76
7	CT	4,43	7,37	-2,94
8	CT	4,52	7,29	-2,77
9	CT	4,8	7,28	-2,48

ANALYSING DATA OF THE LABORATORY COURSE



A. Obtaining Ct values for siCon; siSFPQ

B. Calculation of ΔCt value

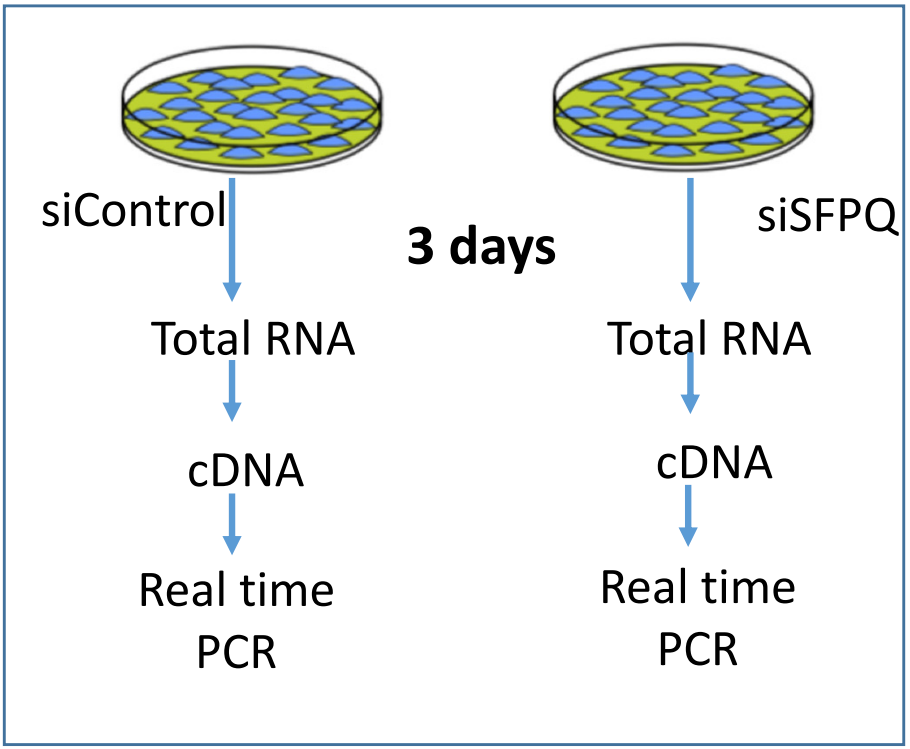
C. Calculation of $\Delta\Delta$Ct values

D. Calculation of fold changes

E. Calculation of StdDev; p-value

F. Generation of Bar-blot diagram with StdDev, p-values, labelling of axes

ANALYSING DATA OF THE LABORATORY COURSE



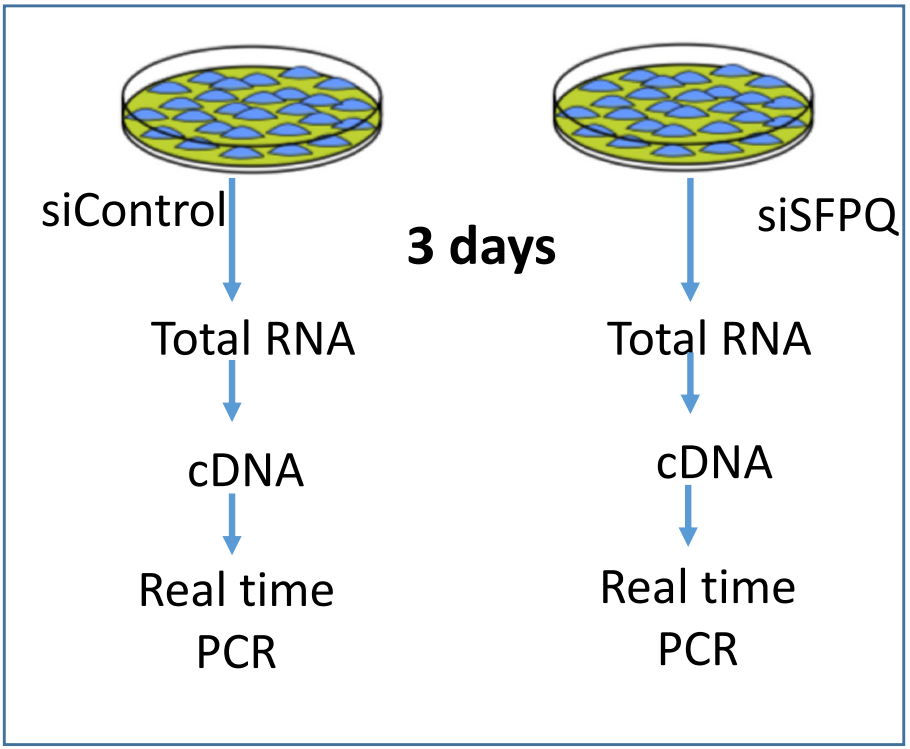
D. Calculation of fold changes

Replicate	siControl delta Ct	siSFPQ delta Ct	delta deltaCt	convert cycle to fold change reduction 2^(deltadeltaCt(*-1))
1	4,9	8,17	-3,27	9,646462622
2	5,21	8,36	-3,15	8,876555777
3	4,7	8,38	-3,68	12,81711804
4	4,14	8,17	-4,03	16,33619401
5	4,76	7,98	-3,22	9,317868692
6	4,85	7,61	-2,76	6,773962499
7	4,43	7,37	-2,94	7,674112955
8	4,52	7,29	-2,77	6,821079134
9	4,8	7,28	-2,48	5,578974665

…remember PCR in exponential phase: amplification per cycle: 2^n (n= numero cicli)

…replica 1: SFPQ is 9,64 fold lower in siControl versus siSFPQ

ANALYSING DATA OF THE LABORATORY COURSE



A. Obtaining Ct values for siCon; siSFPQ

B. Calculation of ΔCt value

C. Calculation of $\Delta\Delta$Ct values

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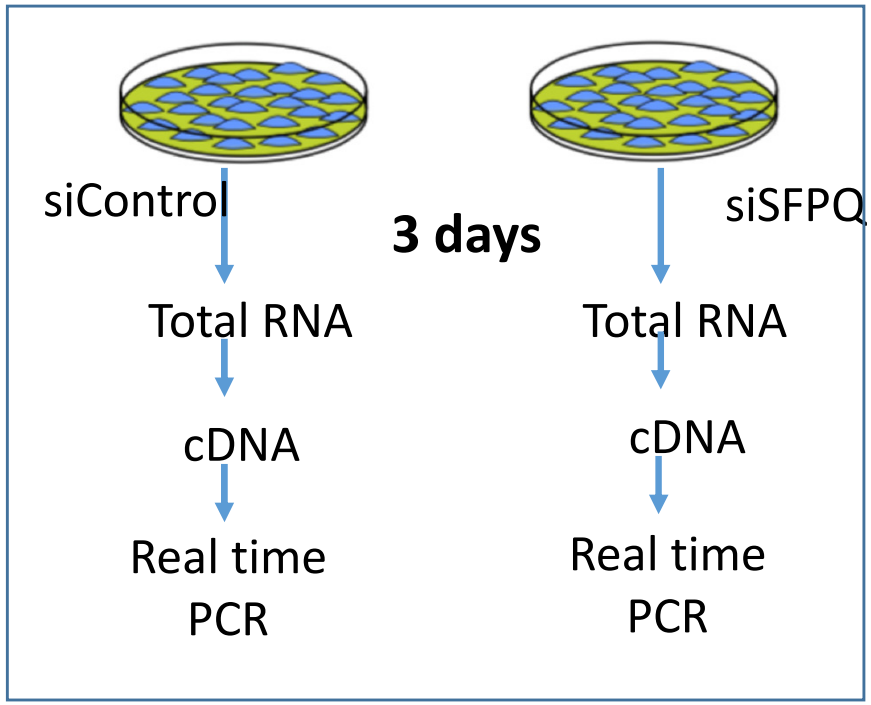
ANALYSING DATA OF THE LABORATORY COURSE



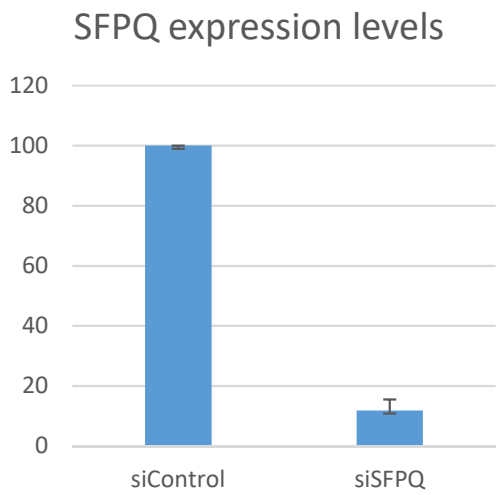
D. Calculation of StdDev, p-values

RELATIVE EXPRESSION LEVELS

	siControl	siSFPQ
1	100	10,3664943
2	100	11,2656308
3	100	7,80206593
4	100	6,12137686
5	100	10,732068
6	100	14,7624083
7	100	13,030822
8	100	14,6604369
9	100	17,9244406
	siControl	**siSFPQ**
Average	**100**	**11,8517493**
StdDev	**0**	**3,66859579**

siControl set ”100”

E. Generation of bar blot with error bars



Insert StdDev

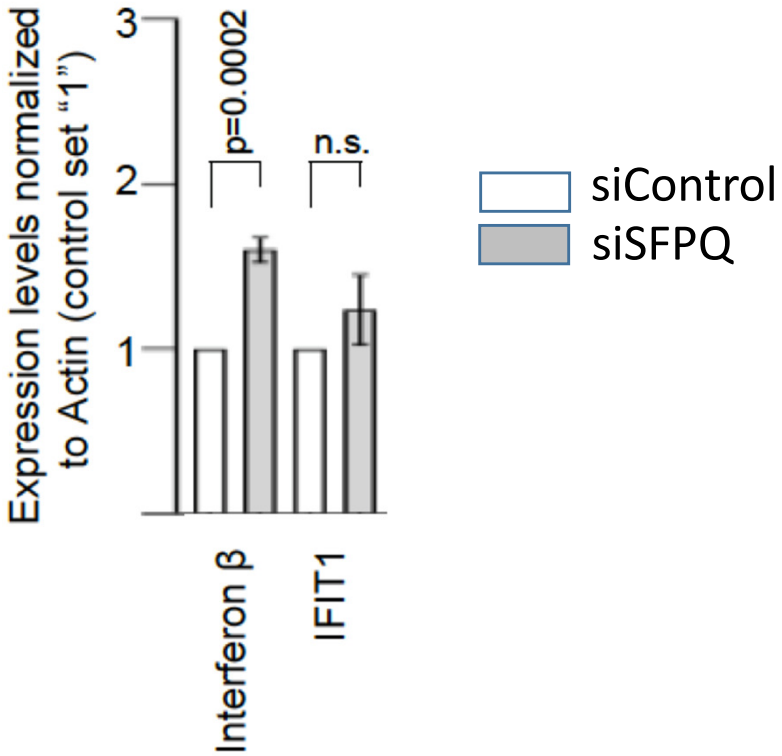
Replicate	siControl delta Ct	siSFPQ delta Ct	delta deltaCt	convert cycle to fold change reduction 2^(deltadeltaCt(*-1))
1	4,9	8,17	-3,27	9,646462622
2	5,21	8,36	-3,15	8,876555777
3	4,7	8,38	-3,68	12,81711804

Replica 1: SFPQ is 9,64 fold lower in siControl versus siSFPQ
→ 100/9,64=10,366

Replica 2: SFPQ is 9,64 fold lower in siControl versus siSFPQ
→ 100/8,87=11,25

ANALYSING DATA OF THE LABORATORY COURSE

Generation of Bar-blot diagram with StdDev, p-values, labelling of axes



Didascalia/Figure legend:
SFPQ expression as detemined by quantitative RT-PCR. A student's t-test was used to calculate statistical singificance. Error bars indicate standard variation. N, number of biological replicates

For example: Paper figure 1B in:
https://www.nature.com/articles/s41467-022-29907-z
(check also didascalia)