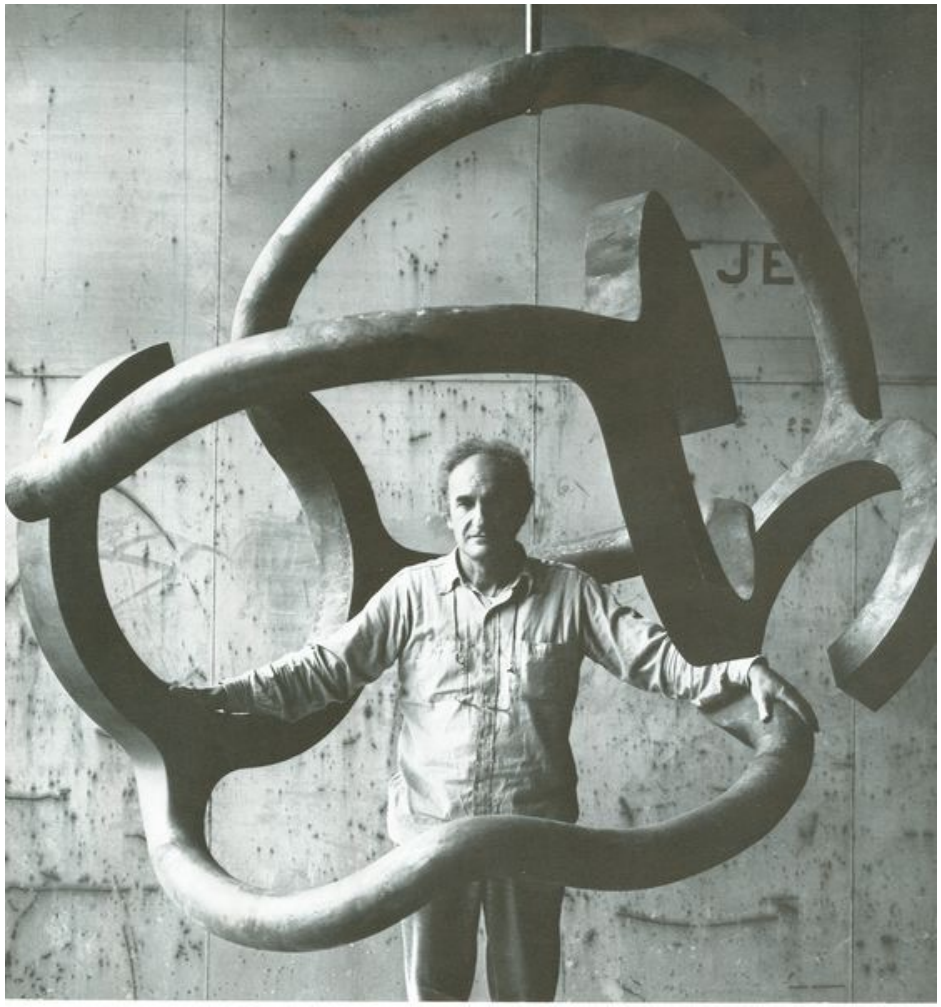


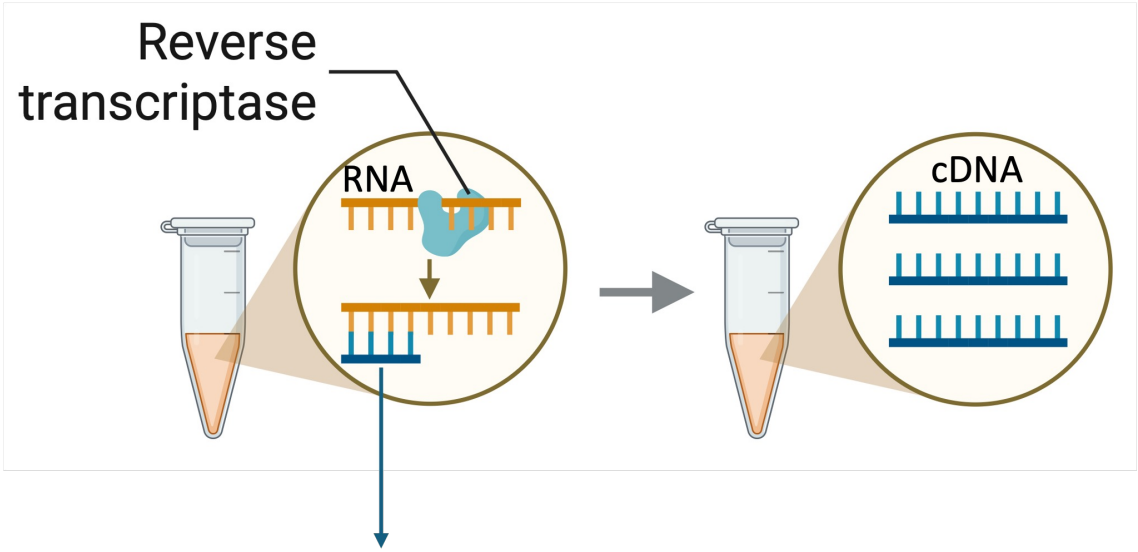
BIO 405L.
Cellular and molecular biology laboratory

Reverse transcription, real-time PCR & differential gene expression analysis

Hugo Castillo, Ph.D.

Gene expression studies start with RNA (mRNA) and PCR works exclusively with DNA, so…

Reverse transcription and complementary DNA (cDNA)



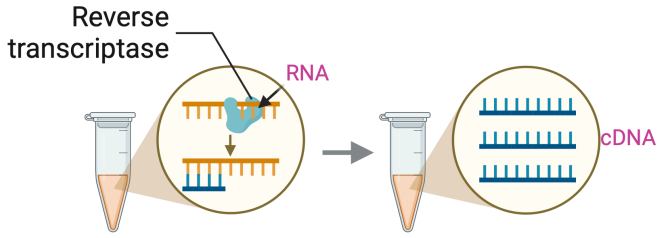
Types of primers

5’-TTTTTTTTTTTT-3’ Oligo dT	Eukaryotic
5’-NNNNNN-3’ Random hexamer	Prokaryotic/eukaryotic
5’-AGTCGTCGTCCT-3’ Gene-specific	Prokaryotic/eukaryotic

cDNA synthesis

Reagent	Volume
Template RNA (1 ug)	?
Random hexamers	2 uL
5X RT buffer	4 uL
Reverse transcriptase	1 uL
Water	Up to 20 uL

Mix the RT and primers mix.
Incubate at 25 C for 10 min
Incubate at 42 C for 60 min
Denature at 70 C for 10 min
Incubate at 4 C forever

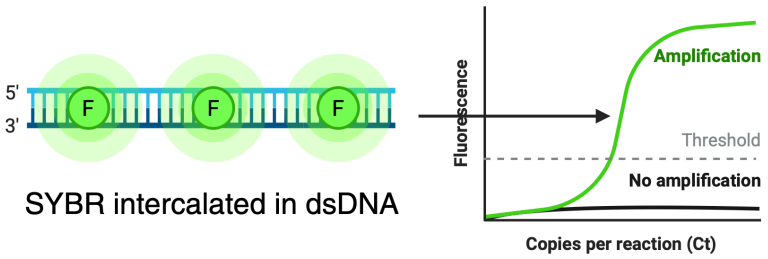


Endpoint vs real-timePCR

Feature	Endpoint PCR	Real-time PCR (qPCR)
Detection Time	After PCR reaction ends (post-PCR)	During the PCR reaction (real-time)
Quantification	Qualitative/Semi-quantitative	Quantitative (accurate)
Sensitivity	Less sensitive	Highly sensitive
Detection Method	Gel electrophoresis	Fluorescence (dye or probe-based)
Cost	Generally lower cost	More expensive due to advanced equipment
Applications	Presence/absence of DNA, genotyping	Gene expression, viral load, precise DNA quantification

Real-time or quantitative PCR

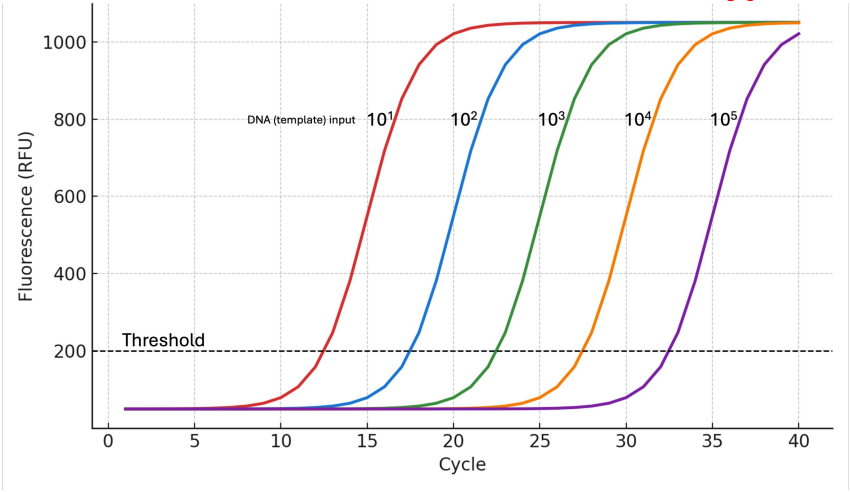
Component	Volume (µL) for One 50-µL Reaction
2X SYBR® Green PCR Master Mix	25
Forward Primer	Variable
Reverse Primer	Variable
Template	Variable
Water	Variable
Total	50

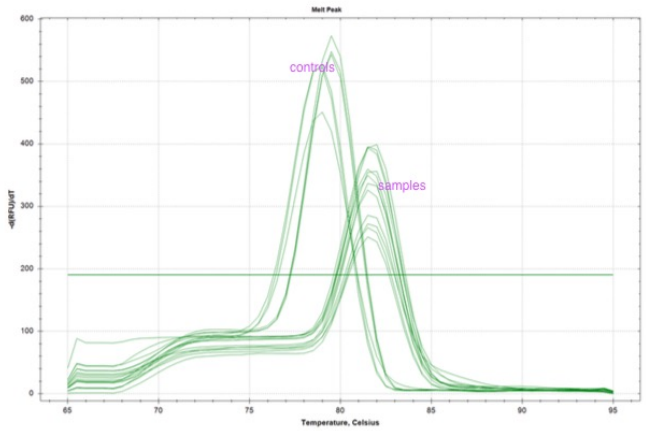


SYBR green detection

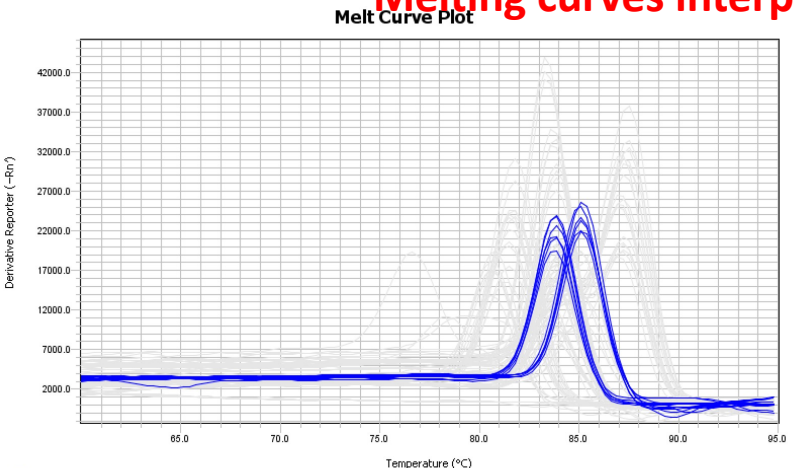
qPCR program

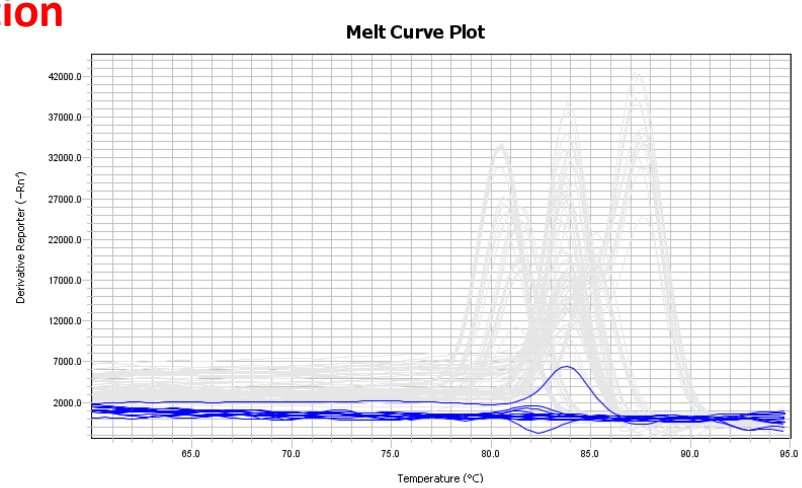
Typical output for real-time

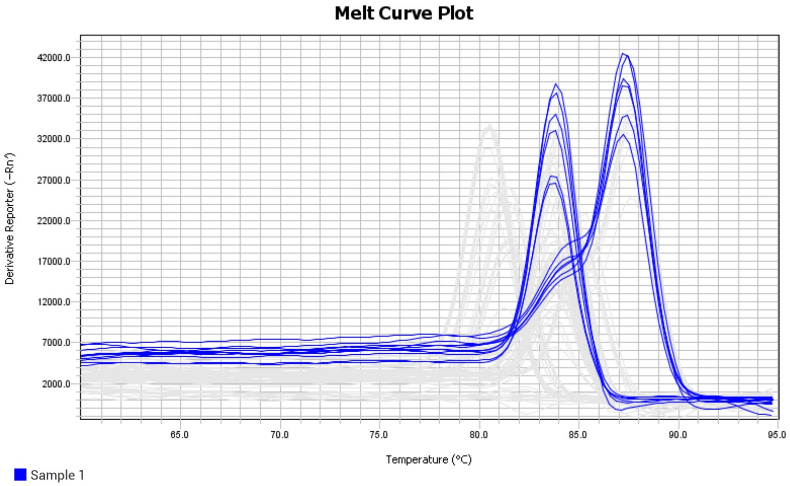


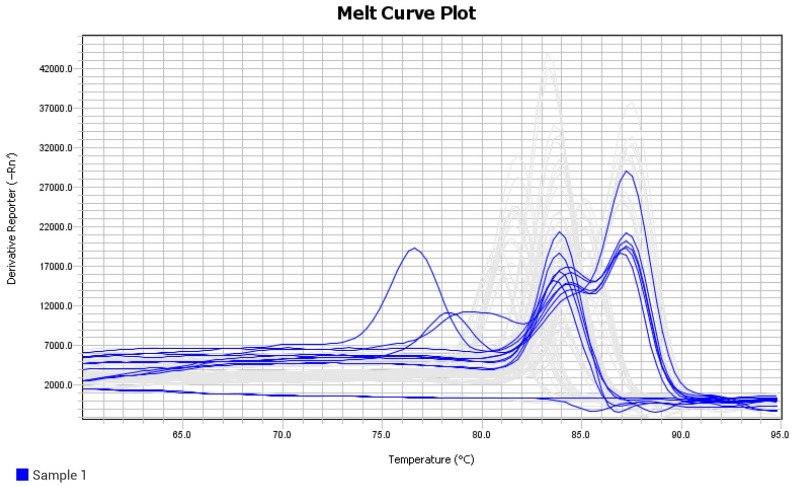


Melting curves interpretation









What you'll get after this experiment…

		Ct value
gapdH	Control	15.2
	Control	15.4
	Control	15.32
	Control	15.26
	Treatment	15.84
	Treatment	15.84
	Treatment	15.65
	Treatment	15.55
luxS	Control	28.52
	Control	28.63
	Control	28.45
	Control	28.46
	Treatment	27.21
	Treatment	27.02
	Treatment	27.41
	Treatment	27.52

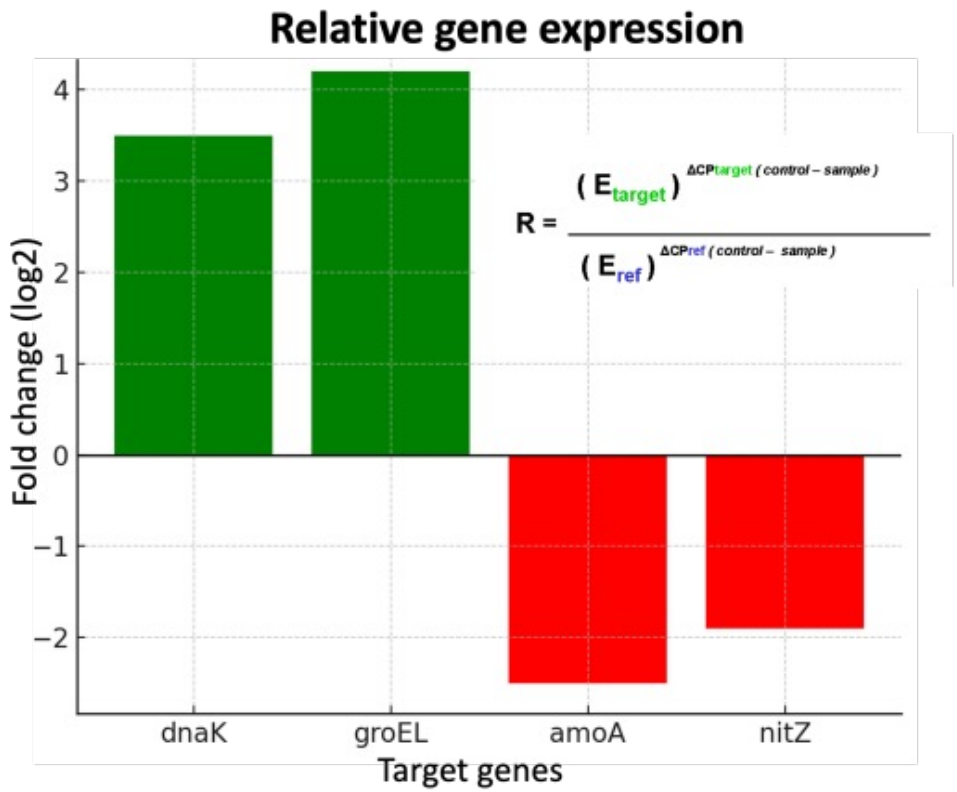
Reference genes	*gapdH*
	16S
Target genes	*luxS*
	cdgI
	motA
	mqsR

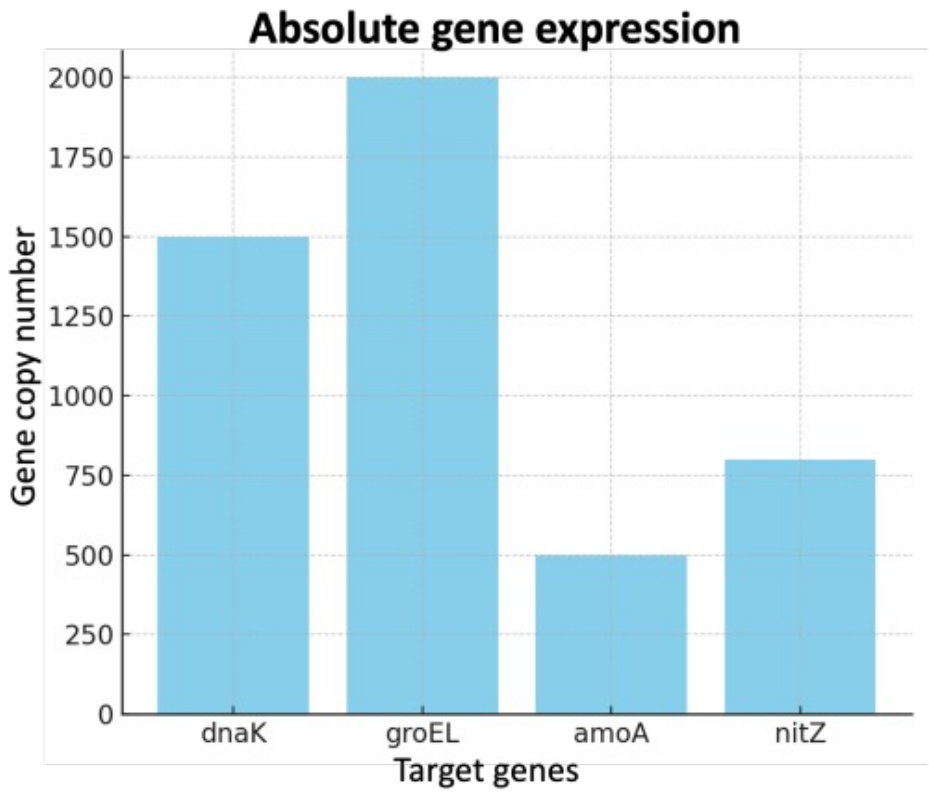
How do we calculate differential gene expression with this data?

Relative gene expression

$$R = \frac{(E_{target})^{\Delta CP target\ (control - sample)}}{(E_{ref})^{\Delta CP ref\ (control - sample)}}$$

	Gene	E	Treatments	Ct	Average	Delta	R
Reference	*gapdH*	1.9	G	20.042	19.935	-1.296	0.41
	gapdH		G	19.630			
	gapdH		G	20.132			
	gapdH		M	21.377	21.231		
	gapdH		M	21.446			
	gapdH		M	20.870			
Target	*clpB*	1.8	G	20.099	20.004	-2.161	0.22
	clpB		G	20.269			
	clpB		G	19.643			
	clpB		M	21.920	22.165		
	clpB		M	22.235			
	clpB		M	22.340			
						Ratio	0.55
						Log2	-0.86


Relative gene expression
Fold change (log2)
4
3
2
1
0
−1
−2
dnaK
groEL
amoA
nitZ
Target genes
R = (E_target)^ΔCPtarget (control − sample) / (E_ref)^ΔCPref (control − sample)


Absolute gene expression
Gene copy number
2000
1750
1500
1250
1000
750
500
250
0
dnaK
groEL
amoA
nitZ
Target genes