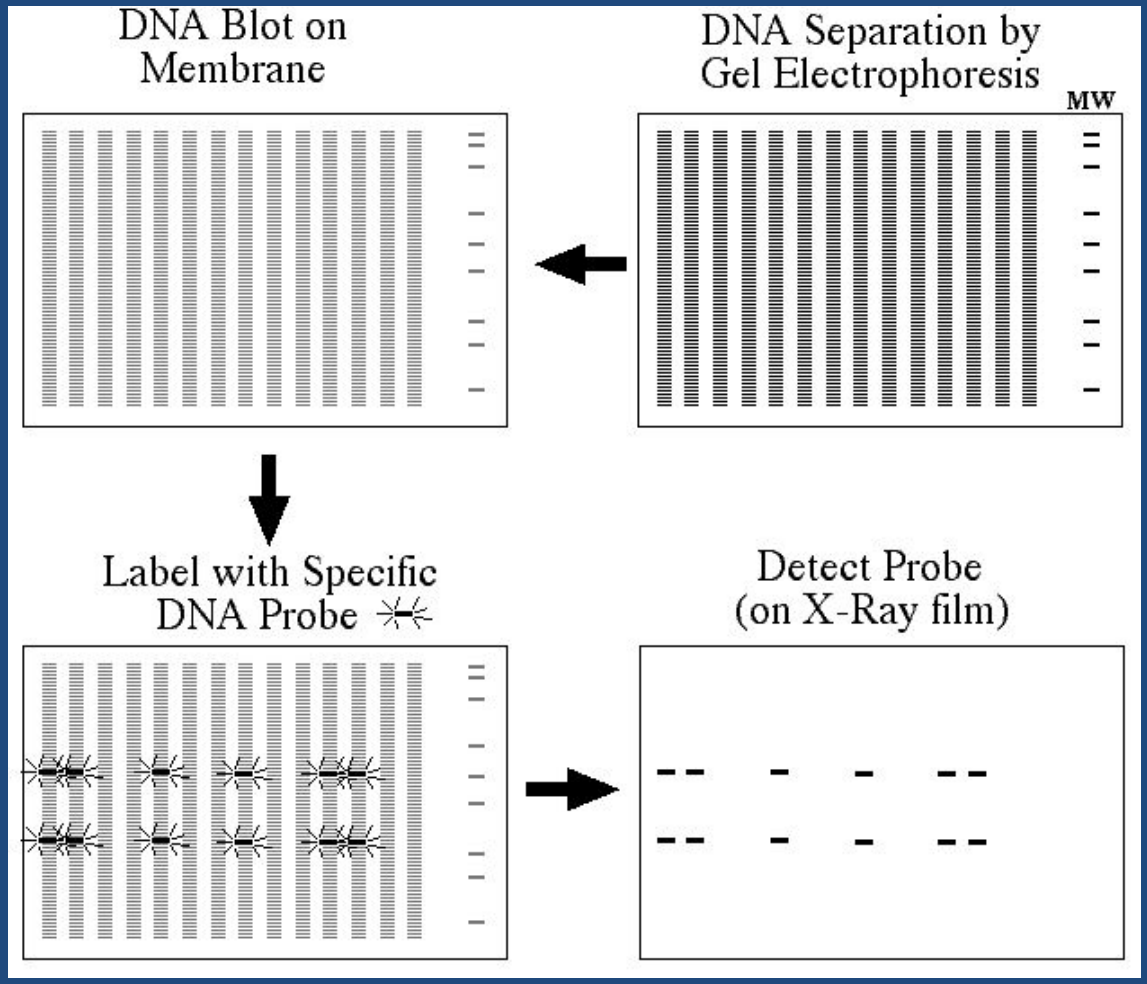
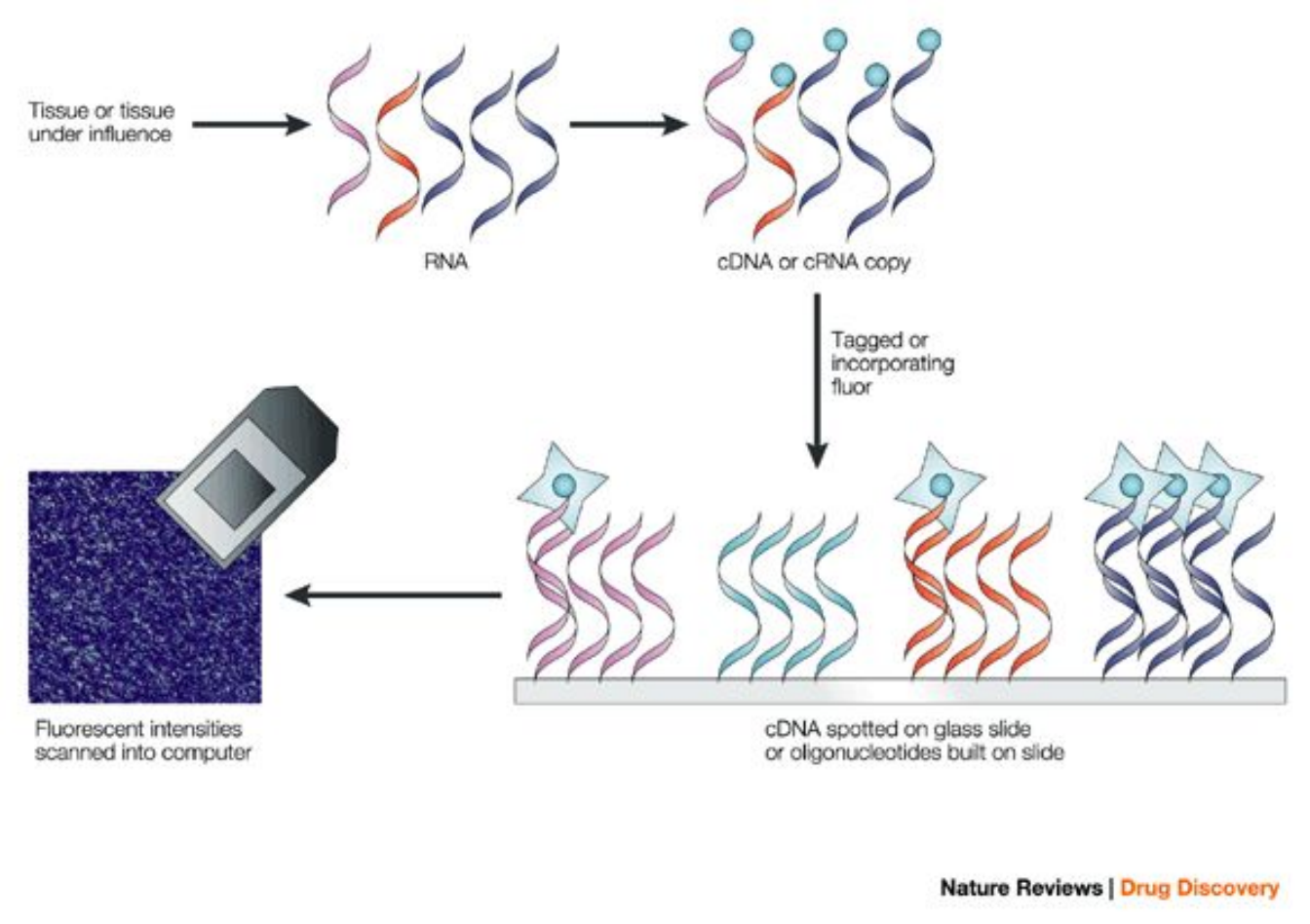
Lab Activity - An introduction to qPCR

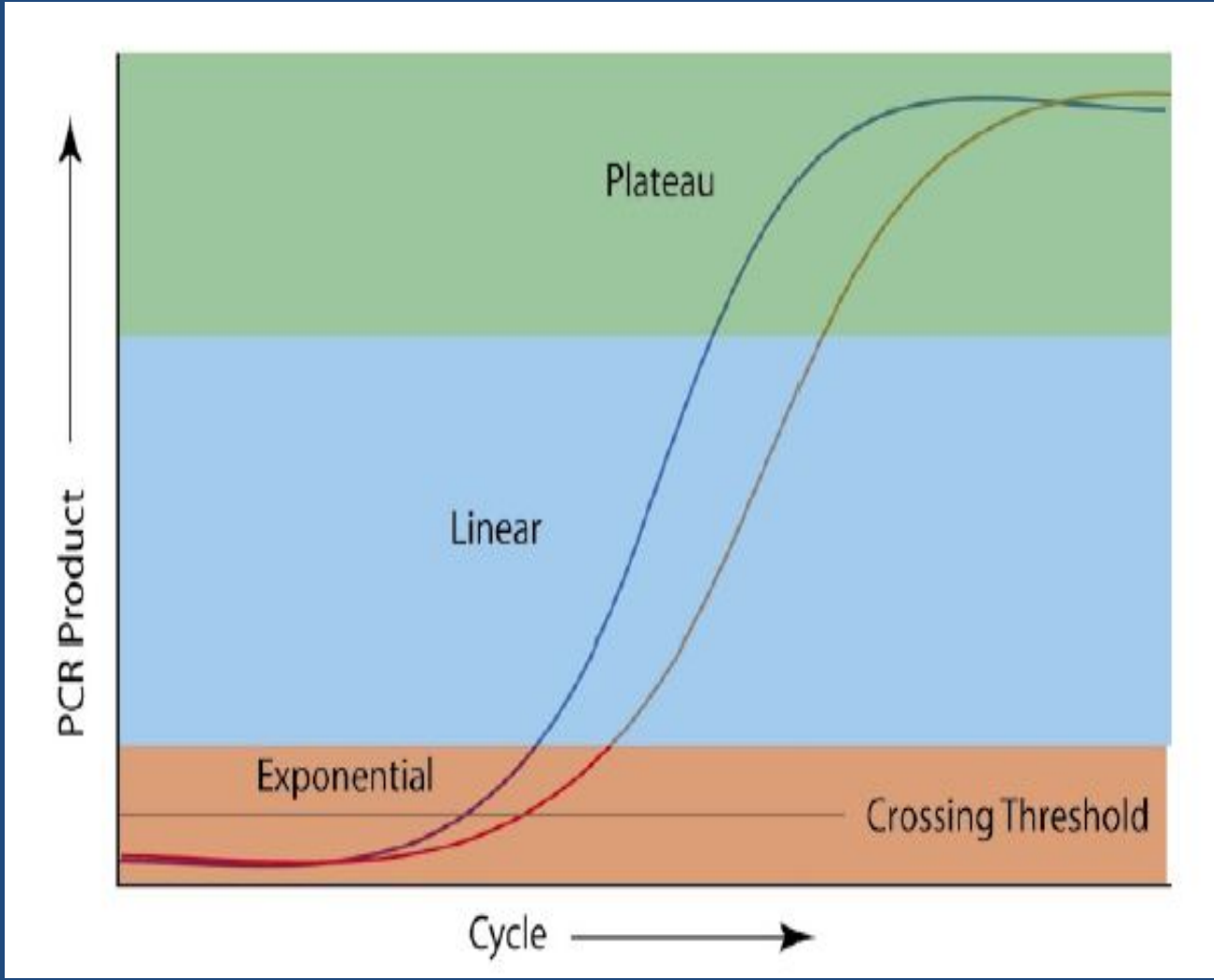
Methods for quantifying DNA and RNA—Blotting



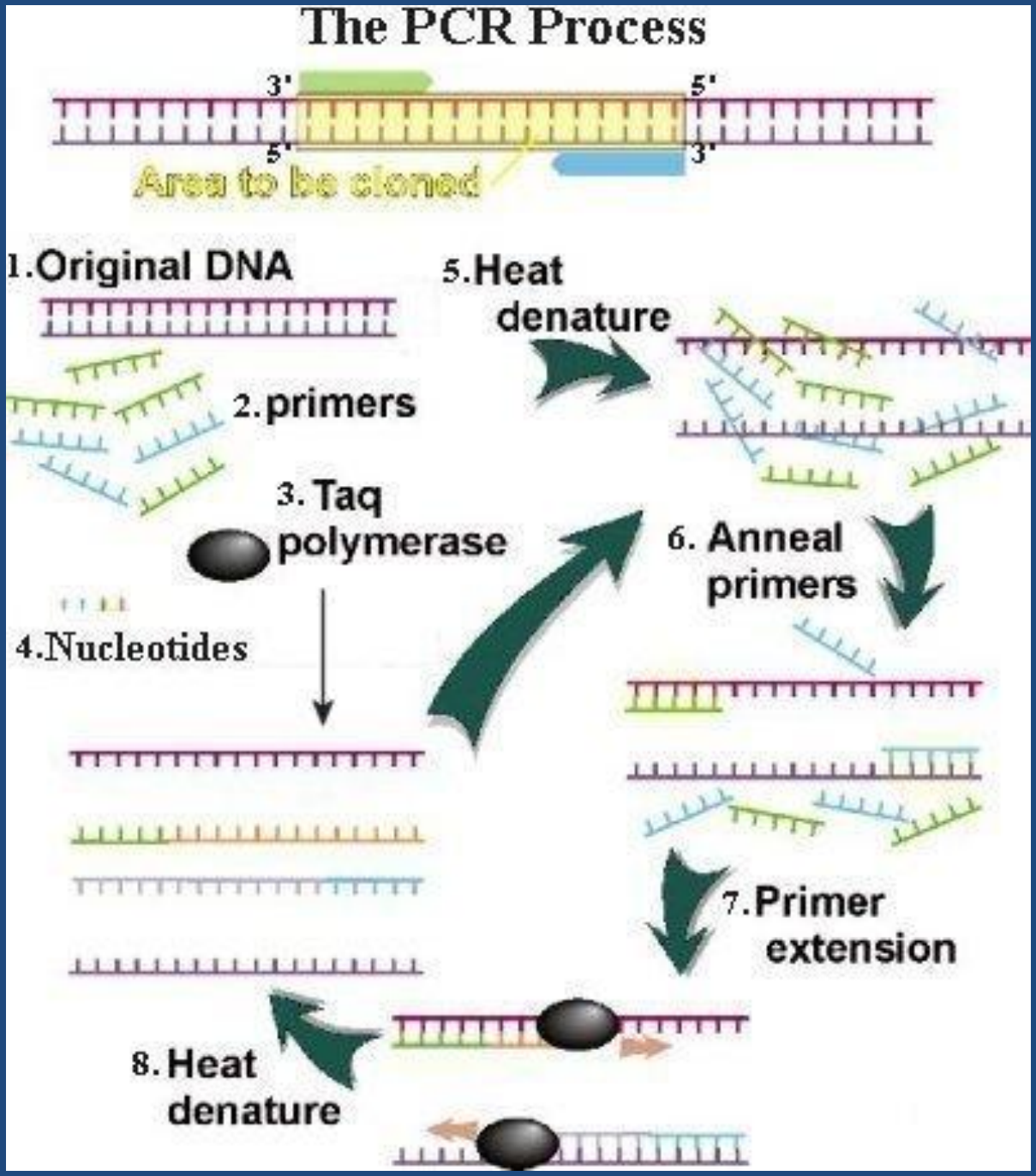
Methods for quantifying DNA and RNA—Microarrays



Quantifying DNA/RNA by qPCR



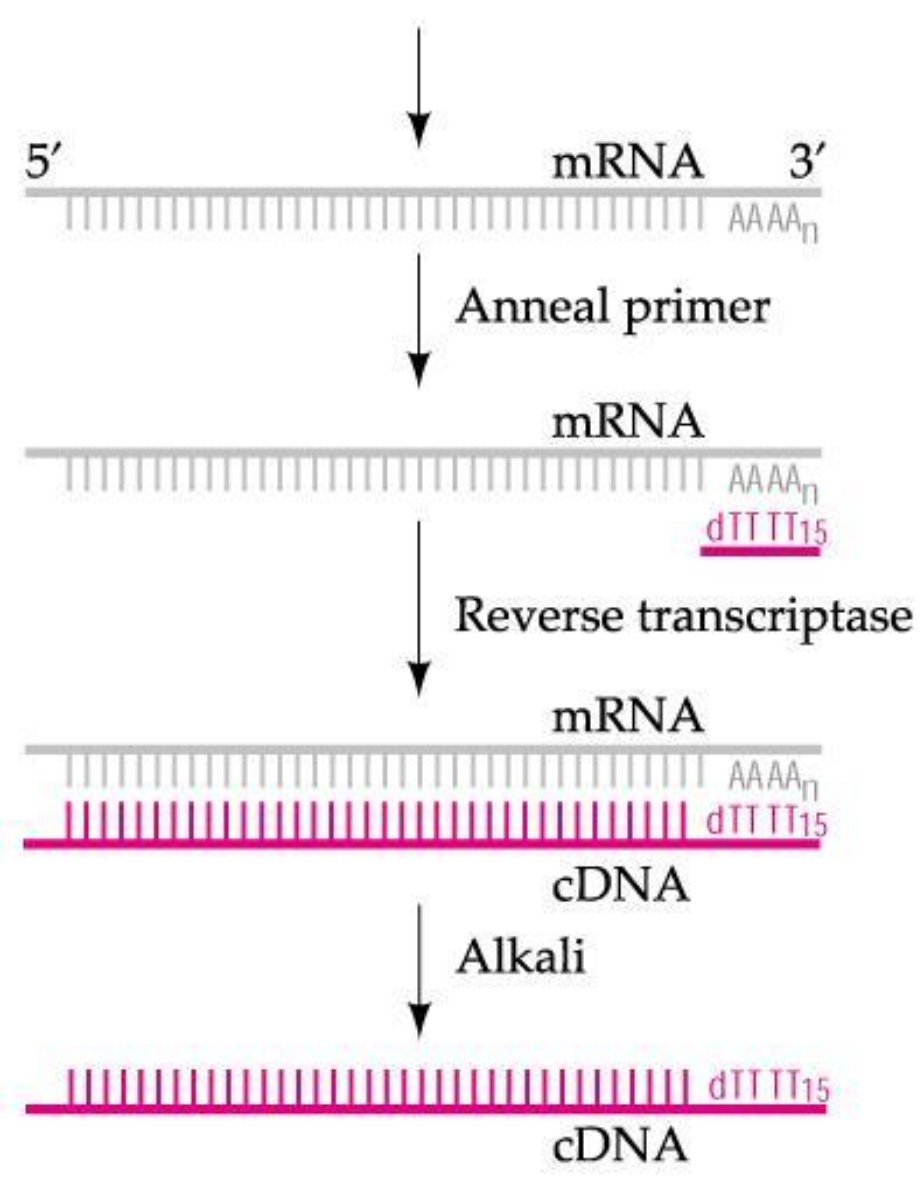
Reminder: PCR



What is qPCR

- “quantitative Polymerase Chain Reaction”
- A method that allows to follow in real time (that is why is also called Real-Time PCR) the amplification of a target.
- The target can be nucleic acids (RNA or DNA).
- Taq polymerase can only synthesize DNA, so how do we study RNA using qPCR?

Reverse Transcription

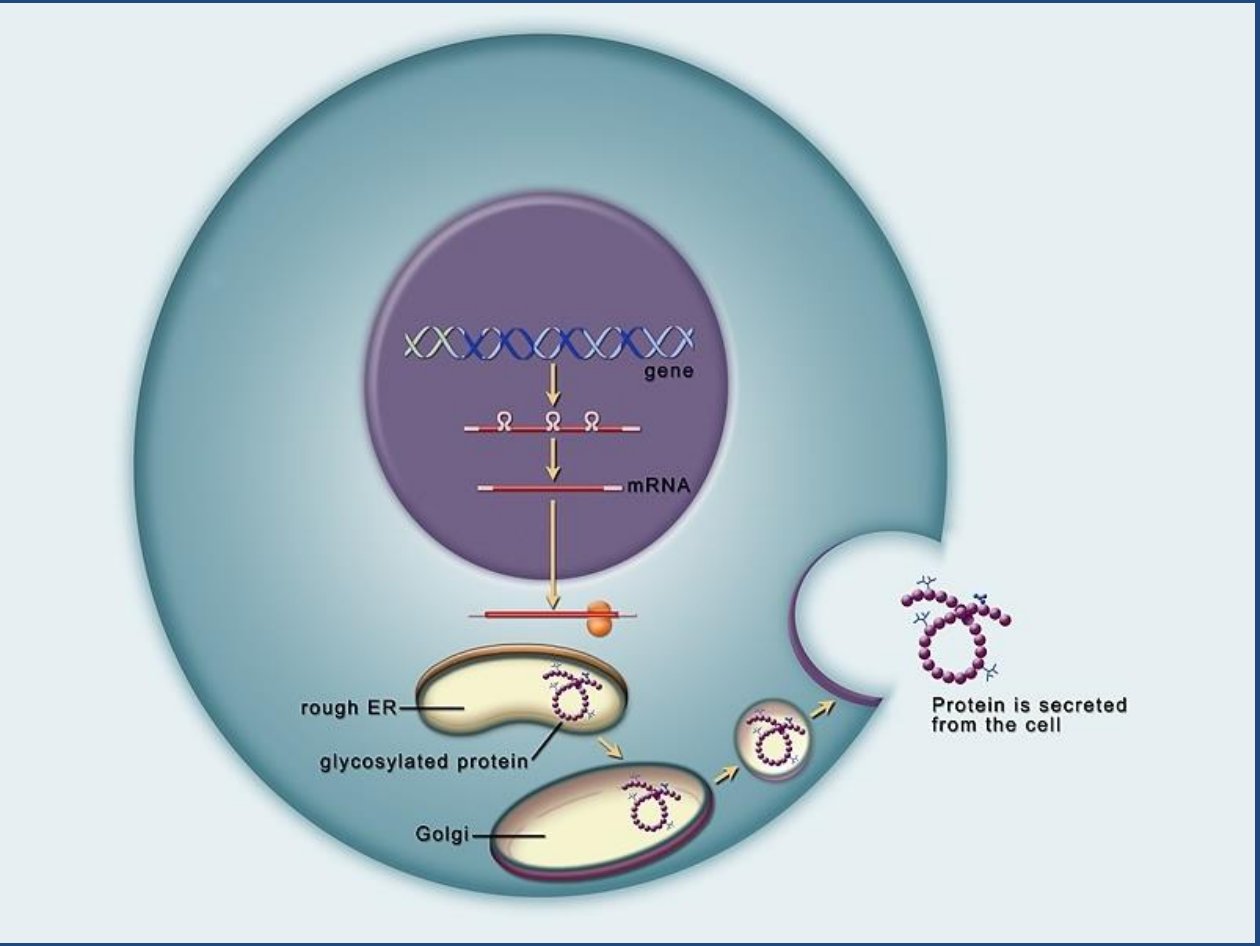


- mRNA can be copied to complementary DNA sequence (cDNA) using reverse transcriptase—a DNA polymerase that uses ssRNA as template.
- Processed mRNA will match protein coding sequence while unprocessed (nuclear) mRNA will contain intron sequences.

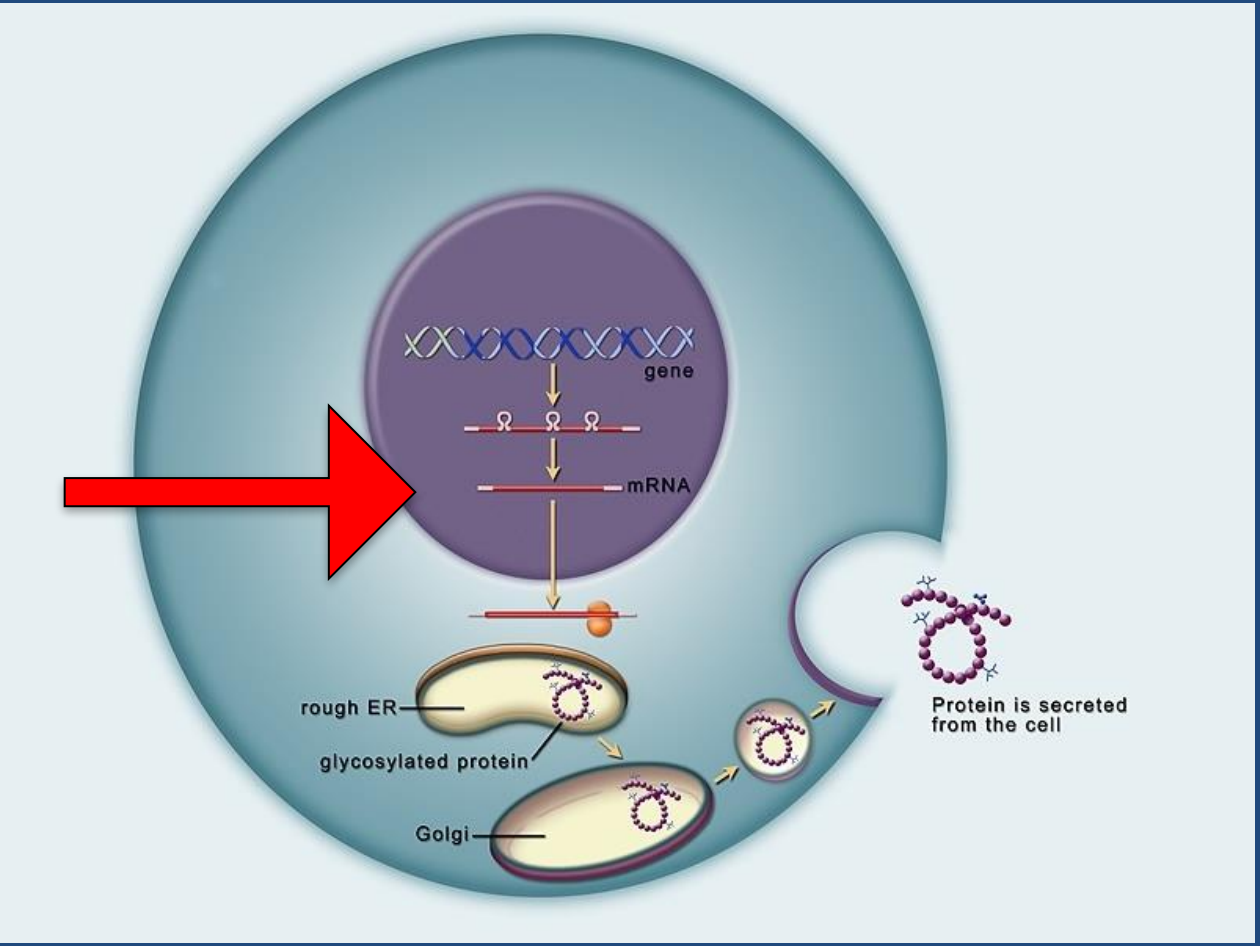
Uses of qPCR

- Precise quantitation of DNA or RNA in samples
- Estimation of gene number
- Gene expression studies by quantification of messenger RNA

Central Dogma of Molecular Biology: From DNA to RNA to protein



Principle of gene expression: From DNA to RNA to protein



What are we doing today?

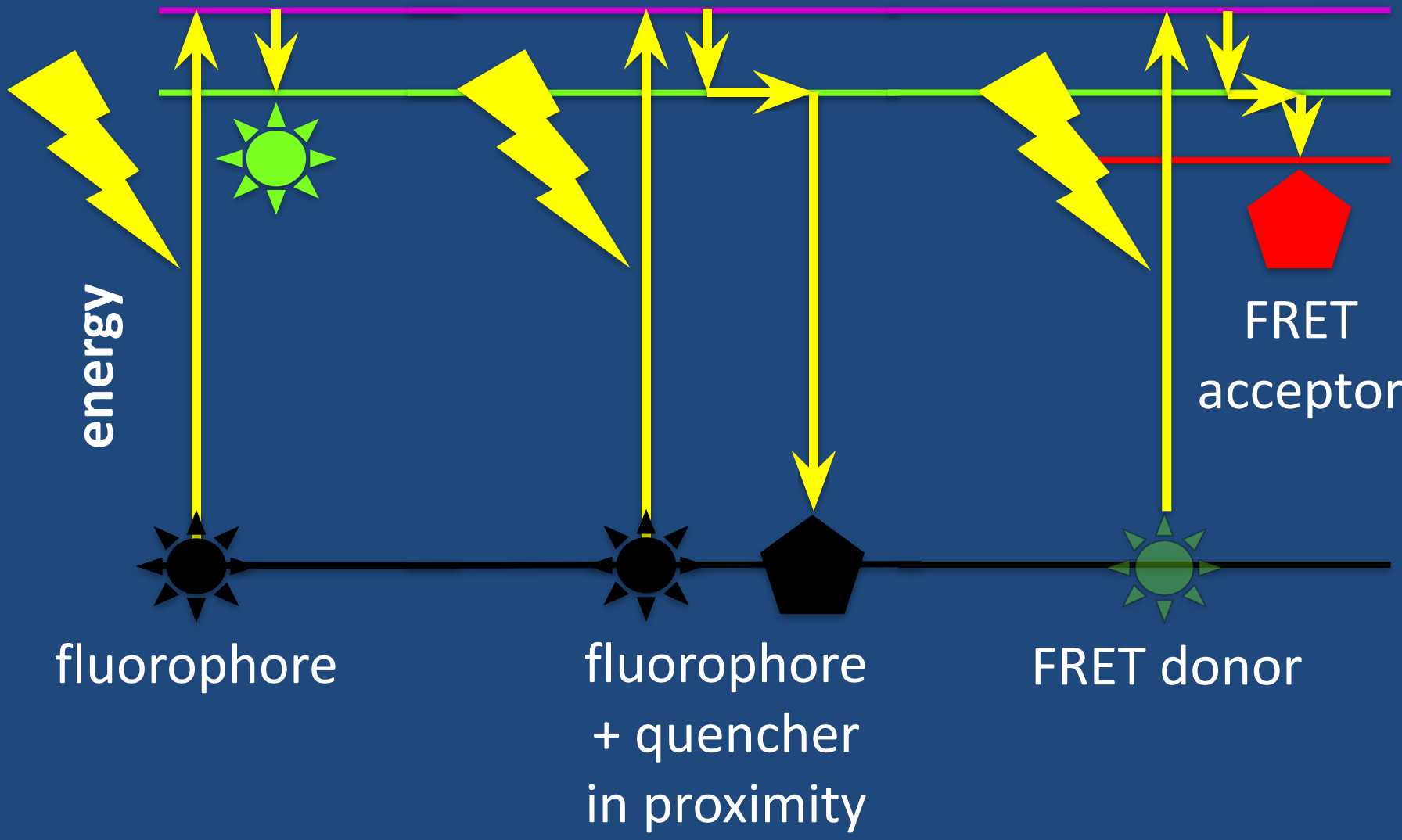
- We are using qPCR as a way to study RNA, in this particular case messenger RNA (mRNA).
- We will test the gene expression response of a plant to a geminivirus infection.
- This can be applied to any gene expression analysis.

Different chemistries involved to obtain quantitative signal

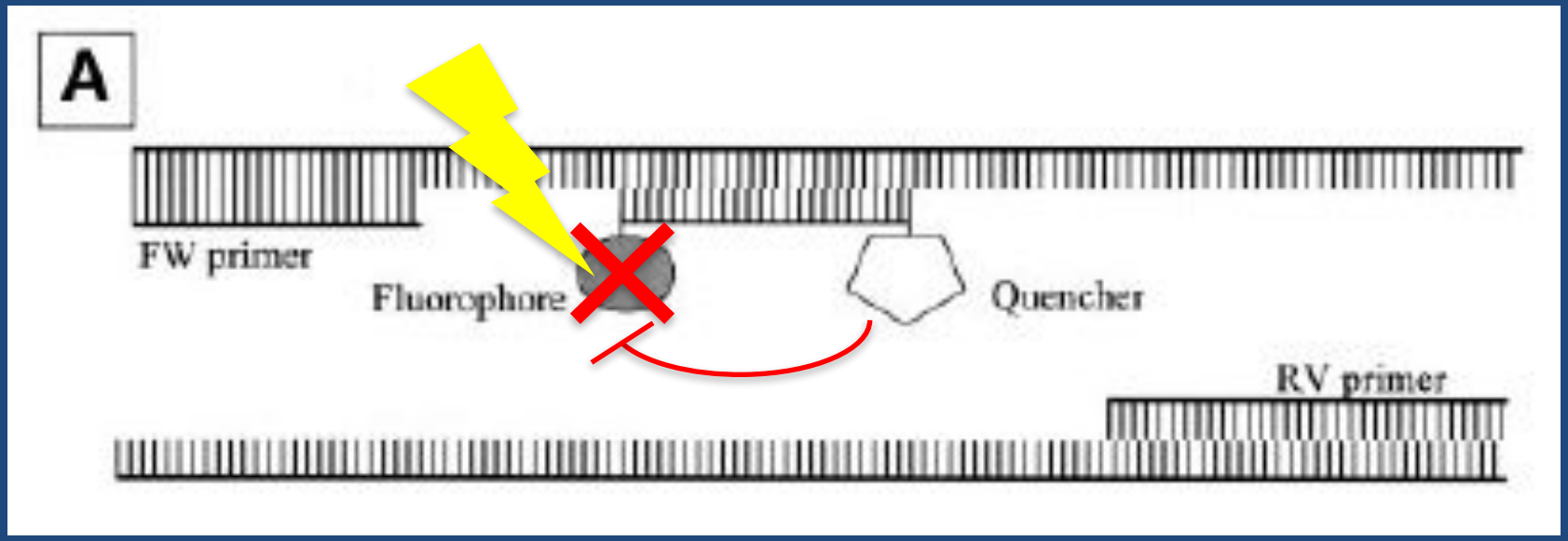
Table 1. Real-time qPCR Technologies

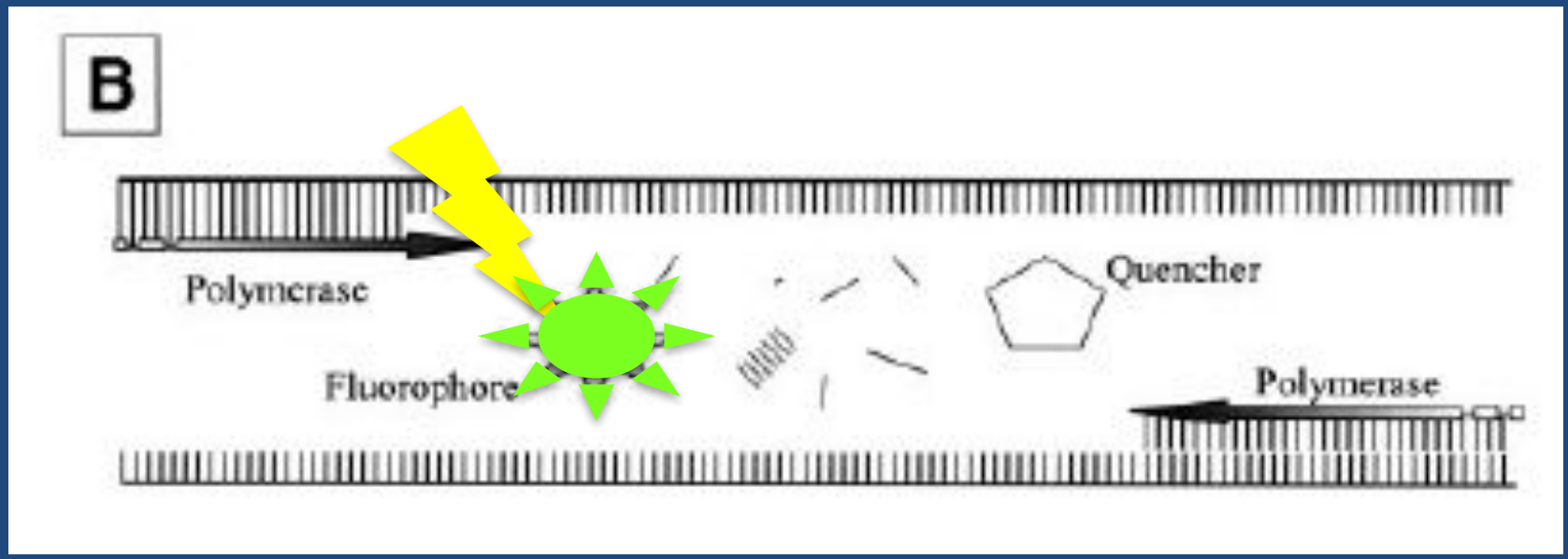
Technology	Supplier	Fluorescent Signal	Multiplexing	Melting Curve Analysis
TaqMan	Applied Biosystems	probe hydrolysis	yes	not necessary
LightCycler	Roche	probe hybridization	yes	not necessary
LUX	Invitrogen	hairpin probe hybridization	yes	necessary
Molecular Beacons	Sigma-Aldrich, IDT	hairpin-loop hybridization	yes	necessary
SYBR Green	Multiple	intercalating dye	no	necessary

qPCR thermalcyclers detect fluorescence

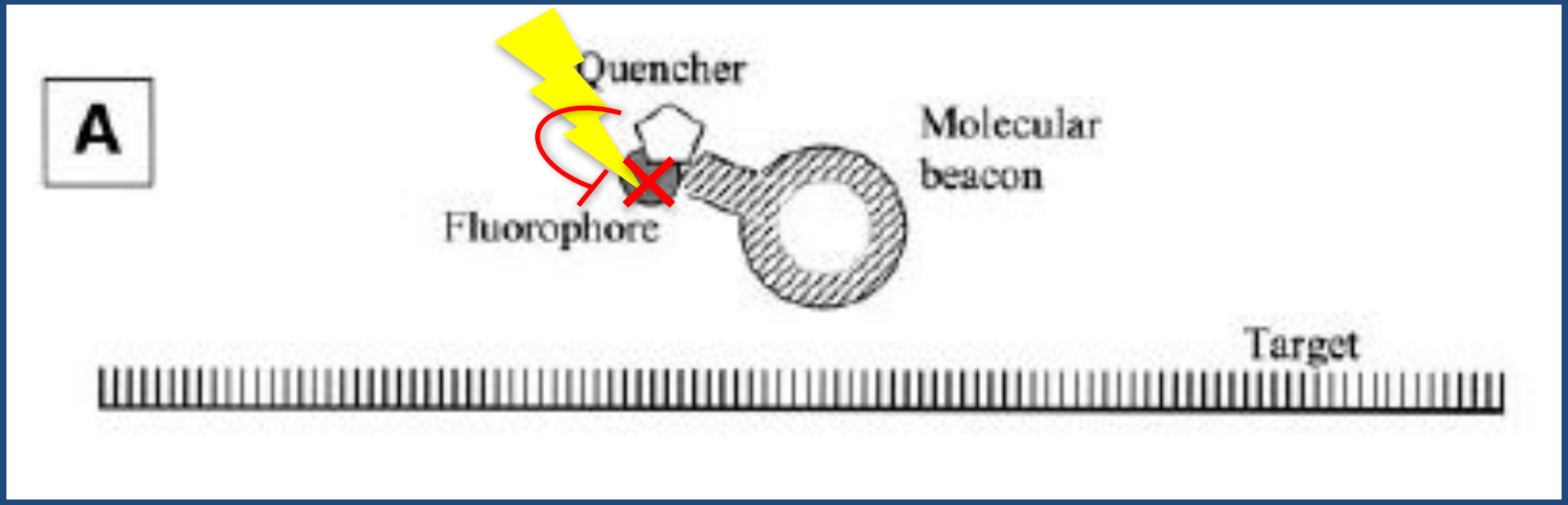


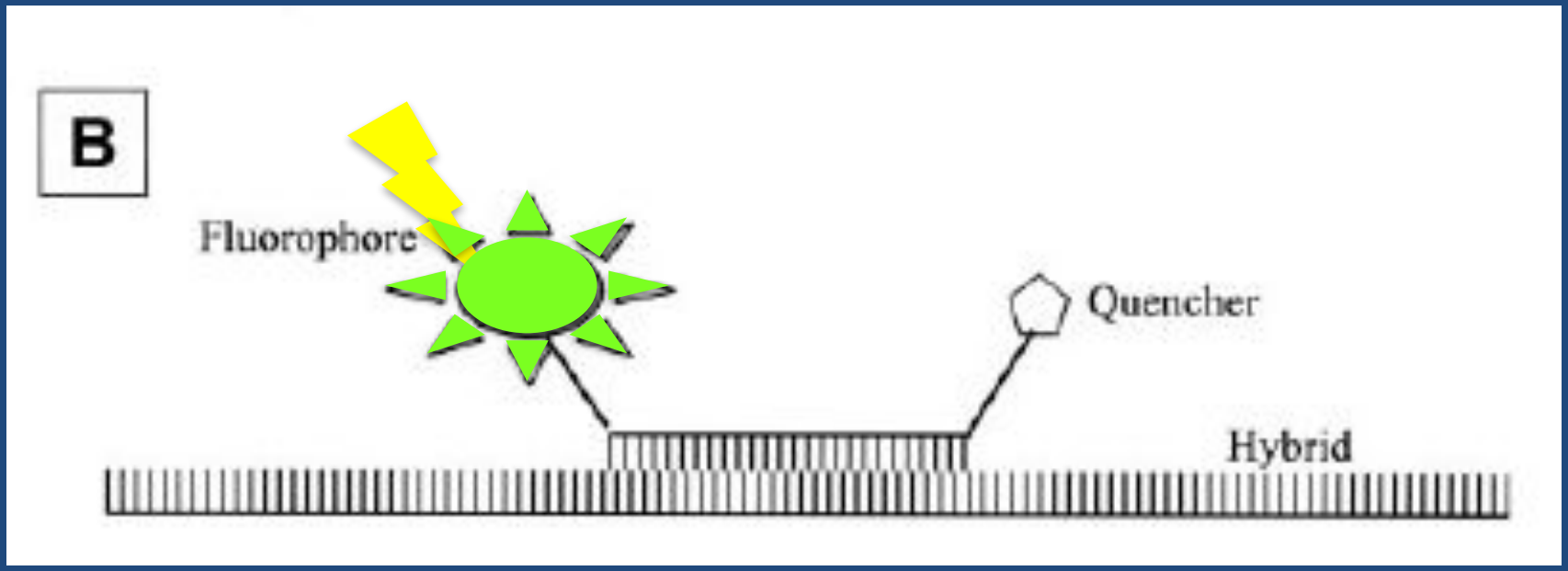
TaqMan


A
FW primer
Fluorophore
Quencher
RV primer

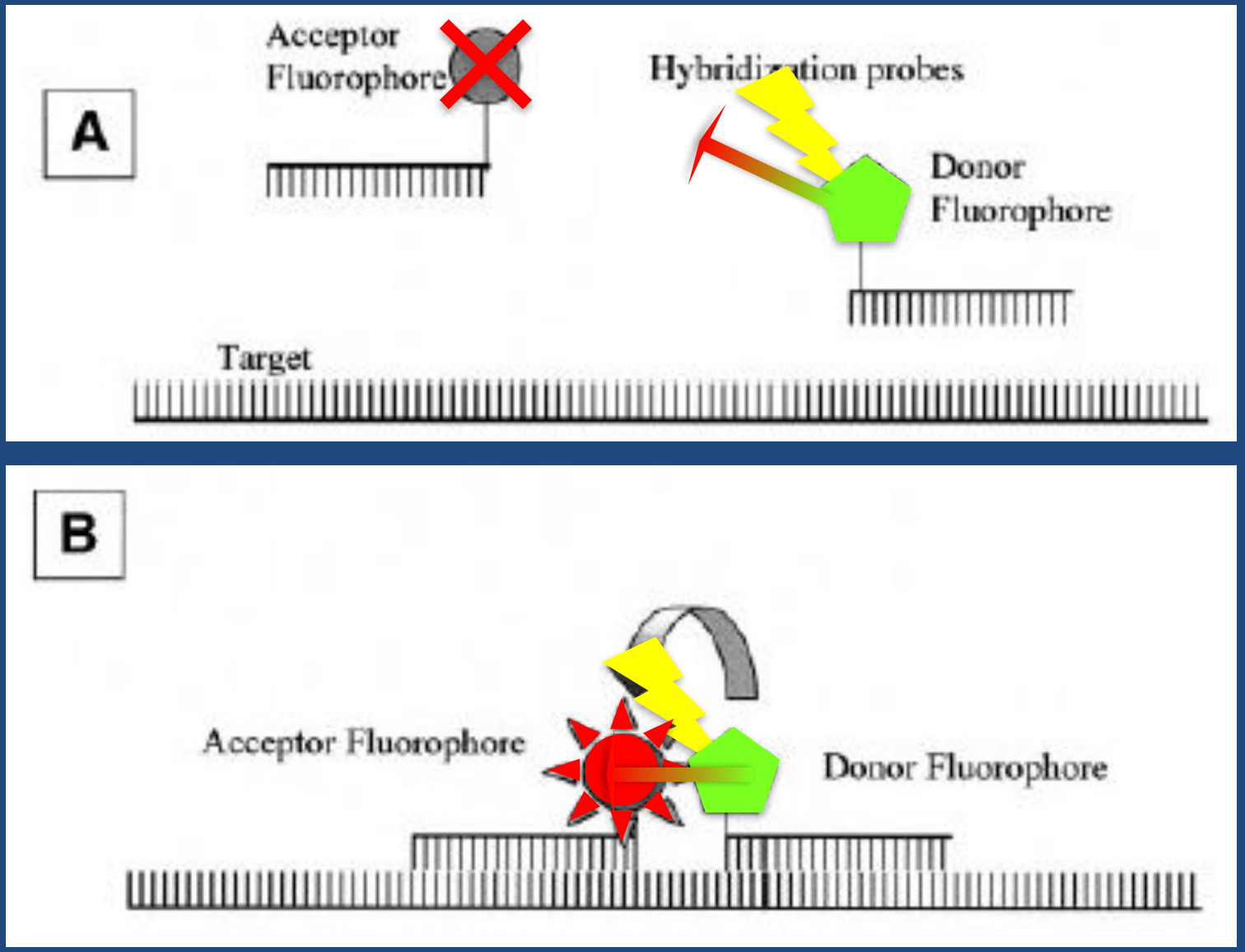

B
Polymerase
Quencher
Fluorophore
Polymerase

Molecular beacons

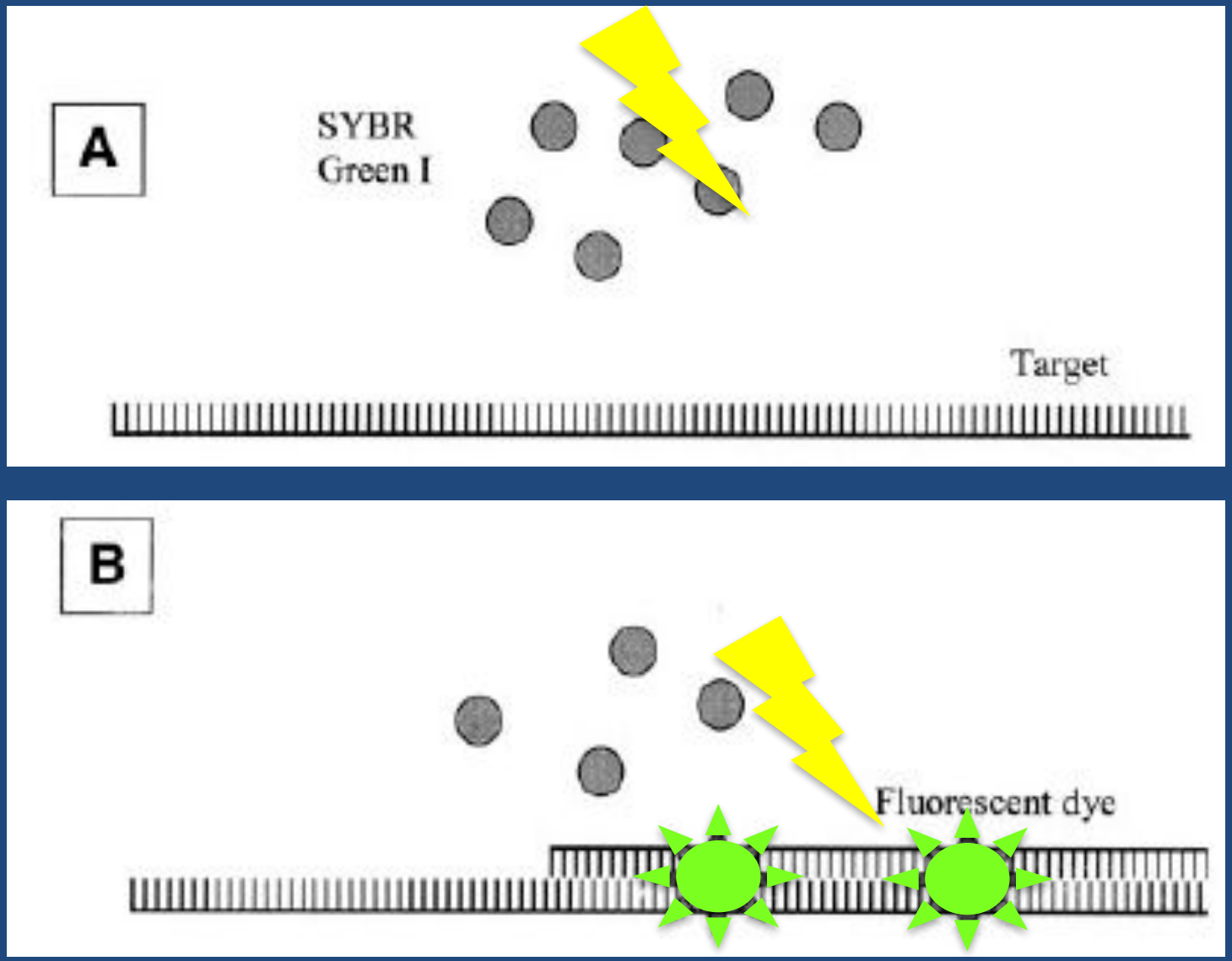




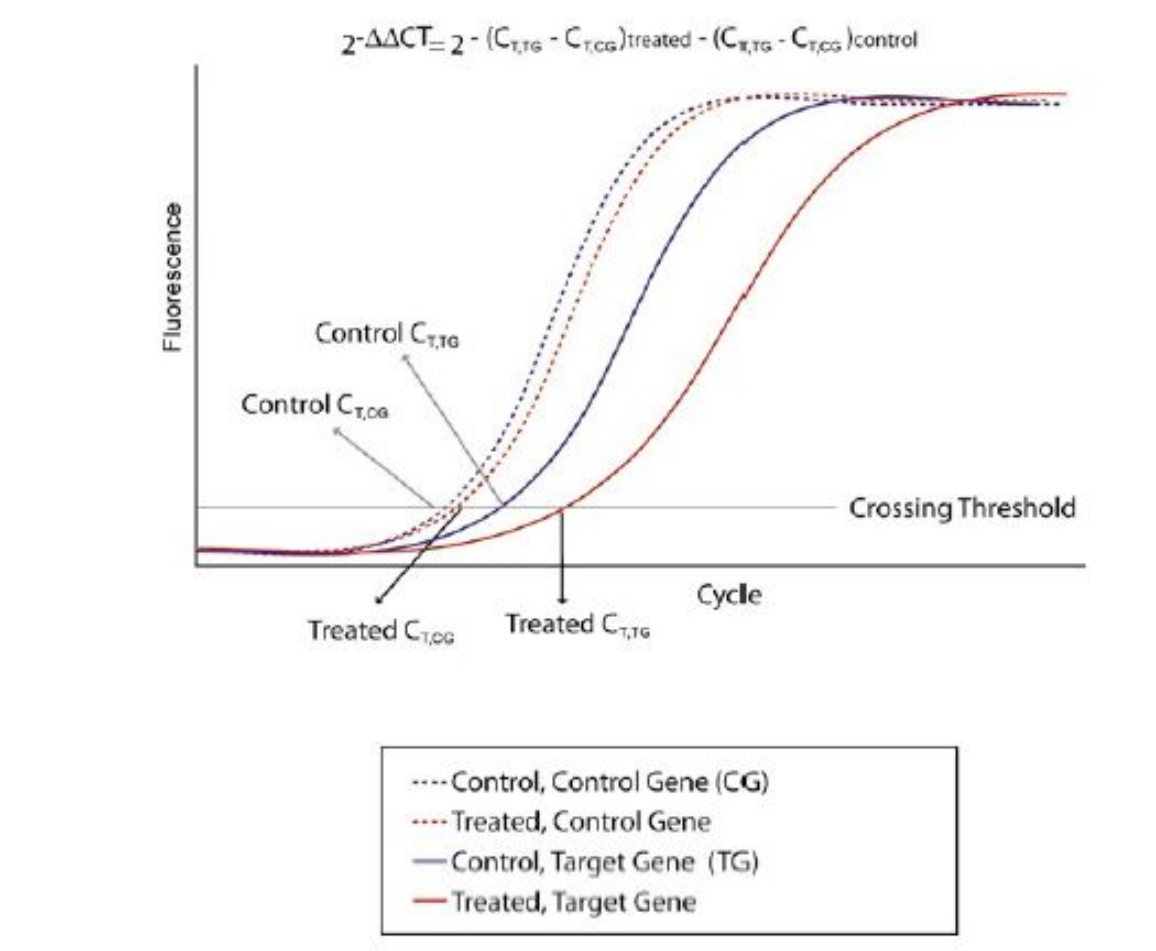
Molecular probes



SYBRGreen



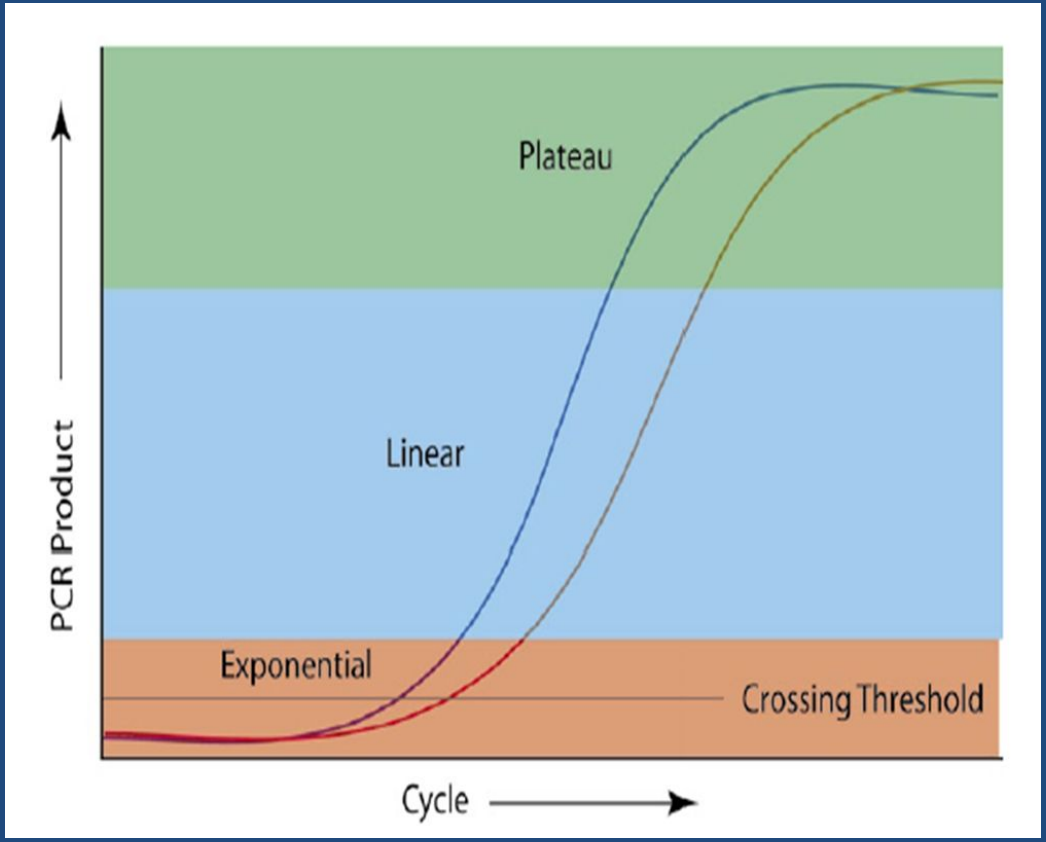
We are using relative expression analysis but absolute expression analysis is possible with qPCR



ΔΔCt method

test reference

$$2^{-[(Ct_{inf}-Ct_{mock})-(Ct_{inf}-Ct_{mock})]}$$



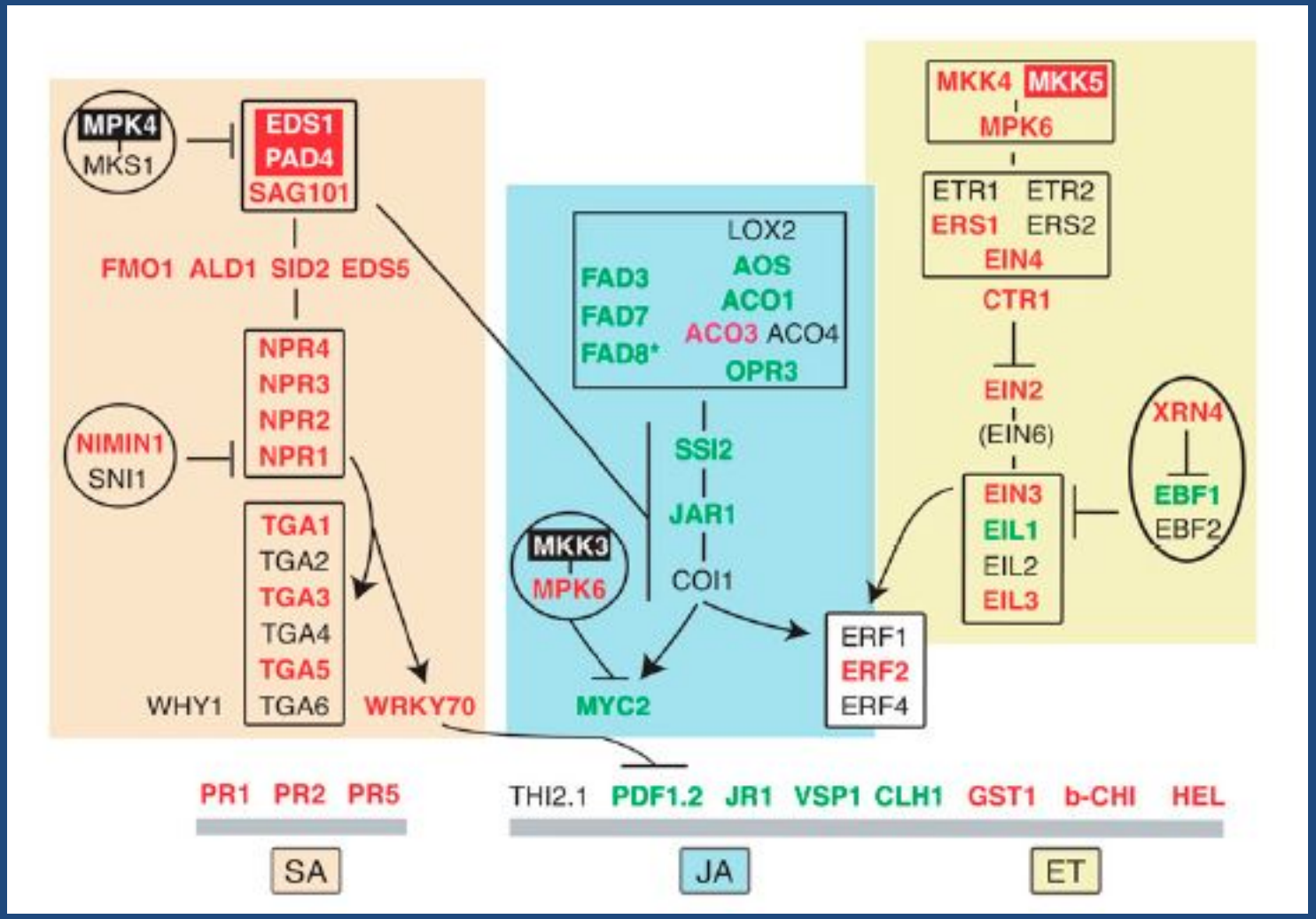
Cabbage Leaf Curl Virus (CaLCuV)

Arabidopsis thaliana

Arabidopsis infected with CaLCuV

- Using qPCR to detect changes in gene expression.
  - Mock tissue (uninfected)
  - Infected tissue
- Looking at genes involved in pathogen response.
- cDNA prepared from RNA purified from *Arabidopsis* plants
  - Reverse transcription using polyT primer and NTPs.

Pathogen response in arabidopsis



Genes of interest for today

- PR1- Pathogenesis-related
- NPR1- Not expressing PR-1
- WRKY53- Transcription factor involved in pathogen response
- FAD8- Fatty-acid desaturase, involved in the production of jasmonate (pathogen response)
- Invertase- Enzyme known to be involved in the pathogen response
- Catalase- Enzyme known to be involved in the pathogen response
- CAB-AB- Protein that takes part in the Chlorophyll complex
- UCE- Ubiquitin conjugating enzyme. Control gene. It has been reported it doesn’t change during infection.

Prior to doing the qPCR

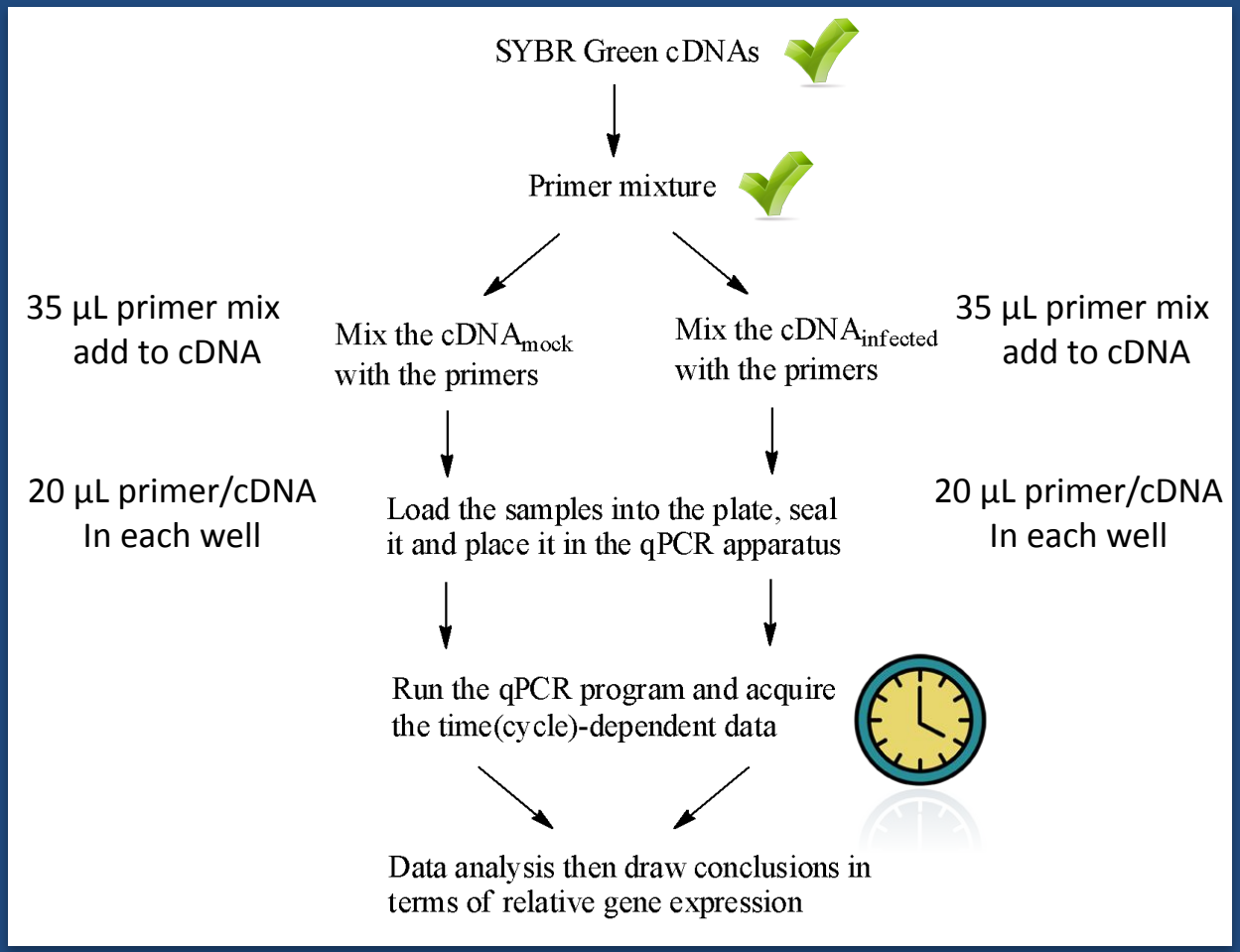
- Total RNA from mock and infected plants was purified
- It was treated with DNAase to get rid of all genomic DNA (will also amplify with primers)
- cDNA was produced and diluted to 50ng/µl

Preparing a qPCR reaction

- Forward and reverse primers (oligos) for each gene of interest
- cDNA of control and treated samples (mock and infected, respectively)
- SYBRGreen PCR mix
- Thermocycler with fluorescence measuring capabilities

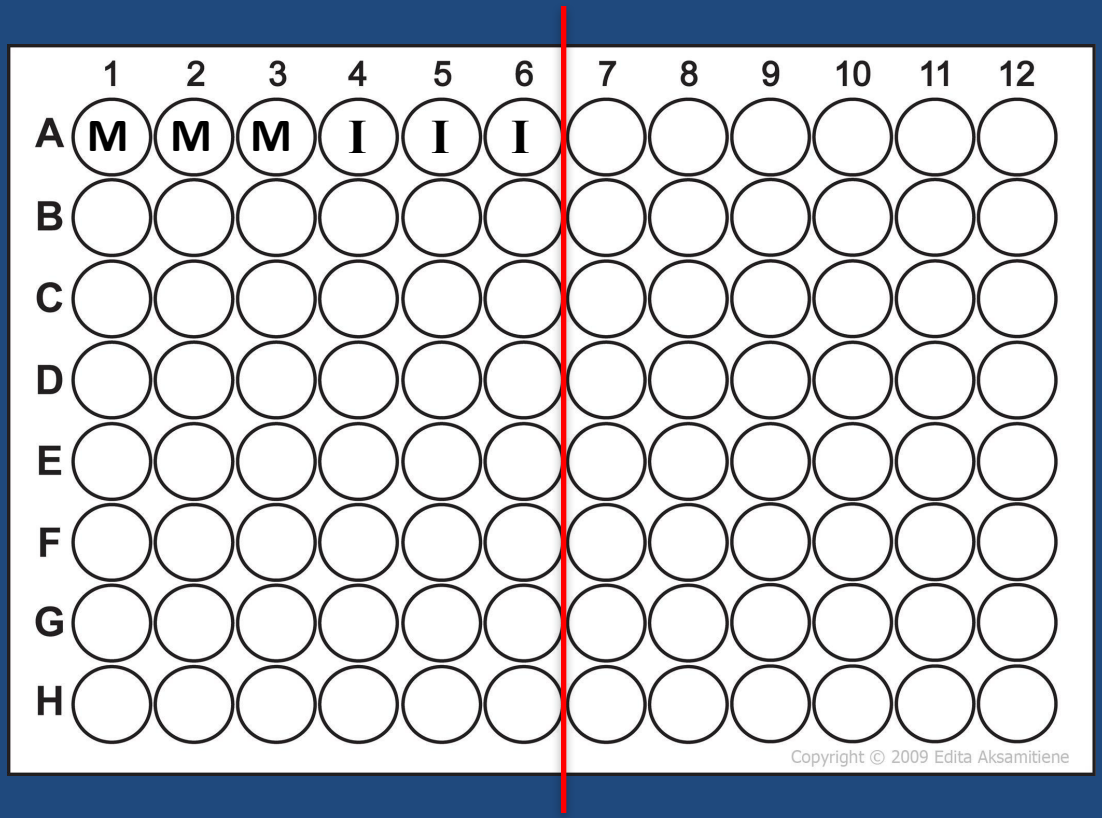
Each team (7 teams total)

- Will prepare mixes with oligos and cDNA for a SINGLE gene (for both mock and infected).



Each team

- The assay will be tested in triplicate (three technical replicates).

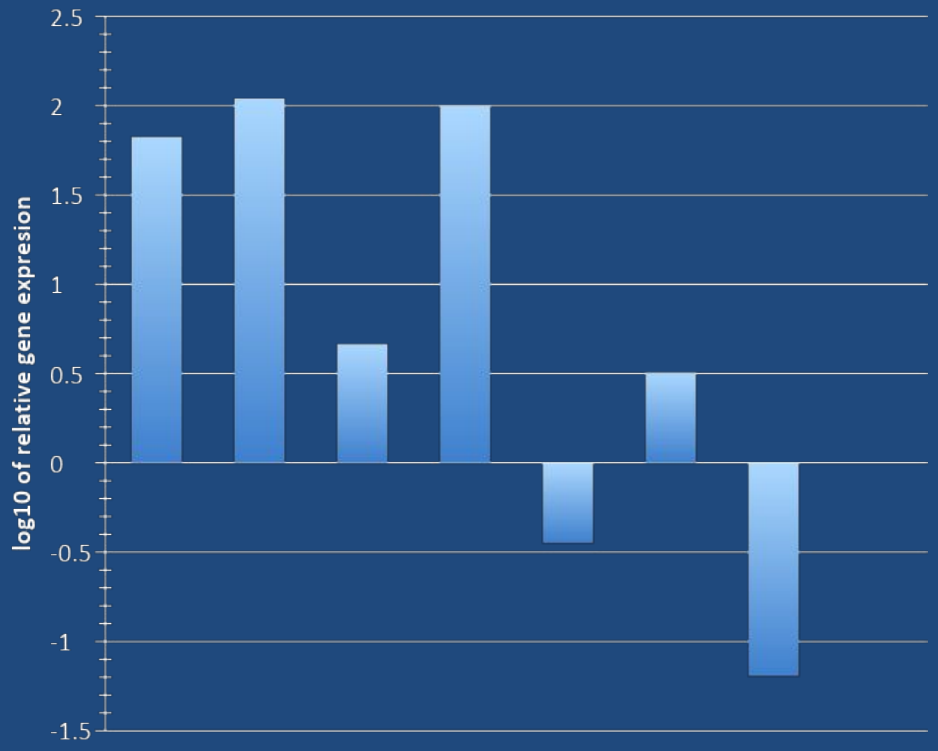


Each team

The whole section will analyze the data, the same way as it was for the pre-lab example.

- Calculate ΔCt for each gene.
- Calculate ΔΔCt for 7 test genes relative to UCE.
- Calculate $\log_{10}(\Delta\Delta Ct)$ for 7 test genes to determine increase or decrease in expression.
- Make bar chart of $\log_{10}(\Delta\Delta Ct)$ for each gene
- Construct table with “call” if gene was upregulated, downregulated or showed no change

Analysis requirements



Gene	Value	Call
A	1.828745739	up regulated
B	2.04179718	up regulated
C	0.666738198	unaffected
D	1.999855536	up regulated
E	- 0.450673805	unaffected
F	0.509327854	unaffected
G	- 1.195371388	down regulated
UCE	0	control gene