Principles and Applications of Real-Time Quantitative PCR

Reverse Summer School

CRUK Cambridge Institute-Uganda Cancer Institute-Makerere 2024

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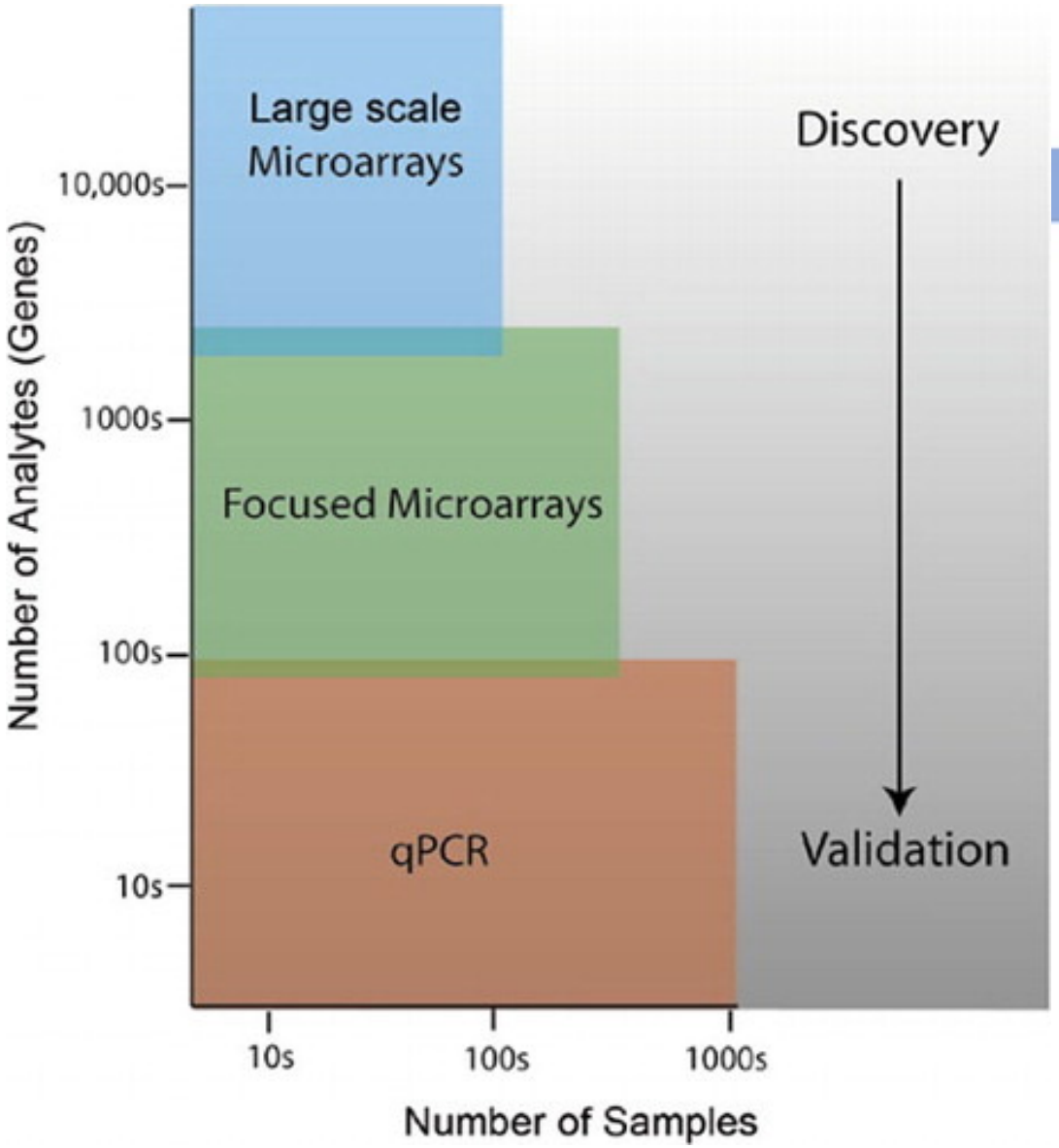
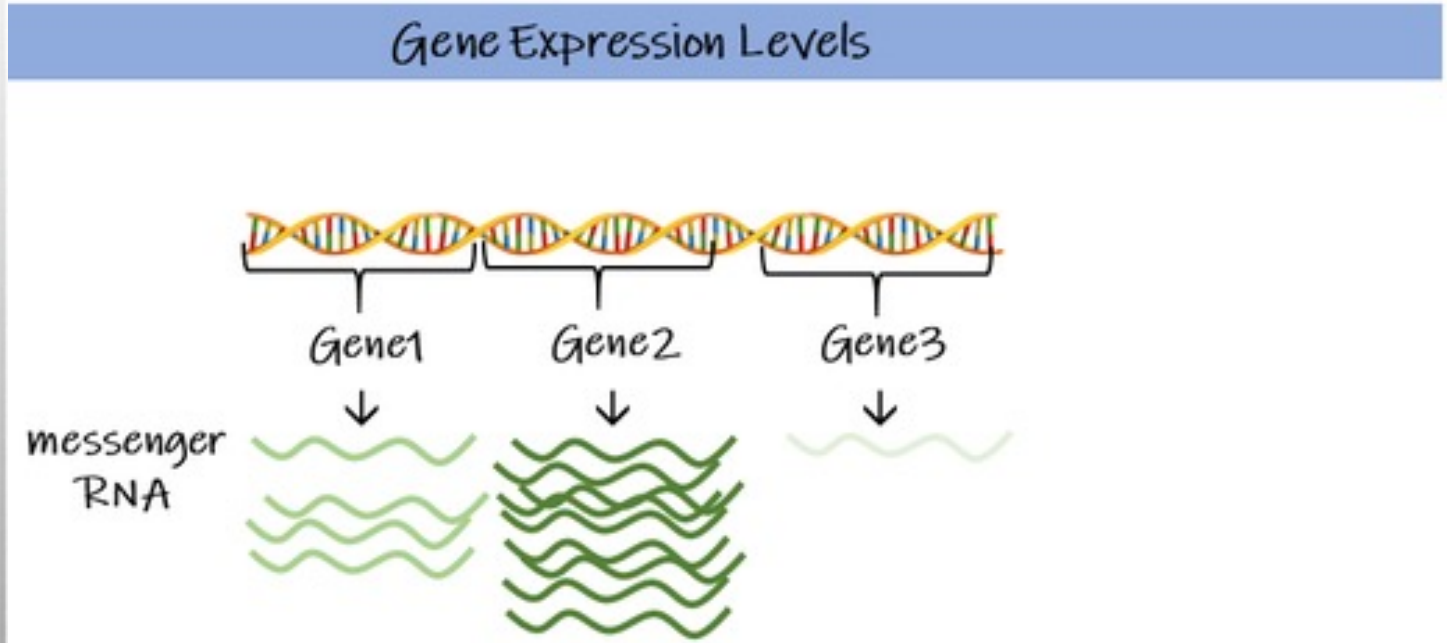
Together we will beat cancer

Measuring gene expression





VanGuilder et al., 2018. *Biotechniques*

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The Polymerase Chain Reaction (PCR)

Kary B. Mullis
Facts

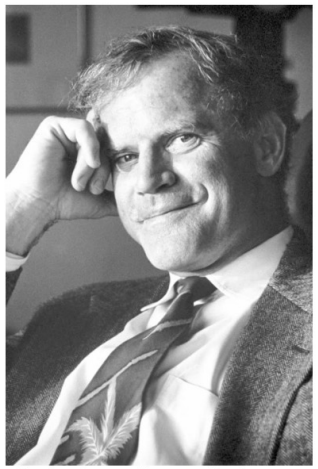


Photo from the Nobel Foundation archive.

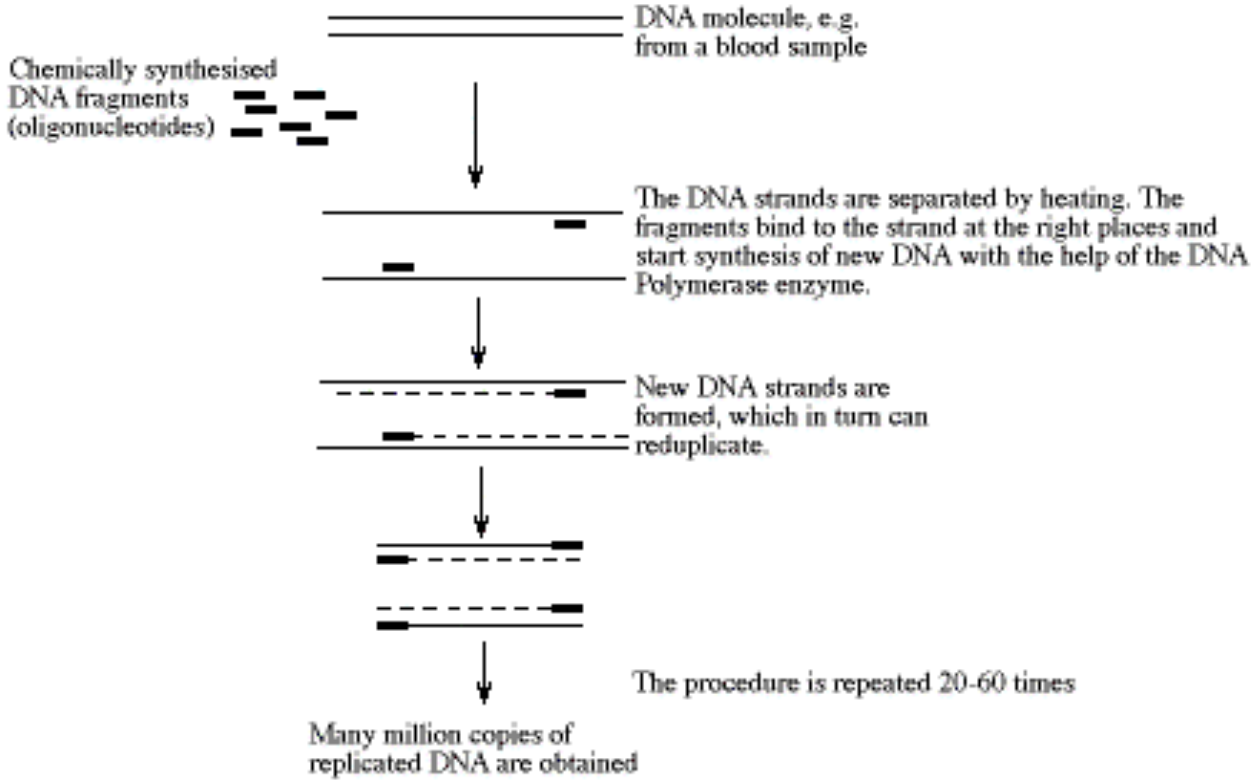
Kary B. Mullis
The Nobel Prize in Chemistry 1993

Born: 28 December 1944, Lenoir, NC, USA

Died: 7 August 2019, Newport Beach, CA, USA

Prize motivation: “for his invention of the polymerase chain reaction (PCR) method”

Prize share: 1/2



The PCR method can be used for reduplicating a segment of a DNA molecule, e.g. from a blood sample. The procedure is repeated 20-60 times, which can give millions of DNA copies in a few hours.

Kary Mullis, Nobel Prize Lecture, 1993.

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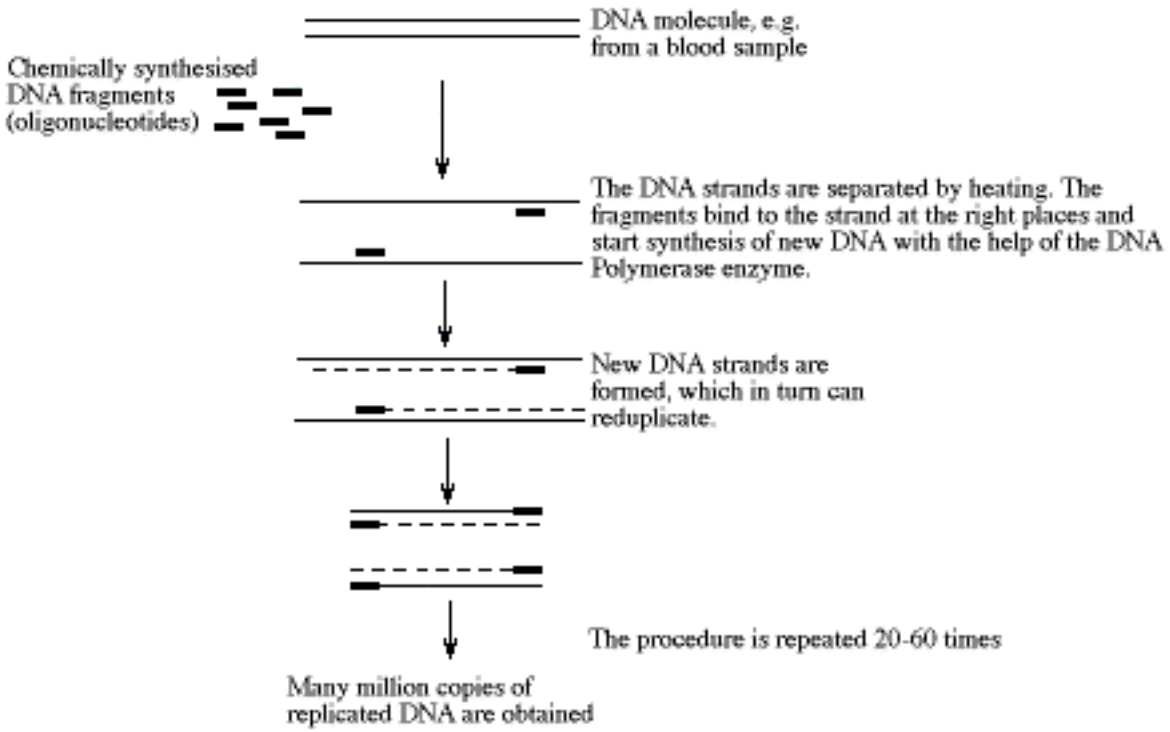
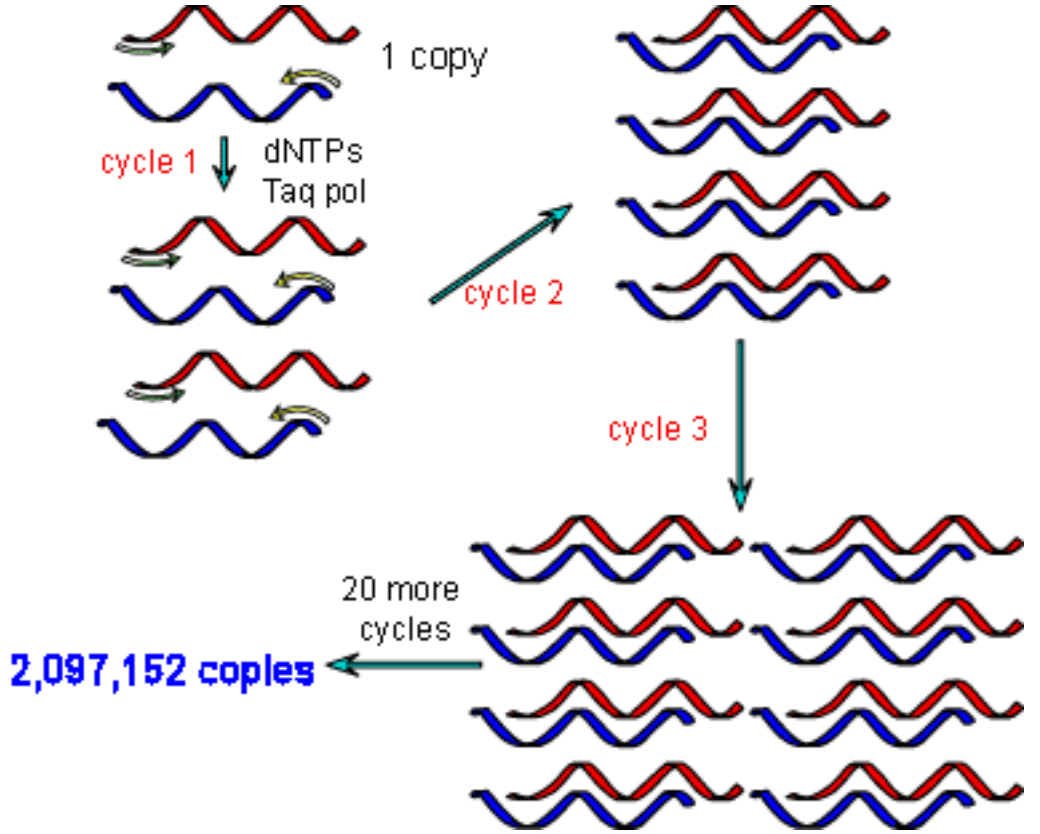
The Polymerase Chain Reaction (PCR)





Kary Mullis, Nobel Prize Lecture, 1993.

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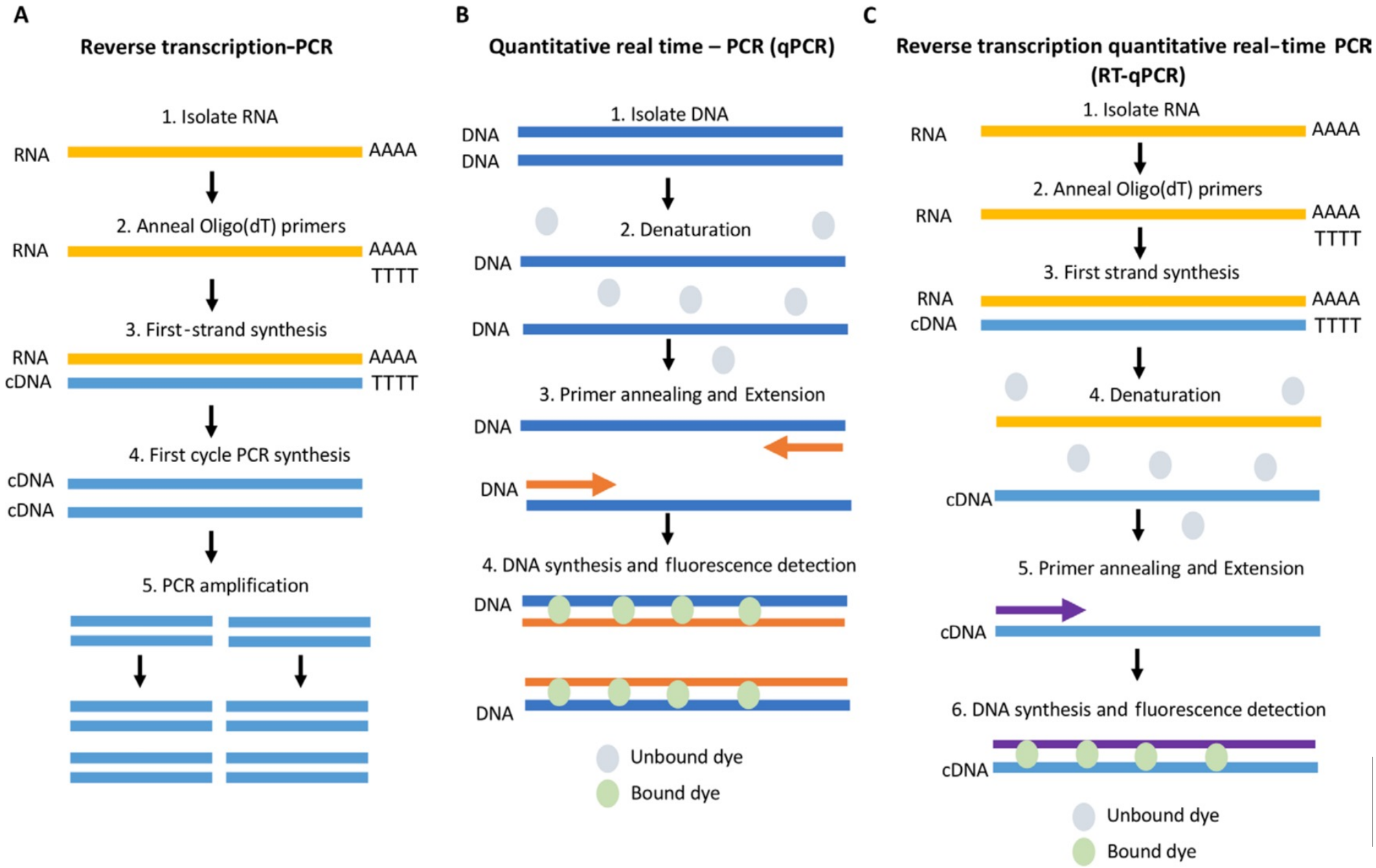
RT-PCR vs qPCR vs RT-qPCR



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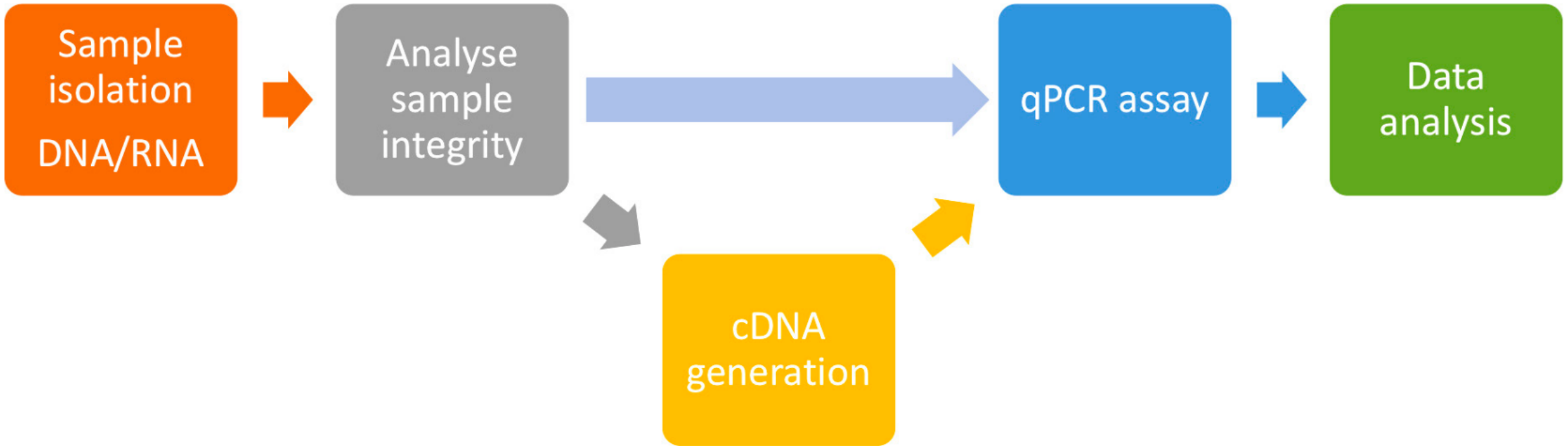
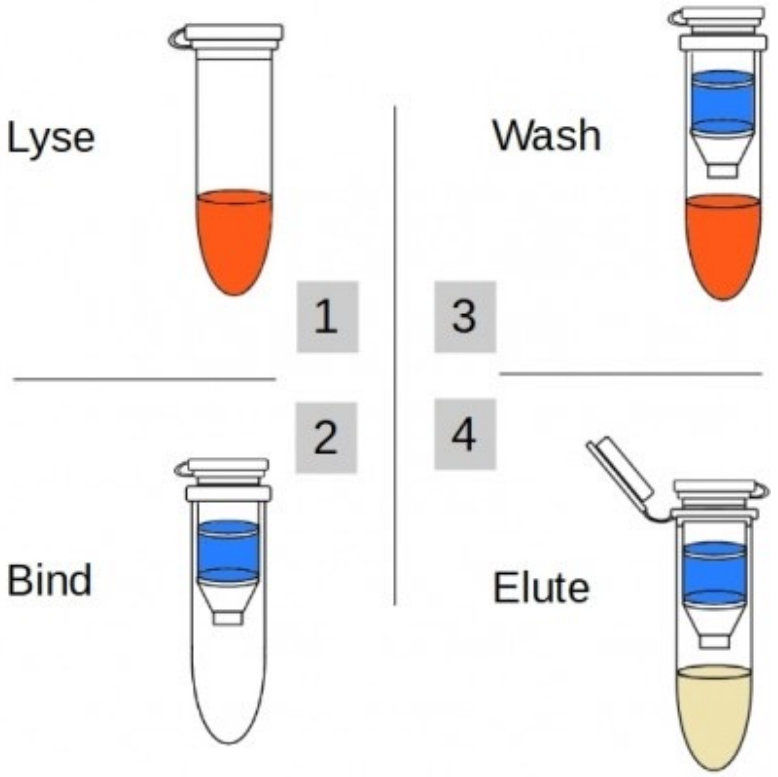
Workflow for qPCR reactions



Workflow- Sample RNA isolation



Handling RNA – The key factors

Working quickly but carefully is key!

General handling

- Always wear latex or vinyl gloves to prevent RNase contamination
- Change gloves frequently and keep tubes closed whenever possible

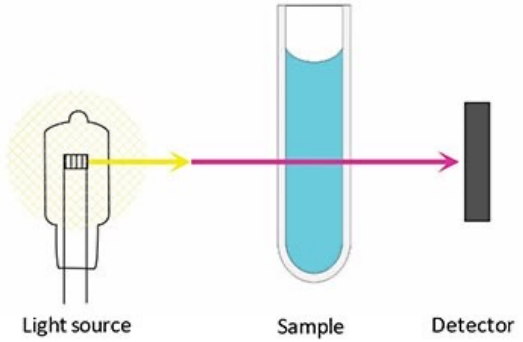
Disposable plasticware

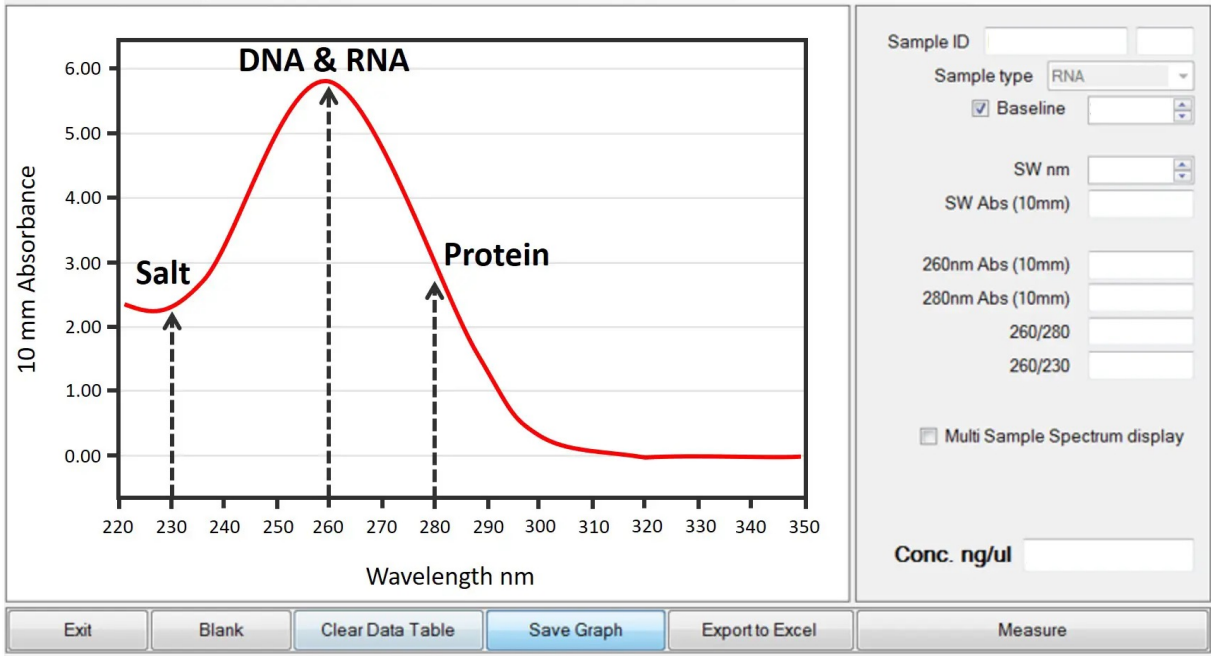
- The use of sterile, disposable polypropylene tubes is recommended
- These tubes are generally RNase-free and do not require pretreatment to inactivate RNases

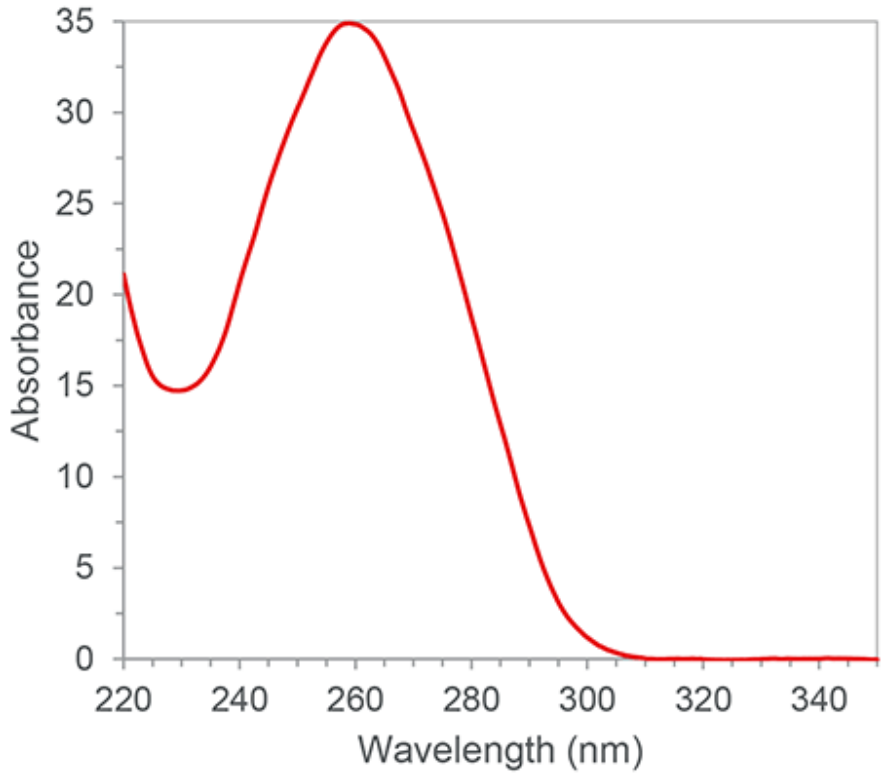
Non-disposable plasticware

- Non-disposable plasticware should be treated before use to ensure that it is RNase-free
- Rinse thoroughly with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water
- Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases

Workflow- RNA quantification and integrity







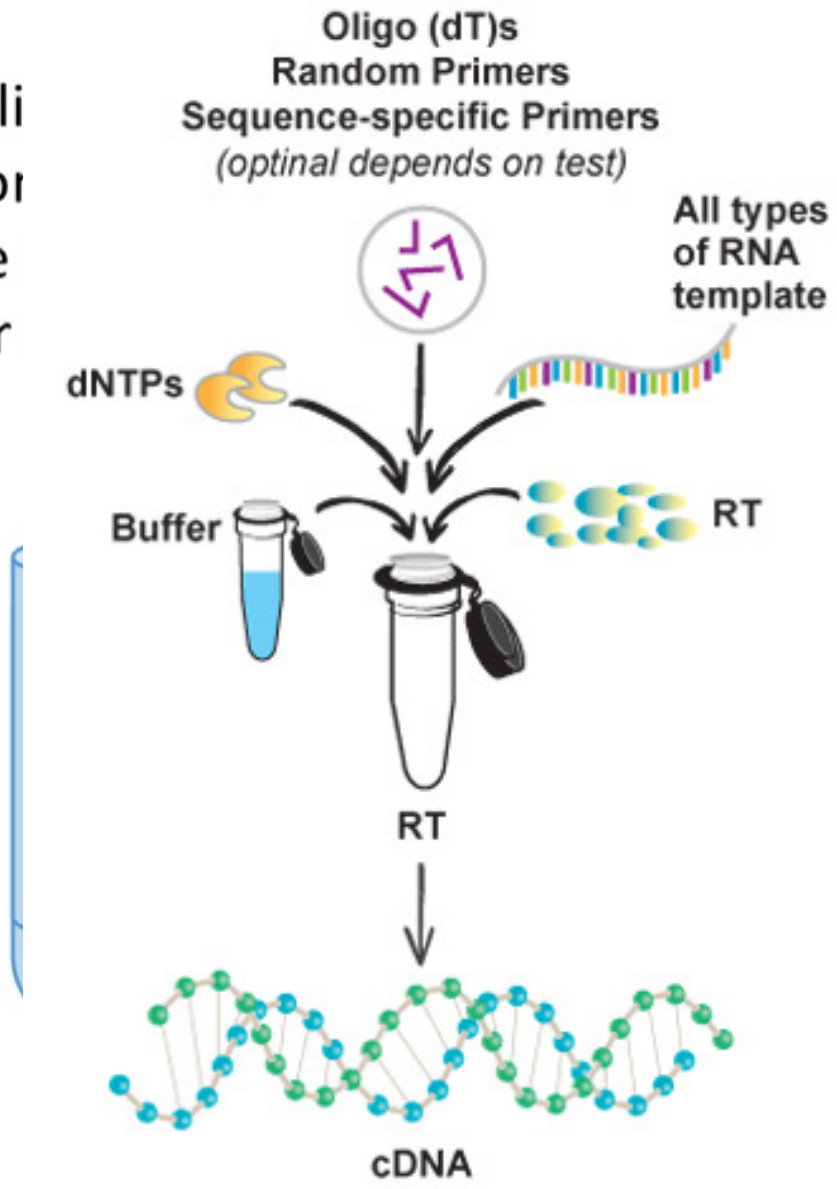
Substance	Absorbance (nm)	260/280 Ratio Values	260/230 Ratio Values
Pure DNA	280 nm	~1.8	2.0–2.2
Pure RNA	280 nm	~2.0	2.0–2.2
EDTA, Carbohydrates, Phenol	230 nm	< 1.5	< 2.0
Guanidine HCL	230 nm	< 1.5	< 2.0

Workflow: One-Step vs Two-Step PCR

RNA
Reverse transcriptase
Sequence-specific primers
DNA polymerase
Buffer and dNTPs

One-step RT-qPCR

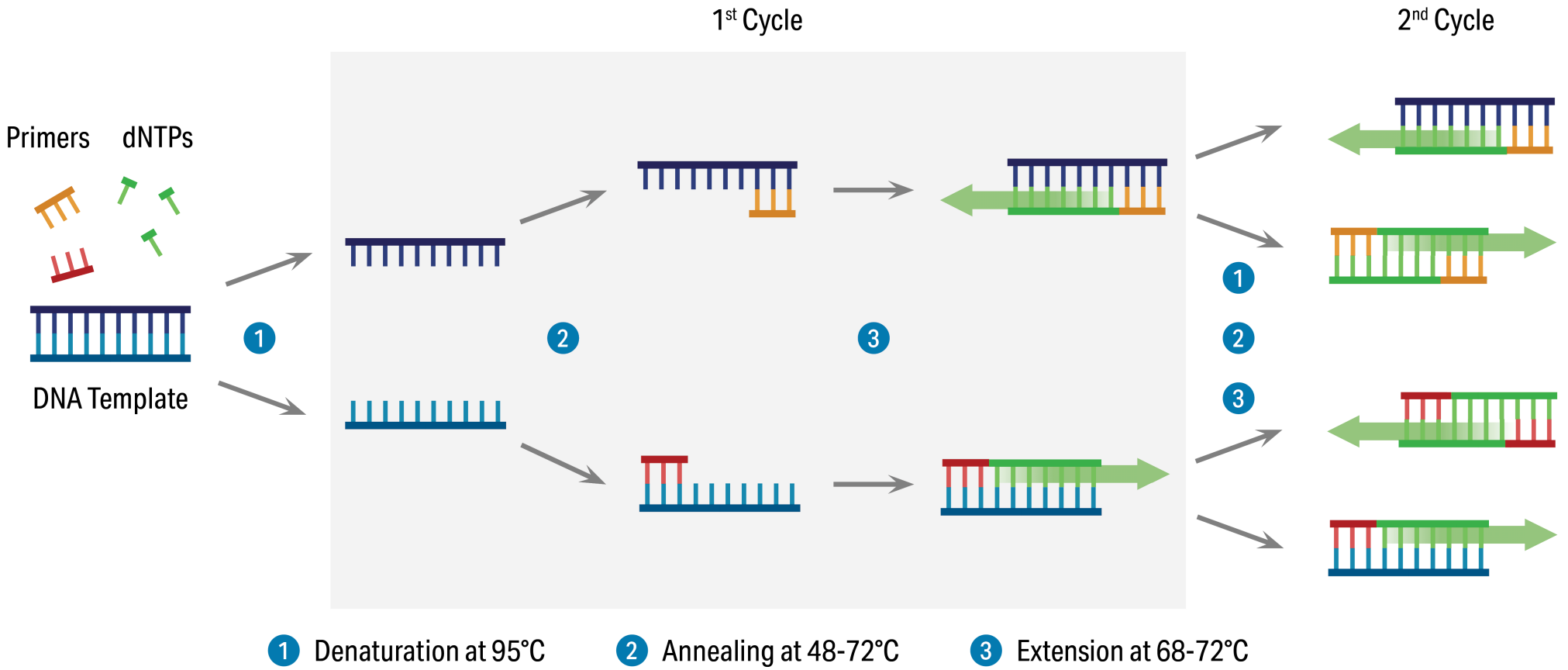
Oli
Rando
Reverse
Buffer



polymerase
-specific primers
and dNTPs

qPCR

Workflow- The PCR reaction



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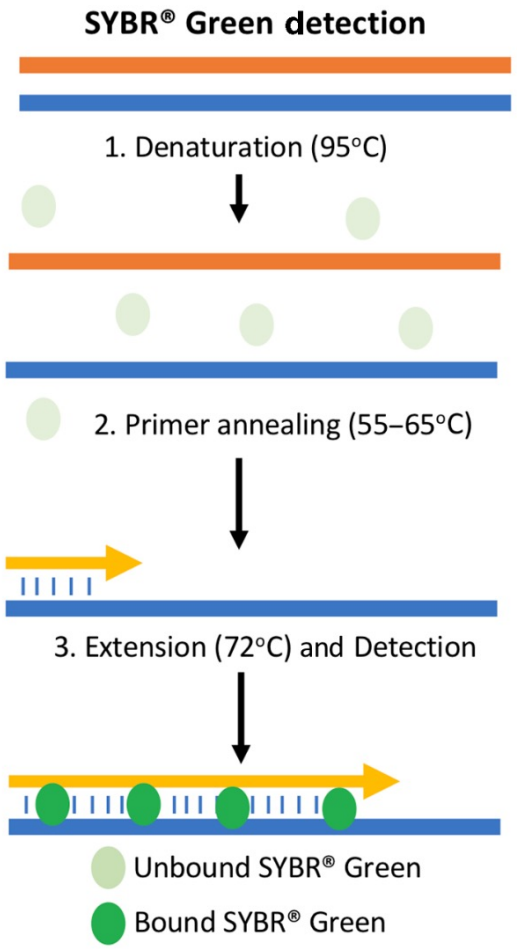
Workflow- SYBR Green detection

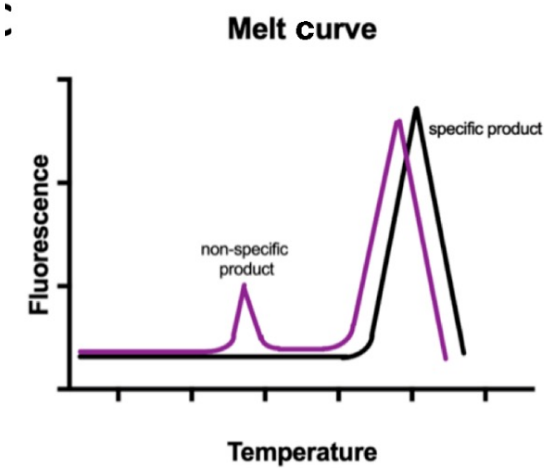


-Intercalating fluorescent dye

-SYBR Green I

-Fluorescence emitted measured after each thermal cycle

PROS:
- Cheaper
- More flexibility with primer design



CONS:
- Dye can bind to any ds-DNA sequence, hence can give non-specific signals…

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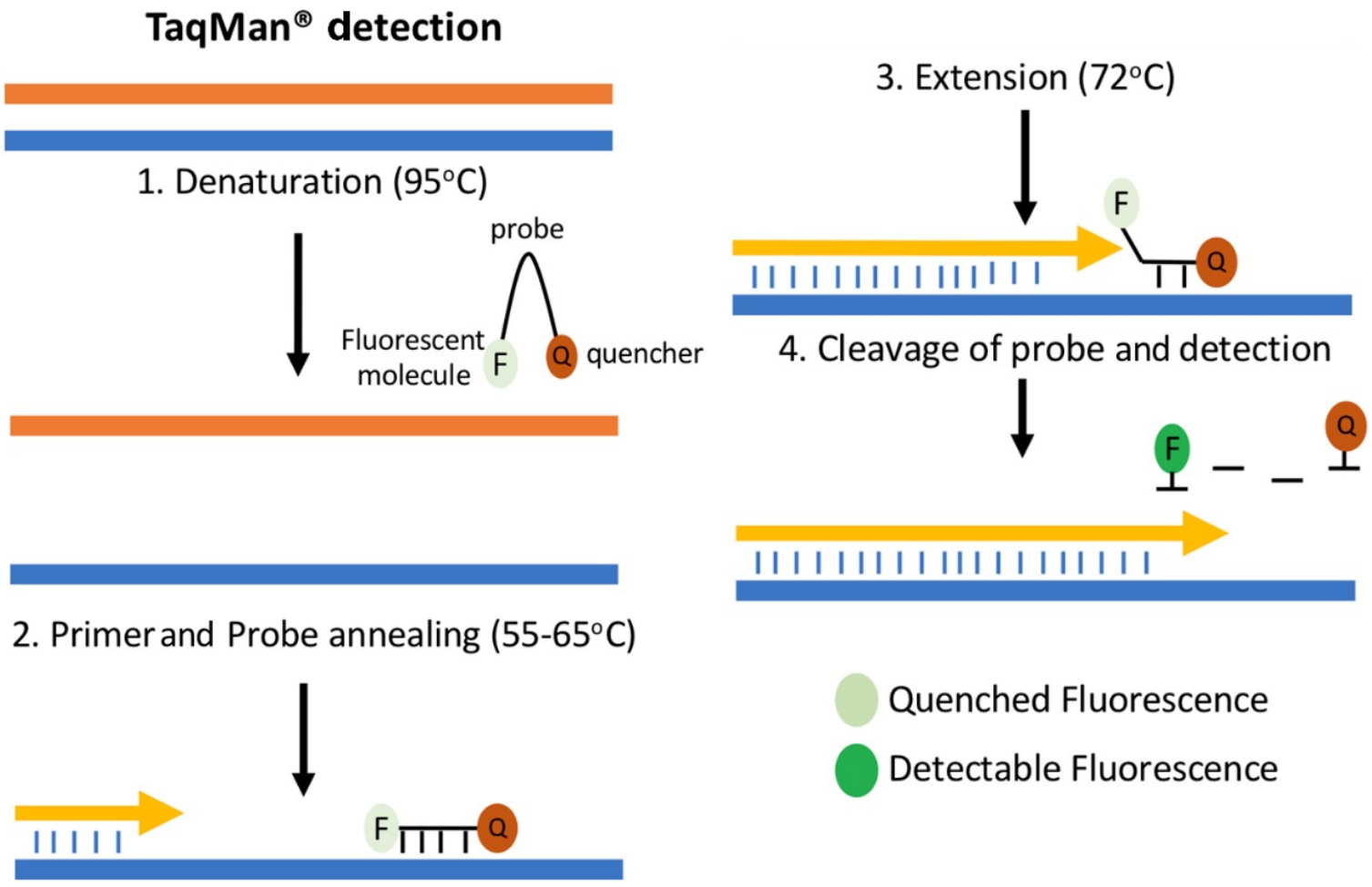
Workflow- Taqman detection



-5’ Fluorescent reporter dye
3’ Quencher dye

-Bind to DNA sequence downstream of primer

-Taq Polymerase hydrolyses linkage, releasing the reporter dye from the quencher, allowing detectable fluorescence

PROS:
-Specific
-Allows multiplexing

CONS:
-More expensive
-Need to have pre-designed assays

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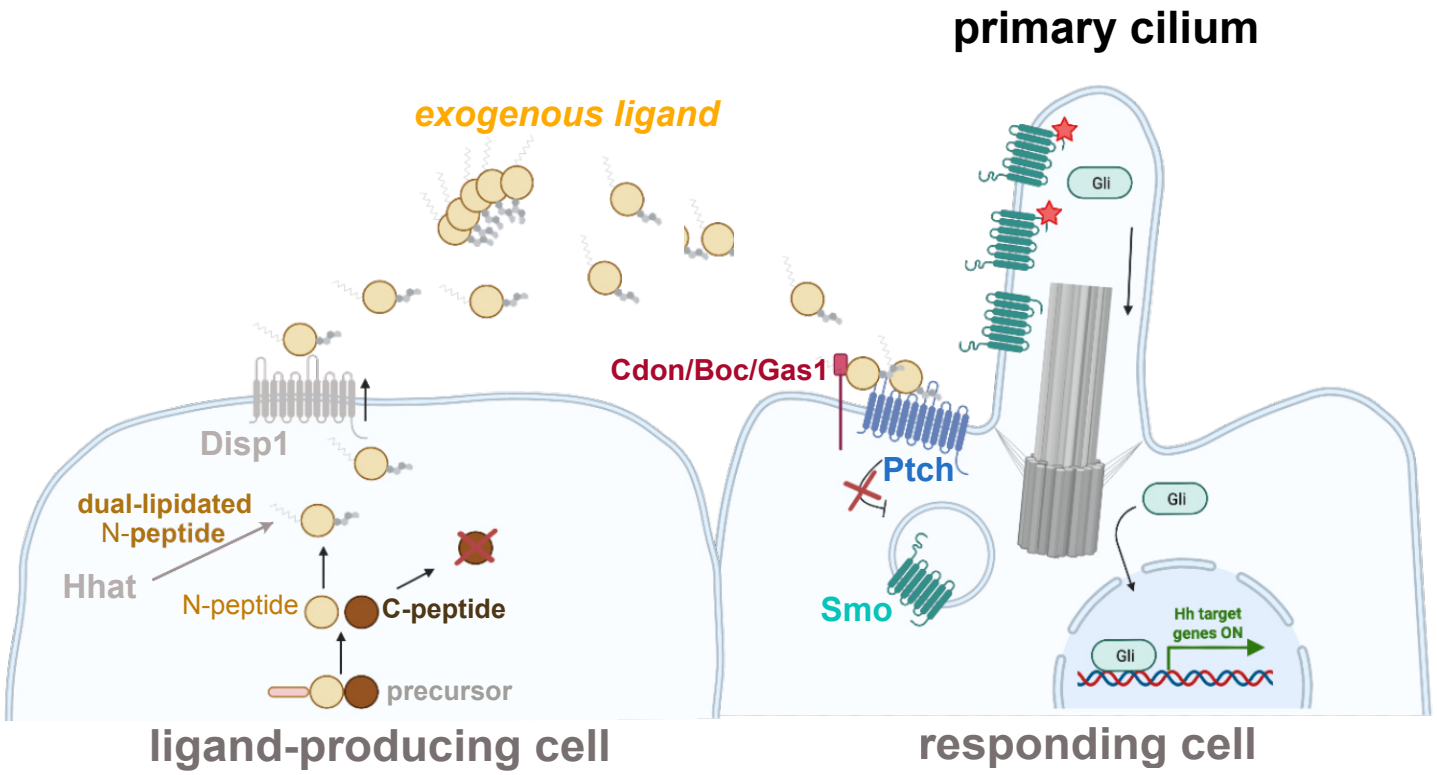
Biological Insights- Hh signalling pathway



Ancient pathway important in:

Embryonic development

Adult tissue maintenance

Tumorigenesis

Immune cell function!

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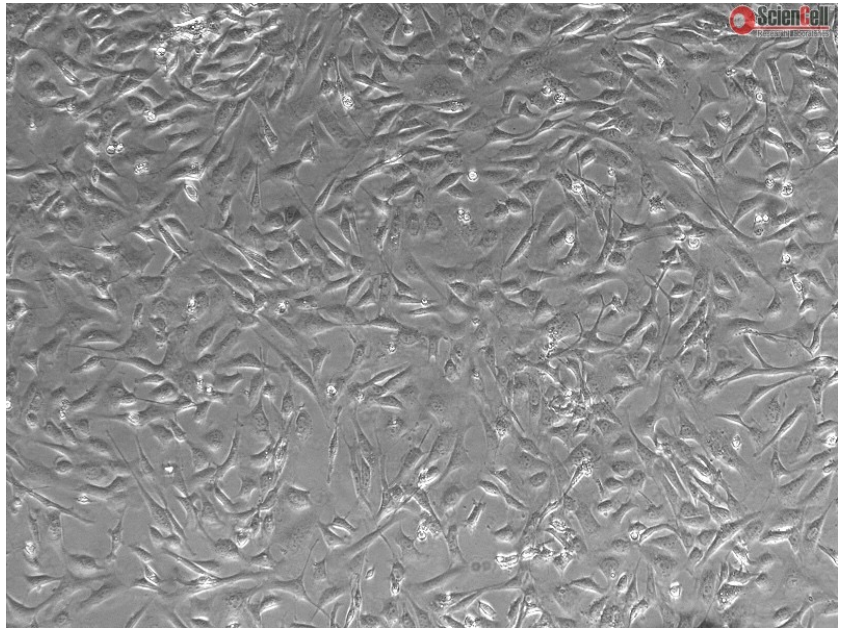
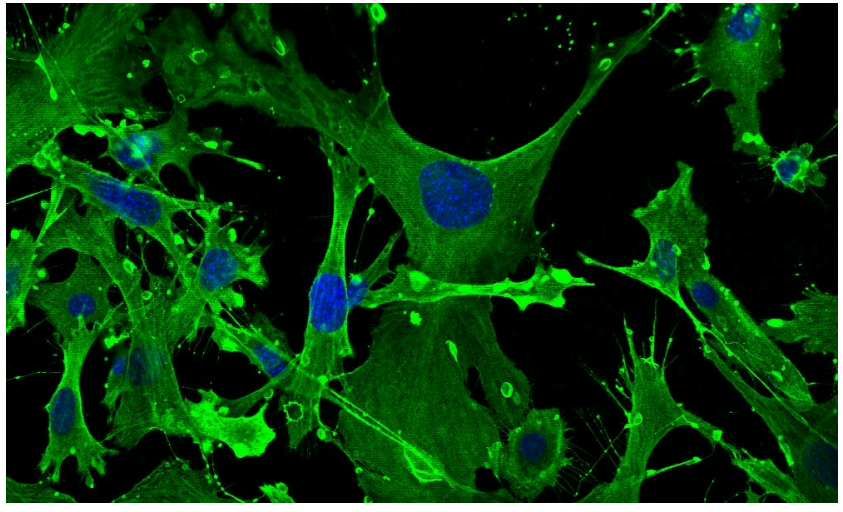
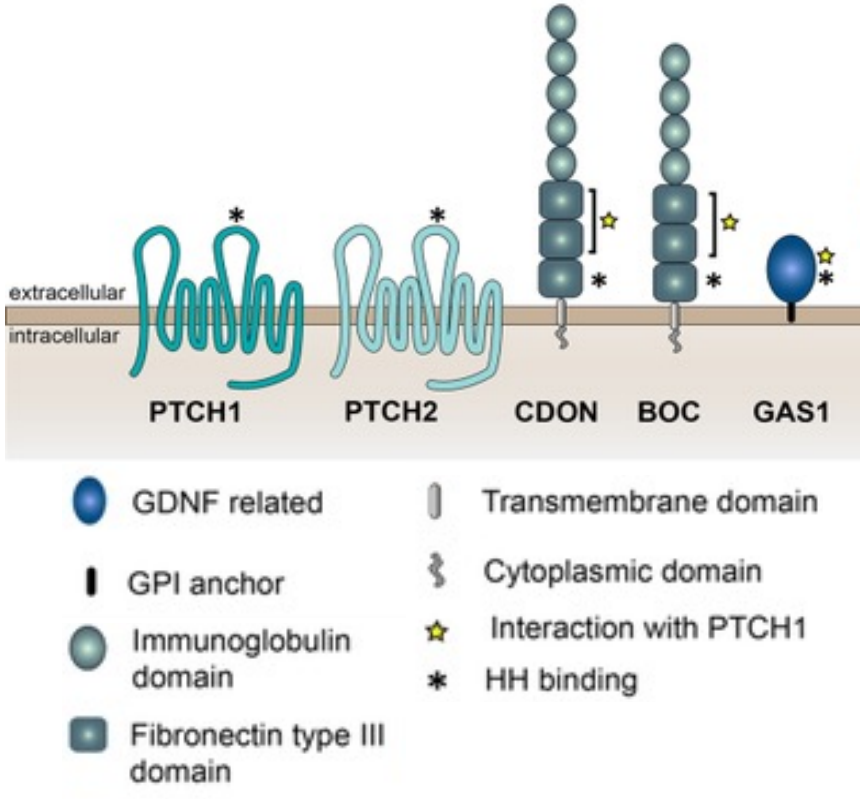
Expression of Hh Co-receptor gene *Gas1* in MEFs

MEFs- Mouse embryonic fibroblasts







Christ et al., *Developmental Dynamics (2016)*

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Expression of Hh Co-receptor gene *Gas1* in MEFs

Question:

What is the effect of serum starvation on the expression of the Hh Co-receptor, Gas1 in MEFs?

Experimental Plan:

1. Culture MEFs with or without serum starvation
2. Collect MEFs and isolate RNA
3. Make cDNA from the isolated RNA
4. Perform a qPCR reaction set-up
5. Run the qPCR on the QuantStudio system
6. Analyse the data and discuss results
7. Discuss ideal follow-up experiments

Expression of *IL-1b* in ….

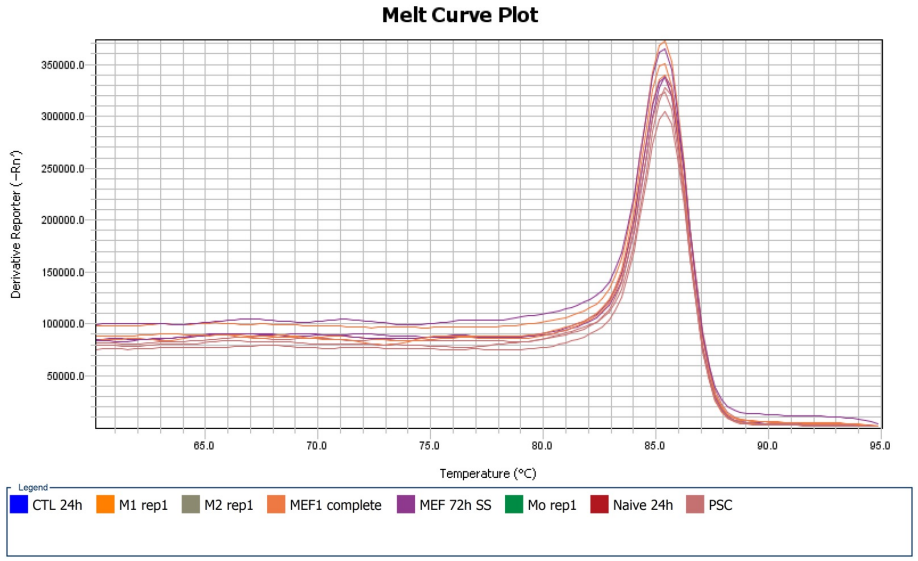
IL-1b and SASP (Senescence Associated Secretory Phenotype)

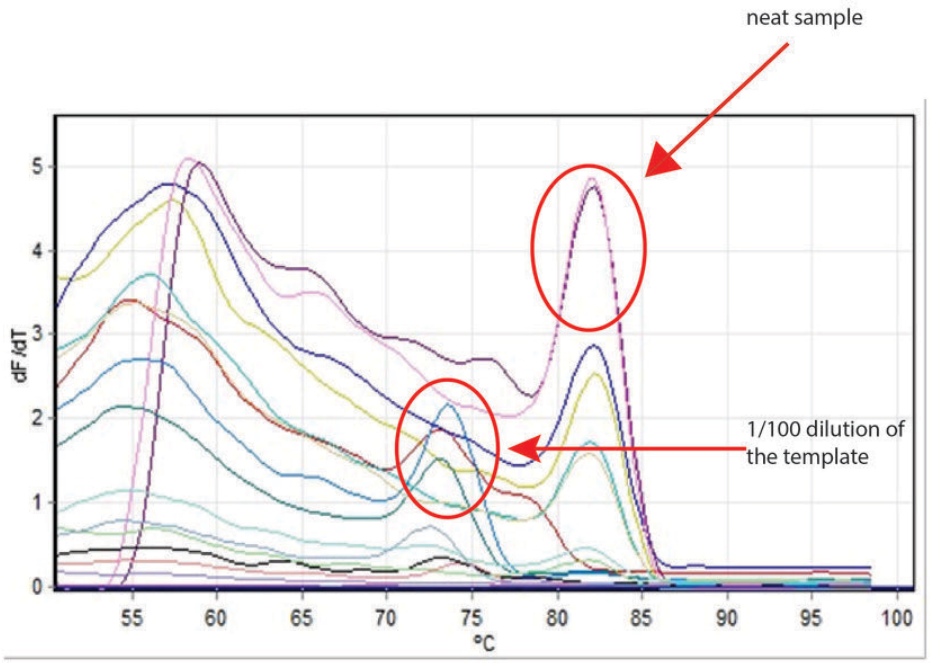
Question:

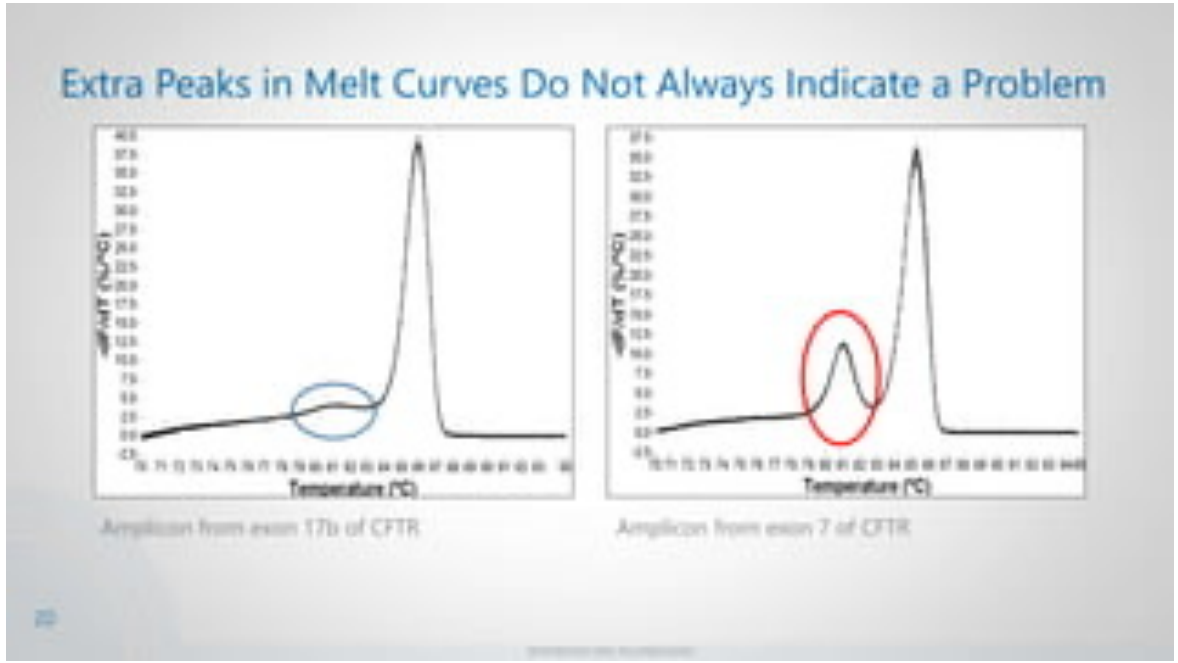
Experimental Plan:

1. Culture
2. Collect cells and isolate RNA
3. Make cDNA from the isolated RNA
4. Perform a qPCR reaction set-up
5. Run the qPCR on the QuantStudio system
6. Analyse the data and discuss results
7. Discuss ideal follow-up experiments

Analysis of qPCR data- Melt curves







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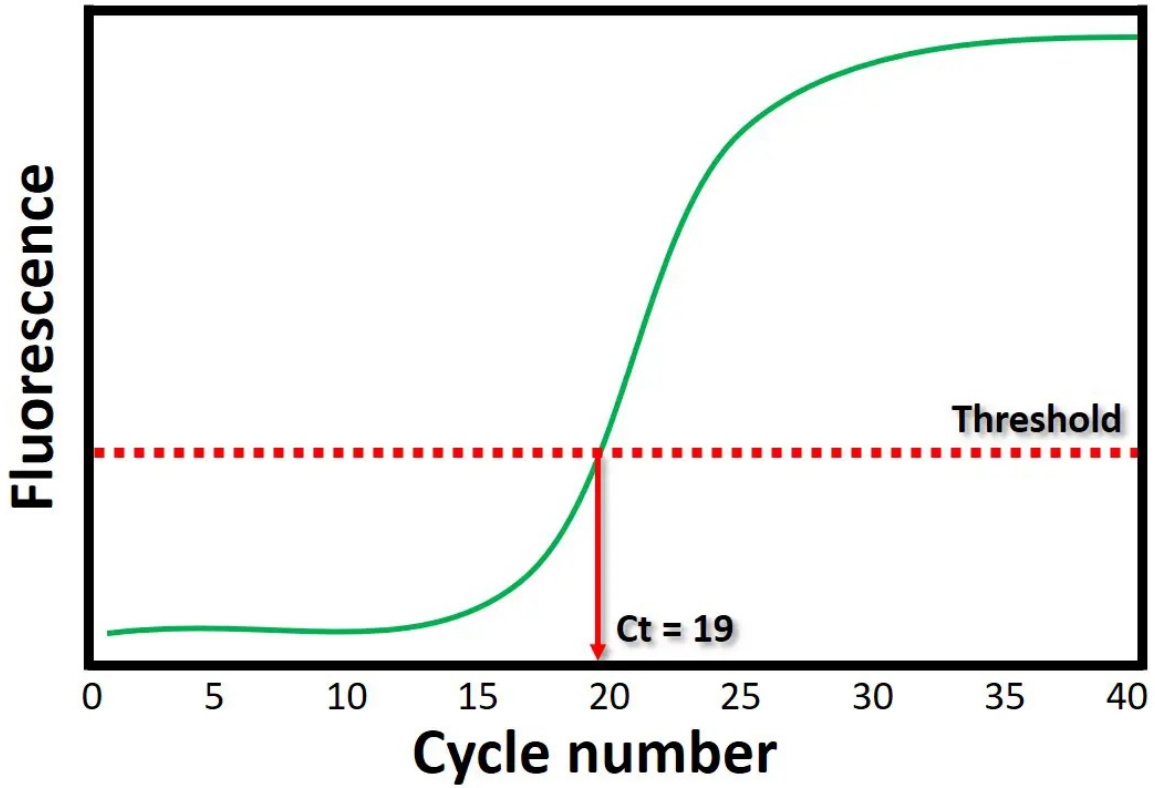
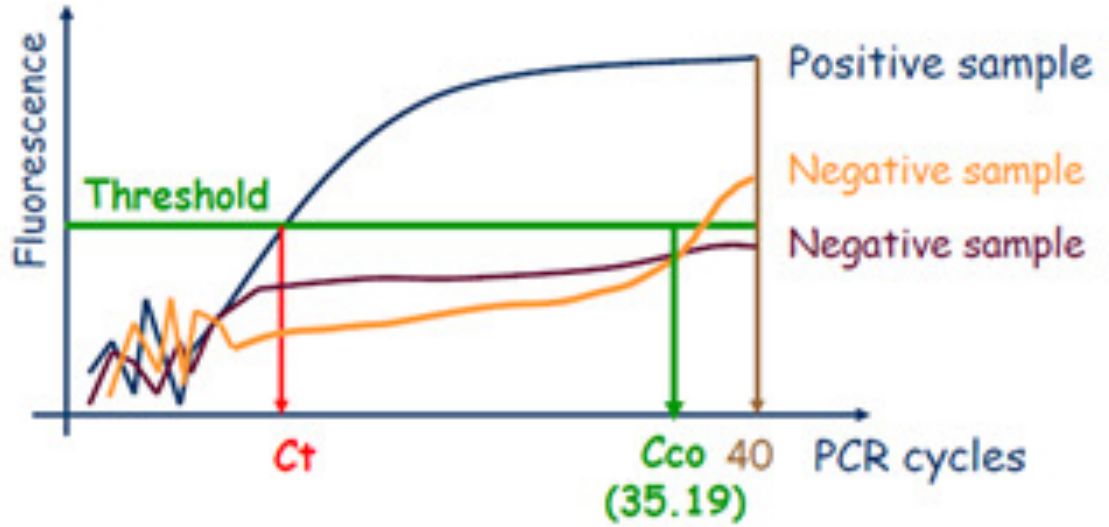
Analysis of qPCR data- Ct value





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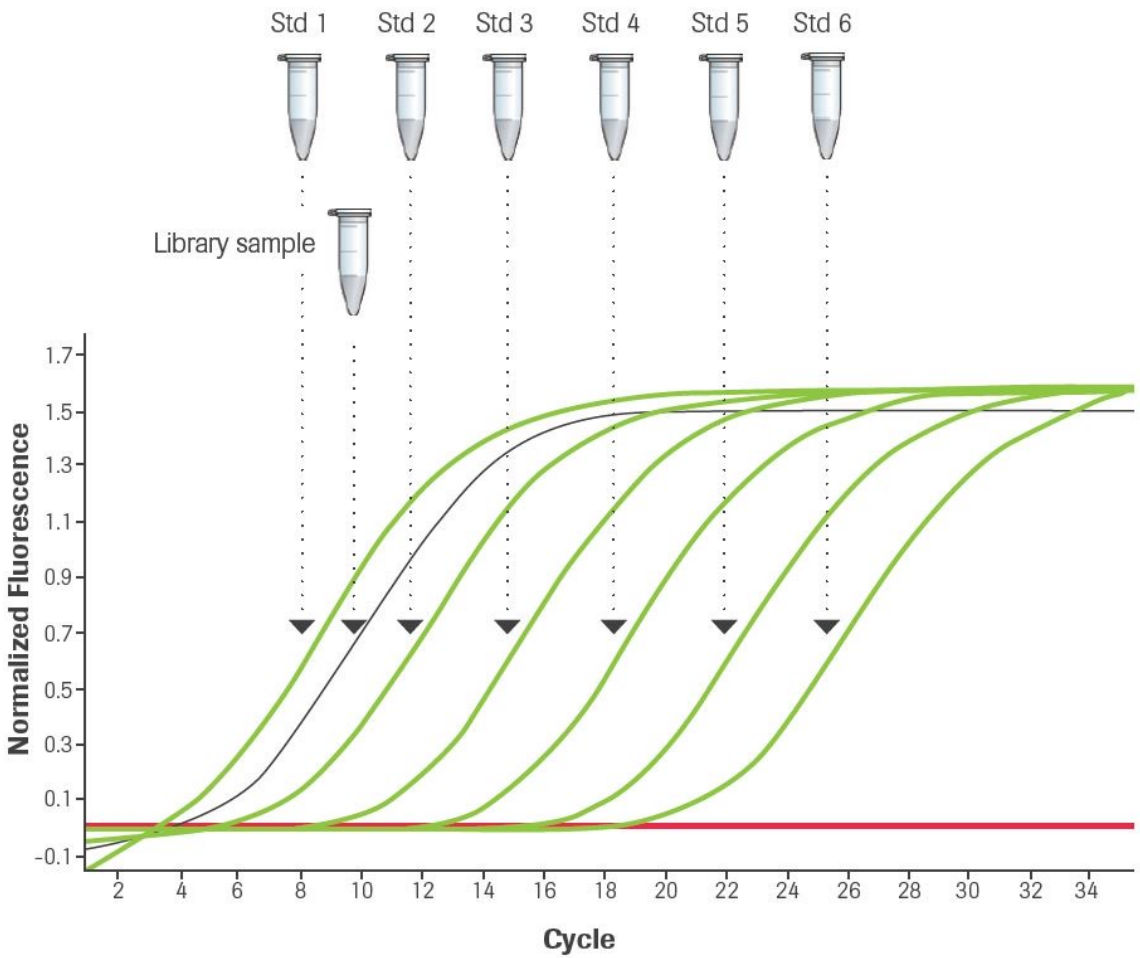
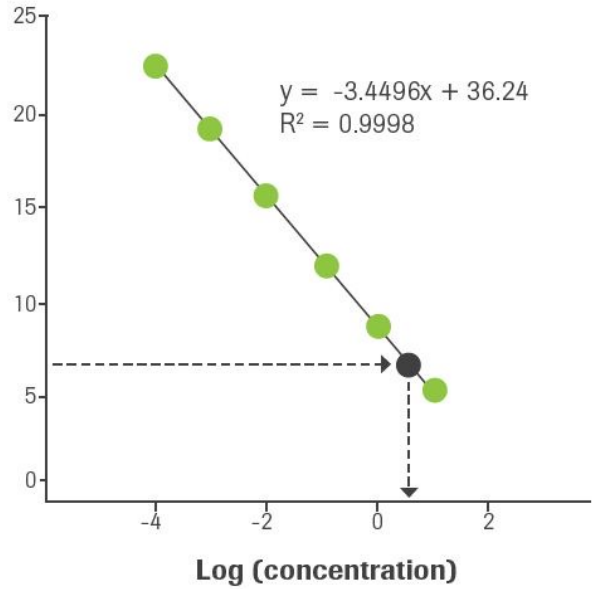
Analysis- Absolute quantification





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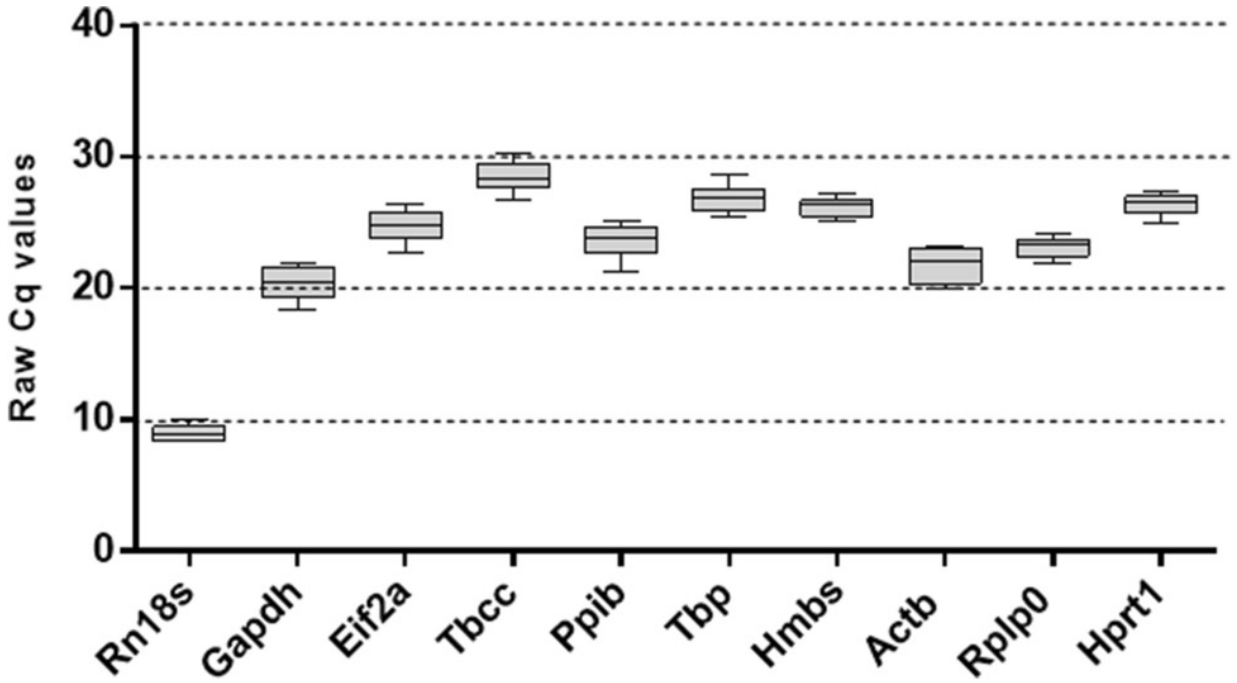
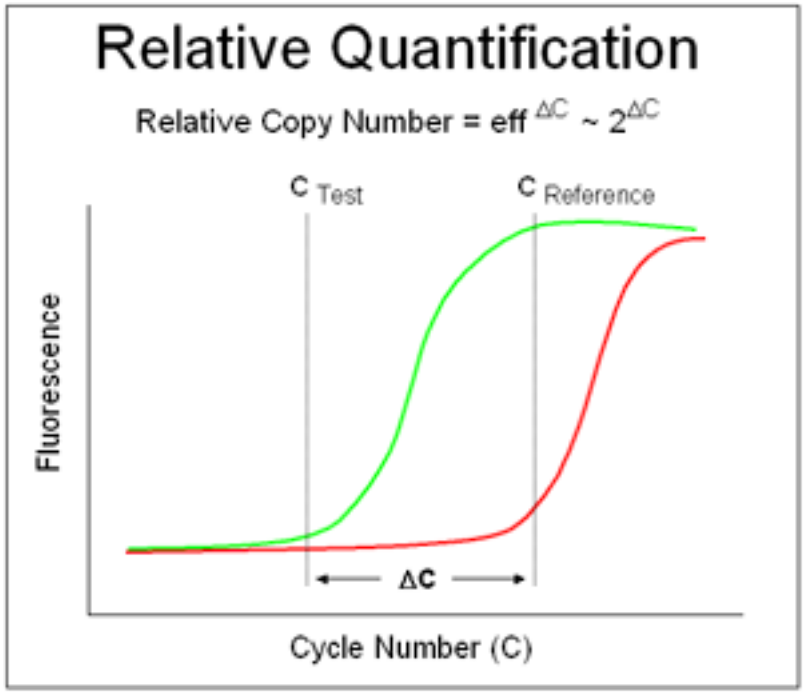
Analysis- Relative quantification

Housekeeping/Reference genes





Giri and Sundar, *Nature Scientific Reports,* 2022.

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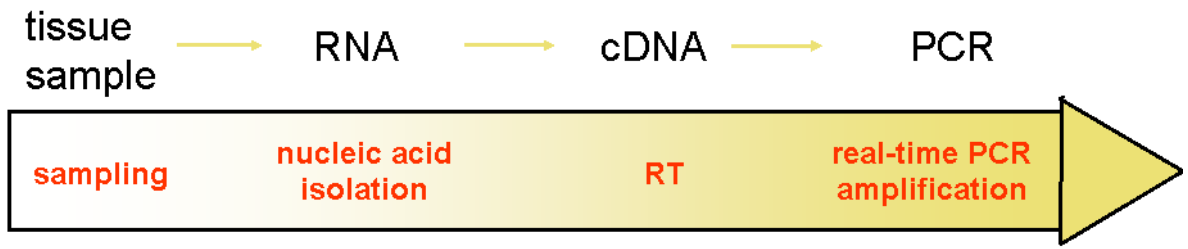
Common challenges and Troubleshooting

Steps and variables of a successful mRNA quantification using real-time RT-PCR (1)



Sampling method:
- Biopsy
- Fixed material
- Fresh blood
- Tissue storage
- Liquid Nitrogen
- RNA Later
- 1st extraction buffer
- RNA storage –80°C

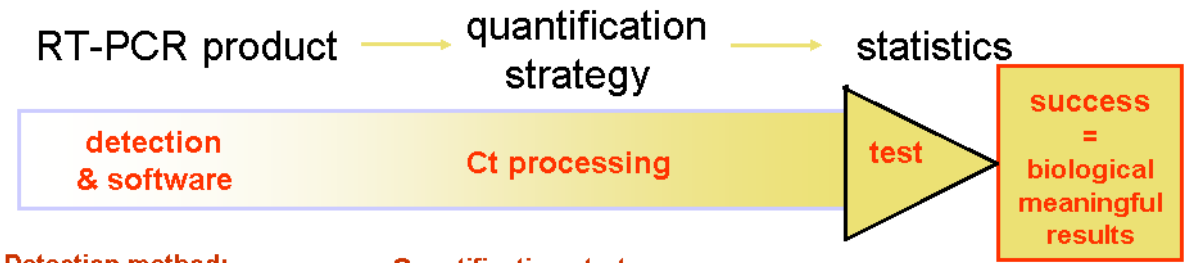
=> native RNA

Extraction method:
- total RNA
  - mRNA
  - microRNA
- liquid-liquid
- columns
- Robot vs. hand made
- **RNA integrity:**
  - Bioanalyzer 2100
  - Experion
  - Nano-Drop
  - mFold algorithm

Efficiency of RT:
- RT enzyme type
- RT temperature
- **Primers:**
  - poly-T Primer
  - Random-hexamers
  - Specific primer
  - Primer mixtures
- **one-step qRT-PCR**
- **two-step RT-qPCR**

PCR Efficiency / Specificity:
- Primer design
  - Primer specificity
  - Consensus Primer
- mRNA abundance
- RNA / cDNA input
- Polymerase types
- Polymerase Mixtures
- PCR Inhibitors & Enhancers
- Robot vs. hand made

Steps and variables of a successful mRNA quantification using real-time RT-PCR (2)



Detection method:
- Intercalating dyes:
  1st, 2nd, 3rd generation
- Probes:
  Taqman, Beacons, Scorpions, …
- raw data vs. background correction
- Fit point method
- TaqMan fitting (10x SD)
- 1st or 2nd derivative maximum
- other models: logistic / sigmoidal / NLR / CalQPlex
- Multiple and/or mixed models
- other curve "manipulations"
- 2-step, 3-step, or 4-step qPCR

Quantification strategy:
- **"absolute" quantification:**
  - type of calibration curve?
  - normalization with RG
- **relative quantification:**
  - total RNA, cells, tissue mass
  - normalization with RG
  - normalization via an RG Index (> 3 RGs)
  - geNorm, REST, BestKeeper, qBASE, qBASE*plus*, Normfinder, etc.

BioStatistics & BioInformatics:
- CP vs. quantified molecules
- Normality of data (???)
- t-Test (?)
- ANOVA (on the ranks ?)
- SAS, SPSS, Excel, Sigma Stat
- Permutation test
- Randomization test (REST 384)
- Bootstrapping (REST 2005; 2008)
- Cluster analysis
- Multiple regression analysis
- Multi-dimensional modeling

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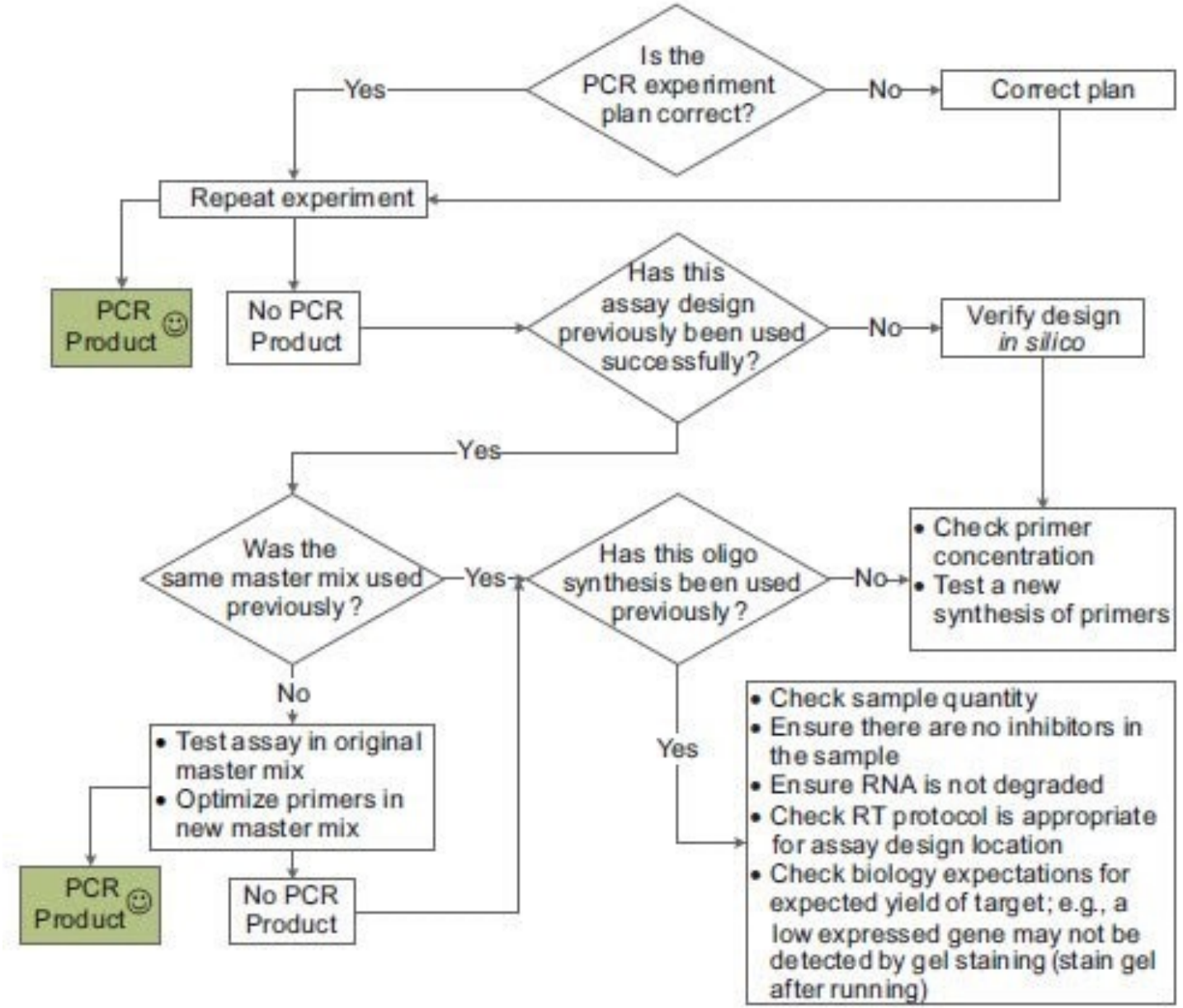
Troubleshooting your qPCR experiment



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Appropriate controls are critical!

Control	Example Material	Expected Result	Possible Reasons for a Positive Result	Possible Reasons for a Negative Result
Positive sample	A sample knwn to contain the assay sequences, e.g. RNA/gDNA expressing/ containing the tatget	Positive	Correct	Assay failure. Any positive data from other samples is unreliabe .
Positive assay control	Any nucleic acid compatible with the PCR assay design e.g..artificial oligo-nucleotide or plasmid con taining the PCR amplicon.	Positive		
Negative control	A sample know not to contain the assay sequences, e.g.RNA/gDNA not expressing/not containing the target.	Negative		
Contamination Negative assay control (No Template Control NTC)	Water	Negative	product or there was contamination of the control during PCR preparation.	Correct

RT-Specific Controls	Example Material	Expected Result
Minus RT enzyme negative control	RNA sample and all components of the RT rection with the exception of the RT enzyme. This should be performed on all samples to verity that they do not contain sequences that amplify under the PCR conditions without the need for RT, e.g. gDNA contamination	Negative

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https://www.sigmaaldrich.com/GB/en/technical-documents/technical-article/genomics/pcr/troubleshooting

De la Roche lab

Thank you!!!

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