Principles and Applications of quantitative Real-Time PCR

Maike de la Roche

Cancer Biology Summer School

Uganda Cancer Institute/Makerere/CRUK Cambridge Institute

29 September 2025

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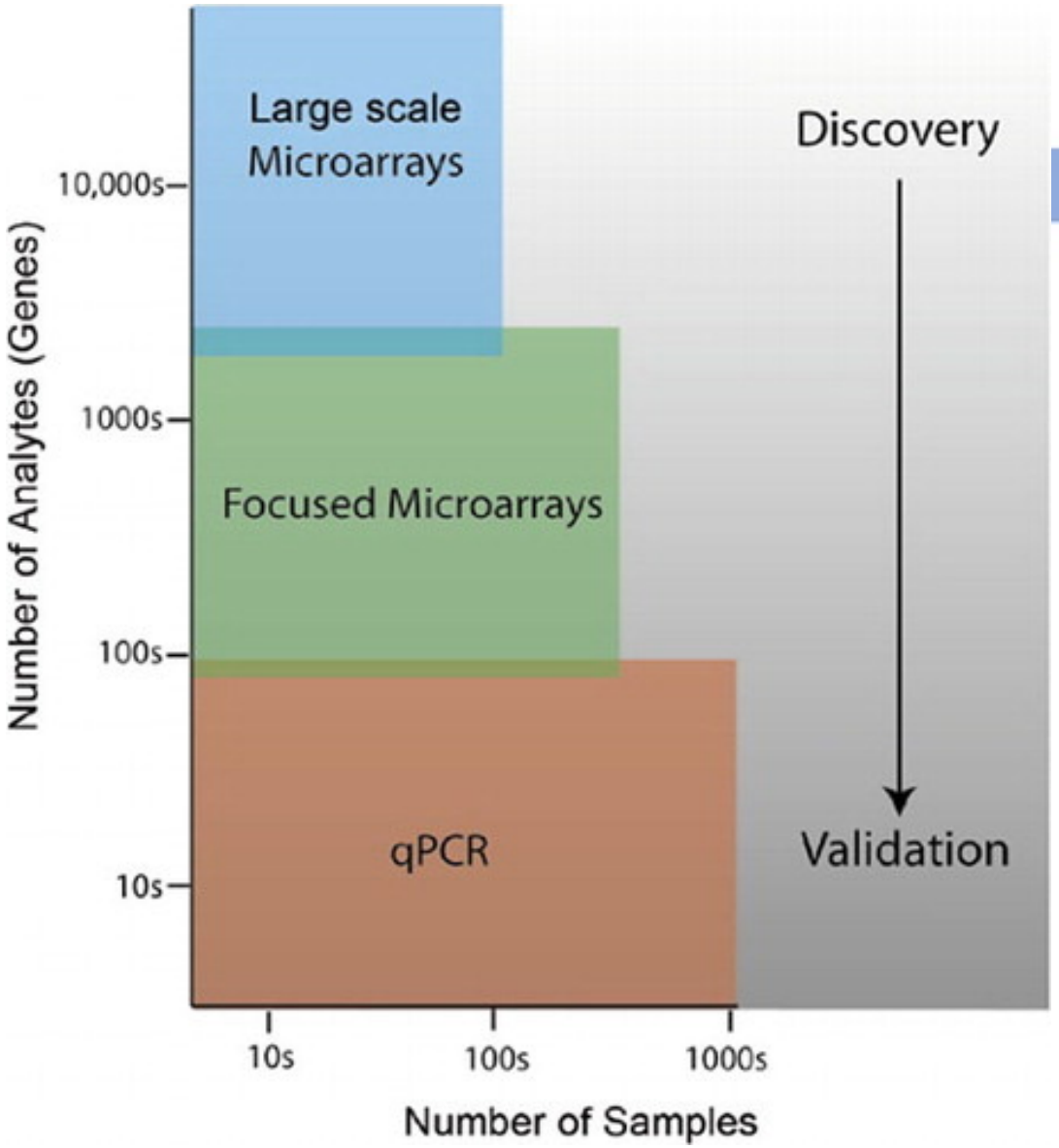
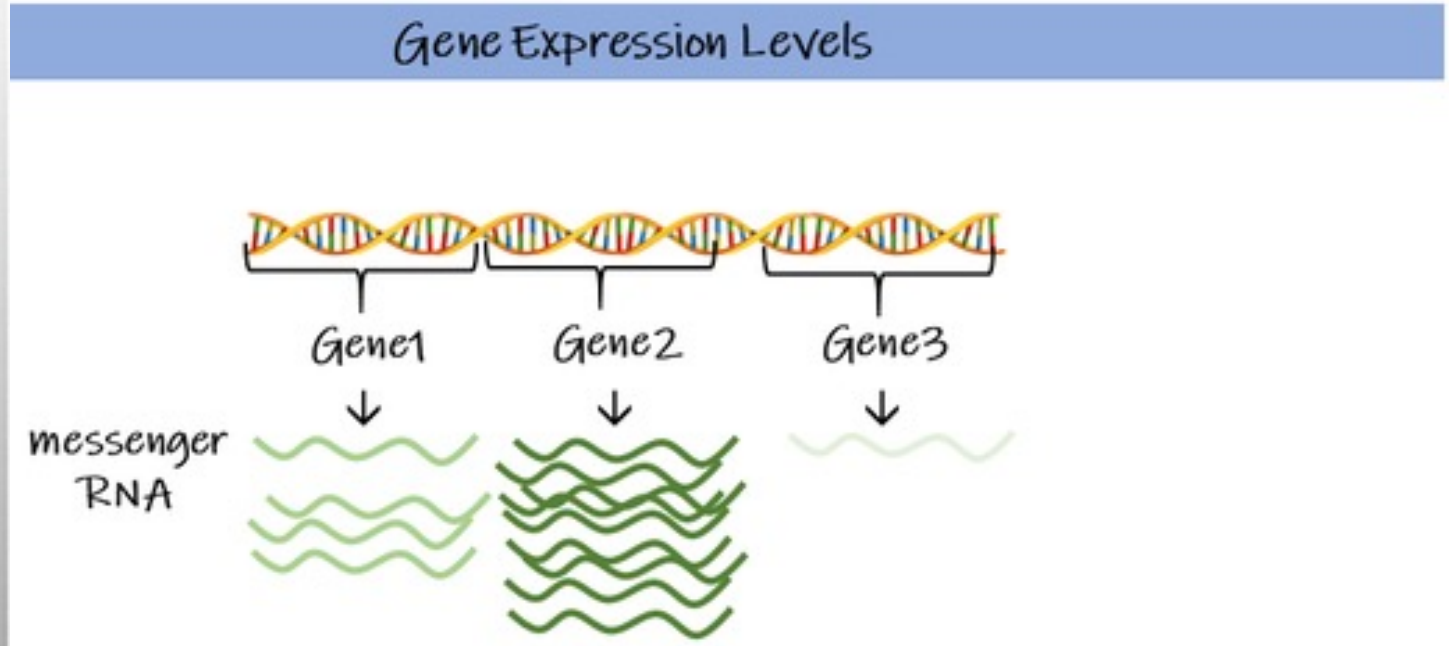
Together we will beat cancer

Measuring gene expression





VanGuilder et al., 2018. *Biotechniques*

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The Polymerase Chain Reaction (PCR)

Kary B. Mullis
Facts

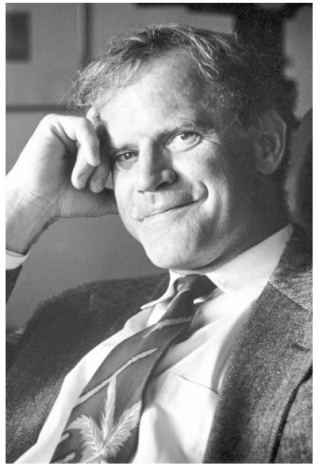


Photo from the Nobel Foundation archive.

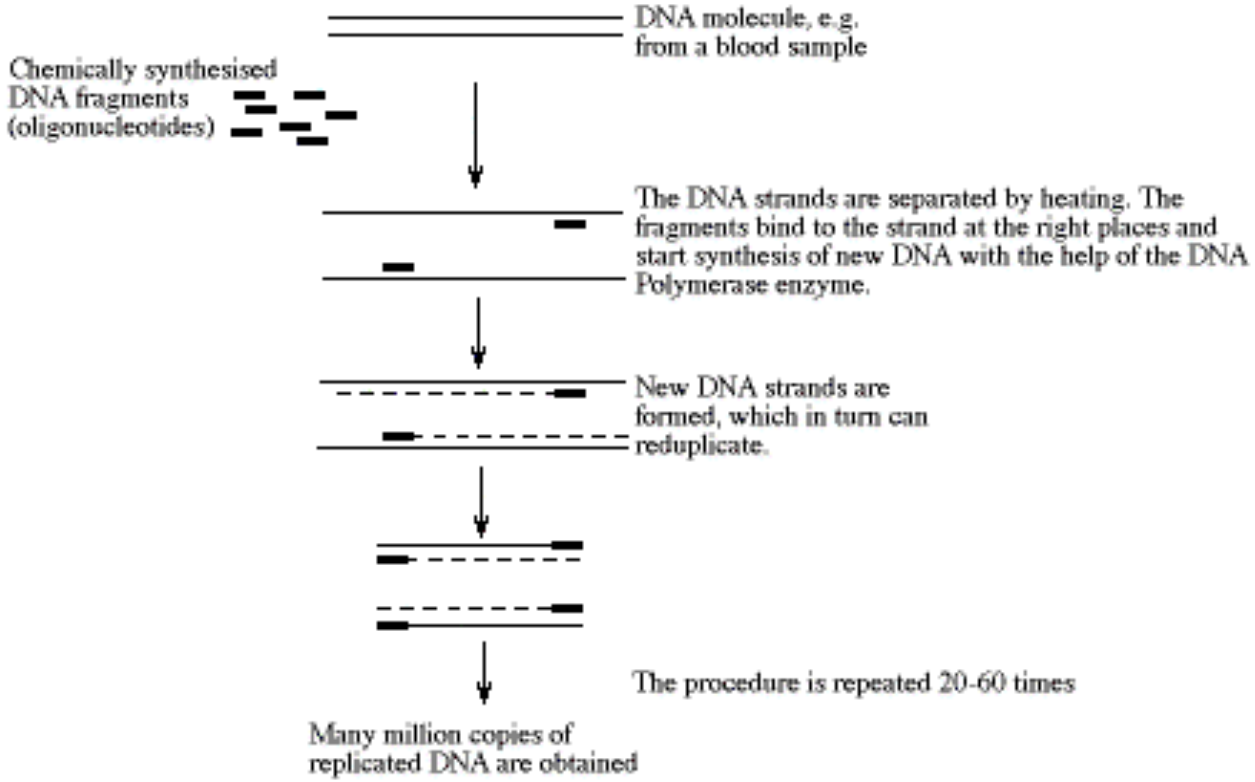
Kary B. Mullis
The Nobel Prize in Chemistry 1993

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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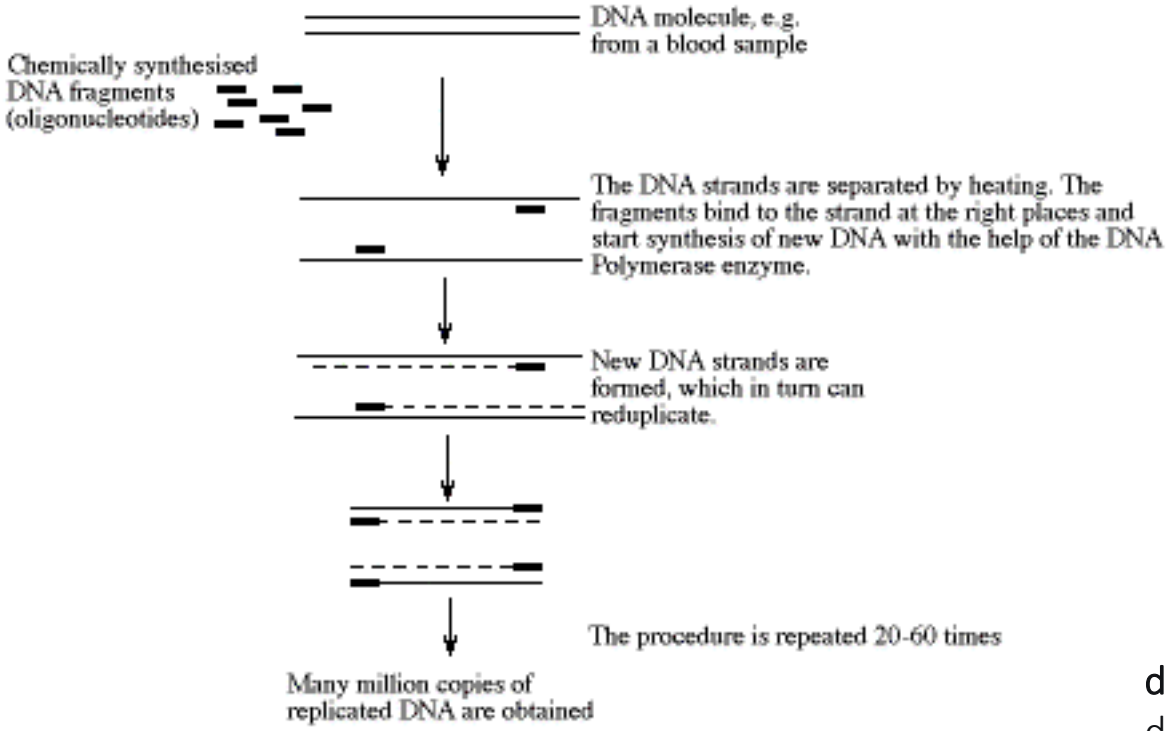
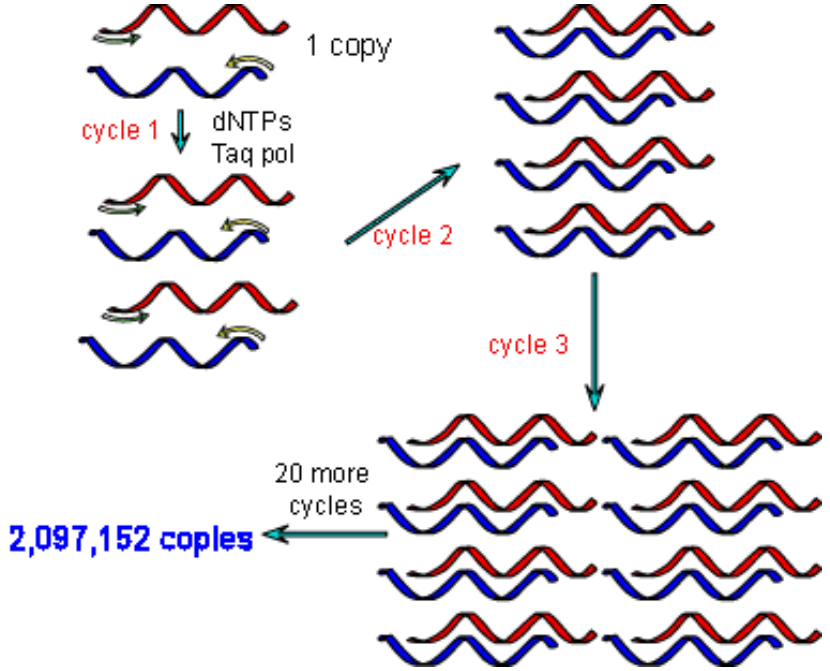
The Polymerase Chain Reaction (PCR)



Kary Mullis, Nobel Prize Lecture, 1993.



dNTPs (deoxynucleoside triphosphates): essential building blocks, dATP, dCTP, dGTP, and dTTP

Polymerase: 1983- isolation of **Taq polymerase** from the thermophilic bacterium Thermus aquaticus (thermostable)
Pfu polymerase (proof-reading capacity)

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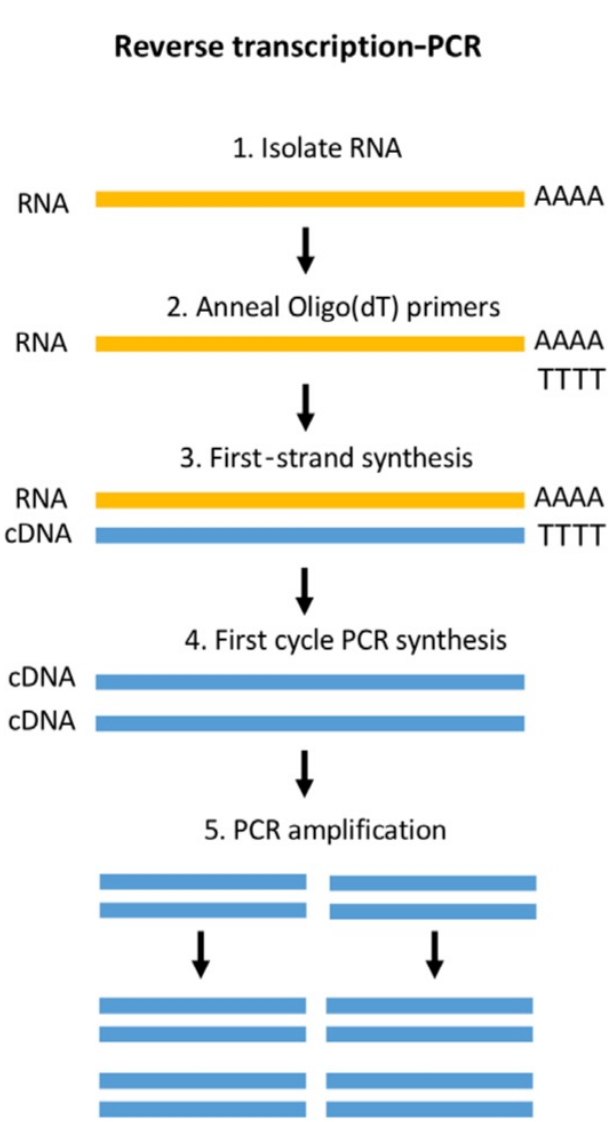
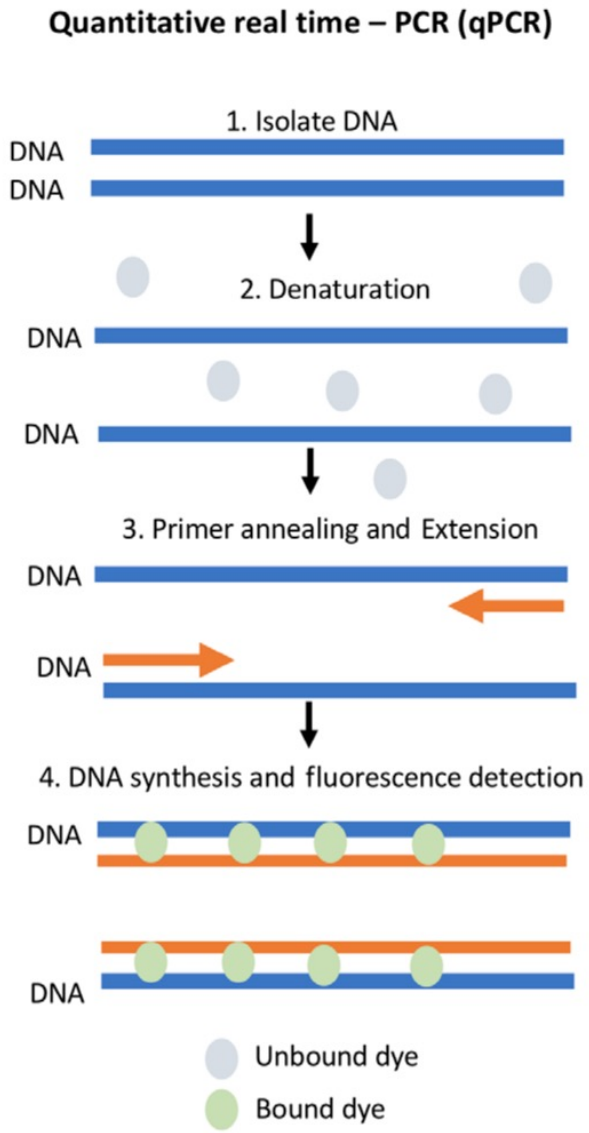
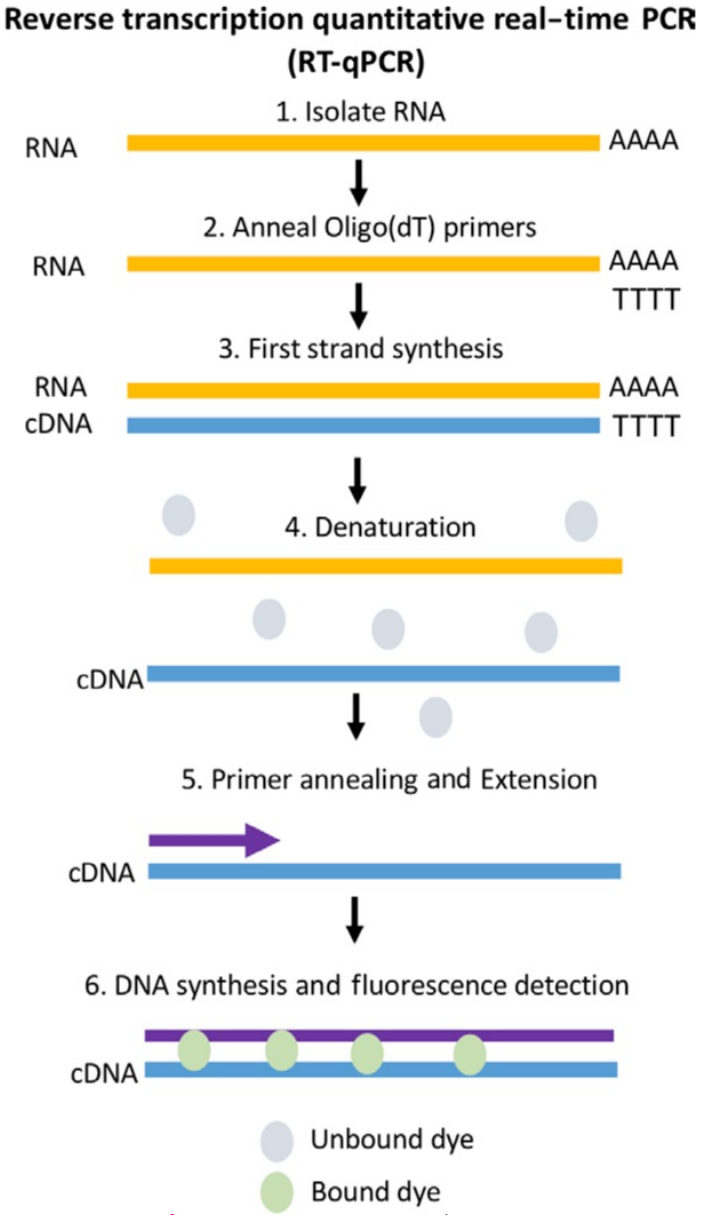
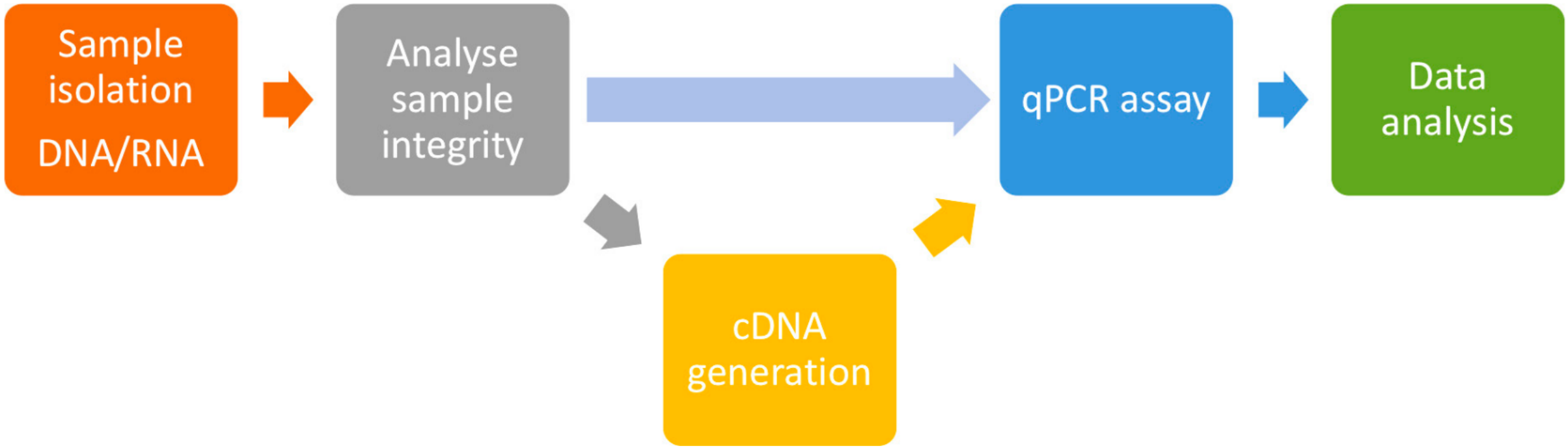
RT-PCR vs qPCR vs RT-qPCR



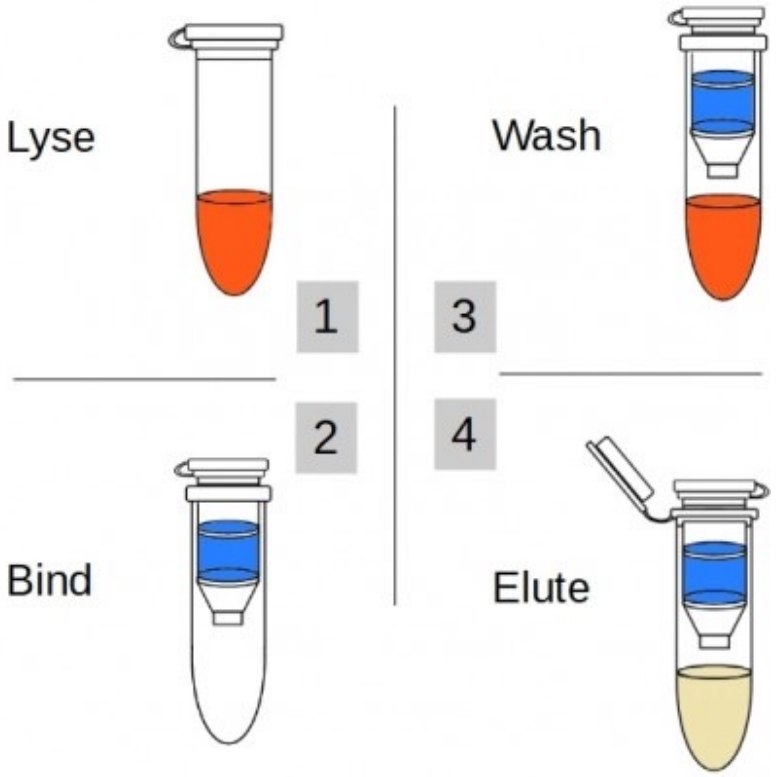




Workflow for qPCR reactions



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

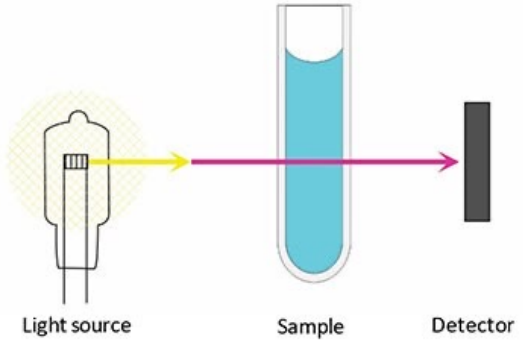
Disposable plasticware

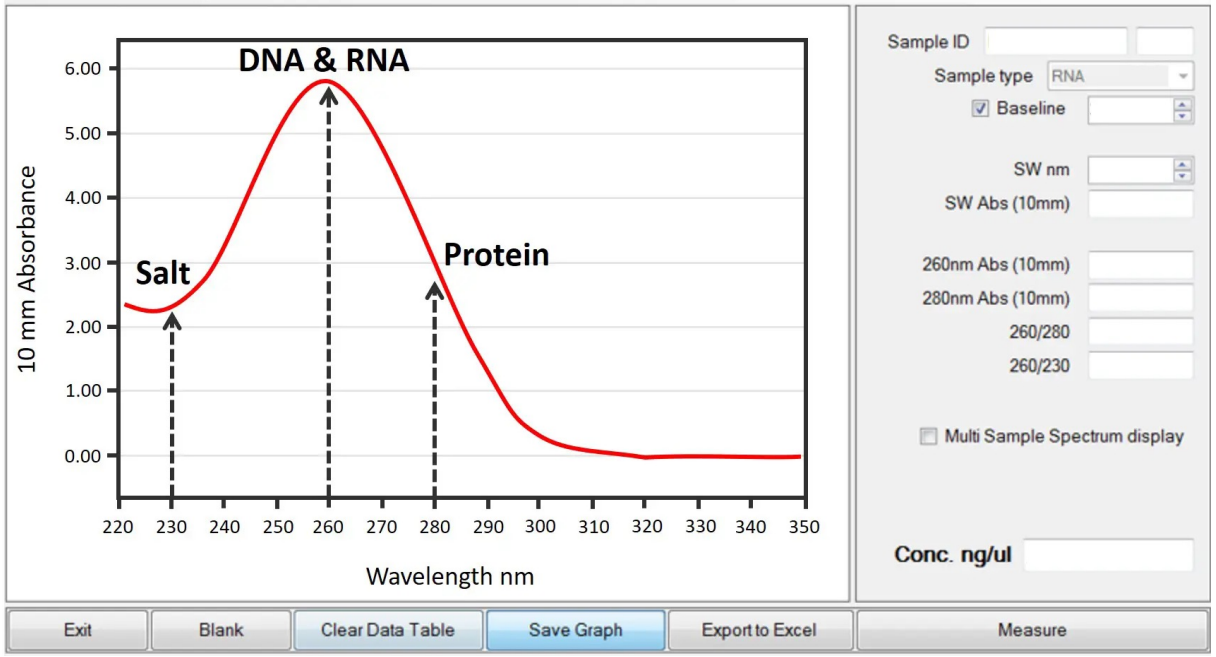
- The use of sterile, disposable polypropylene tubes is recommended
- These tubes are generally RNase-free and do not require pretreatment to inactivate RNases

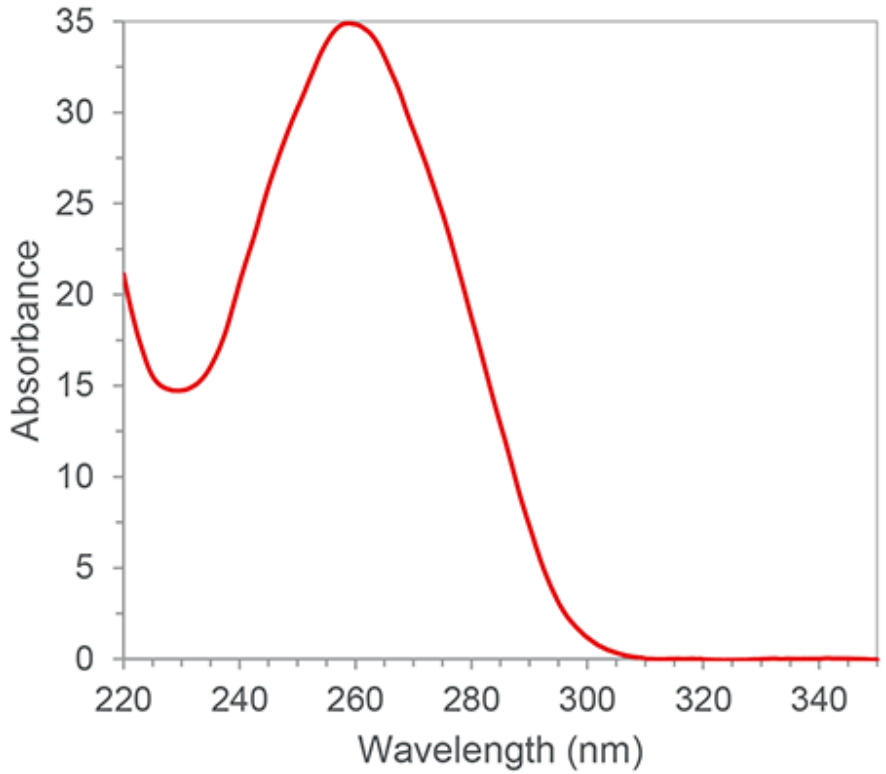
Non-disposable plasticware

- 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

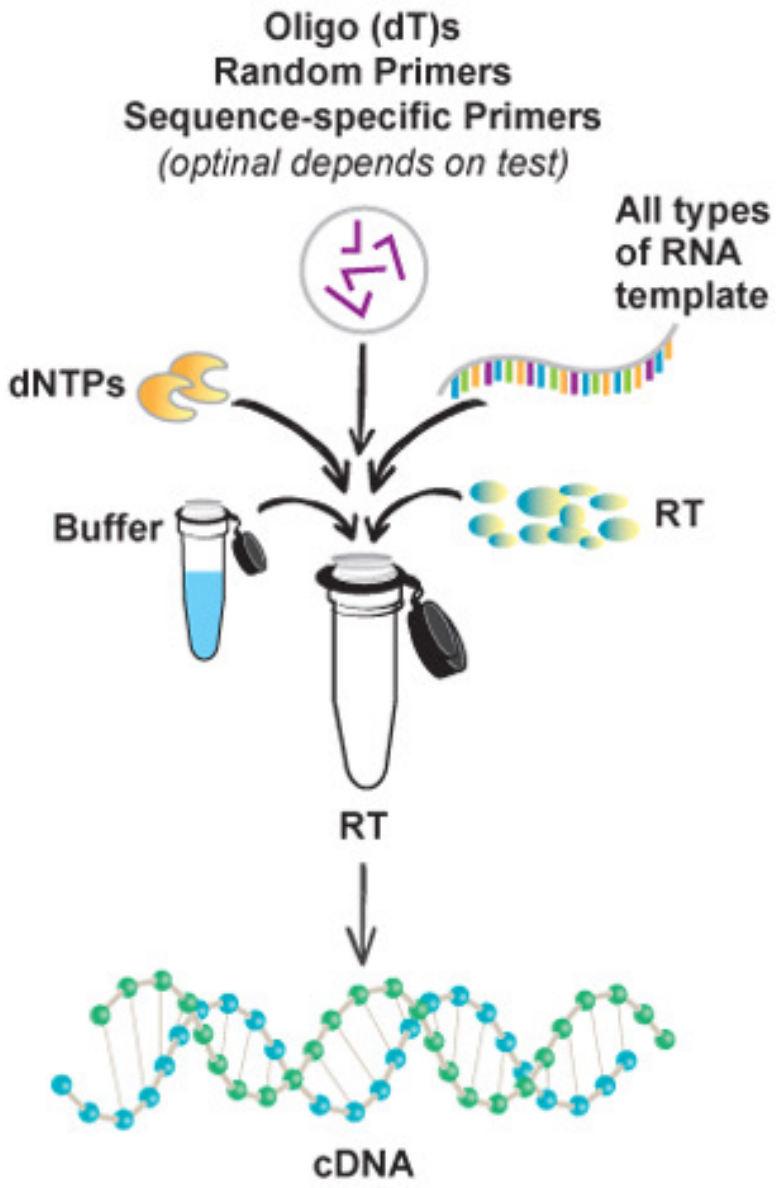
Workflow: One-Step vs Two-Step PCR

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

One-step RT-qPCR

RN
Oligo(
Random h
Reverse tra
Buffer an

R



lymerase
ecific primers
nd dNTPs

CR

Oligo(dT) versus random oligo primers

Oligo(dT) primers:

- specifically bind to the 3' poly(A) tails of eukaryotic mRNA

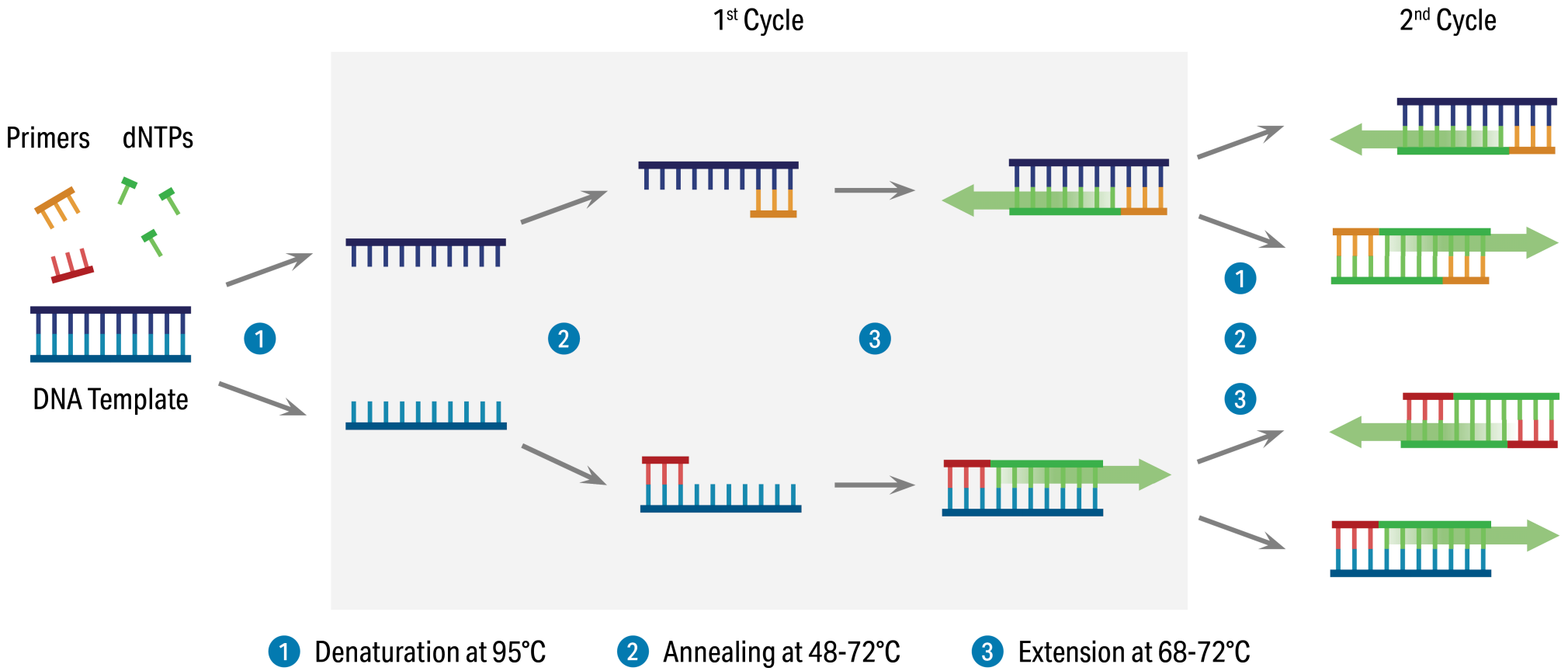
➢ oligo(dT) primers are best for:
full-length cDNA synthesis of mRNAs
constructing cDNA libraries

Random Hexamer primers:

- short, randomly sequenced oligonucleotides that bind anywhere on any RNA molecule.

➢ ideal for amplifying prokaryotic RNA
degraded RNA samples
detecting low-abundance transcripts because they provide more comprehensive priming.

Workflow- The PCR reaction



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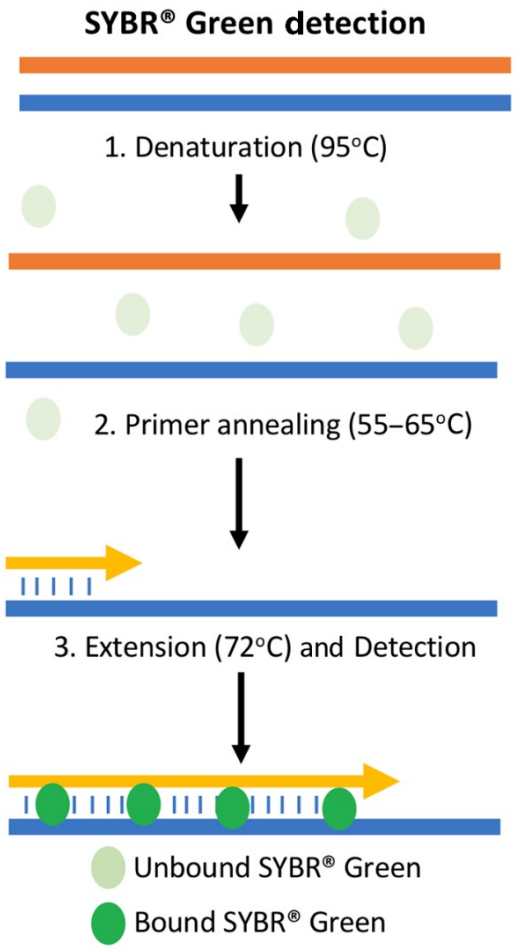
Workflow- SYBR Green detection



-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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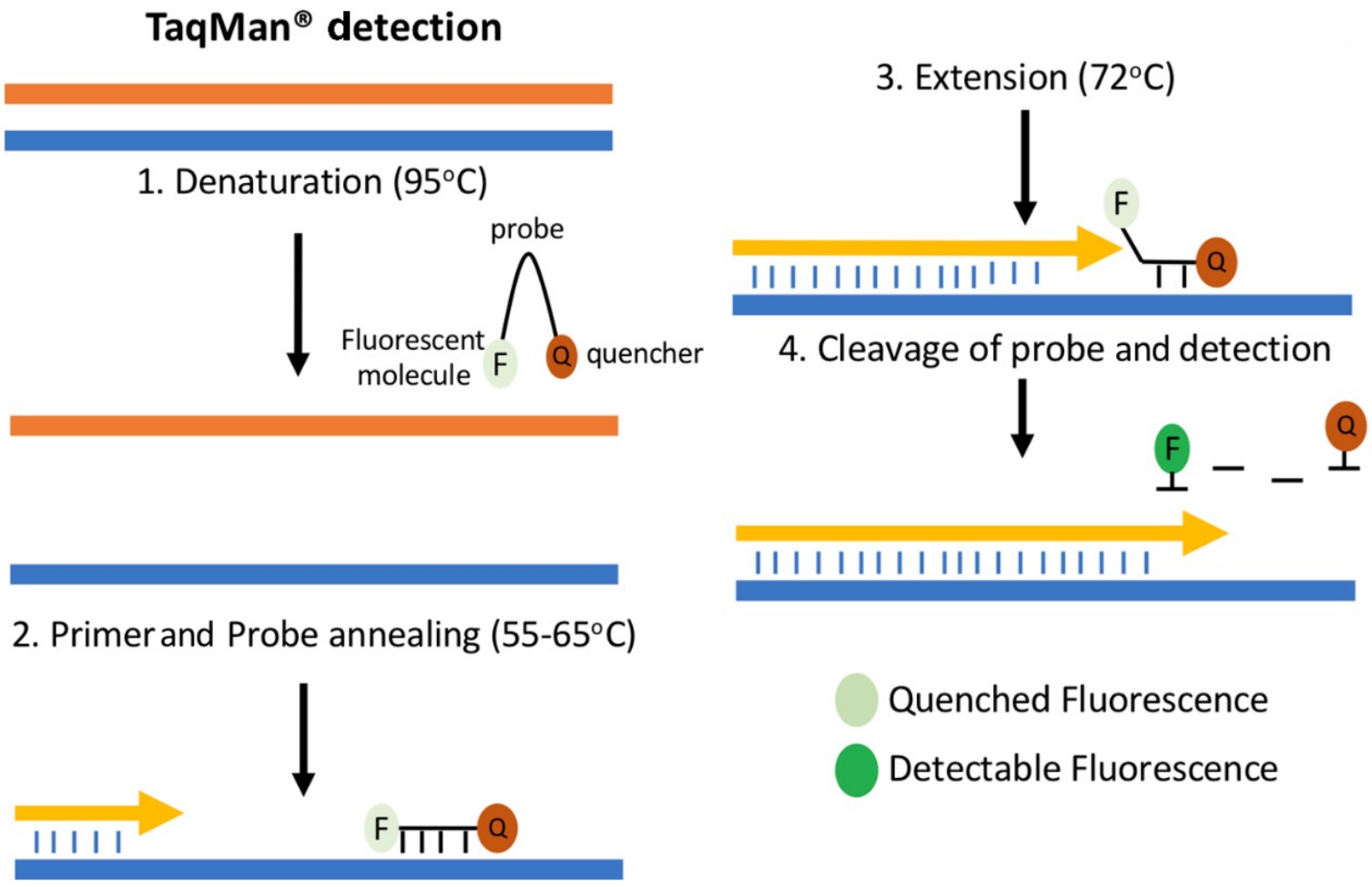
Workflow- Taqman detection



-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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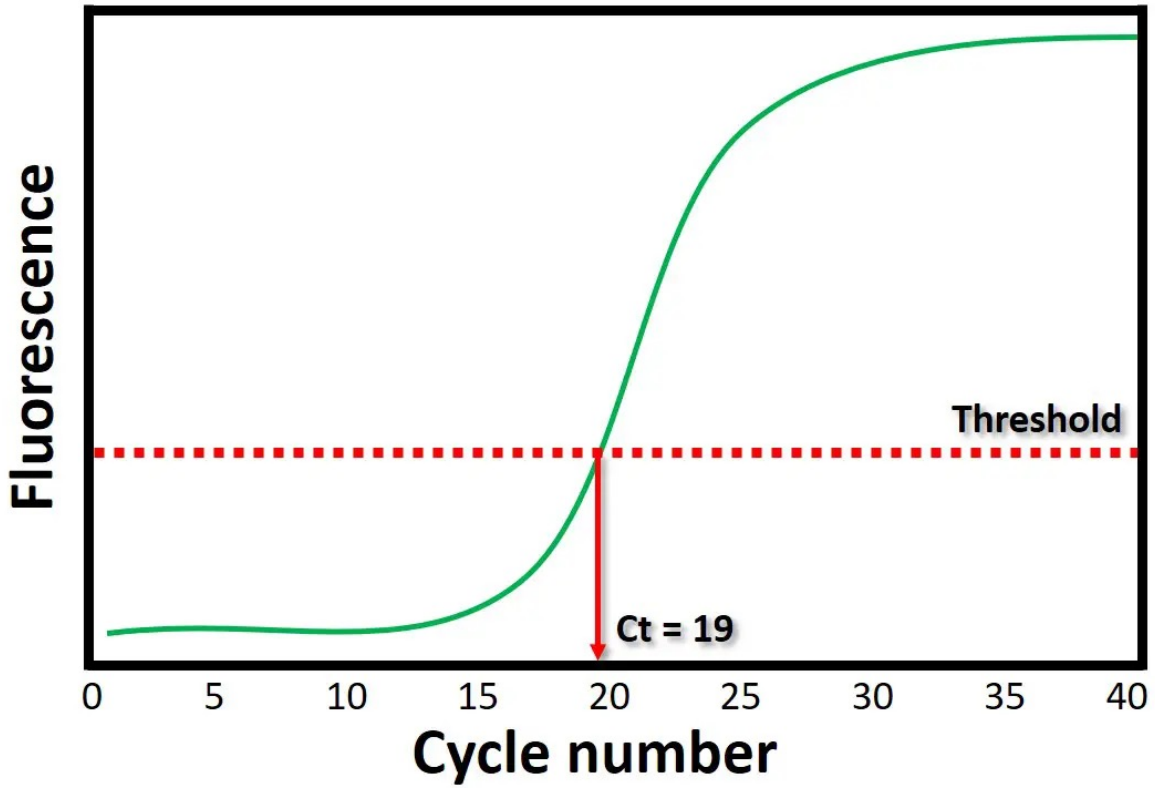
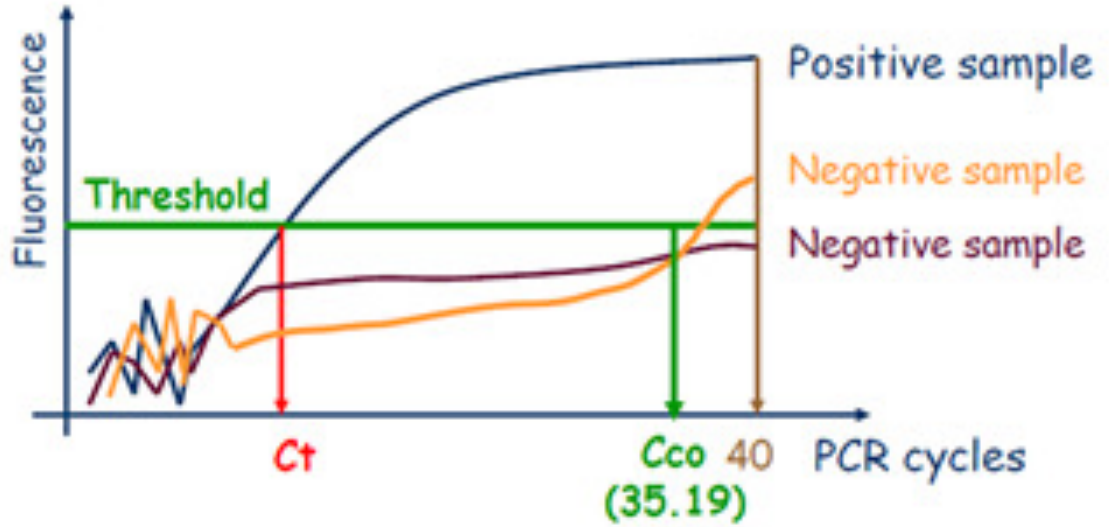
Analysis of qPCR data- Ct value





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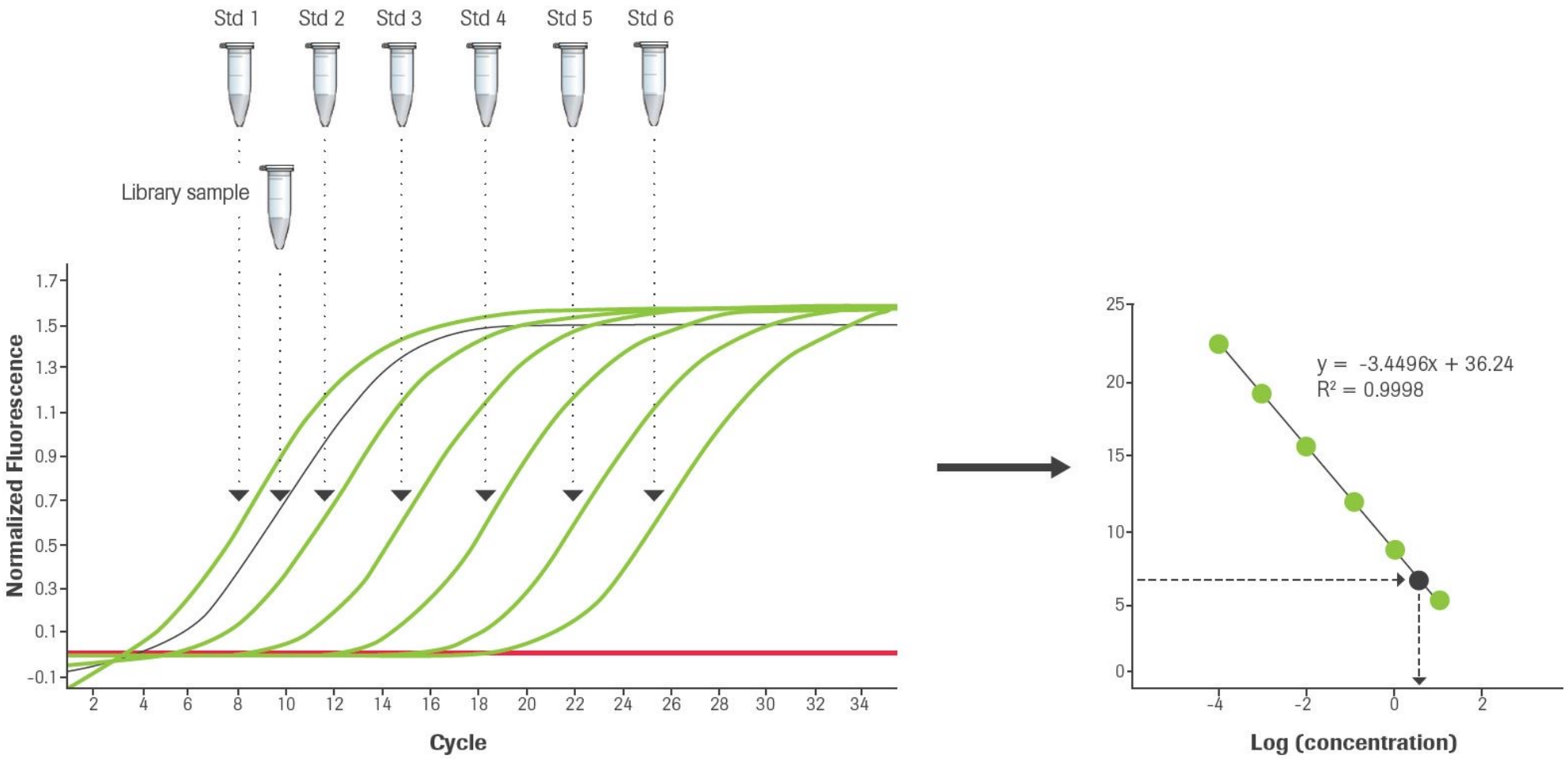
Analysis- Absolute quantification



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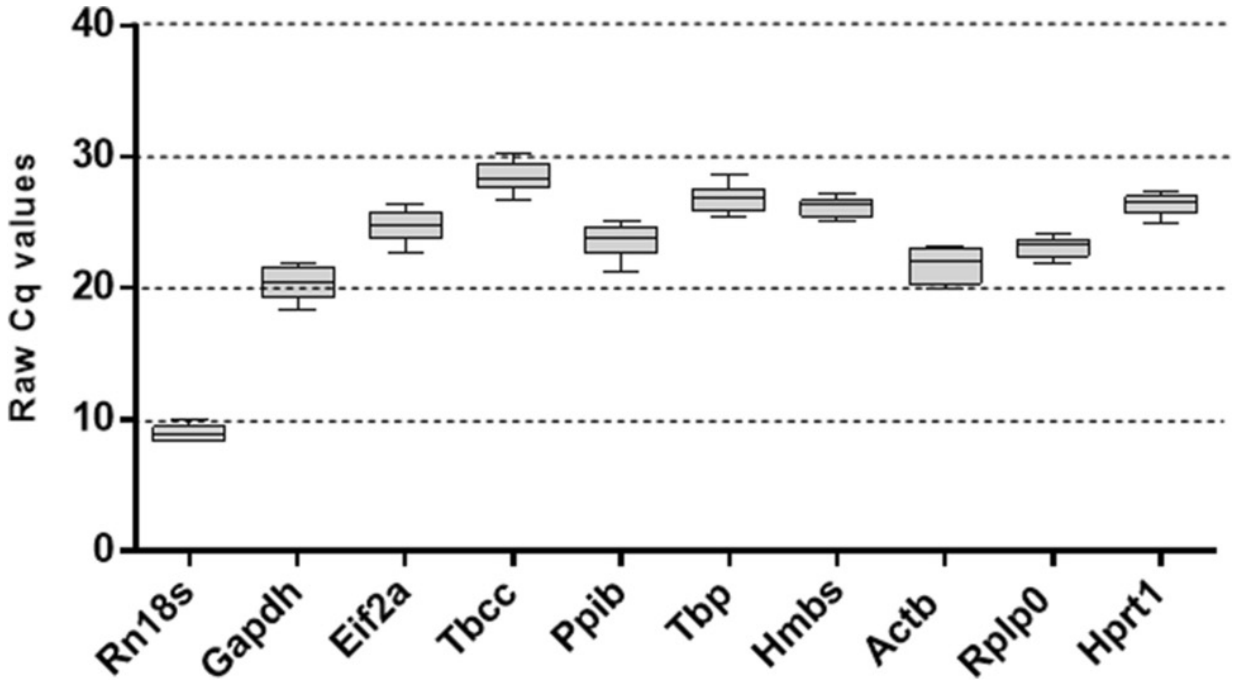
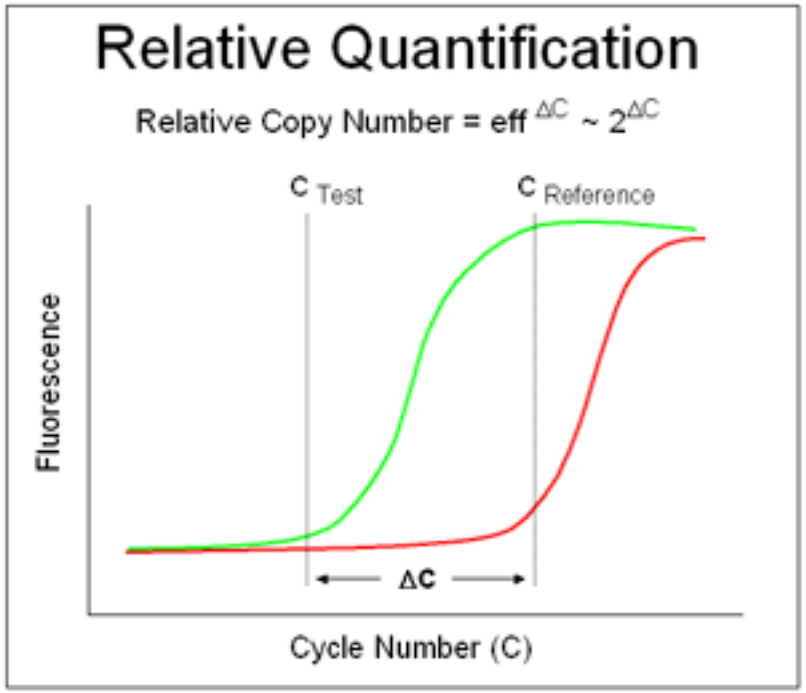
Analysis- Relative quantification

Housekeeping/Reference genes





Historically: genes that are consistently expressed across tissues, essential, carrying out cellular maintenance, and conserved across species

Today: more work, cell type/ tissue specific testing needed, multiple housekeeping genes recommended

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

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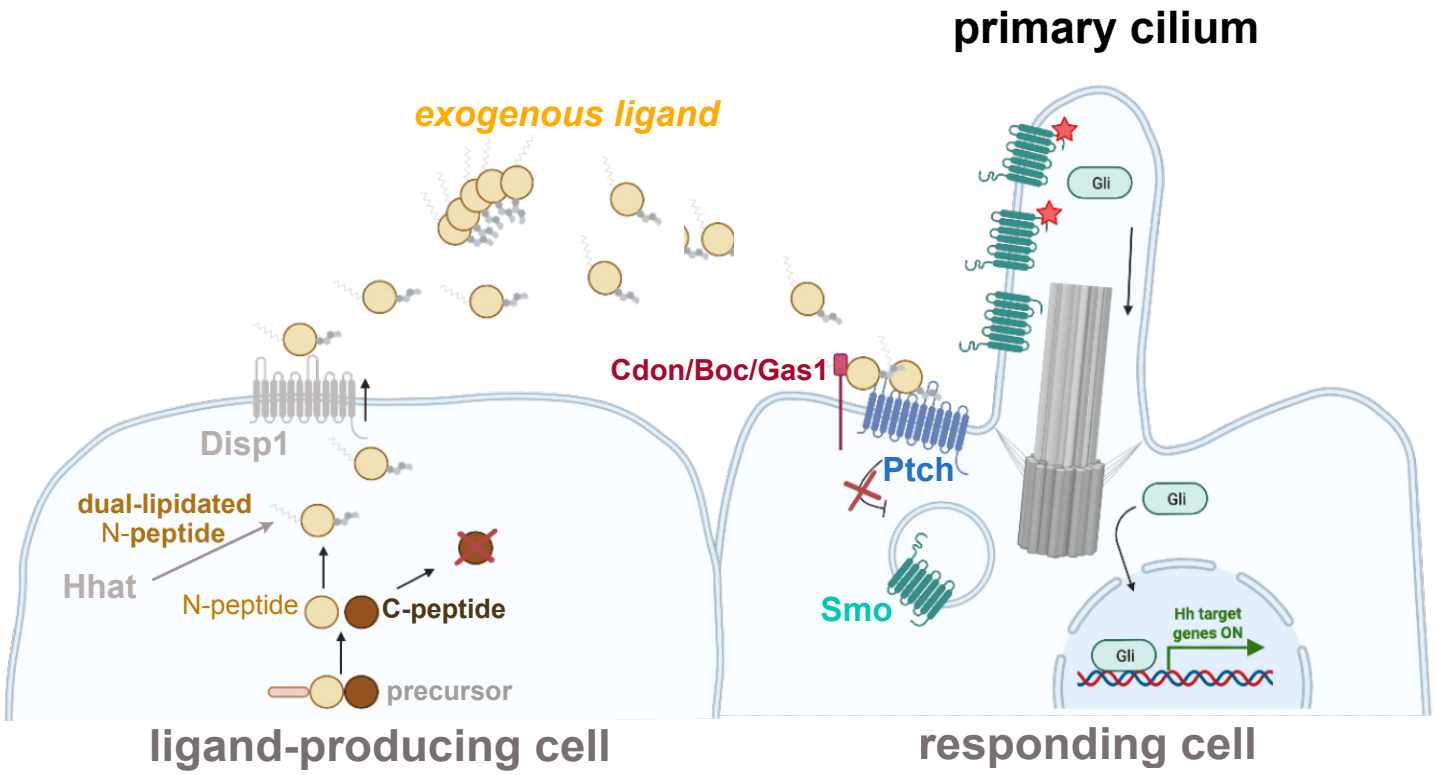
Biological Insights- Hh signalling pathway



Ancient pathway important in:

Embryonic development

Adult tissue maintenance

Tumorigenesis

Immune cell function!

…… but no good antibodies available!

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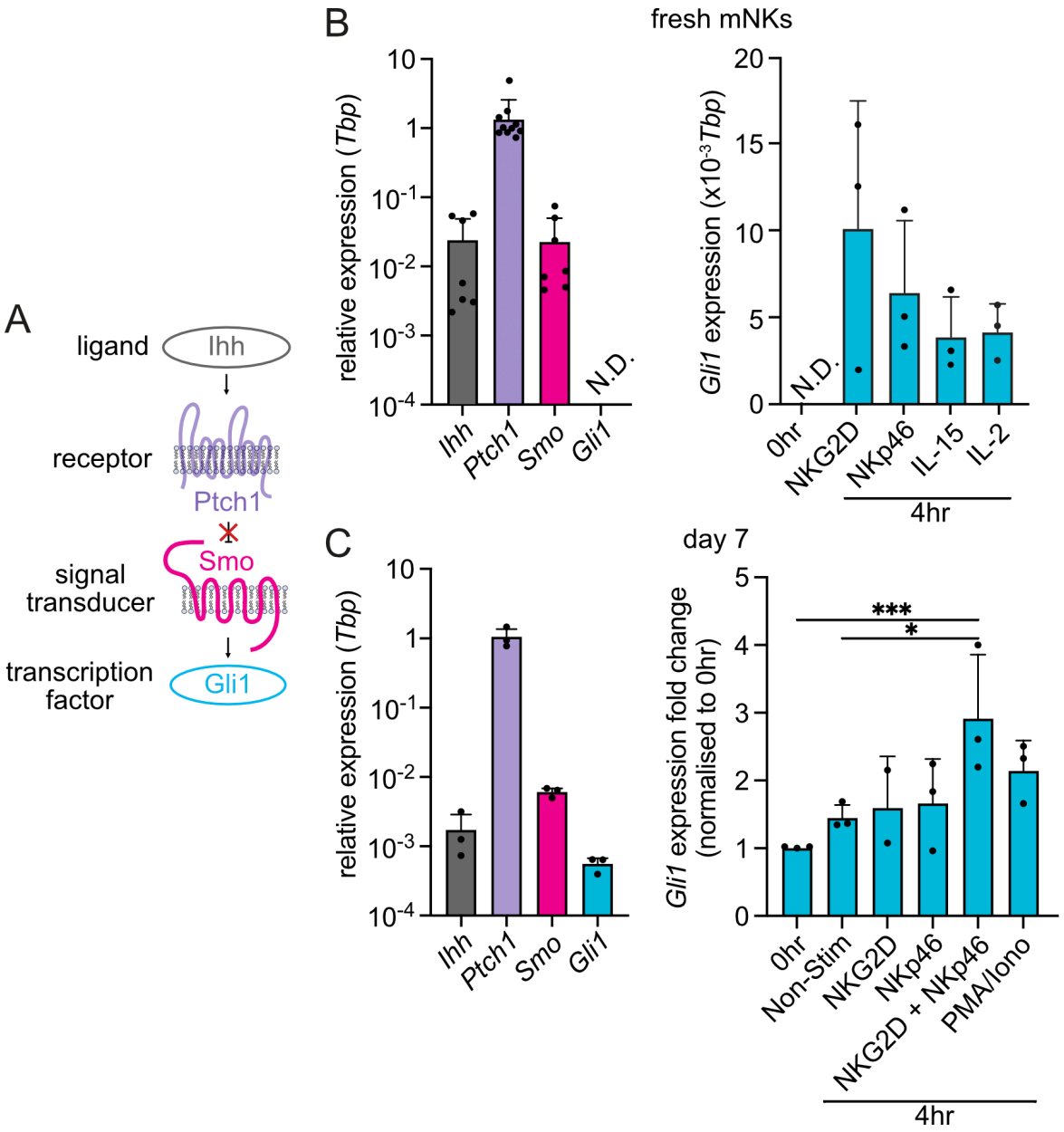

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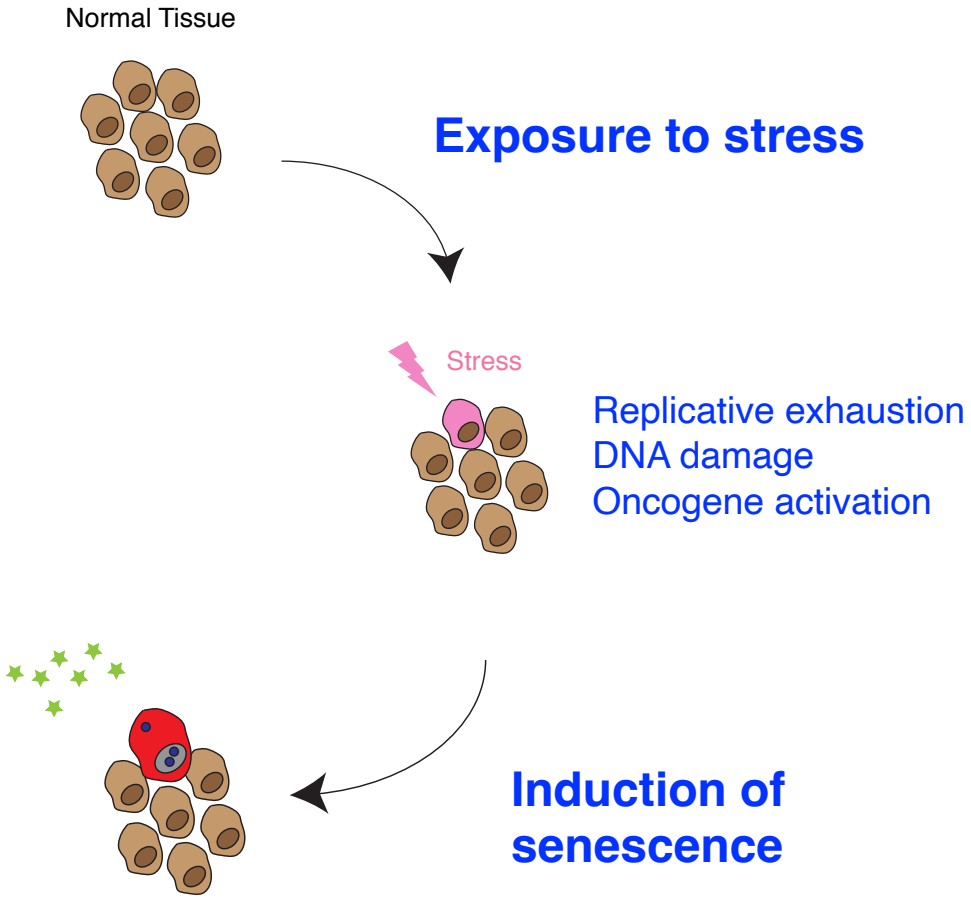
Cellular Senescence: A Chronic Stress Response

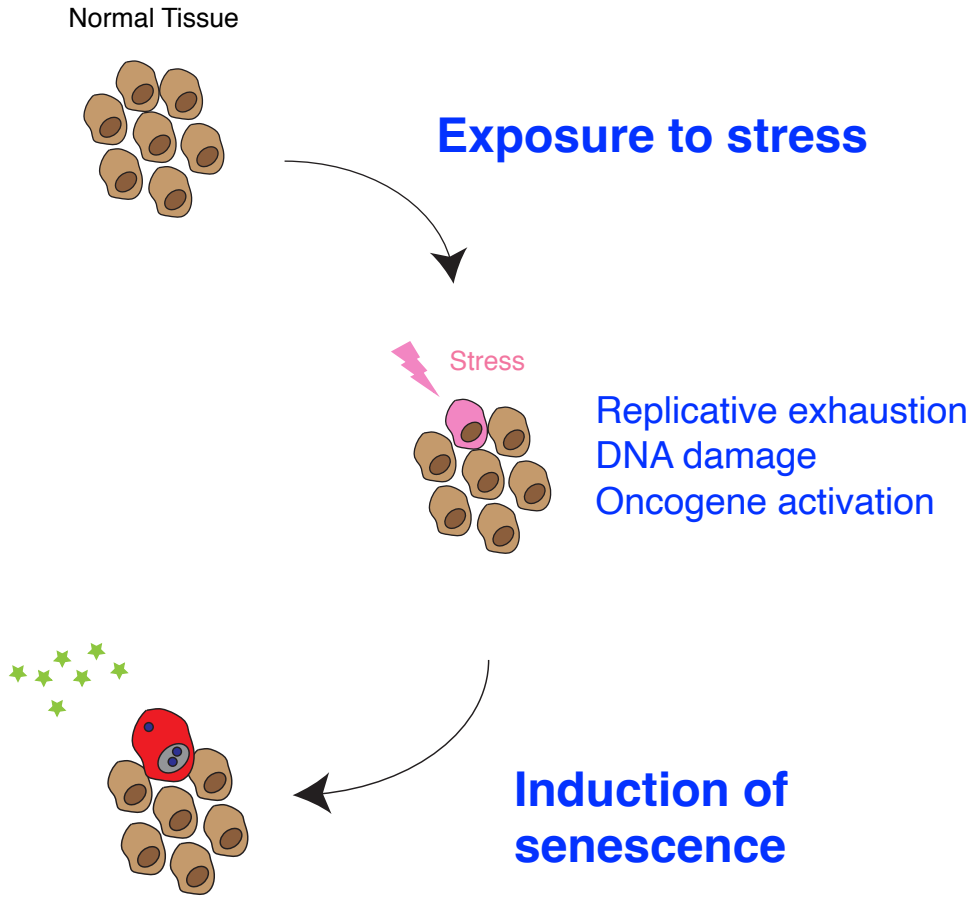


Cell cycle arrest

Chromatin re-organization
Gene expression changes
Senescence-associated secretory phenotype (SASP)

Chan & Narita (2019) Genes Dev.

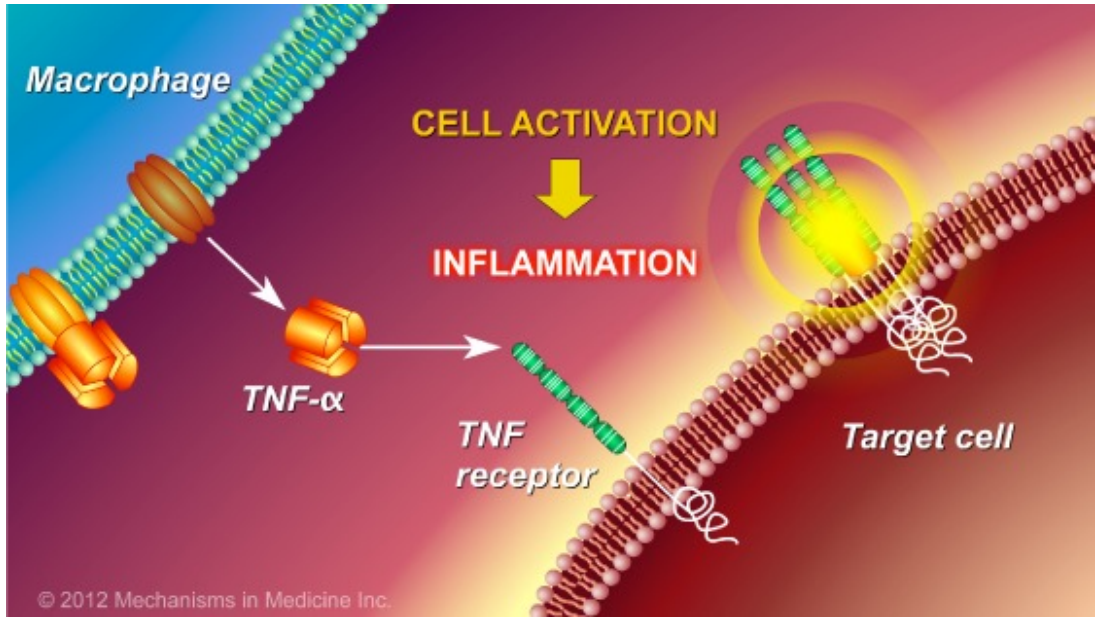
Cellular Senescence: A Chronic Stress Response



Cell cycle arrest

Chromatin re-organization
Gene expression changes
Senescence-associated secretory phenotype (SASP)

SASP components resemble that of acute inflammation



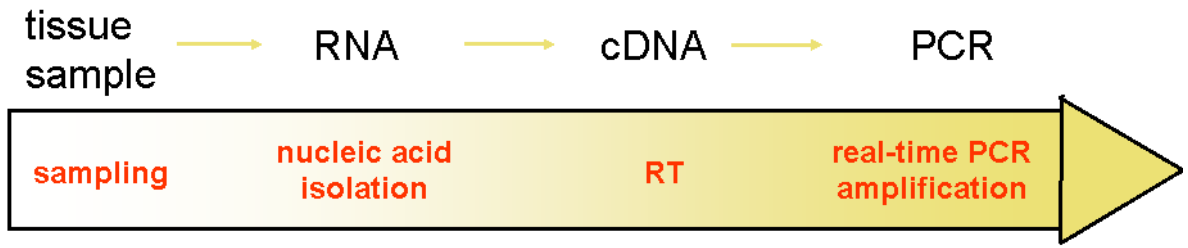
Interleukins e.g. IL1A, IL1B, IL6, IL8
Cytokines and chemokines

In the practical, we will be comparing the degree to which IL1B is upregulated in acute inflammation vs. senescence.

Chan & Narita (2019) Genes Dev.

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

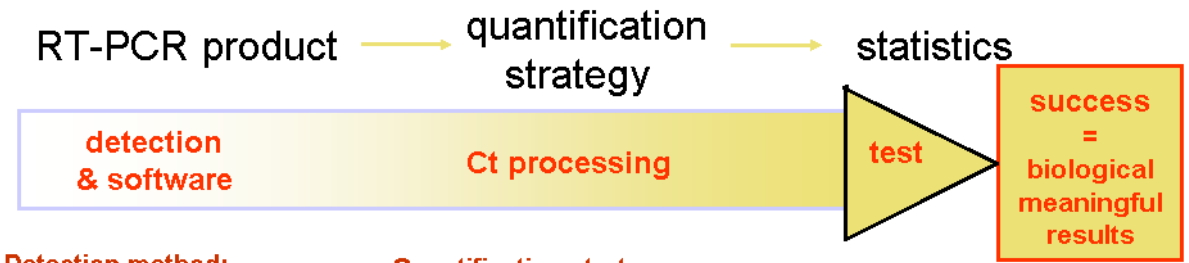
=> native RNA

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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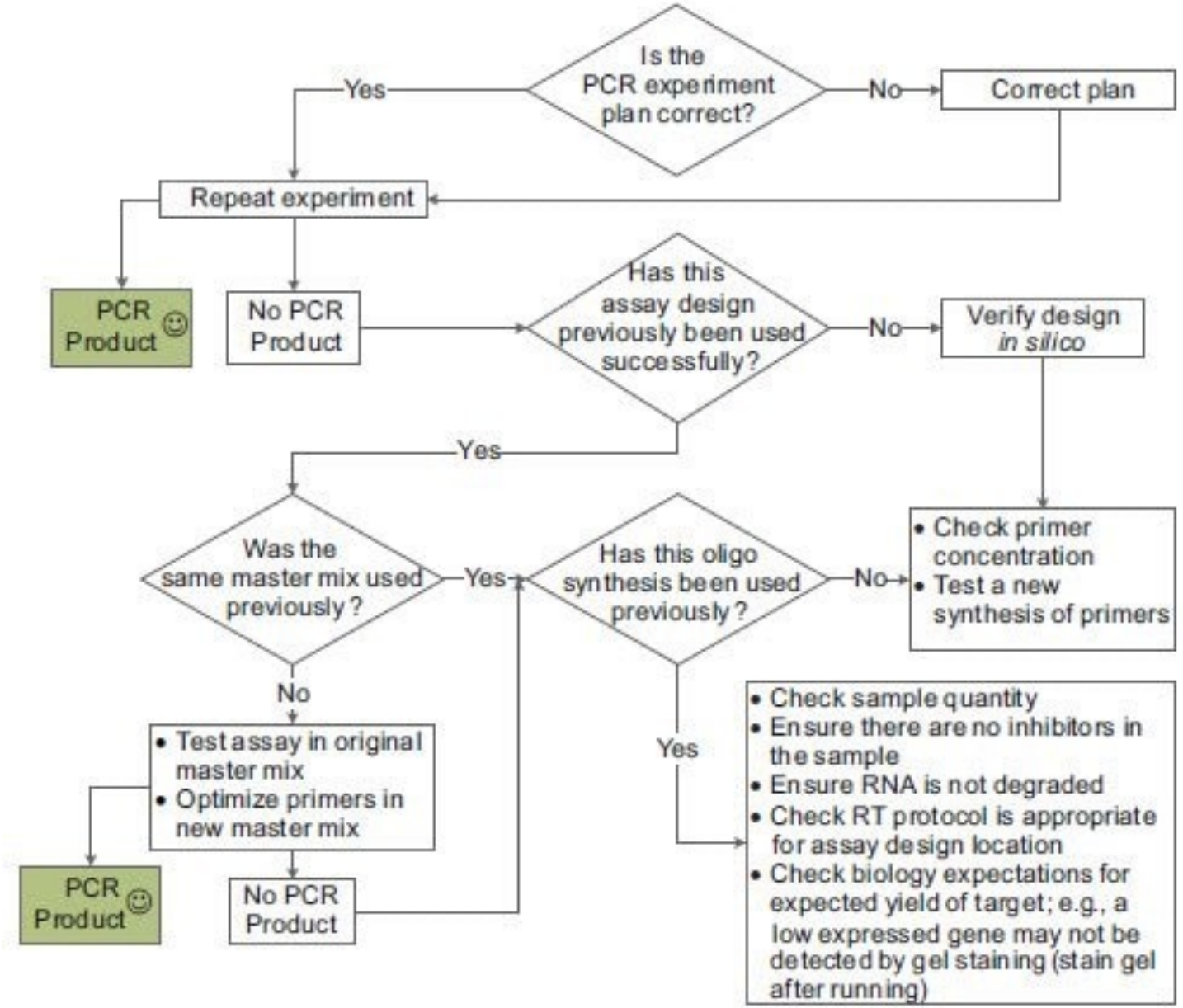
Troubleshooting your qPCR experiment



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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
	A sample knwn to contain the assay			Assay failure. Any positive data from

RT-Specific Controls	Example Material	Expected Result	Possible Reasosn for a Positive Result	Posgble Reasons for a Negative 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	The sample contains gDNA. The reaction became contaminated durirg setup. The primers formed primer dimer products. Analyze in conjuction with NTC	Correct

Contamination Negative assay control (No Template Control NTC)	Water	Negative	The primers are self dimerising resulting in primer dimer product or there was contamination of the control during PCR preparation.	Correct

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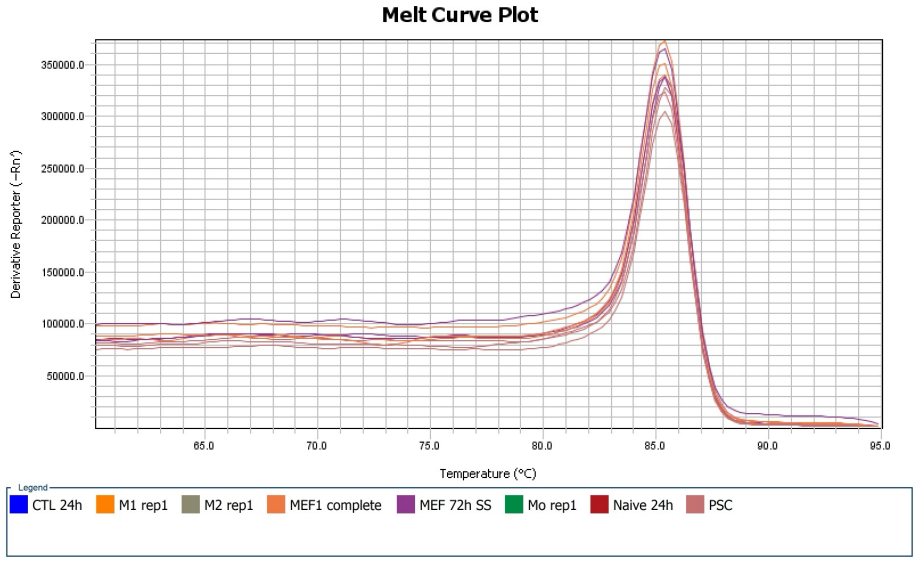
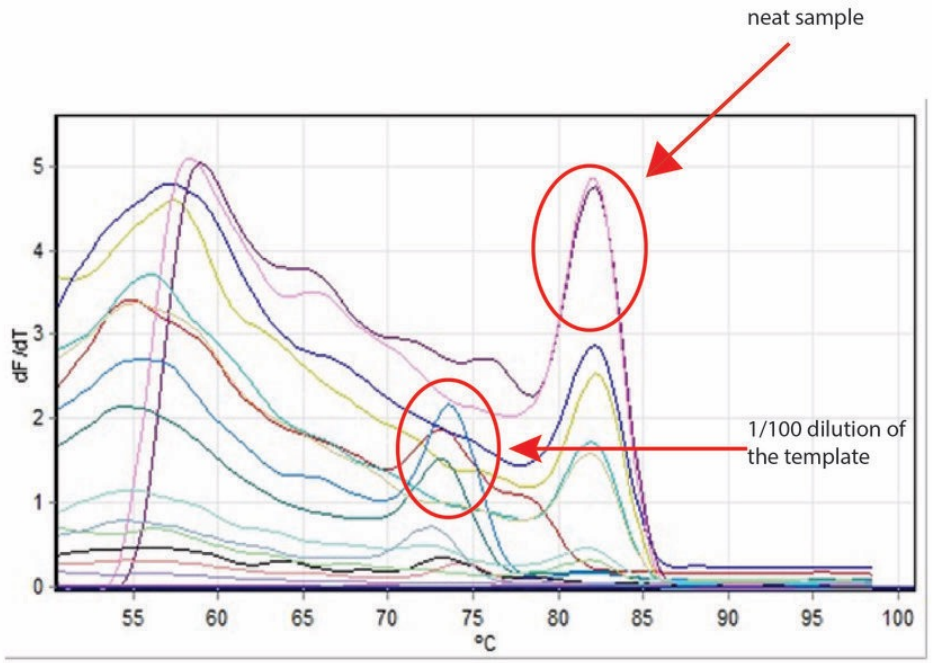
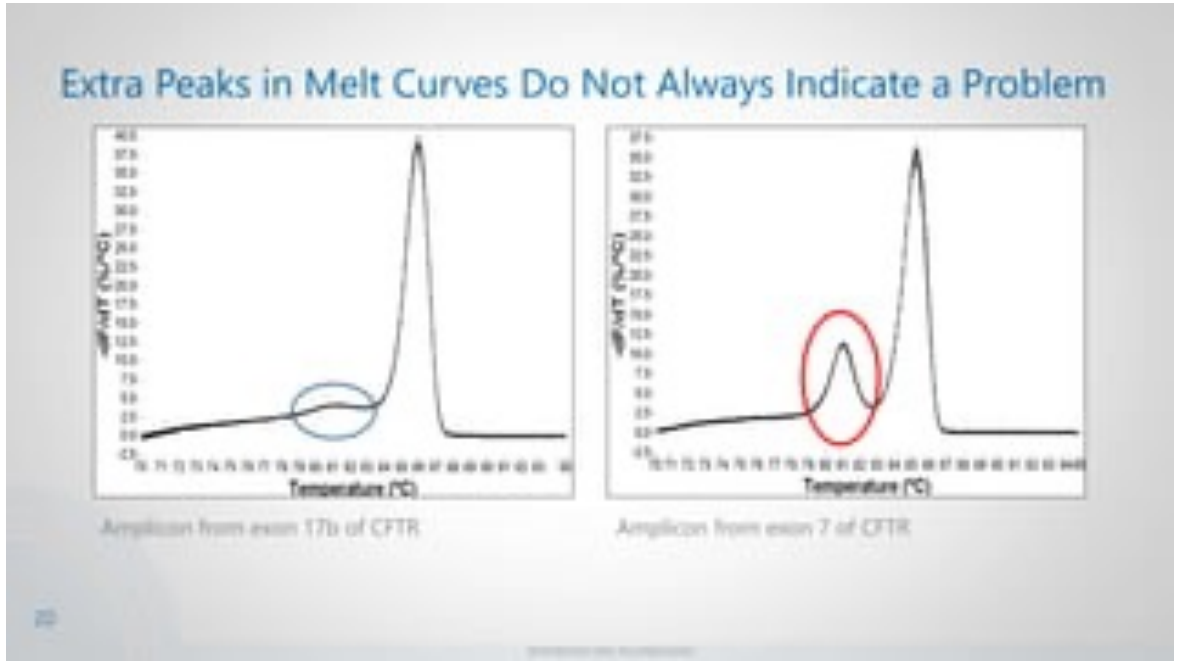
https://www.sigmaaldrich.com/GB/en/technical-documents/technical-article/genomics/pcr/troubleshooting

Analysis of qPCR data- Melt curves







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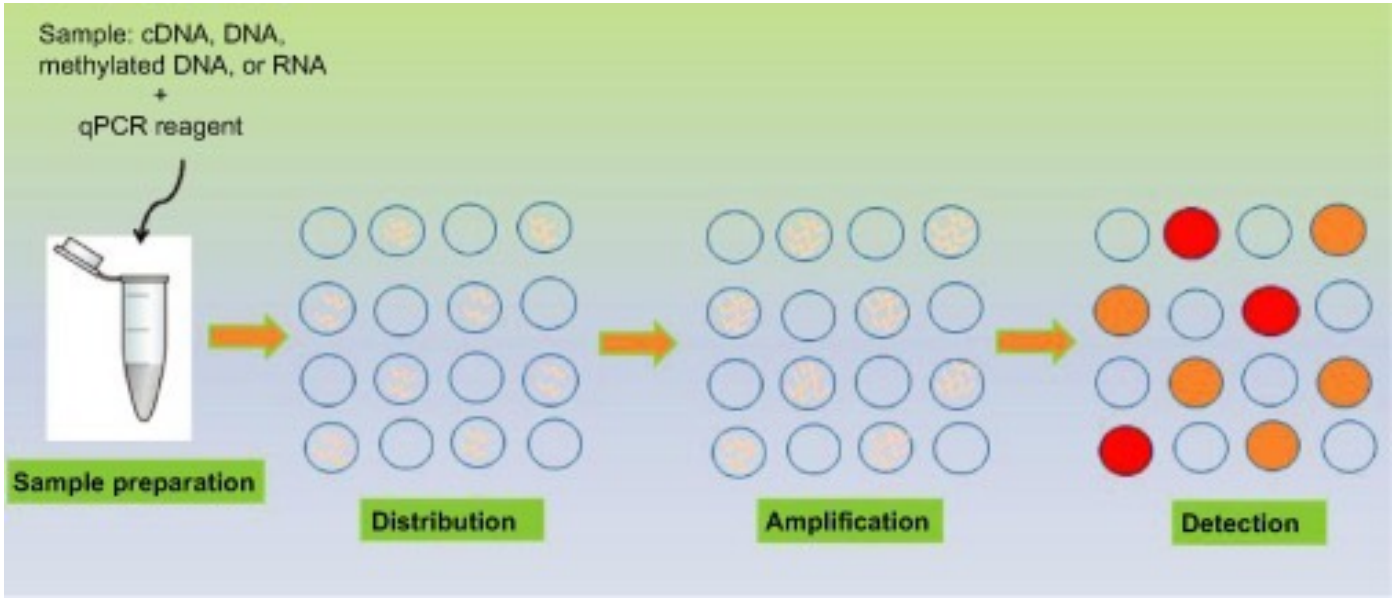
Absolute quantitation using digital PCR

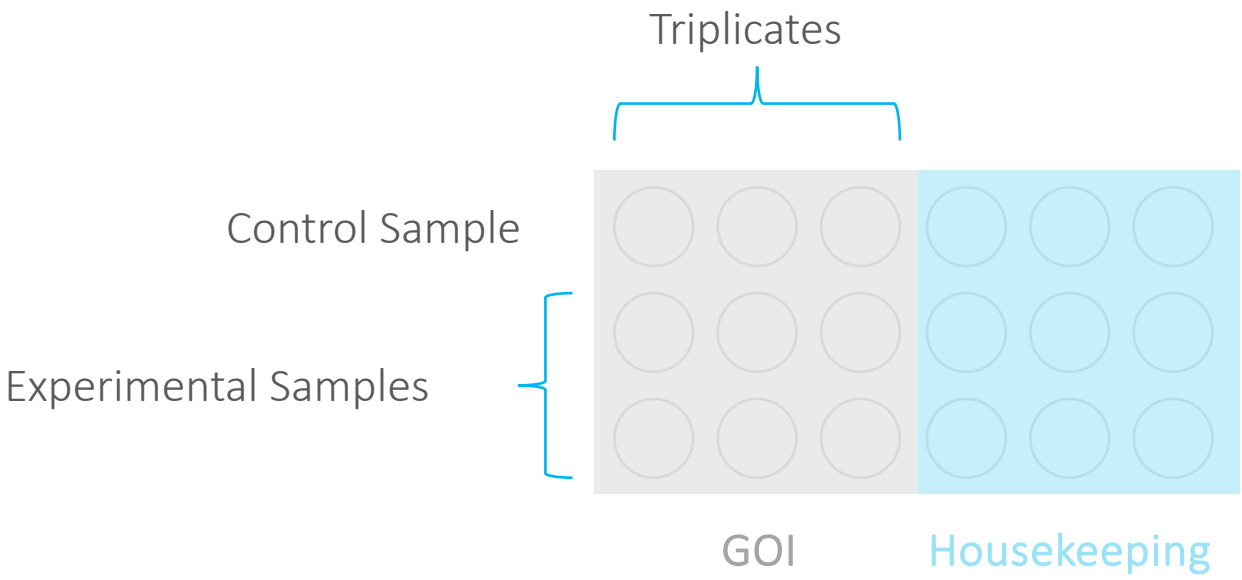


Science Direct, dPCR book chapter

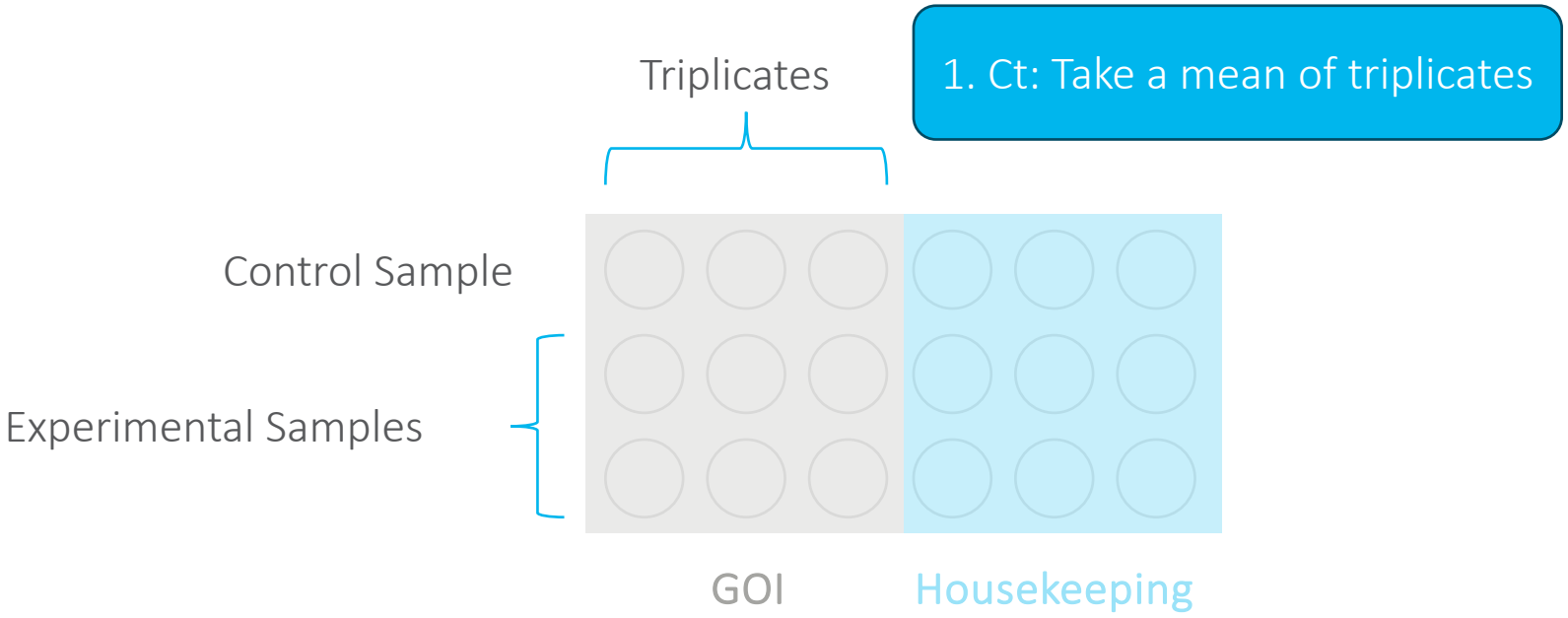
- Partitioning of the PCR reaction into thousands of individual micro-reactions allows absolute quantitation (digital readout)
- Even partitioning of the PCR reaction is achieved by four main ways: droplet-based (oil–water emulsions), microwell-based, channel-based, and printing-based sample dispersion
- Compared to qRT-PCR, digital PCR does not need standard curves and is more sensitive
- Technique is ideally suited for: rare mutation/target detection, pathogen detection (viral load analysis and microbial quantification), detection of viral integration sites (CAR-T cell therapy), microRNA expression, etc

Analysis of qPCR data from the practical workshop

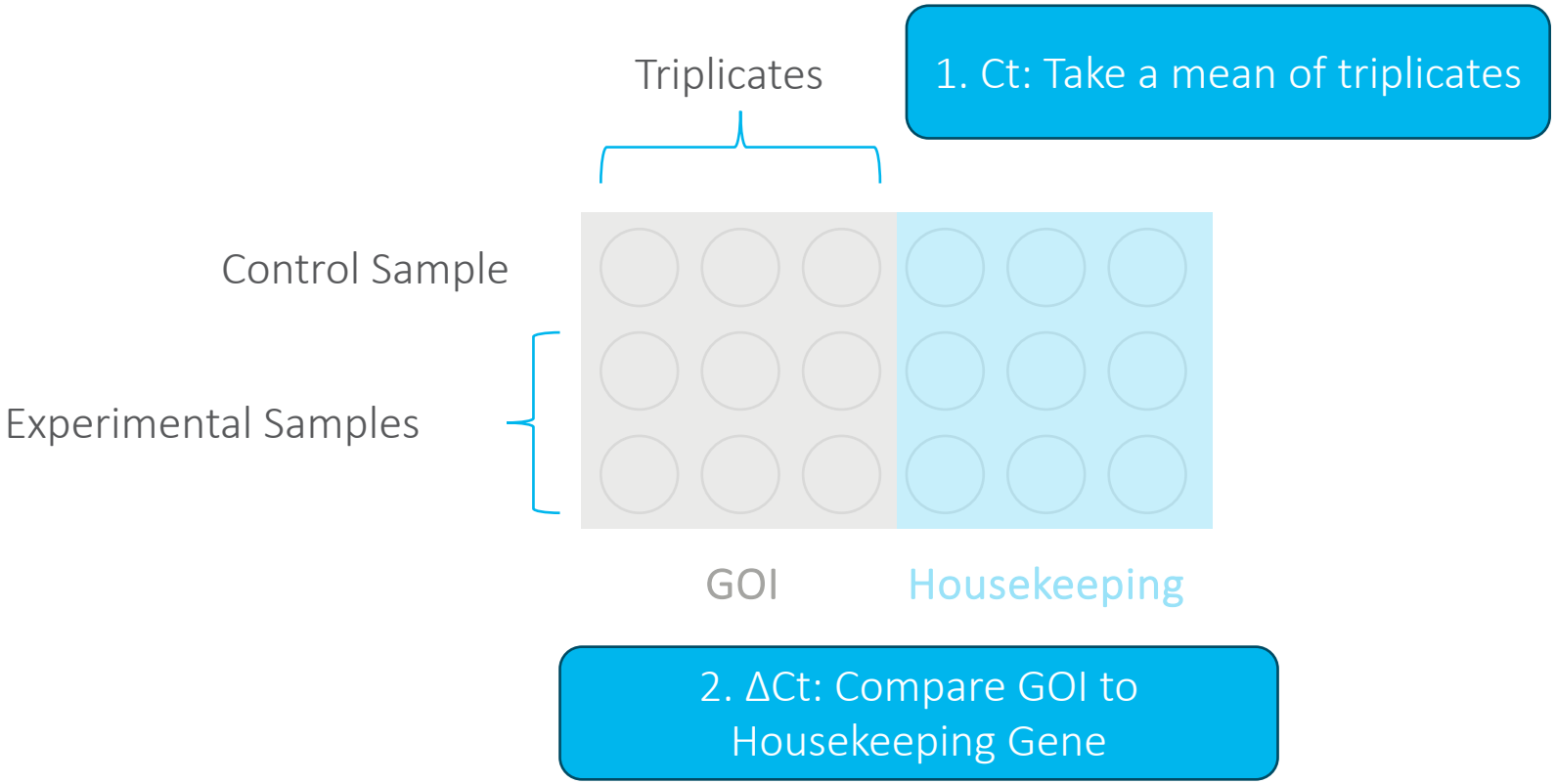
Analysing qPCR Data: The Pipeline



Analysing qPCR Data: The Pipeline

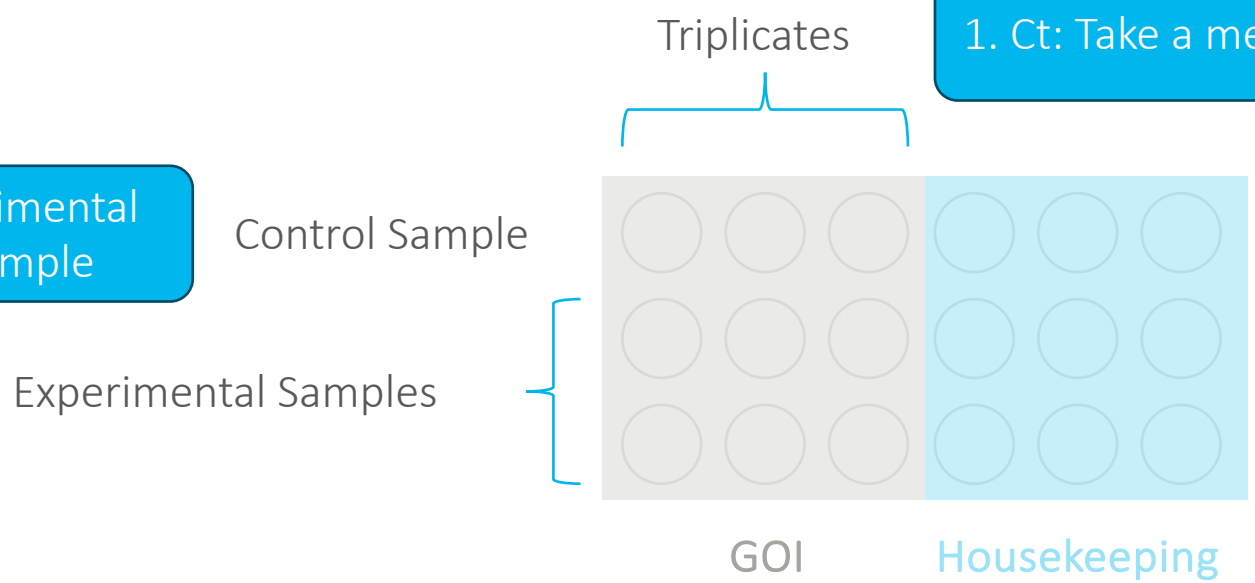


Analysing qPCR Data: The Pipeline



Analysing qPCR Data: The Pipeline

1. Ct: Take a mean of triplicates

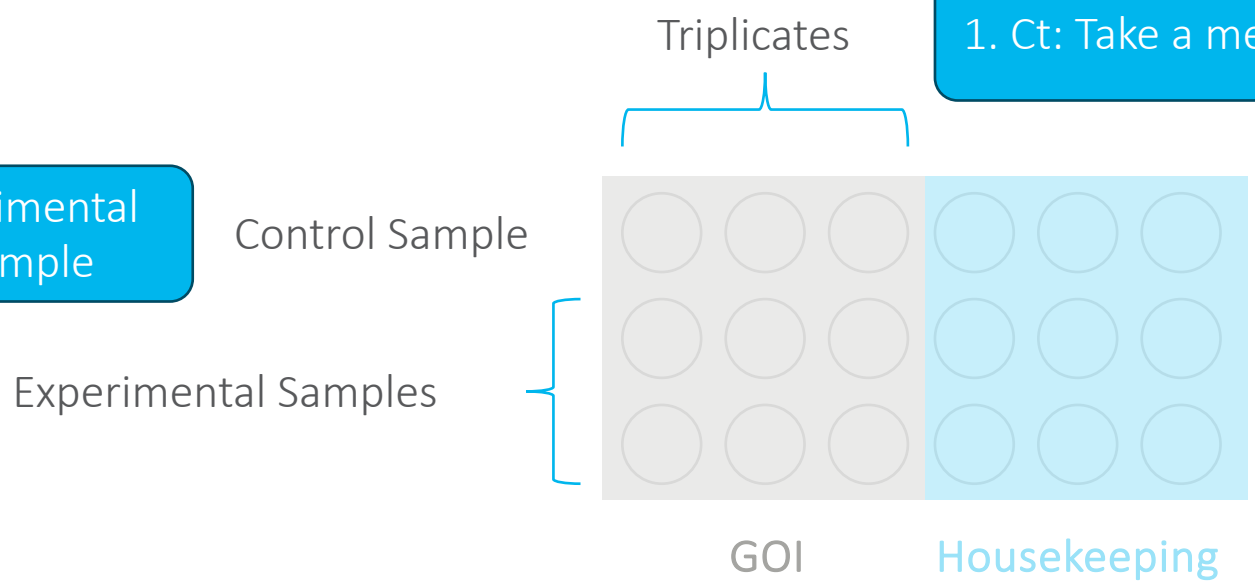


2. ΔCt: Compare GOI to Housekeeping Gene

3. ΔΔCt: Compare Experimental Samples to Control Sample

Analysing qPCR Data: The Pipeline

1. Ct: Take a mean of triplicates



3. ΔΔCt: Compare Experimental Samples to Control Sample

2. ΔCt: Compare GOI to Housekeeping Gene

4. Exp: Converting ΔΔCt to a biologically meaningful value (fold change) with $2^{-\Delta\Delta Ct}$

Analysing qPCR Data: QuantStudio Output

Well	Well Position	Omit	Sample Name	Target Name	Task	Reporter	Quencher	CT	Ct Mean	Ct SD
25	B1	FALSE	Ctrl	IL1B	UNKNOWN	SYBR	None	24.639	24.467	0.173
26	B2	FALSE	Ctrl	IL1B	UNKNOWN	SYBR	None	24.468	24.467	0.173
27	B3	FALSE	Ctrl	IL1B	UNKNOWN	SYBR	None	24.293	24.467	0.173
28	B4	FALSE	TNF	IL1B	UNKNOWN	SYBR	None	19.219	19.210	0.031
29	B5	FALSE	TNF	IL1B	UNKNOWN	SYBR	None	19.175	19.210	0.031
30	B6	FALSE	TNF	IL1B	UNKNOWN	SYBR	None	19.236	19.210	0.031
31	B7	FALSE	Senescence	IL1B	UNKNOWN	SYBR	None	13.020	13.053	0.189
32	B8	FALSE	Senescence	IL1B	UNKNOWN	SYBR	None	13.256	13.053	0.189
33	B9	FALSE	Senescence	IL1B	UNKNOWN	SYBR	None	12.883	13.053	0.189
169	H1	FALSE	Ctrl	bactin	UNKNOWN	SYBR	None	13.572	13.472	0.609
170	H2	FALSE	Ctrl	bactin	UNKNOWN	SYBR	None	14.024	13.472	0.609
171	H3	FALSE	Ctrl	bactin	UNKNOWN	SYBR	None	12.818	13.472	0.609
172	H4	FALSE	TNF	bactin	UNKNOWN	SYBR	None	13.320	13.406	0.121
173	H5	FALSE	TNF	bactin	UNKNOWN	SYBR	None	13.544	13.406	0.121
174	H6	FALSE	TNF	bactin	UNKNOWN	SYBR	None	13.354	13.406	0.121
175	H7	FALSE	Senescence	bactin	UNKNOWN	SYBR	None	13.685	13.737	0.116
176	H8	FALSE	Senescence	bactin	UNKNOWN	SYBR	None	13.869	13.737	0.116
177	H9	FALSE	Senescence	bactin	UNKNOWN	SYBR	None	13.656	13.737	0.116

Analysing qPCR Data: Example Workflow

1. Mean Ct

	IL1B	bactin
Ctrl	24.4669018	13.4715233
TNF	19.2100188	13.4059887
Senescence	13.0531899	13.7366695

2. ΔCt

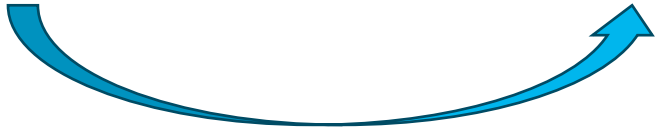
	IL1B	bactin
Ctrl	10.9953785	0
TNF	5.8040301	0
Senescence	-0.6834796	0

3. ΔΔCt

	IL1B	bactin
Ctrl	0	0
TNF	-5.1913484	0
Senescence	-11.678858	0

4. Exp

	IL1B	bactin
Ctrl	1	1
TNF	36.5385732	1
Senescence	3278.58622	1



Value for each gene – Value for housekeeping gene from the same row (bactin in this case)

- Control: 24.467 – 13.472 = 10.995
- TNF: 19.210 – 13.406 = 5.804
- Senescence: 13.053 – 13.737 – -0.683

Note that bactin values are subtracted from itself and are now 0!

Analysing qPCR Data: Example Workflow

1. Mean Ct

	IL1B	bactin
Ctrl	24.4669018	13.4715233
TNF	19.2100188	13.4059887
Senescence	13.0531899	13.7366695

2. ΔCt

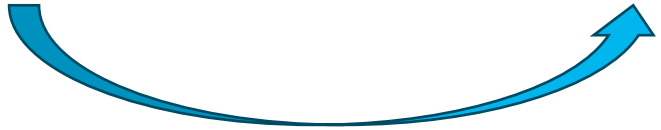
	IL1B	bactin
Ctrl	10.9953785	0
TNF	5.8040301	0
Senescence	-0.6834796	0

3. ΔΔCt

	IL1B	bactin
Ctrl	0	0
TNF	-5.1913484	0
Senescence	-11.678858	0

4. Exp

	IL1B	bactin
Ctrl	1	1
TNF	36.5385732	1
Senescence	3278.58622	1



Value for sample – Value for control sample from the same column (Ctrl in this case)

- TNF: 5.804 – 10.995 = -5.191
- Senescence: -0.683 – 10.995 = -11.679

Note that Ctrl values are subtracted from itself and are now 0!

Analysing qPCR Data: Example Workflow

1. Mean Ct

	IL1B	bactin
Ctrl	24.4669018	13.4715233
TNF	19.2100188	13.4059887
Senescence	13.0531899	13.7366695

2. ΔCt

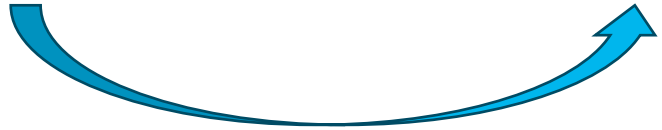
	IL1B	bactin
Ctrl	10.9953785	0
TNF	5.8040301	0
Senescence	-0.6834796	0

3. ΔΔCt

	IL1B	bactin
Ctrl	0	0
TNF	-5.1913484	0
Senescence	-11.678858	0

4. Exp

	IL1B	bactin
Ctrl	1	1
TNF	36.5385732	1
Senescence	3278.58622	1



$2^{-\Delta\Delta Ct}$ for all values

Analysing qPCR Data: Example Workflow

1. Mean Ct

	IL1B	bactin
Ctrl	24.4669018	13.4715233
TNF	19.2100188	13.4059887
Senescence	13.0531899	13.7366695

2. ΔCt

	IL1B	bactin
Ctrl	10.9953785	0
TNF	5.8040301	0
Senescence	-0.6834796	0

3. ΔΔCt

	IL1B	bactin
Ctrl	0	0
TNF	-5.1913484	0
Senescence	-11.678858	0

4. Exp

	IL1B	bactin
Ctrl	1	1
TNF	36.5385732	1
Senescence	3278.58622	1

Biological interpretation

Compared to control, and normalized to b-actin expression:

- IL1B expression is 36.5 fold higher in “TNF inflammation” condition and 3278.5 fold in the “senescence” condition