Principles of Molecular tests

DNA extraction and PCR

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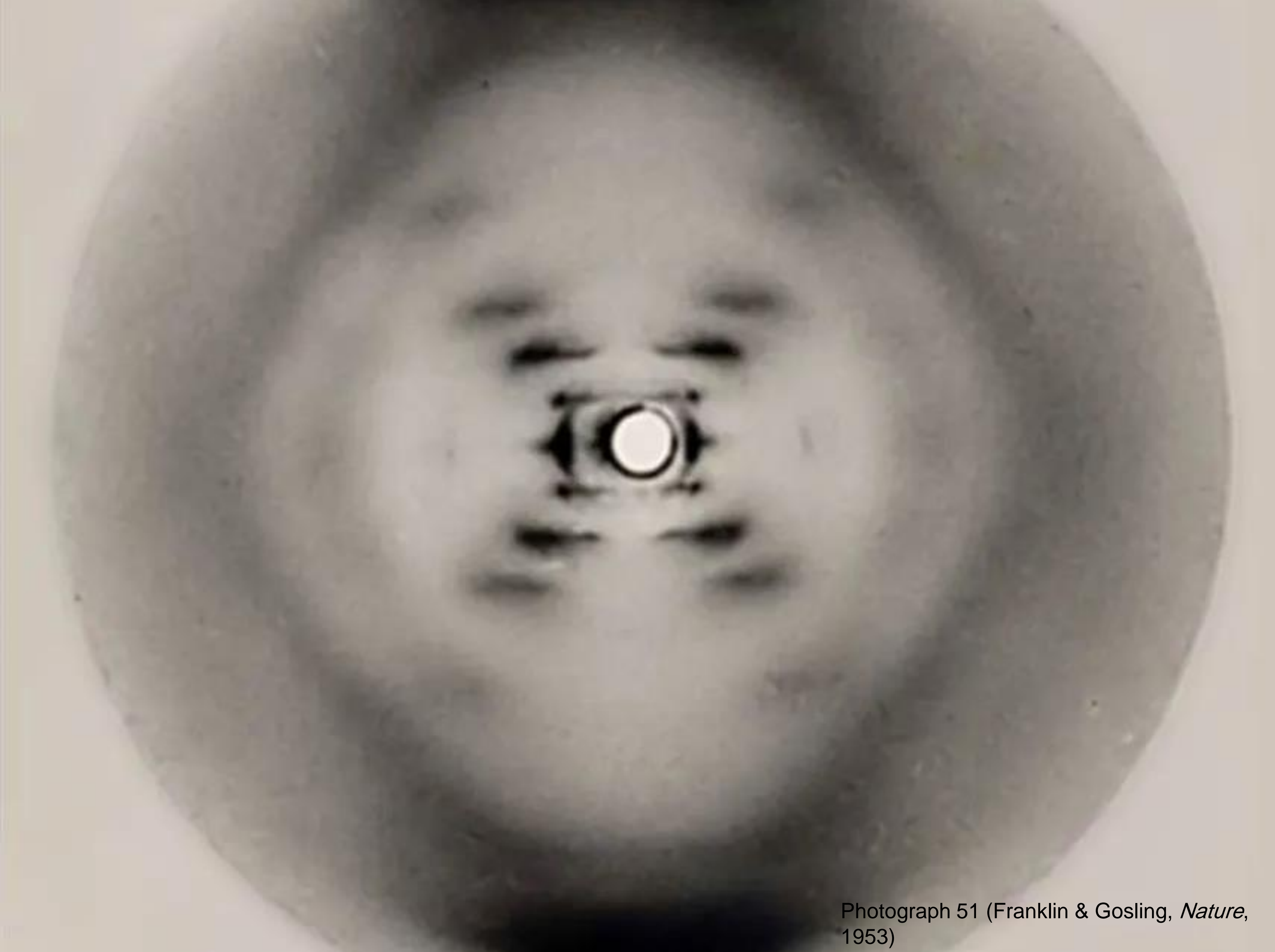
- Sadeghnejad.abdolvahid@gmail.com


Photograph 51 (Franklin & Gosling, *Nature*, 1953)

Student learning goals

1. Students will understand how scientists ask questions.

2. Students will be able to select and/or design quality reagents for experiments.

3. Students will understand the importance of each step of a reaction or experiment.

4. Students will be able to make conclusions about data and connect technical results with biological and societal relevance.

5. Students will be able to use bioinformatics tools to gather information to aid in experimental design.

6. Students will be able to present scientific information in formal and semi-formal environments to their professors and peers.

1. Students will be able to draw each step of PCR accurately and label the temperature of each step, the directionality of the primers, the proper intermediate products, and the final products.

2. Students will be able to make troubleshooting inferences through reflection on the concepts of PCR and gel electrophoresis to determine possible problem areas.

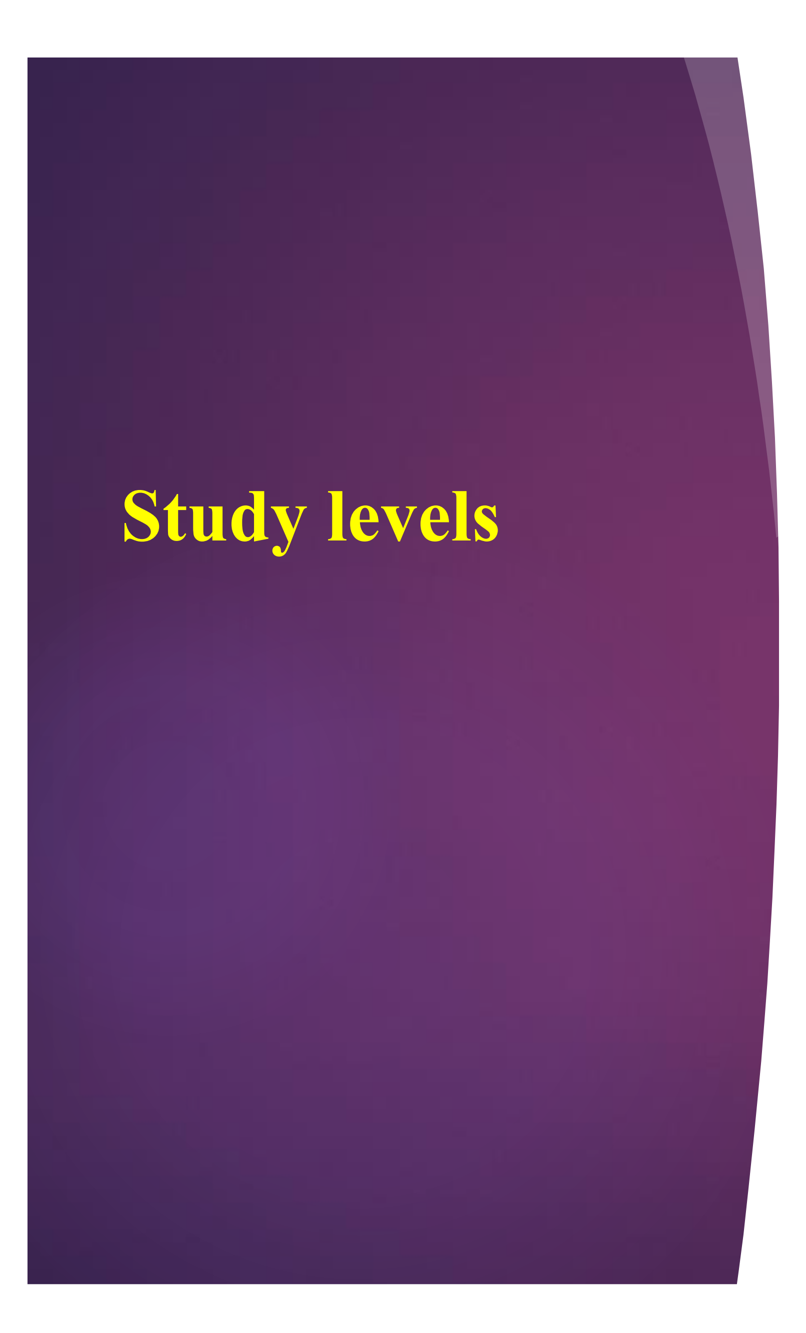
3. Students will be able to explain how DNA molecules move through an agarose gel matrix, that the molecules are separated by size and weight, and why DNA moves toward the positive pole.

4. Students will be able to determine the size of a band in an agarose gel by using a DNA ladder.

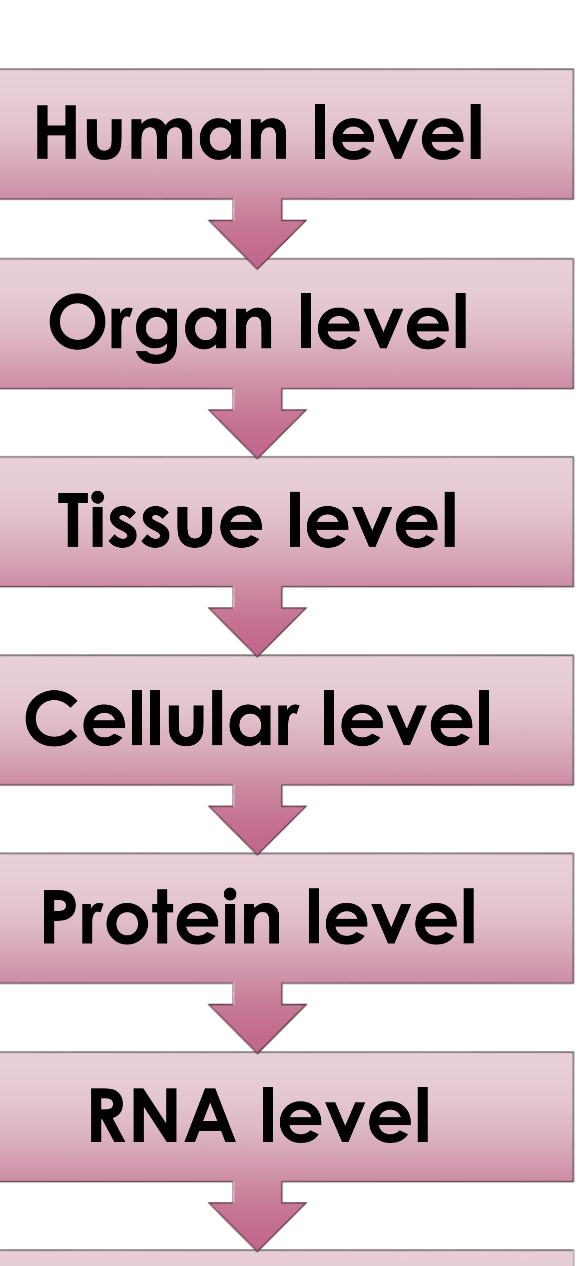
5. Students will be able to interpret positive and negative controls correctly.

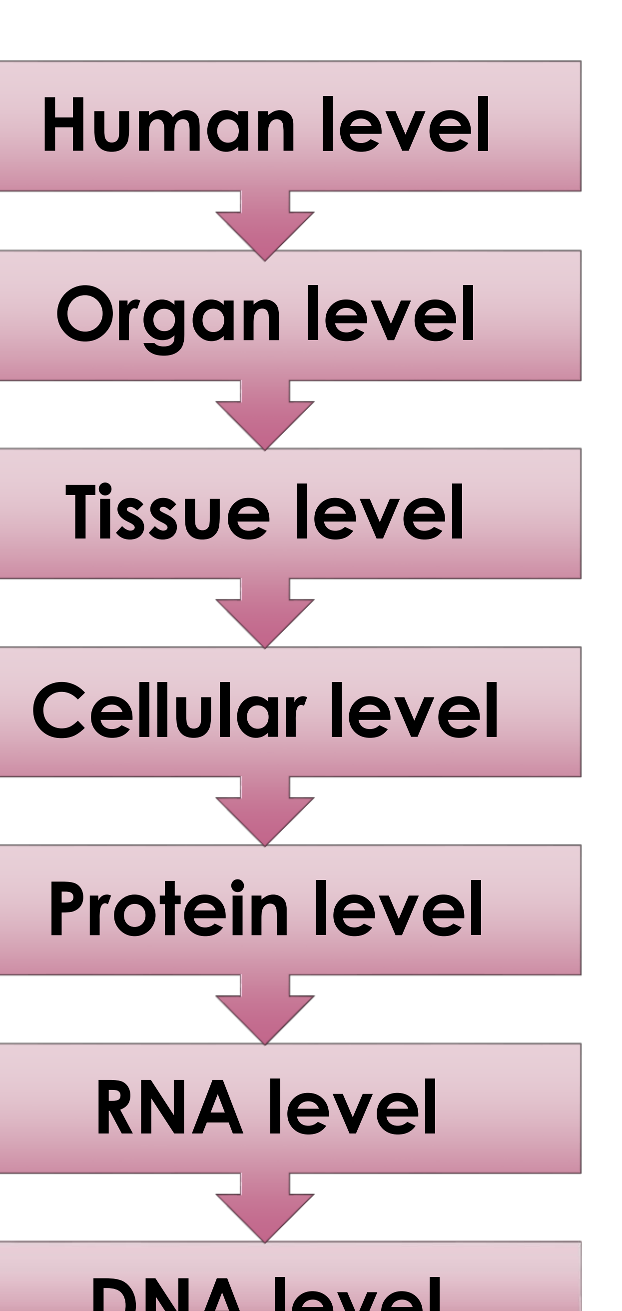
6. Students will be able to design quality PCR primers using bioinformatics databases.

7. Students will be able to analyze data and use the results to support a position for the presence or absence of gmos in food products for a case-based presentation.



Human level
↓
Organ level
↓
Tissue level
↓
Cellular level
↓
Protein level
↓
RNA level
↓
DNA level


Human level
Organ level
Tissue level
Cellular level
Protein level
RNA level
DNA level


Human level
Organ level
Tissue level
Cellular level
Protein level
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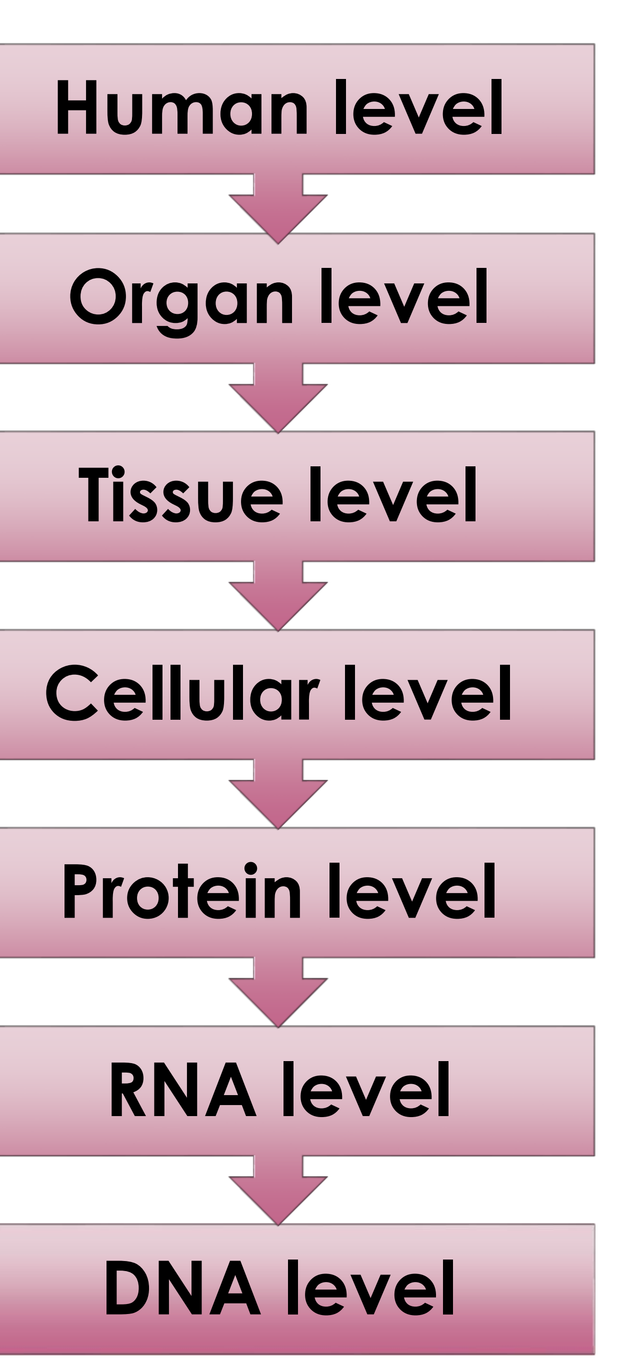
DNA level



Large airway

Alveoli

Human level

Organ level

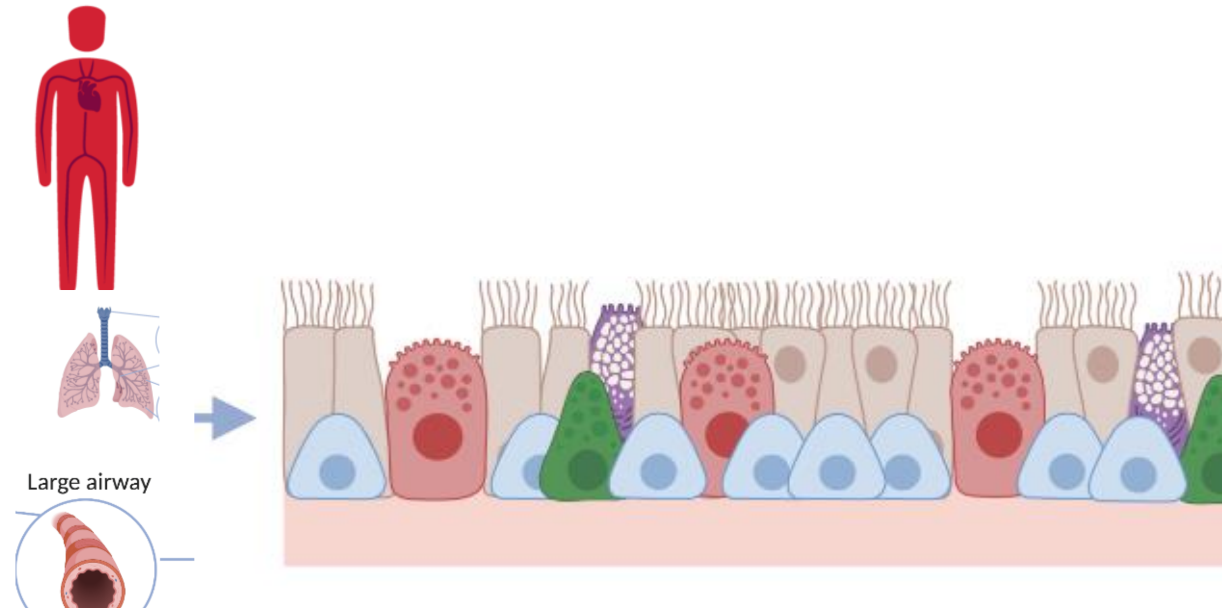
Tissue level

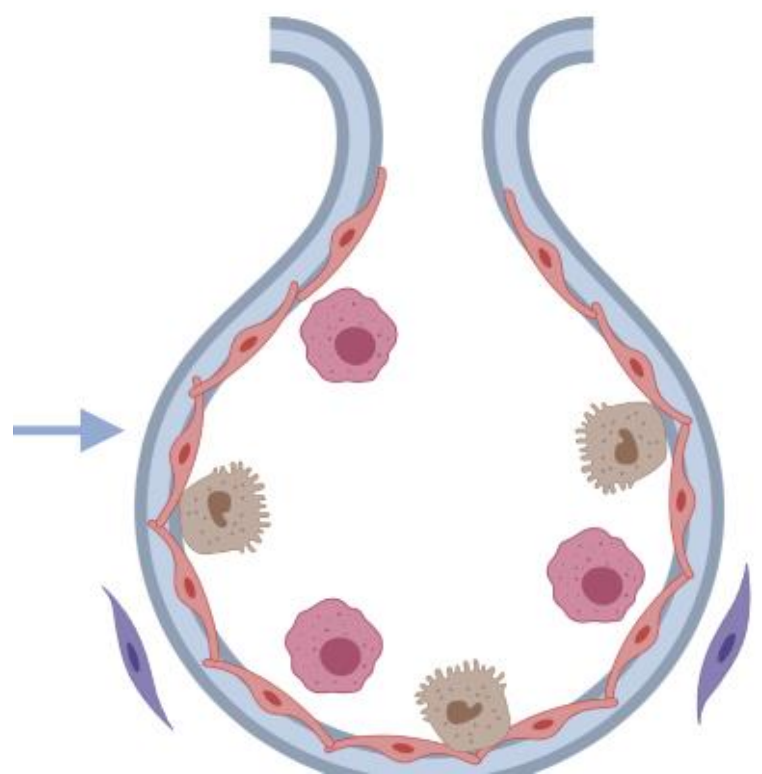
Cellular level

Protein level

RNA level

DNA level





Human level

Organ level

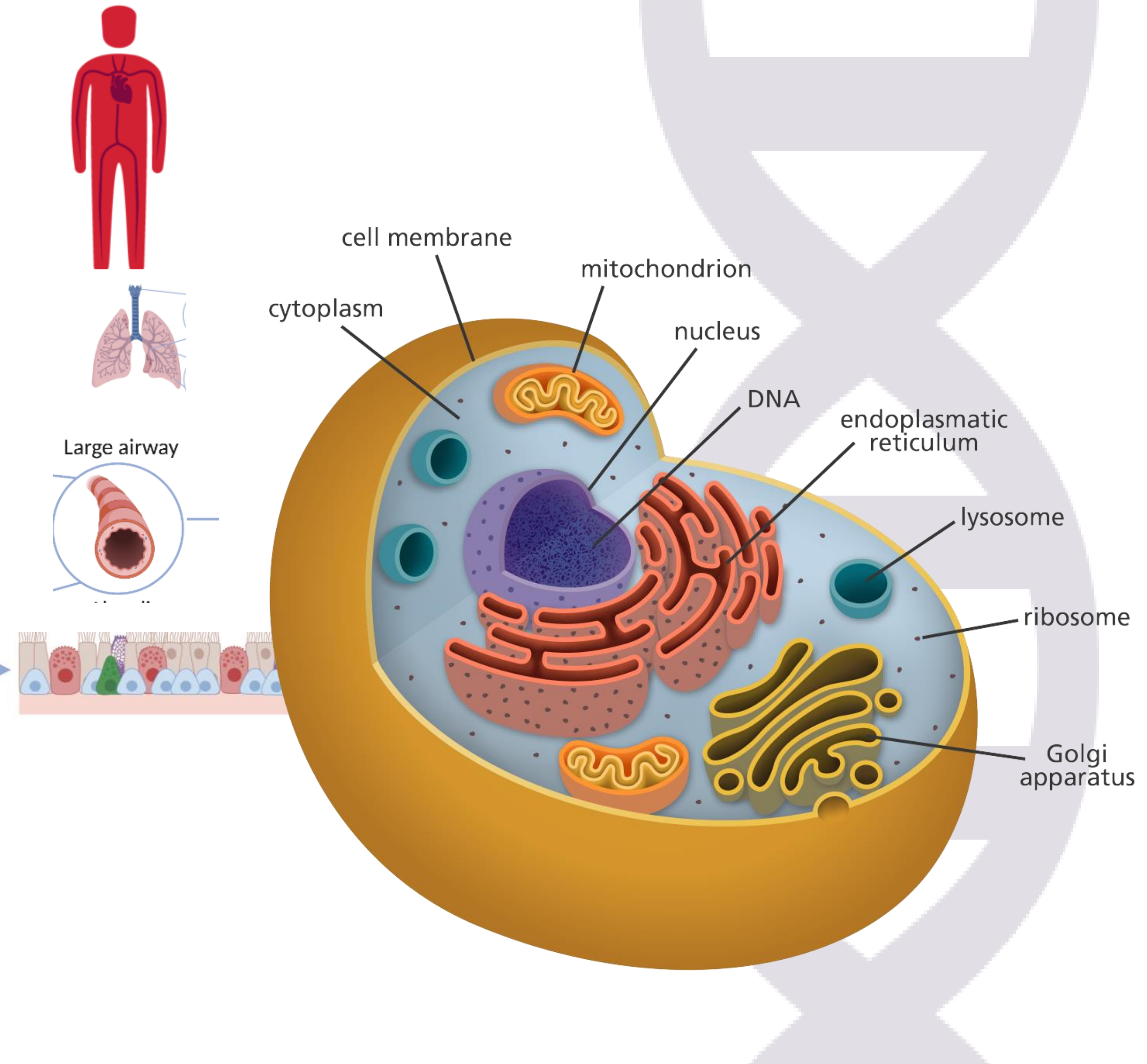
Tissue level

Cellular level

Protein level

RNA level

DNA level



Human level

Organ level

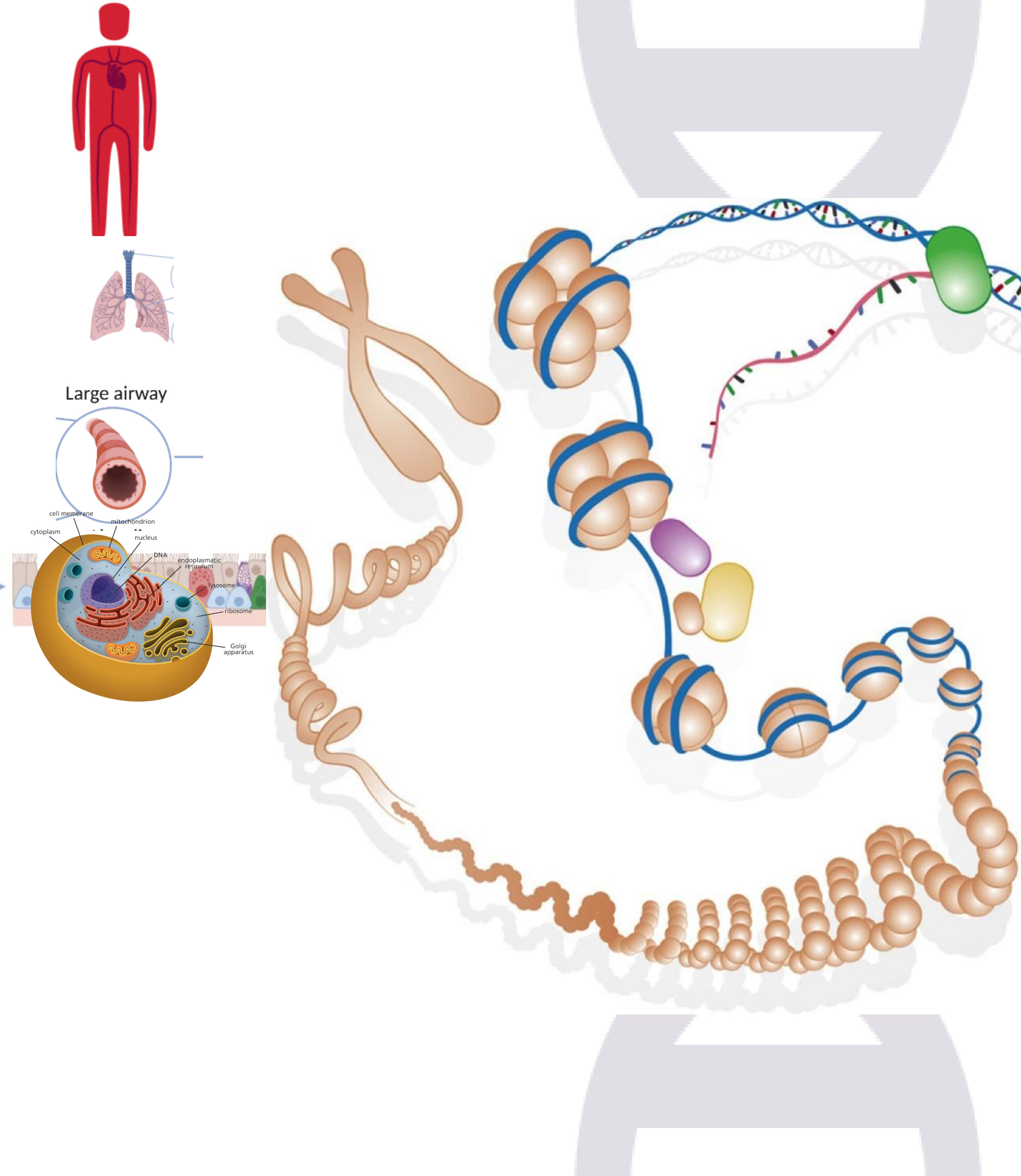
Tissue level

Cellular level

Protein level

RNA level

DNA level



Human level

Organ level

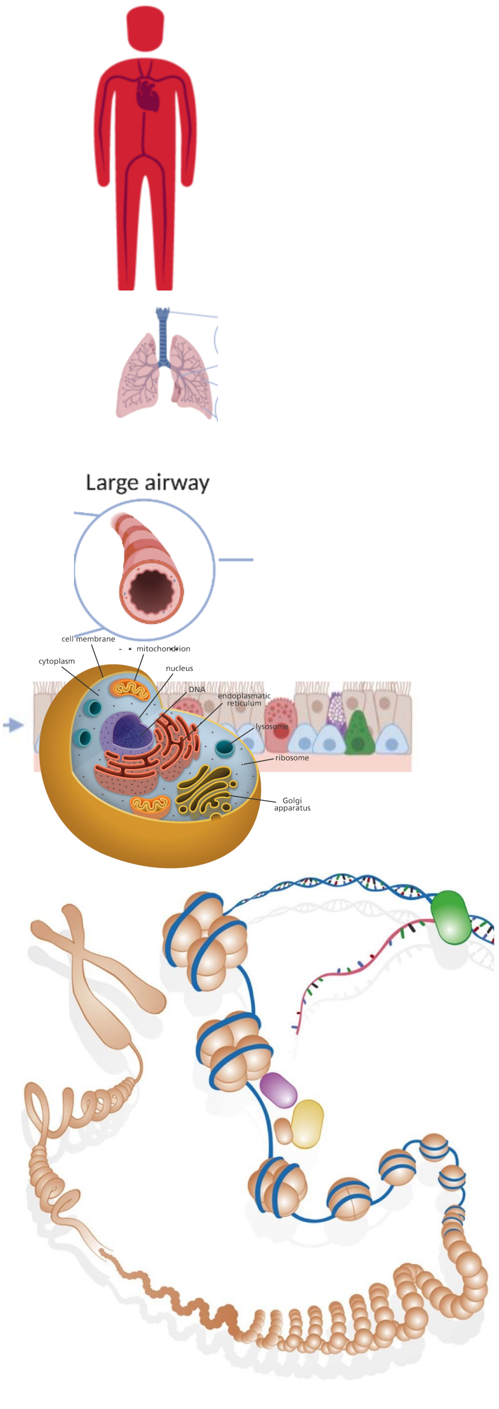
Tissue level

Cellular level

Protein level

RNA level

DNA level



Outline

Basic principles of molecular techniques

Overview PCR and its components

Molecular laboratory setup

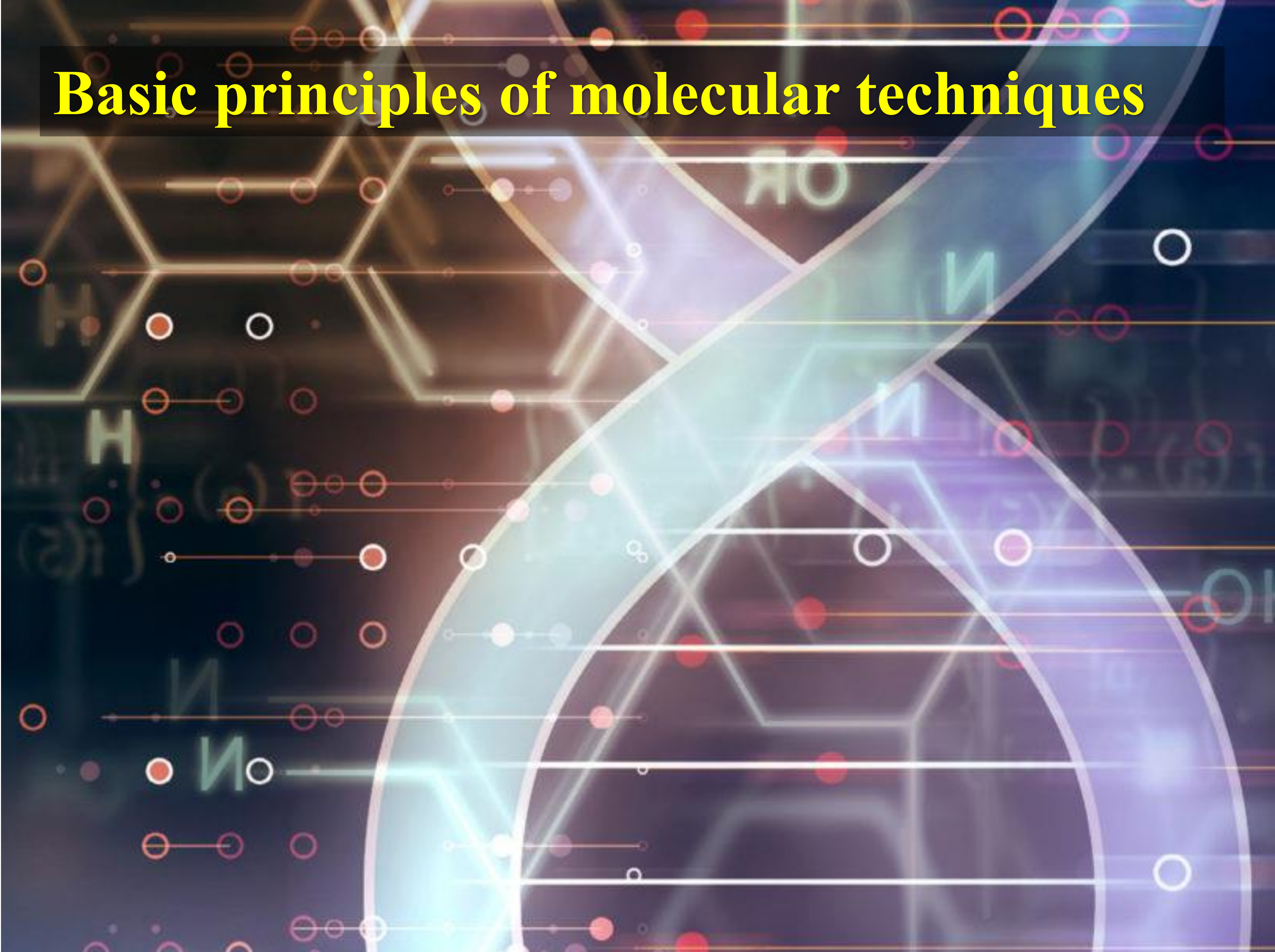
DNA and RNA extraction methods

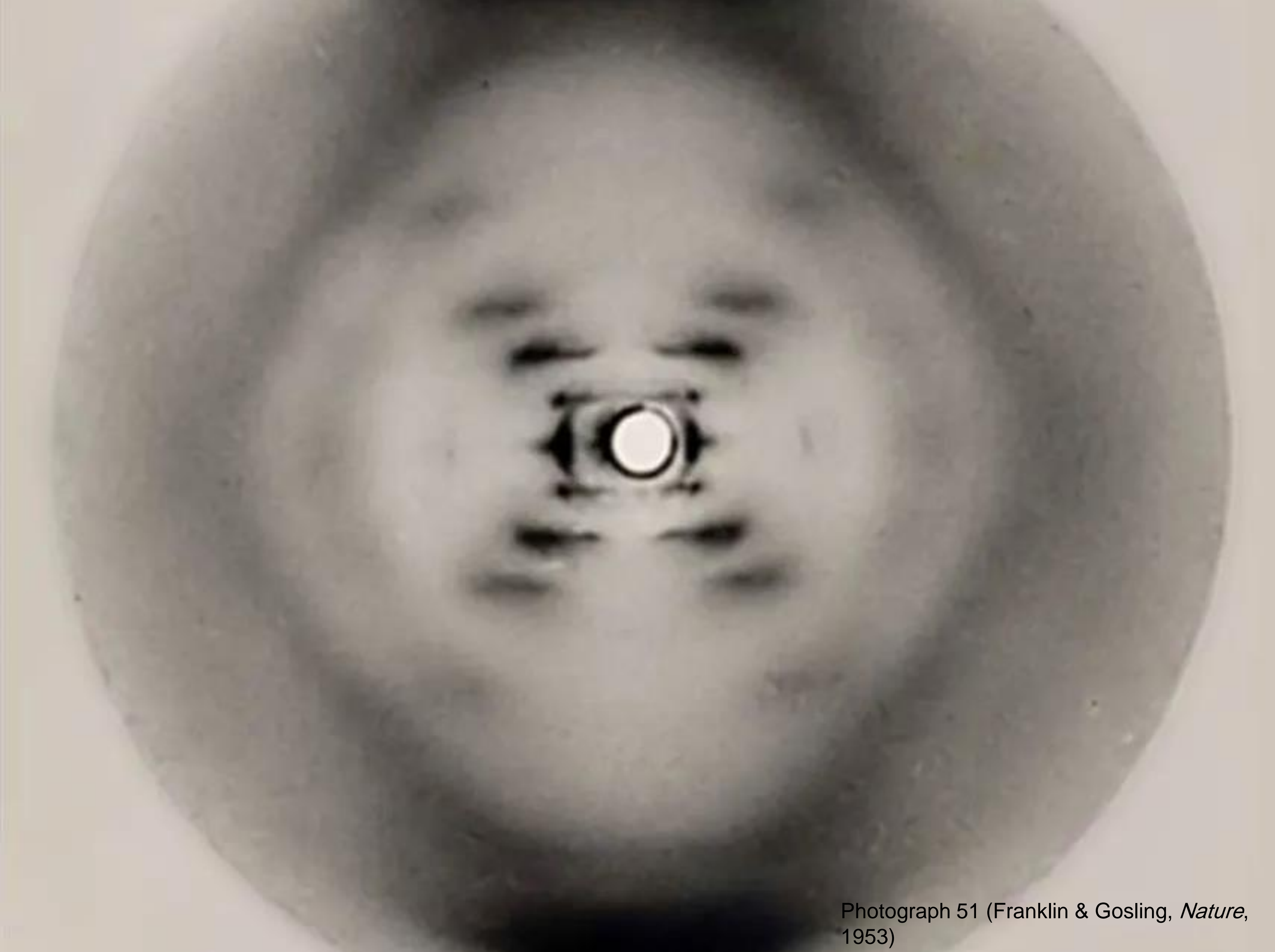
Synthesis of cDNA

Different types of Real-time PCR assays

Troubleshooting common issues in qPCR

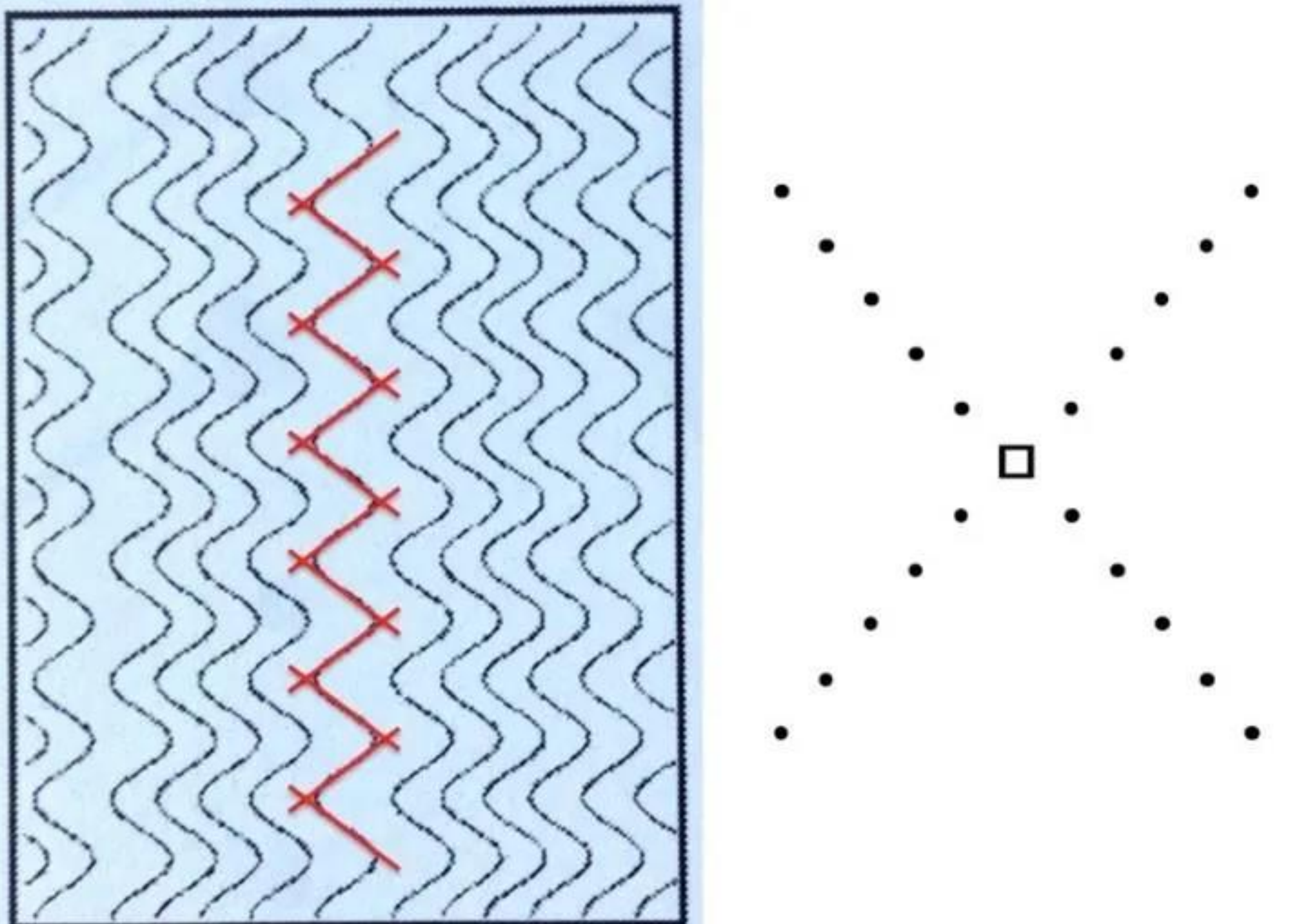
Basic principles of molecular techniques



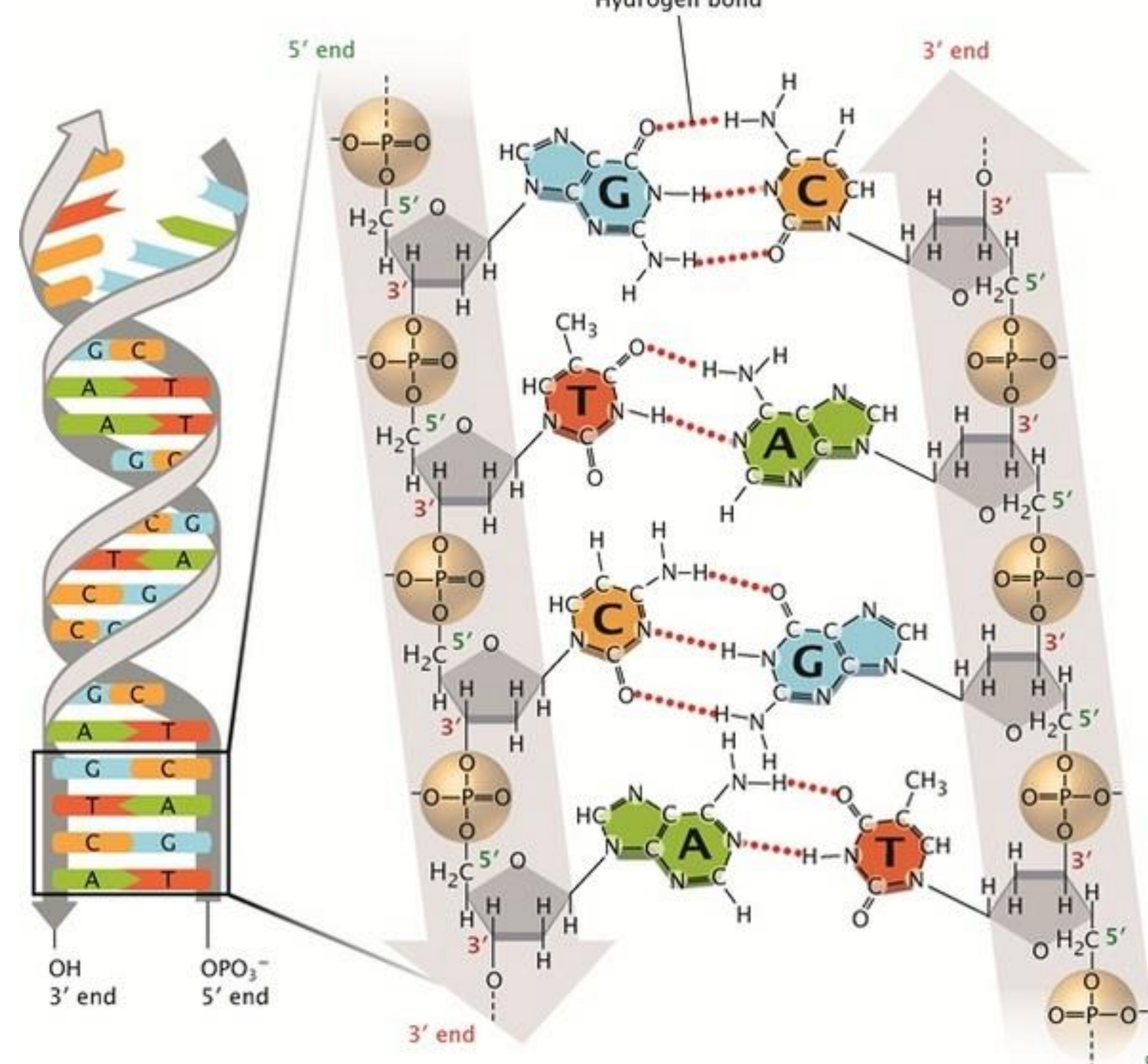


Photograph 51 (Franklin & Gosling, *Nature*, 1953)

Model of the DNA double helix (Watson & Crick, *Nature*, **1953**)



viewed from the side has a “zig-zag” appearance; the repeating zigs and zags give rise to the characteristic cross pattern of diffraction spots.



Discovery of DNA Structure and
Function: Watson and Crick
By: Leslie A. Pray,
Ph.D. © 2008 Nature Education

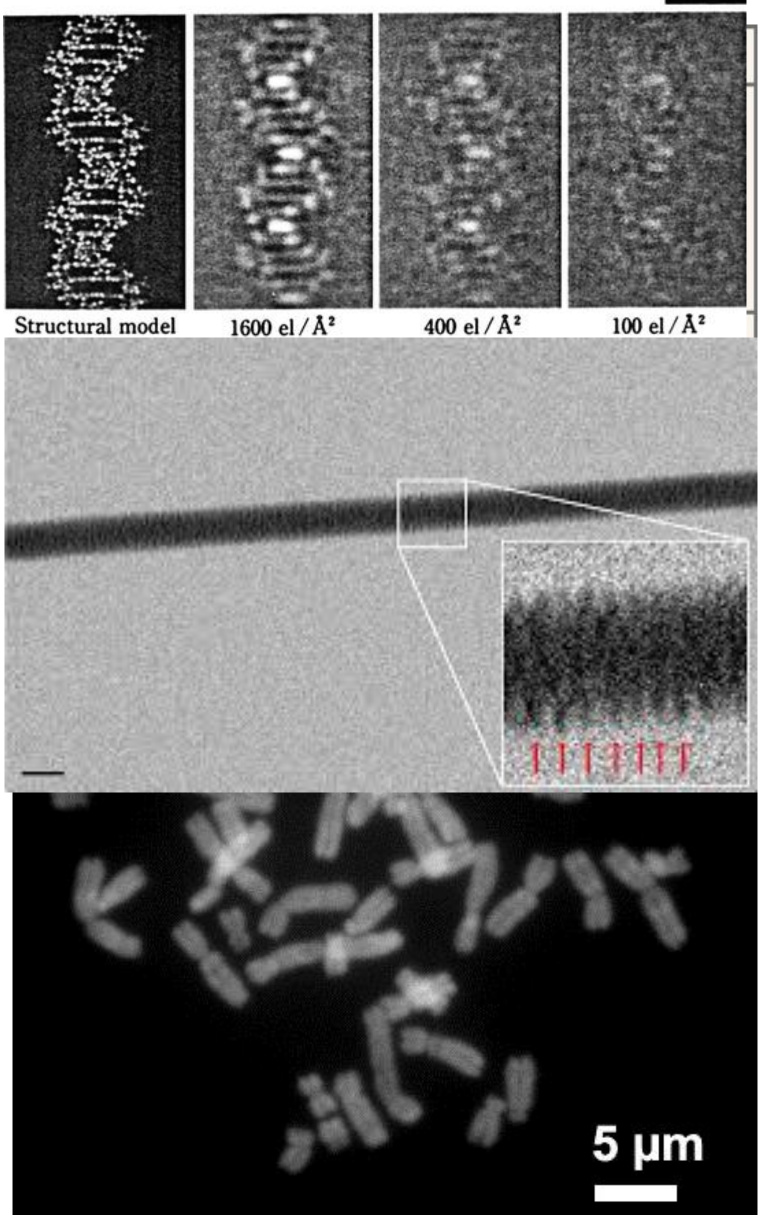
DNA condensation

Chromosome

Chromatin

Nucleosome

Double Helix

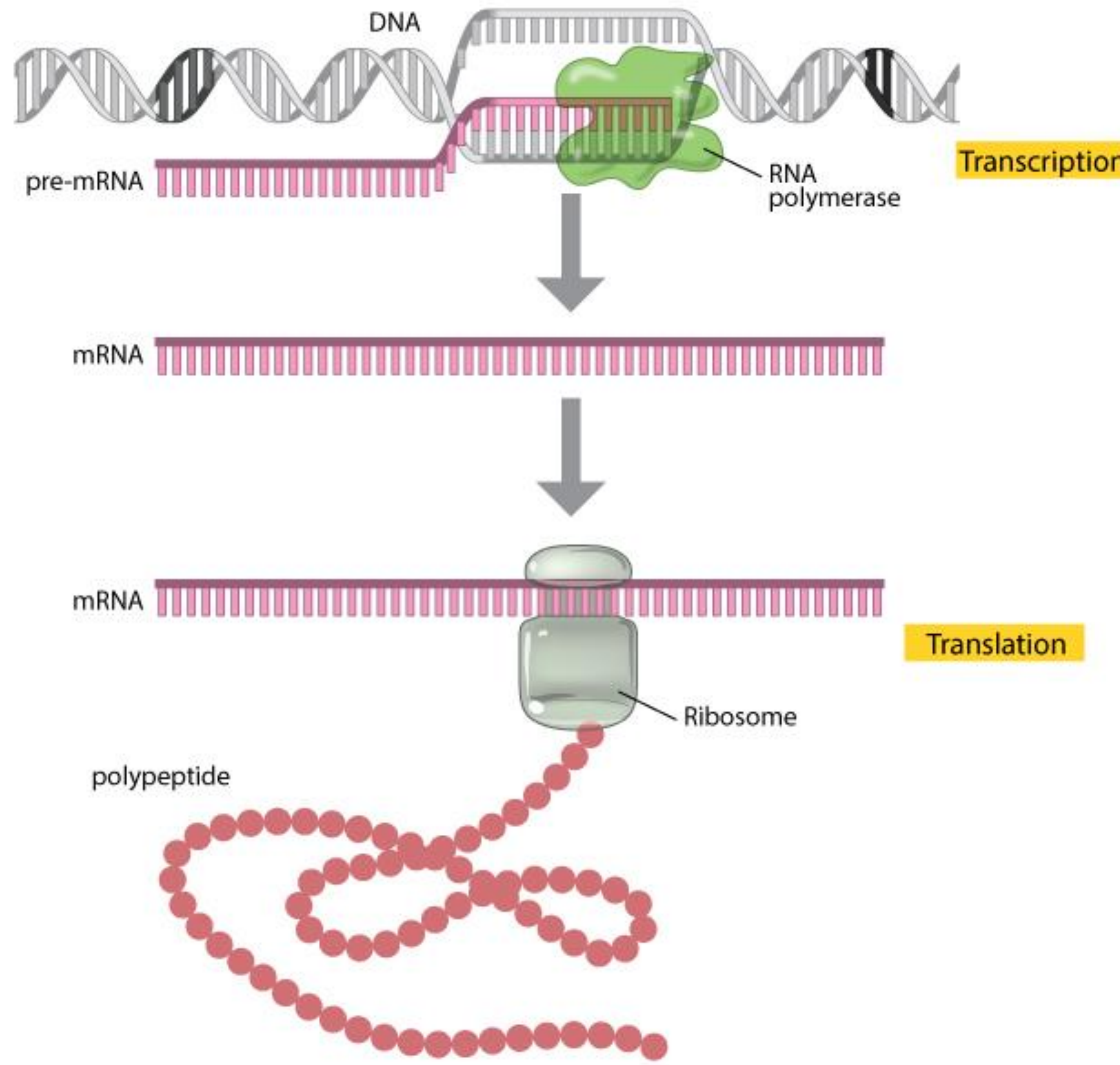


History

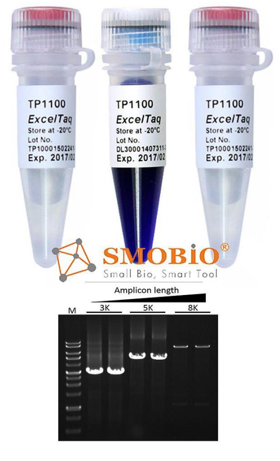
- **Kary B. Mullis** invented the process of **polymerase chain reaction**(PCR) in **1983**. PCR allows the rapid synthesis of designated fragments of **DNA(100-10000 bp)**. Using the technique, **over one billion copies** can be synthesized in a matter of **hours**.
- ***Thermophilus aquaticus* bacterium**
- **Nobel** Prize in Chemistry

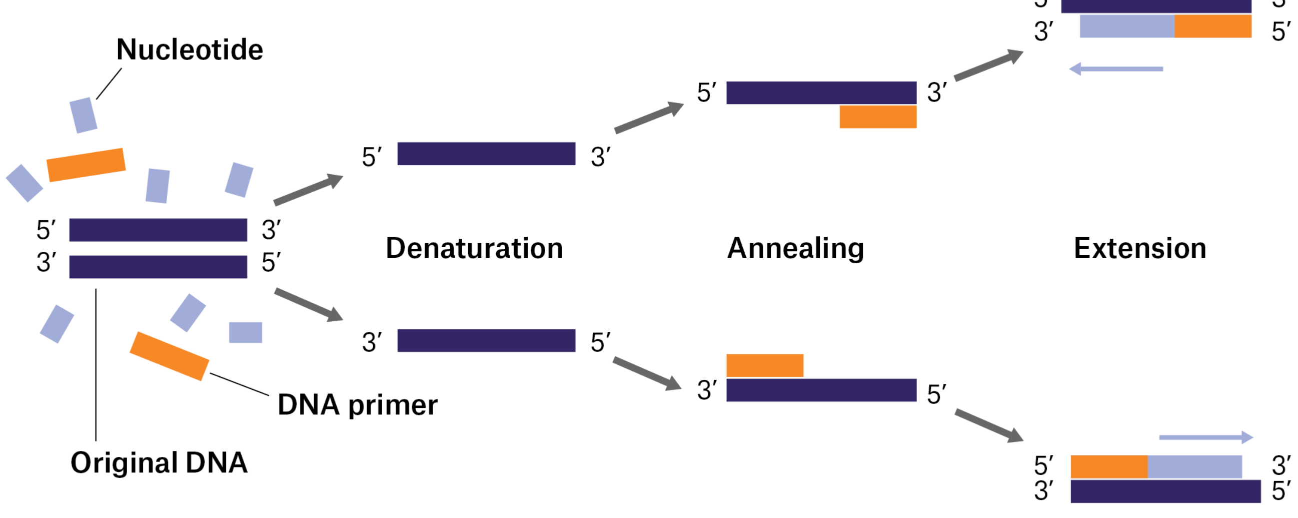
MLA style: Kary B. Mullis – Facts. NobelPrize.org. Nobel Prize Outreach AB 2024. Fri. 26 Jan 2024. <https://www.nobelprize.org/prizes/chemistry/1993/mullis/facts/>

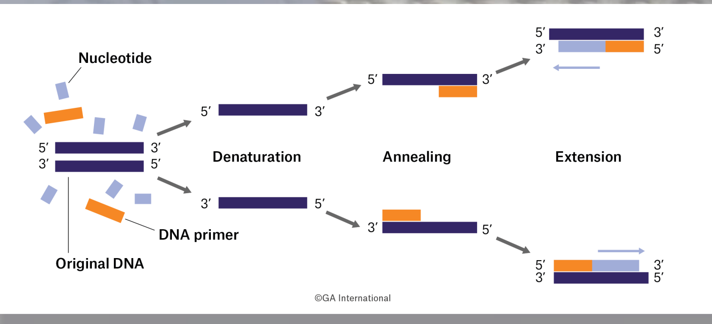
Basic biology DNA replication



Overview PCR and its components


TP1100
ExcelTaq
Store at -20℃
Lot No.
Exp. 2017/
SMOBiO
Small Bio, Smart Tool
Amplicon length
M
3K
5K
8K


Nucleotide
5′
3′
3′
5′
Original DNA
DNA primer
Denaturation
Annealing
Extension
©GA International



PCR

Design- Component- Material- machine

Specificity and contamination

- **Purity of samples:** the extraordinary power of PCR means that contaminants can also be amplified.
- **Primer choice:** if primers anneal to the wrong genomic region, this region will be amplified.
- **Temperature:** Specific base pairing of primers to template DNA only occurs in a narrow range of temperature and salt concentration.

Essential components of PCR mixture

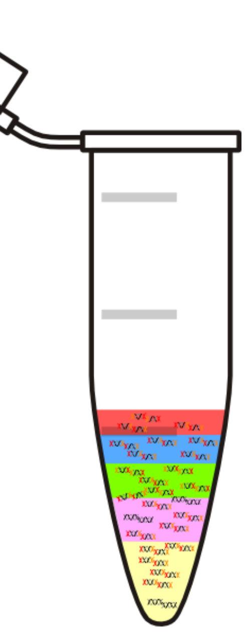
Types of template DNA for PCR:

•**Purified DNA or cDNA**: PCR operates most efficiently when purified DNA is used as a template.

•**Whole cells**: Although less efficient, PCR can be very effective.

•**Environmental samples.**

- **Buffer** (containing Mg++)
- **Template DNA**
- **Two Primers that flank the fragment of DNA to be amplified**
- **dNTPs**
- ***Taq* DNA Polymerase (or another thermally stable DNA polymerase)**



Master mix PCR

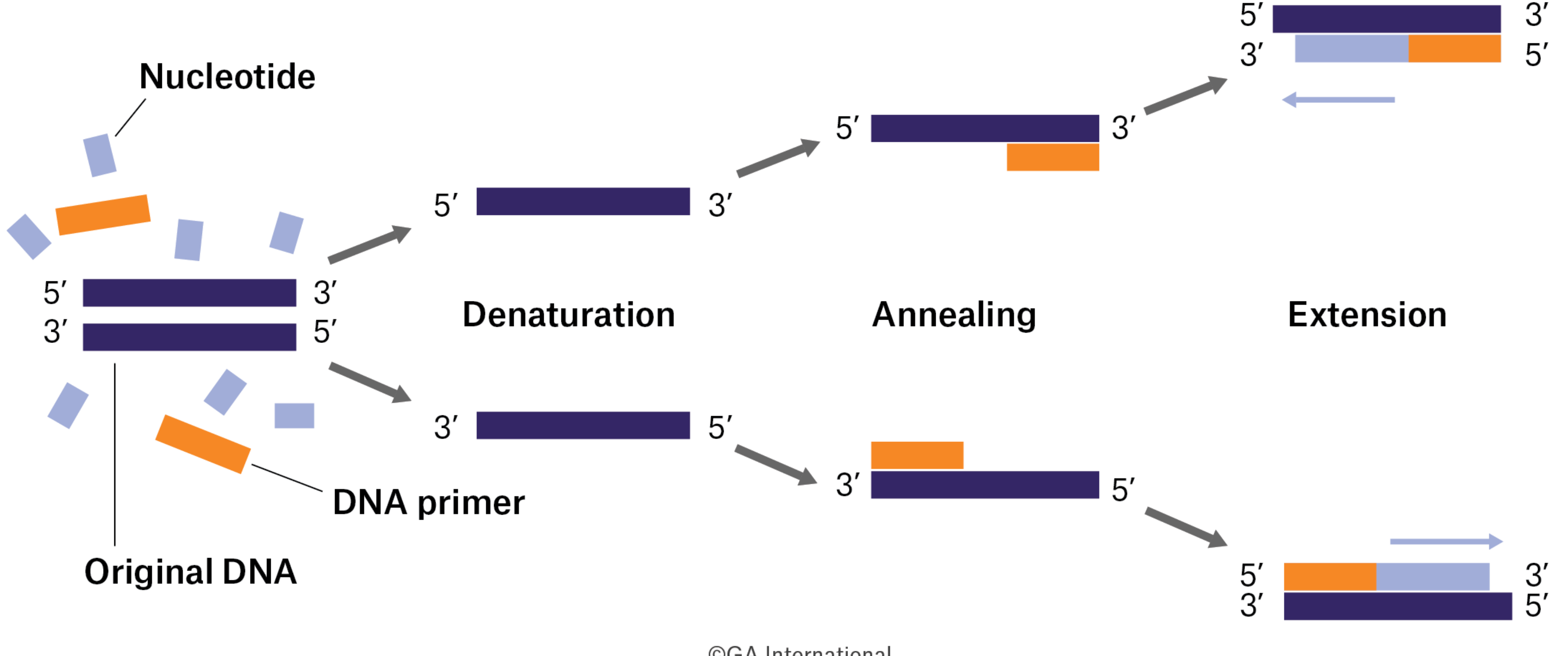
DNA polymerase

dNTPs,

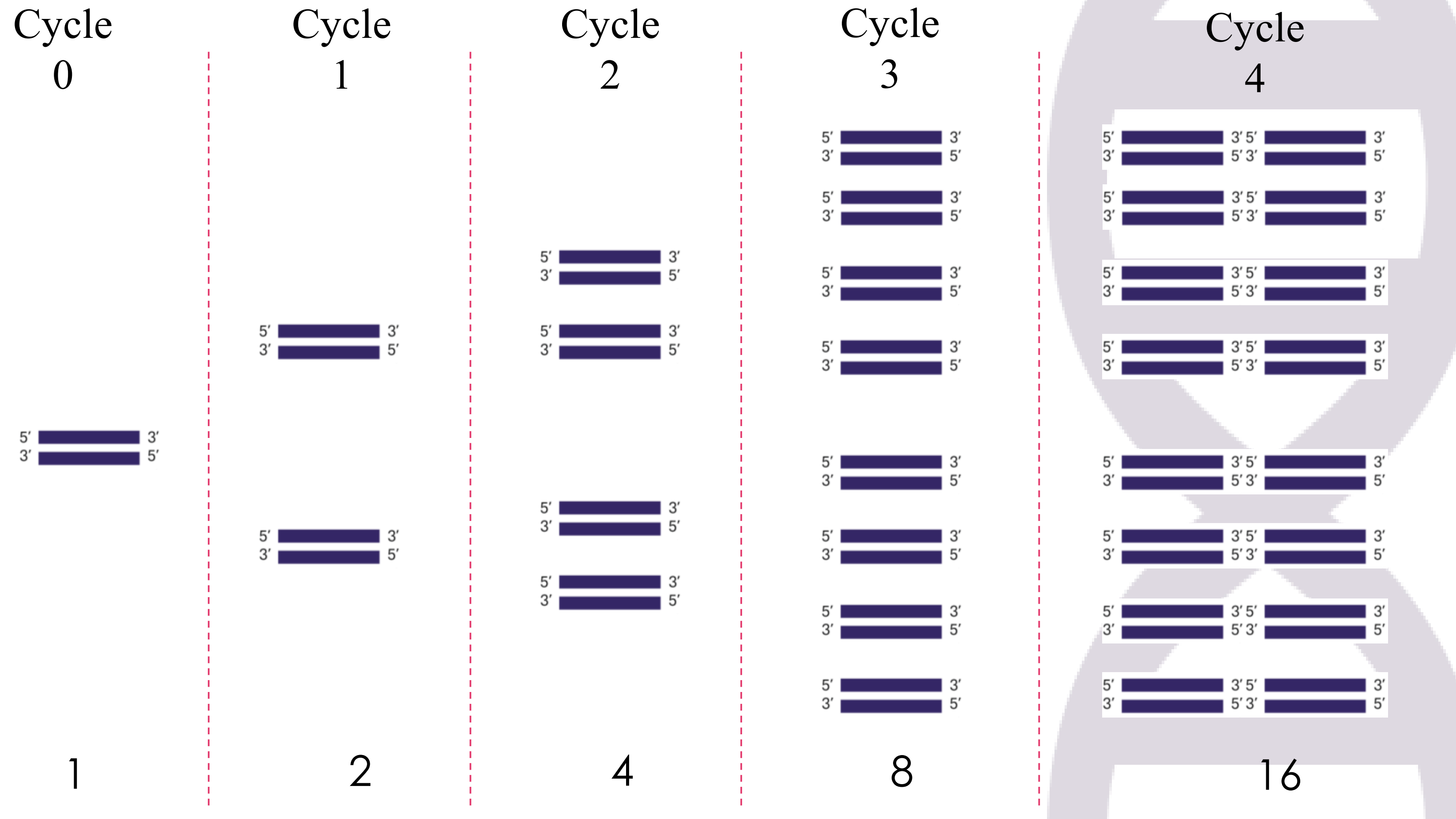
MgCl2

buffer

PCR



DNA Doubles With Each Thermal Cycle



Theoretical Yield Of PCR

Theoretical yield = $2^n \times$ y

- y = starting number of copies
- n = number of thermal cycles

If you start with 100 copies, how many copies are made in 30 cycles?

$$
\begin{aligned}
\text{Theoretical yield} &= 2^n \times y \\
&= 2^{30} \times 100 \\
&= 1{,}073{,}741{,}824 \times 100 \\
&= 107{,}374{,}182{,}400
\end{aligned}
$$

Application

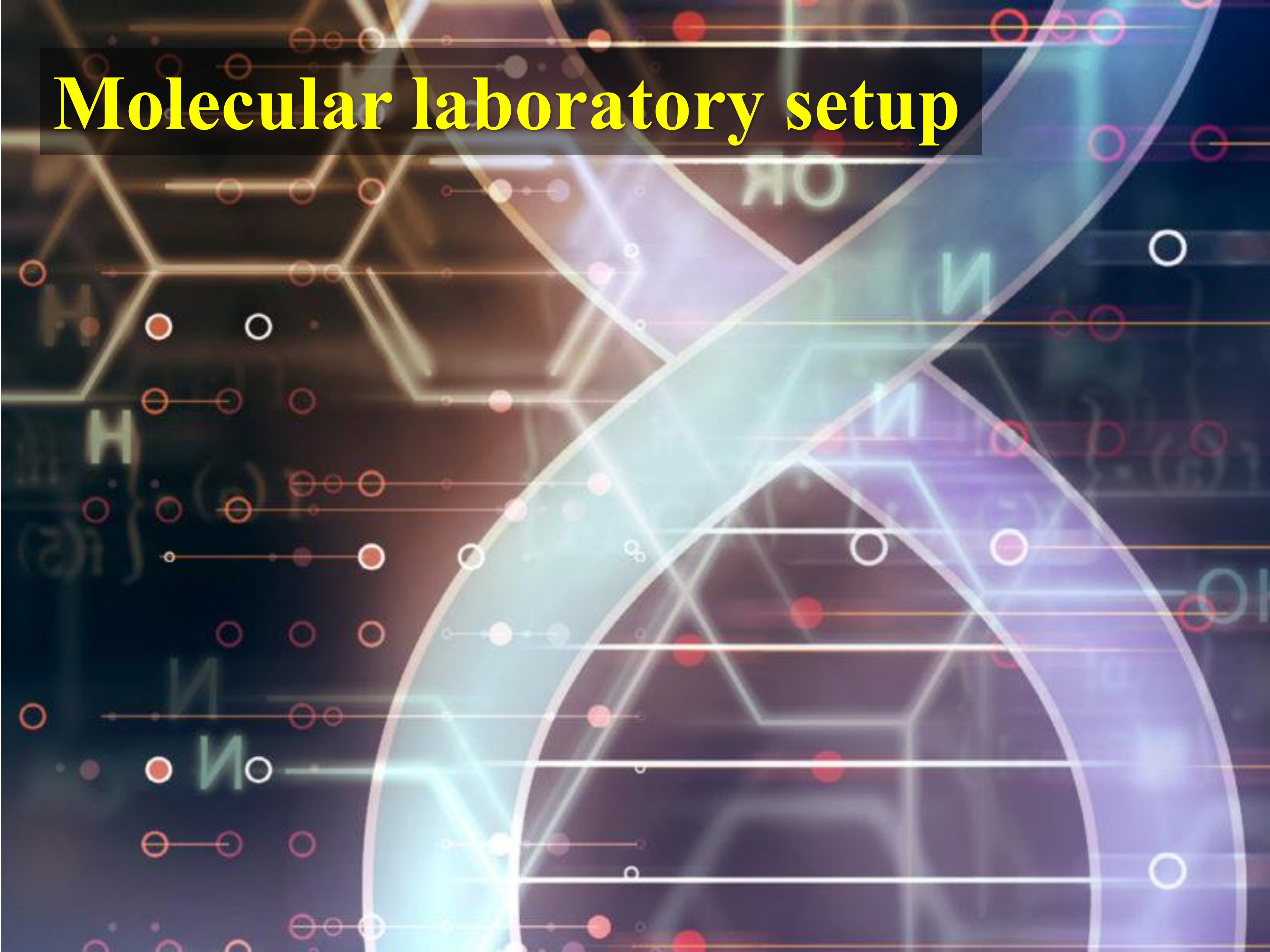
1. **Proteins**
2. **Create modified organisms**
3. **Diagnosis of bacteria and viruses**
4. **Understanding basis of diseases**
5. **Vaccination**
6. **Gene therapy**

Proteins:
Insulin
Interleukins
Interferons
Growth hormones
Rennin
Protease
Lipase
Catalase

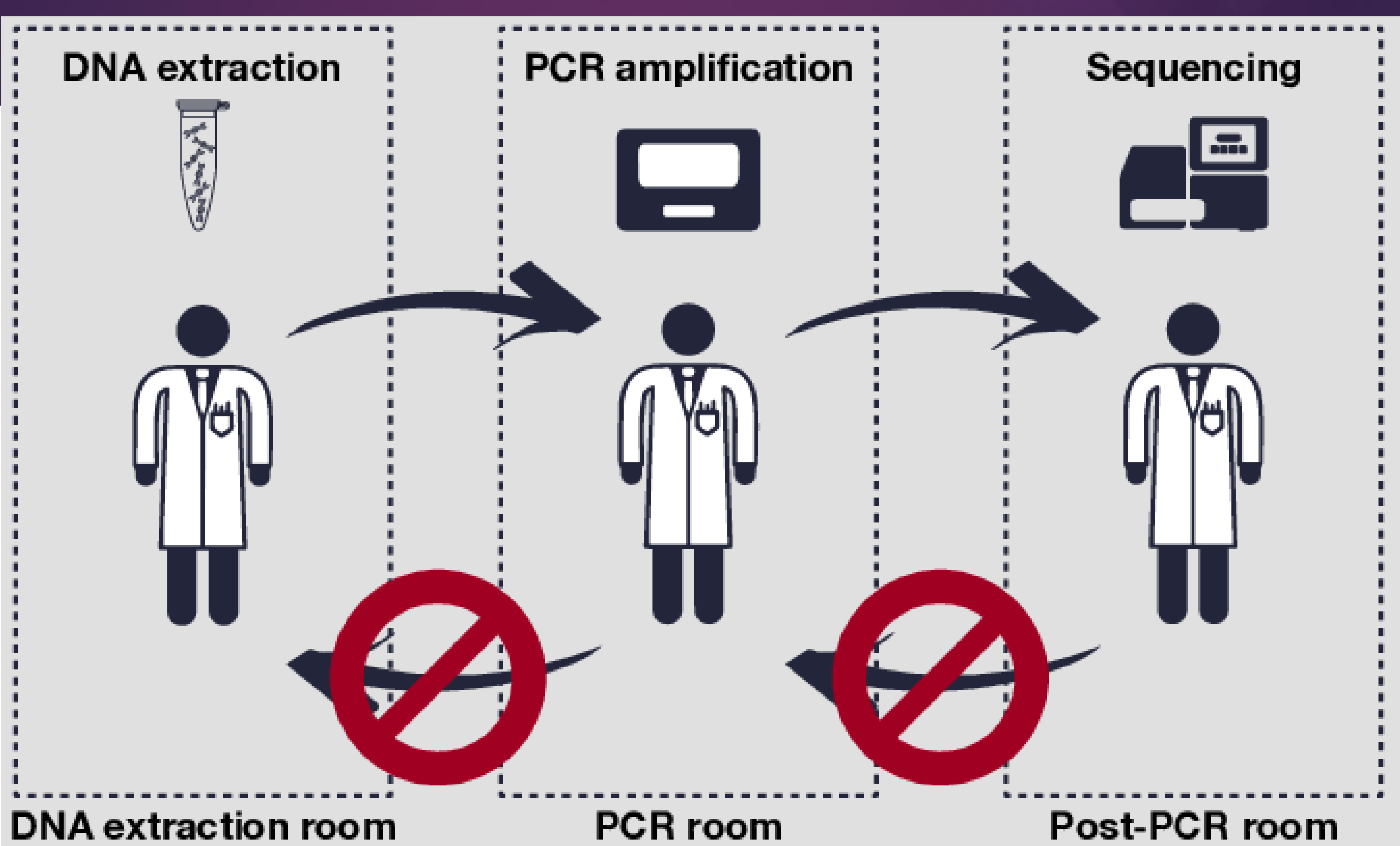
Evaluation

Search on understanding basics of different types of PCR.

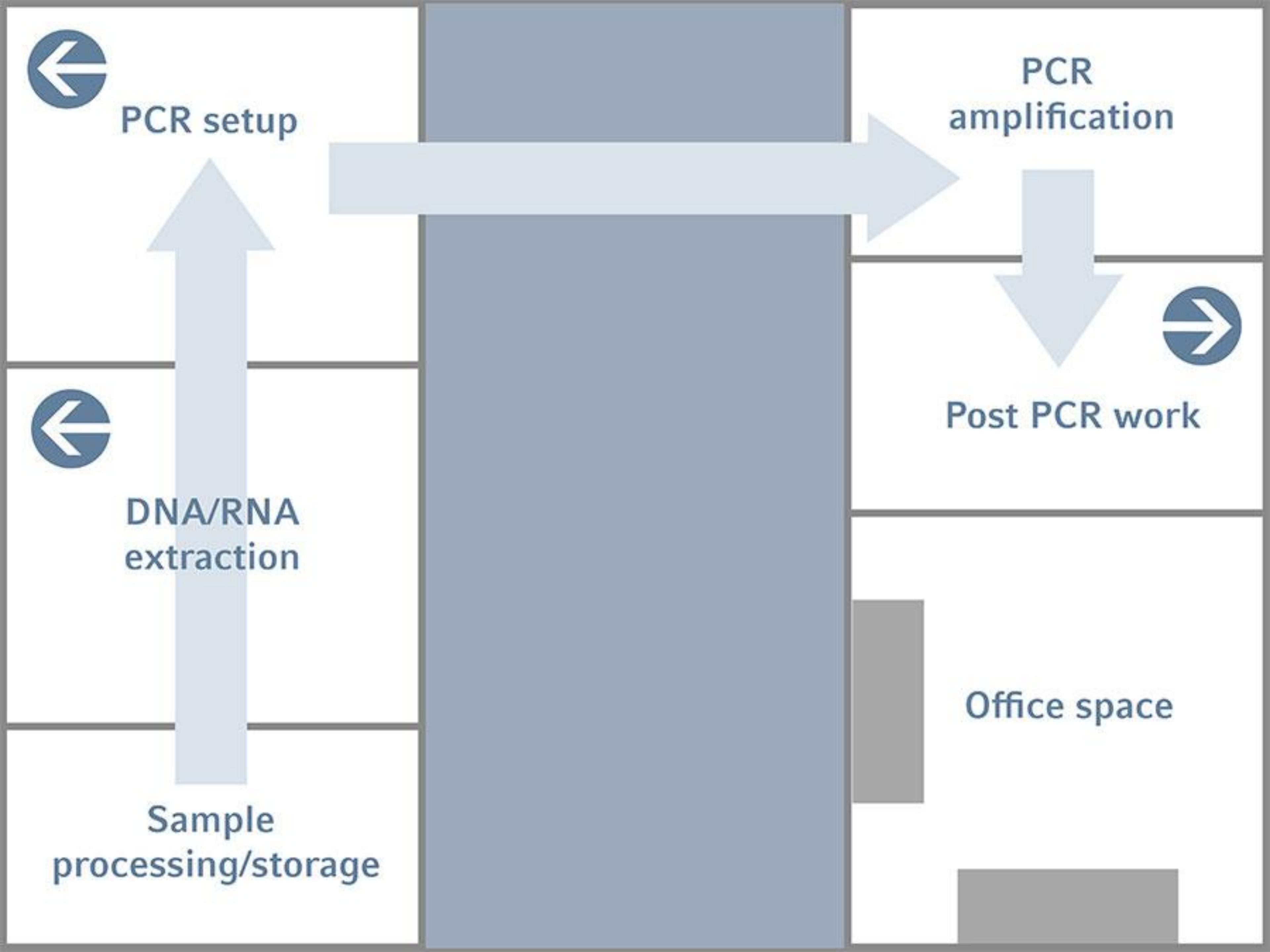
Molecular laboratory setup



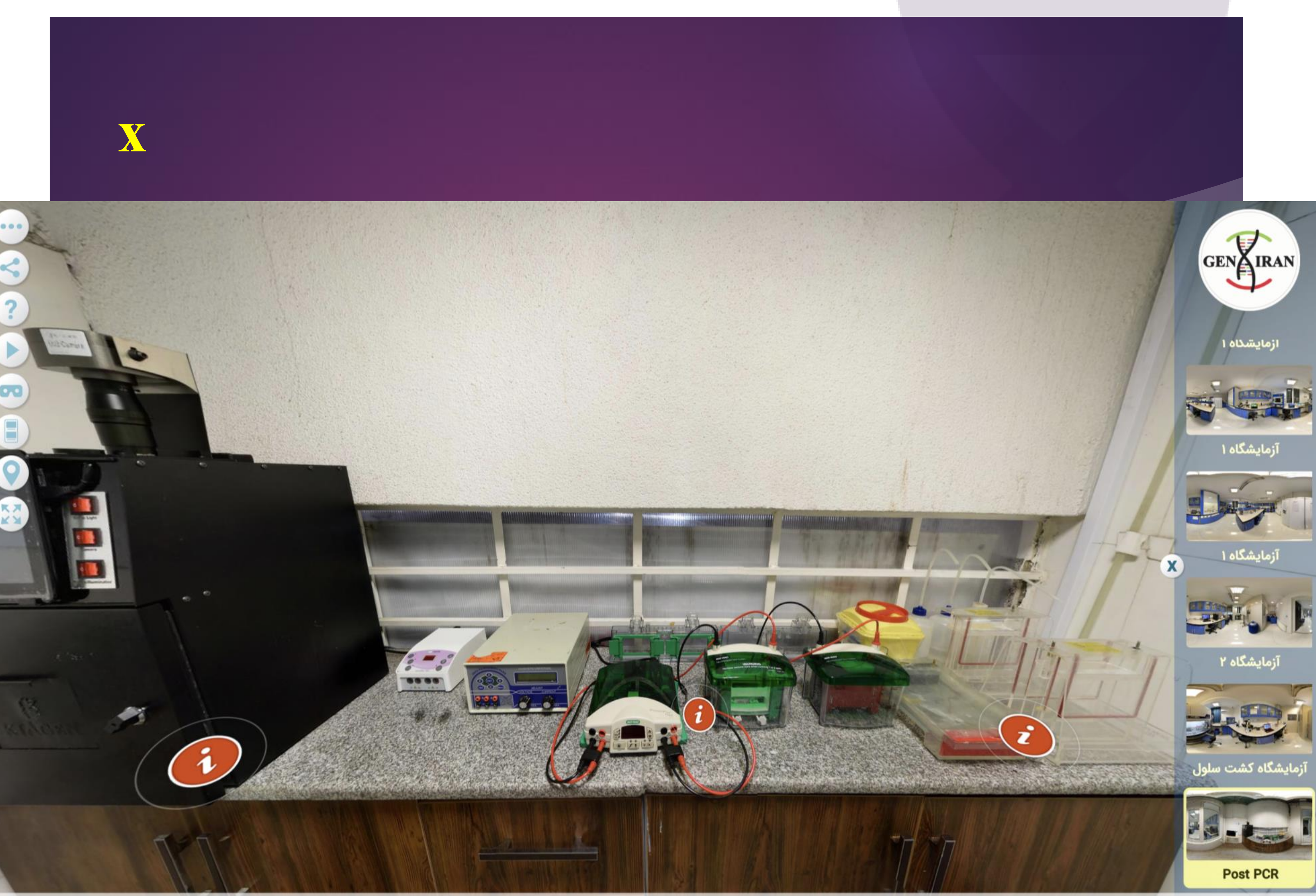
Work flow



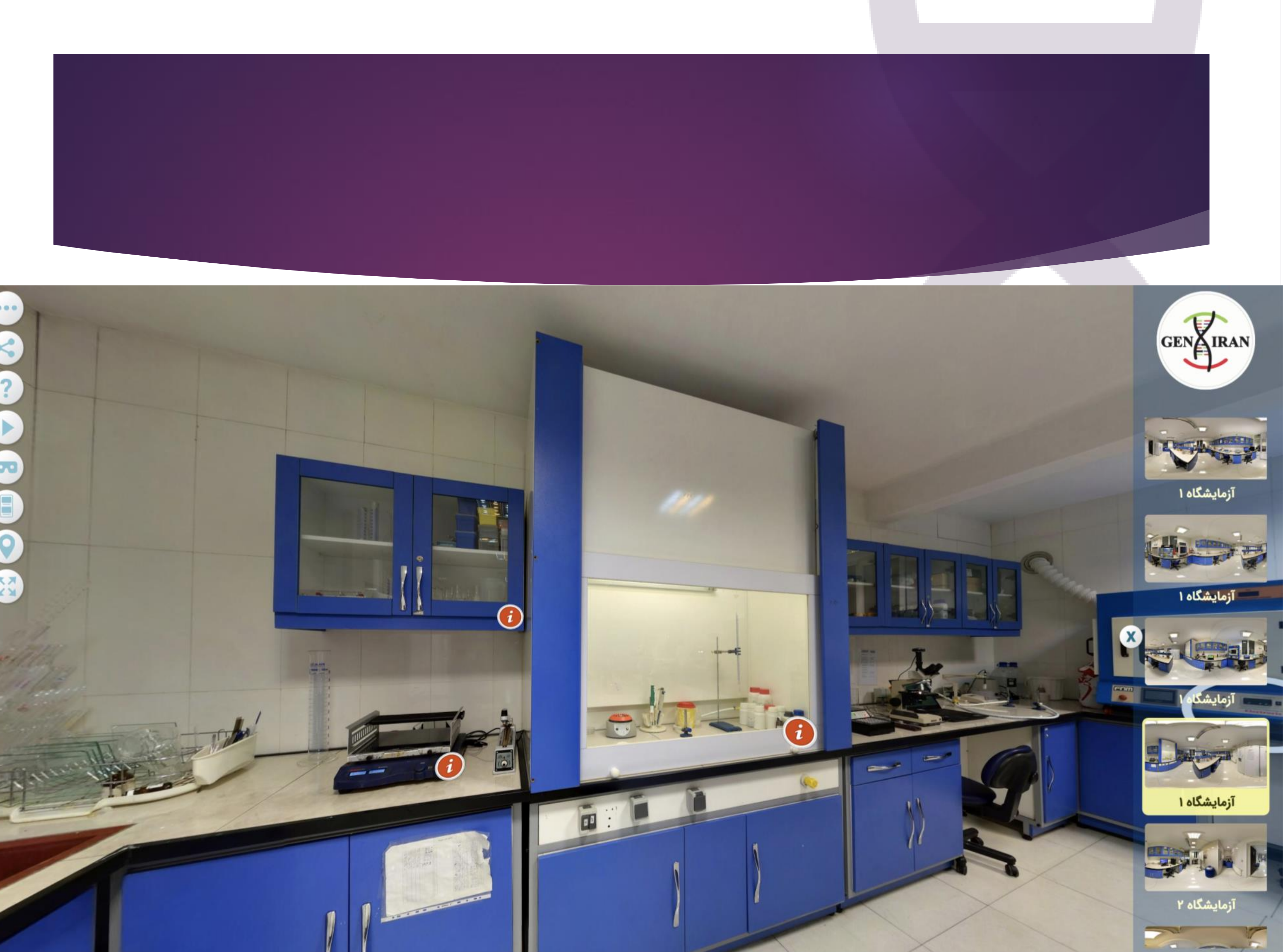
https://askabiologist.asu.edu/sites/default/files/virtual-reality/ASU-lab-VR-360/index.html
https://askabiologist.asu.edu/sites/default/files/virtual-reality/Oncomyx-lab-VR-360/index.html
https://www.geniranlab.ir/vtour/geniran/


PCR setup
PCR amplification
Post PCR work
DNA/RNA extraction
Office space
Sample processing/storage

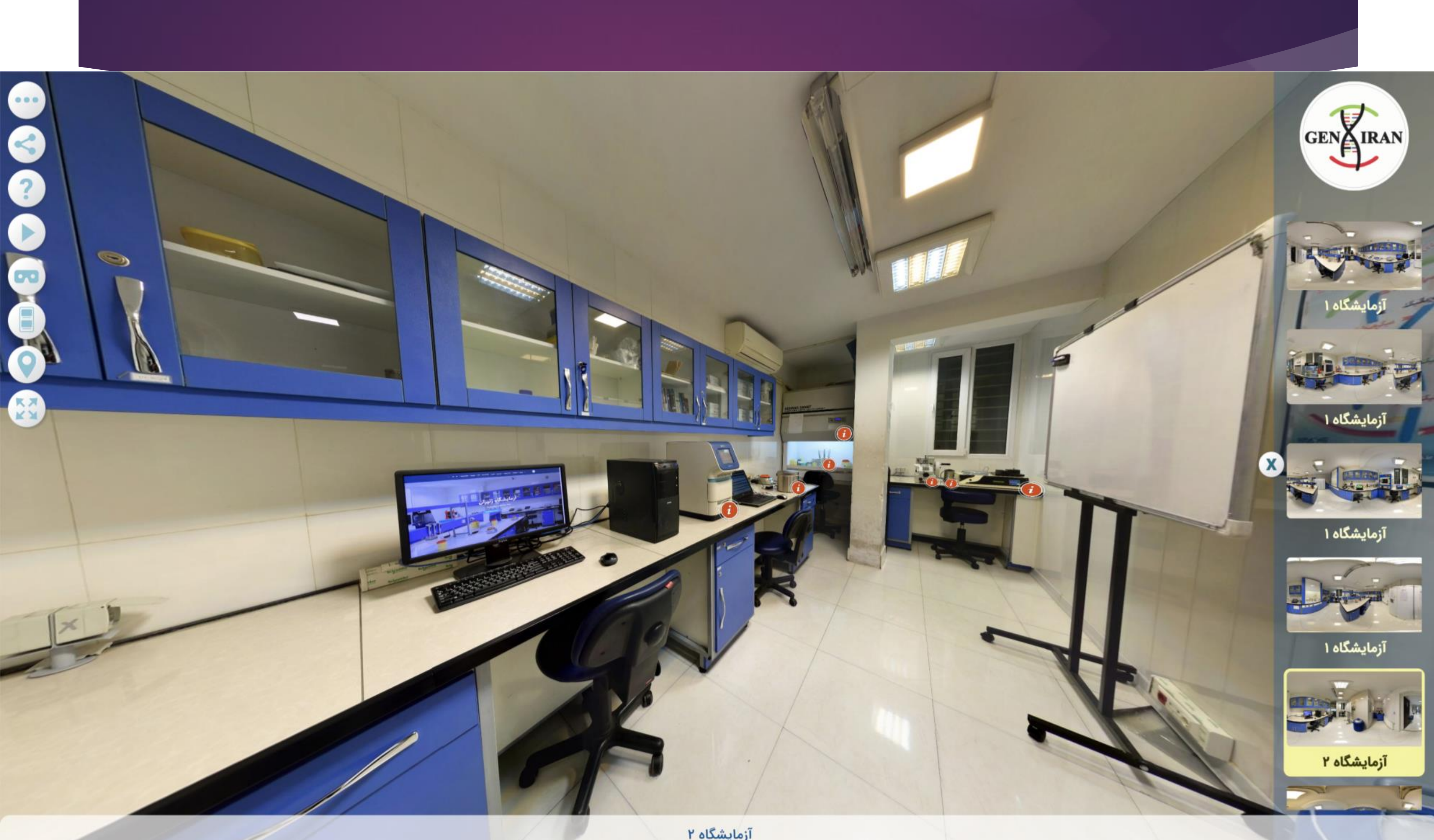
Fume Hood
Thermal cyclers
Gel electrophoresis
Centrifuges, Thermomixer Biophotometer, Pipettes etc.
Working bench – DNA
Laminar flow
PCR Hood
Working bench – RNA
Freezer
Centrifuges Thermomixer Pipettes etc.



X
GEN IRAN
ازمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۲
آزمایشگاه کشت سلول
Post PCR
Post PCR

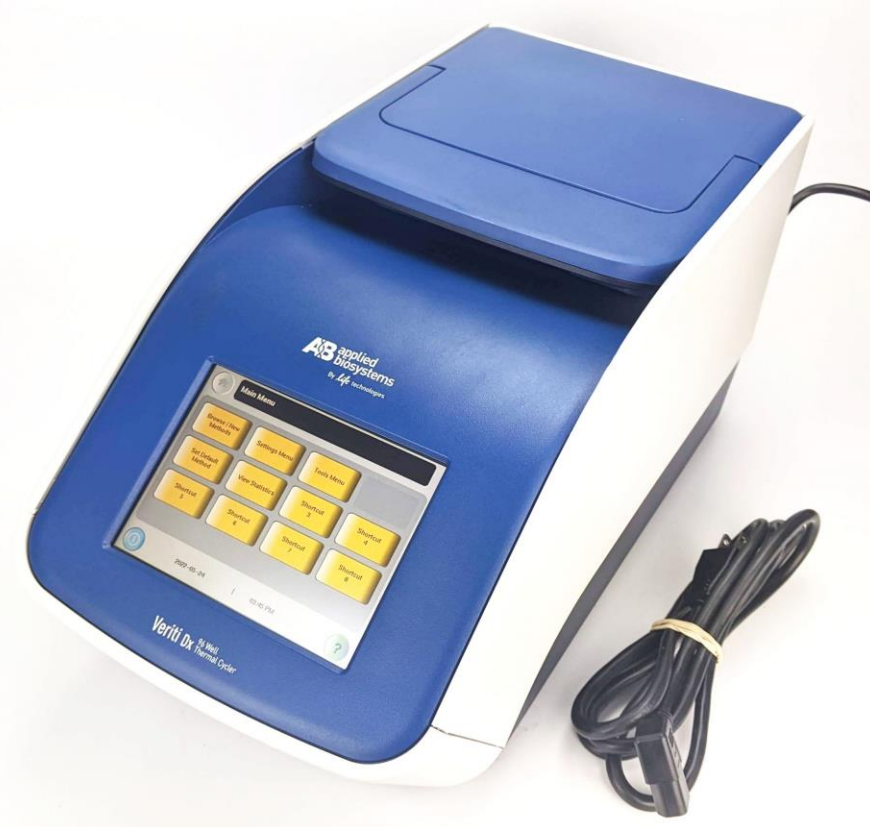


GEN IRAN
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۲


GENXIRAN
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۱
آزمایشگاه ۲
آزمایشگاه ۲

Facilities

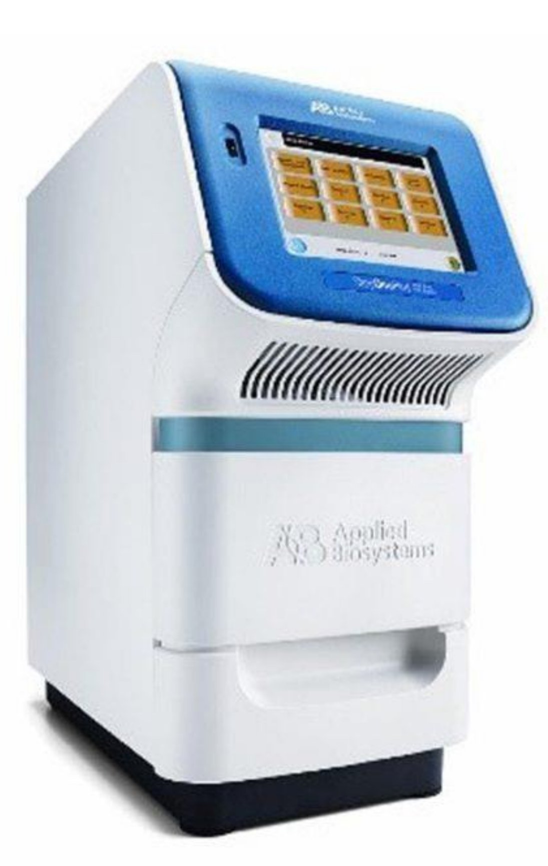
PCR

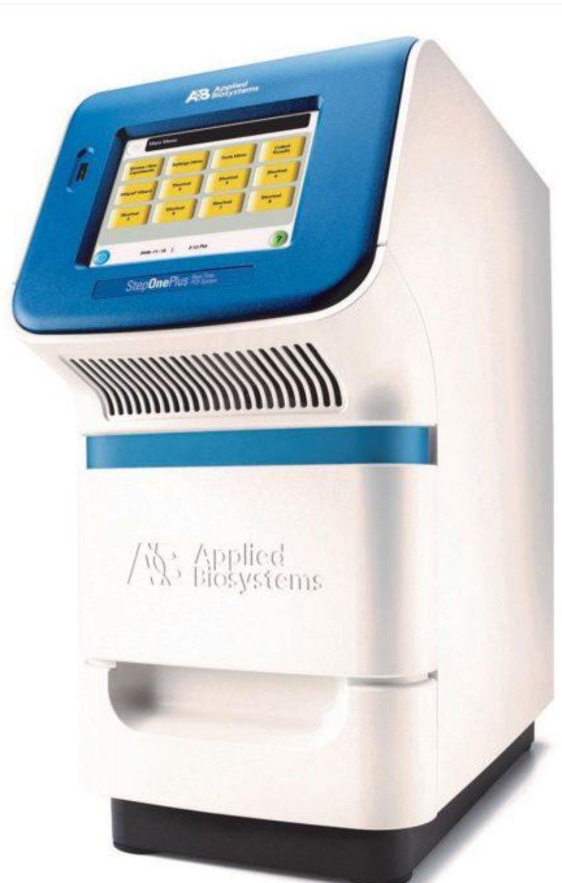


BIO-RAD
T100 Thermal Cycler
RCaH EBS: PCR 1

QX200 Droplet Digital PCR System | Bio-Rad

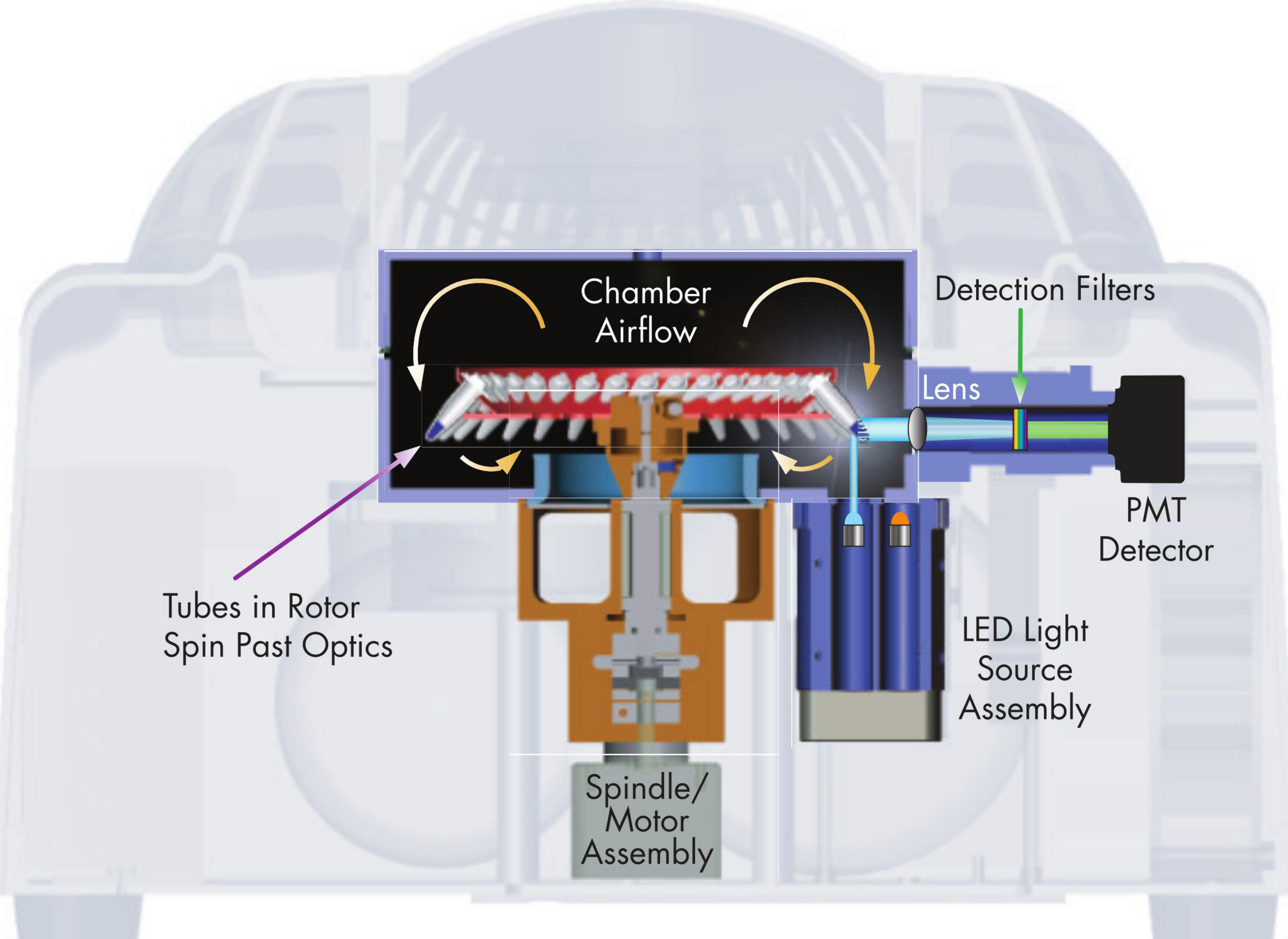
Real-time PCR





7500 fast Real time PCR

Roto-Gene Q Real time PCR



Chamber
Airflow
Detection Filters
Lens
PMT
Detector
Tubes in Rotor
Spin Past Optics
LED Light
Source
Assembly
Spindle/
Motor
Assembly

Gloves

Tips

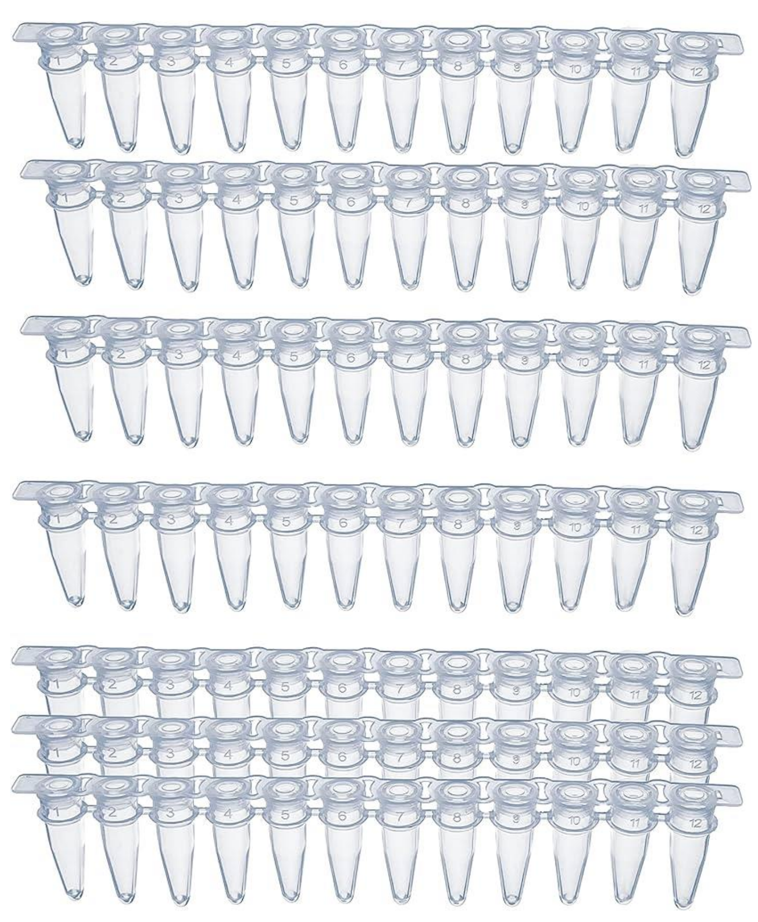
Forceps

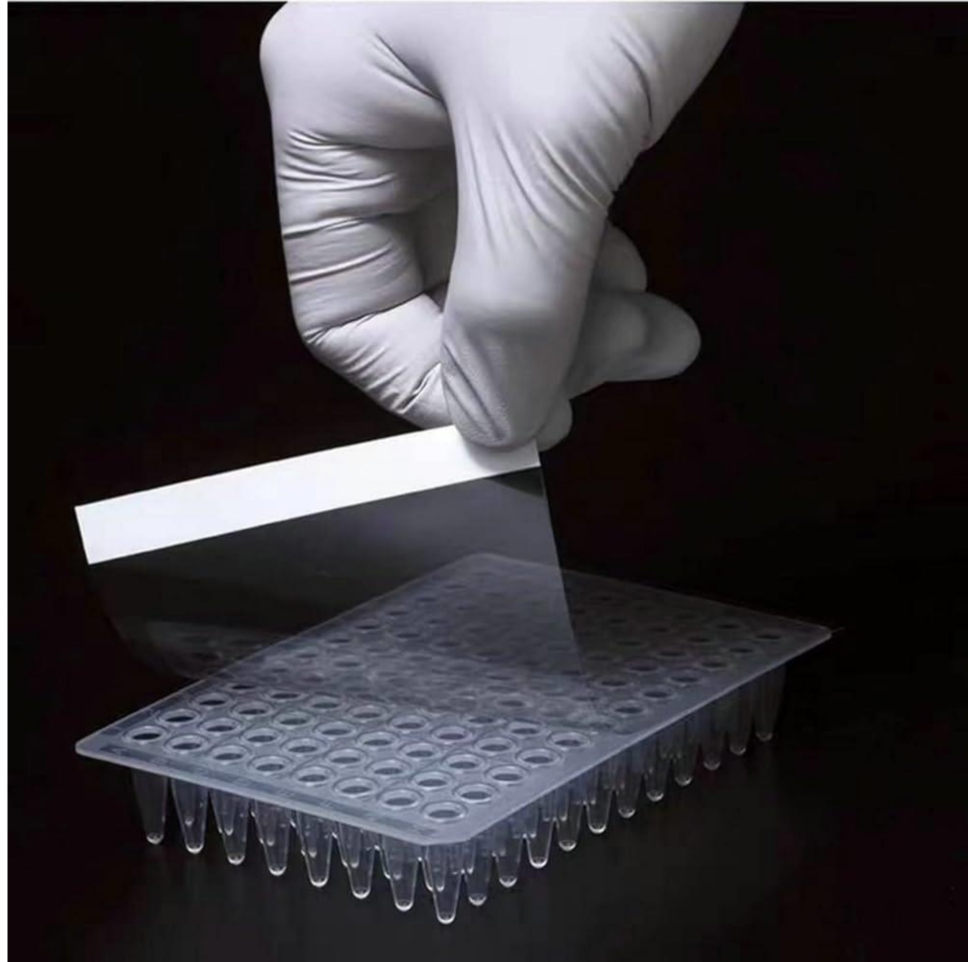
Racks
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Tubes

sciSTAR
GO FURTHER IN SCIENCE
1.5 mL Microcentrifuge Tubes
RNAse and DNAse Free
EO Sterilized
300 Count
Pre-Closed Cap!
1.5
1.5
1.0
Certified
Free

PCR Tubes





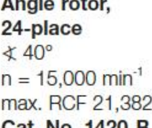
Pipettes

NEXTPETTE
TOPSCIEN
Adjust
Disassemble
10-100ul
Detail Lab

100 - 1000μl
100 - 1000μl
20 - 200μl
20 - 200μl
0.5 - 10μl

Centrifuge 320 R

Rotor

Rotor	Description					
	**Angle rotor, 24-place** ∡ 40° n = 15,000 min^{-1} max. RCF 21,382 **Cat. No. 1420-B**	0.2–2.0 ml		0.5 ml		
	Angle rotor, 30-place ∡ 45° n = 14,000 min^{-1} max. RCF 21,255 **Cat. No. 1689-A**	0.2–2.0 ml		0.5 ml		
	Angle rotor, 8-place ∡ 45° n = 4,500 min^{-1} max. RCF 3,305 **Cat. No. 1418**		5–15 ml		1.6–10 ml	1.1–10 ml
	Angle rotor, 6-place ∡ 35° n = 9,000 min^{-1} max. RCF 9,509 **Cat. No. 1620A**	max. 5 ml	15–94 ml	0.5 ml	8.5–10 ml	7.5–10 ml
	Angle rotor, 12-place ∡ 35° n = 6,000 min^{-1} max. RCF 4,146 **Cat. No. 1613**	max. 5 ml	4–15 ml	0.5 ml	1.6–10 ml	1.1–10 ml
	Angle rotor, 12-place ∡ 35° n = 12,000 min^{-1} max. RCF 16,582 **Cat. No. 1615**	max. 5 ml	4–15 ml	0.5 ml	1.6–10 ml	1.6–10 ml

Angle rotor, 12-place

∢ 45°
n = 15,000 min^{-1}
max. RCF 16,602

Cat. No. 1612

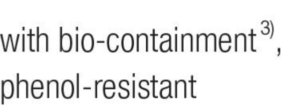
Angle rotor, 24-place

∢ 40°
n = 15,000 min^{-1}
max. RCF 21,382

Cat. No. 1420-B

capacity in ml	0.2	0.4	0.5	0.8	1.5	2.0
Ø x L in mm	6x18	6x45	8x30	8x45	11x38	
Cat. No.	**-**	**-**	**-**	**-**	**2078**	**0536**
lid incl. **rotor Cat. No. 1612**						
Cat. No.	**2024**		**2023**		**2031** [11]	**-**
boring Ø x L in mm	6x40		8x40		10.2x19	11.4x39
tubes per rotor	12					
max. RCF [2]	16,602					
radius in mm	66					
run-up in sec	25					
run-down in sec, braked	23					
temperature in °C [1]	-10					

Angle rotor, 30-place



with bio-containment[3], phenol-resistant

∢ 45°
n = 14,000 min^{-1}
max. RCF 21,255

Cat. No. 1689-A

Angle rotor, 8-place

∢ 45°
n = 4,500 min^{-1}
max. RCF 3,305

Cat. No. (without adapters) **1418**

capacity in ml	0.2	0.4	0.5	0.8	1.5	2.0	0.5
Ø x L in mm	6 x 18	6 x 45	8 x 30	8 x 45	11 x 38		10.7 x 36
Cat. No.	**-**	**-**	**-**	**-**	**2078**	**0536**	**Pediatric**
lid with bio-containment[3] **incl.** **rotor Cat. No. 1689-A**							
Cat. No.	**2024**		**2023**		**2031**[11]	**-**	**0788**[12]
boring Ø x L in mm	6 x 40		8 x 40		10.2 x 19	11.2 x 40.9	11.2 x 39
tubes per rotor	30						15
max. RCF[2]	21,255						20,376
radius in mm	97						93
run-up in sec	35						
run-down in sec, braked	31						
temperature in °C[1]	0						

Cat. No.	-	-	-	-	2078	0536	Pediatric
lid incl. rotor Cat. No. 1420-B							
Cat. No.	2024		2023		2031 [11]	-	0788 [12]
boring Ø x L in mm	6 x 40		8 x 40		10.2 x 19	11.2 x 42.6	11.2 x 39
tubes per rotor	24						12
max. RCF [2]	21,382						20,376
radius in mm	85						81
run-up in sec	25						
run-down in sec, braked	24						
temperature in °C [1]	-4						

[11] For centrifugation at high speeds, we recommend to use form-fitting, phenol-resistant adapters 2031.
[12] Packed in units of 15 pieces.

with bio-containment [3], autoclavable

Cat. No. 2425

with bio-containment [3], autoclavable and phenol-resistant

Cat. No. 2423

Microcentrifuges

Vortex
Benchmark
BRP
SPEED
OFF
ON
TOUCH
lab dancer
IKA®

Contaminations

- Cross-contamination
- RNAase and DNAase
- Ethidium bromide
- PCR inhibitors
- Phenol
- Protein

https://www.neb.com/en/tools-and-resources/usage-guidelines/avoiding-ribonuclease-contamination

DNAOUT™

For Removal of Contaminating DNA

INSTRUCTIONS:

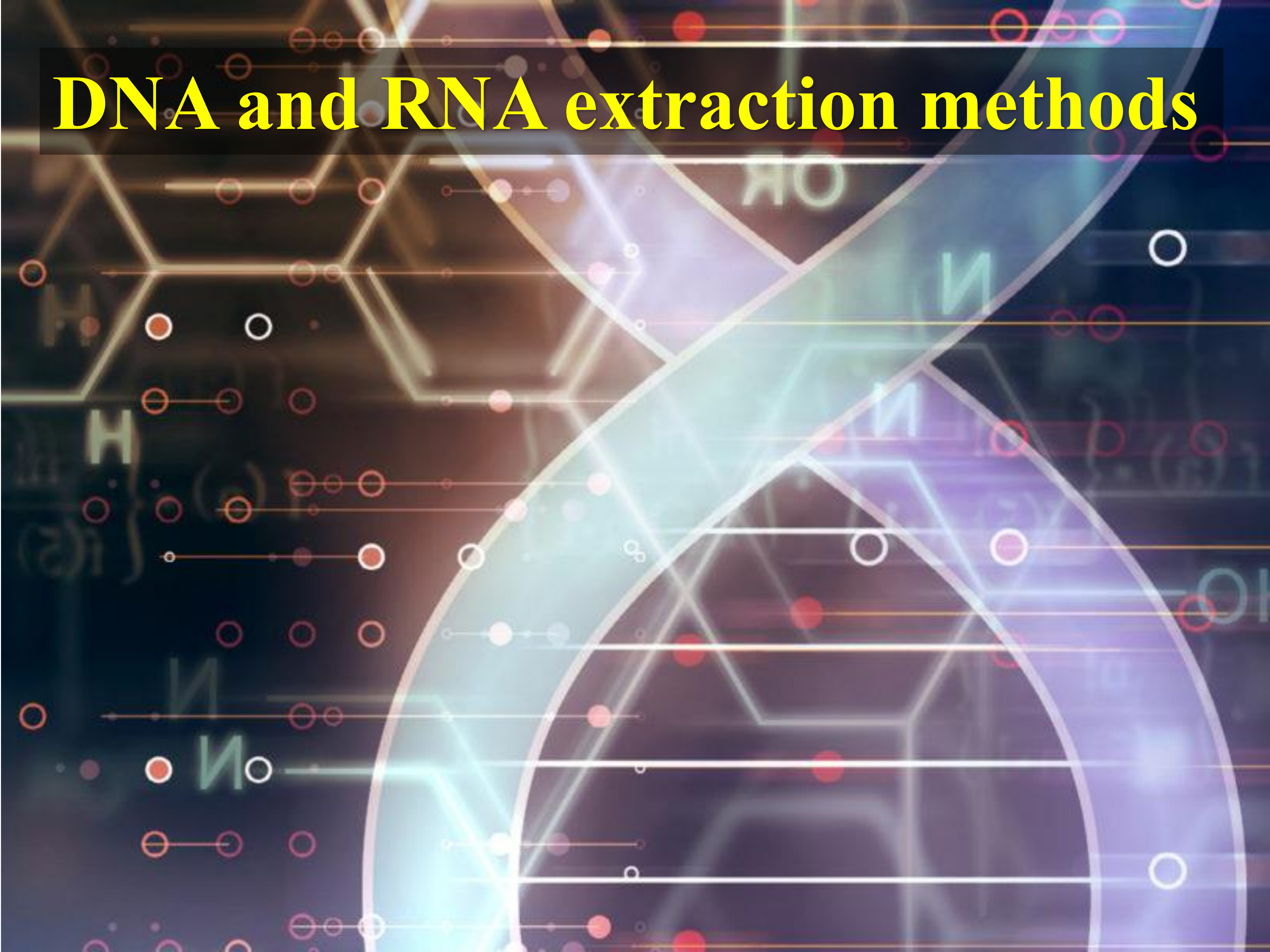
1. Spray or wipe generous amount on surface, allow 5-10 seconds to act

2. Rinse thoroughly with clean molecular grade water

For Research Use Only.

G-Biosciences

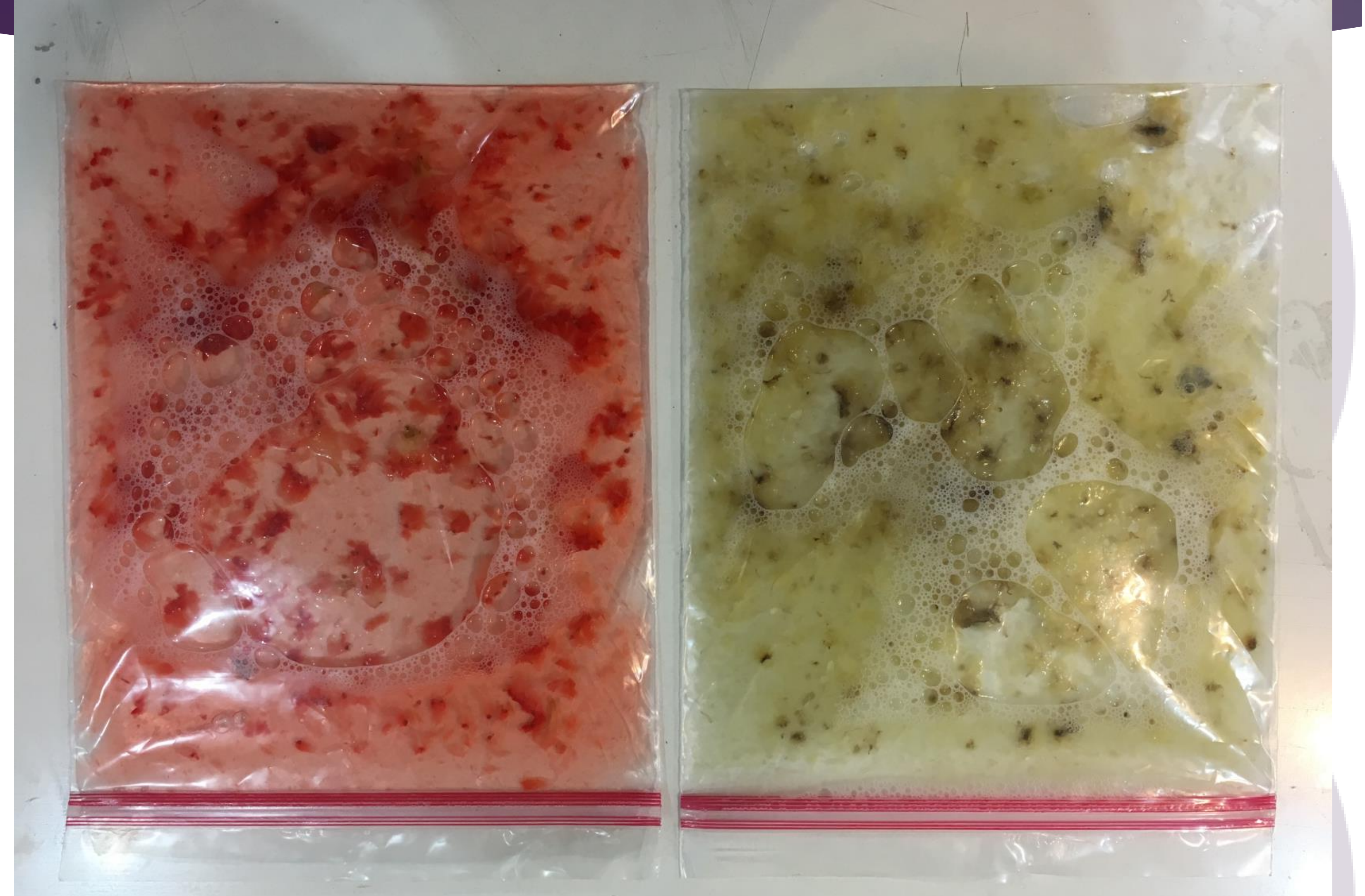
DNA and RNA extraction methods



Strawberry DNA

1-Measure 6T (90 ml) of water into a small glass container.
2-Add 2 tsp (10 ml) dish soap to the water
3-Stir in a ¼-tsp salt and mix until the salt dissolves. This is the extraction mixture.
4-Place one strawberry into a plastic zipper-lock bag.
5-Pour the extraction mixture into the bag with the strawberry.
6-Remove as much air from the bag as possible and seal it closed.
7-Use your hands to smash, and moosh the strawberry inside of the bag.
8-Pour the mixture through a strainer and into a medium glass bowl.
9-Use a spoon to press the mashed bits of strawberry against the strainer forcing even more of the mixture into the container.
10-Add 1 tsp (5 ml) of the chilled isopropyl alcohol to the solution and hold the mixture at eye level.

Acid nucleic extraction

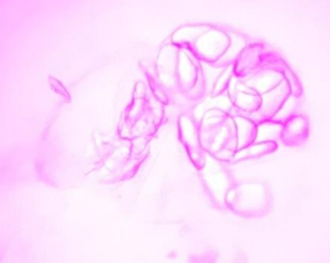


Acid nucleic extraction

37 trillion cells in an adult human body= 3.7×10^{13}

6.5 picograms of DNA per cells = 6.5×10^{-12}

As result : 250 gram DNA or more



الیاف کروماتین (DNA) با ضخامت 700 نانومتر با بزرگنمایی1000x
آزمایشگاه بیولوژی دبیرستان احسان

Basic Steps in Characterization of DNA Profiling and Analysis

The four basic steps involved in DNA Profiling include,

- DNA extraction
- DNA quantification
- DNA amplification
- Detection of the DNA-amplified products

Evaluation

Search on understanding basics of different methods of DNA extraction, quantitation, amplification and detection.

Evaluation

- **Search on understanding basics of different methods of DNA extraction, quantitation, amplification and detection.**

Chromatography-based DNA extraction method.

1- Ethidium bromide–cesium chloride (EtBr-CsCl).
2- gradient centrifugation method.
3- Alkaline extraction method.
4- Silica matrices method.
5- Salting-out method.
6- Cetyltrimethylammonium bromide (CTAB) extraction method.
7- Sodium dodecyl sulfate (SDS)-proteinase K method.
8- Silica column-based DNA extraction method.
9- Cellulose-based paper method.
10- Chelex-100 extraction method.

TRIzol reagent

invitrogen USER GUIDE

TRIzol™ Reagent

Catalog Numbers 15596026 and 15596018

Doc. Part No. 15596026.PPS **Pub. No.** MAN0001271 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product information

Invitrogen™ TRIzol™ Reagent is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour. TRIzol™ Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. TRIzol™ Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. TRIzol™ Reagent allows for simultaneous processing of a large number of samples, and is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987).

TRIzol™ Reagent allows to perform sequential precipitation of RNA, DNA, and proteins from a single sample (Chomczynski, 1993). After homogenizing the sample with TRIzol™ Reagent, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is precipitated from the aqueous layer with isopropanol. DNA is precipitated from the interphase/organic layer with ethanol. Protein is precipitated from the phenol-ethanol supernatant by isopropanol precipitation. The precipitated RNA, DNA, or protein is washed to remove impurities, and then resuspended for use in downstream applications.

- Isolated RNA can be used in RT-PCR, Northern Blot analysis, Dot Blot hybridization, poly(A)+ selection, in vitro translation, RNase protection assay, and molecular cloning.
- Isolated DNA can be used in PCR, Restriction Enzyme digestion, and Southern Blots.
- Isolated protein can be used for Western Blots, recovery of some enzymatic activity, and some immunoprecipitation.

TRIzol™ Reagent can also be used with Phasemaker™ Tubes to isolate RNA. Refer to *TRIzol™ Reagent and Phasemaker™ Tubes Complete System User Guide* (MAN0016163) for the full protocol.

Contents and storage

Contents	Cat. No. 15596026 (100 reactions)	Cat. No. 15596018 (200 reactions)	Storage
TRIzol™ Reagent	100 mL	200 mL	15–30°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 1 Materials required for RNA, DNA, and protein isolation

Item	Source
Equipment	
Centrifuge and rotor capable of reaching 12,000 × *g* and 4°C	MLS
Tubes	
Polypropylene microcentrifuge tubes	MLS
Reagents	
Chloroform	MLS

Table 2 Materials required for RNA isolation

Item	Source
Equipment	
Water bath or heat block at 55–60°C	MLS
Reagents	
Isopropanol	MLS
Ethanol, 75%	MLS
RNase-free water of 0.5% SDS	MLS
(Optional) RNase-free glycogen	MLS

Table 3 Materials required for DNA isolation

Item	Source
Reagents	
Ethanol, 100%	MLS
Ethanol, 75%	MLS
0.1 M sodium citrate in 10% ethanol	MLS
8 mM NaOH	MLS
HEPES	MLS

Table 4 Materials required for protein isolation

Item	Source
Equipment	
(Optional) Dialysis membranes	MLS
Reagents	
Isopropanol	MLS
Ethanol, 100%	MLS
0.3 M Guanidine hydrochloride in 95% ethanol	MLS
1% SDS	MLS

Sample requirements

- Perform RNA isolation immediately after sample collection or quick-freeze samples immediately after collection and store at –80°C or in liquid nitrogen until RNA isolation.

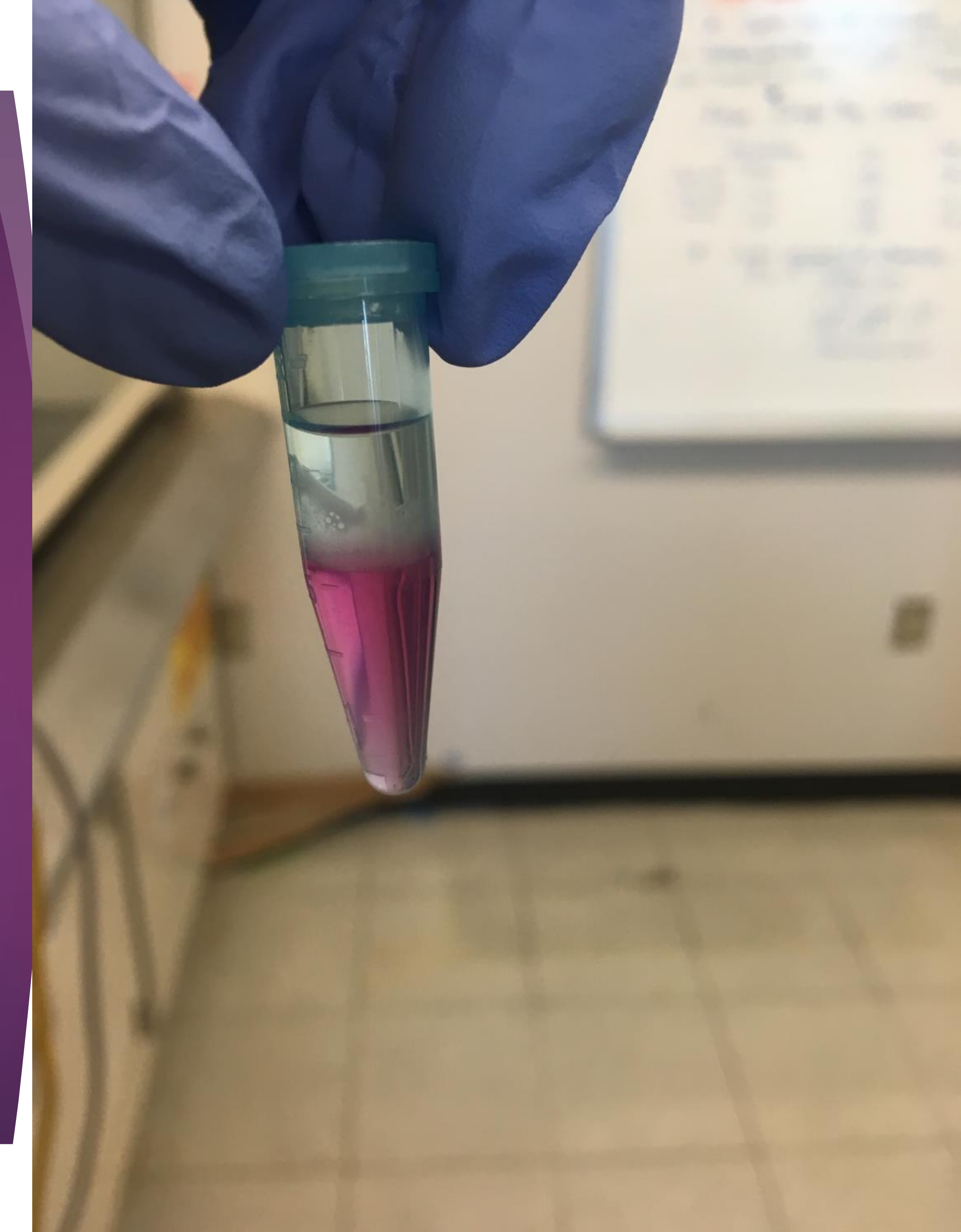
Sample type	Starting material per 1 mL of TRIzol™ Reagent
Tissues[1]	50–100 mg of tissue
Cells grown in monolayer	1×10^5–1×10^7 cells grown in monolayer in a 3.5-cm culture dish ($10\ cm^2$)
Cells grown in suspension	5–10 $\times 10^6$ cells from animal, plant, or yeasty origin or 1 $\times 10^7$ cells of bacterial origin

TRIzol reagent RNA extraction

- **TRIzol** (or TRI Reagent) is a monophasic solution of **phenol** and **guanidinium isothiocyanate** that simultaneously solubilizes biological material and denatures protein.
- **High quality total RNA, DNA and proteins**
- From cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour.
- Inhibition of **RNase** activity

TRIzol reagent RNA extraction

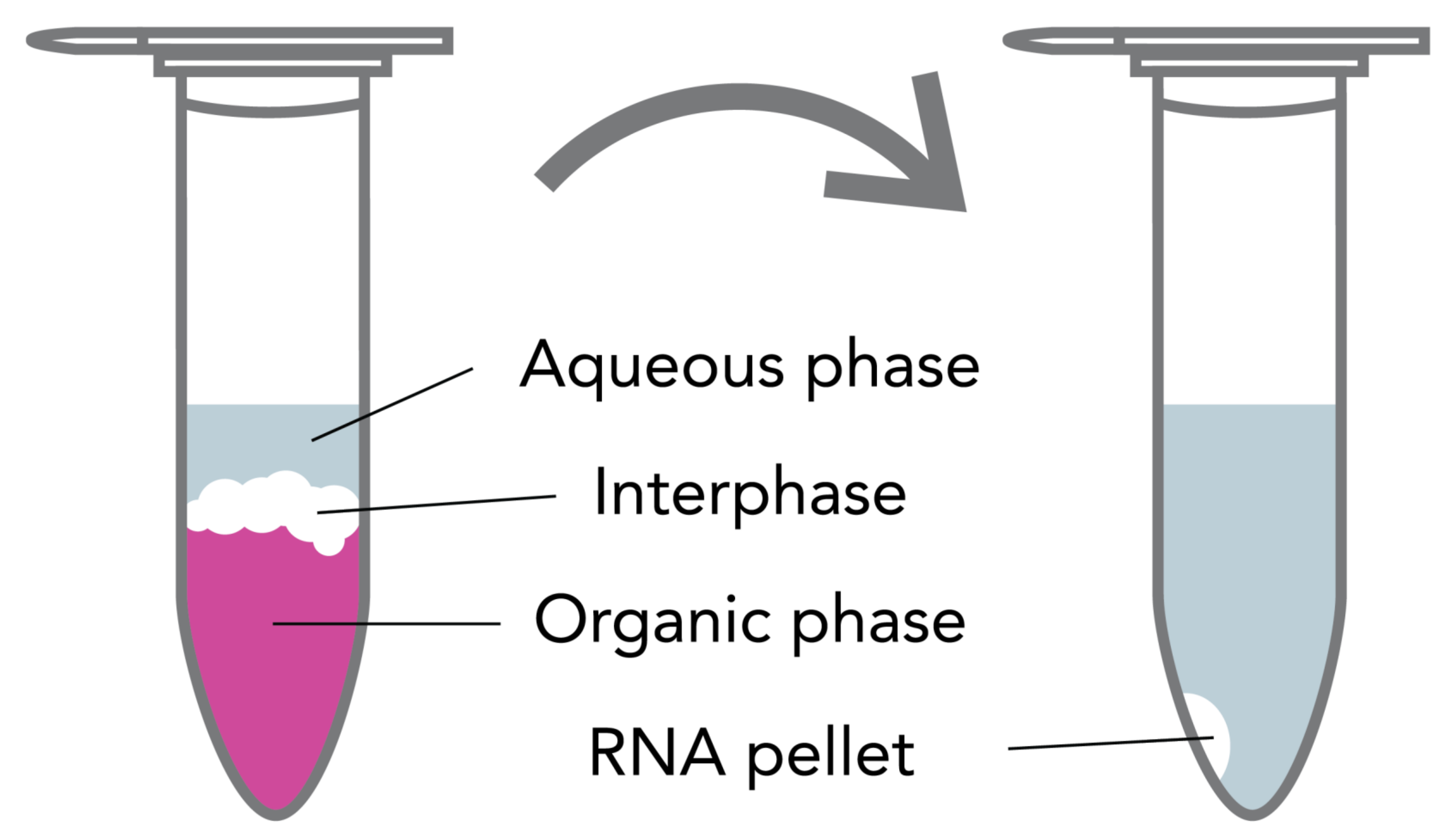
- Clear upper aqueous layer (containing RNA)
- Interphase, and a red lower organic layer (containing the DNA and proteins)
- **RNA** is precipitated from the aqueous layer with **isopropanol**.
- **DNA** is precipitated from the interphase/organic layer with **ethanol**
- **Protein** is precipitated from the phenol-ethanol supernatant by isopropanol precipitation



TRIzol reagent RNA extraction

Phase separation

Isopropanol precipitation



Downstream applications

- Isolated **RNA** can be used in RT-PCR, **Northern Blot** analysis, Dot Blot hybridization, poly(A)+ selection, in vitro translation, RNAase protection assay, and molecular cloning.
- Isolated **DNA** can be used in PCR, Restriction Enzyme digestion, and **Southern Blots**.
- Isolated **protein** can be used for **Western Blots**, recovery of some enzymatic activity, and some immunoprecipitation.

Procedural guidelines

- Perform all steps at **room temperature (20–25°C)** unless otherwise noted.
- Use **cold TRIzol™ Reagent** if the starting material contains high levels of RNase, such as spleen or pancreas samples.
- Use disposable, individually wrapped, sterile plastic ware and sterile, disposable **RNase-free** pipettes, pipette tips, and tubes.
- Wear disposable gloves while handling reagents and RNA samples to prevent **RNase contamination** from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.
- Always use proper **microbiological aseptic techniques** when working with RNA.
- Use RNaseZap™ RNase **Decontamination Solution** (Cat. no. AM9780) to remove RNase contamination from work surfaces and non-disposable items such as centrifuges and pipettes used during purification.

RNA Pellet

a

b

How do we identify and detect a specific sequence in a genome?

- SPECIFICITY
- AMPLIFICATION

TWO BIG ISSUES:

- There are a LOT of other sequences in a genome that we're not interested in detecting. (SPECIFICITY)
- The amount of DNA in samples we're interested in is VERY small. (AMPLIFICATION)

- Figuring 2 characters per base pair (bp), that came to 1,288 bp per page. To accommodate the 3 billion bp of the human genome at that size would therefore amount to about **2,329,000 pages**. That's a pretty thick book.

How many molecules do we need to be able to see them?

- To be visible on an **agarose gel**, need around **10 ng DNA**
- For a **500 bp** product band, weighing **660 g/mol.bp**, therefore need 10e-9 / (500*660) = 3.03×10^{-14} moles
- One base pair = **660 g/mol**
- Avogadro's number = $\mathbf{6.023 \times 10^{23}}$ per mol
- Therefore need **1.8e10 copies**!

- In other words, to "see" **a single "gene"**, the DNA in a sample of **100 cells** would have to be **multiplied 180 million times**!!!!!

Some biology points!

- **Rate**: replication or transcription or translation (Translation rate: 20 acid amin/ second)
- **Ratio**: Protein/mRNA or mRNA/DNA
- DNA transcription produces **a single-stranded RNA** molecule
- DNA contain **25000 genes** coding for **25000 different proteins**
- mRNA degrade after **3 min**
- **mRNA** accounts for only **1–5% of the total** cellular RNA although the actual amount depends on the cell type and physiological state.
- Approximately **360,000 mRNA molecules** are present in **a single mammalian cell**, made up of approximately **12,000 different transcripts** with a typical length of around **2 kb**.
- **Polysome** and Ribosome
- On the average, **a single mRNA** is used to manufacture about **900 copies** of the corresponding **protein**

Some biology points!

- The rate of **DNA replication** varies from 0.2 to 1.2 micron/min, the average of **0.6 micron/min.**
- There are some **42 million protein molecules** in a simple cell.
- Most of the **proteins** assessed exist within a narrow range of between **1000 and 10,000 molecules**.
- **mRNA**, **rRNA**, and **tRNA** are the three main types of RNA involved in protein synthesis.
- In a typical mammalian cell, **mRNA** takes **~4%** of the **total RNA** mass and aside from **80% ribosomal RNA (rRNA**), other operational RNAs make up the rest.

RNA Pellet

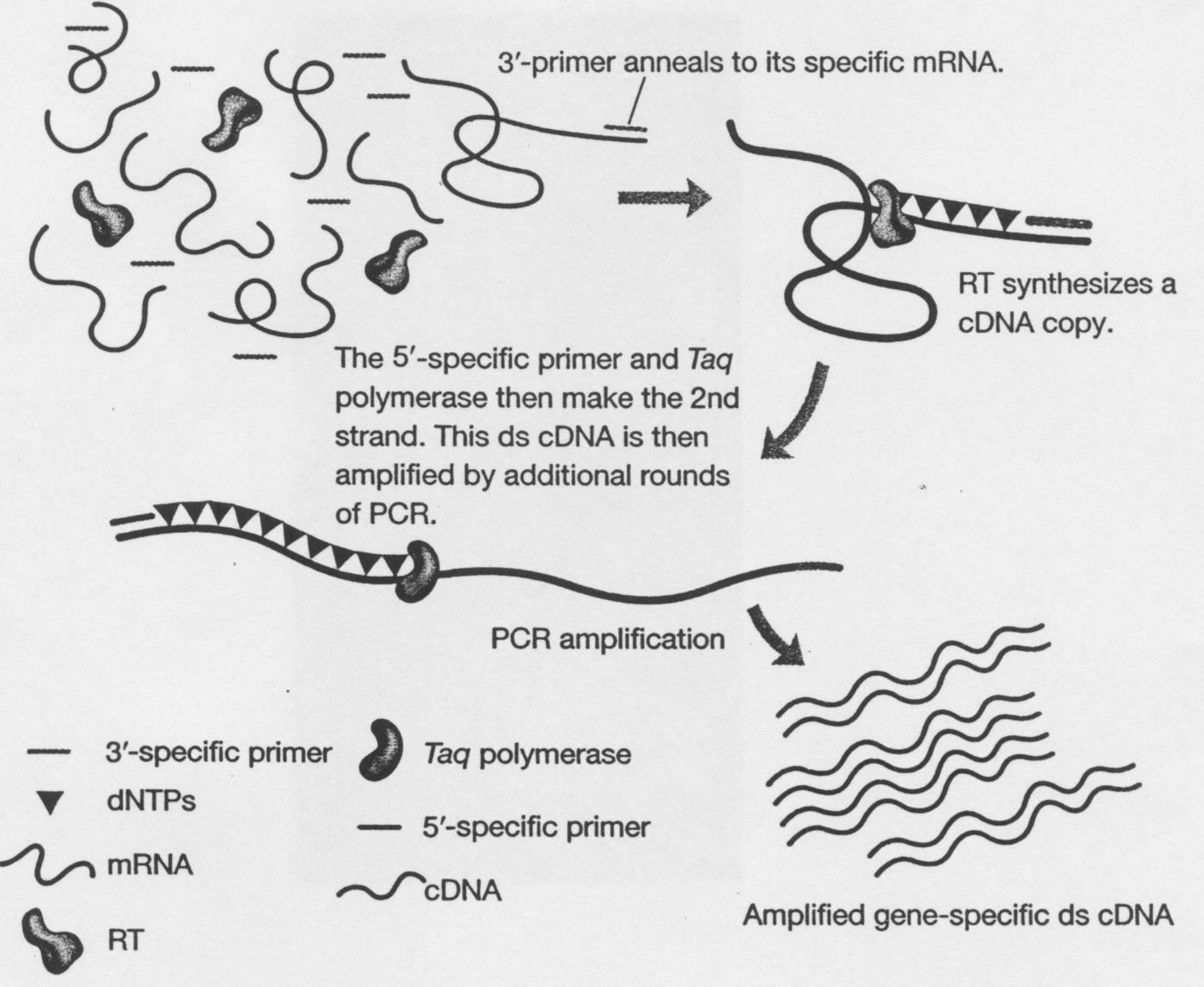
a

b

Evaluation

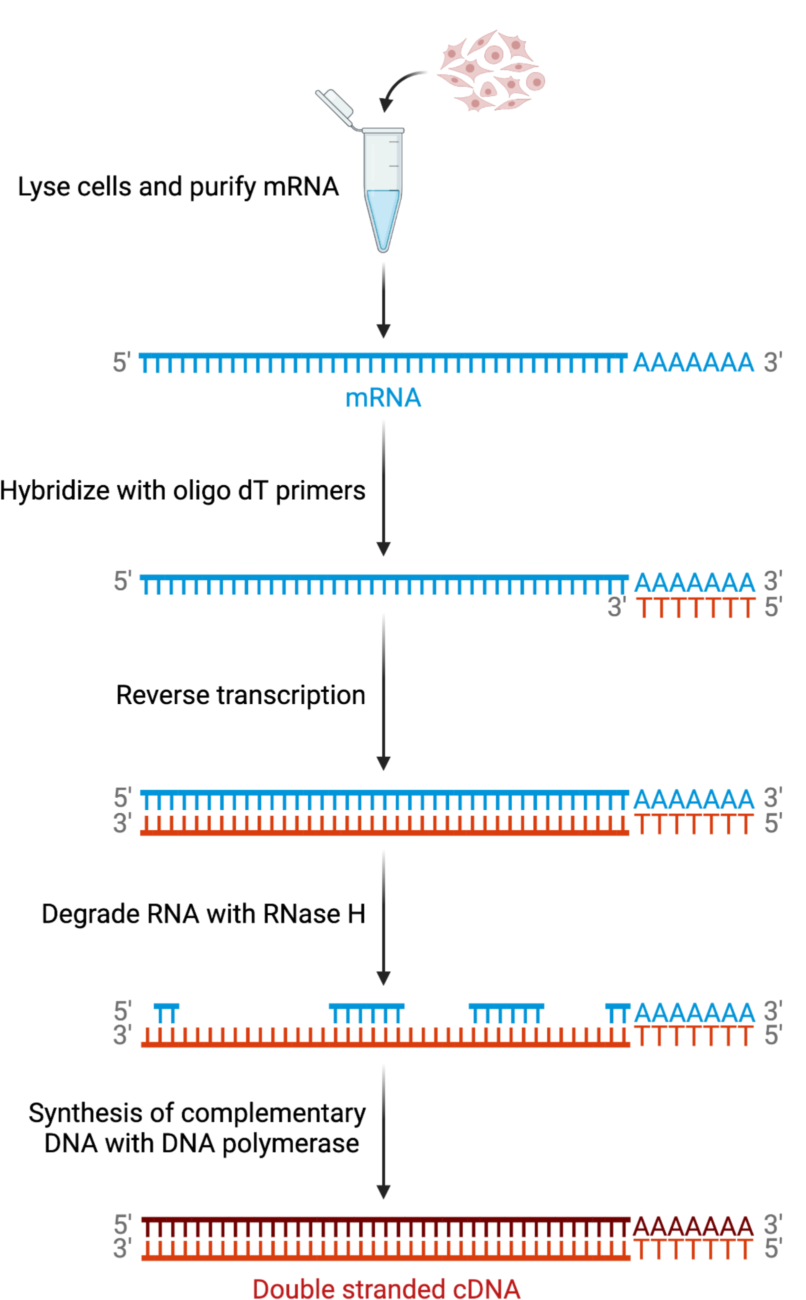
Read and Explain DNA isolation part of Thermo Fisher TRIzol Reagent guideline.

Synthesis of cDNA



Reverse Transcriptase–Polymerase Chain Reaction

cDNA Synthesis



cDNA synthesis kits

cDNA synthesis kits

Product Insert

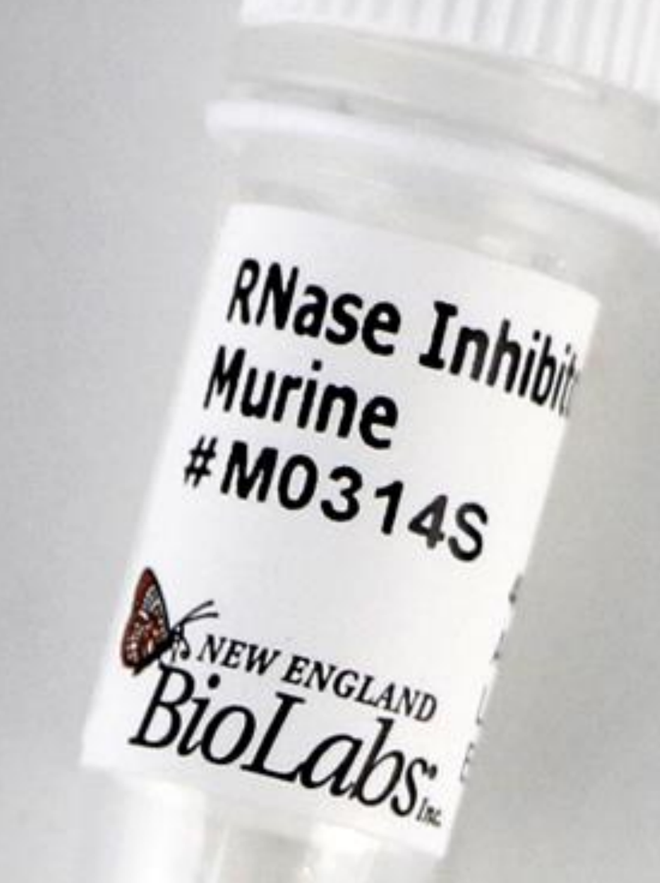
cDNA Synthesis Kit

LOT: See product label

EXPIRY DATE:

ORDERING INFORMATION

CAT. NO.	SIZE	PACKAGE CON
BR0400401	10 rxn of 20 µl	10 µl RevertUP™ II Reverse Transcriptase 100 µl 5× Reverse Transcriptase Buffer 25 µl dNTP Mix (10 mM each) 5 µl RNase Inhibitor 10 µl Hexamer Primer 5 µl Oligo (dT) Primer 1.5 ml PCR Grade Water



cDNA synthesis kits

COMPONENT	COMPOSITION
RevertUP II Reverse Transcriptase	RevertUP II Reverse Transcriptase, 200 U/μl in Storage buffer, containing 50% glycerol
5× Reverse Transcriptase Buffer	Optimized 5× Reverse Transcriptase buffer for cDNA synthesis
dNTP Mix (10 mM each)	Aqueous solution (pH 7.0) containing 10 mM each: dATP, dCTP, dGTP, dTTP sodium salts
RNase Inhibitor	RNase Inhibitor, 40 U/μl, in Storage buffer, containing 50% glycerol
Hexamer Primer	25 μM Random Hexamer Primer
Oligo (dT) Primer	10 μM Oligo (dT) Primer
PCR Grade Water	Ultrapure, sterile filtrated water, DNase-, RNase- and protease-free

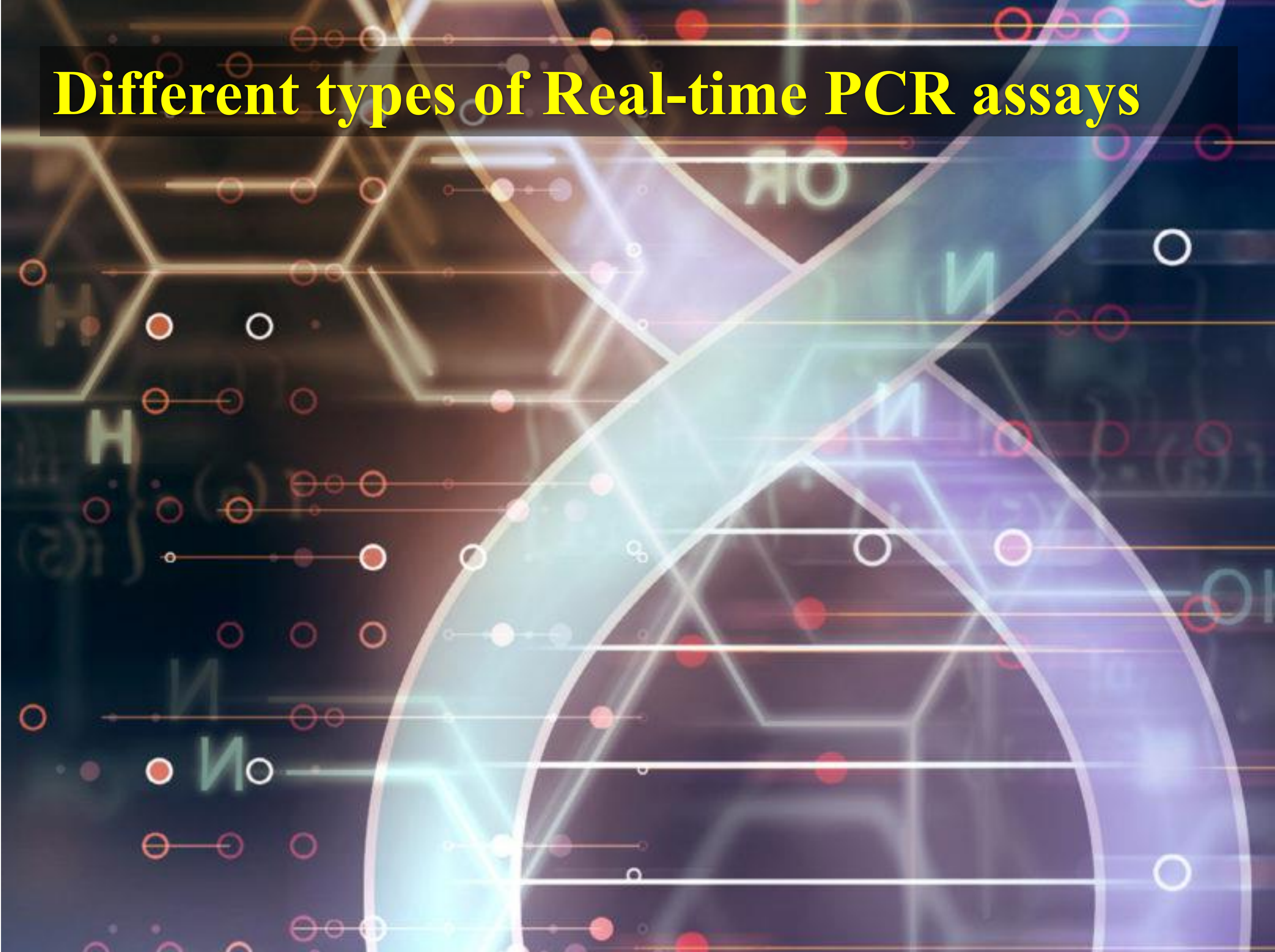
STORAGE -20°C (until expiry date – see product label)

cDNA synthesis kits

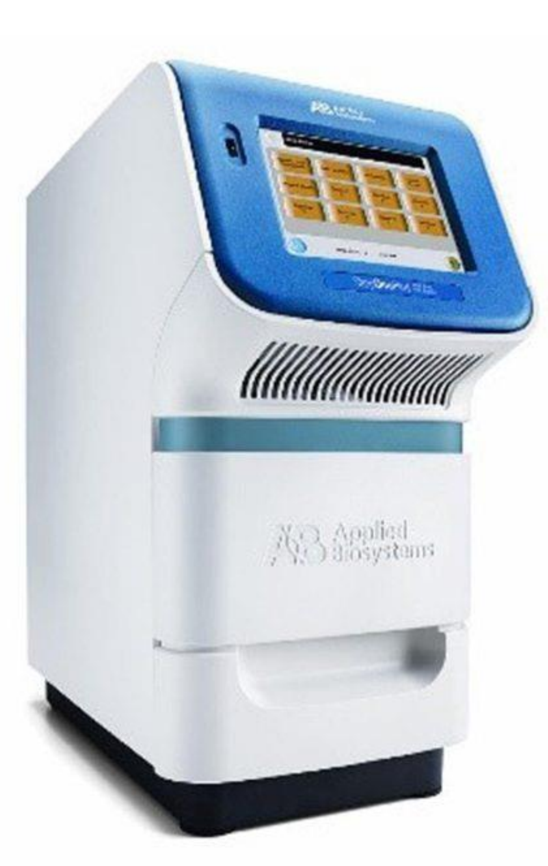
COMPONENT	VOLUME	FINAL CONCENTRATION
dNTP Mix (10 mM each)	2 µl	1 mM (each dNTP)
RNase Inhibitor, 40 U/µl (optional)	0.5 µl	1 U/µl
Oligo (dT)$_{12–18}$ (10 µM) – or *Hexamer Primer (25 µM) – or* *Gene Specific Primer (10 µM)*	*0.5 µl* *1 µl* *0.5 µl*	*0.25 µM* *1.25 µM* *0.25 µM*
5× Reverse Transcriptase Buffer	4 µl	1×
RNA Template	0.1–1 µg total RNA or 50–500 ng mRNA (polyA)	
RevertUP™ II Reverse Transcriptase	1 µl	10 U/µl
PCR Grade Water	Variable	
Total volume	20 µl	

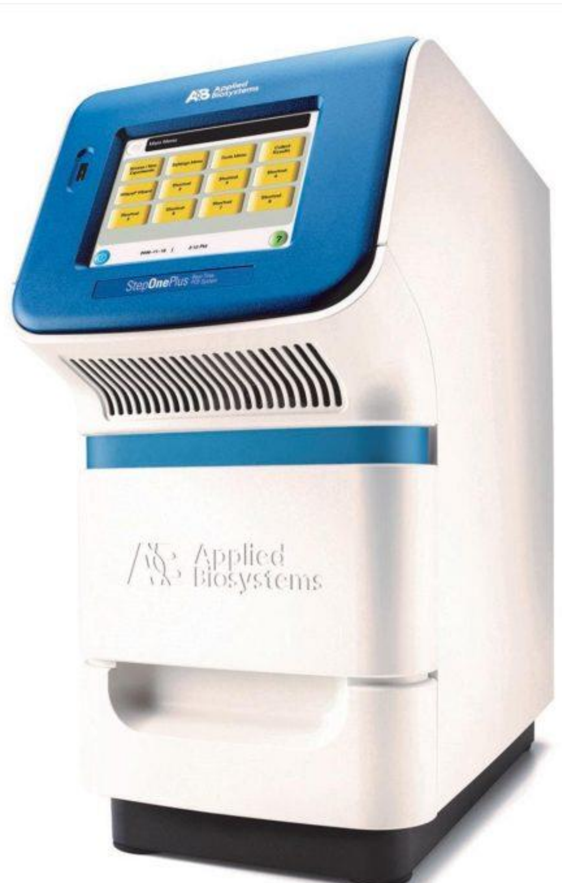
Evaluation

Search and Read different cDNA synthesis kit protocol


Different types of Real-time PCR assays

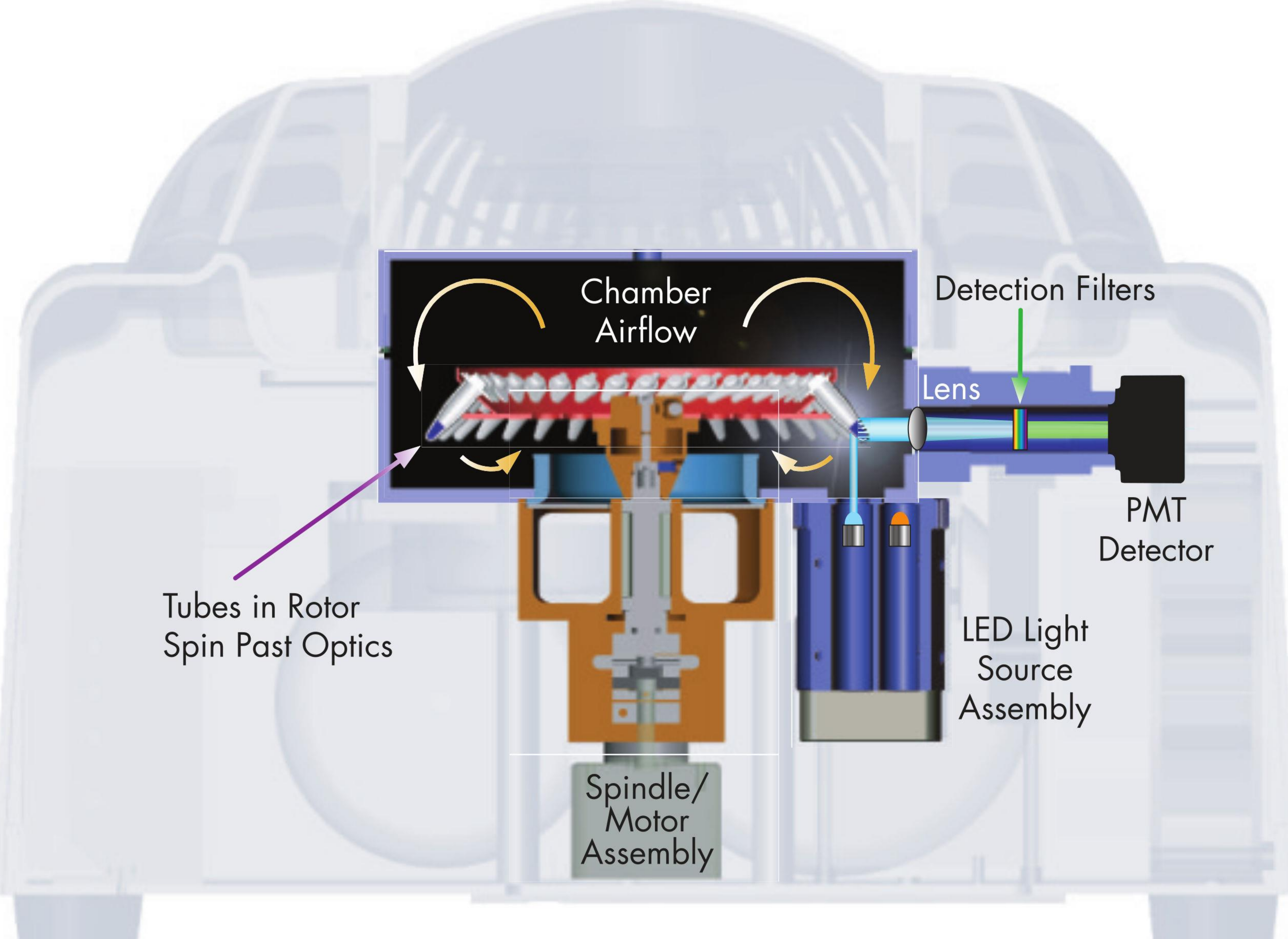
Real-time PCR





7500 fast Real time PCR

Roto-Gene Q Real time PCR


Chamber
Airflow
Detection Filters
Lens
PMT
Detector
Tubes in Rotor
Spin Past Optics
LED Light
Source
Assembly
Spindle/
Motor
Assembly

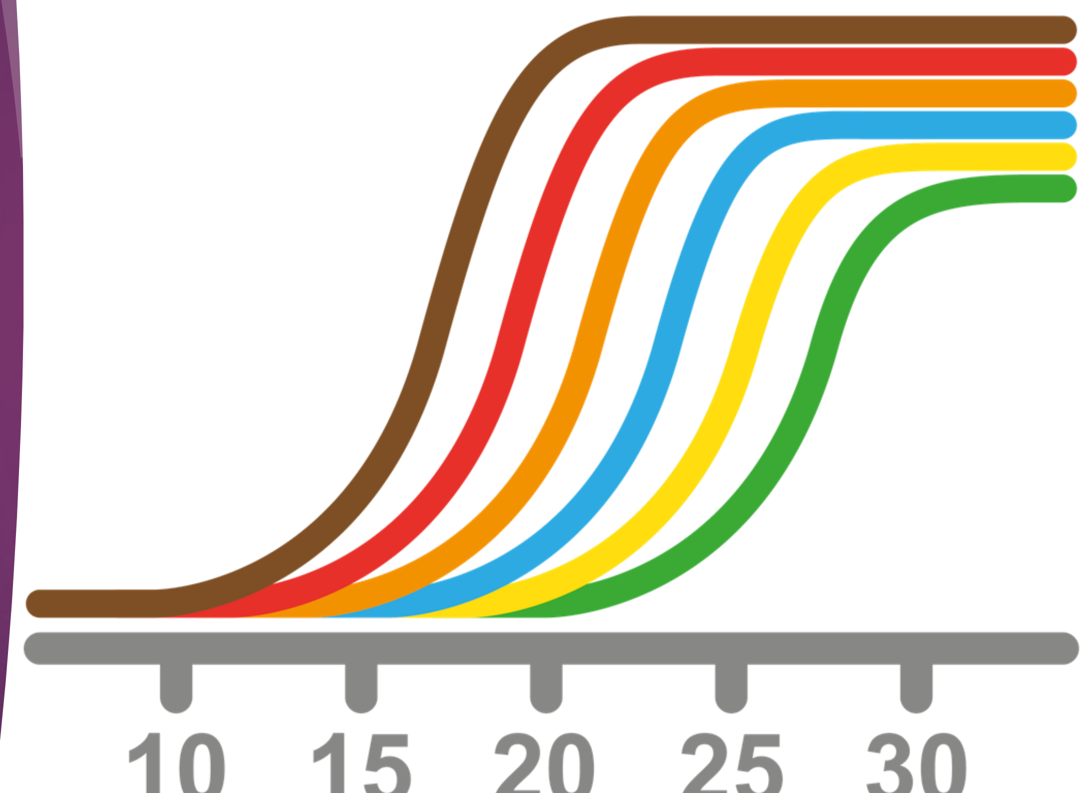
Master Mix qPCR

- Polymerase
- Buffer
- dNTPs
- MgCl2
- Fluorescent dye

double-stranded DNA (dsDNA)- binding dyes

Terminology

-Baseline
-Threshold
-Ct (threshold cycle)
-Standard curve
-Absolute vs relative quantification

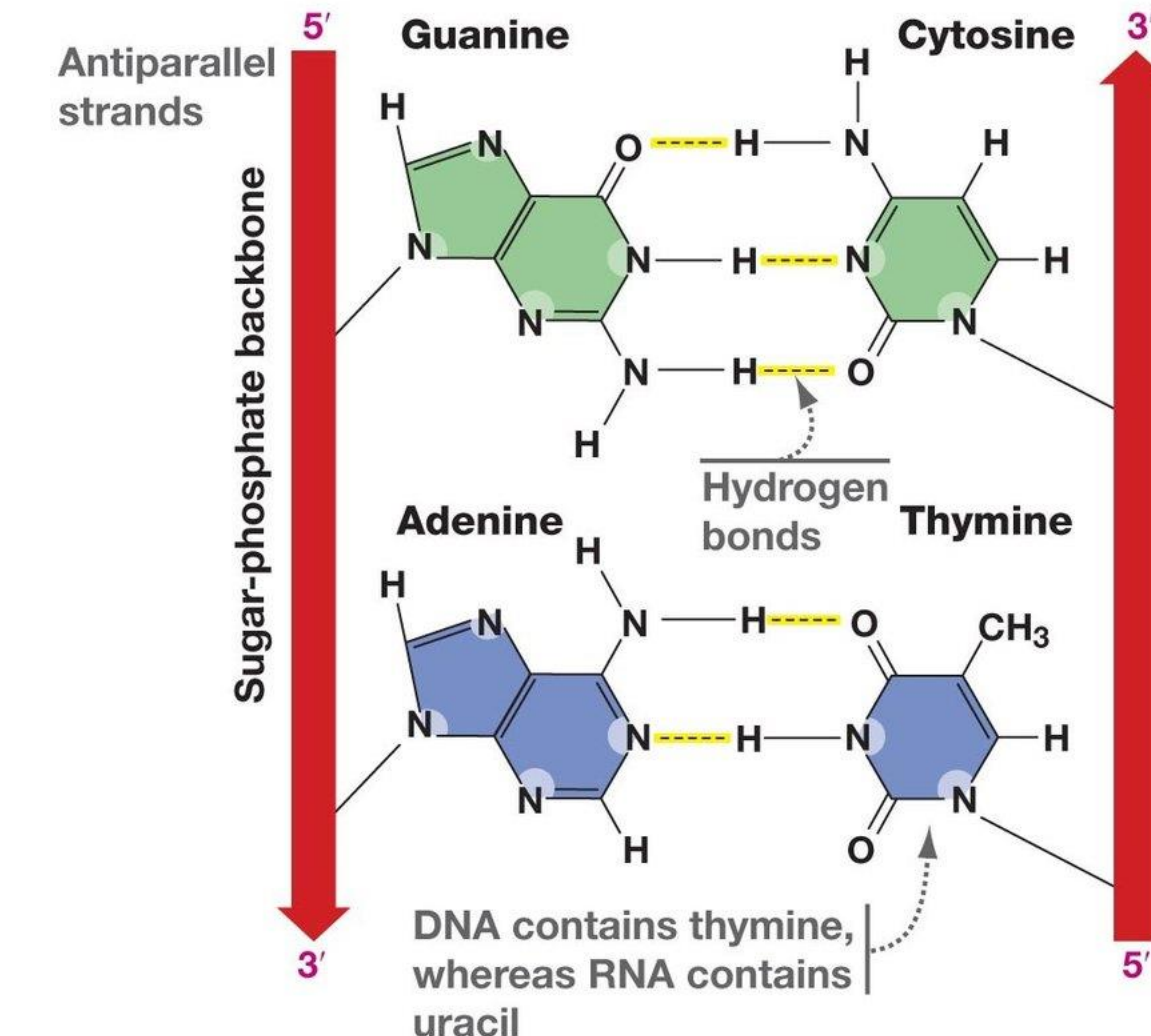


Overview of real-time PCR

DNA polymerase:
thermostable, nonspecific binding at low temperature
Reverse transcriptase: provides high yields of full-length cDNA, good activity at high temperatures
dNTPs:
purchase both the dNTPs and the thermostable DNA polymerase from the same vendor.
Magnesium concentration:
the optimal magnesium concentration may vary between 3 and 6 mM.
Good experimental technique:
cross-contamination problems. Triplicates,
prepare a master mix that contains all the reaction components except sample, pipetting errors.
Template:
Use 10 to 1,000 copies, 100 pg to 1 µg of genomic DNA, or cDNA generated from 1 pg to 100 ng of total RNA. Genomic DNA contamination. DNase I.

Overview of real-time PCR

- Good primer design:
- The amplicon length should be approximately 50–150 bp, since longer products do not amplify as efficiently. 18–24 nucleotides in length.
- specific for the target sequence and be free of internal secondary structure.
- avoid stretches of homopolymer sequences (e.g., poly (dG)) or repeating motifs, as these can hybridize inappropriately
- have compatible melting temperatures (within 5ÅãC) and contain approximately 50% GC content.
- the 3 end of the primer should be GC rich (GC clamp) to enhance annealing of the end that will be extended.
- avoid complementarity and hybridization between primers (primer-dimers).
- Optimal results may require a titration of primer concentrations between 50 and 500 nM. A final concentration of 200 nM for each primer is effective for most reactions.


Antiparallel strands
Sugar-phosphate backbone
5′
3′
3′
5′
Guanine
Cytosine
Adenine
Thymine
Hydrogen bonds
CH3
DNA contains thymine, whereas RNA contains uracil

Real-time PCR steps

1. **Denaturation**:

High temperature incubation is used to “melt” double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95 ˚c). The denaturation time can be increased if template GC content is high.

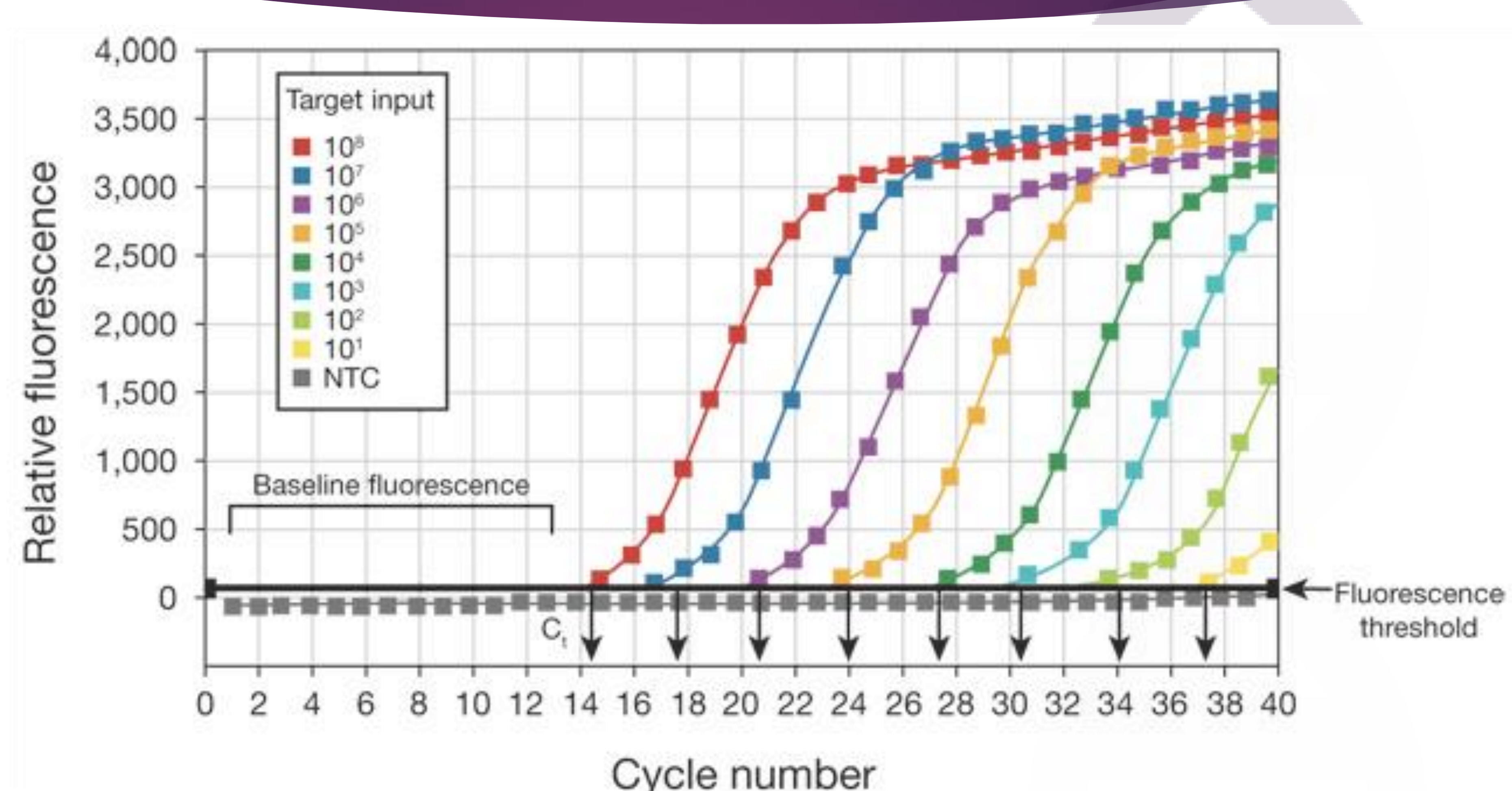
1. **Annealing**:

During annealing, complementary sequences have an opportunity to hybridize, so a appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5 ˚c below the Tm of the primer).

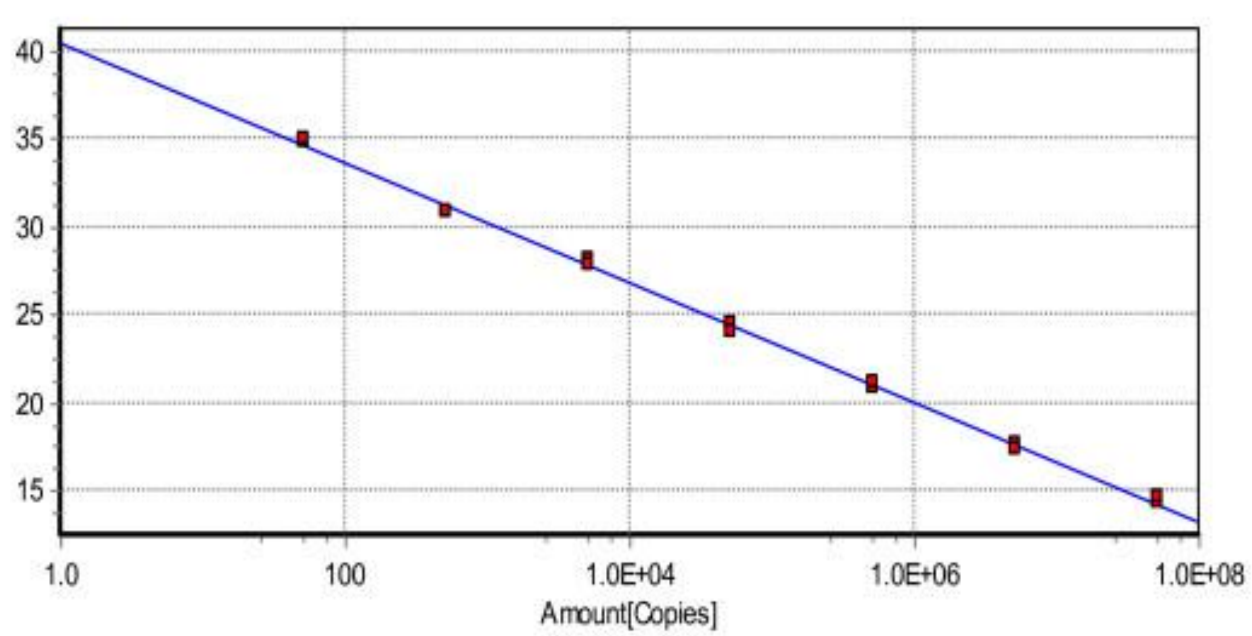
1. **Extension**:

At 70-72 ˚c, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60 ˚c as the temperature.

Relative fluorescence vs. Cycle number.



Standard curve



Slope: -3.395

Y-Intercept: 40.47

Efficiency: 0.97

R^2: 0.998

Standard curve parameters

- **Correlation coefficient (R2):**

  reflects the **linearity** of the standard curve.

- **Y-intercept**:

  the theoretical **limit of detection** of the reaction, or the Ct value expected if the lowest copy number of target molecules

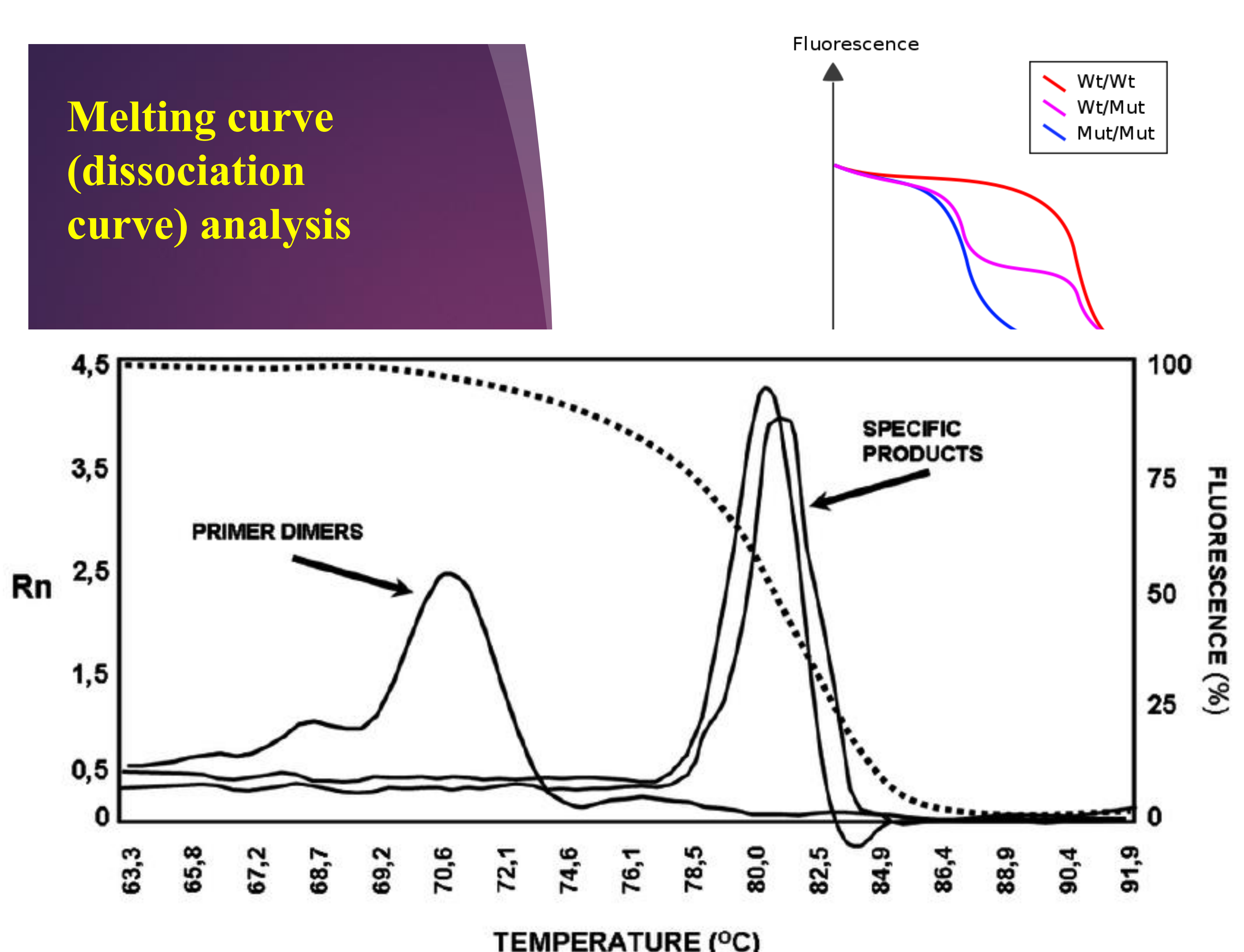
- **Slope**:

  measure of reaction **efficiency**.

- **Efficiency**:

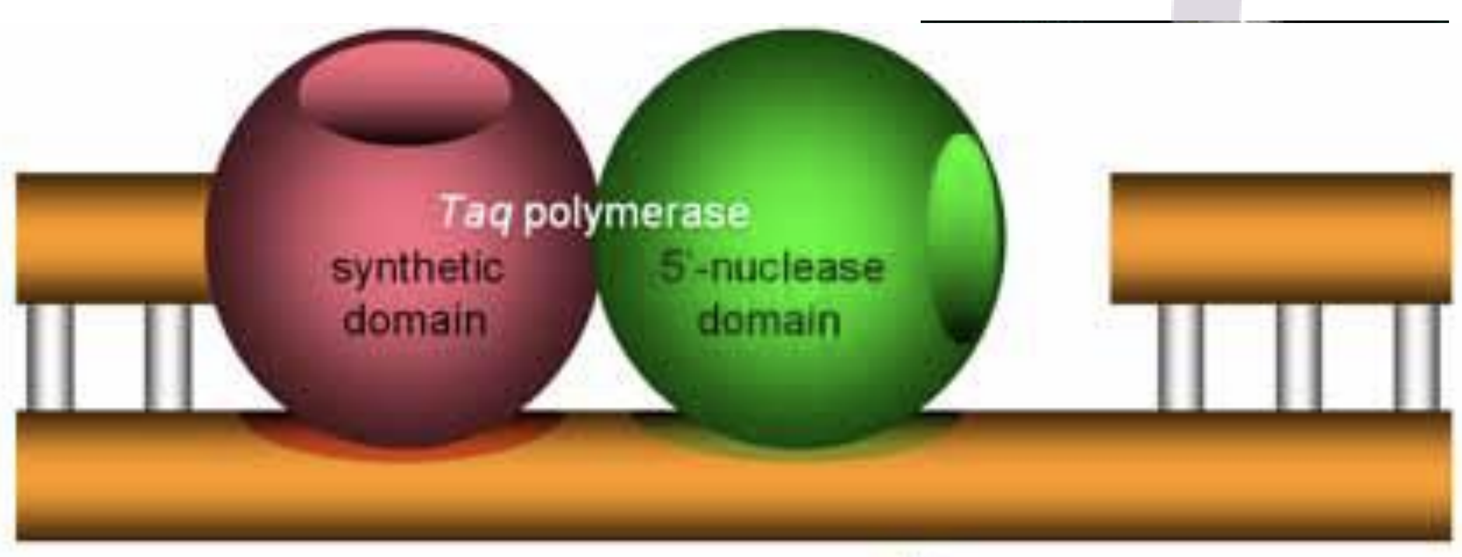
  the efficiency (E) of a PCR reaction should be **100%,** meaning the template **doubles** after each thermal cycle during exponential amplification.

  The presence of PCR inhibitors in one or more of the reagents can produce efficiencies of greater than 110%. A good reaction should have an efficiency **between 90% and 110%,** which corresponds to a **slope** of between **-3.58 and -3.10**.


Melting curve (dissociation curve) analysis
Fluorescence
Wt/Wt
Wt/Mut
Mut/Mut
4,5
3,5
2,5
1,5
0,5
0
Rn
100
75
50
25
0
FLUORESCENCE (%)
PRIMER DIMERS
SPECIFIC PRODUCTS
63,3
65,8
67,2
68,7
69,2
70,6
72,1
74,6
76,1
78,5
80,0
82,5
84,9
86,4
88,9
90,4
91,9
TEMPERATURE (ºC)

Real-time PCR fluorescence detection systems

the TaqMan. Assay and SYBR. Green dye-based assays

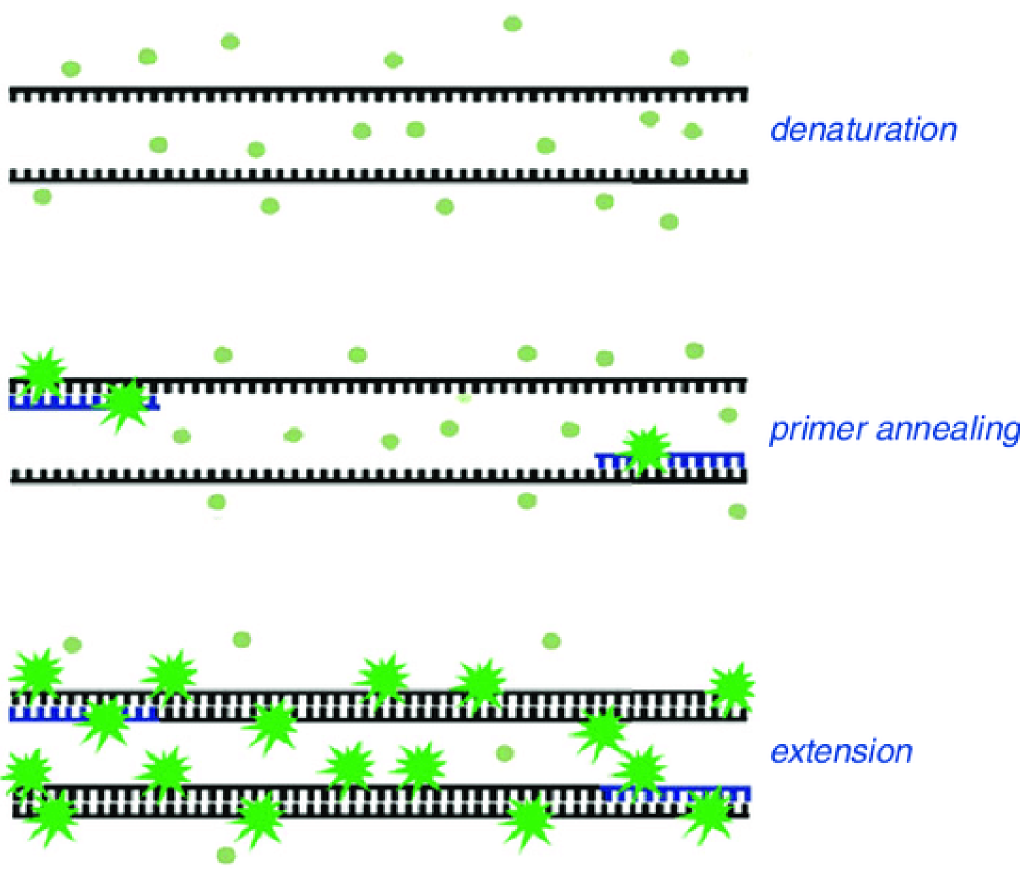


SYBR. Green dye-based assays

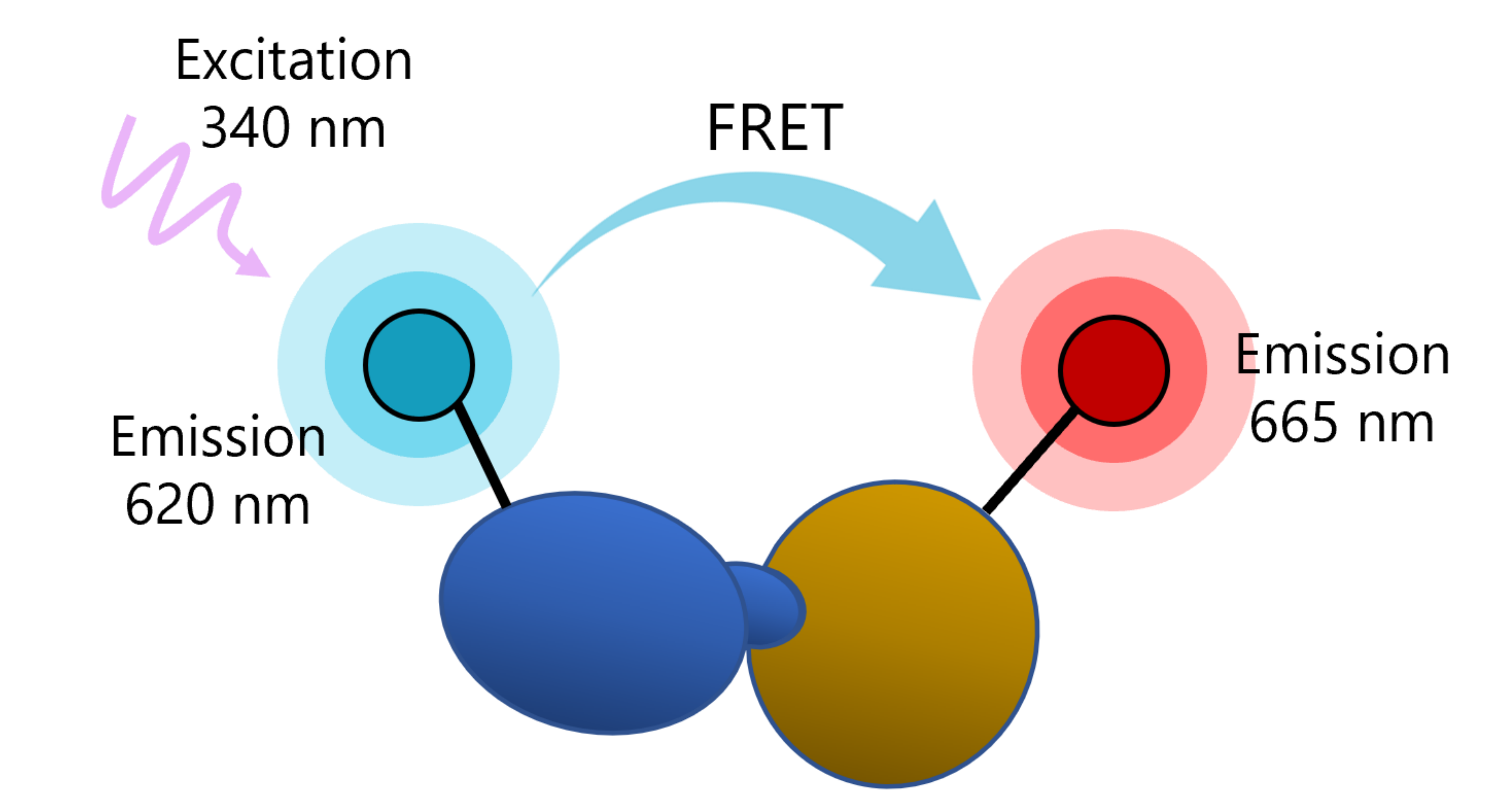
bind to any amplified product, target or non target

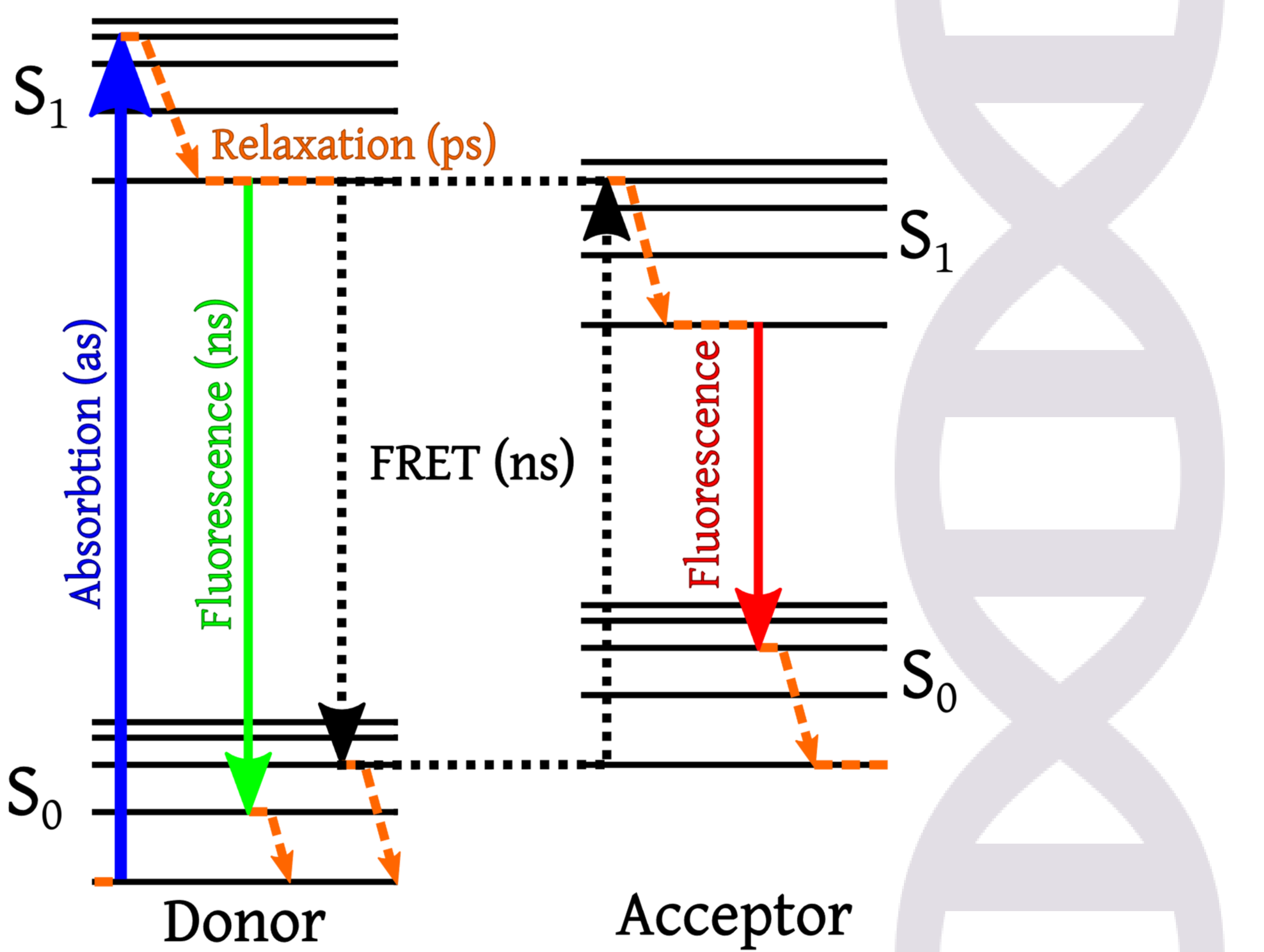
the dissociation analysis.

from target negative samples, sequencing or gels,

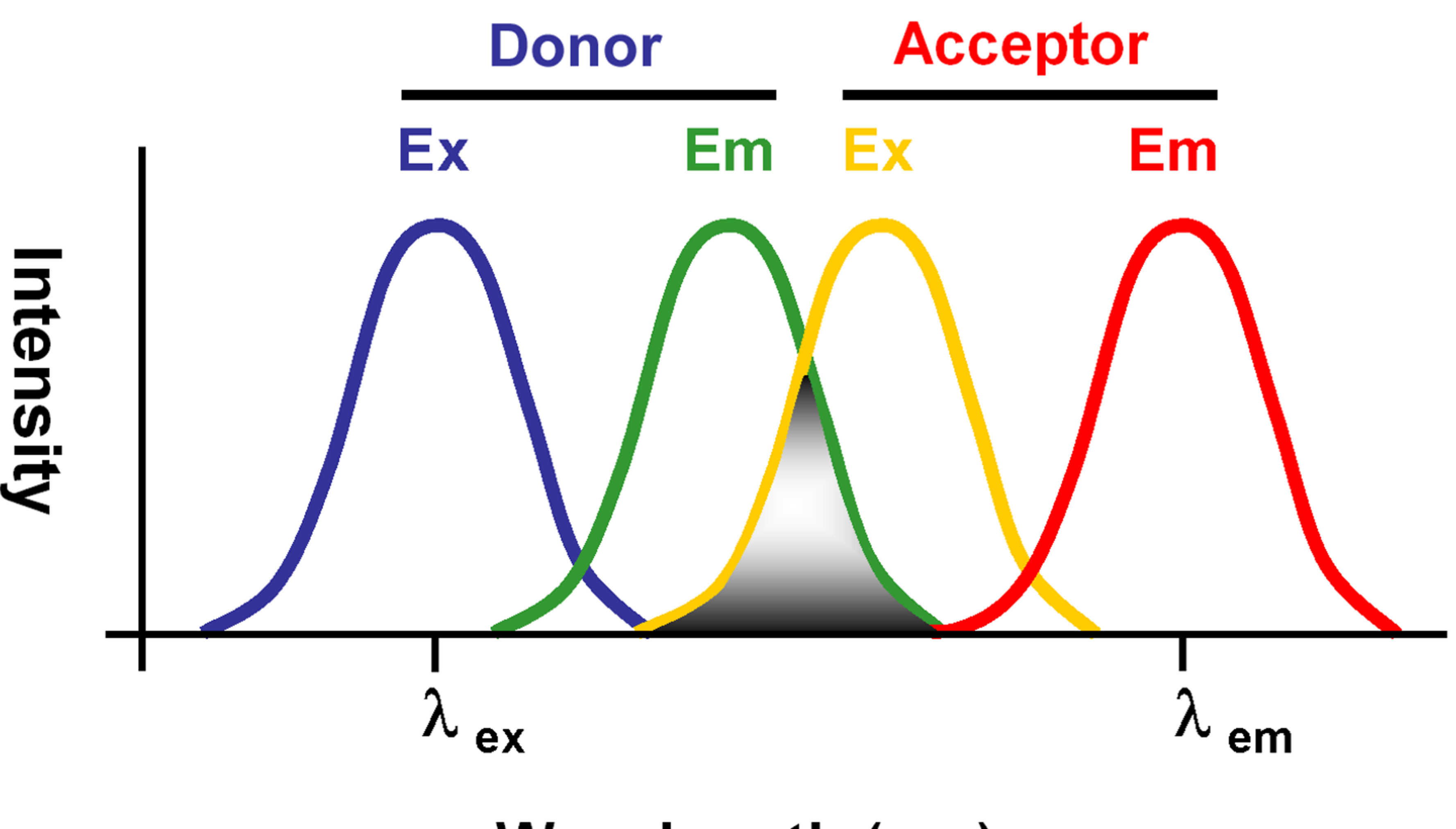


Förster resonance energy transfer




S1
Relaxation (ps)
Absorbtion (as)
Fluorescence (ns)
FRET (ns)
S1
Fluorescence
S0
S0
Donor
Acceptor

Förster resonance energy transfer



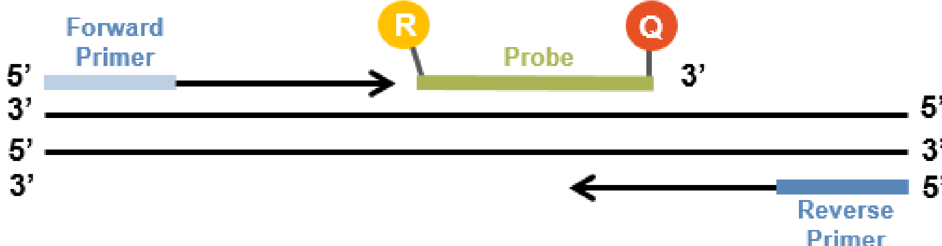
TaqMan assay

The TaqMan. probe has a gene-specific sequence and is designed to bind the target between the two PCR primers.

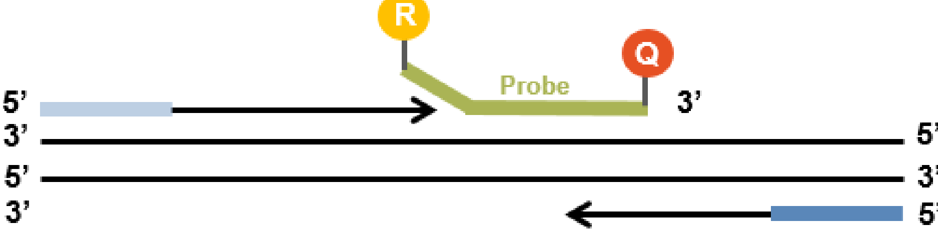
Specificity

Temperature

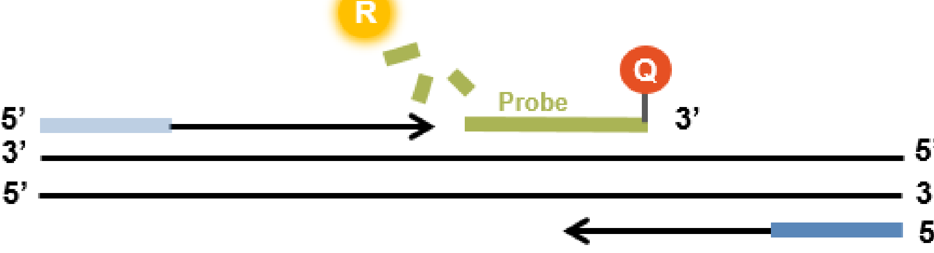
Polymerization



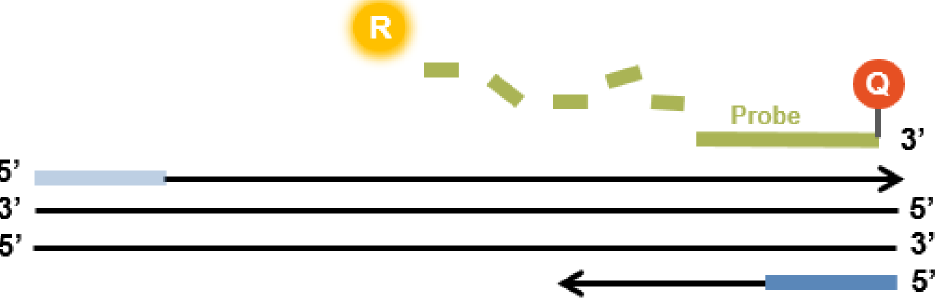
Strand Displacement



Cleavage



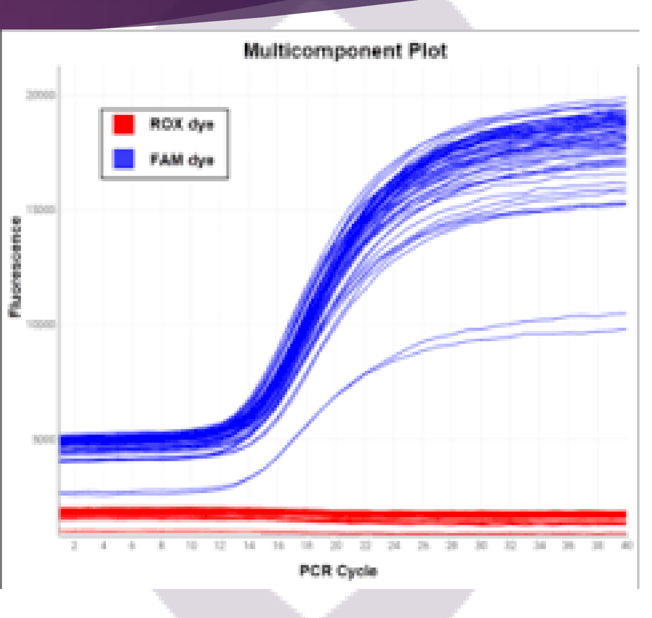
Cycle Complete



Use of passive reference dyes

- ROX™ dye

- Internal control
- Normalize for non-PCR related fluctuations in fluorescence (e.g., caused by pipetting errors)
- Normalize for fluctuations in fluorescence resulting from machine "noise"
- Compensate for variations in instrument excitation and detection
- Provide a stable baseline for multiplex real-time PCR and qRT-PCR



Contamination prevention

- Cross-contamination between samples
- Contamination from laboratory equipment
- Carryover contamination of amplification products and primers from previous PCRs. This is considered to be the major source of false positive PCR results

Uracil DNA glycosylase (UDG)

Internal controls and reference genes

- By using an endogenous control as an active reference, quantification of an mRNA target can be normalized for differences in the amount of total RNA added to each reaction.
- housekeeping genes:

  Ideally, the expression level of the chosen housekeeping gene should be validated for each target cell or tissue type to confirm that it remains constant at all points of the experiment.

Endogenous Controls

- β-actin (BACT): cytoskeletal gene
- 18S Ribosomal RNA (**rRNA**): ribosomal subunit
- Cyclophilin A (CYC): serine-threonine phosphatase inhibitor
- Glyceraldehyde phosphate dehydrogenase (**GAPDH**): glycolysis pathway
- β-2-microglobulin (**B2M**): major histocompatibility complex
- β-glucuronidase (GUS): exoglycosidase in lysosomes
- Hypoxanthine ribosyltransferase (HPRT): purine salvage pathway
- TATA-Box binding protein (TBP): RNA transcription

Troubleshooting common issues in PCR and qPCR

Troubleshooting

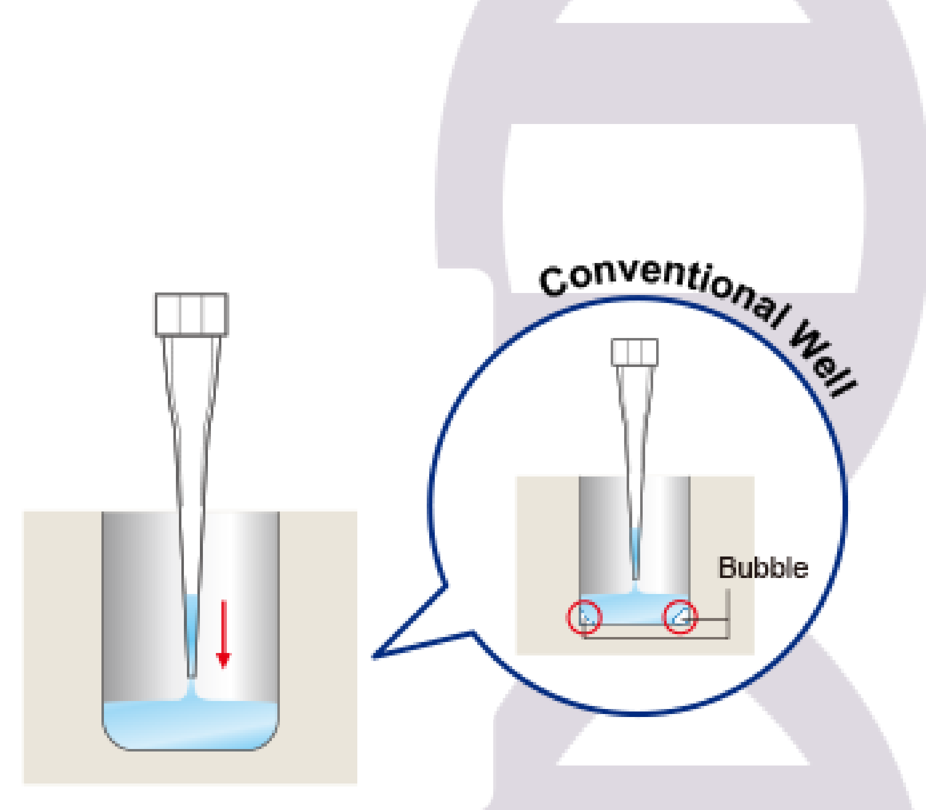
- No amplification
- Not target Amplicon
- Multiple amplicon
  - Contaminated reagents: during opening tubes.
  - Aliquot without changing tips
- Wrong size amplicon
  - Wrong template, primer, Blast search, specificity
- Hidden mutation

No amplicon

- Bad reagent?
- Bad Primers?
- Bad template?
- PCR inhibitors?

- **Bubbles**

- **Positive control**



No amplification, even Pos control

- Test primers, Reagents and sample
- Amplify with new reagent
- Check template quality and quantity($A_{260/280}$)
- Redesign the test primers

Reagents

- Using reagents that were sold separately from the polymerase
- Using reagent that are not completely thawed
- Thawed too many times
- Concentrations ($MgCl_2$, BSA)
- Old probe
- Polymerase
  - High GC template, long amplicons, secondary structures, etc.

Use and test different buffer and test your reaction with Gel electrophoresis

Primers

- Secondary structures
- Mismatch
- Dimer
- Genbank fail
- Annealing temperature
    - Too low or Too high
- Degraded (Dnase, Freeze/thaw)
- Small volume crystalize easily

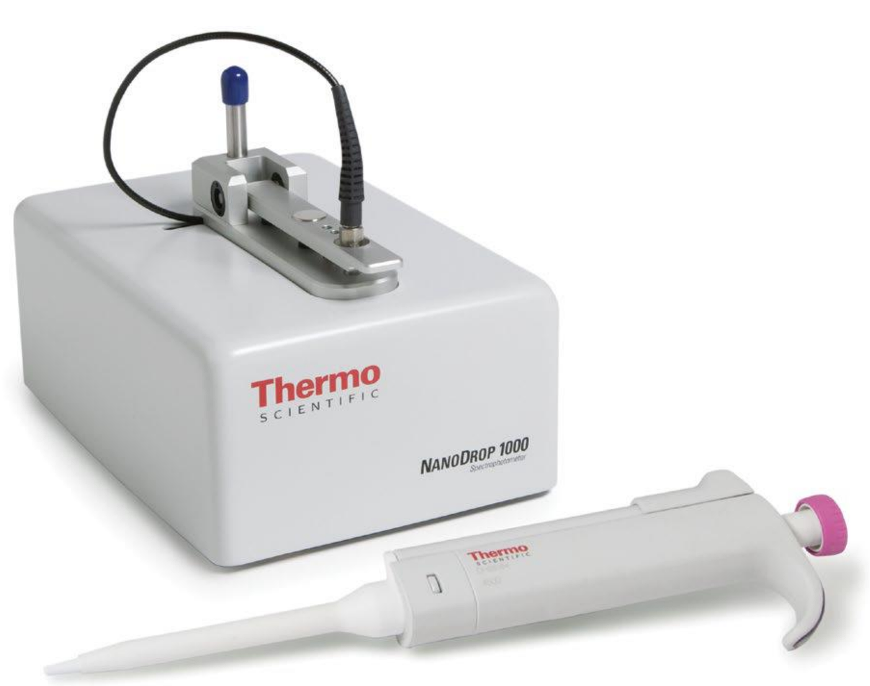
Te

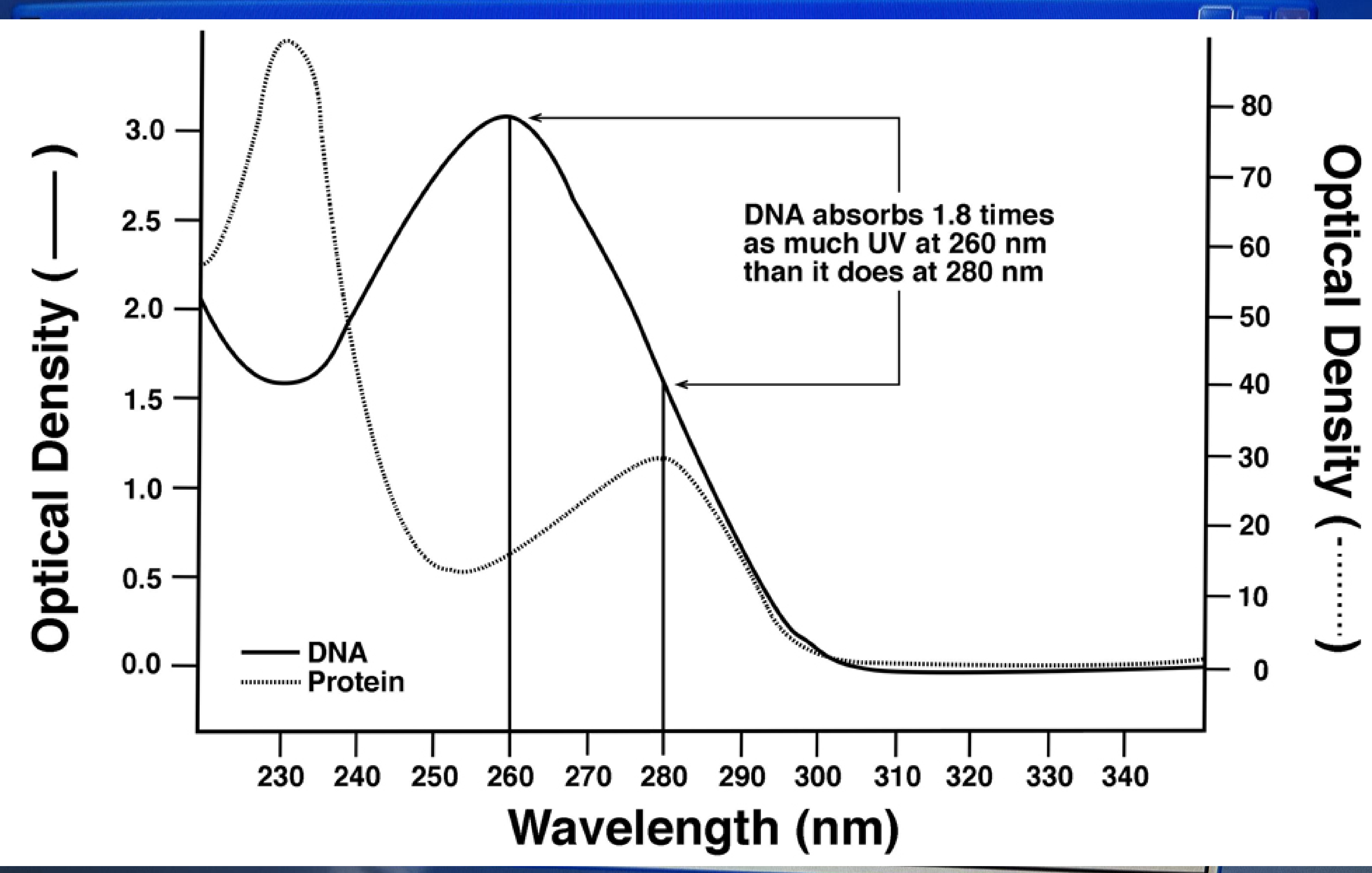
M 1 2 3 4 M

10000
8000
6000
5000
4000
3000
2500
2000
1500
1000
750
500
250

PCR inhibitors

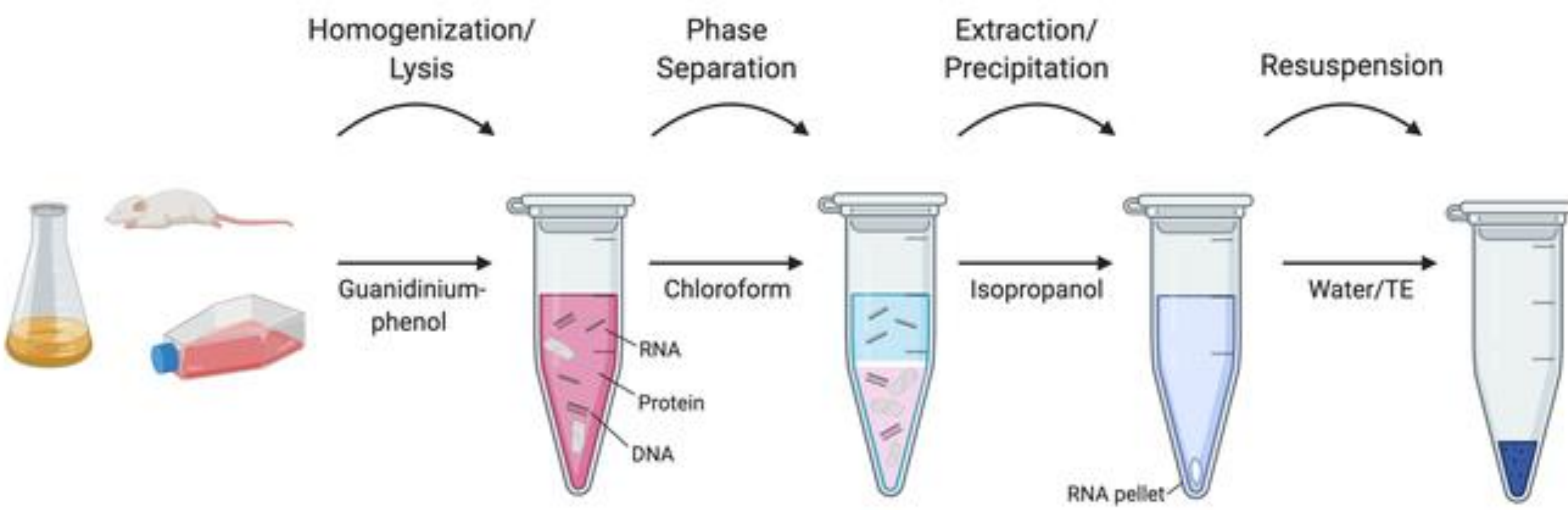
- Co-isolated with the template nucleic acid
- EDTA for Blood DNA extraction
- Heparin
- Phenol
- EtOH
- Silica
- SDS
- Heme
- Polysaccharide
- Acids inn soil
- LPS of bacteria
- Collagen of tissues




Optical Density (——)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DNA
Protein
DNA absorbs 1.8 times
as much UV at 260 nm
than it does at 280 nm
80
70
60
50
40
30
20
10
0
Optical Density (........)
230
240
250
260
270
280
290
300
310
320
330
340
Wavelength (nm)

Review

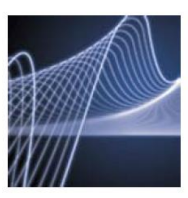
RNA extraction



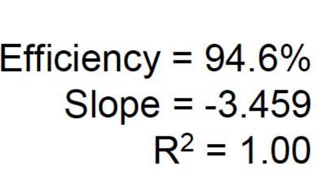
PCR components

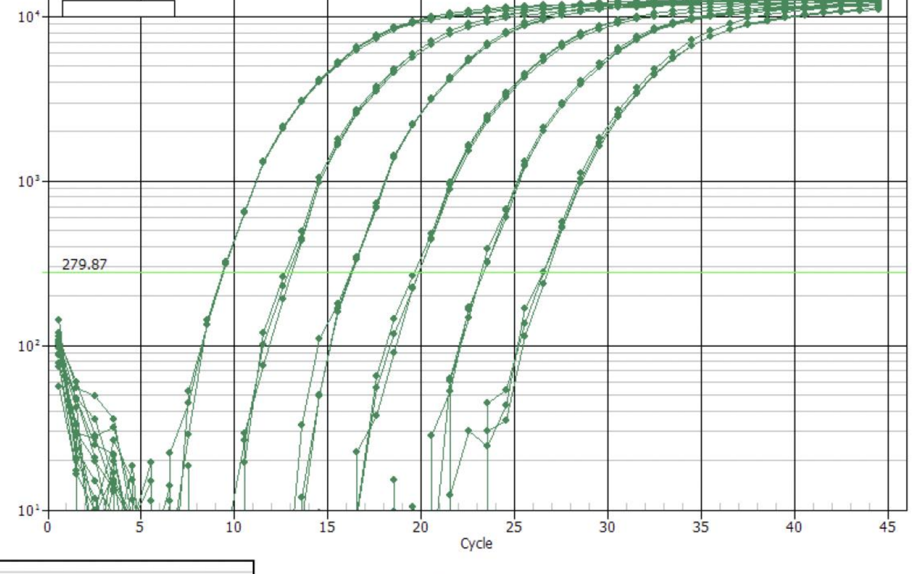
-Baseline
-Threshold
-Ct (threshold cycle)
-Standard curve
-Absolute vs relative quantification

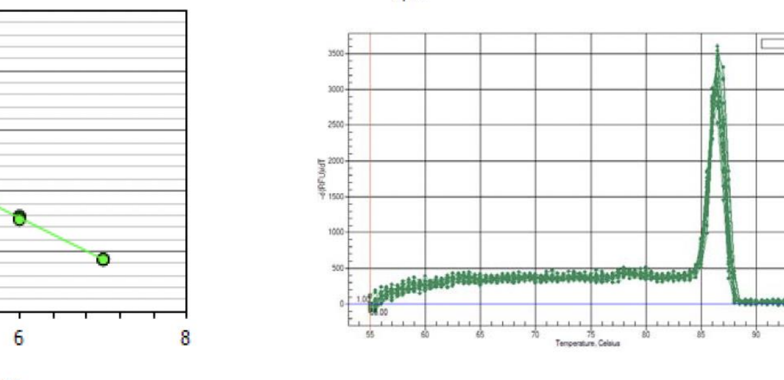
- RNA With Secondary Structure.
- The Exponential Amplification Phase
- The Initial Quantity Of Target
- DNA Or cDNA Template
- Copied, or “Amplified”
- PCR Product Molecules (Amplicons)
- NTC: No- Template Control
- Melting

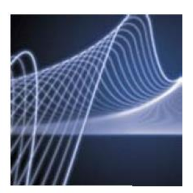


Standard Curve - Good Efficiency, and R^2



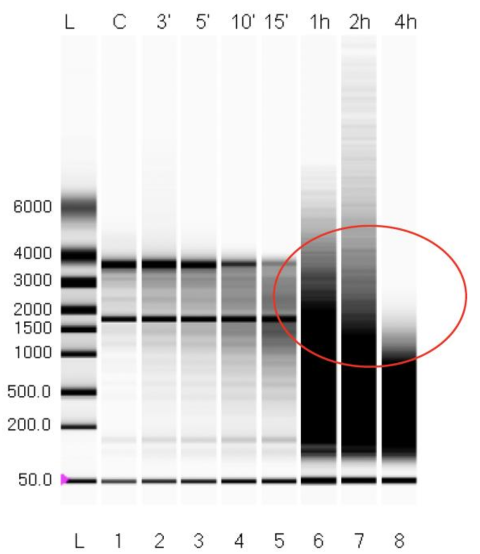






Analysis of RNA purity and integrity

Virtual Gel



Nanodrop Readings indicate all samples were "good"

Sample	A260/280*	A260/230*
1. Control-no heat	1.90	2.44
2. 3 min @ 90C	1.93	2.40
3. 5 min @ 90C	2.06	2.37
4. 10 min @ 90C	2.03	2.37
5. 15 min @ 90C	2.02	2.31
6. 1 hr @ 90C	1.99	2.18
7. 2hr @ 90C	2.00	2.32
8. 4 hr @ 90C	1.89	2.23

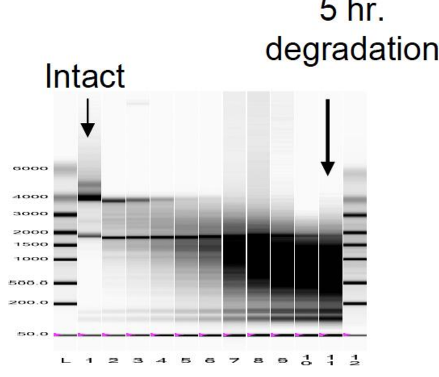
*Note: Generally accepted ratios (A260/280 and A260/230) for good quality RNA are > 1.8.

RQI Value & Color Coded Classification

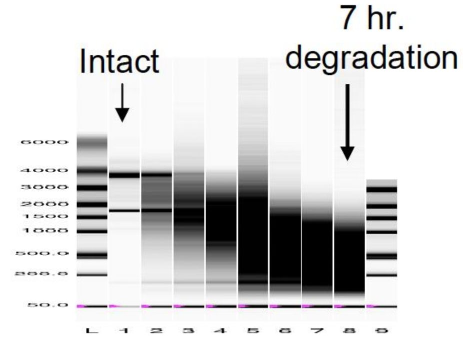
Well ID	Sample Name	RNA Area	RNA Concentration (ng/µl)	Ratio [28S/18S]	RQI	RQI Classification	RQI Alert
1	Control - no heat	278.23	102.60	1.60	9.8		
2	3 min @ 90C	317.12	116.94	1.23	9.2		
3	5 min @90C	306.89	113.17	0.89	8.1		
4	10 min @ 90C	257.56	94.98	0.50	6.5		
5	15 min @ 90C	247.31	91.20	0.15	5.9		
6	1 hour @ 90C	200.94	74.10	0.46	2.2		
7	2 hour @ 90C	252.37	93.07	0.81	2.0		
8	4 hour @ 90C	274.16	101.10	0.00	1.8		

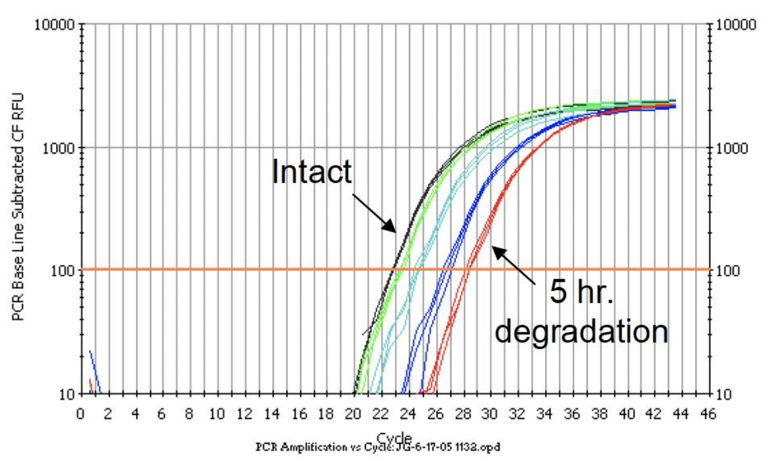
Total RNA Analysis

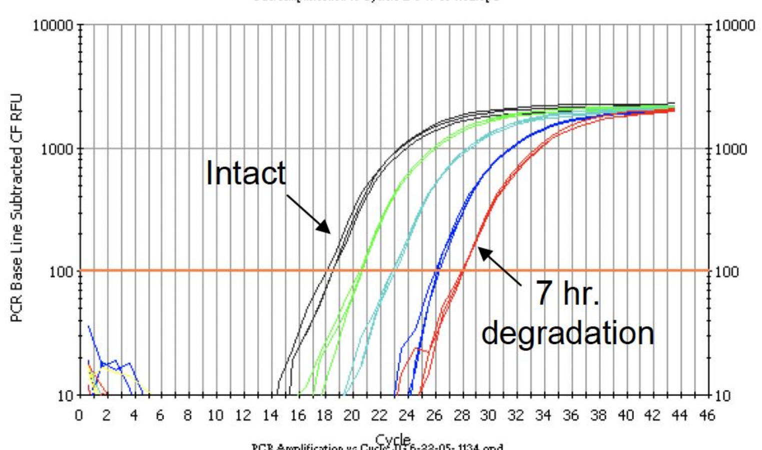
Normal Liver RNA



Carcinoma Liver RNA







qPCR: GAPDH gene

STUDENT WORKSHEET

To be completed prior to beginning the protocol.

Name: ____________________

Date: ____________________

Number of DNA samples to be used: __________

Gene of interest: ____________

_______ DNA samples + 1 for Neg. Control = ____**tubes**

_______ **tubes** + 1 for error = ___ **(MULTIPLIER for master mix)**

TABLE 1 — Quantities of Components to Add to Each Tube

Component	µL per reaction	MULTIPLIER	µL in master mix
Taq Polymerase Mix	12.5		
Molecular biology grade H_2O	9.5		
Primer Mix (eg ACE-F/R)	1		
Total PCR master mix volume	**23.0 µL**		**µL**

TABLE 2 — Labels for PCR Microcentrifuge Tubes

PCR Strip Tube Label	DNA Sample	Primer Mix

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D

Downloaded from https://academic.oup.com/clinchem/arti

Lab book

Thanks for your nice attention

Abdolvahid Sadeghnejad

Sadeghnejad.abdolvahid@gmail.com

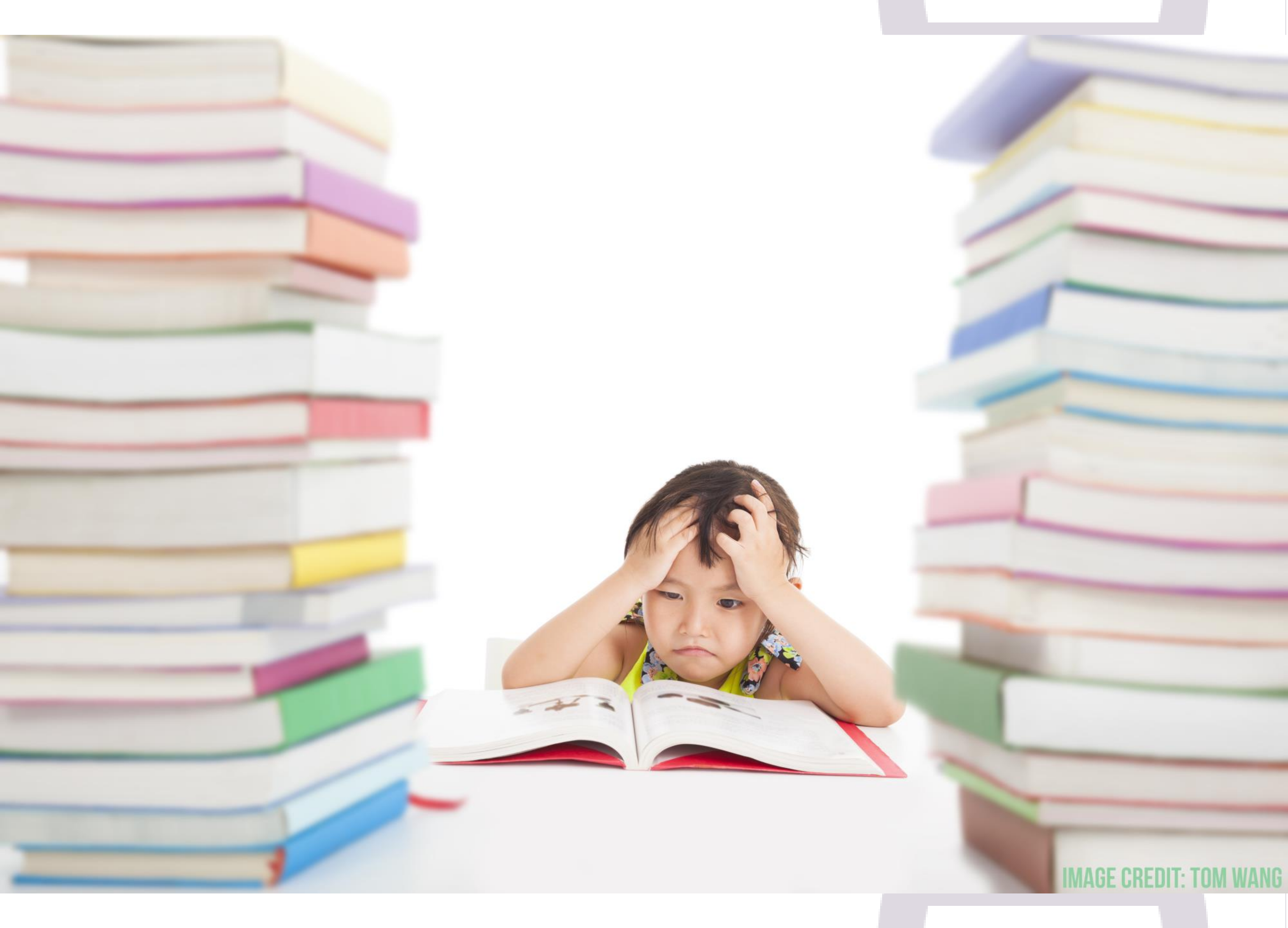
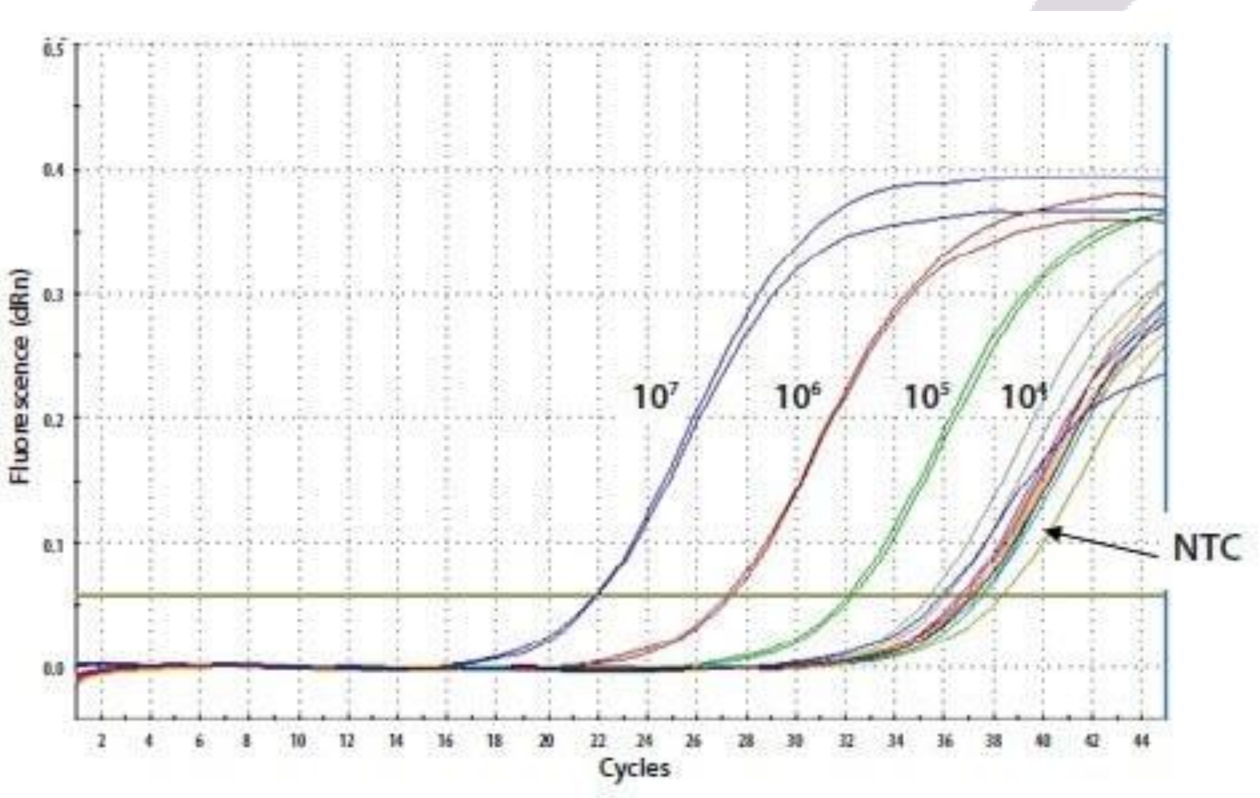


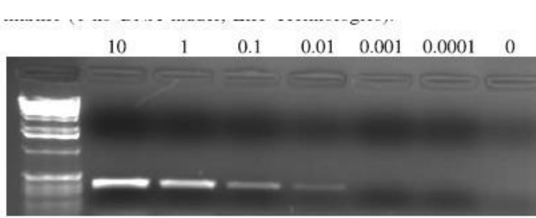
IMAGE CREDIT: TOM WANG

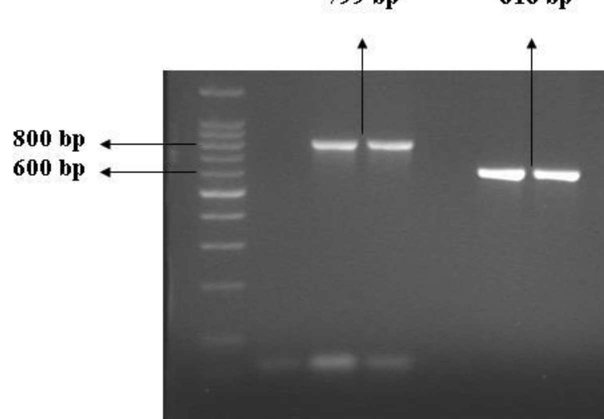
Tools for Design

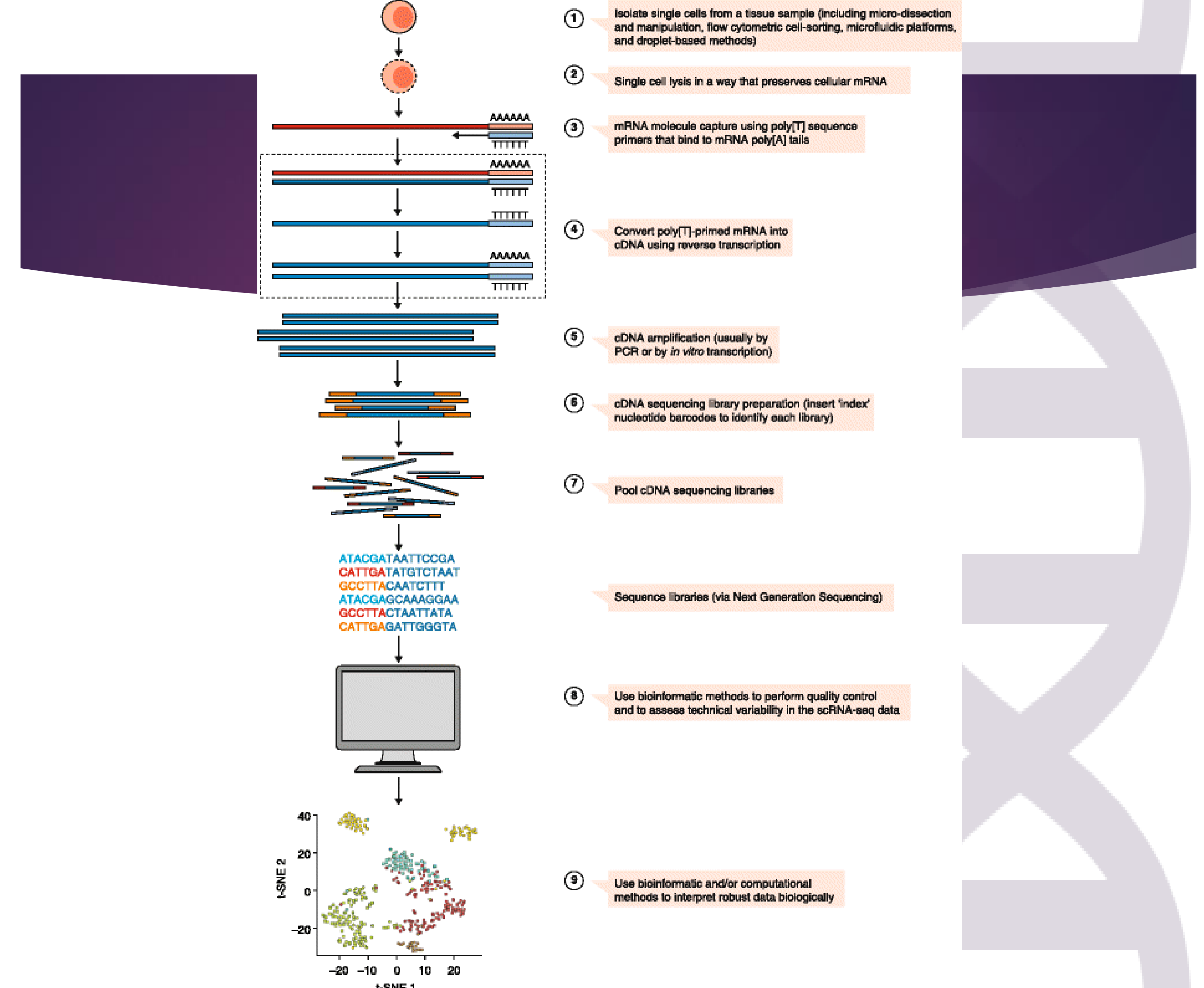
- OligoArchitect
  http://www.sigmaaldrich.com/life-science/custom-oligos/dna-probes/product-lines/probe-design-services.html
- Beacon Designer
- NCBI GenBank http://www.ncbi.nlm.nih.gov/nuccore
- Primer3 http://frodo.wi.mit.edu/primer3/
- RTPrimerDB http://medgen.ugent.be/rtprimerdb/
- PrimerBank http://pga.mgh.harvard.edu/primerbank/
- NCBI Primer Design Tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/
- Mfold webserver http://mfold.rna.albany.edu/
- Other


Fluorescence (dRn)
Cycles
10^7
10^6
10^5
10^4
NTC
0.5
0.4
0.3
0.2
0.1
0.0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44

Digital PCR


10
1
0.1
0.01
0.001
0.0001
0


799 bp
616 bp
800 bp
600 bp


Isolate single cells from a tissue sample (including micro-dissection and manipulation, flow cytometric cell-sorting, microfluidic platforms, and droplet-based methods)
Single cell lysis in a way that preserves cellular mRNA
AAAAAA
TTTTTT
mRNA molecule capture using poly[T] sequence primers that bind to mRNA poly[A] tails
Convert poly[T]-primed mRNA into cDNA using reverse transcription
cDNA amplification (usually by PCR or by in vitro transcription)
cDNA sequencing library preparation (insert 'index' nucleotide barcodes to identify each library)
Pool cDNA sequencing libraries
ATACGATAATTCCGA
CATTGATATGTCTAAT
GCCTTACAATCTTT
ATACGAGCAAAGGAA
GCCTTACTAATTATA
CATTGAGATTGGGTA
Sequence libraries (via Next Generation Sequencing)
Use bioinformatic methods to perform quality control and to assess technical variability in the scRNA-seq data
Use bioinformatic and/or computational methods to interpret robust data biologically
t-SNE 2
t-SNE 1
40
20
0
−20
−20
−10
0
10
20
1
2
3
4
5
6
7
8
9