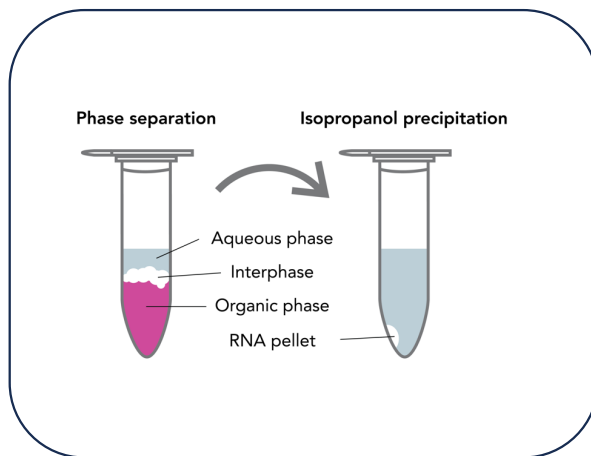


Gene Expression Analysis

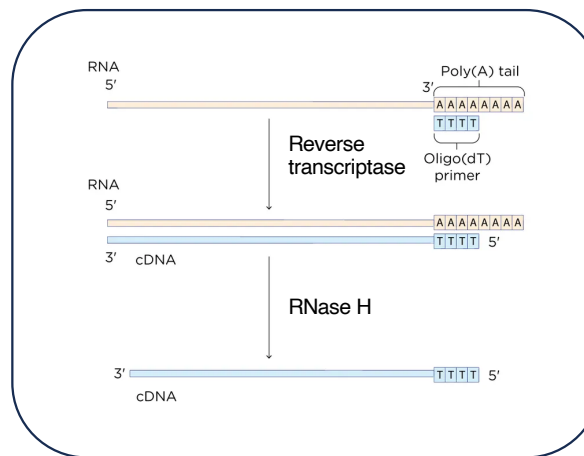
RT-PCR and Real-time PCR

RT-PCR e Real-time PCR

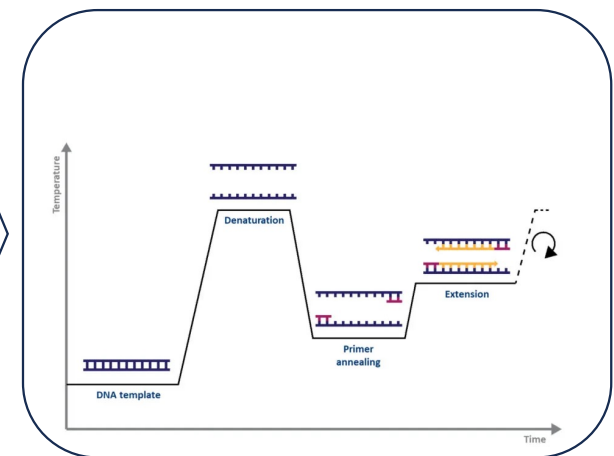
Total RNA isolation



Reverse transcription

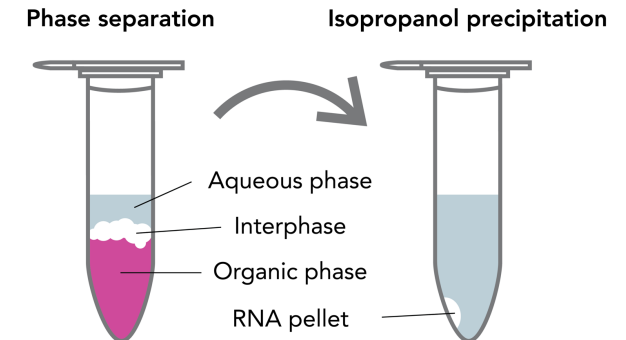


PCR



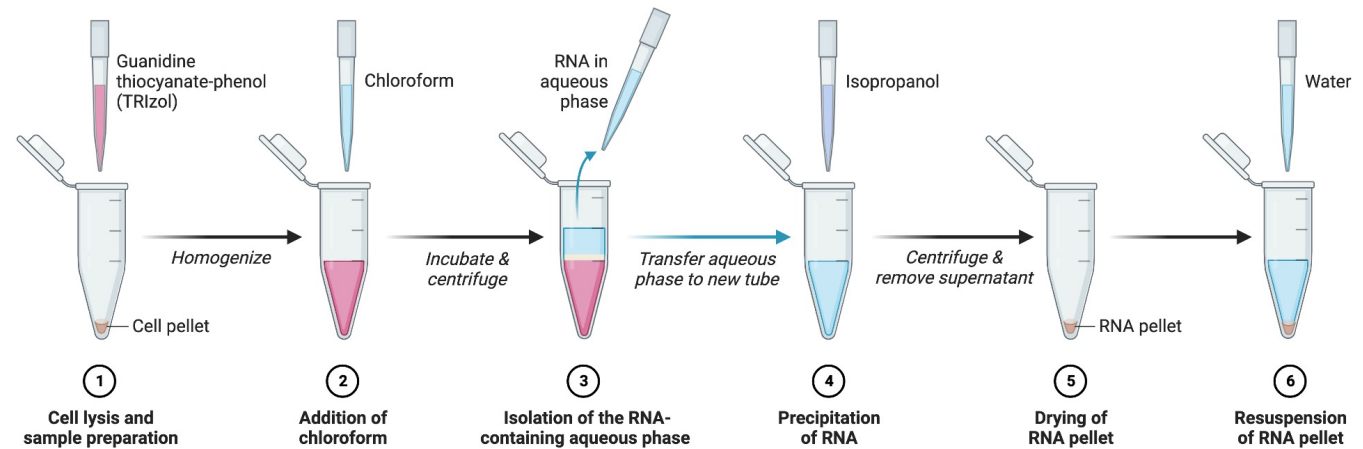
RNA isolation

The most widely used reagents for RNA extraction from biological samples are based on guanidinium thiocyanate and phenol (e.g., TRIzol). The low pH of the reagent allows for the differential separation of RNA from DNA and proteins. The guanidinium salt acts as a chaotropic agent to denature proteins, while phenol is an organic compound used to partition nucleic acids and proteins. Following sample solubilization and homogenization, RNA, DNA, and proteins are differentially partitioned by adding a phase-separation reagent (chloroform). This separates the mixture into distinct layers: a clear upper aqueous phase containing primarily RNA, while the interphase and lower organic phase contain DNA, proteins, and lipids. Subsequently, the RNA in the upper aqueous phase is recovered via alcohol precipitation. The resulting RNA pellet is washed with an ethanol solution, air-dried, and resuspended in deionized water.



RNA isolation

Standard
(with precipitation)



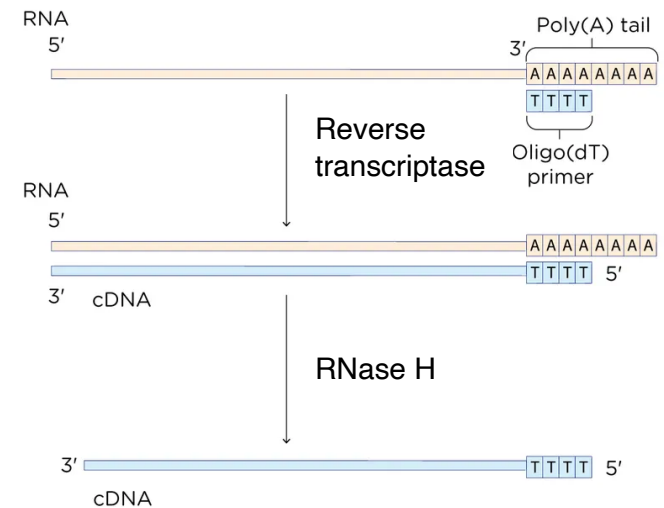
Fast
(with column)



cDNA Preparation

Reverse transcription is the process by which RNA molecules are transcribed into complementary DNA (cDNA). This cDNA synthesis reaction is mediated by enzymes called **reverse transcriptases (RTs)**. Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) is one of the classic RTs used in molecular biology workflows. However, newly engineered RTs are often preferred due to their improved efficiency, enhanced thermostability, and higher cDNA yields.

The reverse transcriptase binds to an RNA template in the presence of a primer. The enzyme's RNA-dependent DNA polymerase activity synthesizes cDNA by incorporating dNTPs from the reaction mixture. This generates a cDNA:RNA hybrid in a process termed first-strand cDNA synthesis. If the reverse transcriptase possesses RNase H activity (as M-MLV RT does), the RNA within the cDNA:RNA hybrid is cleaved during first-strand synthesis. In engineered enzymes, RNase H activity is often reduced or eliminated to prevent premature RNA degradation.



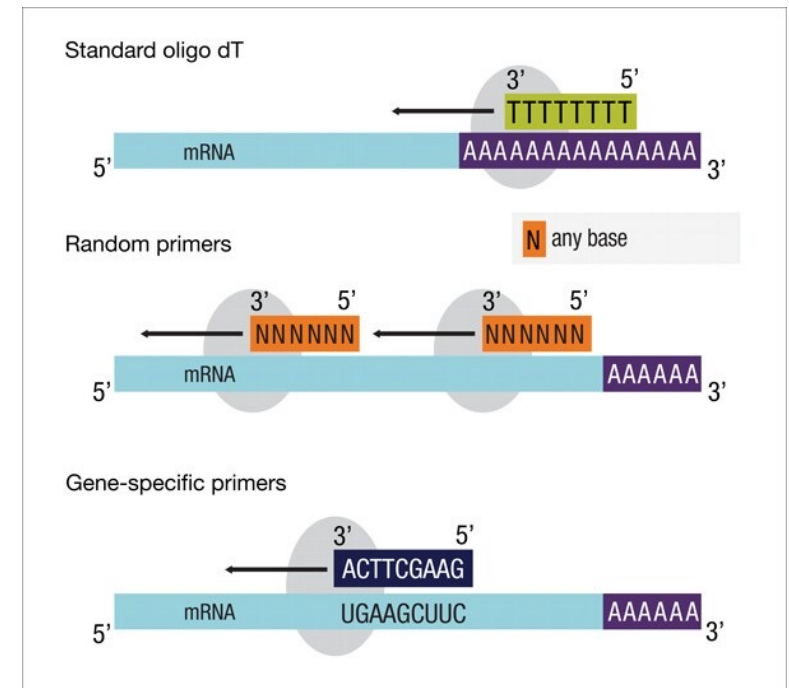
cDNA Preparation

To initiate reverse transcription, a short DNA oligonucleotide called a primer is required to anneal to the template RNA strand and provide reverse transcriptase with a starting point for synthesis.

Three distinct priming strategies can be utilized for cDNA synthesis:

- **standard oligo(dT) primers**
- **random primers**
- **and sequence-specific primers**

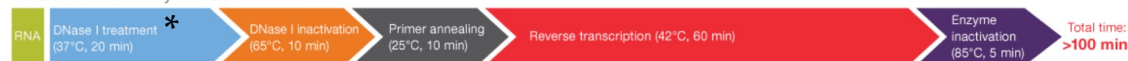
A mixture of oligo(dT) and random primers is frequently employed. Protocols vary depending on the reverse transcriptase (RT) enzyme utilized.



SuperScript IV VILO Master Mix cDNA synthesis workflow



Traditional cDNA synthesis workflow



* Prior to initiating the RT reaction, RNA samples must be treated with DNase to eliminate contaminating genomic DNA.

Semi-quantitative RT-PCR

Semi-quantitative PCR is a gene expression quantification technique based on standard (endpoint) PCR. It relies on the principle that by running identical PCR reactions across samples, using the same primers and cycle number, the resulting band intensity can be quantified to determine the relative initial amount of target cDNA present in each sample.

This technique is used to obtain a general (if rough) estimate of gene expression levels under different biological conditions, such as:

- Healthy cell vs. tumor cell
- Early-stage vs. differentiated cell
- Pre-treatment vs. post-treatment cell
- etc.

PROS

- Speed and ease of use
- High sensitivity (amplification is possible starting from low quantities of DNA, even of low quality)

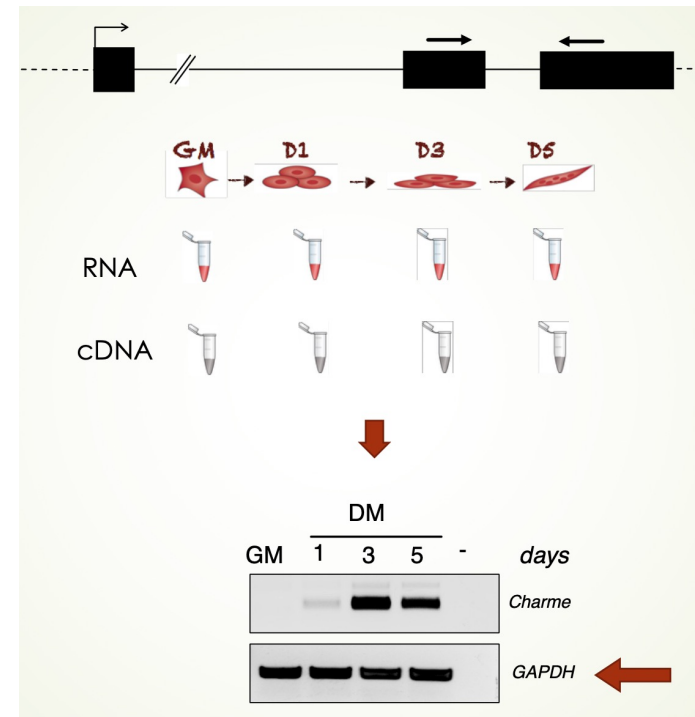
CONS

- Difficulty in obtaining accurate quantification of the final product

Semi-quantitative RT-PCR

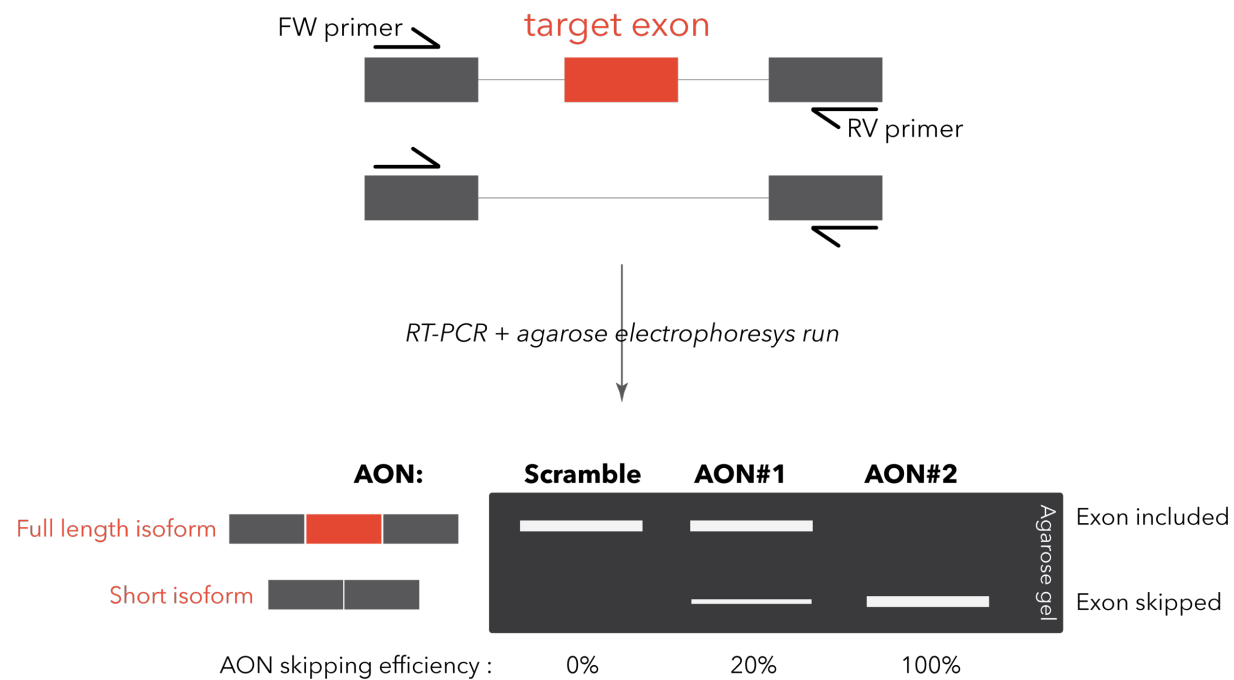
Example: Quantifying the expression of candidate genes involved in muscle differentiation.

- Induce in vitro differentiation
- Extract total RNA from cells
- Reverse transcribe RNA into cDNA (using random hexamers or oligo(dT) primers)
- Perform PCR using primers specific to the gene of interest (e.g., *Charme*) and an endogenous control (e.g., *GAPDH*)
- Run agarose gel electrophoresis
- Quantify the resulting band intensities



Semi-quantitative RT-PCR

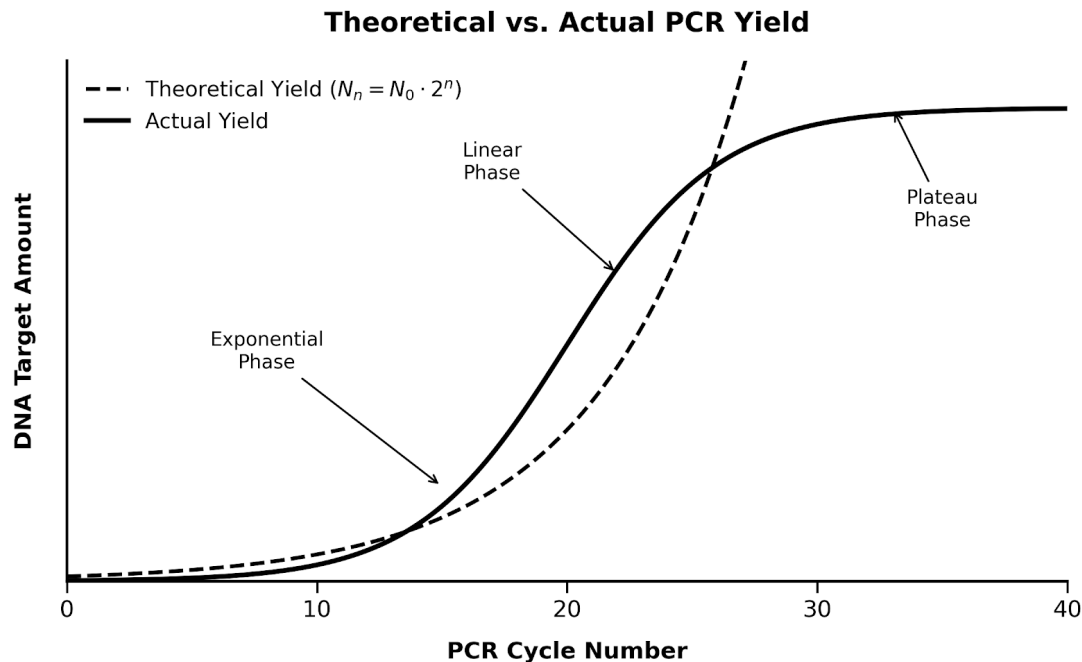
Viene utilizzata per lo studio dello splicing alternativo perchè permette di visualizzare le differenti isoforme con un'unica amplificazione



Semi-quantitative RT-PCR

Limitations:

Although semi-quantitative PCR provides reasonable estimates of relative expression levels by partially capturing the relationship between mRNA abundance and amplification output, its results remain highly approximate and cannot determine absolute transcript concentrations. A primary cause of quantitative unreliability in endpoint RT-PCR is the difficulty in accurately measuring signal intensity once amplification products reach saturation.



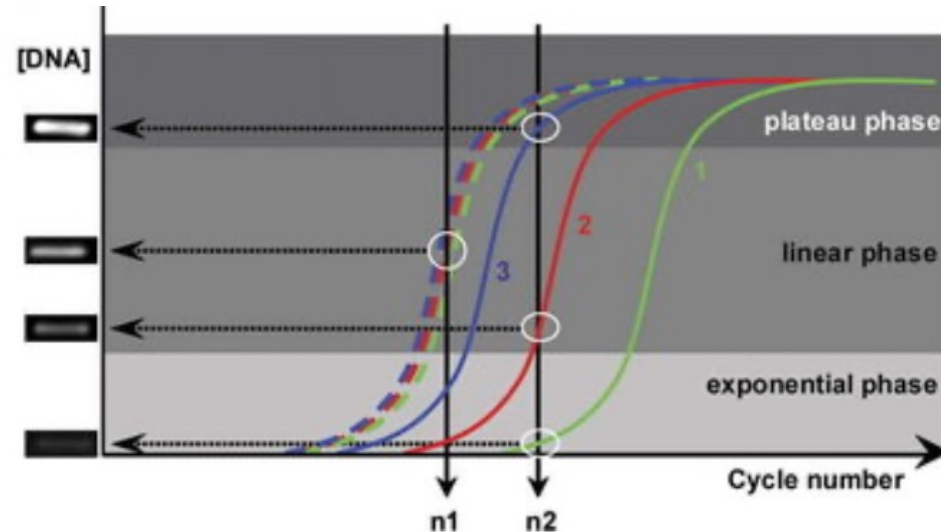
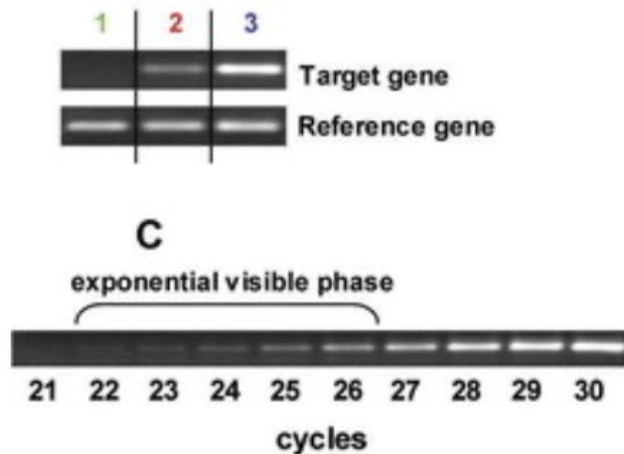
PCR reaction saturation (Plateau phase) depends on:

- Inactivation of Taq polymerase
- Depletion of dNTPs
- Depletion of primers
- Inhibition by pyrophosphate
- Self-annealing of amplified DNA strands

Semi-quantitative RT-PCR

Limitations:

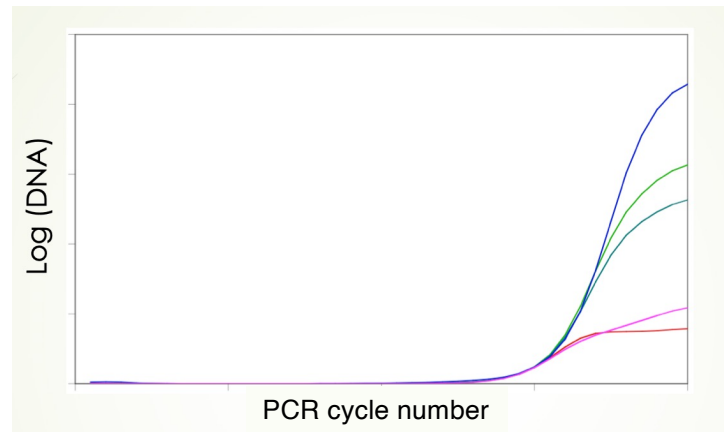
Although semi-quantitative PCR provides reasonable estimates of relative expression levels by partially capturing the relationship between mRNA abundance and amplification output, its results remain highly approximate and cannot determine absolute transcript concentrations. A primary cause of quantitative unreliability in endpoint RT-PCR is the difficulty in accurately measuring signal intensity once amplification products reach saturation.



Semi-quantitative RT-PCR

The Plateau Effect

Even when the initial template amount is identical across samples, the plateau phase is reached at different times and cycle numbers.



At the end of the reaction, the final amplicon yield is NOT reliably proportional to the starting DNA/cDNA concentration.

Consequently, endpoint PCR is primarily a QUALITATIVE (or semi-quantitative) assay, NOT a strictly QUANTITATIVE one.

REAL-TIME PCR (qRT-PCR)

Solution:

For a QUANTITATIVE measurement of the amplified product, data must be collected during the exponential phase of the amplification process, where the PCR product amount is directly proportional to the initial template concentration. This requires real-time monitoring of each reaction at every replication cycle.

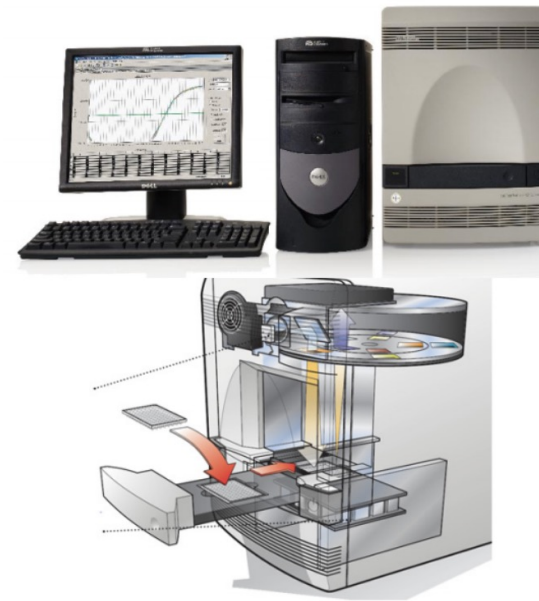
This monitoring is achieved by detecting a fluorescent signal whose accumulation during each amplification cycle follows the exact kinetics of the PCR reaction, making fluorescence directly proportional to the amount of generated PCR product.

REAL-TIME PCR (qRT-PCR)

Components:

- Template DNA (genomic DNA or cDNA derived from RNA reverse transcription)
- DNA Polymerase
- dNTPs
- Reporter dye
- Specific primers

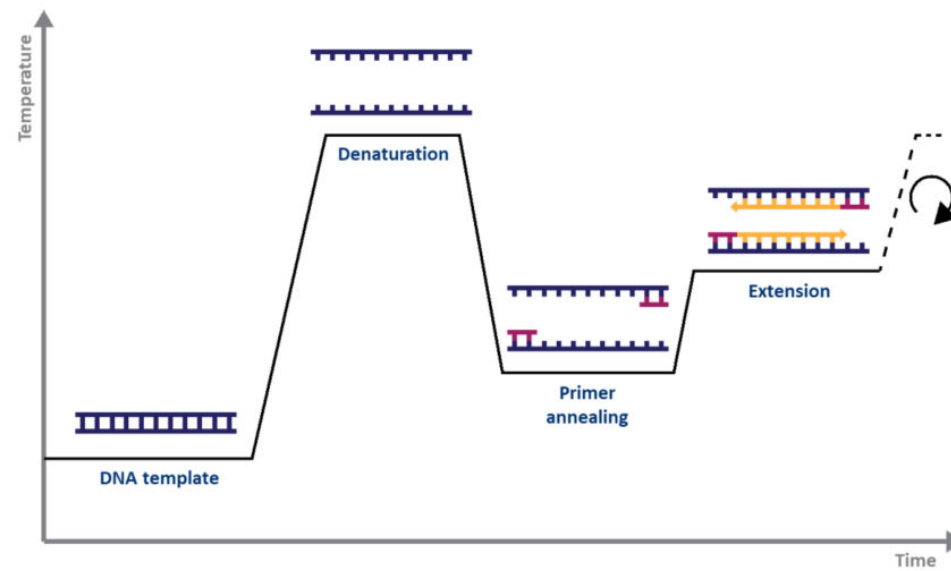
Master Mix



20.000 - 80.000 Euro

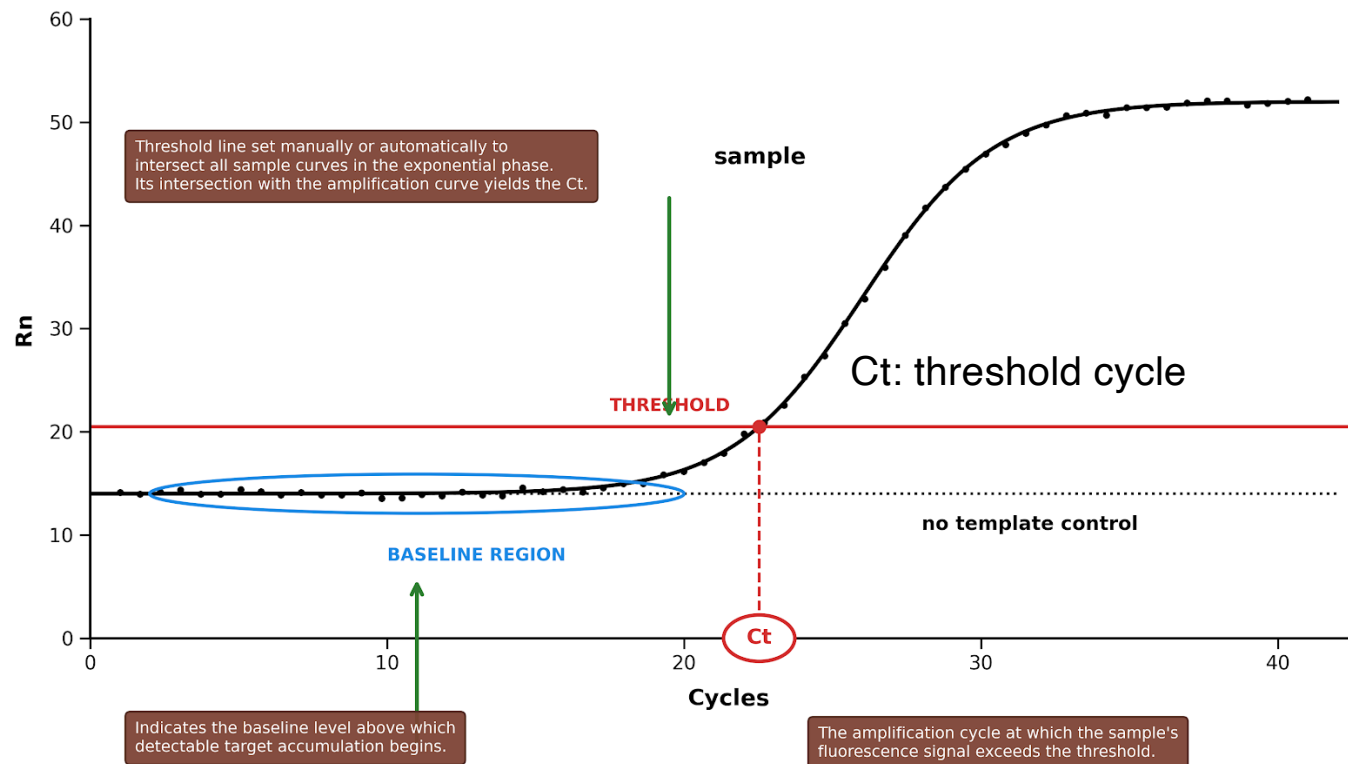
REAL-TIME PCR (qRT-PCR)

Steps:



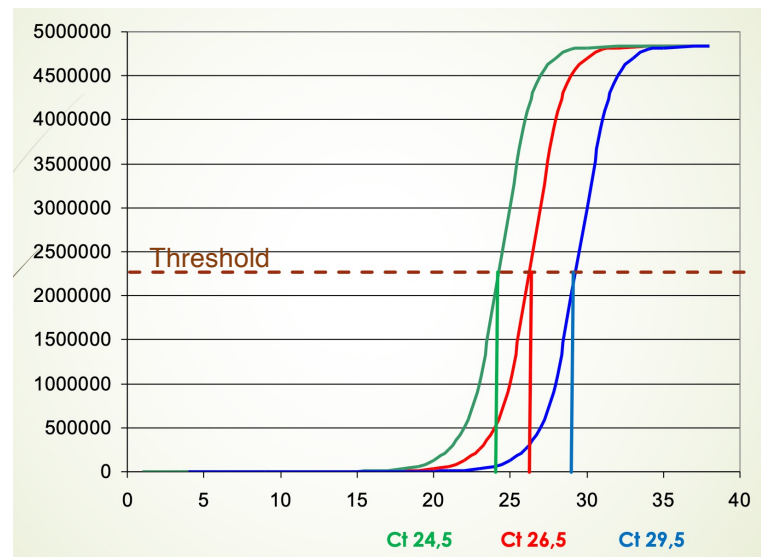
REAL-TIME PCR (qRT-PCR)

The fluorescence (R_n) emitted at each amplification cycle is detected and analyzed by a computer and displayed in the form of an amplification plot.



REAL-TIME PCR (qRT-PCR)

For each sample, an amplification curve is obtained, whose **threshold cycle (Ct)** value is inversely proportional to the initial amount of template. The Ct represents a relative measure of the sample concentration in the PCR reaction



REAL-TIME PCR (qRT-PCR)

Absolute Quantification:

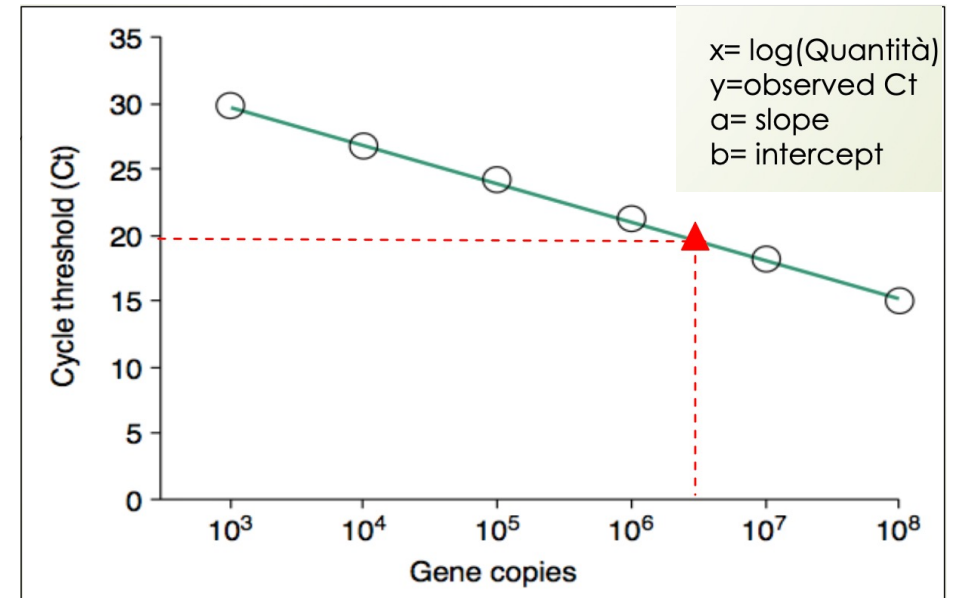
- Requires standards of known concentration (ng/ μ L) to construct a calibration curve or 'standard curve'.
- Identical sample amounts must be assayed for all 'unknowns'.

Relative Quantification:

- Requires endogenous controls (a standard curve is not used).
- Unknowns are 'quantified' by comparing their ΔC_t with that of the endogenous control.

Absolute Quantification

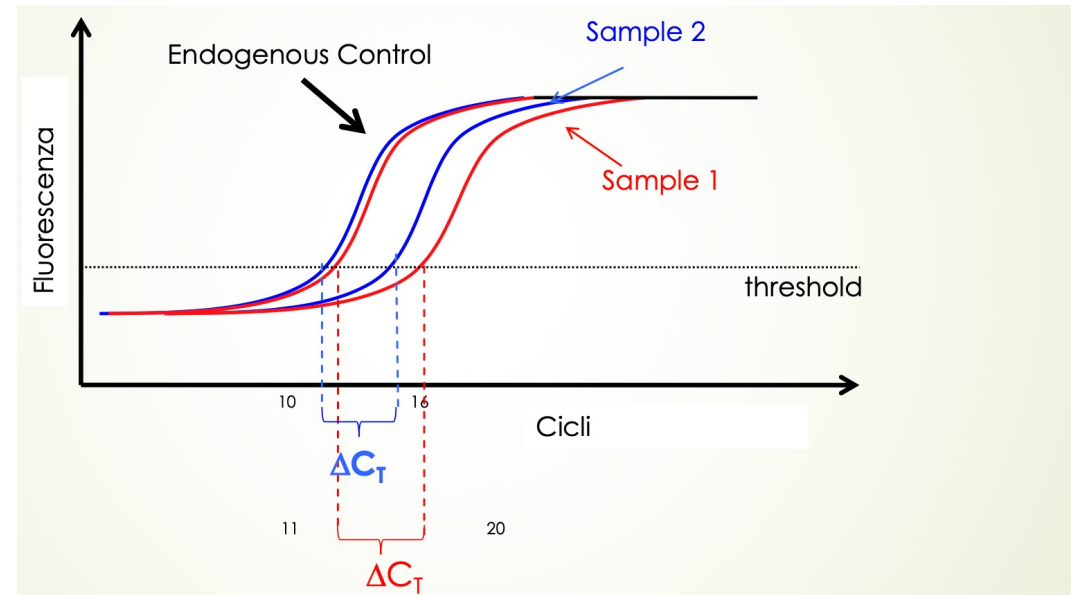
- Serial dilutions of a sample with a known concentration (standard) are used to construct a standard curve, described by the linear equation: $y = ax + b$.
- The concentration of unknown samples is then determined through interpolation against this standard curve.



Relative Quantification

Performed by comparing Ct values to determine the change in expression of a TARGET gene relative to a sample chosen as the CALIBRATOR (housekeeping gene).

The resulting values are normalized using an endogenous control, a constitutively expressed gene present in all analyzed samples—which is necessary to correct for variations in template loading and differences in reaction efficiency.



I valori così ottenuti saranno normalizzati per un **controllo endogeno**, ossia un gene espresso costitutivamente in tutti i campioni analizzati, necessario per normalizzare i dati rispetto alla quantità di DNA caricato e a variazioni di efficienza della reazione

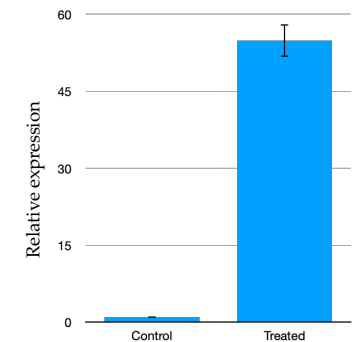
The Comparative Ct Method ($2^{-\Delta\Delta Ct}$)

- $\Delta Ct_{\text{treatment}} = Ct_{\text{gene of interest in treatment}} - Ct_{\text{housekeeping in treatment}}$
- $\Delta Ct_{\text{control}} = Ct_{\text{gene of interest in control}} - Ct_{\text{housekeeping in control}}$
- $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$
- fold change = $2^{-\Delta\Delta Ct}$

	Housekeeping gene				Gene of interest						
Sample	Ct1	Ct2	Avg.Ct	Sample	Ct1	Ct2	Avg.Ct	$\Delta Ct = \text{Avg Ct gene of interest} - \text{Avg Ct housekeeping}$	$\Delta\Delta Ct = \Delta Ct - \text{Avg } \Delta Ct \text{ control}$	$2^{-\Delta\Delta Ct}$	
Control 1	17.19	17.16	17.18	Control 1	30.57	30.53	30.55	13.38	-0.18	1.1	Control 1.0
Control 2	16.96	16.95	16.96	Control 2	30.73	30.37	30.55	13.60	0.04	1.0	
Control 3	17.07	17.15	17.11	Control 3	30.76	30.82	30.79	13.68	0.13	0.9	
Treated 1	18.04	17.95	18.00	Treated 1	26.11	25.54	25.83	7.83	-5.72	52.7	Treated 54.9
Treated 2	17.99	17.91	17.95	Treated 2	25.7	25.56	25.63	7.68	-5.87	58.5	
Treated 3	17.9	17.86	17.88	Treated 3	25.64	25.74	25.69	7.81	-5.74	53.4	
								Avg. ΔCt control			
								13.55			

Biological replicates (n=3)
Technical replicates (n=2)

Gene of interest
(relative expression with SD)



The mean ΔCt (Avg) of the control group is also subtracted from the ΔCt values of individual control replicates to determine the standard deviation (SD) around the normalized baseline value of 1.

REAL-TIME PCR (qRT-PCR)

Monitoring qPCR Amplification in Real Time

Non-Specific Detection (DNA-Binding Dyes)

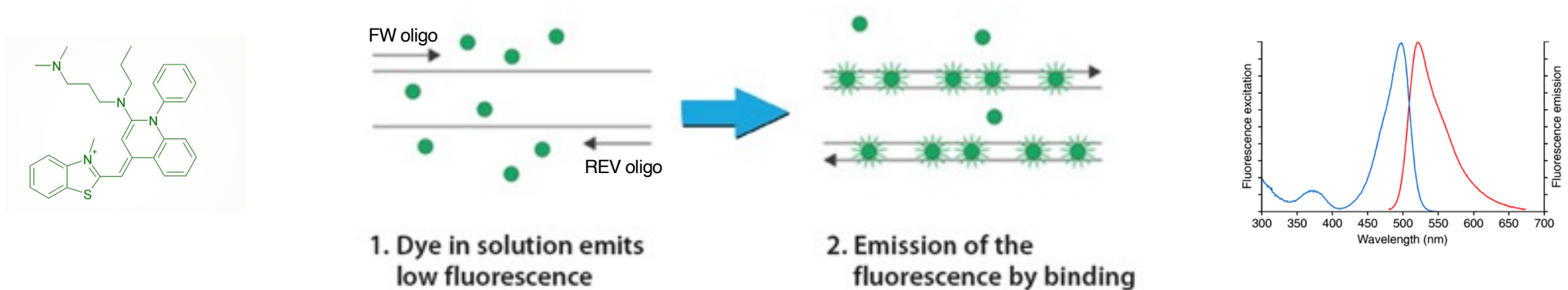
- **Fluorophore:** SYBR Green
- **Mechanism:** Binds non-specifically to the minor groove of all double-stranded DNA (dsDNA).
- **Advantage:** Low cost, versatile (no specific probe required).
- **Limitation:** Detects non-specific products and primer-dimers (requires Melt Curve analysis).

Sequence-Specific Detection (Fluorogenic Probes)

- **Chemistry:** TaqMan® Probes
- **Mechanism:** Uses a target-specific oligonucleotide with a 5' Reporter fluorophore and a 3' Quencher.
- **Advantage:** High specificity, enables multiplexing (detecting multiple targets in one reaction).
- **Limitation:** Higher design cost per target.

SYBR-GREEN

This dye exhibits NO fluorescence when free in solution due to internal vibrations of its aromatic rings, which convert electronic excitation energy into heat that is dissipated into the surrounding solvent. It emits fluorescence only upon binding to the MINOR GROOVE of double-stranded DNA.

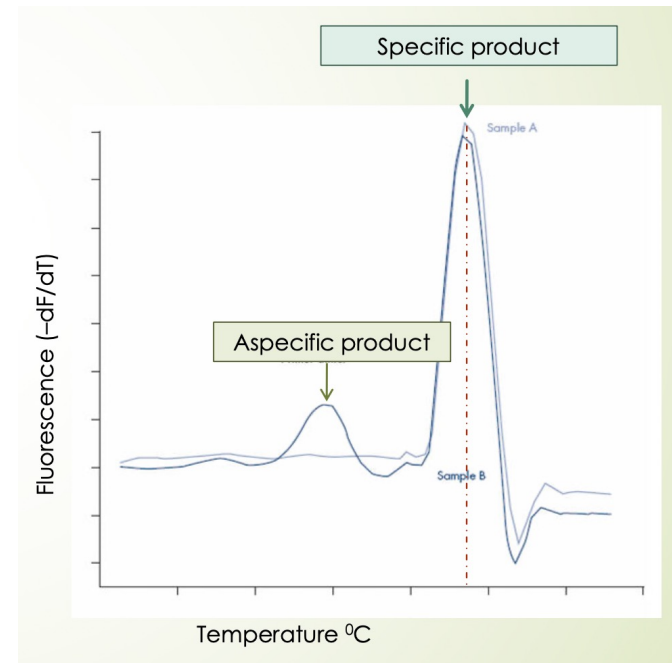
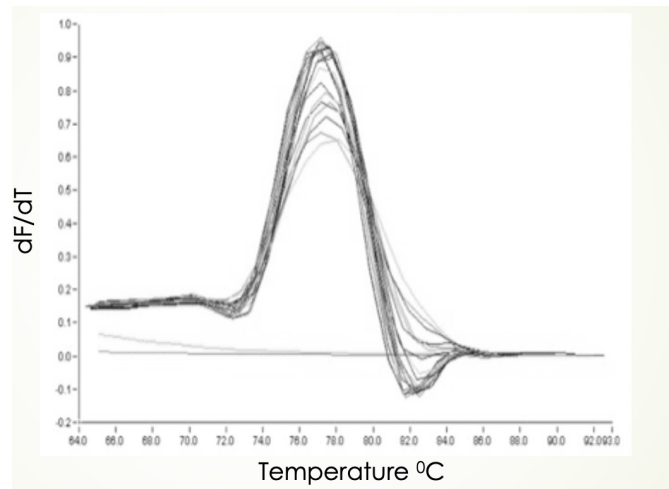


As the PCR reaction progresses, a gradual increase in fluorescence generated by SYBR Green binding to dsDNA can be observed, which directly corresponds to the increase in amplicon copy number.

The DNA-dye complex absorbs blue light at a maximum wavelength of $\lambda_{\text{max}} = 488 \text{ nm}$ and emits green light at $\lambda_{\text{max}} = 522 \text{ nm}$. In qRT-PCR instruments, the excitation light source can be a halogen lamp, an LED, or a laser.

Melt Curve Analysis

At the end of amplification, samples are heated while fluorescence decay is continuously monitored to generate a dissociation or 'melting curve.' Melting curve analysis evaluates the thermal denaturation characteristics of dsDNA as temperature increases.

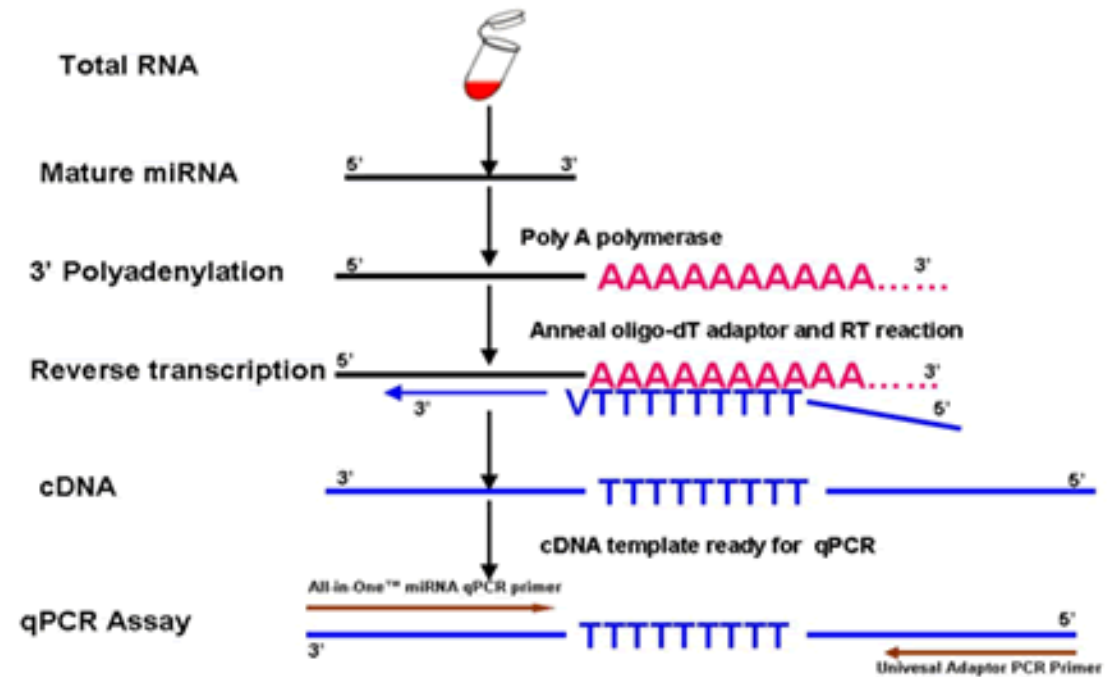


The thermal energy required to disrupt hydrogen bonds between complementary strands depends on amplicon length, GC content, and sequence complementarity. Because SYBR Green binds indiscriminately to all dsDNA, melting curve analysis is essential for detecting non-specific amplification, such as primer-dimers, genomic DNA contamination, or off-target products resulting from primer misannealing, which display dissociation peaks distinct from the specific target amplicon.

microRNA qRT-PCR Detection

MicroRNA (miRNA) detection via qPCR presents unique challenges due to the short length (~22 nucleotides) and lack of a poly(A) tail in mature miRNAs. To enable quantification, miRNAs must be lengthened during reverse transcription (RT).

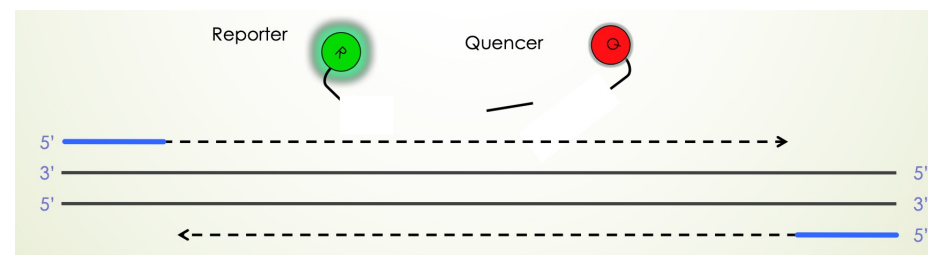
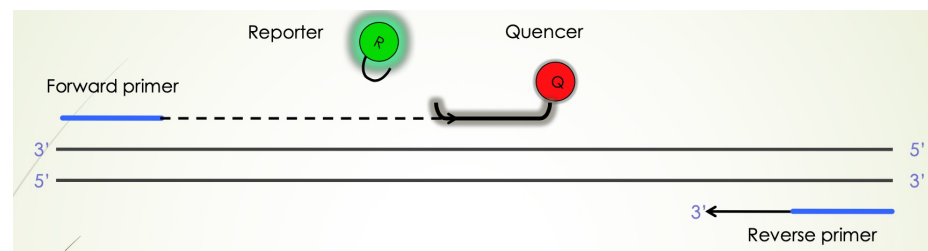
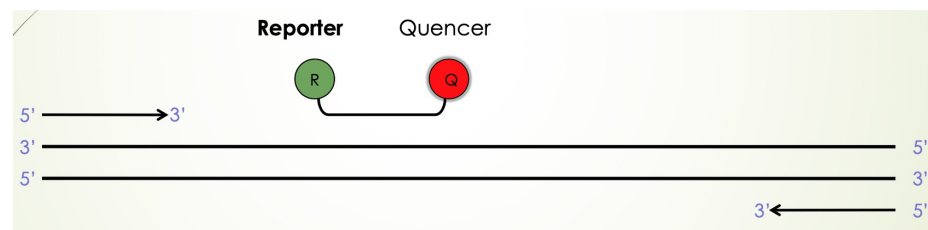
The primary strategy is the Poly(A) Tailing, where Poly(A) Polymerase adds a poly(A) tail to all miRNAs, followed by universal oligo(dT) reverse transcription.



Taqman probe-based assay

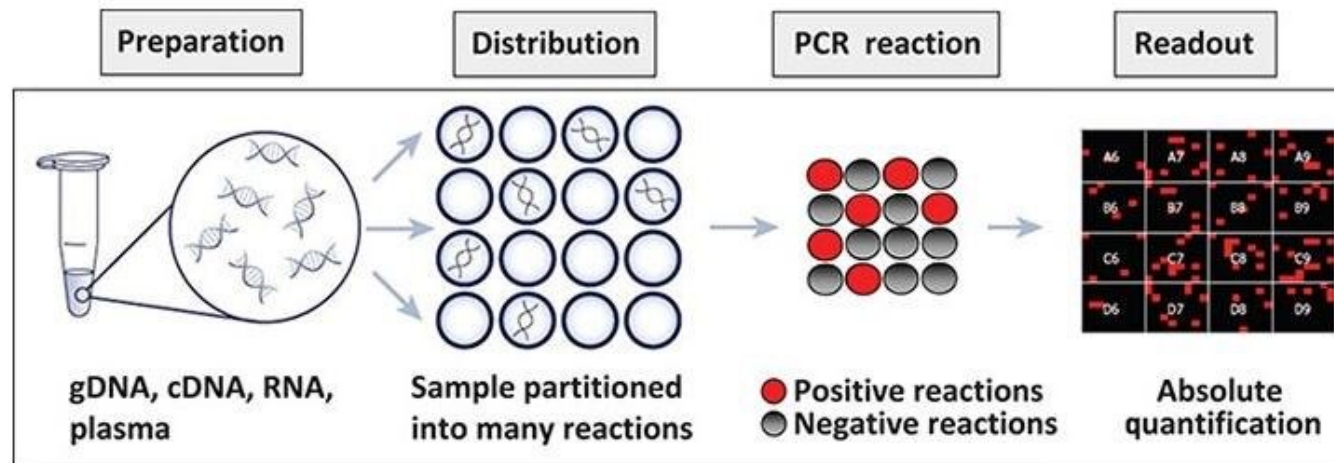
A TaqMan probe is an oligonucleotide designed to be complementary to the target sequence, much like PCR primers. It is engineered to hybridize specifically within the internal region of the amplified PCR fragment.

1. A reporter dye (R) and a quencher (Q) are attached to the 5' and 3' ends of the TaqMan probe, respectively. When the probe is intact, the reporter's fluorescence is quenched due to the close proximity of the quencher.
2. During each extension cycle, DNA polymerase cleaves the probe, releasing the reporter dye.
3. Once separated from the quencher, the reporter emits its characteristic fluorescence. The increase in reporter fluorescence is directly proportional to the number of amplicons generated.

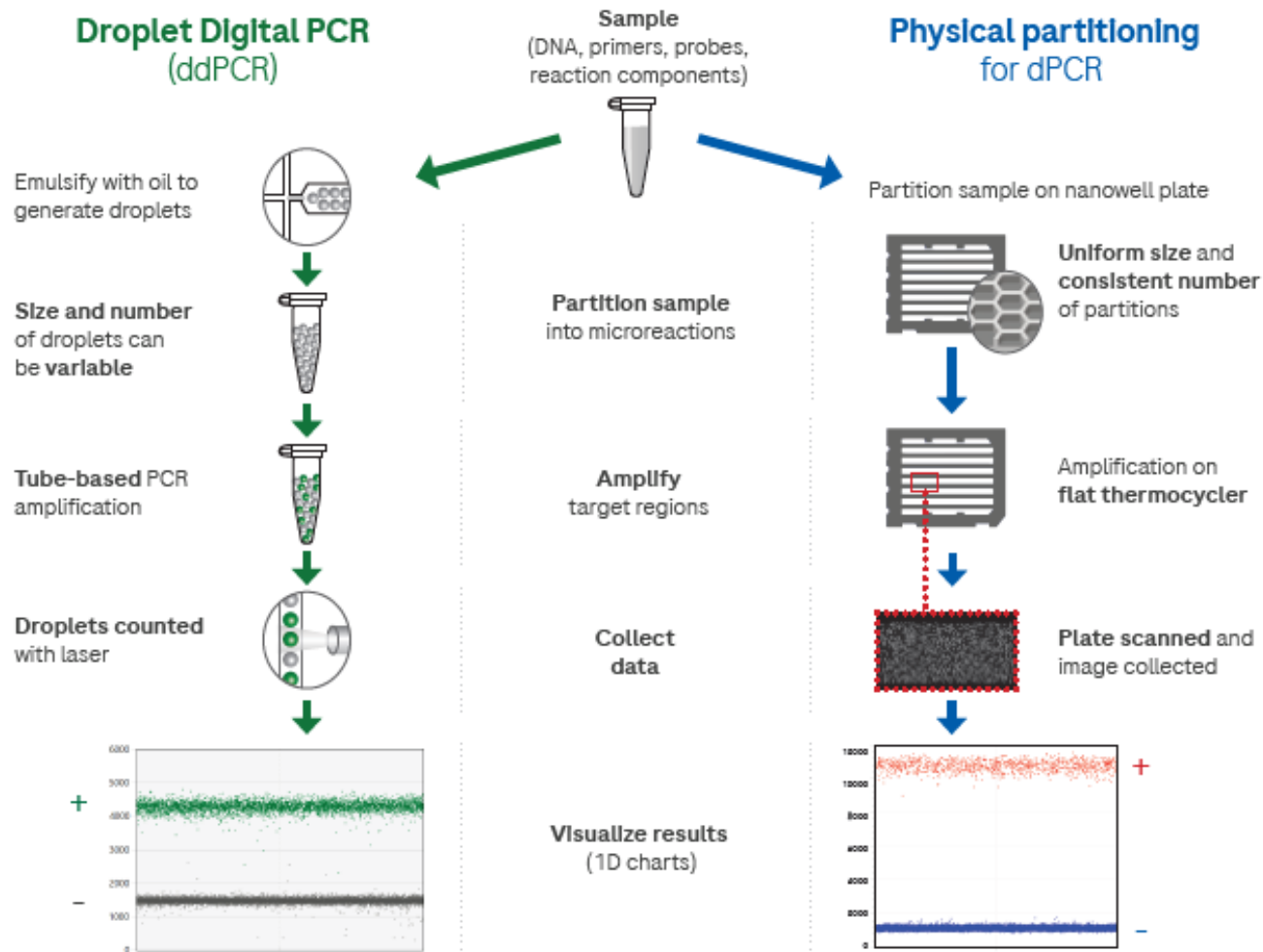


DIGITAL PCR (dPCR)

Digital PCR (dPCR) enables highly sensitive absolute quantification of nucleic acids without the need for standard curves. In the Droplet Digital™ PCR System, a PCR sample is partitioned into 20,000 droplets. After amplification, droplets containing target sequence are detected by fluorescence and scored as positive, and droplets without fluorescence are scored as negative. Poisson statistical analysis of the numbers of positive and negative droplets yields absolute quantitation of the target sequence.



Major Types of Digital PCR (dPCR)



DIGITAL PCR (dPCR)

Contrary to real-time qPCR, digital PCR does not rely on every amplification cycle to determine the relative amount of target molecule; rather, it relies on **Poisson statistics** to determine the absolute target quantity following an end-point amplification.

As the target molecule is distributed randomly across all available partitions, Poisson distribution estimates the average number of molecules per partition (zero, one or more) and calculates the copies of the target molecule per positive partition. Poisson statistical analysis of the number of positive and negative reactions yields precise, absolute quantitation of the target sequence.

λ is the expectation value for these events and will tell what the most likely average value of copies/partitions is

$$\lambda = -\ln \left(\frac{\text{number of valid partitions} - \text{number of positive partitions}}{\text{number of valid partitions}} \right)$$

Computing the estimated concentration of target genes in copies/ μ l

Given:
Valid partitions: 8000
Positive partitions: 4000

$$\lambda = -\ln \left(\frac{\text{number of valid partitions} - \text{number of positive partitions}}{\text{number of valid partitions}} \right)$$

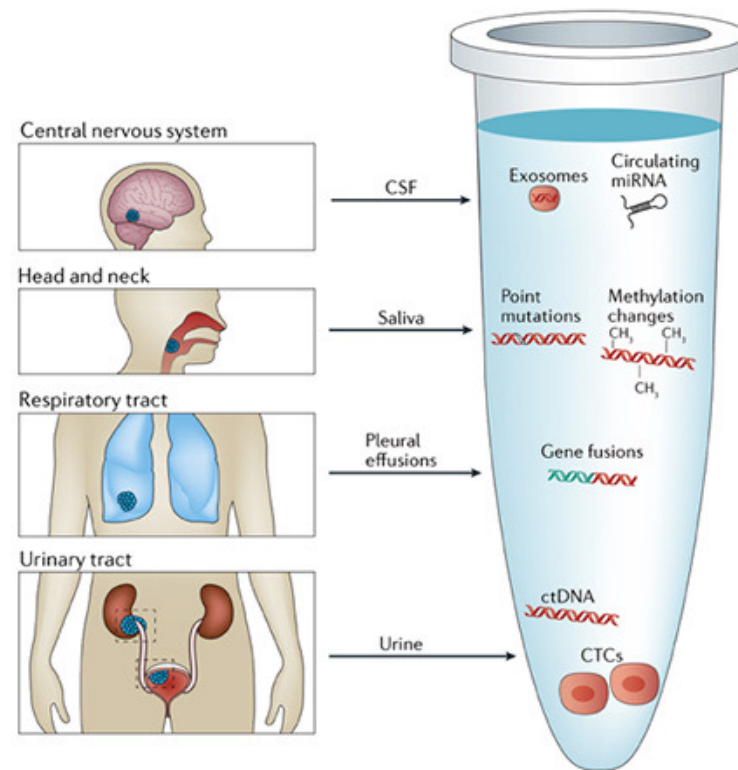
$$\lambda = -\ln \left(\frac{8000 - 4000}{8000} \right) = 0.693 \text{ Calculated target copies per partition}$$

Given:
Partition volume is $V = 0.23 \text{ nl}$

$$\lambda_{\text{volume}} = \frac{\lambda}{V [\mu\text{l}]}$$

$$\lambda_{\text{volume}} = \frac{0.693}{0.23} \times 1000 = 3013 \text{ copies}/\mu\text{l} \text{ Calculated target copies per } \mu\text{l}$$

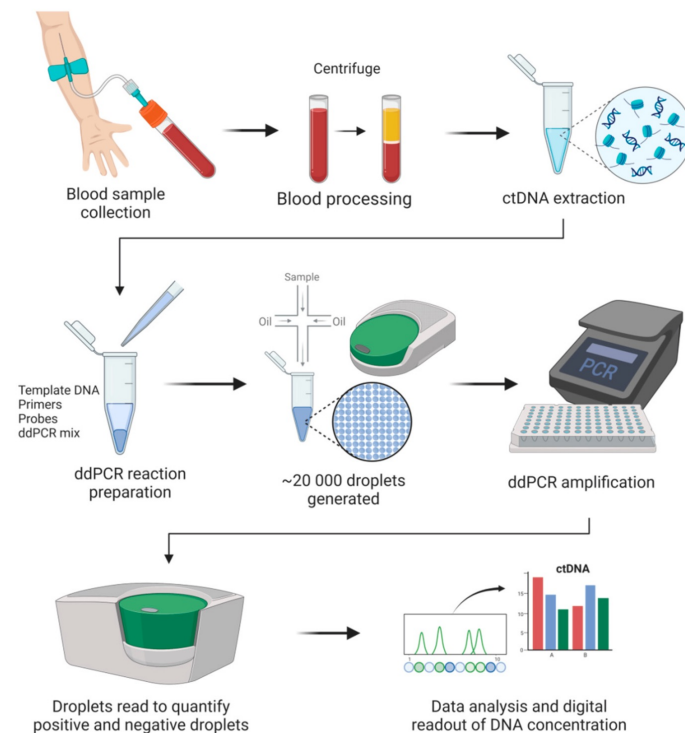
Body fluids as a source of tumour-derived molecular information



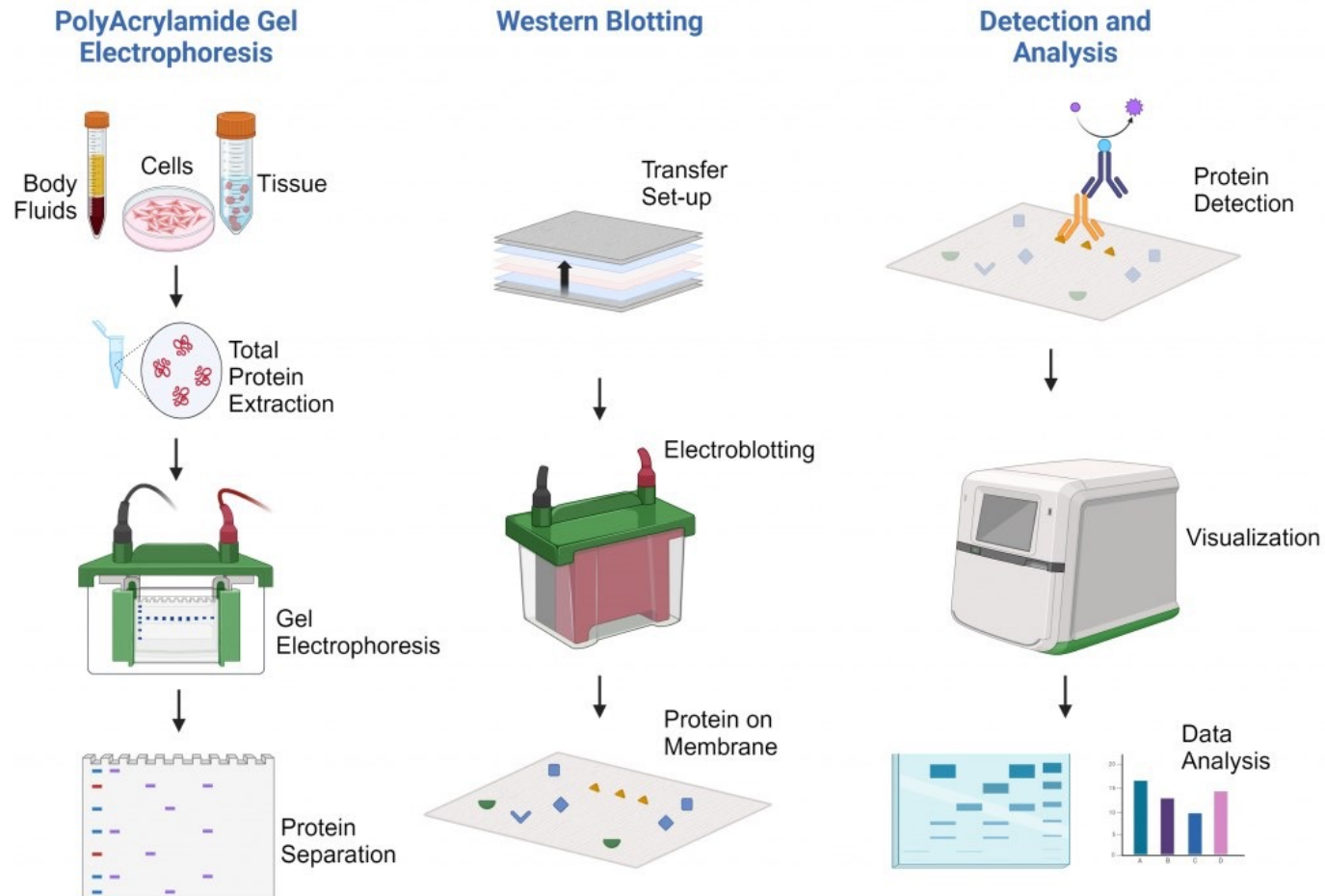
General digital droplet PCR (ddPCR) workflow in cancer liquid biopsies

Circulating tumor DNA (ctDNA), cell-free fragmented tumor-derived DNA in blood plasma, is an invaluable source of specific cancer-associated mutations and holds great promise for the development of minimally invasive diagnostic tests. ddPCR-based monitoring is used for early metastasis detection and therapy modification.

Mutation detection was shown to precede clinical detection of metastasis in 86% of breast cancer patients.

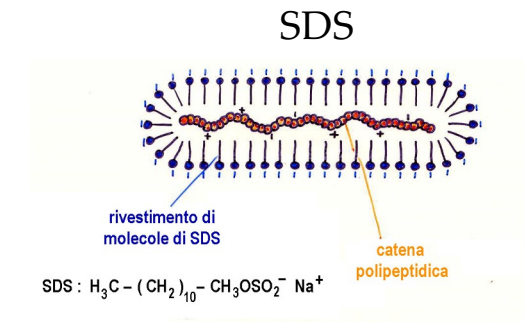
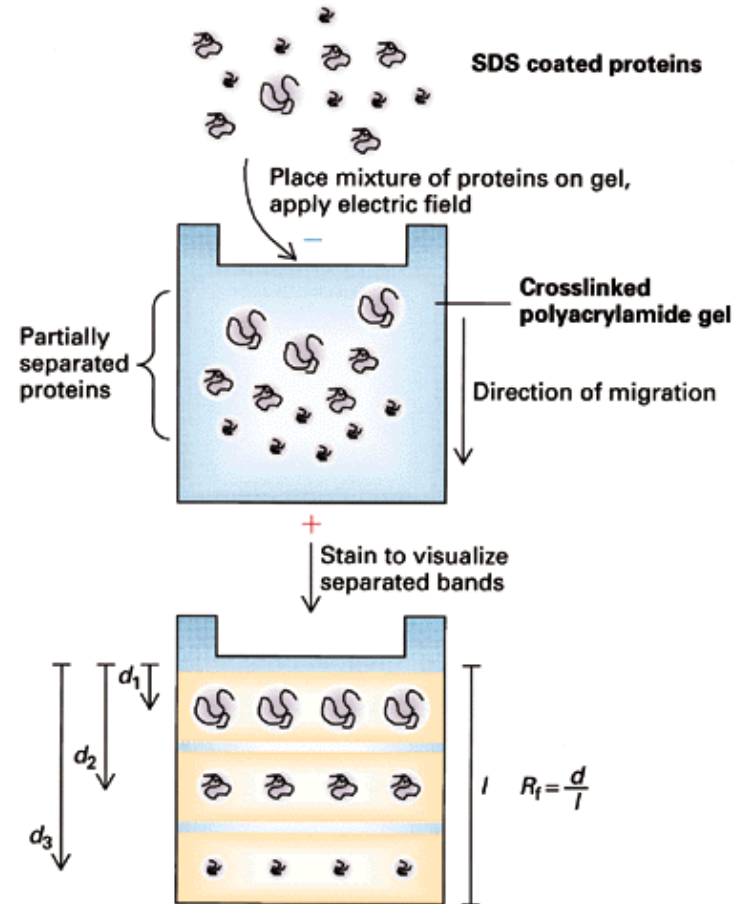


Western blot



SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- Once negatively charged, all proteins run toward +
- The smaller are the proteins, the faster they run
- Denaturing conditions allow proteins to move according to their size only, no influence from conformation.



Anionic detergent tightly binding proteins in a fixed ratio (1 mol SDS/2 mol aminoacids)

Protein Extraction

Steps:

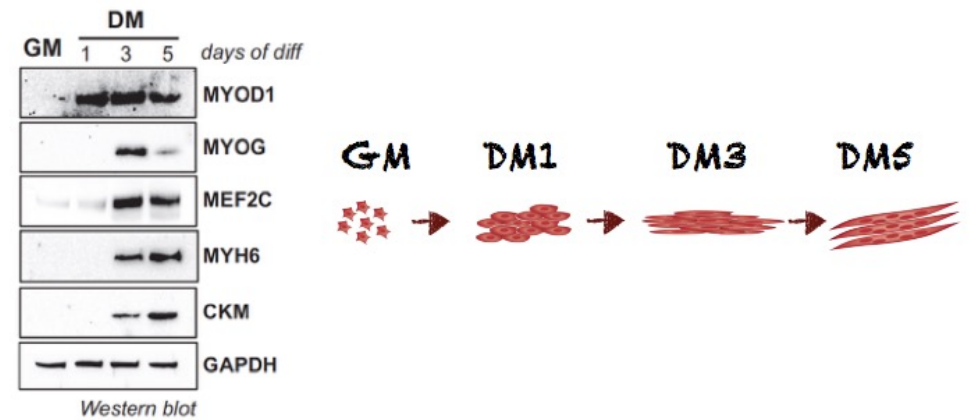
- Cell lysis in conditions ensuring: membrane break, protein dissociation (strength depending on the analysis), protease inhibition.
- Spectrophotometric determination (concentration).

Protease inhibition:

Leupeptin, Pepstatin, PMSF, EDTA, 4° C

Western Blot applications

- Specific protein detection (presence/absence)
- Gene expression analysis
- Comparing different conditions



Blocking

- Saturation of free hydrophobic spots on the membrane
- Avoids unspecific binding of primary Ab to the membrane

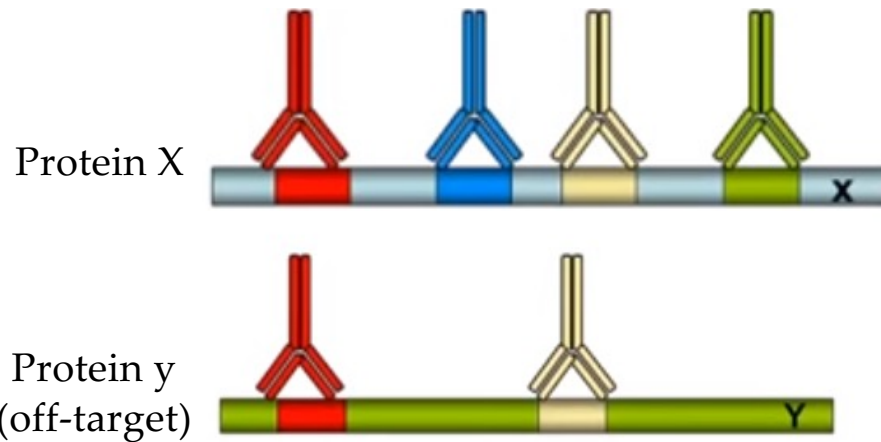


This reduces **background** leading to **clearer results**, and eliminates false positives

Antibodies

polyclonal

Specific to multiple epitopes on a single antigen

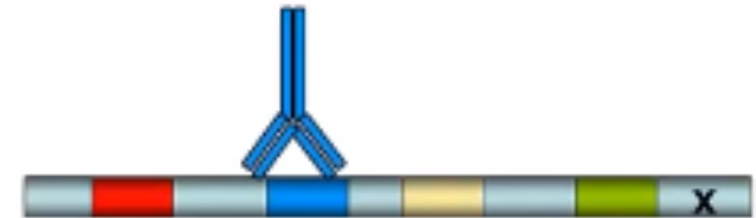


Pro: Signal amplification

Cons: non-specific interactions

monoclonal

Specific to one epitope on a particular antigen



Pro: High specificity

Cons: High sensibility to damage of epitopes

Antibody Bound

SECONDARY ANTIBODIES

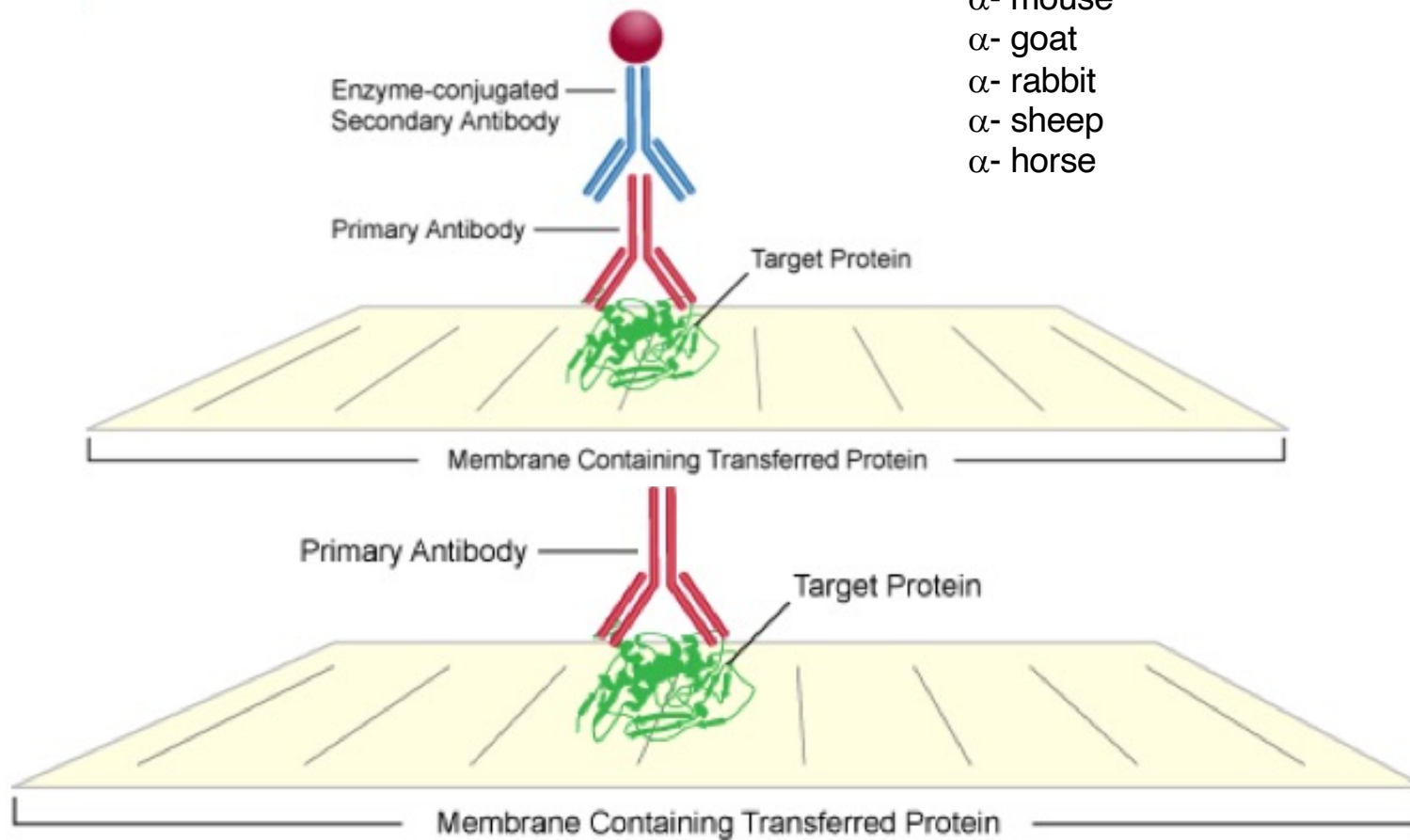
α - mouse

α - goat

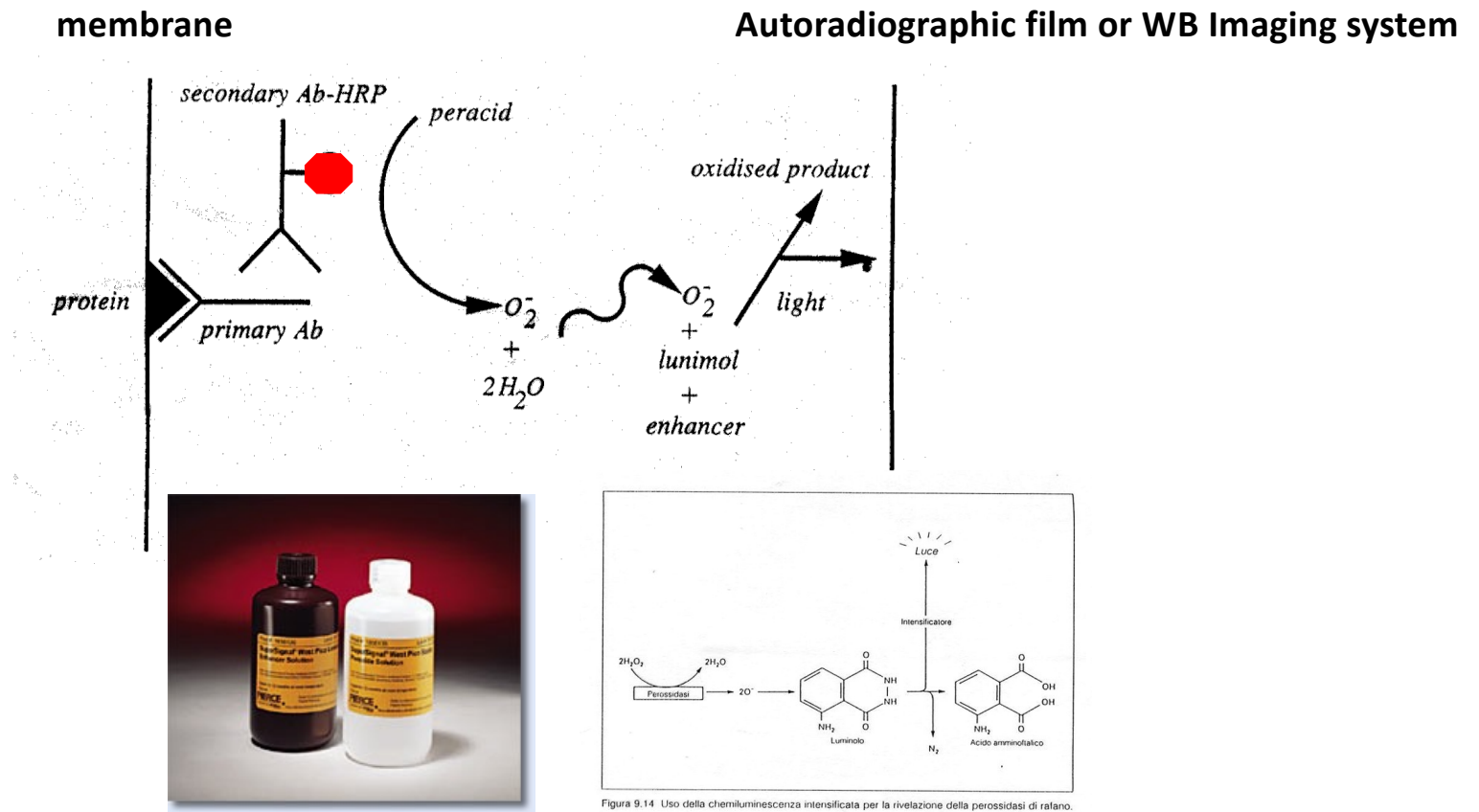
α - rabbit

α - sheep

α - horse

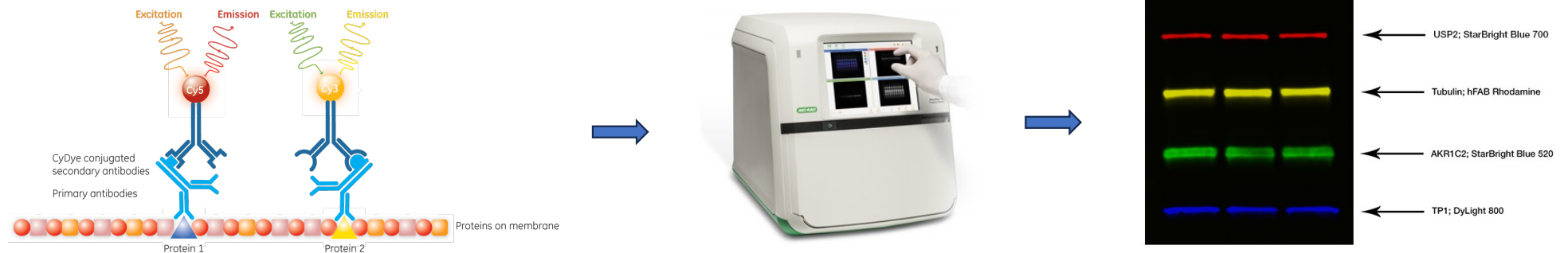


ECL (Enhanced Chemio-Luminescence) method



The substrate metabolized by Horseradish peroxidase (HRP) emits light

Fluorescent Western Blotting Detection



The benefits of fluorescent western blotting multiplexing are:

- Multiple samples are analyzed under the same experimental conditions
- Greater consistency of readout using fluorescent dyes rather than the variability of an enzymatic reaction

Techniques to Study Gene Function

- Gene Expression Analysis (when and where)
- Loss of Function -> Phenotypic Analysis
- Interactions
- Reporter Gene Studies

Molecular techniques for the study of the interaction between:

Protein-protein:

Immunoprecipitation
GST pull-down

DNA-protein:

ChIP (Chromatin immunoprecipitation)
Cut&Run/Cut&Tag
ATAC-seq

RNA-DNA:

ChIRP (Chromatin isolation by RNA purification)

RNA-Protein (RNA centric):

Exogenous RNA pulldown,
Endogenous RNA pulldown
RAP (RNA antisense purification)

Protein-RNA (Protein centric):

RIP (RNA immunoprecipitation)
CLIP (Cross-linked immunoprecipitation)

Immunoprecipitation

Isolation (enrichment) of antigen/antibody complexes

- Requires: specific antibodies directed toward the target protein or its tagged variant
- Allows: identification of protein or ribonucleoprotein (RNP) complexes

Protein Tagging

Protein Tagging is a strategy for fusing a protein to a well-characterized peptide. The peptide (TAG) confers the protein with the possibility to go through easy purification, allowing to isolate it in big amounts or to identify multiprotein or RNA/DNA/protein complexes.

1. Tagged proteins can be obtained by cloning into expression vectors (recombinant protein:

DNA encoding for the protein + DNA encoding for the Tag

2. Tagged protein can be directly expressed within the cell line by using episomal vectors or genome editing

Types of TAG

GST - protein of bacterial origin (16 kDa)

CBP - calmodulin binding protein (6 kDa)

ProtA - protein of bacterial origin that binds IgG (20 kDa)

TAP - CBP+ProtA (30 kDa)

c-myc- 10 aa derived from human c-myc (EQKLISEEDL)

HA - 9 aa derived from "haemagglutinin protein" of influenza virus (YPYDVPDYA)

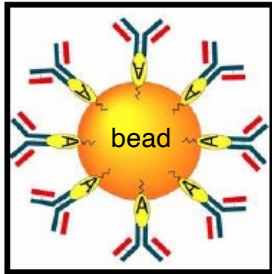
6His tag – synthetic peptide of 6 histidines

FLAG - synthetic peptide of 8 aa (DYKDDDK)

Immunoprecipitation

Isolation (enrichment) of antigen/antibody complexes

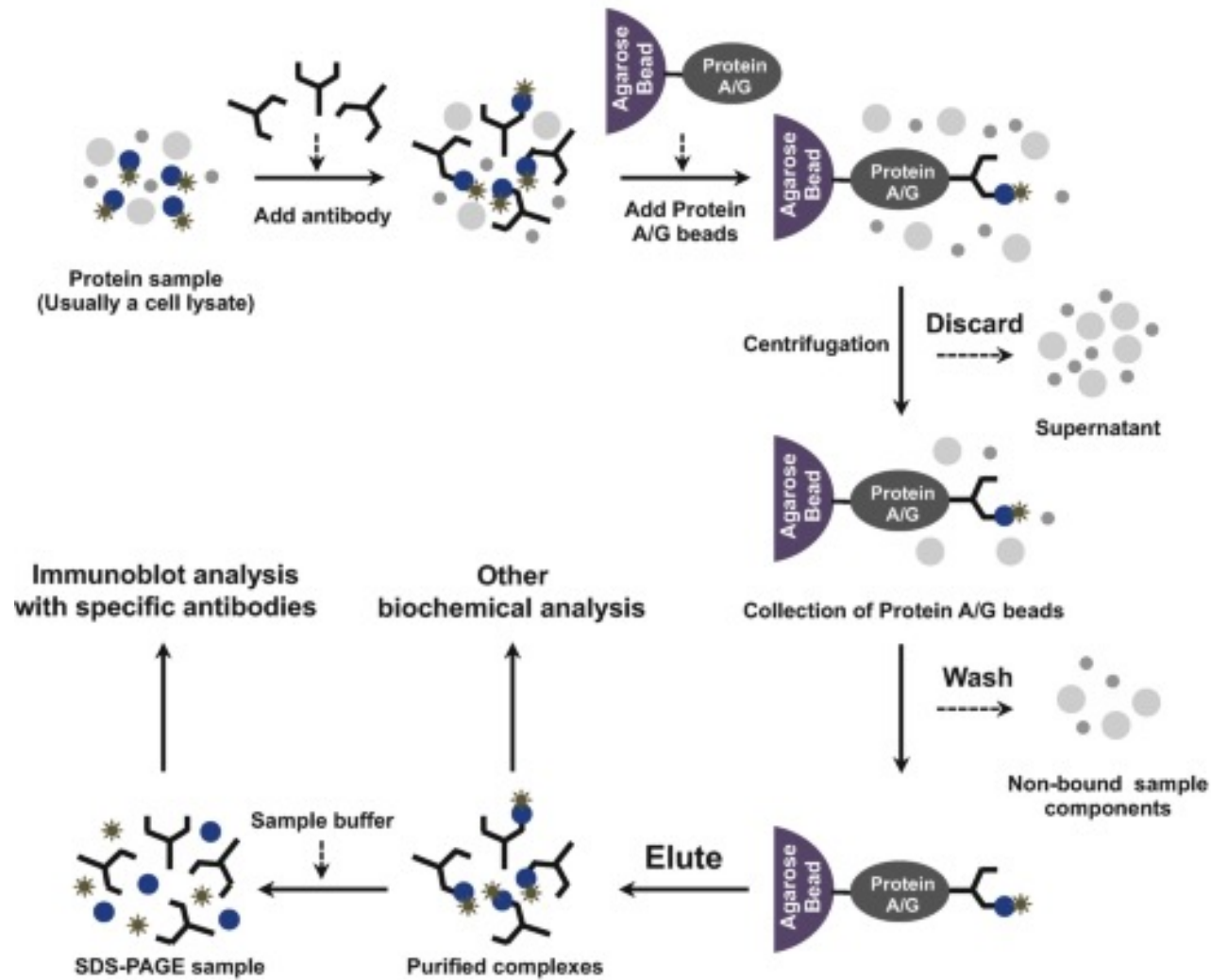
- Requires: specific antibodies directed toward the target protein or its tagged variant
- Allows: identification of protein or ribonucleoprotein (RNP) complexes



Antibodies specifically bind to Protein A or Protein G through their Fc region. These surface proteins, derived from *Staphylococcus aureus* and *Streptococcus* spp., respectively, are covalently bound (or coupled) to agarose or Sepharose beads.

Binding Characteristics of Some Immunoglobulins			
Immunoglobulin		Protein A	Protein G
Mouse	IgG1	+	++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+++
	IgM	-	-
	IgA	-	-
	IgE	-	-
Rat	IgG1	+	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
	IgG4	+	++
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++

Co-Immunoprecipitation (Co-IP)

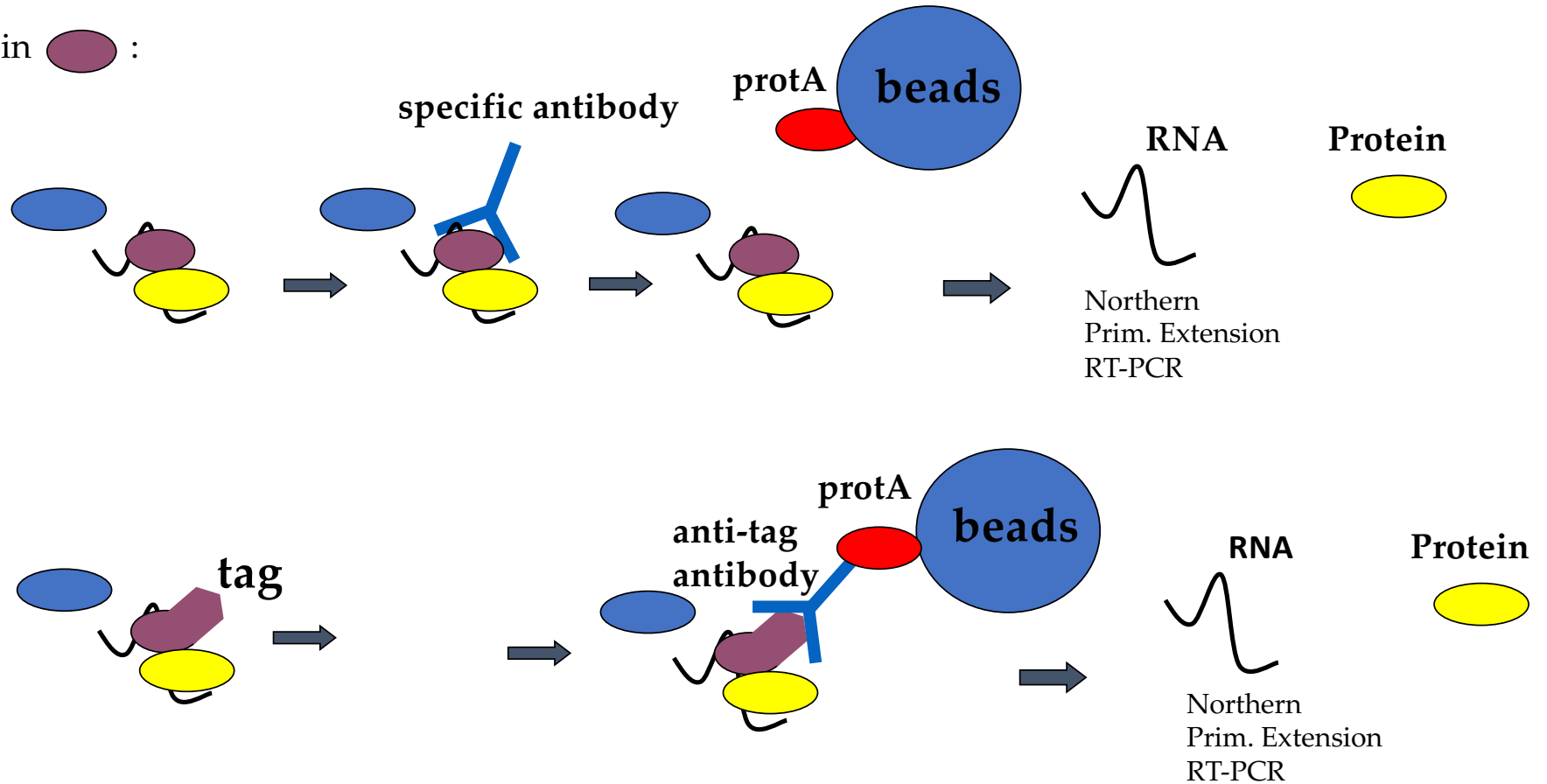


Magnetic beads !

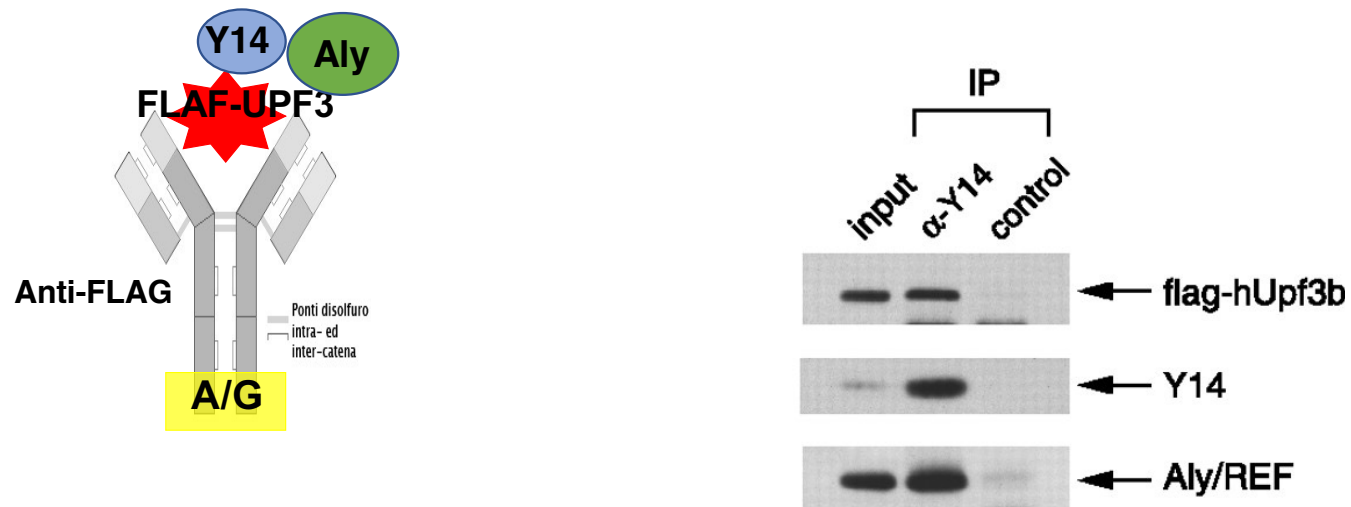


Co-immunoprecipitation: experimental variations

Interactors of protein  :

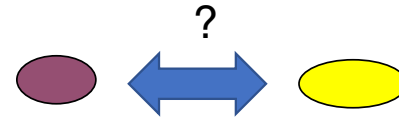


Co-Immunoprecipitation: an example

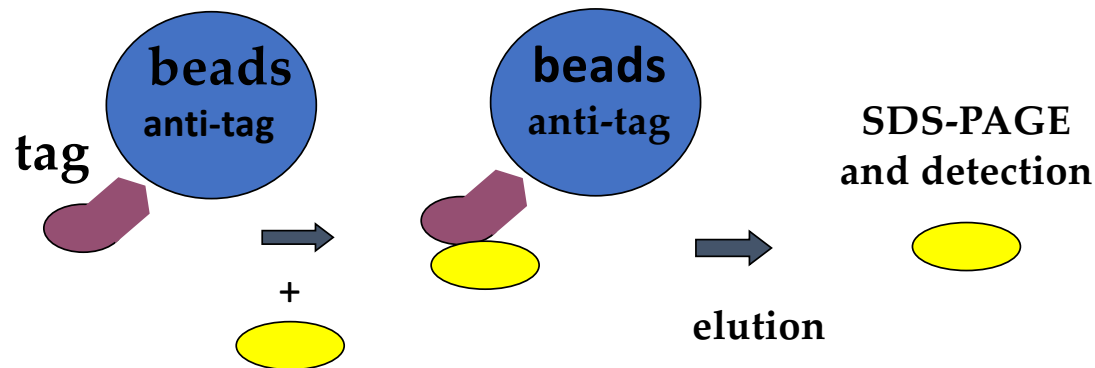


Immunoprecipitations were performed using the protein extract from HeLa cells 24 hours after transfection of **Flag-hUpf3b**

Protein-protein interaction: experimental variations



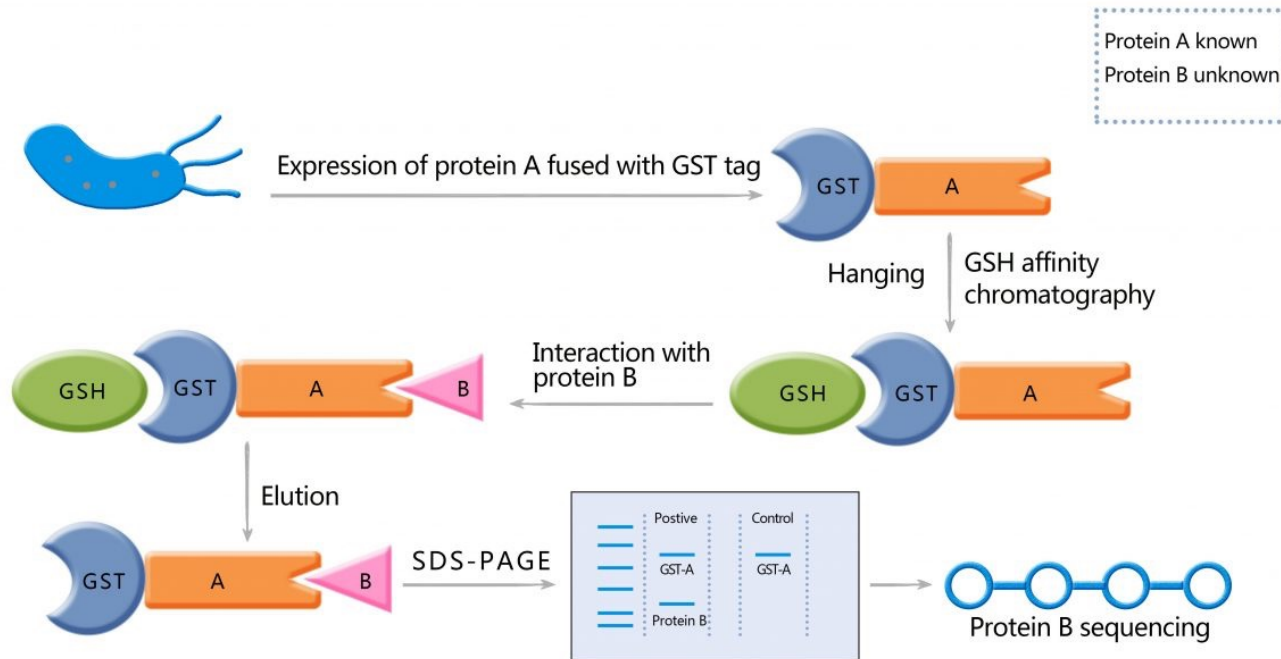
NO protein extract !



Both proteins must be purified (e.g. recombinant or produce by *in vitro* translation)

Tag: GST, His etc.

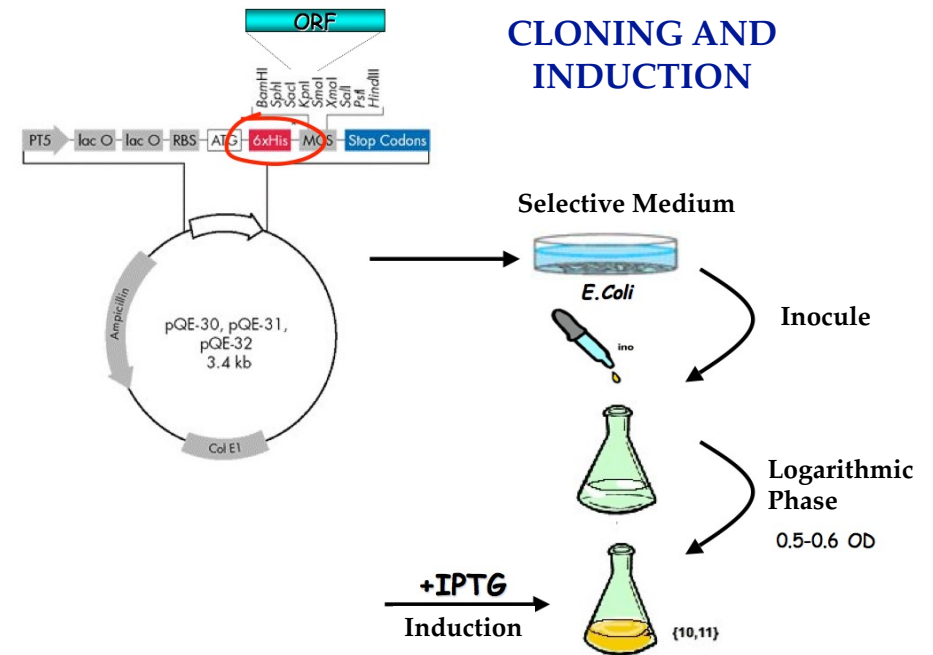
GST pull-down



Expression and Purification of a recombinant protein

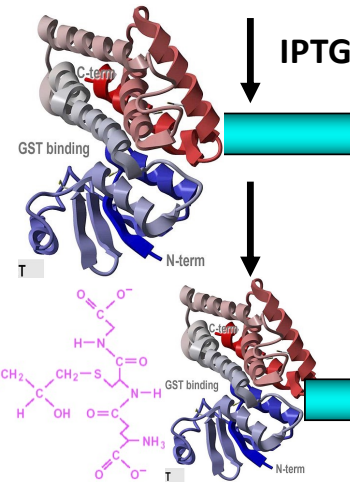
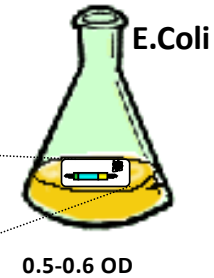
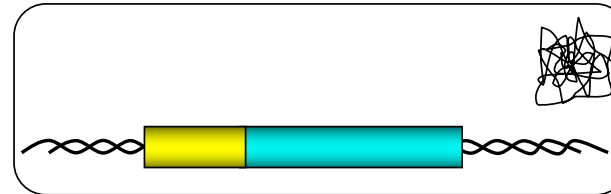
1. TRANSFORMATION (Expression vector in the expression host).
2. AMPLIFICATION of the positive bacterial strain.

1. INDUCTION of the recombinant protein.
2. PURIFICATION of the recombinant protein.



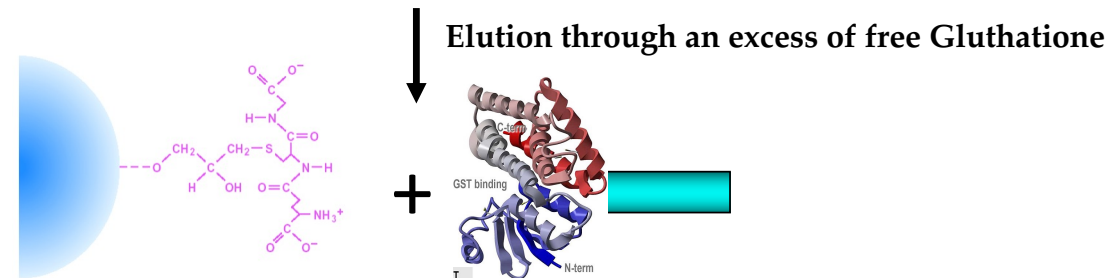
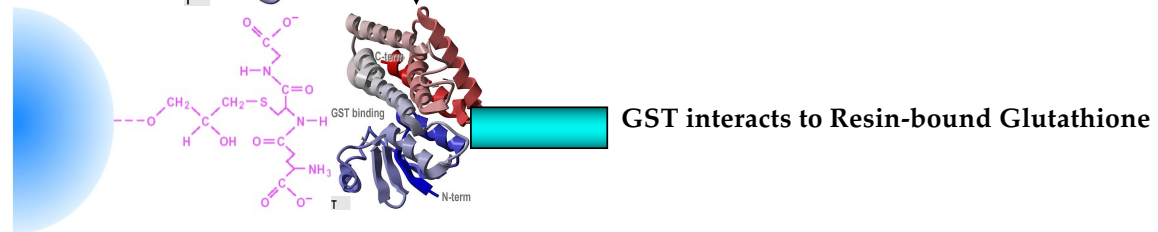
The GST TAG SYSTEM

 GST
 Protein of Interest

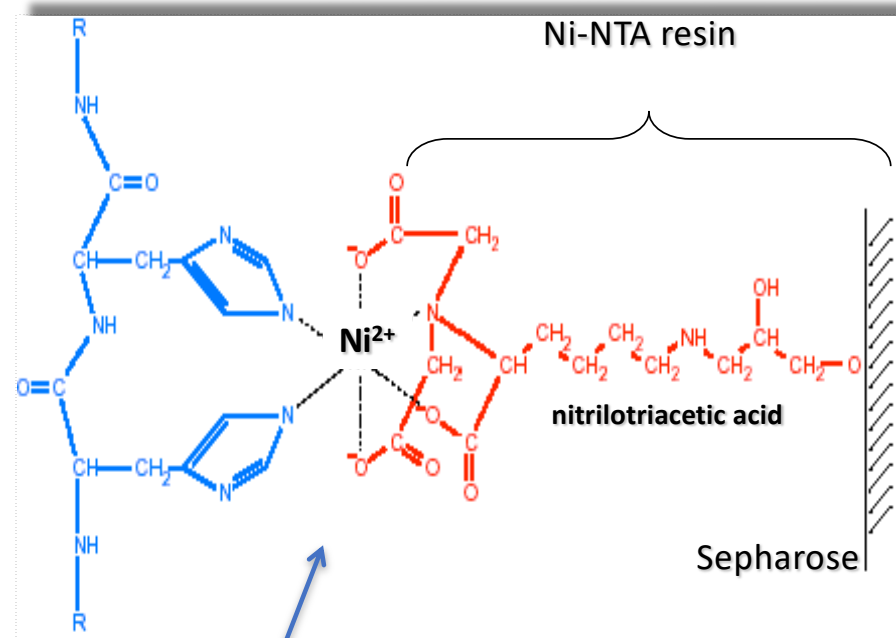
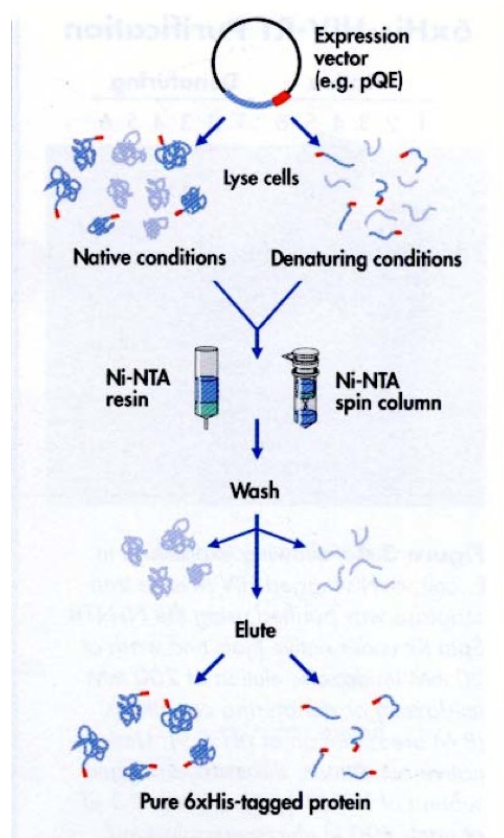


**Protein
Purification by
Affinity
Chromatography**

Glutathione/Sepharose Resin



The HIS TAG System



Elution By Imidazole (a histidine analogue)

Cell-Free Transcription/Translation Systems

- Rabbit Reticulocyte Lysate or Wheat Germ Extract translation systems use RNA synthesized *in vitro*
- Coupled systems bypass many of these steps by incorporating the reagents needed for transcription directly in the translation mix.
- Traditionally, radioactive [³⁵S]methionine has been added to cell-free expression reactions, and the methionine is incorporated into the expressed protein, allowing detection by autoradiography.

