lab 6

Real time PCR

Definition of real-time PCR :

Real-time PCR (also known as Quantitative PCR, Real-time Quantitative PCR, or (RTQ-PCR -Q-PCR/qPCR/qrt-PCR) or *kinetic polymerase chain reaction* (KPCR)-- but not **RT**-PCR) is a method of simultaneous DNA quantification and amplification. DNA is specifically amplified by polymerase chain reaction (PCR). After each round of amplification, the DNA is quantified. Common methods of quantification include the use of fluorescent dyes that intercalate with double-strand DNA and modified DNA oligo nucleotides (called probes) that fluoresce when hybridized with a complementary DNA.

Frequently, real-time PCR is combined with reverse transcription-polymerase chain reaction (RT-PCR) to quantify low abundance messenger RNA, enabling a researcher to quantify relative gene expression at a particular time, or in a particular cell or tissue type. The combined technique is often called quantitative RT-PCR.

The main idea :

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle .

Three general methods for the quantitative assays:

1. Hydrolysis probes

(TaqMan, Beacons) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

2. Hybridization or FRET probes

(Light Cycler)

3. DNA-binding agents

(SYBR Green, Eva Green, LC GReen) non-specific fluorescent dyes that intercalate with any double-stranded DNA .

Principles of Real-Time Quantitative PCR Techniques

(a) **SYBR Green I technique:**

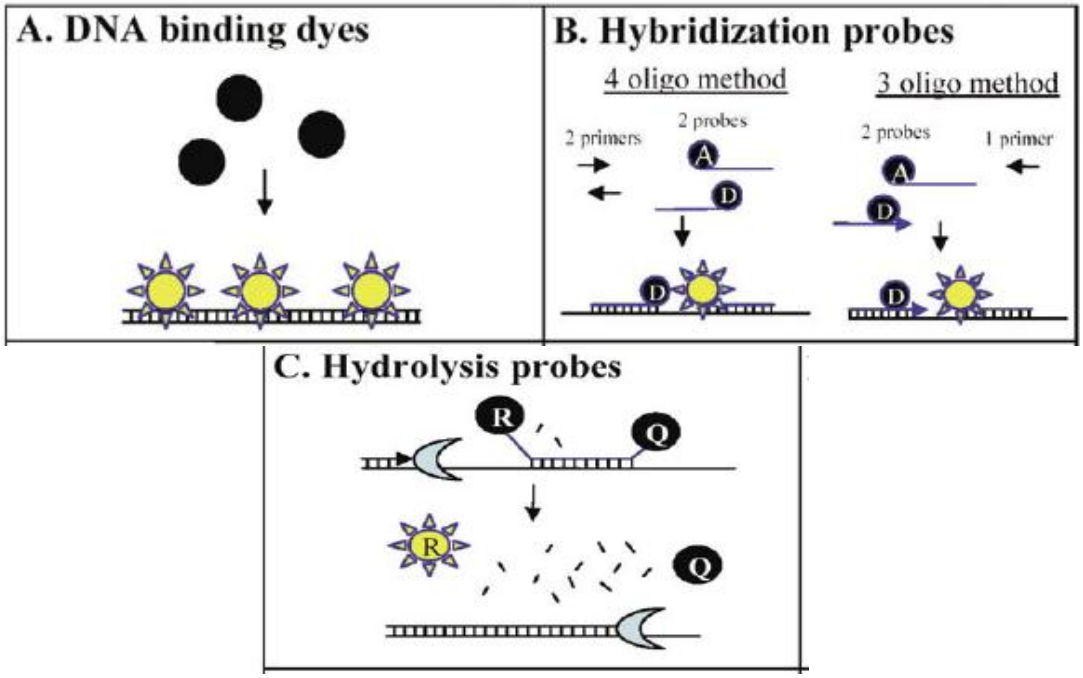
SYBR Green I fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

(b) **Hydrolysis probe technique:**

The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

(c) **Hybridization probes technique:**

In this technique one probe is labelled with a donor fluorochrome at the 3' end and a second – adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

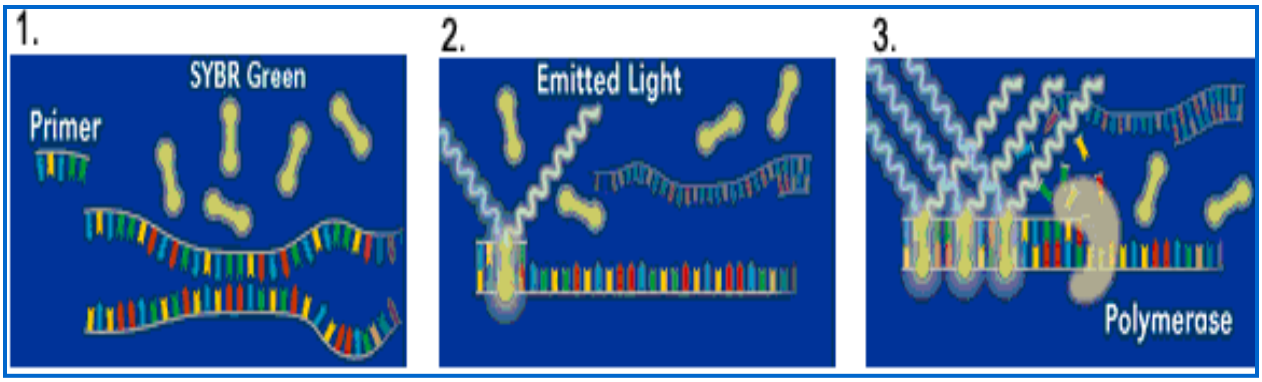


SYBR Green (double-stranded DNA binding dye):

* emits a strong fluorescent signal upon binding to double-stranded DNA
* nonspecific binding is a disadvantage
* requires extensive optimization
* requires melting curve analysis to ensure specificity
* longer amplicons create a stronger signal
* may be multiplexed when coupled with melting curve analysis

SYBR Green

(1) At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers and the SYBR Green. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis. (2) After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green molecules to emit light upon excitation. (3) During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.



When to Choose SYBR Green:

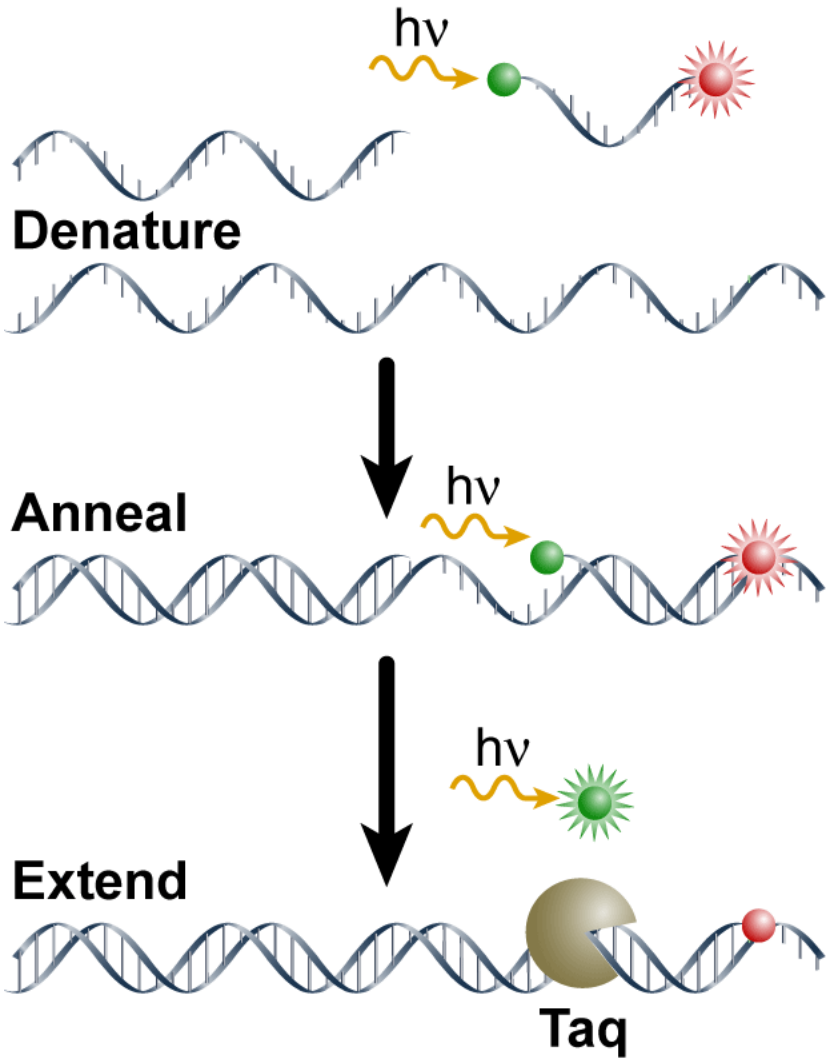
* Assays that do not require specificity of probe based assays. Detection of 1000s of molecules
* General screening of transcripts prior to moving to probe based assays

- When the PCR system is fully optimized -no primer dimers or non-specific amplicons, e.g. from genomic DNA
- * Low level pathogen detection
- Commonly used in gene expression assays

TaqMan method

In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3, is designed to bind specifically to a site on the target sequence downstream of the P1 binding site. P3 is labelled with two fluorophores, a reporter dye (R) is attached at the 5 end, and a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3 end. Because its 3' end is blocked, primer P3 cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by P1 and as the enzyme approaches P3, its 5' exonuclease activity degrades the P3 primer from its 5' end. The end result is that the nascent DNA strand extends beyond the P3 binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye moves away from the quencher, the resulting increase in reporter emission intensity is easily detected.

Commonly used in diagnostic assays



Primer design – key to successful PCR

• Good primer design saves time and money
• Advanced applications require even more stringent primer design
– » Multiplex
– » Single-cell real-time PCR
• Primer Express software

Good primer / Primers should have:

• 18-24 bases
• 40-60% G/C
• Balanced distribution of G/C and A/T bases
• Avoid runs (≥4) of an identical nucleotide (especially G's)
• Tm that allows annealing at 55-65 °C
• No internal secondary structures (hair-pins)
• The five nt at the 3'end should have no more than two G and/or C bases.

Real-time PCR applications

• Quantitation of gene expression
• Pathogen detection
• GMO detection
• Viral quantitation
• Array verification
• Drug therapy efficacy
• DNA damage measurement
• Quality control and assay validation
• Genotyping

One-Step or Two-Step PCR

***** one-step real-time RT-PCR performs reverse transcription and PCR in a single buffer system and in one tube
* in two-step RT-PCR, these two steps are performed separately in different tubes

Real-time PCR is another method for measuring RNA abundance. With currently available real-time PCR machines, one can measure only 96 RNA levels at a time, so this method cannot be considered to be a genome wide analysis. However, its sensitivity is superior to that of microarrays, and it is much less expensive than MPSS; thus it serves an important role as a means of verifying expression of genes detected by other means such as microarrays. There are several different methods used in real-time PCR, but all are based on a fluorescent signal released as the PCR amplification proceeds.

Lab 7
DNA Fingerprinting

The chemical structure of everyone's DNA is the same. The only difference between people (or any animal) is the order of the base pairs. There are so many millions of base pairs in each person's DNA that every person has a different sequence.

Using these sequences, every person could be identified solely by the sequence of their base pairs. However, because there are so many millions of base pairs, the task would be very time-consuming. Instead, scientists are able to use a shorter method, because of repeating patterns in DNA.

These patterns do not, however, give an individual "fingerprint," but they are able to determine whether two DNA samples are from the same person, related people, or non-related people. Scientists use a small number of sequences of DNA that are known to vary among individuals a great deal, and analyze those to get a certain probability of a match.

DNA Fingerprinting

Is a technology using tandem repeats of individuals to identify individuals.

Tandem repeats occur in DNA when a pattern of two or more nucleotides is repeated and the repetitions are directly adjacent to each other.

Example:

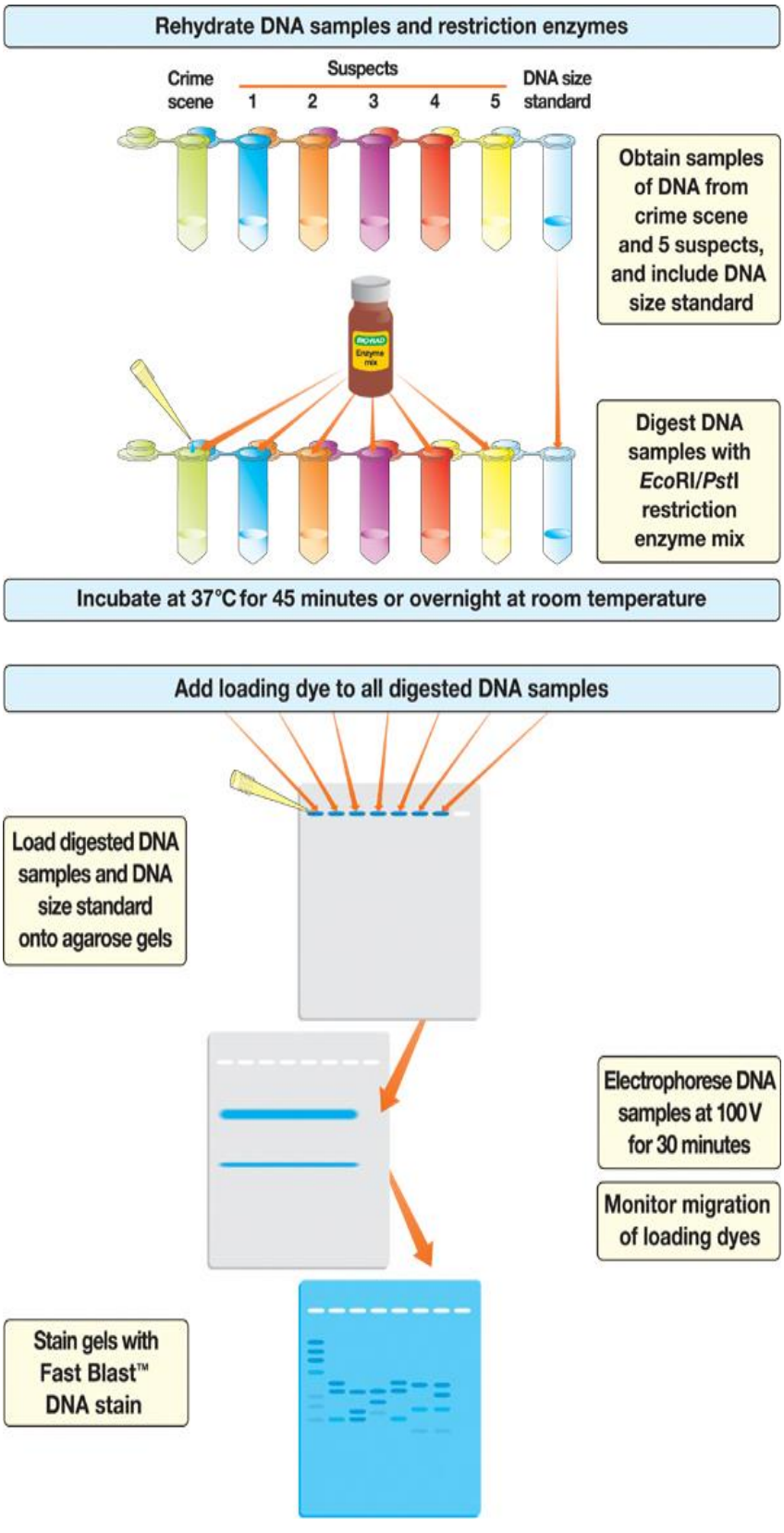
A-T-T-C-G-A-T-T-C-G-A-T-T-C-G

in which the sequence A-T-T-C-G is repeated three times

Procedure Of DNA Fingerprinting:

1. Evidence
   ~DNA is extracted from blood, hair..etc
2. Fragmentation
   ~ cut the DNA into fragments
3. Separation
   ~ gel electrophoresis
4. X-ray
   ~ the separated DNA is transferred to a nylon membrane and radioactively treated.

Technique Of DNA Fingerprinting:



LAb 8
Protein separation and quantitation

SDS gel :

The system most people use for separating proteins by gel electrophoresis was formulated by Laemmli (Nature 227:680-685 [1970]). It is such a commonly used laboratory technique.

SDS-PAGE, officially **sodium dodecyl sulfate polyacrylamide gel electrophoresis**, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding posttranslational modifications and other factors).

Most SDS PAGE sample buffers contain the following:

1- **SDS** (sodium dodecyl sulphate, also called lauryl sulphate), an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass.

2- **b-mercaptoethanol** (BME) : is added to prevent oxidation of cysteines and to break up disulfide bonds.

3-, **bromophenol blue**, : is a dye that is useful for visualizing your sample in the well and tracking its progress through the gel.

4- **Glycerol** : is much more dense than water and is added to make the sample fall to the bottom of the sample well rather than just flow out and mix with all the buffer in the upper reservoir.

5- **Tris-glycine** : the buffer .

6- **Acrylamide** It is a slow spontaneous process by which acrylamide molecules join together by head on tail fashion.

7- **Bisacrylamide** (N,N'-Methylenebisacrylamide) Bisacrylamide is the most frequently used *cross linking agent for poly acrylamide gels.

8-Ammonium persulfate (APS) APS is an initiator for gel formation.

9-TEMED (N, N, N', N'-tetramethylethylenediamine) Chemical polymerisation of acrylamide gel is used for SDS-PAGE.

SDS-PAGE Gel Component Table

Separating/Running Gel (30mL Total Volume; For 35cm gels)				
	5%	**10%**	12.5%	15%
1M Tris HCl, pH 8.8	11.25mL	**11.25mL**	11.25mL	11.25mL
DiH_2O	13.5mL	**8.5mL**	6.0mL	3.5mL
20% SDS	150µL	**150µL**	150µL	150µL
Acrylamide:Bis-Acrylamide 30%:0.8%	5mL	**10mL**	12.5mL	15mL
TEMED	12.5µL	**12.5µL**	12.5µL	12.5µL
10% Ammonium Persulfate	100µL	**100µL**	100µL	100µL
Total Volume	~30mL	**~30mL**	~30mL	~30mL
Separating/Running Gel (24mL Total Volume; For 10.5cm gels)				
	5%	7.5%	8%	**10%**
1M Tris HCl, pH 8.8	9mL	9mL	9mL	**9mL**
DiH_2O	10.75mL	8.75mL	8.35mL	**6.75mL**

20% SDS	120µL	120µL	120µL	**120µL**
Acrylamide:Bis-Acrylamide 30%:0.8%	4mL	6mL	6.4mL	**8mL**
TEMED	10µL	10µL	10µL	**10µL**
10% Ammonium Persulfate	120µL	120µL	120µL	**120µL**
Total Volume	~24mL	~24mL	~24mL	**~24mL**
Stacking Gel (10mL Total Volume)				
	3%		**5%**	
1M Tris HCl, pH 6.8	1.25mL		**1.25mL**	
DiH_2O	7.6mL		**6.9mL**	
20% SDS	50µL		**50µL**	
Acrylamide:Bis-Acrylamide 30%:0.8%	1mL		**1.66mL**	
TEMED	10µL		**10µL**	
10% Ammonium Persulfate	100µL		**100µL**	
Total Volume	~10mL		**~10mL**	
SDS-PAGE Running Buffer (For Electrophoresis Chamber)				
10X Tris Glycine Buffer, pH 8.3	80mL			
20% SDS, pH 8.5	4mL			
DiH_2O	716mL *(800mL Total Volume)*			

Solutions Common to SDS-PAGE Protocol

Acrylamide:Bis-Acrylamide 30%:0.8%

Acrylamide	30g
Bis-Acrylamide	0.8g
DiH_2O	100mL (Total Volume)

*Store in dark container at 4°C. Shelf life is about 3 months.

10% Ammonium Persulfate Solution

	Daily Use	Stock Use
Ammonium Persulfate	0.10g	1g
DiH_2O	1mL	10mL (Total Volume)

*Store in dark container at 4°C. Shelf life is about 1 month.

TEMED

*This item is usually purchased. It is stored at 4°C in a dark bottle.

H_2O Saturated n-Butanol

n-Butanol	50mL
DiH_2O	5mL

Combine in small bottle and shake. Use top layer to overlay gels.
*Store at room temperature indefinitely.

1M Tris HCl

Tris	121.1g
DiH_2O	800mL
Adjust pH to 6.8 or 8.8 as needed	Volume varies

Adjust to final volume of 1 Liter with DiH_2O.
*Store at room temperature. Sterilize by autoclave (If needed) and dispense into aliquots.

Separating/Running Gel Cocktail 10% (Everything except AP and TEMED)

1M Tris HCl, pH 8.8	187.5mL
20% SDS	2.5mL
Acrylamide:Bis-Acryl 30%:0.8%	166.67mL
DiH_2O	Adjust to 500mL total volume

*Store in dark container at 4°C. Shelf life is about 3 months.

Stacking Gel Cocktail (Everything except AP and TEMED)

1M Tris HCl, pH 6.8	31.34mL
20% SDS	1.25mL
Acrylamide:Bis-Acryl 30%:0.8%	41.62mL
DiH_2O	Adjust to 250mL total volume

*Store in dark container at 4°C. Shelf life is about 3 months.

20% SDS Solution

SDS	20g (Measure under hood – irritant to lungs)
DiH_2O	100mL (Total Volume)

Use heat and slow stirring to dissolve completely. Be patient!
*Store at room temperature. Shelf life is 6 months to 1 year.

Protein Cracking Buffer (2X)

Glycerol	2mL
20% SDS	1mL
1M Tris HCl, pH 6.8	400µL
ß-Mercaptoethanol	500µL
Bromophenol Blue	0.5g
DiH_2O	10mL (Total volume)

*Divide into 0.5mL aliquots. Store at -20°C for 6 months to 1 year.

Coomassie Blue SDS-PAGE Staining Solution

Methanol	500mL
Glacial Acetic Acid	100mL
Coomassie Brilliant Blue	1g
DiH_2O	1 Liter (Total Volume)

Dissolve the coomassie blue in methanol first; Then add acid and water.
*Store at room temperature.

SDS-PAGE Destain Solution

Methanol	100mL
Glacial Acetic Acid	100mL
DiH_2O	1 Liter (Total Volume)

*Store at room temperature.

SDS-PAGE Running Buffer (For electrophoresis tank)

10X Tris Glycine	80mL	
20% SDS		4mL
DiH_2O		800mL (Total Volume)

*Made fresh each time it is required.

10X Tris Glycine Solution

Tris		30.3g
Glycine	144.1g	
DiH_2O		1 Liter (Total Volume)

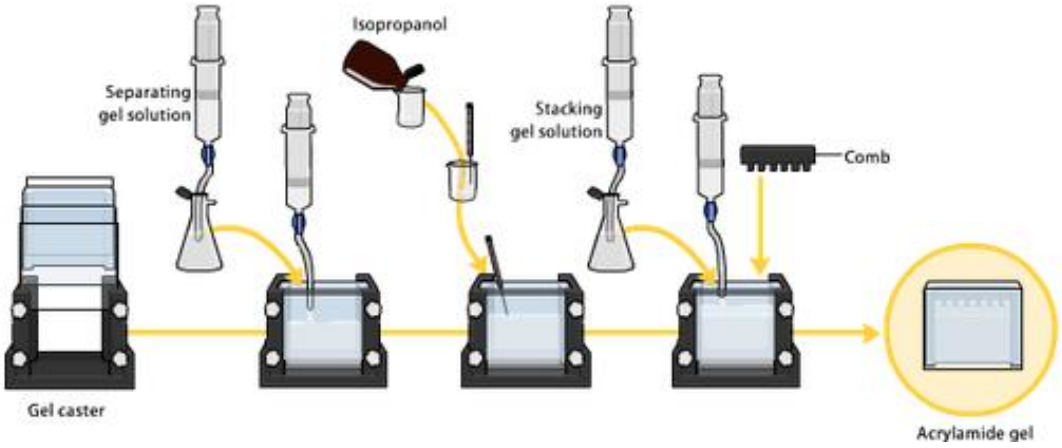
*Store at room temperature. Shelf life is about one year.

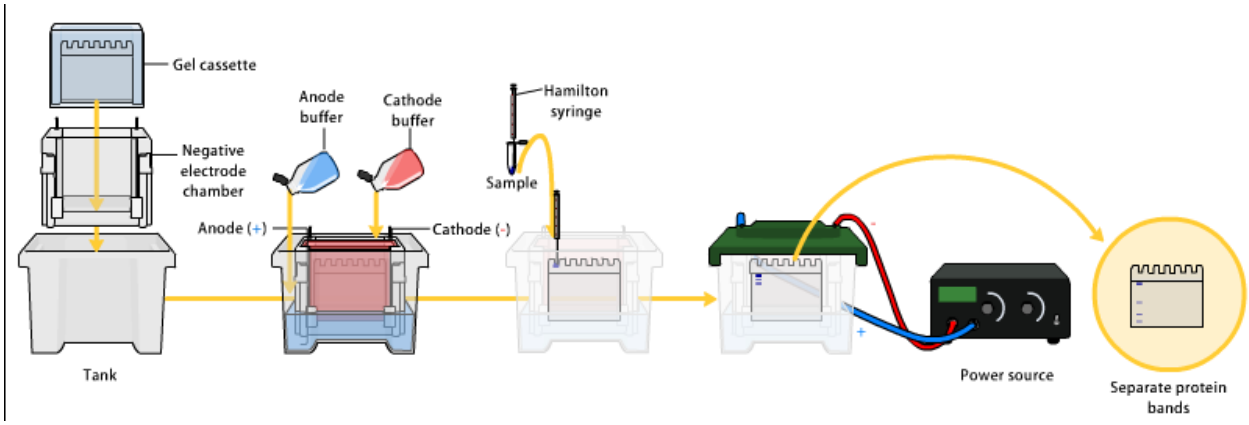
SDS : is an ionic detergent that binds to the vast majority of proteins at a constant ratio of 1.4 gm SDS/gm protein. A few proteins like tubulin do not bind at this ratio and this is one reason why some proteins migrate anomalously (there are other reasons as well so you shouldn't put too much faith in the apparent molecular weight estimated from an SDS PAGE gel).

Since SDS is an anionic detergent it imparts a negative charge to all the proteins in your sample. More importantly, these charges swamp the inherent charge of the proteins and give every protein the same charge-to-mass ratio. Because the proteins have the same charge-to-mass ratio, and because the gels have sieving properties, mobility becomes a function of molecular weight. But what about running gels, stacking gels, electrode buffer, and all these different pHs?

The velocity of a charged particle moving in an electric field is directly proportional to the field strength and the charge on the molecule and is inversely proportional to the size of the molecule and the viscosity of the medium. Adding a gel with sieving properties (that is a gel where the resistance to the motion of a particle increases with particle size) increases the differences in mobility between proteins of different molecular weights. This is the basis of separation. The problem now becomes how to line up all the proteins in an orderly fashion at the starting gate. That's where the discontinuous pH part comes in.

- Laemmli gels are composed of two different gels (stacker and running gel), each cast at a different pH.
- In addition, the gel buffer is at a third, different pH.
- The running gel is buffered with Tris by adjusting it to pH 8.8 with HCl.
- The stacking gel is also buffered with Tris but adjusted to pH 6.8 with HCl.
- The sample buffer is also buffered to pH 6.8 with Tris HCl .
- The electrode buffer is also Tris, but here the pH is adjusted to a few tenths of a unit below the running gel (in this case 8.3) using only glycine – nothing else. We run our gels at constant voltage.





JUST FOR YOUR DENERAL INFORMATION

So here's what happens when you turn on the power. Glycine is a weak acid and it can exist in either of two states, an uncharged zwitterion, or a charged glycinate anion (that is to say, negatively charged). At low pH it is protonated and thus uncharged. At higher pH it is negatively charged. When the power goes on the glycine ions in the running buffer want to move away from the cathode (the negative electrode) so they head toward the sample and the stacking gel. The pH there is low and so they lose a lot of their

charge and slow down. Meanwhile, in the stacker and sample the highly mobile chloride ions (which are also negatively charged) move away from the cathode too. This creates a narrow zone of very low conductance (in other words very high electrical resistance) in the top of the stacking gel. Because V=IR almost all of the voltage that you put across the gel (110 Volts is typical for stacking) is concentrated in this small zone. The very high field strength makes the negatively charged proteins move forward. The trick, however, is that they can never outrun the chloride ions. If they did they would find themselves in a region of high conductance and very low field strength and would immediately slow down. The result is that all the proteins move through the stacker in a tight band just behind the moving front of chloride ions. Behind them, the pokey glycine ions straggle along as best they can (they do move, but with lower mobility than the chloride ions).

When the big caravan of ions hits the running gel everything changes. The pH goes way up and the glycine becomes deprotonated (and thus more negatively charged). The mobility of the glycine goes way up and the mobility of the proteins goes way down (due to the sieving properties of the gel). The result is that the glycine races past the protein and the proteins are no longer in a narrow zone of very high resistance (and very high electric field). They find themselves in a much more relaxed, uniform electric field where they can chill out a bit. Move at their own pace.

Bake to important info :

Safety Notes:

- Acrylimide is extremely toxic, causing central nervous system paralysis. It can be absorbed through unbroken skin. If skin comes in contact with acrylimide solution or powder, wash immediately with soap and a lot of water. Unpolymerized acrylimide should be polymerized with excess catalyst and disposed of with solid waste. DO NOT POUR UNPOLYMERIZED ACRYLIMIDE DOWN THE SINK.
- Amonium Persulfate should be made up fresh or used from a relatively fresh stock. It goes bad after a week or two in the refrigerator. It can be disposed of by dilution with water and pouring down the sink.
- TEMED should be stored in the refrigerator in dark glass bottles. A bottle should be good for about a year, maybe longer.

Lab 9
Protein purification

Protein purification
is a series of processes intended to isolate a single type of protein from a complex mixture.

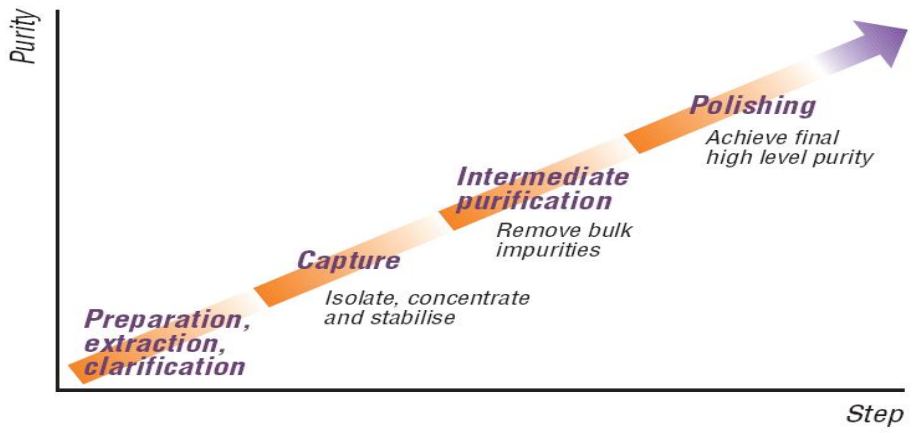
Reasons For Protein Purification

- to identify the function of a protein
- to identify the structure of a protein
- to use the use the purified product to produce a commercial product

Selection of protein source:
Starting material can be from

- Animal tissue
- Plant material
- Biological fluids (e.g. blood, milk, sera)
- cultures (yeast, fungi, bacteria)
- Cell cultures (animal cells, plant cells, insect cells)

Three phase purification strategy:



Protein purification:

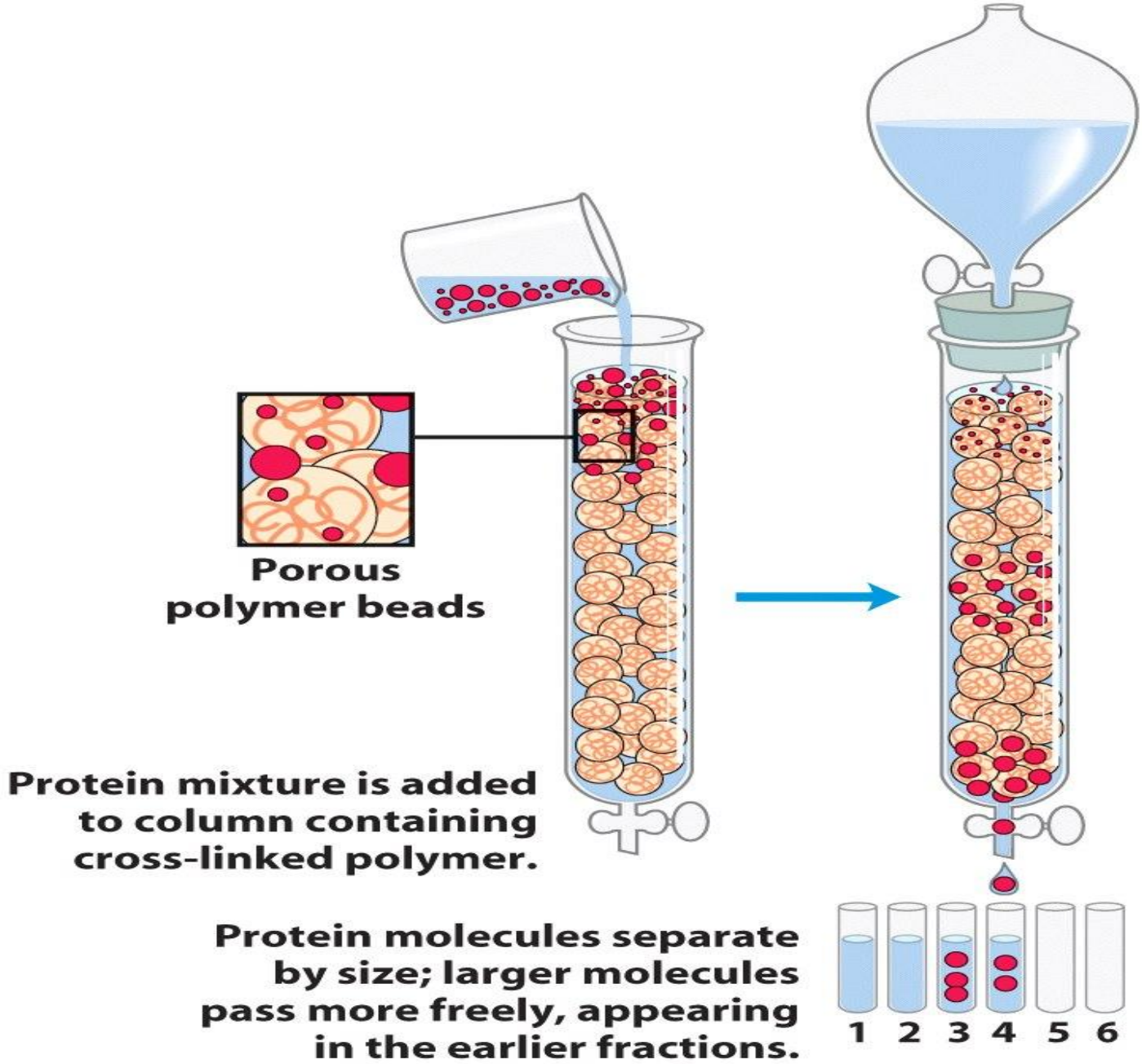
1- Size exclusion chromatography
2- Ion exchange chromatography
3- Electrophoresis
4- Centrifugation

Introduction to Chromatography:

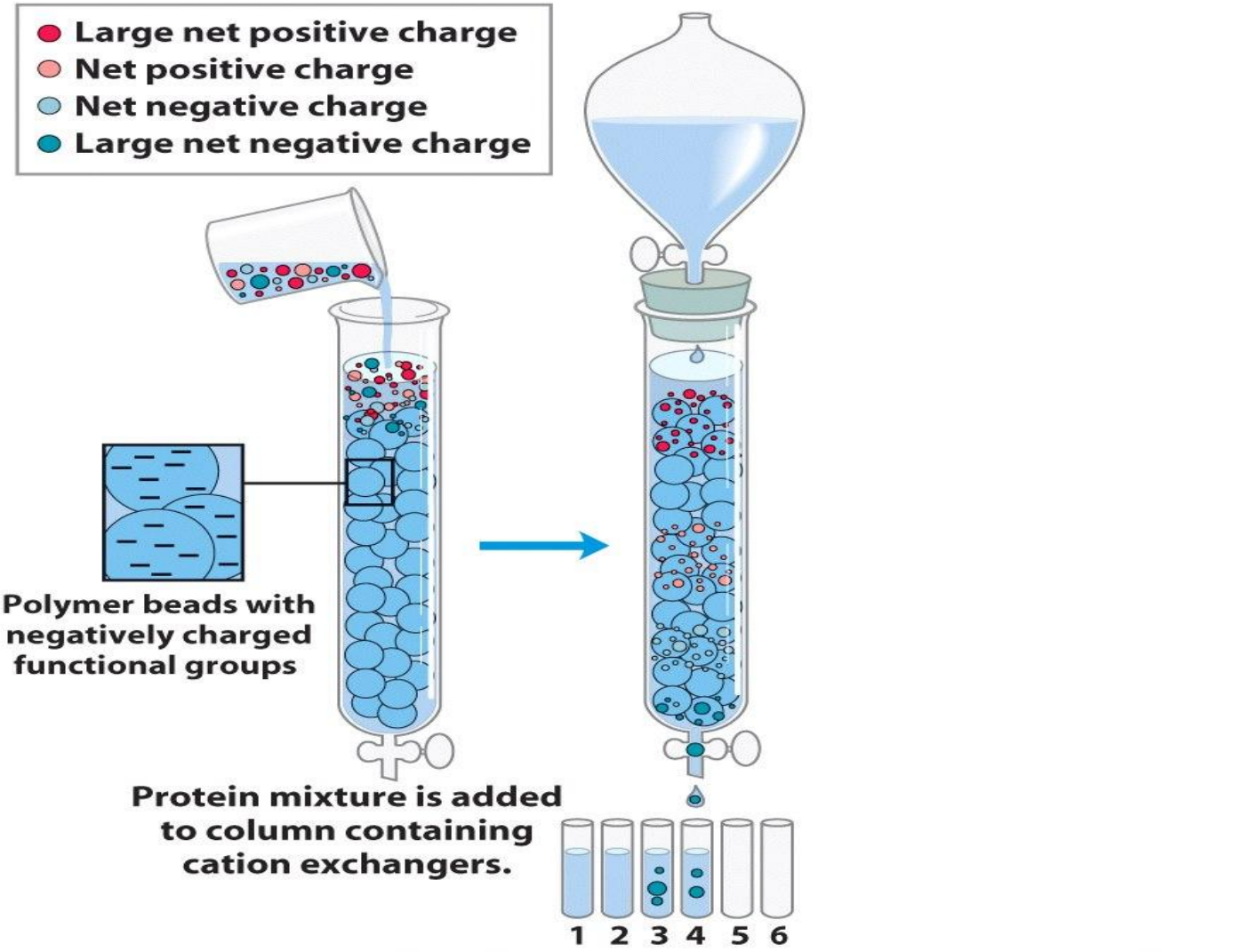
- Chromatography is used to purify complex mixtures.
- We used a resin in a column.
- The resin binds to certain proteins. This purifies these proteins away from other proteins that do not bind to the resin.

- After the other proteins have passed through, the bound proteins can be released.
- There are different resins for different substances you want to purify.

1- Size-exclusion chromatography:

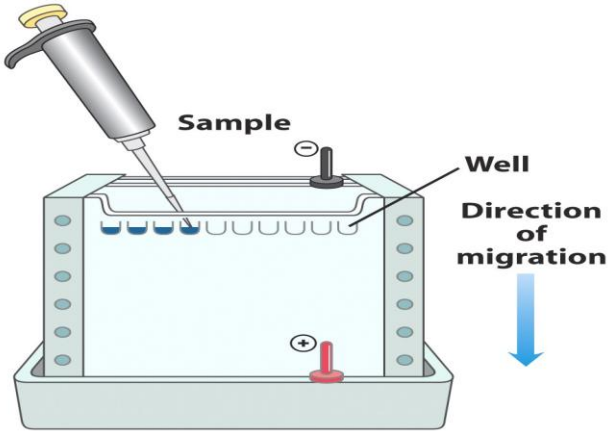


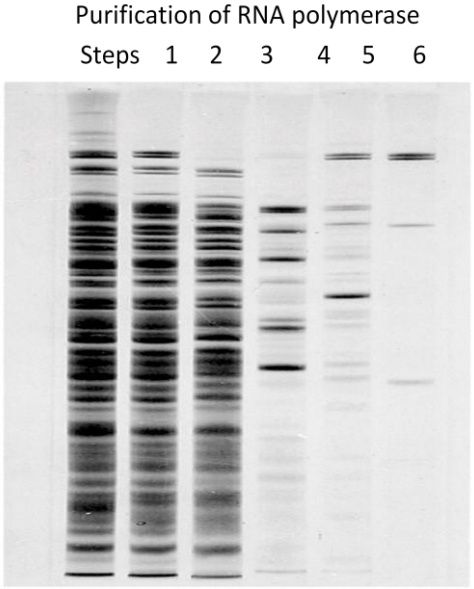
2- Ion-Exchange chromatography:



Proteins move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.

3- **Electrophoresis**:





4- **Centrifugation** :