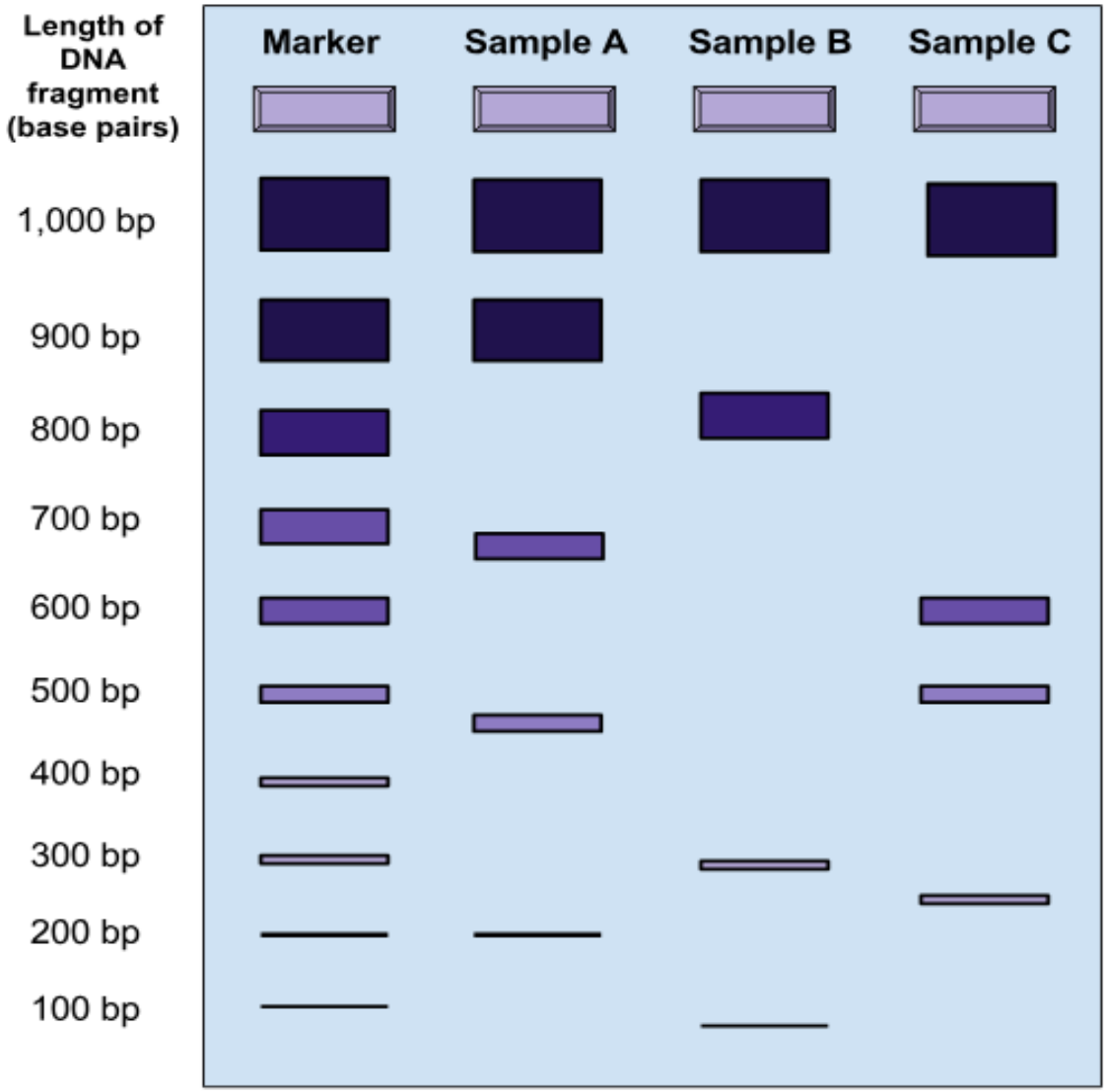
PRACTICAL
DNA TECHNOLOGY



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DNA TECHNOLOGY PRACTICAL

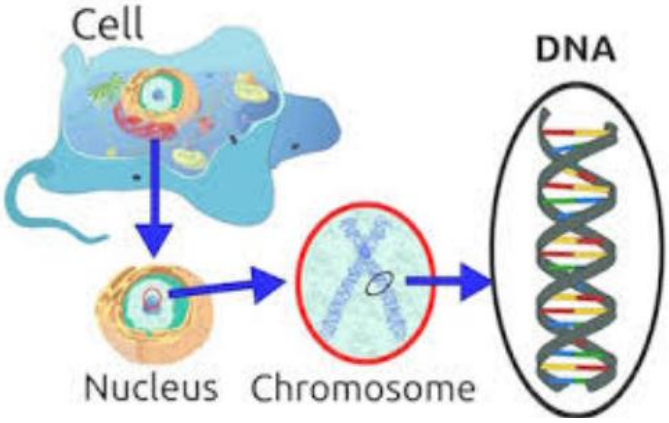
Contents

DNA Extraction

Purpose of DNA Extraction

DNA extraction is a routine procedure to collect DNA for
Analyze forensic evidence.
Study a gene involved in cancer.
DNA fingerprinting to identify individuals (Paternity test).

DNA can be extracted from any nucleated cell: Skin, bone marrow, Blood, Saliva, Semen, hair.



Basic Protocol

• Most DNA extraction protocols consist of two parts

1. A technique to lyse the cells gently and solubilize the DNA

2. Enzymatic or chemical methods to remove contaminating proteins, RNA, or macromolecules.

1- Cell lysis

Cells have an outer membrane – the cell membrane as well as an inner membrane which surrounds the DNA called the nucleus. The membranes are made up of two layers of lipids (fat molecules) with proteins going through them.

The cell membrane and nucleus can be broken apart by chemical means, such as by the addition of a detergent, which separates the lipid molecules to break down the membrane

- Detergent is SDS [Sodium dodeyl sulphate]

2- DNA Isolation (Removal of contaminates as protein , RNA)

a- Protein removal: Proteinase K to digest proteins bound to DNA (histones)

b- RNA ase treatment to digest RNA

c- Phenol Extraction: Forms complexes with lipids and proteins, causing them to precipitate in solution.

d- Centrifuge the sample to pull proteins down and use supernatant for next step

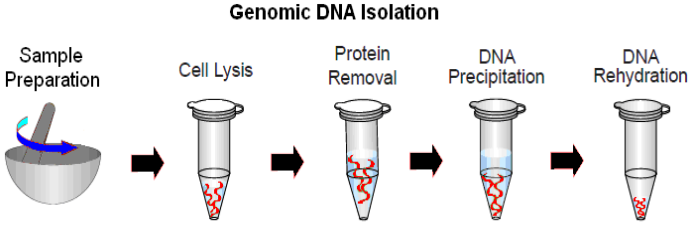
3- DNA precipitation

The DNA now needs to be removed from the liquid solution.

DNA is not soluble in alcohol. So the addition of ethanol or isopropyl alcohol will cause the DNA to clump and form a visible white precipitate.

The most common methods of DNA extraction include organic extraction (also called phenol chloroform extraction), Chelex extraction, and solid phase extraction.

Each method of extraction works well in the laboratory, but analysts typically selects their preferred method based on factors such as the cost, the time involved, the quantity of DNA yielded, and the quality of DNA yielded.

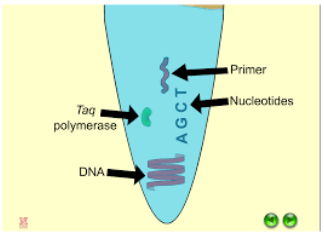


Polymerase chain reaction

To amplify a lot of double-stranded DNA molecules (fragments) with same (identical) size and sequence by enzymatic method and cycling condition.
PCR is DNA replication in a test tube

- PCR targets and amplifies a specific region of a DNA strand.
- It is an *in vitro* technique to generate large quantities of a specified DNA.
- Often, only a small amount of DNA is available eg. A drop of blood, Semen strains, Single hair, vaginal swabs etc.
- Two methods currently exist for amplifying the DNA or making copies.
    - Cloning —takes a long time for enough clones to reach maturity.
    - PCR —works on even a single molecule quickly.

Requirements of PCR



- DNA Template
- Primers
- DNA polymerase: Taq polymerase
- Deoxynucleoside triphosphates (dNTPs)
- Buffer solution
- Divalent cations (eg. Mg^{2+}).

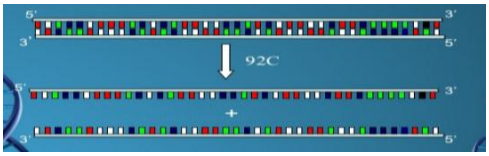
1) DNA sequence of target region must be known.
2) Primers - typically 20-30 bases in size. These can be readily produced by commercial companies. Can also be prepared using a DNA synthesizer.
3) Thermo-stable DNA polymerase - *Taq* DNA polymerase – isolated from a species known as *Thermus aquaticus* that thrives in hot springs.

4) DNA thermal cycler - machine which can be programmed to carry out heating and cooling of samples over a number of cycles.

Steps involved

1. Denaturation of ds DNA template
2. Annealing of primers
3. Extension of ds DNA molecules

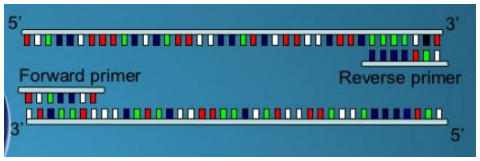
1- DENATURATION:



- The reaction mixture is heated to a temperature between 90-98° C so that the ds DNA is denatured into single strands by disrupting the hydrogen bonds between complementary bases.
- Duration of this step is 1-2 mins.

2- ANNEALING:

- Annealing fancy word for renaturing.

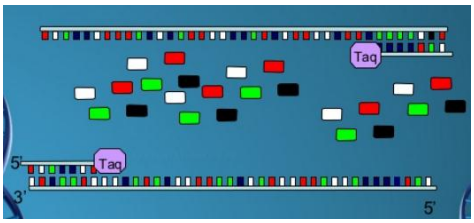


- Temperature of reaction mixture is cooled to 50-70C (dependant on the melting temperature of the expected duplex).
- Primers bind to their complementary sequences
- Hydrogen bonds reform.
- Duration of this step is 45 sec.

3- EXTENSION:

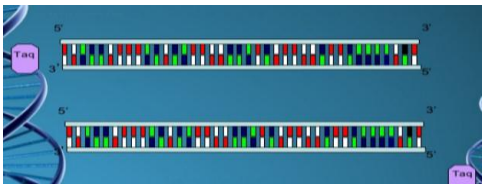
Temperature: ~72°C

Time: 0.5-3min

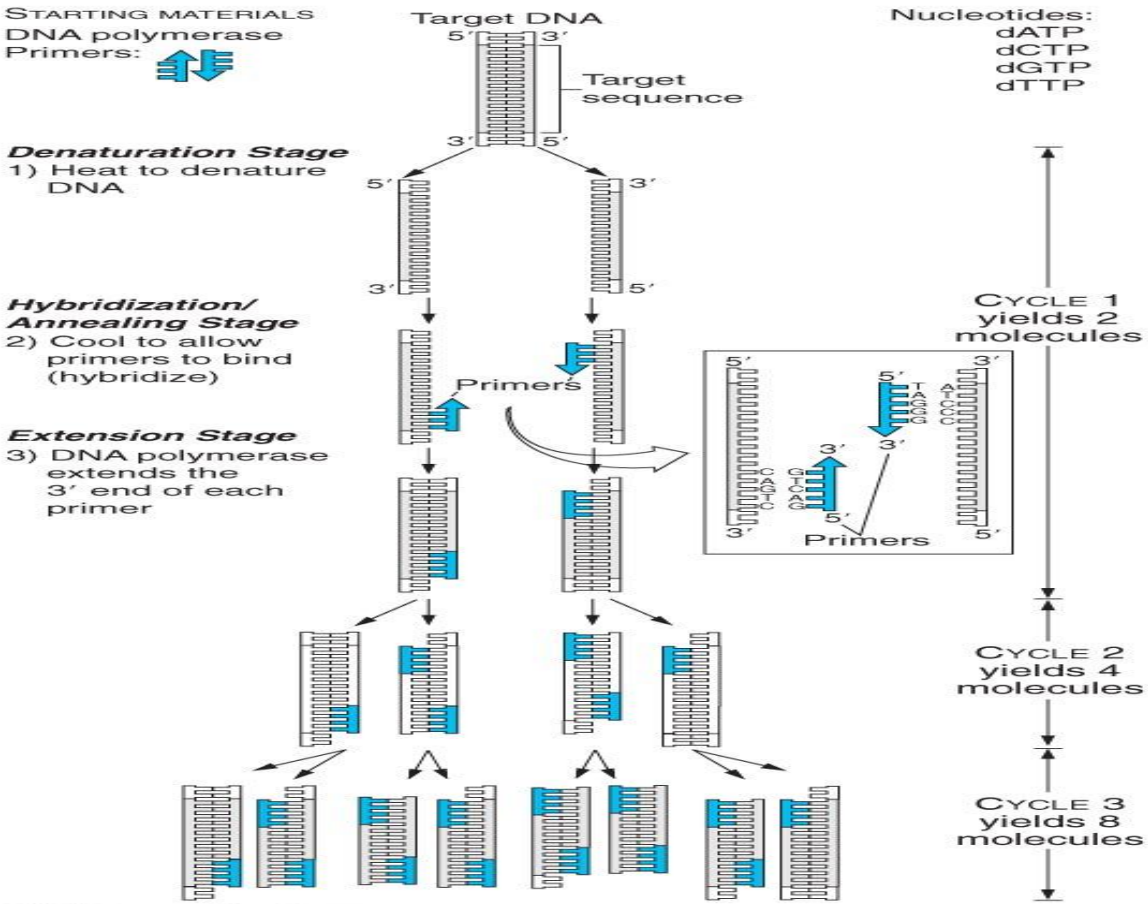


DNA polymerase binds to the annealed primers and extends DNA at the 3' end of the chain adds

dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template.



Now first cycle is over and next cycle is continued, as PCR machine is automated thermocycler the same cycle is repeated up to 30-40 times.



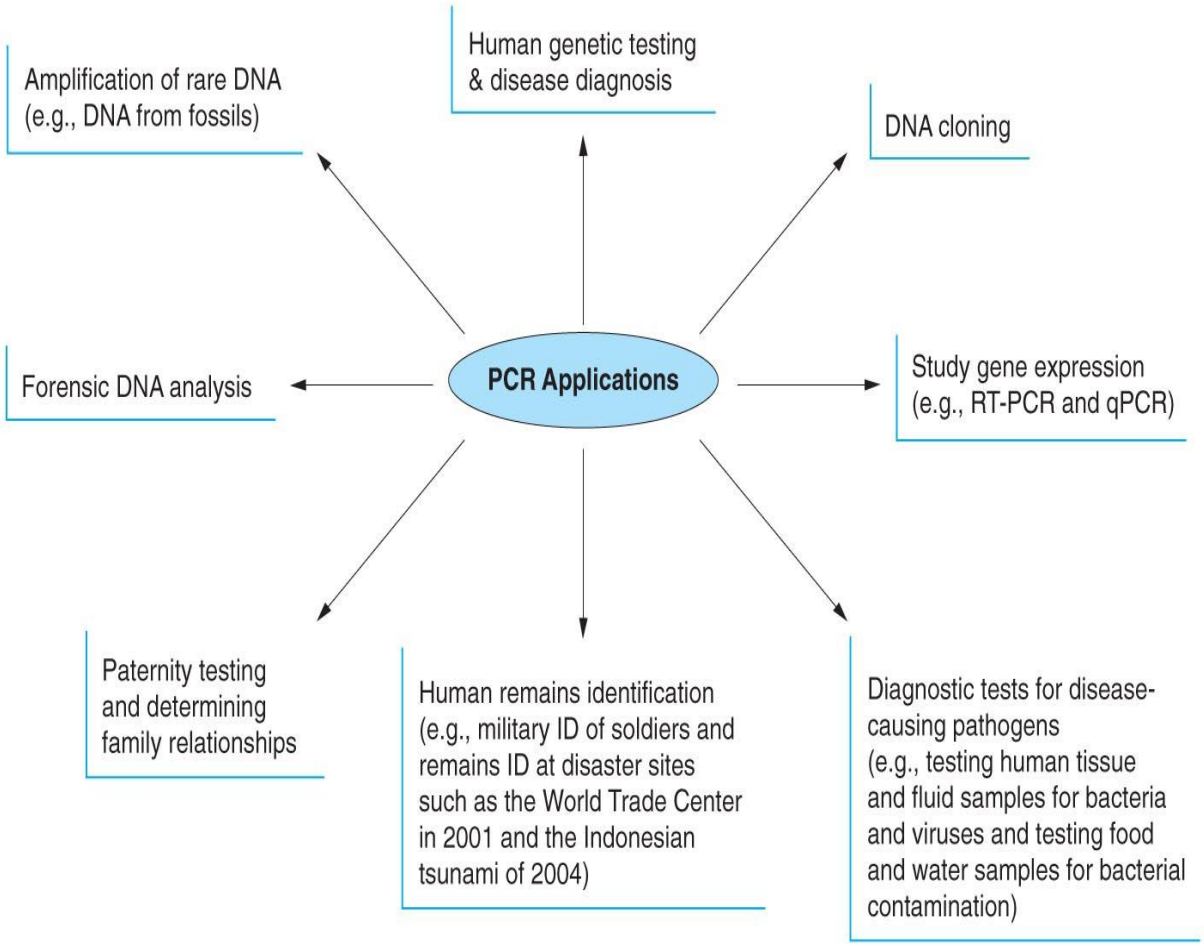
Advantage of PCR:

- Can amplify millions of copies of target DNA from small amount of starting material in short period of time.
- To calculate the number of copies of target DNA starting with 1 molecule of DNA use this equation 2^N in which N represents number of PCR cycles.

Q

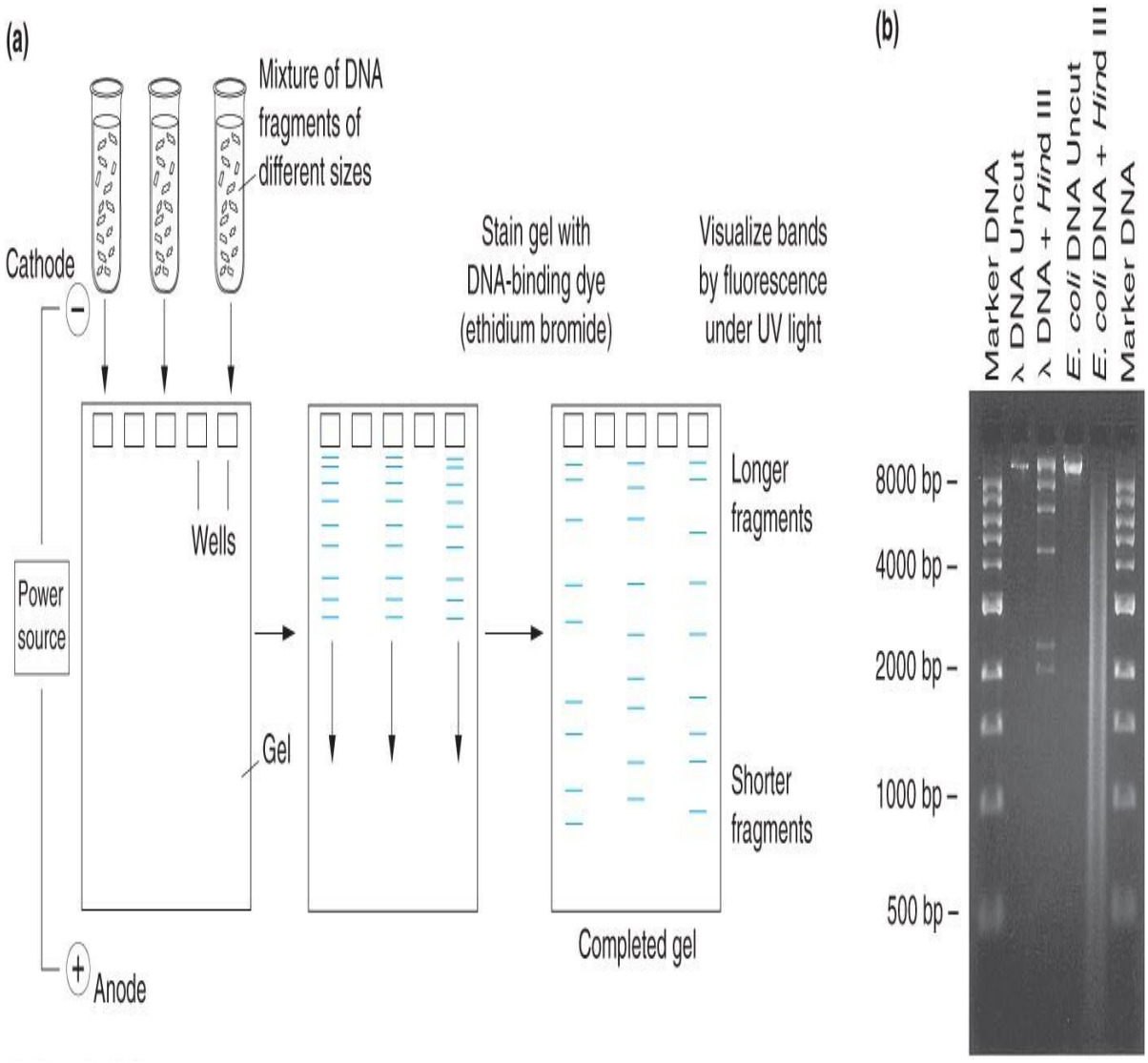
Assume you want to do 22 PCR cycles to amplify your DNA insert, how many copies of DNA will you have at the end of your PCR?

- **Applications**
  - Making DNA probes
  - Studying gene expression
  - Detection of viral and bacterial infections
  - Diagnosis of genetic conditions
  - Detection of trace amounts of DNA from tissue found at crime scene.
  - Detection of DNA from fossilized dinosaur tissue


PCR Applications
Amplification of rare DNA (e.g., DNA from fossils)
Human genetic testing & disease diagnosis
DNA cloning
Forensic DNA analysis
Study gene expression (e.g., RT-PCR and qPCR)
Paternity testing and determining family relationships
Human remains identification (e.g., military ID of soldiers and remains ID at disaster sites such as the World Trade Center in 2001 and the Indonesian tsunami of 2004)
Diagnostic tests for disease-causing pathogens (e.g., testing human tissue and fluid samples for bacteria and viruses and testing food and water samples for bacterial contamination)

Agarose Gel Electrophoresis

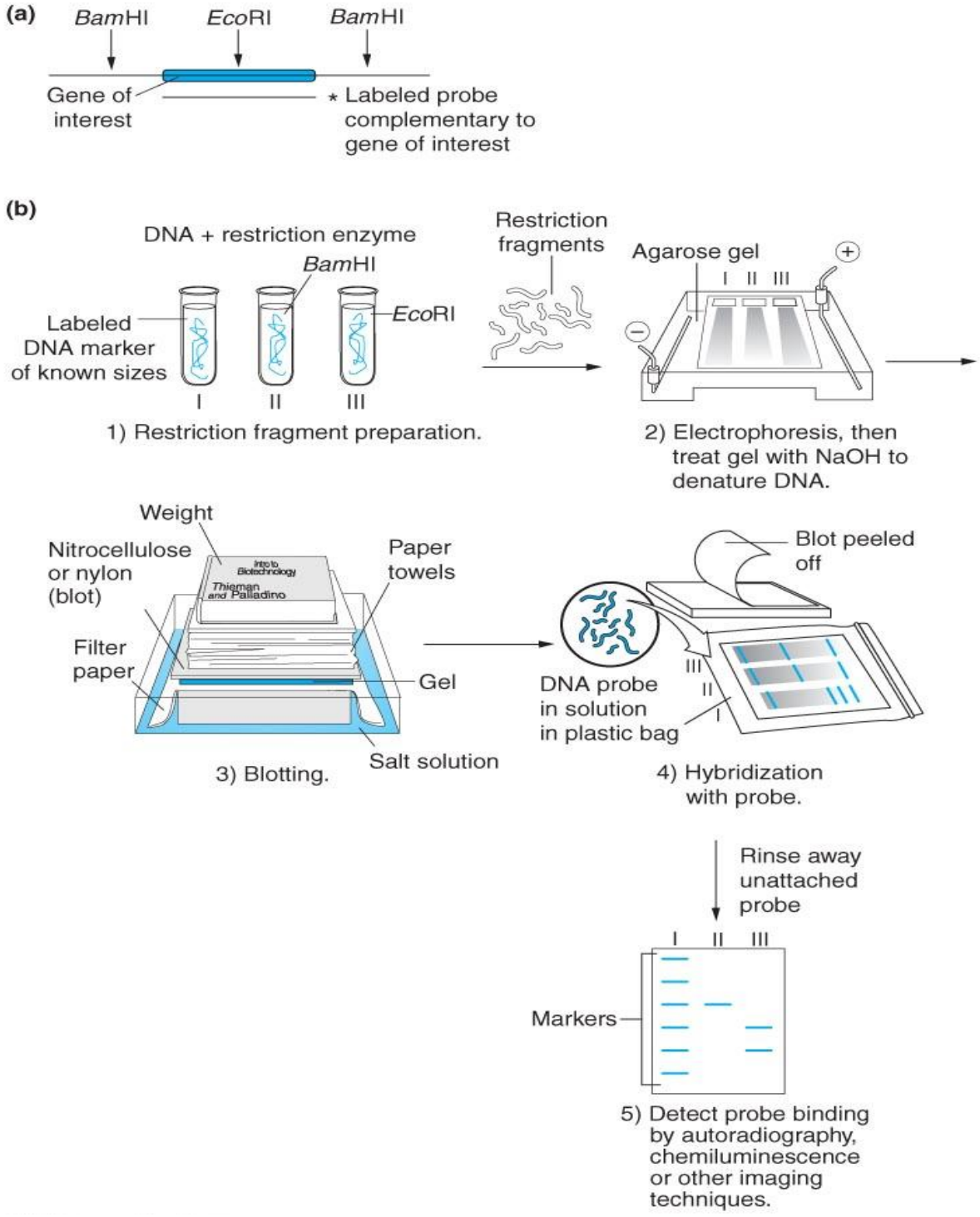
- Separate and visualize DNA fragments based on size
- Agarose is isolated from seaweed and when melted in a buffer solution and poured into a horizontal tray and as it cools it will form a semisolid gel containing small pores through which DNA will travel
  - To run a gel, it is submerged in a buffer solution that conducts electricity
  - DNA is loaded into small depressions called wells at the top of the gel
  - Electric current is applied through electrodes at opposite ends of the gel
- DNA migrates according to its charge and size
- Rate of migration through the gel depends on the size of the DNA because the sugar phosphate backbone makes it always negatively charged
- DNA migrates toward positive pole.
  - Migration distance is inversely proportional to size of DNA fragment
  - Large fragments migrate slowly; smaller fragments migrate faster
  - Tracking dye is added to the samples to monitor DNA migration during electrophoresis
  - DNA can be visualized after electrophoresis by the addition of DNA staining dyes
  - Ethidium bromide: intercalate between DNA base pairs and it fluoresces under ultraviolet light
  - Then a picture can be taken to document the gel results


(a)
Mixture of DNA fragments of different sizes
Cathode
Stain gel with DNA-binding dye (ethidium bromide)
Visualize bands by fluorescence under UV light
Wells
Power source
Gel
Longer fragments
Shorter fragments
Completed gel
Anode
(b)
Marker DNA
λ DNA Uncut
λ DNA + Hind III
E. coli DNA Uncut
E. coli DNA + Hind III
Marker DNA
8000 bp
4000 bp
2000 bp
1000 bp
500 bp

Southern blotting

– **Used to determine gene copy number; gene mapping; gene mutation detection; PCR product confirmation; DNA fingerprinting**

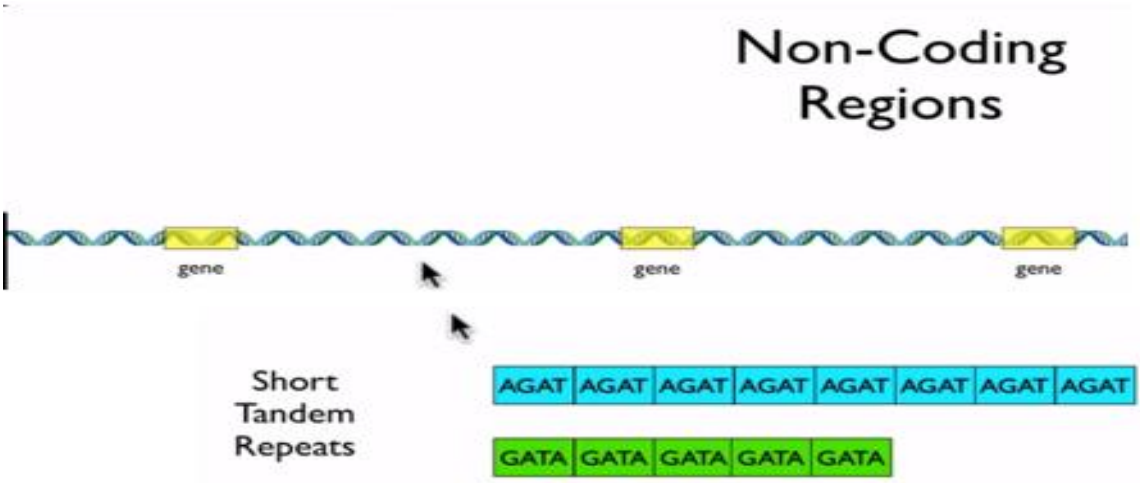
- Digest chromosomal DNA into small fragments with restriction enzymes
- Fragments are separated by agarose gel electrophoresis
- Gel is treated with alkaline solution to denature the DNA
- Fragments are transferred onto a nylon or nitrocellulose filter (called blotting)
- Filter (blot) is baked or exposed to UV light to permanently attach the DNA
- Filter (blot) is incubated with a labeled probe and exposed to film by autoradiography
- Number of bands on film represents gene copy number


(a)
BamHI
EcoRI
BamHI
Gene of interest
* Labeled probe complementary to gene of interest
(b)
DNA + restriction enzyme
BamHI
EcoRI
Labeled DNA marker of known sizes
I
II
III
1) Restriction fragment preparation.
Restriction fragments
Agarose gel
I II III
2) Electrophoresis, then treat gel with NaOH to denature DNA.
Weight
Nitrocellulose or nylon (blot)
Paper towels
Filter paper
Gel
Salt solution
3) Blotting.
Blot peeled off
DNA probe in solution in plastic bag
III
II
I
4) Hybridization with probe.
Rinse away unattached probe
I II III
Markers
5) Detect probe binding by autoradiography, chemiluminescence or other imaging techniques.

DNA Fingerprinting

Also known as DNA Profiling, DNA Restriction analysis

Every individual has a unique genetic make up – DNA fingerprint



STR = VNTR = RFLP

VNTR: Variable number of tandom repeats: Repeats several times

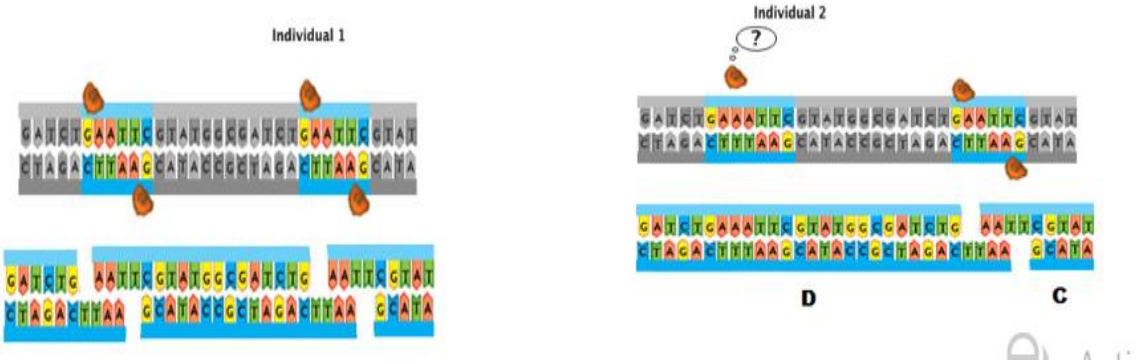
Probability of 2 individuals having same set of VNTR is 1 in 300 million.

More mutation in non coding DNA.

Restriction Fragment Length Polymorphisms, or RFLPs for short, are generated due to mutations in recognition sites. Remember what a recognition site is? It's the specific nucleotide sequence recognized by restriction enzymes. The restriction enzymes bind to the DNA and cut within the recognition site. If the nucleotide sequence has been mutated, then the restriction enzyme will not bind and therefore will not cut at that site.

As this example shows, the change in the recognition site will produce different length fragments in a restriction digest. Individual one has two recognition sites for the EcoRI enzyme. That means EcoRI will cut this DNA fragment twice. What happens when you cut a piece of string twice? You get three pieces. The

same is true when you cut linear DNA with molecular scissors like restriction enzymes. Two cuts generates three fragments labeled here as A, B, and C. In individual 2 the first recognition site is not present. Instead of GAATTC the sequence is now GAAATTC. EcoRI will not bind and cut at this location. That means in the same restriction digest, the restriction enzyme will only cut once and generate two fragments, D and C.



The fragments from the restriction digests can be separated by gel electrophoresis. Because the fragments are different lengths, they will run to different locations on a gel. This creates the unique RFLP pattern, or DNA fingerprint, for each individual.

DNA fingerprinting" is a technique that DNA allows us to see differences in DNA profiling fragments from one person to another in more general form.

Steps of DNA fingerprinting:

- Step 1: DNA (extraction) Isolation: Hair, Blood, Tissue, Semen
- Step 2: DNA Amplification: DNA is amplified by PCR: multiple copies in short times
- Step 3: DNA Fragmentation: DNA sample is subjected to RE (double stranded DNA is broken to many fragments)

- Step 4: Gel electrophoresis: DNA fragments are separated based on their size using agarose gel
- Step 5: Southern blotting: DNA fragments obtained on gel is blotted on Nylon membrane or Nitrocellulose paper. (The gel is fragile and weak so, Southern blotting is formed)
- Step 6: Hybridization: Radioactive DNA probe is prepared
- Step 7: Photography: Nylon membrane is kept on X- ray film

  Documentation is done

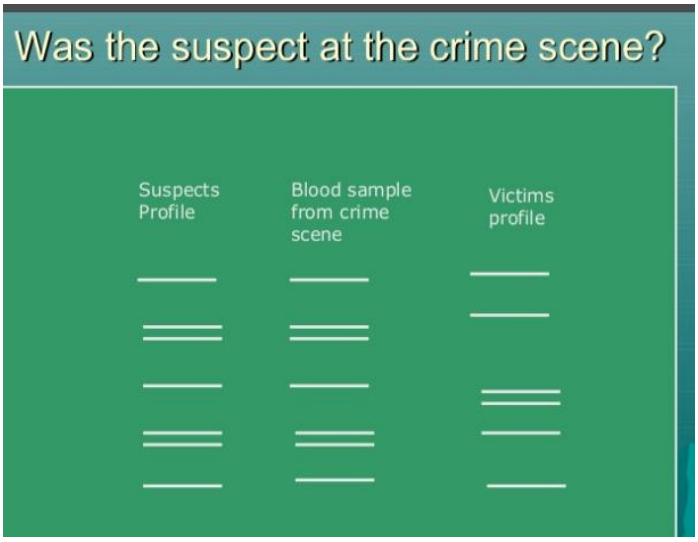
DNA profiling is used in crimes and medical problems:

A crime scene is full of sources of DNA evidence including dirty laundry, a licked envelope, or a cigarette butt. Tiny blood stains, a smear of dried semen, or a trace of saliva is often all it takes to crack a case.

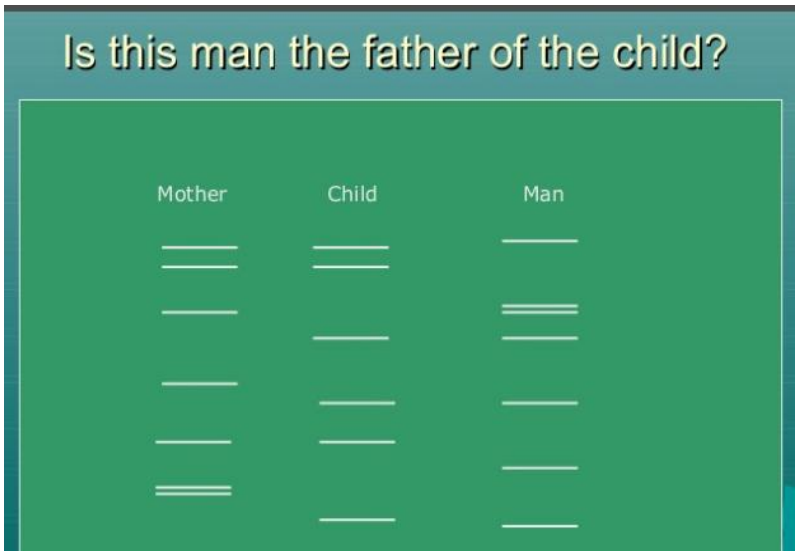
Example 1: A Violent murder occurs.

The forensics team retrieved a blood sample from the crime scene.

They prepared DNA profiles of the blood sample the victim and suspect as follow:



Example2: A Paternity test: By comparing the- DNA profile of mother and her child it is possible to identify DNA fragments in the child which are absent from the mother and must therefore have been inherited from the biological father.



A- Reverse transcription PCR

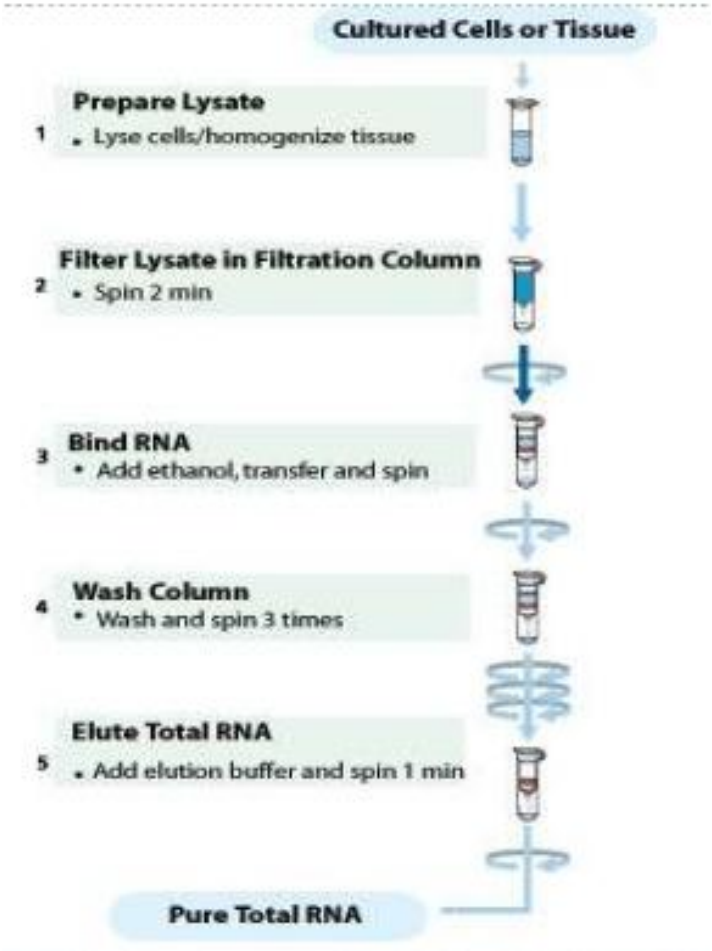
- Used to study mRNA levels when level of detection is below that of Northern.

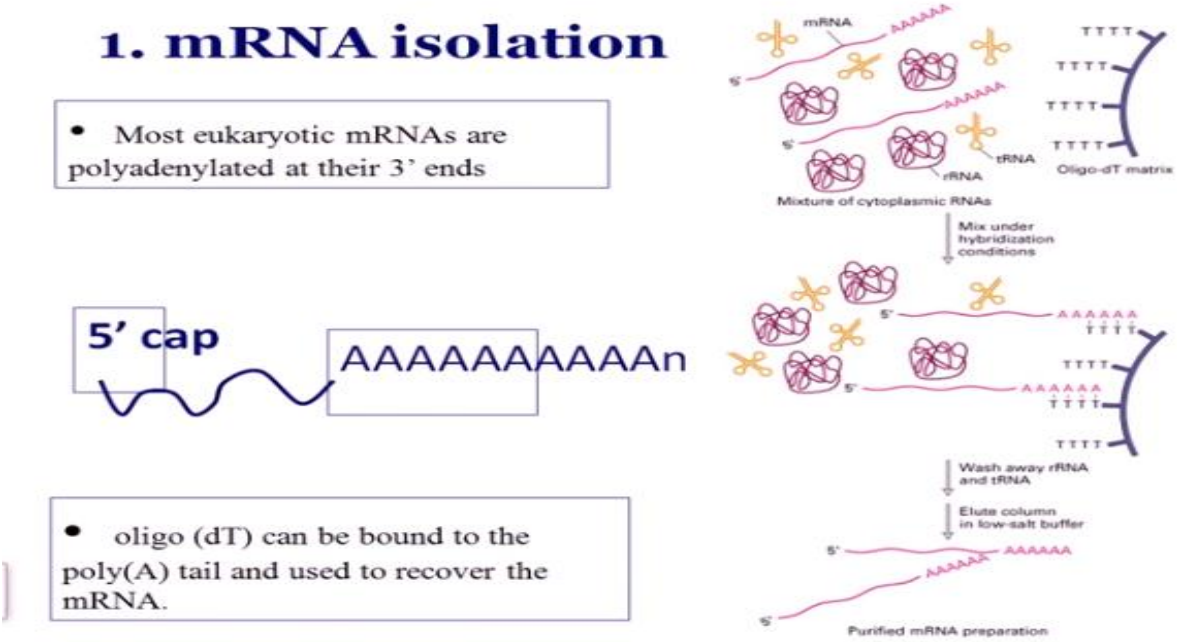
Procedure:

- First Extract RNA then isolate mRNA and use Reverse Transcriptase to make double stranded cDNA.

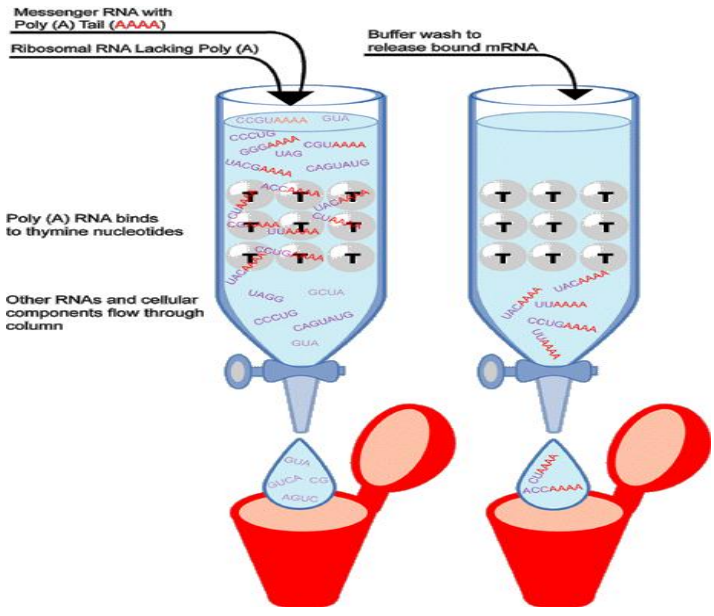
RNA extraction using trizol/tri

- Cell lysis only takes by adding trizol.
- Choloroform is added for phase separation allowing collection of the aqueous phase containing RNA.
- RNA is precipitated with addition of isopropanol.
- RNA precipitate is often invisible before centrifugation.
- Final wash with ethanol.

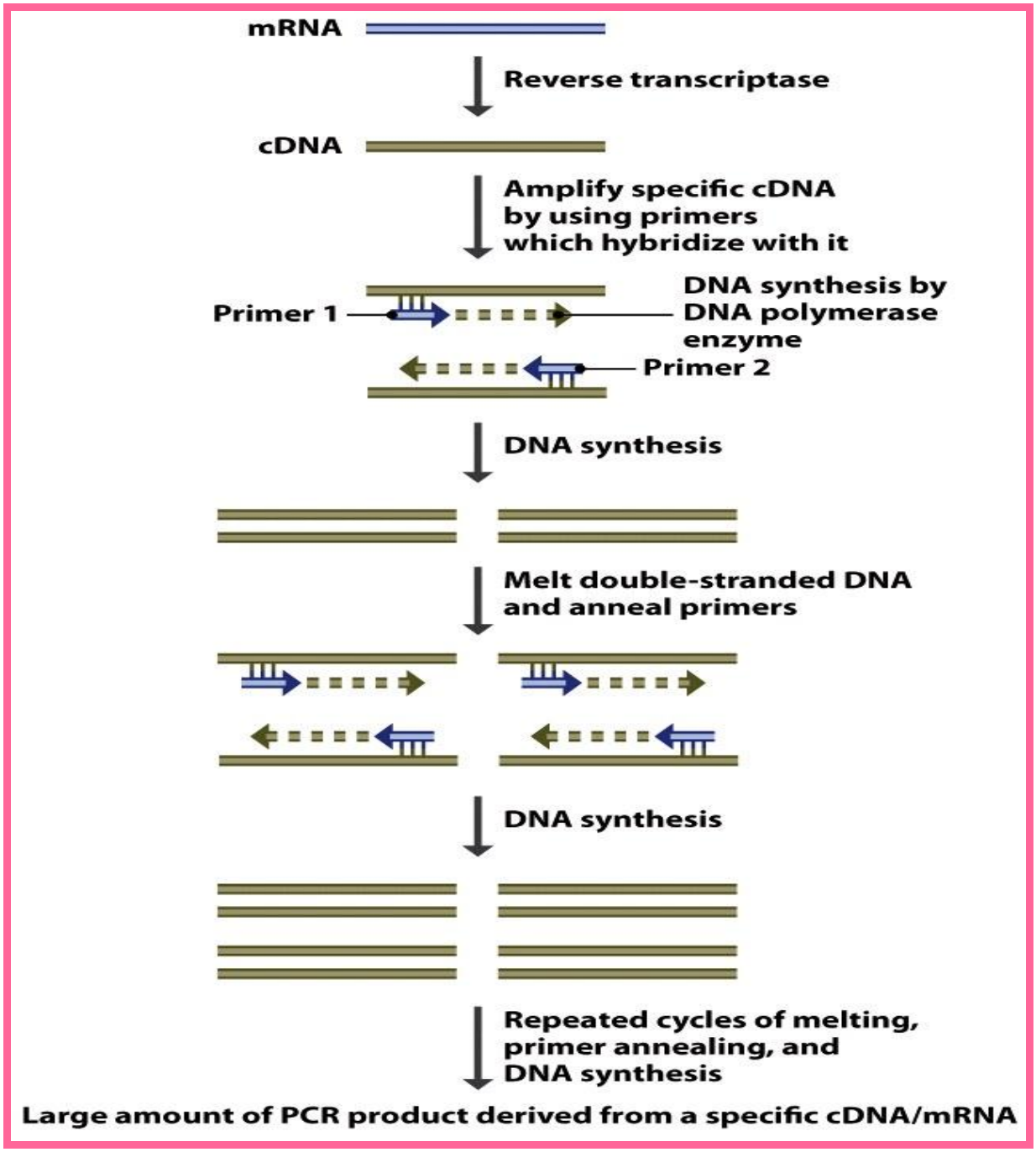




- Mature eukaryote mRNA has a poly-A tail at the 3' end.
- mRNA is isolated by passing cell lysate over a poly-T column composed of oligo dTs (deoxythymidylic acid) .
- Poly-A tails stick to the oligo dTs and mRNAs are retained, all other molecules pass through the column.



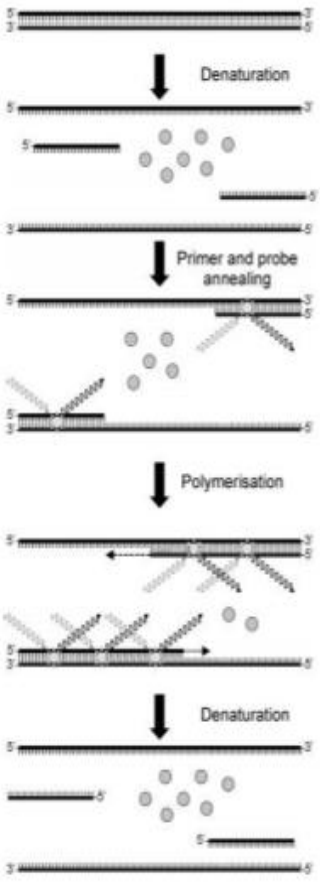
- Use PCR to amplify region of cDNA with set of primers specific for gene of interest.
- Run agarose gel to separate amplified fragments.
- Determine expression patterns in the tissue.
- **Amount of cDNA produced in RT PCR reaction for gene of interest reflects amount of mRNA and level of gene expression.


mRNA
Reverse transcriptase
cDNA
Amplify specific cDNA by using primers which hybridize with it
Primer 1
DNA synthesis by DNA polymerase enzyme
Primer 2
DNA synthesis
Melt double-stranded DNA and anneal primers
DNA synthesis
Repeated cycles of melting, primer annealing, and DNA synthesis
Large amount of PCR product derived from a specific cDNA/mRNA

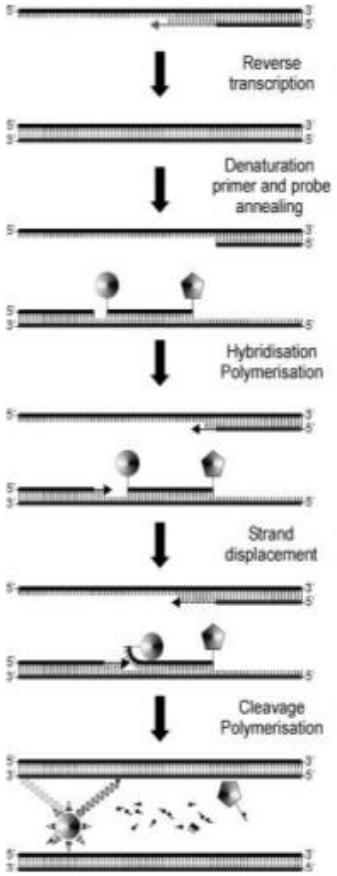
B- Real time or quantitative (qPCR)

- Can quantify amplification reactions as they occur in real time.
- Each reaction tube contains either a dye containing probe or DNA binding dye that emits fluorescent light.
- Light emitted by the dyes correlates with amount of PCR product amplified.
- Light is captured by the detector which relays information to the computer to provide readout on amount of fluorescence.
- Readout is plotted and analyzed to quantify the number of PCR products produced after each cycle.

SybrGreen intercalating dye:



TaqMan™ probe uses FRET (Flueorescent resonance energy transfer):



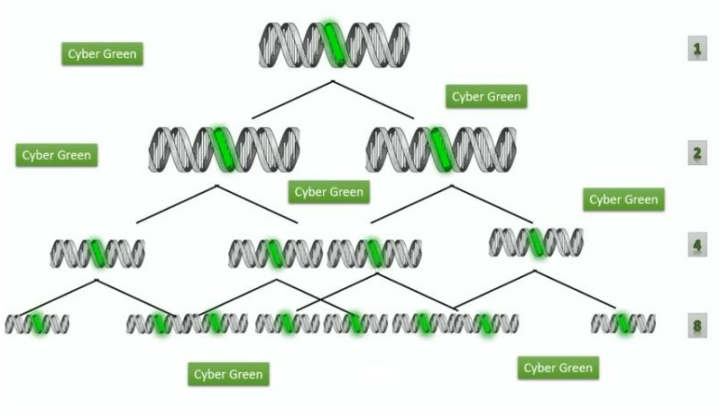
- **Two approaches**

A) SYBR Green I (SG) is an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology.

- SYBR Green I binds double stranded DNA.

- The resulting DNA-dye-complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm).

- Since double stranded DNA can interact quantitatively with SYBR Green 1 preferentially and thus can be used to monitor the amount of DNA synthesized after each cycle of PCR reaction.

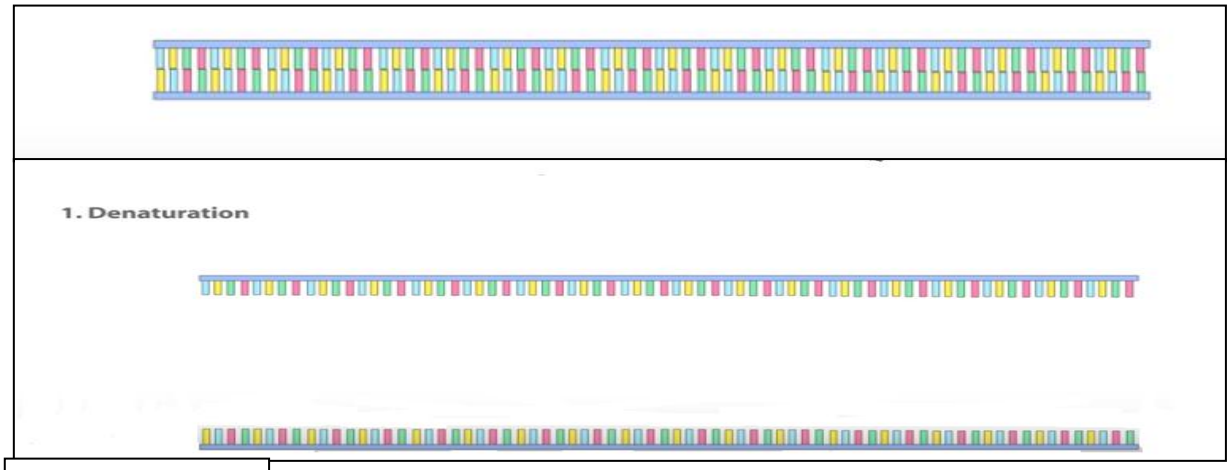


B) Taqman probes are complimentary to specific regions of target DNA between forward and reverse primers for PCR

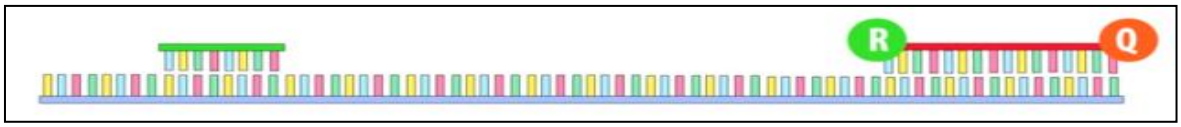
- Taqman probes contain two dyes: reporter located at 5' end of probe and can release fluorescent light when excited by the laser and other dye is quencher which is attached to 3' end of probe.

- Taqman probes is a oligonucleotide that is labeled with a fluorescent reporter and a Quencher

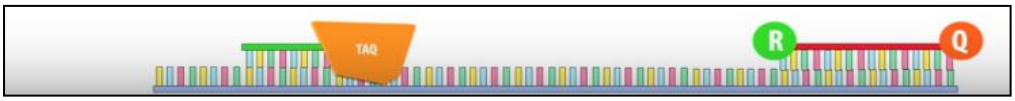
- The Quencher decreases the fluorescence intensity.

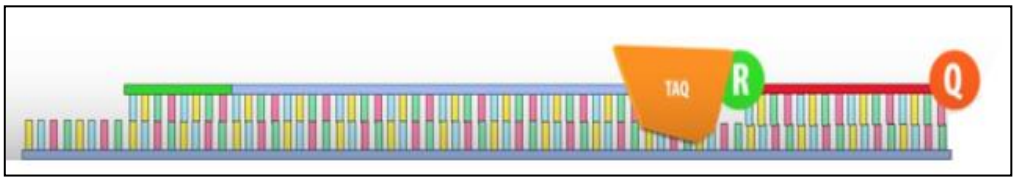


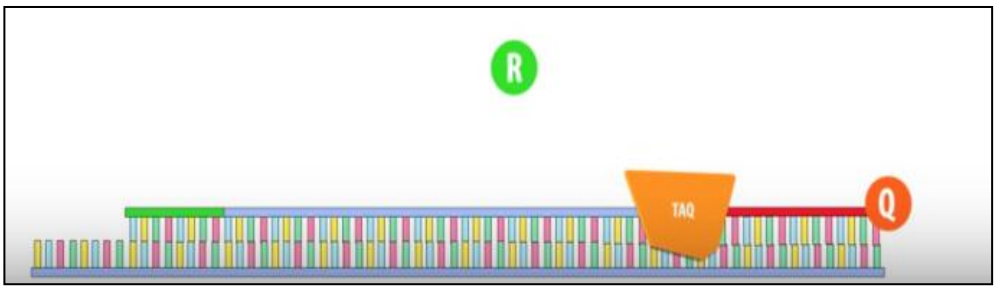
2. Annealing

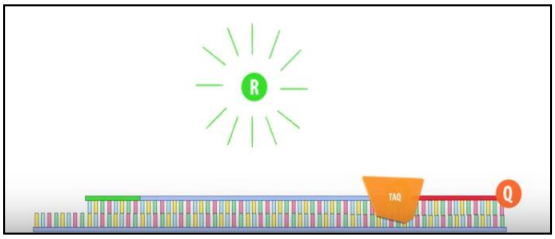


3. Elongation









The reporter molecule is only released when a DNA strand is completely polymerized by TAQ

The Fluorescence of the 30 cycles are measured and converted into an amplification plot/curve.

Pamphlet of RNA extraction

RNA extraction with TRIzol (Invitrogen product name) or the equivalent TRI (Sigma-Aldrich product name) is a common method of total RNA extraction. Iit is generally considered the method that gives the best quality RNA

Principle

- **guanidinium isothiocyanate** (powerful protein denaturant) -> *inactivation of RNases*
- **acidic phenol/chloroform** -> *partitioning of RNA into aqueous supernatant for separation*

Reagents

- TRIzol or TRI reagent
- 0.8 M sodium citrate / 1.2 M NaCl
- isopropanol (2-propanol)
- chloroform
- 75% EtOH in DEPC H2O
- RNase free water (filtered or DEPC)

Steps

1-cell lysis

Cell lysis only takes a few minutes per well, but tissue homogenisation can take 10-20 minutes per sample depending on how tough the tissue is.

add trizol (cell lysis)

1ml / 3.5 cm diameter well (6-well)

5ml / 75 ml bottle

homogenise by pipetting several times (mechanic lysis)

alternative for tubes: vortex 1 min

alternative for tissue: grind 1 g tissue in liquid nitrogen in a motar and pestle, put tissue into plastic screw-cap centrifuge tube + 15 ml TRIzol reagent, incubate samples for 5 min at room temp or 60° C (scaled up as needed)

(5min at RT for complete dissociation of nucleoprotein complexes)

RNA is stable in trizol which deactivates RNases. You can take a break at this point keeping the sample in trizol for a short time or freezing it for a longer one.

2-phase separation

- 15-45 min depending on number of samples and whether an additional chloroform wash is necessary
- add chloroform (1/5 volume of trizol; e.g. 0.2ml to 1ml)
- shake for 15 sec (Eccles protocol: do not vortex)
- incubate 2-5 min at RT
- spin max. 12000g, 5-15 min, 2-8°C
- if centrifugation hasn't been sufficient the DNA-containing interphase will be cloud-like and poorly compacted

( ) OPTIONAL STEP If supernatant appears turbid an additional chloroform cleaning step can be inserted here.

- transfer aqueous upper phase into new tube

Take care not to aspirate the DNA-containing white interface. This quickly happens and will lead to DNA contamination in your RNA prep.

3-RNA precipitation and wash

TIME REQUIRED 20-40 min depending on number of samples

- add isopropanol (70% of aqueous phase or 1/2 trizol volume)
- 0.8 M sodium citrate or 1.2 M NaCl can be added
- (incubate 10min at RT)
- spin max g, 10-15 min, 4ºC
- remove supernatant

(alternative RNA precipitation - RNeasy from Qiagen) better than alcohol precipitation for smaller amounts of RNA (less risk of losing a miniscule nucleic acid pellet); also reduces risk of organic solvent contamination

similar kits to RNeasy: MinElute kit, or Affymetrix sample clean-up

4-RNA wash

TIME REQUIRED 15-30 min depending on number of samples

wash pellet 70% EtOH (add & vortex briefly)

70% ethanol prepared with RNase-free water

( ) OPTIONAL STEP some prefer to wash the pellot more than once with 70% ethanol

spin max g, 2-10 min, 4ºC

air-dry pellet for 5-10 min ! CRITICAL STEP Do not over dry the pellet or you won't be able to redissolve it.

( ) OPTIONAL STEP optional add RNase inhibitor

( ) OPTIONAL STEP incubate at 55-60 C° for 10 min if hard to redissolve

transfer to eppendorf tube

spin 4° C, 5 min (to pellet undissolved material)

5-redissolving of RNA

dissolve pellet in 50-100 µl filtered or DEPC H2O (note: DEPC inhibits RT reaction)

alternatively, 0.5% SDS

pipetting up and down, heat to 55-60°C for 10 min

Common mistakes

use too little trizol; very small volumes are hard to separate and will most likely lead to contamination

aspirate some white interphase (DNA) when removing aqueous supernatant (RNA)

use phenol/chloroform of the wrong pH (has to be acidic)

not working under the hood (phenol is toxic , chloroform is a narcotic)

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