Basic method in molecular biology

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Aims

DNA and RNA

- Structure and synthesis
- Restriction enzyme
- PCR
- Gene expression
- Sequencing

Measurement

- Southern blot analysis: DNA
- Northern blot analysis: RNA

Aims

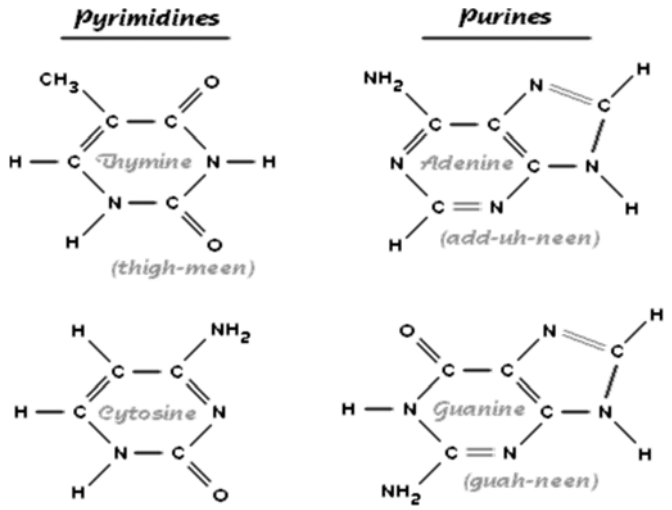
RNA Technology

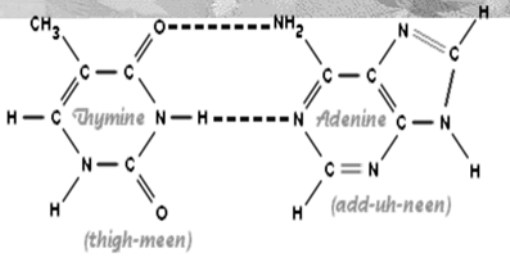
- RT-PCR
- realtime RT-PCR

DNA Technology

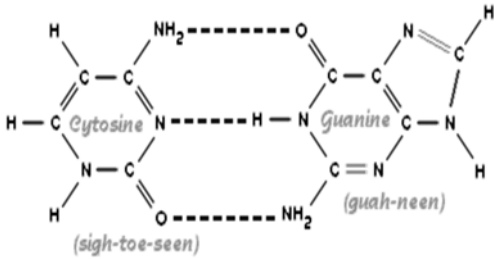
- Cloning

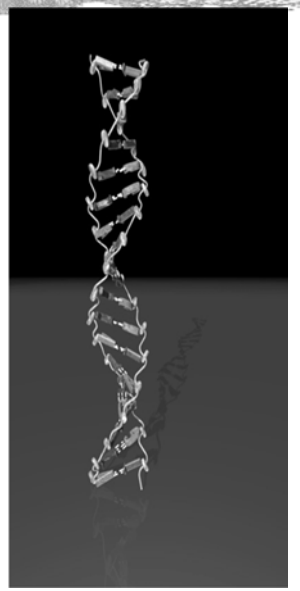
DNA structure





Hydrogen Bonds Between Base Pairs



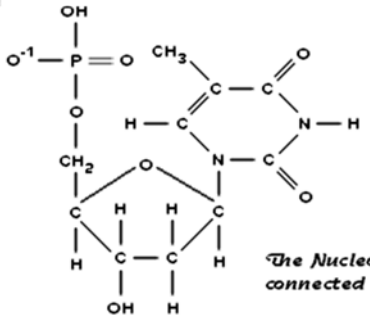


DNA replication

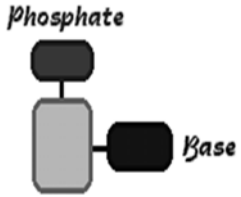
Bases G - C A - T

DNA synthesis from 5'-3'

Reqires 3'-OH for formation phosphate bond



The Nucleotide, connected to thymine



Deoxyribose sugar

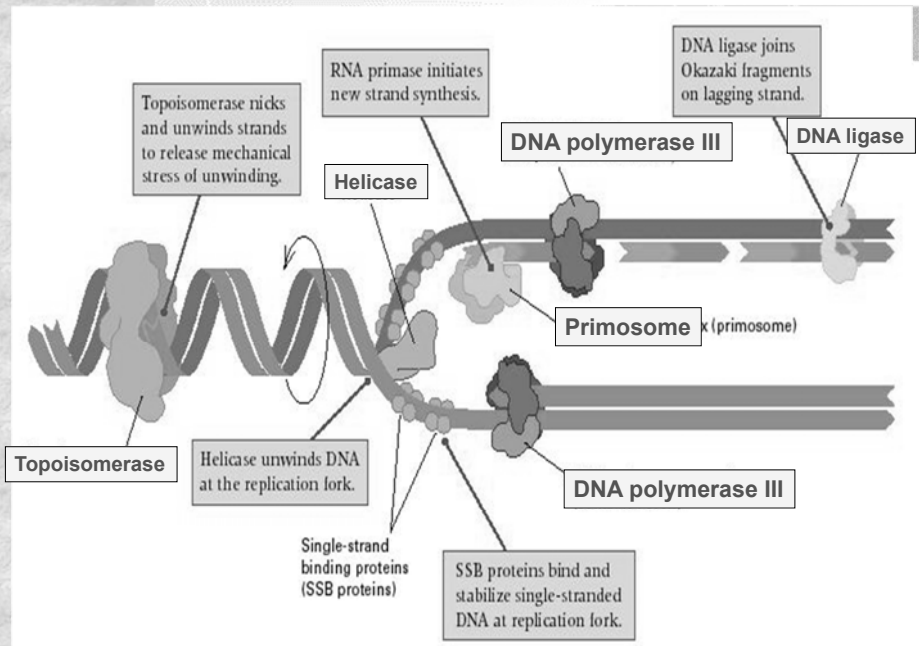
DNA replication

Helicase unwind DNA at replication fork

Newly synthesis DNA

5'-3' : leading strand : DNA polymerase III

3'- 5' : lagging strand : RNA primase
RNA primase complex (primosome)
DNA polymerase III
DNA polymerase I
DNA ligase



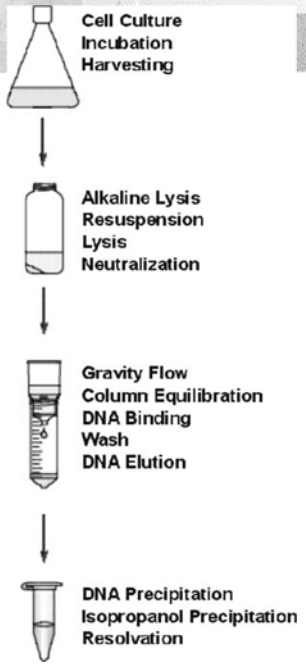
• DNA purification: lysis of cell

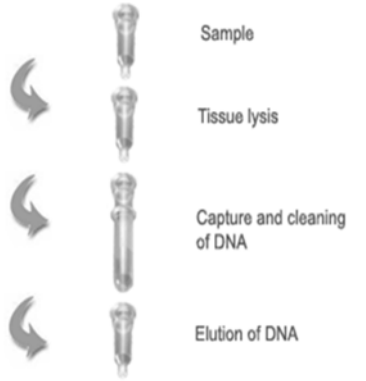
❖ Mini scale

- Alkaline lysis method
- Boiling method
- Sodium dodecyl sulfate (SDS)
- DNA isolation kits:

❖ Large scale

- Polyethylene glycol
- CsCl-ethidium bromide gradient
- Large scale DNA isolation kits:





http://www.epigentek.com/catalog/images/products/p1000/p1009schematic.gif

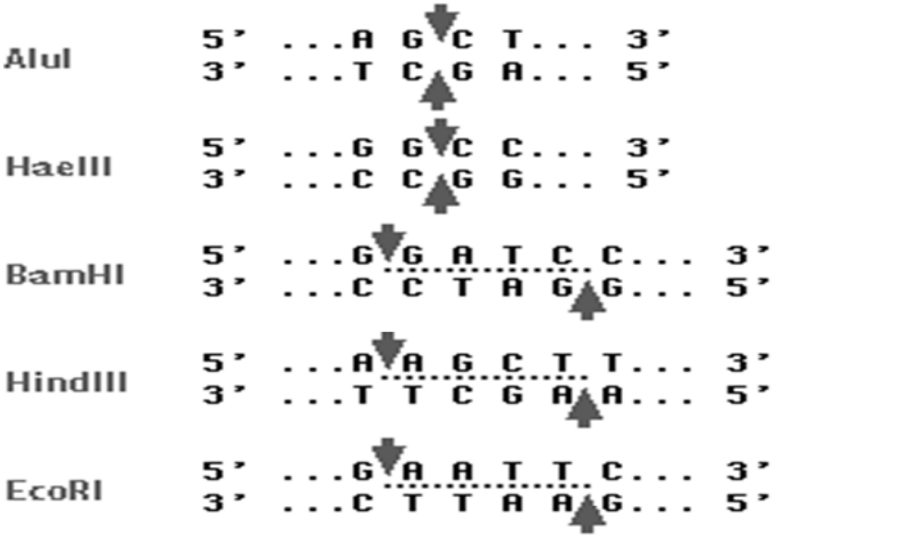
Copy from : http://www.favorgen.com/products_for_res_nae_faPDE_Midi.htm

Restriction enzyme

Restriction enzymes are **DNA-cutting enzymes** found in bacteria. Because they cut within the molecule, they are often called **restriction endonucleases.**

A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides. For example

Animation



AluI and HaeIII produce blunt ends

BamHI HindIII and EcoRI produce "sticky" ends

Polymerase Chain Reaction (PCR)

PCR is the technique for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complimentary strands of DNA.

PCR

Substrates

DNA template

dNTPs: dATP, dCTP, dTTP, dGTP

Primer

Enzyme

Taq DNA polymerase

Primer design

- Specificity
- Stability
- No self dimer

→ length

→ Melting temperature

→ Annealing temperature

Primer design

Manual design

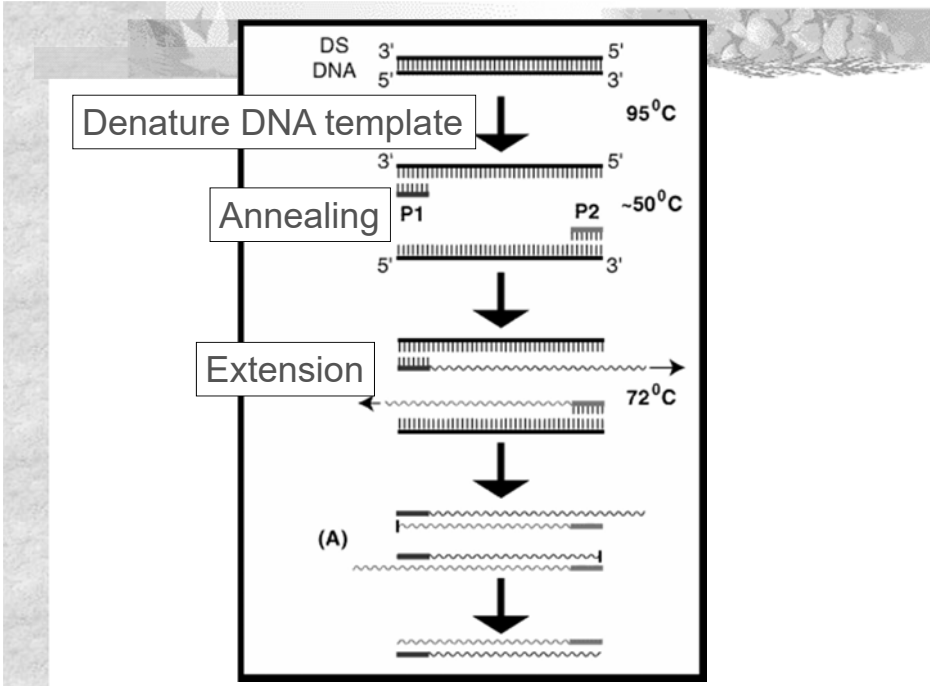
Automatic design

PCR reaction: 3 step

Denature DNA template: 90-95 ºC

Annealing: depend on melting temperature of primers

Extension: Taq DNA polymerase: 72 ºC



PCR application

The detection of hereditary disease

The identification of genetic fingerprint

The cloning of genes

Paternity testing

DNA sequencing

Animation

Restriction fragment length polymorphism (RFLP)
or
DNA fingerprint

• Determine in forensic science

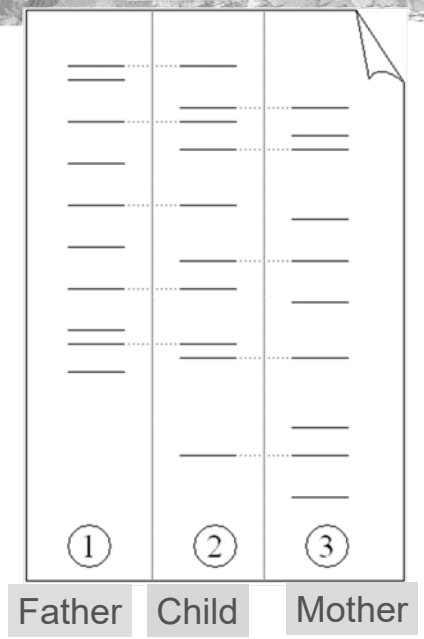
- DNA typing: forensics and paternity disputes

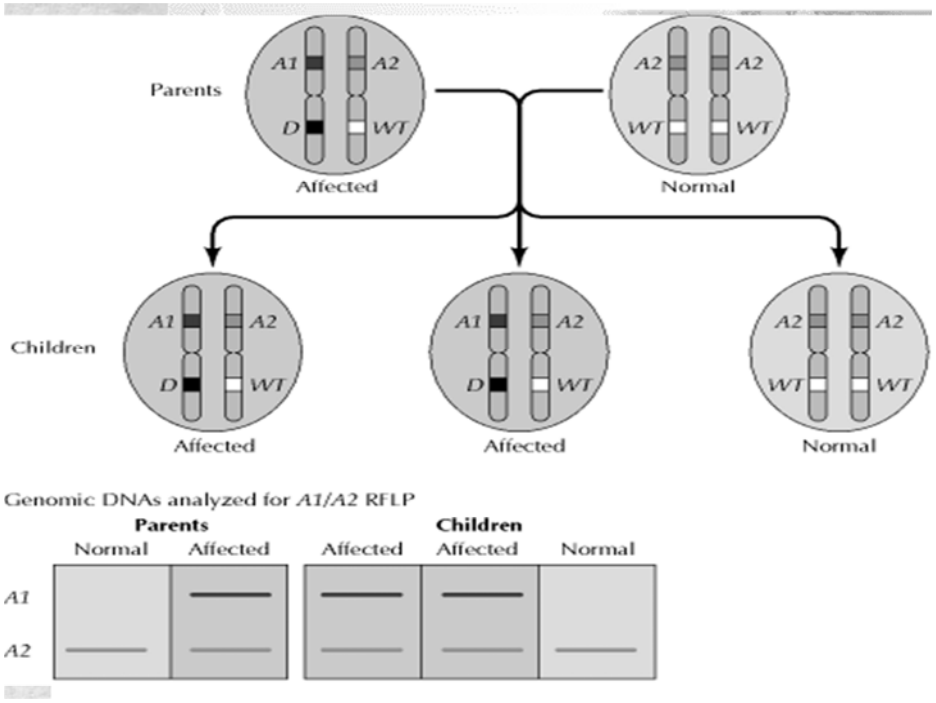
• Isolation of human disease genes

PCR application

Paternity testing

RFLP





DNA sequencing

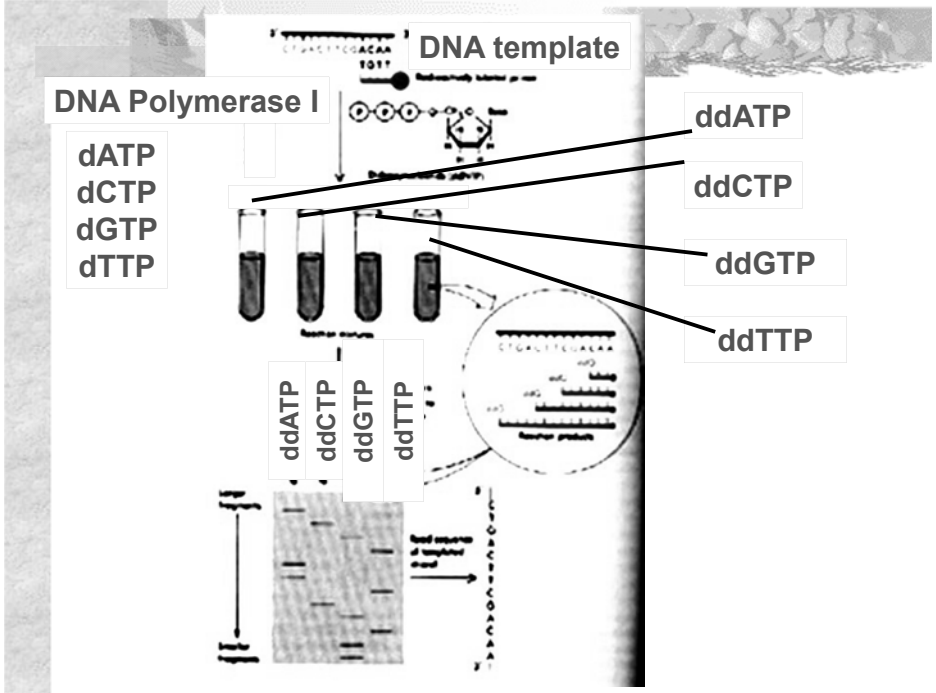
• Chain-terminating dideoxynucleotides

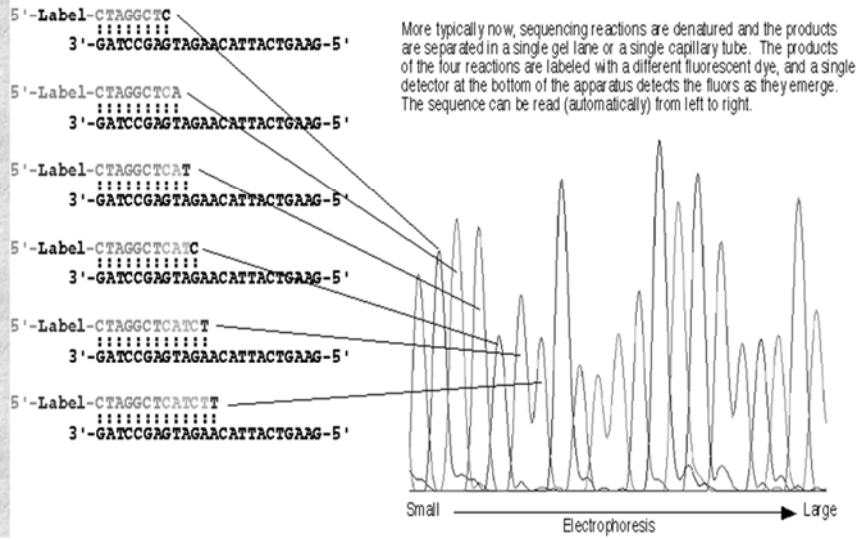
Principle

ddNTP: lack the 3'-OH required for formation of a phosphodiester bond.

ddNTP is labeled

• Automated





Animation

How to detect DNA?

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Southern blot analysis: DNA

General principles

DNA: negative charge at neutral pH

If electrophoresis is performed in a semi-solid matrix, the DNA molecules will be separated by size and/or shape

For double-stranded linear DNA the separation is based mostly on size

Type of gels

Agarose gel

Agarose is a modified polysaccharide extracted from seaweed.

The percentage of agarose in the gel can be varied to optimize separation of particular size ranges.

Polyacrylamide gel

Acrylamide polymerizes to form a highly interlocked meshwork.

Very small DNA molecules can be separated due to the small pore sizes that result.

Detection system

• After fractionation by electrophoresis, nucleic acids in the gel are visualized by staining with a dye whose fluorescence properties change upon binding the nucleic acid.

Ethidium bromide is a common choice. It binds to DNA by intercalating between base pairs

When bound, its fluorescence is greatly enhanced compared to free ethidium. The gel is illuminated with ultraviolet light, and places where DNA is located emit an orange fluorescence with an Intensity that is a reflection of the amount of DNA present. Quantities as small as 1 ng can be visualized this way.

Other dyes

SYBR Green

- potential mutagens

-Like ethidium bromide, these highly sensitive stains fluoresce under UV light.

SYBR Safe

-It is not considered a hazardous waste and can generally be disposed through the regular sewer systems

Other dyes

Eva Green

- inhibit PCR to a lessor extent than other dyes, making it very useful for applications like quantitative real-time PCR

- Eva Green has also been demonstrated to have very low or no cytotoxicity or mutagenicity

Fast Blast

- Non toxic, another alternative dye

• For more sensitive analysis, DNA molecules can be labeled with radioactive isotopes or fluorescent tags before separation, then detected by autoradiography or fluorography.

Southern blot analysis

- DNA purification: lysis of cell
- Quantitation of isolated DNA
- Separation of isolated DNA
- Transfer of DNA from agarose gel to solid support
- Hybridized with DNA probe
- Exposed to x-ray film

- Quantitation of isolated DNA

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Spectophotometers

Basic principle

A solution containing a colored compounded that certain wavelengths of light are selectively absorbed

Quantitative obtained from the absorb light in certain wavelength

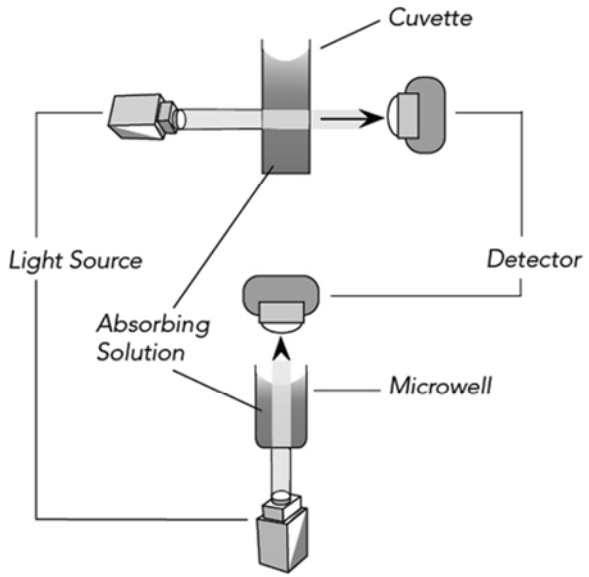
UV light: Wavelength 260

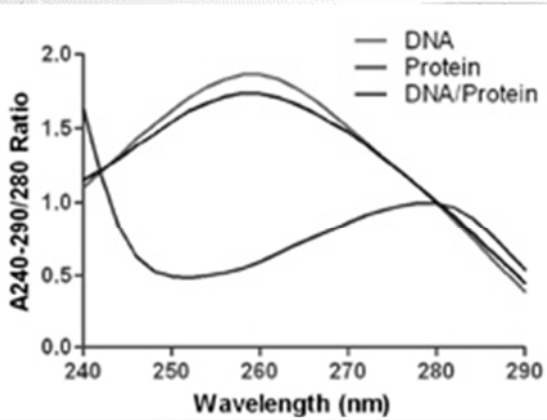
Pure DNA sample give a 260/280 ratio ~ 1.8

Cuvette

Microwell

Principle of light pathway





Nucleic Acid Type	Average Extinction Coefficient (µg/mL)⁻¹ cm⁻¹	Concentration (µg/mL) if OD=1*
Double-stranded DNA	0.020	50
Single-stranded DNA	0.027	37
Single-stranded RNA	0.025	40

Table 1. Commonly accepted extinction coefficients at known concentration.

** Based on a 1 cm path length.*

Electrophoresis

Agarose gel

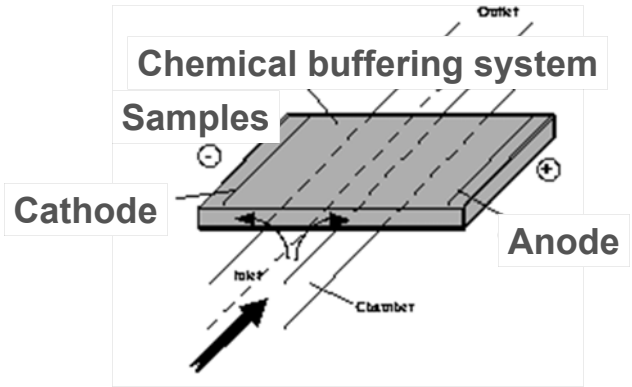
Polyacrylamide gel

Horizontal gel

Resolution: 200bp - 50kb

Vertical gel

Resolution: 5 - 500 bp

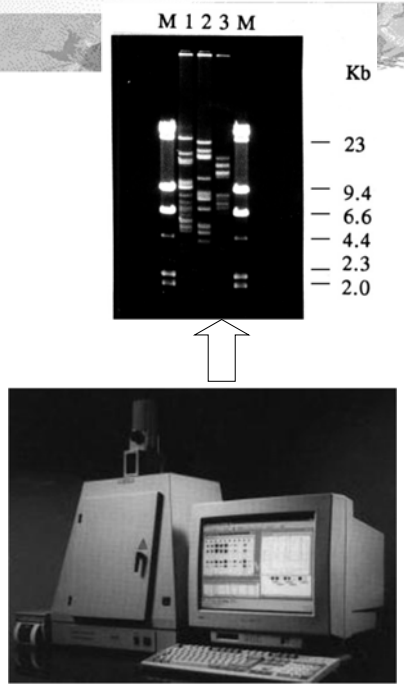


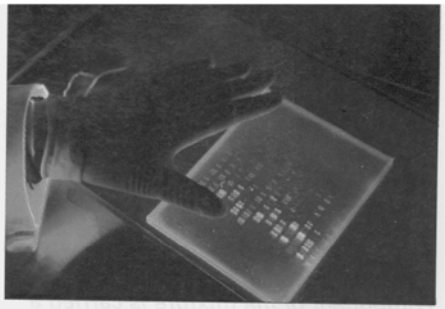
size

DNA: negative charge

Agarose gel

Staining with Ethidium bromide

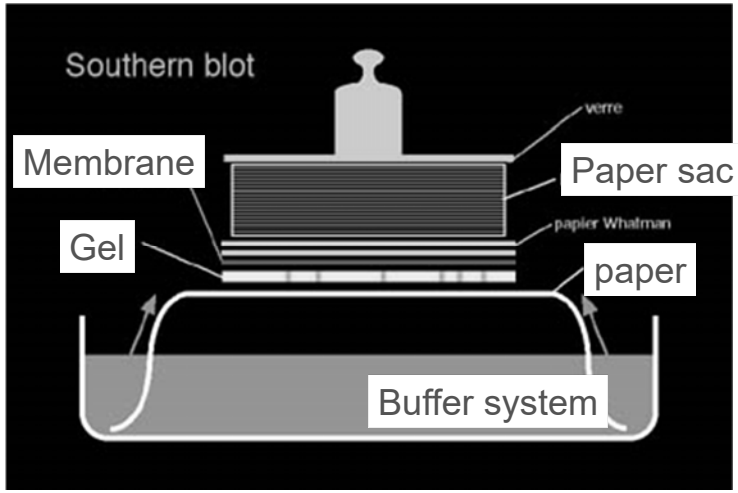


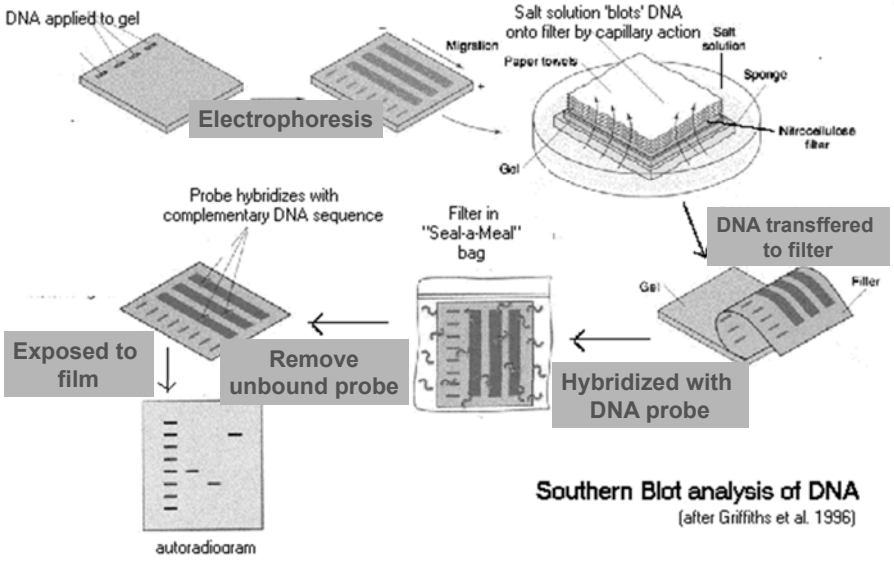


An electrophoresis gel fluorescing to reveal the components of the original mixture. The positions of the constituents are noted. The constituents may be isolated by cutting up the gel and washing each section.

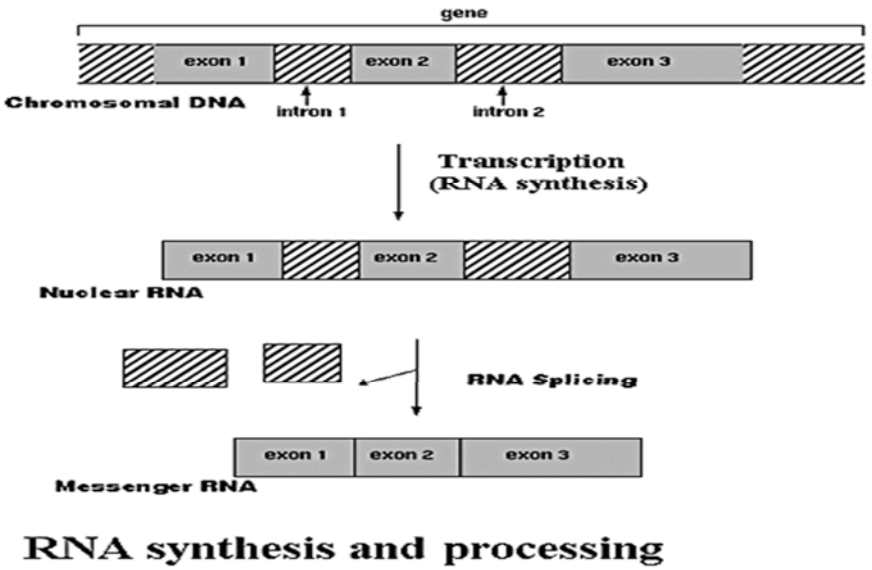
http://ibhumanbiochemistry.wikispaces.com/file/view/electrophoresis+DNA.bmp

• Transfer of DNA from agarose gel to solid support





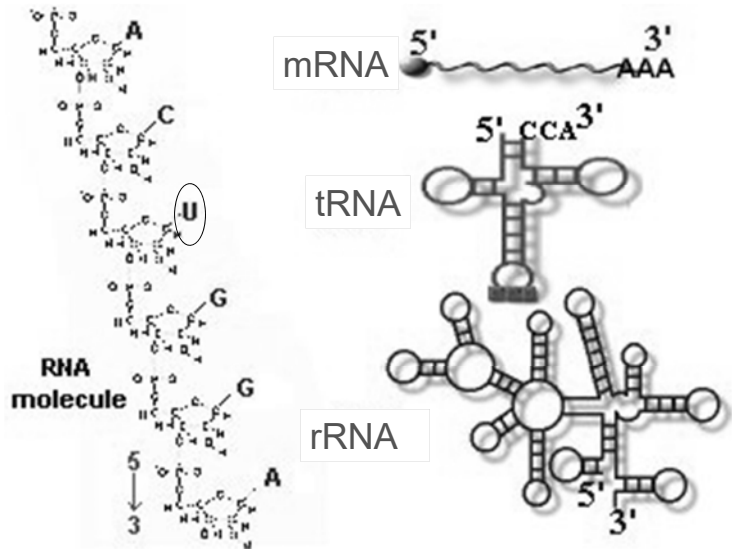
RNA



RNA synthesis and processing

RNA

There are 3 type of RNA



- RNA purification: lysis of cell
  - ❖ Guanidium isothiocyanate (GTC)
  - Diehtyl pyrocarbonate (DEPC)
  - Ribonuclease (RNAases)
- Quantitation of isolated RNA

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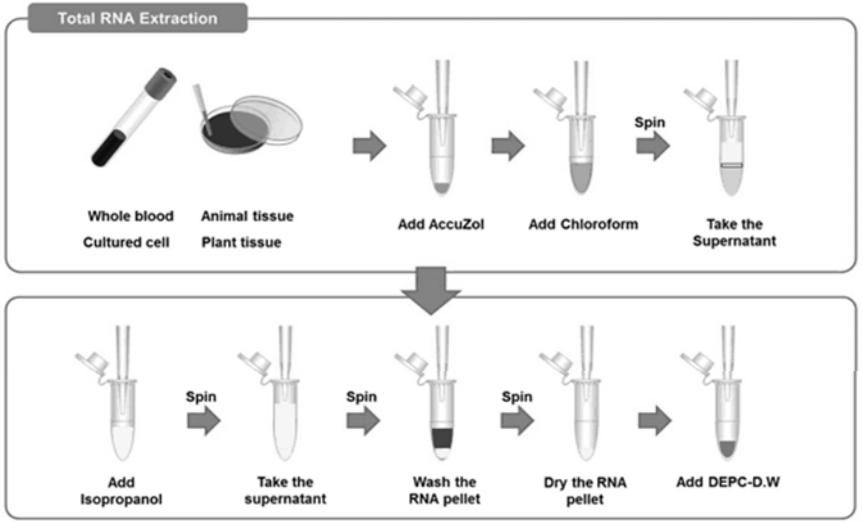
Spectophotometers

UV light: Wavelength 280

Pure RNA sample give a 260/280 ratio ~ 2

RNA extraction kit



• Separation of isolated RNA

Gel containing formaldehyde

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Electrophoresis

• Transfer of RNA from agarose gel to solid support

Nitrocellulose membrane

Nylon membrane: more durable

• Hybridized with RNA probe

Double-stranded DNA labeled by nick translation

Random oligonucleotide primers

• Exposed to x-ray film

Preparation of a radioactive probe

Purified mRNA + DNA primer

^{32}PdNTP

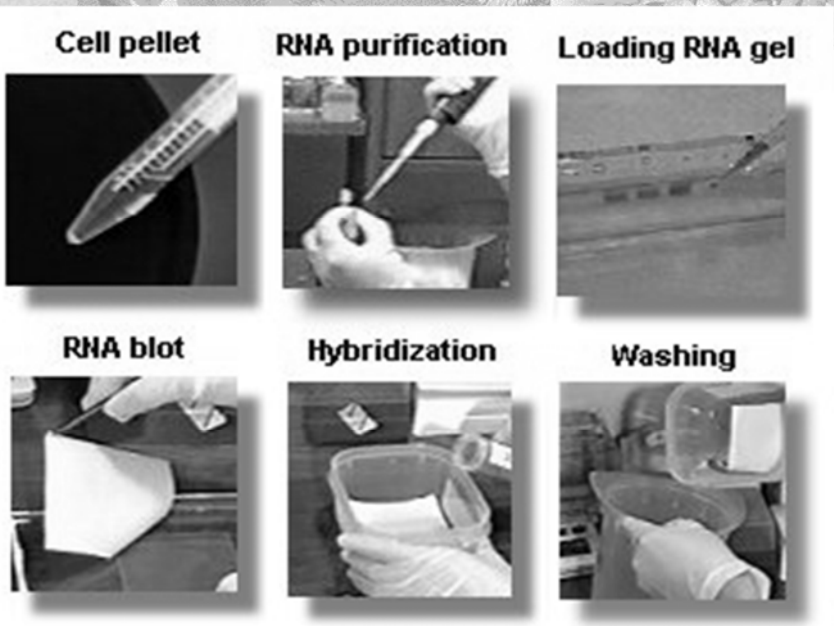
Reverse transcriptase (RT)

DNA polymerase

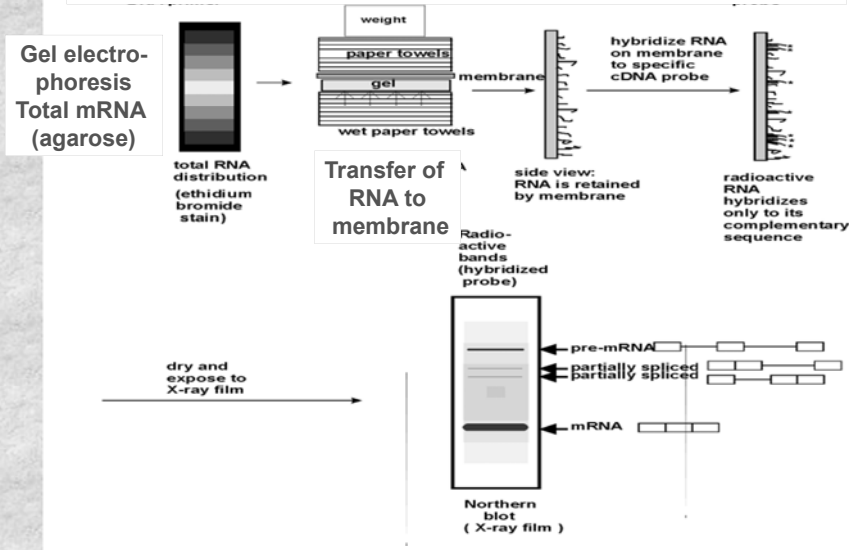
RNA-DNA hybrid (heteroduplex)

RNase

radioactive cDNA probe



Northern blot analysis



Northern blot application

- To examine the developmental or tissue specific expression of particular genes.

Obsolete

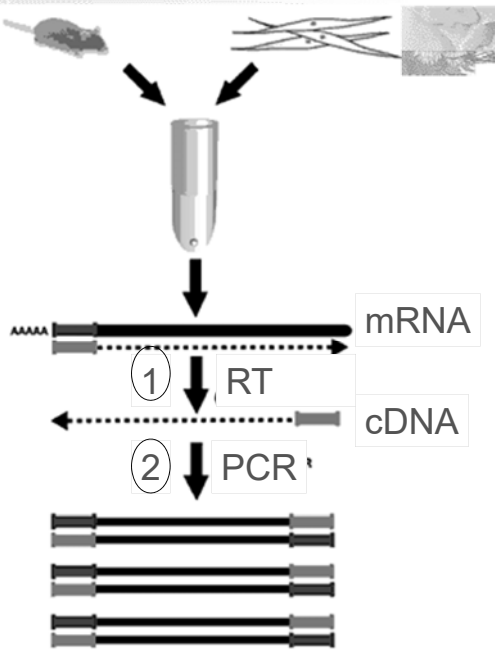
Reverse Transcription Polymerase Chain Reaction (RT-PCR)

- sensitive detection and quatitation of mRNA

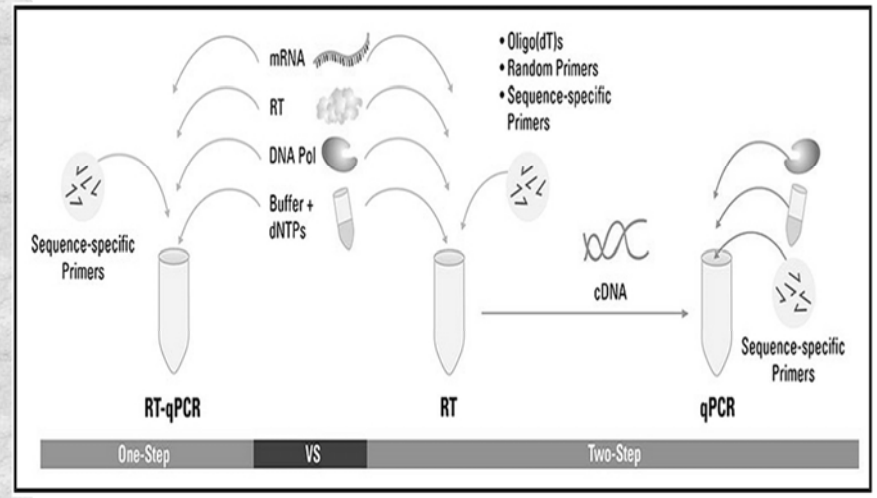
Two parts

- synthesis of cDNA from RNA by reverse transcriptase (RT)

- amplification of a specific cDNA by PCR



(RT-PCR)



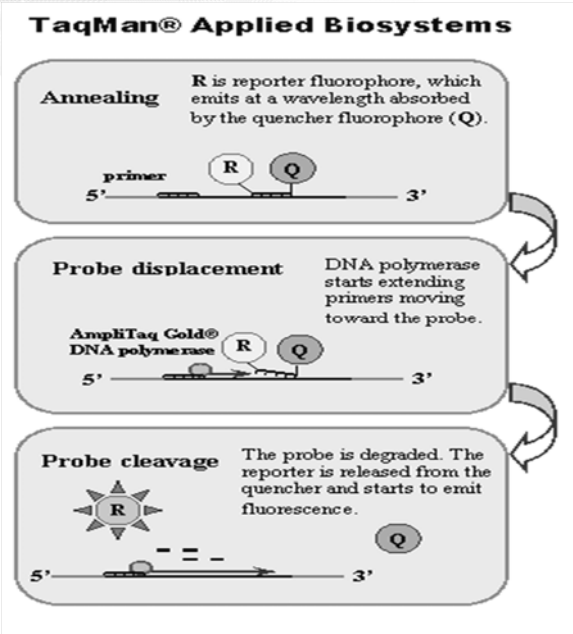
tps://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

	Advantages	Disadvantages
One-step	•Less experimental variation since both reactions take place in the same tube •Fewer pipetting steps reduces risk of contamination •Suitable for high throughput amplification/screening •Fast and highly reproducible	•Impossible to optimize the two reactions separately •Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions •Detection of fewer targets per sample
Two-step	•A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions •The target and reference genes can be amplified from the same cDNA pool without multiplexing •Optimized reaction buffers and reaction conditions can be used for each individual reaction •Flexible priming options	•The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination Time consuming •Requires more opt

tps://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

Real-Time qRT-PCR

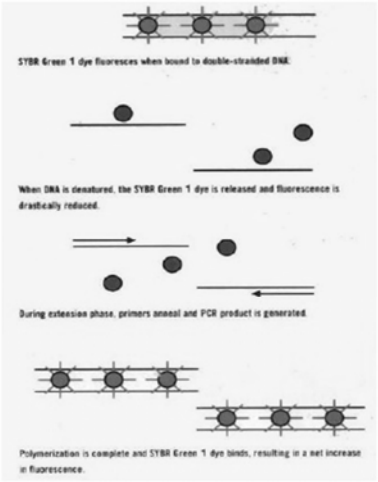
Real-Time Quantitative Reverse Transcription PCR enables reliable detection and measurement of products generated during each cycle of PCR process. This technique became possible after introduction of an oligonucleotide probe which was designed to hybridize within the target sequence. Cleavage of the probe during PCR because of the 5' nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product.



https://www.ncbi.nlm.nih.gov/probe/docs/techqpcr/

Fluorescent dye

Sybr Green PCR Assay

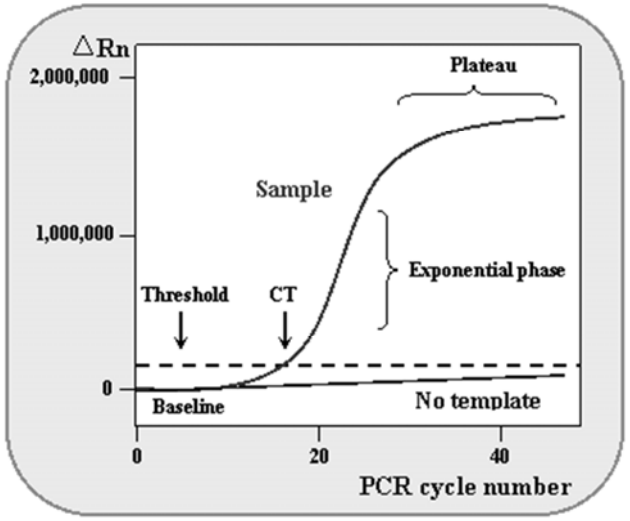


- Stronger signal
- Higher selectivity for dsDNA
- Lesser sequence dependent
- Higher stability
- Lesser inhibitory for *Taq*
- Higher resolution in melting curves
- Less hazardous and mutagenicity)

Binds to

- Non specific PCR product
- Primer dimer

Model of real time quantitative PCR plot



http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml

Calculation

Arithmetic Formula: The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by

$$2^{-\Delta\Delta CT}$$

$$\Delta\Delta CT = CT\,(TAR) - CT\,(REF)_{time\ x} - CT\,(TAR) - CT\,(REF)_{time\ 0}$$

Application

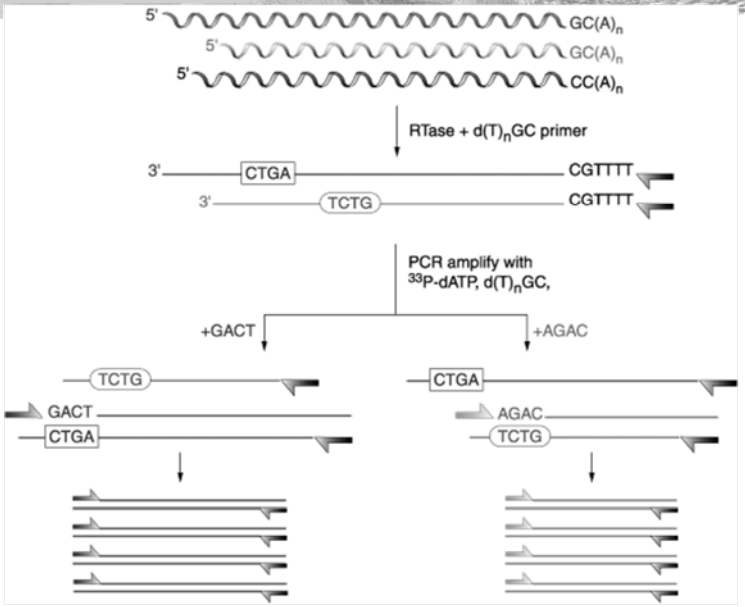
Differential display

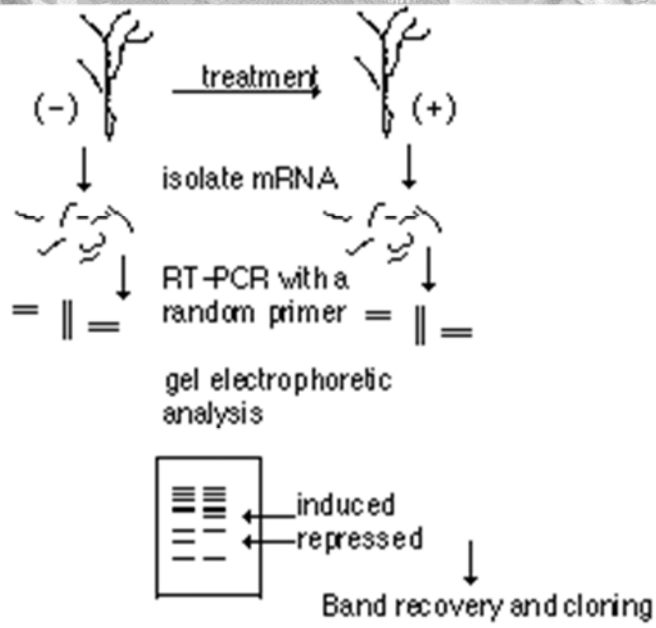
Probe synthesis

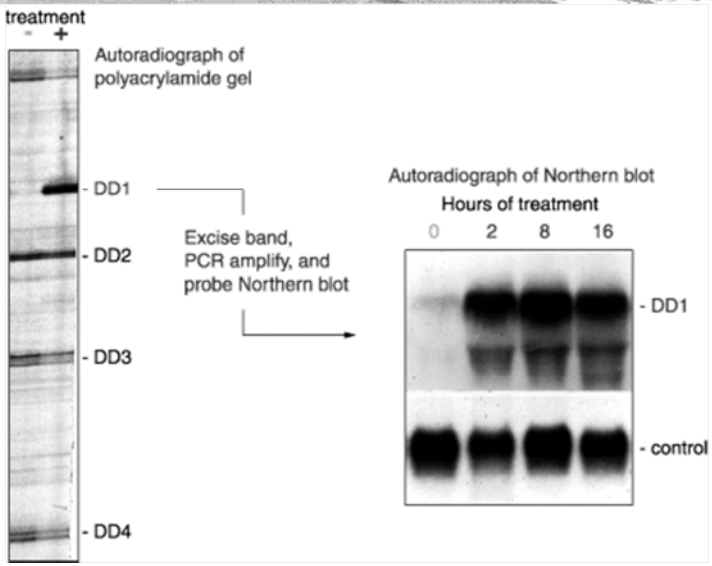
Cloning

cDNA library construction

Differential display

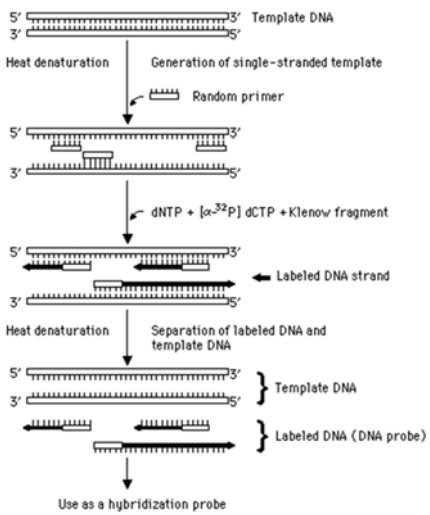




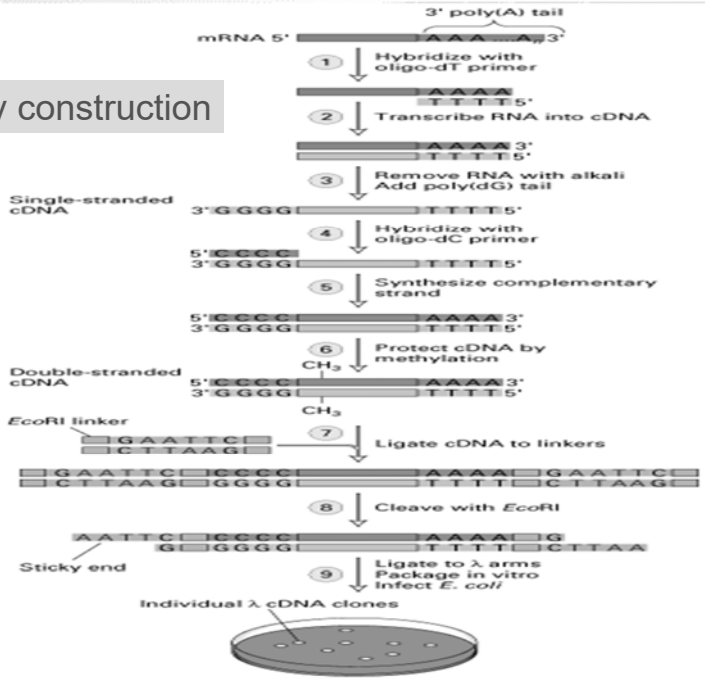


Probe Labeling

- Random prime Labeling



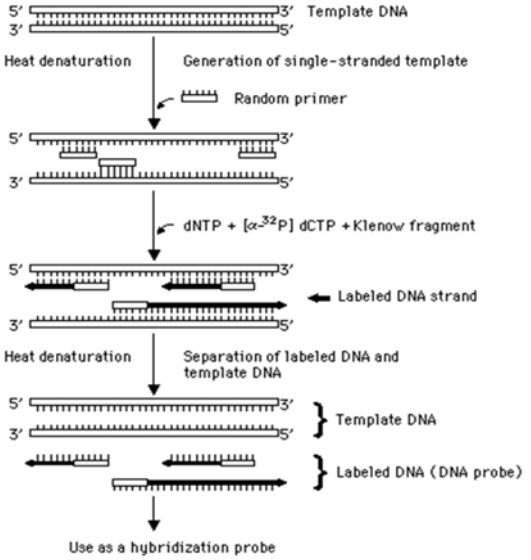
cDNA library construction

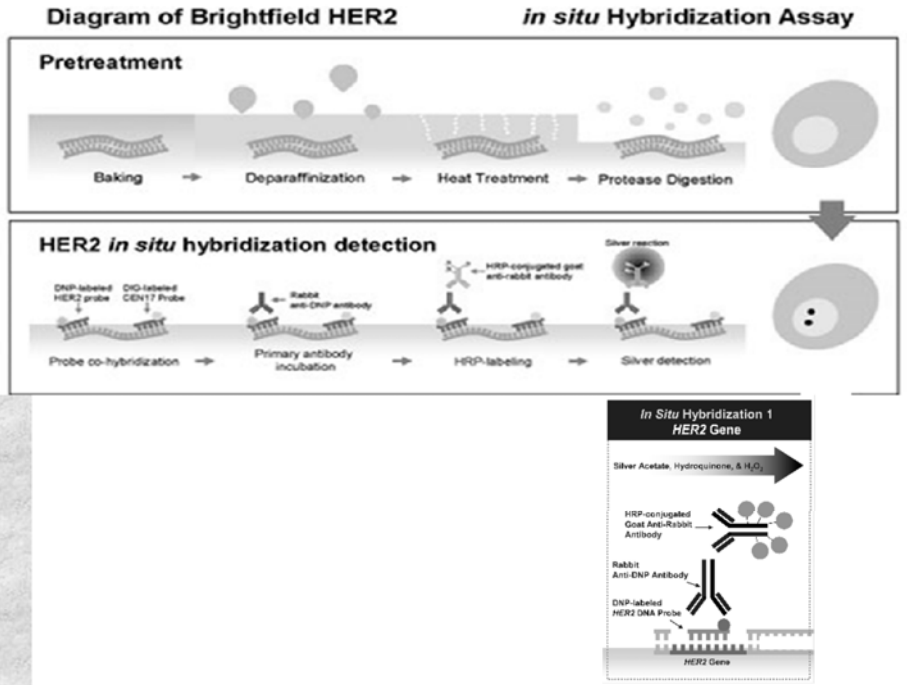


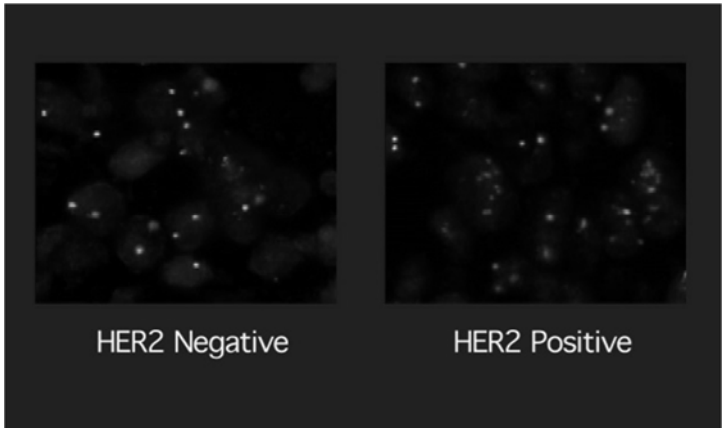
In situ hybridization

Use of a DNA or RNA probe to detect the presence of the complementary DNA sequence in cloned bacterial or cultured eukaryotic cells.

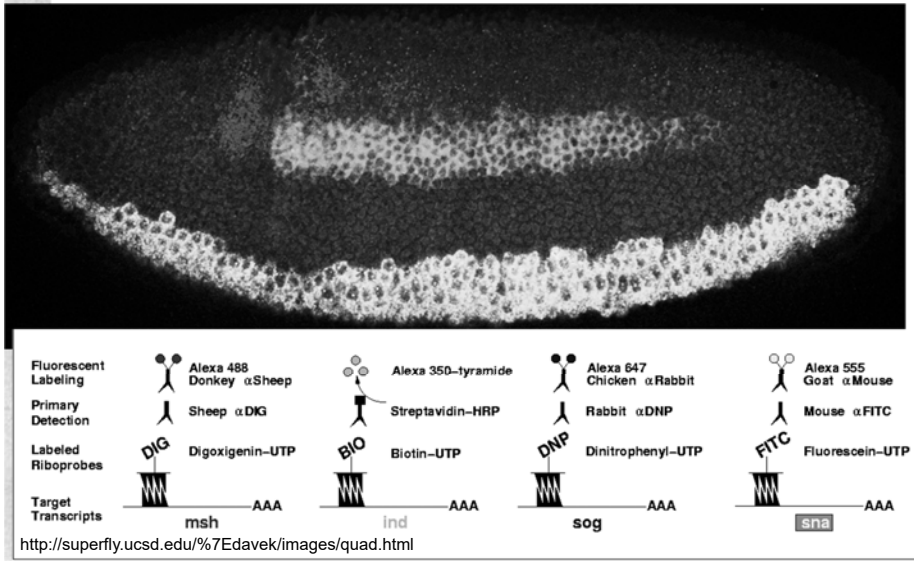
Probe Labeling







Multiplex mRNA detection



http://superfly.ucsd.edu/%7Edavek/images/quad.html