Molecular Biology

2025-2024

DR.Ahmad Al Qawasmi

ARKAN ACADEMY

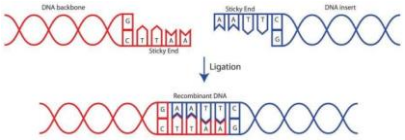
DNA Cloning

- DNA cloning is a technique that allows for:
  - ***Amplifying*** a DNA segment into many, many copies in a biological system
  - ***Expressing*** a gene inside a biological system
- Recombinant DNA is composed of the *gene of interest* (encoding a protein or non-coding RNA) and a *vector* formed using restriction endonucleases and ligase
  - Cut , Join , Insert into a cell

Cloning means that you make several copies of one thing

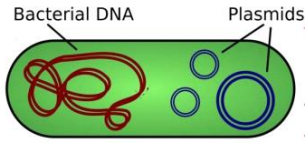
- A clone is a genetically identical population (organisms, cells, viruses, DNA)
- Every member of the population is derived from a single cell, virus, or DNA molecule

- ***Restriction endonuclease*** cut in specific fragments of 4-8 bp palindromic sequences
- There are 2 types of cut can be produced:
  - *Staggered (off-center):* enzymes cut the two DNA strands at *different positions* generating *sticky or cohesive* ends where the fragments have short single stranded overhangs at each end

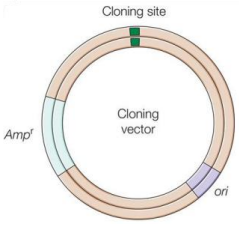


    - ✓ Can form H-bonds with *complementary* sequences, so can be used in cloning
  - *Blunt:* enzymes cut at the *same position* on both strands giving blunt-ended fragments

- ***Vector:*** It is the *carrier* where the gene of interest is added
  - ***Bacterial plasmids*** are considered excellent vectors for cloning (cloning vectors) or expression (expression vectors) where it is a small circular DNA separated from the main bacterial chromosome

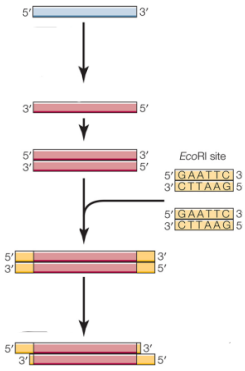


- Features of plasmid cloning vectors:
  - Their *own origin of replication* (OriC) that allows them to replicate *independently* of the bacterial chromosome
  - A *selectable gene* such as an <u>antibiotic resistance gene for ampicillin</u> antibiotic
  - A *restriction site* that allows for insertion of the DNA segment of interest



- The gene of interest is added and joined with the vector by DNA ligase
  - ***DNA ligase:*** It *covalently joins* DNA ends by catalyzing the *ATP*-dependent formation of permanent <u>phosphodiester bonds</u> between 3' hydroxyl of one strand and 5' phosphate of the other strand
- The vector must be previously cut by the **<u>same restriction endonuclease</u>** that the gene of interest cut by
  - To make sticky ends complementary to that in the gene of interest
- DNA libraries are 2 types:
  - ***Genomic library:*** It contains ***all the elements*** of the genome (exons, introns, telomere, …)
    - ✓ A genome is *cleaved* by the <u>same restriction endonuclease</u> as that used for the vector and each fragment is *ligated* into a vector, then each plasmid is *transferred int a bacterial cell* (each cell has 1 plasmid) and can grow into millions of cells and each cell can *make multiple copies* of every plasmid ending up with billions of copies of plasmid with a specific DNA fragment.

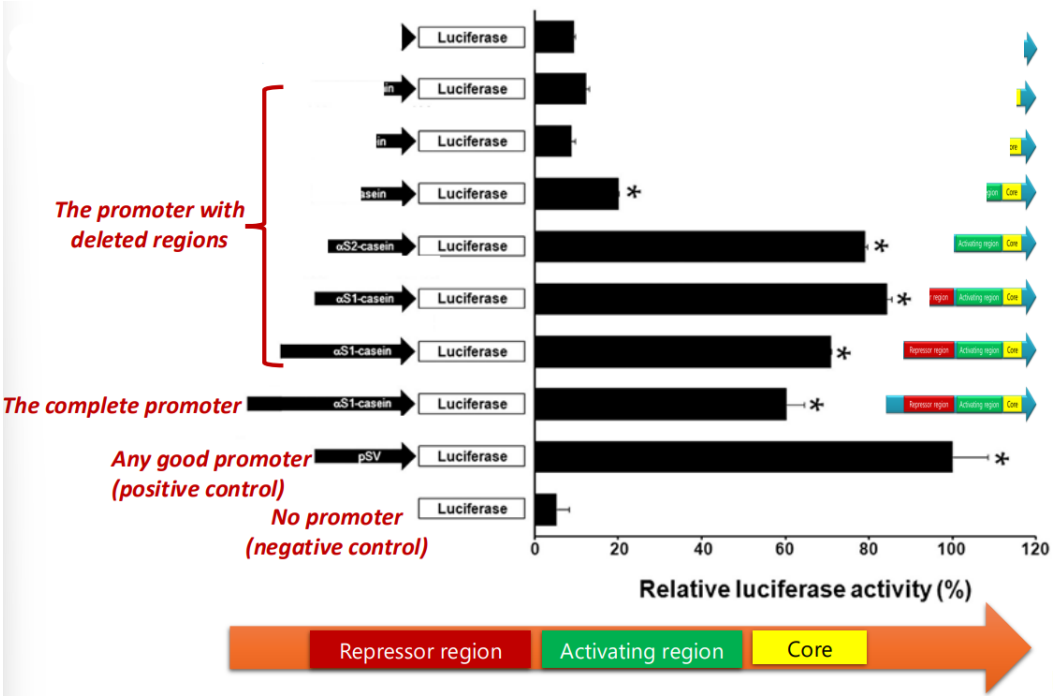
➢ ***cDNA Library***



- ✓ *mRNAs* are isolated and *reverse transcribed* by reverse transcriptase into a cDNA molecule that is *replicated* by DNA polymerase to form a double-stranded cDNA, then *synthetic linkers* containing a restriction site are ligated to the ends of the cDNAs and digested with the *restriction endonuclease* to form overhangs and then cDNAs are *cloned* into a plasmid
- ✓ Contains only the elements present in the *mature mRNA* (exons)
- ✓ ***Only transcribed*** genes can appear

Analysis of transcriptional regulatory sequences

- It is the study of the effect of different conditions on the regulation and level of expression
- It requires a ***reporter gene*** such as *luciferase gene*
  - ➢ Luciferase is an enzyme in fruit flies that convert luciferin into oxyluciferin which is **glowing**
- The reporter gene must be inserted to the plasmid containing the gene of interest, where ***only the regulatory sequences*** (promoter, PPE, enhancers and silencers) are ***upstream*** to it
  - ➢ The plasmid is transfected (inserted) into cells, and the expression level of luciferase (instead of the original gene itself) is ***measured via the strength of the light signal***



Analysis of gene expression

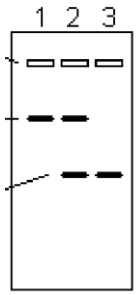
- It is done by measuring RNA levels by basic (northern blotting, in situ hybridization), advanced (real-time PCR, DNA microarray) and very advanced methods (RNA-seq)

Northern blotting

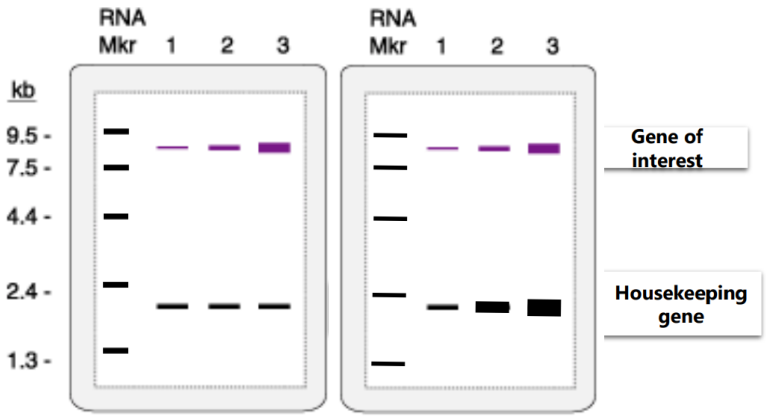
- This is done exactly like Southern blotting with some differences:
  - ***RNA*** from cells is isolated instead of DNA and fractionated based on size by gel electrophoresis
  - RNA molecules are transferred onto a membrane and targeted by a ***labeled DNA probe*** with a sequence that is complementary to a specific RNA molecule

Western blotting: For detection of *proteins* via SDS-PAGE, and using *antibodies* instead of probes

- It can tell us if the gene is ***expressed or not***
- If expressed it can tell us the ***size*** (via the position) and ***amount of RNA*** (via band intensity)

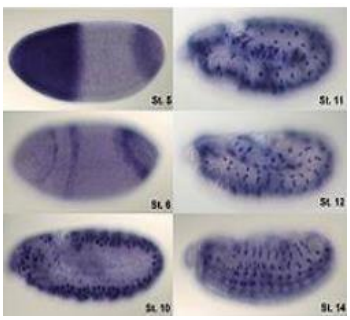


  - If you studied one gene only and 2 bands (with different sized) appeared in the same samples that could be variety in transcription due to ***different promoters***, or different RNA processing (***alternative splicing*** or ***polyadenylation***)
- It can be used to ***compare the expression a gene between different tissues***
  - In this case, house-keeping genes must be assessed also to be sure that the sample is taken properly
  - ***House-keeping genes*** are genes expressed in *all cells*, in the *same levels* (constant expression, do not change) such as ***histones, actin, tubulin*** and some metabolic enzymes



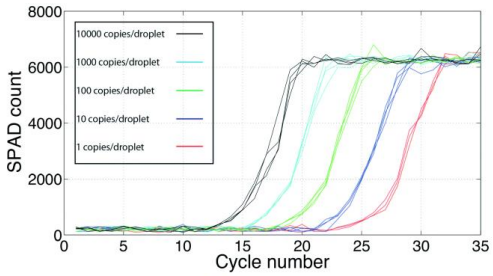
In situ hybridization

- In situ hybridization methods reveals the ***distribution*** of specific RNA molecules in cells in ***tissues***
- RNA molecules can hybridize when the tissue is incubated with a complementary *DNA or RNA probe*
- In this way the patterns of differential gene expression can be observed in tissues, and the location of specific RNAs can be determined in cells
- We use in situ hybridization to know in which cells the gene is expressed, and immunohistochemistry to know where the protein is concentrated

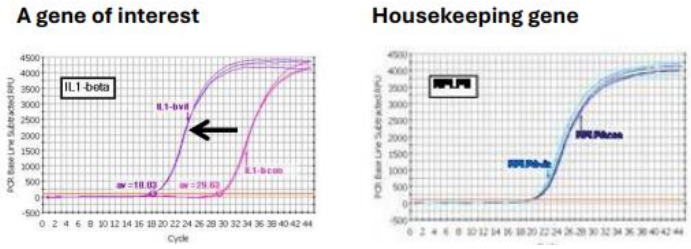


Reverse transcriptase Real time qPCR

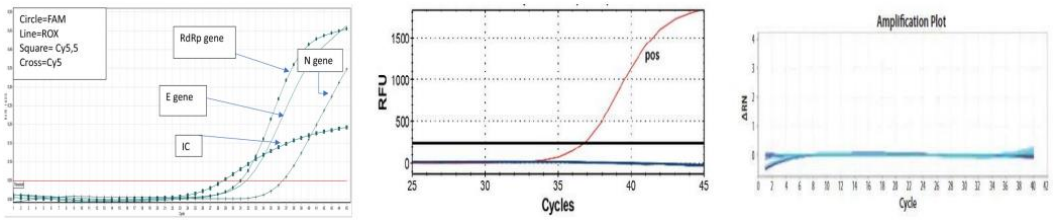
- Quantitative reverse transcriptase RT-qPCR of mRNA is another way of relative quantitation of RNA expression is by *converting RNA into cDNA* followed by PCR in the presence of *SYBR green*
  - The ***higher the amount*** of RNA = higher amount of cDNA, the ***sooner*** detected



- To ***compare expression*** level between 2 tissues, *house keeping* genes analysis is required to ensure proper sample taking



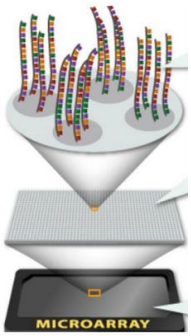
- Detection of ***SARS-Co-2*** is done by qPCR
  - We must analyze the expression of at least 1 viral gene and 1 human gene
  - The analysis of *human gene expression* is to ensure proper sample taking

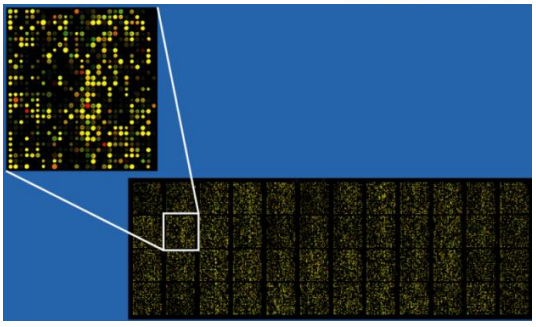


- The study of -omics
  - Genomics: The study of genomes and DNA sequence
  - Transcriptomics: They study of RNA produced by transcription, mainly by DNA microarray
  - Proteomics: The study of proteins
  - Metabolomics: The study of metabolites produced from enzymes

DNA microarrays

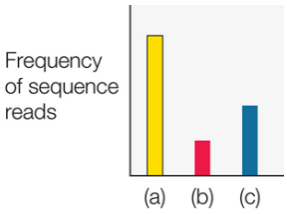
- DNA microarrays are solid surfaces (glass microscope slides or chips) spotted with up to tens of thousands of DNA fragments in an area the size of a fingernail. Where ***the exact sequence and position*** of every DNA fragment on the array is known
- mRNAs are extracted from cells (cancer and normal for example) and *converted to cDNAs*, which are labeled with a *fluorescent dye*, then cDNAs hybridize to a DNA corresponding to distinct human genes
  - The relative level of expression of each gene is indicated by the **intensity of fluorescence** at each position on the microarray
  - Can be used to compare the types of genes expressed between different cell types





RNA sequencing (RNA-seq)

- Cellular RNA is reverse transcribed to ***cDNAs***, which are subjected to next-generation sequencing
  - ➢ The relative amount of initial mRNA is indicated by the ***frequency*** at which its sequence of cDNA is read



- ***RNA-seq*** can be used to characterize *novel transcripts*, identify splicing *variants* and profile the *expression levels* of all transcripts
  - ➢ ***Microarrays*** are limited to detect transcripts corresponding to *known genomic sequences*

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+962 790408805