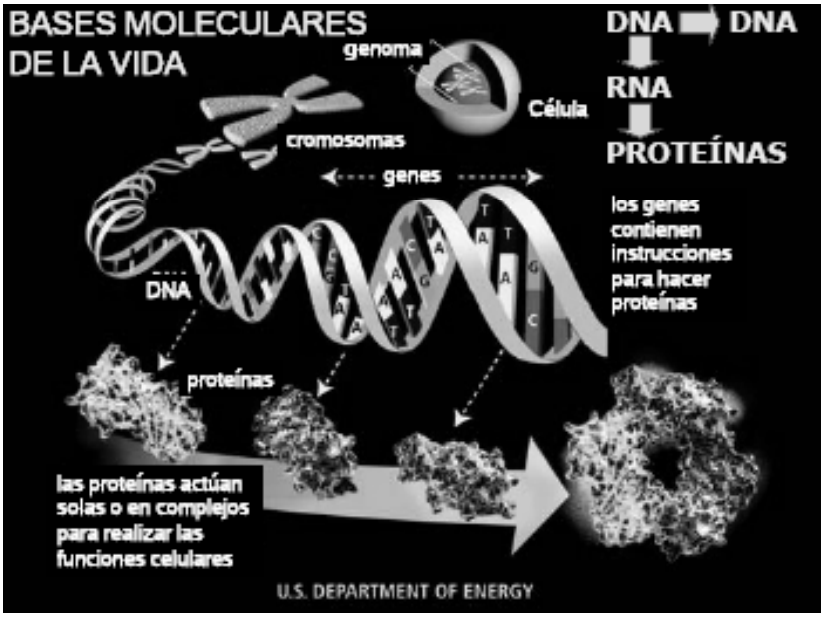
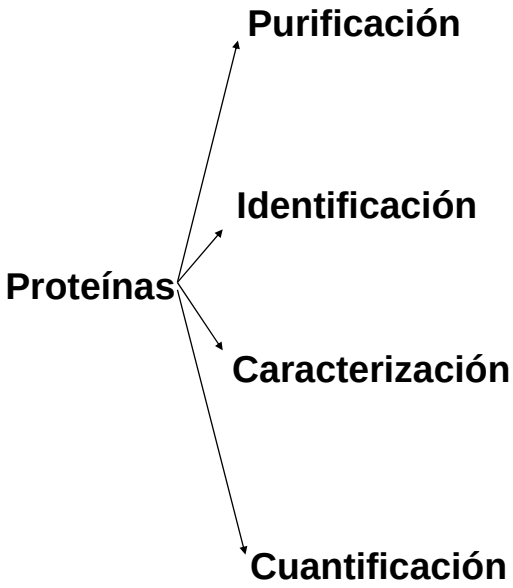
Metodologías Básico-Clínicas





Purificación de Proteínas

- Salting in y salting out
- Centrifugación
- Ultrafiltración y diálisis
- Cromatografía (intercambio iónico, exclusión, afinidad, HPLC)

SEPARACIÓN DE PROTEÍNAS
CENTRIFUGACIÓN DIFERENCIAL

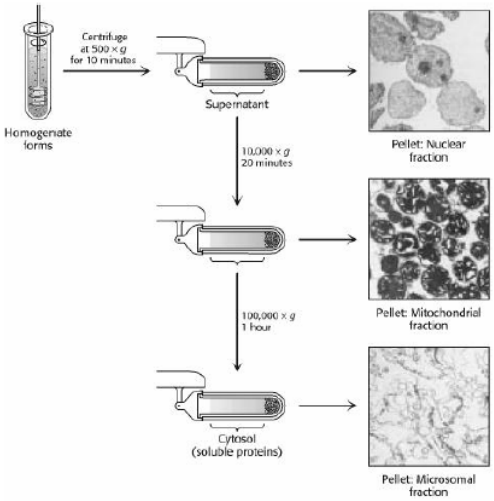


Figure 4.1. Differential Centrifugation. Cells are disrupted in a homogenizer and the resulting mixture, called the homogenate, is centrifuged in a step-by-step fashion of increasing centrifugal force. The denser material will form a pellet at lower centrifugal force than will the less-dense material. The isolated fractions can be used for further purification. [Photographs courtesy of S. Fleischer and B. Fleischer.]

SEPARACIÓN DE PROTEÍNAS
DIÁLISIS

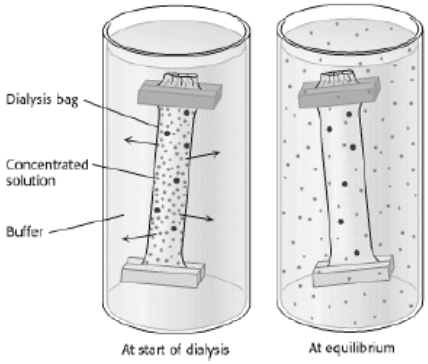


Figure 4.2. Dialysis. Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse

SEPARACIÓN DE PROTEÍNAS
CROMATOGRAFÍA DE FILTRACIÓN

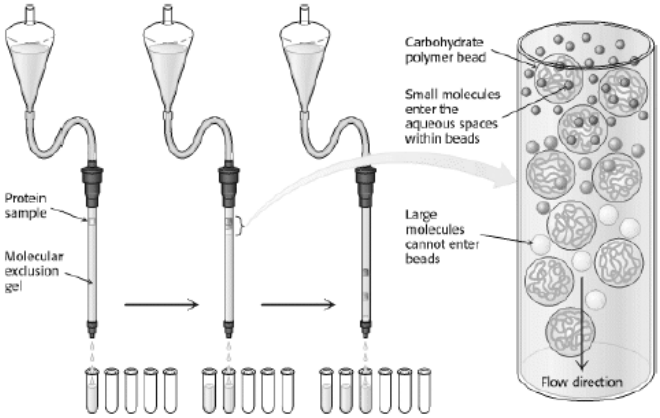


Figure 4.3. Gel Filtration Chromatography. A mixture of proteins in a small volume is applied to a column filled with porous beads. Because large proteins cannot enter the internal volume of the beads, they emerge sooner than do small ones.

SEPARACIÓN DE PROTEÍNAS
CROMATOGRAFÍA CON CARGA ELÉCTRICA

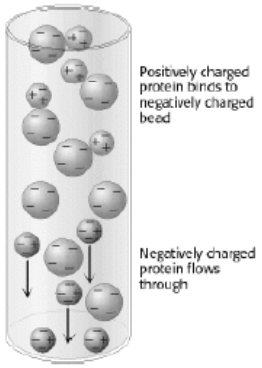


Figure 4.4. Ion-Exchange Chromatography. This technique separates proteins mainly according to their net charge.

SEPARACIÓN DE PROTEÍNAS
CROMATOGRAFÍA DE AFINIDAD

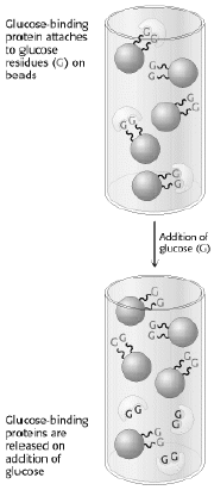


Figure 4.5. Affinity Chromatography. Affinity chromatography of concanavalin A (shown in yellow) on a solid support containing covalently attached glucose residues (G).

SEPARACIÓN DE PROTEÍNAS
CROMATOGRAFÍA LÍQUIDA DE ALTA PRESIÓN-HPLC

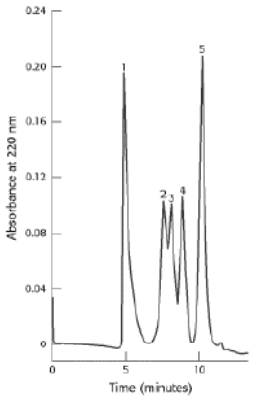
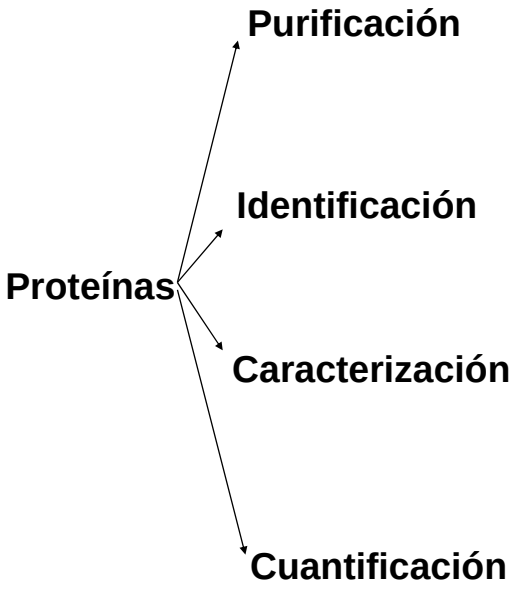


Figure 4.6. High-Pressure Liquid Chromatography (HPLC). Gel filtration by HPLC clearly defines the individual proteins because of its greater resolving power: (1) thyroglobulin (669 kd), (2) catalase (232 kd), (3) bovine serum albumin (67 kd), (4) ovalbumin (43 kd), and (5) ribonuclease (13.4 kd). [After K. J. Wilson and T. D. Schlabach. In *Current Protocols in Molecular Biology*, vol. 2, suppl. 41, F. M. Ausbel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds. (Wiley, 1998), p. 10.14.1.]



Identificación y Caracterización de Proteínas

- Electroforesis
- Electroenfoque
- Western blot
- Inmunodetección
- Técnicas espectroscópicas (NMR, difracción óptica rotatoria, EPR, espectrometría de masa, difracción de rayos X)

SEPARACIÓN DE PROTEÍNAS

ELECTROFORESIS

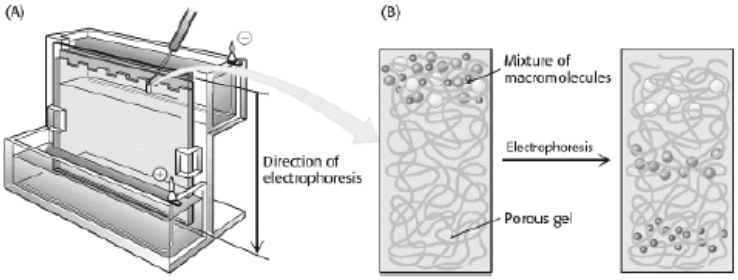


Figure 4.7. Polyacrylamide Gel Electrophoresis. (A) Gel electrophoresis apparatus. Typically, several samples undergo electrophoresis on one flat polyacrylamide gel. A microliter pipette is used to place solutions of proteins in the wells of the slab. A cover is then placed over the gel chamber and voltage is applied. The negatively charged SDS (sodium dodecyl sulfate)-protein complexes migrate in the direction of the anode, at the bottom of the gel. (B) The sieving action of a porous polyacrylamide gel separates proteins according to size, with the smallest moving most rapidly.

SEPARACIÓN DE PROTEÍNAS

ELECTROENFOQUE

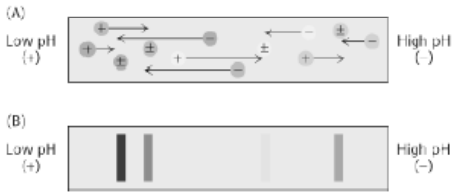
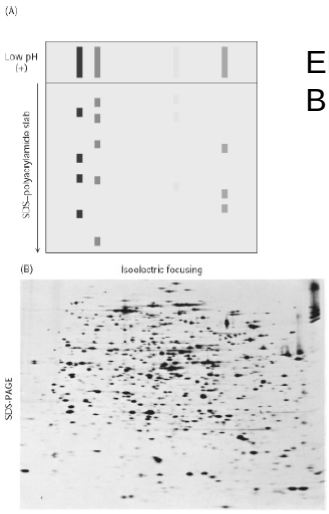


Figure 4.11. The Principle of Isoelectric Focusing. A pH gradient is established in a gel before loading the sample. (A) The sample is loaded and voltage is applied. The proteins will migrate to their isoelectric pH, the location at which they have no net charge. (B) The proteins form bands that can be excised and used for further experimentation.

SEPARACIÓN DE PROTEÍNAS



ELECTROFORESIS BIDIMENSIONAL

Figure 4.12. Two-Dimensional Gel Electrophoresis. (A) A protein sample is initially fractionated in one dimension by isoelectric focusing as described in Figure 4.11. The isoelectric focusing gel is then attached to an SDS-polyacrylamide gel, and electrophoresis is performed in the second dimension, perpendicular to the original separation. Proteins with the same pI are now separated on the basis of mass. (B) Proteins from *E. coli* were separated by two-dimensional gel electrophoresis, resolving more than a thousand different proteins. The proteins were first separated according to their isoelectric pH in the horizontal direction and then by their apparent mass in the vertical direction. [(B) Courtesy of Dr. Patrick H. O'Farrell.]

DETECCIÓN DE PROTEÍNAS
USO DE ANTICUERPOS

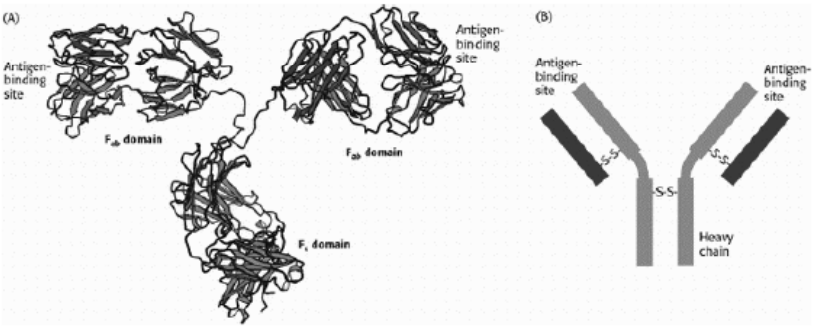


Figure 4.30. Antibody Structure. (A) IgG antibodies consist of four chains, two heavy chains (blue) and two light chains (red), linked by disulfide bonds. The heavy and light chains come together to form Fab domains, which have the antigen-binding sites at the ends. The two heavy chains form the Fc domain. The Fab domains are linked to the Fc domain by flexible linkers. (B) A more schematic representation of an IgG molecule.

DETECCIÓN DE PROTEÍNAS
INTERACCIONES ANTÍGENO-ANTICUERPO

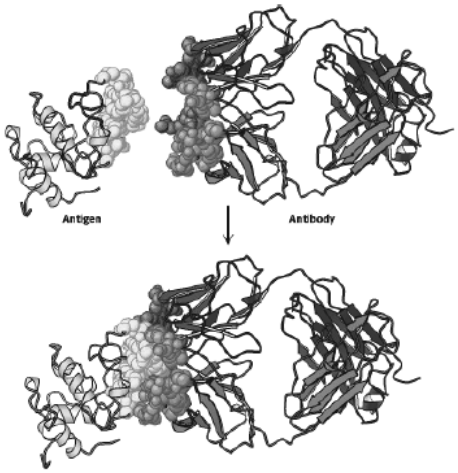
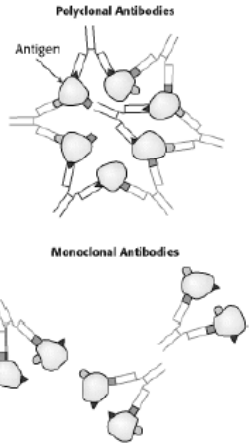


Figure 4.31. Antigen-Antibody Interactions. A protein antigen, in this case lysozyme, binds to the end of an Fab domain from an antibody. The end of the antibody and the antigen have complementary shapes, allowing a large amount of surface to be buried on binding.

DETECCIÓN DE PROTEÍNAS



ANTICUERPOS POLICLONALES
Y MONOCLONALES

Figure 4.32. Polyclonal and Monoclonal Antibodies. Most antigens have several epitopes. Polyclonal antibodies are heterogeneous mixtures of antibodies, each specific for one of the various epitopes on an antigen. Monoclonal antibodies are all identical, produced by clones of a single antibody-producing cell. They recognize one specific epitope. [After R. A. Goldsby, T. J. Kindt, B. A. Osborne, *Kuby Immunology*, 4th ed. (W. H. Freeman and Company, 2000), p. 154.]

DETECCIÓN DE PROTEÍNAS

PREPARACIÓN DE ANTICUERPOS MONOCLONALES

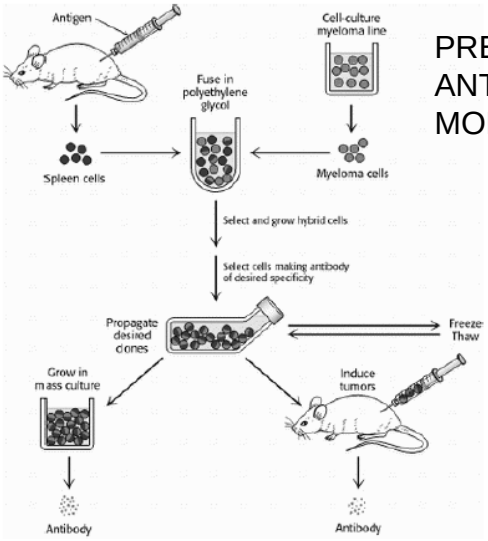


Figure 4.33. Preparation of Monoclonal Antibodies. Hybridoma cells are formed by fusion of antibody-producing cells and myeloma cells. The hybrid cells are allowed to proliferate by growing them in selective medium. They are then screened to determine which ones produce antibody of the desired specificity. [After C. Milstein. Monoclonal antibodies. Copyright © 1980 by Scientific American, Inc. All rights reserved.]

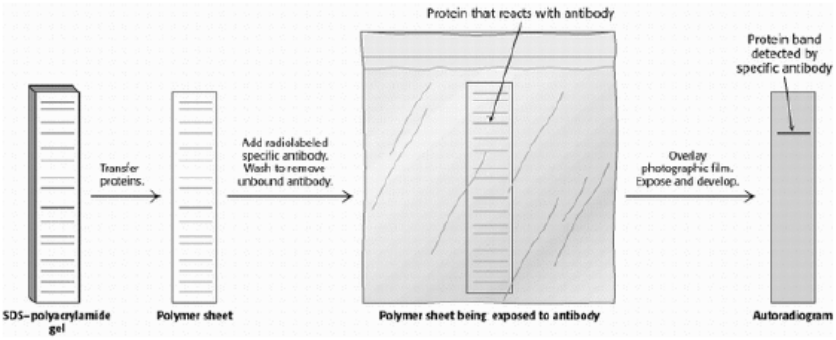
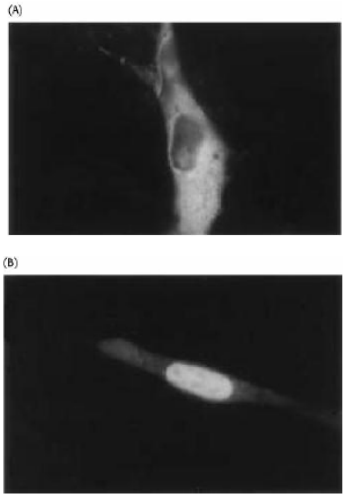


Figure 4.36. Western Blotting. Proteins on an SDS-polyacrylamide gel are transferred to a polymer sheet and stained with radioactive antibody. A band corresponding to the protein to which the antibody binds appears in the autoradiogram.



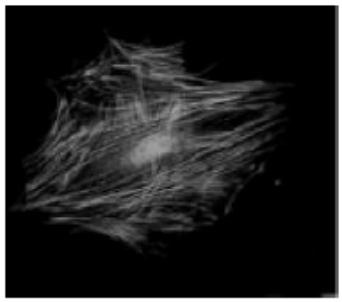
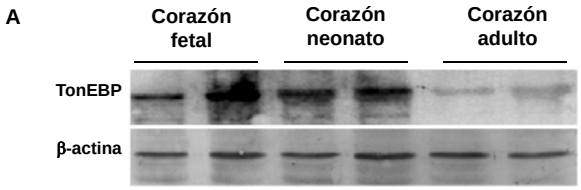
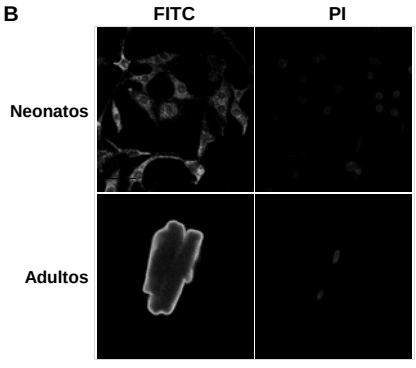


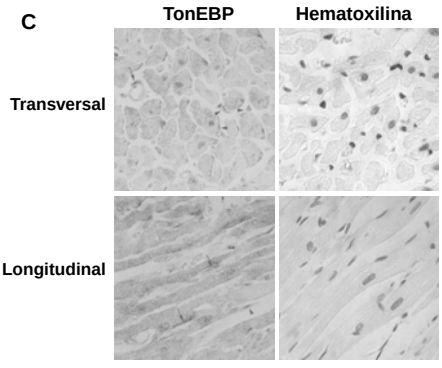
Figure 4.37. Actin Filaments. Fluorescence micrograph of actin filaments in a cell stained with an antibody specific to actin. [Courtesy of Dr. Elias Lazarides.]

Figure 4.38. Nuclear Localization of a Steroid Receptor. (A) The receptor, made visible by attachment of the green fluorescent protein, is located predominantly in the cytoplasm of the cultured cell. (B) Subsequent to the addition of corticosterone (a glucocorticoid steroid), the receptor moves into the nucleus. [Courtesy of Professor William B. Pratt/ Department of Pharmacology, University of Michigan.]

TonEBP está presente en los cardiomiocitos







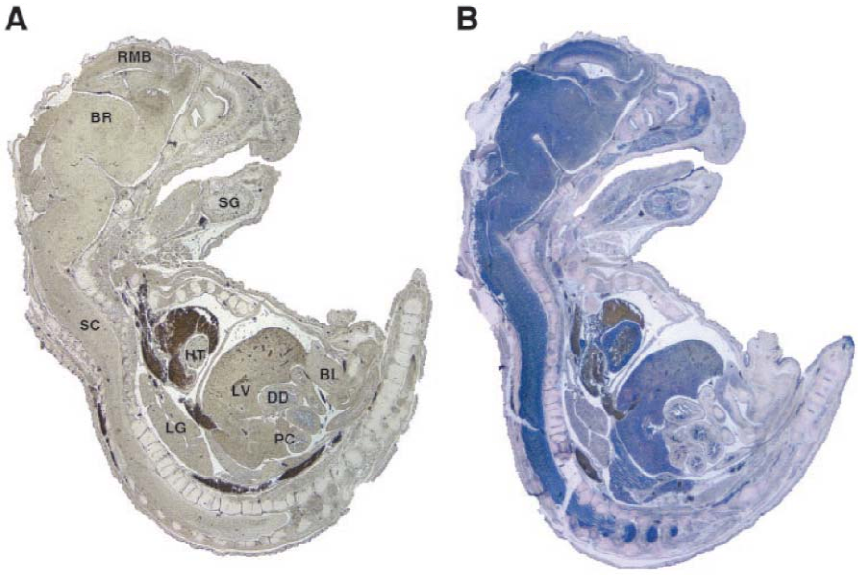
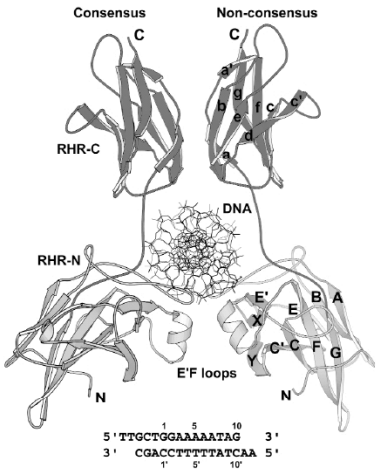
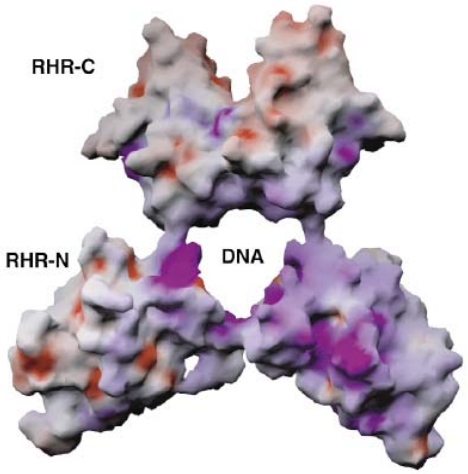
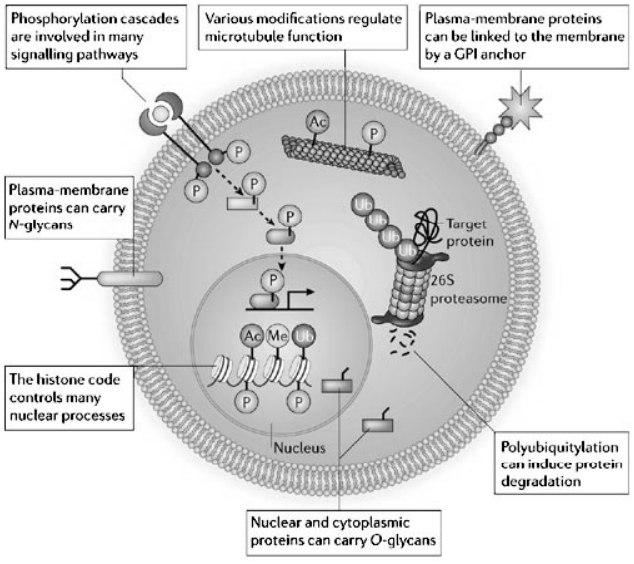


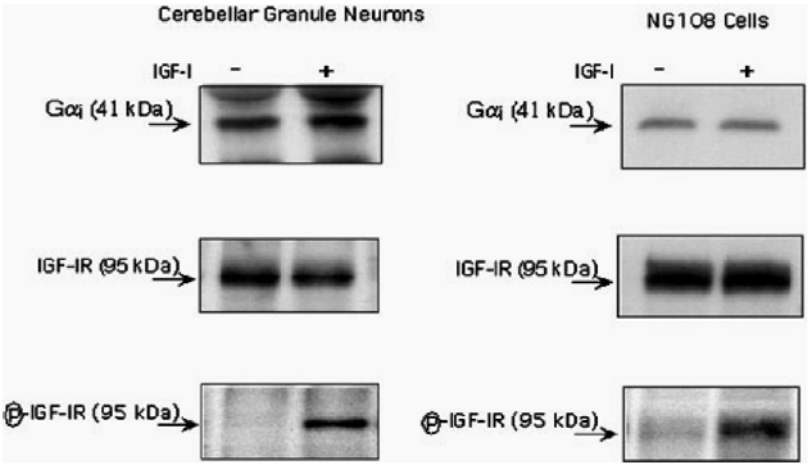
Figure 4.52. Section of the Electron-Density Map of Myoglobin. This section of the electron-density map shows the heme group. The peak of the center of this section corresponds to the position of the iron atom. [From J. C. Kendrew. The three-dimensional structure of a protein molecule. Copyright © 1961 by Scientific American, Inc. All rights reserved.]

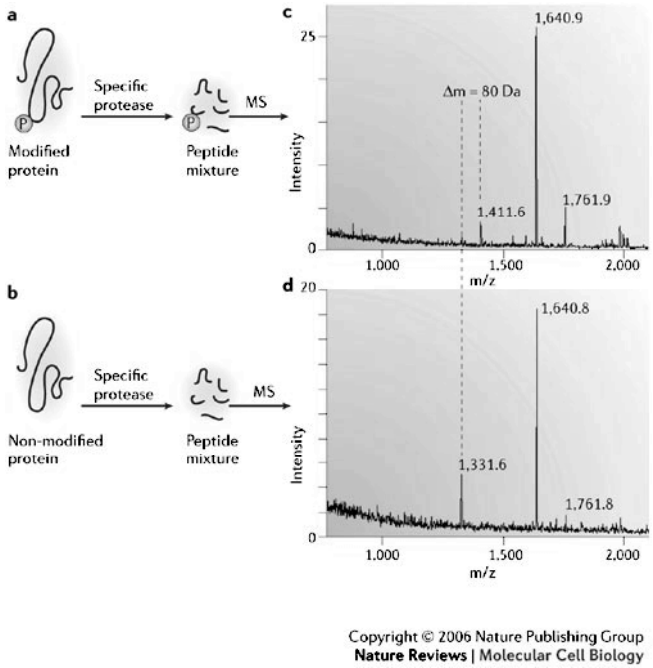


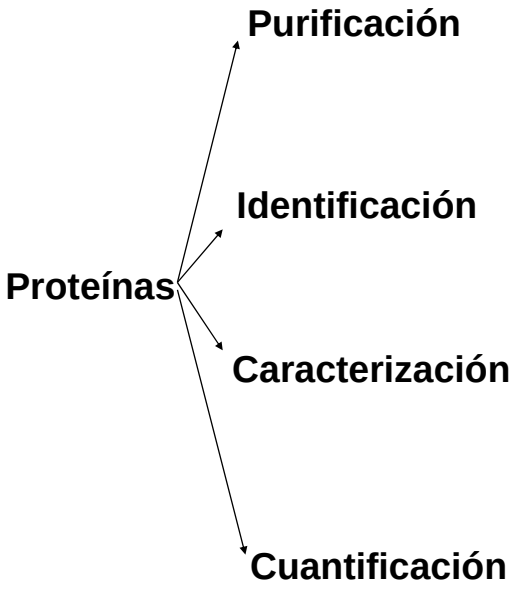




Fosforilación de proteínas







Cuantificación de Proteínas

- Western blot
- ELISA

SEPARACIÓN DE PROTEÍNAS

ELECTROFORESIS-WESTERN BLOT

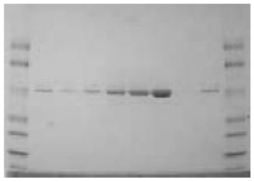


Figure 4.9. Staining of Proteins After Electrophoresis. Proteins subjected to electrophoresis on an SDS-polyacrylamide gel can be visualized by staining with Coomassie blue. [Courtesy of Kodak Scientific Imaging Systems.]

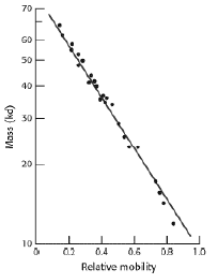


Figure 4.10. Electrophoresis Can Determine Mass. The electrophoretic mobility of many proteins in SDS-polyacrylamide gels is inversely proportional to the logarithm of their mass. [After K. Weber and M. Osborn, *The Proteins*, vol. 1, 3d ed. (Academic Press, 1975), p. 179.]

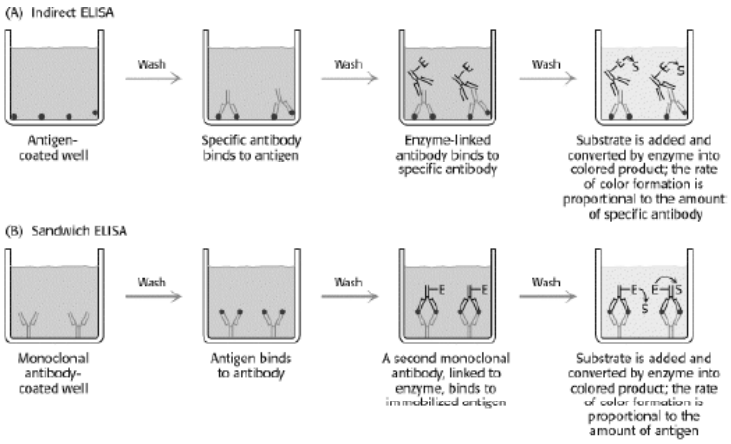
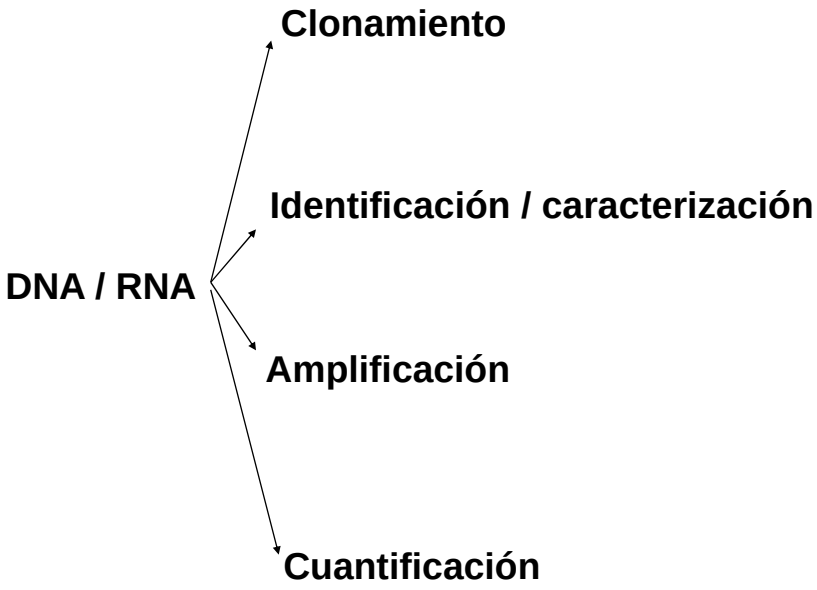


Figure 4.35. Indirect ELISA and Sandwich ELISA (A) In indirect ELISA, the production of color indicates the amount of an antibody to a specific antigen. (B) In sandwich ELISA, the production of color indicates the quantity of antigen. [After R. A. Goldsby, T. J. Kindt, B. A. Osborne, *Kuby Immunology*, 4th ed. (W. H. Freeman and Company, 2000), p. 162.]



Clonamiento de DNA

- Enzimas de restricción
- Vectores de clonamiento
- DNA ligasa

Análisis de DNA

ENZIMAS DE RESTRICCIÓN

Enzimas de restricción

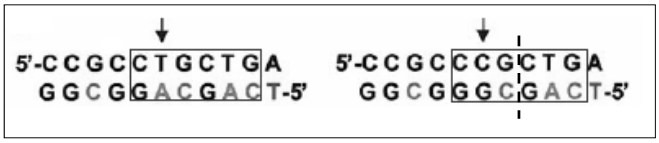
A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA. The enzyme makes two incisions, one through each of the sugar-phosphate backbones (i.e., each strand) of the double helix without damaging the nitrogenous bases. The term restriction comes from the fact that these enzymes were discovered in E. coli strains that appeared to be restricting the infection by certain bacteriophages. Restriction enzymes therefore are believed to be a mechanism evolved by bacteria to resist viral attack and to help in the removal of viral sequences. They are part of what is called the restriction modification system.

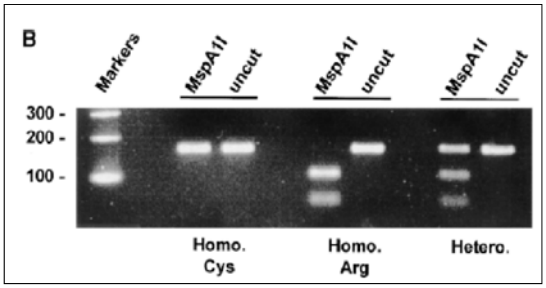
CCC|GGG
GGG|CCC

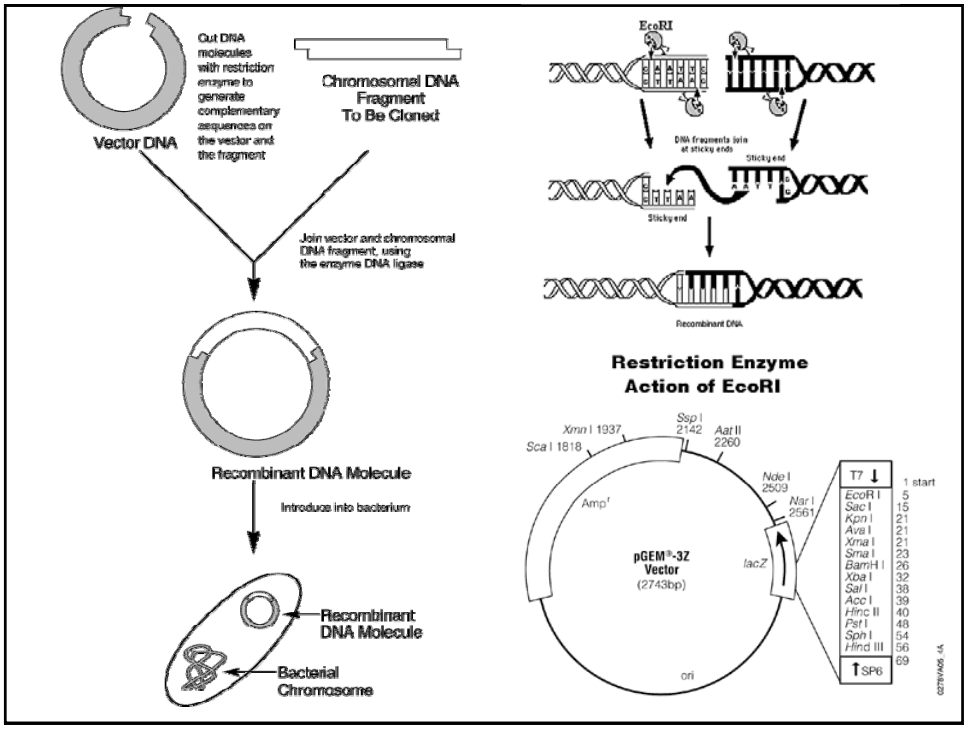
G|AATTC
CTTAA|G

The 1978 Nobel Prize in Medicine was awarded to Daniel Nathans, Werner Arber and Hamilton Smith for the discovery of restriction endonucleases, leading to the development of recombinant DNA technology. The first practical use of their work was the manipulation of E. coli bacteria to produce human insulin for diabetics.

Sitio de restricción MspA11






Cut DNA molecules with restriction enzyme to generate complementary sequences on the vector and the fragment
Vector DNA
Chromosomal DNA Fragment To Be Cloned
Join vector and chromosomal DNA fragment, using the enzyme DNA ligase
Recombinant DNA Molecule
Introduce into bacterium
Recombinant DNA Molecule
Bacterial Chromosome
EcoRI
DNA fragments join at sticky ends
Sticky end
Sticky end
Recombinant DNA
Restriction Enzyme Action of EcoRI
Xmn I 1937
Sca I 1818
Ssp I 2142
Aat II 2260
Nde I 2509
Nar I 2561
Amp
pGEM®-3Z Vector (2743bp)
lacZ
ori
T7
EcoR I 5
Sac I 15
Kpn I 21
Ava I 21
Xma I 21
Sma I 23
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Sal I 38
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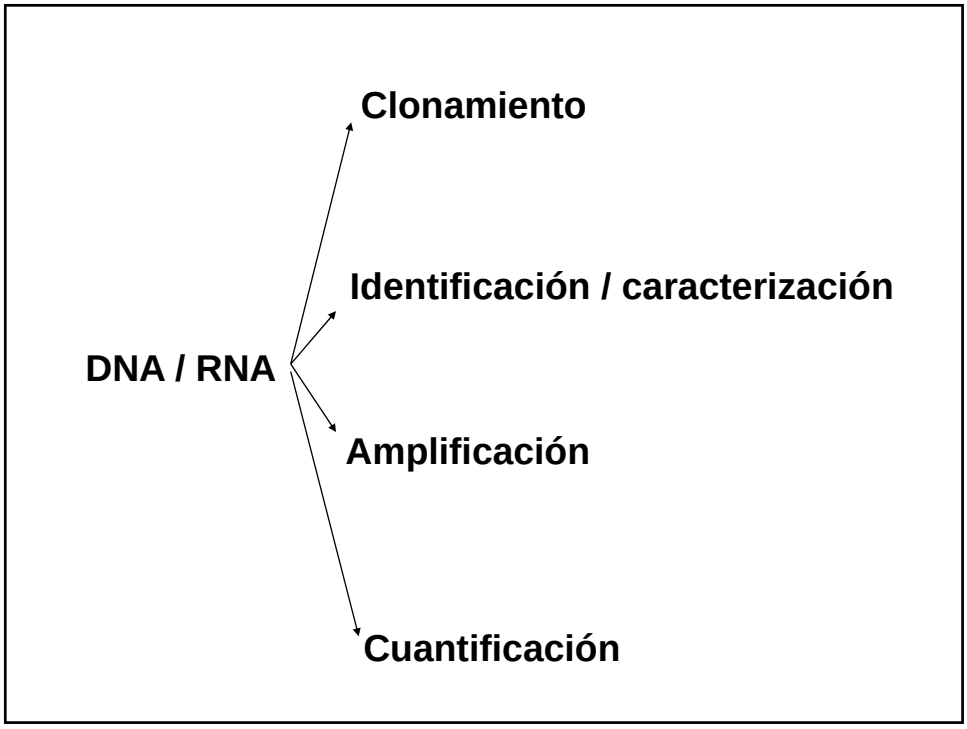
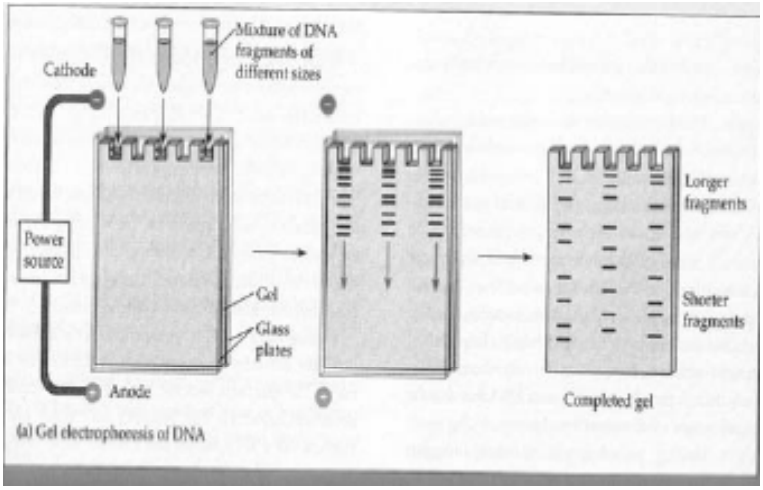
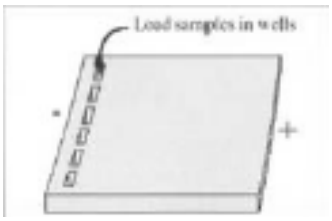
Pst I 48
Sph I 54
Hind III 56
69
SP6
1 start

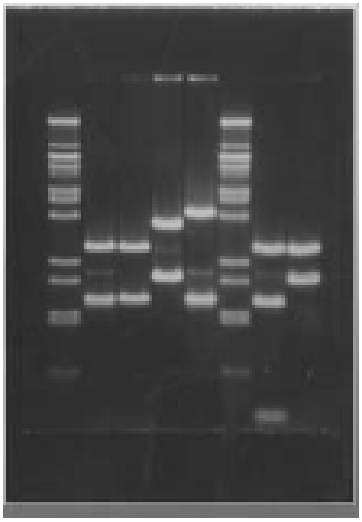

DNA / RNA
Clonamiento
Identificación / caracterización
Amplificación
Cuantificación

Identificación y caracterización de DNA / RNA

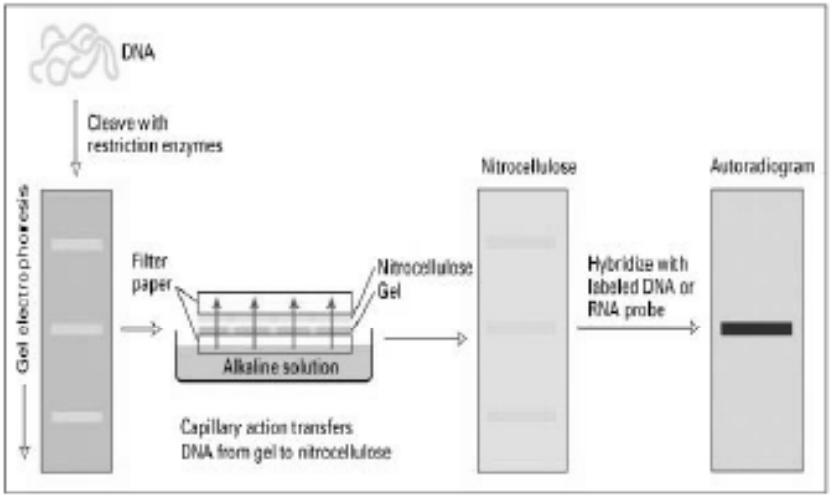
- Electroforesis en agarosa
- Southern blot
- Northern blot
- Microarray
- Secuenciación



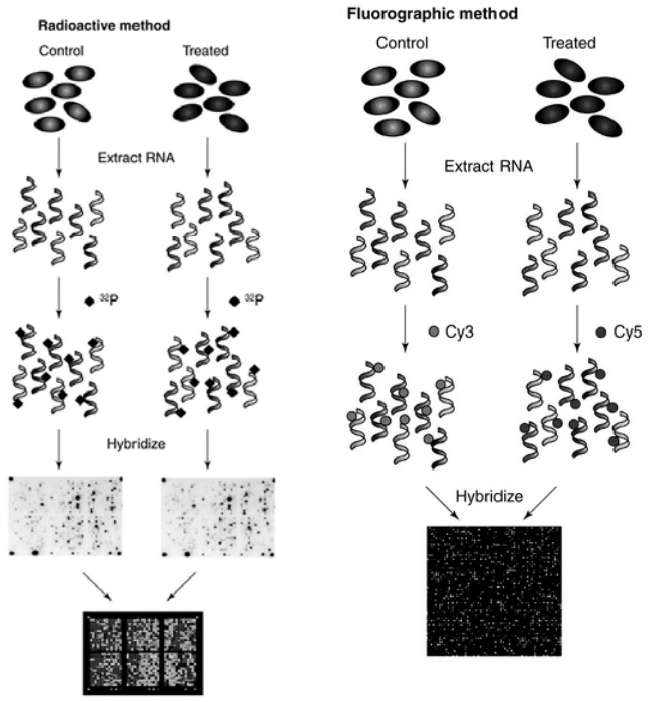




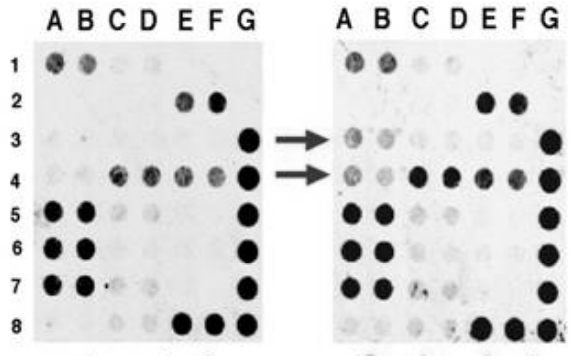
Southern Blotting



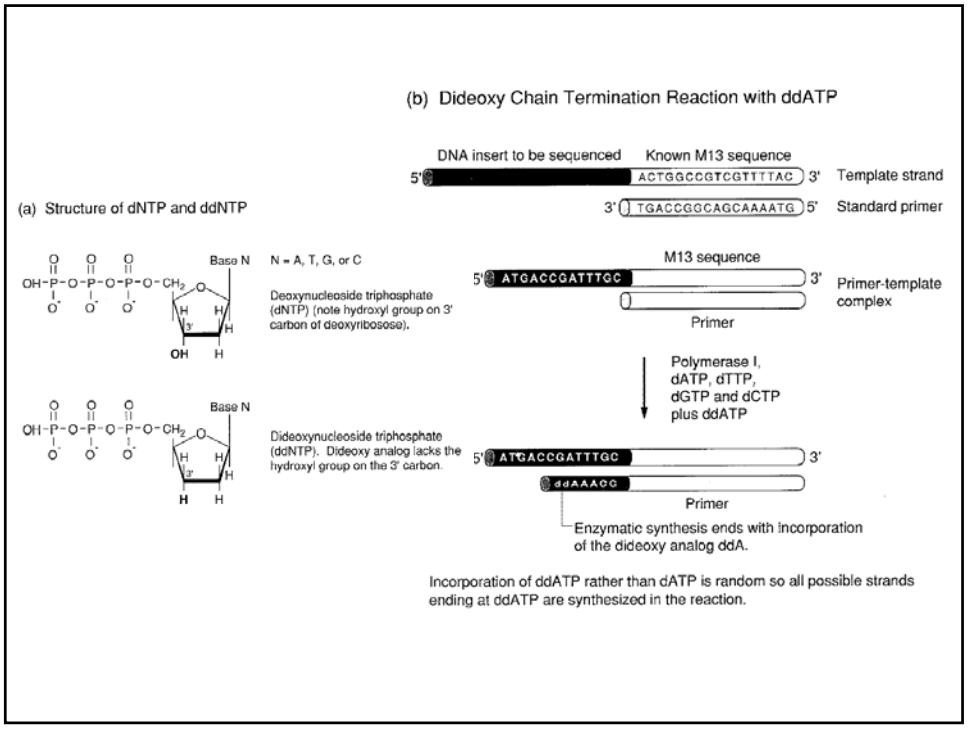
Protocolo macroarray

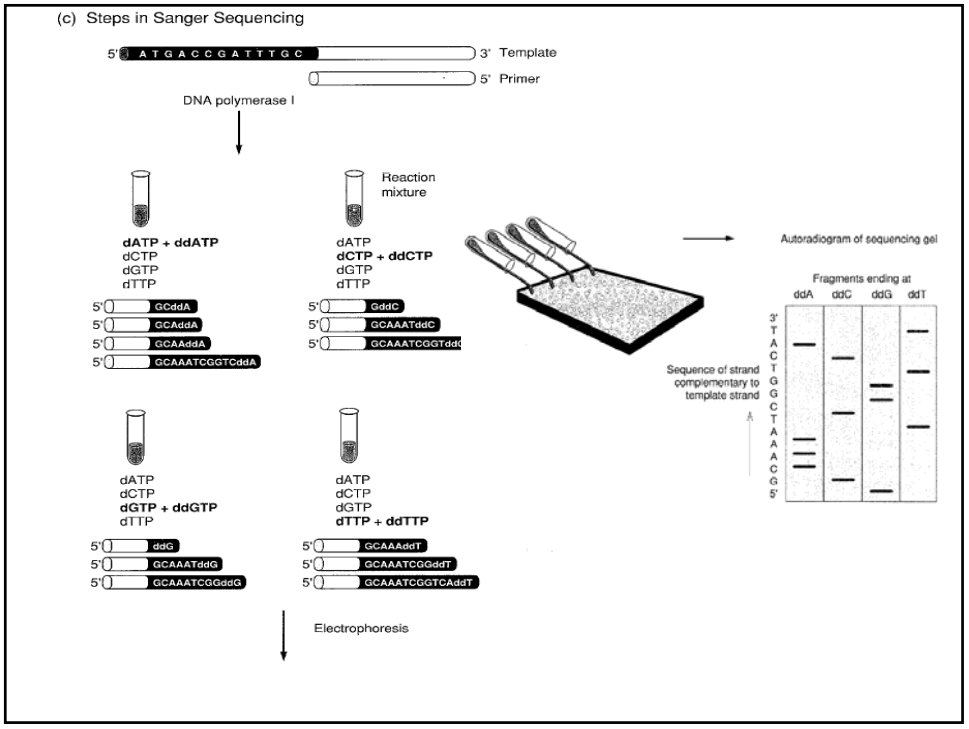


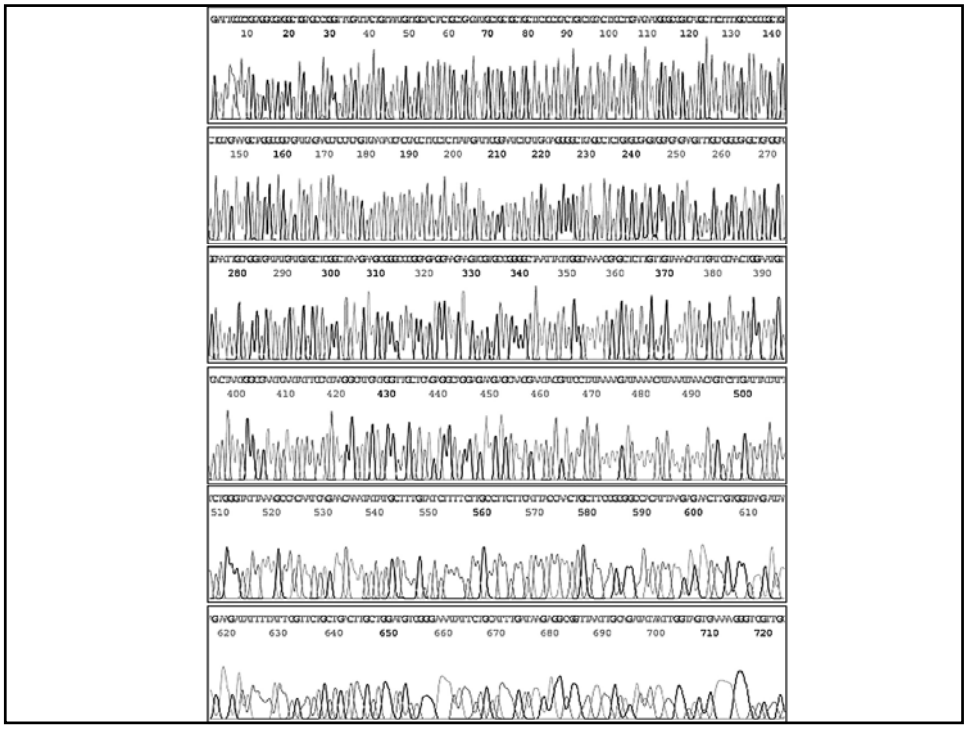
Protocolo de macroarray

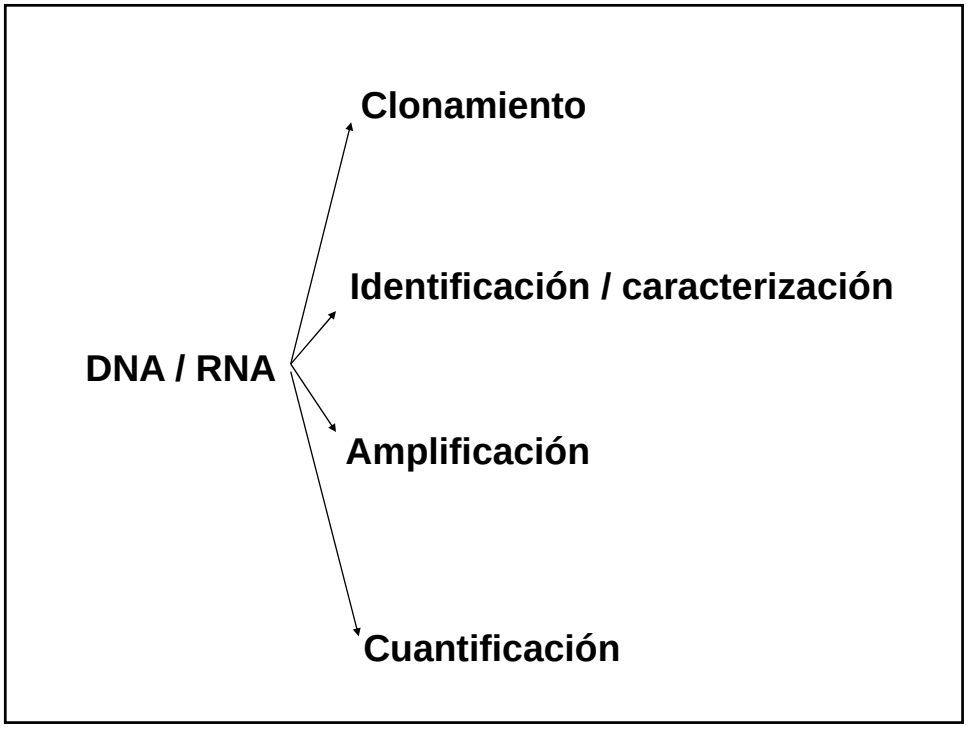


Cell Cycle Genes


(b) Dideoxy Chain Termination Reaction with ddATP
DNA insert to be sequenced
Known M13 sequence
5' ACTGGCCGTCGTTTTAC 3' Template strand
3' TGACCGGCAGCAAAATG 5' Standard primer
(a) Structure of dNTP and ddNTP
Base N
N = A, T, G, or C
Deoxynucleoside triphosphate (dNTP) (note hydroxyl group on 3' carbon of deoxyribosose).
Dideoxynucleoside triphosphate (ddNTP). Dideoxy analog lacks the hydroxyl group on the 3' carbon.
M13 sequence
5' ATGACCGATTTGC 3' Primer-template complex
Primer
Polymerase I, dATP, dTTP, dGTP and dCTP plus ddATP
5' ATGACCGATTTGC 3'
ddAAACG
Primer
Enzymatic synthesis ends with incorporation of the dideoxy analog ddA.
Incorporation of ddATP rather than dATP is random so all possible strands ending at ddATP are synthesized in the reaction.


(c) Steps in Sanger Sequencing
5' ATGACCGATTTGC 3' Template
5' Primer
DNA polymerase I
Reaction mixture
dATP + ddATP
dCTP
dGTP
dTTP
5' GCddA
5' GCAddA
5' GCAAddA
5' GCAAATCGGTCddA
dATP
dCTP + ddCTP
dGTP
dTTP
5' GddC
5' GCAAATddC
5' GCAAATCGGTddC
dATP
dCTP
dGTP + ddGTP
dTTP
5' ddG
5' GCAAATddG
5' GCAAATCGGddG
dATP
dCTP
dGTP
dTTP + ddTTP
5' GCAAAddT
5' GCAAATCGGddT
5' GCAAATCGGTCAddT
Electrophoresis
Autoradiogram of sequencing gel
Fragments ending at
ddA ddC ddG ddT
Sequence of strand complementary to template strand
3' TACTGGCTAAACG 5'

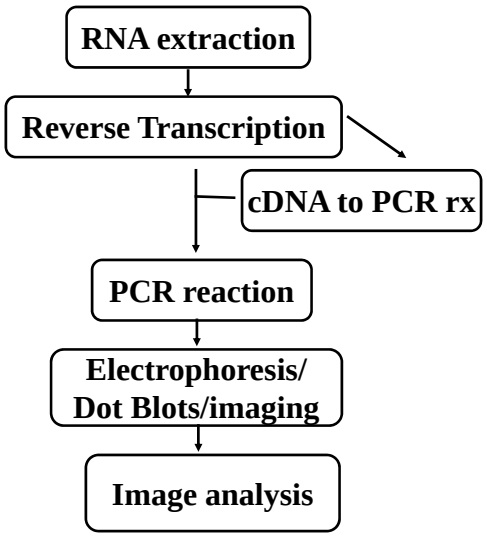



Clonamiento
Identificación / caracterización
DNA / RNA
Amplificación
Cuantificación

Amplificación de DNA

- Reacción de polimerización en cadena (PCR)

Transcriptasa Reversa y Reacción en Cadena de la Polimerasa (RT-PCR)



Reacción en Cadena de la Polimerasa (PCR)

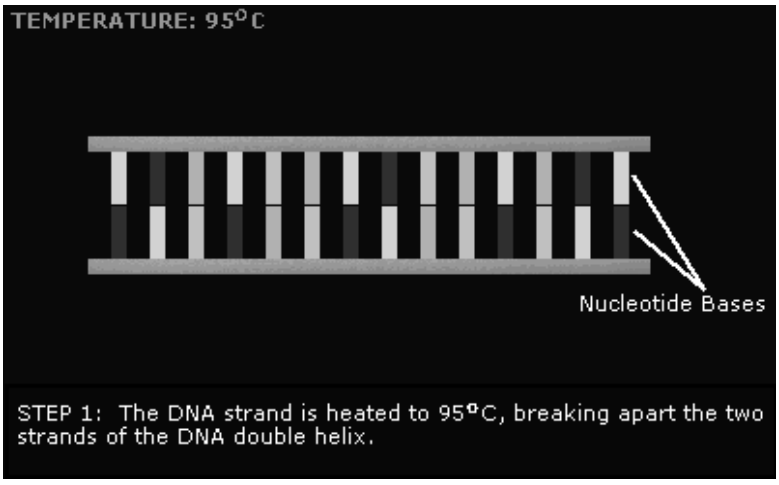
PCR es básicamente una técnica *in vitro* para amplificar una secuencia especifica de DNA.

Método consiste en tres etapas básicas:

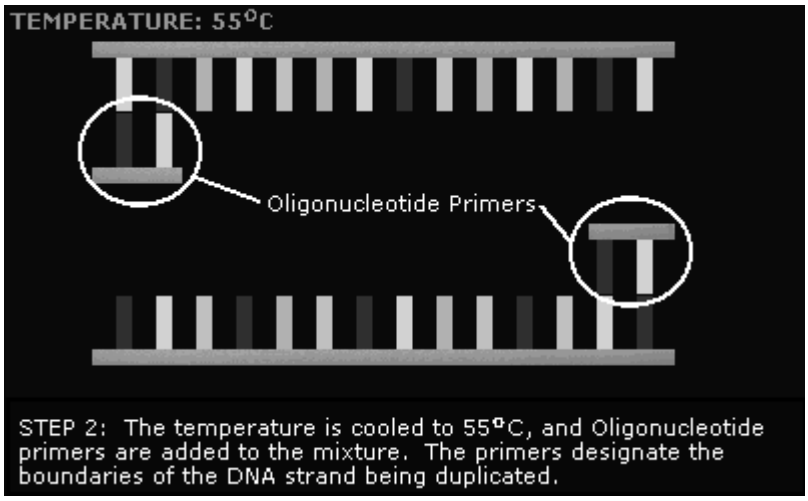
1. **Desnaturación (~95ºC)**
2. **Alineamiento (~55- 60ºC, varía)**
3. **Extensión (~72ºC)**

Las tres etapas se repiten Cíclicamente

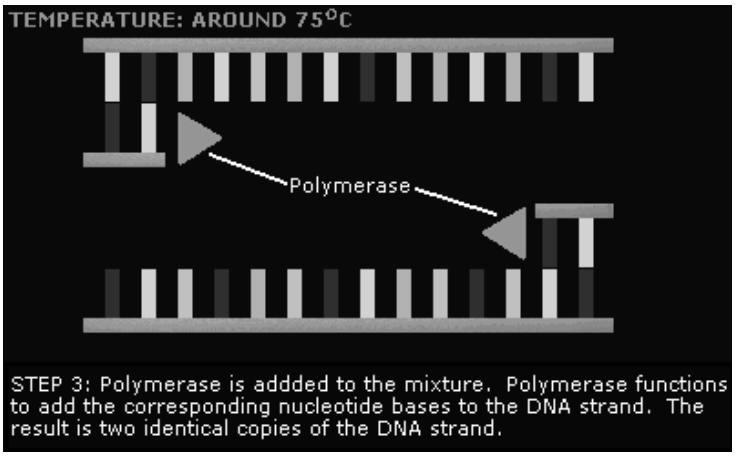
Desnaturación: altas temperaturas separan las dos hebras



Alineamiento: Partidor comúnmente son usados ~20 nucleótidos.

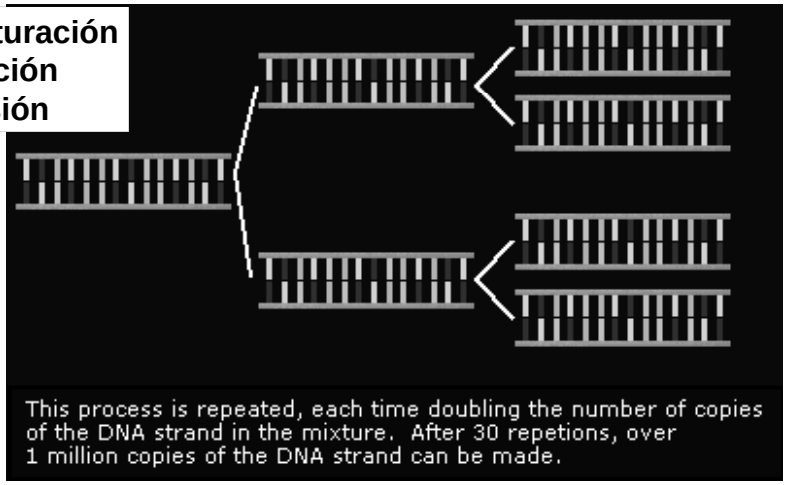


Extensión: La polimerasa agrega dNTPs uno a la vez



Repetición de ciclos

Desnaturación
Alineación
Extensión



Número promedio de ciclos = 30

$2^{30} = 1.07 \times 10^9$ copias

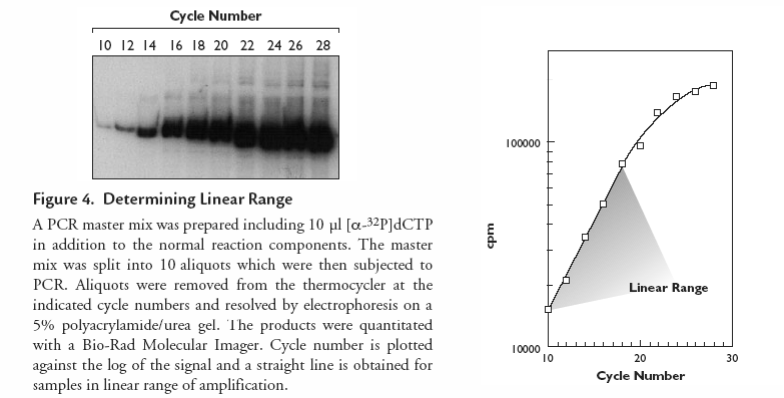
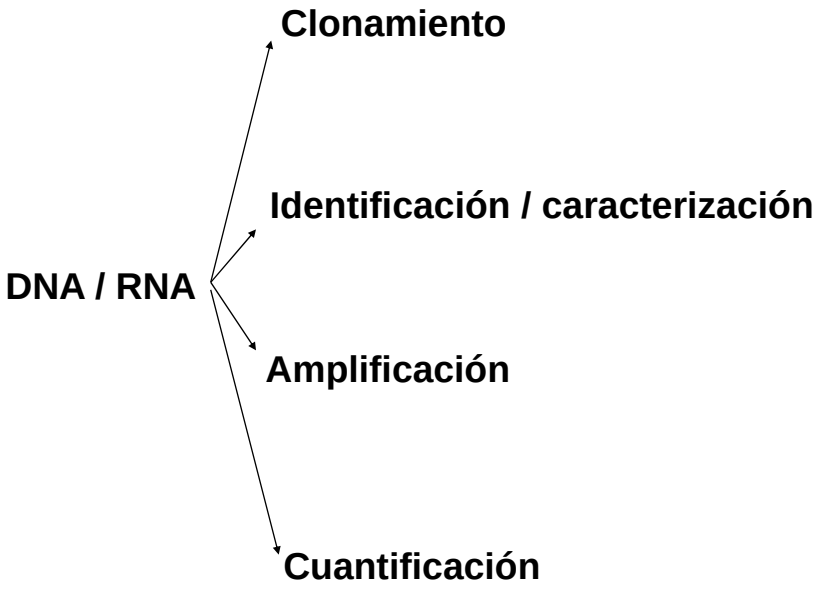


Figure 4. Determining Linear Range

A PCR master mix was prepared including 10 µl [α-^{32}P]dCTP in addition to the normal reaction components. The master mix was split into 10 aliquots which were then subjected to PCR. Aliquots were removed from the thermocycler at the indicated cycle numbers and resolved by electrophoresis on a 5% polyacrylamide/urea gel. The products were quantitated with a Bio-Rad Molecular Imager. Cycle number is plotted against the log of the signal and a straight line is obtained for samples in linear range of amplification.



Cuantificación de DNA / RNA

- Southern y Northern Blot
- Microarray
- RT-PCR en tiempo real

Transcriptasa Reversa y Reacción en Cadena de Polimerasa (RT-PCR)

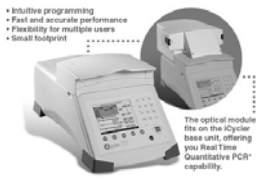
Convencional

- **RNA extraction**
- **Reverse Transcription**
- **cDNA to PCR rx**
- **PCR reaction**
- **Electrophoresis/ Dot Blots/imaging**
- **Image analysis**

Real-time

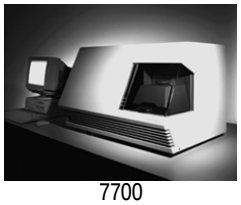
- RNA extraction
- Reverse Transcription
- cDNA to PCR rx
- Real-time PCR

hardware real-time real-time real-time real-time PCR



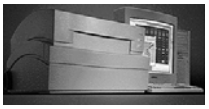
iCycler
BioRad

5700
Applied Biosystems



7700
Applied Biosystems

FluorTracker
Stratagene



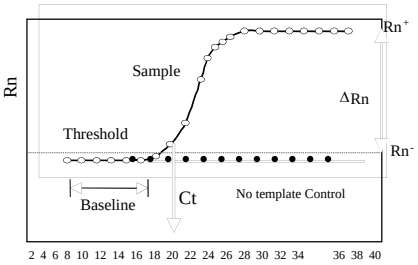
FluorImager
Molecular Dynamics

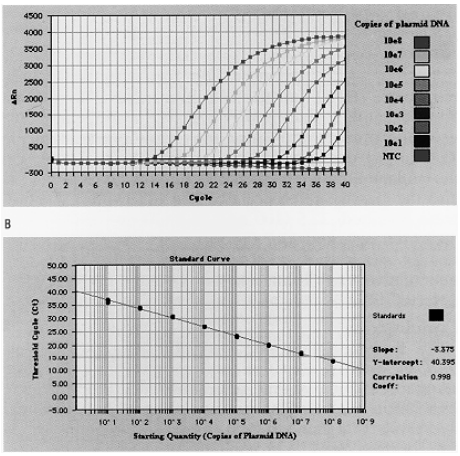
LightCycler
Roche

Opticon 1
MJ research

PCR en tiempo real

Perfil de Amplificación de cDNA





PCR en tiempo real

Los productos de amplificación se observan a medida que transcurren los ciclos del PCR

Esta basado en:

✓ **La detección y cuantificación de un *reportero* fluorescente, cuya señal aumenta en proporción directa a la cantidad de producto de PCR en la reacción.**

✓ **El empleo de un termociclador que tiene acoplado un sistema de detección que es capaz de adquirir y cuantificar la señal emitida por el *reportero* al final de cada ciclo para cada muestra.**