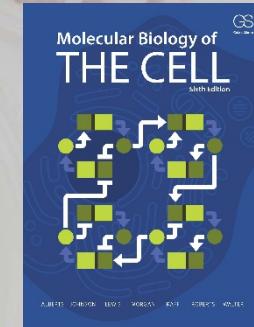


Lecture 3

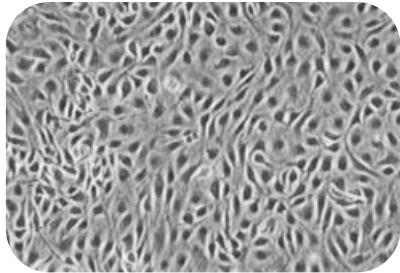
Methods to study cell biology Outline

1. Cell cultures: How to obtain pure cell culture?
2. How are cells cultured?
3. What are cells composed of?
4. How to study the functions of these components in the cell?



Chapter 8

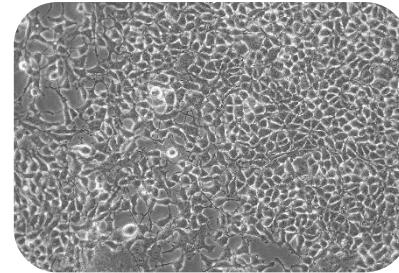
1 Cultivated cells are commonly used to study cell biology



Chinese hamster
ovary (~~COS~~) cells



Tobacco bright
yellow 2 (BY2) cells

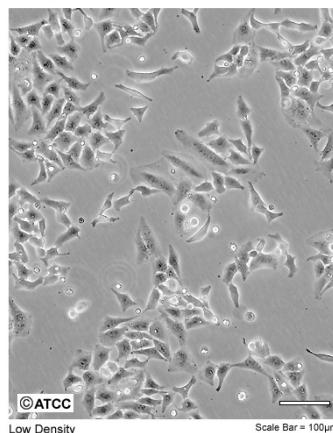


Human embryonic
kidney (HEK) cells



Henrietta Lacks
(1920–1951)

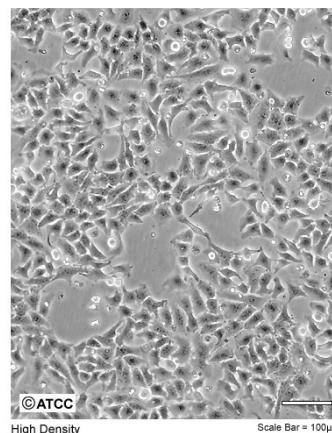
ATCC Number: CCL-2
Designation: HeLa



©ATCC

Low Density

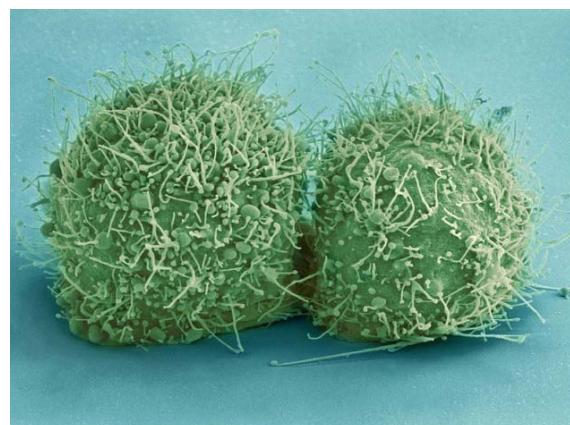
Scale Bar = 100µm



©ATCC

High Density

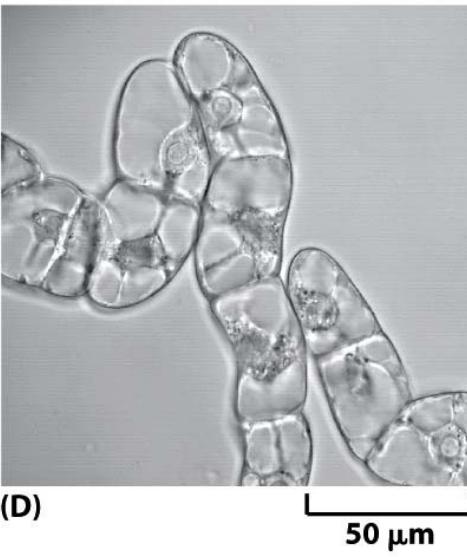
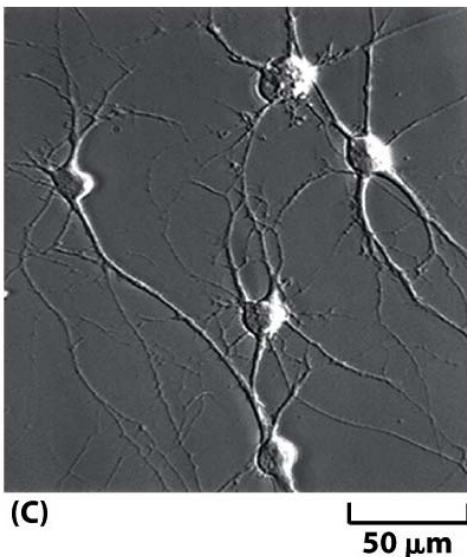
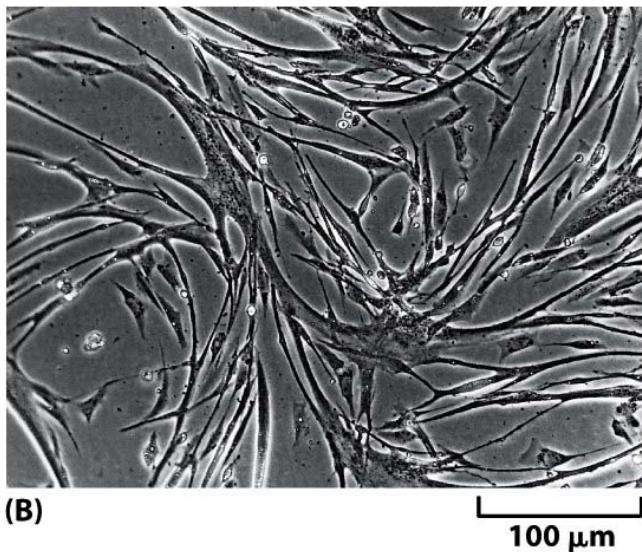
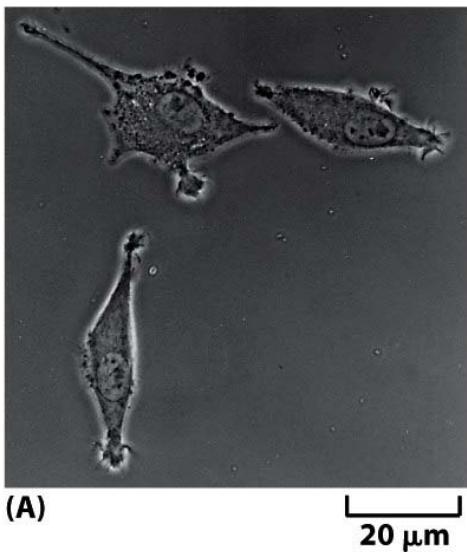
Scale Bar = 100µm



Henrietta Lacks (HeLa) cells
(after division)

- HeLa cells were the first immortalized human cell line (1951) -

Virtually all cells can be grown *in vitro*



- A. **Mouse fibroblasts**
- B. **Chick myoblasts**
(form multinucleate muscle cells by fusing)
- C. **Rat retinal ganglion nerve cells**
- D. **Tobacco BY2 cells**
(suspension culture)
- E. **BY2 liquid culture and callus culture (inset)**

Commonly used cell lines

CELL LINE*	CELL TYPE AND ORIGIN
3T3	fibroblast (mouse)
BHK21	fibroblast (Syrian hamster)
MDCK	epithelial cell (dog)
HeLa	epithelial cell (human)
PtK1	epithelial cell (rat kangaroo)
L6	myoblast (rat)
PC12	chromaffin cell (rat)
SP2	plasma cell (mouse)
COS	kidney (monkey)
293	kidney (human); transformed with adenovirus
CHO	ovary (Chinese hamster)
DT40	lymphoma cell for efficient targeted recombination (chick)
R1	embryonic stem cell (mouse)
E14.1	embryonic stem cell (mouse)
H1, H9	embryonic stem cell (human)
S2	macrophage-like cell (<i>Drosophila</i>)
BY2	undifferentiated meristematic cell (tobacco)

*Many of these cell lines were derived from tumors. All of them are capable of indefinite replication in culture and express at least some of the special characteristics of their cell's of origin.

Authenticated Cell lines can be obtained from stock centers

Table 1. Cell culture banks

Collection	Web site (web addresses accessed July 2014)
American Type Culture Collection (ATCC)	www.atcc.org
CellBank Australia	www.cellbankaustralia.com
Coriell Cell Repository	http://www.ccr.coriell.org
Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)	www.dsmz.de
European Collection of Animal Cell Cultures (ECACC)	www.phe-culturecollections.org.uk/
Health Science Research Resources Bank (HSRRB), Japan	www.jhsf.or.jp/English/index_e.html
Japanese Collection of Research Bioresources (JCRB)	http://cellbank.nihs.go.jp
NIH Stem Cell Unit	http://stemcells.nih.gov/research/nihresearch/scunit/
RIKEN Gene Bank	http://en.brc.riken.jp
UK Stem Cell Bank (UKSCB)	www.ukstemcellbank.org.uk/
WiCell	www.wicell.org



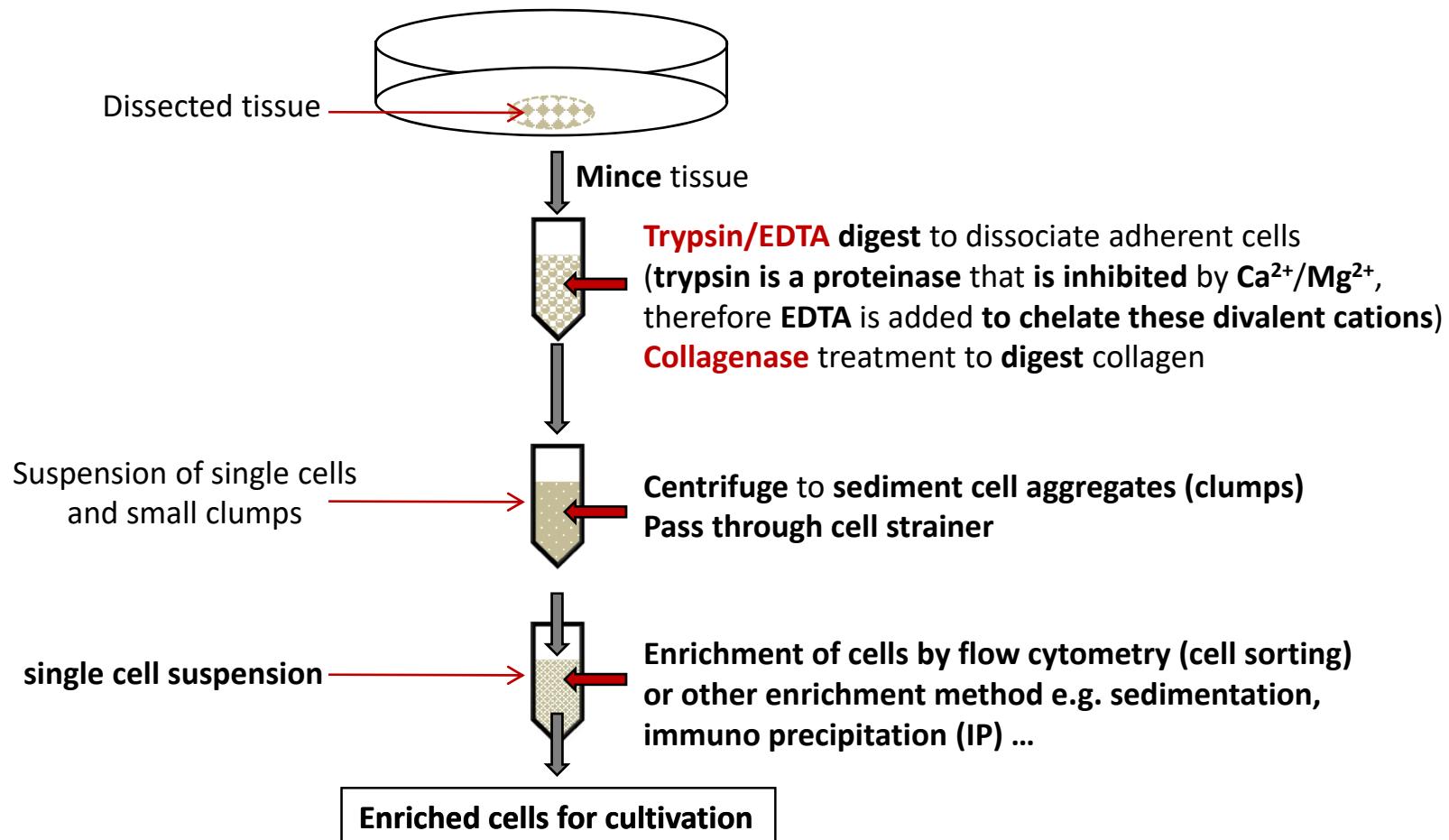
Mission

To acquire, authenticate, preserve, develop, standardize, and distribute biological materials and information for the advancement and application of scientific knowledge.

ATCC serves by characterizing cell lines, bacteria, viruses, fungi and protozoa.

- 4,000 human and animal cell lines and 1,200 hybridoma.
- 18,000 strains of bacteria from 900 genera.
- 2,000 different types of animal viruses and 1,000 plant viruses.
- 49,000 yeast and fungi strains from 1,500 genera and 2,000 strains of protists.

How to start a cell culture?

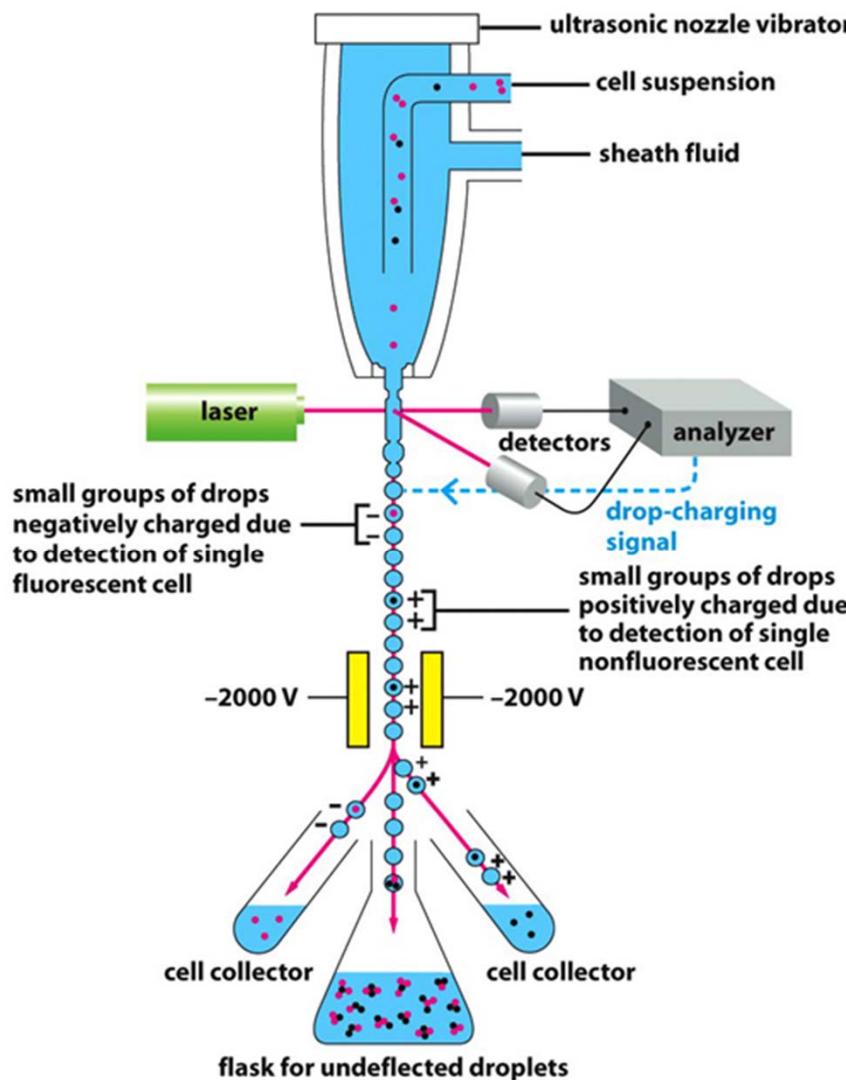


This procedure is also for plants **but cellulase** and **mazerozyme** is used instead of trypsin and collagenase, to digest the cell wall
First you have to isolate cells from tissues...

General strategies to selectively enrich cells

- **Growth/cultivation** of single cell suspensions in selective cultivation media, which support the outgrowth of the selected single cell type over the others.
(e.g. use of antibiotics after transformation of *E. coli* with plasmids that also contain a selectable marker gene)
- **Physical selection** of the cells:
 - **Fluorescence-activated cell sorting (FACS)**
 - **Immunoprecipitation of whole cells using antibody-conjugated beads**
 - the antibody binds to a specific protein at the cell surface and thus attaches the whole cell to the bead (polydextran, magnetic).
 - The cells are then collected by “pull-down”.
 - **Laser capture microdissection**
 - excision of cells using a laser beam

Fluorescence-activated cell sorting (FACS)

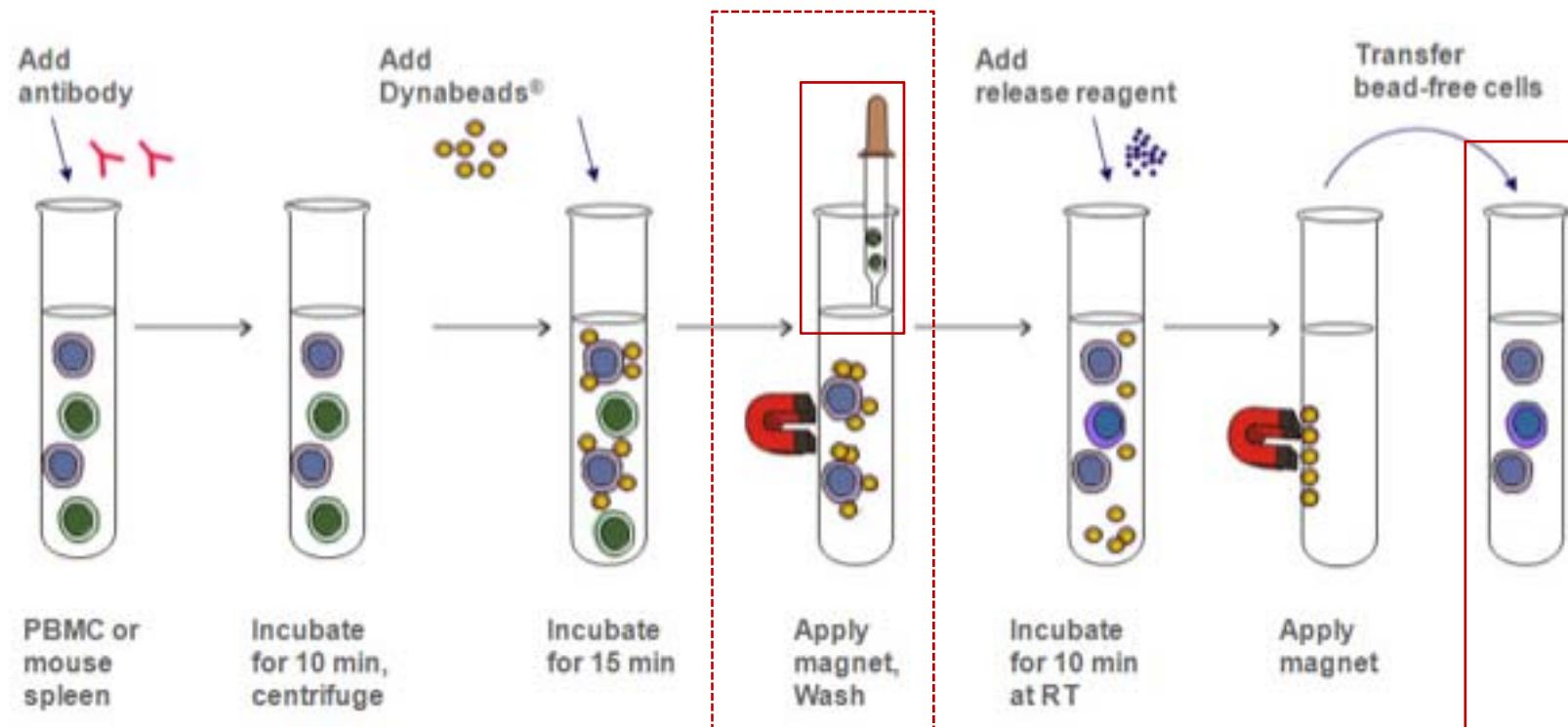


- Prerequisite for sorting is the presence of a fluorescent marker in the cells (e.g. fluorescent dye or fluorescence-conjugated antibody)
- Cells are guided by the **sheath fluid** into the center of the stream to get into the drops (1 cell/drop)
- **Detection of fluorescence** (laser/analyzer)
- Drops are **electrically charged** based the result of analysis of fluorescence.
- Sorting occurs then via deflection of the drop with the cell in an electrical field in different collection tubes

Cell sorting via immunoprecipitation using “magnetic beads”

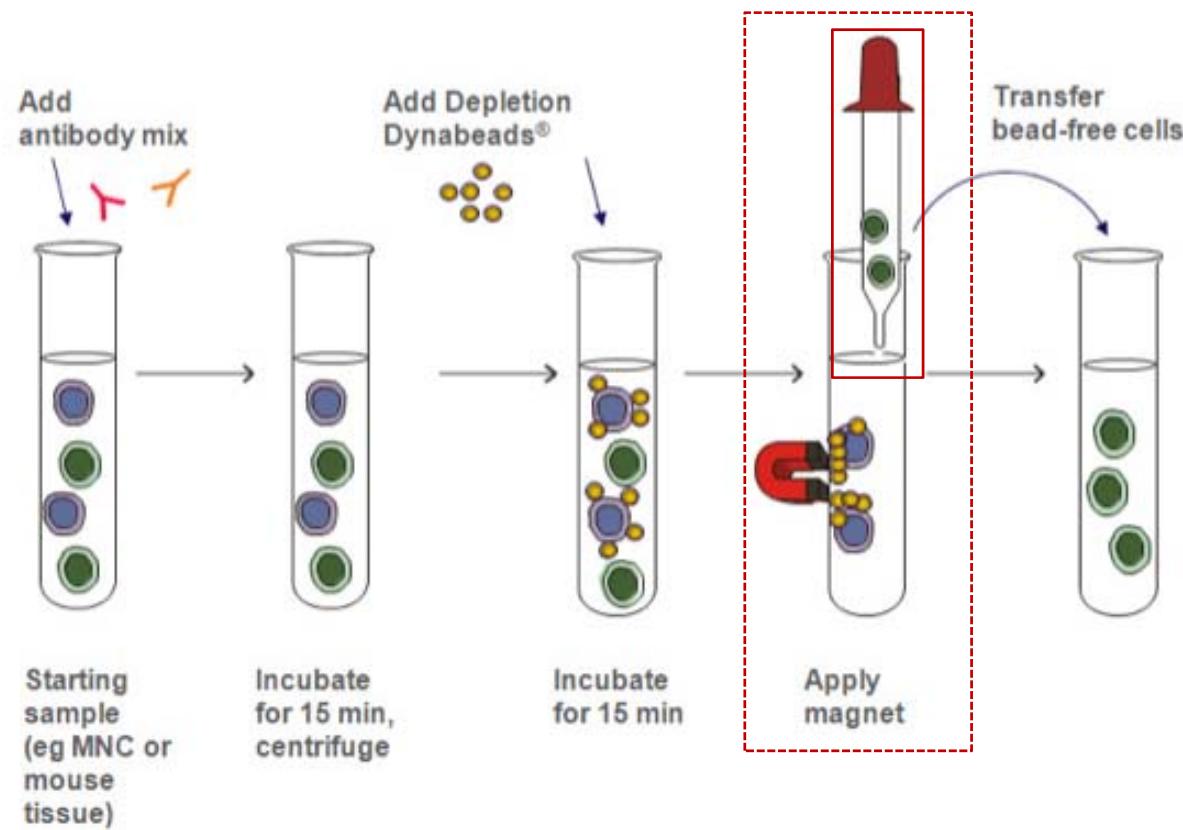
Positive isolation: You immobilize the cells you want

by removing all the other cells



Cell sorting via immunoprecipitation using “magnetic beads”

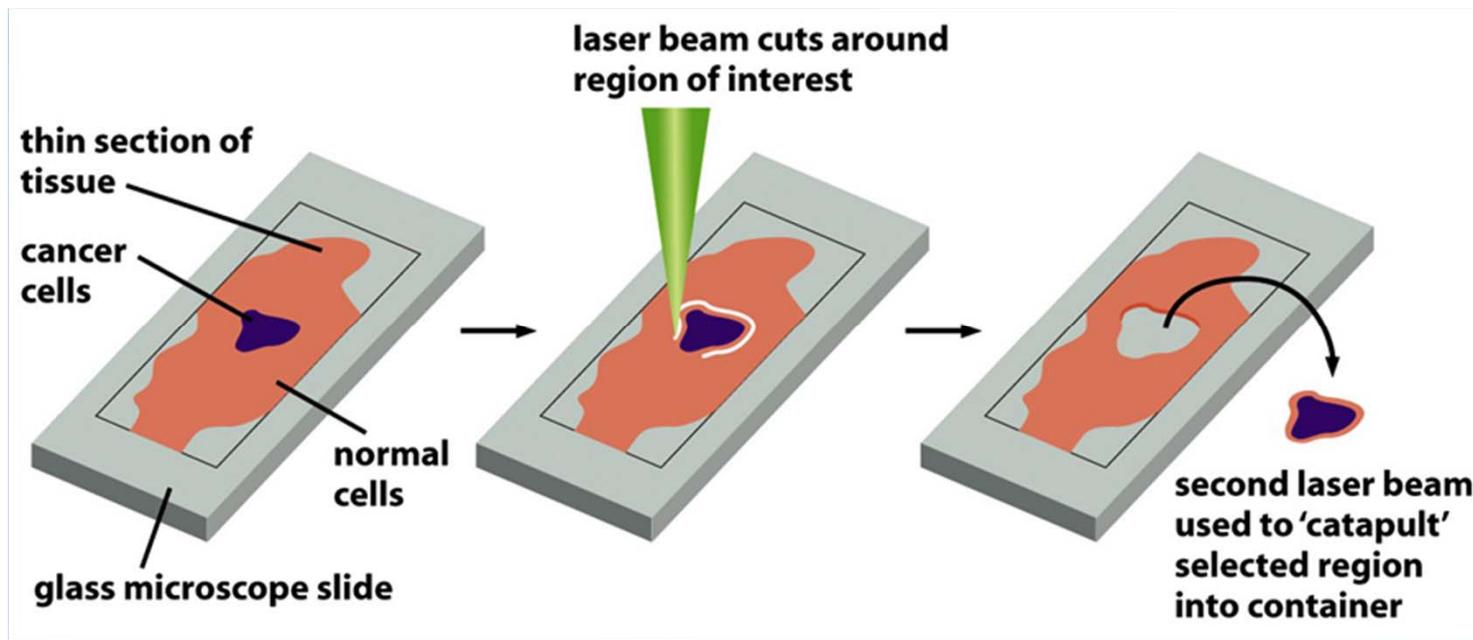
Negative isolation: You immobilize all of the unwanted cells and transfer the wanted “untouched” cells



Example:
Isolation of T cells from thymus:

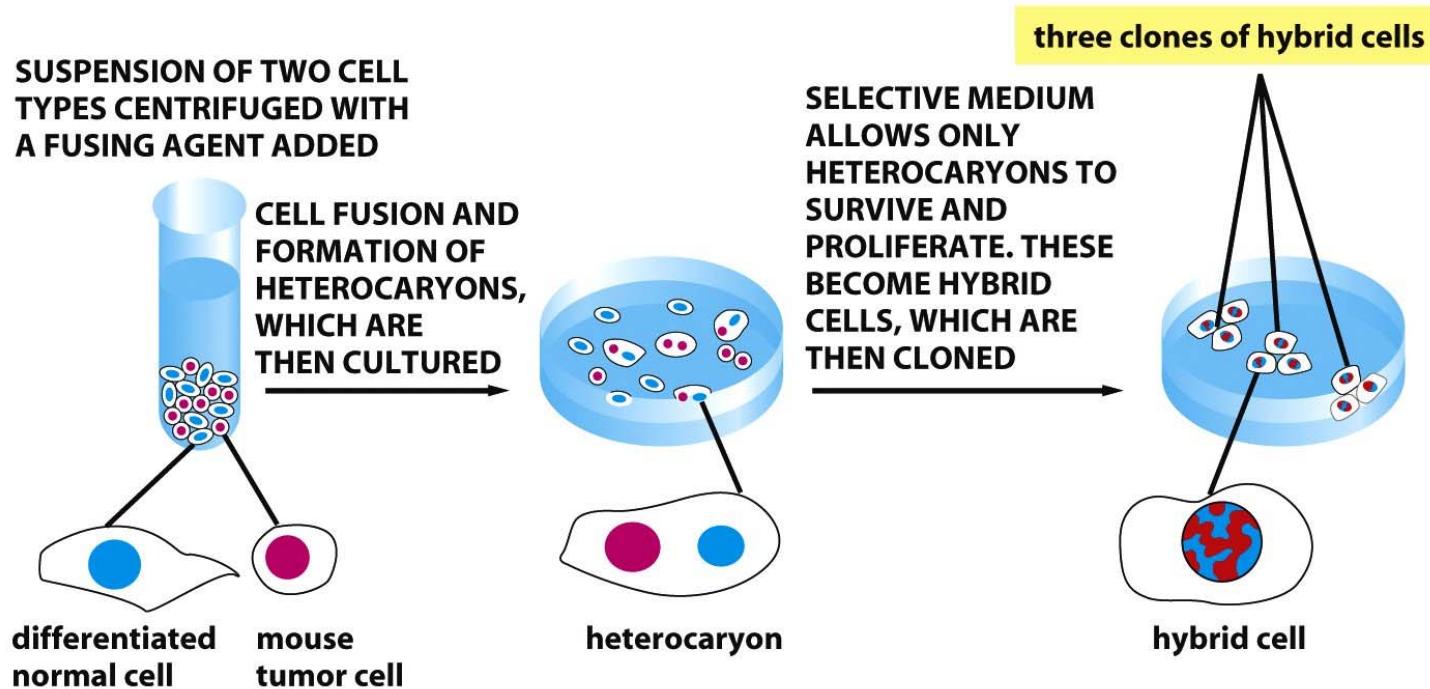
- Homogenize thymus tissue
- Add CD3-conjugated magnetic beads
- Immobilize cells by magnetic force
- Wash immobilized cells
- Mission complete!

Laser capture microdissection



Generation of a “new” cell line by fusion of different cells

Hybridoma cells: fusion of a **differentiated cell** with a **tumor cell**

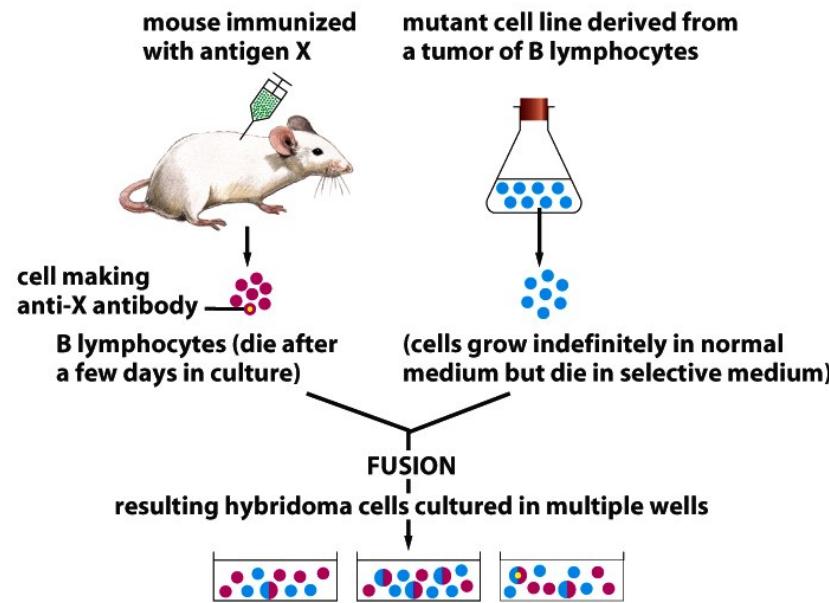


Different types of cells can be fused together to display properties from the two sides...

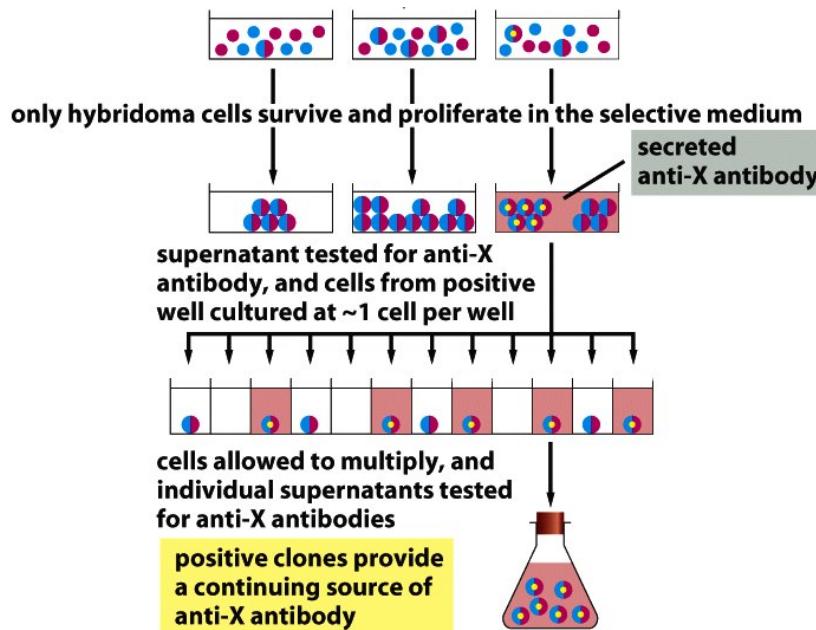
Fusion with a tumor cell line results in immortal cells (immortalization)...

Generation of monoclonal antibodies in hybridoma cells

Generate antibody-secreting B lymphocytes by immunization of a mouse and fuse these cells with B lymphocyte tumor cells to generate a hybridoma line, an immortal, antibody-secreting cell line!!!



Next, screen the individual hybridoma to get the line that produces the best antibody...



All commercially available monoclonal antibodies are produced in hybridoma cells...

Growth & maintenance of cell lines and cell cultures

A basic cell culture media for mammalian cells usually contains:

1. Buffer substance for pH regulation
2. Food source (e.g. glucose, glutamine, amino acids, nucleotides, etc.)
3. Serum/growth stimulant
4. Minerals for metabolic functioning of the cells
5. Antibiotic additives and pH indicator

Some commonly used media:

Dulbecco's modified essential medium (DMEM)
RPMI-1640
McCoy's 5A
F12, etc



A basic cell culture media for plant cells usually contains:

1. Buffer substance for pH regulation
2. Nitrogen source (e.g. NH_4NO_3)
3. Minerals for metabolic functioning of the cells

Commonly used media:

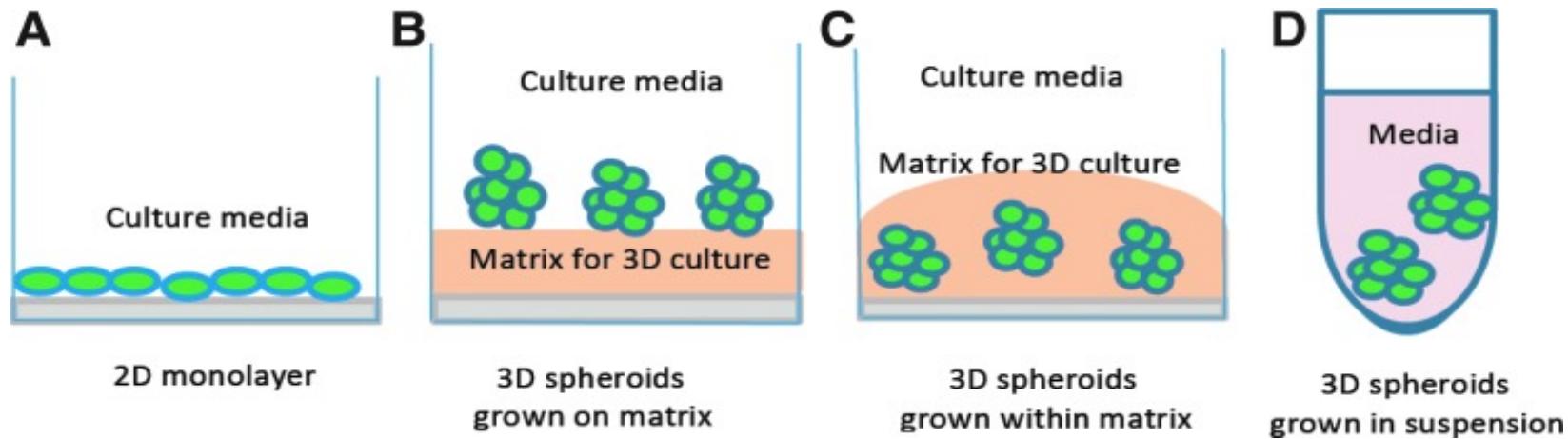
1. Murashige & Skoog (MS) medium in different concentrations
 - MS is perfect for most plants
 - $\frac{1}{2}$ strength is perfect for orchids
 - some cell cultures like up to 2% sucrose

Plant medium very cheap!!!

2-D and 3-D cell culture

3-D cell culture might reflect the *in vivo* condition more accurately.

In the *in vivo* situation, in which cell-cell communication occurs, and cells are embedded in the extracellular matrix, will have effects on cellular activity



Comparison between 2D and 3D culture

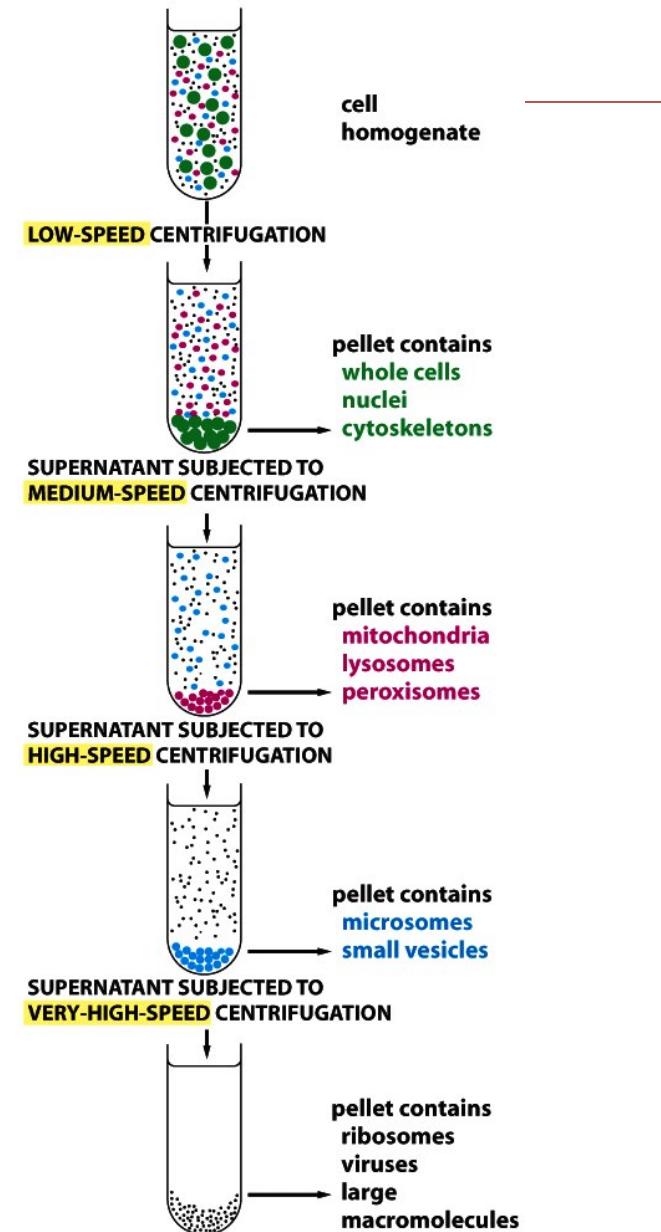
	2D	3D
Cell Shape	Flat and stretched	Natural shape (ellipsoid/polarized) is retained
Cell interface to medium	All cells are equally exposed to media components	As in physiological conditions, there is gradient availability of media components. Upper layer of cells are highly exposed over the lower layer (Heterogeneous exposure)
Cell junction	Cell junctions are less prevalent and does not resemble physiological conditions	Cell junctions are prevalent and enable cell to cell communication.
Cell Differentiation	Moderately and poorly differentiated	Well differentiated
Drug metabolism	Drug metabolism not well observed	Enhanced drug metabolism with increased expression of CYP enzymes
Drug Sensitivity	Cells are sensitive and drugs show high efficacy	Cells often show resistance and drugs show low potency
Cell Proliferation	Higher proliferation rate than in natural environment	Proliferation rate may be high or low, it is based on cell type and 3D-cell culture technique.
Response to stimuli	Poor response to mechanical stimuli of cells	well-established responses to mechanical stimuli of cells
Viability	Sensitive to cytotoxin	Greater viability and less susceptible to external factor
Apoptosis	Highly susceptible to drug-induced apoptosis	Enhanced resistance to drug-induced apoptotic stimuli

Adapted from Sigma/Aldrich

Subcellular fractionation: Separation in functional fractions

Subcellular fractionation via differential centrifugation :

- Isolation of intracellular compartments:
 - Nuclei
 - ER
 - Golgi apparatus
 - Mitochondria
 - Plastids
 - Transport vesicles
 - PM
 - Ribosomes
- Combination of isopycnic and rate-zonal centrifugation steps



4. How to study cell biology? But what do we want to study?

Remember the main objectives?



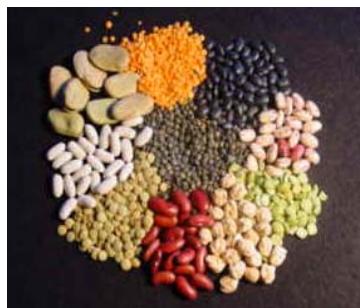
Differentiation & development

- organ development
- neuro biology
- cancer biology
- stem cell research



Stress physiology

- cell-cell interaction
- signaling
- environmental cues
- detoxification



Food production

- food safety
- renewable energy

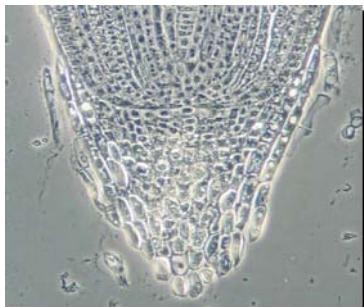


Biotechnology

- molecular pharming
- pharmaceuticals

4. How to study cell biology? But what do we want to study?

Remember the main objectives?



Differentiation & development

- organ development
 - neuro biology
 - cancer biology
 - stem cell research



Stress physiology

- cell-cell interaction
 - signaling
- environmental cues
 - detoxification



Food production

- food safety
- renewable energy



Biotechnology

- molecular pharming
- pharmaceuticals

- **Regulation**
(genes & proteins)

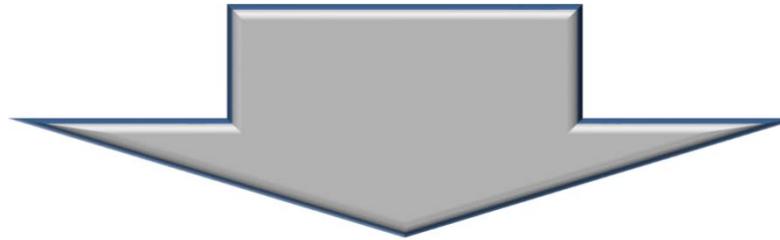
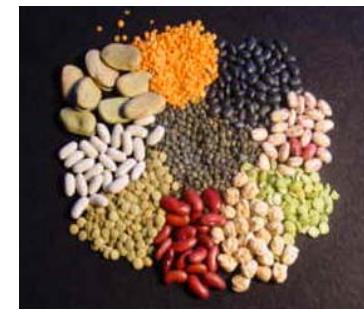
- **Membrane dynamics**
(proteins & lipids)

- **Protein-protein interaction**

- **Selective transport**
(proteins & molecules between compartments & across membranes)

4. How to study cell biology? But what do we want to study?

Remember the main objectives?



It is all about: Proteins

DNA

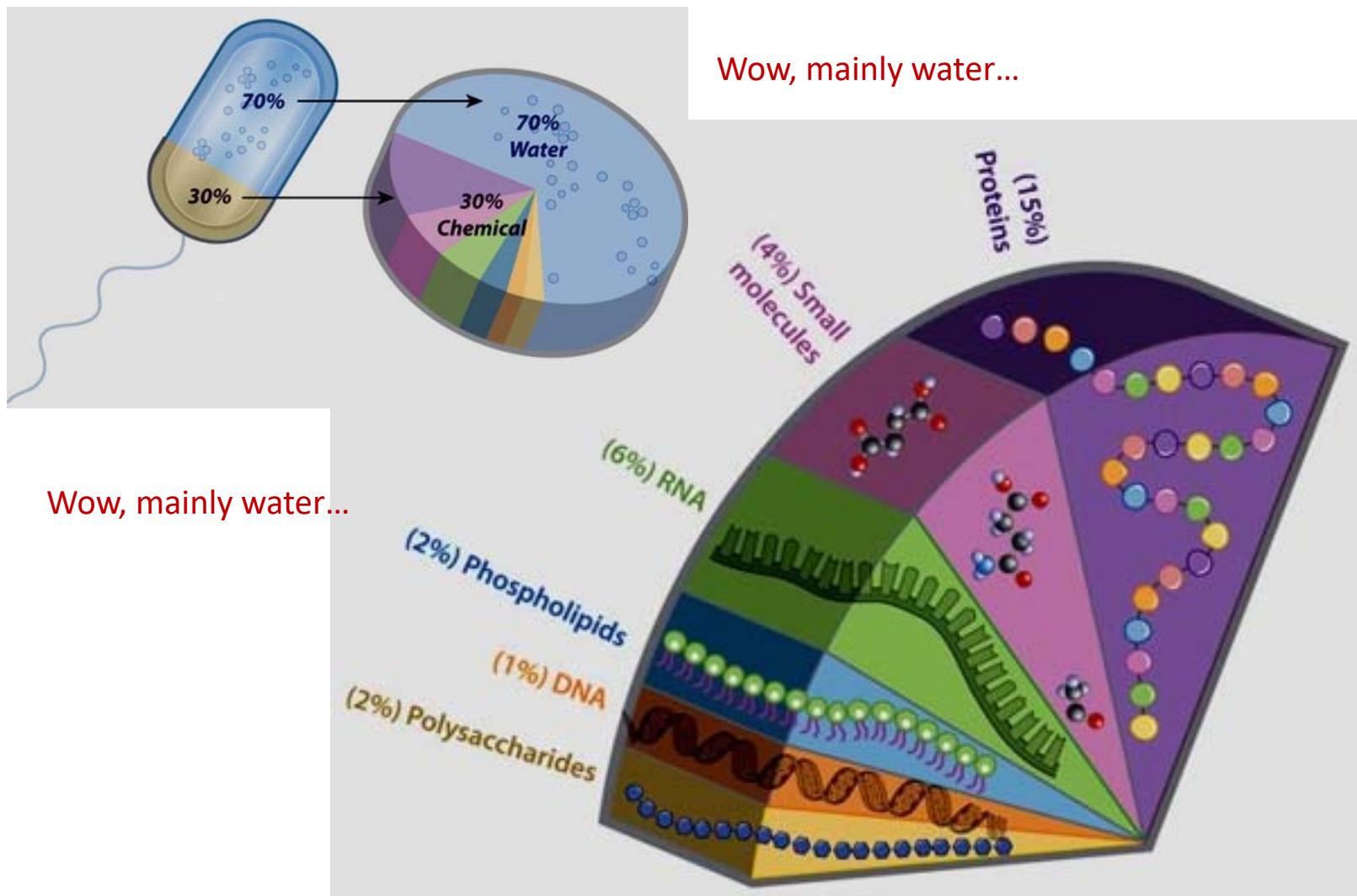
RNA

Lipids

Metabolites

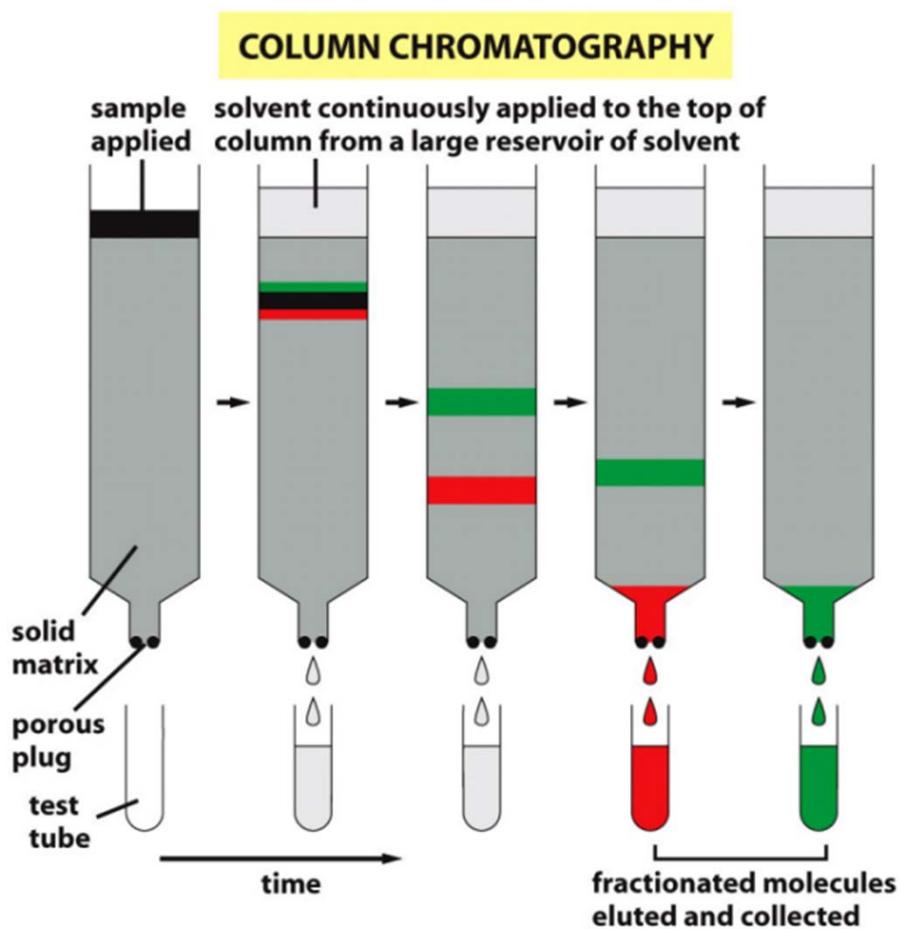
3 The composition of cells

Chemical Components of a cell



4 Proteins can be isolated by chromatography

Various components travel at different rates and can be separated...



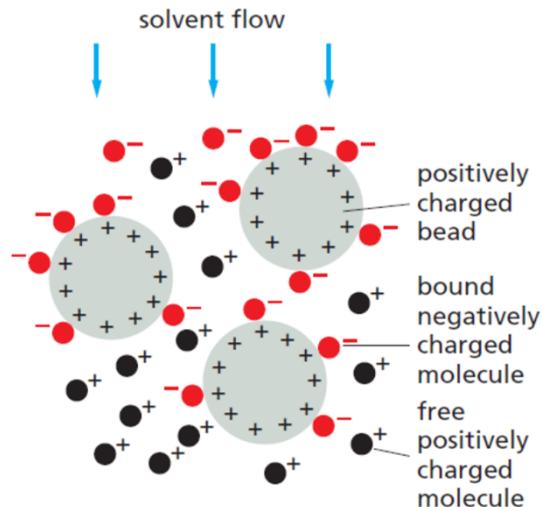
Different types of matrices:

- Gel filtration
- Ion exchange chromatography
- Affinity chromatography

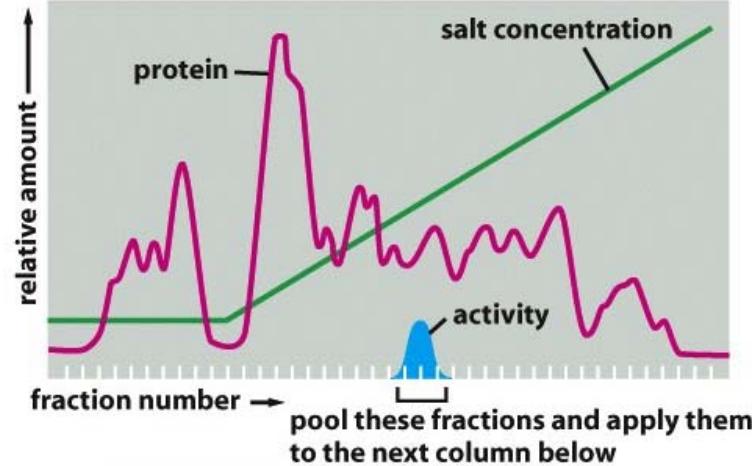
Protein chromatography

Different matrices for different proteins....

Ion-exchange chromatography



(A) ION-EXCHANGE CHROMATOGRAPHY



Principle:

Charged matrix **immobilizes** proteins of **opposite** charge

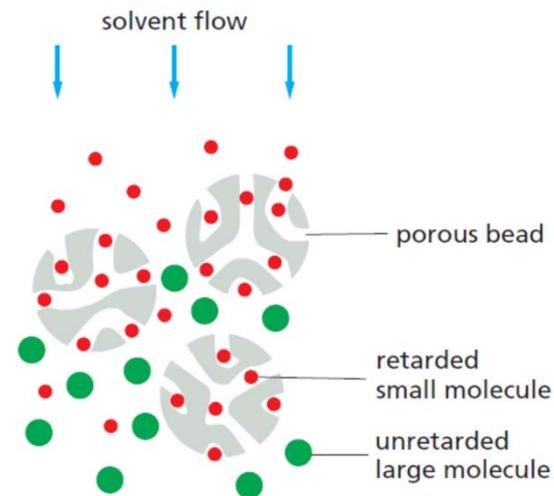
- Strength of association depends on ionic strength and pH and is therefore varied systematically (elution with raising concentrations of salt or pH)

You need a criteria for detection, otherwise you will not find the protein in the fractions
(enzymatic activity or molecular tags (myc, HA, FLAG...))

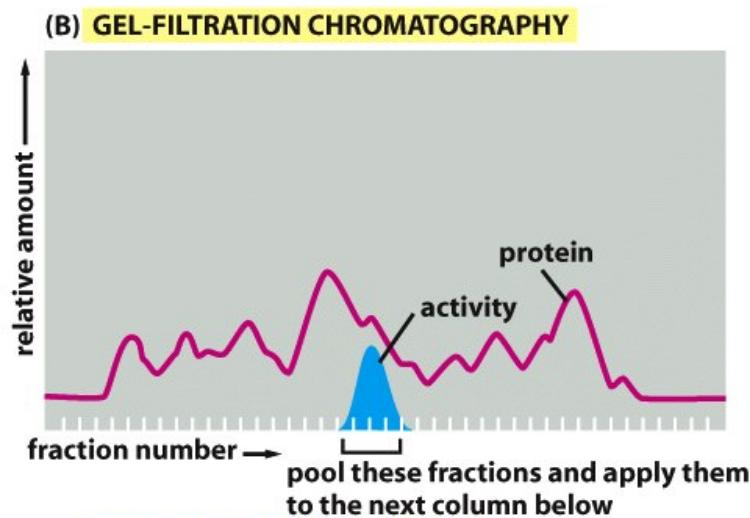
Protein chromatography

Different matrices for different proteins....

Gel-filtration chromatography



(B) GEL-FILTRATION CHROMATOGRAPHY



Principle:

Porous matrix (available with many different sizes of pores)

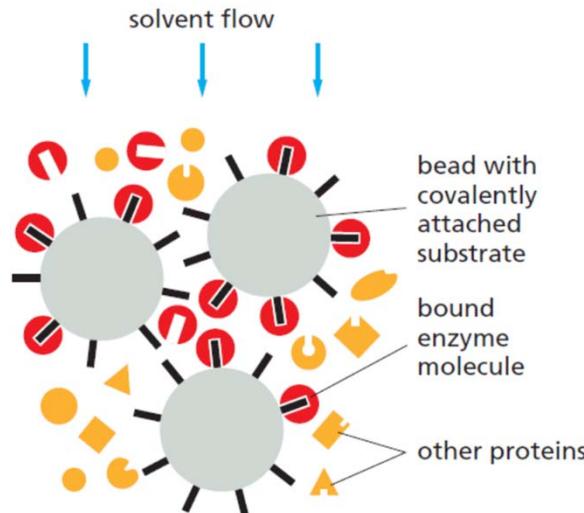
- **Small molecules go into the beads** that is why they are slow; they go from bead to bead. **Large molecules don't enter beads**, they stay in the void and travel fast

You need a criteria for detection, otherwise you will not find the protein in the fractions
(enzymatic activity or molecular tags (myc, HA, FLAG...))

Protein chromatography

Different matrices for different proteins....

Affinity chromatography



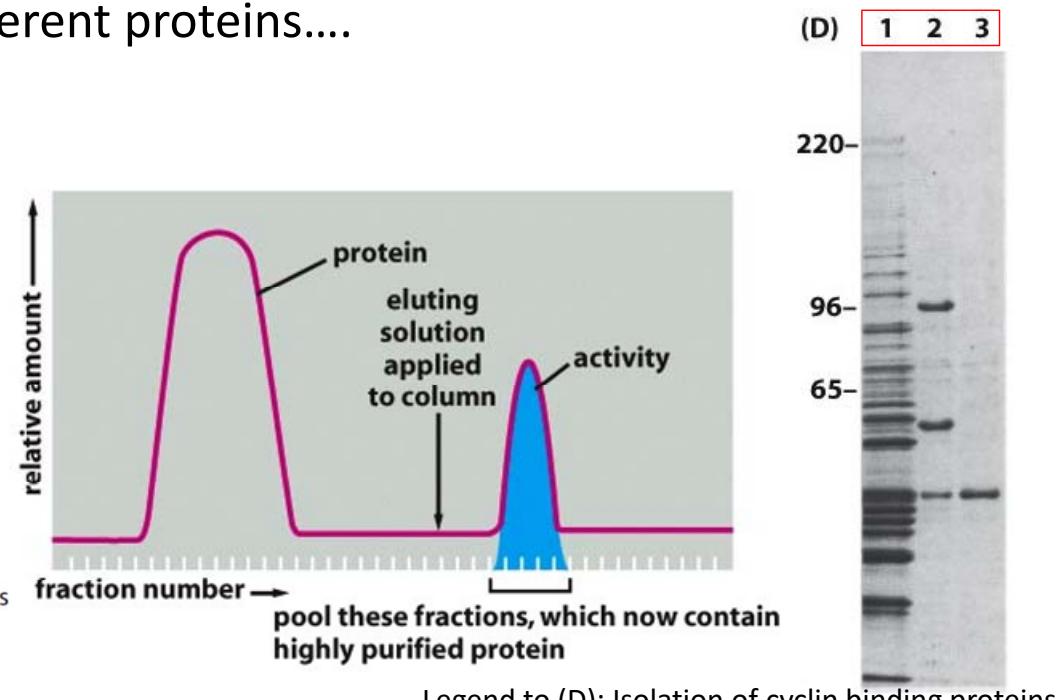
(C) AFFINITY CHROMATOGRAPHY

Principle:

Matrix is coated with either am specific antibody or a substrate, and the protein is immobilized via their direct interaction

- **Elution** requires conditions that dissociate the interaction with antibodies (low/high pH, salt, temperature) and excessive free substrate, respectively

You need a criteria for detection, otherwise you will not find the protein in the fractions (enzymatic activity or molecular tags (myc, HA, FLAG...))

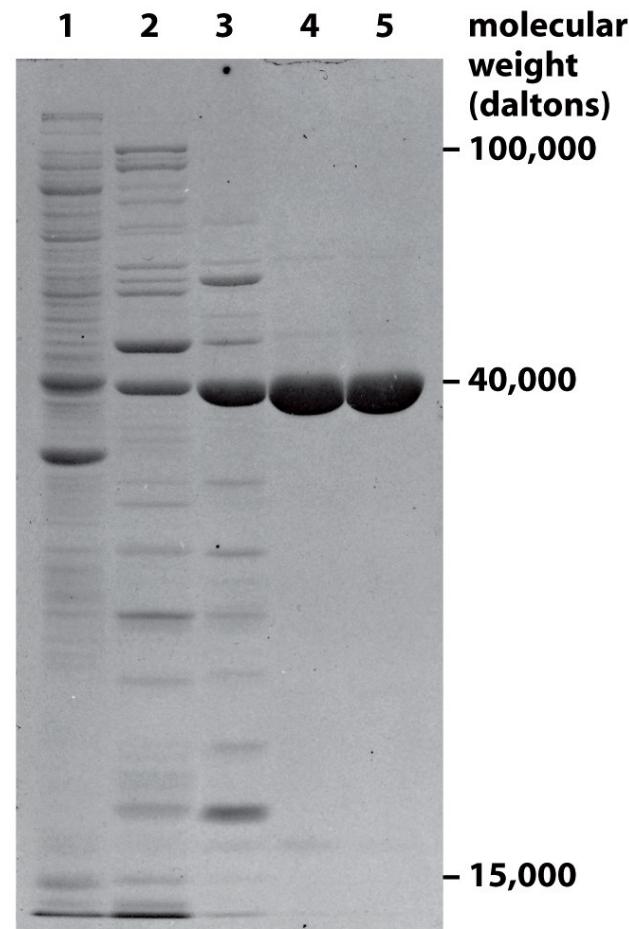
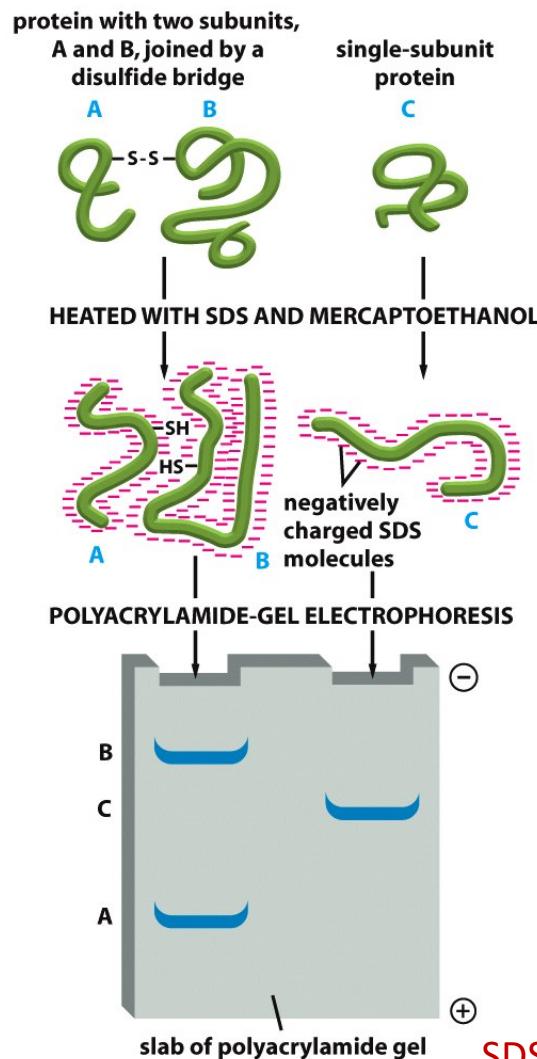


Legend to (D): Isolation of cyclin binding proteins

- 1: total protein extract from cells
- 2: elution from affinity column using cyclin B2
- 3: elution from affinity column using cyclin B3

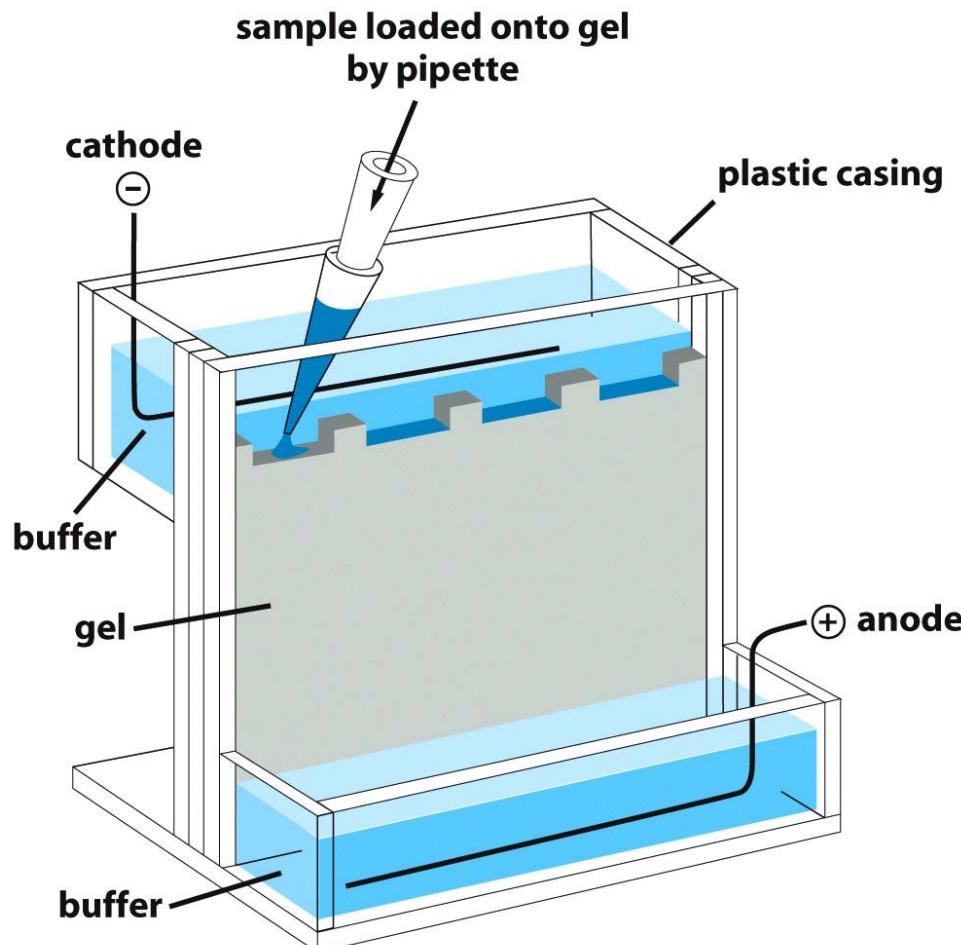
4.2 Protein analysis by SDS-PAGE & Western blot

sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis



SDS-PAGE denatures the proteins - no native proteins anymore!!!

Protein analysis by SDS-PAGE

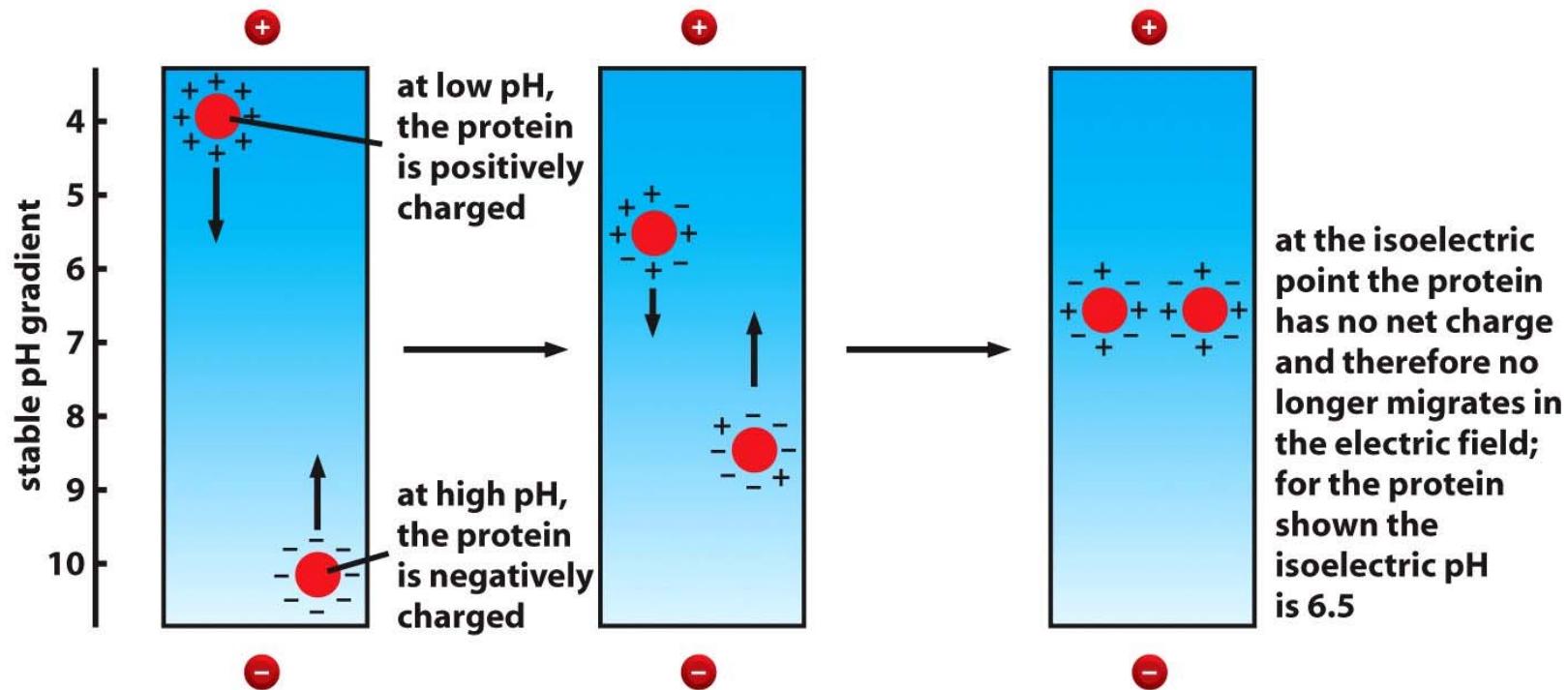


- The gel usually consists of two parts:
 - stacking gel
 - separation gel
- Acrylamide forms a matrix with defined “mesh size”.
- Mesh size is defined by the acrylamide concentration(6-14%) and is chosen according to the size of the proteins.
- Proteins move according to size: the smaller the faster/further

All proteins are negatively charged due to the SDS and move to the anode...

2-D protein analysis

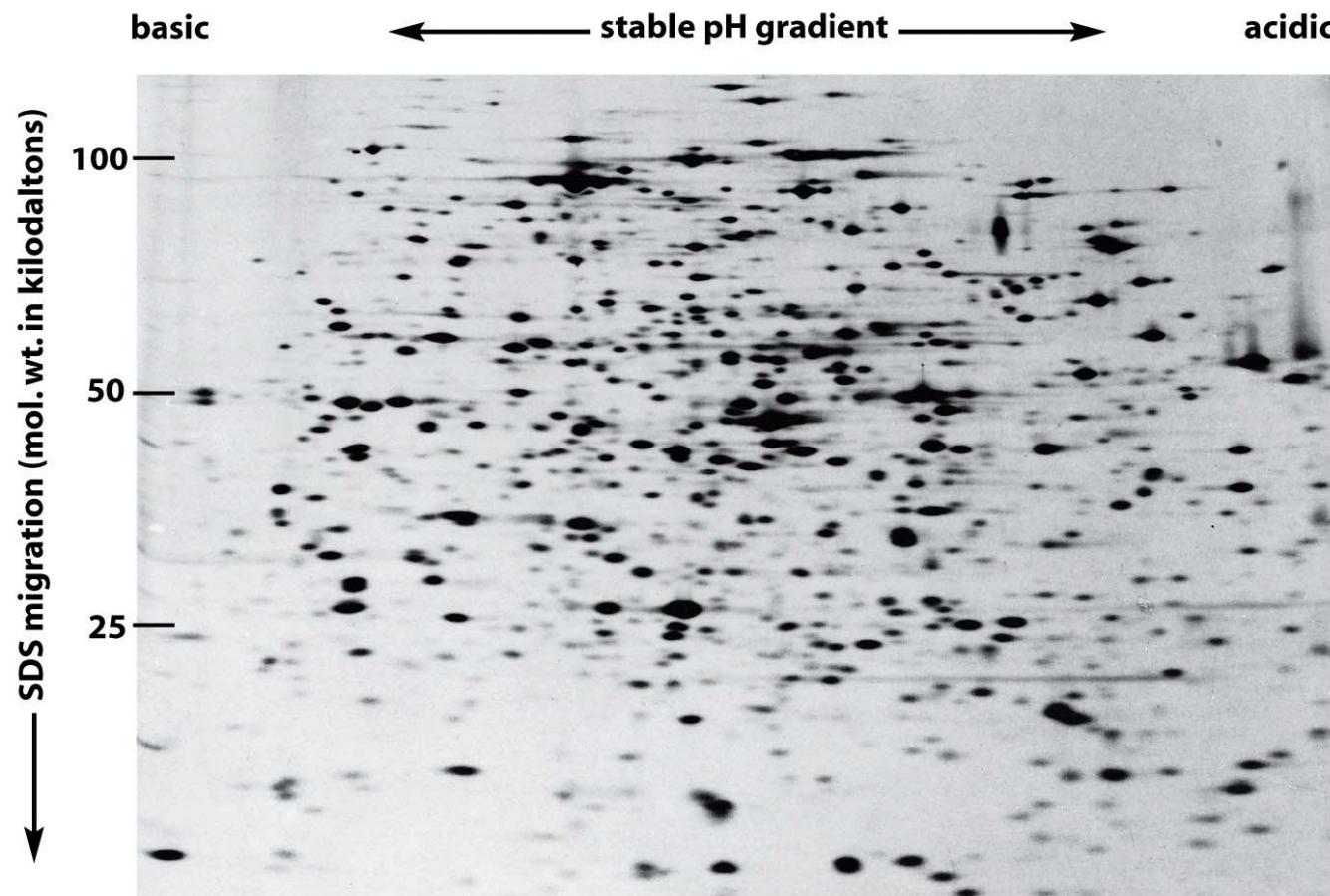
Isoelectric focusing



HERE, the proteins are not denatured by SDS!!!

2-D gel analysis - the second dimension

Most important: FIRST: isoelectric focusing, SECOND: SDS-PAGE



You cannot change the order of these two steps! Guess why....

4.2 How to analyze DNA/RNA?

Agarose-gel electrophoresis: just another matrix

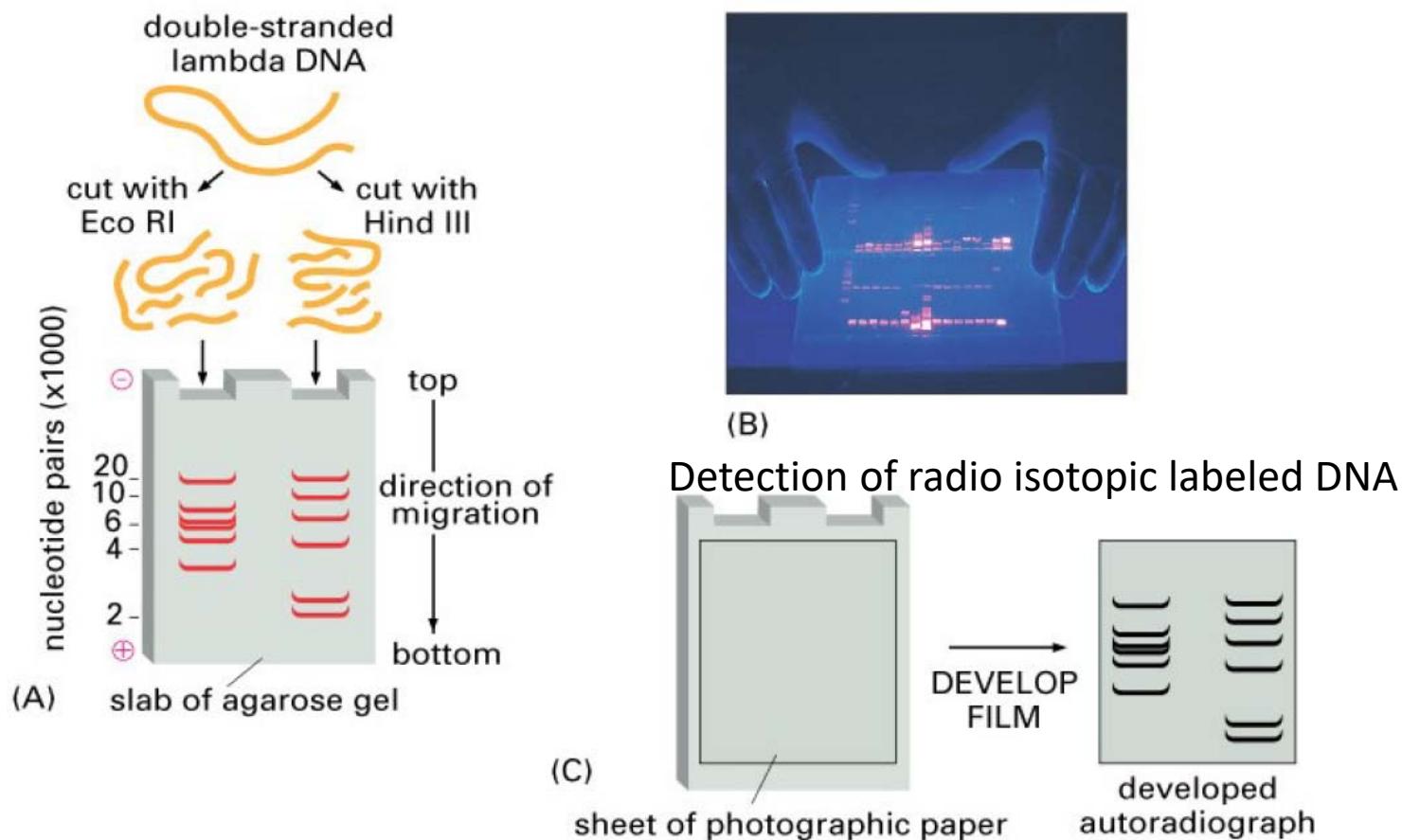
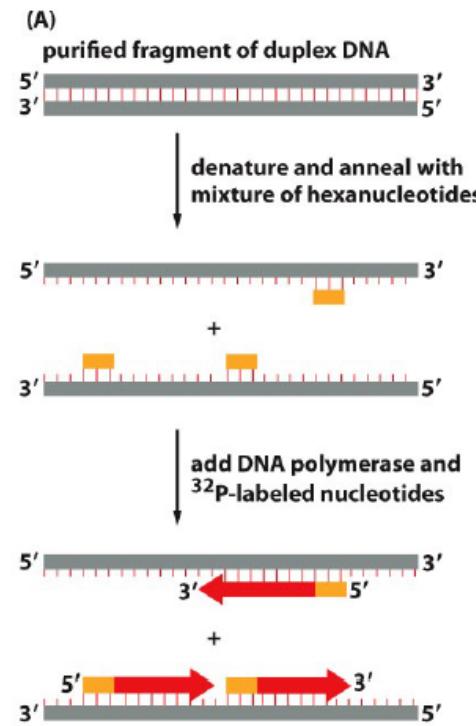


Figure 10-5 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Why are SDS Gels vertical whilst agarose gels are horizontal??? Hahahaha!!!

DNA/RNA labeling: radio isotopes or chemicals

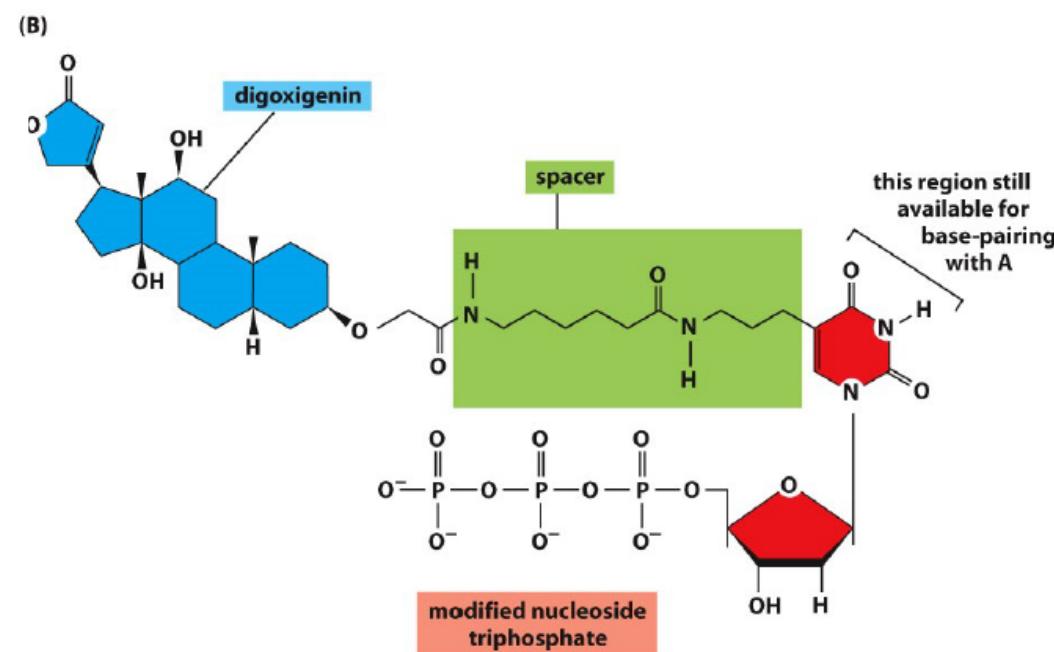
Radio isotope labeling (^{32}P Phosphorus)



DNA polymerase incorporates ^{32}P nucleotides, resulting in a population of radiolabeled DNA molecules that contain sequences from both strands

Figure 8-26 Molecular Biology of the Cell 6e (© Garland Science 2015)

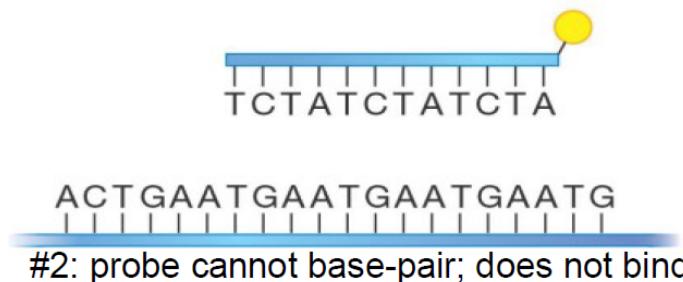
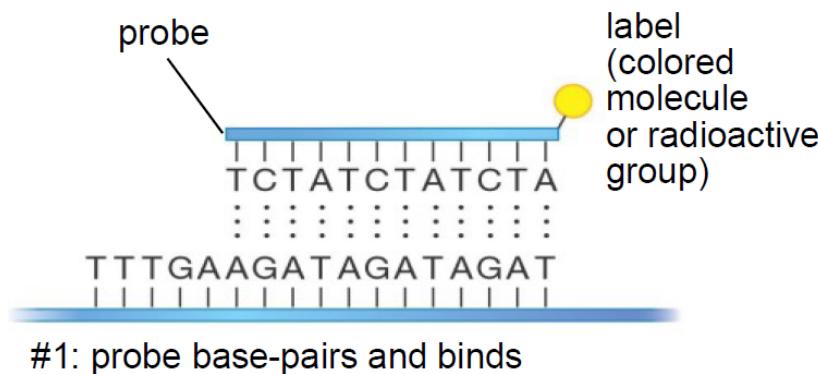
Chemically labeled DNA (digoxigenin)



Digoxigenin labeled DNA is detected by fluorescent dye-conjugated digoxigenin antibodies

Using single strand labeled RNA/DNA as probe

Labeled RNA/DNA is called a probe, which binds specifically to complementary RNA/DNA sequences.



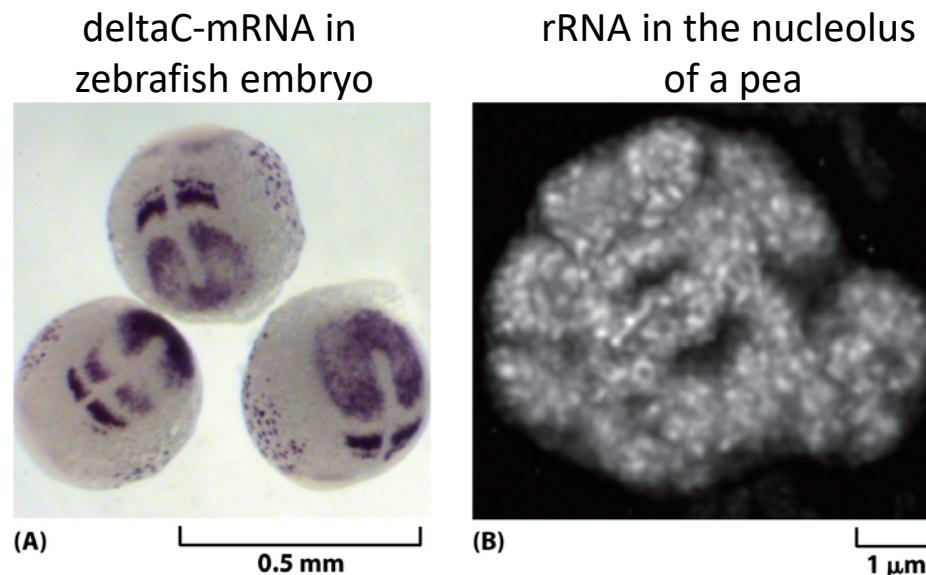
Copyright © 2005 Pearson Prentice Hall, Inc.

Probes are used to search for genes of interest in not yet sequenced organisms

RNA *fluorescent in situ hybridization* targeting (FISH)

RNA FISH is a methodology for detecting and localizing particular **RNA** molecules in fixed cells.

This detection utilizes nucleic acid probes that are complementary to target **RNA** sequences within the cell.



1. Make fluorescence labeled DNA/RNA probe
2. Cells were fixed and RNA can be accessible (permeabilized)
3. Apply the probe on fixed cells to allow hybridization
4. Visualization under fluorescence microscope

FISH works also with DNA: DNA FISH

In situ hybridization to locate specific genes on chromosomes

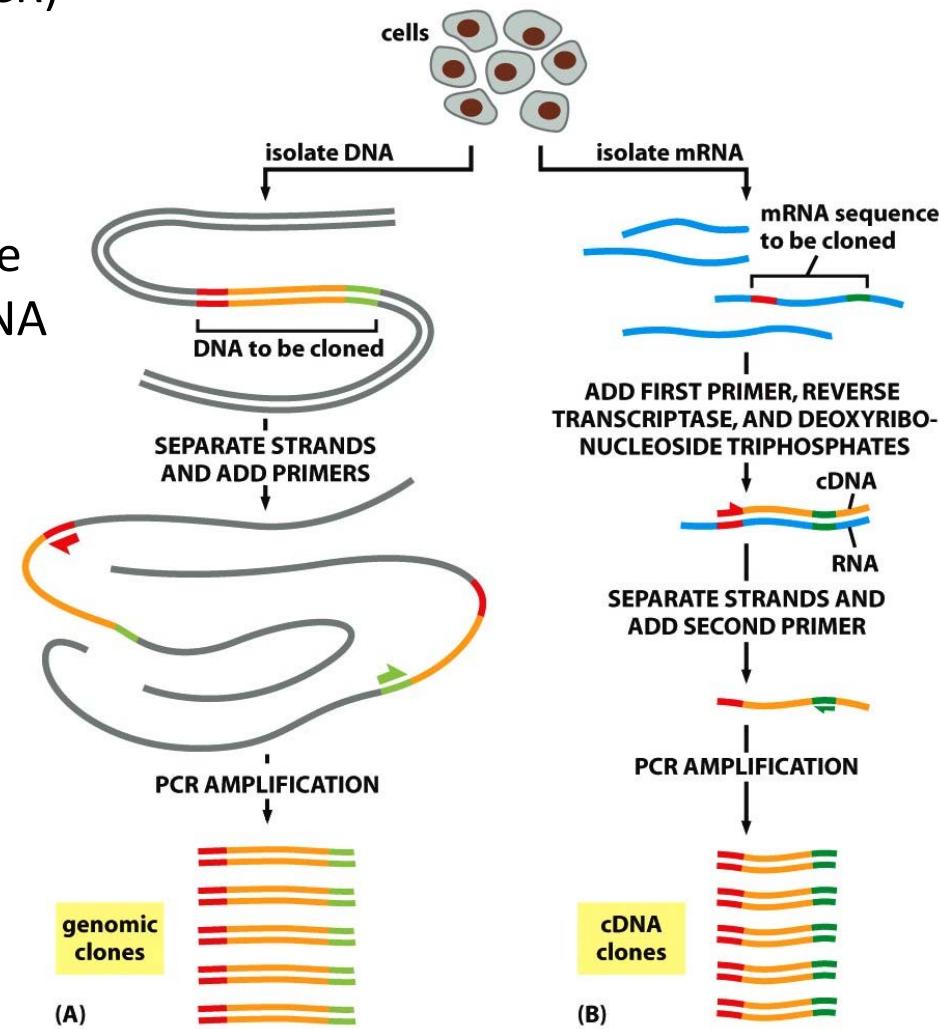


- Parallel use of **six different** probes to label sequences on human chromosome 5
- Both copies of chromosome 5 are shown
- Each probe labels two spots because the **metaphase chromosome has replicated its DNA!**

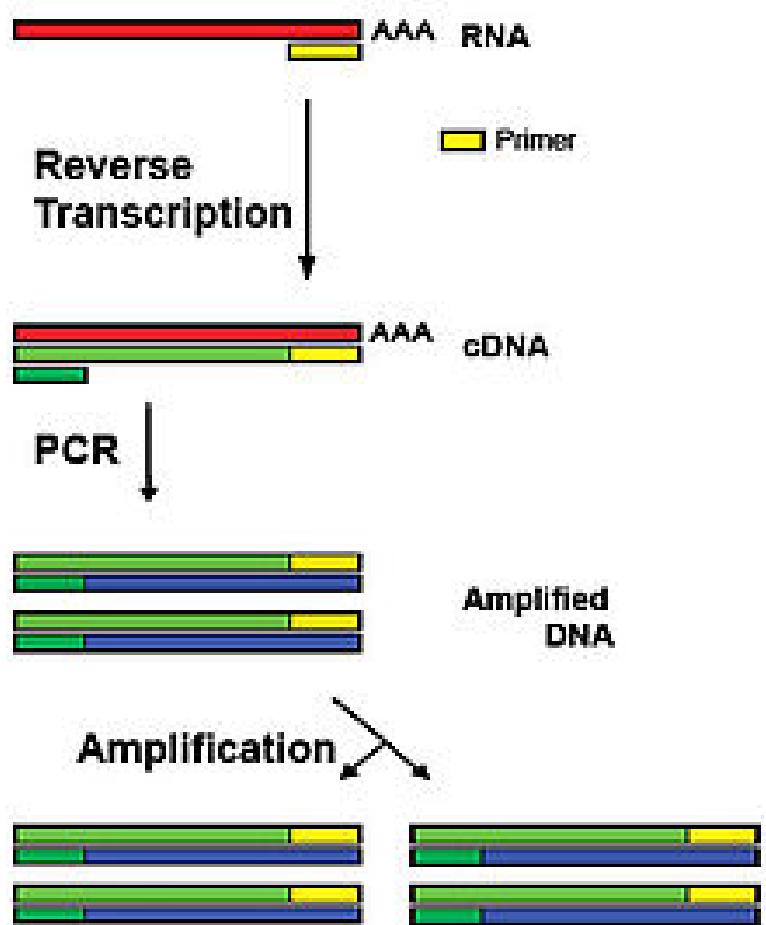
Cloning a specific gene or analyze gene expression levels

Polymerase chain reaction (PCR)

- PCR can be used to clone **genomic sequences** or **cDNA sequences**, depending on the template used: genomic DNA or cDNA



RT-PCR (reverse transcription-PCR)



Procedure:

1. Extract total RNA
2. Reverse transcribe RNA into its complementary DNA (cDNA) with oligo(dT) as primer (or other gene-specific primer)
3. Use cDNA as template, use gene-specific primers to do PCR
4. Analyze PCR products by SYBR green incorporation or by agarose gel analysis

RT-PCR, technique commonly used in molecular biology to detect **RNA expression**.

RT-PCR is often confused with real-time polymerase chain reaction (**qPCR**) but they are separate and distinct techniques.:.

RT-PCR is used to qualitatively detect gene expression through the creation of complementary DNA (cDNA) transcripts from RNA.

qPCR is used to quantitatively measure DNA amplification, using fluorescent dyes.

qPCR is also referred to as quantitative PCR, quantitative real-time PCR, & real-time quantitative PCR.

Cell free system (*In vitro* systems)

Cell free systems are frequently used for three main reasons:

- Identification of the minimal requirements for a specific process to occur
 - Isolation of a specific process from the cellular context (manipulations could be lethal)
 - Elimination of influences from other cellular components/processes and to provide direct evidence for specific functions
-
- An *in vitro* system consists of pure biomolecules, cell homogenates or cell extracts/fractions that are needed to catalyze a biological process
 - Processes that have been analyzed using *in vitro* systems are:
 - DNA replication,
 - DNA transcription,
 - Protein translation,
 - RNA splicing, etc.
 - Vesicle-mediated protein transport