蛋白質體學技術簡介

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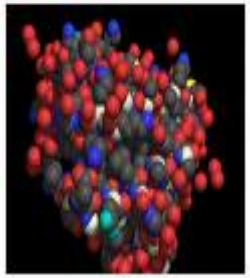
Three properties of proteins

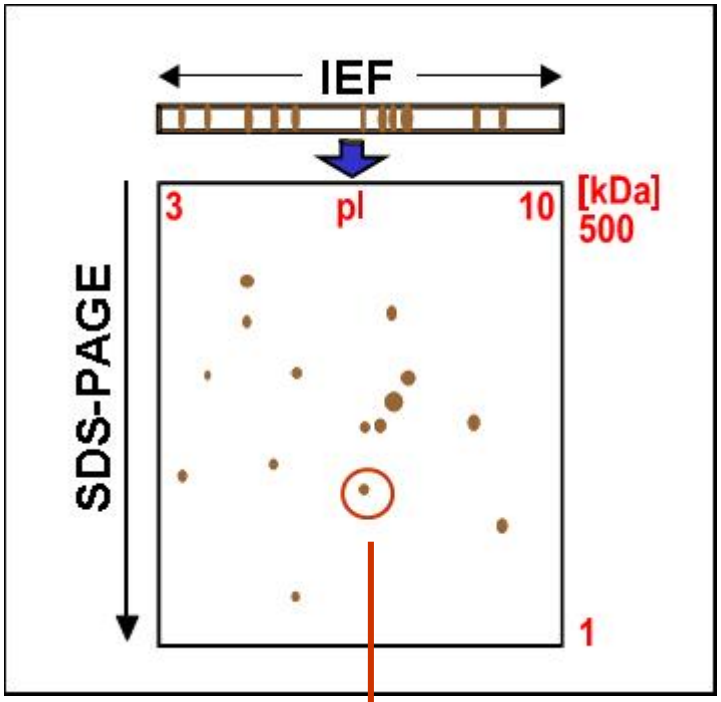
- **Size:** molecular weight (utilized in 2-DE)
- **Charge:** pI (utilized in 2-DE)
- **Hydrophobicity**

Name:
human myoglobin (MYG_HUMAN)



MW: 17183.8 Da
pI: 7.14
Hydrophobicity: -0.467 (gravy score)

What is 2-DE?



1. First dimension:
denaturing isoelectric focusing
separation according to the pI

2. Second dimension:
SDS electrophoresis (SDS-PAGE)
Separation according to the MW

Interested spot

Digest to peptide fragment → **MS analysis**

Two dimensional electrophoresis, 2-DE

- Only “Proteomics” is the large-scale screening of the proteins of a cell, organism or biological fluid, a process which requires stringently controlled steps of sample preparation, 2-D electrophoresis, image detection and analysis, spot identification, and database searches.

- The core technology of proteomics is 2-DE

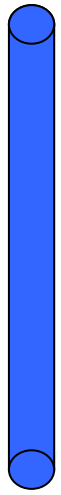
- At present, there is no other technique that is capable of simultaneously resolving thousands of proteins in one separation procedure.

Evolution of 2-DE methodology

Traditional IEF:

- IEF in run in thin poly-acrylamide gel rods in glass or plastic tubes.
- Gel rods containing: 1. urea, 2. detergent, 3. reductant, and 4. carrier ampholytes (form pH gradient).
- Problem: 1. tedious. 2. not reproducible.

In the past



Evolution of 2-DE methodology

The long life SDS-PAGE :

- This “O’Farrell” techniques has been used for 20 years without major modification.

- 20 x 20 cm have become a standard for 2-DE.

  - Assumption: 100 bands can be resolved by 20 cm long 1-DE.
  - Therefore, a 20 x 20 cm gel can resolved 100 x 100 = 10,000 proteins.

Evolution of 2-DE methodology

Problems with traditional 1st dimension IEF

- Works well for native protein, not good for denaturing proteins, because:

OPERATOR DEPENDENT

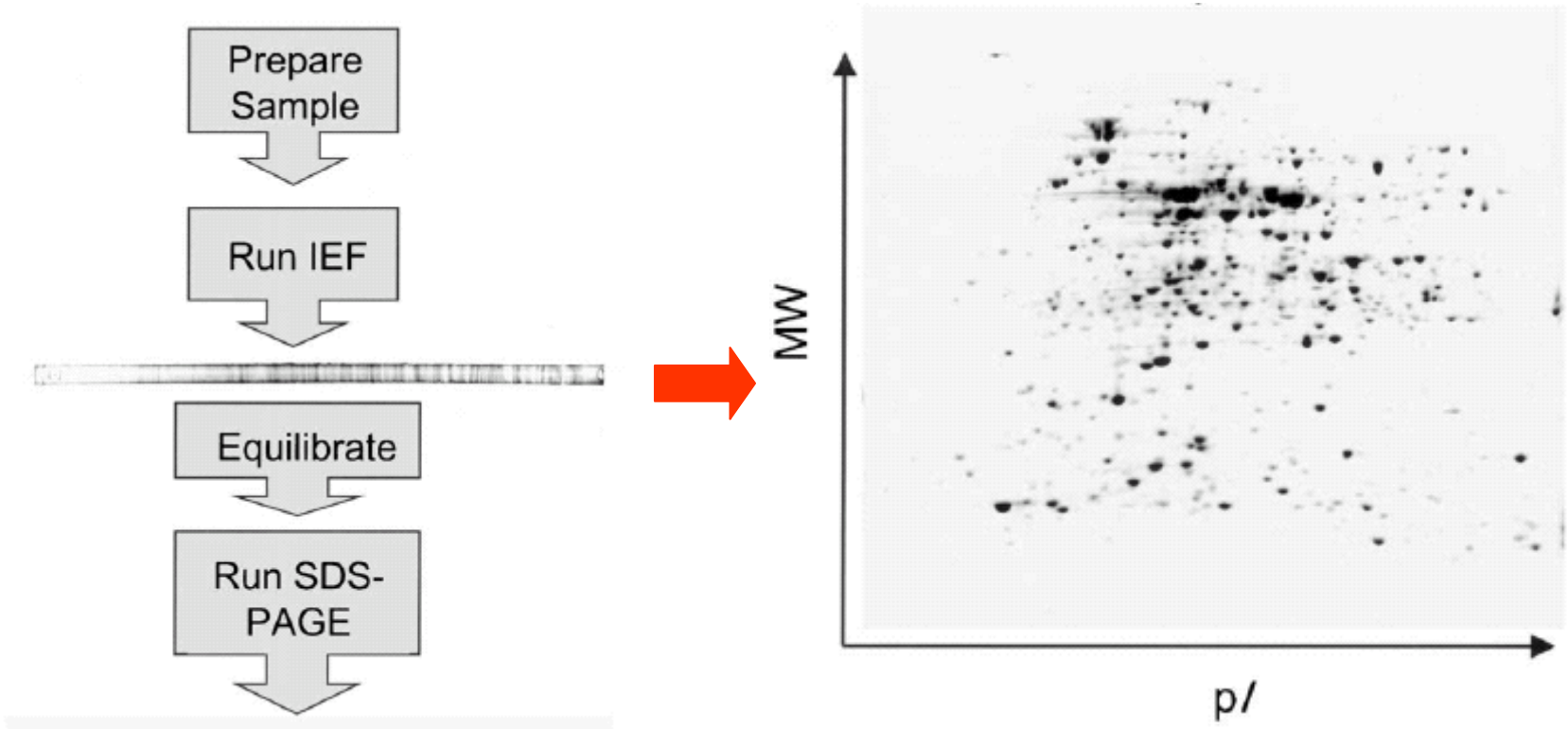
  - Takes longer time to run.
  - Techniques are cumbersome. (the soft, thin, long gel rods needs excellent experiment technique)
  - Batch to batch variation of carrier ampholytes.
  - Patterns are not reproducible enough.
  - Lost of most basic proteins and some acidic protein.

Evolution of 2-DE methodology

Resolution for IEF: Immobilized pH gradients.

- Developed by Bjellqvist (1982, Biochem. Biophys Methods, vol 6, p317)
- PH gradient are prepared by co-polymerizing acrylamide monomers with acrylamide derivatives containing carboxylic and tertiary amino groups.
  - The pH gradient is fixed, not affected by sample composition.
  - Reproducible data are presented.
  - Modified by Angelika Gorg by using thin film to support the thin polyacrylamide IEF gel, named IPG strips. (1988, Electrophoresis, vol 9, p 531)

Run 2-DE, a quick overview

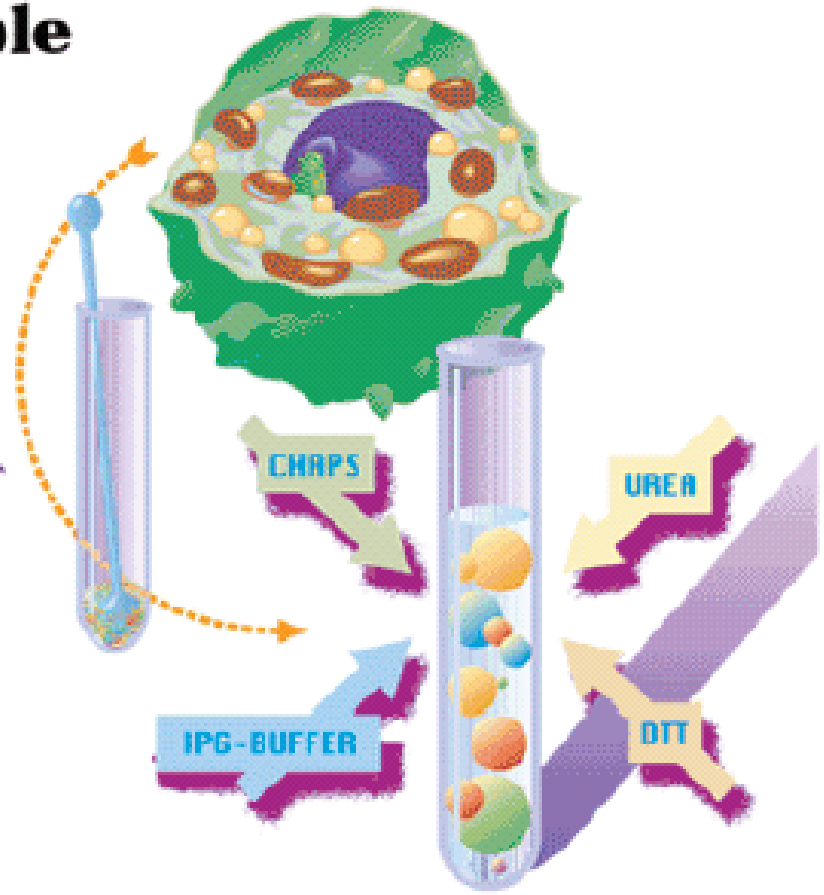


Run 2-DE, step by step

1. Prepare the sample

Samples are denatured and fully solubilized for optimal separation.

Solubilize your cell extract or sample in urea, a non-ionic detergent, IPG-Buffer and a reducing agent.



Run 2-DE step by step

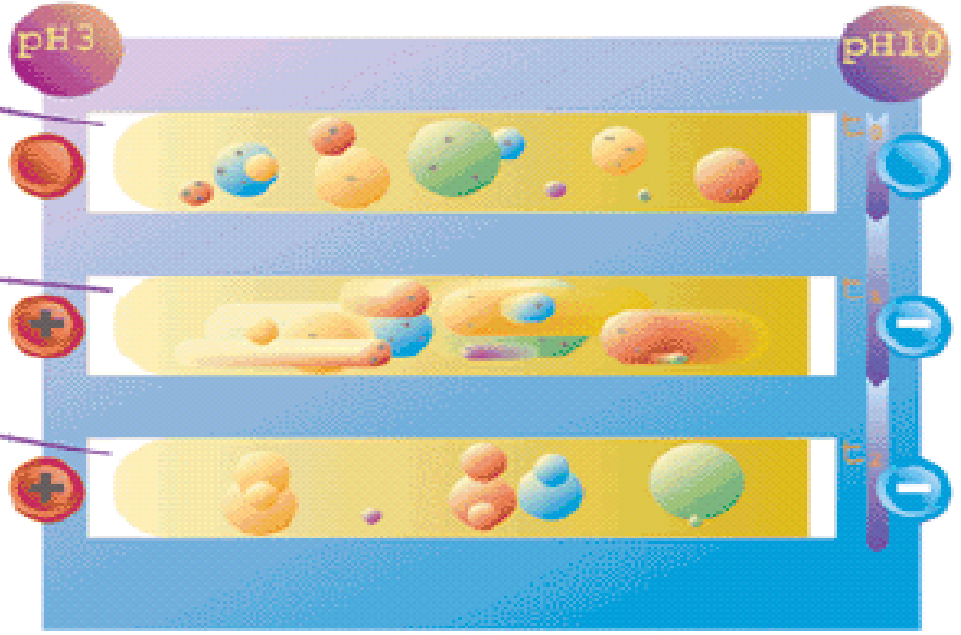
2. Run the first dimension – IEF

In the first dimension, proteins separate by isoelectric point (pI) in the immobilized pH gradient (IPG) of the Immobiline DryStrip gel.

A. Apply the sample to the IPG strip either in a defined zone or distributed throughout the entire strip.

B. Apply the electric field. The charged proteins migrate within the pH gradient.

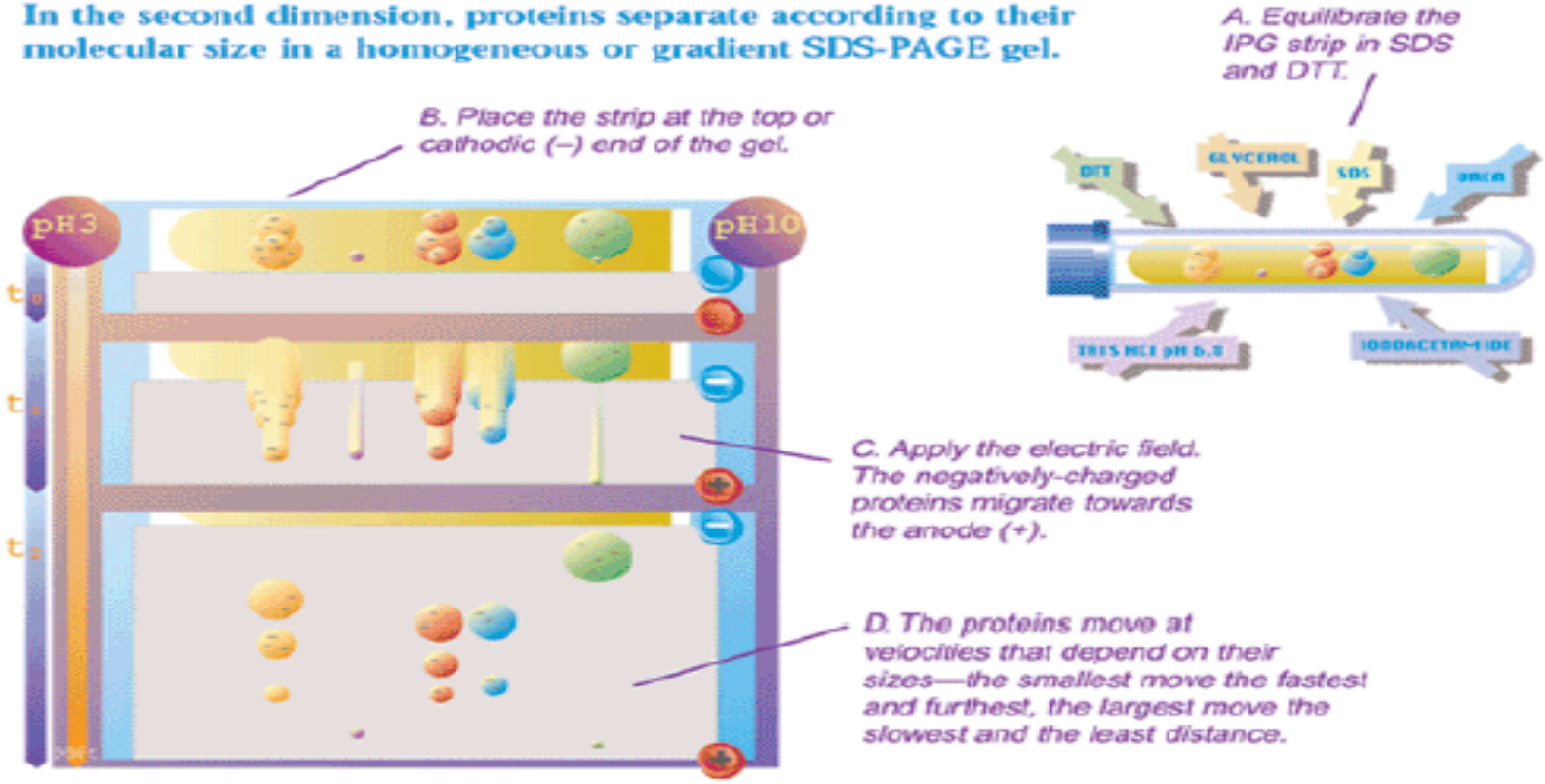
C. When the proteins reach the pH in the gradient equal to their pIs, their net charge drops to zero and they focus as distinct zones. Because the pH gradient in the strip is immobilized, no further migration can occur.



Run 2-DE step by step

3. Run the second dimension – SDS-PAGE

In the second dimension, proteins separate according to their molecular size in a homogeneous or gradient SDS-PAGE gel.

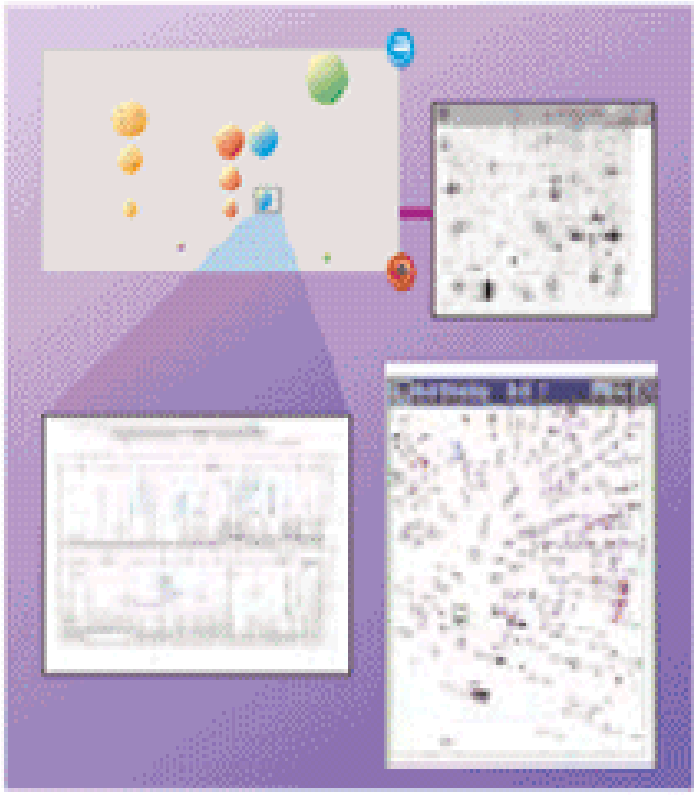


Run 2-DE step by step

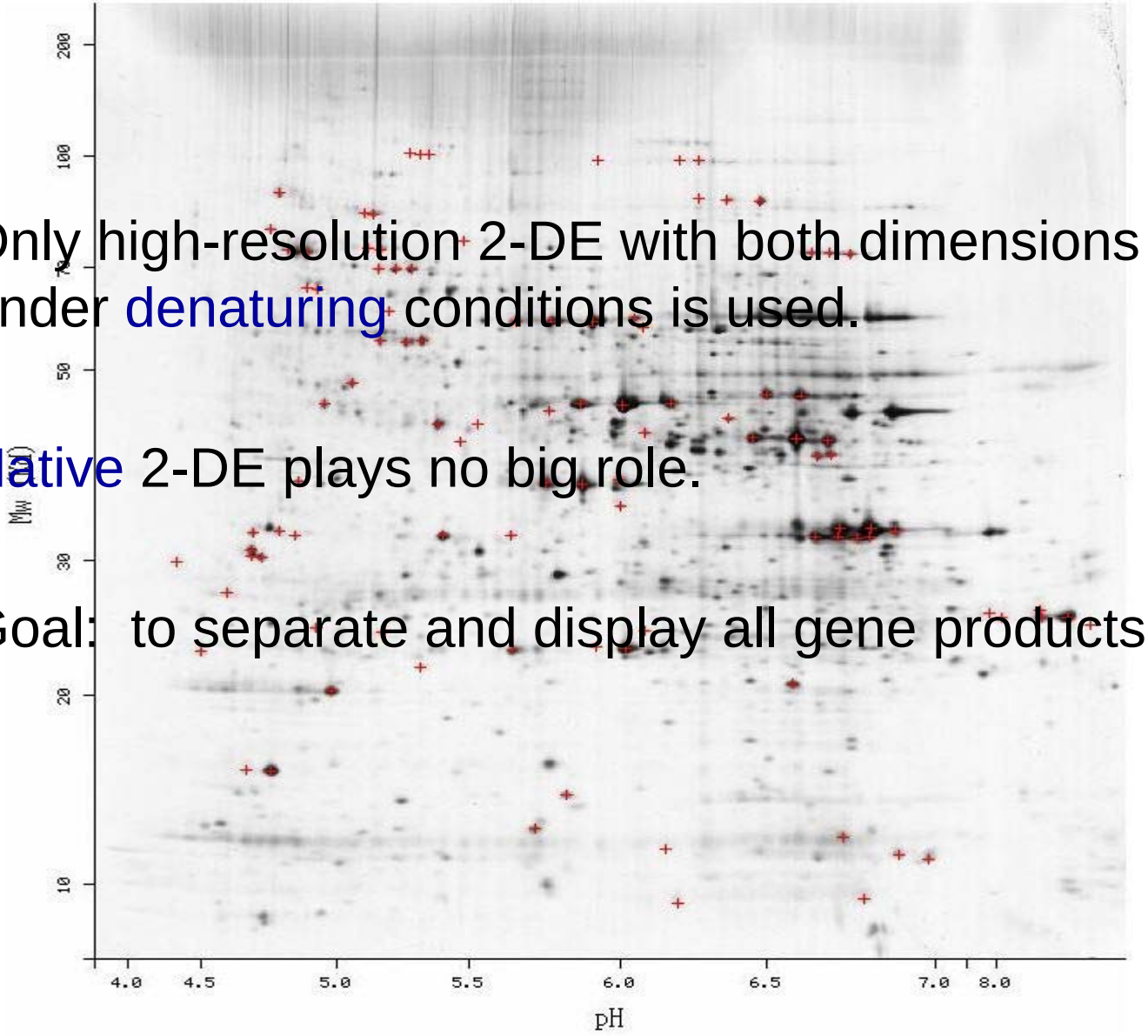
4. Visualize and analyze

Detect separated proteins by autoradiography, staining, or immunodetection after blotting onto a membrane. An array of powerful tools and techniques is available to compare samples and identify proteins of interest:

- Image analysis software to determine spot position and abundance, compare gel images, create databases, and search for patterns;
- Mass spectrometry to determine masses with high precision, peptide fragment fingerprints, amino acid sequences, and nature and site of post-translational modifications.



Today's 2-DE



- Only high-resolution 2-DE with both dimensions run under denaturing conditions is used.
- Native 2-DE plays no big role.
- Goal: to separate and display all gene products present.

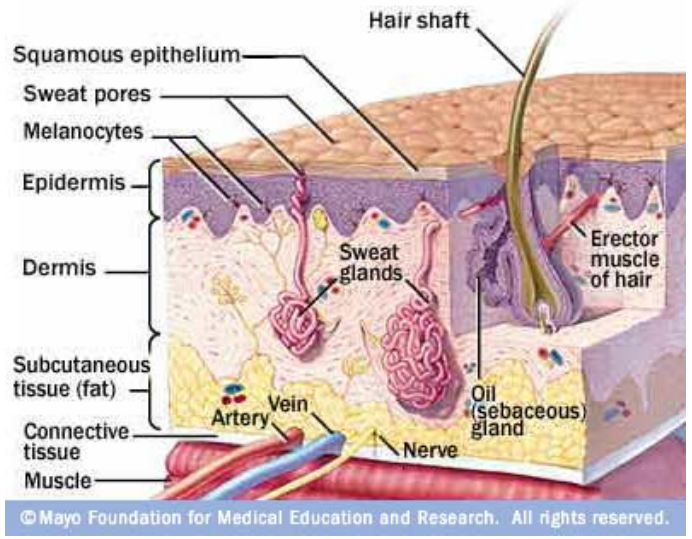
Sample preparation

Some important concepts for sample preparation

1. A good sample preparation is the key to good result.

2. The protein composition of the cell lysate or tissue must be reflected in the patterns of 2-DE.

3. Avoid protein contamination from environment.



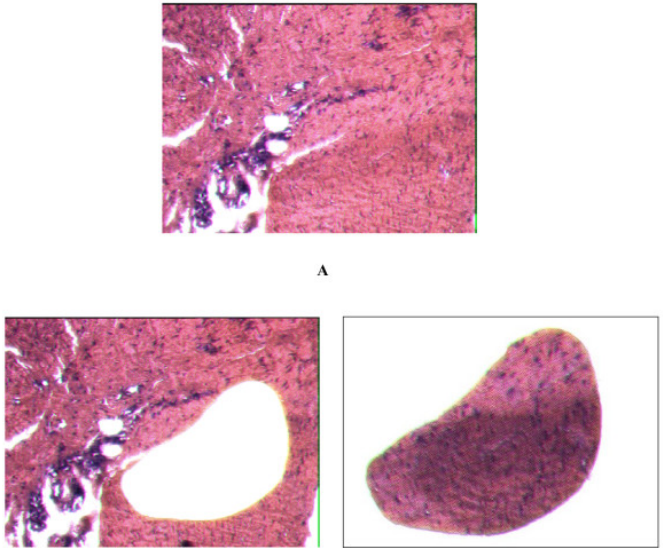
Keratin contamination

Some important concepts for sample preparation

4. Co-analytical modification (CAM) must be avoided (pre-purification sometimes leads to CAM)

5. Highly selective procedure for tissue analysis (Laser capture micro dissection, LCM)

LCM



Some important concepts for sample preparation

6. Treatment of sample must be kept to a minimum to avoid sample loss.

7. Keep sample as cold as possible.

8. Shorten processing time as short as possible.

9. Removal of salts

Frequently applied treatments

1. Cell washing
2. Cell disruption
3. Removal of contaminant
4. Micro-dialysis
5. Electrophoretic desalting
6. Precipitation methods
7. For very hydrophobic protein

1. Cell washing

- To remove contaminant material
- Frequent used buffer

1. **PBS:** phosphate buffer saline, sodium chloride, 145 mM (0.85%) in phosphate buffer, 150 mM pH7.2
2. **Tris buffer sucrose** (10mM Tris, 250 mM sucrose, pH 7,2)

- The utilized buffers must provide enough osmoticum to avoid cell lysis.

2. Cell disruption

Gentle lysis method

1. Osmotic lysis (cultured cells)

- Suspend cells in hypoosmotic solution.

2. Repeated freezing and thawing (bacteria)

- Freeze using liquid nitrogen

3. Detergent lysis (yeast and fungi)

- Lysis buffer (containing urea and detergent)
- SDS (have to be removed before IEF)

4. Enzymatic lysis (plant, bacteria, fungi)

- Lysomzyme (bacteria)
- Cellulose and pectinase (plant)
- Lyticase (yeast)

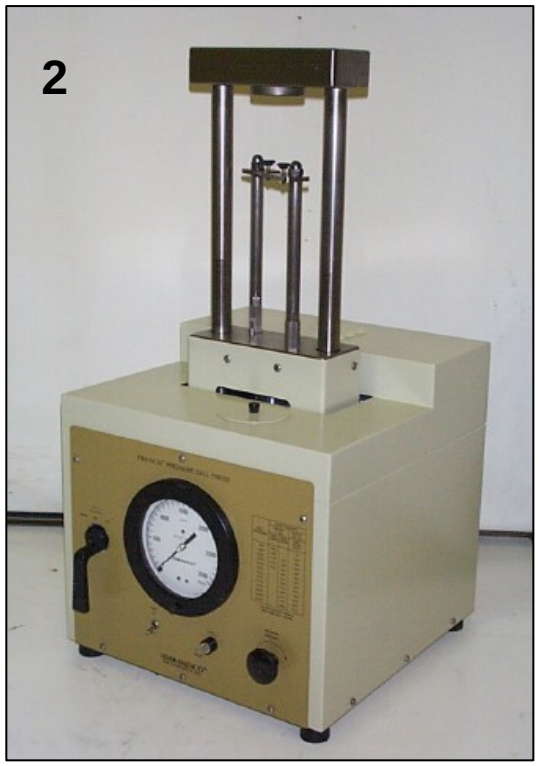
2. Cell disruption (continued)

- **Vigorous lysis method**
  1. **Sonication probe (cell suspension)**
     - Avoid overheat, cool on ice between burst.
  2. **French pressure (microorganism with cell wall)**
     - Cells are lysed by shear force.
  3. **Mortar and pestle (solid tissue, microorganism)**
     - Grind solid tissue to fine powder with liquid nitrogen.
  4. **Sample grinding kit (for small amount of sample)**
     - For precious sample.
  5. **Glass bead (cell suspension, microorganism)**
     - Using abrasive vortexed bead to break cell walls.

2. Cell disruption (continued)

1

2



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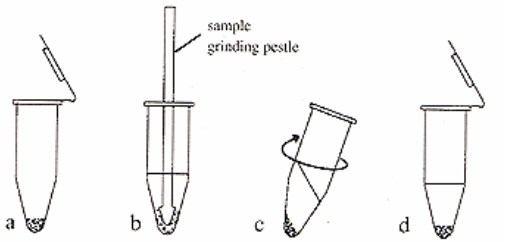

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2. Cell disruption (continued)

- Key variables for successful extraction from crude material, must consider

1. The method of cell lysis
2. The control of pH
3. The control of temperature
4. Avoidance of proteolytic degradation

3. Removal of contaminants

- **Major type of contaminants:**

1. DNA/RNA
2. Lipids
3. polysaccharides
4. Solid material
5. Salt

DNA/RNA contaminant

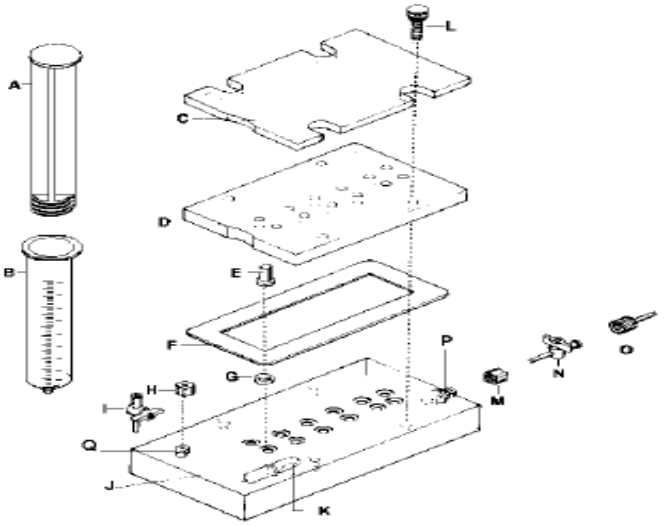
- DNA/RNA can be stained by silver staining.
- They cause horizontal streaking at the acidic part of the gel.
- They precipitate with the proteins when sample applying at basic end of IEF gel
- How to remove:
  - DNase / RNase treatment to digest DNA/RNA
  - Sonication to break DNA/RNA(mechanical breakage)
  - DNA/RNA extraction (phenol-chloroform method)
  - **Precipitation of proteins**

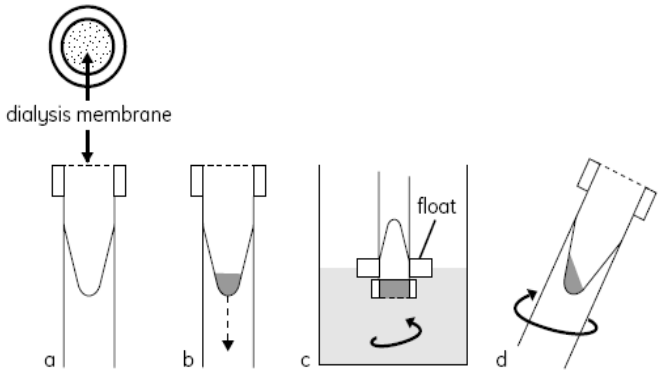
Removal of other contaminants

- Removal of lipids:
  - >2% detergent
  - **Precipitation of proteins**
- Removal of polysaccharides:
  - Enzymatic degradations
  - **Precipitation of proteins**
- Removal of solid material
  - Centrifugation
- Removal of salts
  - Microdialysis
  - **Precipitation of proteins**

4. Microdialysis

- Specially design for small volume samples
- Membrane cut-off is about 8000 Da
- Drawbacks:
  1. Time consuming (some protease might be active and digest proteins during the dialysis)
  2. Some proteins precipitation after dialysis.





5. Electrophoretic desalting

- There are some case where the sample must not be dialysed. (halobacteria lysate)
- Some proteins will gel if desalted. (Bovine vitreous proteins)

✸ Example of electrophoretic desalting: low voltage (100V) for 5 hours before IEF running. (A. Gorg, 1995)

6. Precipitation methods

- The reasons for applying protein precipitation procedure

1. Concentrate low concentrated protein samples.

2. Removal of several disturbing material at the same time.

3. Inhibition of protease activity.

Four precipitation methods

1. Ammonium sulfate precipitation
2. TCA precipitation
3. Acetone precipitation
4. TCA/Acetone precipitation

Ammonium sulfate precipitation

- Proteins tend to aggregate in high concentration of salt (salting out)
  - Add Ammonium sulfate slowly into solution and stir for 10-30 mins
  - Harvest protein by centrifugation.
- Limitation
  - Some proteins are soluble at high salt conc.
  - Ammonium sulfate seriously affect IEF.

TCA precipitation

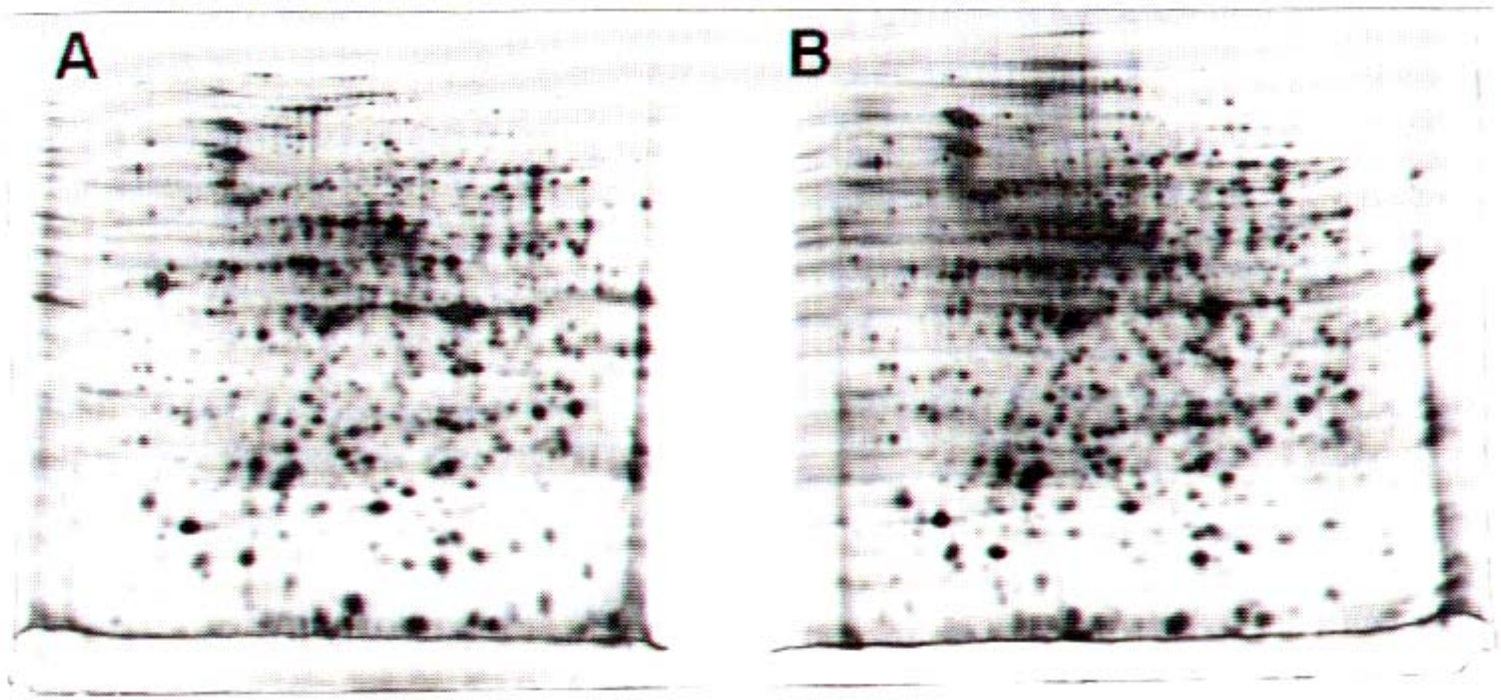
- Trichloroacetic acid (TCA) is a very affective protein precipitant.
  - Add TCA to extract to final conc.10-20%.
  - Add 10-20% TCA directly to tissue or cells.
  - Harvest protein by centrifugation.
  - Wash access TCA by ethanol or acetone.
- Limitation
  - Sometimes the pellet is hard to re-dissolve.
  - TCA must remove complete. (affecting IEF)
  - Some degradation or modification of protein occurs

Acetone precipitation

- The most common organic solvent used to precipitated proteins, lipid and detergent remain in solution.
  - Add at least 3 vol. of ice-cold acetone into extract.
  - Stand on ice for at least 2 hours.
  - Harvest protein by centrifugation.
  - Remove access acetone by air drying.

- Limitation
  - Sometimes the pellet is hard to redissolve.
  - Some proteins would not precipitate.
  - DNA/RNA and glycan also precipitate.

Example (acetone precipitation)



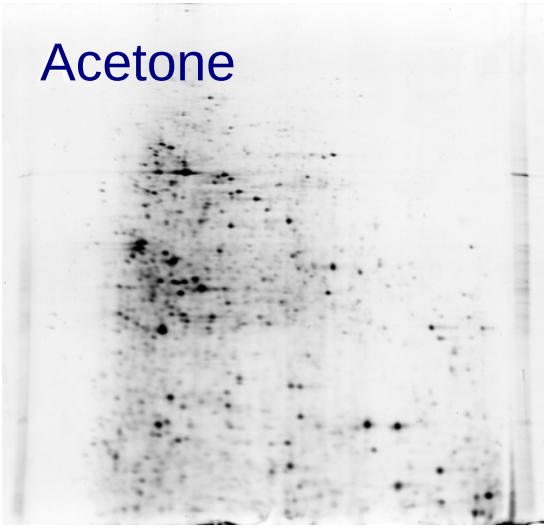
With Acetone precipitation

Crude extract by lysis buffer

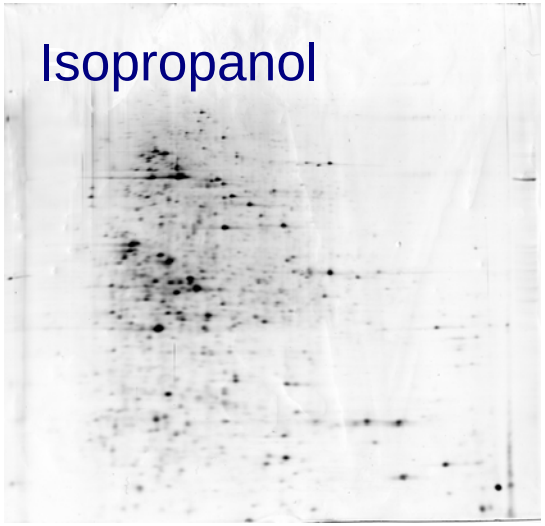
TCA/acetone precipitation

- The method is more active than TCA or acetone alone. Most commonly used in 2-DE exp.
  - Suspension samples in 10% TCA/Acetone with 0.07% 2-mercaptoethanol or 20mM DTT.
  - Stand on -20C for at least 45mins.
  - Harvest protein by centrifugation.
  - Wash the pellet by acetone with0.07% 2-mercaptoethanol or 20mM DTT.
  - Remove access acetone by air dry.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - TCA must remove complete. (affecting IEF)
  - Some degradation or modification of protein occurs

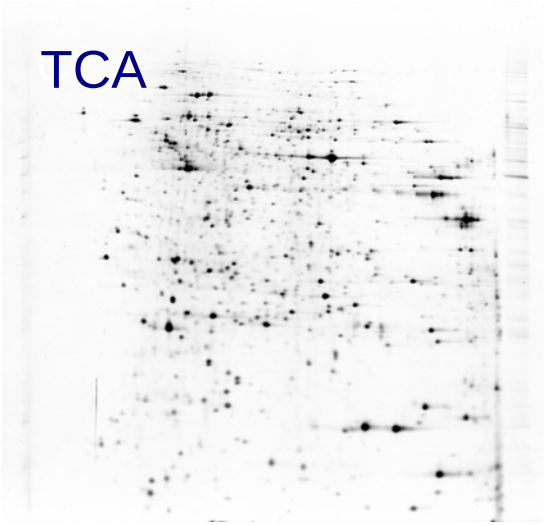
Comparison of the precipitation methods



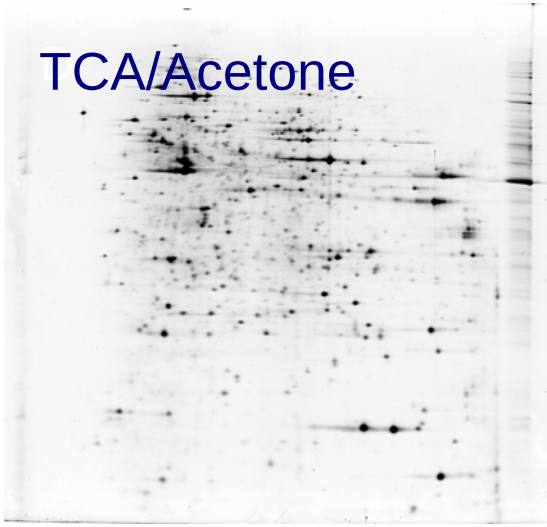
Σ 823 spots



Σ 757 spots



Σ 969 spots

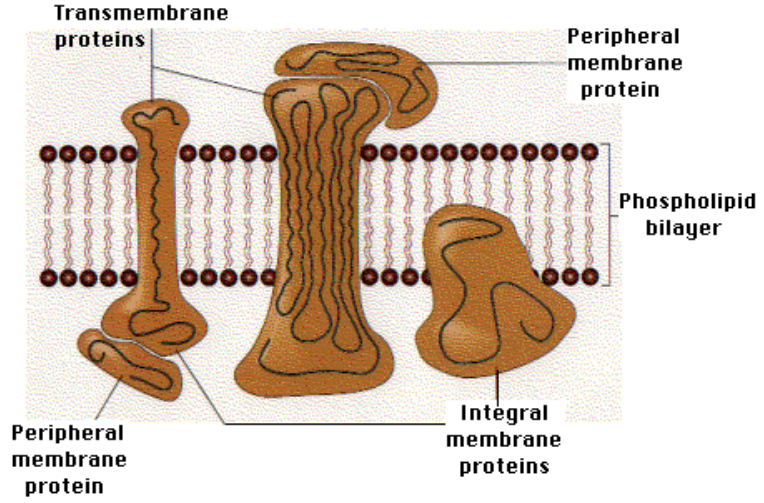


Σ 899 spots

7. For very hydrophobic proteins

- Membrane proteins do not easily go into solution. A lot of optimization work is required.
- Generally applied procedures to solubilize very hydrophobic proteins:

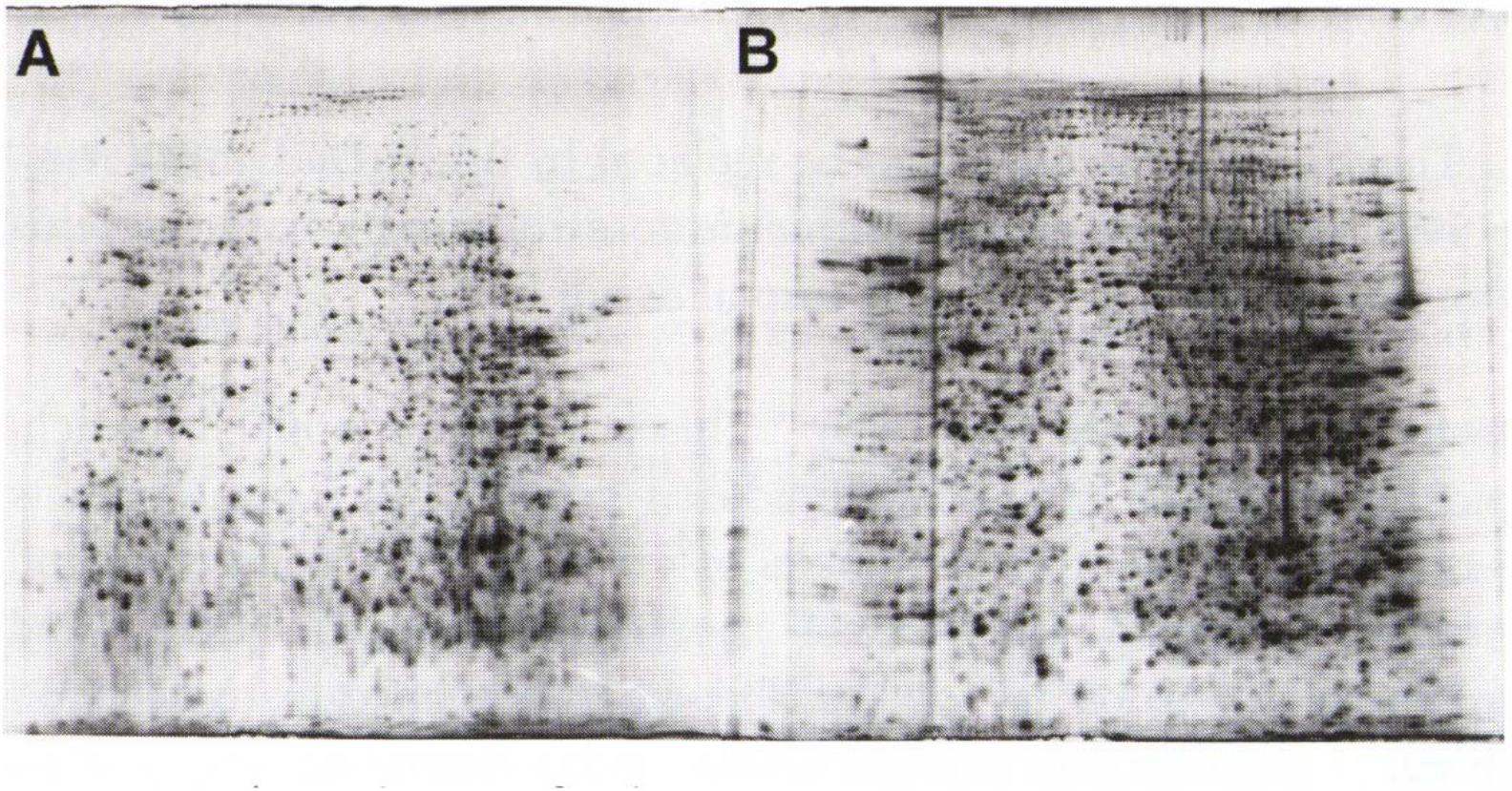
1. Thiourea procedure
2. SDS procedure
3. Utilization of new zwitterionic detergent



Thiourea procedure

- 7M urea + 2M thiourea (Rabilloud, 1998)
  - **Pros:** Increase spot number considerably.
  - **Cons:** Causing artifact spots.
    Causing vertical streaking at acidic area.

Example, thiourea procedure



Lysis buffer, 8M urea

Lysis buffer, 7M urea+ 2M thiourea

SDS procedure

- Good for emergency case.
- Up to 2% SDS can be used.
- Have to dilute SDS samples at least 20 fold with urea an a non or zwitterionic detergent containing solutions.

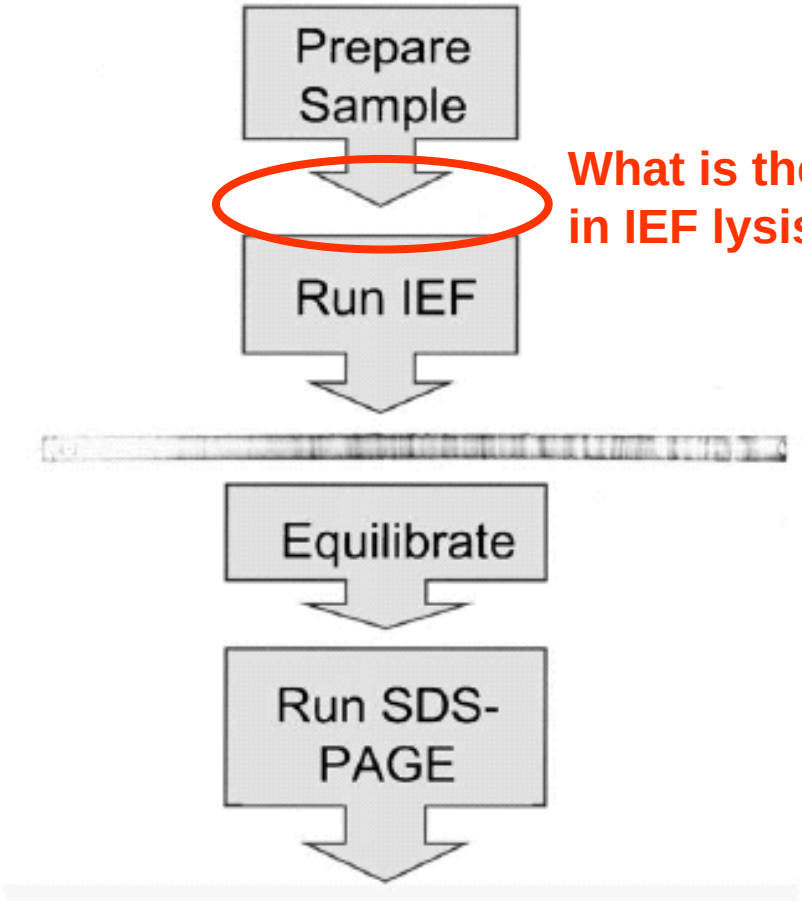
- SDS can
  1. Prevent the formation of protein oligomers.
  2. Dissolve tough cell walls samples (with boiling).
  3. Dissolve very hydrophobic proteins.

Utilization of new zwitterionic detergents

- **Three major types of detergent**

1. Non ionic detergent
   - Triton x-100, Tween 20, Brij-35
2. Ionic detergent
   - SDS, CTAB, Digitonin
3. Zwittergent
   - CHAPS, CHAPSO, Zwittergent 3-08, 3-10, 3-12 e.t.c.

Now, we are ready to dissolve protein samples in IEF lysis buffer

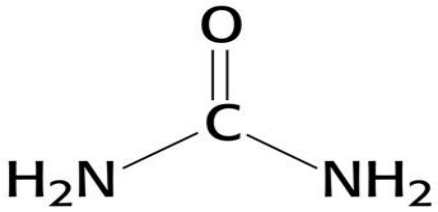


What is the composition in IEF lysis buffer?

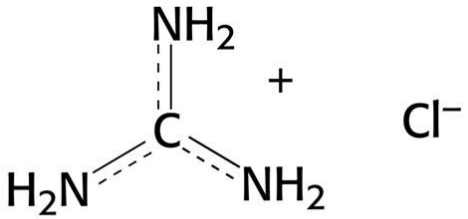
2-DE are in denaturing condition

- The denaturing components must present in 2-DE denaturing condition (namely, in IEF lysis buffer or rehydration buffer), for example,

1. Urea (often > 7M)
2. Reductant (DTT used most widely)
3. Non-ionic or zwitterionic detergent



Urea



Guanidinium chloride

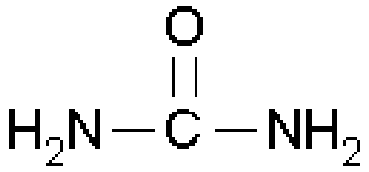
why not using native condition

- Under native condition, a great part of proteins exists in several conformations. This leads to more complex 2-DE patterns.
- Native protein complexes sometimes too big to enter the gel.
- Reduction of protein-protein interactions.
- For match the theoretical pI and MW, all proteins should not have 3D structure or quanternary structure.

Composition of standard IEF buffer (general called lysis or rehydration buffer)

1. 8M urea
2. 4% CHAPS
3. 1% DTT
4. 0.8% carrier ampholyte
5. 0.02% bromophenol blue.

1



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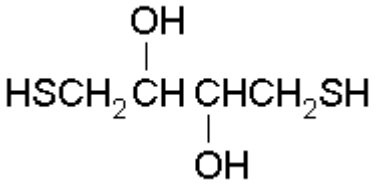

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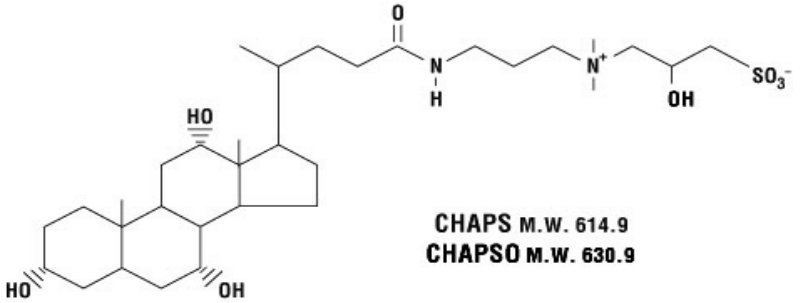

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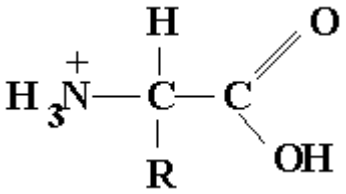

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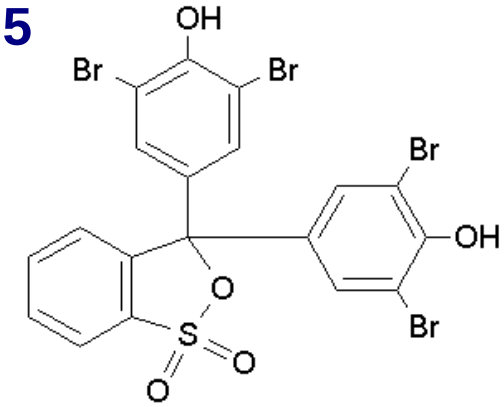
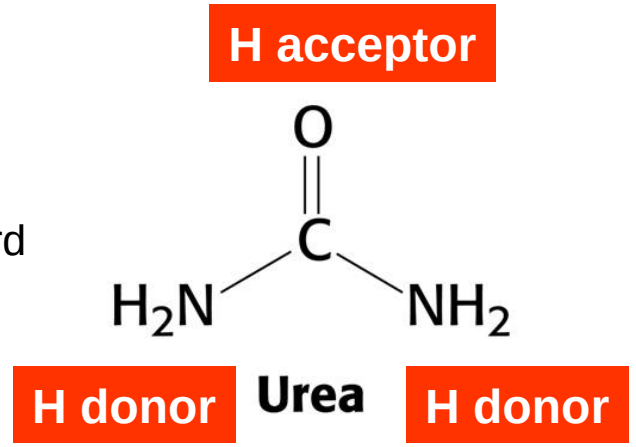
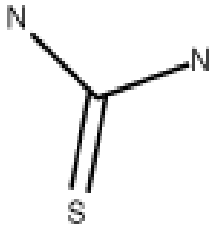


Function of denaturant (urea)

- To convert proteins into single conformation by canceling 2nd and 3rd structure.
- To keep hydrophobic proteins into solution.
- To avoid protein-protein interaction.
- Thiourea: for very hydrophobic proteins only.





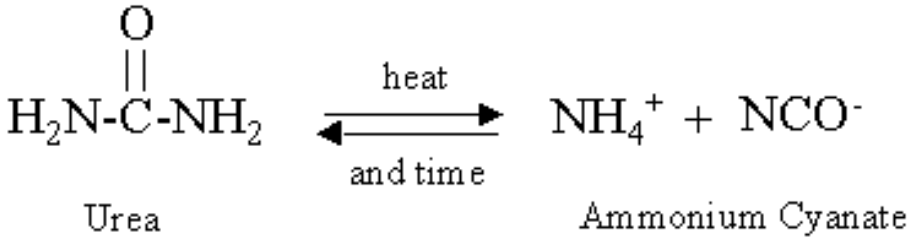
Thiourea

Beware when using urea

- The purity of urea is very critical.
- Isocyanate impurities and heating will cause carbamylation of the proteins.
- It does not seem to make a difference what grade of urea is used because, urea + heat + protein = carbamylation.

Carbamylation of proteins

Decomposition of Urea



Carbamylation of Proteins

(amino terminus of a peptide used as an example)

+ 43 kDa

Isocyanic Acid

Peptide Amino Terminus
(or side chain of Lys or Arg)

Carbamylated Peptide or Protein

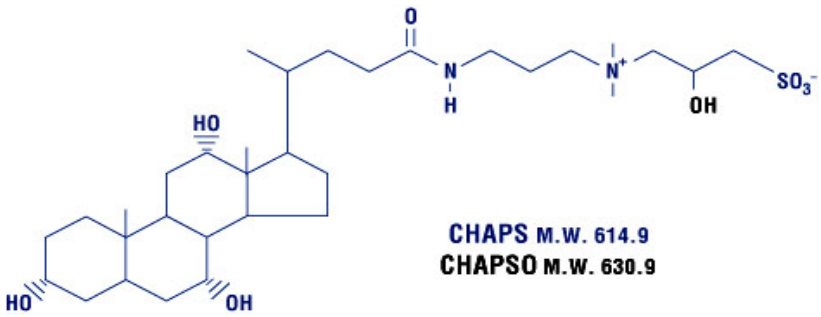
Results of Carbamylation

Amino Acid	Residue Composition	Residue Monoisotopic Mass	Delta Mass
Lysine	$C_6H_{12}N_2O$	128.09496	0
Carbamyl Lysine	$C_7H_{13}N_3O_2$	171.10078	43.00582
Carbamylation	* NHCO	43.00582	-
*Note: A proton is lost from the amino group on the protein during carbamylation and thus the change in composition is NHCO.			

Function of detergent (CHAPS)

- To disrupt nonspecific protein interactions (particularly hydrophobic interactions).

- Zwitterionic detergent (CHAPS):
  1. To combine all the advantages of polar, sulfobetaine-containing detergents and hydrophobic, bile salt, anionic detergents into a single molecule with superior membrane protein solubilization properties
  2. Less protein aggregation than non-ionic detergents
  3. Electrically neutral
  4. Easily removed by dialysis

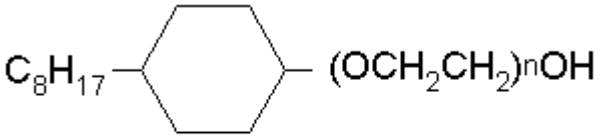


CHAPS M.W. 614.9
CHAPSO M.W. 630.9

Other detergents

1. Triton X-100

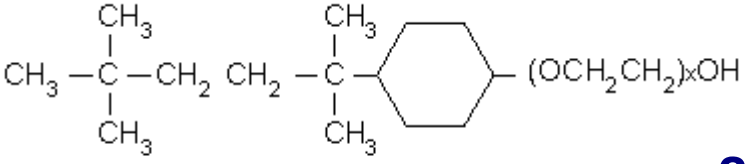
(not easily remove and interfering MS)



n ~ 10

1

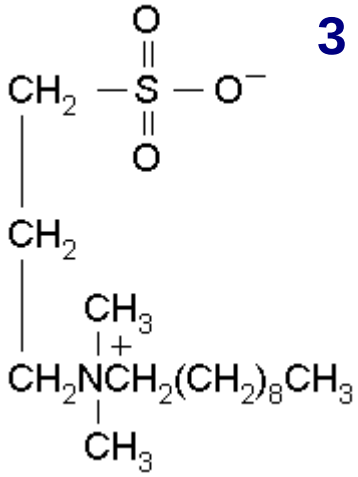
2. Nonidet NP-40



x = 9 - 10 (avg.)

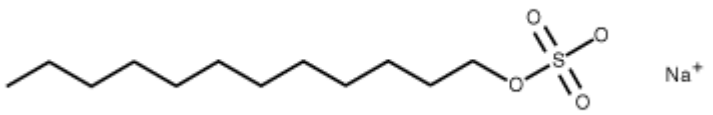
2

3. SB3-10



3

4. SDS



4

Functions of reductant

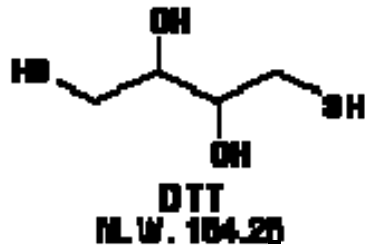
- To prevent different oxidation steps of proteins.

β-mercaptoethanol:

1. should not be used because its **apparent** buffering effect above pH 8.
2. Keratin contamination might from β-mercaptoethanol.

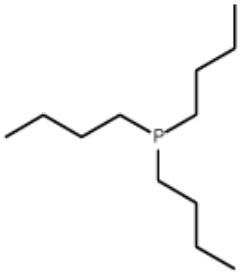
DTT (dithiothreitol) or DTE (dithioerythritol):

1. most used in 2-DE experiments.
   - DTT and DTE also ionizes above pH8. They move toward anode during IEF in basic pH gradient. It leads to horizontal streaking at basic area.



Other reduction methods

- Novel reductant: **TBP (tributylphosphine)**
  no ionization above pH 8, very unstable.



- Alternative reduction procedures.
  1. Addition of higher amount of DTT to the gel
  2. Addition of more DTT to a cathodal paper strip.

Function of carrier ampholyte

- Functions of carrier ampholyte (traditional 2-DE)

  1. To generate pH gradients

- Functions of carrier ampholyte (current 2-DE, **IPG buffer**)

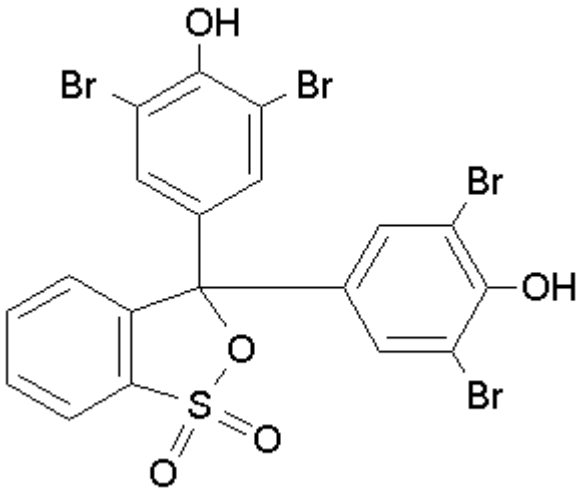
  1. To substituting ionic buffer

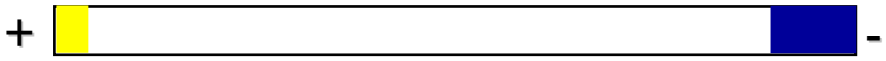
  2. To improve the solubility of protein

Carrier ampholytes do not disturb IEF like buffer addition, because they become uncharged when migrating to their pI.

Function of dyes (bromophenol blue)

- To visualize the sample solution
- To monitor the 2-DE running condition.
- Bromophenol blue is interchangeable with Orange G.





Prevent protease activity

- Some proteases are also active in presence of urea and detergent.
- No complete insurance against protease activity
- Boiling sample in SDS buffer for a few seconds can inactive protease.
- Precipitate protein s with TCA/acetone at -20C might inactivation protease activity.

  - Pefabloc (AEBSF) can also be used but modified proteins.
  - PMSF is frequently used (8mM), toxic and short half-life.

Types of protease inhibitor

Protease inhibitor	Effective against:	Limitations
PMSF (Phenylmethylsulfonyl fluoride) Most commonly used inhibitor. *Use at concentrations up to 1 mM.*	PMSF is an irreversible inhibitor that inactivates: • serine proteases • some cysteine proteases	PMSF rapidly becomes inactive in aqueous solutions: Prepare just prior to use. PMSF may be less effective in the presence of thiol reagents such as DTT or 2-mercapto-ethanol. This limitation can be overcome by disrupting the sample into PMSF-containing solution lacking thiol reagents. Thiol reagents can be added at a later stage. PMSF is very toxic.
AEBSF (Aminoethyl benzylsulfonyl fluoride or Pefabloc SC Serine Protease Inhibitor) *Use at concentrations up to 4 mM.*	AEBSF is similar to PMSF in its inhibitory activity, but is more soluble and less toxic.	AEBSF-induced modifications can potentially alter the pI of a protein.
1 mM EDTA or 1 mM EGTA *Generally used at 1 mM.*	These compounds inhibit metalloproteases by chelating free metal ions required for activity.	
Peptide protease inhibitors (e.g. leupeptin, pepstatin, aprotinin, bestatin) • reversible inhibitors • active in the presence of DTT • active at low concentrations under a variety of conditions *Use at 2–20 µg/ml.*	Leupeptin inhibits many serine and cysteine proteases. Pepstatin inhibits aspartyl proteases (e.g. acidic proteases such as pepsin) Aprotinin inhibits many serine proteases. Bestatin inhibits aminopeptidases.	Peptide protease inhibitors are: • expensive. • small peptides and thus may appear on the 2-D map, depending on the size range separated by the second-dimension gel. Pepstatin does not inhibit any proteases that are active at pH 9.
TLCK, TPCK (Tosyl lysine chloromethyl ketone, tosyl phenylalanine chloromethyl ketone) *Use at 0.1–0.5 mM.*	These similar compounds irreversibly inhibit many serine and cysteine proteases.	
Benzamidine *Use at 1–3 mM.*	Benzamidine inhibits serine proteases.	

Before running IEF, you should…

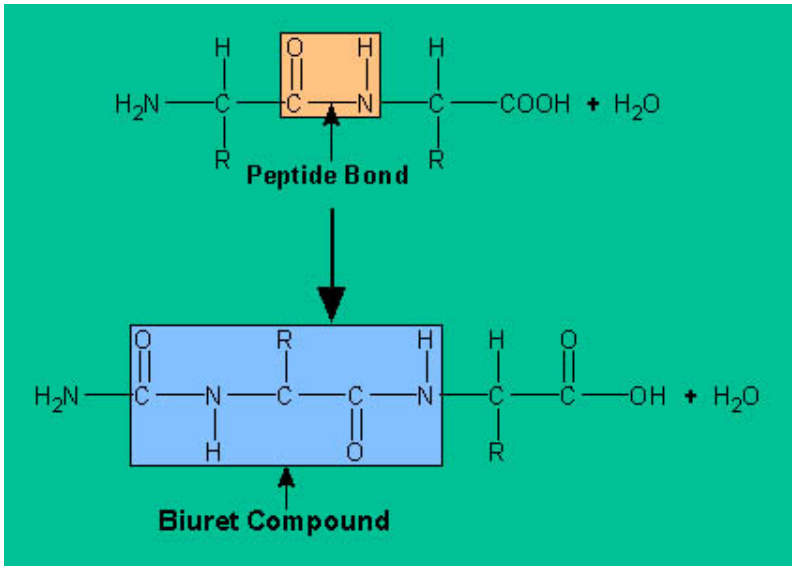
Measure the protein concentrations of your samples !!

- Protein quantitation methods:
  1. Biuret
  2. Lowry methods.
  3. Bradford methods.
  4. UV methods.
  5. Special methods
  6. Other commercial methods.
     - **BCA assay (bicinchoninic acid assay, Pierce)**
     - **DC protein assay (detergent compatible, Bio-rad)**
     - **DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)**

1. Biuret method

- **Principle:** The reactivity of the peptide bonds with the copper [II] ions under alkaline conditions to form purple biuret complex.
- **Interfering substance:** Ammonium sulfate, Tris, etc.
- **Sensitivity:** >mg

A white, crystalline, nitrogenous substance, $C_2O_2N_3H_5$, formed by heating urea. It is intermediate between urea and cyanuric acid.



2. Lowry method

- **Principle:** The reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Try, Try).

- **Interfering substance:** amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids, ammonium ions, zwitterionic buffers, nonionic buffers and thiol compounds.

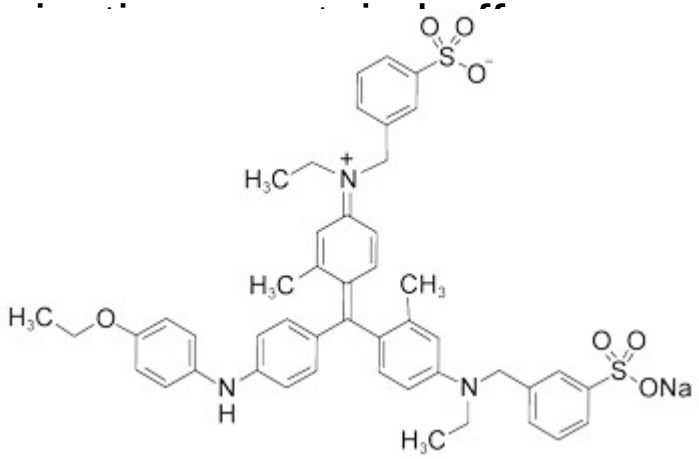
- **Sensitivity:** > 0.1 mg

3. Bradford method

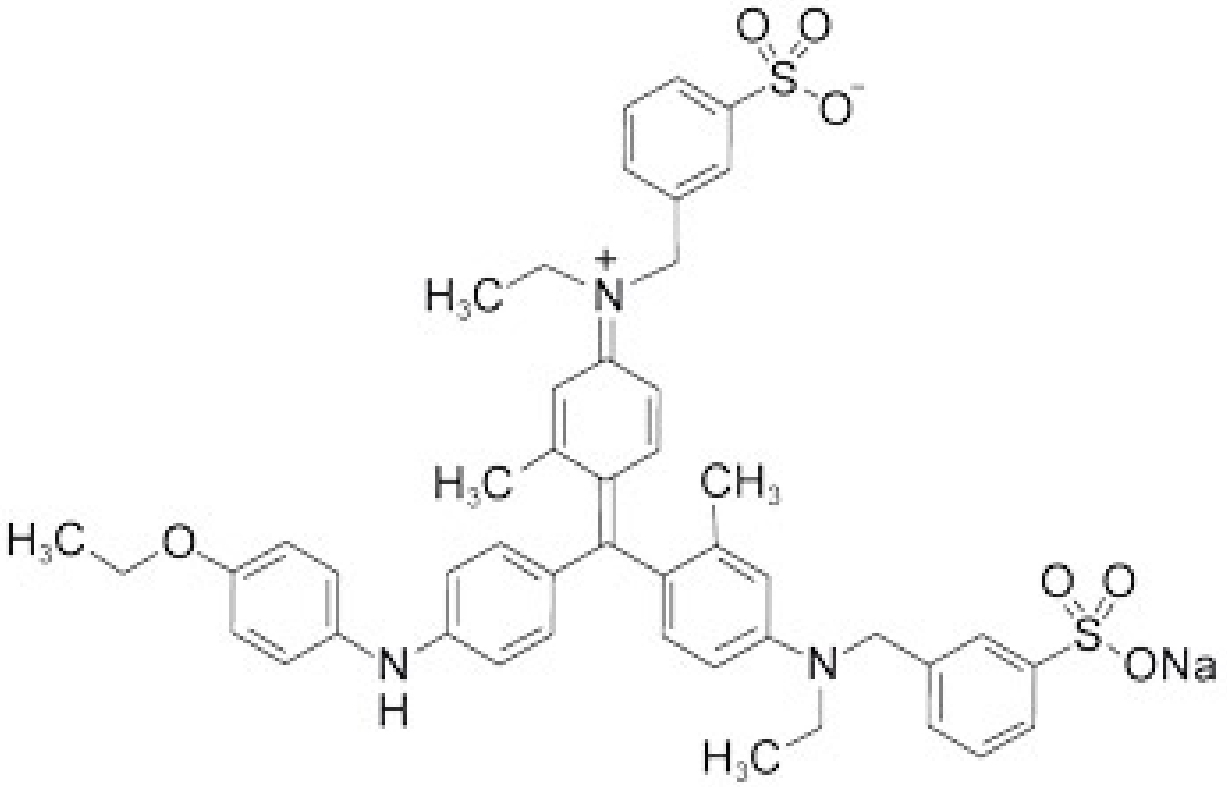
- Principle: The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie® dye binds primarily with basic and aromatic side chains. The interaction with arginine is very strong and less strong with histidine, lysine, tyrosine, tryptophan, and phenylalanine. About 1.5 to 3 molecules of dye bind per positive charge on the protein.

- Interfering substance: amino acid de
  drugs, lipids, sugars, salts, nucleic a
  zwitterionic buffers, nonionic buffers

- Sensitivity: >10 -100 ug



Coomassie brilliant blue G-250



4. UV methods

- **Principle:** The aromatic groups (Phe, Tyr, Trp) and the peptide bonds have maximum UV absorbance around 280nm and 200nm. 280nm was used most frequently.

- **Interfering substance:** anything containing

- **Sensitivity:** >mg

5. Special methods

- **Principle:** Some proteins contain functional groups, eg: Heme in peroidase, hemoglobin and transferrin can be detected at 403nm, Cd2+ in some phytochelatins.

- **Interfering substance:** similar functional groups.

- **Sensitivity:** various

6. Commercial available methods

- BCA assay (bicinchoninic acid assay, Pierce)

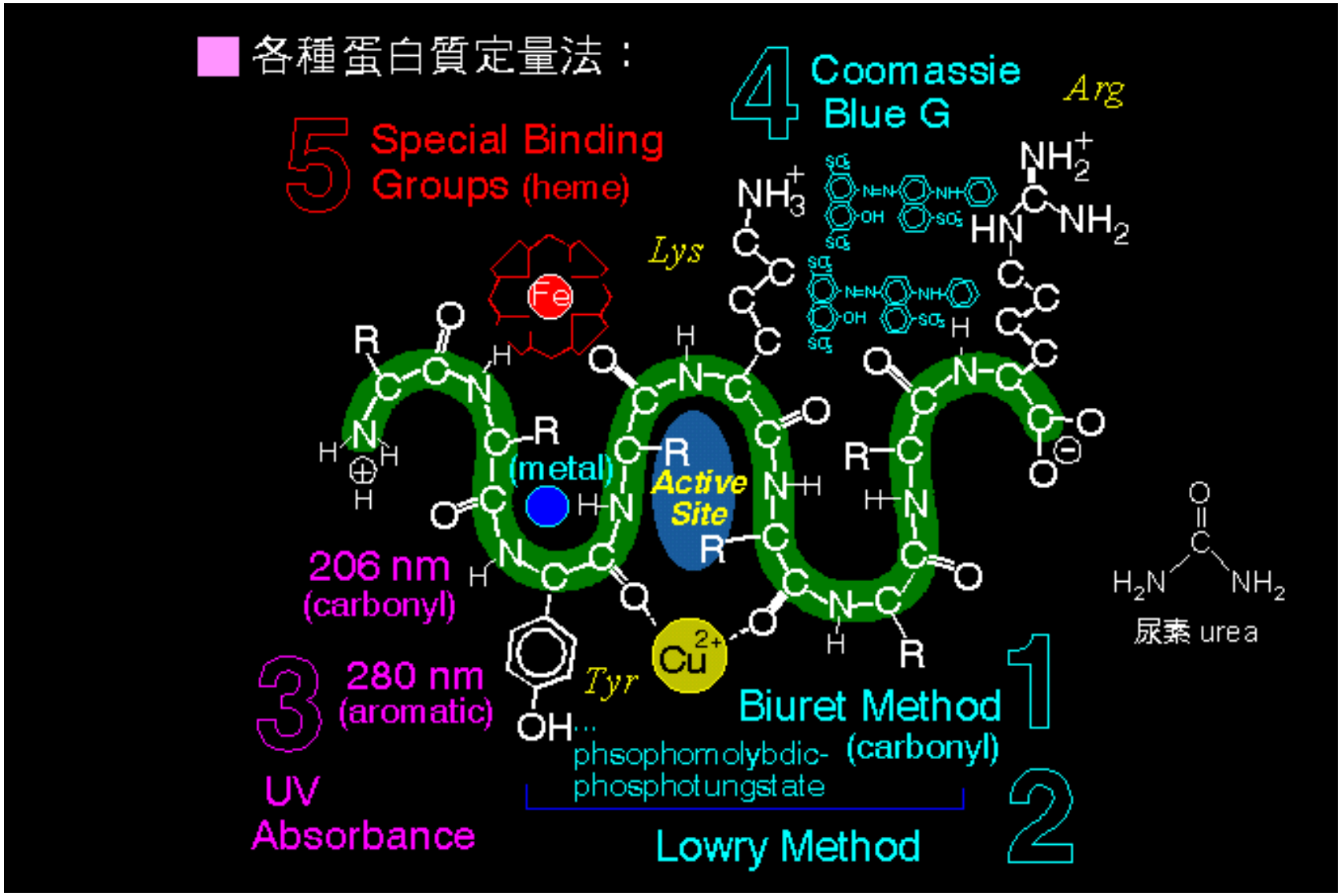
This process is a two-step reaction.

Protein + Cu^{2+} + OH^- $\longrightarrow$ Cu^{1+}

Cu^{1+} + 2 BCA $\longrightarrow$ Cu^{1+}/BCA chromophore (562 nm).

- DC protein assay (detergent compatible, Bio-rad)
- DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)

Summary of protein quantitation methods



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Our conclusions on selecting a proper protein quantitation methods for 2-DE experiments

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TECHNICAL BRIEF

Evaluating the compatibility of three colorimetric protein assays for two-dimensional electrophoresis experiments

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To evaluate compatibility of commonly used colorimetric protein assays for 2-DE experiments, we investigated the interfering mechanisms of major 2-DE component(s) in the Lowry-based assay, the Bradford assay and the bicinchoninic acid (BCA) assay. It was found that some 2-DE components did not directly interfere with the assays' color development reaction, but possibly influenced the quantitation results by interacting with proteins. Generally, simultaneous presence of 2-DE components in the samples demonstrated a cooperative rather than additive interference. Interference by reductants in the Lowry-based assay and the BCA assay were too prominent and could not be completely eliminated by either the reported alkylation procedure or the water dilution procedure. The Bradford assay however, presented a more suitable method for quantitating 2-DE samples because it was less interfered by most 2-DE components. Furthermore, despite slightly compromising protein solubility, utilization of reductant free 2-DE sample buffers conferred application of the Lowry-based and BCA assays in the 2-DE experiments.

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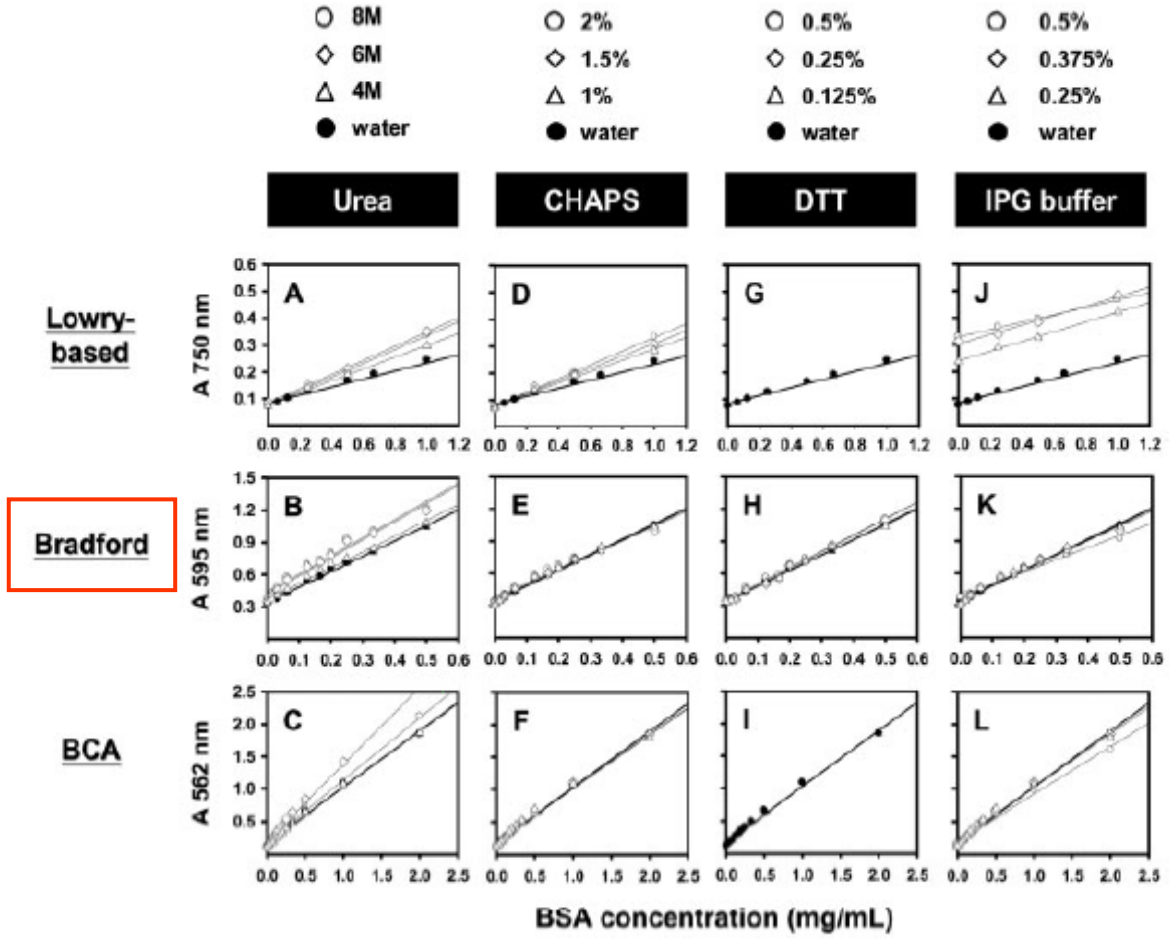
Keywords:
2-DE / BCA assay / Bradford assay / Lowry-based assay

How 2-DE components interfere with different protein quantitation methods



We suggest using Bradford assay to quantitate 2-DE samples.