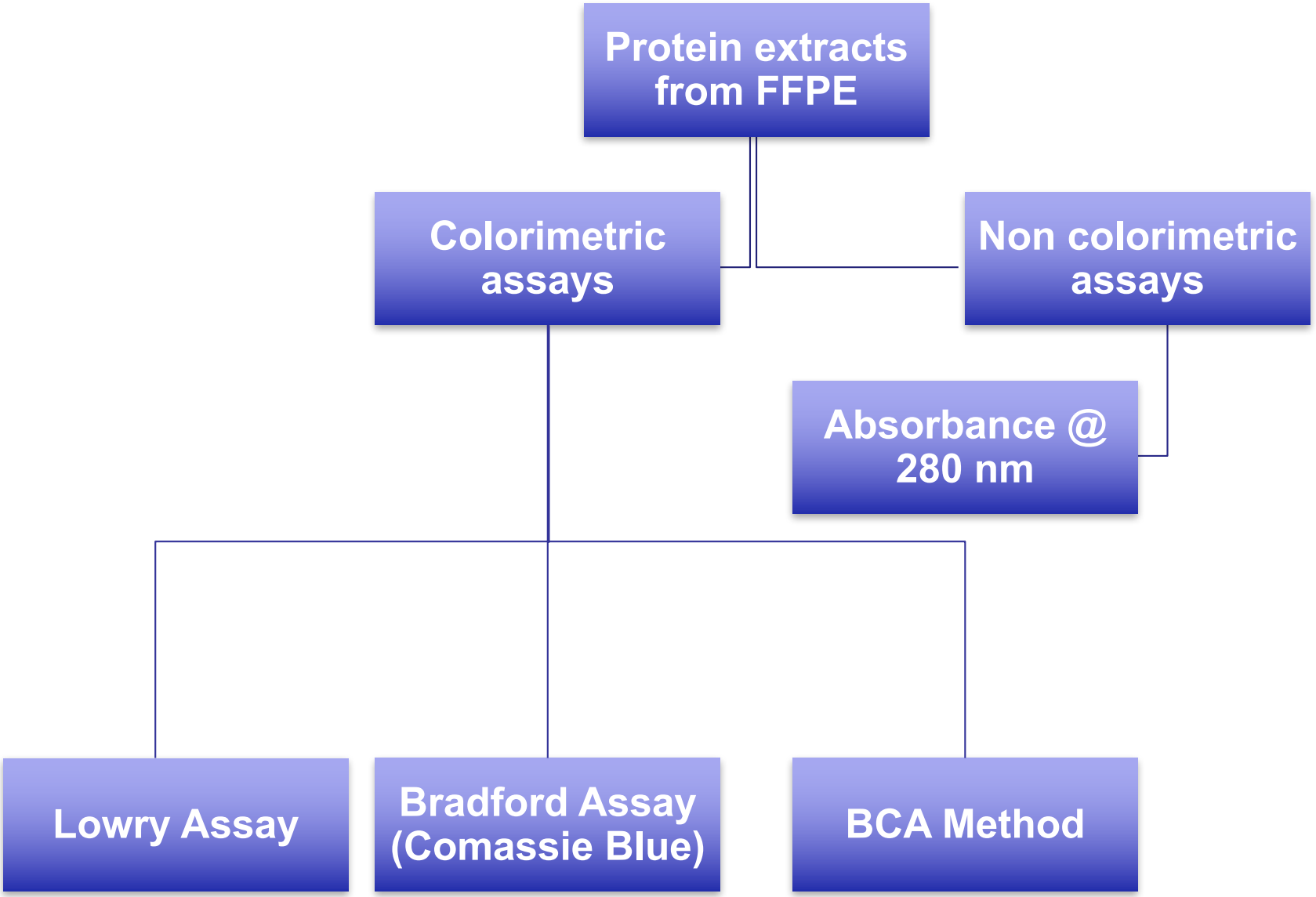
PROTEIN ANALYSIS IN ARCHIVE TISSUES

Methods for extracted proteins

Protein quantification



LAMBERT-BEER LAW

The Beer-Lambert law (or Beer's law) is the linear relationship between absorbance and concentration of an absorbing species where the light path is mainteined constant

$$A = \log_{10}(I_0/I_1) = \varepsilon cl$$

ε is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}

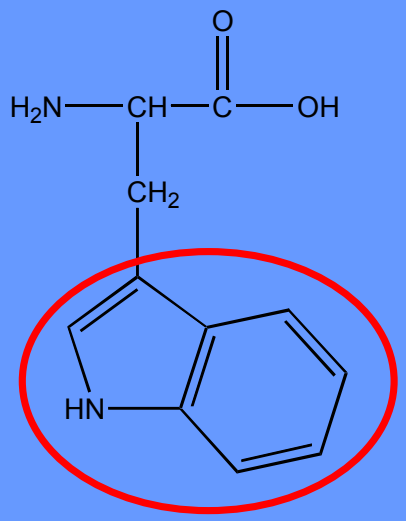
Absorbance is dimensionless.
Usually the concemtration is given as molarity (M) and the length of path in cm (cm), $\varepsilon^{0.1\%}_{280nm}$ is the absorbance @280 nm of a solution 0.1% (1 mg/ml) of the absorbing specie (ie. a protein) in a 1 cm cell.

non colorimetric methods: Absorbance @ 280 nm

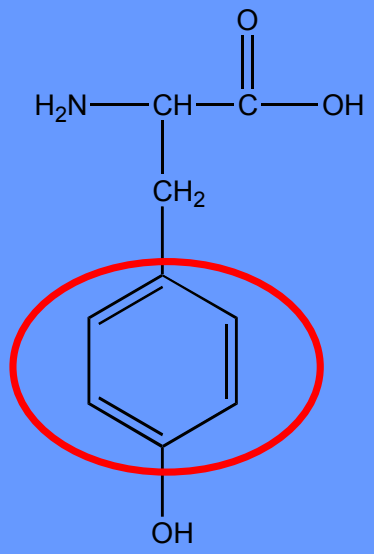
(Warburg and Christian method)

- **Protein concentration can be estimated by measuring the absorbance of the protein solution at 280 nm (UV).**
- **The method is commonly used because it does not destroy the sample and it is very fast**
- **Most proteins have a maximum absorption at 280 nm because of the presence of the aromatic amino acids tryptophan (W), tyrosine (Y) and phenylalanine (F)**
- **As the amino acid composition of proteins varies greatly, the molar absorption will also vary greatly, depending on the content of these amino acids**

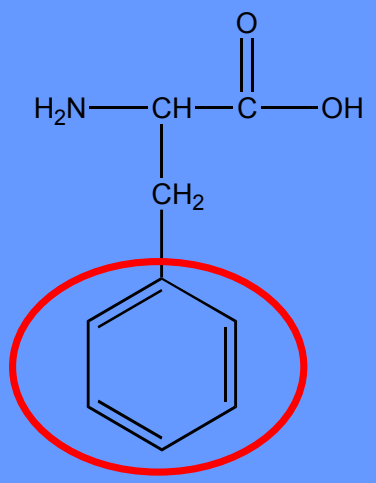
Proteins that do not contain W, Y and F will not have a maximum absorption at 280nm, while proteins that contain many W, Y and F residues will have high values of molar absorption, with a maximum absorption at 280 nm. The method is therefore not very accurate unless the protein is pure and is known molar absorption.



Trp (Triptofano)

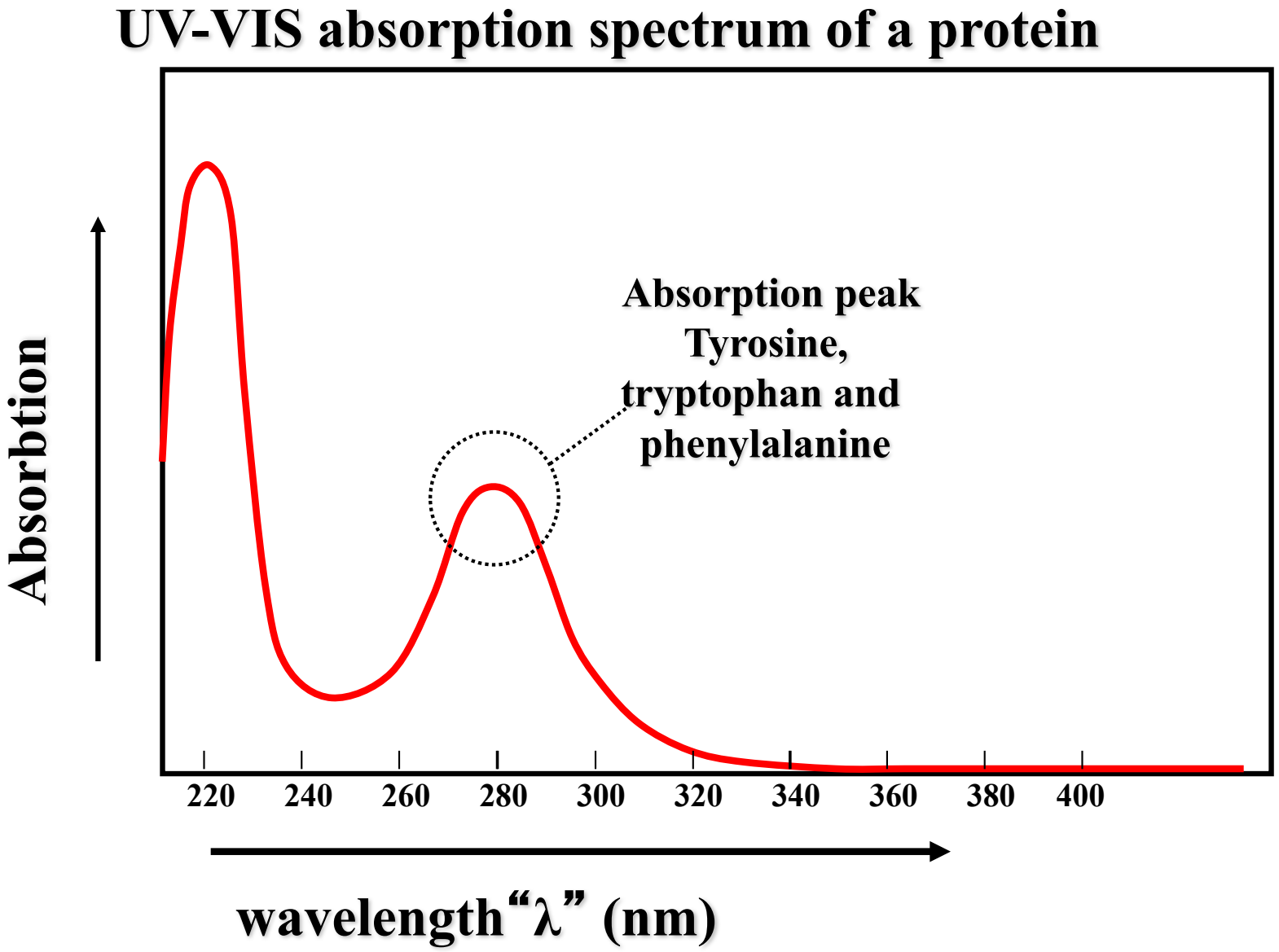


Tyr (Tirosina)



Phe (Fenilalanina)

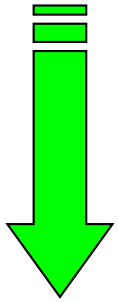
Protein concentration by Spectrophotometry



UV = 200 nm – 350 nm

DISADVANTAGES

- **Unless the protein is free from other light-absorbing compounds at 280 nm, the results will be inaccurate**
- **Nucleic acids interfere because of the purine and pyrimidine residues, which have an absorption maximum close to 260 nm, with a considerable absorption extending up to 280 nm**



If nucleic acids are the only contaminants, protein concentration can be estimated using the formula that corrects reasonably well for the nucleic acid content:

$$[\text{protein}]\ (\text{mg/ml}) = 1{,}55\ A_{280} - 0{,}76\ A_{260}$$

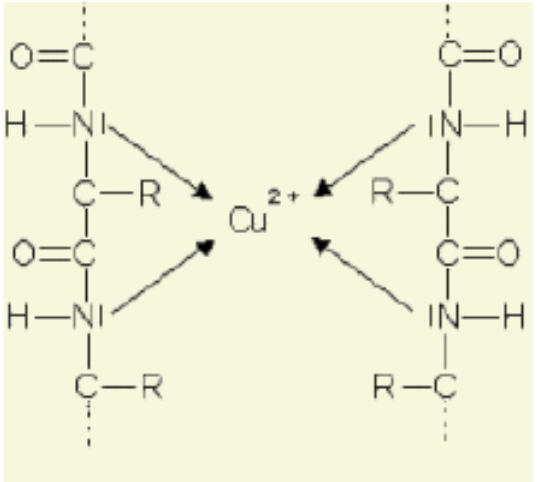
Biuret REACTION

$2\ NH_2\text{-}CO\text{-}NH_2 \xrightarrow{T \geq 160^\circ C} NH_2CONHCONH_2 + NH_3$

The name of this assay is somewhat confusing because assay for proteins using this method does not actually use biuret. Biuret is a small compound that forms when urea is heated which causes two urea molecules to join. In this assay protein samples are combined with Biuret Reagent

which contains copper ions in a basic solution. The

copper ions will complex with the amide groups in the proteins to create a blue color that will be measured using a spectrophotometer. The amount of blue color that forms is directly proportional to the quantity of protein in your samples.



Colorimetric Assays

- **Lowry Assay (BioRad *DC* Protein Assay):**

-The reaction is similar to the well-documented Lowry assay, but with some improvements.

-The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to color development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein.

-Copper ions facilitate the reduction step

-Color development is primarily due to the amino acids tyrosine and trypto-phan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

Section 3 Reagent Compatibility

The listed reagents were tested and found to be compatible with the *RC DC* Protein Assay. The presence of one or more of these substances may change the response of the protein to the assay reagents. Thus the protein standard should always be prepared in the same buffer as the protein sample.

Reagents	One Wash	Two Washes (Optional)
Dithiothreitol (DTT)	100 mM	350 mM
Tributylphosphine (TBP)	2 mM	-
β-mercaptoethanol	5%	10%
Sequential Extraction Buffer 2*	Not Compatible	Full Strength
Sequential Extraction Buffer 3**	Not Compatible	Full Strength
Laemmli Buffer (with 5% β-mercaptoethanol)	Full Strength	-
CHAPS	2%	-
Tween 20°	2%	-
Triton X-100°°	2%	-
EDTA	100 mM	-
Imidazole	500 mM	-
Tris, pH 8.4	500 mM	-
NaOH	2.5 M	-

°Tween is a registered trademark of ICI Americas, Inc.
°°Triton is a registered trademark of Rohm and Haas.
*40 mM Tris, 8 M urea, 4% (w/v) CHAPS, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 2 mM TBP (Catalog #163-2103)
**40 mM Tris, 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 2 mM TBP (Catalog #163-2104)

Colorimetric Assays

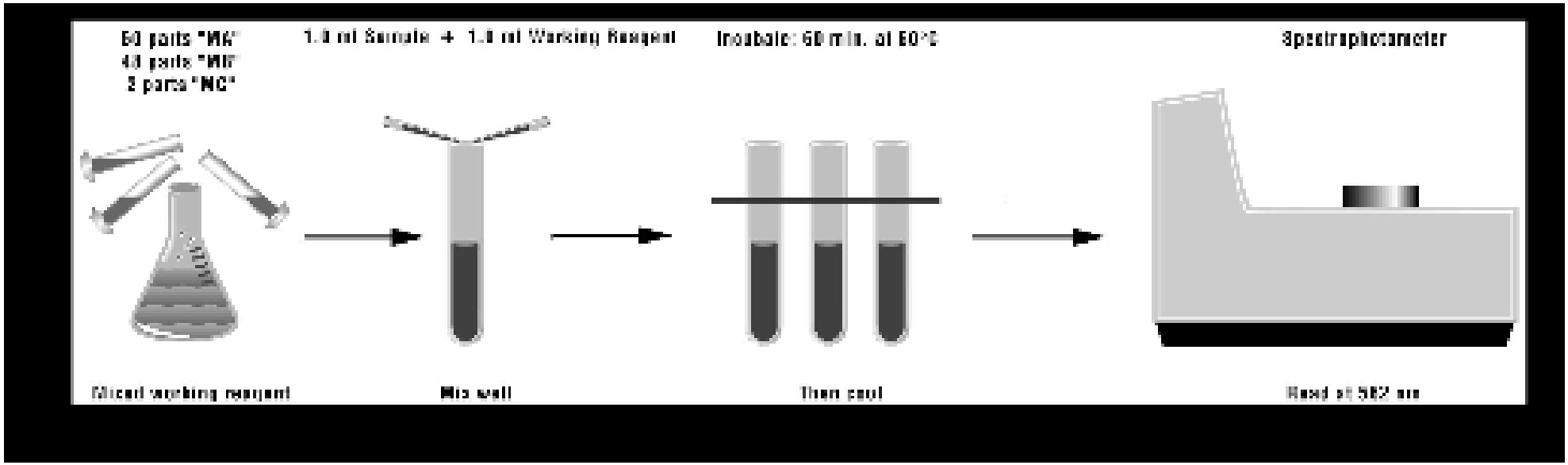
- **BCA method (Pierce micro BCA Protein Assay Kit):**

-It is a detergent-compatible formulation based on bicinchoninic acid (BCA) - This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction)

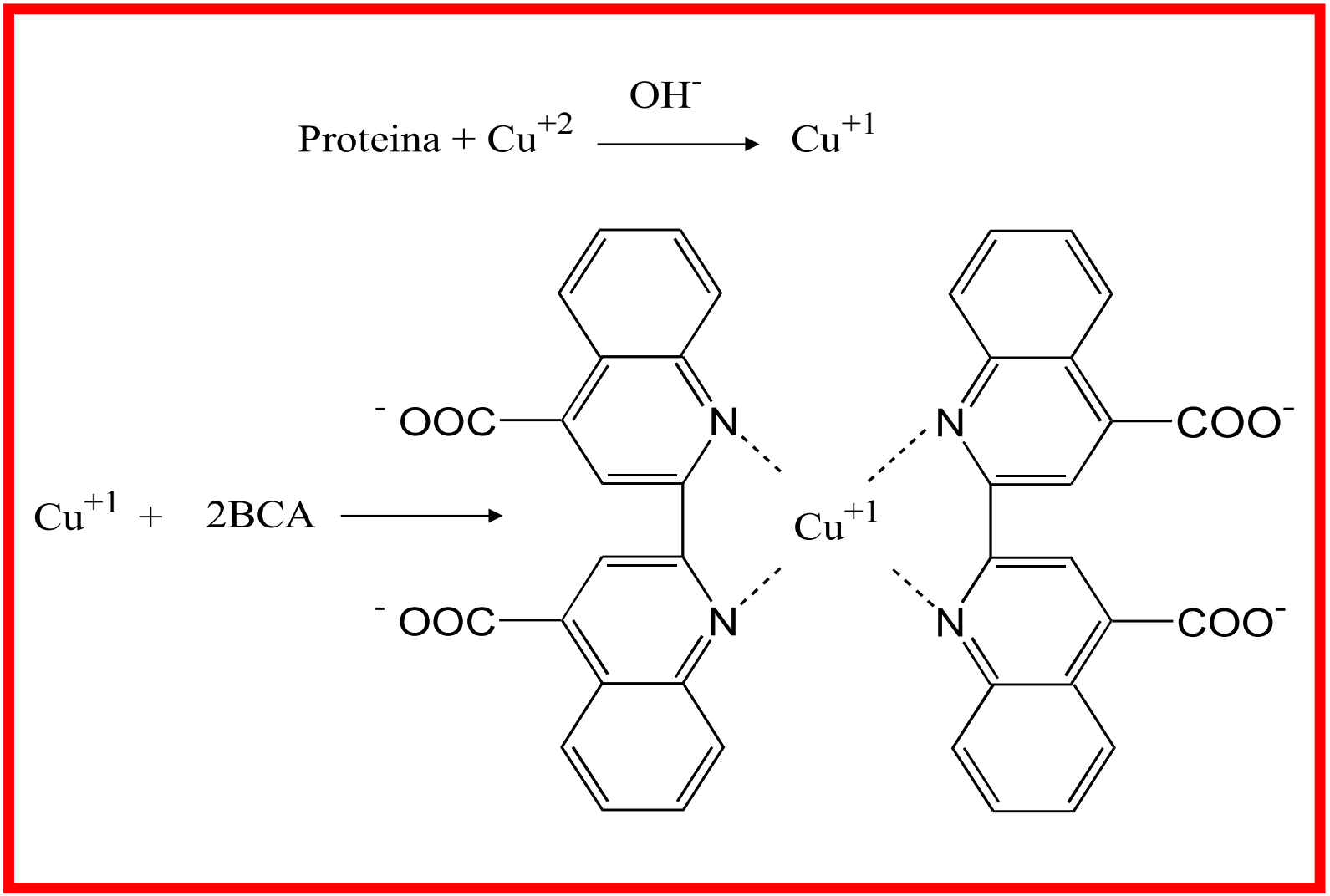
The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion.

This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL)

Incubation @37° C for 2 hours or 60° C for 1 h



BCA REACTION



Colorimetric Assays

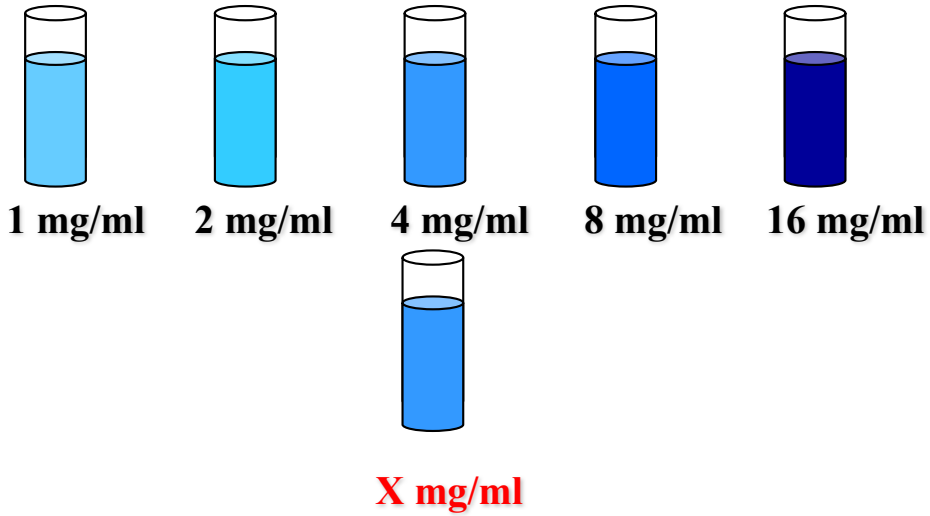
Bradford Assay (BioRad Protein Assay)

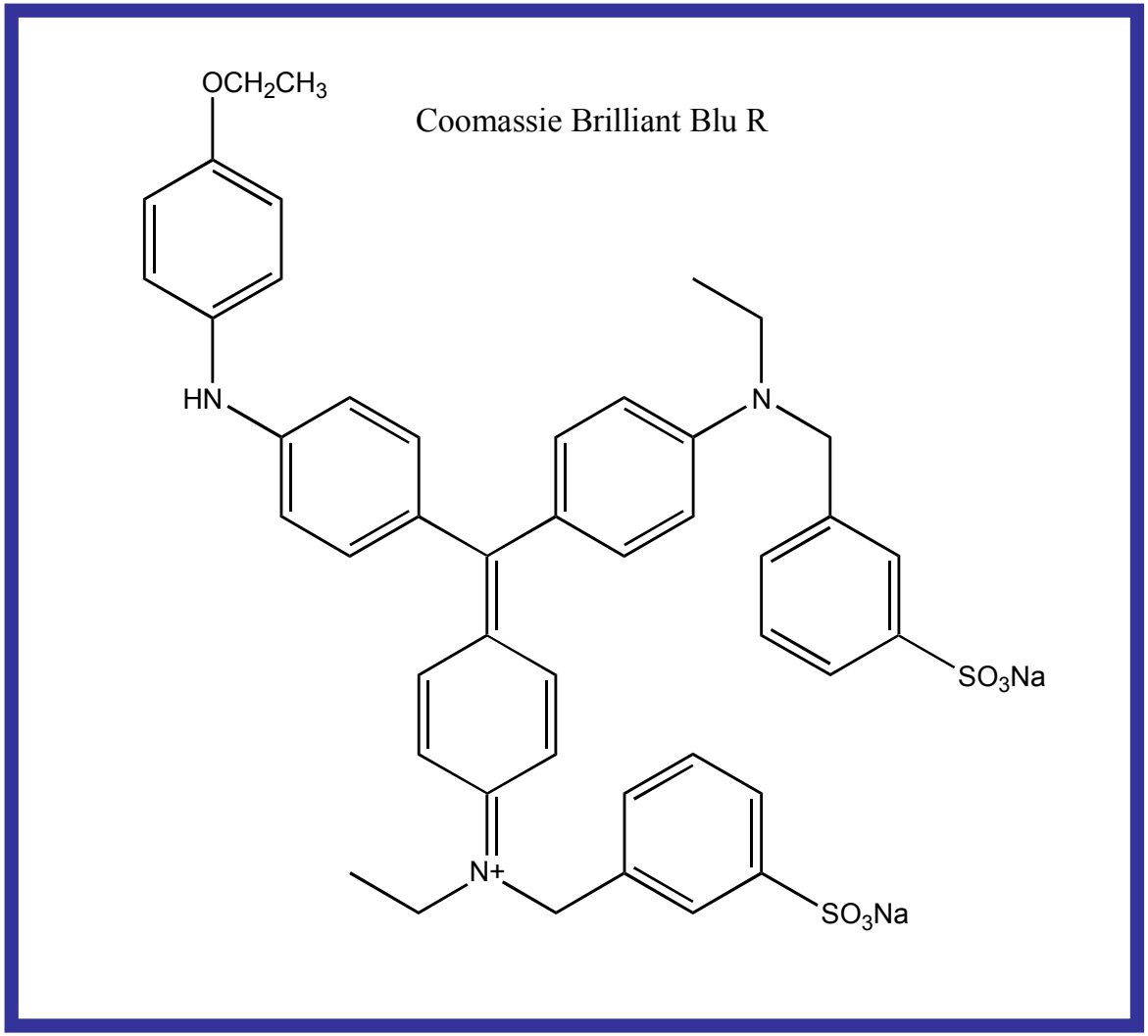
-The binding of *Coomassie Brilliant Blue* G-250 to proteins shifts the absorption peak of the dye from 465 nm (red) to 595 nm (blue) in acidic solutions (Bradford, 1976)

-This dye forms strong non-covalent complexes with proteins by electrostatic interactions with amino and carboxyl groups and by van der Waals forces

-Dye solution is prepared in phosphoric acid

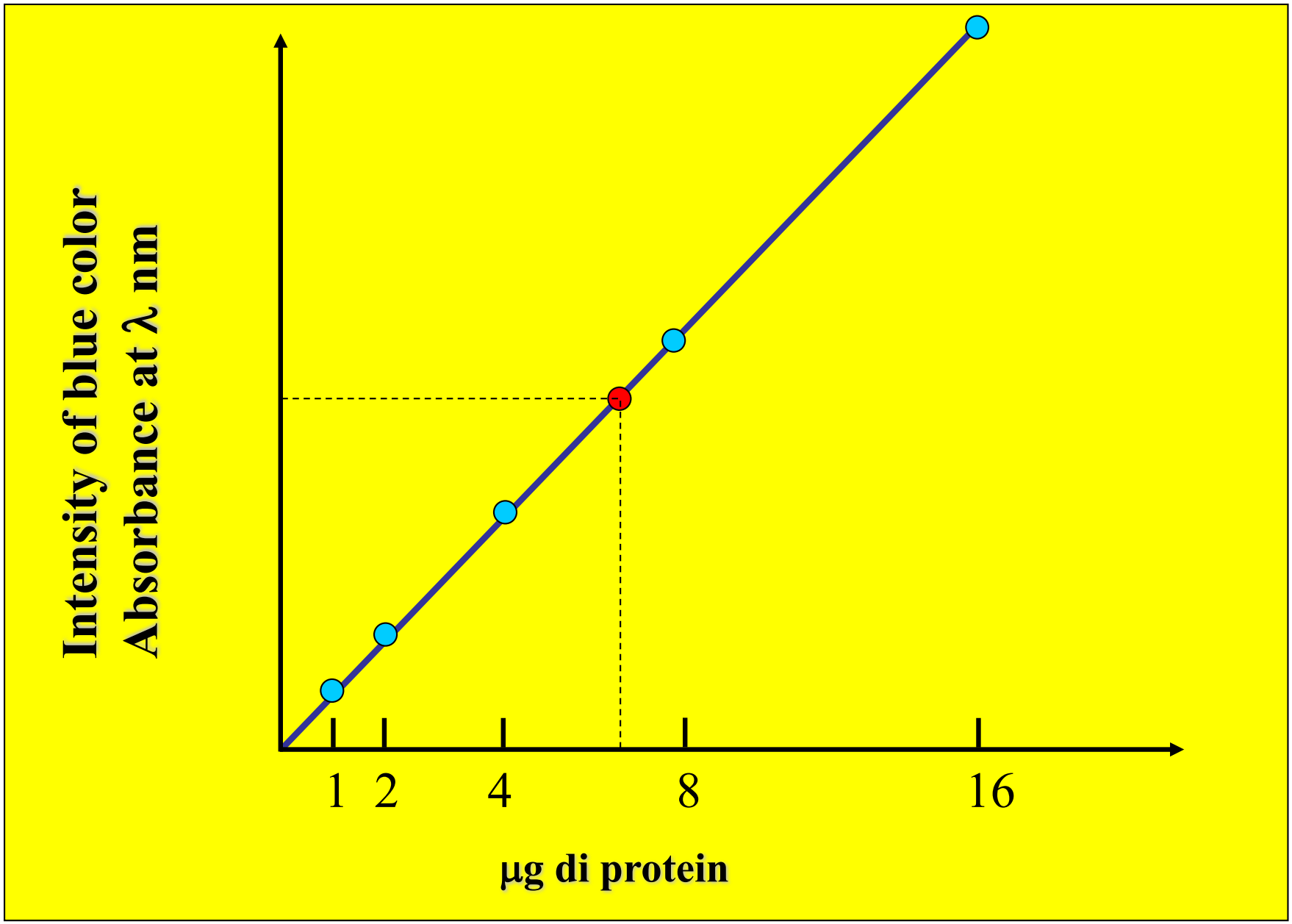
-The method is a simple procedure consisting of a single step where the dye is added to the samples and the absorbance is determined at 595 nm after a brief incubation time



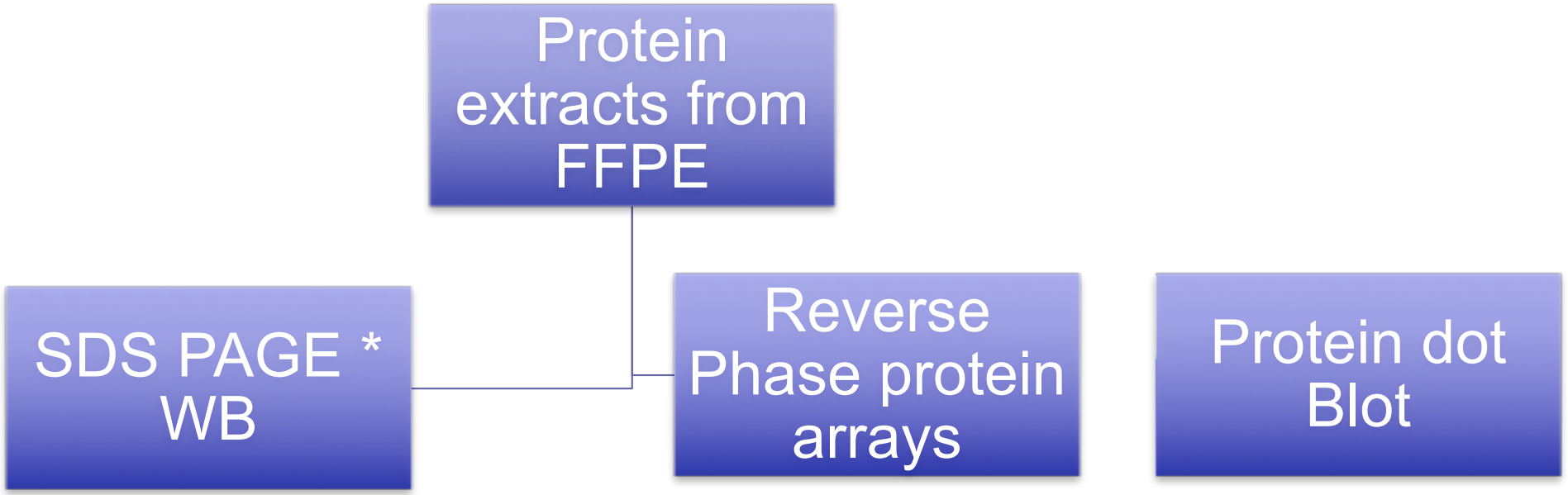


The amount of dye binding the protein is proportional to the amount of protein solution.
The intensity of the blue color (therefore the absorption) is proportional to protein concentration.
Generally equal amounts of different proteins bind the same amount of dye
→ the assay is independent of the type of protein

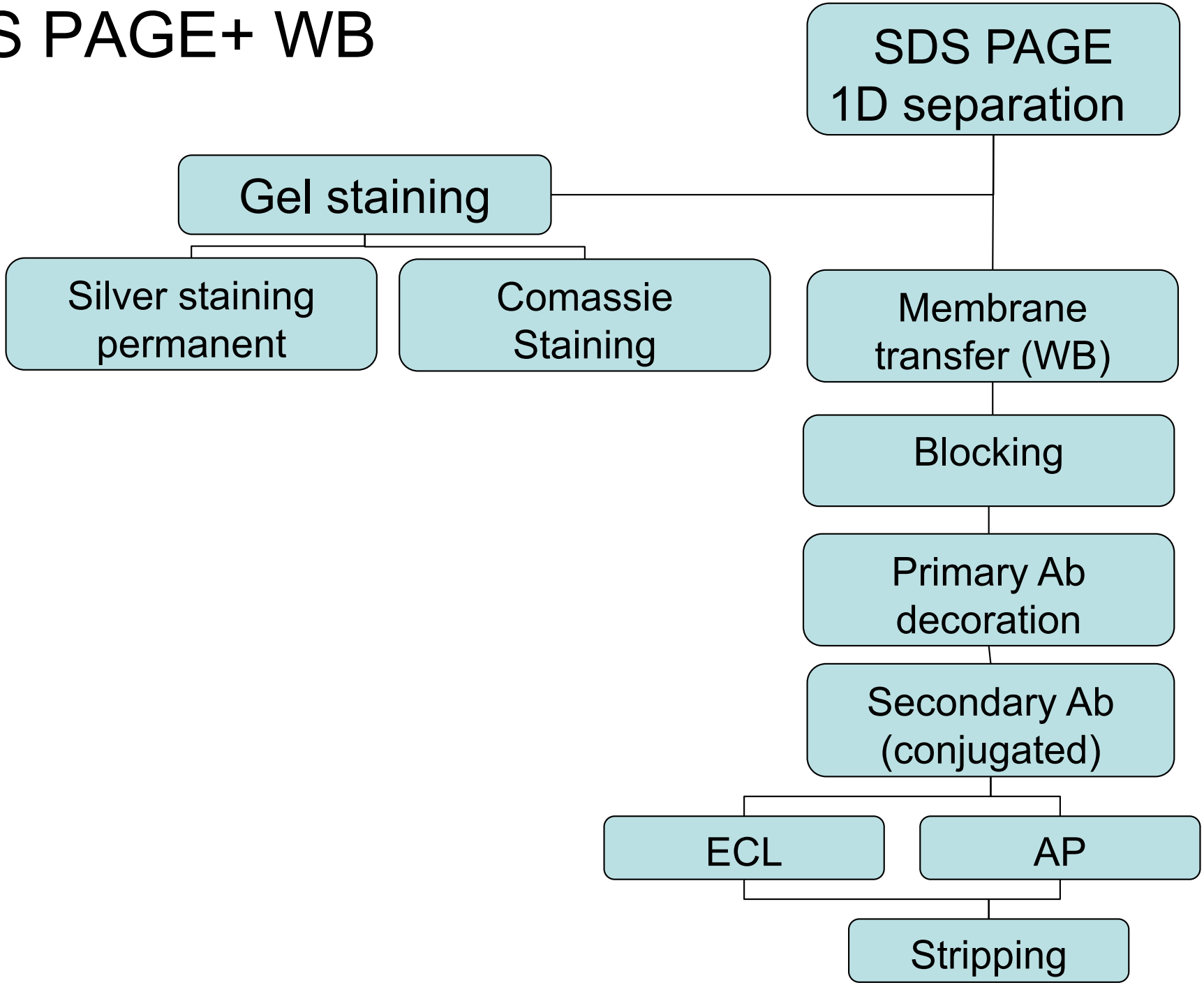
Standardization curve



Immunoassays for protein extracts from FFPE

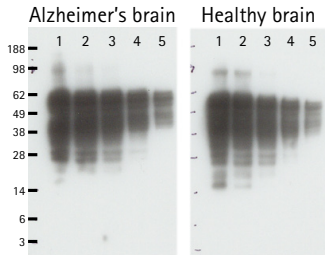
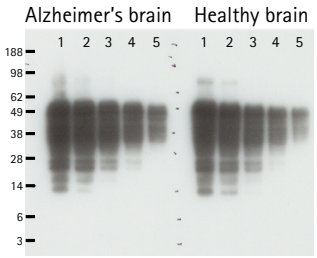
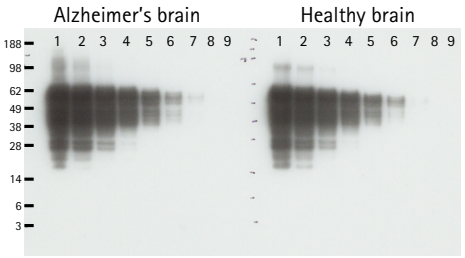
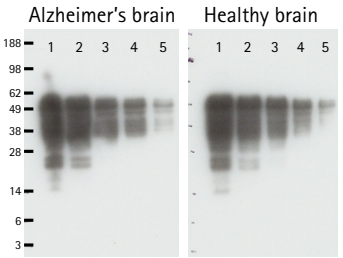


SDS PAGE+ WB


SDS PAGE
1D separation
Gel staining
Silver staining
permanent
Comassie
Staining
Membrane
transfer (WB)
Blocking
Primary Ab
decoration
Secondary Ab
(conjugated)
ECL
AP
Stripping

Snap id

Western blot optimization of anti-Tau-1 antibody in Alzheimer's disease brain and healthy brain samples

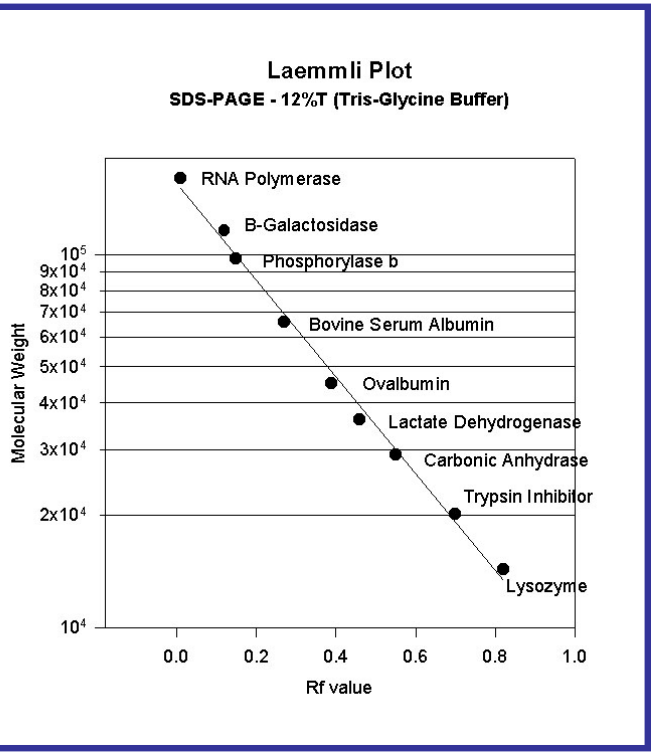
	SNAP i.d.® 2.0 MultiBlot	SNAP i.d.® 2.0 Mini Blot	SNAP i.d.® 2.0 Midi Blot	Standard Western blot detection
MW				
Blocking	0.5% NFDM for 20 sec	0.5% NFDM for 20 sec	0.5% NFDM for 20 sec	0.5% NFDM for 1 hr
Primary Antibody	Anti-Tau1 1: 1,000 for 10 min	Anti-Tau1 1: 1,000 for 10 min	Anti-Tau1 1: 1,000 for 10 min	Anti-Tau1 1: 5,000 for 1 hr
Secondary Antibody	Goat anti-Mouse 1: 10,000 for 10 min	Goat anti-Mouse 1: 10,000 for 10 min	Goat anti-Mouse 1: 10,000 for 10 min	Goat anti-Mouse 1: 50,000 for 1 hr
Total Time	< 30 min	< 30 min	< 30 min	3 hr 30 min

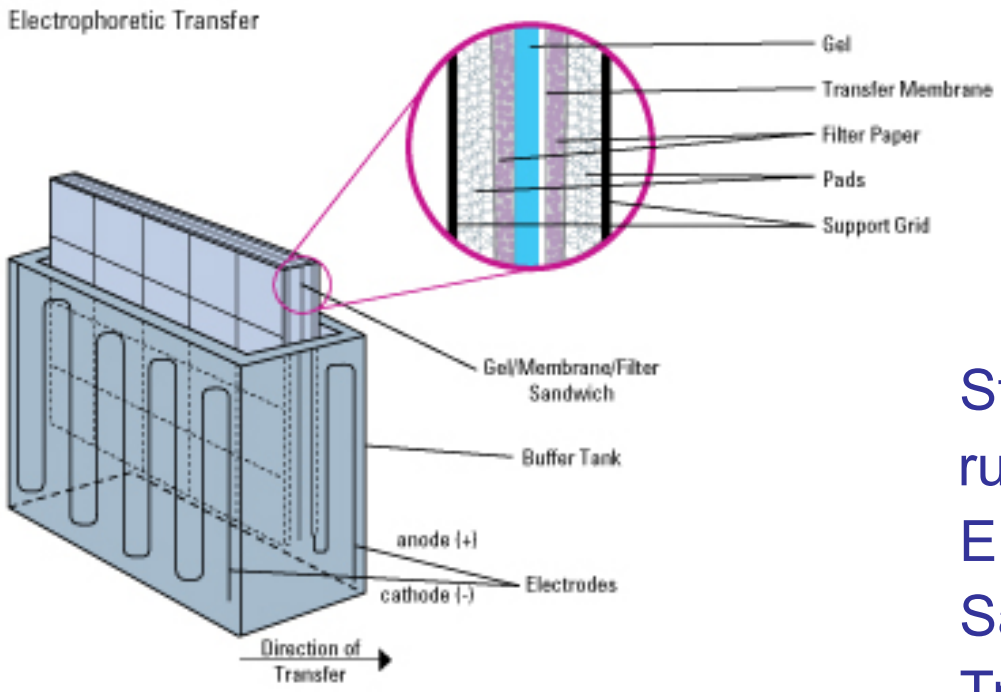
SDS-PAGE + WB

- SDS, anionic detergent, protein denaturation with negative charges ⇒ eliminate the influence of structure and charges.

-Proteins are separted by their molecular weigth.

-there is a linear relationship between proteins log MW and their Rf=distance from gel top to peptide/distance from gel top to dye





Steps

running e stacking gel

Electrophoresis

Sandwich

Transfer

Stacking gel:

Tris-HCl 1M pH6.8	520µl
Acril-Bis (30:0.8)	640 µl
SDS 10%	40 µl
H_2O	2.96 ml

Temed 6 µl, APS 80 µl

Running Gel:

H_2O	3 ml
Tris-HCl 1.5 M pH8.8	4.5 ml
Acril-Bis (30:0.8)	8 ml
SDS 10%	150 µl

Temed 6 µl, APS 150 µl

% PAA	Range di proteine (kDa)
8	40-200
10	21-100
12	10-40

Running Buffer 10x: Tris 0.25 M (30.3 g Tris), Glicina 1.9 M (144.2 g) SDS 1% (10 g). Per 1 l.

Sample buffer: 2 ml glycerol, 4 ml SDS 10%, 0.5 ml Tris 1M pH 6.8, H_2O 3 ml, 50 µl di 2-mercaptoethanol for wach ml solution. Touch BPB with a tip.

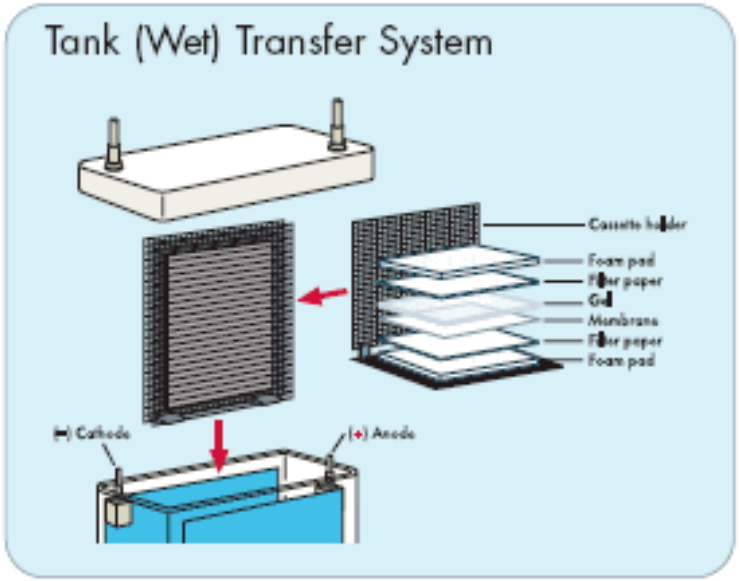
Electrophoresis

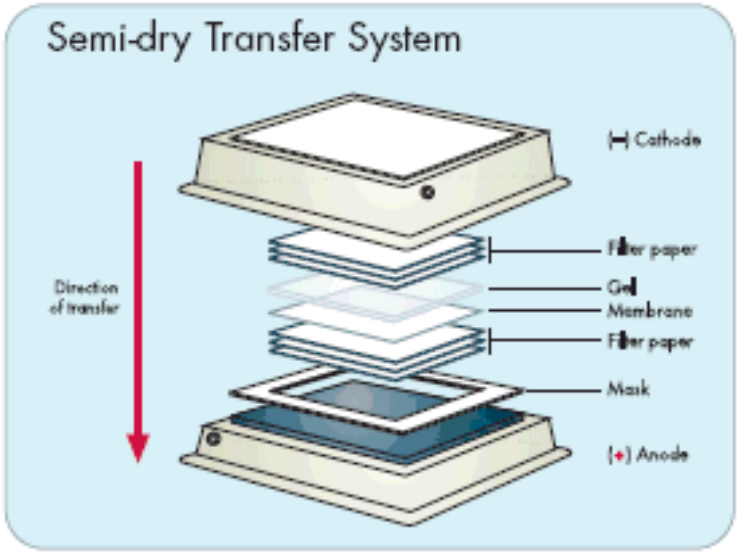
Proteins + sample buffer @95° C 5-10 min

120 V up to the exit from stacking gel, then 180V.

Protein Transfer

- Semidry blotting Horizontal
- Wet blotting Vertical





Tris -Glycine 10x: 30 g Tris, 144 g glycine -1l
Transfer Buffer: Tris - Glycine 1x final-MeOH (20% v/v final)

Wet:

- Gel in transfer buffer 15 min
- Activation of PVDF membrane in MeOH then transfer
- Sandwich- without air bubbles
- Transfer by swirling @ 30 V (small18 V) or 3-4 h @45 V(25mA)
- RT using cold buffer or cold room

Semidry

- Gel in transfer buffer 15-20 min
- Wash anode plate with ddH_2O
- Activation of PVDF membrane in MeOH then transfer
- Sandwich- without air bubbles
- with wet 3MM paper (wet in transfer buffer)
- Cathode plate – wash with ddH_2O
- 0.5 kg over
- Transfer 15V 15 min minigel; 25 V 30 min big

Membrane staining with Ponceau red

Aqueous solution: 0.2% dye in 3% of TCA

Stain for 3-5' R.T

Wash with water up to the bands appear, mark and take a picture

Continue with the usual incubations of immuno blotting.

Red color does not interfere with ECL, but it can be eliminated by PBS or other saline solution washing.

Membrane Stripping

Stripping Buffer : 2% SDS, 100 mM 2-mercaptoethanol, 50 mM Tris, pH 6.8

Procedure:

1. Heat stripping buffer to 50° C in water bath
2. Incubate blot with stripping buffer at 50°C for 15 to 30 minutes with shaking
3. Rinse blot multiple times in TBS
4. Block and blot as normal

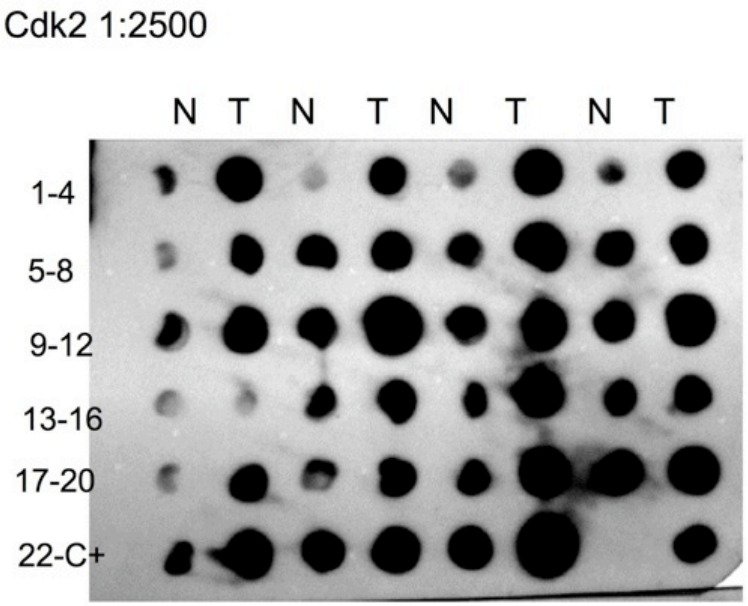
COLORIMETRIC DETECTION

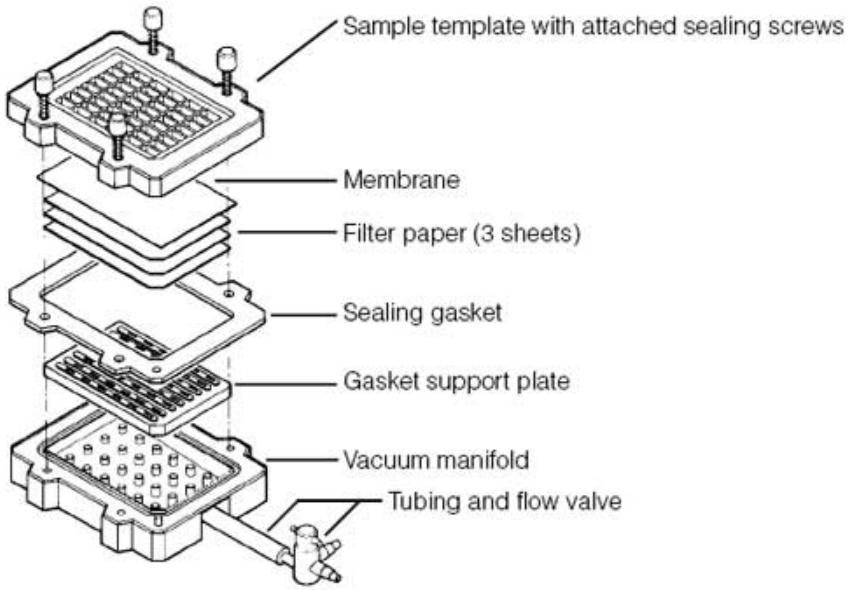
- ✓ The Western blot detection method is based on the binding of the primary Ab to a secondary Ab conjugated with the alkaline phosphatase or peroxidase.

- ✓ For AP the substrate for staining consists of 5-Br-4-Cl-indolyl-phosphate (BCIP) in combination with tetrazolium nitro-blue (NBT).
- ✓ The enzyme converts BCIP to the hydroxyl compound (enol) which precipitates and tautomerizes to ketone
- ✓ Ketone oxidizes and dimers to form the indigo, blue color. The hydride ions released by the dimerization reaction are taken by the NBT. The reduction causes the formation of a purple compound which is deposited with the indigo on the site of enzymatic activity.
- ✓ With AP the color development can continue for hours or ON, while the HRP is inactivated by the substrate, so the actual reaction time is about 30 '.

DOT BLOT PROTEICO

1. PVDF membrane activation
2. Membrane in PBS
3. Assembly of the appartus
4. Spot the protein using the extraction buffer
5. Protein block as for WB

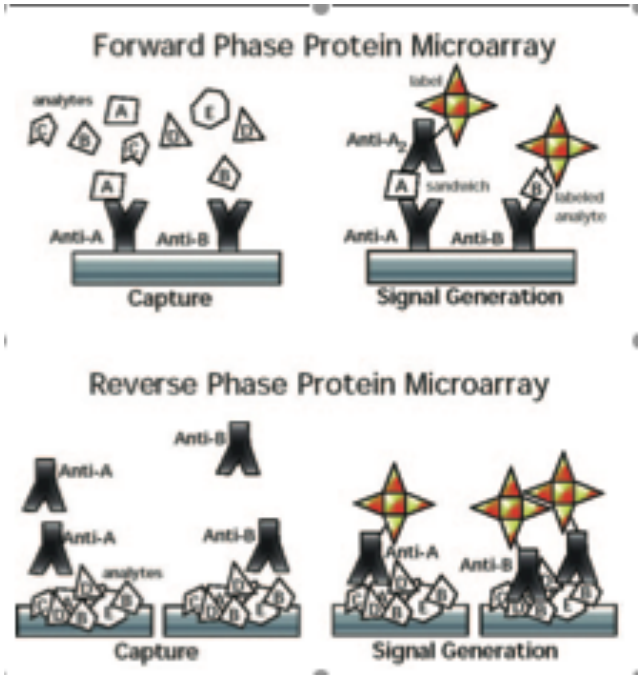




2µg di estratto proteico per ogni campione; 5' di esposizione

Protein microrrays

- Protein microarrays
  - Protein profiling
  - Functional protein Arrays
    - Reverse phase protein Arrays
    - Foward phase protein Arrays



Protein microrrays

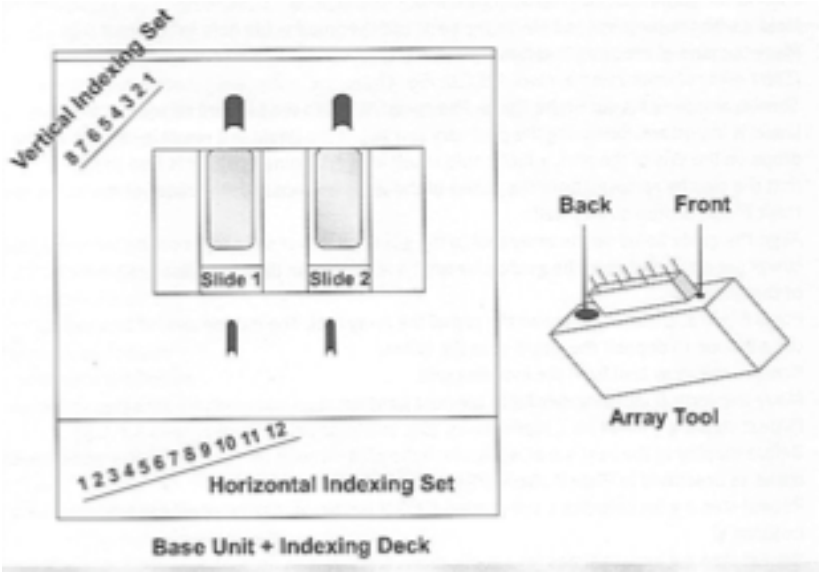
Forward protein microarrays

- *In a forward-phase array format, a large number of immobilized capture molecules enable the analysis of many parameters from a single sample*
- Each array, is incubated using a (lisato o siero di pazienti).
- *More analytes are detected*

Reverse phase protein microarrays

- Micro dot-blot
- *In a reversed-phase array, many samples are immobilized.*
- *highly specific antibody used to analyze the expression of a single parameter in the immobilized samples*.
- *Specificity Ab* $\Rightarrow$ *crossreaction and Ab availability*

MicroCaster System



Orientation of slides and pins

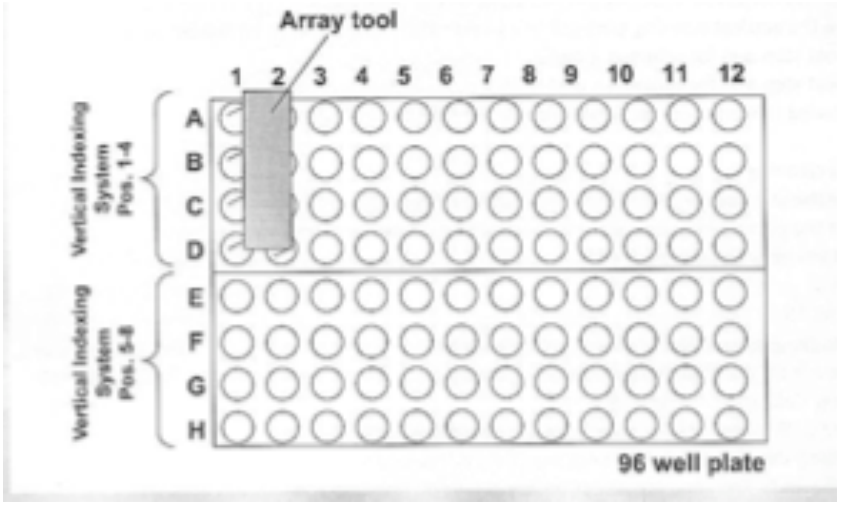
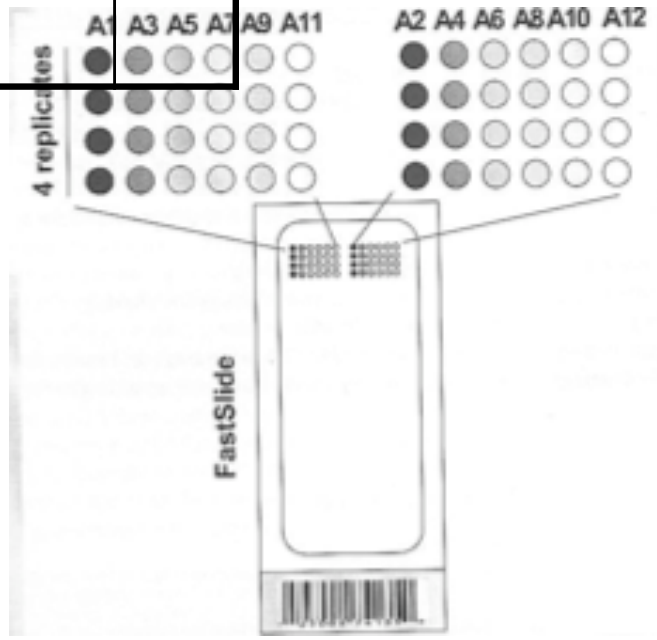


Plate with samples to spot

	1	2	3	4	5	6	7	8	9	10	11	12
A	**S1 undil**	**S2 undil**	**S1 1:2**	**S2 1:2**	**S1 1:4**	**S2 1:4**	**S1 1:8**	**S2 1:8**	**S1 1:16**	**S2 1:16**	Buff.C trl.	Buff.C trl.
B	**S5 undil**	**S6 undil**	**Ect.**									
C												
D												
E	**S3 undil**	**S4 undil**	**S3 1:2**	**S4 1:2**	**S3 1:4**	**S4 1:4**	**S3 1:8**	**S4 1:8**	**S3 1:16**	**S4 1:16**	Buff.C trl.	Buff.C trl.
F	**S7 undil**	**S8 undil**	**Ect.**									
G												
H												

From sample to array



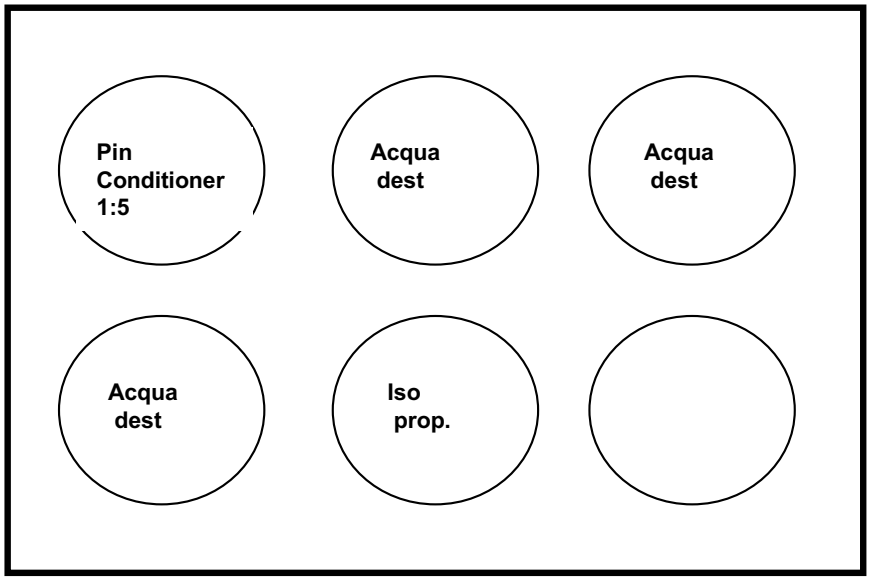


Plate A: for coating

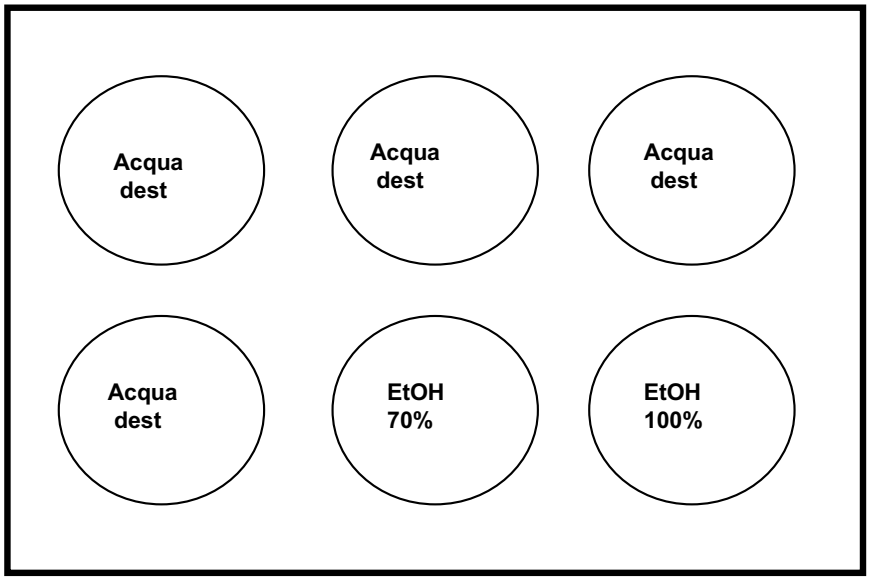


Plate B: for washing

Fasi:

1. **Prepare the plate with the dilutions, seal it, centrifuge it and keep it on ice.**
2. **Wash and balance pins**
3. **Array set up**
4. **Wash between one spot and another**

Protein microarrays