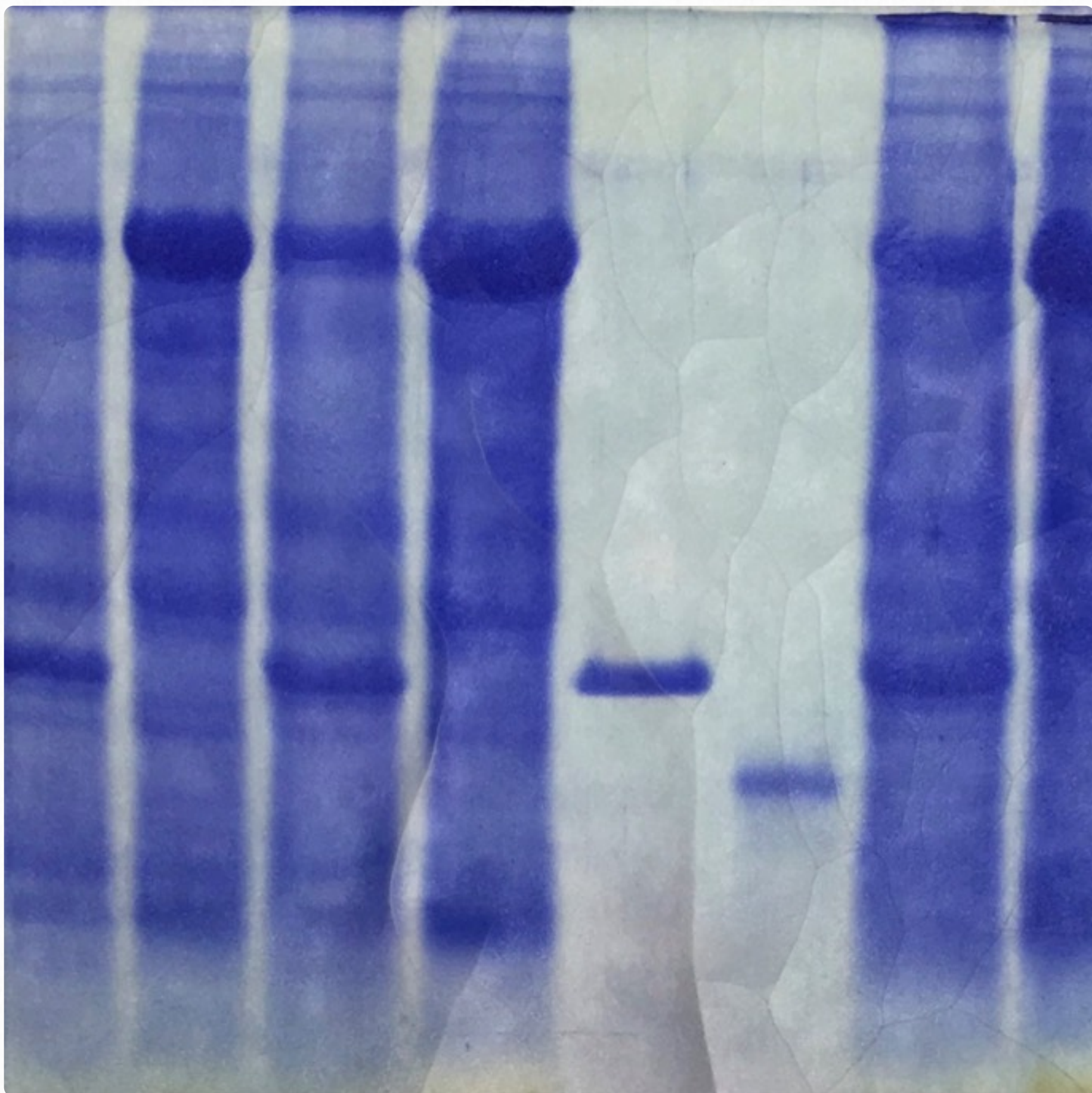


Ed Rybicki's

# Manual of SDS-PAGE and Immunoblotting Techniques



1

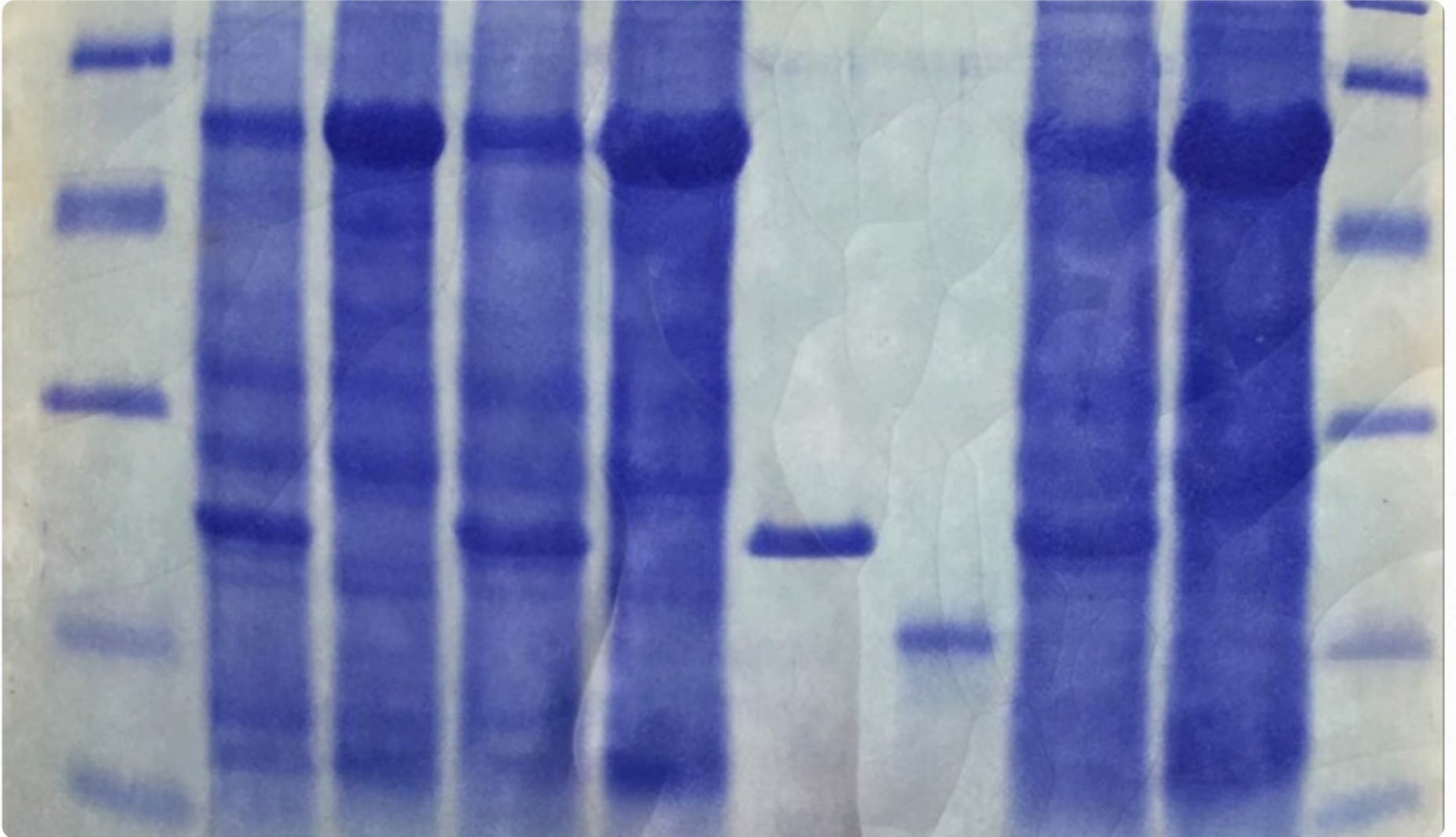
# SDS-PAGE

“If at first you don’t succeed  
- hide all evidence you  
tried”

– anon.



# Introduction to gel electrophoresis



## INTRODUCTION

### Support Matrices

### Separation of Proteins and Nucleic Acids

### SDS-PAGE of Proteins

- Separation of Proteins under Denaturing conditions
- Determination of Molecular Weight

- Continuous and Discontinuous Buffer Systems
- Non-Denaturing Gel Electrophoresis

# INTRODUCTION

Electrophoresis is the **migration of charged molecules in solution in response to an electric field**. Their rate of migration depends on:

- the **strength** of the field;
- the **nett charge, size and shape** of the molecules
- the **ionic strength, viscosity and temperature** of the medium in which the molecules are moving.

As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used **analytically** to study the properties of a single charged species, and as a **preparative separation technique**.

## Support Matrices

Generally the sample is run in a **support matrix such as paper, cellulose acetate, starch gel, agarose or polyacrylamide gel**. The matrix inhibits **convective mixing caused by heating** and **provides a record of the electrophoretic run**: at the end of the run, the matrix can be **stained and used for scanning, blotting, autoradiography or storage**.

In addition, the **most commonly used support matrices - agarose and polyacrylamide** - provide a means of **separating molecules by size**, in that they are porous gels. A **porous gel may act as a sieve by retarding**, or in some cases completely obstructing, **the movement of large macromolecules** while allowing smaller molecules to migrate freely.

Because dilute agarose gels are generally more rigid and easy to handle than polyacrylamide of the same concentration, **agarose is used to separate larger macromolecules such as nucleic acids, large proteins and protein complexes**. **Polyacrylamide**, which is easy to handle and to make at higher concentrations, **is used to separate most proteins and small oligonucleotides that require a small gel pore size for retardation**.

# Separation of Proteins and Nucleic Acids

**Proteins are amphoteric compounds;** their nett charge therefore is determined by the pH of the medium in which they are suspended. **In a solution with a pH above its isoelectric point, a protein has a nett negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode.** The nett charge carried by a protein is in addition independent of its size - ie: the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, **the electrophoretic separation of proteins is determined by both size and charge of the molecules.**

**Nucleic acids however, remain negative at any pH used for electrophoresis** and in addition carry a **fixed negative charge per unit length of molecule**, provided by the  $\text{PO}_4$  group of each nucleotide of the the nucleic acid. **Electrophoretic separation of nucleic acids therefore is strictly according to size.**

## SDS-PAGE of Proteins

### *Separation of Proteins under Denaturing conditions*

**Sodium dodecyl sulphate (SDS)** is an **anionic detergent** which denatures proteins by "wrap-ping around" the polypeptide backbone - and **SDS binds to proteins fairly specifically in a mass ratio of 1.4:1**. In so doing, **SDS confers a negative charge to the polypeptide in proportion to its length** - ie: the denatured polypeptides become "rods" of negative charge cloud with **equal charge or charge densities per unit length**. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with **2-mercaptoethanol or dithiothreitol**. In denaturing SDS-PAGE separations therefore, **migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.**

## ***Determination of Molecular Weight***

This is done by SDS-PAGE of proteins - or PAGE or agarose gel electrophoresis of nucleic acids - of known molecular weight along with the protein or nucleic acid to be characterised. A **linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide, or native nucleic acid, and its R<sub>f</sub>**. The R<sub>f</sub> is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front. A simple way of determining relative molecular weight by electrophoresis (M<sub>r</sub>) is to plot a standard curve of distance migrated vs. log<sub>10</sub>MW for known samples, and read off the logM<sub>r</sub> of the sample after measuring distance migrated on the same gel.

## ***Continuous and Discontinuous Buffer Systems***

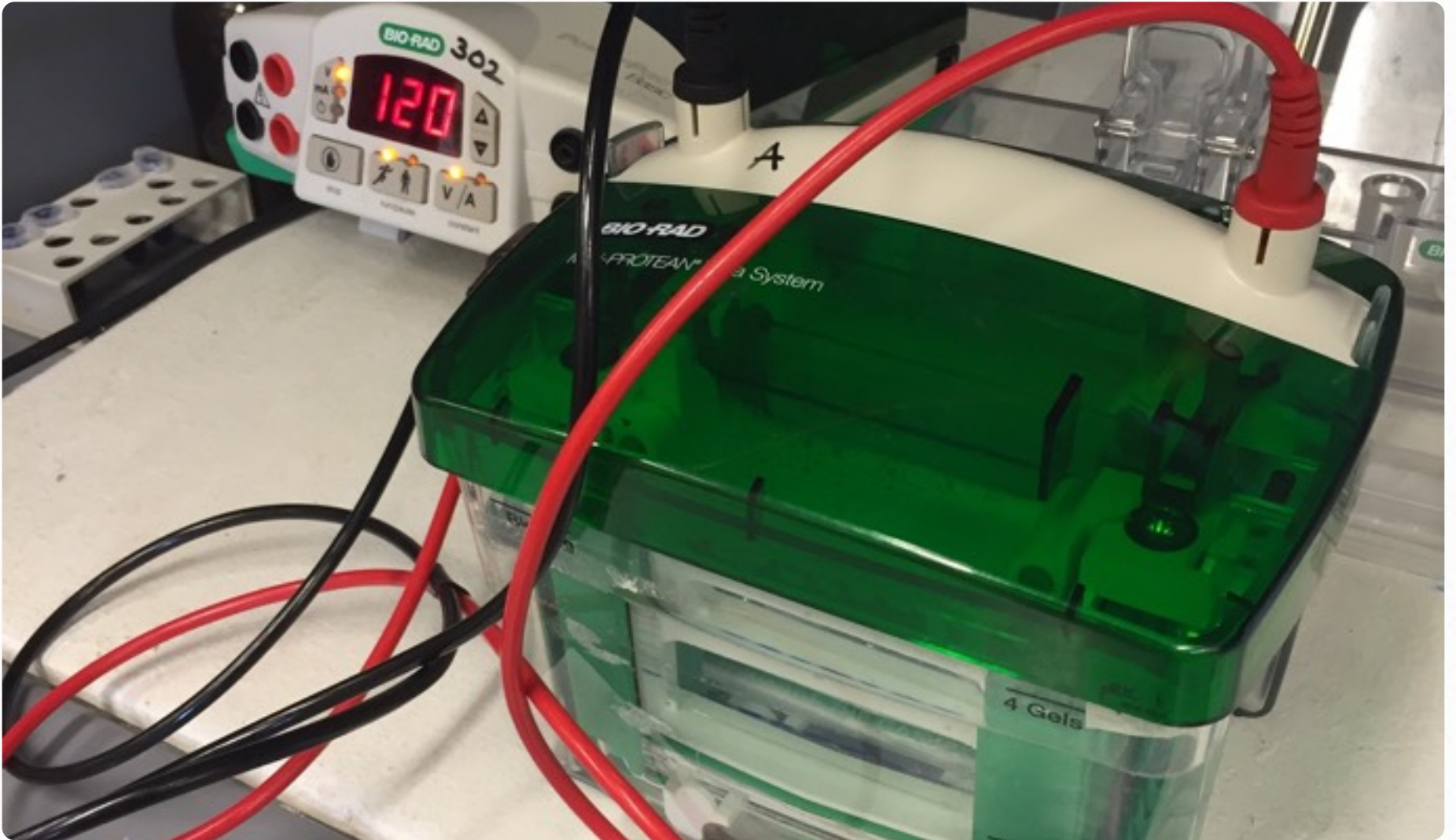
There are two types of buffer systems in electrophoresis, **continuous and discontinuous**. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. **The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system** (read about this in any textbook).

## ***Non-Denaturing Gel Electrophoresis***

It is possible to blot non-denaturing gels - gels run without any SDS - however, **one must be very careful to choose a blotting buffer in which all of the proteins of interest migrate towards the same electrode**.



# SDS-PAGE Protocols



## PROTOCOL

Assembling Gel Apparatus

Resolving Gels

Stacking Gels

Electrophoresis buffer

Sample Preparation

Staining of Gels

**NB: ACRYLAMIDE MONOMER IS A POTENT CUMULATIVE NEUROTOXIN. DO NOT MOUTH PIPETTE ACRYLAMIDE SOLUTIONS, AND WEAR GLOVES WHEN HANDLING UNPOLYMERISED SOLUTIONS.**

## Assembling gel apparatus:

Assemble glass / backing plates with two side spacers, clamps, grease, etc. as shown by demonstrators **or manufacturer instructions**. Stand assembly upright using clamps as supports, on glass plate or in sealed stand.

If you are using a home-made assembly, pour some pre-heated 1% agarose onto glass plate, place assembly in pool of agarose: **this seals the bottom of the assembly**.

## Resolving Gels:

Example: Gel concentration of 12.5% in 0.25 M Tris-HCl pH 8.8

Reagent:	Volume (ml: TO MAKE 30ML)	Volume (ml: TO MAKE 10ML)
40% Acrylamide stock*:	9.4	3.1
water (distilled)	12.3	3.8
1M Tris-HCl pH 8.8	7.5	2.5
10% SDS	0.3	0.1
Peroxydisulphate 1%	0.5	0.5
TEMED (added last)	20ul	20ul

\* = 19:1 - 38:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide

Mix ingredients **GENTLY!** in the order shown above, ensuring no air bubbles form. Pour into glass plate assembly **CAREFULLY**. Overlay gel with **isopropanol** via syringe or Pasteur pipette (with bulb!) to **ensure a flat surface and to exclude air**. Wash off isopropanol with water after gel has set (+15 min).



## Stacking Gels:

Example (good for most uses): **Gel concentration of 4.5%** in 0.125 M Tris-HCl pH 6.8

Reagent:	Volume (ml TO MAKE 15 ML)	Volume (ml TO MAKE 10 ML)
40% Acrylamide stock	1.7	1.1
water	10.8	7.1
1M Tris-HCl pH 6.8	1.9	1.25
10% SDS	0.15	0.1
Peroxydisulphate 1%	0.5	0.5
TEMED (stir quickly)	20ul	20ul

Mix as before, then **pour onto top of set resolving gel**, insert comb, allow to set. Then **CAREFULLY AND SLOWLY** remove comb, fill with electrophoresis buffer. Assemble top tank onto glass plate assembly. Fill with electrophoresis buffer.

## Electrophoresis buffer

The final TANK buffer composition is **196mM glycine / 0.1% SDS / 50mM Tris-HCl pH 8.3**, made by diluting a **10x stock solution**. This goes in both top and bottom tanks.

## SDS-PAGE disruption mix:

This is **125mM Tris-HCl pH 6.8 / 10% 2-mercaptoethanol / 10% SDS / 10% glycerol**, containing a little **bromophenol blue**. Store at 4degC

## Sample Preparation:

**Example:** Grind a little leaf material (eg. 2 grams) in a mortar. Centrifuge in an Eppendorf tube for 3 min. Take supernatant and mix 100ul 1:1 (v:v) with. BE CAREFUL WITH THIS AS IT SMELLS AWFUL and is poisonous to boot!!

For **liquid / purified samples**, take eg. 100 ul and add 50 - 100 ul of disruption mix.

**Heat sample Eppendorfs for 5 min at 95°C** in a polystyrene "float" in a waterbath or in a dedicated heating block. Layer samples **under** the buffer in wells in stacking gels. Connect up apparatus and electrophorese.

## Staining of Gels:

### 1. Coomassie Brilliant Blue/Page-Blue 83

Make up stain: 0.2% CBB (w:v) in **45:45:10% (v:v) methanol:water:acetic acid**.

Cover gel with staining solution, seal in plastic box and leave overnight on shaker (RT) or for 2 to 3 hours at 37°C, also with agitation.

**Destain** with **25:65:10% (v:v) methanol:water:acetic acid** mix, with agitation, for as long as required.

**Coomassie brilliant blue G-250 detects to about 0.3 ug/band**. The stain is not easily reversible, and most often the protein **cannot be recovered intact** for other procedures.

### 2. Copper Chloride (0.3M CuCl<sub>3</sub>) [Lee et al., Anal. Biochem. 166, 303-312; 1987]

Rinse gel in distilled water, **immerse in copper chloride solution with agitation** for about 20 minutes (RT), **rinse with distilled water** and immerse in **sufficient fresh distilled water to cover the gel** (this acts as the destaining step). Seal in a plastic box for storage.

**Copper stained gels are negatively stained**: the bands are transparent and the background is opaque, so they are best viewed lit from above against a dark background. The stain is about as sensitive as Coomassie, but no organic solvents are used.

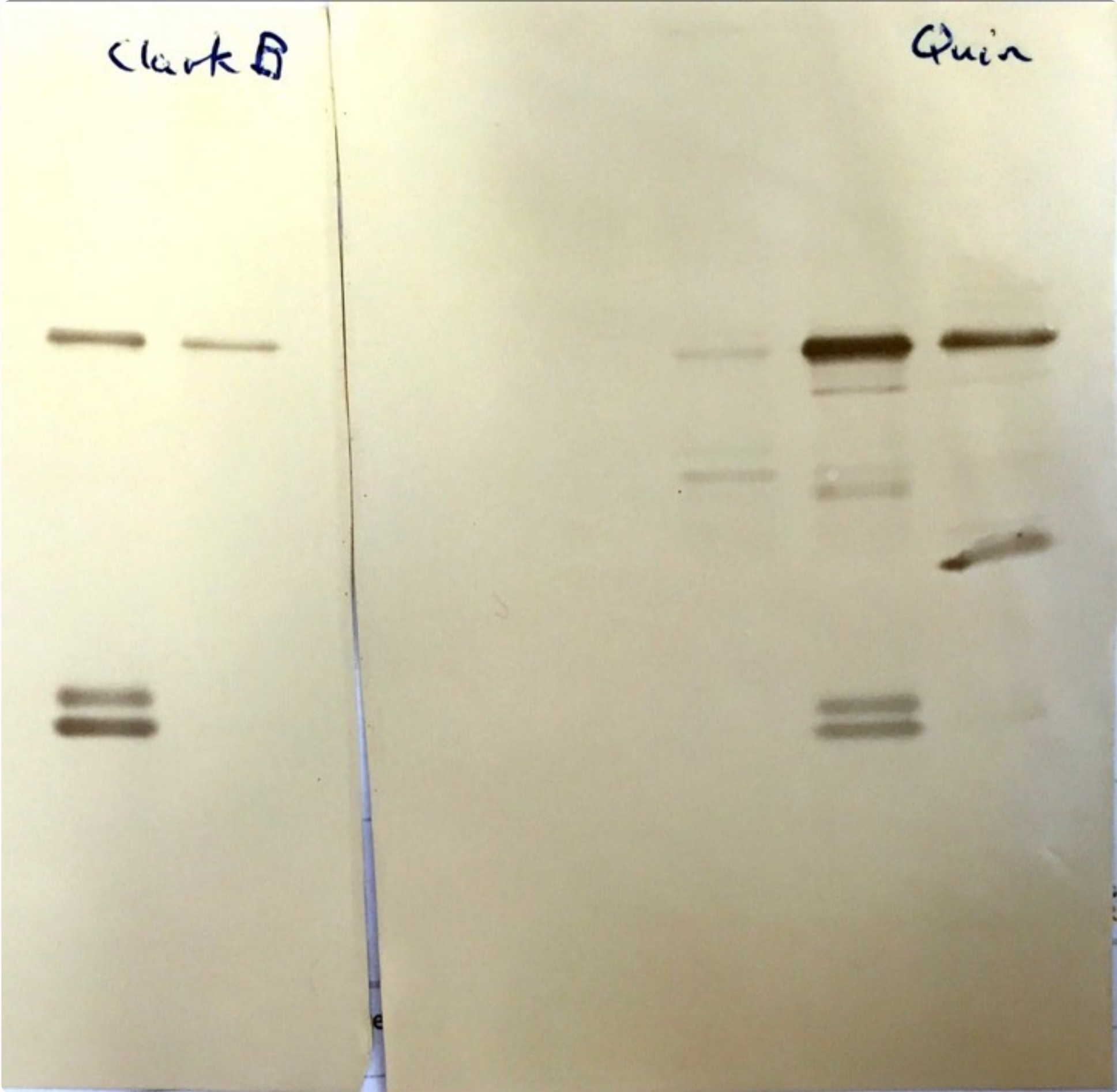
**Gels may be stored in water for up to several months at room temperature** with no problem or fading: proteins are immobilised as a **Cu-SDS-polypeptide complex in the gel**, which remains clear; the colour and background opacity are due to a Cu-SDS-Tris complex.

**Gels may be destained completely by repeated washing in 0.1-0.25 M Tris/0.25 M EDTA pH 8.0**, and then electroblotted, or eluted from the gel for other purposes.

# 2

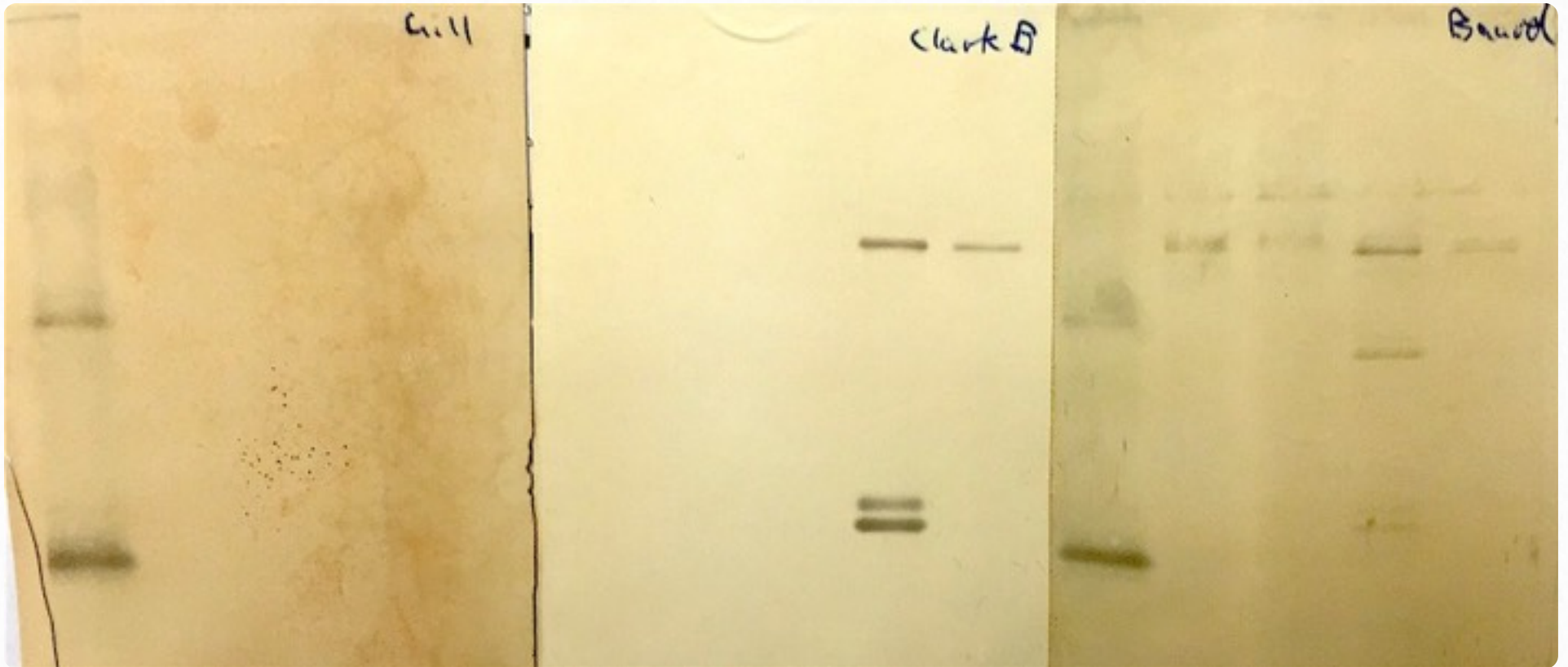
## Western Blotting

“Forget the hypotheses,  
let’s just *discover* some-  
thing!”  
– anon.





# Introduction to western blotting



Western blot of extracts of virus-infected wheat plants, probed with three different rabbit antisera.

## INTRODUCTION

Electroblotting

Immunoassay

# Electroblotting

Electroblotting has been a feature of a large number of laboratories for over 30 years now, and there are a large number of different apparatus around that will **efficiently transfer proteins (or other macromolecules) transversely from polyacrylamide (or other) gel to membrane**. Most of these were based on the design of Towbin et al. (1979): ie., they have **vertical carbon / stainless steel / platinum electrodes larger than the gel in a large tank**.

Some enterprising researchers then turned the whole issue on its side, and have invented **"semi-dry" or "horizontal" blotting**. With this technique, one uses **two plate electrodes** (stainless steel or graphite / carbon) for uniform electrical field over a short distance, and sandwiches between these **up to six gel / membrane / filter paper assemblies**, all well soaked in transfer buffer. The assembly is clamped or otherwise secured **ON ITS SIDE, and electrophoretic transfer effected in this position**, using as transfer buffer only the liquid contained in the gel and filter papers or other pads in the assembly.

There are a number of advantages to this procedure over the conventional upright protocol, not the least of which is that as little as a **couple of hundred millilitres of buffer are all that is needed for electroblotting several gels**, compared to as much as **five litres** for some commercial kits. In addition, several gels can be blotted simultaneously; electrodes can be cheap carbon blocks; less power is required for transfer (and therefore a simpler powerpack).

# Immunoassay

The reason for **transferring proteins to membranes from gels** is so as to be able to get at them more efficiently with various probes, as **polyacrylamide is not particularly amenable to the diffusion of large molecules**.

The immunoassay is normally done by **blocking the transfer membrane** with a concentrated protein solution (eg: **10% foetal calf serum, 5% non-fat milk powder**) to prevent further non-specific binding of proteins; this is followed by **incubation of the membrane in a diluted antiserum / antibody solution**, washing of the membrane, **incubation in diluted conjugated probe antibody or other detecting reagent**, further washing, and the **colorimetric / autoradiographic / chemiluminescent** detection.

The most popular type of probe of immobilised proteins is an **antibody** of one type or another: **the attachment of specific antibodies to specific immobilised antigens can be readily visu-**

**alised by indirect enzyme immunoassay techniques**, usually using a **chromogenic substrate which produces an insoluble product**.

Probes for the detection of antibody binding can be **conjugated anti-immunoglobulins** (eg: goat-anti-rabbit / human); **conjugated staphylococcal Protein A** (which binds IgG of various species of animal); or **probes to biotinylated / digoxigeninylated primary antibodies** (eg: conjugated avidin / streptavidin / antibody).

An example of a blot from 1983 is shown on the Chapter cover: this was **Nitro Blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)** with alkaline phosphatase. **This can achieve sensitivities of 1 ng/band**, or up to 1000x more sensitive than Coomassie staining, and similar to ELISA sensitivities.

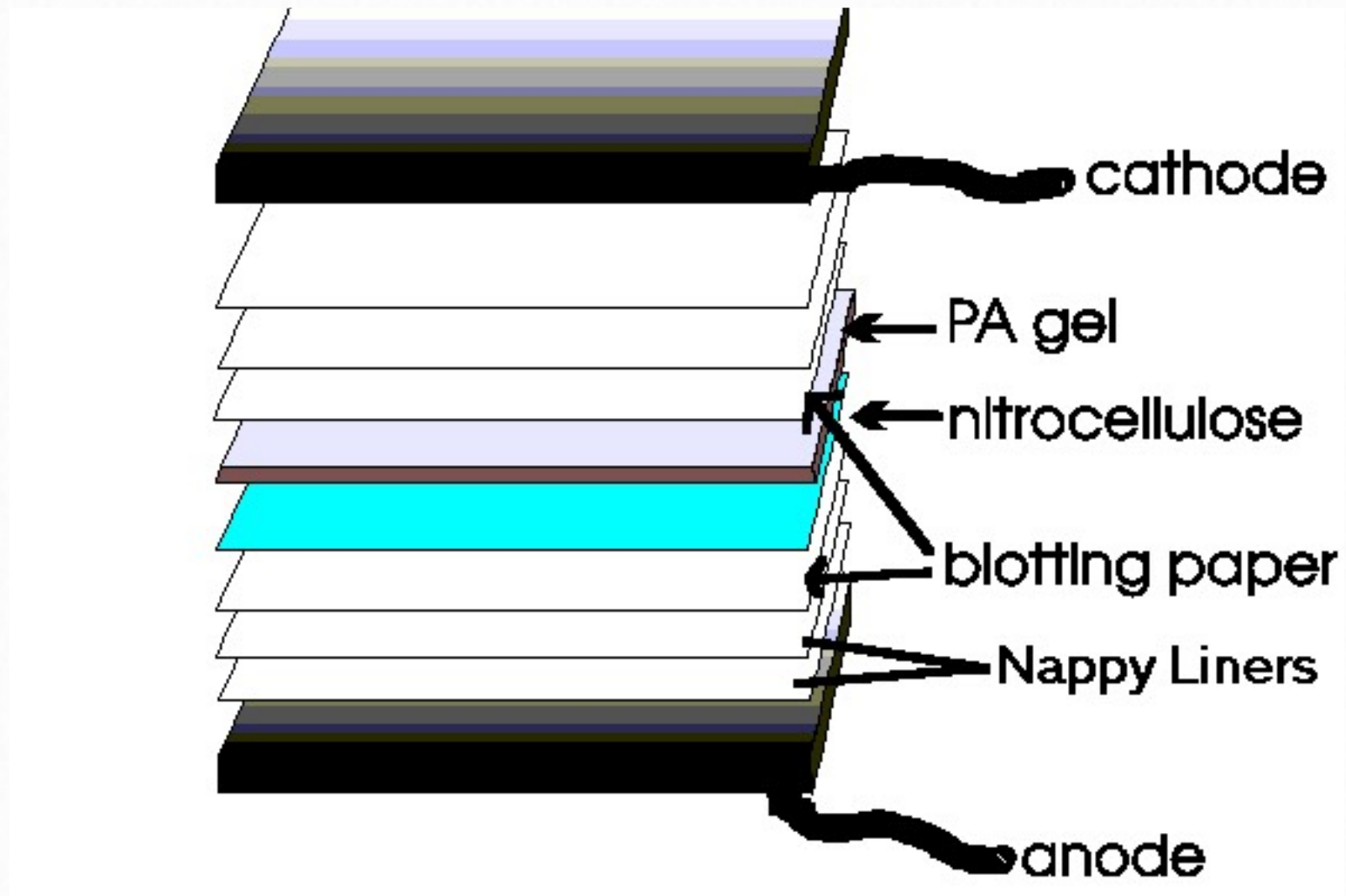
**Chemiluminescent** substrates are also used because of their **greater detection sensitivity**. Other possibilities for probing include the use of **fluorescent or radioisotope labels** (fluorescein,  $^{125}\text{I}$ ).

**The power of the technique lies in the simultaneous detection of a specific protein by means of its antigenicity, and its molecular mass**: proteins are first separated by mass in the SDS-PAGE, then specifically detected in the immunoassay step.

**It is also possible to use a similar technique to elute specific antibodies from specific proteins resolved out of a complex mixture**, many of whose components react with a given antiserum: one can electrophorese a mixture of proteins, cut out a specific band from a gel or membrane, and use this to fish out specific antibodies from a serum (see **Monospecific Antibody preparation**, here).



# Western blot protocol



## PROTOCOL

Apparatus and Equipment

Blotting Buffer

Blotting

Staining Proteins on Membranes

Indirect enzyme immunoassay

- Buffers and Solutions

- Procedure

Notes

This protocol describes the use of **horizontal blotting of simple SDS-PA gels**, and subsequent detection of proteins using **rabbit antisera** and **alkaline phosphatase-conjugated goat-anti-rabbit IgG**, detected using **bromo-chloro-indolyl phosphate (BCIP)** and **Nitro-blue tetrazolium (NBT)** salts.

## Apparatus and Equipment:

The Hoefer SE 600 vertical slab gel apparatus (or other proprietary or custom apparatus) is used for SDS- PAGE.

**Nitrocellulose paper** (0.45 um pore size) is usually supplied as 33 x 300 cm rolls, or possibly as cut pieces; **filter paper** is **Whatman 3MM** or - preferably - **BLOTTING PAPER (cheaper)**. Both are cut to size **USING A CLEAN BLADE AND GLOVES!!!** Finger marks / grease can result in ugly blots.

**Blotting Electrodes** are carbon slabs, 1cm thick x 20 cm long x 15 cm wide, with heavy insulated leads, **OR patent blotting apparatus**.

**Powerpack:** normally a **high-amperage low-voltage apparatus**; the Shandon Southern Destainer powerpack is suitable, if antique. The powerpack **must** be capable of supplying **500mA - 1A current at voltages as low as 5V**.

**DO NOT USE A STANDARD ELECTROPHORESIS POWERPACK: VOLTAGE CONTROL AT HIGH AMPERAGE / LOW VOLTAGE IS INACCURATE AND THEY ARE NOT BUILT TO TAKE THE CURRENT!!**

## Blotting Buffer:

This is often simply **Laemmli electrophoresis buffer** (50mM Tris-HCl / 0.196M glycine pH 8.3) **with 20% methanol added (PREVENTS GEL SWELLING AS IT HEATS)** FOR VERTICAL BIG-TANK BLOTTING; however, this has **too high an ionic strength for horizontal blotting** because of the **short inter-electrode distances used**. We have found that **25mM Tris-Cl pH 8.3/20% methanol (or pH 7.0 - 8.8)** works well; for that matter, one may equally well use **25 - 50mM phosphate/methanol pH 7.0 - 8.0**.

**It is a good idea to use pH values above 7.0**, in order to ensure that **all proteins migrate towards the anode**. The methanol is necessary to prevent gel swelling with heating, and to **keep proteins adsorbed to the membrane**.

**Jorge Meyer** (then at CIAT, Colombia) wrote that one may also use anodal and cathodal buffers as follows:

- **Anodal 1** (for pad nearest anode): 0.3M Tris/20%MeOH pH 10.4.
- **Anodal 2** (for other gel pad assemblies): 25mM Tris/20% MeOH pH 10.4.
- **Cathodal** (for all pads etc. nearest cathode): 25mM Tris/40mM aminocaproic acid/20% MeOH, pH 9.4.

It turns out that **blotting big gels** (eg 14.5 x 11 cm) is quite adequately done (even for two or more stacked gels) at **0.3 A for 60 min or less**: the system does not heat up appreciably, and transfers are good.

**NOTE: you should cut nappy liners / papers to the size of the gel(s) to be blotted, so as to minimise surface area exposed to the electrode**: this reduces the amount of current that needs to be passed in order to effect transfer (and incidentally reduces heating effects).

## Blotting

Freshly-electrophoresed SDS-polyacrylamide gels are **dipped into transfer buffer** (0.025 M Tris-HCl/20% (v/v) methanol, pH 8.3), then **laid flat on pre-wetted nitrocellulose paper supported on three layers of transfer buffer-wetted filter paper resting on the ANODE** (+ve electrode). We have previously made use of nappy (=DIAPER) liners as absorptive buffer-containing pads; however, **blotting paper or (if really necessary) 3MM paper may be used instead**. The gel is overlaid with **three wetted filter papers**, and then either the **CATHODE** (-ve electrode) or **another layer of nitrocellulose / gel / blotting paper**. [See cover picture for detail.](#)

**Care should be taken to exclude bubbles between gel and nitrocellulose, and between nitrocellulose and paper.**

The assembly is placed in a plastic tray resting on the anode. The **ANODE (electrode on the nitrocellulose paper side of the assembly)** is connected to the **anode or RED** connector - and the **gel side CATHODE to the cathode or BLACK connector** - of an appropriate powerpack. A current of 500mA is passed for 20-30 min to effect transfer.



## **DO NOT TOUCH ASSEMBLY WHILE POWER IS ON!!!**

Transfer is found to be **essentially quantitative** - for thin assemblies - after electrophoresis under these conditions. **One hour should be sufficient** for transfer of all but the most recalcitrant proteins. If in doubt, **blot gels in duplicate, remove one and stain for protein after 1 hr** and continue blotting other.

NB: IF USING CARBON ELECTRODES, ALWAYS KEEP SAME ELECTRODES AS CATHODE AND ANODE - THEY WILL FLOOD YOUR SYSTEM WITH COLLOIDAL CARBON IF YOU REVERSE THEM

After blotting, **disassemble the assembly**, saving nappy liner pads if used (put straight back into buffer on tray if to be re-used soon). **Rinse membrane in saline or other buffer before further treatment.**

**NB: ONE CAN USE THE SAME ASSEMBLY FOR BLOTTING NUCLEIC ACIDS - JUST CHANGE BUFFERS TO ONES APPROPRIATE FOR NUCLEIC ACID (eg. 0.025M phosphate pH 6.5 for DNA or RNA)**

## **Staining of proteins in gels**

Staining of proteins in gels may be done using the standard **Coomassie** brilliant blue (or PAGE blue), **Amido Black**, and **silver stain reagents** of different kinds. **All of these protocols are relatively long:** silver staining requires a number of finicky treatment and washing steps and takes at least a couple of hours, while other stains are two-step stain/destain procedures, but require hours (sometimes days) for satisfactory destaining. **Silver staining is (or can be) extremely sensitive - to +1 ng/band** - while of the other commonly used stains, **Coomassie** brilliant blue G-250 is probably the best, **but detects only to about 0.3 ug/band.**

**None of these stains are easily reversible**, and most often the protein cannot be recovered intact for other procedures.

**However, it is possible to reversibly stain gels prior to blotting by a couple of methods:** the simplest is by simply soaking them in ice-cold **1M potassium chloride**: SDS precipitates as KDS, and **proteins are visible as whiter zones in an opaque-to-translucent white background.** The method is not sensitive, however.

The copper staining technique previously described is especially suitable for subsequent blotting: gels can be **completely destained** and proteins will not have been subjected to **acidic or alcoholic washes** or silver fixation..

## Staining Proteins on Membranes:

**Nitrocellulose** or other adsorptive membrane is generally used for transfers: **nitrocellulose is good for adsorption** and gives **low staining backgrounds with India ink, Amido Black, Coomassie Brilliant Blue, colloidal gold and peroxidase and alkaline phosphatase substrates**, but is fragile. Hybond-N (nylon) and similar membranes are amazingly strong but give horrible background with most enzyme-immune substrates.

**No nylon can be stained with the other stains**; however, Millipore claims their **polyvinylidene difluoride (PVDF)** membranes are as good as nylon and can be stained with colloidal gold.

Methods which have been tried here are **Amido Black staining** (not satisfactory); **India ink staining** (OK), and **Ponceau S staining (highly useful)**. The Ponceau S method is as follows:

- Stain: 2 g Ponceau S, 30 g trichloroacetic acid, 30 g sulfosalicyclic acid; water to 100 ml.
- Method: Immerse fresh blot (NOT blocked!!) in stain, leave until bands are visible. The stain can be completely removed for subsequent immunoassay with a normal (saline) wash

## Indirect enzyme immunoassay

### *Buffers and Solutions:*

**Incubation/Blocking Buffer:** 10 mM Tris-Cl/150 mM NaCl containing 1-5% Protea non- fat milk powder and 0.05% Tween-20 or Triton X-100 (=Nonidet P-40), pH 7.4.

**Washing buffer:** 10 mM Tris-Cl/150 mM NaCl pH 7.4 (OR SIMPLY SALINE) containing 0.05% Tween-20 or Triton X-100.

**Substrate buffer:** 100 mM Tris-Cl/100 mM NaCl/5 mM MgCl<sub>2</sub> pH 9.5.

**Substrate stocks:** Nitro Blue tetrazolium (NBT) (Sigma), 75 mg/ml in 70% dimethyl formamide; 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma), 50 mg/ml in formamide (100%).

**NB: KEEP SUBSTRATE STOCKS IN 10 ML ALIQUOTS AT -20°C**

## **Procedure:**

**Blocking:** nitrocellulose blots are briefly rinsed in transfer buffer, then soaked for 1 hr at 37°C, or 2 hr at room temperature in blocking buffer. This procedure allows saturation of all non-specific protein binding sites on the blots.

**Attachment of specific antibodies to proteins:** is achieved by incubation of blots in rabbit antiserum diluted 1/10- 1/1000 in incubation buffer, in sealed boxes for 1 hr on a shaking waterbath at room temperature (22°C).

**Washing:** blots are washed by shaking in +100 ml/wash, 3x5 min., at room temperature. Increase number and duration of washes if background is a problem.

**NB: Recognition of host plant (or other) proteins can be avoided ([see here](#))**

**Probing of antibody binding:** is by means of suitably (eg 1/5000)-diluted alkaline phosphatase - goat anti-rabbit globulins (GAR-AP)(Miles Laboratories, Cape Town) in incubation buffer. Incubation conditions and washing are the same as for rabbit antiserum.

**Probe visualisation:** by the reaction of NBT and BCIP mixture.

**To make 10 ml of substrate:** NB: MAKE FRESH BEFORE EACH ASSAY.

Mix 50 µl of NBT stock with 10 ml of substrate buffer, then add 50 µl of BCIP stock and mix well by swirling.

**NOTE: IF SOLUTION IS FLOCCULENT OR VERY CLOUDY AT THIS STAGE, DISCARD AND MAKE FRESH STOCKS.**

A little cloudiness is normal.

Pre-rinse blot(s) in a little substrate buffer, then add substrate: +10 ml/100 cm<sup>2</sup> blot. **Incubate in the dark with occasional agitation.** The reaction can take several hours, but **is normally complete in 30-60 min.** Leave overnight with gentle agitation IN THE DARK if reactions are very faint.

**Terminate reaction by washing with water.** Blots are dried under weighted filter paper, and stored in the dark. Results may be recorded photographically using a red filter for B&W, or with no filter for colour.



# NOTES

**Two other techniques for studying protein-protein interactions on top of crosslinking and immunoprecipitation.**

**Far western blot:** Similar to a western blot except that **a labelled protein is used as a probe** [to detect specific protein-protein interactions]. The probe can be **biotinylated or labelled with  $^{32}\text{P}$ ,  $^{125}\text{I}$  or  $^{35}\text{S}$** . The following references will be useful: Oncogene (1990), 5, 451-458 Mol Endocrinol (1991), 5, 256-266 Genes & Dev (1992), 6, 439-453 Science (1992), 257, 803-805

**Affinity chromatography:** Express one of your proteins as a **GST (or MBP, or other) fusion** in *E. coli* or other expression system (we would use plants B-). These fusion proteins **will then bind to glutathione sepharose or amylose resins** and can be used to **fish out binding proteins**. Numerous labs have used this technique which was originally described by Kaelin et al. in Cell (1991), 64, 521-532.

## **Immunoabsorption of unwanted antibodies from probe**

**Recognition of host plant (or other) proteins** can be avoided by **diluting the antiserum in buffer containing (for example) a 1:3 mix of sap of healthy plants** (plants crushed 1:1 (w/v) **or *E. coli* lysate** in 0.1 M phosphate, pH 7.0) or other antigen with the buffer used to dilute the antiserum; and **pre-incubating at 37°C for 1 hour**.

**This procedure effectively gets rid of host plant and *E. coli* contaminant reactions.**

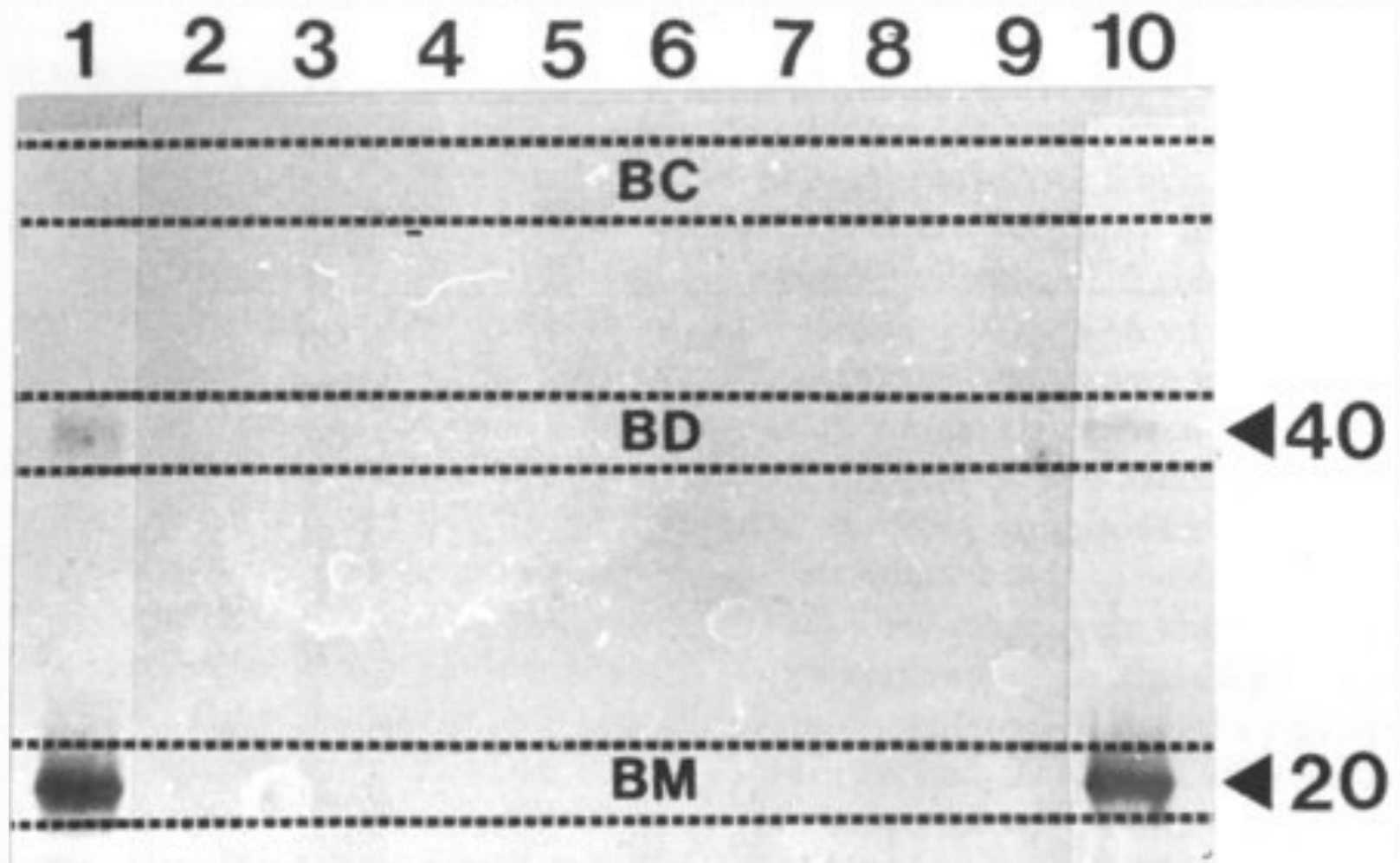
Another procedure more generally used in this laboratory is the removal of anti-host antibodies by **plant or bacterial extracts immobilised on nitrocellulose** (or other membrane): simply soak membrane in a **concentrated extract of whatever it is that you want to remove antibodies with** (+30 min); **block** as for normal blot; **add diluted antibody suspension to this membrane for +30 min** before use on the Western blot proper, **then pour off for use as probe antibody**. This procedure should **remove all antibodies responsible for reacting with antigens that contaminated the inoculum used to immunise rabbits** (or whatever). SEE ALSO HERE FOR MORE DETAILED PROTOCOLS!!!

## **References:**

Rybicki and von Wechmar, 1982; J. Virol. Methods 5:267-278;

Towbin et al. 1979, Proc. Natl. Acad. Sci. USA 76:4350.

# Affinity Purified Monospecific Antibodies to Gel-Purified Proteins



## Introduction

## Elution of monospecific antibodies

## Materials and Methods

## Routine use

## Preparation of immunoadsorbent

## Adsorption of antibody

# Introduction

Since the introduction of **sensitive immune assays**, it has become increasingly apparent that previous criteria of protein purity **are inadequate for the production of truly specific antisera**. For example, I found that rabbit antisera raised against highly-purified Brome mosaic virus (BMV) reacted sufficiently well with plant components as to obscure the virus protein reaction in western blotting tests with infected plant samples ([RYBICKI and VON WECHMAR 1982](#)), even though only traces of plant proteins were visible after silver staining of SDS-PAGE fractionated purified virus used for rabbit immunisation (RYBICKI, unpublished).

Because this problem necessitated the thorough and relatively laborious absorption of all sera to be used in such tests, I decided to investigate the feasibility of using **preparative electroblotting to purify virus capsid protein** to a sufficiently rigorous extent as to ensure the absence of any of the common host protein contaminants, and then to apply the method of [OLMSTED \(1981\)](#) for the **affinity purification of virus-specific antibodies from antisera** that also reacted with plant components.

The latter technique entails using the **virus protein immobilised on a porous support** as a specific immunoabsorbent, from which antibodies may be eluted after specific binding. This investigation was designed to test the applicability of the outlined procedures to the **routine small-scale production of antibodies suitable for use in enzyme-linked immunosorbent assays** (ELISA), for viruses that occur only at very low concentration in plant sap, or which are extremely hard to purify because of their lability. **There are also obviously many other applications!**

## Materials and Methods

Nitrocellulose paper eg: BA 85, 0.45 um (Schleicher and Schuell, Keene, NH, USA). Goat-anti-rabbit IgG horse radish peroxidase conjugate (GAR-HRP) eg: from BioRad Laboratories, as for the 4-chloro-1-naphthol-containing colour reagent. I have also used alkaline phosphatase conjugate and appropriate colour reagents (see [here](#) for methods). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and sample preparation techniques [are described elsewhere](#).

For the **Olmsted technique**, the sample containing the protein of interest **is electrophoresed as identical aliquots in separate gel lanes, as a continuous "smear"** - detectable by immunoblotting or silver staining - results from running samples as one wide lane. Electrophoresis



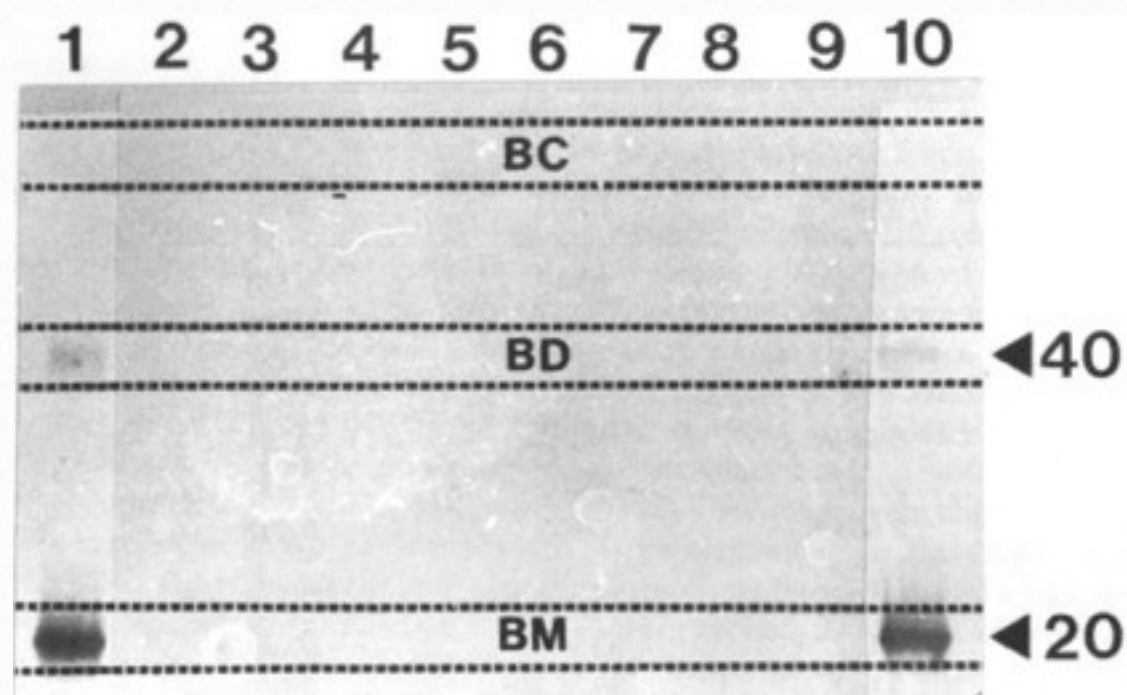
is **best performed at 4 °C in an apparatus with a heat-exchanger** (such as the Hoefer SE-600) so as to avoid the 'smile' effect that results from **uneven heating of the gel during electrophoresis**.

## The routine use of the technique:

**Dilute samples 1 : 3 in SDS-PAGE sample buffer**, heat to 95°C for 5 min, and load 20 µl (20 µg) into each of the 10 slots of a **1.5 mm-thick 4.5 %/12 % discontinuous acrylamide gel**. Samples are run as appropriate with coolant circulating. The gels are notched with a scalpel at the top of each track, the stacking gel is removed and the resolving portion laid onto a sheet of pre-wetted nitrocellulose. [Electrophoretic transfer](#) is then performed.

**Vertical strips corresponding to lanes 1 and 10 (alignment strips)** are excised from the stacked blots. All the nitrocellulose pieces are **blocked** in 10 mM Tris/150 mM NaCl / 2 % BSA / 0.05 % Nonidet P-40 (NP-40 blocking buffer; also use Triton X-100 or Tween 20, and 1-5 % non-fat dry milk suspension) **for 12 h to block protein-adsorption sites**. The **two alignment strips from each blot are then shaken for 90 min with antiserum** diluted (eg) 1/1000 in blocking buffer, **washed** on a shaker with 4 x changes of 150 mM NaCl/0.05 % NP-40 (wash buffer) for 5 min, and **probed** with a 1/ 1000 dilution of GAR-HRP or other conjugate **for 90 min**. The immunoblots are developed in a substrate solution made by mixing eg: 1 volume of 3 mg/ml Bio-Rad colour reagent in methanol, 5 volumes of 50 mM Tris/ 200 mM NaCl pH 7.4, and 1/2000 volumes of 30 % H<sub>2</sub>O<sub>2</sub> (0.015 % final concentration). The reaction is stopped by rinsing in water.

In a test experiment with BMV the **two side strips each had faint protein bands at 40 kd, strong bands at 20 kd, and minor bands smaller than 20 kd** (see Figure 1 below). The 20 kd band is the virus coat protein monomer; the 40 kd band has been presumed to be a coat protein dimer, and the minor bands to be derived by proteolysis from the coat protein ([RYBICKI and VON WECHMAR 1982](#)).





## Preparation of immunoadsorbent:

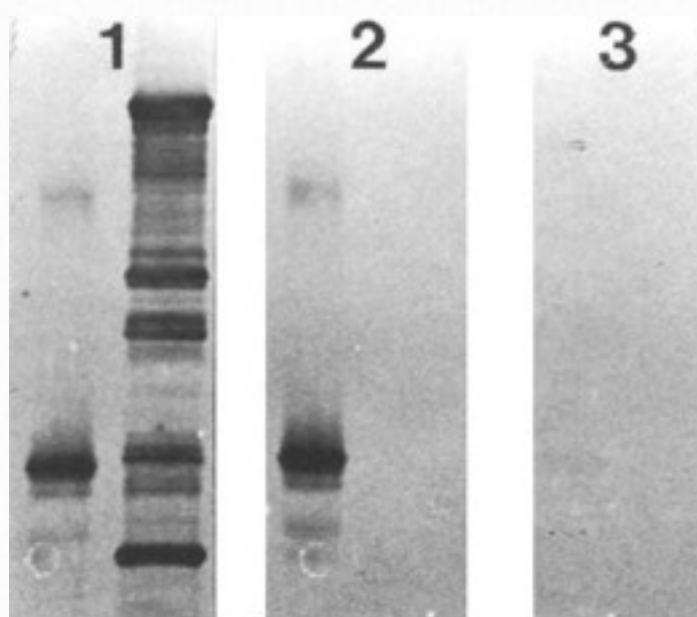
The strips were aligned with the parent blot, and three horizontal strips of 5 mm width - corresponding to the 20 kd capsid protein monomer (strip BM), the 40 kd (presumed) capsid protein dimer (strip BD), and a region of the blot containing no polypeptides (strip BC) (see Figure) - were excised.

## Adsorption of Antibody:

The strips were shaken in **1/40-diluted BMV antiserum for 90 min**, then washed in **4X changes of wash buffer**, and **2X changes of saline**.

## Elution of monospecific antibodies

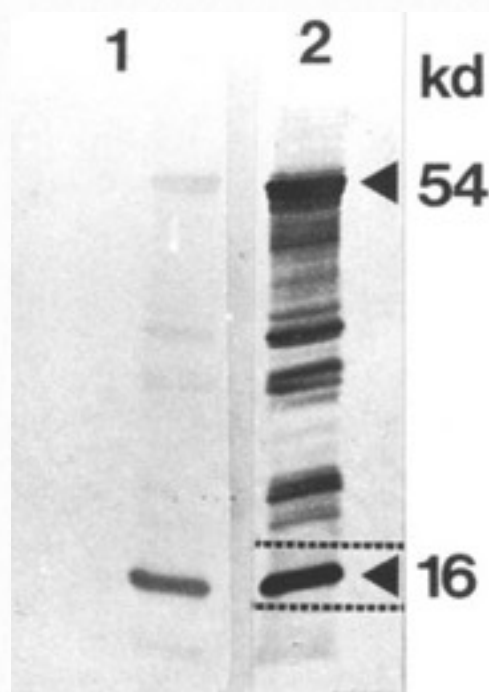
The preparative strips were placed **coiled in 50 ml beakers**, and **7.5 ml of 0.1 M glycine-HCl buffer pH 2.9** added to each. The beakers were **agitated for 10 min**, the contents decanted and **quickly neutralised by the addition of 1.4 ml of 0.1 M NaOH**. The entire process was repeated twice - to give preparations 1, 2 and 3 - **using the same serum dilution as antibody source**. **Essentially all IgG is removed from blots after two elution steps with the glycine-HCl buffer** (result not shown); **blots may be re-used up to six times** (EP Rybicki, unpublished) for successive serum absorption / elution.



**Figure 2: Purification of BMV-specific antibody from a mixed antiserum**

All immunoblot strips had 10 ug of BMV (left lane) and 20 ul of barley homogenate (right lane). Strip 1 was probed with a 1/5-diluted 1:1 mixture of anti-BMV and anti-"Fraction 1" (Rubisco) an-

tisera; strip 2 with antibodies eluted from an electroblotted strip of BMV monomer protein (strip BM, see Fig 1); and 3 with the eluate of control strip BC. Monospecific antibody preparations were used unconcentrated.



**Figure 3: Illustration of the Reactivity of Antibodies Eluted from Low Molecular-Weight Barley Protein.**

Immunoblot 1 contains 5 ug of BMV (left lane) and 10 ul of barley homogenate (right lane), 2 contains only barley homogenate and is the right-hand "alignment strip" from a blot of identical samples used to prepare specific antibodies for low-MW proteins. The dotted lines indicate the region of the whole blot that was excised. 2 was probed with 1/50-diluted anti-'Fraction 1' serum, and 1 with unconcentrated eluate from the excised strip. Molecular weights are indicated at the side of the Figure.

**This result illustrates the most obvious potential use of the technique: using a protein band from an electrophoretically fractionated crude extract to purify antibodies from an antiserum made against the crude extract.**

## Discussion

The technique described here is an **obvious development from "Western blotting" techniques** in general, and a **simple extension of well-established affinity adsorption techniques** that are widely used for either the removal of unwanted antibodies from a serum, or the purification of monospecific antibodies using a highly purified immobilized antigen. The technique has already found application in this laboratory in the production of specific antibodies for the proteins of low-yielding plant viruses, for specific bacterial enzymes, and for the different capsid subunits of an insect picornavirus (E. P. RYBICKI, R. T. MEW, C. WILLIAMSON, unpublished results).

Although I have used nitrocellulose and electroblotting to immobilize SDS-PAGE-resolved polypeptides, there are **no obvious objections to the use of other kinds of electrophoretic fractionation**, or to **non-electrophoretic transfer techniques**, or to the use of **other blotting**

**papers.** My choice of a **glycine-HCl buffer** for antibody elution was based on familiarity only; **other elution media could be equally or more efficient.**

I have found that inclusion of **any of the detergents Tween-20, Triton X-100, and NP-40** helps both **to reduce background staining of electroblots, and to increase the specificity of antibodies eluted from nitrocellulose.**

Incubation of blot strips in **too high a concentration of antiserum leads to greatly increased non-specific adsorption of antibodies:** a useful rule of thumb practised here is to determine **the highest concentration of serum that produces minimal background staining** on blots, and to use **no more than double that concentration for adsorption in preparative experiments.**

It is evident that **the technique cannot be used for large-scale production of antibody.** Accumulation of electroblots from many gels would enable preparation of **at most a few milligrams of specific antibody,** if the blots were **used repeatedly** as immunoadsorbents. Rather, **it is a relatively simple small-scale procedure** best suited to the preparation of the small amounts of specific antibody required for use in **Western blotting or indirect ELISA,** or for **labelling with isotopes, enzymes, fluorescent compounds or other ligands,** for the **sensitive detection of specific proteins in complex mixtures.**

I feel that this type of technique, which utilises relatively simple and even home-made equipment, **provides a viable alternative to the far more expensive and complicated monoclonal antibody technology,** especially for the less well-endowed laboratory.

#### **Source Reference:**

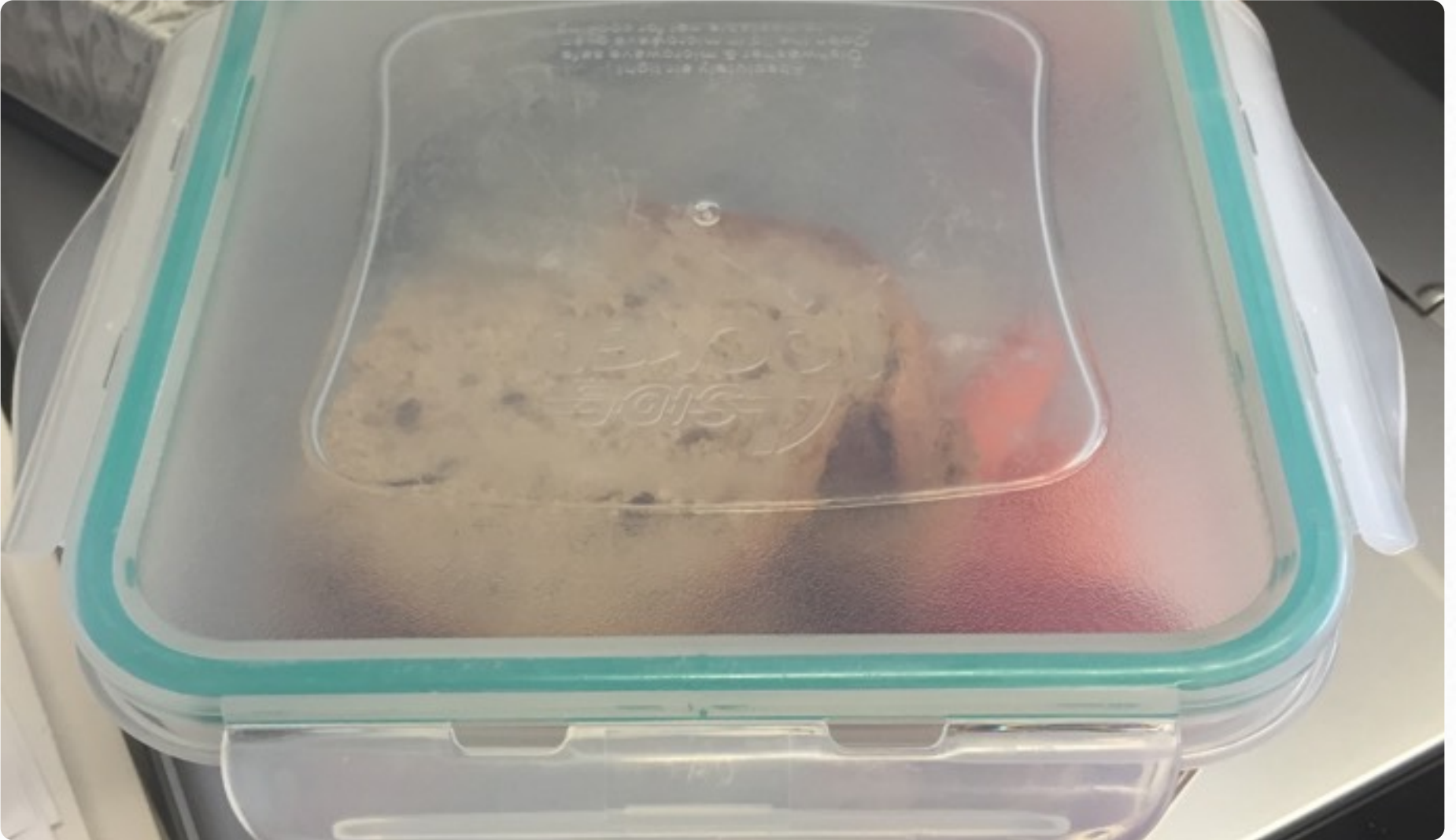
Rybicki, E.P. 1986. Affinity purification of specific antibodies from plant virus capsid protein immobilised on nitrocellulose. *Journal of Phytopathology* 116:30-38.

#### **References**

Olmsted, J.B. 1981. Affinity purification of antibodies from diazotised paper blots of heterogeneous protein samples. *Journal of Biological Chemistry* 256:11955-11957.

Rybicki, E.P. and M.B. von Wechmar. 1982. Enzyme-assisted immune detection of plant virus proteins electroblotted onto nitrocellulose paper. *Journal of Virological Methods* 5:267-278.

# “Lunchbox” Immunoabsorption



## Introduction

Absorption of antigens to membrane

Absorption of antibodies from serum

Attachment and elution of specific antibodies

## Immunoassay techniques

Discussion

References



# Introduction

I have explored the possibilities of using nitrocellulose and other adsorptive membranes as **immunoadsorbents** for simple, reliable "low tech" procedures for the **purification of a specific antibody**. The technique does not require the prior fractionation of an antigen mixture by electrophoresis, and requires only very basic laboratory equipment, including nitrocellulose membrane, simple benchtop centrifuges, a plastic freezer container or "lunch box," and some form of simple immunoassay system. **The suitability of the method for the small-scale preparation of antibodies for immunoassays such as ELISA, immunodotblot, and western blotting, is discussed.** Another techniques page will cover the specific absorption of Ab to, and elution from, electrophoretically-purified proteins ([Monospecific Ab Production](#)).

## Absorption of antigens to membrane ("blotting")

**Nitrocellulose paper** (0.45um pore; Schleicher and Schuell) is cut into 10 x 10 cm squares, which fit into 12.5 x 17 x 8 cm plastic freezer boxes ("**lunchboxes**") with room for movement during shaking. Other membranes such as polyvinylidene difluoride (PVDF, Millipore) or other nylon based material can also be used (E.P. Rybicki, unpublished). I have used crude Brome mosaic virus (BMV) preparations to make monospecific antibody preparations: **semipurified virus was made by resuspension of centrifugally pelleted clarified plant extract** in 0.1M phosphate pH 7.0 (1/10 original volume) and used to coat nitrocellulose. This sort of extract contains a large amount of plant proteins, including cell wall and membrane components. I and others in the lab have used a variety of other crude preparations, including ***E coli* preparations with foreign proteins**, with great success. In the *E coli* case, **a sonicated soup or French-pressed mush** works fine.

**Water-wetted nitrocellulose is added to  $\pm$  25 ml volume of extract**, and the boxes **agitated at room temperature (22°C) for at least 2 hours**. Alternatively, the boxes are left on the bench and agitated by hand every 10 min. **It does not appear necessary to "fix" antigen to the membrane** by heat or other treatment.

**Membranes are washed in 3x changes of saline** containing 0.05% (v/v) Tween-20 or Triton X-100 for 5 min, **then "blocked" overnight** using 20 ml phosphate-buffered saline (PBS) containing 0.05% Tween or Triton and **2% (w/v) bovine serum albumin or 1-5% (w/v) skimmed milk powder** (blocking buffer).

## Absorption of antibodies from serum

**Sera to be absorbed are diluted** - depending on titre, pre-tested using a simple precipitin assay - **in saline/detergent**, and **20 ml added to a drained blot** of healthy plant extract (made exactly as for the virus blot, above) in a freezer box. **The box is agitated at room temperature for 2 hours**, and the **serum then poured off**. This is also a useful way to absorb serum dilutions immediately prior to their use in western blot tests.

## Attachment and elution of specific antibodies

**Appropriately diluted absorbed serum is added to a drained virus extract blot in a freezer box**. This is incubated **at 37°C** (or at room temperature) **for 1-2 hr with shaking**. The blot is then **washed** as above, with a final rinse in water.

**Bound antibodies are eluted using about 20 ml of a 0.1 M glycine/HCl, buffer, pH 2.9**. The box was **agitated for 10 min**, the liquid drained off and **immediately neutralized by addition of a predetermined amount of 0.1 M NaOH**. Essentially **all specifically bound antibody may be removed from a blot by a single elution** (Rybicki, 1986 and unpublished), with yields of **up to 1 mg for BMV extracts**. The antibody attachment and elution steps **may be repeated up to three times** with the same BMV-infected sap blot with only slight drop in yield, **as long as a fresh dilution of serum is used each time** (E.P. Rybicki, unpublished).

Antibody preparations are concentrated by dialysis against water and lyophilisation. Preparations are resuspended in small volumes of saline.

## Immunoassay techniques

Antibody preparations may be tested for specific activity by **indirect ELISA** (eg: Rybicki and von Wechmar 1981) and **western blotting** (eg: Rybicki and von Wechmar 1982). An example of a blot - taken from the source reference below - is shown (left).

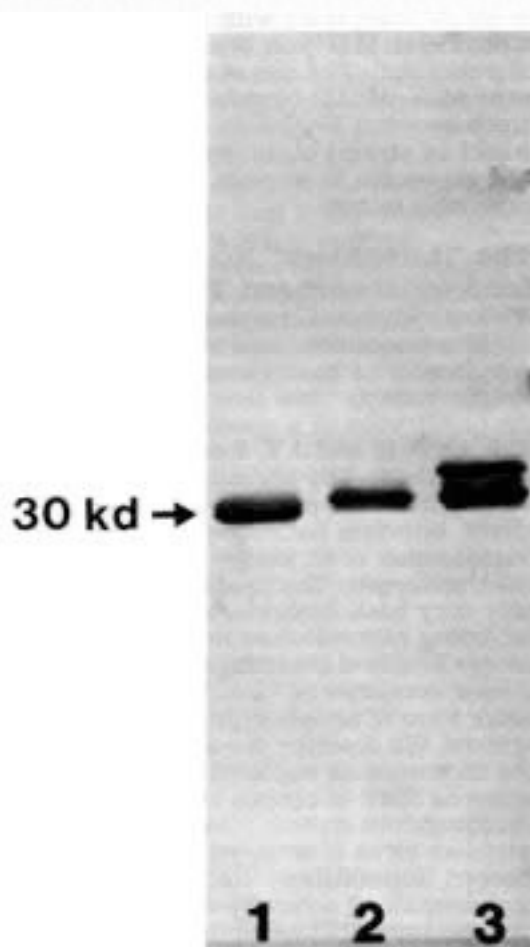


Figure 2. Western blot of three isolates of *Ornithogalum* mosaic virus probed with monospecific antibodies purified from virus adsorbed onto a nitrocellulose sheet.

Crudely purified virus isolates: left to right, OMV; OMV ex *Lachenalia* sp.; OMV 'wild' isolate. Antibodies eluted from virus (OMV) adsorbed onto nitrocellulose as described in the text, and detected using alkaline phosphatase-labelled goat-anti-rabbit antibodies and Nitro blue tetrazolium complex substrate.

## Discussion

As mentioned above, **1 mg or more antibody can be prepared from a single elution** of a single BMV-infected sap extract-coated membrane; **this is sufficient to prepare enzyme conjugates for DAS-ELISA**, and would provide materials for many assays. In any case, re-use of the antigen-coated membrane enables **accumulation of enough antibody to allow many serological tests**. If antisera are absorbed with host plant (or other) antigens prior to application to blots, **eluted antibodies react almost exclusively with virus coat protein**, with very little "background" reaction with other polypeptides in western blot tests (see Figure). Eluted anti-BMV antibody could be diluted by a factor of 1/50 for use in western blots, and up to 1/500 for indirect ELISA. **This is a far better yield than can be obtained with individual excised polypeptides.**

**An important consideration is that cheap materials may be used.** Nitrocellulose or other membrane is the only expensive component, and that is far cheaper, far easier to use, and far better as an adsorbent than CNBr- Sepharose, or other comparable column chromatography materials commonly used as immunoadsorbents. **Up to 100 ug protein/cm<sup>2</sup> may be adsorbed onto nitrocellulose**; polyvinylidene fluoride's capacity is even higher.

**The use of crude extracts to purify antibodies is important when no facilities exist for purification** of (eg) low-yielding viruses: clarified sap extracts could be concentrated by PEG treatment; non-infected extracts could be used to absorb antisera; and infected extracts to purify monospecific antibodies. Even if antibody preparations are not exactly monospecific, their preparation in this way represents an important purification and concentration step, as **relatively specific antibodies are purified in one step from raw serum**, with a consequent increase in activity of the preparation once most of the extraneous serum proteins and immunoglobulins are removed.

This has worked very successfully with detection of plant virus coat protein bands against a background of whole plant, using antisera that reacted with EVERYTHING before absorption; also with antisera raised against purified proteins either from, or cloned into, *E coli*: in latter case, as anyone who has done it knows, when you do a western, ALL the bands light up, as rabbits are immune to *E coli* and related gut microflora - and no-one thought to tell you...!

**You can use the same technique to mass-absorb / elute Ab to a particular purified protein, without having to go to the trouble of making up an expensive column immunoabsorbent:** soak NC or other membrane in protein of interest, wash, block, soak in AS. Wash thoroughly,

then elute Ab with preferred elution mix (I use 0.1M Glycine/HCl/0.15M NaCl pH 2.9). **You can repeat the absorption/elution several times, and yield is quite high** - certainly enough for labelling specific Ab for immunofluorescence, ELISA, etc. We have used it in our labs to make monospecific Ab to plant viruses, and to *E coli* proteins or proteins cloned in *E coli*, **as long as one has a background free of the protein of interest.**

**You can also combine two techniques:** pre-absorb antisera with membrane with complex mixture NOT containing protein of interest, then pour off antisera onto membrane with complex mixture CONTAINING protein of interest, preferably at highish concentration. First absorption takes out Ab reacting with "host protein", in second, what is left is hopefully relatively monospecific, and can be eluted as above, for labelling, etc.

Very simple, very easy, **VERY CHEAP!!!** - and originally published here:

### **Source Reference:**

EP Rybicki, MB von Wechmar and JT Burger (1990). Monospecific antibody preparation for use in the detection of viruses. pp. 149-153 in "World Perspectives on Barley Yellow Dwarf" (PA Burnett, ed.); CIMMYT, Mexico DF, Mexico.

### **References**

Rybicki, E.P. 1986. Affinity purification of specific antibodies from plant virus capsid protein immobilised on nitrocellulose. *Journal of Phytopathology* 116:30-38.

Rybicki, E.P. and M.B. von Wechmar. 1981. The serology of the bromoviruses. 1. Serological interrelationships of the bromoviruses. *Virology* 109:391-402.

Rybicki, E.P. and M.B. von Wechmar. 1982. Enzyme-assisted immune detection of plant virus proteins electroblotted onto nitrocellulose paper. *Journal of Virological Methods* 5:267-278.