TECHNIQUES IN CLINICAL IMMUNOLOGY

EDITED BY R A THOMPSON

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Techniques in Clinical Immunology

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Preface

The establishment of immunology in the clinical arena has resulted in an increased demand for laboratory tests of immune function, as well as the application of immunological techniques in a variety of disciplines, for example endocrinology, microbiology and clinical chemistry etc. Thus many hospital pathology departments have set aside areas or laboratories where some of these tests can be performed. The methods used are usually obtained from a perusal of the specialised literature or reference volumes intended for the research worker.

This book aims to gather together the most useful laboratory tests in clinical immunology with detailed descriptions of the methods (and the problems) by experts in the subject who are also active at the bench, and who carry out the techniques they describe. It is not intended as yet another immunology textbook, although the applications, interpretations and limitations of the methods are indicated where appropriate. Chapter authors have been encouraged to be discriminatory, and to include tests which are their personal preference. Many have included numerous references for the reader who wishes to explore further aspects of the relevant techniques.

The methods are grouped for convenience, either because of the similarity of the technical procedures (e.g. immunoprecipitation), or because they are used to evaluate a particular part of the spectrum of clinical immunology (e.g. analysis of lymphocytes). There is inevitably some overlap although this has been kept to a minimum.

Descriptions of techniques in the literature often fail to indicate the sources of specialised necessary materials. For this reason suppliers are given for all materials and apparatus (except the most common chemicals, reagents and benchware) mentioned in the text of this book. It is implicit that those mentioned represent the personal preference of the authors in each case, and are not necessarily the only suppliers of the relevant material. The addresses of the quoted suppliers are given separately in alphabetical order in the Appendix.

Birmingham 1976 *R. A. Thompson*

Contents

CHAPTER ONE

Immunoprecipitation in the evaluation of the proteins in plasma and body fluids

A. Milford-Ward

INTRODUCTION

Immunoprecipitation techniques have become standard tools for the qualitative and quantitative assessment of proteins in biological fluids. Many good reviews of the subject exist, and the present chapter does not pretend to be a complete review of the techniques involved, but more a practical exposition of certain of the more basic techniques as they are performed in the Supraregional Specific Protein Reference Unit at Sheffield.

The basic principle involved in all the gel phase techniques is the formation of an immune precipitate by combination of antigen and antibody at equivalence. In this context the human protein constituent is usually the antigen and the reagent used in its localisation is antibody raised in rabbits, goats, sheep or horses. However with automated immunoprecipitation, a fluid phase technique, the immune reaction is conducted in antibody excess. Although each technique can be considered in isolation, certain items of equipment and reagents are common to many techniques.

APPARATUS—GENERAL CONSIDERATIONS

Glass plates Various size plates can be used for the casting of gels, the size being in many cases adjusted to the size of any given analytical batch. The sizes in most frequent use are shown in Table 1.1 together with the techniques for which they may be needed and convenient sources of supply.

Gel punch It is convenient to have a range of gel punches for different diameter origin wells. A convenient range of sizes includes 1 mm, 1·2 mm, 2 mm, 3 mm, 4 mm and 6 mm. The wells cut with these punches in 1·5 mm thick gels will contain approximately 1 µl, 1·7 µl, 5 µl, 10 µl, 18 µl and 40 µl respectively. Punches can be constructed from stainless steel tubes ground on the internal aspect to leave a vertical external face. Commercial punches are

Table 1.1 Common sizes of glassplates for gel techniques.

Size	*Technique*	*Supplier*
50 × 50 mm	DID, SRID	Transparency cover glasses, Chance
80 × 80 mm	DID, SRID, EID	Transparency cover glasses, Chance
100 × 100 mm	SRID, EID	Hoechst Pharmaceuticals
200 × 100 mm	EID	Chromatography plates. Hoechst Pharmaceuticals

DID: Double immunodiffusion
SRID: Single radial immunodiffusion
EID: Electroimmunodiffusion

available from Hoechst Pharmaceuticals. To remove the gel plug from the well, the punch should be connected to a suction device.

Templates In many instances perspex or plexiglass templates can be constructed to overlay the gels and allow wells to be cut to various patterns. This will ensure that wells are cut vertically in the gel. An alternative approach is to draw the template pattern on thin card and lay the gel over it.

Horizontal table It is essential to have a horizontal surface for pouring gels. This can be achieved with a levelling table and spirit level. (Hoechst Pharmaceuticals; Shandon Southern).

Water bath 1% solutions of agar and agarose are fluid at 50°C, but antiserum is denatured at 56°C. A water bath maintained at 52°C will be will required to keep antiserum-agar mixtures fluid prior to pouring plates.

Moist chamber Gels must be kept moist during diffusion phases. Suitable moist chambers can be constructed from plastic sandwich boxes. Glass rods should be fixed to the base of the box to support the gels and a little water put in the base of the box to raise the humidity.

Micropipette Although it is possible to fill the wells for single radial immunodiffusion to the brim, application of measured volumes by micropipette (Hamilton or Shandon Terumo) gives more reproducible results.

DOUBLE DIFFUSION
(gel diffusion of Ouchterlony)

The double diffusion technique as described by Ouchterlony (1953) is a qualitative or at best semiquantitative technique derived from the earlier linear immunodiffusion techniques. Agar gel is poured evenly onto glass or

plastic plates and holes or wells cut into the solidified gel. Antigen and antibody solutions are placed in the wells and allowed to diffuse. Visible precipitin lines are formed in the gel at the point of equivalence. The technique can be used to detect the presence of antigen or antibody in a test solution and to show antigenic crossreactivity. By a system of serial dilutions of test samples, an approximate concentration or titre can be derived.

Materials

Glass plates, horizontal table, gel punch and suction device, moist chamber.

Agar, I.D. Agar (Oxoid BR 27) or similar, 0·15 M phosphate buffer pH 7·1: [Solution I: $Na_2HPO_4 2H_2O$ 26·7 g, distilled water to 1 litre. Solution II: KH_2PO_4 20·41 g, distilled water to 1 litre. Mix in proportion Solution I 67 ml; Solution II 33 ml.]

Method

1 Preparation of gels: Dissolve 4 g agar in 100 ml 0·15 M phosphate buffer and 300 ml distilled water. Add 40 mg merthiolate as preservative. Heat to 100°C to dissolve the agar and allow to cool. (Agar can be conveniently dispensed in 9·6 ml aliquots until required.) 9·6 ml of melted agar is poured onto a level 80 × 80 mm glass plate and allowed to gel.

2 Origin wells: for routine purposes 3 mm diameter wells are cut according to the pattern in Fig. 1.1. Smaller or larger wells may be required for certain special techniques.

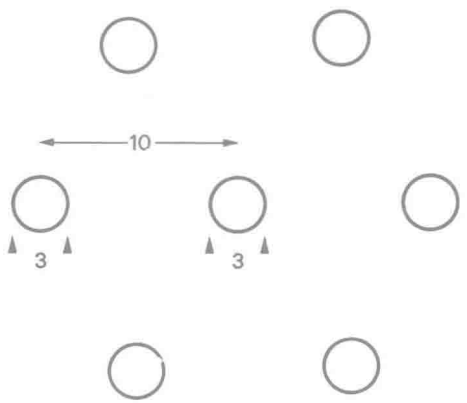

Figure 1.1. Template for double diffusion. Proportionate well dimensions may be adjusted for relative antigen or antibody concentration but centre-to-centre distances should be maintained.

3 Sample application and diffusion: The wells are filled almost to the brim with 10 µl of antigen solution or antiserum. The gel is covered in a moist chamber and allowed to stand at ambient temperature for 2–3 days.
4 Reading of results: Immune precipitates will be visible as lines in the gel, the precise location of which depends on concentration, and rate of diffusion of antigen and antibody. Various patterns of antigenic identity, partial identity, and non-identity can be recognised.
5 Recording of results: If desired the precipitin patterns can be stained with any protein stain or recorded photographically. A convenient and simple technique is to immerse the gel in water and place over photographic paper in a dark room. The plate is lit from above with a 200w lamp and the photographic paper processed.

Modifications

1 Expensive antisera such as antisera to immunoglobulin subclasses call for microtechniques of double diffusion. The same general procedure is followed except that 1 mm diameter wells are cut 4 mm apart to the same general pattern (Fig. 1.1). These wells will accept only 1 µl of antiserum.
2 When testing for the presence of human antibodies to fungal antigens, non-specific precipitates may form between C-reactive protein in serum and fungal antigens. This can be avoided by the use of an agar gel dissolved in acid citrate buffer (0·1 M Citric acid 18 ml, 0·2 M Na_2 HPO_4 $2H_2O$, 82 ml, merthiolate 10 mg). The procedure is as for the standard method except in respect of buffer and well sizes, the serum sample being placed in a 6 mm central well and the test antigen solutions in peripheral 2 mm wells.

Possible causes of error

1 Specific antisera are frequently rendered monospecific by absorption of contaminating antibodies by the addition of soluble antigen. The presence of absorbant may give precipitin lines between *antiserum* wells on double diffusion.
2 Circular precipitates enveloping an antigen well are due to lipaemic or aged samples producing non-specific precipitation of protein in the gel. Haemolysed samples may give a similar appearance on a photographic record.
3 Non-antigen antibody precipitation in agar gels can occur between substances in solution (e.g. C-reactive protein and bacterial polysaccharides, or CIq and DNA, etc.). This should be remembered, particularly when using crude extracts of tissues and organs, or mucoid secretions, e.g. jejunal fluid.

SINGLE RADIAL IMMUNODIFFUSION

The technique of quantitative estimation of proteins by radial diffusion in antibody containing gels was first described by Mancini *et al.* (1965). The original method required diffusion to go to completion and was, therefore, a time consuming technique, small molecular weight proteins reaching completion in 2–3 days whilst large molecular weight proteins like α_2-macroglobulin or IgM requiring up to 6 or 7 days. The modification introduced by Fahey & McKelvey (1965) allowed the results to be read after a limited time, and, whereas this made the technique more useful in a clinical context, it did mean some loss of precision.

The protein antigen diffuses radially from a point application into an antibody containing gel, a circular precipitate being formed at the point of equivalence. With constant or uniform antibody concentration and constant gel thickness, the area encompassed by the precipitin ring is proportionate to the concentration of antigen (*Cag*). It is more convenient to plot the diameter^2 (d^2) against antigen concentration, and, provided diffusion has gone to completion, this will be a straight line which can be expressed by the equation:

$$d^2 = K[Cag] + So$$

The intercept (*So*) is a function of the antigen well diameter and the volume of antigen applied; the slope of the line is inversely proportional to antibody concentration in the gel.

Materials

Glass plates: the size of the plate can be adjusted according to the number of samples to be assayed. (50 × 50 mm, 80 × 80 mm, 100 × 100 mm).

Horizontal table, gel punch and suction device, template, water bath, moist chamber, micropipette, graduated eyepiece (Graticules).

1 % agar solution: Difco Special Agar-Noble or Oxoid Agar tablets (BR 27) dissolved in barbitone acetate buffer pH 8·6 I 0·25 (Oxoid BR 11 G), antiserum, standard, 1 % tannic acid.

Method

1 Preparation of gel: The volumes of buffered 1 % agar needed to produce a 1·5 mm thick gel are shown in Table 1.2. The volume of antiserum required depends on the titre of the relevant batch but is usually in the order of 10–40 µl/ml of gel. Increasing the antibody concentration will reduce the final ring diameter. Pipette the requisite volume of antiserum into a universal or similar container and place in the water bath. Add the measured volume of melted agar and mix thoroughly. With the clean

Table 1.2 Agar or Agarose volumes for various plate sizes for single radial immunodiffusion (SRID) and electroimmunodiffusion (EID).

Plate size (mm^2)	*SRID* (1·5 *mm thick gel*) *agar volume*† (*ml*)	*EID* (1·9 *mm thick gel*) *agarose volume** (*ml*)
50 × 50	3·75(16)	—
80 × 80	9·6 (49)	12·0
100 × 100	15·0 (81)	18·0
200 × 200	—	36·0

* Prepared by mixing equal volumes of agarose (15 g/l in distilled water) and 40 mM barbitone buffer, containing specific antiserum.

† Numbers in brackets indicate the number of sample wells that can be conveniently used on the plate.

glass plate on a horizontal table, pour the agar-antiserum mixture evenly over the whole surface. Air bubbles may be removed by gentle flaming or with a Pasteur pipette. Allow the gel to set and then equilibrate in a moist chamber at 4°C.

2 Origin wells: Locate the template over the gel and cut the origin wells with the gel cutter, the agar plug being removed by suction. The wells

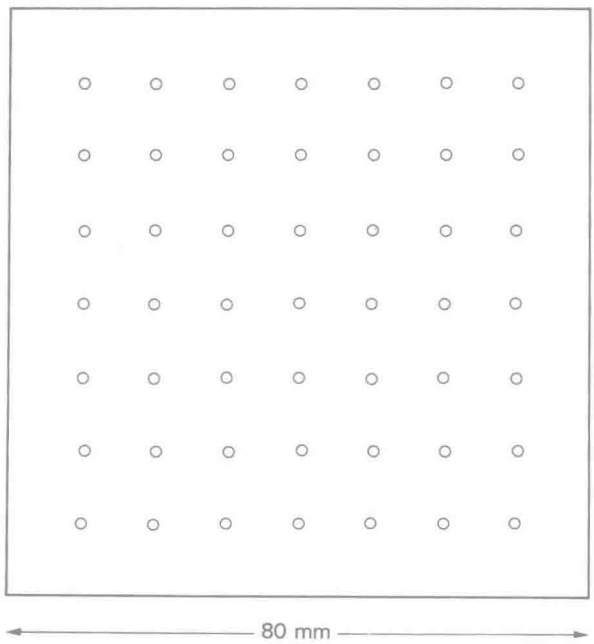

Figure 1.2 Template for single radial immunodiffusion.

should be evenly spaced and follow a regular pattern to facilitate identification (Fig. 1.2).

3 Sample loading: Measured volumes of test and standard solution are applied to the origin wells by micropipette. 2 mm diameter origin wells will accept 5 µl, and 3 mm diameter wells 10 µl.

4 Diffusion: The loaded gels are placed in a moist chamber at room temperature until completion of diffusion. Diffusion is deemed complete when the largest precipitin ring fails to increase in size as judged by daily measurement.

5 Quantitation: Place the gels in 1% tannic acid for 5 min to enhance the precipitin rings (Fig. 1.3). Measure the ring diameter (d) to the nearest

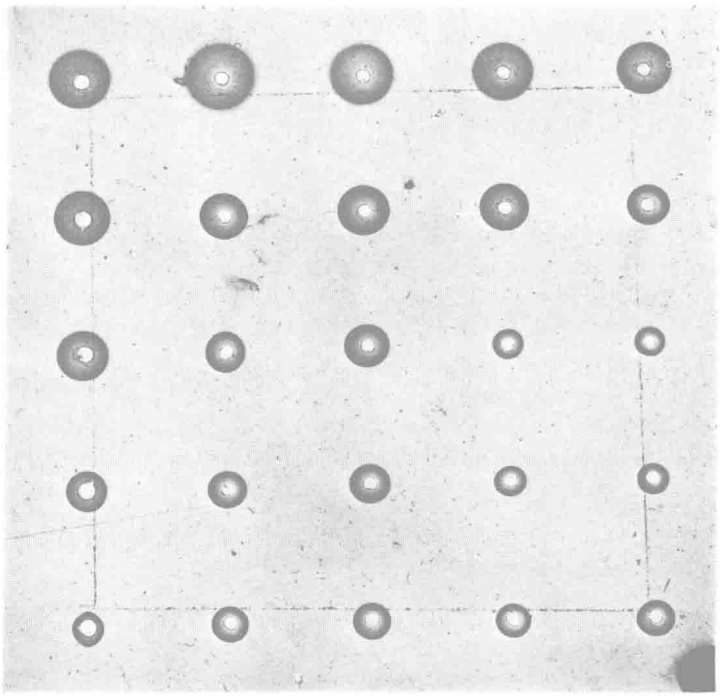

Figure 1.3. Single radial immunodiffusion.

0·1 mm and obtain d^2 from the tables. A standard curve is constructed by plotting d^2 against concentration of the standard solutions, and the concentration of unknowns read from the curve (Fig. 1.4,A).

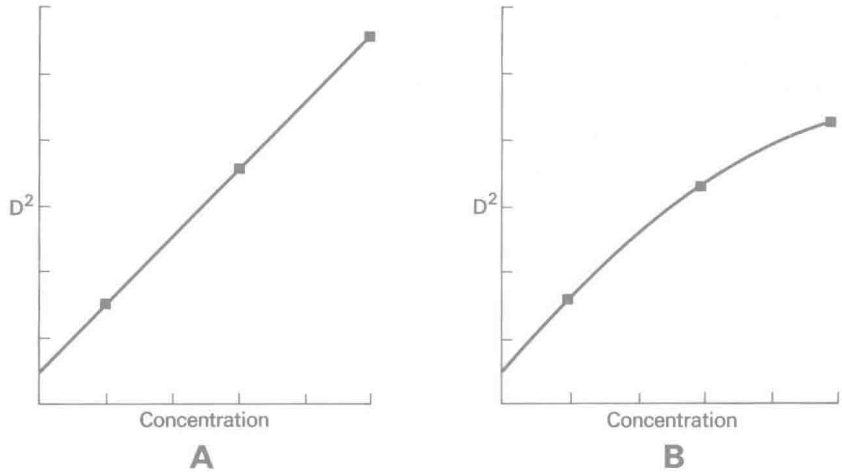

Figure 1.4 Standard curves in single radial immunodiffusion (A) allowing diffusion to proceed to completion (Mancini) (B) after 18 hours diffusion (Fahey).

Modifications

1 The agar gel may be cast between glass plates using a U-frame spacer to ensure uniform and standardised gel thickness.
2 Provided a uniform gel thickness and constant diameter origin wells, the wells can be filled to the brim with only marginal loss of accuracy.
3 The time required for diffusion to reach completion may be considered excessive. The precipitin ring diameters can be read after overnight (18 hours) diffusion but it should be realised that, as completion has not been reached, the standard curve will be non-linear at the upper end and appropriate standards should be included in the assay (Fig. 1.4,B).

Required antiserum concentrations

Although the antiserum concentration is usually determined by experimental observation, the percentage concentration of antiserum which must be incorporated into the gel can be calculated from the equation:

$$P = \frac{4\ Vag\ Cag}{T\pi h(D^2 - d^2)}$$

where P = antiserum concentration (µl/ml gel); Vag = volume of antigen (µl); Cag = maximum concentration of antigen (g/l); T = antibody titre; h = depth of gel; D = maximum precipitin ring diameter (mm); and d = diameter of origin well (mm).

Reversed radial diffusion The incorporation of antigen into the gel pro-

vides a simple method of checking relative antibody titres of different batches of antisera. With known antigen concentration, the antibody titre is proportionate to the precipitin ring diameter.

Sensitivity and precision

The limiting factor in sensitivity of the method is the size of the precipitin ring and its density. Enhancement of the ring can improve sensitivity, and ring diameter may be modified by adjustment of the antiserum concentrations. For practical purposes the lower limit of sensitivity is usually taken as 5 mg/l although problems may be encountered even at this level in whole plasma due to the presence of high levels of protein in the gel. Using low antiserum concentration, and antigens of low total protein concentration the extreme lower limit of sensitivity is 1·5 mg/l.

The coefficient of variation of the method is in the order of ± 5–8% for a practised technician; this will be increased markedly by inexperience. Using the method of diffusion to completion the coefficient of variation can be improved to ± 2–3%.

ELECTROIMMUNODIFFUSION
(rocket immunoelectrophoresis)

The technique of quantitation of proteins by electrophoresis into an antibody containing gel was first described by Laurell (1966). Although originally described as a method for albumin determination, it can be applied to most proteins with an anodic migration at pH 8·6; more recent modifications to the technique, including carbamylation (Weeke, 1968) of either antibody or antigen or formylation (Slater, 1975) of antigen, have allowed the quantitation of cathodic migrating proteins such as the immunoglobulins.

The protein under estimation migrates into the antibody-containing gel under the influence of the electric field; antigen–antibody complexes are formed which aggregate to form visible precipitates at the point of equivalence. The reaction is deemed to have gone to completion when the sample peak with the highest antigen concentration fails to extend. Provided that completion has been reached, there is a linear relationship between peak height and antigen concentration. For optimum results antibody and antigen concentrations should be adjusted so that antibody concentrations are kept at a minimum and peak heights range from 1–4 cm.

Materials

Electrophoresis apparatus: the electrophoresis tank should be equipped with