

Chapter 4

Immunoelectrophoresis for the Characterization of Allergen Extracts

Gitte Nordskov Hansen and Jørgen Nedergaard Larsen

Abstract

Immunoelectrophoresis can be used for analysis of individual proteins in complex mixtures. The conditions involved in immunoelectrophoresis are mild, avoiding the risk of denaturation, and it is possible to perform relative quantification of individual components. The fundamental disadvantage is the dependence on rabbit antisera as reagents. The usefulness of immunoelectrophoresis in allergy research is greatly enhanced by the possibility of identification of allergens to which the individual in question has IgE.

The common principle is characterized by two independent electrophoreses having direction of current perpendicular to each other, i.e., crossed immunoelectrophoresis (CIE). This ultimately results in the formation of characteristic bell-shaped precipitates, each precipitate representing one antigen. There is a linear relationship between the amount of antigen and size of precipitate for a given antibody concentration for each precipitate and so relative quantification can be performed. The sensitivity and resolution power of CIE are very high and there are multiple variations of the technique, some of which will be illustrated in this chapter.

Key words Immunoelectrophoresis, Crossed immunoelectrophoresis (CIE), Allergen, IgE

1 Introduction

Immunoelectrophoresis is the common name for a versatile family of electrophoretic precipitation-in-gel techniques, which can be used for analysis of individual proteins in complex mixtures [1, 2]. Compared to corresponding techniques, the major advantages of immunoelectrophoresis are firstly that experimental conditions are mild avoiding the risk of denaturation, and secondly the possibility of performing relative quantification of individual components. A disadvantage is the dependence on rabbit antisera as reagents.

The common principle is characterized by two independent electrophoreses having direction of current perpendicular to each other, i.e., crossed immunoelectrophoresis (CIE) (Fig. 1a). The first-dimension electrophoresis is performed in an agarose gel at

Penny Lympany and Meinir G. Jones (eds.), *Allergy: Methods and Protocols*, Methods in Molecular Biology, vol. 2020, https://doi.org/10.1007/978-1-4939-9591-2_4, © Springer Science+Business Media, LLC, part of Springer Nature 2019

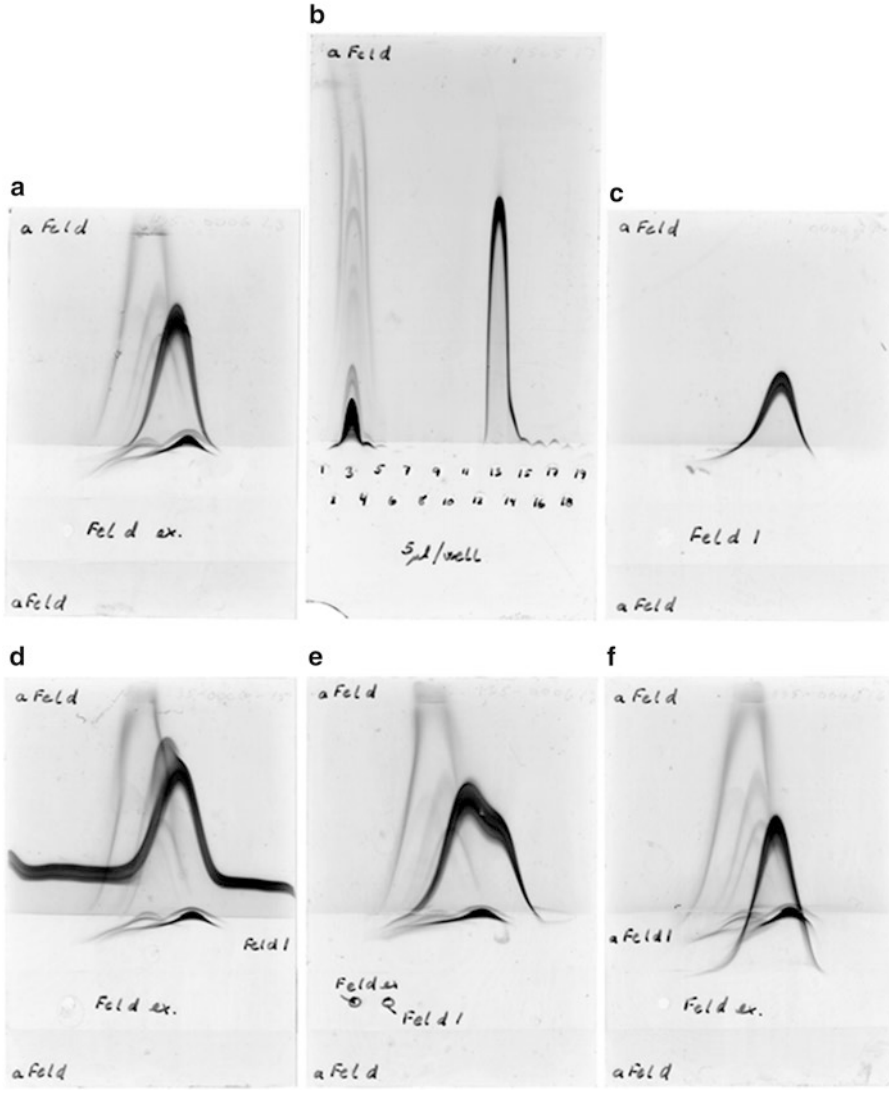


Fig. 1 Different types of immunoelectrophoresis of cat hair extract (Fel d) using a polyclonal rabbit antibody raised against the same extract (a Fel d). (**a**) CIE of Fel d extract, (**b**) FRIE following a purification (immunoabsorption) of the major allergen Fel d 1, (**c**) CIE of purified Fel d 1 against a Fel d, (**d**) CLIE with Fel d 1 in intermediate gel for identification of the allergen, (**e**) TCIE with Fel d extract and Fel d 1 showing a double peak, (**f**) CIIE with monospecific Fel d 1 in intermediate gel

pH 8.6. Protein mixtures are separated into sharp bands according to a complex relation between mass and charge. In the second-dimension electrophoresis performed perpendicular to the first, the gel on both sides of the track of proteins resulting from the first dimension electrophoresis is replaced by a gel containing polyclonal rabbit antiserum. Again, the electrophoresis is performed at pH 8.6, at which pH the average charge of the antibody molecules is zero resulting in minimal mobility of the antibody molecules during electrophoresis. Formation of antigen–antibody complexes

during second-dimension electrophoresis will result in the formation of characteristic bell-shaped precipitates, each precipitate representing one antigen. The size of the precipitate will depend on the ratio between antigen and antibody as well as the average avidity of the interaction and is not a direct measure of antigen concentration. Relative quantification, however, can be performed, as there is a linear relationship between the amount of antigen and size of precipitate for a given antibody concentration for each precipitate. This is a major advantage when applied in allergen extract standardization. Furthermore, the usefulness of immunoelectrophoresis in allergy research is greatly enhanced by the possibility of incubating the electrophoretic plate with allergic patients' serum IgE for simple and reliable identification of allergens to which the individual in question has IgE.

A major advantage of immunoelectrophoresis is the providing of a range of possibilities for immunochemical analyses of individual proteins directly in a complex protein mixture. Experiments are performed under mild conditions without increased risk of protein denaturation, an important aspect when studying allergen–IgE interactions. The sensitivity and resolution power of CIE are very high; at optimal conditions about 50 antigens may be distinguished on one immunoplate, and at least 20 antigens may be quantified. Furthermore, the ease of preparation and stability of immunoglobulins make rabbit antisera useful as reference reagents for the characterization of complex allergen extracts. The dependence on antiserum, however, may also represent a drawback. When used for assessment of allergen extract complexity and for identification of allergic reactivities, it is essential to perform additional experiments to verify that all relevant allergens are precipitated by the antiserum. A limitation of the technique is that protein antigens with pI near 8.6 are difficult to analyze. The buffer pH 8.6 cannot be changed without chemical modifications of the immunoglobulins (e.g., carbamylation).

Multiple variations of immunoelectrophoresis may be designed, some of which will be illustrated in this chapter.

Crossed radio-immunoelectrophoresis (CRIE, Fig. 2a, b) is used for the identification of allergens. The cold dried CIE plate is incubated with allergic patients' IgE and developed using labeled anti-IgE and autoradiography. CRIE analyses of serum samples from several allergic patients may be used to construct an allergogram (Fig. 2d), a graphic representation of the relative importance of individual allergens.

Under carefully controlled experimental conditions the area delimited by the precipitate is proportional to the amount of antigen applied. Thus, quantitative immunoelectrophoresis (QIE, Fig. 3c) can be used for quantification of individual components in complex mixtures, for example, in batch-to-batch standardization. QIE is a method to quantify antigens without the need for monospecific antibodies.

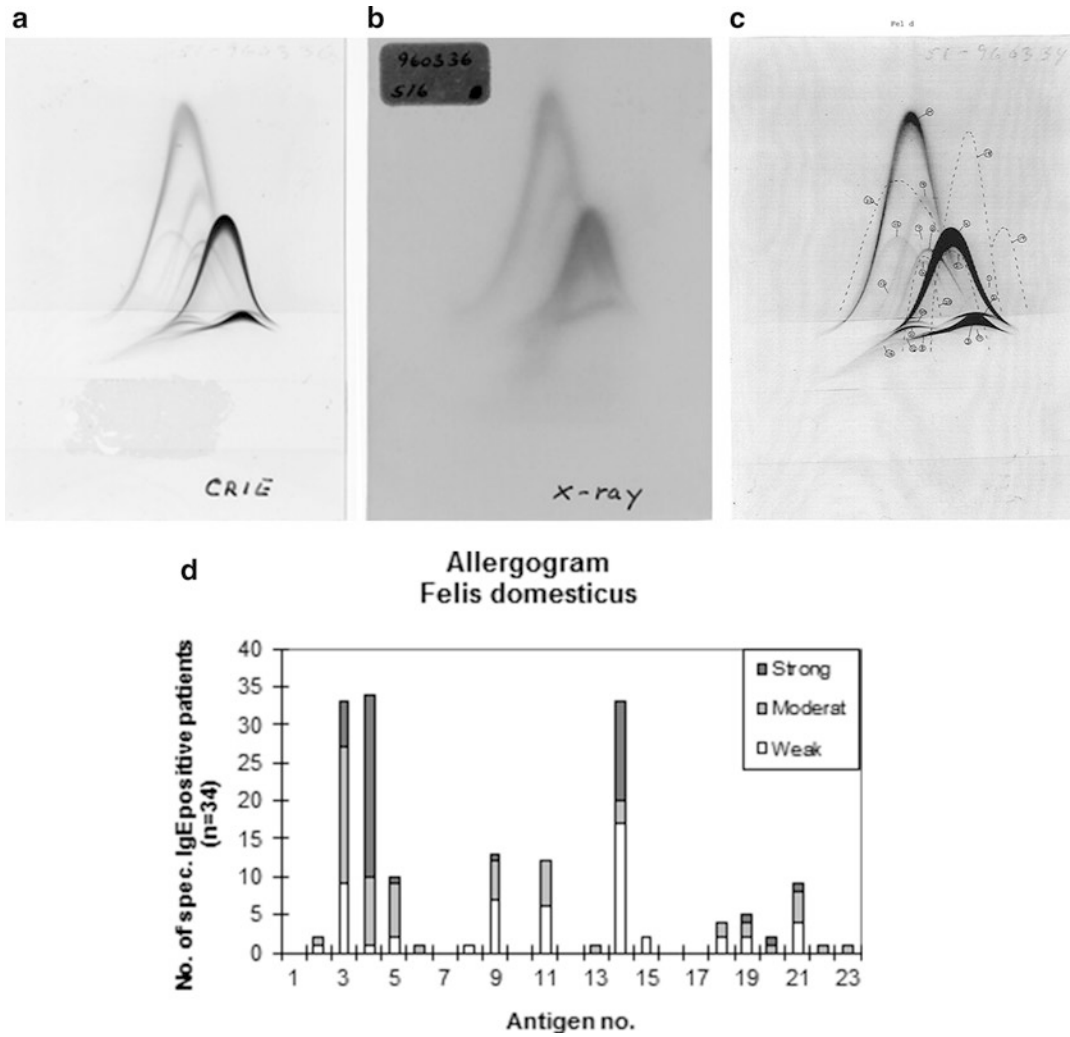


Fig. 2 CRIE and allergogram on Fel d extract. (**a**) Stained CRIE plate and (**b**) corresponding X-ray print from a cat-allergic patient, (**c**) reference picture with numbered precipitates, (**d**) allergogram from CRIE investigation of 34 cat-allergic patients' sera

A unique application is the use of fused rocket immunoelectrophoresis (FRIE, Fig. 1b) for the monitoring of a protein purification procedure. Aliquots of each fraction following a biochemical fractionation (e.g., a chromatographic step) are applied in wells in an agarose gel containing polyspecific rabbit antibody, and electrophoresis is performed in only one dimension. The wells are placed close together resulting in one large fused precipitate. Precipitates on the FRIE plate can be identified using crossed-line immunoelectrophoresis (CLIE, Fig. 1d), in which an aliquot from the relevant fraction is added to an intermediate gel in a CIE design (Fig. 1a).

If only limited amount of material is available tandem crossed immunoelectrophoresis (TCIE, Fig. 1e) can be used as an alternative for this purpose. In TCIE, two wells about 0.75 cm apart are

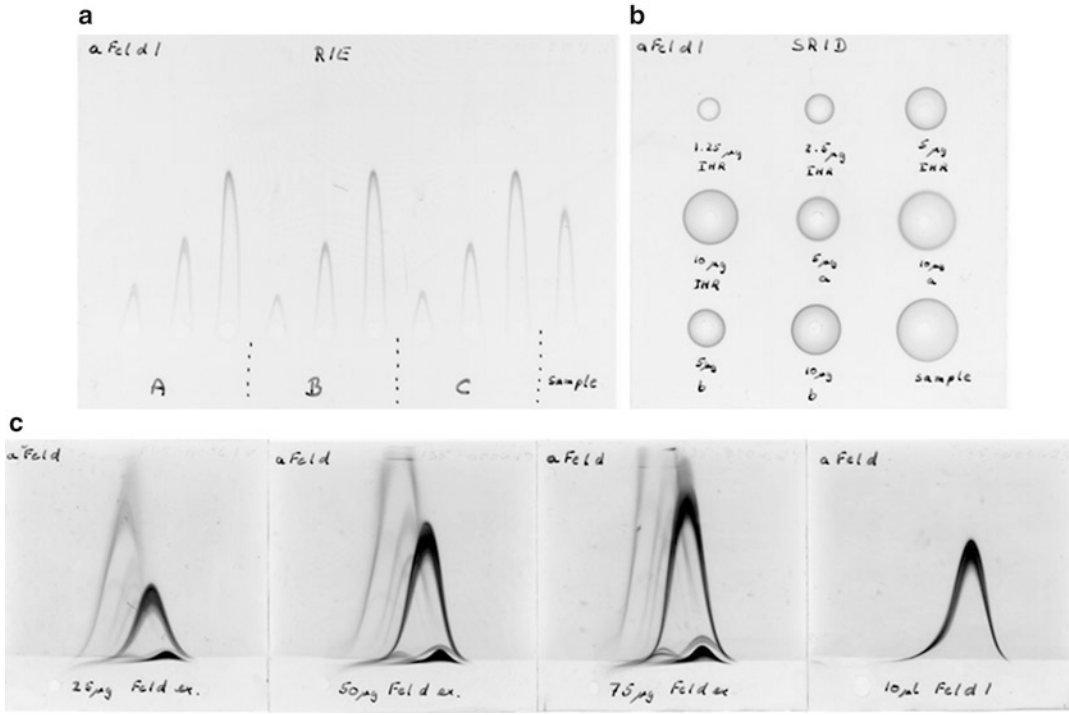


Fig. 3 Quantification methods using monospecific and polyspecific antibodies raised against Fel d 1 (**a**, **b**) and Fel d extract (**c**). (**a**) RIE of three dilutions of three Fel d batches and a sample, (**b**) SRID of four dilutions of in-house reference and different samples, (**c**) QIE of three concentrations of Fel d extract and a sample (Fel d 1)

placed alongside in the first-dimension gel; one well is filled with the reference extract and the other well with the sample to be analyzed. The precipitate formed by the antigen in the sample will make a perfect fusion with the precipitate formed by the identical antigen in the reference extract resulting in a double peak. In addition, TCIE is ideal for the study of partial cross-reactivity from which partially fused precipitates will result.

Having access to the purified protein antigen of interest, Fig. 1c, enables the production of monospecific, polyclonal rabbit antibodies by repeated immunization using the purified antigen. The specificity of the monospecific antiserum can be analyzed in crossed intermediate gel immunoelectrophoresis (CIIE, Fig. 1f) in which the monospecific antiserum is placed in an intermediate gel in a CIE design. The precipitate resulting from the monospecific antiserum will precipitate in the intermediate gel.

The monospecific antiserum can furthermore be used for identification of the antigen in an unknown mixture by CIIE or for quantification by rocket immunoelectrophoresis (RIE, Fig. 3a) or single radial immunodiffusion (SRID, Fig. 3b). In RIE multiple samples can be quantified on the same plate, as only one dimension is performed, as mentioned for FRIE.

In RIE, however, the wells are placed further apart so that individual samples can be analyzed separately. If the antigen has a high pI and the electrophoretic mobility is toward the cathode quantification can be performed using passive diffusion, as in SRID. In both RIE and SRID the quantification is performed relative to an internal standard reference preparation applied as a dilution series on the same plate.

2 Materials

2.1 Equipment

1. Heated thermostat-controlled water bath 56–60 °C.
2. Cooled thermostat-controlled water bath 15 °C.
3. Electrophoresis apparatus (two buffer vessels, two electrodes, cooled surface, chamber).
4. Power supply.
5. Glass plates (std sizes, e.g., 5 × 5, 5 × 7, 10 × 7 cm).
6. Paper wicks: Whatman no. 1 filter paper, standard size: 21 × 10 cm.

2.2 Buffers and Solutions

2.2.1 Buffer for Electrode Vessels and Agarose Gel

1. 5,5-Diethylbarbituric acid (Veronal) Mw 184.20 112.1 g.
2. Tris (Sigma 7–9) Mw 121.14 221.5 g.
3. Calcium lactate (purum) Mw 308.30 2.7 g.
4. Milli-Q H_2O to 5 L.
5. Dissolve overnight by stirring. Store at +5 °C.
6. Dilute 1 + 4 before use to obtain ionic strength of 0.02, pH 8.6.

2.2.2 Agarose Gel

1. 1% (w/v) Litex agarose-type HSA (M_r = −0.13) in the above-mentioned buffer.
2. The agarose is heated under stirring, boiled for 2 min, and kept fluid in a water bath at +56 °C.

2.2.3 Staining Solution

1. Coomassie Brilliant Blue R-250 5 g.
2. Ethanol 96% 450 mL.
3. Milli-Q water 500 mL.
4. Glacial acidic acid 50 mL.
5. Dissolve overnight by stirring. After filtration the solution is ready for use.

2.2.4 Destaining Solution

1. Ethanol 96% 450 mL.
2. Milli-Q water 500 mL.
3. Glacial acidic acid 50 mL.

2.2.5 Buffer for Incubation with Serum (CRIE)

1. Phosphate-buffered saline (PBS), 1 L:
2. NaCl Mw 58.44 8.0 g.
3. KCl Mw 74.55 0.2 g.
4. Na_2HPO_4, $2H_2O$ Mw 175.98 1.44 g.
5. KH_2PO_4 Mw 136.06 0.2 g.
6. Buffer for CRIE incubations (serum, biotin-aIgE, ^{125}I-streptavidin).
7. PBS, 0.1% HSA, 0.05% Tween (5 days' shelf life at +5 °C).
8. Washing buffer for CRIE:
9. PBS, 1% Tween.

2.2.6 Biotin-algE (ALK, Hørsholm, Denmark)

1. 20 µL/Plate of a dilution 10 ng/µL

2.2.7 Isotope for CRIE

1. Isotope: ^{125}I-streptavidin Code IRC-140 (Institute of Isotopes Co., Ltd., Budapest, Hungary).
2. Bottle with 500 µL containing 50 µCi or 0.1 µCi/µL on activity date. Use 0.4 µCi/plate.

2.2.8 Standard Developing and Fixing Solutions for Medical X-Ray Film

1. Processing (e.g., AGFA G150 Developer and AGFA G354 Fixing Bath).

2.2.9 X-Ray

1. X-ray cassettes, e.g., Lanex, Kodak X-Omat with regular screens.
2. Kodak X-ray film, MXG ref. 1666007.

3 Methods (*See* Notes 1 and 2)

3.1 CIE (Crossed Immuno-electrophoresis) (See Note 3)

3.1.1 First-Dimension Electrophoresis (Fixed 1. Dimension)

1. Mark a 5 × 7 cm glass plate with identification numbers (e.g., year, month, and no.).

   Turn the plate upside down and clean with ethanol. Place it on a leveled surface and apply 5.3 mL of hot agarose.
2. After thorough setting, punch a well as indicated on the template (Fig. 4a) and apply antigen.
3. Place the glass plate on the cooled surface (approx. 15 °C) of the electrophoresis apparatus, oriented as indicated in Fig. 4a. Connecting bridges of eight layers of paper wicks moistened with electrode buffer are established. The wicks should overlap the gel plate by approx. 0.5 cm.
4. Switch on the current and adjust the voltage across the gel to 10 V/cm. Continue electrophoresis for 25–30 min.

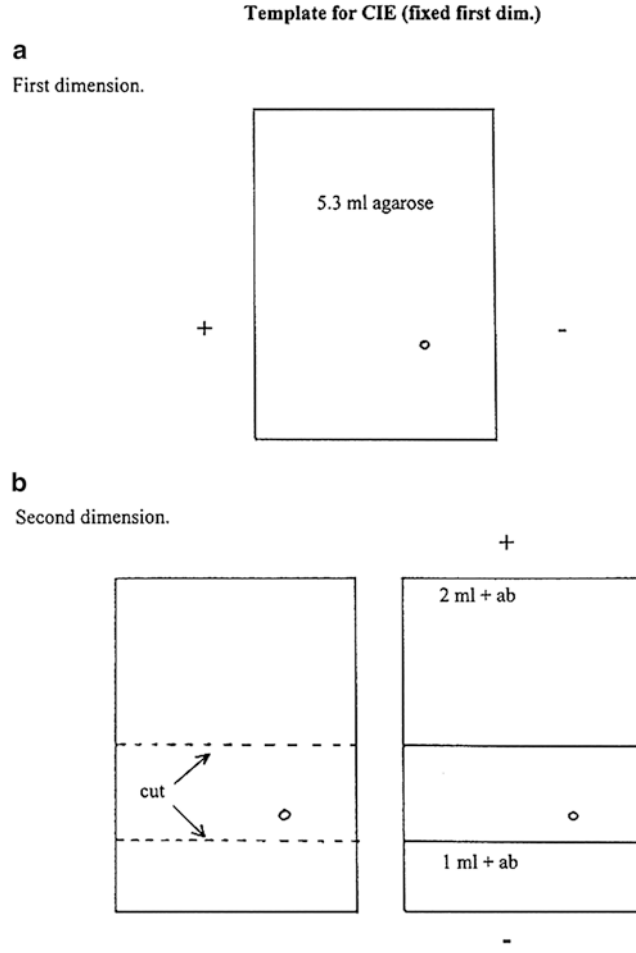


Fig. 4 Template for standard CIE indicating position of well, anode (+), and cathode (–) for the first dimension (**a**) and dimensions of antibody-containing gels for the second dimension (**b**)

3.1.2 Second-Dimension Electrophoresis

1. Cut away gel according to the template (Fig. 4b). Place the plate on the leveled surface.
2. Pour 1 mL agarose mixed thoroughly with the antibodies on the cathodic side of the first-dimensional gel.
3. Pour 2 mL agarose mixed with antibodies on the anodic side of the first-dimensional gel.
4. After thorough setting place the plate on the cooled surface of the electrophoresis apparatus and connect it to buffer reservoirs with five layers of paper wicks. The wicks should overlap the gel by approx. 0.5 cm.
5. Adjust the voltage to 2 V/cm. Place a glass plate on top of the wicks to avoid water to condense on the gel and perform electrophoresis overnight.

3.1.3 Pressing and Washing of the Plates (See **Note 4***)*

1. Place the glass plates on a filter paper and fill all wells with distilled water (avoid air bubbles, which may cause rupture of the gel). Cover the gel with a wet filter paper (paper wick) and press the plates under several layers of filter paper (use large sheets, which can be air-dried and reused), a thick glass plate, and a load of 3–4 kg.
2. Renew the upper layers of filter paper after 10 min and press again.
3. Transfer the plates to a container with excess 0.1 M NaCl for 15–30 min (washing) and press again as above.
4. Dry the plates in a stream of hot air.

3.1.4 Staining and Destaining (See **Note 5***)*

1. Stain the plates for approx. 5 min in Commassie Brilliant Blue staining solution.
2. Immerse the plates for a few seconds in distilled water to remove a great deal of the surplus stain.
3. Destain the plates twice in successive baths until the optimal destaining is reached (approx. 15 min).
4. Dry the plate in hot air.
5. Turn gel side down and write sample numbers and other information on the plate. Only write on the glass side.

3.2 Variations of CIE (See Note 6)

3.2.1 CLIE (Crossed-Line Immunoelectrophoresis)

1. Run the one-dimensional electrophoresis as described in Subheading 3.1.1.
2. Cut and remove the intermediate gel (Fig. 5).
3. Pipet a sample of the protein to be investigated into a test tube and heat it in the water bath. Apply agarose to a total volume of 0.9 mL. Mix the gel and sample and pour it in the slit between the first-dimension strip and the upper gel.
4. Allow the newly cast gel to settle before cutting and removing the anodic part of the gel. Apply the antibody-containing gels and run the electrophoresis as described in Subheading 3.1.2.

3.2.2 CIIE (Crossed Intermediate Gel Electrophoresis)

1. Run the one-dimensional electrophoresis as described in Subheading 3.1.1.
2. Cut and remove the intermediate gel (Fig. 5).
3. Pipet an antibody (polyclonal) into a test tube and heat it in the water bath. Apply agarose to a total volume of 0.9 mL. Mix the gel and antibody and pour it in the slit between the first-dimension strip and the upper gel.
4. Allow the newly cast gel to settle before cutting and removing the anodic part of the gel. Apply the antibody-containing gels and run the electrophoresis as described in Subheading 3.1.2.

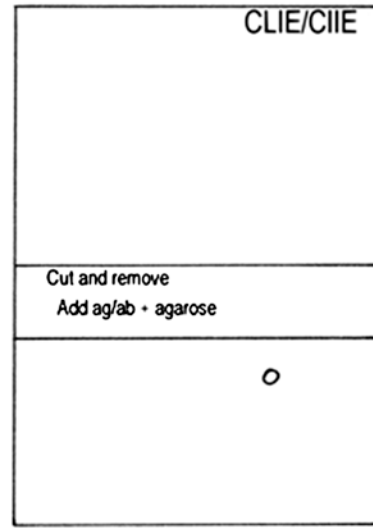


Fig. 5 Template for CLIE and CIIE indicating the position of the intermediate gel during second-dimension electrophoresis

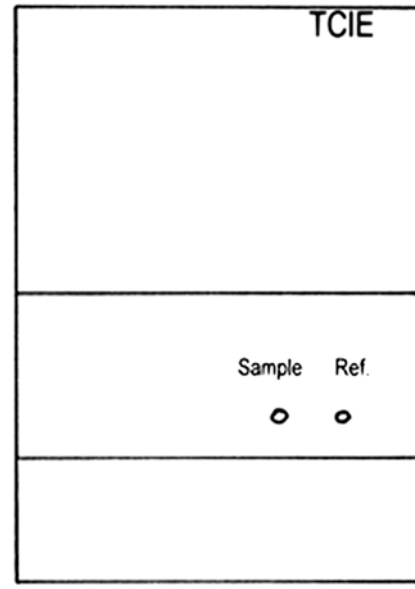


Fig. 6 Template for TCIE indication position of the two parallel wells for the first-dimension electrophoresis

3.2.3 TCIE (Tandem Crossed Immunoelectrophoresis)

1. Cast a plate for the first-dimension electrophoresis as described in Subheading 3.1.1.
2. Punch two wells alongside each other in the first-dimension gel according to the template in Fig. 6.
3. Follow the instructions for CIE as described in Subheading 3.1.

3.3 Crossed Radioimmunoelectrophoresis and Allergogram

3.3.1 CRIE (See Note 7)

1. Choose the optimized CIE standard conditions for the extract in question and perform one CIE for each serum sample to be tested.
2. After pressing and washing (*see* Subheading 3.1.3) the plates are dried in a stream of cold air. This is important, as heat can denature the proteins, which may lead to loss of their biological activity.
3. To make reproducible results run all plates before serum incubation.

3.3.2 Incubations and Autoradiography

1. The plates are placed in small plastic boxes, which fit the size of the plate (5.3 × 7.3 cm) gel side up, and incubated with a mixture of serum (200–500 μL) and PBS, 0.1% HSA, and 0.05% Tween. Each plate should be totally submerged in a total volume of 8 mL. Before incubation each box is labeled with serum or plate number. Incubate overnight at room temperature (RT) without stirring.
2. Wash each plate directly in the incubation box for 4 × 10 min with approx. 4 × 10 mL PBS and 1% Tween.
3. Incubate with biotin-labeled aIgE (ALK, Hørsholm, Denmark); 200 ng/plate, overnight at RT, in a total volume of 8 mL PBS, 0.1% HSA, and 0.05% Tween.
4. Repeat the washing procedure (4 × 10 min with PBS, 1% Tween). Incubate with ^{125}I-streptavidin (Budapest, Hungary) 0.4 μCi/plate ~4 μL; 2 h at RT in a total volume of 8 mL PBS, 0.1% HSA, and 0.05% Tween. Repeat the washing procedure (3 × 10 min with PBS, 1% Tween, 1 × 2 min with distilled water).
5. Remove the plates from the individual boxes and dry them. Clean the glass side with ethanol and place in an X-ray cassette gel side up. In a darkroom place an X-ray film (Kodak MXG) on top of the plates and expose at −80 °C for 1 day, 3 days, and 10 days, for a total of 14 days.

Following autoradiography all CIE plates are washed for approx. 10 min in distilled water dried and stained (Subheading 3.1.4). Mark the position of the CRIE plates on the X-ray film with a pencil and label each autoradiography (well to the left) with plate no., serum ID, and exposure time and cut the individual X-rays apart with a pair of scissors.

3.3.3 Standard Picture, Scoring, and Allergogram

1. Choose one of the stained CIE plates and use it as the reference plate. Draw a standard picture. The precipitates are then numbered from right to left according to the peak of each precipitate (Fig. 2c).
2. A table with patient numbers and precipitate numbers is used to record the scorings. The stained CIE plate and the three

corresponding autoradiograms from each patient are then, in sequence, compared to the standard picture, and the IgE-binding precipitates visible on the X-ray film are identified.

3. When a precipitate is visible after 1 d score 3 (strong) is given, after 3 d score 2 (moderate), and after 10 d score 1 (weak).
4. Finally the allergogram is constructed as a histogram using the precipitate numbers from the standard picture and the number of patients reacting to each precipitate. On each column the number of patients reacting with the three different scores (1, 2, or 3) is indicated (Fig. 2d).
5. An allergen is classified as a major allergen if at least 50% of the patients show strong or moderate IgE binding. Other clearly specific IgE-binding allergens are classified as minor allergens.

3.4 Quantification of Antigens (See Note 8)

3.4.1 QIE (Quantitative Immunoelectrophoresis)

1. Label four 5 × 5 cm glass plates in the upper right-hand corner with identification numbers. Turn the plates upside down and place them on a leveled surface. Clean with ethanol.
2. Pipet 3.5 mL agarose in each plate.
3. After thorough setting, punch wells according to the template (Fig. 7).
4. Apply, e.g., three concentrations of the standard and one concentration of the sample.
5. Perform the electrophoresis as described in Subheadings 3.1.1, 3.1.2, 3.1.3, and 3.1.4.

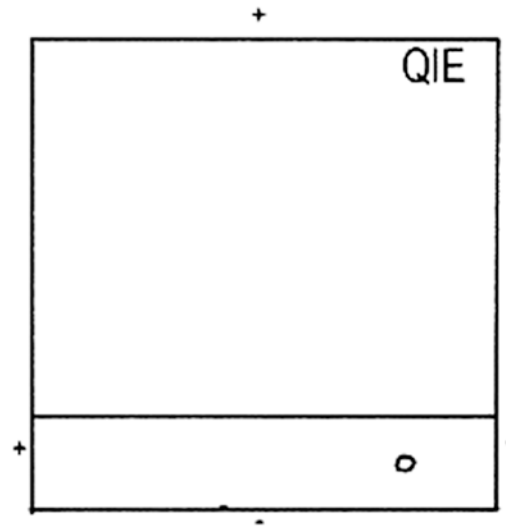


Fig. 7 Template for QIE indicating anode (+) and cathode (–) for both first- and second-dimension electrophoresis

3.4.2 RIE (Rocked Immunoelectrophoresis)

1. Label the glass plate (e.g., 10 × 7 cm) in the upper right-hand corner with identification number. Turn the plate upside down and place it on a leveled surface. Clean the plate with ethanol.
2. Pipet antibody into a test tube and heat it in the water bath (56 °C). Pipet 11 mL of agarose into the tube and mix the gel and the antibody (seal the tube with Parafilm and invert four times). Pour the antibody-containing agarose onto the glass plate (avoid air bubbles).
3. After thorough setting, punch wells 1.5 cm from the lower edge of the plate, Fig. 8a. Well size adjusted to the volume is to be applied.
4. Place the plate on the cooled surface on the electrophoresis apparatus as indicated in the template. Connecting bridges of five layers of filter paper are established. Switch on the current and adjust the voltage across the gel to 2 V/cm.
5. Apply the same volume of all samples. Place a glass plate on top of the wicks to avoid water to condense on the gel. Continue electrophoresis overnight.
6. Press and stain the plate as described in Subheadings 3.1.3 and 3.1.4.

3.4.3 SRID (Single Radial Immunodiffusion)

Prepare the gel as described in Subheading 3.4.2. Punch the wells equally spread on the plate (Fig. 8b), and apply the samples. Place the plate in a humid chamber for approx. 40 h. Press and stain the gel as described in Subheadings 3.1.3 and 3.1.4.

3.4.4 Calculation of Results

RIE and SRID: Measure the area of each precipitate. Draw a standard curve and read each sample.

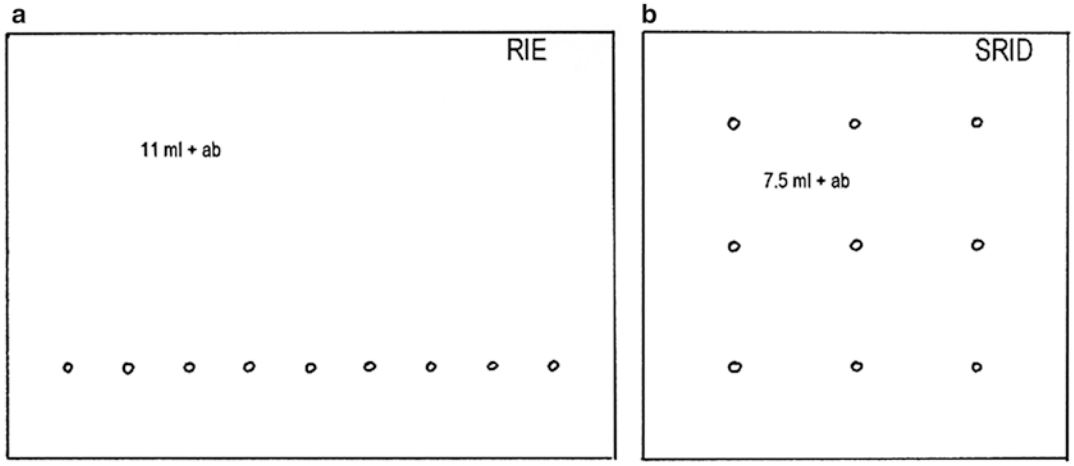


Fig. 8 Template for RIE (**a**) and SRID (**b**)

QIE: Measure the area limited by the precipitate to be quantified on the four plates. Use the line between the first-dimension gel and the antibody-containing gel as a baseline for the precipitate. Draw a standard curve and read the sample.

3.5 Fused Rocket Immunoelectrophoresis (FRIE)

1. Mark and clean a glass plate (e.g., 7 × 10 cm). Pour 11 mL agarose onto the plate.
2. After thorough setting cut away the upper and lower parts of the gel according to the template (Fig. 9).
3. Pipet antibody into two test tubes and heat them in the water bath. Pipet 5.5 mL agarose into the tube for the anodic gel and 1.5 mL agarose for the cathodic gel. Mix the gel and the antibody and pour the mixture on the glass plate.
4. Punch wells in the middle section (not containing antibody) according to the template (Fig. 9) and apply samples from each fraction (from a fractionation experiment) starting as indicated on the template.
5. Place the plate on the cooled surface of the electrophoresis apparatus and connect it to buffer reservoirs with five layers of paper wicks. The wicks should overlap the gel by approx. 0.5 cm.
6. Adjust the voltage to 2 V/cm. Place a glass plate on top of the wicks and continue electrophoresis overnight.
7. Press and stain the gel as described in Subheadings 3.1.3 and 3.1.4.

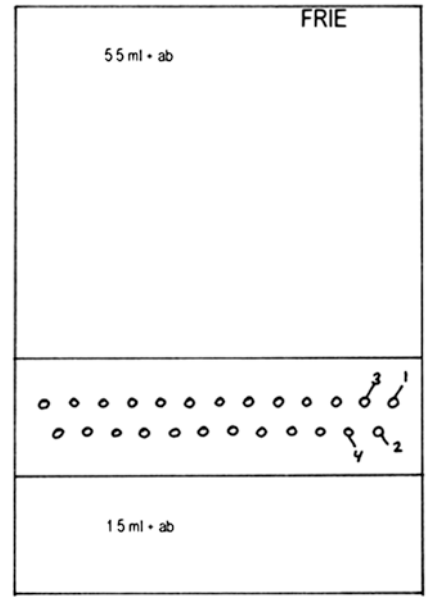


Fig. 9 Template for FRIE indicating position of wells and succession of the fractions applied

4 Notes

1. When starting to work with an unknown antigen/antibody (ag/ab) system, several parameters have to be established before the final conditions for the standard CIE picture are selected. Plates sized 5 × 7 cm have been found convenient to work with. Ag migration in the first dimension: Make sure that no ags run further than 3.5 cm toward the anode or 1.5 cm toward the cathode. As a control run the first dimension for, e.g., 10 and 30 min.

   Ag and ab concentrations: Adjust empirically the ratio between antigen and antibody so that a majority of the precipitates have a suitable size.

   Adjustments of the pattern: Initial examination of the CIE system may lead to adjustment of the gel dimensions. Positioning of the well is dependent on the mobility of the antigens in question. The size of the intermediate gel may be adjusted or a second ab may be included in this. If no antigens run toward the cathode, the antibody-containing cathodic gel can be spared and the first-dimension gel moved to the edge of the glass plate.

   Agarose: The type of agarose can be changed. Usually agarose-type HSA with electroendosmosis (Mr = −0.13) is used. In rare occasions, agarose-type HSB (Mr = −0.07) or HSC (Mr = −0.02) may be more suitable.

2. Instead of glass plates, coated plastic sheets cut in suitable sizes can be used.

   Disposable plastic tubes can replace glass test tubes, but they are difficult to incubate in a rack in the water bath.

   Use a (rotating) diamond pen to scratch the labels into the glass plate for identification.

   After use the agarose gel may be stored at 5 °C and used again after boiling.

   Heat the pipet to 56 °C before pipetting the agarose. When not used, store the pipet in a tall cylinder glass placed in the water bath to keep the pipet hot. Work fast! The agarose will set quite quickly.

   The size of the well must correspond to the sample volume. Use a gel puncher (e.g., 5, 10, or 15 μL size) connected to a vacuum pump (water jet stream or mechanical pump).

   Always keep the anode to the same side in the apparatus. Cut the paper wicks to fit the size of the glass plates to cover. The current is measured in V/cm. Use a probe with a fixed distance between the electrodes connected to a voltmeter for the calculation of V/cm. Place a glass plate on top of the wicks during the overnight electrophoresis. Moisture from the air will condense on top of the upper glass plate instead of on the gel, which may cause fluffy precipitates.

The buffer in the electrophoresis vessels can be used for approximately six runs before renewal is necessary (first and second dimensions included in one run).

All volumes of agarose gel are calculated from the area in cm2 and the thickness of the gel. The thickness is adjusted so that the first cast gel on a plate is a little higher than the next. First-dimension gel: 1.5 mm. Intermediate gel: 1.4 mm. Ab gel (anode): 1.3 mm.

Pipet the antibody into a test tube and heat the tube for approx. 10 s in the water bath before applying the agarose. Let the agarose run along the side of the test tube. Avoid air bobbles when agarose and antibody are mixed. Pay attention to the temperature in the water bath; temperatures above 60 °C will denature the antibodies. If air bubbles are present in the newly cast gel they may be removed (punctured) immediately with a clean pipet tip.

In order to reduce background staining, nonprecipitated proteins are removed by repeated pressing and washing. The use of purified immunoglobulins instead of crude antisera also reduces background staining.

3. The method described above uses a fixed first dimension directly on the glass plate, on which the antibody subsequently is applied. If several plates have to be performed in parallel a transferred one dimension may be preferred. In that case, several one-dimension strips can be performed side by side on a larger plate, separated (with a long razor blade), and transferred to the final plates where intermediate gel and antibody-containing gels are cast. Care should be taken comparing plates run under the two different conditions (transferred and fixed first dimension).

   Check and adjust the voltage once during electrophoresis, e.g., after 15 min. Beware of the shock hazard.

4. The paper wick directly on the plates may stick to the gel. In that case, moisten the paper with distilled water and remove it carefully starting in one corner. Check with a touch of a fingertip that the gels are still on the glass plates. Pour on more water if the gel sticks to the paper.

   Put saline in a suitable box, place a paper wick in the bottom (easier to get the plates up again!), and carefully place the plates in the box. Do not move the box. The gels might slide off the plates.

   Dry in cold air if the proteins should retain their biological activity for subsequent incubation with, e.g., serum (CRIE).

5. Stain with gel side up. Use a special rack accommodating several plates. Keep the staining and destaining solutions in tight plastic boxes for reuse.

The destaining solution can be regenerated passing through a filter with activated charcoal and can be reused several times.

If the gels by accident are destained too excessively, they can be restained. The best result is obtained if the plates are not dried before restaining.

6. Make a 2% agarose gel if more than 400 μL must be applied in the intermediate gel.
7. The dried plates can be stored at +5 °C for several months in a sealed plastic box or wrapped in tinfoil. Place a piece of filter paper or a soft tissue between the plates to avoid scratching of the dried agarose gels.

   The amount of ^{125}I-streptavidin/plate is calculated from the activity date given on the product specification sheet. 4 μL/Plate is used on activity reference date = 0.

Day from activity date	μL/8 per CRIE plate
−10	28
−8	29
−6	30
−4	31
−2	31
0	32 (=4 μL/plate)
2	33
4	34
6	34
8	35
10	36
12	37
14	38

The washed and dried plates are placed edge by edge in the cassette not leaving any space between them. This will ease the cutting of the film later. Start in the upper right-hand corner. Make sure that the plates do not slide in the cassette; keep it horizontal at all times.

Label the film in one edge with a pencil with date and cassette number. Expose the film in the freezer; the low-exposure temperature (−80 °C, alternatively −20 °C) will lead to stronger X-ray staining.

Before each film is developed remove the cassette from the freezer in ample time to allow complete equilibration at RT; otherwise condensed water will damage the X-ray film.

Standard developing and fixing solutions for medical X-ray film processing and procedures from the manufacturer are used.

The standard CIE picture is either drawn during projecting a stained CRIE plate in a photo magnifier or printed from a digitally scanned plate. Check the picture carefully with the stained CRIE plates to ensure that all precipitates are visible on the standard picture.

If it is difficult to recognize a CRIE precipitate on the X-ray film when individual patients are scored, it may help also to look at the stained CIE plate from which the standard picture was drawn.

Very often particular precipitates are visible only on the autoradiogram and not on the Coomassie-stained plates. This is because of lower sensitivity in the Coomassie staining. These precipitates are drawn on the standard picture with dotted lines and numbered.

All autoradiograms should be scored by two individuals. In case of discrepancies, the scoring is re-evaluated.

The CRIE technique can also be performed with biotin-labeled αIgE followed by avidin-AP (alkaline phosphatase-conjugated avidin) and substrate. This method is much faster because autoradiography is avoided; however, the relative intensity in staining is lost. Furthermore, unspecific binding is more pronounced by using this method. The method is, however, useful if a fast analysis of an individual serum or a serum pool is wanted or when monitoring, e.g., an allergen purification.

8. The humid chamber for diffusion of the SRID plate is made by placing paper napkins or filter paper moistened with distilled water in a tight box. Leave at room temperature and beware of one-sided heat from, e.g., laboratory equipment, which will disturb the shape of the precipitates.

   The height of the rockets (RIE) and d × d (diameters perpendicular to each other) of the SRID correlate with the area limited by the precipitates.

   A more precise method to work out the area is to determine it electronically or place the plate in a photo magnifier, draw the precipitates on paper, cut them out, and weigh.

References

1. Løwenstein H (1978) Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. Prog Allergy 25:1
2. Axelsen NH, Krøll J, Weeke B (1973) A manual of quantitative immunoelectrophoresis. Methods and applications. Scand J Immunol 2(Suppl 1):1