PhastSystem™
Technical Note **No. 3**

Crossed Immunoelectrophoresis (CIE) and Rocket Immunoelectrophoresis (RIE) with PhastSystem

Introduction

This note describes a method used in our laboratories for casting thin (0.7 mm) agarose gels for CIE and RIE. Running conditions for performing CIE and RIE are given and the results are illustrated. Human serum is used to demonstrate the techniques. To optimise separation and staining conditions, further development is needed, but this procedure may prove useful as a starting point for your particular application.

The procedure described here is based upon work earlier presented by Jacobsson and Degerman (1), now enriched with more practical details and an easier gel casting technique. Running CIE or RIE with PhastSystem means that you reduce separation and detection times. CIE takes about 60 minutes to run (total) and RIE takes about 35 minutes to run. This is to be compared with 30 minutes + over night and 3 hours respectively which are average times with standard equipment. Our staining procedure, which is automated, takes only 45 minutes to do. The fundamental advantage, apart from time saving and easy handling, is that you can run immunoelectrophoresis with the same equipment that you use for electrophoresis and isoelectric focusing.

Materials and methods

Procedures for performing CIE and RIE on PhastSystem, including agarose gel casting, running conditions and coomassie staining, are described in this section.

The equipment and chemicals that we have used for immunoelectrophoresis are listed here for convenience.

Equipment	Code No
PhastSystem Instrumentation	18-1600-01 (120 volt) 18-1601-01 (220 volt)
Empty buffer strip cases[1]	
Gelbond™	17-0652-01
Levelling plate	19-2871-01
Spirit level	19-3287-01
Gel punch (2.5 mm)	19-2940-01
Holder and template	19-7669-01
Filter paper (127x225 mm)	17-0653-01
Glass plates (82x82 mm)	19-1098-01
Glass tubes (55x10/11 mm)	
Razor blade	
Waterbath	
Thermometer	

Chemicals		Code No.
Agarose A	10 g	17-0422-01
Agarose A	100 g	17-0422-02
Agarose A	1000 g	17-0422-03
Agarose IEF2	10 g	17-0468-01
PhastGel BlueR		17-0517-01
5,5-Diethylbarbituric acid (barbital)		
Tris		
Calcium lactate		
Sodium azide		
Methanol		
Acetic acid		
Sodium chloride		

[1] If you do not use Buffer Strips for other applications please contact Pharmacia for some samples.
[2] Required for making buffer strips.

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Gel and buffer strip solutions

Stock buffer for agarose strips and gels.

Take 22.4 g barbital, 44.3 g Tris,
0.43 g calcium lactate, 0.8 g sodium azide and add distilled water to 1 liter.

Agarose buffer strips.

Make up a 2 % (w/v) agarose solution using Agarose IEF and stock buffer. Boil the solution until it is clear and let it cool down to about 65 °C before pouring it in the empty buffer strip cases. Each strip requires 2.5 ml solution, thus 30 ml will do for 10 strips (one buffer strip frame). Pour agarose properly up to the edge of each buffer strip well.

Agarose for gel casting.

Make up a 1 % agarose solution using agarose A and diluted stock buffer (dilute the stock buffer 1+4 with distilled water). Boil the solution until it is clear and put it in a waterbath keeping 56 °C.

One 43x50 mm, 0.7 mm thin gel requires approximately 1.5 ml agarose solution. For CIE gels 450 µl is used for the 1st dimension zone and 1 ml for the 2nd dimension zone.
The following sections for CIE and RIE contain details about the gel casting.

Crossed Immunoelectrophoresis (CIE) of human serum

1) Cut 43x50 mm squares from a sheet of Gelbond. For convenience and to protect the hydrophilic surface of the Gelbond, draw the 43x50 mm squares on the Gelbond cover paper before you cut it.

2) Put 450 µl of 1 % agarose solution in a small glass tube and keep it warm at 56 °C. Agarose pours more smoothly from glass than from plastic.

3) Remove the cover paper and put the Gelbond on a levelling plate with the hydrophilic side up. Place a glass plate (or similar) as a barrier across the Gelbond as shown in figure 1 to define the first dimension gel (without antibody).

4) Pour the agarose slowly onto the first dimension zone of the Gelbond as shown in figure 2. Let the edge of the glass tube be in contact with the Gelbond during the casting procedure. Ensure that the entire zone is evenly covered with agarose.

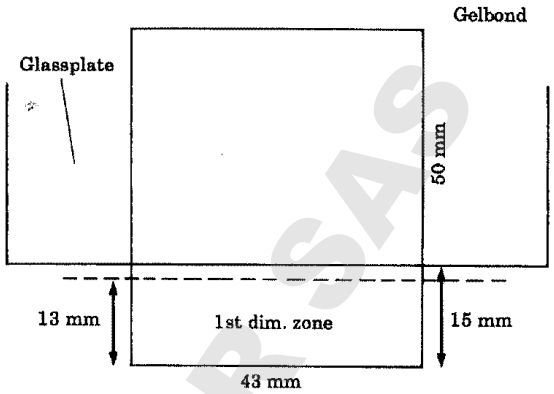


Figure 1: A template for first and second dimension of CIE.

5) Remove the glass plate after 5 minutes and adjust the size of first dimension zone with a razor blade. Remove about 2 mm so that the 1st dimension zone is about 13 mm.

6) For the second dimension zone, mix warm agarose (56 °C) with antibody. We used 100-150 µl[1] of antibody to 1 ml of agarose. The concentration of antibody has to be optimized for every antigen-antibody system. Add the antibody to a glass tube containing warm agarose to avoid congealation.

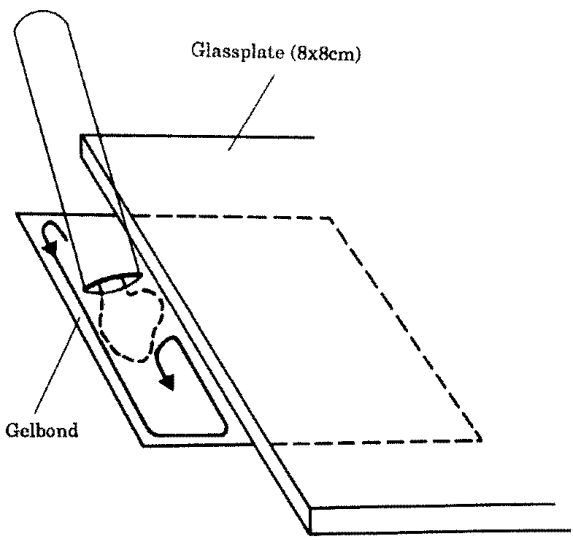


Figure 2: Casting of first dimension gel.

[1] A good starting point for choosing the optimal volume is to take 10 % v/v of the agarose volume.

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7) Cast the second dimension gel in the same way as for the first dimension gel using a small glass tube.

8) After congealation attach a gel punch (2.5 mm i.d.) to a vacuum source and punch a well in the left corner of the first dimension zone, as shown in figure 3.

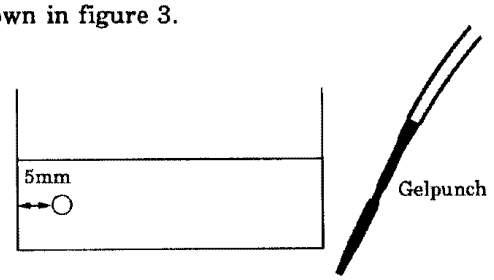


Figure 3: Using a sample well in the first dimension gel.

Running the first dimension

9) Place the gel on the separation bed with a thin film of water between the Gelbond and the separation bed to ensure good thermal contact. Adjust the position of the gel to ensure that the buffer strip will come to rest on the gel. Place the buffer strip holder in and insert the buffer strips.

10) Apply 1-3 µl of sample in the well. Include bromophenol blue (BPB) in your sample so that you can follow the separation. A microsyringe is most suitable for the sample application.

11) Start the separation:

EXTRA ALARM AT 25 Vh
SEP 1.1 70V 25mA 7W 15°C 35Vh

12) The extra alarm was put in as a check point to see how far the BPB has migrated. Stop the run when the sample has migrated about 2.5 cm from the sample well; with serum it takes bout 35 Vh.

Two CIE gels may be run at the same time, but note that different samples and/or sample preparations could have different migration rates.

13) When the first dimension is ready, turn the gel 90° clockwise and run the second dimension:[1]

SEP 1.2 50V 25mA 7W 15°C 70Vh

[1] You might find some crystallisation on the buffer strips after the first dimension. This will normally not affect the performance of the

14) Stain the gel(s) immediately after the separation. Methods for coomassie staining are described in the last section of this note.

Rocket Immunoelectrophoresis (RIE) of human serum

1) Cut a 43x50 mm square from a Gelbond sheet and place it on a levelling plate with the hydrophilic side up as already described above.

2) Add 20ul of antibody (anti human albumin) to 1.5 ml of 1% agarose (56 °C). See above for details how to prepare Agarose A for gel casting. 1.5 ml agarose will give a 0.7 mm gel. As a rule of thumb for choosing the amount of antibody you can start off with 1 % v/v of the agarose volume.

3) Pour the warm (56 °C) agarose-antibody solution onto the entire (43x50 mm) Gelbond surface.

4) Let the gel congeal for 5 minutes. Use the holder and the template when you punch 8 wells with the 2.5 mm gel puncher. See figure 4.

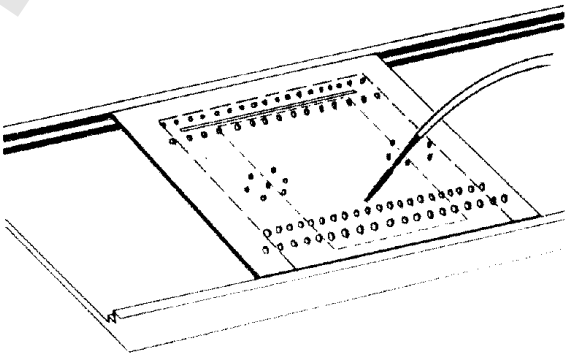


Figure 4: Punching sample wells for RIE using the gel punch together with the holder and the template.

5) Dilute the serum in the range 25x-3200x with 5x diluted stock buffer.

6) Place the gel on the separation bed, put the buffer strip holder in place and insert the buffer strips.

7) Apply 1 µl of sample in each well and start the separation:

SEP 1.1 100V 25mA 7W 15°C 100Vh

Alternatively, another method will be suitable:

SEP 1.1 150V 25 mA 7W 15°C 15Vh
SEP 1.2 100V 25 mA 7W 15°C 85Vh

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8) Stain the gel(s) immediately after the separation. Methods are described at the end of this section.

Staining CIE and RIE gels with coomassie blue (CBB)

1) Remove the gel from the separation bed and place it on a flat surface. Soak a piece of filter paper in saline (0.9 % NaCl) and place it on the gel. Place some dry paper towels on top and finally a weight of about 1 kg (figure 5). Let this stand for 5 minutes.

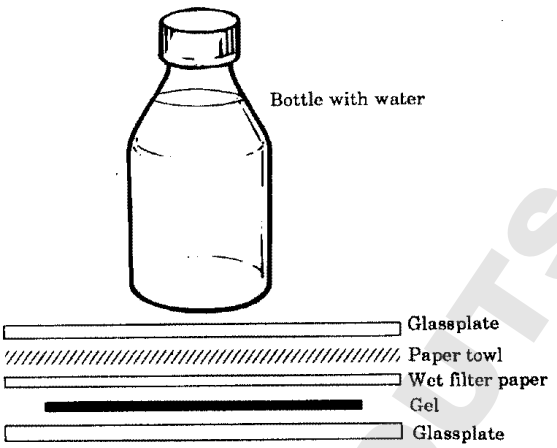


Figure 5: Pretreatment of the agarose gel before the staining procedure in order to remove non-precipitated proteins.

2) Remove the filter papers from the gel and replace them with new filter papers which are soaked in distilled water. Let it stand for 5 minutes.

3) Remove the filter papers from the gel. Place the gel on the separation bed. Set the stand by temperature to 50° C and leave the gel there until it is dry.

4) Put the dried gel in the development unit and stain it using the following method:

Step No	Solution	IN	OUT	Time (min)	Temp (C°)
1	Saline	1	0	15	20
2	Dist.H_2O	2	0	5	20
3	CBB stain	3	0	10	50
4	Destain	4	0	2	50
5	"	4	0	5	50
6	"	4	0	10	50

Use 0.1 % PhastGel Blue R for the coomassie staining.

See Development Technique File No. 200 for preparation of the solutions. The destaining solution is methanol: acetic acid: distilled water in the proportions 3:1:6.

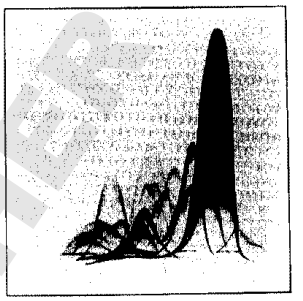


Figure 6: CIE using PhastSystem. The sample is normal human serum and antiserum antibodies (Dako).

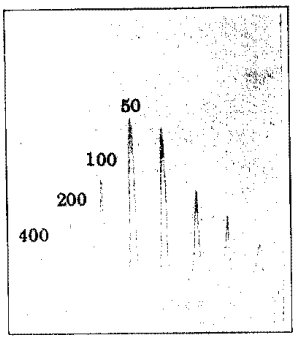


Figure 7: RIE using PhastSystem. The sample is normal human serum and anti-albumin antibodies (Dako). The sample was serially diluted.

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Results and concluding remarks

Figure 6 shows a CIE gel and figure 7 a RIE gel obtained with the procedures described in this note. These results demonstrate the use of home-made agarose gels with PhastSystem. The results resemble those obtained with standard equipment.

Immunoelectrophoresis with PhastSystem has several advantages worth considering. Since both CIE and RIE are quantitative techniques, automatic separations and staining under controlled conditions will contribute to the reliability of the quantitation. Other major advantages with PhastSystem are reduced time, convenience in handling, and the requirements for only small sample and antibody amounts.

The methods described in this note may be useful for other antigen-antibody systems with little or no modification other than those used in the examples above. Since this note primarily gives practical procedures, more detailed theory (2) about these techniques has not been considered here.

References

1. Electrophoresis '86, Ed. M.J. Dunn, p.389-391.

2. A manual of quantitative immuno-electrophoresis. Methods and applications. Edited by N.H. Axelsson., J.Krøll., and B.Weeke. Universitetsforlaget, Oslo.

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