Scandinavian Journal of
Immunology

Supplement No. 10, Volume 17, 1983

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PUBLISHED
BY BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD LONDON EDINBURGH BOSTON MELBOURNE

Published by Blackwell Scientific Publications
Editorial offices:
Osney Mead, Oxford, OX2 0EL
8 John Street, London, WC1N 2ES
9 Forrest Road, Edinburgh, EH1 2QH
52 Beacon Street, Boston
Massachusetts 02108, USA
99 Barry Street, Carlton
Victoria 3053, Australia

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St Louis, Missouri 63141

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Ontario, M1B 2X4

Australia
Blackwell Scientific Book Distributors
31 Advantage Road, Highett
Victoria 3190

British Library
Cataloguing in Publication Data

Handbook of immunoprecipitation-in-gel techniques. —(Scandinavian Journal of Immunology. Supplement ISSN 0301 6323; No. 10)
1. Immunology techniques
I. Axelsen, Nils H.
574'.2'9'028 QR183

ISBN 0-632-01057-6

Printed in Great Britain by the University Press, Cambridge

Cover illustration from Chapter 42.

Acknowledgements

This book has been in preparation for some years and I want to cordially thank all the contributors for their good work and for their tolerance and patience.

I am also grateful to Dorrit Sørensen and Helle Danielsen for their never failing, fast, perfect and kind secretarial assistance. Ellen Johannessen from the Editorial Office of *Scandinavian Journal of Immunology* has been of great help in making the manuscript ready for printing. Finally I want to thank my wife, Elisabeth Bock, for much good advice.

Nils H. Axelsen

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Preface

This handbook is a guide to the performance and use of the most important gel immunoprecipitation techniques. The aim of the book is to provide the reader with sufficient background and up-to-date knowledge to start bench work without further reading.

The book is divided into nine sections, of which Part I deals with chemicals, solutions, equipment and general procedures. Part II deals with electrophoresis and immunofixation, Part III with single radial immunodiffusion and double diffusion-in-gel, and Part IV with classical immunoelectrophoresis and spot immunoprecipitate assay. The largest part of the book—that is, Parts V, VI, VII, VIII—is devoted to the sensitive and highly resolving electroimmunoprecipitation techniques, of which Laurell's crossed immunoelectrophoresis is the flagship. The term 'electroimmunoprecipitation' was coined by Bengt G. Johansson for immunoprecipitation taking place in an electric field. Part IX deals with production of antibodies for use in immunoprecipitation techniques.

Each chapter describes the details of a single method and is divided into the following sections: Principle of the technique; Equipment; Reagent; Procedure; Evaluation of results; and Use and limitations of the technique.

Most of the 54 contributors have unique experience from research and postgraduate teaching of the methods, and several of the contributors also have experience in large-scale application of some of the methods in diagnostic laboratory work.

Copenhagen, August 1982

Nils H. Axelsen

Contents

Part I
Chemicals, Solutions, Equipment and General Procedures

Scand. J. Immunol. Vol. 17, Suppl. 10, 3–20, 1983

1. Chemicals, Solutions, Equipment and General Procedures

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INTRODUCTION

The immunoprecipitation techniques described in this book are generally simple and inexpensive procedures requiring a minimum of technically complicated equipment. Since most of the methods involve one or two electrophoretic steps performed in agarose gel, the most obvious need is an electrophoresis apparatus, including a d.c. power supply and a cooling device, which should fulfil the demands described in detail in this chapter. The other equipment is optional, but it should be remarked that access to it is of considerable help in performing the various immunoprecipitation methods with a maximum resolution and/or minimal imprecision.

The choice of some chemicals or reagents, e.g. antibody preparations, agarose, and buffers, is also of importance, especially in the immunoelectrophoretic methods, and will be described here as well. Special equipment in some procedures will be discussed in other chapters of this book.

0301–6323/83/0300–0003 $02.00

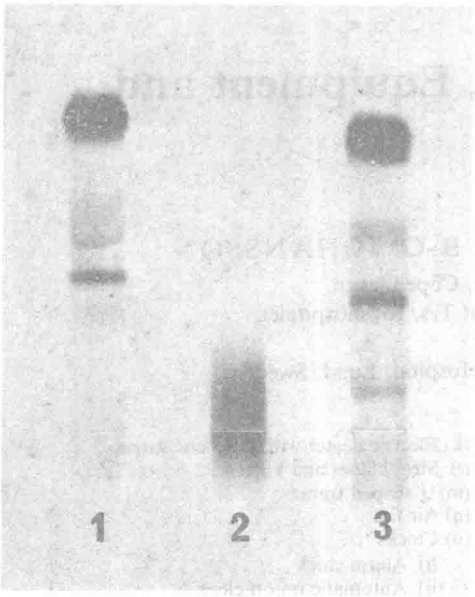

FIG. 1.1. Agarose gel electrophoresis. Buffer: Tris/barbital pH 8.6, ionic strength 0.02. Electrophoresis: 15 V/cm for 60 min at 15°C. Samples: 1, rabbit serum; 2, immunoglobulin fraction of rabbit anti-human serum; 3, normal human serum. Three microlitres were applied. Fixation and staining as described in the text. Note that the average migration of the immunoglobulins is close to zero.

CHEMICALS AND SOLUTIONS

Chemicals

2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)
N-tris(hydroxymethyl)methyl glycine (Tricine)
5,5-diethylbarbituric acid (barbital)
5,5-diethyl-sodium barbiturate (barbital-Na)
Sodium azide
Calcium lactate
Glacial acetic acid
Sodium chloride
Agarose (with an M_r value of -0.13; for definition of M_r see p. 5)
Coomassie brilliant blue R 250
Amido black B
Ethanol 96%
Picric acid
Polyethylene glycol 6000
Potassium cyanate
Boric acid
Sodium tetraborate

Solutions

(*i*) *Buffer.* In quantitative immunoelectrophoresis the electroendosmosis of the agarose gel and the pH and ionic strength of the buffer must be balanced so that the average mobility of the antibodies is close to zero. Most distinct immunoprecipitates are obtained between the ionic strengths 0.01 and 0.03. This low ionic strength facilitates cooling during electrophoresis but requires a buffer with a very high buffering capacity. Rabbit antibodies and 1% agarose gel with an M_r value of -0.13 (see below) in Tris/barbital buffer, ionic strength (I) 0.02 and pH 8.6, fulfill the above-mentioned demands [627] (see Fig. 1.1).

Tris/barbital buffer, pH 8.6, $I = 0.1$ (stock solution):

5,5-diethylbarbituric acid (barbital)	224 g
2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)	443 g
Sodium azide	10 g
Calcium lactate	20 g
Distilled water to make	10 l

(Dilute one part stock solution plus four parts distilled water to obtain ionic strength 0.02. The pH at 25 °C is 8.5.)

The chemicals dissolve rather quickly in distilled water (minimum 5 litres) under magnetic stirring. No heating is necessary. Distilled water is finally added to a total volume of 10 litres.

This buffer will produce the same pattern in quantitative immunoelectrophoresis as the one recommended earlier [692] and exhibits a buffering capacity that is several times higher than that of the earlier buffer, where the buffering capacity was often exhausted. This caused the pH in the cathodic electrode vessel to rise to over 11, resulting in dissolving of the immunoprecipitates from the cathodic side. The new Tris/barbital buffer can be used three to four times with the same polarity, thereby preventing excess antigens —which in a previous run have migrated into the electrode vessel—from migrating back into the agarose gel from the electrode vessel and producing artifacts in the precipitation patterns. An automatic timer could of course be used to control the time of exposure to current flow. The duration of a single run is 18 h at 3 V/cm.

For screening electrophoresis of plasma proteins at high ionic strength the following buffer is recommended [395]:

Barbital/barbital-Na buffer, pH 8.6, I = 0.075 (as used):

5,5-diethylbarbituric acid (barbital)	30 g
5,5-diethyl-sodium barbiturate (barbital-Na)	155 g
Sodium azide	10 g
Calcium lactate	3 g
Distilled water to make	10 l

The barbital dissolves after a few minutes in 1 litre of boiling distilled water under magnetic stirring. Heating is then discontinued and the barbital-Na is added. When this has dissolved, sodium azide and calcium lactate are added. Finally distilled water is added to a final volume of 10 litres.

The handling and distribution of barbital derivatives is restricted by federal drug-control rules in some countries, and the access to these compounds is therefore quite impractical and often rather expensive. A non-barbital buffer for immunoelectrophoresis has therefore been suggested [see Ref. 453] by substituting the barbital with Tricine:

Stock solution, Tris/Tricine, pH 8.6, I = 0.1:

N-tris(hydroxymethyl)methyl glycine (Tricine)	226 g
2-amino-2(hydroxymethyl)propane-1,3-diol (Tris)	443 g
Sodium azide	10 g
Calcium lactate	20 g
Distilled water to make	10 l

(Dilute one part stock solution with four parts distilled water before use. The pH at 25 °C is 8.5.)

Comparison of immunoplates obtained using this buffer with those obtained with the barbital/Tris buffer reveals only minor changes in the precipitation pattern, and these will not confuse the interpretation. The comparison was performed by means of crossed immunoelectrophoresis with 2 µl human serum (pooled) and 1000 µl (12.5 µl/cm²) anti-human serum (DAKO, code 100 SF); the first dimension was run at 15 V/cm and the second dimension was run at 3 V/cm.

(*ii*) *Agarose.* Agarose is a mixture of polysaccharides consisting of repeating units of galactose and 3,6-anhydrogalactose and is obtained by fractionation of agar. Although agarose has a considerably lower content of charged groups (sulphate and pyruvate) than the unfractionated agar, it still contains such groups in various amounts, depending on the quality. The charged groups are responsible for two phenomena, appearing in agarose gel electrophoresis: electroendosmosis and adsorption of certain proteins. The M_r value is a measure of the electroendosmosis and is obtained by subjecting human serum albumin and Dextran 70 to electrophoresis in the actual system. The shift of the dextran is measured (the migration direction taken into consideration: negative for migration to the cathode, positive for migration to the anode), and this distance is related to the total distance between the dextran and human albumin. Agarose qualities with a suitable degree of electroendosmosis should be employed in the various procedures. The agarose used for most of the experiments in this book is of very good strength and guaranteed to maintain the same electroendosmosis from batch to batch, which for type HSA (Litex, Glostrup, Denmark) is −0.13.

1% agarose in Tris/barbital buffer, pH 8.6, I = 0.02:

Two grams of agarose are added to 200 ml of the diluted buffer and dissolved by gentle heating on a magnetic stirrer. The solution should boil for 5–6 min to ensure that the agarose is completely dissolved. (Prolonged boiling should be avoided, as the solution may turn yellow and agarose gel formed afterwards may be impaired.)

The solution is kept fluid at 56°C in a water bath and is ready for use after temperature equilibration. One per cent agarose can be stored for several weeks at 4°C and liquified repeatedly by heating.

(*iii*) *Staining solutions.* Amido black B or Coomassie brilliant blue R 250 can be used for staining the immunoelectrophoresis plates. Coomassie brilliant blue has the advantage of being about three times more sensitive than Amido black B. Weak precipitates may therefore be revealed more clearly or the amount of antibodies can be decreased.

Ethanol 96%	4500 ml
Distilled water	4500 ml
Glacial acetic acid	1000 ml
Coomassie brilliant blue R 250 (or Amido black B)	50 g

The dye is dissolved by heating to 60°C followed by cooling to room temperature and filtering

through filter paper. The time for staining is 5–10 min.

(*iv*) *Destaining solution.*

Ethanol 96%	4500 ml
Glacial acetic acid	4500 ml
Distilled water	1000 ml

After being stained, the immunoplates are washed three times in this solution to remove excess stain and obtain a clear background. Destaining to a faint bluish background is recommended, since this will ensure that too much destaining has not occurred, thereby losing sensitivity unintentionally. The destaining solution can be used repeatedly if filtered through activated charcoal after use.

(*v*) *Solution for protein fixation.* It is occasionally desirable to compare the first-dimension agarose electrophoresis with the second-dimension immunoelectrophoresis, and the proteins must therefore be denatured before staining. The best binding of the dye is achieved when the proteins have been precipitated with picric acid.

Picric acid	14 g
Distilled water	1000 ml
Glacial acetic acid	200 ml

The picric acid is added to the water, which is then heated to 60°C. The warm solution is filtered through filter paper and finally the glacial acetic acid is added.

(*vi*) *Washing solutions.* After immunoelectrophoresis, non-precipitated proteins must be removed from the agarose gel. This is done by washing twice in 0.1 M NaCl and once in distilled water (see below).

(*vii*) *Antibodies.* Crude antisera may always be used directly for the experiments. However, to obtain a low-background staining the crude antibody preparation should be subjected to salting out (250 g ammonium sulphate/1000 ml rabbit antiserum), dialysis, and chromatography on DEAE-Sephadex A-50 at pH 5.0 in sodium acetate/acetic acid buffer with ionic strenth 0.05. The purified gammaglobulin fraction is then dialysed against 0.1 M NaCl/15 mM sodium azide. This preparation will lose less than 2% of its activity per year if stored at 4°C. For further details see Chapter 41.

The immunoprecipitates may be enhanced by including polyethylene glycol (mean molecular weight 6000) in the antibody-containing gel to a final concentration of 3–5% (w/v). This is especially important in the determination of low-molecular-weight proteins or if antibodies in low concentration and/or with low avidity must be used.

APPARATUS AND ACCESSORIES

A variety of equipment for immunoelectrophoresis is available today. The following description therefore aims at outlining the principles and criteria for the individual items to obtain reproducible and successful experiments. Since most of the experiments appearing in this book have been conducted with equipment originally developed by Laurell and his group [49, 400] and by the Protein Laboratory in Copenhagen, such equipment will be described below. However, other types of equipment meeting the same requirements may also be used.

(*a*) *Electrophoresis apparatus*

One-millimetre-thick glass plates are used to support the agarose gels. The following sizes are recommended as standard: 5 × 5 cm, 5 × 7 cm, 7 × 7 cm, 7 × 10 cm, 10 × 10 cm, and 11 × 20.5 cm. The electrophoresis apparatus (1 in Fig. 1.2a) has been designed to accommodate a multitude of these standard glass plates on the cooled surface (1 in Fig. 1.2b). The cooled surface measures 22 × 12 cm. The design is similar to that described by Johansson [340], and the cooling is obtained by cutting a serpentine-shaped cooling channel in a thick perspex plate, leaving 1 mm of material between the cooling channel and the cooled surface, which by this procedure is left perfectly plane to optimize the cooling capacity. The cooling water is circulated by means of a cooling thermostat (2 in Fig. 1.2a) which should be set to maintain a temperature of 10–15°C in the cooling water and a flow rate of approximately 1 litre/min for sufficient cooling capacity for immunoelectrophoresis. Since water restrictions are in effect in most areas, cooling with water from a water tap should be used only in an emergency, and in such cases temperatures below 8°C and above 22°C are not recommended. Too intense cooling may cause water to condense on the gel surface, whereby the immunoelectrophoresis pattern is distorted. Insufficient cooling will increase evaporation from the gel and introduce partial drying of the gel. The cooling plate is

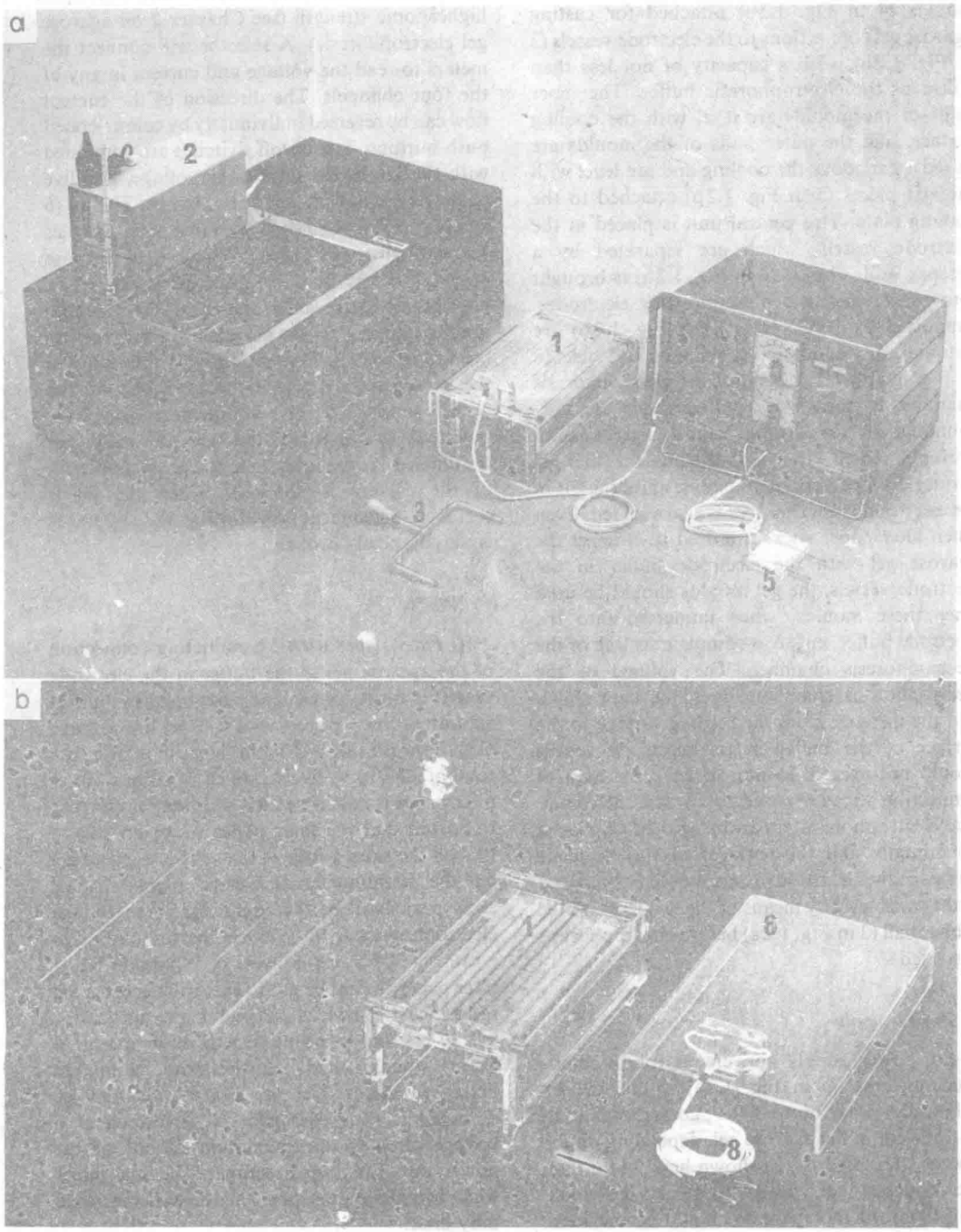

FIG. 1.2. (a) Electrophoresis module. 1, Electrophoresis apparatus; 2, cooling thermostat (sufficient for five to ten electrophoresis apparatuses); 3, U-tube with squeeze-ball for levelling the surface in the electrode vessels; 4, d.c. power supply with four channels, individually adjustable; 5, test probe for measuring and adjusting the potential gradient directly in the agarose gel. (b) Parts of electrophoresis apparatus. 1, Cooling plate; 2, supports for cooling plate; 3, electrode vessels; 4, moulds for gel connections; 5, end pieces; 6, lid with access holes for test probe; 7, supports for platinum wire electrodes; 8, connection wire to power supply.

attached to two supports (2 in Fig. 1.2e) with moulds (4 in Fig. 1.2b) attached for casting agarose gel connections to the electrode vessels (3 in Fig. 1.2b), with a capacity of not less than 1 litre of the electrophoresis buffer. The inner walls of the moulds are level with the cooling surface, and the outer walls of the moulds are raised 1 cm above the cooling and are level with the end pieces (5 in Fig. 1.2b) attached to the cooling plate. This central unit is placed in the electrode vessels, which are separated by a perspex wall. The lid (6 in Fig. 1.2b) is brought into place, and the platinum-wire electrodes, supported by PVC plates (7 in Fig. 1.2b) are automatically connected to the wire and jacks (8 in Fig. 1.2b). Furthermore, the electrophoresis chamber is completely sealed from the surrounding air. This arrangement to a great extent prevents water from condensing on the gel surface even on very humid days, and draught in the electrophoresis chamber is also avoided. Even when filter paper wicks are used to connect the agarose gel with the electrode buffer in the electrode vessels, the gel moulds should be used since these moulds, when immersed into the electrode buffer, ensure a complete sealing of the electrophoresis chamber. The volume of the electrophoresis chamber should be kept small, and the distance from the cooling surface to the surface of the buffer in the electrode vessels should not exceed 30 mm, to keep the area of connection wicks exposed to air at a minimum. The electrophoresis apparatus should be placed horizontally and the surfaces of the electrode buffers in the electrode vessels should be balanced to the same level by means of the U-tube with the rubber ball (3 in Fig. 1.2a) before electrophoresis is started.

(*b*) *Power supply*

A d.c. power supply convenient for immunoelectrophoresis (4 in Fig. 1.2a) should deliver stabilized voltage up to 300 V, corresponding to 10–15 V/cm in the agarose gel, depending on the type of wick used. If, as shown here, more than one channel has been built in, individual adjustment of the channels should be included, since both low-voltage and high-voltage electrophoresis can then be performed at the same time. Three channels have the capacity of delivering 300 V/100 mA d.c., which is sufficient for immunoelectrophoresis. The fourth channel can deliver 300 V/200 mA d.c. and is used with buffers of higher ionic strength (see Chapter 2 on agarose gel electrophoresis). A selector will connect the meters to read the voltage and current in any of the four channels. The direction of the current flow can be reversed individually by colour-coded push buttons. The on/off switches are combined with the knobs for setting the voltage, and live channels are indicated by pilot lamps. The lid (6 in Fig. 1.2b) is furnished with five pairs of holes spaced 4 cm apart. These holes serve the purpose of giving access for a test probe (5 in Fig. 1.2a), which is used to measure and adjust the potential gradient directly in the gel when the current has been switched on. The test probe is connected to the power supply, and a rocker switch on the power supply sets the voltmeter to read the potential gradient in the gel in volts per centimetre. At the same time the correct polarity of the current is checked. When the lid is removed, the current flow through the apparatus is automatically broken.

(*c*) *Wicks*

(*i*) *Filter paper wicks.* Conducting connection of the agarose gel to the buffer in the electrode vessels is most conveniently obtained by buffer-saturated filter paper wicks. For low-voltage electrophoresis at 2–3 V/cm, corresponding to a total of 70–110 V, five layers of Whatman No. 1 filter paper is recommended. It is very important to ensure that the filter paper wicks are cut to exactly the same width as that of the agarose gel on the immunoelectrophoresis plate—if not, skew peaks will be the result. For high-voltage electrophoresis at 10–15 V/cm, corresponding to a total of 300 V, eight layers of Whatman No. 1 filter paper should be used. This will decrease the resistance in the wicks exposed to air; the voltage will be less, and evaporation is therefore to a great extent counteracted. Furthermore, a higher voltage gradient over the agarose gel may be obtained if the experiment is performed at a voltage close to the maximum output of the power unit. The fibre direction of the filter paper wicks is of no importance, and the wicks are used only once.

(*ii*) *Agarose gel connections.* An alternative way of establishing conductance between the electrode buffer and the agarose gel on the plate is to fill the moulds (4 in Fig. 1.2b) with 1.5% (w/v) agarose solution in the electrophoresis buffer at