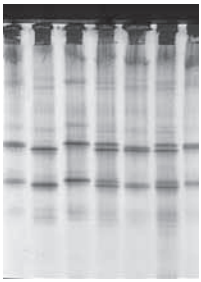
instructions

Immobiline DryPlate



User Manual

ⓘ 71-7030-01 Edition AD

Amersham Biosciences

Important user information

Reading this entire manual is recommended for full understanding of the use of this product.

The exclamation mark within an equilateral triangle is intended to alert the user to the presence of important operating and maintenance instructions in the literature accompanying the instrument.

Should you have any comments on this manual, we will be pleased to receive them at:

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Contents

1. Introduction

Immobiline™ DryPlate offers a convenient and reliable way to obtain the utmost separation power of isoelectric focusing.

The Immobline system has indefinitely stable pH gradients allowing high voltages for maximal separation and, when necessary, long focusing times (1, 2).

The rehydratable dry gels facilitate the use of additives such as urea, detergents, carrier ampholytes etc, for optimal performance, even for samples with poor solubility.

This manual gives general instructions on how to use Immobiline DryPlate for isoelectric focusing. Please consult the Application Notes and/or the articles in the reference list (3–21) for detailed instructions on specific applications.

Immobiline DryPlate is a polyacrylamide gel with an immobilized pH gradient. It is bound to plastic backing and is ready to use for isoelectric focusing after rehydration. The product is available with various pH gradients (See Table 1.) The pH gradients are linear over the stated interval.

Table 1

Code No.	pH interval	Major application	Appl.Note
80-1128-28	4–7	General purpose	—
80-1128-29	4.2–4.9	α_1-antitrypsin	470
80-1128-30	4.5–5.4	Group specific component	471
80-1128-31	5.0–6.0	Transferrin	472
80-1128-32	5.6–6.6	Phosphoglucomutase	473

1.1. Package contents and technical data

Package contents

Each gel package contains 3 gels, filter papers, experimental result forms and instructions.

Designation	Code No.	No. per pack.
Immobiline DryPlate	(See label)	3
Filter paper		50
Experimental result form		3
Instructions	71-7030-01	1

Technical data

Gel dimensions:	Approx. 250×110 x 0.5 mm
Gel matrix:	Polyacrylamide T=4%, C=3%
Buffering capacity:	3 meqv/pH/L
Gel backing:	Polyester film
Storage:	–20° C
Shelf life:	18 months from manufacturing. Please observe “Expiry date” printed on each kit.

2. Preparing the gel

2.1. Preparing the rehydra-tion solution

One of the advantages of the dry gel format is the opportunity to include different additives in the reswelling solution (10, 14–16, 18–19). The three options given in Table 2 should therefore only be regarded as typical examples that will give good results for most applications. However, whenever required these recipes can easily be modified for further optimization. Consult the relevant Application Note (Table 1 and Reference list) or any of the cited references for instructions about specific applications. The rehydration process itself has also been investigated (20, 21).

Table 2.

	Alt.1	Alt.2	Alt.3
Application areas:	Water soluble proteins	Proteins with reduced solubility	Proteins with low solubility, e. g. Membrane proteins Lipoproteins
Composition:			
Distilled water	20.0 ml	19.5 ml	12.0 ml
Pharmalyte 3–10/ Ampholine pH 3.5–9.5		0.5 ml	0.5 ml
Urea			9.6 g
Triton X-100			0.1 ml
DTT			60 mg
Total volume:	20.0 ml	20.0 ml	20.0 ml
Rehydration time:	1–2 h	1–2 h	15–18 h

Comments

Note: All chemicals should be of the highest purity. PlusOne chemicals are highly recommended. Double-distilled water should be used.

The presence of carrier ampholytes not only increases protein solubility but also their electrophoretic migration velocity resulting in shorter focusing times.

Mercaptoethanol (2%) or dithiothreitol (15–50 mmol/l) can be added to avoid oxidation of sensitive proteins.

Glycerol (20–25%) improves solubility of hydrophobic proteins and reduces the risk for urea crystallization.

Lateral band spreading can be reduced by adding acetic acid (2 mmol/l) and applying the sample at the anodic side *or* adding Tris (2 mmol/l) and applying the sample at the cathodic side.

Triton X-100 can be replaced with other non-ionic or zwitterionic detergents, e.g. CHAPS. Other carrier ampholytes than Pharmalyte 3–10/Ampholine pH 3.5–9.5 may also be used.

Alt. 3 in table 2 corresponds to what is used in the first dimension focusing in 2-D electrophoresis. This alternative can be regarded as a standard choice for focusing under denaturing conditions and will normally give high quality results with all kind of samples.

2.2. Opening the package

Note 1: Wear clean gloves to avoid contamination of the gel surface, particularly when using silver stain.

Note 2: The gel is packed so that it is faced down to the aluminium foil backing of the package, and the gel support is uppermost.

Note 3: If only half of the gel is to be used, cut the package in half with sharp scissors, reseal the portion to be saved with tape, and store it at –20°C. Remember to identify the polarity of the remaining part.

Open the gel package from the transparent side. Use scissors to cut around all four sides of the package, taking care not to cut either the gel or its transparent backing film.

To simplify gel handling later on, identify the polarity of the pH gradient. The support film has a precut corner which indicates the anodic side of the pH gradient.

2.3. Rehydrating Immobiline DryPlate

For this procedure the specially designed Reswelling Cassette is highly recommended. It allows fast, convenient, even and reproducible rehydration of the gel. It also facilitates including the additives necessary for optimal performance for each application. Proceed as follows: (See the instruction manual for the Reswelling Cassette or the Multiphor II Electrophoresis System user manual for detailed instructions).

1. To prevent the gel from adhering to the glass plate fitted with the U-frame, coat the plate with Repel-Silane.
2. Mark the cathodic side of the gel.
3. Wet a clean thick glass plate with a few drops of water and place the gel on the glass plate with the gel side up.
4. Roll the gel with a clean rubber roller (Code No. 80-1106-79) to remove all air bubbles from between the glass plate and the support film.
5. Mount the gel in the cassette taking particular care that the U-frame gasket seals also over the cut-off corner of the supporting plastic foil and that the clamps are mounted correctly to avoid leakage.
6. Fill the cassette with the desired rehydrating solution.
7. Leave the gel to rehydrate for the recommended time.
8. Open the cassette and check the gel surface. Remove excess liquid by placing a filter paper moistened in distilled water on top of the gel followed by a dry filter paper on top.
9. Blot the gel by gently rolling the rubber roller under slight pressure over the dry filter paper. Finally remove the filter papers carefully from the gel.

   (Since gels rehydrated in detergent containing solutions have less tendency to stick to dry filter paper, they can be dried with a simpler procedure: Place a piece of dry tissue paper (e.g. Kleenex) on the gel, press gently to ensure contact between tissue and gel, and remove the paper carefully.)

3. Sample treatment

3.1 Sample preparation

Even if Immobiline DryPlate is exceptionally tolerant towards impure samples, best results are still obtained with samples that are free from precipitates. Should aggregation occur at the application point, this can often be overcome by diluting the sample or changing the sample application position.

Best results are generally obtained when the samples are solubilized in the rehydration solution. If this is not possible, the concentration of salt and buffer ions should still be kept at a minimum and, as a general rule, preferrably below 50 mmol/l. Excess buffer and salt ions will cause local overheating due to high local currents, which can result in protein denaturation and/or prolonged running times.

Desalting and buffer exchange can be carried out by dialysis, or, more easily, by gel filtration using a prepacked Sephadex G-25 column (Choose NAP-5 Column, NAP-10 Column or PD-10 Column depending on the sample size. See Ordering information).

3.2. Sample concen-tration

In general, Immobiline gels can take much higher sample loads than corresponding gels with carrier ampholytes. Several factors will determine the optimal sample concentration and volume:

1. pH range
2. Number and relative proportions of the components in the sample
3. Sensitivity of the detection method used.

Guide lines: PhastGel™ Blue R stain detects proteins down to the µg range. Normally 15–20 µl of sample with a concentration of 0.5–10 mg/ml will give good results. Silver staining has about 20 times higher sensitivity. A suitable load in a narrow pH gradient is normally 2–3 times higher than the load in a pH 4–7 gradient.

4. Isoelectric focusing

4.1. Preparing the experiment

Setting the cooling temperature

Connect Multiphor II electrophoresis unit to MultiTemp II thermostatic circulator and set the desired running temperature. A running temperature of 10 °C is often used except for gels containing urea, which are preferably run at somewhat higher temperatures (15 °C or more) to avoid precipitation of the urea.

Switch on the thermostatic circulator 15 minutes before starting the run.

Positioning the gel on the cooling plate

Pipette a few milliliters of insulating fluid (kerosene or light paraffin oil) on the cooling plate of Multiphor II. Position the gel on the cooling plate so that the polarity of the gel corresponds with the polarity marked on the cooling plate. Ensure that no air bubbles are trapped between the gel and the cooling plate.

Electrode strips are used to ensure good electrical contact between the gel and the electrodes. This prevents sparking and allows salt ions from the gel to migrate into the electode strips where they will stay and not interfere with the separation.

Soak the electrode strips evenly with approximately 3 ml distilled water and remove the excess by using a filter paper. The electrode strips should appear very dry before they are applied to the gel.

Lay the electrode strips along the long edges of the gel. Cut off the parts of the strips which protrude beyond the short ends of the gels using a pair of sharp scissors.

4.2. Sample application

There are basically three different methods for sample application. Which method to choose is determined primarily by the sample volume.

1. For 5–20 µl sample volumes: Apply sample directly on the gel, using Immobiline applicator strip (Code No. 18-1002-76). This applicator strip makes it possible to use a multiple syringe which allows quick and simple sample loading, especially when working with large numbers of samples. Check that the contact between the gel and the applicator strip is uniform. Leave the applicator strip on the gel during focusing.
2. For 15–20 µl (and larger) sample volumes: Use sample application pieces (Code No. 80-1129-46). Apply the dry pieces to the gel surface at the desired position(s) in the gradient. Using a micropipette, apply 15–20 µl volumes of sample solution on each piece. To apply larger volumes, use 2 or 3 pieces stacked or laid end-to end. If you want to apply smaller volumes with by this method, trim the paper proportionally before applying it to the gel. Remove the pieces of paper after completing half the total focusing time.
3. For 2–10 µl sample volumes: Apply the sample as droplets directly on the gel surface.

4.3. Running conditions

Place the electrode holder on the Multiphor II electrophoresis unit and align the electrodes with the center of the electrode strips. Finally, connect the two electrodes to the base unit and place the safety lid in position. Connect the electrode leads to the power supply.

Typical running conditions are listed in Table 3.

Table 3.

pH range	Voltage (V)	Current(mA)		Power (W)	Time (KVh)	Time (h)
4–7	3 500	5.0	(1.0)*	15.0	7–15	2–4
4.2–4.9	3 500	5.0	(2.0)	15.0	15–25	4–7
4.5–5.4	3 500	5.0	(2.0)	15.0	15–25	4–7
5.0–6.0	3 500	5.0	(1.0)	15.0	15–25	4–7
5.6–6.6	3 500	5.0	(1.0)	15.0	15–25	4–7

Comments:

- Decrease the power and current settings proportionally if only part of the plate is being used.
- The settings above are only to be regarded as guidelines. Some proteins focus very slowly and may require as much as 50–60 KVh to give optimal sharp bands. This must be determined experimentally in each case: Run the sample for different times.
- Since there is no gradient drift in the Immobiline DryPlate there is no limitation in the electrophoresis system as such as to how long the experiment can be continued. The limits are set only by the risk of drying out the gel, oxidising or denaturing the sample.

  These risks can be minimized by placing a plastic foil on top of the gel, running at low tempera-tures, flushing the unit with inert gas (N_2) and/or including a reducing agent (DTT or b-mercapto-ethanol) in the rehydration mixture. The surface can also be protected with DryStrip Cover Fluid (22).

5. Detection

All currently used detection methods can be applied on Immobiline DryPlate gels, including Coomassie Blue (4, 14), silver staining (23). Possible problems from extensive swelling of the gel can be reduced by adding ethanol (30%) to the washing solutions.

Enzymatic- and immunologically-based staining procedures as well as blotting can also be used (10–12).

5.1 Silver Staining

This silver staining method is according to Heukeshoven and Dernick (23) with some modifications.

Silver staining solutions

Note: 250 ml of solutions are needed per gel. All steps should be done with gentle shaking of the tray.

Fixing solution: 60 min	Trichloroacetic acid Ethanol Dissolve in distilled water and make up to 250 ml.	30.0 g 125 ml
Wash: 2 × 15 min	Ethanol Acetic acid Make up to 500 ml with distilled water	150 ml 50 ml
Incubation solution: minimum 40 min	Ethanol Sodiumacetate • 3 H_2O Glutardialdehyde (25%w/v)* Sodium thiosulfate, $Na_2S_2O_3$ • 5 H_2O Make up to 250 ml with distilled water	75 ml 17.0 g 1.3 ml 0.50 g
Wash: 3 × 5 min	Distilled water	
Silver solution: 20 min	Silver nitrate Formaldehyde* Make up to 250 ml with distilled water	0.25 g 50 µl
Developing solution: 5–15 min	Sodium carbonate Ethanol Formaldehyde* Make up to 250 ml with distilled water	6.25 g 75 ml 25 µl
Stop solution: 10 min	EDTA-Na_2 • 2 H_2O Ethanol Make up to 250 ml with distilled water	3.0 g 75 ml
Wash: 5 min	Ethanol Make up to 500 ml with distilled water	150 ml
Preserving solution: 20 min	Glycerol (87% (w/w) Ethanol Make up to 250 ml with distilled water	25 ml 75 ml

* **Note:** Add these components immediately before use.

Fixation
Remove the electrode strips by using forceps, thereafter immediately immerse the gel in the fixing solution for 60 minutes. This solution precipitates the proteins and allow detergents (if used) to diffuse out.

Washing
Thereafter wash the gel in washing solution for 2 × 15 minutes.

Incubation
Place the gel in incubation solution for a minimum of 40 minutes. The gel can be left over night in the incubation solution.

Washing
Thereafter, wash three times in distilled water, each time for 5 minutes.

Silver reaction
Put the gel in silver solution for 20 minutes.

Developing
Develop the gel in developing solution for 5–15 minutes. The protein bands should become intensively dark.

Stopping
Stop the reaction by placing the gel in stop solution for 10 minutes.

Washing
Wash in washing solution for 5 minutes.

Preserving
To preserve the silver stained gel, place the gel in preserving solution for 20 minutes. Then place the gel on a glass plate with the gelside up, and cover the gel with cellophane preserving sheet soaked in preserving solution. Allow the gel to dry in room temperature (Do not put the gel in a heating cabinett, the silver stain will bleach due to the increased temperature).

5.2 Coomassie staining

This is a general protein stain which detects protein concentrations down to the µg level. 250 ml of solution is used in each step.

Fixing solution: 30–60 min	Trichloroacetic acid Sulphosalicylic acid Make up to 400 ml with distilled water	46 g 14 g
Destaining solution: 5 min	1. Ethanol Make up to 1000 ml with distilled water 2. Acetic acid Make up to 1000 ml with distilled water Mix 1:1 before use	500 ml 160 ml
Coomassie solution: 10 min	PhastGel™ Blue R Dissolve 1 tablet in 400 ml destaining solution. Stir with a magnetic stirrer and heat the solution to 60 °C. Filter before use. Use only once.	1 tablet
Destaining solution: Until the background is clear	See above	
Preserving solution:	Glycerol Add 360 ml destaining solution and mix well.	40 ml

Fixation

Remove the elexctrode strips with forceps. Immediately place DryPlate in Staining Kit containing fixing solution for 30–60 minutes. This solution precipitates the proteins.

Destaining
Before staining, wash DryPlate in destaining solution for 5 minutes.

Staining
Pour off the destaining solution and stain DryPlate for 10 minutes in staining solution which has been preheated to 60 °C.

Destaining
Destain DryPlate with several changes of destaining solution until the background is clear.

Preserving
Place the destained DryPlate in the glycerol preserving solution for 30–60 minutes. Then place the gel on a glass plate with the gel side up, and cover the gel with a cellophane preserving sheet soaked in preserving solution. Allow it to dry at room temperature.

5.3 Electro-phoretic transfer

Before electrophoretic blotting can take place, the support film must be removed to allow the current to pass through the gel. FilmRemover is ideal for this purpose. After the film and the gel have been separated, the proteins can be transferred to an immobilizing membrane by using the Multiphor II NovaBlot transfer kit. Complete information on running conditions is given in Multiphor II User Manual (Code No. 18-1103-43).

6. Evaluation

6.1 Determination of the isoelectric point

Because of the low ionic strength in the gel, the pH gradient cannot be directly measured with a standard surface pH electrode unless carrier ampholytes have been included in the rehydrating solution (21).

An alternative to direct pH measurement is to run pI calibration proteins in parallel with the experimental samples.

For details about the use of pI calibration proteins see the Instruction enclosed with each pI Calibration Kit.

6.2 Densitometric evaluation

ImageMaster 1D Software (Code No. 80-6350-37) is a powerful software package for protein quantitation and data analysis. By using ImageMaster 1D Software together with ImageScanner (Code No. 18-1134-45), you can capture, store, evaluate, and report all the information contained in your electrophoresis gels. ImageMaster 1D Software automatically selects your lanes, bands, subtracts the background, corrects the smiling, and integrates areas and band volume (OD × mm^2). The software calculates relative amounts, percentages of totals, and amounts of proteins in real quantity units using a calibration curve.

ImageMaster 1D Software also calculates isoelectric points or molecular weights, compares bands across different lanes or gels, and produces hierarchial clustering information. Further lane comparision, databasing, and identification using an internal library can be done by using ImageaMaster 1D Database (Code No 80-6350-94) and an add-on package to ImageMaster 1D Software.

7. References

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8. Ordering information

Code No.	Designation
80-1128-28	Immobiline DryPlate pH 4–7
80-1128-29	Immobiline DryPlate pH 4.2–4.9
80-1128-30	Immobiline DryPlate pH 4.5–5.4
80-1128-31	Immobiline DryPlate pH 5.0–6.0
80-1128-32	Immobiline DryPlate pH 5.6–6.6
80-1106-79	Roller
18-1004-40	IEF electrode strips (100)
18-1002-76	Immobiline applicator strip (5)
80-1129-46	IEF sample application pieces (200)
17-0851-01	PD-10 column, Desalting samples <= 2.5 ml (30)
17-0853-01/02	NAP-5 column, Desalting samples <= 0.5 ml (20/50)
17-0854-01/02	NAP-10 column, Desalting samples <= 1.0 ml (20/50)
18-1018-06	Multiphor II electrophoresis unit
18-1130-05	EPS 3500 XL Power Supply
18-1102-77	MultiTemp III Thermostatic Circulator, 115 V AC
18-1102-78	MultiTemp III Thermostatic Circulator, 220 V AC
18-1016-86	NovaBlot electrophoretic transfer kit
18-1013-75	FilmRemover
80-1129-38	Cellophane preserving sheets, 210×320 mm (50)
80-6350-37	ImageMaster 1D Software
18-1134-45	ImageScanner
17-0518-01	PhastGel Blue R
17-0471-01	Broad pI Calibration kit, pH 3–10
17-0472-01	Low pI Calibration kit, pH 2.5–6.5
17-0473-01	High pI Calibration kit, pH 5–10.5

Code No.	Designation
PlusOne chemicals	
17-1319-01	Urea
17-1315-01	Triton X-100
17-1325-01	Glycerol 87% (w/w)
17-1317-01	2-Mercaptoethanol
17-1318-01	Dithiothreitol (DTT)

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