CleanGel IEF

Isoelectric focusing

Instructions for Use

Dry polyacrylamide gels for isoelectric focusing

CleanGel IEF is a dried polyacrylamide gel optimized for analytical isoelectric focusing (IEF). The gel is excellent for separating enzymes with preserved activity. The gel is cast on a polyester support film and carefully washed to remove catalysts and nonpolymerized substances and then dried. Prior to isoelectric focusing, the dried gel is rehydrated in a solution containing Pharmalyte™ carrier ampholytes. Additives such as urea and/or detergents can also be added at this stage. CleanGel IEF is rehydrated in a flat tray (GelPool) to a thickness of 0.43 mm.

Introducing carrier ampholytes and additives into the gel after the polymerization eliminates the risk of copolymerizing chemicals in the gel matrix. Thus, CleanGel IEF gels give low electroendosmosis, resulting in sharp bands and minimum pH gradient drift. Even additives that are sensitive to polymerization reactions, or that inhibit the polymerization can be used.

The gels are preferably run on the MultiPhor™ II electrophoresis unit.

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1 Application areas

CleanGel IEF can be used for all IEF analyses but is particularly well suited in application areas where one or more of the factors listed below are of special significance:

- *Biological activity*

  CleanGel media contain no residual polymerization catalysts or other substances that may interfere with zymogram or immunological staining procedures.

- *Increased sample load and salt tolerance*

  Persulfate/sulfate and other ions that might disturb the pH gradient are absent.

- *Demanding separations*

  Requiring specific pH gradients, special solvent compositions and/or maximal band sharpness.

2 Package contents and technical data

Package contents

Each gel package contains 5 dry gels and instructions.

Code No.	Designation	No. per pack
18-1035-32	CleanGel IEF	5 gels
71714400	Instructions	1

Technical data

Gel matrix	Polyacrylamide T=5%, C=3%
Gel dimensions	250 x 110 x 0.43 mm (after rehydration)

Gel backing	Polyester film
Shelf life	12 months Please observe the Expiry Date printed on each kit
Storage	–20°C

3 Designing the rehydration solution

Mixing carrier ampholytes

To check the composition of an unknown sample a pH gradient from 3 to 10 should be used. To study selected pH intervals in more detail, narrow pH ranges are recommended.

pH gradients are made by incorporating carrier ampholytes in the rehydration solution. The desired pH interval is obtained by using the appropriate Pharmalyte interval. Preblended Pharmalyte gives a uniform buffering capacity, an even conductivity and a linear pH gradient within the range stated.

Standard Pharmalyte ranges can also be mixed to obtain special pH gradients. Table 1 lists examples of mixtures and corresponding pH gradients. The recommended minimum concentration for Pharmalyte is 1 part Pharmalyte and 15 parts of rehydration solution. Disturbances from buffering substances in the sample are reduced by increasing the concentration of carrier ampholytes.

Table 1. Examples of pH gradients obtained with Pharmalyte.

pH gradient	Carrier ampholyte mixture	Amount (ml) of Pharmalyte to 1 1 ml rehydration solution
4.0 to 6.0	Pharmalyte 2.5 to 5	0.56
	Pharmalyte 4.5 to 5.4	0.18
3.5 to 6.5	Pharmalyte 2.5 to 5	0.56
	Pharmalyte 4 to 6.5	0.18
3.5 to 7.5	Pharmalyte 2.5 to 5	0.37
	Pharmalyte 5 to 8	0.37
4.0 to 8.0	Pharmalyte 4.0 to 6.5	0.37
	Pharmalyte 5 to 8	0.37
5.0 to 9.5	Pharmalyte 5 to 8	0.37
	Pharmalyte 8 to 10.5	0.37
6.0 to 10.0	Pharmalyte 8 to 10.5	0.59
	Pharmalyte 5 to 8	0.14
	Arginine	22 mg

Use of chemical spacers

A narrow pH gradient in combination with high voltage ensures high resolution. Resolution may be further improved by flattening the pH gradient over a selected pH interval. This can be done by using chemical spacers together with carrier ampholytes (1). Chemical spacers are amphoteric substances which have ionizable groups with widely separated pK values. Chemical spacers are thus isoelectric over a wide pH interval. To be useful, they must have a high solubility at their isoelectric point.

During IEF chemical spacers are focused at their pI and thereby flatten the pH gradient at this pH. However, within a limited pH interval the expansion tends to take place in the middle of the gel, rather than at the pI of the chemical spacer. Therefore, some experimental work is required to optimize the ampholyte mixture in combination with a suitable chemical spacer and its optimum concentration. A concentration of 100 mg chemical spacer per 11 ml rehydration solution could be used as a first trial. Typical chemical spacers are listed in the Table below.

Table 2. Chemical spacers (separators).

Full name	Abbreviation	pK1	pK2	pI
2-(N-morpholine) - ethanesulphonic acid	MES		6.2	4.1
N-(2-acetamido)-2 - aminoethanesulphonic acid	ACES		6.9	4.5
3-(N-morpholine)- propanesulphonic acid	MOPS		7.2	5.0
N,N-bis-(2-hydroxyethylglycine)	BICINE	2	8.4	5.2
N-Tris(hydroxymethyl)-methyl-				
2-aminoethanesulphonic acid	TES		7.5	5.5
Threonine				5.6
N-2-Hydroxyethylpiperazin-N-2-ethanesulphonic acid	HEPES		7.6	5.7
Glycine		2.3	9.8	6.0
ß-Alanine		3.6	10.2	6.9
4-Aminobutyric acid				7.3

Full name	Abbreviation	pK1	pK2	pI
6-Aminocapronic acid				7.5
L-histidine				7.7

Use of additives

Since some proteins are not soluble in water solutions, or have low solubility close to their isoelectric point, it is sometimes difficult to obtain good focusing results. In these cases, results can be improved considerably by using solubilizing additives. Additives can be introduced into CleanGel IEF.

Additives are used to:

- Solubilize proteins.
- Dissociate protein complexes, subunits or to disperse lipids from proteins.
- Avoid oxidation of proteins and to break disulphide bonds.
- Increase the viscosity to stabilize the pH gradient and sharpen the bands.
- Stabilize the protein or to alter its pI by adding substances forming complex with the protein.

Urea

The solubilizing effect of urea is due to its disrupting effect on both hydrophobic and ionic bonds, resulting in partial unfolding on the protein.

Urea is often used in combination with detergents. The concentration of urea required to solubilize or to denature a protein depends on the protein in question. Concentrations ranging between 2 to 9 M have been used (2).

Urea must be handled with caution since it hydrolyses to ammonia and cyanate (3). This effect is accelerated at high temperature and high pH. Cyanate will carbamylate primary amino groups and thiol groups. Use only freshly prepared solutions of high quality urea.

When high reproducibility is needed, charged ions in the urea solution can be removed by treating the solution with a mixed ion exchange resin, such as PlusOne Amberlite IRN-150L. When using high concentrations of urea (greater than 8 M) avoid running temperatures below 15°C as crystallization of urea can occur.

pH estimations in urea

Apparent pIs and pH gradients measured in urea cannot be compared directly with those obtained in normal aqueous solutions. Not only is there a conformational change in the structure of the protein, which potentially gives rise to changes in surface charge, and hence pI, but urea also significantly influences the pK values of both proteins and the carrier ampholytes. These effects change the pI values of the proteins and the range of the pH gradient. Moreover, errors are introduced by the effect of urea on the glass electrode. However, apparent pH values measured in urea solutions are of value in a relative sense and *Gelsema et. al.*(4) have devised tables for correlation purposes.

The positions of pI Calibration Kit protein bands are difficult to translate into pH values in urea containing gels. However, under identical conditions the patterns can be used for comparison between gels.

Detergents

Most proteins can be solubilized with preserved biological activity using detergents. In this respect, the type, concentration and quality of the detergent is of importance (5).

In IEF, only non-ionic detergents or detergents with a net charge of zero should be used. Detergents often have an optimum concentration. Too low concentrations of detergent are unable to solubilize the protein. Too high concentrations often widen protein bands and cause streaking.

The most commonly used non-ionic detergents in IEF are the very similar Triton™ X-100 and Nonidet P-40. Concentrations ranging between 0.1% to 0.5% are used. Concentrations of Triton X-100 above 0.5% often cause streaking and problems during staining. Other detergents used: Tween™ 20, Tween 40, Tween 80 (polyoxyethylenesorbitolesters), and Lubrol W (polyoxyethylene-alcohols).

Zwitterionic detergents can also be used. Their useful pH range is however limited, due to the net charges obtained as the ionizable groups approach their respective pK values. Examples are: CHAPS (3-[(3-cholamidopropyl-dimethylammonio] -1-propanesulphonate) and Zwittergent 3-14.

CleanGel IEF needs extended rehydration time when detergent is used. In water, detergent micelles of high molecular weight (up to 100 kDa) are formed. These diffuse very slowly into the gel. When rehydrating CleanGel IEF in a detergent solution, all the solution (11 ml) should be absorbed into the gel to ensure that the right amount of detergent has entered. Rehydrating a gel in an excess amount of solution will result in a concentration of detergent below its CMC (critical micell concentration) inside the gel.

Combining urea and detergents

A combination of urea and detergent is one of the most common methods for solubilizing hydrophobic proteins such as membrane proteins.

Reducing agents

Reducing agents break disulphide bonds in proteins. A low concentration of reducing agent (1 mM) in the rehydration solution will counteract the oxidation of proteins, and preserve the biological activity of many enzymes. Chemicals such as ß-mercaptoethanol can be used, but for safety and enviromental reasons, other chemicals e.g. dithiothreitol (DTT) or dithioerythritol (DTE) are preferred.

Stabilizing the pH gradient

The pH gradient can be stabilized by increasing the viscosity in the gel. This is obtained by introducing substances such as glycerol, sorbitol or sucrose (10 to 15%). The increased viscosity reduces electroendosmosis and the increased osmolality reduces the amount of water extruded onto the gel surface. Higher viscosity will also prolong the rehydration time.

Stabilizing proteins

Many proteins are stabilized in the presence of their substrates or other ligands. If these substrates or ligands are non-ionic they can be incorporated in the rehydration solution. Protease inhibitors can also be introduced into the gel provided they are neutral.

Other additives

Protein conformational changes can be studied by the introduction of specific ligand molecules e.g. the binding of specific carbohydrates to lectins. Formamide up to 75% as well as 30% acetonitrile or 25% methanol can be used to solubilize very hydrophobic proteins. The limiting factors regarding the amount of these agents which can be used are the solubility of the carrier ampholytes and the ability of the polyacrylamide gels to swell in the solvent mixture.

Note: *Since both the result and the reproducibility of the experiment depend on the quality of the additives, use only analytical grade chemicals.*

Solutions

Note: *All chemicals should be of the highest purity. Double distilled water should be used. Rehydration solutions for pH gradient 3 to 10:*

IEF Native

1.1 g Sorbitol (10% w/v)

820 µl Pharmalyte pH 3 to 10

Make up to 11 ml with distilled water.

IEF with Urea

5.28 g urea (8 mol/l)

820 µl Pharmalyte pH 3 to 10

Make up to 11 ml with distilled water

4 Sample treatment

Sample preparation

The composition of the sample solution should be as similar as possible to the composition of the rehydration solution. For reproducible results, the ratio between detergents and protein should be kept constant in all samples during preparation. Be aware of the alkylating ability of urea. Samples containing insoluble material should be filtered before use.

Sample concentration

The optimal final sample concentration depends on the volume of sample solution to be applied to the gel, the sensitivity of the detection method and the number and relative proportions of components in the sample. As a guideline when Coomassie staining is used, the protein concentration should be 1 to 3 mg/ml for a 10 µl sample.

Salt tolerance

CleanGel IEF will tolerate salt concentrations up to 50 mmol/l in the sample. At higher concentrations of salt band disturbances will appear. If you see band disturbances, increase the Pharmalyte concentration or desalt the sample.

Desalting can be carried out by dialysis or by gel filtration on a PD-10 or NAP™-10 column or by the use of Vivaspin™ column. If the sample tends to precipitate, dialyse against 1% glycine or a zwitterionic detergent, as these do not interfere with the pH gradient.

5 Rehydration of CleanGel IEF

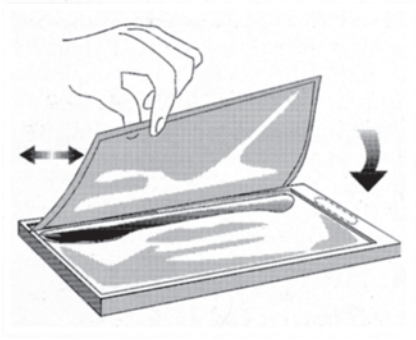
CleanGel IEF can be used in one piece, or depending upon the number of samples, cut into smaller portions, while still dry. If cut, the unused part of the gel should be sealed in an airtight plastic bag and stored in a freezer (–20°C).

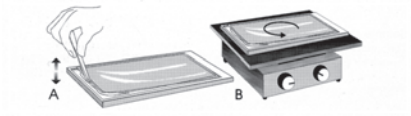
Follow the instructions below or the detailed separate instructions supplied with GelPool (Code No. 71-7142-00). An even rehydration of the CleanGel is critical in order to avoid curved sample lanes.

Step	Action
1	Open the gel package and remove the gel.

2	Select the appropriate reswelling chamber of GelPool.

Step	Action
3	Clean the GelPool with distilled water and dry with tissue paper.
4	Degas the rehydration solution in order to remove CO_2 for sharper bands in the alkaline region.
5	Pipette the rehydration solution into the chamber • Gel, full size (260 x 125 mm) 11 ml • Gel, half size (130 x 125 mm) 5.5 ml
6	Set the edge of the gel film, with the gel surface downwards, into the rehydration solution and slowly lower it.



Step	Action
7	Move the gel to and fro, in order to achieve an even distribution of the liquid and to avoid trapping air bubbles (A). To obtain even rehydration and to avoid the gel sticking to the GelPool, place the GelPool on a rotary shaker and shake (B).  If a shaker is not available, place the GelPool on a horizontal table, preferably a levelling table. In both cases, lift the gel edge repeatedly during the first 15 minutes to prevent the gel sticking to the GelPool in order to obtain an even rehydration of the CleanGel.
8	After 60 minutes the gel has reswollen completely to a thickness of 0.43 mm, and can be removed from the GelPool, and used at once.
9	If additives e.g. urea or non-ionic detergents are used, rehydration time must be prolonged, up to several hours.

6 Isoelectric focusing

Prepare cooling and add the gel

Step	Action
1	Connect the Multiphor II electrophoresis unit to MultiTemp™ II thermostatic circulator and set the temperature to 10°C (15°C for IEF with urea).
2	Switch on the thermostatic circulator 15 minutes before starting the analysis. Isoelectric focusing has to be performed at a defined constant temperature, as the pH gradient and the isoelectric points are dependent on the temperature.
3	Position the gel on the cooling plate. ***Note:*** *Wear clean gloves to avoid contamination of the gel surface, particularly when using sensitive silver stain.*
4	Pipette about 2 ml of insulating fluid (kerosene or light paraffin oil) onto the cooling plate of the Multiphor II.

Step	Action
5	Remove the gel from GelPool and dry the gel surface with the edge of a filter paper.

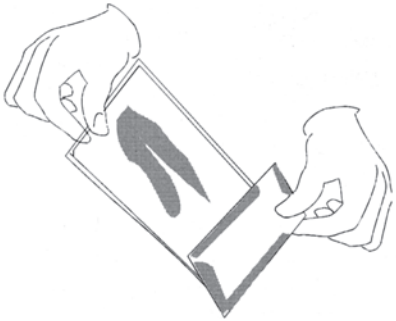


Fig 1. Drying the gel surface with the edge of a filter paper.

Note:
The gel surface should be absolutely dry, otherwise the gel will extrude water during the isoelectric focusing.

6	Position the gel in the centre of the cooling plate, use the screen print as a guide. No air bubbles should be trapped beneath the gel.

Prefocusing

Step	Action
1	Clean the platinum electrode wires, before and after each electrophoresis run, with a wet tissue paper.

Step	Action
2	Place the electrode holder with the IEF electrodes on the electrophoresis unit and align the electrodes so that they rest on the outer edges of the gel. Electrode wicks between the gel and the electrodes are not necessary.
3	Connect the cables of the electrodes to the unit (Figure below).

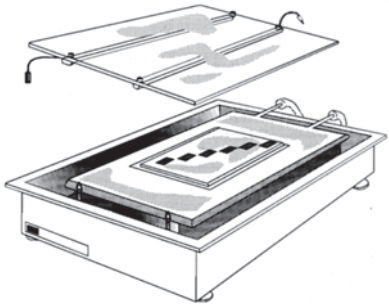


Fig 2. CleanGel IEF (half a gel) placed on the Multiphor II. Samples applied at various positions across the gradient

4	Place the safety lid in position. Connect the power supply. Follow the recommended electrical settings and running conditions given in *Table 3, on page 20*.
5	Prefocus for 20 minutes without samples in order to establish the pH gradient in the gel.

Sample application

Note: *If water is excluded from the gel during the prefocusing procedure it should be removed, see Fig. 1, on page 17.*

In IEF there is, for most samples, one optimal position within the pH gradient for sample application. This should be found by applying the samples at various positions across the gradient, using sample application pieces (Figure above).

There are three different methods for sample application. The optimal method is determined primarily by the sample and the volume to be applied.

- *IEF/SDS applicator strip (Code No. 18-1002-26)*

  Up to 52 samples can be applied with a sample volume of 5 to 20 µl in each well. The applicator strip is made of silicon and is applied directly on the gel surface.

- *Sample application pieces (Code No. 80-1129-46)*

  Recommended sample volumes 15 to 20 µl, (or for smaller volumes cut the paper). The sample application pieces are made of Paratex. These sample application pieces are valuable when the samples are to be applied at different positions on the gel.

- Very small sample volumes (e.g. 2 µl) can be applied as droplets directly onto the gel surface.

When the protein concentration is between 1 to 3 mg/ml apply 10 µl /sample, by using an applicator strip or sample application pieces.

The pH gradient can be determined by using pI markers. Dissolve the Broad pI Kit pH 3 to 10 (Code No. 17-0471-01) according to the instructions supplied and apply 10 µl at two different places on the gel. For determination of the calibration curve please consult the instructions to the pI Kit.

Place the electrode and safety lid in position and start the isoelectric focusing according to the Table below.

Running conditions

Table 3. Recommended running conditions for one CleanGel IEF pH gradient 3 to 10

	Voltage (V)	Current (mA)	Power (W)	Time (min)
Prefocusing	700	12	8	20
Sample entrance	500	8	8	20
Isoelectric focusing	2000	14	14	90
Band sharpening	2500	14	18	10

Note: *If only half of a gel is used, remember to divide the current and power settings by two.*

Narrow gradients

When running narrow pH gradients e.g. pH 5 to 7, use the same electrical settings, but prolong the isoelectric focusing time to 4 hours.

7 Detection

All current detection methods used for isoelectric focusing can be used with CleanGel IEF. In case of background staining, try a prolonged washing step after fixation by 2 hours, or if needed over night, to eliminate this problem. For further information see Multiphor II Electrophoresis System, *Users Manual (Code No. 80131880)*.

Electrophoretic transfer

Eletrophoretic transfer is a way of transferring separated proteins from a gel matrix to an immobilizing membrane prior to detection. When the protein is bound to a membrane the protein is available for specific identification methods. Identification is easier, faster and more sensitive on membranes than on gels. Besides being highly efficient in terms of yield, the technique allows multiple copies of the separation to be obtained. The membranes themselves are easy to handle and store. In electrophoretic transfer the support film must be removed to let the current pass through the gel. The best way to do this is to use FilmRemover (Code No. 18-1013-75). The focused proteins can then be electrophoretically transferred to an immobilizing membrane by using the Multiphor II NovaBlot electrophoresis transfer kit. Complete information is given in the Multiphor II Electrophoresis System, *Users Manual (Code No. 18110343)*.

8 Evaluation

Determination of the isoelectric point

The Isoelectric point can be estimated with the help of marker proteins. The markers are run in parallel with the unknown sample of the isoelectric focusing gel. After focusing and staining, the migration distances from the cathodic edge of the gel to the different marker protein bands are plotted on the X-axis. The calibration curve can then be drawn. By measuring the migration distance of the unknown protein, it is possible to interpolate the isoelectric point of the protein from the curve. Fore more details about pI markers please see the Instruction Sheet enclosed with the pI marker kit.

Automatic evaluation

ImageQuant™ TL Software (Code No. 28-9236-62, 28-9194-45) is a powerful software package for protein quantitation and data analysis. By using ImageQuant TL Software together with ImageScanner III (Code No. 28-9076-07), you can evaluate and report all the information contained in your electrophoresis gels. ImageQuant TL Software automatically selects your lanes, bands, substracts the background, corrects the smiling, and integrates areas and band volume (OD x mm2). The software calculates relative amount, percentages of totals, and amounts of proteins in real quantity units using a calibration curve. ImageQuant TL Software also calculates isoelectric points or molecular weights and compares bands across different lanes or gels.

9 Recommended equipment, carrier ampholytes, chemicals and accessories

Code No.	Designation
18-1031-58	GelPool
18-1018-06	Multiphor II electrophoresis unit
18-1130-05	EPS 3500 XL
18-1102-78	MultiTemp III thermostatic circulator, 220V, 60 Hz
18-1102-77	MultiTemp III thermostatic circulator, 115V, 50 Hz
18-1016-86	Multiphor II NovaBlot electrophoretic transfer kit
18-1013-75	FilmRemover
28-9236-62, 28-9194-45	ImageQuant TL Software
28-9076-07	ImageScanner III

Carrier ampholytes: Pharmalyte (25 ml)

Code No.	Range
17-0451-01	Pharmalyte pH 2.5 to 5
17-0452-01	Pharmalyte pH 4 to 6.5
17-0453-01	Pharmalyte pH 5 to 8
17-0455-01	Pharmalyte pH 8 to 10.5
17-0456-01	Pharmalyte pH 3 to 10
17-0562-01	Pharmalyte pH 4.2 to 4.9
17-0563-01	Pharmalyte pH 4.5 to 5.4
17-0564-01	Pharmalyte pH 5 to 6
17-0566-01	Pharmalyte pH 6.7 to 7.7

Chemicals and accessories

Code No.	Designation	Quantity
17-0518-01	PhastGel Blue R	40 tablets
17-1319-01	Urea	500 g
17-1326-01	PlusOne Amberlite IRN-150L	500 g
17-1325-01	Glycerol (87%w/w)	1 liter
17-1315-01	Triton X-100	500 ml
17-1318-01	Dithiothreithol (DTT)	1 g
17-1318-02		5 g
17-0471-01	Broad pI kit, pH 3 to 10	10 vials
17-0472-01	Low pI kit, pH 2.5 to 6.5	10 vials
17-0473-01	High pI kit, pH 5 to 10.5	10 vials
80-1129-38	Cellophane preserving sheets	50
18-1002-26	IEF/SDS applicator strip, for 52 samples	5
80-1129-46	Sample application pieces	200
17-0851-01	PD-10 columns (for< 2.5 ml samples)	30

Vivaspin columns with 3,000 molecular weight cut off (MWOC)[1]

Code No.	Designation	Quantity
28-9322-18	Vivaspin 500, 3,000 MWCO	25 concentrators
28-9322-40	Vivaspin 2, 3,000 MWCO	25 concentrators
28-9322-93	Vivaspin 6, 3,000 MWCO	25 concentrators
28-9323-58	Vivaspin 20, 3,000 MWCO	12 concentrators

[1]Other MWCO (5,000, 10,000, 30,000, 50,000 and 100,000) are available for all Vivaspin column sizes.

10 References

1. Separator isoelectric focusing, an improved method of protein analysis and purification. Anal. Biochem. 79 (1977) 166-180, Caspes, M.L., Posey, Y., Brown, R.K.
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3. Reactions of the cyanate present in aqueous urea with amino acids and protein. J. Biol. Chem.235 (1960) 3177-3181.Stack, G.R., Sein, W.H., Movie, S.
4. Isoelectric points of proteins determined by isoelectric focusing in the presence of urea and ethanol. J Chromatogr. 171 (1979) 171-181, Gelsema, W.J., DeLigny, C.L., Van Der Veen, M.G.
5. Electrophoresis and electrofocusing in detergent containing media. A discussion of basic concepts. Electrophoresis 2 (1981) 1-11, Hjelmeland,L.N. and Chrambach, A.

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