IEF and electrophoretic titration curve analysis

Separation Technique File No. 100

PhastSystem™

This technique file describes optimized methods for IEF (isoelectric focusing) and electrophoretic titration curve analysis with PhastGel® IEF 3–9, PhastGel IEF 5–8 and PhastGel IEF 4–6.5. These methods are based on studies using crude protein extracts and commercially available proteins. Therefore, they have general applicability and offer a good starting point in designing methods for specific applications.

This file gives only specific method information. Detailed descriptions of how to program separation methods, how to load sample applicators, and how to run PhastGel IEF media are given in the Users Manual of PhastSystem.

Introduction

IEF is a high resolution technique for separating proteins on the basis of their isoelectric points. With PhastSystem and PhastGel IEF media, it is also fast, reproducible and convenient; separations take approximately 30 minutes, run under exact programmed conditions, and require no buffer preparation.

PhastGel IEF media

As their names imply, PhastGel IEF 3–9 covers the pH range 3 to 9, PhastGel IEF 5–8 the pH range 5 to 8, and PhastGel IEF 4–6.5 the pH range 4 to 6.5. PhastGel IEF media are homogeneous polyacrylamide gels (5% T, 3% C) containing Pharmalyte® carrier ampholytes. Pharmalyte generates stable, linear pH gradients in the gels during the run (1). Proteins migrate under an electric field, essentially unhindered by the porous gel, to a point in the pH gradient that corresponds to their pI (isoelectric point).

Applications

PhastGel IEF 3–9 is particularly suitable for screening protein samples. For example, to analyze complex protein mixtures, to check the progress of a purification scheme (a chromatographic peak), or to check the degree of completion of a reaction (digestion or phosphorylation). It is also useful in selecting the best pH interval for pI measurements, and in determining protein charge characteristics using electrophoretic titration curve analysis. Titration curves can aid in predicting optimal separation conditions for chromatography and electrophoresis techniques (2).

Since PhastGel IEF 5–8 and 4–6.5 cover narrower pH intervals, they are well suited for characterizing and analyzing mixtures of proteins with smaller pI differentials, for example, for pI measurements or titration curve analyses of isoenzymes.

Instructions for pI measurements using PhastGel IEF media and the Pharmacia pI calibration kit proteins are given in the Users Manual of PhastSystem.

Sample preparation

Salt and buffer tolerance

Samples may contain salt concentrations up to 0.25 M without causing band disturbance. Samples may contain higher salt concentrations up to 0.5–0.7 M, but slight band disturbances (mainly wavy bands) might occur at or near the point of sample application.

Buffer concentrations up to 0.05 M can be used without affecting results. Higher concentrations may be applicable but this will depend on the buffer in question.

Electrophoretic titration curves may take longer to form with samples containing more than 0.5–0.7 M salt or 0.5 M buffer.

Sample concentration

The lower limit for sample concentration depends on the volume of the sample applied and on the sensitivity of the detection technique used to develop the gel. Generally, the sample must

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contain at least 20 to 30 ng of each protein/μl for Coomassie staining, and at least 1 to 5 ng of each protein/μl for silver staining. Samples containing more than 2 μg/μl (Coomassie) or 100 ng/μl (silver) overload the gel and distort results.

Samples containing insoluble material should be filtered or centrifuged, especially if you plan to use silver staining to develop the proteins.

Methods

The methods presented in tables 1 to 5 give the running conditions for one gel. Methods are always programmed for one gel. When you start the method you enter the number of gels to run. If you enter 2 gels, PhastSystem automatically adjusts the current and power so that both gels run under the same conditions according to the programmed method. See the Users Manual of PhastSystem for details.

Isoelectric focusing

The methods for isoelectric focusing contain three steps: a prefocusing step, a sample application step, and a focusing step. Step 1 is the prefocusing step in which the pH gradient is formed. The sample applicators can be loaded during this step.

You can program the extra alarm to sound at any point during the method. We suggest that you program it to sound about 2 volthours before the applicator arm is lowered. When the extra alarm sounds, you will know that you have 2 volthours left to insert the sample applicators, if you haven't done so.

Once the method is paused, you insert the sample applicators into the sample applicator arm, and continue the method. Samples can be applied in the anodal, cathodal or middle position, depending on the sample. For unknown samples, or for pI determinations, we suggest that you apply the sample at both the anode and cathode position to ensure that the proteins reach their pI. The proteins will focus at the same point in the gel if they are at their pI.

At step 2, the samples are applied to the gel for 15 Vh. The voltage is lower in this step (200 V) to avoid streaking patterns caused by contaminants or poorly soluble proteins.

The applicators are raised at the beginning of step 3 and the proteins migrate to their isoelectric points. At the end of step 3, an alarm will sound to mark the end of the method. However, step 3 will continue to run until you stop the method. This is to prevent diffusion, should the method end when you are beyond hearing distance of the alarm.

Fig. 1 below illustrates the electric field conditions in the gel during a run. This is for PhastGel IEF 3–9 using the method in table 1. However, PhastGel IEF 5–8 and 4–6.5 run under essentially the same conditions.

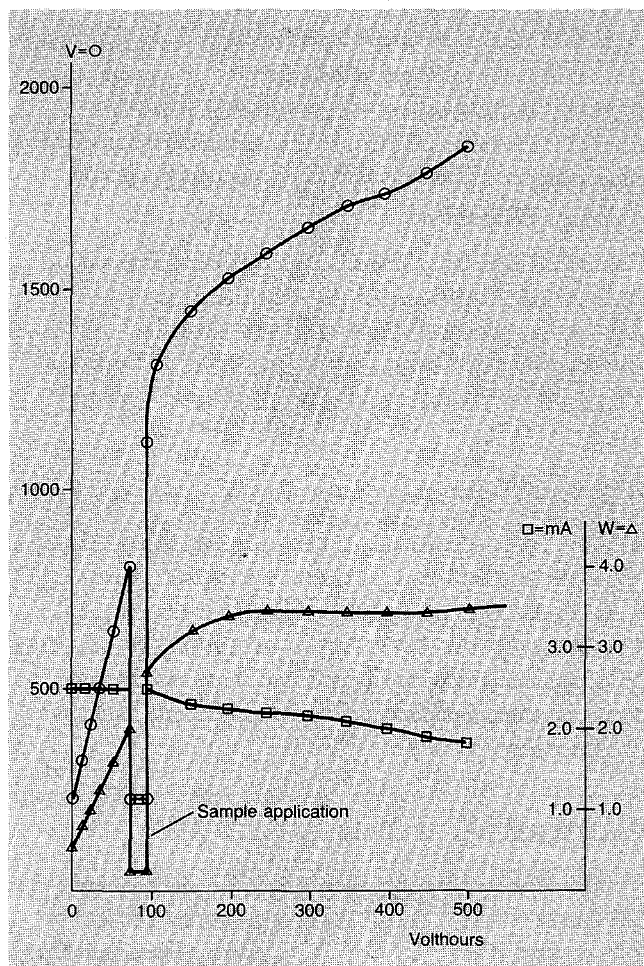
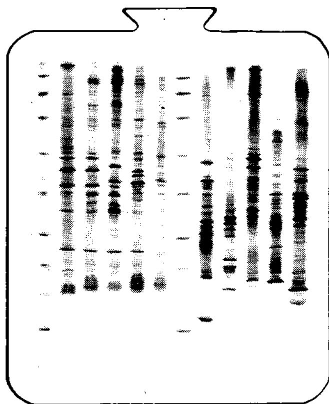


Fig. 1. Running conditions during IEF with PhastGel IEF 3–9 using the method in table 1.

Table 1. Optimized method for IEF with PhastGel IEF 3–9 to program into the separation method file of PhastSystem (given as method 1).

SAMPLE APPL. DOWN AT				1.2	0 Vh
SAMPLE APPL. UP AT				1.3	0 Vh
EXTRA ALARM TO SOUND AT				1.1	73 Vh
SEP 1.1	2 000 V	2.5 mA	3.5 W	15°C	75 Vh
SEP 1.2	200 V	2.5 mA	3.5 W	15°C	15 Vh
SEP 1.3	2 000 V	2.5 mA	3.5 W	15°C	410 Vh

The run takes 500 Vh or approximately 30 minutes. The prefocusing step takes approximately 10 minutes.

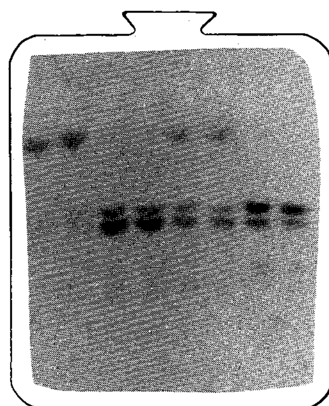


Example of IEF with PhastGel IEF 3—9. The gel was Coomassie stained using the method described in development technique file number 200. From the left, the samples are: Pharmacia Broad pI calibration kit, elk, reindeer, beef, lamb, and horse muscle extracts, Pharmacia broad pI calibration kit, eel, coalfish, salmon, pike and perch muscle extracts.

Table 2. Optimized method for IEF with PhastGel IEF 5—8 to program into the separation method file of PhastSystem (given as method 2).

SAMPLE APPL. DOWN AT				2.2	0 Vh
SAMPLE APPL. UP AT				2.3	0 Vh
EXTRA ALARM TO SOUND AT				2.1	73 Vh
SEP 2.1	2000 V	2.0 mA	3.5 W	15°C	75 Vh
SEP 2.2	200 V	2.0 mA	3.5 W	15°C	15 Vh
SEP 2.3	2000 V	5.0 mA	3.5 W	15°C	510 Vh

The run takes 600 Vh or approximately 30 minutes. The prefocusing step takes approximately 8 minutes.

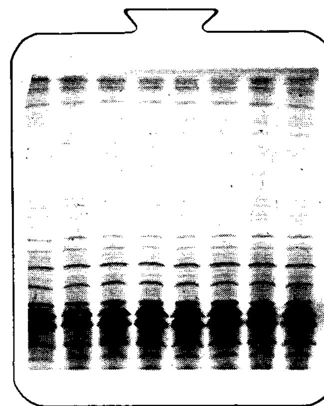


Example of IEF with PhastGel IEF 5—8. The gel was specifically stained for erythrocyte acid phosphatase (EAP) with 4-methylumbelliferyl phosphate. From the left, the phenotypes are: EAPA, A, B, B, BA, BA, CB, and CB.

Table 3. Optimized method for IEF with PhastGel IEF 4—6.5 to program into the separation method file of PhastSystem (given as method 3).

SAMPLE APPL. DOWN AT				3.2	0 Vh
SAMPLE APPL. UP AT				3.3	0 Vh
EXTRA ALARM TO SOUND AT				3.1	73 Vh
SEP 3.1	2000 V	2.0 mA	3.5 W	15°C	75 Vh
SEP 3.2	200 V	2.0 mA	3.5 W	15°C	15 Vh
SEP 3.3	2000 V	5.0 mA	3.5 W	15°C	410 Vh

The runs takes 500 Vh or approximately 25 minutes. The prefocusing step takes approximately 6 minutes.



Example of IEF with PhastGel IEF 4—6.5. The gel was silver stained using the method described in development technique file number 210. The repeating sample is elderberry extract.

Electrophoretic titration curve analysis

This is a two-dimensional technique to analyze protein charge characteristics. In the first dimension, the pH gradient is generated, like the prefocusing step in IEF. The gel is then rotated clockwise 90° and the sample is applied perpendicular to the pH gradient across the middle of the gel. The proteins become negatively or positively charged depending on their pI and start to migrate from the center (or remain at the center) to the cathode or anode. The rate of their migration will depend on the magnitude of their charge. (See reference 2 for more information about the principles and applications of titration curves.)

The titration curve methods below contain two steps divided by an empty (unprogrammed) step. The first step generates the pH gradient. After the first step an alarm will sound to mark the end of the method since the second step is empty. The first step will continue to run until the method is stopped. This step can be allowed to run for an additional 100 Vh without affecting the results.

After that, the iso-pH lines become more pronounced which cause distortions in the curves.

Once the gel(s) is repositioned for the second dimension, the PhastGel sample applicator TC (TC = titration curve) is loaded with sample and inserted into the middle position of the sample applicator arm. This applicator should be filled with 3 to 3.5 μl of sample for optimal results.

At the beginning of step 3, the sample is applied to the gel and the proteins migrate to one of the electrodes or remain at the center of the gel. An alarm will sound to mark the end of step 3 (end of method) but this step will continue until the method is stopped. Unlike the first step, step 3 must be stopped as soon as the alarm sounds. The proteins migate very fast in this step.

The Users Manual of PhastSystem has a section describing the procedures for running titration curves. You should read this before running the method.

Table 4. Optimized method for electrophoretic titration curve analysis with PhastGel IEF 3—9 to program into the separation method file of PhastSystem (given as method 4).

SAMPLE APPL. DOWN AT	4.3	0 Vh			
SAMPLE APPL. UP AT	4.3	6 Vh			
SEP 4.1 2 000 V	2.5 mA	3.5 W	15°C	150 Vh	
SEP 4.2 0 000 V	00.0 mA	0.0 W	00°C	0 000 Vh	
SEP 4.3 1 000 V	2.5 mA	0.2 W	15°C	40–60 Vh[1]	

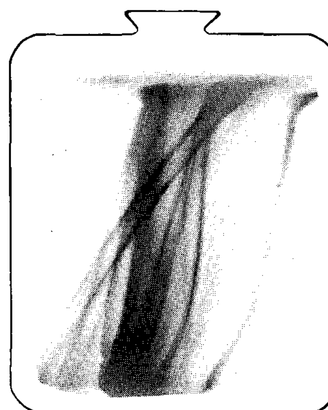
[1] The number of volthours required in step 3 will generally vary between 40 and 60 Vh, depending on the sample. The average running time is approximately 25 minutes, including the time it takes to reposition the gel for the second dimension run.

Example of a titration curve with PhastGel IEF 3—9. The gel was Coomassie stained using the method described in development technique file number 200. The sample is beef muscle extract.

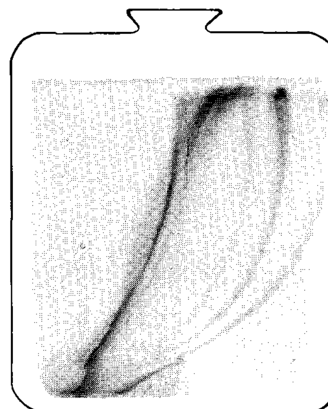
Table 5. Optimized method for electrophoretic titration curve analysis with PhastGel IEF 5—8 and 4—6.5 to program into the separation method file of PhastSystem (given as method 5).

SAMPLE APPL. DOWN AT	5.3	0 Vh		
SAMPLE APPL. UP AT	5.3	6 Vh		
SEP 5.1 2 000 V	2.0 mA	3.5 W	15°C	150 Vh
SEP 5.2 0 000 V	00.0 mA	0.0 W	00°C	0 000 Vh
SEP 5.3 1 000 V	2.0 mA	0.2 W	15°C	40–60 Vh[1]

[1] See the note for table 4 above.



Example of a titration curve with PhastGel IEF 5—8. The gel was silver stained using the method described in development technique file number 210. The sample is beef muscle extract.



Example of a titration curve with PhastGel IEF 4—6.5. The gel was silver stained using the method described in development technique file number 210. The sample is plaice muscle extract.

References

1. A carrier ampholyte for isoelectric focusing. *International Laboratory,* Jan/Feb. (1979), Williams, K. W., Söderberg, L.
2. Use of electrophoretic titration curves for predicting optimal chromatographic conditions for fast ion-exchange chromatography of proteins. *J Chromatogr* 266 (1983) 409—425, Haff, L. A. *et al.*

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