# PhastSystem™ PAGE of basic proteins

# Application File No. 300

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This application file describes three methods for resolving basic proteins using PhastSystem, fitted with the Reversed Polarity Electrode Assembly, and PhastGel™ media. Optimal conditions for separation are obtained by modifying the buffer composition of the buffer strips or PhastGel media. This includes decreasing the buffer pH and incorporating urea and detergent into the gel. The examples show separations of basic proteins with high water-solubility, histones and ribosomal proteins. This file also demonstrates the possibility for engineering the conditions for optimum separation of basic proteins in general.

Key words : Basic proteins, PAGE, PhastSystem, PhastGel, Reverse Polarity Electrode Assembly, acid-urea-Triton.

## Introduction

Proteins with high isoelectric points (generally higher than 8.0) can be separated according to their molecular weight (Stokes radius) by the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). However, if small charge differences are to be detected and/or if the tertiary structure or biological activity is to be preserved then SDS must be omitted.

In the absence of SDS basic proteins are positively charged at physiological pH and many are poorly soluble at the pH levels provided by common buffers used in electrophoresis (approximately pH 8). The electrophoretic separation of such proteins requires acidic buffer systems. With PhastSystem this can be achieved either with modified buffer strips alone, or with modifications to the buffer composition of both buffer strips and PhastGel media. In addition, the polarity of the electrical field across the gel must be reversed (using a Reversed Polarity Electrode Assembly) so that the automatic sample applicator system can still be used.

In the first method described here a number of basic proteins with good solubility at pH 4.2 can be separated using PhastGel media as supplied (containing Tris/acetate buffer pH 6.4). The acidic conditions required for the separation are provided by special buffer strips prepared with a β-alanine - acetic acid buffer at pH 4.1. As shown in this Application File, this method can be used for separations of proteins such as cytochrome c from horse heart (pI 10.3), egg lysozyme (pI > 11), chymotrypsinogen A from bovine pancreas (pI 9.6), and venoms from cobra, honey bee, and yellow jacket vespid (the allergens of insect venoms include basic proteins such as phospholipases and hyaluronidase, see reference 1).

The second and third methods are designed for basic proteins with a low solubility in Tris/acetic acid buffer. Examples of such proteins are histones and ribosomal proteins. These proteins are commonly separated using denaturants which prevent aggregation while preserving the charge differences between protein species - for example a low pH buffer containing acetic acid and urea (method 2 in this Application File ; see references 2 and 3). The separation can be modified by including Triton X-100 in the buffer system (method 3 in this Application File; see references 2, 4 and 5).

Separations using methods 2 and 3 require the equilibration of PhastGel with acetic acid / urea buffer (plus Triton X-100 if required) before use. The equilibration also makes it possible to change the porosity of the gel. The low pH of the buffer prevents the use of agarose buffer strips since agarose will not set in this buffer; alternative buffer strips are used.

These notes should be read in conjunction with the instructions for PhastSystem instrumentation and Development Technique File No. 200.

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# Materials

The major instrumentation and chemicals required for methods 1, 2 and 3 are identified here for easy reference :

| Instrumentation | Cat. no. |
|---|---|
| PhastSystem | 18-1600-01 (120 volt) |
| | 18-1601-01 (220 volt) |
| PhastGel Sample Applicator 8/0.5 | 18-1617-01 |
| PhastGel Sample Applicator 8/1 | 18-1618-01 |
| PhastSystem Reversed Polarity Electrode Assembly | 18-9494-01 |
| Electrode Strips (dry) | 19-3664-01 |

| Chemicals | Cat. no. |
|---|---|
| PhastGel Homogeneous 12.5* | 17-0623-01 |
| PhastGel Homogeneous 20* | 17-0624-01 |
| PhastGel Blue R | 17-0518-01 |

(*) Depending on their size range, basic proteins can also be separated using the following PhastGel media :

| | |
|---|---|
| PhastGel Gradient 10-15 | 17-0540-01 |
| PhastGel Gradient 8-25 | 17-0542-01 |
| PhastGel Homogeneous 7.5 | 17-0622-01 |

### Installation of reversed polarity electrodes

The standard electrode assembly is removed according to the instructions given in the Maintenance section of the System Guide to PhastSystem. The Reverse Polarity Electrode Assembly is inserted in the same way as the standard electrode assembly. (Note that the anode and cathode on the Reversed Polarity Electrode Assembly are marked on the plastic frame).

# Methods

Three methods for preparing samples, running PAGE, and staining are described here.

# Method 1

This method is used for basic proteins with good solubility at pH 4.2 (for example, cytochrome c, egg lysozyme, chymotrypsinogen A, and venoms from cobra, honey bee, and yellow jacket vespid).

### Sample preparation

The sample buffer should be acidic and compatible with the buffer used in the gel. Buffer salt concentrations should be kept low (0.1 - 0.2 M), although concentrations of neutral salts such as sodium chloride may be as high as 0.5 M without significantly affecting the result. The samples used in this method are dissolved in the buffer present in PhastGel media (0.112 M acetate, 0.112 M Tris, pH 6.4) with Pyronin Y as tracking dye.

### Buffer strips

Buffer strips are prepared as follows (for 25 buffer strips ) :

2 g agarose IEF or C (Pharmacia LKB)

4.4 g β-alanine

4.0 ml acetic acid

95 ml distilled deionised water

The components are mixed and boiled with stirring to dissolve the agarose. The solution is cooled to 70 °C and poured into casting moulds (empty buffer strips packages) and allowed to solidify by cooling to room temperature. The buffer strips have a pH of approximately 4.1. They should be stored at 4 - 8 °C and have a shelf-life of several weeks. These buffer strips in combination with the original buffer in PhastGel give a pH of 4.2 behind the buffer front in the gel during electrophoresis.

### PhastGel media

The separation is run on PhastGel Homogeneous 20.

### Running conditions

Once the reversed polarity electrode assembly has been installed, PhastSystem is operated according to the instructions given in the Owners Manual.

The running conditions are shown in Table 1.

*Table 1*: Running conditions to program into the separation unit of PhastSystem for PAGE of basic proteins using method 1.

| | | | | | |
|---|---|---|---|---|---|
| SAMPLE APPL. DOWN AT 1.1 | | | | | 1 Vh |
| SAMPLE APPL. UP AT 1.1 | | | | | 10 Vh |
| SEP 1.1 | 200 V | 10 mA | 2.5 W | 15 °C | 100 Vh* |

(*) or until the tracking dye reaches the buffer strips. The separation takes approximately 30 minutes.

### Staining

The proteins are stained with PhastGel Blue R according to the instructions given in PhastSystem Development Technique File no. 200 "Fast coomassie staining".

After staining some proteins (e.g. myoglobin) may resolubilise within the gel and give rise to diffuse bands. This can be avoided by storing the gels in 10 % trichloroacetic acid.

## Method 2

This method is suitable for proteins with poor solubility in Tris/acetic acid buffer (for example histones and ribosomal proteins).

### Sample preparation

The sample buffer should be acidic, contain 6.25 M urea and be compatible with the buffer used in the gel. Ideally, the samples are dissolved in 0.9 M acetic acid and 6.25 M urea (pH approximately 3) with Pyronin Y as tracking dye.

### Buffer strips

The buffer strips are cut out from dry Electrode Strips (19-3664-01) so that, on swelling in buffer (by 10 to 20 %), they have the same dimensions as those of PhastGel Buffer Strips. The dry buffer strips are soaked in buffer containing 0.9 M acetic acid, excess liquid is removed by briefly placing the strips on filter paper. In order to ensure good contact between electrode, buffer strip and gel the buffer strips should be inserted into the buffer strip holder before the holder is placed in PhastSystem. This allows you to check that the buffer strips protrude above and below the holder by 2-3 mm. The buffer strips can be re-used after washing in distilled water.

### Equilibration of PhastGel media

PhastGel media are equilibrated as follows :

- Remove the protective film from PhastGel media (PhastGel Homogeneous 20).
- Place one gel in a Petri dish containing 15 ml of buffer with the composition : 0.9 M acetic acid and 6.25 M urea. It is important that only one gel is placed in each dish to ensure even equilibration of buffer across the surface of the gel without interference from other gels.
- Incubate for 30 minutes on a shaking table, changing the buffer every 10 minutes.
- Remove the gel from the dish, and remove excess liquid from the surface of the gel with a sheet of thin, flexible plastic.
- Place the gel on the separation bed of PhastSystem according to the instructions in the Owners Manual.

PhastGel swells during the equilibration, as shown in Figure 1. As a result, the concentration of polyacrylamide will decrease. For example, the effective polyacrylamide concentration of PhastGel Homogeneous 20 after an incubation of 30 minutes can be estimated as 14 %.

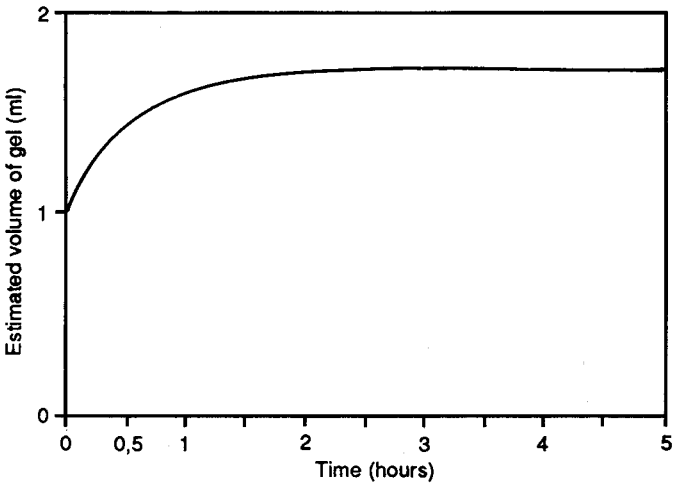


*Figure 1*: The swelling (as indicated by volume change) of PhastGel Homogeneous 20 with buffer containing 6.25 M urea and 0.9 M acetic acid (pH approximately 3). Gels were incubated in 15 ml of buffer on a shaking table for the times shown, with a change of buffer every 10 minutes. Gels were taken from the incubation bath, excess liquid was removed, and the gels were weighed. The weight of the backing film was subtracted and the volume was estimated using the density of the buffer solution. Electrophoresis of basic proteins on gels incubated for different times showed that the separation pattern changed very little with incubations of more than 30 minutes (results not shown). This indicated that the ionic composition of the gel had reached an end point after 30 minutes, although as shown in Figure 1 the maximum volume of the gel is reached after 2 hours.

### Running conditions

The running conditions are shown in Table 2.

*Table 2*: Running conditions to program into the separation unit of PhastSystem for PAGE of basic proteins using methods 2 and 3.

| | | | | | |
|---|---|---|---|---|---|
| SAMPLE APPL. DOWN AT 1.1 | | | | | 1 Vh |
| SAMPLE APPL. UP AT 1.1 | | | | | 6 Vh |
| SEP 1.1 | 50 V | 4 mA | 0.5 W | 15 °C | 10 Vh |
| SEP 1.2 | 150 V | 4 mA | 1.0 W | 15 °C | 30 Vh |
| SEP 1.3 | 200 V | 6 mA | 1.0 W | 15 °C | 100 Vh * |

(*) or until the tracking dye reaches the buffer strips. The separation takes approximately 45 minutes.

### Staining

The proteins are stained with PhastGel Blue R according to the instructions given in PhastSystem Development Technique File no. 200 "Fast coomassie staining".

## Method 3

This method is used for the separation of histones in the presence of Triton X-100.

### Sample preparation

The samples are dissolved in buffer containing 0.9 M acetic acid, 2.5 M urea and 2 % Triton X-100 (pH approximately 3) with Pyronin Y as tracking dye.

### Buffer strips

The buffer strips were identical to those used in method 2.

### Equilibration of PhastGel media

This method requires the introduction of Triton X-100 into the gel. The flow of Triton X-100 into PhastGel is, however, limited by a combination of the large micellar structure of this detergent (relative molecular mass 90,000) in water solutions and the dense polyacrylamide matrix in PhastGel. This means that equilibration of PhastGel media in free solution results in a maximum concentration of Triton X-100 in the gel which is less than the critical micellar concentration of 0.24 mM. A higher concentration of detergent is obtained by drying the gel and then adding only a limited volume of detergent solution so that the whole volume is absorbed by the gel. Method 3 uses a more porous gel than that used in method 2 since some histones with hydrophobic surfaces will bind Triton X-100 and their mobility will be reduced to such an extent that PhastGel Homogeneous 12.5 will give optimal separations.

Drying the gel

- Wash the gel in 15 ml distilled water for 20 minutes, changing the water after 10 minutes.
- Wash the gel in 10 % (w/v) glycerol for 10 minutes.
- Dry the gel with a hair drier or leave at room temperature overnight (the dry gels are stable for several weeks when stored at 4 - 8 °C).

Incubating the gel

- Place 0.9 ml of buffer containing 0.9 M acetic acid, 2.5 M urea and 2 % Triton X-100 on a glass plate. Place the gel upside down on the buffer and cover with a Petri dish. Leave at room temperature for 3 hours.

### Running conditions

The running conditions are shown in Table 2.

### Staining

The proteins are stained with PhastGel Blue R according to the instructions given in PhastSystem Development Technique File no. 200 "Fast coomassie staining".

## Results

Examples of the results obtained are shown in Figure 2 (method 1), Figure 3 (method 2) and Figure 4 (method 3).

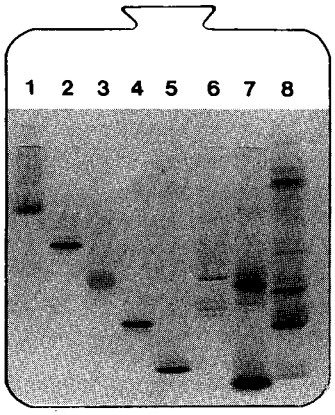


*Figure 2*: PAGE of basic proteins with PhastGel Homogeneous 20 and buffer strips with modified buffer composition (method 1 in text). The proteins were detected using coomassie blue staining. Samples : lane 1, lens lectin ; lane 2, chymotrypsinogen A; lane 3, horse myoglobin; lane 4, lysozyme; lane 5, cytochrome c from horse heart; lane 6, venom from yellow jacket vespid; lane 7, venom from honey bee; lane 8, venom from cobra.

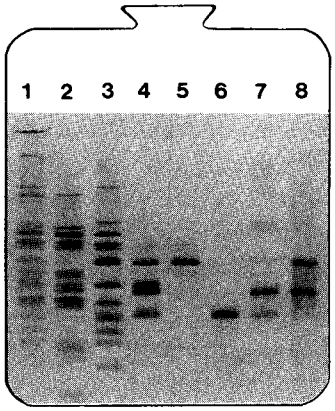


*Figure 3:* PAGE of basic proteins with PhastGel Homogeneous 20 pre-incubated with a buffer containing 0.9 M acetic acid and 6.25 M urea and buffer strips containing 0.9 M acetic acid (method 2 in text). The proteins were detected using coomassie blue staining. Lanes 1 - 3 contain preparations of proteins from the ribosomes of *E. coli* : lane 1, 70S subunit; lane 2, 30S subunit; lane 3, 50S subunit. Lanes 4 - 8 contain histone preparations from calf thymus : lane 4, mixture of H1, H2A, H2B, H3 and H4; lane 5, histone H1 ; lane 6, histone H4 (samples in lanes 4 - 6 were supplied by Boehringer Mannheim); lane 7, Sigma Type II-AS; lane 8, Sigma Type V-S (samples in lanes 7 and 8 were supplied by Sigma Chemical Company).

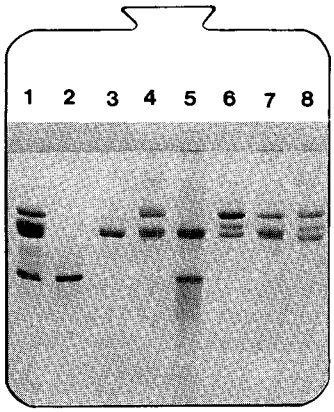


*Figure 4:* PAGE of histones from calf thymus with PhastGel Homogeneous 12.5 pre-incubated with a buffer containing 0.9 M acetic acid, 2.5 M urea and 2 % Triton X-100, and buffer strips soaked in 0.9 M acetic acid (method 3 in text). The proteins were detected using coomassie blue staining. Lanes 1 - 3 contain preparations supplied by Boehringer Mannheim : lane 1, histone mixture ; lane 2, histone H1 ; lane 3, histone H4. Lanes 4 - 8 contain preparations supplied by Sigma Chemical Company : lane 4, Type II-AS ; lane 5, Type V-S ; lane 6, Type VI-S ; lane 7, Type VII-S ; lane 8, Type VIII-S.

Method 1 is suitable for basic proteins which remain soluble in the buffer conditions provided by standard PhastGel media and the modified buffer strips. The low solubility of proteins such as histones and some ribosomal proteins results in poorly-defined, smeared bands when using this method (results not shown). The separations of ribosomal proteins and histones using methods 2 and 3, however, show sharp bands, indicating that the proteins remain soluble in the buffer conditions chosen. A marked result of the addition of Triton X-100 to the buffer system is the reversal in the relative positions of the bands corresponding to histones H1 and H4 (compare Figure 3, lanes 5 and 6 with Figure 4, lanes 2 and 3).

Triton X-100 has been used in standard electrophoresis to reveal the presence of primary structure variant forms which differ in their ability to bind the detergent, and are therefore retarded to different degrees in their electrophoretic migration (2,4,5). The effect of Triton X-100 on mobility is reduced by urea (2). The urea concentration was therefore decreased from 6 M (method 2) to 2.25 M (method 3) to improve the binding of Triton X-100. The results shown here suggest that H4 binds more Triton X-100 than H1 does, and is therefore less mobile in PhastGel media.

## Conclusions

- Water-soluble basic proteins are separated by method 1.
- Hydrophobic basic proteins such as histones and ribosomal proteins are separated by method 2 or 3.
- The buffer composition in PhastGel can be changed by equilibration of the gel with new buffer solutions.
- The porosity of PhastGel can be controlled by equilibration time or by drying the gel and re-swelling it in a limited volume of buffer.
- Substances with a high relative molecular mass, such as detergent micelles, can be introduced into PhastGel by drying and re-swelling the gel.
- The use of detergents offers the possibility of selectively changing the electrophoretic mobility of different proteins and thereby improving resolution.
- The use of PhastSystem, fitted with the Reversed Polarity Electrode Assembly, and PhastGel facilitates the rapid separation of basic proteins.

## References

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