GE Healthcare
Life Sciences

Amersham Calibration Kits for pI Determinations using Isoelectric Focusing

17-0471-01 Broad range pI (ph 3–10)
17-0472-01 Low range pI (ph 2.5–6.5)
17-0473-01 High range pI (ph 5–10.5)

Product Booklet

Codes: 17-0471-01
17-0472-01
17-0473-01

Page finder

1. Legal

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2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only.*

Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

Warning: *Potentially infectious material.*

Human blood products provided as a component of this pack have been obtained from donors who were tested individually and were found to be negative for the presence of Human Immunodeficiency Virus antibody (HIV-Ab)* as well as for Hepatitis B surface antigen (HBsAg) using EIA.

As no test method can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus antibody (HIV-Ab) or other infectious agents are absent, all human blood products should be considered potentially infectious. Handling, use, storage and disposal should be in accordance with the procedures defined by an appropriate National biohazard safety guideline or regulation, where it exists (for example USA Centre for Disease Control/National Institutes of Health manual 'Biosafety in Microbiological and Biomedical Laboratories').

* HIV is the abbreviation used for HTLV-II and LAV.

Instructions relating to handling and use of test materials.

1. All operations should be carried out in restricted areas by trained and authorized persons. Storage of test materials should be specially designated areas accessible only to authorized personnel.
2. Wear appropriate protective laboratory clothing including disposable gloves. Avoid 'sharps' (for example sharp cutting edges, needles, scissors) which may puncture the skin or damage protective clothing. Do not spill or splash reagents or form an aerosol. No smoking, eating or drinking should be allowed in areas where specimens or test materials are handled. Do not pipette by mouth. Wash hands before leaving the laboratory.

Decontamination and disposal of waste

All specimens and test materials should be decontaminated before disposal. An effective way of decontaminating waste is subjecting it to autoclaving for a minimum of 15 minutes at 121ºC or higher. Decontaminating materials may be disposed of as laboratory waste.

Spills and breakages

Cover any spills with absorbent material and saturate with a disinfectant appropriate to the surface and material spilt and use according to the manufacturer's instructions. Carefully gather up all of the materials and decontaminate the area with fresh disinfectant. 'Sharps' should be disposed of in a secure and safe container. Dispose of all materials used to wipe up the spills as though potentially infectious.

2.2. Storage

The kit should be stored at 2–8°C.

2.3. Expiry

The GE Healthcare pI Calibration Kits are stable for at least two years when stored at 2–8°C in unopened vials. Once the vials have been reconstituted, the pI markers should be used within 12 hours. For best results, it is recommended that the Calibration Kit protein standards be reconstituted just prior to use.

3. Components

Broad range (pH 3–10)
17-0471-01

Low range (pH 2.5–6.5)
17-0472-01

High range (pH 5–10.5)
17-0473-01

Each of the three GE Healthcare pI Calibration Kits consists of ten vials containing a lyophilised mixture of well-characterised, purified proteins. Each vial contains 20–50 µg of each pI marker protein (total protein per vial ~200–400 µg).

3.1. Other materials required

- Electrophoresis reagents appropriate to the application being run.
- IEF apparatus for example Multiphor II system.

3.2. Critical parameters

The pI Calibration Kits are not recommended for use under denatured conditions, i.e. 8 M urea. The protein pattern will then be altered.

4. Introduction

Isoelectric Focusing (IEF) is the method of choice for determining protein isoelectric points (pIs). IEF is easy to do, reproducible, fast and requires very small amounts of sample. High resolution is obtained by IEF in gel-stabilized systems, making it possible to determine the pI's of many components in a single run, and to analyse even crude, multi component preparations. Accurate calibration of the pH gradient profile across the IEF gel is essential to obtain precise measurement of protein pIs. By using well characterised pI markers under established conditions well focused and distinct bands are obtained enabling simple and accurate measurements of the pH gradient in IEF gels.

5. Description of pI Calibration Kits

The pI marker proteins are lyophilised in the presence of sucrose (High and Broad pI Kits) or mannitol (Low pI Kit). Reconstitution of the vial contents with 100 µl of distilled or deionized water will give a solution containing about 30% sucrose for the High and Broad pI Kits and 3% mannitol for the Low pI Kit. Upon focusing, the proteins yield distinct bands of known pI's with minimal impurities. The exact protein amounts have been chosen so that each pI marker band is easily distinguishable on staining with Coomassie™ Blue after focusing. Coloured pI markers are included in each pI Calibration Kit enabling one to visually monitor the time and quality of focusing as it is proceeding. Also, the coloured markers are useful for proper identification of the various pI marker bands after focusing. Each product contains ten vials each containing a mixture of the following proteins.

Broad range (pH 3–10) 17-0471-01	
Protein	**pI at 24°C ± 1.5°C**
Amyloglucosidase,	pI 3.50
Methyl red (dye)**,	pI 3.75
Soybean trypsin inhibitor,	pI 4.55
ß-Lactoglobulin A,	pI 5.20
Bovine carbonic anhydrase B,	pI 5.85
Human carbonic anhydrase B,	pI 6.55
Horse myoglobin-acetic band (coloured marker),	pI 6.85
Horse myoglobin-basic band (coloured marker),	pI 7.35
Lentil lectin-acidic band,	pI 8.15
Lentil lectin-middle band,	pI 8.45
Lentil lectin-basic band,	pI 8.65
Trypsinogen,	pI 9.30

** Methyl red is a dye and does not appear in final stained gel.

Low range (pH 2.5–6.5) 17-0472-01 Protein	pI at 24°C ± 1.5°C
Pepsinogen,	pI 2.80
Amyloglucosidase,	pI 3.50
Methyl red (dye)**,	pI 3.75
Glucose oxidase,	pI 4.25
Soybean trypsin inhibitor,	pI 4.55
ß-Lactoglobulin A,	pI 5.20
Bovine carbonic anhydrase B,	pI 5.85
Human carbonic anhydrase B,	pI 6.55

** Methyl red is a dye and does not appear in final stained gel.

High range (pH 5–10.5) 17-0473-01 Protein	pI at 24°C ± 1.5°C
ß-Lactoglobulin A,	pI 5.20
Bovine carbonic anhydrase B,	pI 5.85
Human carbonic anhydrase B,	pI 6.55
Horse myoglobin-acidic band (coloured marker),	pI 6.85
Horse myoglobin-basic band (coloured marker),	pI 7.35
Lentil lectin-acidic band,	pI 8.15
Lentil lectin-middle band,	pI 8.45
Lentil lectin-basic band,	pI 8.65
Trypsinogen,	pI 9.30
Cytochrome C (coloured marker),	pI 10.25

5.1. Method for determining pI values for marker proteins.

The pI values for protein markers were determined by isoelectric focusing in thin slabs of both polyacrylamide and agarose. The method used for determination of marker pI values was based on the procedure suggested by Låås *et al.* (1). The pI values (24°C ± 1.5°C) are accurate to: ± 0.5 pH units for markers with pIs <6. ± 0.08 pH units for markers with pIs between 6–9. ± 0.1 pH units for markers with pIs >9.

6. Protocol for determining protein isoelectric points

If the approximate pI of the protein of interest is known, chose a suitable Ampholine PAGplate pH 3.5–9.5 or select a suitable narrow range Pharmalyte™ interval (see Ordering Information) and prepare a gel plate according to the recommended procedure. If the approximate pI of the protein is unknown, chose Ampholine PAGplate 3.5–9.5 or prepare a gel containing Pharmalyte pH 3–10. Determine the approximate pI of the protein using the procedure described below. Then determine the protein's pI more accurately on a narrow pH range gel.

6.1. Reconstitution of pI Calibration Kit.

Follow the instructions below to reconstitute the pI Calibration Kits.

Step	Action
1.	Dissolve the content of one vial with 100 µl of water. **Note:** For Phast Gels use 50 µl of water.
2.	Pipette mix gently.
3.	**Broad pI kit 17-0471-01:** Use the required volume of the reconstituted vial immediately. Retain the reconstituted vial on ice for duration of use. **Low pI kit 17-0472-01 and High pI kit 17-0473-01:** Use the required volume of the reconstituted vial within 12 hours of reconstitution, but preferably immediately.

6.2. Prepare the IEF gel.

Place the gel on the IEF apparatus and adjust the temperature of the coolant to the value that gives 24°C in the gel at the end of the run.

6.3. Sample application.

The volume of pI markers to apply to the IEF gel depends on the type length and thickness of the gel and the detection method used. Sample volumes recommended for various types of gels stained with Coomassie Blue G-250 are given in Table 1. When using silver staining, which is at least 20 times more sensitive, the amount of sample applied to the gel should be decreased accordingly. The pI markers can be applied to thin layer gel slabs using GE Healthcare analytical IEF sample applicators or with small pieces of filter paper. The pI markers are preferably applied 1/3 from the cathode. It is recommended that the pI markers are applied in sample lanes on both sides of the protein(s) of interest to confirm that the iso-pH lines are straight between the pI markers and the sample of interest.

Table 1.

Approximate type of gel	sample volume (µl)
Thin layer gels (100 x 1 mm) of PAA	10–20
Thin layer gels (100 x 1 mm) of agarose	10–20
Polyacrylamide gel rods (2.7 mm in diameter)	30*

* For larger diameter rods, increase the volume of sample applied.

6.4. Focusing conditions.

Follow the recommended focusing conditions relevant to the IEF equipment you are using. To refer to the pI values at 24°C select a temperature setting (10–20°C) and the maximum power supply settings to result in a gel temperature of 24°C ± 1.5°C upon completion of focusing. Start the focusing. Remove the plastic applicator strip or filter papers used for applying the samples approximately 15–30 minutes after onset of focusing. Mark the cathode end of the gel with a sharp blade at the inside edge of the wick immediately after the run is completed and note the position of the coloured pI markers. Immediately fix, stain, destain, and dry the gel.

6.5. Determine the pH gradient and protein pI.

Using a piece of graph paper with 1 cm markings and 0.1 cm subdivisions, position the gel over the grid so the origin of the grid is lined up with the cathode end of the gel. Using the grid, determine the distance from the cathode to each protein pI marker. Plot the known pI value of each pI marker protein versus its distance from the cathode. Connect the points to obtain the pH gradient profile calibration curve. The identity of the focused bands can be determined by noting the positions of the coloured pI markers before staining and counting the bands from those positions after staining. Note that some pI markers will focus in the wicks when the narrow pH intervals are used. To estimate the pI of the protein of interest, determine the distance from the cathode to the protein(s) of interest. From the pH calibration curve and known distance(s) from the cathode of the protein(s) of interest, determine the pI(s) of the protein(s).

7. Tips for successful and accurate pI determination

Focus until the protein achieves a steady state.

Accurate protein pI determination requires that the protein reaches equilibrium (steady state) (2) in the pH gradient established by the carrier ampholytes. The minimum duration of focusing (volthours) to achieve steady state must be determined for each protein, and may differ from the pI markers in the pI Calibration Kits. This is particularly true when working with high molecular weight proteins in polyacrylamide gels. To test for attainment of steady state in flat bed isoelectric focusing, apply the sample approximately 1 cm from each electrode and determine the minimum volthours required to obtain a coalescence. When the volthours required for steady state focusing of the protein(s) have been determined, experiment to find the optimal point of sample application that gives the best focusing pattern with the sharpest bands in minimum volthours.

Consider the temperature in the gel.

It is important to remember that the pI of the protein is temperature dependent. Fredriksson (3, 4) showed that in the basic region differences as high as 0.6 pH units can be obtained for protein pI values measured at 4°C and 25°C (higher pI is obtained at lower temperature). Proteins with lower pIs show less variation of pI with temperature, typically -0.005 pH units/°C, whereas strongly basic proteins have variations of typically -0.03 pH units/°C. Therefore, one should know the temperature in the gel at the point where the protein has focused. This can present a problem since the temperature is always higher in the gel than in the coolant, and there is always a temperature variation in the gel which follows the field strength distribution profile (1). By using a carrier ampholyte with an even conductivity like Pharmalyte the temperature gradient in the gel is minimized.

8. Results

Protein pattern of the marker proteins and pH gradient profiles as determined by the pI Calibration Kits in thin slabs of PAA containing various Pharmalyte ranges are shown in Figures 1 to 4.

Figures 5 and 6 show typical results and pH profiles for the pI Calibration Kits run in gels made with Agarose IEF from GE Healthcare.

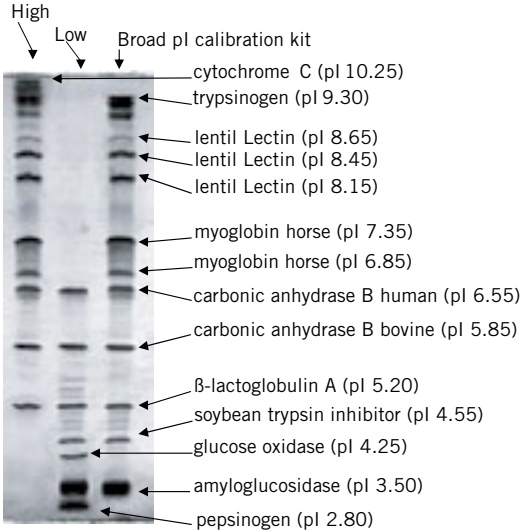

Figure 1. High, Low and Broad pI Calibration Kit run on Ampholine PAG plate pH 3.5–9.5.

Experimental Conditions Gel: Ampholine PAGplate pH 3.5–9.5 5%T, 3%C PAA thin layer 1 mm thick. 10 cm distance between electrodes.

Focusing conditions: Maximum power supply settings – Power 30 W, Volts 1500 V. Current 50 mA, coolant temperature 16°C.

Focusing time: 1700 volt hours about 1.5 hours.

Fixing: 1 hour in aqueous solution of 10% trichloroacetic acid. (w, w, v).

Equilibration: 30 minutes in aqueous solution of 25% methanol, 5% acetic acid (v, v, v).

Staining: 10–20 minutes in 0.1% Coomassie Blue G-250 in aqueous solution of 25% methanol, 5% acetic acid (w, v, v, v).

Destaining: in aqueous solution of 25% methanol, 5% acetic acid (v, v, v) until background is clear.

Preservation: 1 hour in 5% glycerol, 25% methanol, H_2O (v, v, v); let dry at room temperature overnight, then cover with clear plastic sheet.

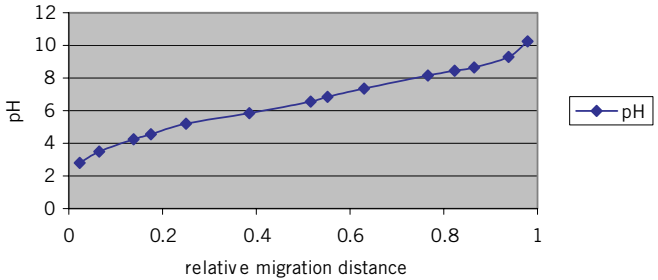

Figure 2. Determination of pH gradient profile using High, Low and Broad pI Calibration Kit on Ampholine PAGplate pH 3.5–9.5.

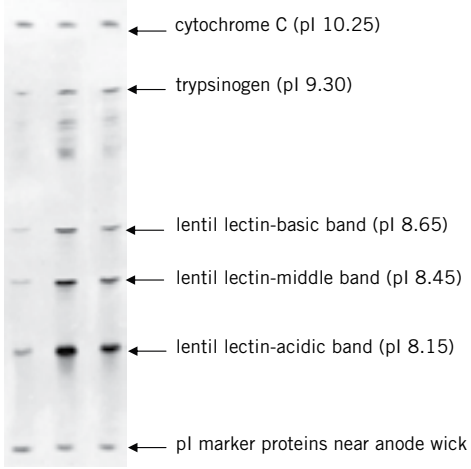

Figure 3. High pI Calibration Kit run on a 5% PAA gel containing Pharmalyte 8–10.5.

Experimental Conditions.

Gel: 5% PAA gel 1 mm thick containing 10 % glycerol and Pharmalyte 8–10.5 final dilution 1:16.

Focusing conditions: Maximum power supply settings – Power 60 W, Volts 3000 V, Current 150 mA, coolant temperature 5°C for 45 minutes then 12°C for the duration of run (N_2 atmosphere).

Focusing time: 1.5 hours.

Fixing, staining, destaining as in Figure 1 .

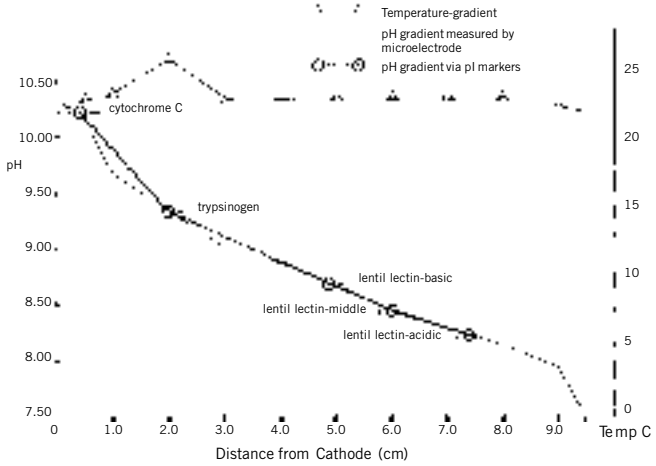

Figure 4. Determination of pH gradient profile using the High pI Calibration Kit on a 5% PAA gel containing Pharmalyte 8–10.5.

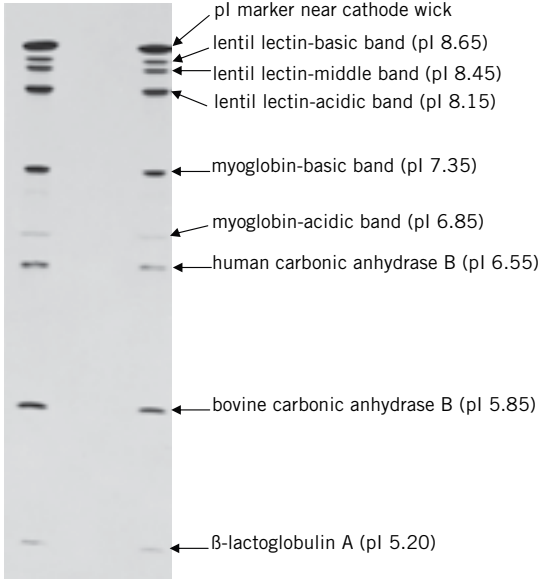

Figure 5. High pI Calibration Kit run on a 1% agarose gel containing Pharmalyte 5–8.

Experimental Conditions.

Gel: 1% Agarose IEF thin layer gel 1 mm thick containing 12% sorbitol and Pharmalyte 5–8 (final dilution 1–16) ~ 10 cm distance between electrodes.

Focusing conditions: Maximum power supply settings – Power 15 W, Volts 1500 V, Current 50 mA, coolant temperature 18°C.

Focusing time: 1.5 hours.

Fixing: 30 minutes in aqueous solution of 10% trichloroacetic acid, 5% sulfosalicylic acid (w, w, v).

Equilibration: 30 minutes in 35% ethanol, 10% acetic acid, H_2O (v, v, v) (add fresh solution after 15 minutes).

Drying: 3 layers of filter paper were placed on gel followed by a glass plate and a weight of about 1 kg. After 15 min all were removed and the gel dried with a hairdryer.

Staining: 5–10 minutes in an aqueous solution of 0.2% Coomassie Blue R250 in 35% ethanol, 10% acetic acid (v, w, v, v).

Destaining: 10–15 minutes in 35% ethanol, 10% acetic acid, H_2O (v, v, v).

Drying: Gel dried to a thin film with a hairdryer.

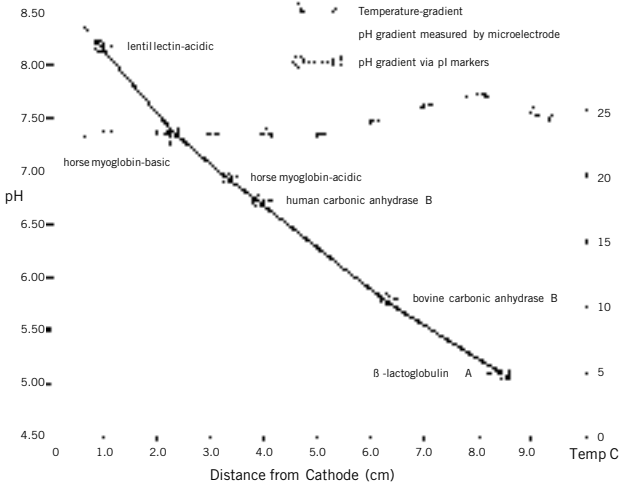

Figure 6. Determination of pH gradient profile using the High pI Calibration Kit on a 1% agarose IEF gel containing.

9. References

1. Låås, T., Olsson, I., and Söderberg, L., High Voltage Isoelectric Focusing with Pharmalyte: Field Strength and Temperature Distribution, Zone Sharpening, Isoelectric Spectra, and pI Determinations, *Analytical Biochemistry* **101**, 449–461, (1980).

2. Delinceé, H., and Radola, B. J., Determination of Isoelectric Points in thin Layer Isoelectric Focusing: The Importance of attaining the steady state and the Role of CO_2 Interference, *Analytical Biochemistry* **90**, 609–623, (1978).

3. Fredricksson, S., in 'Electrofocusing and Isoachophoresis', ed. by Radola, B. J., and Graesslin, D., Walter de Gruyter, Berlin, New York, 71–83, (1977).

4. Fredricksson, S., Temperature Dependance of Ampholine pH Gradients used in Isoelectric Focusing, *Journal of Chromatography* **151**, 347–355, (1978).

10. Related products

Calibration Kits for pI determinations using Isoelectric Focusing

Designation	Code No.
Broad pI kit pH 3–10	17-0471-01
Low pI kit pH 2.5–6.5	17-0472-01
High pI kit pH 5–10.5	17-0473-01

Recommended equipment and accessories

Designation	Code No.
Multiphor™ II Electrophoresis unit	18-1018-06
EPS 3501 XLPower supply	18-1130-05
MultiTemp™ III thermostatic circulator	
115 VAC	18-1102-77
230 VAC	18-1102-78

Chemicals

Designation	Code No.
Pharmalyte pH interval 2.5–5	17-0451-01
Pharmalyte pH interval 4–6.5	17-0452-01
Pharmalyte pH interval 5–8	17-0453-01
Pharmalyte pH interval 8-10.5	17-0455-01
Pharmalyte pH interval 4.2-4.9	17-0562-01
Pharmalyte pH interval 4.5-5.4	17-0563-01
Pharmalyte pH interval 5–6	17-0564-01
Pharmalyte pH interval 6.7–7.7	17-0566-01
Pharmalyte pH interval 3–10	17-0456-01
Agarose IEF	17-0468-01
PlusOne Bis-acrylamide 2% Solution	17-1306-01
PlusOne Ammonium Persulphate	17-1311-01
PlusOne TEMED	17-1312-01

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