Amersham™ pI calibration kits

17-0471-01 Broad range pI (ph 3–10)
29-4435-48 Low range pI (pH 3.5–6.5)
17-0473-01 High range pI (ph 5–10.5)

Product booklet

Codes: 17-0471-01
29-4435-48
17-0473-01

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1. Legal

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2. Handling

2.1. Safety warnings and precautions

Warning: For research use only.

This product is not recommended or intended for diagnosing of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered potentially hazardous. That's why we recommend that this product is only handled by people who have been trained in laboratory techniques. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Take care to avoid contact with skin or eyes. In case of contact with skin or eyes, wash the area immediately with water. Check material data sheets and safety statements 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, you should consider all human blood products to be potentially infectious. Handling, use, storage and disposal should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.

* 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. You should store test materials in specially designated areas that are only accessible to authorized personnel.
2. Wear appropriate protective laboratory clothing including disposable gloves. Avoid sharps like sharp cutting edges, needles, and scissors that can 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 your hands before leaving the laboratory.

Decontamination and disposal of waste

Decontaminate all specimens and test materials before disposal. You can effectively decontaminate waste by subjecting it to autoclaving for at least 15 minutes at 121ºC or higher. You can dispose of decontaminating materials as laboratory waste.

Spills and breakages

Cover any spills with absorbent material and saturate it with a disinfectant appropriate for the surface and type of spill. Carefully gather up all of the materials and decontaminate the area with fresh disinfectant. 'Dispose of any sharps in a secure and safe container. Dispose of all materials you used to wipe up the spill as if they are potentially infectious.

2.2. Storage

You should store the kit between 2°C and 8°C.

2.3. Expiry

The pI Calibration Kits are stable for at least two years when stored at 2ºC–8ºC in unopened vials. After reconstituting the vials, you should use the pI markers within 12 hours. For best results, reconstitute the Calibration Kit protein standards just prior to use.

3. Components

Broad range (pH 3–10) 17-0471-01

Low range (pH 3.5-6.5) 29-4435-48

High range (pH 5–10.5)17-0473-01

Each of the three pI Calibration Kits contains ten vials containing a lyophilised mixture of well-characterized, purified proteins. Each vial contains 20–50 µg of each pI marker protein and the total protein per vial is ~200–400 µg.

3.1. Other materials required

- Electrophoresis reagents appropriate to the application being run
- IEF apparatus (e.g., Multiphor II system)

3.2. Critical parameters

We don't recommend using pI Calibration Kits in denatured conditions like 8 M urea. These conditions will alter the protein pattern.

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. Accurately calibrating the pH gradient profile across the IEF gel is essential to obtaining precise measurement of protein pIs. You can use well-characterized pI markers in established conditions to obtain well-focused and distinct bands, enabling accurate measurements of the pH gradient in IEF gels.

5. Description of pI Calibration Kits

The pI marker proteins are lyophilize in the presence of sucrose (High and Broad pI Kits) or mannitol (Low pI Kit). Reconstituting the vial contents with 100 µL of distilled or deionized water yields 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 pIs 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. Colored pI markers are included in each pI Calibration Kit enabling you to visually monitor the time and quality of focusing as it's underway. Also, the colored markers are useful for properly identifying 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	I 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 3.5–6.5) 29-4435-48	pI at 24°C ± 1.5°C
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

We determined the pI values for protein markers using isoelectric focusing in thin slabs of both polyacrylamide and agarose. The method we used is based on the procedure suggested by Låås et al. (1). The pI values (24ºC ± 1.5ºC) 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 you know the approximate pI of the protein of interest, choose 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 you don't know the approximate pI, choose 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 Kit

Step	Action
1.	Dissolve the content of one vial with 100 µl of water. **Note:** For PhastGel™, 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 29-4435-48 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. We recommend sample volumes types of gels stained with Coomassie Blue G-250 in Table 1. When using silver staining, which is at least 20 times more sensitive, you should decrease the amount of sample applied to the gel accordingly. You can apply the pI markers to thin layer gel slabs using Cytiva analytical IEF sample applicators or with small pieces of filter paper. We recommend applying the pI markers 1/3 from the cathode. Apply the pI markers 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–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 colored 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 lines 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. You can determine the identity of the focused bands can by noting the positions of the colored pI markers before staining and counting the bands from those positions after staining. Note that some pI markers will focus in the wicks when you use narrow pH intervals. To estimate the pI of the protein of interest, determine the distance from the cathode to the protein(s) of interest. Using 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. You must determine the minimum duration of focusing (volthours) to achieve steady state for each protein — it might 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. After determining the volthours required for steady state focusing of the protein(s), 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.

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/ºC, whereas strongly basic proteins have variations of typically -0.03 pH units/°C. This means you should know the temperature in the gel at the point where the protein focuses. 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). Using a carrier ampholyte with an even conductivity like Pharmalyte minimizes the temperature gradient in the gel.

8. Results

We determined protein patternd of the marker proteins and pH gradient profiles as using the pI Calibration Kits in thin slabs of PAA containing various Pharmalyte ranges 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 Cytiva.

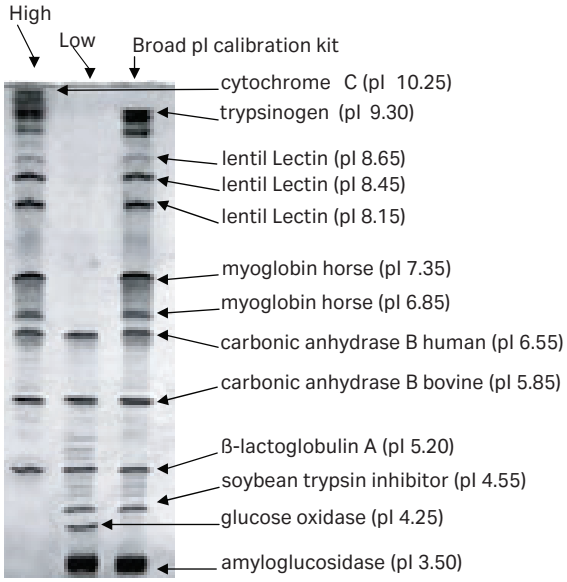

Fig 1. High, Low and Broad pI Calibration Kit run on Ampholine PAG plate pH 3.5–9.5.

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 16ºC.

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, H2O (v, v, v); let dry at room temperature overnight, then cover with clear plastic sheet.

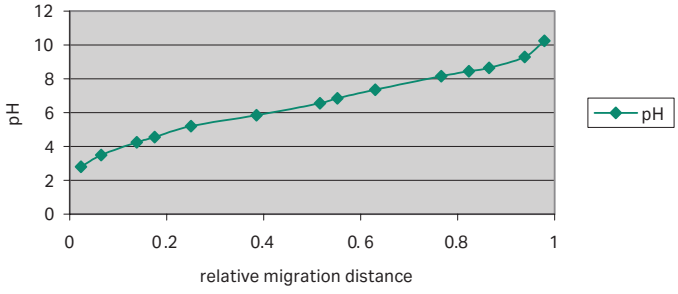

Fig 2. Figure 2. Determination of pH gradient profile using High, Low and Broad pI Calibration Kit on Ampholine PAGplate pH 3.5–9.5.

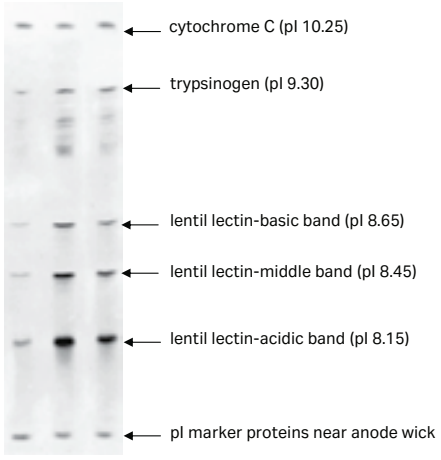

Fig 3. High pI Calibration Kit run on a 5% PAA gel containing Pharmalyte 8–10.5.

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 (N2 atmosphere). Focusing time: 1.5 hours.

Fixing, staining, destaining as in Figure 1 .

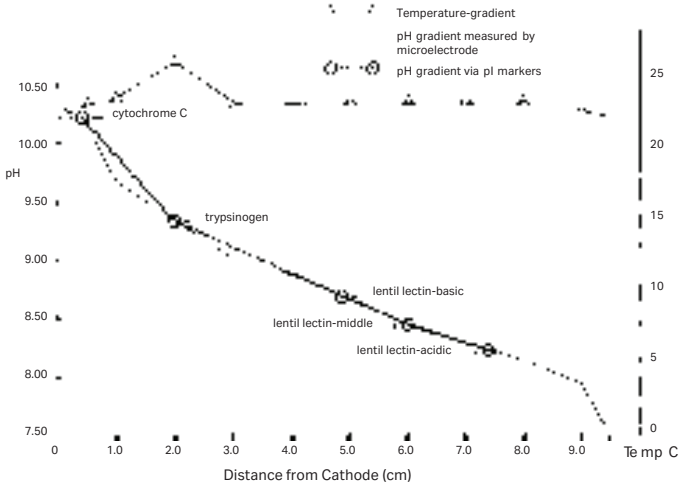

Fig 4. Determination of pH gradient profile using the High pI Calibration Kit on a 5% PAA gel containing Pharmalyte 8–10.5.

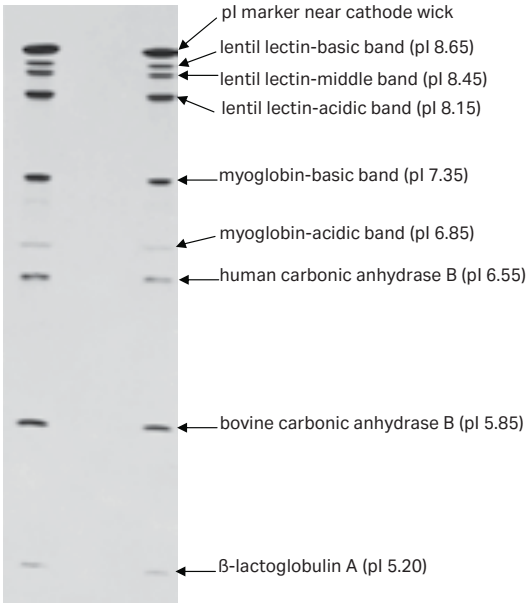

Fig 5. High pI Calibration Kit run on a 1% agarose gel containing Pharmalyte 5–8.

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, H2O (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 hair dryer.

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,

H2O (v, v, v).

Drying: Gel dried to a thin film with a hair dryer.

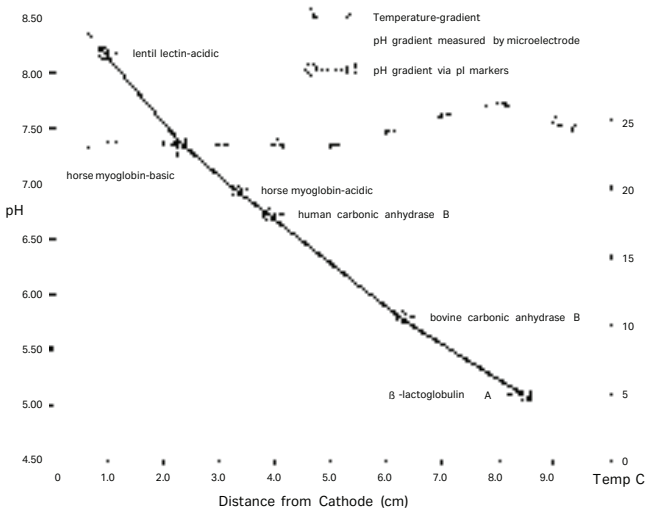

Fig 6. Determination of pH gradient profile using the High pI Calibration Kit on a 1% agarose IEF gel containing. gradient profile using the High pI Calibration Kit on a 5% PAA gel containing Pharmalyte 8–10.5.

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 CO2 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	29-4435-48
High pI kit pH 5–10.5	17-0473-01

Recommended equipment and accessories

Designation	Code No.
EPS 3501 XLPower supply	18-1130-05

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
Agarose IEF	17-0468-01

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