Table 4: Sensitive silver staining protocol

Step	Solution	Volume	Time
1. Fixing	20% trichloroacetic acid (w/v)	200 mL	45 min
2. Washing	H_2O_{dist}.	200 mL	5 min
3. Rinsing I	50% methanol / 10% acetic acid (v/v)	200 mL	40 min
4. Rinsing II	5% methanol / 7% acetic acid (w/v)	200 mL	20 min
5. Incubation	2.5% glutaraldehyde (gels may be kept overnight at this stage)	200 mL	15 min
6 - 9. Washing	H_2O_{dist}.	4 × 200 mL	20 + 15 + 10 + 10 min
10. Silvering *(Freshly prepared solutions)*	Solution 1: Dissolve 250 mg $AgNO_3$ in 1 mL H_2O_{dist}. Solution 2: 40 mL H_20 dist + 4 mL NaOH (1M) + 1.5 mL NH_3 (25%) Drop Solution 1 into 2 while stirring, fill up to 200 mL with H_2O_{dist}.*(or see the Silver-Staining Kit manual)*	200 mL	40 min
11-12 Washing	H_2O_{dist}.	2 × 200mL	1 + 5 min
13. Developing *(Freshly prepared solutions)*	0.0025% citric acid + 100 µL formaldehyde in 200 mL with H_2O_{dist} *(Or see the Silver-Staining Kit manual)*	200 mL Visual control! Set Beep in Step 12	1-5 min
14-16. Stopping & preserving	10% ethanol, 1% acetic acid, 5% glycerol	3 × 200 mL	3 x 10 min

Drying: Air-dry the gel down on the film support, then roll the polyester cover film (supplied with the gel) onto the surface.

DIGE and other Fluorescence Staining

Fluorescence visualization methods can only be performed with gels on non-fluorescent backing film (see www.gelcompany.com).

Gelcompany, Paul Ehrlich Str. 17, 72076 Tuebingen, Germany.
Tel: +49 (7071) 25703-0, Fax: +49 (7071) 25703-69, e-mail: sales@gelcompany.com

Isoelectric Focusing in FocusGels and Staining Methods

FocusGels have been polymerized to produce a matrix optimal for isoelectric focusing. FocusGels are thin gels (0.65 mm) with a gel concentration (T) of 5% and cross-linking (C) of 3%.
Catalysts as well as other toxic and non-polymerized compounds are washed from the matrix resulting in gels that are non-toxic. The gels contain a carrier ampholyte cocktail designed to achieve an optimal pH gradient. No electrode solutions and electrode strips are required, and the electrodes are placed directly on the gel surface. FocusGels 24S contain 24 pre-polymerized sample wells for easy application of protein samples (up to 25µL). The other FocusGel types do not contain sample well and samples are applied with using sample applicators. For reproducible runs the commonly used "Running Conditions" are converted into actively driven "Voltage Ramping Methods" where the power supply follows a given volt /time graph.

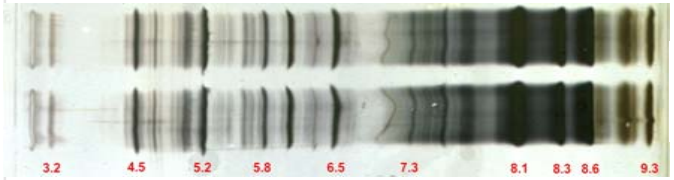

Fig.1a: FocusGel 3-10 IEF of pI Markers, silver staining

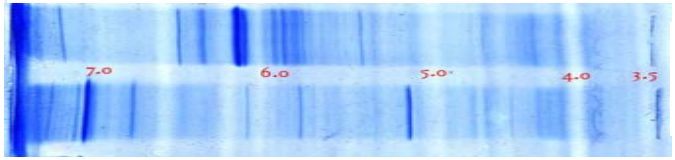

Fig.1b: FocusGel 3-7. IEF of pI Markers, Coomassie-staining.

Materials Required

Consumables and Chemicals

FocusGel 3-10 (gelcompany 1006-01), FocusGel 3-10 24S (**gel**company; cat # 1006-18), FocusGel 3-7 (**gel**company; cat # 1006-02), FocusGel 4-5 24S (**gel**company; cat # 1006-07), FocusGel 4-6 24S (**gel**company; cat # 1006-09). Ammoniacal Silver Staining Kit (**gel**company; cat # 1004-05) contains the $AgNO_3$ and citric acid. Sample Application Pieces (GE-Healthcare), Sample Application Nets (gelcompany; cat # 1003-21) kerosene (Serva 26940), trichloroacetic acid, methanol, ethanol, acetic acid, glutaraldehyde, formaldehyde, sodium acetate, ammonia solution, Coomassie G-250 (Merck, p.A.).

Equipment

FlatTop (**gel**company; cat #1100-01) or Multiphor II (GE-Healthcare), MultiTemp III (GE-Healthcare), EPS 3501 XL power supply (GE-Healthcare).

Sample Preparation

If gels are to be stained using Coomassie Brilliant Blue Staining; to approximately **1 µg** of protein add **1 mL** distilled water.

If the gels are to be stained using Silver Staining; to approximately **0.1 µg** of protein add **5 mL** of distilled water.

We recommend the use of IEF markers pH 4.6—10 from Merck.

Add an aliquot of the sample (25 µL) to each gel slot in the gel or in the sample applicator net.

Loading the Gel

Open the bag with scissors and carefully remove the gels then the protective cover-film from the gel surface. Keep the cover film as it will serve as a protective sheet later. The gel is immediately ready for use.

Gel-application: Spread 1.2 mL of kerosene onto the cooling plate of the focusing chamber to ensure good cooling contact, (Fig. 2). Place the gel (gel side-up) on the center of the cooling plate (Fig. 3); avoid trapping any air bubbles. For the **gel-**company FlatTop match the edges of the backing with the lines 4 and 16 (Fig. 2). For the GE-Healthcare Multiphor II the gel edges should match with the lines 3 and 15

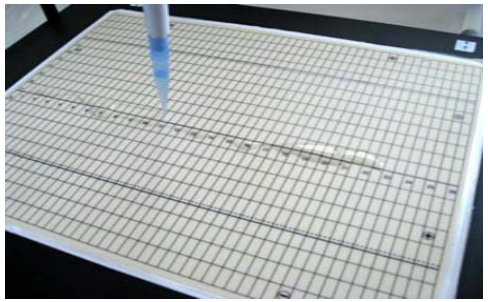

Fig. 2: Applying kerosene onto the cooling plate

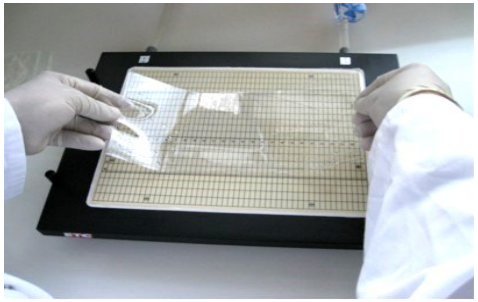

Fig. 3: Placing the gel onto the cooling plate

Sample application

FocusGels are available both with (*FocusGel 24S*) and without sample wells.

Sample application on gels without slots can be performed using various types of commercially available sample applicators or silicone rubber masks. These allow different volumes to be applied.

For IEF it is important to apply the sample at the correct position within the pH gradient. For most samples the optimal application position is in the acidic region, close to the anode. For a new sample type the optimal position can be determined by a step trial test, (Fig. 4). It is important that the sample pieces have a distance of 1 cm from each other.

Sensitive Ammoniacal Silver Staining

This silver staining protocol (adapted from reference 1) provides enhanced detection of basic proteins and can be performed with a Hoefer Processor Plus Autostainer (GE-Healthcare; figure 8) .

Fig. 8: Hoefer Processor Plus.

Program and Ports

All solutions should be at room temperature.

1	Fixing :	*40g TCA, make to 200mL with H_2O_{dist}.*
2	Rinsing I:	*100mL MeOH + 20mL HAc, make to 200mL with H_2O_{dist}*
3	Rinsing II:	*10mL MeOH + 14mL HAc, make to 200mL withH_2O_{dist}*
4	Incubating :	*20mL glutaraldehyde (25%), make to 200mL with H_2O_{dist} then add 3.5g NaAc*
5	Water :	*1000mL H_2O_{dist}.*
9	Water :	*400mL H_2O_{dist}.*
6	Silvering :	*Solution 1: Dissolve 250mg $AgNO_3$ in 1mL $H_20_{dist.}$* *Solution 2: 40mL H_20_{dist} + 4 mL NaOH (1M) + 1.5mL NH_3 (25%)* *Drop Solution 1 into 2 while stirring, make to 200mL with H_2O_{dist}, (or see the silver-staining Kit manual*
7	Developing:	*5mg citric acid + 100 µL formaldehyde, make to 200mL with H_2O_{dist}, or see the silver-staining kit manual*
8	Stopping :	*60mL EtOH + 6 mL HAc + 30 mL glycerol make to 600mL with H_2O_{dist}.*

Abbreviations:

HAc (Acetic acid), NaAc (Sodium Acetate), MeOH (Methanol), EtOH (Ethanol), TCA (Trichloroacetic acid), *H_2O_{dist}. (distilled Water)*

References:

[1] Rabilloud T: A comparison between low background silver diamine and silber nitrate protein stains. Electrophoresis (1992) 13, 429 - 439

Standard Silver Staining

The silver staining described below is commonly used, offers good sensitivity and can be performed in an Autostainer (Fig. 8, GE-Healthcare) at ambient temperature

Table. 3: Staining solutions and program

Step	Solution	Volume	Time
1 Fixing	20% trichloroacetic acid (w/v)	200mL	20min
2+3 Rinsing	20% ethanol/ 8% acetic acid (v/v)	2×200mL	2x10 min
4 Incubation	0.1% sodium thiosulphate; 0.4 mol/L sodium acetate/acetic acid pH 6.5; 0.125 % glutaraldehyde.	200mL	15min
5 Rinsing	20% ethanol / 8% acetic acid	200mL	10 min
6-8 Washing	H_2O_{dist} (place gel into a glass tray, with the gel surface side up)	3×200mL	3×10 min
9 Silvering	0.1% $AgNO_3$ /0.004% formaldehyde (w/v) 20µL formaldehyde (37% w/v) per 200 mL	200mL	30min
10 Developing	2.5% Na_2CO_3 / 0.004% formaldehyde 40µL formaldehyde (37% w/v) per 400 mL Observe until suitably developed	1×200mL 1 x 200mL	0.5min 2 - 3min
11 Stopping/ Preserving	10% HAc, 5% glycerol	200mL	20min

Drying: Air-dry the gel on the film, then roll on the polyester cover sheet (supplied with the gel) onto the surface.

Total staining time*:* Approximately 2.5 hours

Ports

1. TCA: 40g TCA, make up to 200mL with H_2O_{dist}.
2. Rinsing: 40mL EtOH + 16mL HAc, make to 200mL with H_2O_{dist}
4. Incubating: 11g NaAc + 200 mg thiosulphate + 1mL Glutardialdehyde (25%) make to 200 mL with H_2O_{dist}. adjust to pH 6.5 with HAc
5. Water: 600mL H_2O_{dist}.
6. Silvering: 200mg $AgNO_3$ make to 200mL with H_2O_{dist}, then add 20 µL formaldehyde (37%)
7. Developing: 10g Na_2CO_3 make to 400 with H_2O_{dist}, then add 40µL Formaldehyde (37%)
8. Stopping: 20mL HAc + 20 mL glycerol make to 200mL with H_2O_{dist}.

FocusGel 24S: For some sample types, e.g. serum and CSF the position of the pre-formed wells is optimized for anodal application in a pH gradient 6-11 (Fig. 5). This well position might also be suitable for other sample types. The gels can also be turned around for cathodal application. Note: all wells must be filled with liquid; i.e. with sample or water.

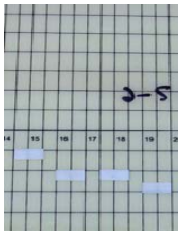

Fig. 4: Step trial test for optimization of sample application point.

Fig. 5: For serum and CSF the wells are orientated towards the anode

Isoelectric Focusing

Clean the platinum wires with moist tissue paper before (and after) IEF runs. Move the platinum electrodes to the correct positions over the edges of the gel. Lower the electrode holder onto the gel surface. The platinum wires should rest directly on the gel edges and not on the support film. Apply the samples, close the safety lid, and start focusing (table 1 or table 2).

Note: There is no requirement to use electrode strips or buffers. Those must not be used.

Temperature: Isoelectric focusing has to be performed at a defined constant temperature because the pH gradient and the isoelectric points depend on the temperature. Switch on the thermostatic circulator, set to 7°C. Recommended temperature range: 5°C - 10°C.

Settings for Power Supply

During isoelectric focusing the electric resistance of the gel changes considerably and current or power values determine the voltage-values. Small variations of the gel conductivity result in high variations of volt-values (Fig. 6.)

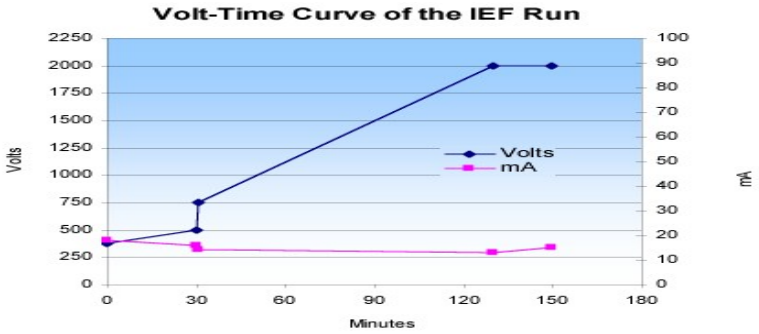

Fig. 6: Voltage / time curve for IEF

The most commonly applied method is to limit the voltage *via* the mA and the Watts achieved in the gel. With non-programmable (manual) power supplies use the settings in table 1. With programmable power supplies use the settings in table 2.

Pre-focusing: For some sample types it is beneficial to start with pre-focusing of the pH gradient before sample application. In this case add a Step 0.1 with the following settings:

1000 V 25 mA 10 W 20 min

Settings for Manual Power Supplies

With manual power supplies the electric conditions are limited by mA and Watts (Table. 1)

Table 1: Manual power supplies

Table 1	SET	*Start Value*[1]	SET	SET	Time	Process
Step 1	500 V	*250 V*	20 mA	10 W	30 min	sample entrance
Step 2	1700 V	*600 V*	20 mA	25 W	90 min	focusing
Step 3	2000 V	*1850 V*	20 mA	30 W	30 min	band sharpening

Note 1: At the start of each step the starting volt-value (in italics) should be adjusted *via* the limiting mA value.
Note 2: For a half gel apply the same voltage and half of the mA and W
Note 3: Remove sample applicators before staining.

Settings Programmable Power Supplies

With programmable power supplies the electric conditions are controlled by the programmed voltage curve (see Tab. 2). This is the better method but not all power supplies are programmable.

Table 2	SET	*Start Value*	SET	SET	Time	Process
Step 1	375 V —> 500 V	*(35 mA)*	35 mA	15 W	30 min	sample entrance
Step 2	750 V —> 2000 V	*(30 mA)*	30 mA	35 W	100 min	focusing
Step 3	2000 V	*(20 mA)*	20 mA	35 W	20 min	band sharpening

Table 2 Programmable power supplies

Note 1: Set “Ramping...on” or “Volt Level...Changing”
Note 2: mA and Watt should not be limiting
Note 3: Remove sample applicators before staining
Note 4: Half gels can run with this method without changing the settings

Staining

Hot Coomassie Blue G-250

Stock solutions:

TCA: 20% TCA: Dilute 40 mL of 100% TCA (w/v) to 200 mL
A: 0.2 % $CuSO_4$ / 20 % acetic acid (2 g of $CuSO_4$ in 1L of 20% HAc).
B: 0.04 % Coomassie G-250 in 60 % methanol (0.4 g Coomassie G250 in 1L, 60 % methanol)
C: 50% (v/v) methanol

Staining protocol:

Fix: 15min in 200 mL 20% TCA (at room temperature)
Wash: 2 × 1 min in 200 mL wash solution (*mix equal amounts of A and C*)
Stain: 45 min in 200 mL of staining solution (*mix equal amounts of A and B*). Heat (50°C) solution while stirring (Fig. 7). Suitable steel trays with lids and grids are available for single gels (**gel**company cat # 1003-25) or multiple gels (**gel**company cat# PGS-001-TI).

Wash: 2 × 5 min in 200mL wash solution (*mix equal amounts of A and C*)
De-stain: 2-3 × 15 min in wash solution (in a tray), A and C
Impregnate: 5 min in 200 mL 5 % (v/v) glycerol
Dry: air-dry (leave at room temperature)

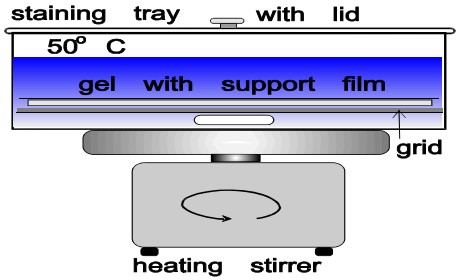

Fig. 7: Hot Coomassie staining