One Dimensional and Double Native Electrophoresis of Peptides and Proteins
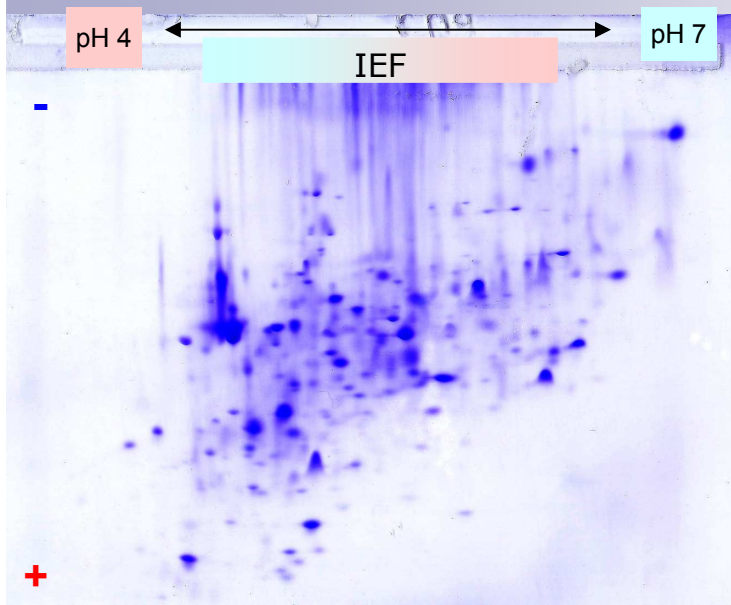
Fig.1: *Double Native separation of acidic and neutral peptides and proteins from E.coli: 1.*
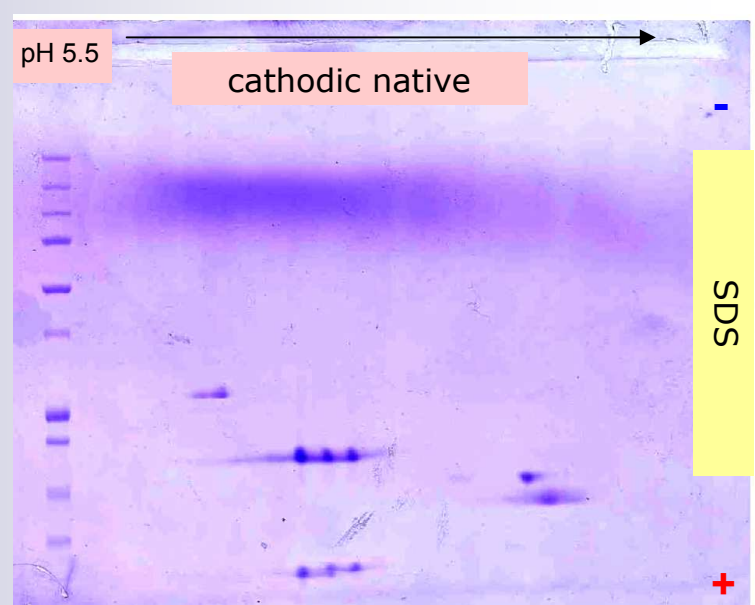

Fig.2: *Double Dimension separation of basic protein marker mix. 1. dim=cathodic native, 2. dim=SDS*

General: *Peptide separations and visualization procedures should be performed in different ways compared to the methods used for proteins.*
SDS is a molecule that aggregates to only longer sequences of lipophilic structures (more than 10-12 amino acids!). Smaller peptides can not offer this condition to this molecule. SDS itself migrates mycellarely in the peptides region (1-5 kD), after the leading ion. No separation will occur there! Because it also disturbs the visualization processes it hinders the recognition of the peptides themselves.
However, without SDS the petides have different charge-properties due to their different isoelectric points. This leads to the decision that the acidic and neutral peptides should be treated different:
1. acidic/neutral peptides = anodal native / and basic peptides = cathodal native electrophoresis.
2. If basic proteins or peptides refuse to show good separation in IEF, it is recommended to use these 2 native electrophoresis methods as first dimension.
3. Peptides show a high tendency to resolve and though disappear in washing and staining solutions. Staining protocols with many steps or only with one step including alcohol will not be able to keep the peptides inside of the gel. It is recommended to use only the Coomassie staining protocols stated here.

The different chapters:

1. **Anodal Native Electrophoresis** (1 dimensional and Double Native)
2. **Cathodal Native Electrophoresis** (1-dimensional, or Double Dimension)
3. **Native electrophoresis as 1st dimension** (Double Dimensional Electrophoresis)
4. **SDS-Electrophoresis as 2nd dimension** (Classical 2D Electrophoresis with IEF on IPG-Strips as 1st dimension)

Equipment:
flatbed professional: horizontal chamber (edc-prof-2836) or flatbed modular: modular horizontal chamber (edc-mod-4856), Power Supply minimum 1000 V, 150 mA, 100 W (IEF needs more volts); thermostatic circulator (lab supply)
Steel Staining Tray: (stainless steel tray with elevated stainless steel grid) (edc-wm-n1)
Optional: Multi 6 Tray: for staining 6 gels simultanous   (edc-wm-m6)
Accessories:
DryPool Combi   (*edc-me-d*)
Tray for rehydration of dry gels (small size) and soaking electrode strips
DryGels:
4 DryGels Elpho 52S or 24S   (*edc-4113 or edc-4112*)
Buffer Kits:
Protein Buffer Kit Cathodic   (*edc-5010*)
Protein Buffer Kit Anodic   (*edc-5003*)
Protein Buffer Kit SDS   (*edc-5002*)
DryGel Kits:
2D-DryGel Kit Small, 4 gels, buffers and strips   (*edc-3002*)

Additionally Required: SDS-standard, Urea, Thiourea, Dithiothreitol (DTT), Iodoacetamide (IAA), Kerosene

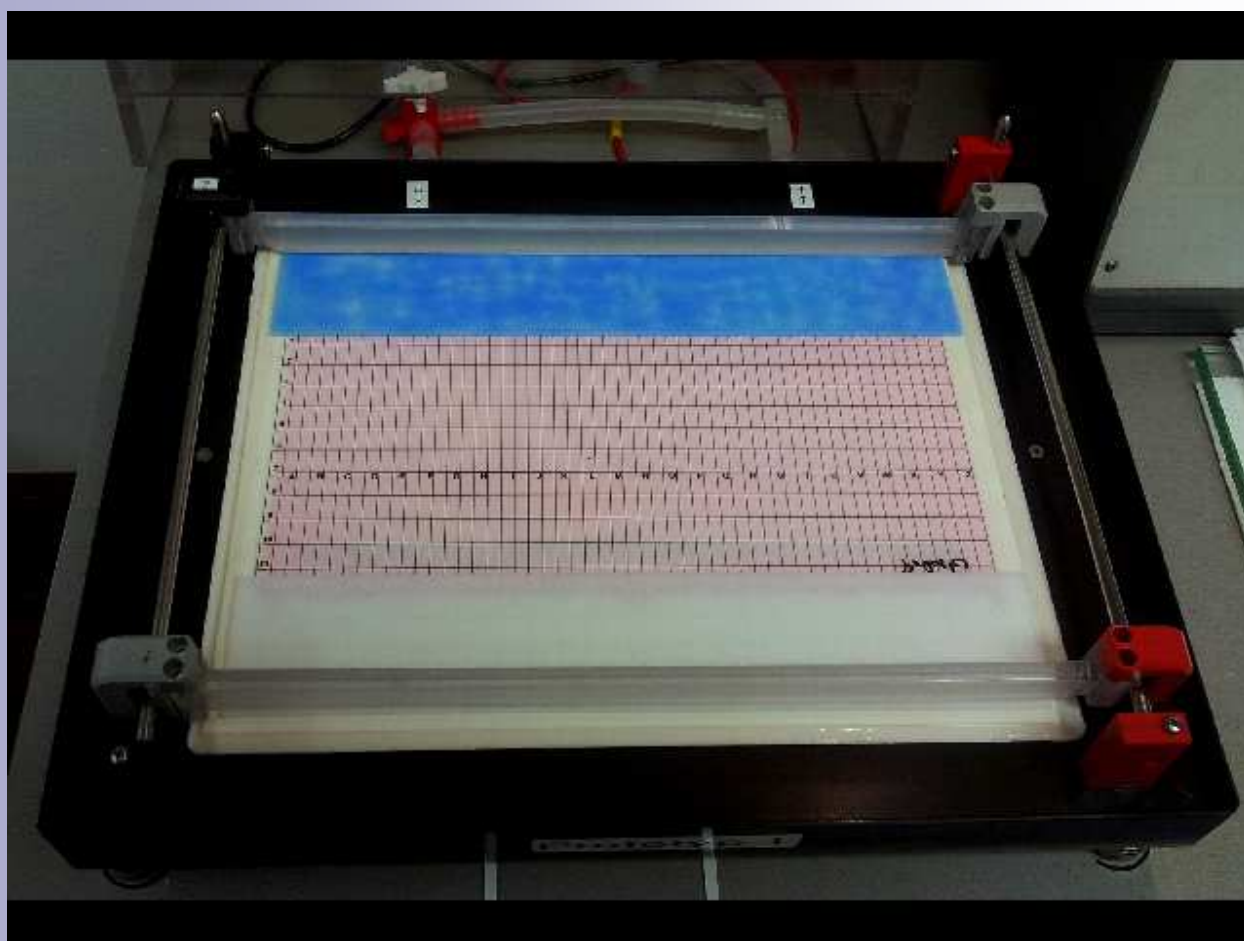**Fig.3:** *flatbed professional running a cathodic electrophoresis*

1. Anodal Native Electrophoresis

(one dimensional and Double Native)

<u>Strategy for 2-dimensional electrophoresis of acidic peptides:</u>

Because the IPG-strips give nice results between pH 4-7 also for peptides, this method can be used as first dimension.

Rehydration of the dry gel (1-dim or 2-dim)

DryGels normally can be used for all techniques.

Lay the DryPool combi onto a horizontal table; pipette the rehydration solution into the chamber (fig. 8),

for a complete DryGel:50 ml.

Lay the edge of the DryGel - with the gel surface facing down - into the rehydration buffer (fig. 5) and slowly lower it, avoiding air bubbles. Lift the film at the edges up to the middle, using foreceps and lower them again without catching air bubbles, in order to achieve an even distribution of the liquid (fig. 6 A). Repeat this procedure during the first 15 min.

Very even rehydration is also obtained when performing it on a shaker at a slow rotation rate (fig. 6 B).

2 hours later the DryGel has rehydrated completely and can be removed from the CleanPool. Remove excess buffer, dry the sample wells and wipe exceed buffer volume off the gel surface with the edge of a drying cardboard (fig. 7) until you can hear a "squeaking".

Important, the gel surface must be total dry!

The rehydrated gels are ready for run or can be placed back in their bags, close them with a Scotch Tape and put them to a fridge (+4°C).
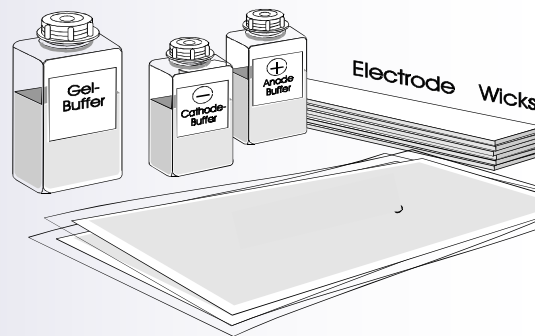
Maximal storage time: 2 weeks.

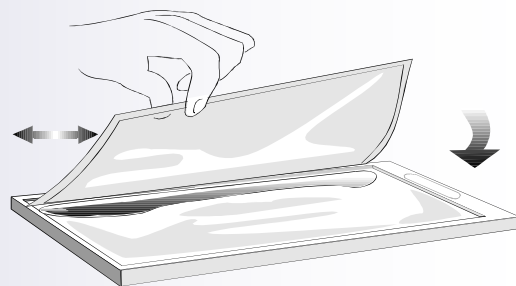**Fig.4:** *The Anodic Native Buffer Kit*

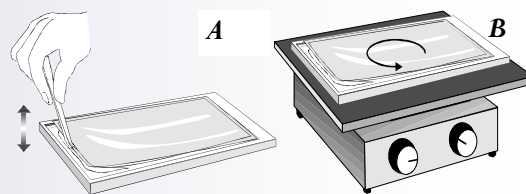**Fig.5:** *Placing the dry gel into the DryPool Combi for rehydration*

Fig.6: *A Lifting the edges for an even distribution of the liquid. B Rehydration on a rocking platform*
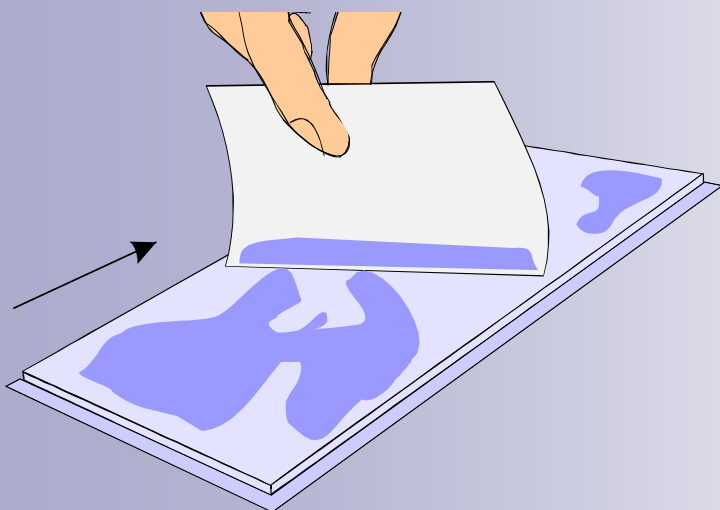
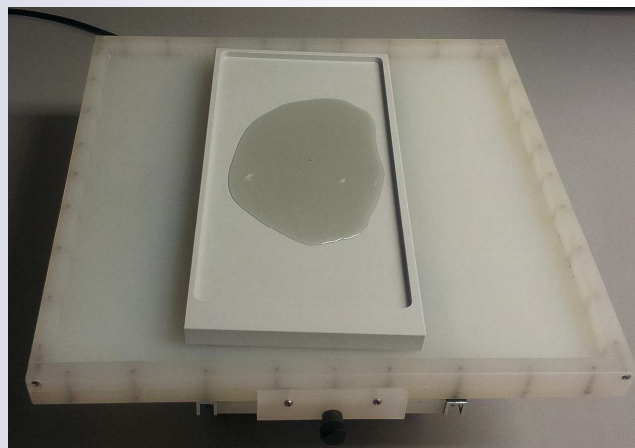**Fig.7:** *Removing the excess buffer from the gel surface using the edges of a drying cardboard*

Fig.8: *Pipet 50 ml Rehydration Buffer*

Sample preparation
Sample and Equilibration buffer: Only for Blue Native electrophoresis:
Add 650 µl 0.1 % (in ethanol) Coomassie G250 (0.0026 %).
Sample treatment, 1-dimensional samples: Dilute the samples with the sample
buffer at least 1 + 1. Dilute as much as possible, to reach the µg/µl region, this gives
best results when stained with Coomassie. To control the samples concentrations: Take
GE´s HMW marker and pipette 400 µl sample buffer to the vial and run at least one lane
per gel. This is the right concentration for Coomassie-stained electrophoresis. Apply 15µl
each lane, don´t leave sample slots unfilled.
E.coli sample (1-dim. or 2-dim): page 11. 2-dimensional marker-slot takes only 5 µl.
IPG-strip treatment, 2-dimensional sample:
The 11 cm IPG-strips were equilibrated in an IPG-Strip Pool, see figure 9.
Shake slowly during the 2 steps. Protocol see table 1.

Step		Sample Buffer	Time
1	per strip	add 3 ml	15 min
2	per strip	add 3 ml	15 min

Table 1: *Equilibration steps of IPG-strips for native anodal electrophoresis 2nd.dimension*
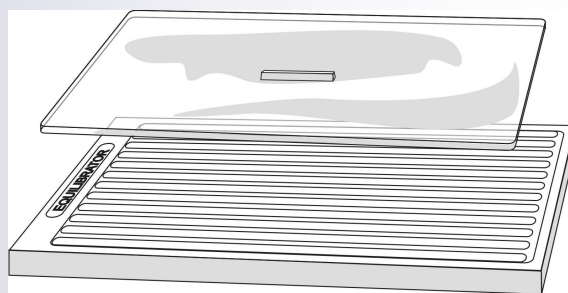

Fig.9: *The IPG-Strip Pool*

Application of the gel and the electrode strips
Switch on the thermostatic circulator, adjusted to 10 °C. Cooling switches set to
„Bypass". Apply a very thin layer of kerosene (ca. 2.5 ml) onto the cooling plate with a
pipette, in order to ensure good cooling contact. Place the gel (surface up) onto the
center of the cooling plate: The side containing the wells must be orientated towards the
cathode
For Blue Native electrophoresis add 5 ml Coomassie G250 (0.1% , in Ethanol) to the
25 ml cathode buffer.
Lay two of the electrode wicks into the compartments of the DryPool Combi. Apply 25 ml
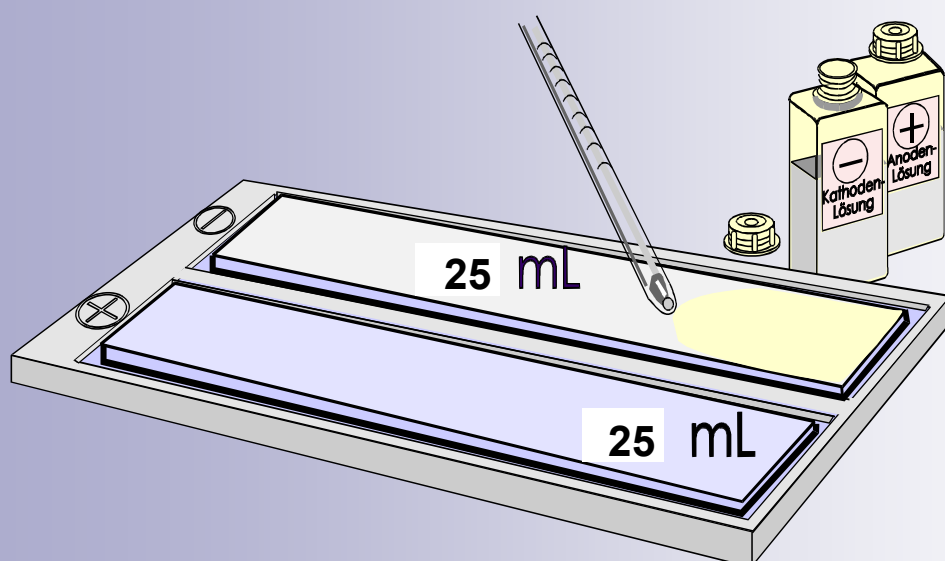of the respective electrode buffer to each wick using a pipette (fig. 10).

Fig. 10: *Soaking the electrode strips with the anode and cathode buffer in the DryPool Combi.*

Place the cathode strip onto the cathodal edge of the gel, edge of the electrode strip overlapping the gel-edge with 2-3 mm, see figure 2. The edge of the strip should lie as close as possible to the sample wells. Otherwise the smaller fragments exhibit less band sharpness. Place the anode strip over the anodal edge overlapping the gel-edge with 2-3 mm, see figure 12. Smooth out air bubbles by sliding bent tip forceps along the edges of the wicks laying in contact with the gel.
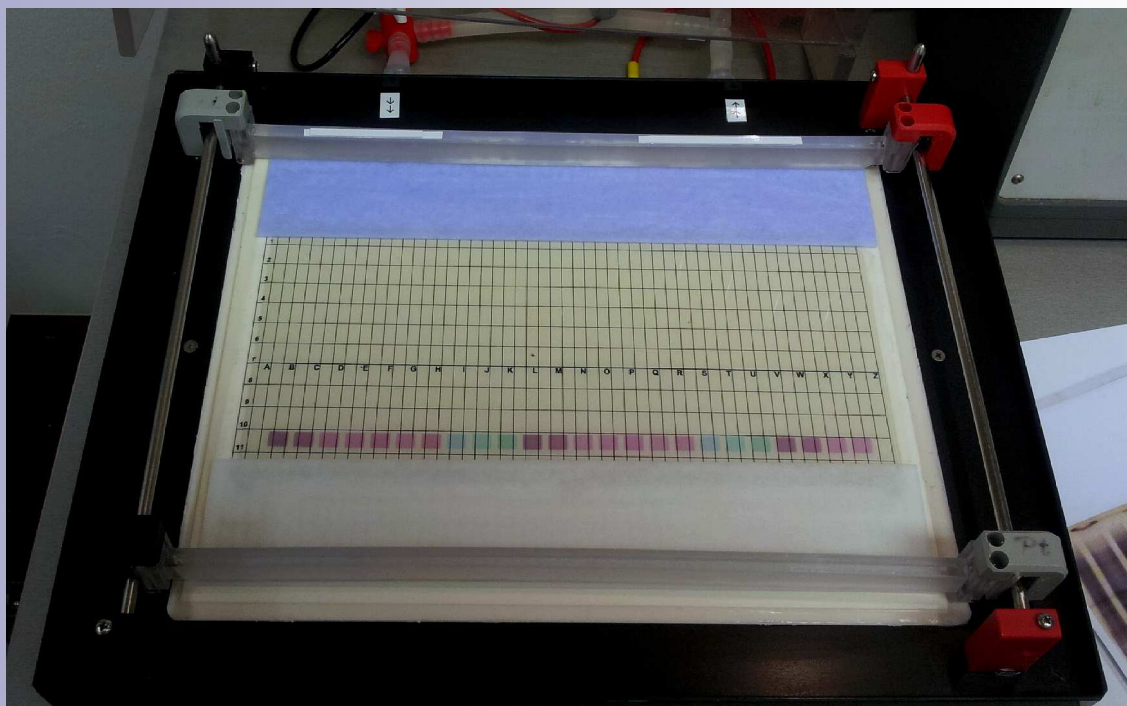

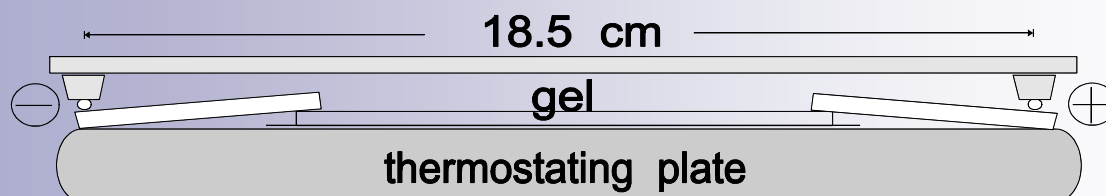**Fig.11:** *A*rrangement of gel (24S) and electrodes on the cooling plate (flatbed professional)

Fig.12: Overlapping of gel and electrodes

1-dimensional sample application
Apply 15 µl (24S) or 5µl (52S) of each sample to the sample wells using a micropipette (or use appropriate multipipette). Clean platinum electrode wires before (and after) each electrophoresis run with a wet tissue paper. Move electrodes so that they will rest on the outer edges of the electrode wicks (fig.11). (Multiphor: Connect the cables of the electrodes to the basic apparatus). Finally lower and close the safety lid.

Application of the IPG-strips, 2-dimensional sample application
Remove the 11 cm IPG strips from the equilibration tubes.
Do not blot them on filter paper. The film support of the strips should be trimmed on both sides to protrude only 2 mm.
Place each strip gel-side down (!) into the slots of the SDSGel using two forceps and push them carefully towards the anode to ensure good contact to the anodal edge of the slot, see figure 13. Slide along the backside of the strips with the forceps to ensure good contact to the gel. Anodal end (+) of each strip should be orientated to the right hand side. Figure 14 shows the final assembling.
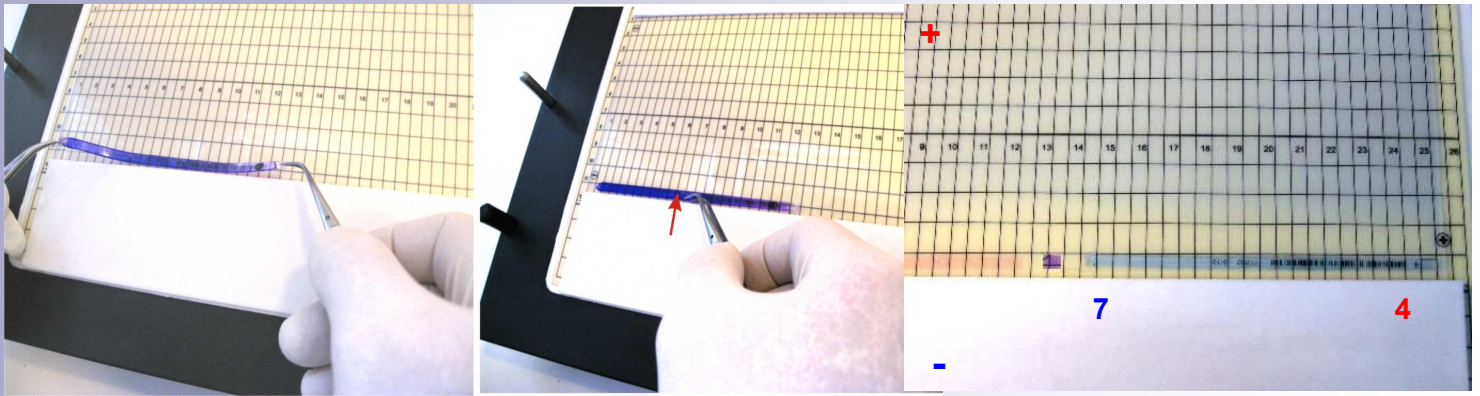
Fig. 13: *Placing the IPG strip into the slot, and pushing it carefully towards the anodal edge of the slot.*

Fig. 14: *Arrangement of the DryGel 2D, cathodal buffer strip, the IPG-strips and the native marker slot.*

Electrophoretic Run (one dimensional and Double Native)
Running conditions (10°C) whole gel:
Set the cooling switches to „ON"

Total running time: 2 hours 30 min

Bio-Rad Power Pac HV: type in this parameter as constant —->
(Select „BASIC" mode) type in the other settings as limits —->

	n V
	n mA

1 Gel:	Set V	*Start Value*	SET mA	Set W	Time	Comment
phase 1 (sample entrance)	200 V	(190 V)	20 mA	10 W	10 min	15%-gel: time=20 min
phase 2 (main electrophoresis 1)	375 V	(320 V)	30 mA	20 W	50 min	after 10 min of this phase: **remove the strips!**
phase 3 (main electrophoresis 2)	450 V	(450 V)	30 mA	20 W	30 min	for a 12.5% gel

Results and Interpretation Native Anodal and Double Native Anodal
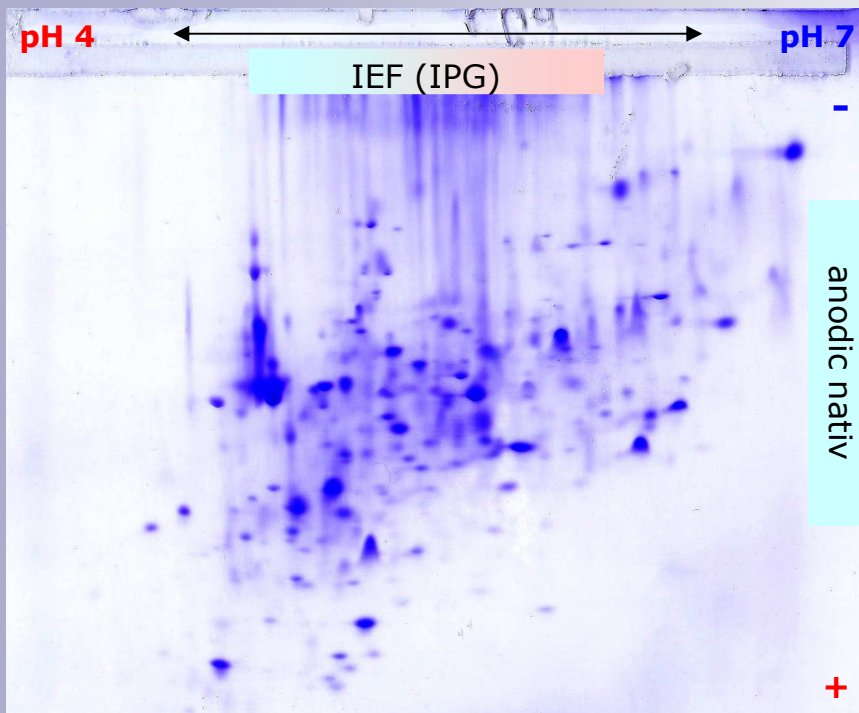A) Results

Fig.**15a**: Double Native electrophoresis of E.coli. 1st dim=11 cm IPG pH 4-7. 2nd dim= native anodal electroph.
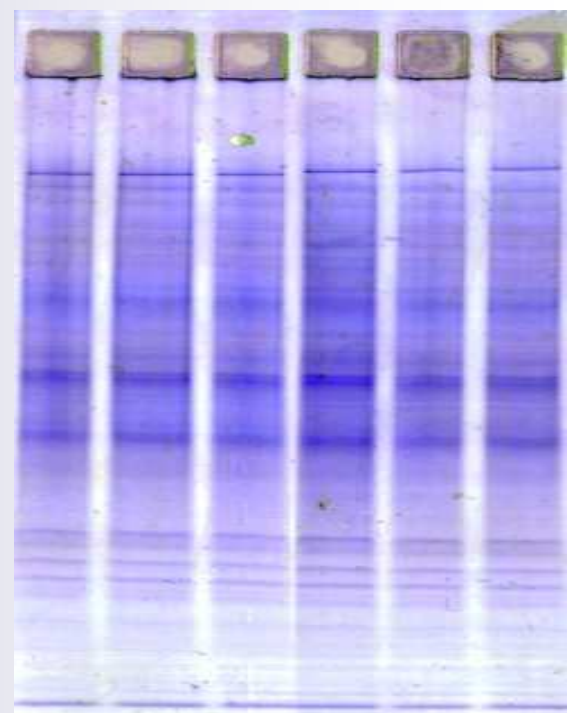
Fig.16: *native anodal electrophoresis of E.coli. DryGel Elpho 24S*

B) Interpretation of Nativ Electrophoresis

pH 4

pH 7

+

🟥	10-15 kD
🟨	15-25 kD
🟩	25-40 kD
🟦	40-60 kD
🟦	60-100 kD

Please note: in native electrophoresis the proteins run not corresponding to their mol-weight. More important for the migration speed seems to be the charges.
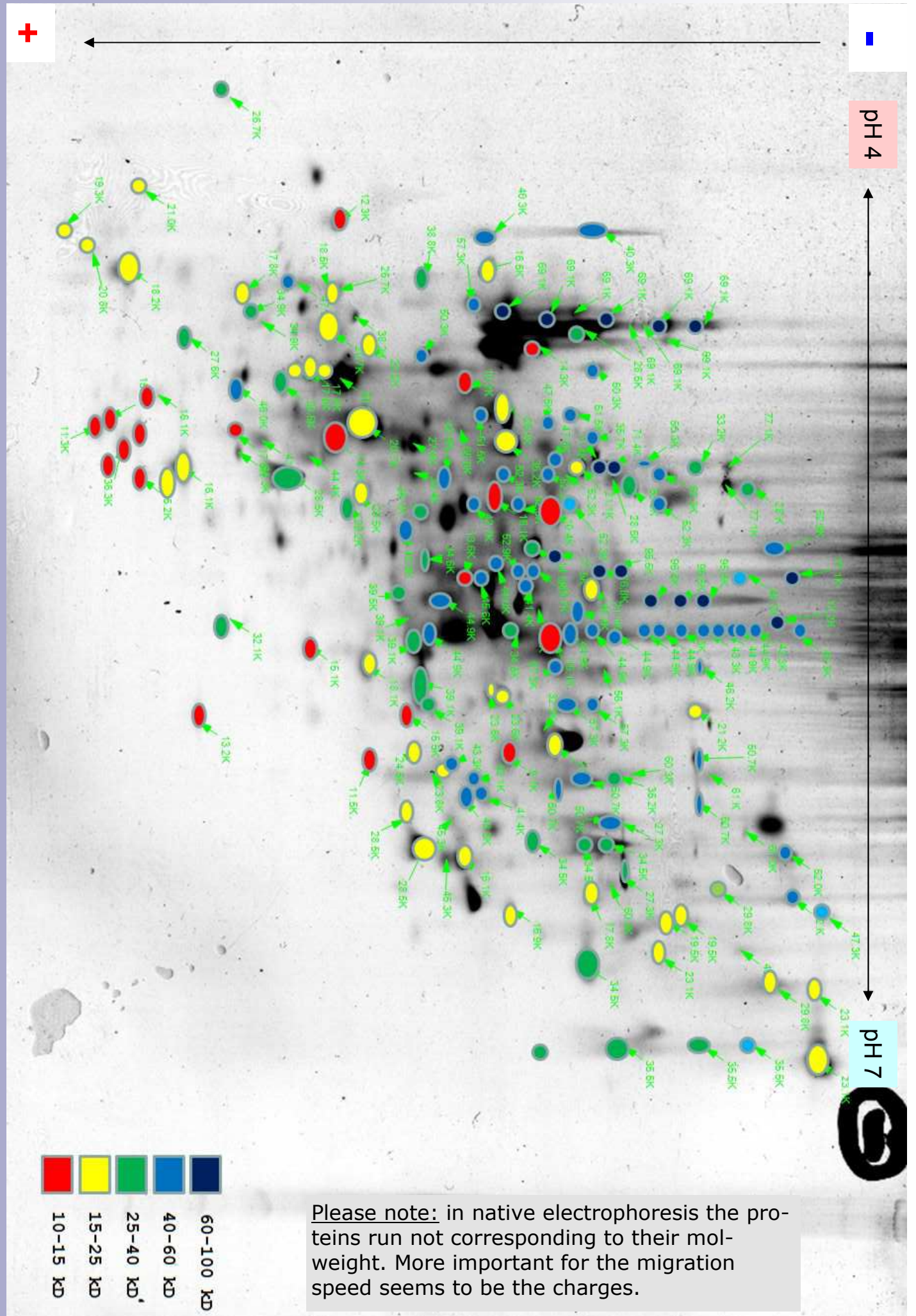
2. Cathodal Native Electrophoresis (one dimensional, or Double Dimension)
Strategy for two dimensional electrophoresis of basic peptides.
 If the IPG-strips will not provide nice results in the basic region: Use this method as first dimension and the SDS electrophoresis as the second.
Rehydration of the DryGels (one dim. or two dim.)
Lay the DryPool Combi onto a horizontal table, fill in 50 ml of the cathodic rehydration solution into the chamber, avoiding air bubbles.
for a complete gel: 50 ml
For the second dimension: Rehydrate the DryGel-2D in 50 ml 2D buffer (see spec.manual). See pictures 4 - 14 in part 1.)

If only one DryPool Combi is available, the 2D-gel should be rehydrated before, dried with the cardboards and put back in its bags.
Sample preparation
Sample buffer: 25 ml cathodic rehydration buffer and if needed + 3 M Urea (4.5 g) + 40 µl 1 % Basic Blue solution + 40 µl 1 % Methyl Green solution + 50 µl 1% Triton X100 (= 0.002%).
Sample treatment, one dimensional samples: Dilute the samples with the sample buffer at least 1 + 1. Dilute as much as possible, to reach the µg/µl region, this gives best results when stained with Coomassie. To control the samples concentrations: Take GE's IEF marker 3-10 and pipette 250 µl sample buffer to the vial and run at least one lane per gel. This is the optimal concentration for Coomassie-stained electrophoresis. Apply 15µl each lane, don´t leave sample slots unfilled.
Two dimensional marker-slot takes only 5 µl!
IPG-strip treatment, two dimensional sample:
The IPG-strips were equilibrated in the IPG-Strip Pool, see table 1 and figure 9.
Shake slowly during the 2 steps.
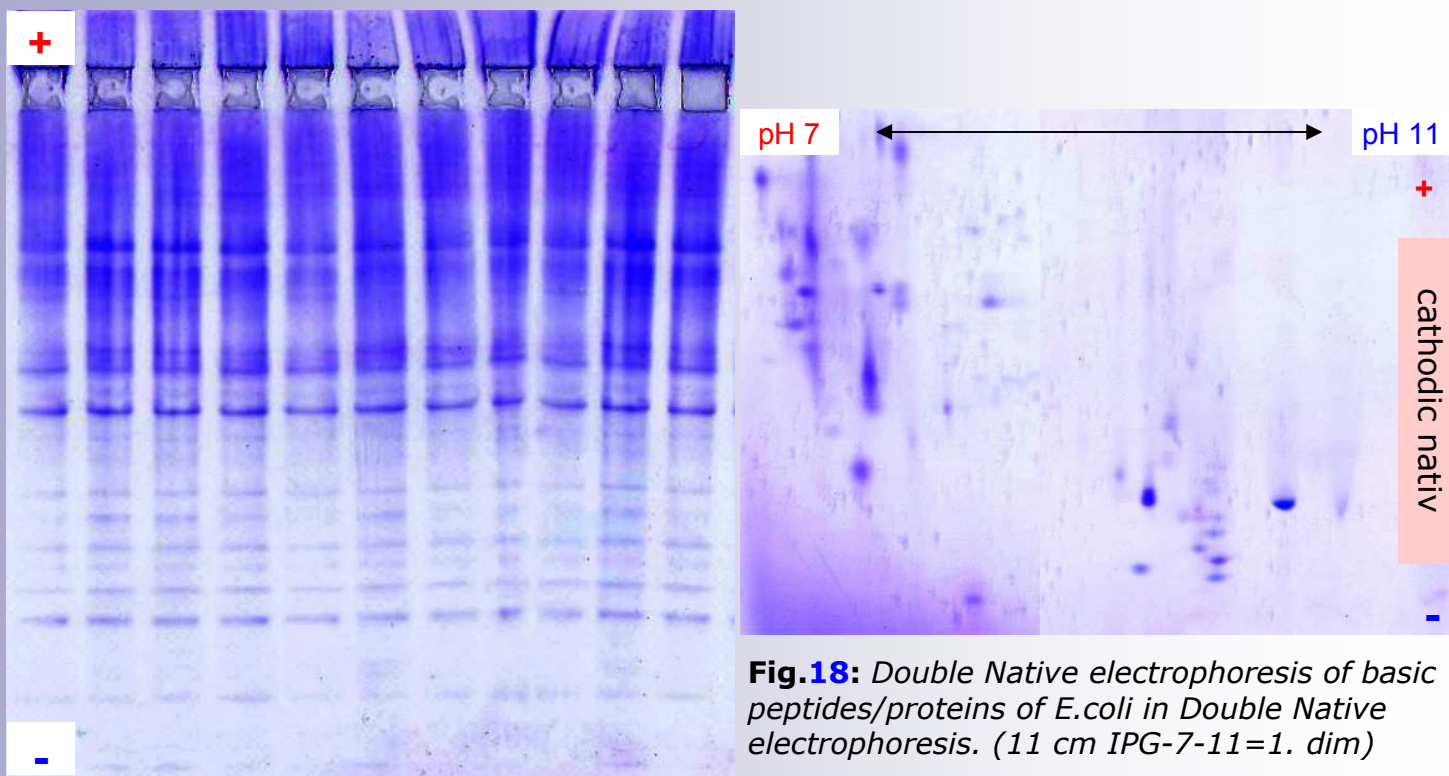E.coli sample (one dim. or two dim.): page 4

Fig.17: *Basic peptides of Collagen-hydrolysates run in native cathodal electrophoresis*

Fig.18: *Double Native electrophoresis of basic peptides/proteins of E.coli in Double Native electrophoresis. (11 cm IPG-7-11=1. dim)*

3. Native electrophoresis as first dimension: Double Dimensional Electrophoresis

If the IPG-strip gives no sufficient results, especially for peptides in the basic region e.g., then as the first dimension this native cathodal electrophoresis should be used, and then the SDS-electrophoresis as the second dimension.

## Cutting out the first dimension

After running and staining of the native cathodal electrophoresis lay the gel face down on a plastic film or a glas-plate. Mark the area that should be cutted out for transferring with an Edding. The size of this area should be 10 cm x 3 mm. See figure 19

cathodic native

Fig.19: *Cut-out of the native electrophoresis lane for transferring to the SDS-electrophoresis*.
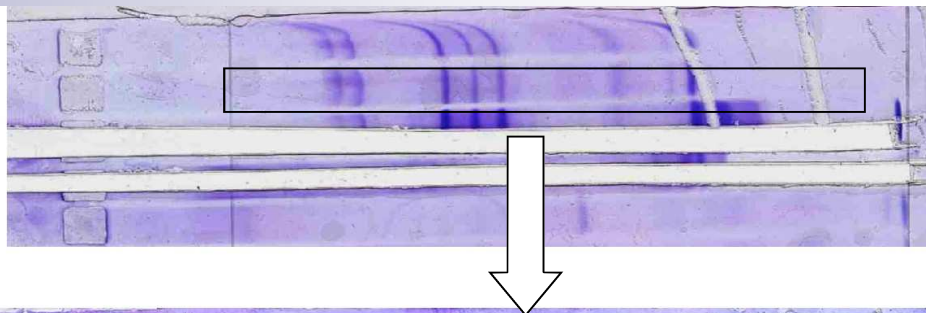
DryGel 2D
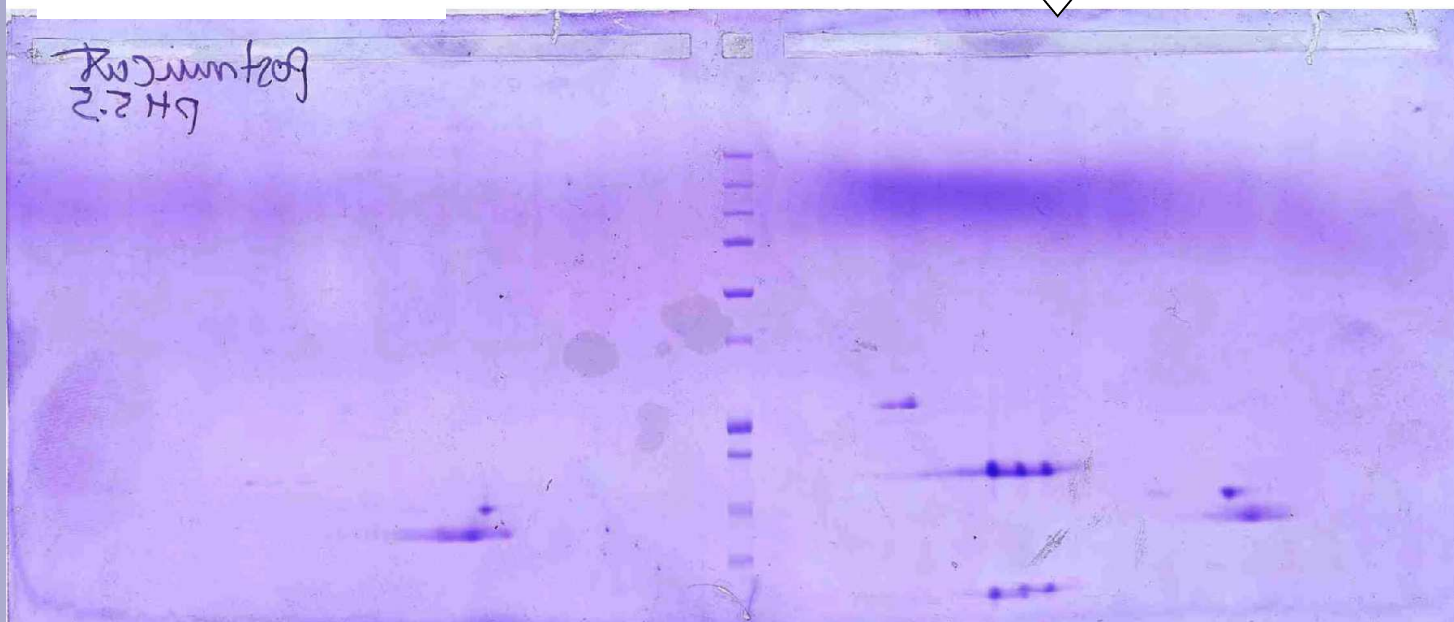
Equilibration of the native electrophoresis strip

The electrophoresis strips are equilibrated in the IPG-Strip Pool, figure 9.
Shake slowly during the 3 steps. Protocol see table 2.

Step		0.02% SDS	SDS Sample Buffer	Time
1	per strip	add 3 mL	—	10 min
2	per strip	—	add 3 ml (+0.05% DTT)	6 min
3	per strip	—	add 3 ml (+ 0.2% IAA)	6 min

Table 2:
Equilibration steps of native electrophoresis strips for the SDS-electrophoresis

4. SDS-Electrophoresis as second dimension:
Classical 2D Electrophoresis with IEF on IPG-Strips

Generally different to the described native methods is the SDS-sample preparation procedure: The SDS-electrophoresis is a denaturing method!

Starting the Electrophoresis

Standards: Dilute the SDS-standard at least 1 + 1 with the gel buffer.

IPG-Strip Equilibration: in 5 ml equilibration buffer per 7/11 cm long strip.

Important: Use EDC's IPG-strip equil. buffer, res. gel-rehydration buffer (see manual).

Step 1, 2: Weight in the reagents, add equilibration buffer, and dissolve them completely

Step 3: For reducing SDS-concentration the strips are shaked in the rehydration buffer.

Step	Urea	Thio-Urea	DTT	IAA	equilibr. Buffer	Time
1	1.5 g*	760 mg*	40 mg*	- - -	add 4.2 ml**	15 min
2	1.5 g*	760 mg*	- - -	105 mg*	add 4.2 ml**	13 min
3	1.5 g*	760 mg*	—–	—–	add 4.2 ml **gel-buffer**	2 min

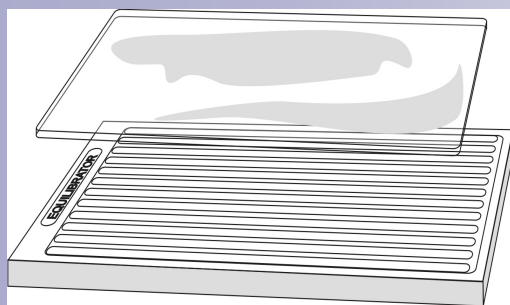* per strip
** makes 5 ml

Table 4

Fig. 20: *Equilibr. steps 1,.and 2*

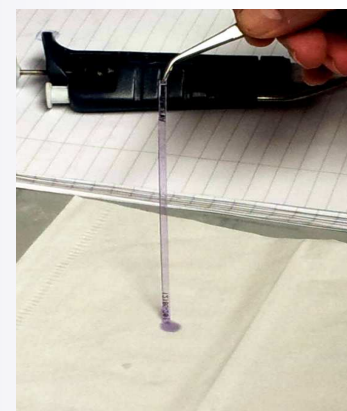Fig. 21: Step 3, shaking strip with gel buffer

Fig. 22: The last droplet is soaked by a paper tissue

See the DryGel-2D manuals

Temp=15°C

Total running time: 3 hours 50 min	*Bio-Rad Power Pac HV: type in this parameter as constant —-> (Select „BASIC" mode) type in the other settings as limits —->*	n mA / n V

1 Gel:	Set V	*Start Value*	SET mA	Set W	Time	Comment
phase 1 (sample entrance 1)	120 V	(~90 V)	8 mA	2 W	40 min	Discontinuity ~5 mm before the slot
phase 2 (sample entrance 2)	190 V	(~150 V)	12 mA	4 W	30 min	Discontinuity through the slot
phase 3 (sample concentration)	300 V	(~240 V)	18 mA	6 W	15 min	after this phase: **remove the strips!**
phase 4 (main electrophoresis)	650 V	(~350 V)	25 mA	16 W	145 min	Bromophenolred + SDS-zone in the Anode

Results and Interpretation

In all two examples the second dimension can be used as mol-weight determination. The marker lane should then be a SDS-standard.

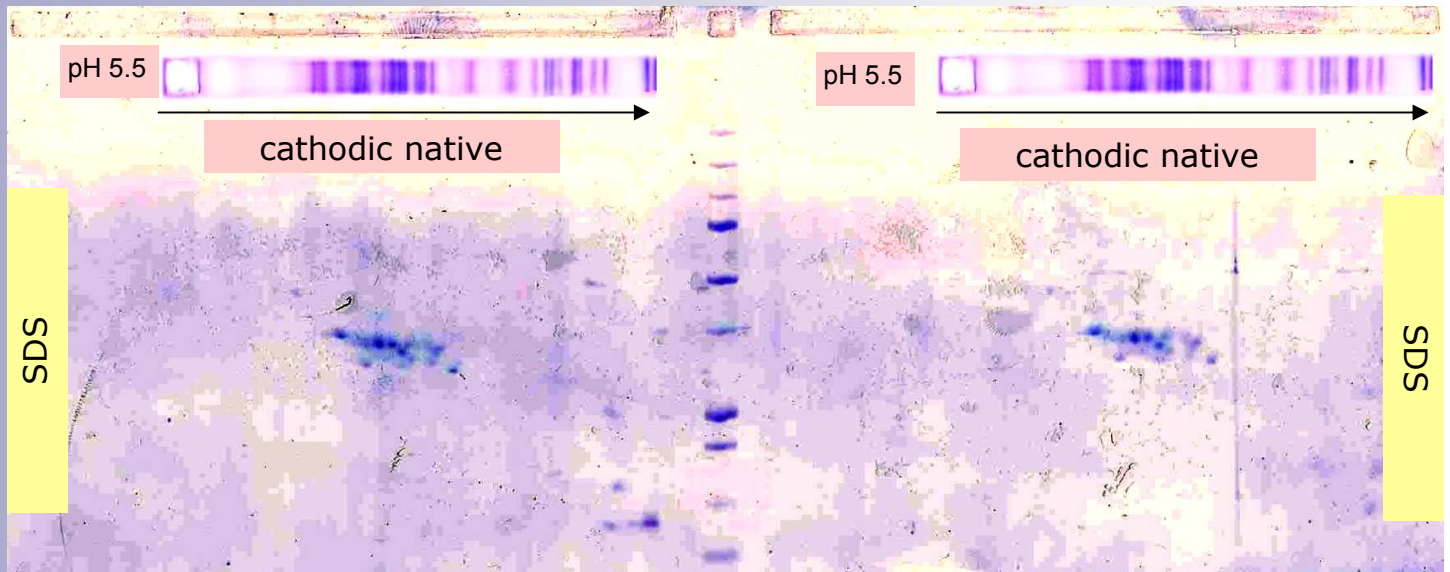**3.)** Native electrophoresis as first dimension

Fig. 23: *2 Barley varieties run in native cathodal electrophoresis as first dimension. SDS is the second dimension.*

4.) IPG-IEF as a first dimension
This is the classical two dimensional electrophoresis:
IEF in the first dimension: Measured as pH-values (the isoelectric points (pI) of the proteins), SDS in the second dimension: measured in kDa (kilo-Dalton)

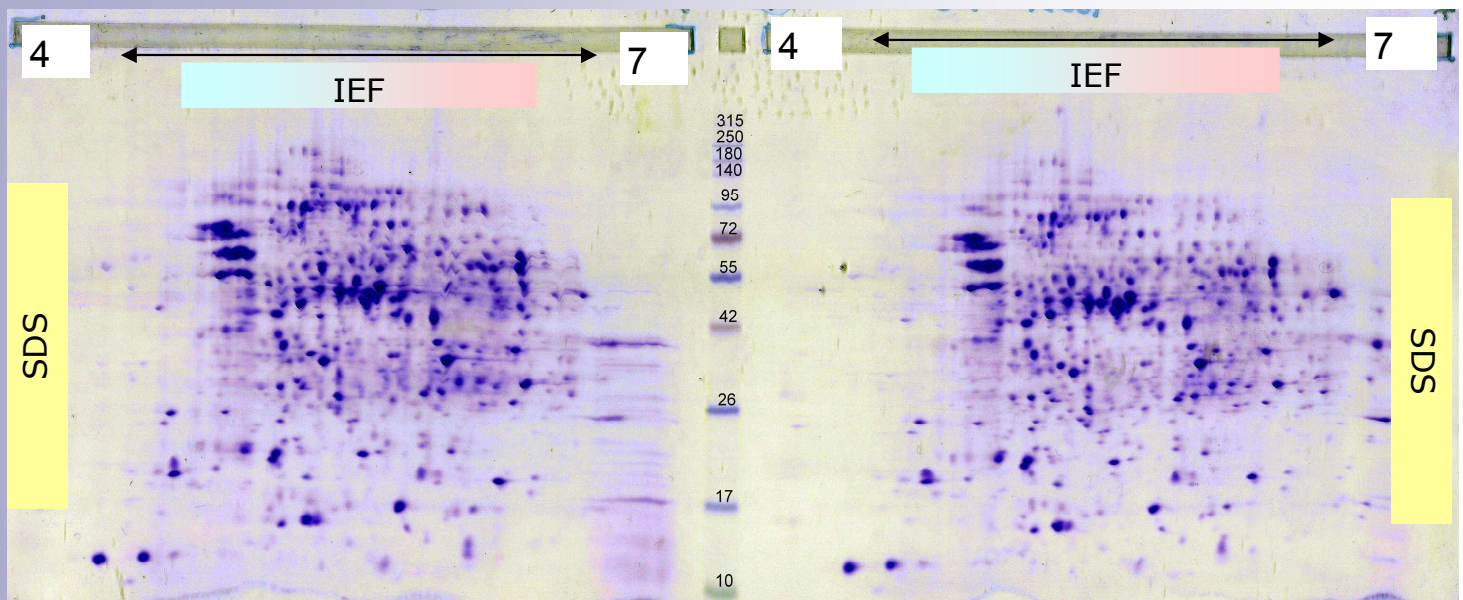
Fig.24: *Left and right side: E.coli extract, 250 µg; 2D-DryGel Kit 2x7/11: 2x11 cm IPG strips pH 4-7,*

<u>Please note:</u> Only when IPG-strips are used as first dimension the equilibration times are total 30 min. See table 1 and 4 for long equilibration times.
Equilibration using normal (ampholyte based) IEF or native electrophoresis is shorter. See table 2 for short equilibration time.

Example: Echerischia coli (E.coli, Aldrich) Sample Preparations

Native anodal one dimensional
10 mg E.coli + 1 ml anodic native sample buffer.
15 min ultra sonic treatment. Centrifuge 5 min 12000 rpm.
Pipet the supernatant in a new vial.
Sample volume: 15 µl

Native Cathodal Marker two dimensional
30 mg E.coli + 1 ml anodic nativ sample buffer.
15 min ultra sonic treatment. Centrifuge 5 min 12000 rpm.
Pipet the supernatant in a new vial.
Sample volume: 5 µl

Native Cathodal one dimensional
40 mg E.coli + 1 ml cathodic native sample buffer +120 mg/ml Urea (2M, freshly added). 15 min ultra sonic treatment. Centrifuge 5 min 12000 rpm.
Pipet the supernatant in a new vial.
Sample volume: 15 µl

Native Cathodal Marker two dimensional
55 mg E.coli + 1 ml cathodic native sample buffer +120 mg/ml Urea (2M, freshly added). 15 min ultra sonic treatment. Centrifuge 5 min 12000 rpm.
Pipet the supernatant in a new vial.
Sample volume: 5 µl

SDS-electrophoresis one dimensional
Extraction Buffer: 250 mM TRIS + 190 mM Glycine + 1 mM EDTA, gives pH of 9.2
10 mg E.coli + 1 ml Extraction Buffer + 180 mg Urea (3M, freshly added!)
15 min ultra sonic treatment, centrifuge 5 min. 400 µl sample + 400 µl SDS-sample buffer 2X +0.05% Dithiothreitol (DTT), heat 3 min to 95°C, then add 0.2% Iodoacetamide (IAA), when cooled down.
Centrifuge 5 min 12000 rpm. Pipet the supernatant in a new vial. Sample volume: 15 µl

SDS-electrophoresis Marker two dimensional
20 mg E.coli + 1 ml Extraction Buffer (see above) + 180 mg Urea (3M, freshly added!)
15 min Ultrasonic treatment, centrifuge 5 min. 400 µl sample + 400 µl SDS-sample buffer 2X +0.05% Dithiothreitol (DTT), heat 3 min to 95°C, then add 0.2% Iodoacetamide (IAA) when cooled down. Centrifuge 5 min 12000 rpm. Pipet the supernatant in a new vial. Sample volume: 5 µl

First dimension IPG-strip pH 4-7, 11 cm
30 mg E.coli + 1 ml sample solution (9 M urea, 2% (v/v) IPG Ampholyte Mix, 4% CHAPS, 1% DTT, 0.005% Bromophenolblue (BPB)). 15 min Ultrasonic treatment, or freeze and thaw 2 times. Centrifuge 5 min 12000 rpm. Then dilute 35 µl sample with 65 µl of the current IPG-strip rehydration cocktail. Apply these 100 µl per strip.

First dimension IPG-strip pH 7-11, 11 cm
40 mg E.coli + 1 ml sample solution (9 M urea, 2% (v/v) IPG Ampholyte Mix, 4% CHAPS, 0.5% DTT, 0.005% Bromophenolblue (BPB)). 15 min Ultrasonic treatment, or freeze and thaw 2 times. Centrifuge 5 min 1200 rpm. Then dilute 45 µl sample with 55 µl of the current IPG-strip rehydration cocktail. Apply these 100 µl per strip.

Procedure first dimension on IPG-strip:
http://www.electrophoresis-development-consulting.de/html/firstdim.html

Semi-Colloidal Coomassie Staining Protocol

Download this protocol:
http://www.electrophoresis-development-consulting.de/html/semicolloidal.html

Hot Coomassie Staining Protocol

This hot Coomassie-staining is staining and fixing simultanuously, (Figure 25).
The acetic acid for staining and destaining can be of technical quality.

Stock solutions:

staining solution: 0.03 % (w/v) Coomassie R-350
in 1250 mL 12.5 % acetic acid

destaining solution: 12.5 % acetic acid

preserving solution: 10% (v/v) glycerol

If Pharmalytes were used in the first dimension these 2 washing steps should be done first:

Washing programm: 40% EtOH/10% HAc for 30 min
(*Pharmalytes in 1st dim.*) 20% EtOH/10% HAc for 30 min

Staining programme: Staining: 2 h *fresh* staining solution at 60 °C (in a fume hood) while stirring (Fig. 25), stain the 15% gels 3 h.
Destaining: Overnight in a small volume destaining solution in a tray - film-side down - on a rocking platform.
Preserving: 10 min preserving solution
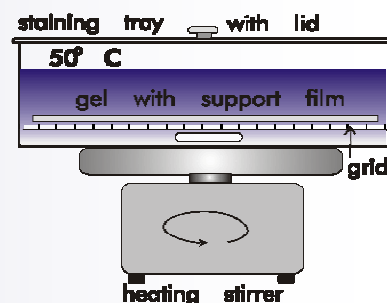Drying: Air-dry overnight

Fig.25: *Hot Coomassie-staining using the „Staining Tray Normal size"*

Silver Staining Protocol

Follow also: http://www.electrophoresis-development-consulting.de/html/2dsilver.html

For all steps: place gel onto the glass tray's bottom, gel side up

step	reagent	volume	time
1 Fixing	40% (v/v) Ethanol / 10% Acetic Acid (v/v)/ 1% Citric Acid *(160 ml EtOH/40 ml HAc/4 g Citric Acid in 400 mL)*	2 x 400 mL	2 x 30 min
2-5 Washing	H_2O dist	4 x 200 mL	4 x 10 min
6 Sensiti-zing*	30% Ethanol; 0.03% (w/v); Sodium Thiosulphate; 10 mmol/l Sodium Acetate *(60 ml EtOH/65 mg Thiosulfate/300 mg NaAc in 200 mL)*	200 mL	30 min
7-10 Washing	H_2O dist	4 × 200 mL	4 × 5 min
11 Silvering	0.25% $AgNO_3$ / 0.03% Formaldehyde (w/v) *(500 mg $AgNO_3$/180 µl Formaldehyde (37%) in 200 mL)*	200 mL	20 min
12-14 Washing	H_2O dist	3 × 200 mL	3 × 1min
15 Developing	2.5 % Na_2CO_3 / 0.03% Formaldehyde/ 0.00075% Sodium Thiosulfat *(5 g $NaCO_3$/180 µl Formaldehyde/ 75 µl Sodium-Thiosulfat (2%) in 200 mL)*	200 mL visual control!	3-5 min
10 Stopping/ Preserving	10% HAc, 10% Glycerol *(20 ml HAc/20 ml Glycerol in 200 ml)*	200 mL	20 min
11 Drying	air dry , on the support-film		16 h

* without Glutardialdehyde, because of the mass-spectrometer!