Isoelectric Focusing on DryGel IEF

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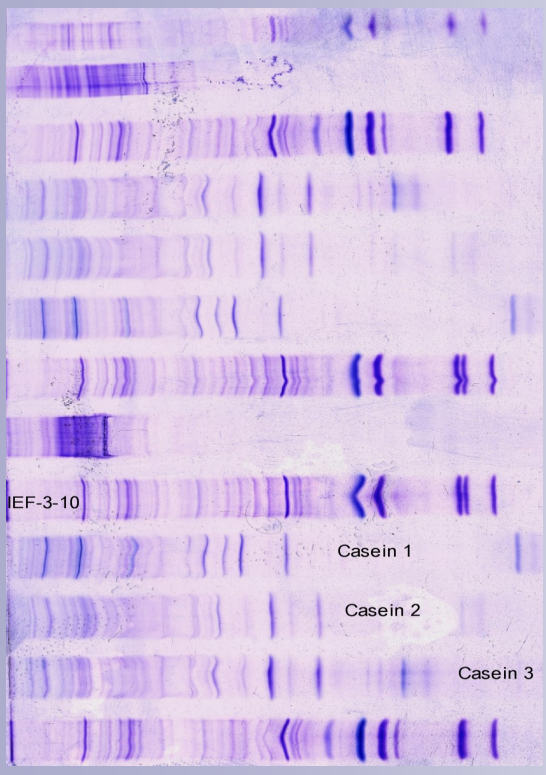

***Fig.1a*:** IEF of markers and different Caseins

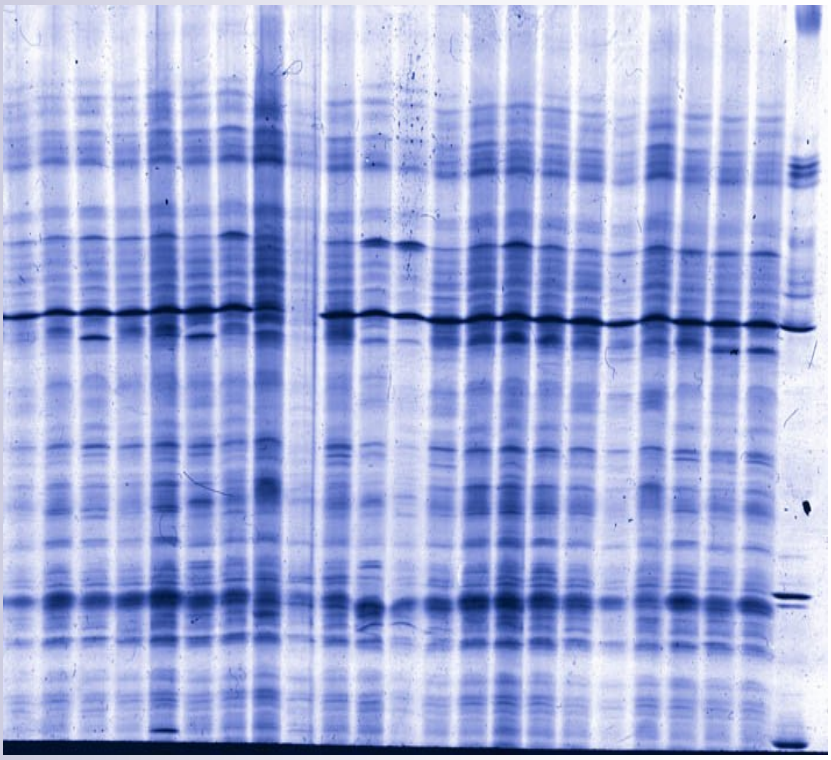

Fig.1b: Denaturing IEF of different cereal cultivars in the presence of 7M Urea

***General**:DryGel IEF is a 0.5 mm thin polyacrylamide gel with T = 5% gel concentration and C = 3% crosslinking for isoelectric focusing (IEF), which has been polymerized under special conditions to obtain an optimal matrix suitable for native and denaturing focusing (7 M Urea), figure 1a and 1b.*
Optional available: with different slot-geometries.
Catalysts and non polymerized substances have been removed by washing the gel. These gels are dried on their film support.
The dry gels must be stored at -20°C, in order to preserve their reswelling properties.
Before use, they are reconstituted in a flat tray (Rehydration Pool) in the appropriate carrier ampholyte solution with or without additives (e.g. urea, nonionic detergents and reducing agents).
As visualization techniques both, Coomassie and silver-staining are described.

All DryGels IEF can also be produced on fluorescence-free NF-support.

Gels

DryGel IEF 4 gels for native and denaturing IEF, no slots, 0,5 mm *(edc-1110)*
DryGel IEF 24S: 4 gels for nat. & denat. IEF, 24 slots à 20 µl, 0,65 mm *(edc-1111)*
DryGel IEF 40S: 4 gels for nat. & denat. IEF, 40 slots à 10 µl, 0,65 mm *(edc-1112)*
DrGels IEF NF on demand.

Additionally required

DryPool Combi (*edc-me-d*)
Tray for rehydration of dry gels (normal size) and soaking electrode strips

Sinulyte (Sinus, Heidelberg 68008), SepaLyte (ProTec, Heidelberg), Urea Ultra Pure (ICN 821519), Serdolit MB2 (Boehringer-Serva 40711)

A. Native Isoelectric Focusing

Rehydration solution for native IEF (example for a gradient pH 3 to 10, 20 ml)
1.5 ml Ampholytes (Servalyte / Biolyte / Pharmalyte) pH 3 - 10 (=3% w/v). Addition of 3.5 % (v/v) Ethylenglycol and 1 M urea increases the salt-tolerance of the gel. Fill up to 20 ml with H_2O bidest.
If a special gradient is needed, f.e. pH 4 to pH 5, please note that 10 % of the total Ampholytes should be pH 3 - 10. This prevents that the acid and the alkaline proteins migrate up to the platinum wires. Fill up to 20 ml.

Rehydration of the DryGel
Depending on the number of samples to be separated, either the entire gels or half a gel or even smaller portions may be used. If only a section of the gel is used the rehydration volume as well as the separation conditions have to be adjusted properly.
Place the Rehydration Pool onto a horizontal table. Clean it with distilled water and tissue paper.

Pipette appropriate volumes of rehydration solution (note the diff.gel-thickness dimensions).

DryGels 24S and 40S (0,65 mm):

entire gel:	18 ml (0.5 mm, no slots)	22 ml (0.65mm, 24/40 slots)
half gel:	9 ml (0.5 mm, no slots)	11 ml (0.65mm, slots)

Place the edge of the gel-film - with the dry gel surface facing downward - into the rehydration solution (fig. 2) and slowly lower the film downward. At the same time move the gel-film to and fro, in order to achieve an even distribution of the liquid and to avoid trapping air bubbles. Lift the film at the edges with tweezers, and slowly lower them down, in order to maintain an even distribution of the liquid (fig. 3a) and to remove air bubbles. After that procedure, the gel must float freely on the rehydration liquid.

Repeat this measure several times during the first 15 min to prevent the gel from sticking to the DryPool Combi. Check, whether the gel can be moved around on its reswelling liquid. Then lift the gel every 20 min to ensure even rehydration. After 60 minutes the rehydration-liquid is almost completely incorporated and the gel will no more float freely on the residual liquid, see picture 3.

End of the rehydration process is after 120 min (no slots - 0,5 mm), 240 min (24S, 40S - 0,65 mm).

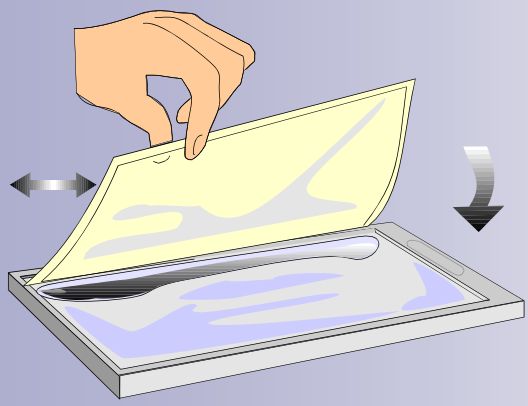

Fig.2: Placing the DryGel into the DryPool Combi tray

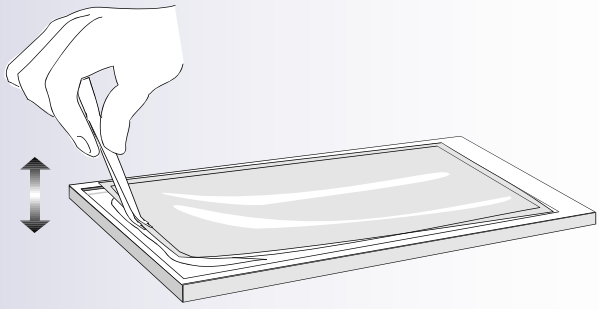

Fig.3: Lifting the edges for an even distribution of the liquid...

At the end of rehydration (240 min), the gel is removed from the DryPool Combi and the carrier backside of the gel is cleaned with a wet laboratory tissue and placed with the carrier plastic side onto a dry filter paper (gel up-face!).

Normally the gel‘s surface should be dry! If not, any exes-sive liquid is wiped off from the surface with the edge of a drying cardboard (blotting paper) according to figure 4. Do not apply the cardboard with the flat side down, it will stuck to the gel.

The rehydration process can also be done overnight. After the 240 min of rehydration was processed according described above, cover the the DryPool with a glasplate.

Note: The gel surface should be absolutely dry, otherwise the gel starts to sweat during isoelectric focu-sing

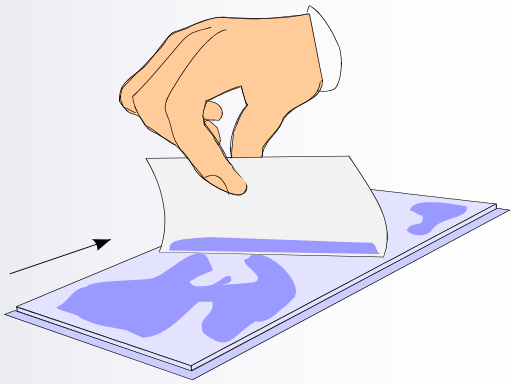

Fig.4: Drying the gel-surface with the edge of a drying cardboard

Isoelectric Focusing and Sample Application

Gel-application: Spread 2 ml of kerosene onto the cooling plate of the focusing chamber, in order to ensure good cooling contact. Place the gel (surface up) on the center of the cooling plate (fig. 5). Avoid trapping of air bubbles. Before (and after) IEF thoroughly clean the platinum wires with wet tissue paper. Move the platinum electrodes to their positions over the edges of the gel. Lower the electrode holder onto the gel surface. Connect the focus-ing electrode cables to the plugs in the chamber, close safety lid and begin with prefocusing (table 1 or table 2).

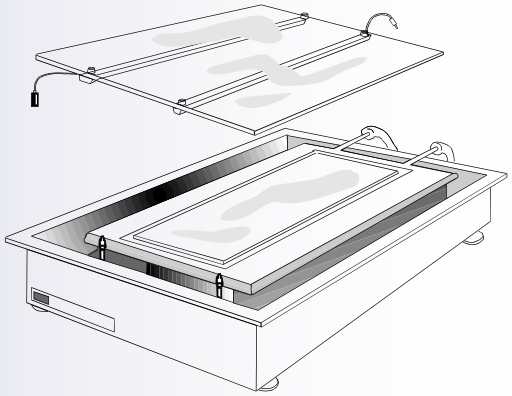

Fig.5: Placing the gel on Multiphor´s cooling plate

Temperature: Isoelectric focusing has to be performed at a defined constant temperaturebecause the pH gradient and the isoelectric points are dependent on the tempera-ture. Switch on the thermostatic circulator, set to 7°C.

Running conditions: During the isoelectric focusing the electric resistance of the gel is changing dramastically and in the end small currant- or power-values determine the voltage -values of the run. The commonly used method is to limit the voltage via the mA and the Watts achieved in the gel during the run, see table 1 and figure 6

Sample concentration: Depends on the visualization process: Coomassie-staining should have around 0.5 µg per sample, silver-staining is 50 times more sensitive.

Prefocusing: Normally samples are applied after a short prefocusing step (step 1). Exception: Salty samples, f.e. serum or cerebrospinal fluid, are applied at the beginning of the IEF.

In case of a prefocusing the IEF is stopped after the first phase to apply the samples. Mod-ern power supplies are set to “Autohold” after the first step. Focusing is then continued with step 2 of table 1.

Sample application positioning: After “Autohold” take place, or after switching off the power manually, open the chamber again and apply 20 µl of sample solution pipetted onto IEF ap-plication pieces normaly at a distance of 1 - 4 cm from the anode. This should be optimized from sample type to sample type.

Running Conditions (7°C)

Table 1 Native run limited by mA and Watt (commonly used method)

Note 1: Set "Autohold" after step 1
Note 2: At the begin of the single steps the starting volt-values (cursive and in parenthesis) should be adjusted via the limiting mA-values.

Table 1	**SET**	***Start Value***	**SET**	**SET**	Time	Process
Step 1:	800 V	*(370 V)*	13 mA	10 W	20 min	prefocusing
Step 2	500 V	*(500 V)*	9 mA	5 W	10 min	sample entrance
Step 3	2000 V	*(730 V)*	13 mA	15 W	100 min	focusing
Step 4	2300 V	*(1800 V)*	14 mA	20 W	20 min	sharpening

Note 3: For a half gel: take the same voltage and half of the mA and W
Note 4: After the run: Immediately take the application pieces away with a pair of forceps and start the staining procedure

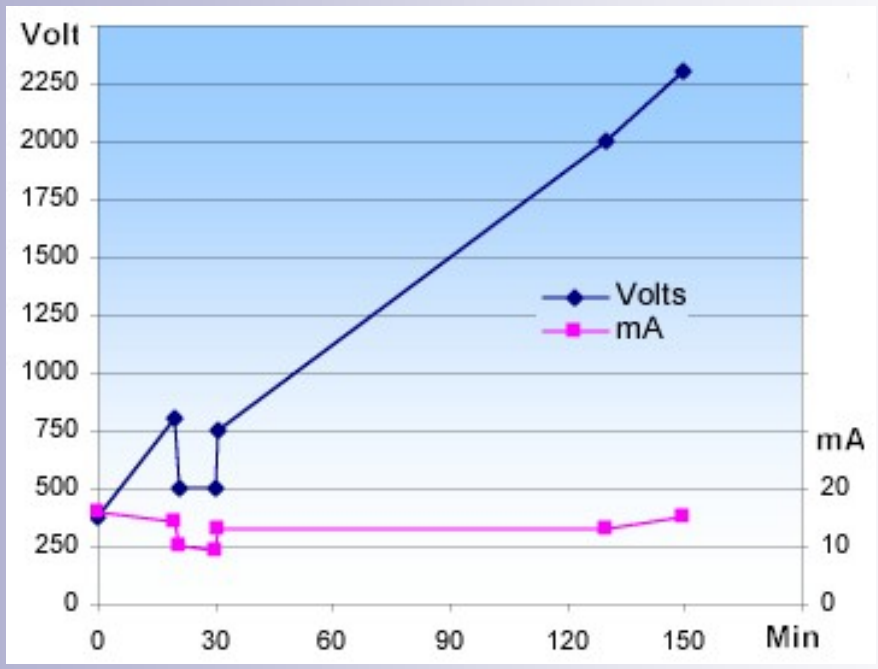

Fig.6: The volt - time curve of a native IEF-run

B. Denaturing Isoelectric Focusing

In this section only the handlings and the recipes differing from section A are described.
Urea-stock solution: Make up 48.05 g urea ultrapure (ICN 821530) to 100 ml with H_2O bidist, add 2-3 g Serdolit MB2 (Boehringer-Serva 40711) and stir for 20 min. Then filtrate through a membrane (0.45 µm) into a brown glas-bottle. Shelf life at 4 °C: 24 h, for continued use deionize once more.
Rehydrating Solution (for 1 complete gel)
17.8 ml Urea Stock Solution (=7M), 2.2 ml Sinulyte pH 3 - 10 (=4.4% w/v)

Running Conditions (12°C)

Table 2 Denaturing run limited by mA and Watt (commonly used method)

Note 1: Set "Autohold" after step 1
Note 2: At the begin of the single steps the starting volt-values (cursive and in parenthesis) should be adjusted via the limiting mA-values.

Table 2	SET	*Start Value*	SET	SET	Time	Process
Step 1:	1200 V	*(675 V)*	14 mA	15 W	30 min	prefocusing
Step 2	800 V	*(800 V)*	10 mA	10 W	20 min	sample entrance
Step 3	2300 V	*(1000 V)*	13 mA	25 W	100 min	focusing
Step 4	2500 V	*(2300 V)*	15 mA	35 W	30 min	sharpening

Note 3: For a half gel (MiniCleanGel): take the same voltage and half of the mA and W
Note 4: After the run: Immediately take the application pieces away with a pair of forceps and start the staining procedure

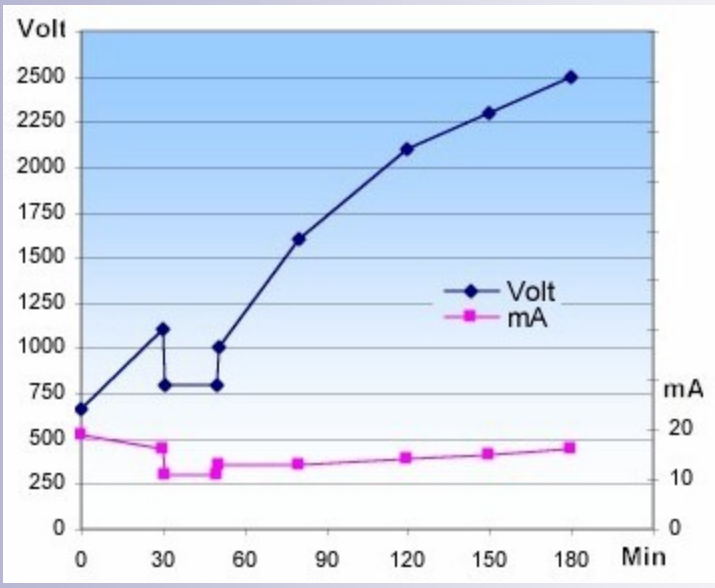

Fig.7:.Volt-time curve of the denaturing run

Detection of protein bands

1. Staining with Coomassie Blue G-250

Stock solutions:

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

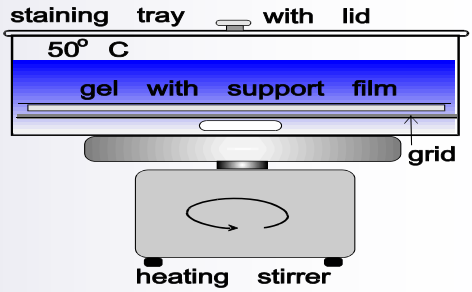

Fig.8:. Hot Coomassie-staining

Staining programme:

Fix:	15 min 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*) (Slighly heat the staining solution to 50°C while stirring, see figure 7)
Wash:	2*5 min in 200 ml wash solution (*mix equal amounts of A and C*)
Destain:	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)

Abbreviations: TCA = Trichloroacetic Acid

A ethanol-based Commassie G250 staining:
http://www.electrophoresis-development-consulting.de/html/iefcoomassie.html

2. Silver Staining

Automated Silver-Staining is performed **at ambient temperature** with the Hoefer Automated Gel Stainer (GE 80-6395-02).
Adapted staining programme [1]

Staining solutions and programme:

Table 4:

step	reagent	volume	time
1 Fixing	20% trichloroacetic acid (w/v)	200 ml	20 min
2+3 Rinsing	20 % ethanol/ 8% acetic acid (v/v)	2 x 200 ml	2 x 10 min
4 Incubation	0.1 % sodium thiosulphate; 0.4 mol/l sodium acetate/acetic acid pH 6.5; 0.125 % glutardialdehyde	200 ml	15 min
5 Rinsing	20 % ethanol / 8% acetic acid	200 ml	10 min
6-8 Washing	H_2O dist (place gel into a glass tray, with the gel surface side up)	3x 200 ml	3x10 min
9 Silvering	0.1% $AgNO_3$ /0.004% formaldehyde (w/v) 20µl HCHO (37% w/v) per 200 ml 0.1% $AgNO_3$	200 ml	30 min
10 Developing	2.5 % Na_2CO_3 / 0.004% formaldehyde 40µl HCHO (37% w/v) per 400 ml 2.5% Na_2CO_3	2x 200 ml observe to stop	0.5 min 2-3 min
11 Stopping/ Preserving	10% HAc, 5% glycerol	200 ml	20 min
Drying	air dry , on the support-film		16 h

Ammoniacal Silver Staining (spezial staining recipe for basic proteins):
http://www.*electrophoresis-development-consulting.de*/download/csffocus.pdf

References:

[1] Krause I.; Forschungszentrum für Milch und Lebensmittel, Weihenstephaner Berg 5 85354 Freising, Germany (I.B.Krause@lrz.tum.de)