Cereal Typing using
IEFGels and DryGels IEF

EDC
V43 02/19

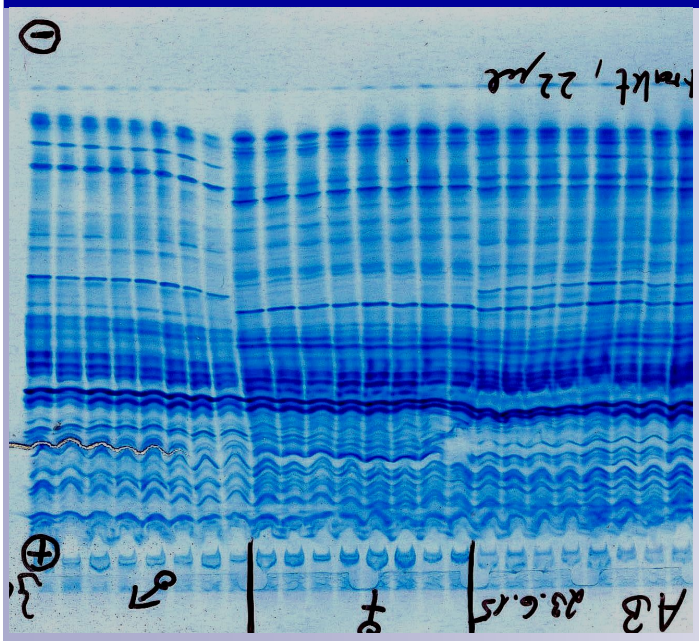


Fig.1a: DryGel IEF cereal.
To be used with 1-7 M Urea. Half gel, Maice-reference samples.
By R. Knoblauch, LTZ Ka.(Germany).

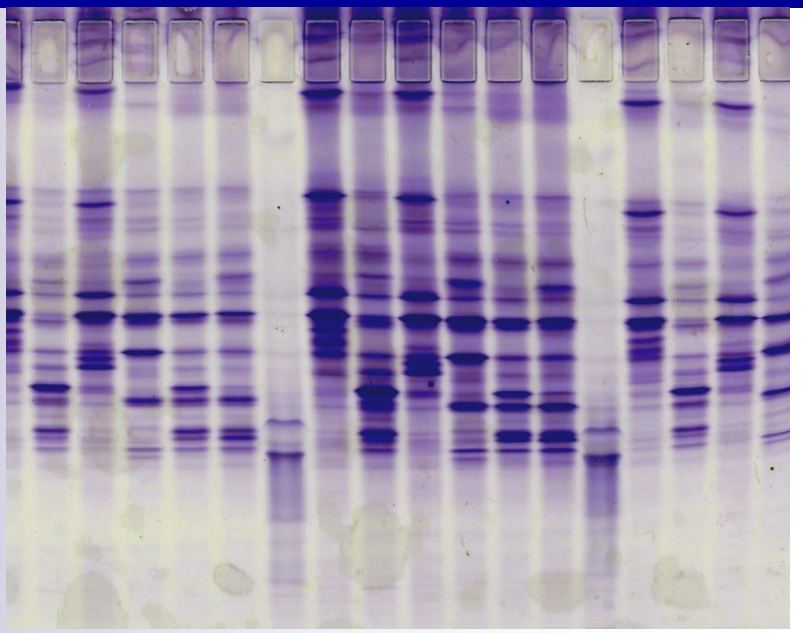


Fig.1b: IEFgel cereal SoftGel *EQ-type*
Buffer-soluble proteins.
Half gel, wheat-reference samples.

***General**:DryGel cereal and IEFGel cereal are 0.5/ 0.65 mm thin polyacrylamide gels (250 mm x 110 mm) with a spezial designed matrix for isoelectric focusing (IEFGel: SoftGel EQ-type) of cereal varieties f.e. from maice or wheat.*
Potatoe-varieties can also be detected.
DryGels have been polymerized under special conditions to obtain an optimal matrix suitable for denaturing focusing (1-7 M Urea), figure 1. Catalysts and non polymerized substances have been removed by washing the gel.
IEFGels are Ready-To-Use gels. DryGels are washed and dried on their film support
Special „two phases" recipe available: On one side there is a „sample application zone" for easy sample entrance followed by a separation zone —> for quick run time: 75 min.
Staining time with Coomassie Violet is ~1 hour.
Alternatively the „high resolution" variant can be ordered: Highest band sharpness with a run time of ~2 hours.
The dry gels must be stored at -20°C. Before use, they are reconstituted in a DryPool combi in the appropriate carrier ampholyte solution with or without additives (e.g. urea, nonionic detergents +/- reducing agents).
Two Coomassie procedures and one silver-staining are described as visualization tec.

Gels

DryGel IEF cereal 40S (4 gels, slot volume: 8 µl)	edc-1123 (–2p– or –hr-)
DryGel IEF cereal 52S (4 gels, slot volume: 12 µl)	edc-1126 (–2p– or –hr-)
DryGel IEF cereal 104S (4 bidirect gels, slot volume: 5 µl)	edc-1117
IEFGel cereal 40S SoftGel *EQ type*	edc-1020
IEFGel cereal 52S SoftGel *EQ-type*	edc-1021
IEFGel cereal 104S bidirect SoftGel *EQ-type*	edc-1022

Additionally required

DryPool Combi (tray for rehydration of dry gels	edc-me-d
Flatbed electrode, 8 mm wide (for cereal IEF)	edc-elec2843

Ampholytes: SepaLyte 3-10, 7-10 EXP, 4-6 EXP (ProTec, Heidelberg), Urea Ultra Pure, Triton X100, Trichloroethanol, Tetramethy urea, Ethylenglycol, Sample Application Strips (54 à 10 µl) (Biostep BS146.668), 2-Chloroethanol, Tetramethyurea (TMU), Kerosene

Rehydration of the DryGel IEF (not necessary for IEFGels)
Place the DryPool Combi (for normal sized gels) on a horizontal table. Clean it with distilled water and tissue paper.
Rehydration volume: 18 ml for a normal sized gel.
Carefully cast this volume into the DryPool Combi.
Place the edge of the gel film - with the dry gel surface facing downward - into the rehydration solution (Fig. 2a, b and c) and slowly lower the film. 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. 3) and to remove air bubbles.

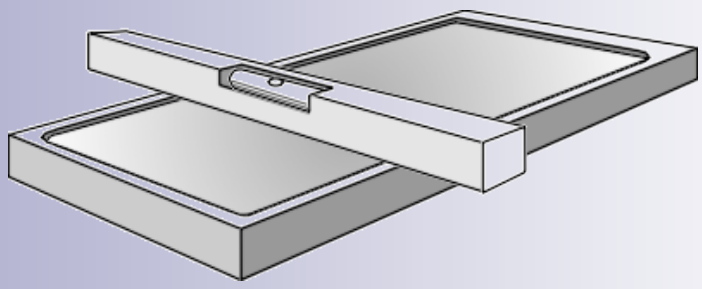


Fig.2a: Adjusting the rehydration pool horizontally

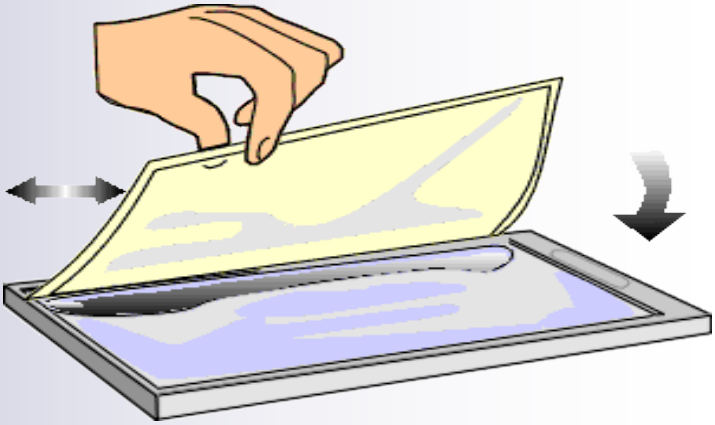


Fig.2c: DryPool Combi (normal sized gels): 18 ml

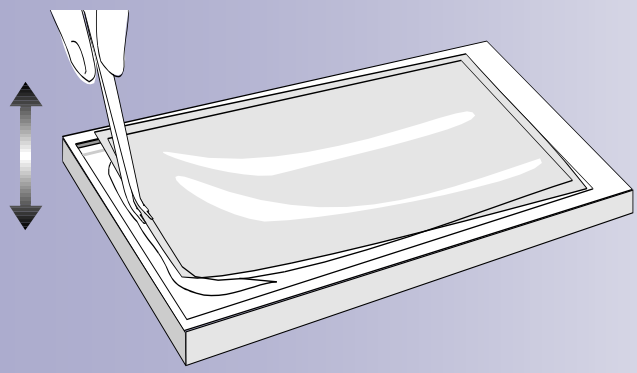


Fig.3: Lifting the edges for an even distribution of the liquid under the gel

Rehydration time is ~2 hours 20 min.
During this time the gel have to be lifted several times with a forceps:

1. Directly after setting the gel onto the liquid
2. Then 2 x after every 5 min and then after 10 min.
3. Finally 6-7 x after every 20 min.

The rehydrated gel must have a dry surface!

Special native procedure for potatoes:
Rehydrating solution normal size: for 1 gel with 3.8% Ampholytes (18 ml)
1.44 ml Sepalyte 7-10 EXTENDED, 295 µl Biolyte 3/10, 900 µl Ethylene glycol
20 µl Triton 10% (v/v). Fill up to 18 ml (+15.4 ml H_2O).

Protein extraction Mix Potatoe: for 10 ml nativ extraction solution
130 µl Sepalyte 3-10, 80 µl Orange G (1%), 10 µl Bromophenol Blue, 15 µl Bromophenol-red, spatula-tip of Dithiothreitol

Protein extraction potatoe: Press potatoe cubes with a squeezer (garlic-press) and collect the sap in a eppendorf centrifuge.
Dilute with the extraction solution according to the visualization procedure: e.g. Coomassie=1+3. Sample volume: 8 - 15 µl. Centrifuge for 5 min at 5000 rpm.

Fig.4: garlic press for sqeezing the potatoe cubes

Special denaturing procedure for maize:

Rehydration solution normal size: : for 1 gel with 4M Urea and 4.4% Ampholytes (18 ml)
4.4 g Urea, 1.4 ml Sepalyte 3-10, 230 µl Biolyte 3/10, 50 µl Serva 9-11, 40 µl Triton 10% (v/v), Fill up to 18 ml (+13 ml H_2O). Stir until the urea has dissolved completely.

Protein Extraction Mix Maize: for 10 ml denaturing extraction solution
0,5 ml Tetramethyl urea (TMU), 0,5 ml 2-Chloroethanol, 100 µl Sepalyte 3-10, 50 µl Triton 10%, 65 µl Pyronin Y, 15 µl Methylene Blue, spatula-tip Dithiothreitol.
Fill up to 10ml (+9 ml H_2O)

Protein Extraction Procedure Maize:

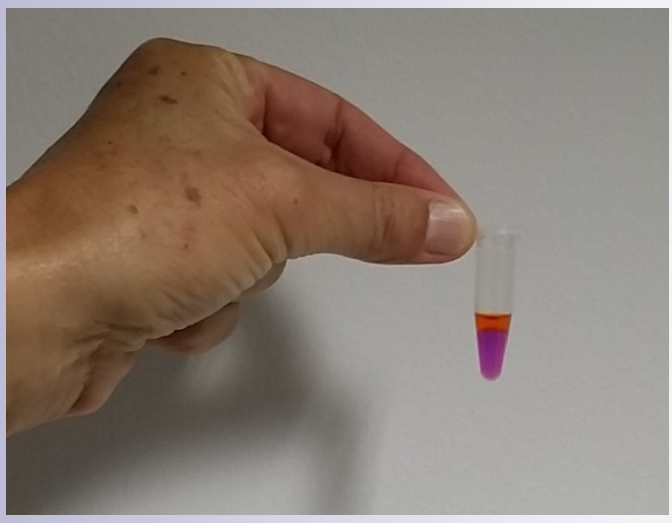
Pulverice one grain with a shredder, or pulverize it in a plastic bag with a hammer (fig. 5).
Extracting the proteins out of one grain maize with the following volumes:

Hybrid: 1000 µl — male: 650 µl — female: — 800 µl of the extraction solution.

Apply 15 min ultrasonic treatment.

Optional: defatting by overlaying 200 µl Kerosene (+ spatula tip of Sudan-red). Shake the cups well and leave for 10 min. Then take the lower layer, see fig 5.
Centrifuge the Eppendorfs 5 min 5000 rpm.

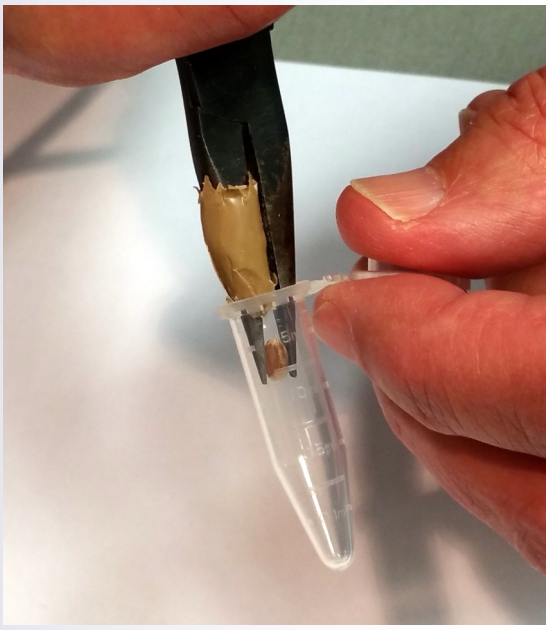
Fig.5: pulverizing a single grain of maize (above)and overlaying with kerosene for defatting (below).



Protein extraction procedure wheat, barley:
Extraction buffer: 60 mg Hepes, 1 tip of a spatula Dithiothreitol, 75 µl Tetramethyurea, 30 µl Ampholyte 3-10 on 5 ml.

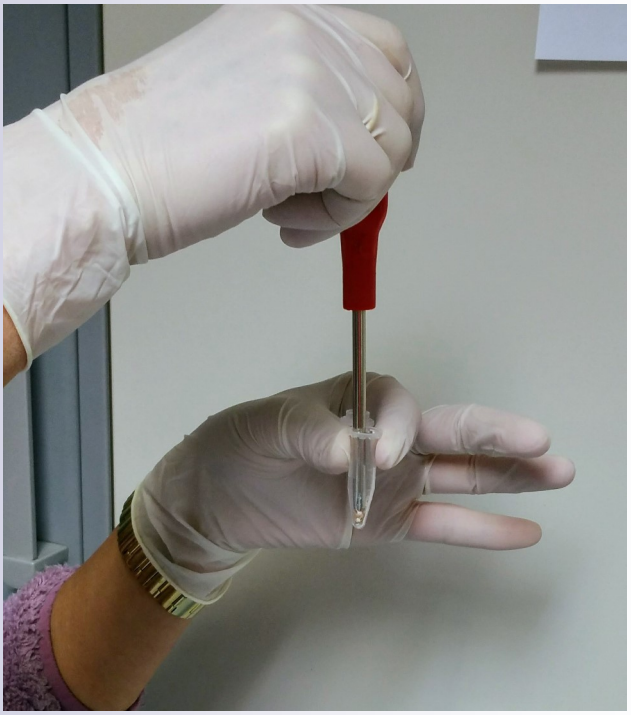
100 µl per one crushed and pulverized wheat grain. See figures 6.
15 min ultra sonic treatment. Centrifuge the Eppendorfs 5 min 5000 rpm.
Gels: IEFGels cereal SoftGel *EQ-type*

Fig.6: *radio pliers for cracking the grain. One side with tape: this prevents splinters splashing out during final grating.*



Cracking the grain ...

….and grating the grain ...



….or using a medium-sized Phillips screwdriver

End of Rehydration (only for DryGels, not necessary for IEFGels)

At the end of rehydration (140 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 upface!).

Normally the gel‘s surface should be dry! If not, any exessive liquid is carefully wiped off from the surface with the edge of a drying cardboard (blotting paper) according to figure 6. Do not apply the cardboard with the flat side down, it will stuck to the gel.
Note: The gel surface should be absolutely dry, otherwise the application strips will leak and the gel starts to sweat during isoelectric focusing!

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. 8). Avoid trapping of air bubbles.
Orientation of the DryGel IEF cereal with 2 phases:
The sample application zone of the gel (on the opposite side of the round corner) should be orientated to the start side electrode: this depends on the samples, fig. 6.
Apply sample slot zone on this side!
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 focusing electrode cables to the plugs in the chamber, close safety lid and begin with prefocusing (table 1 or table 2).
Please note: the central electrode for small bidirect gels should be the 8 mm wide electrode (see page 1). Otherwise the electrode will overlap the slots!
Temperature: Isoelectric focusing has to be performed at a defined constant temperature because the pH gradient and the isoelectric points are dependent on the temperature.
Switch on the thermostatic circulator, set to 15°C.
For complicate samples the first step is performed at ambient temperature. Set the valves to “bypass” and after this step to “cooling”.

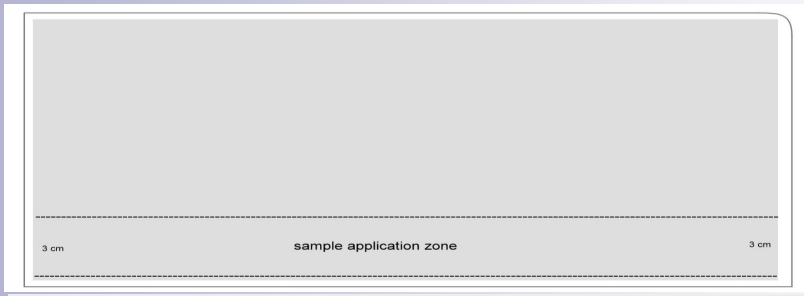


Fig.6: Sample application zone: 3 cm when using

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 2 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: Seed testing samples run without prefocusing.
Other 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. Modern 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 application pieces normaly at a distance of 1 - 4 cm from the anode. This should be optimized from sample type to sample type.

Running Conditions for the IEFGels cereal SoftGel *EQ-type*

Table 1a for native IEFGels 0.5 mm (40S, 52S): 2,5% Ampholytes, total time=2h 50 min
Temp: 25°C in step 1, then 15°C!

Table 1	**SET**	***Start Value***	**SET***	**SET**	Time	Process	temp
Step 1	100 V	*(60 V)*	6 mA	5 W	20 min	sample entrance 1	**25°***
Step 2	300 V	*(140 V)*	8 mA	5 W	20 min	sample entrance 2	15°
Step 3	900 V	*(450 V)*	14 mA	15 W	20 min	slow start	15°
Step 4	1250 V	*(1050 V)*	15 mA	15 W	90 min	main focusing	15°
Step 5	1600 V	*(1600 V)*	10 mA	20 W	20 min	band sharpening	15°

Table 1b for native IEFGels 0.65 mm (24S): 2,5% Ampholytes, total time=2h 50 min
Temp: 25°C in step 1 then 15°C!

Table 1	**SET**	***Start Value***	**SET***	**SET**	Time	Process	temp
Step 1	100 V	*(80 V)*	7 mA	5 W	20 min	sample entrance 1	**25°***
Step 2	300 V	*(140 V)*	9 mA	5 W	20 min	sample entrance 2	15°
Step 3	900 V	*(470 V)*	15 mA	15 W	20 min	slow start	15°
Step 4	1250 V	*(980 V)*	14 mA	15 W	90 min	main focusing	15°
Step 5	1600 V	*(1600 V)*	10 mA	20 W	20 min	band sharpening	15°

** Note: If other rehydration mixes are applied (pH-gradients, ampholyte-concentrations) adjust the starting Volt-values with the mA set-value.*
** Do not forget to set the kryostat to the lower temperature in step 3.*
For a half gel: take the same voltage and half of the mA and W
After the run: Immediately start the staining procedure

Table 2 for denaturing DryGels: Total time=2h

Temp: 15°C

Table 2	**SET**	***Start Value***	**SET**	**SET**	Time	Process
Step 1	450 V	*(120 V)*	15 mA	10 W	30 min	sample entrance
Step 2	1800 V	*(450 V)*	23 mA	30 W	90 min	focusing

** Note: If other rehydration mixes are applied (pH-gradients, ampholyte-concentrations) adjust the starting Volt-values with the mA set-value.*

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

Running Conditions for the –2P- variant

Table 3 for denaturing gels "two phases" recipe gels. Total time=1h 25min

Temp: 15°C

Table 1	SET V	*Start Value*	SET mA	SET W	Time	Process
Step 1:	450 V	*(200 V)*	20 mA*	10 W	15 min	sample entrance
Step 2:	1800 V	*(400 V)*	30 mA*	50 W	50 min	main focusing
Step 3:	2000 V	*(2000 V)*	25 mA*	50 W	~10 min	band sharpening *

* *Note: If other rehydration mixes are applied (pH-gradients, ampholyte-concentrations) adjust the starting Volt-values with the mA set-value.*
* *Note 3: Stop the run when the 2500 Volts are reached*

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

Table 4 for bidirect IEFGels SoftGel *EQ-type* and DryGels. Total time=1h 25min

Temp: 15°C

Table 1	SET V	*Start Value*	SET mA*	SET W	Time	Process	temp
Step 1:	150 V	*(50 V)*	10 mA	5 W	15 min	sample entrance 1	**ambient***
Step 2:	250 V	*(110 V)*	16 mA	6 W	10 min	sample entrance 2	**ambient***
Step 3:	400 V	*(330 V)*	24 mA	8 W	10 min	slow start	15° (switch by-pass off!)
Step 4:	700 V	*(500 V)*	30 mA	12 W	40 min	Main focusing	15°
Step 5:	900 V	*(900 V)*	24 mA	50 W	10 min	band sharpening *	15°

* *Note: If other rehydration mixes are applied (pH-gradients, ampholyte-concentrations) adjust the starting Volt-values with the mA set-value.*

* *ambient temperature: run the cooling apparatus in bypass—not through the cooling plate! Then in step 3: open the cooling circuit*

After the run: Immediately start the staining procedure

Detection of protein bands

1. Staining native gels with Coomassie-Violet

Stock solution

Dye-Concentrate (10 x conc): 0,1% Coom.Violet = 1 g in 1000 ml H_2O dist

Staining solution (Mix shortly before use!)

ca. 170 ml H_2O dist. + 20 ml dye-conc. + 6 ml HAc + 8 ml H_3PO_4

Staining programme

Fix:	30 min in 200 ml 20 % TCA (at room temperature)
Wash 1:	20 min in 200 ml 10% Ethanol
Wash 2:	2 x 10 min in 200 ml H_2O dist.
Stain:	30 min 0.01% Coomassie-Violet in 3,4% (w/v) Phosphoric acid + 3% (v/v) HAc + 2,5% EtOH. Prepare just before use!.
Enhancing:	overnight in 200 ml 2,5% EtOH + 3 ml Dye Concatrate (C.violett 0,0015%) + 3 ml Hot Commassie Dye Concentrate (C.R350 0.00045%)
Impregnate:	20 min in 200 ml 5 % (v/v) Glycerol + 5% (v/v) Ethylenglycol
Dry:	air-dry (leave at room temperature)

Abbreviations: TCA = Trichloroacetic Acid

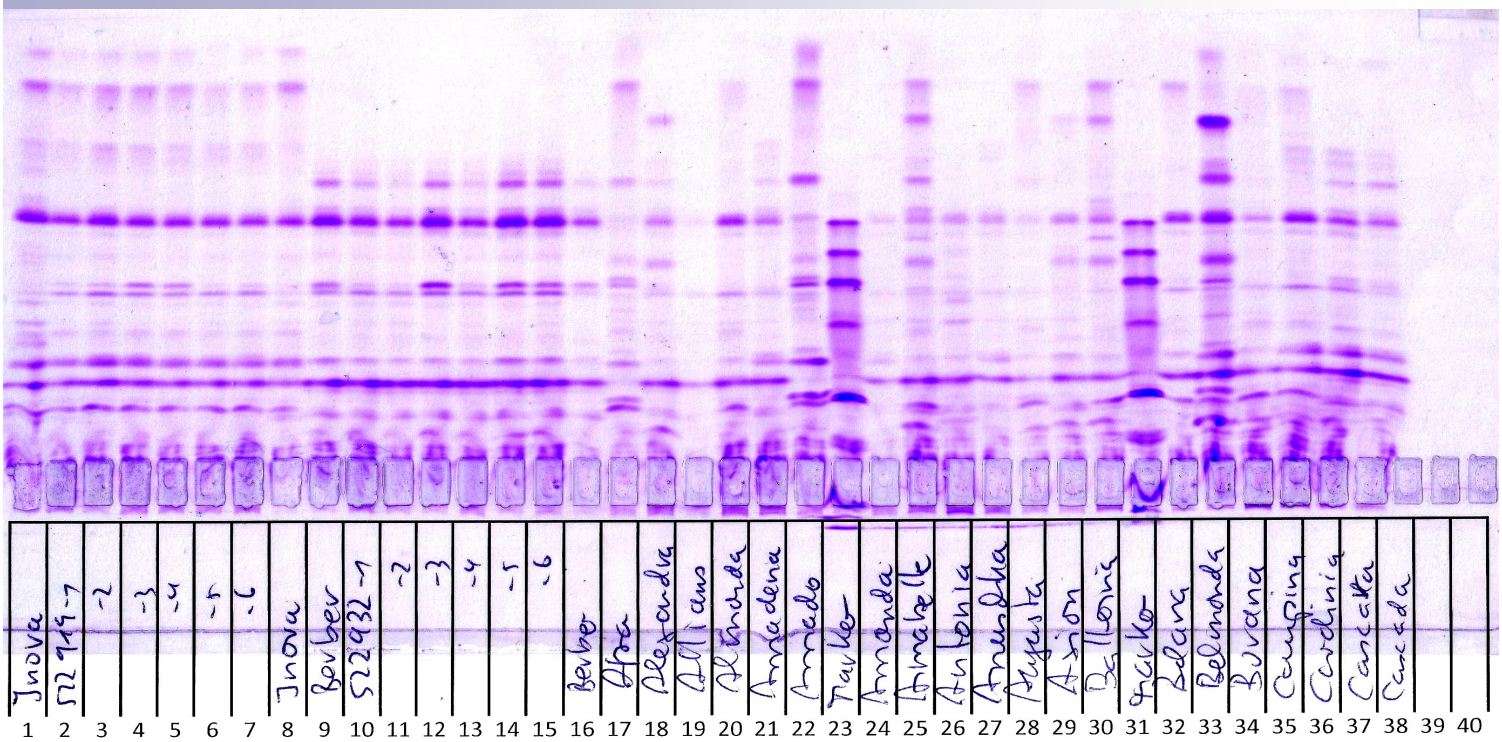


Fig. 7: Diverse potatoe-varieties run with SepaLyte 7-10 EXT on DryGel IEF cereal 40S
By V.Tichy, LUFA -ITL GmbH, AGROLAB GROUP, Kiel (Germany)

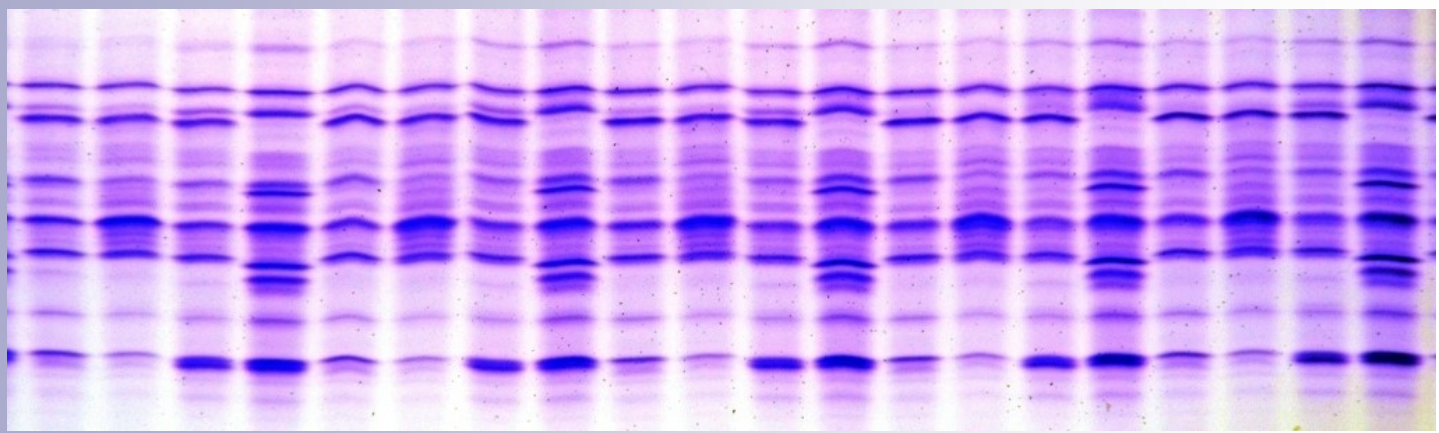


Fig. 8: Four wheat varieties with SepaLyte 4-6 EXPANDED by Manfred Demharter Heidelberg (Germany)

2. Staining denaturing IEF with Blakesley / Roti Blue

Based on „Blakesley-Staining": ANALYTICAL BIOCHEMISTRY 82, 580-582 (1977)

A five fold concentrate staining solution can be purchased from Roth (Karlsruhe): 1 liter **Roti-Blue** (#A152.1). Sensitivity is 3-5 fold higher than other Coomassie-stainings.

Recipe and procedure for urea-containing IEF-gels:
(double volumes for large sized gels)

fixing	100 ml H_2O	+ 12.5g TCA	30 min
washing	70 ml H_2O + 30 ml MeOH	+ 1 ml Phosphoric acid	15 min
staining	mix: 60 ml H_2O + 40 ml MeOH	stir in: 20 ml Roti-Blue	3h-overnight
washing	75 ml H_2O + 25 ml MeOH	- - -	5 min
preserving	3% Glycerol	- - -	20 min

Abbreviations: TCA = Trichloroacetic acid, MeOH = Methanol

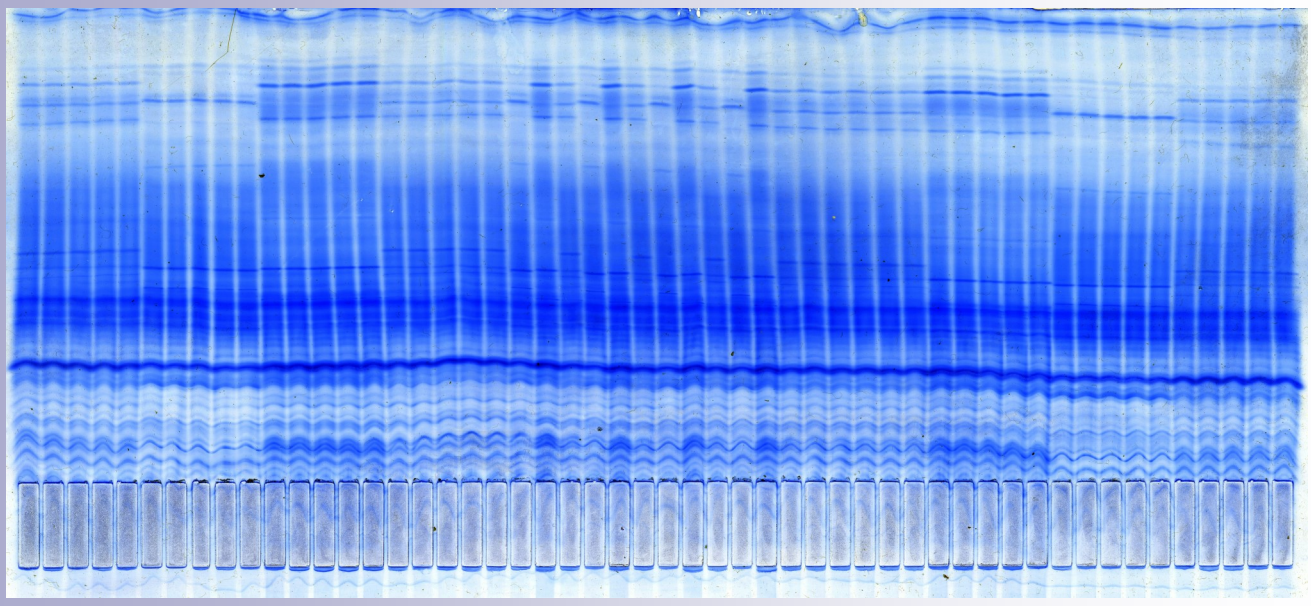


Fig. 9: Maize family: hybrid-male-femal on a DryGel IEF cereal 40S with 4 M urea

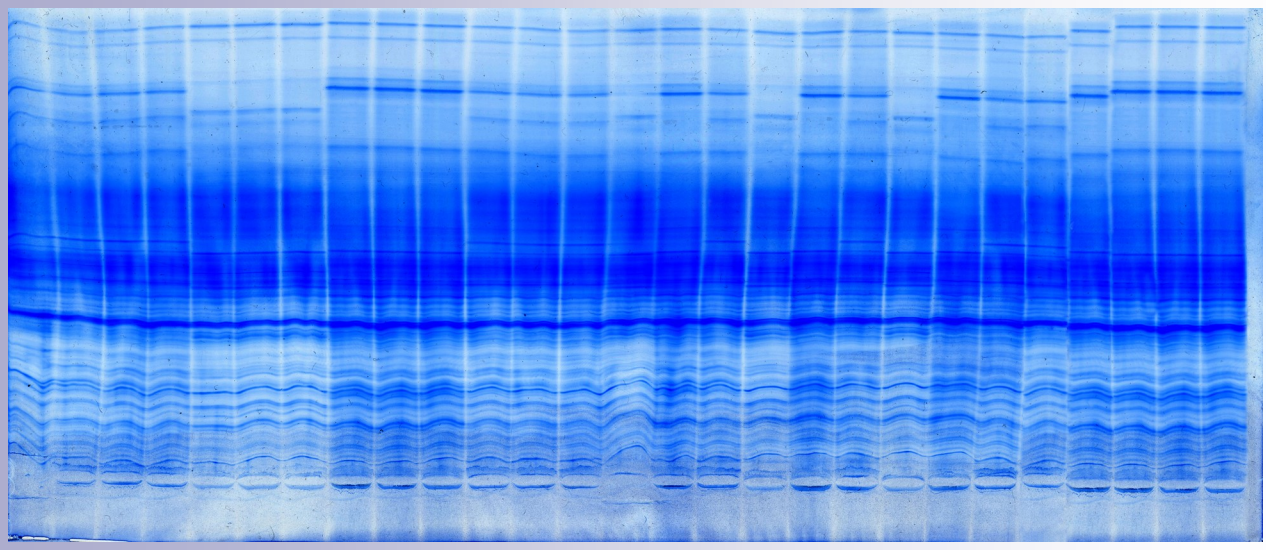


Fig. 10: Maize family: hybrid-male-femal: DryGel IEF, 4 M urea, 2 phases recipe

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 3:

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] A.Jonitz[1], D.Miranda[2], VDLUFA Kongressband 2008
[1] Landwirtschaftliches Technologiezentrum Augustenberg, Karlsruhe, Germany
[2] Universidade de Pelotas, Pelotas, Brazil

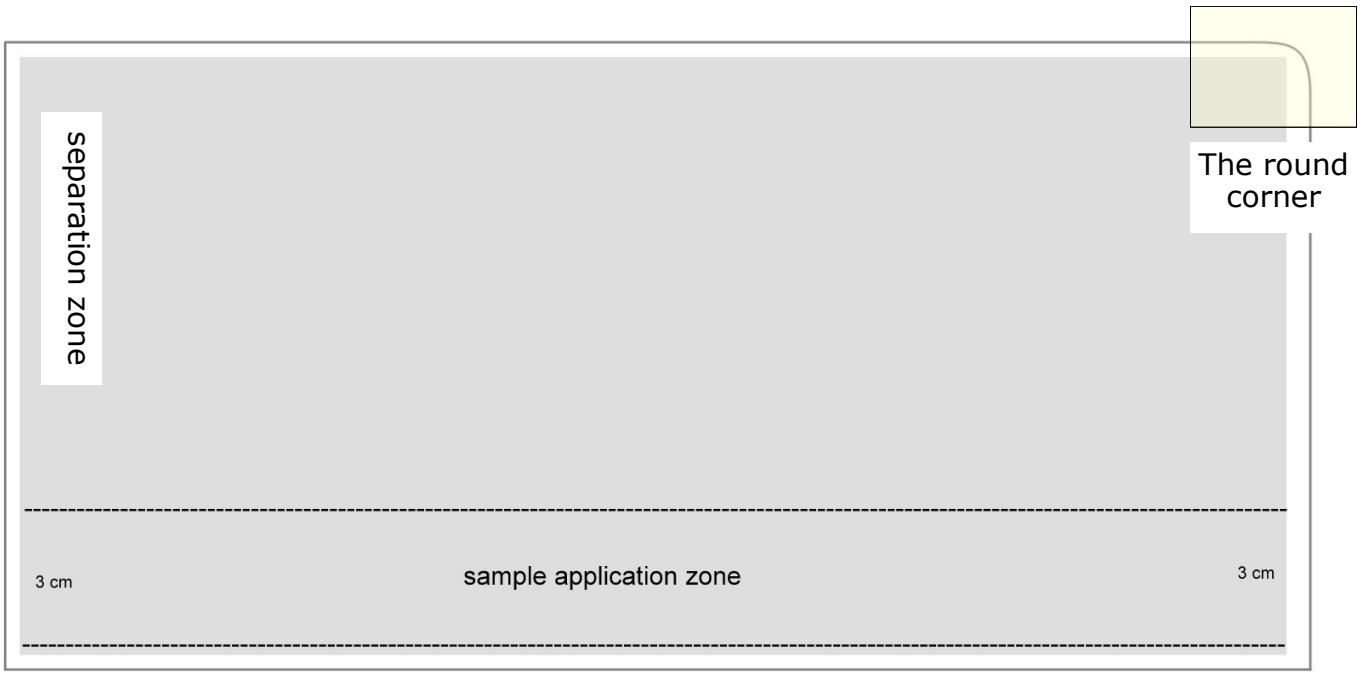


Fig.11: Geometry *of a DryGel IEF cereal „two phases"*

The DryGel IEF cereal „two phases:

A Sample Application Zone
This zone is located on the opposite side of the round corner and can be orientated towards the anode (anodel sample application, or to the cathode (cathodal sample application). Zone lengths: ~3 cm.

B Separation Zone
Here will be the separation of your applied samples. Zone lengths: ~ 8cm.

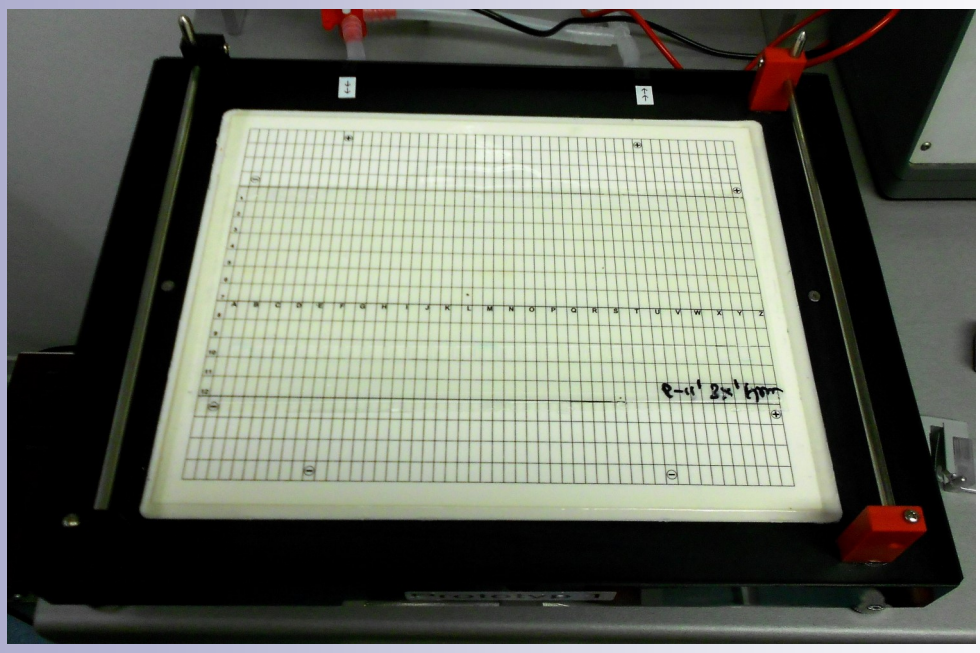


Fig.12: The gel on the cooling plate of a flatbed professional. The samples should be applied on the side without the round corner. See above.