INSTRUCTION: CASEIN-IEF KIT

DETECTION OF COWS´ MILK IN EWES´ AND GOATS´ MILK CHEESE IN THE PRESENCE OF UREA ON DRYGEL IEF

DryGel IEF is a 0.43 mm thin polyacrylamide gel with T = 5% gel concentration and C = 3% crosslinking for isoelectric focusing, which has been polymerized under special conditions to obtain an optimal matrix suitable for native and denaturing focusing (7 M Urea).
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 (DryPool) in the appropriate carrier ampholyte solution with or without additives (e.g. urea, nonionic detergents and reducing agents).
To obtain reproducable runs the commonly used "Running Conditions" are converted into activly driven "Ramping Methods" where the power supply follows a given volt-time curve.
A detailed protocol is given using DryGel IEF for the detection of cows' milk in ewe's and goats' milk cheese via bovine γ_2- and γ_3 -caseins (see fig. 1) as described in [1]

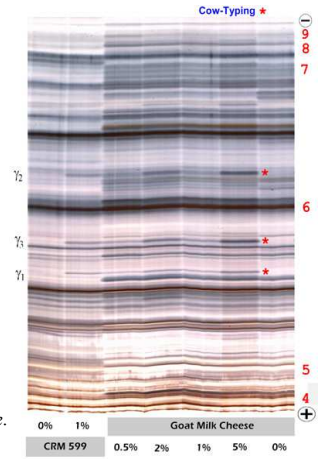

Fig.1: *Detection of cows' milk caseins in ewes'and goats' milk cheeses.g_2 , g_3.= Cow-specific casein indicators; CRM599 = Certified Reference Material (RF0%, RF1%). Amount of cows' milk is given as percentage.*

Kit: Casein-IEF-Kit (EDC 1021-07) consists of: 5 DryGel IEF (EDC 1110) and 1 Casein-IEF Consumables Kit (EDC 1004-06)
Additionally required: CleanPool (1003-20), Sample application pieces (GE 80-1129-46)

Rehydration Solution for IEF of Casein [2]

1. Urea-stock solution*: Make up 48.05 g urea ultrapure (ICN 821530) and 3.5 ml Ethylenglycol (100%) p.A. (Merck) to 100 ml with H_2O bidist, add 2-3 g Serdolit MB2 (Ion-Exchanger, 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. *Not necessary if ETC 1004-06 is used.

2. Rehydrating Solution (for 1 complete gel):
Casein-IEF-Kit: Pipette 6 ml of the detergent-solution to the portioned urea, then pipette 2.35 ml Ampholine-cocktail to this solution and flush pipette-tip also in this solution. Fill up to 20 ml with the detergent-solution. Alternatively, gels may be rehydrated in a solution containing 18 ml urea stock solution (1), 400 µl SinuLyt pH 3-10 (#68008,Sinus Biochemistry, Heidelberg, 1.2 ml SinuLyt pH 5-7 (#68013), and 0.4 ml Servalyt 5-7 (#42905 Serva, Heidelberg).

Rehydration of the Dry Gel

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 DryPool onto a horizontal table. Clean it with distilled water and tissue paper. Pipette the appropriate volume of rehydration solution, e.g. for:

entire gel:	20 ml
half gel:	10 ml

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.

Note: Repeat this measure several times during the first 15 min to prevent the gel from sticking to the DryPool. Check, whether the gel can be moved around on its reswelling liquid. After 60 minutes the rehydration-liquid is almost completely incorporated and the gel will no more float freely on the residual liquid.

Alternatively, a rocking platform can be used (fig. 3b.) during the first 60 minutes of the rehydration.

After 110 min (10 min before the end of rehydration), lifting of the edges should be repeated once again, in order to catch also the remaining liquid droplets from the edges of the DryPool.

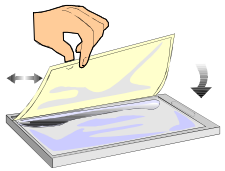

Fig.2: *Placing the DryGel into the DryPool Combi*

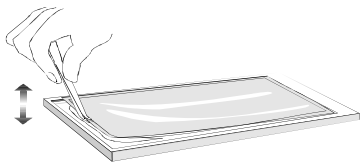

Fig.3a: *Lifting the edges for an even distribution of the liquid…*

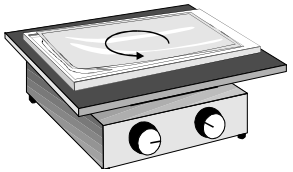

Fig.3b:*….or gently shake it using a rocking platform*

Detection of protein bands

Silver Staining

Automated Silver-Staining is performed **at ambient temperature** with the Hoefer Automated Gel Stainer (Amersham Biosciences 80-6395-02). Adapted staining Programme available upon request [4]
Staining solutions and programme:

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, 3% glycerol	200 ml	20 min
Drying	air dry, on the support-film		16 h

Alternatively, less sensitive Coomassie staining may be applied as described in [5]

Abbreviations: HAc = Acetic Acid,

References:

[1] Commision Regulation (EC) No.213/2001; Off.J.Eur.Comm.(2001) No.L 37, 51-57

[2] Official German Method No. L-01.00-39. BVL (1995)

[3] German Normative DIN 10469. Beuth Verlag, Berlin (1996)

[4] Krause I.; Forschungszentrum für Milch und Lebensmittel, TUM-Weihenstephan, Hochfeldweg 1, D-85354 Freising (I.B.Krause@lrz.tum.de)
[5] Klotz, A.; Krause I.; Einspanier, R. (2000) EurFoodResTechnol (2000) 211:1-5

Note 4: After the run: Immediately take the application pieces away with a pair of forceps and start the staining procedure

Table 2 Denaturing run driven actively by the power supply (better method!)

Note 1: Set "Ramping=on" or "Volt Level=Changing"

Table 2	SET	*Start Value*	SET	SET	Time	Process
Step 1:	650 V –> 1100 V	*(12 mA)*	20 mA	15 W	30 min	prefocusing
Step 2	800 V –> 800 V	*(9 mA)*	15 mA	15 W	20 min	sample entrance
Step 3	1000 V –> 2500 V	*(7 mA)*	15 mA	30 W	100 min	focusing
Step 4	3000 V –> 3000 V	*(11 mA)*	15 mA	40 W	30 min	sharpening

Note 2: Set "Autohold" after step 1

Note 3: mA and Watt should not be limiting!

Note 4: After the run: Immediately take the application pieces away with a pair of forceps and start the staining procedure

Note 5: Half gels can also run with this methode without changing values

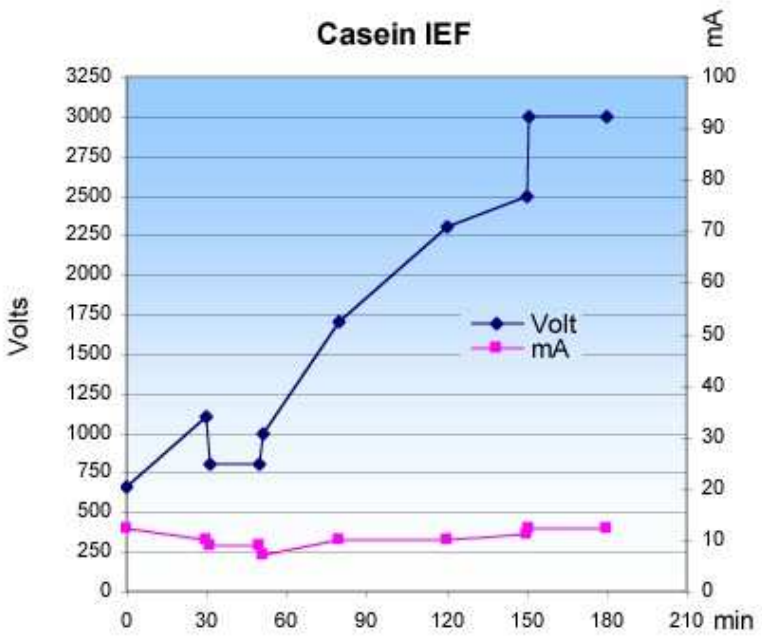

Fig.6: *The Volt- and the mA - values during the denaturating run using a given volt-time curve*

At the end of rehydration (120 min), the gel is removed from the DryPool 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!).

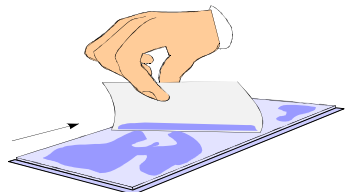

Fig.4: *Drying the gel-surface with the edge of a drying cardboard*

Then any exessive liquid is wiped off from the surface with th edge of a drying cardboard, see figure 4. Do not apply the cardboard flatside-down as it will stuck to the gel. If not used immediatly after rehydration, the gel surface is protected with a plastic covering sheet, and the ′gel sandwich′ is then welded into a film tube and stored in a refrigerator overnight.

Note: The gel surface should be absolutely dry, otherwise the gel starts to sweat during isoelectric focusing.

Sample Preparation: Casein Isolation and Plasminolysis

The procedures described in the EU-Reference Method [1], German Official Method [2] or German Normative [3] are generally referred to. Alternatively, the following rapid procedure developed by the author [4] of the above cited methods may be used:

1.Isolation of Caseins from Milk, Yoghurt or Cheese

To 10 g of homogenized or grated cheese, 5g reference material (CRM599, Institute for Reference Materials and Measurements, Retieseweg, B-2440 Geel, Belgium), 20 g yoghurt or 20 ml milk add 60 ml of 1 mol/l ammonium acetate buffer pH 4.6 (make up 67 ml 96% acetic acid + 43 ml 25% NH_3 to 1000 ml with bidist. H_2O, readjust pH if necessary). Homogenize thoroughly with an Ultra-Turrax and centrifuge (5000 Upm, 5 min). Remove topping fat layer and decant the supernatant whey, wipe away fatty residues from the tube wall with laboratory paper soaked in acetone. Homogenize casein residue in 50 ml 1 mmol/l ammonium acetate buffer pH 4.6 (dilute 1mol/l stock solution by 1:1000), centrifuge again and decant supernatant. Remove any fatty residues as described above and finally homogenize casein residue in 50 ml of acetone and filter through a high speed filter. Wash the casin residue on the filter with acetone applied from a squeezing-bottle (ca. 120 ml). Leave the casein residue to dry in the air for a few minutes, then spread the still acetone containing casein cake with the aid of a spatula and let the finely grated powder dry in a fumehood. Break up any particles formed during the drying step with a glassrod or spatula and store the dry casein powder at room temperature.

2.Modified Plasminolysis Designed for DryGel IEF and Silver Staining

Dissolve 2 mg finely ground casein powder in 0.5 ml 0.1 mol/l ammonium carbonate buffer (titrate a 0.2 mol/l ammonium hydrogencarbonate solution (1.58 g/100 ml water) containing 0.04 mol/l Na_4-EDTA (1.52 g/100 ml), with a 0.2 mol/l ammonium carbonate solution (1.92 g/100 ml) containing 0.04 mol/l Na_4-EDTA to pH 8) in an 1.5 ml Eppendorf-Vial during 10 min of ultrasonic treatment. Incubate at 40 °C for 5 min and

then add 5 µl of Plasmin (5U/ml; Boehringer-Roche 602370). Plasminolysis of caseins is performed during 1 h at 40°C with continous shaking and finally stopped by the addition of 5 µl ε-aminocaproic acid solution (dissolve 2.624 g 6-amino-n-hexanoic acid in 100 ml of 40 % (v/v) ethanol) and the solution is placed in an ice bath to cool at 4°C for several minutes.
To this solution (500 µl) carefully add 50 µl of 1 g/ml (100%) trichloroacetic acid, await the release of CO_2-bubbles, the close and - after gently mixing - carefully reopen the vial again to reduce pressure built up by further release of CO_2 . Repeat this step once and finally leave on the ice bath for 5 min to completely precipitate the protein. Afterwards, the protein is recovered by centrifugation (14000 rpm, 5 min). The supernatant is removed thoroughly and the protein precipitate is shortly washed in the vial with 200µl of bidist.H_2O. After additional centrifugation (14000 rpm, 1 min), the washing liquid is removed and the residue is dissolved in 0.5 ml protein dissolving buffer (dissolve 3.45 g glycerol (87 % w/w), 14.42 g urea ultrapure (ICN or Merck) and 150 mg dithiothreitol (ICN) in bidist. H_2O and make up to 30ml, in case of the *Casein-IEF Consumables Kit*: Add 3 ml Sample Colour and then fill up to 30 ml) and sonificated for 2-5 min. The sample solution may be stored at -20°C until use (max. 1month). If less sensitive CoomassieBlue-staining is preferred, the amount of casein is increased to 25 mg and 50 µl of plasmin and 50µl of ε-aminocaproic acid solution have to be applied accordingly.

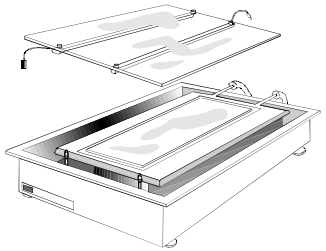

Fig. 5: Placing the gel on Multiphor´s cooling plate

Isoelectric Focusing and Sample Application

Gel-applcation: 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 focusing electrode cables to the plugs in the chamber, close safety lid and begin with prefocusing (table 1 or table 2).

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 12°C. Attention: At colder temperatures the highly concentrated urea will begin to form crystals on the gel-surface.
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. Small variations of the gel´s conductivity yield in highly changed volt-values.
Anyhow, the commonly used method is to limit the voltage via the mA and the Watts achieved in the gel during the run.
With older power supplies you should chose this method given in table 1.
Better and more reproducable results can be achieved by driving the power supply actively along a given volt-time curve. This method is available in all modern power supplies and is called "Ramping" or "Volt Level changing". See table 2 and figure 6.
In both cases the IEF is stopped after the first phase to apply the samples:
After switching off the power, open the chamber again and apply 20 µl of sample solution pipetted onto IEF application pieces at a distance of 5 mm from the anode and less than 1 mm from each other. True internal standard may be added by pipetting 1µl of plasminolyzed bovine γ-casein onto a 1mm sample applicator piece and placing it on top of the unknown sample. This procedure greatly improves identification of bovine γ-caseins.Note: Do not use silicon rubber bands for sample application as the adhesion is reduced in the presence of urea or detergents!
Focusing is then continued with step 2 of table 1 or 2).

Running Conditions (12°C)

Table 1 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 1	SET	*Start Value*	SET	SET	Time	
Step 1:	1200 V	*(675 V)*	12 mA	12 W	30 min	prefocusing
Step 2	800 V	*(800 V)*	10 mA	10 W	20 min	Sample entrance
Step 3	2500 V	*(1000 V)*	10 mA	25 W	100 min	focusing
Step 4	3000 V	*(3000 V)*	12 mA	35 W	30	sharpening

Note 3: For a half gel take the same voltage and half of the mA and W