EPOetins with EPO-IEF DryGel Kits

EDC
V1 10/18

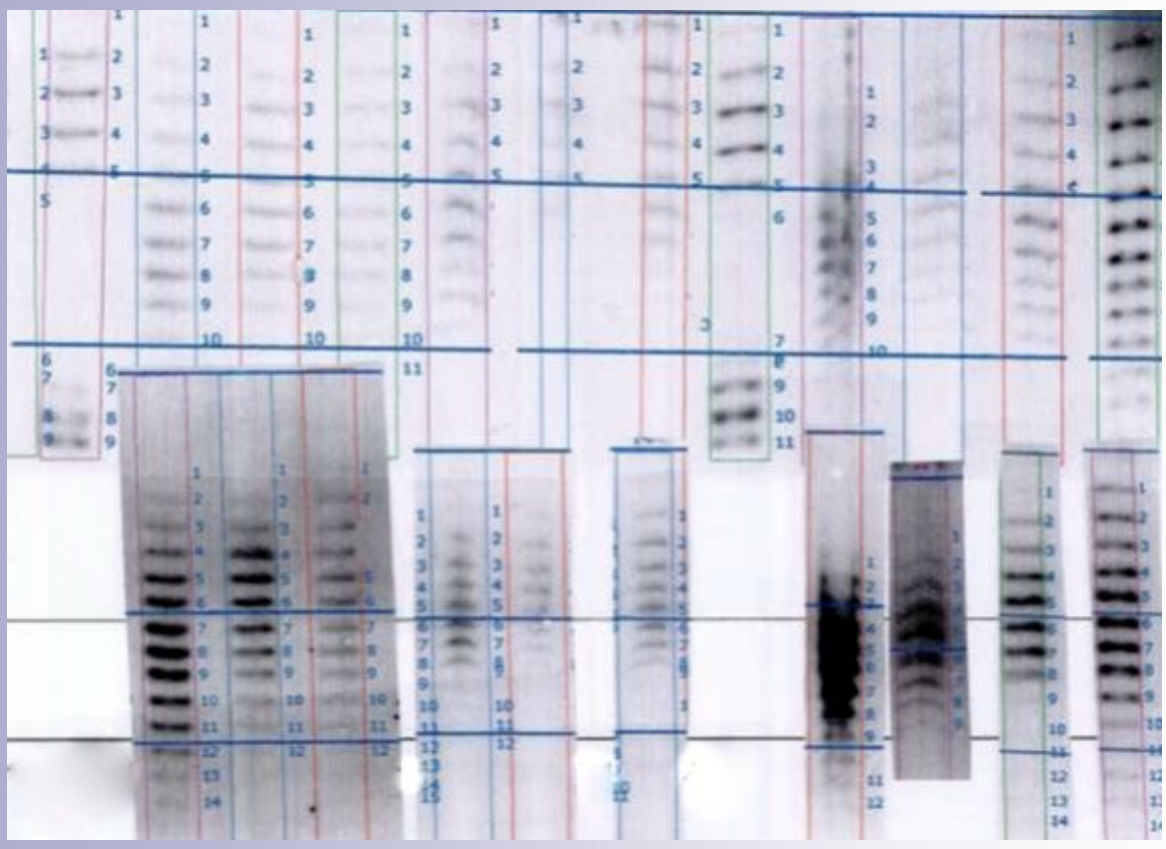


Fig.1: Diagnosis of Epoetins using the EPO-IEF DryGel Kit

***General**:DryGels IEF 40S and 24S are 0.65 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 also for denaturing focusing (7 M Urea), figure 1.*
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 (DryPool Combi) in the delivered carrier ampholyte solution with the added Urea amount.
As visualization technique luminescence is recommended:
Coomassie and Silver-staining are also described.

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

These kits include:
Gels:
DryGel IEF 24S: 4 gels for nat. & denat. IEF, 24 slots à 20 µl, 0,65 mm *(edc-4031)*
or:
DryGel IEF 40S: 4 gels for nat. & denat. IEF, 40 slots à 10 µl, 0,65 mm *(edc-4030)*

DryGels IEF NF on demand.

Chemicals:
4 x Urea, 4 x special SepaLytes epo

Additionally required
DryPool Combi (*edc-me-d*)
Tray for rehydration of dry gels

Denaturing Isoelectric Focusing

1. Take the 15 ml Falcon tube with the carrier-ampholyte solution and cast it into the urea-containing 50 ml Falcon tube.
2. Shake it for 1 h in 37°C until all the Urea is dissolved clearly.
3. Purge the 15 ml tube with this solution and castn it back in the 50 ml tube. All the amholyte-solution will be now in the 50 ml tube.

Volume should be in the end ~23 ml. See fig.2.

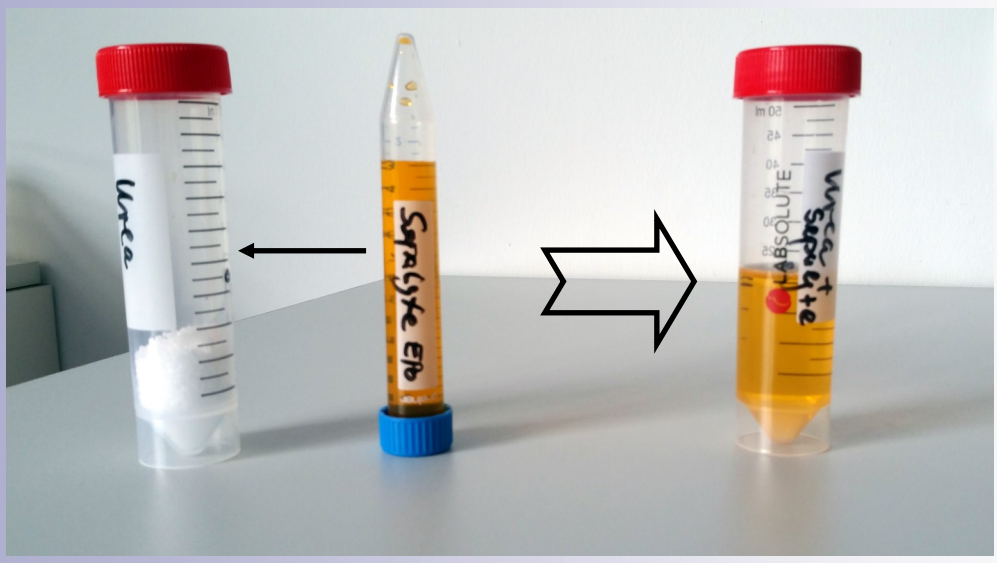


Fig.2: Mixing the rehydration solution (preliminary picture)

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 DryPool Combi onto a horizontal table. Clean it with distilled water and tissue paper.

Carefully cast the 23 ml rehydration volume into the pool.

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. 3) 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 4.

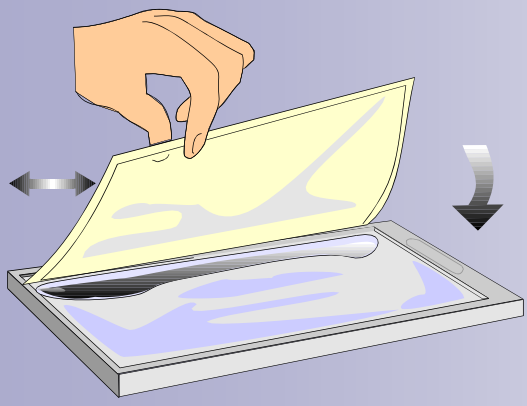


Fig.3: Placing the DryGel into the DryPool Combi tray

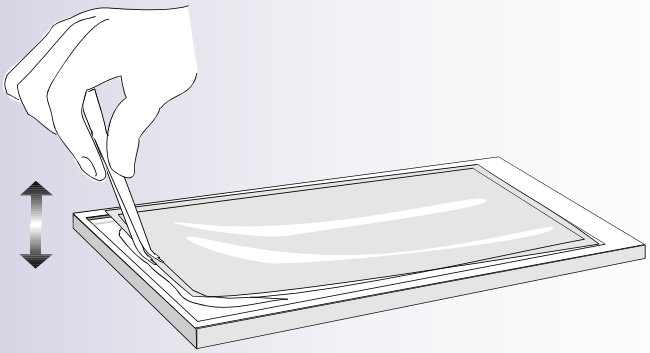


Fig.4: Lifting the edges for an even distribution of the liquid...

End of the rehydration process is after 120 min.

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	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: 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

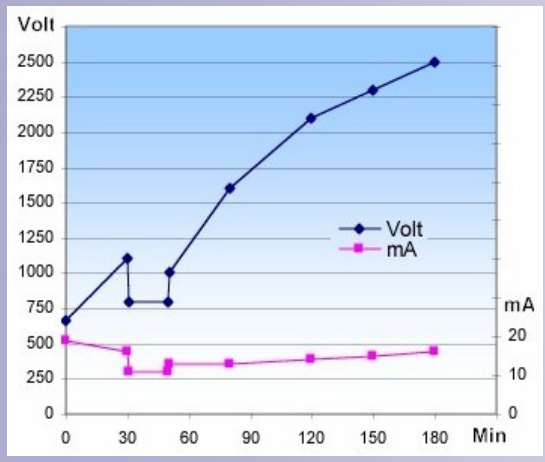
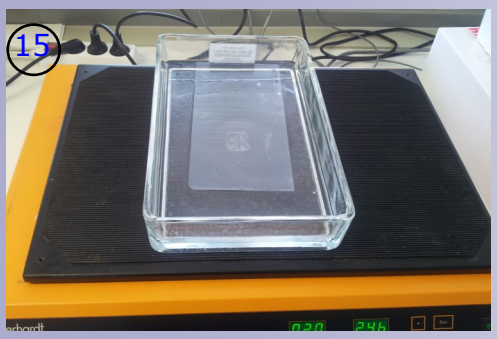


Fig.5:.Volt-time curve of the denaturing run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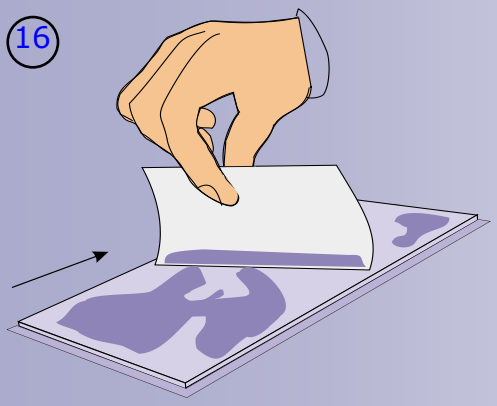
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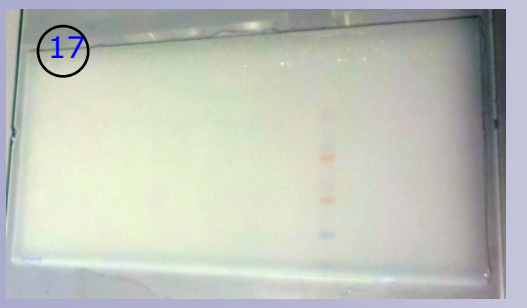
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Contact Blot or Capillary Blot

A) Without the BEO Dry Blotter

The most simple blotting method SAR electrophoresis is the "Contact Blot". Using this transfer-method the plastic -support of the SDS-gel must not be removed.
Because water must pass the membrane, a hydrophilic material is recommended: Nitrocellulose, 0.45 µm pore size (alternatively: PVDF-membranes (Teflon) will work also.
Please note that SAR-gels - have to be equilibrated in dist. water to amend the loss of water during electrophoresis and to flush out the electrophoretic buffer and additives.
This functions best when the gel is floating on the water's surface with the gel down. Figure 15 or 21
After the electrophoretic procedure the blotting membrane is directly layed on the gel-surface. With the help of the BEO-Dry Blotter the SAR-proteins are forced to migrate on the membrane.
For SAR-proteins run in a 10 % T gel nice results are achieved up to 70 –100 kD.
The procedure can be tested using the prestaind MW-size marker: After the blotting ist should be completely on the membrane and out of the gel, see fig. 17.
This blotting method is no quantitative technique, but sufficient for many qualitative tests.
Cut the necessary filter papers (5 x Whatman´s No.1) and the blotting membrane to the size of the gel (25.5 cm x 11.5 cm).

⇒ The gel should be first equilibrated 20 min in dist. Water to amend the gel's lost water content, buffers and additives. Gel floats on the surfacefilm-side up!, Fig 15.
⇒ Then dry the gel's surface with a Drying Cardboard. Figure 16.
⇒ The PVDV-membrane is activated in 100% methanol for 10 minutes. This step is not necessary for Nitrocellulose-membranes. The membrane is then placed in dist. Water for 5 min.
⇒ The SAR-gel is placed on a glassplate with the support-film down. Fig.17.
⇒ The membrane is layed on the gel-surface, avoiding air-bubbles! Fig.18.
⇒ Five dry and clean filter-papers are layed onto the membrane. Fig.19.
⇒ An additional glas-plate is applied on the stack.Fig.19.
⇒ A 2-3 kg weight is standing on the contact-blot for 2h Fig. 20.

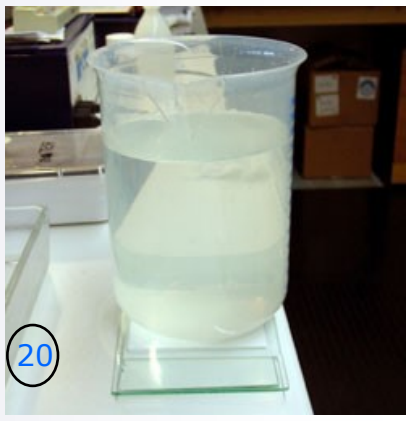
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Fig.21: *Tray for washing the gel*

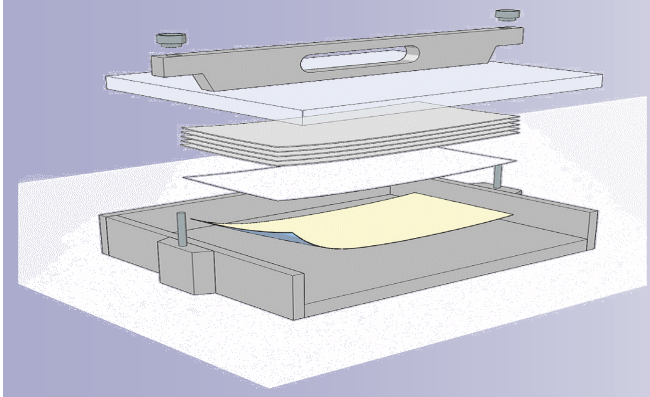

Fig.22: *Blotting batch in the BEO-Blotter*

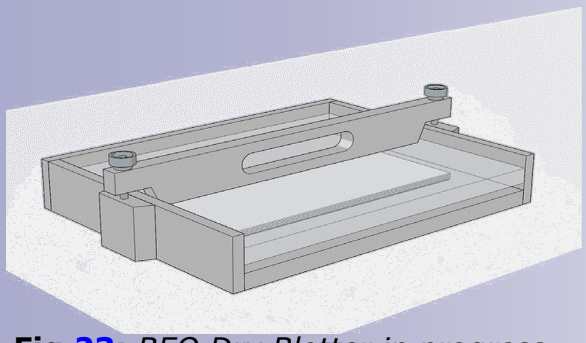

Fig.23: *BEO Dry Blotter in progress*

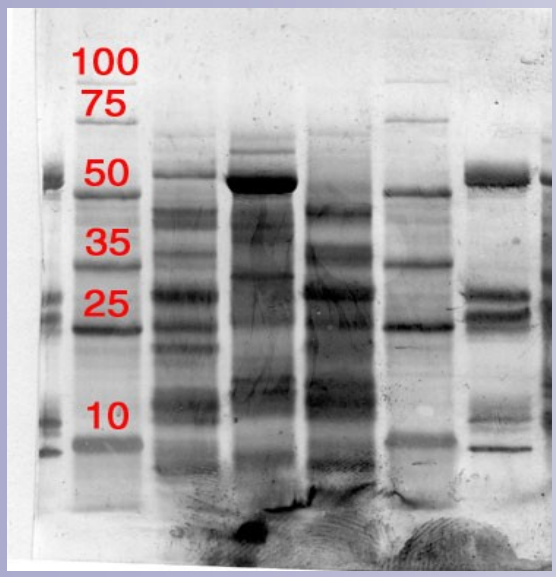

Fig.24: *Contact blot out of a 12.5 % T gel, transfer: 5-75 kD*

B) Using the BEO Dry Blotter (See reference [1])

After electrophoresis the SAR gels were equilibrated in pure water for 15 min and then the proteins were blotted on an Immobilone-P membrane. Figure 21.
Best washing effect is achieved when the gel is swimming on the water's surface—gel-side down

With the help of dry blotting paper and pressure the proteins are forced to migrate on the membrane.

Blotting Procedure (see Figure 22)**:**

The protein transfer was performed for 2 h by capillary blotting using the Beo Dry Blotter.

The membrane was placed on top of the gel with additionally three sheets of wet and 12 sheets of dry blotting paper (Munktell, Falun, Sweden). Figure 23.

Chemoluminescence detection of Epoetins on the Blot-film (from reference [1])
After transfer the membrane was first incubated in 5 M DTT/PBS (45 min, RT), washed with PBS (3 x 1 min) and then placed in the trays of the BlotCycler.
All subsequent steps were performed at 4°C to 8°C.
After blocking in 5% milk/ PBS (70 min) the membranes were washed and incubated for 5 h in a solution of the primary antibody (mouse monoclonal anti-EPO antibody, clone AE7A5, 1 µg/ ml) in 1% milk/PBS.
The membranes were washed (PBS; 9 x 5 min) and incubated with secondary goat anti-mouse IgG (H+L), conjugated with a horseradish peroxidase (1:100.000, 1% milk/ PBS, 11 h).
Immediately after washing with PBS (9 x 5 min) the membranes were incubated in chemiluminescence substrate (Immobilon Western HRP Substrate). Alternatively, these steps can be performed manually at room temperature with reduced incubation time for the secondary antibody.

Scanning
The images were acquired using a CCD camera (LAS-4000; Fujifilm, Tokyo, Japan). Finally, images were analysed using GASepo (version 2.1) software.

References:

[1] Christian Reichel, Friedrich Abzieher and Thomas Geisendorfer:
SARCOSYL-Page: a new method for the detection of Mircera– and EPO-doping in blood. Drug Test. Analysis 2009, 1, 494–504
www.drugtestinganalysis.com, DOI 10.1002/dta.97

[2] Blotting of horizontal gels.
EDC application note:
http://www.electrophoresis-development-consulting.de/blothor.pdf

[3] Blotting with the BEO-Dry-Blotter, Dyeagnostics application note:
http://www.dyeagnostics.com/site/wp-content/uploads/2011/01/Western-Blotting_22_11_13_DE.pdf

[4] Imaging using Octoplus, Dyeagnostics brochures:
http://www.dyeagnostics.com/site/products/octoplus-2/
http://www.dyeagnostics.com/site/wp-content/uploads/2012/05/OctoplusQPLEX_eBrochure_170613.pdf

[5] Dirk Schwenke, Institute of Doping Analysis and Sports Biochemistry Dresden, Kreischa, Germany. dirk.schwenke@idas-kreischa.de
Improved detection of EPO in blood and urine based on novel Velum SAR precast horizontal gels optimized for routine analysis.
Schwenke 2015 Application Note DOC15012015 - Improvements for EPO Detection

General Detection of protein bands

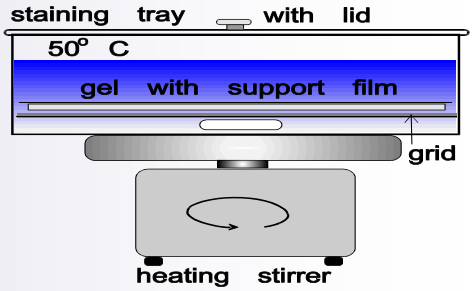

Fig.8:. Hot Coomassie-staining

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)

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

EDC's IEF Coomassie staining: Coomassie Violet
http://www.electrophoresis-development-consulting.de/html/coomassieviolet.html

2. Silver Staining

Silver-Staining is performed **at ambient temperature**
Adapted staining programme [1]

Staining solutions and programme:

Table 2:

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

References:

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