INSTRUCTION MANUAL

SERVALYT™ PRECOTES™ Hp KIT

(Cat. No. 42805.01)

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Contents

1. SERVALYT™ PRECOTES™ Hp KIT

1.1. Kit contents

Kit contains:	5 SERVALYT™ PRECOTES™ Hp gels (Gel format**:** 245 x 125 mm, 300 µm) Anode- and cathode buffer Electrode wicks Applicator strip Manual

1.2. Storage conditions

The recommended storage temperature is 2 - 8 ℃. If stored at the recommended temperature at least usable until: see expiry date on package.

2. Sample preparation

2.1. Solutions

- **Precipitation solution** (solution is stable for 1 month at 4 ℃)
  8.0 g polyethylene glycol 6000 (Cat. No. 33137)
  1.7 g NaCl (Cat. No. 30183)
  0.2 g NaN_3 (Cat. No. 30175)
  fill up to 100 ml with demin. H_2O

- **Sodium chloride solution** (solution is stable for 1 month at 4 ℃)
  0.9 g NaCl (Cat. No. 30183)
  in 100 ml demin. H_2O

- **Reduction reagent (prepare always fresh!!)**
  1.0 g urea (Cat. No. 24524)
  1.2 ml borate buffer (see below)
  22 mg dithiothreitol (Cat. No. 20710)
  fill up to 100 ml with demin. H_2O

- **Borate buffer** (solution is stable for 1 month at 4 ℃)
  0.62 g (Cat. No. 15165) are dissolved in
  80 ml H_2O and titrated to pH 8.8 with
  0.1 N NaOH solution and filled up to 100 ml with H_2O

- **Iodoacetamide solution** (solution is stable for 1 month at 4 ℃)
  100 mg Iodoacetamide (Cat. No. 26710)
  in 1 ml demin. H_2O

2.2. Procedure

1. After thawing, mix serum well. Eventually spin shortly.

2. 160 µl precipitation solution
   100 µl serum
   25 µl Hp-antiserum (Dako, Cat. No. Q 0330, rabbit-anti-human Hp IgG fraction)
   The sample is stored overnight in the refrigerator.

3. Centrifuge the precipitation mixture for 3 minutes at 3000 rpm.

4. Carefully remove the supernatant.

5. Resuspend precipitate in 1 ml sodium chloride solution and mix thoroughly.

6. Centrifuge precipitate for 3 minutes at 3000 rpm.

7. Carefully remove the supernatant.

8. Resuspend precipitate with 40 µl of reduction reagent **(prepare always fresh)**, mix thoroughly and incubate for 45 minutes at 37 ℃.

9. Dilute solution with 16 µl iodoacetamide solution.

10. 15 minutes (at room temperature) after adding iodoacetamide the sample is ready to use for isoelectric focusing or may be frozen at –20 ℃ .

3. Electrophoresis

3.1. Electrophoresis – short protocol

Gel format	245 x 125 mm, 300 µm gel thickness
Electrode fluids	
Anode:	Anode fluid 3 (Cat. No. 42984)
Cathode:	Cathode fluid 10 (Cat. No. 42986)
Cooling	Adjust to + 5 ℃
Focusing parameters	2000 V, 16 mA, 25 W
Pre-focusing (Without sample)	20 minutes
Sample application	1,5 cm away from the outer edge of the Precote cathode
Sample volume	15 - 20 µl
Removal of paper applicator pieces	45 minutes **after** pre-focusing
Total duration including Pre-focusing	4000 Vh or 3 hours

3.2. Step-by-step protocol

1. Pre-cool the cooling plate to 5 ℃.

2. With a pipette, add 2 ml kerosene (Cat. No. 26940) to the centre of the cooling plate (kerosene serves as cooling agent between cooling plate and Precote).

3. Remove the Precote from its carton and cut open the aluthene bag on three sides (two long and one width) using scissors.

Note: ***Rubber gloves must be worn when handling acrylamide gels.***

4. Role the Precote **with cover sheet** onto the cooling plate (with imprint uppermost), avoiding air bubbles. Remove any excess kerosene at margins with tissue paper.

5. Take two paper electrode wicks from the pack and lay them on a smooth, clean surface (e.g. glass plate).

6. Impregnate evenly the electrode wicks: **2 ml** anode fluid 3 (red cap), **2 ml** cathode fluid10 (black cap)

Note: ***Impregnated electrode wicks should not be dabbed with blotting paper. The volume of 2 ml relates to the original length of an electrode wick.***

7. Remove cover sheet with pointed forceps.

8. Place the impregnated electrode wicks on the gel. Anode wick along the **red** line marking, cathode wick along the **black** line marking.

9. Put the **minus** electrode on the cathode wick, and the **plus** electrode on the anode wick. Eventually put a heavy glass plate on top.

10. Close the lid of the chamber and connect to power equipment.

11. In the "Set" modus (limitation of parameters) enter the following settings:

    2000 Volt, 16 mA, 25 Watt

12. Start isoelectric focusing without samples (pre-focusing). **Duration: 20 minutes**

Note: ***After switching on, voltage should be between 250 V and 350 V and should rise to 400 V to 600 V after 20 minutes.***

13. After 20 minutes, electrophoresis is interrupted and the chamber opened.
14. Position the sample application pieces with the **narrow end** in the running direction.

15. The sample application pieces must be placed **exactly 1.5 cm** away from the **outer edge** of the Precote cathode.

16. **15 µl – 20 µl** of the respective samples are added to the paper applicator pieces by means of a pipette (see sample preparation) and electrophoresis is started again.

17. After **45 minutes** electrophoresis is interrupted and the sample applicator pieces are **carefully** removed using pointed forceps.

18. After **4000 Vh** or **a further 120 minutes**, electrophoresis is completed. The electrode wicks are removed from the gel with forceps and the IEF gel is **immediately** transferred to a 20 % trichloroacetic acid.

Note: ***To allow better fixing and any further staining or destaining of the proteins, it is imperative that the Precote is moderately agitated in the respective solutions by a shaker. After being used three times, the trichloroacetic acid should be safely disposed of.***

19. For staining and destaining with SERVA Violet 17 see enclosed instructions.

General note:
If the number of samples does not require a whole gel, the gel may be cut in half with scissors (remove cover sheet before cutting). The remaining gel is then enveloped in the cover sheet and refrigerated at 4 ℃ in a plastic b ag, as airtight as possible, until required.

In this case **reduce** the electrophoresis parameter **amperage and power** to half, **voltage** remains **unchanged**.

4. Protein staining: SERVA Violet 17

4.1. Reagents and solutions

- **Fixing solution**
  200 g trichloroacetic acid (Cat. No. 36910)
  fill up to 1000 ml H_2O dest.

- **Staining stock solution 1**
  1 g SERVA Violet 17 (Cat. No. 35072)
  ad 500 ml H_2O dest.

- **Staining stock solution 2**
  136ml H_3PO_4 86 %, ad 1000 ml H_2O dest.

- **Destaining solution**
  18 ml H_3PO_4 86 %, ad 1000 ml H_2O dest.

4.2. Staining procedure

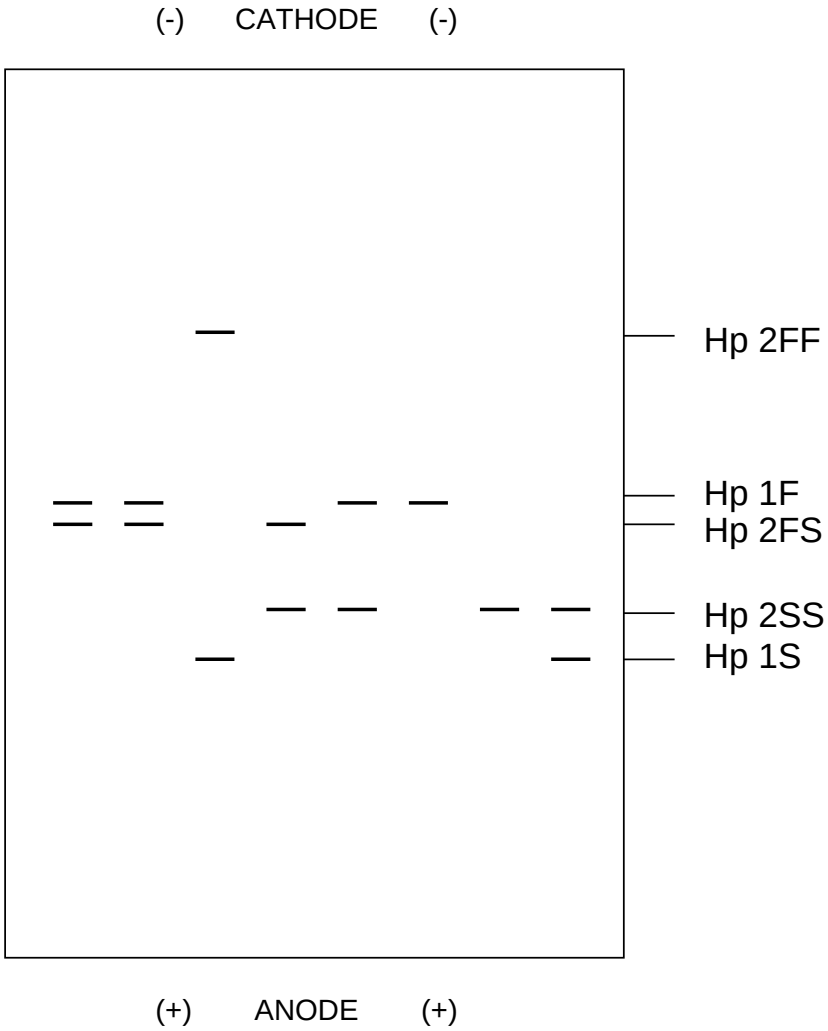
Fixing — On completion of electrophoresis the electrode wicks are removed with forceps and the IEF gel is **immediately** transferred to 200 ml of 20 % trichloroacetic acid.
Duration: 20 minutes
Note: *To allow better fixing and any further staining or destaining of the proteins, it is* ***imperative*** *that the Precote is moderately agitated in the respective solutions by a shaker. After being used three times, the trichloroacetic acid should be safely disposed of.*
Protective goggles and rubber gloves must always be worn when handling trichloroacatic acid.

Rinsing — After fixing, the gel is transferred to 200 ml demin. H_2O.
Duration: 1 minute

Staining	Mix 100 ml staining stock solution 1 und 100 ml stock solution 2 **<u>immediately prior to use</u>** and stain Precote. **Duration: 10 minutes**
Destaining	Destain Precote 2 to 3 times in 200 ml destaining solution until background becomes clear. **Duration: 10 minutes each**
Neutralizing	Neutralize Precote twice in demin. H_2O or 1 % glycerol. **Duration: 2 minutes a time**
Drying	Dry Precote in a warm airstream or overnight at room temperature.

5. Interpretation

The figure below illustrates the pattern of the most common Haptoglobin subtypes after separation by isoelectric focusing using **SERVALYT™ PRECOTES™ Hp**.



Frequencies of the subtypes (2):

Subtype	Frequency	Subtype	Frequency
1F	0.0217	1F-2FS	0.1697
1F-1S	0.0728	1S-2FS	0.2846
1S	0.0610	2FS-2SS	0.0323
1F-2FF	0.0005	2FS	0.3319
1S-2FF	0.0008	1F-2SS	0.0083
2FF-2FS	0.0018	1S-2SS	0.0139
2FF-2SS	0.0001	2SS	0.0008
2FF	0.0001		

6. Literature

1. Javid J (1978)
   Human Haptoglobins
   Current Topics in Hematology **1**, 151 - 192

2. Berghaus G, Pflug W, Madea B (1995)
   Elektrophoretisch nachweisbare Serum-Polymorphismen
   in: Schleyer F, Oepen I, Henke J (eds.): Humanbiologische Spuren. Sicherung, Nachweis und Analyse in Kriminaltechnik und forensischer Medizin
   Kriminalistik Verlag, Heidelberg 1995, 72 – 102

7. Trouble shooting

Problem	**Possible cause**	**Countermeasure**
Isoelectric Focusing		
No current	Circuit not closed	Remove cables from power supply and check electrophoresis chamber
Low current	Bad contact between electrodes and wicks	Weight electrodes e. g. with a glass plate
Condensation	Too much power	Check settings at power supply and reduce power to 20 W (10 W). Extend focusing time
	Insufficient cooling	Check cooling temperature of the chamber's cooling plate and the connected cooling bath. Remove trapped air bubbles between gel and cooling plate if necessary
	Salt concentration of the sample too high	Desalt sample by dialysis, ultra filtration or gel filtration
Gel burns through	See "condensation", gel dries up	Try to stop condensation at the very beginning of the run
	Gel overloaded	Reduce protein concentration or sample volume, dilute sample if necessary
Gel burns through at one edge	Electrode wicks longer than gel size	Electrode wicks must not protrude beyond the gel
Distorted bands	Poor soluble or precipitated proteins, high salt concentration in the sample	Spin sample
	Gel overloaded	See above

Problem	Possible cause	Countermeasure
Blurred bands, not separated or missing bands	Application place too near to isoelectric point	Place sample applicator pieces exactly 1.5 cm away from outer cathodic edge
Staining		
Gel comes loose of supporting film	Gel too long incubated in TCA	If necessary store gel in 50 % Ethanol/10 % glacial acetic acid
Blue background	Carrier ampholytes not completely washed out	Prolong fixation in TCA
Blue precipitation on gel	TCA precipitates dye	Rinse gel with water after fixing thoroughly
Green background	Concentration of dye too low	Prepare a new stock solution I, solve dye completely
Green background, greenish bands that fade during drying	PH value of gel too low after destaining procedure, gel was not neutralized properly	Neutralize and rinse gel with water or glycerol (1 %) or re-stain gel