



Immunoblotting of von Willebrand Factor Polymers in Plasma and Platelets using Peroxidase Conjugated Antibody and Chemiluminescence Detection

Principle

Patient platelet-poor-plasma proteins are partially denatured using heat and an anionic detergent, Sodium Dodecyl Sulfate (SDS). This detergent binds to all plasma proteins. The amount of anion bound to the protein is directly proportional to the size of that protein. Thus, when SDS treated plasma proteins are subjected to an electric current they will migrate towards the positive electrode (anode). If forced to move through an agarose "sieving" medium, smaller proteins will migrate faster than larger proteins, and a size dependent distribution of plasma proteins is the result. After electrophoresis, the proteins are transferred out of the gel onto nitrocellulose membrane. The nitrocellulose is then blocked and probed with a peroxidase-conjugated goat anti-vWF antibody. The vWF bands are then visualized using the Amersham Enhanced Chemiluminescent Detection System. Normal polymers of von Willebrand factor range in size from 1 to 14 million Daltons in molecular weight. In some variants of von Willebrand's disease, the larger polymers are missing from the plasma. The gels of higher concentration agarose (3%) are required if triplet substructure is to be defined, while gels of lower agarose concentration (1.5%) allow more straightforward identification of type II variants.

Equipment Required

- Power supply, capable of delivering up to 100 V., 0.1 A.
- Horizontal slab gel electrophoresis apparatus (LKB Multiphor 2117).
- Boiling water bath or Microwave oven.
- 60°C water bath.
- Anti-condensation lid, Plexiglas, 110 x 250 x 4 mm.
- Gel pouring kit (gel should be poured using a "sandwich" method).
- Whatman 3 MM filter paper.
- Small (1mL) plastic test tubes with caps.
- Container for washing gel, at least 14 x 28 cm.
- PVDF membranes (Millipore Corp.) Used as per manufacturers instructions.
- Transphor Electroblothing Unit (LKB #2005 or Hoefer #TE62X) or equivalent, complete with power supply, cassettes and sponges.



Preparation: (Note: use Milli-Q water or comparable purity for all reagents.)

- Stacking gel buffer: 0.125 M Tris-HCl, pH 6.8
- To make 500 ml: 7.57 grams Tris to 500 ml water. pH to 6.8 with HCl. (Note : add SDS to 0.1% (w/v) just before use.)
- Separating gel buffer: 0.375 M Tris-HCl, pH 8.8
- To make 1 liter: 45.41 grams Tris to 1 liter water. pH to 8.8 with HCl. (Note: add SDS to 0.1% (w/v) just before use.)
- Tray buffer: 0.05 M Tris, 0.384 M Glycine, 2.5 g SDS (0.1% (w/v)), pH 8.35
- To make 2.5 liters: 15.0 grams Tris, 72.0 grams Glycine, 2.5 grams SDS, adjust pH to 8.35 if necessary with 1M HCl or 1M NaOH.
- Sample prep buffer:
 - Stock: 0.1 M Tris-HCl, 0.01M EDTA, pH 8.0 Store at 4°C.
 - To make 100 ml: 1.21 grams Tris, 0.372 g Na₂EDTA, pH to 8.0 with HCl.
 - Working Solution: 0.01 M Tris-HCl, 0.001 M EDTA, 2% (w/v) SDS pH 8.0
 - For 100 ml: 10 ml stock solution, 90 ml water, add 2.0 grams solid SDS.
- PBS: 8.0g NaCl and 1.15g Na₂HPO₄, 0.2g of KH₂PO₄, and 0.2g of KCl, up to 1 litre, pH to 7.4.
- PBS-Tween: 0.25 ml Tween 20 to 500 ml PBS.
- PBS-2% (w/v) BSA (Blocker): 10 g BSA to 500 ml PBS, readjust pH to 7.4.
- Transfer Buffer: To make 6 liters: Add 42.59 g Na₂HPO₄, 2.4 g SDS, pH to 7.4 with 1 M H₃PO₄. Store at room temp.
- Probing Buffer: 5% (w/v) Carnation Skim Milk Powder in PBS + 0.1% (v/v) Tween-20. Adjust pH to 6.5 with 1 M H₃PO₄. Centrifuge at 3500 X g for 30 minutes just before addition of antibody.
- Bromphenol Blue: 1% (w/v) Bromphenol blue in water.

Collection and Handling of Specimens

- A. 4.5 ml of venous blood is drawn into a plastic syringe and anticoagulated with 3.8% trisodium citrate (4.5 ml blood to 0.5 ml trisodium citrate). Patient must be fasting.
- B. Platelet poor plasma (PPP) is prepared by centrifuging the specimen in table-top centrifuge at 3000 rpm (100 x g) for 10 minutes at room temperature.
- C. PPP is carefully removed with a plastic transfer pipette, placed in a plastic test tube and frozen and stored at -20°C or below.
- D. Sample preparation for platelet vWF multimeric analysis:
 - 1) Collect blood as above, but spin for 5 min @ 180 X G
 - 2) Perform platelet count on the PRP and calculate volume required to give total of 2.5x10⁸ platelets.
 - 3) Spin required volume of PRP in a microfuge for 1 minute to achieve pellet of 2.5x10⁸ platelets.
 - 4) Save supernatant and freeze (for plasma vWF analysis) and freeze platelet pellet
 - 5) Thaw pellet and add 900 ul H₂O.
 - 6) Freeze & thaw 5 times (to lyse platelets).
 - 7) Spin in microfuge, 1 min & remove supernatant (contains released vWF)
 - 8) Add equal volume of 0.5% Triton X-100 (v/v) in 5% SDS (w/v) and incubate for 15 minutes at 60°C.
 - 9) Spin in microfuge and apply to gel as for plasma.
(Note: Can use 1x10⁸ platelets but scale everything down.)



Method

A. Pouring the gel ("sandwich" method):

1. The sandwich or mold consists of two glass plates, a Ushaped spacer, 1 mm thick and four clamps.
2. Place 1 mm spacer between the two glass plates. Clamp assembly together and place mold(s) in 60°C oven for 15 minutes.
3. For the separating gel, weigh appropriate amount of agarose (see below) and place in 50 mL glass tube containing 20 ml separating gel buffer. Cap tube to prevent evaporation. Place in boiling water bath or microwave to thoroughly dissolve the agarose. Add 0.2mL 10% (w/v) SDS. Next place tube in 60°C water bath for at least 10 minutes.

For desired gel, add the following weight agarose to 20 ml separating gel buffer:

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| 1.6% gel: | 320 mg agarose (routinely used) |
| 2.0% gel: | 400 mg agarose |
| 2.25% gel: | 450 mg agarose (best for triplets) |

4. Remove mold from oven. Place mold vertically on bench with opening facing up. Pour 20 ml agarose into mold. Do not inject air bubbles. Allow gel to cool to room temperature for a minimum of 40 minutes.
5. Disassemble mold and remove top plate. Cut gel to a length of 8.6 cm. Reassemble gel in mold.
6. Prepare stacking gel (0.8% agarose): place 80 mg agarose in a 40 ml centrifuge tube containing 10 ml stacking gel buffer. Cap tube and place in boiling water bath or microwave. After agarose is melted, cool in a 60°C water bath for a minimum of 10 minutes. Add 0.1mL 10% (w/v) SDS.
7. Fill the mold with agarose solution using a pre-warmed disposable pipette. Reserve an aliquot of stacking gel solution to fill the wells of the gel during electrophoresis (keep at 60°C). Allow gel to cool 40 minutes.
8. Disassemble mold and remove top plate. Cut gel to 12 cm length. Carefully cut five evenly spaced wells, each 2 x 11 mm, and remove agarose by suction. Wells are 10 cm from and parallel to the stacking gel separating gel interface.

B. Preparation of the samples:

1. Frozen samples are thawed at 37°C. 25 ul of sample is added to tube containing 70 ul of sample prep buffer and 5 ul of bromophenol blue.
2. Mix diluted sample and place in 60°C water bath for 15 minutes.



C. Preparation of electrophoresis unit and running gel:

1. Place 2.5 liters of tray buffer in beaker. Add 2.5 grams solid SDS. Adjust pH to 8.35 if necessary using 5 M NaOH. Place 1.2 liters in each of the two reservoirs of electrophoresis unit.
2. Pipette a milliliter or two of tray buffer onto the electrophoresis unit's glass cooling plate. Place agarose slab (still on bottom glass plate of mold) on cooling plate so that the stacking gel and the wells are on cathode (-) side. Turn on cooling water. Cooling water should be either running tap water or preferably refrigerated circulation at 10°C. Use five pieces of filter paper on each side as wicks. Dip wicks in tray buffer and place them on gel so that they are parallel to each other. The wicks are placed 10 cm apart.
3. Carefully pipette 14 ul of sample into each well. Install anti-condensation lid and electrophoresis unit cover. Current will not flow without the cover installed; also protects operator from shock. Turn power supply on and set to 12 mA/gel for 45 minutes; this moves the plasma proteins from the well into the agarose. Shut off power and remove unit's cover and anti-condensation lid; fill in wells with stacking gel buffer/agarose using a plastic transfer pipette. Turn on power and continue electrophoresis until dye front reaches anodal wick. Electrophorese at 10 mA/gel for 6 hours or 4.5 mA/gel for about 16 hours.

D. Electroblotting onto nitrocellulose membrane:

1. When tracking dye has reached bottom of gel, turn off power supply.
2. The cassette is assembled per manufacturer's instructions with sponge on bottom, then 3MM filter paper, then gel, nitrocellulose membrane, filter paper and top sponge. Each item should be soaked by dipping in electroblot buffer prior to assembly of the cassette, and kept wet during the assembly. Do not allow air bubbles to become trapped between the gel and nitrocellulose. The cassette is closed and placed in Transphor unit with the nitrocellulose on the cathode side (red) of the gel.

Transfer of protein from the gel to the membrane is performed for 2 hours at 16°C at 500 mAmps.

E. Probing and detection:

1. Turn off power supply. Remove cassette from Transphor unit and trim membrane to the same size as gel. Put a small nick in the membrane to mark the point of application of the first sample. Rinse the membrane briefly in transfer buffer then place in a Tupperware dish containing 50 ml of PBS-2%BSA (blocking solution).
2. Block membrane for 1 hour at ambient temperature on a platform shaker.
3. Rinse briefly with PBS-Tween.
3. Incubate membrane with 50 mL Probing buffer containing peroxidase conjugated antibody (#GAVWF-HRP) at approximately 0.2 ug/ml for 1 hour at ambient temperature on a rocker. The optimal antibody concentration should be determined by titration.
5. Wash membrane with PBS-Tween three times, 15 minutes each.
6. Develop as per instructions for the ECL Western Blotting Detection System, starting at step #12 on page #12 in the ECL Instruction Manual. Typically a 2 min exposure time is sufficient using Amersham Hyperfilm-MP.



References

Budde, U., Schneppenheim, R., Plendl, H., Dent, J., Ruggeri, Z.M., Zimmerman, and T.S.: Luminographic Detection of von Willebrand Factor Multimers in Agarose Gels and on Nitrocellulose Membranes. *Thrombosis and Haemostasis*, 63(2): 312-315, 1990.

Ruggeri, Z. M., Zimmerman, T.S.: Variant von Willebrand's Disease. Characterization of two subtypes by analysis of multimeric composition of FVIII/vWF in plasma and platelets. *J. Clin. Invest.*, 65:1318, 1980.

Towbin, H., Gordon, J.: Immunoblotting and Dot Immunobinding - Current Status and Outlook. *J. Immunol. Meth.*, 72:313, 1984.