

# A rapid and cost-effective method to visualize von Willebrand factor multimers in plasma

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## Abstract

The multimeric analysis of von Willebrand factor (VWF) is utilised for von Willebrand factor disease (VWD) subclass identification and is important for treating purposes. A highly sensitive and rapid method for the visualisation of the multimeric structure of VWF in plasma and platelets was described by Krizek et al in 2000 [1]. This method uses a western blot technique where horizontal agarose electrophoresis is followed by the transfer of the VWF onto a polyvinylidene fluoride (PVDF) membrane. The multimeric pattern of VWF is visualized by immunolocalisation and luminographic detection and no radioactivity is used. We modified this method comprehensively to increase its sensitivity and to reduce the cost and duration of the test. We used one instead of two localisation antibodies and thereby reduced the immunolocalisation time by more than two hours. This also reduced the cost of the procedure. We further reduced the cost by using two carbon plates for blotting in stead of a blotter instrument. A total cost reduction of 40% could be achieved. A higher sensitivity was obtained by degassing the agarose before the casting process. The higher sensitivity is reflected by the fact that differences between the multimer patterns of type 2M and normal patients could be detected.

## Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein that plays a dual role in haemostasis. Firstly, it forms a complex with coagulation factor VIII and it protects it from degradation. Secondly, VWF contributes to platelet adhesion and aggregation by acting as a molecular bridge between sub endothelial collagen and platelets [2]. The ability of VWF to support platelet adhesion and aggregation increases with multimer size. In the circulation multimer size is controlled by several mechanisms including proteolytic cleavage by the VWF cleaving protease, ADAMTS-13 [3, 4]. The steady state concentration of plasma VWF reflects the equilibrium between synthesis and clearance. In addition, the multimer distribution of plasma VWF depends on competition between the mechanisms of clearance and of proteolysis by ADAMTS-13 [4, 5].

Inherited VWD has been subdivided into three types that reflect its pathophysiology. Types 1 and 3 VWD reflect respectively, the partial or virtually complete deficiency of VWF. Type 2 VWD is a qualitative defect that is subdivided into 4 subtypes (2A, 2B, 2M and 2N). Type 2A refers to variants with decreased platelet dependent function and is associated with the absence of high molecular weight multimers. Type 2B refers to variants with increased affinity for platelet glycoprotein 1b. Type 2M refers to variants with decreased platelet dependent function not caused by the absence of high molecular weight multimers and Type 2N to variants with markedly decreased affinity for factor VIII.

No single test is available that provides appropriate information about the various functions of VWF and the laboratory diagnosis of VWD is based on a panel of tests that includes the bleeding time, the measurement of factor VIII coagulant activity (VIII:C), VWF antigen (VWF:Ag), VWF activity as measured by the ristocetin cofactor activity (VWF:RCo) and the collagen binding assay (VWF:CBA), VWF multimer analysis, ristocetin induced platelet agglutination (RIPA) and the factor VIII binding assay of plasma VWF.

The evaluation of the multimeric analysis of VWF is utilised for subclass identification of VWD. It is important to identify the specific subtype, because treatment of VWD is based on the classification of the disease. A highly sensitive and rapid method for the visualisation of the VWF multimers is described [1], which we modified significantly to make it even more rapid, sensitive and cost-effective.

## Materials and Methods

### Preparation of SDS-Agarose Gel

A 0.65% agarose gel is prepared in 100ml Tris-acetate electrophoresis buffer (40 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.8) by melting the agarose until clear. After degassing by vacuum suction, the agarose is poured into a horizontal gel apparatus with a 20 tooth comb in place and after solidification, the gel is placed at 4°C for 30 min.

### Sample preparation

Whole blood of patients with a history of a bleeding tendency who was referred to our Haematology Clinic is collected into two vacutainer tubes containing 0.105 M sodium citrate in a ratio of 1:9 with blood. Platelet-poor plasma is prepared by centrifugation of whole blood at 2000 g for 20 minutes at room temperature. Samples are stored in 200  $\mu$ l aliquots in polypropylene tubes at -70°C until analysed. All tests are performed on aliquots that were not previously thawed. Each plasma sample is thawed at 37°C and diluted 1:30 in sample buffer (0.01M Na<sub>2</sub>HPO<sub>4</sub>, 37 mM iodoacetamide and 1% SDS, pH 7.0). After incubation at 37°C for 60 minutes, 10  $\mu$ l bromophenol blue is added 1:10 to the diluted plasma and centrifuged at 14000 rpm for 1 minute in an Eppendorf centrifuge

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### Electrophoresis

The gel is set in place and the comb removed. Pre cooled electrophoresis buffer is poured onto the gel to overlay it with no more than 2-3 mm and 10  $\mu$ l of diluted sample is loaded into each well. The power is set on 30 mamps (constant amp) for 30 minutes where after it is set at 50 mamps and run for a further 4-6 hours until the dye front had migrated ( $\pm$ 8cm).

### Western Blot

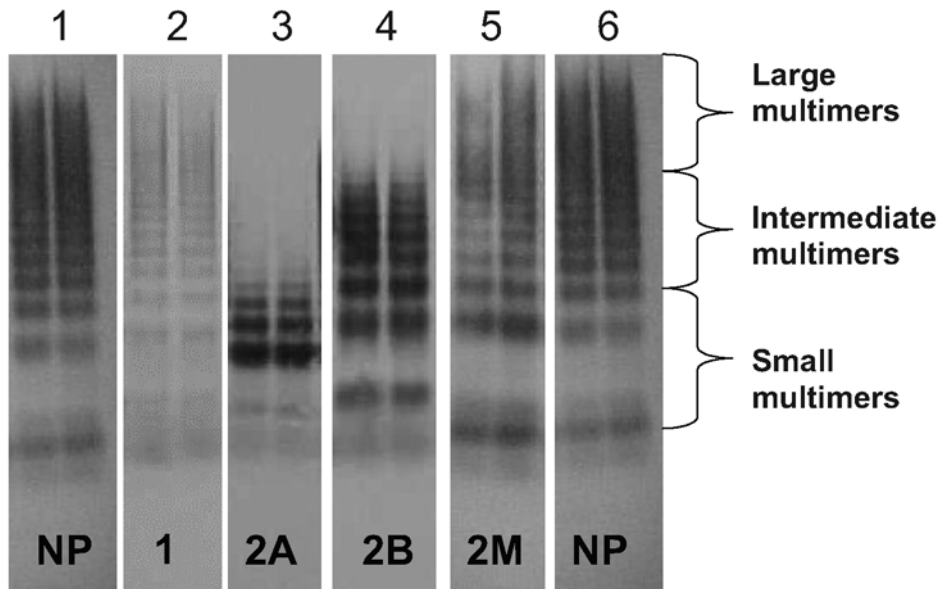
Two litres of transfer buffer (2.5 mM Tris, 19.2 mM glycine, 20% methanol, 0.01% SDS, pH 8.8) are prepared and stored at 4°C. After electrophoresis is complete, the gel is placed in transfer buffer and equilibrated for 30 minutes. Polyvinylidene fluoride (PVDF) 0.45  $\mu$ m membrane are cut only a bit smaller than the size of the gel, pre-soaked in methanol for 1-2 minutes and stored in transfer buffer until use. A western blot "sandwich" is assembled by placing the cathode carbon block with black electrode at the bottom, 2 Scotch Brite pads on top of it, 2 thick filter papers (Whatman CHR3, Whatman International, England), 2 thin filter papers (Whatman no 1), the gel, the PVDF membrane, 2 thin filter papers, 2 thick filter papers, 2 Scotch Brite pads and the anode block with red electrode on top. The pads and filter papers are soaked in transfer buffer before the assembly. The whole sandwich is placed into a plastic container at 4°C and covered with plastic wrap to prevent evaporation. The transfer conditions are 70 mille-ampere (constant ampere) overnight (15-17 hours).

### Blocking and immunolocalization of VWF multimers

After blotting, the PVDF membrane is placed in the blocking agent that contains 5% skimmed milk powder in TBS for 1.5 hours at room temperature. Three washing steps are done with 50 ml TBS-Tween for 1 minute and 3 times 100 ml TBS-Tween for 7.5 minutes each. The membrane is then placed into a 1:4000 dilution of an anti-human VWF-HRP conjugated antibody in TBS-Tween for 1 hour 15 minutes. Washing steps are done as before where after the membrane was placed onto a mat surface with the protein side upwards. Equal volumes of ACL western blot detection reagent 1 and 2 (AEC Amersham, UK) are mixed and poured onto the membrane. The reagent must cover the entire surface of the membrane and must be held to the surface by surface tension. After 1 minute incubation, the excess detection reagent is drained off, the membrane wrapped into plastic wrap and placed in the dark on an x-ray film and exposed for 1-2 minutes. The film is then removed and developed in an automated film developer (Kodak, USA). The density of the high, intermediate and low molecular weight multimers are determined using a Geldoc XR geldocumentation system (Bio-Rad, CA, USA). The total duration of this multimer analysis procedure is less than 28 hours.

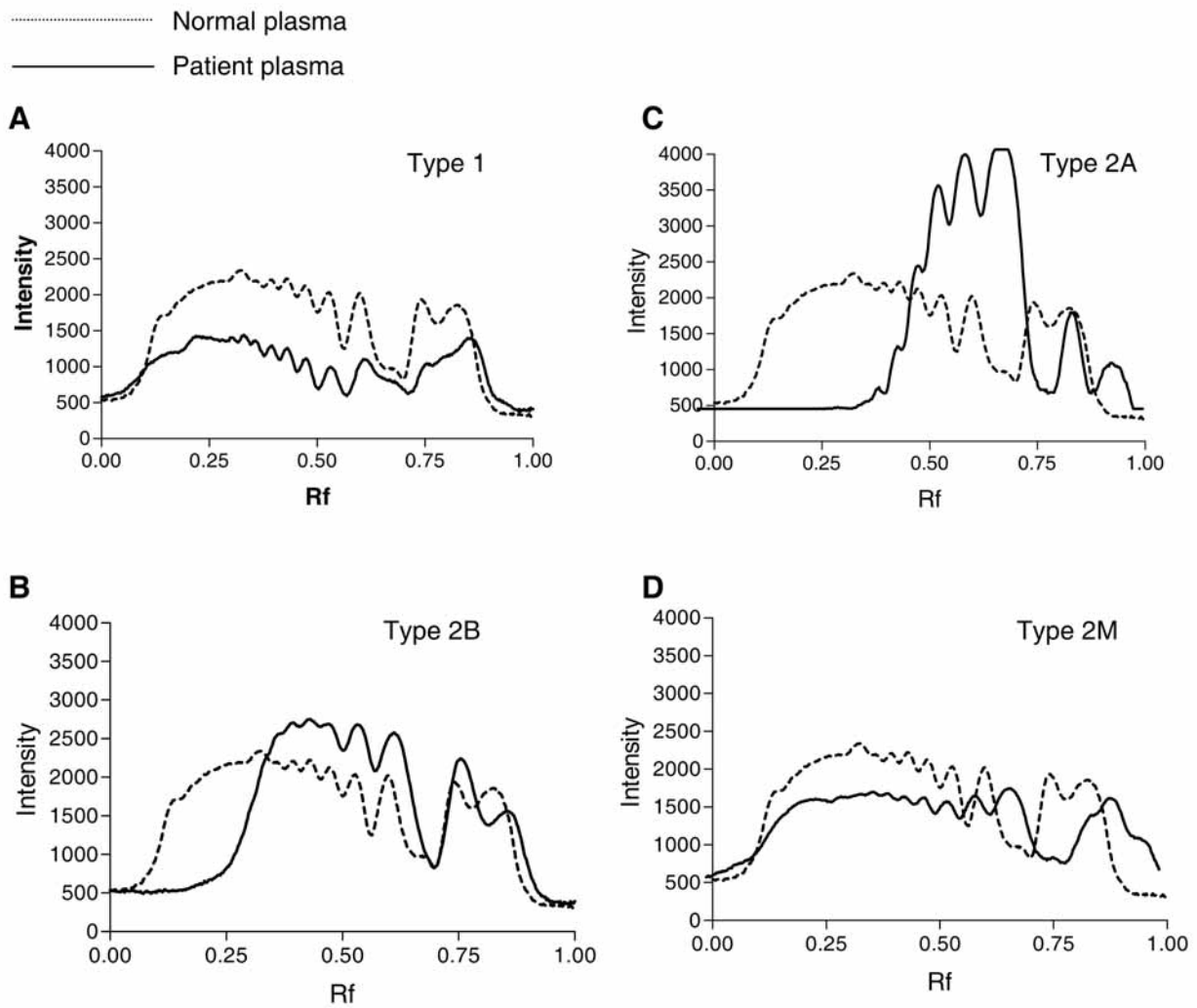
## Results

Typical patterns of VWF multimers of normal pooled plasma and vWD types 1, 2A, 2B, and 2M are shown in figure 1. The density of the different bands was plotted in figure 2 against the relative front (Rf value) of the different lanes. The relative front is the distance of a band from the top of its lane, divided by the total length of the lane. The larger multimers are represented by the smallest values on the x-axis in figure 2. The first set of lanes in figure 1 contains plasma from a pool of 20 normal human volunteers. The highest density is in the region of the larger multimers (broken line in figure 2).



**Figure 1**

The multimeric structure of VWF in normal plasma (NP) (lane sets 1 and 6), type 1VWD (lane set 2). Type 2A VWD (lane set 3), Type 2B VWD (lane set 4) and type 2M VWD (lane set 5).



**Figure 2**

Densitometric tracing of lane sets 2 to 5. The density of the different bands is plotted against the relative front (Rf value) of the different lanes.

Plasma from a patient with type 1 VWD was run in the second set of lanes in figure 1. The highest density is also in the range of the larger multimers and due to the low VWF:Ag levels, the bands are much lighter (lower density) than those of normal persons (figure 2A). Plasma from a patient with type 2A vWD was run in the lane set 3 (figure 1). No high molecular weight multimers can be detected and an increase in the small multimers is visible. The low molecular weight multimers also show a higher density than those in normal plasma (figure 2C). Plasma from a patient with type 2B VWD was run in lane set 4 in figure 1. Only the intermediate and small multimers are visible. The density of these multimers is also higher than those in normal plasma (figure 2B). Lane set 5 in figure 1 contains plasma from a type 2M patient. It is interesting to note the difference in distribution of the different size multimers compared to that of normal plasma. The density profile shows an almost even distribution of all multimers (figure 2D). The density of the small multimers is higher than those of normal plasma and those of the large multimers less.

**Discussion**

This method distinguishes type 1 from type 2A and 2B VWD. Type 2A shows a total absence of the high and intermediate molecular weight multimers and type 2B shows an absence of only the highest molecular weight multimers. Type 1 VWD shows a multimer pattern similar to that of normal plasma. Plasma from type 2M VWD patients contains a higher concentration of small multimers than normal plasma, although large, intermediate and small multimers are present. Perhaps the greatest advantages of our method are the rapid processing (less than 28 hours), high sensitivity to low concentrations of VWF, no radioactivity and the low cost of this procedure. A cost comparison between this method and those described in the literature shows a reduction of 40% in favour of our method when all the apparatus and reagent costs are taken into account. We could also demonstrate the difference in multimer pattern between normal plasma and that of type 2M

patients, which is not the case with the method of Krizek et al in 2000 [1]. By using only one antibody to VWF instead of the two that is described in the literature, we shortened the duration of the test with more than 2 hours and further reduced the cost of the method.

In conclusion, our modified luminographic method is a rapid, highly sensitive and cost-effective visualisation method for VWF multimers in plasma and it will be wise to include the density profiles in the diagnostic work-up of von Willebrand disease.

**References**

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