

## METHODOLOGICAL ARTICLE

# Commonly used anti-von Willebrand factor antibody for multimer analysis cross-reacts with fibronectin, which is difficult to distinguish from von Willebrand factor

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**Abstract**

Von Willebrand factor (VWF) is a 500- to 15 000-kDa multimeric protein circulating in the blood. When VWF has a higher molecular weight, its hemostatic activity is greater. The size distribution of VWF multimers is usually analyzed by SDS-agarose gel electrophoresis followed by immunoblotting. We found that the most commonly used anti-VWF antibody cross-reacted with fibronectin in VWF multimer analysis. In addition, since the apparent molecular weights of VWF and fibronectin are almost identical, these molecules were difficult to distinguish by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. Cross-reactivity between the anti-VWF antibody and fibronectin was inhibited by pretreating the antibody with fibronectin-coated plates. To obtain accurate data using anti-VWF antibodies, it is necessary to be aware of the possibility of cross-reactivity with fibronectin.

**KEYWORDS**

agarose gel electrophoresis, antibody, fibronectin, immunoblotting, SDS-PAGE, von Willebrand factor

**Essentials**

- Von Willebrand factor (VWF) multimer analysis is important for both basic research and clinical diagnosis.
- The commonly used antibody in VWF multimer analysis cross-reacted with fibronectin.
- Cross-reactivity was inhibited by pretreatment of the antibody with fibronectin.
- In research involving VWF, it is necessary to be aware of the possibility of cross-reactivity.

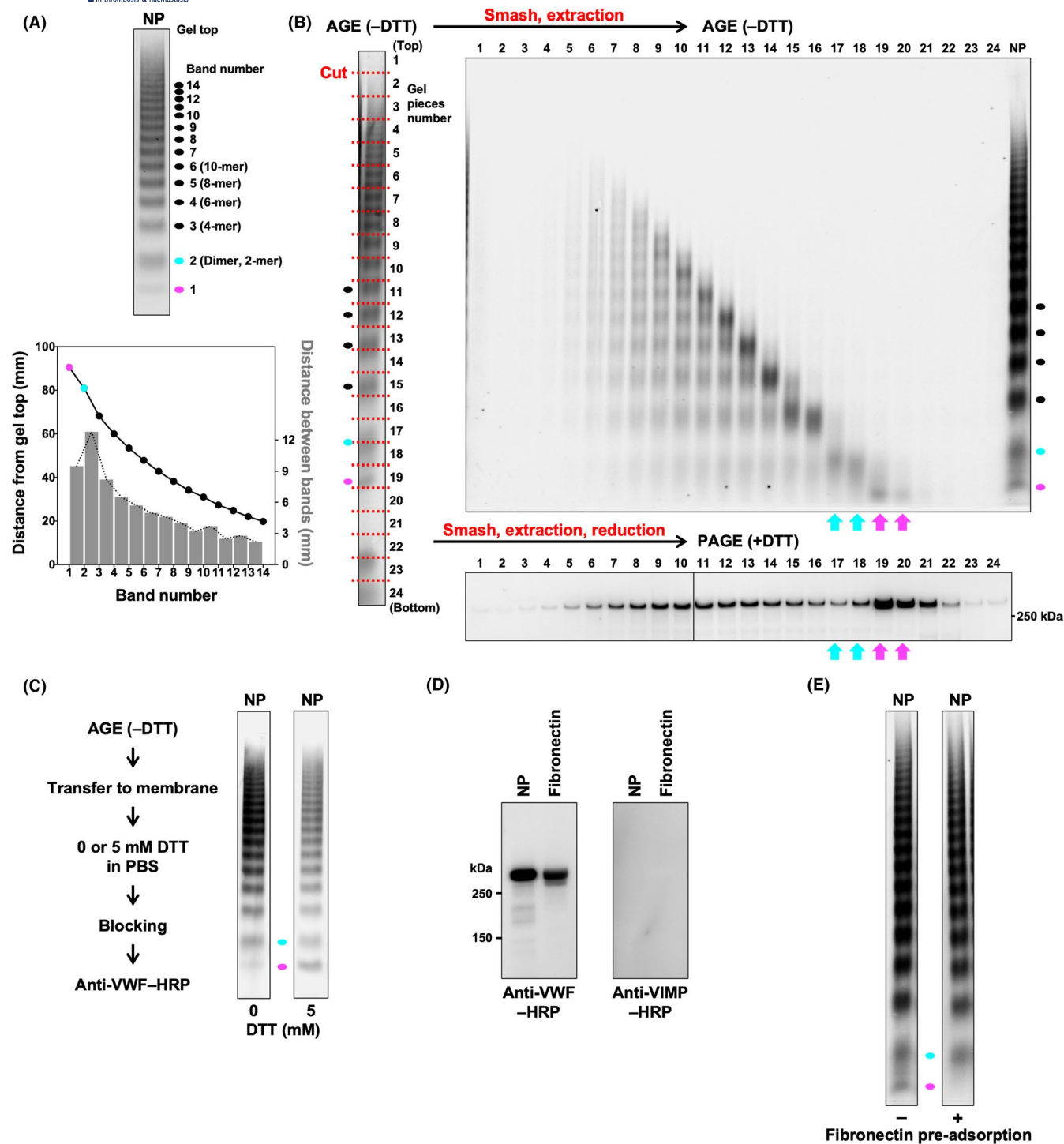
## 1 | INTRODUCTION

Von Willebrand factor (VWF), a plasma glycoprotein essential for hemostasis, circulates in the blood as homomultimers of 500 to 15 000 kDa, with higher molecular weights leading to higher hemostatic capacities.<sup>1,2</sup> It is crucial to track plasma VWF as a decrease in the levels and the high-molecular-weight VWF multimers

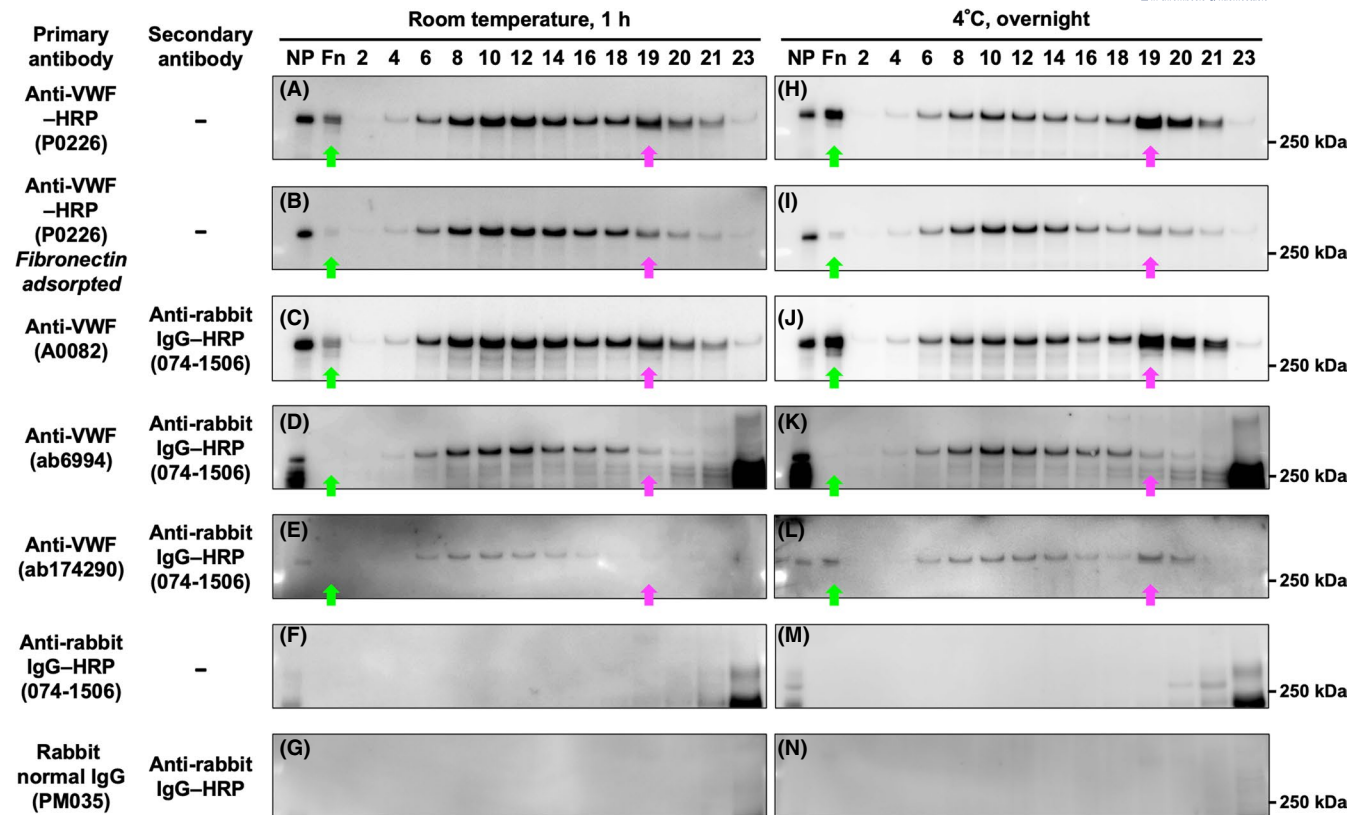
in the plasma might be indicative of von Willebrand disease or von Willebrand syndrome.<sup>3,4</sup> In contrast, the presence of unusually large VWF multimers is indicative of thrombotic thrombocytopenic purpura.<sup>5,6</sup> Hence, VWF multimer analysis forms a crucial aspect of basic research and clinical diagnosis. The size distribution of VWF multimers is analyzed by SDS-agarose gel electrophoresis followed by immunoblotting. In this method (VWF multimer

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**FIGURE 1** Von Willebrand factor (VWF) multimer analysis using anti-VWF antibody reveals the lowest band to be fibronectin. (A) VWF multimer analysis (upper) using human normal plasma (NP, GCH-100A; Sysmex, Kobe, Japan). The mobility (distance from the gel top) of each band (1-14) and the distance between adjacent bands (lower) showed band 1 (magenta) behaved differently from band 2 (cyan, dimer) and the other bands (black). (B) The agarose gel after electrophoresis of NP was cut into 24 pieces from top to bottom (left). Liquid in gel pieces was reanalyzed by VWF multimer analysis (right upper). Liquid in gel pieces was reduced with dithiothreitol (DTT) and analyzed by western blotting (right lower). Band 1 was recovered from gel piece 19 (magenta). (C) AGE-separated NP on the membrane was treated with 0 or 5 mM DTT in phosphate-buffered saline (PBS) for 10 minutes. The DTT treatment strengthened the signal of band 1 (magenta) but faded the signals of band 2 (cyan) and other bands. (D) NP and purified human fibronectin (33016-023, Gibco; Thermo Fisher Scientific, Waltham, MA, USA) were detected by western blotting using horseradish peroxidase (HRP)-conjugated anti-VWF antibody (Dako P0226) and anti-VIMP antibodies (negative control, V6639; Merck Sigma-Aldrich, St. Louis, MO, USA) by Peroxidase Labeling Kit-NH<sub>2</sub> (LK11; Dojindo, Kumamoto, Japan). (E) Multimer analysis of NP was performed using anti-VWF antibodies without (-) or with (+) fibronectin preadsorption. AGE, agarose gel electrophoresis under nonreducing conditions



**FIGURE 2** Cross-reactivity of anti-von Willebrand factor (VWF) antibodies to fibronectin. Human normal plasma (NP), human purified fibronectin (Fn), and liquid samples recovered from gel pieces (pieces numbers correspond to Figure 1B) were subjected to western blotting using indicated antibodies. Primary antibodies were incubated at room temperature for 1 h (A-G) or at 4°C overnight (H-N). Signal intensities of purified fibronectin (green arrows) were correlated with those of gel piece 19 (magenta arrows) that contain fibronectin. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (074-1506, KPL) and unconjugated rabbit normal IgG (PM035; MBL, Woburn, MA, USA) were used as negative control primary antibodies

analysis), the target sample's multimeric state is evaluated by separating the VWF multimer into ladders and comparing them to the standard sample.<sup>7</sup> This report highlights the characteristics of anti-VWF antibodies, which are most commonly used in VWF analysis.

## 2 | METHODS AND RESULTS

We often performed VWF multimer analyses as described previously,<sup>8</sup> which necessitates the initial identification of the dimeric band to quantify the band intensities. Human normal plasma analysis revealed that the second-lowest band was dimeric because the lowest-molecular-weight (highest-mobility) band size did not match the calibration curve based on each band's mobility (Figure 1A). The possibility that the lowest band could be VWF monomer led us to analyze it further.

First, proteins were extracted from the agarose gel after electrophoresis to quantify the amount of VWF in each multimer band more accurately. The agarose gel was cut into 24 pieces from top to bottom (Figure 1B, left) and crushed, the protein-containing supernatant was recovered by a spin column, and the successful recovery

of the multimer bands was confirmed by multimer reanalysis using agarose gel electrophoresis (AGE) (Figure 1B, right upper). SDS-PAGE, and western blotting of the recovered proteins after dithiothreitol (DTT)-mediated denaturation showed that contrary to our expectation, the VWF amount in gel pieces containing the lowest band was the highest among all samples (Figure 1B, lower right). The membrane was then treated with 0 or 5 mM DTT after AGE and probed with an anti-VWF antibody to investigate if the reduction by DTT affected the reactivity of the antibodies. DTT treatment strengthened the lowest band's signal and conversely weakened the signal of the other bands (Figure 1C). Based on these results, we focused on the lowest band detected by the anti-VWF antibody, assuming that it might have a different structure and function from the higher-molecular-weight VWF multimers.

Surprisingly, mass spectrometry of the supernatant of gel piece 19 identified the lowest band as fibronectin. Next, western blotting revealed that the anti-VWF antibody strongly cross-reacted with fibronectin, thereby explaining the mass spectrometry data (Figure 1D). Prior incubation of the anti-VWF antibody on wells of ELISA plates coated with purified fibronectin to adsorb fibronectin-reactive molecules abolished the signal of the lowest band in VWF multimer analysis (Figure 1E).

### 3 | DISCUSSION

The cross-reactivity between the anti-VWF antibodies and fibronectin may lead to inappropriate interpretation of experimental results. Cao et al<sup>9</sup> noticed that in western blotting of plasma-derived VWF, there was a nonspecific band recognized by the anti-VWF antibody, which was suggested to be fibronectin in the present study. We found several papers in which the fibronectin signals might influence the VWF signals in the agarose gel multimer analysis and western blotting. Although uninvestigated, the cross-reactivity between anti-VWF antibodies and fibronectin may affect the outcomes of ELISA and tissue staining as well.

In this study, we used the most commonly used antibody against VWF (P0226; Agilent Dako, Carpinteria, CA, USA). The 100 most recent articles in the National Library of Medicine PubMed database describing the use of VWF antibodies showed that 62% of the studies used Dako's anti-VWF antibodies (P0226 and A0082) for the following purposes: immunoblotting, including multimer analysis (30%), ELISA (27%), immunostaining of cells (19%), and tissue immunostaining (15%). The second most commonly used antibody (31%) was Abcam's products (ab6994 and ab174290; Cambridge, UK).

We performed western blotting using primary antibodies, two each from Dako and Abcam, to detect VWF recovered from the agarose gel pieces (Figure 2). The membranes were incubated with the antibodies for 1 hour at room temperature or overnight ( $\approx 16$  hours) at 4°C. Chemiluminescence was detected using horseradish peroxidase (HRP)-conjugated secondary antibodies, except for HRP-conjugated primary antibodies (P0226 and 074-1506). The anti-VWF (P0226)-derived signals of purified fibronectin and gel piece 19 were weaker relative to other gel pieces upon antibody preadsorption with fibronectin (compare A and B, and H and I in Figure 2), suggesting that gel piece 19 contained fibronectin. Fibronectin-derived signals were clearer after overnight incubation at 4°C than after 1 hour of incubation at room temperature (compare A and H in Figure 2). Prolonged incubation at low temperatures increased the affinity between fibronectin and the antibodies. Other antibodies, Dako's A0082 and Abcam's ab174290, showed similar results, but Abcam's ab6994 did not cross-react with fibronectin.

According to the attached documents, the immunizing antigen of the P0226, A0082, and ab174290 products was plasma-derived VWF. It is highly likely that the immunizing antigen contains fibronectin because it has been recently reported that fibronectin binds to VWF with relatively high affinity through the VWF A1 domain.<sup>10</sup> It should be noted that our data do not distinguish between "the anti-VWF antibody products contain anti-fibronectin antibodies" and "a part of antibodies that react with VWF also react with fibronectin."

### 4 | CONCLUSION

Our study does not dismiss Dako's anti-VWF antibodies as unreliable; therefore, we will continue using them because of their good reactivity with VWF. However, it is noteworthy that certain anti-VWF

antibodies may cross-react with fibronectin under certain conditions. Absorbing the antibodies with fibronectin before use might reduce such cross-reactivity. We hope that this report will contribute to the growing field VWF experiments.

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#### RELATIONSHIP DISCLOSURE

The authors have no conflicts of interest to disclose.

#### AUTHOR CONTRIBUTIONS

YE designed the research, performed the experiments, and wrote the manuscript. KK contributed to data interpretation and wrote the manuscript.

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