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Note

# Production of functional recombinant human tissue factor for the prothrombin time test

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**Tissue factor (TF) triggers extrinsic blood coagulation and is used for the prothrombin time test to investigate blood disorders. TF consists of the N-terminal extracellular, the transmembrane, and the C-terminal cytoplasmic domains. Here, a convenient method for preparing active recombinant TF for the prothrombin time test is described. The sole extracellular domain (TF219) and the transmembrane domain which is linked to TF219 (TF243) were produced in *Escherichia coli.* The recombinant TF243 was retained on the 300 kDa molecular weight cut-off filter membrane, though TF219 passed through it. The retained TF243 increased the stability to be stable at room temperature for a week probably due to removing contaminants, whereas TF243 in crude extract was inactivated after the incubation for three days at the same conditions.**

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**Keywords:** tissue factor; prothrombin time test; extracellular domain; transmembrane domain.

## Introduction

Tissue factor (TF) is a transmembrane protein on the surface of extravascular cells and plays an important role in blood clotting when the cells are exposed to blood at the site of injury [1]. The prothrombin time (PT) test is a screening method for hemorrhagic disorders caused by hemophilia and acquired coagulopathy including vitamin K deficiency, liver disease, and disseminated intravascular coagulation, and also used to measure the effect of blood-thinning medicine warfarin. PT test measures the time to clot after adding a mixture of TF and phospholipid to a patient's plasma.

The recombinant TF expressed in *Escherichia coli* is mainly produced as inclusion bodies and the refolding is reported [2]. The recombinant TF shows coagulation activity, though the glycosylated native TF obtained from the human placenta shows 4 times higher activity [3]. TF consists of 263 amino acid residues and is divided into three parts, the N-terminal extracellular domain (TF219, residues 1-219), transmembrane domain (residues 220-242), and cytoplasmic domain (residues 243-263). The extracellular domain is responsible for the activity.

In this study, a useful method using conventional techniques for the production of the recombinant active TF for the PT test is described.

## Materials and Methods

### *The PT test*

The recombinant TF (2 μl) was lipidized in PT buffer (100 μl) consisting of 1 wt% lecithin, 1 mM dithiothreitol, 0.01 %v/v triton X-100, 50 mM HEPES (pH 7.5), 400 mM glycine, 25 mM $CaCl_2$, 40 mM NaCl, and 15 mM $NaN_3$. The PT time was measured until clotting occurred after adding the

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lipidized TF solution (100 μl) to the coagulation control (50 μl, Xiehe Shengwu, Chengdu, China).

***Production of TF219 and TF243***

The genes encoding TF219 and TF243 were cloned into the expression vector pET22b and produced as a fusion protein with the C-terminal hexa-histidine tag in *E. coli* BL21 (DE3). The transformant cells were incubated for 16 h at 37˚C or for 40 h at 25˚C, and the expression was induced by adding 0.5 mM isopropyl 1-thio-β-galactoside (IPTG) with further cultivation for 2 h or 4 h, respectively. The cells were harvested by centrifugation and sonicated in 10 mM tris/HCl (pH 7.5). The cell-free extract was centrifugated at 6,000 g for 10 min.

The supernatant was filtrated in a centrifugal ultrafiltration unit (Vivaspin, Sartorius Lab Instruments GmbH & Co. KG., Germany) with 0.2 μm, 300,000 MW, and 3,000 MW cut-off according to the manufacturer's instructions, and the PT time of each eluant was measured.

**Table 1. The activity of TF219 and TF243 expressed in *E. coli*.** The clotting times of the PT test of the crude TF219 and TF243 expressed in *E. coli* were shown.

| | Clotting times (s) | |
|---|---|---|
| | 25˚C | 37˚C |
| TF219 | 35±1.2 | 33±0.58 |
| TF243 | 29±3.5 | 26±1.0 |

The commercial TF showed 29.0 s at the same assay conditions.

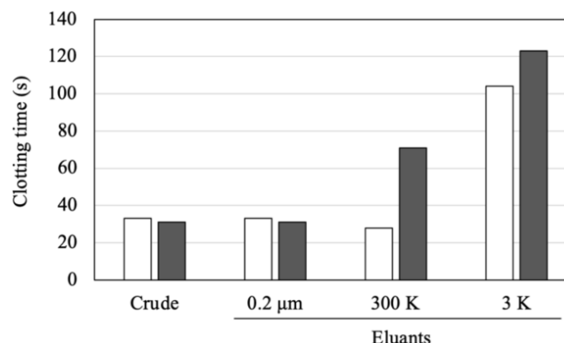


**Fig. 1. The clotting times of the PT test using TF219 and TF243 passed through an ultra-filtration membrane.** The clotting times of the PT test of TF219 (white bar) and TF243 (grey bar) produced at 37˚C passed through the ultrafiltration filter were shown.

**Results and Discussion**

Both TF219 and TF243 were produced in *E. coli* with the activity equivalent to the commercial TF. The activity of TF243 was slightly higher than that of TF219 (Table 1). Since TF219 and TF243 were produced both in the soluble and insoluble fractions based on the result of SDS-PAGE, the cultivation temperatures were decreased from 37 to 25˚C. However, the cultivation temperatures had no significant effect on the activity of the recombinant proteins. It is worth noting that *E. coli* cells expressing TF219 or TF243 gene in the presence of 0.5 mM IPTG showed no growth at both 25 and 37˚C probably due to the toxicities of the proteins. Thus, the recombinant proteins were induced for a few hours before the harvest.

The crude extract from cells expressing the TF219 or TF243 gene was fractionated by ultrafiltration, and the activity of the eluants was assayed. The clotting time decreases as the activity increases, and the clotting time was more than 10 min when the crude extract from *E. coli* cells harbored pET22b as a negative control was used for the assay. TF219 passed through the 300 kDa cut-off filter and the activity was measured in the eluent. Lower activity was observed in the eluent from TF243 (Fig. 1) suggesting some TF243 of which the calculated molecular weight is 27,285 Da, retained on the filter. TF243 has a transmembrane domain which is hydrophobic to bind to the membrane. This hydrophobic transmembrane domain may be aggregated to retain on the 300 kDa cut-off ultrafilter membrane. The retained TF243 was stable for 1 week at room temperature whereas the untreated crude TF243 lost entire activity. The contaminants from *E. coli* cells including proteases should be removed by this ultrafiltration. This rapid purification technique can be practiced to prepare active recombinant TF for the PT test without any purification step including immobilized metal affinity chromatography.

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