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An in-house ELISA method for quantification of circulating tissue factor

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Abstract

Tissue Factor (TF) is a low molecular weight transmembrane glycoprotein that initiates the clotting protease cascade. It is considered to be the principal regulator of the extrinsic coagulation pathway, hemostasis and thrombosis, as well as inflammation and cellular immune response. An in-house two-step direct sandwich ELISA *(enzyme-linked immunosorbent assay)* for immunological quantification of plasma TF was successfully developed. The assay employed a monoclonal antibody against human TF (1:400 dilution; 1250 ng/ml) and peroxidase-conjugated anti-TF IgG (1:1000 dilution; 2000 ng/ml) as capture and detecting antibodies respectively, whilst tetramethylbenzidine/H_2O_2 were utilized as substrates. Titration curves of recombinant TF were linear within 10 to 4000 pg/ml, with a detection limit of 36.31 pg/ml. It demonstrated low intra- (2.50 – 9.23 CV%) and inter-assays (5.65 – 13.57 CV%) variability, as well as satisfactory analytical recovery (91.55 – 103.95%) and good parallelism. The assay developed was intended to be applied for measurement of plasma TF levels in patients with thrombotic disorders.

Key words: Tissue Factor, enzyme-linked immunosorbent assay, thrombotic disorders

INTRODUCTION

Cellular initiation of the extrinsic coagulation serine protease cascade is mediated by Tissue Factor (CD142; Factor III; tissue thromboplastin, TF), which is a ubiquitous membrane-anchored low molecular weight (–47kD) glycoprotein. It is a high-affinity cellular receptor and cofactor for Factor VII and its activated form, VIIa. TF expression *in vivo* is highly tissue and cell-type specific[1]. As initiation of coagulation is not physiologically desirable within the vasculature, vascular cells are notably devoid of TF except in extraordinary circumstances. Constitutive expression of TF particularly by perivascular cells and epithelia that encapsulate many organs, provides a hemostatic barrier against blood loss into the extravascular spaces.[1,2] However, monocytes,[3] endothelial cells,[4] vascular smooth muscle cells[5] and fibroblasts[6] can be induced directly to express TF by various soluble mediators.

TF is implicated in having important roles in thrombogenesis, inflammation and the cellular immune response.[4,7,8] There is now persuasive evidence demonstrating that TF contributes in some capacity to thrombotic manifestation in coronary artery disease, atherosclerosis, malignancy, septicemia, inflammation, and more generally disorders associated with disseminated intravascular coagulation.[8,9] Additionally, there are reports implicating the association of TF with glomerulonephritis[10] and metastasis and neovascularization in human tumors.[11] Hence, in view of the harmful effects of aberrant expression of TF, regulation of this integral membrane protein seems to be of paramount importance in a variety of pathological conditions. In this regard, there are mounting studies to explore the potential pathophysiological role of TF as well as intense research into the structural biology of TF-FVIIa complex. Owing to the limited availability and high cost of commercial kits, the present study was designed to develop an in-house ELISA for quantification of plasma TF.

MATERIALS AND METHODS

Commercial antibodies and antigen

The capture antibody, mouse monoclonal antibody against human TF (McAb-TF; 0.5 mg/ml) was obtained from Enzyme Research Laboratories Inc. (Indiana, USA) whilst the detecting antibody, purified sheep anti-human TF IgG-horseradish peroxidase conjugate (IgG-TF-HRP; 2 mg/ml) was supplied by Affinity

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Biologicals Inc. (Ontario, Canada). Meanwhile, the recombinant TF (rTF; 25 µg/ml) was purchased from American Diagnostica Inc. (Greenwich, CT, USA). All were stored and reconstituted according to the manufacturers' instructions.

Buffers

The following chemicals were purchased from Sigma Co. (USA), unless otherwise indicated.

(a) Phosphate-buffered saline (PBS, pH 7.4): 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl.
(b) Coating buffer (pH 9.6): 50 mM carbonate-bicarbonate buffer.
(c) Washing buffer (PBST, pH 7.4): PBS with 0.05% (v/v) Tween 20 (USB).
(d) Blocking buffer: PBS with 5% (w/v) skimmed milk (SM) and 0.5% (v/v) Tween 20. Freshly prepared.
(e) Dilution buffer: PBS with 1.5% (w/v) SM and 0.5% (v/v) Tween 20. Freshly prepared.
(f) Substrate buffer: 10 ml of 50 mM phosphate-citrate buffer (pH 5.0), containing 0.03% sodium perborate (H_2O_2 substitute) and 1 mg 3,3',5,5'-tetramethylbenzidine (TMB). Prepared within 30 minutes of use.
(g) Stopping solution: 1 M sulphuric acid (H_2SO_4; BDH).

Plasma pool

Venous blood samples from 4 apparently healthy individuals (2 males, 2 females) were drawn atraumatically into 3.8% (129 mM) trisodium citrate (9:1 v/v). Platelet-poor-plasma was prepared by centrifugation at 3000 rpm, 4°C for 30 minutes. The plasma was then pooled, aliquoted into polypropylene microtubes, snap-frozen and stored at -80°C for subsequent optimization and validation of TF ELISA method.

ELZSA procedure

The assay principle was based on the two-step direct sandwich ELISA,[12] and employed two antibodies against human TF. Briefly, the microtiter plate (Immulon® 2HB Removawell®; Dynex Technologies, USA) was coated at 4°C overnight with 100 pllwell of McAb-TF (1250 ng/ml in coating buffer), and washed with PBST. The remaining unbound plate surfaces were then blocked for 2 hours at 27°C by floating each well with 300 pl of blocking buffer. The plate was then washed and thoroughly tap-dried before it was reacted for 1 hour at 27°C with 100 µl/well of samples or standards, which were diluted 1:2 in dilution buffer. After another washing cycle, the presence of TF antigen was detected by incubation at 27°C for 1 hour with 100 µl/well of IgG-TF-HRP (2 µg/ml) in dilution buffer. Finally, 100 µl/well of freshly prepared substrate buffer was added, and incubation was carried out for another 10 minutes at 27°C in the dark. The reaction was then terminated by acidification with 100 µl/well of stopping solution before the absorbance was measured spectrophotometrically. During each washing step, the microtiter plate was washed 5 times with 300 µl/well of PBST and soaked for 1 minute each cycle. Residual moisture was thoroughly tap-dried from the wells onto absorbent papers before the plate was subjected to the next step. All incubations were done with agitation (30 rpm) in VorTemp 56 microplate mixer (Labnet; NJ, USA), except when the microtiter plate was coated overnight in the fridge. The plasma pool was assayed in triplicate, and the respective TF levels were interpolated from the linear portion of the standard curve. Absorbance was read in Multiskan MS Version 8.0 microplate reader (Labsystem; Finland) at dual-wavelength 450 nm/630 nm. Results were expressed as delta optical density (OD).

Assay optimization

Optimization of the assay was achieved by altering three different variables separately: McAb-TF, IgG-TF-HRP and rTF. Each time a criss-cross serial dilution of two variables was done across the microwell plate in the opposite way to obtain a chequerboard or chessboard titration. Each well represented a different concentration of the two reagents. Hence, the dilutions that yielded the optimal signal-to-noise ratio could readily be ascertained.

Standard curve and dynamic range

A two-fold serial dilution of rTF was assayed in quadruplicate under the optimized conditions, and the color developed was measured as detailed above. Standard curves were constructed and the dynamic range was determined. Then, a standard curve with its determined dynamic range would be run in parallel with the samples every time the assay is performed.

Assay validation

The overall goal of assay validation is to determine whether the obtained values of the potency estimates for unknowns are accurate and correct. For this purpose, reference plasma of high (H; 2000 pg/ml), medium (M; 1000 pg/

ml) and low (L; 100 pg/ml) levels of TF, covering the upper, middle and bottom parts of the dynamic range were prepared by spiking the plasma pool with rTF.

(a) High-dose hook effect
The stock solution of rTF (25 µg/ml) was serially diluted twofold in dilution buffer, and assayed under the optimized conditions to study the high-dose hook effect.

(b) Assay sensitivity
As the limiting factor in non-competitive immunoassays is nonspecific binding, "zero" standard (dilution buffer) was assayed 12 times and its concentrations were read from the standard curve. The detection limit can be then defined statistically as the concentration corresponding to 2 standard deviation (SD) different from the signal at "zero" standard."

(c) Assay accuracy
The interference of matrix effects, which may compromise the accuracy of an immunoassay, can be evaluated by virtue of recovery and dilution studies. Analytical recovery of the assay was evaluated by measuring the concentrations of the unspiked plasma pool, H, M and L reference plasma. Recovery rates were calculated as below to assess the influence of interference between sample and calibrator matrices:

$$\text{Recovery Rate} = \frac{\text{Measured increase in concentration}}{\text{Predicted increase in concentration}} \times 100\%$$

Two-fold serial dilutions (ranging from 1:2 to 1:32) of standard rTF (1000 pg/ml), H, M and L reference plasma were performed under the optimized conditions. Then, final concentrations of each dilution for each sample were plotted against the dilutions, to demonstrate whether the analyte in the standards and samples reacted with the reactants in a similar manner.

(d) Assay precision
Precision that comprises within-run (intra-assay) and between-run (inter-assay) variability is expressed as:

$$\text{Coefficient of Variation (CV)} = \frac{\text{SD}}{\text{Mean}} \times 100\%$$

Intra-assay precision was evaluated by assaying the 3 reference plasma 10 times in one run, whereas the plasma was tested 10 times over 6 consecutive days to derive the inter-assay precision.

Statistical analysis
Microsoft[a] Excel 97 was applied to generate titration plots, and standard curves using linear regression. Results were statistically analyzed by SPSS[a] 9.0 for Windows'.

RESULTS

Optimization experiments revealed that capture McAb-TF and detecting IgG-TF-HRP at dilutions around 1:400 (1250 ng/ml) and 1:1000 (2000 ng/ml) respectively gave reproducible standard curves with a dynamic range from 10 to 4000 pg/ml of TF (Figure 1). The phenomenon of 'high-dose hook effect' was observed when TF concentration exceeded 3.125 x 10^6 pg/ml (Figure 2).

Table 1 clearly compared the observed and expected concentrations of the three reference plasma samples spiked with known concentrations of rTF. Analytical recoveries of the assay ranged from 91.55 to 103.95% (Table 1). Meanwhile, the intra- and inter-assay variability varied from 2.50 – 9.23% and 5.65 – 13.57% respectively (Table 2). The reference plasma diluted out the same way as the standard rTF, if only the plasma was diluted at least 1:2 in the dilution buffer. Parallelism or similarity was shown when the data of the diluted plasma were graphed in linear-linear coordinates (Figure 3a) or in linear-log coordinates (Figure 3b). Undiluted plasma showed dissimilarity, i.e. failure to generate a curve of the shape as standard.

Study of the effectiveness of various blocking agents revealed that PBS supplemented with 0.5% Tween 20 and 5% SM was the most effective in controlling the assay background (0.05 OD). As graphically depicted in Figure 4, comparative study demonstrated that 1.5% SM and 0.5% Tween 20 in PBS was the optimal dilution buffer.

DISCUSSION

An in-house TF ELISA with a wide dynamic range and great sensitivity was successfully developed, which is comparable to the TF assay developed by Albrecht *et al.*[14] and Koyama *et al.*[15] In this study, the assay design was based upon two-site (sandwich type) and two-step immunoassay because of its advantages over the one-step sandwich assay. The cross-reacting

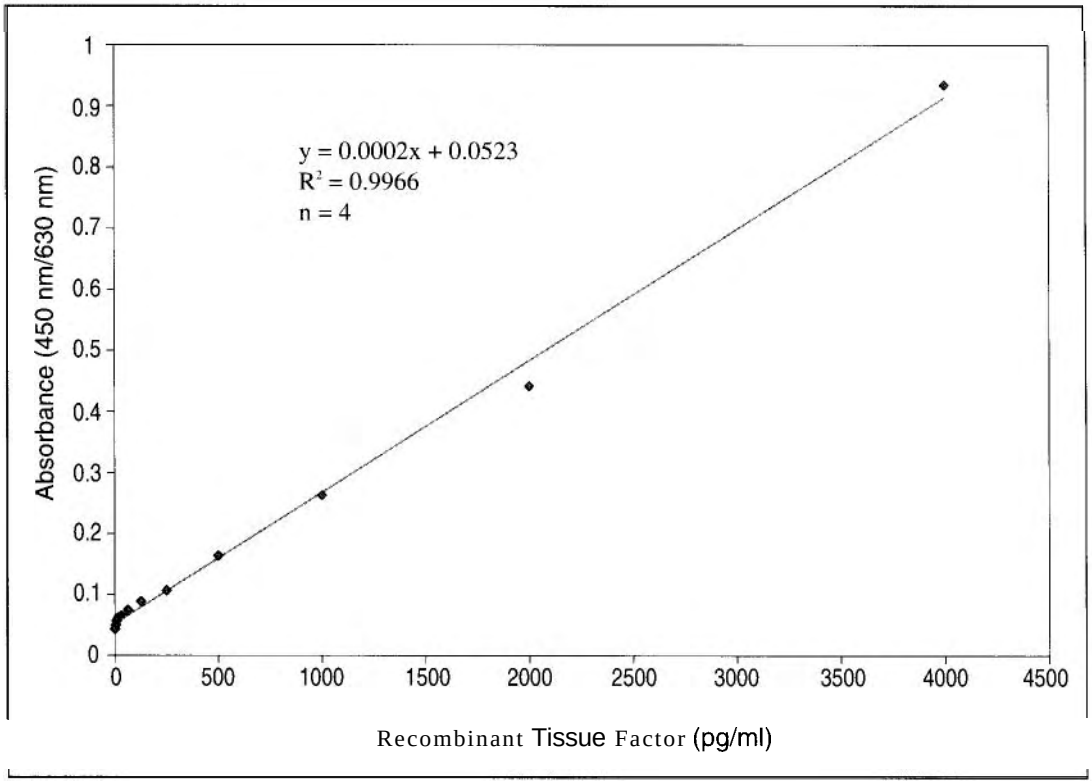

FIG. 1: Standard curve for plasma TF assay covering the dynamic range from 10 to 4000 pg/ml. Each point represents the mean of 4 determinations.

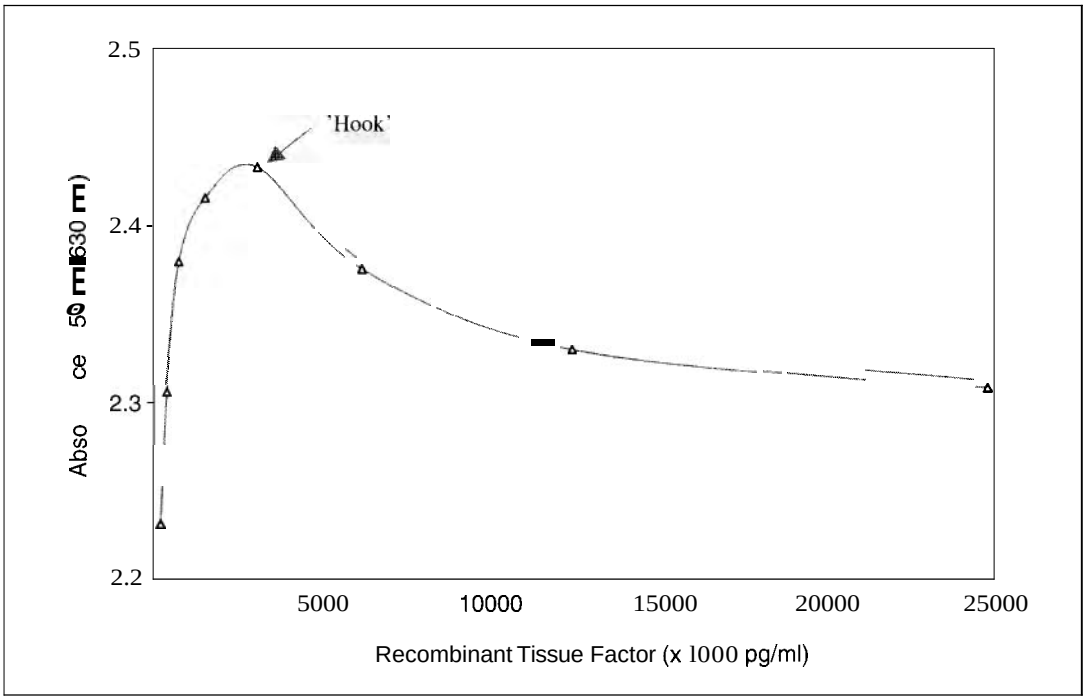

FIG. 2: TF at very high concentrations (>3.125 x 10^6 pg/ml) demonstrate high-dose hook effects.

TABLE 1: Analytical recovery of the rTF added to spiked plasma pool.

Reference Plasma	TF Concentrations (pg/ml)[a]				Recovery (%)
	Endogenous	Added	Expected	Measured	
Low	*88.50*	*100.00*	*188.50*	*191.50*	*103.00*
Medium	88.50	1000.00	1088.50	1004.00	91.55
High	88.50	2000.00	2088.50	2167.00	103.95

[a] Recorded values depict the mean of 10 measurements.

TABLE 2: Intra-assay and inter-assay precision.

	TF Concentrations (pg/ml)		
	Low	***Medium***	***High***
Intra-assay (n = 10) [a]			
Mean	185.00	1016.50	2087.00
SD	17.07	25.41	87.85
Variance	291.39	645.56	7718.06
CV (%)	**9.23**	**2.50**	**4.21**
Inter-assay (n = 6) [b]			
Mean	163.50	1008.37	2056.47
SD	22.19	103.67	116.17
Variance	492.50	10748.33	13496.53
CV (%)	**13.57**	**10.28**	**5.65**

[a] Samples are assayed 10 times in one run, and recorded values depict the means of 10 replicate determinations.

[b] Samples are tested 10 times over 6 consecutive days, and values obtained for each day are the means of 10 replicate determinations.

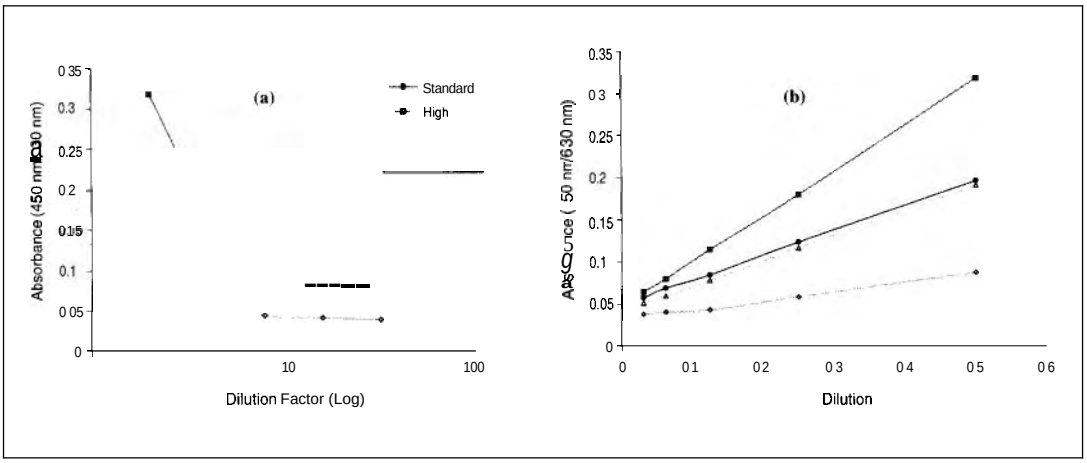

FIG. 3: Demonstration of parallelism when data are graphed in (a) linear-log or (b) linear-linear coordinate systems.

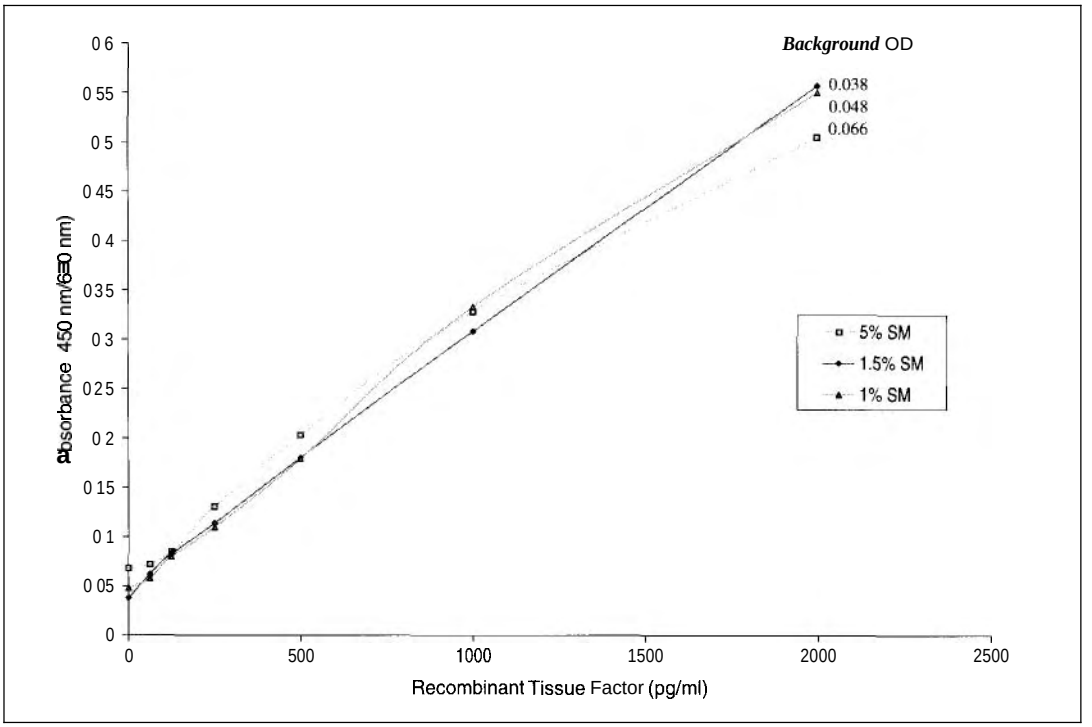

FIG. 4: Comparative study of three different concentrations of SM as dilution buffers.

substances that react with the labelled antibody but not with the capture antibody can be removed with a washing step. Two-step assay eliminates interference of sample constituents with the labelled antibody, and is less prone to interference due to closely related substances, which share antigenic determinants.[16] Extreme sensitivity with a detection limit of 36.31 pg/ml was attained by the combination of monoclonal and polyclonal antibodies. Best results were obtained by using monoclonal antibody for solid phase coating, and polyclonal antibody in the enzyme-labelled form.'' When affinity-purified IgG anti-TF was applied for coating antibody, very high background staining was noted. This might stem from the contaminated IgG with ligand that had leached from the affinity column, resulting in increasing background noise through immunochemical 'short-circuits', which were often encountered in sandwich assays.[18]

The flat-bottomed well was preferred since it was recommended where spectrophotometric reading was employed to assess color development,[19] whereas preparation of dilutions was done in round-bottomed well. During washing steps, a l-minute soaking cycle was practised as this improved the washing efficiency and reduced background variance.[20] Agitation of plates was carried out throughout the incubation steps to enhance the reaction rate and improve the assay kinetics,[13] besides eliminating viscosity and temperature effects, as well as time and edge-well differences.[19]

In order to eliminate the difference of time for signal generation in the first well and the last, a stopping reagent (H_2SO_4) was used.[13] Absorbance was measured at dual-wavelength since this would correct for any optical interference due to imperfections in the microplates.[21] Optimal dilution should yield an optical response within the absorbance range that could be measured by the spectrophotometer, i.e. below 2.0. In this regard, the optimal amount of titrate antibody should range around 1 – 1.5 OD.[19]

Meanwhile, the incubation time for antigen-immunoconjugate complex formation was set to 1 hour in the standard procedure, as this duration was found to be sufficient to reach a steady state that yielded satisfactory precision (Table 2). Prolongation of incubation time (1+ hours) increased the absorbance of blank wells (>0.1 OD) with antigen omitted (dilution buffer only), compounding the nonspecific binding of immunoconjugate to the well surfaces. This resulted in unsatisfactory detection limit and poor sensitivity.

A combination of 0.5% Tween 20 and 5%

SM in PBS was the most effective in blocking the vacant protein binding sites on the well surfaces. SM turned out to be the most effective blocking agent. This may be due to the predominance of small proteins in SM. However, the same buffer was not used for dilution of samples or reagents, as SM at this concentration caused clot formation in the plasma. This phenomenon may be due to the presence of high calcium ions in SM that plays a pivotal role in the clotting cascade. Hence, in order to maintain the fluidity of the samples, 1.5% SM and 0.5% Tween 20 in PBS was used as dilution buffer. As could be seen in Figure 4, 1.5% SM was preferable to 1% SM as the former gave a better titration curve with extremely low background (0.038 OD).

The assay demonstrated a nearly 100% recovery rate (Table 1), denoting that plasma components did not interfere with the TF-binding characteristics of the McAb-TF. Good parallelism (Figure 3a, 3b) indicated that the assay fulfilled one of the fundamental principles of immunoassay, i.e. the unknown antigen gave the same response as the standard antigen. In order to achieve this, a dilution of 1:2 was necessary, which was the minimal dilution required for the reduction of matrix effects in the samples. This finding was in line with Albrecht et al.[14] and Koyama et al.[15]. Precision of the method was satisfactory with fairly good within-batch and between-batch reproducibility (Table 2). Hence, this assay was compatible with the criterion that these variances should be <10% for clinical routine work.[22]

Plasma pool was chosen for assay validation as this is the body fluid targeted for the determination of TF. We intend to make use of this in-house ELISA method to investigate the clinical significance of plasma TF antigen in thrombotic disorders.

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