Optimisation of antithrombin resistance assay as a practical clinical laboratory test: development of prothrombin activator using factors Xa/Va and automation of assay

Shogo Tamura [a], Yuina Suga [a, *], Mei Tanamura [a, *], Moe Murata-Kawakami [b], Yuki Takagi [a, c], Yuna Hottori [a], Misaki Kakihara [a], Sachiko Suzuki [a], Akira Takagi [a], and Tetsuhito Kojima [a]

[a] Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan

[b] Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan

[c] Japan Society for the Promotion of Science, Tokyo, Japan

Short running title: Optimisation for practical ATR test

Corresponding author

Tetsuhito Kojima, MD, PhD

Department of Medical Technology, Nagoya University School of Health Sciences,

1-1-20, Daiko-Minami, Higashi-ku, Nagoya, 461-8673, Japan

E-mail: kojima@met.nagoya-u.ac.jp

Summary

Introduction: Antithrombin resistance (ATR) is a novel thrombotic risk in abnormal prothrombins. A manual ATR assay using *Oxyuranus scutellatus* (Ox) venom as a prothrombin activator was established for detecting antithrombin-resistant prothrombin. However, this assay was limited because of Ox snake venom availability and its throughput capacity. Here we have improved the ATR assay using bovine factors Xa and Va (FXa/Va) as prothrombin activators and have optimised assay conditions for an automated instrument (ACL TOP 500). **Methods**: Diluted plasma was incubated with a prothrombin activator mix (phospholipids, $CaCl_2$, and bovine FXa/Va), followed by inactivation with antithrombin for 10, 20, and 30 min. We added a chromogenic substrate S-2238, and assessed changes in absorbance/min at 405 nm. We also adapted assay conditions for ACL TOP 500. **Results**: Optimum conditions for FXa/Va treatment were 6.25% phospholipids, 5 mM $CaCL_2$, 0.01 μg/mL FXa, and 0.1 μg/mL FVa. ATR assay kinetics with the FXa/Va activator was comparable with that with the Ox activator in heterozygous reconstituted plasma with the recombinant wild-type or antithrombin-resistant prothrombin. Using ACL TOP 500, optimum conditions for the FXa/Va treatment were 10.0% phospholipids, 5 mM $CaCl_2$, 0.02 μg/mL FXa, and 0.2 μg/mL FVa. The automated ATR assay with the FXa/Va activator demonstrated good detectability for antithrombin-resistant prothrombin in plasma from a heterozygous carrier with prothrombin Yukuhashi or Belgrade. **Conclusion**: We optimised the ATR assay with the FXa/Va activator and adapted the assay for ACL TOP 500; the assay showed the ability to clearly detect antithrombin-resistant prothrombin in manual and automated procedures.

Keywords: antithrombin resistance, prothrombin, Ox venom, FXa/Va activator, ACL TOP 500

1. Introduction

Venous thromboembolism (VTE) is a multifactorial disease resulting from a complex association between genetic predisposition and acquired risks (e.g. ageing, obesity, surgery, pregnancy, puerperium, use of oral contraceptives, and/or cancer).[1] Loss-of-function mutations of natural anticoagulants, such as antithrombin (*SERPINC1*),[2] protein C (*PROC*),[3] and protein S (*PROS1*),[4] are known to be risk factors for VTE.[4] In addition, gain-of-function mutations of procoagulant factors, such as prothrombin (*F2*) and factor V (*F5*), are common causes related to an increased risk for thrombosis.[5] *F2* mutation at 3′-untranslated region (prothrombin G20210A) is a mild risk for VTE development.[5,6] Several *F5* mutations, such as Arg506Gln (FV Leiden[7]), Trp1920Arg (FV Nara[8]) and Ala512Val (FV Bonn[9]), exhibit a reduced susceptibility to activated protein C (known as activated protein C resistance), resulting in severe and recurrent VTE.

In 2012, we postulated antithrombin resistance (ATR), a novel thrombophilic concept, using a prothrombin mutant analysis.[10] Prothrombin Yukuhashi (*F2* c.1787G>T, p.Arg596Leu) is the first case of antithrombin-resistant prothrombin with familial thrombophilia.[10] A propositus carrying the heterozygous mutation had suffered from recurrent and severe VTE from a young age. Thrombin derived from prothrombin Yukuhashi showed impaired inactivation by antithrombin, resulting in a prolonged clotting function that led to recurrent thromboembolism. Following the report on prothrombin Yukuhashi, prothrombin Belgrade (*F2* c.1787G>A, p.Arg596Gln)[11] and prothrombin Padua 2 (*F2* c.1786C>T, p.Arg596Trp)[12] were also reported as an antithrombin-resistant prothrombin. Currently, patients with the antithrombin-resistant prothrombin mutation are found in seven unrelated families worldwide (prothrombin Yukuhashi: one Japanese family[10]; prothrombin Belgrade: two in Serbia,[11] one in India,[13] and one in Japan[14]; and prothrombin Padua 2: two Italian families[12]).

Diagnosis of antithrombin-resistant prothrombin depends on a history of provoked or unprovoked VTE and subsequent definitive gene sequencing. Global coagulation tests (e.g. prothrombin time or activated partial thromboplastin time) cannot detect the antithrombin-resistant prothrombin abnormality in heterozygote patients.[10,12,15] Previously, we have established a manual operation assay specialised in detecting ATR.[16] This manual operation assay has a prothrombin activator step by *Oxyuranus scutellatus* (Ox) venom; however, currently it is difficult to obtain Ox venom because it is not marketed in some countries including Japan. In addition, the previous manual assay is sufficient to detect antithrombin-resistant prothrombin abnormality in plasma, but there is a limit in handling throughput.

In this study, we have improved the ATR assay to overcome problems of the previous assay. First, we evaluated the performance of the combination of bovine factors Xa (FXa) and Va (FVa) as an alternative prothrombin activator compared with that of Ox venom in a manual operation procedure. Second, we developed an automated ATR assay using the ACL TOP 500 instrument to apply it as a clinical laboratory test in hospital practice.

2. Materials and Methods

2.1 Reagents

Bovine FXa and FVa were obtained from Haematologic Technologies (Essex Junction, VT, USA) and PTT-Reagent RD (lyophilised cephalin) was obtained from Roche Diagnostics KK (Tokyo, Japan). In this study, we used reconstituted cephalin solution with 2 mL distilled water as 100% phospholipids (PL). We obtained prothrombin-deficient plasma (prothrombin activity is <1%) from Mitsubishi Chemical Medience Co. (Tokyo, Japan). The chromogenic substrate H-D-Phe-Pip-Arg-p-nitroranilide (S-2238) was purchased from Sekisui Medical Co. (Tokyo, Japan). Ox

venom also known as taipan snake venom, which is a high-molecular weight (approximately 250 kDa) prothrombin activator, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Human AT was obtained from Mitsubishi Tanabe Pharma Co. (Osaka, Japan).

2.2 Recombinant prothrombins

Stable transfectants of HEK293 expressing WT or mutant recombinant prothrombins were established as previously described.[10] Stable transfectants were cultured for 24 h in a serum-free Dulbecco's Modified Eagle Medium containing 5 μg/mL vitamin K1 (Isei, Yamagata, Japan). Then, cultured media were collected, centrifuged at 2000 ×*g* for 10 min to remove cellular debris, and concentrated using Vivaspin Turbo 15 (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Concentrated media were stored at −80°C. Prothrombin antigen levels were evaluated by ELISA composed of anti-prothrombin antibodies (FII-EIA, Enzyme Research Laboratories, South Bend, IN, USA) and O-Phenylenediamine substrate (Wako, Tokyo, Japan).

2.3 Manual ATR kinetic assay

2.3.1 Ox activator-based manual ATR assay

The Ox activator-based manual ATR kinetic assay was conducted as reported previously.[16] The assay comprised the following three steps: (a) prothrombin activation, (b) thrombin inactivation, and (c) residual thrombin activity assessment. Then, normal pooled plasma or reconstituted plasma, prepared by mixing prothrombin-deficient plasma with recombinant prothrombins on the assumption that the prothrombin concentration was 100 μg/mL in normal plasma (100%), was subjected to the assay. Plasma was diluted to 1/100 with a sample dilution buffer. The sample dilution buffer

was composed of 50 mM Tris-HCl, 200 mM NaCl, pH 8.1. The Ox activator comprises 50% PL, 15 mM $CaCl_2$, and 0.1 mg/mL Ox venom. Before the prothrombin activation step, 500 μL of the sample was warmed at 37°C for 2 min. In the prothrombin activation step, 200 μL of activator was added, and the mixture was incubated for 2 min at 37°C. In the thrombin inactivation step, 100 μL of the antithrombin solution (or saline) was added to the mixture, followed by incubation for 0, 10, 20, and 30 min at 37°C. In the assessment step, the residual thrombin activity was determined using a chromogenic substrate (S-2238, 200 μL). A change in the absorbance/min (ΔABS/min) was evaluated at 405 nm using TBA-180 (Toshiba Medical System Co., Tokyo, Japan). As an assessment parameter on TBA-180, lag and measurement time were 10 and 15 s, respectively. The relative residual thrombin activity (RRTA) was compared to the 0 min data using the following formula:

$$\text{RRTA}\ (\%) = \frac{(\text{inactivated thrombin activity at each time point})}{(\text{thrombin activity at 0 min})} \times 100$$

2.3.2 FXa/Va activator-based manual ATR assay

The assay procedure of the FXa/Va activator-based manual ATR assay was identical to the Ox activator-based manual ATR assay, except for the activator reagent. The FXa/Va activator comprises PL, $CaCl_2$, bovine FXa, and bovine FVa. Optimal conditions of the sample dilution buffer and FXa/Va activator were determined as described in the Results section. Besides, the details of optimizing protocol was shown in supporting information.

2.4 Automated ATR kinetic assay

The automated ATR kinetic assay was performed using ACL TOP 500 CTS (Instrumentation Laboratory, Tokyo, Japan). In the ACL TOP assay, plasma was diluted

with 50 mM Tris-HCl-based dilution buffer. We warmed 100 μL of the sample at 37°C for 2 min. In the prothrombin activation step, the sample was mixed with 40 μL of activator mixture at 37°C. In the thrombin inactivation step, 20 μL of antithrombin solution (or saline) was added and incubated at 37°C for 0, 10, 20, and 30 min. In the assessment step of the residual thrombin activity, S-2238 was used as a thrombin substrate, and ΔABS/min was evaluated at 405 nm. Detailed assessment conditions and parameters for the ACL TOP ATR assay were determined as discussed in the Results section.

3. Results

3.1 FXa/Va prothrombin activator performance assessment

We investigated the usability of bovine factors Xa and Va (FXa/Va activator) as an alternative to the Ox venom-based prothrombin activator (Ox activator). We detailed compositions of the sample dilution buffer and FXa/Va activator in the optimised FXa/Va activator-based manual assay procedure (Fig. 1). The optimal sample dilution buffer of FXa/Va activator-based ATR assay was determined as 50 mM Tris-HCl, 200 mM NaCl, pH 8.2 (Fig. 1A and B). The FXa/Va activator comprises PL, $CaCl_2$, bovine FXa, and bovine FVa. Optimal composition of each component in the FXa/Va activator were 6.25% PL, 5 mM $CaCL_2$, 0.01 μg/mL FXa, and 0.1 μg/mL FVa (Fig. 1C–F).

We manually compared the prothrombin activation kinetics with the FXa/Va activator to that with the Ox activator to assess the performance of the FXa/Va activator (Fig. 2). On the FXa/Va activator-based ATR assay, we observed the optimal activation time for 2 min in the prothrombin activation step (Fig. 2A) and the equivalent performance to that of the Ox activator in the thrombin inactivation step (Fig. 2B). Based on these results, we determined the detailed manual assay procedure with the FXa/Va activator (Fig. 2C). We subsequently investigated the ATR detection ability of

the FXa/Va activator system using the reconstituted plasma model (Fig. 2D and E). Reconstituted plasma was prepared by mixing prothrombin-deficient plasma and recombinant prothrombin as described previously,[16] and recombinant Arg596Leu (prothrombin Yukuhashi) or Arg596Gln (prothrombin Belgrade) prothrombin was mixed with wild-type prothrombin in a 1:1 ratio to prepare the reconstituted plasma mimicking the plasma from each heterozygote patient. In the Ox activator-based ATR assay, RRTAs at 10, 20, and 30 min were 29.6%, 10.6%, and 6.1% for WT (Arg/Arg: R/R); 70.3%, 59.9%, and 53.9% for prothrombin Yukuhashi heterozygote (Arg/Leu: R/L); and 67.4%, 53.1%, and 47.5% for prothrombin Belgrade heterozygote (Arg/Gln: R/Q), respectively (Fig. 2D). In the FXa/Va activator-based ATR assay, RRTAs at 10, 20, and 30 min were 40.5%, 17.6%, and 10.0% for WT (R/R); 77.0%, 66.9%, and 62.8% for prothrombin Yukuhashi heterozygote (R/L); and 74.5%, 63.0%, and 57.6% for prothrombin Belgrade heterozygote (R/Q), respectively (Fig. 2E). These results suggested that the FXa/Va activator-based assay has an equivalent ability to detect ATR as the Ox activator-based assay.

3.2 Application of the FXa/Va activator-based ATR assay to automation using the ACL TOP500 instrument

For practical clinical use, we adjusted the FXa/Va activator-based ATR assay for an automated operation using ACL TOP 500. Optimum conditions of the FXa/Va activator-based ATR assay were re-investigated with 1/100 or 1/200 diluted normal pooled plasma for automated ACL TOP 500 (Fig. 3). The optimal composition for sample dilution buffer was 50 mM Tris-HCl, 200 mM NaCl, pH 8.3 (Fig. 3A and B) and the optimal condition for prothrombin activation using the FXa/Va activator comprised 10.0% PL, 5 mM $CaCl_2$, 0.02 μg/mL bovine FXa, and 0.2 μg/mL bovine FVa (Fig. 3C–F).

Furthermore, we examined other parameters of the ATR assay, activation duration in the prothrombin activation step, antithrombin concentration to be added during the thrombin inactivation step, and substrate concentration in the assessment step (Fig. 4). The optimal time for prothrombin activation demonstrating high throughput was 75–85 s (Fig. 4A). Because ACL TOP 500 has only one probe arm, short intervals in the multianalyte assay are mechanically restricted. Optimal concentrations of antithrombin and S-2238 substrate were determined to be 75 μg/mL and 0.5 mM, respectively (Fig. 4B and C). The plasma dilution linearity using ACL TOP 500 was investigated at dilution from 1/50 to 1/600 (Fig. 4D); however, data instability, which seems to be due to the interference of the fibrin clot in the cuvette, was observed at 1/100 and 1/50 dilution. Therefore, in the FXa/Va activator-based ATR assay using ACL TOP 500, the 1/200 plasma dilution was determined to be optimal.

Finally, we assessed the ATR detection ability of the optimised procedure of the ACL TOP 500 assay using the FXa/Va activator (Fig. 5). The reconstituted plasma with recombinant antithrombin-resistant prothrombin (Arg596Leu or Arg596Gln) or plasma from an antithrombin-resistant patient (prothrombin Yukuhashi or prothrombin Belgrade) was subjected to the performance assessment test. The optimal automated assay procedure is summarised in Fig. 5A. Although RRTA at 30 min of reconstituted plasma with WT prothrombin (R/R: WT) was approximately 10%, those of reconstituted plasmas with recombinant antithrombin-resistant prothrombin (L/L: Arg596Leu Homo, and Q/Q: Arg596Gln Homo) were over 90% (Fig. 5B). In addition, we examined the performance of the automated ATR assay using the FXa/Va activator for plasma from a patient carrying a heterozygote of prothrombin Yukuhashi or Belgrade (Fig. 5C). RRTAs at 10, 20, and 30 min were 41.3%, 14.2%, and 7.9% for normal pooled plasma; 66.2%, 53.7%, and 45.8% for plasma from the patient with the prothrombin Yukuhashi mutation; and 70.9%, 53.1%, and 54.3% for plasma from the patient with the

prothrombin Belgrade mutation, respectively. These results revealed that the automated ACL TOP 500 ATR assay using the FXa/Va activator clearly detected the antithrombin-resistant property of prothrombin Yukuhashi (p.Arg596Leu) and Belgrade (p.Arg596Gln).

4. Discussion

In this study, we developed an ATR assay using the FXa/Va activator. The optimised ATR assay with the FXa/Va activator represented a clear distinction between antithrombin-resistant prothrombins and normal plasma, which was a comparable performance to the previous assay with the Ox activator. Advantages of the assay are as follows: (1) bovine FXa and FVa are commercially available; (2) the ATR assay with the FXa/Va activator is optimised for manual and automated operation procedures; (3) automatisation of the assay can result in a high throughput; and (4) because ACL TOP series is a common coagulation analyser distributed globally, this optimised assay protocol can be easily referenced and used.

Antithrombin-resistant prothrombin is a mutant presenting paradoxical features in thrombosis and haemostasis *in vivo*, i.e. the mutant thrombin derived from antithrombin-resistant prothrombin displays a mild decline in the procoagulant activity. However, a substantial impairment in its inactivation by antithrombin results in a prolongation of the procoagulant activity, causing thromboembolism in some patients.[10,12,15,17] In clinical laboratory tests, plasma from the patient with a heterozygous ATR mutation does not exhibit a significant alteration in PT and APTT levels.[12,15] Miljic et al. reported a significant decline in the PT-based prothrombin activity and an increase in the endogenous thrombin potential (ETP) in the thrombin generation assay in heterozygous carriers of prothrombin Belgrade.[15] In contrast, in heterozygous carriers of prothrombin Padua 2, PT-based prothrombin activities were

slightly reduced or at borderline levels, and ETP of patients did not significantly increase compared with that of the pooled normal plasma.[12] These previous insights suggest that clinical significance of the PT-based prothrombin activity and ETP in the thrombin generation assay seems to be controversial. Conversely, the ATR assay with the FXa/Va activator developed in this study showed a performance to clearly detect antithrombin-resistant prothrombins. Furthermore, the general versatility of the developed assay is one of the advantages for clinical laboratory test, implying that optimised assay conditions and parameters can be readily adapted to a general purpose automated analyser installed in a hospital clinical laboratory unit. Cost-effectiveness is also an advantage of the FXa/Va activator-based assay (Supporting Table). If a general coagulation analyser is not available, the ATR assay with the FXa/Va activator can be manually conducted. In addition, the ATR assay can detect ATR even in the plasma from a patient undergoing warfarin treatment[16].

The thrombotic clinical penetrance of antithrombin-resistant prothrombinemia is estimated to be equivalent or higher than that of antithrombin deficiency. With respect to prothrombin Belgrade (Arg596Gln), 60% heterozygous carriers of this mutation in the reported family developed venous thrombosis during their lifetime, and the annual incidence rate of the first thrombosis from birth was 2.2%.[15] Conversely, the prevalence of an antithrombin-resistant prothrombin carrier with this mutation has not been precisely estimated among patients with VTE. In their preliminary study, Bulato et al. described that the prevalence of other antithrombin-resistant prothrombin (Padua 2: Arg596Trp) was 5 carriers out of 1000 patients with VTE (0.5%), although with a quite selective and arbitrary screening strategy based only on the personal history of VTE and the presence of a mild reduction in the prothrombin activity with normal PT.[12] This implies that antithrombin-resistant prothrombin is rare but present as a cause of VTE and has been possibly overlooked as a cause of unexplained VTE. It is necessary

to conduct an antithrombin-resistant prothrombin screening on a large scale for estimating the actual prevalence of antithrombin-resistant prothrombins, such as prothrombins Yukuhashi, Belgrade, and Padua 2, in patients with VTE. At this point, the high-throughput system of the automated ATR assay with the FXa/Va activator can contribute to the extensive screening of antithrombin-resistant prothrombin.

To investigate the sensitivity and specificity of the FXa/Va activator-based ATR assay, follow-up studies to examine the performance in a large population are clearly needed. Especially, the specificity of this assay should be strictly tested in the plasmas containing other thrombophilias (e.g., antithrombin deficiency), or potential confounders (e.g., lupus anticoagulants) or various therapeutic anticoagulants (e.g., warfarin). The cut-off value based on receiver operating characteristic (ROC) curve and the reference range on a large donor population should be also investigated.

In conclusion, we developed a more convenient ATR assay using bovine FXa and FVa. The ATR assay with the FXa/Va activator is optimised for both manual operation and automated instrument system and is now ready for its widespread global usage. Although antithrombin-resistant prothrombin mutations (prothrombins Yukuhashi, Belgrade, and Padua 2) are recognised as a rare thrombophilic risk, the prevalence of antithrombin-resistant prothrombin might be more frequent than expected before. The accurate diagnosis of antithrombin-resistant prothrombin is essential for therapeutic planning and/or genetic counselling of patients. We hope that the developed ATR assay significantly contributes to the investigation and diagnosis of antithrombin-resistant prothrombin-associated VTE.

Authors' Contributions

S.T. designed and performed the research, analysed data, and drafted the manuscript. Y.S., M.T., and M. M-K. performed the research, analysed data, and drafted the

manuscript. Y.S. and M.T. contributed equally to this work and shared second authorship. Y.T., Y.H., M. K., and S.S. interpreted data and contributed to the analytic methodology. A.T. developed and supervised the project and edited the manuscript. T.K. designed the project, analysed data, and drafted the manuscript. All authors were involved in critical reading of the manuscript prior to submission.

Competing interests

The authors declare no competing financial interests.

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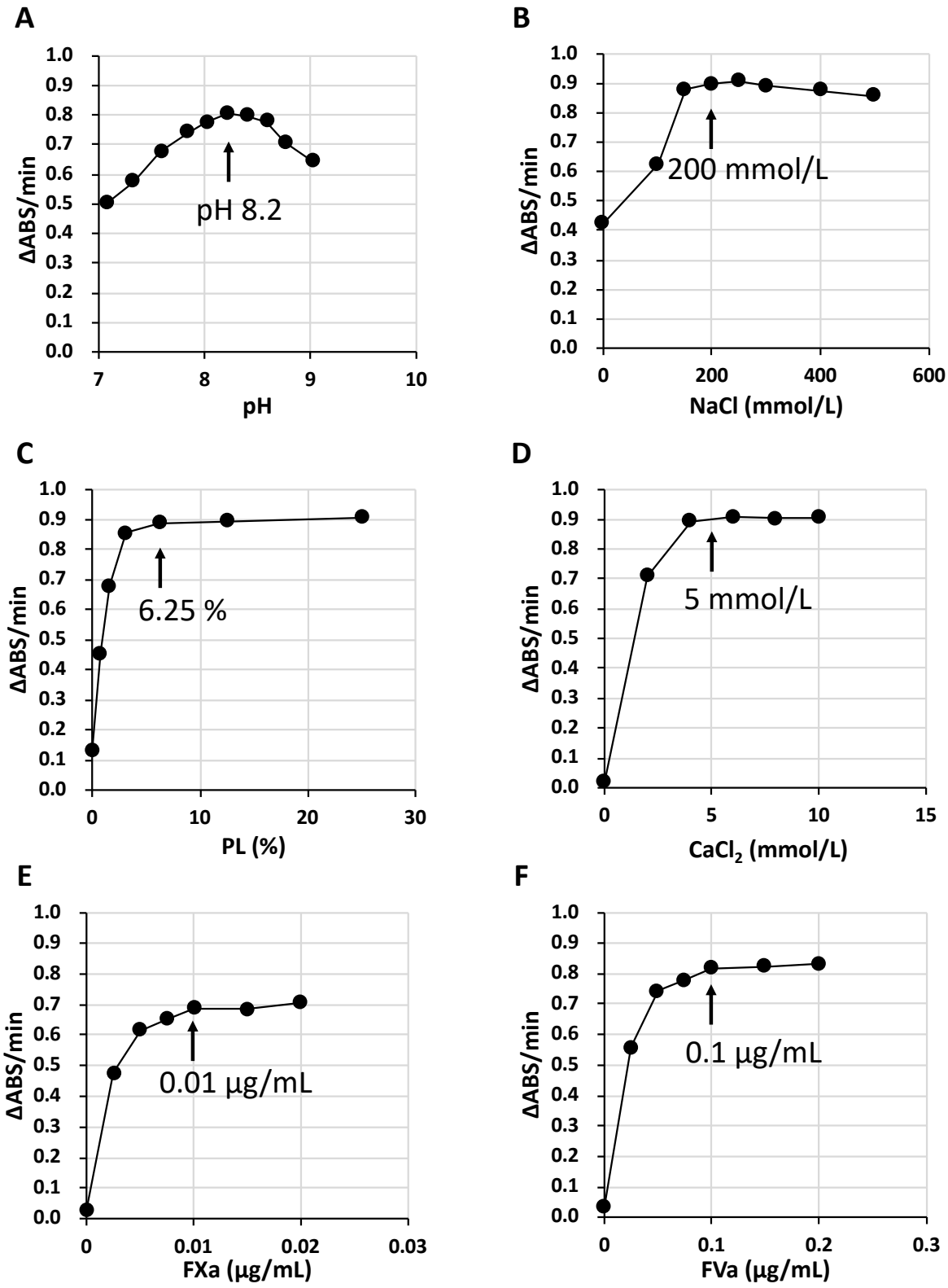
Figure. 1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ΔABS/min
pH
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B
ΔABS/min
NaCl (mmol/L)
200 mmol/L
C
ΔABS/min
PL (%)
6.25 %
D
ΔABS/min
CaCl2 (mmol/L)
5 mmol/L
E
ΔABS/min
FXa (μg/mL)
0.01 μg/mL
F
ΔABS/min
FVa (μg/mL)
0.1 μg/mL

Figure. 2

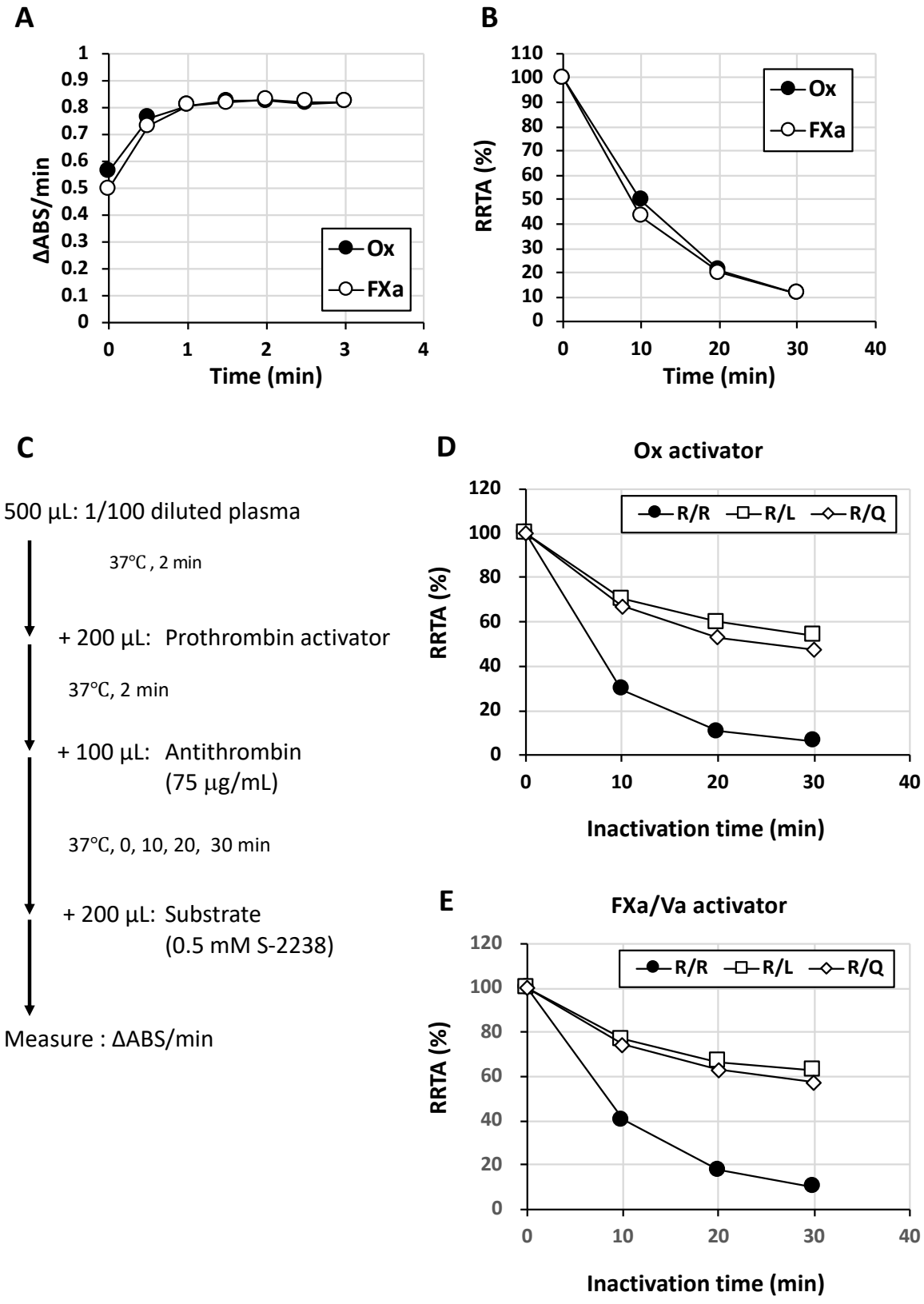
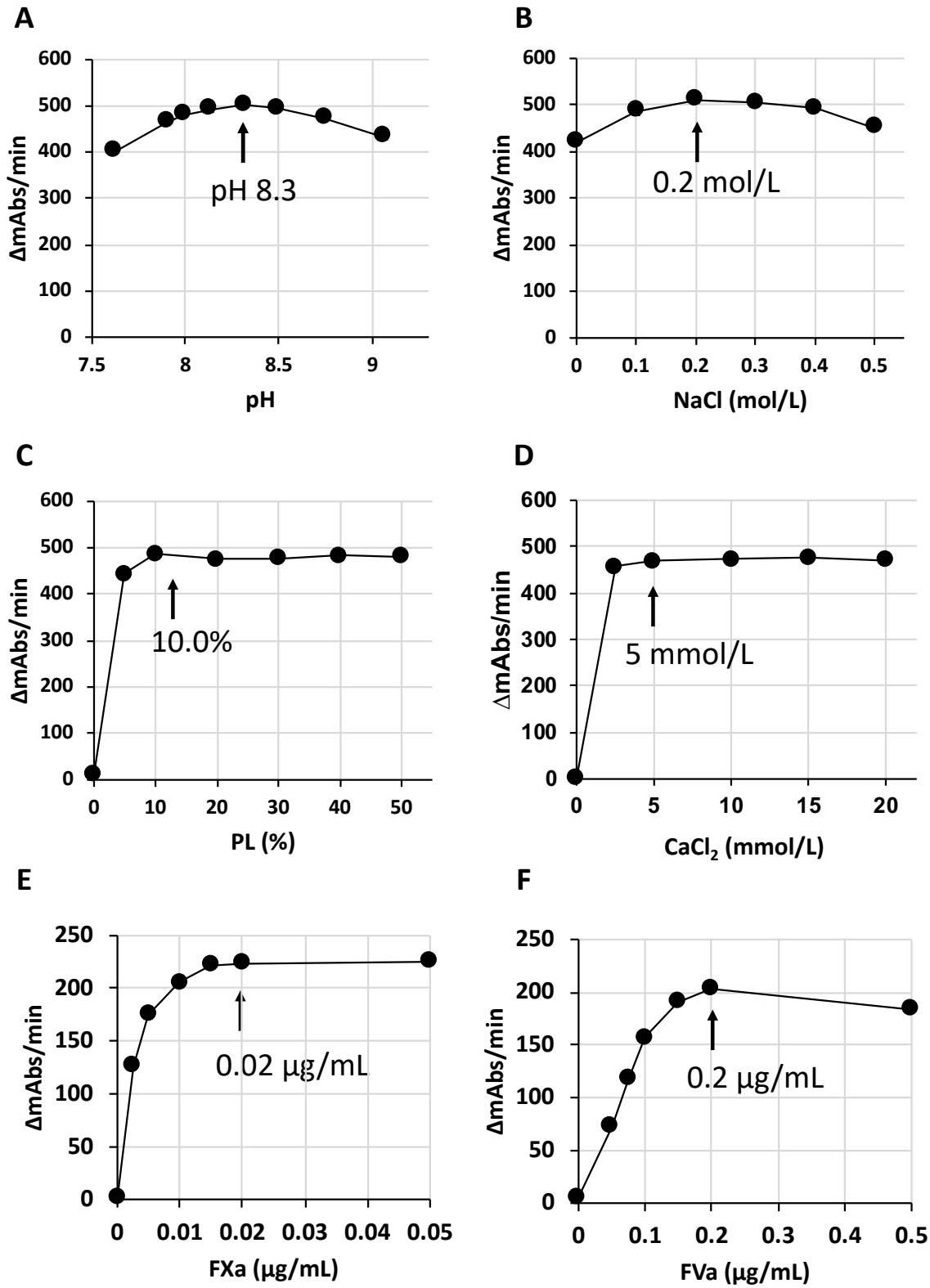

A
ΔABS/min
Time (min)
Ox
FXa
B
RRTA (%)
Time (min)
Ox
FXa
C
500 μL: 1/100 diluted plasma
37℃, 2 min
+ 200 μL: Prothrombin activator
37℃, 2 min
+ 100 μL: Antithrombin (75 μg/mL)
37℃, 0, 10, 20, 30 min
+ 200 μL: Substrate (0.5 mM S-2238)
Measure : ΔABS/min
D
Ox activator
R/R
R/L
R/Q
RRTA (%)
Inactivation time (min)
E
FXa/Va activator
R/R
R/L
R/Q
RRTA (%)
Inactivation time (min)

Figure. 3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pH
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ΔmAbs/min
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PL (%)
D
ΔmAbs/min
5 mmol/L
CaCl2 (mmol/L)
E
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FXa (μg/mL)
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FVa (μg/mL)

Figure. 4

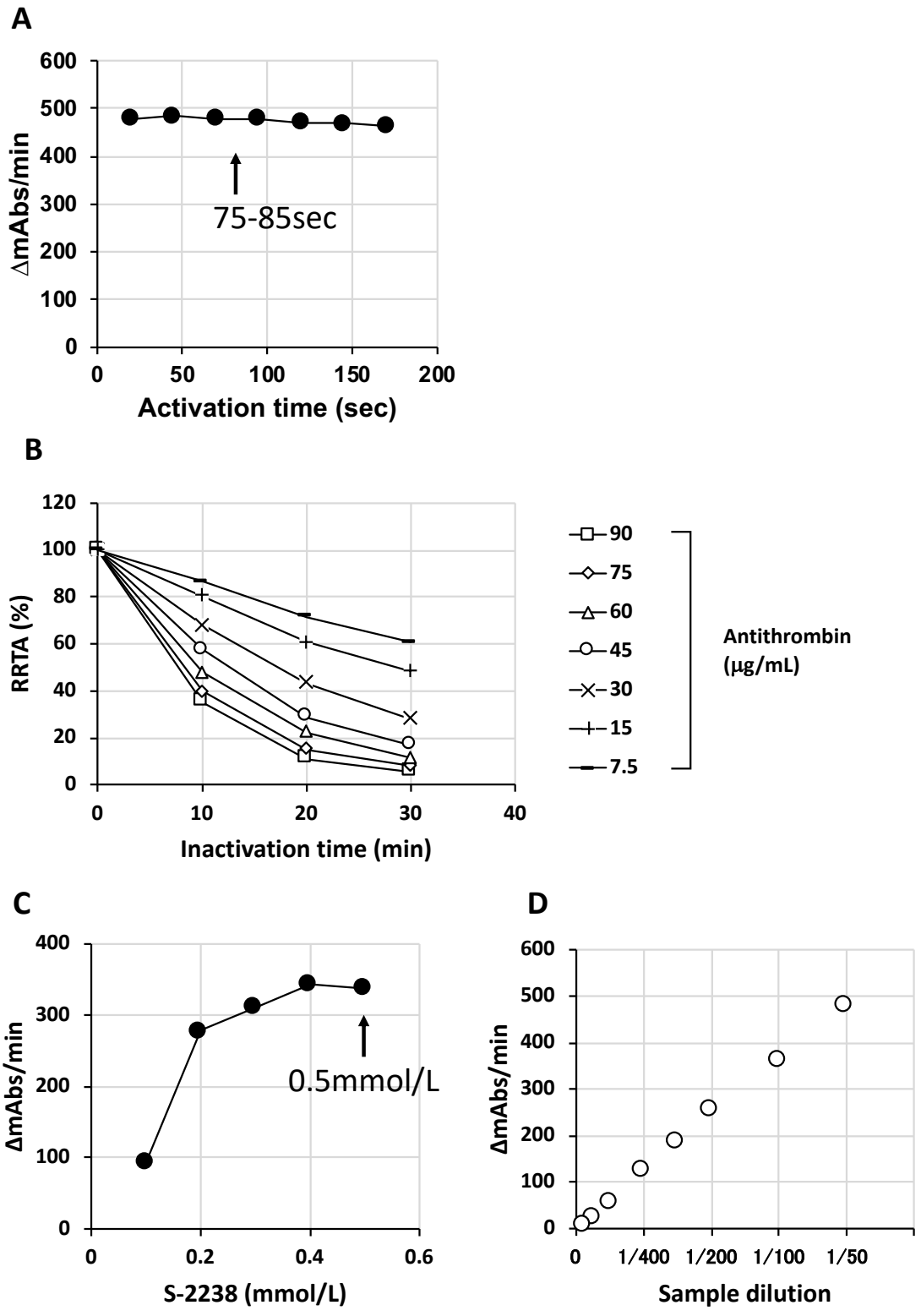
A
600
500
400
300
200
100
0
ΔmAbs/min
75-85sec
0
50
100
150
200
Activation time (sec)
B
120
100
80
60
40
20
0
RRTA (%)
0
10
20
30
40
Inactivation time (min)
90
75
60
45
30
15
7.5
Antithrombin
(μg/mL)
C
400
300
200
100
0
ΔmAbs/min
0.5mmol/L
0
0.2
0.4
0.6
S-2238 (mmol/L)
D
600
500
400
300
200
100
0
ΔmAbs/min
0
1/400
1/200
1/100
1/50
Sample dilution

Figure. 5

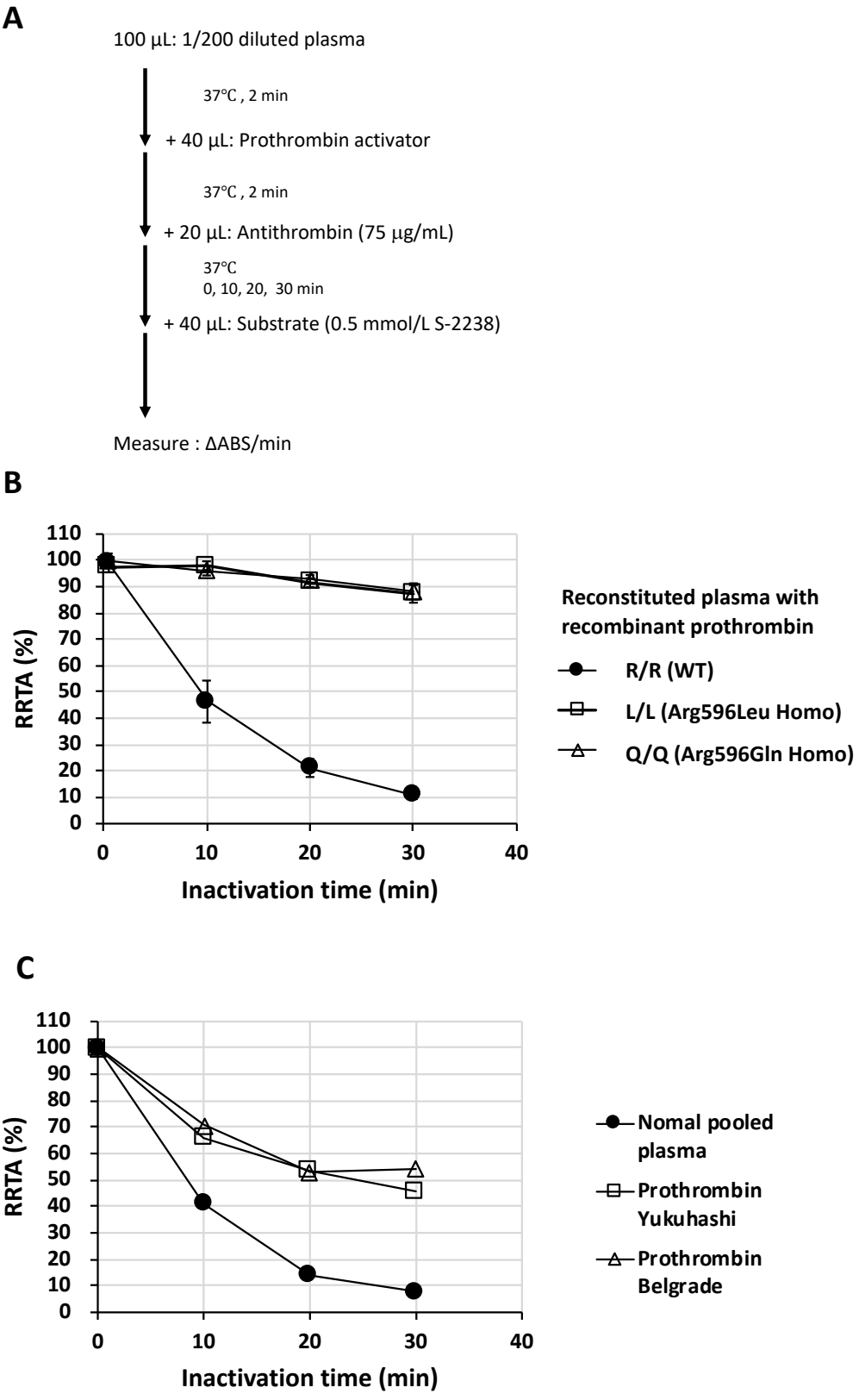
A
100 µL: 1/200 diluted plasma
37℃ , 2 min
+ 40 µL: Prothrombin activator
37℃ , 2 min
+ 20 µL: Antithrombin (75 µg/mL)
37℃
0, 10, 20, 30 min
+ 40 µL: Substrate (0.5 mmol/L S-2238)
Measure : ΔABS/min
B
RRTA (%)
Inactivation time (min)
Reconstituted plasma with recombinant prothrombin
R/R (WT)
L/L (Arg596Leu Homo)
Q/Q (Arg596Gln Homo)
C
RRTA (%)
Inactivation time (min)
Nomal pooled plasma
Prothrombin Yukuhashi
Prothrombin Belgrade

Figure legends

Fig. 1. Optimal conditions of the ATR assay with the FXa/Va activator in the manual operation procedure.

A, B: pH (A) or NaCl concentration (B) in the sample dilution buffer (50 mM Tris-HCl). C–F: the concentration of each component in the FXa/Va activator for the manual assay; phospholipid (PL) (C), $CaCl_2$ (D), bovine FXa (E), and bovine FVa (F).

Fig. 2. Kinetics of thrombin activity in the manual ATR assay with the Ox or FXa/Va activator.

A: Thrombin activity profiles in prothrombin activation. B: Thrombin activity profiles in inactivation by antithrombin. C: The optimised ATR assay with the FXa/Va activator in the manual operation procedure. D: RRTA kinetics in the ATR assay with the Ox activator for the reconstituted plasma with recombinant prothrombins prepared as described in Materials and Methods. E, RRTA kinetics in the ATR assay with the FXa/Va activator for reconstituted plasma with recombinant prothrombins prepared as described in Materials and Methods. R, Arg596; L, Arg596Leu; Q, Arg596Gln prothrombins. R/R, WT reconstituted plasma; R/L and R/Q, heterozygous Arg596Leu and Arg596Gln plasma, respectively.

Fig. 3. Optimal conditions of the ATR assay with the FXa/Va activator in the ACL TOP 500 automated system.

A, B: pH (A) and NaCl concentration (B) in the sample dilution buffer (50 mM Tris-HCl). C–F: Concentration of each component in the FXa/Va activator for the ACL TOP 500 automated system; PL (C), $CaCl_2$ (D), bovine FXa (E), and bovine FVa (F).

Fig. 4. Parameter optimisation for the automated ATR assay with the FXa/Va activator. A: Optimisation of the condition for the automated ATR assay and the duration of prothrombin activation. B: Adding antithrombin concentration. C: Chromogenic substrate concentration. D: Plasma dilution linearity.

Fig. 5. The ATR assay profile with the FXa/Va activator on automated ACL TOP 500. A: The optimal ATR assay procedure on automated ACL TOP 500. B: RRTA kinetics of the homozygote plasma model for wild-type (R/R), prothrombin Yukuhashi (Arg596Leu: L/L), and prothrombin Belgrade (Arg596Gln: Q/Q). Reconstituted plasma were prepared as described in Materials and Methods. R, Arg596; L, Arg596Leu; Q, Arg596Gln. C, RRTA kinetics of normal pooled plasma, plasma from patient with prothrombin Yukuhashi (Arg596Leu, heterozygote), and plasma from patient with prothrombin Belgrade (Arg596Gln, heterozygote).

Supporting information

Optimisation protocol for sample dilution buffer and prothrombin activator compositions.

For optimization of the FXa/Va activator-based manual ATR assay, we used Tris–HCl (50 mM) and NaCl as sample dilution buffer, and FXa, FVa, PL and $CaCl_2$ as prothrombin activator.

1. pH of sample dilution buffer:
   When testing pH of sample dilution buffer, other components of dilution buffer and prothrombin activator were tentatively fixed (NaCl 150 mM, PL 10%, $CaCl_2$ 10 mM, FXa 0.02 μg/ml and FVa 0.2 μg/mL). As a result, the optimal pH of sample dilution buffer was determined to be 8.2.
2. NaCl of sample dilution buffer:
   NaCl concentration was tested under the determined pH 8.2 sample dilution buffer and other tentatively fixed prothrombin activator conditions of PL 10%, $CaCl_2$ 10 mM, FXa 0.02 μg/mL and FVa 0.2 μg/mL. As a result, the optimal NaCl concentration was determined to be 200 mM.
3. PL of prothrombin activator:
   Concentration of PL was tested under the determined sample dilution buffer conditions of pH 8.2 and NaCl 200 mM, and other tentatively fixed prothrombin activator conditions of $CaCl_2$ 10 mM, FXa 0.02 μg/ml and FVa 0.2 μg/mL. As a result, optimal PL concentration was determined to be 6.25%.
4. $CaCl_2$ of prothrombin activator:
   $CaCl_2$ concentration was tested under the determined conditions of pH 8.2, NaCl 200 mM and PL 6.25%, and other tentatively fixed conditions of FXa 0.02 μg/mL and FVa 0.2 μg/mL. As a result, optimal $CaCl_2$ concentration was determined to be 5 mM.
5. FXa of prothrombin activator:
   FXa concentration was tested under the determined conditions of pH 8.2, NaCl 200 mM, PL 6.25% and $CaCl_2$ 5 mM, and other tentatively fixed condition of FVa 0.2 μg/mL. As a result, optimal FXa concentration was determined to be 0.01 μg/mL.
6. FVa of prothrombin activator:
   FVa concentration was tested under the determined conditions of pH 8.2, NaCl 200 mM, PL 6.25%, $CaCl_2$ 5 mM and FXa: 0.01 μg/mL. As a result, optimal FVa concentration was determined to be 0.1 μg/mL.

Subsequently, these determined conditions of pH 8.2 and NaCl 200 mM for sample dilution buffer as well as PL 6.25%, $CaCl_2$ 5 mM, FXa 0.01 μg/mL and FVa 0.1 μg/mL for prothrombin activator were re-examined as follows respectively (Figure. 1).

1. Sample dilution buffer pH (ranged from 7.0 to 9.0) was re-tested under other determined conditions for sample dilution buffer and prothrombin activator, and pH 8.2 was confirmed as the optimmal condition for sample dilution buffer.
2. NaCl concentration (ranged from 0 to 500 mM) of sample dilution buffer was re-tested under the determined conditions for sample dilution buffer and prothrombin activator, and NaCl 200 mM was confirmed as the optimal condition for sample dilution buffer.
3. PL concentration (ranged from 0 to 25%) of prothrombin activator was re-tested under the determined conditions for sample dilution buffer and prothrombin activator, and PL 6.25% was confirmed as the optimal condition for prothrombin activator.
4. $CaCl_2$ concentration (ranged from 0 to 10 mM) of prothrombin activator was re-tested under the determined conditions for sample dilution buffer and prothrombin activator, and $CaCl_2$ 5 mM was confirmed as the optimal condition for FXa/Va activator
5. FXa concentration (0 to 0.02 μg/mL of prothrombin activator was re-tested under the determined conditions for sample dilution buffer and prothrombin activator, and FXa 0.01 μg/mL was confirmed as the optimal condition for FXa/Va activator.
6. FVa concentration (0 to 0.2 μg/mL of prothrombin activator was re-tested under the determined conditions for sample dilution buffer and prothrombin activator, and FVa 0.1 μg/mL was confirmed as the optimal condition for FXa/Va activator.

After performing all of the above procedures, optimum conditions for sample dilution buffer (Tris–HCl 50 mM, pH 8.2; NaCl 200 mM) and prothrombin activator (PL 6.25%, $CaCl_2$ 5 mM, FXa 0.01 μg/mL and FV 0.1 μg/mL) were determined.

For optimisation of the FXa/Va activator-based automated ATR assay, we also carried out a similar procedure using the ACL TOP 500 analyzer as described above, and determined the optimum conditions for sample dilution buffer (Tris–HCl 50 mM, pH 8.3; NaCl 200 mM) and prothrombin activator (PL 10.0%, $CaCl_2$ 5 mM, FXa 0.02 μg/mL and FV 0.2 μg/mL) as shown in Figure 3.

Supporting Table.

sTable. Assay cost in each manual ATR assay

Maker	Venom	Content	Price (Jpn Yen)	Conc. of stock solution	Used amount	Cost/Assay
Latoxan	Ox	10 mg (powder)	¥47,500 (in 2013)	1 mg/mL	0.2 mL of 0.1 mg/mL	¥95.0
SIGMA-ALDRICH	Ecarin	44-55 U/vial	¥95,100	55 U/vial	0.05 mL of 1.1 U/mL*	¥95.1
Haematologic Technologies Inc.	Bovine FXa	100 μg	¥9,720	2.9 mg/mL	0.2 mL of 0.01 μg/mL	¥0.19
Haematologic Technologies Inc.	Bovine FVa	100 μg	¥46,656	6.5 mg/mL	0.2 mL of 0.1 μg/mL	¥9.33

The exchange rate is 113.45 yen/US dollar as of December 13, 2017.

* Bulato C et al. Arterioscler Thromb Vasc Biol. 2016;36:1022-1029.