「医薬品に果たす品質の役割」に関する米国薬局方(USP)-MHLW/PMDA共同ワークショップ

General Information: Bacterial Endotoxins Test and Alternative Methods using Recombinant Protein-reagents for Endotoxin Assay <*G4-4-180*>

参考情報：エンドトキシン試験法と測定試薬に遺伝子組換えタンパク質を用いる代替法 <*G4-4-180*>

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Disclaimer

The views and opinions are those of the author and do not necessarily reflect the official policy or position of the Japanese Pharmacopoeia.

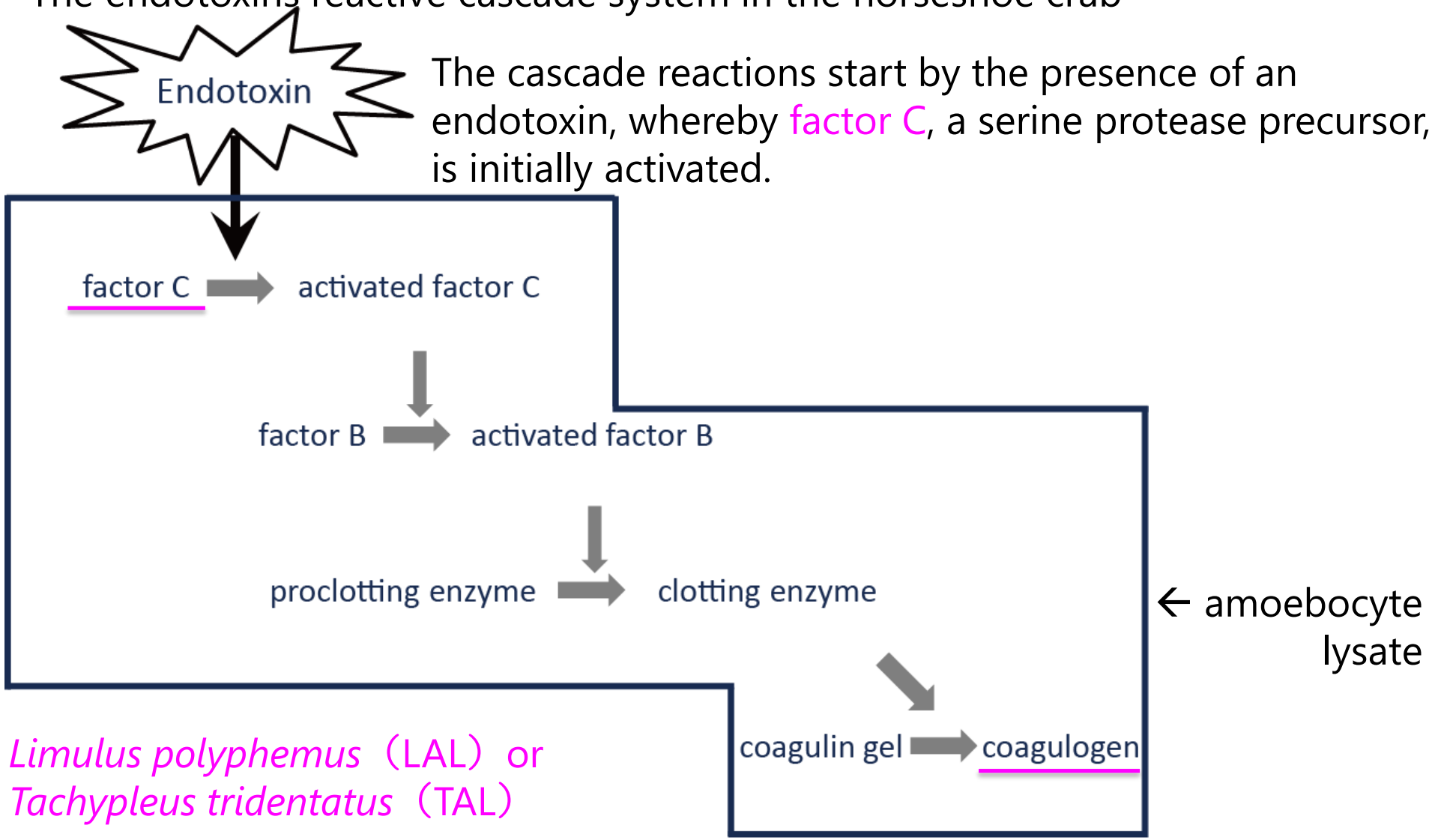
Today's presentation is an overview of the 18th edition of Japanese Pharmacopeia's general information "Bacterial Endotoxins Test and Alternative Methods using Recombinant Protein-reagents for Endotoxin Assay <*G4-4-180*>."

General tests "Bacterial Endotoxins Tests (BET)" was published in the 11th edition, supplement, of Japanese Pharmacopoeia (JP) in October of 1988 and harmonized with the U.S. Pharmacopeia (USP) and the European Pharmacopoeia (Ph.Eur.) in the 16th edition of the JP in March of 2011.

New chapter is modification of theirs using recombinant Factor C-based procedure in June of 2021.

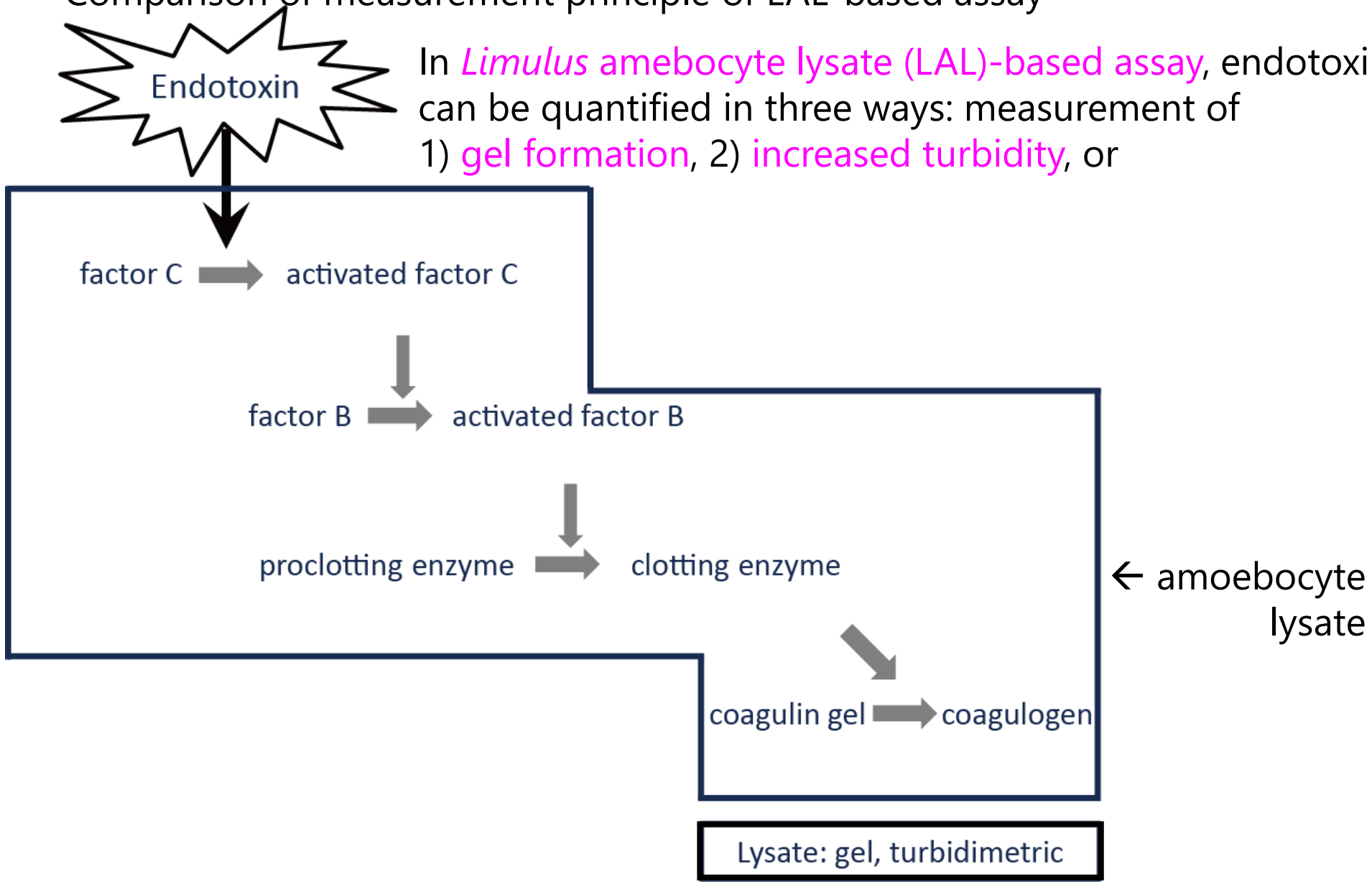
Date	JP	note
1988	11, suppl.	Gel-clot techniques (BET, General Tests)
1996	13	Turbidimetric technique and Chromogenic technique (BET, General Tests)
2011	16	harmonized with USP and Ph.Eur. (4.01 BET, General Tests)
2021	18	recombinant Factor C-based procedure (<*G4-4-180*>, General Informations)

The endotoxins reactive cascade system in the horseshoe crab

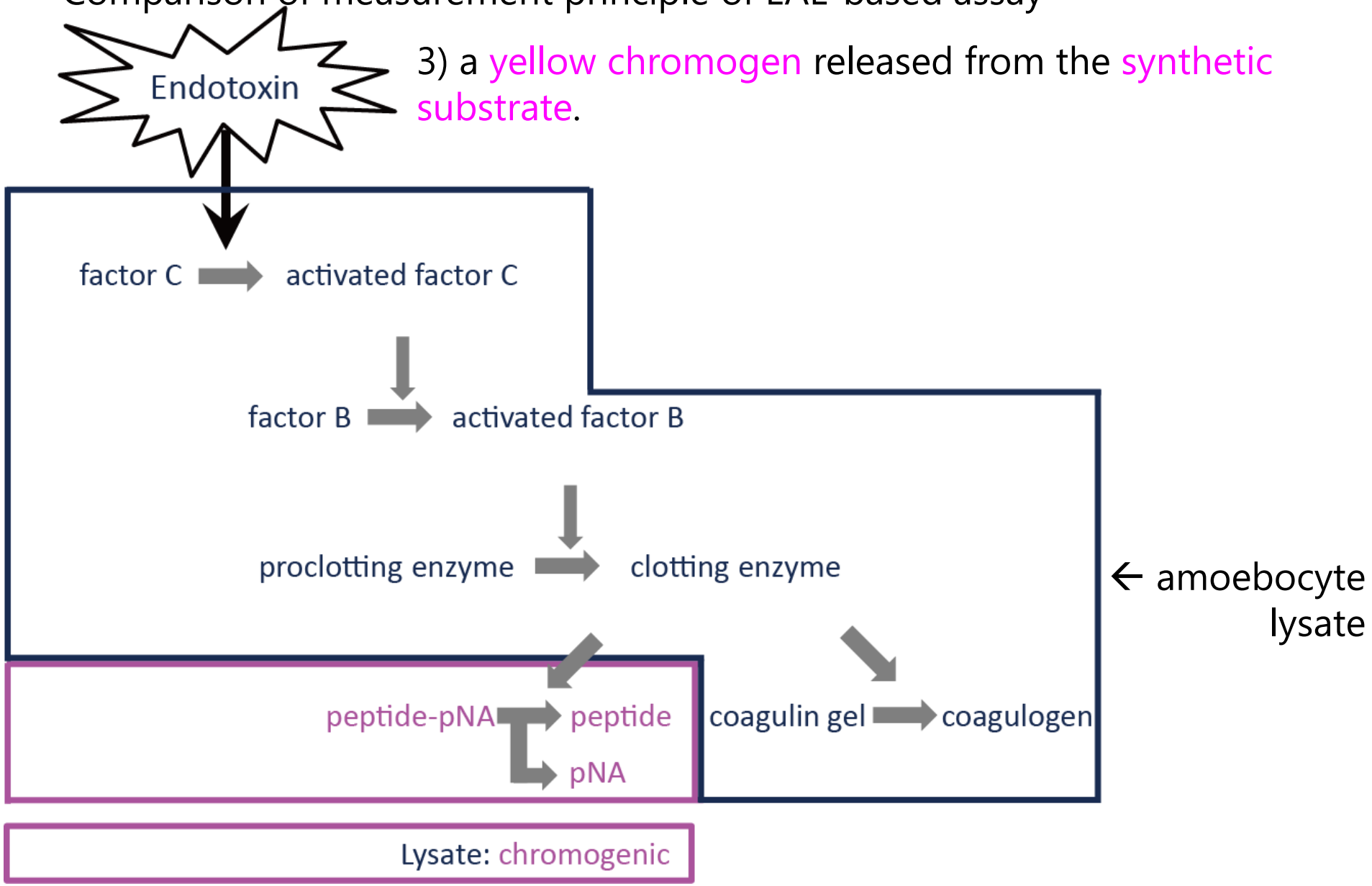


Comparison of measurement principle of LAL-based assay

In *Limulus* amebocyte lysate (LAL)-based assay, endotoxin can be quantified in three ways: measurement of 1) gel formation, 2) increased turbidity, or



Comparison of measurement principle of LAL-based assay



Recombinant factor C-based procedure

1st generation

PyroGene, Lonza (since 2003)

Recombinant factor C of Singapore horseshoe crab, *Carcinoscorpius rotundicauda*.

2nd generation

EndoZyme/EndoZyme II, bioMérieux/Hyglos

Recombinant factor C of Japanese horseshoe crab, *Tachypleus tridentatus*.

3rd generation

PyroSmart/PyroSmart NextGen, Seikagaku corporation/Associates of Cape Cod

Comprising recombinant cascade reagents: factor C, factor B and proclotting enzyme of *T. tridentatus* / American horseshoe crab *L. polyphemus*.

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1st generation

PyroGene, Lonza (since 2003)

Recombinant factor C of Singapore horseshoe crab, *Carcinoscorpius rotundicauda*.

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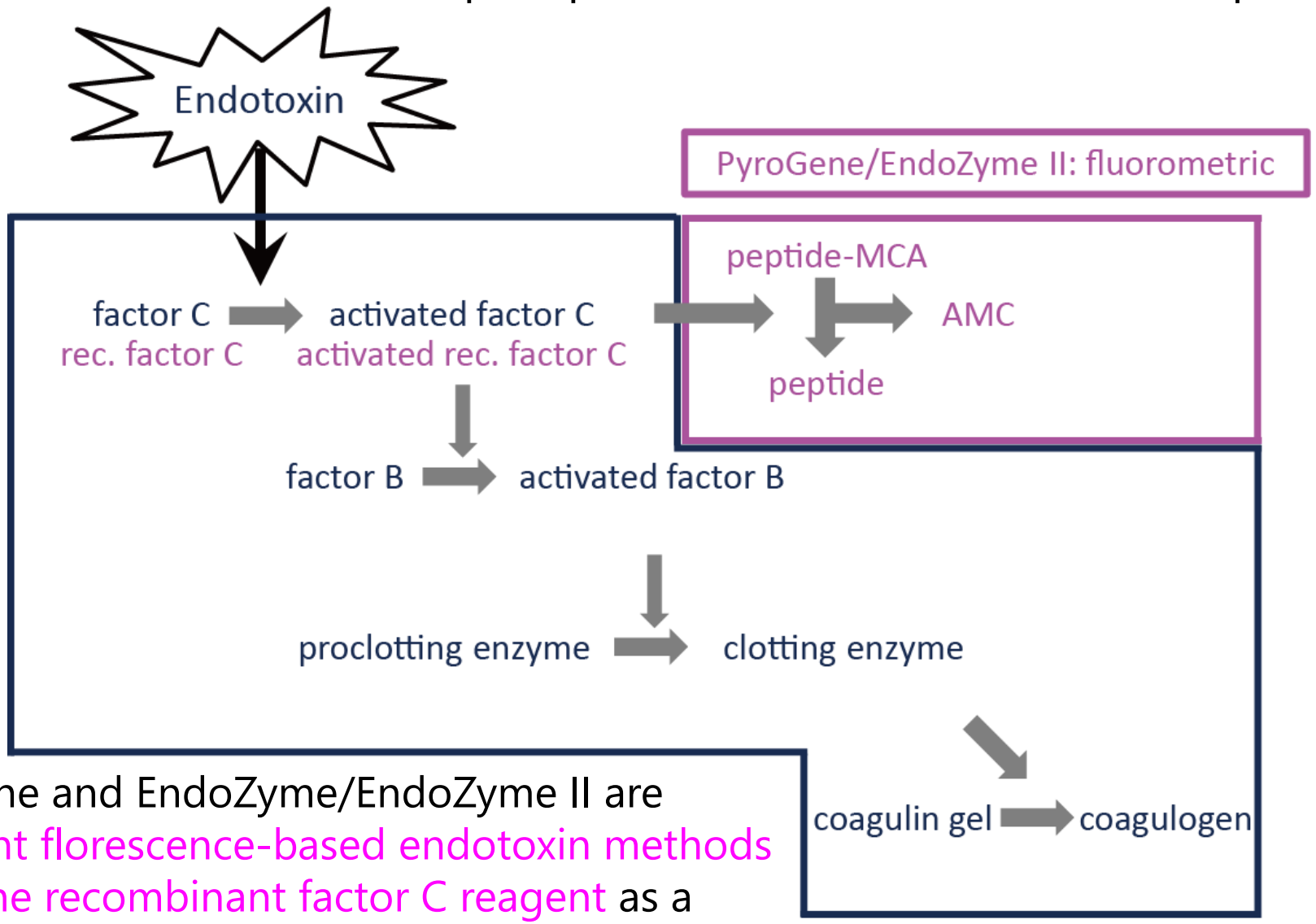
Comprising recombinant cascade reagents: factor C, factor B and proclotting enzyme of *T. tridentatus* / American horseshoe crab *L. polyphemus*.

Comparison of measurement principle of recombinant factor C-based procedure



PyroGene and EndoZyme/EndoZyme II are endpoint florescence-based endotoxin methods using the recombinant factor C reagent as a replacement to the amoebocyte lysate.

Recombinant factor C-based procedure

1st generation

PyroGene, Lonza (since 2003)

Recombinant factor C of Singapore horseshoe crab, *Carcinoscorpius rotundicauda*.

2nd generation

EndoZyme/EndoZyme II, bioMérieux/Hyglos

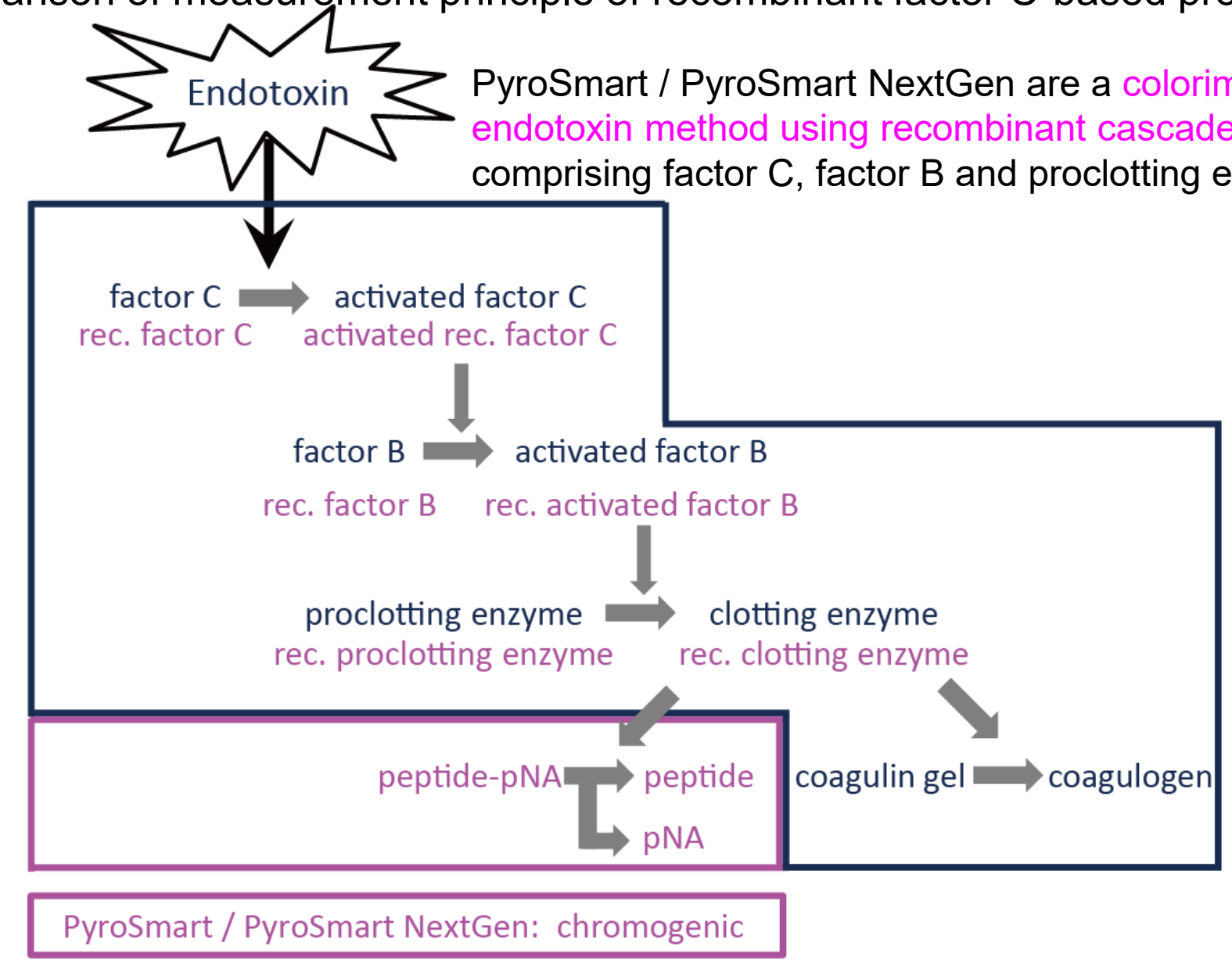
Recombinant factor C of Japanese horseshoe crab, *Tachypleus tridentatus*.

3rd generation

PyroSmart/PyroSmart NextGen, Seikagaku corporation/Associates of Cape Cod

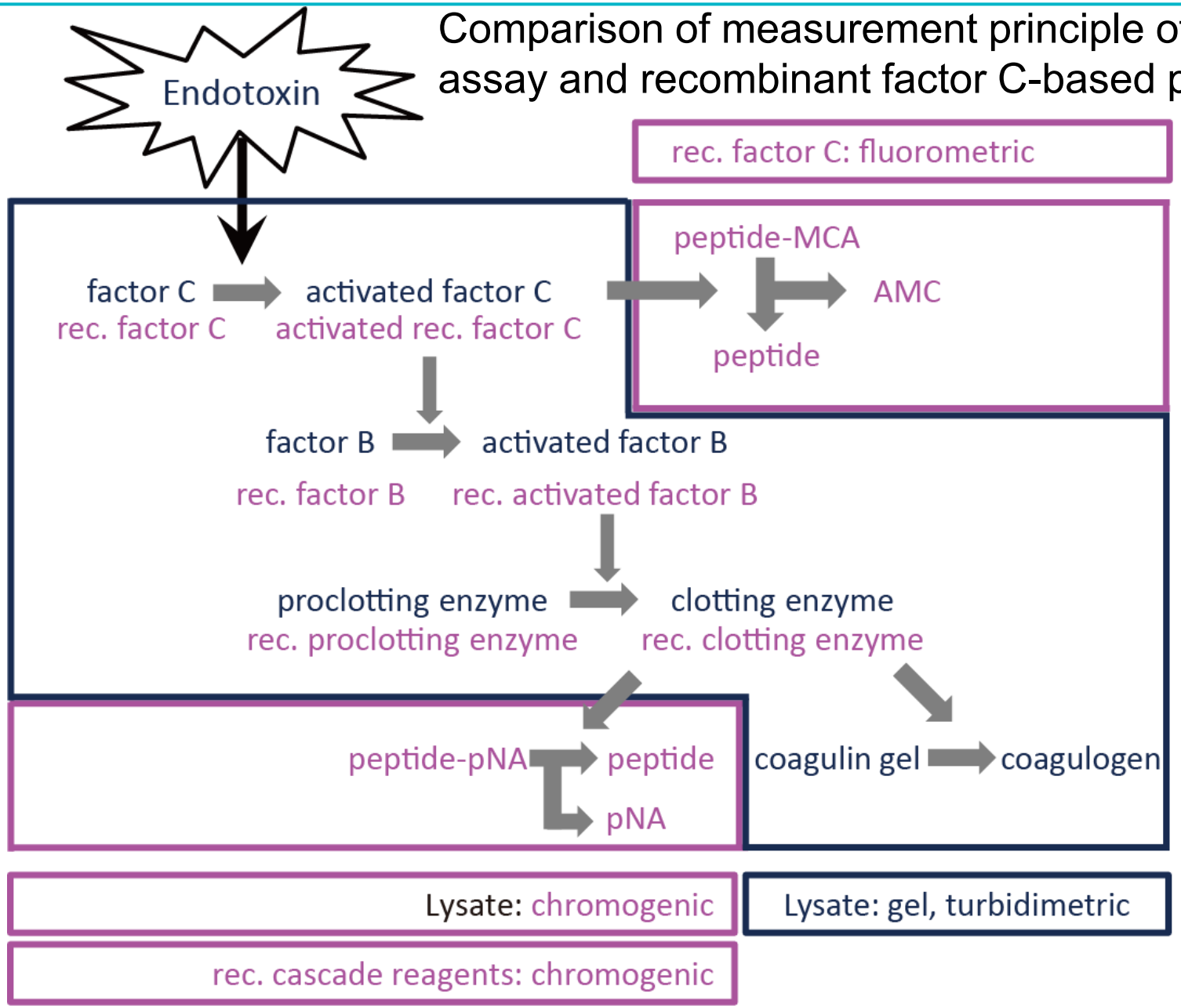
Comprising recombinant cascade reagents: factor C, factor B and proclotting enzyme of *T. tridentatus* / American horseshoe crab *L. polyphemus*.

Comparison of measurement principle of recombinant factor C-based procedure



PyroSmart / PyroSmart NextGen are a colorimetric-based endotoxin method using recombinant cascade reagents comprising factor C, factor B and proclotting enzyme.

Comparison of measurement principle of LAL-based assay and recombinant factor C-based procedure



In case of USP

U.S. Department of Health and Human Services, U.S. Food and Drug Administration.
Guidance for industry-pyrogen and endotoxins testing: questions and answers. 2012.

5. May a firm use alternative assays to those in the USP for a compendial article? Below are two examples of alternative assays.

(1) Recombinant Horseshoe Crab Factor C Assay

If a manufacturer chooses to use a recombinant factor C-based assay, then method validation should be in accordance with the requirements of USP Chapter <85>, Bacterial Endotoxins Test, as described in the section for Photometric Quantitative Techniques, and USP Chapter <1225>, Validation of Compendial Procedures.

In-Process Revision: USP Chapter <1085.1>

Use of Recombinant Reagents in the Bacterial Endotoxins Test–Photometric and Fluorometric Methods Using Recombinantly Derived Reagents.

In case of Ph.Eur.

European Pharmacopoeia 10.3
01/2021:20632
General Chapter 2.6.32 Endotoxins using recombinant factor C

The test for bacterial endotoxins using recombinant factor C (rFC) is carried out to quantify endotoxins from gram-negative bacteria. It is performed using rFC based on the gene sequence of the horseshoe crab (*Limulus polyphemus*, *Tachypleus tridentatus*, *Tachypleus gigas* or *Carcinoscorpius rotundicauda*), using a fluorometric method.

In case of JP

The Japanese Pharmacopoeia Eighteenth Edition. Official in June of 2021

General Notices:

14. The test methods specified in the Japanese Pharmacopoeia can be replaced by alternative methods which give better accuracy and precision. However, where a difference in test results is suspected, only the result obtained by the procedure given in the Pharmacopoeia is effective for the final judgment.

General Tests:

4. Biological Tests/Biochemical Tests/Microbial Tests

4.01 Bacterial Endotoxins Test

General Information:

Bacterial endotoxins test and alternative methods using recombinant protein-reagents for endotoxin assay *<G4-4-180>*

Introduction

·····This General Information describes procedures and consideration in measurement when using recombinant protein-reagents for endotoxin assay as alternative methods, in addition to lysate reagents and test methods in Bacterial Endotoxins Test *<4.01>*.

1. Measurement principle of the Bacterial Endo-toxins Test

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2. Measurement method in the Bacterial Endotoxins Tests

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3. Reagents used for the Bacterial Endotoxins Test

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4. Measurement by alternative methods using recombinant protein-reagents for endotoxin assay and points to consider in the measurement

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Regarding recombinant factor C based on the gene sequence of the horseshoe crab (e.g. *C. rotundicauda* or *T. tridentatus*), using a fluorometric method.

Regarding recombinant cascade reagents based on the gene sequence of factor C, factor B and proclotting enzyme of the horseshoe crab (e.g. *T. tridentatus*) , using a photometric method.

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Regarding reagents prepared using the gene sequence of the protein of different species of horseshoe crab from horseshoe crab (*L. polyphemus* or *T. tridentatus*) specified in the Bacterial Endotoxins Test <*4.01*>, it should be noted that the difference of the recombinant proteins may affect the reactivity to endotoxins.

The recombinant protein-reagents for endotoxin assay do not identical to “an amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab” specified in Bacterial Endotoxins Test <*4.01*>. If these reagents for endotoxin assay are used as an alternative method, confirm that accuracy, precision, sensitivity, specificity, etc. are equal or better compared to Bacterial Endotoxins Test <*4.01*> using lysate reagents.

How about recombinant factor C reagent in JP?

Members of JP formed a workgroup to develop a collaborative study on the bacterial endotoxin test using recombinant Factor C-based procedure for detection of LPS.

Reports

1. Kikuchi, Y. *et al.*, Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides. *Pharmaceutical and Medical Device Regulatory Science* **48** (4), 252-260 (2017)

2. Kikuchi, Y. *et al.*, Collaborative Study on the Bacterial Endotoxins Test Using Recombinant Factor C-based Procedure for Detection of Lipopolysaccharides, Part 2. *Pharmaceutical and Medical Device Regulatory Science* **49** (10), 706-718 (2018)

Materials

LAL-based assay (black symbols)

1. Endospecy ES-50M set was purchased from Seikagaku corporation.
   kinetic-chromogenic method
2. ES-II was purchased from FUJIFILM Wako Pure Chemical Corporation
   kinetic-turbidimetric method
3. Kinetic-QCL was purchased from Lonza Japan Ltd.
   kinetic-chromogenic method

recombinant factor C-based procedure (white symbols)

4. PyroSmart was purchased from Seikagaku corporation
   kinetic-chromogenic method
5. PyroGene was purchased from Lonza Japan Ltd.
   kinetic-fluorometric method
6. EndoZyme/EndoZyme II was purchased from Hyglos/bioMérieux Japan Ltd.
   kinetic-fluorometric method

Japanese Pharmacopeia reference standard endotoxin (JP-RSE)

LPS from *Escherichia coli* O113:H10:K negative (ENH15000001, ENH16000001, ENH18000001) were purchased from Pharmaceutical and Medical Device Regulatory Science Society of Japan.

Endotoxin Panel

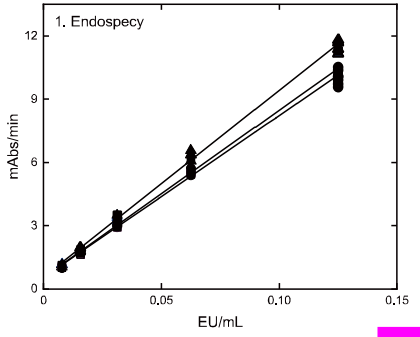
LPS from *Burkholderia cepacia* was kindly provided from Prof. Kazuyoshi Kawahara, Kanto Gakuin University. Other LPS were purchased from suppliers.

Comparison of the reactivities of endotoxin panel in LAL-based assay and recombinant factor C-based procedure

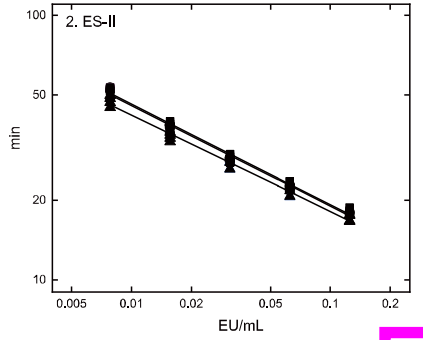
LPS		endotoxin activity (EU/μg)*1					
no.	strain	Endospecy	ES-II	Kinetic-QCL	PyroSmart	PyroGene	EndoZyme
1	*Burkholderia cepacia*	9267 ± 219	13032 ± 2353	15633 ± 504	18267 ± 3384	6693 ± 1233	6170 ± 1356
	ratio*2	37068.0	52128.0	62532.0	73068.0	26772.0	24680.0
3	*Escherichia coli* J5	4903 ± 625	26870 ± 1245	12125 ± 1024	4118 ± 384	9293 ± 452	6868 ± 925
		19612.0	107480.0	48500.0	16472.0	37172.0	27472.0
10	*Helicobacter pylori* GU2	2063 ± 163	9.82 ± 1.67	1086 ± 99	32.5 ± 4.23	4.45 ± 0.25	0.25 ± 0.04
	ratio	8252.0	39.3	4344.0	130.0	17.8	1.0
12	*Porphyromonas gingivalis* ATCC 33277	0.99 ± 0.07	0.46 ± 0.1	0.88 ± 0.05	0.44 ± 0.06	0.34 ± 0.05	0.34 ± 0.16
	ratio	4.0	1.8	3.5	1.8	1.4	1.4
14	*Pseudomonas aeruginosa* 10	1093 ± 56	6754 ± 172	3323 ± 449	658 ± 70	2620 ± 315	1970 ± 330
	ratio	4372.0	27016.0	13292.0	2632.0	10480.0	7880.0
16	*Salmonella enterica* serotype Typhimurium 1114	4904 ± 441	4895 ± 229	5573 ± 349	6086 ± 429	5190 ± 497	6313 ± 313
	ratio	19616.0	19580.0	22292.0	24344.0	20760.0	25252.0

*1 Values are the mean ± standard error of each participating institutes.
*2 Relative ratio among the assay (value/minimum value).

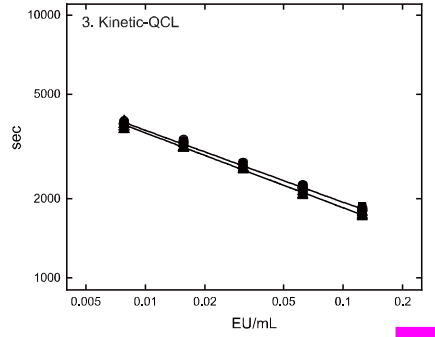
Comparison of standard curve between LAL-based assay and recombinant factor C-based procedure in independent assay laboratories



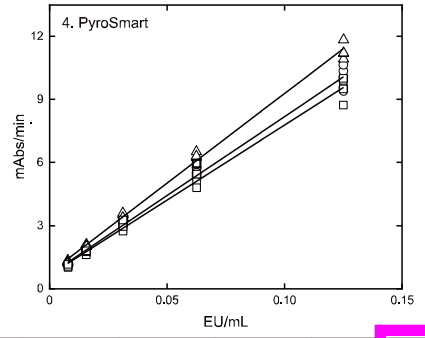
plot	equation	*n*	| *r* |
● (A)	y = 0.546 + 76.786*x	35	0.998
▲ (B)	y = 0.565 + 88.493*x	20	0.999
■ (C)	y = 0.541 + 79.362*x	20	0.995



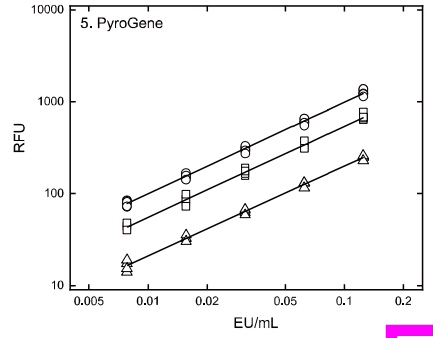
plot	equation	*n*	| *r* |
● (A)	log (y) = 0.896 - 0.382 log (x)	35	0.996
▲ (B)	log (y) = 0.895 - 0.364 log (x)	20	0.993
■ (C)	log (y) = 0.902 - 0.381 log (x)	20	0.995



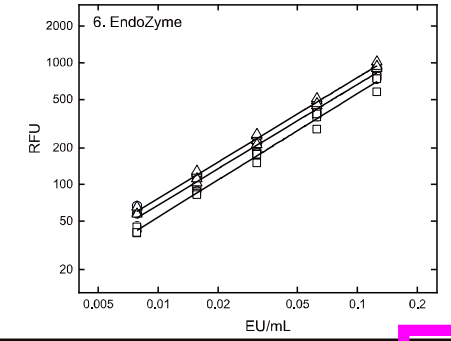
plot	equation	*n*	| *r* |
● (A)	log (y) = 3.014 - 0.274 log (x)	35	0.997
▲ (B)	log (y) = 2.982 - 0.285 log (x)	20	0.998
■ (C)	log (y) = 3.011 - 0.274 log (x)	20	0.995



plot	equation	*n*	| *r* |
○ (A)	y = 0.672 + 75.158*x	35	0.997
△ (B)	y = 0.756 + 85.268*x	20	0.998
□ (C)	y = 0.642 + 71.353*x	20	0.994



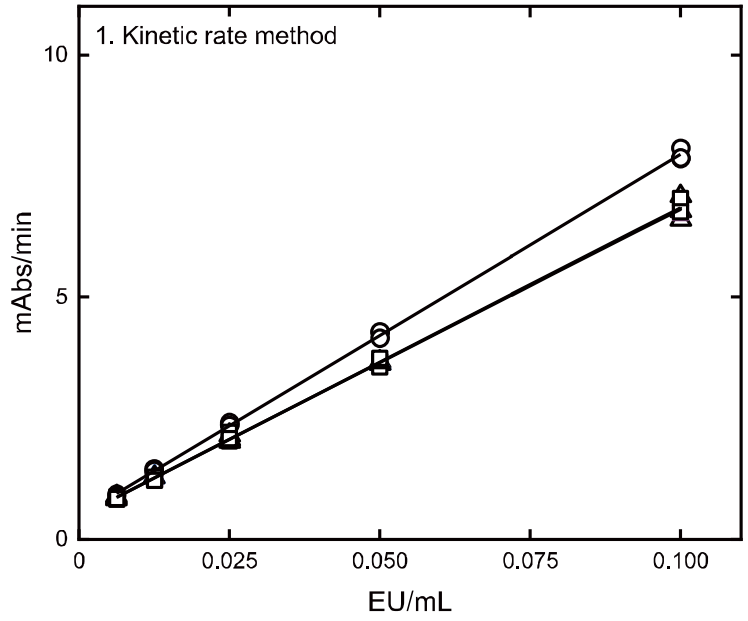
plot	equation	*n*	| *r* |
○ (A)	log (y) = 3.993 + 0.999 log (x)	35	0.998
△ (B)	log (y) = 3.279 + 0.980 log (x)	20	0.997
□ (C)	log (y) = 3.722 + 0.990 log (x)	20	0.996

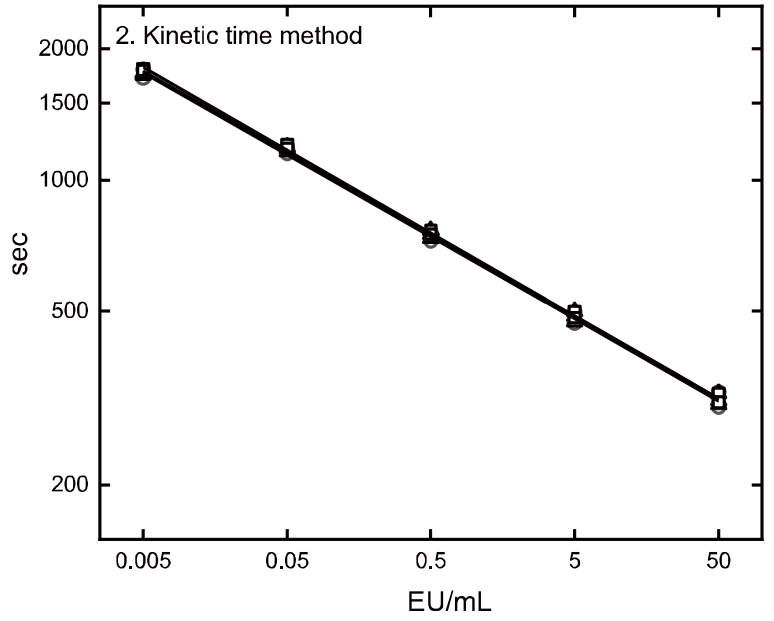


plot	equation	*n*	| *r* |
○ (A)	log (y) = 3.811 + 0.989 log (x)	35	0.993
△ (B)	log (y) = 3.871 + 0.992 log (x)	20	0.998
□ (C)	log (y) = 3.761 + 1.014 log (x)	20	0.996

JP-RSE was analyzed by LAL-based assay (panel 1-3) and recombinant factor C-based procedure (panel 4-6). The mean standard curve of 3 participating laboratories are shown. The absolute value of the correlation coefficient, |r|, was determined by Pearson's correlation coefficient using the subjects' means within 7 (A) or 4 (B and C) independent assay runs.

Evaluation of PyroSmart lot-to-lot variation test in single laboratory



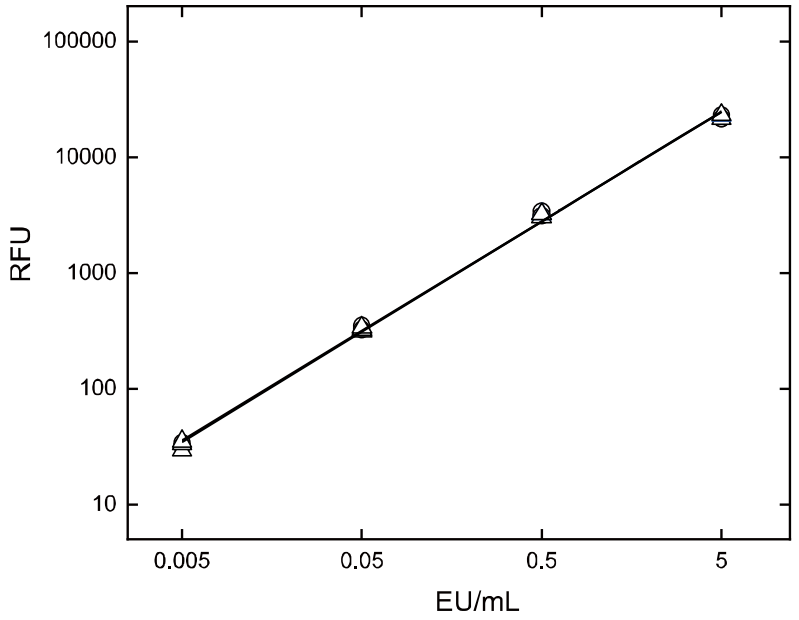


lot	plot	equation	*n*	| *r* |
RS15001	○	y = 0.482 + 74.687*x	15	1.000
RS15002	△	y = 0.480 + 63.425*x	15	0.999
RS15003	□	y = 0.465 + 63.893*x	15	0.999

lot	plot	equation	*n*	| *r* |
RS15001	○	log (y) = 2.813 - 0.188 log (x)	15	0.999
RS15002	△	log (y) = 2.818 - 0.188 log (x)	15	0.999
RS15003	□	log (y) = 2.821 - 0.190 log (x)	15	1.000

JP-RSE was analyzed by kinetic rate method (panel 1) and kinetic time method (panel 2) using 3 lots of PyroSmart in a participating laboratory. Each of 3 standard curves are shown. The absolute value of the correlation coefficient, |r|, was determined by Pearson's correlation coefficient using subjects' means by 3 independent assay runs.

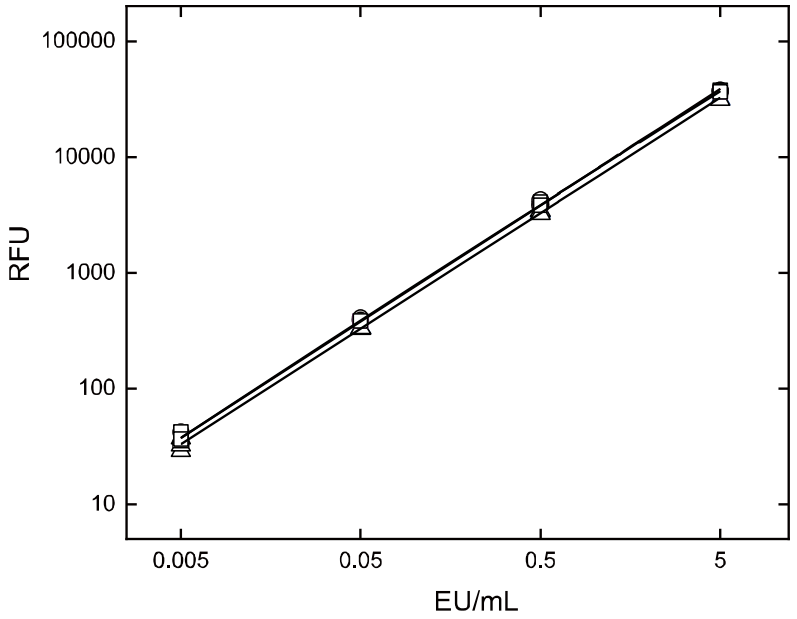
Evaluation of PyroGene lot-to-lot variation test in single laboratory



lot	plot	equation	*n*	| *r* |
0000591576	○	log (y) = 3.730 + 0.946 log (x)	12	0.999
0000680544	△	log (y) = 3.729 + 0.950 log (x)	12	0.999

JP-RSE was analyzed by endpoint fluorescence assay using 2 lots of PyroGene in a participating laboratory. Each of 2 standard curves are shown. The absolute value of the correlation coefficient, |r|, was determined by Pearson's correlation coefficient using subjects' means by 3 independent assay runs.

Evaluation of EndoZyme II lot-to-lot variation test in single laboratory



lot	plot	equation	*n*	| *r* |
18238	○	log (y) = 3.883 + 1.005 log (x)	12	1.000
18244	△	log (y) = 3.811 + 0.996 log (x)	12	1.000
18434	□	log (y) = 3.877 + 0.998 log (x)	12	1.000

JP-RSE was analyzed by endpoint fluorescence assay using 3 lots of EndoZyme II in a participating laboratory. Each of 3 standard curves are shown. The absolute value of the correlation coefficient, |r|, was determined by Pearson's correlation coefficient using subjects' means by 3 independent assay runs.

Conclusions and future directions:

- Recombinant factor C-based procedures have almost same reactivities against endotoxin panel as LAL-based assay.
- Standard curve of recombinant factor C-based procedures show same absolute value of the correlation coefficient in independent assay laboratories.
- Variation of recombinant factor C-based procedures are small when changing lots of reagents.
- Recombinant factor C-based procedure might be LAL-based assay replacement for BET.
- Pharmaceutical products-specific method validation and robustness study using recombinant factor C-based procedure are needed to replace LAL-based assay.

Acknowledgements
I would like to thank Drs. Masashi Muroi (Musashino University) and Yukari Nakagawa (Pharmaceutical and Medical Device Regulatory Science Society of Japan) for helpful discussions.

Thank you for your attention!
御静聴ありがとうございました