More flexible MAT assay design by taking advantage of reporter assay-based MAT

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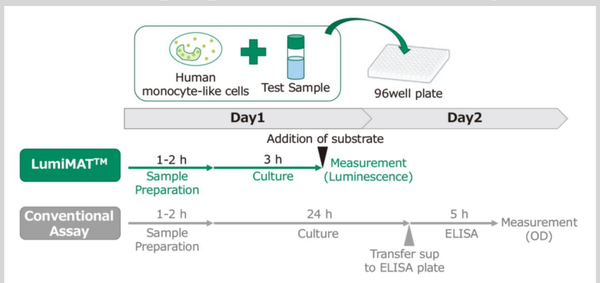
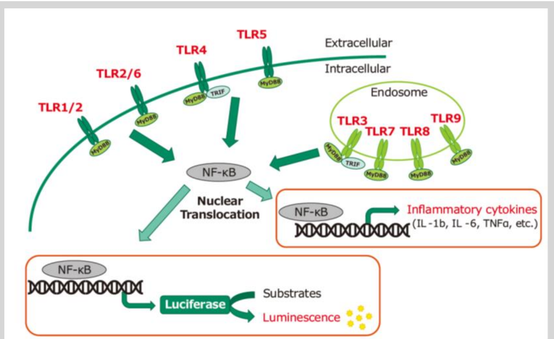
Abstract & Introduction

The LumiMAT pyrogen detection kit detects the transcriptional activity of NF-κB, a transcription factor that induces the expression of inflammatory cytokines, using a luciferase reporter assay. Compared to ELISA-based MAT, the advantages of the reporter assay-based MAT include a significant reduction in cell incubation time due to increased sensitivity (from 24 hours to 3 hours) and the removal of labor-intensive steps (several hours for ELISA to a few minutes for the addition of luminescent substrates).
For practical application in pyrogen testing, we conducted preliminary tests in accordance with EP 2.6.30. guidelines on several medicinal products.

Additionally, our method allows for reduced coefficient of variation, enabling the reduction in the number of replicates (n=4 to n=3) and increasing throughput (from a 96-well plate to a 384-well plate). These features demonstrate the potential for designing more cost-effective test protocols.



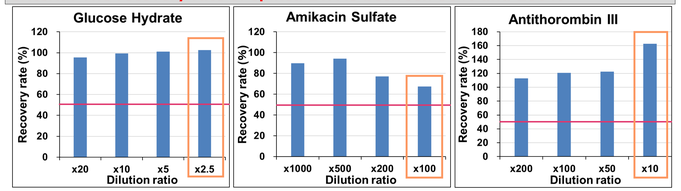


Results

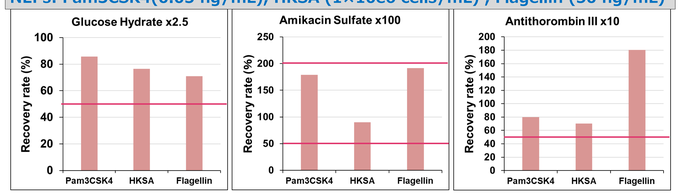
Preparatory test for pharmaceuticals using LumiMAT™ Pyrogen Detection kit

1. Glucose Hydrate (5%)
2. Amikacin Sulfate
3. Human antithrombin III *Inhibit the Limulus reaction

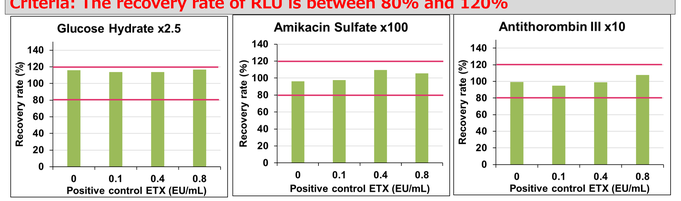
A. TEST FOR INTERFERING FACTORS (EP2.6.30. 6-2.)
Sample vs Sample + ETX
Criteria: The recovery rate of spiked ETX is between 50% and 200%



B. METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS (EP2.6.30. 6-5.)
NEPs vs NEPs + Sample
Criteria: The recovery rate of spiked NEP is between 50% and 200%
NEPs: Pam3CSK4(0.05 ng/mL), HKSA (1×10e6 cells/mL) , Flagellin (50 ng/mL)



C. INTERFERENCE IN THE DETECTION SYSTEM(EP2.6.30. 6-4.)
Positive control (0, 0.1, 0.4, 0.8 EU/mL ETX) vs Positive control + Sample
Criteria: The recovery rate of RLU is between 80% and 120%



The above three pharmaceuticals passed the preliminary tests specified by the EP2.6.30. using LumiMAT.

Flexibility of calibration curve

A. Arrangement of standard ETX concentrations

Regarding the number of calibration standard concentrations, LumiMAT recommends seven concentrations plus a blank; however, for a 4PL curve, the EP2.6.30. requires a minimum of five concentrations.

Blank + 7 concentrations

ETX(EU/mL)	n=4
0	R0
0.0125	R1
0.025	R2
0.05	R3
0.1	R4
0.2	R5
0.4	R6
0.8	R7

5 concentrations ①

ETX(EU/mL)	n=4
0	R0
0.0125	R1
0.025	
0.05	R2
0.1	
0.2	R3
0.4	R4
0.8	R5

5 concentrations ②

ETX(EU/mL)	n=4
0	R0
0.0125	R1
0.025	R2
0.05	
0.1	R3
0.2	
0.4	R4
0.8	R5

Calibration curves using a 4PL model were created and compared under the conditions of blank plus 7 ETX concentrations or 5 ETX concentrations.

ETX (EU/mL)	Accuracy(%) of back calculated concentration (EE/mL)		
	7 conc.	5 conc.①	5 conc.②
0.0125	99.2	99.4	109.3
0.025	100.6	99.7	109.6
0.05	105.7	104.7	110.1
0.1	99.3	98.6	98.8
0.2	96.8	96.4	92.5
0.4	107.5	107.5	102.0
0.8	99.9	100.0	109.4

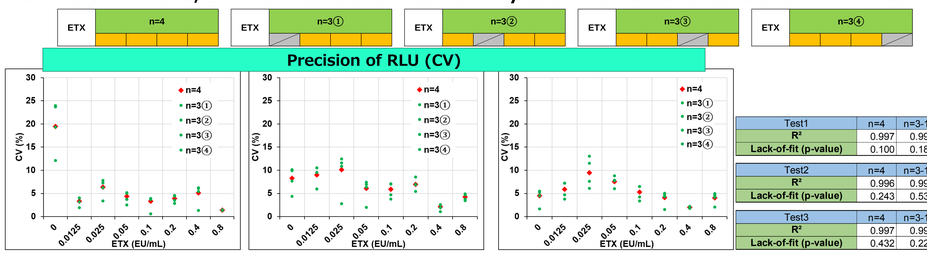
Standard curve criteria (EP2.6.30.)
R^2>0.975 p(lack of fit)>0.05

ETX concentration	7	5①	5②	Criteria
R^2	0.996	0.996	0.998	>0.975
Lack-of-fit (p-value)	0.243	0.115	0.481	>0.05

A sufficiently accurate calibration curve was also created for the case of five concentrations.

B. Arrangement of sample replicates

The EP requires at least four replicates for each concentration.
On the other hand, LumiMAT can achieve sufficiently accurate measurements even with three replicates.



Test1	n=4	n=3-1	n=3-2	n=3-3	n=3-4	Criteria
R^2	0.997	0.997	0.997	0.997	0.999	>0.975
Lack-of-fit (p-value)	0.100	0.188	0.284	0.141	0.119	>0.05

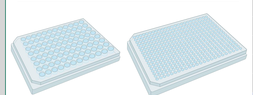
Test2	n=4	n=3-1	n=3-2	n=3-3	n=3-4	Criteria
R^2	0.996	0.997	0.996	0.996	0.995	>0.975
Lack-of-fit (p-value)	0.243	0.530	0.160	0.675	0.165	>0.05

Test3	n=4	n=3-1	n=3-2	n=3-3	n=3-4	Criteria
R^2	0.997	0.999	0.997	0.997	0.997	>0.975
Lack-of-fit (p-value)	0.432	0.222	0.747	0.527	0.464	>0.05

Luminescence measurements exhibit low variability, allowing sufficiently accurate measurements even with three replicates.

Measurements using a 384-well plate can maintain sufficient accuracy.

	96-well	384-well
well/kit	96	240
Sample+Cell (μL/well)	50 + 50	20 +20
Testable samples	3	8



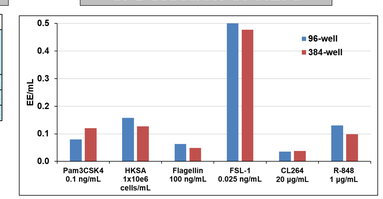
A. CV of standard curve

ETX (EU/mL)	BCC CV (%)	
	96-well	384-well
0.0125	8.4	3.8
0.025	8.7	11.6
0.05	9.2	7.1
0.1	10.1	15.9
0.2	4.6	3.3
0.4	4.0	11.2
0.8	6.7	14.5
Average	7.4	9.6

B. Accuracy of spiked ETX

Spiked ETX (EU/mL)	96-well BCC (EE/mL)	96-well Accuracy (%)	384-well BCC (EE/mL)	384-well Accuracy (%)
0.0375	0.0343	91.6	0.0357	95.3
0.15	0.160	106.7	0.155	103.5
0.6	0.625	104.1	0.579	96.5

C. Detection of NEPs



Luminescence measurements can be handled as reliably with a 384-well plate manually as with a 96-well plate.