Detection of Bacterial Endotoxins with Thermo Scientific Multiskan FC Microplate Photometer

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Application Notes

Key Words

Endotoxin, turbidimetric, kinetic, photometry, Limulus Amebocyte, microplate

Goal

This application note describes how the bacterial endotoxin assay can be performed with Thermo Scientific™ Multiskan™ FC microplate photometer. The assay is a turbidimetric measurement of the reaction between possible endotoxins and Limulus Amebocyte Lysate (LAL) reagent.

Introduction

Endotoxins are lipopolysaccharide molecules located in the cell wall of gram-negative bacteria. Endotoxins are not secreted by the bacteria, but they are released to the environment after cell lysis. The molecules may be toxic to humans and animals, and may cause fever, diarrhea, sepsis and altered resistance to bacterial infections. Endotoxins are very stable and can withstand very high temperature. Because endotoxins are toxic to individual cells, it is important in cell based laboratory work that all materials (consumables, containers, reagents, liquids, etc.) are free of endotoxins. Even small amounts of the molecules can have severe effects in all experiments that use cell lines or primary cells. Endotoxin assays are therefore commonly used for quality control purposes to confirm that materials are endotoxin-free and safe to be used. The bacterial endotoxin assay is based on using the LAL reagent. The reagent is an amebocyte extract from the Horseshoe Crab (Limulus polyphemus).

Researchers have demonstrated that when a gram-negative bacterium infects a Horseshoe Crab, the immune response is an intravascular clotting reaction. This coagulation results from a reaction between endotoxin and a clottable protein secreted by amebocytes. The same endotoxin clotting reaction is utilized in commercial LAL endotoxin assays. The formation of clot causes increase in turbidity of the sample. This process can be measured in kinetic format on a microplate photometer.

Materials and methods

Reagents

- PYROGENT™–5000 Test Kit, code N384, Lonza corp. Switzerland.
- E. Coli O55:B5 Endotoxin Standard, code N186, Lonza corp. Switzerland.

Instrument and plastics

- Multiskan FC with incubator equipped with 1.01.06 (or later) internal software (previous versions should not be used).
- Thermo Scientific™ SkanIt™ Software (version 4.1 or later). SkanIt software was used to control the microplate photometer and to perform all calculations.
- Thermo Scientific™ NUNC™ 96-well flat bottom cell culture plate.

Thermo SCIENTIFIC

Assay procedure

Multiskan FC was pre-heated to +37 °C prior to the assay. The kinetic measurement was started by mixing a 100 µl aliquot of each sample (blank, calibrator or unknown sample) and 100 µl of the LAL reagent together in a clear 96-well microplate well. The kinetic measurement was started immediately with a 405 nm filter at 37 °C. The formation of the endotoxin clot increases turbidity of the sample and this process was followed kinetically. Each well was measured once in every 30 seconds for one hour.

Result calculations

The turbidimetric endotoxin assay produces kinetic curves as optical density (OD) values increases due to clot formation. The higher the endotoxin concentration in the sample, the faster is the clotting formation and the sooner the reaction curve reaches a plateau, as seen in **Figure 1**.

Reaction time calculation

The first calculation step is to reduce the kinetic data into one value per sample that can then be used for both the generation of the standard curve and concentration calculations of unknown samples. **Time to change** calculation in the **Kinetic Reduction** menu searches the time point when the kinetic curve reaches a defined threshold value. The settings to calculate this threshold time with time to change function are shown in **Figure 2**. The resulting time is referred as “reaction time”. The requested threshold value to be used is given in the kit instructions, but it is typically 0.03 OD units above the baseline.

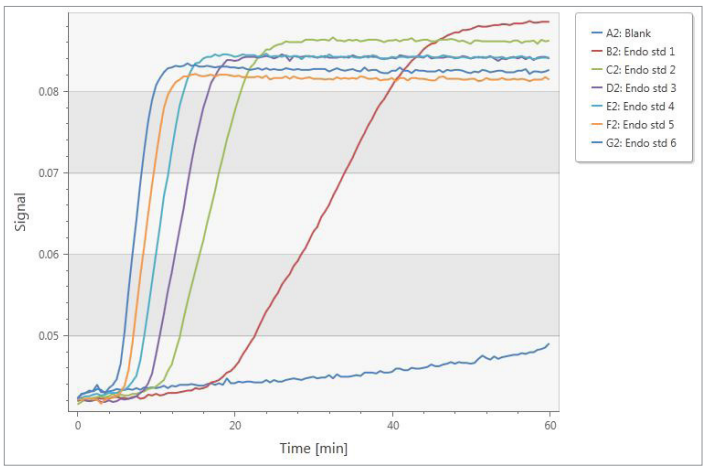


Figure 1: Typical kinetic curves of the turbidimetric endotoxin assay with Multiskan FC.

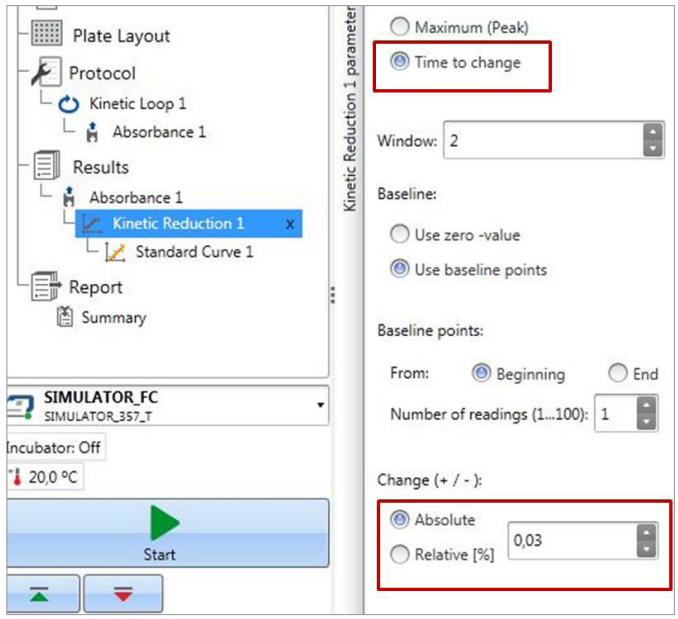


Figure 2: Typical time to change settings for calculating reaction times in a bacterial endotoxin assay.

There is normally very little of spontaneous clotting in this assay without endotoxins. Blanks, clean samples and sometimes even the lowest calibrators do not cause remarkable increase in the OD, therefore never reaching the requested threshold limit value. The time to change calculation gives a marking “NaN” (Not a Number) as an indicator of this situation where sample does not reach the threshold value. An example of result data set with NaN results is shown in **Figure 3**. Samples giving “NaN” will be automatically excluded from all further calculations and marked as well with “NaN”.

Name: Plate 1

	1	2	3	4	5	6	7	8
A	NaN Blank	NaN Blank	3 182 Blank	NaN Blank	NaN Sterile water	NaN Sterile water	NaN Sterile water	NaN Sterile water
B	2 037 Endo std 1	2 105 Endo std 1	2 069 Endo std 1	2 027 Endo std 1	2 072 Std 1 as sample	2 125 Std 1 as sample	2 072 Std 1 as sample	2 087 Std 1 as sample
C	1 097 Endo std 2	1 106 Endo std 2	1 101 Endo std 2	1 114 Endo std 2	1 121 Std 2 as sample	1 108 Std 2 as sample	1 102 Std 2 as sample	1 104 Std 2 as sample
D	873.9 Endo std 3	884.6 Endo std 3	887.6 Endo std 3	891.0 Endo std 3	873.5 Std 3 as sample	893.5 Std 3 as sample	891.9 Std 3 as sample	879.3 Std 3 as sample
E	711.7 Endo std 4	717.9 Endo std 4	721.7 Endo std 4	723.7 Endo std 4	733.0 Std 4 as sample	730.1 Std 4 as sample	726.9 Std 4 as sample	716.5 Std 4 as sample
F	601.2 Endo std 5	608.8 Endo std 5	608.7 Endo std 5	610.3 Endo std 5	611.3 Std 5 as sample	610.0 Std 5 as sample	611.1 Std 5 as sample	600.7 Std 5 as sample
G	507.3 Endo std 6	512.8 Endo std 6	516.7 Endo std 6	520.4 Endo std 6	523.4 Std 6 as sample	520.6 Std 6 as sample	522.6 Std 6 as sample	517.8 Std 6 as sample
H	NaN Blank	NaN Blank	NaN Blank	NaN Blank	535.5 Tap water	530.2 Tap water	668.7 Tap water	630.2 Tap water

Figure 3: Example of the calculated reaction times. Samples that do not reach the threshold value are marked with “NaN”.

Concentration calculation

SkanIt software calculates endotoxin concentrations by plotting reaction times against known endotoxin standard concentrations to create a standard curve. Then unknown sample concentrations are solved based on the produced standard curve. The most common curve fit formula to be used in endotoxin assays is linear regression using logarithmic transformations and data extrapolation. It is recommended to use extrapolation in linear regression fit because heavily contaminated samples are likely to be outside calibration range. SkanIt software also calculates basic fitting statistics from the standard curve. Settings for the standard curve fitting calculation are given in **Figure 4** and typical endotoxin standard curve is shown in **Figure 5**.

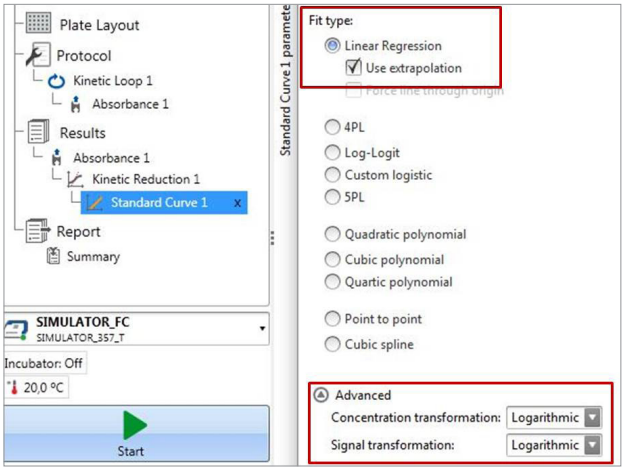


Figure 4: Typical settings of the standard curve calculation in endotoxin assay.

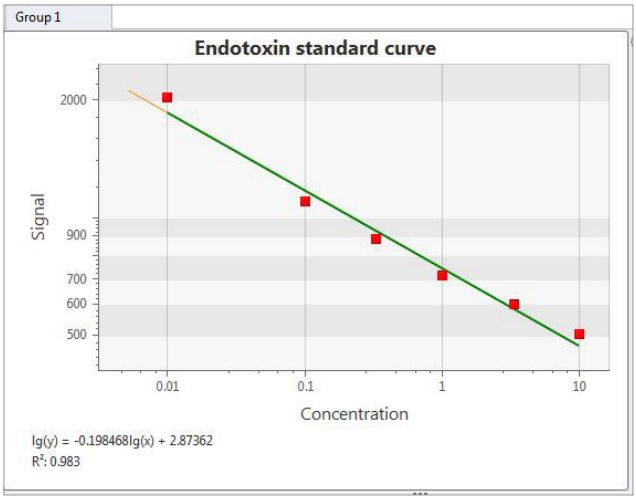


Figure 5. Endotoxin standard curve. Calculated reaction times are plotted against endotoxin standard concentrations.

When concentrations of the unknown samples are calculated, all those samples that gave “NaN” in the reaction time are then classified with “NaN” in the concentration calculation. See **Figure 6**.

Plate | List

Type	Sample	Signal	Concentration
Unknown	Distilled water	NaN	NaN
Unknown	Sterile water	NaN	NaN
Unknown	Sample A	2 087	0.005666
Unknown	Sample B	1 106	0.1389
Unknown	Sample C	881.4	0.4360
Unknown	*Sample D*	*722.2*	*1.189*
Unknown	*Sample E*	*603.2*	*2.948*
Unknown	*Tap water*	*583.9*	*3.471*
Unknown	*Sample F*	*514.7*	*6.556*

Figure 6: Calculated endotoxin concentrations.

Sample classification

The next step is to classify the samples as contaminated or clean. This classification is done in Microsoft® Excel® using SkanIt software report as a calculation template. The classification feature inside SkanIt software cannot classify those “NaN” results and is therefore not sufficient for the final classification. Classification divides samples into two categories with limit value separating the categories. All samples showing endotoxin concentration below the lowest calibrator are classified as “Clean” samples and all showing concentrations higher than the lowest calibrator are considered as “Contaminated”.

Classification is quite simple to perform in Excel when one uses linear regression fitting with extrapolation. All samples giving “NaN” as a result in curve fit can be reliably considered to be totally clean. The only possible source for those “NaN” results is during reaction time calculation. Even still, “NaN” can come only when the sample gives very slow response in the assay.

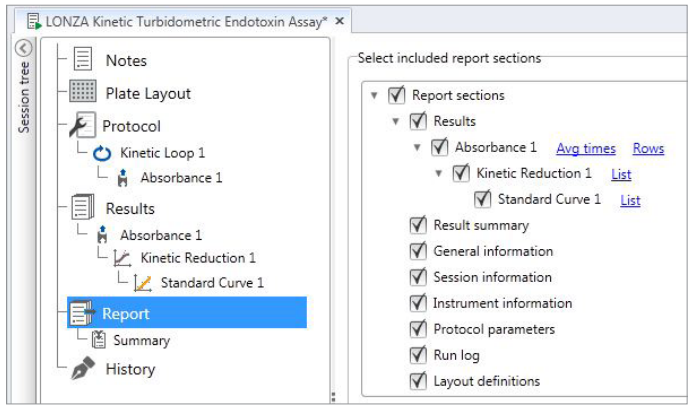
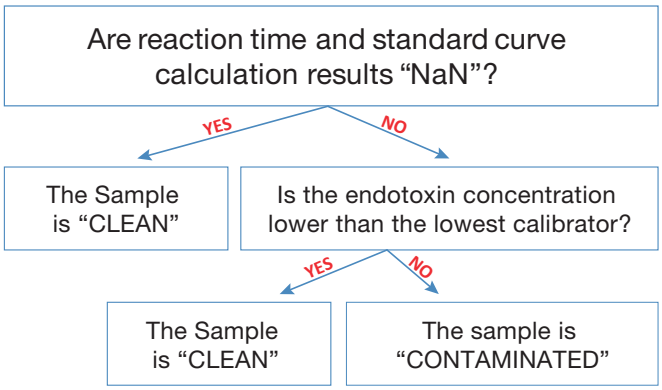


Figure 7: Report export settings when all report sections are added into the created Excel file.

Classification in Excel is performed as follows:

- Create a full report including all sections using SkanIt software as shown in **Figure 7**.
- Report file is automatically opened with Excel. Go to the Result Summary sheet.
- Create the classification formula into a new column. Classification is based on logical "IF" functions that search in which category each sample belongs to. The functions are set according to logical procedure below:



- The test is done using two combined "IF" functions. "=IF(F2="NaN";"CLEAN";IF(F2<0.01;"CLEAN";"CONTAMINATED")) where "F2" refers to the result from the standard curve calculation and "0.01" is the lowest calibration concentration in the assay.
- This formula checks if the reaction time value in column C is "NaN" and in such case the sample is classified as "CLEAN". If the result is not "NaN" then result is classified based on the calculated endotoxin concentration. An example of the classification results is shown in **Figure 8**.

G2 =IF(F2="NaN";"CLEAN";IF(F2<0.01;"CLEAN";"CONTAMINATED"))

	A	B	C	D	E	F	G	H
1	**Plate**	**Well**	**Type**	**Sample**	**Signal**	**Conc.**	**Classification**	
2	Plate 1	H01	Unknown	Distilled water	NaN	NaN	CLEAN	
3	Plate 1	B06	Unknown	Sample A	2123	0.005399	CLEAN	
4	Plate 1	C06	Unknown	Sample B	1105	0.1427	CONTAMINATED	
5	Plate 1	D05	Unknown	Sample C	871.3	0.4705	CONTAMINATED	
6	Plate 1	E08	Unknown	Sample D	712.0	1.297	CONTAMINATED	
7	Plate 1	F05	Unknown	Sample E	606.1	2.909	CONTAMINATED	
8	Plate 1	G05	Unknown	Sample F	517.1	6.451	CONTAMINATED	
9	Plate 1	A08	Unknown	Sterile water	NaN	NaN	CLEAN	
10	Plate 1	H06	Unknown	Tap water	523.0	6.096	CONTAMINATED	

Figure 8: Result summary sheet in Excel with the result interpretation column where classification has been performed using IF function. The colours have been added using conditional formatting.

Result analysis notes

- These calculations and interpretations shown here are based on the instructions of LONZA Pyrogent endotoxin assay kit.
- If curve fit is done using other fitting formula, as recommended with certain other assay kits, this same result interpretation of "NaN" cannot be used.
- Using for example logistic (4PL) or polynomial fitting formulas will change the calculations so that contaminated samples can also give "NaN" results.
- With these non-linear fitting formulas one has to do final result interpretation using more complicated analysis formulas investigating the root cause of those "NaN" values.

Conclusions

This study shows that the turbidimetric kinetic endotoxin assay is very straightforward to perform and reliable results can be received with this system. The assay provides a sensitive method for detection of varying concentrations of endotoxins. Multiskan FC together with the SkanIt Software offers a handy and high-quality package for easy detection and analysis system of dozens of samples simultaneously in a microplate format.

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