1.0 PURPOSE

The purpose of this procedure is to provide a protocol that can be used to determine protein interference and provide validation for users of the PYROSTAR™ ES-F/Plate kinetic turbidimetric assay. The EndoPrep™ treatment kit is useful in providing a user with a strategy to overcome interference when the MVD is met, and heat treatment does not remove the interference. The effects of protein interference have been advised on by the USP and detailed through research. Since the cascade reaction of the LAL chemistry involves a cascade of protease enzymes, proteins that cause potent interference to the LAL cascade are protease or protease inhibitor proteins. As catalyzing proteins often resist denaturation and have a potent effect in small concentrations, dilution and heat treatment, the traditionally recommended procedures for overcoming protein interference are often ineffective.

The cascade reaction of LAL initiates the first serine protease precursor (Factor C) from its inactivated form; this in turn activates the second serine protease precursor (Factor B). The activated Factor B cleaves the proclotting enzyme, creating the active clotting enzyme. The clotting enzyme cleaves peptide bonds within coagulogen to yield coagulin, an insoluble protein that precipitates from solution. The amount of time it takes turbidity to reach a preset threshold absorbance value is recorded as the activation time. The relationship between activation time and endotoxin concentration leads to the calculation of the relationship relative to the concentration of endotoxin. Protease and protease inhibiting proteins can respectively shorten or lengthen the activation time, leading to enhancement or inhibition of the assay.

Endoprep™ is a sample treatment system that neutralizes the inhibitory effects of proteins/peptides on endotoxin and results in more accurate detection and quantification of endotoxin. To accomplish this, the sample to be tested is subjected to an hour-long treatment with the Endoprep™ digestion enzyme. Predictive Oncology and FUJIFILM Wako have validated that a 100-fold dilution after the treatment is sufficient to subsequently remove the digestion enzyme's effects on the LAL protein cascade. The FDA, AAMI allow and welcome treatment such as this that will not interfere with the LAL protein cascade.

2.0 SCOPE

Refer to the References Section for more guidance and industry documents to review. It is important to read over the documents prior to testing.

3.0 DEFINITIONS

3.1 LAL: Limulus Amebocyte Lysate
3.2 LRW: LAL Reagent Water (Endotoxin-free)
3.3 EU/ml: Endotoxin Units per milliliter
3.4 λ (lambda): The lowest concentration used in the standard curve for the KTA method.
3.5 CSE: Control Standard Endotoxin
3.6 LAL Reagent: PYROSTAR™ ES-F/Plate

3.7 KTA: Kinetic Turbidimetric Assay. During a KTA reaction, the turbidity of the test solutions is continually monitored by the microplate reader.

3.8 Ta: Activation time. The time required for a sample to reach a predetermined threshold absorbance level over background noise. Gelation time (Tg) may also be used interchangeably.

3.9 Assurance of Criteria for the Standard Curve: Must be performed for each lot of reagent. A CSE standard curve is tested to confirm linearity of the endotoxin dilutions that will be routinely used during testing.

3.10 Irl: Absolute value of r, the correlation coefficient, for the standard curve. This value is used to confirm linearity of the standard curve.

3.11 Test for interfering Factors: A test that determines if the product has factors that cause inhibition or enhancement in the LAL reaction.

3.12 MVD: Maximum Valid Dilution

3.13 Endotoxin limit: The amount of endotoxin that the product must be less than to be released to the public.

3.14 Reconstitution: Adding aqueous solution to dissolve dried material to bring the material back to a solution.

3.15 Certificate of Analysis: A document with information about the LAL or CSE Reagent.

3.16 NC: negative control, BET water only

3.17 PC: positive control, BET water spiked with CSE

3.18 Treated NPC (Sample): negative product control, sample only, treated with EndoPrep

3.19 Treated PPC: positive product control, sample with endotoxin spike to the midpoint of the standard curve, treated with EndoPrep

3.20 Untreated NPC: NPC without EndoPrep treatment

3.21 Untreated PPC: PPC without EndoPrep treatment

3.22 NCEP: negative control EndoPrep, no protein, no endotoxin spike, EndoPrep diluted with water or digestion buffer

3.23 BET: Bacterial Endotoxin Test

4.0 MATERIALS AND EQUIPMENT

4.1 Materials

- 4.1.1 PYROSTAR™ ES-F/Plate Multi test kit
- 4.1.2 EndoPrep™ Treatment kit
- 4.1.3 Lysate Reagent Water
- 4.1.4 Endotoxin-free 96 well microplate
- 4.1.5 Endotoxin-free dilution tubes
- 4.1.6 Endotoxin-free pipette tips

4.2 Equipment

- 4.2.1 Incubating microplate reader that can perform kinetic measurements at 405 nm

4.2.2 1000 uL and 100 uL pipettors

4.2.3 Vortexer

5.0 GENERAL PRECAUTIONS

5.1 Rigid aseptic techniques are required. All glassware and disposable supplies (i.e., pipette tips, dilution tubes, etc.) in direct contact with lysate must be endotoxin-free.

5.2 Keep the workstation clear of all items that will not be used for testing.

5.3 Vibrations in the microplate surroundings can interfere with gel formation during testing.

5.4 It is recommended to wear disposable Nitrile or other powder-free gloves while handling glass tubes and biological products.

5.5 For storage information, refer to the package insert for each reagent. Once reconstituted, the LAL reagent should be kept refrigerated or frozen until ready to use.

5.6 Endotoxin is added to the microplate wells first, followed by LAL solution.

5.7 Do not dispense lysate until ready to perform testing. The LAL reaction begins when the lysate is dispensed. If too much time passes between when the LAL is dispensed and when the microplate reader is started, inaccurate results may be obtained.

5.8 Avoid smudging the microplate with dirt, oil, or debris as this may affect light transmission.

6.0 DETERMINING THE CORRECT DILUTION FACTOR

6.1 List the product endotoxin limit in EU/mL. (If the endotoxin limit is listed in EU/mg, list the concentration of the product as well in mg/mL).

6.2 List the lysate sensitivity or lower quantitative limit (λ) of the reagent.

6.3 Use the following equation to calculate the MVD. MVD = Endotoxin limit x product concentration / lysate sensitivity

6.4 Divide the MVD by 1,000. Confirm that this value is greater than 1. This is the maximum dilution that the sample should be at before the EndoPrep™ treatment begins.

7.0 DETERMINING THE SPIKED SAMPLE AND STANDARD CURVE RANGE

7.1 Take the endotoxin limit of the product and divide by 1,000. This is the treatment-adjusted lysate sensitivity limit for the product.

7.2 Refer to the package insert of the reagent to create at least a series of three 10-fold dilutions, with the finest concentration being greater or equal to the lysate sensitivity limit.

7.3 Find the midpoint of the standard curve. This is the value that the PPC needs to be spiked to. Multiply this number by 10,000. This is the CSE concentration that will need to be used to spike the product before the initiation of the EndoPrep™ treatment.

8.0 PREPARATION OF CONTROL STANDARD ENDOTOXIN DILUTIONS

8.1 Prepare the Control Standard Endotoxin (CSE)

8.1.1 Remove the plastic cap and foil seal by lifting the plastic cap at the arrow, then pulling the cap and foil seal to the left and around the rim until the foil seal is off.

8.1.2 Gently remove the rubber stopper from the vial, using caution to avoid contaminating the stopper.

8.1.3 Aseptically add the appropriate volume of LAL Reagent Water to yield a 1,000 EU/mL solution according to the Certificate of Analysis.

8.1.4 Replace the rubber stopper, invert the vial, and then vortex vigorously for at least 2 minutes at room temperature.

8.2 Preparation of Endotoxin Dilutions from CSE

8.2.1 Add and label the appropriate number of depyrogenated dilution tubes to the test tube rack. Cover each tube with an aluminum cap.

8.2.2 Open the LAL Reagent Water. Add 1.8 ml of LRW to each dilution tube.

8.2.3 Open the CSE aseptically, using caution to avoid contaminating the stopper.

8.2.4 Prepare the dilution series:

8.2.4.1 Using the 1,000 EU/mL CSE solution, prepare the endotoxin dilution series created in previous steps. An example dilution series is shown in Table 2 below.

8.2.4.2 Observe the volume of the solution in each dilution tube to ensure that each is at the proper level before proceeding. Vortex each solution for 30 seconds in between dilutions.

8.2.4.3 Dilutions may be prepared using different volumes as long as the same ratios are maintained.

Table 2. – CSE Standard Curve Solutions

Initial Endotoxin (EU/mL)	Volume Endotoxin (mL)	Volume LRW (mL)	Final Endotoxin (EU/mL)
1000 (CSE)	0.2	1.8	100
100	0.2	1.8	10
10	0.2	1.8	1
1	0.2	1.8	0.1
0.1	0.2	1.8	0.01

9.0 PREPARING THE MICROPLATE READER SOFTWARE

9.1 Before beginning any assay, first confirm the test group, instrument settings, and workflow. Refer to the software manual for details on how to change these settings.

9.2 Confirm that the instrument and measurement parameters are as follows:

Read speed: Normal
Shake before measurement: yes
Duration: 20 seconds
Speed: medium
Turn off temperature control after measurement: no

9.3 Confirm that the protocol that will be used has the following settings:

Test Mode: General test
Reaction type: WL1
Onset OD: 0.015
Count: 1
Wait: 40 seconds
Baseline count: 1
Activation time type: Ta
Regression type: Linear
Time axis type: log(x)log(y)
Endotoxin unit: EU/mL
Use mean time: no
Positive control: midpoint
Interpolate: yes
Measurement wavelength: 405 nm
Reference wavelength: 0 nm
Time interval: 40 seconds
Target temperature: 37 °C

10.0 PH MEASUREMENT

10.1 Take the pH reading of the product to ensure it is in the intended range (pH 6.0 - 8.0) for testing.

10.2 If it is out of the range, mix the product dilution with the LAL and test the pH again. LAL has the buffering capability to bring most products back in the appropriate pH range for testing.

10.3 If a pH adjustment is necessary, the pH of the product should be adjusted with endotoxin-free HCl or NaOH.

11.0 PREPARATION OF ASSAY REAGENTS

11.1 Product Preparation

11.1.1 The sample to be tested should be the product in its final formulation and packaging.

11.1.2 Typically, the product will not be diluted before the procedure. However, the user should confirm that the product has an MVD greater than 1000 as 6.0 outlines.

11.2 Sample

11.2.1 Add 60 µL of the product to 540 µL of POAI Digestion Buffer

11.2.2 Remove 270 µL and transfer to a sterile, endotoxin-free borosilicate tube
11.2.3 Add 30 µL POAI Protease Solution to the tube from the previous step, label the tube "Sample".
11.2.5 Cover tubes with parafilm, incubate tubes in a 37°C water bath for 60 minutes
11.2.6 After digestion, dilute both samples 1:100 in LRW, prior to performing the kinetic turbidimetric assay.

11.3 Positive Product Control

11.3.1 For each protein sample. Label a dilution tube "PPC"
11.3.2 Dispense 450 µL of the product into the dilution tube.
11.3.3 Add 50 µL of the CSE concentration determined previously.
11.3.4 Add 60 µL of the Spiked Product to 540 µL of POAI Digestion Buffer
11.3.5 Remove 270 µL and transfer to a sterile, endotoxin-free borosilicate tube
11.3.6 Add 30 µL POAI Protease Solution to the tube from step 11.5.2, label the tube "PPC"
11.3.7 Cover tubes with parafilm, incubate tubes in a 37°C water bath for 60 minutes
11.3.8 After digestion, dilute both samples 1:100 in LRW, prior performing the kinetic turbidimetric assay

11.4 Standard Concentrations

11.4.1 The same concentrations used for the Assurance Criteria for the Standard Curve test must be used for the controls.

11.4.2 Prepare the determined CSE solutions (an example is in Table 2). Prepare enough of each solution to test in duplicate.

11.5 Negative Controls

11.5.1 Prepare the negative control (LRW only, NC) in duplicate.
11.5.2 If desired, 270 µL of POAI digestion buffer with 30 µL of POAI protease solution can be tested as a negative control for the EndoPrep™ Kit (NCEP)

12.0 PREPARATION OF THE REAGENT, MICROPLATE, AND READER

12.1 Reconstitution of PYROSTAR™ ES-F/Plate

12.1.1 Remove the plastic cap and foil seal by lifting the plastic cap at the arrow, then pulling the cap and foil seal to the left and around the rim until the foil seal is off.

12.1.2 Using clean forceps, a cap opener, or a freshly opened pipette tip, aseptically break the vacuum seal so that the lyophilized powder is not released.

12.1.3 Carefully remove the stopper and place it on the bench top with the inside portion facing up. Be sure to avoid contaminating the inside of the stopper.

12.1.4 Reconstitute the LAL with the appropriate volume of LRW based on the label (2.0 mL or 5.2 mL). Be sure to rehydrate multiple vials of LAL if needed.

12.1.5 Carefully place the stopper back on the LAL vial(s) and swirl the LAL gently until the lyophilized product has fully dissolved. Try to avoid contacting the stopper with the LAL solution, and do NOT vortex the vial.

12.2 Preparation of the microplate

12.2.1 If desired, use a fine-tipped permanent marker to outline on the lid the microplate wells with the standards/lot numbers that will be tested. Be careful to avoid marking over the wells themselves - this may affect light transmission during measurement if the lid is used during measurement. To avoid such issues, the lid can be removed immediately before placing the plate into the microplate reader for measurement.

12.2.2 Use a mechanical pipette to dispense 0.05 mL of the sample (CSE solution, negative control, spiked sample, or NPC) directly into the bottom of each microplate well. For clarity, a sheet of black paper may be placed under the microplate, so it is easier to see which wells contain the solution that was dispensed.

12.2.3 When the microplate reader has reached the target temperature and the measurement is ready to be performed, add 0.05 mL of the LAL reagent into the wells containing the samples. A repeater pipette fitted with a 2-200 µL tip at the end of a 2.5 mL or 5 mL pipette tip can be used to dispense the small volume of the reagent more quickly and accurately.

12.2.4 Complete testing as previously determined.

Reagent: PYROSTAR™ ES-F/Plate
Sample Volume: 50 µL reagent+ 50 µL sample

Onset OD:	0.015
Count:	1
Wait:	40 seconds
Wavelength:	405 nm (no reference wavelength)
Time interval:	40 seconds
Assay time:	80 minutes
Shaking:	20 seconds, medium speed

12.3 After the reaction is completed, save the data, and calculate the standard curve and correlation coefficient according to the applicable software manual.

13.0 ASSAY LAYOUT/INTERFERENCE VALIDATION

13.1 Prepare the following solutions. Solutions A and B will be multiplied by each lot tested.

13.1.1 Solution A: Test in duplicate the EndoPrep™ treated sample (Treated NPC)

13.1.2 Solution B: Test in duplicate the EndoPrep™ treated spiked sample (Treated PPC)

13.1.3 *For purposes of initial method validation, solution of A and B identically diluted but without the addition of digestion enzyme can act as a non-treated control comparison. (untreated NPC and untreated PPC)*

13.1.4 Solution C: Prepare CSE solutions as outlined by the standard curve determined in this procedure.

13.1.5 Solution D: Prepare the Negative Control (NC) as water for BET.

13.1.6 Test all unique samples in duplicate.

13.2 Interpret the test result. All replicates of Solution D should be negative. The standard curve should have an $|r|>0.980$. Recovery rates for the spiked samples should be between 50-200%. If this is the case, the sample does not cause product interference and is suitable for testing according to the Bacterial Endotoxin Test.

14.0 ACCEPTANCE CRITERIA

14.1 Assurance Criteria of the Standard Curve

14.1.1 $|r| \geq 0.980$

12.1.1.1 If $|r|$ is less than 0.980, repeat the test with new glassware and reagents to confirm the result.

14.1.2 Ta of NC > Tg of λ + 10 min. (600 sec.)

14.1.2.1 If the negative control does not meet this criteria, repeat the test with new glassware and reagents to confirm the result.

14.2 Test for Interfering Factors

14.2.1 $|r| \geq 0.980$ for the CSE controls

12.2.1.1 If $|r|$ is less than 0.980, repeat the test with new glassware and reagents to confirm the result.

14.2.2 Ta of NC > Tg of λ + 10 min. (600 sec.)

12.2.2.1 If the negative control does not meet this criteria, repeat the test with new glassware and reagents to confirm the result.

14.2.3 For the Positive Product Control, the mean endotoxin recovery must be between 50-200% of the spiked value.

14.2.3.1 If the mean endotoxin recovery is outside of the range of 50-200%, the product dilution that was tested is thought to contain interfering factors. Repeat the test using a larger dilution, but not exceeding the MVD.

14.2.4 Interference may also be eliminated by filtration, neutralization, dialysis, or heat if such method(s) are validated by the customer.

15.0 REFERENCES

15.1 RM- 411 PYROSTAR™ ES-F Multi Package Insert (with CSE)

15.2 RM-412 PYROSTAR™ ES-F Single Package Insert (with CSE)

15.3 USP <85> Bacterial Endotoxins Test

15.4 USP<1085> Guidelines on Endotoxins Testing

15.5 USP <161> Medical Devices-Bacterial Endotoxin and Pyrogen Tests

15.6 ANSI/AAMI ST72:2019 Bacterial Endotoxins - Test methods, routine monitoring, and alternatives to batch testing

15.7 HS-004.00 FUJIFILM Wako Kinetic Turbidimetric Assay on the Microplate Reader

15.8 Predictive Oncology EndoPrep™ Instruction Booklet