NCL Method STE-4

Detection and Quantification of β-(1,3)-D-Glucan Contamination in Nanoparticle Formulations by Factor C Depleted LAL (Glucatell®) Assay

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This document describes a protocol for the quantitative detection of β-(1,3)-D-glucans in nanoparticle preparations using a chromogenic endpoint Glucatell assay. β-(1,3)-D-glucans are products of cell walls of some microorganisms, including yeast and fungi. The most common source of β-(1,3)-D-glucans is fungi *Candida* and *Aspergillus*. B-(1,3)-D-glucans are also found at low levels (< 60 pg/mL) in the blood of healthy humans where they are believed to distribute from the gastrointestinal tract following the consumption of β-(1,3)-D-glucan-containing foods (plants, grains, cereal, mushrooms etc.,). In patients with an invasive fungal infection, these levels increase at or above 80 pg/mL [1-5]. β-(1,3)-D-glucans may become undesirable contaminants in pharmaceutical products, where they are introduced during manufacturing through filtration processes utilizing cellulose-based filters or contaminated starting materials or common excipients such as sucrose. β-(1,3)-D-glucans are not as immunologically potent as bacterial endotoxins. However, they are pro-inflammatory and can also activate the immune system. There is an increasing amount of information suggesting that β-(1,3)-D-glucans may exaggerate endotoxin-mediated toxicities as well as synergize with other immunologically active impurities introduced into pharmaceutical products during manufacturing and, therefore, lead to adverse immune effects [6-8]. Moreover, FDA immunogenicity guidance for industry suggests minimizing the levels of β-(1,3)-D-glucans in therapeutic protein formulations to decrease the immunogenicity of these products [9].

Unlike bacterial endotoxins, β-(1,3)-D-glucans are currently not regulated; there is no compendial standard for their detection and no harmonized approach to acceptable levels. Nevertheless, there is a growing trend in industry and among regulatory authorities worldwide to detect β-(1-3)-D-glucans and understand their safety levels [6,8].

Since many nanotechnology platforms are not immunologically inert, understanding the presence of immunologically reactive contaminants besides endotoxin becomes an essential step in understanding the safety margins for formulations containing such materials [10]. This becomes especially important when such nanomaterials are intended for immunotherapy involving the intentional application of immune checkpoint inhibitors to enhance the immune response.

The assay used in this protocol is based on the commercial kit approved for the detection of β-(1,3)-D-glucans in human serum to diagnose fungal infection. We adapted an R&D version of

this assay for the screening of nanomaterial formulations not to disqualify nanoparticles, but to inform the formulation of immunologically safe nanomaterials.

2. Principles

The Glucatell® assay is a derivative of the Limulus amebocyte lysate (LAL) assay. The lysate in the LAL assay contains two main proteases named Factor C and Factor G, which are activated specifically by bacterial endotoxins and β-(1,3)-D-glucans, respectively (Figure 1). The presence of β-(1,3)-D-glucans in test nanoparticle samples results in a false-positive result in the LAL assay. To avoid such false-positive results, a Glucashield reagent is used to block Factor G (please see details in the NCL STE-1 assay series). In contrast, to make the LAL assay specific to β-(1,3)-D-glucans, Factor C is removed from the lysate. The resultant lysate, also known as Glucatell Reagent, contains Factor G and, therefore, is specific to β-(1,3)-D-glucans. β-(1,3)-D-Glucans in the test nanoparticle sample causes the activation of serine proteases. An activated protease cleaves p-nitroaniline (pNA) from the peptide substrate. The generation of pNA results in a change in sample color (along with addition of the Diazo Reagents) and is measured at 540 nm. The intensity of the color is proportional to the amount of β-(1,3)-D-glucans in the test sample.

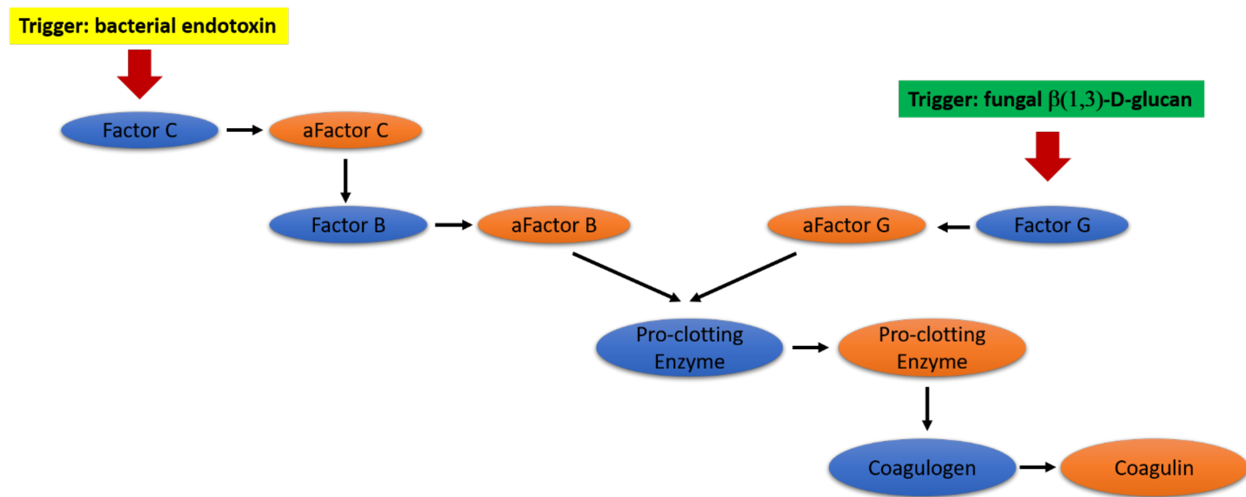

Figure 1. Key elements of the LAL enzymatic reaction and their specificity to endotoxin and β-(1,3)-D-glucans. LAL is a mixture of serine proteases (Factor C, Factor B, Factor G, Pro-clotting enzyme, and coagulogen), which form an enzymatic cascade. There are two arms in the cascade. One arm is triggered by bacterial endotoxins, which specifically activate Factor C. The second arm is triggered by β-(1,3)-D-glucans, which specifically activate Factor G. The activated Factor C and activated Factor G, in turn, cleave their respective targets, and the proteolytic cascade continues until it culminates with the production of coagulin which results in turbidity and clotting of the lysate. The presence of β-(1,3)-D-glucans in test samples, therefore, interferes with the accurate quantification of bacterial endotoxins by the LAL assay. To avoid this interference, the Factor G pathway is either blocked by glucan masking reagents (e.g., Glucashield) or by changing the assay format to the recombinant Factor C. To avoid similar interference from endotoxin during quantification of β-(1,3)-D-glucans using the LAL assay, Factor C is depleted from the LAL lysate. The LAL without Factor C is known as Glucatell reagent, which is used in our protocol. Note: a = activated.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Test nanomaterial

3.1.2 Glucatell Kit (Associates of Cape Cod, GT003)

3.2 Materials

3.2.1 Repeating pipette with Combi tips, pyrogen free (250 mL; 5 mL)

3.2.2 Test tubes for sample dilution (13 x 100 mm)

3.2.3 Pyrogen free pipette tips (1000, 250 µL)

3.2.4 Parafilm®

3.2.5 Disposable endotoxin-free glass dilution tubes, 12x75 mm (ACC, TB240)

3.3 Equipment

3.3.1 Microcentrifuge

3.3.2 Refrigerator, 2-8°C

3.3.3 Freezer, -20°C

3.3.4 Vortex

3.3.5 Plate Reader capable of reading at 540 nm

3.3.6 Block Heater w/ 96-well plate insert (37ºC)

4. Reagent Preparation

4.1 Preparation of standard solution: The β-(1,3)-D-glucanscontent is stated on the vial label. Add the appropriate volume of Reagent Grade Water (RGW) to the vial of glucan standard (Pachyman) to make a 100 pg/mL solution (see vial label for volume to add). Vortex for at least one minute to resuspend homogeneously.

4.2 Reconstitute one vial of Glucatell reagent with 2.8 mL of Pyrosol. Swirl the vial gently to dissolve completely (**DO NOT VORTEX**.) Use the reconstituted Glucatell reagent within 10 minutes or place it at 2-8°C and use within 2 hours.

4.3 Preparation of the diazo coupling reagents:

4.3.1 Add the 1N HCl solution (vial 1A) to the sodium nitrite. This is vial 1 for use in step 7.6.

4.3.2 Add 4.0 mL of Reagent Grade Water to the ammonium sulfamate. This is vial 2 for use in step 7.6.

4.3.3 Add the contents of the of N-methyl-pyrrolidinone to the N-(1-napthyl) ethylenediamine dihydrochloride (NEDA). This is vial 3 for use in step 7.6.

Note: All of these solutions should be used the same day.

5. Preparation of Standard Curve and Quality Controls

5.1 Calibration Standards

Calibration standards are prepared by spiking a known amount of β-(1,3)-D-glucan standard into reagent grade water (RGW). Example reagent volumes are provided in Table 1 below.

Table 1. Calibration Standards

Sample	Nominal Concentration (pg/mL)	Preparation Procedure
1	40	600 µL RGW + 400 µL Stock β-(1,3)-D-glucan Standard (100 pg/mL)
2	20	400 µL RGW + 400 µL Solution 1
3	10	400 µL RGW + 400 µLSolution 2
4	5	400 µL RGW + 400 µL Solution 3

5.2 Quality Control (QC)

The QC is prepared by spiking a known amount of β-(1,3)-D-glucan standard into reagent grade water (RGW). Example reagent volumes are provided in Table 2 below.

Table 2. Quality Control

Sample	Nominal Concentration (pg/mL)	Preparation Procedure
QC	15	567 µL RGW + 100 µL Stock β-(1,3)-D-glucan Standard (100 pg/mL)

5.3 Inhibition/Enhancement Control (IEC)

The IEC is prepared for each dilution of the tested nanomaterial (NP). Example reagent volumes are provided in Table 3 below.

Table 3. Inhibition/Enhancement Control

Sample	Nominal Concentration (pg/mL)	Preparation Procedure
IEC	15	283.5 µL NP + 50 µL Stock β-(1,3)-D-glucan Standard (100 pg/mL)

6. Preparation of Study Samples

Samples are tested at three dilutions (1:5, 1:50, and 1:500) prepared directly from the stock nanomaterial (NP). Prepare the initial 5-fold-dilution using RGW. Next, perform two serial 10-fold dilutions. Example volumes are provided in Table 4 below.

Table 4. Study Samples

Sample	Dilution	Preparation Procedure
Nanoparticle A	5	100 µL NP stock + 400 µL RGW
Nanoparticle A	50	50 µL Dil 5 + 450 µL RGW
Nanoparticle A	500	50 µL Dil 50 + 450 µL RGW

7. Experimental Procedure

7.1 Prepare calibration standards, quality controls, inhibition enhancement controls, and test nanomaterials as described in Sections 5 and 6 above.

7.2. Plate 50 µL of RGW (blank) or calibration standards in duplicate into the corresponding wells of a 96-well plate (an example plate map is available in the Appendix).

7.3 Plate 50 µL of samples and IEC in duplicate to the corresponding wells of a 96-well plate (see example plate map in Appendix).

7.4 Add 50 µL of Glucatell reagent to each well using a repeater pipette. Cover the plate with a lid and shake by tapping the edge.

7.5 Place the plate on a heating block or a plate reader preheated to 37°C ± 1°C for the recommended incubation time (see Certificate of Analysis) for the curve chosen.

7.6 Stop the reaction by adding 50 µL of sodium nitrite (vial 1 from step 4.3.1) with a repeater pipette. Then add in sequence 50 µL of ammonium sulfamate (vial 2 from step 4.3.2), and then 50 µL of N-(1-napthyl) ethylenediamine dihydrochloride (NEDA) (vial 3 from step 4.3.3), using a new pipette tip each time. The color development starts immediately.

7.7 Place the uncovered plate in the microplate reader and read the optical density at 540-550 nm.

7.8 Use the plate reader software to plot a standard curve and to calculate the concentration of β-(1,3)-D-glucan in the test-samples, quality controls, and IECs.

8. Assay Acceptance Criteria

8.1 The linear regression algorithm is used to construct the standard curve. Precision (% CV) and accuracy (percent difference from theoretical) of each calibration standard and quality control should be within 25%.

8.2 At least three calibration standards should be available for the assay to be considered acceptable.

8.3 If quality controls fail to meet the acceptance criterion described in 8.1, run should be repeated.

8.4 If the standard curve fails to meet the acceptance criterion described in 8.1-8.2, the run should be repeated.

8.5 Precision of the study sample should be within 25%.

8.6 Precision of the inhibition/enhancement control should be within 25%.

8.7 Spike recovery indicative of the accuracy of the inhibition/enhancement control should be between 50-200% [2]. Spike recovery less than 50% is indicative of inhibition; that above 200% is indicative of either contamination or enhancement.

8.8 If sample interference is detected, the assay results for this sample are invalid.

9. Data Interpretation

No compendial procedure or criteria is currently available for estimation of acceptable levels of β-(1,3)-D-glucans in pharmaceutical products. Below we discuss the approach used in our laboratory [11].

This approach relies on several assumptions: a) an average adult weight is 70 kg; b) the blood volume of such an adult is 5.6 L (or 8% of the bodyweight); and c) the entire injected dose stays in the circulation. The detected amount of β-(1,3)-D-glucans per 1 mL of the test-nanomaterial is converted into the β-(1,3)-D-glucan dose using the API concentration in the stock nanomaterial and the intended API dose. Next, the β-(1,3)-D-glucans dose is converted into the concentration in one milliliter of blood; the latter is compared to the limit (70 pg/mL) used in the clinical diagnostic Fungitell assay where beta-glucan levels are indicative of fungal infection. For example, if this assay determines that the level of β-(1,3)-D-glucans is 1000 pg/mL of a nanoformulation containing 10 mg/mL of API and the API dose is 10 mg/kg, then the beta-glucan dose is 1000 pg/kg. After conversion to the amount per milliliter of blood (1000 pg x 70 kg = 70000 pg of beta-glucan per 5600 mL of blood), the result is 12.5 pg/mL, which is less than 70 pg/mL. Therefore, we would consider this result normal, as it is within healthy levels of beta-glucans present in the human blood from dietary sources.

Other approaches are available in the literature [12, 13] and discussed in detail elsewhere [14].

10. References

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11. Abbreviations

API	active pharmaceutical ingredient
CV	coefficient of variation
FDA	Food and Drug Administration
HCl	hydrochloric acid
IEC	inhibition/enhancement control
LAL	Limulus Amebocyte Lysate
NEDA	N-(1-napthyl) ethylenediamine dihydrochloride
NP	nanomaterial
pNA	p-nitroaniline
QC	quality control
RGW	reagent grade water

12. Appendix

Example plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Std 1	Std 1	Sample 1 Dil 5	Sample 1 Dil 5	Sample 1 Dil 500 IEC	Sample 1 Dil 500 IEC	Sample 2 Dil 50 IEC	Sample 2 Dil 50 IEC	Sample 3 Dil 5 IEC	Sample 3 Dil 5 IEC	
C		Std 2	Std 2	Sample 1 Dil 50	Sample 1 Dil 50	Sample 2 Dil 5	Sample 2 Dil 5	Sample 2 Dil 500 IEC	Sample 2 Dil 500 IEC	Sample 3 Dil 50 IEC	Sample 3 Dil 50 IEC	
D		Std 3	Std 3	Sample 1 Dil 500	Sample 1 Dil 500	Sample 2 Dil 50	Sample 2 Dil 50	Sample 3 Dil 5	Sample 3 Dil 5	Sample 3 Dil 500 IEC	Sample 3 Dil 500 IEC	
E		Std 4	Std 4	Sample 1 Dil 5 IEC	Sample 1 Dil 5 IEC	Sample 2 Dil 500	Sample 2 Dil 500	Sample 3 Dil 50	Sample 3 Dil 50	RGW QC IEC	RGW QC IEC	
F		NC	NC	Sample 1 Dil 50 IEC	Sample 1 Dil 50 IEC	Sample 2 Dil 5 IEC	Sample 2 Dil 5 IEC	Sample 3 Dil 500	Sample 3 Dil 500			
G												
H												

Std = standard; NC = negative control; IEC = inhibition/enhancement control; dil = dilution; RGW QC = reagent grade water quality control