Setting Endotoxin In-Process Controls For Recombinant Therapeutic Proteins

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Introduction

Recombinant therapeutic proteins (e.g. monoclonal antibodies) are manufactured using biotechnological processes, which typically consist of the following:

- Inoculation of the genetically modified host (most commonly Chinese Hamster Ovary cells or *E. coli*) in nutrient media and expansion of the cell culture to production scale. The host produces the protein of interest, which is either secreted into the medium or retained within the host cell.
- Separation of the host cells and media (commonly achieved by centrifugation)
- Capture and purification of the protein using column chromatography
- Viral clearance by filtration
- Ultrafiltration/Diafiltration and dilution to place the protein in the final formulation at the target concentration
- Dispensing of the protein into bulk storage containers for long term storage, which typically occurs at either 2-8°C or -20°C
- Aseptic processing of the bulk to fill into the final drug product containers

While the filling process occurs under sterile conditions, the other processes occur under non-sterile conditions. Though these processes are non-sterile, because microbes can produce components that may have deleterious effects on product quality (e.g. proteases) and patient safety (e.g. endotoxin and other Pathogen Associated Molecular Pattern (PAMP) or exotoxins), microbial control is paramount. Maintaining microbial control is challenging because the processes are conducive to microbial growth as they utilize nutrient rich media, buffers of neutral pH and occur at ambient or warmer temperatures. Therefore a robust microbial control strategy must be employed consisting of both prevention and detection controls. The primary prevention control is an appropriate process design to prevent the ingress and proliferation of microorganisms (e.g. effective equipment sanitization, filtration of raw materials and in-process intermediates, validated hold times). The other key element of an effective microbial control strategy is a testing program for both bioburden and endotoxin with appropriate limits that trigger remedial action when exceeded to restore the desired level of microbial control.

Bioburden monitoring performed on representative sampling points allows determination of the total microbial load of the product and serves as a surrogate test for toxins and non-endotoxin PAMPs. The potential load of toxins and non-endotoxin PAMPs can be calculated based on the bioburden concentration (e.g. CFU/10 mL), cell characteristics of the contaminating organism, process step, and

microbiological factors of toxins and non-endotoxin PAMPs. The calculated load can be compared to safety limits specific for toxins and non-endotoxin PAMPs. Bioburden analysis can also indicate the potential presence of degradative enzymes (e.g. extracellular proteases, or endoglycosidases), which could cause product degradation or modification.[1]

Endotoxins are potent pyrogens, which should be monitored throughout the manufacturing process to ensure the total endotoxin load of the final product is below the pyrogenic threshold. In addition, endotoxin testing of representative in-process samples serves as an orthogonal assay to bioburden testing, as it allows detection of non-culturable gram negative bacteria.

Problem Statement

While the compendia contain guidance on setting final product specifications for endotoxin, there is currently no compendial or health authority guidance on setting of in-process limits for either bioburden or endotoxin. It is critical that the limits are set in a manner to ensure an appropriate level of control while not triggering superfluous investigations. In a survey conducted by the BioPhorum Operations Group, it was noted that most companies set bioburden action limits ranging from 1-10 CFU/mL for bulk biologics manufacturing.[2] While setting of appropriate bioburden control limits is achievable by analyzing historical data from similar processes, setting in-process endotoxin controls presents the following special challenges:

- **Variance in inputs from raw materials:** Unlike bioburden, which can be removed via 0.2µm filtration, endotoxin will pass through filtrations into the process. Therefore, the endotoxin load from raw materials will vary from process to process.
- **Endotoxin method capability:** The Limulus Amebocyte Lysate (LAL) Assay is the most commonly used assay for endotoxin quantification. The matrix (e.g. protein concentration, pH, etc.) of the sample tested may cause inhibition or enhancement of the LAL assay, which impacts the limit of quantification (LOQ) achievable for that specific sample. The LOQ of the endotoxin assay may vary widely from sample to sample.

Procedure for Setting Endotoxin In-Process Limits

The following procedure applies to endotoxin testing of in-process controls (process intermediates including column equilibration samples) of non-sterile materials intended for sterile dosages in the manufacturing process streams for Roche-Genentech Biologics commercial and investigational medicinal products (IMPs). It does not apply to non-product endotoxin testing (e.g., fermentation media, process buffers, process waters, etc.) or setting of endotoxin specifications of Drug Substance, Drug Product and Direct Materials.

General Requirements

- Endotoxin in-process control comprises alert levels and action limits.
- For products with less than 30 data points, a provisional action limit of > 3.0 EU/mL is defined.* The provisional action limit applies for all in-process steps except if a process control step is not expected to provide endotoxin clearance downstream of a given sampling point. In cases where no further downstream clearance is expected, the action limit at that sample point must not exceed the Drug Substance or Drug Product release criterion. Process steps between Drug Substance and Drug Product (e.g. dilution or addition of excipients) must be considered. For products with less than 30 data points no alert level is defined.

Rationale: The provisional action limit of > 3.0 EU/mL was derived by evaluating historical in-process endotoxin data from different Roche-Genentech products. Most of the current in-process data are within this 3.0 EU/mL. A tighter limit may be set. However, setting a limit below 3.0 EU/mL would be prone to false positive results for some products that have in-process samples associated with a higher Limit of Quantitation. Therefore the provisional action limit of > 3.0 EU/mL is considered to be a compromise between setting a limit that will detect a drift from normal operating conditions, and LAL method capability.

Establishing Endotoxin Alert Levels and Action Limits

A minimum of 30 data points is needed for setting a two-tier in-process control system (alert levels and action limits). Only data obtained from GMP batches must be included to ensure proper data quality. To ensure differences in raw material endotoxin load are considered, the 30 batches used for calculation of limits should have been manufactured with differing batches of raw materials. Endotoxin data which represent limit excursions, adverse trends or secondary contaminations must not be included in the data set because these data would erroneously increase calculated limits.

Endotoxin Alert Levels

- Endotoxin alert levels of in process control steps are calculated from a minimum of 30 data points (see reference section for rationale documents) per process control step by determining the 95th percentile. For endotoxin values below LOQ (e.g. <0.6 EU/mL) the LOQ (e.g. 0.6 EU/mL) should be used for calculation. In order to calculate the 95 th percentile an appropriate software is used. The alert level is defined as > 95th percentile.

Endotoxin Action Limits

- Endotoxin action limits are calculated using the following formula:

Endotoxin action limit (EU/mL) = Endotoxin alert level (EU/mL) x 6

Rationale: The LAL assay has an intrinsic method variability of factor 2. This factor 2 is multiplied by a common statistical factor (factor 3, e.g. 3SD) to achieve an action limit, which is meaningfully different than the alert level, given the inherent variability of the LAL assay.

- Calculated endotoxin action limits cannot exceed provisional action limits or action limits which have already been established.
- The minimum action limit is set at > 0.3 EU/mL, which is the specified endotoxin limit for water for injection (WFI) rounded to one digit (> 0.25 EU/mL). Any in-process limit below 0.3 EU/mL would trigger superfluous investigations because the WFI endotoxin load is regarded as generally safe.

*Note: For products manufactured using an endotoxin-producing host (e.g. *E. coli*), endotoxin is a process impurity. The provisional action limit of 3.0 EU/mL is not applicable to the early purification process steps because you cannot distinguish the endotoxin derived from the host and a microbial contamination event. Therefore, assaying for endotoxin at the early purification steps does not provide value for detection of microbial contamination and should not be performed routinely. However, it does provide value during process validation to understand the capability of the process to remove endotoxin derived from the host, which is critical to ensuring patient safety.

- If a process control step is not expected to provide endotoxin clearance downstream of a given sampling point, the action limit for that sampling point may not exceed the Drug Substance or Drug Product release specification. Process steps between Drug Substance and Drug Product (e. g. dilution or addition of excipients) need to be considered.

Case Study

The procedure for establishing endotoxin alert levels and action limits described above was applied to a biotech manufacturing process consisting of CHO cell fermentation, cell harvest, and six protein purification steps. In-process alert levels were calculated from historical data sets with a minimum of 35 data points by determining the 95th percentile. Action limits were calculated from unrounded 95th percentiles by multiplying with a factor of 6. Calculated alert levels were rounded to meaningful values (one digit). Calculated action limits were rounded to >0.3 EU/mL, if values were < 0.3 EU/mL.

Sample	Calculated Limits		Number of data points	Applied Limits	
	Alert Level	Action Limit		Alert Level	Action Limit
cell harvest step 1	> 0.022	> 0.131	101	> 0.1	> 1.9
cell harvest step 2	> 0.096	> 0.575	35	> 0.1	> 1.9
Protein purification step 1	> 0.320	> 1.920	100	> 0.3	> 1.9
Protein purification step 2	> 0.080	> 0.480	101	> 0.1	> 0.5
Protein purification step 3	> 0.020	> 0.120	100	> 0.1	> 0.3
Protein purification step 4	> 0.040	> 0.240	101	> 0.1	> 0.3
Protein purification step 5	> 0.020	> 0.120	36	> 0.1	> 0.3
Protein purification step 6	> 0.016	> 0.098	36	> 0.1	> 0.3

Note: Although the "cell harvest step 1" and "cell harvest step 2" demonstrate greater process capability than the action limit applied, the action limit of >1.9 EU/mL was selected to align with the action limit calculated for the "protein purification step 1" because limits applied to earlier steps should not be tighter than steps further downstream.

Guideline for Investigating Excursions

Every limit excursion must be reviewed holistically and all findings and information that may impact product quality and patient safety must be considered before determining acceptability of the batch. Table 1 provides an overview of proposed actions.

Table 1. Proposed actions for endotoxin in-process limit excursions.

Alert level excursions	
Step	Actions
A1	Conduct a laboratory investigation which includes, but is not limited to, the following areas: • Sample – verify that the correct sample was tested, stored under proper conditions and tested within expiry (if applicable). Ensure that the appropriate sample container was used and that container integrity was not compromised. • Analyst – ensure that the analyst was qualified to perform the assay. Interview the analyst for abnormal events or problems during testing of impacted samples. • Method – ensure that the correct method was followed, applicable controls met acceptance criteria and that calculations were accurate. • Materials – ensure that the correct reagents were used, met qualification criteria and were used within expiration. • Equipment – ensure that equipment was functioning as intended and that they were maintained and calibrated per local procedures. If a laboratory error is identified, invalidate the test result and conduct a retest. If no lab error is found, the test result is considered valid. Proceed to Step A2.
A2	Review data for an adverse trend. An example of adverse trends includes: When three consecutive microbiological alert level excursions for the same process step across three batches or three alert level excursions within a production run. If an adverse trend is identified refer to step B2.
Action limit excursions	
Step	Actions
B1	Conduct a lab investigation as described in Step A1.
B2	Determination of Cause Perform an evaluation to determine the cause of the excursion. Items that should be reviewed include, but are not limited to: • Manufacturing process • Manufacturing SOPs and other production control records • Sampling procedures • Equipment cleaning, sanitization, and sterilization records • Personnel qualification and training records • Equipment maintenance information
	Disposition of Batch and Identification of CAPAs
	Assess the data provided, in collaboration with appropriate technical experts, and disposition the impacted product batch. Consider the following, as appropriate: • Impact on product safety, quality and efficacy. • Impact on equipment and its suitability for further use. • Impact on other product batches. CAPAs may be identified as a result of an investigation for the cause of the endotoxin excursion. These actions may be immediate to prevent recurrence of the excursion (e.g., equipment repair, procedural modifications, additional training) or long-term for overall process enhancement (e.g., process or equipment modifications).

Discussion

The described procedure for setting endotoxin in-process limits provides a statistically justifiable approach, based upon historical data, which will ensure appropriate responses are taken in response to endotoxin data. However, because the limits are generated based upon historical data obtained from a specific endotoxin method, caution must be exercised when making changes to the endotoxin method that impact the LOQ. Additionally, because Health Authorities expect in-process action limits to be the same for products manufactured at multiple sites, it's recommended to use the same method across manufacturing sites to ensure a consistent LOQ.

Equally important to setting appropriate limits is having adequate procedures in place to respond to excursions. The two-tier endotoxin control system outlined may provide early warning of drifts in the process performance or a serious microbial control failure, triggering actions commensurate with the risk to product quality and patient safety.

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