Pharmaceutical Microbiology
Working Group

ECA Foundation
Fostering harmonisation of GMP/GDP regulations

Guidelines for the Evaluation and Investigation of Microbiological Deviations

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Chapter 1: Deviation Handling of Microbiological Environmental Monitoring Excursions in Non-Sterile Pharmaceutical Manufacturing

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Chapter 1: Deviation Handling of Microbiological Environmental Monitoring Excursions in Non-Sterile Pharmaceutical Manufacturing

1. Purpose

This document provides examples of controlled room requirements (action and alert levels) and serves as a guide for the handling of deviations occurring during microbiological environmental monitoring in facilities manufacturing non-sterile pharmaceutical products.

2. Procedures

2.1 Examples of controlled room requirements

Little guidance is available to establish microbiological levels for non-sterile manufacturing. In general, each company has to define its own microbiological requirements for their facilities based on the activities in the controlled room and criticality of the product using a risk-based approach. I.e. if rectal suppositories are produced, obviously less stringent requirements would be needed than for an aqueous nasal product. Actually, the USP chapter <1115> states "In general, environments for tablet and powder- and liquid-filled capsule manufacturing should require no monitoring or infrequent monitoring."

Tables 1-3 in the Appendix show some examples of microbiological requirements for non-sterile manufacturing. These levels are not mandatory. It is common practice to set action levels based on regulatory guidance or criticality of the controlled room following a risk assessment. In addition to the action level, an alert level is often defined in order to assess unusual high microbial counts that may signal a potential drift from expected microbial counts. If enough data is available, the initial alert level (e.g. 50% of the action level) can be replaced with a calculated alert level based on historical data. Generally, for non-sterile product manufacturing the monitoring frequency is quite low and for each sampling location not enough data points are available to calculate historically-based alert levels per sampling location. Therefore, it is recommended to group sampling points of controlled rooms of similar activity for which similar environmental conditions are expected (e.g. grouping all air sampling points of granulation rooms or all the ones from wash rooms). In addition, since microbiological data from environmental monitoring are in general not normally-distributed, higher sophisticated statistical models must be used whereby the alert levels may be defined by establishing a threshold of acceptance using percentiles with Gamma- or Negative-Binomial-distributions. These alert levels must be re-assessed at pre-defined time intervals or if sufficient additional results can be integrated in the calculation. For further details see e.g. Gordon *et al.* (2015), ZLG (2014), Rieth (2012, 2016), PDA TR No. 13 (2014).

Beside the total aerobic microbial count, it might be advisable to have another more stringent level for molds. E.g. 10-50% of the total aerobic microbial count might be used (see Table 3 in the Appendix for examples).

The following list gives an overview of most often monitored parameters in non-sterile manufacturing; this list might not be exhaustive:

- Active air[1]
- Surfaces (product contacting, non-product contacting, wall, floor, personnel in special cases)
- Cleaning and disinfectant solutions and utensils
- Utilities such as water, pressurized air, gases, etc.

Sampling frequency and locations: Sampling frequency and locations should be based on a risk assessment evaluating the general hygiene of the environment as well as proximity to the unprotected

[1] Passive air monitoring might be used in special cases, e.g. for close to open product monitoring for critical products such as inhalation products.

product formulation. Also, the frequency of testing should take into account the criticality of the product (e.g. higher testing frequency for inhalation products as compared to oral dosage forms). It is up to the company to use a representative testing frequency to record any trends which indicate a drift from the norm, i.e. routine microbiological monitoring should provide sufficient data to ascertain that the controlled environment is operating within an adequate hygiene level. The frequency can range from twice a year up to weekly or even daily for very critical areas. Examples are given in Table 1 and 4 of the Appendix.

New or modified controlled rooms: New or modified controlled rooms need an initial qualification. Normally the qualification is performed in "at rest" stage and the levels should be more stringent than the ones from the routine monitoring "in operation" (examples are given in Table 3a and 3b of the Appendix). The qualification can include e.g. one "at rest" run followed by three "in operation" runs or over a certain time period several measurements are performed to show that the room is running in the pre-defined conditions.

The sampling points should be defined by risk assessment (e.g. open product, high personal or material flow, central positions, difficult to clean areas, position of high exposure). For the qualification an increased number of sampling points is tested while for the routine testing with the received results the number of sampling points can be reduced risk based.

It is advisable to perform the microbiological controlled room qualification after the technical qualification phase including HVAC qualification.

2.2 Procedure if the alert level or action level is exceeded

If the microbiological counts of a controlled room are above the action level, the hygiene performance might not be adequate for its grade. Corrective and possibly preventive actions should be implemented.

In general, when the microbiological level is exceeded, a thorough investigation needs to be performed. In the following paragraph a possible procedure is described which is depicted in Figure 1.

In this concept the first step is to define if a critical or non-critical deviation occurred.

Non-critical deviation: Exceeding of sampling points (surface or air) with no direct product contact (e.g. floor, wall, non-product contacting surfaces, gowning area, cleaning solutions, disinfectant) or product contacting surfaces for non-critical product categories (e.g. non-aqueous oral application). Exceeding of alert levels.

Critical deviation: For critical product categories (e.g. aqueous application, inhalation products) when sampling points have direct product contact (e.g. product contacting equipment, air samples close to physically unprotected product, disinfectant). Exceeding of recurring events or an adverse trend for product contacting surfaces or air sampling close to the product or for critical product categories.

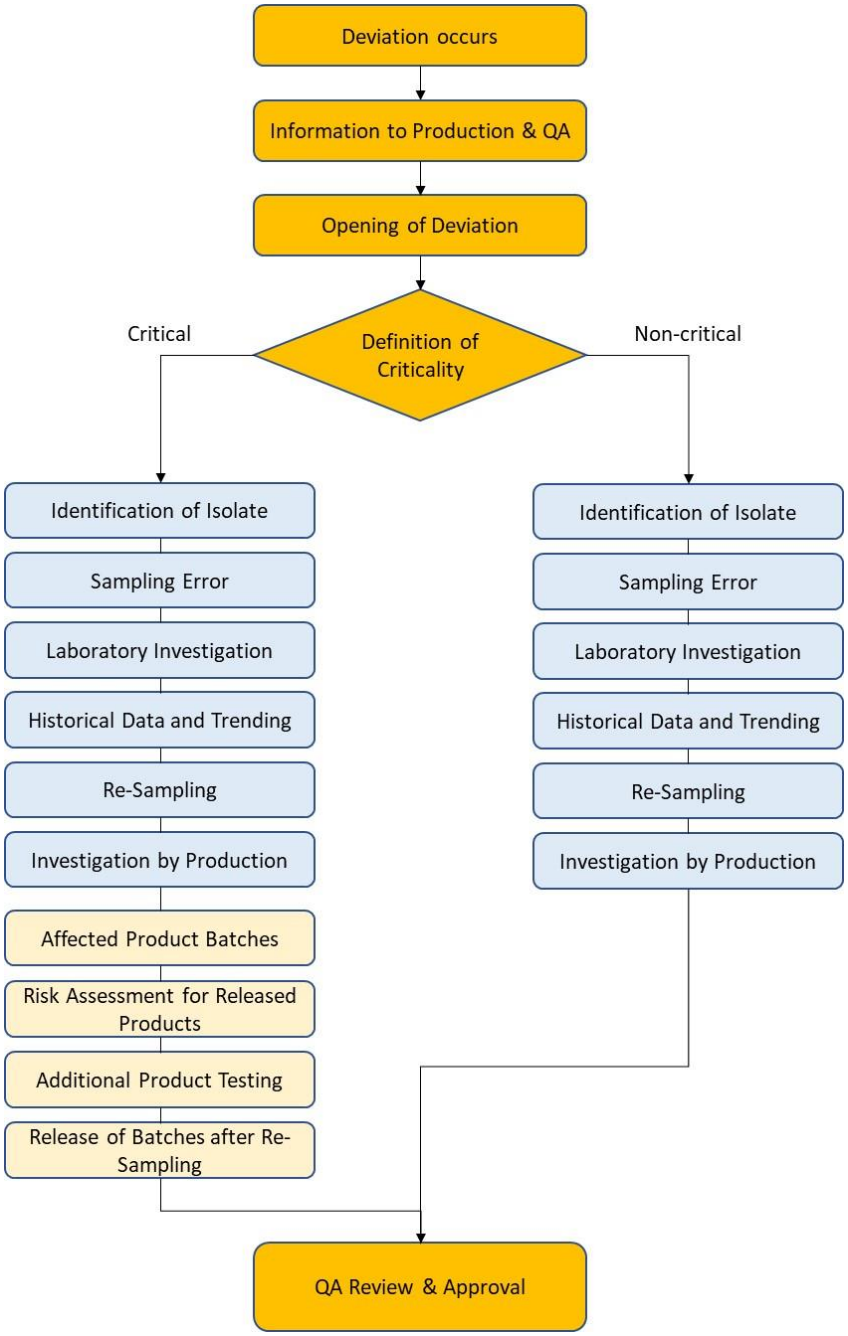

Figure 1: Example of a possible flowchart for the deviation procedure of non-critical and critical deviations from the microbiological monitoring of non-sterile manufacturing controlled rooms. It must be mentioned that the actions need to be opened contemporaneously to provide a closure of the deviation within a certain timeline (e.g. 20 working days).

2.2.1 Identification of isolate

For the root cause investigation, it is of high importance to know the type of contaminants present. At first it should be checked if a monoculture (i.e. colonies of similar morphological aspect) is present or rather a mixture of isolates. If several morphological distinguishable colonies are present it can be sufficient to identify the most frequent isolate (i.e. the most representative colony type). Modern identification methods that can identify to the species level (e.g. DNA sequencing, MALDI-TOF, biochemical test kits) should preferably be used; however, in less critical cases as long as the monitoring

process does not require special attention to objectionable microorganisms, an identification to the genus level is enough. The identification of the isolate helps to provide information of the origin of contamination e.g. gram-positive cocci are mostly human related bacteria, presence of gram-negative rods may indicate contamination from water. In critical cases (e.g. manufacturing of liquid products) it might be advisable to check if the isolated microorganism is objectionable or not. For more insight on objectionable microorganisms refer to PDA TR No. 67.

The micro-organisms which are frequently isolated in the ambient air or on critical surfaces of production areas might be stored as so-called "in house isolates" in the internal culture collection for possible usage (e.g. growth promotion tests, fingerprint analysis).

2.2.2 Sampling error

It must be clarified if any sampling error happened. E.g. has the person that took the sample been appropriately qualified? Were there any special issues during sampling? Were there any concerns with the released agar plates? Was the sample hold time followed? Did a mistake occur during transportation from the sampling site until the incubation? Etc.

Agar plates which are not properly closed can contract secondary contaminations quite easily. If plates are transported in boxes, the microbiological status of the boxes must be impeccable.

If repeated sampling errors occur corresponding CAPAs must be implemented (e.g. revision of sampling instructions, re-qualification of sampling staff, sampling performed under QA oversight).

All investigated points are documented and it is advisable to take a picture of the sample/plate with the exceeded count for documentation.

2.2.3 Laboratory investigation (Lab error)

As for the sampling error, it must be clarified if an error during incubation happened. Here some examples:

- Petri dish lid mistakenly displaced
- Plates fallen by transferring
- Humidity problem of the plate
- Problems with the incubator
- Any other issues during sample handling in the laboratory

All investigated points are documented and it is advisable to take a picture of the sample/plate with the exceeded count.

2.2.4 Historical data and trending

To evaluate if the microbial excursion is a single incidence or a recurring issue, the historical data of the room/equipment should be checked. A sufficient number of samples (e.g. 6 if the rules below are used) should be evaluated. Not only should the concerned sampling point locations be trended but also the complete room or equipment.

An adverse trend is an early warning of a potential degradation or loss of control within the environment. Only a single excursion above a defined microbiological alert or action level is not considered an adverse trend. An adverse trend can be defined as repeating higher than usual counts or increasing number of microorganisms or contamination occurrences over a certain time period. To perform an adequate trending some predefined rules for the trending should be defined. Here some examples of such rules are given:

- 3 or 2 times exceeding the alert level in a row
- 2 times exceeding the action level in a row
- 3 times in a row exceeding the contamination recovery rate limit
- Concerned sampling point is exceeded more than 30% during the considered time period
- Sampling point group (complete equipment or room) 3 consecutively exceeded levels

- All sampling points (room resp. equipment) more than 10% are exceeded during considered time period
- The number of cfu increases at least four times in a row
- The number of exceeding occurrences in one interval is 50% higher than in the preceding interval
- Increased number of exceeding occurrences during three consecutive intervals
- Increased counts or frequency of occurrence in the graphical interpretation of data
- Repeated (e.g. three times) occurrence of a specific microorganism

If an adverse trend is present, corresponding CAPAs (e.g. modifying cleaning/disinfection procedure, increase air rate exchange, optimize personnel/material flow, QA oversight, etc.) should be initiated.

Furthermore, the microbiological data of the neighboring rooms might be evaluated and it should be checked if other comparable deviations occurred during this time period.

2.2.5 Re-Sampling

In some cases, re-sampling may be performed. In case the re-sample of the concerned sampling point result is within requirements, it is insufficient to consider that the problem has been spontaneously solved. Actually, the environmental condition at the time of re-sampling is probably totally different than at the time of the initial deviating sample (e.g. many air changes, different personnel/material flow, cleaning/disinfection of surfaces, etc.). This means that re-sampling should actually serve to acquire more information for the investigation of the root cause and/or help to determine if an adverse trend is present. Re-sampling alone is not sufficient to decide the outcome of the investigation.

If a sampling error might have been the root cause in the first instance, another analyst should perform the sampling or if the same person performs the re-sampling she/he must be observed by QA oversight.

Depending on the criticality of the deviation it must be decided if the complete room/equipment or just the concerned sampling point (with just a few additional locations if necessary) is re-sampled.

2.2.6 Investigation by production

In dependence of the criticality of the deviation, it might be advisable to setup an investigation team composed of production personnel, microbiological experts and QA, in order to investigate the environmental monitoring deviation efficiently. In Table 5 of the Appendix a checklist for production investigation is provided.

In cases of single events of microbial deviations, it is not evident to determine a definitive root cause. Nonetheless it is recommended to verify all possible hypotheses, if applicable, such as those listed in Table 5 to rule them out as a potential root cause.

2.2.7 Affected product batches

For critical deviations (see above for definition) the impact on the produced batches should be evaluated. Thereby two different batch populations should be taken into consideration. The first population of batches is the one before the current batch for which the deviation occurred and the second one is composed of the batches manufactured after the deviation occurred (Figure 2). All affected batches must be listed and must be assigned to the corresponding batch population. In certain cases, e.g. when non-critical human skin microorganisms are found and the microorganism cannot proliferate in the product, a reduced risk assessment might be enough for product assessment.

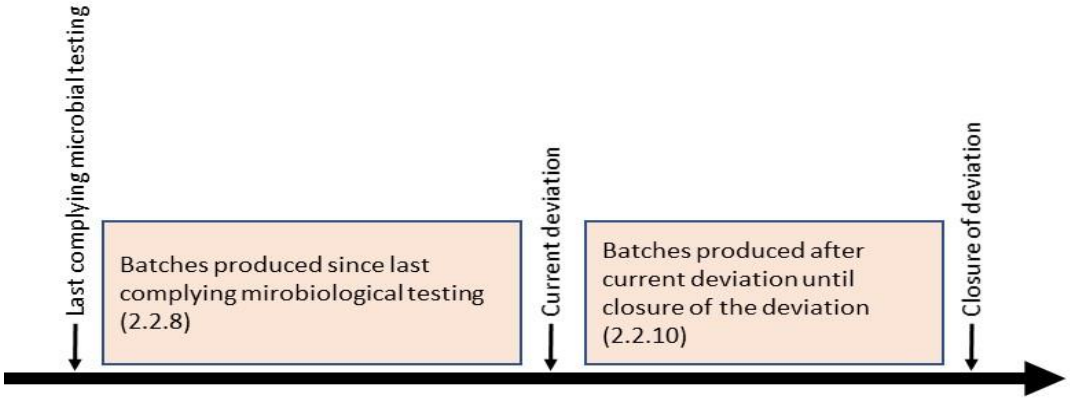

Figure 2: Schematic figure to differentiate the affected batches.

2.2.8 Risk assessment for released products

For critical deviations and/or critical product categories, as depicted in figure 2, the first batch population is the one which was already released and might be on the market. For these batches a risk assessment within 48 hours should be carried out to find out if any products on the market are affected. Typically, the risk for these batches is assessed using the product characteristics (water activity, pH, preservatives, etc.) and its application (oral, inhalation, topical, liquid, solid, etc.). Additional information such as a calculation of the number of microorganisms from the deviating sampling location that would theoretically be present in the product, or product bioburden testing results (microbial enumeration test and test for specified microorganisms) can be used for the assessment. If a relevant risk is acknowledged, a potential recall must be initiated.

2.2.9 Additional product testing

A further possibility for handling critical deviations or product categories is an increased product testing, i.e. ideally the batch just before and after the current deviation sampling can be tested for bioburden (microbial enumeration test and testing for specified microorganisms) to evaluate its microbiological quality. If the bioburden level is above the specification, a rejection of the batch must be considered and a further thorough investigation and risk evaluation for the batches on the market needs to be started (see 2.2.8). If growth occurs and the same species are found as in the deviation of the environmental monitoring, further investigations are needed (e.g. evaluation if there is a path of contamination and if the contaminant is objectionable or not).

2.2.10 Release of batches after re-sampling of environmental monitoring

For critical deviations and/or critical product categories as mentioned above (chapter 2.2.7), in general the batches after the deviating sampling are released within the closure of the deviation investigation. However, in certain cases (e.g. due to extensive root cause investigation or CAPAs) batches might need to be released before the deviation investigation has been completed. For example, all batches which did comply with the requirements after resampling can be released with a written statement signed by the quality unit. In this statement it must be shown that the deviation has no impact on the product's quality attributes and batches can be released from a microbiological point of view.

2.2.11 Final decision by QA

Finally, the entire deviation, for critical as well as non-critical cases, must be challenged and approved by the QA responsible person. Here some points which should be challenged by the QA as an example are listed:

- The report is correct filled in and completed

- The deviation is properly and comprehensively documented
- All decision-relevant corrective actions are completed
- The investigation is complete and the root cause is scientifically sound and based on evidence. If the root cause cannot be found then all investigated parameters must be listed or documented to show the exclusion of possible root causes
- The appropriate corrective actions have been identified, assigned and agreed
- Preventive actions are identified, assigned and defined in a time schedule
- The deviation is correctly categorized (e.g. criticality, root cause classification for trending)
- QA closure of deviation
- For adverse trends, an increase in sampling frequency might need to be initiated to monitor the hygiene status more closely or to check the effectiveness for implemented CAPAs

Furthermore, the following points might be addressed for critical deviations:

- All relevant corrective actions for the release of the batch are complete
- Risk assessment for concerned and potentially concerned batches were carried out and approved by responsible person (e.g. QA)
- The investigation was extended to other batches if relevant
- Decision on batch release

3. Appendix

Table 1: Examples of controlled room requirements (air) and testing frequencies for non-sterile manufacturing from ZLG (2010). Remark: "at rest" testing is in general only required for qualification purposes.

Area	Suggested requirement "in operation"		Suggested requirement "at rest"	Suggested testing frequency
	Alert level (cfu/m³)	Action level (cfu/m³)	(cfu/m³)	
Production of non-sterile, semi-solid[1)] and liquid applications	**250**	**500**	**100**	**Quarterly**
Production of tablets, capsules and sugar-coated tablets	**500**	**800**	**400**	**Quarterly to annually**

1) For liquid semi-solid applications, more stringed requirements might be needed.

Table 2: Examples of controlled room requirements (total aerobic microbial counts) for non-sterile manufacturing from Rieth & Krämer (2016; adapted). Zone "1" is defined as non-sterile products: microorganism reduced liquids (e.g. nasal use). Zone "2" is defined as non-sterile products: liquids (aqueous or non-aqueous), topical use. Zone "3" is defined as non-sterile products: solids and raw material. n.a. = not applicable

	Zone «1» levels		Zone «2» levels		Zone «3» levels	
	Alert	Action	Alert	Action	Alert	Action
Active air (cfu/m³)	**100**	**200**	**100**	**200**	**500**	**1000**
Passive air (cfu/60 cm²)	**20**	**30**	**50**	**100**	**50**	**100**
Product contacting surface (cfu/25 cm²)	**15**	**25**	**25**	**50**	**50**	**100**
Surface close to product (cfu/25 cm²)	**25**	**50**	**50**	**100**	**100**	**200**
Wall (cfu/25 cm²)	**25**	**50**	**50**	**100**	**100**	**200**
Floor (cfu/25 cm²)	**50**	**100**	**100**	**200**	**300**	**500**

Remarks:

- Passive air monitoring is in general not needed for routine purposes, especially in Zone "2" and Zone "3".
- Testing of walls is in general not needed.

Table 3a and 3b: Examples of action and alert levels "at rest" and "in operation" for two different controlled room qualities for non-sterile manufacturing (user example). TAMC = Total aerobic microbial count (all cfus found on contact plates with CASO agar), Molds = Number of Molds found in TAMC. n.a. = not applicable.

	Room is «at rest»			
	Zone «I»		Zone «II»	
	Action level		Action level	
	TAMC	Mold	TAMC	Mold
Active air (cfu/m^3)	**100**	**20**	**250**	**50**
Product contacting surface (cfu/25 cm^2)	**12**	**2**	**25**	**5**
Non critical surfaces (cfu/25 cm^2)	**25**	**5**	**50**	**10**
Floor (cfu/25 cm^2)	**50**	**10**	**100**	**20**

	Room is «in operation»							
	Zone «I»				Zone «II»			
	Alert level		Action level		Alert level		Action level	
	TAMC	Mold	TAMC	Mold	TAMC	Mold	TAMC	Mold
Active air (cfu/m^3)	**100**	**20**	**200**	**40**	**250**	**50**	**500**	**100**
Non critical surfaces (cfu/25 cm^2)	**25**	**5**	**50**	**10**	**50**	**10**	**100**	**20**
Floor	**50**	**10**	**100**	**20**	**100**	**20**	**200**	**40**

Remarks:

- For the "at rest" testing (Table 3a) no alert levels are defined.
- Passive air monitoring is in general not needed for routine purposes, especially in Zone "2" and Zone "3".
- Product contacting surfaces "in operation" (Table 3b) are not tested since this is not really possible due to the presence of product which might inhibit or influence microbial growth or recovery.
- The alert level should be based on historical data analysis and trending as an indication of unusual high counts or potential hygiene problems. The alert level counts provided in the table are used as initial levels if sufficient data are not available. As an alternative other means to include historical data in the assessment of the environment microbiological quality may be used (e.g. target value system).

Table 4: Example of testing frequencies for non-sterile manufacturing from Rieth & Krämer (2016; adapted). In general, it must be mentioned, that the frequency must be evaluated by risk assessment and can therefore deviate significantly from the examples given in the table.

Zone "1" is defined as non-sterile products: microorganism reduced liquids (e.g. nasal use). Zone "2" is defined as non-sterile products: liquids (aqueous or non-aqueous), topical use. Zone "3" is defined as non-sterile products: solids and raw material.

	Zone «1» levels	Zone «2» levels	Zone «3» levels
Active air	**Weekly**	**Monthly**	**Biannually**
Passive air	**Weekly**	**Monthly**	**Monthly**
Product contacting surface	**Monthly**	**Monthly**	**Quarterly**
Close to product	**Monthly**	**Quarterly**	**Biannually**
Wall	**Monthly**	**Quarterly**	**Biannually**
Floor	**Monthly**	**Quarterly**	**Biannually**

Remarks:

- Passive air monitoring is in general not needed for routine purposes, especially in Zone "2" and Zone "3".

Table 5: Example of a checklist for investigation by production for deviations occurring in non-sterile manufacturing.

1. General information on the room or equipment, such as:
   a. Which process is running in the room / on the equipment?
   b. For which product is it used?
   c. Are there other comparable rooms or equipment of this type?

2. General investigation by production:
   a. Interview with operator: Were there some anomalies/special activities on this day or during the cleaning and disinfection?
   b. Checking the logbook: Were there some anomalies on that day or the days before which could explain the deviation?
   c. Were there some other deviations during this time period? Might there be a correlation?
   d. Was the material introduced into the controlled room according to procedure?
   e. Was there an uncommon material flow?
   f. Were there any cardboard or wooden pallets in the room?
   g. Where validated standing times for aqueous intermediate solutions exceeded?

3. Sampling:
   a. Was the sampler correctly qualified?
   b. Were there some issues evident during sampling?
   c. Was the sampling performed during special activities (e.g. repair, service or cleaning)?

4. Cleaning & Disinfection:
   a. Was the cleaning/disinfection correctly performed according to the SOP? E.g. cleaning frequency, correct product and dilution, within expiry date, correct equipment, and correct storage.
   b. Could a re-contamination of the cleaned equipment happen during transport?
   c. Which quality of water was used for the cleaning? Is the water controlled for microbial quality? Where there any exceeding microbial levels of the water testing?
   d. Is there any antimicrobial treatment of the equipment (e.g. drying at high temperatures)?
   e. Was the contact time of the disinfection followed?
   f. Was the room optically clean and dry?
   g. Are there some obvious leaks (e.g. hose, silicon joints)?

5. Personnel (operators, cleaning staff, visitors):
   a. Are all concerned people correctly qualified and trained?
   b. Were the people correctly gowned (overall, shoes, hood, mouth protection, gloves)?
   c. Were the hands correctly disinfected?
   d. Was the personnel flow executed according to the procedure?
   e. Did the operator clean the equipment/room beforehand? Is she/he experienced?
   f. Were there more people in the room than usual?
   g. Were there technicians or visitors present?
   h. Was there any misbehavior of the people observed?

6. Disturbance, maintenance, technique, etc.
   a. Were there some maintenance activities performed?
   b. Were there some disturbances (e.g. temperature, humidity, pressures, HVAC, facility alarms HAVC, air lock)?
   c. Did the HVAC run correctly?
   d. Did the room (air locks, emergency door, and windows) remain airtight?
   e. Was the material flow executed according to the procedure?
   f. Where there any other microbiological deviations in the concerned area?

4. References

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5. Abbreviations

CAPA	:	Corrective Action Preventive Action
cfu	:	Colony-forming units
CASO	:	Casein Soy Bean Digest Agar
DNA	:	Deoxyribonucleic Acid
HVAC	:	Heating, Ventilation and Air Conditioning
QA	:	Quality Assurance
QC	:	Quality Control
SOP	:	Standard Operating Procedure
MALDI-TOF	:	Matrix-Assisted Laser Desorption/Ionization Time Of Flight
TAMC	:	Total Aerobic Microbial Count
n.a.	:	Not applicable

Chapter 2: Lab Investigations – Endotoxin Out of Specification (OOS)/ Out of Trend (OOT)/ Atypical Results Investigations

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Chapter 2: Lab Investigations – Endotoxin Out of Specification (OOS)/ Out of Trend (OOT)/ Atypical Results Investigations

– FOR INFORMATIONAL PURPOSES ONLY –

1. Out of Specification (OOS)/Out of Trend (OOT)/Atypical Results Investigations

Out of specification, out of trend, and atypical results all necessitate investigation. The primary objective of an OOS investigation is to determine either if an assignable cause exists or that no assignable cause can be identified. An assignable cause must be a documented and scientifically sound determination that an OOS result can be traced to an error. The depth and scope of the investigation will be dependent on the type of deviation encountered.

A clear and well-defined pre-approved documented procedure should be followed for all OOS investigations. To be meaningful, the investigation should be thorough, timely, unbiased, well-documented, and scientifically sound. The investigation should start in the laboratory, moving out to include all other relevant departments if no clear and obvious lab error is identified. The initial OOS must be considered valid unless a comprehensive investigation clearly demonstrates otherwise.

Investigations of "Out of Specification (OOS) / Out of Trend (OOT) / Atypical results" must be done in cases of:

- Batch release testing and testing of starting materials
- In-Process Control testing: if data is used for batch calculations/decisions and if in a dossier and on Certificates of Analysis
- Stability studies on marketed batches of finished products and/or active pharmaceutical ingredients, on-going/follow up stability (no stress tests)
- Previous released batch used as reference sample in an OOS investigation showing OOS or suspect results
- Batches for clinical trials

1.1 Creating the Investigation Process Parameters

If an OOS result is detected, it must be reported promptly and an immediate investigation conducted.

Description of the testing should be written, and then approved by QA prior to initiating investigational testing. The requirements of investigational testing are listed below:

The description must fully document:

- The hypothesis to test the root cause being investigated
- What samples will be tested
- The exact execution of the testing
- How the data will be evaluated

This Investigational/Hypothesis testing may continue from the re-measurement of the original preparations if stable. Investigational/Hypothesis testing may not be used to replace an original suspect analytical result. It may only be used to confirm or discount a probable cause.

1.2 OOS vs. Invalid

If no obvious lab error is uncovered, the OOS result must be reported to QA within a predetermined period of time as stated in your organization's SOP. This reporting of the OOS result to QA will then lead to a formal investigation, comprising a multi-disciplined team from all relevant departments.

Finding an OOS test result requires a laboratory investigation to assess the accuracy of the data. However, invalid tests should not be considered OOS results.

Invalid tests are those where system suitability parameters, such as those shown below, do not function as expected and therefore may affect the accuracy of the test results.

- Negative controls (positive gel or reaction time within the range of the standard curve)
- PPCs (outside 50 – 200%)
- Confirmation of label claim in gel clot
- Generation of a linear standard curve
- %CV (outside set limits if applicable)

Invalid tests should be tracked and trended to look for patterns and trends that may indicate a corrective action is required; a true OOS only exists when a valid assay has been performed and generates results that exceed the endotoxin specification.

An OOS can only be generated by a valid assay that shows the product does not meet endotoxin specifications.

Valid assay includes:

- Spike Recovery 50-200% or 2 lambda gel clot PPC is positive
- % CV within the client's specifications or according to the manufacturer's package insert
- R-Value is ≥ to the absolute value of 0.980
- Gel clot label claim is confirmed
- There are no regulatory requirements for slope or y-intercept

An invalid assay, which fails the validity criteria above, would result in a re-test from the original sample. Confirmed invalids should still be investigated, but as a lab investigation only, not an OOS. Confirmation based on a retest that proved the same result as the first test, as in Spike Recovery lying outside the 50-200% range, would require further investigation.

1.3 Performing the Laboratory Investigation

At any point in the laboratory investigation, investigational testing can be carried out in order to challenge theories relating to the cause of the sample material to fail to meet its specification. It is vital to note that any investigational tests are not considered retests and must not be used to release the product. They must only be used to test the theory of what might have caused an inaccuracy in the original testing of the product. A well-defined, justified, and pre-approved SOP or policy for investigational testing should exist that describes the procedure that must be followed for investigational testing.

In order to assist with possible investigations, analysts should not discard any original samples, sample or endotoxin dilutions, or other reagents used in an assay until the results of the assay are known and have been evaluated against product specifications. Original samples, dilutions, and reagents from an OOS assay can provide important information to assist with laboratory investigations.

If an analyst believes that an error has been made during the setup of an assay, including how the sample was prepared, how dilutions were made, aliquoting out of all solutions and reagent additions, calculations, instrument parameters, or setting, the assay should be stopped and the reason for doing so documented at the time the error is recognized and reported to a supervisor.

The intended purpose of the laboratory investigation is to determine the accuracy of the original data set. A checklist should be prepared that includes, for example, at least the following:

▪ Is the analyst trained on the method?	▪ Any previous issues with this assay?
▪ Interview analyst to assess knowledge of the correct procedure.	▪ Review of other data for other batches performed within the same analysis set.
▪ Correct test methodology followed, e.g., version number?	▪ Consideration of any other OOS results obtained on the batch of material under test.
▪ Correct sample(s) taken/tested (check labels - was it taken from correct place)?	▪ Assessment of sample validation method, e.g. lot-to-lot variability.
▪ Was sample integrity maintained, correct container, and chain of custody (was there an unusual event or problem)?	▪ Was there contamination present in other tests (or related tests) performed at the same time, including environmental controls?
▪ How were sample containers stored prior to use?	▪ Were negative and positive controls satisfactory?
▪ Correct sampling procedure followed, e.g., version number?	▪ Were the correct reagents used?
▪ Assessment of the possibility that the sample contamination has occurred during the testing/retesting procedure (e.g., sample left open to air or unattended).	▪ Any issues with environmental temperature/humidity within the area whilst the test was conducted?
▪ All equipment used in the testing is within calibration date.	▪ Were the samples stored correctly (refrigerated)?
▪ Review equipment logbooks.	▪ Were the samples held for the correct time before being tested?
▪ Appropriate standards used in the analysis?	▪ Was the reagent stored correctly before use?
▪ Standard(s) and/or control(s) performed as expected?	▪ Were the incubation conditions satisfactory?
▪ Were system suitability conditions met?	▪ Consider taking photographs or screen shots to document the samples at time of reading.
▪ Correct depyrogenated glassware and endotoxin-free accessories used?	▪ Correct specification applied?
▪ Correct volume pipette and non-filtered endotoxin-free tips used?	▪ Reagents prepared according to manufacturer's recommendations.
▪ All items were within expiry date.	▪ Examination of the raw data graphs; any anomalous or suspect data.
▪ A visual examination reveals normal or abnormal appearance.	▪ Other potentially interfering testing/activities occurring at the time of the test.
▪ Data acceptance and assay suitability criteria met.	

If the laboratory investigation is unable to rule out the OOS result, the OOS result must be considered valid. In this instance, the investigation must expand to include manufacturing and any other relevant departments.

When the obvious error theory is discarded then investigational testing could be applied to search for the root cause

If no assignable cause that could explain the results can be identified during the manufacturing investigation or the assay failure investigation, **retesting** may be considered. Part of the investigation may involve retesting a portion of the original sample.

1.4 Conclusion

- If no laboratory or calculation errors are identified in the laboratory investigations, there is no scientific basis for invalidating initial OOS results in favour of passing retest results. All test results, both passing and suspect, should be reported in all QC documents and all data has to be considered in batch release decisions.
- If the investigation determines that the initial sampling method was inherently inadequate, a new accurate sampling method must be developed, documented, and reviewed and approved by the Quality Assurance responsible for release. A consideration should be given to other lots sampled by the same method.
- An initial OOS result does not necessarily mean the subject batch fails and must be rejected. The OOS result should be investigated, and the findings of the investigation, including retest results, should be interpreted to evaluate the batch and reach a decision regarding release or rejection which should be fully documented.
- In those cases where the investigation indicates an OOS result is caused by a factor affecting the batch quality (i.e., an OOS result is confirmed), the result should be used in evaluating the quality of the batch or lot. A confirmed OOS result indicates that the batch does not meet established standards or specifications and should result in the batch's rejection and proper disposition. Other lots should be reviewed to assess impact.
- For inconclusive investigations – in cases where an investigation:

    1. does not reveal a cause for the OOS test result and
    2. does not confirm the OOS result

The OOS result should be given full consideration (most probable cause determined) in the batch or lot disposition decision by the certifying Qualified Person (QP) and the potential for a batch specific variation also needs considering.

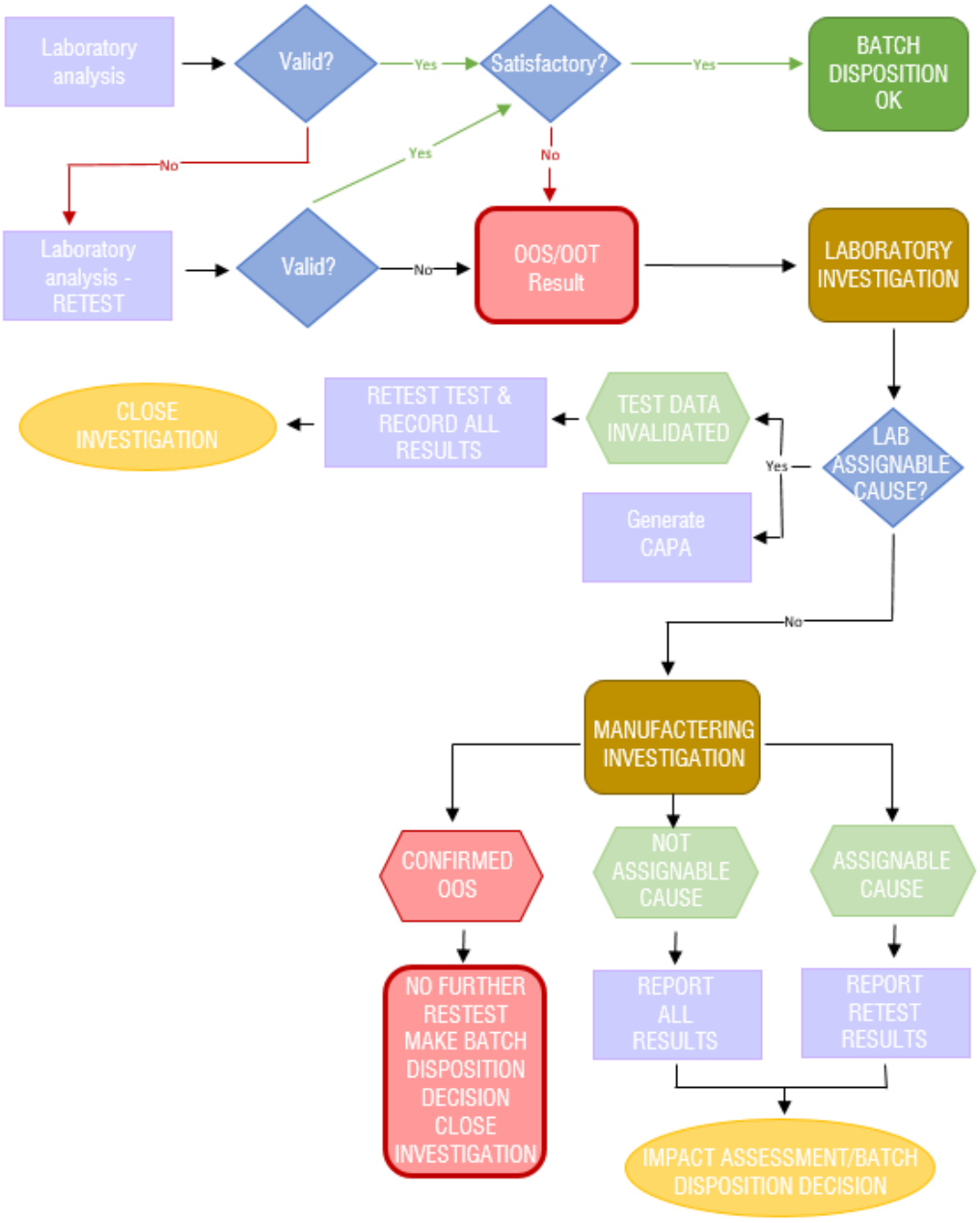
- Any decision to release a batch, despite an initial OOS result that has not been invalidated, should come only after a full investigation has shown that the OOS result does not reflect the quality of the batch. In making such a decision, Quality Assurance/Qualified Person should always follow the path of caution.

APPENDICES

Appendix 1

OOS Investigation Flow Chart

Below is an example OOS investigation flow chart. Charts like this, when included in investigational procedures, provide a useful tool for assisting in investigations and decision making.



Appendix 2

Organizational Tools for Conducting Investigations

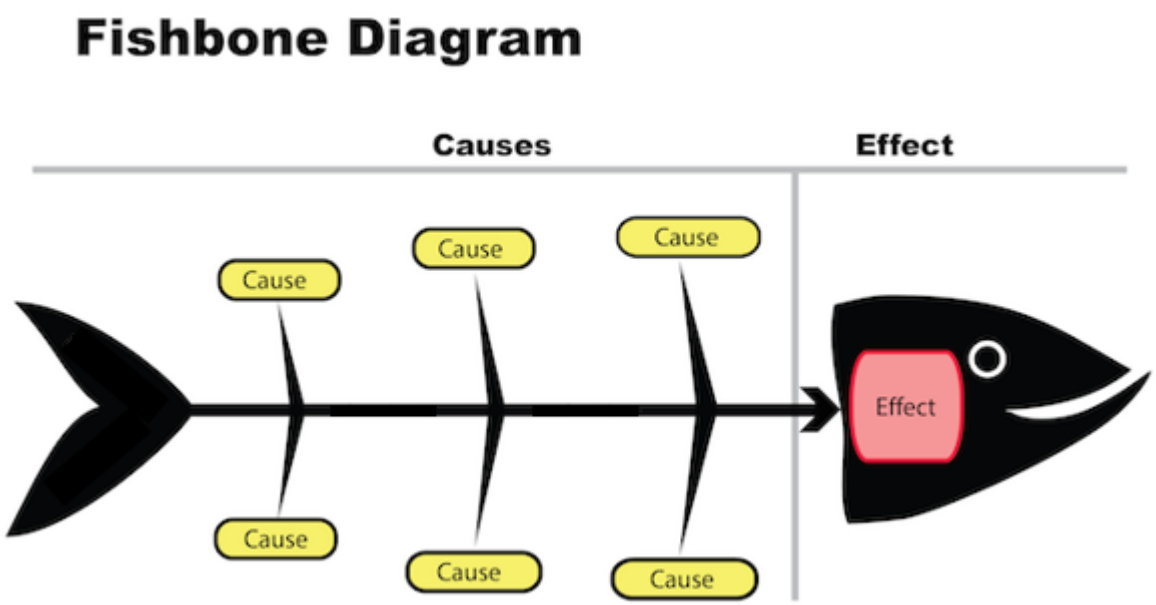
DMAIC

One way we can organize investigations is by using tools such as a Six Sigma principal called DMAIC all the way through the investigation lifecycle. DMAIC is an acronym that stands for Define, Measure, Analyze, Improve, and Control. DMAIC provides an organized way to tell a story, define the problem, brainstorm, and drive to root cause using data and facts. The methodology is very well defined and has been utilized for this purpose in many industries including the pharmaceutical and biotechnology industries. It is a well utilized tool which auditors are familiar with.

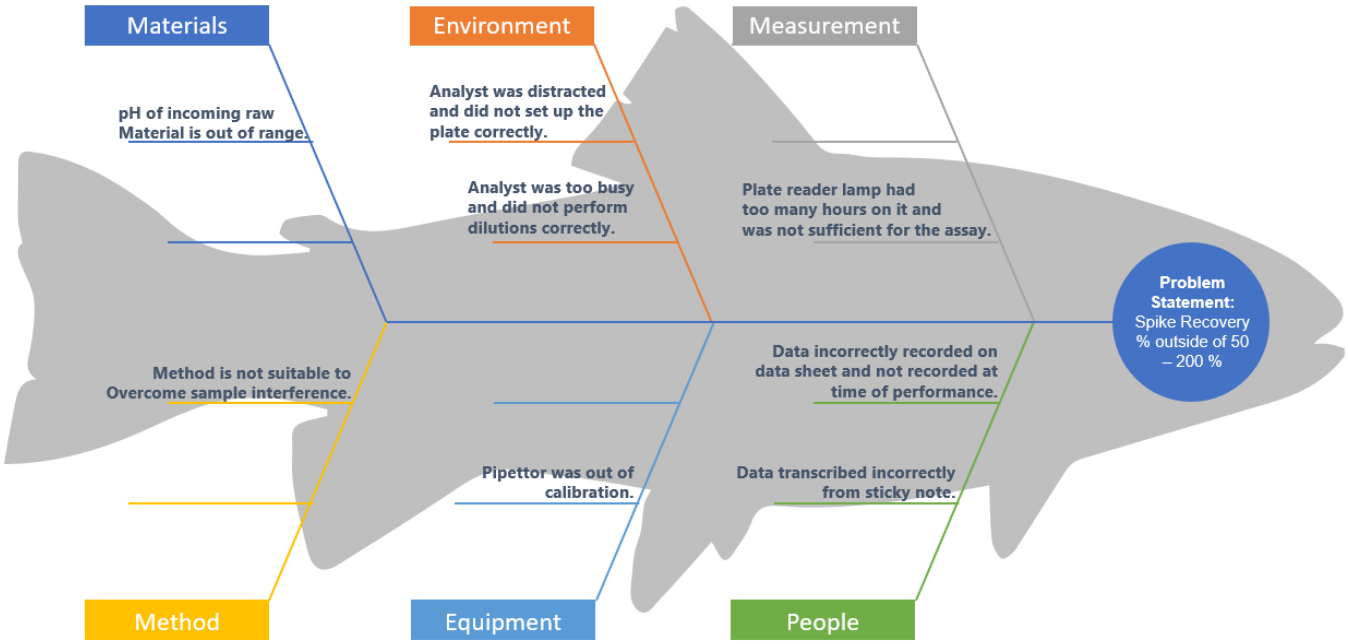
- **Define** – Answer the following questions about the event or the problem: what, when, where, and who was involved? Keep in mind that when attempting to define the problem to be solved, make sure detailed relevant information is included that helps determine the scope and impact of the investigation.
- **Measure** – Make sure to look at historical data in the context of the problem. Ensure the data is relevant and representative of the problem and the current process. Also ensure that the data is visualized in a chart or graph format. Common practices here are to utilize time-series plots, bar graphs, and control charts.
- **Analyze** – This part of the investigation is all about brainstorming potential root causes and drilling down to the true root cause of the problem. Common tools utilized by many organizations here include the fishbone diagram and asking the "5 whys". It's important to remember that any potential root cause needs to be either confirmed or disproved with data and sound scientific theory.
- **Improve** – Once the true root cause of the investigation is determined, it's time to make improvements to the process. Improvements to any process in a regulated environment are usually captured and recorded with a Corrective and Preventative Action (CAPA).
- **Control** – Once the process and the problem are fixed, it's important for organizations to use data to prove to all stakeholders that the process remains under control into the future. When making improvements, also consider the human element in your processes, in terms of reducing the risk for human error.

The Fishbone Diagram

The fishbone diagram, also commonly called the cause and effect diagram, is a visual tool that aids investigative teams in brainstorming potential root causes to a variety of different problems. Common categories that form the "backbone" and "spine" of the fish include equipment, process, people, materials, measurement, and environment. These categories can be changed depending on the process and the problem being addressed. It's important to note that exercises like completing a fishbone diagram are best accomplished as part of an investigation or brainstorming team.

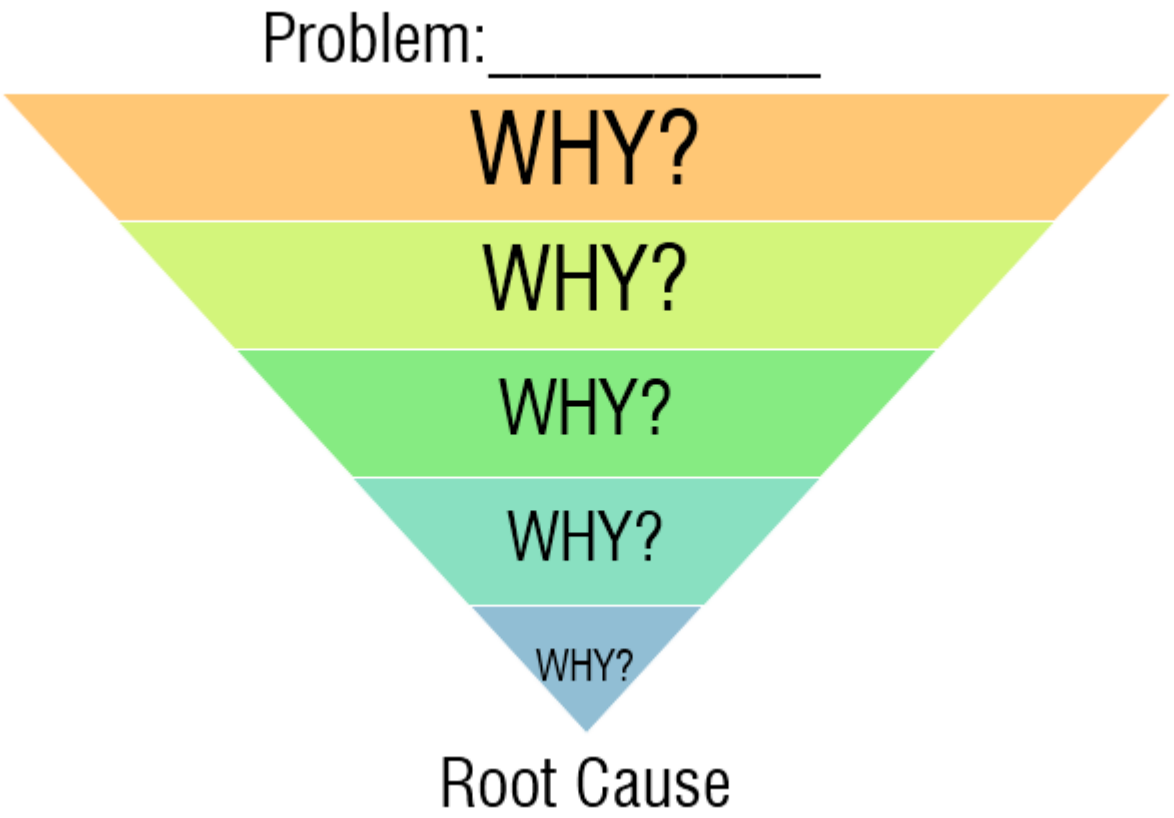


Example Fishbone Diagram



The "5 Whys" Tool:

Once potential root causes have been identified, it's important that investigators attempt to drive to the true root cause of the problem. Determining the true root cause ensures that any improvement to the process is fixed so that it does not reoccur in the future. One commonly used tool to determine the true root cause is asking why, about the potential root cause, until that why cannot be answered any longer; this method is called the 5 Whys technique. Each time why is answered it should be supported or refuted with data and sound science along the way. This tool is also best utilized with the entire investigative team.



Appendix 3

OOS Check List

The following is an example OOS check list, which could be used to assist with an investigation.

OOS Check list -*EXAMPLE*-			
Laboratory, Materials, Reagents, Personnel and Equipment			
QUESTIONS	**ANSWER**		**COMMENTS**
Is the analyst trained on the method?	☐ **YES**	☐ **NO**	
Was correct test methodology followed, e.g., version number?	☐ **YES**	☐ **NO**	
Were all correct reagents used and within expiration date?	☐ **YES**	☐ **NO**	
Were all reagents prepared according to manufacturer's recommendations?	☐ **YES**	☐ **NO**	
Were all reagents stored properly?	☐ **YES**	☐ **NO**	
Were all accessories depyrogenated or released from quarantine?	☐ **YES**	☐ **NO**	
Does all equipment meet calibration/qualification requirements?	☐ **YES**	☐ **NO**	
Was all equipment (reader/heat blocks) at proper temperature?	☐ **YES**	☐ **NO**	
Were problems seen in other assays performed the same day by the analyst?	☐ **YES**	☐ **NO**	
Has equipment logbook been reviewed?	☐ **YES**	☐ **NO**	
Were correct pipettes and tips used?	☐ **YES**	☐ **NO**	
Was correct depyrogenated glassware and endotoxin-free accessories used?	☐ **YES**	☐ **NO**	
Were any environmental issues or other activities noted in area analysis was performed?	☐ **YES**	☐ **NO**	
Was reader/heat block/water bath at required temperature before analysis was started?	☐ **YES**	☐ **NO**	

Was reader/heat block/water bath at required temperature at the end of analysis?	☐ **YES**	☐ **NO**	
Has raw data (data graphs) been reviewed for any anomalous or suspect plots?	☐ **YES**	☐ **NO**	
For Standard Curve or Negative Water Controls (NWC) Invalidity			
QUESTIONS	**ANSWER**		**COMMENTS**
Were correct standard and CoA used in analysis?	☐ **YES**	☐ **NO**	
Were standard curve dilutions correctly prepared?	☐ **YES**	☐ **NO**	
Was the CSE rehydrated properly?	☐ **YES**	☐ **NO**	
Do the test tubes/wells have the proper volume?	☐ **YES**	☐ **NO**	
For kinetic, compare curve onset times to previously run curve.	☐ **SIMILAR**	☐ **NOT**	
Were all negative controls satisfactory?	☐ **YES**	☐ **NO**	
Were all positive controls satisfactory?	☐ **YES**	☐ **NO**	
For CV, invalidity on standard curve point or NWC (where only one replicate is in the range of the curve):	☐ **YES**	☐ **NO**	
Check tubes/wells for visible contamination/particulate matter/missing reagent/bubbles.	☐ **YES**	☐ **NO**	
For kinetic standard curve point CV invalidity, compare curve onset times to previously run curve.	☐ **SIMILAR**	☐ **NOT**	
For NWC contamination:	☐ **YES**	☐ **NO**	
For kinetic, did all NWC replicates have similar reaction times?	☐ **YES**	☐ **NO**	
For gel clot, were all NWC replicates positive?	☐ **YES**	☐ **NO**	
Test the water used that showed contamination. Is endotoxin present?	☐ **YES**	☐ **NO**	
For Product Invalidity			
	ANSWER		**COMMENTS**

Were correct samples taken/tested?	☐ **YES**	☐ **NO**	
Was sample integrity maintained?	☐ **YES**	☐ **NO**	
Were samples stored correctly prior to testing?	☐ **YES**	☐ **NO**	
Were samples tested within validated storage time limits	☐ **YES**	☐ **NO**	
Were samples prepared according to validated procedures?	☐ **YES**	☐ **NO**	
Could samples have been contaminated prior to or during testing, e.g., were they left open or unattended?	☐ **YES**	☐ **NO**	
Has data for other analysis for the same batch shown any issues?	☐ **YES**	☐ **NO**	
Have other batches of the same product shown any issues?	☐ **YES**	☐ **NO**	
Has product validation data been reviewed for variability?	☐ **YES**	☐ **NO**	
For Invalid CV:			
Check tubes/wells for visible contamination/particulate matter/missing reagent/bubbles.	☐ **OK**	☐ **NOT**	
For Invalid Spike Recovery			
Check % CV:	☐ **OK**	☐ **NOT**	
Check product dilution:	☐ **OK**	☐ **NOT**	
Check that the correct standard was used for spiking.	☐ **OK**	☐ **NOT**	
Compare PPC reaction time to the other reaction times on the plate and to the standard curve point used for spiking.	☐ **OK**	☐ **NOT**	
For Product Failure			
QUESTIONS	**ANSWER**		**COMMENTS**

Were samples prepared according to validated procedures?	☐ **YES**	☐ **NO**	
Test the LRW or sample diluent for contamination and attach results.			

Appendix 4

Glossary of Terms

Out of Specification (OOS) result: An OOS result is any test result that does not meet the product's pre-defined specification. The product's pre-defined acceptance criteria could be from a filed application, drug master file, approved marketing submission, official compendia or the manufacturer's internal acceptance criteria, such as alert or action limits depending on company procedure. This would include not only final product test results, but also raw material and in-process test results.

Out of Trend (OOT) result: An OOT result is any test result that does not follow the expected trend, either in comparison with other batches or with respect to previous results collected. However, the trends of starting materials and in-process samples may also yield out of trend data. The result is not necessarily OOS but it does not look like a typical data point.

Atypical/Aberrant/Anomalous/Out of Expectation result: Results that are still within specification but are unexpected, questionable, irregular, deviant, or abnormal. Examples include borderline linearity or negative controls, higher or lower spike recoveries.

Incorrect instrument parameters: For example, setting the reader at the wrong wavelength or optical density, analyst and supervisor document the event, annotate "incorrect instrument parameter"; analysis to be repeated" on all associated analytical documentation.

Specification: A specification is defined as an acceptance criterion which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product. or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. "Conformance to specification" means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval**.**

Regulatory approved specification: Specifications for release testing. If no release specifications have been established, then the internal specification becomes the release specification.

Acceptance criteria: Numerical limits, ranges, or other suitable measures for acceptance of the results of analytical procedures which the drug substance or drug product or materials at other stages of their manufacture should meet.

Internal specification: Are action and alert limits within regulatory specifications.

Assignable cause: An identified reason for obtaining an OOS or aberrant/anomalous result.

No assignable cause: When no reason could be identified.

Invalidated test: A test is considered invalid when the investigation has determined the assignable cause (not to be confused with an invalid test due to invalid suitability criteria).

Reportable result: Is the final analytical result. This result is appropriately defined in the written approved test method and derived from one full execution of that method, starting from the original sample.

Investigative/hypothesis testing: Testing performed to help confirm or discount a possible root cause i.e., what might have happened that can be tested; for example, it may include further testing regarding sample dilution, vortexing/extraction, and potential equipment failures, etc. Multiple hypotheses can be explored.

Most probable cause: Scientifically justified determination that the result appears to be erroneous.

Retest: Performing the test over again using material from the original sample or composite (pooled sample), if it has not been compromised and/or is still available. If not, a new sample will be used.

Retesting:

- Performed on the original sample, not a different sample.
- Can be a second aliquot from the same sample that was the source of the original failure.
- If insufficient quantity of the original sample remains to perform all further testing, then the procedure for obtaining a resample must be discussed and agreed by QA. The process of obtaining the resample should be recorded within the laboratory investigation.
- The decision to retest should be based on sound scientific judgement. The test plan must be approved before retesting occurs.
- The maximum number of retests allowed should be documented within the procedure and be based upon scientifically sound principles.
- The retests should be performed by a different analyst where possible. The second analyst should be at least as experienced and qualified in the method as the original analyst.

Re-sample: A new sample from the original container, where possible, required in the event of insufficient material remaining from original sample or composite or proven issue with original sample integrity.

Re-sampling:

- If insufficient quantity of the original sample remains to perform all further testing, then the procedure for obtaining a resample must be discussed and agreed by QA. The process of obtaining the resample should be recorded within the laboratory investigation.
- Re-sampling should be performed by the same qualified methods that were used for the initial sample. However, if the investigation determines that the initial sampling method was in error, a new accurate sampling method shall be developed, qualified, and documented.
- Involves collecting a new sample from the batch.
- Will occur when the original sample was not truly representative of the batch or there was a documented/traceable lab error in its preparation.
- Evidence indicates that the sample is compromised or invalid.
- Sound scientific justification must be employed if re-sampling is to occur.

Lab investigation: Endeavours to determine root cause and assign a suitable Corrective and Preventative Action (CAPA).

References:

MHRA Presentation; Out of Specification & Out of Trend Investigations, October 2017

USP Chapter <1085>

FDA Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results For Pharmaceutical Production, October 2006

FDA Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers, June 2012

Chapter 3: Guidance for Sterility Test Failures

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Version

Version 1.0 -

Technical Review

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Chapter 3: Guidance for Sterility Test Failures

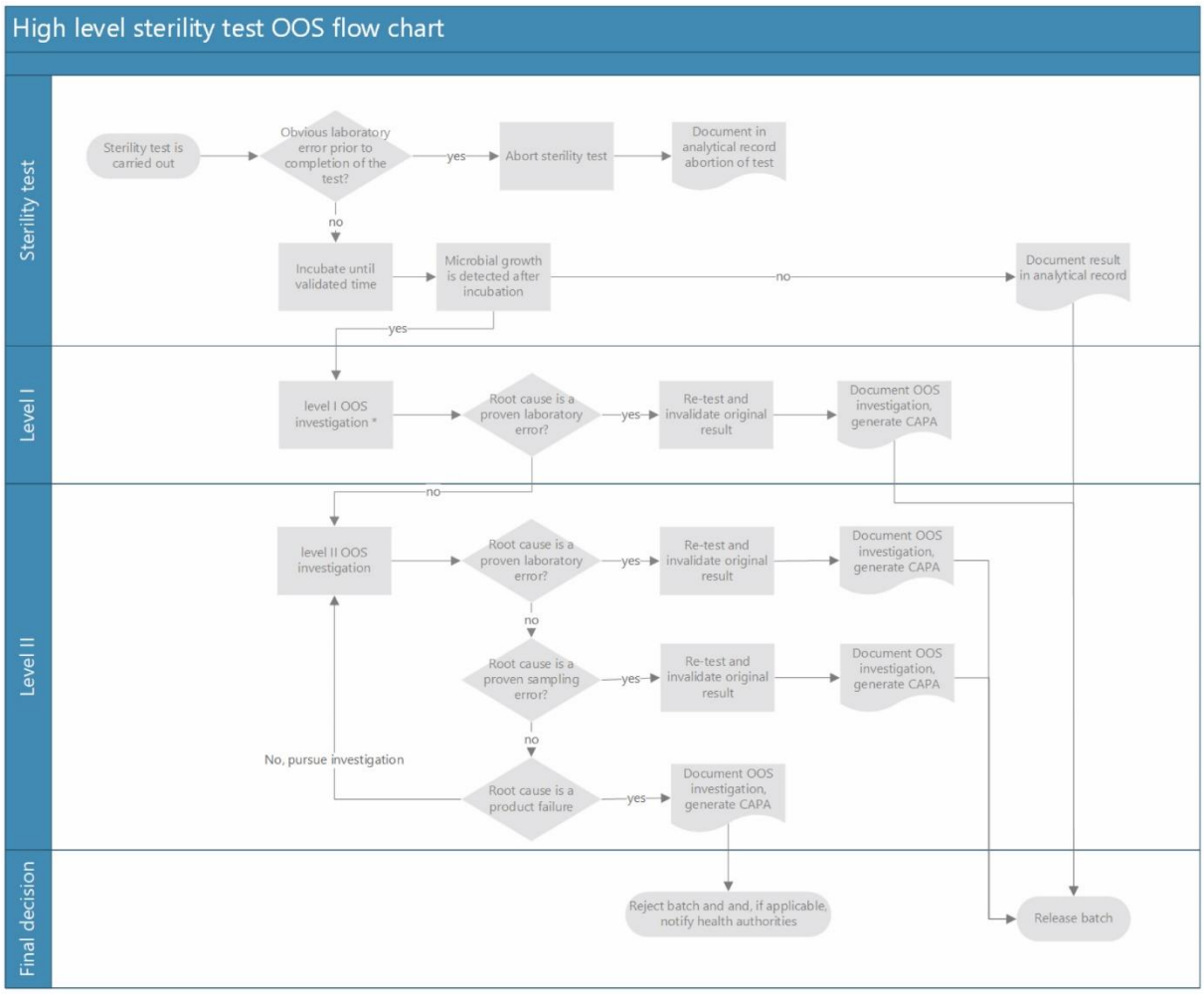
1. Purpose and scope

The sterility test is part of the quality control for products, substances or materials that require to be "sterile", hence that comply to the sterility test. Taking into consideration the statistical and methodological limitations of the sterility test, detection of microorganisms in a sterility test is normally a rare event. However when microbial growth detected in a sterility test the item tested must be considered "non-sterile" and an immediate intention and full dedication to the investigation must be provided. Considering the route of administration, products declared as sterile, should be investigated thoroughly and holistically.

This document provides a guide for the handling of activities following a sterility test failure of products that require absence of microbial growth in the sterility test.

2. Procedures

2.1 General procedure



* For sterility test failures in products, it is common industry practice that a level II investigation is be immediately opened in parallel to the level I investigation

Sterility test failures are conventionally considered as out of specification (OOS) deviations. These excursions require a full investigation, root cause assessment and corrective or preventive actions to resolve the deviation, ensure decision to release the product or not is based on scientific evidence and prevent future re-occurrence.

OOS deviations are typically performed in a 2-step approach:

- Level I investigation which is composed of a laboratory investigation to determine if an analytical error or contamination of the sample occurred during the laboratory testing
- Level II investigation is a full scale investigation and investigates the whole sampling, further laboratory investigation and manufacturing process of the product in question.

The responsibility of a contract testing laboratory in performing OOS deviations is equivalent to that of a manufacturing firm. Contract laboratories should share the finding, relevant data, and support documentation to the manufacturing firm's quality control/assurance unit, who should then initiate the full-scale OOS investigation if a laboratory error is not confirmed.

2.2 Investigation Timelines

The investigation of a sterility test failure must initiated as soon as possible and kept under a reasonable timeframe in order to examine the conditions as close as possible to those during which the failed batch was manufactured, to remain focused with a core investigation team and for patient safety and business reasons. The product batch that failed the sterility test must be put under quarantine, and an initial assessment on the potential extension of contamination (e.g. same manufacturing) carried out and documented.

The FDA Pharmaceutical Microbiology Manual, 2014 actually suggests that sterility test failures are reported to the manager and investigated without delay.

Due to its criticality, a product sterility test OOS is escalated immediately to the qualified person in charge of the market release, local QA head and to the global QA functions in larger companies. If required, further experts have to be involved and/or notified (e.g. regulatory, supply chain, etc.) since market supply may be impacted and the potential batches affected must be clearly allocated.

Table 1 suggests investigation timelines based on the common practice of many pharmaceutical companies and which are generally accepted by health authorities.

Table 1 Recommended timelines to perform sterility test failure investigations.

Phase	Timeline
Initiate investigation	Without delay (e.g. within 1 working day)
Level I investigation	7 calendar days if no hypothesis or re-test is performed May be extended as per minimum time necessary for a hypothesis or re-test.
Level II investigation	45 calendar days

Actually, for sterility test failures in products, it is common industry practice that a level II investigation is be immediately opened in parallel to the level I investigation as soon as the failed sterility test result

is read and documented by the analyst. This enables to gain time in starting the root cause investigation for this critical quality attribute.

When the OOS investigation cannot be completed within the predefined timelines in case of complex cases, an *interim report* may be established, which would include the following information:

- the current status of the investigation with the actions taken to date;
- the impact of extending the due date and if, applicable, any additional interim controls required;
- the justification / rationale for extending the investigation and for how long it will be extended (new due date specified);

2.3 Obvious laboratory failure prior to completion of the test

This event would not be considered an OOS results since no valid result has occurred as the incubation did not take place. If during the sterility test session there is an analytical deviation (e.g. test samples fallen down & broken, isolator glove not integer during the sterility test session, wrong culture medium used), the sterility testing session or the respective sterility test performance must be aborted and an analytical deviation opened. In this case an analytical deviation must be opened and the sterility test is regarded as invalid and need to be repeated with the same sample size. It is not recommended to incubate sterility tests as for instance "for information only" if there is a breach in the asepsis practice during the sterility test session (e.g. not integer isolator glove). If the results of this "for information only" sterility test shows microbial growth then an OOS with verification of the product manufacturing conditions would anyways have to take place as there is not necessarily proof that the failed test is due to the issue during testing (especially if the negative control remains without growth).

2.4 Level 1 Laboratory Investigation

The level I investigation is carried out to determine if an analytical error or contamination of the sample occurred during laboratory testing. It is mainly composed of a microbial identification and laboratory checklist to verify if an obvious laboratory error occurred. In addition, supportive microbiological testing may be applicable) in support of the level I investigation into an obvious laboratory error. They may consist for instance of:

- Investigating environmental monitoring of the testing environment including historical data (e.g. isolator and surrounding area)
- Further investigation testing of original sample (if remaining material is available and was manipulated aseptically). It must be clearly stated that the results of this investigation test would not replace the original deviating sterility test result.
- Verification of sterilization/decontamination/disinfection procedures with the isolated microorganism from the sterility test

2.4.1 Microbial identification

The first milestone of a microbial investigation is to perform an adequate isolation and identification of the sterility test contaminants and the environmental contaminants of the sterility laboratory during the performance of the sterility test. There are multiple identification systems on the market that may fit these purposes (table 2) but the genotypic identification is commonly used microbial identification in case of sterility test failures.

Table 2: Examples of identification methods used for sterility test failures

Phenotypic fatty acid profiling	Genotypic sequencing	Proteotypic MALDI-TOF
Low cost, low throughput time	High cost, medium throughput time	Low cost, high throughput time

Discrimination to species level only	Discrimination also to strain level depending on the test method used (e.g. ribotyping, whole genome sequencing)	Discrimination also to strain level for some species
Not valid for mould ID		Not ideal for mould ID

If the company or QC lab does not have the appropriate tools, the isolates may be sent to a contract laboratory with specialized expertise in microbial identification.

In addition, strain typing methods may be of advantage especially if the investigation must define a true root cause to invalidate the original result. The strain typing method may be used to compare the original sterility test isolate with an isolate from a potential source of contamination.

The PIC/S 2007 guideline actually specifies that molecular typing technique or other techniques similar to those used for epidemiological studies should be used if it should be demonstrated that isolates from the material or environment are the same as the ones detected in the product. The FDA Guidance for Industry on aseptic processing (FDA, 2014) also requires sterility test isolates should be identified to the species level using advanced identification methods such as nucleic-acid based technology.

2.4.2 Laboratory checklist

The following list contains essential points requiring verification and whose failure might have impacted directly or indirectly the test session of the failed sterility test. This list is not exhaustive.

Sampling
Was the correct sample container taken? Is a sample mix-up suspected?
Was the sample container visibly damaged, non-integer?
Is the sample labelling correct?
Does the sample volume correspond to the one defined?
Were the samples stored and transported under suitable conditions (e.g. temperature, light exposure) in the laboratory?
Where applicable, were the hold times from sampling to testing within defined timelines?
Testing environment
Was the laboratory isolator/ biosafety cabinet, qualified calibrated, and maintained in accordance to the validated procedure?
Was there a loss of integrity of the isolator during the test period of concern?
Were physical parameters of isolator/biosafety cabinet/surrounding clean room area compliant? (e.g. particles, overpressure, humidity, air flow)
Where the isolator gloves integer at the time of testing?
Where the analysts gloves integer at the time of testing in case of testing in biosafety cabinets?
Has the routine verification of isolator glove integrity program been followed?
Was the validated H_2O_2 decontamination cycle used in the isolator?
Was the concentration and expiry date of H_2O_2 compliant?
Was there a different loading pattern in the isolator that might not be covered with the patterns used in the decontamination validation runs?
Was the validated disinfection procedure used in the biosafety cabinet?
Could there have been non-exposed/hidden surfaces due to incorrect loading of the isolator?
Has the surrounding clean room disinfection procedure been followed (validated disinfectant, concentration, expiry date, method applied)?
Has the HVAC system functioned according to the qualified status?
Where environmental controls during sterility test session compliant (active air, settling plates, surfaces, personnel (if conventional clean room is used)?

Were the environmental controls of the isolator surrounding area and incubators compliant?
Testing equipment
Was the autoclave/dry heat sterilizer/material locks used for sterilizing/decontaminating equipment compliant (e.g. loading pattern, temperature, time, and program)?
Was the testing equipment used sterilized, integer and maintained in aseptic conditions (e.g. glassware, test tubes, forceps, micropipettes)?
If applied, were the materials/equipment prior to decontamination/disinfection adequately disinfected to reduce the bioload prior to decontamination?
Was the qualification/calibration status of the testing equipment compliant?
By electronic systems was there an anomaly observed in the audit trails?
Was there a data integrity concern?
Was the incubator used in a clean and qualified status?
Were the incubation conditions compliant (e.g. temperatures, times)?
Was the filtration equipment clean and functioning correctly (e.g. no backdraft through pipe system, qualification status of filtration equipment, if applicable)?
Was the sterilization of the sterility test filter units confirmed (supplier CoA or internal procedure)?
Have all testing equipment used under the isolator been part of the H_2O_2 decontamination validation?
If an alternative rapid or automated microbiological test system was used, did the customized system function as intended?
Dilution solutions and nutrient media
Were the dilution solutions and nutrient media used sterile?
Were the dilution solutions and nutrient media used within the validated expiry dates?
Were the dilution solutions and nutrient media stored under controlled conditions? (time, temperature, light exposure)?
Was any deficiency (e.g. failed autoclave cycle) observed during the dilution solution and culture media preparation
Were the containers in which the nutrient media and dilution solutions are stored integer?
Were the culture media incoming tests (e.g. growth promotion test, pH, absence of microbial contamination) compliant?
Where applicable, was the certificate of analysis of the supplier compliant?
Were positive control or growth promotion test results compliant?
Were negative control results of the testing session and further test session compliant?
Was the nutrient media container containing the product integer (e.g. leakage has been observed)?
Analytical test procedure
Was the correct testing procedure/SOP used and the testing performed as per the suitable test method?
Were the suitable dilution solutions and nutrient media used?
Were the suitable number of rinsing steps followed?
If a rapid microbiological method was used as alternative to the compendial sterility test, the method's specificities should be investigated, if necessary, with support of the system's supplier
Was there a data integrity concern?
Analyst
Was the training status of personnel performing the preparation, testing and reading out results of samples compliant (e.g. current SOP/testing procedure, re-/qualification)?
Is the analyst who performed the testing experienced?
Has a microbial contamination already been observed with the analyst that carried out the test?
Were any other anomalies noticed/mentioned during analyst interview (e.g. during sampling, during incubation, during read out of samples)?
If more than one sample was taken at the same time or processed at the same time, were any anomalies detected for these?
Trends
Have any adverse trend occurred with regards to sampling, environment, equipment used, analyst)?
Were there any changes in the execution of the test since the previous non contaminated product batches tested (e.g. changed working technique/ new apparatus/ new employee)?

2.4.3 Conclusion level I investigation

If the level I investigation concludes that an obvious laboratory error has been identified with data-driven evidence, a re-test can be performed. The re-test must be carried out with exactly the same amount of units and/or sample volume as per the original test following a predefined retest plan. The original result obtained may be invalidated if the root cause is in accordance with the compendial guidances and a re-test may be carried out if sufficient material is available.

USP <71> and Ph. Eur. 2.6.1 consider the following conditions to invalidate an original sterility test result:

> " *a. The data of the microbiological monitoring of the sterility testing facility show a fault.*
>
> *b. A review of the testing procedure used during the test in question reveals a fault.*
>
> *c. Microbial growth is found in the negative controls.*
>
> *d. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure."*

As per the PIC/S September 2007 guideline, "*when conditions (a), (b) or (c) apply then the test should be aborted prior to the completion of the incubation period*" if these have been detected prior to the end of incubation.

Only in very rare cases a failed sterility test result may be invalidated due to an obvious laboratory contamination as an unequivocal scientific evidence may not be easily demonstrated. Such cases occur when cross contamination of the testing environment occurs, when aseptic laboratory practices have dramatically failed or when a crucial error occurred during the test performance (e.g. non-sterile equipment has been used).

For instance even if there is growth in the negative control, would that be sufficient to prove that no anomaly occurred during the product manufacturing? This, especially if the strain found in the negative control is different than the one found on the product sample.

If the level I investigation is not conclusive then the level II investigation must be carried out. In the level II investigation additional laboratory testing can be done and may take the form of further hypothesis testing.

2.5 Level II Full scale investigation

The level II investigation has in scope the sampling procedures and production processes carried out for the product of concern. Even if no evident laboratory error was concluded in the level I investigation, further and deeper investigation in the laboratory also can take place during the level II investigation.

Level II investigations should be performed in a holistic manner verifying all potential failures that may occur that would have led to a failed sterility test, even if there is a suspicion of a laboratory-related contamination. As long as no root cause can invalidate a suspicion of laboratory-related contamination, the scope of investigation should not only consider the product batch(es) which failed the sterility test but all batches of the same production campaign produced before and after the failed batch(es) in question and also the ones manufactured on the same production line. All these batches should remain in scope even if their sterility test complied because of the weak statistical sample size of the sterility test.

Level II investigations, especially for sterility test failures, may be quite complex to handle and require a multidisciplinary team at best with an independent facilitator coordinating and structuring the deviation.

Typically an investigation team may be composed of:

- Facilitator
- Microbiological Expert

- Manufacturing process experts
- Engineering
- Quality assurance and Quality Control representatives

Due to the complexity and high amount of information gathered it is recommended to use root cause investigation tools such as a Fishbone diagram to structure the investigation.

The following steps may be followed:

1. Problem statement.
2. Gather the facts.
3. Define the timelines.
4. Brainstorming and categorizing root cause assumptions.
5. Compare possible causes with the facts
6. Confirm root cause and define CAPAs

The FDA 2004 Aseptic guideline recommends in addition to the laboratory investigation a comprehensive evaluation of the production with focus on:

- Monitoring of production area environment with focus on both short- and long-term environmental trend analyses.
- Monitoring Personnel including personnel practices and training
- Product Presterilization Bioburden
- Production record review including special events, batch and trending data of utilities and support systems
- Manufacturing history of the product or similar product

For further details on root cause investigation with microbial deviations refer to e.g. ECA Roesti and Goverde, 2020; Goverde and Roesti 2019; Gapp 2014.

2.5.1 Checklist for a level II investigation

The following list contains essential points requiring verification and whose failure might have caused directly or indirectly microbial contamination of the item in scope of the sterility test deviation. This list is not exhaustive.

Scope
Which product is affected?
Which batches are potentially affected by the deviation (these include also batches released on the market)?
Which manufacturing lines would be potentially affected by the case?
Sampling
Was the training status of personnel performing the sampling compliant?
Was the sampling equipment used sterilized, integer and maintained in an aseptic manner?
Was the correct sampling procedure in terms of aseptic technique and gowning used and executed correctly?
Was the sampling area contaminated?
Production environment
Was the production isolator/ biosafety cabinet, qualified calibrated, and maintained in accordance to the validated procedure?
Was there a loss of integrity of the production isolator during the test period of concern?
Were physical parameters of isolator/biosafety cabinets compliant? (e.g. overpressure, humidity, air flow)
Where the isolator gloves integer at the time of testing?
Has the routine verification of isolator glove integrity program been followed?
Has the HVAC system functioned according to the qualified status? Last qualification status of HVAC should be checked.

Where environmental controls during batch/campaign manufacturing compliant (active air, settling plates, surfaces, personnel if conventional clean room is used)? Focus especially on sterile manufacturing and aseptic filling operations.
Was an anomaly observed with regards to the amount of particles required in the grade A and B areas during manufacturing?
Is there an adverse trend in microbial or particle counts in the environment of the manufacturing line?
Sterilization/decontamination processes
Where the bioburden results prior to microbial or sterile filtration compliant?
Where the sterilization filters used validated for sterile filtration?
Did all the sterile filters used in the process pass the filter integrity test?
Before isolator and transfer chamber decontamination, were the preparation steps such as cleaning and idle time compliant to the validated procedure?
Was the validated H_2O_2 decontamination cycle used in the production isolator and transfer chamber?
Was the concentration and expiry date of H_2O_2 compliant?
Was there a different loading pattern in the isolator/transfer chamber that might not be covered with the patterns used in the decontamination validation runs?
Could there have been non-exposed/hidden surfaces due to incorrect loading?
When was the last successful isolator or transfer chamber revalidation?
Was the validated disinfection procedure used in the biosafety cabinet and/or surrounding cleanroom (validated disinfectant, concentration, expiry date, method applied)?
Were the disinfectants used in the grade A/B area sterilized prior to use and where they maintained in a manner to prevent contamination?
Where the disinfectants used validated for antimicrobial efficacy against the surfaces to which they were applied?
For terminal sterilized products, check of the terminal sterilization cycle should be made (e.g. verifying of the autoclave records, qualification status, if loading patterns comply)
Was there an anomaly in the cleaning and sterilization of equipment and materials used for the aseptic filling?
When applicable, were the washing and sterilization/depyrogenation processes of primary packaging compliant to the validated procedure?
If primary packaging are supplied sterilized by the supplier without further treatment (e.g. stoppers), were the aseptic manipulations related to their utilization in the manufacturing line followed?
Has the primary packaging declared as "sterile" passed the sterility test?
Aseptic processes
Review of production records such as batch record for anomalies, non-usual events, interventions maintenance or construction activities during the whole batch filling process and including the primary packaging components used
The last relevant media fill must be checked to verify if there was an anomaly
Have interventions taken place that were not included in the media fill program?
Were the personnel involved in the aseptic processes qualified?
Have interventions taken place for which the operators were not qualified?
If applicable, did the quality oversight observe an irregularity in the aseptic processing of operators?
Did operator interview provide a lead to potential miss-handling/non-aseptic working practice or an unusual event that was not captured in the validated process?
Was the personnel monitoring of operators involved in the aseptic filling and sterile filtration compliant?
What was the experience of the operators that executed the aseptic processes and what was the last contamination related to these operators?
Utilities, product drug substance and excipients
Where utilities (e.g. WFI, sterile-filtered gas) used microbiologically conform?
Is there an adverse trend in the utilities microbiological monitoring?
Were the filters of the sterile filtered-gases integer?
Other points to consider

For cell therapy products from which cells were originally sourced from patients, was the collection center investigated?
Were there any recent changes in filling environment or sterile filtration environment (e.g. new working technique, new operators, new equipment)?
Are endotoxin results compliant?
Is there an adverse trend in terms of microbial contamination /media fill failure with the product and manufacturing line in scope?
If additional analysis have been carried out for investigation purposes, what are the results? These may include for instance an additional media fill or enhanced environmental monitoring
Trends
Product or similar product
Manufacturing line or site

2.5.2 Root cause assessment and Corrective and preventive actions (CAPA)

As described in Roesti and Goverde, 2020, "The root cause is defined as the fundamental reason or cause for the excursion. Contributing factors may be associated to the root cause. Contributing factors consist of conditions that would affect the likelihood, the intensity or severity of the excursion. Eliminating a contributing factor would not eliminate the excursion."

As a root cause is identified actions have to be carried out to correct or eliminate the anomaly (corrective action) or to prevent its re-occurrence and reduce the probability that an excursion occurs again (preventive action).

The effectiveness of CAPAs may also be demonstrated with an improvement in microbiological trend or additional verification aseptic process simulations/media fills. For aseptic filling lines, in case of sterility test failures due to product contamination, it is recommended to revalidate the line with media fills prior to re-start of production.

Table 3 Examples of root causes, contributing factors and CAPAs

Root cause of the sterility test deviation	**Contributing factor**	**CAPAs**
Sample bottle was uptaken by personnel that did not wear gloves during transportation from sampling site to the laboratory.	Microorganisms embedded in sebum are harder to kill	CA: Revise procedures to limit the manipulation of the sterility test samples such as use of a closed transport box and ensure the right gowning is used by all personnel involved (gloves)
Contamination during blood collection for cell therapy products	Less experienced staff performed the collection	CA: Blood collection aseptic protocol in hospitals tightened. PA: Only experienced personnel to collect blood from patients
Bioload in the isolator was too high prior to H_2O_2 decontamination due to unsatisfactory cleaning and disinfection	H_2O_2 decontamination not efficient to reduce such a high bioload	CA: Revise cleaning and disinfection procedure in the isolator prior to H_2O_2 decontamination. PA: Revise and re-qualify the isolator H_2O_2 decontamination procedure.
Isolator gloves were not integer (re-occurence)	Forerarm of analyst was not completely covered by personnel gloves	CA: Replace defect isolator gloves PA: Introduce a tighter isolator glove qualification program and monitoring of integrity

		PA: Analysts to wear longer gloves over hand and forearm
Contamination during compounding in Pharmacy by mixed-up utilisation of non-sterilized primary packaging	Compounding aseptic procedure re-training frequency is too low Great pressure on staff due to understaffing and sudden increase in product demand	CA: Remove all non-sterilized primary packaging from the compounding area of aseptic products PA: Revise the qualification and training procedure of all staff PA: Revise the cGMP procedure and organisation in the compounding pharmacy

2.5.3 Documentation

The deviation report is reviewed and approved by QA.

The deviation report should be written by the investigation lead and should contain:

- Clear and comprehensive writing
- Classification of criticality
- Product batches in scope
- A statement evaluating if the event is a re-occurance on the product or manufacturing line
- All reportable results generated during the investigation supporting the root cause and contributing factors including trending data.
- All elements that were investigated even if they have no direct contribution to the root cause/contributing factor. This demonstrates that the investigation was performed in a holistic manner and that no potential loss of sterility assurance was overseen.
- The true root cause
  - Direct evidence demonstrates that the excursion is truly triggered by the root cause
  - Only sound scientific data are able to justify a true root cause
- The most probable root cause
  - Indirect evidence that the excursion may be triggered by the root cause
- Corrective and preventive actions (CAPAs) (description, responsibility, timeline for completion)
- Decision on batch release or not as well as other potentially affected batches (e.g. same campaign) with corresponding rationales
- Decision to stop the filling line or not with corresponding rationales
- Decision to let the analyst pursue sterility testing or not with corresponding rationales
- The actions opened for a follow-up of the effectiveness of the CAPAs

For a sterility test failure, root cause "unknown" should not be allowed as a re-occurance of the failure may happen and there is no guarantee that future production batches would always meet the sterility test acceptance criteria. In these cases, a deep investigation would need to prove a proper production and test performance and prevent a reoccurrence due to improper conditions; but nevertheless, the batch would need to be discarded.

2.6 Batch disposition and health authority notification

The product batches in scope of the deviation may be released or not depending on the outcome of the investigation. An unequivocal root cause such as a laboratory contamination not related to any default observed in the sterility assurance and asepsis status of the product would allow release on the market without having to notify health authorities. As explained above in the level I investigation, the invalidation of a failed sterility test result requires very strict criteria that are difficult to fulfill with certainty. Exceptional release of products that do not comply with the specification require health authority approval in advance (e.g. might be necessary for some life-saving or cell therapies products).

The decision to inform regulatory authorities in which the product is marketed is under the responsibility of the quality assurance department based on local regulations (see examples in table 4). As drug product sterility test failures may potentially mean a loss of sterility assurance, sterility test failures are seen as critical by health authorities and would require a notification in case other product batches already distributed might be affected or that the sterility test failure affects the market supply.

Table 4: Examples of health authority notification procedures and timelines for sterility test failures of sterile drug products

Heath Authority	Type of notification	Timeline	Example Guidelines
FDA	Field alert report (FAR), Submitted via Form FDA-3331a	For full and abbreviated new drug applications, regulations require submitting within **3 working days** a field alert report (FAR) of information concerning any failure of a distributed batch to meet any of the specifications established in an application.	FDA Guidance for Industry Sterile Drug Products Produced by Aseptic Processing — Current Good Manufacturing Practice, 2004
FDA	Biological Product Deviation Reports (BPDRs), Submitted via Form FDA-3486	Reporting time frame of 45 days from the date the event was discovered	FDA Guidance for Industry Biological Product Deviation Reporting for Licensed Manufacturers of Biological Products Other than Blood and Blood Components 2006
EMA	EMA quality defect reporting	Quality defects should be reported in a timely manner by the manufacturer to the marketing authorization holder/sponsor and all concerned Competent Authorities in cases where the quality defect may result in the recall of the product or in an abnormal restriction in the supply of the product.	Eudralex Volume 4 EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use Part 1 Chapter 8: Complaints, Quality Defects and Product Recalls

3. References

- Eudralex (2014) Volume 4 EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use Part 1 Chapter 8: Complaints, Quality Defects and Product Recalls
- FDA (2004) Guidance for Industry Sterile Drug Products Produced by Aseptic Processing — Current Good Manufacturing Practice, 2004
- FDA (2006) Guidance for Industry Biological Product Deviation Reporting for Licensed Manufacturers of Biological Products Other than Blood and Blood Components
- FDA (2006) Guidance for Industry Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production

- FDA (2014) Pharmaceutical Microbiology Manual
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- USP42-NF37 2S chapter <71> Sterility Tests

ECA Guidance Documents

This document is part of the ECA Guidance Documents series. So far the following documents have been issued:

European QP Association
- European QP Association Code of Practice for QPs – Duties, Responsibilities and Continuous Training for Qualified Persons in the EU" – Version 7.0

European GDP Association
- ECA/PQG Guidance on the Interpretation and Implementation of European Good Distribution Practice for Active Substances
- ECA/PQG Guidance on the Interpretation and Implementation of European Good Distribution Practice
- ECA Code of Practice for the Responsible Person for GDP

Validation Group
- Integrated Qualification and Validation – A guide to effective qualification based on Customer - Supplier Partnership
- ECA Good Practice Guide on Validation – Version 02

Analytical Quality Control Group
- Analytical Procedure Lifecycle Management Guideline
- ECA Standard Operating Procedure (SOP): Laboratory Data Management - Out of Specification (OOS) Results
- Laboratory Data Management Guidance: Out of Expectation (OOE) and Out of Trend (OOT) Results

Visual Inspection Group
- Good Practice Paper "Visual Inspection of Medicinal Products for Parenteral Use" – Version 3.2
- Container Closure Integrity testing of medicinal products for parenteral use – Position Paper, Version 2.0

Pharmaceutical Microbiology Group
- ECA Guidelines for the Evaluation and Investigation of Microbiological Deviations
  Chapter 1 – Deviation Handling of Microbiological Environmental Monitoring Excursions in Non-Sterile Pharmaceutical Manufacturing
  Chapter 2 – Lab Investigations – Endotoxin Out of Specification (OOS)/ Out of Trend (OOT)/ Atypical Results Investigations
- Chapter 3 – Guidance for Sterility Test Failures

Data Integrity & IT Compliance Group
- ECA Audit Checklist - PaaS Service Providers
- ECA Audit Checklist - SaaS Service Providers
- ECA SOP - Selection Process for Cloud Service Providers
- Data Integrity Guide – Version 2.0

ATMP Group
- AGORA Toolbox

Cannabis Group
ECA Roadmap – GMP Requirements and Regulatory Information on Medicinal Cannabis (and CBD Products) – Version 1