Title **Increase level of reject rates for protein particles in Immune Globulin 10% solution Final Containers**

Reference: **PR#2428796**

Document type: Final investigation report

Executive Summary:

An increased level of visible IgG particles in GAMMAGARD LIQUID/KIOVIG/HYQVIA (IgI 10% (Human)) finished products were observed during the 100% visual inspection process, on lots manufactured since September 2021 up to October 2022 (purification date). With an average of ~1.5% reject rate for protein particles at 100% visual inspection in the past year.

The particles have been identified and confirmed as being of proteinaceous nature with size from 170 µm to 2785 µm.

A cross functional team with internal and external experts has been working on this investigation since the detection of the trend to further evaluate all influencing factors and potential causes of protein particle formation, including learnings from previous events. As protein particles have been observed in lots manufactured starting from different Precipitate G (PptG) sources, on all purification PL1/PL4 and filling FL1/FL3 lines, the investigation covered the different process steps from Fractionation to 100% visual inspection of final containers, including utilities. Batch records, events, changes, process parameters, maintenance activities, calibrations, and raw materials of the IgI 10% manufacturing process were assessed for the related batches.

Starting from the known aggregation mechanisms, hypotheses for the presence of protein particles were proposed and assessed. In parallel documentation records and process parameters were reviewed to identify potential changepoints in the process. Despite the thorough investigation concerning a total of 19 hypotheses performed, with a special deep dive on four hypotheses namely, designated raw materials, final pH adjustment practices, CM chromatography and Filling parameters/sequence, no formal root cause was confirmed that could explain the increased reject rates observed since September 2021. Note that in August 2022, five IgI 10% lots produced higher reject rates, with one lot up to 14%. Based on this cluster, a focused investigation was performed to identify the specific drivers, however no contributive factors could be identified.

Robustness actions concerning the CM chromatography step, the pH adjustment practices and the duration between incubation and visual inspection were nonetheless implemented to further reduce the stress factors identified in the process liable to participate in the protein aggregation mechanism. Process robustness improvement efforts will continue to improve current knowledge regarding protein aggregation in IgG manufacturing process and reduce further protein particle occurrence at visual inspection.

Even if no clear link were established, since implementation of several actions to reduce the stress factors, from November 2022 visible protein particle reject rate levels are back within normal trend, with more than 30 consecutively produced lots with visible protein particles reject levels below the action limit and comparable to the period before the start of the issue.

Based on the 100 % visual inspection and AQL results, sub-visible particles testing, final container testing, additional characterization, pre-clinical data, and stability data, preclinical studies, health hazard assessment and pharmacovigilance review confirm that the protein particles do not affect quality, safety and efficacy of IgI 10%.

Final Investigation Report
PR#2428796
10 February 2023

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1. PURPOSE

The purpose of this report is to document the investigation and product impact performed regarding the slightly increased level of visible protein particles detected during the 100% visual inspection in Gammagard Liquid/Kiovig/HyQvia final container from September 2021 to October 2022 (purification date) and covered under deviations records PR#2318024, PR#2321997, PR#2329863, PR#2348128 and PR#2428796.

2. BACKGROUND

Gammagard Liquid/Kiovig and HyQvia are Immune Globulin Infusion 10% (Human, IgI 10%) products for intravenous and subcutaneous administration, used to treat patients with primary immunodeficiency diseases.

Human IgI 10% is obtained from human plasma and its manufacturing process is divided into fractionation (upstream) and purification (downstream) processes. The plasma used can come from different sources depending on the type of extraction process and the geographical origin. Source plasma and recovered plasma refer to the plasma obtained respectively directly from plasmapheresis or obtained by recovering the plasma from whole blood donation. The geographical origin of the plasma is also of importance as some countries only accept products made from plasma obtained in certain areas. Takeda Lessines only processes plasma from US or EU origins. The fractionation of human blood plasma is performed at Los Angeles, Rieti and Vienna Takeda's plants and at Amsterdam Prothya's plant (CDMO) facilities to produce Precipitate G (PptG) pastes. The further processing of PptG pastes into Gammagard Liquid/Kiovig/HyQvia, i.e. the downstream purification process, is then performed by Lessines facility till the packaging of final containers.

Gammagard Liquid/Kiovig are IgI 10% products for intravenous administration while HyQvia is the subcutaneous administration product done by the combination of IgI 10% with Recombinant Human Hyaluronidase (rHuPH20).

An overview of the IgI 10% manufacturing process is presented in Figure 1. After low pH incubation (step 19), the final containers are 100% visually inspected for solution defect detection, glass defect detection, and container closure defect detection. In addition to the 100% visual inspection, an AQL sampling is performed on every batch by the Quality department.

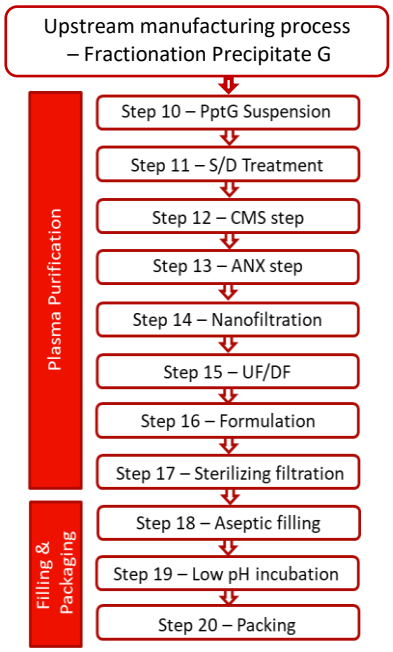


***Figure 1**. Overview of Gammagard Liquid manufacturing process in Lessines*

3. DESCRIPTION OF THE EVENT

Since the visual inspection of batch LE12X208 on 08-Oct-2021, see Figure 2, in Lessines Facility, slightly increased levels of protein particle have been observed at the visual inspection of IGI 10% final container lots following the procedure SOP-026399. At first individual deviations were opened for the first batches exceeding the action limit (see PR#2318024, PR#2321997, PR#2329863 and PR#2348128) before a general deviation was opened covering the general trend (PR#2428796).

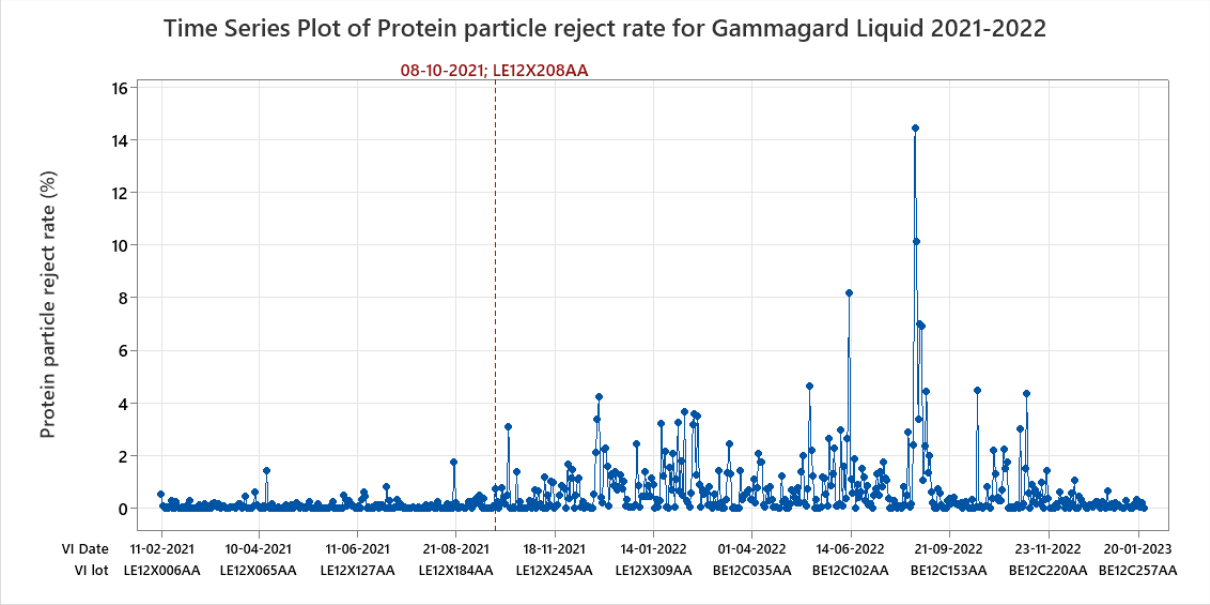


Figure 2*. Evolution of the protein particles reject rate for Gammagard Liquid/Kiovig in 2021 and 2022, sorted by visual inspection date*

In total 115 batches exceeded the action limit for protein particles observed at 100% visual inspection, as per SOP-005032. These batches will be referred as OOL batches in the next sections. The last batch related was batch BE12C219, visually inspected on 23-Nov-2022. Refer to attachment #01 for the detailed list.

4. PARTICLES IDENTIFICATION AND CHARACTERIZATION

The identification of protein particles observed in the vials was performed according to SOP "*Global SOP, Particle Identification Submission and Analysis*" (G-TAK-001530), based on visual inspection and particle identification.

Visual inspections observation performed per SOP-026399 ("Méthode d'inspection visuelle – Packing") identified particles in the vials as protein particles. The operators have been trained to categorize particles of this nature as proteinaceous as per SOP-048674 ("Formation à l'inspection visuelle au Packing").

Protein particles identifications were confirmed by Scanning Electron Microscopy (SEM) with Dispersive X-ray Spectroscopy (EDX), and FTIR analysis. These tests were performed by the Particle Laboratory in Vienna (Takeda laboratory) or the Certech contract laboratory. In both cases, particles were isolated from the solution by fishing method. The identification results confirmed the proteinaceous nature of the particles.

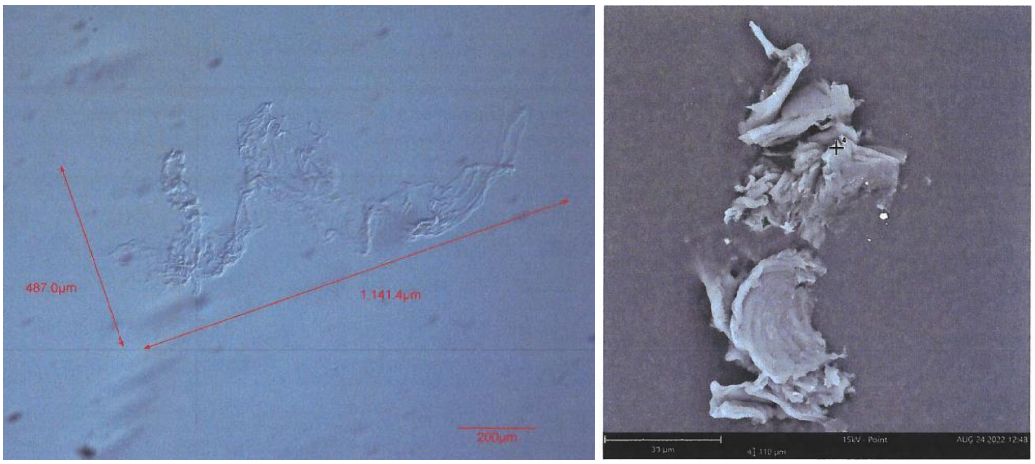


***Figure 3**. Representative microscopy pictures of a protein particle (extracted from batch BE12C150). Left: optical microscopy; Right: SEM.*

The particle sizes ranged from 170µm to 2785µm. All particle characterizations have been summarized in the memo ME-TS-21-086 (see attachment #02).

Complementary analysis using capillary electrophoresis (Caliper) was also performed on isolated particles and demonstrated that the protein composing the particles migrates like IgG. Samples tested in spin trap mode showed no protein bands. The Caliper results confirmed that the protein particles are composed of IgG.

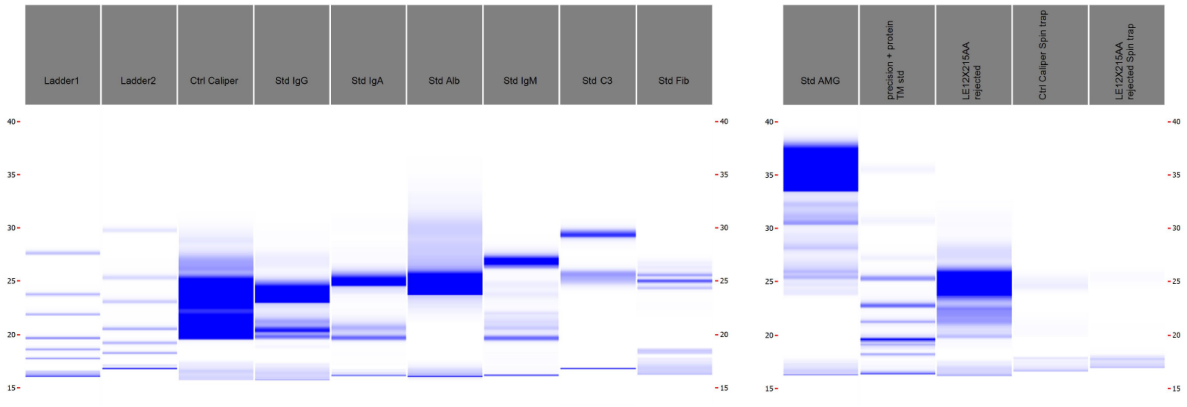


***Figure 4**. Electrophoresis profiles by Caliper – obtained on batch LE12X215*

A series of extra testing was performed on some related batches with the aim to provide more insights on the aggregates and solutions. For each of the batch subjected to the tests summarized in Table 1, solution was aliquoted from a conform and a rejected vial and compared along with a reference sample from a conform vial of a batch which did not exceed the rejection limit for protein particles. Extra testing results are available in attachment #03.

Technique	Purpose	Result
Headspace GC-QQQ	Analysis of volatile organic compounds	No difference between solution samples from conform and rejected vial as well as reference sample
MFI	Particles characterization by size (1µm - 100µm)	
DLS	Particles characterization by size (0.3nm - 10µm); complementary to MFI	
ICP-OES	Elemental analysis; indication for contaminants (e.g. leachable/extractable)	

***Table** 1. Extra testing*

5. MECHANISMS OF PROTEIN AGGREGATION

The following protein particle generation pathways have been identified as explained based on bibliography reviews and were considered during the different investigations:

- Exposure to conditions (mechanical / shear forces, thermal, chemical) during process and storage (storage time) which may result in protein morphology change and can increase the amount of protein aggregates formed.
- Surface adsorption, which can be driven by a combination of electrostatic forces, hydrophobic binding interactions, and entropy changes due to contributions from both water and protein. These surface adsorption processes may be reversible or irreversible and may lead to either complete unfolding or partial unfolding of the adsorbed protein. In specific cases protein adsorption could nucleate leading to further aggregation and particle formation.
- Agglomeration which may occur with protein coated particles or silicone oil droplets.
- Protein damage, as caused by directly reacting with leachable, potentially creating an aggregation competent protein species.
- Exposure to air/water interface (also involving mixing and agitation).

6. ROOT-CAUSE INVESTIGATION

6.1. Investigation scope and methodology

6.1.1. Retrospective review

In 2017, higher level of reject rates during the 100% visual inspection process were encountered in Lessines on Gammagard Liquid product, due to particles identified as IgG protein particles (covered by PR#1171566 and PR#1200576).

The main root cause was identified to be the raw material configuration for the glycine excipient (glycine produced by Yuki Gosei Kogyo Co. conditioned in 500kg tote big bags supplied by Greif) in conjunction with some filling parameters as contributing stress factors.

Although multiple tests on different glycine sources confirmed appropriate quality of glycine, historical investigation, including nanoparticles tracking analysis, indicated a higher level of nanoparticles for the Yuki/Greif tote compared to the other glycine configurations. Those nanoparticles were considered to serve as a nucleus point for protein aggregation.

The corrective action consisted of changing to another glycine configuration (Yuki glycine conditioned in 25kg containers, or Chattem glycine conditioned in 500kg Big Bags supplied by Dover) and adapting the filling process parameters, resulting in satisfactory outcome. Additionally, manufacturing an additional IgI 10% lot with Greif tote big bag confirmed the root cause. Nowadays, Yuki Glycine is again used but in combination with big bags supplied by Dover.

Note that, nowadays, filling process parameters that were adapted in 2017 have not changed in the course of the current investigation.

Between March and November 2019, an increased levels of reject rates for particles during the 100% visual inspection process were also encountered. Particles were identified as IgG protein particles (covered by PR#1399546) and were connected to sources of protein stresses: final pH adjustment practice confirmed to be a contributing factor.

The identified stress factor was improved by implementing a change of diafiltration buffer pH to reduce the frequency of pH adjustments during the final formulation step (covered by PR#1433777). After implementation of corrective action in November 2019, satisfactory results with the decreasing of protein particle reject rate were observed till the period investigated in this report.

Additional improvement opportunities to further reduce stress on proteins were identified for (1) the UF/DF step, related to the equipment configuration and operations (reduction of post wash duration, design of UF retentate piping) and (2) for the storage time and conditions of the vials after 30-32°C incubation and before visual inspection. Several of these robustness actions were already implemented in PL4: Optimization of UF/DF postwash (covered by PR1499209) and extension of the UF retentate pipe in the formulation tank (covered by PR1473601).

Average particle reject rate related to the current trend is significantly lower than in 2017.

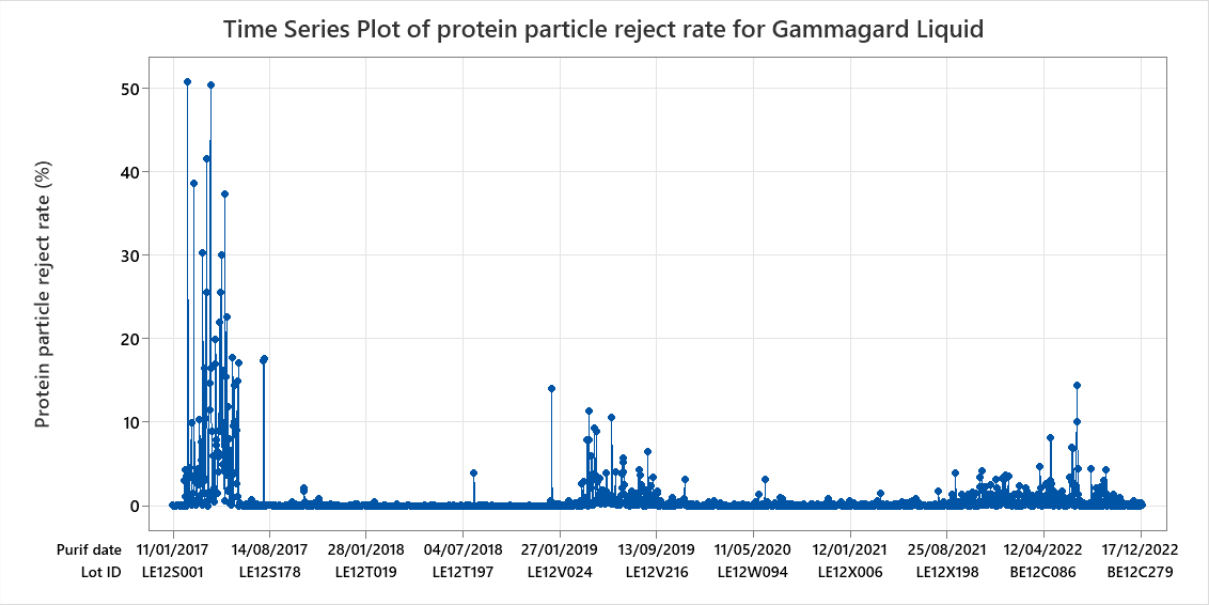


***Figure 5**. Protein particles reject rates on Gammagard Liquid manufactured in Lessines from 2017 to 2022 (purification date)*

6.1.2. Investigation scope and timeframe

The fractionation of human blood plasma is performed at Los Angeles, Rieti, Vienna and Prothya (CMO) facilities to produce Precipitate G (Ppt G) pastes. The purification process of Ppt G pastes into IgI 10% products is performed at the Lessines facility on two (2) bulk purification lines, PL1 and PL4. The product is filled on two (2) different filling lines, FL1 (RABS technology) and FL3 (Isolator technology). The product is aseptically dispensed into vials of sizes ranging from 10 mL to 300 mL.

Most of the IgI 10% batches and in particular the biggest formats (100mL, 200mL and 300mL) are mainly processed on PL4 and FL3 lines which have highest capacities. While Purification Line PL1 and Filling Line FL1 are more dedicated to other products. To get data on a bigger picture some batches were expressly purified and filled in the other configurations PL1/FL3 and PL4/FL1 in the frame of the investigation (in January and February 2022).

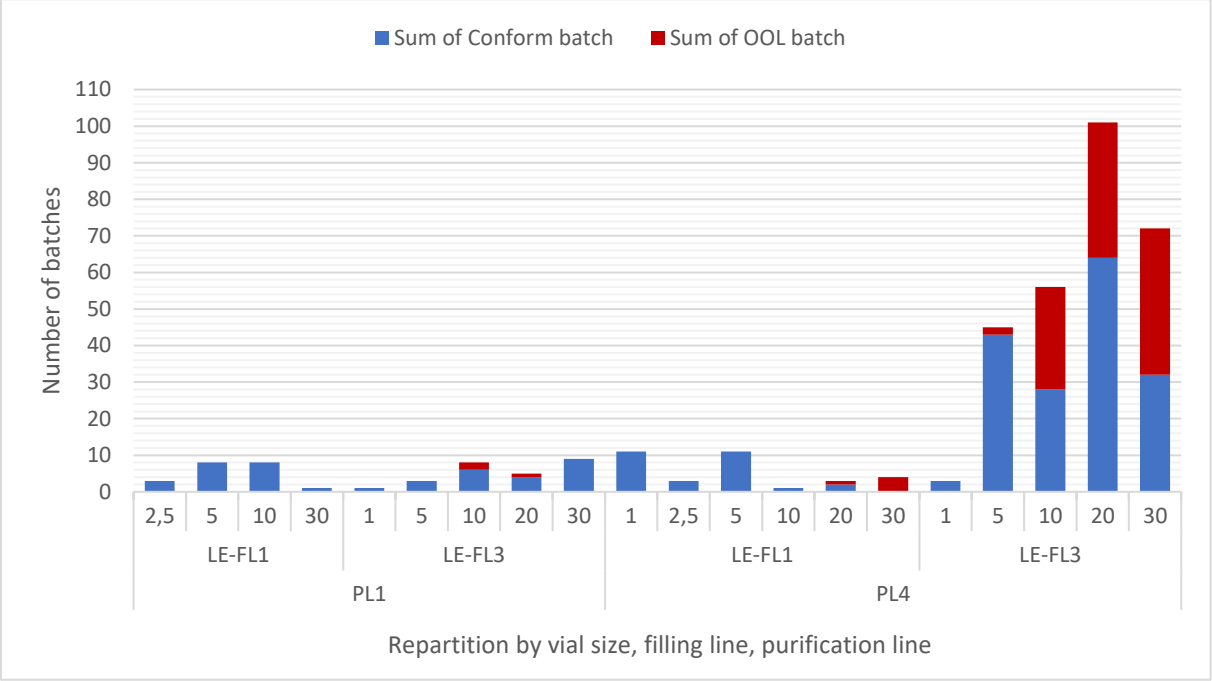


***Figure** 6. Repartition of the OOL batches between the different purification and filling lines in Lessines since the visual inspection date of the batch LE12X208 (visual inspection date 08-Oct-2021 to 10-Dec-2022)*

107 batches out of a total of 115 OOL batches have been processed on the PL4 and FL3 lines, therefore the investigation focused on those manufacturing lines. But OOL batches were also detected for Purification Line PL1 as well as Filling Line FL1.

The general investigation was documented in the frame of the PR#2428796 and was carried on continuously from October 2021 to December 2022. Four distinct stages can be established as shown on Figure 7:

1. Initial investigation: focusing on the first batches showing higher protein particle reject rates. This part covered mainly the period from September 2021 to December 2021. The work performed in this stage drove the consecutive investigation efforts that continued in 2022. This stage started with the batch LE12X208 that was visually inspected on 08-Oct-2021 and for which the purification process started on 01-Sep-2021. The review of the protein particle rejects rate trend showed however an increasing trend starting from the batch LE12X173 (purification started on 03-Jul-2021 and visually inspected on 10-Aug-2021) onwards.
2. Dedicated investigation on batch BE12C080 due to the presence of PDMS within the protein particles of this batch.
3. Dedicated investigation on batch BE12C117 due to the atypical reject rate observed at that time (8,17%).
4. Post summer shutdown investigation from August 2022: focusing on the series of batches visually inspected after the summer shutdown 2022 whose protein particle reject rates up to 14,45% appeared atypical with regards to the reject rates observed up to this date. This stage includes 11 OOL batches from BE12C138 to BE12C154. The investigation demonstrated that those batches do not form a specific cluster and are consistent with the other related batches despite the higher reject rates.

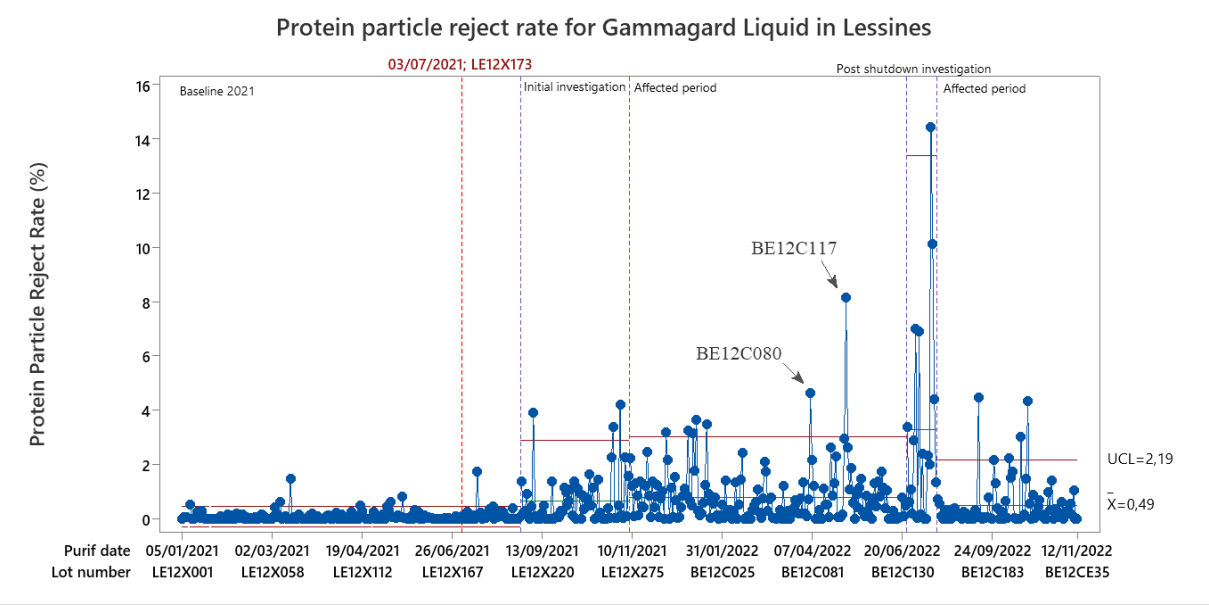


Figure 7. *Overview of the different stages of the investigation and evolution of the protein particle reject rate for Gammagard Liquid in Lessines from 2021*

All associated reports for the different stages described above can be found in the PR#2428796. Regular status updates on the progress of the investigation were also issued.

6.1.3. Investigation approach

The investigation consisted of:

- Overall documentation review (section 6.2):
    1. Batch records
    2. Events
    3. Changes implemented by Lessines
    4. Changes implemented by suppliers
    5. Maintenance activities
    6. Raw materials

- Review of each manufacturing step and focusing on generated hypotheses and with special deep dive on 4 hypotheses namely, designated raw materials, final pH adjustment practices, CM chromatography and Filling parameters/sequence (sections 6.3 to 6.8):
    3. Visual inspection
    4. Upstream process - Fractionation
    5. Purification
    6. Filling & Set-Up
    7. Post-filling steps (storage and incubation)
    8. Utilities

6.2. Overall Documentation Review

As part of the investigation, batch records, events, changes, maintenance interventions and raw material lots were reviewed during the 4 stages of the overall investigation:

1. At the beginning of the investigation for the batches LE12X208 to LE12X273
2. For batch BE12C080 and associated batches BE12C081 and BE12C082 for the finishing part only
3. For batch BE12C117
4. During the post shutdown investigation for batches from BE12C138 to BE12C154
5. For batches BE12C76, BE12C201 and BE12C206 in a system impact assessment of December 2022

An index of the general review document is provided in attachment #04 with the location of the documents within PR#2428796, a summary is provided below.

6.2.1. Review of batch records

A review of the batch record documentation for the batches included in the stages described above was performed from purification steps to the visual inspection.

No common event or issue was identified that could explain the occurrence of protein particles.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to batch records review of purification, finishing and visual inspection/packing processes.

6.2.2. Review of deviations

Events related to the lots involved in the scope of the investigation were assessed to identify any potential causality with the occurrence of protein particles.

The review and analysis of related deviations demonstrated that no relationship could be established with the current issue. They were eventually ruled out as potential root cause for the occurrence of protein particles.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to deviations review of purification, finishing and visual inspection/packing processes.

6.2.3. Review of changes implemented by Lessines

All changes implemented at Lessines plant between 01-Jun-2021 to 15-Nov-2022 were reviewed in the course of the different stages of the investigations as described above, covering all process steps of IgI 10% products.

There was no change identified that could explain the increased protein particles levels.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to change records review of purification, finishing and visual inspection/packing processes.

6.2.4. Review of maintenance activities

Corrective and preventive maintenance activities were reviewed at the different stages described above covering the manufacturing periods of the different batches impacted.

The first general review was performed during the initial investigation between 12-Jun-2021 to 27-Sep-2021. A second general review was performed during the August 2022 investigation between 01-May-2022 to 31-Jul-2022. Additional ad hoc reviews were also conducted for specific batches such as BE12C080, BE12C117 BE12C176, BE12C201 and BER12C206 and were limited to the production of the batches considered.

None of those reviews allowed to identify specific maintenance activities that could be linked with the increase in protein particles occurrence. More specifically, no work order related to residues found in the suspension tank P8 was initiated.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to maintenance activities review.

6.2.5. Review of calibration

Calibration activities were reviewed during the initial investigation and the post summer shutdown investigation, covering the periods from 12-Jun-2021 to 27-Sep-2021 and from 01-May-2022 to 31-Jul-2022 respectively.

None of those reviews allowed to identify specific calibration activities that could be linked with the increase in protein particles occurrence.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to calibration review.

6.2.6. Raw materials review

Mappings of the different raw material lots used for the production (purification and filling) of IgI 10% batches was performed at the different stages of the investigation.

The first review highlighted four raw materials for which there was a potential timely coincidence between the use of a new lot number and the increase of protein particles reject rate: (1) Octoxynol 9 or Triton X-100 (Part Number PN 0200690), (2) Polysorbate 80 (PN 0200185), (3) acetic acid 1N (PN 0200008) and (4) tromethamine or Tris base (PN 0200177). Further investigations detailed in section 6.5.1.2 concluded that none of those raw materials could have contributed to the formation of protein particles.

For the investigation related to batch BE12C080 (stage 2), the raw materials review was done only on the bromobutyl 32mm Omniflex plus stoppers (PN 3000420) and the different types of tubing present on the filling kit (PN 3200531, 3201170, 3201169, 3201168 and 3201167), those materials being identified as potential sources of PDMS. The comparison analysis of lots of stoppers as well as tubing between IgI 10% conform and OOL batches did not however allow to explain the increased rate of protein particles observed nor the presence of PDMS within the protein particles of batch BE12C080.

Finally, a third mapping was performed during the post shutdown investigation on the different raw materials lots used during the year 2022. Although this review did not highlight any direct correlation between the use of a specific batch of raw material, a special focus was placed on the following raw materials for which a change of batch occurred during the impacted period: glycine Yuki 500kg (PN 0201006), glacial acetic acid (PN 0200099), bromobutyl stopper 32 mm (PN 3000420) and RX-65 tubing (PN 3201168). For those raw materials, the certificates of analysis and receiving inspection results that where applicable for the batches used during the impacted period were reviewed and found satisfactory.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to raw material evaluation.

6.2.7. Review of changes implemented by suppliers

A review of the Supplier Notification of Changes (SNC) received and implemented in 2021 was initially performed. Based on the implementation dates or the nature of the changes, there was only one change concerning the Triton X-100 that was further investigated to confirm the first use of post change material in comparison to the increase of protein particles (PR#1699436, see section 6.5.1.2).

A second SNC review was performed during the post shutdown investigation for 4 raw materials for which a timely coincidence with an increase in protein particle reject rate was identified: glycine Yuki 500kg (PN 0201006), glacial acetic acid (PN 0200099), bromobutyl stopper 32 mm (PN 3000420) and RX-65 tubing (PN 3201168). For each raw material, the review period extended from one year before the production date of each of the identified batches. No SNC was identified on those raw materials that could explain an increase in protein particle. Corresponding suppliers were also contacted and confirmed that no change was implemented at their respective sites in the periods considered.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to SNC documentation review.

6.3. Visual inspection process review

To determine if the increase in protein particles reject rate was due to a measurement system/method change or specific events, elements related to the visual inspection (VI) were reviewed in the initial investigation on a period starting from 01-Aug-2021 to 30-Nov-2021. Additional verifications were performed for the OOL batches up to 18-Jan-2022. Finally, VI process was reviewed during the August 2022 investigation focusing on the period from 01-Jul-2022 to 28-Aug-2022 which includes the summer shutdown.

The following elements were reviewed:

- No change was implemented related to VI method as per SOP-026399 (Méthode d'inspection visuelle – Packing).
- No change was implemented to VI criteria included in SOP-005032 (Critères et méthode d'inspection visuelle pour les produits liquides et lyophilisés – Packing), that could have resulted in the observed increase of protein particles identified.
- The calibrations of the visual inspection booths were reviewed and were satisfactory (see section 6.2.5).
- All operators are qualified according to SOP-048674 (Formation à l'inspection visuelle au packing)
- All visual inspection shifts reported the presence of protein particles in finished product vials.

It is concluded that the increase in protein particles reject rate is not due to a measurement system/method change or specific events during the visual inspection step.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to the visual inspection process review.

6.4. Upstream process review – Fractionation

The different hypotheses related to upstream process are summarized in Table 2 below.

Hypothesis	Investigation outcome
Paste origins: a change in PptG protein composition parameters lead to protein aggregation.	All sources of PptG are impacted similarly.
Fractionation process ethanol concentration: Ppt G produced with 25% ethanol process induce more protein aggregation.	Final container lots produced from both 20% Ethanol and 25% Ethanol PptG are impacted similarly.

Table 2. Upstream process hypotheses of protein particle generation

The source of the PptG for the batches processed during the affected period was analyzed to determine if there had been a change in the fractionation process that could have resulted in the observed increase of protein particles formation in the IgI 10% products vials.

The analysis presented in Figure 8 below shows the rejection rates of all the batches exceeding the action limit in function of the origin of the PptG paste (fractionation site, plasma origin and plasma collection method (source or recovered)). It demonstrates that protein particles are observed whatever the origin of the paste and that the variability in rejection rates is also similar between pastes.

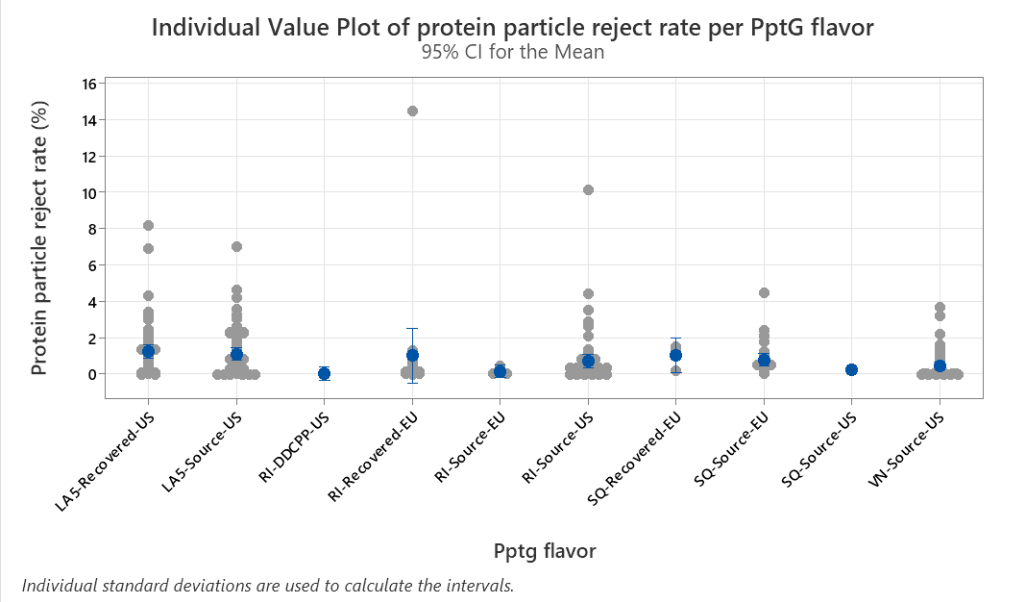


Figure 8*. Reject rates per paste source from 01-Sep-2021 to 05-Dec-2022*
(LA : Los Angeles – RI: Rieti – SQ : Sanquin – VN : Vienna)

To further evaluate whether if the origin of the pastes could be the related to protein particles observed at the visual inspection, the dryness of pastes was analyzed. Dryness is not monitored on the precipitate G pastes so an indirect measurement has been used through the relative protein content of the pastes: the weight of protein content reported to the weight of the paste (g protein / kg paste), giving an indirect evaluation of the paste dryness.

The study has been performed three-times through a three-year timeframe from batch LE12V001Z (January 2019) to batch BE12C151Z (July 2022) and documented in report DIV-TS-21-001, DIV-TS-22-002, and DIV-TS-22-029. The study demonstrated that the dryness slight variations occur through time among the different paste origins with variable tendencies (stable, positive, negative). Protein particles reject rate increased level has been identified independently of the paste origins and dryness variations. Therefore, it is considered that no correlation can be made between the dryness variations and protein particle rejection level.

Based on the previous analyses it is concluded that no significant difference in the protein particle reject rates can be seen between the different fractionation sites and plasma origins. As it is highly improbable that all upstream sites would have experience the same change at the same time, it is concluded that the root cause of increased reject rates for protein cannot be attributed to the upstream manufacturing processes.

The same exercise as the one performed for the paste origin and presented in Figure 8 was conducted by comparing the batches manufactured with PptG pastes from the 20% ethanol and 25% ethanol processes. Those processes differ by the concentration of ethanol that is used during the upstream process, this difference is known to produce PptG pastes with slightly different compositions in proteins profiles.

As shown on Figure 9, Although a slight difference in average protein particles reject rate can be observed between the two processes, the variability is similar and OOL batches are observed for both ethanol process variation.

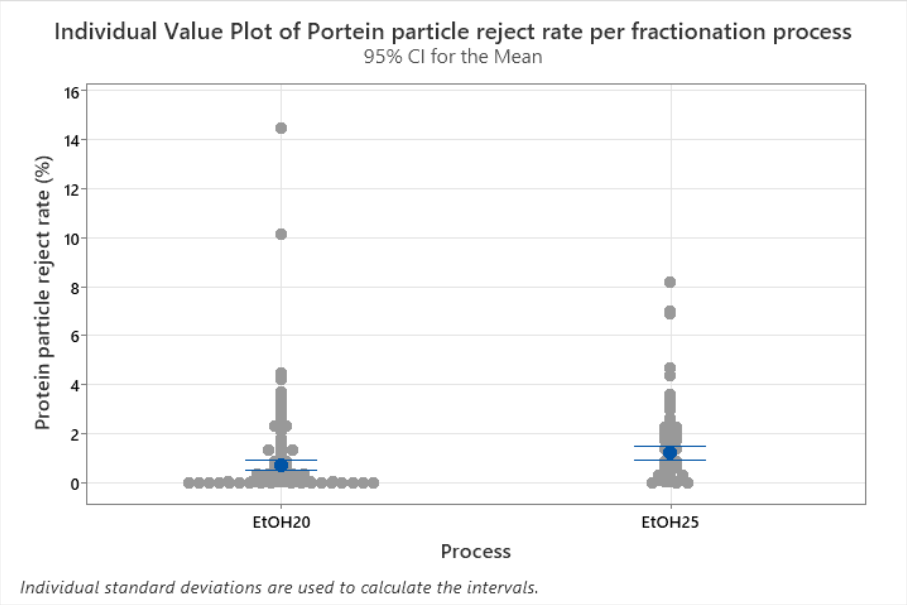


Figure 9. *Reject rate per fractionation process from 01-Sep-2021 to 05-Dec-2022*

It was concluded that fractionation processes did not contribute to the higher rejection rates for protein particles observed at the 100% visual inspection of IgI 10% products at the Lessines facility.

6.5. Purification manufacturing process review

The purification process of PptG pastes into IgI 10% product is performed at Takeda Lessines facility on the two purification lines (PL1 and PL4). These lines are independent of each other and have separate equipment but use similar raw materials. The different hypothesis investigated are summarized in the Table 3 below.

Hypothesis	Investigation outcome
Main Hypothesis: Purification raw materials: a change in raw material quality have occurred	No raw material, including the excipient, could be linked with protein particle generation
Purification process parameters: one or several process parameters have drifted	Shifting parameters were identified by MVDA. Only the parameters related to pH adjustments and CM chromatography were found relevant and further investigated.
Main Hypothesis: pH adjustment practices: the proteins have been exposed to high pH during the purification process	Improved pH adjustment practices on dialysis buffer have been implemented although the protein particle reject rates was not formally linked with pH adjustments.
Main Hypothesis: CM chromatography: the cause of drift observed on CM eluate parameters, or its consequences contribute to protein aggregation	Mitigation actions have been conducted although the protein particle reject rates cannot be formally linked with CM eluate increased parameters.
Cleaning of purification equipment: contamination of the process equipment due to insufficient cleaning	No evidence that CIP cycles of the diverse equipment could be linked with an increase in protein particles generation was found.
Purification process times: proteins are exposed to additional stress due to increased process steps duration	No change or shift in purification process time could be linked with increased protein particle occurrence

***Table** 3. Purification process hypothesis of protein particle generation*

6.5.1. Purification raw materials

6.5.1.1. Glycine

In addition to the raw materials analysis documented in section 6.2.6, a specific analysis was performed for glycine, as this raw material is an excipient driving the stability of the IgI 10% products formulation, and as it was identified as the main root cause of the 2017 investigation.

In the IgI 10% manufacturing process two sources (manufacturers) of glycine are qualified, Yuki and Chattem. For Yuki glycine, two packaging sizes are qualified, 500 kg big bags (using bags supplied by Dover) and 25 kg containers, while for Chattem only 500 kg Big Bags is qualified using big bag supplied also by Dover. In Lessines, only the Yuki glycine with a packaging size of 500 kg Big Bags (Takeda part number 0201006) is used on a routine basis and was therefore the one used for batches during the period covered by this investigation.

The initial review for this material did not reveal the existence of a related supplier change nor a timely coincidence between the use of a specific batch of raw material and the increase in protein particles reject rate.

An investigational experimental plan was designed to evaluate if the source of the glycine material used could influence the level of protein particles reject rate in final container (refer to PR#2446119).

The experiment was performed between the 12-Dec-2021 and 02-Mar-2022 (see PR#2560002 and, see ME-TS-23-007 in Attachment #05) and consisted in processing IgI 10% batches on PL4 with glycine buffer prepared using the different qualified sources:

- Glycine from Yuki in 25kg bags containers.
- Glycine from Chattem in 500kg big bags near its expiry date.
- Glycine from Chattem in 500kg big bags with a recent production date.

All batches produced followed the usual purification, fill/finishing process up to visual inspection. The rejection rates for protein particles were then compared to the rejection rates obtained with the routine process that uses the glycine from Yuki in 500kg big bags. The result of the experiment is provided in Figure 10 below

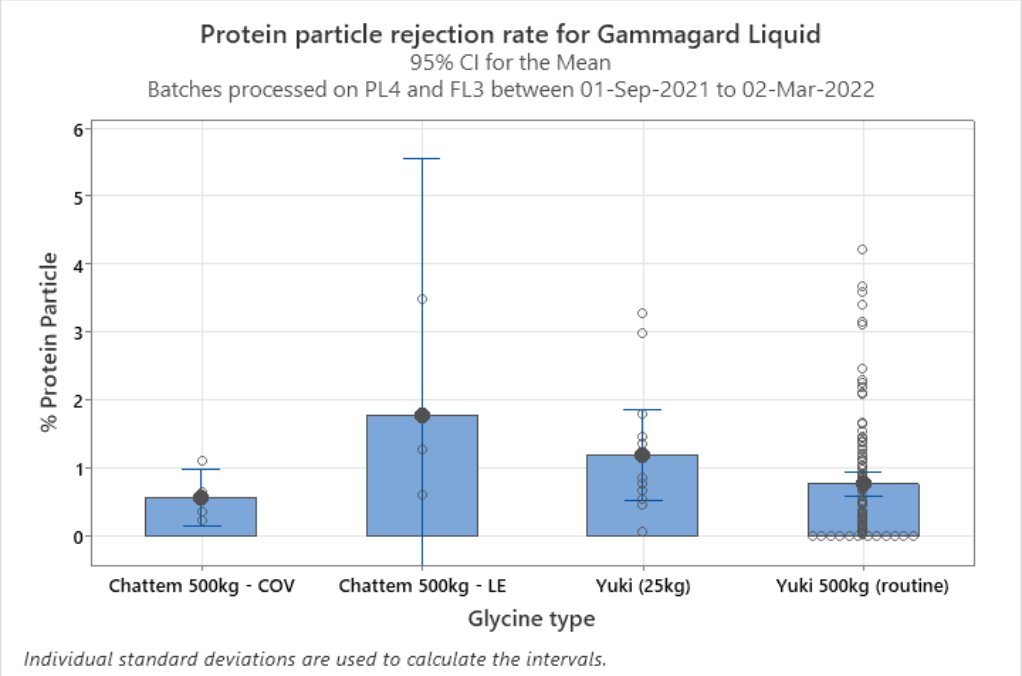


*Figure **10**. Protein particles reject rates with different sources of glycine*

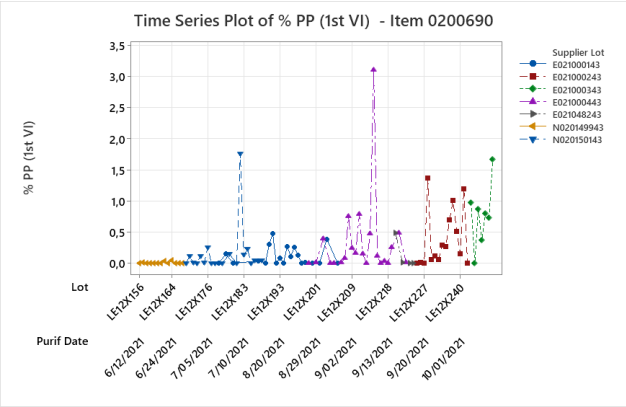
All sources of glycine tested resulted in conform and OOL batches. No meaningful statistical difference could be established regarding the protein particles rejection rates of the different test groups. The experiment plan concluded that neither the supplier of the glycine nor its packaging type contributed to the formation of protein particle in finished drug product.

6.5.1.2. Triton X-100, Tween 80, Base Tris, Acetic Acid 1N

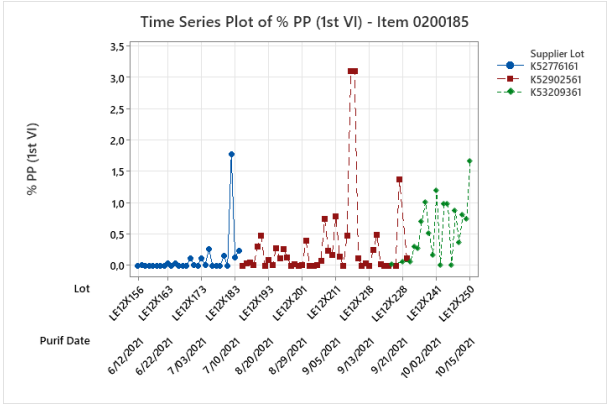
As explained in section 6.2.6, the following raw materials were identified to have a timely coincidence between the use of a new batch and the increase in protein particles:

- Octoxynol 9 or Triton X-100 (Takeda part number 0200690)
- Polysorbate 80 or Tween 80 (Takeda part number 0200185)
- Tromethamine or Tris Base (Takeda part number 0200177)
- Acetic Acid 1N (Takeda part number 0200008)

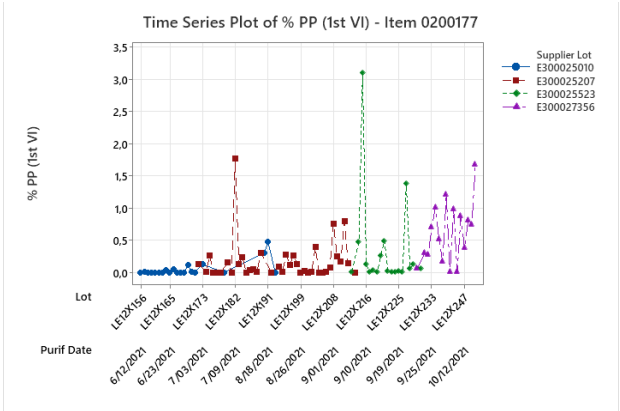
Triton X-100



Tween 80



Tris Base



Acetic Acid 1N

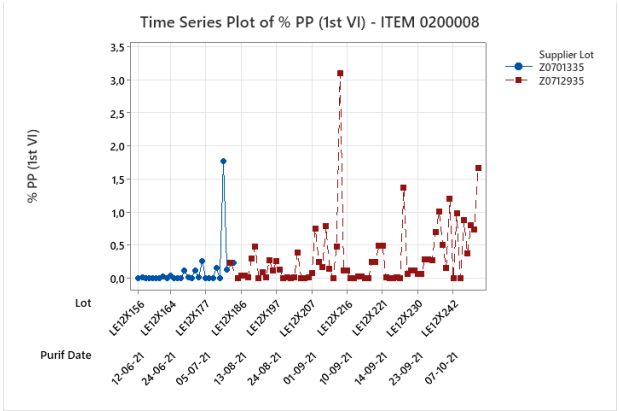


Figure 11. Protein particle reject rates depending on the raw material batches processed for Triton X-100

For all raw materials the certificates of analysis and the SNC were reviewed, and suppliers were contacted. For Tween 80, Tris Base and acetic acid 1N, no supplier change was reported to be potentially linked with protein particles generation. The quantities of Triton X-100 and Tween 80 in final container are also controlled during QC release testing and reported to be conform for all IgI 10% batches.

The SNC review indicated that one supplier change affecting the Triton X-100 required further investigation: the SNC PR#1699436 related to the transfer of Triton X-100 production process to a dedicated vessel at Merck's manufacturing site in Darmstadt, Germany. The certificates of analysis of 6 batches of Triton X-100 were analyzed: 3 batches before the implementation of the change and 3 batches after. All parameters were within their respective specifications and no shift before and after the change could be observed. Moreover, the supplier confirmed that the equipment was like for like and that the manufacturing process as well as the raw materials, product specifications and the quality of material remained unchanged (refer to attachment #04 "raw material review" section for document location). This SNC was ruled out as potential root cause for protein particles generation.

To confirm the absence of relationship between those materials and the protein particles rejection rate in final containers, an experiment plan was designed to use new batches of each raw material. On one hand 4 batches of IgI 10% batches were processed with a new lot of Triton X-100 and on the other hand 4 batches were processed using new lots of Tween 80, Tris Base and acetic acid at once. All batches were processed on PL4 between 23-Jan-2022 and 03-Feb-2022.

All batches produced followed the usual purification, fill/finishing process up to visual inspection. The protein particles rejection rates obtained on those batches were compared with the rejection rates obtained on IgI 10% batches produced between January 2021 and July 2021 ("routine" test group) with overall low protein particles level. Results are displayed on Figure 12 and ME-TS-23-007 in Attachment #05.

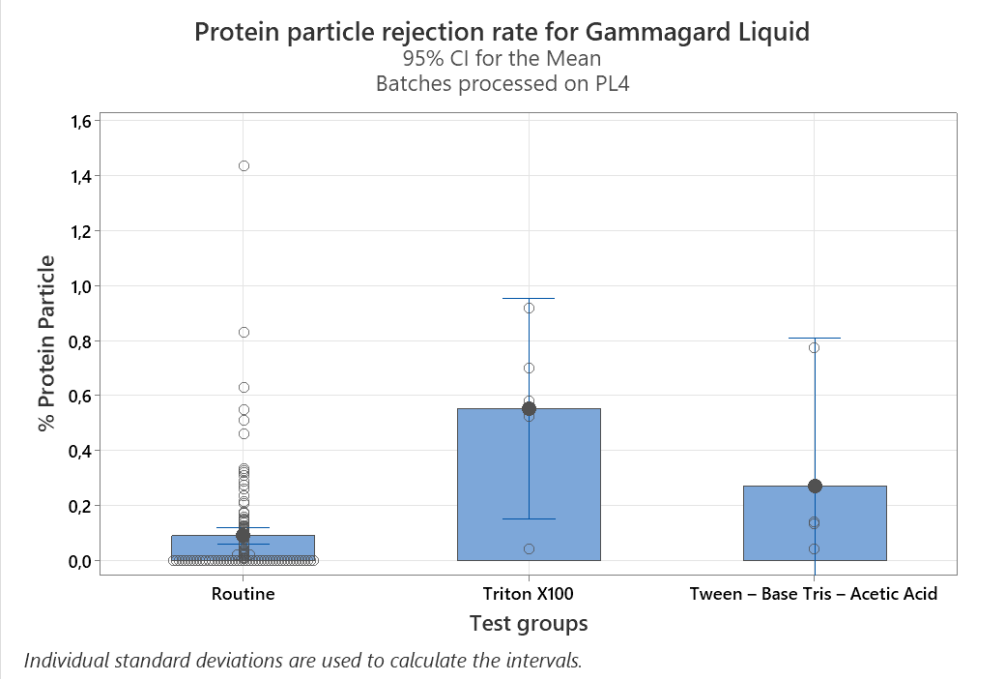


Figure 12. Comparison of the protein particles rejection rates obtained with different lots of raw materials

Despite the use of new lots, both test groups gave conform and OOL batches for reject rate for protein particle at 100% visual inspection. There is no statistical difference between the mean rejection rates of the different populations.

To conclude, the different raw materials reviews and the experiment plans results exclude that the four raw materials above may have contributed to the generation of protein particles in final container.

6.5.2. Purification process parameters – Multivariate data analyses

Analyses of the purification process parameters was performed at different stages of the investigation, relying on multivariate statistical tool (MVDA).

In the first stage, a database was built containing as many as 706 features per batch composed of purification process parameters and attributes extracted from Discoverant data system. Additionally, minimum, maximum, average, and standard deviation features were extracted from continuous data (flowrate, temperature, pressure) related to UF/DF step as this step was identified as potential stress contributor for the protein particles due to the recirculation in the tangential flow filtration system. The dataset was restricted to Purification Line PL4. An initial model was proposed (see ME-TS-21-084) and updated on a broader dataset in January 2022.

The initial model compared batches from a "golden period" that had low protein particles reject rates and batches from the "affected period". The golden period extends from 12-Jun-21 to 01-Jul-21 and covers 14 batches. For the MVDA, the considered affected period started from batch LE12X173 and the start of the slight increasing trend in protein particles reject rate. This period extends from 03-Jul-21 to 10-Dec-21 and involves a total of 124 lots.

The second iteration of the model included all the batches manufactured in 2021 and refined the classification by separating the batches in three groups: (g1) the batches for which there was no protein particles, (g2) the batches with protein particles reject rate comprised between 0 and 1%, and (g3) the batches with reject rate for protein particle higher than 1%.

For both iteration, two kinds of models were built:

- Partial Least Square models (PLS) which compared the batches from the different groups and identified statistical differences between each group.
- Orthogonal Partial Least Square (OPLS-DA) which aimed at identifying the correlations between all the parameters and given output parameters, in this case the protein particle reject rates at visual inspection.

The models identified a series of parameters for which statistical differences could be found. The results were reviewed by the multidisciplinary team to investigate further the parameters for which an actual causality could be anticipated. Based on the MVDA conclusions, parameters were grouped as follow:

- pH at final formulation.
- CMS chromatography parameters: CMS column equilibration volumes, CMS eluate conductivity, pH and adjustments and ANX load duration.
- UF/DF parameters: filtrate and retentate pressures, inlet pressure during postwash, outlet flowrate skid 2, P5 protein tank weight at start of the 2nd concentration and UF/DF temperature.

The following parameters were also evaluated as isolated signals:

- MSD – Monomers + Dimers
- AC-titer
- Tween concentration
- Weight C1
- Temperature Equilibration II ANX buffer
- Osmolality

Thorough review of each of the parameters above except the ones related to the CMS chromatography step (refer to section 6.5.2, 6.5.3 and 6.5.4) did not allow to identify a drift or shift that could have likely been associated with an increase in protein particles reject rate in final container.

During the August 2022 investigation, a complementary MVDA (see ME-TS-22-086) was performed by Global Manufacturing Sciences group that aimed at comparing the process data of the two following populations: (1) IgI 10% batches with the highest levels of reject rate observed in August and (2) historical process data. This analysis only considered batches processed on PL4 and FL3 lines in the biggest filling format sizes: 100 ml, 200 ml and 300 ml. Historical process data covered batches processed between June 2021 and August 2022.

In this case, the approach was modified: process SMEs reduced the dataset to approximately 200 parameters of interest regarding the aggregation of protein. Discrete and continuous data were taken into consideration and analyzed thanks to multivariate statistical tools.

This analysis highlighted that the two populations differences were driven by variations in the CM performance and the UF/DF temperature but that there was no correlation with the protein particle reject rate. Which confirms the findings of the first analysis.

Minor signals related to buffer properties were also identified although they were also not correlated with protein particle reject rates. The UF/DF temperature shift was later attributed to the calibration of the temperature probe of the tank P5. No major signal was identified either for vials cleaning or filling steps.

To conclude, although this work highlighted a few varying process parameters none appeared to be correlated with protein particles reject rates. It is however not possible to exclude that those parameters do not lead to an increased risk for protein aggregation as a result of cumulative effect of different parameters.

Refer to ME-TS-22-086 attached to PR#2428796 (see exact location in attachment #04) for further details.

For the CMS and the UF/DF robustness actions are being implemented, see section 8.

To conclude, excepted the parameters related to CMS chromatography, no atypical behaviour of one or several of the purification process parameters monitored was observed that could explain the increase in protein particle reject rate in final container for IgI 10% products.

6.5.3. pH at final formulation and adjustment practices

In the IgI 10% manufacturing process, the concentrated protein solution recovered at the end of the first UF concentration is diafiltered against a 0,25M glycine buffer with a specific pH (license range 4,2 ± 0,2, as per SOP-042623). After diafiltration, the protein solution is further concentrated to a protein concentration of minimum 11% w/v. The protein concentration is finally adjusted to 10,0 ± 0,1% w/v with diafiltration buffer during final formulation and the pH is adjusted to 4,4-4,9 if necessary (run-sheet range 4,45-4,55).

The exposition of proteins to low pH is a known stress factor ultimately leading to aggregation.

Following the protein particles investigation of 2019, the target pH range of the dialysis buffer was modified to 4,05 ± 0,05 (refer to PR#1433777). The objective was to standardize the pH adjustment practices and avoid having to adjust the final product solution pH with 1N HCl during the final formulation step by meeting the target pH range of the protein solution directly at the end of the second concentration. The dialysis target pH was revised on 10-Aug-2020 to 4,10 ± 0,05 based on the review of the data collected under change control PR#1604019 to further reduce the need for final pH adjustments.

In the present case, from lot LE12X178Z onwards, variability of HCl concentration in the dialysis buffer composition induced higher variability in the native pH of proteins at final formulation step and coincided with the start of increased protein particles reject rate period (see Figure 13).

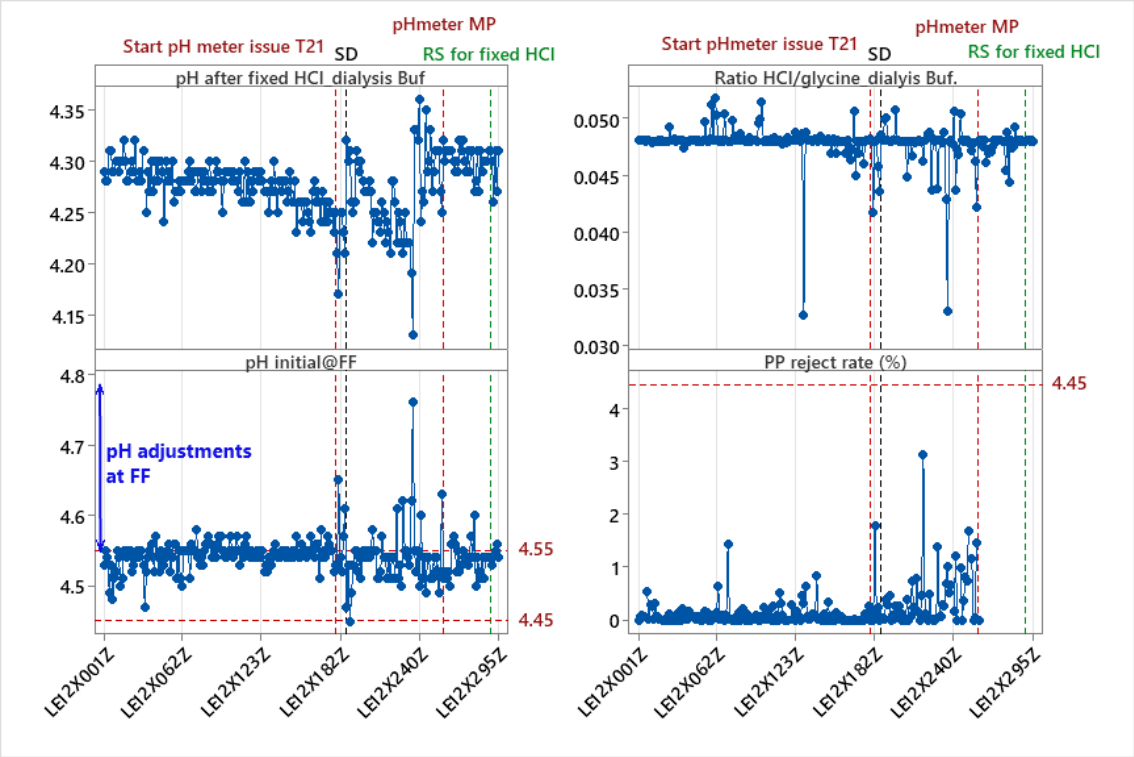


Figure 13. Evolution of the dialysis pH buffer preparation and respective protein particle reject rates

This variability in dialysis buffer composition was attributed to a drifting pH-meter probe in the dialysis buffer tank (T21). This probe issue was solved as part of the preventive maintenance performed before the production of the batch LE12X256Z (WO5160919).

After correction of the pH measure, some variability in the pH of the dialysis buffer remained. An additional mitigation action was implemented with a checklist (see PR#2560003) to prepare the dialysis buffer with constant HCl 6N quantities; this checklist is applied in purification area since the batch LE12X289Z.

The definitive implementation of this action is covered by change control PR#2529017 (PL4) and PR#2528924 (PL1).

This standardized practice reduced the number of lots that were adjusted for pH at final formulation as well as the amplitude of pH adjustment if needed (see Figure 13). Nevertheless, this practice, which secured the process by reducing stress on protein, did not induce a reduction of protein particles level as illustrated in the Figure 14 below.

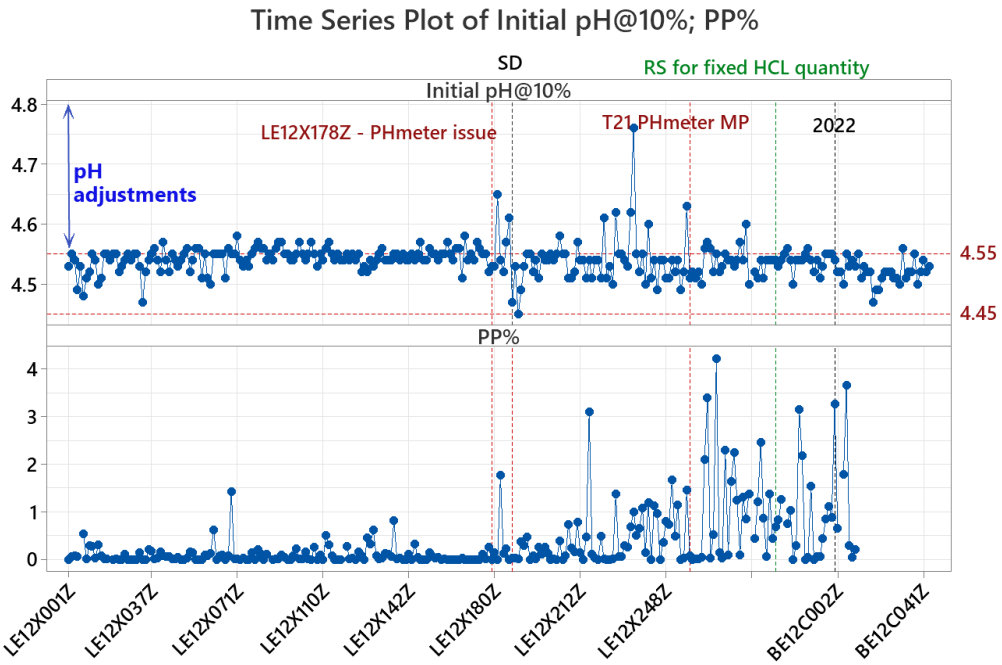


Figure 14. Analysis of initial pH at Final Formulation 10% and protein particles, period from January 2021 to February 2022 (purification date)

To conclude, the different investigation and mitigation actions showed that pH adjustment practices alone cannot explain the high level of protein particles in the IgI 10% final containers. Actions to improve the robustness of this pH adjustment practice are being evaluated.

6.5.4. CMS chromatography investigation: eluate initial conductivity

An upward shift of the CM eluate's initial conductivity has been identified around batch LE12X178Z (see Figure 15) which coincides nearly with the beginning of the slightly increased level of protein particle observed during the 100% visual inspection process.

As the conductivity in the CMS eluate pool is known to be correlated to the elution volume (see Figure 15), the larger the elution volume the higher the conductivity, the investigation focused on determining the root cause of the increase in elution volumes and, determining if there is potential relationship between both signals. All four CM columns were affected but mainly CM1 (LECM9292), CM3 (LECM14868) and CM4 (LECM14896) while CM2 (LECM9293) was more stable. This trend was investigated in the deviation PR#2589645.

The CM chromatography process step itself cannot really explain the protein aggregation. However, bigger CM eluate volumes induce additional stresses on the proteins later in the process by increasing process times. Increased mechanical stresses could occur due to longer nanofiltration or UF/DF steps.

Even if an increase is observed on the initial conductivity of the CM eluate, before further processing the conductivity is adjusted. Therefore, there is no impact of the conductivity on the next process steps.

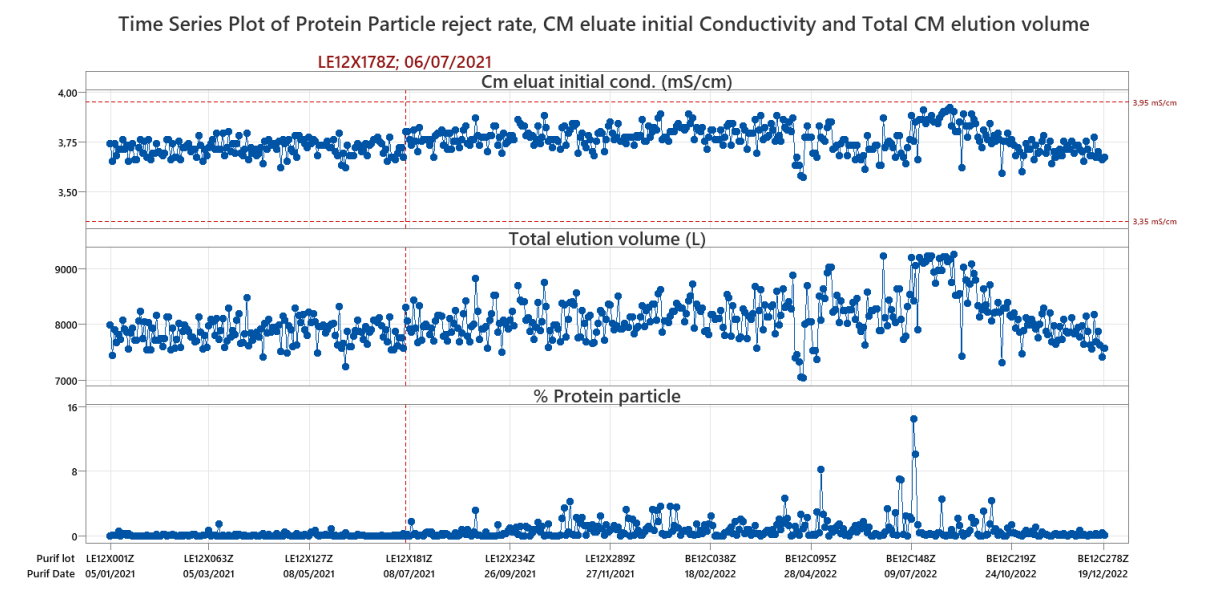


Figure 15. Evolution of protein particles reject rate, CMS eluate initial conductivity and CMS elution volumes on PL4 between 5-Jan-2021 to 2-Nov-2022

As a result of the brainstorming performed by a multidisciplinary investigation team, a list of potential root causes of increasing elution volume has been elaborated.

In the first stage, the presence of air in the equipment was reported after a Gemba. As corrective action the control valves for CMS purification line 4 were replaced (PR#2379026) during the winter shutdown 2021 but this modification had no direct impact neither on elution volumes nor on protein particles reject rates.

To further investigate the potential effect of the increased elution volume on the purification process and the protein particles generation a temporary change was implemented on a limited number of batches (7) on PL4 (see PR#2673047). For these batches, the elution step of the CMS chromatography was cut when the optical density at 280 nm of the eluate reached a value of 0,8 instead of a value of 0,4 as it is defined in routine process. This resulted in smaller elution volumes which did not demonstrate any impact on the protein particles occurrence as the protein particles rate varied from 0,08% to 2,98%.

Following the absence of improvement consecutively to the replacement of the control valves, the resin bed of the CMS chromatography columns was repacked with fresh resin. The activities were performed on 02-May-2022 to 04-May-2022 for the columns CM3 and CM4 and on 09-May-2022 to 12-May-2022 for the columns CM1 and CM2 (refer to PR#2767831). The first batches produced after repacking of the columns were BE12C099Z for the columns CM3 and CM4 and BE12C101Z for the columns CM1 and CM2.

The columns CM1 and CM2 were again repacked during the 2022 summer shutdown to further improve their performances. The first batch produced after those repacking was batch BE12C153Z. However, it was observed that, after this repacking, CM2 performances were different compared to others CM. Therefore, the repacking of this column was repeated on 20-sept and 21-sept-2022. The first batch produced afterwards was lot BE12C182Z. See PR#2740867 and PR#2951987.

No direct effect of repacking activities on the protein particles reject rate can be observed (see Figure 16) Even if the second repacking of the CM1 and CM2 is followed by a series of batches with low protein particles level, the relationship between the protein particles observed and the repacking itself is not confirmed.

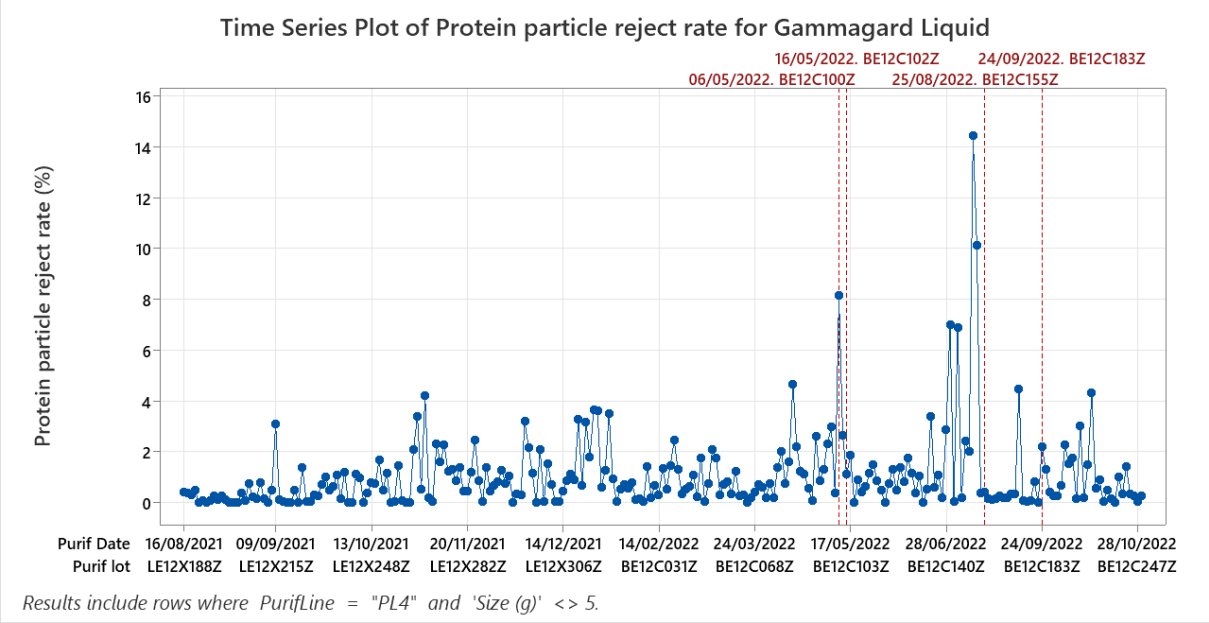


Figure 16. Evolution of protein particles over time. The first lots after repacking of CMS columns are highlighted. The first batch produced after the last repacking of the CM2 was BE12C182Z, it does however not appear on this figure as the graph excludes the batch filled in 5g vial size and the next batch was BE12C183Z.

Following the last repacking activities, CMS eluate volumes have decreased on PL4 and are back to the levels of early 2021 (see Figure 15). Nevertheless, no link could be formally established between the protein particle reject rate and the mitigation actions on the CM chromatography, which is further confirmed by the results of the MVDA (see 6.5.2).

6.5.5. Cleaning of purification equipment

The cleaning of the purification equipment involved in the final formulation step was reviewed twice: at the beginning of the investigation and during the investigation of August 2022. The following equipment were covered:

Purification Line	PL4
Final Formulation vessel	P5
pH glycine buffer vessel	T20
Dialysis buffer vessel	T21
Rinsing UF tampon vessel	T9

Table 4. Identification of process equipment used for UF/DF and final formulation in PL4

Analysis of the microbiological count (CFU and molds), conductivity, pH, LAL and TOC trends concluded that there was no shift, trend or atypical value within the investigation period for any of the vessels.

For the UF656 system, a similar trending is performed with microbiological count (CFU and molds), LAL and TOC.

In each of the periods considered, CIP (cleaning in Place) cycles were reviewed and there was no evidence that the cleaning of the diverse equipment is related to the increase observed for the reject rate for protein particles.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to cleaning of purification equipment.

6.5.6. Purification process times

A review of the process times for which longer duration could generate additional stress on the protein was performed at the different stages of the investigation. The following parameters were identified:

- Sterile filtration duration (including refiltration in the specific case of the batch BE12C117)
- CMS process time
- ANX process time
- Nanofiltration process time
- Total purification process time

The analyses could not identify a shift, nor a trend of purification process times correlated to the increase of reject rate for protein particles. Process times were also part of the different MVDA performed which did not highlight any significant or relationship with reject rate for protein particles.

Shorter batch production times in PL4 were reported after the summer shutdown of 2021. Due to shorter time, more activities are performed in parallel to the batch processing such as CIP of tanks or chromatography cleaning steps. An analysis was conducted by the Digital and Data Science group to assess if the concomitance between several process steps could be linked to the generation of protein particles. Potential relationships were identified for the following overlapping process steps:

- CMS elution vs CIP UF
- ANX cleaning NaCl vs nanofiltration
- CIP UF vs P5 protein material addition
- Nanofiltration vs CIP P10

Corresponding installations were then verified by Engineering and Process SMEs. No physical relationship between the different installations and piping was found in any of the cases above. Therefore, the concomitance of cleaning equipment steps and process steps was excluded as a potential root cause for protein stress that could lead to protein aggregation.

6.6. Filling & Set-Up manufacturing process review

The hypothesis investigated are summarized in the table below.

Hypothesis	Investigation outcome
Main Hypothesis: Filling raw materials: a change in raw material quality has occurred	No raw material could be linked with protein particle generation.
Filling process parameters: one or several process parameters have drifted	Shifting parameters were identified by MVDA. No parameter was found relevant after investigation.
Reject over filling campaign: specific campaigns or positions of the batch within a campaign generate more protein particles	Protein particles reject rates are homogeneously distributed between filling campaigns and batch positions.
Reject over filling sequence: specific sequences in the filling process generate more protein particles	Non-random distributions of rejected vials are observed along the filling sequence but are not reproducible from one batch to another and not related to protein particle clusters
Filling equipment: one or several pieces of equipment used in filling process generate more protein particles	No specific equipment was identified as preferential protein particle contributor.
Presence of particles in the environment: particles from the environment have contaminated the solution and triggered aggregation as nuclei	No shift or drift in particle monitoring data in filling and setup environment was observed.
Main Hypothesis: Influence of filling stops: stops of the filling process generate protein aggregation potentially due to increased shear stress or air/liquid exposition	Rejected vials were not specifically distributed around stops of the machine.
Influence of empty mode: machine settings at the end of the process generate protein aggregation potentially due to increased shear stress or air/liquid exposition	Rejected vials were not specifically distributed in the vials filled while the machine functioned in empty mode.

Table 5. Filling process hypotheses of protein particle generation

6.6.1. Filling raw materials

As described is the analysis performed on raw materials and documented above in section 6.2.6 and 6.2.7, no timely coincidence between the increase of protein particles reject rates and the use of a specific lot number was found for any of the filling raw materials.

However, an additional analysis was performed for glass vials, as these components are in direct contact with the final product.

Highest protein particles rates are observed for IgI 10% batches filled in filling line FL3 in the bigger vial sizes having wide necks: 100 mL, 200 mL, 300 mL from FL3.

Empty vials are visually inspected before filling to eliminate any potential defective vial following the local procedure SOP-048772. The hypothesis was that the presence of specific defects in the glass vials could induce aggregation of protein.

The reject rates for critical and major defects for the empty vials were checked and compared between the conform and OOL batches for protein particles. Using the supplier batch number, no coherent relationship could be established between the reject rate of empty vials and the reject rate for protein particles in final container for the different vial sizes.

To confirm this observation, a hypothesis testing was performed on batch BE12C028 which was partially filled on FL3 with conform empty vials that came from a batch that had higher reject rate for stains defect while the rest of the batch was filled with a batch of empty vials that had low reject rate for the same defect. For this batch BE12C028, 1,4% of the vials were rejected for protein particles and the defects were evenly distributed between the two groups of vials.

It demonstrates that there is no relationship between the stain defect on empty vials, and in general any defect observed on empty vials, and the protein particles reject rate at the 100% visual inspection.

6.6.2. Filling process parameters – Multivariate data analyses

As for purification process parameters, a statistical analysis of filling process parameters was performed in a Multi-Variate Data Analysis (MVDA), see ME-TS-21-085.

The initial model compared batches from a “golden period” and batches from the “affected period”. The golden period extends from 18-Jun-21 to 07-Jul-21 (filling date) and covers 15 batches. The considered affected period started from batch LE12X173 and the start of the slight increasing trend in protein particles reject rate. This period extends from 08-Jul-21 to 27-Oct-21 and involves a total of 57 lots.

The database was composed of 161 features for each batch (continuous parameters such as HVAC, inline particles counting, temperature, pressure, etc.). The dataset was restricted to Filling Line FL3.

Batch Evolution Model (BEM) with Partial Least Square (PLS) model analysis was performed for the prediction of protein particles defects for feature importance calculations. The model identified the following parameters:

- Shift in the MetOne #1, #2 and #3 flowrates (air particles counter).
- Shift in the air differential pressure of the isolator filling probes PT1842 & PT1843 and of the capping probe PT2842.

Further investigation was conducted with filling SMEs. Those shifts were attributed to maintenance interventions and could not be related to the generation of protein particles.

In the frame of the investigation conducted in August 2022, two analyses of filling parameters were performed.

First, an analysis was conducted based on a dataset of filling parameters for the Filling Line FL3 including process and environmental parameters. The objective was to identify differences between the batches presenting the highest reject rates compared to the ones with no or low reject rates and filled during the same period.

Secondly, the MVDA performed by the Global MS group in August 2022 and described above in section 6.5.2 also included a dedicated review of filling parameters. This review followed the same approach as the first one and compared the two batches with the highest protein particles reject rate (batches BE12C149 and BE12C150) with the other batches produced during the same period that had lower protein particles reject rates.

This analysis included the following parameters for both groups:

- Vial cleaning and depyrogenation process parameters, including environmental parameters recorded during the cycles of the different batches considered.
- Filling equipment used.
- Filling process parameters during filling, including environmental parameters recorded during the cycles of the different batches considered.

For both analyses, no significant difference was found for any of the parameters considered between the groups.

To conclude, no atypical behaviour of one or several of the filling process parameters monitored was observed that could explain the increase in protein particles reject rate in final container for IgI 10%.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to process parameters analysis.

6.6.3. Analysis of reject over filling campaigns

IgI 10% batches are mainly filled in campaigns in filling line FL3. For the batches filled between 09-Sep-2021 (LE12X204) and 06-Dec-2021 the distribution of the protein particles reject rate per campaign was plotted. The objective was to identify if filling campaigns systems contribute to the formation of protein particles by assessing if the protein particles reject rate of the batches is homogeneous within a filling campaign. Protein particle rates were also compared from one filling campaign to another to assess if the specific position of the batches in the campaign showed preferential protein aggregation.

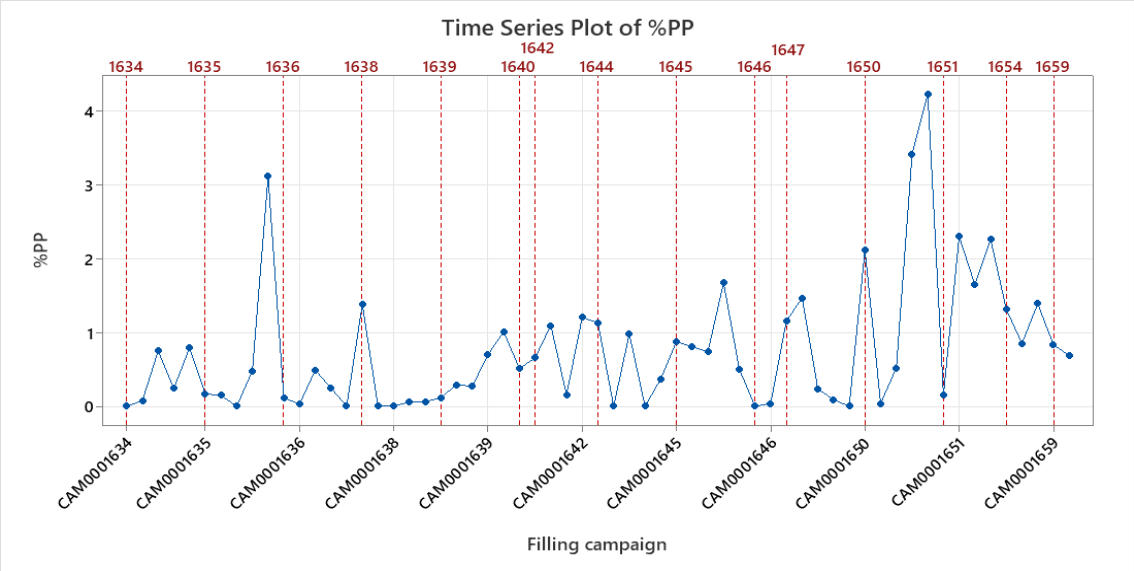


Figure 17. Repartition of the protein particles reject rates per filling campaign on FL3 from 09-Sep-2021 to 06-Dec-2021. Every first batch of a filling campaign is highlighted with a red dotted line

As shown on Figure 17, OOL batches are not grouped in specific campaigns. Large differences in protein particles reject rates can be seen from one batch to another within the same campaign. Similarly, no specific position in a campaign shows reproducible reject rate from one campaign to another.

To conclude, no specific link between filling campaigns and protein particles reject rate can be established.

6.6.4. Analysis of rejects over the filling sequence

On the filling line FL3, every vial of product receives a unique sequential identifier during the filling process. The unique IDs of all vials rejected for protein particles were recorded for 19 IGI 10% batches covering the whole range of reject rates segregated by formats 10g, 20g and 30g.

The statistical analysis of data collected suggests that the phenomenon at the origin of the protein agglomeration generates particles in clusters during the filling sequence independently from the total reject rate of a batch.

An example of distribution from the batch LE12X263 (20g, 3,4% reject rate) is provided on Figure 18 below. The left graph shows the repartition of the defective vial marked for a y value of 1. On the right, the run chart displays the number of conform vials between two rejected vials in the filling order. Smallest values indicate sequences of consecutive defective vials while highest values indicate series of conform vials. On the example given, the distribution is nonrandom and the p-value for clustering is 0,000.

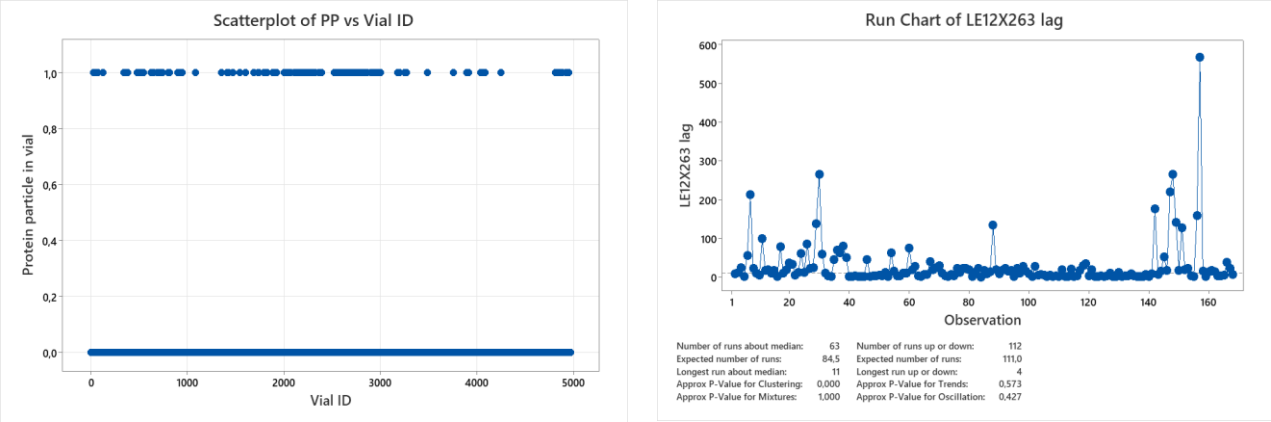


Figure 18. Distribution of the defects in the filling sequence of batch LE12X263

It was also demonstrated that the position of the clusters in the filling sequence is not repeatable from one batch to another. Therefore, there is no predominant moment or common patterns during the filling sequence for the generation of protein particles.

Hypothesis related to the clustering was that more protein particles are generated following a stop in the filling line due to increased shear forces, see section 6.5.5.

Note that the same analysis was reproduced for the two batches BE12C149 and BE12C150 with the highest protein particle reject rates observed in August 2022. For both batches the defects were distributed along the whole filling sequence. No specific pattern was identified by this analysis.

6.6.5. Evaluation of filling stops on protein particles generation

It was hypothesized that process stops during filling could generate protein aggregation and therefore explain to some extent the presence of the clusters observed along the filling sequence as presented in previous section 6.6.4.

To verify this hypothesis, the filling of the batch LE12X309 was stopped for two hours in the middle of the filling process. The distribution of the defective vials is displayed on Figure 19, each point represents a vial with conform vials identified at y=0 and rejected vials at y=1.

The result of the statistical analysis performed showed that the defects were spread before and after the stop and that no cluster of defects could be observed after the stop.

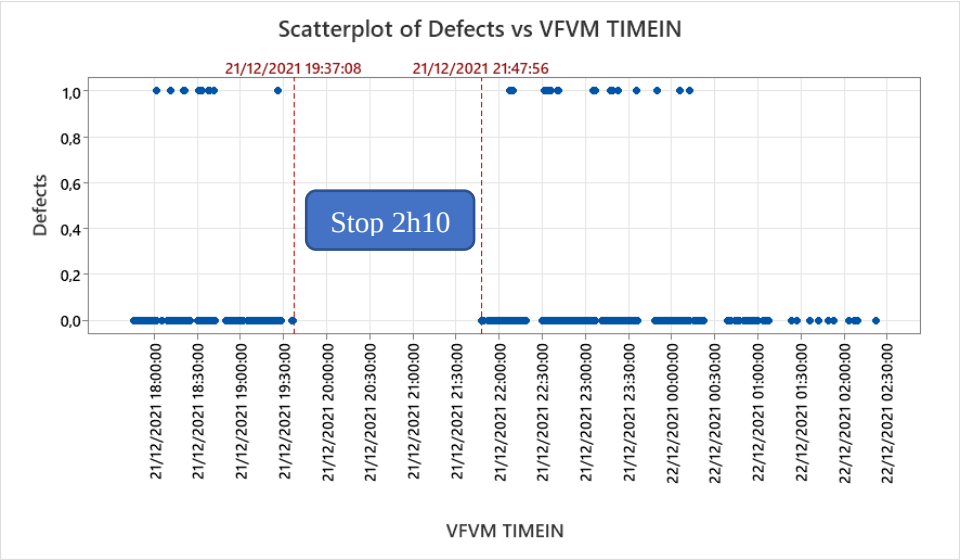


Figure 19. Distribution of defective vials in the filling sequence of batch LE12X309

Therefore, it was concluded that the stops did not contribute to a clustering effect and in the protein particle formation

6.6.6. Review of filling equipment

6.6.6.1. Mobile bulks, filling kit and stoppers processor

The different mobile bulks, filling kits (LTC) and stoppers processor tanks used during the period investigated in this report were reviewed to assess if the protein particles can be linked with one or several pieces of equipment specifically. This analysis was performed in the frame of the initial investigation and for the investigation of August 2022 covering the periods from June 2021 to December 2021 and from May 2022 to July 2022 respectively.

These reviews showed that conform and OOL batches were evenly filled between the different pieces of equipment considered.

The mobile bulks, filling kits and stoppers processors cannot be linked with the generation of protein particles.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to finishing processes.

6.6.6.2. Filling needles and orifices

The potential sources of mechanical stresses on the filling line were assessed by the investigation team. Due to their reduced diameter, filling needles and orifices were identified as potential sources of shear stress for the proteins.

The filling kit in Filling Line 3 is set-up with four large needles and two small needles which are connected via Silastic (platinum cured silicone) tubing and orifices with manifold. The manifold is thus filled with protein solution from mobile bulk via Silastic tubing. Note also that protein solution is filtered prior to filling the manifold with a 1.2 µm filter (see Figure 20). The vials filling is performed by applying the pressure in the manifold of 0,5 bar.

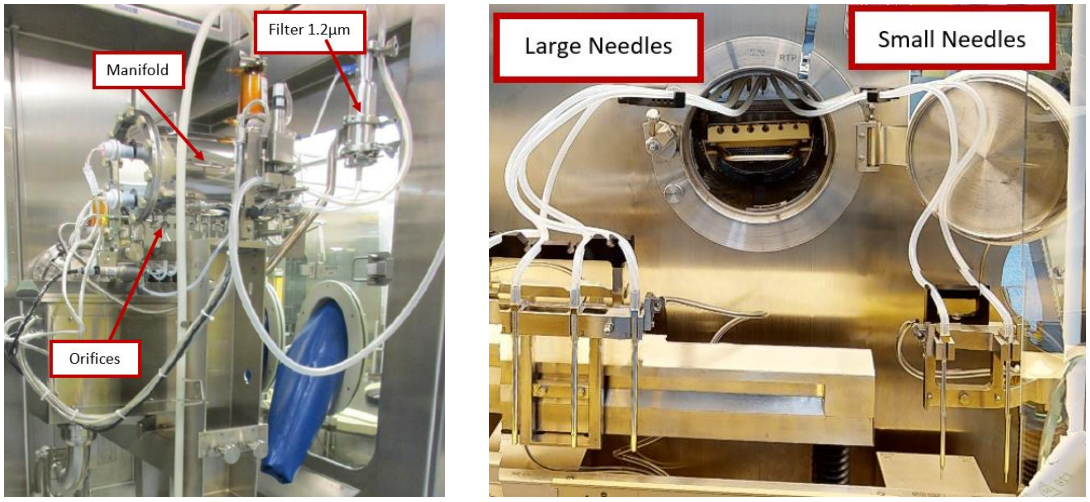


Figure 20. Pictures of the Filling Line 3 filling kit

A Study was conducted to evaluate if the needles, orifices and/or the needle position have any correlation with protein particles generation in the filled vials. For that, for five batches of IGI 10% filled in 300 mL vial size on the filling line FL3 between 09-Nov-2021 and 23-Nov-2021, the unique equipment ID of the set of needles, orifices and needle/orifice position used for filling of each vial was recorded.

A binary logistic regression analysis was performed and demonstrated a p-value ≥ 0.05 and a R-square value of 1,76%. Which concluded that there is no significant statistical correlation between a given set of equipment and the formation of protein particles in the vials. Therefore, the protein particles presence cannot be associated with needles ID, orifices ID and needle positions.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to finishing process.

6.6.7. Cleaning and sterilization of equipment

A review of the cleaning and sterilization of equipment used in the filling line FL3 for the production of IGI 10% batches that could contribute to the generation of protein particles was performed. This review was performed in the frame of the initial investigation and for the investigation of August 2022, covering the periods from June to December 2021 and from May 2022 to July 2022 respectively.

The following pieces of equipment were considered:

- Cleaning Out of Place (COP) stations used in Filling line 3 complex.
- Stopper processor equipment used to clean the stopper tanks.
- Cleaning In Place (CIP) stations: CIP 196.1, CIP 638.5 and CIP 431.1. Those stations are used to clean mobile bulks and are located in building A (SU2) and building C (SU3).

For all the equipment above, inline conductivity values and TOC, available values were reviewed. For all cycles within the considered periods no shift or trend was observed, and all values remained within their acceptable ranges.

During the initial investigation, a complementary review was performed concerning the Aniosteril cleaning solution, but no correlation was observed between the use of a specific batch of Aniosteril solution, and the protein particles reject rate.

Regarding the sterilization processes, the review covered:

- Sterilization In Place (SIP) of mobile bulk tank: this is performed on two stations LESIP683.1 and LESIP918
- Sterilization cycles for the stoppers processor
- Sterilization cycles of Belimed autoclave

The SIP stations used for the different mobile tanks were analyzed. Conform and OOL batches used mobile tanks that were evenly sterilized between the two stations. Since the stations are located in different buildings it was deemed unlikely that the same issue would have impacted both stations at the same time. A defect in the sterilization of the mobile bulks was ruled out.

The temperature and pressure parameters of the sterilization cycles for the stoppers processor and the Belimed autoclave were reviewed. For both considered periods, no shift or atypical values in those parameters were observed.

To conclude the reviews of all cleaning and sterilization processes from FL3 did not bring evidence that they are linked with the increase in protein particles levels in finished drug product.

See attachment #04 for the list and location within PR#2428796 of the documents providing further details related to cleaning and sterilization of equipment.

6.6.8. Presence of particles in the Filling/Set-Up environment

Review of the environmental monitoring data was performed to confirm absence of increase of particles that could be present in the vials and serve as nuclei for protein particle formation. This evaluation was performed in the frame of the initial investigation and for the investigation of August 2022, covering the periods from June to December 2021 and May 2022 to July 2022 respectively.

Due to the presence of filters in the product path on the filling system, this analysis was performed on the following set-up and filling area, including the continuous monitoring of air particles on the filling line:

- Filling Line 3 (FL3): rooms R901 and Filling Line 3
- Set-Up 3 (SU3): rooms 915, 918 and 924

This analysis was performed to confirm absence of increase of particles that could be present in the vials and serve as nuclei for protein particle formation.

No trend nor shift in particle counting was identified on the considered periods. Therefore, the presence of

particles coming from the filling or set-up environment and present in the vials is unlikely to be the cause of to the increase of protein particles.

See attachment #04 for the list and location within PR#2428796 of the documents providing further details related to environmental monitoring.

6.7. Post filling process review

Following the filling process, IgI 10% vials are kept in stainless steel cages and incubated prior to visual inspection. An overview of the process flow is detailed on Figure 21.

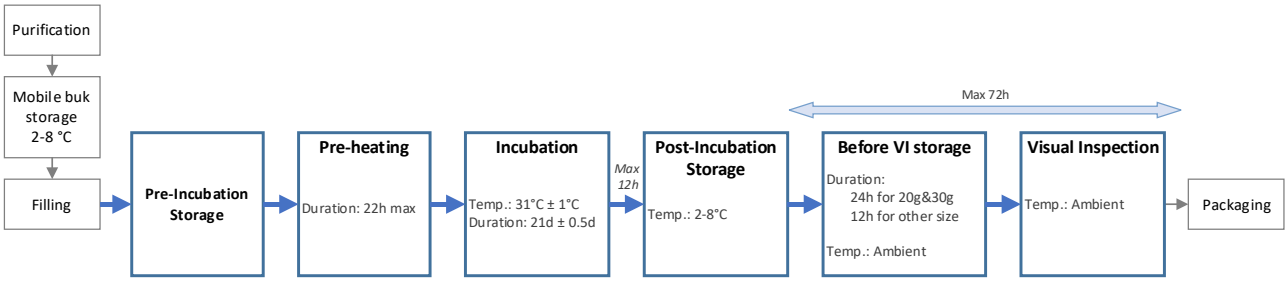


Figure 21. Post-filling flow of GAMMAGARD LIQUID/KIOVIG Final Container

To collect data regarding the different duration and temperature of the different steps, two tracking studies were conducted by installing temperature data loggers to each cage of each batch for:

1. 19 IGI 10% lots filled between 23-Nov-2021 and 23-Dec-2021: LE12X277 to LE12X309
2. 22 IGI 10% lots filled between 30-Aug-2022 to 26-Oct-2022: BE12C153 to BE12CD62

The hypotheses investigated are summarized in the table below.

Hypothesis	Investigation outcome
Storage between end of filling and incubation: storage conditions and duration of the product could generate more protein particles due to increased thermal stress or favorable kinetics conditions	No correlation was observed between protein particles reject rates and storage conditions.
Incubation process: differences in the incubation parameters and duration generate more protein particles due to increased thermal stress or favorable kinetics conditions	No correlation was observed between protein particles reject rates and incubation conditions.
Storage in cold room between incubation and visual inspection: storage conditions and duration of the product could generate more protein particles due to increased thermal stress or favorable kinetics conditions	Data analysis showed a weak relationship between protein particles reject rates and post-incubation storage conditions and durations. Investigations are still on-going to gather data to confirm or not the correlation.

Table 6. Post filling process hypothesis of protein particle generation

6.7.1. Pre-incubation storage

Based on data gathered thanks to temperature data loggers, a regression analysis was performed to identify if a correlation exists between the storage time before start of incubation (duration between end filling and start of incubation) and the reject rate of protein particles for the considered batches. As shown on Figure 22 no statistical correlation could be found.

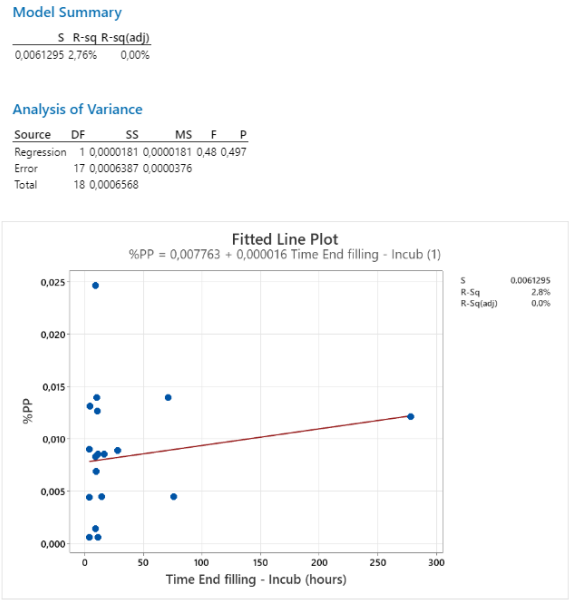


Figure 22. Regression analysis between the duration "end filling - start incubation" and the protein particles reject rate

The duration between filling and preheating was also assessed in the frame of the investigation of August 2022. The dataset for this analysis consisted in 30 batches processed on PL4 and FL3 visually inspected between 01-Jun-2022 to 17-Aug-2022. The results did not show any correlation between end of filling and start of the pre-heating before incubation. The lots with higher protein particles reject rates were comparable with the ones that did not exceed the limit.

Depending on the availability of the preheating chambers, some batches can be stored in refrigerated conditions (2-8°C) while some just remain at ambient temperature before pre-heating. To evaluate if this difference can influence the formation of protein particles, a comparison of the protein particles reject rate was done for both storage conditions. Using a 2-Sample T test showed that there is no statistical difference between the two populations.

A 2-Sample % defective test showed that there is no significant statistical difference between the vials that were stored in ambient conditions and the ones that were stored in refrigerated conditions, see DIV-TS-22-040.

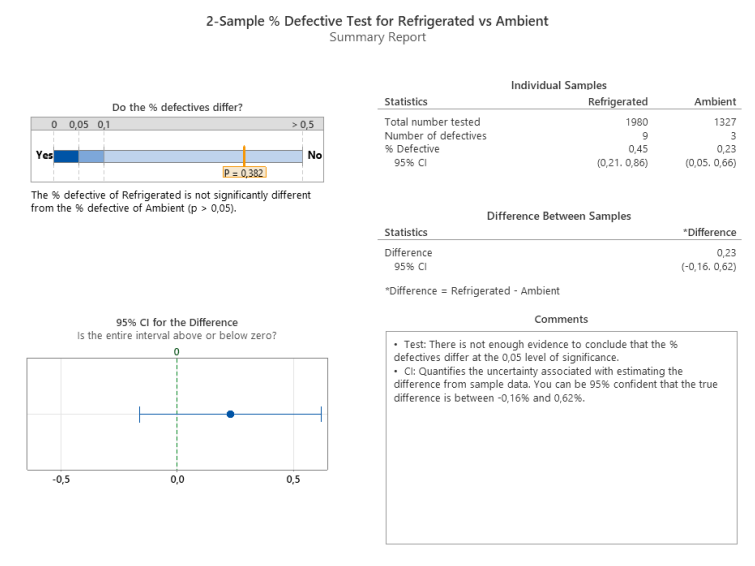


Figure 23. Comparison of the defect rates between the vials that were stored in ambient or refrigerated conditions

Based on the analyses previous presented it is concluded that duration and conditions of storage before the incubation is unlikely to be the root cause of the occurrence of protein particles.

6.7.2. Incubation process

Following the filling, the vials are incubated for 21 days at 30°C – 32°C for the low pH viral inactivation process (process step #19). Prior to entering the incubator, the vials are pre-heated to bring the temperature up to 30°C – 32°C. Three preheating chambers are available: the chambers 1 and 2 for which the preheating duration is 22h and the chamber 3 for which the preheating duration is 7h.

During the investigations, reviews of the pre-heating and incubation processes were performed to determine if any changes or unusual events occurred or if there was a correlation of increased protein particles to a specific pre-heating chamber or incubator location. In particular, the following systems and parameters were reviewed:

- Preheating chamber, duration, and temperature.
- Incubation duration and temperature.

For each of the parameters above, no evidence of a correlation with protein particles reject rate was found.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to incubation review.

6.7.3. Post incubation storage

The overall duration between end of incubation and start of visual inspection covers several transfers to different storage locations and conditions (sub-steps). Therefore, the final product is submitted before visual inspection to several thermal transitions, see Figure 21:

1. The transfer of the finished product cages from incubator to the cold room (ambient temperature)
2. The storage in the cold rooms (2-8°C)
3. The transfer of the finished product cages to the visual inspection area and the standby time before visual inspection (ambient temperature)

This overall duration was identified as a contributive factor to protein particle occurrence during the particle investigation from 2019 (see PR#1399546). Even if during the investigation no strong correlation/regression model could be established, it was however noticed that batches for which this duration was below 9 days tend to produce more OOL batches than the others with longer storage duration before visual inspection. Therefore, the visual inspection planning was setup to have minimum 9 days between the end of the incubation and the start of visual inspection and a routine monitoring was performed to evaluate the efficacy of this organization.

This duration was assessed end of 2021 as part of the root cause investigation covered by the present report. At that time, it was concluded that this duration was unlikely to be the root cause of the protein particle formation as the efficacy of having 9 days between end of incubation and start of the visual inspection was not demonstrated based on the trends presented in Figure 24.

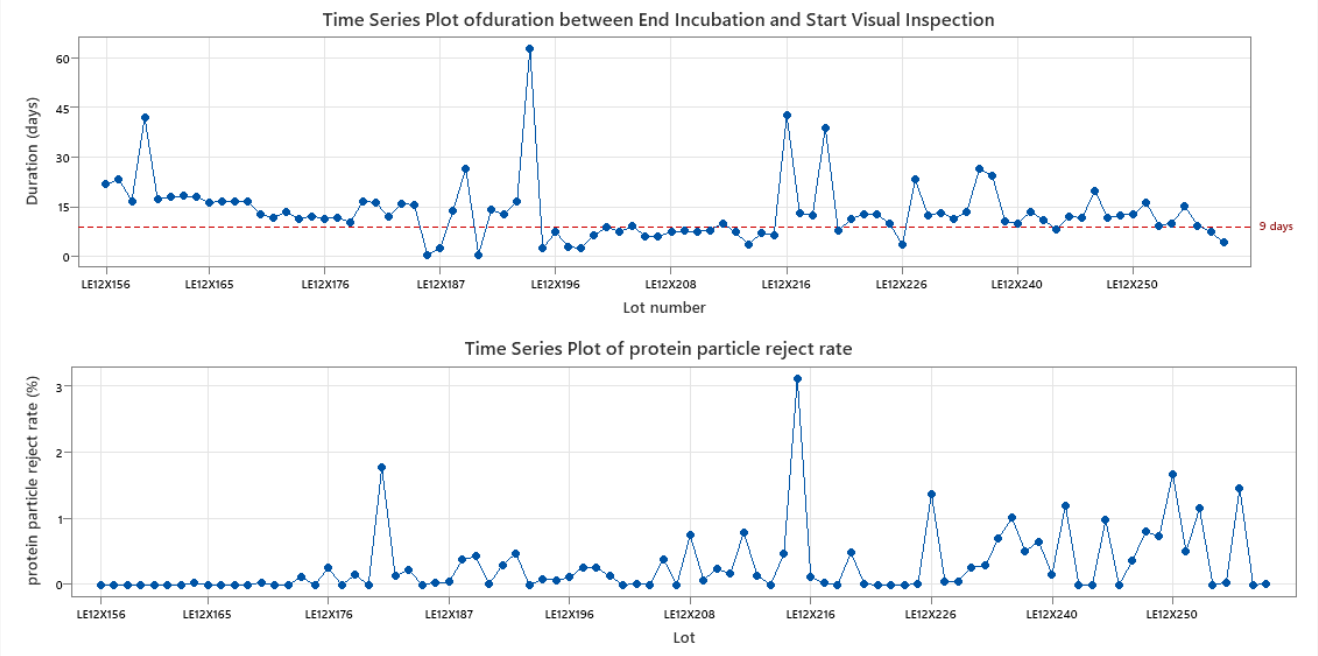


Figure 24. Protein particles reject rate and duration between end of incubation and start visual inspection from lot LE12X156 to LE12X259

In order to evaluate whether if one of the sub steps could be the root cause of the protein particle formation, the data collected thanks to the temperature data logger installed on each cage of 19 batches in the frame of the first study presented in section 6.7 were analyzed. The reject rate for protein particle for each cage was determined.

After incubation the cages of finished product are transferred to the cold room R503 or R504 where they are kept at 2-8°C, see Figure 21. In the cold room each rack has nine positions with unique identification.

To assess if the cold rooms themselves could influence the protein particles formation, a 2- Sample T test was performed to determine whether the population means differ between lots stored in cold room R503 and lots stored in cold room R504. It concluded that the means of protein particles reject rates for each cage were statistically not different between the batches that were stored in the cold room R503 versus that were stored in the cold room R504. Therefore, the cold room used does not explain the occurrence of protein particle at 100% visual inspection.

A second analysis, that included statistical analysis, comparative analysis and Gemba walk, was performed to evaluated whether if a specific position of a cage within the cold room could lead to higher protein particles occurrence. For the different batches of the study, the protein particles reject rates of each cage was compared with its position in the cold room and the rack it was placed on. No specific rack or position in a rack could be identified as contributor for protein particles as the reject rates were comparable between all positions and racks. The analysis showed that the pallets having the highest protein particles reject rates were evenly distributed within the cold room. In addition, a review of temperature trends of the cold rooms R503 and R504 was also performed between July 2021 and December 2021. The temperature remained within target ranges and the trends had similar patterns from month to month. The maximum delta between the hot and cold points of the cold rooms was 2°C. Based on the previous elements it was concluded that the location within the cold room does not explain the occurrence of protein particle at 100% visual inspection.

For the transfer and storage durations, based on data loggers of each cage of the 19 batches, regression models were performed to identify for each cage any correlation (1) between the duration between end of incubation and the beginning of storage in cold room and the protein particles reject rates and (2) between the storage time in cold rooms and the protein particles reject rate. No significant correlations could be established for these two durations and the protein particle reject rate of each cage (see Figure 25).

Based on these analyses it was concluded that the protein particle reject rate for each cage is not explained by the transfer to or the storage in the cold rooms.

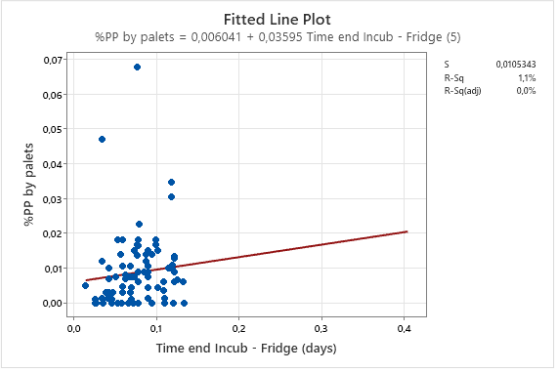
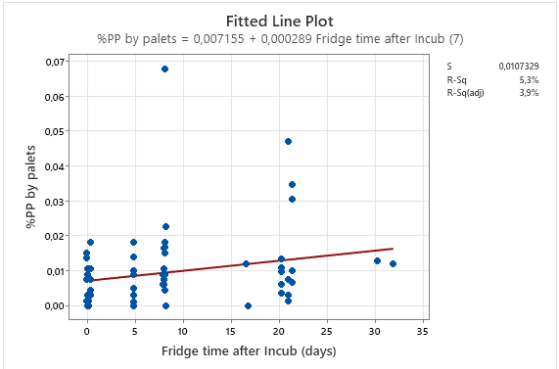
Time between end of incubation to storage in cold room	Storage time in cold room after incubation
Model Summary S R-sq R-sq(adj) 0,0105343 1,07% 0,00% **Analysis of Variance** Source DF SS MS F P Regression 1 0,0000981 0,0000981 0,88 0,350 Error 82 0,0090997 0,0001110 Total 83 0,0091978  	**Model Summary** S R-sq R-sq(adj) 0,0107329 5,26% 3,91% **Analysis of Variance** Source DF SS MS F P Regression 1 0,0004481 0,0004481 3,89 0,053 Error 70 0,0080636 0,0001152 Total 71 0,0085116 

Figure 25. Regression analysis models between protein particles reject rates for each cage and post incubation conditions and duration

After the storage in cold room, the cages are moved out and let to warm up at ambient temperature before visual inspection. This is done to avoid having condensation on the glass vials that would infer with the visual inspection.

A regression analysis was performed between the duration between the time out of the cold and the start of the visual inspection and the protein particle reject rate of each cage. Over the group of 19 batches considered no significant correlation could be established.

The overall duration was again assessed in the frame of the investigation. The dataset for this analysis consisted in 30 batches processed on PL4 and FL3 visually inspected between 01-Jun-2022 to 17-Aug-2022. The results did not show any strong correlation between the protein particle reject rate and the duration between end of incubation and start of visual inspection. However, due to different thermal transitions that occurred during this step it was decided to continue to investigate.

As described in section 6.7, a second tracking study, ME-TS-22-118 was performed on 22 IGI 10% batches produced on Purification Line PL4 and filled on Filling Line FL3 from 30-Aug-2022 to 26-Oct-2022, with a focus on 10, 20 and 30g formats. This tracking study consisted in recording the temperature data between end of incubation and start of visual inspection by installing temperature data logger on each cage. For 22 batches, the duration between the end of incubation and the start of visual inspection was compared to the protein particle reject rate. As shown on Figure 26, again no strong correlation could be identified on this dataset. However higher reject rates are mainly observed for the shorter storage time (< 48 hours).

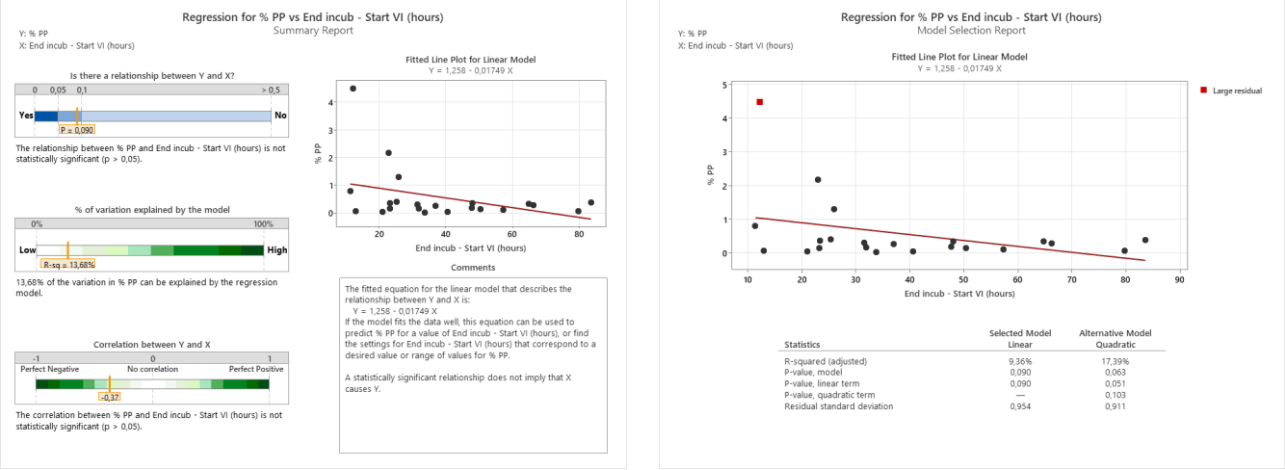


Figure 26. Regression model between end of incubation and visual inspection and the protein particle reject rate.

The exact duration of each sub-step between end of incubation and start of visual inspection is not tracked in routine and might vary from one batch to another. Those variations were not analyzed globally for a lot but for each cage of a lot as described above. From this perspective, no definitive conclusion can be drawn from this analysis regarding the exact role of the intermediate durations and thermal transitions between incubation and visual inspection regarding the occurrence of protein particles.

Based on the latest analysis performed and described above it was decided as a mitigation to implement a minimum storage time in the fridge after incubation of 48h, see CAPA related to PR#2428796. The thermal transitions and their potential effect on Protein particle occurrence will be further investigated from end of Filling to start of visual inspection.

6.7.4. Reject reinspection

During the August 2022 investigation, since a few batches experienced higher level of reject rate for protein particles than before the packing shutdown, it was decided for investigational purpose to reinspect the rejected vials from the batches BE12C149 and BE12C150, see attachment #06.

On a significant number of vials, the visual inspectors were not able to detect protein particles anymore. Moreover, no vial containing a large number of protein particles was reported for any of the two batches.

To better characterize this phenomenon multiple re-inspections of the rejected vials stored at 2-8°C were requested, both from Quality Operations (QO) and Production teams.

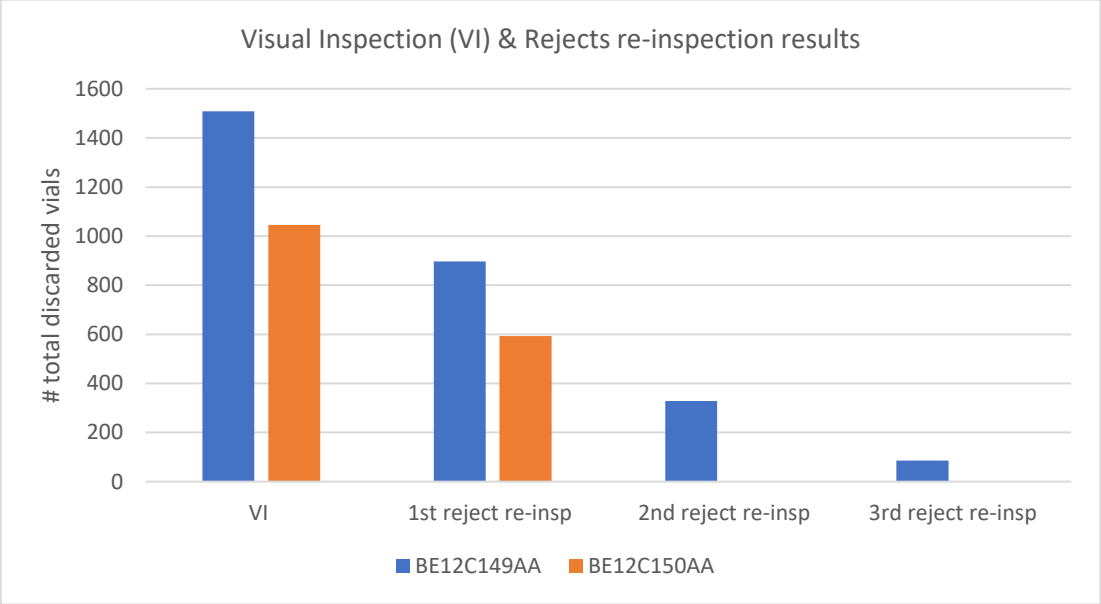


***Figure 27**. Evolution of the observation of protein particles over multiple inspections*

As shown on Figure 27, the number of vials presenting protein particles decreased after each visual inspection. It demonstrates the potential transient formation of visible particles and morphology change mechanism of protein particles. The temperature and/or the manipulation of the vials during visual inspection are suspected to contribute to the morphology change and the re-solubilization of the particles. Further experimental studies are ongoing under DIV-TS-22-036 to better understand this phenomenon.

6.8. Utilities

A review of the utilities was performed at the initial investigation and during the August 2022.

All changes related to utilities and implemented at Lessines plant between 01-Jun-2021 and 31-Nov-2021 and between 25-Jun-2022 and 20-Jul-2022 were reviewed (see section 6.2.3). There was no change identified that could explain the occurrence of protein particles.

Additionally, all corrective and preventive maintenance activities related to utilities feeding PL4 and FL3 and performed before the manufacture of the lots above action limit for protein particles were reviewed (see section 0). All reviewed Work Orders have been evaluated to represent only a low risk for the generation of protein particles.

During the initial investigation, analyses results performed on the WFI production system used during the formulation step on PL4 (WFI 110) over a time period going from 12 June 2021 to 03 December 2021 were reviewed to confirm the absence of change and/or shifts and trends. Considering that online conductivity, online TOC, microbiological counts, LAL, TOC, nitrate, and conductivity controls do not show any trend, it was concluded that the quality of WFI produced on site and used for CIP activities and during the formulation process was unlikely to have contributed to the generation of protein particles.

During the August 2022 investigation, the scope of the utilities review was extended and included:

- City water
- Ekopak water
- Deionized water (DIW)
- Water for injection (WFI)
- Compressed air

Analyses results performed on the WFI production system over the period going from 01-May-2022 to 20-Jul-2022 were reviewed. None of the measured parameters showed shifts or concerning trends.

Based on the above, the quality of the utilities used for the purification or filling processes cannot have contributed to the generation of protein particles in the finished product.

Refer to attachment #04 for the list and location within PR#2428796 of the documents providing further details related to utilities review.

7. TIMELINE AND MITIGATION ACTIONS

After set-up of the investigational multi-disciplinary team, multiple investigation actions have been conducted starting from October 2021 to investigate different hypotheses and improve the understanding of the issue. It must be noted that due to incubation duration (step 19 - minimum 21 days) and to operational planning constraints between purification, filling, incubation and visual inspection, the effect of an action on the protein particles reject rate is generally of one month.

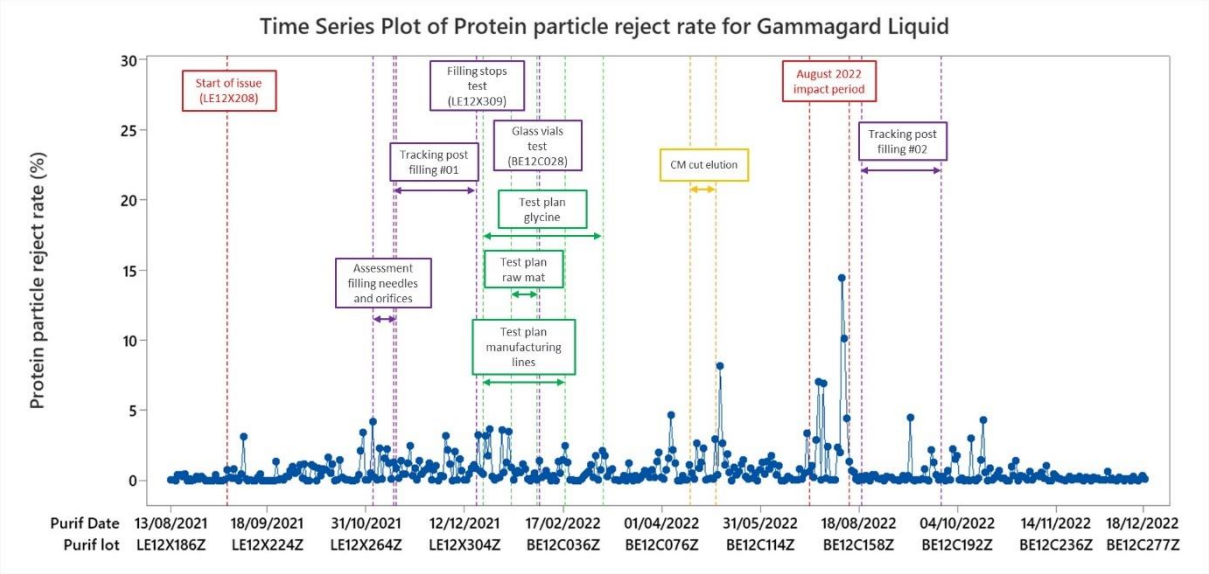


Figure 28. Timeline of the investigation experiments relative to batch purification process date

The different investigation actions are detailed in Table 7 below.

Action	Date/period of realization
Assessment filling needles and orifices: evaluation of the influence of filling equipment	09-Nov-2021 to 23-Nov-2021 (filling date)
Tracking post filling: assessment of finished product cages flow after filling	23-Nov-2021 to 23-Dec-2021 (filling date)
Test plan glycine: assessment of different sources and packaging of glycine	12-Dec-2021 to 02-Mar-2022
Filling stops test: assessment of the influence of process stops during filling in FL3	21-Dec-2021 (filling date)
Test plan manufacturing lines: comparison of the different purification and filling lines	18-Jan-2022 to 18-Feb-2022
Test plan raw materials: assessment of different raw materials batches for Triton X-100, Tween 80, Tris Base, Acetic Acid 1N	23-Jan-2022 to 03-Feb-2022
Glass vials test: assessment of the influence of glass vials lots	09-Feb-2022 (filling date)
CM cut elution: assessment of the influence of reduced CMS chromatography eluate volumes	21-Apr-2022 to 30-Apr-2022
Tracking post filling #2: assessment of finished product cages flow after filling	30-Aug-2022 to 04-Oct-2022 (filling date)

Table 7. Details of the different investigation actions and experiments performed

Following the progress of the investigation, mitigation actions have been implemented in the course of the investigation.

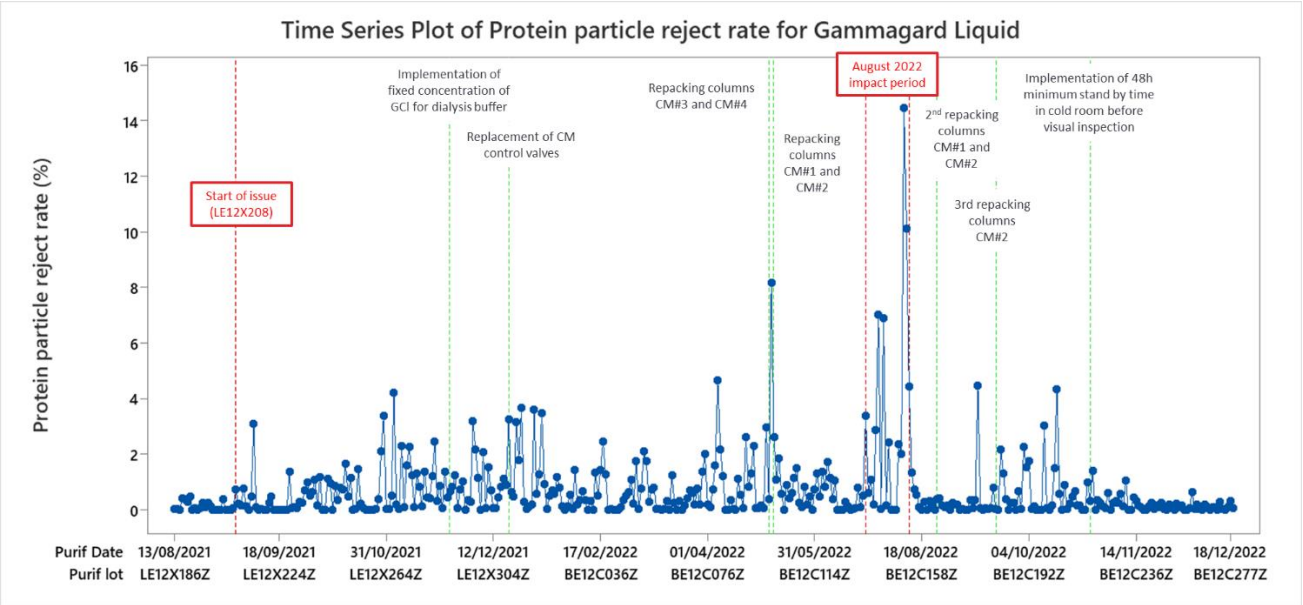


Figure 29. Timeline of mitigation actions relative to batch purification process date

The different mitigation actions are detailed in the Table 8 below.

Action	Date of implementation	Date of visual inspection results
Implementation of fixed concentration for dialysis buffer preparation using HCl 6N	27-Nov-2021	03-Jan-2022
Replacement of control valves on CMS columns	11-Jan-2022 Winter Shutdown 2021	10-Feb-2022
Repacking CM#3 and CM#4	02/04-May-2022	03-Jun-2022
Repacking CM#1 and CM#2	09/12-May-2022	10-Jun-2022
Second repacking CM#1 and CM#2	11 to 16-Aug-2022 Summer Shutdown 2022	21-Sep-2022
Third repacking CM#2	20/21-Sep-2022	22-Oct-2022
Implementation of 48h minimum stand by time in cold room before visual inspection.	22-Nov-2022	22-Nov-2022

Table 8. Mitigation actions implemented in the frame of the investigation

8. ACTIONS (CAPA)

Because of the absence of formal root cause identified by the investigation, no CAPA plan could be defined.

The following actions are nonetheless considered to reduce the different stresses that can be applied to the proteins during Lessines' process and eventually lead to protein aggregation.

- Preparation of the dialysis buffer with fixed quantities in HCl 6N: implemented on 27-Nov-2021 and refer to PR#2529017 (PL4) for the definitive implementation.
- Modification of the Trans Membrane Pressure (TMP) regulation for UF/DF. This change aims at reducing the number of recirculation within the tangential flow filtration system and therefore minimize the exposure of the proteins to shear stress during UF/DF step. Refer to PR#2754707.

Process robustness efforts will continue in Lessines to keep reducing stress contributors within the process. Further evaluation and limitation of thermal stress on final product will be followed as a CAPA type: Continuous Improvement.

9. PRODUCT IMPACT ASSESSMENT

Throughout the investigation, several intermediary reports were issued to document Product Impact Assessments of lots having shown initial visual inspection results above the action limit of protein particles (documented in PR#2428796).

Each of those assessments indicated that any potential impact on the quality, safety, purity and efficacy of the concerned Ig 10% lots was negligible based upon the different elements discussed here below.

9.1. Particles identification and characterization

As described in section 4 above, particles have been identified and confirmed of proteinaceous nature per SEM-EDX and FTIR analyses. Capillary electrophoresis testing further confirmed the protein nature of the particles as being IgG. This is consistent with the other protein particles characterized within previous investigations (ME-TS-21-086 V2)

Additional characterization was performed on vials of impacted lots (with and without particles) to generate a further understanding of the particles composition on the impacted samples. The extended characterization included: MFI, DLS and Elemental Analysis through Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES). The test results showed similar elemental composition, contradicting any potential external agent based on the protein particles generation.

The index of the different PIAs and associated batches is described in Attachment #07;

9.2. 100% FDP Visual Inspection

For the visual inspection, operators are trained and qualified to reject vials showing different defects, including «Protein Particles» as per SOP-048674.

All IgI 10% batches are 100% visually inspected, followed by an AQL (Acceptance Quality Level) performed by quality (according to SOP-005032). All AQL (0.65% for major defects) were satisfactory, except for lot BE12C149 for which the reinspection and tightened AQL performed were found satisfactory. For each batch that exceed the established limit, reinspection was evaluated according to SOP-005032.

The index of the different PIAs and associated batches is described in Attachment #07.

9.3. In-Process parameters

The Process Performance Indicators (PPIs) for the IGI 10% process have been defined in the global CPV plan VV-00919304 and the acceptance criteria are documented in pFMEA VV-00676447. All the PPIs included in the CPV plan were reviewed.

The review of PPIs performed in previous versions of Product Impact Assessments demonstrated that each OOL lot (in which atypical reject rates for protein particles were observed) was not associated with an impact on the consistency of the process. The analysis also demonstrated that OOL lots are comparable in terms of normal process variability to non-OOL lots. The review of selected CPP demonstrated that critical process parameters were all found within their validated ranges.

As critical process parameters remain under control and no impact on the PPIs was observed, no impact on critical quality attributes was expected at final container level, that was confirmed by the review discussed here above.

Details of in-process parameters for those lots can be found in the different intermediary PIA documented in PR#2446118. The index of the different PIAs and associated batches is described in Attachment #07.

9.4. Final container testing

All final container testing of IgI 10% lots were performed and reviewed against specifications. All testing results were within acceptable ranges and within trends.

Each lot was also covered by a trending analysis that did not highlight out of trend (OOT) results that could be due to the observation of protein particles

Results of all QC assays on final container were satisfactory and within control limits.

Documentation can be found in in each different intermediary PIA (see Attachments in PR#2446118). The index of the different PIAs and associated batches is described in Attachment #07.

9.5. Sub-visible particles

Subvisible particle testing were monitored as per USP <788> and European Pharmacopoeia (EP) 2.9.19 and collected for IgI 10% lots produced since July 2020.

All IgG 10% lots were compliant to the USP 788 and EP2.9.19 and are within historical trends.

Table below summarizes applied limits for subvisible particles testing.

Particle size	Format ≤ 100mL	Format > 100mL
Particles ≥ 10 µm	Limit: ≤ 6000 part./vial	Limit: ≤ 25 part./ml
Particles ≥ 25 µm	Limit: ≤ 600 part./vial	Limit: ≤ 3 part./ml

Table 9. Light obscuration particles count test limits

Results are documented in intermediate PIAs (see PR#2446118). The index of the different PIAs and associated batches is described in Attachment #07.

9.6. Root cause investigation

The root cause investigation (see section 6) rejected potential external contamination source for OOL batches. This never indicated a potential presence of extraneous agent, nor any specific indication that would have voided the release of the lots.

In each intermediary Product Impact Assessment, an update on the progress of the root cause analysis was provided. Elemental Analysis (ICP-OES) and volatile impurities analysis by Headspace Gas Chromatography (Headspace C/QQQ) on impacted and non-impacted samples versus reference lots demonstrated similar compositions, contradicting any potential external agent on the basis of the protein particle generation.

9.7. Additional characterization & extra testing

Additional characterization has been performed on several lots in the course of the investigation. Testings performed were related to the following:

- IgG functional activity, such as IgG subclasses distribution and Fc function
- Product related impurities, such as C3, Albumin, Plasminogen and IgE
- Process related impurities, such as Alcohol, Silicon and Aluminium
- Tests for thrombogenic potential evaluation, such as PL-1 and FXI protein
- Additional antibody titer, i.e. *Haemophilus influenzae*

All the results are conform and within the historical range.

Results are documented in intermediate PIAs (see PR#2446118). The index of the different PIAs and associated batches is described in Attachment #07.

9.8. Stability study

In the past, 11 historical lots impacted between 2017 (2020 report GGL-R-083594) and 2019 (2022 final report GGL-R-200320) were put onto stability for:

- 36 months at 5°C (long-term)
- 24 months at 25°C / 60% RH (long-term)
- 3 months at 40°C / 75% RH (accelerated)

The reports conclude that results are within acceptance criteria and demonstrate the unimpaired product quality over the monitoring period of 36 months, whatever the initial level of protein particles rejection rate.

Since 2021, five extra lots (LE12X215; LE12X268; LE12X250; BE12C117; BE12C149) were put onto stability (protocol # GGL-D-162277 and protocol GGL-D-216202) to evaluate the accelerate and long-term stability, because of protein particles observed.

The study is ongoing but the results available to date indicate that there are no differences in stability behavior from the stability indicating attributes (see Attachment #08):

- Lots LE12X215; LE12X268; LE12X250:
  - after 12 months at + 5 °C,
  - after 12 months at + 25 °C / 60 % RH,
  - and after 3 months at + 40 °C / 75 % RH

- Lot BE12C117:
  - after 6 months at + 5 °C,
  - after 6 months at + 25 °C / 60 % RH,
  - and after 2 months at + 40 °C / 75 % RH

9.9. Retain samples reinspection

In addition, 5 retain samples stored at 2-8°C of 10 OOL batches were visually inspected. See Table 10 below and Attachment #09, and no particles were found in the vials.

Lot number	Protein Particle found
BE12C149AA	0
BE12C150AA	0
BE12C117AA	0
BE12CC41AA	0
BE12C142AA	0
BE12C080AA	0
BE12C176AA	0
BE12C154AA	0
BE12C206AA	0
LE12X268AA	0

Table 10. Retain samples visual inspection results

9.10. Health Hazard Evaluation

A medical assessment was established in 2017 to document potential health / safety risks associated with protein particles intravenous injection. Rating was concluded as serious only for hypersensitivity reactions, but these can occur with any number of proteinaceous particles, serious hypersensitivity reactions are very rare. Rating was concluded as negligible as the harm from a very limited number of visible proteinaceous particles that get trapped in the lungs is clinically insignificant (see Attachment #10):

Extract from HHE for GGLQ/Kiovig indicated that:

Visible and subvisible particles (> 5-7 micrometers in diameter) regardless of their nature that enter the blood stream via intravenous infusion will be trapped in arterioles or the capillary vessels of the pulmonary arterial tree, depending on their caliber. A permanent obstruction of an affected arteriole or capillary is more likely to occur if the particles consist of cellulose, teflon, or other extraneous materials. Collaterals at the alveolar level can compensate for obstructions at the capillary level.

Intravenous solutions meeting USP 790 are essentially free of visible particulates, i.e. the number of units detected to contain visible particulates upon inspection must not exceed a specified limit. For products such as GGL that may contain inherent visible protein particles or agglomerates, additional specifications are included in the respective approved regulatory application documents. GGL is also in compliance with the USP 788 specifications for sub-visible particulates. GGL is not expected to cause any clinically significant impairment of lung perfusion / function from visible or sub-visible proteinaceous particulate matter.

The potential of protein aggregates to elicit immunogenic reactions is an established fact. In vaccines this property is exploited for medical benefit. Less clear is a presumed correlation of immunogenicity and protein aggregate size. As the example of adjuvants in vaccines demonstrates, immunogenicity can be very high in sub-visible particles that allow orderly presentation of peptides on their surfaces. Sub-visible and visible aggregates in plasma-derived solutions manufactured in the 1950's and 1960's have been shown to be associated with hypersensitivity reaction (see discussion below). In summary, the incidental presence of a visible protein aggregate in addition to the sub-visible aggregates that are deemed acceptable per USP 788 is not expected to substantially alter the hypersensitivity potential of a biologic.

Extract from HHE for HyQvia indicated that (refer to attachment #10):

HyQvia (GGL plus hyuluronidase) is administered by subcutaneous injection/infusion (hypodermoclysis) and absorbed into the circulatory system predominantly via lymphatic capillaries in the subcutaneous tissue. While the wall of a lymphatic capillary is very tenuous and permeable to fluids, it is practically impenetrable for any size of particular matter. Thus, the issue of micro-embolism as discussed for GGL does not apply to HyQvia and will not be discussed.

The potential of protein aggregates to elicit immunogenic reactions is an established fact. In vaccines this property is exploited for medical benefit. Less clear is a presumed correlation of immunogenicity and protein aggregate size. As the example of adjuvants in vaccines demonstrates, immunogenicity can be very high in sub-visible particles that allow orderly presentation of peptides on their surfaces. Sub-visible and visible aggregates in plasma-derived solutions manufactured in the 1950's and 1960's have been shown to be associated with hypersensitivity reaction (see discussion below). In summary, the presence of a visible protein aggregate in addition to the sub-visible aggregates that are deemed acceptable per USP 788 is not expected to substantially alter the hypersensitivity potential of a biologic.

9.11. AE and non-medical complaints assessment

In 2022 pharmacovigilance review were performed in relation to proteinaceous particles impacted lots and potentially related adverse events. To assist with the evaluation of protein particles which have exceeded current limits an assessment of the impact of the protein particles on the safety, efficacy, and quality of the product has been established (see Attachment #11).

Review and analysis were performed from the period JAN 2019-MAY 2022 on the potential adverse reactions and potential health impact that may occur as a result of the detection of protein particles (exceeding current limits) considering the entire population and any subgroups of the population that might be at higher risk for adverse reactions. Exercise was done on:

- 100 lots where no vials were rejected due to protein particles during visual inspection in Lessines (rejection rate 0%) and
- 100 lots with a rejection rate > 1%

The search of the Takeda Global Safety Database (GSD) identified a total of 123 cases:

- 71 cases with the 0% rejection rate lots
- 52 cases with the lots with > 1% protein particle rejection rates.

Overall, the events were consistent with the known safety profile of immunoglobulins therapy. Of note, no thromboembolic events were reported for either the impacted or non-impacted batches in relation to protein particles rejection rate.

In conclusion the risk of thromboembolic events is low. The chronic administration and the potential for many particles to be administered over time, could result in potential clinical consequences. However small numbers of particles are not likely to result in any adverse effects on patient safety.

9.12. GPS assessment

A Global Pathogen Safety (GPS) was performed for both Gammagard Liquid/Kiovig and HyQvia products and concluded that the virus clearance data on file for low pH inactivation was valid for the affected lots by proteins particles (see Attachment #12 “Proteinaceous Particles Found in Final Container – Virus Safety Impact Assessment for low pH treatment”)

9.13. Preclinical studies performed with IGI 10% protein particle

Two nonclinical GLP studies were conducted to compare the bronchospastic anaphylactoid potential (Study 8439891) and the local tolerance (Study 8441553) of two different lots of GAMMAGARD LIQUID/KIOVIG, lots with or without visible protein complexes, see ME-TS-22-055 in Attachment #13.

For the two preclinical studies performed in 2020, test vials were selected with several proteinaceous particles and or large proteinaceous particles during a visual inspection process by qualified and trained operators. Operators identified vials with large particle (≥1mm) or with more particles <1mm that can be reliably counted i.e., more than 4 protein particles.

8439891 study “*Anaphylactoid bronchospastic potential in anaesthetized guinea pigs*” was performed in order to document the absence of specific effect of IGI 10% products containing proteinaceous particles. Intravenous administration of several IGI 10 % lots with particles <1 mm in size and with particles ≥1 mm in size at a target dose level of 1000 mg/kg showed no bronchospastic anaphylactoid potential in the guinea pig. Positive bronchospastic anaphylactoid reactions were defined as an increase in pulmonary inflation pressure of ≥30% lasting for ≥1 minute within 10 minutes of injection.

8441553 study "*Local tolerance study in the rabbit following intravenous and subcutaneous administration*" was also performed in order to document the absence of specific effect of IGI 10% products containing proteinaceous particles. Administration of IGI 10 % several lots with particles <1 mm in size and with particles ≥1 mm in size was locally well tolerated (based on observations at injection site and body weight recording) and was not associated with test item related change at the injection sites (necroscopy, histology and pathology evaluations) following a single intravenous and/or subcutaneous injection, compared to an IGI 10% without particle.

These two nonclinical GLP studies were conducted to compare the bronchospastic anaphylactoid potential and the local tolerance of two different lots of GAMMAGARD LIQUID, lots with or without visible protein complexes. For all tested lots no anaphylactoid potential was detected, and all lots were well-tolerated. In conclusion, based on the presented nonclinical studies, no increased anaphylactoid potential or potential for local irritation could be detected for the lots with visible particles compared to the lots without particles.

10. CONCLUSION

An increased level of visible IgG particles in GAMMAGARD LIQUID/KIOVIG/HYQVIA (IgI 10% (Human)) finished products were observed during the 100% visual inspection process, on lots manufactured since September 2021 up to October 2022 (purification date). With an average of ~1.5% reject rate for protein particles at 100% visual inspection in the past year.

The particles have been identified and confirmed as being of proteinaceous nature with size from 170 µm to 2785 µm.

A cross functional team with internal and external experts has been working on this investigation since the detection of the trend to further evaluate all influencing factors and potential causes of protein particle formation, including learnings from previous events. As protein particles have been observed in lots manufactured starting from different Precipitate G (PptG) sources, on all purification PL1/PL4 and filling FL1/FL3 lines, the investigation covered the different process steps from Fractionation to 100% visual inspection of final containers, including utilities. Batch records, events, changes, process parameters, maintenance activities, calibrations, and raw materials of the IgI 10% manufacturing process were assessed for the related batches.

Starting from the known aggregation mechanisms, hypotheses for the presence of protein particles were proposed and assessed. In parallel documentation records and process parameters were reviewed to identify potential changepoints in the process. Despite the thorough investigation concerning a total of 19 hypotheses performed, with a special deep dive on four hypotheses namely, designated raw materials, final pH adjustment practices, CM chromatography and Filling parameters/sequence, no formal root cause was confirmed that could explain the increased reject rates observed since September 2021. Note that in August 2022, five IgI 10% lots produced higher reject rates, with one lot up to 14%. Based on this cluster, a focused investigation was performed to identify the specific drivers, however no contributive factors could be identified.

Robustness actions concerning the CM chromatography step, the pH adjustment practices and the duration between incubation and visual inspection were nonetheless implemented to further reduce the stress factors identified in the process liable to participate in the protein aggregation mechanism. Process robustness improvement efforts will continue to improve current knowledge regarding protein aggregation in IgG manufacturing process and reduce further protein particle occurrence at visual inspection.

Even if no clear link were established, since implementation of several actions to reduce the stress factors, from November 2022 visible protein particle reject rate levels are back within normal trend, with more than 30 consecutively produced lots with visible protein particles reject levels below the action limit and comparable to the period before the start of the issue.

Based on the 100 % visual inspection and AQL results, sub-visible particles testing, final container testing, additional characterization, pre-clinical data, and stability data, preclinical studies, health hazard assessment and pharmacovigilance review confirm that the protein particles do not affect quality, safety and efficacy of IgI 10%.

11. ATTACHMENTS

#	**Description of attachment**
01	List of all Gammagard Liquid batches exceeding the action limit
02	Particle Characterization
03	Extra testing
04	Index of attachments of PR#2428796
05	ME-TS-23-007_Summary report of the experiment plan related to protein particle deviation PR#2428796
06	Memos_Reject reinspection of lot BE12C149AA and BE12C150AA
07	Index of Product Impact Assessments for PR#2428796
08	Stability reports for lot LE12X215, LE12X268, LE12X250, and BE12C117
09	Memo_Retain Samples reinspection
10	Health Hazard Evaluations
11	Medical assessment Protein Particle 2022
12	Global Pathogen Safety (GPS) assessment
13	ME-TS-22-055_Preclinical studies on rabbit and guinea pig for protein particles in Gammagard Liquid / Kiovig / HyQvia