



Life Sciences

Validation Guide

USTR2969

Allegro™ 50 L Single-Use Mixer Biocontainers



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1 Validation Overview

1.1 Introduction

This document contains data applicable to the Allegro 50 L single-use mixer biocontainer. The validation guide documents testing that has been performed to demonstrate the suitability of Allegro 50 L single-use mixer biocontainer for use in biotechnology and pharmaceutical applications. The mixer biocontainer has the same manufacturing process and identical materials of construction to other 3D biocontainers and mixers in the Allegro range. Some of the information related to validation can also be found in the latest revision of the validation guide for the Allegro 200 L single-use mixer (Pall document reference number USTR2777), and the Allegro 2D and 3D biocontainers and totes (Pall document reference numbers USTR2475 and USTR2527). Any additional materials are documented in this guide.

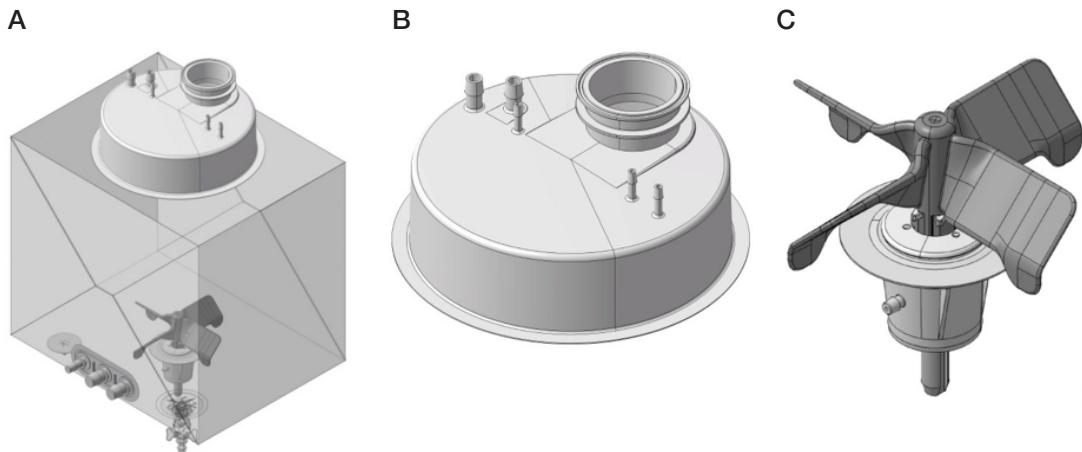
The Allegro 50 L single-use mixer biocontainer includes some unique design features that significantly improve the ease of use and robustness of the single-use system while enabling efficient mixing of a wide range of solutions and high product recovery. There are four (4) inlet ports and two (2) outlet ports. There is one inlet and outlet port on the top of the biocontainer for controlling the air flow to and from the biocontainer, the three additional inlet ports on the top of the biocontainer are for acid/base addition and initial fluid filling, and the last outlet port is for fluid draining. The outlet/drain port incorporates a flush port valve. When the outlet port is closed, it is flush with the bottom of the biocontainer therefore no solids or concentrated solution can get trapped in the outlet line.

Figures 1a, 1b and 1c show a typical Allegro 50 L single-use mixer biocontainer.

Various powder addition bags are also available along with a powder bag support frame. Details on the operation of the Allegro single-use mixer are provided in Pall document reference number USD2915: Allegro 50 L single-use mixer instructions for use.

Figure 1

Typical Allegro 50 L single-use mixer biocontainer (A) with powder port (B) and seal housing/impeller (C) shown in more detail



1.2 Summary of Conclusions

1.2.1 Gamma Irradiation Dose

Unless otherwise stated, components were irradiated using gamma radiation at a dose of 45-55 kGy prior to any testing.

- 1.2.2 Water Leak Test**
This test is aimed at allowing a service life claim of one (1) day. The leak tests confirmed that the manufacturing process produces Allegro 50 L single-use mixer biocontainers that are leak-proof and able to withstand normal use for at least 48 hours continuous service, which is twice the claim of 1 day continuous mixing at maximum impeller speed with appropriate mixer totes.
- 1.2.3 Drainage and Drain Valve Operation**
The drain valve operation test showed good drainage and a high level of product recovery (water) from the Allegro single-use mixer biocontainer.
- 1.2.4 Resistance to Gamma Sterilization**
The tests conducted on the Allegro 3D biocontainers, which use the same materials and methods of construction of the film as the mixer biocontainers, confirmed that the welds maintained their tensile strength after being subjected to gamma-irradiation up to 100 kGy.
The tests conducted on the Allegro 50 L single-use mixer biocontainers showed that the biocontainers were leak proof after gamma irradiation up to a maximum dose of 50 kGy.
- 1.2.5 Connections Testing**
The tests conducted for the Allegro 50 L single-use mixer biocontainers verified that the tubing attached to the molded connection piece of the biocontainers does not pull off when attached using an appropriately tensioned cable tie. No leakage was detected at the connection when subjected to a maximum applied pressure of 1.03 barg (15 psig) for 15 seconds before and after gamma irradiation. The connections withstood a maximum 6.5 kg pull test force for 15 seconds before and after gamma irradiation.
On the Allegro 50 L single-use mixer biocontainers the drain valve flange connection was also tested. It worked properly without leaks when tested before and after gamma irradiation. Testing was conducted during mixing mode and static mode.
- 1.2.6 Extractables Study**
The purpose of this study was to quantify and characterize the chemicals that may be extracted out from the materials of construction of Allegro mixer biocontainers when exposed to different solutions and time periods. The analysis of the extractant after 14 days (low density polyethylene (LDPE) components), 28 days (high density polyethylene (HDPE) components) and 91 days (biocontainer film), identified compounds which are detailed in Tables 6, 7 and 8.
- 1.2.7 Biological Safety Tests**
The materials used in the Allegro mixer biocontainers, when tested after after gamma sterilization, meet the requirements of the USP <88> Biological Reactivity Tests *In Vivo* for Class VI Plastics. The materials used also meet the requirements of the USP <87> Biological Reactivity Tests *In Vitro*, Cytotoxicity.
- 1.2.8 Physicochemical Tests**
The purpose of these tests was to evaluate the physicochemical suitability of the Allegro 50 L mixer biocontainer when tested to USP <661> Containers. The materials used in the Allegro 50 L mixer biocontainer meet the requirements of USP<661>.

1.2.9 Particle Content

Particle testing was performed to demonstrate that Allegro 50 L mixer biocontainer meets the requirements of USP <788> Particulate Matter in Injection when analyzed for particle count and sizing by optical microscopy.

1.2.10 Bacterial Endotoxin

USP <85> Bacterial Endotoxins testing was performed on components used in the construction of the Allegro 50 L mixer biocontainer. The components of the Allegro 50 L mixer biocontainers meet the requirements of USP<85>.

1.2.11 Shelf Life Studies

Shelf life studies have established a three (3) year shelf life for the Allegro 2D and 3D biocontainers as well as the Allegro 200 L single-use mixer biocontainers all subjected to 45-55 kGy gamma irradiation, stored under controlled accelerated aging conditions and at ambient conditions for real time aging. Tests on the influence of gamma sterilization on the mechanical strength of connectors, seal weld, and cross crease fold were performed. Leak testing was also performed as a further test of the integrity of the seals.

The Allegro 50 L single-use mixer biocontainers use the same manufacturing methods and material of construction for the main biocontainer film as for other 2D and 3D biocontainers and mixers in the Allegro range. For information on the Allegro biocontainer film please refer to USTR2527 (validation guide for Allegro 3D biocontainers and totes). For information on the seals, bearings, connections and impeller, please also refer to USTR2777 (validation guide for Allegro single-use mixer biocontainers).

A three (3)-year full shelf life study is currently underway on the Allegro 50 L single-use mixer biocontainer. Samples of representative Allegro 50 L single-use mixer biocontainers will be subjected to a leak test, tensile strength test of the outer welds, and peel test on welded connections after 6 months, 1 year and 3 years of accelerated aging and 3 years of real time aging. Reports will be available upon request as developed.

2. Water Leak Test

2.1 Overview

This testing has been performed as part of the validation of the Allegro 50 L single-use mixer biocontainers. The aim of the test is to establish that the manufacturing process has produced biocontainers that are leak-free and able to withstand normal use. For this test, three different manufacturing batches were tested. Unless otherwise stated, components were irradiated using gamma radiation at a dose of 45-55 kGy prior to testing.

2.2 Summary of Methods

The Allegro 50 L single-use mixer biocontainers were leak tested with the impeller running. Thirty (30) biocontainers from 3 batches were tested. All biocontainers were gamma irradiated prior to testing. The biocontainers were then filled with water to 50 L. The impeller speed was set to 200 rpm and run for a total of 48 hours, with periodic checks for leaks.

2.3 Results

There were no leaks seen at any time point or at any locations on the Allegro mixer biocontainer during the 48 hours of testing in the mixing mode, as summarized in Table 1.

Table 1
Water leak test results

Batch Number	Biocontainer Number	Test Temperature (°C)	Test Time (hrs)	Pass/Fail
W131526	2	2	48	Pass
W131526	3	2	48	Pass
W131526	4	2	48	Pass
W131526	10	2	48	Pass
W131526	11	2	48	Pass
W131526	5	40	48	Pass
W131526	7	40	48	Pass
W131526	8	40	48	Pass
W131526	9	40	48	Pass
W131526	13	40	48	Pass
W131527	3	2	48	Pass
W131527	4	2	48	Pass
W131527	5	2	48	Pass
W131527	8	2	48	Pass
W131527	9	2	48	Pass
W131527	14	40	48	Pass
W131527	15	40	48	Pass
W131527	16	40	48	Pass
W131527	18	40	48	Pass
W131527	19	40	48	Pass
W131528	12	Ambient	48	Pass
W131528	13	Ambient	48	Pass
W131528	14	Ambient	48	Pass
W131528	15	Ambient	48	Pass
W131528	17	Ambient	48	Pass
W131528	1	Ambient	48	Pass
W131528	16	Ambient	48	Pass
W131528	18	Ambient	48	Pass
W131528	19	Ambient	48	Pass
W131528	20	Ambient	48	Pass

2.4 Conclusions

The tests confirmed that gamma irradiated Allegro 50 L single-use mixer biocontainers remained integral and leak free after 48 hours of running from 2 °C to 40 °C. Applying a 100% safety margin on operational life, the maximum continuous operational life claim for the Allegro mixer biocontainers is therefore 1 day between 2 °C and 40 °C.

3. Residual Drainage Testing

3.1 Overview

Testing was performed to observe the drainage of the Allegro single-use mixer biocontainers and to measure the remaining fluid volume in the biocontainer after gravity drainage.

3.2 Summary of Methods

An Allegro 50 L single-use mixer biocontainer, filled with 50 L of RO (reverse osmosis) water was drained through the drain valve. Following complete drainage and without manual intervention, the residual volume of water in the biocontainer was measured.

3.3 Results

Hold up volume of Allegro 50 L mixer biocontainer after drainage was 60 mL.

3.4 Conclusions

Only a minimal amount of fluid remains in the biocontainer following drainage without any operator intervention.

4. Resistance to Gamma Irradiation

4.1 Overview

Validation of the Allegro 50 L single-use mixer biocontainer's resistance to gamma irradiation is confirmed based on work conducted previously on the standard Allegro 3D biocontainer products and the testing outlined in this validation guide. For more information, please refer to the latest revision of Allegro 3D biocontainers and totes validation guide (Pall document reference number USTR2527). The material of construction and manufacturing processes for the Allegro 50 L single-use mixer biocontainers are the same as for the standard Allegro 3D biocontainers which are used in general purpose fluid management applications.

4.2 Summary of Methods

As part of the Allegro 3D biocontainers validation, ten (10) samples of a 240 L volume 3D biocontainer were manufactured for these tests using the same polymer film and manufacturing methods used for the manufacture of all Allegro biocontainers, including the Allegro 50 L single-use mixer biocontainer. Prior to the gamma irradiation, all of the biocontainers were tested for leaks using a forming gas. The biocontainers were then gamma irradiated between 35 and 50 kGy. Five (5) of the biocontainers were then gamma irradiated for a second time between 35 and 50 kGy (total dose between 70-100 kGy). The tensile strength of the welded seal of each biocontainer was checked using a tensile tester. The sample strip was 25 mm wide and the tensile force was applied at an elongation rate of 200 mm/min. During this test the force at which the film broke must exceed 70 Newtons (N).

The water leak testing, detailed in section 2, was used to determine whether or not the Allegro 50 L single-use mixer biocontainers could withstand the maximum recommended dose of 50 kGy.

4.3 Results – Allegro 3D Biocontainers

The tensile strength of the film used to make all Allegro 3D biocontainers exceeded 70 Newtons. The strength of the welded regions exceeded 70 Newtons.

4.4 Results – Allegro 50 L Single-use Mixer Biocontainers

There were no leaks seen at any time point at any location on the Allegro 50 L single-use mixer biocontainer during the 48 hours when the motor/impeller was running.

4.5 Conclusions

The tests confirmed that gamma irradiated Allegro 50 L single-use mixer biocontainers remained integral and leak free after 48 hours of running. Applying a 100% safety margin on operational life, the maximum continuous operational life claim for the Allegro mixer biocontainers is therefore 1 day.

5. Connections Testing

5.1 Overview

The purpose of this test was to verify that the tubing connections to the molded connection pieces of the Allegro 50 L single-use mixer biocontainers are leak free and strong when using an appropriately tensioned cable tie. Unless otherwise stated, components were irradiated using gamma radiation at a dose of 30-50 kGy prior to testing.

In strength testing, the connection was subjected to various mechanical loads as appropriate to the type of connection, please see table below. All connections held securely during this test. The leak test consisted of holding the tubing on the inlet/outlet port under water for 15 seconds with the connection at various internal pressures, please see table below. This test was also repeated on these connections after gamma irradiation. All connections passed with no leakage.

In operation, connection validation was confirmed for the outlet drain valve through the extensive operational filling and drainage testing conducted as part of the design verification.

This is described below.

5.2 Summary of Methods

The connections of the Allegro 50 L single-use mixer biocontainer have also been tested for leaks and subjected to a mechanical strength test. The actual test parameters have been varied to be appropriate to the components under test – for example the connection to the probes has been tested at a lower pressure as this seal will not actually be under pressure in service. All tests were performed pre and post gamma irradiation and at temperatures from 2 to 40 °C. These are standard tests and are performed on all Allegro single-use systems prior to confirmation of design of the single-use system and approval to manufacture. Figure 2 shows schematic of the Allegro 50 L single-use mixer biocontainer with the various ports labelled.

Figure 2

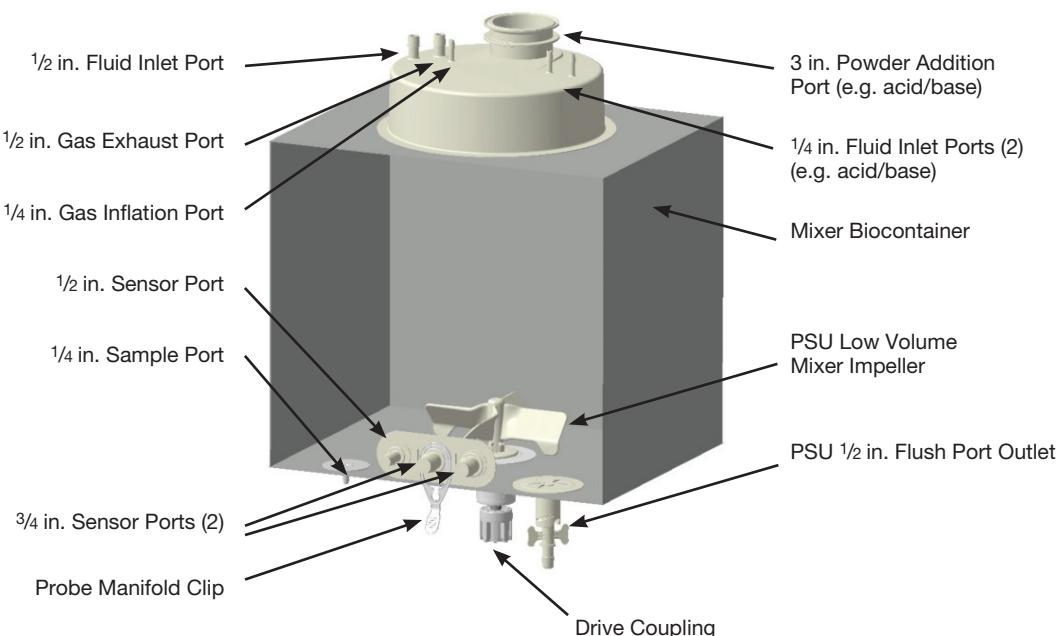


Table 2
Junction test parameters

Junction	Pressure	Time	Pull Force
½ inch sensor probe seal to ½ inch sensor port on manifold	1 bar (15 psi)	30 seconds	6.5 kgf
½ inch Kleenpak® sterile connector to ¾ inch sensor probe seal	0.55 bar (8 psi)	30 seconds	2.0 kgf
¾ inch sensor probe seal to ¾ inch sensor port on manifold	0.55 bar (8 psi)	30 seconds	2.0 kgf
½ inch Kleenpak sterile connector to silicone bellows	0.2 bar (3 psi)	30 seconds	0.5 kgf
¼ inch sample port to tubing	1 bar (15 psi)	30 seconds	2.0 kgf
¼ inch fluid/gas inlet port to tubing	1 bar (15 psi)	30 seconds	2.0 kgf
½ inch fluid/gas inlet port to tubing	1 bar (15 psi)	30 seconds	6.5 kgf
½ inch drain valve to tubing	1 bar (15 psi)	30 seconds	6.5 kgf

Individual connections were tested under pressure with tensile force applied for the time stated as detailed in Table 2. If the connection remained integral under the pressure and force conditions stated for the required time then the connection was deemed to be secure and appropriate for use.

In addition to this, thirty (30) biocontainers were water leak tested, as described in Section 2, and then drained, providing ‘in operation’ testing of the drain valve connection when cable tied with appropriate tension to tubing. During drainage the drain valve/tubing interface was monitored visually to ensure there was no leakage from the drain valve or from the valve/tubing connection.

No observed leakage from the interface between the drain valve and the connected tubing confirms a secure connection.

5.3 Results

During leak testing (section 2) and drainage testing (section 3) of the Allegro mixer biocontainer all drain valve/tubing connections remained leak proof as detailed in Table 3.

Table 3
Results of drain/tubing connection leak test during drainage

Batch #	Biocontainers Per Batch	Test Temperature	Testing Time (hours)	Drain/Tubing Leak Test – Pass/Fail?
W131526	10	2 °C and 40 °C	48 hrs	Pass
W131527	10	2 °C and 40 °C	48 hrs	Pass
W131528	10	Ambient	48 hrs	Pass

All individual connections, when tested at the conditions detailed in Table 2, remained secure and integral for the duration of the test.

5.4 Conclusions

When using appropriately sized tubing according to the hose barb sizes on the Allegro 50 L single-use mixer biocontainer inlet and probe ports and drain valve, the connections are leak free when using cable ties at appropriate tension. All connection ports can withstand normal use for a period of 1 day.

6. Extractables Testing

6.1 Overview

The purpose of the extractables/leachables study was to quantify and characterize the volatile and non-volatile residues or components that may be extracted/leached out from typical Allegro biocontainers, which includes the Allegro 50 L single-use mixer biocontainer, when exposed to different solutions.

All Allegro 2D and 3D biocontainers use the same manufacturing method and materials of construction for the film and molded connection pieces. Therefore, the extractables/leachables test results are applicable for all types of Allegro biocontainers which includes the Allegro 50 L single-use mixer biocontainer. The data presented are taken from the validation of 2D and 3D Allegro biocontainer testing.

6.2 Summary of Extractables/Leachables Methods for Allegro Biocontainer Film and Polyethylene Material Used for Molded Connection Pieces

The Allegro biocontainer film along with LDPE molded pieces were gamma irradiated at a dose 26-32 kGy. These were extracted for 91 days as described in Table 4.

Table 4
Contact fluids and tests applied to tested Allegro biocontainers

Analytical Test Methods	Contact Fluid						
	PBS - pH3	WFI	PBS - pH11	NaCl 3 M	Tween 80 - 1% in WFI	Ethanol 96%	DMSO 10%
Allegro biocontainer filling and recording of weight (empty + filled)	ABC	ABC	ABC	ABC	ABC	BC	BC
pH measurement	ABC	ABC	ABC	ABC	ABC	BC	BC
Conductivity	ABC	ABC	ABC				
Total organic carbon (TOC)	ABC	ABC	ABC				
Metals (ICP-OES)	ABC	ABC	ABC	ABC	ABC	BC	BC
Headspace GC-MS	ABC	ABC	ABC	ABC	ABC	BC	BC
Solvent extraction + GC-MS	ABC	ABC	ABC	ABC	ABC	BC	BC
Solvent extraction + LC-MS	ABC	ABC	ABC	ABC	ABC	BC	BC
Ion chromatography	ABC	ABC	ABC	ABC	ABC	BC	BC
Derivatization GC-MS	ABC	ABC	ABC	ABC	ABC	BC	BC
	40 °C 75% RH	25 °C	20 °C				
Storage conditions							

Key

- Biocontainer - 30 day incubation (A)
- Biocontainer - 90 day incubation (B)
- Control - Glass bottle (C)
- PBS – phosphate buffered saline
- WFI – water for injection
- DMSO – dimethyl sulfoxide
- RH – relative humidity
- GC – gas chromatography
- LC – liquid chromatography
- MS – mass spectrometry.

Seven bags made using the Allegro biocontainer film, to give an internal surface area of 1625 cm² and a maximum volume of 5 liters, and the polyethylene material used for Allegro molded connection pieces, were each filled with 3.25 L of contact fluid via a peristaltic pump to provide an extraction ratio of 2 mL per cm² of test film material.

The extractable/leachable test fluids from the bags made using the Allegro biocontainer film were compared with control samples from a glass bottle. The glass bottle only samples were stored at the same conditions as the test samples. These samples were analyzed as described in Table 4.

6.3 Summary of Extractable/Leachables Methods for Additional Materials Used in the Biocontainers Used for the Allegro 50 L Single-Use Mixer

Extractables/leachables tests on components were conducted by submerging each material part into either ethanol or water for injection (WFI), then storing in the control environment of 40 °C for 14 days (30 days in the case of HDPE material) before analysis. Blank solutions were stored in glass bottles used as a reference solution. All samples are irradiated at 50 ± 5 kGy prior to the test. The same surface area/volume ratio of 2 cm²/mL of extraction solvent for each material is applied. The samples were then subjected to the analytical tests described in Table 5.

Table 5
Extractables testing on additional Allegro 50 L single-use mixer biocontainer components

	Connection Ports, Top Hat (LDPE)	Seal Housing (HDPE)		Endcap,Impeller, Clip (Polysulfone)		Seal* (UHMW PE)		O-ring* (Silicone)	
Extraction Solution	Ethanol, 99%	WFI	Ethanol 99%	WFI	Ethanol, 99%	WFI	Ethanol 99%	WFI	Ethanol 99%
pH	—	✓	—	✓	—	—	—	—	—
Conductivity	—	✓	—	✓	—	—	—	—	—
TOC	—	✓	—	✓	—	✓	—	✓	—
Metals (ICP)	✓	✓	✓	✓	✓	—	—	—	—
Headspace									
GC/MS	✓	✓	✓	✓	✓	—	—	—	—
GC/MS	✓	✓	✓	✓	✓	—	—	—	—
LC/MS (NVR)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anions (IC)	✓	✓	✓	✓	✓	—	—	—	—

* not in fluid contact in normal use.

✓ Test conducted

— Test not conducted

6.4 Extractables/Leachables Testing Results

6.4.1 Extractables/Leachables Results for Allegro Biocontainer Film and Polyethylene Material Used for Molded Connection Pieces.

The bags made using the Allegro biocontainer film, together with LDPE material used for the molded connection pieces (after gamma irradiation dose of 26-32 kGy), were filled with 7 different contact fluids. No significant loss in weight (evaporation), change in pH or change in conductivity was observed after 30 days and 91 days exposure to the contact fluids. The fluid analysis after 91 days of contact revealed low concentration of extractables in comparison to the glass bottle used as a control.

Virtually all identified extracted/leached chemical entities attributed to the biocontainer see Table 6, are explainable as either oligomers of the polymer used or degradation products from the antioxidants inherent therein.

Table 6

Analytical summary of extractables/leachables analysis for Allegro biocontainers, containing film molded connection pieces

Extractant

Level	WFI	PBS-pH3	PBS-pH 11	3M NaCl	96% Ethanol	1% Polysorbate 80	DMSO 10%
2-10 ppm					1-3-di-tert-butylbenzene		
1-2 ppm	TOC	TOC			C8-alkenes		
0.1-1 ppm		Acetate Hexanal	TOC	Fatty acids	Acetate, Antioxidant degradation, Alkenes (C9+)	1-octene C8-alkenes Methylcyclo pentane 1-3-di-tert- buty lbenzene	1-3- di-tert- butylben zene Antioxidant
10-100 ppb	Acetate Antioxidant degradation/ Di-tert- butylphenol	Antioxidant degradation/ Di-tert- butylphenol	2-methyl- 1-propene Di-tert- butylphenol	Di-tert- butylphenol	Alkanes		
5-10 ppb	2-methyl- 1-propene 2-octanone		Antioxidants	Hexanal			
<5 ppb	Antioxidant			Antioxidants 2-methyl-1- propene			

TOC - Total Organic Carbon

WFI – Water for Injection

PBS – Phosphate Buffered Saline

DMSO - Dimethylsulphoxide

6.4.2 Extractables//Leachables Results for the Additional Materials Used in the Biocontainers for the Allegro 50 L Single-Use Mixer.

Contact fluids did not show a significant change in pH or conductivity after 14 days of contact (30 days in the case of HDPE material). Additionally, virtually all identified extracted/leached chemical entities are explainable as oligomers of polymer used, polymer additives or degradation products from the antioxidants inherent therein (Tables 7 and 8).

Table 7

Summary of extractables/leachables data for additional parts used for the Allegro 50 L single-use mixers

LDPE Tested as Part of Allegro Single-Use Mixer Validation		Polysulfone Tested as Part of Allegro Single-Use Mixer Validation				HDPE Tested as Part of Allegro STR 200 Biocontainer Validation				
% of Fluid Contact Surface Area	26.40%	6.30%				0.94%				
Contact Solution (extraction time)	Ethanol, 99% (14 days)		WFI (14 days)		Ethanol, 99% (14 days)		WFI (30 days)		Ethanol, 99% (30 days)	
Time Point	T=0	T=3 yrs	T=0	T=3 yrs	T=0	T=3 yrs	T=0	T=3 yrs	T=0	T=3 yrs
pH	—	—	6.11	5.5	—	—	4.9	—	—	—
Conductivity	—	—	<50 µS/cm	<5 µS/cm	—	—	7 µS/cm	—	—	—
TOC	—	—	0.4 ppm	2.7 ppm	—	—	10 ppm	Data Not Available	—	Data Not Available
Metals (ICP)	Al: <0.14 ppm Na: 0.3 ppm	Ba: 0.2 ppm Ca: 1.8 ppm Cr: 0.2 ppm Fe: 0.2 ppm Mg: 0.2 ppm Mn: 0.03 ppm Na: 3 ppm Ni: 0.16 ppm	Ca: 0.94 ppm Mg: 0.04 ppm Na: 3.28 ppm Zn: 0.22 ppm	Ca: 0.18 ppm Na: 0.24 ppm Zn: 0.42 ppm	Al: 0.07 ppm Zn: 0.05 ppm	Cr: 0.05 ppm Mg: 0.03 ppm Na: 0.01 ppm	Ca: 0.73 ppm Fe: 0.09 ppm Mg: 0.044 ppm Na: 0.1 ppm Zn: 0.24 ppm	Ca: 0.82 ppm Fe: 0.009 ppm Mg: 0.01 ppm Zn: 0.018 ppm	Ca: 0.82 ppm Fe: 0.009 ppm Mg: 0.01 ppm Zn: 0.018 ppm	Ca: 0.82 ppm Fe: 0.009 ppm Mg: 0.01 ppm Zn: 0.018 ppm
Volatile Organic Compounds (Headspace GC/MS)	Alkanes: 28.9 ppm	None Detected	None Detected	None Detected	None Detected	None Detected	Tert-Butanol 0.020 ppm	Alkanes 128 ppm Alkenes 0.37 ppm	Alkanes 128 ppm Alkenes 0.37 ppm	
Semi-volatile Organic Compounds (GC/MS)	4 Hydrocarbons detected with total conc: 38 ppm	13 Hydrocarbons detected with total conc: 59 ppm	None Detected	None Detected	None Detected	None Detected	2,4-Di-tert-butylphenol 0.23 ppm	Alkanes ⁴ 229 ppm 1,3-Di-tert-butylbenzene 49 ppm ⁴ 2,4-Di-tert-butylphenol 5.9 ppm Stearic Acids 14.5 ppm	Alkanes ⁴ 229 ppm 1,3-Di-tert-butylbenzene 49 ppm ⁴ 2,4-Di-tert-butylphenol 5.9 ppm Stearic Acids 14.5 ppm	
Non-volatile Organic Compounds (LC/MS)	None Detected	None Detected	None Detected	Erucamide 0.057 ppm	None Detected	None Detected	Unknown compounds 0.41 ppm 2,4-Di-tert-butylphenol ³ 0.23 ppm 7,9-Di-tert-butyl-oxaspiro ^(4,5) deca-6,9-diene-2,8-dione ³ 0.22 ppm	Fatty Acids 57 ppm 2,4-Di-tert-butylphenol ³ 4.4 ppm	Fatty Acids 57 ppm 2,4-Di-tert-butylphenol ³ 4.4 ppm	

Table 7 (continued)

	LDPE Tested as Part of Allegro Single-Use Mixer Validation	Polysulfone Tested as Part of Allegro Single-Use Mixer Validation				HDPE Tested as Part of Allegro STR 200 Biocontainer Validation		
		Acetate: 6 ppm	None Detected	Formate: 0.1 ppm Phosphate: 0.2 ppm	Acetate 0.6 ppm Formate 0.8 ppm	Acetate: 0.1 ppm	Nitrate 1 ppm Phosphate 12 ppm	Acetate 2.9 ppm Formate 2 ppm
Anions (IC)								

¹Concentration between the detection limit and the quantification limit, therefore only indicative.²Of the 13 compounds detected 7 compounds were close to the reporting limit of the equipment of 2.4 ppm³Most probable compound⁴Most probably oligomers of HDPE due to chain scission by gamma radiation**Table 8**

Summary of extractables/leachables data for additional parts used for the Allegro 50 L single-use mixers

	UHMW PE				Silicone Seal O-rings			
% of Fluid Contact Surface Area	0.02%				0.06%			
Contact Solution	WFI Ethanol, 99%				WFI Ethanol, 99%			
Time Point	T=0	T=3 yrs	T=0	T=3 yrs	T=0	T=3 yrs	T=0	T=3 yrs
pH	-	-	-	-	-	-	-	-
Conductivity	-	-	-	-	-	-	-	-
TOC	34 ppm	22 ppm	-	-	63 ppm	86 ppm	-	-
Metals (ICP)	-	-	-	-	-	-	-	-
Volatile Organic Compounds (Headspace GC/MS)	-	-	-	-	-	-	-	-
Semi-volatile Organic Compounds (GC/MS)	-	-	-	-	-	-	-	-
Non-volatile Organic Compounds (LC/MS)	None Detected	None Detected	Erucamide: 1.1 ppm *Palmitic acid: 1 ppm	Erucamide: 10 ppm Palmitic acid: 57 ppm Stearic acid: 37 ppm	None Detected	None Detected	Palmitic acid: 12 ppm Stearic acid: 9 ppm	Erucamide: 5.3 ppm Palmitic acid: 8.4 ppm
Anions (IC)	-	-	-	-	-	-	-	-

^{*}Concentration between the detection limit and the quantification limit, therefore only indicative.

- Not tested due to very small surface area in fluid contact.

6.5 Extractables/Leachables Testing Conclusions

The results from the Allegro biocontainer testing after 30 and 91 days indicate that the level of extractables/leachables for tested contact fluids was extremely low (Table 6), especially for the WFI and PBS contact fluids, which more closely match actual application conditions.

Summary Tables 7 and 8 show the findings from 2 extractables studies conducted on the 5 additional polymeric components used in the construction of the Allegro 50 L single-use mixer. The T=0 study was conducted on parts which were irradiated (50 kGy) and sent for analysis. The T=3 yrs study was conducted on parts which were irradiated and subsequently aged, for the equivalent of 3 years, at 40 °C 75% RH prior to analysis. All identified extracted chemical entities are either oligomers from the polymers used or degradation products from antioxidants.

As expected, the ethanol extraction showed higher concentrations of the various detected compounds for most parts tested. The levels seen in the water extract are much lower which suggests that the compounds have limited solubility in water. The components tested account for a very small proportion of the total surface area of the biocontainer and therefore the concentration of the detected compounds should be viewed in that context. For example TOC detected for HDPE component was 10 ppm in extraction volume. The fluid contact surface area of the seal housing, as a percentage of the whole system, is 0.94% therefore the seal housing would contribute only extremely low TOC to the whole biocontainer.

7. Biological Safety Tests

7.1 Overview

The purpose of these tests was to evaluate the biological suitability of the materials of construction of the Allegro 50 L single-use mixer biocontainer. The materials of construction for the Allegro single-use mixer biocontainer are detailed in Table 9.

For information on the Allegro biocontainer film (low density polyethylene film) please refer to Pall document reference number USTR2527 (validation guide for Allegro 3D biocontainers and totes) and for the information on the PSU, UHMW PE, and silicone please refer to the Allegro 200 L mixer validation guide (Pall document reference number USTR2777). The Allegro single-use mixer biocontainers use the same manufacturing method and material of construction for the main biocontainer film as do the Allegro 2D and 3D biocontainers.

Unless otherwise stated, components were irradiated using gamma radiation at a dose of 45-55 kGy prior to any testing.

Table 9

Materials of construction

Biocontainer Part	Material
Biocontainer film	Low Density Polyethylene (LDPE)
Inlet port plate	Low Density Polyethylene (LDPE)
Powder addition port and impeller cover	
Drain valve flange	
Impeller	Polysulfone (PSU)
Endcap	
Drain valve	
Seal housing	High Density Polyethylene (HDPE)
Seal	Ultra-high Molecular Weight Polyethylene (UHMW PE)
Seal O-ring	Silicone
Drain valve O-ring	
Shaft	Stainless Steel 316

7.2 Summary of Methods

Tests include USP Biological Reactivity Tests, *In Vivo*, for Class VI Plastics as described in the current United States Pharmacopeia Chapter <88>, USP Biological Reactivity tests, *In Vitro*, as described in the current United States Pharmacopeia Chapter <87>, and tests on Bacterial Endotoxins as described in USP <85>.

7.2.1 USP <88> Biological Reactivity Test, *In Vivo*, for Class VI Plastics

The Biological Reactivity Tests *In Vivo* for Class VI-50 °C Plastics as described in the United States Pharmacopeia include:

- Injection of extracts of plastic materials
- Implantation of the solid material into animal tissue

The four (4) extracting media listed in the USP simulate parenteral solutions and body fluids. These include:

- 0.9% Sodium Chloride for Injection
- 1 in 20 Solution of Ethanol in Sodium Chloride Injection
- Polyethylene Glycol 400
- Vegetable Oil (sesame or cottonseed oil).

All polymer materials including Allegro biocontainer film and molded connection pieces were gamma irradiated and then extracted with these solutions at 50 +/- 2 °C for 72 +/- 2 hours. The extracts were then used in the following tests to determine the biological effects they have:

- Acute Systemic Injection Tests

An Acute Systemic Injection test was performed to evaluate the potential of a single injection of an extract to produce systemic toxicity. Extracts in Sodium Chloride Injection and 1-in-20 Solution of Ethanol in Sodium Chloride Injection were injected intravenously. Cottonseed oil extract and Polyethylene Glycol 400 extracts were injected intraperitoneally.

- Intracutaneous Tests

An Intracutaneous test was performed to evaluate the potential of a single injection of an extract to produce tissue irritation. All four of the extracts listed above were used for these tests.

- Implantation Tests

Implantation tests were performed, in order to subject the Allegro biocontainer materials of construction to the most stringent conditions included in the USP.

7.2.2 USP <87> Biological Reactivity Test, *In Vitro*, Cytotoxicity

The purpose of this study is to assess cytotoxicity (i.e., the effect of extractables from test material on the test cells) as per USP <87> guidelines. The materials tested under USP <87> are the drain valve flange, endcap, seal, and o-ring. For each material part, an extract of the gamma-irradiated test article, is prepared using single strength Minimum Essential Medium (MEM) supplemented with 5% serum and 2% antibodies (1X MEM). This test extract was placed onto two separate monolayers of L-929 mouse fibroblast cells propagated in 5% CO₂. Two separate monolayers were prepared for the negative control (high density polyethylene) and the positive control (tin stabilized polyvinylchloride).

All monolayers were incubated at 37 °C in the presence of 5% CO₂ for 48 hours and were examined microscopically after 48 hours to determine any change in the cell morphology. After 48 hours, both the negative and positive controls should perform as anticipated. The 1X MEM test extract is analyzed to see if there has been any cell lysis or toxicity.

7.2.3 USP <85> Bacterial Endotoxins Test

This test is to establish that endotoxins level of the disposable mixer in accordance with USP <85> Bacterial Endotoxins Test. The test was performed on individual components, which is added to the LAL Endotoxin level from Allegro biocontainers tested to show the result for the Allegro 50 L single-use mixer biocontainer. The endotoxin tests were analyzed using chromogenic endpoint techniques. Each component material (low density polyethylene drain valve flange, polysulfone endcap, ultra-high molecular weight polyethylene seal, and silicone o-ring) was extracted with endotoxin free water and the fluid was then tested for endotoxins. As per US Pharmacopeia <85>, the endotoxin level should be < 0.25 EU/mL for each component material. All samples passed the specified tests.

7.3 Results

All materials used in the construction of the Allegro 50 L single-use mixer biocontainer passed USP <88> Biological Reactivity Test, *In Vivo*, for Class VI Plastics, USP <87> Biological Reactivity Test, *In Vitro* and the USP <85> endotoxin test. The film of the Allegro single-use mixer biocontainer has the same manufacturing method and materials of construction as the Allegro 2D and 3D biocontainers, so please refer to Pall document reference number USTR2527 (validation guide for Allegro 3D biocontainers and totes) for biological safety results of the film. Detailed copies of the reports are available upon request.

7.4 Conclusions

All materials used in the Allegro 50 L single-use mixer meet the requirements of the USP Biological Reactivity Tests, *In Vivo*, for Class VI Plastics (USP <88>), USP Biological Reactivity Tests, *In Vitro*, Cytotoxicity (USP <87>), and the endotoxin determination tests as specified in USP <85>.

8. Physicochemical and Particulate Tests

8.1 Overview

The purpose of these tests is to evaluate the physicochemical suitability of Allegro 50 L single-use mixer biocontainers when tested to USP <661> and to determine if, under normal operating conditions, any particulate matter generated is below the limits according to USP <788>

For information on the Allegro biocontainer film please refer to USTR2527 (validation guide for Allegro 3D biocontainers and totes). The Allegro 50 L single-use mixer biocontainers use the same manufacturing method and material of construction for the Allegro biocontainer film as the Allegro 2D and 3D biocontainers therefore the the USP <661> testing conducted previously is valid for the Allegro 50 L single-use mixer biocontainer.

Unless otherwise stated, components were irradiated using gamma radiation at a dose of 45-55 kGy prior to any testing.

8.2 Summary of Methods

8.2.1 USP <661> Containers - Plastic

Plastic containers that are intended for packaging products for parenteral use must meet the requirements of physicochemical testing - plastics found in the current USP. These tests are designed to measure the properties of impurities extracted from plastics when leached with extraction medium over a specified period and temperature. The value of these tests becomes important to ensure the efficacy of product within the container.

Samples of LDPE drain valve flange, polysulfone endcap, UHMW PE seal, and silicone rubber were gamma irradiated and extracted at 70 °C for 24 hours in purified water and isopropyl alcohol. Samples of the liquids were tested under the following conditions under USP <661> guidelines:

- Non-volatile Residue: This test measures organic/inorganic residues soluble in extraction media. A volume of the extract is taken to dryness at 105 °C alongside a blank of the liquid. The individual residue weights are recorded and the difference in weight is calculated. The limit for the test is an NVR of <15 mg.
- Residue on Ignition: This test is only performed when the NVR is greater than 15 mg. The sample and blank residues are heated to 800 °C. The difference in weight of the residues is again calculated. The difference must be less than 5 mg.
- Heavy Metals: This test detects the presence of metals such as lead, tin, and zinc. A volume of the sample extract is brought to a pH of 3.0 – 4.0. The same is done to a dilute lead solution of a known concentration. Both solutions are then treated with hydrogen sulfide TS in separate comparison tubes. Any color produced in the sample tube is compared to the color in the standard tube. The limit is <1 ppm as indicated by the color.
- Buffering Capacity: This test measures the alkalinity or acidity of the extract. A volume of the sample extract is brought to pH of 7.0 using dilute acid or alkali. A blank of purified water is also brought to pH 7.0. The amount of titrant used is recorded for each and compared. There must be no more than 10.0 mL difference in the titrant volume used for each sample.

8.2.2

USP <788> Particulate Matter In Injections

The particle count analysis, based on USP <788>, is to determine the particulates level within the Allegro 50 L single-use mixer biocontainer before, during, and after mixing. One gamma irradiated Allegro 50 L single-use mixer biocontainer was filled with five (5) liters of ultra-pure water. Three samples of water were taken at Time =0 (immediately after filling), 1 hour, 24 hours, 48 hrs, plus negative control samples. Air, filtered (through a 0.2 µm sterilizing grade Pall® Mini Kleenpak capsule filter with Emflon® II filter medium, part number KA02V002P2G) was used to inflate and maintain the pressure inside the mixer biocontainer before and during the mixing process. Mixing speed was set at 200 rpm. At each interval, samples of 50 mL taken from the drain valve of the mixer for measurement. The samples were submitted for microscopic analysis to determine if they met with the requirements of USP <788>. The number of particles of sizes ≥25 µm must be <2 /mL, and for particles ≥10 µm must be <12 /mL.

8.3 Results

All materials of the Allegro 50 L single-use mixer biocontainer tested (LDPE drain valve flange, polysulfone endcap, polyethylene seal, and silicone o-ring) passed all the tests specified under USP <661> Physicochemical Test. The results from the particulate analysis met with the requirements of USP<788>. Table 10 shows the results of the particulate analysis.

Table 10

Results of particulate analysis

Sample	Blank 1		Blank 2		Blank 3	
	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$
Blank	<1	<1	<1	<1	<1	<1
Sample	Sample 1		Sample 2		Sample 3	
	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$
0 hours	<1	<1	<1	<1	<1	<1
Sample	Sample 1		Sample 2		Sample 3	
	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$
1 hour	<1	<1	<1	<1	<1	<1
Sample	Sample 1		Sample 2		Sample 3	
	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$
24 hours	<1	<1	<1	<1	<1	<1
Sample	Sample 1		Sample 2		Sample 3	
	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$	$\leq 10 \mu\text{m}$	$\leq 25 \mu\text{m}$
48 hours	<1	<1	<1	<1	<1	<1

8.4 Conclusions

The components of the Allegro 50 L single-use mixer biocontainers meet the requirements of the Physicochemical Test-Plastics USP <661> and USP <788> Particulate Testing.

9. Shelf Life Studies

9.1 Overview

9.1.1 Shelf Life Analogy to Allegro 200 L Single-Use Mixer Biocontainer

Shelf life studies have established a three (3) year shelf life for the Allegro 200 L single-use mixer biocontainers subjected to 45-55 kGy gamma irradiation, when stored under controlled accelerated aging conditions at 40 °C 75% relative humidity. Tests on the influence of gamma sterilization on the mechanical strength of connectors, seal weld, and cross crease fold were performed. Leak testing was also performed as a further test of the integrity of the seal housing.

For shelf life information on the biocontainer film please refer to Pall document reference number USTR2527 (validation guide for Allegro 3D biocontainers and totes). The Allegro 50 L single-use mixer biocontainers use the same manufacturing method and material of construction for the main biocontainer film as for the Allegro 2D and 3D biocontainers. The Allegro 50 L mixer single-use biocontainer uses the same manufacturing methods and materials of construction as the Allegro 200 L mixer biocontainer. For shelf life information relating to seals, bearings, connections and impeller, please refer to Pall document reference number USTR2777 (validation guide for Allegro single-use mixer biocontainers).

9.1.2 Long Term Shelf Life Study

A full shelf-life study is under way to establish a 3-year shelf life specific to the Allegro 50 L single-use mixer biocontainers subjected to 45-55 kGy gamma irradiation, stored under ambient conditions (real time) and accelerated aging (40 °C / 75% RH) conditions. Interim reports will be available on request as developed.

At each interval during the 3-year shelf life study, tests on the influence of gamma sterilization on the mechanical strength of connectors, seal weld, and cross crease fold will be performed. Leak testing as per section 2 is also performed as a further test of the integrity of the impeller seal housing.

Table 11

Shelf-life plan matrix – Quantity to be tested at each time point

Sample Description	50 °C Ambient Humidity		Accelerated Aging 40 °C 75% RH		Real Time (Ambient Conditions)
	1 month (6 months)	4.3 months (1 year)	13 months (3 years)		
Allegro 50 L single-use biocontainer	3	3	3	3	3



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