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validation Guide

LeThenea® Single-use Freezing Bag

LePure Biotech

Shanghai LePure Biotech Co., Ltd.

CONTENT

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1. Overview

Single use freezing bags are used for blood and cell storage, its safety and reliability are extremely important. The LeThenea ® single use freezing bag developed by LePure has good performance in biological safety, chemical compatibility and physical properties. It has passed quality tests and validation, which can provide biopharmaceutical companies with safe and reliable single use products.

This "Validation Guide" describes the physical and chemical characteristics, key controls, quality standards and key quality parameters in the manufacturing process of the LeThenea ® single use freezing bags made of EVA film, which manufactured by Shanghai LePure Biotech Co., Ltd. (here in after referred to as LePure Biotech) This "Validation Guide" can provide customers with basic information for evaluating and testing this product before use.

1.1 Supply chain safety and security

As the key consumables for biological products, suppliers of single-use systems need to provide comprehensive solutions to ensure the safety and controllability of their supply chains and avoid instability caused by extreme conditions.

LeThenea ® single use freezing bags provide stable supply chain safety and security by:

- localized production

The main production site is located in Shanghai, China and the holding subsidiary of LePure Biotech is responsible for production. So, LePure Biotech controls the entire production process and film quality.

- multi-center production

In order to avoid the risks caused by a single production site, LePure Biotech is verifying the second overseas production site and will complete the layout of the second global production site in 2023 to achieve the purpose of multi-center production.

1.2 Manufacturing site

Single use freezing bags are produced in a Class C clean room and dynamic environmental monitoring

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is carried out continuously during production. The test data shows that the air environment (dust particles and sedimentation bacteria) around the equipment can meet the dynamic Class C standard.

The single use freezing bags which made of EVA film is produced at Zhengbo Road, Lingang Free Trade Zone, Shanghai. The basic situation is as follows:

The total area is 10078m^2, the Class D is 450m^2, the Class C is 3400m^2, and the Class C+A is 600m^2.

2 . Quality assurance

2.1 Quality management system

LePure follows the ISO 9001: 2015 and ISO 13485:2016 to develop a quality management system and has passed the ISO9001:2015 and ISO13485: 2016 system certification. The LeThenea ® single use freezing bags are manufactured in compliance with strict performance and industry safety standards. Key production is carried out in a clean room environment that meets Class C and is continuously monitored. Besides, clean room personnel management, material management, process specifications, equipment management and verification are carried out in accordance with the requirements of Good Manufacturing Practice (GMP) to ensure that the standards and requirements of each regulation.

2.2 Production management

LeThenea ® single use freezing bags are produced in Class C environment.

The production equipment and instruments have passed perfect verification and the calibration is completed before they are put into production. Through regular maintenance, the good condition of production equipment is guaranteed.

Production plans are managed through a unified internal process, taking orders, drawings, customer requirements and production schedules are all confirmed by relevant personnels.

The production materials are inspected before entering the warehouse and they are released for the production of single use products after meeting the requirements.

During the production, we will check and confirm the production process and materials, including but not limited to: consistency with technical drawings, material inspection, visual inspection (component

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cleanliness, sealing, foreign matter, firmness, assembly, etc.), size inspection, packaging, labeling, etc.

Each batch of semi-finished products and finished products has corresponding batch production records, which can trace the production history and quality.

2.3 Material control and supplier management

According to the characteristics of the single-use system, the selection criteria, management methods and audit specifications for suppliers have been established. The quality of raw materials and components used is strictly controlled. Raw materials and components for production are controlled according to risk levels. Typical raw materials and components should meet the following basic requirements:

- Meets USP<87> and USP<88> Class VI, and / or ISO10993
- Free of TSE/BSE
- USP661 compliance and / or EP related compliance
- Other Regulatory Compliance Documents

All raw materials and components are to be confirmed by internal QC testing, including supplier's quality documents, packaging and labeling, appearance and dimension inspection, etc.

Suppliers are regularly audited to review the quality of raw materials and components to ensure consistency and reliability.

2.4 Personnel

LePure Biotech has a group of production, quality, management and technical personnel with corresponding professional knowledge of single-use systems. Ongoing staff training and qualifications to ensure employees have awareness of the required standards. Employee qualifications are measured through rigorous initial screening and ongoing competency testing.

2.5 Change Management

Based on science and risk, LePure Biotech has fully evaluated the factors that affect the performance of the production process and the quality of the final product and has established a systematic change

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management strategy.

In case of situations that affecting product consistency and quality reliability, such as changes in film material formulations or key production process parameters, etc., in order to ensure that users have sufficient time for evaluation, under normal circumstances, LePure Biotech will notify users at least 6 months in advance.

2.6 Gamma sterilization process

According to ISO11137, use the VDmax verification method of the lowest sterilization dose (such as 25kGy or 40kGy) and the method of verification of the sterilization cycle. The average microbial load level is determined by selecting a representative product family and experiments are carried out to finally select and confirm that the gamma irradiation dose range of 25-40 kGy can reach the sterility assurance level SAL $< 10^{-6}$.

2.7 Batch management and traceability management

The production of EVA film and single use freezing bags have established operating procedures for dividing product batches, so that the division of production batches can ensure the uniformity and consistency of the quality and characteristics of the same batch products. Besides, LePure Biotech formulate the Standard operating procedures of batch number and production dates, so that each batch of products has unique batch number.

The raw and auxiliary materials, semi-finished products, finished products, main equipment and necessary production workshops used in the production of single use freezing bags are all labeled and marked. Besides, specifications and batch numbers of the materials in production are marked in other ways and the realization of the products can be traced in the whole process.

3. EVA film physical performance test

3.1 Film material

EVA is Polyethylene vinylacetate, a polyethylene-polyvinyl acetate copolymer. Compared with polyethylene (PE), EVA reduces high crystallinity, improves toughness, impact resistance as well as low

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temperature resistance due to the introduction of vinyl acetate monomer in the molecular chain.

LeThenea ® single use freezing bag is made of high-safety medical-grade raw materials and the thickness is 350μm, which provides a solid guarantee for liquid nitrogen -196°C cryopreservation.

3.2 Film parameters

The following data describe the basic physical parameters of EVA film that form the basis film performance.

Table 1 EVA film parameters

Performance	Unit	Test value (before irradiation)	Test value (after irradiation)	Guideline
Film thickness	μm	350	350	/
Light transmittance	%	92	92	ASTM D1003
Haze	%	30	33	ASTM D1003
Tensile strength at break	MPa	15	17	ASTM D882
Elongation at break	%	922	892	ASTM D882
Heat seal strength	N/cm	49	48	ASTM F88
Puncture Strength	N	73	79	ASTM F1306
Puncture elongation	mm	48	52	ASTM F1306
water vapor transmission rate	g/ (m^2 ·day)	25	25	ISO 15106-2
Oxygen transmission rate	cm^3/(m^2 ·day ·bar)	1554	1534	ASTM D1434
Carbon dioxide transmission rate	cm^3/(m^2 ·day ·bar)	2 682	2678	ASTM D1434

*Note: This test result is a typical value of the film under certain processing conditions and test

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environment.

3. Specifications of LeThenea ® single use freezing bag

3.1 Application and operating unit

LeThenea ® single use freezing bags can be widely used in ultra-low temperature cryopreservation of blood and various cells. A variety of specifications meet the application scenarios of different cryopreservation volumes. The following table shows the recommended minimum and maximum liquid volumes for different specifications of cryopreservation bags.

Table 2 LeThenea ® Single Use Freezing Bag Specifications

Specification / describe	50mL	200mL	500mL	750mL	1000mL
Minimum filling volume (mL)	10	30	55	80	125
Maximum filling volume (mL)	30	70	100	190	270

3.2 List of main components and materials

Table 3 LeThenea ® Single Use Freezing Bag Component Materials

Part Name	material
Connection components	I pipeline is EVA tube, and the branch pipeline is PVC tube
Bag	EVA film
Sampling port	Luer port, silicone needle-free sampling valve

4. LeThenea ® single use freezing bag application test

LeThenea ® single use freezing bags have completed complex biocompatibility, chemistry, physics, freeze-thaw effect tests, virus impermeability tests, extractables tests, etc., providing users with reliable performance in the biopharmaceutical process.

Depending on the nature of the validation method, validation is performed on individual components,

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representative test bags or the final product containing all relevant components.

4.1 LeThenea® Single Use Freezing Bag Validation Test Summary

Table 4 LeThenea ® Single Use Freezing Bag Test Summary

Application Test Project	Description	Reference	chapter
Freezing effect test of LeThenea® single use freezing bag	This study aimed to determine the cryogenic performance of LeThenea ® single use freezing bags in liquid nitrogen	NA	4.1.1 _
Validity verification	Of EVA film and bags, after the accelerated aging is completed, the physical properties of the samples are tested	ASTM F1980-2016	4.1.2 _
Freeze virus impermeability test	This study aimed to determine the barrier properties of LeThenea ® single use freezing bags against viruses		4.1.3
LeThenea ®Single-Use Freezing Bags	NA	NA	4.2 _
Product release and production control	LeThenea ® single use freezing bags has established raw materials, production process, and finished products.	USP<85>, CHP<1143> USP<788> CHP<0903> ASTM F88 ASTM F88 CHP <0631> ISO11137	4.2.1.
Biosecurity	Biosafety is a necessary passing condition, LeThenea ® single use freezing bags have been tested in accordance with the relevant items required by the United States Pharmacopoeia	ISO 10993-4 ISO 10993-5 ISO 10993-6 ISO 10993-10 ISO 10993-11 USP<87>	4 .3

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	USP and ISO10993	USP<88>	
Pharmacopoeia compliance	NA	NA	4 .4
Physical and chemical test	After 45-55kGy-gamma irradiation before testing, evaluate whether it meets the USP<661> standard after irradiation sterilization	USP<661>	4.4.1 _
Bacterial Endotoxin Test	Test whether the LeThenea® single use freezing bag complies with the Chinese Pharmacopoeia and the United States Pharmacopoeia, as well as LePure Biotech's internal control standards for bacterial endotoxin control standards	CHP<1143> USP<85>	4.4.2
Insoluble Particulate Test	The purpose of this test is to investigate the level of insoluble particulates in single use freezing bags made with EVA film	USP<788> CHP<0903>	4.4.3
chemical compatibility	Fill the liquid storage bag with 45-55kGy gamma irradiated solution first, and place it at 30±2.5°C for 7 days, then conduct appearance, drop, film thickness, heat and strength, connection firmness, infrared and other tests	ASTM, China Food and Drug International Exchange Center, "Single-use System Application and Technical Guidelines"	4.5
Gamma Sterilization Validation	Verified that the radiation sterilization dose of LeThenea ® single use freezing bags is set at 25~40kGy, which can reach the sterility assurance level of SAL<10	ISO11137	4.6

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4.1.1 Freezing effect test

Purpose

The purpose of this study is to determine the integrity of the LeThenea ® single use freezing bag under the condition of recommended liquid volume and repeated freezing and thawing through testing. The repeated freezing and thawing is to simulate the long-term stability freezing bag in liquid nitrogen in the actual process.

Materials and methods

For this verification, representative samples of 50mL, 500mL and 1000mL were selected for freeze-thaw test according to small, medium and large. To ensure simultaneous verification of the maximum recommended filling capacity of LeThenea ® single use cryopreservation bags, the filling volume in the test will be slightly larger than the recommended filling volume and each bag will be sterilized by high-dose gamma irradiation of 45-55kGy.

Pre-cool the prepared samples in a -80°C ultra-low temperature refrigerator for 60 minutes, then take out the samples, put them into a liquid nitrogen tank filled with -196°C liquid nitrogen, and freeze for 15 minutes. After freezing, take the sample out of the liquid nitrogen and put it directly into a 40-degree water bath for rethawing; after completing rethawing, check whether there is any leakage in the cryopreservation bag; if not, proceed to the next round of freezing experiments and repeat 10 times until the end; if there is leakage, the test of the sample is terminated and the cause is analyzed.

Table 5 Freezing experiment table

Freezer Bag Specifications	Recommended volume	Experimental test volume	Radiation dose	Number of bags (pieces)
50mL cryopreservation bag	1 0-30mL	3 5mL	4 5-55kGy	5 0
500mL cryopreservation bag	5 5-100mL	1 20mL	4 5-55kGy	5 0
1000mL cryopreservation bag	1 25-270mL	3 00mL	4 5-55kGy	5 0

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The results show

A total of 150 cryopreservation bags, under the condition of slightly overfilling and high-dose irradiation, one of the 500mL cryopreservation bags may be scratched by the cryopreservation box. A small crack appeared at the mouth, and the other 149 still maintained the integrity of the liquid after repeated freezing and thawing 10 times.

Conclusion

LeThenea ® single use freezing bags have excellent low temperature resistance and can bring customers a safe and stable freeze-thaw experience under the recommended filling volume and normal radiation dose.

4.1. 2 Validity verification

According to ASTM F1980-2016, the accelerated aging of EVA film and bags is carried out by increasing the temperature, simulating natural aging for 2 years and testing the product validity period. After the accelerated aging was completed, the physical properties of the samples were tested.

Table 6 The validity period of EVA film and bag

product name	validity period
EVA film	3 years
LeThenea ® Single Use Freezing Bags	2 years

A longer validity period test and an aging test simulating real conditions are in progress and LePure Biotech will further update the data regularly according to the test results.

4.1. 3 Virus impermeability test

Purpose

The anti-penetration ability of LeThenea® single use cryopreservation bags was tested by biological culture.

Materials and methods

5 samples of 50mL cryopreservation bags sterilized by irradiation, put them into 50mL LB broth , freeze, unpack and soak them in λ phage suspension for 30 minutes and take them out after soaking. Mix the

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extract and the bacterial solution thoroughly, incubate at 37°C for 15 minutes , transfer to 3 mL of molten LB agar and pour on the LB agar plate. Incubate at 37 °C for 24 hours, and observe the virus plaque forming units (pfu) on the culture dish .

Table 7 Virus penetration test results

Group	Plaque forming units (pfu)
Negative control	0
Blank control	0
Positive control	235
Sample 1	0
Sample 2	0
Sample 3	0
Sample 4	0

The results show

The negative control and the blank control had a plaque forming unit of 0, the positive control >0, and the plaque forming units of the test samples were all 0.

Conclusion

The experimental results are valid and LeThenea ® single use freezing bags can play a role in preventing virus penetration.

4.2 Integrity of LeThenea ® single use cryopreservation bags

The production process has been verified, and the raw materials, production process and final products have been monitored and controlled accordingly.

Table 8 Integrity Test

Project	Test content	Reference Method/Standard	Result
Connection firmness	Component connection strength	100% visual inspection and internal acceptance criteria	qualified

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Leak check	By the pressure decay method, the bag and the device are tested	100% leak checked, internal method	qualified
Package integrity	Check the package after vacuuming to confirm whether the outer package is complete	100% visual inspection	qualified

4.2.1 Product release and production control

LeThenea ® single use freezing bags has established a quality control system for raw materials, production processes and finished products.

Table 9 Production Raw Material Control Standards

Project	Guideline	Acceptance Criteria Defined by Regulations	LePure Biotech internal release standard
Physical dimension	Internal method	/	Internally Accepted Specifications
Bacterial endotoxin	USP<85>, CHP<1143>	＜ 0.25EU/mL	＜ 0.125EU/mL
Insoluble particles	USP<788> CHP<0903>	≥ 10 μm：≤2 5 cells / mL ≥ 25 μm：≤3 pcs / mL	≥ 10 μm：≤ 10 / mL ≥ 25 μm：≤ 1 / mL
Visible foreign matter	Visual inspection, internal standard method	/	≤3pcs /50mL

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Table 10 Production Process Control Standards

Project	Guideline	Le pure biological internal release standard
Physical dimension	Internal method	Internally Accepted Specifications
Heat seal strength	ASTM F88	Film thickness ⩾ 300μm: The strength of each heat sealing part is not less than 30N/15mm
Product production conforms to drawings	Drawing	100% products conform to drawings
Production Process	Internal procedures	100% meet production requirements

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Table 11 Release Standards for Finished Products

Project	Guideline	Acceptance Criteria Defined by Regulations	LePure biotech internal release standard
Integrity	Pressure Decay, ASTM F 2095-07	/	100% pass pressure decay to ensure integrity
Heat seal strength	ASTM F88	/	Film thickness ⩾ 300μm: the strength of each heat-sealing part is not less than 30N/15mm
Connection firmness	internal method	/	Withstand a net pulling force of 15N for 15 s without falling off
Bacterial endotoxin	USP<85>, CHP<1143>	＜ 0. 25EU/mL	＜ 0.125EU/mL
Insoluble particles	USP<788> CHP<0903>	⩾ 10 μm：⩽2 5 cells / mL ⩾ 25 μm：⩽3 pcs / mL	⩾ 10 μm：⩽ 20 / mL ⩾ 25 μm：⩽ 2 / mL
Visible foreign matter	Visual inspection, internal standard method	/	⩽3pcs /50mL
pH	CHP <0631>	/	5.0-7.0

4.3 Biosafety

Biological safety is a necessary condition for passing. LeThenea ® single use cryopreservation bags have been tested in accordance with the relevant items required by the United States Pharmacopoeia USP and ISO10993. The chart summarizes the biological safety test results of LeThenea® single use cryopreservation bags.

Table 12 Biosafety test results

Standard test	Test items	Test Results
ISO 10993-4	Hemolysis test	no hemolysis
ISO 10993-5	Cytotoxicity	non-cytotoxic
ISO 10993-6	Implantation test	No skin irritation

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ISO 10993-10	Sensitization test (Irritation and Sensitization tests)	No allergies
ISO 10993-11	Acute systemic poisoning test (Acute Systemic Toxicity test)	non-toxic
USP <8 7 >	in vitro biological response (Biological reactivity testing, in vitro)	non-cytotoxic
USP<88>	biological response in vivo (Biological reactivity testing, in vivo, class VI)	No in vivo toxicity

4.4 Pharmacopoeia compliance

4. 4. 1 USP <661> physical and chemical tests

USP <661> stipulates the physical and chemical tests that must be carried out for plastics used in high-risk areas (inhalants, injections, ophthalmic preparations, etc.) to ensure that no harmful substances are released.

The LeThenea ® single use freezing bags were irradiated with a maximum irradiation dose of 45~ 55kGy before the test and the test items and corresponding results after irradiation sterilization are as follows

Table 1 3 USP < 661> physical and chemical test results

Test Methods	Test items	Judgment criteria	Result
USP <661>	Cushioning performance	⩽1 0.0 mL	qualified
	Heavy metal	⩽1ppm	qualified
	Non-volatile residue	⩽15mg	qualified
	Residue on ignition	⩽5mg	qualified

After testing, LeThenea® single use freezing bags can meet the requirements of the United States Pharmacopoeia for plastics used in high-risk areas.

4.4.2 USP<85> Bacterial Endotoxin Test

The internal control standard of Le Pure Biotech is < 0.125EU / mL.

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The test requirements of CHP< 1143> and USP< 85> , LeThenea ® single use freezer bags have passed all tests and are in line with the Chinese Pharmacopoeia, the United States Pharmacopoeia, and the control standards for bacterial endotoxins in LePure’ s internal control standards .

4.4.3 USP<788> Insoluble Particulate Test

The purpose of this test is to investigate the level of insoluble particulates in single-use cryopreservation bags made of EVA film. EVA film are produced on a large scale. In order to avoid excessive liquid filling, dilute particles and cause deviations in test results, the enterprise standard has detailed regulations on the liquid volume (the ratio of sample surface area to extraction medium is 6 cm^2 /mL).

The internal control standards of LePure Biotech for insoluble particles are as follows:

⩾ 5μm : ⩽ 100 / mL

⩾10μm : ⩽ 20 cells / mL

⩾ 25μm : ⩽ 2 cells/ mL

According to the test requirements of CHP<0903> and USP<788>, LeThenea® single use cryopreservation bag has passed all the tests and can meet the control standards of CHP< 0903>, USP< 788> and the internal control standards of insoluble particles, which is from LePure Biotech.

4.5 Chemical Compatibility

There are many possible chemical interactions between single-use systems and contact fluids, and pharmaceutical manufacturers should fully consider the consequences of these interactions. If the data on chemical compatibility (including from manufacturers, literature, industry experience, etc.) can be directly cited, pharmaceutical companies can make evaluations based on these chemical compatibility data① . If other project data in the overall verification project portfolio can be effectively used to assess the risk against chemical compatibility, the industry will usually also conduct reference assessments based on these data. If pharmaceutical companies need to test for chemical compatibility, they can also refer to the relevant test methods of the American Society for Testing and Materials (ASTM) to establish appropriate methods ② .

Notes: ① ② China Center for Food and Drug International Exchange: Chapter 3.6 of "Guidelines for

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Application and Technology of Single-use Systems".

Bags equipped with EVA tubing were used. Before the test, fill in solutions into the liquid storage bag which irradiated by 45-55kGy gamma, and then place it at 30±2.5°C for 7 days , then conduct appearance, drop, film thickness, heat seal strength, connection firmness, infrared and other tests. The test results are shown in the following table.

Table 1 4 EVA film chemical compatibility list

The solution		Experimental results
Ethanol 75%	75% ethanol	compatible
Glycerol 50%	50% glycerin	compatible
Ethylene glycol 50%	50% ethylene glycol	compatible
N,N-Dimethylformamide (DMF) 10%	10% N,N -Dimethylformamide	compatible
N,N-Dimethylformamide (DMF) 10%	20% N,N -Dimethylformamide	compatible
N,N-Dimethylacetamide (DMA) 20%	20% N,N -Dimethylacetamide	compatible
N- methylpyrrolidone 20%	20% Methylpyrrolidone	compatible
Phosphate buffer solution pH10	pH10 Phosphate Buffer	compatible
HCl/ KCl buffer pH3	pH3 HCl/ KCl buffer	compatible
Tween 80 10%	10% Tween 80	compatible
Ammonium hydroxide 5M (pH14)	5M (pH14) ammonia water	compatible
Dimethyl sulfoxide (DMSO) 20%	20% dimethyl sulfoxide	compatible
Methanol 50%	50% Methanol	compatible
Hydrogen Peroxide 10%	10% hydrogen peroxide	compatible
L-Histidine 5g/L	5g/L L - histidine	compatible
Ethylene Diamine Tetraacetic Acid (EDTA)10%	10% EDTA	compatible
Calcium Chloride ($CaCl_2$)	saturated calcium chloride	compatible
Magnesium Sulfate ($MgSO_4$-7H_2O)	saturated magnesium sulfate	compatible
Potassium Chloride (KCl)	saturated potassium chloride	compatible
Sodium Bicarbonate ($NaHCO_3$)	saturated sodium bicarbonate	compatible
Sodium Chloride (NaCl)	saturated sodium chloride	compatible

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Sodium Phosphate monobasic ($NaH_2PO_4H_2O$)	Saturated sodium dihydrogen phosphate	compatible
Glucose 10g/L	10g/L glucose	compatible
Sucrose 5M	5M sucrose	compatible
Ethanol 50%	50% ethanol	compatible
Peptone 10g/L	10g/L peptone	compatible

Note: This table is the test results of the frozen storage bag placed at 30±2.5°C for 7 days

4.6 Validation of gamma sterilization

Biological single-use system includes three steps: product definition, process definition and dose field test. The implementation process is as follows:

- Product definition

Select typical single-use system components for microbial load testing to obtain initial microbial load data.

According to the definition of ISO11137 regulations, the single-use system series products are classified as a product family during the irradiation sterilization process.

- Process definition

Includes precise dosing of radiation, sterility testing methods, validation of bioburden recovery, and more. It proved that the pharmaceutical single-use system is suitable for the $VD_{max}25$ radiation sterilization dose establishment method, and the minimum sterilization dose of the product is 25kGy.

According to the radiation verification results of pharmaceutical single-use system products, during the Gamma sterilization process, there may be up to 1.6 times the unevenness of radiation, so the set radiation dose range is 25~40kGy

- Dose Field Distribution Test

LePure Biotech entrusts external irradiation processing enterprises to carry out gamma irradiation sterilization processing on products. The radiation dose field distribution test was carried out on the external irradiation device and the test results showed that the radiation dose of the product in each irradiation device was in the range of 25~40kGy.

- Summary

According to the above verification process, the results show that the irradiation sterilization dose of the

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single-use system is set at 25~40kGy, which can reach the sterility assurance level of S AL < 10^{-6}.

5 Conclusion

This guide summarizes the key quality attributes in the design, development, production, inspection and validation of EVA film and LeThenea® single use freezing bags made of EVA film. For further needs, please contact the LePure Biotech local sales representative for original data test report and other information.

LePure Biotech

微信公众号

官方网站

Shanghai Lechun Biotechnology Co., Ltd.

Shanghai LePure Biotech Co., Ltd.

Website: www.LePure-bio.com

Building 3, No. 41, Yunzhen Road, Songjiang District, Shanghai

Email: marketing@LePure-bio.com

LePure Biotech