Validation Guide

LeKrius® Single-use Bioreactor Bag（50 L-2000 L）

Shanghai LePure Biotech Co., Ltd

Catalogue

1. Overview

Single-use systems are widely used in the operation of biopharmaceutical processes, involving the manufacturing processes of antibodies, vaccines, recombinant proteins, cell and gene therapies, nucleic acid drugs, etc. The application scope of common single-use systems in pharmaceutical processes includes cell culture, buffer, media, intermediates, drug substance, final drug product, sampling, etc. With the rapid development of the biopharmaceutical industry, it has also boosted the demand for quality consistency, supply assurance, business continuity and change management of single-use products.
To meet such demands, LePure Biotech has developed the brand-new LeKrius® film (code: K) and the corresponding single-use process bag product line, covering single-use process applications from upstream and downstream of the biological industry to drug product filling. The film mainly boasts the following features:

- The film formula and injection molding process parameters of the film can ensure the continuous supply of single-use products;
- The antioxidant formula has been optimized to ensure excellent cell culture performance;
- The composition of the film material has been improved, enhancing the heat seal strength and reducing the risk of leakage;
- Very low extractable levels;
- It can be used in cell culture, fluid storage and shipping, freezing at -80 °C, drug products and filling;

1.1. Manufacturing Site

The LeKrius® film is manufactured in a Grade D cleanroom with real-time continuous dynamic environmental monitoring®. The test data indicate that the air environment (number of dust particles and passive airborne viable) in contact with the film around the co-extruded-film equipment can meet the dynamic Grade C criteria.
The basic conditions of the manufacturing site of single-use bioreactor bags made of LeKrius® film are as follows:
The total area of the Lingang Factory is 10,078 m^2, including the Grade D clean area of 450 m^2, the Grade C clean area of 3,400 m^2, and the Grade C + A clean area of 600 m^2.

2. Quality Assurance

2.1. Quality Management System

The current quality systems of all manufacturing sites of LePure Biotech are in compliance with ISO 9001 standards. At present, LePure Biotech has been upgraded in accordance with ISO 13485 requirements for their quality system to further meet the needs of biopharmaceutical enterprises.
The LeKrius® single-use process bag product line has completed the FDA Drug Master File (DMF) registration (DMF No.: 037284).

2.2. Manufacturing Management

The production of single-use systems of LePure Biotech is performed in the environment of ISO 7 clean room, and the critical production steps are carried out in the ISO 5 clean area.

Both the production equipment and instruments have passed 3Q validation, and they cannot be put into production until their calibration is completed. The production equipment is kept in a good condition through regular maintenance.

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

The production materials are inspected before warehousing and they cannot be released for the production of single-use products only after meeting the requirements.

During the production of single-use products, quality management personnel or designated personnel shall perform real-time testing and qualification of the production process and materials, including but not limited to: consistency with technical drawings, material inspection, visual inspection (cleanliness of components, sealing, foreign matter, firmness, assembly, etc.), dimensional inspection, packaging, labeling, etc.

Corresponding batch production records should be kept for each batch of semi-finished products and finished products, so that the production history and quality related status of that batch can be traced.

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 used for production are controlled according to risk grades. Typical raw materials and components should meet the following basic requirements:

- Conform to USP < 87 > and USP < 88 > Class VI, and/or ISO 10993
- TSE/BSE-free
- Conform to USP 661, and/or EP
- Conform to other regulatory compliance documents

All raw materials and components, including supplier's quality documents, packages and labels, need to be confirmed by in-house QC testing, spot check by infrared spectroscopy (FTIR), appearance inspection, dimensional inspection, etc.

Regular supplier audits are conducted to review the quality of raw materials and components to ensure consistency and reliability.

2.4. Personnel

LePure Biotech has a number of production, quality, and management personnel and technicians with corresponding expertise in the single-use system, and all meet the relevant personnel qualification requirements specified in ISO 9001 Requirements of Quality Management System. Continuous personnel training and qualification assessment are performed to ensure that employees have a clear understanding of the required criteria. Employee qualifications are measured through strict initial screening

and continuous competency testing.

2.5. Change Management

Based on a scientific and risk-based approach, LePure Biotech has fully assessed the factors affecting the performance of the manufacturing process and the quality of the final product, and established a systematic change management strategy.
In case of situations that affect the consistency and quality reliability of the product, such as changes in film material formula or important manufacturing process parameters, LePure Biotech will generally notify the user at least 1 year in advance to ensure that the user has sufficient time to perform relevant assessment.

2.6. Gamma Sterilization Process

The VDmax validation method using the minimum sterilization dose (e.g. 25 kGy or 40 kGy) and the method for validating the sterilization cycle are used, as specified in Part 1 & 2: 2013 of ISO11137. A representative product family is selected to determine the average microbial load and carry out the experiment. The gamma irradiation dose range of 25-40 kGy is finally selected and confirmed to be able to achieve the sterility assurance level SAL = 10^{-6}.

3. Physical Property Testing of LeKrius® Film

LeKrius film® is developed specifically for biopharmaceutical processes. In order to adequately ensure good cell culture performance, the antioxidant Irgafos ® 168 is not used in the selection of LeKrius® resin particles. At the same time, the physical properties of the film have been improved by optimizing the formula of the film, so that the tensile strength, heat seal strength, puncture resistance and anti-twisting property of the film can be improved.

3.1. Film Structure

The LeKrius® film is a medical grade multilayer co-extruded film with a thickness of 400 μm (Figure 3):
The outer layer is made of low density polyethylene (LDPE), which can resist the damage caused by external puncture, friction, tear and twisting;
The material of the inter-layer is ethylene-vinyl alcohol copolymer (EVOH), which is sandwiched between the outer layer and the fluid contact layer and produced by co-extrusion at the same time, and has a better gas barrier property;
The material of the fluid contact layer is ultra-low density polyethylene (ULDPE), with good weldability and excellent chemical inertness.

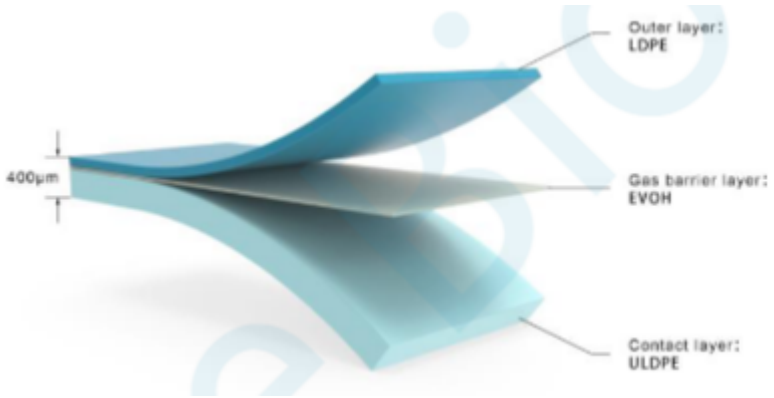


Figure 3. Structure of LeKrius® Film

3.2. Film Parameters

The following data are the physical parameters of the LeKrius® film, which form the basis of the film properties.

Table 1. LeKrius® Film Parameters

Property	Unit	Test value (Before irradiation)	Test value (After irradiation)	Reference standard
Film thickness	μm	400	400	/
Transmittance	%	87	87	ASTM D1003
Haze	%	42	42	ASTM D1003
Tensile strength at break	MPa	14	14	ASTM D882
Elongation at break	%	406	432	ASTM D882
Elastic modulus	MPa	220	210	ASTM D882
Heat seal strength	N/cm	54	54	ASTM F88
Puncture strength	N	48	50	ASTM F1306
Puncture elongation	mm	13	13	ASTM F1306
Falling dart impact strength	g	1300	1300	ASTM D1709
Anti-twisting	number	0.3	0.5	ASTM F392 B
Density	g/cm^3	0.9	0.9	ASTM D792
Water vapor transmission rate	$g/m^2.day$	0.8	0.8	ISO 15106-2

Oxygen transmission rate	$cm^3/(m^2.day.bar)$	1.1	1.1	ASTM D1434
Carbon dioxide transmission rate	$cm^3/(m^2.day.bar)$	0.8	0.8	ASTM D1434

3.3. Physical Strength

According to ASTM E3051-16, some strength indicators of the film are tested, including:

- Tensile strength at break
- Anti-puncture
- Anti-twisting
- Impact resistance
- Heat seal strength

Three different batches of film samples are used for each performance test to compare the performance indicators before and after irradiation. Irradiation is performed in accordance with ISO 11137 with an irradiation dose of 45-55 kGy.

3.3.1. ASTM D882 Tensile Strength at Break

Tensile testing is a test of the stress produced by the film under different strains (stretch deformation length). The purpose of this test is to detect the counter force produced by the film when it is stretched with an external force and the energy required when the film is stretched and broken.

3.3.1.1. Test Method

This test is performed according to ASTM D882 standard. A 15mm-wide film is fixed on a tensile tester by an upper clamp and a lower mechanical clamp with a distance of 50mm, and then stretched at a constant rate of 500 mm/min until it breaks (Figure 4).

Figure 4. Tensile Sample Stripe on Clamps

In this test, the elastic modulus represents the proportional relationship between stress and strain (Hooke's law) during the elastic deformation stage of the film, and the proportional coefficient is the elastic modulus. Elastic modulus is a physical quantity describing the ability of the material to resist deformation, and the greater numerical value indicates the higher film rigidity; conversely, it indicates the higher ductility of the

film (Figure 5). Elongation at break represents the strain of the film when it breaks in tension, usually expressed as a percentage. The breaking strength represents the stress strength of the film when it breaks in tension, usually expressed in MPa or N (Figure 5).

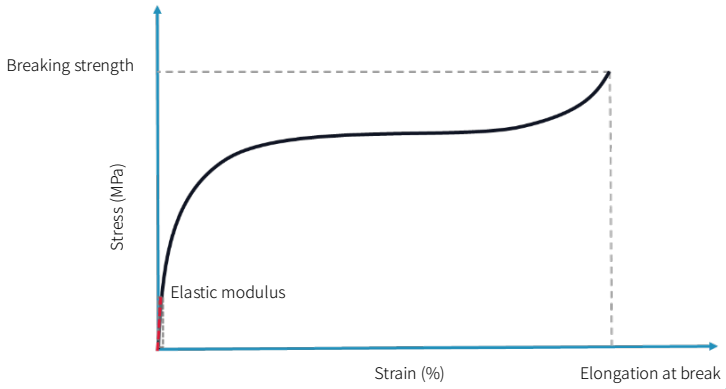


Figure 5. Tensile Curve of Sample Film Stripe

In the process of film processing and molding, the film winding direction is the MD-Machine Direction, also called the Longitudinal Direction; while the TD-Transverse Direction is perpendicular to the MD-Machine Direction, also called the Transverse Direction (Figure 6).

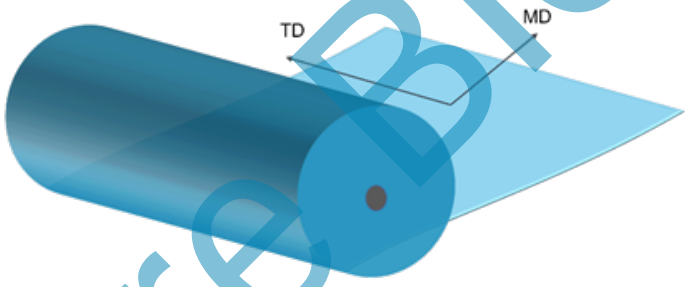


Figure 6. Schematic Diagram of Film's MD and TD

3.3.1.2. Test Results

Table 2. Test Results of Tensile Strength of LeKrius™ Film at Break

Standard	Test	Unit		Test value (Before irradiation)	Test value (After irradiation)
ASTM D882	Elongation at break	%	MD	420	458
			TD	392	408
	Breaking strength	MPa	MD	15	15
			TD	14	14
	Elastic modulus	MPa	MD	230	220
			TD	210	200

3.3.2. ASTM F1306 Anti-puncture Property

The anti-puncture test is performed by applying an external force to the film at a certain

speed with a probe in the direction perpendicular to the film until the film is perforated (Figure 7). This test predicts the risk of single-use bags being punctured by sharp objects during packaging, shipping, or use.

3.3.2.1. Test Method

This test is performed according to ASTM F1306 standard. A conical needle is used in the test and the maximum puncture force and puncture distance during the test are recorded. The maximum puncture force represents the maximum stress produced when the film is strained during the application of an external force to the film by a probe until the film is perforated, usually expressed in the unit of N. The maximum puncture distance represents the strain length of the film at the time of being perforated by a probe, usually expressed in mm.

The test probe is tapered, with a needle diameter of approximately 3.2 mm, and the film is a circular sample with a diameter of 35 mm and is fixed by clamps. The probe gradually penetrates the film at a rate of 25 mm/min at a position perpendicular to the central point of the film.

Figure 7. Puncture Test Procedure

3.3.2.2. Test Results

Table 3. Test Results of Anti-puncture of LeKrius® Film

Standard	Test	Unit	Test value (Before irradiation)	Test value (After irradiation)
ASTM F1306	Maximum puncture force	N	48	50
	Maximum puncture distance	mm	13	13

3.3.3. ASTM F392 B Anti-twisting Property

The anti-twisting test detects whether the film is still able to maintain integrity after repeated twisting and extrusion in a spatial dimension. This test can evaluate the resistance of the film against twisting and bending damage during production, processing, shipping, use, etc.

3.3.3.1. Test Method

The test is performed in accordance with the ASTM F392 B standard with a torque of 15 cm and a torsion angle of 440° in the vertical direction for a total of 900 twists (Figure 8). At the end of the test, the number of micropores is determined by ink penetration method, and the smaller the number represents the better anti-twisting performance.

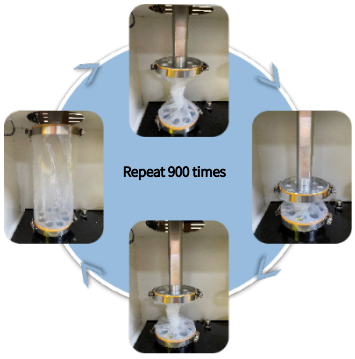


Figure 8. Anti-twisting Test

3.3.3.2. Test Results

Table 4. Test Results of Anti-twisting of LeKrius® Film

Standard	Test	Unit	Test value (Before irradiation)	Test value (After irradiation)
ASTM F392 B	Anti-twisting: number of pinholes produced after 900 times of twists	Number per piece of film	0.3	0.5

3.3.4. ASTM D1709 Impact Resistance

The impact resistance of the film is one of the important indicators of the film. The intensity of the impact strength of the film directly affects the ability of the film to withstand external impact, indicating whether the single-use bag can maintain its integrity when impacted.

3.3.4.1. Test Method

This test is performed according to ASTM D1709 standard using Method A. The diameter of the test sample is 150 mm. The weight falls freely from a fixed height of 660 mm (Figure 9), if the film is not damaged, increase the weight of the weights, and conversely, if the film is damaged, reduce the weight of the weights. Repeat the test for no less than 20 times and calculate the impact strength when the breakage rate is right 50%.
Impact strength indicates the impact force of the film at 50% breakage, usually expressed in g.

Figure 9. Falling Dart Impact Test

3.3.4.2. Test Results

Table 5. Test Results of Impact Resistance of LeKrius® Film

Standard	Test	Unit	Test value (Before irradiation)	Test value (After irradiation)
ASTM D1709	Impact strength	g	1300	1300

3.3.5. ASTM F88 Heat Seal Strength

The heat seal strength has the most direct effect on the integrity of the single-use bag. Reasonable heat sealing process parameters have been validated to ensure the stability of the bag body welding process.

3.3.5.1. Test Method

Seal two pieces of 15-mm wide film by heat according to ASTM F88. After complete cooling (for at least 24 h), stretch the two pieces of heat sealed film in opposite directions with a tensile tester until peel or break at the heat seal. During this process, the maximum stress tested by the tensile machine is the heat seal strength, usually expressed in unit of N/cm or N/15 mm.

3.3.5.2. Test Results

Table 6. Test Results of Heat Seal Strength of LeKrius® Film

Standard	Test	Unit	Test value (Before irradiation)	Test value (After irradiation)
ASTM F88	Heat seal strength	N/15mm	81	81
		N/cm	54	54

3.4. Barrier Properties

Barrier property test items include gas (oxygen, carbon dioxide, etc.) and vapor transmission properties.

3.4.1. ASTM D1434 Gas Barrier

According to ASTM D1434 standard, place the processed film between the upper and lower chambers by the pressure differential method, and under the action of the pressure

gradient, the gas passes through the film from the upper chamber to the lower chamber (Figure 10). The amount of gas passes (Figure 14) can be calculated based on the pressure change in the lower chamber, usually expressed in $cm^3/(m^2.day.bar)$.

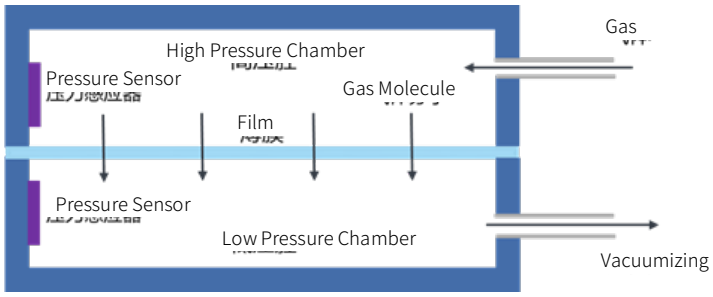


Figure 10. Principle of Gas Transmission Rate Tester

The test results are shown in the table below:

Table 7. Test Results of Oxygen/Carbon Dioxide Transmission Rates

Standard	Test	Unit	Test value (Before irradiation)	Test value (After irradiation)
ASTM D1434	Oxygen transmission rate	$cm^3/(m^2.day.bar)$	1.1	1.0
	Carbon dioxide transmission rate	$cm^3/(m^2.day.bar)$	0.8	0.8

3.4.2. ISO15106-2 Water Vapor Barrier

According to ISO15106-2, fix the pre-processed film in the middle of the test chamber by using the infrared method principle, nitrogen with a relatively stable humidity flows in the lower chamber, and dry nitrogen flows in the upper chamber. Due to the presence of a humidity gradient, water vapor will diffuse from the high humidity chamber to the low humidity chamber. The water vapor that passes through the sample is carried by the flowing dry nitrogen to the infrared sensor to measure the water vapor transmission rate (Figure 11), usually expressed in $g/(m^2.day)$.

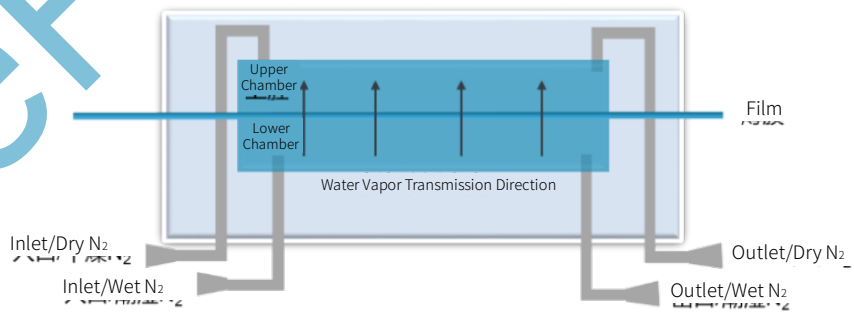


Figure 11. Water Vapor Transmission- by Principle of Infrared Method

The test results are shown in the table below:

Table 8. Test Results of Water Vapor Transmission Rate

Standard	Test	Unit	Test value (Before irradiation)	Test value (After irradiation)
ISO15106-2	Water vapor transmission rate	g/(m².day)	0.8	0.9

3.5. High Temperature Use Tolerance Test

Three finished products of 1 L and 5 L of LeKrius® single-use storage bags are prepared each, stored at 60 °C for 21 days after being filled with pure water, then transferred to room temperature for cooling, and the physical properties of these single-use storage bags are tested. Test items included: bag body inspection, pipeline inspection, connector inspection, fluid flow regulating clamp inspection, integrity test, connection firmness test, and heat sealing strength test.

The test results show that the medical single-use system manufactured from LeKrius® film could be used for 21 days at 60 °C.

3.6. Biocompatibility Test

The biocompatibility test of LeKrius® film is performed after gamma irradiation (45-55 kGy). At the same time, the film after accelerated aging for 3 years is also tested.

	Item	Result
ISO 10993-4	Hemolysis test	Pass
ISO 10993-5	Cytotoxicity test	Pass
ISO 10993-6	Implantation test	Pass
ISO 10993-10	Irritation and sensitization test	Pass
ISO 10993-11	Acute systemic toxicity test	Pass
USP <87>	In vitro biological response	Pass
USP < 88 > Class VI	In vivo biological response	Pass
EP 5.2.8	TSE/BSE risk	Pass

4. Size of LeKrius® Single-use Bioreactor Bags

4.1. Application and Operation Unit

- Installed in the LePhinix® Single-use Bioreactor
- For culture of mammalian and insect cells (antibodies, cell therapy/gene therapy, vaccines, etc.)

4.2. Process Operating Range:

In-process parameters	Range
Operating temperature	Cooling water temperature + 8 °C to 40 °C
Maximum operating pressure	50mbar
Maximum stirring condition	50L： 240rpm 200L: 200rpm 500L: 160rpm

	1000L: 140rpm 2000L: 115rpm
Operating time	It has been validated that it could be used for 21 days at a pressure of 30 mbar under the maximum process condition

4.3. Working Volume:

Strength / Description	50L	200L	500L	1000L	2000L
Total volume (L)	68	280	700	1300	2800
Minimum working volume (L)	22	40	100	200	400
Maximum working volume (L)	50	200	500	1000	2000

4.4. List of Main Components and Materials:

Connection components	Luer taper, CPC sterile quick connector/Pall Kleenpak
Capsule air filter	0.2 μm sterile PVDF air filter element
Port	Sampling port * 1 Feed port * 6 Harvest port * 1 Customizable ATF connector
Compatible pH/DO electrode	Single-use pH electrode Single-use DO electrode Reusable traditional analog pH electrode Reusable optical DO electrode
Bottom ventilation	316L stainless steel
Mixing paddle	Polypropylene
Pipeline material	TPE and platinum vulcanized silicone tubing

5. Application Test of LeKrius® Single-use Bioreactor Bags

The LeKrius® single-use bioreactor bag has completed complex biocompatibility test, chemical test, physical test, cell growth test, extractable test, etc., providing reliable performance for users in the biopharmaceutical process.

Depending on the nature of the validation method, each component, representative test bag or final product containing all relevant components is validated.

5.1. Summary of Validation Tests of LeKrius® Single-use Bioreactor Bags

Application Test Item	Description	Reference	Section
Medium Storage and Cell Growth Compatibility	Compatibility of cell growth: The biological reaction bag is extracted through the culture medium, the extracted culture medium is used for cell culture, and compared with the control group		4.1.1
Shelf Life Validation	Accelerated aging of LeKrius® film and bags is performed. After completion of accelerated aging, the sample is tested for physical properties	ASTM F1980-2016	4.1.1.1
CHO Fed-batch Culture and Control in LeKrius® Single-use Bioreactor Bags	The objective of this study is to determine the performance of the LeKrius® single-use bioreactor bag in the high cell density fed-batch process of recombinant CHO cell lines producing monoclonal antibodies		4.1.1.2
Integrity of LeKrius® Single-use Bioreactor Bags	NA	NA	4.2
Integrity of Bag Body and Assembly	The manufacturing process has been validated, and raw materials, manufacturing processes and final products also have been monitored and controlled accordingly		4.2.1
Product Release and Manufacturing Control	Manufacturing of LeKrius® film and LeKrius® single-use bioreactor bags made of the film has established raw material, manufacturing process, and finished product control	USP<85>, CHP<1143> USP<788> CHP<0903> ASTM F88 ASTM F88 CHP <0631> ISO11137	4.2.2
LeKrius® Single-use Bioreactor Bag: Physicochemical Test	NA	NA	4.3
USP <661>: Containers, Physicochemical Tests - Plastics	Gamma irradiation (45-55 KGy) prior to testing to assess compliance with USP < 661 > criteria after irradiation sterilization	USP<661>	4.3.1
EP 3.1.5 Polyethylene with Additives	Gamma irradiation (45-55 KGy) prior to testing to assess compliance with EP 3.1.5 criteria after irradiation sterilization	EP 3.1.5	4.3.2
Chemical	The storage bags irradiated by 45-55 kGy gamma	ASTM、	4.3.3

Compatibility	are filled with solution first and then placed at 30 ± 2.5 °C for 7 days, then appearance inspection, drop testing, film thickness, heat strength, connection firmness, infrared tests, etc. are carried out	China Center for Food and Drug International Exchange Guidance on Application and Technology of Single-Use System	
Compliance with Other Regulatory Guidelines	Our LeKrius® single-use bioreactor bags comply with the latest regulations and there is no BSE/TSE source in the raw materials and manufacturing process of the film	NA	4.4

5.1.1. Medium Storage and Cell Growth Compatibility

Compatibility of cell growth: The biological reaction bag is extracted through the culture medium, the extracted culture medium is used for cell culture, and compared with the control group.

Test Method

A 0.6 L (total volume) 2D bag is used in this study for gamma sterilization at 50 kGy. 200 mL of medium (CHO MaxC Production Medium from Medium Bank) is filled into the 0.6 L bag under the worst-case condition of a surface area to volume ratio of 3 cm^2/mL. Extraction is performed in a sterile environment at 37 °C without stirring for 3 days, protected from light. Glass bottles are selected as reference standard. Subsequently, the medium extraction solution is transferred to flasks for cell culture testing. See Figure 12.

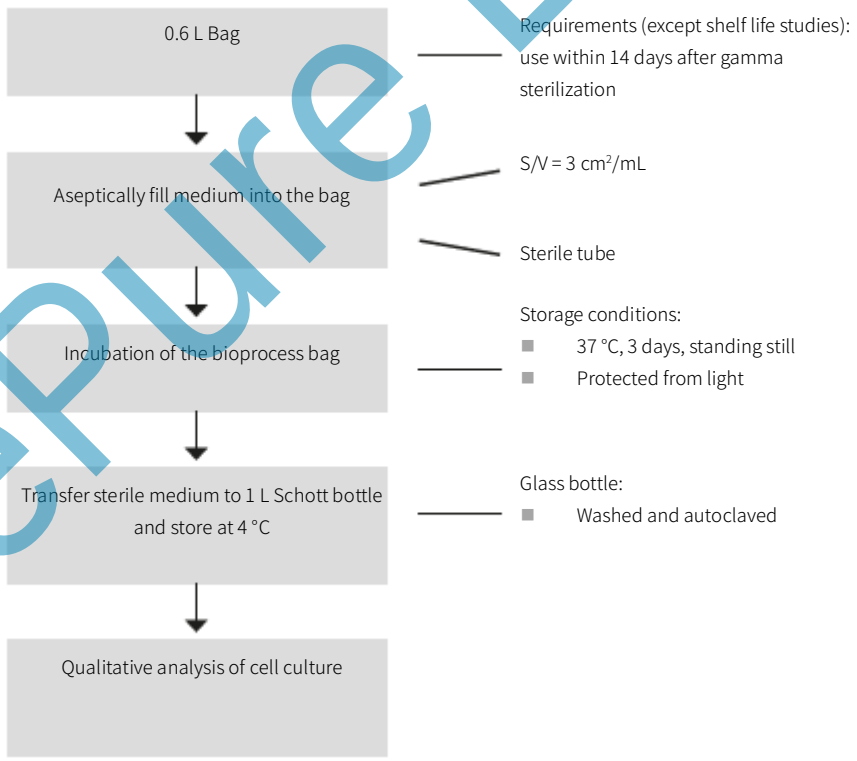


Figure 12: Flow Chart of Medium Extraction Bag

Human IgG1 secreted by the Chinese hamster ovary cell line CHO-DG44 (Cellca GmbH, Germany) is selected as the CHO model cell line. The seeding cell density is 0.3 × 10^6

cells/mL. The culture parameters used for cell culture analysis are shown in the table below. Viable cell concentration and activity are measured daily with a counter.

Table 1: Culture Parameters Selected based on Determination

Parameter	Set value
Rotation speed	120 rpm
Tempature	37.0°C
Partial pressure of carbon dioxide	5.0%
Humidity	80 %

5.1.1.1. Shelf Life Validation

In accordance with ASTM F1980-2016, accelerated aging of LeKrius® film and bags is performed by raising the temperature. After completion of accelerated aging, the physical properties of the sample is tested.

Table 1 Shelf Life of LeKrius® Film and Bags

Product Name	Shelf life
LeKrius® film	3 years
LeKrius® single-use bags and assembly	2 years

LePure Biotechnology is currently conducting the tests for longer shelf life and simulating real-time aging tests. The company will further update the data based on the test results obtained.

5.1.1.2. CHO Fed-batch Culture and Control in LeKrius® Single-use Bioreactor Bags

Objective

The objective of this study is to determine the performance of the LeKrius® single-use bioreactor bag in the high cell density fed-batch process of recombinant CHO cell lines producing monoclonal antibodies. Fed-batch runs are performed in a 200 L LePhinix® single-use bioreactor. This result is compared with the results of control cultures in shake flasks.

Test Method

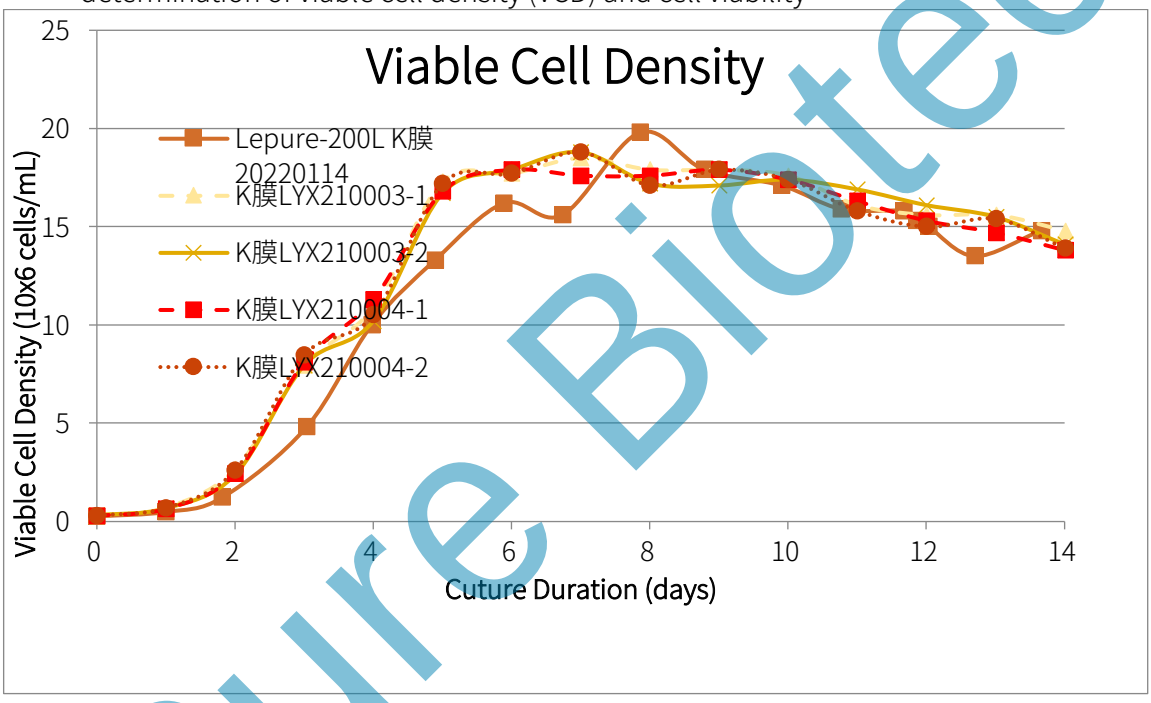
Type of the bag used in this experiment:

- LeKrius® 200 L Single-use Bioreactor Bag
- Chemically defined cell culture medium provided by Medium Bank is used: CHO MaxC Production Medium, MaxFA Feed Medium, MaxFB Feed Medium 7.5% $NaHCO_3$, 250 g/L glucose
- Transgenic mammalian cell line: CHO-K1 expressing human immunoglobulin IgG1
- Initial cell density: 0.25 * 10^6 cells/mL
- Working volume: 100 mL (500 mL shake flask), 50 L (LeKrius® single-use bioreactor bag 200 L)

- Incubation time: 14 days
- Incubation conditions

Temperature (℃)	D1-D6	37.0
	D6-End	33.0
pH	D1-D6	7.20±0.3
	D6-End	7.05±0.1
DO（%）	40	
Agitation rate(rpm)	D1-D6	60
	D6-End	70

  - Rotation speed: D1-D6: 60 rpm D6-D14: 70 rpm
  - Angle: 7-10°
  - Temperature: D1-D6: 37.0 °C D6-D14: 33.0 °C
  - pH: D1-D6: 7.20 D6-D14: 7.20
  - DO：40 %
- Cedex HiRes (Roche Diagnostics, Switzerland) is used to monitor cell growth and determination of viable cell density (VCD) and cell viability



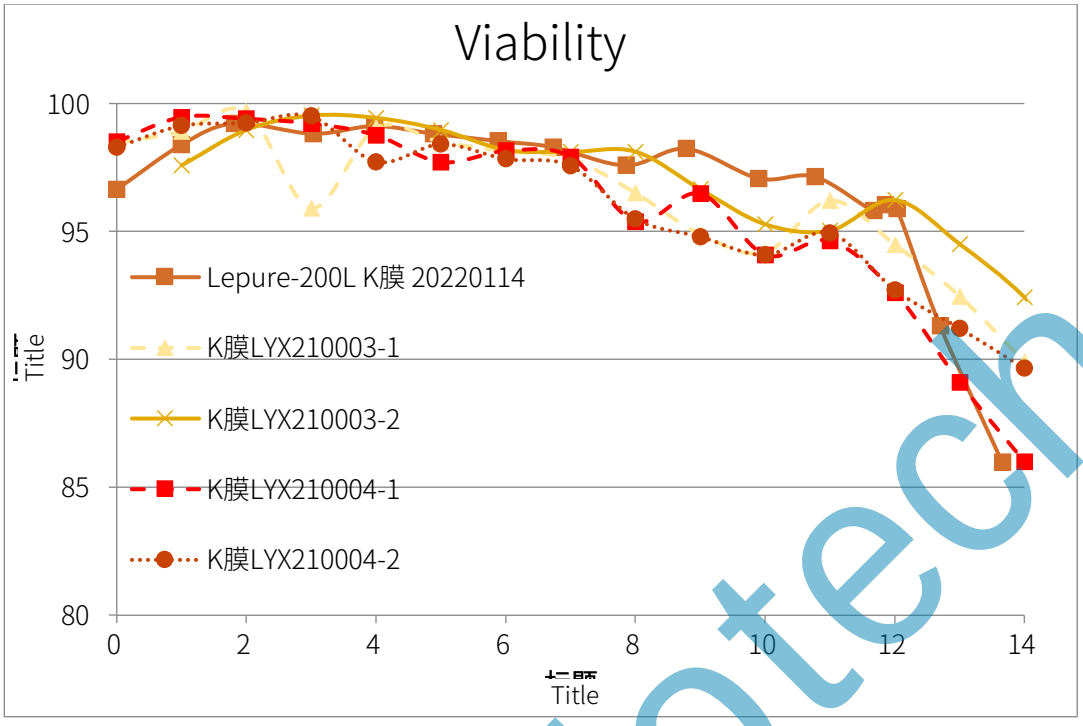


Figure 2: Cell Density and Viability in the LePhinix® Bioreactor

Result

This figure shows the CHO cell density and viability after 14 days of culture in a LePhinix® single-use bioreactor. In this reactor, the cell density peaks after day 6, then enters the plateau phase and is maintained at ~ 15 x 10^6 cells/mL; the cell viability maintains very well throughout the culture cycle, with viability maintained at more than 90% before day 12 and > 85% at harvest on day 14. Comparing the previous shake flask culture results with the culture results of this reactor, there is a very high culture consistency in terms of viable cell density and cell viability.

The results show comparable cell growth and viability between LeKrius® single-use bioreactor bags and shake flasks.

5.2. Integrity of LeKrius® Single-use Bioreactor Bags

5.2.1. Integrity of Bag Body and Assembly

The manufacturing process has been validated, and raw materials, manufacturing processes and final products also have been monitored and controlled accordingly.

Item	Test Content	Reference Method/Standard	Result
Connection firmness		100% visual inspection and in-house acceptance criteria	
Clamp/strip firmness		100% visual inspection and in-house acceptance criteria	
Leak check	Leak check is performed on the bag and device by pressure attenuation method	100% leak check, in-house method	
Package integrity	The integrity of the vacuumed packaging is performed to confirm whether the secondary packaging is complete	100% visual inspection	

5.2.2. Product Release and Manufacturing Control

Manufacturing of LeKrius® film and LeKrius® single-use bioreactor bags made of the film® has established raw material, manufacturing process, and finished product control.

Control of raw materials

Item	Reference standard	Acceptance criteria as defined by regulations	In-house release criteria of LePure Biotech
Appearance, size	In-house method	/	In-house acceptance size
Material identification	FTIR	/	Conformance with FTIR spectrum
Bacterial endotoxins	USP<85>, CHP<1143>	＜0.25EU/mL	＜0.125EU/mL
Sub-visible particles	USP<788> CHP<0903>	⩾ 10 μm: ⩽ 25 particles/mL ⩾ 25 μm: ⩽ 3 particles/mL	⩾ 10 μm: ⩽ 10 particles/mL ⩾ 25 μm: ⩽ 1 particle/mL
Visible particles	Visual inspection, in-house method	/	⩽ 3 particles/50 mL

In-process control

Item	Reference standard	In-house release criteria of LePure Biotech
Appearance, size	In-house method	In-house acceptance size
Heat seal strength	ASTM F88	⩾50N / 15mm
Product manufacturing complies with the drawing	Drawing	100% product complies with the drawing
Manufacturing flow	Internal procedure	100% meets manufacturing requirements

Final product testing

Item	Reference standard	Acceptance criteria as defined by regulations	In-house release criteria of LePure Biotech
Integrity	Pressure attenuation, ASTM F2095-07	/	Ensure integrity 100% through pressure attenuation
Heat seal strength	ASTM F88	/	⩾50N / 15mm
Connection firmness	In-house method	/	In-house acceptance size
Bacterial endotoxins	USP<85>, CHP<1143>	＜0.25EU/mL	＜0.125EU/mL
Sub-visible particles	USP<788> CHP<0903>	⩾ 10 μm: ⩽ 25 particles/mL ⩾ 25 μm: ⩽ 3 particles/mL	⩾ 10 μm: ⩽ 10 particles/mL ⩾ 25 μm: ⩽ 1 particle/mL
Visible particles	Visual inspection, in-house method	/	⩽ 3 particles/50 mL
pH	CHP <0631>	/	5.0-7.0

Sterility assurance

The loading form and dose of the irradiation have been validated according to the acceptance criteria specified in ISO11137 for VDmax^{25}. The irradiation dose range used in actual manufacturing is 25-40 kGy and the sterility assurance level is SAL = 10^{-6}. At the same time, representative product will undergo bioburden testing and dose review every 3 months, and each batch of products has an irradiation certificate to confirm that the irradiation dose meets the validation requirements.

5.3. LeKrius® Single-use Bioreactor Bag: Physicochemical Test

5.3.1. USP <661>: Containers, Physicochemical Tests - Plastics

Conduct gamma irradiation (45-55 KGy) prior to testing to assess compliance with USP < 661 > criteria after irradiation sterilization.

Test Method	Test Item	Judgment Criteria	Result
USP<661>	Buffer performance	⩽10.0mL	Acceptable
	Heavy metals	⩽1ppm	Acceptable
	Non-volatile residuals	⩽15mg	Acceptable
	Residue on ignition	⩽5mg	Acceptable

5.3.2. EP 3.1.5 Polyethylene with Additives

Conduct gamma irradiation (45-55 KGy) prior to testing to assess compliance with EP 3.1.5 criteria after irradiation sterilization.

Test Method	Test Item	Judgment Criteria	Result
EP 3.1.5	Appearance of solution	Clear, colorless	Acceptable
	pH	Consumed volume of 0.01 M NaOH solution ⩽ 1.5 mL, indicator turns blue Consumption volume of 0.01 M HCl solution ⩽ 1.0, mL indicator turns orange	Acceptable
	Absorbance	⩽0.2	Acceptable
	Reducing substance	Volume difference of volumetric solution ⩽ 0.5 mL	Acceptable
	Aluminum	⩽1ppm	Acceptable
	Chromium	⩽0.05ppm	Acceptable
	Titanium	⩽1ppm	Acceptable
	Vanadium	⩽0.1ppm	Acceptable
	Zinc	⩽1ppm	Acceptable
	Zirconium	⩽0.1ppm	Acceptable
	Heavy metals	⩽2.5ppm	Acceptable
	Sulfated ash	⩽1.0%	Acceptable

5.3.3. Chemical Compatibility

A 1000 mL 2D storage bag fitted with platinum vulcanized silicone tubing, PE assembly, PP assembly, PC assembly is used in this test. The storage bags irradiated by 45-55 kGy gamma are filled with solution first and then placed at 30 ± 2.5 °C for 7 days, then appearance inspection, drop test, film thickness measurement, heat strength test, connection firmness test, infrared test, etc. are carried out. The test results are shown in the table below.

Bag Assembly Compatibility List (7 days at 30 ± 2.5 °C)

Solution	Test Result
Ethanol 95%	Compatible
Ethanol 70%	Compatible
Glycerol 100%	Compatible
Propylene Glycol 100%	Compatible
Glycols 100%	Compatible
Methanol 100%	Compatible
Ethyl Acetate 100%	Compatible
DMSO 10%	Compatible
DMF10%	Compatible
Heptane100%	Compatible
Hydrochloric Acid 3%	Compatible

Hydrochloric Acid 10%	Compatible
Hydrochloric Acid 20%	Compatible
Hydrochloric Acid 37%	Compatible
Citric acid 1%	Compatible
Sulfuric Acid＜10%	Compatible
Sulfuric Acid 20%	Compatible
Boric Acid	Compatible
Phosphoic Acid 25%	Compatible
Ammonium Hydroxide(5M)	Compatible
Sodium Hydroxide 1%	Compatible
Sodium Hydroxide 20%	Compatible
Sodium Hydroxide 50%	Compatible
Acetate buffer solution pH4.6	Compatible
Acetate buffer solution pH10	Compatible
Phosphate buffer solution pH7.2	Compatible
Phosphate buffer solution pH10	Compatible
pH 3 HCl/KCl buffer	Compatible
Calcium Carbonate	Compatible
Barium sulfate	Compatible
Sodium Bicarbonate	Compatible
Sodium acetate 27%(50mM)	Compatible
Sodium Chloride 5M	Compatible
X-100 Trion X-100 100%	Compatible
Hydrogen Peroxide 10%	Compatible
Tween 80 100%	Compatible
Oliver oil 100%	Compatible

5.4. Compliance with Other Regulatory Guidelines

Our LeKrius® single-use bioreactor bags comply with the latest regulations and there is no BSE/TSE source in the raw materials and manufacturing process of the film.

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