Isolere Bio™

IsoTag™ AAV User Manual

for AAV Purification

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1. About This Manual

This manual is part of the product; it must be read in full and retained. This manual applies to the following versions of the product:

IsoTag™ AAV, 10mL Evaluation Kit (Research Grade)

IsoTag™ AAV, 10mL Reagent (Research Grade)

IsoTag™ AAV 125mL Reagent (Research Grade)

IsoTag™ AAV, 500mL Reagent (Manufacturing Grade)

2. Intended Use

IsoTag™ AAV reagent (Research Grade) is intended for **research use only.** It is **not** for diagnostic use or direct administration to humans or animals.

IsoTag™ AAV reagent (Manufacturing Grade) is intended **for research use or further manufacturing.**

The products are intended exclusively for use in accordance with this manual. Any other use is considered improper.

3. Product Description

The IsoTag™ AAV reagent is a fusion protein engineered for the demanding requirements of small and large-scale downstream purification. It enables a robust, efficient, and consistent purification process for a broad spectrum of adeno-associated virus (AAV) serotypes. IsoTag™ AAV combines the principles of affinity capture with liquid-liquid phase separation (ALPS) using a proprietary fusion protein. The single protein reagent has two domains: (1) an AAV-specific binding domain and (2) a stimulus-responsive biopolymer.

4. Reagent Specifications

Characteristic	Description
Appearance	Clear, colorless, liquid
Formulation buffer	20mM Histidine, pH 7.0
Concentration*	11.3 mg/mL (+20%, -0%)
Serotype affinity**	AAV9
Recommended concentration for use	For ALPS-TFF: 6.3% v/v IsoTag™ AAV "seed" material, 0.5% v/v IsoTag™ AAV "Feed" material For ALPS-CF: 2.3% v/v IsoTag™ AAV
Buffer additives	Histidine, PBS, and water.
Incompatible buffers	Urea, EDTA
Storage conditions	-80˚C until use

** For specific IsoTag™ AAV reagent lot concentration, see the Certificate of Analysis*
*** Binding efficacy observed with small-scale capture tests with AAV9, 8, 6, 2, 1, PHP.B and Rhs.10*

5. Methods

The following section describes common applications of IsoTag™ AAV reagent in the downstream purification of AAV and baseline methods. Optimization may be required, see section 6 for optimization information.

For the centrifugation process (ALPS-CF), see section 5.1.
For the tangential flow filtration process (ALPS-TFF), see section 5.2.

5.1 Centrifugation (ALPS-CF)

The ALPS-CF method offers a small-scale method to screen IsoTag™ AAV reagent compatibility with specific capsids and buffers. The results of this method are directionally translatable to the ALPS-TFF processes.

5.1.1 Equipment

- Fixed angle centrifuge (5,000 x g, 22°C)
- Tube rotator or shaker at 4°C
- Heat block at 37°C (recommended), or water bath at 37°C

5.1.2 Materials

Component	Composition
AAV harvest material	Lysed and clarified AAV harvest (containing 0.01% poloxamer 188 recommended)*
IsoTag™ AAV reagent	IsoTag™ AAV reagent, 20mM histidine, pH 7.0
Phase Transition Buffer 1	5M NaCl
Elution Buffer	Elution buffer optimization may be necessary. The following buffers have been shown to work with AAV9: • 100mM Glycine, pH 3.0 • 0.5M Arginine, pH 7.0
Neutralization Buffer (For use with low pH Elution)	1M Tris-HCl, pH 7.5
Phase Transition Buffer 2	5M MgCl2
0.25X PBS (recommended)	Phosphate Buffered Saline
Appropriately sized centrifuge tubes	N/A
Ice bucket	N/A

** Addition of poloxamer 188 may prevent AAV from adhering to centrifuge tubes.*

5.1.3 Material Prep

1. Thaw IsoTag™ AAV reagent at room temperature (20°C - 25°C). Once fully thawed, store on ice for the duration of the experiment. Allow IsoTag™ AAV reagent to chill on ice for at least 20 minutes prior to use and ensure reagent is completely clear before proceeding.
2. Equilibrate AAV lysate to 4°C and place on ice for the duration of the experiment.
3. Pre-chill Elution Buffer and 0.25X PBS at 4°C or on ice prior to use.

5.1.4 Capture

1. Prepare and label centrifuge tubes for each sample and experimental replicate.
2. Invert AAV harvest material at least 5 times to mix, then add harvest material to each labeled tube. Reserve and set aside an aliquot of each harvest material in separately labeled tubes for determining initial titers during subsequent analysis.

3. Chill tubes containing AAV lysate on ice for 10 minutes.
4. Mix the IsoTag™ AAV reagent by inverting at least 5 times or briefly vortex (3-5 times). Add 2.3% v/v IsoTag™ AAV reagent to AAV lysate into each tube. Mix by inverting tubes at least 5 times.
5. Remove the tubes from ice and incubate at room temperature (20°C-25°C) for 10 minutes.
6. Add Phase Transition Buffer 1 (5M NaCl) to achieve a final concentration of 0.6M NaCl to each tube of AAV lysate material containing IsoTag™ AAV reagent. Invert tubes at least 5 times to mix.
7. Incubate the tubes at room temperature (20°C-25°C) for 10 minutes. Sample should appear visibly cloudy
8. Place tubes in centrifuge and spin at 5,000 x g at room temperature (20°C-25°C) for 10 minutes.
9. Following centrifugation, a pellet should be visible at the bottom of the tube. Remove supernatant (capture supernatant), using caution not to aspirate the pellet.

   Recommended: save a sample of capture supernatant for analytics to determine capture efficiency.

9.1. If a pellet is not visible, place the tube(s) on a heat block (37°C) for 3 minutes. If the pellet(s) is still not visible and/or the solution in the tube(s) appears cloudy, spin for an additional 10 minutes at 5,000 x g and room temperature (20°C-25°C).

5.1.5 Elution

1. Using the corresponding volumes, carefully pipette 4°C Elution Buffer into each tube with a pellet, using caution not to touch the pellet with the pipette tip.

   DO NOT pipette up and down to mix, as you risk aspirating the pellet.

- Low pH (2.5 – 3.5) Elution Buffer – 80% of desired final volume into each tube with a pellet
- Neutral pH Elution Buffer (6.5 – 7.5) – 90% of desired final volume into each tube with a pellet

2. Briefly vortex each tube (3-5 times), then place on a rotator at 4°C for 1 hour to resuspend the pellet.
3. After removing the tubes from the rotator, briefly vortex (3-5 times). Verify that the pellets have completely resuspended by placing the tubes on a heat block at 37°C for 3 minutes. A visible pellet at the bottom of a tube indicates incomplete resuspension. If a pellet is visible, briefly vortex the tube and return to the rotator at 4°C for an additional 30 minutes until pellet is no longer visible, then proceed to the next step.
4. Once the pellet is completely resuspended and the sample is hazy throughout at room temperature (20°C-25°C), add Phase Transition Buffer 2 (5M $MgCl_2$) to a concentration of 0.6M $MgCl_2$ to each tube. Invert tubes at least 5 times to mix. The samples should become visibly cloudy.
5. Place tubes in centrifuge and spin at 5,000 x g and room temperature (20°C-25°C) for 10 minutes.
6. Following centrifugation, a pellet should be visible at the bottom of the tube. Transfer elution supernatant (eluted AAV) from each tube into a new labeled centrifuge tube, using caution not to aspirate the pellet.

6.1. If using low pH elution buffer, add Neutralization Buffer to each elution supernatant to achieve desired pH.

7. ***Recommended: Pipette cold 0.25X PBS equal to starting volume into each tube with a pellet (if retaining for analysis).***

   DO NOT pipette up and down to mix as you risk aspirating the pellet.

Briefly vortex the tubes (3-5 times) and place on ice to resuspend the pellets.

8. All samples (AAV lysate, capture supernatant, elution supernatant, and resuspended pellet) should be stored at -80°C until ready for analysis.

8.1. *Recommended: If performing analytical assays such as ddPCR then buffer exchange eluted AAV (elution supernatant) into a final formulation buffer for analytics using a centrifugal filter or similar. The high conductivity of the neutralized elution buffer may interfere with analytics such as ddPCR.*

5.1.6 Common ALPS-CF Process Volumes

The IsoTag™ AAV centrifugation protocol can be scaled linearly, provided that the volume can be accommodated in a fixed-angle centrifuge capable of 5,000 x g. The volume for the elution step can also be reduced to concentrate the elution sample; however, resuspension of the pellet may be more difficult and require more time. See the table below for common process volumes.

Capture			1X Elution Concentration		10X Elution Concentration	
AAV Harvest (mL)	IsoTag™ AAV (mL)	5M NaCl (mL)	Elution Buffer (mL)	5M $MgCl_2$ (mL)	Elution Buffer (mL)	5M $MgCl_2$ (mL)
1	0.023	0.137	0.80	0.10	0.08	0.01
5	0.115	0.685	4.00	0.50	0.40	0.05
10	0.230	1.37	8.00	1.00	0.80	0.10
50	1.15	6.85	40.0	5.00	4.00	0.50

5.2 Tangential Flow Filtration (ALPS-TFF)

The ALPS-TFF method enables scalable, affinity-based filtration of AAV capsids using IsoTag™ AAV reagent. This approach leverages the phase-separating properties of IsoTag™ to retain AAV capsids on a microfiltration TFF filter, allowing for capture, concentration, and washing. Purified AAV is then eluted through the permeate, while the IsoTag™ reagent remains retained. Additionally, ALPS-TFF employs a second auxiliary pump in permeate control mode to regulate flux across the filter—an essential feature for microfiltration TFF processes. The process can be performed using either hollow fiber filters or flat sheet cassettes. Recommended parameters for each are provided in Sections 5.2.6 and 5.2.7, respectively

5.2.1 Equipment

- TFF system with two auxiliary pumps (necessary for permeate control mode for microfiltration)
- Automated back pressure valve or pinch valve
- TFF pressor sensors (3)
- Retentate vessel that can accommodate working volumes of 2% - 10% of starting AAV lysate volume

5.2.2 Materials

Component	Composition
0.2µm PES filter	With sufficient surface area (recommended loading <70L/m2)
Tubing	Of appropriate internal diameter to achieve desired flow rates. • See section 5.2.6 for recommended hollow fiber TFF parameters. • See section 5.2.7 for recommended flat sheet parameters.
IsoTag™ AAV Reagent	IsoTag™ AAV, 20mM histidine, pH 7.0
Phase Transition Buffer 1	5M NaCl
Wash Buffer	20mM Tris, 0.6M NaCl, 0.01% poloxamer 188, pH 7.5
Elution Buffer	Elution buffer optimization may be necessary. The following buffers have been shown to work with AAV9: • 100mM Glycine, pH 3.0 • 0.5M Arginine, pH 7.0
Phase Transition Buffer 2	5M $MgCl_2$
Neutralization Buffer (For use with low pH Elution)	1M Tris-HCl, pH 7.5

5.2.3 System Setup

1. Prepare an aliquot >10% starting AAV lysate volume of Elution buffer with salt by adding Phase Transition Buffer 2 (5M MgCl2) to a final concentration of 0.6M. Set aside at Room temperature (20°C-25°C) until the elution step.
2. Place the remainder of Elution Buffer at 4°C until elution step (5.2.5).

3. Setup the TFF system as shown in Figure 1 below.
    - Place retentate vessel on feed scale.
    - Connect main hold-up loop tubing from retentate vessel to pressure sensors that have been connected to the filter ends
    - Ensure that the Automatic Back Pressure Valve is connected to tubing returning to the retentate vessel.
    - Connect diafiltration line to retentate vessel with first auxiliary pump.
    - Connect tubing to the pressure sensor connected to the permeate port of the filter and a second auxiliary pump to vessel on the permeate scale.
    - Flush filter with water as necessary according to manufacturer recommendations.
4. Equilibrate the TFF system by pumping a minimum of 3X system hold up volume of Wash Buffer through the retentate and permeate lines and send to waste to fully prime the TFF filter and all tubing.
5. Tare feed and permeate scales.

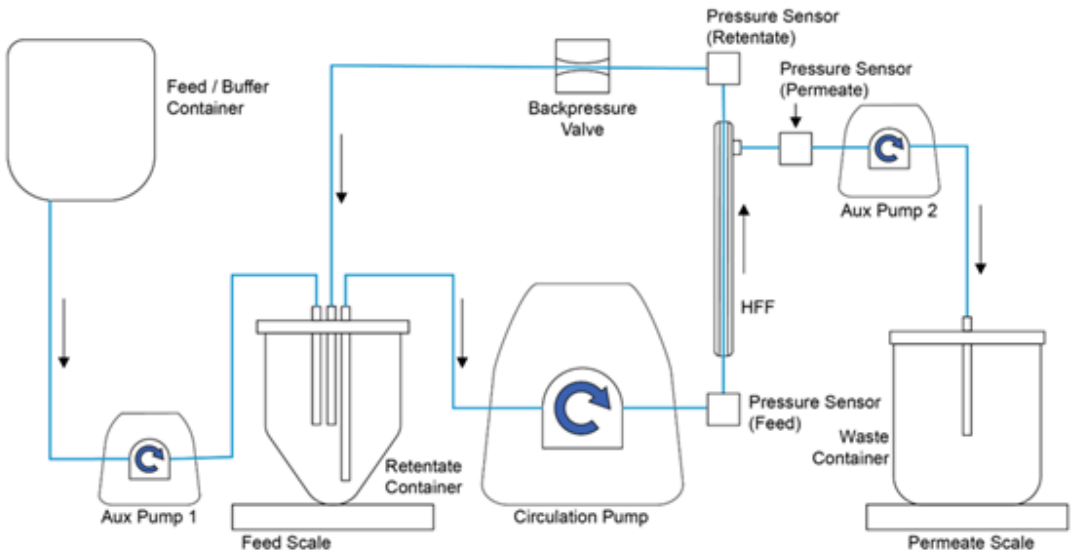

Figure 1 - TFF System set up utilizing 2 auxiliary pumps and a stir plate on the feed scale.

5.2.4 Concentration and Wash

1. Lyse, nuclease treat and clarify AAV harvest material prior to IsoTag™ AAV purification. Recommended starting material turbidity is <15 NTU.
2. Add Phase Transition Buffer 1 (5M NaCl) to room temperature (20°C-25°C) lysate to a final concentration of 0.6M NaCl and mix.
3. Thaw IsoTag™ AAV reagent at room temperature (20°C - 25°C). Once fully thawed, store on ice for the duration of the experiment. Allow IsoTag™ AAV reagent to chill on ice for at least 20 minutes prior to use and ensure reagent is completely clear before proceeding.
4. Aliquot 2% of the starting volume of the AAV lysate material. To this aliquot, add IsoTag™ AAV reagent to achieve a final concentration of 6.3% v/v relative to the aliquot volume. This high concentration IsoTag™ AAV material will be known as the "seed" and will be added to the retentate vessel to start the run.
   (Note: solution will turn cloudy when IsoTag™ reagent is added, this is due to the phase separation of the reagent.)
5. To the remaining AAV lysate, add IsoTag™ AAV reagent to 0.5% v/v. This will be considered the "feed" and will be used as the diafiltrate for the concentration of the AAV.
   (Note: solution will turn cloudy when IsoTag™ reagent is added, this is due to the phase separation of the reagent.)
6. Incubate "seed" and "feed" solutions with IsoTag™ AAV reagent for at least 20 minutes at room temperature (20°C-25°C) prior to beginning the run.
7. Add "seed" (2% AAV harvest material, NaCl, and IsoTag™ AAV solution) to retentate vessel.
8. Prime the diafiltration line with "feed" material (bulk AAV harvest, NaCl and IsoTag™

AAV solution).

9. Set the TFF system up to run in *permeate control* mode with the back pressure valve set to control the retentate pressure and the second auxiliary pump set to control the permeate flow. The permeate pump should be set to the minimum possible starting flow rate. See Recommended TFF Parameters section 5.2.6 or 5.2.7 depending on filter geometry.
10. Using the AAV "feed" as the diafiltrate, run the TFF in diafiltration mode until all the "feed" has been added to the retentate vessel. Ensure the concentration factor is set to 1 and the two aux pumps are maintaining equal flow rates such that the volume in the retentate vessel (2% of starting volume) remains constant, concentrating the bulk harvest or "feed" into the retentate vessel.
11. When retentate pressure reaches the setpoint and TMP is stable, permeate flow rate can be increased slowly to achieve target flux. (See Recommended TFF Parameters section 5.2.6 or 5.2.7 depending on filter geometry.)
12. Following the concentration step, transfer the diafiltration line to Wash Buffer and perform a wash with 6 diavolume (DVs) buffer exchange to remove additional contaminants.
13. After the wash step (DVs) are completed stop the pumps. Record the weight of the flow through and wash waste volumes and sample for mass balance analysis. Add collection vessels for the elution on the permeate scale and tare.

(Note: the total elution volume will be ~20% starting harvest volume. It is recommended to collect elution concentrate (~10% starting harvest volume) and elution DVs (8x ~1.25% starting harvest volume) separately for analytical purposes.)

5.2.5 Elution

1. Ensure permeate pump is off and line is clamped and open the back pressure valve.
2. Remove the Elution Buffer from 4°C storage, ensure the buffer is <10°C, and immediately add 4X retentate vessel volume to the "seed" or retentate vessel. Recirculate at the same flow rate used for capture/wash process for 5 minutes to mix the solution and facilitate AAV elution. (See Recommended TFF Parameters section 5.2.6 or 5.2.7 depending on filter geometry.)
3. After 5 minutes of recirculation, add Phase Transition Buffer 2 (5M MgCl2) to a final concentration of 0.6M MgCl2 to the retentate reservoir and continue recirculation of the material for an additional 10-15 minutes, allowing the solution to warm up to room temperature (20°C-25°C).

   (Note: the retentate should become cloudy after Phase Transition Buffer 2 is added and solution reaches room temperature (20°C-25°C). The visible cloudiness indicates phase separation.)
4. Prime the diafiltration line with the room temperature (20°C-25°C) solution prepared in Step 1 of Section 5.2.3, containing Elution Buffer and Phase Transition Buffer 2. After priming, ensure the diafiltration line is connected to the Elution and Phase Transition Buffer aliquot designated for diavolume washes (DVs).
5. Once the retentate solution has reached room temperature (20°C-25°C), stop the recirculation. Ensure the permeate scale contains the elution collection vessel(s) and is tared.
6. Concentrate the retentate 10X using the same TFF parameters as outlined in Section 5.2.4, Step 9 (Concentration/Wash Step). Allow the retentate pressure to reach the target set point (10psi) before gradually ramping up the permeate flow rate to the desired flux.

   (Note: The elution step may be ramped more quickly and operated at a higher flux than

the concentration/wash step.)

7. Once the concentration factor is reached, proceed with the diafiltration step using 8 DVs of the solution containing Elution Buffer and Phase Transition Buffer 2.
8. If applicable, add sufficient neutralization buffer to achieve desired final pH.

8.1. For some analytics such as ddPCR it may be necessary to buffer exchange the elution samples prior due to the high conductivity of the elution buffer.

5.2.6 Recommended TFF Parameters - Hollow Fiber

Parameter	Recommended Setting
Filter Loading	≤70 L/m^2
Shear Rate	8000 sec^{-1}
Cross Flow Rate	Sufficient to maintain shear rate, see filter manufacturers guidelines
Retentate Pressure	10 PSI (0.7 bar)
Starting Flux	8 LMH
Target Flux	40-50 LMH

5.2.7 Recommended TFF Parameters - Flat Sheet

Parameter	Recommended Setting
Filter Loading	≤50 L/m^2
Cross Flow Rate	5-10 LMM
Retentate Pressure	10 PSI (0.7 bar)
Starting Flux	8 LMH
Target Flux	35-45 LMH

5.2.8 Common ALPS-TFF Process Volumes

AAV Harvest (L)	Min. Filter Area (m^2)	Seed Volume (L)	IsoTag™ AAV Volume Seed (mL)	IsoTag™ AAV Volume Feed (mL)	Phase Transition Buffer 1 (L)	Wash Buffer (L)	Elution Buffer (L)	Phase Transition Buffer 2 (L)
1	0.014	0.02	1.26	5.00	0.134	0.12	0.18	0.014
5	0.07	0.10	6.30	25.00	0.668	0.60	0.90	0.07
10	0.14	0.20	12.61	50.00	1.33	1.20	1.80	0.14
50	0.70	1.00	63.03	250.00	6.68	6.00	9.00	0.70
100	1.40	2.00	126.06	500.00	13.36	12.0	18.0	1.40

6. Process Development

Process Step	Optimization
Harvest material treatment	The method described in this document uses lysate that has been clarified and treated to remove cellular debris and DNA. Lysate turbidity should be <15 NTU.
Concentration and wash	The recommended shear rate for the hollow fiber process is 8000 sec^{-1}, and the recommended crossflow rate for the flat sheet process is 5-10LMM to prevent fouling of the membrane. Increasing the flux as described in the protocol should also proceed at a slow rate to prevent fouling of the membrane. If fouling persists, it may be necessary to run at a lower flux, decrease the filter loading or improve the clarification of the lysate.
AAV serotype compatibility	The method described in this document was optimized for AAV9. For other serotypes, increasing the concentration of IsoTag™ AAV reagent to 1.0% v/v in the bulk material is recommended. Further increasing IsoTag™ AAV reagent concentrations, may improve capture but could have impacts on the elution. Capsid specific optimization of IsoTag™ AAV concentrations used may be necessary.
Elution	Elution buffer optimization may be necessary. The following buffers have been shown to work with AAV9. • 100 mM Glycine pH 3.0 • 0.5 M Arginine pH 7.0 • 0.5 M Proline pH 2.5

7. Troubleshooting

Initial Harvest Treatment and Preparation

Observation	Possible Cause	Recommended Action
Lysate does not appear cloudy after IsoTag™ AAV reagent addition	Incorrect temperature	Confirm that the lysate is at room temperature (20°C-25°C)
	Incorrect salt concentration OR Phase Transition buffer is not prepared to the correct conductivity	Confirm conductivity of all solutions
	Incorrect IsoTag™ AAV reagent concentration	Confirm IsoTag™ AAV transition in saline solution at similar dilution and salt concentration

Capture Step (ALPS-TFF)

Observation	Possible Cause	Recommended Action
IsoTag™ AAV permeate is not clear	Incorrect temperature	Confirm transition in saline solution at similar dilution and salt concentration **AND** Confirm conductivity of all solutions
	Incorrect salt concentration	
	Incorrect IsoTag™ concentration	
	Phase transition buffer is not prepared to the correct conductivity	
	Incorrect filter pore size is used	Confirm correct filter is used

Observation	Possible Cause	Recommended Action
Fouling of TFF filter	Process not run in permeate control mode	Microfiltration process must be run with a pump on the permeate line to maintain constant flux
	Contaminant profile of harvest material	Consider additional clarification or nuclease treatment, recommended turbidity <15 NTU
	Incorrect filter loading	Reduce filter loading on a volume to meter squared basis
	Incorrect flux (LMH)	Confirm TFF run settings, consider running at a lower flux or ramping the flux at a lower rate
	Incorrect retentate pressure	Confirm TFF run settings, consider replacing pressure sensors
	Running at an improper shear rate	Calibrate pumps
Incomplete capture, loss of material.	Insufficient IsoTag™ AAV reagent concentration	Increase IsoTag™ AAV reagent working concentration to 2X % v/v amount recommended in protocol
	Interference from harvest material additives	Confirm absence of EDTA in harvest material
	pH of harvest is outside of tested range	Adjust harvest pH to 7.2-7.4
Pressure spikes during TFF run	Fouling of TFF filter	(See above 'Fouling of TFF filter' for recommended actions
	Insufficient harvest material clarification	Consider additional clarification or nuclease treatment

Elution Step (ALPS-TFF)

Observation	Possible Cause	Recommended Action
Retentate does not turn clear upon addition of Elution Buffer	Elution Buffer temperature is too high	Ensure Elution Buffer is chilled to 4°C prior to use
	Elution Buffer is at the wrong conductivity	Ensure no salt has been added to Elution Buffer
	Residual contaminants are causing the turbidity	Improve clarification prior to starting IsoTag™ AAV process. Replace TFF filter with fresh filter for the elution step.
Retentate does not turn cloudy upon addition of Phase Transition Buffer 2	Insufficient amount of Phase Transition Buffer 2 is added	Confirm correct buffer and volume is added
	Retentate temperature is too low	Ensure retentate is allowed to warm to room temperature (20°C-25°C)

8. Order Information

Item Number	Description
100003083	IsoTag™ AAV, 10mL (Research Grade)
100003084	IsoTag™ AAV, 10mL Evaluation Kit (Research Grade)
100003085	IsoTag™ AAV, 125mL (Research Grade)
100003100	IsoTag™ AAV, 500mL (Manufacturing Grade)

9. Support

For technical support or to obtain a Certificate of Analysis, please contact us at IsolereSupport@donaldson.com

10. Limited Product Warranty

Isolere Bio, Inc and/or its affiliate(s) warrant their products as set forth in the Isolere Bio General Terms and Conditions of Sale found on Isolere Bio's website at www.isolerebio.com/terms-and-conditions-of-sale

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