Isolere Bio™
By Donaldson

IsoTag™ AAV User Manual

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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
IsoTag™ AAV, 10mL Reagent

2. Intended Use

The product is intended for **research use only.** It is **not** for diagnostic use or direct administration to humans or animals. The product is intended exclusively for use in accordance with this manual. Any other use is considered improper.

3. Product Description

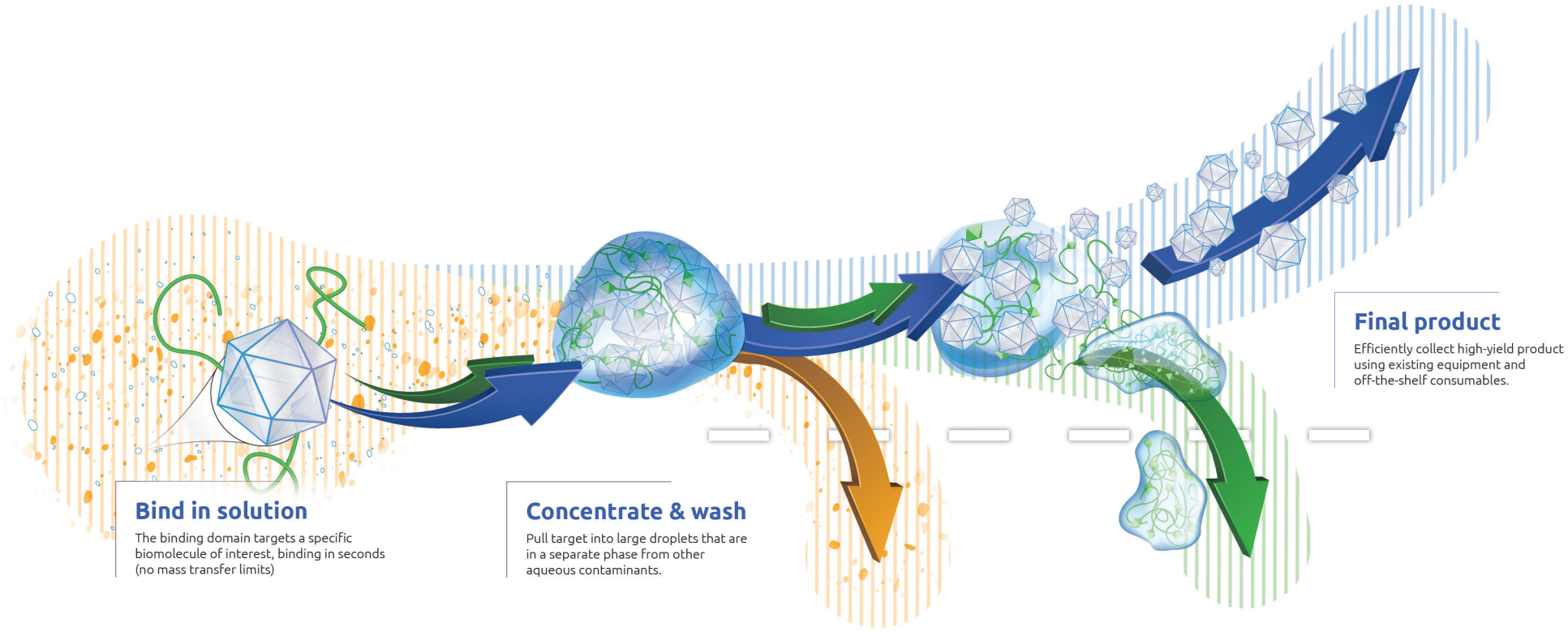
IsoTag™ AAV combines the principles of affinity capture with liquid-liquid phase separation using a proprietary fusion protein. The single protein reagent has two domains: (1) an AAV-specific binding domain and (2) a stimulus-responsive biopolymer.

IsoTag™ AAV is a specialized reagent, 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.

Features of IsoTag™ AAV include:

- Single-step purification with high purity and yield
- Linear scalability based on culture volume, rather than AAV titer
- Lower AAV aggregation than traditional affinity chromatography
- Compatibility with existing, familiar TFF equipment and off-the-shelf consumables

4. Affinity Liquid Phase Separation Overview



5. Specifications

Characteristic	Description
Appearance	Clear, colorless, liquid
Formulation buffer	20mM Histidine, pH 7.0
Concentration	11.3 mg/mL (+20%, -0%)
Serotype affinity*	AAV9 AAV8
Recommended concentration for use	0.67 mg/mL in seed volume, 0.045 mg/mL in harvest material for AAV9
Buffer additives	Histidine, PBS, and water. *The use of urea may cause inhibition of the phase behavior. Addition of EDTA will inhibit affinity activity of the reagent.*
Storage conditions	-80˚C until use

**Binding efficacy observed with small-scale capture tests with AAV9, 8, 6, 2, 1, PHP.B and Rhs.10 – see IsoTag™ AAV white paper for more info (www.isolerebio.com/aavpaper)*

6. 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 7 for optimization information. For the centrifugation process, see section 6.1. For the tangential flow filtration process, see section 6.2.

6.1 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.

6.1.1 Equipment

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

6.1.2 Materials

- AAV harvest material, lysed and clarified (containing 0.01% poloxamer 188 recommended)
- IsoTag™ AAV reagent
- Appropriately sized centrifuge tubes
- Phase Transition Buffer 1 (5M NaCl)
- Elution Buffer (see section 7 for recommended buffers)
- Phase Transition Buffer 2 (5M $MgCl_2$)
- Ice bucket
- 0.25X PBS (recommended)

6.1.3 Material Prep

1. Thaw IsoTag™ AAV reagent at room temperature. 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.

6.1.4 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 on ice for 10 minutes.
4. Mix the IsoTag™ AAV reagent by inverting at least 5 times or briefly vortex. Add IsoTag™ AAV reagent to achieve a final concentration of 0.25mg/mL to each tube. Pipette up and

down 10 times to mix the reagent, then invert tubes at least 5 times to mix.

5. Remove the tubes from ice and incubate at 22°C for 10 minutes.
6. Add Phase Transition Buffer 1 to achieve a final concentration of 0.6M NaCl to each tube of AAV harvest material containing IsoTag™ reagent. Invert tubes at least 5 times to mix.
7. Incubate the tubes at 22°C for 10 minutes.
8. Place tubes in centrifuge and spin at 5,000 x g and 22°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.

6.1.5 Elution

1. Pipette 4°C Elution Buffer equal to 80% desired final volume into each tube with a pellet, using caution not to touch the pipette tip to the pellet. DO NOT pipette up and down to mix as you risk aspirating the pellet.
2. Briefly vortex each tube, then place on a rotator at 4°C for 1 hour.
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, add Phase Transition Buffer 2 to a concentration of 0.6M $MgCl_2$ to each tube. Invert tubes at least 5 times to mix. The samples should become cloudier.
5. Place tubes in centrifuge and spin at 5,000 x g and 22°C for 10 minutes.
6. Following centrifugation, a pellet should be visible at the bottom of the tube. Transfer

supernatant (elution supernatant) from each tube into a new labeled centrifuge tube, using caution not to aspirate the pellet.

7. Add Neutralization Buffer to each elution supernatant as necessary to neutralize samples.
8. 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 and place on ice to resuspend the pellets.
9. All samples (harvest material, capture supernatant, elution supernatant, and resuspended pellet) should be stored at -80°C until ready for analysis.

6.1.6 Recommended Buffer Exchange

It is recommended to buffer exchange eluted AAV into a final formulation buffer for analytics using a centrifugal filter or similar. The high conductivity of the neutralized elution buffer may interfere with some analytics such as ddPCR.

6.1.7 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	0.8	0.1	0.08	0.01
5	0.115	0.685	4	0.5	0.4	0.05
10	0.23	1.37	8	1	0.8	0.1
50	1.15	6.85	40	5	4	0.5

Note: IsoTag™ AAV volumes calculated assuming 11.3 mg/mL stock solution of reagent.

6.2 Tangential Flow Filtration (ALPS-TFF)

The ALPS-TFF method allows for scalable affinity-based filtration of AAV capsids using IsoTag™ AAV reagent. The ALPS-TFF method utilizes the phase separating properties of the IsoTag™ AAV reagent to retain AAV capsids on a microfiltration TFF filter to capture, concentrate and wash AAV; purified AAV is then eluted through the permeate while the IsoTag™ reagent is retained. ALPS-TFF utilizes a second auxiliary pump for permeate control mode to control the flux across the filter, this is essential for microfiltration TFF processes. The process can be run on hollow fiber filters (see 6.2.6 for recommended parameters) or flat sheet cassettes (see section 6.2.7)

6.2.1 Equipment

- TFF system with *two auxiliary pumps (necessary for permeate control mode for microfiltration)*
- Recommended: small stir plate and stir bar for retentate vessel

6.2.2 Materials

- 0.2µm mPES filter of sufficient surface area (recommended loading <70L/m^2)
- Tubing of appropriate ID to achieve desired flow rates. See section 6.2.6 for recommended hollow fiber TFF parameters and section 6.2.7 for recommended flat sheet parameters.
- Retentate vessel that can accommodate working volumes of 2%-10% of starting AAV harvest volume

Kit Component	Composition
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	100mM glycine, 0.01% poloxamer, pH 3.0 (see section 7 for additional recommended buffers)
Phase Transition Buffer 2	5M $MgCl_2$
Neutralization Buffer	1M Tris-HCl, pH 7.5

6.2.3 System Setup

1. Aliquot 10% starting harvest volume worth of Elution Buffer. Add Phase Transition Buffer 2 to a concentration of 0.6M $MgCl_2$. Set aside at RT until elution step.
2. Place remainder of Elution Buffer at 4°C until elution step.
3. Setup the TFF system as shown in Figure 1 below.
    - Place retentate vessel (with stir bar) and stir plate on feed scale
    - Connect main hold-up loop tubing from retentate vessel to filter with ABV pressure valve on tubing returning to the retentate vessel.
    - Connect diafiltration line to retentate vessel with first auxiliary pump
    - Connect tubing to permeate port of filter and a second auxiliary pump to vessel on permeate scale.
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.

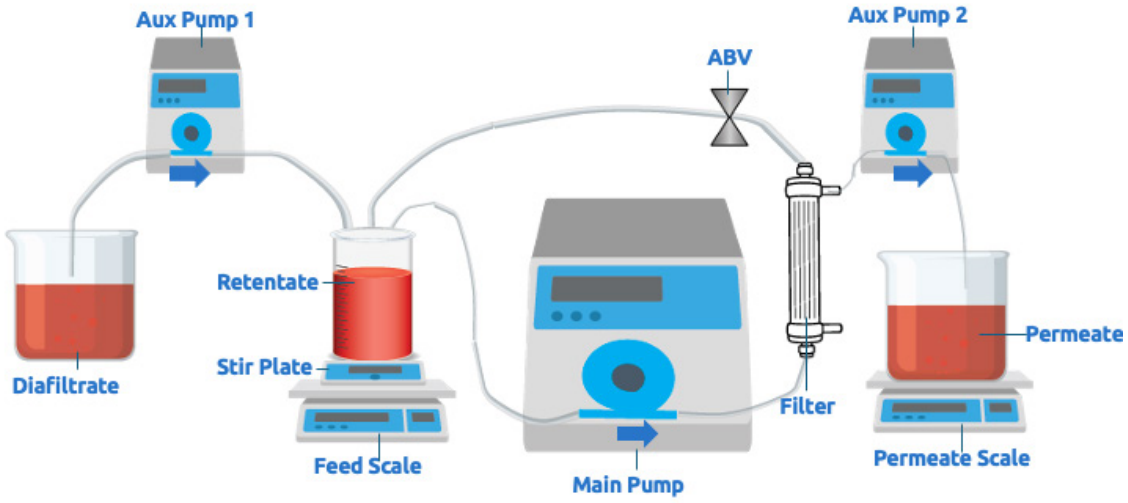


Figure 1 - TFF System set up utilizing 2 auxiliary pumps and a stir plate on the feed scale.

6.2.4 Concentration and Wash

1. Lyse, nuclease treat and clarify AAV harvest material prior to IsoTag™ AAV purification. Recommended starting material turbidity is <45 NTU.
2. Add Phase Transition Buffer 1 to lysate to a final concentration of 0.6M NaCl and mix. Filter solution using a 0.2 µm filter.
3. Ensure IsoTag™ AAV reagent is fully thawed and then placed on ice until ready to use. IsoTag™ AAV reagent should be clear when adding to AAV lysate. If reagent appears cloudy, do not proceed, return to ice until reagent is clear.
4. Aliquot 2% starting volume of AAV material and add IsoTag™ AAV reagent to achieve a 0.67 mg/mL final concentration. 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™ is added, this is due to the phase separation of the reagent.)
5. To the remaining AAV lysate, add IsoTag™ AAV to achieve a 0.045mg/mL final concentration. 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™ is added, this is due to the phase separation of the reagent.)
6. Incubate "seed" and "feed" solutions with IsoTag™ AAV for at least 20 minutes at room temperature 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). Connect the primed line to the retentate vessel.
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 6.2.6 or 6.2.7 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 remains constant, concentrating the bulk harvest 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 6.2.6 or 6.2.7 depending on filter geometry.)
12. Following the concentration step, transfer the diafiltration line to Wash Buffer and perform 6 diavolumes (DVs) to remove additional contaminants.

6.2.5 Elution

1. Stop the permeate pump, ensure the permeate line is clamped and open the back pressure valve.
2. Remove Elution Buffer from 4°C storage 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. (See Recommended TFF Parameters section 6.2.6 or 6.2.7 depending on filter geometry.)
3. After 5 minutes of recirculation, add Phase Transition Buffer 2 to a final concentration of 0.6M $MgCl_2$ 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.

(Note: the retentate should become cloudy after Phase Transition Buffer 2 is added and

solution reaches room temperature.)

4. Prime the diafiltration line with the room temperature solution containing Elution Buffer and Phase Transition Buffer 2 aliquoted in 6.2.3 step 1.
5. Once the solution has reached room temperature, stop the recirculation, and place a new collection vessel(s)on permeate scale to collect the eluted AAV. Tare the permeate scale.
   (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.)
6. Start the run for 10X concentration of the retentate using the same TFF parameters as the concentration/wash step, allowing the retentate pressure to reach the set pressure before beginning the permeate flow rate ramp to desired flux.
   (Note: elution step maybe ramped quicker and run at higher flux than the concentration/ wash.)
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 necessary, add sufficient neutralization buffer to achieve desired final pH

(Note: it may be necessary to buffer exchange elution samples prior to analytics. The conductivity of the elution sample has been seen to interfere with ddPCR tittering.)

6.2.6 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

6.2.7 Recommended TFF Parameters - Flat Sheet

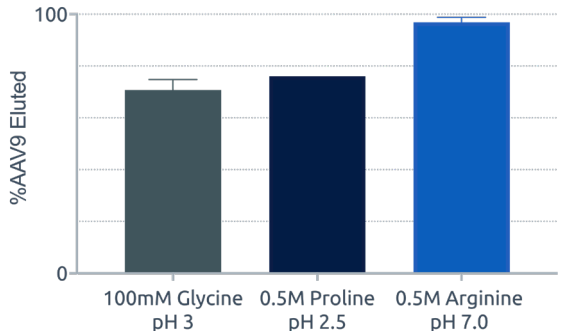
Parameter	Recommended Setting
Filter Loading	≤50 L/m^2
Cross Flow Rate	5-10 LMM
Retentate Pressure	10 PSI (0.7 bar)
Starting Flux	8 LMH
Target Flux	35-45 LMH

6.2.7 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	4.53	0.134	0.12	0.18	0.014
5	0.07	0.1	6.30	22.66	0.668	0.6	0.9	0.07
10	0.14	0.2	12.61	45.33	1.33	1.2	1.8	0.14
50	0.7	1	63.03	226.63	6.68	6	9	0.7
100	1.4	2	126.06	453.25	13.36	12	18	1.4

Note: IsoTag™ AAV volumes calculated assuming 11.3 mg/mL stock solution of reagent. See CoA for lot specific concentration.

7. Process Optimization

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 <45 NTU. Depending on the feedstock used, it may be possible to remove the pre-treatment. Further development may be needed for each feedstock.
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 recommended 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 0.09mg/mL in the bulk material and 0.9mg/mL in the seed volume. Further increasing IsoTag™ AAV concentrations, may improve capture but could be detrimental for 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  *Figure 2 - AAV9 viral genome TFF recovery using recommended elution buffers. Results produced by internal laboratory testing. Results may vary depending on methods and parameters followed.*

8. Troubleshooting

Troubleshooting: **Initial Harvest Treatment and Preparation**

Observation	Possible Cause	Recommended Action
Lysate does not appear cloudy after IsoTag™ AAV reagent addition	• Incorrect temperature • Incorrect salt concentration • Incorrect IsoTag™ AAV reagent concentration • Phase Transition buffer is not prepared to the correct conductivity	• Confirm IsoTag™ AAV transition in saline solution at similar dilution and salt concentration • Confirm conductivity of all solutions

Troubleshooting: **Capture Step**

Observation	Possible Cause	Recommended Action
IsoTag™ AAV permeate is not clear	• Incorrect temperature • Incorrect salt concentration • Incorrect IsoTag™ concentration • Phase transition buffer is not prepared to the correct conductivity • Incorrect filter pore size is used	• Confirm transition in saline solution at similar dilution and salt concentration • Confirm conductivity of all solutions • Confirm correct filter is used
Fouling of TFF filter	• Process not run in permeate control mode • Contaminant profile of harvest material • Running at an improper shear rate • Incorrect flux (LMH) • Incorrect retentate pressure • Incorrect size/volume ratio	• Microfiltration process must be run with a pump on the permeate line to maintain constant flux • Consider additional clarification or nuclease treatment • Reduce filter loading on a volume to meter squared basis • Confirm TFF run settings, consider running at a lower flux or ramping the flux at a lower rate • Calibrate pumps and replace pressure sensors

Observation	Possible Cause	Recommended Action
Incomplete capture, loss of material.	• Incorrect IsoTag™ AAV reagent concentration • Presence of harvest material additives • pH of harvest is incorrect	• Increase IsoTag™ AAV reagent working concentration to 2X amount recommended in protocol • Confirm absence of EDTA in harvest fraction
Pressure spikes during TFF run	• Fouling of TFF filter • Insufficient harvest material clarification	• Consider additional clarification or nuclease treatment

Troubleshooting: **Elution Step**

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

9. Order Information

Item Number	Description
100003069	IsoTag™ AAV, 10mL
100003070	IsoTag™ AAV, 10mL Evaluation Kit

For more information, please contact us at IsolereSupport@donaldson.com

10. Support

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

11. 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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