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Fluorescent Dye Labeling of rAAV

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We use this protocol and it's working

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Abstract

This protocol describes the labeling of recombinant adeno-associated virus (rAAV) with a fluorescent amine-reactive dye (DyLightTM 550 NHS (N-hydroxysuccinimide) ester). The ester dye molecules form stable covalent amide bonds with primary amines on the rAAV capsid proteins VP1, VP2 and VP3, enabling tracking of the fluorescently labeled rAAV in (live) cell imaging applications.

This AAV labeling protocol is based on a protocol used for labeling of antibodies (ThermoFisher Scientific, 2015). It is adjusted and optimized for: labeling of rAAV particles at low protein concentrations, incubation temperatures to keep rAAV particles stable, maximal removal of unbound excess dye and labeling incubation times.

Materials

Reagents	Article number	Supplier	Storage temperature
Recombinant AAV	/	Any supplier or made in-house	-80 °C
Dulbecco's Phosphate Buffered Saline (DPBS (1×))	14190-94	Gibco	4 °C
1.5 ml micro tubes	72.690.001	Sarstedt AG & Co. KG	Room temperature
Amber 1.5 ml micro tubes	10160431	ThermoFisher Scientific	Room temperature
DyLight™ Antibody Labeling Kit ▪ DyLight NHS Ester dye (3 vials) ▪ Sodium Borate buffer (0.67 M) ▪ Purification resin (not used) ▪ Spin columns (not used) ▪ Microcentrifuge collection tubes (not used)	Depending on fluorophore (e.g. DL550; cat.84530) 	ThermoFisher Scientific	DyLight NHS Ester dye (-20 °C) Sodium Borate Buffer 0.67 M (4 °C)
Zeba™ Dye and Biotin Removal Spin Column - 2 ml	A44298	ThermoFisher Scientific	4 °C
15 ml tubes	18827	Greiner Bio-One	Room temperature
Bolt™ Bis-Tris Plus Mini Protein Gel, 4-12%, WedgeWell™ format (SDS-PAGE)	NW04125BOX	ThermoFisher Scientific	4 °C
Precision Plus Protein™ unstained Protein standard	1610363	Bio-Rad	-20 °C
SYPRO™ Ruby protein gel stain	S12000	ThermoFisher Scientific	Room temperature
Pierce™ Bovine Serum Albumin (BSA) standard ampules (2 mg/ml)	23209	ThermoFisher Scientific	Room temperature
Dithiothreitol (DTT)	BP172-25	ThermoFisher Scientific	4 °C
Laemmli sample buffer (4×)	1610747	Bio-Rad	Room temperature
Invitrogen™ NuPAGE™ MOPS SDS running buffer (20×)	NP0001	ThermoFisher Scientific	4 °C
Methanol	20846.361	VWR Chemicals	Room temperature
Acetic acid	A/0360/PB15	Fisher Chemical	Room temperature

Devices	Model	Supplier
Gel imaging system (Fluorescence)	Typhoon FLA 9500	GE Healthcare Bio-Sciences AB
Gel imaging system (SYPRO™ Ruby stained gel)	E-Box CX5	Vilber
Overhead Tube rotator	L28	Labincio B.V.
Tube centrifuge	MEGA STAR 1.6R	VWR
Heating block	QBT4	Grant
Orbital shaker	Type 3011	GFL (Gesellschaft für Labortechnik)

Before start

Various volumes of recombinant adeno-associated virus (rAAV) diluted in DPBS (Dulbecco's Phosphate Buffered Saline) can be fluorescently labeled depending on the amount of labeled virus needed. Our protocol described in detail here, can be used for labeling **400 µl of rAAV diluted in DPBS (8.35E12 viral particles/ml) with 7281-fold molar excess of DyLight™ 550 ester dye (MW= 1040 g/mol)**. Volumes of reagents needed during the different steps of the labeling protocol are listed in **Table 1**.

rAAV before labeling			DyLight™ NHS Ester dye labeling of rAAV		
Volume (µl) of rAAV diluted in DPBS (8.35E12 vp/ml)	Volume (µl) Sodium Borate buffer (0.67 M)	Volume (µl) total reaction mixture	Volume (µl) dye-DPBS solution (0.25 mg/ml)	Volume (µl) Sodium Borate buffer (0.67 M)	Volume (µl) total reaction mixture
1	0.08	1.08	0.42	0.04	1.54
100	8	108	42	4	154
200	16	216	84	8	308
400	32	432	168	16	616
500	40	540	210	20	770

Table 1: Volumes of reagents needed during the different steps of the rAAV DyLight™ labeling protocol.

This labeling protocol was tested and successfully implemented for a range of conditions;

1. Different **recombinant AAV serotypes**; we successfully labeled rAAV2, rAAV9, and rAAV8. We further assume that the protocol is applicable for other rAAV serotypes and (parvo)viruses similar in capsid structure and size such as minute virus of mice, bovine parvovirus and human parvovirus B19.
2. Various **DyLight™ ester dyes**; rAAV2, rAAV9 and rAAV8 were effectively labeled with DyLight™ 550 NHS ester dye, as well as with DyLight™ 488 NHS ester dye. We expect that other fluorescent dyes, linked to an NHS ester functional group (e.g. Alexa Fluor™ NHS ester dyes), are compatible with this protocol.
3. Different **concentrations of rAAV**; rAAV diluted in DPBS with a titer ranging between 8.35E12 - 8.35E13 viral particles (vp)/ml were effectively labeled. Other concentrations might work as well, but might require further optimization.
4. Various **molar excess dye**; The rAAV2 that was used in some of our initial experiments was labeled with 7281-fold molar excess dye. We labeled other serotypes and rAAV2 preparations with the same dye concentration throughout our experiments, but lower molar excess (~500-, ~600-, 728-fold) were also tested and all resulted in successful labeling.

rAAV before labeling

- 1 Thaw an aliquot of rAAV from the -80 °C freezer on ice. Spin thawed vials briefly.

Note: Our in-house produced rAAV stock are typically supplemented with 0.001% Pluronic F-68 (Art. 24040-032, Gibco), a non-ionic surfactant, to limit capsid aggregation and sticking to the plastic micro tubes in which they are stored. We did not test the effect of Pluronic F-68 on labeling since the vector is further diluted in DPBS during the labeling (Step 2), and labeling has been shown to be effective with the current method used.

- 2 Dilute the rAAV stock to 8.35E12 vp/ml in DPBS.

For example, for a rAAV stock of 4.40E13 vp/ml, the rAAV needs to be diluted 5.27 times in DPBS to reach a titer of 8.35E12 vp/ml. Take a 1.5 ml tube and add 85.4 µl of rAAV stock to 364.6 µl of DPBS. The total volume will be 450 µl of rAAV in DPBS, of which 400 µl is used for the labeling reaction. The remaining 50 µl unlabeled, diluted rAAV (8.35E12 vp/ml) will serve as control during quality control steps (Step 16, 23-24).

- 3 Take 2 light protective 1.5 ml tubes:
 - Tube 1: 400 µl rAAV diluted in DPBS (8.35E12 vp/ml)
 - Tube 2: 400 µl DPBS (serves as negative control for the labeling; process this sample the same as the rAAV sample throughout all further steps of the labeling protocol)
- 4 Add 32 µl of Sodium Borate buffer (0.67 M) to Tube 1 and Tube 2. Sodium Borate buffer enhances the binding of NHS esters to amines.

Note: if a precipitate is formed in the Sodium Borate buffer stock solution during storage, dissolve it by warming to 37 °C and vortexing the vial.

DyLight™ NHS Ester dye labeling of rAAV

- 5 Take the vial(s) with lyophilized DyLight™ (DL) dye & briefly centrifuge to ensure that the dye is at the bottom of the vial.
- 6 Dissolve the dye by adding 200 µl DPBS to each vial containing 50 µg DL dye to create a dye-DPBS solution of 50 µg/200 µl (= 0.25 mg/ml dye-DPBS).

Note: The amount of lyophilized dye in the DL containing reagent vials vary depending on the fluorophore. Vials can contain 50 µg or 65 µg lyophilized dye. In this protocol, the DL 550 dye is used (50 µg lyophilized dye). If using other dyes, recalculate the amount of dye-DPBS to add to the labeling reaction mixture.



- 7 Add 168 µl of the 0.25 mg/ml dye-DPBS solution to the samples (rAAV & DPBS negative control, Tube 1 & 2 respectively) containing 432 µl of (rAAV in) DPBS and Sodium Borate Buffer.
- 8 Incubate the reaction mixture(s) at 4 °C on an overhead tube rotator (10 rpm) for 18-19 h. This step is ideally done overnight.
- 9 Remove the tubes from the overhead tube rotator and centrifuge briefly to ensure the reaction mixture solution is at the bottom of the tubes.
- 10 Add an additional 16 µl of Sodium Borate Buffer (0.67 M) to each tube.
- 11 Incubate the reaction mixtures for 30 min at 4 °C on an overhead tube rotator (10 rpm).
- 12 Remove the tubes from the overhead tube rotator. Take ~ 25 µl of each reaction mixture (= PRE-dye removal samples) and store at 4 °C.



Removal of unbound excess dye with Zeba™ Dye and Biotin Removal Spin Column

- 13 Cool the centrifuge to 4 °C.
- 14 **Prepare the Zeba™ dye spin column.**

Note: Step 14 'Prepare the Zeba™ dye spin column' and step 15.1-15.3 'Process the sample' are based on the 'Zeba™ Dye and Biotin Removal Spin Columns' user guide (ThermoFischer Scientific, 2023).

Note: Depending on the total volume of the reaction mixture (rAAV + Sodium Borate Buffer + dye-DPBS solution), you can use smaller or larger Zeba™ dye spin columns. For total reaction mixture volumes between 200 µl - 1 ml, use 2 ml Zeba™ spin columns (Art. A44298, ThermoFisher Scientific). Smaller spin columns (Art. A44296S, ThermoFisher Scientific) might be used for reaction mixtures < 200 µl.

 - 14.1 Remove the bottom plug of the Zeba™ dye spin column by twisting. Loosen the cap, but do not remove it from the column.
 - 14.2 Place the column in a 15 ml tube. Remove the storage buffer by centrifuging the column-tube assembly at 1000x g for 2 minutes at 4 °C. Discard the flow-through.

- 14.3 Check if the column resin is slanted upwards. If so, place a mark on the side where this occurs. For all subsequent centrifugation steps, place the column in the centrifuge with the mark facing away from the rotor center. Improper orientation of the column during centrifugation can result in reduced dye removal.

15 Process the sample

- 15.1 Place the prepared column in a fresh 15 ml tube and remove the cap.
- 15.2 Gently apply the sample to the center of the resin.
- 15.3 Centrifuge the column-tube assembly at 1000x g for 2 minutes at 4 °C. Discard the column. The sample (labeled rAAV and labeled negative control (DPBS)) is in the 15 ml tube, with excess dye retained on the column (**Figure 1**). Check that the resin is not saturated with dye. This can lead to incomplete removal of excess dye.

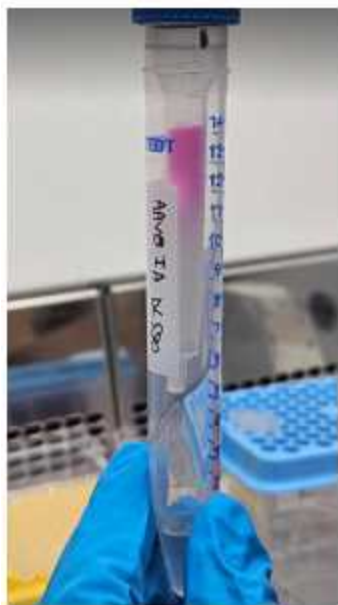


Figure 1: Column-tube assembly after centrifugation of DL 550 labeled rAAV. Excess dye is captured by the resin in the column. The column is not saturated as evidenced by the remaining white resin materials at the bottom of the column. Flow-through contains the DL 550 labeled rAAV.



- 15.4 Store the labeled rAAV and DPBS negative control (= POST-dye removal samples) protected from light at 4 °C and analyze the sample (Step 16-26) within 5 days.

Check for successful unbound excess dye removal & labeling of rAAV viral proteins

- 16 Run an SDS-PAGE gel(s) to separate rAAV viral proteins VP1, VP2 and VP3 according to molecular weight.

Note: A detailed step-by-step protocol for running an SDS-PAGE (Step 16) and performing a SYPROTM Ruby protein staining (Step 18) are not provided here, but key information and information specifically related to running rAAV samples, is included.

16.1 Preparation of reagents:

- MOPS SDS running buffer (1×): 50 ml MOPS SDS running buffer (20×) + 950 ml demineralized water (dH₂O)
- Sample loading buffer (5×): 40 µl Dithiothreitol (DTT) (1 M) + 160 µl 4× Laemmli sample buffer
- BSA standard solution (100 ng/µl): 40 µl stock BSA standard (2 mg/ml) + 760 µl DPBS
- Fixing solution: 100 ml methanol + 14 ml acetic acid + 86 ml dH₂O
- Washing solution: 10 ml methanol + 7 ml acetic acid + 83 ml dH₂O

16.2 Sample preparation:

Note: e.g. for using a final sample volume of 15 µl/well;

3 µl Sample loading buffer (5×) + x µl sample (rAAV/DPBS ctrl/ DPBS+DL ctrl) + (12-x) µl DPBS = 15 µl

- Dilute BSA standard solution (100 ng/µl) further in DPBS (e.g. 100 - 750 ng range).
- Dilute rAAV to be in range of the BSA standards: the amount of rAAV sample volume (µl) and corresponding viral proteins (ng) to be loaded on the SDS-PAGE gel can be calculated from the vp/ml titer (see **Table 2**).
- Spin samples briefly.
- Heat all samples at 95 °C in a heating block for 2 minutes, and spin them briefly.



1) Converting molecular weight of AAV into nanogram

AAV	kDa	mg	ng
1	1	1.66E-18	
1	3800	6.31E-15	6.31E-09

1 kilodalton is $\sim 1.66\text{E-}18$ mg1 (empty) AAV capsid is ~ 3.8 Mda* = 3800 kDa = $6.31\text{E-}15$ mg = $6.31\text{E-}9$ ng* (Ebberink, E.H.T.M. *et al.*, 2022)

2) Calculating the amount of rAAV sample volume to load on the SDS-PAGE gel

Example

	vp/ml	vp/ul	Volume (ul)	Viral particles (vp)	Viral proteins (ng) (= vp x 6.31E-09 ng/vp)
rAAV2XDL550	5.42E+12	5.42E+09	1	5.42E+09	34.2
			5	2.71E+10	171.0
			10	5.42E+10	342.0
rAAV sample volume to load on gel:			7.3	3.96E+10	250

Table 2: Calculation for the amount of rAAV sample volume to load on the SDS-PAGE gel.**16.3 Sample loading on gel:**

Note: BoltTM Bis-Tris Plus Mini Protein, WedgeWellTM 4-12% gels, have wide wells, that allow easy sample loading.

- Mount the gel in the electrophoresis device and add MOPS SDS running buffer (1×).
- Remove the combs from the gel and rinse the wells with MOPS SDS running buffer (1×).
- Load the samples in the wells of the gel. Combine these samples on gel(s);
- Gel 1: PRE- & POST-dye removal samples of DL 550 labeled rAAV and labeled DPBS control (**Figure 2**).
- Gel 2: Bovine serum albumin (BSA) standards, unstained protein standard, unlabeled diluted (8.35E12 vp/ml) rAAV and DL 550 labeled rAAV (POST-dye removal) (**Figure 3**).

16.4 Gel run:

- Protect the electrophoresis device from light (e.g. by wrapping in aluminum foil or putting in a dark room) to avoid photobleaching of the fluorescently labeled rAAV samples.
- Run the gel at 120 volt (V) for the first 10 minutes.
- Afterwards, increase the voltage to 150 V and run for an additional hour to enable clear separation of proteins.
- Remove the gel from the plastic mold and transfer to a container with dH₂O.

17 Image the SDS-PAGE gel(s) with a fluorescence gel imaging system (e.g. TyphoonTM FLA9000, GE healthcare Bio-Science Ab) to visualize the fluorescent signals.

18 Perform a **SYPROTM Ruby protein staining** on the SDS-PAGE gel(s).

- Transfer the gel(s) to a container, cover the gel(s) with fixing solution and agitate on an orbital shaker for 30 minutes at low speed. Protect the container from light.
- Remove fixing solution, add fresh fixing solution and agitate the gel(s) on an orbital shaker for an additional 30 min. Remove fixing solution.

- Add enough SYPROTM Ruby to cover the gel(s). Protect the container from light and agitate on an orbital shaker for 1 hour.
- Rinse the gel(s) two times with dH₂O to prevent possible corrosive damage to the imaging system.

19 Image the gel(s) with an imaging system.

20 **Check for successful unbound excess dye removal**

In contrast to the PRE-dye removal samples, no fluorescent signals of excess DL dye should be visible at the bottom of the gel for POST-dye removal samples (**Figure 2**). This indicates successful excess dye removal. Furthermore, the DL 550 labeled DPBS control should not show protein bands.

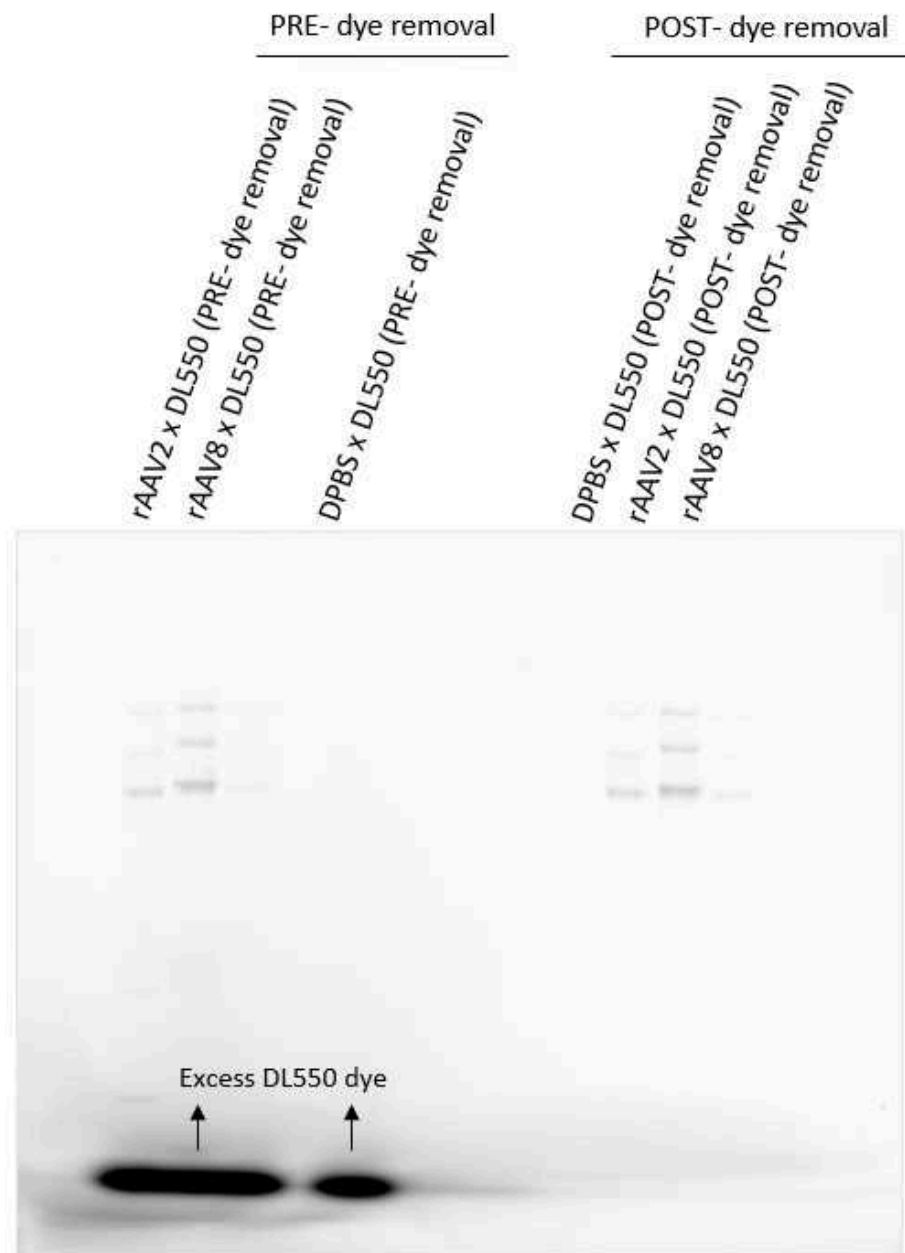


Figure 2: Check for successful unbound excess dye removal. PRE- & POST-dye removal samples of DL 550 labeled rAAV and DPBS are loaded on an SDS-PAGE gel (Gel 1). The gel is imaged with a fluorescent gel imaging system. Fluorescent signals of rAAV unbound excess dye are visible for the PRE-dye removal samples, and not for the POST-dye removal samples.

21 Check for successful labeling of rAAV viral proteins.

Labeling of rAAV is successful if the detected fluorescent signals (**Figure 3A**) match the apparent molecular weight and relative abundance of the DL 550 labeled rAAV capsid

proteins (VP1: 87 kDa, VP2: 73 kDa, VP3: 62 kDa) (**Figure 3B**). Samples of unlabeled (unl) rAAV (diluted 8.35×10^{12} vp/ml) should not show fluorescent signals (**Figure 3A**).

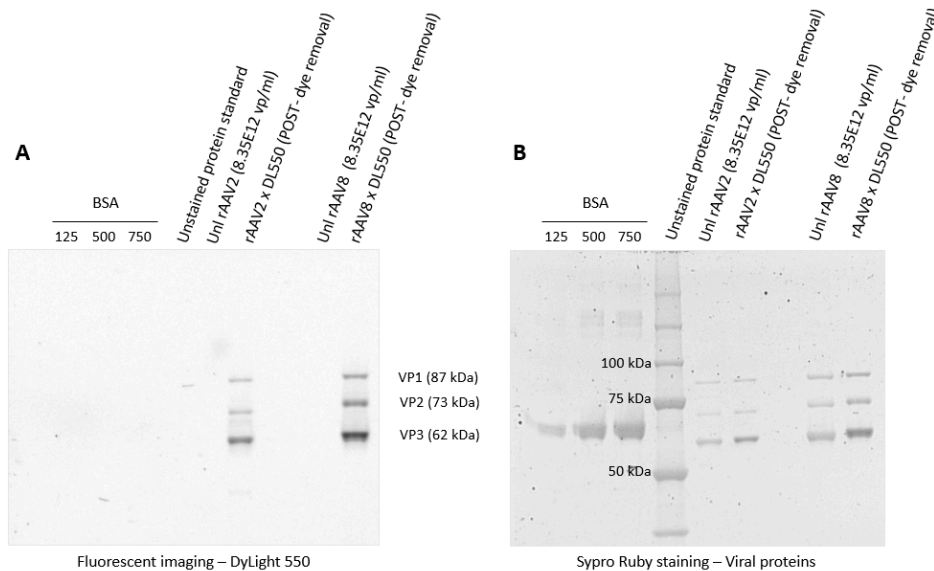


Figure 3: Check for successful DyLight™ labeled rAAV viral proteins.

A) Region from fluorescently imaged SDS-PAGE gel (Gel 2) loaded with BSA (125 ng, 500 ng, 750 ng), unstained protein standard, unlabeled (unl) diluted rAAV2 and rAAV8 (8.35×10^{12} vp/ml), and DL 550 labeled rAAV2 and rAAV8 (POST-dye removal samples). **B)** Same region of SDS-PAGE gel (Gel 2) after SYPRO™ Ruby protein staining and imaging. The DL 550 fluorescent signals match with rAAV capsid protein signals VP1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa).

Determine the titers of the (DyLight™ labeled) rAAV

- 22 During the labeling process, the rAAV titer is diluted by;
1. Adding DPBS & Sodium Borate Buffer (rAAV before labeling)
 2. Adding dye-DPBS solution & Sodium Borate Buffer (DyLight™ NHS Ester dye labeling of rAAV)
 3. Potential remaining storage buffer in the Zeba dye spin column that was not completely centrifuged out of the resin. (Removal of unbound excess dye with Zeba™ Dye and Biotin Removal Spin column)



Some rAAV might stick to the resin in the column. This implies that the titers (vg/ml and vp/ml) of the labeled rAAV will be affected. Therefore it is important to re-determine the titers after labeling. Starting from the rAAV diluted in DPBS (8.35×10^{12} vp/ml) (Step 2), the labeling process might typically dilute the sample 2-5 times.



- 23 Determine the vg/ml titer of the DL labeled rAAV sample (e.g. perform a qPCR or droplet digital Polymerase Chain Reaction (ddPCR) (Van den Berghe, N. *et al.*, 2024)), including proper controls: DPBS, DL labeled DPBS, unlabeled rAAV diluted in DPBS ($8.35E12$ vp/ml).
- 24 Determine the vp/ml titer of the DL labeled rAAV sample (e.g. using ELISA (Progen, 2018), or use densitometry based on the BSA standards). Include proper controls: DPBS, DL labeled DPBS, unlabeled rAAV diluted in DPBS ($8.35E12$ vp/ml).

Store DyLight™ labeled rAAV

- 25 Make single-use aliquots of the DL labeled rAAV. Use light protective amber 1.5 ml tubes.
- 26 Freeze the aliquots at -80°C .

Protocol references

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- Dr. Benjamien Moeyaert (Trellis Research Group, Department of Cellular and Molecular Medicine, KU Leuven, Belgium)