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Version 2

Library tissue handling, viral DNA extraction, and NGS sample preparation V.2

Forked from a private protocol

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Protocol status: Working

We use this protocol and it's working

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Abstract

This protocol describes the procedure to isolate viral DNA from AAV-transfected tissue and prepare it for next-generation sequencing.



Protocol materials

- ✕ TRizol Reagent **Thermo Fisher Scientific Catalog #15596026**
- ✕ RNase Cocktail™ Enzyme Mix **Thermo Fisher Catalog #AM2286**
- ✕ SmaI - 2,000 units **New England Biolabs Catalog #R0141S**
- ✕ CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #B7204S**
- ✕ Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #D4014**
- ✕ Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**
- ✕ NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) - 96 rxns **New England Biolabs Catalog #E7600S**
- ✕ UltraPure™ Low Melting Point Agarose **Thermo Fisher Scientific Catalog #16520050**
- ✕ Sodium Acetate 3M, pH 5.2 **Thermo Scientific Catalog #R1181**
- ✕ GlycoBlue™ Coprecipitant **Thermo Scientific Catalog #AM9516**
- ✕ UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #10977-015**



Marmoset tissue extraction (library selections)

- 1 Raise and house marmosets in compliance with your local Institutional Animal Care and Use Committee (IACUC) and ensure that all protocols and procedures have been approved by the appropriate ethical and regulatory committees.

For adult marmosets, ensure that there is no detectable neutralizing antibodies for AAV9. This can be conducted by The Penn Vector Core at the University of Pennsylvania (<https://gtp.med.upenn.edu/intranethome/core-facilities-internal/immunology-core>)

- 2 Inject marmosets with desired dose of library (e.g. 2×10^{12} vector genomes of library) intravenously (e.g. via the femoral vein).
- 3 At four weeks post-injection, euthanize marmosets and perfuse with 1X phosphate buffered saline.
- 4 Flash freeze tissue (e.g. using 2-methylbutane chilled with dry ice). Separate the brain into coronal blocks and flash freeze the blocks. Store tissue at -80°C until ready for processing.

DNA Extraction

- 5 Add  100 mg tissue sample (brain, liver, or spinal cord) and  1 mL TRIzol reagent  TRIzol Reagent **Thermo Fisher Scientific Catalog #15596026** to bead homogenizer tubes. Use prefilled tubes with 1.5 mm Zirconium beads or 2.8 mm stainless steel beads.

Equipment

Prefilled 2.0ml tubes, Zirconium Beads, 1.5mm Triple-Pure - High Impact, 50pk	NAME
Homogenizer tubes (1.5 mm Zirconium beads)	TYPE
Benchmark Scientific	BRAND
D1032-15	SKU
https://www.benchmarkscientific.com/product/d1032-group/	LINK

Equipment

Prefilled 2.0ml tubes, Stainless Steel, 2.8mm Acid Washed, 50pk	NAME
Homogenizer tubes (2.8 stainless steel)	TYPE
Benchmark Scientific	BRAND
D1033-28	SKU
https://www.benchmarkscientific.com/product/d1032-group/	LINK

6 Homogenize tissue in using the following settings:

- Speed: 5.0 m/s
- Time: 30 seconds
- Pause: 1 minute
- Cycles: 2

Incubate for  00:05:00 .

5m

Equipment

BEADBUG 6, SIX POSITION HOMOGENIZER, 115V^{NAME}

Tissue homogenizer (6 position) ^{TYPE}

Benchmark Scientific ^{BRAND}

D1036 ^{SKU}

<https://www.benchmarkscientific.com/product/d1036/> ^{LINK}

Equipment

BEADBLASTER 24 MICROTUBE HOMOGENIZER, 115V^{NAME}

Tissue Homogenizer (24 position) ^{TYPE}

Benchmark Scientific ^{BRAND}

D2400 ^{SKU}

<https://www.benchmarkscientific.com/product/d2400/> ^{LINK}

Note

Samples can be stored at -20 °C for up to year in TRIzol.

- 7 Centrifuge the homogenizer tubes containing the TRIzol solution and homogenized tissue using the following parameters:  12000 x g, 4°C, 00:05:00 . Transfer the

5m

supernatant to a new tube (microcentrifuge tube or similar).

Equipment

Centrifuge 5425/5425 R - Microcentrifuge

NAME

Refrigerated centrifuge

TYPE

Eppendorf

BRAND

2231000909

SKU

<https://www.eppendorf.com/us-en/eShop-Products/Centrifugation/Microcentrifuges/Centrifuge-5425-5425R-p-PF-934144>

LINK

Equipment

DNA LoBind® Tubes

NAME

Microcentrifuge tubes

TYPE

Eppendorf

BRAND

022431021

SKU

<https://www.eppendorf.com/us-en/eShop-Products/Laboratory-Consumables/Tubes/DNA-LoBind-Tubes-p-PF-56252>

LINK

- 8 Add  600 µL chloroform to each tube for every  1 mL TRIzol used for lysis, vortex briefly, and incubate for  00:03:00 .

3m



- 9 Centrifuge  12000 x g, 4°C, 00:15:00 to separate the nucleic acid phase from the protein phase. Transfer the top aqueous phase to a new tube (approximately  500 µL aqueous phase)

15m

Equipment

Centrifuge 5425/5425 R - Microcentrifuge

NAME

Refrigerated centrifuge

TYPE

Eppendorf

BRAND

2231000909

SKU

<https://www.eppendorf.com/us-en/eShop-Products/Centrifugation/Microcentrifuges/Centrifuge-5425-5425R-p-PF-934144>

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Equipment

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SKU

<https://www.eppendorf.com/us-en/eShop-Products/Laboratory-Consumables/Tubes/DNA-LoBind-Tubes-p-PF-56252>

LINK

- 10 Add 1 equivalent volume of isopropanol, 1/10 volume of sodium acetate, and co-precipitant (e.g.  500 µL isopropanol ,  50 µL sodium acetate ,

10m



🧪 2-3 μ L co-precipitant) and vortex briefly. Incubate for ⌚ 00:10:00 .

⊗ Sodium Acetate 3M, pH 5.2 **Thermo Scientific Catalog #R1181**

⊗ GlycoBlue™ Coprecipitant **Thermo Scientific Catalog #AM9516**

- 11 Centrifuge ⚙ 12000 x g, 4°C, 00:10:00 to pellet nucleic acids. Discard supernatant and wash pellet with 🧪 1 mL 75% ethanol . Centrifuge again

⚙ 7500 x g, 4°C, 00:05:00 and discard supernatant.

15m

- 12 Air dry pellet and resuspend in 🧪 84 μ L PCR clean water

⊗ UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #10977-015**

- 13 Remove RNA by digestion with 🧪 3 μ L RNase cocktail and digest with 🧪 3 μ L SmaI . Supplement reaction with 🧪 10 μ L CutSmart . Incubate at

🌡 Room temperature for 2-3 hours and 🌡 37 °C overnight .

⊗ RNase Cocktail™ Enzyme Mix **Thermo Fisher Catalog #AM2286**

⊗ SmaI - 2,000 units **New England Biolabs Catalog #R0141S**

⊗ CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #B7204S**

- 14 Purify with ⊗ Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #D4014**

Genome Recovery

30m 40s

- 15 Amplify the region around the diversified genome insertion by PCR with 25 cycles of

🌡 98 °C for ⌚ 00:00:10 , 🌡 60 °C for ⌚ 00:00:30 and 🌡 72 °C for

⌚ 00:30:00 using

⊗ Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

and 50% of the total extracted viral DNA as a template using primers XF (ACTCATCGACCAATACTTGACTATCTCTCTAGAAC) and 588-R2lib-R (GTATTCCTTGTTTTGAACCCAACCG).

30m 40s



Index addition

1m 40s

- 16 Dilute PCR product 1:100 and use as template for an additional round of PCR amplification around the variable region with primers containing Read1 and Read2 sequences by 10 cycles of 98 °C for 00:00:10 , 59 °C for 00:00:30 , and 72 °C for 00:00:10 using



Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

and primers 588i-lib-PCR1-6bpUID-F (CACTCATCGACCAATACTTGACTATCTCTCT) and 588i-lib-PCR1-R (GTATTCCTTGTTTTGAACCCAACCG).

- 17 Purify with Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #D4014**

- 18 Append Illumina flow cell adapters and unique indices by PCR amplification with



NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) - 96 rxns **New England Biolabs Catalog #E7600S**

by 10 cycles of 98 °C for 00:00:10 , 59 °C for 00:00:30 , and 72 °C for 00:00:10 using



Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

50s

Clean up and validation

- 19 Run PCR products on a freshly-prepared 2%



UltraPure™ Low Melting Point Agarose **Thermo Fisher Scientific Catalog #16520050**

gel.

- 20 Verify the expected size of the band and extract from the gel.

- 21 If desired, verify the nucleotide diversity at the randomized insertion site by Sanger sequencing.



Note

If additional material is needed for Sanger sequencing, perform an additional PCR amplification using 15-20 cycles of  98 °C for  00:00:10 ,  60 °C for  00:00:30 and  72 °C for  00:00:10 with primers NGS-QC-F (AATGATACGGCGACCACCGAG) and NGS-QC-R (CAAGCAGAAGACGGCATACGA).