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

Forked from a private protocol

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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 status: Working
We use this protocol and it's working

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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 x 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 ºC 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: 5m

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

Incubate for 00:05:00 .

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 ºC 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 supernatant to a new tube (microcentrifuge tube or similar). 5m

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	LINK

Equipment

DNA LoBind® Tubes	NAME
Microcentrifuge tubes	TYPE
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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, 2-3 µL co-precipitant) and vortex briefly. Incubate for 00:10:00. 10m

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 µL PCR clean water

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

13 Remove RNA by digestion with 3 µL RNase cocktail and digest with 3 µL SmaI. Supplement reaction with 10 µL 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 30m 40s 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 (ACTCATCGACCAATACTTGTACTATCTCTCTAGAAC) and 588-R2lib-R (GTATTCCTTGGTTTTGAACCCAACCG).

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 (CACTCATCGACCAATACTTGTACTATCTCTCT) and 588i-lib-PCR1-R (GTATTCCTTGGTTTTGAACCCAACCG).

50s

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).