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DAT-TRAP Protocol

 In 1 collection**Peter Kilfeather**¹¹University of Oxford1 *Works for me* Sharedx.doi.org/10.17504/protocols.io.6qpvr4eo2gmk/v1 Peter Kilfeather

ABSTRACT

This protocol describes the capture of eGFP-L10a-tagged ribosomes and mRNA from DAT-expressing cells in mouse ventral midbrain.

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PROTOCOL CITATION

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COLLECTIONS

 **Protocols from Kilfeather, Khoo et al., 2022**

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Prepare all reagents under RNase-free conditions, preferably with the use of a PCR hood.

- **Tissue-lysis buffer, Low-salt buffer and High-salt buffer made according to the *Reagent Setup* section of Heiman et al., 2014:**
- ***Tissue-lysis buffer:*** Mix 20 mM HEPES KOH (pH 7.4), 150 mM KCl and 10 mM MgCl₂ in RNase-free water. Store it at 4 °C for several months. Add EDTA-free protease inhibitors, 0.5 mM DTT, 100 µg/ml cycloheximide and 10 µl/ml rRNasin and Supersin to an aliquot immediately before use.
- ***Low-salt buffer:*** Mix 20 mM HEPES KOH (pH 7.3), 150 mM KCl, 10 mM MgCl₂ and 1% (vol/vol) NP-40 in RNase-free water. Store the buffer at 4 °C for up to several months. Add EDTA-free protease inhibitors (one mini tablet per 10 ml), 0.5 mM DTT, 100 µg/ml cycloheximide and 10 µl/ml rRNasin and Supersin to an aliquot immediately before use.
- ***High-salt buffer:*** Mix 20 mM HEPES KOH (pH 7.3), 350 mM KCl, 10 mM MgCl₂ and 1% (vol/vol) NP-40 in RNase-free water. Store it at 4 °C for up to several months. To an aliquot, add DTT to a final concentration of 0.5 mM and cycloheximide to a final concentration of 100 µg/ml immediately before use.

Reagent list

A	B	C	D
Item	Specific name	Vendor	Code
anti-eGFP antibody (clone 19C8)	anti-eGFP antibody (clone 19C8)	Memorial Sloan Kettering	Heintz Lab TRAP anti-GFP 19C8
anti-eGFP antibody (clone 19F7)	anti-eGFP antibody (clone 19F7)	Memorial Sloan Kettering	Heintz Lab TRAP anti-GFP 19F7
RNaseZap	RNaseZap RNase Decontamination Wipes-100 sheets	Life Technologies	AM9786

HEPES	HEPES, 1 M, 100 mL, pH 7.3, RNase-free	Fisher	10041703
KCl	KCl, 2M, 100 mL, RNase-free	Life Technologies	AM9640G
MgCl ₂	MgCl ₂ , 1M, 100 mL, RNase-free	Life Technologies	AM9530G
RNase-free water			
EDTA-free Protease Inhibitors	cOmplete, Mini, EDTA-free Protease I	Sigma	11836170001
DTT	DL-DTT	Sigma	D9779-1G
Cycloheximide	Cycloheximide from <i>Streptomyces griseus</i> , 1g	Sigma	D769801G
rRNasin	Recombinant RNasin Ribonuclease Inhibitor, 10,000u	Promega	N2515
Supersasin	SUPERase In RNase Inhibitor (20 U/μL)-10,000 units	Life Technologies	AM2696
HBSS	HBSS (10X), calcium, magnesium, no phenol red-500	Life Technologies	14065056
Glucose	D-(+)-GLUCOSE BIOXTRA	Sigma	G7528-250G
NaHCO ₃	SODIUM BICARBONATE BIOXTRA		S6297-250G
NP-40			
Streptavidin Dynabeads	Dynabeads MyOne Streptavidin T1-2 mL	Life Technologies	65601
Streptavidin Dynabeads	Dynabeads MyOne Streptavidin T1-10 mL	Life Technologies	65602
NaOH			
NaCl			

BSA IgG-free, RNase-free	Bovine Serum Albumin (IgG-Free, Protease-Free)	Strattech Scientific	001-000-162-JIR-50g
Proclin 300	PROCLIN 300, 50ML	Sigma	48912-U
DEPC water		Autoclave	
Graeff forceps	Size 5 Graeff Forceps		
Cutting Blades			
Dounce homogenisers	DOUNCE TISSUE GRINDER, ALL-GLASS, 2 ML	Sigma	D8938-1SET
1.5 mL DNA-LoBind Eppendorf Tubes	Eppendorf® DNA LoBind tubes	Sigma	EP0030108051-250EA
Cervical dislocation tool			
Surgical scissors			
Fine scissors for skull			
Spatula for brain			
PBS			
Wide orifice P200 tips			
DHPC	1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine (DHPC) - Purity: $\geq 99\%$ (by HPLC analysis)	Generon (Non-catalogue)	D607-250MG
B-Mercaptoethanol			
70% Ethanol			
80% Ethanol			
RNeasy Micro Columns	RNeasy Plus Micro Kit (50)	Qiagen	74034
DNA Eliminator Columns			
Buffer RLT+			
Buffer RW1			

Buffer RPE			
Elution water			
Ribogreen Kit	Quant-iT RiboGreen RNA Assay Kit-1 kit	Life Technologies	R11490
Magnetic rack	DynaMag-2	ThermoFisher	12321D
PCR Hood HEPA Filter	HEPA Filter for 32 Laminar Flow PCR Cabinet	Starlab	N3942-7000
PCR Hood HEPA Pre-Filter	Pre-Filters for 32 Laminar Flow PCR Cabinet	Starlab	N3942-7500

SAFETY WARNINGS

Toxicity: Contact your departmental safety office for specific advice about handling and safe disposal of cycloheximide.

Matrix preparation

- 1 Prepare anti-GFP-coated paramagnetic bead matrix according to Box 1 of *Heiman et al., 2014*.

Heiman M, Kulicke R, Fenster RJ, Greengard P, Heintz N (2014). Cell type-specific mRNA purification by translating ribosome affinity purification (TRAP).. *Nature protocols*.
<https://doi.org/10.1038/nprot.2014.085>

The quantity of beads/antibody must be titrated according to the eGFP-content of the sample of interest. See the Supplementary text of *Dougherty et al., 2010* further information.

Dougherty JD, Schmidt EF, Nakajima M, Heintz N (2010). Analytical approaches to RNA profiling data for the identification of genes enriched in specific cells.. *Nucleic acids research*.
<https://doi.org/10.1093/nar/gkq130>

Tissue Collection

- 2 Prepare all dissection instruments and collection tubes on ice. Set a refrigerated centrifuge to 4 °C. Be prepared to work swiftly, to minimise changes in translation occurring after death. Collection materials should be prepared in an RNase-free manner, to minimise the risk of

sample degradation.

- 3 Cull the mouse by cervical dislocation. Extract the brain and perform rapid chilling by submersion in **Dissection Buffer**. Place the brain into a matrix or onto a flat surface for sectioning.
- 4 From each section, dissect the target brain region. Immediately homogenise dissected tissue in **Tissue Lysis Buffer** using a dounce homogeniser. The volume of tissue lysis buffer used should be decided in optimisation experiments and should scale with the total mass of tissue dissected. *For information on choosing an appropriate volume, see Dougherty et al., 2010.*

Dougherty JD, Schmidt EF, Nakajima M, Heintz N (2010). Analytical approaches to RNA profiling data for the identification of genes enriched in specific cells.. Nucleic acids research.
<https://doi.org/10.1093/nar/gkq130>

To ensure consistent and thorough disruption, use a fixed number of strokes for every sample (e.g 20, 30) and select a pestle that provides minimal clearance. Avoid foaming by keeping the pestle below the surface of the buffer at all times.

Note: Tissue can be snap-frozen immediately after dissection, to facilitate collection of large numbers of samples. Alternatively, homogenized contents can be stored on ice while additional samples are collected. **Tissue Lysis Buffer** contains cycloheximide to stall translation.

Immunoprecipitation

- 5 Transfer each lysate into ice-cold Eppendorf tubes and centrifuge at 2,000 x *g* at 4 °C for 10 minutes. Carefully transfer the supernatant to a new tube.
- 6 To the supernatant, add 1/8th volume of **300 mM DHPC** and 1/8th volume of **10 % NP-40**. Mix the solutions by inversion. Hold the mixtures on ice for 5 minutes before centrifugation at 20,000 x *g* at 4 °C for 10 minutes. Carefully transfer the supernatant to a new tube.
- 7 Transfer 50 ul of lysate into a separate tube to be used as a paired 'Input' sample. To ensure the same conditions are kept, hold this sample at 4 °C until the 'IP' sample is processed for RNA extraction the following day.
- 8 Add titrated volume of anti-eGFP-coated paramagnetic bead matrix to the 'IP' sample. Rotate overnight at 4°C.

Washing and extraction

- 9 On day 2, proceed to washing the bead matrix: Place each IP sample on a magnetic rack to pellet beads on the sidewall. Aspirate and discard all supernatant. Resuspend the bead matrix in 1 mL of ice-cold High Salt buffer and dispense into a fresh tube.
- 10 Incubate for 5 minutes on ice, repeat pelleting, resuspension and transfer to a fresh tube. Perform this washing step 6 times in total.
- 11 After the final wash, pellet the bead matrix using a magnet rack, remove the supernatant, warm the tube to room temperature and resuspend in 100 μ L of room temperature Buffer RLT-Plus with 1 % β -mercaptoethanol. Vortex vigorously and incubate for 10 minutes.
- 12 Pellet the bead matrix using a magnetic rack and transfer the supernatant to a Qiagen RNEasy Micro collection column (Qiagen, #74034). Follow manufacturer's instructions for RNA extraction. Use 14 μ L of nuclease-free water for RNA elution and divide the elute into 2 μ L and 10 μ L (allowing for 2 μ L loss) volumes. Store the 10 μ L volume immediately at -80 °C.
- 13 Hold the 2 μ L volume at 4 °C and proceed to RNA yield quantification using the Quant-it™ RiboGreen RNA Assay Kit (ThermoFisher #R11490). Measure RNA integrity using the Agilent 2100 RNA Pico BioAnalyzer.