DAT-TRAP Protocol V.2

In 1 collection

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VERSION 2

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

Created: Jan 10, 2024

ABSTRACT

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

GUIDELINES

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

MATERIALS

- **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 $MgCl_2$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 Superasin to an aliquot immediately before use.*
- ***Low-salt buffer:*** *Mix 20 mM HEPES KOH (pH 7.3), 150 mM KCl, 10 mM $MgCl_2$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 Superasin to an aliquot immediately before use.*
- ***High-salt buffer:*** *Mix 20 mM HEPES KOH (pH 7.3), 350 mM KCl, 10 mM $MgCl_2$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

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	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
	MgCl2	MgCl2, 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 Steptomyces griseus, 1g	Sigma	D769801G
	rRNasin	Recombinant RNasin Ribonuclease Inhibitor, 10,000u	Promega	N2515
	Superasin	SUPERase In RNase Inhibitor (20 U/uL)-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
	NaHCO3	SODIUM BICARBONATE BIOXTRA		S6297-250G

	A	B	C	D
	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)	Stratech 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: ≥ 99% (by HPLC analysis)	Generon (Non-catalogue)	D607-250MG

	A	B	C	D
	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.

ETHICS STATEMENT

This protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC)

Matrix preparation

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

CITATION

Heiman M, Kulicke R, Fenster RJ, Greengard P, Heintz N (2014). Cell type-specific mRNA purification by translating ribosome affinity purification (TRAP).. Nature protocols.
LINK
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.

CITATION

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.
LINK
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.*

CITATION

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.
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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 µL 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 µL of nuclease-free water for RNA elution and divide the elute into 2 µL and 10 µL (allowing for 2 µL loss) volumes. Store the 10 µL volume immediately at -80 °C.

13 Hold the 2 µL 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.