TRAP-SEQ_Sympathetic chain ganglia_Protocol

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ABSTRACT

Protocol describes steps to perform TRAP-SEQ with individually dissected sympathetic chain ganglia.

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MATERIALS TEXT

- Regular ice in ice box
- Nuclease free water
- Microcentrifuge tube rack
- Microcentrifuge tubes
- Sterptavidin MyOne T1 Dynabeads (in the -4 °C fridge) (300×4×1.2=1440 µl)
- Magnet (1)
- 1×PBS (10 ml)
- Biotinylated protein L (120×4=480 µl)

Biotinylated protein L 0.5 mg, (instruction says reconstitute in 100 µl), but to get 1 µg/µl, we need to add 500 µl 1×PBS; 500 µg/500 µl (final volume) = 1 µg/µl

- Low salt buffer (5 ml)
- 1×PBS containing 3% BSA (6 ml)
- Use 14 ml sterile tube as a container to measure 0.18 g (6 ml×3%=0.18 g) BSA; then add 1×PBS till 6 ml; vortex till BSA is completely dissolved; filter the solution for sterilization; place the tube in ice.
- 4 tubes of 50 µg antibody 19C8 and 4 tubes of 50 µg antibody 19F7
- On the day of use, thaw the aliquot to be used that day on ice, spin the tubes at maximum speed (>13,000g) in a microcentrifuge for 10 min at 4 °C and transfer the supernatants (antibody) to new tubes. Add sodium azide as needed. The antibody can be kept at 4 °C for a few days.
- Another round ice box with cover
- 3 microcentrifuge tubes labeled as desired
- Tissue-lysis buffer:

1. mix 20 mM HEPES KOH (pH 7.4), 150 mM KCl and 10 mM MgCl2 in RNase-free water.
2. Store it at 4 °C for several months.
3. 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.

For 1 ml tissue-lysis buffer, 1/10 piece of EDTA-free protease inhibitor tablet, 0.5 µl DTT (1M), 1 µl cycloheximide (100 mg/ml), 10 µl rRNAasin and 10 µl Superasin

Lysates can be prepared from tissue collected from TRAP transgenic mice

- 100 mg/ml cycloheximide
- High salt buffer (6 ml)
- 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.

- *For making 1 ml high-salt buffer, add 1 ul cycloheximide (100 mg/ml); If needed volume is x, then 100 µg/ml= (x×100 mg/ml)/1ml, so x=1 µl;*

- *For making 1 ml high-salt buffer, add 0.5 ul DTT (1M); If needed volume is x, then 0.5 mM×1 ml=1M×x, so x=0.5 µl*

- *For making 6 ml high salt buffer, add 6 ul cycloheximide (100 mg/ml) and 3 ul DTT (1M)*
  - Absolutely RNA Nanoprep Kit
- Ice and ice boxes
- Minicentrifuge
- Water bath (60C)
- Magnet

Day 1 (Preparation of the affinity matrix)

1 Resuspend the Streptavidin MyOne T1 Dynabeads thoroughly in the original bottle by gentle hand mixing.

2 Calculate the amount of Dynabeads required on the basis of the ratios above. Note that the affinity matrix to be used in one particular experiment should be aliquotted to samples from a common source: either prepare all matrix in one larger tube or prepare batches in smaller tubes and combine them into a larger tube for mixing before aliquotting. As recommended by the manufacturer, throughout all manipulations, keep the volume of beads close to the original

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volume from the source bottle.

3 Transfer the beads to be used to a tube(s) and collect them on a magnet (30–60 s).

Troubleshooting: Problem (Unequal amount of beads appear in equivalent aliquots); Possible reason (Beads are not fully resuspended before being aliquoted); Solution (Mix beads well by swirling bottle and by manual pipetting. Ensure that no bead clumps exist in bottle or are seen on pipette tip during aliquotting step)

4 Wash the beads with 1× PBS once (1 ml for all washes if a 1.5-ml tube is used).

5 Collect the beads on the magnet and resuspend them in the appropriate volume of 1× PBS (original bead volume minus the volume of biotinylated protein L to be added).

6 Incubate the beads with biotinylated protein L in 1× PBS (aim for a 1.5-ml total volume if you are using a 2-mL tube) for 35 min at room temperature by using gentle end-over-end mixing in a tube rotator.

7 Collect the protein L–coated beads on the magnet.

8 Wash the coated beads 5 times with 1× PBS containing 3% (weight/volume) BSA (IgG and protease-free).

6×0.03g BSA=0.18 g BSA

9 Proceed to antibody binding in low-salt buffer, by binding 50 µg each of 19C8 and 19F7 (100 µg total, in 1 ml total volume) (needs to ×n if there are n samples) for 1 hr at room temperature or in the cold room over night by gentle end-over-end rotation in a tube rotator. Do not vortex affinity matrix after antibody binding.

10 After antibody binding, wash the beads three times with low-salt buffer (1 ml each time). After washing, resuspend the beads in a volume of low-salt buffer such that each IP will receive an aliquot of the components listed above—beads/protein L/Ab (the affinity matrix in ratios listed above)—in a 200-µl final aliquot volume.

- *add filtered cycloheximide and DTT into low-salf buffer immediately before use*
- *3 mlLlow-salt buffer for washing*
- *200 µl low-salt buffer need for resuspension*
- *For making 1 ml low-salt buffer, add 0.5 µl DTT (1M), 1 µl cycloheximide (100 mg/mL)*
- *For making 4 ml low-salt buffer, add 2 µl DTT (1M), 4 µl cycloheximide (100mg/mL)*

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Once it is prepared, the affinity matrix can be used immediately, or it can be stored for up to 2 weeks at 4 °C with the addition of 0.02% sodium azide. If pre-prepared affinity matrix is stored in sodium azide; it should be washed three times quickly in low-salt buffer before use. Pre-prepared affinity matrix may be difficult to resuspend quickly, and it may be carefully resuspended by gentle agitation overnight on a tube rotator. Do not vortex the affinity matrix after antibody binding.

Day 2 Cell Lysis and Immunopurification (Tissue lysate preparation 4hr)

12 Collect desired ganglia

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Dissect the desired tissue region rapidly, and place the tissue into ice-cold dissection buffer quickly to rinse it.

Dissected tissue can be stored for several years at −80 °C if it is immediately flash-frozen in liquid nitrogen upon dissection. To freeze the tissue, immediately upon dissection wash the tissue in dissection buffer for 2 s; transfer it to a clean, empty microcentrifuge tube; cap the tube and fully immerse the tube immediately into liquid nitrogen; and transfer the frozen tubes to the storage location at −80 °C, without allowing the tubes to warm above −80 °C. When the tissue is ready to use, without allowing the tissue to thaw, use cold forceps to transfer the frozen tissue piece(s) quickly into a cold homogenizer containing prechilled tissue lysis buffer and immediately homogenize it, so that the tissue thaws as it is being homogenized.

14

Transfer the tissue to a prechilled homogenizer on ice that contains tissue-lysis buffer. Homogenize ~25-50 mg of tissue per 1 ml of tissue-lysis buffer. The homogenizer listed in the Equipment section is ideal for 1-ml volumes, but similar models of Teflon-glass homogenizers are available for smaller or larger volumes (scale according to a 25-50 mg: a ml tissue-lysis buffer ratio). *(add 1/5 the tissue-lysis buffer to the ganglia tube each time, and gradually transfer all the ganglia to the Teflon-glass)*

If more than one dissection is being performed, homogenize each sample as it is dissected.

15 Homogenize the samples in a cold room, or at room temperature, by placing the homogenizer in a 50-mlconical tube with ice. Homogenize the samples in a motor-driven Teflon-glass homogenizer, at 900 r.p.m, with 12 full strokes. First insert the Teflon pestle into the glass tube until the solution submerges the entire Teflon pestle, start to stir at 300 r.p.m and then raise the speed slowly to 900 r.p.m. Lower the glass tube but do not let the Teflon pestle rise to the air-solution interface because it will produce significant aeration, which may lead to protein denaturation.

Troubleshooting: Problem (Tissue is difficult to homogenize); possible reason (Too much tissue is used for volume of lysis buffer chosen); Solution (use a Teflon-glass homogenizer and volume of lysis buffer that keeps a ratio of ~25-50 mg tissue:1 ml tissue lysis buffer)

16 Transfer the lysate into a prechilled microcentrifuge (labeled as lysate) tube on ice.

17 Prepare a postnuclear supernatant (S2) by centrifuge at 4 °C for 10 min at 2,000g.

18 Transfer S2 to a new, prechilled microcentrifuge tube on ice.

19 Add 1/9 sample volume of 10% np-40 to S2 (final concentration=1%), and mix it gently inverting the tube. Pulse-centrifuge the sample in a minifuge to collect the liquid at the bottom of the tube. (around 100 µl 10% np-40)

20 Add 1/9 sample volume of 300 mM DHPC (final concentration= 30 mM), mix it gently by inverting the tube and incubate the mixture on ice for 5 min. (around 100 ul 300 Mm DHPC)

Troubleshooting: problem (DHPC is difficult to resuspend); possible reason (insufficient time has passed for complete

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hydration); solution (reconstitute DHPC with water to 300 mM; it will need to sit at room temperature with occasional vortexing for ~30 min to fully go into solution. Once reconstituted in water, use it up to 7 d later, stored at 4 in a glass bottle. Don't store in plastic.)

21 Prepare the postmitochondrial supernatant (S20) by centrifuge at 4 °C for 10 min at 20,000g.

22 Take S20 to a new, prechilled microcentrifuge tube and proceed immediately to IP (Step 2). Lysates can be stored on ice for several hours while additional samples are collected, before proceeding to Step 2. A small aliquot of S20 (e.g., 1% total volume; 1000×1%=10 µl; we will use 100 ul) can be saved at this point to compare it with the sample collected after enrichment of transcripts in purified material by downstream assays. If so, we recommend incubating this S20 aliquot at 4 °C for the same length of time as the TRAP IP (16-18 h) before freezing the aliquot at -80 °C, to ensure that all samples are incubated for equal durations.

Day 2 Cell lysis and Immunopurification cont. (Immunopurification 18-20hr)

23 Thoroughly resuspend the pre-prepared affinity matrix. If the pre-prepared affinity matrix was stored in sodium azide, wash it three times quickly in low-salt buffer before use.

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Add 200 ul freshly resuspended beads to each S20 sample (~1,000 ul).

Always resuspend the affinity matrix thoroughly by gentle pipetting immediately before use.

Troubleshooting: Problem (Affinity matrix is difficult to resuspend); Possible reason (Affinity matrix has been stored at 4 °Cfor an extended period of time.); Solution (On a tube rotator, rotate tube containing matrix gently for several hours until beads are completely resuspended).

25 Incubate the samples at 4 °C for 16-18 h with gentle end-over-end mixing in a tube rotator.

Day 3 RNA extraction, purification, and quantification (RNA extraction)

26 After incubation, collect the beads with a magnet (well-chilled in an ice bucket). Use a minifuge to spin down beads from the caps between washes. The whole fraction, or an aliquot of the unbound fraction, can be saved at -80 °C at this point to compare it with the sample collected after enrichment of transcripts in purified material by downstream assays.

27 ⚠

Resuspend the beads in 1,000 ul of high-salt buffer, and collect them with a magnet as described above.

All washes should be performed by careful pipetting that avoids the introducing of bubbles. After the beads are visibly resuspended, the beads should be mixed by pipetting at least four more times. High background could result from insufficient bead resuspension during washes.

28 Repeat the wash three times (1,000 ul of high-salt buffer each time, a total four washes).

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After the fourth wash with high-salt buffer, remove all remaining wash buffer, remove the tubes from the magnet and warm the tubes to room temperature. Resuspend the beads in 100 ul Nanoprep lysis buffer with beta-mercaptoethanol (β-ME) (use lysis buffer from the Stratagene Absolutely RNA Nanoprep kit or equivalent), vortex the mixture, incubate it for 10 min at room temperature, remove the RNA (now in Nanoprep lysis buffer) from the beads with the magnet and proceed immediately to RNA cleanup, according to the kit manufacturer's instructions.

Buffers from other RNA purification kits may be used, but the buffer that is used to release bound RNA from affinity matrix must contain the denaturant guanidine thiocyanate.

Guanidine thiocyanate can form crystals at low temperature. Be sure to extract and clean up RNA at room temperature to avoid crystallization and carry-through to downstream applications.

30

Eluted RNA (in lysis buffer and removed from beads) can be kept frozen in lysis buffer with β-ME at -80 °C before column cleanup. Warm it to room temperature upon thawing, before resuming purification with the kit.

Day 3 RNA extraction, purification, and quantification (RNA cleanup and quantification 4-6hr)

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Follow the manufacture's instruction for the Agilent Nanoprep kit, or a similar kit, to clean up RNA (including the optional DNase digestion and the optional two RNA elutions with elution buffer heated to 60 °C, all optional steps per kit instructions). RNA purified in this manner is of high enough purity for use in most downstream applications, and our studies have indicated that subsequent acidic phenol purification of these samples does not improve the purity of the samples (as judged by spectrophotometer readings and use in downstream implication reactions, e.g. reverse transcription).

After column purification at room temperature, return the samples to ice and keep them on ice at all times; purified RNA should be kept at -80 °C for long-term storage.

Perform RNase DNase digestion at this step if it is required for downstream applications.

32

After column purification, RNA can be stored at -80 °C for several years.

33 To assay the integrity of the RNA, analyze 1 ul of each sample on a Bioanalyzer 2100 by using an RNA Pico/Nano chip (follow Agilent's protocol for running chips). The qualitative range of the PicoChip assay is 200-5,000 pg/ul, and the qualitative range of the NanoChip assay is 5-500 ng/ul.

Troubleshooting:
1) Problem (RNA is degraded); Possible reason (RNase contamination); Solution (Use only RNase-free reagents, use aerosol-resistant filter pipette tips, change gloves often an keep samples on ice at all times. If working with tissue, homogenize tissue sample immediately collection and wash tissue before homogenization to remove blood.).

2) Problem (RNA yields are low); Possible reason (Inefficient immnoaffinity purification caused by RNase contamination, EDTA present in buffers, inefficient cell/tissue lysis); Possible solutions (Ensure that polysome integrity is maintained by adding cycloheximide, $MgCl_2$ and RNase inhibitors to all solutions, and by keeping samples on ice at all times; Ensure that protease inhibitors used do not contain EDTA; Ensure that a tight-fitting homogenizer similar to the one listed in the equipment section is used. Use a volume of lysis buffer that keeps a ratio of about 25-50 mg tissue:1 ml tissue lysis buffer. Save aliquots of and perform western blots on each step of the purification. Use a non-mouse GFP antibody for western blot analysis: efficiency of purifications can be checked by running western blots (against EGFP) with IP input, unbound and immunopurified samples).

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3) Problem (RNA is not of high enough concentration for use in downstream applications); Possible reason (RNA is diluted during cleanup); Solution (Concentrate RNA samples using a vacuum concentrator, with no or low heating settings).

34 Quantify the samples on a small-volume spectrophotometer (e.g., NanoDrop, Thermo Scientific). For precise quantification of low-concentration samples (<10 ng/ul), run a RiboGreen (or similar) fluorescence-based assay. The use of a spectrophotometer to quantify samples that have been column-purified and that also are of a concentration less than ~10 ng/ul is not recommend, as debris from the columns leads to inaccurate readings (silica shed from the column scatters light).

35

After quantification of RNA and after checking its integrity, RNA can be kept at -80 °C for serval years

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