# Direct Polysome IP from Brain Tissue

Myriam Heiman: bonillm@rockefeller.edu

Protocol below is for 1 IP, scale accordingly

**General Notes:**
-7 mouse striata pooled per IP
-IP with 50 µg 19C8 and 50 µg 19F7 (monoclonal anti-GFP from Sloan-Kettering) and 375µl Dynal Protein G magnetic beads
-IP for 30 minutes in cold room
-10% NP-40 solution purchased from Calbiochem
-DHPC purchased from Avanti Polar Lipids and dissolved in water
-Use Qiagen's Rneasy Micro/Mini Kit for cleanup of RNA
-I purchase RNase-free reagents from Ambion for these experiments

-For the first experiment, I very strongly recommend to run triplicate test runs from mice that are the same age as those to be used in actual experiments. The test will indicate any RNA degradation you may have to address and also the reproducibility of the protocol in your hands. You can save aliquots at each step in the purification to use in Western blot or Bioanalyzer anaysis.

-Purified RNA should be run on an Agilent Bioanalyzer to determine integrity (absence of degradation and RIN number).

-The amount of tissue that you will require will depend upon the strength of the BAC driver, the brain region being analyzed, the age of the mice, as well as the amplification method and assay you will be using. I usually recover ~50ng of total RNA from the pooled striata (both hemispheres) of 7 adult mice, 8-12 weeks old, C57BL/6J background. Stronger BAC drivers from less myelin-rich regions can give much higher yields.

- To maintain the samples always RNAse-free: use aerosol-resistant pipette tips, use RNase-free reagents, decontaminate bench with RnaseZap, and change gloves often (use powder-free gloves). Always keep samples well-chilled on ice.

-The high-salt wash of beads after IP indicated below in the protocol is critical to reduce background protein and RNA binding

- Whenever you are washing beads, pipette up and down several times with a P1000 to make sure that the beads are completely resuspended. I always pipette up and down 5 times after the initial resuspension. Never pipette directly onto the collected beads, rather to the side. Insufficient resuspension of the beads during washes raises background RNA purification. To reduce contamination, I always use a picofuge to quickly spin down any liquid/beads that are on the tube cap before opening the tube.

**GFP Antibodies:**
Order from the Memorial Sloan-Kettering Monoclonal Antibody Facility
Contact Dr. Frances Weis-Garcia (f-weis-garcia@ski.mskcc.org)
Order just “bioreactor supernatant” purity and you will need equal amounts of HtzGFP_04 (clone19F7) and HtzGFP_02 (clone 19C8), see protocol for amounts

19C8 and 19F7, GFP monoclonal antibodies:
Upon receipt, record the two antibodies’ concentrations (concentrations will vary by batch). If the antibodies arrive unfrozen, aliquot each bioreactor supernatant into small aliquots (e.g. amounts you might use within a week), snap-freeze aliquots in liquid nitrogen, and store the aliquots at –80°C. If the antibodies arrive frozen, store immediately at –80°C; they should be thawed on ice and aliquoted before or at first use.

On the day of use, thaw aliquot to be used that day on ice, spin tubes at maximum speed (>13,000 x *g*) in microcentrifuge for 10 minutes, 4 °C, and take supernatants (antibody) to new tubes. Add sodium azide as needed. Can keep antibody at 4 °C for a few days. If interval between IPs is longer than this, aliquot, snap-freeze in liquid nitrogen, and store in single-use aliquots at –80 °C.

## BEAD PREPARATION

Note: Bind antibody to beads before brain lysate preparation, so that beads are ready to use as soon as the S20 (see below) is prepared. Bead/Ab amounts below are ***per IP***.

1) Resuspend Dynal Protein G magnetic beads thoroughly
2) Transfer 375 µl beads into a microcentrifuge tube at room temperature
3) Place tube in Dynal MPC-S magnet for 1 minute and pipet off supernatant
4) Remove tube from magnet and resuspend beads in 1000 µl 0.15M KCl IP wash buffer; repeat washing procedure 2 more times
5) Resuspend beads in 275 µl 0.15M KCl IP wash buffer
6) Add 100 µg total of anti-GFP (50 µg each 19C8 and 19F7) antibody to the tube of beads
7) Incubate with slow end-over-end mixing for 1 hour at room temperature
8) Place tube in magnet for 1 minute and pipet off supernatant
9) Remove tube from magnet and resuspend beads in 1000 µl 0.15M KCl IP wash buffer
10) Repeat washing procedure 2 more times, each time with 1000 µl 0.15M KCl IP wash buffer.
11) Resuspend washed beads in 200 µl 0.15 M KCl IP wash buffer

## BRAIN LYSATE PREPARATION

1) Anesthetize mice with $CO_2$ or nembutal and decapitate
2) Perform hand dissection of striata and place in dissection buffer on ice
3) When finished with each set of mice, transfer the striata into a glass homogenizer on ice that contains 1 ml Lysis Buffer

Homogenize in cold room with the motor-driven Teflon-Glass homogenizer at 900 rpm, 12 strokes. Make sure that the Teflon pestle hits the bottom of the tube on each stroke. First insert the Teflon pestle into the glass tube; when the solution submerges the entire Teflon part of the pestle, start to stir at 300 rpm and then raise speed slowly to 900 rpm. Lower glass tube but do not let Teflon pestle rise to air-solution interface, because it will produce a lot of bubbles.

4) Transfer the lysate into a microcentrifuge tube on ice
5) Prepare a post-nuclear supernatant (S2) by centrifugation at 4°C, 10 minutes, 2,000 x *g*
6) Transfer S2 to a new microcentrifuge tube on ice
7) Add 1/9 sample volume 10% NP-40 to S2 (final concentration = 1%); mix gently by inversion of tube
8) Add 1/9 sample volume 300 mM DHPC (final concentration = 30 mM); mix gently by inversion and incubate on ice for 5 minutes
9) Prepare post-mitochondrial supernatant (S20) by centrifugation at 4°C, 10 minutes, 20,000 x *g*
10) Take S20 to a new microcentrifuge tube on ice and proceed with IP

**IMMUNOPRECIPITATION (*n.b.* bead/Ab amounts below are per IP)**

1) Add 200 µl freshly-prepared antibody-bound beads to IP (~800-1000 µl S20)
2) Incubate at 4 °C for 30 minutes with end-over-end mixing
3) Collect beads with magnet in ice bucket (keep beads cold!) for 1 minute, save supernatant (unbound fraction) if desired; use picofuge to spin down beads from caps in between each wash.
4) Resuspend beads in 1000 µl 0.35 M KCl IP wash buffer and collect with magnet as above
5) Repeat wash 3 times (1000 µl wash buffer each time, total 4 washes)

**RNA EXTRACTION AND PURIFICATION**

After the last wash, remove all wash buffer from beads on magnet at room temperature. Resuspend room-temperature (temperature is critical!) beads in the RLT buffer (with B-ME) from Qiagen's RNeasy kit (choose micro or mini kit depending on expected yields). Incubate in RLT buffer for 5 minutes at room temperature, and then remove RLT carefully from beads (it now has the RNA that has been eluted) and follow Qiagen's protocol, including in-column DNase digestion.

**RNA QUANTITATION**

To assay the integrity of the RNA, run 1 µl of each sample on the Bioanalyzer 2100 using an RNA Pico/Nano chip (follow Agilent's protocol for running chips).

PicoChip assay qualitative range: 200-5000 pg/µl
NanoChip assay qualitative range: 5-500 ng/µl

If you want *exact* quantification, the Bioanlayzer is not extremely accurate and I recommend running a RiboGreen (or similar) fluorescence-based assay. Use of a Nanodrop for samples that have been column-purified and are of a concentration of less than ~10ng/µl is not recommended, as debris from the columns leads to inaccurate readings (silica shed from the column scatters light).

**BUFFER RECIPES**

Stock cycloheximide (CHX), powder from Sigma, 1000X: 100mg/ml in methanol, always freshly prepared. Caution: cycloheximide is very toxic and all waste should be collected for proper disposal by lab safety. Do not use cycloheximide if it has a very yellow color.

Rnasin is sensitive to denaturation, add last, just before use of buffer and do not vortex tube once it has been added (only swirl gently)

| **Lysis Buffer** | **For 10 ml** |
|---|---|
| 20mM HEPES KOH [pH 7.4] | 200 µl 1M |
| 5mM $MgCl_2$ | 50 µl 1M |
| 150mM KCl | 750 µl 2M |
| Water | 9 mls |
| | |
| Immediately before use add | |
| 0.5mM DTT | 5 µl 1M |
| Protease Inhibitors (Roche Mini Complete, EDTA-Free) | 1 tablet |
| 100 µg/ml CHX | 10 µl 1000x |
| 40 U/ml Rnasin (Promega, Recombinant) | 10 µl of 40 U/µl |

| **Dissection buffer** | **For 50 mls** |
|---|---|
| 1 x HBSS | 5 ml 10x HBSS (Invitrogen # 14065056) |
| 2.5 mM HEPES-KOH [pH 7.4] | 125 µl 1M |
| 35 mM Glucose | 1.75 ml 1M |
| 4 mM $NaHCO_3$ | 200 µl 1M |
| water | 42.93 mls |
| | |
| Immediately before use add | |
| 100 µg/ml CHX | 50 µl 1000x |

| **0.15 M KCl IP Wash Buffer** | **For 30 mls** |
|---|---|
| 20 mM HEPES-KOH [pH 7.4] | 600 µl 1M |
| 5 mM $MgCl_2$ | 150 µl 1M |
| 150 mM KCl | 2.25 ml 2M |
| 1% NP-40 | 3 mls 10% |
| Water | 24 mls |
| | |
| Immediately before use add | |
| 0.5 mM DTT | 15 µl 1M |
| 100 µg/ml CHX | 30 µl 1000x |

| **0.35 M KCl IP Wash Buffer** | **For 30 mls** |
|---|---|
| 20 mM HEPES-KOH [pH 7.4] | 600 µl 1M |
| 5 mM $MgCl_2$ | 150 µl 1M |
| 350 mM KCl | 5.25 ml 2M |
| 1% NP-40 | 3 mls 10% |
| Water | 21 mls |
| | |
| Immediately before use add | |
| 0.5 mM DTT | 15 µl 1M |
| 100 µg/ml CHX | 30µl 1000x |