Mats Ljungman, Ph.D.
Department of Radiation Oncology
University of Michigan
ljungman@umich.edu

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Document authors: Michelle Paulsen (tenbroeke@umich.edu), Brian Magnuson (bmagnuso@umich.edu), Ishwarya Venkata Narayanan (ivenkat@umich.edu)

Bru-seq Experiment Protocol

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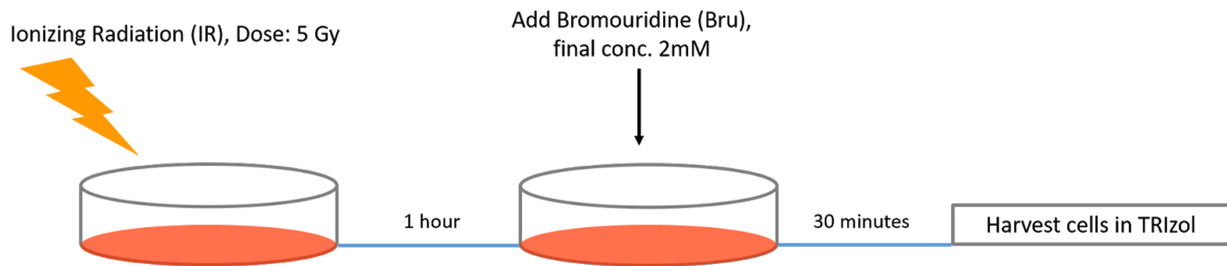
This protocol is optimized for 150mm dish (1 per sample) with the intent to collect a minimum of 5 million cells (more is better). Bru-RNA makes up ~1% of total RNA, requiring a large amount of starting material for a Bru-RNA library. For BruUV-seq and BruChase-seq, the % Bru-RNA is expected to be even less.

The main differences in protocols between Bru-seq and the derivative techniques BruUV-seq and BruChase-seq are found in the labeling protocol. Bru-seq with IR treatment protocol is identical to Bru-seq (no treatment) beginning with the bromouridine labeling section.

Visual Protocol



IR Treatment

Equipment

IC-320 Biological Irradiator (Kimtron Inc., Oxford, CT)

Steps

1. Following standard safety and operating practices for the irradiator, dose cells with 5 Gy x-rays (66 sec at dose rate of 4.5223 Gy/min).
2. Incubate at 37˚C for 1 hour.
3. Immediately label cells with bromouridine (next section).

Bromouridine Labeling of Cells

Reagents

50mM Bromouridine (Aldrich; cat # 850187) in PBS

Steps

1. Add Bru.
    a. Adherent cells: Remove 10ml of media from each plate of cells to a sterile tube and add BrU to a final concentration of 2mM. Discard remaining media. Add back BrU-containing media to the plate.
    b. Suspension cells: Measure media volume and add Bru to a final concentration of 2mM.
2. Incubate at 37˚C for 30 minutes.
3. Lyse cells immediately (next section).

Cell Lysis and RNA Extraction

Reagents/Materials

Round bottom polypropylene test tube (e.g. Falcon 352059)
TRIzol (Invitrogen; cat # 15596-026)
Chloroform
Isopropanol
75% Ethanol in DEPC-H20
DEPC water
Turbo DNA free kit (ThermoFisher; cat #AM1907)

1. Lyse cells in TRIzol.
    a. Adherent cells: Aspirate media and immediately add 3ml TRIzol to the plate to lyse cells. Scrape cells and transfer lysate to a round bottom tube.
    b. Suspension cells: Transfer cells and media to a round bottom tube, spin down cells, aspirate media, and resuspend pellet in 3ml TRIzol.
2. Store at -80°C.
3. Thaw to room temperature.
4. Follow TRIzol protocol for RNA extraction.
5. Resuspend pellet in 100µl DEPC-water and incubate at 55°C for 10min to fully dissolve RNA.
6. Follow Turbo DNA free kit protocol.
7. Store total RNA at -80°C.

Bru-RNA Isolation

Reagents/Materials

Goat anti-mouse Dynabeads (Invitrogen cat# 110.33)
Magnetic stand for Eppendorf tubes
Anti-BrdU (BDPharmingen 555627)
0.1% BSA in DEPC-PBS
RNaseOUT Ribonuclease inhibitor (Invitrogen; Cat #10777019) : Diluted to 0.5x using 0.1% BSA in DEPC-PBS
RNA spike-in cocktail (see spike-in reference documentation)

1. Use 50µl aliquot of magnetic beads per sample. Capture beads with magnet and aspirate storage buffer.

2. Wash 3x with 200µl 0.1% BSA in PBS, capturing beads between each wash.
3. Resuspend in 200µl 0.1% BSA in PBS
4. Add 2.5µg (5µl) anti-BrdU monoclonal antibody to beads, then add 1.0µl diluted RNaseOUT.
5. Conjugate by incubating at room temperature with gentle rotation for 1 hour.
6. Wash beads 3x with 200µl 0.1% BSA in PBS, capturing beads between each wash.
7. Resuspend beads in 200µl 0.1% BSA in PBS plus 1.0µl diluted RNaseOUT.
8. Thaw aliquot of RNA spike-in cocktail (on ice) and select appropriate volume to add per 100µg of sample total RNA:
    a. For Bru-seq, use 25µl per 100µg of sample total RNA.
    b. For BruUV-seq, use 20% of the Bru-seq amount (5µl).
    c. For BruChase-seq (6h chase), use 25% of the Bru-seq amount (6.25µl).
    d. For BruChase-seq (2h chase), use the same amount as for Bru-seq (25ul).
9. Combine total RNA, spike-ins, and water to a total volume of ~200µl.
10. Incubate isolated RNA to 80˚C for 10 minutes.
11. Add RNA solution to conjugated beads, incubate at room temp with gentle rotation for 1 hour.
12. Wash beads with 400µl 0.1% BSA in PBS, rotating for 1 minute.
13. Briefly centrifuge, capture beads, and discard supernatant.
14. Add 200µl 0.1% BSA in PBS, flick to mix. Capture beads, discard supernatant.
15. Add 200µl DEPC-PBS, flick to mix. Capture beads, discard supernatant.
16. Add 35µl DEPC-H2O to beads. Use less for a more concentrated sample. Immediately transfer to a new (siliconized or LoBind) tube.
17. Incubate at 96˚C for 10 min in a thermomixer set to 550rpm. Briefly centrifuge, then capture beads and keep the supernatant, which contains the Bru-RNA.
18. Measure RNA concentration and store at -80˚C.

Stranded Library Preparation

Reagents/Materials

200ng Bru-RNA in 16µl (can use more)
5x First-Strand buffer
Actinomycin D
DTT
Random Primers (3ug/µl)
Fast Select (Qiagen; cat# 333180)
Superscript II (Invitrogen; cat # 18064-014)
Random primer (Invitrogen; cat # 48190-011)
NEBuffer 2 (NEB; cat # B7002S)
RNase H (Invitrogen; cat # 18021-071)
dUTP (Roche; cat # 11934554001)

DNA polymerase 1 (Invitrogen; cat # 18010-025)
T4 Ligase Buffer (NEB; cat # B0202S)
T4 DNA polymerase (NEB; cat # M0203S)
T4 PNK (NEB; cat # M0201S)
Klenow 3' - 5' exo (NEB; cat # M0212S)
Quick Ligation Kit (NEB; cat # M2200S)
NuSieve 3:1 agarose (Lonza; cat # 50090)
Accugene 10X TAE (Lonza; cat # 50844)
Qiaex II Kit (Qiagen; cat # 20021)
Phusion Master Mix (ThermoFisher; cat # F548S)
USER (NEB; cat # M5505L)
RNA Clean beads (Fisher; cat # NC0068576)
AmPure beads (Fisher; cat # NC9933872)
dNTP set (Invitrogen; cat # 10297-018)
RNaseOUT (Invitrogen; cat # 10777-019)
100bp DNA ladder (NEB; cat # N3231S)

Notes

Steps denoted with *** are stopping points where samples can be stored at -20˚C for up to seven days.

1. Remove rRNA withFastSelect.
    a. Prepare pre-mix: 8µl First-Strand buffer, 1µl Random Primers, 0.10µl Fast Select (per sample).
    b. Add 9.10 µl of pre-mix, then 16µl RNA, to each PCR tube. Mix well.
    c. Run FastSelect protocol in thermal cycler. Cool to 4˚C.
2. Synthesize First Strand cDNA.
    a. Mix (per sample): 6.9µl ddH2O, 4.0µl 100mM DTT, 0.8µl 25mM dNTP, 0.8µl ActinomycinD (2.5µg/µl), 0.5µl RNaseOUT, 2.0µl Superscript II
    b. Run thermal cycler program: 1) 25˚C for 10 minutes, 2) 42˚C for 50 minutes, 3) 70˚C for 15 minutes, 4) hold at 4˚C.
    c. Clean up with AMPure RNAclean beads.
        i. Add 72µl AMPure beads in a microcentrifuge tube, then add cDNA mixture.
        ii. Bind at 25˚C for at least 10min (with shaking) and capture beads.
        iii. Remove and discard supernatant from each sample using micropipettor.
        iv. Without disturbing the beads, add ~200µl freshly prepared 80% ethanol.
        v. Incubate at room temperature for 30sec, then discard supernatant.
        vi. Repeat ethanol wash.
        vii. Spin briefly and remove any remaining ethanol.
        viii. Dry beads at 37˚C, until just cracked.

   d. Resuspend the dried pellet with 32μl of 5mM Tris pH 8.0. Mix well. Incubate at 28˚C for 6min (with shaking).
   e. Capture beads and transfer 30μl of the supernatant to a new PCR tube.
3. Synthesize Second Strand cDNA.
   a. Mix (per sample): 26.48μl ddH2O, 15μl NEBuffer 2, 0.9μl 25mM dG+dA+dU+dC mix, 0.75μl RNase H, 1.875μl DNA polymerase
   b. Add 45μl mixture to each 30μl First Strand product (from Step 2) and mix well.
   c. Incubate using thermal cycler at 16˚C for 2 hours.
   d. Clean up with 112.5μl AMPure beads. Proceed as per step 2.c. above.
   e. Resuspend the dried pellet with 27μl 5mM TRis pH 8.0. Mix well. Incubate at 28˚C for 6min (with shaking).
   f. Capture beads and transfer 25μl of the supernatant (now ds cDNA) to a new microcentrifuge tube.***
4. Perform End Repair.
   a. Mix (per sample): 14.7μl ddH2O, 5μl T4 DNA Ligase Buffer, 0.8μl 25mM dNTPs, 2μl T4 DNA Polymerase, 0.5μl Klenow, 2μl T4 PNK
   b. Add 25μl mixture to each 25μl Second Strand product (from Step 3) and mix well.
   c. Incubate samples at 20˚C for 30 minutes.
   d. Clean up with 75μl AMPure beads. Proceed as per step 2.c. above.
   e. Resuspend the dried pellet in 32 μl 5mM Tris pH 8.0. Incubate samples at 28˚C for 6min (with shaking).
   f. Capture beads and transfer 30μl of the supernatant to a new microcentrifuge tube.***
5. Adenylate 3' Ends.
   a. Mix (per sample): 11.5μl ddH2O, 5μl NEBuffer 2, 1μl 10mM dATP, 2.5μl Klenow Exo(-)polymerase
   b. Add 20μl mixture to 30μl eluted cDNA (product of Step 4) and mix well.
   c. Incubate samples in a thermomixer at 37˚C for 30min.
   d. Clean up with 75μl AMPure beads. Proceed as per step 2.c. above.
   e. Resuspend the dried pellet in 20 μl 5mM Tris pH 8.0. Incubate samples at 28˚C for 6min (with shaking).
   f. Capture beads and transfer 18μl of the supernatant to a new microcentrifuge tube.***
6. Ligate Adapters.
   a. Anneal ligation adapters: thaw adapter aliquot, incubate at 55˚C for 1min in a thermomixer, and cool to 22˚C.
   b. Mix (per sample): 20μl 2x Quick Ligase Buffer, 1μl Quick ligase
   c. Add 1.5μl annealed ligation adapter to 18μl eluted cDNA (product of step 5), then add 20μl ligase mix. Mix well.
   d. Incubate at room temp for 30 minutes.
   e. Clean up with 40μl AMPure beads. Proceed as per step 2.c. above.
   f. Resuspend the dried pellet in 32 μl 5mM Tris pH 8.0. Incubate samples at 28˚C for 6min (with shaking).

   g. Capture beads and transfer 30µl of the supernatant to a new microcentrifuge tube.
   h. Add 5µl gel-loading buffer.***
7. Perform size selection by Agarose gel (3% gel using NuSieve 3:1 agarose).
   a. Remove buffer from wells before loading.
   b. Run gel in TAE (do not cover gel with buffer) at 75V for 80 min.
   c. Excise gel slices at 500bp. Reserve a larger slice as a backup.
   d. Purify gel slices using the Qiagen QIAEXII kit. Elute in 42µl resuspension buffer to recover 40µl. Reserve 20µl as a backup.
8. Perform uridine digestion and enrich DNA fragments.
   a. Add 1µl USER to each 20µl sample. Incubate in PCR machine 37˚C for 15min, then cool to 4˚C.
   b. Prepare MasterMix/TIPP: Add 1µl TIPP per 250µl MasterMix.
   c. Put samples on ice.
   d. Add 1.5µl adapter primer mix.
   e. Add 25µl Phusion Master Mix/TIPP and mix well.
   f. Incubate samples (hot start) in a thermal cycler using the program:
      i. 98˚C for 30sec
      ii. 11-14 cycles of:
         1. 98˚C for 10sec
         2. 60˚C for 30sec
         3. 72˚C for 30sec
      iii. 72˚C for 5min
      iv. Hold at 10˚C
   g. Clean up with 38µl AMPure beads. Proceed as per step 2.c. above.
   h. Resuspend the dried pellet in 36µl 5mM Tris pH 8.0. Incubate samples at 28˚C for 6min (with shaking).
   i. Capture beads and transfer 36µl of the supernatant to a new low-bind microcentrifuge tube.
9. Measure DNA concentration (Nanodrop or similar).
10. Run 3µl of each library on a confirmation gel (1.5% agarose).