NASCENT RNA LABELING AND PURIFICATION

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1. For 4sU tagging/pulsing, cells growing in 10cm or 15cm dishes were pulsed with a growth medium containing 4-thio-uridine (4sU), for as little as 5min, 15min, 1 hr, to 4 hrs, in the 37deg CO2 incubator; avoid from strong light.
    a. Determine the concentration of 4sU: as 4sU higher than **200uM** causes noticeable growth toxicity, when prolonged pulsing is needed, a lower 4sU concentration can be used.
        i. for HeLa and human ESCs, we pulse with **250uM for 1hr** for shorter or **50uM for 4 hrs** for longer pulsing.
        ii. Higher concentration of 4sU pulsing tends to recover RNA species with lower uridine ratio, but different cell line's tolerance varies – we usually titrate 4SU from 50 uM to 1M to measure the % of nascent / total RNA
            1. The goal is to **minimize** the pulsing time and 4sU dosage to avoid the nonspecific effect.
            2. The expected recovery of nascent RNA is ~ 1% of total RNA, e.g. input of 100ug of total RNA for the biotinylating step (step 5) will yield about 1ug of nascent RNA
2. For chase experiments, 4SU containing medium was removed, wash cells once with PBS, add 2.5 mM Uridine containing media.
3. To harvest, Trizol can be directly applied on cells, or alternatively, perform trypsinization-> centrifugation -> resuspend pellets in Trizol
4. Extract RNA with standard Trizol procedure-> resuspend RNA pellet with ddH2O with nanodrop at 260nm – final concentration around 1ug/ul.
5. To biotinylate 4SU-tagged RNAs, treat 100ug of total RNA with Biotin HPDP [Pierce 21341] at 0.5 mg/mL in 40% DMF and 10 mM Tris pH 7.4, 1 mM EDTA, for 1.5 hours at room temperature in a thermomixer at 1000 rpm.
    a. RNA was extracted with an equal vol of chloroform twice -> combined both aqueous phase into a 2ml Phase Lock Gel tube, followed by isopropanol/ethanol precipitation.
    b. Resuspend RNA pellets in ddH2O; check RNA quality by 1% agarose gel electrophoresis.
    c. As a biotinylated standard across samples, in vitro-transcribed (IVT) luciferase RNA transcribed in the presence of 4SU was spiked into the biotinylation mixture for a final concentration of 0.01 ng/uL.
6. To separate nascent from steady-state RNAs, biotinylated RNA was immobilized onto the column (as the bound fraction) from total steady-state RNA (as the flowthrough fraction), using mMacs Streptavidin Kit (Miltenyi 130-074-101).
    a. mix equal vol of streptavidin bead slurry and RNA solution, mixing on a nutator for 15min at RT.
    b. The flowthrough fraction was recovered using isopropanol/ethanol precipitation.
    c. Biotinylated RNA was released from beads using two sequential elutions with 100 mM freshly-made DTT and cleaned using RNeasy MinElute Kit (QIAGEN 74204) -> eluted in 25ul ddH2O
7. Quality Control: The flowthrough fractions contain the steady-state total RNAs; the column-eluates contain nascent RNAs.
    a. Nascent RNAs should account for ~ 1% of the total RNA
    b. Nascent RNA abundance across experimental samples can be compared by normalizing target vs. IVT-luciferase spike-in, using qRT-PCR assays.
        i. Immediate early genes (e.g. c-myc, c-fos, c-jun) produce transcripts tends to be short-lived, and enriched for up to 10-fold in nascent fraction vs. long lived mRNA (GAPDH)
    c. Nascent and steady-state RNAs can also be QCed by agarose gel electrophoresis or by Agilent Bioanalyzer, where the rRNA should be prominent in the steady-state, but not the nascent fraction.
    d. RNA may need to be further fragmented into 60-200 nt using RNA fragmentation reagents (Ambion AM8740) before proceeding to make cDNA libraries for next-generation sequencing.

REFERENCE:

Chen et al. TGS1 controls snRNA 3' end processing, prevents neurodegeneration and ameliorates SMN-dependent neurological phenotypes in vivo | bioRxiv 2020.10.27.356782

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