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HIGH-QUALITY RNA EXTRACTION FROM SMALL NUMBER (40,000-250,000) OF SORTED T CELLS

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- Sort cells by flow cytometry, pellet at 350 x g for 20 mins, aspirate supernatant, and resuspend in 1ml freshly mixed RLT plus (Qiagen #1053393) + 10 ul undiluted BME
- Mix well by vortexing at maximum speed for 0.5 – 1 min
- Quick spin to get sample back to bottom of the tube
    - If necessary, can store here at -80˚C. However, it is optimal to isolate RNA fresh. If freeze-down, do quick thaw at 37˚C and mix well again by vortexing at maximum speed for 0.5 – 1 min
- RNase-zap work area (Argos #D6002), reagents, and gloves
- Bring the RNeasy miniElute column (Qiagen #74204) to room temperature
- Add to QIAshredder (Qiagen #79654) to homogenize and spin at maximum speed 2.5 mins at room temperature
    - If centrifuge has a "soft" mode, turn OFF as many of the subsequent spins are very short and this helps get rid of contaminants
- Transfer to new tube. Avoid cellular debris pelleted during spin above.
    - Use low-binding tubes (Eppendorf #022431021) and tips to minimize RNA loss during isolation
- Add 70% EtOH 1:1 (Fisher 200 proof molecular biology grade #BP2818-500) and shake vigorously ~15 seconds to mix
    - Make 70% EtOH fresh
    - Minimize vortex steps to avoid shearing DNA (and long non-coding RNA if interested) as this makes contaminating DNA harder to detect
- Quick spin to get sample back to bottom of the tube
- Transfer samples to Qiagen RNeasy MiniElute column
    - Preferred because of ability to DNase treat on the column, small elution volume, and subsequent higher RNA concentration
- Spin at 10,000 x g 1 min at room temperature
- Discard flow through and repeat two steps above for remainder of samples to run through the same column
    - Aspirate flow through (vs. decant) to reduce salt contamination on outside of RNeasy column that can contaminate sample later upon elution
- DNase treat on column (Qiagen RNase free DNase set recommended by RNeasy column packet #79254):

- Wash with 350 ul RWI from the Qiagen RNeasy MiniElute kit at 10,000 x g for 15 seconds at room temperature
- Add 10 ul DNase + 70 ul RDD mixed by gentle inversion if any. DNase very sensitive to physical denaturation – do not vortex or pipet up and down to mix
- Incubate at room temperature 15 minutes
- Wash by adding 350 ul RWI (Qiagen #1014567) at 10,000 x g for 15 seconds
    - If necessary, repeat DNase treatment or increase incubation time to 20 mins
- Put column into new wash tube to prevent getting residual waste/salt on the outside of the column that can contaminate at a later time. Add 500 ul RPE to the column, and invert for ~2 mins before to eliminate salt from the inside of the column. Spin at 10,000 xg 1 min at room temperature.
- Repeat above for a total of two RPE washes
    - If a lot of salt contamination, repeat wash step multiple times, increase volume of RPE to 700 ul, and increase speed of centrifuge
- Wash column with 500 ul of 80% EtOH, spin 10,000 x g 1 minute at room temperature
- Transfer column to a new tube
- To dry tube, spin with tube lid open at 16,000 x g 5 mins at room temperature
    - Any hint of EtOH will inhibit RNA elution
- Transfer column to new tube
- Add 14 ul of water to the column, close lid, incubate at room temperature for 1 min, and spin at 16,000 x g for 1 min
- Repeat elution step above with the 14uL flow-through
    - This can yield up to twice as much RNA

RNA isolation from 40,000 - 250,000 sorted T cells should yield 500 pg/ul – 11 ng/ul x 14 ul for gene expression profiling

Useful to optimize technique through inspection of RNA samples via NanoDrop for UV identification of contaminants and quantity estimate if have a high enough RNA concentration (Aranda *et al.,* 2009), 2100 Bioanalyzer for quality of RNA and quantity estimate of low RNA concentrations, Qubit for specific quantitation of low amounts or RNA (5 ng in variable volume), and RT-PCR for identification of DNA