RT-PCR WILDseq prep Protocol

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RNA Extraction

Notes before starting
Use *Quick*-RNA Microprep Kit (cat# R1050/R1051)

- Use Pico-Pure for small cell pellets
- Check that all kit components have been properly constituted
- Discard flow through after every spin
- Spin columns for 1 min at max rpm (13.2rpm)
- Prepare DNAse mix
  - 5 μL DNAse I + 35μL DNA Digestion Buffer per sample
  - Make master mix at 1.1n

Protocol:

1. Add RNA Lysis buffer
   - For cell amount 10^5 use 100μL
   - For cell amount 10^6 use 300μL
2. Add 1 volume of 100% Ethanol and mix well
   - Pipette up and down
   - Vortex for <30sec
   - Repeat until pellet is fully dissolved
3. Transfer mix into spin column
4. Wash column with 400μL RNA wash buffer
5. Add 40μL DNAse I to spin column and incubate at RT for 30C for 15min
6. Add 400 μL RNA Prep Buffer and spin
7. Add 700 μL RNA Wash Buffer and spin
8. Add 400 μL RNA Wash buffer and spin
9. Spin column to dry and transfer to nucelase-free tube
10. 15 μL DNAse/RNAse-Free water to column matrix and centrifuge

Reverse Transcription

Notes Before Starting:

- Label PCR strip tubes
- Thaw RT reagents
- Uses WILDseq specific RT primer
- Dilute RT primer to 2μM
  - Do this in a PCR hood
  - **IMPORTANT** Do not mix primers and carefully note which RT primer is added to which sample
- Make RT and ExoI master mixes (MM) before starting
  - MM amounts are 1.1n

Protocol:

Note: Use "rt" protocol in callum folder on thermocycler for heating steps

1. In PCR strip tubes dilute 1-5ug RNA in a total volume of 10 μL of RNAse/DNAse-free water
2. Add 1 ul of RT primer
3. Add 1 ul of dNTPs
4. Denature at 65 C for 5 mins in PCR machine
5. Add straight to ice for 2 mins
6. Spin to get liquid to bottom of tube
7. Prepare master mix of RT enzyme and buffers (Make prior to starting)

Component	Volume	MM volume
5x SSIV Buffer	4 μL	
SSIV RT	1 μL	
100 mM DTT	1 μL	
RNAse Out	1 μL	

8. Add 7 μL of RT MM prepared above to each sample and mix
   - Spin briefly to get liquid to bottom of the tube
9. In PCR Machine: incubate at 53C for 10 mins followed by 80C for 10 mins
10. Add 3 μL of ExoI MM to each sample

Component	Volume	MM volume
Thermolabile Exonuclease I	1 μL	
NEBuffer r3.1*	2 μL	

* Most PCR buffers are compatible

11. In PCR Machine: heat at 37C for 4 mins followed by 80C for 1 min
    - Spin briefly to get liquid to bottom of the tube
12. Add 1 μL of RNAse H and incubate at 37C for 20 mins
13. Dilute final cDNA 1 in 2 with DNAse/RNAse-free water

PCR Amplification of cDNA

1. Prepare mastermix of below reagents at 1.1x the number of samples

Component	Volume
5x Q5 Reaction Buffer	10 μL
GC Enhancer	10 μL
dNTPs (10mM)	1 μL
P5_R1_fwd (100μM)	2.5 μL
P7_rev	2.5 μL
DNAse/RNAse H20	13.5 μL
Q5 polymerase	0.5 μL
Total Master Mix volume	**40 μL**
cDNA from above	10 μL

2. Perform PCR using the following parameters:

Steps	Time
Step 1: 98C	30s
Step 2: 98C	**10s**
Step 3: 61C	**30s**
Step 4: 72C	**30s**
Step 5: 72C	2 min
Step 6: 12C	Hold

20-25 cycles of steps 2-4

3. Run out 5 μL of each PCR reaction on 1.5% agarose gel

- Amplicon should be ~200bp

Running PCR Gel protocol

1.