

CRaft-ID Detailed Supplemental Protocol:

Cell Infection:

- Seed 4.2×10^6 cells on 4 10cm plates the day before infection
- Add X μ L of virus (to obtain an MOI of 0.15) to the cells in media supplemented with $8 \mu\text{g/mL}$ polybrene
- 24 hours later, remove viral media and replace with media supplemented with $2 \mu\text{g/mL}$ puromycin and select for 3 days

MicroRaft preparation:

***NOTE – do not allow microRaft to dry in between washes or media changes**

- Wash microRaft array three times with 2ml 1x PBS, incubating for 5 minutes each wash
- Coat array with 2ml 0.001% w/v poly-D-lysine (PDL) hydrobromide (Millipore Sigma #P6407) resuspended in sterile H_2O for 1hr at 37°C
- Aspirate and remove PDL coating
- Wash twice with 3ml 1x PBS, 10 minutes each
- Add 2ml DMEM+10%FBS and store in 37°C until cells are ready for plating

Cell Dissociation (volumes used are for T75 flask):

- Remove and discard culture medium
- Rinse cells with 15ml 1x PBS buffer
- Add 10ml TrypLE to flask and incubate for 5 minutes at 37°C . Gently tap flask to detach cells
- Add 20ml complete growth media (DMEM+10%FBS) and gently pipette across flask to dissociate cells
- Transfer cell suspension to 50ml conical tube
- Centrifuge at $200 \times g$ for 5mins to pellet
- Aspirate supernatant and resuspend in 2ml media
- Pipette 20x with P1000 micropipette to break up colonies
- Add 18ml media and strain cell suspension through 40um cell strainer (Fisher #087711)

Cell Plating:

- Count cells and resuspend to a concentration of 1.2×10^4 cells/ml
- Remove and discard final DMEM+10%FBS wash from microRaft array and immediately add 1ml cell suspension across array (1.2×10^4 cells/array)
 - o Do not allow rafts to dry in between aspirating wash media and seeding cells
- Secure microRaft in swinging-bucket centrifuge and spin for 4 minutes at $400 \times g$
- Carefully move and place microRaft in 37°C , 5% CO_2 incubator
- After 24hrs, add 2ml fresh media

Stressing cells with sodium arsenite:

- 72hrs after seeding, remove culture medium from microRaft array and add 2ml 500uM sodium (meta)arsenite in DMEM+5%FBS. Incubate for 1hr at 37°C .

Fixing and Staining on microRaft:

- Add 1ml of 12% PFA to microRaft, for a final fixation concentration of 4% PFA. Incubate for 15mins at room temperature
- Remove and discard media
- Wash fixed cells three times with 2ml Wash Buffer (0.01% Triton-X in 1x PBS)

- Simultaneously block and permeabilize cells:
 - o 0.1% Triton-X with 5% goat serum in 1x PBS for 45mins at room temperature
- 1X wash with 1ml Wash Buffer
- Incubate overnight in 1ml primary antibody solution at 4°C:
 - o Wash buffer + 5% goat serum and rabbit anti-G3BP1 (1:1000, MBL #RN08PW)
- 3X washes with 1ml Wash buffer, 5 minutes each
- Incubate in 1ml secondary antibody solution at room temperature:
 - o Wash buffer + 5% goat serum and Alexa Fluor 555 goat anti-rabbit (1:1000, Invitrogen #A21429)
- 3X washes with 1ml Wash buffer, 5 minutes each
- Incubate in 1ml Hoescht33342 (1ug/ml in PBS, Thermo Scientific #62249) for 30 minutes at room temperature
- 2X washes with 1ml Wash buffer, 5 minutes each
- Store in 2ml 1x PBS with 1% PenStrep

gRNA Library Preparation Protocol:

*NOTE – It is important to perform DNA extraction and set up first PCR reaction in a pre-AMP PCR clean space

DNA Isolation:

- Collect raft into 6ul Quick Extract Buffer (Lucigen #QE09050) on ice and proceed to DNA extraction prior to storage at -20°C
- Extract DNA with the following protocol:
 - o Vortex 15 seconds
 - o Heat at 65°C for 6 minutes
 - o Vortex 15 seconds
 - o Heat at 98°C for 2 minutes
 - o Chill on Ice

First PCR (25ul rxn) – Add directly to tube with collected microRaft:

- Reaction components:
 - o 12.5 ul 2X Q5 High-Fidelity DNA polymerase (NEB #M0492L)
 - o 2.5 ul 10uM PCR1-rev primer
 - o 2.5 ul 10uM PCR1-fwd indexed primer
 - o 2.5 ul H2O
 - o 5 ul DNA extract (already in tube)
- Protocol:
 - o 98°C 30 sec
 - o 98°C 15 sec
 - o 68°C 1 min
 - o 72°C 1 min
 - o GOTO step 2 – 21X (22 cycles total)
 - o 72°C 2 min
 - o Hold 4°C

Pool First PCR and cleanup:

- Pool 20ul from each sample from 4 unique PCR1 indices (save 5ul at -20°C)
- Cleanup pool with MinElute PCR Purification Kit (Qiagen)

*all spin steps performed at 13,000rpm, 30 sec

- Add 400ul (5X) buffer PB to PCR1 pool
- Spin through MinElute column
- Transfer flow-through back onto the column for double-binding and spin again
- Add 750ul buffer PE to wash, spin, discard flow-through
- Spin column an additional 1 min to remove residual ethanol
- Elute with 12.5ul Buffer EB. Let column stand for 1min prior to spin
- Transfer flow-through back to column and spin again for double elution

Second PCR (25ul rxn):

- Reaction components:
 - 12.5 ul 2X Q5 High-Fidelity DNA polymerase (NEB #M0492L)
 - 1.25 ul 20uM PCR2 Fwd Primer
 - 1.25 ul 20uM PCR2 Rev Primer
 - 5ul template cleaned from PCR1 (only use half, save other half at -20°C)
 - 5ul H2O
- Protocol:
 - 98°C 30 sec
 - 98°C 15 sec
 - 72°C 1 min
 - GOTO step2 9X (10 cycles total)
 - 72°C 2 min
 - Hold at 4°C

Gel Extraction of Final Library:

- Prepare 3% Low-melt agarose gel (NuSieve GTG #50080) in 1% TBE
- Add 8ul Orange DNA loading dye (Thermo R0631) to 25ul rxn
- Load on gel, run 95V for 50 mins
- PCR will yield 3 products, the longest of which contains the correct gRNA insert (249bp)
- Gel extract top band (249bp) from gel (Qiagen Gel Extraction Kit)
 - Add 600ul Buffer QG to melt gel. Melt at room temperature on benchtop (do not heat)
 - After gel is melted, add 200ul isopropanol and mix well
 - Load on column and spin max speed, 30 sec
 - Wash column with 500ul buffer QG, spin max speed 30 sec
 - Wash with 750ul buffer PE, spin max speed 30 sec
 - Discard flow-through and spin max speed, 2 min to dry column
 - Elute in 12.5ul buffer EB. Let column stand for 1min prior to spin
 - Transfer flow-through back to column and spin again for double elution
 - Quantify purified library on D1000 tapestation
- **NOTE – alternatively, users may quantify all libraries on the Tapestation prior to gel extraction. After quantification of the desired produce, libraries can be pooled in equimolar ratios and loaded in a single well of an agarose gel to remove unwanted adapter dimer products. This is necessary for users who are preparing many sequencing libraries in parallel.

Recovery of rafts that failed sequencing:

*Some of the rafts pooled for sequencing did not yield a gRNA insert in the sequencing data. To recover those samples, the remainder of material saved before pooling from PCR1 was used with additional cleanup steps to make a new library.

Redo PCR1 with 10 additional cycles:

- Reaction components:
 - o 12.5 ul 2X Q5 High-Fidelity DNA polymerase (NEB #M0492L)
 - o 2.5 ul 10uM PCR1-rev primer
 - o 2.5 ul 10uM PCR1-fwd indexed primer (same index used from first PCR1 reaction)
 - o 2.5 ul H2O
 - o 5 ul PCR1 product saved before pooling
- Protocol:
 - o 98°C 30 sec
 - o 98°C 15 sec
 - o 68°C 1 min
 - o 72°C 1 min
 - o GOTO step 2 – 9X (10 cycles total)
 - o 72°C 2 min
 - o Hold 4°C

Cleanup PCR1 reaction with TBE gel isolation:

- Add 8ul Orange DNA loading dye (Thermo R0631) to PCR reaction and load on 8% TBE gel (Thermo #EC6215). Run at 200V in 1X TBE buffer.
- There will be two products, a small one without the insert (~130bp) and a larger one with the insert (~160bp). Using a razor, isolate the top band from the gel.
- Gel Extraction Procedure
 - o Using a plastic pestle in an Eppendorf tube, crush the gel piece in 300ul PAGE buffer (300nM NaCl, 10mM TRIS PH8, 1mM EDTA).
 - o Rotate overnight at 4°C
 - o Mix in thermomixer 37°C, 1hr, 1200rpm
 - o Transfer all contents of the tube to a SpinX column fitted with a 0.45 uM filter. Spin with lid open 13,000xg for 10 min, room temperature. Discard the gel pieces trapped in the column.
 - o Add 1ul GlycoBlue (Thermo AM9515) and 50ul 3M NaOAc to the flow-through.
 - o Add 1mL 100% EtOH, vortex, store at -80°C for 2hrs (or overnight)
 - o Spin 13,000 x g, 4°C, 15 min
 - o Decant the supernatant and wash the pellet 2X with 1mL 70% EtOH
 - o After the last spin, remove residual liquid with fine pipette tip, dry at room temp 5 mins
 - o Resuspend pellet in 5ul H2O.
- Pool the purified DNA from uniquely indexed samples at equal ratios.
- Perform PCR2 and final library gel extraction with the same PCR2 method outlined above, using 1ul of the PCR1 pool as the template and 9ul H2O in the reaction.