CRISPR-Cas9 screening protocol (two-vector system sgRNA library

Infection with library virus for screening

Always maintain a library coverage of ≥ 150-fold throughout the entire experiment!

1. Transduce 2.1 x 10^8 HAP1 Cas9:
    a. Prepare 14x 6-well plates with each well containing 2.5 x 10^6 cells in total 5 ml complete IMDM
    b. Add x µl virus supernatant to each well
        i. x = amount of virus that gives MOI = 0.3-0.5
2. Spinfect:
    a. Centrifuge plate 1,800 RPM for 1 hour at 37 ℃
3. Transfer cells to incubator and grow for 48 hours at 37 ℃
4. Wash cells with PBS
5. Add 500 µl trypsin and incubate for 5-10 minutes at 37 ℃
6. Add 1.5 ml complete IMDM and resuspend the cells
7. Pool all wells and count cells
8. Add 1.5 x 10^7 infected cells to a T175 flask in a total volume of 50 ml complete medium containing 3.5 µg/ml puromycin (15x T175 flasks in total)
9. Grow cells for 72 hours at 37 ℃
10. (Optional grow cells for another 2-3 days, split first)
11. Wash each T175 flask with PBS
12. Add 2 ml trypsin and incubate 5-10 minutes at 37 ℃
13. Add 8 ml complete IMDM and resuspend
14. Pool all flasks and count cells
15. Take three 2 x 10^7 cells samples in 15 ml tubes for sequencing (pre-treatment sample)
    a. See DNA extraction protocol for sample handling
16. Transfer 9 x 10^7 cells to a 50 ml tube (once for each condition) and centrifuge (5 min 1,200 RPM)
17. Resuspend cells from each tube in medium containing the appropriate treatment
18. Transfer 15 x10^7 cells to a T175 flask (6x) and grow for 48 hours at 37 ℃
19. Repeat step 11-18 until about 14 population doublings have been achieved

DNA extraction (QIAGEN Gentra Puregene)

1. Centrifuge the 2 x 10^7 cell samples in 15 ml tubes at 1,200 RPM for 5 minutes
2. Remove all but ~200 µl medium
3. Vortex cell to resuspend completely
4. Add 3 ml Cell Lysis Solution and vortex for 10 seconds
   a. This solution can be stored for at least 2 years at RT
5. Add 15 µl RNase A Solution and incubate ≤ 1 hour at 37 ℃
6. Add 1 ml Protein Precipitation Solution and vortex for 20 seconds
7. Place sample on ice for > 5 minutes
8. Centrifuge 2000 x *g* for 10 minutes in a swing bucket rotor
9. Add 3 ml isopropanol to a new 15 ml tube
10. Carefully add supernatant from step 8 to this tube
11. Invert tube 50 times
12. Centrifuge 2000 x *g* for 3 minutes in a swing bucket rotor
    a. A white pellet should be visible
13. Remove supernatant and dry tube inverted on a piece of paper
14. Add 3 ml 70 % ethanol and invert tube several times to wash the pellet
15. Centrifuge 2000 x *g* for 1 minute in a swing bucket rotor
16. Remove supernatant and dry tube inverted on a piece of paper briefly
17. Air dry pellet for 5-10 minutes
18. Add 400 µl DNA Hydration Solution and vortex for 5 seconds
19. Incubate at 65 ℃ for one hour
20. Incubate O/N at RT while gently shaking
    a. Incubating O/N at 4 ℃ after this step will improve DNA measurements
21. Measure DNA concentration with Nanodrop

Amplification lentiviral inserts for sequencing sgRNA sequences

A nested PCR approach is used to amplify viral inserts

1. Prepare reaction mix for PCR1 (48 reactions/sample):
    i. 960 µl GC enhancer 5x
    ii. 960 µl Q5 buffer 5x
    iii. 48 µl Q5 polymerase
    iv. 24 µl Forward primer (5'- aggcttggatttctataacttcgtatagcatacattatac)
    v. 24 µl Reverse primer (5'- ACAtgcatggcggtaatacggttatc)
    vi. 96 µl dNTPs 10 mM
    vii. 374 µl gDNA (if [DNA] ≤ 513 ng/µl)
    viii. 2314 µl MPW

   Run 48 reactions of 100 µl (each reaction contains 4 µg gDNA)
2. PCR program:
    i. 98 ℃ 0:30
    ii. 98 ℃ 0:10
    iii. 63.2 ℃ 0:20
    iv. 72 ℃ 0:20, repeat ii – iv 17 times
    v. 72 ℃ 2:00
3. Pool all PCR1 reactions and prepare PCR2:
    i. 20 µl GC enhancer 5x
    ii. 20 µl Q5 buffer 5x
    iii. 1 µl Q5 polymerase
    iv. 0.5 µl Forward primer (contains Illumina P5 sequence)
    v. 0.5 µl Reverse primer (contains Illumina P7 sequence and index)
    vi. 2 µl dNTPs 10 mM
    vii. 3 µl pooled PCR1
    viii. 53 µl MPW
4. PCR program:
    vi. 98 ℃ 0:30
    vii. 98 ℃ 0:10
    viii. 68.2 ℃ 0:20
    ix. 72 ℃ 0:20, repeat ii – iv 11-19 times
    x. 72 ℃ 2:00
5. Multiple PCRs can be run with increasing numbers of cycles.
    i. Continue with reaction with least amount of cycles that gives a measurable product on the Bioanalyser (next steps)

Clean-up of sgRNA amplicons

1. Transfer 75 µl PCR2 to 1.5 ml tube
2. Add 60 µl AMPure XP beads and mix by pipetting up and down 5 times
3. Place on magnet for at least 2 minutes
4. Aspirate and discard supernatant
5. Add 200 µl 70 % ethanol
6. Remove ethanol after 30 seconds
7. Repeat wash
8. Remove tube from the magnet and dry for 2 minutes
9. Add 35 µl dH_2O
10. Incubate for at least 30 seconds
11. Put the tube back on the magnet for 2 minutes
12. Remove 30 µl purified PCR2 to a new 1.5 ml tube
13. Analyse purified PCR2 on Bioanalyser (Agilent DNA 1000 kit) according to standard protocol

Quantification amplicons

1. Quantify purified PCR2 with DNA1000 kit on the Agilent Bioanalyser according to standard protocol
2. PCR2 product should be 276 bp and ≥ 5 nmol/l

Preparation of amplicons for NGS

1. Set concentration of each sample to 5 nmol/l
2. Mix all indexed samples together and send 15 µl of this mix for sequencing on HiSeq400 at CRUK
3. Include sequencing primer (Bassik seq, agactataaGtatcccttggagaaCCAcctTGTTG) in 1.5 ml tube
    a. 50 µM
    b. 30 µl per lane

Bioinformatic analysis of NGS data