FREQ-Seq condensed protocol

Locus-specific primer design

Forward primer: 5'-GTAAAACGACGGCCAGT + ~20 bp primer sequence homologous to region adjacent to mutation

Reverse primer: 5'- CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT + ~20 bp primer sequence homologous to region ~250 bp downstream of the mutation

Stage 1: Locus amplification

1. Prepare crude genomic DNA extraction from sample population (boil prep or other lysis preparation)
2. Desired loci are amplified in a 50 µL reaction using the locus-specific FREQ-Seq primers with conditions:
    a. 5-10 µL of crude DNA template from Step 1.
    b. 0.5 µM of each locus specific primer, 200 µM dNTP, and appropriate buffering conditions
    c. PCR thermal cycling: 10-15x cycles using conditions optimized for the locus specific FREQ-Seq primer set.
3. Primers from this first reaction are removed by:
    a. Treatment with 5U of exonuclease I (*Exo*I) and 1U of *Dpn*I for 30 minutes at 37 °C and 20 minutes at 80 °C to heat inactivate these enzymes.
    b. Purification using Ampure XP™ magnetic beads, eluting in 27 µL of ddH_2O

Stage 2: Bar-coding and library purification

1. For amplified loci, samples can be bar-coded as follows:
    a. For samples treated as in Step 1.3a, 25 µL of the *Exo*I/*Dpn*I treated reaction can be mixed with 25 µL of fresh PCR reaction mixture at 1X concentration and enrichment primers at 2X concentrations (e.g. 0.1 µM Primer A and Primer B).
    b. For samples treated as Step 1.3b, 25 µL of the purified reaction can be mixed with 25 µL of fresh PCR master mix at 2X concentration and enrichment primers at 2X concentrations (e.g. 0.1 µM Primer A and Primer B).
2. To each reaction, add $1/10^{th}$ the molar equivalent of a single FREQ-Seq bridging primer to a single reaction (ex. for ~50 µL reaction add 10-20 ng of dsDNA FREQ-Seq primer).
3. PCR amplify the mixture under the following conditions:
    a. Cycling conditions:
    b. 98 °C – 60 s
    c. 98 °C – 20 s
    d. 53 °C – 20 s
    e. 72 °C – 30 s
    f. Cycle c-e 10-15X depending on the amplicon
    g. 4 °C – <3 hrs
4. The resulting reactions can be treated with QIAGEN Buffer PB (5M Gu-HCl (aq), 30% Isopropyl alcohol) to stop polymerase activity.
5. Pool samples according to the loci of interest, followed by purification using QIAGEN columns or generic silica gel equivalents. This is a complete FREQ-Seq library.
6. (Optional) To ensure FREQ-Seq product size and purity, products can be run on a 2% agarose/1X TAE gel followed by standard gel extraction.
7. Determine the concentration of each allele pool.
8. Dilute and mix all allele pools together to give a final concentration of 10-20 nM depending on sequencing facility requirements.