TCR Ampli**Cot** Protocol 5 September 2006

**Materials**

RNeasy Mini Kit (Qiagen)
Qiashredder columns (Qiagen)
MMLV (NEB)
Exonuclease I (NEB)
Rnasin plus (Promega)
SYBR Green Real time PCR mastermix without ROX (MCLab)
$ddH_2O$
200 mM MOPS pH 7.5
500mM EDTA
5M NaCl
Brij-700 (5% solution in water)
SYBR Green dye (Invitrogen)
$dT_{16}$ primer
V, J and C oligonucleotides (see spreadsheet)

Equipment

Real time PCR machine capable of multiple reads during annealing step

We recommend the MJ Research Opticon [Bio-Rad] but other machines such as the ABI7700 may be used as well.

Consumables for Opticon:
Optical caps
Strip tubes (white)
96 well plate (white)

Optional:

a. Nanodrop spectrophotometer or Agilent Bioanalyzer
b. Regular PCR machine for RT and non-real time amplification

**Method**

1. Start with lymphocyte samples ($10^5$ to $10^7$ cells), obtained via
    a. Dissociated spleen or thymus
    b. Ficoll prepped PBMCs from blood or a buffy coat
    c. FACS or Miltenyi sort
2. Pellet cells in eppendorf tubes by spinning 10 minutes at 3000 rpm in refrigerated microfuge. Aspirate supernatant
3. Disrupt pellet in buffer RLT (Qiagen). Use 350μl if less than $5x10^6$ cells in sample, 600μL if $5x10^6$ cells or more in sample. Place RLT suspension through Qiashredder column (1 minute spin at top speed in microfuge) to homogenize. Samples may be frozen at –80C at this point.
4. Prepare RNA using Rneasy columns following the Qiagen protocol. RNA should be eluted with 34μL RNase-free water; final volume will be about 30μl. Expected yield will be at least 1mg/$10^6$ cells. Freeze RNA at –80°C.
5. Optional ways to check RNA yields:

a. Nanodrop spectrophotometer
b. Oligogreen assay (Molecular Probes) according to manufacturer's directions. Use a 100 μl assay volume and read FAM fluorescence with the real time PCR machine.
c. Agilent bioanalyzer chip.

6. Combine 14 μl RNA with 1 μl dT18 (15uM) and 1 μl Rnasin plus (40 units/μl; Promega) in a 200μL strip tube. [Note: regular Rnasin is not heat-resistant and must be added in step 7 instead.] Heat to 65°C for 10 minutes in PCR machine then remove promptly and chill on wet ice.
7. To each tube add:
   2 μl 10x MMLV buffer
   1 μl 10mM dNTPs
   1 μl MMLV RT (200 units/uL; NEB)
8. Incubate 37°C x 60 minutes, then 95°C x 5 minutes. Freeze leftover RTs at –80°C.
9. First round V-C amplification
   50 μl mastermix (MCLab)
   2 μl V-specific primer (15μM)
   2 μl universal C primer (15μM)
   4 μl RT reaction
   42 μl water
   →100 μL total reaction

   Program:
   95°C 2 minutes
   35 cycles of
   95°C 15 seconds
   60°C 30 seconds
   final extension
   72°C 10 minutes
   Freeze leftover VC reactions at –20°C.
10. Second round V-J amplification

    Set up this reaction for each J gene required:
    25 μl mastermix (MCLab)
    1 μl V-specific primer (15uM)
    1 μl J primer (15uM)
    0.5 μl V-C PCR reaction
    22.5 μl water
    →50 μL total reaction

    Program:
    95°C 5 minutes
    10 cycles of
    95°C 15 seconds
    60°C 30 seconds
    final extension

72°C 10 minutes

Follow fluorescence to make sure reactions did not reach saturation.
If desired, clonal standards may also be amplified from plasmids, in order to create standards for the annealing reactions.

9. Remove single stranded DNA from reaction (excess primers, asymmetric PCR products, cDNA remnants):
   Add 1 μl Exonuclease I (NEB) to each tube.
   Incubate at 37°C for 60 minutes.
10. To each well add 12.5 μl of reannealing buffer. Mix well; spin plate if there are bubbles. Protect from light as possible.
    For 1 mL annealing buffer:
    552 μl 200mM MOPS pH 7.5
    110 μl 500mM EDTA
    276 μl 5M NaCl
    34 μl 5% Brij-700 (protect stock from light)
    28 μl dye concentrate (10,000x SYBR stock from Molecular Probes/Invitrogen, diluted 1:10 in DMSO; protect from light and store at –20°C)

    Final concentrations in annealing reaction:
    20mM MOPS
    10mM EDTA
    250mM NaCl
    0.03% Brij-700
    5x SYBR

11. Check samples by doing 70-99°C melting curve on 10μl aliquots
12. Perform annealing reaction, at Tm-3.5°C (just below edge of peak on negative first derivative plot of melting curve). Split reaction into 25 μl sample and 25 μl reference on either side of a 96 well plate. Up to 32 samples may be measured at once, using the left 4 columns and the right 4 columns of the plate. Fluorescence reads should be every 5 seconds to every 20 seconds, and annealing time can be 1-6 hours depending on expected diversity of sample.
    a. Make sure that the gradient is set so the rightmost reference well will stay below the annealing temperature.
    b. Make sure the machine's temperature regulation is set to block and not sample.
    c. Do melting curves at conclusion of annealing step.