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## Unbiased molecular analysis of T cell receptor expression using template-switch anchored RT-PCR

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### Keywords

T cell receptor; polymerase chain reaction; antigen-specific T cell

This protocol describes the use of a template-switch anchored reverse transcription-polymerase chain reaction (RT-PCR) to quantify and characterize all expressed T cell receptor (TR) gene products within any defined T cell population. The approach is based on a switching mechanism at the 5'-terminus of the RNA transcript (SMART) (Chenchik, 1998) and the rapid amplification of cDNA ends (RACE) (Matz et al., 1999), which subsequently enable the unbiased and linear amplification of rearranged TR-specific transcripts using primers that bind an anchor sequence and a conserved 3' region within the TR constant (C) domain. Combined with amplicon subcloning and high throughput sequencing, a comprehensive dataset is generated that defines all constituent clonotypes and their relative frequencies within the template pool (Douek et al., 2002). The procedure is optimized for direct *ex vivo* analysis, such that *in vitro* amplification is unnecessary and outgrowth frequency bias is avoided (Price et al., 2004). Materials and methods are provided for each of the following steps: (1) isolation of antigen-specific T cell populations for analysis of TR gene expression; (2) mRNA extraction; (3) cDNA synthesis; (4) cDNA clean-up; (5) PCR amplification of rearranged TR products; (6) gel extraction and purification of amplicons; (7) amplicon ligation into TA cloning vector; (8) vector transformation into competent *E. coli*; and, (9) colony PCR and sequencing.

### 1. ISOLATION OF ANTIGEN-SPECIFIC T CELL POPULATIONS FOR ANALYSIS OF TR GENE EXPRESSION

The success of this protocol depends greatly on high quality mRNA, which in turn depends greatly on cell preparation and handling. Cells must be isolated viably and not subjected to fixation buffers or azide; wash steps are best conducted using standard cell medium with a serum component. If fixation is required for safety reasons, then an alternative protocol should be followed to preserve RNA template quality (van Bockel et al., 2007). A high degree of purity (>98%) is essential to prevent the artefactual detection of non-specific clonotypes and overestimation of clonality. For this reason, high definition flow cytometric sorting with purity checking is imperative. Other methods for cell enrichment, such as magnetic beadbased procedures, should be avoided in conjunction with this protocol. In

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addition, detailed phenotypic information can be collected during the flow cytometric sorting procedure. Specificity can be determined either physically with pMHC multimers (Chattopadhyay et al., 2008; Wooldridge et al., 2009) or functionally using the detection of cell surface activation markers after stimulation with the antigen of choice (Casazza et al., 2006). Clean separation from non-specific background is the primary consideration in the selection of an appropriate sorting strategy. In all cases, cells should be handled carefully, processed as rapidly as possible and sorted immediately after surface staining into an RNA protectant solution. CAUTION: Biosafety practices should be followed rigorously when working with viable blood or tissues.

## Materials

Single cell suspension of live T cells defined with an appropriate staining procedure  
RNAlater (Applied Biosystems)  
1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)  
Flow-cytometric cell sorter  
Microcentrifuge

## Procedure

1. Sort T cells (up to 10,000) directly into a 1.5ml polypropylene O-ring microcentrifuge tube containing 100  $\mu$ l RNAlater. For greater cell numbers, additional aliquots of up to 10,000 cells should be sorted to prevent excessive dilution of the RNAlater; alternatively, cells can be sorted into 100  $\mu$ l of standard cell medium or PBS, pelleted and layered with RNAlater after aspiration of the supernatant. To minimize sampling bias, at least 500–1,000 cells should be collected for each analysis.
2. Vortex gently or flick the tube to mix, and then spin at 14,000 rpm in a microcentrifuge for 30 sec to collect all cells in the RNAlater solution. Freeze immediately and store at  $-80^{\circ}\text{C}$ .

## 2. mRNA EXTRACTION

Purification of mRNA is carried out using the Oligotex Direct mRNA Mini Kit (Qiagen), with small alterations to the manufacturer's protocol. This method is based on the isolation of pure polyadenylated RNA (polyA+ RNA) from cell lysates in an RNase-free environment using particles coated with oligo(dT), which binds to the polyA+ tail. The beads are then used to pull down the mRNA for washing.

## Materials

QIAshredders with spin columns (Qiagen)  
 $\beta$ -mercaptoethanol (BME; Sigma-Aldrich)  
Frozen vial of sorted cells in RNAlater (Applied Biosystems)  
1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)  
Oligotex Direct mRNA Mini Kit (Qiagen)  
Buffer OL1 (lysis buffer)  
Buffer ODB (dilution buffer)  
Buffer OW1 (wash buffer 1)

Buffer OW2 (wash buffer 2)

Buffer OEB (elution buffer; incubate at 70°C)

Oligotex suspension (incubate at 37°C)

Spin columns

Microcentrifuge

Two heating blocks or thermomixers (37°C, 70°C)

Freezer

## Procedure

1. Prepare reagents for later steps: (i) heat Oligotex bead suspension to 37°C in a heating block, vortex and place at room temperature; (ii) incubate buffer OEB in a 70°C heating block; (iii) for every 2 samples, add 30 µl of BME to 1 ml of buffer OL1.
2. Partially thaw the sample and centrifuge immediately at 14,000 rpm for 7 min at 4°C. There should be a whitish pellet, containing both cells and salts. Remove supernatant immediately, leaving the pellet and a minimal residual volume.
3. Add 0.4 ml of buffer OL1 (containing BME) to the pelleted white precipitate and vortex for 20–30 sec.
4. Pipette the lysate on to a QIAshredder spin column placed in a 2 ml collection tube and centrifuge for 2 min at 14,000 rpm. Discard spin column and pipette flow-through into a new 1.5 ml tube.
5. Add 0.8 ml of buffer ODB to the lysate and vortex thoroughly.
6. Vortex Oligotex suspension thoroughly and add 20 µl to the sample. Vortex thoroughly and incubate at room temperature for 10 min to hybridize the oligo dT<sub>30</sub> on the beads with the polyA<sup>+</sup> RNA.
7. Centrifuge for 5 min at 14,000 rpm to pellet the beads. Carefully remove and discard the supernatant, leaving a residual volume of approximately 10–20 µl with the pellet.
8. Add 350 µl of buffer OW1 and vortex thoroughly for 30 sec to resuspend the beads.
9. Pipette the sample on to a small spin column placed in a 1.5 ml tube without touching the bottom of the column. Centrifuge for 1 min at 14,000 rpm. Discard the collection tube containing the flow-through.
10. Transfer the spin column into a new 1.5 ml collection tube. Pipette 350 µl of buffer OW2 on to the column. Centrifuge for 1 min at 14,000 rpm. Discard the collection tube containing the flow-through. Repeat this step.
11. Transfer the spin column into a new 1.5 ml tube and place in a 70°C heating block. Pipette 20 µl of hot (70°C) buffer OEB on to the column. Pipette up and down several times to resuspend the beads, taking care not to break the membrane. Centrifuge for 1 min at 14,000 rpm.
12. To ensure maximal yield, incubate the tube again for 1 min at 70°C and pipette the same 20 µl of hot eluate from step 11 on to the column. Pipette up and down several times to resuspend the beads as above. Centrifuge for 1 min at 14,000 rpm. Discard the column.

13. Proceed to cDNA synthesis immediately and store the remaining mRNA at  $-80^{\circ}\text{C}$ .

### 3. cDNA SYNTHESIS

This section describes the synthesis of cDNA with anchor sequence incorporation. The method is based on the template-switching ability of RNase H- point mutants of MMLV reverse transcriptase (Figure 1).

#### Materials

PolyA+ mRNA

1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)

5'CDS oligo(dT) primer (10  $\mu\text{M}$ ): 5'-(T)<sub>25</sub>XY-3' (X=A, C, G; Y= A, C, G, T)

SMART II anchor oligo (10  $\mu\text{M}$ ): 5' -  
AAGCAGTGGTATCAACGCAGAGTACGCGGG-3' containing three G  
ribonucleotides (not dG) at the end

5 $\times$  RT buffer: 250 mM Tris-HCl (pH 8.3), 375 mM KCl and 30 mM MgCl<sub>2</sub>

Dithiothreitol (DTT, 20 mM; Invitrogen)

Tricine buffer: 10 mM Tricine-KOH (pH 8.5), 1.0 mM EDTA

dNTP mix (10 mM; Invitrogen)

RNaseOUT (RNase inhibitor; Invitrogen)

Superscript II RNase H<sup>-</sup> Reverse Transcriptase (RT, 200U/ $\mu\text{l}$ ; Invitrogen)

Microcentrifuge

Two heating blocks ( $42^{\circ}\text{C}$ ,  $70^{\circ}\text{C}$ )

Freezer

The SMARTer™ PCR cDNA Synthesis Kit (Clontech) contains several components required for cDNA synthesis, including a proprietary modified SMART oligo, which can be successfully used in this method. However, the 5'CDS oligodT should be synthesized as described above. The 5' PCR primer II A from the SMARTer™ PCR cDNA Synthesis Kit (Clontech) can be used as an alternative to the Universal Primer Mix in Section 5 – *PCR amplification of rearranged TR products*.

#### Procedure

1. Before starting, turn the heating blocks to  $42^{\circ}\text{C}$  and  $70^{\circ}\text{C}$ , respectively. Keep enzymes frozen until just before use.
2. Add the following reagents in the order listed into a 1.5 ml tube:
 

5'CDS	1 $\mu\text{l}$
SMART II oligo	1 $\mu\text{l}$
mRNA	3 $\mu\text{l}$
3. Place the tube in the  $70^{\circ}\text{C}$  heating block for 1 min (to break any double strands) then at  $-20^{\circ}\text{C}$  for 1 min (to anneal).
4. Add:
 

5 $\times$ RT Buffer	2.2 $\mu\text{l}$
DTT	1 $\mu\text{l}$
RNaseOUT	1 $\mu\text{l}$

dNTP mix            1  $\mu$ l  
 Superscript II RT   1  $\mu$ l

5. Vortex, spin briefly (30 sec at 14,000 rpm) and place in the 42°C heating block for 2 hr.
6. Spin briefly at top speed, then add 10  $\mu$ l of Tricine buffer and mix gently with a pipette.
7. Place in a heating block at 72°C for 7 min (to deactivate RT), then spin briefly at top speed.

Approximately 20  $\mu$ l of cDNA is made; it can be stored at -80°C or used immediately in the clean-up procedure prior to PCR.

#### 4. cDNA CLEAN-UP

In the NucleoSpin® Extract II procedure, cDNA binds to a silica membrane in the presence of chaotropic salt added by the NT binding buffer. The binding mixture is loaded directly on to NucleoSpin® Extract II Columns. Contaminants, such as salts and soluble macromolecular components, are removed by a simple washing step with ethanolic wash buffer NT3. Pure cDNA is finally eluted under low ionic strength conditions with the slightly alkaline elution buffer NE (5 mM Tris-HCl, pH 8.5).

#### Materials

cDNA  
 ≥ 99.5% ethanol  
 1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)  
 DNase/RNase-free water, molecular biology grade (Sigma)  
 1.5 ml Eppendorf tubes  
 NucleoSpin® Extract II kit (Clontech):  
     Buffer NT  
     Buffer NT3 (requires addition of 24 ml ethanol)  
     Buffer NE  
     NucleoSpin® Extract II Column with clear collection tube  
 Microcentrifuge

#### Procedure

1. Adjust DNA binding conditions by dissolving the cDNA in buffer NT and H<sub>2</sub>O in the following volumes:  
     20  $\mu$ l cDNA  
     11.4  $\mu$ l buffer NT  
     68.6  $\mu$ l H<sub>2</sub>O
2. Pipette the 100  $\mu$ l sample on to the binding column. Spin the column at 10,000 rpm for 1 min to bind cDNA to the column membrane. Remove and discard flow-through from the collection tube by pipetting. Replace the column in the same collection tube.

3. Pipette 600  $\mu$ l of NT3 buffer on to the column. Keep the NT3 bottle closed as much as possible to avoid ethanol evaporation. Spin at 10,000 rpm for 1 min to wash the cDNA. Remove and discard flow-through from the collection tube by pipetting. Replace the column in the same collection tube.
4. Spin the empty tube at 10,000 rpm for 2 min. Residual NT3 buffer will collect at the bottom of the tube. Remove column from the collection tube and place in a 1.5 ml Eppendorf.
5. Add 25  $\mu$ l of NE buffer directly on to the center of the column and incubate at room temperature for 1 min. Spin at 10,000 rpm for 1 min to elute the cDNA.

## 5. PCR AMPLIFICATION OF REARRANGED TR PRODUCTS

Touchdown PCR is performed on clean single-stranded cDNA using TRC-specific and anchor-complementary primers. The TRC-specific primers are designed to enable optimal sequencing across the CDR3 including unequivocal identification of the proximal J region. In the protocol described below, the human TRBC primer MBC2 is used; this primer binds both C1 and C2. Universal primer mix contains two oligonucleotides of different lengths that allow an initial specific extension from the adapter to reduce subsequent non-specific binding. The PCR product is then run on a 1% agarose gel with a 100 bp DNA ladder and excised. Bands containing amplified TR genes are 500–700 bp in length.

### Materials

cDNA (stored at  $-80^{\circ}\text{C}$ )

1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)

0.2 ml PCR tubes

10 $\times$  UPM (Universal Primer Mix)

MBC2 primer (25  $\mu\text{M}$ )

Advantage<sup>®</sup>2 PCR Kit (Clontech):

- 10 $\times$  PCR buffer
- AdvanTaq2 (a mixture of two enzymes, polymerase and proofreading)
- PCR-grade water
- dNTP mix (10 mM)

SYBR Gold (Invitrogen)

UltraPure Agarose (Invitrogen)

TAE buffer

Disposable scalpel

Microcentrifuge

UV illuminator or blue light transilluminator

Thermocycler

Gel box/thick combs/power source

The Advantage<sup>®</sup>2 PCR Kit contains all necessary reagents except the TRC-specific primer and the 10 $\times$  UPM. In this protocol, MBC2 is used for illustration; all TRC-specific  $\alpha$  and  $\beta$

primer sequences for mouse, macaque and human are detailed in Table 1. The 10× UPM can be prepared by mixing the following primers at the indicated concentrations:

Long (0.4 μM): 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT  
 Short (2 μM): 5'-CTAATACGACTCACTATAGGGC

## Procedure

1. Make a PCR mastermix of all reagents except for template in a 1.5 ml microcentrifuge tube. Mix well by pipetting when adding the water. Sufficient mastermix should be made to cover all samples, one negative control (water instead of template) and one extra tube (to account for pipetting error). The volumes for one sample are shown below:

10× PCR buffer	5 μl
10× UPM	5 μl
MBC2 primer	1 μl
dNTP mix	1 μl
cDNA	7–13 μl
AdvanTaq2	1 μl
Water to total 50 μl (depending on template volume)	

2. Mix cDNA template with mastermix in a thin-walled 0.2 ml PCR tube with a pipette. Spin briefly; do not vortex.
3. Run reactions in a thermocycler under the following conditions:

For 1 cycle:	95°C	30 sec
For 5 cycles:	95°C	5 sec
	72°C	2 min
For 5 cycles:	95°C	5 sec
	70°C	10 sec
	72°C	2 min
For 30 cycles:	95°C	5 sec
	68°C	10 sec
	72°C	2 min

4. Load and run the entire product with a 100bp DNA ladder on a 1% agarose gel made with 1× TAE buffer (UNIT 10.4). Leave an empty well between each sample to minimize the chances of cross-contamination. Stain the gel with SYBR Gold and visualize with low intensity UV light or a blue light transilluminator, which minimizes DNA mutagenesis. There should be a bright band in the 500–700 bp range.
5. Carefully excise the band of interest using a scalpel. Avoid taking excess agarose. Transfer the gel to a clean 1.5 ml microcentrifuge tube.

## 6. GEL EXTRACTION AND PURIFICATION OF AMPLICONS

Amplified TR products are extracted from the gel and cleaned up for sub-cloning.

### Materials

Gel-bound PCR product  
 ≥ 99.5% ethanol

1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)

1.5 ml Eppendorf tubes

NucleoSpin® Extract II kit (Clontech):

Buffer NT

Buffer NT3 (requires addition of 24 ml ethanol)

Buffer NE

NucleoSpin® Extract II Column with clear collection tube

Microcentrifuge

Thermomixer (50°C)

### Procedure

1. Weigh the 1.5 ml conical tube containing your gel-bound PCR product and subtract the weight of an empty tube. The gel piece should weigh from 100 mg to 500 mg.
2. Dissolve the gel by adding NT buffer to the 1.5 ml tube containing the gel piece. Add 2 volumes of NT buffer for every 1 volume of gel (i.e. add 200  $\mu$ l buffer per 100 mg gel). Incubate the tube in a thermomixer at 50°C/750 rpm for 10 min. If any solid phase remains visible after this period, vortex periodically and incubate for a few more minutes.
3. Pipette up to 600  $\mu$ l of the NT solution on to the binding column. Spin the column at 10,000 rpm for 1 min to bind DNA to the column membrane. If the volume is more than 600  $\mu$ l, repeat this step. Discard flow-through from the collection tube by pipetting. Replace column in the same collection tube.
4. Proceed as for cDNA clean-up above (section 4, steps 3–5).

## 7. AMPLICON LIGATION INTO TA CLONING VECTOR

Amplicons are ligated into plasmids designed to capture adenine overhangs for sub-cloning and sequencing.

### Materials

Gel-extracted PCR product

pGEM-T Easy Vector System I kit (Promega), containing pGEM vector, T4 DNA ligase, 2 $\times$  ligation buffer #1 and control insert

1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)

DNase/RNase-free water, molecular biology grade (Sigma)

Microcentrifuge

Refrigerator

### Procedure

1. For experimental samples, add the following reagents in a 1.5 ml microcentrifuge tube: 5  $\mu$ l gel-extracted DNA product, 1  $\mu$ l pGEM-T Easy vector, 1.5  $\mu$ l T4 Ligase and 7.5  $\mu$ l 2 $\times$  buffer #1. Set up a negative control tube using 5  $\mu$ l water in place of the DNA product and a positive control using 2  $\mu$ l of control insert and 3  $\mu$ l water.



2. Vortex and spin briefly, then incubate overnight (12–18 hrs) at 4°C. Shorter incubations can be performed, but efficiency is generally suboptimal.
3. Proceed to transformation; residual ligation products can be stored at –20°C.

## 8. VECTOR TRANSFORMATION INTO COMPETENT *E. coli*

The pGEM-T Easy vector contains an ampicillin resistance gene; thus, only transformed bacterial colonies will grow on agar plates in the presence of ampicillin. Blue/white colony screening is used to identify transformants that contain insert-positive vectors; these appear white due to insertional inactivation of the beta-galactosidase gene in the vector.

### Materials

Ligation product

Transformation-competent, ampicillin-sensitive *E. coli* cells (e.g. Max Efficiency DH5 $\alpha$  Competent Cells; Invitrogen)

SOC medium (Invitrogen)

LB agar plates containing 100  $\mu$ g/ml ampicillin, 50  $\mu$ g/ml isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and 50  $\mu$ g/ml X-galactosidase (X-gal)

1.5 ml polypropylene O-ring microcentrifuge tubes (Sarstedt)

Bench ethanol

Bunsen burner

Plate spinner

Glass spreader

Thermomixer (37°C)

Heating block (42°C)

### Procedure

1. Pipette 7.5  $\mu$ l of each ligation reaction, including positive and negative controls, into separate 1.5 ml microcentrifuge tubes. A PUC19 plasmid control may also be transformed to act as a control for bacterial viability.
2. Thaw *E. coli* cells gently on ice, taking care not to agitate. Gently tap the vial of *E. coli* cells to mix, then pipette 50  $\mu$ l into each tube. Gently flick the mixture; do not mix by pipetting as competent cells are highly sensitive to shear stress and heat. Incubate on ice for 30 min to allow DNA to adhere to the bacteria.
3. Heat-shock the *E. coli* cells by placing in a 42°C heat block for 50 sec to enhance plasmid uptake. Place the samples on ice for 2 min.
4. Using sterile technique, add 950  $\mu$ l of SOC medium (room temperature) to each tube. Replace the caps loosely on the sample tubes and incubate in a thermomixer at 37°C/750 rpm for 2 hr.
5. Set up Bunsen burner, plate spinner, glass spreader and beaker containing ethanol. Place a labeled LB plate on the plate spinner. Flame a pipette tip and transfer 200  $\mu$ l of the bacterial culture on to the LB plate. Flame the glass spreader in ethanol and allow it to cool for a few seconds. Spread bacteria evenly over the surface of the LB plate using the glass spreader while rotating the plate spinner several times. Repeat for each sample.

6. Incubate LB plates inverted at 37°C overnight (16–24 hrs). Expect ~100 colonies to form. Blue colonies are negative and white colonies contain inserts. Plates can be stored for up to 4 weeks at 4°C.

## 9. COLONY PCR AND SEQUENCING

Single bacterial colony plasmid inserts are amplified in a 96-well plate format using primers flanking the insertion site. White colonies contain the insert and should be selected for amplification and sequencing. It is important to pick only single colonies and to avoid splashing between wells.

### Materials

Transformation plates

Sterile toothpicks

Sterile plastic disposable reservoir enabling multi-channel pipette access (Costar, Corning Inc.)

LB plate containing ampicillin, IPTG and X-gal as above

Platinum Taq DNA Polymerase High Fidelity (HiFi; Invitrogen) with 10× HiFi buffer and MgSO<sub>4</sub> (50mM)

dNTP mix (10 mM; Invitrogen)

M13F and M13R primers, 5 μM each (M13F: 5'-TTTTCCCAGTCACGAC-3'; M13R: 5'-CAGGAAACAGCTATGAC-3')

DNase/RNase-free water, molecular biology grade (Sigma)

15 ml polypropylene tube

Skirted 96-well PCR plate (AB1000, Applied Biosystems)

Snap caps (MicroAmp 8-strip cap; Applied Biosystems)

Cap installing tool (Applied Biosystems)

Aluminum foil seals (Seal & Sample; Beckman Coulter)

Benchtop centrifuge

Thermocycler

### Procedure

1. After overnight incubation, check negative (few blue colonies only) and positive (mostly white colonies) control plates and discard.
2. Prepare PCR mastermix for each required plate in a 15 ml polypropylene tube by adding the reagents as follows to cover 100 reactions:

10× HiFi buffer	250 μl
MgSO <sub>4</sub>	100 μl
dNTPs	50 μl
M13F primer	100 μl
M13R primer	100 μl
HiFi Taq	14 μl
Water	1,886 μl

3. Pour the PCR mastermix into a sterile reservoir and pipette 25  $\mu$ l into each well of a 96-well PCR plate by using a multi-channel pipette.
4. Move to a laboratory space outside the dedicated PCR room.
5. Draw a 96-well grid on an LB plate and label with date and sample identification code; this plate is termed the restreak plate.
6. Place the PCR plate on ice. Harvest individual white colonies by spearing with a sterile toothpick. Make a gentle imprint on the restreak plate, then place the toothpick in the first well of the PCR plate and twirl it around to dislodge the bacteria. Continue with separate colonies for each of the remaining wells. Pick one dark blue colony (plasmid without insert) as a control in the last well.
7. Place the restreak plate in 37°C incubator overnight, then store at 4°C.
8. Place snap-cap lids on the plate, taking care not to touch the insides of the lids. Seal tight using the cap installing tool.
9. Briefly vortex the PCR plate and centrifuge at 1,000 rpm for 30 sec to bring down any splashes from the sides of the wells.
10. Run the PCR in a thermocycler as follows:
 

For 1 cycle	95°C	5 min
For 35 cycles	95°C	30 sec
	57°C	30 sec
	68°C	3 min
HOLD	4°C	
11. Once the PCR programme is completed, remove the plate from the thermocycler and spin at 1,000 rpm for 1 min to pull down any condensation.
12. Carefully remove the snap-cap strip from the first column and discard. Add 25  $\mu$ l of DNase/RNase-free water to each well with a multi-channel pipette and mix well by pipetting up and down. Using the same tips, transfer 25  $\mu$ l of the diluted PCR product to a new 96-well plate.
13. Repeat this process for the entire plate. Place an aluminium foil seal securely on one plate and store at -20°C until ready to sequence. The other plate serves as a back-up in case sequencing problems occur and is also used to check that the PCR worked.
14. Run 5  $\mu$ l of several individual products from the back-up plate on an agarose gel to assess the efficiency of amplification (Figure 2). If the colony PCR failed, then perform a new PCR using colonies from the restreak plate. Seal the plate as above and store at -20°C
15. Send the one plate per sorted T cell population for Sanger sequencing at a high throughput facility. Use the insert-specific TRC primer for sequencing. Sequencing facilities vary in their template requirements and procedures for clean-up, so check local specifications and adjust accordingly.

Sequences can be aligned, checked and compiled manually using specific analysis software such as Sequencher (Gene Codes Corporation). Any samples with base ambiguities or sequence quality <80% should be discarded. Sequences encoding pseudogenes, frame-shifts or premature stop codons should also be excluded from the analysis; the latter two scenarios originate from non-productive rearrangements that can maintain basal levels of expression. In addition, erroneous sequences (e.g. GIRLS or GINAEYA) can arise from the universal

primer; these should similarly be disregarded. A minimum of 50 functional sequences should be compiled per sorted T cell population. In all cases, chromatograms should be checked visually. The V and J gene segments are identified from their 3' and 5' ends, respectively, using the tables from <http://imgt.cines.fr/>. As an alternative, the concatenated sequences can be exported as a FASTA file into IMGT/V-QUEST, which can identify the V and J segments. The CDR3 should be displayed as a sequence, as TRBD gene usage is difficult to assign due to N-diversity. Grouped sequences can then be ordered according to their frequency, which reflects the original clonotypic hierarchy in the sorted T cell population due to the linear and unbiased nature of the initial PCR. Further details on bioinformatics tools can be found in Appendix 1W.

## COMMENTARY

### Background Information

The  $\alpha\beta$  T cell receptor (TR) is a membrane-anchored heterodimer expressed on the surface of T cells that mediates specific recognition of antigen in the form of major histocompatibility complex (MHC) molecules presenting endogenously-derived peptides bound in the  $\alpha 1\alpha 2$  groove. Surface expression of such peptide-MHC (pMHC) antigens provides a display library that enables T cells to detect abnormal intracellular processes through TR-mediated surveillance, which in turn is the pivotal event that dictates the initiation of a T cell response. Each  $\alpha$  and  $\beta$  chain comprises three hypervariable complementarity-determining regions (CDRs), which determine the antigen specificity of the heterodimeric TR. Three mechanisms govern the generation of diversity within the TR repertoire that is necessary to recognize the myriad potential antigenic peptides that a host may encounter (Nikolich-Zugich et al., 2004). First, each TR chain is formed by the genetic rearrangement of variable (V), diversity (D;  $\beta$  chain only) and joining (J) germline gene segments, which vary in number between species, with a TRC gene. Second, nucleotide insertions and deletions at the V(D)J junctions (N-diversity) provide an additional level of variability within the CDR3 of each chain; the CDR1 and CDR2 regions are germline-encoded by the V genes. Third, pairing of individual  $\alpha$  and  $\beta$  chains further amplifies the potential number of TRs that can be generated. After thymic selection, which operates to ensure a degree of MHC bias and delete autoreactive TRs, it has been estimated that approximately  $2.5 \times 10^7$  unique human (Arstila et al., 1999) and  $2 \times 10^6$  murine (Casrouge et al., 2000) TRs populate the periphery and are available to respond to antigenic challenges. However, these numbers are dwarfed by the potential number of antigenic pMHC combinations. By necessity, then, an intrinsic degree of cross-reactivity is incorporated within the TR recognition system to enable sufficient coverage and also to increase the likelihood that a given antigen will be recognized by a cognate TR within a time frame that facilitates an effective response (Mason, 1998). Thus, an individual clonotype, i.e. a T cell defined by the singular expressed TR, can recognize multiple ligands; similarly, individual pMHC ligands can productively engage multiple TRs. As a consequence, any antigen-specific T cell response can comprise multiple clonotypes, which in turn dictate the functional qualities of the T cell population. The role of this protocol is to enable the accurate and quantitative analysis of constituent clonotypes within T cell populations specific for defined antigens. A detailed understanding of clonal selection within the memory and effector T cell pools is essential to further our understanding of the factors that influence effective T cell immunity and has direct implications for the rational design of vaccines and immunotherapies (Appay et al., 2008; Price et al., 2009).

### Critical Parameters and Troubleshooting

Meticulous attention to detail is essential for the successful and reliable application of this protocol. Reagents should be DNase/RNase-free and PCR-grade plastics should be used

throughout. Dedicated hoods in a "clean room" free from high copy number plasmids should be used for mRNA extraction, cDNA synthesis and PCR set-up. Benches and pipettes should be cleaned with 10% bleach solution and an RNase-inactivator (e.g. RNase AWAY; Sigma-Aldrich) both before and after each procedure to minimize contamination. Disposable sleeves and gloves should be used throughout. Refer to *UNIT 10.20* for detailed recommendations on optimal work practices in a molecular biology laboratory.

### Template purity and quality

As discussed in section (1) above, this protocol is entirely dependent on the isolation of good quality RNA from viable, healthy and pure T cell populations. The sequence output, due to its sensitivity and linearity, will reflect the nature of the starting template. Thus, erroneously captured T cells will lead to the overestimation of clonality and diversity. Similarly, exclusion of specific T cells, for example due to antigen-induced cell death, can lead to the loss of true clonotypes from the starting template and subsequent underestimation of clonality and diversity.

### Sampling issues

Related to template quality, adequate sampling of the repertoire under investigation is necessary to ensure reproducibility. Thus, as many cells as possible should be sorted from the initial pool of T cells and the number of sequences generated should reflect the heterogeneity of constituent TRs within the pool. In practice, based on empirical replicate analyses, adequate sampling is achieved for largely oligoclonal pMHC tetramer-sorted T cell populations with >500 cells and >50 sequences. These minimal guidelines should be adjusted, however, according to the nature of the population under investigation. Thus, lower frequency clonotypes will be detected as more sequences are generated; however, the intrinsic sequence error rate will limit the reliable detection of very low frequency clonotypes. Sequence comparisons should account for such issues (Venturi et al., 2008a; Venturi et al., 2007).

### Unsuccessful PCR amplification

If the 5'-RACE PCR does not generate bands of the expected size, repeat the procedure using more cDNA (e.g. 13  $\mu$ l). Further cDNA can be made from the stored RNA for additional attempts. However, unsuccessful amplification generally reflects a poor starting template, in which case RNA concentration can be useful (see below). Do not use additional PCR cycles or nested approaches to amplification, as these procedures increase the error rate.

### Materials

- Microcon Centrifugation Filters (Millipore)
- DNase/RNase-free water, molecular biology grade (Sigma)
- Microcentrifuge

### Procedure

1. Bring the remaining mRNA up to 20  $\mu$ l with water.
2. Place the Microcon filter into a collection tube and add the sample to the red side of the column. This contains a glycerol-coated filter that allows solutes and fluid through, but retains the RNA.
3. Close the lid and spin at 14,000 rpm for 3 min.

4. Transfer the Microcon filter to another clean collection tube.
5. Add 6  $\mu$ l of water to the red side.
6. Flip over the filter into the collection tube so that the red side is now facing the bottom of the tube.
7. Spin the filter and collection tube for 3 min at 3,300 rpm. The concentrated mRNA is now in the collection tube.
8. Use all 6  $\mu$ l of mRNA for cDNA synthesis, doubling reagent volumes accordingly.

### Poor transformation efficiency

If the 5'-RACE PCR gave a distinct band of the expected size but few bacterial colonies contained an insert, product may have been lost during gel extraction. Most notably, ensure that buffer NT3 contains the appropriate quantity of ethanol; this can be reduced due to differential evaporation after reconstitution, especially if the bottle is opened frequently. Ligation efficiency can also be compromised due to loss of adenine overhangs if the PCR product is not processed promptly; thus, always proceed to ligation as quickly as possible once the 5'-RACE PCR is completed. Finally, the quality of the competent bacteria should be verified.

### Anticipated Results

This protocol allows the rapid and comprehensive analysis of all expressed TRs within any given T cell population and offers significant advantages over previous approaches (Rufer, 2005). Multiple facets of TR usage can be assessed depending on the particular issues under investigation. Indeed, fundamental aspects of T cell immunobiology have already been illuminated with this approach (Davenport et al., 2007; Venturi et al., 2008b). It should be noted that allelic exclusion does not operate at the  $\alpha$  locus; thus, clonotypic assessment is defined by molecular analysis of TRB gene products.

### Time Considerations

With experience and good organization, samples can be processed from mRNA extraction to overnight ligation in one day. An approximate time-guide is: mRNA extraction, 90 min; cDNA synthesis and clean-up, 3 hr; RACE-PCR amplification of rearranged TR products, 2 hr 45 min; gel extraction and ligation, 2 hr plus overnight incubation; transformation, 3 hr; colony PCR, 4 hr; preparation of samples for sequencing, 1.5 hr. Up to 4 samples can comfortably be handled in parallel. However, caution should be exercised as scaling up increases the risk of cross-contamination.

### Abbreviations

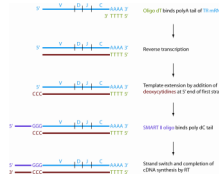
<b>cDNA</b>	complementary DNA
<b>mRNA</b>	messenger RNA
<b>PCR</b>	polymerase chain reaction
<b>RACE</b>	rapid amplification of cDNA ends
<b>SMART</b>	switching mechanism at the 5'-terminus of the RNA transcript
<b>polyA+</b>	poly-adenylated
<b>RT</b>	reverse transcription
<b>TR</b>	T cell receptor

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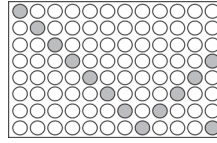
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**Figure 1. Schematic representation of the 5'-SMART-RACE PCR**

During cDNA synthesis, an anchor of known sequence is incorporated into the 5' end of full-length mRNA transcripts. PolyA+ RNA is reverse transcribed from a modified oligo(dT) primer. When the RT reaches the 5' end of the RNA, its terminal transferase activity adds a short deoxycytidine (dC) sequence. This dC sequence then hybridizes to the G ribonucleotide sequence at 3' end of the SMART oligonucleotide. The RT then jumps strands to transcribe the 5' portion of the SMART oligonucleotide. As the addition of deoxycytidine nucleotides is more efficient for full-length cDNA-RNA hybrids, full-length products are selectively enriched. PCR is then performed on the single-stranded cDNA using primers complementary to TRC genes of interest and the SMART oligonucleotide.



**Figure 2. Outline of well selection for checking colony PCR efficiency**

From each of the highlighted wells, 5  $\mu$ l of diluted PCR product is harvested and run on a 1% agarose gel with a 1 kb ladder. Bands should be visible at approximately 800 bp, while the bottom right well (blue colony input) should yield a band at approximately 300 bp.

**Table 1**  
**TR constant regions primers for use in RACE PCR**

RM, rhesus macaque.

Species	Gene	Primer sequence (5'-3')	Primer name
Human/RM	TRBC	TGCTTCTGATGGCTCAAACACAGCGACCT	MBC2
Human	TRAC	GGAAC TTCTGGGCTGGGGAAGAAGGTGTCTTCTGG	MAC2
RM	TRAC	AATAGGCAGACAGACTTGTC ACTGGA	3CA3
Mouse	TRBC	TGGCTCAAACAAGGAGACCT	MuMBC
Mouse	TRAC	TCAACTGGACCACAGCCTCAGCGTCA	MuCalpha3