Master Protocol for
Recovery of Paired T Cell Receptors from Seq-Well Libraries

As outlined in:

"TCR Sequencing Paired with Massively-Parallel 3' RNA-Seq Reveals Clonotypic T Cell Signatures"

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Table of Contents

Definition of terms:

WTA- Whole transcriptome amplified products. Full length transcriptome products that are single-cell barcoded on the 3' side. In this protocol, WTA libraries were constructed via Seq-Well, which has identical WTA structure as DropSeq.

Pull-down probes- Oligonucleotide probes with biotinylated 5' end. This protocol uses probes targeting the constant region of the TCRs. Used in conjunction with the IDT xGen enrichment kit to target for TCR whole transcripts. See Supplementary Table 1 in the original publication for exact sequences.

UPS primers- Universal priming site primers. UPS and UPS2 primers are used to uniformly amplified all products and construct Illumina-compatible libraries. See Supplementary Table 1 for exact sequences.

I. TCR Enrichment from 3' Barcoded Single-cell Libraries:

The protocol assumes that whole transcriptome amplified products generated by the Seq-Well/DropSeq platform are being used.

Before starting:

1. Make sure to dilute all the necessary buffers from the xGen kit (IDT; Cat.No.1072281). Extra can be kept at 4°C, and brought to 37°C before use to make sure all salts go into solution.
2. Pull-down-probes should be diluted ahead of time. We highly suggest aliquot the probes in multi-channel compatible format (i.e. in strip tubes with caps).
    a. TCRα and TCRβ probes should be mixed and diluted to 1.5uM (each, or 3 uM total)
3. Thaw both the 2x hybridization buffer and the xGen buffer enhancer from the xGen kit, and bring to room temperature.

A. Make the following master mix (per sample/reaction):

UPS (50 uM)	0.8 uL
Cot-1 DNA	0.5 uL
2X Hybridization Buffer	8.5 uL
xGen Buffer Enhancer	2.7 uL
Total	12.5 uL

1. Mix 3.5 uL of WTA product with the master mix (for a total of 16 uL)
    a. We recommend using 0.2mL PCR strip tubes with caps.
2. Incubate mixture at room temperature for 5 minutes
3. Then incubate mixer at 95 degree for 10 minutes (could be done in thermocycler, with lid set to 105 degrees)
4. Remove mixture to room temperature, and add 1uL of pull-down probes mix (see above). Vortex to mix, and centrifuge to collect liquid.
5. Incubate the mixture at 65 degree for one hour to allow for hybridization of pull-down probes to WTA libraries.

B. During the incubation, prepare the streptavidin beads (Invitrogen; Cat.No.65306)

1. Aliquot 50uL of beads per sample into a 1.5 mL microcentrifuge tube.
2. Place the tube on magnetic stand and allow for the beads to pellet.
3. Remove supernatant, and add equal volume of bead wash buffer into the tube
4. Vortex to mix, and place back on the magnetic stand
5. Repeat 3-4
6. Take tube off of magnet, and add equal volume of bead was buffer
7. Aliquot 50uL (per sample) of the mixture into PCR strip tubes.
8. Place tubes back on magnetic stands.
9. Remove the supernatant. The beads are now ready for hybridized mixes from A.

C. After the pull-down probes have been hybridized to WTA libraries

1. Add mixes from A into the prepared beads from B.
2. Vortex to mix.
3. Incubate mixture at 65 degrees for 45 minutes.
    a. Intermittently vortex the mixture every 10 minutes to keep the streptavidin beads suspended in the mixture.
    b. If possible, we recommend using a thermal shaker (e.g. Eppendorf ThermoMixer C) to automate intermittent vortexing.

D. Prepare buffer for 65 degree wash steps.

1. During incubation of C, aliquot and preheat Wash Buffer 1 (WB1) and Stringent Wash Buffer (SWB) at 65 degree.
2. Preheat at least 100 uL/sample of WB1 and 400uL/sample of SWB (2x200uL).
    a. We recommend aliquot the buffers into PCR strip tubes, and preheat on thermocycler/thermoshaker for fast preheating.

E. Wash hybridized beads at 65 degrees.

1. After C, add 100uL of heated WB1 into each hybridized mixture, and place onto magnetic stand.
2. Aspirate supernatant, and take mixtures off of magnetic stand.
3. Add 200uL of heated SWB into each reaction, and resuspend the bead pellets
4. Inbcubate mixture at 65 degrees for 5 minutes
5. Place mixture back on magnetic stand, and repeat 2-4
6. Aspirate the final wash of SWB.

F. Wash hybridized beads at room temperature

1. Add 200uL of room temperature WB1 into each reaction
2. Vortex for 2 minutes to mix, spin to collect liquid, and place on magnetic stand.
3. Aspirate, and add 200uL of room temperature WB2.
4. Vortex for 1 minutes to mix, spin to collect liquid, and place on magnetic stand.
5. Aspirate, and add 200uL of room temperature WB3.
6. Vortex for 30 seconds to mix, spin to collect liquid, and place on magnetic stand.
7. Aspirate, and add 20uL of water.

G. PCR amplify pull-down TCR products

For each reaction, we will perform 5 PCR reactions, using 2uL of mixture from F in each reaction (using a total of 10 out of 20 uL).

Make the following master mix (per reaction).

Kapa Hifi Hotstart Readymix 2X (Kapa Biosystems)	12.5 uL
UPS (10uM)	2uL
Water	8.5uL

1. Aliquot 5x 23uL of master mix for each reaction
2. Add 2uL of mixture from F into each of the aliquots
3. PCR amplify using the following condition

1 cycle of
[95^{o}C for 3 minutes]
25 cycles of
[98^{o}C for 40 seconds
67^{o}C for 20 seconds
72^{o}C for 1 minute]
1 cycles of
[72^{o}C for 5 minutes]
Hold at 4^{o}C

4. After amplification, pool all 5 PCR reactions (20uL for each sample) into a single tube (for 100uL total)
5. Perform SPRI purification, aiming to remove all products < 1kbp
    a. We recommend using AmpureXP or homemade Serapure SPRI reagent (see appendix for protocol)
    b. Elute the samples into a final volume of 13-15 uL of water.
    c. The final product contains both full-length TCR alpha and beta products.
    d. We recommend assessing the quality of the enrichment via fragment analyzer.
    e. See example below for expected size distribution.
    f. Take note of estimated concentration
    g. If amplification was unsuccessful (either due to low concentration of product, or technical error), the rest of the beads from F can be used to repeat G.

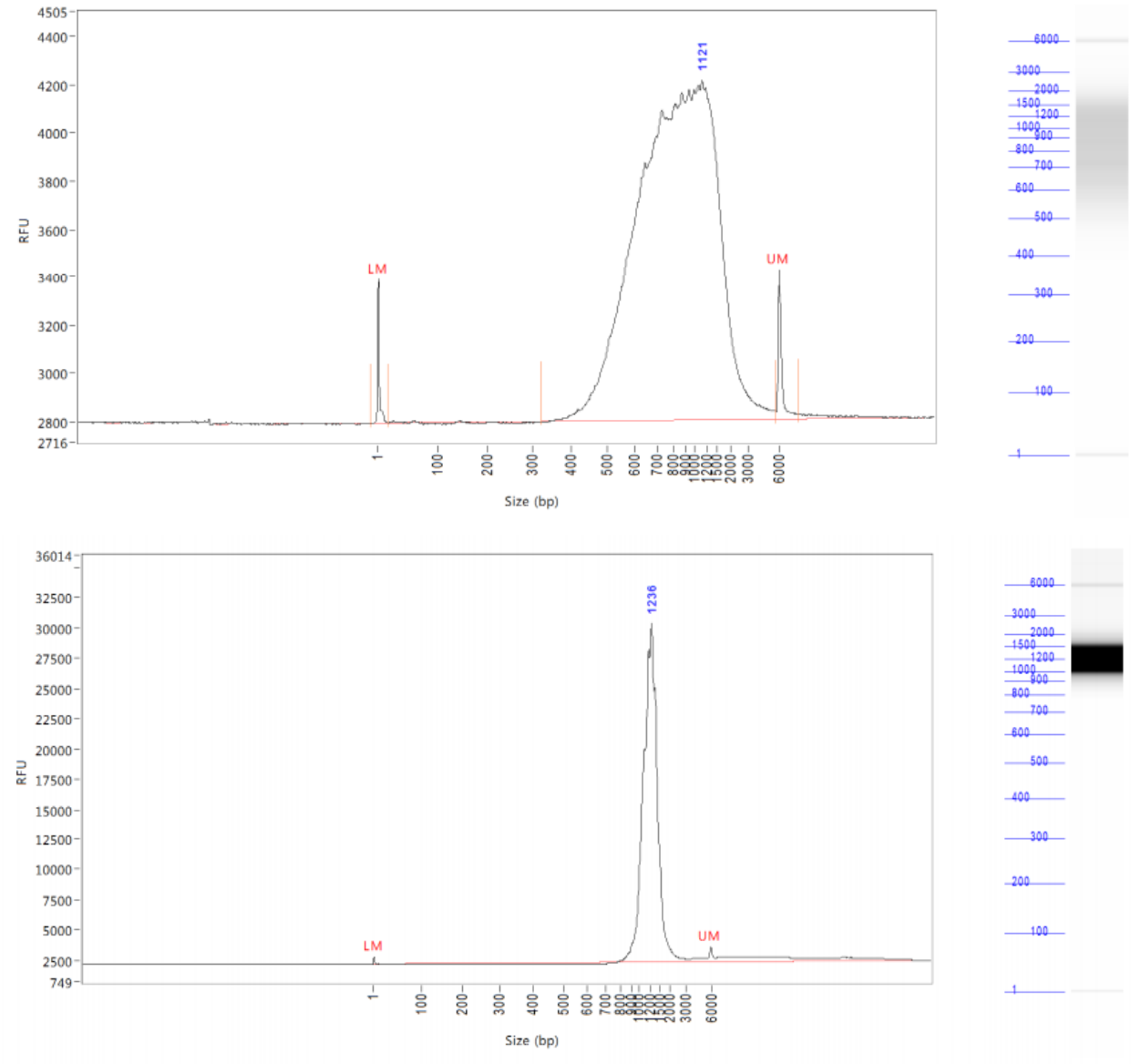

Figure 1. (top) Typical size distribution of starting WTA product and (bottom) expected size distribution of amplified TCRα and TCRβ pull-down products. Slight variation between samples is acceptable and expected.

Note: Certain samples often need to be enriched for TCR transcripts multiple times, especially for samples with low gene recovery, or low number of αβ T cells. Part **I**. could be repeated if necessary, resulting in multiple rounds of TCR enrichment. For subsequent rounds of enrichment, we recommend decreasing the cycles of PCR amplification from 25 to 12.

II. TCR V-region primer extension:

After enriching for full-length TCR products, we then perform primer-extension using TCRV-UPS2 primers (see **Supplementary Table 1** for exact sequences).

Before starting, prepare separate TCRV-UPS2 alpha and beta mixes by equimolar pool each of the primers, and dilute as necessary to 10uM (total concentration).

Make the following master mix (per reaction). Make two separate mixes (one for TCRα and one for TCRβ chain):

Kapa Hifi Hotstart Readymix 2X	12.5µL
TCRV-UPS2 TCRα or TCRβ(10uM)	2.5 µL
Water	6µL

Aliquot 21 uL (per reaction) of mastermix into PCR tubes.

Add 4uL of TCR enrich product, for a total of 25uL per reaction.

Perform primer extension using the following condition

1 cycle of
[95^{o}C for 5 minutes]
1 cycles of
[55^{o}C for 30 seconds]
1 cycles of
[72^{o}C for 2 minutes]
Hold at 4^{o}C

After extension, add 25uL of water, to bring each reaction to a total of 50uL

SPRI clean as before. Elute into 11uL of water.

III. TCR UPS2 amplification:

After primer-extension, we use UPS2-N70x and UPS-mod-N50x primers to amplify the resulting products, and add Illumina flanking sequences for sequencing.

See **Supplementary Table 1** for primer sequences. Thaw and dilute the primers into 10uM aliquots.

To minimized barcode swapping, we will split each sample into 4 PCR reactions, and pool them again after amplification.

Make the following master mix (per reaction. prepare 4 reactions for each sample):

Kapa Hifi Hotstart Readymix 2X	12.5µL
UPS-mod-N50x	0.5µL
UPS2-N70x	0.5µL
Water	9µL

Add 2.5µL of products from **II.** To each reaction, for a total of 25µL per rection.

Amplify using the following condition:

1 cycle of
[95ºC for 2 minutes]
9-18 cycles (see below) of
[95ºC for 30 seconds
60ºC for 30 seconds
72ºC for 1.5 minute]
1 cycles of
[72ºC for 5 minutes]
Hold at 4ºC

The precise cycle would need to be tested and adjusted for each sample. We recommend the following guideline, based on the concentration estimation of amplified pull-down product from **I.:**

Concentration of products from **I.** (ng/µL)	Recommended cycle number for TCRβ	Recommended cycle number for TCRα
< 1	16-18	17-18
1-10	14	15
10-20	13	14
20-30	12	13
30-40	11	12
>40	9-10	9-11

Take 12.5 uL of each reaction and pool for each sample (for a total of 50uL per sample)

SPRI clean as previously described. The final product is ready for Illumina Sequencers.

We recommend assessing the size distribution (figure2) and concentration of the final product using fragment analyzer and Kapa quant qPCR.

If amplification was unsuccessful after SPRI purification, the remaining half of the unpurified amplified product can be amplified further for 2-4 more PCR cycles.

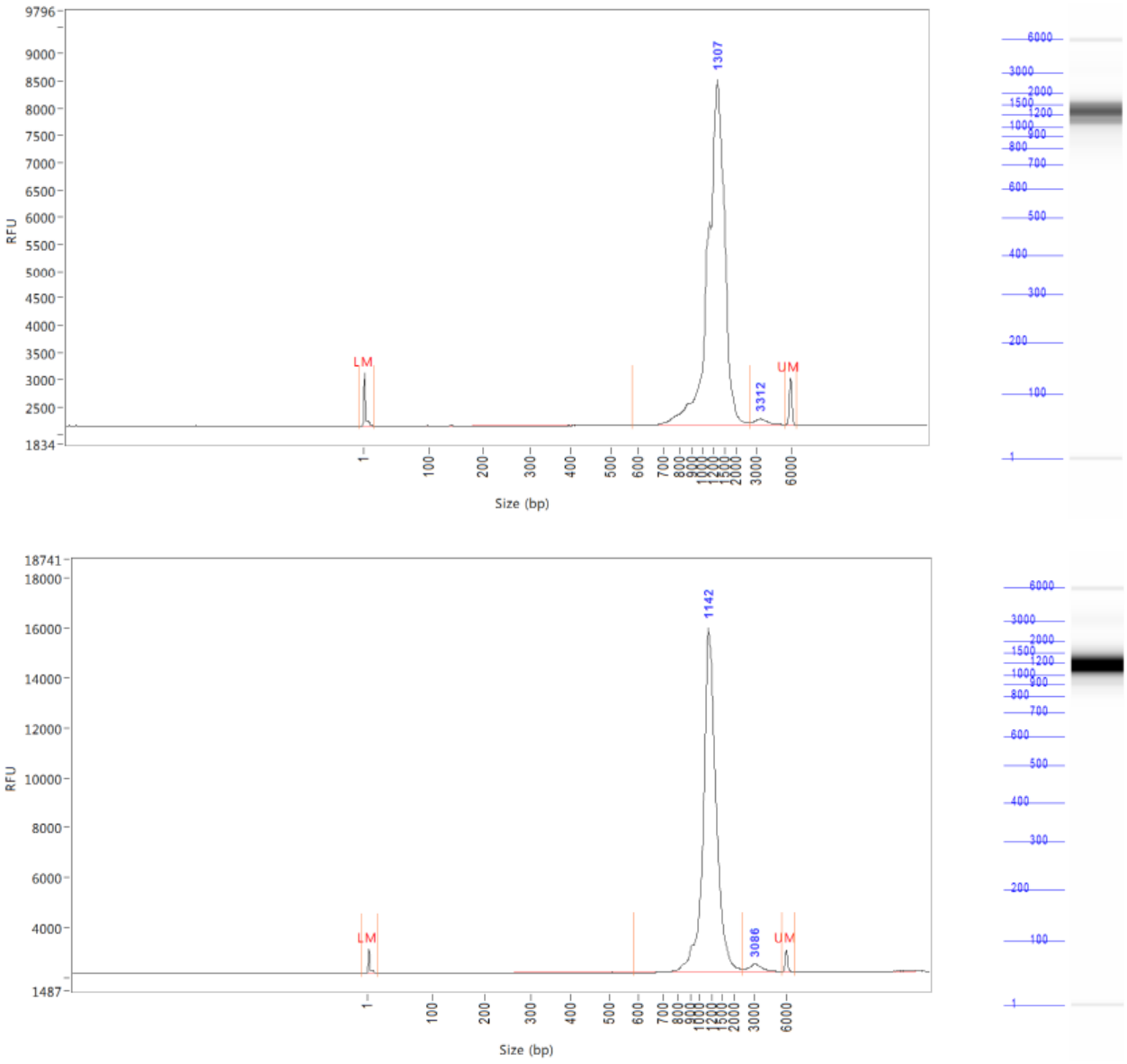

Figure 2. Expected size distribution of UPS2 amplified (top) TCRα and (bottom) TCRβ products. Slight variation between samples is acceptable and expected. TCRα products are expected to be longer than TCRβ products.

IV. Sequencing of final TCR libraries:

Here we describe the sequencing condition for sequencing the prepared TCR libraries. For a detailed loading protocol, please consult Illumina.

We require the use of two custom sequencing primers (see **Supplement**). Due to the large size of the final libraries, we aim to load the flow cells to a lower density

We recommend sequencing on the Illumina MiSeq using the 150 cycle kit. For libraries consisting of high number of cells, a NextSeq could also be used.

Sequencing specification:

Read 1: 150bp
Index 1: 20bp

MiSeq:
Sequencing primers were used at a final concentration of 2.5µM. We aimed for 8-12 * 10^6 pass filter reads per lane (cluster density of roughly 450K/mm2). Based on the whole-transcriptome data, we allotted ~6,000 T cells per lane.

NextSeq:
Sequencing primers were used at a final concentration of 2.5µM. We aimed for 150-200 * 10^6 pass filter reads per lane (cluster density of roughly 100K/mm2). Based on the whole-transcriptome data, we allotted ~80,000 T cells per lane.

Final fastq will contain TCR sequences in Read 1, and cell barcode with UMI in Index 1.

Appendix. SPRI protocol and notes

Throughout this protocol, we require purification of amplified products at various steps. In all cases, we aim to purify TCR products > 1kbp in length. Standard SPRI reagents could be used following manufacturer's instructions (Ampure XP, etc.). Here we provide a homemade alternative taken from Nadin Rohland (Doi: 10.1101/gr.128124.111). Heretofore referred to as Serapure reagent.

To make homemade SPRI reagent:
Materials:
Sera-mag SpeedBeads (Fisher # 09-981-123)
PEGN8000 (Amresco 0159)
0.5 M EDTA, pH 8.0 (Amresco E177)
1.0 M Tris, pH 8.0 (Amresco E199)
Tween 20 (Amresco 0777)
5 M NaCL
Fermentas ladder(s) (Ultra-low range: Fisher # FERSM1211, 50 bp: FERSM0371)

1. In a 50 mL conical using sterile stock solutions, prepare TE (10 mM TrisNHCl, 1 mM EDTA = 500 µL 1 M Tris pH8 + 100 µL 0.5 M EDTA, fill conical to 50 mL mark with dH20).
2. Mix SeraNmag SpeedBeads and transfer 1 mL to a 1.5 mL microtube.
3. Place SpeedBeads on magnet stand until beads are drawn to magnet.
4. Remove supernatant with P200 or P1000 pipetter.
5. Add 1 mL TE to beads, remove from magnet, mix, return to magnet.
6. Remove supernatant with P200 or P1000 pipetter.
7. Add 1 mL TE to beads, remove from magnet, mix, return to magnet.
8. Remove supernatant with P200 or P1000 pipetter.
9. Add 1 mL TE to beads and remove from magnet. Fully resuspend and set microtube in rack (i.e. not on magnet stand).
10. Add 9 g PEGN8000 to a new 50 mL, sterile conical.
11. Add 10 mL 5 M NaCL (or 2.92 g) to conical.
12. Add 500 µL 1 M TrisNHCL to conical.
13. Add 100 µL 0.5 M EDTA to conical.
14. Fill conical to ~ 49 mL using sterile dH20. You can do this by eye, just go slowly.
15. Mix conical for about 15 minutes until PEG goes into solution (solution, upon sitting, should be clear).
16. Add 27.5 uL Tween 20 to conical and mix gently.
17. Mix 1 mL SpeedBead + TE solution and transfer to 50 mL conical.
18. Fill conical to 50 mL mark with dH20 (if not already there) and gently mix 50 mL conical until brown.
19. Test against AMPure XP using aliquots of ladder (Fermentas GeneRuler). I recommend the 50 bp ladder in place of the ultra-low range ladder.
20. Wrap in tinfoil (or place in dark container) and store at 4°C.

Testing:

You should test the Serapure mixture to ensure that it is working as expected. You can do this using DNA ladder (Fermentas GeneRuler – NEB ladders may cause problems):

1. Prep fresh aliquots of 80% EtOH.
2. Mix 2 µL GeneRuler with 18 µL dH20.
3. Add 20 µL GeneRuler mixture to a volume of Serapure and/or AMPure (the specific volume depends on whether you are trying exclude small fragments or not; see the figure on the next page).
4. Incubate mixture 5 min. at room temperature.
5. Place on magnet stand.
6. Remove supernatant.
7. Add 200 µL 80 % EtOH.
8. Incubate on stand for 1 min.
9. Remove supernatant.
10. Add 200 µL 70% EtOH.
11. Incubate on stand for 1 min.
12. Remove supernatant.
13. Place beads on 37°C heat block for 3-4 min. until dry.
14. Rehydrate with 20 µL dH20.
15. Place on magnet stand.
16. Transfer supernatant to new tube.
17. Mix supernatant with 1 µL loading dye.
18. Electrophorese in 1.5 % agarose for 60 min. at 100 V.

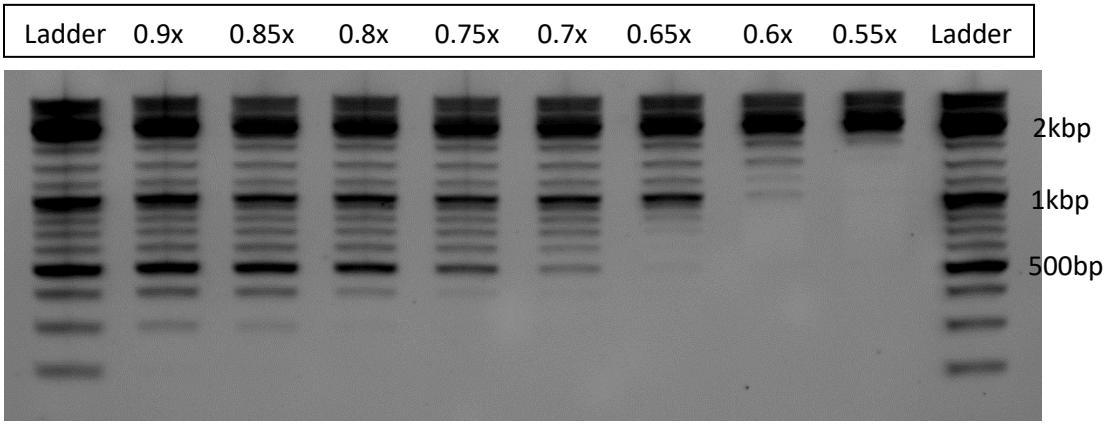

Figure 3. Example results of varying the ratio of Serapure reagent to GeneRuler DNA ladder. The ratio is denoted as ratio of the Serapure reagent to the sample. For example, for a 50µL sample, 0.9x of serapure reagent means 50*0.9 = 45µL of Serapure reagent, for a total mixture of 50+45 = 95µL of Serapure + original sample.

Purifying PCR products with Serapure Reagent:

Before starting: Determine the optimal ratio of Serapure reagent to use to purify the target sequence of interests. For TCRαβ transcripts in this protocol, we usually use 0.64x ratio of Serapure reagent to PCR products (i.e. 32uL of Serapure to 50µL of PCR product).

It is also important to bring the Serapure reagent to room temperature before starting.

1. Prep fresh aliquots of 80% EtOH.
2. Mix 32µL of Serapure to 50µL of PCR product (for a 0.64x ratio)
3. Incubate at RT for 15 minutes
4. Place mixture on magnetic stand for 1-5 minutes, or until the magnetic beads pellet.
5. Remove supernatant
6. Add 200µL of 80% EtOh
7. Remove Supernatant
8. Repeat 6-7
9. Allow bead pellets to dry. Roughly 5-10 minutes
10. Add 10-15uL of water
11. Vortex to mix
12. Incubate at RT for 10-15 minutes
13. Place back on magnetic stand for beads to pellet
14. Collect the supernatant and discard the magnetic beads. The supernatant now contains purified DNA.

Optional: to ensure that the product is as clean as possible, a beadless version of the Serapure reagent (wash reagent) can be made (replace the volume of speedmag beads with TE) to supplement the protocol above. The wash reagent should be diluted to the same ratio as the beads to PCR product ratio (i.e. 0.64x) with water. After supernatant is removed at step 5, add 100uL of the diluted wash reagents and pipette to mix. Then place back on magnetic stand to collect the pellet. Remove the supernatant, and continue with step 6.