1) **Add custom primers into RT Mastermix, then perform droplet capture with the 10x Genomics Chromium Single Cell 5' Chemistry Kits.**

Prior to rt, add 0.5uL of 10uM Barcode RT primer directly into the 68.3uL of Mastermix for each lane.

2) **cDNA amplification.**

Each of the custom primers has an adapter identical to the adapter sequence on the Poly-dT RT Primer (10x Genomics, PN-2000007). This adapter servers as a primer binding site for the NonPoly(dT) primer (10x Genomics, PN-220106) during cDNA amplification, and thus allows amplification of reverse transcribed TCR and barcode products. Therefore, the cDNA amplification can be performed according to the 10x kit instructions.

We expect a 1,000 bp amplicon on a BioAnalyzer from the custom primer.

3) **PCR amplification of each transcript and sample indexing**

Prepare TCR amplicons according to the standard V(D)J kit instructions.

<u>For the mCherry Barcode constructs:</u>

First PCR to add i7 handle for each sample **(do 4 parallel reactions for each sample and then pool)**:

Reagent	**Volume for 1 Reaction**	**Volume for 4.25 Reactions**
10uM mChBC_ui7_r	1	4.25
10uM P5	1	4.25
Amplified cDNA: 10ng	Depends on DNA conc	Depends on DNA conc
2x KAPA Hi-Fix Master Mix	25	106.25
Water	Depends on DNA conc	Depends on DNA conc
Total (uL)	50	212.5

<u>PCR 1 conditions:</u>

Step	**Time**	**Temperature**	**Cycles**
Initial Denaturation	3 min	95°C	1
Denature	15 s	98°C	25
Anneal	15 s	57°C	
Extend	1 min	72°C	
Final Elongation	5 min	72°C	1
Hold	Indefinitely	-	1

<u>Purification for PCR 1</u>

Perform 0.6x left-sided SPRI cleanup (taking the elution from the beads) to recover amplified DNA and remove primers.

Expected size is ~900 bp. Refer to this as mCherryBC-i7 and carry each forward to next step.

Second PCR to add sequencing index to barcodes (do 4 parallel reactions for each sample and then pool):

Reagent	Volume for 1 Reaction	Volume for 4.25 Reactions
10uM BC0X (X = 1 for sample 1, 2 for sample 2)	1	4.25
10uM P5	1	4.25
Amplified mCherryBC-i7: 10ng	Depends on DNA conc	Depends on DNA conc
2x KAPA Hi-Fix Master Mix	25	106.25
Water	Depends on DNA conc	Depends on DNA conc
Total (uL)	50	212.5

PCR 2 conditions:

Step	Time	Temperature	Cycles
Initial Denaturation	3 min	95°C	1
Denature	15 s	98°C	25
Anneal	15 s	57°C	
Extend	1 min	72°C	
Final Elongation	5 min	72°C	1
Hold	Indefinitely	-	1

Purification for PCR 2 and Sequencing Prep

Perform 0.6x left-sided SPRI cleanup (taking the elution from the beads) to recover amplified DNA and remove primers. Expected size is ~920bp.

This is now indexed DNA ready for QC and sequencing on the MiSeq, with read length 150nt.

4) Sequencing

TCRs from all V(D)J preps should be pooled and run on a MiSeq or NextSeq.

The mCherry Amplicon samples should be analyzed on 2 MiSeq runs (4 pooled samples each). For the mCherryBC amplicon: 150nt (75+75) paired end. Sequencing primers are Solexa i5 and i7. Primer sequences are below for verification.

Name	Full Primer Sequence (5' to 3')	Barcode Sequence
RT Spike-in	AAGCAGTGGTATCAACGCAGAGTACGAGGAGAAAATGAAAG CCATACGGGAAGC	-
mCh_BC_ui7_r	GCTGAACCGCTCTTCCGATCTNNNNNNNNCAGAGGTTGATTA CCGATAAGCTTGATATCG	-
BC01	CAAGCAGAAGACGGCATACGAGATATCACGCGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	CGTGAT

BC02	CAAGCAGAAGACGGCATACGAGATCGATGTCGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	ACATCG
BC03	CAAGCAGAAGACGGCATACGAGATTTAGGCCGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	GCCTAA
BC04	CAAGCAGAAGACGGCATACGAGATTGACCACGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	TGGTCA
BC05	CAAGCAGAAGACGGCATACGAGATACAGTGCGGTCTCGGCA TTCCTGCTGAACCGCTCTTCCGATCT	CACTGT
BC06	CAAGCAGAAGACGGCATACGAGATGCCAATCGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	ATTGGC
BC07	CAAGCAGAAGACGGCATACGAGATCAGATCCGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	GATCTG
BC08	CAAGCAGAAGACGGCATACGAGATACTTGACGGTCTCGGCAT TCCTGCTGAACCGCTCTTCCGATCT	TCAAGT
P5 (fwd)	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTA CACGACGCTCTTCCGATCT	-