EuroClonality-NGS Working group

Standard Operating Procedure (SOP) for

Two-step IG/TR NGS-based marker identification for the Illumina MiSeq platform

Version 1.0 (11 June 2019)

Updated in Version 1.0

- Not applicable (new SOP).

For complementary information or questions,
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EuroClonality-NGS Working group SOP for **Two-step IG/TR NGS-based marker identification for the Illumina MiSeq platform**

Content

1. Working definitions, material references and general remarks

- This SOP provides detailed instructions on how to prepare amplicon sequencing libraries for **target screening using EuroClonality-NGS primer sets** for IGH (VJ + DJ), IGK (VJ-Kde + intron-Kde), TRB (VJ + DJ), TRG and TRD on Illumina MiSeq.
- Besides patient samples it is highly recommended to include **negative control** (water) and **positive control** (buffy coat) libraries in each run.
- Please comply with all recommendations. Even small deviations will impact the results.

Equipment:

- Thermocycler
- 1 full set of pipettes comprising P10µl, P20µl, P100µl, P200µl and P1000µl
- Heat Block
- Gel electrophoresis chamber
- UV lamp
- Vortex
- Microfuge
- Plate centrifuge
- Light Cycler LC480/Qubit Fluorometer
- Laminar flow hood
- Illumina MiSeq sequencer

Reagents & Consumables:

- dNTPs (ROTH, Cat No: L785.3)
- EagleTaq DNA Polymerase (Roche, Cat No: 05206952190) **or** AmpliTaq Gold DNA Polymerase with Buffer II and $MgCl_2$ (ThermoFisher Scientific, Cat No: N8080241)
- In combination with Eagle Taq DNA Polymerase: FastStart PCR Buffer (Roche, Cat No: 05917166103) and $MgCl_2$ (Roche, Cat No:11600770103)
- Fast Start High Fidelity PCR System (Roche, Cat No: 04738292001)
- HPLC-H_2O (Merck, Cat No: 1153331000, or other)
- Gel Red (VWR, Cat No: 41003-1)

- Gel loading dye (self-made)
- Agarose
- TBE (or TAE) Buffer
- TE Buffer
- 10M NaOH
- Scalpel
- PCR plate caps
- Pipette tips
- 1,5 ml Low Binding Tubes
- 0.2 ml PCR tubes
- Ice block
- MinElute Gel extraction kit (Qiagen, Cat No: 28606, or other)
- Qubit® dsDNA HS Assay Kit (Thermofisher Scientific, Cat No: Q32854) **or** QuantiT PicoGreen dsDNA Assay Kit (Thermofisher Scientific, Cat No: 50107677)
- Illumina PhiX spike in control v3 (Illumina, Cat No: FC1103001)
- MiSeq Reagent Kit v2, 2×251bp (Illumina, Cat No: MS1022003)
- Tween 20 (ROTH, Cat No: 9127.1, or other)
- Buffy coat DNA
- Spike-in DNA (described in Knecht et al., Leukemia, accepted)
- 1st and 2nd step primers (see Appendix)

2. 1st step PCR

2.1. Prepare your samples:

2.1.1. Normalize patients' diagnostic DNA to 100 ng/µl (dilute with TE buffer).

2.1.2. Dilute buffy coat (BC) DNA to 100 and 10 ng/µl (dilute with TE buffer).

Note: Pipetting BC into each PCR serves as control of the PCR concerning detection of a band after gel electrophoresis and can be skipped if a user is experienced with the technique. Preparing the BC control library serves as the quality control of run performance.

2.2. Prepare the master mixes: prepare the individual master mix for each target-specific PCR (see Table 1 below). For IGK and TRB, prepare the double amount.

2.3. Vortex and centrifuge the master mixes.

Note: IGK and TRB targets are amplified in two separate reactions (TRB-VJ and DJ and IGK-VJ-Kde and intron-Kde).

Table 1. Composition of 1st step PCR master mixes.

		1st PCR											
	Stock concentration	IGH-VJ		IGH-DJ		IGK-VJ-Kde, intron-Kde		TRB-VJ, TRB-DJ		TRG		TRD	
		Final concentration	µl/library	Final concentration	µl/library	Final concentration	µl/library	Final concentration	µl/library	Final concentration	µl/library	Final concentration	µl/library
PCR Buffer II	10x	1x	5	1x	5	1x	5	1x	5	1x	5	1x	5
MgCl2	25 mM	2,5 mM	5	3 mM	6	1,5 mM	3	4 mM	8	4 mM	8	2 mM	4
dNTP-Mix	10 mM	0.2 mM	1	0.4 mM	2.0	0.2 mM	1	0.2 mM	1	0.2 mM	1	0,2mM	1
EagleTaq/AmpliTaq Gold	5 U/µl	1U/rxn	0.2	1.5U/rxn	0.3	1U/rxn	0.2	1U/rxn	0.2	1U/rxn	0.2	1 U/rxn	0.2
reaction volume: 50µl													

2.4. Pipette the master mix into the PCR tubes.

2.5. Pipette the 1st step primers into all tubes.

2.6. Add 1 µl of patient DNA (100 ng) into the appropriate tube(s).

2.7. Add 1 µl of HPLC-H_2O or 1 µl of buffy coat (100 ng) into the appropriate tubes.

2.8. Add 1 µl of buffy coat (10 ng) DNA and 720 copies of spike-in DNA into all tubes containing patient's DNA.

2.9. Add the appropriate amount of HPLC-H_2O into all tubes to a total reaction volume of 50 µl.

2.10. Vortex and centrifuge the PCR strips.

2.11. Place the strips into a thermocycler and select the correct program to run (see Table 2 below).

Note: PCR conditions are the same for all target-specific PCRs.

Table 2. 1st step PCR program.

1 cycle	Initial denaturation	94°C	10 min
35 cycles	**Denaturation**	**94°C**	**1 min**
	Annealing	**63°C**	**1 min**
	Extension	**72°C**	**30 sec**
1 cycle	Final extension	72°C	30 min
		12°C	∞

2.12. Once the run has finished, either store the products at 20°C, or continue with gel electrophoresis to check the successful PCR amplification of all targets (IGH-VJ, IGH-DJ, IGK-VJ-Kde, intron-Kde, TRG and TRD). If you identify positive bands, move forward to the next steps, otherwise repeat the 1st PCR.

3. Purification of TRB (VJ, DJ) PCR products by gel extraction

Note: As the TRB PCR (both VJ and DJ) leads to unspecific products (for instance TRB-VJ around 188-190bp), a gel extraction prior to the 2nd round PCR is necessary for TRB PCR products. You can use any commercial kit. We prefer the MinElute Gel extraction kit.

3.1. For the gel electrophoresis, use 21 µl of the TRB PCR product + 9 µl gel loading dye and mix it.

3.2. Pipette the mix (30 µl) into a big-sized gel (2% agarose).

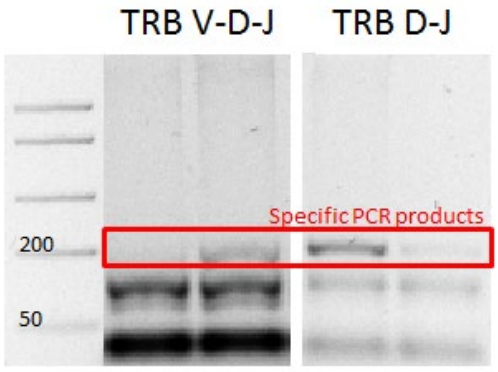
3.3. Run the gel at 130V for 55 minutes.

3.4. Purify the bands according to the manufacturer's instructions for your commercial kit.

Table 3. Expected fragment lengths of TRB target products and unspecific products.

	Fragment length	Unspecific product
TRB-VJ	200 bp	180 bp
TRB-DJ	210 bp	190 bp

Figure 1. Example of TRB PCR products after gel electrophoresis.



4. 2nd step PCR

4.1. Dilute the 1st round PCR products (TRG, TRD, IGH-VJ, IGH-DJ, IGK-VJ-Kde and intron-Kde) 1:50. If the band on the gel is too weak (for example as the first and the fourth sample in Figure 1), do not dilute the PCR product before the 2nd round. TRB (both VJ and DJ) PCR products should never be diluted after the gel extraction.

4.2. Prepare the master mix for the 2nd round PCR (see Table 4 below).

4.3. Vortex and centrifuge the master mix.

Note: Keep the polymerase and the master mix on ice.

Table 4. Composition of 2nd step PCR master mixes.

	Stock concentration	all genes	
		Final concentration	µl/library
PCR Buffer with	10x	1x	5
MgCl2	18 mM	1.8 mM	0
dNTP-Mix	10 mM	0.2 mM	1
see the central primer table for concentrations			
Fast Start High Fidelity polymerase	5 U/µl	2.5U/rxn	0.5

reaction volume: 50µl

4.4. Pipette the master mix into the PCR tubes.

4.5. Into each tube, add the unique combination of the barcoded forward and reverse primers (see Table 5 below, primer concentrations in the central primer table).

Table 5. Barcodes of forward and reverse primers.

reverse barcodes

forward barcodes	D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712	
D501													IGH-VJ
D502													IGH-DJ
D503													IGK-VJ-Kde
D504													intron-Kde
D505													TRB-VJ
D506													TRB-DJ
D507													TRD
D508													TRG
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	cPTC	water	

96 combinations

4.6. Add the 1st round PCR products into the PCR tubes: 1 µl of 1:50 diluted TRG, TRD, IGH-VJ, IGH-DJ, IGK-VJ-Kde and intron-Kde products (unless the band was too weak, see 4.1) and 3 µl of undiluted TRB products.

4.7. Vortex the PCR tubes.

4.8. Place the plate into a thermocycler and select the correct program to run (see Table 6 below).

Table 6. 2nd step PCR program.

1 cycle	Initial denaturation	95°C	2 min
20 cycles	**Denaturation**	**94°C**	**30 sec**
	Annealing	**63°C**	**30 sec**
	Extension	**72°C**	**30 sec**
1 cycle	Final extension	72°C	5 min
		12°C	∞

4.9. Check the length of all the 2nd round PCR products by gel electrophoresis (see Figure 2).

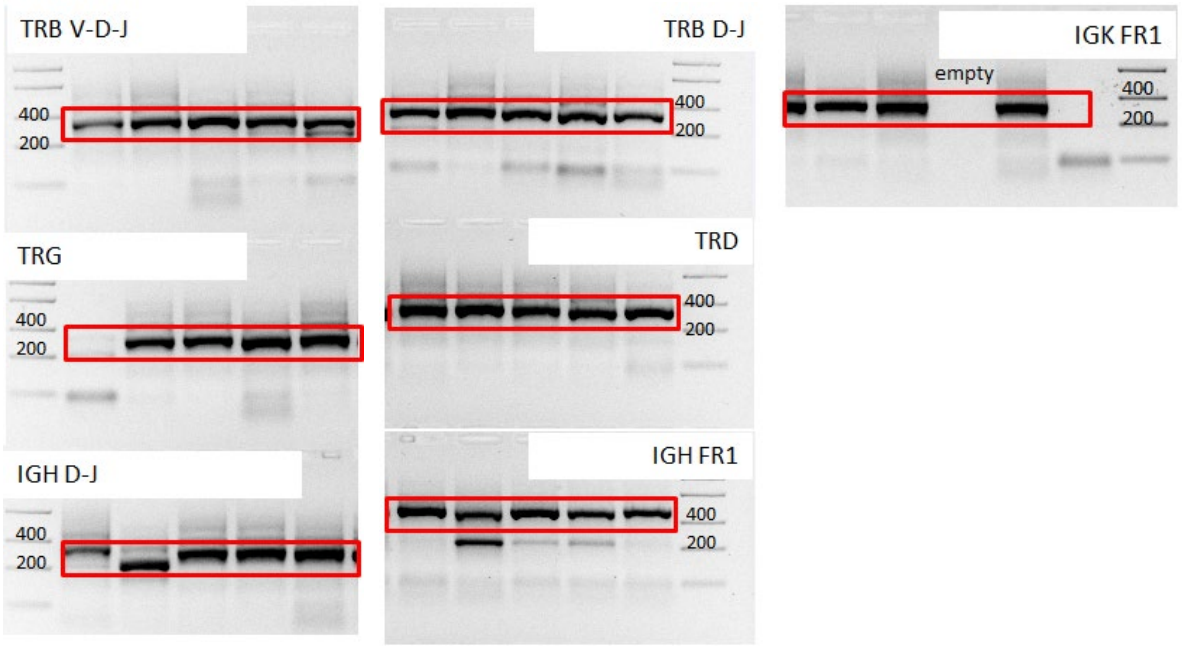
4.10. Quantify all 2nd round PCR products using either Qubit® dsDNA HS Assay Kit or QuantiT PicoGreen dsDNA Assay Kit, according to the manufacturer's instructions.

4.11. Create targetwise subpools by pooling all libraries covering the same target gene.

Note: This will result in 8 subpools, each containing libraries created by the same mix of 1st round PCR primers.

4.12. Perform the gel extraction of the 8 subpools with 21 µl of the PCR product mixed with 9 µl gel loading dye (see also the instructions in Section 3).

Figure 2. Example of 2nd step PCR products after gel electrophoresis.



4.13. Quantify all subpools using either Qubit® dsDNA HS Assay Kit, or QuantiT PicoGreen dsDNA Assay Kit, according to the manufacturer's instructions.

4.14. Pool the subpools in equimolar ratios into one final pool.

4.15. Perform sequencing on the MiSeq instrument employing the 2×250 MiSeq reagent Kit, according to the manufacturer's instructions.

5. Appendix

Table 7. 1st step primers.

1st step PCR	Primer nomenclature	µM in PCR	Primer direction	Primer sequence with M13 adapter (forward/reverse) 5' to 3'
TRB V-J	TRB-V-C-1	0.00625µM	5'	GTAAAACGACGGCCAGTTCGCTTCTCACCTGAATGCCC
	TRB-V-A-1	0.0125µM	5'	GTAAAACGACGGCCAGTCTCAGTTGAAAGGCCTGATGGA
	TRB-V-X-1	0.0125µM	5'	GTAAAACGACGGCCAGTGGAAGCATCCCTGATCGATTCT
	TRB-V-AA-1	0.0125µM	5'	GTAAAACGACGGCCAGTTCAGCTAAGTGCCTCCCAAATT
	TRB-V-B-1	0.025µM	5'	GTAAAACGACGGCCAGTAGTTCCAAATCGCTTCTCACCT
	TRB-V-F-1	0.025µM	5'	GTAAAACGACGGCCAGTTTCCCTAATCGATTCTCAGGGC
	TRB-V-J-1	0.025µM	5'	GTAAAACGACGGCCAGTTACAACTGCCAAAGGAGAGGTC
	TRB-V-L-1	0.025µM	5'	GTAAAACGACGGCCAGTTAAAGGAGAAGTCCCGAATGGC
	TRB-V-M-1	0.025µM	5'	GTAAAACGACGGCCAGTGGAGAAGTTCCCAATGGCTACA
	TRB-V-S-1	0.025µM	5'	GTAAAACGACGGCCAGTATAAAGGAGAAGTCCCCGATGG
	TRB-V-W-1	0.025µM	5'	GTAAAACGACGGCCAGTCTCTAGATGATTCGGGGATGCC
	TRB-V-Z-1	0.025µM	5'	GTAAAACGACGGCCAGTTGAAGCAGACACCCCTGATAAC
	TRB-V-AE-1	0.025µM	5'	GTAAAACGACGGCCAGTTGAGCGATTTTTAGCCCAATGC
	TRB-V-AG-1	0.025µM	5'	GTAAAACGACGGCCAGTACAAAGGAGAGATCTCTGATGGA
	TRB-J-A-1	0.025µM	3'	TAATACGACTCACTATAGGGCTACAACTGTGAGTCTGGTGCC
	TRB-J-B-1	0.025µM	3'	TAATACGACTCACTATAGGGCTACAACGGTTAACCTGGTCC
	TRB-J-C-1	0.025µM	3'	TAATACGACTCACTATAGGGTACAACAGTGAGCCAACTTCCC
	TRB-J-D-1	0.025µM	3'	TAATACGACTCACTATAGGGCAAGACAGAGAGCTGGGTTCC
	TRB-J-E-1	0.025µM	3'	TAATACGACTCACTATAGGGCTAGGATGGAGAGTCGAGTCCC
	TRB-J-F-1	0.025µM	3'	TAATACGACTCACTATAGGGCTGTCACAGTGAGCCTGGTC
	TRB-J-G-1	0.025µM	3'	TAATACGACTCACTATAGGGCCTTCTTACCTAGCACGGTGAG
	TRB-J-H-1	0.025µM	3'	TAATACGACTCACTATAGGGTTACCCAGTACGGTCAGCCTAG
	TRB-J-I-1	0.025µM	3'	TAATACGACTCACTATAGGGCTTACCGAGCACTGTCAGCC

	TRB-J-J-1	0.025µM	3'	TAATACGACTCACTATAGGGCTTACCCAGCACTGAGAGCC
	TRB-J-K-1	0.025µM	3'	TAATACGACTCACTATAGGGTCACCGAGCACCAGGAGCC
	TRB-J-N-1	0.025µM	3'	TAATACGACTCACTATAGGGGAATCTCACCTGTGACCGTGAG
	TRB-V-D-1	0.05µM	5'	GTAAAACGACGGCCAGTGGAAACTTCCCTGGTCGATTC
	TRB-V-N-1	0.05µM	5'	GTAAAACGACGGCCAGTCAACGATCGGTTCTTTGCAGTC
	TRB-V-O-1	0.05µM	5'	GTAAAACGACGGCCAGTTAAATCAGGGCTGCTCAGTGAT
	TRB-V-P-1	0.05µM	5'	GTAAAACGACGGCCAGTCAGTGATCGGTTCTCTGCAGAG
	TRB-V-R-1	0.05µM	5'	GTAAAACGACGGCCAGTCTTGAACGATTCTCCGCACAAC
	TRB-V-V-1	0.05µM	5'	GTAAAACGACGGCCAGTCCGAGGATCGATTCTCAGCTAA
	TRB-V-AB-1	0.05µM	5'	GTAAAACGACGGCCAGTGCCAAAGGAACGATTTTCTGCT
	TRB-V-AI-1	0.05µM	5'	GTAAAACGACGGCCAGTAGGGAGATGTTCCTGAAGGGTA
	TRB-V-AJ-1	0.05µM	5'	GTAAAACGACGGCCAGTCCTGAGGGGTACAGTGTCTCTA
	TRB-V-AL-1	0.05µM	5'	GTAAAACGACGGCCAGTCAGAATCTCTCAGCCTCCAGAC
	TRB-V-E-1	0.1µM	5'	GTAAAACGACGGCCAGTACTTCCCTGATCGATTCTCAGC
	TRB-V-H-1	0.1µM	5'	GTAAAACGACGGCCAGTCTCAGGTCACCAGTTCCCTAAC
	TRB-V-I-1	0.1µM	5'	GTAAAACGACGGCCAGTCCTAGATTTTCAGGTCGCCAGT
	TRB-V-Q-1	0.1µM	5'	GTAAAACGACGGCCAGTCTCAACTAGACAAATCGGGGCT
	TRB-V-U-1	0.1µM	5'	GTAAAACGACGGCCAGTATCGATTTTCTGCAGAGAGGCT
	TRB-V-Y-1	0.1µM	5'	GTAAAACGACGGCCAGTCGGTATGCCCAACAATCGATTC
	TRB-V-AC-1	0.1µM	5'	GTAAAACGACGGCCAGTCTGAAGGGTACAGCGTCTCTC
	TRB-V-AH-1	0.1µM	5'	GTAAAACGACGGCCAGTTCCTCTGAGTCAACAGTCTCCA
	TRB-V-AK-1	0.1µM	5'	GTAAAACGACGGCCAGTCTGAGGCCACATATGAGAGTGG
	TRB-J-L-1	0.1µM	3'	TAATACGACTCACTATAGGGGAAAACTCACCCAGCACGGTC
	TRB-J-M-1	0.1µM	3'	TAATACGACTCACTATAGGGTCACCCAGCACGGTCAGCC
	TRB-V-G-1	0.15µM	5'	GTAAAACGACGGCCAGTGATTCTCAGGTCTCCAGTTCCC
	TRB-V-K-1	0.15µM	5'	GTAAAACGACGGCCAGTTACCACTGGCAAAGGAGAAGTC
	TRB-V-T-1	0.15µM	5'	GTAAAACGACGGCCAGTCAAAGGAGAAGTCTCAGATGGC
	TRB-V-AD-1	0.15µM	5'	GTAAAACGACGGCCAGTTTTCTCATCAACCATGCAAGCC
	TRB-V-AF-1	0.15µM	5'	GTAAAACGACGGCCAGTGGAGATGCACAAGAAGCGATTC

TRB D-J	TRB-J-A-1	0.025µM	3'	TAATACGACTCACTATAGGGCTACAACTGTGAGTCTGGTGCC
	TRB-J-B-1	0.025µM	3'	TAATACGACTCACTATAGGGCTACAACGGTTAACCTGGTCC
	TRB-J-C-1	0.025µM	3'	TAATACGACTCACTATAGGGTACAACAGTGAGCCAACTTCCC
	TRB-J-D-1	0.025µM	3'	TAATACGACTCACTATAGGGCAAGACAGAGAGCTGGGTTCC
	TRB-J-E-1	0.025µM	3'	TAATACGACTCACTATAGGGCTAGGATGGAGAGTCGAGTCCC
	TRB-J-F-1	0.025µM	3'	TAATACGACTCACTATAGGGCTGTCACAGTGAGCCTGGTC
	TRB-J-G-1	0.025µM	3'	TAATACGACTCACTATAGGGCCTTCTTACCTAGCACGGTGAG
	TRB-J-H-1	0.025µM	3'	TAATACGACTCACTATAGGGTTACCCAGTACGGTCAGCCTAG
	TRB-J-I-1	0.025µM	3'	TAATACGACTCACTATAGGGCTTACCGAGCACTGTCAGCC
	TRB-J-J-1	0.025µM	3'	TAATACGACTCACTATAGGGCTTACCCAGCACTGAGAGCC
	TRB-J-K-1	0.025µM	3'	TAATACGACTCACTATAGGGTCACCGAGCACCAGGAGCC
	TRB-J-N-1	0.025µM	3'	TAATACGACTCACTATAGGGGAATCTCACCTGTGACCGTGAG
	TRB-D-A-1	0.1µM	5'	GTAAAACGACGGCCAGTCCTCCACTCCCCTCAAAGGA
	TRB-D-B-1	0.1µM	5'	GTAAAACGACGGCCAGTCAGACTAACCTCTGCCACCTG
	TRB-J-L-1	0.1µM	3'	TAATACGACTCACTATAGGGGAAAACTCACCCAGCACGGTC
	TRB-J-M-1	0.1µM	3'	TAATACGACTCACTATAGGGTCACCCAGCACGGTCAGCC
TRG	TRG-V-E-1	0.05µM	5'	GTAAAACGACGGCCAGTCAAGCATGAGGAGGAGCTGGAAATTG
	TRG-V-F-1	0.05µM	5'	GTAAAACGACGGCCAGTACGTCTACATCCACTCTCACC
	TRG-V-A-1	0.1µM	5'	GTAAAACGACGGCCAGTGCACAAGGAACAACTTGAGATTG
	TRG-V-B-1	0.1µM	5'	GTAAAACGACGGCCAGTTGGAAGCACAAGGAAGAACTTGAGAA
	TRG-V-C-1	0.1µM	5'	GTAAAACGACGGCCAGTGCACAGGGAAGAGCCTTAAATT
	TRG-V-D-1	0.1µM	5'	GTAAAACGACGGCCAGTCAGGAGGTGGAGCTGGATATT
	TRG-V-G-1	0.1µM	5'	GTAAAACGACGGCCAGTCTCTCACTTCAATCCTTACCATCAA
	TRG-V-H-1	0.2µM	5'	GTAAAACGACGGCCAGTGCTCACACTTCCACTTCCACTTTGAAAATAAAGT
	TRG-J-A-1	0.2µM	3'	TAATACGACTCACTATAGGGAGTGTTGTTCCACTGCCAAAG
	TRG-J-B-1	0.2µM	3'	TAATACGACTCACTATAGGGGTTCCGGGACCAAATACCTTG
	TRG-J-C-1	0.2µM	3'	TAATACGACTCACTATAGGGGAGCTTAGTCCCTTCAGCAAATA
	TRG-J-D-1	0.2µM	3'	TAATACGACTCACTATAGGGCCTAGTCCCTTTTGCAAACG
TRD	TRD-V-A-1	0.2µM	5'	GTAAAACGACGGCCAGTGAATGCAAAAAGTGGTCGCTATTC

	TRD-V-B-1	0.2µM	5'	GTAAAACGACGGCCAGTTGCAAAGAACCTGGCTGTACT
	TRD-V-C-1	0.2µM	5'	GTAAAACGACGGCCAGTTGCAGATTTTACTCAAGGACGG
	TRD-V-D-1	0.2µM	5'	GTAAAACGACGGCCAGTGCAAAATGCAACAGAAGGTCG
	TRD-V-E-1	0.2µM	5'	GTAAAACGACGGCCAGTGATAAAAATGAAGATGGAAGATTCACTGT
	TRD-V-F-1	0.2µM	5'	GTAAAACGACGGCCAGTCTCCTTCAATAAAAGTGCCAAGC
	TRD-V-G-1	0.2µM	5'	GTAAAACGACGGCCAGTATTGAAAAGAAGTCAGGAAGACTAAGT
	TRD-V-H-1	0.2µM	5'	GTAAAACGACGGCCAGTTCCAGAAAGCAGCCAAATCC
	TRD-D-A-1	0.2µM	5'	GTAAAACGACGGCCAGTAGGGGTATTGTGGATGGCAG
	TRD-J-A-1	0.2µM	3'	TAATACGACTCACTATAGGGTTCCACAGTCACACGGGT
	TRD-J-B-1	0.2µM	3'	TAATACGACTCACTATAGGGGGTTCCACGATGAGTTGTGTT
	TRD-J-C-1	0.2µM	3'	TAATACGACTCACTATAGGGCACGAAGAGTTTGATGCCAGT
	TRD-J-D-1	0.2µM	3'	TAATACGACTCACTATAGGGGTTGTTGTACCTCCAGATAGGTT
	TRD-J-E-1	0.2µM	3'	TAATACGACTCACTATAGGGTGGCTAGAAACACTTACTTGCA
	TRD-D-B-1	0.2µM	3'	TAATACGACTCACTATAGGGCCCAGGGAAATGGCACTTTTG
IGH D-J	IGH-D-A-1	0.2µM	5'	GTAAAACGACGGCCAGTGATTCYGAACAGCCCCGAGTCA
	IGH-D-B-1	0.2µM	5'	GTAAAACGACGGCCAGTGATTTTGTGGGGGYTCGTGTC
	IGH-D-C-1	0.2µM	5'	GTAAAACGACGGCCAGTGTTTGRRGTGAGGTCTGTGTCA
	IGH-D-D-1	0.2µM	5'	GTAAAACGACGGCCAGTGTTTRGRRTGAGGTCTGTGTCACT
	IGH-D-E-1	0.2µM	5'	GTAAAACGACGGCCAGTCTTTTTGTGAAGGSCCCTCCTR
	IGH-D-F-1	0.2µM	5'	GTAAAACGACGGCCAGTGTTATTGTCAGGSGRTGTCAGAC
	IGH-D-G-1	0.2µM	5'	GTAAAACGACGGCCAGTGTTATTGTCAGGGGGTGYCAGRC
	IGH-D-H-1	0.2µM	5'	GTAAAACGACGGCCAGTGTTTCTGAAGSTGTCTGTRTCAC
	IGH-J-A-1	0.4µM	3'	TAATACGACTCACTATAGGGCTTACCTGAGGAGACGGTGACC
IGH V-J	IGH-V-FR1-B-1	0.1µM	5'	GTAAAACGACGGCCAGTGCAGTCTGGAGCAGAGGTGAAAA
	IGH-V-FR1-E-1	0.1µM	5'	GTAAAACGACGGCCAGTGAGGTGCAGCTGTTGGAGTC
	IGH-V-FR1-G-1	0.1µM	5'	GTAAAACGACGGCCAGTCAGTGGGGCGCAGGACTGTT
	IGH-V-FR1-H-1	0.1µM	5'	GTAAAACGACGGCCAGTCCAGGACTGGTGAAGCCTCC
	IGH-V-FR1-K-1	0.1µM	5'	GTAAAACGACGGCCAGTCCTCAGTGAAGGTTTCCTGCAAGG
	IGH-V-FR1-L-1	0.1µM	5'	GTAAAACGACGGCCAGTAAACCCACAGAGACCCTCACGCTGAC

	IGH-V-FR1-M-1	0.1µM	5'	GTAAAACGACGGCCAGTCTGGGGGGTCCCTGAGACTCTCCTG
	IGH-V-FR1-N-1	0.1µM	5'	GTAAAACGACGGCCAGTCTTCACAGACCCTGTCCCTCACCTG
	IGH-V-FR1-O-1	0.1µM	5'	GTAAAACGACGGCCAGTTCGCAGACCCTCTCACTCACCTGTG
	IGH-J-A-1	0.1µM	3'	TAATACGACTCACTATAGGGCTTACCTGAGGAGACGGTGACC
	IGH-J-B-1	0.1µM	3'	TAATACGACTCACTATAGGGCTCACCTGAGGAGACGGTGACC
	IGH-V-FR1-A-1	0.2µM	5'	GTAAAACGACGGCCAGTCTGGGGCTGAGGTGAAGAAG
	IGH-V-FR1-C-1	0.2µM	5'	GTAAAACGACGGCCAGTTCACCTTGAAGGAGTCTGGTCC
	IGH-V-FR1-D-1	0.2µM	5'	GTAAAACGACGGCCAGTAGGTGCAGCTGGTGGAGTC
	IGH-V-FR1-F-1	0.2µM	5'	GTAAAACGACGGCCAGTCCAGGACTGGTGAAGCCTTC
	IGH-V-FR1-I-1	0.2µM	5'	GTAAAACGACGGCCAGTGTACAGCTGCAGCAGTCAGG
	IGH-V-FR1-J-1	0.2µM	5'	GTAAAACGACGGCCAGTGCTGGTGCAATCTGGGTCTG
IGK-A	IGK-V-A-1	0.1µM	5'	GTAAAACGACGGCCAGTAAGTGGGGTCCCATCAAGGTTCAG
	IGK-V-B-1	0.1µM	5'	GTAAAACGACGGCCAGTAGTCCCATCTCGGTTCAGTGGCAG
	IGK-V-C-1	0.1µM	5'	GTAAAACGACGGCCAGTGAAACAGGGGTCCCATCAAGGTTC
	IGK-V-D-1	0.1µM	5'	GTAAAACGACGGCCAGTTCCCAGACAGATTCAGTGGCAGTG
	IGK-V-E-1	0.1µM	5'	GTAAAACGACGGCCAGTCTGGAGTGCCAGATAGGTTCAGTG
	IGK-V-F-1	0.1µM	5'	GTAAAACGACGGCCAGTCCCTGGAGTCCCAGACAGGTTCAG
	IGK-V-G-1	0.1µM	5'	GTAAAACGACGGCCAGTGCATCCCAGCCAGGTTCAGTG
	IGK-V-H-1	0.1µM	5'	GTAAAACGACGGCCAGTGTCCCTGACCGATTCAGTGGCA
	IGK-V-I-1	0.1µM	5'	GTAAAACGACGGCCAGTAATCCCACCTCGATTCAGTGGC
	IGK-V-J-1	0.1µM	5'	GTAAAACGACGGCCAGTCTCAGGGGTCCCCTCGAGGTT
	IGK-V-K-1	0.1µM	5'	GTAAAACGACGGCCAGTAGACACTGGGGTCCCAGCCA
	IGK-DE-A-1	0.1µM	3'	TAATACGACTCACTATAGGGGCAGCTGCAGACTCATGAGGAG
	IGk-J-A-1	0.1µM	3'	TAATACGACTCACTATAGGGACGTTTGATCTCCACCTTGGTCCC
	IGK-J-B-1	0.1µM	3'	TAATACGACTCACTATAGGGACGTTTGATATCCACTTTGGTCCC
	IGK-J-C-1	0.1µM	3'	TAATACGACTCACTATAGGGACGTTTAATCTCCAGTCGTGTCCC
IGK-B	IGK-INTR-A-1	0.1µM	5'	GTAAAACGACGGCCAGTGAGTGGCTTTGGTGGCCATGC
	IGK-DE-A-1	0.1µM	3'	TAATACGACTCACTATAGGGGCAGCTGCAGACTCATGAGGAG

Table 8. 2nd step primers.

2nd step PCR	Primer nomenclature	µM in PCR	Primer direction	Barcoded primer sequence with M13 adapter (forward/reverse) 5' to 3'
forward	Ill-D501-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D502-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D503-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D504-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D505-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D506-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D507-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
	Ill-D508-F	0.2µM	5'	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAAAACGACGGCCAGT
reverse	Ill-D701-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D702-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D703-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D704-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D705-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D706-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D707-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D708-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D709-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D710-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D711-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG
	Ill-D712-R	0.2µM	3'	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAATACGACTCACTATAGGG