

EuroClonality Standard Operating Procedure (SOP) for **One-step IG/TR NGS-based MRD detection**

Version 1.0 (July 2024)

This SOP is based on the following publication of the EuroClonality-NGS Working Group, with some modifications to optimize the library preparation for MRD quantitation.

Brüggemann et al., Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study. Leukemia 2019;33:2241-2253. doi: 10.1038/s41375-019-0496-7.

For complementary information or questions, please go to www.EuroClonality.org
or contact Eva Froňková (eva.fronkova@lfmotol.cuni.cz)

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Attachments:

- Primers for one-step IG/TR NGS-based MRD detection
- Index sequences

1. Introduction

This SOP provides detailed instructions how to prepare amplicon sequencing libraries for **MRD detection using EuroClonality-NGS IGH-VJ-FR1, IGH-DJ, IGK-VJ, intron-Kde, TRB-VJ, TRB-DJ, TRG and TRD primer sets**, on the Illumina MiSeq platform. This SOP is also suitable for **marker screening with only 100 ng of DNA** input used.

2. Equipment and reagents

2.1 Equipment required:

- Thermocycler
- 1 full set of pipettes comprising: P10 μ l, P20 μ l, P100 μ l, P200 μ l and P1000 μ l
- HeatBlock
- Gel electrophoresis chamber
- UV lamp
- Vortex
- Microfuge
- Light Cycler LC480/Qubit Fluorometer
- Laminar flow hood
- Illumina MiSeq sequencer

2.2 Reagents & Consumables:

- Product purification kit (Agencourt SPRISelect, Agencourt AMPure XP, MinElute Gel)
- Fast Start HiFi Polymerase/EagleTaq DNA Polymerase/AmpliTaq Gold DNA Polymerase
- 10x PCR buffer II, dNTPs (10mM), MgCl₂ (25mM)
- ddH₂O
- EuroClonality-NGS Primers (Brüggemann et al., 2019)
- PCR tubes
- Pipette tips
- Gel Red
- Gel loading dye

- Scalpel, Agarose, TBE (or TAE) Buffer (for Gel Purification)
- 10M NaOH
- 1,5 ml Low Binding Tubes
- Ice block
- Qubit® dsDNA HS Assay Kit/QuantiT PicoGreen dsDNA Assay Kit
- Illumina PhiX spike in control v3 (Illumina: FC1103001)
- MiSeq Reagent Kit v2, 2×250bp (Illumina, order no. MS1022003)
- Tween 20 (SigmaAldrich: P13791L)

3. Primer mix preparation

Suggested primer design follows this scheme, so that each system shares a unique barcode in the Forward primer and the Reverse primers can be used to barcode individual samples. This can be used for Marker screening using all 8 primer sets per patient or with selected systems for NGS MRD quantitation in follow-up samples.

		<i>Reverse primers</i>												
		D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712	
<i>Forward primers</i>	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	Patient 11	Patient 12	

The concentration of individual primers in the final PCR reaction mix is specified in the Primer e c ed s S nd c cu ed s e c e se e s ec n e added to the final reaction mix.

4. PCR Library preparation

4.1 Prepare your samples: DNA samples should have a concentration of at least 100 ng/μl. (Adjust the final volume of H₂O and DNA, so that **2 μg** of DNA is used in each PCR; this protocol was tested with DNA volume of up to **20 μl**, please be aware that increasing the amount of DNA can inhibit PCR).

4.2 Prepare the master mixes: Prepare the individual master mix for each target-specific PCR (see table below).

Primer mixes should be prepared with respective concentrations from the Primer Table.

	Stock concentration	IGH-VJ-FR1		IGH-DJ		IGK-VJ-Kde		Intron-Kde	
		Final concentration	μl/sample	Final concentration	μl/sample	Final concentration	μl/sample	Final concentration	μl/sample
PCR Buffer II	10x	1x	10 μl	1x	10 μl	1x	10 μl	1x	10 μl
MgCl ₂	25 mM	1.5 mM	6 μl	3 mM	12 μl	1.5 mM	6 μl	1.5 mM	6 μl
dNTP-MIX	10mM	0.2 mM	2 μl	0.2 mM	2 μl	0.2 mM	2 μl	0.2 mM	2 μl
Forward Primer MIX			4 μl		4 μl		4 μl		4 μl
H ₂ O			51.5 μl		45.5 μl		51.5 μl		51.5 μl
Roche HF/AmpliTaQ Gold	5U/μl	2.5U/rxn	0.5 μl	2.5U/rxn	0.5 μl	2.5U/rxn	0.5 μl	2.5/rxn	0.5 μl
Total			74.0 μl		74.0 μl		74.0 μl		74.0 μl

	Stock concentration	TRB-VJ		TRB-DJ		TRD		TRG	
		Final concentration	μl/sample	Final concentration	μl/sample	Final concentration	μl/sample	Final concentration	μl/sample
PCR Buffer II	10x	1x	10 μl	1x	10 μl	1x	10 μl	1x	10 μl
MgCl ₂	25 mM	4 mM	16 μl	4 mM	16 μl	2 mM	8 μl	4 mM	16 μl
dNTP-MIX	10mM	0.2 mM	2 μl	0.2 mM	2 μl	0.2 mM	2 μl	0.2 mM	2 μl
Forward Primer MIX			4 μl		4 μl		4 μl		4 μl
H ₂ O			41.5 μl		41.5 μl		49.5 μl		41.5 μl
Roche HF/AmpliTaQ Gold	5U/μl	2.5U/rxn	0.5 μl	2.5U/rxn	0.5 μl	2.5U/rxn	0.5 μl	2.5U/rxn	0.5 μl
Total			74.0 μl		74.0 μl		74.0 μl		74.0 μl

- 4.3 Pipette the master mix into PCR tubes.
- 4.4 Into all tubes add:
4 µl of reverse primer mix.
2 µl of cIT-QC (spike-ins)
up to 20 µl of DNA sample (2 µg).
- 4.5 Final volume of each PCR reaction is 100 µl.
- 4.6 Place the strips into a thermocycler and select the correct program to run.

PCR conditions are NOT the same for all systems (see below).

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	10 min
		12°C	

* For the **TRB-VJ** system, an annealing temperature of **65°C** should be used.

Once the run has finished, either store the products at 4°C, or continue with **gel electrophoresis** to check the successful PCR amplification of all tubes. If you identify positive bands, move forward to the next steps, otherwise repeat.

Expected size range of the specific library for each system:

System	Size (bp)
IGH-VJ-FR1	440 - 640
IGH-DJ	220 - 320
IGK-VJ-Kde	250 - 350
intron-Kde	270 - 340
TRB-VJ	270 - 370
TRB-DJ	260 - 370
TRG	210 - 320
TRD	270 - 410

4.7 Perform the purification of the libraries:

You can use a commercial kit of your preference (Agencourt SPRISelect, Agencourt AMPure XP beads, MinElute Gel extraction kit). We recommend the Agencourt SPRISelect, because it allows precise selection of fragment lengths validated for each lot. Recommended ratios of PCR product and SPRISelect beads are:

- **1:1** for **IGK-VJ-Kde, intron-Kde, TRG** and **TRD** systems (30 µl PCR sample + 30 µl beads);

- **1:0.9** for **IGH-DJ** and **TRB-DJ** (30 µl PCR sample + 27 µl beads);
 - **1:0.7** for **IGH-VJ-FR1** and **TRB-VJ** (30 µl PCR sample + 21 µl beads).
- 4.8 **Quantify all purified products** using either Qubit® dsDNA HS Assay Kit, or QuantiT PicoGreen dsDNA Assay Kit, according to the manufacturer's instructions.
- 4.9 **Pool the products** in equimolar ratios into **1 final pool with the concentration of 4 nM** based on the median size of the libraries and concentrations.

5. Sequencing on MiSeq

- 5.1 **Perform the sequencing run** according to the MiSeq System Guide and Library preparation instructions. Use **2 x 250 bp v2 chemistry** and add **10-15 % PhiX** control library to avoid low-complexity library issues. We recommend a final concentration of **7 pM** for the amplicon library (this can be adjusted if you get optimal results with a different concentration on your MiSeq instrument, but please be cautious to avoid overclustering).
- 5.2 **For clarity, consistency, and simplified analysis:**
- a. Use **Sample name (CODE)**, and the relevant EuroClonality-NGS **primer set names** (IGH-VJ-FR1, IGH-DJ, IGK-VJ-Kde, intron-Kde, TRB-VJ, TRB-DJ, TRD, TRG) for sample names of the respective tubes, bookended by underscores:
e.g. 1234-FU1_IGH-DJ or 3456-FU2_TRD
 - b. If using a polyclonal run control, add **"runQC"** to the **polyclonal** sample name, since these will serve as run quality controls and will not be analysed for spike-ins:
e.g. 220130_runQC_IGH-VJ-FR1

6. Data analysis

We recommend to use **ARResT/Interrogate** (<http://arrest.tools/interrogate-latest>) to analyze your NGS data. Please follow the instructions on the website.

Index sequences

D501 TATAGCCT
D502 ATAGAGGC
D503 CCTATCCT
D504 GGCTCTGA
D505 AGGCGAAG
D506 TAATCTTA
D507 CAGGACGT
D508 GTACTGAC
D701 CGAGTAAT
D702 TCTCCGGA
D703 AATGAGCG
D704 GGAATCTC
D705 TTCTGAAT
D706 ACGAATTC
D707 AGCTTCAG
D708 GCGCATT
D709 CATAGCCG
D710 TTCGCGGA
D711 GCGCGAGA
D712 CTATCGCT

A501 TGAACCTT
A502 TGCTAAGT
A503 TGTTCTCT
A504 TAAGACAC
A505 CTAATCGA
A506 CTAGAACA
A507 TAAGTTCC
A508 TAGACCTA
A701 ATCACGAC
A702 ACAGTGGT
A703 CAGATCCA
A704 ACAAACGG
A705 ACCCAGCA
A706 AACCCCTC
A707 CCCAACCT
A708 CACCACAC
A709 GAAACCCA
A710 TGTGACCA
A711 AGGGTCAA
A712 AGGAGTGG