EuroClonality-NGS Working group

Standard Operating Procedure (SOP) for

NGS-based clonality assessment of immunoglobulin gene rearrangements for the Ion Torrent 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 **NGS-based clonality assessment of immunoglobulin gene rearrangements for the Ion Torrent platform**

Content

1. Working definitions, material references and general remarks

- Besides the patient samples, it is highly recommended to include a polyclonal tissue specimen (e.g. tonsil DNA) to check the performance of the multiplex PCR.
- 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
- Vortex
- Microfuge
- Plate centrifuge
- DynaMag-96 Side Magnet (ThermoFisher Scientific, Cat No: 12331D) or Chemagic Magnetic Stand 96 (PerkinElmer, Cat no: CMG-301)
- Light Cycler LC480, Qubit Fluorometer or any equivalent equipment
- Laminar flow hood
- Ion Torrent template preparation (Ion One Touch system, Ion Chef system) and sequencing equipment (Ion PGM, Ion GeneStudio S5)

Reagents & Consumables:

- AmpliTaq Gold DNA Polymerase, kit with GeneAmp 10x Gold Buffer and $MgCl_2$ (Applied Biosystems, Cat No: 4311818)
- dNTPs (GE Healthcare, Cat No: 28-4065-51)
- Milli-Q (MQ)
- Nuclease-free water
- 70% analytical ethanol; freshly made
- Low TE ($T_{10}E_{0.1}$)
- PCR plate caps
- Pipette tips
- 0.2 ml PCR tubes/strips
- 1.5 ml Low Binding Tubes
- Qubit dsDNA High Sensitivity Assay Kit (Thermofisher Scientific, Cat no:

Q32854)

- Qubit Assay tubes (Thermofisher Scientific, Cat no: 32856)
- Ion Plus Fragment Library kit (Life Technologies, Cat No: 4471252)
- Ion Xpress Barcode Adapters 1-16 and/or 17-32 kit (Life Technologies, Cat No: 4471250/4474009)
- Deepwell plate 96/500 µl, white border (Eppendorf, Cat No: 951032000)
- Agencourt AMPure XP Beads (Beckham Coulter, Cat No: A63882)
- DNA LoBind plates
- Ion 318 Chip Kit; Ion 520 Chip Kit (Larger-sized Chip Kits can be used for > 24-32 samples)
- Primers (see Appendix)

2. Reaction 1: Amplification of IGH-FR3, IGHD and IGK

2.1. Prepare three different 0.2 ml PCR tubes per sample: IGH-FR3, IGHD, and IGK.

2.2. Add 40 ng DNA (Qubit measured; preferable DNA quality with fragment length >200 bp) and the other components of the reaction, according to Table 1. Adjust the total PCR volume to 25 µl with MQ.

2.3. Set a 100 µl pipette to 20 µl and pipette the entire volume up and down at least 10 times to mix thoroughly (setting the pipette to a lower volume avoids creating bubbles during pipette mixing). Perform a quick spin to collect all liquid from the sides of the tube.

Table 1. Composition of PCR reaction 1. Primer sequences and final concentrations are given in Tables 8-10.

Component	IGH-FR3	IGHD	IGK
Input DNA	40 ng	40 ng	40 ng
GeneAmp 10x Gold Buffer	2.5 µl	2.5 µl	2.5 µl
Primers	**0.2/0.4 µM**	**0.2 µM**	**0.2 µM**
dNTP (2 mM)	2.5 µl	2.5 µl	2.5 µl
$MgCl_2$	**1.5 mM**	**2.0 mM**	**1.5 mM**
AmpliTaq Gold DNA Polymerase	0.5 U	0.5 U	0.5 U
MQ	Adjust to 25 µl	Adjust to 25 µl	Adjust to 25 µl

2.4. In a thermocycler, with the heated lid set to 105°C, run the PCR according to the program in Table 2.

Table 2. PCR reaction 1 program.

1 cycle	Initial denaturation	94°C	10 min
30 cycles	**Denaturation**	**92°C**	**30 sec**
	Annealing	**60°C**	**40 sec**
	Extension	**72°C**	**40 sec**
1 cycle	Final extension	72°C	30 min
		20°C	10 min
		12°C	∞

2.5. After completing the PCRs, combine tube IGH-FR3, IGHD, and IGK per sample (~75 µl total volume).

3. Clean-up of IGH-FR3, IGHD and IGK amplicons

3.1. Allow the AMPure XP magnetic beads to warm to room temperature for at least 30 minutes before use. Ensure that the beads are homogeneous prior to use by mixing the tube by hand for 20 seconds.
3.2. Pipet the pooled samples in a DNA LoBind plate and add 1.8 times (135 µl) volume Agencourt AMPure XP magnetic beads per sample.
3.3. Set a 200 µl pipette to 200 µl and then pipette the entire volume up and down at least 5 times until the beads are homogeneously mixed.
3.4. Incubate for 5 minutes at room temperature.
3.5. Place samples for 2-5 minutes in a magnetic stand until the solution is clear.
3.6. Carefully remove supernatant using a 200 µl pipette.
3.7. Add 150 µl freshly made 70% ethanol per sample.
3.8. Move plate in the magnetic stand approximately 4 times from left to right, make sure the bead pellet migrates and is washed clean.
3.9. Carefully remove supernatant using a 200 µl pipette.
3.10. Repeat steps 3.7 to 3.9 once.
3.11. Carefully remove any remaining supernatant using a 10 µl pipette.
3.12. Air-dry beads for 5 minutes to allow complete evaporation of residual ethanol, but avoid the beads from overdrying (overdrying the beads may result in lower recovery of DNA).
3.13. Resuspend samples in 25 µl Low TE.
3.14. Set a 100 µl pipette to 20 µl and pipette the entire volume up and down at least 5 times to generate a homogeneously mixed brown solution and leave for 2 minutes at room temperature.
3.15. Place samples for 2 minutes in the magnetic stand until the solution is clear.
3.16. Collect the purified DNA by pipetting the solution (~25 µl) into a new PCR strip.

Note: After this step the product can be stored at -20°C.

4. Reaction 2: End repair of amplicons

4.1. Measure the DNA concentration of every individual sample using Fluorimetric Quantitation (using 2 µl of the sample for the Qubit high sensitivity assay).

4.2. Transfer max 40 ng DNA to a 0.2 ml PCR tube for the end repair step. In case the total yield is less than 40 ng, use as much as possible. Adjust the volume to 39.5 µl with Low TE.

4.3. Add the reagents for the end repair reaction from the Ion Plus Fragment Library kit to the amplicons according to Table 3.

4.4. Set a 100 µl pipette to 45 µl and pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: This protocol uses half the amount of reagents provided with the Ion Xpress kit per sample.

Table 3. Composition of the end repair reaction.

Component	Volume (µl)
Pooled amplicons (40 ng) adjusted to 39.5 µl with Low TE	39.5
5x End Repair Buffer	10
End Repair Enzyme	0.5
Total volume	*50*

4.5. Incubate 30 minutes at room temperature.

5. Clean-up of end repaired amplicons

5.1. Allow the AMPure XP magnetic beads to warm to room temperature for at least 30 minutes before use. Ensure that the beads are homogeneous prior to use by mixing the tube by hand for 20 seconds.

5.2. Pipet the end repaired samples in a DNA LoBind plate and add 1.8 times (90 µl) volume Agencourt AMPure XP magnetic beads per sample.

5.3. Set a 200 µl pipette to 130 µl and then pipette the entire volume up and down at least 5 times until the beads are homogeneously mixed.

5.4. Incubate for 5 minutes at room temperature.

5.5. Place samples in a magnetic stand for 2-5 minutes, or until the solution is clear.
5.6. Carefully remove supernatant using a 200 µl pipette.
5.7. Add 150 µl freshly made 70% ethanol per sample.
5.8. Move plate in the magnetic stand approximately 4 times from left to right, make sure the bead pellet migrates and is washed clean.
5.9. Carefully remove supernatant using a 200 µl pipette.
5.10. Repeat steps 5.7 to 5.9 once.
5.11. Carefully remove any residual supernatant using a 10 µl pipette.
5.12. Air-dry beads for 5 minutes to allow complete evaporation of residual ethanol, but avoid the beads from overdrying.
5.13. Resuspend samples in 25 µl Low TE.
5.14. Set a 100 µl pipette to 20 µl and pipette the entire volume up and down at least 5 times to generate a homogeneously mixed brown solution and leave for 2 minutes at room temperature.
5.15. Place samples for 2 minutes in the magnetic stand until the solution is clear.
5.16. Collect the purified DNA by pipetting the supernatant (~25 µl) into a new PCR strip.

Note: After this step the product can be stored at -20°C.

6. Reaction 3: Adapter ligation

6.1. To ligate adapters to the amplicon and to perform nick repair, for each sample, add the amplicons and reagents from the Ion Plus Fragment Library kit and Ion Xpress Barcode Adapter kit to a 0.2 ml PCR tube according to Table 4. Check that for each sample a different adapter is used.

Note: Barcoded libraries (For 24-32 samples, 24-32 different library barcodes are required).

Table 4. Composition of the adapter ligation/nick repair reaction.

Component	Volume (µl)
Pooled amplicons (40 ng)	24.5
10x Ligase Buffer	5
Ion P1 Adapter from the Barcode Kit (not Library kit)	1
dNTP Mix	1
Nuclease-free water	12.5
DNA Ligase	1
Nick Repair Polymerase	4
Ion Xpress Barcode X	1
Total volume	*50*

6.2. Run the adapter ligation program according to Table 5.

Table 5. Adapter ligation program.

1 cycle	25°C	15 min
1 cycle	72°C	5 min
1 cycle	4°C	10 min
	4°C	∞

7. Clean-up of adapter-ligated amplicons

7.1. Allow the AMPure XP magnetic beads to warm to room temperature for at least 30 minutes before use. Ensure that the beads are homogeneous prior to use by mixing the tube by hand for 20 seconds.

7.2. Pipet the adapter-ligated samples in a DNA LoBind plate and add 1.8 times (90 µl) volume Agencourt AMPure XP magnetic beads per sample.

7.3. Set a 200 µl pipette to 130 µl and then pipette the entire volume up and down at least 5 times until the beads are homogeneously mixed.

7.4. Incubate 5 minutes at room temperature.

7.5. Place samples 2-5 minutes in a magnetic stand until the solution is clear.

7.6. Carefully remove supernatant using a 200 µl pipette.

7.7. Add 150 µl freshly made 70% ethanol per sample.

7.8. Move plate in the magnetic stand approximately 4 times from left to right, make sure the bead pellet migrates and is washed clean.

7.9. Carefully remove supernatant using a 200 µl pipette.
7.10. Repeat steps 7.7 to 7.9 once.
7.11. Carefully remove any residual supernatant using a 10 µl pipette.
7.12. Air-dry beads for 5 minutes to allow complete evaporation of residual ethanol, but avoid the beads from overdrying.
7.13. Resuspend samples in 13 µl Low TE.
7.14. Set a 100 µl pipette to 20 µl and pipette the entire volume up and down at least 5 times to generate a homogeneously mixed brown solution and leave for 2 minutes at room temperature.
7.15. Place samples for 2 minutes in the magnetic stand until the solution is clear.
7.16. Collect the purified DNA by pipetting the 12.5 µl supernatant into a new PCR strip to ensure that the pellet is not disturbed.

Note: After this step the product can be stored at -20°C.

8. Reaction 4: Library amplification

8.1. To amplify the libraries, for each sample, add the purified adapter-ligated amplicons and reagents from the Ion Plus Fragment Library kit to a 0.2 ml PCR tube according to Table 6.
8.2. Set a 100 µl pipette to 55 µl and pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Table 6. Composition of the library amplification reaction.

Component	Volume (µl)
Pooled amplicons (unamplified library)	12.5
Platinum PCR SuperMix High Fidelity	50
Library Amplification Primer Mix	2.5
Total volume	*65*

8.3. In a thermocycler, with the heated lid set to 105°C, run the PCR according to the program in Table 7.

Table 7. Library amplification PCR program.

1 cycle	Initial denaturation	95°C	5 min
8 cycles	**Denaturation**	**95°C**	**15 sec**
	Annealing	**58°C**	**15 sec**
	Extension	**70°C**	**1 min**
1 cycle	Hold	4°C	∞

9. Clean-up of amplified libraries

9.1. Allow the AMPure XP magnetic beads to warm to room temperature for at least 30 minutes before use. Ensure that the beads are homogeneous prior to use by mixing the tube by hand for 20 seconds.

9.2. Pipet the amplified samples in a DNA LoBind plate and add 1.4 times (90 µl) volume Agencourt AMPure XP magnetic beads per sample.

9.3. Set a 200 µl pipette to 140 µl and pipette the entire volume up and down at least 5 times until the beads are homogeneously mixed.

9.4. Incubate for 5 minutes at room temperature.

9.5. Place samples in a magnetic stand for 2-5 minutes until the solution is clear.

9.6. Carefully remove supernatant using a 200 µl pipette.

9.7. Add 150 µl freshly made 70% ethanol per sample.

9.8. Move plate in the magnetic stand approximately 4 times from left to right, make sure the bead pellet migrates and is washed clean.

9.9. Carefully remove supernatant using a 200 µl pipette.

9.10. Repeat steps 9.7 to 9.9 once.

9.11. Carefully remove any residual supernatant using a 10 µl pipette.

9.12. Air-dry beads for 5 minutes to allow complete evaporation of residual ethanol, but avoid the beads from overdrying.

9.13. Resuspend samples in 25 µl Low TE.

9.14. Set a 100 µl pipette to 20 µl and pipette the entire volume up and down at least 5 times to generate a homogeneously mixed brown solution and leave for 2 minutes at room temperature.

9.15. Place samples for 2 minutes in the magnetic stand until the solution is clear.

9.16. Collect the purified DNA by pipetting the supernatant (~25 µl) into a new PCR strip.

Note: After this step the product can be stored at -20°C.

10. Ion Torrent sequencing run

10.1. Measure the DNA concentration of all samples using Fluorimetric Quantitation.

10.2. Pool all samples at an equivalent DNA amount and measure the total pool DNA concentration with Fluorimetric quantitation.

10.3. Dilute each pooled sample to a final DNA concentration of 12 ng/ml with Low TE. Alternatively, library quantification can be performed with the Ion Library TaqMan Quantification Kit (220-250 pM final concentration).

10.4. Run Ion Torrent on 318- or 5S Chip for a total of 24-32 samples.

Note: Follow protocol according to your local Sequence Facility

- *Ion PGM Template OT2 200 Kit*
- *Ion 510™ & Ion 520™ & Ion 530™ Kit Chef*
- *Ion Chef (or Ion OneTouch 2 System)*

11. Appendix

Table 8. Primers included in the multiplex PCR reaction for NGS-based clonality assessment: Tube IGHV (IGH-FR3).

Primer nomenclature	Final concentration	Primer direction	Primer Sequence
IGH-V-FR3-A-1	0.4 µM	5’	AAGTTCCAGGGCAGAGTCAC
IGH-V-FR3-B-1	0.4 µM	5’	GTCCATCAGCACAGCCTACA
IGH-V-FR3-C-1	0.4 µM	5’	GACATGTCCACAAGCACAGC
IGH-V-FR3-D-1	0.2 µM	5’	TCTCCAAGGACACCTCCAAGA
IGH-V-FR3-E-1	0.2 µM	5’	CAGGCTCACCATCTCCAAGG
IGH-V-FR3-F-1	0.2 µM	5’	CCATCTCTGAAGAGCAGGCT
IGH-V-FR3-G-1	0.4 µM	5’	TGAAGGGCCGATTCACCATC
IGH-V-FR3-H-1	0.4 µM	5’	AGGCAGATTCACCATCTCAAGA
IGH-V-FR3-I-1	0.4 µM	5’	AGCGCCGATTCATCATCTCC
IGH-V-FR3-J-1	0.2 µM	5’	CCAAAAGCATCACCTATCTGCA
IGH-V-FR3-K-1	0.2 µM	5’	GAAGGGCCGGTTCACCATC
IGH-V-FR3-L-1	0.2 µM	5’	ACCTCCAGAGATAACGCCAAG
IGH-V-FR3-M-1	0.2 µM	5’	CAGGAAGGGCAGATTCACCA
IGH-V-FR3-N-1	0.2 µM	5’	GAAGGGCCGATTGACCATCTC
IGH-V-FR3-O-1	0.4 µM	5’	CTCCGTGAAGGGCAGATTCA
IGH-V-FR3-P-1	0.2 µM	5’	GATGATTCAAAGAACACGGCGT
IGH-V-FR3-Q-1	0.4 µM	5’	CCGTCCCTCAAGAGTCGAGT
IGH-V-FR3-R-1	0.4 µM	5’	CCGTCCCTCAAGAGTCGAAT
IGH-V-FR3-S-1	0.2 µM	5’	GTCACCATCTCAGCCGACAA
IGH-V-FR3-T-1	0.2 µM	5’	CAAGTCCATCAGCACTGCCT
IGH-V-FR3-U-1	0.4 µM	5’	CAGTTCTCCCTGCAGCTGAA
IGH-V-FR3-V-1	0.4 µM	5’	GGCTTCACAGGACGGTTTGT
IGH-J-A-1	0.2 µM	3’	CTTACCTGAGGAGACGGTGACC
IGH-J-B-1	0.2 µM	3’	CTCACCTGAGGAGACAGTGACC
IGH-J-C-1	0.2 µM	3’	CTCACCTGAGGAGACGGTGACC

Table 9. Primers included in the multiplex PCR reaction for NGS-based clonality assessment: Tube IGHD.

Primer nomenclature	Final concentration	Primer direction	Primer Sequence
IGH-D-A-1	0.2 µM	5'	GATTCYGAACAGCCCCGAGTCA
IGH-D-B-1	0.2 µM	5'	GATTTTGTGGGGGYTCGTGTC
IGH-D-C-1	0.2 µM	5'	GTTTGRRGTGAGGTCTGTGTCA
IGH-D-D-1	0.2 µM	5'	GTTTRGRRTGAGGTCTGTGTCACT
IGH-D-E-1	0.2 µM	5'	CTTTTTGTGAAGGSCCCTCCTR
IGH-D-F-1	0.2 µM	5'	GTTATTGTCAGGSGRTGTCAGAC
IGH-D-G-1	0.2 µM	5'	GTTATTGTCAGGGGGTGYCAGRC
IGH-D-H-1	0.2 µM	5'	GTTTCTGAAGSTGTCTGTRTCAC
IGH-J-A-1	0.2 µM	3'	CTTACCTGAGGAGACGGTGACC
IGH-J-B-1	0.2 µM	3'	CTCACCTGAGGAGACAGTGACC
IGH-J-C-1	0.2 µM	3'	CTCACCTGAGGAGACGGTGACC

Table 10. Primers included in the multiplex PCR reaction for NGS-based clonality assessment: Tube IGK.

Primer nomenclature	Final concentration	Primer direction	Primer Sequence
IGK-V-A-1	0.2 µM	5'	AAGTGGGGTCCCATCAAGGTTCAG
IGK-V-B-1	0.2 µM	5'	AGTCCCATCTCGGTTCAGTGGCAG
IGK-V-C-1	0.2 µM	5'	GAAACAGGGGTCCCATCAAGGTTC
IGK-V-D-1	0.2 µM	5'	TCCCAGACAGATTCAGTGGCAGTG
IGK-V-E-1	0.2 µM	5'	CTGGAGTGCCAGATAGGTTCAGTG
IGK-V-F-1	0.2 µM	5'	CCCTGGAGTCCCAGACAGGTTCAG
IGK-V-G-1	0.2 µM	5'	GCATCCCAGCCAGGTTCAGTG
IGK-V-H-1	0.2 µM	5'	GTCCCTGACCGATTCAGTGGCA
IGK-V-I-1	0.2 µM	5'	AATCCCACCTCGATTCAGTGGC
IGK-V-J-1	0.2 µM	5'	CTCAGGGGTCCCCTCGAGGTT
IGK-V-K-1	0.2 µM	5'	AGACACTGGGGTCCCAGCCA
IGK-INTR-A-1	0.2 µM	5'	GAGTGGCTTTGGTGGCCATGC
IGK-DE-A-1	0.2 µM	3'	GCAGCTGCAGACTCATGAGGAG
IGK-J-A-1	0.2 µM	3'	ACGTTTGATCTCCACCTTGGTCCC
IGK-J-B-1	0.2 µM	3'	ACGTTTGATATCCACTTTGGTCCC
IGK-J-C-1	0.2 µM	3'	ACGTTTAATCTCCAGTCGTGTCCC