EuroClonality Standard Operating Procedure (SOP) for the

EuroClonality-NGS DNA Capture strategy for comprehensive immunogenetics

Version 1.0 (September 2024)

For complementary information or questions, please contact
Dr. Peter Stewart: p.stewart@qub.ac.uk
or go to www.EuroClonality.org

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1. Summary

In the era of genomic medicine, targeted next-generation sequencing (NGS) strategies are becoming increasingly adopted by clinical molecular diagnostic laboratories to identify genetic diagnostic and prognostic biomarkers in hemato-oncology. This SOP describes how to use the EuroClonality-NGS DNA Capture (EuroClonality-NDC) assay, which is designed to simultaneously detect B and T cell clonal rearrangements, translocations, copy number alterations, and sequence variants associated with lymphoproliferative disorders (Stewart et al., Blood Adv. 2021;5:3188-98). The accompanying validated bioinformatics pipeline enables production of an integrated report. The combination of the EuroClonality-NDC laboratory protocol and bioinformatics pipeline minimizes the potential for human error, reduces economic costs compared to current molecular testing strategies, and should improve diagnostic outcomes. The commercial assay is available through Univ8 Genomics (https://univ8genomics.com) and users of the EuroClonality-NDC assay should refer to the website up to date information on the EuroClonality-NDC assay.

2. Materials

2.1 DNA Quantification

The following products and equipment from Thermo Fisher Scientific (Waltham, MA, USA) are required:

- Qubit dsDNA broad-range (BR) assay.
- Qubit dsDNA high-sensitivity (HS) assay.
- Qubit Assay Tubes.
- Qubit Fluorometer.

2.2 DNA Integrity Assessment

The following products and equipment from Agilent Technologies (Santa Clara, CA, USA) are required:

- Genomic DNA Reagents.
- Genomic DNA ScreenTape.
- D1000 Reagents.
- D1000 ScreenTape.
- High Sensitivity D1000 Reagents.
- High Sensitivity D1000 ScreenTape.
- 4150/4200 TapeStation System.

2.3 DNA library preparation

The following products from Roche Sequencing Solutions (Pleasanton, CA, USA) are required:

- KAPA HyperPlus Kit.
- KAPA UDI Primer Mixes.
- KAPA Universal Adapter.
- KAPA HyperPure Beads.

The following items will be required for the multiple bead clean up steps that are performed in both a pre- and post-PCR environment:

- 96 well magnetic plate for pre-PCR.
- Magnetic stands for 0.2 mL PCR strips for both pre- and post-PCR.
- Magnetic stands for 1.5 mL microfuge tubes for both pre- and post-PCR.

2.4 DNA hybridization

The following products from Roche Sequencing Solutions (Pleasanton, CA, USA) are required:

- KAPA HyperCapture Reagent Kit
- KAPA HyperCapture Bead

The following product from Univ8 Genomics Ltd (Belfast, UK) is required:

- EuroClonality-NDC

2.5 Sequencing of enriched DNA library

The following products from Illumina, Inc (San Diego, CA, USA) are required:

- PhiX Sequencing Control V3
- NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles)
- NextSeq 500/550 Sequencing System

3. Methods

3.1 Genomic DNA evaluation and preparation for DNA library generation

3.1.1 If the extraction of genomic DNA leads to the DNA being eluted into a buffer containing EDTA (see Note 1) a column or bead-based purification should be performed prior to performing any additional steps as the fragmentation enzyme is sensitive to EDTA.

Note 1: An alternative method to address EDTA-containing genomic DNA samples is to add 5 µL of a conditioning solution, provided in the KAPA HyperPlus Kit to genomic DNA in a total volume of 30 µL. The conditioning solution, provided in the KAPA HyperPlus Kit, is diluted to a concentration dependent on EDTA concentration in the DNA sample.

3.1.2 The gDNA concentration is assessed using the Qubit broad range assay. Manufacturer guidelines are followed with two modifications; (1) the standard/sample is added to the Qubit assay tubes first followed by the Qubit working solution and (2) the incubation time prior to reading the standard/sample is 20 minutes.

3.1.3 The gDNA integrity assessment is performed using the Genomic DNA ScreenTape Assay. Manufacturer guidelines are followed without any modifications.

3.1.4 For the EuroClonality-NDC protocol a positive control, a no template control (NTC) and 22 samples are processed in each batch (see Note 2). In well A1 of a 96-well PCR plate place 100 ng of the positive control in a total of 35 µL and in well A2 place 35 µL of the NTC.

Note 2: NTC is either PCR grade water or the same buffer used to elute the gDNA following DNA extraction. Users can use commercially sourced high molecular weight gDNA as a positive control to monitor the consistency in performance of library preparation across multiple batches.

3.1.5 For the EuroClonality-NDC assay 100 ng of high molecular weight genomic DNA is required or for genomic DNA extracted from formalin fixed DNA 100 ng (average fragment size >1000 bp) or 200 ng (average fragment size <1000 bp) is used in a total of 35 µL. Each sample to be prepared should be placed into a separate well of a 96-well PCR plate (see Note 3).

Note 3: It is worthwhile generating a template to record the order the samples are added to the 96-well plate and for later stages of library preparation to record the UDI primer mix assigned to each sample. Plates can be prepared the day before to minimize set up times on the day of library preparation.

3.2 DNA library generation

3.2.1 Remove the following products from the KAPA HyperPlus Kit and thaw on ice:

3.2.1.1 KAPA Frag Buffer (10X)

3.2.1.2 End Repair & A-Tailing Buffer

3.2.1.3 Ligation Buffer

3.2.1.4 KAPA HiFi HotStart ReadyMix (2X)

3.2.1.5 Library Amplification Primer Mix (10X)

3.2.2 Prepare a thermocycler by selecting the fragmentation program (Table 1) and pausing prior to the commencement of the first step to ensure the block is pre-cooled to 4°C (see Note 4).

Table 1. Fragmentation program

Step	***Temperature*** *(°C)*	***Time*** *(min)*	***Heated Lid*** *(°C)*
Pre-cool block	4	2	50
Fragmentation	37	22	
Hold	4	∞	

Note 4: Fragmentation is key to the size distribution of the final library and is impacted by fragmentation time and temperature. With the wide range of different thermocyclers on the market, optimization of the fragmentation time is advised to ensure the ideal size distribution profile following fragmentation is achieved.

3.2.3 While keeping the reagents on ice, prepare a mastermix which contains 5 µL KAPA Frag Buffer (10X) and 10 µL KAPA Frag Enzyme for each reaction to be performed.

3.2.4 While on ice, add 15 µL of the fragmentation mastermix to the well containing 35 µL double-stranded genomic DNA to achieve a total volume of 50 µL (see Note 5). Vortex gently before spinning down briefly.

Note 5: Maintaining the temperature of the reaction at 4°C during the set-up of the fragmentation reaction is critical and it is advised using a PCR cooler to ensure fragmentation does not being prior to loading the plate on the thermocycler.

3.2.5 Place the reaction in the pre-cooled thermocycler and start the paused fragmentation program.

3.2.6 While samples are undergoing fragmentation prepare the End Repair & A-Tailing Buffer mastermix which contains 7 µL KAPA Frag Buffer (10X) and 3 µL HyperPlus ERAT Enzyme Mix for each reaction to be performed.

3.2.7 Following completion of the fragmentation reaction, place samples onto the plate cooler.

3.2.8 Add 10 µL of the End Repair & A-Tailing Buffer mastermix to the well containing 50 µL of fragmented genomic DNA to achieve a total volume of 60 µL (see Note 6). Vortex gently before spinning down briefly.

Note 6: After thawing, the End Repair & A-Tailing buffer may contain precipitates which may require incubation at 37°C and thorough vortexing before use to ensure they have been completely resuspended.

3.2.9 Incubate samples on a thermocycler using the selected End Repair & A-Tailing Buffer program (Table 2).

Table 2. End Repair & A-Tailing Buffer program

Step	***Temperature*** *(°C)*	***Time*** *(min)*	***Heated Lid*** *(°C)*
End Repair & A-Tailing	65	30	85
Hold	4	∞	

3.2.10 While the End Repair & A-Tailing program is underway prepare the Ligation mastermix which contains 30 µL Ligation buffer and 10 µL DNA Ligase for each reaction to be performed.

3.2.11 Also, while the End Repair & A-Tailing program is underway KAPA HyperPure Beads are removed from 4°C to ensure they are equilibrated to room temperature in time for later paramagnetic bead clean up steps (see Note 7).

Note 7: Aliquots of KAPA HyperPure Beads can be made to reduce the number of times the KAPA HyperPure Beads are removed from storage at 4°C.

3.2.12 Following completion of the End Repair & A-Tailing reaction, place samples onto the plate cooler.

3.2.13 Add 10 µL of the Universal adapter followed by 40 µL Ligation mastermix to each well to achieve a total volume of 110 µL (see Note 8). Vortex gently before spinning down briefly.

Note 8: Universal adapter stocks are aliquoted to avoid repeated freeze/thaw cycles.

3.2.14 Incubate samples on a thermocycler using the selected Adapter Ligation program (Table 3).

Table 3. Adapter Ligation program

Step	***Temperature*** *(°C)*	***Time*** *(min)*	***Heated Lid*** *(°C)*
Adapter Ligation	20	15	50
Hold	4	∞	

3.2.15 While the Adapter Ligation program is running remove the required number of UDI primer mixes from the freezer and thaw on ice.

3.2.16 Following completion of the Adapter Ligation thermocycler program remove samples from the thermocycler.

3.2.17 Resuspend the room temperature KAPA HyperPure beads by vortexing vigorously.

3.2.18 Perform a 0.8X bead cleanup by adding 88 µL of KAPA HyperPure beads to each well to achieve a total volume of 198 µL before pipette mixing ten times taking care not to generate bubbles.

3.2.19 Incubate the bead/sample mixture for 15 minutes at room temperature to allow the DNA to bind to the beads (see Note 9).

Note 9: We use a 15-minute incubation period with beads to ensure maximal recovery of library in samples with a poor gDNA integrity.

3.2.20 While the bead/sample mixture is incubating prepare 20 mL of fresh 80% ethanol by adding 4 mL PCR grade water to 16 mL molecular grade ethanol. Vortex and leave at room temperature until required.

3.2.21 While the bead/sample mixture is incubating prepare 5 mL of fresh 10mM Tris-HCl, pH 8.0 by adding 50 µL 1M Tris-HCl, pH 8.0 to 4.95 mL PCR grade water. Vortex and leave at room temperature until required.

3.2.22 Place samples onto a magnetic stand and wait approximately 5 minutes for the solution to clear (see Note 10).

Note 10: Various plate magnets are available on the market with different locations of the magnets and variable magnetic strengths. This variation can impact on the time required for beads to pellet and it is worthwhile determining optimal times for incubation of bead containing samples for specific magnetic plates.

3.2.23 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.2.24 With the plate remaining on the magnetic stand perform an ethanol wash by adding 200 µL of freshly prepared 80% ethanol (see Note 11).

Note 11: Steps such as ethanol washes of beads and elution of beads should be performed using a multichannel pipette to ensure consistency and prevent beads drying out.

3.2.25 Incubate the sample in 80% ethanol for 30 seconds.

3.2.26 Carefully remove the ethanol taking care not to disturb the pellet.

3.2.27 Repeat steps 3.2.24 to 3.2.26 until a total of two ethanol washes have been performed.

3.2.28 Remove residual ethanol without disturbing the beads.

3.2.29 Air-dry the beads at room temperature to enable evaporation of any remaining ethanol (see Note 12).

Note 12: Air-drying of beads is dependent on room temperature. The bead pellet should still be dark brown and glossy but show little sign of excess liquid. Over-drying of beads can lead to poor elution of DNA from the beads and therefore lower yields.

3.2.30 Remove the sample from the magnetic stand.

3.2.31 Resuspend each bead pellet in 22 µL of 10 mM Tris-HCl, pH 8.0 (see Note 11).

3.2.32 Incubate the sample for 2 minutes to enable DNA to elute from the beads.

3.2.33 Place the sample on the magnetic stand to pellet the beads and for the solution to clear.

3.2.34 With the plate remaining on the magnetic stand transfer 20 µL of the eluate to a new 200 µL PCR plate.

3.2.35 To the 20 µL of eluate add 5 µL of KAPA UDI Primer mix to each individual sample library followed by 25 µL of KAPA HiFi HotStart ReadyMix. Vortex gently before spinning down briefly (see Note 3).

3.2.36 Incubate samples on a thermocycler using the selected Pre-Capture PCR Amplification program (Table 4).

Table 4. Pre-Capture PCR Amplification program

Step	***Temperature*** *(°C)*	***Time*** *(sec)*	***Cycles***	***Heated Lid*** *(°C)*
Initial Denaturation	98	45	1	105
Denaturation	98	15	6	
Annealing	60	30		
Extension	72	30		
Final Extension	72	60	1	
Hold	4	∞	1	

3.2.37 Following completion of the Pre-Capture PCR Amplification program remove samples from the thermocycler.

3.2.38 To the 50 µL PCR reaction add 70 µL of KAPA HyperPure beads before pipette mixing ten times taking care not to generate bubbles.

3.2.39 Incubate the bead/sample mixture for 15 minutes at room temperature to allow the DNA to bind to the beads (see Note 9).

3.2.40 Place samples onto a magnetic stand and wait approximately 3 minutes for the solution to clear (see Note 10).

3.2.41 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.2.42 With the plate remaining on the magnetic stand perform an ethanol wash by adding 200 µL of freshly prepared 80% ethanol (see Note 11).

3.2.43 Incubate the sample in 80% ethanol for 30 seconds.

3.2.44 Carefully remove the ethanol taking care not to disturb the pellet.

3.2.45 Repeat steps 3.2.42 to 3.2.44 until a total of two ethanol washes have been performed.

3.2.46 Remove residual ethanol without disturbing the beads.

3.2.47 Air-dry the beads at room temperature to enable evaporation of any remaining ethanol (see Note 12).

3.2.48 Remove the sample from the magnetic stand.

3.2.49 Resuspend each bead pellet in 32 µL of 10 mM Tris-HCl, pH 8.0 (see Note 11).

3.2.50 Incubate the sample for 2 minutes to enable DNA to elute from the beads.

3.2.51 Place the sample on the magnetic stand to pellet the beads and for the solution to clear.

3.2.52 With the plate remaining on the magnetic stand transfer 31 µL of the eluate to a new 200 µL PCR plate, labelled 'Master Plate' which is to be retained for preparation of the hybridization.

3.2.53 From the 31 µL of the transferred eluate, remove 4 µL of the eluate and transfer to a new plate, labelled 'QC Plate' for the purposes of quality control assessment. The Master Plate can be stored at -20°C until ready to perform the hybridization. Proceed to step 3.3 with the QC Plate.

3.3 Quality control of DNA libraries

3.3.1 The concentration of each individual library is assessed using the Qubit broad range assay (see Note 13). Manufacturer guidelines are followed with two modifications; (1) the standard/sample is added to the Qubit assay tubes first followed by the Qubit working solution and (2) the incubation time prior to reading the standard/sample is 20 minutes.

Note 13: With this version of the library preparation method, the concentration of individual libraries tends to lie within the range of the Qubit broad range assay.

3.3.2 The average fragment size of each individual library is assessed using the TapeStation D1000 assay. Manufacturer guidelines are followed without any modifications.

3.4 DNA hybridization

3.4.1 Thaw reagents required for the DNA hybridization step which include: COT Human DNA, Universal Enhancing Oligos, Hybridization Buffer and Hybridization Component H.

3.4.2 Remove the KAPA HyperPure beads from 4°C and allow to equilibrate to room temperature for 30 minutes.

3.4.3 For the EuroClonality-NDC protocol 22 clinical samples are pooled, in equal amounts, into one hybridization reaction to achieve a total of 1.5 µg of DNA (i.e. 68.2 ng of each individual library). To achieve this, calculate the volume of each library to enable 68.2 ng of each library to be added to the hybridization reaction. For the NTC, which should not have a measurable DNA concentration, the average volume of library being added from the 22 samples is calculated to determine the amount of volume of the NTC library to add (see Note 14).

Note 14: Inclusion of the positive control for the hybridization steps is not required. While the NTC is included in the hybridization reaction, the NTC DNA concentration should be negligible and not factored into the calculations for the combined DNA mass of 1.5 µg.

3.4.4 Label a LoBind DNA 1.5 mL tube and add the required volume of each of the 22 libraries and the NTC to this tube.

3.4.5 To the pooled libraries add 20 µL COT Human DNA. Vortex gently before spinning down briefly.

3.4.6 Calculate the total volume of the 22 pooled libraries, the NTC library plus the 20 µL of COT DNA. If the total volume of libraries is < 45 µL (ie libraries then PCR grade water is added to adjust volume to 45 µL. The volume of beads required in the next step is 2x this total volume (i.e. if the total volume was calculated to be 75 µL then 150 µL KAPA HyperPure beads will be required).

3.4.7 Vortex the KAPA HyperPure beads until a homogenous solution is achieved.

3.4.8 To the pooled libraries add the volume of KAPA HyperPure beads calculated in the step 3.4.6. Seal the tube and vortex vigorously for 10 seconds.

3.4.9 Incubate the bead/sample mixture for 10 minutes at room temperature to allow the pooled libraries and COT Human DNA to bind to the beads.

3.4.10 Place samples onto a magnetic stand and wait approximately 3 minutes for the solution to clear (see Note 10).

3.4.11 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.12 With the plate remaining on the magnetic stand perform an ethanol wash by adding 200 µL of freshly prepared 80% ethanol.

3.4.13 Incubate the sample in 80% ethanol for 30 seconds.

3.4.14 Carefully remove the ethanol taking care not to disturb the pellet. Remove residual ethanol with an additional pipetting step without disturbing the beads.

3.4.15 Air-dry the beads at room temperature for approximately 5 minutes to enable evaporation of any remaining ethanol (see Note 12).

3.4.16 Remove the sample from the magnetic stand.

3.4.17 Add 13.4 µL of Universal Enhancing Oligos (UEO) to the tube, before sealing the tube and vortexing vigorously for 10 seconds to ensure a homogeneous mixture is achieved.

3.4.18 To the library pool and UEO mixture, add 43 µL of mastermix prepared using the following components: 28 µL Hybridization Buffer, 12 µL Hybridization Component H and 3 µL PCR grade water.

3.4.19 Vortex before spinning down briefly. Incubate for 2 minutes at room temperature.

3.4.20 Place samples onto a magnetic stand and wait approximately 3 minutes for the solution to clear.

3.4.21 Transfer 56.4 µL of the eluate into a new well containing 4 µL of the EuroClonality-NDC panel.

3.4.22 Vortex vigorously before spinning down briefly.

3.4.23 Incubate samples on a thermocycler using the selected 'Hybridization' program (Table 5).

Table 5. Hybridization program

Step	***Temperature*** *(°C)*	***Time***	***Heated Lid*** *(°C)*
Denaturation	96	5 min	105
Hybridization	55	16-20 h	

3.4.24 Dilute wash buffers provided in the KAPA HyperCapture Reagent Kit using the volumes of stock buffer solution and PCR grade water detailed in Table 6.

Table 6. Preparation of post-hybridization wash buffers

Step	*Volume of Stock Buffer (µL)*	*Volume of PCR Grade Water (µL)*	*Temperature (°C)*
10X Stringent Wash Buffer	40	360	55
10X Wash Buffer I	10	90	55
	20	180	RT
10X Wash Buffer II	20	180	RT
10X Wash Buffer III	20	180	RT
2.5X Bead Wash Buffer	120	180	RT

3.4.25 Split the 400 µL of 1X Stringent wash buffer into two aliquots of 200 µL in 0.2 mL PCR tubes and incubate on the thermocycler at 55°C for at least 15 minutes.

3.4.26 Place the 100 µL aliquot of 1X Wash Buffer I into the thermocycler at 55°C for at least 15 minutes.

3.4.27 Vortex the Capture Beads from the KAPA HyperCapture Bead kit thoroughly to ensure a homogenous solution.

3.4.28 Remove 50 µL of Capture Beads for each pool and place into a 1.5 mL tube and equilibrate to room temperature for 30 minutes.

3.4.29 Following the 30-minute incubation place the tube containing 50 µL of Capture beads onto a magnetic stand and wait approximately 3 minutes for the solution to clear.

3.4.30 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.31 Add 100 µL of 1X Bead Wash Buffer to the pelleted Capture Beads.

3.4.32 Remove from the magnetic stand and vortex for 10 seconds before spinning down briefly.

3.4.33 Place the tube back onto the magnetic stand and wait until the beads have pelleted and the solution is clear.

3.4.34 Carefully remove and discard the supernatant taking care not to disturb the pellet

3.4.35 Perform a second wash of the Capture Beads by performing steps 3.4.31 to 3.4.34 again.

3.4.36 Add 50 µL of 1X Bead Wash Buffer to the pelleted Capture Beads.

3.4.37 Remove from the magnetic stand and vortex for 10 seconds before spinning down briefly.

3.4.38 Aliquot 50 µL of the resuspended Capture Beads into a 0.2 mL tube for each capture to be performed.

3.4.39 Place the tube onto the magnetic stand and wait until the beads have pelleted and the solution is clear (see Note 15).

Note 15: Different magnetic stands will be required for the workflow and some will be required in both pre- and post-PCR environments. From our experience sourcing good magnetic stands is essential to the success of the workflow.

3.4.40 Carefully remove and discard the supernatant taking care not to disturb the pellet

3.4.41 The Capture beads are now ready to bind the hybridized DNA.

3.4.42 Proceed to the next step immediately to prevent the Capture Beads from drying out.

3.4.43 Transfer the hybridization sample (60.4 µL) to the tube containing the pelleted Capture Beads from the previous step (see Note 16)

Note 16: Keep the 'Hybridization' program on thermocycler running for incubations with the Capture Beads and subsequent wash steps.

3.4.44 Vortex for 10 seconds before spinning down briefly (see Note 17).

Note 17: This step is to ensure all the sample-bead mix is at the bottom of the well for the incubation steps on the thermocycler. Do not spin long enough for the beads to pellet at the bottom of the tube.

3.4.45 Incubate the tube now containing the hybridized DNA and the Capture Beads on the thermocycler at 55°C for 15 minutes.

3.4.46 After the 15-minute incubation remove the samples from the thermocycler and the tube containing the 100 µL aliquot of 1X Wash Buffer.

3.4.47 Add the 100 µL of 55°C 1X Wash Buffer I before vortexing for 10 seconds and spinning down briefly.

3.4.48 Place the tube onto the magnetic stand and wait until the beads have pelleted and the solution is clear.

3.4.49 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.50 Remove a tube containing a 200 µL aliquot of 1X Stringent Wash Buffer from the thermocycler and add the 200 µL of 1X Stringent Wash Buffer to the sample.

3.4.51 Remove the sample from the magnet before vortexing for 10 seconds.

3.4.52 Incubate the sample on the thermocycler at 55°C for 5 minutes.

3.4.53 After the incubation, spin the sample briefly before placing the sample onto the magnetic stand. Wait until the beads have pelleted and the solution is clear.

3.4.54 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.55 Repeat steps 3.4.50 to 3.4.54 with the only remaining 200 µL aliquot of pre-warmed 1X Stringent Wash Buffer.

3.4.56 Add 200 µL of room temperature 1X Wash Buffer I to the sample followed by vortexing for 10 seconds.

3.4.57 Incubate the sample for 1 minute at room temperature.

3.4.58 After the incubation, spin the sample briefly before placing the sample onto the magnetic stand. Wait until the beads have pelleted and the solution is clear.

3.4.59 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.60 Add 200 µL of room temperature 1X Wash Buffer II to the sample followed by vortexing for 10 seconds.

3.4.61 Incubate the sample for 1 minute at room temperature.

3.4.62 After the incubation, spin the sample briefly before placing the sample onto the magnetic stand. Wait until the beads have pelleted and the solution is clear.

3.4.63 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.64 Add 200 µL of room temperature 1X Wash Buffer III to the sample followed by vortexing for 10 seconds.

3.4.65 Incubate the sample for 1 minute at room temperature.

3.4.66 After the incubation, spin the sample briefly before placing the sample onto the magnetic stand. Wait until the beads have pelleted and the solution is clear.

3.4.67 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.68 Add 20 µL of PCR grade water to the sample followed by vortexing for 10 seconds and subsequently spin the sample briefly.

3.4.69 Remove KAPA HyperPure Beads (70 µL required for each capture) for use in later steps within a post-PCR area and allow to equilibrate to room temperature.

3.4.70 For each hybridization add to a fresh 0.2 mL tube 25 µL of KAPA HiFi HotStart ReadyMix and 5 µL of Post-Capture PCR Oligos.

3.4.71 Add the 20 µL of bead-bound captured DNA from step 3.4.68 to the 0.2 mL tube containing the PCR reagents to achieve a total volume of 50 µL. Mix thoroughly by pipette mixing.

3.4.72 Within a post-PCR designated area, place samples on a thermocycler and run the selected Post-Capture PCR Amplification program detailed in Table 7 (see Note 18).

Note 18: It is advised the post-capture PCR and all further steps are performed in an area designated for post-PCR activities to minimize contamination risks.

Table 7. Post-Capture PCR Amplification program

Step	***Temperature*** *(°C)*	***Time*** *(sec)*	***Cycles***	***Heated Lid*** *(°C)*
Initial Denaturation	98	45	1	105
Denaturation	98	15	11	
Annealing	60	30		
Extension	72	30		
Final Extension	72	60	1	
Hold	4	∞	1	

3.4.73 While the Post-Capture amplification is underway prepare 80% ethanol by adding 200 µL PCR grade water to 800 µL molecular grade ethanol in a 1.5 mL microfuge tube. Vortex gently before spinning down briefly.

3.4.74 Following completion of the Post Capture amplification thermocycler program vortex the KAPA HyperPure Beads which are now equilibrated to room temperature.

3.4.75 Add 70 µL KAPA HyperPure Beads to each 50 µL PCR reaction which contains the amplified and enriched DNA library pool before vortexing for 10 seconds and spinning down briefly.

3.4.76 Incubate the bead/sample mixture for 5 minutes at room temperature to allow the sample to bind to the beads.

3.4.77 Place samples onto a magnetic stand and wait approximately 3 minutes for the solution to clear.

3.4.78 Carefully remove and discard the supernatant taking care not to disturb the pellet.

3.4.79 With the plate remaining on the magnetic stand perform an ethanol wash by adding 200 µL of freshly prepared 80% ethanol.

3.4.80 Incubate the sample in 80% ethanol for 30 seconds.

3.4.81 Carefully remove the ethanol taking care not to disturb the pellet.

3.4.82 Repeat steps 3.4.79 to 3.4.81 for a total of 2 ethanol washes.

3.4.83 Carefully remove any residual ethanol with an additional pipetting step without disturbing the beads.

3.4.84 Air-dry the beads at room temperature for approximately 5 minutes to enable evaporation of any remaining ethanol (see Note 12).

3.4.85 Remove the sample from the magnetic stand.

3.4.86 Resuspend the bead pellet in 22 µL of PCR grade water before vortexing for 10 seconds and spinning down briefly (see Note 17).

3.4.87 Incubate the sample for 2 minutes to enable DNA to elute from the beads.

3.4.88 Place the sample on the magnetic stand to pellet the beads and for the solution to clear.

3.4.89 With the plate remaining on the magnetic stand transfer 20 µL of the eluate to a new 200 µL PCR plate.

3.4.90 The amplified and enriched library is now ready for the final quality control steps prior to sequencing.

3.5 Quality control of enriched DNA library

3.5.1 The concentration of the amplified and enriched library is assessed using the Qubit high sensitivity assay. Manufacturer guidelines are followed with two modifications; (1) the standard/sample is added to the Qubit assay tubes first followed by the Qubit working solution and (2) the incubation time prior to reading the standard/sample is 20 minutes.

3.5.2 The average fragment size of each individual library is assessed using the High Sensitivity D1000 TapeStation assay. Manufacturer guidelines are followed without any modifications.

3.6 Sequencing of enriched DNA library

3.6.1 Prepare the amplified and enriched pooled library for sequencing on the NextSeq 500/550 by adhering to the Illumina 'Denature and Dilute Guidelines' using the following parameters (see Note 19):

3.6.1.1 Protocol A (Standard Normalization Method)

3.6.1.2 Final dilution of library is to 1.5 pM for Mid Output kits

3.6.1.3 Final PhiX (sequencing control) spike-in percentage is 1% of the final library and PhiX composition.

Note 19: The EuroClonality-NDC protocol was developed and validated to achieve an optimal mean target coverage depth for the detection of clonal rearrangements, translocation, copy number alterations and SNV/indels in 22 samples, using a single hybridization reaction and sequenced on an Illumina NextSeq 500/550 system with the Mid Output kit sequencing reagents. It is at the readers' discretion if they want to adapt the protocol to:

a. Employ different Illumina sequencing platforms

b. Utilize sequencing reagents with increased output

c. Alter the number of samples being applied to the flow cell for sequencing

3.6.2 To simplify the subsequent bioinformatic analysis, when setting up the sequencing of the enriched DNA library it is recommended the 'Run Monitoring and Storage' option for BaseSpace Sequence Hub can be selected (see Note 20). This enables conversion of BCL files to FASTQ files and demultiplexing of the sequencing data, on BaseSpace Sequence Hub, to generate sample specific FASTQ files.

Note 20: The NextSeq 500 System Guide provides further information on configuring the run set up. Selecting the 'Run Monitoring and Storage' option for BaseSpace Sequence Hub will require access to a completed sample sheet.

3.7 Bioinformatic Analysis of the EuroClonality-NDC Sequencing Data

3.7.1 The bioinformatics pipeline is now available as a EuroClonality-NDC analysis application on the Illumina BaseSpace Sequence Hub (see Note 20).

Note 20: Univ8 Genomics Ltd (https://univ8genomics.com) provide a comprehensive EuroClonality-NDC Assay Analysis Guide. This provides information on:

1. The requirement for a Professional BaseSpace Sequence Hub (BSSH) account to run the EuroClonality-NDC analysis application.
2. Accessing and launching the EuroClonality-NDC application through the BSSH Apps tab.
3. Selection of samples for analysis through the EuroClonality-NDC application.
4. Downloading the analysis files to a specified local directory.
5. Description of the output files and how to interpret the data.