Clinical Exome Sequencing (CES) NGS Assay

compatible with Illumina Platform

REF 9-241 RUO

Σ 4x4 Reactions

Upon arrival, please store components according to their labels!

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rev 1.0

Instructions for use

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Covered Genes

The ViennaLab Clinical Exome Sequencing NGS Assay covers more than 7500 genes (whole coding sequence) which are known contributors of disease pathogenesis. In addition, the mitochondrial genome as well as intronic hotspot regions are included to offer a comprehensive solution for even challenging phenotypes.
Target size 19.7 Mb
A list of all genes covered by the ViennaLab Clinical Exome Sequencing NGS Assay is available at www.viennalab.com/products/genetic-disorders

Provided Materials

Table I. Library Preparation Box #1

Product Name	Storage Condition
FEAT Buffer FEAT Enzyme Ligation Buffer Ligation Enzyme CES Adapter 2x PCR Mix	-30°C to -15°C

Table II. Library Preparation Box #2

Product Name	Storage Condition
Dual Index Primer i5 (4 different primers) Dual Index Primer i7 (4 different primers)	-30°C to -15°C

Table III. Target Capture Box #1

Product Name	Storage Condition
Block #1 Block #2 Block #3 Capture Probe #2 Post Capture PCR Forward Primer Post Capture PCR Reverse Primer	-30°C to -15°C

Table IV. Target Capture Box #2

Product Name	Storage Condition
Wash Buffer #1 (4 tubes) Wash Buffer #2 (3 tubes) Wash Buffer #3 (10 tubes) Library Hyb Buffer	+19°C to +25°C

Table V. Clinical Exome Sequencing Target Capture Box #3

Product Name	Storage Condition
CES Capture Probe #1	-90°C to -60°C

Table VI. Additional Provided Reagents – Beads

Product Name	Storage Condition
Clean-up Beads Streptavidin Beads	+2°C to +8°C

Additional Required Materials (Not Provided)

Table VII. Additional Reagents and Consumables (Not Provided)

Product Name	Suggested Supplier
Nuclease-free water (not DEPC-treated)	General lab supplier
Ethanol (200 proof)	General lab supplier
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
P10, P20, P200 and P1000 pipettes	General lab supplier
Multichannel pipette	General lab supplier
Sterile, nuclease–free aerosol barrier pipette tips	General lab supplier
0.2 ml PCR Tube Strips, Low Profile and Cap Strips or 96-well PCR plates and adhesive seals	General lab supplier
Powder-free gloves	General lab supplier
DNA LoBind Tubes, 1.5 ml PCR clean	Eppendorf (p/n: 022431021 or equivalent)

Table VIII. Additional Equipment (Not Provided)

Description	Suggested Supplier
Thermal Cycler (with adjustable lid temperature)	e.g. Eppendorf X50s
Fluorometric DNA quantification device	e.g. Qubit Invitrogen
Centrifuge (for 1.5 ml tubes, PCR strips, 96-well PCR plates)	General lab supplier
Vertical rotator	e.g. bioSan Multi Bio RS-24
CoolBox™ XT PCR Strip Workstation, alternatively ice bucket	BioCision
Vortex mixer	General lab supplier
Thermo-mixer	e.g. Eppendorf ThermoMixer F1.5
Fragment Analyzer NGS / TapeStation / BioAnalyzer	Agilent
Magnetic separator (for 1.5ml tubes, 0.2ml PCR strips or 96-well plates)	General lab supplier
Optional: vacuum concentrator	e.g. Eppendorf Concentrator plus

Before you begin

General

- Read the manual carefully to understand the experimental procedure and make sure that you have all the required equipment before starting an experiment.
- Possible stopping points, where samples may be stored at -30°C to -15°C, are indicated in the protocol.
- Important steps which affect the quality of the data produced are highlighted.

Important

The accurate quantification and quality of the input DNA have a large impact on the results obtained. For accurate quantification use dsDNA specific assays (e.g. Qubit). Do not use Nanodrop or other spectrophotometric methods. The protocol has been optimized for high quality DNA input amounts between 50-100 ng.

Directions for the use of this product

1. This product is for Research Use Only.
2. Use pipettes dedicated to PCR setup only. Use aerosol-guarded pipette tips.
3. Adhere to recommended storage temperatures, which are: room temperature (RT, +19°C to +25°C), fridge (+2°C to +8°C), freezer (-30°C to -15°C) and deep freezer (-90°C to -60°C).
4. Always handle enzymes with care and avoid vortexing them. Mix them by slowly pipetting up and down. Keep enzymes on ice during reagent setup and store them back at -30°C to -15°C as soon as possible after each use.
5. Never freeze Clean-up beads or Streptavidin beads. Store them at +2°C to +8°C and keep Clean-up beads at +19°C to +25°C for at least 30 minutes before use.
6. Use 1.5 ml LoBind tubes instead of regular 1.5 ml tubes whenever low concentrated DNA samples are involved.

Safety information

1. Do not drink, eat, smoke, or apply cosmetics in designated work areas. Always use disposable powder-free gloves and wear suitable laboratory coat when handling specimens and reagents. Wash hands thoroughly afterwards.
2. Handle specimens as if capable of transmitting infectious agents. Thoroughly clean and disinfect all materials and surfaces that have been in contact with specimens. Discard all waste associated with clinical specimens in a biohazard waste container.
3. Avoid contact of DNA with skin, eyes, or mucous membranes. If contact does occur, immediately wash with large amounts of water. If irritation persists: Get medical advice/attention. If spilled, dilute with water before wiping dry.
4. Adhere to all local and federal safety and environmental regulations which may apply.

If you have any questions related to the protocol or the product, please contact techhelp@viennalab.com.

Protocol Overview

The Clinical Exome Sequencing (CES) NGS Assay offers a complete solution to detect and analyze any coding mutation in more than 7500 genes (whole CDS) which are known contributors of disease pathogenesis. First, DNA is converted into an Illumina compatible NGS-ready library. Second, libraries of 4 samples are pooled and processed together for the remaining experimental steps. Next, targeted enrichment of selected genomic regions by biotinylated probes allows to maximize the accurate identification of disease-causing germline variants in a cost-efficient manner. Finally, sequencing data can be uploaded to a tailored bioinformatic pipeline via a secure web-interface. Provided analysis includes automated processing and QC of the data, identification and annotation of variants as well as selection of variants for the generation of a meaningful report.

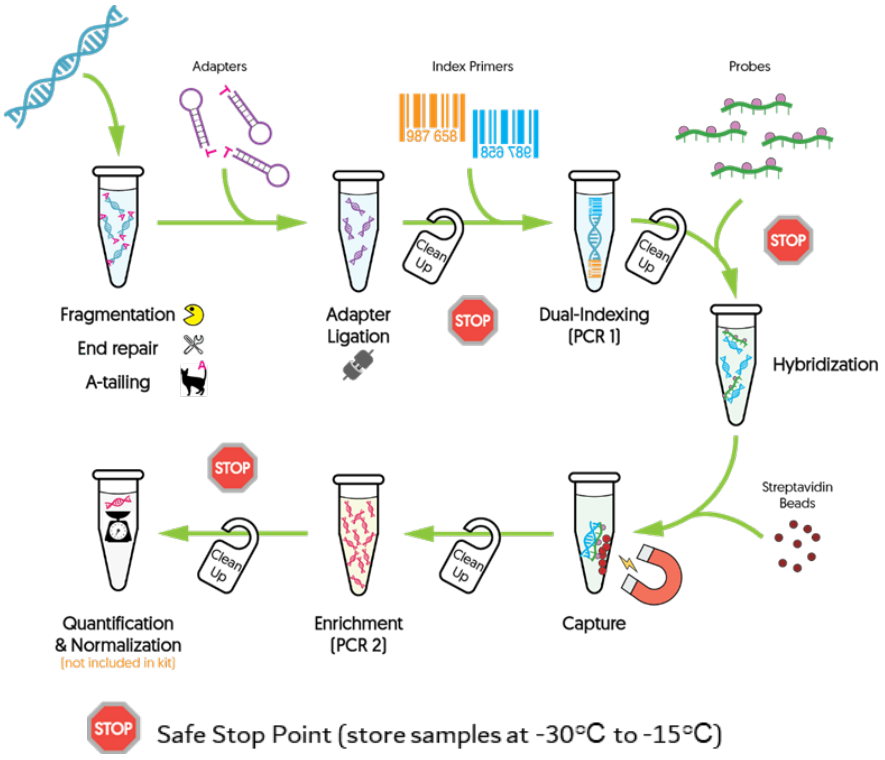


Figure 1. Graphical workflow overview

	Workflow	Total time	Hands on time
Part 1	Fragmentation, End repair, A-tailing	1 hour	15 min
	Adapter ligation & Activation / Bead Clean-up	1 hour 15 min	30 min
	Dual Indexing / Bead Clean-up	1 hour 30 min	30 min
	Quality Assessment and Quantification	30 min to 1 hour	20 min
Part 2	Hybridization	16 hours	1 hour
	Capture	1 hour 15 min	20 min
Part 3	Enrichment / Bead Clean-up	1 hour 30 min	30 min
	Quality Assessment and Quantification	30 min to 1 hour	20 min

Part 1. Generation of Indexed Library

Step 1. Enzymatic DNA Fragmentation, End repair and A-Tailing (FEAT)

Note: It is recommended to perform experiments using multiples of 4 samples as the later hybridization step requires 4 samples to be pooled and processed together. This kit provides sufficient reagents to perform 4x4 reactions.
This protocol has been optimized for high quality genomic DNA inputs between 50 ng and 100 ng and will generate libraries with an average fragment size of 350-450 bp. Optimal fragment length will depend on the read length used for sequencing. The fragmentation time of high-quality samples is set to 20 minutes. However, DNA samples with lower quality (e.g. genomic DNA with low DNA Integrity Number) may require optimization of fragmentation time.

[Required Material]

- Accurately quantified DNA (gDNA)
- Library Preparation Box #1 → FEAT Buffer, FEAT Enzyme
- 0.2 ml PCR tubes and caps
- Nuclease-free water

[Procedure]

1. Transfer **50-100 ng of DNA** into a new 8 strip PCR tube (or 96-well plate) on ice, and adjust the volume of the sample to **21 µl** according to Table 1.

Table 1. Input sample adjustment

Reagent	Volume per sample (µl)
DNA sample	x
Nuclease-free water	21 - x
Total volume	21

2. Prepare a master mix on ice, as described in Table 2 (examples are given for 1 reaction as well as for 4 or 8 samples including excess volume). Mix well by pipetting up and down 5-10 times.

Table 2. Composition of Enzymatic Prep Master Mix. Prepare on ice.

Reagent	1 Reaction (µl)	4.4 Reactions (µl)	8.5 Reactions (µl)
FEAT Buffer	3	13.2	25.5
FEAT Enzyme	6	26.4	51
Total volume	9	39.6	76.5

Critical: Prepare master mix on ice and keep on ice until placed in the thermal cycler!

3. **Add 9 µl of Enzymatic Prep Master Mix** to each well containing the DNA sample, mix well by pipetting 5-10 times (setting pipette to 20 µl) and quick-spin the tubes. Keep reaction mix on ice until ready to avoid over-fragmentation as FEAT Enzyme is active at room temperature.

4. Start the following thermal cycler program:

Table 3. Fragmentation, End-repair and A-Tailing (lid temperature: 70°C, vol = 30 µl)

Step	Temperature	Time
1	4°C	Hold
2	32°C	20 minutes*
3	65°C	30 minutes
4	4°C	Hold

Important: Start program and hold at 4°C before putting the reaction mix into the thermal cycler.

***** In case that the fragmentation profile does not look as expected, incubation times at 32°C can be modified +/- 5 min accordingly.

5. Once the program has reached the initial 4°C, add the prepared sample mix into the thermal cycler and continue with the program. In the meantime, start preparations for the Adapter Ligation and Activation step (Part 1, Step 2).

Important: Fragmented samples should not be kept at 4 °C longer **than one hour before continuing**.

Step 2. Adapter Ligation

[Required Material]

- Library Preparation Box #1 → Ligation Buffer, Ligation Enzyme, CES Adapter

  Tip: Thaw Adapter and Ligation Buffer on ice in advance. Make sure that Ligation Buffer is fully thawed (if needed leave at room temperature for 1 min) and mix well before proceeding.

- 0.2 ml PCR tubes and caps
- Nuclease-free water

[Procedure]

1. Take out tubes from cycler (Step 1.5) and store on ice.

2. **Add 10 µl of CES Adapter** directly into the PCR tube (DNA sample) where the Fragmentation, End repair and A-Tailing reaction was performed. Mix well by pipetting up and down 5-10 times or by gentle vortexing. Spin down briefly.

3. Prepare Ligation Master Mix on ice in a new 1.5 ml LoBind tube as described in Table 4.

   Important: Ligation Buffer is highly viscous. Make sure to thaw completely and mix slowly but thoroughly with a P200 pipette before use.

Table 4. Composition of Ligation Master Mix. Prepare on ice.

Reagent	1 Reaction (µl)	4.4 Reactions (µl)	8.5 Reactions (µl)
Nuclease-free water	4	17.6	34
Ligation Buffer	12	52.8	102
Ligation Enzyme	4	17.6	34
Total volume	20	88	170

4. **Add 20 µl of Ligation Master Mix** into the PCR tube containing DNA sample and adapter. Mix well by pipetting up and down 10 times or gentle vortexing. Spin down briefly.

5. Incubate the Ligation mix at 20°C for 20 minutes (Table 5) in the thermal cycler (no heated lid, volume = 60 µl). After incubation **continue directly with bead clean-up**.

Table 5.

Temperature	Time
20°C	20 minutes

Step 3. Bead clean-up after Adapter Ligation

[Required Material]

- Clean-up Beads
  Important: Keep the beads at room temperature for at least 30 minutes before use. Store Clean-up Beads at +2°C to +8°C after use and do not freeze.
- 80% ethanol solution (always prepare fresh on the day of the experiment, e.g. 8 ml 100% EtOH + 2 ml nuclease free water)
- Nuclease-free water

[Procedure]

1. Let the Clean-up Beads come to room temperature for at least 30 minutes. Mix well so that they appear homogenous and consistent in color.
2. **Add 48 µl of homogenous Clean-up Beads (0.8x volume)** directly to the 60 µl Adapter-Ligated sample. Mix well by pipetting/vortexing and spin down briefly (make sure not to pellet the beads, they need to stay homogeneously in solution). **Incubate at room temperature for 5 minutes**.
3. Place the tube in a magnetic separator for **3-5 minutes**, or until the solution is clear.
4. Keep the tube in the magnetic separator and carefully discard the supernatant (do not touch the beads as this might result in sample loss).
5. Keeping the tube in the magnetic separator, **add 200 µl of freshly prepared 80% ethanol** to each tube.
6. **Incubate for 30 seconds at room temperature** and carefully discard the 80% ethanol solution (do not touch the beads as this might result in sample loss).
7. Repeat the 80% ethanol wash (steps 5-6) one more time **for a total of two washes**.
8. Spin down briefly, place the tube back into a magnetic separator and discard residual ethanol with a P10 or smaller pipet.
9. **Dry the sample tube at room temperature for 3-5 minutes** or until residual ethanol has completely evaporated (avoid excessive bead drying until beads appear cracked, as this might reduce elution efficiency).
10. **Add 22 µl of nuclease-free water** to the sample and mix well by pipetting/vortexing. Pulse-spin down and incubate the sample **at room temperature for 2 minutes**.
11. Place the tube into the magnetic separator for **2 minutes** or until the solution is clear.
12. **Transfer the supernatant (20 µl)** to a new 0.2 ml PCR tube and continue with the next step. Alternatively, transfer the 20 µl supernatant into a new 1.5 ml LoBind Tube and store at -30°C to -15°C until needed.

Stopping Point: If not continuing with the next step, samples can be stored at -30°C to -15°C.

Step 4. Amplification of Adapter-Ligated Library (Dual Index)

Note: Select the appropriate Dual Index Primers for each sample (Table 7) and use a different Dual Index Primers combination for each sample. In case of low number of samples to multiplex, consider balanced barcode combinations following these rules: use primers in ascending order and use at least two different forward (i5) and reverse primers (i7) (e.g. 501 + 701, 501 + 702; 502 + 701 and 502 + 702 in case of 4 samples).

[Required Material]

- Library Preparation Box #2 → Dual Index Primer i5, Dual Index Primer i7
- 2x PCR mix
- 0.2 ml PCR tubes and caps

[Procedure]

1. Make sure that the sample from Step 3.12 is in a 0.2 ml PCR tube and keep on ice during setup.
2. **Add 2.5 µl of Dual Index Primer i5 and 2.5 µl of Dual Index Primer i7** individually to each tube from Step 3.12. Use different Dual Index Primer combinations for each sample.

   Important: Select appropriate dual index combinations (Table 7) as described above.
3. **Add 25 µl of 2x PCR mix** to each tube, mix thoroughly by pipetting up and down 5-10 times, spin down briefly and put back on ice.
4. Transfer tubes to the thermal cycler and run the PCR program shown in Table 6.

Table 6. PCR program (lid: 105°C, final volume = 50 µl, limit ramp rates to max. 4ºC/sec for heating and max. 2.5ºC/sec for cooling)

Step	Temperature	Time
Step 1	98°C	45 seconds
Step 2	98°C	15 seconds
Step 3	65°C	30 seconds
Step 4	72°C	1 minute
Step 5	-	Repeat step 2 to step 4 for a total of 9-10 cycles*
Step 6	72°C	10 minutes
Step 7	12°C	Hold

* We recommend to perform the first experiment with 10 cycles. If library yield is at least twice as high as needed for hybridization in all samples (e.g., >1 µg and vacuum concentrator is available) then reduce to 9 cycles for next experiments.

Table 7. Dual Index sequences

Index	Sequence	Index	Sequence
Dual index 501 primer	TATAGCCT	Dual index 701 primer	ATTACTCG
Dual index 502 primer	ATAGAGGC	Dual index 702 primer	TCCGGAGA
Dual index 503 primer	CCTATCCT	Dual index 703 primer	CGCTCATT
Dual index 504 primer	GGCTCTGA	Dual index 704 primer	GAGATTCC
Dual index 505 primer	AGGCGAAG	Dual index 705 primer	ATTCAGAA
Dual index 506 primer	TAATCTTA	Dual index 706 primer	GAATTCGT
Dual index 507 primer	CAGGACGT	Dual index 707 primer	CTGAAGCT
Dual index 508 primer	GTACTGAC	Dual index 708 primer	TAATGCGC
Dual index 509 primer	GACCTGTA	Dual index 709 primer	CGGCTATG
Dual index 510 primer	CGGTGGTA	Dual index 710 primer	TCCGCGAA
Dual index 511 primer	GTTGGACT	Dual index 711 primer	TCTCGCGC
Dual index 512 primer	CTAAGATC	Dual index 712 primer	AGCGATAG
Dual index 513 primer	AATGGTTC	Dual index 713 primer	GCATAGTG
-	-	Dual index 714 primer	CATTCCAG
-	-	Dual index 715 primer	GGCAGGAT
-	-	Dual index 716 primer	CAACAACA

Step 5. Bead clean-up after indexing PCR

[Required Material]

- Clean-up Beads
  Important: Keep the beads at room temperature for at least 30 minutes before use. Store Clean-up Beads at +2°C to +8°C after use and do not freeze.
- 80% ethanol solution (always prepare fresh on the day of the experiment, e.g. 8 ml 100% EtOH + 2 ml nuclease free water)
- Nuclease-free water

[Procedure]

1. Let the Clean-up Beads come to room temperature for at least 30 minutes. Mix well by vortexing so that they appear homogenous and consistent in color.
2. **Add 50 µl Clean-up Beads (1:1 volume ratio)** directly into the 50 µl amplified Adapter-Ligated sample. Mix well by pipetting/vortexing and spin down briefly (make sure not to pellet the beads, the solution has to be homogeneous). **Incubate at room temperature for 5 minutes**.
3. Place the tube in a magnetic separator for **3-5 minutes** or until the solution is clear.
4. Keep the tube in the magnetic separator and discard the supernatant (do not touch the beads as this might result in sample loss).
5. Keeping the tube in the magnetic separator, **add 200 µl fresh 80% ethanol** to each sample tube.
6. **Incubate for 30 seconds at room temperature** and then discard the 80% ethanol (be careful not to touch the beads while you remove the supernatant).
7. Repeat 80% ethanol wash (steps 5-6) one more time **for a total of two washes**.
8. Spin down and place the tube in the magnetic separator and discard residual ethanol with a P10 or smaller pipet.
9. **Dry the sample tube at room temperature for 2 minutes** or until residual ethanol has completely evaporated (avoid drying beads until pellet appears cracked, as excessive drying decreases elution efficiency).
10. **Add 17 µl of nuclease-free water** to the sample and mix well on a vortexer. Pulse-spin down and incubate the sample **at room temperature for 2 minutes**.
11. Place the tube in the magnetic separator for **2 minutes** until the solution is clear.
12. **Transfer the supernatant (15 µl)** into a new 1.5 ml LoBind Tube.

Stopping Point: If not continuing with the next step, samples can be stored at -30°C to -15°C.

Step 6. Quality Assessment and Quantification of Index-Tagged Libraries

[Procedure]

1. Quantify amplified libraries from Part 1, Step 5.12 using a fluorometric method such as the Qubit dsDNA kit. If a vacuum concentrator is available, the protocol requires an input of 500 ng of indexed library per sample for the upcoming hybridization. However, if no vacuum concentrator is available, 1000 ng of indexed library per sample are required.

2. Determine the fragment size profile (e.g. with instruments such as TapeStation, Bioanalyzer or Fragment Analyzer) to check for appropriately fragmented and adapter-ligated DNA. A typical library displays a single peak between 200-800 bp with the maximum at ca. 350-450 bp (see Figure 2).

   In addition, no or only minimal primer dimers at around 120 bp should be visible. If the primer dimer peak is clearly visible (see Appendix, Figure 9-1), an additional bead clean-up step using a [bead] : [sample] ratio = 0.8 : 1 is recommended (see Appendix).

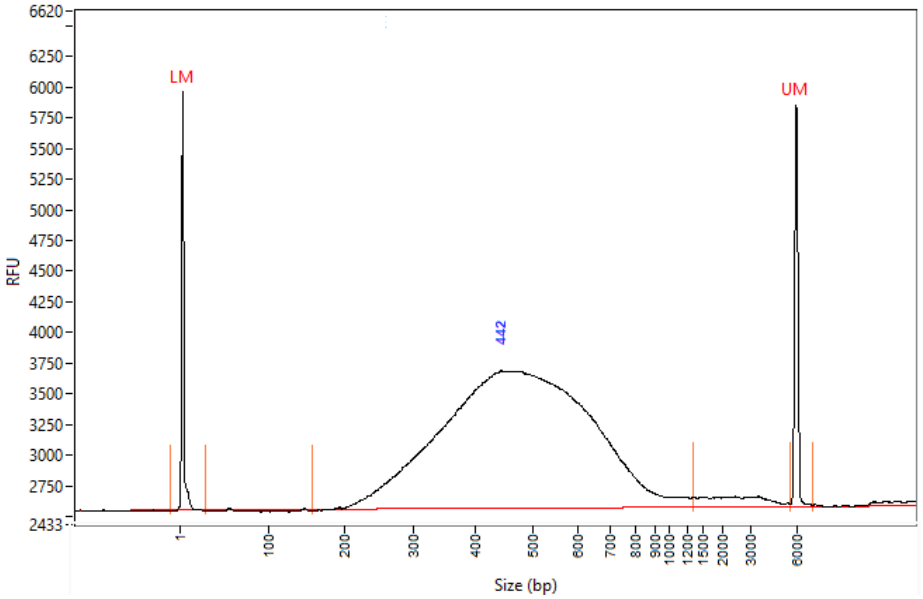


Figure 2. Electrophoresis diagram detected by Fragment Analyzer with the main peak ranging from 200-800 bp and the maximum at 442 bp.

Part 2. Hybridization

Recommendation:
We recommend to perform a pilot test to determine if the plasticware and PCR machine seal tightly and avoid excessive evaporation:

1. Pipet 32 µl of nuclease-free water into multiple wells of a PCR plate or 0.2 ml PCR tubes intended to be used. After sealing the PCR plates or tubes with sealing tape or PCR caps, incubate at 65°C for 2 hours (lid temperature is 105°C). If using PCR tubes, place them centrally in the cycler and put empty tubes on the edge positions to enable equally distributed pressure from the heating lid.

2. Ensure that there is no excessive evaporation by measuring the volume afterwards and observe if there are equal volumes in all filled wells. It is necessary that evaporation does not exceed 4 µl from the total to guarantee optimal assay performance.

Step 1. Hybridization of Capture Probes to the Library

Important:
- The hybridization requires pooling of 4 libraries for each hybridization reaction. The following protocol has been optimized with the use of a vacuum concentrator (Part 2, Step 1, Section 1.1). In this case, 4x 500 ng of indexed library (2000 ng total) are required. If no vacuum concentrator is available, 4x 1000 ng of indexed library are required which are concentrated through an extra bead clean-up step described in the alternative protocol (Part 2, Step 1, Section 1.2).
- Thaw the CES Capture Probe #1 on ice slowly and put back at -90°C to -60°C after use. Avoid too many freeze/thaw cycles.
- Do not vortex CES Capture Probe #1 (tapping or pipetting is allowed).
- Library Hyb Buffer should be pre-warmed for at least 15 minutes at 37°C (check for any precipitates) before use.
- Keep the samples in the cycler at 65°C during the whole hybridization process.

[Required Material]
- Vacuum concentrator *or*
  Clean-up Beads, 80% ethanol solution (freshly prepared) and nuclease-free water
- Target Capture Box #1→ Block #1, Block #2, Block #3, Capture Probe #2
- Target Capture Box #2→ Library Hyb Buffer
- Target Capture Box #3 → CES Capture Probe #1

[Overview]

To perform hybridization, three parts are needed:

1. Block reagents mixed together with the DNA library
2. Library Hyb buffer
3. Capture probe mix targeting the desired region to be sequenced

The three components are sequentially incubated in a thermal cycler (either in a 96-well PCR plate or in multiple 0.2 ml 8-well PCR strips) and later mixed together in a specific order as displayed in Figure 3. At the end of the procedure, all reagents will be in column 3.

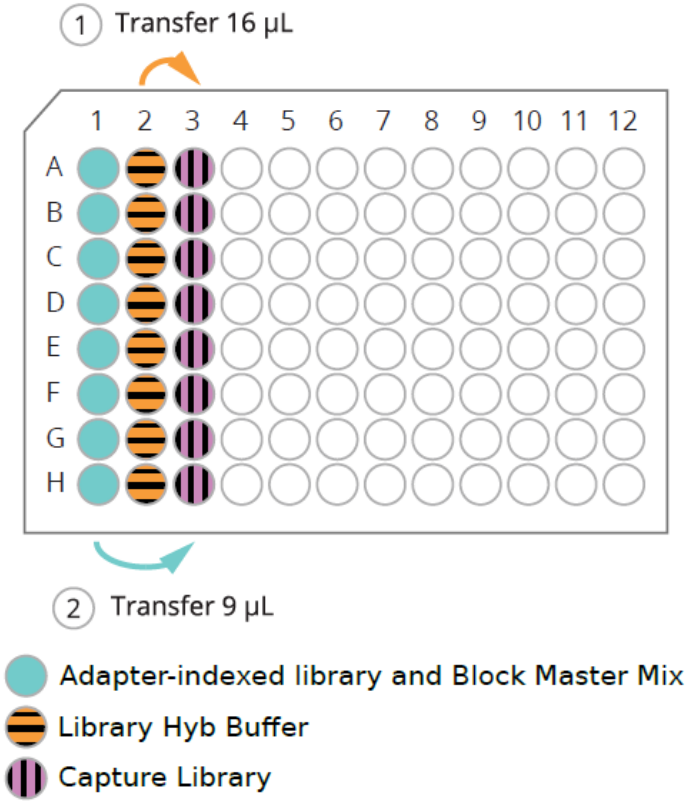


Figure 3. Overview of final plate setup

[Procedure]

1. Preparation of Block Mix with amplified Indexed Library

Note: The required amount of DNA for hybridization is **500 ng** per sample if using a vacuum concentrator, or **1000 ng** if none is available. Do not exceed this amount per sample. If less input for hybridization is used, final library yield will be reduced and the sensitivity of detection of low frequency variants might be decreased. In that case, use as much as possible (up to the required amount), for each sample to be pooled.

Important: For the preparation of the Block Mix with amplified Adapter-Ligated DNA library follow Step 1.1 if you have a vacuum concentrator or 1.2 if you do not.

1.1 *Block Mix procedure with vacuum concentrator*

Table 8-1. Pre-capture pooling for hybridization

Number of samples	Sample amount (ng) for each sample	Total amount after pooling (ng)
4	500	2000

a. Based on the concentration of the sample from PCR 1, add the corresponding volume **of 500 ng of 4 amplified Indexed Libraries** into a new tube compatible with the vacuum concentrator (either 1.5 ml LoBind tube or 0.2ml PCR strip).

b. Prepare Block Mix as shown in Table 9-1.

Table 9-1 Block Mix (with vacuum concentrator)

Reagent	Volume for 1 capture pool (µl)
Block #1	2.5
Block #2	2.5
Block #3	0.6
Total volume	5.6

c. **Add 5.6 µl of Block Mix** to each tube containing the library pool of 4 samples.

d. **Completely dry** the amplified indexed library and Block Mix using a vacuum concentrator at ≤ 45°C. (Depending on the total volume this should take between 30-60 minutes).

e. **Add 9 µl of nuclease-free water** into the tube to resuspend the completely dried sample-blocker mix. Pipet up and down 5-10 times to carefully mix and spin down briefly.

Note: If needed, transfer the entire Block Mix DNA library (9 µl) into a new 8 strip PCR tube (or 96-well PCR plate), seal and spin down briefly.

f. Keep the PCR tube (or plate) **on ice** before hybridization. Continue to step 2.

1.2. *Bead concentration procedure without vacuum concentrator*

Table 8-2. Pre-capture pooling for hybridization

Number of samples	Sample amount (ng) for each sample	Total amount after pooling (ng)
4	1000	4000

a. Let the Clean-up Beads come to room temperature for at least 30 minutes. Mix well by vortexing so that it appears homogenous and consistent in color.

b. If the resulting volume of the pooled library is below 40 µl, add nuclease-free water to have a final volume of 40 µl.

c. **Add 1.8x volume of Clean-up Beads (e.g. 72 µl beads to 40 µl library pool)** directly into the library pool. Mix well by pipetting/vortexing and spin down briefly (make sure not to pellet the beads, the solution has to be homogeneous). **Incubate at room temperature for 5 minutes**.

d. Place the tube in a magnetic separator for **3-5 minutes** or until the solution is clear.

e. Keep the tube in the magnetic separator and discard the supernatant (do not touch the beads as this might result in sample loss).

f Keeping the tube in the magnetic separator, **add 200 µl fresh 80% ethanol** to each sample tube.

g **Incubate for 30 seconds at room temperature** and then discard the 80% ethanol (be careful not to touch the beads while you remove the supernatant).

h. Repeat 80% ethanol wash (steps f-g) one more time **for a total of two washes**.

i. Spin down and place the tube in the magnetic separator and discard residual ethanol with a P10 or smaller pipet.

j. **Dry the sample tube at room temperature for 2 minutes** or until residual ethanol has completely evaporated (avoid drying beads until pellet appears cracked, as excessive drying decreases elution efficiency).

k. **Add 8 µl of nuclease-free water** to the sample and mix well on a vortexer. Pulse-spin down and incubate the sample **at room temperature for 2 minutes**.

l. Place the tube in the magnetic separator for **2 minutes** until the solution is clear.

m. **Transfer the supernatant (7 µl)** into a new 1.5 ml LoBind Tube.

n. Determine the library concentration with a fluorometric method (e.g. Qubit).

1.3 *Block mix procedure without vacuum concentrator*

a. Prepare Block Mix as shown in Table 9-2.

Table 9-2. Block Mix (without vacuum concentrator)

Reagent	Volume for 1 capture pool (µl)
Block #1	2.5
Block #2	2.5
Block #3	0.6
Total volume	5.6

b. **Add 5.6 µl of Block Mix** into a new 8 strip PCR tube (or 96-well PCR plate).

c. **Add 3.4 µl of concentrated Indexed Library pool** (from step 1.2n, containing ca. 2000 ng total) to each well or tube containing Block Mix. If the DNA library pool amount exceeds 2000 ng in 3.4 µl, use the corresponding volume for 2000 ng and then fill up to 3.4 µl with water.

d. Mix well by pipetting.

e. **Seal the tubes (or plate)** and spin down briefly.

f. Keep the PCR tube (or plate) **on ice** before hybridization. Continue to step 2.

2. Preparation of Library Hyb Buffer (continue here for workflow with and without vacuum concentrator)

a. Pre-warm Library Hyb Buffer at 37°C at least 15 minutes before use.

Note: Check for any precipitation in the Library Hyb Buffer and make sure to properly mix directly before use.

b. Pipet 25 µl of Library Hyb Buffer per sample into a new 8 strip PCR tube called “B” (or 96-well PCR plate), seal and spin down briefly.

Note: the prepared Library Hyb Buffer at room temperature, do not place on ice.

3. Preparation of Capture Library Mix

Critical
- Thaw CES Capture Probe #1 on ice slowly.
- Do not vortex CES Capture Probe #1 and Capture Library Mix (tapping and pipetting are allowed) and put back at -90°C to -60°C after use.

The capture probes are shipped lyophilized, and need to be dissolved with nuclease-free water before their first use.

Add 33.6 µl of nuclease-free water to CES Capture Probe #1. Mix gently by pipetting up and down 5-10 times, incubate for at least 5 minutes on ice, and keep on ice at all times.

Important: 6 µl of the reconstituted solution will be used per hybridization reaction, store the remaining probe solution at -90°C to -60°C after use.

a. Prepare Capture Library Mix in a 1.5 ml LoBind tube on ice as shown in Table 10. Mix-well by gentle pipetting.

Table 10. Capture Library Mix (keep on ice)

Reagent	Volume for 1 capture pool (µl)
CES Capture Probe #1	6
Capture Probe #2	1
Total volume	7

b. **Put 7 µl of Capture Library Mix** into a new 8 strip PCR tube (or 96-well PCR plate), seal and spin down briefly.

c. Keep the Capture Library Mix tube **on ice** at all times.

4. Perform the hybridization reaction in a thermal cycler.

a. Set up the following program on a thermal cycler (lid temperature: **105°C**).

Table 11. Hybridization Program

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

b. Put the Block Mix with indexed library tube (or plate) in the thermal cycler (e.g. in column 1) and start the Hybridization program.

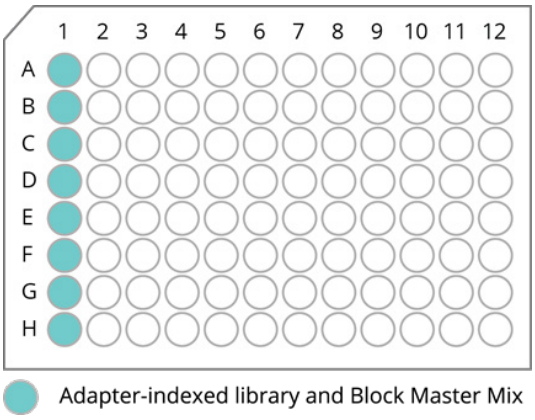


Figure 4. Adapter-Indexed DNA library and Block Master Mix in 'Column 1' (total 9 µl).

Note: From this step on, always keep tubes or plate in the thermal cycler during the entire procedure.

c. Once the temperature of the thermal cycler reaches 65°C, place the previously prepared **Library Hyb Buffer** tube (**Step 2b**)* into the thermal cycler (e.g. column 2, see Figure 5) and incubate at **65°C** for **3 minutes**.
*When using a 96-well PCR plate, transfer Library Hyb Buffer to an empty column (e.g. column 2) and completely seal the PCR plate quickly.

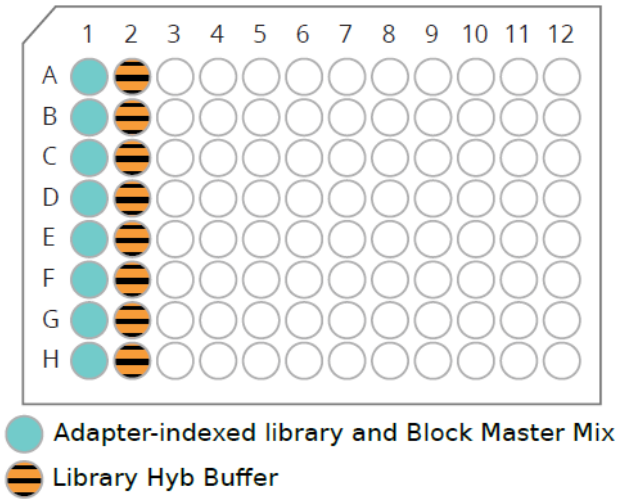


Figure 5. Library Hyb Buffer in “Column 2”.

d. After the incubation of Library Hyb Buffer at 65°C for 3 minutes, put the previously prepared **Capture Library Mix PCR tube*** (**Step 3c**) into the thermal cycler (e.g. column 3, see Figure 6) and incubate at **65°C** for **2 minutes**.

*When using a 96-well PCR plate, transfer 7 µl Capture Library Mix to an empty column (e.g. column 3) and completely seal the PCR plate quickly.

After Step d, there should be three lanes of reagents in the thermal cycler, all at 65°C.

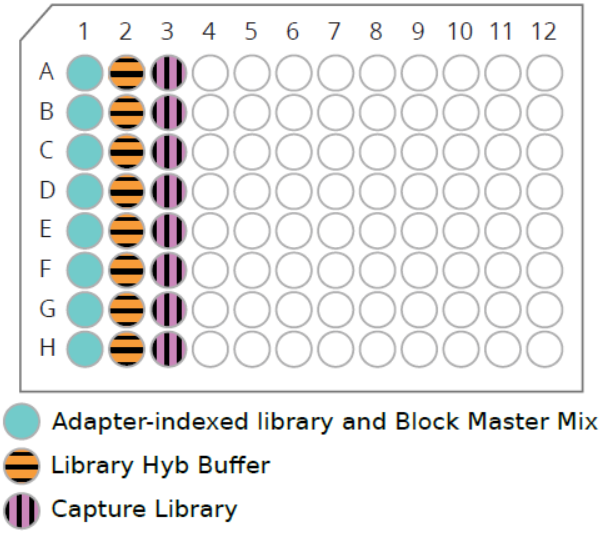


Figure 6. Capture Library in “Column 3”.

Note

- Subsequent steps must be performed quickly and efficiently from the moment the cap (or plate sealing film) is opened. Prolonged exposure to high temperature causes evaporation of reagents.
- Prepare a new cap (or a new sealing film) for the final hybridization step (h) and set the volume of a pipette (or a multichannel pipette) to 16 µl.

Caution: The PCR lid is very hot and serious burns can occur. Avoid touching the lid or PCR block as much as possible.

e. **Open** the thermal cycler and remove the plate seal/tube caps of the appropriate columns.

f. Quickly transfer **16 µl** of the **Library Hyb Buffer** (**column 2**) into the Capture Library Mix (column 3) and mix well by pipetting up and down 2-3 times (Figure 7. ①). Avoid generating air bubbles.

g. Transfer **9 µl** (entire amount) of **Block Mix with DNA library** (**column 1**) into column 3 containing the remaining reagents and mix well by pipetting up and down 5-10 times (Figure 7. ②). Avoid generating air bubbles.

h. **Quickly seal** the PCR tube (or PCR plate) with a new cap (or a new sealing film) completely and continue incubation at **65°C overnight** (**15-18 h, optimal time: 16 h**). All reagents should now be combined in 1 tube per sample (e.g. column 3). Remove all empty PCR tubes, if necessary.

i. Make sure that the PCR tube (or PCR plate) is completely sealed and the lid temperature is 105°C.

Important: The volume of hybridization mixture should be 29 to 32 µl, depending on the degree of evaporation during incubation.

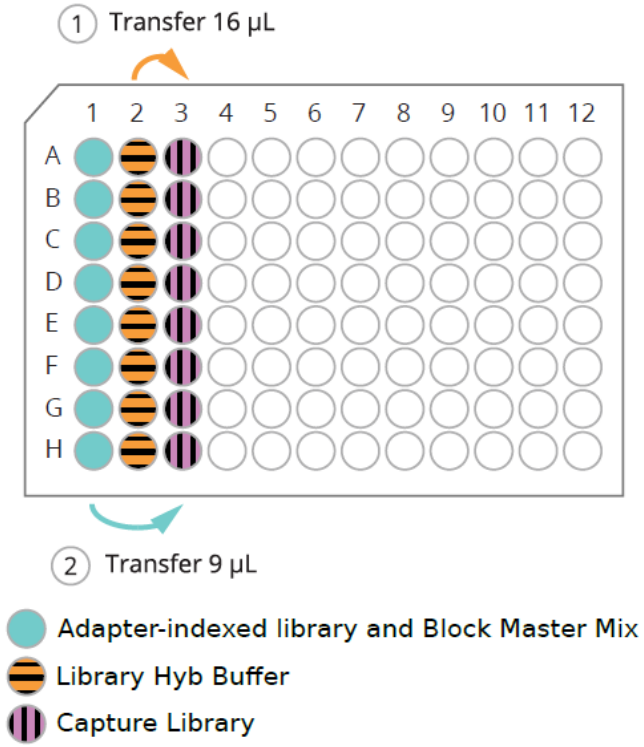


Figure 7. Transfer 16 µl from Column 2 to Column 3 and 9 µl from Column 1 to Column 3.

Step 2. Preparation of Streptavidin Beads

[Required Material / Preparation]

- Keep the hybridization reaction at 65°C at all times.
- The preparation of the Streptavidin Beads takes ca. 30 minutes and should be started before the hybridization reaction is complete.
- Target Capture Box #2→ Wash Buffer #1, Wash Buffer #2, Wash Buffer #3
- Streptavidin beads
- Nuclease-free water
- Preheat thermo-mixer at 70°C. Measure that temperature is accurate with a thermometer.

[Procedure]

1. **Pre-heat Wash Buffer #3 at 70°C** in the thermo-mixer for the next step: Step 3. Selection of the Target Captured Library.
2. Vortex Streptavidin beads to achieve a homogeneous solution.
3. Prepare **50 µl of the Streptavidin beads** per sample pool in a new 1.5 ml LoBind tube.
4. Wash the Streptavidin beads as follows:
    a. **Add 200 µl of Wash Buffer #1** per sample pool to each tube containing the Streptavidin beads.
    b. Mix well by vortexing/pipetting and spin down briefly.
    c. Put the tube in the magnetic separator and incubate for **2-4 minutes** or until the solution is clear.
    d. Keep the tube in the magnetic separator and discard the supernatant (be careful not to touch the beads while removing the supernatant).
    e. Remove the tube from the magnetic separator and repeat **steps a-d** two more times (for a **total of 3 times**).
5. **Add 200 µl of Wash Buffer #1** to the beads, mix well and spin down briefly.

Step 3. Selection of Target-Captured Library

[Procedure]

1. Keep the sample-hybridization mixture from **Part 2, Step 1. Hybridization of the Library** in the thermal cycler at 65°C.
2. Gently unseal the PCR tube (or PCR plate) in the thermal cycler and **immediately transfer all of each sample-hybridization mixture** (**29-32 µl depending on evaporation**) to the corresponding bead solution from **Part 2**, **Step 2.5**. Immediately invert the tube 3-5 times and mix thoroughly by pipetting 5-10 times.
3. **Rotate the sample** in a rotator (25 rpm) for **30 minutes at room temperature**. Check occasionally that the liquid is moving well within the tube and that the beads do not dry.

   Important: Do not vortex the tube vigorously, as it may damage the probes.
4. Spin down briefly.
5. Put the tube in a magnetic separator for **2-4 minutes** or until the solution is clear. Carefully remove the supernatant.
6. Take the tube out of the magnetic separator and add **500 µl** (180 µl for PCR tube or 96-well plate) of **Wash Buffer #2** to each tube. Mix well on a vortexer and pulse-spin down.
7. Incubate the sample for **15 minutes at room temperature** and occasionally mix by vortexing or incubate on a rotator (25 rpm).
8. Briefly mix the sample on a vortexer and spin down briefly. Put the tube in the magnetic separator for **2-4 minutes** or until the solution is clear and remove the supernatant.
9. High stringency wash with Wash Buffer #3 at 70°C:

   Important: Work fast, keep the sample and Wash buffer #3 at 70°C as much as possible and keep times at room temperature as short as possible.

   a. **Add 500 µl** (180 µl for PCR tube or 96-well plate) of 70°C pre-heated Wash Buffer #3 to each tube and mix well on a vortex mixer. Spin down briefly.

   b. **Incubate the solution for 10 minutes at 70°C** in a thermo-mixer at 500 rpm. Check every 2-3 minutes if the solution is homogenous, and gently mix by tapping if necessary.

   c. Briefly mix the sample by gentle vortexing or pipetting 5-10 times and spin down.

   d. Put the tube in the magnetic separator until the solution is clear and carefully remove the supernatant.

   e. Repeat washing steps **a-d** twice with Wash Buffer #3 for a **total of 3 washes**.

   f. Spin down the tube for 3-5 seconds and put in the magnetic separator. Remove any residual liquid completely with a P10 or smaller pipette.

      Important: Wash Buffer #3 must be completely removed.

g. Add **30 µl of nuclease-free water** to each tube, resuspend the beads well and spin down briefly. This now contains the target enriched DNA bound to magnetic beads. **Do not discard the beads!** Continue directly to the next step.

Part 3. Amplification of Target-Captured Library

Step 1. Amplification of the Target-Captured Library

[Required Material]

- Target Capture Box #1 → Post Capture PCR Forward Primer, Post Capture PCR Reverse Primer
- 2x PCR Mix
- 0.2 mL PCR tubes and caps

Note: In this step only half of the on-bead captured DNA will be used for the amplification reaction. Store the remaining half at -30°C to -15°C as a safety backup.

Important: Combine all reagents on ice.

[Procedure]

a. Mix bead-library solution from Part 2, Step 3 point “g” well by pipetting up and down 5-10 times, and **transfer 15 µl of homogenous solution** into a new 0.2 mL PCR tube. Store remaining bead-DNA solution at -30°C to -15°C.

b. Prepare a PCR master mix as described in Table 12 (add 0.5x additional amount of reagents for multiple library pools).

Table 12. Composition of PCR mix (setup on ice)

Reagent	Volume for 1 Library pool (µl)
Nuclease-free water	5
Post Capture PCR Forward Primer	2.5
Post Capture PCR Reverse Primer	2.5
PCR Polymerase	25
Total volume	35

c. Add **35 µl of the PCR mix** to each PCR tube and mix well by pipetting up and down 5-10 times. Spin down briefly and keep on ice.

d. Run the PCR program shown in Table 13 in a thermal cycler.

Note: At the end of the PCR program, the beads will have settled to the bottom of the tube. This is normal and does not impact the overall performance, as long as the input was homogenous at the start of the PCR.

Table 13. PCR program (lid: 105°C, vol = 50 µl, limit ramp rates to max. 4ºC/sec for heating and max. 2.5ºC/sec for cooling)

Step	Temperature	Time
Step 1	98°C	45 seconds
Step 2	98°C	15 seconds
Step 3	60°C	30 seconds
Step 4	72°C	1 minute
Step 5		Repeat step 2 to step 4 for a total of 10 cycles*
Step 6	72°C	10 minutes
Step 7	4°C	Hold

* We recommend 10 cycles for the first experiment. If final library yield in Part 3 Step 3 is > 20 ng/µl, PCR cycles for the next experiments could be reduced to 9 cycles. Optimal final library concentrations should be between 5-20 ng/µl.

Step 2. Bead clean-up of Amplified Target-Captured Library

[Required Material]

- Clean-up Beads
  Important: Keep the beads at room temperature for at least 30 minutes before use. Store Clean-up Beads at +2°C to +8°C after use and do not freeze.
- 80% ethanol solution (always prepare fresh on day of experiment, e.g. 8 ml 100% EtOH + 2 ml nuclease-free water)
- Nuclease-free water

[Procedure]

1. Let the Clean-up Beads come to room temperature for at least 30 min. Mix well so that they appear homogenous and consistent in color.
2. **Add 90 µl Clean-up Beads** (**1.8x volume**) directly to the **50 µl amplified captured DNA library**. Mix well by pipetting/vortexing and spin down briefly (make sure not to pellet the beads, they need to stay homogeneously in solution). **Incubate at room temperature for 5 minutes**.
3. Place the tube in a magnetic separator for **3-5 minutes** or until the solution is clear.
4. Keep the tube in the magnetic separator and carefully discard the supernatant (do not touch the beads as this might result in sample loss).
5. Keeping the tube in the magnetic separator, **add 200 µl fresh 80% ethanol** to each sample tube.
6. **Incubate for 30 seconds at room temperature** and then discard the 80% ethanol (be careful not to touch the beads while you remove the supernatant).
7. Repeat the 80% ethanol wash (step 5-6) one more time **for a total of two washes**.
8. Spin down briefly, put the tube back into the magnetic separator and discard residual ethanol with a P10 or smaller pipet.
9. **Dry the sample tube at room temperature for 3-5 minutes** or until residual ethanol has completely evaporated (avoid drying beads until beads appear cracked, as this might reduce elution efficiency).
10. **Add 21 µl of nuclease-free water** to the sample and mix well on a vortexer. Pulse-spin down and incubate the sample **at room temperature for 2 minutes**.
11. Place the tube in the magnetic separator for **2 minutes** until solution is clear.
12. **Transfer the supernatant** (**20 µl**) to a new 1.5 ml LoBind Tube. This is the final target-enriched library.

Stopping Point: Samples can be stored at -30°C to -15°C here.

Step 3. Quality Assessment and Quantification of Final Libraries

[Procedure]

1. Quantify the final, target-enriched Libraries from Part 3, Step 2.12 using a fluorometric method such as the Qubit dsDNA High Sensitivity kit.

2. Additionally, determine the average fragment size with instruments such as TapeStation, Bioanalyzer or Fragment Analyzer (expected: single peak between 200-600 bp and the maximum at ca. 350-450 bp).

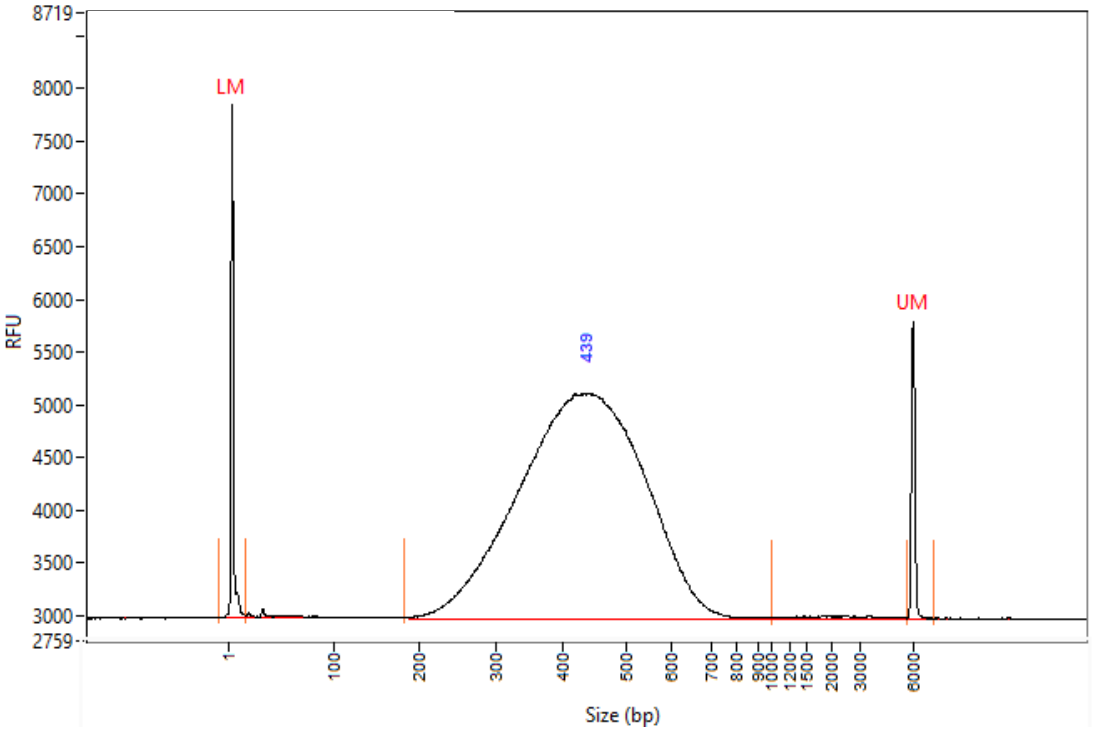


Figure 8. The electrophoresis diagram shows a peak in the region of 439 bp of the captured library.

In addition, none or only minimal primer dimers at ca. 120 bp should be visible (see Figure 8). If a clear primer dimer peak is visible, an additional bead clean up step using a [bead] : [sample] ratio = 0.8 : 1 is recommended (see **Appendix**).

Accurate quantification and at least 4 nM of each final library are required for sequencing. A template for calculation of final library molarity is provided in the NGS assays support section at www.viennalab.com//support/ngs-assays.

Instruction for Pooling and Illumina Sequencing

In this step, all target captured samples which are intended to be sequenced together are pooled.

Critical: Only samples with a unique index combination can be pooled and sequenced at the same time.

Note: The final library sizing profile represents the DNA insert length plus the length of the adapter sequences (approximately 120 bp for dual indexed adapters). For example, this means that the actual average DNA fragment length, that contains information about the sample, is ca. 320 bp, although the final library size is 440 bp.

For Illumina sequencing, 2x150 bp paired end sequencing mode is recommended. In case the average fragment size of the final library is <325 bp, 2x100 bp paired end sequencing can also be used. The number of samples which can be sequenced together depends on the desired target coverage and the Illumina machine available. In more detail, in case of testing for hereditary mutations with the Clinical Exome Sequencing (CES) NGS Assay, 100x average sequencing coverage (with >95% of targets at 30x) is recommended and can be achieved with 30-40 M read pairs (60-80 M reads) per sample. For example, a Novaseq SP (2x150 bp) flow cell allows pooling of at least 16 samples.

Refer to the NGS assays support section at www.viennalab.com//support/ngs-assays for help in preparing information for the sample sheet required for sequencing.

Bioinformatic Analysis

NGS sequencing data can be analysed with ViennaLab's proprietary bioinformatic analysis software: GENOVESA. We recommend to access our bioinformatic solution GENOVESA from a computer with at least 6 GB of RAM, a screen resolution of at least 1920x1080 (Full HD) and a minimum internet upload/download speed of 10Mbps.

Getting started

Access the software through the weblink **https://ngs-genovesa.biovendor.group**, choose ViennaLab NGS Assay and **complete the registration form**. Note that it is possible to register up to 3 users and get shared access to the uploaded data of the main account. Once your account has been approved, log in into GENOVESA with your username (email) and password. It is recommended to initiate account registration in advance.

Managing projects

After successful log in into the platform, click "Add project" to create a new project and upload fastq files by choosing the appropriate analysis workflow depending on the purchased kit. Once upload is completed, analysis will start automatically. All projects are displayed in the **"Projects"** table together with the analysis status and project details. More information about the uploaded samples can be accessed by clicking on the corresponding project once processing of the data has finished.

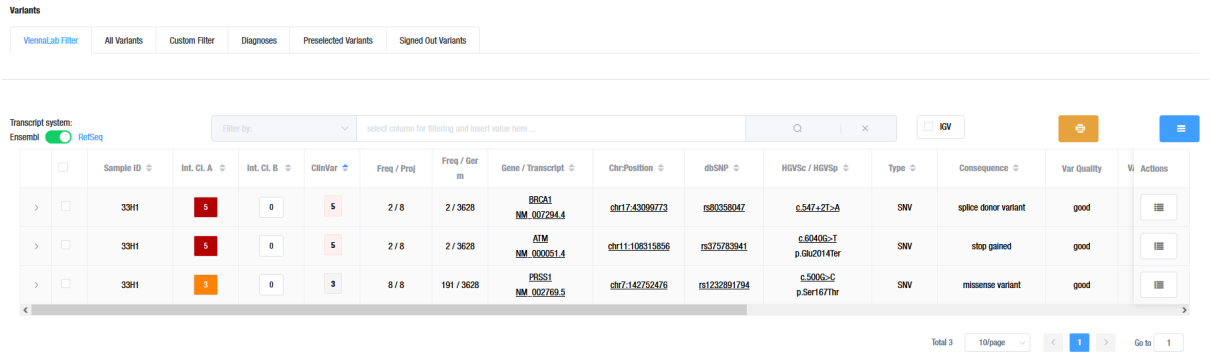
Examining your data

The "**Samples of the project" section** contains information about the sample status, sequencing statistics and the Quality Control (QC) of the data. The "Actions" field on the right allows inspection of detailed Mapping QC and FASTQ QC data, as well as management of sample relevant information.

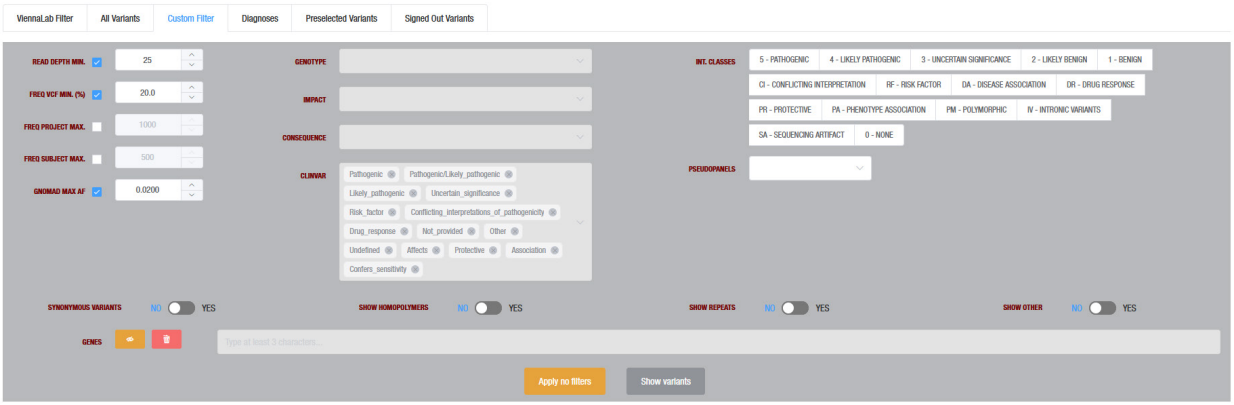
ID		SampleID	Status	Total reads	Total Variants	Median depth	% Duplicates	% Depth 30X	% Depth 100X	Actions
7171		21S8	PASSED	2 850 684	64	916	23	99	99	
7172		21S10	PASSED	3 499 112	63	1142	22	99	99	
7555		36S3	PASSED	2 674 850	107	805	13	99	98	

Assessing variants

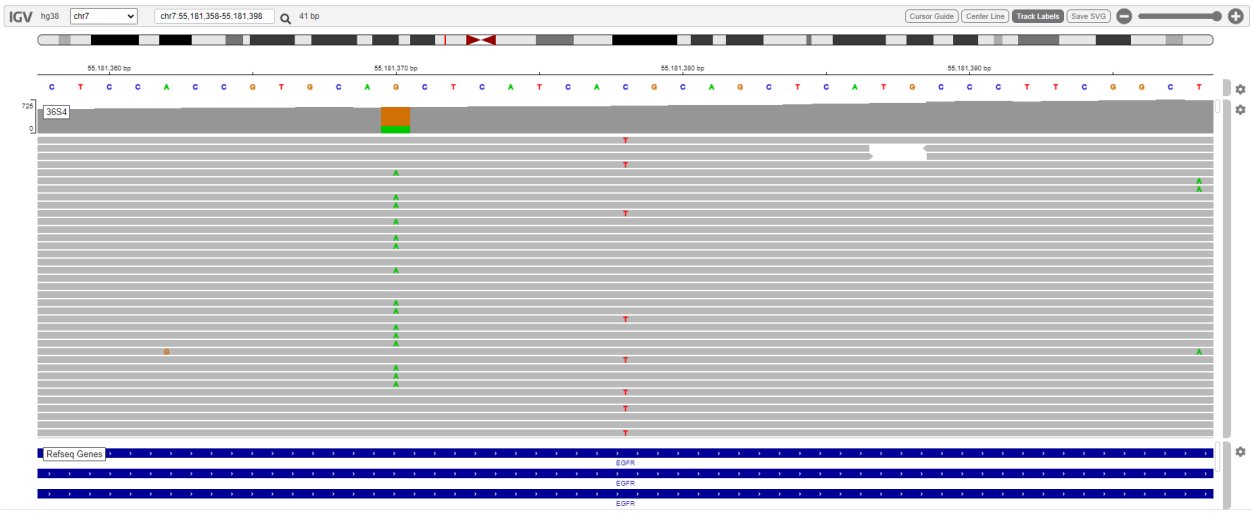
Every assessed sample has the potential to carry clinically relevant variants. The **"Variants" section** contains a list of variants found in a particular sample, including useful information from various databases. In particular, this section comprises different sub-sections such as:



- **ViennaLab Filter**: shows a list of variants after pre-set filters optimized for simplified and time-saving identification of clinically relevant variants were applied.
- **All Variants**: contains an unfiltered list of variants.
- **Custom Filter**: allows flexible tailoring of various filtering parameters.
- **Preselected Variants**: Allows the preselection of a subset of variants by the user before being finalized into the report. This might be helpful especially in case of a large number of identified variants (e.g. Clinical Exome Sequencing NGS Assay data).

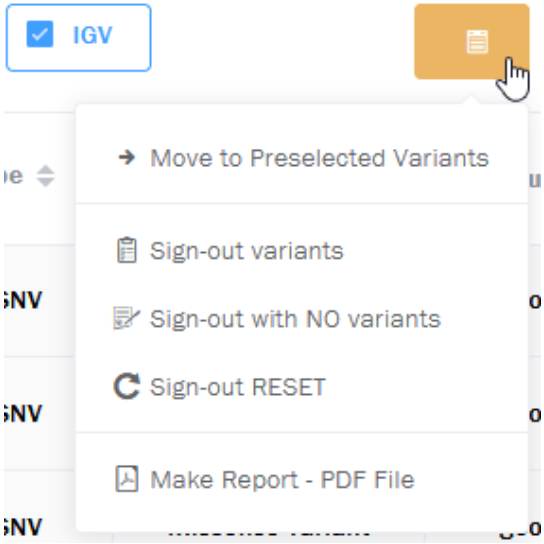


Visual display and inspection of SNVs and InDels through a one-click integrated viewer is achieved by ticking the “IGV” box and clicking on the IGV symbol below the chromosomal coordinate of the variant of interest.



Variant reporting

Relevant **variants are scored** by up to two users in the “Internal Classification by user A (Int. Cl. A)” and B column utilizing the provided information. Clinically significant variants which should be included in the **genetic variant report need to be selected and signed out**. Do this by ticking the box on the left of the variant, then click on the document symbol, select “Sign-out variants” and “Make Report” in the drop-down menu.



Additional remarks

Copy number variation (CNVs) and structural variation (SVs) analysis and visualization are available upon request for the Hereditary Cancer NGS Assay and Somatic Mutations NGS Assay, respectively.

For technical support on our NGS products, including troubleshooting of the experimental library preparation as well as help with the bioinformatic analysis and requests for CNVs and SVs analysis contact ViennaLab through the local distributor or directly at techhelp@viennalab.com.

Appendix

Removal of Primer-Dimers

Note: Detection of primer-dimers can be done by gel-electrophoresis based instruments such as TapeStation, Fragment Analyzer and Bioanalyzer.

1. If the primer-dimer peak is clearly visible at ca. 125 bp, an additional bead clean-up step is recommended.

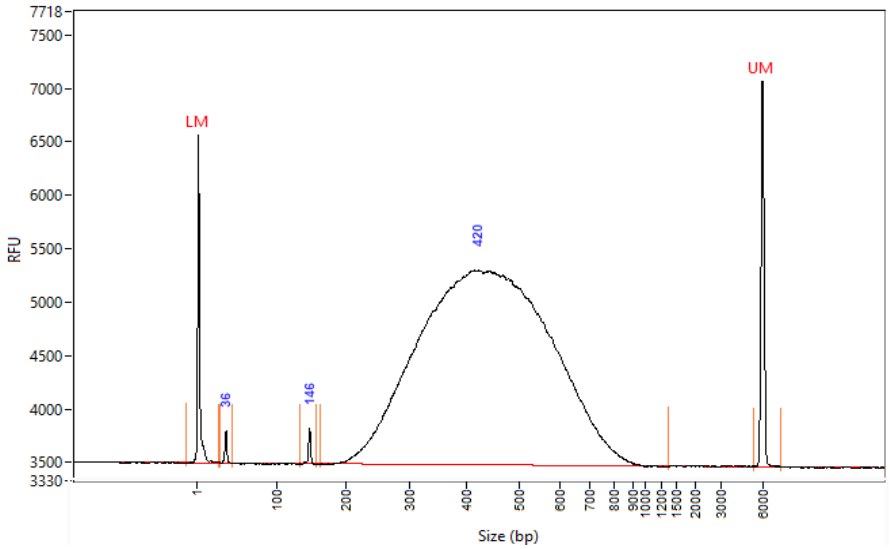


Figure 9-1. The electrophoresis diagram shows the primer-dimer peak at 146 bp.

2. Purify the sample using the procedure shown in **Part 1, Step 5** with a [bead] : [DNA sample] = 0.8 : 1.

3. Confirm the removal of primer-dimers through available instrumentation and re-quantify the purified final libraries.

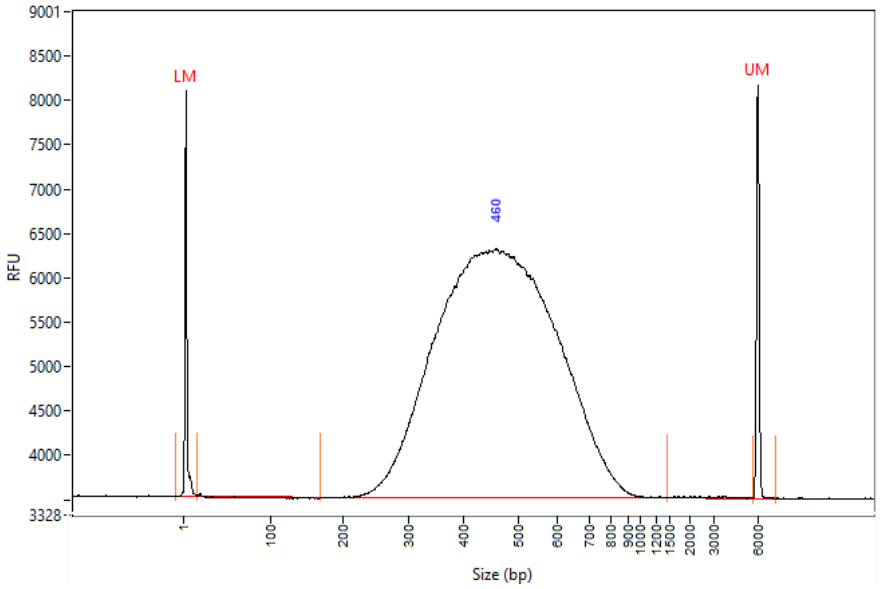


Figure 9-2. The electrophoresis diagram shows that most primer-dimers at 146 bp were removed successfully.

Revision History

Version	Date	Description
rev 1.0	2022-07	Initial version

Notes

Notes

REF		Σ
9-221	Hereditary Cancer NGS Assay	16 reactions
9-231	Somatic Mutations NGS Assay	16 reactions
9-241	Clinical Exome Sequencing (CES) NGS Assay	4x4 reactions

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