HHS Public Access

Author manuscript

Cold Spring Harb Protoc. Author manuscript; available in PMC 2015 July 03.

Published in final edited form as:

Cold Spring Harb Protoc. ; 2015(7): pdb.prot084855. doi:10.1101/pdb.prot084855.

Whole-Exome Enrichment with the Roche NimbleGen SeqCap EZ Exome Library SR Platform

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Abstract

Multiple platforms are available for whole-exome enrichment and sequencing (WES). This protocol is based on the Roche NimbleGen SeqCap EZ Exome Library SR platform, which enriches for ~44 Mb of the human exonic regions. The SeqCap system uses 55- to 105-base DNA probes to capture known coding DNA sequences (CDS) from the NCBI Consensus CDS Database, RefSeq, and Sanger miRBase. The protocol can be performed at the benchside without the need for automation, and the resulting library can be used for targeted next-generation sequencing on an Illumina HiSeq 2000 sequencer.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Agarose gels, precast (E-Gel EX, 2% [Life Technologies G4020–02; 20 gels])

Agencourt AMPure XP magnetic bead-based purification system (Beckman Coulter A63881; 60 mL) Bioanalyzer reagents (Agilent)

DNA 1000 Kit (5067-1504)

High-Sensitivity DNA Kit (5067-4626)

COT human DNA, fluorometric grade (Roche 05 480 647 001)

dATP (100 mM; New England BioLabs N0440S)

Dynabeads M-270 Streptavidin (Life Technologies 653-06; 10 mL)

End-It DNA End-Repair Kit (Epicentre ER0720)

Ethanol, molecular biology grade (Sigma-Aldrich E7023)

Prepare a solution of 70% ethanol.

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Genomic DNA, human (high-quality, nondegraded, with an average fragment size of 40 kb or greater and an A260/A280 of 1.8–2.0)

Klenow Fragment (3′→5′ exo-) with NEB Buffer #2 (New England BioLabs M0212S)

LigaFast Rapid DNA Ligation System (Promega M8221)

MinElute PCR Purification Kit (QIAGEN 28006)

Nimblegen SeqCap EZ Human Exome Library v2.0 (Roche, 05 860 504 001; 48 reactions)

NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche 05 634 253 001; 96 reactions)

The hybridization and wash kit contains the following reagents: 10× buffer I, 10× buffer II, 10× buffer III, 10× stringent wash buffer, 2× hybridization buffer, hybridization component A, 2.5× bead wash buffer. Immediately before use (Steps 30–37), dilute the 10× wash buffers (I, II, III, and stringent) and the 2.5× bead wash buffer to 1× working solutions. Prepare the following volumes for each sample: 400 µL of 1× stringent wash buffer, 300 µL of 1× wash buffer I, 200 µL of 1× wash buffer II, 200 µL of 1× wash buffer III, and 500 µL of 1× bead wash buffer.

Nuclease-free water (UltraPure Distilled Water [Life Technologies 10977-015] or equivalent)

PE Adapter Oligo Mix (Illumina 1001782)

PE oligonucleotides

PE-HE1: 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T-3′ (1000 µM)

PE-HE2: 5′-CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC* T-3′ (1000 µM)

PE-POST1: 5′-AAT GAT ACG GCG ACC ACC GAG A-3′ (100 µM)

PE-POST2: 5′-CAA GCA GAA GAC GGC ATA CGA G-3 (100 µM)

PE-PRE1: 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T-3′ (100 µM)

PE-PRE2: 5′-CAA G CA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC* T-3′ (100 µM)

The asterisk symbolizes the addition of phosphorothioate bond between the last two deoxyribonucleotides to prevent degradation.

Phusion High-Fidelity PCR Master Mix (New England BioLabs F-531L; 500 reactions) TE buffer (Life Technologies 12090-015)

Equipment

2100 Bioanalyzer (Agilent)

Adhesive film (MicroAmp Clear [Life Technologies 4306311] or equivalent)

AFA microTUBES with snap caps (Covaris 520045)

DNA LoBind tubes, 1.5-mL (Eppendorf 022431021 or equivalent)

Heat blocks at 37°C, 47°C, 70°C, and 95°C

Laboratory stickers (see Step 27)

Magnetic plate (Dynal DynaMag-96 Side Magnet [Life Technologies 12331D] or equivalent)

Magnetic stand (Dynal DynaMag-2 [Life Technologies 12321D] or equivalent)

Microcentrifuge (Fisher Scientific accuSpin Micro 17 or equivalent)

Mini LabRoller (Labnet H5500 or equivalent)

PCR plates, 96-well (Agilent 410088 or equivalent)

PCR tubes, 0.2-mL (sterile)

Alternatively, the protocol can be performed using only 96-well PCR plates.

Pipette tips (sterile, nuclease-free with aerosol barrier)

Thermal cycler (DNA Engine Tetrad 2 [BioRad PTC-0240G])

Ultra-sonicator (Covaris S-series Single Tube Sample Preparation System, Model S2)

Set up the ultrasonicator following the manufacturer's instructions. Make sure the tank is filled with fresh deionized H_2O chilled to 2°C–5°C and degassed for least 30 min before use.

Vacuum concentrator (Thermo Scientific Savant SPD111V or equivalent) at 60°C

Vacuum manifold (QIAvac 24 Plus; QIAGEN 19413)

Vortex (Fisher Scientific 02-215-370 or equivalent)

METHOD

The general scheme of DNA preparation for hybridization-based whole-exome capture and sequencing is diagrammed in Figure 1. The following protocol is based on the original method provided by Roche (NimbleGen SeqCap EZ Exome Library SR User's Guide, version 2.2), with minor modifications to streamline the process based on our experience. A detailed performance evaluation of this method as well as a comparison to alternative platforms can be found in our previous publication (Clark et al. 2011); see Related Information.

Fragmenting Genomic DNA

1. In a 1.5-mL LoBind tube, dilute 3 µg of human genomic DNA in TE buffer to a total volume of 120 µL. Transfer the diluted DNA to an AFA microTUBE.
2. Load the microTUBE into the tube holder of the ultrasonicator and shear the DNA using the following settings: mode, frequency sweeping; duty cycle, 10%; intensity, 5; cycles per burst, 200; duration, 60 sec × 6 cycles; temperature, 4°C–7°C.
3. Transfer the sheared DNA to a fresh 1.5-mL LoBind tube. Check the shearing by running 2 µL of the sample on a 2% E-Gel EX gel.

   The average DNA size should be ~150–200 bp.

   It is safe to stop here and store the sample at −20°C.

Purifying DNA with Agencourt AMPure XP Magnetic Beads

4 Equilibrate the Agencourt AMPure XP reagent for at least 30 min to room temperature and mix well by vortexing.

5 Add 216 µL of homogenous AMPure XP reagent to the sheared DNA sample (~120 µL) and mix well by vortexing. Incubate the mixture on the Mini LabRoller at room temperature for 5 min.

6 Place the tube in the magnetic stand. Wait for the solution to clear (~2 min) and carefully discard the cleared solution without touching the beads.

7 Wash the beads twice with 500 µL of 70% ethanol for 1 min per wash.

8 Dry the beads for 5 min in a heat block at 37°C.

9 Add 34 µL of nuclease-free water to the beads and mix thoroughly by vortexing. Incubate the mixture on the Mini LabRoller for 5 min at room temperature.

10 Place the tube back into the magnetic stand and wait for the solution to clear (~2 min). Transfer the supernatant containing the eluted DNA to a fresh 1.5-mL LoBind tube.

It is safe to stop here and store the sample at −20°C.

Preparing the Adaptor-Ligated DNA Library

Repairing DNA Ends

11 For each sample of sheared DNA, prepare the following reaction in a 1.5-mL LoBind tube using the reagents provided in the End-It DNA End-Repair Kit.

Reagent	Amount to add
DNA sample from Step 10	34 µL
10× End-Repair Buffer	5 µL
2.5 mM dNTP mix	5µL

Reagent	Amount to add
10 mM ATP	5μL
End repair-enzyme mix	1μL
Total volume	50 μL

12 Incubate the reaction for 45 min at room temperature, and then inactivate the enzymes for 10 min at 70°C.

13 Purify the end-repaired DNA using one MinElute column according to the manufacturer's instructions. Briefly, add 250 μL of Buffer PB to the reaction, mix well, load the mixture onto a MinElute column and filter on the QIAvac 24 Plus vacuum manifold, wash the column with 750 μL of Buffer PE, centrifuge the column at maximum speed (17,000g) for 1 min, and elute the DNA in 32 μL of Buffer EB.

Adding an Adenine Base to End-Repaired Fragments

14 For each sample, prepare the following reaction in a 0.2-mL PCR tube.

Reagent	Amount to add
DNA sample from Step 13	32 μL
NEB Buffer #2	5μL
1 mM dATP	10μL
Klenow fragment (3′→5′ exo-; 5 U/μL)	3 μL
Total volume	50 μL

15 Incubate the reaction in a thermal cycler for 30 min at 37°C.

16 Purify the DNA using a MinElute column as described in Step 13, but elute the purified DNA in 19 μL of Buffer EB.

Ligating the Paired-End Adaptor

17 For each sample, prepare the following reaction in a 1.5-mL LoBind tube.

Reagent	Amount to add
DNA sample from Step 16	19 μL
2× Rapid Ligation Buffer (LigaFast)	25 μL
PE Adaptor Oligo Mix (15 μm)	1 μL
T4 DNA ligase (3 U/μL) (LigaFast)	5 μL
Total volume	50 μL

18 Incubate the reaction for 15 min at room temperature.

19 Purify the ligated DNA with AMPure XP beads as described in Steps 4–10, with the following modifications: add 90 μL of homogenous AMPure XP reagent to the reaction, and elute the purified DNA in 30 μL of nuclease-free water.

Amplifying the Adaptor-Ligated Library

20 For each sample, prepare two identical reactions in 0.2-mL PCR tubes as follows.

Reagent	Amount to add
Adaptor-ligated library DNA from Step 19	15 µL
Phusion high-fidelity PCR master mix (2×)	50 µL
Nuclease-free water	31 µL
PE-PRE1 (100 µm)	2 µL
PE-PRE2 (100 µm)	2 µL
Total volume	100 µL

21 Place the tubes in a thermal cycler and amplify the library DNA using the following program: 30 sec at 98°C, 12 cycles of (10 sec at 98°C, 30 sec at 65°C, 30 sec at 72°C), 5 min at 72°C.

22 Combine the two reactions for each sample for a total volume of 200 µL. Purify the amplified sample library using one MinElute column according to the manufacturer's instructions. Briefly, add 1000 µL of Buffer PB to the reaction, mix well, and load the mixture onto a MinElute column in multiples of 750 µL and filter on the QIAvac 24 Plus vacuum manifold. Wash the column with 750 µL of Buffer PE, centrifuge the column at maximum speed (17,000g) for 1 min, and elute the DNA in 50 µL of nuclease-free water.

23 Check the quality of the amplified preenrichment library by running 1 µL on a DNA 1000 chip using a 2100 Bioanalyzer following the manufacturer's instructions. Record the measured concentration of the library.

The amplified library should have a peak size of ~350 bp.

It is safe to stop here and store the sample at −20°C.

Hybridizing the Library

24 For each sample, aliquot 4.5 µL of the SeqCap EZ Human Exome Library and thaw on ice.

25 Add the following reagents to a 1.5-mL LoBind tube.

Reagent	Amount to add
COT DNA (1 mg/mL)	5 µL
Amplified library DNA from Step 22	2 µg
1,000 µm PE-HE1	1 µL
1,000 µm PE-HE2	1 µL

26 Close the cap of the tube and poke a hole in the center of the tube with a 21-gauge needle. Lyophilize the mixture in a vacuum concentrator (~30 min) at 60°C.

27 Add 7.5 µL of 2× hybridization buffer and 3 µL of hybridization component A to the lyophilized sample and pipette up and down to mix well. Seal the hole in the cap with a laboratory sticker, vortex the tube for 10 sec and centrifuge at maximum speed for 10 sec.

28 Denature the mixture in a heat block for 10 min at 95°C. Centrifuge at maximum speed for 10 sec at room temperature to collect the denatured sample.

29 Transfer the denatured sample to a 0.2-mL PCR tube or a well in a 96-well PCR plate. Add 4.5 µL of the SeqCap EZ Human Exome Library, mix thoroughly by pipetting up and down 10 times, and incubate for 72 h at 47°C in a thermal cycler with the heated lid set to 57°C. Complete Steps 30–33 immediately before the end of this incubation and then proceed to Step 34.

It is critical to keep the temperature of the hybridization mixture at 47°C before Step 34.

Performing Hybrid Capture Selection with Dynabeads M-270 Streptavidin

30 For each captured library, prewarm 400 µL of 1× stringent wash buffer and 100 µL of 1× Wash buffer I in separate tubes to 47°C in a heat block.

31 Equilibrate the Dynabeads M-270 Streptavidin for 30 min to room temperature. Thoroughly resuspend the beads by vortexing for 1 min. Transfer 100 µL of beads (per captured library) to a 1.5-mL LoBind tube.

Beads for up to six captures (600 µL) can be processed in one 1.5-mL LoBind tube.

32 Separate the Dynabeads using a magnetic stand and discard the supernatant when it becomes clear. Wash the beads twice with 200 µL of 1× bead wash buffer per wash. For each wash, vortex for 10 sec, separate the beads with a magnetic stand, and discard the supernatant when it becomes clear.

33 Resuspend the beads in 100 µL of 1× bead wash buffer (per captured library) and transfer to one well of a 96-well plate. Separate the beads on a magnetic plate, and discard the supernatant when it becomes clear.

34 Immediately following Step 33, transfer the hybridization mixture from Step 29 to the washed beads, and pipette up and down 10 times to mix. Seal the plate with adhesive film, and incubate the mixture for 45 min at 47°C in a thermal cycler with the heated lid set to 57°C. Briefly vortex the sample every 15 min to maintain the beads in suspension.

35 Once the incubation is complete, without separating the beads from the reaction, add 100 µL of preheated (47°C) 1× wash buffer I. Transfer the entire contents of the reaction to a 1.5-mL LoBind tube and vortex the mixture for 10 sec. Separate

the beads on a magnetic stand, and discard the supernatant when it becomes clear.

36 Wash the beads twice with 1× stringent wash buffer as follows.

- **i.** Add 200 µL of preheated (47°C) 1× stringent wash buffer to the beads and pipette up and down 10 times to mix.
- **ii.** Incubate the mixture for 5 min at 47°C.
- **iii.** Separate the beads on a magnetic stand, and discard the supernatant when it becomes clear.

37 Wash the beads as follows.

- **i.** Add 200 µL of room-temperature 1× wash buffer I to the beads and vortex for 2 min. Separate the beads on a magnetic stand, and discard the supernatant when it becomes clear.
- **ii.** Add 200 µL of room-temperature 1× wash buffer II to the beads and vortex for 1 min. Separate the beads on a magnetic stand, and discard the supernatant when it becomes clear.
- **iii.** Add 200 µL of room-temperature 1× wash buffer III to the beads and vortex for 30 sec. Briefly centrifuge the tube, separate the beads on a magnetic stand, and discard the supernatant when it becomes clear.

38 Add 50 µL of nuclease-free water to the beads.

There is no need to separate the beads before postcapture amplification (below).

It is safe to stop here and store the sample at −20°C.

Performing Post-Capture Amplification

39 For each sample, prepare two identical reactions in 0.2-mL PCR tubes as follows.

Reagent	Amount to add
Bead-bound captured DNA from Step 38	20 µL
Phusion high-fidelity PCR master mix (2×)	50 µL
Nuclease-free water	26 µL
PE-POST1 (100 µm)	2 µL
PE-POST2 (100 µm)	2 µL
Total volume	100 µL

40 Place the tubes in a thermal cycler and amplify the DNA using the following program: 30 sec at 98°C, 18 cycles of (10 sec at 98°C, 30 sec at 60°C, 30 sec at 72°C), 5 min at 72°C.

41 Combine the two reactions for each sample in a fresh 1.5-mL LoBind tube for a total volume of 200 µL. Separate the beads on a magnetic stand, and transfer the supernatant containing the amplified library to a fresh 1.5-mL LoBind tube when it becomes clear.

42 Purify the amplified library with AMPure XP beads as described in Steps 4–10, with the following modifications: add 360 µL of homogenous AMPure XP reagent to the reaction, and elute the purified DNA in 50 µL of QIAGEN buffer EB.

43 Check the quality of the final library as follows.

i. Run 1 µL on a high-sensitivity DNA chip using the Agilent 2100 Bioanalyzer following the manufacturer's instructions.

The amplified library should have a peak size of ~350 bp.

ii. Record the measured concentration of the library for reference when pooling multiple libraries.

We recommend pooling libraries based on their molar concentration as determined by the Bioanalyzer.

The library is now ready for paired-end sequencing on an Illumina HiSeq 2000 sequencer. Alternatively, the library can be placed at −80°C for long-term storage.

RELATED INFORMATION

Multiple platforms are available for WES, particularly for use with Illumina next-generation sequencers. In our previous publication (Clark et al. 2011), we compared the performance of three of the most popular hybridization-based whole-exome capture and sequencing platforms for the human exome, including the Roche NimbleGen system described here; protocols for the two other platforms are provided in Whole-Exome Enrichment with the Agilent SureSelect Human All Exon Platform (Chen et al. 2015a) and Whole-Exome Enrichment with the Illumina TruSeq Exome Enrichment Platform (Chen et al. 2015b). In summary, the performance of each platform was generally on par with the others, with specific, but minor, pros and cons. Researchers would be wise to choose among the platforms based on their specific experimental needs (e.g., genomic regions of interest, coverage vs. depth, etc.). The size of the targeted regions for each platform is flexible: By designing custom probes with Agilent, Roche, or Illumina, each protocol is suitable for enrichment of a lower number of custom targets for Illumina sequencing.

ACKNOWLEDGMENTS

This publication is supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under Award Number 3U54DK10255602S2.

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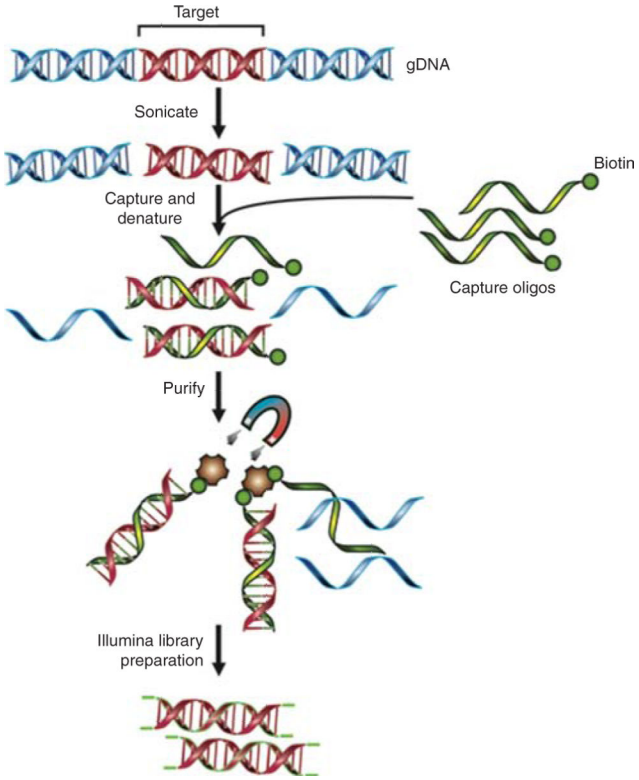

FIGURE 1.

Common scheme for hybridization-based whole-exome enrichment methods. In summary, genomic DNA is fragmented, denatured, and hybridized with capture oligos during library preparation for high-throughput sequencing. The captured sequences are then enriched with streptavidin-conjugated paramagnetic beads and further amplified before being subjected to Illumina sequencing. Diagrammed DNA sequences in red, on-target regions; sequences in blue, off-target regions; single-stranded oligos in green, capture probes labeled with biotin; brown particles, streptavidin-conjugated paramagnetic beads.