SMRT RenSeq protocol

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Method Article

Keywords: RenSeq, PacBio, SMRT sequencing, targeted enrichment

Posted Date: April 26th, 2016

DOI: https://doi.org/10.1038/protex.2016.027

Abstract

R gene enrichment and Sequencing \(RenSeq, Jupe et al. 2013) is a genome complexity reduction method which allows to enrich for nucleotide-binding, leucine reach repeat \(NLR) type plant disease resistance genes prior to sequencing. RenSeq was established and successfully used with Illumina platforms \(Jupe et al. 2013, Andolfo et al. 2014), however the repetitive nature of NLR genes hampered de novo assembly of this family. Here we describe a protocol which enables to prepare long enriched libraries that are suitable for Pacific Biosciences Single-Molecule Real Time \(SMRT) sequencing. Reads Of Inserts \(ROI) generated with this protocol are around 3-4 kb in length \(longer than the average NLR sequence). These long reads are especially well suited for de novo assembly of whole NLR genes including their regulatory elements \(Witek et al. 2016). The protocol presented here is not restricted to NLR genes; it can be used to generate long \(>3 kb) enriched libraries for any multigene family from any type of tissue and organism.

Introduction

This protocol is used to prepare long gDNA and cDNA libraries for targeted enrichment followed by PacBio SMRT sequencing. In the first step, Illumina adaptors are ligated to sheared template DNA, which allows subsequent amplification of long libraries prior to and post enrichment. The post-enrichment library is used as an input to PacBio SMRT library preparation. The protocol is designed to yield 3-5 kb libraries that can be sequenced with the PacBio P6-C4 chemistry \(>14 kb average read length). To avoid sequencing of smaller molecules \(<3 kb), an additional size selection step is recommended after shearing \(2.1.2). The target enrichment protocol is optimized for the MYbaits sequence capture kit, using halved reaction volumes; however, it can be simply adjusted to be compatible with other enrichment kits \(e.g. Agilent SureSelect).

Reagents

Except from standard laboratory reagents \(like molecular biology grade water, agarose gels, 96% ethanol), the following kits and reagents are necessary to complete this protocol: • NEBNext Ultra DNA Library Prep Kit for Illumina, New England Biolabs, Cat No. E7370S and NEBNext Multiplex Oligos for Illumina \(Index Primers Set 1), New England Biolabs, Cat No. E7335S • MYbaits customized target enrichment kits for next-gen sequencing, MYcroarray \(http://www.mycroarray.com/). Sequences of 20,000 Solanum baits can be found in attachment File 1. • KAPA HiFi HotStart ReadyMix, KAPA Biosystems, Cat. No. KK2601 • Agencourt AMPure XP Reagent, Beckman Coulter, Cat. No. A63880 • Dynabeads MyOne Streptavidin C1, Thermo Fisher Scientific, Cat. No. 65001 • SeqCap EZ Developer Reagent, Roche, Cat. No. 06684335001 • Covaris Red miniTUBEs \(5.0 kb), Covaris, Cat. No. 520066

Equipment

• 0.2 ml PCR tubes, 1.5 ml Eppendorf tubes • Magnetic particle collector for ~1.5 ml tubes • Covaris S2 sonicator or a different suitable model • Thermal cycler \(any model) • Eppendorf ThermoMixer • Water bath • Optionally: SpeedVac vacuum concentrator and a qPCR system

Procedure

Long library preparation In this step, long libraries are prepared and Illumina adaptors are ligated. **DNA shearing** We tested two Covaris-based options to shear DNA; Red miniTUBE \(this protocol) and gTUBE for longer fragments \(>5 kb). **1. Red miniTUBE on Covaris S2.** Shear 2-4 µg of gDNA with the following settings \(5 kb): • Intensity – 1 • Duty Cycle – 20% • Cycles per Burst – 1000 • Treatment time – 600 s • Temperature – 20°C • Water level – 15 • Sample volume – 200 µl Check whether DNA was sheared to the desired size on 0.8% agarose gel with a suitable DNA marker. **2. Purification and size selection on AMPure XP beads** This purification step includes also first size selection with 0.4X AMPure XP beads. • To the sheared DNA \(190 µl) in a sterile, nuclease-free Eppendorf tube, add 76 µl \(0.4X) resuspended by vortexing AMPure XP beads and mix well by pipetting up and down at least 10 times. • Incubate for 5 minutes at room temperature. • Spin briefly \(1-2 s) and place the tube on a magnetic stand to separate beads from supernatant. After the solution is clear \(about 5 minutes), carefully transfer the supernatant to a new tube. Be careful not to disturb the beads that contain the target DNA fragments. • Add 200 µl of freshly prepared 80% ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. • Repeat wash step twice, to three washes in total. • Briefly spin the tube \(1-2 s) and put it back on the magnetic stand. • Completely remove the residual ethanol and air dry beads for up to 10 minutes while the tube is on the magnetic stand with lid open. • Elute the DNA target from the beads with 65 µl nuclease-free water. Mix well by pipetting up and down and put the tube on the magnetic stand until the solution is clear. • Transfer the supernatant to a sterile, nuclease-free Eppendorf tube. Check size-selected fraction on 0.8% agarose gel \(it should contain fragments >2 kb). Size selection is optional for longer libraries \(>3 kb). **3. Enrichment library construction** To construct a DNA library for sequence capture, the NEBNext Ultra DNA Library Prep Kit for Illumina is used according to the manufacturer’s protocol, with minor modifications. The recommended input is 500 ng-1 µg of sheared and size-selected DNA. Lower amounts are acceptable, but will require some adjustments \(see NEBNext protocol for details). **Note:** Other kits can also be used, but check for compatibility with downstream oligo blocks and adaptors. **4. End repair** Before starting, make sure that DTT in End Repair Reaction buffer is fully dissolved. • Mix the following components in a sterile, nuclease-free Eppendorf tube: End Prep Enzyme Mix – 3.0 µl End Repair Reaction Buffer \(10X) – 6.5 µl Fragmented DNA – 55.5 µl ——————————- Total volume – 65 µl • Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube. • Incubate as below: 30 minutes at 20°C 30 minutes at 65°C 5 minutes on ice. **5. Adaptor ligation** • Add the following components directly to the End Prep reaction mixture and mix well: Blunt/TA Ligase Master Mix – 15 µl NEBNext Adaptor for Illumina – 2.5 µl Ligation Enhancer – 1 µl ——————————- Total volume – 83.5 µl • Mix by pipetting, followed by a quick spin to collect all liquid from the sides of the tube. • Incubate at 20°C for 30 minutes. • Add 3 µl of USER enzyme to the ligation mixture above. • Mix well and

incubate at 37°C for 20 minutes. **6. Library purification** For this step, use ~0.5X AMPure beads as there is no need to perform a more stringent size selection step here. • Add 43 µl resuspended by vortexing AMPure XP beads to the ligation reaction. Mix well by pipetting up and down at least 10 times. • Incubate for 5 minutes at room temperature. • Spin briefly \(1-2 s) and place the tube on a magnetic stand to separate beads from supernatant. After the solution is clear \(about 5 minutes), carefully transfer the supernatant to a new tube. Be careful not to disturb the beads that contain the target DNA fragments. • Add 200 µl of freshly prepared 80% ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. • Repeat wash step twice, to three washes in total. • Briefly spin the tube \(1-2 s) and put it back on the magnetic stand. • Completely remove the residual ethanol and air dry beads for up to 10 minutes while the tube is on the magnetic stand with lid open. • Elute the DNA target from the beads with 25 µl nuclease-free water. Mix well by pipetting up and down and put the tube on the magnetic stand until the solution is clear. • Transfer the supernatant to a sterile, nuclease-free Eppendorf tube. **7. PCR amplification** Mix the following components in a sterile, nuclease-free PCR tube: Adaptor Ligated DNA Fragments – 10 µl* 2X KAPA HiFi HotStart ReadyMix – 25 µl Index Primer – 1 µl Universal PCR Primer – 1 µl Water – 13 µl ——————————- Total volume – 50 µl PCR cycling conditions: Initial denaturation: 98°C, 4 minutes 8-15 cycles with the following settings: Denaturation: 98°C, 30 seconds Annealing: 65°C, 30 seconds Extension: 72°C, 4 minutes Final extension 72°C, 10 minutes. ==*== The amount of the library used for amplification may require optimization for different libraries. • First perform 8 cycles and run 5 µl of the reaction on 0.8% agarose gel next to 1 µl of unamplified library \(as a control of input DNA). In case there is not enough DNA, put the reaction back to thermocycler for additional 2-5 cycles. **8. Library size selection and purification** • Transfer the PCR reaction \(45 µl) to a sterile, nuclease-free Eppendorf tube, add 18 µl \(0.4X) of resuspended by vortexing AMPure XP beads and mix well by pipetting up and down at least 10 times. • Incubate for 5 minutes at room temperature. • Spin briefly \(1-2 s) and place the tube on a magnetic stand to separate beads from supernatant. After the solution is clear \(about 5 minutes), carefully transfer the supernatant to a new tube. Be careful not to disturb the beads that contain the target DNA fragments. • Add 200 µl of freshly prepared 80% ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. • Repeat wash step twice, to three washes in total. • Briefly spin the tube \(1-2 s) and put it back on the magnetic stand. • Completely remove the residual ethanol and air dry beads for up to 10 minutes while the tube is on the magnetic stand with lid open. • Elute the DNA target from the beads with 20 µl nuclease-free water. Mix well by pipetting up and down and put the tube on the magnetic stand until the solution is clear. • Transfer the supernatant to a sterile, nuclease-free Eppendorf tube. Check size-selected fraction on 0.8% agarose gel. **9. Hybridization** The enrichment of target DNA fragments is achieved through hybridization of the PCR amplified genomic libraries \(Section 2) with complementary RNA \(cRNA) baits \(bait library). The following protocol is based on the MYbaits protocol and kit \(Agilent SureSelect can be easily adapted as well). The high capture efficiency observed over time allowed to perform the hybridization in a half of reaction volume. **Note:** Hybridization setup is based on MYbaits protocol v3. For more information, see MYbaits protocol: www.mycroarray.com/pdf/MYbaits-manual-v3.pdf Before starting, equilibrate HYB #4 tube at room temperature to fully dissolve SDS that

may have precipitated during storage at 4°C. **10. Library Master Mix Preparation** **Note 1:** The recommended input for half a reaction volume is 250-350 ng of library DNA in 3.5 µl. It may be necessary to concentrate the genomic library before further processing by reducing the volume using a SpeedVac. **Note 2:** Although Block #1 and Block #2 from MYbaits kit can be used for plant based enrichment, enrichment efficiency is higher when these blockers are replaced by Roche SeqCap EZ Developer Reagent. **Note 3:** Make sure that Block #3 matches adapters used for long library preparation. In a sterile, nuclease-free PCR tube, mix: SeqCAP – 2.5 µl Block #3 – 0.3 µl Long Illumina library from step 2.2.5 \ (250-500 ng) – 3.5 µl ——————————- Total volume – 6.3 µl Transfer the tube containing the Library Master Mix to the thermocycler and incubate at 95°C for 5 minutes and then hold at 65°C, with the 'heated lid' option enabled. While Library Master Mix is incubated in the thermocycler, proceed to Hybridization Master Mix preparation. **11. Hybridization Master Mix Preparation** In a sterile, nuclease-free PCR tube, mix: Hyb #1 – 4.5 µl Hyb #2 – 0.25 µl Hyb #3 – 1.75 µl Hyb #4 – 0.25 µl RNase Block – 0.5µl Baits – 2.5 µl ——————————- Total volume – 9.25 µl • Mix the components by vortexing, followed by a quick spin to collect all liquid from the sides of the tube. • Transfer the tube containing the Hybridization Master Mix to the thermocycler and incubate at 65°C for 5 minutes. • While keeping the tube at 65°C, transfer 9 µl of Hybridization Master Mix to the Library Master Mix and mix by pipetting. • Hybridize solution at 65°C for 16-24 hours. **12. Recovery of captured targets** Before starting, preheat Wash Buffer 2 to 65°C for at least 1 hour, then prepare Wash Buffer 2.2 as follows: • In a 50 ml tube, combine 400 µl HYB #4, 39.6 ml nuclease-free water and 10 ml Wash Buffer 2. • Heat the Wash Buffer 2.2 to 65°C for at least 45 minutes before use. The prepared volume of Wash Buffer 2.2 is sufficient for washing 33 samples. It can be stored at 4°C for up to 6 weeks. **13. Capture and washing** • Transfer 20 µl of MyOne Streptavidin C1 magnetic beads to a sterile, nuclease-free Eppendorf tube. • Place the tube on a magnetic stand to separate beads from supernatant. After the solution is clear, carefully remove and discard the supernatant. • Add 200 µl Binding Buffer to wash the beads. Vortex the tube for 5-10 seconds, place on the magnetic stand for 2 minutes, and then carefully remove and discard the supernatant. • Repeat wash step twice for a total of three washes. • Resuspend the beads in 35 µl Binding Buffer and incubate at 65°C for 2 minutes. • Transfer the hybridization solution to the Binding Buffer/Beads and incubate 45min at 65°C, mixing solution every 5-10 minutes. • Pellet the beads on the magnetic stand for 2 minutes and carefully remove and discard the supernatant. • Add 500 µl of the Wash Buffer 2.2 from 65°C to the beads and mix by pipetting. Incubate for 10 minutes at 65°C in a thermal block. Flick the tube occasionally to resuspend the beads. Pellet the beads on the magnetic stand for 2 minutes and carefully remove and discard the supernatant. • Repeat washing step twice for a total of three 65°C washes. After the third wash, make sure all additional buffer is removed by giving the tube a quick spin after the supernatant has been removed, and re-pelleting the beads with the magnetic stand. • Resuspend the beads in 20 µl molecular biology grade water. **14. Amplification of the captured library** This step consists of amplifying the captured DNA while it is still attached to the streptavidin beads. It is important to limit the number of cycles to get just enough material for sequencing while minimizing PCR amplification bias. For this step, KAPA HiFi DNA Polymerase was used which compares favourably to other available DNA polymerases. • Prepare a 50 µl PCR reaction as follows on ice in a nuclease-free PCR tube, and mix by pipetting: Captured Library – 1 µl* 2X KAPA HiFi HotStart ReadyMix – 25 µl P5 primer \

(10 µM) – 1.5 µl P7 primer \(10 µM) – 1.5 µl Water – 22.5 µl ————————— Total volume – 50 µl • PCR cycling conditions: Initial denaturation: 98°C, 4 minutes 25 cycles with the following settings: Denaturation: 98°C, 30 seconds Annealing: 65°C, 30 seconds Extension: 72°C, 4 minutes Final extension: 72°C, 10 minutes. ==*== The amount of the enriched library \(from step 3.2.1) used for amplification may require optimization for different libraries. • Check 5 µl of PCR product on the 0.8% agarose gel – usually, 25 cycles yield 1-2 µg of the amplified enriched library; therefore, 5 µl should be well visible on gel. If amplification is weak, perform 2-5 additional cycles. • It is highly recommended to perform enrichment efficiency check using qPCR. Primer should be designed in a few genes used for enrichment. Difference between enriched and non enriched samples will oscillate between 8-11 cycles.

Timing

1 week

Anticipated Results

This protocol should yield an enriched 3-5 kb library. It is crucial to perform Blue Pippin size selection after enrichment and prior to PacBio library construction, as otherwise short fragments are preferentially sequenced. Agarose gel based or AMPure XP size selection are generally not sufficient for removal of small DNA fragments. AsBlue Pippin size selection recovers only a fraction of the input DNA, usually 5-10 individual, 50 µl PCR reactions need to be performed. This should yield >10-15 µg of amplified DNA, resulting in at least 500 ng of size selected DNA. Final PacBio library preparation requires an input of 500-750 ng of size selected DNA.

References

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