**PROCEDURE DUPLEX SEQUENCING**

(Adapted from ﻿doi:10.1038/nprot.2014.170)

**LIST OF REAGENTS**:

Enzymes:

* NEBNext® End Repair Module (NEB #E6050)
* NEBNext® dA-Tailing Module (NEB # E6053)
* NEBNext® Quick Ligation Module (NEB #E6056)
* KAPA HiFi HS RM (6.25ml) 2X

Others:

* Tubes for sonication: Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm SKU: 520045
* gBlock with Luciferase sequence flanked by P5 and P7 illumina tags for qPCR standard curve
* ﻿12,5x SYBR Green (10,000×; Life Technologies, cat. no. S-7563) ﻿Dilute 1 µl of 10,000× SYBR Green in 799 µl of ddH2O, and divide the solution into 50-µl aliquots; store the aliquots at −20 °C for up to 6 months.
* DNA chips for bioanalyzer
* ddH2O, ethanol 80%, low TE buffer
* Duplex sequencing adapters (2µM)
* MWS21 primer and indexing primers
* Eppendorfs, PCR tubes, pipettes, tips, long tips for Covaris tubes
* Ampure or Clean NGS beads

Equipment:

* Covaris Sonicator
* Bioanalyzer 2100
* Vortex
* Thermocycler
* qPCR machine
* Magnetic rack

**A. SONICATION OF DNA**

**critical** The following procedure is presented for a single sample; however, the protocol can be scaled up for an arbitrary number of DNA samples.

**1|** For each library, dilute the DNA into a final volume of 130 µl of TElow. For samples that do not use targeted capture, we typically start with 300–500 ng of DNA (do calculations for **300 ng**). For targeted capture, we typically start with 1–3 µg of DNA.

**2|** Transfer the DNA to a Covaris sonication tube and use the following settings o shear nuclear DNA to ~300 bp (range 100–500 bp): duty cycle, 10%; intensity, 5; cycles/burst, 200; and time, 20 s × 6. For small genomes, such as mtDNA, viral or plasmid DNA, the following settings should be used: duty cycle, 10%; intensity, 5; cycles/burst, 100; and time, 20 s × 3.

* Put the sonication tube in the tube holder
* Select duplex run
* Click start -> start
* Wait 2 min till run finishes

**critical step** Ensure that there are no air bubbles in the bottom of the tube after loading the sample. In the case of air bubbles, gently tap or shake to bring the solution to the bottom of the well. **Use long tips** for loading the sample into the tube. In addition, **the water bath temperature should be 4 °C, and the water bath should be degassed for 30 min before shearing.**

**Beads purification: 2:1 ratio**

**3|** Vortex the room-temperature AMPure XP bead mixture to resuspend any magnetic particles that may have settled.

**critical step** Beads must be at room temperature before use. A cold bead mixture significantly reduces yield.

**4|** Transfer the 130 µl of sonicated DNA into a 1,5 ml tube.

**5|** Add **260 µl** of AMPure XP (2:1 bead:sample ratio) and mix well by vortexing or by pipetting up and down. The 2:1 ratio is used to ensure the maximal retention of all DNA.

**6|** Incubate the mixture for 5 min at room temperature.

**7|** Place tubes onto the magnetic rack for at least 2 min or until all the beads are out of solution. Visually confirm that the beads have moved to the side of the tubes and that the solution is clear.

**8|** Aspirate the supernatant from each tube and discard it. Keep the tubes on the magnetic rack.

**9|** Carefully dispense 400 µl of room-temperature 80% (vol/vol) ethanol to each tube and incubate the tubes for 30 s at room temperature.

**10|** Aspirate out the ethanol and discard it. Repeat Steps 9 and 10 once more for a **total of two washes.**

**11|** Allow the beads to dry for 5 min at room temperature until there are no visible traces of ethanol.

**critical step** Overdrying or underdrying the beads reduces yield.

**12|** Remove the tubes from the magnetic rack, add **42 µl** of **low TE buffer** to each sample and resuspend.

**13|** Let the bead mixture sit for at least 2 min.

**14|** Place the tube back on the magnetic rack for 2 min or until the supernatant is clear. Transfer the supernatant to a **0.2-ml PCR tube**. Discard the beads.

Analyze size of fragments in Bioanalyzer: we expect a broad peak centered at 230-280bp. Example:

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**PAUSE POINT** Samples can be stored at −20 °C for several weeks.

**B.END REPAIR OF SONICATED DNA AND SIZE SELECTION**

**15|** Combine the components in the table below in a 0.2-ml PCR tube and mix them carefully by pipetting up and down. Incubate the mixture in a thermocycler for **30 min at 20 °C**. Lid heated at 30 ºC or off.

|  |  |  |
| --- | --- | --- |
| **reagent** | **Volume (µl)** | **Final concentration** |
| DNA from Step 14 | 40 | Variable |
| 10× NEBNext end-repair buffer | 5 | 1× |
| NEBNext end-repair enzyme Mix | 5 | N/A |

Transfer the content of the 0.2-ml PCR tubes to **1,5 mL tubes** for the beads purification.

**Beads purification: double size selection**

**16|** Add **35 µl** of AMPure XP beads (0.7:1 bead:sample ratio) and incubate the tube at room temperature for 5 min. This selects against large (>500 bp), poorly sheared DNA fragments.

**17|** Place the tube containing the end-repaired DNA onto the magnetic rack for at least 2 min or until all the beads are out of solution, and then **transfer the supernatant** (should be 85 µl) to a new tube. Discard the beads.

**18|** Add 55 µl of AMPure XP beads to the sample and incubate the tube at room temperature for 5 min. Place the tube onto the magnetic rack for at least 2 min or until all the beads are out of solution, and discard the 140-µl supernatant. Keep the beads.

**19|** Repeat the double 80% (vol/vol) ethanol wash outlined in Steps 9–11.

**20|** Remove the tube from the magnet and add **45 µl** of low TE buffer to each sample and gently pipette up and down until the beads are resuspended. Let the bead mixture sit for at least 2 min.

**21|** Place the tube back onto the magnetic rack for at least 2 min or until all the beads are out of solution, and then transfer the 45 µl supernatant to a new **0.2-ml PCR tube**.

**PAUSE POINT** Samples can be stored at −20 °C for up to several weeks.

**C. 3**’**A-TAILING OF BLUNT-ENDED DNA LIBRARY**

**22|** Combine the components in the table below in a 0.2-ml PCR tube and mix them carefully by pipetting up and down. Incubate the mixture in a thermocycler for **30 min at 37 °C** with the lid heated >= 45 ºC.

|  |  |  |
| --- | --- | --- |
| **reagent** | **Volume (µl)** | **Final concentration** |
| DNA from Step 21 | 42 | Variable |
| NEBNext A-tailing buffer(10x) | 5 | 1× |
| Klenow fragment | 3 |  |

Transfer the content of the 0.2-ml PCR tubes to **1,5 mL tubes** for the beads purification.

**Beads purification: 1.8:1 ratio**

**23|** Vortex AMPure XP beads to resuspend. Add **90 μl** (1.8X) of resuspended AMPure XP Beads to the ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

**24|** Incubate for 5 minutes at room temperature.

**25|** Put the tube in the magnetic rack to separate beads from supernatant. After the solution is clear (~2 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

**26|** Add 400 μl of 80% freshly prepared ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Repeat the wash step for a total of two washes.

**27|** Air dry beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

**Caution:** Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

**28|** Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding **35 μl** of low TE buffer. Mix well on a vortex mixer or by pipetting up and down 10 times and incubate for 2 minutes at room temperature.

**29|** Put the tube in the magnetic rack until the solution is clear. Without disturbing the bead pellet, carefully transfer 35 μl of the supernatant to a **0.2-ml PCR tube**.

**D. LIGATION OF DS ADAPTERS TO SAMPLE DNA**

**30|** Mix the following components, in order, in a 0.2-ml PCR tube, and mix them carefully by pipetting up and down.

|  |  |
| --- | --- |
| **reagent** | **Volume (µl)** |
| DNA A tailed | 33 |
| Duplex Sequencing adapters (2 µM) | 2,5 |
| NEBNext Quick ligation buffer (5x) | 10 |
| Quick T4 ligase | 5 |

**31**| Incubate at **20 ºC for 15 min** with **lid heated to 30º C** or off. No stop buffer is needed with this kit.

Transfer the content of the 0.2-ml PCR tubes to **1,5 mL tubes** for the beads purification.

**Beads purification: 1.2:1 ratio**

**32| Complete with 9,5 µl of low TE buffer** and add **72** **µl** of AMPure beads (1.2× bead:sample ratio) and mix. This ratio selects against DNA fragments <150 bp in size. Incubate the tube for 5 min at room temperature.

**33|** Place the tube containing the ligated sample onto the magnetic rack for at least 2 min or until all the beads are out of solution. Remove and discard the supernatant.

**34|** Repeat the double 80% (vol/vol) ethanol washes outlined in Steps 9–11, with the exception that **the beads be fully resuspended in the ethanol by vortexing before being placed on the magnetic separator.**

**critical step** Resuspending the magnetic beads during the ethanol washes is essential for removing adapter dimers that form during ligation. Failure to do so will adversely affect the downstream PCR steps. Removal of any residual ethanol by drying at 37 °C is also important, as ethanol inhibits PCR.

**35|** Remove samples from the magnetic separator and add **25 µl** of TElow to each sample. After 2 min, place the samples back on the magnetic separator for 2 min or until the supernatant is clear. Transfer the 20 µl of supernatant to a new **0.2-ml PCR tube**.

**PAUSE POINT** Samples can be stored at −20 °C for several weeks.

**E. Quantification and PCR amplification**

**36|** Quantify the molar amount of adapter-ligated DNA library from Step 35 on an Agilent TapeStation 2200 or Bioanalyzer 2100. The ligation reaction can result in multiple peaks; we quantify all DNA seen between 200 and 900 bp. This quantification will be useful to determine purity and quality.

**Quantitative PCR:**

Quantify the number of copies/µl by qPCR. Dilute samples 1/50 to get more accurate results. The standard curve for this qPCR is FinalG (Luciferase sequence (gBlock) tagged with the long primers at a known concentration).

**Long primers:** D501+D701 for example (any tag can be used).

\*It is important to dilute the samples. The range of the standard curve is not very wide, so diluting the samples will help to be within it. Moreover, this dilution can be used later on to fix the number of copies that will be used as input for the PCR.

* 1. Mix the components in the table below in a qPCR plate.

|  |  |  |  |
| --- | --- | --- | --- |
| **reagent** | **Volume (µl)** | **Final concentration** | |
| ddH2O | 9 |  |
| 2× KAPA HiFi MasterMix | 12.5 | 1× |
| 10 µM Primer 1 | 1 | 0.4 uM |
| 10 µM Primer 2 | 1 | 0.4 uM |
| 12.5× SYBR Green | 0.5 | 0.25× |
| DNA (diluted 1/100) | 1 | Variable |
| Total volume | 25 |  |

* 1. Incubate the mixture in a qPCR thermocycler as follows:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **cycles** | **denature** | **anneal** | | **extend** |
| 1 | 95 °C for 4 min |  | — | — |
| 2–(35-40) | 98 °C for 20 s | 60 °C for 20 s | | 72 °C for 15 s |

After knowing the actual correctly-tagged copies/mL, adjust samples to use 4.8E+06 copies as input for the PCR.

**PCR amplification and tagging**

Use a different set of tags for each pair of samples if possible: D50X and D70X.

**37|** Mix the components in the table below in a qPCR plate or PCR tubes.

|  |  |  |
| --- | --- | --- |
| **reagent** | **Volume (µl)** | **Final concentration** |
| ddH2O | 8.5 |  |
| 2× KAPA HiFi MasterMix | 12.5 | 1× |
| 10 uM Primer 1 | 1 | 0.4 µM |
| 10 uM Primer 2 | 1 | 0.4 µM |
| DNA (4.8\*10E6 copies) | 2 | Variable |
| Total volume | 25 |  |

**critical step** The number of PCR cycles is a crucial variable; a difference of a single cycle can result in nonspecific, higher-molecular-weight products appearing.

**38|** Incubate the mixture in a qPCR thermocycler as follows (can be performed in a regular thermocycler):

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **cycles** | **denature** | **anneal** | | **extend** |
| 1 | 95 °C for 4 min |  | — | — |
| **16** | 98 °C for 20 s | 60 °C for 20 s | | 72 °C for 15 s |

**Beads purification: 1:1 ratio or double size selection if needed**

**39|** Add 0.8 volumes of AMPure XP beads to the PCR (typically 25 µl of beads for every 25 µl of PCR) and leave in a 1.5 mL tube. The bead ratio selects against DNA fragments <200 bp in size. Incubate the mixture for 5 min at room temperature.

**40|** Place the PCR sample in the magnetic rack for at least 2 min or until all the beads are out of solution, and then remove and discard the supernatant.

**41| Remove the beads from the magnetic rack and resuspend them** in 400 µl of room-temperature 80% (vol/vol) ethanol by vortexing.

**critical step** Failure to completely resuspend the beads during this step can lead to the retention of nonspecific PCR products (supplementary Fig. 6). Also use recently prepared 80% EtOH

**42|** Place the microcentrifuge tube in the magnetic rack for at least 2 min or until all the beads are out of solution, and then remove and discard the supernatant.

**43|** Repeat for a total of two washes. Let the beads dry for 5 min.

**44|** Remove the tube from the magnetic rack and resuspend the beads in 20 µl of ddH2O. Let the bead mixture sit for at least 2 min.

**45**| Place the sample in the magnetic rack for at least 2 min or until all the beads are out of solution, and then transfer the 20 µl of eluent to a new 0.2-ml PCR tube.

**PAUSE POINT** Samples can be stored at −20 °C for several weeks.

**Quantification of tagged libraries**

**46|** Quantify the final bead-purified PCR products with **Qubit.**

**47|** Quantify the final bead-purified PCR products with between 200 and 900 bp using an Agilent TapeStation 2200 or **Bioanalyzer 2100.**

**48|** Quantify the product by qPCR using the ilumina primers (P5 and P7) to determine the nmol that will be sent to the sequencing service. This can be done with the SYBR master mix, no need to use the KAPA HiFi enzyme.

Chart, histogram, box and whisker chart

Description automatically generated

**THE SAMPLE CAN BE SUBMITTED FOR SEQUENCING AT THIS POINT.**