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Generating Exome Enriched Sequencing Libraries from Formalin-Fixed, Paraffin-Embedded Tissue DNA for Next Generation Sequencing

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Abstract

This unit describes a protocol for generating exome enriched sequencing libraries using DNA extracted from Formalin Fixed Paraffin Embedded (FFPE) samples. Utilizing commercially available kits, we present a low input FFPE workflow starting with 50ng of DNA. This procedure includes a repair step to address damage caused by FFPE preservation that improves sequence quality. Subsequently, libraries undergo an in-solution targeted selection for exons, followed by sequencing using the Illumina next generation short read sequencing platform.

Keywords

FFPE; DNA Repair; DNA Library Preparation; Whole Exome Sequencing; Next Generation Sequencing

INTRODUCTION

DNA quantity and quality are a major issue for a variety of targeted selection methods, in particular, for NGS platforms (Clark et al. 2011; Mamanova et al. 2010; Teer et al. 2010). Irrespective of input quantity, not all samples are of sufficient quality for sequencing. Formalin Fixed Paraffin Embedded (FFPE) samples are a valuable source of DNA for NGS studies (Frampton et al. 2013; Hedegaard et al. 2014; Schweiger et al. 2009; Van Allen et al. 2014) but present challenges due to age-related damage and the fixation process itself, resulting in changes to the nucleotide sequence and fragmentation (Do and Dobrovic 2015).

In this unit, we describe an optimized workflow for generating exome enriched sequencing libraries using commercially available reagents suitable for FFPE-derived DNA, inclusive of optimized steps to reduce DNA input requirements. These steps include (i) the use of a qPCR kit that amplifies several fragment sizes to accurately assess the quality and quantity of the DNA, (ii) utilization of a DNA repair enzyme mix that addresses the most common damage effects resulting from formalin fixation, and (iii) ‘with-bead’ clean ups to minimize sample loss and improve ligation efficiency (Fisher et al. 2011). Protocols are based on the manufacturer’s methods for commercially available kits with some modifications where indicated.

STRATEGIC PLANNING

DNA Isolation from FFPE Tissue

This process requires deparaffinization (removal of paraffin) of the tissue section, digestion of the resultant tissue pellet, followed by DNA extraction. Several commercial kits are available, in addition to traditional methods (phenol-chloroform extractions), which may provide an increased yield and higher quality of isolated DNA. Two recent articles (Janecka, Adamczyk, Gasinska 2015; Senguven et al. 2014) compare the performance of commercial kits to standard methods and offer considerations when choosing the best option for isolating DNA from FFPE samples.

Basic Protocol 1 – QUANTITATION AND QUALITY ASSESSMENT OF FFPE DNA

Accurate sample quantitation is a key step in generating libraries for sequencing FFPE-derived DNA. Quantitation methods that use optical density or fluorescent dyes can over estimate the amount of DNA or underestimate the extent of DNA damage to the sample. Quantitative PCR (qPCR) yields higher sensitivity and specificity than other methods. This protocol is based on the successful use of the KAPA hgDNA Quantification and QC kit to perform both a qPCR assay for the quantitation of genomic DNA (gDNA) extracted from FFPE samples as well as quality assessment.

Materials

FFPE derived gDNA

Elution Buffer (Qiagen; pn: 19086)

Human Genomic DNA Quantification and QC Kit
(KapaBiosystems; pn: KK4961)

KAPA SYBR® FAST qPCR Master
Mix (2×)

41bp Primer Premix (10×)

129bp Primer Premix (10×)

305bp Primer Premix (10×)

KAPA DNA Standards (1-5)

Nuclease Free Water (ThermoFisher Scientific; pn: AM9930)

MicroAmp Optical 96 well Reaction Plate (ThermoFisher Scientific; pn: N8010560)

MicroAmp Clear Adhesive Film (ThermoFisher Scientific; pn: 4306311)

MicroAmp Optical Adhesive Film (ThermoFisher Scientific; pn: 4311971)

Vortexer

Centrifuge

Mini-centrifuge

qPCR instrument (ABI 7900 or ABI Quantflex)

Dilution of gDNA and preparation of reagents

1. Thaw all components of the KAPA Human Genomic DNA Quantification and QC kit on ice. Once all reagents are thawed, vortex for 3-5 sec at high speed and spin briefly in a mini-centrifuge.
2. Prepare a fresh working stock of each FFPE derived gDNA sample in a total of 50 μ L, normalized to between 0.1ng/ μ L – 1ng/ μ L using Elution Buffer as diluent. This stock will be used as input for the qPCR assay.
3. Prepare each individual Primer Master Mix as separate aliquots. Steps 3-6 only need to be done the first time the kit is opened.
4. Add 200 μ L of the 41bp Primer Premix to one tube containing 1mL of KAPA SYBR FAST qPCR mix. Vortex for 3-5 sec at high speed and spin briefly in a mini-centrifuge.
5. Add 200 μ L of the 129bp Primer Premix to one tube containing 1mL of KAPA SYBR FAST qPCR mix. Vortex for 3-5 sec at high speed and spin briefly in a mini-centrifuge.
6. Add 200 μ L of the 305bp Primer Premix to one tube containing 1mL of KAPA SYBR FAST qPCR mix. Vortex for 3-5 sec at high speed and spin briefly in a mini-centrifuge.

qPCR Amplification

7. For each Primer mix being processed, include a set of standards (1-5), NTC (no template control) and diluted sample in triplicate (Figure 1).
8. Dispense 12 μ L of the KAPA qPCR master mix to each well of a MicroAmp Optical 96 reaction plate that will contain a sample, standard or NTC.
9. Dispense 4 μ L of each standard into the appropriate well.
10. Dispense 4 μ L of Nuclease Free Water to the appropriate well for NTC.
11. Dispense 4 μ L of diluted sample into the appropriate well.
12. Once all reagents and samples are added, seal plate with a MicroAmp Optical Adhesive Film, then centrifuge at 2000 rpm for 1 min.
13. Keep plate on ice and avoid direct light until ready for qPCR.
14. Use the following parameters for the qPCR run: 95°C for 3 min; 40 cycles of 95°C for 10 sec, 62°C for 30 sec. Refer to the instrument manual for additional information regarding set up of the instrument and related software.

gDNA concentration determination and quality assessment

15. Confirm the quality of each standard curve by verifying that the R^2 value is 0.985, the Slope is between -3.10 to -3.59, and the Efficiency is between 90-110% using the software on the qPCR instrument. Refer to the instrument manual for additional information regarding the software related to the qPCR instrument.
16. Calculate the concentration (pg/ μ L) of each sample per primer using steps 17-21.
17. Determine which replicates to use in the calculation. Select sample data points where the quantitation cycle (C_q) values fall within the range of the standard curve and exclude any sample data points that are outside the standard curve range.
18. Calculate the average quantitation cycle (AvC_q) for each sample.
19. Apply the following formula: [(AvC_q – Intercept)/Slope] to determine the Log(conc).
20. Determine the Dilution factor (Df), if one was used to generate the initial input stock from step 2.

21. Calculate the final concentration of the undiluted sample using the following formula: $(10^{\log(\text{conc})})Df$.
22. The final concentration derived from the 41bp primer set is used to determine the sample quantity and to calculate the volume required for 50ng of input DNA.
23. The final concentration derived from the 41, 129 and 305 bp primer sets is used to determine sample quality. Calculate the ratio between the concentration values of 129/41 and 305/41, independently. This represents the Q-ratio. Ratio values closer to 1 indicate a high quality sample, while ratio values closer to 0 indicate poor quality samples.

NOTE: Samples with low quality may require a larger amount of DNA input in order to generate enough library for exome capture, and could indicate the need for additional sequencing.

Basic Protocol 2 – DNA REPAIR

Sample age and use of Formalin Fixed Paraffin Embedded (FFPE) DNA to prepare libraries for sequencing present several challenges that can affect overall library yield and sequence quality. Types of damage caused by the formaldehyde used in fixation include depurination (producing abasic sites), cytosine deamination and fragmentation of DNA due to crosslinks. DNA damage inhibits amplification (reducing yield, increasing duplication rate) and generates sequence artifacts that affect the overall quality of the sequencing data (Do et al. 2013). Low quality libraries may require additional sequencing, but in the case of very poor libraries increasing the amount of sequencing will not overcome the deficiencies in the library. In this step, DNA damage is repaired by using a combination of enzymes (PreCR Repair Mix, New England Biolabs) that facilitate a variety of repair mechanisms to reverse damage from nicks, abasic sites and deaminated cytosines, in addition to repairing thymidine dimers, blocked 3' ends, oxidized guanine and pyrimidines. Repairing DNA increases the yield of the amplified library, increases the number of unique molecules, reduces the rate of age related artifacts and improves data quality. However, fragmented DNA is not repairable using this step. Standard DNA input for low input processing is 50ng. Lower quality DNA may require higher inputs (e.g. 500ng) in order to obtain sufficient yields for exome enrichment.

Materials

Nuclease Free Water (ThermoFisher Scientific; pn: AM9930)

PreCR repair kit (New England Biolabs; pn: M0309L)

ThermoPol Buffer

NAD

PreCR Repair Enzyme Mix

dNTP Solution Mix (Enzymatics; pn: N2050L)

Agencourt Ampure XP (Beckman Coulter; pn: A63881)

Elution Buffer (Qiagen; pn: 19086)

Ethanol 100% molecular biology grade

MicroAmp Optical 96 well Reaction Plate (ThermoFisher Scientific; pn: N8010560)

MicroAmp Clear Adhesive Film (ThermoFisher Scientific; pn: 4306311)

Vortex

Centrifuge

Thermal cycler

DynaMag-96 Side Skirted Plate Magnet (Invitrogen; pn: 12027 or 12331D)

Repair DNA

1. Allow Agencourt Ampure XP beads to come to room temperature for 30min.
2. Thaw all components of the PreCR repair kit on ice. Once all reagents are thawed, vortex for 3-5 sec at high speed and spin briefly in a mini-centrifuge.
3. Prepare 50ng (based on the qPCR quantitation) of each FFPE derived gDNA sample in a total of 30µL, using Nuclease Free Water as diluent. Dispense each sample into a corresponding well of a MicroAmp Optical 96 well reaction plate.
4. Prepare the DNA repair master mix using the following recipe for 1 reaction:
 - a. 8.5µL Nuclease Free Water
 - b. 5µL ThermoPol Buffer
 - c. 5µL 100uM dNTP Solution Mix
 - d. 0.5µL NAD
 - e. 1µL PreCR Repair Mix Enzyme
5. Vortex for 3-5 sec at high speed the DNA repair master mix, then spin briefly in a mini-centrifuge and keep on ice.
6. Add 20µL of DNA repair master mix to each sample in the MicroAmp Optical 96 well reaction plate.

7. Seal with a MicroAmp Clear Adhesive Film. Vortex at high speed for 3-5 sec and centrifuge at 2000rpm for 1 min.
8. Incubate plate on a thermal cycler for 20 min at 37C, 4C hold. Do not use a heated lid.

Ampure XP Bead Clean Up

9. Add 90µL of Agencourt Ampure XP beads to each sample well. Pipette up and down 10 times with a pipet to ensure complete mixing. Color should appear even across the wells when mixed properly.
10. Incubate at room temperature for 2 min.
11. Place plate on the plate magnet and incubate for 4 min. Wells should appear clear.
12. While plate is on the plate magnet, remove and discard the supernatant.
13. Add 100µL of freshly prepared 70% ethanol.
14. Incubate on plate on the plate magnet for 30 sec at room temperature.
15. While plate is on the plate magnet, completely remove and discard the ethanol.
16. Air-dry the samples at room temperature for 2 min, after removal from the plate magnet.
17. Add 50µL of Elution Buffer. Pipette up and down 10 times with pipet to ensure complete mixing.
18. Remove plate from the plate magnet and incubate for 2 min at room temperature.
19. Place plate on the plate magnet and incubate for 2 min at room temperature.
20. Transfer the supernatant to a new MicroAmp Optical 96 well reaction plate, confirming that supernatant transferred is clear and does not contain beads.
21. Seal plate with a MicroAmp Clear Adhesive Film. This is a safe stopping point. Plate can be stored at 4C for up to 7 days or at –20C for long-term storage.

Basic Protocol 3 – FRAGMENTATION

In preparation for library construction and NGS, genomic DNA is fragmented into molecule sizes that are optimal for the run lengths of the sequencer. Fragmentation is performed using a focused-ultrasonicator, which employs acoustic energy, to mechanically fragment samples.

Parameters for fragmentation can be adjusted to create the desired fragment length. This sequencing protocol requires paired end 100bp reads for use with the Illumina HiSeq sequencer. The sample fragment length should be no smaller than 2× the total read length (i.e. >200bp), otherwise paired reads will overlap and result in double sequencing the same molecule. Fragment sizes should not exceed 800 bp as they will generate clusters on the flowcell that have large diameters which could overlap with adjacent clusters. This overlap reduces the efficiency and yield of the sequencing.

Materials

Agencourt Ampure XP (Beckman Coulter; pn: A63881)

Ethanol 100% molecular biology grade

Nuclease Free Water (ThermoFisher Scientific; pn: AM9930)

High Sensitivity DNA Kit (Agilent; 5067-4626)

Focused-ultrasonicator (Covaris; model: E220)

96 MicroTube plate or MicroTube AFA Fiber Snap-Cap
(Covaris; pn: 520069 or 520045)

MicroAmp Optical 96 well Reaction Plate (ThermoFisher
Scientific; pn: N8010560)

MicroAmp Clear Adhesive Film (ThermoFisher Scientific; pn:
4306311)

DynaMag-96 Side Skirted Plate Magnet (Invitrogen; pn:12027
or 12331D)

2100 BioAnalyzer (Agilent)

Fragmentation of gDNA

1. Allow Agencourt Ampure XP beads to come to room temperature for 30 min.
2. Transfer all (~ 50µL) of the DNA Repaired sample to a Covaris Tube. For higher sample through put, use the Covaris 96 microTube plate.
3. Fragment DNA using the Covaris Series E220 and the following settings: Duty Factor = 10, Peak Power = 140.0, Cycles per Burst = 200, Time = 50 sec.
4. Transfer all (~50µL) of the fragmented DNA from the Covaris tube into a new MicroAmp Optical 96 well Reaction Plate.

Ampure XP Bead Clean Up

5. Add 88 μ L of Agencourt Ampure XP beads to each sample. Pipette up and down 10 times with a pipet to ensure complete mixing.
6. Incubate at room temperature for 2 min.
7. Place plate on the plate magnet and incubate for 4 min. Wells should appear clear.
8. While plate is on the plate magnet, remove and discard supernatant.
9. Add 100 μ L of freshly prepared 70% ethanol.
10. Incubate on plate magnet for 30 sec.
11. While plate is on the plate magnet, completely remove and discard the ethanol.
12. Air dry the samples for 2 min, after removal from the plate magnet.
13. Add 50 μ L of Nuclease Free Water. Pipette up and down 10 times with pipet to ensure complete mixing. Do not transfer the sample.
14. A sample may be taken at this point to QC the fragmentation step, using the 2100 BioAnalyzer. A 1:5 dilution of sample can be run using the Agilent High Sensitivity DNA kit. Refer to the instrument manual for additional information regarding set up of the instrument and related software. See Figure 2 for an example electropherogram.
15. Seal plate with a MicroAmp Clear Adhesive Film. This is a safe stopping point. Store plate at 4C for up to 3 days.

Basic Protocol 4 – LIBRARY PREPARATION (End Repair, A-Tailing, Ligation and Amplification)

Library preparation consists of 3 molecular modification steps (End Repair, A-Tailing, Ligation) to produce sequencing libraries followed by amplification. Fragmentation of the DNA molecules from the previous step produces jagged edges or overhangs. During the process of End Repair, nucleotides are filled in to create blunt ends. Next, an 'A' base is added to the 3' end of each blunt ended fragment to create an overhang. The third step is ligation, in which synthetic oligonucleotide indexed adapters containing a 'T' base overhang are ligated to the DNA fragment. The use of the 'A' or 'T' base overhang in the fragmented DNA or adapter prevents concatemerization or formation of adapter dimers of the DNA during the ligation step. The adapters also include sequences specific for sequencing on the Illumina platform and a unique 8bp index. After ligation, the libraries are amplified by PCR to enrich for molecules that contain both ends properly ligated to adapters.

Materials

Hyper Prep Kit (KapaBiosystems; pn: KK8504)

End Repair/A Tailing Buffer

End Repair/A Tailing Enzyme

Ligation Buffer

Kapa T4 DNA Ligase

Kapa HiFi Master Mix

Kapa Primer Premix

Indexed Adapter oligonucleotide (Integrated DNA Technology)
– See Reagents and Solutions

20% PEG/2.5M NaCl solution – See Reagents and Solutions

Ethanol 100% molecular biology grade

Nuclease Free Water (ThermoFisher Scientific; pn: AM9930)

Agencourt Ampure XP (Beckman Coulter; pn: A63881)

MicroAmp Optical 96 well Reaction Plate (ThermoFisher Scientific; pn: N8010560)

MicroAmp Clear Adhesive Film (ThermoFisher Scientific; pn: 4306311)

2100 BioAnalyzer (Agilent)

Elution Buffer (Qiagen; pn: 19086)

High Sensitivity DNA Kit (Agilent; 5067-4626)

Vortex

Centrifuge

Thermal cycler

DynaMag-96 Side Skirted Plate Magnet (Invitrogen; pn:12027 or 12331D)

End Repair and A-Tailing

1. Thaw all components of the Hyper Prep kit on ice. Once all reagents are thawed, vortex for 3-5 sec at high speed, then spin briefly in a mini-centrifuge.
2. Prepare a 20% PEG/2.5M NaCl solution (See Reagents and Solutions), and allow solution to come to room temperature for 30 min.

3. Prepare the EndRepair/A-Tailing Master Mix using the following recipe for 1 reaction:
 - a. 7 μ L End Repair/A-Tailing Buffer
 - b. 3 μ L End Repair/A-Tailing Enzyme Mix
4. Vortex at high speed for 3-5 sec the End Repair/A-Tailing master mix, then spin briefly in a mini-centrifuge and keep on ice.
5. Add 10 μ L of End Repair/A-Tailing master mix to each sample in the MicroAmp Optical 96 well Reaction Plate.
6. Seal with a MicroAmp Clear Adhesive Film, vortex at high speed for 3-5 sec, then centrifuge at 600 rpm – allowing the centrifuge to ramp up to the expected speed then immediately ramping down to stop.

Note: Centrifugation speed may require optimization depending on centrifuge model used. Desired results are for solution to be spun to the bottom of the tube/plate without the beads pelleting from solution, but maintaining suspension in the liquid.
7. Incubate plate on a thermal cycler with the following parameters:
 - a. 20°C for 30 min
 - b. 65°C for 30 min
 - c. 4°C hold. Use a heated lid (105°C).

Ligation of Indexed Adapters

8. Prepare the Ligation Master Mix using the following recipe for 1 reaction:
 - a. 5 μ L Nuclease Free Water
 - b. 30 μ L Ligation Buffer
 - c. 10 μ L KAPA T4 DNA Ligase
9. Add 45 μ L of the Ligation Master Mix to each sample in the MicroAmp Optical 96 well Reaction Plate.
10. Add 5 μ L of the specific Indexed Adapter oligonucleotide [25 μ M] to each corresponding sample.

11. Seal plate with a MicroAmp Clear Adhesive Film. Vortex at high speed for 3-5 sec, then centrifuge at 600 rpm – allowing the centrifuge to ramp up to the expected speed then immediately ramping down to stop.
12. Incubate plate on a thermal cycler with the following parameters:
 - a. 20°C for 15 min
 - b. 4 °C hold. Do not use a heated lid.

Ampure XP Bead Clean Up

13. Add 88µL of 20% PEG/2.5M NaCl solution to each sample. Pipette up and down 10 times with a pipet to ensure complete mixing.
14. Incubate at room temperature for 2 min.
15. Place plate on the plate magnet and incubate for 4 min. Wells should appear clear.
16. While plate is on the plate magnet, remove and discard supernatant.
17. Add 100µL of freshly prepared 70% ethanol.
18. Incubate on the plate magnet for 30 sec.
19. While plate is on the plate magnet, completely remove and discard the ethanol.
20. Air-dry the samples for 2 min, after removal from the plate magnet.
21. Add 25µL of Nuclease Free Water. Pipette up and down 10 times with pipet to ensure complete mixing.
22. Incubate off magnet for 2 min at room temperature.
23. Place plate on the plate magnet and incubate for 2 min.
24. Transfer the supernatant to a new MicroAmp Optical 96 well Reaction Plate.

Note: It is important to remove the beads prior to amplification, as the presence of beads will prevent the amplification reaction from occurring.

Library Amplification

25. Prepare the Amplification Master Mix using the following recipe for 1 reaction:
 - a. 25µL KAPA HiFi Master mix

- b.** 5 μ L KAPA Primer PreMix
- 26.** Add 30 μ L of the Amplification Master Mix to each sample in the MicroAmp Optical 96 well Reaction Plate.
- 27.** Seal with a MicroAmp Clear Adhesive Film, vortex at high speed for 3-5 sec, then centrifuge for 1 min at 2000 rpm.
- 28.** Incubate plate on a thermal cycler with the following parameters:
- a.** 98°C 45 sec
- b.** 8 cycles of
- i.** 98
°C
15
sec
- ii.** 60
°C
30
sec
- iii.** 72
°C
30
sec
- c.** 72°C 1 min
- d.** 4°C hold

Ampure XP Bead Clean Up

- 29.** Add 40 μ L of Agencourt Ampure XP beads to each sample. Pipette up and down 10 times with pipet to ensure complete mixing. Color should appear even across the wells when mixed properly.
- 30.** Incubate at room temperature for 2 min.
- 31.** Place plate on the plate magnet and incubate for 4 min. Wells should appear clear.
- 32.** While plate is on the plate magnet, remove and discard supernatant.
- 33.** Add 100 μ L of freshly prepared 70% ethanol.

34. Incubate on the plate magnet for 30 sec.
35. While plate is on the plate magnet, completely remove and discard the ethanol.
36. Air-dry the samples for 2 min, after removal from the plate magnet.
37. Add 30 μ L of Nuclease Free Water. Mix up and down 10 times with pipet to ensure complete mixing.
38. Incubate off magnet for 2 min at room temperature.
39. Place plate on the plate magnet and incubate for 2 min.
40. Transfer the supernatant to a new MicroAmp Optical 96 well Reaction Plate.
41. A sample may be taken at this point to QC the amplification step, using the 2100 BioAnalyzer. A 1:100 dilution of sample can be run using the Agilent High Sensitivity DNA kit. Refer to the instrument manual for additional information regarding set up of the instrument and related software. See Figure 3 for example electropherogram.
42. Seal plate with a MicroAmp Clear Adhesive Film. This is a safe stopping point. Plate can be stored at -20°C for long-term storage.

Basic Protocol 5 – EXOME ENRICHMENT AND CAPTURE

DNA libraries are hybridized to exon specific RNA baits which are in turn biotinylated. Following hybridization the biotinylated RNA baits are captured with streptavidin-coated magnetic beads. DNA fragments bound to the exon specific baits are pulled down and unbound DNA fragments are washed away. The remaining captured DNA fragments are amplified. The protocol described in this unit is based on the Agilent target enrichment system for whole exome sequencing and is optimized for use in conjunction with low input/ FFPE libraries. Modifications to the Agilent process include the addition of indexed blockers and changes in the hybridization blocker mix. Steps in the capture process have been formatted for small sample processing. However, higher sample through-put is possible with some additional instruments and consumable changes.

Materials

Universal Blocker Oligonucleotide (Integrated DNA Technology) – See Reagents and Solutions

Indexed Blocker Oligonucleotide (Integrated DNA Technology)
– See Reagents and Solutions

Nuclease Free Water (ThermoFisher Scientific; pn: AM9930)

SureSelect XT Human All Exon Kit (Agilent; pn:5190-8863)

SureSelect Hyb #1, 2, 3, 4

SureSelect Block # 1, 2

RNase Block

SureSelect Human All Exon Capture

Sure Select Binding Buffer

Sure Select Wash Buffer I

Sure Select Wash Buffer II

Dynabeads My One Streptavidin T1 (ThermoFisher Scientific; pn: 65602)

HiFi HotStart ReadyMix (2×) (KapaBiosystems; pn: KK2612)

PCR Primer 1 (Integrated DNA Technology) – See Reagents and Solutions

PCR Primer 2 (Integrated DNA Technology) – See Reagents and Solutions

Agencourt Ampure XP (Beckman Coulter; pn: A63881)

Ethanol 100% molecular biology grade

Elution Buffer (Qiagen; pn: 19086)

2100 BioAnalyzer (Agilent)

Vacuum concentrator

MicroAmp Optical 8-Cap Strips (ThermoFisher Scientific; pn: 4323032)

High Sensitivity DNA Kit (Agilent; 5067-4626)

MicroAmp Optical 96 well Reaction Plate (ThermoFisher Scientific; pn: N8010560)

MicroAmp Clear Adhesive Film (ThermoFisher Scientific; pn: 4306311)

DynaMag-2 Magnet (Invitrogen; pn: 12321D)

DynaMag-96 Side Skirted Plate Magnet (Invitrogen; pn: 12027 or 12331D)

DNA LoBind Tubes (Eppendorf; pn: 22431021)

Tube Rocker BD Clay Adams Nutator Mixer (BD Diagnostics; pn: 421105)

Torrey Pines Echotherm Heat Block (Torrey Pines)

Prepare DNA Library and sequence specific blockers

1. Prepare 500-750ng of each library in a MicroAmp Optical 96 well Reaction Plate. Note: For low quality samples with less than 500ng use all available library (down to 100ng) in the assay. Quality of sequencing data may vary with lower inputs.
2. Add 4 μ L of the Universal Blocker Oligonucleotide [250uM] to each sample.
3. Add 4 μ L of the specific Indexed Blocker Oligonucleotide [250uM] to each sample matching to the indexed adapter sequence.
4. Dry down samples using a vacuum concentrator with a heat setting no higher than 45°C.
5. Resuspend in 3.4 μ L of Nuclease Free Water.
6. Seal the plate with a MicroAmp Clear Adhesive Film. This is a safe stopping point. Plate can be stored at -20°C for short-term storage.

Prepare Enrichment reagents

7. Thaw all components of the Sure Select XT Human All Exon kit on ice. Once all reagents are thawed, vortex at high speed for 3-5 sec, then briefly spin in a mini-centrifuge. Hyb #3 can be thawed on the bench.
8. Prepare the Blocker mix using the following recipe for 1 reaction:
 - a. 6.6 μ L Nuclease Free Water
 - b. 2.5 μ L Agilent SureSelect Block #1
 - c. 2.5 μ L Agilent SureSelect Block #2

Note: Blocker #3 from the Agilent kit is not used in this protocol. The Index specific blocker is substituted in step 3.

9. Vortex at high speed for 3-5 sec the Blocker mix, then briefly spin in a mini-centrifuge and keep on ice.
10. Prepare the Hybridization Buffer mix using the following recipe for 1 reaction:
 - a. 6.63 μ L SureSelect Hyb #1
 - b. 0.27 μ L SureSelect Hyb #2

- c. 2.65µL SureSelect Hyb #3
 - d. 3.45µL SureSelect Hyb #4
11. Vortex at high speed for 3-5 sec the Hybridization Buffer mix, then briefly spin in a mini-centrifuge.
12. Heat the Hybridization Buffer mix at 65°C for 5 min, then keep at room temperature until use.
13. Prepare the Exome RNA Bait mix using the following recipe for 1 reaction:
 - a. 1.5µL Nuclease
 - b. 0.5µL RNase Block
 - c. 5µL SureSelect Capture Library (Bait)

Exome Enrichment

14. Add 11.6µL of the Blocker mix to each sample (library, indexed blocker and universal blocker).
15. Seal plate with a MicroAmp Clear Adhesive Film. Vortex at high speed for 3-5 sec, then centrifuge for 1 min 2000rpm for 1 min.
16. Incubate on thermal cycler for 5 min at 95°C; 65°C hold.
17. For each sample that will be hybridized combine 13µL of Hybridization buffer and 7µL of Exome RNA Bait mix into a separate plate.
18. Seal plate with a MicroAmp Clear Adhesive Film. Vortex at high speed for 3-5 sec, then centrifuge for 1 min 2000rpm for 1 min.
19. After the plate with library, indexed blocker, universal blocker and blocker mix has been at 65°C for at least 5 min, add all (~20µL) of the Hybridization and Exome RNA Bait mix to each sample, keeping plate on thermal cycler at 65°C.
20. Seal plate using the MicroAmp Optical 8-Cap Strips, while plate remains on the thermal cycler at 65°C.
21. Hybridize plate overnight (16-24 hrs) at 65°C.

Note: If alternative plates and seals are used for the hybridization incubation, it is important to test for evaporation prior to processing samples.

Prepare Exome Capture Beads

22. After 16-24 hrs, pre-warm Wash Buffer II at 65°C until use.

23. Prepare Dynabeads My One Streptavidin T1 Beads.
24. Add 200 μ L of Binding buffer to a 1.5mL LoBind tube for each sample that is being processed.
25. Vortex the Dynabeads vigorously to completely resuspend beads.
26. Add 50 μ L of Dynabeads to each tube containing Binding buffer.
27. Vortex at high speed for 3-5 sec, then briefly spin in a mini-centrifuge. Place in the tube magnet and incubate at room temperature for 2 min. Wells should appear clear.
28. While the tubes are on the magnet, remove and discard supernatant.
29. Repeat washes for a total of 3 $\times$.
30. After final wash, resuspend beads in 200 μ L of binding buffer.

Exome Capture

31. While keeping samples on the 65°C block, transfer all of the volume (~30 μ L) from each sample to the corresponding tube designated for that sample which contains the pre-washed Dynabeads.
32. Pipette the sample-bead mixture up and down 3 $\times$, close the tube and invert 3 $\times$.
33. Place the tubes on a rocker and incubate for 30 min at room temperature.

Note: A minimal amount of evaporation can be expected. However, if a large amount of evaporation is observed (e.g. <20 μ L of reaction volume remaining), poor enrichment performance can be expected.
34. Following the 30 min incubation, briefly spin tubes in a mini-centrifuge and place on the tube magnet. Incubate at room temperature until solution is clear (~2 min).
35. While the tube is on the tube magnet, remove and discard the supernatant.

Exome Wash

36. Resuspend beads in 500 μ L of Wash Buffer I.
37. Incubate for 15 min at room temperature off of the magnet. Vortex (3-5 sec at high speed) at 5 min intervals.
38. Place tube on the tube magnet and incubate at room temperature until solution is clear (~1-2 min).

39. While the tube is on the tube magnet, remove and discard the supernatant.
40. Resuspend beads in 500 μ L of pre-warmed Wash Buffer II (pre-warmed at 65°C).
41. Incubate for 10 min at 65°C, using a tube heat block. Vortex (3-5 sec at high speed) the tubes at 5 min intervals.
42. Place tube on the tube magnet and incubate at room temperature until solution is clear (~ 1-2 min).
43. While tube is on the tube magnet, remove and discard the supernatant.
44. Repeat steps 37-40 with Wash Buffer II for a total of 3 washes.
45. After final removal of Wash Buffer II, ensure that Wash Buffer II is completely removed.
46. Add 21 μ L of Nuclease Free Water to each sample, pipetting up and down 10 $\times$ to resuspend the beads.

Post Capture Amplification

47. Allow Agencourt Ampure XP beads to come to room temperature for 30 min.
48. Thaw all reagents on ice. Once thawed vortex at high speed for 3-5 sec, then briefly spin in a mini-centrifuge.
49. Prepare the Amplification Master Mix using the following recipe for 1 reaction:
 - a. 25 μ L KAPA HiFi Master Mix
 - b. 2 μ L PCR Primer 1 [20uM]
 - c. 2 μ L PCR Primer 2 [20uM]
50. Add 29 μ L of the Amplification Master Mix to a new MicroAmp Optical 96 well Reaction Plate.
51. Transfer all of the resuspended beads from each tube to the corresponding well designated for each sample containing amplification master mix.
52. Seal plate with a MicroAmp Clear Adhesive Film. Vortex at high speed for 3-5sec, then centrifuge at 600 rpm – allowing the centrifuge to ramp up to the expected speed then immediately ramping down to stop.
53. Incubate plate on thermal cycler with the following parameters:
 - a. 98°C 45 sec

- b.** 10 cycles of
- i.** 98
°C
15
sec
 - ii.** 60
°C
30
sec
 - iii.** 72
°C
30
sec
- c.** 72°C 1 min
- d.** 4°C hold

Note: Presence of Dynabeads will not interfere with the amplification reaction.

Post Capture Ampure XP Bead Clean Up

- 54.** Add 90µL of Agencourt Ampure XP beads to each sample. Pipette up and down 10 times with pipet to ensure complete mixing. Color should appear even across the wells when mixed properly.
- 55.** Incubate at room temperature for 2 min.
- 56.** Place plate on the plate magnet and incubate for 4 min. Wells should appear clear.
- 57.** While plate is on the plate magnet, remove and discard supernatant.
- 58.** Add 100µL of freshly prepared 70% ethanol.
- 59.** Incubate on the plate magnet for 30 sec.
- 60.** While plate is on plate magnet, completely remove and discard the ethanol.
- 61.** Air dry the samples for 2 min, after removal from the plate magnet.
- 62.** Add 30µL of Nuclease Free Water. Pipette up and down 10 times with pipet to ensure complete mixing.

63. Incubate off magnet for 2 min at room temperature.
64. Place plate on the plate magnet and incubate for 2 min.
65. Transfer the supernatant to a new MicroAmp Optical 96 well Reaction Plate.
66. A sample may be taken at this point to QC the amplification step, using the 2100 BioAnalyzer. A 1:10 dilution of the sample can be run using the Agilent DNA High Sensitivity kit. Refer to the instrument manual for additional information regarding set up of the instrument and related software. See Figure 4 for an example electropherogram.
67. Seal plate with a MicroAmp Clear Adhesive Film. This is a safe stopping point. Plate can be stored at -20°C for long-term storage.
68. Captured libraries are now ready for sequencing using the Illumina sequencing platform. These libraries were prepared to support 2×100 read lengths. Refer to Illumina guidelines and protocols for denaturing, diluting and sequencing libraries on Illumina sequencers.

REAGENTS AND SOLUTIONS

20% PEG/2.5M NaCl Solution

Polyethylene glycol 40% (w/w) in water (Sigma-Aldrich; pn: P1458-50mL)

5M NaCl (ThermoFisher Scientific; pn: AM9759)

Equal parts Polyethylene glycol to NaCl

Store at 4C for up to 12 months

Oligonucleotides

Figure 5 lists the oligonucleotide sequences for indexed adapters, the corresponding indexed blockers, universal blockers and amplification primers (Oligonucleotide sequences © 2007-2012 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.). The list contains 24 unique indexes (Agilent Technologies), but can be scaled to accommodate higher indexing (e.g. 96 unique indexes). The following modifications are required when synthesizing the oligonucleotides: Universal adapters require a 3' end C->T phosphorothioate bond; Indexed adapters require a 5' phosphate group and should be duplexed to the universal adapter oligonucleotide; Indexed blockers require an inverted-dT 3' terminator modification. HPLC purification is recommended. Resuspend lyophilized oligonucleotides in low TE at a concentration of 100uM for adapters and PCR primers, and 1000uM for blockers for long-term storage. Working stocks of the

adapters (25uM), PCR primers (20uM) and blockers (250uM) can be prepared by diluting the oligonucleotides in low TE as required by the appropriate step. Store at -20°C for up to 12 months.

COMMENTARY

Background Information

Short read sequencing technology (Bentley et al. 2008) has opened the door to determining the base order from a variety of sample sources, including formalin-fixed paraffin-embedded tissue (FFPE) derived DNA that would otherwise be problematic, especially if using conventional sequencing (e.g., Sanger, Capillary electrophoresis based sequencing). Short read sequencing is performed by reducing the gDNA down to small fragments, where, through several molecular steps, a synthetic oligonucleotide is attached to each end of the fragmented DNA generating a library. The attached synthetic oligonucleotide or adapter contains specific sequences for a variety of purposes including: amplification of the adapter-ligated fragment using universal primers, incorporating an index sequence to allow for multiple libraries to be combined together during sequencing, immobilization of the fragment on a flowcell (a glass slide containing channels fixed with oligonucleotide sequences complementary to the adapters), and sequencing by synthesis (SBS) using specific primers to facilitate sequencing. Once a library is created, it can be loaded on to the flowcell channels. The flowcell architecture allows SBS reagents to be delivered to the libraries by flow through vacuum sealed ports on both sides of the channel. Library molecules are clonally amplified on the flowcell to produce single stranded cluster colonies. The generation of clusters across a flowcell allows for parallelization of the sequencing events that greatly increases the amount of sequencing data generated at one time. Sequencing primers are dispensed to the channels and hybridized to the clusters. The SBS reaction is performed by adding a mixture of fluorescently labeled dNTPs to the flowcell channel with a DNA polymerase to generate a complementary sequence. After each cycle of nucleotide incorporation, the sequencers imaging module excites the fluorescent labels and captures a picture of the cluster fluorescence. Proprietary software on the sequencer computer will convert these images to base calls used for downstream analysis.

In-solution target enrichment technology expanded on the advancements made by short read sequencing, enabling capture of short fragments dependent on complementary sequences that could be designed specifically for regions of interest (Gnirke et al. 2009). This provided a more cost effective way of studying the exome without having to sequence an entire genome. Compared to other technologies (e.g. array based capture, MIPs, PCR) in-solution hybridization provides a number of advantages including the ability to capture larger numbers of targets or genes and processing samples in scale (few to many) (Mamanova et al. 2010). Prepared libraries (fragmented, adapter ligated DNA) are denatured and combined with biotinylated RNA baits. These baits are complementary to specific sequences intended for capture (exonic regions). The library and bait mixture is hybridized overnight (16-24 hrs). Subsequently, streptavidin coated magnetic beads are used to capture the DNA-RNA bound biotinylated baits and any unbound DNA is washed away. Universal primers specific

to the adapters are used to amplify the captured DNA library from the streptavidin bead. The resulting enriched library is then ready for sequencing.

The use of FFPE derived DNA in short read sequencing is useful for a variety of clinical and basic research studies. However the nature of FFPE as a DNA source comes with challenges that may require additional care to produce data of good quality. Common types of DNA damage caused by the process of formalin fixation include the formation of DNA cross-links, DNA fragmentation and deamination. Fragmentation is a common problem with FFPE samples. However, since short read sequencing relies on reducing the gDNA to smaller fragments many previously un-usable FFPE samples are now amenable to sequencing. Severe fragmentation (<100bp size) is still problematic and is a consequence of acidic conditions produced by formalin during long-term storage of samples. Lowering the pH causes denaturation of the DNA which destabilizes the DNA over time and promotes damage to the DNA. This also increases the presence of abasic sites which can prevent amplification or result in the mis-incorporation of primarily adenine bases at that site producing sequencing artifacts. Hydrolytic deamination of cytosine to form uracil producing a G:C>A:T sequencing artifact is another common type of damage produced by FFPE fixation (Do and Dobrovic 2015). Treatment with DNA repair enzymes can improve the quality of the DNA by filling in gaps and repairing base changes resulting from oxidation and deamination. Still, some types of DNA damage, such as cross links and fragmentation, may not be repairable using any known method (Briggs and Heyn 2012; Do et al. 2013; Skage and Schander 2007).

Accurate sample quantitation is a key step in generating successful sequencing libraries. Inaccuracies from quantitation can result in less than optimal amounts of input DNA resulting in poor yields. A variety of methods for double strand DNA (dsDNA) quantitation are available including UV spectrophotometry, fluorescent DNA binding dyes and Quantitative PCR (qPCR). FFPE derived DNA, however, is difficult to quantitate using standard methods (Georgiou and Papapostolou 2006; Hedegaard et al. 2014; Nakayama et al. 2016; Sedlackova et al. 2013; Simbolo et al. 2013). For instance, UV Spectrophotometry (NanoDrop) can overestimate DNA concentration for NGS purposes as it does not distinguish between dsDNA and single-stranded DNA. Fluorescent based assays, such as Picogreen and Qubit, are more reliable, but the increase in DNA fragmentation results in increased binding of fluorescent dyes, thus underestimating the amount of dsDNA in the sample. Quantitative PCR (qPCR) has been the gold standard for DNA quantitation due to its high sensitivity and specificity, which exceeds all other methods. Modification of the qPCR method to include primer sets for specific fragment lengths provides both qualitative and quantitative assessment of FFPE derived DNA and is better suited to more accurately measure 'viable' concentrations of DNA (Umetani et al. 2006).

Critical Parameters

Sample Quantitation—This is a key step for using a low input process; if the sample quantitation is inaccurate it can result in less DNA input resulting in lower than expected library yields after amplification. This is especially crucial when the quality of the DNA sample is low.

Post Fragmentation QC—This step could become optional with an established protocol due to the consistent nature of the Covaris shearing platform. If sample quantitation is unclear this step will confirm the amount of DNA input with regard to subsequent steps of the protocol. Some DNA loss after the DNA Repair and Fragmentation clean up steps is expected.

Buffers and Enzymes—The End Repair buffer may have crystals when thawed. This reagent can be thawed on bench if needed or hand warmed to ensure that all crystals have gone into solution. Enzymes and the Ligation buffer are viscous, be careful to slowly pipette these reagents to ensure accurate volume transfer when preparing master mixes and distributing reagents to sample reactions.

A-Tailing—dATPs are sensitive to storage temperature and repeat freeze thaw cycles. It is recommended that single use aliquots be prepared to prevent multiple freeze thaw cycles. Poor A-Tailing can result in in-efficient ligation (not enough molecules have the A' base overhang to compliment the T' base overhang from the adapter) and generation of a higher proportion of chimeric molecules (blunt ends are ligated together between two molecules that did not undergo A-Tailing) thus lowering the overall yield of library after amplification. This, in turn, will effect downstream library complexity and increase the percent of duplicate reads that are sequenced.

Master Mix Preparation—Make sure all reagents are completely thawed. Vortex (3-5 sec at high speed) reagents and quickly spin down tubes prior to pipetting into a master mix. Enzymes should not be vortexed, but can be mixed by inverting the tube, 'flicking' the bottom of the tube to aide in mixing. All enzymes should be kept at -20°C and removed from the freezer just prior to pipetting them into the master mix, and returned to the freezer immediately after use. When assembling a master mix, always add the least sensitive reagent first (i.e. water, buffers) and the most sensitive reagent last (i.e. Enzymes). Once a master mix has been completely assembled, vortex (3-5 sec at high speed) and spin briefly, keeping the tube on ice before distribution to the sample reactions. When pipetting reagents, always dispense the reagent to the side and bottom of the tube or directly into the solution pipetting up and down a few times to ensure all reagent has been dispensed – especially for viscous reagents. Recipes do not include overage to account for dead volume that may be required for pipetting. Additional reactions may be needed when calculating the final recipe to account for small volume reagents.

Ampure XP Beads—These beads require vigorous vortexing prior to use, as settling can occur. They should also be brought to room temperature before use. Bead color should always be a chocolate brown, if the color of the beads is yellow discard.

Indexed Blockers—The standard Agilent targeted enrichment kit utilizes non-indexed adapters during ligation. Subsequently, the blockers required during the hybridization are specific to the universal adapters used during the process. The samples are indexed post-capture using indexed primers. In the protocol described in this unit, indexed adapters are used during ligation and require the corresponding indexed blocker to be added during the hybridization steps. If the incorrect blocker (or no blocker) is used, two molecules can

hybridize together via the adapter sequences and form dimers. If one of the molecules also binds to an RNA bait, the duplexed molecule can be pulled down, resulting in enrichment of non-targeted molecules in the library. The percent selection metric is an indicator of the proportion of reads that carry bases matching the expected targets. If additional non-targeted molecules are also captured, this metric will decrease.

Exome Enrichment Library DNA Input—Optimally 500-750ng of amplified library should be used as input for the enrichment reaction. Lower quality libraries with yields less than 500ng can be attempted in the enrichment reaction (we have successfully used as low as 100ng of input), however sequencing metrics may be affected (e.g. percent duplication) and additional sequencing depth may be required.

Troubleshooting

See Table 1 for troubleshooting tips.

Anticipated Results

For any new process, it is always important to validate overall performance and quality control within a laboratory. Other commercial kits may be available for different steps that render benefits specific to the needs of an individual laboratory (e.g. cost differences, streamlined reagents and specific chemistry).

FFPE samples can vary in quality, even with DNA repair. Metrics generated during the library preparation and sequencing process may also vary depending on the capture product used and the amount of sequence data generated. The following performance was observed using FFPE samples of different quality as input for the protocol described above:

Yield at pre capture PCR: 100-800ng

Yield at post capture PCR: >2nM for captured libraries.

% selection: 60-80%. Less than 50% may indicate a problem with hybridization.

% duplication = 5-25%, for extremely damaged or older samples 30-50%

Insert size: 170-260bp for higher quality samples, 140-220 for lower quality samples.

Time Considerations

A typical workflow schedule:

- Day 1 (total time ~7 hrs)
 - qPCR quantitation and Quality Assessment (3 hrs hands on, 1.25 hrs instrument time)

For manual processing, up to 8 samples is recommended for this workflow. With experience, more samples could be processed manually (up to 16), but would require adaptation of the protocol to using a plate format for the exome capture and wash portion and would require additional equipment. Automation can be applied to these steps to increase throughput using the same workflow schedule. The number of plates processed in a week is scalable and will depend on the amount of instrumentation and technician support available.

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Primer 41bp

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Sample A	Sample A	Sample A	Sample I	Sample I	Sample I	Sample Q	Sample Q	Sample Q
B	Std 2	Std 2	Std 2	Sample B	Sample B	Sample B	Sample J	Sample J	Sample J	Sample R	Sample R	Sample R
C	Std 3	Std 3	Std 3	Sample C	Sample C	Sample C	Sample K	Sample K	Sample K	Sample S	Sample S	Sample S
D	Std 4	Std 4	Std 4	Sample D	Sample D	Sample D	Sample L	Sample L	Sample L	Sample T	Sample T	Sample T
E	Std 5	Std 5	Std 5	Sample E	Sample E	Sample E	Sample M	Sample M	Sample M	Sample U	Sample U	Sample U
F	NTC	NTC	NTC	Sample F	Sample F	Sample F	Sample N	Sample N	Sample N	Sample V	Sample V	Sample V
G				Sample G	Sample G	Sample G	Sample O	Sample O	Sample O	Sample W	Sample W	Sample W
H				Sample H	Sample H	Sample H	Sample P	Sample P	Sample P	Sample X	Sample X	Sample X

Primer 129bp

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Sample A	Sample A	Sample A	Sample I	Sample I	Sample I	Sample Q	Sample Q	Sample Q
B	Std 2	Std 2	Std 2	Sample B	Sample B	Sample B	Sample J	Sample J	Sample J	Sample R	Sample R	Sample R
C	Std 3	Std 3	Std 3	Sample C	Sample C	Sample C	Sample K	Sample K	Sample K	Sample S	Sample S	Sample S
D	Std 4	Std 4	Std 4	Sample D	Sample D	Sample D	Sample L	Sample L	Sample L	Sample T	Sample T	Sample T
E	Std 5	Std 5	Std 5	Sample E	Sample E	Sample E	Sample M	Sample M	Sample M	Sample U	Sample U	Sample U
F	NTC	NTC	NTC	Sample F	Sample F	Sample F	Sample N	Sample N	Sample N	Sample V	Sample V	Sample V
G				Sample G	Sample G	Sample G	Sample O	Sample O	Sample O	Sample W	Sample W	Sample W
H				Sample H	Sample H	Sample H	Sample P	Sample P	Sample P	Sample X	Sample X	Sample X

Primer 305bp

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Sample A	Sample A	Sample A	Sample I	Sample I	Sample I	Sample Q	Sample Q	Sample Q
B	Std 2	Std 2	Std 2	Sample B	Sample B	Sample B	Sample J	Sample J	Sample J	Sample R	Sample R	Sample R
C	Std 3	Std 3	Std 3	Sample C	Sample C	Sample C	Sample K	Sample K	Sample K	Sample S	Sample S	Sample S
D	Std 4	Std 4	Std 4	Sample D	Sample D	Sample D	Sample L	Sample L	Sample L	Sample T	Sample T	Sample T
E	Std 5	Std 5	Std 5	Sample E	Sample E	Sample E	Sample M	Sample M	Sample M	Sample U	Sample U	Sample U
F	NTC	NTC	NTC	Sample F	Sample F	Sample F	Sample N	Sample N	Sample N	Sample V	Sample V	Sample V
G				Sample G	Sample G	Sample G	Sample O	Sample O	Sample O	Sample W	Sample W	Sample W
H				Sample H	Sample H	Sample H	Sample P	Sample P	Sample P	Sample X	Sample X	Sample X

Figure 1.

An example qPCR plate map including well assignment for standards, no template controls (NTC) and diluted samples in triplicate.

Figure 2.

DNA fragmentation quality check. 50ng of FFPE derived DNA was sheared using the Covaris E220 instrument. Following clean up each sample was diluted 1:5 and run on a 2100 BioAnalyzer High Sensitivity DNA chip to check the DNA fragment size distribution and sample concentration. A broad distribution of sizes should be expected between 100-2000bp is typical, with the majority of the fragments in the 200-800bp range.

Figure 3.

Pre-Capture amplification quality check. Adapter ligated libraries were amplified prior to hybridization capture. Following clean up each sample was diluted 1:100 and run on a 2100 BioAnalyzer High Sensitivity DNA chip to confirm library fragment size and concentration. A focused size distribution between 200-800bp is typical. Quality of FFPE samples may affect how this distribution appears (average size between 250-550bp). Fragment size includes the added length of bases (123bp) from the ligated adapters.

Figure 4. Post-Capture amplification quality check. Enriched libraries were amplified prior to sequencing. Following clean up each sample was diluted 1:10 and run on a BioAnalyzer High Sensitivity chip to determine library molarity.

Oligonucleotide	Sequence*
Universal Adapter	5' AATGATACGGCGACACCGAGATCTACACTCTTTCCTACACGACGCTCTCCGATCT
Indexed Adapter 1	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AACTGATA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 2	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AACTCGA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 3	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ATGCTAA ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 4	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AGTGGTCA ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 5	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACCACTG ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 6	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACATTGGCA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 7	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACAGATCTG ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 8	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ATCAAGTAT CTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 9	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C CGCTGATC ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 10	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACAAGCTAA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 11	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C CTGTAGCC ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 12	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AGTACAA GATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 13	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AAACAA CTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 14	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AAAGAGAA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 15	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AAAGCTTAA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 16	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AGAGCGAA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 17	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AGGTACA ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 18	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACAGAAAT CTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 19	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACAGAGAA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 20	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACCTCAA ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 21	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACGCTCGAA TCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 22	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AGTATCA ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 23	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C ACTATGCA ATCTCGTA TGCCGCTCTTCGCTTG
Indexed Adapter 24	5' GATCGGAA GAGCA CACGTCGAAC TCCAGTCA C AGAGTCAA ATCTCGTA TGCCGCTCTTCGCTTG
PCR Primer 1.0	5' AATGATA CGGCGACCA CCGAGA
PCR Primer 2.0	5' CAAGCAGAAGAC GGCATACGAG
Universal Blocker	5' AATGATA CGGCGACCA CCGAGATCTACACTCTTTCCTACACGACGCTCTCCGATCT
Indexed Blocker 1	5' CAA GCA GAAGA CGGCA TACGAGATATCA CGTTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 2	5' CAA GCA GAAGA CGGCA TACGAGATCGATGTTTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 3	5' CAA GCA GAAGA CGGCA TACGAGATTAGGCATGTGA CTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 4	5' CAA GCA GAAGA CGGCA TACGAGATTGACCA CTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 5	5' CAA GCA GAAGA CGGCA TACGAGATACAGTGGTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 6	5' CAA GCA GAAGA CGGCA TACGAGATGCCAA TGTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 7	5' CAA GCA GAAGA CGGCA TACGAGATCA GATCTGGTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 8	5' CAA GCA GAAGA CGGCA TACGAGATAC TTGATGGTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 9	5' CAA GCA GAAGA CGGCA TACGAGATGATCAGCGGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 10	5' CAA GCA GAAGA CGGCA TACGAGATTA GCTTGTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 11	5' CAA GCA GAAGA CGGCA TACGAGATGGCTACAGGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 12	5' CAA GCA GAAGA CGGCA TACGAGATCTGTACTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 13	5' CAA GCA GAAGA CGGCA TACGAGATTGTTTGTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 14	5' CAA GCA GAAGA CGGCA TACGAGATTCTCGGTTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 15	5' CAA GCA GAAGA CGGCA TACGAGATTAAGC GTTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 16	5' CAA GCA GAAGA CGGCA TACGAGATTCTGTCTTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 17	5' CAA GCA GAAGA CGGCA TACGAGATTGTA CCTTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 18	5' CAA GCA GAAGA CGGCA TACGAGATTCTGTGTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 19	5' CAA GCA GAAGA CGGCA TACGAGATTCTGCTGTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT
Indexed Blocker 20	5' CAA GCA GAAGA CGGCA TACGAGATTGGAGGTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 21	5' CAA GCA GAAGA CGGCA TACGAGATTGAC GCTGTGTGACTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 22	5' CAA GCA GAAGA CGGCA TACGAGATTGATC GTGTGA CTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 23	5' CAA GCA GAAGA CGGCA TACGAGATTGCA TA GTGTGA CTGGAGTTCA GACGTGTGCTCTCCGATCT
Indexed Blocker 24	5' CAA GCA GAAGA CGGCA TACGAGATTGACTCTGTGACTGGA GTTCAGACGTGTGCTCTCCGATCT

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Figure 5.
Oligonucleotide sequences for indexed adapters, indexed blockers, universal blockers and PCR primers.

Table 1

Troubleshooting

Problem	Possible Cause	Solution
Low yield for qPCR/amplification is not within standard curve	Dilution prepared improperly, or initial quantitation value over/under estimated amount of DNA present, extremely poor quality DNA	Remake the dilution adjusting the concentration based on where the amplification is detected from the initial qPCR data (i.e. if the sample is too high on the amplification plot, then the sample was too dilute. If the sample was too low for the amplification plot, then the sample was not diluted enough). Confirm quality using the qPCR assessment. If low quality, higher DNA input may be required.
DNA fragment size distribution	Improper Covaris shearing parameters	Check that the proper program and parameters are set correctly on the instrument. If necessary, process test samples to optimize the settings.
Low yield at library amplification (prior to exome enrichment)	Less than 50ng of sample added into assay	Check the qPCR results, ensuring proper quantitation of sample and calculation for proper dispensing of DNA into the assay.
	Improper Ampure XP bead clean up leading to sample loss	Check quantity of sample after each clean up to ensure limited amount of loss. Follow clean up steps exactly, ensuring that water and ethanol are not swapped and ethanol is prepared fresh. Ensure that the beads are properly mixed prior to dispensing and are chocolate brown in color.
	Inefficient ligation of adapter	For End Repair/A-Tailing and Ligation steps - Do not vortex enzyme. Ensure that master mixes are mixed well and that all reagents are stored properly, especially the dATP.
	PCR Failure	Ensure that no Ampure XP beads are transferred into the PCR reaction and the master mix is properly mixed and reagents are stored properly. If needed, the number of cycles can be increased to increase the yield. Note that as the cycle number increases the amount of molecular duplicates will also increase (less unique molecules).
Excessive adapter-dimer or primer-dimer present in BioAnalyzer trace	Inefficient ligation of adapter, improperly diluted adapter oligonucleotides, inefficient Ampure XP Bead clean up	For End Repair/A-Tailing and Ligation steps - Do not vortex enzymes. Ensure that master mixes are mixed well and that all reagents are stored properly, especially the dATP. Check oligonucleotide stock concentrations and dilutions. Follow clean up steps exactly, ensuring that water and ethanol are not swapped and ethanol is prepared fresh. Ensure that the beads are properly mixed prior to dispensing and are chocolate brown in color.
Over amplification	Improperly diluted primers, incorrect PCR cycles used.	Check oligonucleotide stock concentrations and dilutions. Ensure the correct program is used.
Low yield at post capture amplification (after exome enrichment) - Hybridization Failure	Buffers/bait not properly made	Check all buffers and reagents.
	DNA is not denatured	Ensure that instruments are functioning at the proper temperatures and have the correct programs.
	Temperature is not correct during wash process	Ensure that instruments are functioning at the proper temperatures and have the correct programs.
	Incorrect streptavidin beads used for capture	Ensure that the correct streptavidin beads are used for capture.
	Swapping of the wash buffers	Ensure that all buffers and reagents are correct and stored properly.

Problem	Possible Cause	Solution
	PCR Failure	Ensure that the master mix is properly mixed and reagents are stored properly. Ensure that instruments are functioning at the proper temperature and using the correct programs.
High percent duplication rate	Inefficient ligation of adapter or less than 50ng of sample added into assay	Reflects a low number of unique molecules. For End Repair/A-Tailing and Ligation steps - Do not vortex enzymes. Ensure that master mixes are mixed well and that all reagents are stored properly, especially the dATP. Check the qPCR results, ensuring proper quantitation of sample and calculation for proper dispensing of DNA into the assay. Also a reflection of DNA damage.
Low percent selection rate/Hybridization Failure	Buffers/bait not properly made	Check all buffers and reagents.
	DNA is not denatured	Ensure that instruments are functioning at the proper temperatures and are using the correct programs.
	Temperature is not correct during wash process	Ensure that instruments are functioning at the proper temperatures and have the correct programs.
	Inefficient blocking during hybridization or incorrect indexed blocker was used	Ensure that the appropriate indexed blocker is matched to the indexed adapter used for each individual sample. Ensure that blockers are diluted to proper concentration and were added to the hybridization reaction.
Incorrect Insert Size	Improper Covaris shearing parameters	Check that the proper program and parameters are set correctly on the instrument. If necessary, process test samples to optimize the parameter settings.