## PCR amplify genomic DNA targets (1st step)

A singleplex PCR reaction is described in this section. To save time and reagents, you can perform multiplex PCR if the primers have been specifically designed for multiplex PCR.

IMPORTANT! Multiplexing may increase the probability of unwanted PCR products and of over- or underrepresented amplicons in the pool.

Note: Remember that for bidirectional sequencing, each target region has two sets of primers, and must be amplified in separate PCR reactions.

### Materials required for this procedure

10 μM of forward and reverse primers mix (HLA-A only and Multiplex w/o HLA-A)

0.2-mL strip tubes or 96-well EppendorfR plate

NEB Q5 HS master mix (2x)

High-quality genomic DNA

### 1. Thaw the PCR primers, NEB Q5HS, and high-quality genomic DNA on ice.

### 2. Add the following reagents to 0.2-mL strip tubes or to the wells in a 96-well PCR plate (you may make a master mix first):

Component Volume

100 ng genomic DNA 5 μL

primer mix 0.4 μL

Q5 HS master mix 5 μL

Total 10 μL

### 3. Load the tubes or plates in a thermal cycler and run the program to amplify the target DNA (about 20 min, prepare next step reaction during PCR):

Stage Step Temperature Time

Holding Activate the enzyme 98 oC 30sec

Cycling (25 cycles)

Denature 98oC 10 sec

Anneal 68oC 15 sec

Extend 72oC 1 min/kb (20 sec)

Extend 72 oC 2 min

Holding — 4oC ∞

## Purify the 1st step amplicon libraries

IMPORTANT! If the total fragment size, including amplicon and fusion primer sequence, is <100 bp, use a different purification method such as Qiagen MinElute PCR Purification Kit.

Note: Use 1.5 volumes of Agencourt® AMPure® XP Reagent for every volume of pooled DNA.

### Materials required for this procedure

Agencourt AMPureR XP Reagent

Amplified genomic DNA

lowTE,(10mM Tris pH 8.0, 0.1mM EDTA)

AgencourtR SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator (20-200ul) or Alpaqua LE Magnet Plate for low volume elution (8-10ul)

70% ethanol

### 1. Resuspend the Agencourt® AMPure® XP Reagent and allow the mixture to come to room temperature (~30 minutes).

### 2. Prepare 70% ethanol:

Component Volume

Nuclease-Free Water 3 mL

Ethanol, Absolute 7 mL

Total 1000 μL

IMPORTANT! Use *freshly prepared* 70% ethanol. A higher percentage of ethanol causes inefficient

washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

### 3. Add Agencourt® AMPure® XP Reagent to each sample as indicated below, mix the bead suspension with the DNA by pipetting up and down several times, and incubate the samples at room temperature for 5 minutes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Size (bp) | 100-300 | 301-705 | 750-3k | 3k-10k |
| Volume AMPure | 1.8X | 1.0X | 0.60X | 0.45X |

Component Volume

Amplified genomic DNA 10 μL

AgencourtR AMPureR XP Reagent 15 μL\*

\* Equal to 1.5 volumes of PCR product

### 4. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator or Alpaqua LE Magnet Plate for low volume elution) for 2 minutes. After the solution clears, carefully remove and discard the supernatant from each sample without disturbing the pellet.

### 5. Without removing the samples from the magnet, add 100 μL of freshly prepared 70% ethanol to each well or tube at opposite site of the pellet and incubate the samples at room temperature for 30 seconds. After the solution clears, remove and discard the supernatant without disturbing the pellet.

### 6. Repeat step 5 for a second wash.

### 7. To remove residual ethanol, keep the samples on the magnet and carefully aspirate remaining supernatant with a 20-μL pipet without disturbing the pellet.

### 8. Air-dry the beads on the magnet at room temperature for ≤5 minutes. (do not overdry)

### 9. Remove the samples from the magnet, add 25 μL of water to each well or tube, and pipet the samples up and down to mix.

### 10. Place the plate or tube on the magnet for at least 2 minute until the solution clears and the beads are pelleted. Transfer 22.6μL supernatant containing the amplicon library to a new well or tube.

## PCR adding indexed adaptor to DNA targets (2nd step)

### Materials required for this procedure

10 μM of forward and reverse primers mix (indexed adaptor primer D50X and D70X)

0.2-mL strip tubes or 96-well EppendorfR plate

NEB Q5 HS master mix (2x)

High-quality genomic DNA

### 1. Add the following reagents to 0.2-mL strip tubes or to the wells in a 96-well PCR plate (you may make a master mix first) (D50X and D70X premix can keep in a 96well or strip tubes):

Component Volume

Step1 PCR purified 22.6 μL

D50X primer 1.2 μL

D70X primer 1.2 μL

Q5 HS master mix 25 μL

Total 50 μL

### 2. Load the tubes or plates in a thermal cycler and run the program to amplify the target DNA (about 40 min):

Stage Step Temperature Time

Holding Activate the enzyme 98 oC 30sec

Cycling (13 cycles)

Denature 98oC 10 sec

Anneal 68oC 15 sec

Extend 72oC 1 min/kb (20 sec)

Extend 72 oC 2 min

Holding — 4oC ∞

## Purify the amplicon libraries

IMPORTANT! If the total fragment size, including amplicon and fusion primer sequence, is <100 bp, use a different purification method such as Qiagen MinElute PCR Purification Kit.

Note: Use 1.8 volumes of Agencourt® AMPure® XP Reagent for every volume of pooled DNA.

### Materials required for this procedure

Agencourt AMPureR XP Reagent

Amplified genomic DNA

lowTE,(10mM Tris pH 8.0, 0.1mM EDTA)

AgencourtR SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator

70% ethanol

### 1. Resuspend the Agencourt® AMPure® XP Reagent and allow the mixture to come to room temperature (~30 minutes).

### 2. Prepare 70% ethanol: (can use step II left over)

Component Volume

Nuclease-Free Water 3 mL

Ethanol, Absolute 7 mL

Total 1000 μL

IMPORTANT! Use *freshly prepared* 70% ethanol. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

### 3. Add Agencourt® AMPure® XP Reagent to each sample as indicated below, mix the bead suspension with the DNA by pipetting up and down several times, and incubate the samples at room temperature for 5 minutes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Size (bp) | 100-300 | 301-705 | 750-3k | 3k-10k |
| Volume AMPure | 1.8X | 1.0X | 0.60X | 0.45X |

Component Volume

Amplified genomic DNA 50 μL

AgencourtR AMPureR XP Reagent 50 μL\*

\* Equal to 1.0 volumes of PCR product

### 4. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 2 minutes. After the solution clears, carefully remove and discard the supernatant from each sample without disturbing the pellet.

### 5. Without removing the samples from the magnet, add 100 μL of freshly prepared 70% ethanol to each well or tube at opposite site of the pellet and incubate the samples at room temperature for 30 seconds. After the solution clears, remove and discard the supernatant without disturbing the pellet.

### 6. Repeat step 5 for a second wash.

### 7. To remove residual ethanol, keep the samples on the magnet and carefully aspirate remaining supernatant with a 20-μL pipet without disturbing the pellet.

### 8. Air-dry the beads on the magnet at room temperature for ≤5 minutes. (do not overdry)

### 9. Remove the samples from the magnet, add 40 μL of lowTE to each well or tube, and pipet the samples up and down to mix.

### 10. Place the plate or tube on the magnet for at least 2 minute until the solution clears and the beads are pelleted. Transfer 40 μL supernatant containing the amplicon library to a new well or tube.

IMPORTANT! The ***supernatant*** contains the amplicon library. **Do not discard! Beads interfere Bioanalyzer reading.**

consider dilute the DNA (add 50 to100 μL low TE) first before doing Bioanalyzer, high concentration may cause error readings.

**If a lot of dimers exist in gel or bioanalyzer, consider a second round of purification.**

## Prepare an equimolar pool of the amplicon libraries (by Bioanalyzer or qPCR)

Pooling the amplicon libraries in equimolar amounts for library construction ensures even coverage of the target regions.

Materials required for this procedure

KK4854 – 07960298001 qPCR Master Mix optimized for LightCycler® 480 (Complete Kits with: DNA Standards 1 – 6 (80 μL each) Primer Mix (1 mL) KAPA SYBR® FAST qPCR Master Mix (5 mL))

Roche Light Cycler 480

KAPA SYBR FAST qPCR Master Mix (2X) 10 μL

Primer Premix (10X) 2 μL

PCR-grade water 4 μL

Purified Library DNA or Standard 4 μL (library diluted 1:1000 - 10000)

Total volume: 20 μL

qPCR

Initial denaturation 95ºC 5 min 1 cycle

Denaturation 95ºC 30 sec

Annealing/Extension 60ºC 45 sec 35 cycles

Data acquisition

Melt curve analysis 65 – 95°C

Data analysis (absolute quantification) with light cycler or KAPA calculation sheet for the concentration in nM

Make equal amount of DNA mix with 2nM library.

In the following procedure, each amplicon library is analyzed on the Agilent Bioanalyzer™ instrument to:

Quantitate each amplicon library for pooling

Assess the size distribution of each library

Materials required for this procedure

Agilent Bioanalyzer™ 2100 instrument

Agilent DNA 1000 Kit (HS DNA kits)

Amplified genomic DNA

lowTE, pH 8.0

### 1. Analyze an aliquot of each amplicon library with a Bioanalyzer™ instrument and Agilent DNA HS Kit.

Follow the manufacturer’s instructions.

If a library is too dilute to detect with a DNA1000 Kit, use an Agilent High Sensitivity Kit.

IMPORTANT! Ensure that excessive amounts of primer-dimers (immediately adjacent to the marker) or over-amplification products (concatemers) are not present.

### 2. Determine the molar concentration (nmol/L) of each amplicon library using the Bioanalyzer™ software.

If necessary, use manual integration to place the entire range of library fragments within a single peak.

### 3. Prepare an equimolar pool of amplicon libraries at the highest possible concentration.

### Optional: 4. Calculate the combined concentration of the pooled amplicon library stock.

Alternatively, analyze an aliquot of the library pool on the Bioanalyzer™ with an Agilent DNA 1000 Kit,and use the Bioanalyzer™ software to determine the molar concentration of the pooled library stock. If necessary, use manual integration to place the entire distribution of library molecules within a single peak.

Follow the manufacturer’s instructions.

STOPPING POINT Store the pooled library stock at –20°C. Before use, thaw the storage stock on ice. To reduce the number of freeze-thaw cycles, store the pooled stock in several aliquots.

## Sequencing on Illumina Miseq machine

Sequencing reagent: Miseq v2 micro 300 cycle kits or v3 150 cycle kits

Thaw the reagent cartridge prior to sequencing

Perform a post-wash on the Miseq machine

### Pooling the amplicon libraries in equimolar amounts (2nM library) for library construction ensures even coverage of the target regions.

### Fill the desired sample sheet (cartridge number, date, dual-index: Truseq-HT, 2x 151 (or 1x151), mode: fastq)

### Prepare fresh 0.2 N NaOH

### Mix 5 ul 2nM library with 5 ul 0.2 N NaOH, spin down (280g) for 1 min, room temperature denature for 5 min,

### 10ul denature DNA with 990ul HT1 (supplied with Miseq catridge)

### Mix with denatured PhiX DNA (5%-20% depend on the diversity of library), final total volume 600ul, keep on ice till loading.

### Clean the flow cell, water rinse, gently wipe, alcohol wipe (not touch inlet), lens paper wipe and clean (make sure flow cell dry, not dirt or fingerprint on lanes)

### Fill the 600ul mixed denatured DNA to cartridge

### According to the guide on Miseq screen, load the flow cell and cartridge and start the run.