Biotinylated baits for capturing mitochondrial genome

Introduction

Mitochondrial genome can be amplified using long-range PCR. The PCR product can be sheared to make library, and sequenced subsequently using Illumina sequencing. The long-range PCR product can also be used to make homemade biotinylated baits to capture those individuals that don't work for the long-range PCR.

Library prep procedure

I. long-range PCR

Mitochondrial genome can be amplified using one or more pair of primers. Using more primer pairs that amplify different overlapping parts of the mt genome means more work, but sometime may produce cleaner PCR products. Use 5' blocked primers, such as /5SpC3/ from IDT to increase the homogeneity of capturing (Harismendy and Frazer, 2009).

1. Prepare a master mix for the number of samples needed as the follow. Add 0.4 μL of total sample DNA to each tube.

Reagent	Volume (μL) per sample	× *n*	Final concentration in 20-μL reaction
Takara LA Taq Hot Start Version (cat# RR042A)	0.2		0.05 U/μL
Buffer (10×)	2		1×
$MgCl_2$ (25 mM)	2		2.5 mM
dNTPs (25 mM)	0.32		0.4 mM each
Forward primer (10 μM)	1		0.5 μM
Reverse primer (10 μM)	1		0.5 μM
H_2O	13.08		

2. Load the tubes onto a thermo cycler and run the following program:

    1. 94 °C for 1 min
    2. 98 °C for 10 sec
    3. 68 °C for 15 min
    4. goto step 2 for 34 more times
    5. 72 °C for 30 min
    6. 4 °C for 10 min
    7. end

3. Check 2 μL of the PCR product on a mini agarose gel. Prepare more PCR for the products with clean band and expected size to obtain enough DNA for shearing. Note: product > 10 kb may be stuck in the well of the agarose gel.

II. Shearing the genomic DNA

1. "Dry" SPRI beads using a magnetic plate. Please read "*DNA Clean-Up Using Solid Phase Reversible Immobilization (SPRI)*" for how to use the SPRI system. Add the PCR product and 10 μL PEG to each tube with dried beads. Clean the PCR products using SPRI methods. Elute the samples with 20 μL of water.
2. Measure the concentration of the elution. If there are multiple amplified reactions for one sample, mix them in equal molar, i.e., the short fragment should be at lower concentration than the large fragment.
3. Load 3 to 6 μg PCR product in 130 μL volume and shear it to ~ 200 bp range.

III. Size selection

Select DNA that has size between 150 bp and 200 bp.

1. Add 100 μL AMPure XP beads to a 200 μL tube. "Dry" the beads using a magnetic plate. Please read "*DNA Clean-Up Using Solid Phase Reversible Immobilization (SPRI)*" for how to use the SPRI system.
2. Add 50 μL sheared samples and 37.5 μL 20% PEG buffer to the dried beads, vortex the tube for several times. Let the tube set for 10 min, collect the liquid at the bottom of the tube by briefly centrifuging.
3. Place the tube on a magnetic plate, and let it stand for 10 min to separate the beads from the solution. Transfer the supernatant to a new tube with dried beads prepared as step 1.
4. Add 12.5 μL 20% PEG buffer to the sample tube. Prepare one positive and one negative control as the following: Add 50 μL AMPure XP beads to a tube with 30 μL nuclease free water and to a tube with 30 μL positive DNA (1 to 100 diluted PCR product of any gene with a size 100 bp - 200 bp). Vortex all tubes for several times. Let all tubes set for 10 min, collect the liquid at the bottom of the tube by briefly centrifuging.
5. Place the tube on a magnetic plate, and let it stand for 10 min to separate the beads from the solution. Pipette off and discard the supernatant without removing the beads.
6. Leave the tube on the magnetic rack, and wash the beads by adding 186 μL of freshly prepared 70% ethanol. Let stand for 1 min and remove the supernatant. *Keep the tube on the magnetic rack, do not disturb the beads!!!
7. Repeat Step 6 one more time.
8. Remove residual traces of ethanol. Let the beads air-dry for 5 min at room temperature without caps.
9. Proceed to the next step immediately.

IV. Blunt-end repair

1. Prepare a master mix for the number of samples needed as the follow. Add 20 µL of the master mix to each sample tube. Mix the sample by pipetting it up and down.

Reagent	Volume (µL) per sample × *n*	Final concentration in 20-µL reaction
Buffer Tango (10×)	2	1×
dNTPs (25 mM each)	0.08	100 µM each
ATP (100 mM)	0.2	1 mM
T4 polynucleotide kinase (10 U/µL)	1	0.5 U/µL
T4 DNA polymerase (5 U/µL)	0.4	0.1 U/µL
H_2O	16.32	

2. Incubate the samples in a thermal cycler for 15 min at 25 °C followed by 5 min at 12 °C.
3. Add 20 µL 20% PEG to the sample and clean up the reaction according to the SPRI protocol. Keep the dried beads. Proceed immediately to the ligation step.

V. Adapter ligation (**using M13 adapter, not the adapter for the target lib!!!**)

1. Prepare a master mix for the number of samples needed. Add 39 µL of the master mix to each sample tube. Mix the samples well by vortexing.

Reagent	Volume (µL) per sample × *n*	Final concentration in 40-µL reaction
T4 DNA ligase buffer (10×)	4	1×
PEG-4000 (50%)	4	5%
Adapter mix (50 µM each)	2	2.5 µM each
H_2O	29	

2. Spin down the liquid by brief centrifuging. Add 1 µL T4 DNA ligase (5 U/µL) to the sample. Briefly vortex to mix the sample and collect the liquid at the bottom of the tube by briefly centrifuging, then incubate for 30 min at 22 °C in a thermal cycler.
3. Cleanup the reaction using SPRI methods (adding 40 µL PEG). Keep the dried beads. Proceed immediately to the next step.

VI. Fill-in

1. Prepare a master mix for the number of samples needed.

Reagent	Volume (µL) per sample × *n*	Final concentration in 40-µL reaction
ThermoPol reaction buffer (10×)	4	1×
dNTPs (25 mM each)	0.4	250 µM each
Bst polymerase, large fragment (8 U/µL)	1.5	0.3 U/µL
H_2O	34.1	

2. Add 40 µL of master mix to the samples. Briefly vortex to mix the sample. Collect the liquid at the bottom of the tube by briefly centrifuging. Incubate the samples for

20 min at 37 °C.

3. Cleanup the samples using SPRI method (adding 40 µL PEG). Elute the samples with 20 µL of nuclease free water. Transfer the supernatant to a new tube labeled as "sample name + BaitsLib". The libraries can be kept frozen at -20 °C for a short period.

VII. PCR to make the biotinylated baits.

1. Prepare a master mix as the follow. More master mix can be made if more bait is needed.

Reagent	Volume (µL) per sample	× *n*	Final concentration in 50-µL reaction
Gold Buffer (10×)	5		1×
$MgCl_2$ (25 mM)	5		2.5 mM
dNTP/UTP (25 mM ATP, UTP, CTP and GTP)	0.5		0.25 mM
bioM13F (10 µM)	2		0.4 µM
bioM13R (10 µM)	2		0.4 µM
AmpliTaq Gold 360	0.25		1.25 U/reaction
H_2O	29.25		

2. Add 44 µL of master mix to empty tubes, and then add 6 µL of "BaitsLib" samples. Mix well and amplify the samples using the following thermal profile: 95 °C for 9 min, ~ 32 cycles of 95 °C for 15 sec, 55 °C for 30 sec and 72 °C for 45 sec, followed by 72 °C for 7 min, and hold at 4 °C for 10 min. Check the PCR product on a mini agarose gel. There should be no amplification in the negative sample. There should be a size shift for the positive sample compare to the positive DNA insert.

3. Cleanup the PCR product using SPRI method (adding 100 µL PEG). Elute the DNA using 200 µL of water and transfer it to a new tube labeled as "sample name + HomeBaits". Measure the concentration using a Qubit 2.0 Fluorometer.

Recipe for M13 adapter mix

1. Assemble the following hybridization reactions in separate PCR tubes:

Hybridization mix for adapter 1 (100 μM):

Reagent	Volume (μL)	Final concentration in 100-μL reaction
Adaptor1 (500 μM)	20	100 μM
Id697 (500 μM)	20	100 μM
Oligo hybridization buffer (10×)	10	1×
H_2O	50	

Hybridization mix for adapter 2 (100 μM):

Reagent	Volume (μL)	Final concentration in 100-μL reaction
Adaptor2 (500 μM)	20	100 μM
Id697 (500 μM)	20	100 μM
Oligo hybridization buffer (10×)	10	1×
H_2O	50	

2. Mix and incubate the reactions in a thermal cycler for 10 sec at 95 °C, followed by a ramp from 95 °C to 12 °C at a rate of 0.1 °C/sec. Combine both reactions to obtain a ready-to-use adapter mix (50 μM each adapter).

Recipe for oligo hybridization buffer (10X)

Reagent	Volume (μL)	Final concentration in 10 ml
NaCl (5 M)	1 ml	500 mM
Tris-Cl, pH 8.0 (1 M)	100 μL	10 mM
EDTA, pH 8.0 (0.5 M)	20 μL	1 mM
H_2O	8.88 ml	

Sequences of adapters, and primers

* indicates a PTO bond

Name	Sequences
Adaptor1	G*T*TTTCCCAGTCACGACTTCATA*C*G
Adaptor2	C*A*GGAAACAGCTATGACTTCATA*C*G
Id697	C*G*TATG*A*A
M13_Forward(-40)	GTTTTCCCAGTCACGAC
M13_Reverse	CAGGAAACAGCTATGAC
bio*M13_Forward(-40)	/52-Bio/GT TTT CCC AGT CAC GAC
bio*M13_Reverse	/52-Bio/CA GGA AAC AGC TAT GAC