Whole-mitogenome sequencing of *Oncorhynchus masou masou* by next-generation sequencing

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MATERIALS TEXT

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PrimeSTAR GXL DNA Polymerase **Contributed by users Catalog #R050A**

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

KAPA Library Quantification Kit for Illumina® Platforms **Kapa Biosystems Catalog #KK4835**

QIAseq FX DNA Library Kit **Qiagen Catalog #180475**

MiSeq v3 Sequencing Reagents (600 cycles) **Illumina, Inc. Catalog #MS-102-3003**

Preparation of the mitogenome DNA

1 Amplify the *Oncorhynchus masou masou* whole-mitogenome fragment (16.6 kbp) with the following PCR mixture and PCR program:

Component	Amount
5× PrimeSTAR GXL Buffer (TaKaRa Bio Inc.)	3 µl
PrimeSTAR GXL DNA polymerase (TaKaRa Bio Inc.)	0.3 µl
2.5 mM dNTP mix	1.2 µl
10 µM primer MT-F	0.3 µl
10 µM primer MT-R	0.3 µl
Genomic DNA	20–100 ng
Nuclease-free water	Variable
Total volume	15 µl

PCR mixture

Step	Temperature	Time	Number of cycles
1	98°C	10 sec	1
2	98°C	10 sec	40
3	60°C	15 sec	
4	68°C	14 min	
5	68°C	5 min	1
6	8 °C	Hold	N/A

PCR program

2 Electrophorese the total PCR product mixture through a 0.8% agarose–TAE gel.

3 Cut the band containing the mitogenome from the agarose gel.

4 Extract the mitogenome from the excised band by using the Wizard SV Gel and PCR Clean-up System (Promega), and elute with 20 µl 10 mM Tris-HCl, pH 8.0 warmed to 60°C.

5 Measure the concentration of the purified mitogenome DNA by using a spectrophotometer. Also, check the quality of the purified mitogenome DNA by conducting agarose gel electrophoresis.

Preparation of the mitogenome libraries for next-generation sequencing

6 Amplify and purify the mitogenome libraries by using a QIAseq FX DNA Library Kit (QIAGEN) and Agencourt AMpure XP beads (Beckman Coulter), respectively. To reduce reagent use, library preparation can be performed on a quarter of the

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scale described in the manufacturer's manual (i.e., by using 25 ng of template DNA) if the following conditions are used:

- Thermocycling for the fragmentation, end repair, and “A” addition steps is conducted under the following program:

Step	Temperature	Time
1	4°C	1 min
2	32°C	320 sec
3	65°C	30 min
4	4°C	Hold

- FX reaction mix is prepared following table without FX Enhancer.

Component	Amount
FX Buffer, 10x	1.25 µl
Purified DNA (in 10 mM Tris, pH 8.0)	25 ng
Nuclease-free water	Variable
Total volume without FX Enhancer and FX Enzyme Mix	10 µl

- Amplification of library DNA is performed with the following condition.

Step	Time	Temperature	Number of cycles
1	2 min	98°C	1
2	20 sec	98°C	6
3	30 sec	60°C	
4	30 sec	72°C	
5	1 min	72°C	1
6	Hold	8°C	N/A

7 For each library, dissolve the amplified and purified library DNA in 10 mM Tris-HCl, pH 8.0.

8 Take an aliquot of each library and dilute it 1:3 with 10 mM Tris-HCl, pH 8.0 for quality check and quantification (described below). Store the rest of the libraries at −20°C until use.

9 Check the quality and fragment size of each diluted library by using an Agilent BioAnalyzer (Agilent) and Agilent High Sensitivity DNA Kit (Agilent).

Note: The quality (fragment size and presence of adaptor dimer) can also be checked in the next step 10.

10 Quantify the molar concentration of each library by conducting qPCR using an KAPA Library Quantification Kit (KAPA Biosystems)(please refer to the protocol (dx.doi.org/10.17504/protocols.io.bmgck3sw) for details)

11 Pool equal amounts of each library adjusted to 2 nM to obtain the final library.

Note: It is recommended to check by qPCR whether the molar concentration of the pooled library is appropriate.

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12 Sequence the pooled and denatured library (8 pM) containing 5% volume of PhiX (control library; Illumina) by using an Illumina MiSeq system and MiSeq Reagent Kit V3 (300 bp paired-end reads) (Illumina).

Sequence analysis

13 Check the quality of the obtained next generation sequencing (NGS) data (i.e., FASTQ data) by using FastQC software.

14 Trim the FASTQ data by using Trimmomatic ver. 0.36 as follows: (a) apply a cleanup adapter and (b) filter out reads with low quality (Q score, <28) and short-length (<50).

15 Map the reads to the reference mitogenome sequence (*O. m. masou* accession No: NC_008747) by using Burrow–Wheeler Aligner ver. 0.7.12.

16 Convert the obtained SAM (Sequence Alignment Map) files to BAM (Binary Alignment Map) files using SAMtools ver. 1.4.1.

17 Visualize the resulting reads with Integrative Genomics Viewer version 2.3.83 and identify SNP (single nucleotide polymorphism) sites with TASSEL ver. 5.0.

18 Replace the primer sites and those outside the region of interest (mitogenome positions: 13950–14022) with the predetermined *ND5* sequence.

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