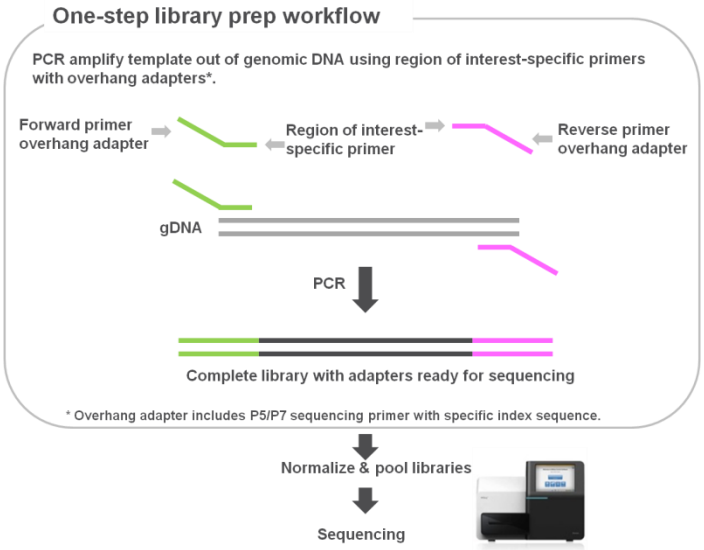
SAFESeqr kit

1 Introduction

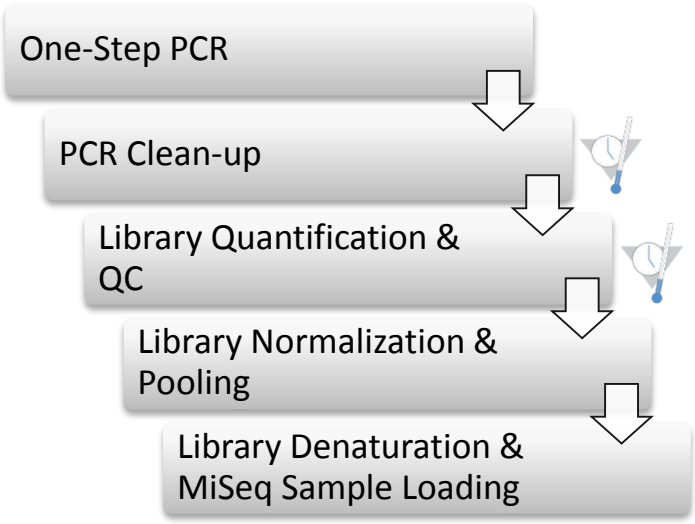
SAFESeqr Sequencing Library Preparation Guide provides the complete guidelines to amplify a short targeted region (~100 bp) in mitochondrial 12S rRNA gene. This region of interest (ROI) was determined using the proprietary bioinformatics pipeline from *BioEasy Sdn Bhd* (Malaysia). This specific ROI is capable of distinguishing more than 1537 genus and 2867 species of eukaryotes.

SAFESeqr provides all the sensitivity and specificity to target sample authenticity in highly processed food, feeds or any samples. Combining SAFESeqr one-step PCR library preparation workflow with Illumina MiSeq Next-Generation Sequencing platform, it is capable of detecting most-to-all major and minor contributors in a mixture sample. Thus, it enables the limit of detection of as low as 0.1pg with the input of 2 ng DNA sample.

SAFESeqr one-step PCR library preparation workflow reduces the handling time from sample-to-results in less than 1 day.



The following diagram illustrates the workflow for SAFESeqr Sequencing Library Preparation Protocol. Safe stopping points are marked between steps.



2. Protocol

2.1 One-step PCR

This step uses polymerase chain reaction (PCR) to amplify out of the region of interest (ROI) from gDNA sample using ROI-specific primers with Illumina adapters attached. The Illumina adapters attached is ready for cluster generation and sequencing on a MiSeq.

All DNA samples must be in high purity (A260/280 = 1.8 - 2.0 and A260/230 ≥ 2.0) and quantified using fluorescence-based method, e.g. Qubit or PicoGreen.

Consumables

No	Item	Quantity	Storage
1	SAFESeqr Kit	2 µL of 5 µM primer per sample	- 20°C
2	FailSafe PCR Enzyme Mix (2.5 U/µL)	0.5 µL per sample (1.25 U)	- 20°C
3	FailSafe PCR 2x PreMix F	12.5 µL per sample	- 20°C
4	Nuclease-Free Water	1 mL	RT

Equipments

No	Item
1	0.2-mL PCR tube or 96-well PCR plate
2	Hot-lid Thermal Cycler
3	Microseal film for 96-well PCR plate

1. Two nanogram of gDNA sample is used as the input DNA. In a 0.2 mL PCR tube / 96-well plate, prepare the following reaction mixture. Combine on ice, all of the following:

Ingredient	Volume (µL)
Primer F (5µM)	2.0
Primer R (5µM)	2.0
gDNA sample (0.25ng/µL)	8.0
FailSafe PCR Enzyme Mix	0.5
Total	**12.5**

! Each library/sample must have unique combination of primers/indexes.

2. Triplicates of non-template control (NTC) must be prepared for every batch of processing. The volume for gDNA sample in NTC is substituted by Nuclease-Free Water.
3. On ice, aliquot 12.5 µL of FailSafe PCR 2x PreMix F into the PCR mixture from Step 1. Mix well.
4. Briefly spin down the PCR mixture.
5. Perform One-step PCR in a hot-lid thermal cycler using the following program:
    - 96ºC for 5 minutes
    - 25 cycles of:
        - 96ºC for 45 seconds
        - 45ºC for 45 seconds
        - 72ºC for 45 seconds
    - 72ºC for 3 minutes
    - Hold at 10ºC
6. Proceed immediately to Section 2.2.

2.2 PCR Clean-Up

This step uses AMPure XP beads to purify the PCR amplicon away from free primers and primer dimer species.

Consumables

No	Item	Quantity	Storage
1	AMPure XP Beads	45 µL per sample	4ºC
2	Freshly Prepared 80% Ethanol	400 µL per sample	RT
3	Resuspension Buffer (RSB) or Nuclease-Free Water	20 µL per sample	RT

Equipments

No	Item
1	Magnetic Stand
2	1.5-mL tubes or 96-well plate
3	Microseal film for 96-well plate

1. Bring the AMPure XP beads to room temperature 30 minutes before use. While the beads warm, prepare 400 µL of fresh 80% ethanol for each sample used in Step 9 later.

! Vortex the AMPure XP beads for 1 minute to make sure that the beads are evenly dispersed before use.

2. Add 45 µL of AMPure XP beads to each 1.5-mL tube or each well of the 96-well plate.
3. Transfer 25 µL of PCR amplified products from Section 2.1 to each 1.5-mL tube or each well of the 96-well plate containing AMPure XP beads.
4. Mix thoroughly by gently pipetting the entire volume of each tube/well up and down 10 times.
5. Incubate at room temperature without shaking for 10 minutes.
6. Place the 1.5-mL tubes or the 96-well plate on a magnetic stand at room temperature for 2 minutes or until the supernatant is clear.
7. Remove and discard the supernatant from each tube/well using a pipette.

! Do not disturb the beads.

8. With the 1.5-mL tubes or the plate remaining on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

a. Add 200 µL of freshly prepared 80% ethanol to each sample tube.
b. Incubate the tube on the magnetic stand for 30 seconds.
c. Carefully remove and discard the supernatant.

! Do not disturb the beads.

9. Repeat Step 8 one more time for a total of two 80% ethanol washes.
10. Allow the tubes or plate to air-dry on their magnetic stands for 10 - 15 minutes at room temperature.
11. Add 20 µL of resuspension buffer (RSB) or Nuclease-Free Water to each tube/well and removed from the magnetic stand.
12. Thoroughly resuspend the beads by gently pipetting 10 times.
13. Incubate the tubes or plates at room temperature for 5 minutes.
14. Place the tubes or plate on the magnetic stand at room temperature for at least 5 minutes, until the supernatant appears clear.
15. Carefully transfer 18 µL of the clear supernatant, from each tube/well to a new tube or plate.
16. Place the tubes or plate on ice and proceed to Section 2.3 or place at -20°C for storage.

Safe stopping point!

2.3 Library Quantification and QC

Consumables

No	Item	Storage
1	Qubit® dsDNA HS Assay Kit	4°C
2	Agilent High Sensitivity DNA Kit	4°C

Equipments

No	Item
1	Qubit® Fluorometer
2	Agilent 2100 Bioanalyzer System
3	0.5-mL clear tubes

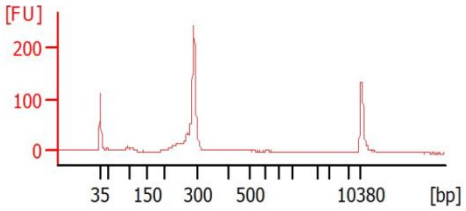
1. Run 2 µL of the products from Section 2.2 on Qubit® Fluorometer or any similar fluorometric quantification method to quantify the library.

! The optimum library will give range of 1 - 10 ng/µL from the 18 µL collected from Section 2.2 PCR Clean-Up step.

2. Run 1 µL of the same product from Section 2.2 on Bioanalyzer High Sensitivity DNA kit to check on the size distribution. The expected size is ~280 bp.
3. Once the library has passed QC, proceed to Section 2.4 or place at -20°C for storage.

Safe stopping point!

FIGURE: Example of HS-DNA Bioanalyzer trace



2.4 Library Normalization and Pooling

Consumables

No	Item	Storage
1	10mM Tris-Cl, pH 8.5 or elution buffer (EB)	RT
2	Tween-20	RT

1. Calculate the DNA concentration in nM based on the concentration determined from Qubit quantification and the size of PCR amplicons as determined from the Bioanalyzer trace:

$$\frac{\text{(concentration in ng/µL)}}{\text{(660 g/mol x average library size)}} \times 10^6 = \text{concentration in nM}$$

For example:
Qubit reading= 10ng/µL;
Bioanalyzer trace= 300bp

$$\frac{10 \text{ ng/µL}}{\text{(660 g/mol x 300)}} \times 10^6 = 50.5 \text{ nM}$$

2. Dilute each concentrated final library using elution buffer (EB) added with 0.1% Tween-20 to 4 nM library.
3. Aliquot 5 µL of each diluted library (4 nM) and pool them into a 1.5-mL tube.

! Each library must have unique indexes. Depending on coverage requirements, up to 96 libraries can be pooled for one MiSeq run.

2.5 Library Denaturation and MiSeq Sample Loading

In preparation for cluster generation and sequencing, pooled libraries are denatured with freshly prepared 0.2 N NaOH, heat denatured and then diluted with hybridization buffer before loading into MiSeq for sequencing.

! At least 5% of PhiX spike-in is crucial because of the low diversity amplicon libraries.

Refer to the complete guidelines for Library Denaturation and MiSeq Sample Loading recommended by Illumina.
http://support.illumina.com/downloads/prepare_libraries_for_sequencing_miseq_15039740.ilmn

2.6 Run settings and setup

1. Create a sample sheet with the Illumina Experiment Manager as follows:

   "MiSeq" > "Next" > "Other" > "FASTQ only" > "Next"

2. The workflow parameter as below need to set accordingly:

   - Sample Prep Kit: select "Nextera V2"
   - Index Reads: select "2"
   - Read Type: select "Single End"
   - Cycles Read 1: set 181

3. Proceed with the run setup steps using the MiSeq Control Software (MCS) interface.

SAFESeqr Sequencing Library Preparation consumables:

No	Item	Supply
1	SAFESeqr Kit	ScienceVision
2	FailSafe PCR Enzyme Mix (2.5 U/µL)	Epicentre
3	FailSafe PCR 2x PreMix F	Epicentre
4	Nuclease-Free Water	User-supplied
5	AMPure XP Beads	User-supplied
6	Freshly Prepared 80% Ethanol	User-supplied
7	Resuspension Buffer (RSB) or Nuclease-Free Water	User-supplied
8	Qubit® dsDNA HS Assay Kit	User-supplied
9	Agilent High Sensitivity DNA Kit	User-supplied
10	Elution Buffer (EB)	User-supplied
11	Tween-20	User-supplied

SAFESeqr Sequencing Library Preparation equipments:

No	Item
1	Hot-lid Thermal Cycler
2	Microcentrifuge
3	Agilent 2100 Bioanalyzer System
4	Qubit® Fluorometer
5	Magnetic Stand
6	0.2-mL PCR tube or 96-well PCR plate
7	0.5-mL clear tubes
8	1.5-mL tubes or 96-well plate
9	Microseal film for 96-well PCR plate

Best Practices:

1. Avoid Cross-Contamination/Carry-Over

- Filter tips are recommended throughout the whole course of experiment.
- Always use fresh pipette tips between samples, between reagents and between dispensing adapter primers, unless specified otherwise.
- Only handle one primer / sample tube at a time.
- Clean pipettes and change gloves between handling different DNA samples.
- Clean workbench with 0.5% Bleach thoroughly before and after the procedure.
- Triplicates of NTC must be prepared in parallel with each batch of the processing library and run in MiSeq to monitor the cross-contamination/carry-over.
- Best to have pre-PCR and post-PCR area.

2. Handling Magnetic Beads

- Before use, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed and the color of the liquid is homogeneous.
- When pipetting the beads, pipette slowly and disperse slowly due to the viscosity of the solution.
- Take care to minimize bead loss, which can affect final yields.
- Change the tips for each sample, unless specified otherwise.
- To prevent the carryover of beads after elution, approximately 2 µL of supernatant is left when the eluates are removed from the bead pellet.
- Keep the tubes / reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, because the presence of ethanol affects the performance of the subsequent steps. A minimum minute of drying time is recommended, but longer drying time can be required.
- Avoid over drying the beads (crack), which can impact final yields.