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Ver 1.0

PrimeAmp ASFV Enrichment Panel Handbook

(KIT-2010)

Molecular Biology Kits

Contents

Kit Contents

No	Product	16 reactions
1	PrimeAmp Hot Start 2x MM	2 x 900 µL
2	ASFV Primer Pool 1	60 µL
3	ASFV Primer Pool 2	60 µL
4	ASFV Primer Pool 3	60 µL
5	ASFV Primer Pool 4	60 µL
6	Nuclease-free Water	2 x 800 µL

Storage

This kit will be delivered and stored at temperature ranging from -30 °C to -15 °C.

Intended Use

For Research Use Only. Not for use in Diagnostic Procedures.

Safety Information

- ✓ All due care and attention should be exercised in the handling of the DNA templates that contains viral particles. We recommend all users to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.
- ✓ Always wear a suitable lab coat, disposable gloves, surgical mask, and protective goggles.
- ✓ For more information, please refer the appropriate safety data sheets (SDS).

Quality Control

Each lot of PrimeAmp Target Enrichment Panel is tested against predetermined specifications to ensure consistent product quality.

Product Specification

	KIT-2010
Targeted Genome Size (i) Pool 1 (ii) Pool 2 to Pool 4	192 kb 4 kb & 7 kb 7 kb each
Duration of PCR Amplification	~ 4 hours
Duration of PCR Purification & Pooling	~ 1 hour

Additional Required Products

1. Agencourt® AMPure® XP Beads (Beckman Coulter®, Cat# A63880 or A63881) for bead-based library purification.
2. Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat# Q32851 or Q32854); iQuant™ Broad Range dsDNA Quantification kit (ABP Biosciences, Cat# N012 or N013; or equivalent reagent
3. Long-read sequencing by Oxford Nanopore Technologies (ONT) MinION or GridION platform:
    - ✓ NEBNext FFPE Repair Mix (NEB, Cat# M6630S or M6630L)
    - ✓ NEBNext Ultra II End Repair/dA-Tailing Module (NEB, Cat# E7546S or E7546L)
    - ✓ NEB Blunt/TA Ligase Master Mix (NEB, Cat# M0367S or M0367L)
    - ✓ NEBNext Quick Ligation Module (NEB, Cat# E6056S or E6056L)
    - ✓ Native Barcoding Kit 96 V14 (ONT, Cat# SQK-NBD114.96)
    - ✓ R10.4.1 flow cell (ONT, Cat# FLO-MIN114)
    - ✓ Flow Cell Priming Kit (ONT, Cat# EXP-FLP004)
    - ✓ Flow Cell Wash Kit (ONT, Cat# EXP-WSH004)
4. Short-read sequencing by Illumina MiSeq® platform:
    - ✓ Agilent High Sensitivity DNA Kit (Agilent, Cat# 5067-4626)
    - ✓ JetSeq™ Library Quantification Lo-ROX Kit (Bioline, Cat# BIO-68029); or equivalent reagent
    - ✓ xGen™ DNA Lib Prep EZ UNI Kit (IDT, Cat# 10009864 or 10009822) or similar short-insert DNA library preparation kit
    - ✓ xGen™ UDI-UMI Adapters (IDT, Cat# 10006914 or 10005903)
    - ✓ 1.0M Sodium Hydroxide, Biotechnology Grade (1st BASE Biochemicals, Cat# BUF-1115-500ml)
    - ✓ 1.0M Tris Buffer, pH 8.5, Biotechnology Grade, 500 mL (1st BASE Biochemicals, Cat# CUS-1416-500mL-pH8.5)
    - ✓ MiSeq Reagent Nano Kit v2 (500-cycle) (Illumina, Cat# MS-103-1003)
    - ✓ PhiX Control Kit v3 (Illumina, Cat# FC-110-3001)

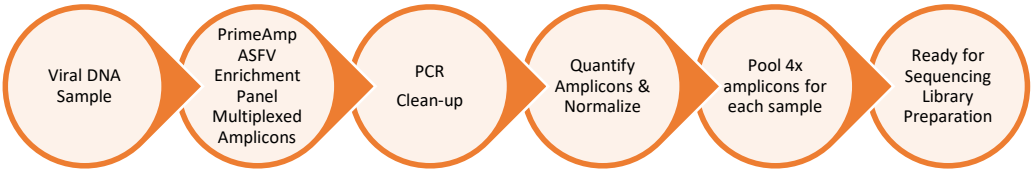
Introduction

African Swine Fever Virus (ASFV) is a highly infectious viral disease with high mortality rate across swine species such as domestic pigs and wild boars. ASFV has large genome size, 170 kb to 194 kb and the extracted DNA often mixed with high prevalence of host DNA, making it challenging to reconstruct whole genome and requires baits/ probe to enrich ASFV DNA. Commonly, *B646L* gene (~400 bp) is used to study the genetic variation for ASFV. Studies of virus characterization and epidemiological require whole genome sequencing of the virus. PrimeAmp ASFV Enrichment Panel is specifically designed and to overcome the difficulties by amplifying whole genome using multiplexed ASFV PCR primers sets, which produce ~4 kb and ~7 kb long amplicons in 4 different PCR enrichment pools and assembled through tiled sequencing approach.

The amplicons are pooled followed by DNA repair, end-prep and clean-up for Next-Generation Sequencing (NGS) library construction, either using long-read sequencing (Oxford Nanopore Technologies, ONT) or short-read sequencing (Illumina® platforms). For long-read sequencing using ONT, it is recommended to use Native Barcoding method (SQK-NBD114.96). Meanwhile, short-read sequencing by Illumina requires enzymatic DNA fragmentation and size selection prior to ligation of Illumina adaptors.

Principle and Procedure

PrimeAmp ASFV Enrichment Panel utilizes a 3-stage PCR workflow for targeted enrichment of the entire ASFV genome (Figure 1).



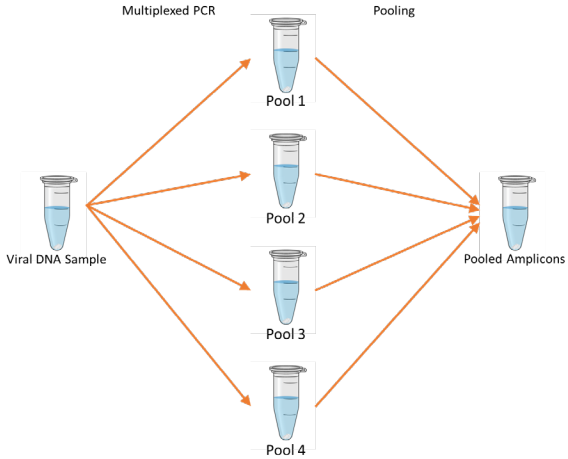


Figure 1. Schematic of optimized PrimeAmp ASFV Enrichment Panel. The PrimeAmp ASFV Enrichment Panel workflow illustrates the amplification of the targeted ASFV DNA from viral DNA sample. The amplified amplicons are then purified and pooled. The purified amplicons from the multiplexed PrimeAmp ASFV Enrichment Panel are then ready for sequencing library preparation by employing a one-tube library construction step for each sample.

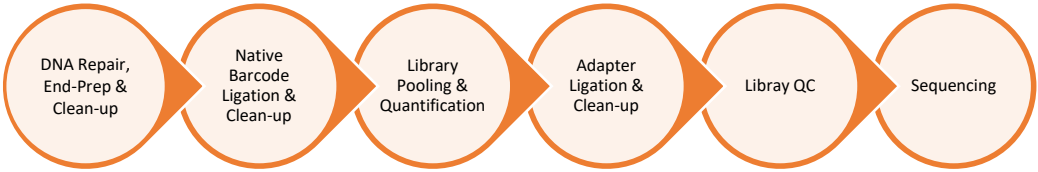


Figure 2A. Schematic of ONT Native Barcoding DNA Library Preparation Workflow for Long-Read Sequencing

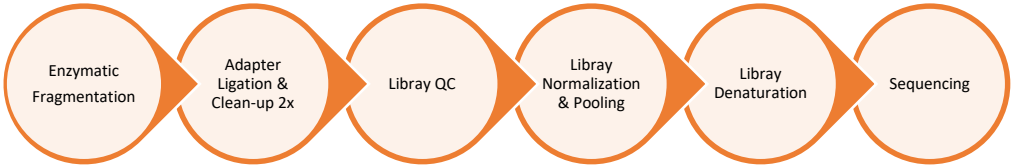


Figure 2B. Schematic of Illumina DNA Library Preparation Workflow for Short-Read Sequencing

Figure 2. Scheme of optimized sequencing workflow, which is not included in this kit. Purified amplicons from the multiplexed PrimeAmp ASFV Enrichment Panel are converted to sequencing libraries by employing a one-tube library construction step either using 2A) long-read sequencing by ONT; or 2B) short-read sequencing by Illumina.

Whole Genome Enrichment

PrimeAmp ASFV Enrichment Panel workflow begins with high-fidelity multiplex PCR amplification using Hot Start PCR MasterMix to prepare four (4) pools of amplicons at the size range from 4 kb to 7 kb. 8 µL (2 µL for each PCR pool) of DNA input is the recommended as starting amount; DNA volume can be adjusted with not more than 5 µL for each PCR pool as there may be present of PCR inhibitors. Only the DNA sample that show positive amplification results for all four (4) PCR pools is recommended to continue.

The qualified amplicons are purified and quantified individually. Based on the quantification data, four (4) amplicon pools per sample are pooled into a single tube according to the assigned proportion (Table 8). The pooled amplicons are then subjected to the DNA library construction of the preferred Next-Generation Sequencing (NGS) approach using either ONT or Illumina platform.

Supplementary Methodology for Long-Read Sequencing by ONT MinION or GridION Platform

DNA Library Construction

a) DNA Repair and End-prep
A total of 700 ng purified and pooled amplicons are subjected to DNA repair and end-prep. Next, the prepared DNA is purified and quantified.

b) Native Barcode Ligation, Library Pooling and Adapter Ligation
500 ng end-prepped DNA are subjected to ligation of Native Barcodes. Next, the barcoded DNA are purified and quantified. Equimolar of barcoded samples are pooled to total amount of 700 ng in a single tube. The pooled DNA library is subjected to ligation of sequencing adapter. The treated DNA library is purified and washed with Long Fragment Buffer to remove fragments less than 3 kb.

c) Final Library QC
The DNA library is quantified with expected recovery are more than 300 ng.

d) Third-Generation Sequencing
PrimeAmp ASFV Enrichment Panel is compatible with ONT MinION and GridION platforms. DNA library is mixed with sequencing buffer and loading beads right before loading. Prime the R10.4.1 flow cell and load the prepared DNA library according to the manufacturer's protocol for sequencing.

Important: As a starting point, we recommend allocating 24 samples per flow cell in MinION or GridION sequencer. Through testing, it is possible to increase number of samples per flow cell. Table 1 describes the number of multiplex samples per flow cell (assumes 10% duplicates).

Table 1: Number of multiplex samples per flow cell recommendations

Instrument	Flow Cell Version	Output/ Flow Cell (Gb)	Samples/ Flow Cell
MinION (single flow cell)	R10.4.1	10 to 20	24
GridION (5x flow cells)	R10.4.1	10 to 20	24

e) Data Analysis
The datasets can be assembled separately using LILO[1] with the ASFV reference sequence and primer sets. All assembled contigs can then be aligned with the reference ASFV genome using MAFFT V7.4.9[2], to reveal regions with gaps.

For variant calling, the cleaned reads from the above process can be mapped to ASFV reference genome using Minimap2[3]. Variant call and annotation are called using GATK[4].

Supplementary Methodology for Short-Read Sequencing by Illumina MiSeq® Platform

a) Enzymatic Fragmentation and Adaptor Ligation

500 ng of purified and pooled amplicons from the PrimeAmp ASFV Enrichment Panel are first enzymatically sheared into smaller fragments. The desired median fragment size is ~ 400 bp. The fragmented DNA is then ready for adapter ligation. Illumina platform-specific adapters are ligated to both ends of the DNA fragments.

b) Library QC, Normalization and Pooling

Size selection and purification are performed on the ligated DNA using two different ratios of magnetic beads. The DNA Library is qualified and quantified using DNA Labchip and qPCR quantification kit respectively. Based on the quantification data, each individual DNA library will be diluted to 4 nM. Next, equal volume of each 4 nM library will be pooled together to produce a pooled DNA library with up to 30 libraries.

c) Library Denaturation

The pooled DNA Library and PhiX Control DNA are denatured separately and diluted to desired loading concentration. Spike in 5% PhiX Control DNA into diluted, denatured DNA Library. The final library is then sequenced with MiSeq Reagent v2 chemistry, 250 paired-end sequencing on MiSeq platform.

d) Next-Generation Sequencing

PrimeAmp ASFV Enrichment Panel is compatible with Illumina MiSeq® platform. When using Illumina MiSeq®, 251 bp paired-end reads are required.

Important: As a starting point, we recommend allocating 30 samples per flow cell in MiSeq®. Through testing, it is possible to increase number of samples per flow cell. Table 2 shows the number of samples that can be multiplexed (assumes 10% duplicates).

Table 2: Sample multiplexing recommendations

Instrument	Version	Output/ flow cell	Samples/flow cell
MiSeq®	v2 Nano (500 Cycle)	500 Mb	30

e) Data Analysis

The sequencing adapters and sequence clean-up can be done using BBDuk of the BBTools Packages. The cleaned reads were independently assembled via *de novo* approach using SPAdes V3.11.1[1] and Megahit v1.1.2[2]. Both assemblies merged using MAC[3] and polished using Pilon V1.23[4].

For variant calling, the cleaned reads from the above process can be mapped to ASFV reference genome using Bowtie2[5]. Variant call and annotation were called using GATK[6].

Starting Materials

Quantification of the DNA template that contains both host DNA and viral DNA do not directly reflect actual amount of viral DNA. General guideline of the required DNA template is provided as Table 3 below.

Table 3: Sample requirements

Sample Type	Volume	Concentration*	qPCR titer (Ct value)
gDNA Contains African Swine Flu Virus (ASFV)	≥ 10 µL	≥ 40 ng/µL	≤ 20.0

*Based on fluorometric quantification

DNA Integrity and amount of the genomic DNA (gDNA) do not provide guarantee that DNA template contains sufficient viral DNA for whole genome enrichment. Therefore, if the DNA amount meeting the requirements of Table 3, you may proceed to whole genome enrichment (refer Protocol A) followed by qualification and quantification check of the enriched viral DNA (refer Protocol B) for downstream applications.

Equipment and Reagents to be Supplied by User

Consumables and Reagents

- ✓ Nuclease-free, filter tips
- ✓ 0.2 mL thin-walled PCR tubes (Watson® Bio Lab, Cat# 137-211C) or 8-well tube strips (Watson® Bio Lab, Cat# 137-231C)
- ✓ 1.5 mL microcentrifuge tubes (Watson® Bio Lab, Cat# 131-7155C)
- ✓ 99.8% or absolute ethanol (ACS grade, Undenatured)
- ✓ Freshly prepared 70% ethanol (from ethanol absolute 99.8%, undenatured) in nuclease-free water
- ✓ Nuclease-free Water (1st BASE Biochemicals, Cat# BUF-1180-100ml or Qiagen, Cat# QIAG-129114)
- ✓ Ice

Laboratory Equipment and Apparatus

- ✓ Pipette (P10, P20, P200, P1000)
- ✓ Magnetic separation rack suitable for 1.5 mL tube
- ✓ Agarose Gel Electrophoresis Set
- ✓ Centrifuge for short spin
- ✓ Vortex mixer
- ✓ Thermal Cycler with heated lid
- ✓ Timer
- ✓ Heat block/ water bath set to 37°C
- ✓ Cool Block set to 25°C
- ✓ Qubit 4 Fluorometer; Qubit Flex Fluorometer (Thermo Fisher Scientific); or equivalent
- ✓ Rotator mixer (e.g., Elmi Intelli-Mixer™ RM-2M)

Important Notes

- ✓ It is highly recommended to thoroughly read the manual prior to starting, especially for first-time user.
- ✓ Take note of required product, notes, recommendations, and stopping points.

Protocol A: Whole Genome Enrichment

Preparation Prior to Starting

- ✓ 8 µL (2 µL for each PCR Pool) of the Viral DNA Sample is required as the starting material for the Whole Genome Enrichment.
- ✓ Thaw PrimeWay ASFV Enrichment Panel on ice.
- ✓ Flick and/ or invert reagent tubes to ensure they are well mixed.
- ✓ Always spin down tubes before opening for the first time each day.
- ✓ Set up the PCR on ice.
- ✓ Use a thermal cycler with a heated lid.
- ✓ Use filter tips when dispensing the reagents.

1. PCR setup
   i) Prepare the PCR MasterMix for each primer pool according to Table 4 without the Viral DNA Sample. There will be a total of four PCR reactions per sample.

   Table 4: Preparation of PCR MasterMix.

Reagent	ASFV Primer Pool 1	ASFV Primer Pool 2	ASFV Primer Pool 3	ASFV Primer Pool 4
Nuclease-free Water	20 µL	20 µL	20 µL	20 µL
PrimeAmp Hot Start 2x MasterMix (MM)	25 µL	25 µL	25 µL	25 µL
Primer Pool	3 µL	3 µL	3 µL	3 µL
Viral DNA Sample*	2 µL	2 µL	2 µL	2 µL
Total	**50 µL**	**50 µL**	**50 µL**	**50 µL**

*For no template control (NTC) of each primer pool, replace the viral DNA sample with Nuclease-free Water.

   ii) Mix the PCR MasterMix thoroughly by vortex and briefly centrifuge.
   iii) Aliquot 48 µL PCR MasterMix into each 0.2 mL thin-walled PCR tube.
   iv) Add 2 µL viral DNA sample into the tube. For NTC, use 2 µL Nuclease-free Water instead.

2. Setup the PCR cycling condition on thermal cycler as Table 5.

Table 5: PCR cycling conditions.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	1 min	1
Denaturation	98 °C	15 sec	40
Annealing	60 °C	15 sec	
Extension	72 °C	4 min 40 sec	
Final Extension	72 °C	5 min	1
Hold	10 °C	∞	

3. Place the PCR reactions into the thermal cycler and start the PCR amplification according to Table 5. The PCR amplification will take ~ 4 hours.

Safe Stop Point: The pooled amplicons can be stored at -20°C for a maximum of 2 weeks, if downstream processes are not conducted immediately.

Protocol B: Whole Genome Enrichment Validation, Quantification and Normalization

Preparation Before Start

- ✓ Agencourt® AMPure® XP beads (not provided), equilibrate to room temperature (15 – 25 °C) for at least 30 minutes before use.
- ✓ Freshly prepared 70% ethanol (from ethanol absolute 99.8%, undenatured) in nuclease-free water: 400 µL per PCR product, 1600 µL per sample.
- ✓ Heat block/ water bath set to 37 °C
- ✓ Magnetic separation rack for 1.5 mL tubes.

1. Validation of PCR Amplification via Gel Electrophoresis

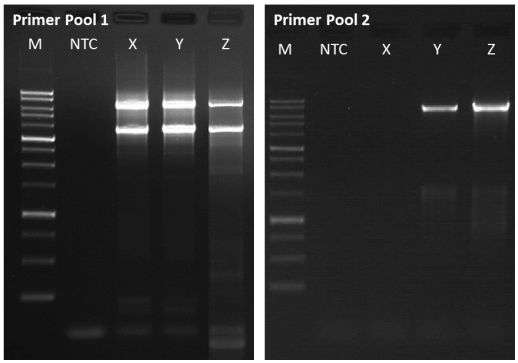
   i) Analyse 2 µL of each PCR product for each sample on 1% agarose gel, 100 V for 60 minutes.

   ii) Refer to Table 6 for the expected PCR Product Size from Whole Genome Enrichment.

   Table 6: Expected PCR product size from Whole Genome Enrichment.

Primer Pool	PCR Product Size
1	4 kb & 7 kb
2	7 kb
3	7 kb
4	7 kb

   iii) Samples that show positive PCR amplification for all primer pools like Sample Y and Z shown in Figure 3, are recommended to continue with their downstream applications. Refer to Table 7 for Assessment of PCR Product after Whole Genome Enrichment.



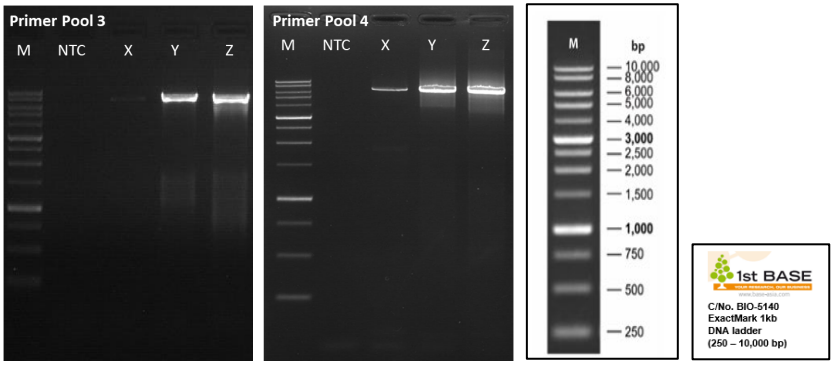


Figure 3. Three viral DNA samples, namely X, Y and Z, are processed using PrimeAmp ASFV Enrichment Panel. After the end of whole genome enrichment protocol, 2 µL of each PCR product for each primer pool is run on 1% agarose gel at 100 V for 60 minutes.

Table 7: Assessment of PCR products after Whole Genome Enrichment.

Sample Name	PCR Results
X	Only Primer Pool 1 shown strong positive PCR result. Process this sample to sequencing will be at risk to have gaps during genome asssembly.
Y	Pass
Z	Pass

2. Purification of PCR Products

   i) Equilibrate Agencourt® AMPure® XP beads to room temperature (15 – 25 °C) for at least 30 minutes before use.

   ii) Transfer each PCR reaction to new 1.5 mL microcentrifuge tube (total of 4 tubes/sample).

   iii) Vortex the Agencourt® AMPure® XP beads to ensure the beads are evenly dispersed.

   iv) Add 20 µL AMPure® XP beads (0.4X ratio) to each PCR reaction. Mix gently by pipetting.

   v) Incubate at room temperature for 10 minutes.

   vi) Briefly centrifuge to bring down the mixture.

   vii) Place the sample on the magnetic rack for 5 minutes or until the supernatant is clear.

viii) With the tube remaining on the magnetic rack, carefully remove and discard the supernatant.

ix) With the tube remaining on the magnetic rack, wash the beads with 200 µL freshly prepared 70% ethanol without disturbing the beads.

a) Rotate the tube by 180°. Wait until the beads are fully attracted to the magnet.

b) Rotate the tube by 180° (back to the starting position) and wait until the beads are fully attracted to the magnet.

c) Remove the ethanol with pipette.

x) Repeat step (ix) for a total of 2 washes.

xi) Briefly centrifuge and place the tube on the magnetic rack. Remove any residual ethanol.

xii) With the tube remaining on the magnetic rack, allow the beads to air dry for 30 seconds.

xiii) Remove the tubes from the magnetic rack and resuspend the beads with 17 µL Nuclease-free water. Incubate at 37 °C for 5 minutes.

xiv) Place the tube on the magnetic rack for 2 minutes or until the supernatant is clear.

xv) Carefully transfer 16 µL of the eluate to a new 1.5 mL microcentrifuge tube (total of 4 purified PCR product/sample).

3. Quantification and Normalization of PCR Products

i) Take 1 µL of purified PCR product for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent.

ii) Based on the quantification result, pool the purified PCR product according to the proportion stated in Table 8 and Table 9 for the total amount of pooled PCR product into a new 1.5 mL microcentrifuge tube.

Table 8: Normalisation of PCR products and pool into a single tube.

Primer Pool	Proportion
1	3%
2	43%
3	46%
4	8%

Table 9: Total amount of pooled PCR product required for each type of sequencing platform.

Total pooled PCR Product Amount	
Long-Read Sequencing	Short-Read Sequencing
700 ng	500 ng

Safe Stop Point: The pooled amplicons can be stored at -20°C for a maximum of 2 weeks if downstream processes are not conducted immediately.

Supplementary Protocols for Long-Read Sequencing by ONT MinION/ GridION Platform

Library preparation and sequencing reagents for ONT platforms are not included in this kit. Refer page 3.

Additional reagents and equipment for this option of sequencing

- ✓ Freshly prepared 70% ethanol (from Ethanol absolute 99.8%, undenatured) in nuclease-free water
- ✓ Rotator mixer (e.g., Elmi Intelli-Mixer™ RM-2M)
- ✓ Heat block/ water bath at 37 °C
- ✓ Cool block at 25 °C

Protocol C1: DNA Repair and End-Prep

Preparation Prior to Starting

- ✓ Pooled amplicons of each sample are required.

  Note: If there are insufficient pooled amplicons for 700 ng, the end-prep reaction in this protocol can be carried out at half of the volume.
- ✓ Equilibrate Agencourt® AMPure® XP beads at room temperature (15 – 25 °C) for at least 30 minutes before use.
- ✓ Freshly prepared 70% ethanol (from ethanol absolute 99.8%, undenatured) in nuclease-free water.
- ✓ Thaw NEBNext FFPE Repair Mix and NEBNext Ultra II End Repair/dA-Tailing Module on ice before use.
- ✓ Flick and/ or invert reagent tubes to ensure they are well mixed.
- ✓ Briefly centrifuge to bring down the liquid before opening the tube for the first time of the day.
- ✓ Ultra II End-Prep Buffer and FFPE DNA Repair Buffer
  - A small amount of precipitate may be observed. Allow the buffer to reach room temperature, then break up the precipitate by pipetting up and down, followed by vortexing to ensure thorough mixing of the buffers.
  - Yellow tinge may be observed in FFPE DNA Repair Buffer, this will not affect the buffer performance.
- ✓ Use filter tips when dispensing the reagents.

1. DNA Repair and End-Prep Reaction Setup

   i) Prepare the reaction in a 0.2 mL tube according to Table 9.

   Table 9: DNA Repair and End-Prep Reaction setup

Reagent	Volume
Pooled Amplicons (700 ng from each sample)	48.0 µL
NEBNext FFPE DNA Repair Buffer	3.5 µL
NEBNext Ultra II End-Prep Reaction Buffer	3.5 µL
NEBNext Ultra II End-Prep Enzyme Mix	3.0 µL
NEBNext FFPE DNA Repair Mix	2.0 µL
Total	**60 µL**

   a) After adding each reagent, mix thoroughly by pipetting up and down for 10 - 20 times.

   b) If volume of pooled amplicon is less than 48 µL, top up to 48 µL with nuclease-free water.

   ii) Briefly centrifuge and place the reaction in thermal cycler with setting as shown in Table 10.

   Table 10: DNA Repair and End-Prep incubation program.

Temperature	Time
20 °C	5 min
65 °C	5 min
10 °C	∞

   iii) Once the incubation is completed, transfer the End-Prep products into a new 1.5 mL microcentrifuge tube.

   iv) Immediately proceed to purification of the End-Prep products using Agencourt® AMPure® XP beads.

2. Purification of End-Prep DNA Products

   i) Equilibrate Agencourt® AMPure® XP beads at room temperature (15 – 25 °C) for at least 30 minutes before use.

   ii) Vortex the Agencourt® AMPure® XP beads to ensure the beads are evenly dispersed.

   iii) Add 60 µL AMPure® XP beads (1X ratio) into each End-Prep product and mix thoroughly by pipetting.

   iv) Incubate on rotator mixer, 20 rpm (e.g., Elmi Intelli-Mixer™, Mode F4) at room temperature for 5 minutes.

   v) Briefly centrifuge to spin down the volume.

vi) Place the sample on the magnetic rack for 2 minutes or until the supernatant is clear. Remove the supernatant.

vii) With the tube remaining on the magnetic rack, add 200 µL freshly prepared 70% ethanol without disturbing the beads.

a) Rotate the tube by 180°. Wait until the beads are fully attracted to the magnet.

b) Rotate the tube 180° (back to the starting position) and wait until the beads are fully attracted to the magnet.

c) Remove the ethanol using a pipette.

viii) Repeat step (vii) for a total of 2 washes.

ix) Briefly centrifuge and place the tube on the magnetic rack. Remove any residual ethanol.

x) With the tube remaining on the magnetic rack, allow the beads to air dry for 30 seconds.

xi) Remove the tubes from the magnetic rack and resuspend the beads with 27 µL nuclease-free water.

xii) Incubate at 37 °C for 5 minutes.

xiii) Place the tube on the magnetic rack for 2 minutes or until the supernatant is clear.

xiv) Transfer 26 µL of eluate into a clean 1.5 mL microcentrifuge tube.

xv) Take 1 µL of eluted DNA for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent.

Note: Expected concentration will be ≥ 23 ng/µL.

Protocol D1: Native Barcode Ligation, Pooling and Adapter Ligation

Important Points Before Start

- ✓ 500 ng of End-Prep DNA Products is required.
- ✓ Thaw NEB Blunt/TA Ligase Master Mix on ice before use.
- ✓ Thaw required number of Native Barcodes (NB01-96) from Native Barcoding Kit 96 V14 at room temperature before use.

1. Native Barcode Ligation Setup
   - i) Choose a unique barcode to each sample that will be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one run.
   - ii) Individually mix the barcodes by tapping, spin down, and place them on ice.
   - iii) Dilute 500 ng of each End-Prep DNA Product into a final volume of 22.5 µL by using nuclease-free water.
   - iv) Add the reagents according to the sequence shown in Table 11. After adding each reagent, mix by flicking the tube.

     Table 11: Native barcode ligation setup

No.	Reagent	Volume
1	500 ng End-Prep DNA for each sample	22.5 µL
2	Native Barcode	2.5 µL
3	Blunt/ TA Ligase MasterMix	25.0 µL
	Total	**50.0 µL**

   - v) Pipette the mixture 10 - 20 times between each addition of reagents. Briefly centrifuge to bring down the mixture.
   - vi) Incubate the reaction at 25 °C for 20 minutes.
   - vii) Immediately proceed to Purification of Barcoded DNA, Step 2 using Agencourt® AMPure® XP beads.

2. Purification of Barcoded DNA
   - i) Equilibrate Agencourt® AMPure® XP beads at room temperature (15 – 25 °C) for at least 30 minutes before use.
   - ii) Resuspend the Agencourt® AMPure® XP beads by vortex.
   - iii) Add 50 µL AMPure® XP beads (1X ratio) to each tube and mix by pipetting.
   - iv) Incubate on rotator mixer (e.g., Elmi Intelli-Mixer™, Mode F4, 20 rpm) at room temperature for 5 minutes.
   - v) Briefly centrifuge to bring down the mixture.

vi) Place the sample on the magnetic rack for 2 minutes or until the supernatant is clear.

vii) With the tube remain on the magnetic rack, carefully remove and discard the supernatant.

viii) With the tube remaining on the magnetic rack, wash the beads with 200 µL freshly prepared 70% ethanol without disturbing the beads.

a) Rotate the tube by 180°. Wait until the beads are fully attracted to the magnet.

b) Rotate the tube by 180° (back to the starting position) and wait until the beads are fully attracted to the magnet.

c) Remove the ethanol with pipette.

ix) Repeat step (viii) for one more time.

x) Briefly centrifuge and place the tube on the magnetic. Remove any residual ethanol.

xi) With the tube remain on the magnetic rack, allow the beads to air dry for 30 seconds.

xii) Remove the tubes from the magnetic rack and resuspend the beads with 28.5 µL nuclease-free water.

xiii) Incubate at 37 °C for 5 minutes.

xiv) Place the tube on the magnetic rack for 2 minutes or until the supernatant is clear.

xv) Carefully transfer 28 µL of eluate into a clean 1.5 mL microcentrifuge tube.

xvi) Take 1 µL of eluted DNA for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent.

Note: Expected concentration ≥ 10 ng/µL.

3. DNA Pooling before Adaptor Ligation

i) Pool equimolar of each Barcoded DNA (take the lowest concentration of Barcoded DNA as base) to a clean 1.5 mL microcentrifuge tube. The total amount of pooled Barcoded DNA is 700 ng.

ii) Take 1 µL of eluted DNA for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent.

Note: Expected concentration ≥ 10.8 ng/µL.

iii) Dilute the 700 ng pooled Barcoded DNA with final volume of 65 µL, using nuclease-free water.

Note: If pooled Barcoded DNA is more than 65 µL, DNA purification and concentration is required using AMPure® XP beads (2.5X ratio) followed by elution with 65 µL nuclease-free water.

Pooled Barcoded DNA volume x 2.5 = Volume of AMPure® XP beads

4. Adaptor Ligation Setup

Important Points Before Start

- ✓ 700 ng of the pooled Barcoded DNA.
- ✓ Thaw the buffer/ reagent below at room temperature. Mix by vortex, briefly centrifuge and keep on ice. Check the contents of the buffer to ensure no precipitation.
  - Elution Buffer (EB) from Native Barcoding Kit 96 V14
  - NEBNext Quick Ligation 5X Reaction Buffer from NEBNext Quick Ligation Module
  - Long Fragment Buffer (LFB) from Native Barcoding Kit 96 V14
- ✓ Briefly centrifuge and keep on ice.
  - T4 Ligase from NEB Blunt/TA Ligase Master Mix
  - Native Adapter (NA) from Native Barcoding Kit 96 V14

i) Add the reagents according to the sequence in Table 12. After adding each reagent, mix by flicking the tube.

Table 12: Adaptor ligation setup

No.	Reagent	Volume
1	700 ng of pooled Barcoded DNA	65 µL
2	Native Adapter (NA)	5 µL
3	NEBNext Quick Ligation 5X Reaction Buffer	20 µL
4	Quick T4 DNA Ligase	10 µL
	Total	**100 µL**

ii) Pipette 10 - 20 times to mix and briefly centrifuge to bring down the liquid.

iii) Incubate the reaction at 25 °C for 20 minutes.

iv) Immediately proceed to Purification of DNA Library using Agencourt® AMPure® XP beads.

Protocol E1: Library Purification and Quantification

1. Purification of DNA Library
   - i) Equilibrate Agencourt® AMPure® XP beads at room temperature (15 – 25 °C) for at least 30 minutes before use.
   - ii) Resuspend the Agencourt® AMPure® XP beads by vortex.
   - iii) Add 40 µL AMPure® XP beads (0.4X ratio) to the unpurified DNA Library and mix by pipetting.
   - iv) Incubate on rotator mixer (e.g., Elmi Intelli-Mixer™, Mode F4, 20 rpm) at room temperature for 5 minutes.
   - v) Briefly centrifuge to bring down the mixture.
   - vi) Place the sample on the magnetic rack for 2 minutes or until the supernatant is clear.
   - vii) With the tube remain on the magnetic rack, carefully remove and discard the supernatant.
   - viii) With the tube remain on the magnetic rack, wash the beads with 250 µL Long Fragment Buffer (LFB) without disturbing the beads.
     - a) Rotate the tube by 180°. Wait until the beads are fully attracted to the magnet.

     - b) Rotate the tube by 180° (back to the starting position) and wait until the beads are fully attracted to the magnet.

     - c) Remove the LFB with pipette.
   - ix) Repeat step (viii) for one more time.
   - x) Briefly centrifuge and place the tube on the magnetic. Remove any residual LFB.

xi) With the tube remaining on the magnetic rack, allow the beads to air dry for 30 seconds.

xii) Remove the tubes from the magnetic rack and resuspend the beads with 16 µL Elution Buffer (EB).

xiii) Incubate at 37 °C for 10 minutes.

xiv) Place the tube on the magnetic rack for 2 minutes or until the supernatant is clear.

xv) Carefully transfer 15 µL of eluate into a clean 1.5 mL microcentrifuge tube.

Note:

- When storing the DNA Library, recommended to use 1.5 mL Eppendorf DNA LoBind® tubes (Eppendorf®, Cat# 0030108051).
- For short-term storage or repeated use (e.g., reloading flow cells between washes), store at 4 °C.
- For long-term storage (> 3 months) and single use, store at -80 °C.

xvi) Take 1 µL of eluted DNA for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent.

Note: Expected concentration ≥ 20 ng/µL.

Protocol F1: Sequencing Setup

Important Points Before Start

- ✓ 300 ng of purified DNA Library per flow cell.
- ✓ Thaw the items at room temperature. Mix by vortexing and briefly centrifuge.
  - Flow Cell Tether (FCT)
  - One tube of Flow Cell Flush (FCF) from Flow Cell Priming Kit (ONT, Cat# EXP-FLP004)
  - Sequencing Buffer (SB)
  - Library Beads (LIB)
- ✓ Thaw R10.4.1 flow cell (ONT, Cat# FLO-MIN114) at room temperature for 30 minutes before use. Complete a flow cell check to assess the number of pores available before loading the library.
- ✓ Prepare Flow Cell Wash Kit (ONT, Cat# EXP-WSH004) only if you would like to reuse the flow cell.

1. Priming the SpotON flow cell according to manufacturer's protocol of MinION or GridION.
2. In a new 1.5 mL microcentrifuge tube, prepare the DNA library for loading according to Table 13 below.

   Table 13: Prepare DNA library for loading.

Reagent	Volume
Sequencing Buffer (SB)	37.5 µL
Library Beads (LIB), mix immediately before use	25.5 µL
DNA Library	12.0 µL
Total	**75.0 µL**

   Note: Immediately proceed to Step 3 after preparation as the component in the buffer will start to react with the adapter.

3. Complete the flow cell priming according to manufacturer's protocol.
4. Mix gently by pipetting up and down prior to loading.
5. Load 75 µL of the prepared DNA Library (from Table 13) into the flow cell according to manufacturer's protocol.
6. Start the run according to manufacturer's protocol.
7. Check the sequencing reads when run duration is reaching 40 hours. If acquiring > 150k reads for all libraries, you may stop the run and re-use the flow cell or continue the run until its maximum setting of 72 hours.

   Notes: Flow cell that has been run for 72 hours will not be eligible for re-use.

8. Ending the experiment:
    9. Once the sequencing run is completed and you wish to reuse the flow cell, follow the Flow Cell Wash Kit's protocol to wash the flow cell. After washing, store the flow cell at 2 – 8 °C; or
    ii) Follow ONT return procedure by washing out the flow cell.

Protocol G1: Data Analysis

The datasets can be assembled separately using LILO (1) with the ASFV reference sequence and primer sets. All assembled contigs can then be aligned with the reference ASFV genome using MAFFT V7.4.9 (2), to reveal regions with gaps.

References

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Supplementary Protocols for Short-Read Sequencing by Illumina MiSeq® Platform

Library preparation and sequencing reagents for Illumina MiSeq® platform are not supplied with this kit. Refer page 3.

Additional reagents and equipment for this option of sequencing

- ✓ Freshly prepared 80% ethanol (from Ethanol absolute 99.8%, undenatured) in nuclease-free water
- ✓ 1.0M Tris Buffer, pH 8.5, Biotechnology Grade, 500 mL (1st BASE Biochemicals, Cat# CUS-1416-500mL-pH8.5)
- ✓ 1.0M Sodium Hydroxide, Biotechnology Grade (1st BASE Biochemicals, Cat# BUF-1115-500ml)

Protocol C2: Enzymatic Fragmentation

Important Points Before Start

- ✓ 500 ng of pooled amplicons from each sample is required.
- ✓ Ensure the pooled amplicons is not dissolved in standard TE buffer. EDTA inhibits enzymatic fragmentation.
- ✓ Thaw xGen™ DNA Lib Prep EZ UNI Kit on ice before use.
- ✓ Flick and/ or invert reagent tubes to ensure they are well mixed.
- ✓ Briefly centrifuge to bring down the liquid before opening the tube for the first time of the day.
- ✓ Keep xGen™ Low EDTA TE Buffer (from xGen™ DNA Lib Prep EZ UNI kit) at room temperature.

1. If the pooled amplicon is less than 19.5 µL, adjust the volume to 19.5 µL using Low EDTA TE (Table 14) in a clean 0.2 mL PCR tube. Keep the tube on ice.

   Table 14: Pooled amplicons volume adjustment.

Reagent	Volume
Low EDTA TE Buffer	19.5 - X µL
Pooled Amplicons	X µL
Total	**19.5 µL**

2. Prepare the Enzymatic Prep MasterMix on ice according to Table 15 in a new 0.2 mL PCR tube.

   Table 15: Enzymatic Prep MasterMix setup.

Reagent	Volume
Buffer K1	3.0 µL
Reagent K2	1.5 µL
Enzyme K3	6.0 µL
Total	**10.5 µL**

3. Mix gently by pipetting up and down 8 times. Briefly centrifuge the tube and keep the MasterMix on ice.
4. Add 10.5 µL Enzymatic Prep MasterMix to each volume adjusted pooled amplicons to final total volume 30 µL for each sample.
5. Mix gently by pipetting up and down for 8 times, avoids creating bubbles.
6. Briefly centrifuge and keep the tube on ice until fragmentation starts.
7. Set up the thermal cycler program according to Table 16 below. Set the heated lid to 70 °C.

   Table 16: Enzymatic Prep program.

Step	Temperature	Time
Cold_Hold	4 °C	∞
Incubation	4 °C	1 min
Fragmentation	20 °C	30 min
Inactivation	65 °C	30 min
Hold	4 °C	Less than 1 hour

8. Start the Enzymatic Prep program. When the thermal cycler reaches 4 °C during the "Cold_Hold" step, place the tube in the chilled thermal cycler and proceed to the next step of the Enzymatic Prep Program.

   Note: The fragmented DNA can be kept at 4 °C for not longer than 1 hour.

Protocol D2: Adapter Ligation

Important Points Before Start

- ✓ Thaw Buffer W1 at room temperature. Buffer W1 is viscous, pipette slowly to draw accurate quantity.
- ✓ Thaw xGen™ UDI-UMI Adapters on ice.

  Note: xGen™ UDI-UMI Adapters are full-length adapters contain 8-base unique dual indexes (UDI) that minimize read misassignment. An optional 9-base unique molecular identifier (UMI) can be used for quantitative assays or low-frequency variant detection. xGen™ UDI-UMI Adapters, 1-96 is ready in 15 µM with a volume of 9 µL per well.

1. Prepare the Ligation MasterMix in a 0.2 mL PCR tube on ice. Add the reagents according to the sequence in Table 17 below.

   Table 17: Ligation MasterMix setup.

No.	Reagent	Volume
1	Buffer W1	12 µL
2	Enzyme W3	4 µL
3	Low EDTA TE Buffer	9 µL
	Total	**25 µL**

   Note: Gently aspirate & dispense Buffer W1 to avoid bubbles and ensure accuracy.

2. Gently vortex the Ligation MasterMix for 5 seconds and briefly centrifuge.
3. When the Enzymatic Prep Programs is completed, keep the tube on ice for 1 minute.
4. Add 5 µL of Full-length Adapter (15 µM) to the tubes containing the fragmented DNA.
5. Add 25 µL of Ligation MasterMix to the tube. Total mixture volume is 60 µL.
6. Mix gently by pipetting up and down for 8 times and briefly centrifuge.

   Note: Avoid creating bubbles in this step.
7. Set up the thermal cycler program according to Table 18 below with the heated lid to 40 °C.

Table 18: Ligation program.

Step	Temperature	Time
Hold	20 °C	∞
Ligation	20 °C	20 min
Hold	4 °C	∞

8. Begin the Ligation Program.
9. Start the Enzymatic Prep program. Once the thermal cycler reaches 20 °C during the "Hold" step, place the tube in the thermal cycler and proceed to the Ligation step.
10. Immediately, proceed to Purification of DNA Library using Agencourt® AMPure® XP beads.

Protocol E2: Library Purification, Quantification, Normalization and Pooling

Important Points Before Start

- ✓ Equilibrate Agencourt® AMPure® XP beads at room temperature (15 – 25 °C) for 30 minutes before use.
- ✓ Freshly prepared 80% ethanol (from ethanol absolute 99.8%, undenatured) in nuclease-free water.
- ✓ 10 mM Tris buffer (pH 8.5).

1. Purification of DNA Library

i) Equilibrate Agencourt® AMPure® XP beads at room temperature (15 – 25°C) for 30 minutes before use.

ii) Resuspend the Agencourt® AMPure® XP beads by vortex.

iii) Add 48 µL Agencourt® AMPure® XP beads (0.8X ratio) to the unpurified DNA Library and mix by pipetting 10 times.

iv) Briefly centrifuge to bring down the mixture.

v) Incubate at room temperature for 5 minutes.

vi) Place the sample on the magnetic rack for 2 minutes or until the supernatant is clear.

vii) With the tube remain on the magnetic rack, carefully remove and discard the supernatant.

viii) With the tube remaining on the magnetic rack, add 180 µL freshly prepared 80% ethanol without disturbing the beads. Incubate at room temperature for 30 seconds. Carefully remove and discard the supernatant.

ix) Repeat step (viii) for a total of 2 washes.

x) With the tube remaining on the magnetic rack, allow the beads to air dry for 3 minutes.

xi) Remove the tubes from the magnetic rack.

xii) Add 50 µL Low EDTA TE Buffer, mix gently by pipetting 10 times. Incubate at room temperature for 5 minutes.

xiii) Place the tube on the magnetic rack for 2 minutes or until the supernatant is clear.

xiv) Transfer the eluate into a clean 1.5 mL microcentrifuge tube. Ensure no bead carryover.

Safe Stop Point: The DNA library can be stored at -20°C overnight if you wish to proceed second purification the next day.

xv) Perform second purification of the DNA Library using 32.5 µL (0.65X ratio) Agencourt® AMPure® XP beads. Mix by pipetting.

xvi) Repeating Step (iv) to Step (xi) above.

xvii) Add 20 µL Low EDTA TE Buffer, mix gently by pipetting 10 times. Incubate at room temperature for 5 minutes.

xviii) Place the tube on the magnetic rack for 2 minutes or until the supernatant is clear.

xix) Transfer the eluate into a clean 1.5 mL microcentrifuge tube. Ensure no bead carryover.

xx) Take 5 µL of each purified DNA Library for Library Quantification to avoid repeat freeze-thaw cycles. Then store the remaining purified DNA Libraries at -20°C until it is ready to use for sequencing.

xxi) Store the DNA Library at 4 °C for short-term storage or -80 °C for long-term storage.

2. Determine the Median Fragment Size of DNA Library by DNA LabChip

i) Take 1 µl of 20X diluted DNA library for DNA LabChip analysis with Agilent High Sensitivity DNA Kit or equivalent method.

ii) Run the LabChip according to the manufacturer's protocol.

iii) Record the median fragment size in bp.

Note: Actual product size can be larger in DNA LabChip analysis. For example, DNA libraries containing 350 bp inserts should migrate to ~800 bp peak on the Agilent High Sensitivity DNA LabChip.

3. Library Quantification by qPCR

i) Quantify the DNA Library using JetSeq™ Library Quantification Lo-ROX Kit or equivalent reagent.

ii) Follow the manufacturer's protocol to obtain the DNA Library Ct value.

iii) Run the qPCR program and collect the Ct value of each DNA Library.

iv) Calculate the DNA Library concentration by using the standard curve following the formula below (for JetSeq™ Library Quantification Lo-ROX Kit):

$$\mathrm{L(pM)} = 10^{\left(\frac{\mathrm{Ct} - b}{a}\right)} \times \frac{342}{\text{Fragment Median Size}} \times \text{Dilution Factor}$$

L (pM) = DNA Library concentration

a = slope of standard curve

b = y-intercept of standard curve

The formula above uses a fragment length for the standards of 342 bp.

4. Library Normalization & Pooling

   i) Dilute each DNA Library into 4 nM using 10 mM Tris (pH 8.5).

   ii) For each diluted DNA Library (4 nM), transfer 5 µL into a new 1.5 mL tube, pool the DNA Libraries with different unique indexes into a tube.

   iii) Depending on coverage needs, up to 30 libraries can be pooled for one MiSeq run using Reagent v2 Nano flow cell.

Safe Stop Point: The pooled DNA library can be stored at -20°C for not more than 2 weeks prior to sequencing.

Protocol F2: Library Denaturation and Sequencing Setup

Important Points Before Start

- ✓ 4 nM pooled DNA Library.
- ✓ Freshly prepare 0.2 N NaOH in nuclease-free water.
- ✓ Set water bath at 96 °C.
- ✓ Thaw the MiSeq reagent cartridge at room temperature.
- ✓ In an ice bucket, prepare an ice water bath (3x ice: 1x water).
- ✓ Chill the HT1 Buffer (Hybridization Buffer) that provided from the MiSeq Reagent Kit on the prepared ice bucket.

1. Denature the pooled DNA Library
   i) Prepare the pooled DNA denaturation reaction in a new 1.5 mL microcentrifuge tube according to the Table 19.

   Table 19: Pooled DNA Library denaturation setup.

Reagent	Volume
0.2 N NaOH (freshly prepared)	5 µL
4 nM pooled DNA Library	5 µL
Total	**10 µL**

   Note: Keep the remaining 0.2 N NaOH for preparation of PhiX control within 12 hours.

   ii) Briefly vortex to mix and centrifuge at 280 x *g* for 1 minute, at 20 °C.

   iii) Incubate at room temperature for 5 minutes to denature DNA.

   iv) Add pre-chilled HT1 Buffer to the denatured DNA Library according to Table 20.

   Table 20: Preparation of 20 pM denatured DNA Library.

Reagent	Volume
Denatured DNA Library	10 µL
Pre-chilled HT1 Buffer	990 µL
Total	**1000 µL**

   v) Keep the denatured DNA on ice until you are prepared to proceed to the next step.

2. Dilute Denatured DNA Library

   i) Perform dilution on denatured DNA Library to the desired loading amount stated in Table 21.

   Table 21: Prepare different concentration of the denatured DNA Library

No. of Sample per flow cell	≤30	>30
Recommended Loading Concentration	**10 pM**	**8 pM**
20 pM Denatured Library	300 µL	240 µL
Pre-chilled HT1 Buffer	300 µL	360 µL
Total	**600 µL**	**600 µL**

   Note: You may adjust the loading concentration of each flow cell according to total number of samples that multiplex in a single flow cell.

   ii) Invert several times to mix and briefly centrifuge.

   iii) Keep the diluted, denatured DNA Library (Amplicon Library) on ice.

3. Denaturation and Dilution of PhiX Control DNA

   i) Dilute the PhiX Control DNA to the same loading concentration as the Amplicon library according to Table 22 below.

   Table 22: Dilution of PhiX Control DNA to 4 nM.

Reagent	Volume
10 mM Tris (pH 8.5)	3 µL
10 nM PhiX Control DNA	2 µL
Total	**5 µL**

   ii) Prepare the PhiX Control DNA denaturation reaction in a new 1.5 mL microcentrifuge tube according to the Table 23.

   Table 23: PhiX Control DNA Denaturation Setup.

Reagent	Volume
0.2 N NaOH	5 µL
4 nM PhiX Control DNA	5 µL
Total	**10 µL**

   Note: Use the remaining 0.2 N NaOH from Step 1.

   iii) Vortex briefly to mix and then spin down.

   iv) Incubate for 5 minutes at room temperature to denature the PhiX Control DNA into single strands.

   v) Add pre-chilled HT1 Buffer to the denatured DNA Library according to Table 24.

Table 24: Preparation of 20 pM of Denatured PhiX Control DNA

Reagent	Volume
Denatured PhiX Control DNA	10 µL
Pre-chilled HT1 Buffer	990 µL
Total	**1000 µL**

vi) Dilute the denatured PhiX Control DNA to be the same concentration as Amplicon Library, prepared in 2(i) as Table 25 below.

Table 25: Prepare the same loading concentration of the Denatured PhiX Control DNA with the Amplicon Library. For example, if the prepared Amplicon Library is 10 pM according to Table 21, the Denatured PhiX Control DNA will also prepare in 10 pM.

Loading Concentration of Amplicon Library	10 pM	8 pM
20 pM denatured PhiX Control DNA	300 µL	240 µL
Pre-chilled HT1 Buffer	300 µL	360 µL
Total	**600 µL**	**600 µL**

vii) Invert several times to mix and briefly centrifuge.

viii) Keep the diluted, denatured PhiX Control DNA on ice.

4. Final preparation of Amplicon Library and PhiX Control DNA

i) Prepare the Final Library with 5% PhiX Control DNA in a new 1.5 mL microcentrifuge tube according to Table 26.

Table 26 Final Library with 5% PhiX Control DNA Setup

Reagent	Volume
Denatured and Diluted PhiX Control DNA	30 µL
Denatured and Diluted Amplicon Library	570 µL
Total	**600 µL**

Note: The recommended PhiX Control DNA spike-in is ≥ 5%.

ii) Invert to mix and briefly centrifuge.

iii) Keep the Final Library on ice until you are ready to heat denature the mixture and immediately load it onto the MiSeq v2 reagent cartridge.

iv) Incubate the Final Library at 96 °C (water bath) for 2 minutes.

v) IMMEDIATELY, invert the tube 2 times to mix.

vi) IMMEDIATELY, keep the tube in the ice-water bath for 5 minutes.

Note: Heat denaturation step (iv) to (vi) must be performed immediately before loading into the MiSeq reagent cartridge to ensure efficient DNA loading on the MiSeq flow cell.

5. Start the run according to manufacturer's protocol. The run duration for MiSeq Reagent Kit v2 Nano flow cell 250PE is ~ 28 hours.
6. After the sequencing run is completed, perform the post-run wash according to manufacturer's protocol. The washed flow cell cannot be reused.

Protocol G2: Data Analysis

The sequencing adapters and sequence clean-up can be done using BBDuk of the BBTools Packages. The cleaned reads are assembled de novo separately using SPAdes V3.11.1 (1) and Megahit v1.1.2 (2). Both assemblies merged using MAC (3) and polished using Pilon V1.23 (4).

For variant calling, the cleaned reads from the above process can be mapped to ASFV reference genome using Bowtie2 (5). Variant call and annotation were called using GATK (6).

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Troubleshooting Guidelines

Problems	Possible Reason	Recommended Action
Low yield or no PCR product	Insufficient primer	Ensure that you use pipettors that has been recently calibrated.
		Increase the amount of primer pool in the PCR reaction by 0.5 - 2 µL.
	Insufficient viral DNA template or abundance of host DNA	Use a host depletion method such as NEBNext® Microbiome DNA Enrichment Kit (NEB, Cat# E2612S or E2612L) before PCR enrichment. This method however requires intact gDNA (≥ 15 kb, 1 µg) for optimal results.
		Use undiluted DNA, increase the amount of DNA template by 0.5 - 2 µL.
	Thermal cycler faulty	Ensure the thermal cycler used is calibrated.
NTC shown amplicon(s)	Contamination during preparation	Ensure the PCR MasterMix is prepared freshly and use sterile consumables.
Low recovery of purified PCR product	Loss of DNA due to a low AMPure® XP beads-to-sample ratio used	Ensure AMPure® XP beads are well resuspended right before adding into the PCR products.
		DNA fragments of any size will be lost during the clean-up when the AMPure® beads-to-sample ratio is lower than 0.4.
	Ethanol used in washing step is not freshly prepared and is less than 70%	DNA will be eluted from the beads when using ethanol less than 70%. Make sure to use the correct percentage and prepare the ethanol freshly.

Please contact us at https://base-asia.com/contact/ for more information.

Product and Service Ordering Information

Product

Agency	Product Number	Product Description
1st BASE Kits	KIT-2010-16	PrimeAmp ASFV Enrichment Panel, 16 reactions
1st BASE Biochemicals	BUF-1180-100ml	Nuclease-free Water, Biotechnology Grade, 100 mL
Beckman Coulter®	A63880/ A63881	Agencourt® AMPure® XP Beads
Thermo Fisher Scientific	Q32851/ Q32854	Qubit® dsDNA HS Assay Kit
ABP Biosciences	N012/ N013	iQuant™ Broad Range dsDNA Quantification kit
-	General Lab Supply	Ethanol absolute 99.8%, Undenatured
Watson® Bio Lab	137-211C; or 137-231C	0.2 mL thin-walled PCR tubes; or 8-well tube strips
	131-7155C	1.5 mL microcentrifuge tubes
Eppendorf®	0030108051	1.5 mL DNA LoBind® tubes
Supplementary Protocols for Nanopore Sequencing		
Beckman Coulter®	A63880/ A63881	Agencourt® AMPure® XP Beads
New England Biolabs (NEB)	M6630S/ M6630L	NEBNext FFPE Repair Mix
	E7546S/ E7546L	NEBNext Ultra II End repair/dA-tailing Module
	M0367S/ M0367L	NEB Blunt/TA Ligase MasterMix
	E6056S/ E6056L	NEBNext Quick Ligation Module
Oxford Nanopore Technologies (ONT)	SQK-NBD114.96	Native Barcoding Kit 96 V14
	FLO-MIN114	R10.4.1 flow cell
	EXP-FLP004	Flow Cell Priming Kit
	EXP-WSH004	Flow Cell Wash Kit
Supplementary Protocols for Nanopore Library Quantification		
ABP Biosciences	N012/ N013	iQuant™ Broad Range dsDNA Quantification kit
Supplementary Protocols for Illumina Sequencing		
Beckman Coulter®	A63880/ A63881	Agencourt® AMPure® XP Beads
1st BASE Biochemicals	CUS-1416-500mL-pH8.5	1.0M Tris Buffer, pH 8.5, Biotechnology Grade, 500 mL
	BUF-1115-500ml	1.0M Sodium Hydroxide, Biotechnology Grade
Integrated DNA Technologies (IDT)	10009864/ 10009822	xGen™ DNA Lib Prep EZ UNI Kit
	10006914/ 10005903	xGen™ UDI-UMI Adapters
Illumina	MS-103-1003	MiSeq Reagent Nano Kit v2 (500-cycle)
	FC-110-3001	PhiX Control v3

Agency	Product Number	Product Description
Supplementary Protocols for Illumina Library Quantification		
Agilent	5067-4626	High Sensitivity DNA Kit
Bioline	BIO-68029	JetSeq™ Library Quantification Lo-ROX Kit

Service

Agency	Product Number	Product Description
Optional Nanopore Sequencing Services		
1st BASE NGS	NGS-2007	Library Preparation - Nanopore platform (Amplicon-Native) Service includes: 1. Amplicon library preparation using Native Barcoding Kits 2. Library quantification
	NGS-3015	Nanopore MinION/ GridION Sequencing Service includes: 1. Nanopore run in 1x Flow Cell (R10.4.1) - 1x Flow Cell Kit (FLO-MIN114) 2. Data output is subject to nature of sample and preparation methods 3. Raw data only.
Optional Illumina Sequencing Services		
1st BASE NGS	NGS-2002	Library Preparation - Illumina platform Service includes: 1. Library preparation using xGen™ DNA Lib Prep EZ UNI Kit 2. Library quantification and Library QC
	NGS-3012	Illumina MiSeq Sequencing v2 (250 PE) Nano Output Service includes: 1. Illumina MiSeq 250 PE run, 1 flow cell 2. PhiX Spike-in 5%, unless advised by customer 3. Reagent Kit v2 (MS-103-1003) to deliver 1 million PE reads per flow cell 4. Data output is subject to final PhiX spike-in amount and nature of pre-made libraries: ~ 500Mb per flow cell. 5. Raw data only.

Agency	Product Number	Product Description
Optional Bioinformatics Analysis Services		
1st BASE NGS	NGS-0106	Standard Bioinformatics Analysis (Re-sequencing) included: - 1. Sequence QC 2. Alignment up to 2 reference genomes with SNP/ InDel calling, annotation and statistics 3. De novo assembly to assemble contigs and identify genome re-arrangement (if there is any)