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Ver 1.0

PrimeAmp Custom Thalassemia Enrichment Panel Handbook

(KIT-2020)

Contents

Kit Contents

No	Product	KIT-2020-16
1	PA Hot Start Master Mix (2X)	2 x 1.4 mL
2	Nuclease-free Water	2 x 1.1 mL
3	Primer Mix, Chr11-1 – Chr11-9	9 x 10 µL
4	Primer Mix, Chr16-1 – Chr16-17	17 x 10 µL
5	Positive Control, 20 ng/µL	1 x 40 µL

Storage

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

Intended Use

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

Safety Information

- ✓ Always wear a suitable lab coat, disposable gloves, surgical mask, and protective goggles.
- ✓ For more information, please refer to the appropriate safety data sheets (SDS).

Quality Control

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

Product Specification

	KIT-2020-16
Duration of PCR Amplification (ramping 5°C/ sec)	~ 3 to 5 hours
Duration of PCR Purification & Pooling	~ 1 hour

Additional Required Products

1. MagSi-NGS^{PREP} Plus (Magtivio, Cat# MDKT00010005, MDKT00010075 or MDKT00010500); or equivalent reagent 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)
    - ✓ Native Barcoding Kit 96 V14 (ONT, Cat# SQK-NBD114.96)
    - ✓ NEB Blunt/TA Ligase Master Mix (NEB, Cat# M0367S or M0367L)
    - ✓ NEBNext Quick Ligation Module (NEB, Cat# E6056S or E6056L)
    - ✓ 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)

Introduction

Thalassemia is a type of monogenic blood disorder caused by mutations in the β-globin gene of chromosome 11 and α-globin gene of chromosome 16. This is a customized panel for Thalassemia, specifically target regions at chromosome 11 and chromosome 16.

Principle and Procedure

PrimeAmp Custom Thalassemia Enrichment Panel utilizes PCR workflow for targeted enrichment of Chromosome 11 and Chromosome 16 for Thalassemia disease (Figure 1).

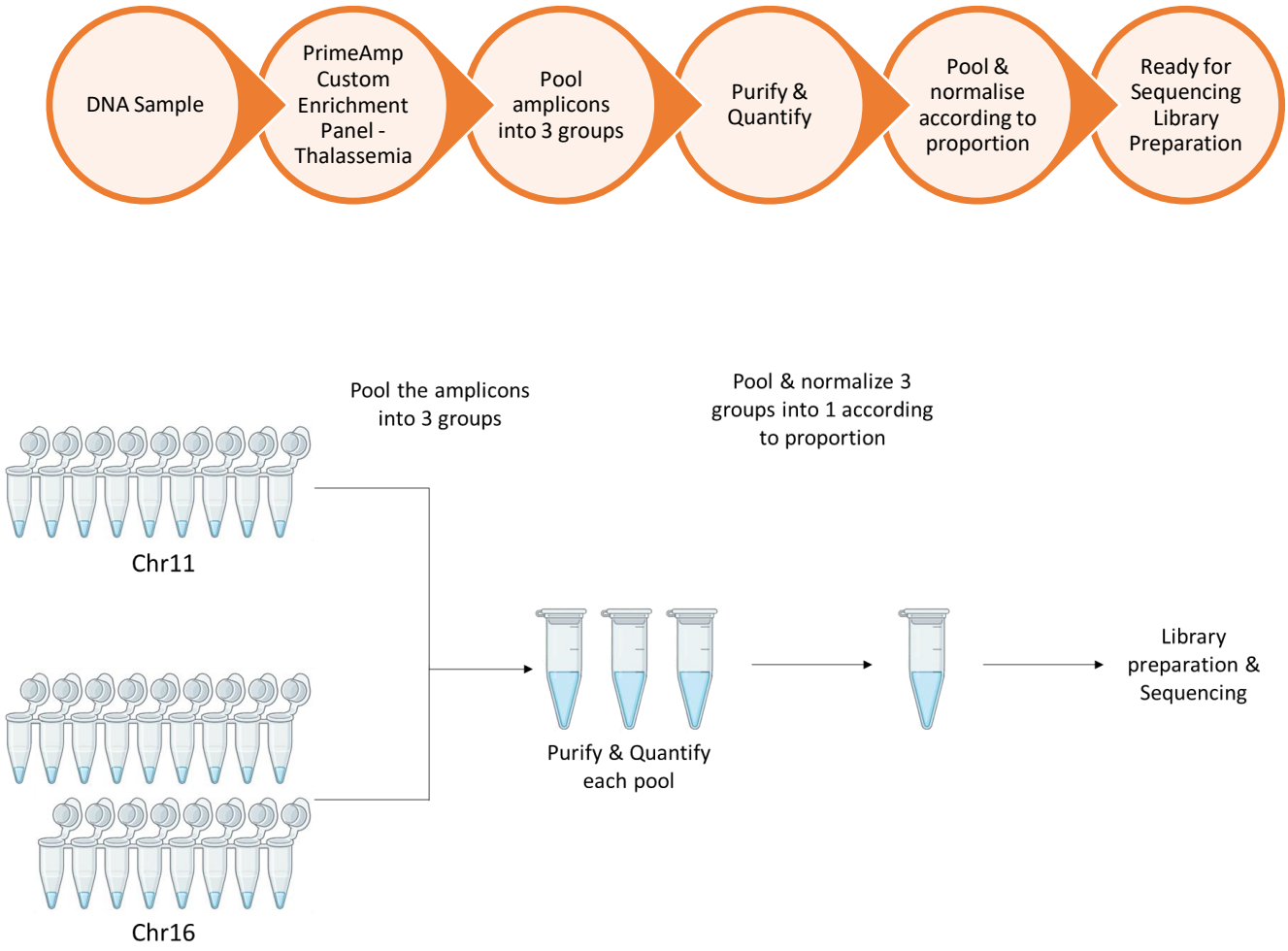

Figure 1. Scheme of optimized PrimeAmp Custom Thalassemia Enrichment Panel. The workflow illustrates the amplification of the targeted region of Thalassemia from DNA sample. The amplified amplicons are pooled into 3 groups, purified and quantified. The purified amplicons are further pooled into one tube according to proportion and ready for sequencing library preparation. Library preparation is employing one-tube library construction method for each sample.

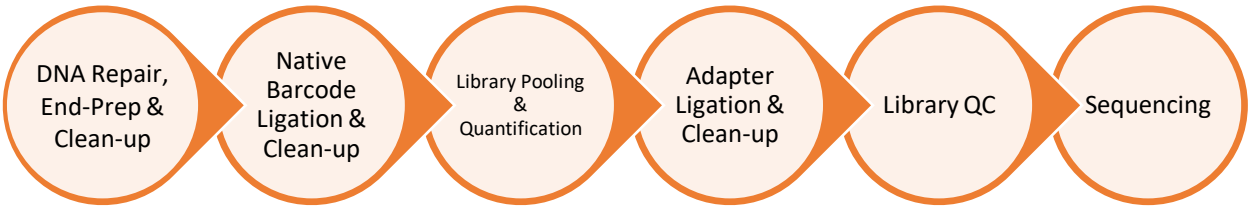

Figure 2. Scheme of optimized sequencing workflow, which is not provided with this kit. Purified pooled amplicons from the PrimeAmp Custom Thalassemia Enrichment Panel are converted to sequencing libraries by employing a one-tube library construction step using long-read sequencing by ONT.

Target Enrichment

PrimeAmp Custom Thalassemia Enrichment Panel workflow begins with high-fidelity PCR amplification of targeted region at the size range from ~2 kb to ~10 kb (Table 1). The PCR products are pooled into 3 groups, purify and quantify each group. Based on the quantification data, perform second pooling into a single tube according to the assigned proportion (Table 7). The pooled amplicons are then subjected to the DNA library construction for Next-Generation Sequencing (NGS) using ONT platform.

Table 1: Primer information.

	Primer Mix	Amplicon Size (bp)	Target ID	GRCh 38	
				Start	End
Chromosome 11	Chr11-1	4255	BPhi45_F	5112282	5113282
	Chr11-2	3604	Bsiriraj_F	5131273	5132273
	Chr11-3	4740	BChi100Del_F BFPFH6_F BFPFH6_F2	5169319 5170370 5172144	5170319 5171170 5173144
	Chr11-4	9805	HPFH3_F B101.3Del_F BAsian49.3Del_F	5193859 5199425 5200644	5194859 5200425 5201644
	Chr11-5	8690	BThai_F B3.5_F B619_F HBB BLPWB_F BLPBALT_F	5220023 5223703 5224789 5225466 5226325 5226625	5221023 5224703 5225789 5226310 5226605 5227990
	Chr11-6	9977	BSEA27_R BPhi45_R B150HBD_F HBD	5228458 5230758 5232164 5232829	5229458 5231758 5233164 5234628
	Chr11-7	8802	BHPFH3_R HBG1 Bsiriraj_R	5243626 5248272 5249566	5244626 5250170 5250566

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	Primer Mix	Amplicon Size (bp)	Target ID	GRCh 38	
				Start	End
Chromosome 11	Chr11-8	9464	HBG1 Bsiriraj_R HBG2full	5248272 5249566 5250614	5250170 5250566 5255170
	Chr11-9	3097	HBG2partial	5253100	5254800
Chromosome 16	Chr16-1	7599	AE261Del_F	46181	47581
	Chr16-2	2409	AE360Del_F	112541	114541
	Chr16-3	7341	AE158Del_F	142653	144653
	Chr16-4	4503	AThai_F APhi_F ADel1_F	149262 151019 152023	150262 152019 153023
	Chr16-5	1897	A20_F	153700	154700
	Chr16-6	5663	ADel2_F2 ADel2_F1	154801 158886	158801 159886
	Chr16-7	5121	AGB_F	161302	162302
	Chr16-8	2576	AMed_F ASEA_F	163200 164800	164200 165800
	Chr16-9	2637	ADel3_F	166693	167693
	Chr16-10	8823	A4.2_F AW_F AM3.5_F HBA123.7_1:Part1 HBA123.7_2	169218 170679 171242 172201 173251	170501 171679 172242 177701 173401
	Chr16-11	6239	HBA123.7_1:Part2 AM3.5_R AMed_R ADel1_R1	172201 174801 178001 180815	177701 175901 181401 181615
	Chr16-12	4587	AThai_R ASEA_R	183251 184001	184251 185001
	Chr16-13	8120	AE158Del_R	239435	241435
	Chr16-14	7644	AE360Del_R	472940	474940
	Chr16-15	6907	A4.2_F AW_F AM3.5_F HBA123.7_1:Part1 HBA123.7_2	169218 170679 171242 172201 173251	170501 171679 172242 177701 173401
	Chr16-16	2804	AE158Del_R	239435	241435
	Chr16-17	2088	A20_F	153700	154700

Notes:

1. Chr16-5 and Chr16-17 are amplifying the same Target ID.
2. Chr16-10 and Chr16-15 are amplifying the same Target ID.
3. Chr16-13 and Chr16-16 are amplifying the same Target ID.

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 is 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 Short Fragment Buffer to retain all fragments.

c) Final Library QC

The DNA library is quantified with expected recovery are more than 300 ng.

d) Third-Generation Sequencing

PrimeAmp Custom Thalassemia 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 2 describes the number of multiplex samples per flow cell (assumes 10% duplicates).

Table 2: 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

Sequence obtained from Nanopore sequencing can be mapped to human reference genome using Minimap21 with the option -ax map-ont -t8. Mapped reads are sorted into coordinate-order using the SortSam function (https://broadinstitute.github.io/picard/) subsequently proceed to variant calling using Freebayes V1.3.62.

Starting Materials

General guidelines of the required DNA template are provided as Table 3 below.

Table 3: Sample requirements

Sample Type	Volume	Concentration*
DNA Sample	≥ 40 µL	≥ 20 ng/µL

*Based on fluorometric quantification

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) or 96-well plate
- ✓ 1.5 mL microcentrifuge tubes (Watson® Bio Lab, Cat# 131-7155C)
- ✓ 99.8% or absolute ethanol (ACS grade, Undenatured)
- ✓ Freshly prepared 85% ethanol (from ethanol absolute 99.8%, undenatured) in nuclease-free water
- ✓ Nuclease-free Water (1st BASE Biochemicals, Cat# BUF-1180-100ml)
- ✓ Ice

Laboratory Equipment and Apparatus

- ✓ Pipette (P10, P20, P200, P1000)
- ✓ Magnetic separation rack/ plate
- ✓ Agarose Gel Electrophoresis Set
- ✓ Centrifuge for short spin
- ✓ Vortex mixer
- ✓ Thermal Cycler with heated lid
- ✓ Timer
- ✓ Qubit 4 Fluorometer; Qubit Flex Fluorometer (Thermo Fisher Scientific); or equivalent

Important Notes

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

Protocol A: Target Enrichment

Preparation Before Start

- ✓ 20 ng/µL (minimum required 40 µL) of gDNA is required as the starting material for the Whole Genome Enrichment.
- ✓ Thaw PrimeAmp Custom Thalassemia 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. Target Enrichment setup

i) Set up 5 target enrichment cycling conditions with different annealing temperature on thermal cycler (e.g., Eppendorf Mastercycler® nexus GSX1, ramp rate 5 °C) as Table 4 for all primers.

ii) Dilute DNA template to 20 ng/µL.

iii) Prepare the target enrichment reaction for each primer mix according to Table 4. Mix the PCR reactions thoroughly by vortex and briefly centrifuge. There will be total of 26 target enrichment reactions per DNA sample.

Table 4: Target Enrichment setup for all primers and example of 16 reactions aliquots and labelling in plate format.

<table>
<tr><th colspan="2">Target Enrichment</th><th>Primer Mix</th></tr>
<tr><td colspan="2">A. Annealing temperature 60 °C

Target Enrichment Cycling Condition
<table><tr><th>Step</th><th>Temperature</th><th>Time</th><th>Cycles</th></tr><tr><td>Initial Denaturation</td><td>95 °C</td><td>1 min</td><td>1</td></tr><tr><td>Denaturation</td><td>98 °C</td><td>15 sec</td><td rowspan="3">35</td></tr><tr><td>Annealing</td><td>60 °C</td><td>15 sec</td></tr><tr><td>Extension</td><td>72 °C</td><td>8 min</td></tr><tr><td>Hold</td><td>10 °C</td><td>∞</td><td></td></tr></table></td><td>Orange cap
Chr11-1
Chr11-2
Chr11-3
Chr11-4
Chr11-6
Chr11-7
Chr11-9

Chr16-1
Chr16-2
Chr16-3
Chr16-4
Chr16-5
Chr16-6
Chr16-7
Chr16-9
Chr16-12
Chr16-14
Chr16-16</td></tr>
</table>

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Target Enrichment	Primer Mix

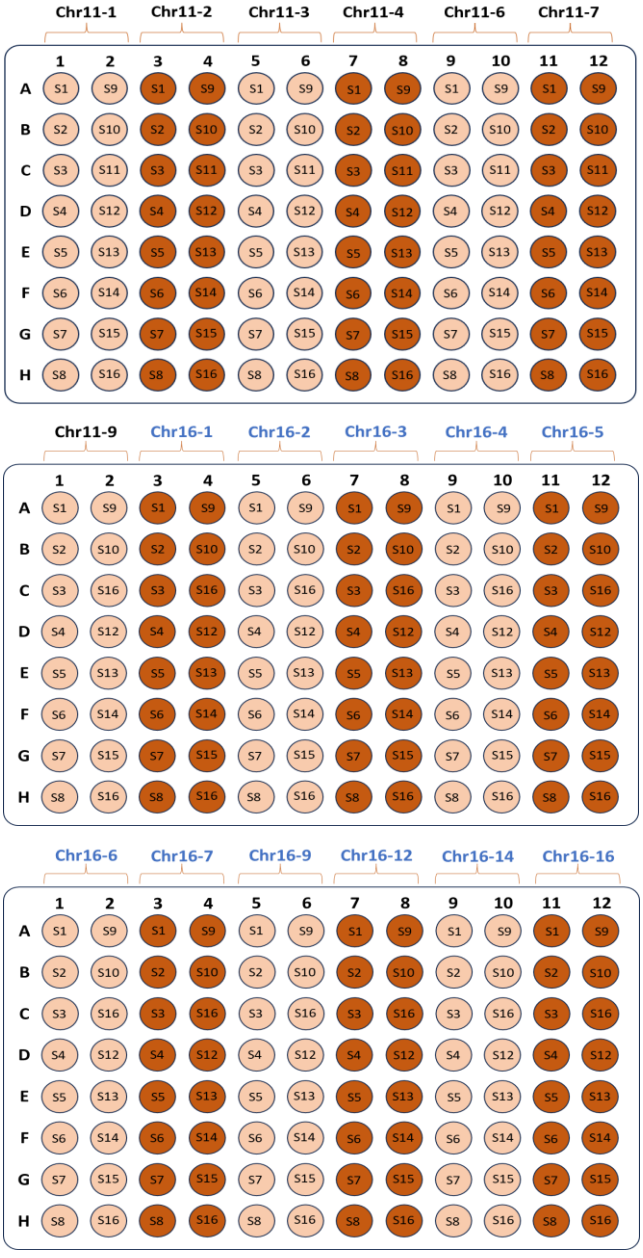
Target Enrichment Reactions

Reagent	Each Primer Mix
Nuclease-free Water	4.5 µL
PA Hot Start Master Mix (2X)	6.0 µL
Primer Mix	0.5 µL
DNA Template, 20 ng/µL	1.0 µL
Total	**12.0 µL**

Note:

(a) If there are more than 1 sample, prepare a target enrichment master mix without the DNA template. Aliquot 11 µL target enrichment master mix into 0.2 mL thin-walled PCR tube/ plate, followed by DNA template.

(b) For no template control (NTC), replace the DNA template with 1 µL Nuclease-free water.



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Target Enrichment	Primer Mix
B. Annealing temperature 61 °C	White cap Chr16-15 Chr16-17

Target Enrichment Cycling Condition

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	1 min	1
Denaturation	98 °C	15 sec	35
Annealing	61 °C	15 sec	
Extension	72 °C	8 min	
Hold	10 °C	∞	

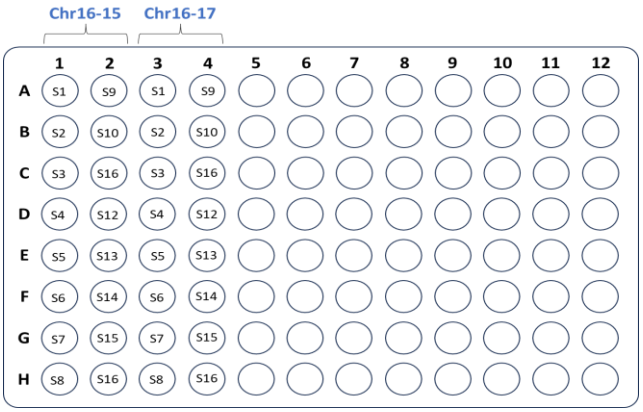
Target Enrichment Reactions

Reagent	Each Primer Mix
Nuclease-free Water	4.5 µL
PA Hot Start Master Mix (2X)	6.0 µL
Primer Mix	0.5 µL
DNA Template, 20 ng/µL	1.0 µL
Total	**12.0 µL**

Note:

(a) If there are more than 1 sample, prepare a target enrichment master mix without the DNA template. Aliquot 11 µL target enrichment master mix into 0.2 mL thin-walled PCR tube/ plate, followed by DNA template.

(b) For no template control (NTC), replace the DNA template with 1 µL Nuclease-free water.



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Target Enrichment	Primer Mix
C. Annealing temperature 65 °C	Blue cap Chr11-5 Chr11-8 Chr16-13

Target Enrichment Cycling Condition

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	1 min	1
Denaturation	98 °C	15 sec	35
Annealing	65 °C	15 sec	
Extension	72 °C	8 min	
Hold	10 °C	∞	

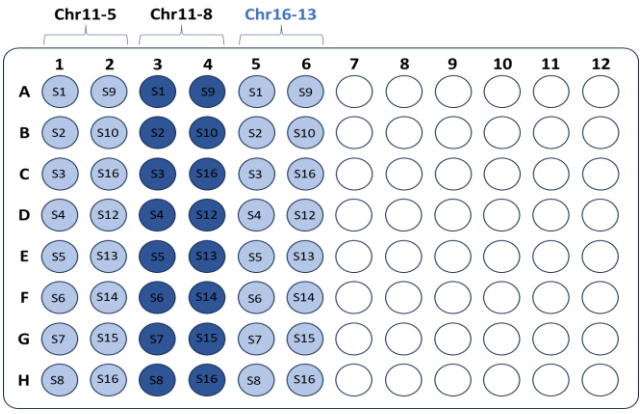
Target Enrichment Reactions

Reagent	Each Primer Mix
Nuclease-free Water	2.5 µL
PA Hot Start Master Mix (2X)	6.0 µL
Primer Mix	0.5 µL
DNA Template, 20 ng/µL	3.0 µL
Total	**12.0 µL**

Note:

(i) If there are more than 1 sample, prepare a target enrichment master mix without the DNA template. Aliquot 11 µL target enrichment master mix into 0.2 mL thin-walled PCR tube/ plate, followed by DNA template.

(ii) For no template control (NTC), replace the DNA template with 3 µL Nuclease-free water.



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Target Enrichment	Primer Mix
D. Annealing temperature 72 °C	Green cap Chr16-10 Chr16-11

Target Enrichment Cycling Condition

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	1 min	1
Denaturation	98 °C	30 sec	35
Extension	72 °C	8 min	
Hold	10 °C	∞	

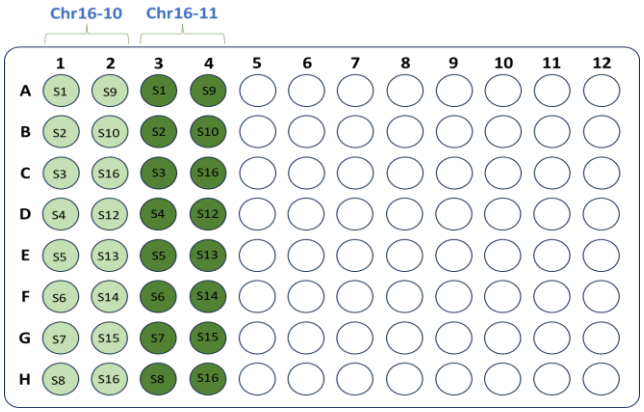
Target Enrichment Reactions

Reagent	Each Primer Mix
Nuclease-free Water	2.5 µL
PA Hot Start Master Mix (2X)	6.0 µL
Primer Mix	0.5 µL
DNA Template, 20 ng/µL	3.0 µL
Total	**12.0 µL**

Note:

(i) If there are more than 1 sample, prepare a target enrichment master mix without the DNA template. Aliquot 11 µL target enrichment master into 0.2 mL thin-walled PCR tube/ plate, followed by DNA template.

(ii) For no template control (NTC), replace the DNA template with 3 µL Nuclease-free water.



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Target Enrichment	Primer Mix

E. Stepdown PCR

Red cap
Chr16-8

Target Enrichment Cycling Condition

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	1 min	1
Denaturation	98 °C	30 sec	5
Annealing	62 °C (ΔT= -1.5 °C)	30 sec	
Extension	72 °C	3 min	
Denaturation	98 °C	30 sec	
Annealing	62 °C (ΔT= -1.5 °C)	30 sec	
Extension	72 °C	3 min	
Denaturation	98 °C	30 sec	30
Annealing	57 °C	30 sec	
Extension	72 °C	3 min	
Final extension	72 °C	5 min	1
Hold	10 °C	∞	

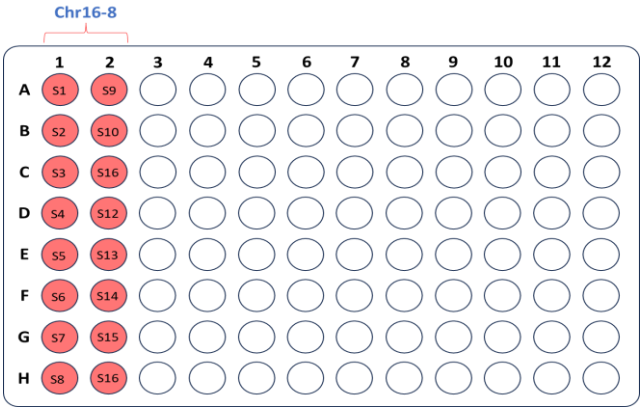
Target Enrichment Reactions

Reagent	Each Primer Mix
Nuclease-free Water	4.5 µL
PA Hot Start Master Mix (2X)	6.0 µL
Primer Mix	0.5 µL
DNA Template, 20 ng/µL	1.0 µL
Total	**12.0 µL**

Note:

(i) If there are more than 1 sample, prepare a target enrichment master mix without the DNA template. Aliquot 11 µL target enrichment master into 0.2 mL thin-walled PCR tube/ plate, followed by DNA template.

(ii) For no template control (NTC), replace the DNA template with 1 µL Nuclease-free water.



iv) Place the PCR reactions into the thermal cycler according to their respective cycling condition (Table 4) and start the PCR amplification. The PCR amplification takes ~ 3 to 5 hours.

Safe Stop Point: The amplicons can be stored at -20 °C for not more than 2 weeks if do not immediately proceed to downstream processes.

Protocol B: Target Enrichment Validation, Quantification and Normalization

Preparation Before Start

- ✓ Equilibrate MagSi-NGS^{PREP} Plus beads at room temperature (15 – 25 °C) for 30 minutes before use.
- ✓ Freshly prepared 85% ethanol (from ethanol absolute 99.8%, undenatured) in nuclease-free water.
- ✓ Magnetic separation rack/ plate.

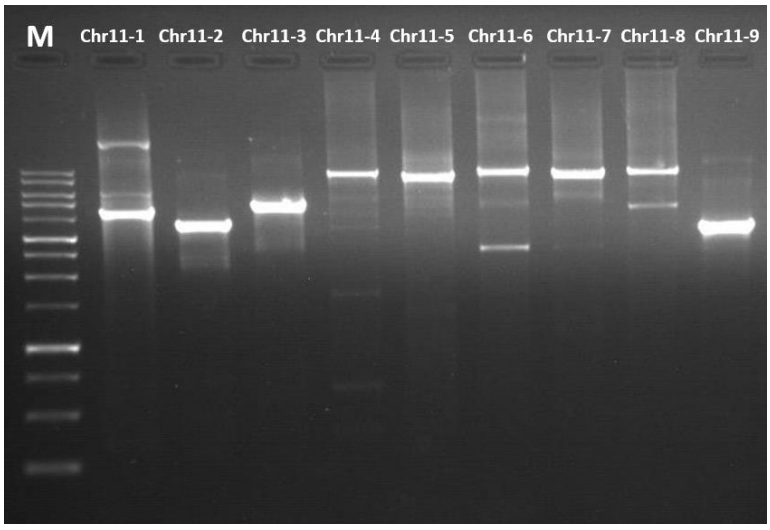
1. Validation of Amplicons via Gel Electrophoresis

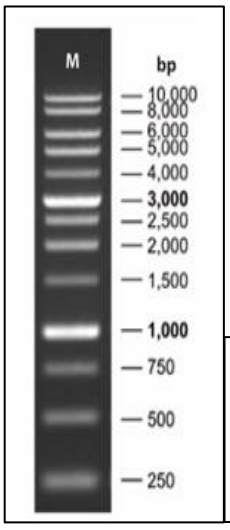
i) Analyse 1 µL of each amplicon (total 26) for each sample on 1% agarose gel, 100 V for 70 minutes. Refer Table 5 for the expected amplicon size from targeted enrichment.

Table 5: Expected amplicon size from target enrichment.

Chromosome 11	Amplicon Size (bp)
Chr11-1	4255
Chr11-2	3604
Chr11-3	4740
Chr11-4	9805
Chr11-5	8690
Chr11-6	9977
Chr11-7	8802
Chr11-8	9464
Chr11-9	3097

Chromosome 16	Amplicon Size (bp)
Chr16-1	7599
Chr16-2	2409
Chr16-3	7341
Chr16-4	4503
Chr16-5	1897
Chr16-6	5663
Chr16-7	5121
Chr16-8	2576
Chr16-9	2637
Chr16-10	8823
Chr16-11	6239
Chr16-12	4587
Chr16-13	8120
Chr16-14	7644
Chr16-15	6907
Chr16-16	2804
Chr16-17	2088





1st BASE
C/No. BIO-5140
ExactMark 1kb
DNA ladder
(250 – 10,000 bp)

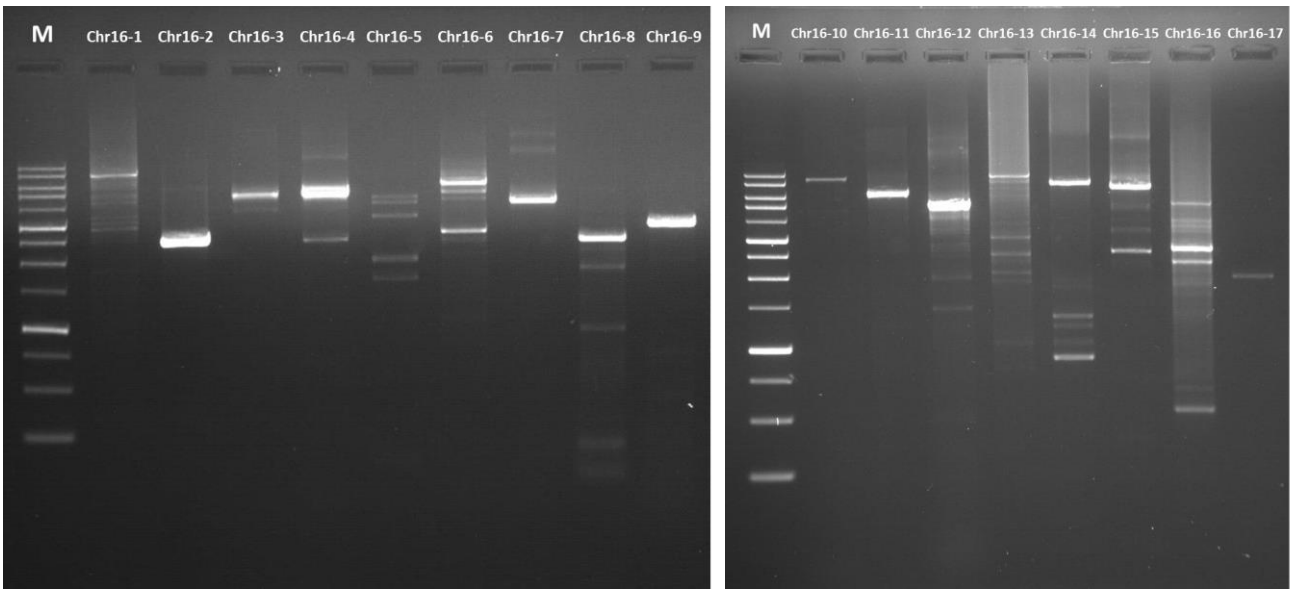


Figure 3. Targeted regions of chromosomes 11 and 16 are amplified using PrimeAmp Custom Thalassemia Enrichment Panel. 1 µL of each amplicon from each primer is run on 1% agarose gel at 100 V, 70 minutes.

Note: Some primers may yield multiple amplicons and results vary from individuals. If the gel analysis shows the targeted amplicon size, NGS result is not affected.

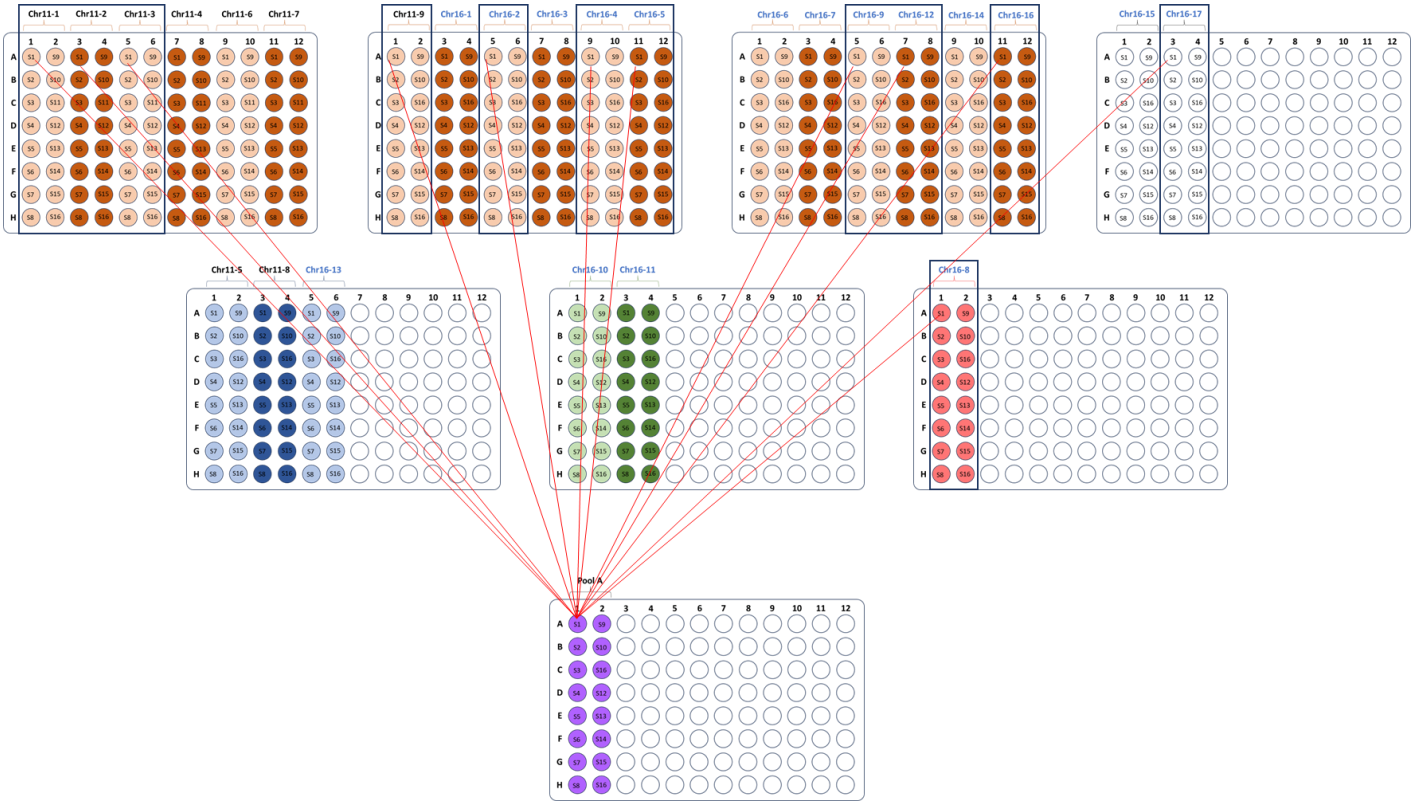
2. Pooling of amplicons into 3 groups: Group A, B and C.

   i) For each sample, pool the amplicons into new 1.5 mL microcentrifuge tube/ 0.2 mL PCR plate according to the Table 6, and Figure 4 is the illustrative example of pooling 16 samples into 3 groups in plate format. Each sample will have 3 groups of pooled amplicons.

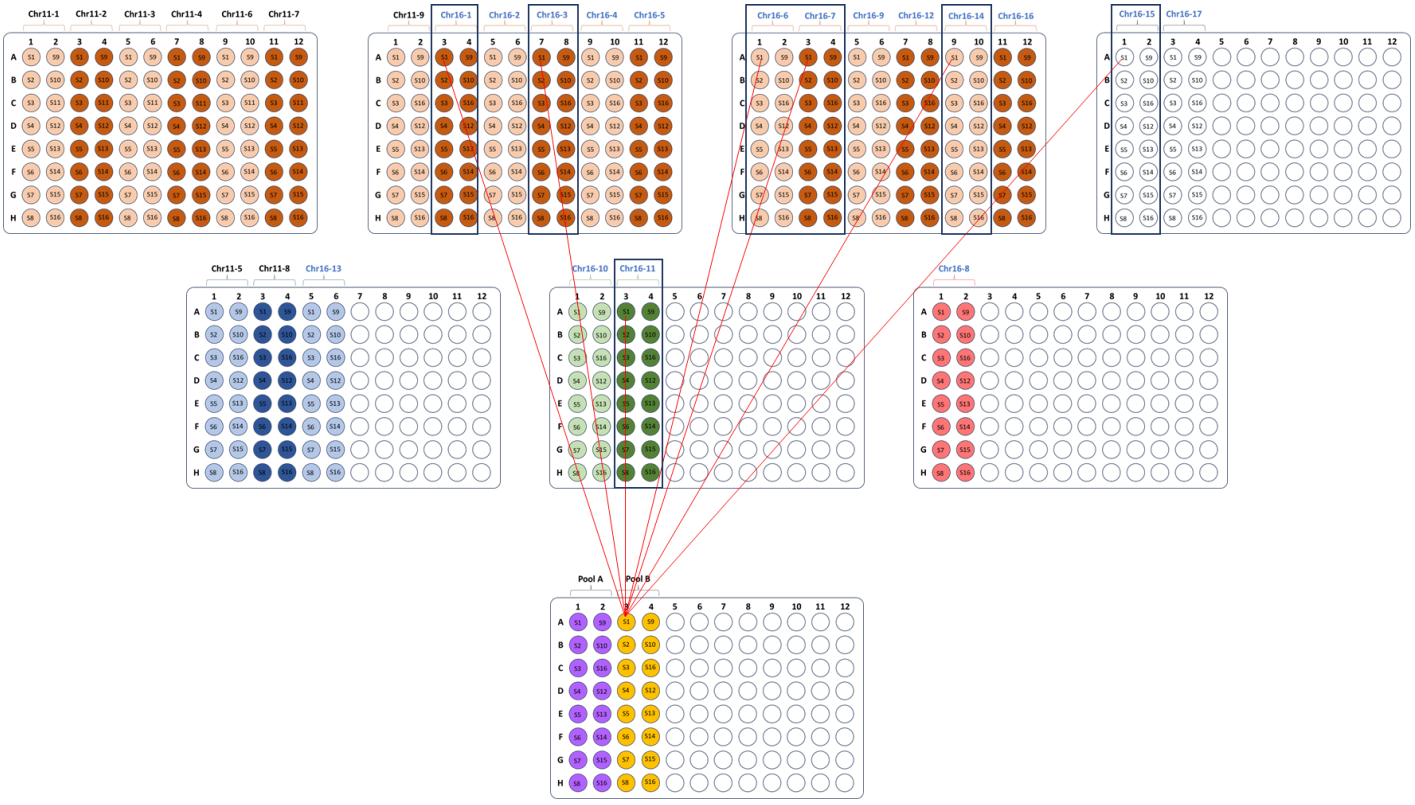
Table 6: Pool of amplicons from each sample into 3 groups.

Group	Primer Mix	Expected total volume
A (12 PCR products)	Chr11-1 Chr11-2 Chr11-3 Chr11-9 Chr16-2 Chr16-4 Chr16-5 Chr16-8 Chr16-9 Chr16-12 Chr16-16 Chr16-17	120 µL
B (7 PCR products)	Chr16-1 Chr16-3 Chr16-6 Chr16-7 Chr16-11 Chr16-14 Chr16-15	70 µL
C (7 PCR products)	Chr11-4 Chr11-5 Chr11-6 Chr11-7 Chr11-8 Chr16-10 Chr16-13	70 µL

Group A



Group B



Group C

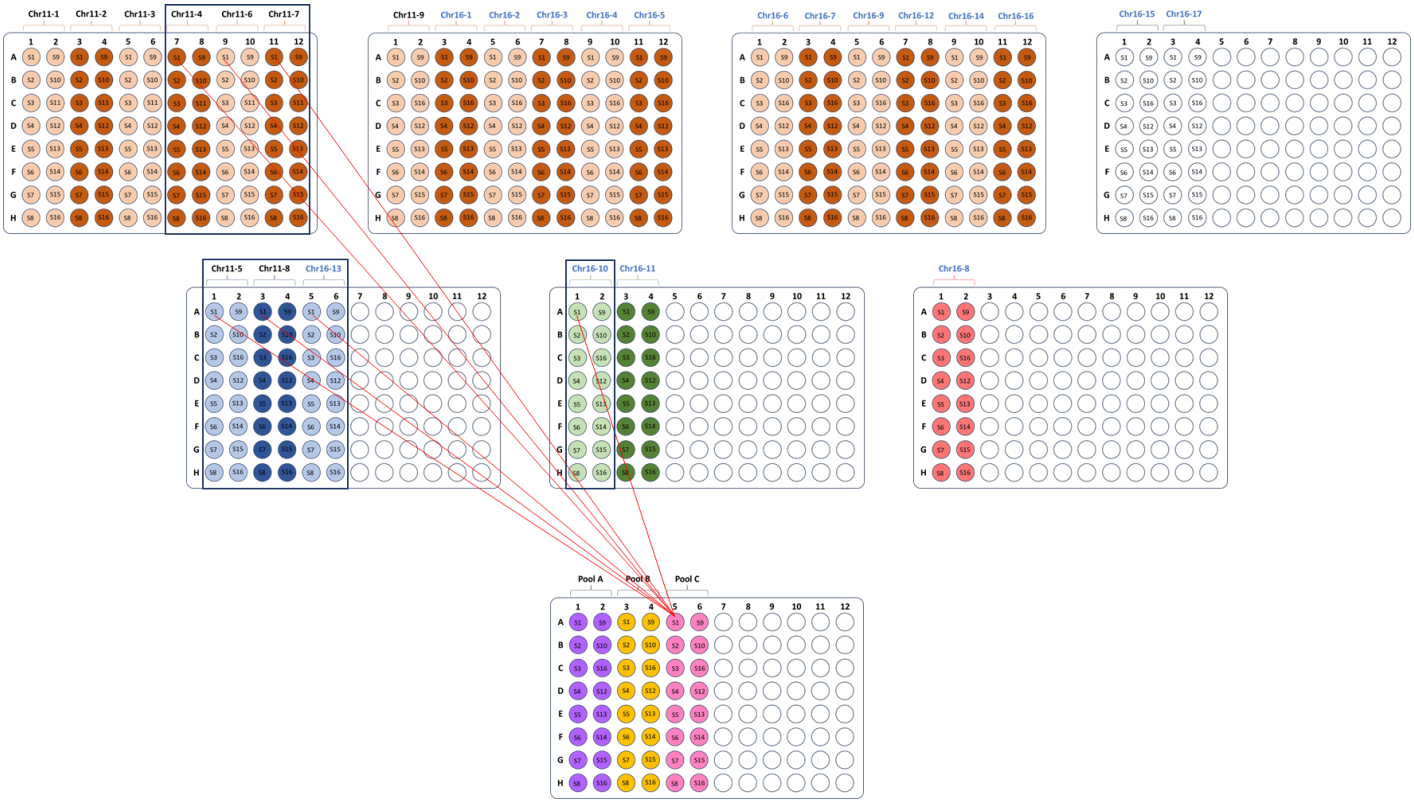


Figure 4: Illustrative example of pooling 16 samples into 3 groups in plate format.

3. Purification of Pooled Amplicons

i) Equilibrate MagSi-NGS^{PREP} Plus beads to room temperature (15 – 25 °C) for at least 30 minutes before use.

ii) Vortex the MagSi-NGS^{PREP} Plus beads for 1 minute to ensure the beads are evenly dispersed.

iii) Add 0.45X MagSi-NGS^{PREP} Plus beads to each group of amplicons. For example:

- Group A (120 µL): Add 54 µL MagSi-NGS^{PREP} Plus beads
- Group B (70 µL): Add 31.5 µL MagSi-NGS^{PREP} Plus beads
- Group C (70 µL): Add 31.5 µL MagSi-NGS^{PREP} Plus beads

iv) Mix gently by pipetting up and down 10 times. Briefly centrifuge to bring down the mixture.

v) Incubate at room temperature for 5 minutes.

vi) Place the sample on the magnetic rack/ plate for 3 minutes or until the supernatant is clear.

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

viii) With the tube remain on the magnetic rack/ plate, perform washing steps without disturbing the beads as follows:

a) Add 180 µL freshly prepared 85% ethanol into each tube.

b) Incubate at room temperature for 30 seconds.

c) Carefully remove and discard the supernatant.

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

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

xi) With the sample remain on the magnetic rack, allow the beads to air dry for 3 minutes.
Note: Do not over dry the beads (appears cracked).

xii) Remove the tube/ plate from the magnetic rack/ plate and resuspend the beads with 32 µL Nuclease-free Water.

xiii) Incubate at room temperature for 2 minutes.

xiv) Place the tube on the magnetic rack for 1 minutes or until the supernatant is clear.

xv) Carefully transfer 30 µL of the eluate to a new 1.5 mL microcentrifuge tube/ 0.2 mL PCR plate.

Safe Stop Point: The pooled amplicons can be stored at -20°C for not more than 2 weeks if do not immediately proceed to downstream processes.

4. Quantification of Purified Amplicons

i) Take 1 µL of each purified amplicons for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent. 3 quantification per sample.

Expected concentration:

a) Group A: ≥ 4.3 ng/µL

b) Group B: ≥ 13.1 ng/µL

c) Group C: ≥ 26.2 ng/µL

5. Pooling and Normalization of Amplicons

i) Based on the quantification result, pool and normalize the amplicons (Group A, B and C) into a new 1.5 mL microcentrifuge tube/ 0.2 mL PCR tube, in accordance with the proportion stated in Table 7. The total amount of final pooled amplicons is 700 ng, in 48 µL.
Note: If the total volume is less than 48 µL, top up the volume with Nuclease-free Water.

Table 7: Pool and normalize the amplicons (Group A, Group B and Group C) in the stated proportion.

	Proportion	Amount
Group A	10%	70 ng
Group B	30%	210 ng
Group C	60%	420 ng

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

Library preparation and sequencing reagents for ONT platforms are not provided with this kit. Refer page 2.

Additional reagents and equipment for Long-Read sequencing

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

Protocol C: DNA Repair and End-Prep

Preparation Before Start

- ✓ Each sample of 700 ng, 48 µL pooled and normalized amplicons are required.
- ✓ 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 tube 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
  - Small amount of precipitate may observe. Let the buffer come to room temperature, break up the precipitate by pipetting up and down, followed by vortexing to ensure the buffers are well mixed.
  - Yellow tinge may observe 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 thin-walled PCR tube/ plate according to Table 8.

Table 8: DNA Repair and End-Prep Reaction setup

Reagent	Volume
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
Normalized Amplicons (700 ng for each sample)	48.0 µL
Total	**60 µL**

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

b) If more than 1 sample, prepare a master mix (without Normalized Amplicons), and make aliquots, 12 µL followed by adding Normalized Amplicons.

ii) Briefly centrifuge and place the reaction in thermal cycler with setting as Table 9.

Table 9: 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 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) Set up thermal block/ water bath at 37 °C.

iii) Vortex the Agencourt® AMPure® XP beads for 1 minute to ensure the beads are evenly dispersed.

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

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

vi) Briefly centrifuge to bring down the mixture.

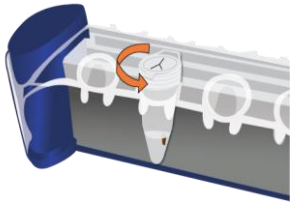
vii) Place the sample on the magnetic rack for 2 minutes or until the supernatant is clear.

viii) Carefully remove and discard the supernatant.

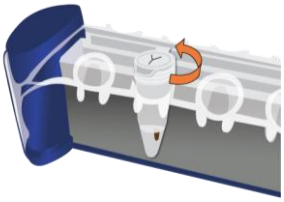
ix) With the tube remaining on the magnetic rack, wash the beads as follows:

a) Add 200 µL freshly prepared 70% ethanol into each tube.

b) Rotate the tube by 180°. Wait until the beads are fully attracted towards the magnet.



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



d) Remove the ethanol using a 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 remain 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 27 µL Nuclease-free Water.

xiv) Incubate at 37 °C for 5 minutes.

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

xvi) Transfer 26 µL of eluate (purified End-prep DNA) into a new 1.5 mL microcentrifuge tube or 0.2 mL PCR plate.

xvii) Take 1 µL of purified End-prep DNA for quantification using iQuant™ Broad Range dsDNA Quantification kit or equivalent reagent.

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

Protocol D: Native Barcode Ligation, Pooling and Adapter Ligation

Important Points Before Start

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

1. Native Barcode Ligation Setup

i) Choose a unique barcode to each sample that will be run together on the same flow cell. 24 samples or more (<= 96 samples) can be barcoded and combined in one run.

ii) Mix the thawed barcodes by tapping, spin down, and place them on ice.

iii) Dilute each End-Prep DNA Product into 500 ng with final volume of 22.5 µL by using nuclease-free Water in 0.2 mL thin-walled PCR tube/ plate.

iv) Add the reagents according to the sequence in Table 10. After adding each reagent, mix by flicking the tube.

Table 10: Native barcode ligation setup

No.	Reagent	Volume
1	500 ng End-Prep DNA	22.5 µL
2	Native Barcode	2.5 µL
3	Blunt/ TA Ligase Master Mix	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) Add 1 µL EDTA to each tube and mix thoroughly by pipetting and spin down briefly.

viii) **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) Set up thermal block/ water bath at 37 °C.

iii) Resuspend the Agencourt® AMPure® XP beads by vortex for 1 minute.

iv) Add 50 µL AMPure® XP beads (1X ratio) to each tube and mix by pipetting.

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

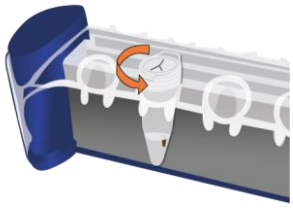
vi) Briefly centrifuge to bring down the mixture.

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

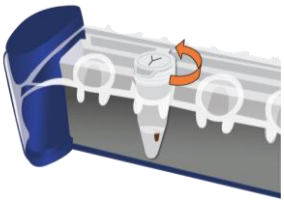
viii) With the tube remain on the magnetic rack, carefully remove, and discard the supernatant.

ix) With the tube remain 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 towards the magnet.



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



c) Remove the ethanol with pipette.

x) Repeat Step (ix) for one more time.

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

xii) With the tube remain 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 28.5 µL Nuclease-free Water.

xiv) Incubate at 37 °C for 5 minutes.

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

xvi) Carefully transfer 28 µL of eluate (Barcoded DNA) into a new 1.5 mL microcentrifuge tube.

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

Note: Expected concentration ≥ 10.8 ng/µL.

3. DNA Pooling before Adaptor Ligation

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

ii) Take 1 µL of pooled Barcoded 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 to final volume, 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 pooled Barcoded DNA at 65 µL.
- ✓ Thaw the buffer/ reagent 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
  - One tube of Short Fragment Buffer (SFB) from Native Barcoding Kit 96 V14
- ✓ Briefly centrifuge and keep on ice.
  - Quick T4 Ligase from NEBNext Quick Ligation Module
  - Native Adapter (NA) from Native Barcoding Kit 96 V14

i) According to the sequence in Table 11, add the reagents into 1.5 mL microcentrifuge tube containing 700 ng, 65 µL pooled barcoded DNA (prepared in Step 3). After adding each reagent, mix by flicking the tube.

Table 11: 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 E: 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) Set up thermal block/ water bath at 37 °C.

iii) Resuspend the Agencourt® AMPure® XP beads by vortex in 1 minute.

iv) Add 40 µL AMPure® XP beads (0.4X ratio) to the unpurified DNA Library and mix by pipetting.

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

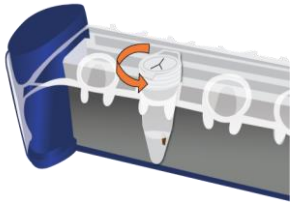
vi) Briefly centrifuge to bring down the mixture.

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

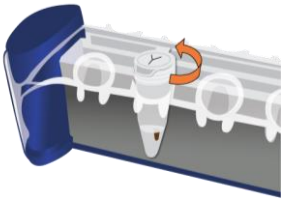
viii) With the tube remain on the magnetic rack, carefully remove, and discard the supernatant.

ix) Add 250 µL Short Fragment Buffer (SFB) and flick the tube to resuspend evenly, spin down briefly and then return the tube to the magnetic rack. Allow beads to pellet on the rack for 2 minutes.

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



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



c) Remove the SFB with pipette.

x) Repeat Step (ix) for one more time.

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

xii) With the tube remain 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 15 µL Elution Buffer (EB).

xiv) Incubate at 37 °C for 10 minutes.

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

xvi) 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.

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

Note: Expected concentration 21 - 28 ng/µL.

Protocol F: Sequencing Setup

Important Points Before Start

- ✓ Purified DNA Library per flow cell.
- ✓ Thaw the items at room temperature. Mix by vortexing and briefly centrifuge.
  - UltraPure™ BSA, 50 mg/ mL
  - Sequencing Buffer (SB)
  - Library Beads (LIB)
  - Flush Cell Tether (FCT)
  - One tube of Flow Cell Flush (FCF) from Flow Cell Priming Kit (ONT, Cat# EXP-FLP004)
- ✓ 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 12 below.

   Table 12: 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 the prepared DNA library gently by pipetting up and down prior to loading.
5. Load 75 µL of the prepared DNA Library (from Table 12) 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:
   i) After the sequencing run is completed and wish to reuse the flow cell, wash the flow cell according to Flow Cell Wash Kit's protocol and store the washed flow cell at 2 – 8 °C; or
   ii) Follow ONT return procedure by washing out the flow cell.

Protocol G: Data Analysis

Sequence obtained from Nanopore sequencing can be mapped to human reference genome using Minimap2[1] with the option -ax map-ont -t8. Mapped reads are sorted into coordinate-order using the SortSam function (https://broadinstitute.github.io/picard/) subsequently proceed to variant calling using Freebayes V1.3.6[2].

References

1. Li H. 2018. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics 34:3094–3100.
2. Garrison E, Marth G. 2012. Haplotype-based variant detection from short-read sequencing.

Troubleshooting Guidelines

Problems	Possible Reason/ Recommended Action
Control is positive; but my sample fails to produce the target amplicon(s)	• Most likely the sample do not have binding sites for the involved primer pair.
Sample does not produce the target amplicon(s) for a few primers	• The integrity of DNA is sub-optimal, thus long PCR product not able to amplify. • Sample do not have binding sites for the primer mix (s).
Unspecific amplification/ DNA bands	• Some primer pairs will give both target and unspecific DNA bands. This will not interfere the NGS result.
PCR clean-up of amplicon pools	• PCR clean-up with 0.45X volume of beads able to remove <1.5 kb amplicon. *Do not use 0.4X volume of bead which might remove the smallest amplicon, 1897 bp from Chr16-5 primer mix.
Amplicons from Chr16-15 and/ or Chr16-17 are inconsistent	• The performance of these primer pairs may vary from thermocyclers. Kindly contact technical support if you need assistance to optimize the PCR.

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

Appendix

	Primer Mix	Target ID	ULSO Sequence (5' to 3')	DLSO Sequence (5' to 3')
Chromosome 11	Chr11-1	BPhi45_F	TCGTTCATTCTGCAGGTTGTCTC	GAGATGGCTGAATTGACAGAAGTAGG
	Chr11-2	Bsiriraj_F	GTTTGGTTCTAAGTCTTGACTAAAGTCC	ATGCTTCTGTAATTGCTGGAGATAGC
	Chr11-3	BChi100Del_F BFPFH6_F BFPFH6_F2	TACAGGACTGTAATGGAGGTTACC	TCAAAGTTCATGAAGATTTACCCATGC
	Chr11-4	HPFH3_F B101.3Del_F BAsian49.3Del_F	GCCTTCTAATATGTGTGTAGTGGTTGG	TGATGGAGAGAGGAAAGGGAAGG
	Chr11-5	BThai_F B3.5_F B619_F HBB BLPWB_F BLPBALT_F	GTGGGCATCTAAGTCTCTTTGTAGG	GCTGACCTCATAAATGCTTGCTACC
	Chr11-6	BSEA27_R BPhi45_R B150HBD_F HBD	GTCTGTTTCCCATTCTAAACTGTACCC	CCTATTCTTGTGTTCACGACTGACATC
	Chr11-7	BHPFH3_R HBG1 Bsiriraj_R	TCCCATTCTCCAACATTCTCATTTCC	CAGCCAGCACACACACTTATCC
	Chr11-8	HBG1 Bsiriraj_R HBG2_R	AACTGGACTCCTTCCTTATACCTTATCC	TCGGGTGCCTACATACATACCT
	Chr11-9	HBG2partial	GCTGGCTCATTTCCTTCACCTAAG	TCGGGTGCCTACATACATACCT

Continued next page.

	Primer Mix	Target ID	ULSO Sequence (5' to 3')	DLSO Sequence (5' to 3')
Chromosome 16	Chr16-1	AE261Del_F	GCAAGAACCACAGGAGGGAATAG	CCGCGAGAGCATAAAGTGAATTATACC
	Chr16-2	AE360Del_F	CAGCAGTGTGTGGGAAGAAAGA	GTGGTGTCTTTATGTCTCTGCCAATC
	Chr16-3	AE158Del_F	GGATGGTCTGGCTGTGTTCTTT	TCCAGGCTGGAGTGTACTTGAT
	Chr16-4	AThai_F APhi_F ADel1_F	TCACTGTCTTCTCTGCCAGTCT	GGGGATCAGATGCTGGGAATTT
	Chr16-5	A20_F	CAGGGTAAGGAGGGAACGATTAGG	TTGCCTATCAGGGAGGTGAGAC
	Chr16-6	ADel2_F2 ADel2_F1	CGCGTAATGCGCCAATAAACC	ACCTCTCACAACCTGTGTCCCACAAGCC
	Chr16-7	AGB_F	CCACTTGCTCTTTCTCTCGGAG	CCTCAGGAGTGACTGTCCTTGA
	Chr16-8	AMed_F ASEA_F	TGGAGAGGTGAGTGTCAGATGGGACTGCC	CGCGTGGGCACCTTTACTGCAGACTTT
	Chr16-9	ADel3_F	CTGTGTTCTCAGTATTGGAGGGAAGG	GGGCCAAGTTCTCCAAACTACC
	Chr16-10	A4.2_F AW_F AM3.5_F HBA123.7_1:Part1 HBA123.7_2	GGTCACTATTGTTGGCCAGTGAAGCTCA	CTGGAGGAGGTGAGACTTAAGGATATG
	Chr16-11	HBA123.7_1:Part2 AM3.5_R AMed_R ADel1_R1	CATATCCTTAAGTCTCACCTCCTCCAG	CAGCAAGCCTTGTTTTCGGAAG
	Chr16-12	AThai_R ASEA_R	AAGACCTACTTCTCCCACCTGG	CTCGGAGATATATGGGTCTGGAAGTG
	Chr16-13	AE158Del_R	GTGGAAGAATGTGGATGAGAAGCC	CCTTGCTCTACTTTAGCATCCATACC
	Chr16-14	AE360Del_R	CCACAGGCCTTGTTTCAGGAATAG	GACAGGATGGTTTGGGATGGAAAG
	Chr16-15	A4.2_F AW_F AM3.5_F HBA123.7_1:Part1 HBA123.7_2	GGAGAAAGTTGGCAACCACACT	CTGGAGGAGGTGAGACTTAAGGATATG
	Chr16-16	AE158Del_R	GCTGAGATTGCACCACTACATTCC	GAATACCACAGTCCATGCAGAAAGG
	Chr16-17	A20_F	CAGGGTAAGGAGGGAACGATTAGG	CAGTCGAGCTCTCCATGGTG

Notes:

1. Chr16-5 and Chr16-17 are amplifying the same Target ID.
2. Chr16-10 and Chr16-15 are amplifying the same Target ID.
3. Chr16-13 and Chr16-16 are amplifying the same Target ID.

Product and Service Ordering Information

Products

Agency	Product Number	Product Description
1st BASE Kits	KIT-2020-16	PrimeAmp Custom Thalassemia Enrichment Panel, 16 reactions
1st BASE Biochemicals	BUF-1180-100ml	Nuclease-free Water, Biotechnology Grade, 100 mL
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		
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 Master Mix
	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
ABP Biosciences	N012/ N013	iQuant™ Broad Range dsDNA Quantification kit

Services

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 Bioinformatics Analysis Services		
1st BASE NGS	NGS-0601	Standard Analysis Custom Target Capture Standard Bioinformatics Analysis included: - 1. Sequence QC 2. Mapping onto reference genome; statistics of sequencing depth and coverage 3. Variant (SNP & InDel) calling, annotation and statistics