MYTHO

NBS custom panel

HANDBOOK

Next Generation Sequencing custom panel

New Born Sequencing Panel

RUO MYT011-096-A
MYT011-096-B

REF MYT011-096-C
MYT011-096-D
MYT011-384

CONTENTS

MYT011 AVAILABLE FORMATS

Commercial Name	REF	Number of test	Size type
NBS panel	MYT011-96	96	Standard
NBS panel	MYT011-384	384	4 boxes of MYT011-96

1. PRODUCT DESCRIPTION

Intended use

The NBS panel is a Next-Generation Sequencing (NGS) custom panel designed for the sequencing of specific genes of interest in blood samples.

This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition.

The product is intended for professional use only.

Principles and procedure overview

The NBS panel utilizes Next-Generation Sequencing (NGS) technology. NGS is a highly sensitive and specific sequencing technique employed across diverse applications. This panel specifically enables the identification of various types of genetic variations within targeted genomic regions of interest.

Storage and handling

Please read the product packaging and storage recommendation carefully for each kit and store components as recommended upon arrival. All components are stable under the recommended storage conditions until the expiry date on the label.

Material provided

REF# MYT011-096					
Box N	**Box content**	**Description**	**Volume (µL)**	**Size**	**Storage**
Box 1	NBS library preparation	Fragmentation enzyme 5x	960	96 rxn	-20°C
		Fragmentation buffer 10x	480		
		DNA ligation buffer	1900		
		DNA ligation mix	960		
		Universal adapters	480		
		Library amplification mix 2x	2400		
	NBS hybridization reagents	Hybridization mix	240	12 rxn	
		Hybridization enhancer	360		
		Amplification primers	30		
		NBS panel probes	48		
		Library amplification mix 2x	400		
		Universal blockers	96		
		Blocker solution	60		
Box 2	NBS library preparation	DNA Purification beads	12500	96 rxn	2-8°C
	NBS hybridization reagents	Binding Buffer	9600	12 rxn	
		Streptavidin binding beads	1200		
		Wash Buffer 1	2400		
		Wash Buffer 2	8400		
		DNA purification beads	2200		

REF	Box	Box content	Description	Volume (µL)	Size	Storage
MYT011-096-A	Plate Box	UDI Primers plate	UDI Primers plate A*	10	96 rxn	-20°C
MYT011-096-B	Plate Box	UDI Primers plate	UDI Primers plate B*	10	96 rxn	-20°C
MYT011-096-C	Plate Box	UDI Primers plate	UDI Primers plate C*	10	96 rxn	-20°C
MYT011-096-D	Plate Box	UDI Primers plate	UDI Primers plate D*	10	96 rxn	-20°C

*each plate type contains different UDI primers

REF# MYT011-384						
N	**Box N**	**Box content**	**Description**	**Volume (µL)**	**Size**	**Storage**
4	Box 1	NBS library preparation	Fragmentation enzyme 5x Fragmentation buffer 10x DNA ligation buffer DNA ligation mix Universal adapters Library amplification mix 2x	960 480 1900 960 480 2400	96 rxn	-20°C
		NBS hybridization reagents	Hybridization mix Hybridization enhancer Amplification primers NBS panel probes Library amplification mix 2x Universal blockers Blocker solution	240 360 30 48 400 96 60	12 rxn	
4	Box 2	NBS library preparation	DNA Purification beads	12500	96 rxn	2-8°C
		NBS hybridization reagents	Binding Buffer Streptavidin binding beads Wash Buffer 1 Wash Buffer 2 DNA purification beads	9600 1200 2400 8400 2200	12 rxn	
1	Box 3	UDI Primer Plates	UDI Primers Plate A UDI Primers Plate B UDI Primers Plate C UDI Primers Plate D	10	96 rxn	-20°C

Gene list

The list of genes included in this panel is available in the attached Annex NBS panel gene list.pdf

Materials required but not provided

The following materials or their equivalent are required to use NBS panel

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8	—
Buffer EB	Qiagen
1.5mL microcentrifuge tubes	VWR
Thin-walled PCR 0.2mL strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
1.5mL compatible magnetic stand	Beckman Coulter
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Agilent DNA 7500 kit	Agilent Technologies
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
EQUIPMENT	
Pipettes and DNase, RNase free tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2mL tubes	—
Thermomixer for 1.5mL tubes	Eppendorf
Thermal cycler (96 well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies
Vacuum concentrator (if unavailable see appendix)	-

General lab equipment

Personal Protective Equipment such as but not limited to:

- Full length lab coat,
- Disposable gloves
- Protective glasses or goggles

2. WARNING AND PRECAUTIONS

This product is for professional use only; it must be used by trained professionals in molecular biology.

General warnings and precautions

- Read all instructions that come with the product carefully before performing the test.
- When performing the test, follow the instructions provided with the product.
- Test the compatibility of your thermal cycler and PCR tubes by incubating them at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.
- This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.
- To avoid contamination of reagents, use PCR clean or DNase/RNase free tubes, filter tips
- Do not use the product after the stated expiration date.
- Do not use the product if the packaging is damaged or the seal is broken upon receipt.
- Use only the reagents supplied with the product and those recommended by the manufacturer.
- Do not combine reagents from different batches or from different tubes of the same batch.
- Handle and dispose of all biological specimens, reagents and all material that has come into contact with the biological specimens as if they were capable of transmitting infectious agents. Avoid direct contact with biological samples, reagents and material. Avoid splashing or splashing. Waste must be handled and disposed of in accordance with appropriate safety regulations.
- Wear protective clothing and appropriate gloves; protect your eyes and face.
- Never pipette solutions with your mouth.
- Do not eat, drink, smoke, or apply cosmetic products in work areas.
- Wash your hands thoroughly before and after handling samples and reagents.

Guidelines for gDNA samples

- Use the Thermo Fisher Scientific Qubit dsDNA HS to accurately quantify the purified gDNA input.
- Input DNA should be suspended in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- It is important to remove all cations and chelators from the starting gDNA sample. The presence of cations and chelators may affect the initial fragmentation reaction.
- For genomic (gDNA) samples, correct input quantity is critical for achieving optimal yield and library fragment length.
- The recommended DNA input is 100 ng of purified gDNA.
- For germinal sample: 100ng of high quality gDNA (minimum input 30ng).
- For somatic sample: 100ng of high quality gDNA (minimum input 30ng).
- Using higher or lower mass input may require optimization of the following steps in library preparation to achieve optimal performance.
- Measuring DNA concentration by absorbance at 260 nm is not recommended.

3. PROTOCOL

Sample information

Use any commercial kit to obtain DNA from biological tissues. Determine the starting DNA concentration by fluorometric methods for accuracy.
The reagents are compatible with mass inputs from 1 ng to 500 ng but may require optimization of the following steps in library preparation for optimal performance.

Protocol overview

This protocol begins after the genomic DNA (gDNA) purification. Determine the starting DNA concentration by fluorometric methods for accuracy.

	Phase	Result	Execution time
	Library preparation		
1	**DNA Fragmentation, End Repair and dA-tailing**	dA-tailed dDNA fragments	1 hour
2	**Ligation with universal adapters and Purification**	gDNA libraries ready for indexing	1 hour
3	**PCR amplification using UDI primers, Purification and QC**	Amplified indexed libraries	1 hour
	Target enrichment		
4	**Library preparation for hybridization**	Indexed library pool	1 hour
5	**Capture probes hybridization with pools**	Hybridized targets in solution	16 hours
6	**Bind hybridized targets to streptavidin beads**	Captured targets on beads	1.5 hour
7	**Post-capture PCR amplify, purification, and QC**	Enriched libraries	1 hour
8	**Sequencing**	Libraries ready for sequencing on Illumina platform or GeneMind platform	

Library preparation

Automated protocol

Please refer to the technical note for your specific workstation model.

Phase 1: DNA Fragmentation, End Repair and dA-tailing

Perform enzymatic fragmentation of input gDNA and subsequent end repair and dA-tailing to generate dA-tailed DNA fragments.

Reagents Required

- Genomic DNA (gDNA): 100 ng per sample
- Molecular biology grade water (chilled)
- Optional 10 mM Tris-HCl pH 8 or Buffer EB
- Qubit dsDNA Broad Range Quantitation Assay (or equivalent)
- Fragmentation Enzyme 5x
- ·Fragmentation Buffer 10x

Preliminary operations

- Thaw the Fragmentation enzyme and gDNA samples and mix gently.
- Thaw Fragmentation buffer and mix by vortex for 2 sec.
- Program the thermal cycler with the following conditions:

Step	Temperature	Time
1	4	HOLD
2	37	25 minutes
3	65	30 minutes
4	4	HOLD

Lid temperature 70°C

Workflow

1. Start the program to pre-chill the thermal cycler
2. Use the Qubit dsDNA Broad Range Quantitation Assay to determine the concentration of your gDNA samples.
3. Dilute the gDNA samples to 5 ng/µL with water, 10 mM Tris-HCl pH 8, or Buffer EB. Mix well with gentle pipetting.
4. Add up to 35 or minimum 10 µL of each diluted gDNA sample (100 ng total gDNA) into a thin-walled PCR 0.2mL strip-tube or well of a 96-well thermal cycling plate, and place on ice.
5. Pulse-spin to ensure all of the solution is at the bottom of the tube.
6. Prepare an enzymatic fragmentation master mix in a 1.5mL microcentrifuge tube on ice. Use the volumes listed in the table below. Mix thoroughly by gentle pipetting

Reagent	Volume per rxn (µL)
DNA sample	From 10 to 35µL
Fragmentation buffer 10x	5
Fragmentation enzyme 5x	10
Total*	**40**

*Bring to the final volume with chilled water if using less than 35 µL of sample.

7. Add 40 µL enzymatic fragmentation master mix (from point 5) to each 10 µL gDNA sample well or tube and mix well by gentle pipetting. Cap the tube and keep the reaction on ice.
8. Pulse-spin the sample plate or tubes and immediately transfer to the pre-chilled thermal cycler.
9. Proceed to steps 2–4 of the thermal cycler program.
10. When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place on ice.

Note: While the thermal cycle program is running, prepare the reagents for phase 2: Ligation with universal adapters and purification.

Phase 2: Ligation with universal adapters and Purification

Ligate the Universal Adapters to the dA-tailed DNA fragments from phase 1 and purify to generate gDNA libraries ready for index introduction through amplification in phase 3.

Reagents Required

- dA-tailed DNA fragments (from phase 1 point 10)
- Ethanol
- Molecular biology grade water (chilled)
- Optional 10 mM Tris-HCl pH 8 or Buffer EB
- DNA Ligation Mix
- DNA Ligation Buffer
- Universal Adapters
- DNA Purification Beads

Preliminary operations

- Thaw Universal adapters, DNA ligation mix, DNA ligation buffer.
- Prepare 1 mL 80% ethanol for each sample.
- Equilibrate DNA purification beads to room temperature for at least 30 minutes.
- Program a thermal cycler to incubate the samples at 20°C with the heated lid set to minimum temperature or turned off. Start the program so that the cycler is at 20°C when the samples are prepared.

Workflow

1. Add 5 µL of Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments for Step 1. Mix gently by pipetting and keep on ice.
2. Prepare the ligation master mix in a 1.5mL microcentrifuge tube on ice as indicated in the table below. Mix well gently.

Reagent	Volume per rxn (µL)
Water (chilled)	15
DNA ligation buffer	20
DNA ligation mix	10
Total	**45**

3. Add 45 µL of the ligation master mix to the sample from Step 2.1 and mix well by gentle pipetting.
4. Seal or cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.
5. Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, then move the samples to the bench top. Proceed to the Purify step.

Turn off the heated lid or set to minimum temperature

Note: While the thermal cycler program is running; prepare the reagents for phase 3.

6. Vortex the pre-equilibrated DNA Purification Beads until well mixed.
7. Add 80 µL (0.8x) of homogenized DNA Purification Beads to each ligation sample from Phase 2 point 5. Mix well by vortexing.
8. Incubate the samples for 5 minutes at room temperature.
9. Place the samples on a magnetic plate for 1 minute or until the supernatant is clear.
10. The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing plate or tubes from the magnetic plate, remove and discard the supernatant.
11. Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.
12. Repeat the wash once, for a total of two washes, while keeping the samples on the magnetic plate.
13. Carefully remove all remaining ethanol with a 10 µL pipet, making sure not to disturb the bead pellet

Note: Spin briefly the bead pellet to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate

14. Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.
15. Remove the plate or tubes from the magnetic plate and add 17 µL water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.
16. Incubate at room temperature for 2 minutes.
17. Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
18. Transfer 15 µL of the clear supernatant containing the ligated and indexed libraries to a clean thin-walled PCR 0.2mL strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

Phase 3: PCR amplification using UDI primers, Purification and QC

Amplify the adapted gDNA libraries with UDI Primers, purify them, and perform quality control (QC) analysis to complete the protocol.

Reagents Required

- Ligated, adapted libraries from phase 2 point 18
- 80% Ethanol from phase 2
- Equilibrated DNA Purification Beads from phase 2
- Molecular biology grade water
- Library Amp Mix 2x
- UDI Primers
- Optional 10 mM Tris-HCl pH 8 or Buffer EB

Preliminary operations

Thaw UDI (plate with single use primers) and Library Amp Mix (2x).

Program the thermal cycler with the following conditions:

	Step	T (°C)	Time(s)	Cycles
1	Initialization	98	45	1
2	Denaturation	98	15	8
3	Annealing	60	30	
4	Extension	72	30	
5	Final extension	72	60	1
6	Final hold	4	HOLD	-

*6-8 cycles is recommended when starting with 50-100 ng of high quality gDNA

Workflow

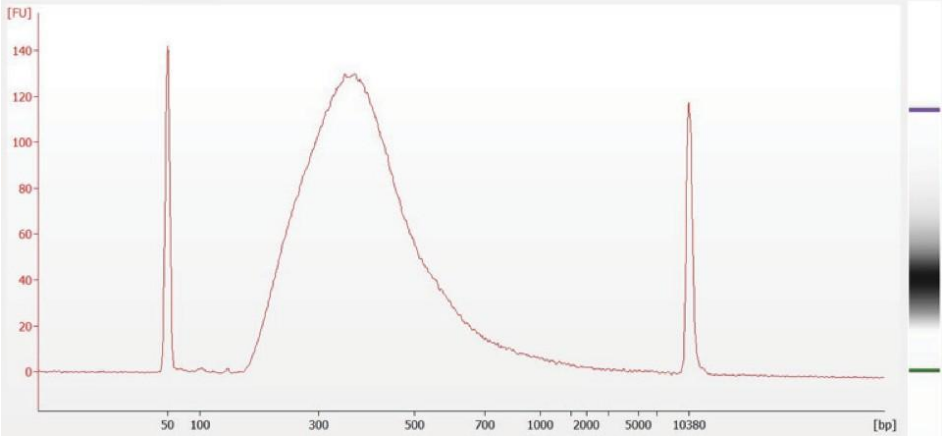
1. Add 10 µL of UDI Primer from the provided 96-well plate to each of the gDNA libraries and mix well by gentle pipetting.
2. Add 25 µL of Library Amp Mix 2x to the gDNA libraries and mix well by gentle pipetting.
3. Pulse-spin sample plate or tube and

immediately transfer to the thermal cycler. Start the program.

4. Remove the sample(s) from the block when the thermal cycler program is complete. Proceed to purification.
5. Vortex the pre-equilibrated DNA Purification Beads until mixed.
6. Add 50 µL of homogenized DNA Purification Beads 1x to each ligation sample from point 4. Mix well by vortexing.
7. Incubate the samples for 5 minutes at room temperature.
8. Place the samples on a magnetic plate for 1 minute.
9. The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing plate or tubes from the magnetic plate, remove and discard the supernatant.
10. Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.
11. Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.
12. Carefully remove all remaining ethanol with a 10- µL pipet, making sure not to disturb the bead pellet.

Note: Before pipetting, spin down the bead pellet to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate

13. Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.
14. Remove the plate or tubes from the magnetic plate and add 22 µL water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.
15. Incubate at room temperature for 2 minutes
16. Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
17. Transfer 20 µL of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2mL strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.
18. Quantify and validate the size range of each library using Thermo-Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 7500 Assay. Final concentration values should be ≥80 ng/ µL, and average fragment length should be 375–425 bp using a range setting of 150–1000 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of gDNA library samples that were prepared as described. Note the single prominent peak at ~400 bp.

Note: If the average fragment length is not in the range of 375–425 bp: input DNA concentration may be inaccurate. Typically, using too much DNA leads to shorter fragments, while not using enough DNA leads to larger fragments.
The presence of cations and chelators may also affect the average fragment length.
If neither of the above factors apply, optimize the 32°C fragmentation in Phase 1 point 1 by changing the time in 3 minute increments—increase time to produce shorter fragments, and decrease time to produce longer fragments.

Stopping point: If not proceeding immediately to the Target Enrichment, store the amplified indexed libraries at -20°C.

The Target Enrichment generates enriched DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems or GeneMind systems.

Automated protocol

Please refer to the technical note for your specific workstation model

Phase 4: Libraries preparation for hybridization

This step involves aliquoting the appropriate amount of amplified, indexed libraries and preparing the hybridization reaction solution.

Reagents required

Amplified, indexed library.

Workflow

This protocol supports a single or multiplex (up to 8-plex) hybridization capture. The amount of indexed library to use depends on the number of indexed samples per pool.

1. Use the concentration of each amplified, indexed library to calculate the volume (in µL) of each library needed for hybridization:
   - Determine the amount of each indexed library per pool from the table below.
   - Divide the amount of each indexed library per pool by the concentrations measured in ng/µL from the library preparation QC. For example: If multiplexing eight libraries per hybridization reaction, the amount of each library will be 187.5 ng and the total mass of the pool will be 1,500 ng.
2. Transfer the calculated volumes from each amplified indexed library to an indexed library pool reaction tube for each hybridization being performed. Clean, thin-walled PCR 0.2mL strip-tube or well of a 96- well thermal cycling plate are recommended to avoid unnecessary transfers in downstream steps.
   Pulse-spin the indexed library pool tube(s) to minimize the amount of bubbles present.

Note: if a vacuum concentrator is available, dry the indexed library pool(s)using a vacuum concentrator using low or no heat. Follow the instruction in the appendix

Indexed samples per pool	Amount of each indexed library per pool	Total mass per pool
1	500 ng	500 ng
2	500 ng	1,000 ng
3	500 ng	1,500 ng
4	375 ng	1,500 ng
8	**187.5 ng**	**1,500 ng**

Note: More than 1,500 ng (1.5 µg) total DNA can be used; do not, however, use more than 4 µg total DNA as this might lead to reduced performance of the enrichment. If the amount of library is insufficient, you can use a smaller amount, however may result in decreased library complexity

Phase 5: Capture probes hybridization with pools

Reagents required

- Indexed library pool(s) from phase 4
- NBS panel probes
- Hybridization Mix
- Hybridization Enhancer
- Universal Blockers
- Blocker Solution
- Molecular biology grade water
- Ethanol
- DNA Purification Beads

Preliminary operations

- Thaw all required reagents, then mix with a-vortex for 2 seconds to mix then spin down.
- Set a heat block to 65°C.
- Program a 96-well thermal cycler to 95°C and set the heated lid to 105°C
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes
- Vortex the pre-equilibrated DNA Purification Beads until well mixed
- Prepare 500 µL fresh 80% ethanol for each sample to be processed.

Workflow

1. Add 1.5x homogenized DNA Purification Beads to the tube(s) containing the DNA library(ies) from Phase 4, point 2. Mix well by vortexing.
2. Incubate for 5 minutes at room temperature.
3. Pulse spin to ensure all the solution is at the bottom of the tube(s) and place the tube(s) on a magnetic plate or rack for 3 minutes or until the solution is clear.
4. The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube(s) from the magnetic plate or rack, remove and discard the clear supernatant.
5. Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.
6. Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic plate.
7. Carefully remove all remaining ethanol using a 10 µL pipette, making sure not to disturb the bead pellet.

Note: Pulse spin if necessary to ensure complete removal of ethanol**.**

8. Air-dry the bead pellet on a magnetic plate for 1–5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.
9. Remove the tube(s) from the magnetic plate or rack and add 7 µL Universal Blockers and 5 µl Blocker Solution. Mix by pipetting until homogenized.
10. Heat the Hybridization Mix at 65°C in the heat block for 10 minutes, or until all precipitate is dissolved, then cool to room temperature on the benchtop for 5 minutes.
11. Prepare a probe solution in a clean thin-walled PCR 0.2mL strip-tube or well of a 96-well thermal cycling plate as indicated in the table below. Mix by flicking the tube(s).

Reagent	Volume (µL)
Hybridization Mix	20
NBS panel probes	4
Water (up to total volume)	4
Total	**28**

Note: Hybridization Mix is very viscous. Pipette slowly to ensure accurate pipetting. Small white particles may be

present in the Twist Fixed or Custom Panel tube(s), this will not affect the final capture product

12. Heat the probe solution to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.
13. While probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool at 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.
14. Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.
15. Pulse-spin the tube(s) to ensure all solution is at the bottom of the tube(s).
16. Add 30 µL Hybridization Enhancer to the top of the entire capture reaction.
17. Pulse-spin the tube(s) to ensure there are no bubbles present.
18. Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C.

Note: Halting hybridization between 15-17 hours will not affect the downstream capture quality.

Phase 6: Bind hybridized targets to streptavidin beads

Reagents required

Hybridization reactions
Amplification Primers
Binding Buffer
Wash Buffer 1
Wash Buffer 2
Streptavidin Binding Beads
DNA Purification Beads

Preliminary operations

Preheat the following tubes at 48°C until any precipitate is dissolved:
- Binding Buffer
- Wash Buffer 1
- Wash Buffer 2

For each hybridization reaction:
- Equilibrate 800 µL Binding Buffer to room temperature
- Equilibrate 200 µL Wash Buffer 1 to room temperature
- Leave 700 µL Wash Buffer 2 at 48°C

Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes

In preparation for Post-Capture PCR Amplify, Purification and QC:
- Thaw Library Amp Mix (2x)
- Thaw Amplification Primers
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes

Workflow

1. Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.
2. Add 100 µL Streptavidin Binding Beads to a 1.5mL microcentrifuge tube. Prepare one tube for each hybridization reaction.
3. Add 200 µL Binding Buffer to the tube(s) and mix by pipetting.
4. Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure not to disturb the bead pellet. Remove the tube from the magnetic stand.
5. Repeat the wash (point 3 and 4) two more times for a total of three washes.
6. After removing the clear supernatant from the third wash, add a final 200 µL Binding Buffer and resuspend the beads by vortexing until homogenized.
7. After the hybridization (Phase 5 point 10) is complete, open the thermal cycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of washed Streptavidin Binding Beads from Step 3.6. Mix by pipetting and flicking.

Rapid transfer directly from the thermal cycler at 70°C is a critical step for minimizing off-target binding. Do not remove the tube(s) of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binding Beads. Allowing to cool to room temperature for less than 5 minutes will result in as much as 10–20% increase in off-target binding.

8. Mix the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 30 minutes at room temperature on a shaker, rocker, or rotator at a speed sufficient to keep the solution mixed.

Note: Do not vortex, aggressive mixing is not required

9. Remove the tube(s) containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube(s).
10. Place the tube(s) on a magnetic stand for 1 minute.
11. Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

Note: Some Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product

12. Remove the tube(s) from the magnetic stand and add 200 µL Wash Buffer 1. Mix by pipetting. Pulse-spin to ensure all solution is at the bottom of the tube(s).
13. Pulse-spin to ensure all solution is at the bottom of the tube(s)
14. Transfer the entire volume from Phase 6, Step 12 (~200 µL) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.
15. Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
16. Remove the tube(s) from the magnetic stand and add 200 µL of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube(s).
17. Incubate the tube(s) for 5 minutes at 48°C.
18. Place the tube(s) on a magnetic stand for 1 minute.
19. Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.
20. Repeat the wash (point 16–19) two more times, for a total of three washes.
21. After the final wash, use a 10 µL pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.

Note: Before removing supernatant, the bead pellet may be briefly spun to collect supernatant at the

bottom of the tube or plate and returned to the magnetic plate

22. Remove the tube(s) from the magnetic stand and add 45 µL water. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

Phase 7: post capture PCR amplification, purification and QC

Reagents required

- Streptavidin Binding Bead slurry (from Phase 6 point 22).
- Ethanol.
- Molecular biology grade water.
- Reagents thawed and equilibrated in Phase 6:
  - DNA Purification Beads
  - Library Amp Mix 2x
  - Amplification Primers
- Agilent Bioanalyzer High Sensitivity DNA Kit (or equivalent).
- Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay.

Preliminary operations

Prepare 500 µL 80% ethanol for each Streptavidin Binding Bead slurry to be processed.

Workflow

1. Program a thermal cycler with the following conditions. Set the heated lid to 105°C.

	Step	T (°C)	Time(s)	N. Cycles
1	Initialization	98	45	1
2	Denaturation	98	15	11
3	Annealing	60	30	
4	Extension	72	30	
5	Final extension	72	60	1
6	Final hold	4	HOLD	-

Variable table		
Panel size	**N. Cycles singleplex**	**N. Cycles multiplex**
>100 Mb	6	5
50–100 Mb	8	7
10–50 Mb	9	8
1–10 Mb	10	9
500–1,000 kb	**12**	**11**
100–500 kb	14	13
50–100 kb	15	14
<50 kb	16	15

2. If the Streptavidin Binding Bead slurry has settled, mix by pipetting.
3. Transfer 22.5 µL of the Streptavidin Binding Bead slurry to a 0.2-ml thin-walled PCR strip-tube(s). Keep on ice until ready to use in the next step.

Note: Number of amplification.

4. Prepare a PCR mixture by adding the following reagents to the tube(s) containing the Streptavidin Binding Bead slurry. Mix by pipetting.

Reagent	Volume (µL)
Streptavidin Binding Bead Slurry	22.5
Amplification Primers,	2.5
Library Amp Mix 2x	25
Total	**50**

5. Pulse-spin the tubes, transfer them to the thermal cycler and start the cycling program.
6. When the thermal cycler program is complete, remove the tube(s) from the block and immediately proceed to the next step.
7. Vortex the pre-equilibrated DNA Purification Beads until well mixed.
8. Add 50 µL (1.0x) homogenized DNA Purification Beads to the tube(s) from Point 6. Mix well by vortexing.

Note: it is not necessary to recover the supernatant or remove Streptavidin Binding Beads from the amplified PCR product

9. Incubate for 5 minutes at room temperature.
10. Place the tube(s) on a magnetic plate for 1 minute or until the supernatant is clear.
11. The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube(s) from the magnetic plate, remove and discard the clear supernatant.
12. Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.
13. Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic plate. Carefully remove all remaining ethanol using a 10 µL pipette, making sure to not disturb the bead pellet.

Note: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate

14. Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.
15. Remove the tube(s) from the magnetic plate and add 32 µL water, 10 mM Tris-HCl pH 8, or Buffer EB to each capture reaction. Mix by pipetting until homogenized.
16. Incubate at room temperature for 2 minutes.
17. Place the plate or tube(s) on a magnetic plate and let stand for 3 minutes or until the beads fully pellet.
18. Transfer 30 µL of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure to not disturb the bead pellet.
19. Validate and quantify each enriched library using an Agilent Bioanalyzer High Sensitivity DNA Kit and a Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay.

Note: When using the Agilent Bioanalyzer High Sensitivity DNA Kit, load 0.5 µL of the final sample. Average fragment length should be 375–425 bp using a range setting of 150–1,000 bp. Final concentration may vary and is dependent on panel size, library input, hybridization reaction size, and the number of PCR cycles

If not proceeding immediately, store the enriched library sample at -20°C for up to 24 hours

Phase 8: Sequencing

Sequence the enriched libraries on an Illumina platform or GeneMind platform. Sequencing protocols and settings depend on the application and instrumentation used.

ANNEX

Pre-capture concentration with concentrator

Use Eppendorf Concentrator plus (or equivalent) to concentrate the pools.

Workflow

1. Set at 45°C for 30 minutes.
2. To the concentrated amplified indexed libraries add the reagents described in the following Table

Reagent	Volume (µL)
Amplified indexed libraries	-
Blocker solution	5
Universal blockers	7
Water	4
Total	**16**

3. After adding the last component, carefully mix the entire resuspension by pipetting, making sure not to generate bubbles. Spin to make sure all of the solutions go to the bottom of the tube.
4. Prepare the hybridization reaction referring to table below

Reagent	Volume (µL)
Hybridization Mix	20
Probes	4
Total	**24**

5. Incubate the hybridization reaction obtained in thermal cycler for 2 minutes, as indicated in table below.

Step	Temperature	Time
1	70°C	HOLD

6. At the end of the 2 minutes incubation transfer the hybridization mix on ice for 5 minutes.

Do not stop the thermal cycler program.

7. At the same time place the resuspended indexed library in the thermal cycler for 5 minutes as indicated in the table at point 5.
8. At the end of the 5 minutes transfer the 2 tubes, one from the ice and one from the thermal cycler, at RT (Room Temperature) for 5 minutes.
9. In the meantime, program the thermal cycler, with lid at 85°C, according to the following protocol:

Step	Temperature	Time
1	70°C	HOLD

10. Start the thermal cycler.
11. After incubation at room temperature, combine the hybridization reaction (24 µL) with the indexed libraries (16 ul) for a total volume of 40 µL.
12. Add 30 µL of Hybridization Enhancer on top of the capture reaction.
13. Spin to remove any bubbles.
14. Transfer the hybridization reaction to the preheated thermal cycler and start the program with the infinite setting.
15. Incubate the reaction for 16 hours.

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6. LEGEND OF THE SYMBOLS USED IN THE MANUAL AND ON THE LABELS

Symbol	Explaination
	Batch number
	Catalog Number
	Expiration date
	Recommended temperature limits
	Consult the instruction for use
	Manufacturer
	Enough content for <n> test
	Do not use if the packaging is not intact and consult the instructions for use

7. MANUFACTURER AND CONTACT FOR ASSISTANCE

Contact your local Ulisse Biomed representative for assistance.

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