GenFisher Hybridization and Wash Kit User Guide

For Illumina Sequencing Platforms

- 24 Reactions, Cat. No. NGS02409
- 96 Reactions, Cat. No. NGS09609
- 384 Reactions, Cat. No. NGS38409

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Content

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Overview

Introduction

This protocol includes the steps of how to capture custom target regions of a Human DNA library, using custom probes panel and reagents provided in the GenScript GenFisher Hybridization & Wash kit. The captured libraries are ready for subsequent DNA sequencing on Illumina Sequencing Platform.

DNA Input Recommendations

The GenFisher Hybridization & Wash kit is verified with libraries prepared from Next Generation Sequencing (NGS) library preparation kits, including GenTrack Library Preparation Kit and GenNature Library Preparation Kit, as well as other DNA library preparation kits, such as KAPA HyperPlus® Kit from Roche, TruSeq or Nextera DNA library preparation kits from Illumina. This kit is optimized for 500 ng of prepared DNA library. Quantifying the starting DNA library is highly recommended.

Input DNA Library Quantification

For optimal results, we recommend to quantify the starting DNA library using a fluorometric based method specific for double-stranded DNA such as the Qubit dsDNA HS Assay system. We recommend to use 1 µL of each DNA sample with 199 µL of the Qubit working solution for sample quantification.

Assessing DNA Library Size Distribution

Run the starting DNA library on an Agilent Technologies 2100 Bioanalyzer using a DNA High Sensitivity Chip. For optimal results, the distribution of DNA fragments with a main size range from about 300 bp to 800 bp is recommended.

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Kit Contents

Before proceeding, please make sure that all of the reagents identified in the kit are ready. The beads, including CB and PB, must be stored at 4°C.

Component	Description	24-Reaction	96-Reaction	Storage
HRD	Human Repeat DNA	132 µL	500 µL	-20°C
TEHB-1	Target Enrichment Hybridization Buffer-1	546 µL	2.19 mL	-20°C
TEHB-2	Target Enrichment Hybridization Buffer-2	145 µL	580 µL	4°C Protect from light
2× BWB	2× Beads Wash Buffer	4.32 mL	17.3 mL	4°C
2× WB I	2× Wash Buffer I	3.75 mL	15 mL	4°C
2× WB II	2× Wash Buffer II	4.5 mL	18 mL	4°C Protect from light
5× WB III	5× Wash Buffer III	830 µL	3.32 mL	4°C
5× WB IV	5× Wash Buffer IV	810 µL	3.23 mL	4°C
DNAP	DNA Polymerase	27 µL	106.5 µL	-20°C
5× PCR Buffer	5× PCR Buffer	270 µL	1.1 mL	-20°C
Primer Mix	Primer Mix	54 µL	215 µL	-20°C
dNTP	dNTP Mix	26.5 µL	105 µL	-20°C
CB	Capture Beads	640 µL	2.5 mL	4°C
PB	Purification Beads	1.25 mL	5 mL	4°C

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Protocol

Introduction

This section describes the GenFisher hybridization and wash protocol.

- Follow the protocol in the order described using the specified parameters for each reagent.
- Before proceeding, confirm the kit contents and the required equipment and consumables.

Pooling Preparation

If you plan to pool libraries for hybridization, add a total of 500 ng pooled libraries.

Tips and Techniques

We recommend to proceed each step immediately to the next step, unless a ***SAFE STOPPING POINT*** is marked.

How to Avoid Cross-Contamination

- When adding or transferring samples or mixture containing samples, new tips must be used after each sample.
- When using a plate for this process, we recommend to always seal the plate with a new Microseal B Seal after an old one is removed.

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Sample or Reagent Transfers

Transfer the accurate volume mentioned in this protocol from each well or tube to the corresponding well or tube.

Handling Beads

- Pipet bead suspension up and down slowly.
- When mixing, mix thoroughly.
- Fully resuspend the beads before use.
- If the beads are aspirated into the pipette tips, dispense them back to the plate or tube(s) on the magnetic stand and wait until the liquid is clear again (~2 min).
- When washing the beads:
  - Use the appropriate magnet for the plate or tube(s).
  - Dispense liquid so that the beads on the side of the wells are wet.
  - Keep the plate or tube(s) on the magnet until instructed to remove it.
  - Do not agitate the plate or tube(s) while on the magnetic stand.

User-Supplied Consumables

- Prepare absolute ethanol and Nuclease-Free Water.

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GenFisher Hybridization and Wash Kit Workflow

The following diagram illustrates the workflow using GenFisher Hybridization and Wash Kit.

⛔ ***SAFE STOPPING POINTS*** are marked as follows. At this point, you can seal the sample plate or tube(s) and store at 4°C overnight or -20°C for up to 1 week.

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Hybridization

This process mixes the DNA library (or libraries) with capture probes library of targeted regions.

Consumables

- TEHB-1
- TEHB-2
- Capture Probes Library

  Note: The Capture Probes Library is not supplied in this kit, we recommend to use GenScript GenFisher Capture Probes Library (Cat. No.: SC2043).
- Universal Blockers

  Note: The Universal Blockers is supplied separately. We recommend to use GenTrack Universal Blockers (Cat. No.: NGS02406i/ NGS09606i/ NGS38406i) for 8 base, dual-index schemes compatible with ligation-based library, such as library prepared by GenScript GenTrack Library Preparation Kit (Cat. No. : NGS01201/ NGS09601/ NGS38401), Illumina TruSeq® library kits, Roche KAPA HyperPlus® kits, et al; or using GenNature Universal Blockers (Cat. No.: NGS02407i/ NGS09607i/ NGS38407i) for 8 base, dual-index schemes compatible with tagment-based library, such as library prepared by GenScript GenNature Library Preparation Kit (Cat. No.: NGS01203/ NGS09603), Illumina Nextera® library kits, et al.
- HRD
- Nuclease-Free Water (User-Supplied)

A. Mix and Dry Down Universal Blockers, HRD and DNA Library

1. Thaw the Universal Blockers and HRD at room temperature and then keep them on ice. Gently vortex and centrifuge briefly before use.
2. In the order listed below, prepare the *DNA Mix* in each well or tube. We recommend using wells in the middle of a plate or an 8-Strip PCR tube. Avoid using wells on edges because evaporation is more likely to occur in the outer wells if it is not sealed properly.

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Component	Volume (μL) per Reaction
DNA Library	Total 500 ng DNA library
HRD	5.0
Universal Blockers	2.0

3. Vortex or pipet the mixture up and down for 15~20 times to mix well.
4. Dry down the *DNA Mix* using a vacuum concentrator (e.g. Eppendorf® Concentrator Plus) set at 45°C, until there is no visible residual liquid.

B. Hybridize Capture Probes Library with the Dried *DNA Mix*

1. Thaw and keep the TEHB-1 on ice. Gently vortex and centrifuge briefly before use.
2. Thaw and keep the TEHB-2 at room temperature away from light, and then gently vortex and centrifuge briefly.
3. Thaw and keep the Capture Probes Library on the ice, and then gently vortex and centrifuge briefly.
4. In the order listed below, prepare the *Hybridization Mix* in a tube. Multiply by the number of samples and add a 10% over fill.

Components	Volume (μL) per Reaction
TEHB-1	10.16
TEHB-2	2.70
Capture Probes Library	x (3 pmol)*
Nuclease-Free Water	4.14 - x
Total	**17.00**

*The optimal input of probes depends on the number of probes and traits of capture regions. It is suggested to try various inputs as preliminary tests, and 3 pmol could be tested for > 6,000 probes at first.

5. Vortex or pipet the mixture up and down for 15~20 times to mix well.
6. Add 17 μL of *Hybridization Mix* to each well or tube containing dried *DNA Mix*.

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7. Gently pipet the entire mixture up and down for 15~20 times to mix thoroughly.
8. **Important!** Securely seal the plate with a Microseal B Seal or tightly close the lid. If possible, surround the samples with well or tubes full of water to avoid evaporation.
9. Incubate at least 5 min at room temperature.
10. After incubation, place the plate or tube(s) on the thermal cycler and start the Hybridization Program. If using tube(s), we recommend placing the tube(s) in the middle of the module of thermal cycler, and if possible, surround the tube(s) with tubes full of water.

Hybridization Program (Lid Set at 100°C; Volume Set at 17 µL)	
Temperature	**Duration**
95°C	30 sec
65°C	16~18 hours or overnight
65°C	Hold

Bead Capture and Wash

This process captures the probes binding fragments of target region.

Consumables

- TEHB-1
- TEHB-2
- 2× BWB
- 2× WB I
- 2× WB II
- 5× WB III
- 5× WB IV
- CB

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- Nuclease-Free Water (User-Supplied)

A. Prepare Required Buffers

1. Thaw the CB at room temperature for at least 30 min before performing the washes. Thoroughly vortex to mix well before use.
2. Thaw and keep the TEHB-1 on ice. Gently vortex and centrifuge briefly before use.
3. Thaw the 2× BWB, 5× WB III and 5× WB IV at room temperature, and then gently vortex.
4. Thaw and keep the TEHB-2 and 2× WB II at room temperature away from light, and then gently vortex.
5. Heat the 2× WB I in a 65°C water bath until clear, and then gently vortex.
6. Dilute the following buffers to prepare the 1× working buffers at room temperature. Multiply by the number of samples and add 10%~20% overfill:

Components	Buffer (µL)	Nuclease-Free Water (µL)	Total Volume (µL) per Reaction
2× BWB*	150	150	300
2× WB I**	125	125	250
2× WB II**	150	150	300
5× WB III***	30	120	150
5× WB IV***	30	120	150

Add *15%, **20% and **10% overfill for each buffer.

7. Prepare the *Bead Resuspension Mix* in a tube, and then keep it on ice. Multiply by the number of samples and add a 10% overfill.

Components	Volume (µL) per Reaction
TEHB-1	10.16
TEHB-2	2.70
Nuclease-Free Water	4.14
Total	**17.00**

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B. Wash CB

Warning! Only wash CB that is equilibrated to room temperature for at least 30 min.

1. Mix the beads thoroughly by vortexing for 15 sec.
2. Add 25 µL of CB to a new PCR plate or tube(s) for every sample being captured, and then add 100 µL of 1× BWB from the buffers prepared at Step A.6.
3. Gently pipet the mixture up and down for 15~20 times.
4. Place the plate or tube containing the beads on a magnet at room temperature for approximately 1 min until the liquid is clear.
5. Carefully remove and discard the clear supernatant. Do not discard the beads.
6. Perform the following wash:
    a. Remove the plate or tube containing the beads from the magnet.
    b. Add 100 µL of 1× BWB to each well or tube containing the beads, and then gently pipet the mixture up and down for 15~20 times to resuspend the beads.
    c. Place the plate or tube on the magnet for approximately 1 min until the liquid is clear.
    d. Carefully remove and discard the clear supernatant. Do not discard the beads.
7. Perform an additional wash by repeating Step B.6 (above).
8. Remove the plate or tube from the magnet, and then add 17 µL of *Bead Resuspension Mix* from the buffers prepared at Step A.7 to each well or tube containing the beads.
9. Pipet up and down to ensure no beads are left to dry in the well or tube(s).

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C. Bead Capture

1. Set the Wash Program in a second thermal cycler.

Wash Program (Lid Set at 70°C; Volume Set at 34 µL)	
Temperature	**Duration**
65°C	Hold

2. After the 16~18 hours incubation, remove the sample plate or tube(s) from the thermal cycler and start the Wash Program.
3. Gently vortex the samples and centrifuge briefly, and then transfer all of the samples to the beads from Step B.9.
4. Gently pipet the mixture up and down for 15~20 times until the beads are fully resuspended and mixed well.
5. Securely seal the plate with a Microseal B Seal or tightly close the lid.
6. Place the sample plate or tube(s) on the thermal cycler running Wash Program for 45 min at 65°C. During incubation, remove the plate or tube(s) every 10~12 min and immediately perform a gentle vortex (2500 rpm for 2 sec) for a total of 4 times of vortexing, without splashing onto the plate the seal or the lid.
7. Place the prepared 1× WB I and 1× WB II from Step A.6 on a heat machine set at 65°C for at least 15 min before performing the next washes.

D. Heated Washes

1. After 45 min of incubation, remove the sample plate or tube(s) from the thermal cycler. Do not stop the Wash Program.
2. Add 100 µL of heated 1× WB I to each well or tube containing the sample and pipet the mixture up and down for 15~20 times. Be careful to minimize bubble formation.

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Note: After this step, remove the 1× WB I from the heat machine to room temperature, but keep the 1× WB II remaining on the heat machine.

3. Place the sample plate or tube(s) on the magnet for at least 1 min until the liquid is clear.
4. Carefully remove and discard the supernatant. Do not discard the beads.
5. Remove the sample plate or tube(s) from the magnet, and then add 150 µL of heated 1× WB II to each well or tube containing the sample.
6. Pipet the mixture up and down for 15~20 times. Be careful to minimize bubble formation.
7. Securely seal the plate with a Microseal B Seal or close the lid of sample tube(s) tightly, and then incubate for 5 min on the thermal cycler running Wash Program.
8. After incubation, place the sample plate or tube(s) on the magnet for at least 1 min until the liquid is clear.
9. Carefully remove and discard the supernatant. Do not discard the beads.
10. Remove the sample plate or tube(s) from the magnet, and then add 150 µL of heated 1× WB II to each well or tube containing the sample.
11. Pipet the mixture up and down for 15~20 times. Be careful to minimize bubble formation.
12. Securely seal the plate with a Microseal B Seal or close the lid of sample tube(s) tightly, and then incubate for 5 min on the thermal cycler running Wash Program.
13. After incubation, place the sample plate or tube(s) on the magnet for at least 1 min until the liquid is clear.
14. Remove and discard the supernatant. Do not discard the beads.

    Note: At this time, you can stop the Wash Program, and the following wash steps should be performed at room temperature.

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E. Room Temperature Washes

1. Remove the sample plate or tube(s) from the magnet, and add 150 µL of 1× WBI that is already equilibrated to room temperature from Step D.2 to each well or tube containing the sample.
2. Securely seal the plate with a Microseal B Seal or close the lid of sample tubes tightly, and then vortex at full-speed thoroughly, until the beads are fully resuspended.
3. Incubate at room temperature and vortex every 30 s to ensure the mixture remains homogenous for a total of 4 times of vortexing.
4. After incubation, briefly centrifuge the sample plate or tube(s) for 2 sec at 1000 rpm to avoid contamination.
5. Place the sample plate or tube(s) on the magnet for at least 2 min until the liquid is clear.
6. Carefully remove and discard the supernatant. Do not discard the beads.
7. Remove the sample plate or tube(s) from the magnet, and then add 150 µL of 1× WB III from Step A.6 to each well or tube containing the sample.
8. Securely seal the plate with a Microseal B Seal or tightly close the lid of sample tube(s), and then vortex at full-speed thoroughly until the beads are fully resuspended.
9. Incubate at room temperature and vortex every 30 s to ensure the mixture remains homogenous for a total of 4 times of vortexing.
10. After incubation, briefly centrifuge the sample plate or tube(s) for 2 sec at 1000 rpm to avoid contamination.
11. Place the sample plate or tube(s) on the magnet for at least 3 min until the liquid is clear.
12. Carefully remove and discard the supernatant. Do not discard the beads.

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13. Remove the sample plate or tube(s) from the magnet, and then add 150 µL of 1× WB IV from Step A.6.
14. Securely seal the plate with a Microseal B Seal or tightly close the lid of sample tube(s), and then vortex at full-speed thoroughly until the beads are fully resuspended.
15. Incubate at room temperature and vortex every 30 s to ensure that the mixture remains homogenous for a total of 4 times of vortexing.
16. After incubation, briefly centrifuge the sample plate or tube(s) for 2 sec at 1000 rpm to avoid contamination.
17. Place the sample plate or tube(s) on the magnet for at least 3 min until the liquid is clear.

Post-Capture PCR and Clean-Up

This process amplifies the capture target enrichment libraries and then purifies them for sequencing.

Consumables

- 5× PCR Buffer
- Primer Mix
- DNAP
- dNTP
- PB
- Fresh 80% (v/v) ethanol and Nuclease-Free Water (User-Supplied)

A. Post-Capture PCR

1. Thaw the 5× PCR Buffer, dNTP and Primer Mix at room temperature and then keep them on ice. Gently vortex and centrifuge briefly before use.

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2. In a tube, prepare the *PCR Reaction Mix* as follows on ice, and multiplied by the number of samples on the tubes and add 10% overfill.

Components	Volume (μL) per Reaction
5× PCR Buffer	10.0
Primer Mix	2.0
DNAP	1.0
dNTP	1.0
Nuclease-Free Water	36.0
Total	**50.0**

3. Carefully remove and discard the supernatant from the beads until there is no visible residual 1×WB IV. Do not discard the beads.

4. Remove the plate or tube(s) from the magnet and add 50 μL of the *PCR Reaction Mix* to each well or tube(s) containing beads.

5. Gently pipet the mixture up and down for 15~20 times until the beads are fully resuspended.

6. Securely seal the plate with a Microseal B Seal or close the lid of the sample tube(s) tightly.

7. Place the plate or tube(s) on a thermal cycler, and run the following Post-PCR Program:

Post-PCR Program (Lid Set at 105°C; volume set at 50 μL)		
Temperature	**Duration**	**Cycles**
98°C	3 min	1
98°C	20 sec	
62°C	30 sec	Variable*
72°C	30 sec	
72°C	3 min	1
4°C	Hold	1

*The number of PCR cycles should be optimized according to the probes panel size, to ensure that there is enough yield for sequencing and the minimum number of cycles. Recommended number of cycles when using GenScript GenFisher Capture Probes Library is listed below:

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Probes Panel Size	Cycles
>100,000 probes	10 cycles
10,000~100,000 probes	12 cycles
500~10,000 probes	13 cycles
1~500 probes	14 cycles

B. Clean-Up

Warning! Only perform the clean-up with the PB equilibrated to room temperature.

1. Thaw the PB at room temperature for at least 30 min before performing the clean-up, and prepare 450 µL of fresh 80% (v/v) ethanol per sample.
2. Make sure to mix the PB thoroughly by vortexing for 15 sec.
3. After the Post-PCR Program ends, remove the samples, and then add 50 µL of PB into each sample.
4. Thoroughly pipet the mixture up and down for 15~20 times.
5. Incubate at room temperature for 5~10 min.
6. Place the tubes on the magnet for at least 2 min until the liquid is clear.
7. Carefully remove and discard the supernatant. Do not discard the beads.
8. While keeping the plate or tube(s) on the magnet, add 200 µL of fresh 80% (v/v) ethanol. Incubate for 1 min, and then remove the ethanol.
9. Repeat another ethanol wash by performing Step B.8 (above).
10. While keeping the plate or tube(s) on the magnet, allow the beads to air dry for 1~3 min. Do not over dry the beads.
11. Remove the sample plate or tube(s) from the magnet and elute in 22 µL of Nuclease-Free Water or equivalent buffer.
12. Thoroughly pipet the mixture up and down for 15~20 times.
13. Incubate for 5 min at room temperature.

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14. Place the plate or tube(s) on a magnet for at least 2 min until the liquid is clear.
15. Transfer 20 µL of elution (captured library) to a new plate or tube(s). Make sure that no beads are carried over.
16. Store the captured library at -20°C for the subsequent quantification and sequencing.

Check Libraries

This is a quality control step, which will quantify the captured library you prepared.

Quantify

To achieve data with the highest quality, quantification of DNA library is needed to load an appropriate input on Illumina sequencing platforms and create optimum cluster densities. Preliminary quantification of your prepared DNA library will be performed using a fluorometric based method specific for double stranded DNA such as the Qubit dsDNA HS Assay System. We recommend using 1 µL of each DNA sample with 199 µL of the Qubit working solution for sample quantification. For a more accurate quantification, a real-time qPCR quantification is needed to be performed.

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