



GenoBaits

Library Preparation & Hybridization Protocol

Molbreeding Biotechnology LLC

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1. Overview

1.1 Introduction

This manual delineates the protocol for constructing Genotyping-by-Targeted Sequencing (GBTS) libraries from genomic DNA samples. The GenoBaits workflow consists of three main parts:

Part 1: Library Preparation

- Fragmented genomic DNA is ligated with Illumina adapters at both ends.
- PCR amplification adds **platform-specific adapters and indexes**.
- The constructed DNA library (fragment size: 250–500 bp) is ready for direct DNA hybridization.

Part 2: Target Capture & Enrichment

- Prepared libraries are pooled and concentrated.
- **Biotinylated probes** hybridize with the DNA libraries.
- Target fragments are captured using **streptavidin magnetic beads**, while uncaptured fragments are washed away.
- A final PCR amplification generates the **sequencing-ready library**.

Part 3: QC of the Libraries & Perform Sequencing

- Qubit detection reagents or dsDNA fluorescence quantitation reagent is recommended for accurate quantification of the captured libraries.
- Gel electrophoresis is recommended to check the size distribution of the captured libraries. Alternatively, Agilent Technology 2100 Bioanalyzer or a similar instrument can be used for evaluation.
- Perform sequencing according to the instructions for NGS sequencer.
Total sequencing data= Total number of samples * Data amount per sample (depending on panel density & genome size).

1.2 Kit components

Component	GB000104 96 rxns	GB000125 960 rxns	GB000126 9600 rxns	Storage
Part 1 GenoBaits DNA Library Prep Kit for Illumina				
GenoBaits End Repair Enzyme	250 µL	2.5 mL	25 mL	-15~-25°C
GenoBaits End Repair Buffer	384 µL	3.84 mL	38.4 mL	-15~-25°C
GenoBaits Ultra DNA Ligase	192 µL	1.92 mL	19.2 mL	-15~-25°C
GenoBaits Ultra DNA ligase Buffer	768 µL	7.68 mL	76.8 mL	-15~-25°C
GenoBaits Adapter for ILM	384 µL	3.84 mL	38.4 mL	-15~-25°C
GenoBaits PCR Master Mix	1920 µL	19.2 mL	192 mL	-15~-25°C
GenoPrep DNA Clean Beads	11040 µL	110.4 mL	1104 mL	2~8°C

Component	GB000600 10 rxns	GB000605 100 rxns	Storage
Part 2 GenoBaits DNA Hybridization and Wash Kit for Illumina			
GenoBaits Block II for ILM	40 µL	400 µL	-15~-25°C
GenoBaits 2× Hyb Buffer	160 µL	1.6 mL	-15~-25°C
GenoBaits Hyb Buffer Enhancer	40 µL	400 µL	-15~-25°C
GenoBaits Primer Mix for ILM	12 µL	120 µL	-15~-25°C
GenoBaits PCR Master Mix	150 µL	1.5 mL	-15~-25°C
GenoBaits 2× Beads Wash Buffer	2.25 mL	22.5 mL	2~8°C
GenoBaits 10× Wash Buffer I	250 µL	2.5 mL	2~8°C
GenoBaits 10× Wash Buffer II	150 µL	1.5 mL	2~8°C
GenoBaits 10× Wash Buffer III	150 µL	1.5 mL	2~8°C
GenoBaits 10× Stringent Wash Buffer	300 µL	3 mL	2~8°C
GenoBaits DNA Probe Beads	500 µL	5 mL	2~8°C
GenoPrep DNA Clean Beads	450 µL	4.5 mL	2~8°C

1.3 Genomic DNA input requirements

- The minimum input DNA amount is 20 ng , we recommend to use 100 - 300 ng per sample for library preparation.
- High-purity DNA (OD260/OD280 ratio of 1.8 - 2.0) with intact integrity (no visible degradation) is recommended for optimal library preparation.

1.4 Required reagents

The following kits and materials are essential for successful execution of this protocol. All items listed with catalog numbers are available through Molbreeding.

Item	Catalog Number	Description
Part 1 Library Preparation		
GenoBaits DNA Library Prep Kit for Illumina	GB000104 (96 rxns) GB000125 (960 rxns) GB000126 (9600 rxns)	For Library Preparation
GenoBaits CDI Barcode Mix for Illumina	GB001304 P071-P130	Indexing plate A total of 5760 combinations
Part 2 Target Capture & Enrichment		
GenoBaits DNA Hybridization and Wash Kit for Illumina	GB000600 (10 rxns) GB000605 (100 rxns)	For DNA hybridization
GenoBaits probe Panel	Predesigned or Custom Design	Predesigned probe Panel Custom probe Panel
Other Reagents		
GenoPrep dsDNA Quantitation Kit	DN000109	For library quantification
Nuclease-free Water	Various supplier	For volume adjustment
10 mM Tris-Cl (pH 8.0)	Various supplier	For library elution
Fresh 80% ethanol	Various supplier	Washes during library prep

1.5 Required equipment & consumables

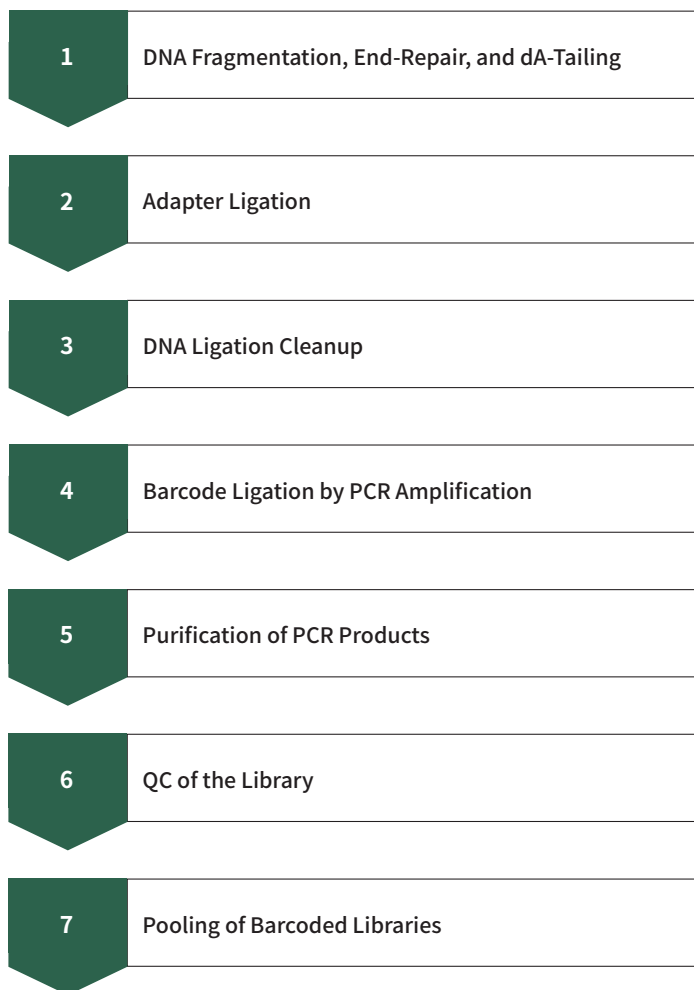
■ Essential Equipment and Accessories

- Pipettes & tips (0.1 - 100 µL range): Single- or multi-channel (for 96-well workflows).
- Thermal cycler (heated lid, compatible with 0.2 mL tubes).
- Centrifuge (adapters for 0.2 mL tubes/strips/96-well plates and 1.5 - 2.0 mL tubes).
- Magnetic rack (e.g., DynaMag™-96 Side Magnet, Thermo Fisher #12331D).
- Fluorometer (e.g., Qubit®, QIAxpert®) for DNA/library quantification.
- 65°C water bath or oven (to dissolve wash buffer precipitates).
- Vortex mixer (for reagent mixing).
- Vacuum concentrator (e.g., Savant™ SpeedVac™ #SPD1030, for library drying).

■ Essential Consumables

- 1.5 - 2 mL Low-binding microcentrifuge tubes
- 0.2 mL Low-binding microcentrifuge tubes/strips/96-well PCR plates (with compatible sealing caps).

2. Workflow



Part 1. Library Preparation Workflow

1 Combine pooled libraries with probe panel and Concentration

2 Hybridization

3 Preparation of Wash Buffers

4 Preparation of GenoBaits DNA Probe Beads

5 Perform Bead Capture

6 Perform Washes

7 Perform Post-capture PCR

8 Purify Post-capture PCR fragments

9 QC of the Captured Library and Perform Sequencing

2.1 DNA Library Preparation Workflow

Step 1: DNA Fragmentation, End-Repair, and dA-Tailing

- Prepare Reaction Mix (**Total Volume: 20 μ L**):
4 μ L GenoBaits End Repair Buffer
2.6 μ L GenoBaits End Repair Enzyme (with Fragmentation Enzyme)
13.4 μ L Genomic DNA (200 ng) + Nuclease-free Water
- Run the following thermal program:

Step	Temperature	Incubation time
1	37°C	20 min
2	72°C	20 min
3	4°C	Hold

Step 2: Adapter Ligation

- Prepare Reaction Mix (**Total Volume: 20 μ L**):
2 μ L GenoBaits Ultra DNA Ligase
8 μ L GenoBaits Ultra DNA Ligase Buffer
4 μ L GenoBaits Adapter for ILM
6 μ L Nuclease-free Water
- Add the adapter reaction mix to the product from Step 1 (**Total Volume: 40 μ L**).
- Run the following thermal program:

Step	Temperature	Incubation time
1	22°C	60 min
2	4°C	Hold

Step 3: DNA Ligation Cleanup

- Add 48 μ L (1.2 $\times$) GenoPrep DNA Clean Beads to each well, mix thoroughly (**Total Volume: 88 μ L**).
- Incubate 5 min (RT), spin briefly.
- Separate beads on magnet ≥ 3 min until clear, discard supernatant.
- Wash beads on magnet with 100 μ L 80% ethanol (30 sec), then fully remove ethanol (DO NOT disturb beads).
- Air-dry beads ≥ 5 min (until cracked surface). Then proceed to Step 4.



Note: Avoid residual ethanol or over-drying to prevent low yield.

Step 4: Barcode Ligation by PCR Amplification

- Prepare reaction mix (**Total Volume: 40 μ L**):
20 μ L GenoBaits PCR Master Mix
10 μ L GenoBaits CDI Barcode
10 μ L Nuclease-free Water
- Add reaction mixture to the air-dried beads from Step 3, run the following thermal program:

Step	Temperature	Incubation Time	Number of cycles
1	98°C	2 min	1
2	98°C	30 sec	7
	65°C	30 sec	
	72°C	40 sec	
3	72°C	4 min	1
4	4°C	∞	Hold

- Safe Stop:** When the program reaches 4 °C, store the PCR product at -20 °C overnight or proceed to Step 5.

Step 5: Purification of PCR Products

- Add 40 μ L (1.0 $\times$) GenoPrep DNA Clean Beads to each well, mix thoroughly (**Total Volume: 80 μ L**).
- Incubate 5 min (RT), spin briefly.
- Separate beads on magnet ≥ 3 min until clear, discard supernatant.
- Wash beads on magnet with 100 μ L 80% ethanol (30 sec), then fully remove ethanol (DO NOT disturb beads).
- Air-dry beads ≥ 5 min (until cracked surface).

Note: Avoid residual ethanol or over-drying to prevent low yield.

- Elute the beads using 45 μ L of 10 mM Tris-HCl (pH 8.0).

- Safe Stop:** The libraries can be stored at -20°C for no more than one month or processed for hybrid capture.

Step 6: QC of the Library

- Quantify:
Qubit reagents or equivalent dsDNA fluorescence kit (e.g., GenoPrep DN000109).
The concentration of the DNA library should be higher than 15 ng/ μ L.
- Size check:
Gel electrophoresis (visual) or Agilent 2100 Bioanalyzer (or equivalent).
The optimal size of the DNA library should be between 250bp - 500 bp.

Step 7: Pooling of Barcoded Libraries

- Pool the 48 libraries with different barcodes into a 1.5 mL centrifuge tube, with an input of 400 ng per DNA library.
- Proceed to 2.2 DNA Hybridization Workflow.

2.2 DNA Hybridization Workflow

Step 1: Combine pooled libraries with probe panel and Concentration

- Prepare Probe Panel Mix:
 - x μ L GenoBaits Panel (Refer to reagent label for dosage details)
 - x μ L GenoBaits Block I (Refer to reagent label for dosage details)
 - 4 μ L GenoBaits Block II
- Add the probe panel mix to the pooled libraries of Step 7 in Part 1.
- Dry the mixture using a vacuum concentrator at $\leq 30^{\circ}\text{C}$.

- II Safe Stop:** The dry mixture could be stored at 4°C overnight or centrifuged at 12,000 rpm for 1 minute before proceeding to Step 2.

Step 2: Hybridization

- Prepare Hybridization Reaction Mix (**Total Volume: 32 μ L**):
 - 16 μ L GenoBaits 2 $\times$ Hyb Buffer
 - 4 μ L GenoBaits Hyb Buffer Enhancer
 - 12 μ L Nuclease-free Water
- Add the prepared 32 μ L Hybridization reaction mix to the product from Step 1.
- Mix the mixture thoroughly, centrifuge at 12,000 rpm for 1 minute, and incubate for 5 minutes.
- Repeat mixing the mixture, centrifuge at 12,000 rpm for 5 seconds.
- Transfer the entire mixture into a 0.2 mL 8-well PCR tube strip.
- Run the following thermal programs:

Step	Temperature	Incubation Time	Heated lid
Denaturation	95°C	10 min	105°C
Hybridization	65°C	2 h	75°C
Hold	65°C	∞	75°C



Note: Perform Steps 3 and 4 before the PCR program finishes, then immediately proceed to Step 5 upon completion of these steps.

Step 3: Preparation of Wash Buffers

- Prepare Working Buffer solutions (The prepared reagent will be used in Step 6):

225 μ L GenoBaits 2 $\times$ Beads Wash Buffer + 225 μ L Nuclease-free Water	Total Volume: 450 μ L
25 μ L GenoBaits 10 $\times$ Wash Buffer I + 225 μ L Nuclease-free Water	Total Volume: 250 μ L
15 μ L GenoBaits 10 $\times$ Wash Buffer II + 135 μ L Nuclease-free Water	Total Volume: 150 μ L
15 μ L GenoBaits 10 $\times$ Wash Buffer III + 135 μ L Nuclease-free Water	Total Volume: 150 μ L
30 μ L GenoBaits 10 $\times$ Stringent Wash Buffer + 270 μ L Nuclease-free Water	Total Volume: 300 μ L



Note: 1 $\times$ working buffer can be stored at 2 ~ 8 $^{\circ}$ C for 4 weeks.

Step 4: Preparation of GenoBaits DNA Probe Beads

- Wash the GenoBaits DNA Probe Beads **three times** on the magnetic rack, using GenoBaits 1 $\times$ Beads Wash Buffer (The prepared beads will be used in Step 5).

Step 5: Perform Bead Capture

- Add 32 μ L hybridization solution to prepared GenoBaits DNA Probe Beads from Step 4. Incubate at 65 $^{\circ}$ C for 45 min. Immediately proceed to Step 6 when the program finishes.

Step 6: Perform Washes

- **Heated washes (65 $^{\circ}$ C):**
1 $\times$ Wash Buffer I (100 μ L, preheated at 65 $^{\circ}$ C) $\rightarrow$ 1 wash.
1 $\times$ Stringent Wash Buffer (150 μ L, preheated at 65 $^{\circ}$ C) $\rightarrow$ 2 washes.
- **Room temperature washes:**
1 $\times$ Wash Buffer I (150 μ L, at RT) $\rightarrow$ 1 wash.
1 $\times$ Wash Buffer II (150 μ L, at RT) $\rightarrow$ 1 wash.
1 $\times$ Wash Buffer III (150 μ L, at RT) $\rightarrow$ 1 wash.
- **Elution:**
Add 20 μ L nuclease-free water to elute.
Split 20 μ L eluent: 10 μ L for Step 7, 10 μ L as backup.

Step 7: Perform Post-capture PCR

- Prepare Reaction Mix (Total Volume of 20 μ L per Sample):
15 μ L GenoBaits PCR Master Mix
1.2 μ L GenoBaits Primer Mix
3.8 μ L Nuclease-free Water
- Add the prepared 20 μ L reaction mixture to 10 μ L of eluent DNA (from Step 6).
- Run the following thermal programs:

Step	Temperature	Incubation Time	Number of cycles
1	98°C	45 sec	1
2	98°C	15 sec	See the GenoBaits Panel product instructions
	60°C	30 sec	
	72°C	30 sec	
3	72°C	1 min	1
4	4°C	∞	Hold

- II Safe Stop:** PCR product can be stored at -20 °C overnight or proceed to step 8.

Step 8: Purify Post-capture PCR fragments

- Add 45 μ L GenoPrep DNA Clean Beads to purify the product from Step 7.
- Transfer supernatant to a new 0.2 mL low-bind tube (avoid beads). The captured libraries can be stored at -20 °C for up to 1 week before sequencing.

Step 9: QC of the Captured Library and Perform Sequencing

- **Quantify:**
Qubit reagents or equivalent dsDNA fluorescence kit (e.g., GenoPrep DN000109).
The optimal concentration of the captured library should be 10 - 20 ng/ μ L. We do not recommend to proceed with sequencing if the concentration is below 5 ng/ μ L.
- **Size check:**
Gel electrophoresis (visual) or Agilent 2100 Bioanalyzer (or equivalent).
The optimal size of the captured library should be between 250bp - 500 bp.
- **Perform sequencing**
Perform sequencing according to the instructions for NGS sequencer.
Volume of captured library mixture = Total sequencing data $\div$ Concentration of captured library. For example, a 48-sample captured library with 48 Gb total sequencing data and 15 ng/ μ L concentration: mixing volume = 48 Gb $\div$ 15 ng/ μ L = 3.2 μ L.

3. DNA Library Preparation Protocol

ⓘ Notes before starting

- Equilibrate GenoPrep DNA Clean Beads at room temperature (20 - 25°C) for 30 min prior to use. Other reagents should be thawed at 4°C.
- Prepare 200 µL of 80% ethanol solution and 45 µL of 10 mM Tris-HCl (pH 8.0) per sample.

Step 1: DNA Fragmentation, End-Repair, and dA-Tailing

1. **Prepare Reaction Mix:** Refer to Table 1 for guidance.

Table 1. Fragmentation Enzyme Reaction Mix

Component	Volume/Per Reaction (µL)
GenoBaits End Repair Buffer	4
GenoBaits End Repair Enzyme	2.6
Total volume	6.6

2. **Dispensing Reaction Mix:** Add 6.6 µL of the prepared reaction mix to each well of a 96-well PCR plate.
3. **Adding Input DNA and Water:** Add 13.4 µL of Input DNA (20-300 ng) along with nuclease-free water to reach a **total volume of 20 µL per well**.
4. **Sealing and Mixing:** Seal the plate with a clear adhesive film, mix thoroughly and briefly centrifuge to collect the contents at the bottom of the wells.
5. **Thermal cycling:** Place the 96-well plate in the thermal cycler, and run the following program with the heated lid set to 82 °C.

Table 2. Fragmentation Enzyme Program

Step	Temperature	Incubation time
1	37°C	20 min*
2	72°C	20 min**
3	4°C	Hold

* 20 min at 37 °C generates library sizes that are approximately 250 bp to 500 bp in length.

** 20 min at 72 °C step is necessary to stop the reaction from fragmenting.

- After the program reaches 4°C, mix thoroughly and briefly centrifuge, immediately proceed to Step 2.

Step 2: Adapter Ligation

- Prepare Reaction Mix:** Refer to Table 3 for guidance.

Table 3. Adapter Ligation Reaction Mix

Component	Volume/Per Reaction (μL)
GenoBaits Ultra DNA Ligase	2
GenoBaits Ultra DNA Ligase Buffer	8
GenoBaits Adapter for ILM	4
Nuclease-free water	6
Total volume	20

- Dispensing Reaction Mix:** Add 20 μL of adapter ligation reaction mix to the product from Step 1 to reach a **total volume of 40 μL per well**.
- Sealing and Mixing:** Seal the plate with a clear adhesive film, mix thoroughly and briefly centrifuge to collect the contents at the bottom of the wells.
- Thermal cycling:** Place the 96-well plate in the thermal cycler, and run the following program with the heated lid OFF.

Table 4. Adapter Ligation Program

Step	Temperature	Incubation time
1	22°C	60 min
2	4°C	Hold

- After the program reaches 4°C, mix thoroughly and briefly centrifuge, immediately proceed to Step 3.

Step 3: DNA Ligation Cleanup

1. Add 48 μL (1.2 $\times$) GenoPrep DNA Clean Beads to each well of the 96-well plate from Step 2, mix thoroughly and incubate at room temperature for 5 minutes. Briefly centrifuge to collect the contents.
2. Place the 96-well plate on the magnetic rack and allow it to sit until the liquid turns completely clear, which should take at least 3 minutes.
3. While keeping the 96-well PCR plate on the magnetic rack, carefully discard the supernatant, ensuring that the beads remain undisturbed.
4. With the plate still on the magnetic rack, add 100 μL of 80% ethanol to each well and incubate for 30 seconds at room temperature. Discard the supernatant, ensuring no residual ethanol remains.
5. Keep the 96-well plate on the magnetic rack, and allow the beads to air dry at room temperature for at least for 5 minutes until they appear dry on the surface.



Note: Ensure no residual ethanol is present, and the beads have just cracked on the surface. The residual ethanol, and over-drying beads may result in low library yield.

6. Remove the 96-well plate from the magnet rack, immediately add Step 4 reaction mix (Contains magnetic beads to amplify).

Step 4: Barcode Ligation by PCR Amplification

1. **Prepare Reaction Mix:** Refer to Table 5 for guidance.

Table 5. PCR Amplification Reaction Mix

Component	Volume/Per Reaction (μL)
GenoBaits PCR Master Mix	20
GenoBaits CDI Barcode Mix for Illumina	10
Nuclease-free water	10
Total volume	40



Note: We recommend you choose GenoBaits Barcode. Please select different Barcode for different samples. Only the samples with different Barcodes can be mixed together for hybrid capture.

2. **Dispensing Reaction Mix:** Add 40 μL of PCR amplification reaction mix to the air-dried beads from Step 3 in each well.

3. **Sealing and Mixing:** Seal the plate with a clear adhesive film, mix thoroughly and briefly centrifuge to collect the contents at the bottom of the wells.
4. **Thermal cycling:** Place the 96-well plate in the thermal cycler, and run the following program with the heated lid set to 105 °C.

Table 6. Barcode ligation program

Step	Temperature	Incubation Time	Number of cycles
1	98°C	2 min	1
2	98°C	30 sec	Based on DNA input (see Table7)
	65°C	30 sec	
	72°C	40 sec	
3	72°C	4 min	1
4	4°C	∞	Hold

Table 7. Corresponding relationship between input and cycle

DNA Input	Cycle
20~100 ng	9
101~150 ng	8
151~200 ng	7
201~250 ng	6
251~300 ng	5



Safe Stop: When the program reaches 4 °C, store the PCR product at -20 °C overnight or proceed to Step 5.

Step 5: Purification of PCR Products

1. Add 40 μL (1.0 $\times$) GenoPrep DNA Clean Beads to each well of the 96-well PCR plate from Step 4, mix thoroughly and incubate at room temperature for 5 minutes. Briefly centrifuge to collect the contents.
2. Place the plate on the magnetic rack and allow it to sit until the liquid turns completely clear, which should take at least 3 minutes.
3. While keeping the 96-well PCR plate on the magnetic rack, carefully discard the supernatant, ensuring that the beads remain undisturbed.
4. With the plate still on the magnetic rack, add 100 μL of 80% ethanol to each well and incubate for 30 seconds at room temperature. Discard the supernatant, ensuring no residual ethanol remains.
5. Keep the plate on the magnetic rack, and allow the beads to air dry at room temperature for at least for 5 minutes until they appear dry on the surface.



Note: Ensure no residual ethanol is present, and the beads have just cracked on the surface. The residual ethanol, and over-drying beads may result in low library yield.

6. Remove the plate from the magnetic rack, add 45 μL 10 mM Tris-HCl to each well. mix thoroughly and incubate at room temperature for 5 minutes to elute DNA from the beads. Briefly spin down to collect the contents.
7. Place the 96-well PCR plate on the magnetic rack and wait until the liquid turns completely clear, which should take at least 3 minutes.
8. Transfer the elute DNA into a new 96-well PCR plate, take care not to disturb the beads.
9. Purified libraries can be stored at $-20\text{ }^{\circ}\text{C}$ for hybrid capture.



Note: The libraries stored at -20°C for no more than one month.

Step 6: QC of the Library

1. **Quantify:** Use Qubit reagents or equivalent dsDNA fluorescence kit (e.g. GenoPrep dsDNA Quantitation Kit, Catlog DN000109) for concentration determination. The concentration of the DNA library should be higher than 15 ng/μL.
2. **Size check:** Use Gel electrophoresis or Agilent 2100 Bioanalyzer to validate the size range of each library. The optimal size of the DNA library should be between 250bp - 500 bp.

Step 7: Pooling of Barcoded Libraries

All libraries are mixed in equal mass. The pooling volume of a library (μL) = $400 \text{ ng} \div \text{concentration of the library (ng/μL)}$. Combine all pooling volumes in a new 1.5 mL centrifuge tube. We recommend a maximum of 48 libraries be accommodated.

4. DNA Hybridization Protocol

⚠ Notes before starting

- Three thermal cyclers, set at different incubation temperatures, are used for hybrid capture in this protocol.
 - Program the first thermocycler according to Table 9 with the heated lid set at 105°C.

Table 9. Denaturation program

Step	Temperature	Incubation Time	Number of cycles
Denaturation	95°C	10 min	1
Hold	65°C	∞	1

- Program the second thermocycler according to Table 10 with the heated lid set at 75°C.

Table 10. Hybridization program

Step	Temperature	Incubation Time	Number of cycles
Hybridization	65°C	2 h	1
Hold	65°C	∞	1

- Program the three thermocycler according to Table 11 with the heated lid set at 75°C.

Table 11. Wash program

Step	Temperature	Incubation Time	Number of cycles
Wash	65°C	∞	1

- For GenoBaits 10× Wash Buffer I and GenoBaits 10× Stringent Wash Buffer, dissolve in an incubator at 65°C if white precipitate is present prior to use.
- Equilibrate GenoPrep DNA Clean Beads and GenoBaits DNA Probe Beads at room temperature for 30 min prior to use.
- Prepare 100 µL of 80% ethanol solution and 35 µL of 10 mM Tris-HCl (pH 8.0) per sample.
- It has been verified that optimal capture efficiency is achieved with hybridization at 65°C for 2 hours. Avoid hybridization reactions shorter than 2 hours.

Step 1: Combine pooled libraries with probe panel and concentration

1. **Prepare Probe Panel Mix:** Refer to Table 12 for guidance.

Table 12. Panel and Block Mixture

Component	Volume/Per Reaction (μL)
GenoBaits Block I	Refer to reagent label for dosage details
GenoBaits Block II for ILM	2
GenoBaits Panel	Refer to reagent label for dosage details

2. **Dispensing Mixture:** Transfer the probe panel mix to the pooled libraries of Step 7 in Part 1.
3. **Concentrate and dry:** Seal the tube with a sealing film, dry down the mixture in vacuum concentrator ($\leq 30^{\circ}\text{C}$).



Safe Stop: The dried products could be stored at 4°C overnight.

4. Centrifuged at 12,000 rpm for 1 minute before proceeding to Step 2.

Step 2: Hybridization

1. **Prepare Hybridization Reaction Mix:** Refer to Table 13 for guidance.

Table 13. Hybridization Reaction Mix

Component	Volume/Per Reaction (μL)
GenoBaits 2X Hyb Buffer	16
GenoBaits Hyb Buffer Enhancer	4
Nuclease-Free Water	12
Total Volume	32

2. **Dispensing Mixture:** Add 32 μL of the hybridization reaction mix to 1.5 mL centrifuge tube from Step 2.
3. Pipette up and down or mix the mixture thoroughly, centrifuge at 12,000 rpm for 1 minute, and incubate at room temperature for 5 minutes.
3. Repeat mixing of the mixture, then centrifuge at 12,000 rpm for 5 seconds.
4. Transfer the entire 32 μL of the mixture into 0.2 mL 8-well PCR tube strip.

- Place the PCR tube strip on the first thermal cycler and start the Denaturation program (see Table 9).
- After the Denaturation program reaches 65°C, immediately transfer the PCR tube strip to the second thermal cycler and start the Hybridization program (see Table 10). Ensure incubation for at least 2 hours.

Step 3: Preparation of wash buffer

- Dilute the following wash buffer to create 1× working solutions according to Table 14. Multiply volumes by the required number of samples and add 10% overfill.

Table 14. Wash Buffer Dilutions

Component	Nuclease-free Water (μL)	Buffer (μL)	Total (μL)
GenoBaits 2× Beads Wash Buffer	225	225	450
GenoBaits 10× Wash Buffer I	225	25	250
GenoBaits 10× Wash Buffer II	135	15	150
GenoBaits 10× Wash Buffer III	135	15	150
GenoBaits 10× Stringent Wash Buffer	270	30	300

- For 8 hybridization reactions, as an example, aliquot and label the plate as follows:
 - Row A: 110 μL of GenoBaits 1× Wash Buffer I
 - Row C: 160 μL of GenoBaits 1× Stringent Wash Buffer
 - Row E: 160 μL of GenoBaits 1× Stringent Wash Buffer

		1	2	3	4	5	6	7	8	9	10	11	12
65°C 1× Wash Buffer I	A												
	B												
65°C 1× Stringent Wash Buffer	C												
	D												
65°C 1× Stringent Wash Buffer	E												
	F												
	G												
	H												

- Seal the buffer plate and set aside.



Note: Do not discard the remaining Wash Buffer I. The remaining buffer is needed to perform the Room temperature washes later in the protocol.

Step 4: Preparation of GenoBaits DNA Probe Beads



Note: Only perform bead washes with beads that have equilibrated to room temperature.

1. Mix the GenoBaits DNA Probe Beads thoroughly by vortexing for 15 seconds.
2. Add 50 μ L of streptavidin beads to a new PCR tube strip, filling a well for every sample to be captured.
3. Place the PCR tube strip on the magnet rack and wait for the liquid to turn clear completely.
4. Discard the supernatant, make sure not to disturb the beads. Take the plate off the magnet rack.
5. Add 150 μ L of GenoBaits 1 $\times$ Beads Wash Buffer to each well of the PCR tube strip, mix thoroughly and briefly spin down.
6. Place the PCR tube strip on the magnet rack and wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
7. Perform an additional wash by repeating the above steps for a total of 3 washes.

Step 5: Perform bead capture

1. Start the wash program (Table 11) in the three thermal cycler to start warming the buffer plate prepared in step 3. Make sure the heated lid temperature is set to 75°C.



Note: The buffer plate needs to warm up for at least 15 minutes. We recommend starting incubation at the same time as the bead capture.

2. After the 2 hour incubation is complete, use a multichannel pipette to transfer the fully hybridization mixture into the prepared GenoBaits DNA Probe Beads in Step 4. Note the correspondence between wells.
3. Vortex for 10 seconds to mixed thoroughly and briefly spin down.
4. Place the PCR tube strip in the 65°C thermal cycler for 45 minutes. During incubation, remove the tube every 10 - 12 minutes to quickly and gently vortex.

Step 6: Perform washes



Note: When performing the heated washes, always keep the buffer plate on the thermal cycler during washes.

Heated washes

1. After 45 minutes, remove the PCR tube strip from the thermal cycler.
2. While the buffer plate is inside the thermal cycler, transfer 100 μ L of heated Wash Buffer I to each well of the PCR tube strip. Pipet the mixture 10 times, being careful to minimize bubble formation.
3. Place the PCR tube strip on the magnet rack for 1 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
4. Take the PCR tube strip off the magnet rack. Add 150 μ L of heated Stringent Wash Buffer to each well. Pipet the mix 10 times and be careful to minimize bubble formation.
5. Seal the PCR tube strip, then incubate for 2 minutes in the 65°C thermal cycler.
6. Place the PCR tube strip on the magnet rack for 1 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
7. Take the PCR tube strip off the magnet rack. Add 150 μ L of heated Stringent Wash Buffer to each well. Pipet the mix 10 times and be careful to minimize bubble formation.
8. Seal the PCR tube strip, then incubate for 2 minutes in the 65°C thermal cycler.
9. Place the PCR tube strip on the magnet rack for 1 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.

Room temperature washes

1. Take the PCR tube strip off the magnet rack. Add 150 μ L of room-temperature 1 $\times$ Wash Buffer I, vortex for 2 minutes, briefly spin down.

2. Place the PCR tube strip on the magnet rack for 1 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
3. Take the PCR tube strip off the magnet rack. Add 150 μL of room-temperature GenoBaits 1 $\times$ Wash Buffer II, vortex for 1 minutes, briefly spin down.
4. Place the PCR tube strip on the magnet rack for 1 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
5. Take the PCR tube strip off the magnet rack. Add 150 μL of room-temperature GenoBaits 1 $\times$ Wash Buffer III, vortex for 30 seconds, briefly spin down.
6. Place the PCR tube strip on the magnet rack for 1 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
7. Take the PCR tube strip off the magnet rack. Add 20 μL of Nuclease-free Water to each well.
8. Pipette up and down slowly 10 times to ensure that all beads are resuspended.
9. Transfer 10 μL of beads to a new 8-well PCR tube strip for Step 7 Post-capture PCR, reserve the remaining 10 μL for backup.

Step 7: Perform Post-capture PCR

1. **Prepare Reaction Mix:** Refer to Table 15 for guidance.

Table 15. Post-capture PCR Reaction Mix

Component	Volume/Per Reaction (μL)
GenoBaits PCR Master Mix	15
GenoBaits Primer Mix for ILM	1.2
Nuclease-Free Water	3.8
Total volume	20

2. **Dispensing Reaction Mix:** Add 20 μL of the prepared reaction mix to each well of the 8-well PCR tube strip in Step 6.

3. **Sealing and Mixing:** Seal the PCR tube strip, mix thoroughly and briefly centrifuge to collect the contents at the bottom of the wells.
4. **Thermal cycling:** Place the PCR tube strip in the thermal cycler, and run the following program with the heated lid set to 105 °C.

Table 16. Post-capture PCR program

Step	Temperature	Incubation Time	Number of cycles
1	98°C	45 sec	1
2	98°C	15 sec	See the GenoBaits Panel product instructions
	60°C	30 sec	
	72°C	30 sec	
3	72°C	1 min	1
4	4°C	∞	Hold



Safe Stop: When the program reaches 4 °C, store the PCR product at -20 °C overnight or proceed to Step 8.

Step 8: Purify Post-capture PCR fragments



Note: Ensure GenoPrep DNA Clean Beads have been equilibrated to room temperature before proceeding.

1. Place the PCR tube strip from Step 7 on the magnet rack for 1 minute, wait for the liquid to turn clear completely.
2. Transfer the supernatant into a new PCR tube strip.
3. Add 45 µL GenoPrep DNA Clean Beads, and vortex to mix thoroughly. Incubate at room temperature for 5 minutes. Briefly centrifuge to collect the contents.
4. Place the PCR tube strip on the magnet rack for 5 minute, wait for the liquid to turn clear completely. Discard the supernatant, make sure not to disturb the beads.
5. With the PCR tube strip still on the magnetic rack, add 100 µL of 80% ethanol to each well and incubate for 30 seconds at room temperature. Discard the supernatant, ensuring no residual ethanol remains.

6. Keep the plate on the magnetic rack, and allow the beads to air dry at room temperature for at least for 5 minutes until they appear dry on the surface.



Note: Ensure no residual ethanol is present, and the beads have just cracked on the surface. The residual ethanol, and over-drying beads may result in low library yield.

7. Remove the PCR tube strip from the magnetic rack, add 35 μ L 10 mM Tris-HCl to each well. mix thoroughly and incubate at room temperature for 5 minutes to elute DNA from the beads. Briefly spin down to collect the contents.
8. Place the PCR tube strip on the magnetic rack and wait until the liquid turns completely clear, which should take at least 3 minutes.
9. Transfer the elute DNA into a new PCR tube strip, take care not to disturb the beads.
10. Store purified PCR fragments per your established, internal laboratory procedures.

Step 9: QC of the captured library and perform sequencing

1. **Quantify:** Use Qubit reagents or equivalent dsDNA fluorescence kit (e.g. GenoPrep dsDNA Quantitation Kit, Catlog DN000109) for concentration determination. The optimal concentration of the captured library should be 10 - 20 ng/ μ L. We do not recommend to proceed with sequencing if the concentration is below 5 ng/ μ L.
2. **Size check:** Use Gel electrophoresis or Agilent 2100 Bioanalyzer to validate the size range of each library. The optimal size of the DNA library should be between 250bp - 500 bp.
3. Perform sequencing according to the instructions for NGS sequencer.

5. Appendix

Instructions for GenoBaits CDI Barcodes

- The 8-base GenoPlexs CDI Barcodes are arranged in 96-well plates, with 1 reactions per well and 96 barcodes per plate, for a total of 5,760 combinations across 18 plates.
- For the base sequence information of GenoBaits CDI Barcodes, please directly contact the technical support.

Molbreeding Biotechnology LLC

Address: 10 Pasteur, Suite 150,
Irvine, CA 92618
United States
<http://www.molbreeding.com>
T: +1-515-508-9174
E: cs@molbreeding.com