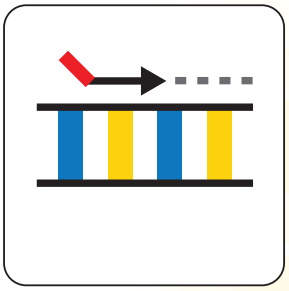
USER GUIDE



Allegro Targeted Genotyping

Catalog Numbers: 10030, 10031, 10032, 10036

Publication Number: M01455

Revision: v1

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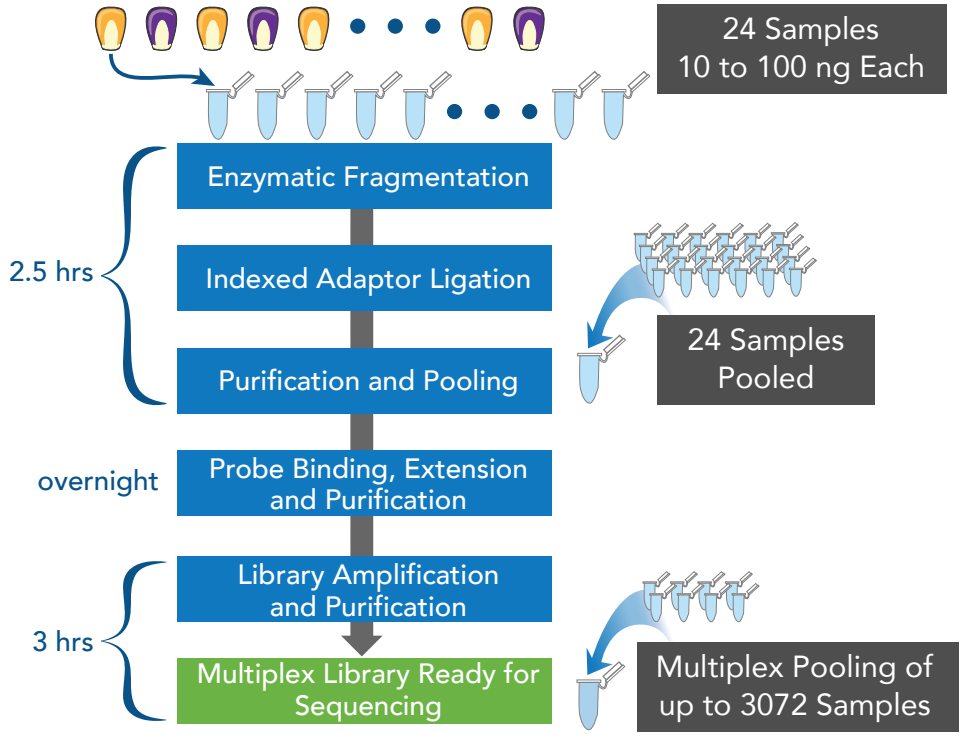
I. Introduction

A. Background

The Allegro Targeted Genotyping system is designed to produce custom target-enriched, multiplexed libraries suitable for sequencing on Illumina NGS platforms. It is a fast, robust and simple system capable of producing target-enriched libraries from 10 to 100 ng of genomic DNA from intact samples. In the simple, add and incubate workflow shown in Figure 1, enzymatically-fragmented gDNA samples are individually ligated with barcoded adaptors. Following ligation and purification, 24 samples are pooled together and combined with the targeting probes for an overnight annealing and extension step, creating amplifiable library molecules. Following a second bead purification, an optional qPCR step determines the optimal number of PCR cycles to use in the final library amplification step. After library amplification, final bead purification prepares the target-enriched library for sequencing.

Figure 1. Allegro Targeted Genotyping Workflow



B. Storage and Stability

The Allegro Targeted Genotyping core kit and probe mix are shipped on dry ice and should be unpacked immediately upon receipt. All components should be stored at –20 °C on internal shelves of a freezer without a defrost cycle.

Every lot of Allegro Targeted Genotyping undergoes functional testing to meet specifications for library generation performance. The kit has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months.

I. Introduction

C. Safety Data Sheet (SDS)

If applicable, an SDS for this product is available on the NuGEN website at www.nugen.com/products/allegro-targeted-genotyping.

D. Before You Start

Please review this User Guide before using this kit for the first time, including the "Reagents Provided," "Planning the Experiment," "Protocol" and "FAQ" sections. For more information, visit the Allegro Targeted Genotyping page at www.nugen.com/products/allegro-targeted-genotyping

New to NGS? Contact NuGEN Technical Support at techserv@nugen.com for tips and tricks on getting started.

II. Components

A. Kit Components

Allegro Targeted Genotyping kit (Part Nos. 10030, 10031, 10032, and 10036) contains the reagents listed in Table 1 and Table 2. Allegro Metaplex Module (Part No. 0413) is available separately.

Table 1. Allegro Targeted Genotyping Core Reagents (Part Nos. 0411 and 0412)

PART NUMBER	DESCRIPTION	VIAL LABEL	VIAL NUMBER
S02370	Fragmentation Buffer Mix	Orange	F1 VER 5
S02371	Fragmentation Enzyme Mix	Orange	F2 VER 4
S01689	Ligation Buffer Mix	Yellow	L1 VER 4
S01690	Ligation Enzyme Mix	Yellow	L3 VER 4
S02362	Allegro 96-plex Adaptor Plate 1 (Part No. 0411 only)	Yellow	---
S02376	Allegro 96-plex Adaptor Plate 2 (Part No. 0412 only)	Yellow	---
S01900	Target Extension Buffer Mix	Clear	TX1
S01902	Target Extension Enzyme Mix	Clear	TX2
S01903	Amplification Buffer Mix	Red	P1 VER 4
S02372	Amplification Primer Mix	Red	P2 VER 17
S01764	Amplification Enzyme Mix	Red	P3 VER 2
S02373	Allegro Custom R1 Primer	Clear	---
S01901	DNA Resuspension Buffer Mix	Clear	DR1
S01113	Nuclease-free Water	Green	D1

Table 2. Allegro Custom Probe Mix

PART NUMBER	DESCRIPTION	VIAL LABEL	VIAL NUMBER
S02374	Allegro Custom Probe Mix	Clear	Varies by custom design, e.g. ST_XXXX

II. Components

Table 3. Part Numbers for Metaplex Module (Available as Part No. 0413)

PART NUMBER	DESCRIPTION	VIAL LABEL	VIAL NUMBER
S02435	Allegro Metaplex Primer Mix 01	Red	MP2-BC01
S02436	Allegro Metaplex Primer Mix 02	Red	MP2-BC02
S02437	Allegro Metaplex Primer Mix 03	Red	MP2-BC03
S02438	Allegro Metaplex Primer Mix 04	Red	MP2-BC04
S02439	Allegro Metaplex Primer Mix 05	Red	MP2-BC05
S02440	Allegro Metaplex Primer Mix 06	Red	MP2-BC06
S02441	Allegro Metaplex Primer Mix 07	Red	MP2-BC07
S02442	Allegro Metaplex Primer Mix 08	Red	MP2-BC08
S02443	Allegro Metaplex Primer Mix 09	Red	MP2-BC09
S02444	Allegro Metaplex Primer Mix 10	Red	MP2-BC10
S02445	Allegro Metaplex Primer Mix 11	Red	MP2-BC11
S02446	Allegro Metaplex Primer Mix 12	Red	MP2-BC12
S02447	Allegro Metaplex Primer Mix 13	Red	MP2-BC13
S02448	Allegro Metaplex Primer Mix 14	Red	MP2-BC14
S02449	Allegro Metaplex Primer Mix 15	Red	MP2-BC15
S02450	Allegro Metaplex Primer Mix 16	Red	MP2-BC16
S02451	Allegro Custom Index 2 Primer	Clear	---

B. Additional Equipment, Reagents and Labware

- **Equipment**
  - Agilent 2100 Bioanalyzer or 2200 TapeStation Instrument, or other equipment for electrophoretic analysis of nucleic acids
  - Real-time PCR system capable of SYBR® Green detection
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge for 0.2 mL volume individual tubes, strip tubes and plates
  - Single channel and multi-channel pipettors
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Qubit® 2.0 or 3.0 Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA and amplified libraries

II. Components

- **Reagents**
  - Agencourt® AMPure XP Beads (Beckman Coulter, Cat. A63881)
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - EvaGreen® Dye, 20X in water (Biotium, Cat. #31000) (recommended)
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793), for diluting nucleic acids
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 8 X 0.2 mL thin-wall PCR strip tubes or 0.2 mL thin-wall PCR plates
  - Magnetic stand for 0.2 mL strip tubes or plates (Thermo Fisher Scientific Cat. #12027 or 12332D; Promega Cat. #V8351; SPRIPlate Ring Super Magnet Plate, Beckman Coulter, Cat. #A32782).
  - Amicon® Ultra-0.5 mL Centrifugal Filter Unit with Ultracel®-30K Membrane (Millipore, Cat. #UFC503024)
  - AlumaSeal II (Excel Scientific, Cat. #AFS-25)

III. Planning the Experiment

A. Input DNA Requirements

Allegro Targeted Genotyping is designed to work with 10 to 100 ng intact (non-degraded) DNA samples. Lower inputs may impact library complexity for larger genomes. Inputs greater than 100 ng will exceed the capacity of the system and are not recommended.

B. Working with the Adaptor Plate

The adaptor plate included with Allegro Targeted Genotyping contains adaptor mixes, each with a unique eight-base barcode. Each well contains sufficient volume for preparation of a single library. Allegro Targeted Genotyping adaptor plates are sealed with a foil seal designed to provide airtight storage.

Thaw adaptor plate on ice and spin down. Do NOT warm above room temperature.

Make sure all adaptor mixes are collected at the bottom of the wells and place the adaptor plate on ice after centrifuging. When pipetting the adaptor mixes, puncture the seal for each well you wish to use with a fresh pipet tip, and follow the protocol for mixing and transfer of the contents. The remaining wells of the plate should remain sealed for use at a later date. Cover used wells with 96-well plate sealing foil to prevent any remaining ligation adaptor mix from contaminating future reactions.

III. Planning the Experiment

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid and a 100 µL reaction volume capacity. Prepare thermocycler programs as shown in Table 4, below.

Table 4. Thermal Cycler Programming

FRAGMENTATION		VOLUME
Program 1 Fragmentation	25 °C – 15 min, 75 °C – 20 min, hold at 4 °C	20 µL
LIGATION		
Program 2 Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	35 µL
PROBE HYBRIDIZATION AND EXTENSION		
Program 3 Hybridization and Extension	95 °C – 5 min, 200 cycles (80 °C – 10 s, decrease 0.1 °C each cycle), hold at 60 °C*, 72 °C – 10 min, hold at 4 °C	50 µL
LIBRARY AMPLIFICATION		
Program 4 Amplification	95 °C – 3 min, N** (95 °C – 30 s, 62 °C – 15 s, 72 °C – 20 s), 72 °C – 2 min, hold at 4 °C	100 µL

*** Important Note regarding Program 3:** Do not proceed to the next step in the program until the Target Extension Enzyme has been added.
****Important Note regarding Program 4:** Cycle number should be optimized using the Optional Real-Time PCR protocol in Appendix A.

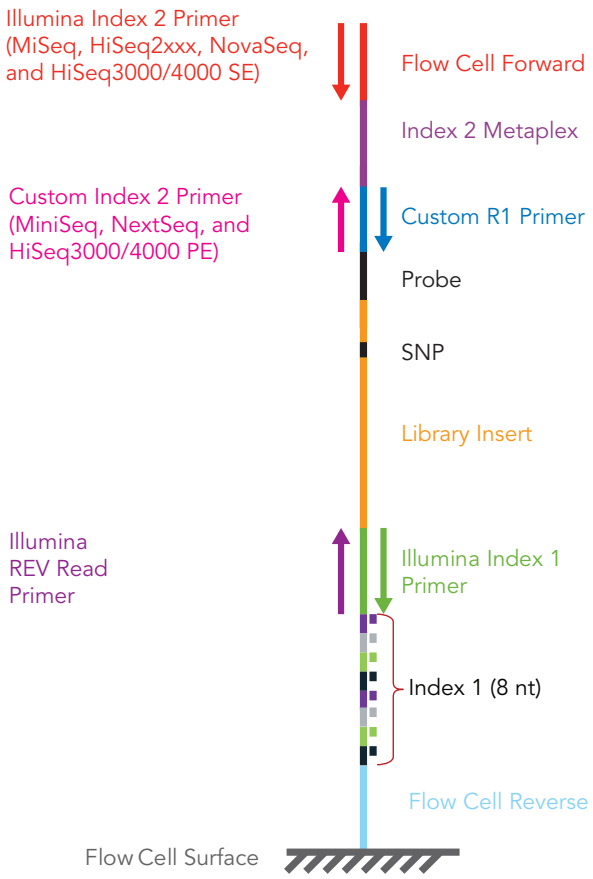
D. Library Quantitation

We strongly recommend using a qPCR quantification method for the most accurate quantification of libraries produced with the Allegro Targeted Genotyping system. Depending on the input DNA quality and size, these libraries may produce a broad profile when analyzed using the Agilent Bioanalyzer. In order to avoid underestimating the library concentration in this case, we recommend using a library fragment size approximately 50–100 bp smaller than the actual peak determined by the Agilent Bioanalyzer to calculate the size-adjusted concentration. Additionally, we recommend loading approximately 60% of the Illumina-recommended maximum input onto the flowcell during initial sequencing. The amount of library loaded can be adjusted for subsequent sequencing runs in order to optimize the cluster density.

III. Planning the Experiment

E. Sequencing Recommendations and Guidelines

Figure 2. Allegro Targeted Genoyping Library Structure.



Allegro Targeted Genotyping produces libraries compatible with Illumina NGS platforms. A Custom Read 1 primer (supplied) is **required**; the libraries cannot be sequenced using the standard Illumina Read 1 primer. The Custom Read 1 primer is supplied at 100 µM and should be diluted to the appropriate concentration for the chosen sequencing instrument per the manufacturer's instructions. If using the Allegro Metaplex Module, a Custom Index 2 primer (supplied) may be required. Refer to Table 5. for a list of requirements by Illumina platform. It is strongly recommended to consult Illumina support for the latest recommendations. Both the Custom Read 1 primer and the Custom Index 2 primer can be mixed with standard sequencing primers.

A minimum 100 bp forward read is recommended to efficiently capture the target variants. A reverse read may be performed but is not required. For more information on analysis of data from paired end reads, please see section F. Data Analysis Guidance.

Standard libraries contain an 8 bp Index 1/i7 barcode to enable multiplexed sequencing. These libraries should be sequenced using the Illumina protocol for multiplex sequencing, following the recommendations for the specific sequencer. The barcode sequences must be entered into the Illumina software prior to parsing and analysis, and can be found in Appendix section C. Barcode Sequences.

III. Planning the Experiment

Table 5. Custom Index 2 Primer Guidelines

PLATFORM	REQUIREMENTS (Obtained from Illumina Document #15057455 v03)
MiSeq	Dual indexing can be performed with the standard Illumina Index 2 primer. See Illumina Doc #15041638 for instructions on loading a Custom R1 primer.
HiSeq 2xxx	Dual indexing can be performed with the standard Illumina Index 2 primer. See Illumina Doc #15061846 for instructions on loading a Custom R1 primer.
NovaSeq	Dual indexing can be performed with the standard Illumina Index 2 primer. See Illumina Doc #1000000022266 for instructions on loading a Custom R1 primer.
MiniSeq	Use of Custom R1 primer requires use of Custom Index 2 primer. See Illumina Doc #1000000002700 for instructions on using Custom R1 and Index 2 primers.
NextSeq	Using the v1 reagent cartridge, a Custom Read 1 primer is possible with single-indexed runs only. Using the v2 reagent cartridge, a Custom Read 1 primer is possible with both single-indexed and dual-indexed runs. For a dual indexed run using the v2 reagent cartridge, a Custom Read 1 primer and Custom Index 2 primer is possible only with NCS v1.4, or later. See Illumina Doc #15057456 for instructions on loading custom primers.
HiSeq 3000/4000	Dual indexing for single read runs does not require any custom indexing primers. Dual indexing for paired end reads requires use of a Custom Index 2 primer, and must be applied to all lanes in the sequencing run. See Illumina Doc #15061846 for instructions on loading custom primers.

F. Data Analysis Guidance

Single end reads produced from Allegro Targeted Genotyping libraries may be processed with standard DNA analysis (ie: Illumina adaptor trimming, genome alignment and SNP analysis). Please note that the first 40 bp of each forward read will begin with probe-derived sequence that may be trimmed. While the probe-derived sequence is suitable for alignment purposes, it should not be used to derive any information about the sequence of the original DNA sample, such as mutations, insertions, or deletions.

Paired end reads require a specialized trimming command to remove the 15 bp probe linker from the 3' end of the reverse read, in addition to the Illumina adaptor from the Forward reads. The following command may be used with Trim Galore! to remove the linker and adaptor sequences from paired end data:

```
trim_galore -a2 GAGAGCGATCCTTGC --paired R1.fq R2.fq
```

For the reverse read, this command will remove any occurrence of the linker sequence and all downstream (3') bases, which will be derived from the adaptor; and for the forward read, this command will remove the standard Illumina adaptor. Removal of the linker sequence will enable more accurate alignment.

If you have any questions regarding how to perform this analysis, please contact NuGEN Technical Support (techserv@nugen.com).

III. Planning the Experiment

G. Multiplexing Guidelines for Allegro Metaplex Module (Part No. 0413)

If a small number of Index 2/i5 barcodes are used they may require color balancing, depending on the sequencing platform.

For sequencing on the HiSeq 3000/4000 platforms with only two Index 2/i5 barcodes, the following barcode combinations may be used:

MP2-BC01 & MP2-BC07

MP2-BC02 & MP2-BC08

MP2-BC03 & MP2-BC06

MP2-BC08 & MP2-BC09

MP2-BC13 & MP2-BC14

Numerous combinations of 3-plex Index 2/i5 barcodes are available. Please contact NuGEN Technical Support (techserv@nugen.com) for the allowable combinations.

Because 24 Index 1/i7 barcodes are required for each pool prior to hybridization similar considerations for the i7 Index Read are not necessary.

IV. Protocol

A. Input

Samples must be processed in batch increments of 24 samples (24, 48, 72, etc.). Each batch of 24 samples to be processed together should be of equivalent quantity and quality in order to maintain equal representation in the final library. Each individual genomic DNA sample should be 10 to 100 ng in 10 µL water or low-EDTA TE buffer (pH 8.0) in a 0.2 mL PCR strip tube or plate.

B. Fragmentation

1. Remove Fragmentation Buffer Mix (orange: F1) and Fragmentation Enzyme Mix (orange: F2) from –20 °C and thaw on ice. Mix F1 by vortexing. Mix F2 by pipetting. Spin both tubes briefly and place on ice.
2. Place DNA samples on ice.
3. Make Fragmentation Master Mix by combining F1 and F2 on ice as shown in Table 6.

Table 6. Fragmentation Master Mix

REAGENT	FRAGMENTATION BUFFER MIX (ORANGE: F1 VER 5)	FRAGMENTATION ENZYME MIX (ORANGE: F2 VER 4)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	7.0 µL	3.0 µL

4. Add 10 µL Fragmentation Master Mix to each DNA sample in a PCR strip tube or PCR plate on ice or chilled block.
5. Mix by pipetting up and down several times with a volume larger than half the total reaction volume (20 µL).
6. Seal tubes/plate and place in a thermal cycler programmed to run the Fragmentation Program:

   25 °C – 15 min, 75 °C – 20 min, hold at 4 °C
7. Remove samples from thermal cycler, spin down and place on ice.

C. Adaptor Ligation

1. Retrieve Agencourt beads from 4 °C and DNA Resuspension Buffer (clear: DR1) from –20 °C and place at room temperature for use in the next step. Remove Ligation Buffer Mix (yellow: L1), Ligation Enzyme Mix (yellow: L3) and Nuclease-free Water (green: D1) from –20 °C storage and thaw on ice. Mix L1 and L3 by pipetting. Spin down L1 and L3 briefly and place on ice.

   Note: Mix L1 **THOROUGHLY** by pipetting up and down carefully with a large volume pipettor (200 µL or 1000 µL pipettor).
2. Remove adaptor plate from –20 °C storage and thaw on ice.
3. Spin down the plate completely to ensure that all adaptors are in the bottom of the wells (for more information on handling adaptor plates, see Section III. Planning the Experiment: B. Working with the Adaptor Plate).

IV. Protocol

4. Carefully pierce or remove the portion of the plate seal covering the set of barcoded adaptors to be used.
5. Using a multichannel pipette, transfer the first column of fragmented samples (20 µL) into the first column in the barcoded adaptor plate. Mix the sample and adaptors well and then transfer the entire volume (23 µL) back into the original reaction tubes or plate.
6. Continue with each column of samples and column of barcoded adaptors (i.e. add the second column of samples to the second column of adaptors) until each sample has been mixed with a different barcoded adaptor.
7. Make Ligation Master Mix on ice by combining L1, L3 and D1 as shown in Table 7.

Important: It is critical that the Ligation Master Mix be thoroughly and carefully mixed!

Table 7. Ligation Master Mix

REAGENT	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	6.0 µL	1.5 µL	4.5 µL

8. Add 12 µL Ligation Master Mix to each sample.
9. Mix by pipetting up and down several times with a large volume pipettor using a volume larger than half the total reaction volume (35 µL).

Important: It is critical that the Ligation Master Mix and sample be thoroughly and carefully mixed!

10. Seal tubes/plate on ice and place in a thermal cycler programmed to run the Ligation Program:

    25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
11. Remove samples from thermal cycler, spin down and place on ice.

D. Purification, Pooling and Volume Reduction

1. Ensure Agencourt beads and DR1 are at room temperature before proceeding.
2. Add 65 µL of room-temperature Nuclease-free water (green: D1) to each ligation reaction.
3. Add 80 µL room temperature Agencourt beads (0.8 volumes) to each sample. Mix thoroughly by pipetting 10 times.
4. Incubate bead-sample mixture for five minutes at room temperature.

Note: Mixing by pipetting each minute during the incubation can help improve recovery.

5. Transfer the tubes to the magnet plate and let stand 5 minutes to completely clear the solution of beads.
6. With the tubes still on the magnet, carefully remove only 175 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into Target Enrichment, so ensure beads are not removed with the binding buffer or the wash.

7. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for at least 30 seconds but no more than 1 minute.
8. Remove the ethanol wash using a pipette.
9. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
10. Remove the tubes from the magnet.
11. Add 21 µL of DNA Resuspension buffer (DR1) to the dried beads. Mix thoroughly by pipetting to ensure all the beads are resuspended. Incubate for 2 minutes at room temperature.
12. Transfer the tubes to the magnet and let stand for 3 minutes to completely clear the solution of beads.
13. Carefully collect 20 µL of the eluate, ensuring as few beads as possible are carried over.

Optional stopping point: Store samples at –20 °C.

14. Combine the eluted DNA of 24 samples into a single fresh 1.5 mL tube. Combined volume should be approximately 480 µL.
15. Concentrate the pooled sample using an Amicon Ultra 0.5 mL Centrifugal Filters Unit with Ultracel-30K following the manufacturer's recommendations. If final volume falls below 32 µL after the concentration step, add water to bring the volume up to 32 µL.

Optional stopping point: Store samples at –20 °C.

E. Probe Hybridization

1. Remove Target Extension Buffer Mix (clear: TX1) and Allegro Targeted Genotyping Custom Probe Mix from –20 °C storage and thaw on ice.
2. Mix the TX1 and Allegro Targeted Genotyping Custom Probe Mix by vortexing.
3. Make Target Extension Master Mix by combining TX1 and Allegro Targeted Genotyping Custom Probe Mix as shown in Table 8.

IV. Protocol

Table 8. Target Extension Master Mix

REAGENT	TARGET EXTENSION BUFFER MIX (CLEAR: TX1)	ALLEGRO TARGETED GENOTYPING CUSTOM PROBE MIX (CLEAR: Varies by custom design)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	7.0 µL	10.0 µL

4. Add 17 µL Target Extension Master Mix to each 32 µL pool.
5. Mix by pipetting up and down several times with a volume larger than half the total reaction volume (49 µL).
6. Seal tubes completely and place in a thermal cycler programmed to run the Hybridization and Extension Program:

   95 °C – 5 min, 200 cycles (80 °C – 10 s, decrease temp 0.1 °C each cycle), 60 °C hold*, 72 °C 10 min, hold at 4 °C

Important: *Incubate at 60 °C for a minimum of 12 hours. Do not advance to 72 °C until after Target Extension Enzyme (TX2) is added in step 7 below!

7. With PCR tubes still in the thermal cycler at 60 °C, open the thermal cycler lid and PCR tube lids and add 1 µL TX2 to each tube. Mix thoroughly by pipetting with a volume larger than half the total reaction volume (50 µL).
8. Close the PCR tube lids, close the thermal cycler lid and advance the thermal cycler to the 72 °C step in the Hybridization and Extension Program.
9. Remove samples from thermal cycler, spin down and place on ice.

F. Post-enrichment Purification

1. Add 50 µL room temperature Nuclease-free water (green: D1) to each tube, bringing the total volume to 100 µL.
2. Add 80 µL room temperature Agencourt beads (0.8 volumes) to each sample. Mix thoroughly by pipetting 10 times.
3. Incubate bead-sample mixture for five minutes at room temperature.

Note: Mixing by pipetting each minute during the incubation can help improve recovery.

4. Place tubes on a magnetic plate for five minutes at room temperature to allow the beads to clear the solution.to disturb or remove any beads.
5. While the tubes are still on the magnetic plate, carefully remove 175 µL supernatant, taking care not to disturb or remove any beads.

IV. Protocol

6. With the tubes remaining on the magnetic plate, add 200 µL 70% Ethanol to each tube, incubate for 30 seconds and then remove ethanol to waste.
7. After completely removing all traces of ethanol with a pipette, allow the tubes to dry at room temp for ten minutes.
8. Remove the tubes from the magnet plate.
9. Add 100 µL DNA Resuspension Buffer Mix (DR1) to each tube and fully resuspend the beads by pipetting up and down several times.
10. Allow tubes to incubate for 3-5 minutes at room temperature.
11. Place tubes on magnetic plate and allow beads to separate for 2 minutes.
12. Remove 100 µL eluted material into fresh PCR tubes.
13. Add 80 µL room temperature Agencourt beads (0.8 volumes) to each sample. Mix thoroughly by pipetting 10 times.
14. Incubate bead-sample mixture for five minutes at room temperature.

Note: Mixing by pipetting each minute during the incubation can help improve recovery.

15. Place tubes on a magnetic plate for five minutes at room temperature to allow the beads to clear the solution.
16. While the tubes are still on the magnetic plate, carefully remove 175 µL supernatant, taking care not to disturb or remove any beads.
17. With the tubes remaining on the magnetic plate, add 200 µL 70% ethanol to each tube, incubate for 30 seconds and then remove ethanol to waste.
18. After completely removing all traces of Ethanol with a pipette, allow the tubes to dry at room temp for ten minutes.
19. Remove the tubes from the magnet plate.
20. While the tubes are still on the magnetic plate, carefully remove 175 µL supernatant, taking care not Add 25 µL DR1 to each tube and fully resuspend the beads by pipetting up and down several times.
21. Allow tubes to incubate for 2 minutes at room temperature.
22. Place tubes on magnetic plate and allow beads to separate for 2 minutes.
23. Remove 24 µL eluted enrichment pool into fresh PCR tubes.

Optional stopping point: Store samples at –20 °C.

IV. Protocol

G. Library Amplification

Note: Whenever using a sample for the first time with the kit, or using a new amount of input, perform real-time PCR as described in Appendix A to determine the appropriate number of library amplification cycles for your sample.

1. Remove Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2), and Amplification Enzyme Mix (red: P3) from –20 °C storage and thaw on ice.

Important: If you are using the dual index Allegro Metaplex Module, please follow instructions for Library Amplification as described in Appendix B. Using the Allegro Metaplex Module.

2. Mix P1 and P2 by vortexing. Mix P3 by pipetting.
3. Make Library Amplification Master Mix by combining P1, P2, P3 and D1 as shown in Table 9..

Table 9. Library Amplification Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 17)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	50.0 µL	20.0 µL	8.0 µL	2.0 µL

4. Add 80 µL Library Amplification Master Mix to 20 µL of enrichment pool in a fresh PCR tube.
5. Mix by pipetting up and down several times with a volume larger than half the total reaction volume (100 µL).
6. Seal tubes completely and place in a thermal cycler programmed to run the Amplification Program:

   95 °C 3 min, N (95 °C 30 s, 62 °C 15 s, 72 °C 20 s), 72 °C 2 min, hold at 10 °C

Note: The number of library amplification cycles (N) should be determined empirically by qPCR.

7. Remove samples from thermal cycler, spin down and place on ice.

H. Final Library Purification

1. Add 80 µL (0.8x volume) room temperature Agencourt beads to each tube and mix well.
2. Incubate bead-sample mixture for five minutes at room temperature.

Note: Mixing by pipetting each minute during the incubation can help improve recovery.

IV. Protocol

3. Place tubes on a magnetic plate for five minutes at room temperature to allow the beads to clear the solution.
4. While the tubes are still on the magnetic plate, carefully remove 175 µL supernatant, taking care not to disturb or remove any beads.
5. With the tubes remaining on the magnetic plate, add 200 µL 70% Ethanol to each tube, allow to incubate for 30 seconds and the remove Ethanol to waste.
6. After completely removing all traces of Ethanol with a pipette, allow the tubes to dry at room temperature for 10 minutes.
7. Remove the tubes from the magnet plate.
8. Add 100 µL DR1 to each tube and fully resuspend the beads by pipetting up and down several times.
9. Allow tubes to incubate for 3-5 minutes at room temperature.
10. Place tubes on magnetic plate and allow beads to separate for 2 minutes.
11. Remove 100 µL eluted library into fresh PCR tubes.
12. Add 80 µL (0.8x volume) room temperature Agencourt beads to each tube and mix well.
13. Incubate bead-sample mixture for five minutes at room temperature.

Note: Mixing by pipetting each minute during the incubation can help improve recovery.

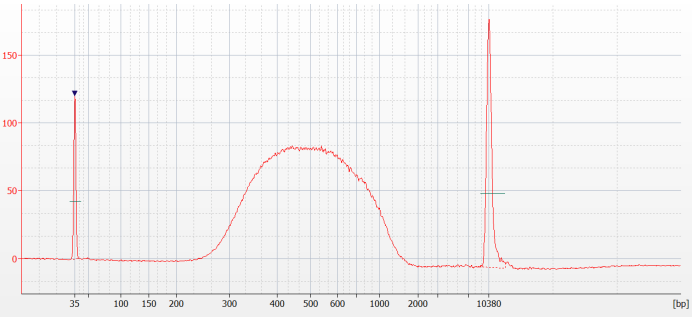
14. Place tubes on a magnetic plate for five minutes at room temperature to allow the beads to clear the solution.
15. While the tubes are still on the magnetic plate, carefully remove 175 µL supernatant, taking care not to disturb or remove any beads.
16. With the tubes remaining on the magnetic plate, add 200 µL 70% Ethanol to each tube, allow to incubate for 30 seconds and the remove Ethanol to waste.
17. After completely removing all traces of Ethanol with a pipette, allow the tubes to dry at room temp for ten minutes.
18. Remove the tubes from the magnet plate.
19. Add 25 µL DNA Resuspension buffer (DR1) to each tube and fully resuspend the beads by pipetting up and down several times.
20. Allow tubes to incubate for 2 minutes at room temperature.
21. Place tubes on magnetic plate and allow beads to separate for 2 minutes.

22. Remove 24 µL eluted library into fresh PCR tubes.
23. Amplified libraries may be stored at –20 °C.

I. Quantitative and Qualitative Assessment of the Library

1. Run 1 µL of 5 ng/µL library on a Bioanalyzer High Sensitivity DNA Chip or other electrophoretic methods. Typical fragment distributions are shown in Figure 3.
2. Measure library concentration by qPCR.

Figure 3. Fragment distribution when 1 µL of a target enriched library is loaded into a Bioanalyzer High Sensitivity DNA Chip.



Important: Concentration calculation of broad libraries should be performed using a library fragment size approximately 50-100 bp smaller than the peak determined by the Agilent Bioanalyzer to avoid underestimating the library concentration. We also recommend initially loading approximately 60% of the Illumina recommended input onto the flow cell to avoid potential over-clustering. The amount of library loaded can be optimized in subsequent sequencing runs.

3. Validate the library as described in the most recent Illumina user guides for DNA library construction, e.g., Genomic DNA Sample Prep Manual.

V. Appendix

A. Optional Real-Time PCR

This step should be performed the first time using a new set of samples or targeted panel. Once the optimal range of PCR cycles is determined for a given sample type and panel, this section does not need to be performed. Sufficient reagent is supplied to perform this reaction for each pooled sample in addition to the PCR step that follows.

An intercalating dye (EvaGreen or SYBR Green I) is added to the PCR reaction in order to monitor the production of double stranded amplicon during thermal cycling.

EvaGreen (recommended) is supplied by the manufacturer in a convenient 20X stock in water. It does not inhibit PCR at 1X concentration. SYBR Green I is supplied by the manufacturer in DMSO at 10,000X stock concentration, and must be diluted with Low-EDTA TE Buffer to 10X working concentration just prior to use. This working stock is not stable and must be used immediately. Any unused working stock of SYBR Green should be discarded. SYBR Green I is inhibitory to PCR at 1X concentration, and is therefore used at 0.5X concentration in the protocol below. When preparing qPCR reactions, be sure to use EvaGreen or SYBR Green I dye; do not use a 2X SYBR Green Master mix.

1. Remove Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2) and Amplification Enzyme Mix (red: P3) from –20 °C storage and thaw on ice.
2. Remove 20X EvaGreen reagent from 4 °C storage and place on ice.

Note: If using SYBR Green I, 10,000X in DMSO, thaw completely, then prepare a working stock of 10X SYBR Green in Low EDTA TE, pH 8.0 and use 0.5 ul 10X SYBR Green per 10 µL reaction for a final concentration of 0.5X in order to reduce the inhibitory effect of SYBR Green on PCR.

3. Mix P1, P2 and 20X EvaGreen reagent by vortexing. Mix P3 by pipetting.
4. Make real-time PCR Master Mix by combining components as shown in Table 10. Prepare enough master mix for 3 reactions per sample plus 3 No Template Control reactions.
5. Dilute each enrichment pool 1:8 by combining 2 µL of enrichment pool with 14 µL DR1. Mix well.

Table 10. Real-Time PCR Master Mix (volumes shown are for a single reaction)

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 17)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)	20X EVAGREEN	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	2.4 µL	0.8 µL	0.2 µL	0.5 µL	4.1 µL

6. In triplicate, add 8 µL real-time PCR Master Mix to 2 µL of each diluted enrichment pool in a qPCR plate.
7. Mix by pipetting up and down several times.
8. Seal plate completely, spin down and place in a qPCR instrument programmed to run the following program:

   95 °C 3 min; 35 cycles (95 °C 30 s, 62 °C 15 s, 72 °C 20 s)

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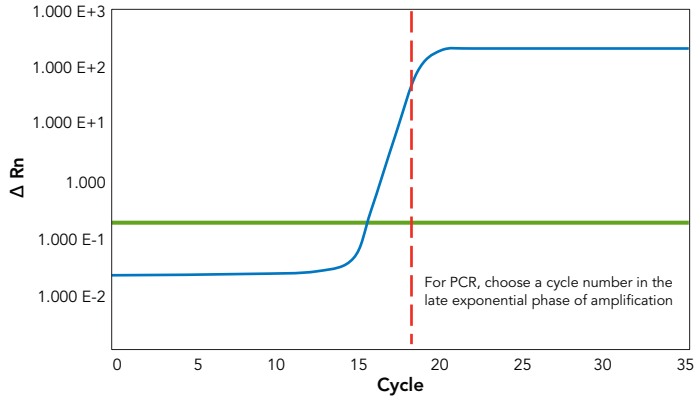
Examine the amplification plot and determine the cycle number for late exponential phase amplification. In Figure 4, this would be 18 cycles. Subtract 3 cycles to account for the 1:8 dilution of the enrichment pool made in step 5. Use this number of PCR cycles for Library Amplification (step G.6.). In this example, you would run 18-3 = 15 cycles of PCR.

Figure 4. Stylized qPCR amplification plot.



V. Appendix

B. Allegro Metaplex Module Barcode Sequences

The Allegro Metaplex Module contains 16 PCR primer mixes that each contain a single Index 2/i5 barcode. The sequences for these barcodes can be found in Table 11. For sequencing runs that require the Custom Index 2 sequencing primer, you must parse based on the reverse complement of the sequence shown in Table 11. The primer mix can be substituted in place of the Amplification Primer Mix (P2 VER 17) in order to add a second barcode for each batch of 24 pooled samples. In addition to the Amplification Primer mix, there is an additional tube that contains Custom Index 2 sequencing primer, a custom sequencing primer that is required on some platforms and configurations. For more information on how to use the Custom Index 2 primer, please see G. Multiplexing Guidelines for Allegro Metaplex Module (Part No. 0413) and Table 11.

Table 11. Index 2/i5 barcode sequences for Allegro Metaplex Module

PRIMER MIX	BARCODE SEQUENCE	REVERSE COMPLEMENT FOR PARSING WITH CUSTOM INDEX2 PRIMER
MP2-BC01	TCCACAGA	TCTGTGGA
MP2-BC02	AGACTGCT	AGCAGTCT
MP2-BC03	CAGACATC	GATGTCTG
MP2-BC04	CGACGATA	TATCGTCG
MP2-BC05	AACAACCG	CGGTTGTT
MP2-BC06	TGAGGTAG	CTACCTCA
MP2-BC07	CTGTGTCT	AGACACAG
MP2-BC08	GATGCAGA	TCTGCATC
MP2-BC09	AGCATGAG	CTCATGCT
MP2-BC10	AGCTCATG	CATGAGCT
MP2-BC11	CCTCGAAT	ATTCGAGG
MP2-BC12	ATCAAGGC	GCCTTGAT
MP2-BC13	GTGTGAAG	CTTCACAC
MP2-BC14	ACACAGGA	TCCTGTGT
MP2-BC15	GATCCGAA	TTCGGATC
MP2-BC16	ACCATAGG	CCTATGG

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C. Barcode Sequences

Barcode sequences for the Allegro Targeted Genotyping 96-plex Adaptor Plates are given below.

Table 12. Index 1/i7 barcode sequences for Allegro Targeted Genotyping 96-plex Adaptor Plate 1

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CGCTACAT	A05	AGGTTCCT	A09	GCCTTAAC
B01	AATCCAGC	B05	GAACCTTC	B09	ATTCCGCT
C01	CGTCTAAC	C05	AAGTCCTC	C09	ATCGTGGT
D01	AACTCGGA	D05	CCACAACA	D09	GCTACAAC
E01	GTCGAGAA	E05	ATAACGCC	E09	TCTACGCA
F01	ACAACAGC	F05	CCGGAATA	F09	CTCCAATC
G01	ATGACAGG	G05	CCAAGTAG	G09	ACTCTCCA
H01	GCACACAA	H05	AAGGACCA	H09	GTCTCATC
A02	CTCCTAGT	A06	ACGCTTCT	A10	GCCAGAAT
B02	TCTTCGAC	B06	CTATCCAC	B10	AATGACGC
C02	GACTACGA	C06	TGACAACC	C10	GTACCACA
D02	ACTCCTAC	D06	CAGTGCTT	D10	ACGATCAG
E02	CTTCCTTC	E06	TCACTCGA	E10	TAACGTCG
F02	ACCATCCT	F06	CTGACTAC	F10	CGCAACTA
G02	CGTCCATT	G06	GTGATCCA	G10	AACACTGG
H02	AACTTGCC	H06	ACAGCAAG	H10	CCTGTCAA
A03	GTACACCT	A07	TGCTGTGA	A11	TCCTGGTA
B03	ACGAGAAC	B07	CAACACAG	B11	CATCAACC
C03	CGACCTAA	C07	CCACATTG	C11	AGCAGACA
D03	TACATCGG	D07	TAGTGCCA	D11	GAAGACTG
E03	ATCGTCTC	E07	TCGTGCAT	E11	TCTAGTCC
F03	CCAACACT	F07	CTACATCC	F11	CTCGACTT
G03	TCTAGGAG	G07	CATACGGA	G11	CTAGCTCA
H03	CTCGAACA	H07	TGCGTAAC	H11	TCCAACTG
A04	ACGGACTT	A08	CAGGTTCA	A12	GACATCTC
B04	CTAAGACC	B08	AGAACCAG	B12	ACTGCACT
C04	AACCGAAC	C08	GAATGGCA	C12	GTTCCATG
D04	CCTTAGGT	D08	AGGCAATG	D12	ACCAAGCA
E04	CCTATACC	E08	TAGGAGCT	E12	CTCTCAGA
F04	AACGCCTT	F08	CGAACAAC	F12	ACTCTGAG
G04	TCCATTGC	G08	CATTCGTC	G12	GCTCAGTT
H04	CAAGCCAA	H08	AGCCAACT	H12	ATCTGACC

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Table 13. Index 1/i7 Barcode sequences for Allegro Targeted Genotyping 96-plex Adaptor Plate 2

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	AACCTACG	A05	TCGAACCT	A09	CCGTTATG
B01	GCATCCTA	B05	CAAGGTAC	B09	TGTCGACT
C01	CAACGAGT	C05	AGCTACCA	C09	CTCTATCG
D01	TGCAAGAC	D05	CATCCAAG	D09	ACTGCTTG
E01	CTTACAGC	E05	CTCACCAA	E09	CGCCTTAT
F01	ACCGACAA	F05	TCAGTAGG	F09	ATAGGTCC
G01	ACATGCCA	G05	GAACGTGA	G09	TGATCACG
H01	GAGCAATC	H05	AGGAACAC	H09	CGGATCAA
A02	CCTCATCT	A06	CCTAAGTC	A10	TACTAGCG
B02	TACTGCTC	B06	AACGCACA	B10	TGGACCAT
C02	TTACCGAC	C06	GTCAACAG	C10	GCGCATAT
D02	CCGTAACT	D06	ACACCTCA	D10	ATCGCAAC
E02	TTCCAGGT	E06	TATGGCAC	E10	TCAGCCTT
F02	CCATGAAC	F06	CGCAATGT	F10	CATTGACG
G02	TTCCTCCT	G06	ACTCAACG	G10	ACAGGCAT
H02	CCAACTTC	H06	GTCTGCAA	H10	AGGTCTGT
A03	GAGACCAA	A07	CACGATTC	A11	CAGATCCT
B03	ACAGTTCG	B07	AGAAGCCT	B11	CTCCTGAA
C03	CTAACCTG	C07	TACTCCAG	C11	AGAGGATG
D03	TCCGATCA	D07	CGTCAAGA	D11	CACCATGA
E03	AGAAGGAC	E07	CTGTACCA	E11	CGGTAATC
F03	GACGAACT	F07	TCACCTAG	F11	GAGTGTGT
G03	TTGCAACG	G07	AACACCAC	G11	AACTGAGG
H03	CCAACGAA	H07	CGTCTTCA	H11	TGTGTCAG
A04	ATCGGAGA	A08	AACGTAGC	A12	TGTCACAC
B04	CCTAACAG	B08	GCAACCAT	B12	AGATCGTC
C04	CATACTCG	C08	GATCCACT	C12	CAATGCGA
D04	TGCCTCAA	D08	ACCTAGAC	D12	TGCTTGCT
E04	TACAGAGC	E08	CTAGCAGT	E12	AATGGTCG
F04	CGAGAGAA	F08	TCGATGAC	F12	AGTTGTGC
G04	AGGTAGGA	G08	TTGGTGCA	G12	GTATCGAG
H04	GAACGAAG	H08	AGTGCATC	H12	GTACGATC

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D. Frequently Asked Questions (FAQs)

Input Recommendations

Q1. How much DNA should I use as input? Can I use degraded DNA as input?
Allegro is designed to work with 10 to 100 ng of high quality DNA. All samples MUST be normalized prior to starting the protocol in order to obtain equal read representation after pooling.

Q2. Can I pool more than 24 samples? Can I pool fewer than 24 samples?
The protocol has been optimized for hybridization of up to 24 samples at 10 to 100 ng input. There is sufficient probe for a maximum of 4 hybridizations for each 96-reaction kit, or 8 hybridizations in a 192-reaction kit.

General Workflow

Q3. Can I substitute my own fragmentation solution or use a mechanical/sonication method to fragment my DNA?
The fragmentation cocktail has been optimized for use with downstream processing steps. Substitution with other fragmentation methods is not recommended.

Q4. Can I pool the samples prior to bead purification?
It is not recommended to pool the samples until after the bead purification step following Ligation.

Q5. Can I take the sample pools off the thermocycler to add the TX2 enzyme mix?
It is critical that the temperature is maintained at 60 °C while adding the Target Extension Enzyme Mix (TX2) to the hybridization. Mix thoroughly at this step prior to advancing to the 72 °C incubation.

Q6. Where can I stop during the protocol?
You may stop after the bead purification and/or sample concentration steps, as marked in the User Guide by a coffee cup.

Q7. Are there any special considerations when working with the adaptor plate?
Store the adaptor plate at –20 °C and keep on ice at all times, even when thawing the adaptor mixes. It is best practice to re-seal the wells after use to minimize the risk of any cross-contamination.

Sequencing Recommendations

Q8. Can I use the standard Illumina sequencing primers with the Allegro system?
Because Allegro has been designed for the most efficient sequencing of desired targets, it requires the use of a Custom Read 1 sequencing primer. The primer is supplied at 100 µM and should be used per the manufacturer's instructions for custom primers for the specific sequencing platform. A standard Illumina reverse sequencing primer, and standard Illumina Index 1 sequencing primer should be used. For systems with dual indexing, the use of a Custom Index 2 primer will depend upon the platform and run configuration. For more information, please see User Guide section III. Planning the Experiment E. Sequencing Recommendations and Guidelines.

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Q9. What is the optimal sequencer configuration for Allegro?
The system is designed to capture desired targets within a 100 base forward read. A reverse read is not required, but can be used to enhance alignment. Index 1 (and Index 2 in dual indexing configurations) is 8 bases in length.

Data Analysis

Q10. What are your recommendations for read trimming?
See User Guide section III. Planning the Experiment F. Data Analysis Guidance of the User Guide for detailed trimming recommendations. We recommend that the first 40 nt of R1 be trimmed to remove probe-derived sequence.

Users are advised to take precaution when using R2 for variant calling to ensure probe-derived sequence is removed. For more information, contact NuGEN Technical Support.

E. Update History

This document, the Allegro Targeted Genotyping user guide (M01455 v1), has been updated from the previous version to address the following topics:

Description	Section	Page(s)
P6 is an unnecessary reagent to make successful libraries and thus is removed from the kit; protocol is updated to reflect this change.	Throughout	Throughout
Added FAQ section for questions commonly addressed in Technical Support.	V. D.	24
Allegro Targeted Genotyping Library Structure is added to Planning the Experiment	III. E.	8
Allegro Metaplex Module information and guidelines are added to Kit Components, Planning the Experiment and Appendix	II. A., III. G., and V. B.	3, 10, and 21

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