

USER GUIDE

Allegro[®] Targeted Genotyping V2

REF 10050, 10051, 10052, and 10053

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








SYMBOL	MEANING
	Catalog Number
	Consult instructions for use
	Contains sufficient for <n> tests
	Warning
	Important
	Expiration Date
	Temperature limitation
	Protect from light
	Optional stopping point
	Note
	Manufacturer

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I. Introduction

A. Overview

Intended Use

The Allegro Targeted Genotyping V2 system is an end-to-end solution designed to produce custom target-enriched, multiplexed libraries suitable for sequencing on Illumina NGS platforms. This kit is intended for Research Use Only and not for use in diagnostic procedures.

Features

The Allegro Targeted Genotyping V2 kit features a robust enzymatic fragmentation of genomic DNA from intact samples, Tecan's proprietary DimerFree® ligation technology to eliminate adaptor dimer formation, and patented Single Primer Enrichment Technology (SPET) for capturing SNP-specific data point for every on-target sequencing read. The Allegro V2 kit is compatible with the Allegro Metaplex® Module (Part No. 0413, available separately) for multiplexing of up to 3,072 samples to enable large scale genotyping. The Allegro V2 kit is designed to be run as a fully automatable workflow with the DreamPrep™ NGS system, with capability to process up to 75,000 samples/year.

Specifications

Input type:	high quality genomic DNA
Input amount:	10-100 ng
# Reactions available:	192, 384, and 768

B. Storage and Stability



All kits should be unpacked immediately upon receipt and stored as directed below.

The Allegro Targeted Genotyping V2 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.



- The Allegro core kit should be stored at -20 °C on internal shelves of a freezer without a defrost cycle.




Every lot of Allegro Targeted Genotyping V2 undergoes functional testing to meet specifications for library generation performance. This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines are warranted to perform to specifications for 6 months from the date of shipment. Expiration dates listed on the kit label may be longer than the warranty period. Do not use kits that have passed the expiration date.

I. Introduction

C. Warnings and Precautions

1. Unpack and inspect the kits immediately upon receiving. In case of severe kit package damage, no dry ice left in the package or ice pack melted, and/or missing components, please contact Tecan NGS Technical Support immediately. Please provide the kit(s) and/or component(s) part number, and lot number information. Do not use damaged components.
2. Follow your institution's safety procedures for working with chemicals and handling of biological samples. Follow good laboratory practices and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary. Changing gloves between handling samples is recommended to avoid contamination of sample or reagents.
3. Consult your institution's environmental waste personnel on proper disposal of unused reagents. Check state and local regulations as they may differ from federal disposal regulations. This material may exhibit characteristics of hazardous waste requiring specific disposal requirements. Institutions should check their country hazardous waste disposal requirements.
4. If appropriate, an SDS for this product is available on the Tecan website at <https://lifesciences.tecan.com/allegro-targeted-genotyping-v2>

D. Before You Start

 Please review this User Guide before using this kit for the first time, including the “Overview”, “Kit Components”, “Planning the Experiment”, “Protocol” and “FAQ” sections. For more information, visit the Allegro Targeted Genotyping V2 page at <https://lifesciences.tecan.com/allegro-targeted-genotyping-v2>

New to NGS? Contact Tecan NGS Technical Support at techserv-gn@tecan.com for tips and tricks on getting started.

II. Components

A. Reagents Provided

Σ Allegro Targeted Genotyping V2 kit (Part Nos. 10050, 10051, 10052, 10053) contains the reagents listed in Table 1 and Table 2. Allegro Metaplex Module (Part No. 0413) contains the reagents listed in Table 3 and is available separately.

Table 1. Allegro Targeted Genotyping V2 core reagents

COMPONENT	PART NUMBER 192-REACTION	PART NUMBER 384-REACTION	PART NUMBER 768-REACTION	VIAL LABEL	VIAL NUMBER
Fragmentation Buffer Mix	S02370	S02370 (2)	S02452	Orange	F1 VER 5
Fragmentation Enzyme Mix	S02371	S02371 (2)	S02453	Orange	F2 VER 4
Ligation Buffer Mix	S01689	S01689 (2)	S02454	Yellow	L1 VER 4
Ligation Enzyme Mix	S01690	S01690 (2)	S02455	Yellow	L3 VER 4
Allegro V2 96-plex Adaptor Plate 1	S02684	S02684 (2)	S02684 (4)	Yellow	—
Allegro V2 96-plex Adaptor Plate 2	S02685	S02685 (2)	S02685 (4)	Yellow	—
Target Extension Buffer Mix	S01900	S01900 (2)	S01912	Clear	TX1
Target Extension Enzyme Mix	S01902	S01902 (2)	S02181	Clear	TX2
Amplification Buffer Mix	S01903	S01903 (2)	S01893	Red	P1 VER 4
Amplification Primer Mix	S02372	S02372 (2)	S02456	Red	P2 VER 17
Amplification Enzyme Mix	S01764	S01764 (2)	S02407	Red	P3 VER 2
Amplification Enhancer Mix	S01904	S01904 (2)	S02683	Red	P6
Allegro Custom R1 Primer	S02373	S02373 (2)	S02373	Clear	—
Nuclease-free Water	S01113 (2)	S01113 (4)	S01113 (2)	Green	D1
Agencourt Beads	S01502	—		Clear	—

II. Components

Table 2. Allegro Custom Probe Mix

COMPONENT	PART NUMBER	VIAL LABEL	VIAL NUMBER
Allegro Custom Probe Mix (192-reaction only)	30186698	Clear	Varies by custom design, e.g. ST_XXXX
Allegro Custom Probe Mix (384-reaction only)	30186698 (2)	Clear	Varies by custom design, e.g. ST_XXXX
Allegro Custom Probe Mix (786-reaction only)	30186700	Clear	Varies by custom design, e.g. ST_XXXX

Table 3. Allegro Metaplex Module (available separately, Part No. 0413)

COMPONENT	PART NUMBER	VIAL LABEL	VIAL NUMBER
Metaplex Primer Mix	S02435	Red	MP2-BC01
Metaplex Primer Mix	S02436	Red	MP2-BC02
Metaplex Primer Mix	S02437	Red	MP2-BC03
Metaplex Primer Mix	S02438	Red	MP2-BC04
Metaplex Primer Mix	S02439	Red	MP2-BC05
Metaplex Primer Mix	S02440	Red	MP2-BC06
Metaplex Primer Mix	S02441	Red	MP2-BC07
Metaplex Primer Mix	S02442	Red	MP2-BC08
Metaplex Primer Mix	S02443	Red	MP2-BC09
Metaplex Primer Mix	S02444	Red	MP2-BC10
Metaplex Primer Mix	S02445	Red	MP2-BC11
Metaplex Primer Mix	S02446	Red	MP2-BC12
Metaplex Primer Mix	S02447	Red	MP2-BC13
Metaplex Primer Mix	S02448	Red	MP2-BC14
Metaplex Primer Mix	S02449	Red	MP2-BC15
Metaplex Primer Mix	S02450	Red	MP2-BC16
Allegro Custom Index 2 Primer	S02451	Clear	—

II. Components

B. Additional Equipment, Reagents and Labware

Required Materials

- **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 individual tubes, tube strips 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[®] Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA and amplified libraries
- **Reagents**
 - Agencourt[®] AMPure XP or RNAClean XP Beads (Beckman Coulter, Cat. #A63881 or A63987)
 - Ethanol, Absolute (200 Proof), Molecular Biology Grade (Sigma-Aldrich, Cat. #E7023; Fisher Scientific Cat. #BP2818), for purification steps
 - Low-EDTA TE Buffer, 1X, pH 8.0 (Thermo Fisher Scientific, Cat. #J75793)
 - EvaGreen[®] Dye, 20X in water (Biotium, Cat. #31000)
- **Supplies and Labware**
 - Nuclease-free pipette tips
 - 0.5 mL and 1.5 mL nuclease-free microcentrifuge tubes
 - 2.0 mL Eppendorf DNA LoBind tubes (Thermo Fisher Scientific Cat. #O22431048)
 - 8 X 0.2 mL thin-wall PCR strip tubes or 0.2 mL thin-wall plates
 - Magnetic separation plate or rack for 0.2 mL strip tubes or plates (Beckman Coulter Cat. #A29164 or A32782; Thermo Fisher Scientific Cat. #12331D, 12027, or 12332D; Promega Cat. #V8351). Other magnetic stands may be used as well, although their performance has not been validated by Tecan
 - DynaMag[™]-2 Magnet for 1.5 mL tubes (Thermo Fisher Scientific Cat. #12321D)
 - 96-well plate sealing foil (Thermo Fisher Scientific, Cat. #AB1720)
 - Disposable gloves
 - Kimwipes
 - Ice bucket

To Order:

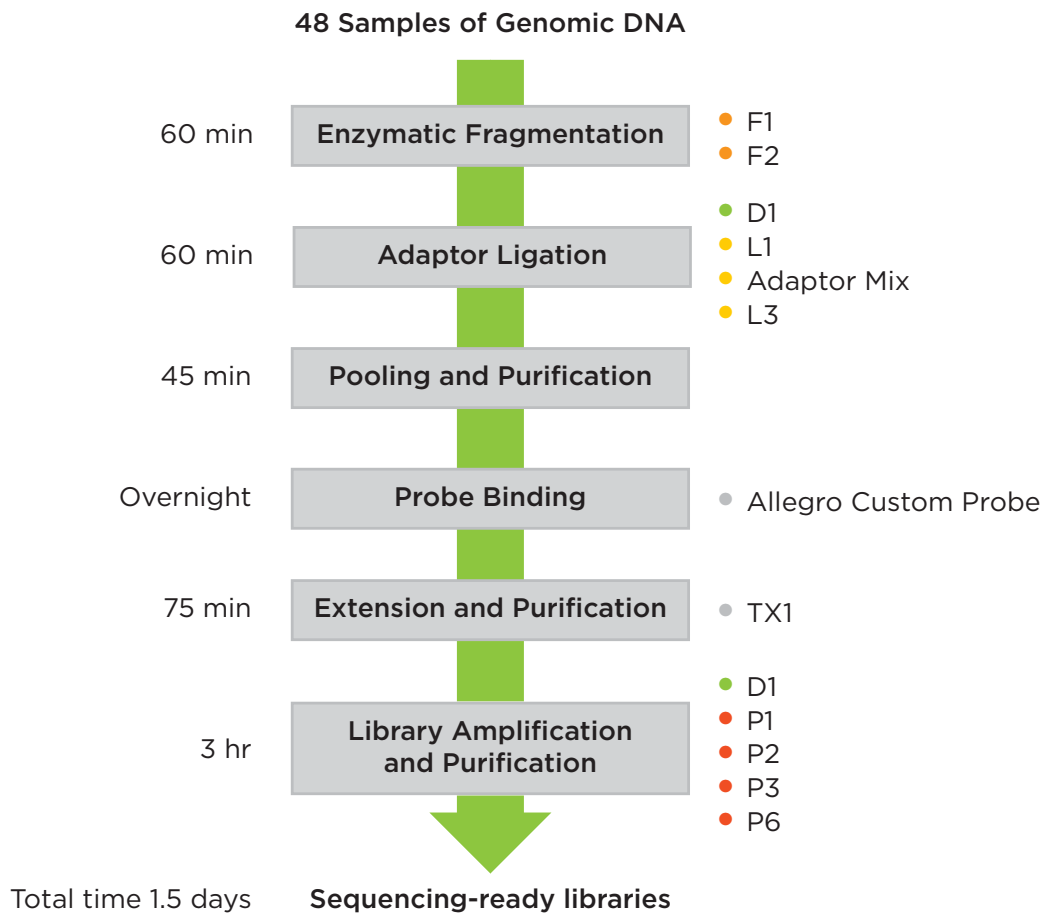
- Agilent, www.agilent.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Fisher Scientific, www.fishersci.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Thermo Fisher Scientific, www.thermofisher.com

III. Planning the Experiment

A. Workflow and Time Required

As shown in **Figure 1**, the simple, add and incubate workflow generates enzymatically-fragmented gDNA samples that are individually ligated with barcoded adaptors. Following ligation, 48 samples of equivalent quantity and quality are pooled together, purified 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 is used to determine 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. Schematic of the Allegro Targeted Genotyping V2 Workflow



III. Planning the Experiment

B. Input DNA Requirements

DNA Quantity

Allegro Targeted Genotyping V2 is designed to work with 10 to 100 ng of intact (non-degraded) DNA samples. Each batch of 48 samples to be processed together should be of equivalent quantity and quality in order to maintain equal representation in the final library. 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.

DNA Purity

DNA samples must be free of contaminating proteins, RNA, organic solvents (including phenol and ethanol) and salts. We recommend using a commercially available system for gDNA isolation. The A260:A280 ratio for DNA samples should be in excess of 1.8 and the A260:A230 ratio in excess of 2.0. Using DNA samples with lower ratios may compromise your results.

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	10 µL
LIGATION		
Program 2 Ligation	25 °C - 30 min, 70 °C - 10 min, hold at 4 °C	19 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	37 °C - 10 min, 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 Library Amplification Optimization protocol in **Appendix B**.

III. Planning the Experiment

D. Working with the 96-Plex Adaptor Plate

The adaptor plates included with Allegro Targeted Genotyping V2 contains adaptor mixes, each with a unique eight-base barcode. Each well contains sufficient volume for preparation of a single library. Allegro Targeted Genotyping V2 adaptor plates are sealed with a foil seal designed to provide airtight storage.

Allegro 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 a new foil seal (e.g., AlumaSeal II) to prevent any remaining ligation adaptor mix from contaminating future reactions.

For details regarding barcode color balancing for multiplex sequencing, please see **Appendix A** on page 23.

E. Magnetic Beads

Ampure XP or RNA Clean XP Beads (Agencourt beads) are suitable for use with this kit. There are modifications to the Agencourt beads' standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads. The bead purification process used for DNA purification before amplification consists of:

- Binding of DNA to beads
- Magnetic separation of beads from supernatant
- Wash of bound beads to remove contaminants
- Elution

Tips and Notes

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes prior to use. Cold beads and buffer will result in reduced recovery.
- Prior to use, ensure beads are fully resuspended by vortexing or inverting and tapping the tube.
- Note that our recommendations in the bead protocols may differ from the standard magnetic bead protocols. Please follow the protocol as written in this guide.
- If using strip tubes or partial plates, ensure they are firmly placed on the magnetic plate. The use of individual tubes is not advised as they are not very stable on the magnetic plates.
- It is critical to let the beads separate on the magnet for the full time indicated at each step. Removing the binding buffer before the beads have completely separated will impact yields.
- After the binding step has been completed, take care to minimize bead loss when removing the binding buffer. Loss of beads at this step may impact yields. With the samples placed on the magnetic plate, carefully remove the specified quantity of binding buffer from each sample to avoid disturbing the beads.
- Ensure that the 70% ethanol wash and 80% acetonitrile wash are freshly prepared from fresh stocks. Lower percent mixes will reduce recovery.
- Unless otherwise directed, do not allow beads to disperse during washes. Keep the samples on the magnet in order to keep the beads on the walls of the plate wells or tubes.


III. Planning the Experiment

- It is critical that all residual ethanol or acetonitrile be removed prior to elution. Therefore, when removing the final wash, first remove most of the supernatant, then allow the excess to collect at the bottom of the tube before removing the remaining supernatant. This also reduces the required bead air-drying time.
- After drying the beads, inspect each tube carefully and make certain that all the ethanol or acetonitrile has evaporated before proceeding with the next step.

F. Library Quantitation

We strongly recommend using a qPCR quantification method for the most accurate quantification of libraries produced with the Allegro Targeted Genotyping V2 system. Depending on the input DNA quality and size, these libraries may produce a broad profile when analyzed with the Agilent Bioanalyzer. In order to avoid underestimating the library concentration, 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.

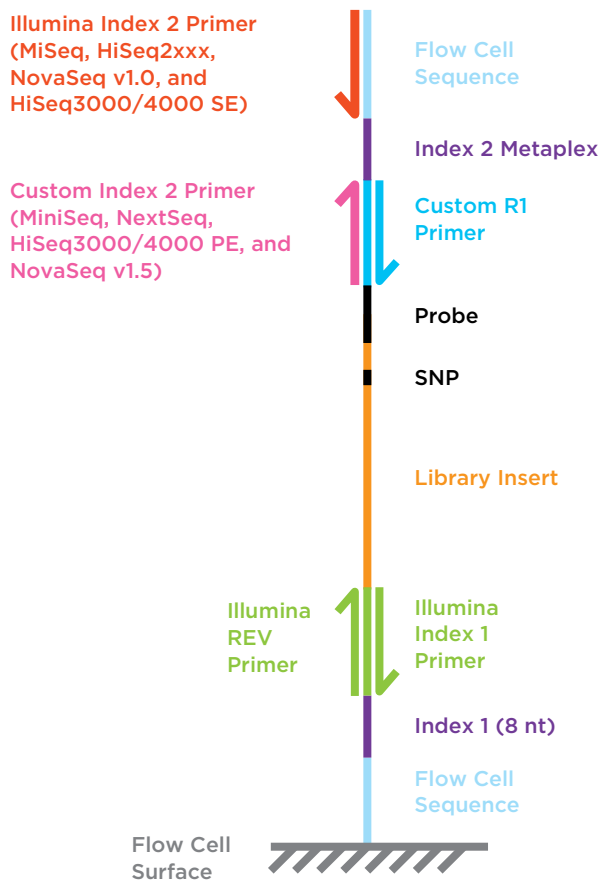
G. Amplified Library Storage

 ^{-20 °C} Amplified libraries may be stored at -20 °C.

III. Planning the Experiment

H. Sequencing Recommendations and Guidelines

Figure 2. Allegro Targeted Genotyping V2 Library Structure



Allegro Targeted Genotyping V2 produces libraries compatible with Illumina NGS platforms. 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. The barcode sequences are found in **Appendix A**.



Important: Allegro Custom R1 primer is **required**; the libraries cannot be sequenced using the standard Illumina Read 1 primer.

The Custom Read 1 primer is supplied at 100 μ M and should be diluted to the appropriate concentration for the chosen sequencing instrument per the manufacturer's instructions. If using the Allegro Metaplex Module (described in **section I. Multiplexing Guidelines for Allegro Metaplex Module (Part No. 0413)**), 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.

III. Planning the Experiment

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 J. 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 A. Barcode Sequences and Guidelines for Multiplex Experiments**.

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. Custom Index 2 primer is required when using NovaSeq v1.5 reagents. See Illumina Doc #1000000022266 for instructions on loading Custom R1 and Index 2 primers.
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.

III. Planning the Experiment

I. 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 Tecan NGS Technical Support (techserv-gn@tecan.com) for the allowable combinations.

Because 48 Index 1/i7 barcodes are required for each pool prior to hybridization, similar considerations for the i7 Index Read are not necessary.

J. Data Analysis Guidance

Single end reads produced from Allegro Targeted Genotyping V2 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 Tecan NGS Technical Support (techserv-gn@tecan.com).

Protocol Notes

General Workflow

- Set up no fewer than 48 reactions at a time to ensure that you are not pipetting very small volumes and to ensure sufficient reagent recoveries for the full nominal number of samples from the kit. Making master mixes for fewer than 48 samples at a time may affect reagent recovery volumes.
- Thaw components used in each step and immediately place them on ice. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed. Do not thaw all reagents at once.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing.
- Keep enzyme mixes on ice after briefly spinning to collect the contents. Do not vortex enzyme or adaptor mixes nor warm any enzyme, adaptor or primer mixes.
- When preparing master mixes, use the minimal amount of extra material to ensure you are able to run the maximum number of reactions using the components provided in the kit.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer from the pipet tip into the reaction mix.
- When instructed to mix via pipetting, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.

Reagents

- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Components and reagents from other Tecan products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the purification protocols.
- Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

IV. Protocol

For each section of the protocol, remove reagents from storage as listed. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

A. Input

Samples must be processed in batch increments of 48 samples (48, 96, 144, etc.). Each batch of 48 samples to be processed together should be of equivalent quantity and quality in order to maintain equal representation in the final library. Dilute each genomic DNA sample to be processed to 10-100 ng in 5 μ L of 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)
1X REACTION VOLUME	3.5 μ L	1.5 μ L

4. Add 5 μ L 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 (10 μ L).
6. Seal tubes/plate and place in a thermal cycler programmed to run Program 1 (Fragmentation, see Table 4):
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 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 to ensure that all adaptors are in the bottom of the wells (for more information on handling adaptor plates, see section III.D.).
4. Using a multichannel pipette, transfer the first column of fragmented samples (10 µL) into the first column in the barcoded adaptor plate. Mix the sample and adaptors well and then transfer the entire volume (13 µL) back into the original reaction tubes or plate.
5. Repeat step 4 for each column of samples and column of barcoded adaptors (i.e. add the second column of samples to the second column of adaptors) until all samples have been mixed with a different barcoded adaptor.
6. Make Ligation Master Mix on ice by combining L1, L3 and D1 as shown in Table 7.



Important: The L1 Ligation Buffer Mix is very viscous. It is critical to pipet this reagent slowly and mix thoroughly.

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)
1X REACTION VOLUME	3.0 µL	0.75 µL	2.25 µL

7. Add 6 µL Ligation Master Mix to each sample.
8. Mix by pipetting up and down several times with a large volume pipettor using a volume larger than half the total reaction volume (19 µL).



Important: The L1 Ligation Buffer Mix is very viscous. It is critical to pipet this reagent slowly and mix thoroughly.

9. Seal tubes/plate on ice and place in a thermal cycler programmed to run Program 2 (Ligation; see Table 4):
25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
10. Remove samples from thermal cycler, spin down and place on ice.

D. Pooling, Purification and Volume Reduction

1. Ensure Agencourt beads are at room temperature before proceeding.
2. Combine 48 reactions with equivalent input quantity and quality into a single 2.0 mL Eppendorf LoBind tube. Total volume should be approximately 900 μ L. If necessary, bring volume up to 900 μ L.



Note: Ensure all samples in the pool have unique barcoded adaptors.

3. Add 450 μ L room temperature Agencourt beads (0.5 volumes) to each 900 μ L pool of 48 samples. Mix thoroughly by repeated pipetting using a mix volume of at least 750 μ L.
4. Incubate bead-sample mixture for 5 minutes at room temperature.



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

5. Transfer the tubes to a magnetic stand and let stand 5 minutes to completely clear the solution of beads.
6. With the tubes still on the magnetic stand, carefully remove 1.3 mL 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 1.5 mL 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. Ensure all the ethanol is removed from each tube.
9. With the tubes still on the magnet, add 1.5 mL of freshly prepared 70% ethanol and allow to stand for at least 30 seconds but no more than 1 minute.
10. Remove the ethanol wash using a pipette. Ensure all the ethanol is removed from each tube.
11. Air dry the beads on the magnet for 5-10 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.
12. Remove the tubes from the magnet.
13. Add 34 μ L of low-EDTA TE buffer to the dried beads. Mix thoroughly by pipetting to ensure all the beads are resuspended. Incubate for 2 minutes at room temperature.
14. Transfer the tubes to the magnetic stand and let stand for 3 minutes to completely clear the solution of beads.

IV. Protocol

- Carefully collect 32 μL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh 0.2 mL strip tube or plate.



Optional stopping point: Store samples at $-20\text{ }^{\circ}\text{C}$.

E. Probe Hybridization

- Remove Target Extension Buffer Mix (clear: TX1) and Allegro Custom Probe Mix from $-20\text{ }^{\circ}\text{C}$ storage and thaw on ice.
- Mix the TX1 and Allegro Targeted Genotyping Custom Probe Mix by vortexing.
- Make Target Extension Master Mix by combining TX1 and Allegro Targeted Genotyping Custom Probe Mix as shown in Table 8.

Table 8. Target Extension Master Mix

REAGENT	TARGET EXTENSION BUFFER MIX (CLEAR: TX1)	ALLEGRO CUSTOM PROBE MIX (CLEAR: VARIES BY CUSTOM DESIGN)
1X REACTION VOLUME	7 μL	10 μL

- Add 17 μL Target Extension Master Mix to each 32 μL pool.
- Mix by pipetting up and down several times with a volume larger than half the total reaction volume (49 μL).
- Seal tubes completely and place in a thermal cycler programmed to run Program 3 (Hybridization and Extension Program; see Table 4):
95 $^{\circ}\text{C}$ – 5 min, 200 cycles (80 $^{\circ}\text{C}$ – 10 s, decrease temp 0.1 $^{\circ}\text{C}$ each cycle), 60 $^{\circ}\text{C}$ hold*, 72 $^{\circ}\text{C}$ 10 min, hold at 4 $^{\circ}\text{C}$



Important: *Incubate at 60 $^{\circ}\text{C}$ for a minimum of 12 hours. Do not remove tubes from the thermocycler. Do not advance to 72 $^{\circ}\text{C}$ until after Target Extension Enzyme (TX2) is added in step 7 below!

- With tubes still in the thermal cycler at 60 $^{\circ}\text{C}$, add 1 μL TX2 to each tube. Mix thoroughly by pipetting with a volume larger than half the total reaction volume (50 μL).
- Reseal the tubes tightly, close the PCR tube lids, close the thermal cycler lid and advance the thermal cycler to the 72 $^{\circ}\text{C}$ step in the Hybridization and Extension Program.
- Remove samples from the thermal cycler, spin down and place on ice.

F. Post-enrichment Purification

- Add 50 μL room temperature Nuclease-free Water (green: D1) to each tube, bringing the total volume to 100 μL .

IV. Protocol

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 5 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 5 minutes at room temperature to allow the beads to clear the solution.
5. While the tubes are still on the magnet, carefully remove 175 μL supernatant, taking care not to disturb or remove any beads.
6. With the tubes on the magnet, add 200 μL 70% Ethanol to each tube, incubate for 30 seconds and then discard ethanol.
7. After completely removing all traces of ethanol with a pipette, allow the tubes to dry at room temp for 10 minutes.
8. Remove the tubes from the magnet.
9. Add 100 μL low-EDTA TE buffer 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 the magnet 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 5 minutes at room temperature.



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

15. Place tubes on the magnet for 5 minutes at room temperature to allow the beads to clear the solution.
16. While the tubes are still on the magnet, carefully remove 175 μL supernatant, taking care not to disturb or remove any beads.
17. With the tubes remaining on the magnet, 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 10 minutes.
19. Remove the tubes from the magnet.
20. Add 25 μL low-EDTA TE buffer 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 the magnet and allow beads to separate for 2 minutes.

IV. Protocol

23. Transfer 24 μL eluted enrichment pool into fresh PCR tubes.



Optional stopping point: Store samples at $-20\text{ }^{\circ}\text{C}$.

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 B** to determine the appropriate number of library amplification cycles for your sample.

1. Remove Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2), Amplification Enzyme Mix (red: P3), and Amplification Enhancer Mix (red: P6) from $-20\text{ }^{\circ}\text{C}$ 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 A. Barcode Sequences and Guidelines for Multiplex Experiments.**

2. Mix P1, P2 and P6 by vortexing. Mix P3 by pipetting. Spin down and place on ice.
3. Make Library Amplification Master Mix by combining P1, P2, P3, P6 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)	AMPLIFICATION ENHANCER MIX (RED: P6)
1X REACTION VOLUME	46 μL	20 μL	8 μL	2 μL	4 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 Program 4 (Amplification; see Table 4):
37 $^{\circ}\text{C}$ – 10 min, 95 $^{\circ}\text{C}$ – 3 min, N(95 $^{\circ}\text{C}$ – 30 s, 62 $^{\circ}\text{C}$ 15 s, 72 $^{\circ}\text{C}$ – 20 s), 72 $^{\circ}\text{C}$ – 2 min, hold at 10 $^{\circ}\text{C}$



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 5 minutes at room temperature.



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

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 magnet, add 200 μL 70% Ethanol to each tube, allow to incubate for 30 seconds and then 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.
8. Add 100 μL low-EDTA TE buffer 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 5 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 5 minutes at room temperature to allow the beads to clear the solution.
15. While the tubes are still on the magnet, carefully remove 175 μL supernatant, taking care not to disturb or remove any beads.
16. With the tubes remaining on the magnet, add 200 μL 70% Ethanol to each tube, allow to incubate for 30 seconds and then remove Ethanol to waste.
17. After completely removing all traces of Ethanol with a pipette, allow the tubes to dry at room temp for 10 minutes.
18. Remove the tubes from the magnet.
19. Add 25 μL low-EDTA TE buffer 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.

IV. Protocol

22. Transfer 24 μL eluted library into fresh PCR tubes.

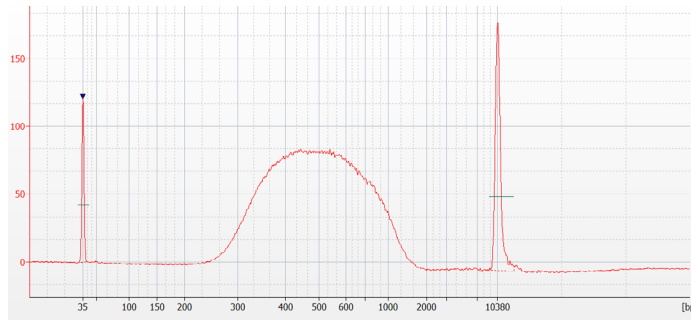


Optional stopping point: Store samples at $-20\text{ }^{\circ}\text{C}$.

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. Normalize and pool libraries following the Illumina guidelines “Best practices for manually normalizing library concentrations” for your specific sequencer. Prepare libraries for sequencing following the Illumina “Denature and Dilute Libraries Guide” for your specific sequencer.

V. Technical Support

For help with any of our products, please contact Tecan NGS Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only) or email techserv-gn@tecan.com.

In Europe contact Tecan NGS Technical Support at +31.13.5780215 (phone) or email europe-gn@tecan.com.

In all other locations, contact your Tecan NGS reagent distributor for technical support.

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A. Barcode Sequences and Guidelines for Multiplex Experiments

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

Table 10. 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	AGGTTCTT	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	TCCAAGTG
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	AACGCCCT	F08	CGAACAAAC	F12	ACTCTGAG
G04	TCCATTGC	G08	CATTGCTC	G12	GCTCAGTT
H04	CAAGCCAA	H08	AGCCAACCT	H12	ATCTGACC

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Table 11. 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	AATGGTCC
F04	CGAGAGAA	F08	TCGATGAC	F12	AGTTGTGC
G04	AGGTAGGA	G08	TTGGTGCA	G12	GTATCGAG
H04	GAACGAAG	H08	AGTGCATC	H12	GTACGATC

Allegro Metaplex Module

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 12.

To use Metaplex sequencing for Allegro libraries, a second barcode must be added to each batch of 48 pooled samples during the Library Amplification step. For each sample pool, substitute the desired metaplex primer mix in place of the Amplification Primer Mix (P2 VER 17).

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 sequencing runs that require the Custom Index 2 sequencing primer,

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you must parse based on the reverse complement of the sequence shown in Table 12. For more information on how to use the Custom Index 2 primer, please see Section III H, I and J of this user guide (pages page 10-12).

Table 12. 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	CTGTGTCT	AGACACAG
MP2-BC03	AGCTCATG	CATGAGCT
MP2-BC04	CGACGATA	TATCGTCG
MP2-BC05	CAGACATC	GATGTCTG
MP2-BC06	TGAGGTAG	CTACCTCA
MP2-BC07	GATCCGAA	TTCGGATC
MP2-BC08	CCTCGAAT	ATTCGAGG
MP2-BC09	GATGCAGA	TCTGCATC
MP2-BC10	AGCATGAG	CTCATGCT
MP2-BC11	ACCATAGG	CCTATGGT
MP2-BC12	ATCAAGGC	GCCTTGAT
MP2-BC13	GTGTGAAG	CTTCACAC
MP2-BC14	ACACAGGA	TCCTGTGT
MP2-BC15	AACAACCG	CGGTTGTT
MP2-BC16	AGACTGCT	AGCAGTCT

B. Library Amplification Optimization with qPCR

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.

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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 substitute Amplification Primer Mix P2 VER 17 with the appropriate Metaplex Primer Mix for each set of samples.

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 µl 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, P6 and 20X EvaGreen reagent by vortexing. Mix P3 by pipetting.
4. Make real-time PCR Master Mix by combining components as shown in Table 13 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 low-EDTA TE buffer. Mix well.

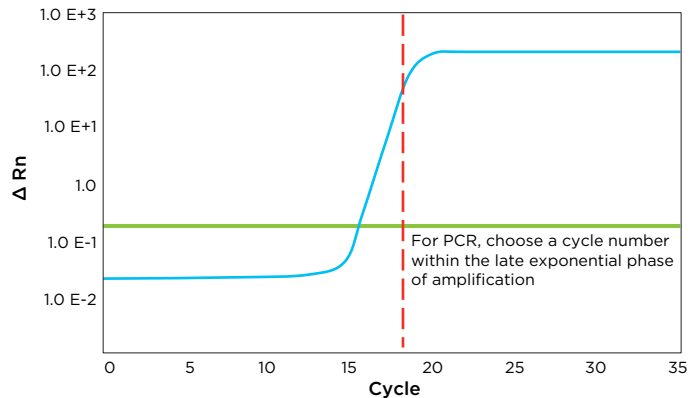
Table 13. Real-Time PCR Master Mix (volumes shown are for a single reaction)

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 17)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)	AMPLIFICATION ENHANCER MIX (RED: P6)	20X EVAGREEN	NUCLEASE-FREE WATER (GREEN: D1)
1X REACTION VOLUME	2.0 µL	0.8 µL	0.2 µL	0.4 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:
37 °C - 10 min; 95 °C - 3 min; 35 cycles (95 °C - 30 s, 62 °C - 15 s, 72 °C - 20 s)

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 section **IV. G. Library Amplification** (step 6). In this example, you would run 18-3 = 15 cycles of PCR.

Figure 4. Stylized qPCR amplification plot.



C. 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. For certain small panels or large genomes, some optimization may be required; please contact Tecan NGS Technical Support.

Q2. Can I pool more than 48 samples? Can I pool fewer than 48 samples?

The protocol has been optimized for hybridization of up to 48 samples of 10 to 100 ng input each. There is sufficient probe for a maximum of 4 hybridizations for each 192-reaction kit, 8 hybridizations in a 384-reaction kit, or 16 hybridizations in a 768-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 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.

Q5. 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.

Q6. 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

Q7. 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 (supplied at 100 µM) will depend upon the platform and run configuration. For more information, please see User Guide section **III. Planning the Experiment H. Sequencing Recommendations and Guidelines**.

Q8. 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

Q9. What are your recommendations for read trimming?

See User Guide section **III. Planning the Experiment J. 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 Tecan NGS Technical Support.

D. Update History

This document, the Allegro Targeted Genotyping V2 user guide (M01501 v2), has been updated from the previous version to address the following topics:

DESCRIPTION	SECTION	PAGE(S)
Changed NuGEN/NuGEN Technologies to Tecan/Tecan Genomics.	Throughout	Throughout
Minor changes and corrections made throughout.	Throughout	Throughout
Content reorganized to streamline User Guide.	Throughout	Throughout
Tecan branding updated throughout.	Throughout	Throughout

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