USER GUIDE

iontorrent

Ion AmpliSeq™ Library Preparation for Human Identification Applications

for use with:
HID-Ion AmpliSeq™ Library Kit
HID-Ion AmpliSeq™ Identity Panel
HID-Ion AmpliSeq™ Ancestry Panel
Ion Xpress™ Barcode Adapters 1–96 Kits
Ion Library Quantitation Kit

Catalog Numbers A26435, A25643, A25642, 4468802

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ThermoFisher SCIENTIFIC

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Not for use in diagnostic procedures.

The HID-Ion AmpliSeq® Identity Panel and the HID-Ion AmpliSeq® Ancestry Panel have been internally tested but have not been validated under SWGDAM guidelines.

The information in this guide is subject to change without notice.

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About This Guide

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

Revision history

Revision	Date	Description
A.0	June 2014	New document.
B.0	September 2014	Change product name to HID-Ion AmpliSeq™ Library Kit.
C.0	May 2015	Include the following bundled kits: • HID-Ion AmpliSeq™ Identity and Library Kit (Cat. no. A26808) • HID-Ion AmpliSeq™ Ancestry and Library Kit (Cat. no. A26807)

Purpose

This guide provides information about the Life Technologies instruments and chemistries associated with the HID-Ion AmpliSeq™ Kits and Panels.

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Product description

This guide covers the following products:

- HID-Ion AmpliSeq™ Library Kit (Cat. no. A26435)
- HID-Ion AmpliSeq™ Identity Panel (Cat. no. A25643)
- HID-Ion AmpliSeq™ Ancestry Panel (Cat. no. A25642)
- HID-Ion AmpliSeq™ Identity and Library Kit (Cat. no. A26808)
- HID-Ion AmpliSeq™ Ancestry and Library Kit (Cat. no. A26807)
- Ion Xpress™ Barcode Adapters 1-96 Kits (various Cat. nos.)
- Ion Library Quantitation Kit (Cat. no. 4468802)

Ion AmpliSeq™products include library preparation reagents and pools of PCR primers for amplification of genomic target regions for sequencing on the Ion PGM™ System.

HID-Ion AmpliSeq™ Library Kit contains reagents for the rapid preparation of libraries containing 12–24,576 amplicons per reaction. These library kits use a plate-based protocol for easy sample handling and tracking, and for compatibility with automation and high-throughput laboratories. When used for Human Identification Applications, this kit requires 1 ng of DNA per target amplification reaction. DNA from a variety of sources, including body fluid and bone sample, can be used as the starting material.

HID-Ion AmpliSeq™ Identity Panel and HID-Ion AmpliSeq™ Ancestry Panel contain pools of PCR primers for amplification of forensically relevant genomic target regions. The primers contain proprietary modifications that enable removal of primer sequences during library preparation, for efficient target assessment during sequencing. HID-Ion AmpliSeq™ Panels are available for order from **www.lifetechnologies.com**

HID-Ion AmpliSeq™ Identity and Library Kit includes the HID-Ion AmpliSeq™ Identity Panel and the HID-Ion AmpliSeq™ Library Kit.

HID-Ion AmpliSeq™Ancestry and Library Kit includes the HID-Ion AmpliSeq™ Ancestry Panel and the HID-Ion AmpliSeq™ Library Kit.

Ion Xpress™ Barcode Adapters 1–96 Kits enable the preparation of barcoded libraries using the above-mentioned library kits. Multiple barcoded libraries can be combined and loaded onto a single Ion Chip to minimize the sequencing run time and cost.

Ion Library Quantitation Kit contains reagents to quantify Ion AmpliSeq™ libraries using quantitative real-time PCR (qPCR) and enables accurate measurement of extremely low quantities of DNA, allowing quantitation of libraries with insufficient material for detection by other methods.

HID-Ion AmpliSeq™ Library Kit

The HID-Ion AmpliSeq™ Library Kit (Cat. no. A26435) is tailored for HID needs and provides reagents for 96 libraries. Customers requiring additional library amplification can purchase Ion AmpliSeq™ Library Kits 2.0 (Cat. nos. 4475345, 4480441, 4480442); for information about amplifying libraries using any of these three kits, refer to the *Ion AmpliSeq™ Library Preparation Guide* (Pub. no. MAN0006735).

Component	Cap color	A26435 (96 reactions)	Storage†
5✕ Ion AmpliSeq™ HiFi Mix	Red	4 × 384 µL	–30°C to –10°C
FuPa Reagent	Brown	4 × 192 µL	
Switch Solution	Yellow	4 × 384 µL	
DNA Ligase	Blue	4 × 192 µL	
Low TE	Clear	4 × 12 mL	Room temp. (15°C to 30°C) *or* –30°C to –10°C

† The kit is shipped on frozen gel packs; store as indicated.

Ion AmpliSeq™ Panels

The HID-Ion AmpliSeq™ Library Kit 2.0 supports the two HID-Ion AmpliSeq™ Panels (available separately or bundled with the HID-Ion AmpliSeq™ Library Kit):

HID-Ion AmpliSeq™ Panels						
Name and catalog number	Conc	Avg library size with barcode adapters	Qty	Volume	No. of primer pairs	Storage
HID-Ion AmpliSeq™ Identity Panel (Cat no. A25643)	2✕	218 bp	1 tube, 96 rxns	960 µL	124	–30°C to –10°C (shipped at room temp.)
HID-Ion AmpliSeq™ Ancestry Panel (Cat no. A25642)	2✕	207 bp	1 tube, 96 rxns	960 µL	165	
HID-Ion AmpliSeq™ Identity and Library Kit (Cat. no. A26808)	For contents and storage information, see the HID-Ion AmpliSeq™ Identity Panel (this table) and the HID-Ion AmpliSeq™ Library Kit (above).					
HID-Ion AmpliSeq™ Ancestry and Library Kit (Cat. no. A26807)	For contents and storage information, see the HID-Ion AmpliSeq™ Ancestry Panel (this table) and the HID-Ion AmpliSeq™ Library Kit (above).					

Ion Xpress™ Barcode Adapters

One or more Ion Xpress™ Barcode Adapters Kits are required for preparing HID-Ion AmpliSeq™ libraries. Each kit includes reagents sufficient for preparing up to 40 HID-Ion AmpliSeq™ libraries per barcode (40 x 16 libraries per kit):

- Ion Xpress™ Barcode Adapters 1–16 Kit (Cat. no. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 Kit (Cat. no. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 Kit (Cat. no. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 Kit (Cat. no. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 Kit (Cat. no. 4474520)
- Ion Xpress™ Barcode Adapters 81–96 Kit (Cat. no. 4474521)

And the complete set of adapters:

- Ion Xpress™ Barcode Adapters 1–96 Kit (Cat. no. 4474517)

Ion Xpress™ Barcode Adapters (each kit includes individually numbered barcodes)				
Component	**Cap color**	**Qty**	**Volume**	**Storage**
Ion Xpress™ P1 Adapter	Violet	1 tube	320 µL	–30°C to –10°C (shipped at room temp.)
Ion Xpress™ Barcode X†	White	1 tube per barcode	20 µL for each barcode	

† Barcode chosen

Required materials and equipment

Description	Supplier	Catalog no.	Qty
One of the following PCR instruments: • GeneAmp® PCR System 9700 Single or Dual 96-well Thermal Cycler • Veriti® 96-well Thermal Cycler • Proflex™ 96-well PCR Systems • AB 2720 Thermal Cycler	Life Technologies	See web product pages	1
MicroAmp™ Optical 96-well Reaction Plates	Life Technologies	N8010560 4306737 (with barcode)	10 plates 20 plates
MicroAmp™ Clear Adhesion Film	Life Technologies	4306311	1
MicroAmp™ Optical Film Compression Pad	Life Technologies	A312639	1 set
Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
DynaMag™-96 Side, or other plate magnet	Life Technologies	12331D	1
Nuclease-free Water	Life Technologies	AM9932	1000 mL
Absolute ethanol	MLS†	N/A	≈ 15 mL
Pipettors, 2-200 µL, and low-retention filtered pipette tips	MLS	N/A	1 each
Ion Library Quantitation Kit	Life Technologies	4468802	1
One of the following Real-time PCR instruments: • Applied Biosystems® 7900 HT • Applied Biosystems® 7500 • StepOne™ • StepOnePlus™ • ViiA™ 7 System • Quant Studio™ 12K Flex Real-Time PCR System	Life Technologies	Various	1
(*Optional*) Eppendorf® MixMate™ tool with PCR 96 Tube holder, for library purification and elution.	Eppendorf	5353 000.014	1

† Major laboratory supplier

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Procedure overview

The HID-Ion AmpliSeq™ Library Kit is used with HID-Ion AmpliSeq™ Panels to amplify target regions using 1 ng of genomic DNA (gDNA).

The resulting amplicons are treated with FuPa Reagent to partially digest the primers and phosphorylate the amplicons. The amplicons are then ligated to Ion Adapters with barcodes and purified.

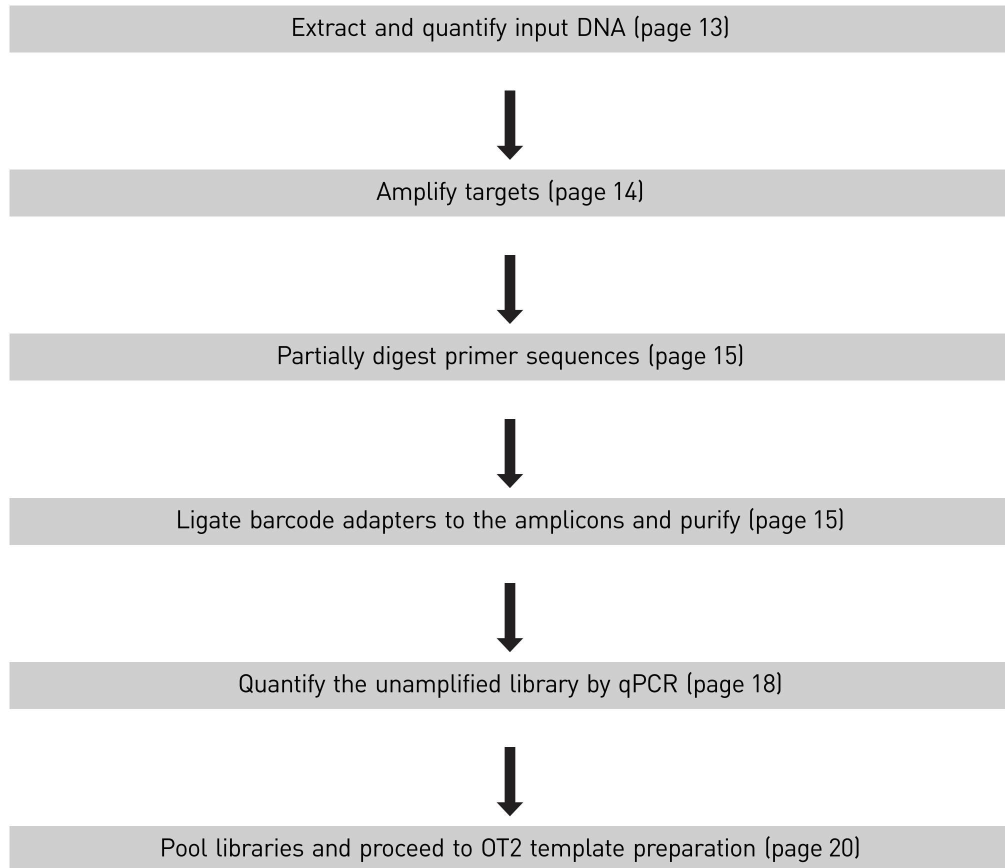
Libraries must be quantified by qPCR using the Ion Library Quantitation Kit.

Completed barcoded libraries can be combined before Ion template preparation and sequencing. Combining libraries maximizes chip use while minimizing cost and labor.

Procedure guidelines

- Thaw components that contain enzymes (such as 5✕ Ion AmpliSeq™ HiFi Mix, FuPa Reagent, and DNA Ligase on ice), and keep on ice during the procedure. All other components, including the HID-Ion AmpliSeq™ Panels, can be thawed at room temperature. Gently vortex and spin down before use.
- If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is separate from template preparation. Always change pipette tips between samples.
- Use a calibrated thermal cycler and real-time PCR instrument specified in "Required materials and equipment" on page 10.
- Pipet viscous solutions slowly; ensure complete mixing by vigorous vortexing or by pipetting up and down several times.

Workflow



Extract and quantify input DNA

Extract DNA

We recommend the PrepFiler Express™ and PrepFiler Express BTA™ Forensic DNA Extraction Kits for extracting and purifying DNA from a variety of forensic sample types:

- The **PrepFiler Express™ Forensic DNA Extraction Kit** (Cat. no. 4441352) is designed for common forensic sample types, including body fluid stains and swabs of body fluids.
- The **PrepFiler Express BTA™ Forensic DNA Extraction Kit** (Cat. no. 4441351) is designed for challenged forensic sample types such as bone, teeth, and adhesive-containing substrates including cigarette butts, chewing gum, and tape lifts.

The kits are appropriate for use with samples containing potential inhibitors of PCR.

Quantify DNA

A number of commercially available kits are suitable for quantifying human DNA. We recommend one of the following kits for quantifying DNA from forensic samples:

- Quantifiler® Duo DNA Quantification Kit (Cat. no. 4387746)
- Quantifiler® Trio DNA Quantification Kit (Cat. no. 4482910)
- Quantifiler® Human DNA Quantification Kit (Cat. no. 4343895)
- Quantifiler® HP DNA Quantification Kit (Cat. no. 4482911)

DNA per reaction

We recommend using 1 ng of input DNA per target amplification reaction for the HID-Ion AmpliSeq™ Identity Panel and the HID-Ion AmpliSeq™ Ancestry Panel.

Amplify targets

1. Using the table below, add the following components to a single well of a 96-well PCR plate. Prepare a master mix for multiple reactions.

HID-Ion AmpliSeq™ Panels (Cat. nos. A25642 and A25643)	
Component	**Volume**
5✕ Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
HID-Ion AmpliSeq™ Identity Panel *or* HID-Ion AmpliSeq™ Ancestry Panel	10 µL
gDNA, 1 ng†	✕ µL
Nuclease-free Water	6 – ✕ µL
Total	**20 µL**

† > 1 ng of gDNA input can be used. Adjust the number of PCR cycles in step 3 accordingly.

2. Seal the plate with MicroAmp® Clear Adhesion Film; ensure a tight seal by applying pressure with an applicator to prevent evaporation. Vortex thoroughly then centrifuge the plate to collect droplets.

3. Place a MicroAmp® Compression Pad on the plate (unless you are using the Veriti® thermal cycler), load the plate in the thermal cycler, then run the following program to amplify target genomic regions.

Stage	Step	Temperature	Time
Hold	Activate enzyme	99°C	2 min
21 cycles†	Denature	99°C	15 sec
	Anneal/Extend	60°C	4 min
Hold	—	10°C	Hold‡

† For > 1 ng of gDNA input, use 18 cycles.
‡ Samples can be held at 10°C overnight.

STOPPING POINT PCR products can be stored at 10°C overnight in the thermal cycler. For longer time periods, store at –20°C.

Partially digest primer sequences

1. Carefully remove the plate seal, then add 2 µL of FuPa Reagent (brown cap) to each amplified sample. The total volume is 22 µL.
2. Seal the plate with MicroAmp® Clear Adhesion Film, vortex thoroughly, then spin-down to collect droplets.
3. Place a MicroAmp® Compression Pad on the plate (unless you are using the Veriti® thermal cycler), load the plate in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

Ligate barcode adapters to the amplicons and purify

Combine and dilute the adapters

1. For each barcode adapter to be used, prepare a 1:4 dilution of Ion P1 Adapter and Ion Xpress™ Barcode X as indicated in the table below. Scale volumes as necessary.

   Note: Diluted barcode adapter mix can be prepared ahead of time and stored at –20°C.

Example of diluted barcode adapter mix for up to 4 reactions	
Component	**Volume**
Ion P1 Adapter	2 µL
Ion Xpress™ Barcode X	2 µL
Nuclease-free Water	4 µL
Total	**8 µL**

Set up and run the ligation reaction

1. If there is visible precipitate in the Switch Solution (yellow cap), vortex at room temperature to resuspend.
2. Carefully remove the plate seal and add the following components to each well that contains digested sample. The Switch Solution and diluted barcode adapter mix can be combined before adding.

Component	Volume
Switch Solution (yellow cap)	4 µL
Diluted barcode adapter mix	2 µL
Total (includes 22 µL of digested amplicon)	**28 µL**

3. Add 2 µL of DNA Ligase (blue cap) to each well.
4. Seal the plate with MicroAmp® Clear Adhesion Film, vortex thoroughly, then spin-down to collect droplets.
5. Place a MicroAmp® Compression Pad on the plate (unless you are using the Veriti® thermal cycler), load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 min
72°C	10 min
10°C	Hold (for up to 1 hour)

STOPPING POINT Samples can be stored at –20°C.

Purify and elute the unamplified library

IMPORTANT! Bring the AMPure® XP Reagent to room temperature, then vortex thoroughly to disperse the beads before use. Pipet the solution slowly.

Freshly prepare 70% ethanol for the next steps: Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.

Do **NOT** substitute a Dynabeads®-based purification reagent for the Agencourt® AMPure® XP Reagent.

1. Carefully remove the plate seal and add 45 µL (1.5✕ sample volume) of Agencourt® AMPure® XP Reagent to each library.
2. Seal the plate with MicroAmp® Clear Adhesion Film, vortex thoroughly, then perform a quick spin to collect droplets. Incubate the mixture for 5 minutes at room temperature.

Alternatively, use a plate mixer (such as the Eppendorf® MixMate™ tool with the Tube Holder PCR 96) to mix the bead suspension at room temperature. Seal the plate, then use the following program:

Eppendorf® MixMate™ tool parameter	Setting
Time	5 min
Speed	2000 rpm

After mixing the bead suspension, spin the plate to collect droplets.

3. Place the plate in a magnetic rack (such as the DynaMag™-96 Side Magnet) and incubate for 2 minutes or until the solution clears. Carefully remove and discard the supernatant without disturbing the pellet.
4. Add 150 µL of freshly prepared 70% ethanol. To wash the beads, move the plate from side-to-side in the two positions of the magnet, then remove and discard the supernatant without disturbing the pellet.

   Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up-and-down five times (with the pipette set at 100 µL). Return the plate to the magnet and incubate for 2 minutes or until the solution clears.
5. Repeat step 4 for a second wash.
6. Perform a quick spin, then place the plate in the magnetic rack until the solution clears. Remove residual ethanol drops using a pipette set at 20 µL.
7. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. **Do not overdry**.

   Note: Residual ethanol drops will inhibit library amplification. Ensure that all ethanol droplets have been removed from the wells.
8. Remove the plate from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads. Seal the plate with MicroAmp® Clear Adhesion Film, vortex thoroughly, then spin-down to collect droplets.
   Alternatively, use a plate mixer (such as the Eppendorf® MixMate™ tool with the Tube Holder PCR 96) to mix the bead suspension at room temperature. Seal the plate, then use the following program:

Eppendorf® MixMate™ tool parameter	Setting
Time	5 min
Speed	2000 rpm

After mixing the bead suspension, spin the plate to collect droplets.

STOPPING POINT Samples can be stored with beads at 4°C for up to 1 month. For long-term storage at –20°C, place the plate in the magnet and transfer the purified samples to a new plate to prevent the beads from shattering.

Quantify the unamplified library by qPCR

Dilute the unamplified library

After eluting the unamplified Ion AmpliSeq™ library, determine concentration by qPCR using the Ion Library Quantitation Kit (Cat. no. 4468802). After quantification, determine the dilution factor that results in a concentration of ~20 pM.

1. If samples have been stored at 4°C, vortex the plate and spin-down to collect droplets.
2. Place the plate in the magnet for 2 minutes or until the supernatant clears.
3. Prepare a 1:100 dilution by removing 2 µL of supernatant and combining with 198 µL of Nuclease-free Water for quantification.
4. After removing the aliquot, store the plate at 4°C.

Prepare the qPCR reactions

After eluting the unamplified Ion AmpliSeq™ library, determine the concentration by qPCR following the steps below. Each sample, standard, and no-template control (NTC) should be analyzed in duplicate 20-µL reactions.

1. Thaw the *E. coli* DH10B Ion Control Library on ice.
2. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library according to the table below. Mark these as standards and use these concentrations in the qPCR instrument software.

Standard	Control Library volume	Nuclease-free Water volume	Concentration
1	5 µL (undiluted)	45 µL	6.8 pmol
2	5 µL Std 1	45 µL	0.68 pmol
3	5 µL Std 2	45 µL	0.068 pmol

3. Thaw the frozen TaqMan® reagents on ice. Gently but thoroughly mix each thawed component, then briefly centrifuge to bring contents to the bottom of the tube.
4. Prepare duplicate reaction mixtures for each sample, standard, and NTC. The qPCR reaction mix for one reaction is described in the table below. Scale the volumes based on the number of qPCR reactions.

Component	Volume (1 reaction)
Ion Library TaqMan® qPCR Mix	10 µL
Ion Library TaqMan® Quantitation Assay, 20X	1 µL

5. Dispense 11-µL aliquots of the qPCR reaction mix into each well of a PCR plate.
6. Add 9 µL of the diluted (1:100) HID-Ion AmpliSeq™ Library to each appropriate well (2 reaction wells per sample library as noted previously) for a total reaction volume of 20 µL.
7. Add 9 µL of the diluted control library to each appropriate well (2 reaction wells per standard) for a total reaction volume of 20 µL.

8. Add 9 µL of Nuclease-free Water to the NTC wells for a total reaction volume of 20 µL.
9. Seal the plate and spin-down to collect droplets.

Run the real-time PCR reactions

1. Program the real-time PCR instrument as follows:
   - Enter the concentrations of the control library standards.
   - Use ROX™ Reference Dye as the passive reference dye.
   - Select a reaction volume of 20 µL.
   - Select FAM™ dye/MGB as the TaqMan® probe reporter/quencher.
   - Use the appropriate thermal cycling conditions according to the following table:

Life Technologies Real-Time PCR System	Stage	Temperature	Time
7500 System *or* 7500 Fast System with SDS Software v1.2.3	Hold	50°C	2 min
	Hold	95°C	20 sec
	40 cycles	95°C	3 sec
		60°C	32 sec
7500 System *or* 7500 Fast System with HID Real-Time PCR Analysis Software v1.1/v.1.2	Hold	50°C	2 min
	Hold	95°C	20 sec
	40 cycles	95°C	3 sec
		60°C	30 sec
7900 HT System 7900 HT Fast System ViiA™ 7 System StepOne™ System StepOnePlus™ System	Hold	50°C	2 min
	Hold	95°C	20 sec
	40 cycles	95°C	1 sec
		60°C	20 sec

2. Place the plate in the real-time PCR instrument, run the reactions, and collect the real-time data.

Determine the template dilution factor (TDF)

1. After the run is completed, calculate the average concentration of the undiluted library.

   Avg concentration of the undiluted library = (qPCR quantity mean) x (library dilution)

   For Example:

   - The qPCR quantity mean is 3 pM.
   - The sample library dilution is 100.

     The average concentration of the undiluted library = (3 pM) x (100) = 300 pM

2. Based on the average library concentration, calculate the template dilution factor (TDF) that results in a concentration of ~20 pM for each library.

   TDF = [Avg concentration of undiluted library] / 20 pM

   Using the example above:

   - The average concentration of the undiluted library is 300 pM.
   - The TDF = [300 pM]/20 pM = 15

3. Dilute the sample library in Nuclease-free Water using the calculated TDF.

   Using the example above:

   If the TDF is 15, then the sample library is diluted 1:15 before performing template preparation using the OT2.

Note: To ensure accurate dilution of sample library, avoid pipetting volumes of 1 µL or less. For the above example of a 1:15 dilution, dilute 2 µL of sample library in 28 µL of Nuclease-free Water.

Pool the libraries (Optional)

After diluting the sample library to 20 pM, multiple diluted libraries can be pooled equi-volume before running on the OT2 and PGM.

Store the libraries

Libraries can be stored at 4°C with AMPure® XP Reagent beads still in the wells for up to 1 month.

For long-term storage, place the plate in the magnet for 2 minutes, then transfer supernatant containing the library to a new plate. Seal the new plate and store at –20°C.

Dilute libraries for OT2 template preparation

IMPORTANT! HID-Ion AmpliSeq™ Libraries must be diluted immediately before preparing the OT2 amplification solution.

1. Ensure that sample library (or pooled sample libraries) has been previously diluted to 20 pM using the TDF calculated by qPCR.
2. Aliquot 10 µL of the 20-pM sample library into a new Eppendorf® LoBind® tube.
3. Add 15 µL of Nuclease-free Water to the Eppendorf® LoBind® tube for a total volume of 25 µL.
4. Add the entire 25 µL of diluted library to the amplification solution for OT2 template preparation.

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Overview

About this chapter

This chapter describes the results of the developmental experiments performed using the HID-Ion AmpliSeq™ Identity Panel and the HID-Ion AmpliSeq™ Ancestry Panel for use with forensic samples on the Ion Personal Genome Machine™.

About the technology

Ion AmpliSeq™ technology enables complex PCR multiplexing while minimizing primer-dimer formation. Both the HID-Ion AmpliSeq™ Identity Panel and the HID-Ion AmpliSeq™ Ancestry Panel contain PCR primers for amplification and use a recommended sample input of 1 ng of genomic DNA. We chose conditions that produced optimum PCR product yield and that met reproducible performance standards.

HID-Ion AmpliSeq™ Panel design

Identity Panel

The HID-Ion AmpliSeq™ Identity Panel is made up of 124 markers; 90 autosomal markers and 34 upper Y-clade markers. The autosomal SNPs are from Ken Kidd's 45-unlinked set (Pokstis, et al. 2010) and from the SNPforID set (Kosoy, R., et al. 2009). The average size for autosomal markers is 132 bp. The average size for upper Y-clade markers is 141 bp (Karafet, T.M. et al. 2008). Amplicon size distributions are shown in Figure 1. The chromosomal location of the SNPs in the HID-Ion AmpliSeq™ Identity Panel is shown in Figure 2.

Figure 1 Amplicon size distributions of SNP subsets in the HID-Ion AmpliSeq™ Identity Panel.

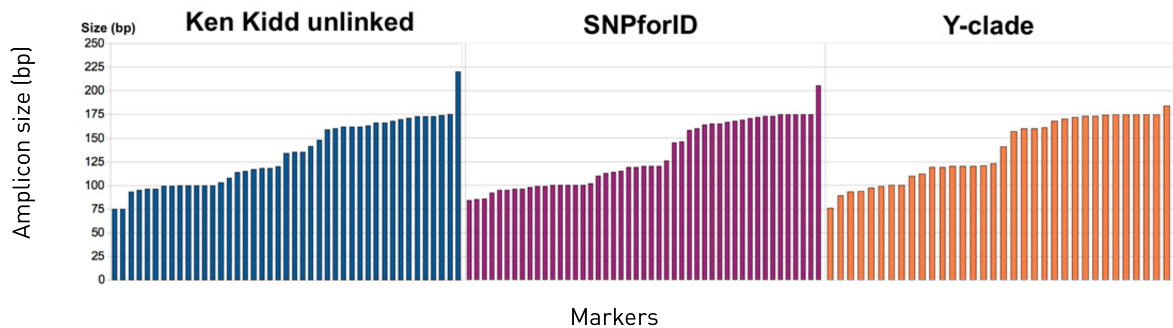
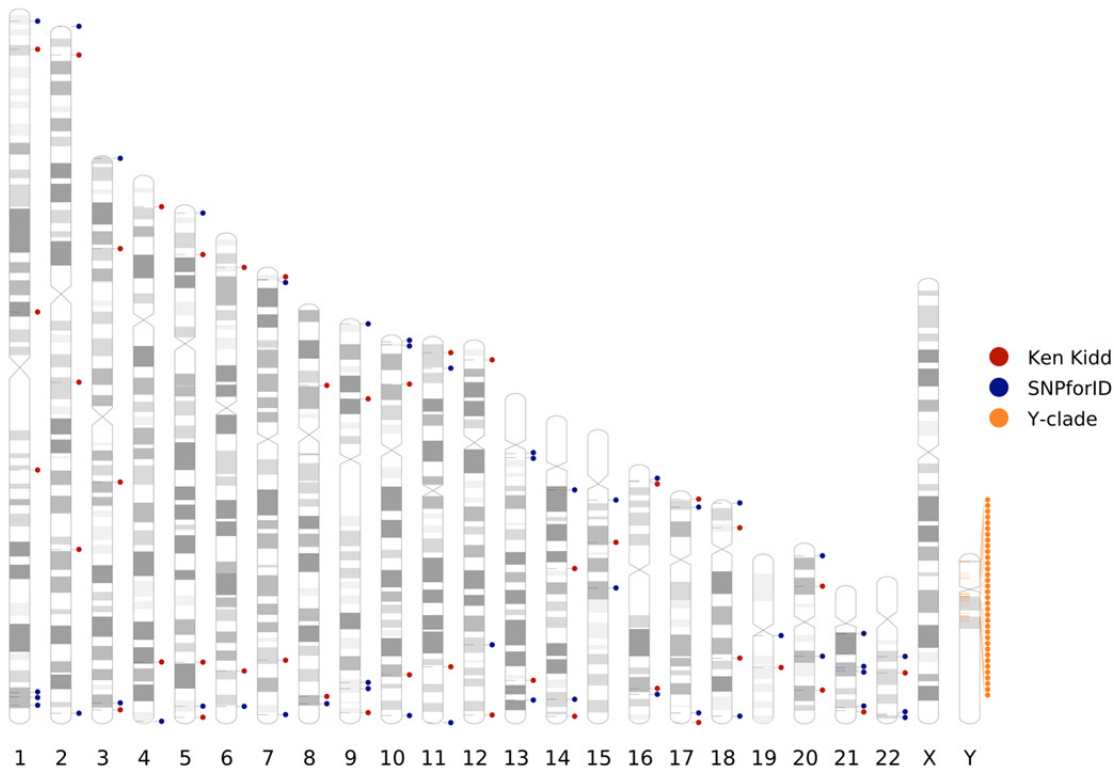


Figure 2 Chromosomal location of identity SNPs in the HID-Ion AmpliSeq™ Identity Panel.



Ancestry Panel

The HID-Ion AmpliSeq™ Ancestry Panel is made up of 165 autosomal markers; 55 from Ken Kidd's AIM set (Kidd, K.K., et al. 2012) and 123 Seldin markers (Nassir, R. et al. 2009). The average size of the Ken Kidd markers is 130 bp. The average size of the Seldin markers is 122 bp. Amplicon size distributions are shown in Figure 3. The chromosomal location of the SNPs in the HID-Ion AmpliSeq™ Ancestry Panel is shown in Figure 4.

Figure 3 Amplicon size distributions of SNP subsets in the HID-Ion AmpliSeq™ Ancestry Panel.

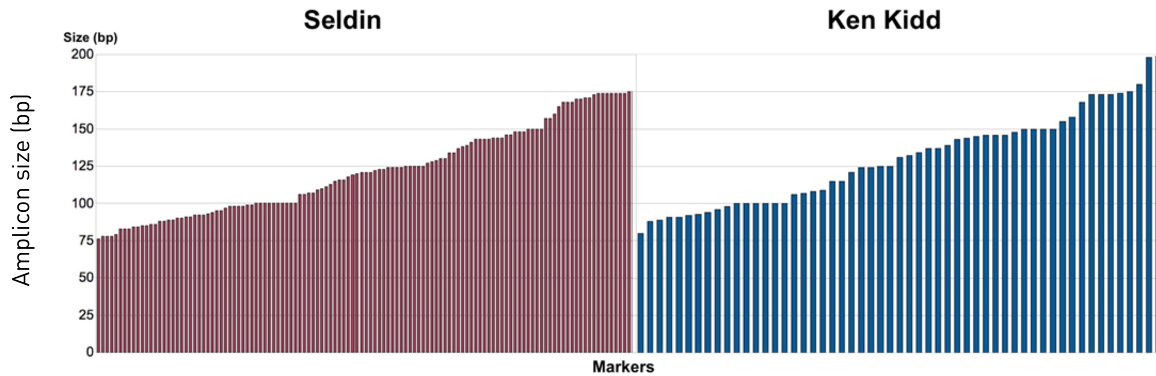
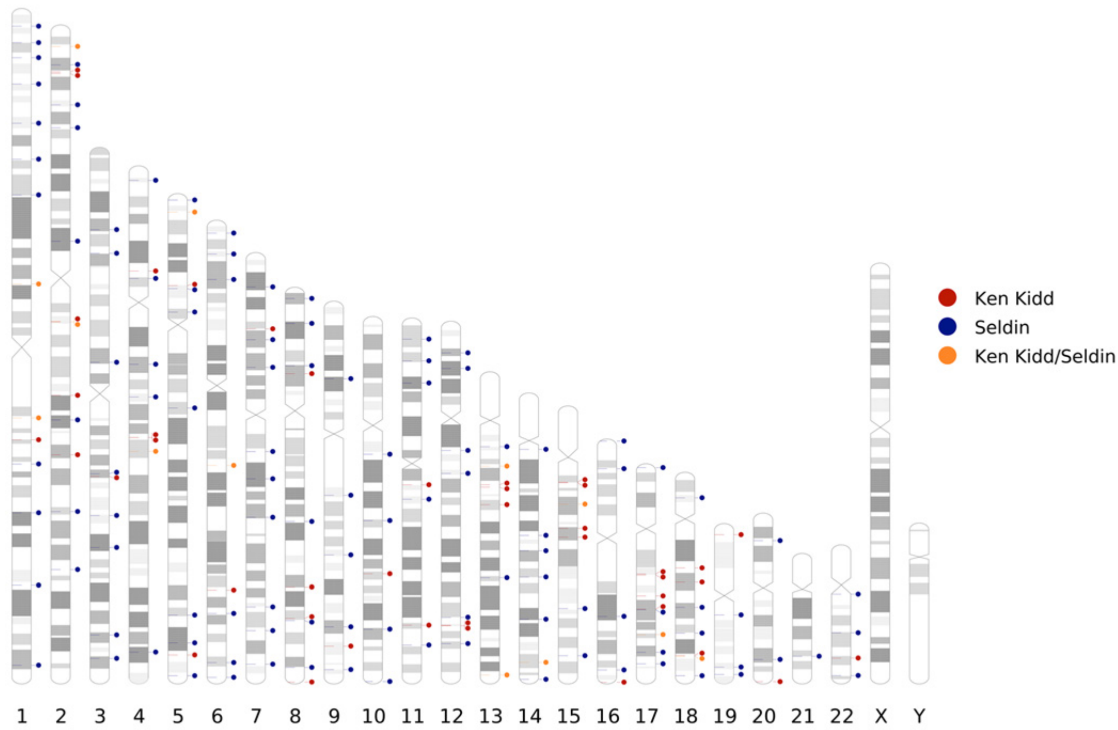


Figure 4 Chromosomal location of identity SNPs in the HID-Ion AmpliSeq™ Ancestry Panel.



Reference DNA samples

Identity Panel

Thirty identity DNA samples and corresponding TaqMan® genotype data for 46 Ken Kidd SNPs were provided by Dr. Ken Kidd (Pakstis, A.J. et al. 2010). These samples were used in the development of the HID-Ion AmpliSeq™ Identity Panel.

The input genomic DNA per PCR reaction ranged from 180 pg to 1 ng. The resulting 30 library samples were quantitated by qPCR using the Ion Library Quantitation Kit. Library samples were diluted and pooled before template preparation and sequenced on a single Ion 318™ Chip v2.

Ancestry Panel

A total of 24 ancestral DNA samples and corresponding TaqMan® genotype data for 55 Ken Kidd SNPs were provided by Dr. Ken Kidd (Yale University). These samples were used in the development of the HID-Ion AmpliSeq™ Ancestry Panel.

The input genomic DNA per PCR reaction was 1 ng. Using the same workflow as in testing of the identity samples, the resulting ancestral library samples were quantitated by qPCR, diluted, pooled, and sequenced on a single Ion 318™ Chip v2.

Performance metrics and panel evaluation

For the development of the HID-Ion AmpliSeq™ panels, a metric system was developed to assess marker selections and panel robustness.

The metrics used in evaluating marker selections and overall panel performance are:

- Amplicon Strand Balance (Figures 5 and 6 on page 25)
- Amplicon Heterozygosity Balance (Figure 7 on page 25 and Figure 8 on page 26)
- Amplicon Coverage (Figures 9 and 10 on page 26)

These metrics were evaluated using data sets generated from the 30 samples provided by Ken Kidd.

The Amplicon Strand Balance is defined as the ratio of the minimum coverage strand counts to the maximum coverage strand counts. The average Amplicon Strand Balance should be 50±20% and have a strand ratio of >0.45 (see Figure 11 on page 27). The target average Amplicon Heterozygosity Balance is 50±15%. The average Amplicon Coverage across all markers should be within 2 standard deviations from the mean (see Figure 12 on page 27).

Sequence coverage for both forward and reverse strands was checked. To minimize heterozygosity bias, any amplicon where sequencing of one strand was favored over the other strand was removed from the panel. Minimizing heterozygosity bias is essential for ensuring equal representation of alleles, and eliminating false allele calls. Variability in amplicon coverage across the panel was minimized by removing markers with lower sequencing performance as compared to other markers.

Figure 5 Amplicon Strand Balance for the autosomal SNPs in the HID-Ion AmpliSeq™ Identity Panel across all markers. Target average Amplicon Strand Balance is 50±20%. 95% of all markers were between 50–90%.

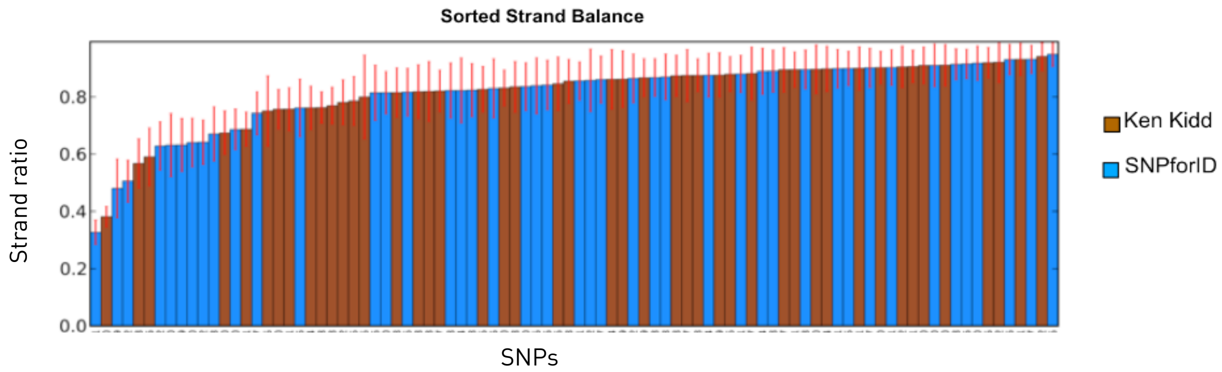


Figure 6 Amplicon Strand Balance for the HID-Ion AmpliSeq™ Ancestry Panel across all markers. Target average Amplicon Strand Balance is 50±20%. All markers were between 60–90%.

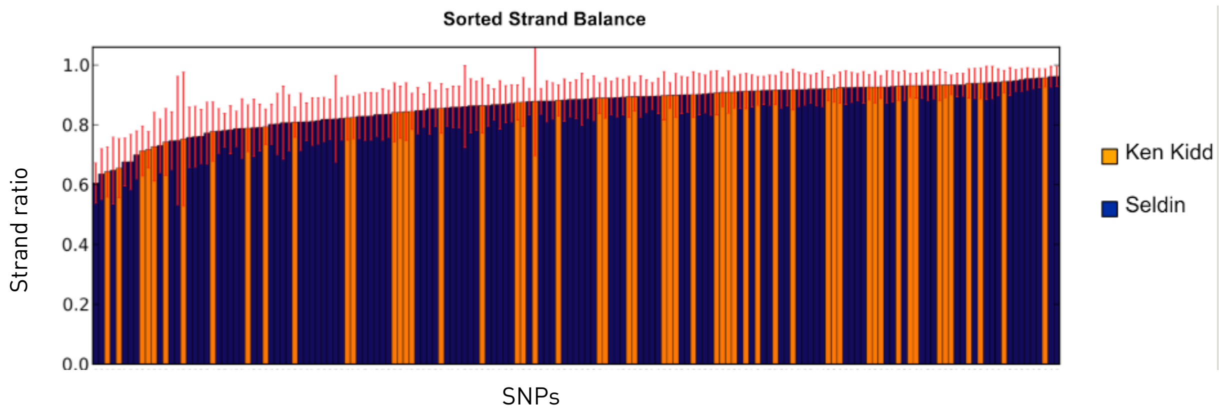


Figure 7 Average Amplicon Heterozygosity balance of autosomal SNPs in the HID-Ion AmpliSeq™ Identity Panel across all markers. Target average heterozygosity is 50±15%. The average heterozygosity across all markers was 35–45%.

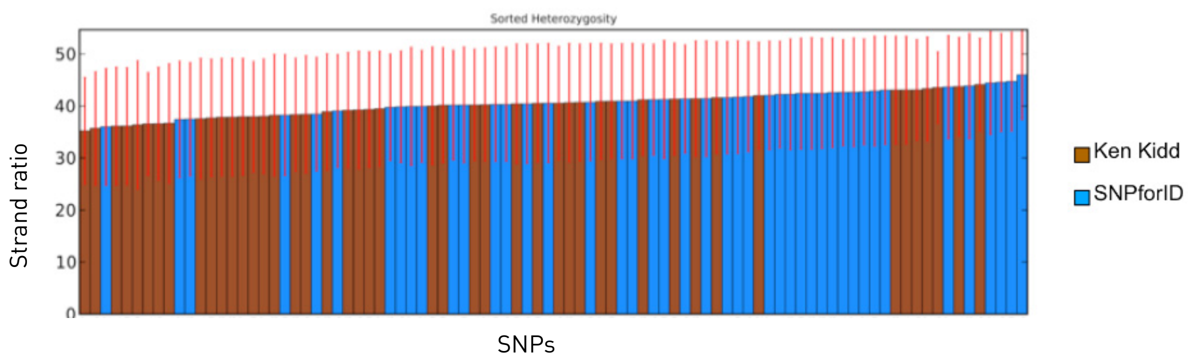


Figure 8 Average Amplicon Heterozygosity balance for the HID-Ion AmpliSeq™ Ancestry Panel across all markers. Target average heterozygosity is 50±15%. The average heterozygosity across all markers was 37–50%.

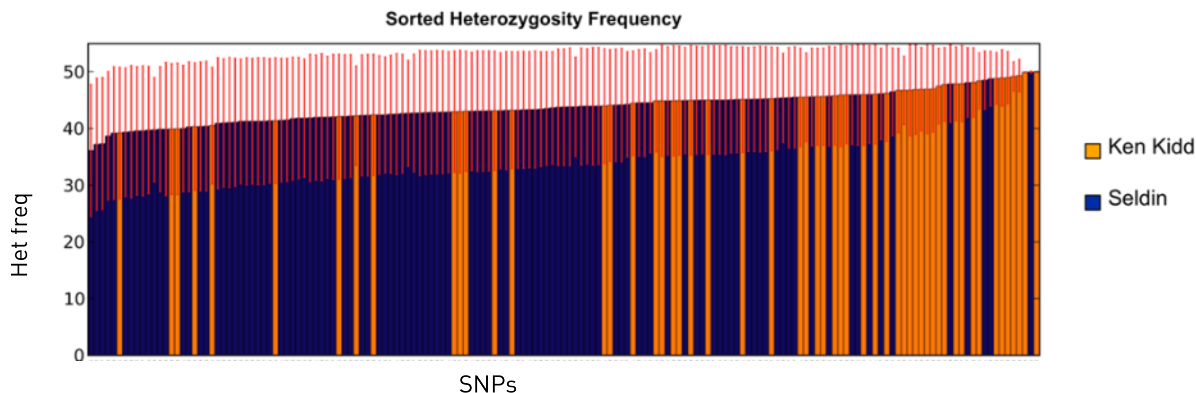


Figure 9 Amplicon Coverage for autosomal SNPs in the HID-Ion AmpliSeq™ Identity Panel across all markers. 96% of all markers were within 2 standard deviations of the mean.

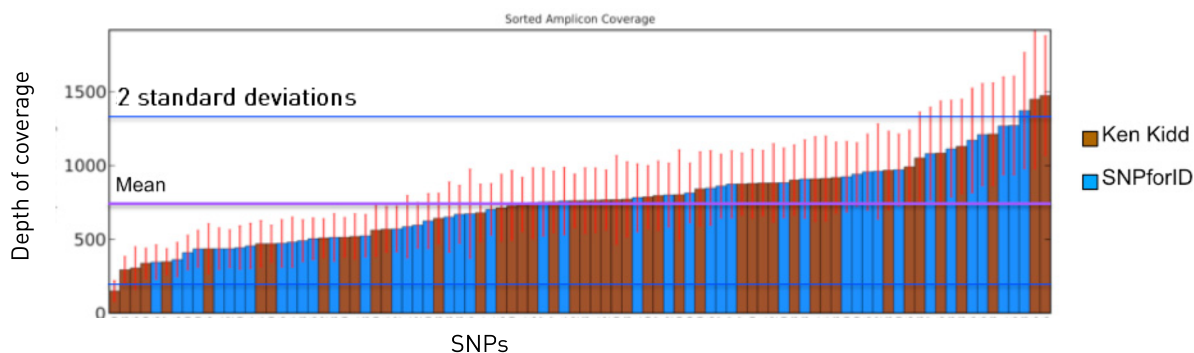


Figure 10 Amplicon Coverage for the HID-Ion AmpliSeq™ Ancestry Panel across all markers. 98% of all markers were within 2 standard deviations of the mean.

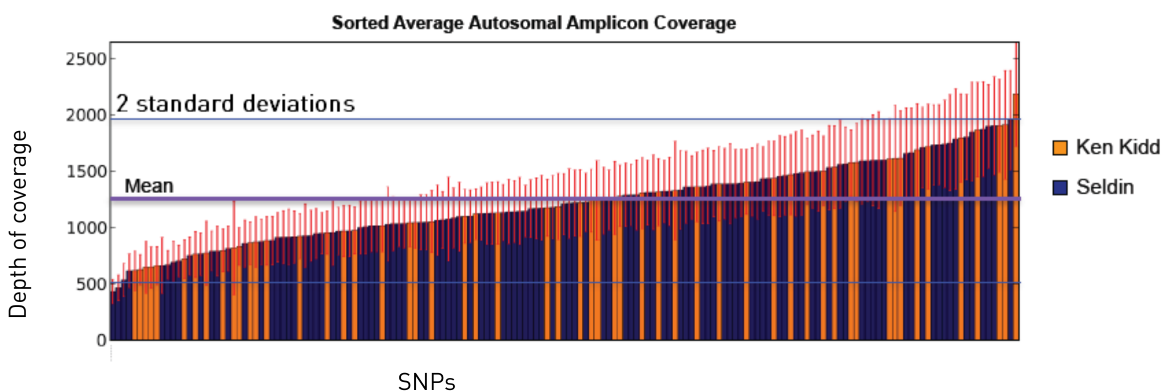


Figure 11 Amplicon Strand Balance for Y-Clade Markers in the HID-Ion AmpliSeq™ Identity Panel. 95% of the markers have an average amplicon strand balance between 70-90%.

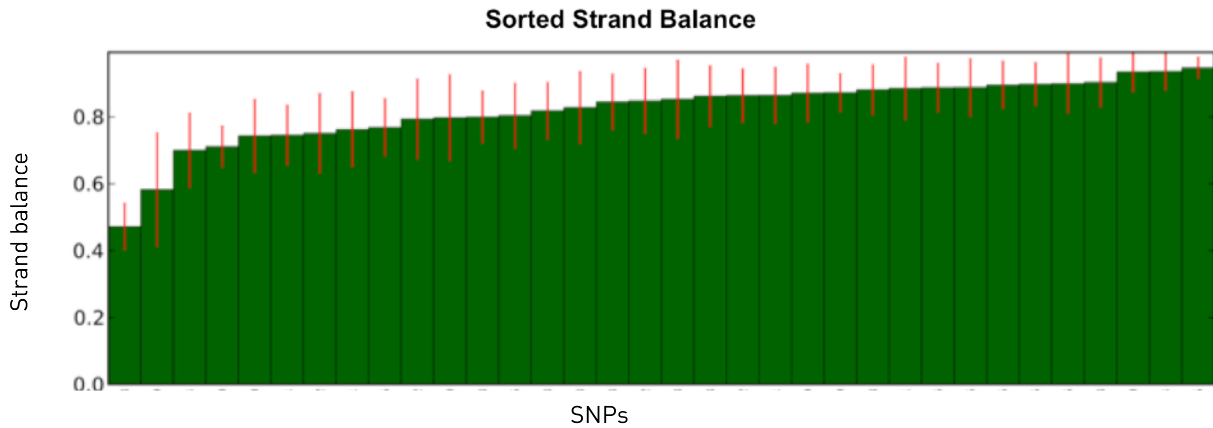
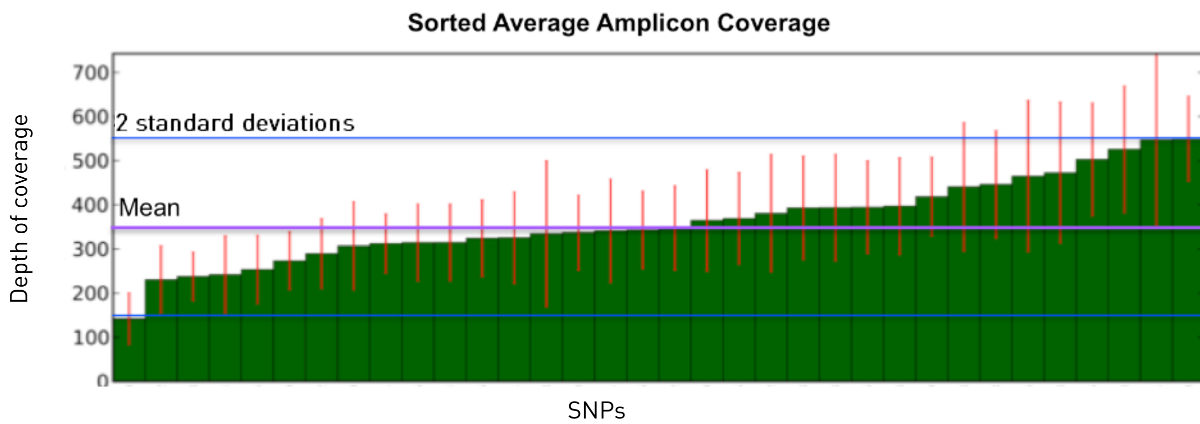


Figure 12 Average Amplicon Coverage for Y-Clade Markers in the HID-Ion AmpliSeq™ Identity Panel. 97% of the markers have an average amplicon coverage within 2 standard deviations of the mean.



Concordance results

The HID SNP Genotyper Plugin was used to analyze sequencing data for both the HID-Ion AmpliSeq™ Identity Panel and the HID-Ion AmpliSeq™ Ancestry Panel. Concordance results from genotype calls made using the plugin analysis output were compared to the reference TaqMan® genotype data provided by Ken Kidd. Concordance data was analyzed in the following subgroups:

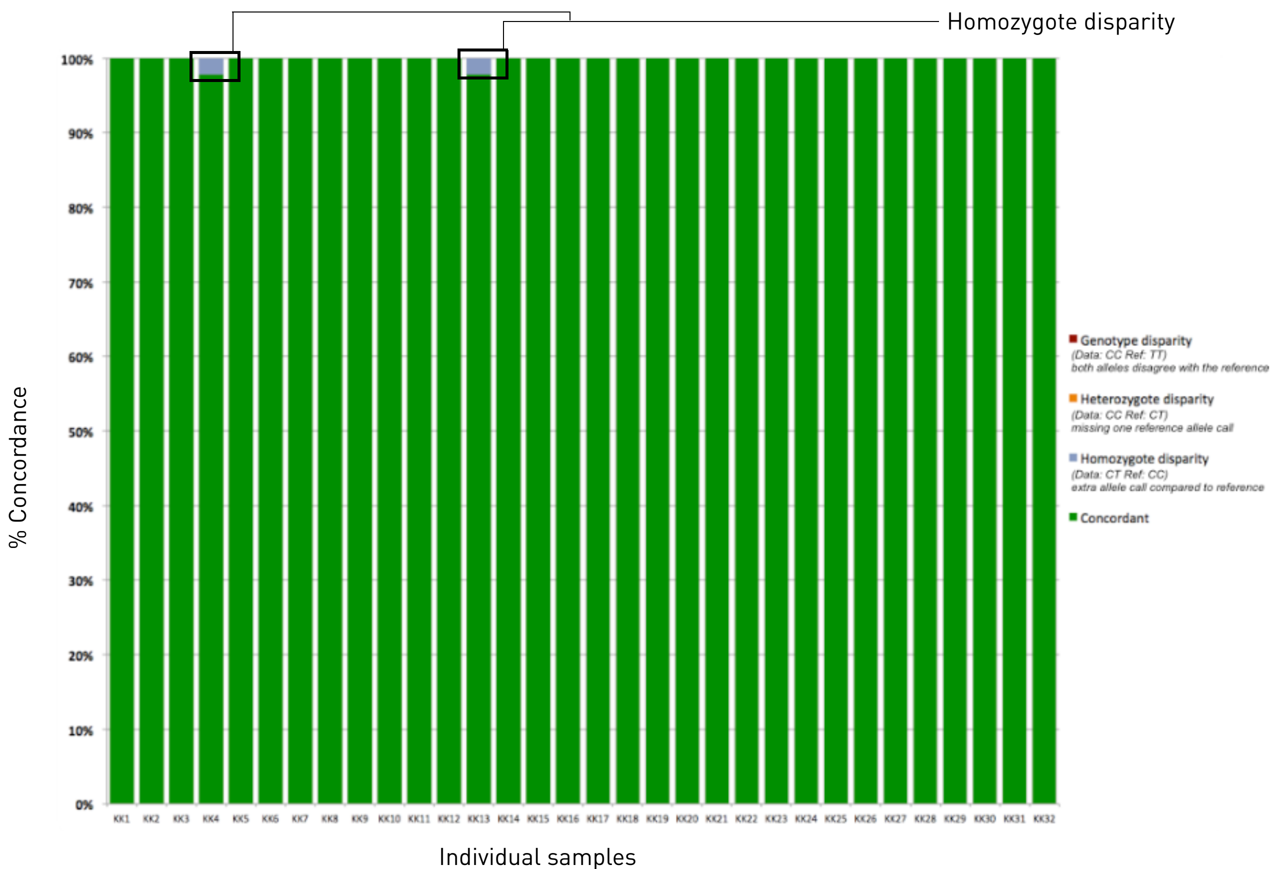
- Genotype disparity (both alleles in the result disagree with the reference)
- Heterozygote disparity (data appears to have a dropout/the loss of one allele)
- Homozygote disparity (data has an additional allele)
- Concordant (both alleles match the reference)

A 10% minor allele frequency threshold was applied to genotype calls made using the plugin.

Identity Panel

Genotype data for 32 individuals was analyzed for concordance (see Figure 13). Library samples were prepared using 180 pg to 1 ng of input gDNA per PCR reaction, and were sequenced on a single Ion 318™ Chip v2. TaqMan® genotype data for the 32 individuals was provided for 46 Ken Kidd SNPs. Two homozygote disparities were observed. Genotype data between calls made using the plugin and the TaqMan® data was 99.99% concordant.

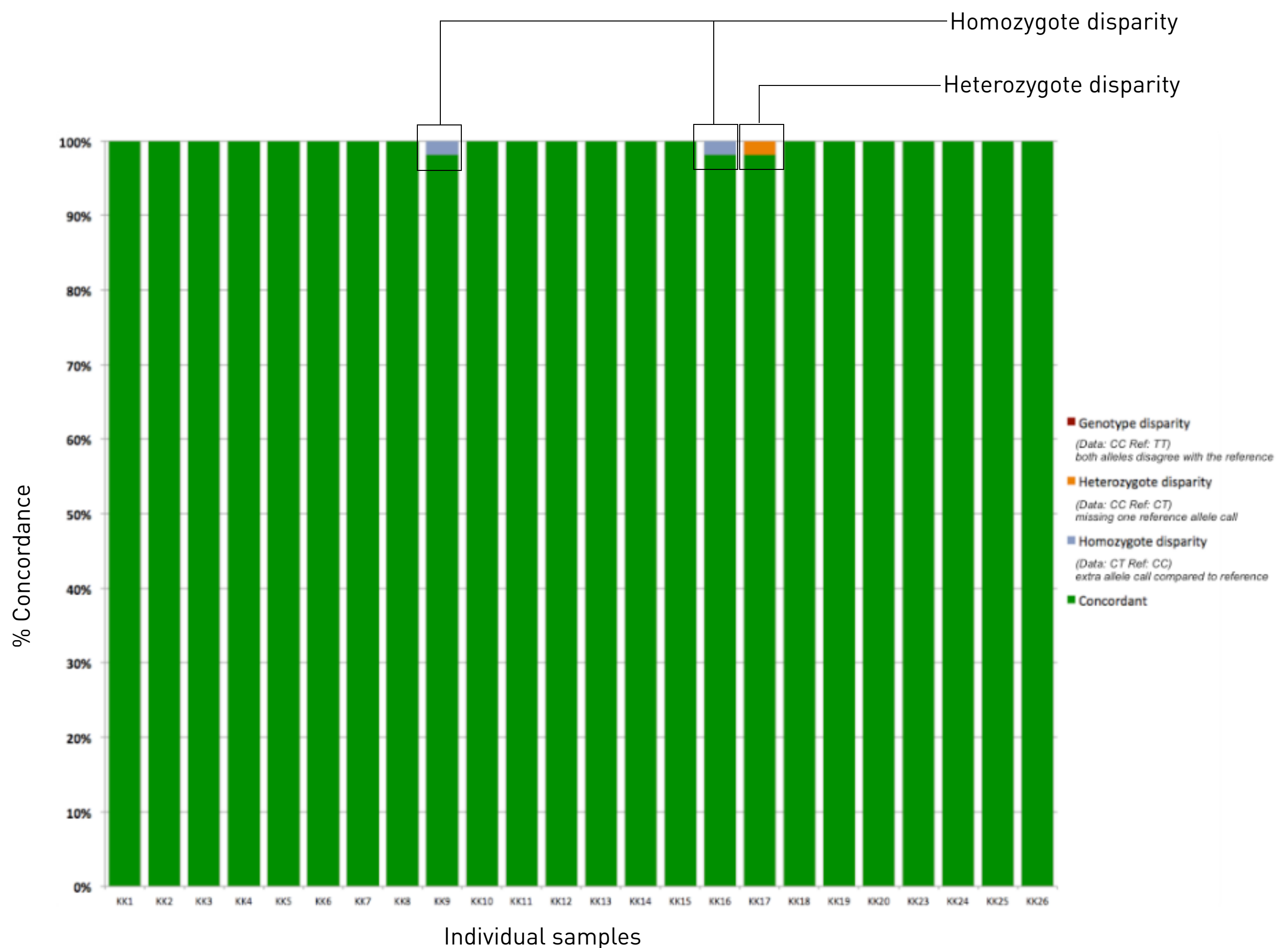
Figure 13 HID-Ion AmpliSeq™ Identity Panel concordance results



Ancestry Panel

Genotype data for 24 individuals was analyzed for concordance (see Figure 14). Library samples were prepared using 1 ng of input gDNA per PCR reaction, and were sequenced on a single Ion 318™ Chip v2. TaqMan® genotype data for the 24 individuals was provided for 55 Ken Kidd SNPs. Two homozygote disparities and one heterozygote disparity were observed. Genotype data between calls made using the plugin and the TaqMan® data was 99.77% concordant.

Figure 14 HID-Ion AmpliSeq™ Ancestry Panel concordance results

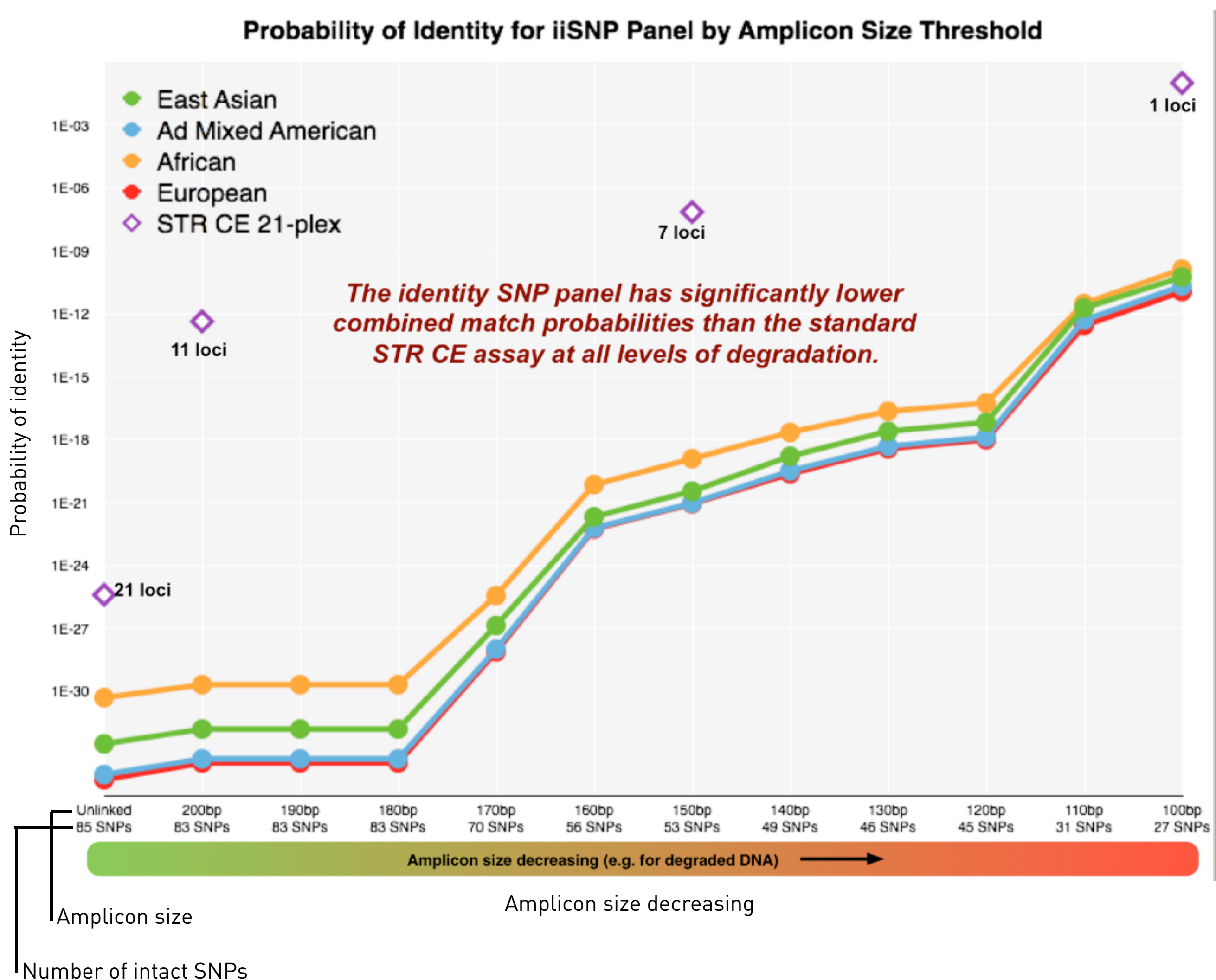


Effect of degradation on the probability of identity

Identity Panel

Using data from the 1000 Genomes database, it was determined that 85 of 90 autosomal identity SNPs were unlinked. The unlinked SNP data set was degraded *in silico* to determine the effect of degradation on the probability of identity. Data for four populations from the 1000 Genomes database (East Asian, Ad Mixed American, African, and European) along with values for a CE-based 21-plex STR assay were plotted to detect changes in probability of identity with decreasing amplicon size (see Figure 15). Based on this data, the identity SNP panel has lower combined match probabilities than the standard CE-based STR assay at all levels of degradation.

Figure 15 Effect of degradation on probability of identity for the HID-Ion AmpliSeq™ Identity Panel using probability of identity calculated from the 1000 Genomes database.



Panel throughput

Using 80% chip loading and 60% usable reads, the minimum amount of coverage and, therefore, the number of individuals per Ion chip type can be determined for each SNP panel.

Identity Panel

To obtain a minimum of 300× coverage for 97% of the autosomal SNPs, an average coverage depth of 738× is required. To obtain a minimum of 150× coverage for 97% of the Y-clade SNPs, an average coverage of 236× is required. Using the condition of 80% chip loading and 60% usable reads, this translates to the following run recommendations by chip type:

- Ion 314™ Chip v2: 8 individuals
- Ion 316™ Chip v2: 38 individuals
- Ion 318™ Chip v2: 77 individuals

Ancestry Panel

To obtain a minimum of 300× coverage for 97% of the autosomal SNPs, an average coverage depth of 594× is required. Using the condition of 80% chip loading and 60% usable reads, this translates to the following run recommendations by chip type:

- Ion 314™ Chip v2: 6 individuals
- Ion 316™ Chip v2: 30 individuals
- Ion 318™ Chip v2: 59 individuals

Troubleshooting

Follow the actions recommended in this appendix to troubleshoot problems that occur during analysis.

Table 1 Troubleshooting

Observation	Possible causes	Recommended actions
The library concentration is < 20 pM	Mis-quantification of input DNA	Requantify input DNA using one of the Quantifiler® Kits (see page 13).
	Less than 1 ng of input DNA	Add more DNA or add up to four PCR cycles to the target amplification.
	Inefficient PCR digestion or PCR ligation	Ensure proper mixing and dispensing of viscous components at each step.
	Overdrying of AMPure XP® beads	Do not dry the beads longer than 5 minutes.
	Residual ethanol drops inhibiting library amplification	Carefully remove all the ethanol drops. If necessary, use an additional spin and removal step.
	Any of the above	If the amount of gDNA is limited: **1.** Calculate the template dilution factor (TDF) that results in a concentration of ~10 pM for each library. **2.** After diluting the sample library to 10 pM, you can pool multiple diluted libraries equi-volume before running on the OT2 and PGM. **3.** Aliquot 20 µL of the 10-pM library into a new Eppendorf®LoBind® tube and add 5 µL of Nuclease-free Water for a total volume of 25 µL. **4.** Add the entire 25-µL volume of diluted library to the amplification solution for OT2 Template Preparation.
Loss of amplicons	Poor purification	Vortex the AMPure® XP Reagent thoroughly before use, and dispense the full volume.
		Increase the AMPure® XP Reagent volume from 1.5X to 1.7X (see"Purify and elute the unamplified library" on page 16).
	Denaturation of digested amplicon	Verify the use of the 60°C/20-minute temperature incubation during the primer digestion step (see "Partially digest primer sequences" on page 15).

Observation	Possible causes	Recommended actions
Uneven barcoded library representation	Inaccurate library quantification	Ensure that the sample library concentrations are within the control library concentration range (within the standard curve) as measured by qPCR.
	Inaccurate library combination	Dilute the libraries to 20 pM, then combine equal volumes. (If the library concentration is <20 pM, dilute all the sample libraries to 10 pM, then combine equal volumes. Aliquot 20 µL of the 10-pM library pool into a new Eppendorf®LoBind® tube and add 5 µL of Nuclease-free Water for a total volume of 25 µL. Add the entire 25-µL volume to the amplification solution for OT2.)
Adapter dimers occur during sequencing	Inefficient purification	Decrease the AMPure® XP Reagent volume from 1.5X to 1.0X (see"Purify and elute the unamplified library" on page 16).
	Barcode adapter dimer formation	Do not combine Switch Solution, diluted barcode adapter mix, and DNA ligase before adding to the ligation reaction.
	Barcode adapter concentration too high	Ensure that barcode adapters are diluted properly.
High polyclonal ISPs (>40%)	Overseeding of the library	Decrease the amount of sample library added to the OT2 amplification solution by 50%. (That is, aliquot 5 µL of the 20-pM sample library instead of 10 µL. See"Dilute libraries for OT2 template preparation" on page 20.
	Inaccurate library quantification	Ensure that the qPCR method was performed correctly.
	Incorrect calculation of the TDF	Ensure that the TDF is calculated correctly.
Low ISP loading (<60%) and high enrichment (>90%)	The liquid is not removed after the chip check	Use a pipette to remove as much liquid as possible. Place the chip upside down in a centrifuge bucket and perform a 5-second quick spin with the chip tab pointing inward.
	Too much liquid is remaining in the chip after loading	Perform a 5-second quick spin with the chip tab pointing outward and remove any liquid. If some liquid remains in the chip after the quick spin, lightly and rapidly tap the point of the chip tab against the benchtop a few times, and remove any liquid. Do NOT spin the chip upside-down.
Low ISP loading (<60%) and low quality ISPs >40%	Underseeding of the library	Double the amount of sample library added to the OT2 amplification solution.
	Inaccurate library quantification	Ensure that the qPCR method was performed correctly.
	Incorrect calculation of the TDF	Ensure that the TDF was calculated correctly.
	Neutralization solution was omitted during ES, and ISPs remained in melt-off solution for >15 min.	Perform a new OT2 200 Template Preparation. Ensure that you add neutralization solution before starting the ES run. Promptly remove samples from ES when the run completes.

Observation	Possible causes	Recommended actions
Extremely low ISP loading (<30%). (Sequencing failure)	Sequencing primer step omitted	If half the volume of enriched ISPs was saved before the sequencing failure, redo Sequencing protocol steps - Ion 314™ Chip v2. If no ISPs were saved, start over from OT2 200 Template Preparation.
	Sequencing polymerase step omitted	If half the volume of enriched ISPs was saved before the sequencing failure, redo Sequencing protocol steps - Ion 314™ Chip v2. If no ISPs were saved, start over from OT2 200 Template Preparation.
	No recovery of ISPs after enrichment	Confirm OT2 and ES operated correctly. Verify the quantity and quality of the library that went into the OT2 amplification solution. Ensure that ~200 µL was present in a 0.2-mL PCR tube after the ES run completed.

B Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Specific chemical handling

CAS	Chemical	Phrase
26628-22-8	Sodium Azide	Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

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Documentation and Support

Related documentation

Document title	Pub. no.
HID-Ion AmpliSeq™ *Library Preparation Quick Reference*	MAN0010638
The HID SNP Genotyper Plugin User Guide	MAN0010641

Portable document format (PDF) versions of this guide and the documents listed above are available at **www.lifetechnologies.com**.

Note: To open the user documentation available from the Life Technologies web site, use the Adobe® Acrobat® Reader® software available from **www.adobe.com**.

Customer and technical support

Visit **www.lifetechnologies.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
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  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Ion contact information

Web site: **www.lifetechnologies.com/iontorrent**

Ion Community: **ioncommunity.lifetechnologies.com**

Support email: **ionsupport@lifetech.com**

Phone numbers
In North America: 1-87-SEQUENCE (1-877-378-3623)
Outside of North America: +1-203-458-8552

Limited product warranty

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For support visit lifetechnologies.com/support or email techsupport@lifetech.com
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