Precision ID SNP Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System: Manual Library Preparation

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Precision ID SNP Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System Application Guide* (Pub. No. MAN0017767). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Extract, then quantify input DNA

1. Extract gDNA using one of the recommended genomic DNA extraction kits listed in the *Precision ID SNP Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System Application Guide* (Pub. No. MAN0017767).

2. Quantify gDNA using one of the recommended DNA quantification kits listed in the *Precision ID SNP Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System Application Guide*.

   Note: Use 1 ng gDNA in target amplification reactions.

Prepare the SNP target amplification reaction

1. Add the following components to each sample well of a 96-well PCR plate.

   Note: Prepare a master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
Precision ID Identity Panel *or* Precision ID Ancestry Panel	10 µL
gDNA, 1 ng[1]	X µL[2]
Nuclease-free Water	6 – X µL
Total	**20 µL**

[1] Less than 1 ng of gDNA can be used, but appropriately adjust the number of PCR cycles in "Amplify the targets".

[2] ≤6 µL

2. Seal the plate with a MicroAmp™ Clear Adhesive Film. To prevent evaporation, create a tight seal by applying pressure with an applicator.

3. Vortex the plate thoroughly, then centrifuge to collect droplets. Place a MicroAmp™ Compression Pad on the plate, then go to "Amplify the targets".

Amplify the targets

The cycle number for target amplification depends on the panel and the amount of input DNA. Cycle numbers can be increased if the quality or quantity of input DNA is uncertain.

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that the input DNA across the samples is roughly equivalent, or the PCR cycle number is based on the sample with the *lowest* quantity. This ensures that the selected cycle number for target amplification is optimal for all the samples in the run.

To amplify target regions, run the following program:

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 minutes
Cycle number (see following table)	Denature	99°C	15 seconds
	Anneal and extend	60°C	4 minutes
Hold	—	10°C	Hold [1]

[1] Store reactions at 10°C overnight on the thermal cycler. For longer-term storage, store covered at –20°C for up to one month.

Table 1 Cycle numbers for each panel depending on input DNA

Panel	Amount of input gDNA	Number of cycles
Precision ID Ancestry Panel *or* Precision ID Identity Panel	1 ng (~300 copies)	21 cycles
	<1 ng (<~300 copies)	21 cycles + 1 to 5 cycles

STOPPING POINT The target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer-term storage, store at –20°C for up to one month.

Partially digest amplicons

1. 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 a clear adhesive film, vortex thoroughly, then spin down to collect droplets.
3. Load in the thermal cycler, then setup and run the following thermal cycling conditions:

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

STOPPING POINT Store the plate at –20°C.

Ligate adapters to the amplicons, then purify

You *must* ligate a different barcode to each library when:

- sequencing multiple libraries on a single chip
- sequencing multiple replicates of DNA libraries from the same sample on a single chip

The barcode adapters included in the Precision ID IonCode™ 1–96 Kit in 96 Well PCR Plate are provided at the appropriate concentration, and include forward and reverse adapters in a single well. No further handling is necessary.

IMPORTANT! When handling barcode adapters, avoid cross-contamination. After use, reseal the barcode adapter plate with adhesive film and store at –30°C to –5°C.

Perform the ligation reaction

IMPORTANT! If there is visible precipitate in the Switch Solution, vortex or pipet up and down at room temperature to resuspend.

1. Carefully remove the plate seal, then add the following components to each well containing digested amplicons in the order listed.

   IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and adapters before adding to digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Barcode adapter (Precision ID IonCode™ Barcode Adapter *or* IonCode™ Barcode Adapter)	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total volume**	**~30 µL**

2. Seal the plate with a new MicroAmp™ Clear Adhesive Film, vortex thoroughly, then centrifuge to collect droplets.
3. Load the plate in the thermal cycler, then perform the ligation reaction using the following temperature program:

Panel	Temperature	Time
Precision ID Ancestry Panel *or* Precision ID Identity Panel	22°C	30 minutes
	68°C	10 minutes
	10°C	Hold (for up to 1 hour)

Purify the libraries

1. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library.
2. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly, then incubate the mixture for 5 minutes at room temperature.

   Alternatively, use a plate mixer (such as the Eppendorf™ MixMate™ mixer with the 96 × 0.2-mL PCR tube holder) to mix the bead suspension. Seal the plate, mix for 5 minutes at 2,000 rpm at room temperature, then centrifuge the plate briefly to collect droplets.
3. Place the plate in a magnetic rack (such as the DynaMag™–96 Side Magnet; Cat. No. 12331D), then incubate for 2 minutes or until solution clears.
4. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.
6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keep the plate in the magnet, then air-dry the beads at room temperature for 5 minutes.

Elute the libraries

1. Remove the plate containing the library from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads.
2. Seal the plate with a MicroAmp™ Clear Adhesive Film, then vortex thoroughly.
3. Incubate for 5 minutes at room temperature, then centrifuge to collect droplets.

   IMPORTANT! For maximum recovery, ensure that the suspension incubates for at least 5 minutes at room temperature.
4. Place the plate on the magnet for at least 2 minutes.

STOPPING POINT Samples can be stored with beads at 4°C for up to one month. For long-term storage at −20°C, place the plate in the magnet, then transfer the sample supernatants to a new plate. Do not store libraries at −20°C in the presence of beads.

Quantify the libraries by qPCR

After eluting each Precision ID library, determine concentration by qPCR with the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802).

Dilute the libraries for quantification

1. If samples have been stored at 4°C, vortex the plate, then centrifuge to collect droplets.
2. Place the plate in the magnetic rack for 2 minutes, or until the supernatant clears.
3. Prepare 1:100 dilutions by removing 2 µL of supernatant, then combine with 198 µL of Nuclease-free Water.
4. After removing the aliquots, store the plate at 4°C.

Quantify the libraries

Use the Ion Library TaqMan® Quantitation Kit to analyze each sample, standard, and negative control in duplicate 20-µL reactions.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Control Library (~68 pM; provided in the kit) at the concentrations listed in the following table. Label them as standards, then use these concentrations in the qPCR experiment setup.

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

2. Prepare sufficient reaction mixture for replicate reactions for each sample, negative control, and control library dilution. Add an extra reaction to compensate for pipetting error. For each reaction, combine 10 µL of Ion Library qPCR Master Mix and 1 µL of Ion Library TaqMan® Quantitation Assay, 20X in a tube, then mix thoroughly.

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

3. Aliquot 11 µL into each reaction well (two wells per reaction) of a PCR plate.
4. Add 9 µL of the diluted (1:100) sample library, each control library dilution, or negative control to reaction wells, for a total reaction volume per well of 20 µL.
5. Set up the real-time PCR instrument.
   a. Enter the concentrations of the control library standards.
   b. Select ROX™ Reference Dye as the passive reference dye.
   c. Enter a reaction volume of 20 µL.

d. Select FAM™ dye/MGB as the TaqMan® probe reporter/quencher.

e. Enter the following run parameters, depending on your system.

Real-time PCR System	Stage	Temperature	Time
7500 Real-Time PCR Instrument with SDS Software v1.2.3	Hold	50°C	2 minutes
	Hold	95°C	20 seconds
	40 Cycles	95°C	3 seconds
		60°C	32 seconds
7500 Real-Time PCR Instrument with HID Real-Time PCR Analysis Software v1.1 or v1.2	Hold	50°C	2 minutes
	Hold	95°C	20 seconds
	40 Cycles	95°C	3 seconds
		60°C	30 seconds

6. Run the reactions, then collect the real-time data.

See for library concentrations required for template preparation. Depending on your quantification results, proceed with one of the following options:

- If sufficient library was prepared, continue to .
- If insufficient library was prepared, continue to "(Optional) Amplify and purify the libraries".
- Continue with less than optimal library concentration. See "Troubleshooting" in the *Precision ID SNP Panels with the HID Ion S5™ /HID Ion GeneStudio™ S5 System Application Guide* (Pub. No. MAN0017767) for effects of using low library concentration.

(Optional) Amplify and purify the libraries

A library that yields less than the recommended concentration can be rescued by library amplification. See the *Precision ID SNP Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System Application Guide* (Pub. No. MAN0017767) for the full procedure.

Amplify the libraries

1. Combine 25 µL of each unamplified library (total undiluted library is ~50 µL, from "Elute the libraries" on page 3) with 72 µL of Platinum™ PCR SuperMix HiFi and 3 µL of Library Amplification Primer Mix from the Precision ID Library Kit in one well of a 96-well PCR plate.

2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets.

3. Load the plate in a thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5–10 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold (for up to 24 hours)

STOPPING POINT Samples can be held overnight or up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the amplified libraries

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- **First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- **Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

First-round purification

1. Tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, then remove the plate seal.

2. **Add 50 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** to each plate well containing ~100 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.

3. Incubate the mixture for 5 minutes at room temperature.

4. Place the plate in a magnet such as the DynaMag™–96 Side Magnet for at least 5 minutes, or until the solution is clear.

5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from "First-round purification" on page 4 above, **add 120 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent**. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

   IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. **Add 150 µL of freshly prepared 70% ethanol** to each well, then move the plate side to side in the magnet to wash the beads. Remove and discard the supernatant without disturbing the pellet.
5. Repeat step 4 for a second wash.
6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not overdry.**
7. Remove the plate from the magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
8. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets.
9. Incubate at room temperature for at least 2 minutes.
10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant as described in "Quantify the libraries by qPCR" on page 3.

    IMPORTANT! The **supernatant** contains the desired amplicons. **Do not discard!**

Dilute, pool, and store the libraries

Dilute the libraries

1. After the run is complete, calculate the average concentration of each undiluted library using the following equation:

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

   For example:
   - qPCR quantities mean: 3 pM
   - Sample library dilution: 100

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

2. Dilute libraries as described in the following table.

   Recommended library dilutions for the Ion Chef™ System

Panel	Dilute to	Minimum volume	Templating size in Planned Run setup
Precision ID Ancestry Panel *or* Precision ID Identity Panel	30 pM	25 µL	200 bp

(Optional) Pool the libraries

After diluting the sample library to its target concentration (pM), combine equal volumes of multiple diluted libraries. Use the pooled libraries in template preparation reactions on the Ion Chef™ Instrument.

Use the following recommendations for the number of manually prepared sample libraries loaded per chip. The recommendations are based on at least 100X coverage of 97% of markers. You may need to adjust the number of samples per chip based on your individual coverage requirements, sample quality, and throughput.

Panel	Samples per Ion S5™ Chip		
	Ion 510™ Chip	Ion 520™ Chip	Ion 530™ Chip
Precision ID Ancestry Panel	48	72	362[1]
Precision ID Identity Panel	54	81	362[1]

[1] If using Precision ID IonCode™ Barcode Adapters, the number of barcode adapters is limited to 96.

Store the libraries

Store both diluted and undiluted libraries at 2°C to 8°C for up to 1 month. For long-term storage, store libraries at –30°C to –10°C.

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