Perform niPGT-A reseach analysis using Ion ReproSeq™ PGS Kits

Pub. No. MAN1000343 Rev. A

Noninvasive Preimplantation Genetic Testing for Aneuploidy (niPGT-A) is a technique that evaluates embryos for chromosomal abnormalities in a non-invasive manner. This method is a modified version of the traditional Preimplantation Genetic Testing (PGT-A), that involves the invasive process of extracting cells from the embryo for analysis. In contrast, niPGT-A uses the analysis of DNA fragments released by the embryo into the culture medium during its development.

This user bulletin describes how to prepare next-generation sequencing libraries for niPGT-A from embryo culture media and perform aneuploidy analysis using Ion ReproSeq™ PGS Kits. The Ion ReproSeq™ PGS Kits provide reagents and materials for high-throughput whole-genome amplification and sequencing to detect chromosomal aneuploidies, chromosome arm events, and copy number variations. The kits are used with the Ion Chef™ System, and the Ion S5™/Ion S5™ XL System or one of the Ion GeneStudio™ S5 Systems, which enable automated template preparation, chip loading, and sequencing to minimize hands-on time.

Note: For safety and biohazard guidelines, see the "Safety" appendix in the following product documentation: *Ion ReproSeq™ PGS Kits – Ion S5™/Ion GeneStudio™ S5 Systems User Guide* (Pub. No. MAN0016712). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Instrument compatibility

The Ion ReproSeq™ PGS Kits described in this user guide are compatible with the Ion S5™/Ion S5™ XL Sequencers and Ion GeneStudio™ S5 Series Sequencers. We recommend using the Ion S5™ XL, Ion GeneStudio™ S5 Plus, or Ion GeneStudio™ S5 Prime Sequencer for rapid turnaround between runs. Use of the kits with the Ion S5™ or Ion GeneStudio™ S5 Sequencer requires more analysis time between runs.

Software compatibility

Ion ReproSeq™ PGS Kit with Ion 510™ Chips, Ion ReproSeq™ PGS Kit with Ion 520™ Chips, and Ion ReproSeq™ PGS Kit with Ion 530™ Chips are compatible with Torrent Suite™ Software 5.4.0 and later. Be sure to update your Torrent Server to the latest available version of Torrent Suite™ Software before using these kits. Update instrument software to the latest available version. For data analysis, Ion Reporter™ Software 5.10 or later is recommended for the latest features and workflows.

Required kits

Kit configurations

Three configurations of the Ion ReproSeq™ PGS Kits for use with Ion GeneStudio™ S5 Systems are available. The kits vary in the number and size of Ion SingleSeq™ Kits provided, the maximum number of samples processed per kit, and the type of Ion S5™ sequencing chip provided.

Ion ReproSeq™ PGS Kit	Amount per kit	Samples per kit
Ion ReproSeq™ PGS Kit with Ion 510™ Chips (16 samples/run); Cat. No. A34899		
Ion SingleSeq™ Kit (24 reactions)	3 boxes	64[1]
Ion S5™ ExT Chef Supplies	4 boxes	
Ion S5™ Chef Solutions	4 cartridges	
Ion S5™ ExT Chef PGS Reagents	4 cartridges	
Ion S5™ ExT Sequencing Solutions	1 box	
Ion S5™ ExT Sequencing Reagents	4 cartridges	
Ion 510™ Chip Kit	4 chips	
Ion ReproSeq™ PGS Kit with Ion 520™ Chips (24 samples/run); Cat. No. A34900		
Ion SingleSeq™ 96 Kit (96 reactions)	1 x 2 boxes	96
Ion S5™ ExT Chef Supplies	4 boxes	
Ion S5™ Chef Solutions	4 cartridges	
Ion S5™ ExT Chef PGS Reagents	4 cartridges	
Ion S5™ ExT Sequencing Solutions	1 box	
Ion S5™ ExT Sequencing Reagents	4 cartridges	
Ion 520™ Chip Kit	4 chips	
Ion ReproSeq™ PGS Kit with Ion 530™ Chips (96 samples/run); Cat. No. A34901		
Ion SingleSeq™ 96 Kit (96 reactions)	4 x 2 boxes	384
Ion S5™ ExT Chef Supplies	4 boxes	
Ion S5™ Chef Solutions	4 cartridges	
Ion S5™ ExT Chef PGS Reagents	4 cartridges	
Ion S5™ ExT Sequencing Solutions	1 box	
Ion S5™ ExT Sequencing Reagents	4 cartridges	
Ion 530™ Chip Kit	4 chips	

[1] We recommend loading 16 sample libraries per Ion 510™ Chip. However, 3 boxes of the Ion SingleSeq™ Kit provide reagents and barcode adapters to prepare 72 libraries. The leftover reagents and barcode adapters can be used with the next purchased kit.

Ion SingleSeq™ Kit

Use the Ion SingleSeq™ Kit (Part No. A28955; 24 reactions/kit) or Ion SingleSeq™ 96 Kit (Part No. A34763; 96 reactions/kit) to extract, amplify, and barcode genomic DNA. Immediately before use, thaw tubes on ice as needed.

Note: The extraction reagents included with the kit are not used for the niPGT-A protocol.

Component	Amount		Storage
	Ion SingleSeq™ Kit	Ion SingleSeq™ 96 Kit	
Cell Extraction Buffer[1] (green cap)	120 µL	480 µL	–30°C to –10°C
Extraction Enzyme Dilution Buffer[1] (violet cap)	115.2 µL	460.8 µL	
Cell Extraction Enzyme[1] (yellow cap)	4.8 µL	19.2 µL	
Pre-Amplification Buffer (red cap)	115.2 µL	460.8 µL	
Pre-Amplification Enzyme (white cap)	4.8 µL	19.2 µL	
Amplification Buffer (orange cap)	648 µL	3 × 864 µL	
Amplification Enzyme (blue cap)	12 µL	48 µL	
Nuclease-free Water (clear cap)	432 µL	432 µL	
Barcode Plate (Ion SingleSeq™ Barcode Adapters loaded in wells A1–H3 or wells A1–H12)	1 plate; 5 µL/well (Barcodes 1–24)	1 plate; 5 µL/well (Barcodes 1–96)	

[1] Not used for the niPGT-A protocol.

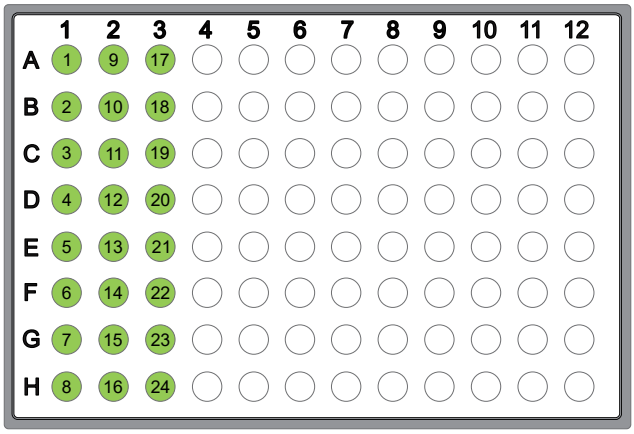
About the Ion SingleSeq™ Barcode Adapters

The Ion SingleSeq™ Kit is designed for high throughput applications and includes a 96-well Barcode Plate containing Barcode Adapters 1–24 or 1–96. Each well contains sufficient volume for a single use. The plate is sealed with foil that can be pierced with a pipet tip to collect the required amount of the Barcode Adapter for a reaction.

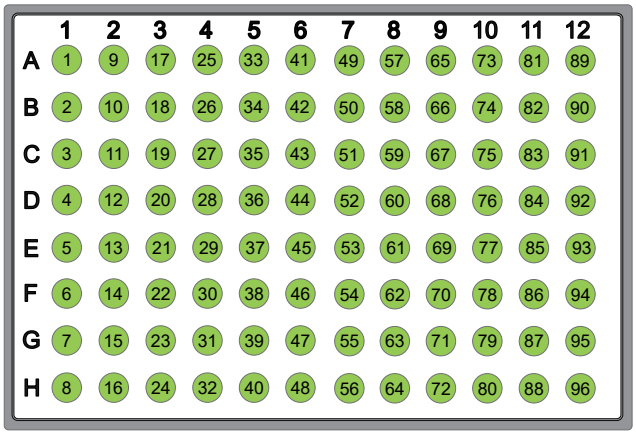
To minimize cross-contamination, either use all barcodes in a plate at once, or if you are using a subset, seal the pierced wells with laboratory tape.

IMPORTANT!

- Do not substitute barcode oligonucleotides from any other source for the Barcode Adapters contained in this kit.
- Avoid repeated freezing and thawing of the plate. Do not exceed 4 freeze/thaw cycles.



Barcode Plate containing Ion SingleSeq™ Barcodes 1–24. Twenty-four unique Barcode Adapters are loaded in wells A1 through H3.



Barcode Plate containing Ion SingleSeq™ Barcodes 1–96. Ninety-six unique Barcode Adapters are loaded in wells A1 through H12.

Note: Barcodes 1–24 in the Ion SingleSeq™ Barcodes 1–96 plate are identical to the Barcode Adapters in the Ion SingleSeq™ Barcodes 1–24 plate.

Ion S5™ ExT Kit components

IMPORTANT! Do not substitute components from any other Ion sequencing kits. We have verified this protocol using these specific materials. Substitution can adversely affect system performance.

IMPORTANT! Store all consumables and cartridges under the recommended conditions and in an upright position. Do NOT store the Ion S5™ ExT Sequencing Reagents (Part No. A30522) on dry ice or in a closed environment where dry ice is present.

The Ion ReproSeq™ PGS Kit with Ion 510™ Chips, Ion ReproSeq™ PGS Kit with Ion 520™ Chips, and Ion ReproSeq™ PGS Kit with Ion 530™ Chips support 4 initializations, and contain all materials required for preparing and sequencing 4 Ion 510™, Ion 520™, or Ion 530™ Chips. On arrival, inspect all consumables and contact Technical Support if any of the products have been damaged during shipping.

Contents	Amount/box	Storage
Ion S5™ ExT Chef Supplies (Part. No. A51932)		
Chip Adapter	1	15°C to 30°C
Enrichment Cartridge v2	1	
Ion S5™ ExT Tip Cartridge	1	
PCR Plate and Frame Seal v2	1 each	
Recovery Station Disposable Lid v2	2	
Recovery Tube	12	
Ion S5™ Chef Solutions (Part. No. A51933)		
Ion S5™ Chef Solutions	4 cartridges	15°C to 30°C
Ion S5™ ExT Chef PGS Reagents (Part. No. A51934)		
Ion S5™ ExT Chef PGS Reagents	4 cartridges	–30ºC to –10ºC
Ion S5™ ExT Sequencing Solutions (Part. No. A51935)		
Ion S5™ ExT Wash Solution	4 x 1.5 L	15°C to 30°C
Ion S5™ Cleaning Solution	250 mL	
Ion S5™ ExT Sequencing Reagents (Part. No. A51936)		
Ion S5™ ExT Sequencing Reagents	4 cartridges	–30ºC to –10ºC

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Materials and equipment required for Ion SingleSeq™ library preparation	
ViiA™ 7 Real-Time PCR System with 96-Well Block (recommended), *or* Veriti™ 96-Well Thermal Cycler (0.2-mL block; for standard PCR amplification), *or* equivalent real-time or standard thermal cycler with heated lid	4453534 4452300
MicroAmp™ Optical 96-Well Reaction Plate MicroAmp™ Optical Adhesive Film (for real-time PCR) MicroAmp™ Clear Adhesive Film (for standard endpoint PCR)[1] *or equivalent*	N8010560 4360954 4306311
(Optional) MicroAmp™ Optical 8-Tube Strip, 0.2 mL[2] MicroAmp™ Optical 8-Cap Strip *or* MicroAmp™ Reaction Tube with Cap, 0.2 mL *or equivalent* 96-Well Tray/Retainer Set specific to your instrument	4316567 4323032 N8010540
Agencourt™ AMPure™ XP Reagent	Fisher Scientific NC9959336
DynaMag™-2 Magnet	12321D
1.5-mL Eppendorf™ DNA LoBind™ Microcentrifuge Tubes[3]	Fisher Scientific 13-698-791
Qubit™ 4 Fluorometer[4]	Q33238
Qubit™ dsDNA HS Assay Kit	Q32851 Q32854
Qubit™ Assay Tubes	Q32856
Ethanol	MLS
Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA)	MLS
Phosphate-buffered saline (1X PBS, Ca^{2+}-free, Mg^{2+}-free, BSA-free)	MLS
Nuclease-free Water	AM9932
SYBR™ Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO[5]	S-7567
ROX™ Reference Dye[5]	12223-012
2, 10, 20, 200, 1000 µL pipettor set and filtered tips	MLS
Multichannel pipettors (2–20 µL and 20–200 µL; 8 channel)	MLS
(Optional) E-Gel™ Agarose Gels, 2%, and E-Gel™ iBase™ Power System, or E-Gel™ PowerBase™ System, *or equivalent*	Various
Additional materials required for templating and sequencing	
Ion Chef™ S5 Series Chip Balance	A29022
Non-interruptible Power Supply (UPS)[6]	MLS
Gloves, powder-free nitrile	MLS
Isopropanol, 70% solution	MLS
Wipes, disposable lint-free	MLS

[1] Recommended for all plate sealing other than real-time amplification reactions for easier application and removal.
[2] For standard end-point PCR amplification, standard tube strips and caps can be substituted.
[3] Can substitute 2.0-mL Eppendorf LoBind™ Tubes for 1.5-mL tubes.
[4] Qubit™ 2.0 Fluorometer and later are supported.
[5] Required if monitoring amplification by real-time PCR.

[6] For laboratories that experience frequent power outages or line voltage fluctuations, we recommend that you use a non-interruptible power supply that provides 2500 W output or higher.

General procedural guidelines

- Use good laboratory practices to minimize cross-contamination of products. When designing the laboratory layout, consider the need for space separation of pre- and post-amplification activities. Dedicate laboratory supplies and/or equipment to the appropriate space to reduce significantly the potential for contamination.
- MicroAmp™ Clear Adhesive Film is easier to apply and remove from amplification plates than MicroAmp™ Optical Adhesive Film, and is recommended for sealing plates in preamplification and standard endpoint amplification reactions. Use MicroAmp™ Optical Adhesive Film to seal plates for real-time PCR amplification reactions.
- Pipet viscous solutions slowly and ensure complete mixing.
- Ensure that all reagents are completely thawed at room temperature, that is, no ice crystals are visible.
- Vortex all reagents, *except* for enzymes, for 5 seconds (mix enzymes by flicking the tube with your finger 4 times). Briefly centrifuge in a microcentrifuge for 3–5 seconds before use.

Guidelines for the number of libraries per run

The maximum number of Ion SingleSeq™ libraries you can pool and sequence on one chip depends on the type of chip you use. Follow these guidelines:

Ion Chip	Recommended maximum libraries per run
Ion 510™ Chip	16
Ion 520™ Chip	24
Ion 530™ Chip	96

Guidelines for sample handling

- Add liquids above the top of liquid in a tube, do not submerge the tip.
- Keep samples and amplification reactions on ice or a cold block during reaction setup, and keep Ion SingleSeq™ libraries on ice during library pooling and quantification.

Amplify the gDNA

Materials required

Sample:

10 µL of Spent Cell Media (SCM)

Provided in the Ion SingleSeq™ Kit (Cat. No. A28955 or A34763):

- Preamplification Buffer
- Preamplification Enzyme
- Amplification Buffer
- Amplification Enzyme
- Nuclease-free Water
- Barcode Plate containing Ion SingleSeq™ Barcodes 1–24 or Ion SingleSeq™ Barcodes 1–96

Other materials:

- MicroAmp™ 96-well Optical Reaction Plate
- MicroAmp™ Clear Adhesive Film
- MicroAmp™ Optical Adhesive Film (if performing real-time PCR amplification.)
- *or* 0.2-mL MicroAmp™ 8-tube PCR tube strips, individual MicroAmp™ PCR tubes, caps, and MicroAmp™ 96-Well Tray/Retainer Set
- Pipettors, multichannel pipettors
- Pipette tips
- Real-time or standard PCR thermal cycler that is equipped with heated lid

- SYBR™ Green I and ROX dyes (Cat. Nos. S-7567 and 12223-012), if monitoring amplification by real-time PCR
- If performing troubleshooting, one of the following for diluting gDNA:
  - Low TE
  - PBS (Ca^{2+}-, Mg^{2+}-, BSA-free)
  - Nuclease-free Water

Preamplify the gDNA

1. Prepare a preamplification master mix in a 1.5-mL tube on ice according to the following table. Scale the volume according to the number of samples (N) and NTCs you have. Vortex briefly and centrifuge for 30 seconds to collect liquid at the bottom of tube.

   Table 1 Preamplification master mix

Component	Volume per reaction	Volume for N reactions [1]
Preamplification Buffer (red cap)	4.8 µL	N × 4.8 µL × 1.1
Preamplification Enzyme (white cap)	0.2 µL	N × 0.2 µL × 1.1

[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 µL.

   Note: To dispense preamplification master mix with an 8-channel multichannel pipettor, aliquot the master mix to 8 wells of a 96-well plate to serve as a reservoir. For example, aliquot 65 µL/well if you are processing 96 samples.

2. Add 10 µL of SCM to each sample well of a 96-well plate.

3. Add 5-µL preamplification master mix to each sample well (15-µL final volume).

   IMPORTANT! Do not mix. Vortex or pipetting up and down can cause loss of DNA on the wall of the tube or pipette tip.

4. Apply a new adhesive film, then centrifuge at 1,000 × *g* for 30 seconds to collect liquid at the bottom of the wells.

5. Cycle samples in a thermal cycler according to the following program:

Step	Temperature	Time[1]	Number of cycles
1	95°C	2 minutes	1
2	95°C	15 seconds	15[2]
	15°C	50 seconds	
	25°C	40 seconds	
	35°C	30 seconds	
	65°C	40 seconds	
	75°C	40 seconds	
3	4°C	Hold	1

[1] Cycling time is about 1 hour and 15 minutes.
[2] Optimization may be required.

   Note: In step 2, 15 cycles should be used as a starting point. Optimization may be required. If the per–sample success rate is lower than desired, you can increase the number of cycles to 16. If the success rate is acceptable, you can decrease the number of cycles to 14 cycles.

   IMPORTANT! The thermal cycler must be equipped with a heated lid.

6. Remove the plate, centrifuge at 1,000 × *g* for 30 seconds to collect liquid at the bottom of the wells, and place on ice or a cold block.

Amplify the libraries

You can perform the amplification reaction on a real-time PCR instrument to monitor amplification during the run. Monitoring can give an early indication of amplification failure in a particular sample, or that a library will be over- or under-represented after pooling.

1. If you are performing real-time PCR, prepare SYBR™ Green I/ROX dye mix. If you are performing standard endpoint PCR, proceed to step 2, then use Nuclease-free Water instead of the dye mix, as indicated in step 3.
   a. Dilute the stock SYBR™ Green I reagent 1:1000 with Low TE buffer to make a 10X working solution.
   b. Combine 10X SYBR™ Green I working solution and 25 µM ROX Reference Dye for the number of reactions (N) according to the following table:

Component	Volume per reaction	Volume for N reactions[1]
10X SYBR™ Green I working solution	0.5 µL	N × 0.5 µL × 1.1
25 µM ROX Reference Dye (or 2.5 µM, see below)	1.0 µL	N × 1.0 µL × 1.1
Nuclease-free Water (clear cap)	1.0 µL	N × 1.0 µL × 1.1

[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 µL.

IMPORTANT! Use ROX Reference Dye at 25 µM for the following real-time PCR instruments: Applied Biosystems™ 7300, 7900HT, StepOne™, StepOnePlus™, ABI PRISM™ 7000, and 7700 instruments.

Dilute ROX Reference Dye to 2.5 µM with Nuclease-free Water and use 1.0 µL per reaction for the following instruments: Applied Biosystems™ 7500 and ViiA™ 7 instruments, and Agilent™ Mx3000P™, Mx3005P™, and Mx4000™ instruments.

2. Prepare the Ion SingleSeq™ Barcodes 1–24 or 1–96 plate:
   a. Thaw the plate for 10 minutes at room temperature.
   b. Centrifuge the plate briefly to collect contents at the bottom of the wells.
   c. Wipe the foil seal with 70% ethanol, then allow it to dry.

3. Prepare an amplification master mix in a 5-mL tube on ice according to the following table. Scale the volume according to the number (N) of samples and NTCs you have. Vortex briefly, then centrifuge to collect liquid at the bottom of tube.

Table 2 Amplification master mix

Component	Volume per reaction	Volume for N reactions[1]
Amplification Buffer (orange cap)	27 µL	N × 27 µL × 1.1
Amplification Enzyme (blue cap)	0.5 µL	N × 0.5 µL × 1.1
SYBR™ Green I/ROX dye mix[2]	2.5 µL	N × 2.5 µL × 1.1

[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 µL.
[2] Replace with Nuclease-free Water if performing endpoint PCR.

Note: To dispense amplification master mix with an 8-channel multichannel pipettor, aliquot the master mix to eight wells of a 96-well plate to serve as a reservoir. For example, aliquot 396 µL/well if you are processing 96 samples.

4. Remove the film seal from the sample plate, then add 30 µL amplification master mix to each well (45-µL final volume).

5. Pierce the foil above the desired well of the Barcode Plate (see Figure 1) with a pipette tip. With a new tip, withdraw 5 µL of the Ion SingleSeq™ Barcode Adapter, then add to the appropriate sample (50-µL final volume). Repeat for each sample. Each Barcode Adapter is single-use only.

Note: To avoid sample misidentification later, be careful to track the correspondence of each sample and its Barcode Adapter. For barcode plate handling guidelines, see “About the Ion SingleSeq™ Barcode Adapters” on page 3

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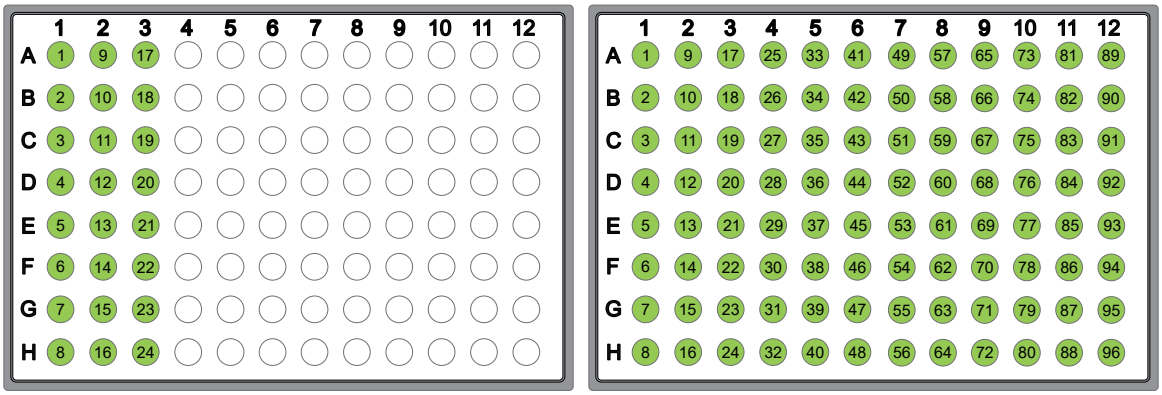


Figure 1 Plate map
Ion SingleSeq™ Barcode Plates containing Barcodes 1–24 and 1–96. Barcode Adapters are loaded in the indicated wells.

6. Adjust a pipettor to 30 µL, then mix the samples by pipetting up and down, using a new tip for each sample. Do not introduce bubbles into the samples.

   Note: If you used a non-optical reaction plate or PCR tube strip for cell lysis and preamplification, you can transfer samples to an optical plate at this point to perform real-time PCR amplification.

7. Apply a new film seal to the plate and briefly centrifuge to collect liquid at the bottom of the wells.

8. Cycle the samples in the thermal cycler using the following program:

Step	Temperature	Time[1]	Number of cycles
1	95°C	3 minutes	1
2	95°C	20 seconds	4
	50°C	25 seconds	
	72°C	40 seconds	
3[2]	95°C	20 seconds	15[3]
	72°C	55 seconds	
4	4°C	Hold	1

[1] Cycling time is approximately 36 minutes.
[2] Acquire fluorescence data at this step if monitoring amplification in real-time.
[3] Optimization may be required.

   Note: In step 3, 15 cycles should be used as a starting point. Optimization may be required. If the per–sample success rate is lower than desired, you can increase the number of cycles to 16. If the success rate is acceptable, you can decrease the number of cycles to 14 cycles.

9. Remove the plate, centrifuge at 1,000 × *g* for 30 seconds to collect liquid at the bottom of the wells, and place on ice or in a cold block.

10. *(Optional)* We recommend that new users run 10 µL of each amplified library on an E-Gel™ 2% Agarose Gel, or equivalent agarose gel, to check the quality of amplified libraries.

    Note: Unpurified library fragments typically migrate nearer to 350 bp instead of the expected 250 bp.

Proceed to “Pool, purify, and quantify the libraries”.

STOPPING POINT Sample libraries can be stored at –30°C to –10°C before proceeding to the next step.

Pool, purify, and quantify the libraries

Note: We recommend that you perform library pooling, purification, and quantification in one session. Do not store library pools before quantification.

Materials required

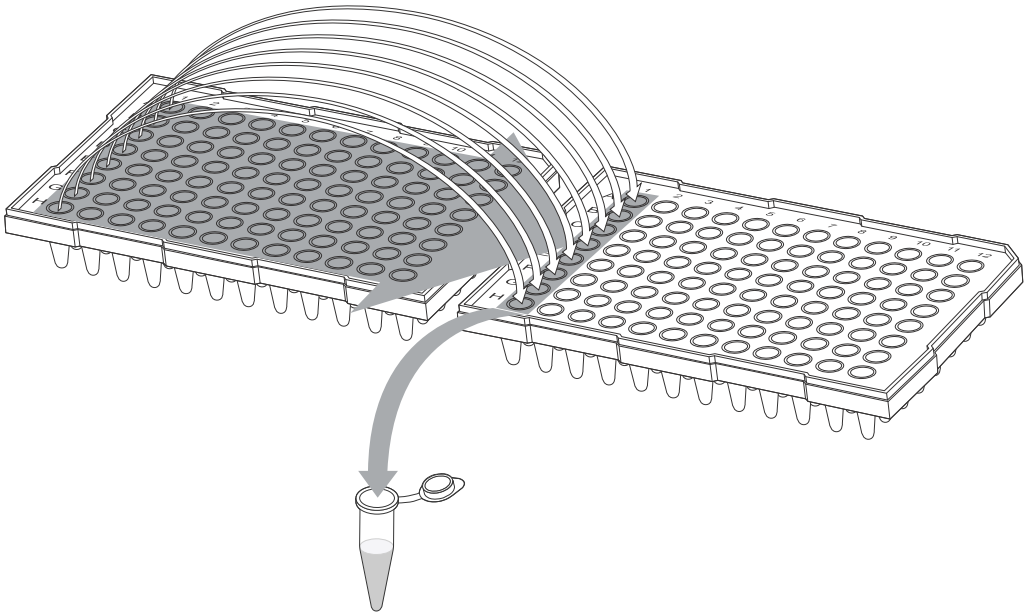
- 70% Ethanol - freshly prepared
- AMPure™ XP Reagent - warmed to room temperature
- DynaMag™-2 Magnet or DynaMag™–96 Side
- Low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- Qubit™ 4 Fluorometer (Qubit™ 2.0 Fluorometer and later are supported)
- Qubit™ dsDNA HS (High Sensitivity) Assay Kit
- Qubit™ Assay Tubes

Pool the libraries

1. Pool the libraries according to the PCR method you used to amplify the libraries. Before pooling, vortex the amplification reactions to mix, then centrifuge briefly to collect contents at the bottom of the wells or tubes.

If you used	Action
Standard endpoint PCR amplification	Add 5 µL of each library to a new 1.5-mL tube to create an equi-volume pool. Vortex the tube to mix, then centrifuge briefly to collect contents at the bottom of the tube.
Real-time PCR amplification	Calculate the median C_t value of the libraries you want to pool and adjust the volumes of libraries deviating from the median C_t following these guidelines: • Libraries that fall >1 C_t of the median should be added at the normal volume (5 µL). • Libraries that fall >3 C_ts later than the median should not be included in the pool. It is likely that the input material was not present in the tube or well or was absorbed to the tube side. • Libraries that fall ≥1 but ≤3 C_ts later than the median should be added at 2X the normal volume (10 µL). • Do not adjust more than 25% of the libraries in a pool (excluding libraries >3 C_ts later than median). If more than 25% of libraries fall 1 or more C_ts away from the median, adjust libraries with the most extreme C_t deviations until the 25% threshold is reached.

Note: You can use the following technique to rapidly pool 5-µL aliquots of a large number of libraries. First use an 8-channel pipettor to transfer aliquots from up to 96 libraries into 8 wells of a new plate, then use a standard pipettor to combine the contents of the 8 wells into a 1.5-mL Eppendorf™ LoBind™ tube. Use a new tip for every well when you transfer the 5-µL aliquots.



2. Transfer 40 µL of the library pool to a 0.2-µL tube for purification and quantification.

   Note:
   - When pooling fewer than 8 libraries, the pool volume is less than 40 µL. Add Nuclease-free Water to bring the final volume to 40 µL before library pool purification.
   - When pooling more than 8 libraries, remove 40 µL from the pool for purification and quantification. If the remaining volume is ≥40 µL, store at –30°C to –10°C to use if a repeat purification is needed.

Purify the library pool

1. Transfer 40 µL of the library pool to a fresh PCR tube.
2. Heat the 40-µL aliquot of library pool in a thermal cycler using the following program.

Step	Temperature	Time	Number of cycles
1	70°C	2 minutes	1
2	22°C	Hold	1

3. Centrifuge the tube briefly to collect the contents, then transfer to a new 1.5-mL Eppendorf™ LoBind™ tube.
4. Add 40 µL (1X volume) of room temperature AMPure™ XP beads to the library pool.
5. Vortex the tube, then centrifuge briefly to collect contents, then incubate for 5 minutes at room temperature.
6. Place the tube in the DynaMag™-2 Magnet, then wait 5 minutes for beads to aggregate to the side of the tube or well of the plate.
7. Carefully remove, then discard the supernatant without disturbing the pellet.
8. Wash beads with 250 µL of freshly prepared 70% ethanol while the tube or plate is still on the magnet.
9. Incubate for 30 seconds.
10. Aspirate the wash solution carefully, then discard.
11. Repeat step 8 through step 10, thoroughly removing all ethanol after the second wash.
12. Allow the beads to dry at room temperature for 3–4 minutes with the tube on the magnet.
13. Remove the tube from the magnet, add 40 µL of Low TE, then resuspend the beads by pipetting up and down.
14. Incubate the tube at room temperature for 1 minute.
15. Place the tube in the DynaMag™-2 Magnet, then wait 2–3 minutes for the beads to aggregate to the side of the tube.
16. Transfer 35 µL of the supernatant containing the purified library pool to a new 1.5-mL Eppendorf™ LoBind™ tube and place on ice. Avoid carryover of beads.

Quantify the library pool

Quantify the Ion SingleSeq™ library pool with the Qubit™ dsDNA HS (High Sensitivity) Assay Kit.

For more information, see the *Qubit™ dsDNA HS Assay Kits User GuideQubit™* (Pub. No. MAN0002326).

1. Prepare a Qubit™ working solution by diluting the Qubit™ dsDNA HS Reagent (Component A) 1:200 in Qubit™ dsDNA HS Buffer (Component B) in a plastic tube.

   Prepare sufficient working solution to quantify one or more purified pools plus two standards (volume required = (number of pools + 2) × 200 µL × 1.1).

   IMPORTANT! Do not prepare the working solution in a glass container.

2. Prepare the standards.
   a. Add 190 µL Qubit™ working solution to two labeled Qubit™ Assay Tubes used for standards.
   b. Add 10 µL of Qubit™ standard (Components C) to the one tube and 10 µL of Qubit™ standard (Components D) to the other tube.

c. Mix by vortexing 2–3 seconds. Do not create bubbles.

3. Prepare the sample or library.
   a. Aliquot 198 µL Qubit™ working solution to labeled Qubit™ Assay Tubes used for samples.
   b. Add 2 µL of the library pool to the appropriate sample tube.
   c. Mix by vortexing 2–3 seconds. Do not create bubbles.

4. Incubate the tubes in the dark at room temperature for 2 minutes.

5. Measure standards first to generate a standard curve, then measure the library pool.

6. Convert ng/µL to nM by multiplying the ng/µL values obtained in step 5 by 6.06 nmol/mg.

   Example: Library pool concentration is 10 ng/µL

   10 ng/µL (or 10 mg/L) × 6.06 nmol/mg = 60.6 nmol/L = 60.6 nM

   Note: For DNA segments of 250-bp average length:

   MW = (250 bp × 660,000 mg/mol/bp) = 1.65×10^8 mg/mol

   Inverting 1.65×10^8 mg/mol and multiplying by 1×10^9 nmol/mol gives 6.06 nmol/mg.

7. Dilute the library pool to 1 nM.

   Note: In the preceding example, add 298 µL of Low TE to 5 µL of 60.6-nM pooled library stock.

   See the *Ion ReproSeq™ PGS Kits – Ion S5™/Ion GeneStudio™ S5 Systems User Guide* (Pub. No. MAN0016712) if library yield and concentration is lower than expected.

STOPPING POINT Diluted library pools and non-diluted library pool stock solutions can be stored for 1 week at 4°C. For longer term, store at –20°C.

Guidelines for templating and sequencing and data analysis

Proceed to Chapter 5 of the *Ion ReproSeq™ PGS Kits – Ion S5™/Ion GeneStudio™ S5 Systems User Guide* (Pub. No. MAN0016712).

IMPORTANT!

- You can multiplex up to 16 libraries on an Ion 510™ Chip.
- You can multiplex up to 24 libraries on an Ion 520™ Chip.
- You can multiplex up to 96 libraries on an Ion 530™ Chip.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN1000343 A

Revision	Date	Description
A	24 July 2024	New document for niPGT-A analysis techniques.