iontorrent

Ion ReproSeq™ PGS View Kits

USER GUIDE

Single cell library preparation and sequencing on the Ion PGM™ System

Catalog Numbers A32243, A32249, A32250, A32251, A32252, A32253

Publication Number MAN0016158

Revision E.0

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ThermoFisher SCIENTIFIC

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Revision history: Pub. No. MAN0016158

Revision	Date	Description
E.0	7 June 2018	• Chapter 6, "Create a Planned Run" updated for Torrent Suite™ Software 5.10. • Guidance added for adjusting the transition penalty parameter to improve detection of small segmental CNV events. • Guidance added for adjusting the expected normal ploidy buffer to improve filtering of false positives. • Guidance added for adjusting for a smaller tile size to increase sensitivity to small deletions.
D.0	21 March 2018	• Chapter 6, "Create a Planned Run" updated for Torrent Suite™ Software 5.8 • Recommendations for Ion Reporter™ workflows in Chapter 9, "Analyze the run" corrected, and the chapter updated for Ion Reporter™ Software 5.6
C.0	26 December 2017	• Updated for use of the Qubit™ 4 Fluorometer • Guidance added in Chapter 9, "Analyze the run" for editing IRGV viewing preferences (see "Edit the IRGV preferences" on page 85) • Troubleshooting expanded for libraries showing high MAPD values (see "High MAPD value (>0.3) is observed" on page 116)
B.0	31 July 2017	• Human CEPH Genomic DNA Control removed from the Ion SingleSeq™ Kit • In "Pool, purify, and quantify the libraries" on page 30, guidance for volume of library pool to purify changed from entire library pool volume to a 40-µL aliquot. • Supplemental procedures removed from Appendices B and C. Users referred to the *Ion PGM™ System Reference Guide* (Pub. No. MAN0009783) for further information • Chapter 9 "Analyze the run" updated for new workflows available in Ion Reporter™ Software 5.4 • Web links and references updated
A.0	25 October 2016	New User Guide provides detailed, step-by-step instructions on how to use the Ion ReproSeq™ PGS View Kits to extract gDNA from single cells, prepare libraries, and generate templated Ion Sphere™ Particles for sequencing using the Ion PGM™ System.

Contents

About this guide

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

Purpose

The *Ion ReproSeq™ PGS View Kits User Guide* (Pub. No. MAN0016158) provides protocols and reference information for using an Ion ReproSeq™ PGS View Kit to prepare whole genome pooled libraries from single cell samples, and prepare template-positive Ion Sphere™ Particles from them using an isothermal amplification reaction. A protocol for using the Ion PGM™ Hi-Q™ View Sequencing Kit with Ion 314™, Ion 316™ and Ion 318™ Chips on the Ion PGM™ System is also provided in this guide.

Prerequisites

This guide also assumes that you have:

- A general understanding of Ion Torrent™ sequencing chemistry and workflow
- Knowledge of techniques for handling and preparing DNA libraries

1 Product information

Product description

The Ion ReproSeq™ PGS View Kits are bundles of four or five sub-kits which, when used in conjunction with the Ion PGM™ System, provide reagents and materials for whole genome amplification and sequencing to detect chromosomal aneuploidies, chromosome arm events (>48 Mb), and copy number variations starting from a single cell.

The Ion SingleSeq™ Kit (24 reactions/kit) includes reagents to extract, amplify, and barcode genomic DNA. The Ion PGM™ Template IA 500 Kit includes reagents for preparing 4 reactions of template-positive Ion PGM™ Template IA Ion Sphere™ Particles (ISPs) for sequencing with the Ion PGM™ System. Template preparation is performed using Ion IA technology, whereby DNA is clonally amplified onto a bead surface through a non-emulsion, isothermal reaction. The Ion PGM™ Hi-Q™ View Sequencing Kit, and Ion PGM™ Wash 2 Bottle Kit include reagents and materials for 4 initializations and up to 12 sequencing runs on the Ion PGM™ Sequencer.

Software compatibility

Ion ReproSeq™ PGS View Kits are compatible with Torrent Suite™ Software 5.2.0 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.

Note: If you use Torrent Suite™ Software 5.2 or earlier, you will need to add custom analysis parameters to the Planned Run, since the Ion ReproSeq™ PGS settings are overwritten after selecting Ion PGM™ Hi-Q™ View Sequencing Kit in run planning. See "Add custom analysis parameters (Torrent Suite™ Software 5.2 or earlier users only)" on page 53 for more information, or contact your local field application specialist for assistance.

Kit contents and storage

Kit configurations

Six configurations of the Ion ReproSeq™ PGS View Kit are available. The kits vary in the number of Ion SingleSeq™ Kits provided, the maximum number of samples processed per kit, the type of Ion sequencing chip provided, and whether Ion sequencing chips are included.

Ion ReproSeq™ PGS View Kit	Cat. No.	Samples per kit
Ion ReproSeq™ PGS 314 View Kit, with chips • Ion SingleSeq™ Kit (1 Kit) • Ion PGM™ Template IA 500 Kit • Ion PGM™ Hi-Q™ View Sequencing Kit • Ion PGM™ Wash 2 Bottle Kit • Ion 314™ Chip Kit v2 BC (8 chips)	A32243	16[1]
Ion ReproSeq™ PGS 316 View Kit, with chips • Ion SingleSeq™ Kit (5 kits) • Ion PGM™ Template IA 500 Kit • Ion PGM™ Hi-Q™ View Sequencing Kit • Ion PGM™ Wash 2 Bottle Kit • Ion 316™ Chip Kit v2 BC (8 chips)	A32249	120
Ion ReproSeq™ PGS 318 View Kit, with chips • Ion SingleSeq™ Kit (8 kits) • Ion PGM™ Template IA 500 Kit • Ion PGM™ Hi-Q™ View Sequencing Kit • Ion PGM™ Wash 2 Bottle Kit • Ion 318™ Chip Kit v2 BC (8 chips)	A32250	192
Ion ReproSeq™ PGS 314 View Kit, without chips • Ion SingleSeq™ Kit (1 Kit) • Ion PGM™ Template IA 500 Kit • Ion PGM™ Hi-Q™ View Sequencing Kit • Ion PGM™ Wash 2 Bottle Kit	A32251	16
Ion ReproSeq™ PGS 316 View Kit, without chips • Ion SingleSeq™ Kit (5 kits) • Ion PGM™ Template IA 500 Kit • Ion PGM™ Hi-Q™ View Sequencing Kit • Ion PGM™ Wash 2 Bottle Kit	A32252	120

Ion ReproSeq™ PGS View Kit	Cat. No.	Samples per kit
Ion ReproSeq™ PGS 318 View Kit, without chips • Ion SingleSeq™ Kit (8 kits) • Ion PGM™ Template IA 500 Kit • Ion PGM™ Hi-Q™ View Sequencing Kit • Ion PGM™ Wash 2 Bottle Kit	A32253	192

[1] Although an Ion SingleSeq™ kit provides reagents for preparing 24 barcoded libraries, we recommend that no more than 2 libraries be loaded on a Ion 314™ Chip v2. See "Guidelines for the number of libraries per run" on page 23 for the recommended maximum number of libraries per sequencing run for each kind of chip.

Ion SingleSeq™ Kit

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

Component	Amount	Storage
Cell Extraction Buffer (green cap)	120 µL	–30°C to –10°C
Extraction Enzyme Dilution Buffer (violet cap)	115.2 µL	
Cell Extraction Enzyme (yellow cap)	4.8 µL	
Pre-Amplification Buffer (red cap)	115.2 µL	
Pre-Amplification Enzyme (white cap)	4.8 µL	
Amplification Buffer (orange cap)	648 µL	
Amplification Enzyme (blue cap)	12 µL	
Nuclease-free Water (clear cap)	108 µL	
Barcode Plate (Ion SingleSeq™ Barcodes 1–24 loaded in wells A1–H3)	1 plate; 5 µL/well	

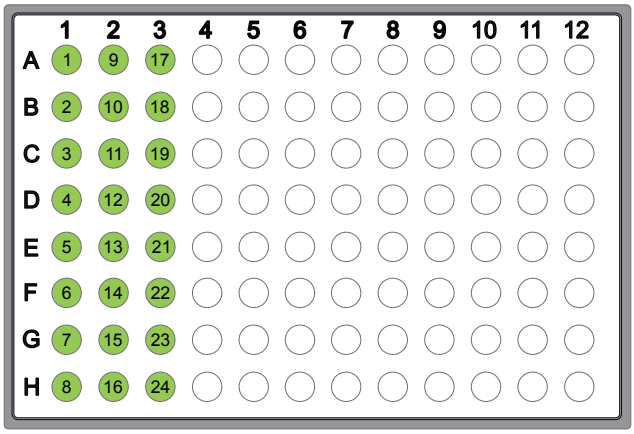
About the Ion SingleSeq™ Barcode Adapters

The Ion SingleSeq™ Kit is designed for high throughput applications and includes a 96-well Barcode Plate containing 24 unique Barcode Adapters. 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 24 barcodes 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.

Ion PGM™ Template IA 500 Kit

IMPORTANT! Use only the Ion PGM™ Template IA 500 Kit with the procedure in this guide. Do not use the Ion OneTouch™ 2 Instrument. Do not mix reactions or disposables, including plates, solutions, and kit reagents from other template preparation kits. We have verified this protocol using this specific material. Substitution can adversely affect performance.

IMPORTANT! Ion PGM™ Template IA Reactions 500 is shipped at 4°C to 8°C. On receipt, store at –30°C to –10°C. Immediately before use, thaw tubes on ice as needed.

Kit contents

Contents[1]	Amount	Storage
Ion PGM™ Template IA Supplies 500 (Part No. A24618)		
Ion OneTouch™ ES Supplies, including: • Eppendorf™ LoRetention Dualfilter, 300 µL PCR pipette tips (5) • 8-well strips (12)	1 bag	15°C to 30°C
Ion PGM™ Template IA Reagents 500 (Part No. A24619)		
Ion PGM™ Template IA Pellets 500	4 foil pouches [2]	4°C to 8°C
Ion PGM™ Template IA ISP Dilution Buffer (yellow cap)	1 mL	
Ion PGM™ Template IA Start Solution (purple cap)	2 × 1200 µL	
Ion PGM™ Template IA Reactions 500 (Part No. A24620)		
Ion PGM™ Template IA Ion Sphere™ Particles (orange cap)	86 µL	–30°C to –10°C
Ion PGM™ Template IA Primer Mix S (black cap)	40 µL	
Ion PGM™ Template IA Primer Mix L (blue cap)	40 µL	
Ion PGM™ Template IA Rehydration Buffer (white cap)	2 × 1.5 mL	
Ion PGM™ Template IA Solutions 500 (Part No. A24621)		
Ion PGM™ Template IA Stop Solution	2.8 mL	15°C to 30°C
Ion PGM™ Template IA Recovery Solution	7 mL	
Ion PGM™ Template IA Wash Solution	4 mL	
MyOne™ Beads Wash Solution (green cap)	1.04 mL	
Neutralization Solution (red cap)	40 µL	
Tween™ Solution	3.5 mL	

[1] We have verified this protocol using this specific material. Substitution can adversely affect performance.

[2] One Ion PGM™ Template IA Pellet 500 per tube per pouch. One pellet used per IA 500 amplification reaction.

Ion PGM™ Hi-Q™ View Sequencing Kit

The Ion PGM™ Hi-Q™ View Sequencing Kit (Cat. No. A30044) contains sufficient reagents and materials for 4 initializations of the Ion PGM™ Sequencer. Using 250 flows per run, the kit enables up to 3 runs per initialization, and up to 12 runs per kit.[1] The kit includes the following components:

Component	Amount	Storage
Ion PGM™ Sequencing Supplies (Part No. A25587)		
Wash 1 Bottle w/ label (250 mL)	1 bottle	15°C to 30°C
Wash 3 Bottle w/ label (250 mL)	1 bottle	
Ion PGM™ Reagent Bottle Sipper Tubes (blue)	16 tubes	
Ion PGM™ Wash Bottle Sipper Tubes (gray)	8 tubes for 250-mL bottles 4 tubes for 2-L bottles	
Reagent Bottles w/ labels (50 mL)	25 bottles	
Ion PGM™ Hi-Q™ View Sequencing Reagents (Part No. A30043)		
Ion PGM™ Hi-Q™ View Sequencing Polymerase (yellow cap)	36 µL	–30°C to –10°C
Ion PGM™ Hi-Q™ View Sequencing Primer (white cap)	144 µL	
Control Ion Sphere™ Particles (clear cap)	60 µL	
Ion PGM™ Hi-Q™ View Sequencing Solutions (Part No. A30275)		
Ion PGM™ Hi-Q™ View Sequencing W2 Solution	4 × 125 mL	2°C to 8°C (store W2 Solution protected from light)
Ion Cleaning Tablet	4 tablets	
Annealing Buffer	12 mL	
Ion PGM™ Hi-Q™ View Sequencing W3 Solution	2 × 100 mL	
Ion PGM™ Hi-Q™ Sequencing dNTPs (Part No. A25590)		
Ion PGM™ Hi-Q™ Sequencing dGTP (black cap)	80 µL	–30°C to –10°C
Ion PGM™ Hi-Q™ Sequencing dCTP (blue cap)	80 µL	
Ion PGM™ Hi-Q™ Sequencing dATP (green cap)	80 µL	
Ion PGM™ Hi-Q™ Sequencing dTTP (red cap)	80 µL	

[1] If you are using a kit that provides chips, and you wish to perform 3 runs per initialization, you will need to order additional sequencing chips separately.

Ion PGM™ Wash 2 Bottle Kit

The Ion PGM™ Wash 2 Bottle Kit (Cat. No. A25591) includes the following components:

Component	Amount	Storage
Wash 2 Bottle w/ label (2 L) **Note:** Must be conditioned at least 8 hours before use as described in "Condition the Wash 2 Bottle for first use" on page 56	1 bottle	15°C to 30°C
Wash 2 Bottle Conditioning Solution	125 mL	

Ion Chip Kits

The following Ion Chip Kits are compatible with the Ion ReproSeq™ PGS View Kits, and can be ordered separately if your Ion ReproSeq™ PGS View Kit does not include chips:

Component	Quantity	Catalog No.	Storage
Ion 314™ Chip Kit v2 BC	8 pack	4488144	15°C to 30°C
Ion 316™ Chip Kit v2 BC	4 pack	4488145	
	8 pack	4488149	
Ion 318™ Chip Kit v2 BC	4 pack	4488146	
	8 pack	4488150	

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other 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
(Optional) Control DNA (from CEPH Individual 1347-02) (50 ng/µL)	403062
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, *or* Qubit™ 3.0 Fluorometer[4], *or* Qubit™ 2.0 Fluorometer[4]	Q33226
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

Item	Source
SYBR™ Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO[5]	S7567
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*	G5018-02 G8008-02 G7008-02 G6400 G6000-02
Materials and equipment required for templating	
Ion OneTouch™ ES Instrument	4473574
2.0-mL Eppendorf™ DNA LoBind™ Microcentrifuge Tubes	Fisher Scientific 13-698-792
Heat block set to 40°C	MLS
Ion PGM™ Enrichment Beads (Dynabeads™ MyOne™ Streptavidin C1 Beads)	4478525 65001, 65002
DynaMag™-2 magnet	12321D
Materials and equipment required for sequencing	
Tank of compressed nitrogen (grade 4.5, 99.995% or better) (see "Gas cylinders" on page 21)	MLS
Multistage (dual-stage) gas regulator (0-50 PSI, 2-3 Bar output)	Fisher Scientific NC0393866 or MLS
Choose from one of the following systems: ELGA™ PURELAB™ Flex 3 Water Purification System Equivalent 18-MΩ water purification system	 4474524 MLS
Microcentrifuge (capable of >15,500 × g, fits 1.5-mL and 0.2-mL microcentrifuge tubes)	MLS
0.22-µm or 0.45-µm vacuum filtration system and filters (nylon or PVDF filters, 1-L volume)	MLS
Rainin™ Pipet-Lite™ LTS Pipette L-100XLS 10–100 µL[6] (Alternatives from Gilson and Eppendorf may be used)	Rainin 17014384
Rainin™ Pipet-Lite™ LTS Pipette L-20XLS 2–20 µL (Alternatives from Gilson and Eppendorf may be used)	Rainin 17014392

Item	Source
Rainin™ LTS pipette tips, 200 µL, SR-L200F (Alternatives from Gilson and Eppendorf may be used)	Rainin 17005859
Rainin™ LTS pipette tips, 20 µL, SR-L10F (Alternatives from Gilson and Eppendorf may be used)	Rainin 17005860
PCR tubes, Flat Cap, 0.2-mL (do not use polystyrene tubes)	Fisher Scientific 14-222-262
Vortexer with a rubber platform	MLS
Thermal cycler with a heated lid	MLS
Graduated cylinders (1 L or 2 L volume)	MLS
Glass bottle (1 L)	MLS
15-mL conical tubes	MLS
NaOH (10 M), molecular biology grade	MLS
Pipette set and filtered tips, P2, P20, P200, and P1000 µL	MLS
Microcentrifuge tubes, 1.5-mL or 1.7-mL	MLS
Syringe, 10 CC, Female Luer-Lok™ (used for clearing lines)	Provided with the Ion PGM™ Sequencer, or MLS
Optional materials	
Ion PGM™ Controls Kit v3 [7]	A30046
Ion PGM™ Sequencing Sippers Kit[8]	4478682
Non-interruptible Power Supply (UPS)[9]	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 strip and caps may be substituted.

[3] Can substitute 2.0-mL Eppendorf LoBind™ Tubes for 1.5-mL tubes.

[4] Supported but no longer available for purchase.

[5] Required if monitoring amplification by real-time PCR.

[6] Ensure tips from any vendors are low binding tips. Required for loading samples onto the Ion PGM™ Chips.

[7] Not commonly needed, but available for troubleshooting.

[8] Contains additional sipper tubes; not commonly needed.

[9] We recommend using a non-interruptible power supply (UPS) for laboratories that experience frequent power outages or line voltage fluctuations. The UPS must be compatible with 1500 W output or higher. The 1500 VA unit from APC provides ~11 minutes of backup power for an Ion PGM™ System.

Optional materials and equipment

The materials in the following table are optional and required only to verify and adjust the pH of the W2 Solution prior to sequencing the Ion chips.

✓	Item	Source
	Thermo Scientific™ Orion Star™ A111 pH Benchtop Meter Kit with electrode, electrode stand, and calibration buffers (or equivalent)	Fisher Scientific 13-645-503
	1 N HCl	MLS
	Magnetic stirrer (must hold 2-L bottle)	MLS
	Magnetic stir bar (4 cm)	MLS
	Squirt bottle	MLS

Workflow overview

Library preparation	"Extract and amplify the gDNA" ▼ "Pool, purify, and quantify the libraries" ▼
Template preparation	Chapter 4, "Prepare template-positive Ion PGM™ Template IA ISPs" ▼ Chapter 5, "Enrich the template-positive Ion PGM™ Template IA ISPs" ▼
Sequencing	Chapter 6, "Create a Planned Run" ▼ Chapter 7, "Clean and initialize the Ion PGM™ Sequencer" ▼ Chapter 8, "Load the chip and start the sequencing run" ▼
Analysis	Chapter 9, "Analyze the run"

2 Before you begin

Before you begin

For additional safety information, see Appendix C, "Safety".

Update the software

IMPORTANT! Before proceeding, make sure that you have updated the Torrent Suite™ and Ion PGM™ System software to version 5.2.1 or later. See the *Ion PGM™ System Reference Guide* (Pub. No. MAN0009783) for more information.

Instrument installation by trained personnel only

IMPORTANT! The Ion PGM™ System is installed by trained service personnel and must not be relocated without assistance from trained service personnel. See "Customer and technical support" on page 121.

Nucleic acid contamination

IMPORTANT! A primary source of contamination is DNA fragments from previously processed samples. Do not introduce amplified DNA into the library preparation laboratory or work area.

IMPORTANT! Possible contamination can occur during the transfer of dNTPs into Reagent Tubes. Be careful to avoid cross contamination of dNTP stocks. Barrier tips are required for all pipetting steps. Change gloves after handling concentrated dNTP stocks.

CO_2 contamination

IMPORTANT! Dry ice (solid CO_2) must be kept away from areas where buffers, wash solutions, or sources of molecular biology grade water for the Ion PGM™ System are used. High air concentrations of subliming CO_2 may change the pH of such buffers during or after their preparation. The stability of the pH of these buffers is a critical factor in the performance of the Ion PGM™ System.

Instrument vibration and clearances

IMPORTANT! Significant vibration during sequencing may add noise and reduce the quality of the measurements. The Ion PGM™ Sequencer must be installed on a bench that is free from vibrations or in contact with equipment that can cause vibrations to the bench, such as freezers, pumps, large benchtop centrifuges, and other similar equipment. Mini and microcentrifuges may be used near the sequencer. An air table is not required, nor is securing the sequencer to the bench.

IMPORTANT! Position the Ion PGM™ Sequencer so that the front bezel is a minimum of 12 in. (30.5 cm) and the Reagent Tubes containing dNTPs are a minimum of 8 in. (20.3 cm) from the front of the laboratory bench. Place the instrument at least 40 in. (1 meter) away from major sources of electronic noise such as refrigerators or microwaves.

Static electricity

IMPORTANT! To avoid possible damage to chips from static electricity, see "Guidelines for chip handling and use" on page 23.

Ventilation requirements

WARNING! Instrumentation must be installed and operated in a well-ventilated environment, defined as having a minimum airflow of 6–10 air changes per hour. Assess the need for ventilation or atmospheric monitoring to avoid asphyxiation accidents from inert gases and/or oxygen depletion, and take measures to clearly identify potentially hazardous areas through training or signage. Please contact your Environmental Health and Safety Coordinator to confirm that the instruments will be installed and operated in an environment with sufficient ventilation.

Gas cylinders

You must supply the required nitrogen gas cylinder and accessories for the installation. This instrument requires a pressurized house line or one size 1-A nitrogen gas cylinder that holds approximately 7.2 m^3 (257 ft^3) of gas when full. Use only prepurified nitrogen of 99.995% (grade 4.5) or greater purity.

CAUTION! Damage to the instrument and its products can result from using impure gas, gases other than nitrogen, or an inadequate amount of gas.

WARNING! EXPLOSION HAZARD. Pressurized gas cylinders are potentially explosive. Always cap the gas cylinder when it is not in use, and attach it firmly to the wall or gas cylinder cart with approved brackets or chains.

WARNING! Gas cylinders are heavy and may topple over, potentially causing personal injury and tank damage. Cylinders should be firmly secured to a wall or work surface. Please contact your Environmental Health and Safety Coordinator for guidance on the proper installation of a gas cylinder.

Perform a leak test

To perform a leak test on the gas cylinder:

1. Open the main tank shutoff valve. The high-pressure gauge of the gas tank regulator reads approximately 2,000–2,500 psi for a full tank.
2. Adjust the pressure to the instrument by slowly turning the pressure adjustment valve clockwise until the low-pressure gauge reads 30 psi.
3. Close the needle valve, then close the main tank valve.
4. Monitor the high-pressure gauge of the gas tank regulator for 5 minutes. There should be no noticeable drop in pressure.

If the pressure	Action
Drops in 5 minutes	There can be a leak at either the needle valve or the gas tank regulator itself. Check the fittings and resolve any problems, then continue with step 5.
Does not drop in 5 minutes	The instrument passes the leak test. Reopen the main tank valve and skip the following steps.

5. Open the main tank valve and the needle valve for at least 15 seconds to pressurize the instrument.
6. Close the main tank valve.
7. Monitor the high-pressure gas tank regulator gauge. There should be no more than a 100-psi drop in pressure after 5 minutes. Locate, then resolve any leaks. Turn the main tank valve back on.

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 gDNA extraction, pre-amplification, 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 the guidelines in the table below:

Ion Chip	Recommended maximum libraries per run
Ion 314™ Chip v2 BC	2
Ion 316™ Chip v2 BC	15
Ion 318™ Chip v2 BC	24

Guidelines for chip handling and use

IMPORTANT! To avoid damage to Ion 318™ Dx Chips or the Ion PGM™ Dx Sequencer due to electrostatic discharge:

- **Remove your gloves when handling chips**, especially before transferring chips on or off the instrument. Follow the steps in the sequencing procedure for taking off and putting on gloves.
- **Hold chips by their edges when handling.**
- **Do not place chips directly on the bench or any other surface.** Always place chips either on the grounding plate on the Ion PGM™ Dx Sequencer or in the Ion PGM™ Dx Chip Minifuge bucket.

Note: Ion 318™ Dx Chips can be handled without gloves during all stages of chip preparation, loading, and sequencing without risk of contamination.

Used chips cannot be reused for sequencing. Used chips must be discarded or clearly marked for cleaning and initialization.

3 Prepare and pool Ion SingleSeq™ libraries

Extract and amplify the gDNA

Materials required

Provided in the Ion SingleSeq™ Kit (Part No. A28955):

- Cell Extraction Buffer
- Extraction Enzyme Dilution Buffer
- Cell Extraction Enzyme
- Pre-Amplification Buffer
- Pre-Amplification Enzyme
- Amplification Buffer
- Amplification Enzyme
- Nuclease-free Water
- Barcode Plate containing Ion SingleSeq™ Barcodes 1–24

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

Guidelines for sample handling

- **Keep the cells on ice.**
- For single cells or low-concentration control input gDNA, do not insert pipette tip into sample. The single cell could adhere to the tip and get removed from the sample tube.
- Add liquids above the top of liquid in a tube, do not submerge the tip.
- **Do not vortex the cells.**
- After adding Extraction Enzyme Master Mix to samples, do not mix. Vortexing or pipetting up and down can cause loss of cells on the wall of the well or pipette tip.
- Keep cell lysis and amplification reactions on ice or a cold block during reaction setup, and keep Ion SingleSeq™ libraries on ice during library pooling and quantification.

Extract genomic DNA

1. Prepare, by FACS or micro-manipulation, 1–10 cells per sample in up to 2.5 µL 1X PBS or Low TE, then pipet the cells into wells of a 96-well optical reaction plate.

   Note: Instead of a 96-well reaction plate, a PCR tube strip, or individual PCR tubes can be used.

2. Add Cell Extraction Buffer (green cap) to each sample well to bring the total volume to 5 µL.

   Note:
   - You can also sort cells by FACS directly into Cell Extraction Buffer at a density of 1–10 cells/5 µL, then add 5 µL aliquots to reaction plate wells.
   - If using gDNA for troubleshooting, mix 2 µL of user-supplied control human gDNA at a concentration of 7.5 pg/µL with 3 µL Cell Extraction Buffer. You can use Control DNA (from CEPH Individual 1347-02), Cat. No. 403062, as control human gDNA. To dilute a 50 ng/µL gDNA stock solution to 7.5 pg/µL,
     a. Add 2 µL of stock solution to 198 µL of Low TE. Vortex to mix, then centrifuge briefly.
     b. Add 2 µL of this solution to 131 µL of Low TE. Vortex to mix, then centrifuge briefly.

   Instead of Low TE, 1X PBS or Nuclease-free Water can be used.

3. (Optional) Prepare a Non-Template Control (NTC) by adding 2.5 µL 1X PBS to 2.5 µL Cell Extraction Buffer.

4. Prepare an Extraction Enzyme 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, then centrifuge to collect liquid at the bottom of the tube.

   Extraction Enzyme master mix

Component	Volume per reaction	Volume for N reactions[1]
Extraction Enzyme Dilution Buffer (violet cap)	4.8 µL	N × 4.8 µL × 1.1
Cell Extraction Enzyme (yellow 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.

5. Add 5 µL Extraction Enzyme master mix to each cell or gDNA sample (10-µL final volume).

   IMPORTANT! Do not mix. Vortexing or pipetting up and down can cause loss of cells on the wall of the well or pipette tip.

6. Seal the plate, then centrifuge at 1,000 × *g* for 30 seconds to collect liquid at the bottom of the wells.

7. Incubate the samples in a thermal cycler using the following temperature program:

Temperature	Time
75°C	10 minutes
95°C	4 minutes
22°C	Hold

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

Pre-amplify the gDNA

1. Prepare a Pre-amplification 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.

   Pre-amplification master mix

Component	Volume per reaction	Volume for N reactions [1]
Pre-Amplification Buffer (red cap)	4.8 µL	N × 4.8 µL × 1.1
Pre-Amplification 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.

2. Carefully remove the film seal from the plate, then add 5-µL Pre-amplification master mix to each sample well (15-µL final volume).

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

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

4. 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	12
	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 approximately 1 hour.

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

5. 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 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 1.5-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.

   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

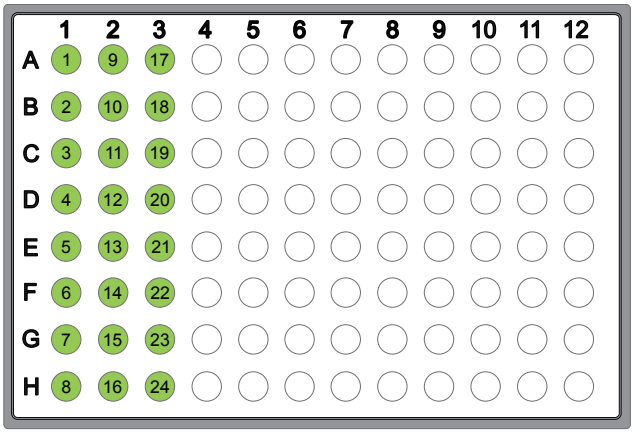
[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 µL.
[2] Replace with Nuclease-free Water if performing endpoint PCR.

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 plate map) 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.

   Note: See "About the Ion SingleSeq™ Barcode Adapters" on page 11 for barcode plate handling guidelines.



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

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 pre-amplification, 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	12
	72°C	55 seconds	
4	4°C	Hold	1

[1] Cycling time is approximately 30 minutes.
[2] Acquire fluorescence data at this step if monitoring amplification in real-time.

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
- Low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- Qubit™ dsDNA HS Assay Kit
- Qubit™ 2.0, Qubit™ 3.0, or Qubit™ 4 Fluorometer
- 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 0.2-mL tube to create an equi-volume pool. Vortex the tube to mix and pulse-centrifuge 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 within 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.

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

   Note:
   - When pooling fewer than 8 libraries, the pool volume drops below 40 µL. Do not transfer, but 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. 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

2. Briefly centrifuge the tube to collect contents, then transfer the heated library pool to a new 1.5-mL Eppendorf DNA LoBind™ tube.
3. To the 40 µL of heated library pool, add 40 µL (1X volume) of room temperature AMPure™ XP beads.
4. Vortex briefly, pulse-centrifuge the tube to collect contents, then incubate for 5 minutes at room temperature.
5. Place the tube in the DynaMag™-2 magnet, then wait 5 minutes for beads to aggregate to the side of the tube.
6. Aspirate the supernatant carefully, then discard.
7. Wash beads with 250 µL of freshly prepared 70% ethanol while the tube is still on the magnet.
8. Incubate for 30 seconds.
9. Aspirate, then discard the wash solution.
10. Repeat steps 7–9, thoroughly removing all ethanol after the second wash.
11. Allow the beads to dry at room temperature for 3–4 minutes with the tube on the magnet.
12. Remove the tube from the magnet, then resuspend beads in 40 µL of Low TE by pipetting up and down.
13. Incubate the tube at room temperature for 1 minute.
14. Place the tube in the DynaMag™-2 magnet, then wait 2–3 minutes for beads to aggregate to the side of the tube.
15. Transfer 35 µL of the supernatant containing the purified library pool to a new 1.5-mL Eppendorf DNA 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. See the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326) for more information.

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 each Qubit™ standard (Components C and D) to the appropriate tube.
   c. Mix by vortexing 2–3 seconds. Do not create bubbles.

3. Prepare the unknown:
   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. For the Qubit™ 2.0 Fluorometer:
   a. For the library pool: on the Qubit™ instrument, press **Calculate Stock Conc.**.
   b. Turn roller wheel to 2 (µLs original sample added to reagent).
   c. Adjust units to ng/µL, then record this value.

      Note: See the *Qubit™ dsDNA HS Assay Kits User Guide* for the measurement protocol if using the Qubit™ 3.0 or Qubit™ 4 Fluorometer.

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) × 6.061 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.

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

   See "Library yield is low" on page 101 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. After 1 week, we recommend that you thaw the individual Ion SingleSeq™ libraries and repeat the pooling, purification, quantification, and dilution steps starting from "Pool the libraries" on page 30.

4 Prepare template-positive Ion PGM™ Template IA ISPs

Materials required

Provided in the Ion PGM™ Template IA Reagents 500 (Part No. A24619):

- Ion PGM™ Template IA Pellet (one pellet per reaction)
- Ion PGM™ Template IA ISP Dilution Buffer
- Ion PGM™ Template IA Start Solution

Provided in the Ion PGM™ Template IA Reactions 500 (Part No. A24620):

- Ion PGM™ Template IA Ion Sphere™ Particles (ISPs)
- Ion PGM™ Template IA Primer Mix S
- Ion PGM™ Template IA Rehydration Buffer

Provided in the Ion PGM™ Template IA Solutions 500 (Part No. A24621):

- Ion PGM™ Template IA Stop Solution
- Ion PGM™ Template IA Recovery Solution
- Ion PGM™ Template IA Wash Solution

Other Materials and equipment

- Nuclease-free Water
- 2-mL Eppendorf LoBind™ Tubes
- Vortexer
- Microcentrifuge
- Low retention barrier pipette tips
- Pipettors
- Heat block set to 40°C (with water added to wells)

Isothermal amplification (IA) reaction procedural guidelines

Guidelines for preventing contamination

The IA reaction is highly sensitive to contaminating DNA. Follow the guidelines below to prevent introduction and carryover of contaminating DNA sequences in the work area.

- A primary source of contamination is spurious DNA fragments from previous sample processing steps. Do not introduce amplified DNA into the work area.
- To prevent carryover contamination, wipe down pipettors, hood and other work surfaces with a 70% ethanol-moistened Kimwipes™ disposable wipe before each experiment.
- After performing the reaction, rinse tube racks with Milli-Q™-quality water and dry.
- If possible, perform Ion PGM™ Template IA ISP preparation/reaction assembly and amplification/ISP recovery in two rooms: a pre-PCR clean room for ISP preparation/reaction assembly and a "dirty" post-PCR room for amplification and ISP recovery.
- If two rooms are used, ISP preparation and reaction assembly in a laminar flow hood is recommended but not required.
- If the entire procedure needs to be performed in one room, prepare the Ion PGM™ Template IA ISPs in a clean pre-PCR laminar flow hood.

IA reaction guidelines

- Use Ion PGM™ Template IA Primer Mix S (black cap) in the Ion ReproSeq™ PGS procedure. Do not use Primer Mix L (blue cap).
- To achieve optimal results, do not agitate tubes after the pulse-centrifugation step that follows addition of the Ion PGM™ Template IA Start Solution to the IA reactions.
- The length of the IA reaction (25 minutes at 40°C) is important. Follow these guidelines if you are performing multiple reactions:
  - Limit the number of reactions you perform at the same time to 4.
  - If you need to perform more than 4 reactions, stagger the start and termination of each additional reaction by 5 minutes.

Before you begin

1. Set a heat block to 40°C, with water added to wells.
2. Thaw the Ion PGM™ Template IA Primer Mix S (black cap), and keep it and the Ion PGM™ Template IA Start Solution (purple cap) on ice while setting up the reaction.
3. Thaw the Ion PGM™ Template IA ISPs (orange cap) and Ion PGM™ Template IA Rehydration Buffer (white cap) and keep on ice while setting up the reaction.

Prepare the final library pool dilution

1. Dilute the 1-nM library pool prepared in the previous chapter 1:100 with Nuclease-free Water to generate a 10-pM library pool.
2. Transfer 50 µL of the 10-pM library pool to a new 0.2-mL PCR tube.
3. Heat the tube in a thermal cycler using the following program:

Step	Temperature	Time	Number of cycles
1	70°C	2 minutes	1
2	4°C	Hold	1

4. Remove the tube from the thermal cycler and place on ice.

Perform the IA reaction

1. Prepare Templating Solution in a 2-mL Eppendorf LoBind™ Tube on ice (or a cold block):

   Templating Solution

Order of addition	Component	Volume per reaction
1	Ion PGM™ Template IA ISP Dilution Buffer (yellow cap)	122 µL
2	Ion PGM™ Template IA Primer Mix S[1] (black cap)	8 µL
3	Ion PGM™ Template IA ISPs[2] (orange cap)	21 µL
4	Library pool (10 pM)[3]	18 µL
—	**Total**	**169 µL**

[1] Do not use Primer Mix L (blue cap).
[2] Vortex 30 seconds at maximum speed to resuspend immediately before addition.
[3] From step 4 of the previous section.

2. Vortex the tube containing the Templating Solution for 2 seconds at maximum setting to mix, pulse-centrifuge, then return the tube to ice.
3. Invert the Ion PGM™ Template IA Rehydration Buffer (white cap) 3 times to mix, then use 720 µL to rehydrate the Ion PGM™ Template IA Pellet. Vortex for 2 seconds at maximum setting, then pulse-centrifuge to collect contents at the bottom of the tube. Place the rehydrated pellet on ice or a cold block.

   Note: The rehydrated Ion PGM™ Template IA Pellet is opaque.

4. Transfer the rehydrated Ion PGM™ Template IA Pellet to Templating Solution on ice, vortex for 2 seconds at maximum setting, then pulse-centrifuge.

   IMPORTANT! The rehydrated pellet solution is viscous. Ensure that you transfer the entire volume by pulse-centrifuging the rehydration tube after transfer and pipetting any remaining volume into the Templating Solution.

5. Invert the Ion PGM™ Template IA Start Solution (purple cap) 3 times to mix, then add 300 µL to the Template/IA Solution using the reverse pipetting technique.

   Note: If you are setting up more than one IA reaction, follow steps 5 through 7 for each reaction before starting the next reaction.

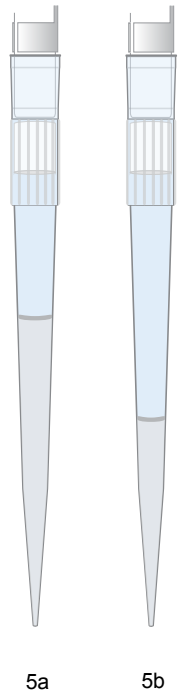
   To use the reverse pipetting technique, perform the following steps:

   a. Set a 1-mL pipette to 300 µL.

   b. Press the pipette knob to the second stop, then dip the tip into the Start Solution.

   c. Slowly release the pipette knob until it returns to the starting position. Allow 10 seconds for Start Solution to be fully drawn up into the tip (Fig. 5a).

   d. Dispense the solution into the Templating Solution tube by gently pressing the pipette knob to the first stop point only. Wait at least 5 seconds until the liquid in the pipette tip stops moving. Some liquid remains in the tip (Fig. 5b).



   e. Withdraw the tip from the tube. If any liquid adheres to the outer surface of the tip, touch the tip to the inner wall of the tube to transfer the liquid to the tube.

6. Vortex the tube 10 times in 1-second pulses at the maximum vortexer setting. Invert the tube, then repeat the ten 1-second pulses.

7. Pulse-centrifuge the tube to collect contents, then immediately place the tube on ice.

   IMPORTANT! Handle the tube gently after centrifuging. To achieve optimum results, do not agitate the tubes from this point on.

8. Start the IA reaction by gently placing the tube in the 40°C heat block. Ensure that the tube is immersed in water.

9. Incubate the IA reaction for 25 minutes at 40°C.

Recover the template-positive ISPs

1. Stop the IA reaction by removing the tube from the heat block and adding 650 µL of Ion PGM™ Template IA Stop Solution.
2. Vortex the tube well to mix contents thoroughly, then centrifuge the tube at 7,500 × *g* for 3 minutes.
3. Aspirate, then discard the supernatant, being careful not to disturb the pellet. Leave ~100 µL in the tube.
4. Resuspend the pellet in 1 mL Ion PGM™ Template IA Recovery Solution.
   a. Pipette up and down to resuspend the pellet.
   b. Add an additional 700 µL Ion PGM™ Template IA Recovery Solution, then vortex thoroughly.
5. Incubate for 5 minutes with vortexing 5 seconds every minute.
6. Centrifuge for 3 minutes at 12,000 × *g*.
7. Immediately remove, then discard all of the supernatant without disturbing the ISP pellet. Remove any bubbles before removing the bulk of the liquid to avoid frothing in subsequent steps.

   Note: The ISPs form a glassy pellet that is barely visible. Note the orientation of the tube in the centrifuge so that the position of the ISP pellet is known. The supernatant must be removed immediately to minimize the resuspension of ISPs.
8. Add 100 µL of the Ion PGM™ Template IA Wash Solution to the ISP pellet.
9. Resuspend the templated ISPs completely by vortexing for 4 seconds at maximum speed, then pipet the ISP suspension up and down 4 times. Proceed to Chapter 5, "Enrich the template-positive Ion PGM™ Template IA ISPs".

STOPPING POINT Store templated ISPs in Ion PGM™ Template IA Wash Solution at 4°C for up to one week.

5 Enrich the template-positive Ion PGM™ Template IA ISPs

Materials required

Provided in Ion PGM™ Template IA Solutions 500 (Part No. A24621):

- Ion OneTouch™ Template IA Wash Solution
- MyOne™ Beads Wash Solution
- Tween™ Solution
- Neutralization Solution

Provided in Ion PGM™ Template IA Supplies 500 (Part No. A24618):

- 8-well strip
- Eppendorf™ LoRetention Dualfilter Tips (P300)

Other Materials and Equipment:

- Ion OneTouch™ ES Instrument
- Ion PGM™ Enrichment Beads (Cat. No. 4478525; Dynabeads™ MyOne™ Streptavidin C1 Beads)
- 1.5-mL Eppendorf LoBind™ Tubes
- 0.2-mL PCR tubes
- Nuclease-free Water
- 1 M NaOH
- Pipettes
- Vortexer
- DynaMag™-2 magnet
- Microcentrifuge

Determine if a residual volume test is necessary

IMPORTANT! Ensure that the AC line voltage module is installed correctly into the Ion OneTouch™ ES Instrument. See the *Ion OneTouch™ 2 System User Guide* (Pub. No. MAN0014388) for information regarding instrument setup, calibration, and maintenance.

To determine if a residual volume test is necessary, follow these guidelines:

Condition	Action
First use of the instrument and during monthly maintenance	Perform a residual volume test (see "Ion OneTouch™ ES Instrument installation, setup, and maintenance" in the *Ion OneTouch™ 2 System User Guide* Pub. No. MAN0014388).
Routine use and residual volume in Well 1 and Well 8 is >5.0 µL	
Routine use and residual volume in Well 1 and Well 8 is ⩽5.0 µL	Operate the instrument without performing the residual volume test. Proceed to "Prepare reagents then fill the 8-well strip" on page 41.

Prepare reagents then fill the 8-well strip

Prepare Melt-Off Solution

Prepare fresh Melt-Off Solution by combining the components in the following order:

Order	Component	Volume
1	Tween™ Solution	280 µL
2	1 M NaOH	40 µL
—	**Total**	**320 µL**

IMPORTANT! Prepare Melt-Off Solution as needed, but appropriately dispose of the solution after 1 day.

The final composition of the Melt-Off Solution is 125 mM NaOH and 0.1% Tween™ 20 detergent.

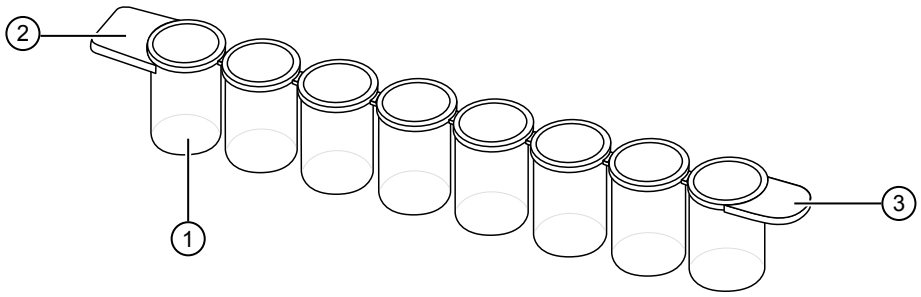
Wash and resuspend the Dynabeads™ MyOne™ Streptavidin C1 Beads

1. Vortex the tube of Dynabeads™ MyOne™ Streptavidin C1 Beads for 30 seconds to resuspend the beads thoroughly, then centrifuge the tube for 2 seconds.
2. Open the tube, then use a new tip to pipet the dark pellet of beads up and down until the pellet disperses. Immediately proceed to the next step.
3. Transfer 13 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind™ Tube.
4. Place the tube on a magnet such as a DynaMag™-2 magnet for 2 minutes, then carefully remove and discard the supernatant without disturbing the pellet of Dynabeads™ MyOne™ Streptavidin C1 Beads.
5. Add 130 µL of MyOne™ Beads Wash Solution to the Dynabeads™ MyOne™ Streptavidin C1 Beads.
6. Remove the tube from the magnet, vortex the tube for 30 seconds, then centrifuge for 2 seconds.

Fill the 8-well strip

Note: If you stored template-positive ISPs at 2°C to 8°C, vortex the tube to resuspend the ISPs and pulse-centrifuge to collect contents. Pipet the solution up and down to resuspend the Ion PGM™ Template IA ISPs and transfer to Well 1 of the 8-well strip.

1. Add the entire volume (~100 µL) of template-positive ISPs from the amplification reaction into Well 1 of the 8-well strip. Well 1 is nearest to the square-shaped tab:



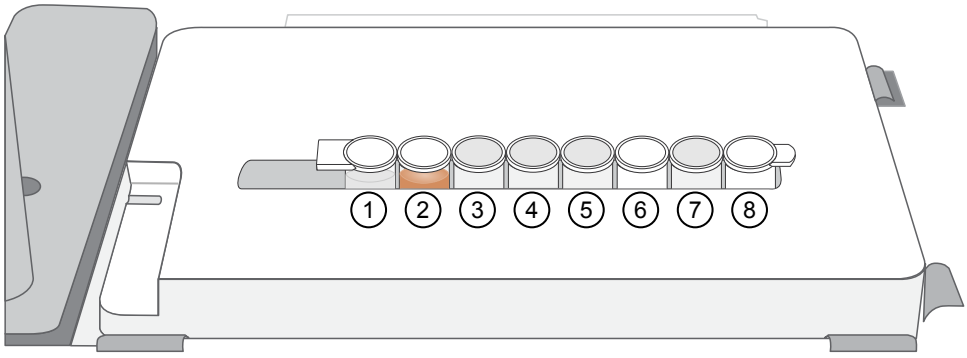
① Well 1
② Square-shaped tab
③ Rounded tab

2. If you have not already assessed the quality of the unenriched, template-positive ISPs, use the following method:

Quality assessment by	Action
Guava™ easyCyte 5 Benchtop Flow Cytometer	Transfer a 1.0-µL aliquot of the unenriched ISPs to a 1.5-mL Eppendorf LoBind™ Tube. See the *Ion Sphere™ Particles (ISPs) Quality Assessment Using the Guava™ easyCyte 5 Benchtop Flow Cytometer User Bulletin* (Pub. No. MAN0015799), available at: **http://tools.thermofisher.com/content/sfs/manuals/MAN0015799.pdf**
Demonstrated protocol: Quality assessment by the Applied Biosystems™ Attune™ Acoustic Focusing Cytometer	Transfer a 1.0-µL aliquot of the unenriched ISPs to a 1.5-mL microcentrifuge tube. Put the sample on ice, then see the *Demonstrated Protocol: Ion Sphere™ Particles (ISPs) Quality Assessment using the Applied Biosystems™ Attune™ Acoustic Focusing Cytometer User Bulletin* (Pub. No. 4477181), available at: **http://tools.thermofisher.com/content/sfs/manuals/4477181A.pdf**

3. Fill the remaining wells in the 8-well strip as follows (see the following figure):

Well number	Reagent to dispense in well
Well 1 (well nearest to the square-shaped tab)	Entire template-positive ISP sample (100 µL; prepared in step 1 of this procedure)
Well 2	130 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads resuspended in MyOne™ Beads Wash Solution [prepared in "Wash and resuspend the Dynabeads™ MyOne™ Streptavidin C1 Beads" on page 41]
Well 3	300 µL of Ion PGM™ Template IA Wash Solution
Well 4	300 µL of Ion PGM™ Template IA Wash Solution
Well 5	300 µL of Ion PGM™ Template IA Wash Solution
Well 6	Empty
Well 7	300 µL of freshly-prepared Melt-Off Solution (prepared in "Prepare Melt-Off Solution" on page 41)
Well 8	Empty



4. Ensure that the square-shaped tab is on the left, then insert the filled 8-well strip with the 8-well strip pushed all the way to the right end of the slot of the Tray.

Prepare the Ion OneTouch™ ES

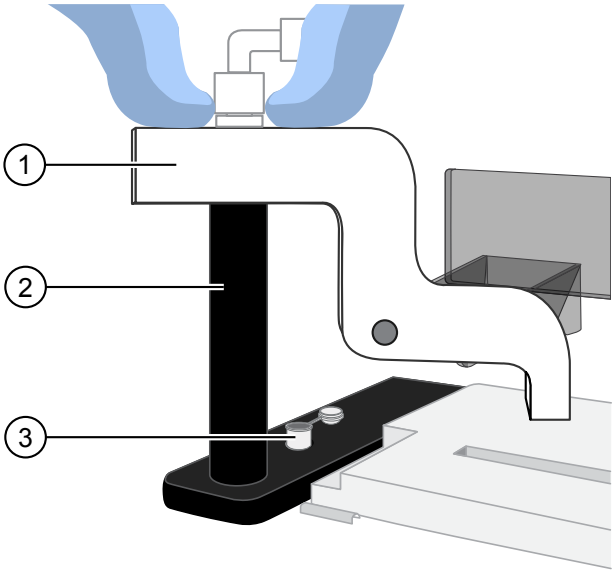
Before every enrichment performed on the Ion OneTouch™ ES Instrument, install a new PCR collection tube and a new Eppendorf™ LoRetention Dualfilter P300 pipette tip.

1. Add 10 µL of Neutralization Solution to a new 0.2-mL PCR tube.

2. Insert the open 0.2-mL PCR tube containing Neutralization Solution into the hole in the base of the Tip Loader, as shown in the figure in step 4.

3. Place a new tip in the Tip Loader. Remove the Tip Arm from the cradle, then align the metal fitting of the Tip Arm with the tip.

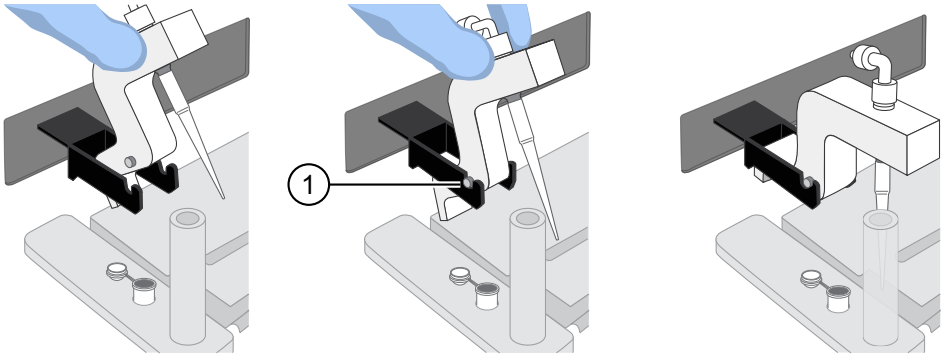
4. Keeping the fitting on the Tip Arm vertical, firmly press the Tip Arm down onto the new tip until the Tip Arm meets the Tip Loader. Hold the Tip Arm to the Tip Loader for ~1 second to ensure proper installation of the tip.



① Tip Arm
② Tip Loader
③ 0.2-mL PCR collection tube containing Neutralization Solution

5. Lift the Tip Arm *straight* up to pull the installed tip from the Tip Loader tube.

6. Return the Tip Arm to the cradle.
   a. Tilt the Tip Arm back (below left), then align the pins with the round notches in the cradle (below center).
   b. Lower the Tip Arm into position (below center).
   c. Move the Tip Arm forward into the working position (below right).



① Tip Arm pins resting in the notches in the cradle

IMPORTANT! Ensure that the back/bottom end of the Tip Arm is not resting on top of the thumb screw, causing the Tip Arm to tilt forward.

Perform the Ion OneTouch™ ES run

Ensure that a new tip and opened 0.2-mL PCR tube with the Neutralization Solution have been loaded. Ensure that Well 1 (ISP sample) is the left-most well and that the 8-well strip is pushed to the far-right position in the slot.

1. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run. Do not introduce bubbles into the solution.

2. If needed, power on the Ion OneTouch™ ES, then wait for the instrument to initialize. The screen displays "rdy". The Tip Arm performs a series of initialization movements and returns to the home position (~5 seconds).

3. Press **Start/Stop**. The screen displays "run" during the run. The run takes ~35 minutes.

   Note: If necessary to stop a run, press **Start/Stop**. The instrument completes the current step, then stops the run and displays "End". Press **Start/Stop** again to return the Tip Arm to the home position. It is not possible to restart (where you left off) after stopping a run.

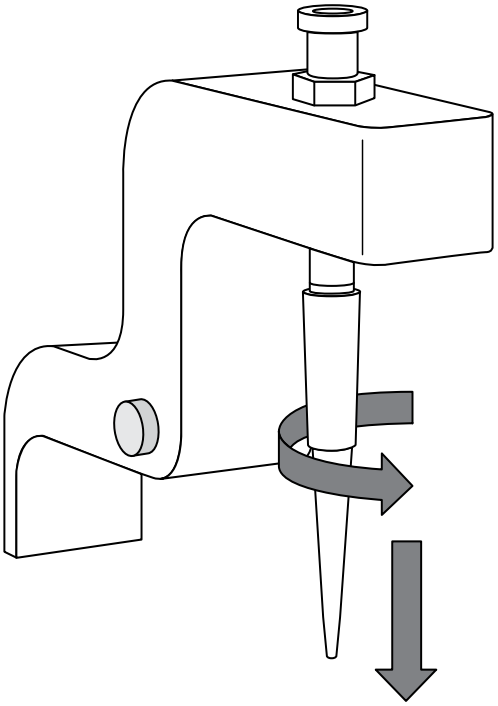
4. At the end of the run, the instrument displays "End" and beeps every 60 seconds. Press the **Start/Stop** button to silence this alarm, then reset the Ion OneTouch™ ES for the next run. The instrument can be left on between runs.

5. ***Immediately after the run***, securely close and remove the PCR tube containing the enriched ISPs.

6. Mix the contents of the PCR tube by gently inverting the tube five times.

   Ensure that the 0.2-mL PCR tube has >200 µL of solution containing the enriched ISPs. After a successful run on the instrument, the sample is in ~230 µL of Melt-Off Solution, Ion PGM™ Template IA Wash Solution, and Neutralization Solution. If the tube has <<200 µL of solution containing the enriched ISPs, contact Technical Support.

7. Remove the used tip: While you are standing above the Tip Arm, and with the Tip Arm in its cradle, twist the tip counterclockwise (as viewed from above), then pull it downward to remove. Discard the tip:



IMPORTANT! Improper removal of tips can loosen the metal tip adapter fitting on the Tip Arm and affect instrument operation.

8. Remove, then discard the used 8-well strip.

Sequence or store the template-positive ISPs

- Sequence using the Ion PGM™ Hi-Q™ View Sequencing Kit, bundled in the Ion ReproSeq™ PGS View Kits. Proceed to Chapter 6, "Create a Planned Run".

  or

- Store the enriched ISPs at 2°C to 8°C for up to 3 days.

Perform quality control on enriched ISPs

You can determine the enrichment efficiency using one of the following methods:

Quality assessment by	Action
Guava™ easyCyte 5 Benchtop Flow Cytometer	Transfer a 1.0-µL aliquot of the enriched ISPs to a 1.5-mL Eppendorf LoBind™ Tube. Refer to the *Ion Sphere™ Particles (ISPs) Quality Assessment Using the Guava™ easyCyte 5 Benchtop Flow Cytometer User Bulletin* (Pub. No. MAN0015799), available at: **http://tools.thermofisher.com/content/sfs/manuals/MAN0015799.pdf**
Attune™ NxT Acoustic Focusing Cytometer	Transfer a 1.0-µL aliquot of the enriched ISPs to a 1.5-mL Eppendorf LoBind™ Tube. Put the sample on ice, then refer to *Demonstrated Protocol: Ion Sphere™ Particles (ISPs) Quality Assessment using the Applied Biosystems™ Attune™ Acoustic Focusing Cytometer User Bulletin* (Pub. No. 4477181), available at: **http://tools.thermofisher.com/content/sfs/manuals/4477181A.pdf**

6 Create a Planned Run

IMPORTANT! This sequencing kit is compatible with Torrent Suite™ Software v5.2.1 and later. Before proceeding, check for updates to the Torrent Suite™ and sequencing instrument software, and install the updates if available.

About Planned Runs

Planned Runs contain all the settings that are used in a sequencing run, including number of flows, kit types, barcodes used (if any), run type (for example, DNA, RNA, amplicons), and reference file (if any). They provide a fast and convenient way to set up and organize your runs.

You create a Planned Run using the Torrent Browser on the Torrent Server connected to your sequencer, then select the appropriate plan in the **Select Planned Run** screen of the sequencer touchscreen when you start the run.

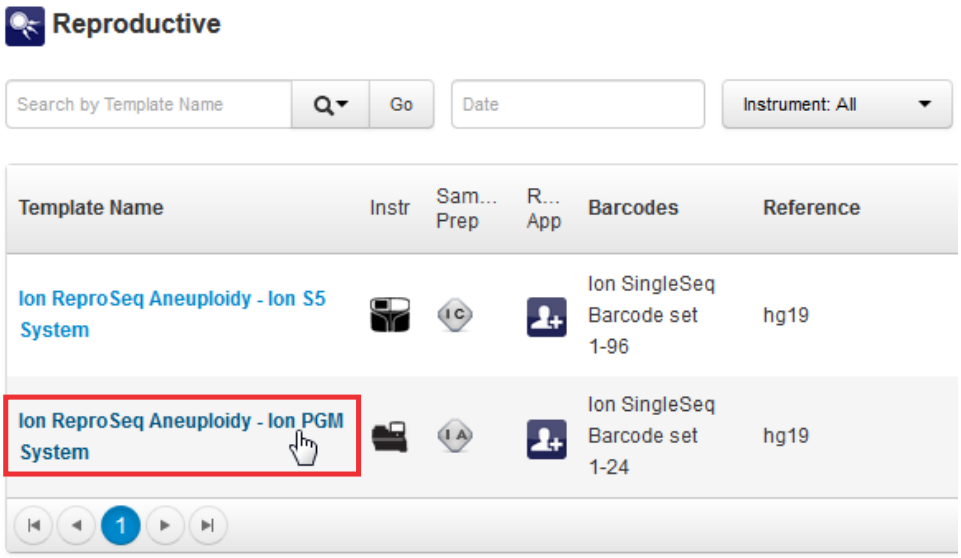
You can also create a Planned Run on one Torrent Server and then transfer it to another server for sequencing. See the *Ion PGM™ Hi-Q™ View Sequencing Kit User Guide*, (Pub. No. MAN0014583; Appendix B), for more information.

Note: For additional information on creating a Planned Run or Planned Run template, see *Torrent Suite™ Software Help*, available at **thermofisher.com**.

Create a Planned Run

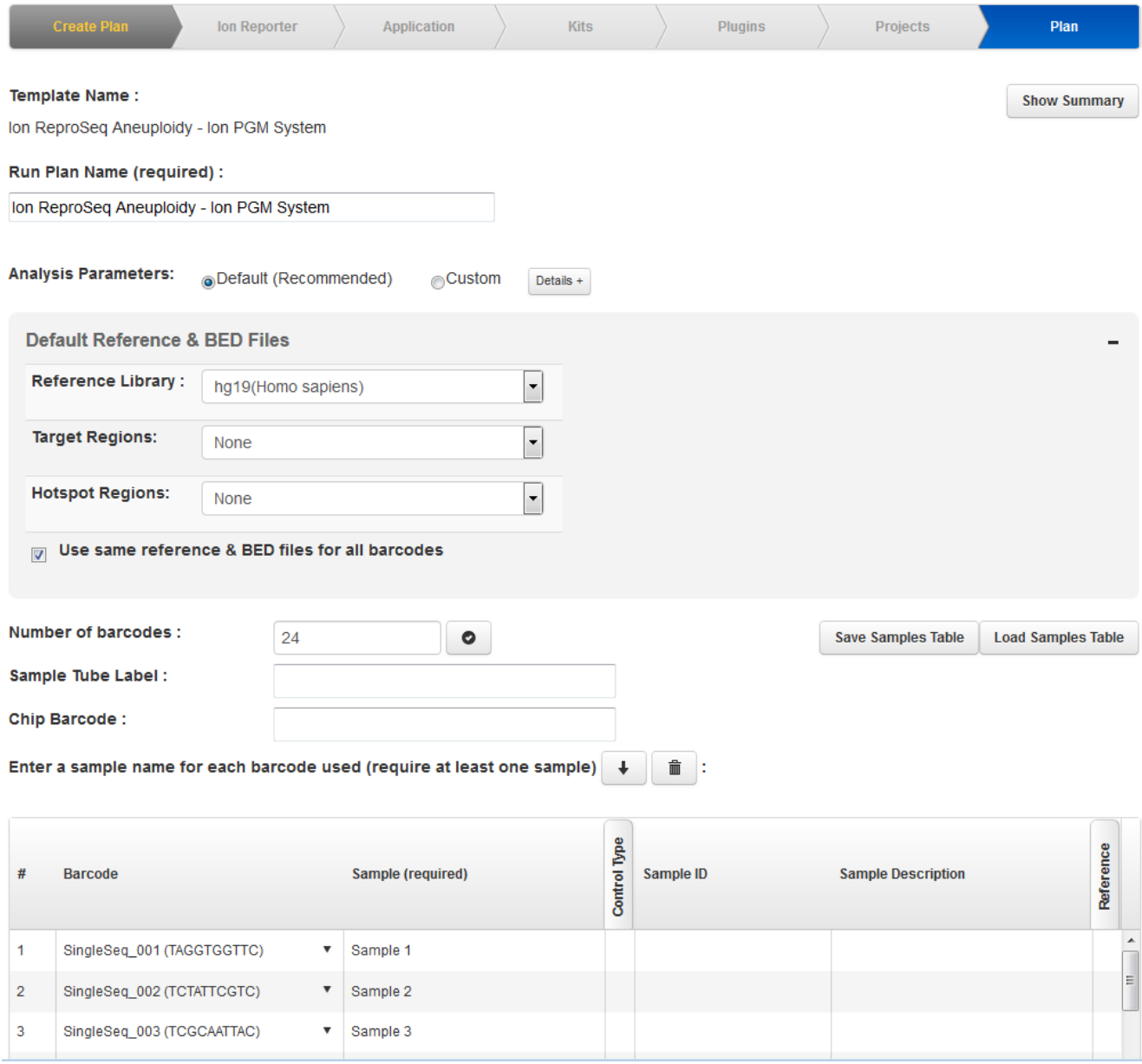
1. Sign in to the Torrent Server in Torrent Suite™ Software.
2. Select the **Plan** tab, select **Plan Template Run**, then click **Reproductive** from the list of applications on the left side of the screen.

3. Select **Ion ReproSeq Aneuploidy - Ion PGM System** from the list of Planned Run templates.



4. The Ion ReproSeq Aneuploidy - Ion PGM System template auto-populates settings in the Application, Kits, and Plugins steps in the Planned Run wizard appropriately. Key fields are described in "Planned Run wizard: key fields" on page 55. In the **Plan** step, enter or make the following selections:
   a. Enter a new **Run Plan Name**, if desired.
   b. Select **hg19(Homo sapiens)** from the **Reference Library** dropdown list.
   c. Select **None** from the **Target Regions** and **Hotspot Regions** dropdown lists.
   d. Enter the number of barcodes you are using in your Sample Set.

e. Select a Barcode from the dropdown list for each sample, if different from the Barcode shown. Enter a unique, descriptive name, such as one including one or more sample-specific or grouping attributes you want to track, for each Ion SingleSeq™ Barcode used. Avoid use of the default "Sample 1", "Sample 2", and so on.

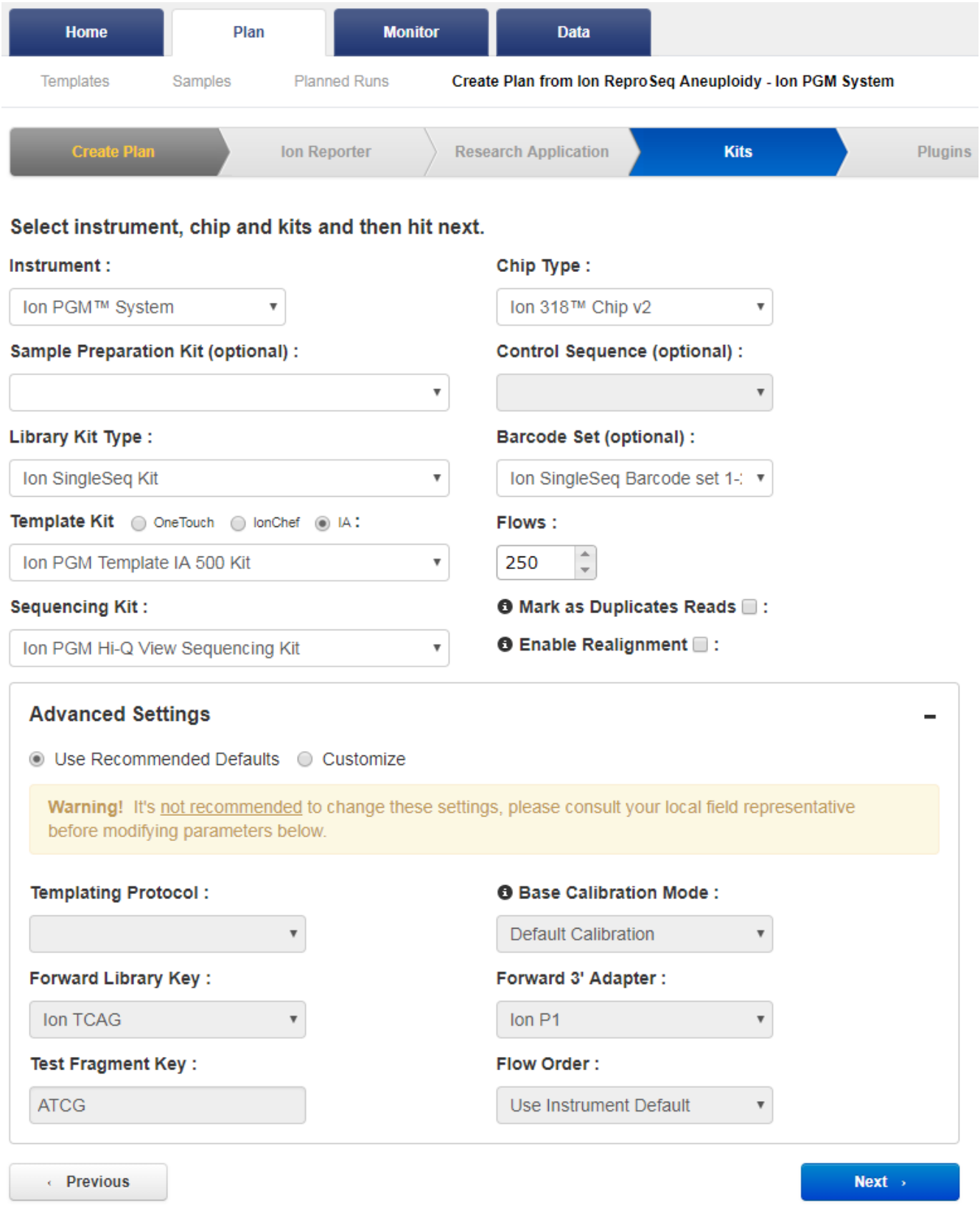


IMPORTANT! We strongly recommend that you assign unique sample names for each barcode and experiment. This enables easy identification and retrieval of results in Ion Reporter™ analyses based on experiment name, plate code, and so on. Do not reuse sample names from experiment to experiment.

5. To set up auto-analysis using a Ion Reporter™ workflow, click the **IonReporter** step, then
   a. Select your account.
   b. Select the appropriate Ion ReproSeq™ workflow from the dropdown list.
6. In the **Kits** step, verify the selections, or make changes appropriate for your run:
   a. Select **Ion PGM™ System** from the **Instrument** dropdown list, if it is not autopopulated.
   b. Select the chip type that you are using. Chip type is set to Ion 318™ Chip v2 by default.
   c. Select **Ion SingleSeq Kit** from the **Library Kit Type** dropdown list.
   d. Select **Ion PGM Template Kit IA 500 Kit** from the **Template Kit** dropdown list.
   e. Select **Ion PGM Hi-Q View Sequencing Kit** from the **Sequencing Kit** dropdown list.
   f. Select the **Ion SingleSeq Barcode Set 1–24** from the **Barcode Set** dropdown list:
   g. Enter **250** flows.
   h. Click the **-/+** toggle to expand the **Advanced Settings** pane. Ensure that **Ion P1** is selected from the **Forward 3' Adaptor** dropdown list.

      Note: Recommended default settings are automatically selected in the **Advanced Settings** pane.

i. Click **Next** twice to proceed to the **Projects** step.



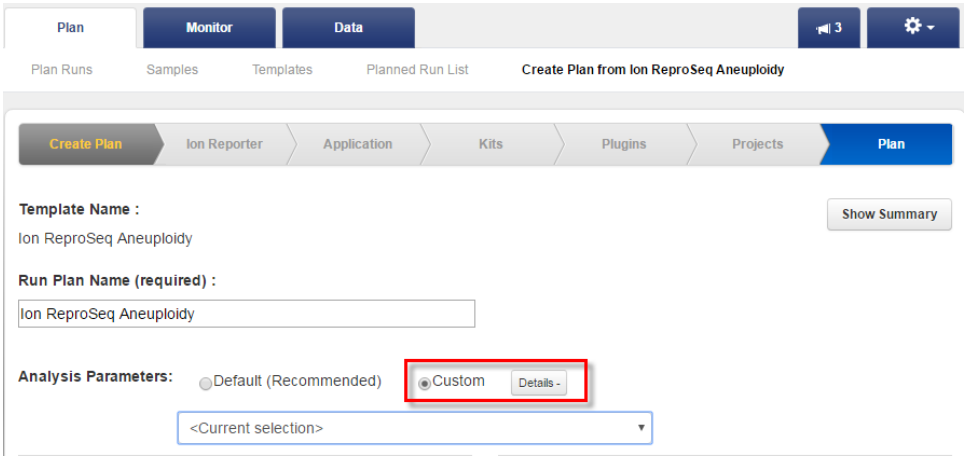
7. Complete your selections in the **Projects** step, then click **Next**.

8. In the **Plan** step, click **Plan Run** in the lower right corner to save the Planned Run. The run is listed on the Planned Runs screen under the name you entered.

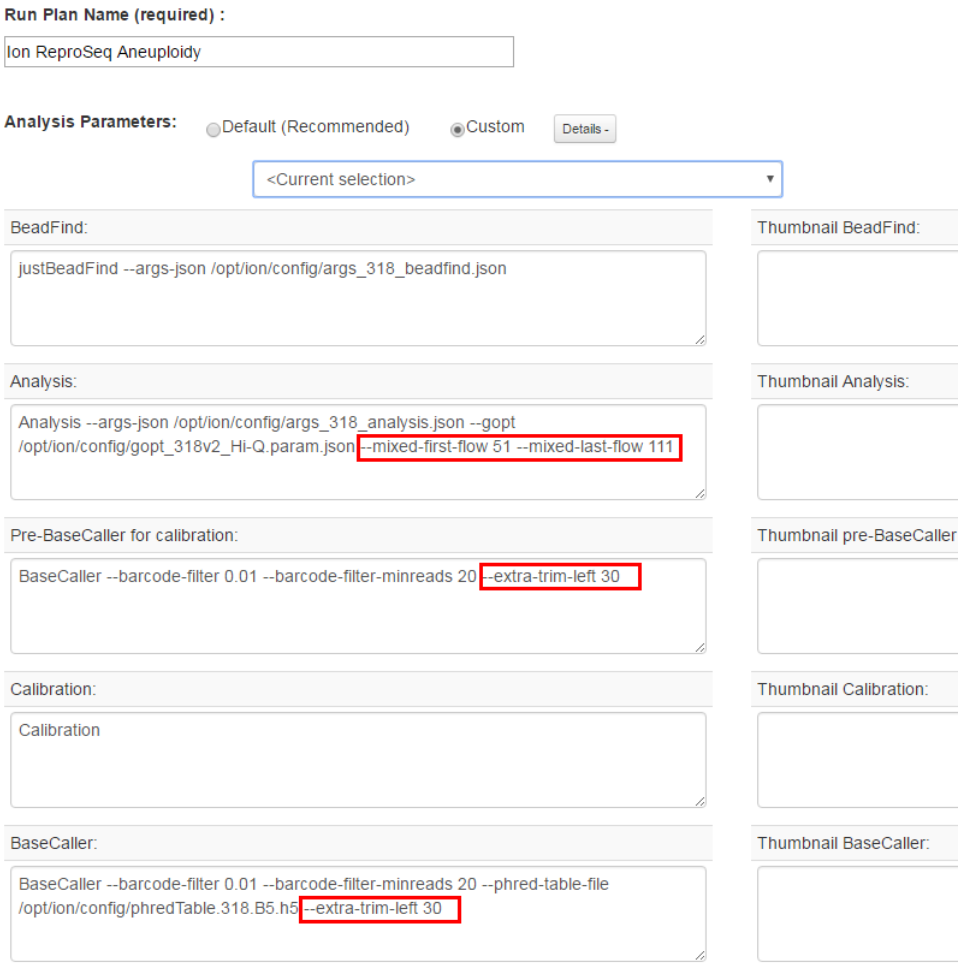
Add custom analysis parameters (Torrent Suite™ Software 5.2 or earlier users only)

If you use Torrent Suite™ Software 5.2 or earlier, you need to add custom analysis parameters for Ion SingleSeq™ libraries after selecting the Ion PGM™ Hi-Q™ View Sequencing Kit in run planning. In Torrent Suite™ Software 5.2.1 and later, these analysis parameters are populated automatically.

1. In the **Plan** tab, select the **Custom** button to the right of **Analysis Parameters:**. The Details fields will expand.



2. Add the following text in the listed fields:
   - **Analysis:** --mixed-first-flow 51 --mixed-last-flow 111
   - **Pre-BaseCaller for calibration:** --extra-trim-left 30
   - **BaseCaller:** --extra-trim-left 30



3. After completing the other selections and entering required information in the Plan tab, click **Plan Run** to save the Planned Run.

Planned Run wizard: key fields

Field name	Description
IonReporter	Select the account, then select the **ReproSeq PGS w1.1** workflow, or other Ion ReproSeq™ w1.1 workflow from the **Existing Workflow** dropdown list. To create a new workflow, click **Create New Workflow**.
Application	Select the sequencing application you are performing: **DNA Whole Genome**
Library Kit Type	Select **Ion SingleSeq Kit**.
Template Kit	Select **Ion PGM Template IA 500 Kit**.
Sequencing Kit	Select **Ion PGM™ Hi-Q™ View Sequencing Kit**.
Flows	Enter the appropriate number of flows for the sequencing kit and read length: ***250 flows***
Chip Type	Select the Ion chip type you are using.
Forward 3' Adapter	Select **Ion P1**.
Barcode Set	Select **Ion SingleSeq Barcode set 1–24**.
Project	Select or add a project within which to group your run data: Ion ReproSeq™ PGS
Run Plan Name	Enter a name for the Planned Run.
Reference Library	Select a reference library uploaded to the Torrent Server: **hg19**
Target Regions and Hotspot Regions	Set to "None" (default).
Enter a sample name	Enter a Sample Name, set relation to "Self", and assign unique Analysis IDs for each sample in the run (number of samples will change based on the number of barcodes selected). Avoid using the default names "Sample 1", "Sample 2", etc.
Monitoring Thresholds	Set thresholds for Bead Loading, Key Signal, and Usable Sequence. In the Torrent Browser **Monitor ▸ Runs in Progress** tab, an alert is displayed if the values for a run fall below the selected thresholds.

7 Clean and initialize the Ion PGM™ Sequencer

Condition the Wash 2 Bottle for first use

New Wash 2 Bottles must be conditioned with Wash 2 Bottle Conditioning Solution for at least 8 hours before first use.

Note: If necessary, you can reuse an existing Wash 2 Bottle while you condition a new bottle. Bottles can be used for sequencing up to 40 times before they must be replaced.

To condition the Wash 2 Bottle:

1. Fill the bottle to the mold line with 18 MΩ water, add the entire container of Wash 2 Bottle Conditioning Solution, then cap the bottle and invert it 5 times to mix.

2. Allow the bottle to sit at room temperature for at least 8 hours and preferably overnight, then dispose of the contents. The bottle is now ready for use.

Clean the Ion PGM™ System

Materials required

- 18 MΩ water (e.g., the ELGA™ PURELAB™ Flex Water Purification System)
- Cleaning bottles and collection trays (provided with the Ion PGM™ System)
- Old chip that has been used for sequencing, marked for cleaning
- Used sipper tubes (from the previous run)
- Squirt bottle
- **Chlorite cleaning:** Ion Cleaning Tablet (provided in the kit)
- **Chlorite cleaning:** 1 M NaOH, diluted fresh each week from 10 M NaOH
- **Chlorite cleaning:** Glass bottle (1 L)
- **Chlorite cleaning:** 0.22-µm or 0.45-µm vacuum filtration system and filters

Cleaning schedule

The Ion PGM™ Sequencer requires cleaning with either 18-MΩ water or a chlorite solution every time the instrument is initialized.

Clean with	Schedule
18 MΩ water	• Daily, when instrument is in use (e.g., not necessary on weekends) • After one or more runs totaling ⩽1,100 flows • If more than 27 hours but less than 48 hours have elapsed between the last cleaning/initialization and the start of a run • If you cleaned with chlorite a week ago and have not used the instrument since then
Chlorite solution	• Once a week, unless the instrument has not been used since the last chlorite cleaning (in which case, clean with 18 MΩ water before using) • If the instrument has been left with reagents for more than 48 hours (for example, over the weekend)

Cleaning setup

IMPORTANT! For all the following steps, use 18 MΩ water directly from the purification system. Do not use water that has been collected or stored in any other containers.

- Remove any wash and reagent bottles that are attached to the Ion PGM™ System before cleaning.
- Do not remove old sippers before cleaning. The sippers are used as part of the cleaning procedure.
- Old chips that have been used for sequencing can be marked and used in the cleaning procedure.
- Wash bottles (250 mL and 2 L) provided as part of instrument installation can be marked and used for cleaning. After you have used the wash bottles provided with the sequencing kit for the specified number of runs, you can use them as extra cleaning bottles. Mark them for cleaning use only.

18 MΩ water cleaning

1. Empty any remaining solution from each cleaning bottle (two 250-mL bottles and one 2-L bottle) and rinse each bottle twice with ~100 mL of 18 MΩ water.
2. Press **Clean** on the touchscreen, and select the **18-MOhm water cleaning** checkbox. Press **Next**.
3. Using ungloved hands, secure a used chip designated for cleaning in the chip clamp.

   IMPORTANT! Always make sure that both red rubber gasket port fittings are securely in place when securing chips with the chip clamp. Failure to do so can result in a spill hazard and instrument damage.
4. Remove all wash and reagent bottles attached to the instrument. Keep the sippers in place at all positions. Press **Next**.
5. Add 250 mL of 18 MΩ water to an empty 250-mL cleaning bottle.

6. Rinse the outside of the sipper tube in the W1 position on the instrument with a squirt bottle containing 18 MΩ water.
7. Attach the 250-mL bottle containing 18 MΩ water to the W1 position, ensuring that the W1 cap is screwed on tightly. Press **Next**.
8. Place the empty 2-L cleaning bottle in the W2 position and the empty 250-mL bottle in the W3 position, and insert the sippers into the bottles. Do not screw on the caps.
9. Place collection trays below the reagent sippers in the dNTP positions. Press **Next** to begin cleaning.
10. When cleaning is complete, remove the bottles and sippers from the W1, W2 and W3 positions. Leave the reagent sippers and collection trays in place. Press **Next** to return to the main menu and proceed to initialization.

Chlorite cleaning

Note: Prepare a stock of 1 M NaOH each week by diluting 10 M NaOH with 18 MΩ water.

1. Empty any remaining solution from each cleaning bottle (two 250-mL bottles and one 2-L bottle), then rinse each bottle twice with ~100 mL of 18 MΩ water.
2. Fill a glass bottle with 1 L of 18 MΩ water, then add an Ion Cleaning tablet (chlorite tablet). Allow the tablet to dissolve completely (~10 minutes).
3. When the tablet has dissolved, add 1 mL of 1 M NaOH and filter the solution using a 0.22-µm or 0.45-µm filter. Use the chlorite solution within 2–3 hours. Discard any unused solution after this time.
4. Press **Clean** on the touchscreen, then select the **Chlorite cleaning** checkbox. Press **Next**.
5. Using ungloved hands, secure a used chip designated for cleaning in the chip clamp.

   IMPORTANT! Always ensure that both red rubber gasket port fittings are securely in place when securing chips with the chip clamp. Failure to do so can result in a spill hazard and instrument damage.

6. Remove all wash and reagent bottles that are attached to the instrument. Keep the sippers in place at all positions. Press **Next**.
7. Add 250 mL of the filtered chlorite solution to an empty 250-mL cleaning bottle.
8. Rinse the outside of the sipper tube in the W1 position on the instrument with a squirt bottle containing 18 MΩ water.
9. Attach the 250-mL bottle with the filtered chlorite solution to the W1 position. Ensure that the W1 cap is tight. Press **Next**.
10. Place the empty 2-L cleaning bottle in the W2 position and the empty 250-mL bottle in the W3 position, then insert the sippers into the bottles. Do not screw on the caps.

11. Place collection trays below the reagent sippers in the dNTP positions. Press **Next** to start cleaning.
12. When prompted, remove the bottle containing the chlorite solution from the W1 position.
13. Rinse the outside of the W1 sipper tube with a squirt bottle containing 18 MΩ water.
14. Fill a clean 250-mL bottle with 250 mL of 18 MΩ water, then attach the bottle in the W1 position. Ensure the cap is tight. Press **Next** to start the water rinse.
15. When cleaning is complete, remove the bottles and sippers from the W1, W2 and W3 positions. Leave the reagent sippers and collection trays in place. Press **Next** to return to the main menu, then proceed to initialization.

Initialize the Ion PGM™ System

Initialization takes ~1 hour. As part of the initialization process, first prepare the Wash and Reagent Bottles as described in this section.

Materials required

Materials provided in the kit

- Ion PGM™ Hi-Q™ Sequencing dGTP
- Ion PGM™ Hi-Q™ Sequencing dCTP
- Ion PGM™ Hi-Q™ Sequencing dATP
- Ion PGM™ Hi-Q™ Sequencing dTTP
- Ion PGM™ Hi-Q™ View Sequencing W2 Solution (stored protected from light)
- Ion PGM™ Hi-Q™ View Sequencing W3 Solution
- Wash 1 and Wash 3 Bottles and sipper tubes
- Wash 2 Bottle and sipper tubes (bottle must be conditioned prior to first use, as described in "Condition the Wash 2 Bottle for first use" on page 56)
- Wash 2 Bottle Conditioning Solution
- Reagent Bottles and sipper tubes

Other materials and equipment

- Used chip (leave chip on the instrument during initialization)
- 18 MΩ water
- 100 mM NaOH (prepared daily)
- Ice
- 5-mL and 25-mL pipettes
- Filtered and unfiltered pipette tips and pipettes
- Vortex mixer
- Microcentrifuge
- **Optional:** Ion PGM™ Sequencing Sippers Kit (Cat. No. 4478682)

Initialization guidelines

IMPORTANT! Handle nucleotides carefully to avoid cross-contamination. Always change gloves after removing used sipper tubes from the Ion PGM™ System to avoid cross contamination of the nucleotides. Also change gloves after handling concentrated dNTP stocks.

For each initialization, the first run should be started within 1 hour after initialization, and the last run must be started within 24 hours after initialization.

Bottle usage

- Wash 2 Bottles can be used for up to 40 initializations, after which you can use them in the cleaning procedure.
- Wash 1 and Wash 3 Bottles can be used for up to 4 initializations, after which you can reuse them in the cleaning procedure.
- Replace the Reagent Bottles and sipper tubes every time you initialize.

Before initialization

1. Remove the dNTP stock solutions from the freezer and begin thawing on ice.
2. Check the tank pressure for the nitrogen gas. When the tank pressure drops below 500 psi, change the tank.

Prepare the Wash 2 Bottle

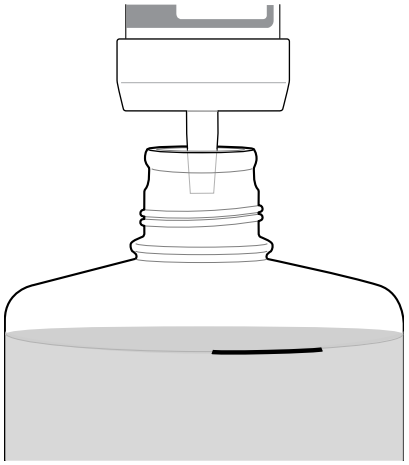
Note:

- Do not remove the old sippers from the dNTP ports until instructed to do so.
- Load the bottles as quickly as possible to prevent atmospheric CO_2 from reducing the pH of the Wash 2 solution.
- For all the following steps, pour the 18 MΩ water directly from the purification system into the Wash 2 Bottle. Do not use water that has been collected or measured in any other containers.

IMPORTANT! Do not let the new sippers touch any surfaces.

1. Rinse the Wash 2 Bottle (2 L) 3 times with 200 mL of 18 MΩ water.
2. Prepare 500 µL of 100 mM NaOH by diluting 50 µL of 1 M NaOH in 450 µL of nuclease-free water.

3. If your 18 MΩ water system has a spigot, extend it into **but not below** the neck of the Wash 2 Bottle. Otherwise, position the nozzle as close to the mouth of the bottle as possible.



Note: If your water system has a digital display, ensure it reads "18 MΩ" throughout filling the bottle. If not, see Appendix A, "Troubleshooting".

4. Fill the bottle to the mold line with 18 MΩ water. The volume of water is ~2 liters. (You can mark the mold line on the bottle for clarity.)

   Note: If you are preparing bottles for multiple sequencers, cap each bottle immediately after filling, and leave capped until you are ready to add Ion PGM™ Hi-Q™ View Sequencing W2 Solution.

5. Add the entire bottle of Ion PGM™ Hi-Q™ View Sequencing W2 Solution to the Wash 2 Bottle.

Note: Keep the Ion PGM™ Hi-Q™ View Sequencing W2 Solution bottle to scan the barcode during the initialization procedure.

6. Using a P200 pipette, add 70 µL of 100 mM NaOH to the Wash 2 Bottle.

   Note: Different sites can require adding different volumes of 100 mM NaOH. Some sites, for example, can require doubling the volume to 140 µL. See "Error message: Added too much W1 to W2" on page 114 for information on determining the volume of 100 mM NaOH to add.

7. Cap the bottle and invert 5 times to mix, and immediately proceed through the remainder of the initialization procedure.

   IMPORTANT! Do not store the mixed Wash 2 Bottle.

Prepare the Wash 1 and Wash 3 Bottles

Note: For the following steps, label the Wash 1 and Wash 3 Bottles to avoid confusion.

1. Rinse the Wash 1 and Wash 3 Bottles 3 times with 50 mL of 18 MΩ water.

2. **Wash 1 Bottle:** Add 350 µL of freshly prepared 100 mM NaOH to the Wash 1 Bottle, then cap the bottle.

3. **Wash 3 Bottle:** Add Ion PGM™ Hi-Q™ View Sequencing W3 Solution to the 50-mL line marked on the Wash 3 Bottle, then cap the bottle.

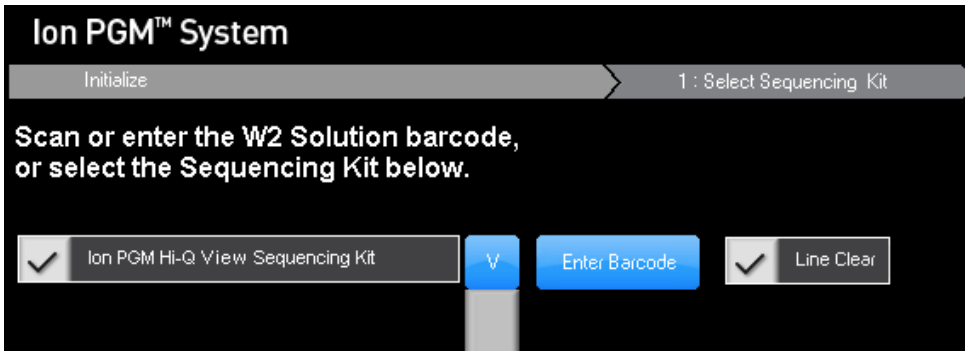
Begin the initialization

Note:

- Do not remove the old sipper tubes from the dNTP ports until instructed to do so.
- Load the bottles as quickly as possible to prevent atmospheric CO_2 from reducing the pH of the Wash 2 Bottle solution.

IMPORTANT! Do not let the new sipper tubes touch any surfaces.

1. On the main menu, press **Initialize**.
2. Make the following selections in the next screen, then press **Next**:
   - Click **Enter barcode** to scan or enter the barcode on the Ion PGM™ Hi-Q™ View Sequencing W2 Solution bottle, or the 2D barcode on the Ion PGM™ Hi-Q™ View Sequencing Solutions box.
   - Alternatively, select the checkbox for the **Ion PGM™ Hi-Q™ View Sequencing Kit** from the dropdown list.
   - In the same screen, if you routinely experience clogging during initialization, select the **Line Clear** checkbox to clear any blockage in the fluid lines before initialization. This is optional.



IMPORTANT! Be careful to select the correct kit, to ensure proper pH adjustment.

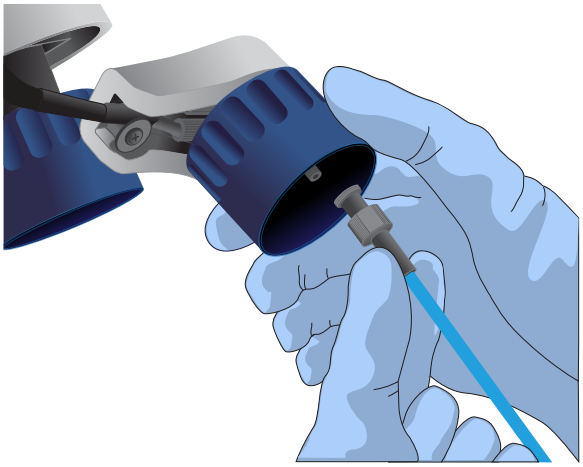
After you press **Next**, the system will check the gas pressure.

3. Following the gas pressure check:

Result	Action
If the pressure is sufficient	Ensure that the cleaning chip, reagent sipper tubes, and collection trays are in place, and press **Next** to start the initialization.
If the pressure is low	Press **Yes** to re-check the pressure. If the pressure remains low, contact Technical Support.

4. Wearing clean gloves, firmly attach a new, long gray sipper to the cap in the W2 position.

   IMPORTANT! Do not let the sipper touch any surfaces, and firmly attach the sipper to the port. Loosely attached sippers can adversely affect results.



5. Immediately attach the prepared Wash 2 Bottle in the W2 position, then tighten the cap. Press **Next**.

6. Change gloves and firmly install new sipper tubes (short gray) in the caps in the W1 and W3 positions.

7. Immediately attach the prepared Wash 1 and 3 Bottles, then tighten the caps. Press **Next**.

8. If you selected the **Line Clear** checkbox in the earlier screen, press **Next**, then follow the touchscreen prompts to perform the line clear procedure. At the beginning and end of the procedure, you are prompted to select one of the following:

Option	Description
Press **Line Clear**	To start a new line clear procedure
Press **Re-flow**	To retest the lines after you have performed a line clear
Press **Auto pH**	If the lines are clear and you are ready to continue with initialization

9. Following line clear, or if you did not select that option, the sequencer begins adjusting the pH of the W2 Solution, which takes ~30 minutes. After 15 minutes, check the instrument touchscreen to confirm that initialization is proceeding normally.

   Note:
   - If an error occurs during the automatic pH process, note the error message and proceed to "Initialization—Auto pH errors" on page 110.
   - During the process, you can start preparing the Reagent Bottles with dNTPs as described in the next section.

Prepare the 50-mL Reagent Bottles with dNTPs

1. Use the labels provided with the kit to label four new Reagent Bottles as dGTP, dCTP, dATP, and dTTP.
2. Confirm that no ice crystals are visible in each thawed dNTP stock solution. Vortex each tube to mix, and centrifuge to collect the contents. Keep the dNTP stock solutions on ice throughout this procedure.

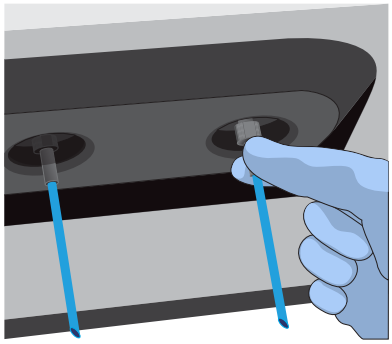
   IMPORTANT! To avoid cross-contamination in the next step, open only one dNTP stock tube at a time and use a fresh pipette tip for each aliquot.

3. Using separate filtered pipette tips and clean gloves, carefully transfer 20 µL of each dNTP stock solution into its respective Reagent Bottle.
4. Cap each Reagent Bottle and store on ice until you are ready to attach it to the instrument. Place the remaining dNTP stocks back into –20°C for storage.

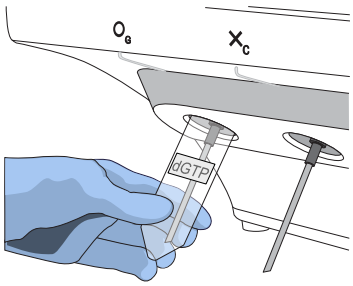
Attach the sipper tubes and Reagent Bottles

1. After the wash solutions have initialized, follow the touchscreen prompts to remove the used sipper tubes and collection trays from the dNTP ports.
2. Change gloves, then firmly insert a new sipper tube (blue) into each dNTP port. Do not let the sipper touch any surfaces.

   IMPORTANT! Be careful to firmly push each sipper onto the port. Loosely attached sippers may adversely affect results.



3. Attach each prepared Reagent Bottle to the correct dNTP port (e.g., the dGTP tube on the port marked "G") and tighten firmly by hand until snug. Press **Next**.



Note: The instrument checks the pressure of the Reagent Bottles and Wash Bottles. If a bottle leaks, check that it is tightly attached to the instrument. If it continues to leak, replace it. If the instrument still does not pass the leak check, contact Technical Support.

4. Follow the touchscreen prompts to complete initialization. The instrument will fill each Reagent Bottle with 40 mL of W2 Solution.

5. At the end of initialization, Ion PGM™ System will measure the pH of the reagents:
   - If every reagent is in the target pH range, a green **Passed** screen will be displayed.
   - If a red failure screen appears, see Appendix A, "Troubleshooting".

6. Press **Next** to finish the initialization process and return to the main menu.

7. Proceed to the appropriate sequencing protocol for your chip type.

8 Load the chip and start the sequencing run

Use the following chip loading and sequencing protocol for all Ion PGM™ chip types.

Materials required

Materials provided in the kit

- Annealing Buffer
- Ion PGM™ Hi-Q™ View Sequencing Polymerase

Other materials and equipment

- Ion Chip kit: Ion 318™ Chip Kit v2 BC, Ion 316™ Chip Kit v2 BC, or Ion 314™ Chip Kit v2 BC
- Enriched template-positive ISPs
- 0.2-mL PCR tube (non-polystyrene)
- Rainin™ SR-L200F pipette and tips
- Vortex mixer
- Ion Chip™ Minifuge
- Thermal cycler with heated lid (programmed at 95°C for 2 minutes and 37°C for 2 minutes)
- Barcode scanner (included with the Ion PGM™ System)

Guidelines for sequencing

- Ion PGM™ Sequencer: When performing multiple sequencing runs in the same instrument initialization, the first run should be started within 1 hour after initialization, and the last run must be started within 24 hours after initialization.
- Ion PGM™ Sequencer: If you press the **Abort** button on the touchscreen during any procedure, the touchscreen can freeze, requiring that you restart the sequencer.
- The ISPs are difficult to see. To avoid aspirating the particles:
  - When centrifuging the ISPs, orient the tab of the tube lid so that it is pointing away from the center of the centrifuge, to indicate where the pellet will be formed.
  - Always remove supernatant from the tube from the top down.

Before you begin

1. Thaw the Sequencing Primer on ice.
2. Update the Torrent Suite™ Software and Ion PGM™ System software to the latest version available, if needed.

Add controls to the enriched, template-positive ISPs

1. Vortex the Control Ion Sphere™ Particles, then pulse-centrifuge in a picofuge for 2 seconds before taking aliquots.
2. Add 5 µL of Control ISPs directly to the entire volume of enriched, template-positive ISPs (prepared using your template preparation method) in a 0.2-mL non-polystyrene PCR tube.

Proceed to "Anneal the Sequencing Primer".

Anneal the Sequencing Primer

1. Mix the tube containing the ISPs by thoroughly pipetting up and down.
2. Place the tube in a microcentrifuge with an appropriate tube adapter. Orient the tab of the tube lid so that it is pointing away from the center of the centrifuge, to indicate where the pellet will be formed.
3. Centrifuge for 2 minutes at 15,500 × *g*.

4. Keeping the pipette plunger depressed, insert a pipette tip into the tube containing the pelleted ISPs and carefully remove the supernatant from the top down, avoiding the side of the tube with the pink ISP pellet (that is, the side with the tab on the tube lid). Discard the supernatant. Leave ~15 µL in the tube (visually compare to 15 µL of liquid in a separate tube).

   Note: ISPs are visible up until chip loading, although the intensity of the pink color decreases as ISPs are processed during the workflow.

5. Ensure that the Sequencing Primer is completely thawed before use (no ice crystals should be visible).

6. Vortex the primer for 5 seconds, then pulse-centrifuge in a picofuge for 3–5 seconds to collect the contents. Leave on ice until ready to use.

7. Add 12 µL of Sequencing Primer to the ISPs, then confirm that the total volume is 27 µL (add Annealing Buffer if needed).

8. Pipet the mixture up and down thoroughly to disrupt the pellet.

   IMPORTANT! Ensure that the pipette tip is at the bottom of the tube during mixing to avoid introducing air bubbles into the sample.

9. Program a thermal cycler for 95°C for 2 minutes and then 37°C for 2 minutes, using the heated lid option.

10. Place the tube in the thermal cycler, then run the program. After cycling, the reaction can remain in the cycler at room temperature (20–30°C) while you set up the sequencing run.

Perform Chip Check

Chip Check tests the chip and ensures that it is functioning properly before loading the sample.

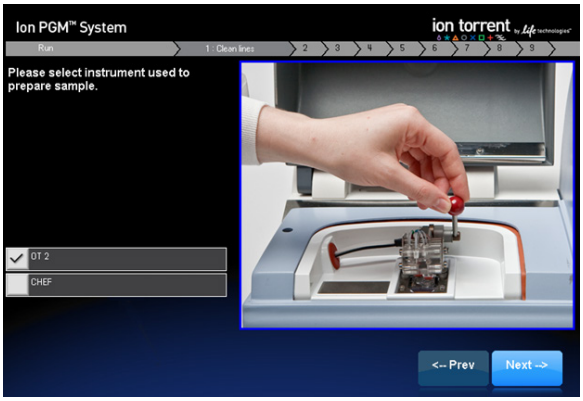
IMPORTANT!

- To avoid damage due to electrostatic discharge (ESD), **do not place the chip directly on the bench or any other surface.** Always place the chip either on the grounding plate on the Ion PGM™ Sequencer or in the Ion Chip™ Minifuge adapter bucket.
- To avoid ESD damage, **do not wear gloves** when transferring chips on and off the instrument.

1. On the main menu of the Ion PGM™ Sequencer touchscreen, press **Run**. Remove the waste bottle and completely empty it. Press **Next**.

2. When prompted to insert a cleaning chip, use the same used chip that was used for initialization. Press **Next** to clean the fluid lines.

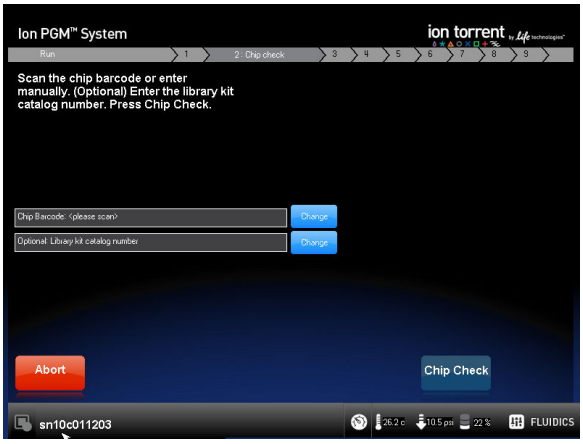
3. When prompted, select the instrument that you used to prepare the template-positive ISPs. Then press **Next**.



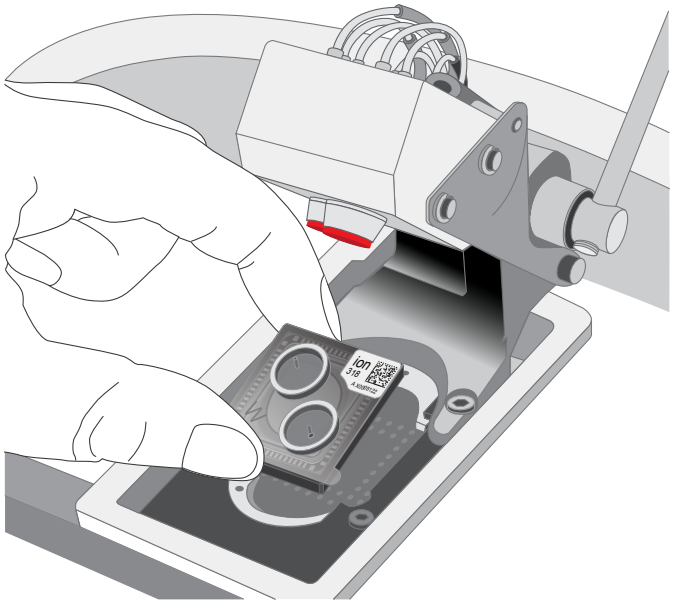
4. Remove gloves, then ground yourself by touching the grounding pad on the sequencer. Remove a new chip from its packaging, then label it to identify the experiment (save the chip package). Press **Next**.

5. When prompted, use the scanner to scan the barcode located on the new chip, or press **Change** to enter the barcode manually. Optionally, you can also enter the library kit catalog number.

   Note: A chip cannot be run without scanning or entering the barcode.



6. Replace the old chip in the chip socket with the new one. Close the chip clamp, then press **Next**.



7. Press **Chip Check**. During the initial part of Chip Check, visually inspect the chip in the clamp for leaks.

   Note:
   - If there is a leak, press the **Abort** button immediately to stop the flow to the chip. Proceed to Appendix A, "Troubleshooting".
   - **The chip socket can be damaged by rubbing or wiping its surface.** Never rub or wipe the socket to clean up leaks. See Appendix A, "Troubleshooting" for more information.

8. When Chip Check is complete:
   - If the chip passes, press **Next**.
   - If the chip fails, open the chip clamp, reseat the chip in the socket, close the clamp, and press **Calibrate** to repeat the procedure. If the chip passes, press **Next**. If the chip still fails, press **Main Menu** and restart the experiment with a new chip. See Appendix A, "Troubleshooting" for more information.

     Note: To return *damaged* chips, contact Technical Support.

9. Following a successful Chip Check, empty the waste bottle, then select the **Waste bottle is empty** checkbox on the touchscreen. Press **Next**.

Bind the Sequencing Polymerase to the ISPs

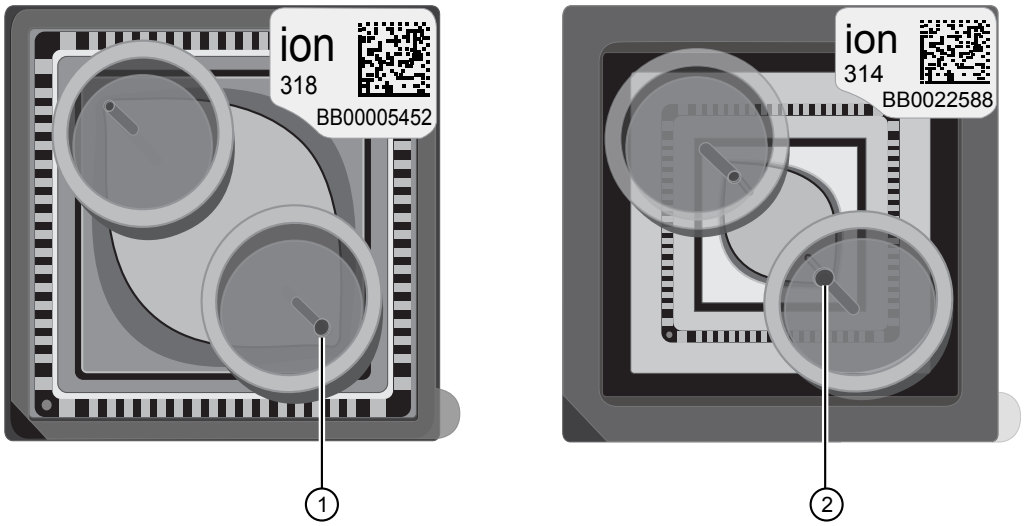
1. Remove the Ion PGM™ Hi-Q™ View Sequencing Polymerase from storage and flick mix with your finger tip 4 times. Pulse-centrifuge for 3–5 seconds. Place on ice.

2. After annealing the Sequencing Primer, remove the ISPs from the thermal cycler, then add 3 µL of Ion PGM™ Hi-Q™ View Sequencing Polymerase to the ISPs, for a total final volume of 30 µL.

3. Pipet the sample up and down to mix, then incubate at room temperature for 5 minutes.

Prepare and load the chip

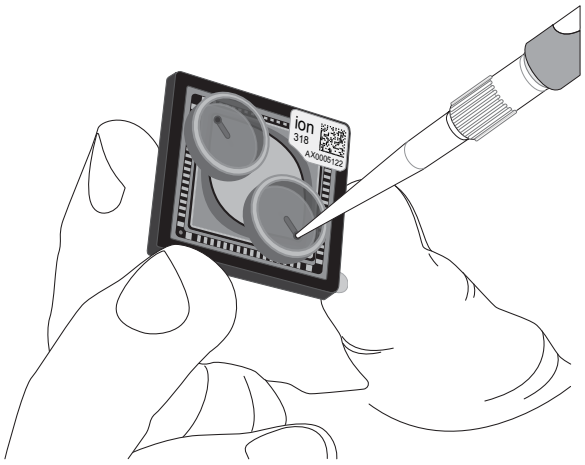
Remove liquid from the chip



① Ion 318™ Chip Kit v2 BC/Ion 316™ Chip Kit v2 BC loading port
② Ion 314™ Chip Kit v2 BC loading port

1. Following chip calibration, remove the new chip from the Ion PGM™ Sequencer. Insert a used chip in the chip clamp while loading the new chip.

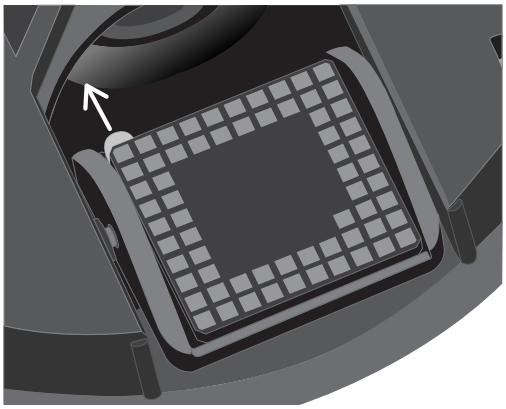
2. Tilt the new chip at a 45° angle so that the loading port is the lower port.



3. Insert the pipette tip firmly into the loading port, then remove as much liquid as possible from the loading port. Discard the liquid.

IMPORTANT! For the next steps, if you are preparing one chip at a time, balance the chip in the Ion Chip™ Minifuge rotor with a used chip of the same chip type and orientation. Be careful to balance an upside-down chip with another upside-down chip. Mark the used chip with a laboratory marker to differentiate it from the new chip.

4. Place the chip **upside-down** in the minifuge bucket, then transfer the bucket **with the chip tab pointing in** (toward the center of the minifuge). Balance the bucket with another chip.



5. Centrifuge for 5 seconds to empty the chip completely.

CAUTION! Allow the minifuge to come to a complete stop before opening the lid.

6. Remove the chip from the bucket, then wipe the bucket with a disposable wipe to remove any liquid. Place the chip right-side up in the bucket.

Load the chip

1. Place the chip in the bucket on a firm, flat surface. Following polymerase incubation, load the chip with following volume of prepared ISPs using the listed pipettes, or equivalent, depending on your chip type. We recommend using a P20 pipette for Ion 314™ Chips for optimal loading.

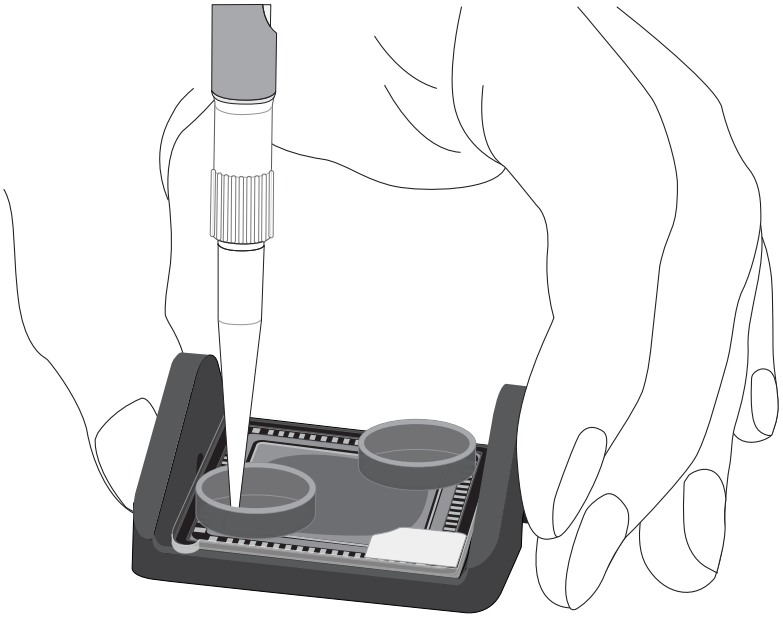
Chip	Volume to load	Recommended pipette[1]
Ion 316™ or Ion 318™ Chip	Entire volume (~30 µL)	Rainin™ Pipet-Lite™ LTS L-100XLS, 10–100 µL
Ion 314™ Chip	10 µL	Rainin™ Pipet-Lite™ LTS L-20XLS, 2–20 µL

[1] Alternatives from Gilson and Eppendorf can be used.

Note: For Ion 314™ Chips, the remaining volume of ISPs can be used to prepare another chip, provided the chip can be loaded and sequenced in parallel with the first chip. After polymerase incubation, ISPs should be loaded and sequencing initiated within ~15 minutes.

2. Insert the tip firmly into the loading port of the chip.

   Note: When loading the ISPs into the chip, keep the pipette tip at a 90° angle to the chip, press the tip firmly into the circular loading port, and apply gentle pressure between the pipette tip and chip.

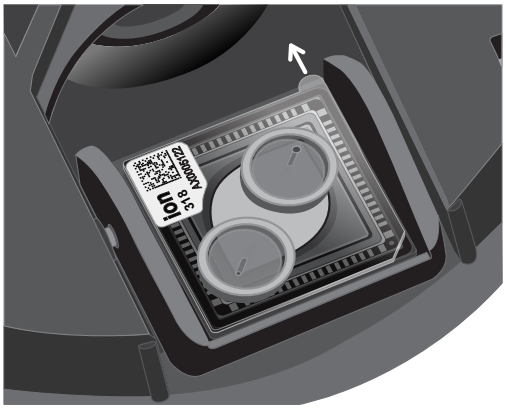


3. With the pipette unlocked, apply gentle pressure between the tip and chip and slowly dial down the pipette (~1 µL per second) to deposit the ISPs. To avoid introducing bubbles into the chip, leave a small amount in the pipette tip (~0.5 µL).

   Note: Do not remove the pipette tip from the port during the dial-down process, because removal can introduce air bubbles and inhibit loading.

4. Remove, then discard any displaced liquid from the other port of the chip.

5. Transfer the chip in the bucket to the minifuge with the chip tab **pointing in** (toward the center of the minifuge), then centrifuge for 30 seconds.



6. Turn the chip so that the chip tab is **pointing out** (away from the center of the minifuge), then centrifuge for 30 seconds.

7. Remove the bucket from the minifuge, then place it on a flat surface. Set the volume of the pipettor as follows, depending on your chip type:
   - **Ion 316™ or Ion 318™ Chip**: 25 µL
   - **Ion 314™ Chip**: 5 µL
8. Tilt the chip at a 45° angle so that the loading port is the lower port, then insert the pipette tip into the loading port.
9. Without removing the tip, slowly pipet the sample out and then back into the chip one time. **Pipet slowly to avoid creating bubbles.**
10. Slowly remove as much liquid as possible from the chip by dialing the pipette. Discard the liquid.
11. Turn the chip upside-down in the bucket, transfer it back to the minifuge, then centrifuge upside-down for 5 seconds. Remove and discard any liquid.

12. If some liquid remains in the chip, lightly and rapidly tap the point of the chip tab against the benchtop a few times, then remove and discard any collected liquid. Do not flush the chip.

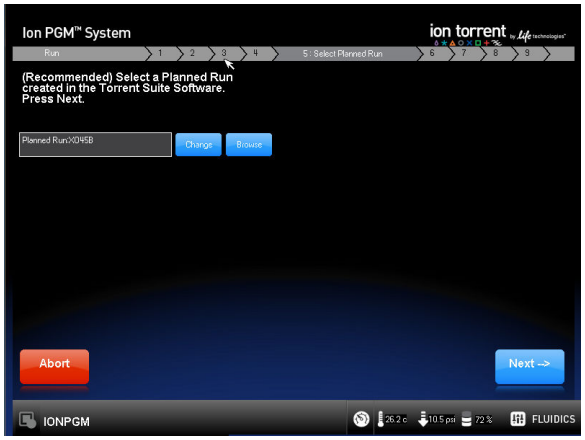
13. When chip loading is complete, press **Next** on the touchscreen, then proceed immediately to performing the run.

Select the Planned Run and perform the run

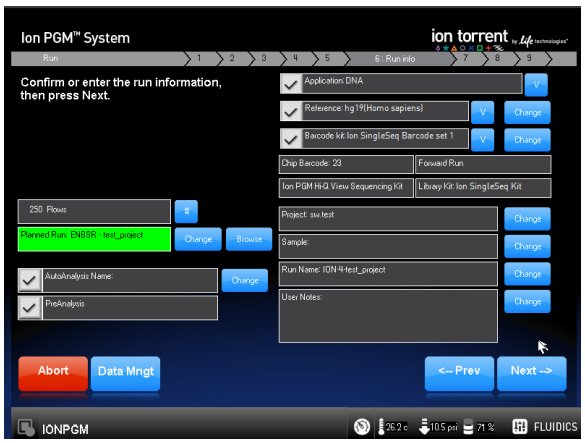
Select the Planned Run

1. Press **Browse** next to the **Planned Run** field and select the name of the plan you created, then touch **Next.**

   Note: The Ion PGM™ Sequencer automatically populates this field for barcoded Ion chips.



2. Confirm that the settings are correct. If necessary, make any changes using the touchscreen controls.



   Note: If the number of flows (cycles) to be run cannot be selected, there may not be enough disk space to store the experiment data. Touch **Data Mngt** to start the Data Management application (this can also be accessed from the Tools Menu) and delete old runs from the Ion PGM™ System.

Perform the run

1. After you enter the Planned Run, press **Next** to verify the experimental setup. Press **OK** to confirm the settings or press **Cancel** to return to the touchscreen to adjust the settings.
2. When prompted by the instrument, load and clamp the chip, then press **Next**.
3. At the beginning of the run, visually inspect the chip in the clamp for leaks before closing the cover. The instrument will flush any loose ISPs from the chip and begin calibrating the chip.
4. When the calibration is complete (~1 minute), the touchscreen will indicate whether calibration was successful.
   - If the chip passes calibration, press **Next** to proceed with the sequencing run.
   - If the chip fails calibration, see "Error message: Calibration FAILED" on page 109.
5. After 60 seconds, the run will automatically begin, or press **Next** to begin the run immediately.

   IMPORTANT! During a run, avoid touching the instrument and any of the attached bottles or tubes, as this may reduce the quality of the measurements.
6. When the run is complete, leave the chip in place, then touch **Next** to return to the Main Menu. You can then remove the chip and proceed with another run or perform a cleaning/initializing if required.

   Note: See "Cleaning schedule" on page 57 to determine whether cleaning is required after the run.

9 Analyze the run

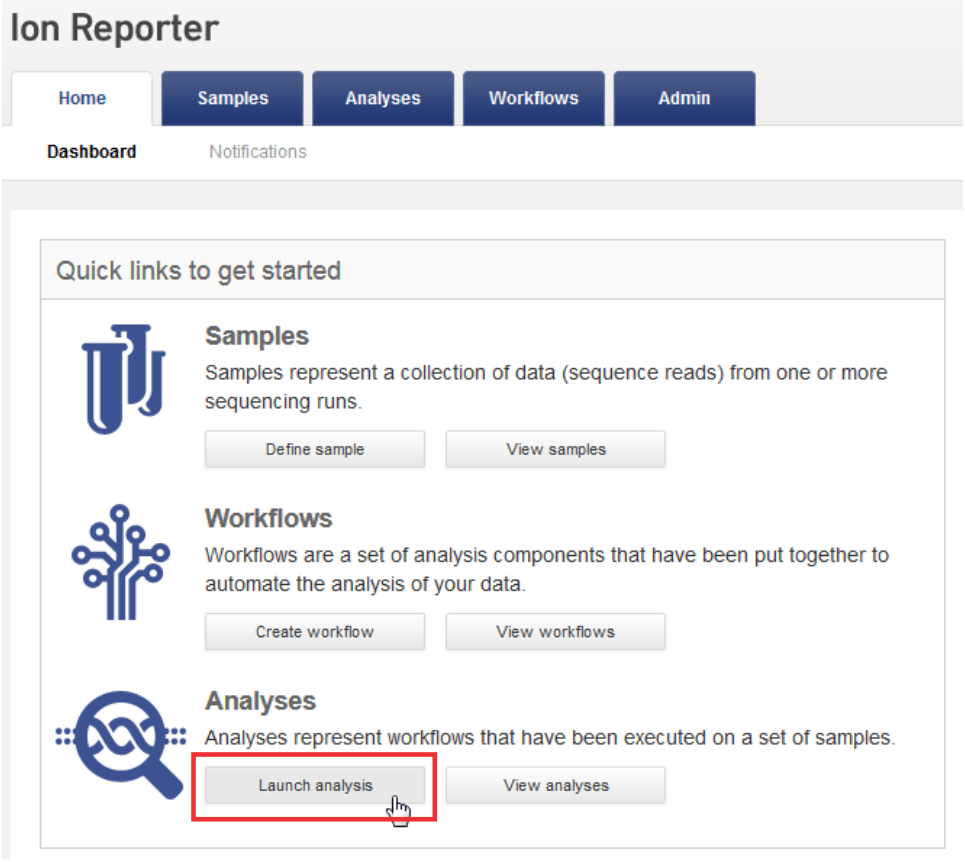
Launch an Ion Reporter™ analysis

If you are analyzing your samples manually, follow the instructions below. If you planned your run for automatic analysis with Ion Reporter™ Software, proceed to step 6.

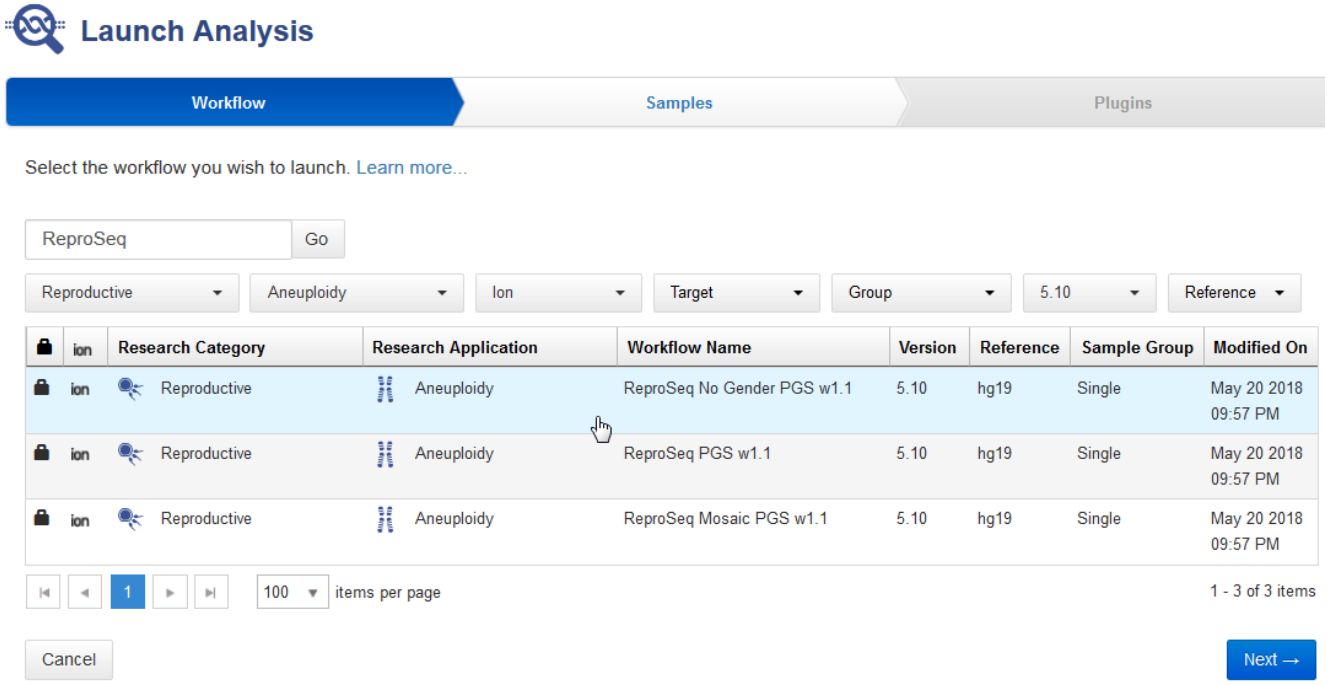
1. Import your samples into Ion Reporter™ Software using the Ion Reporter™ Uploader plugin.

   See the *Ion Reporter™ Software Help* for detailed instructions for importing your samples and defining them in Ion Reporter™ Software.

2. In the Ion Reporter™ **Home** tab, click **Launch analysis** after the Ion Reporter™ Uploader plugin has completed.



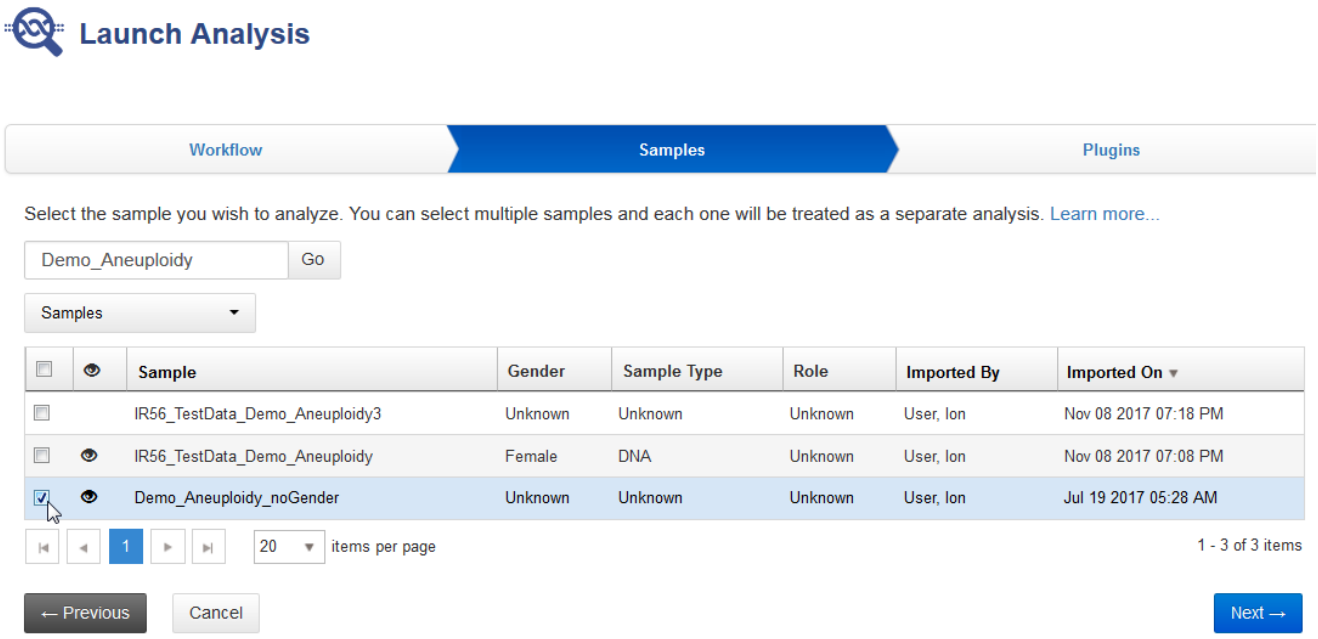
3. In the **Workflow** step, select **Reproductive** from the **Research Category** menu to view the Aneuploidy workflows. Select one of the three ReproSeq w1.1 workflows from the list, then click **Next**.
   - ReproSeq PGS w1.1
   - ReproSeq No Gender PGS w1.1
   - ReproSeq Mosaic PGS w1.1



Note:

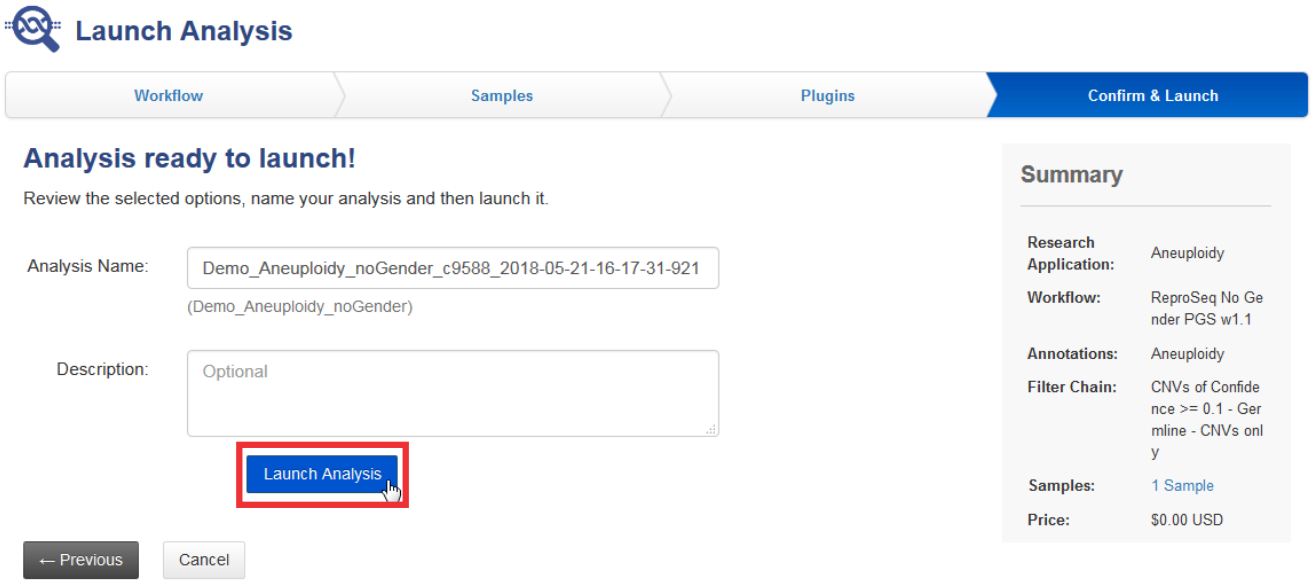
- The ReproSeq Low-Coverage Whole-Genome Baseline is auto-loaded in the aneuploidy workflows, making creation of a custom baseline for your Ion ReproSeq™ analyses unnecessary.
- To customize one of the preloaded aneuploidy workflows, see "Copy and edit a workflow" on page 88.
- The Low-pass whole-genome aneuploidy w1.0 workflow should be used for non-Ion SingleSeq™ libraries only.

4. In the Samples step, select the sample(s) you want to run in your analysis from the list.



5. Click **Next** twice to advance through the **Plugins** step to **Confirm & Launch**.

6. In the **Confirm & Launch** step, enter a name for your analysis if you want to change the default name, then click **Launch Analysis**.

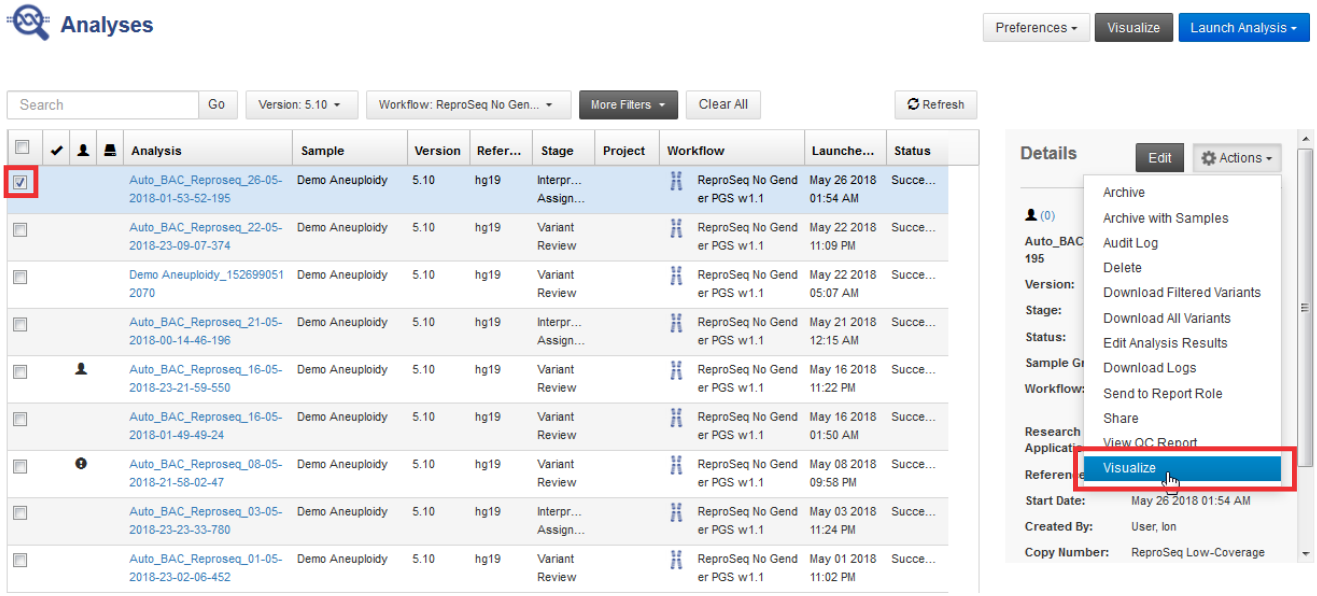


7. Review your results by selecting from the Analyses list after navigating to **Analysis ▸ Overview**. See *Ion Reporter™ Software Help* for further details.

Visualize results with IRGV

You can visualize aneuploidy results and generate reports with the IRGV viewer. See *Ion Reporter™ Software Help* for further details.

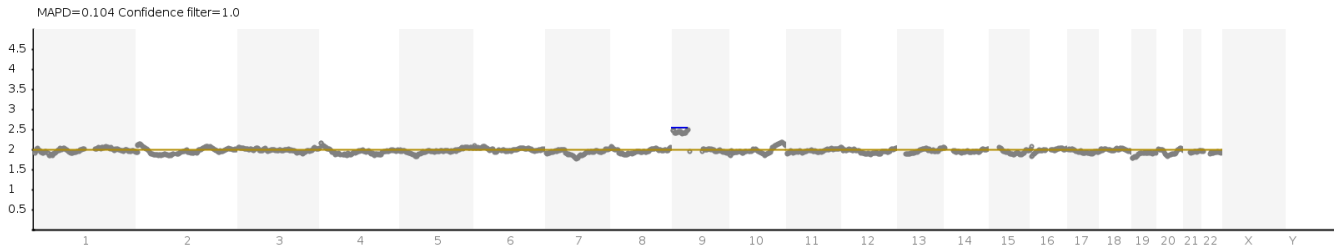
1. On the Ion Reporter™ home screen, navigate to **Analyses** ▸ **Overview**.
2. Select one or more aneuploidy analyses, then click **Visualize** from the **Actions** dropdown list.



Note: You can also click **Visualize** (to the left of the **Launch Analysis** button) after selecting one or more analyses.

The **Analysis Visualization** screen opens to the **IRGV** tab. A copy number histogram for each selected analysis appears, along with ploidy maps for selected chromosomes or chromosome regions, and karyograms showing copy number gains and losses.

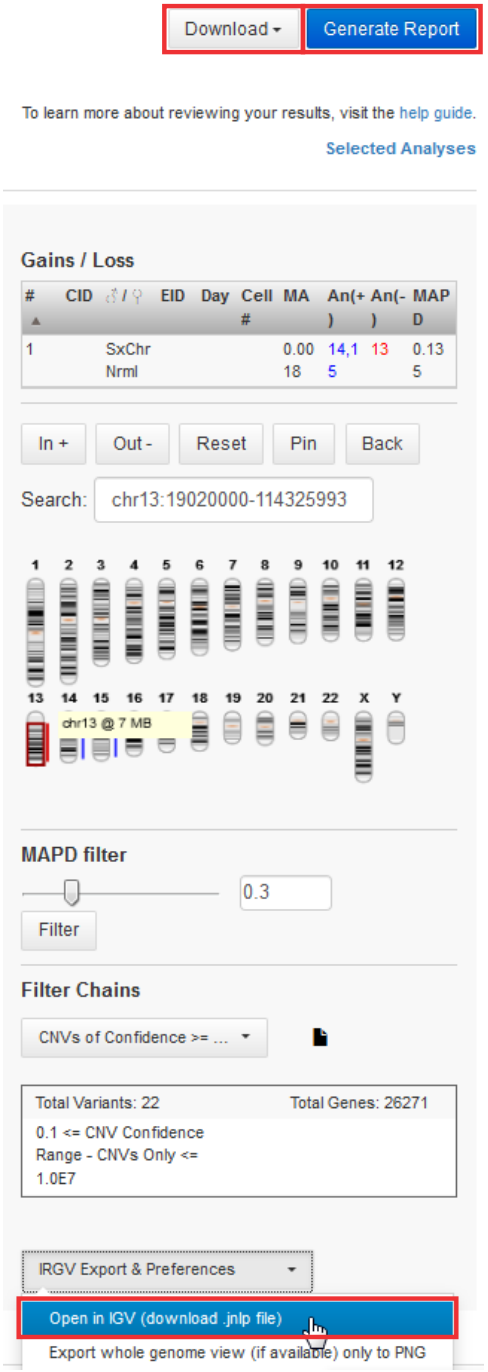
Example of an IRGV plot showing non-integer ploidy of the short arm of chromosome 9, indicating that the sample was mosaic.



Note:

- You can adjust the MAPD filter with the slider control in the lower right corner of the screen. The MAPD (Median of the Absolute values of all Pairwise Differences) metric is an estimate of coverage variability between adjacent genomic tiles. The default threshold is set to 0.3. CNV calls at a MAPD above this value can be inaccurate due to high noise in the sample.
- See “High MAPD value (>0.3) is observed“ on page 116 for troubleshooting high MAPD values.
- Confidence filtering can be applied or changed by selecting from the **Filter Chains** dropdown list.

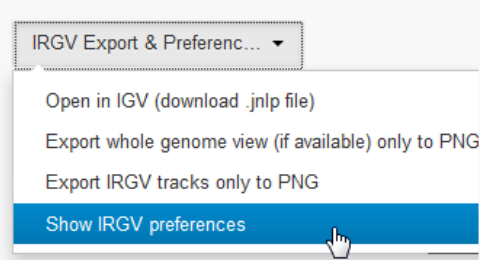
3. Select **Open in IGV (download .jnlp file)** from the **IRGV/Export & Preferences** dropdown list, or click **Download** at the upper right to download a file from which to launch the IGV application. You can also click **Generate Report** to produce a full report with graphics that can be downloaded as a PDF.



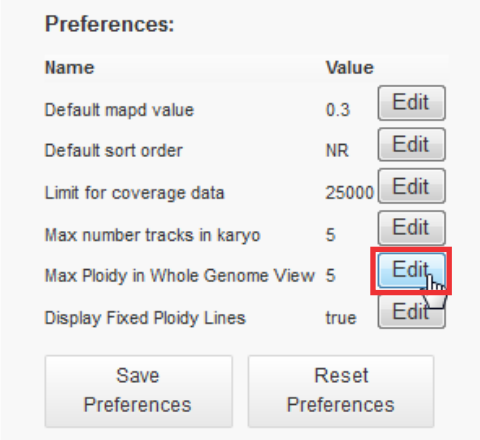
Edit the IRGV preferences

You can edit the IRGV preferences to customize your analysis and reporting. For example, the maximum ploidy in the whole-genome view (y-axis) is set to 5 by default. Setting this parameter to a lower value lowers the range of the y-axis and can enable easier detection and visualization of mosaicism events. The following steps show how to make this edit.

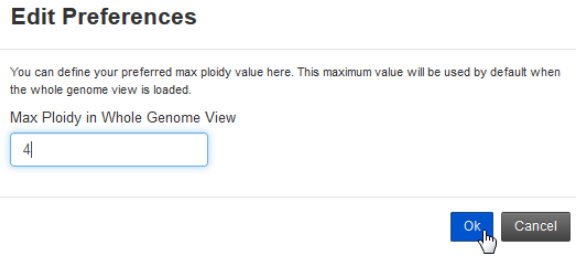
1. Click **IRGV Export & Preferences**, then select **Show IRGV preferences** from the dropdown list.



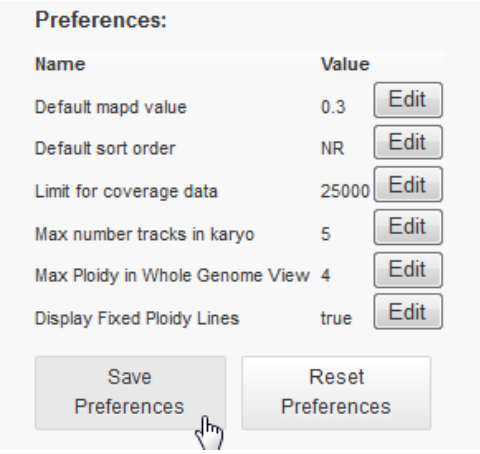
2. Click **Edit** for the **Max Ploidy in Whole Genome View** parameter.



3. In the **Edit Preferences** dialog box, enter 4, then click **Ok**.



4. Click **Save Preferences**.

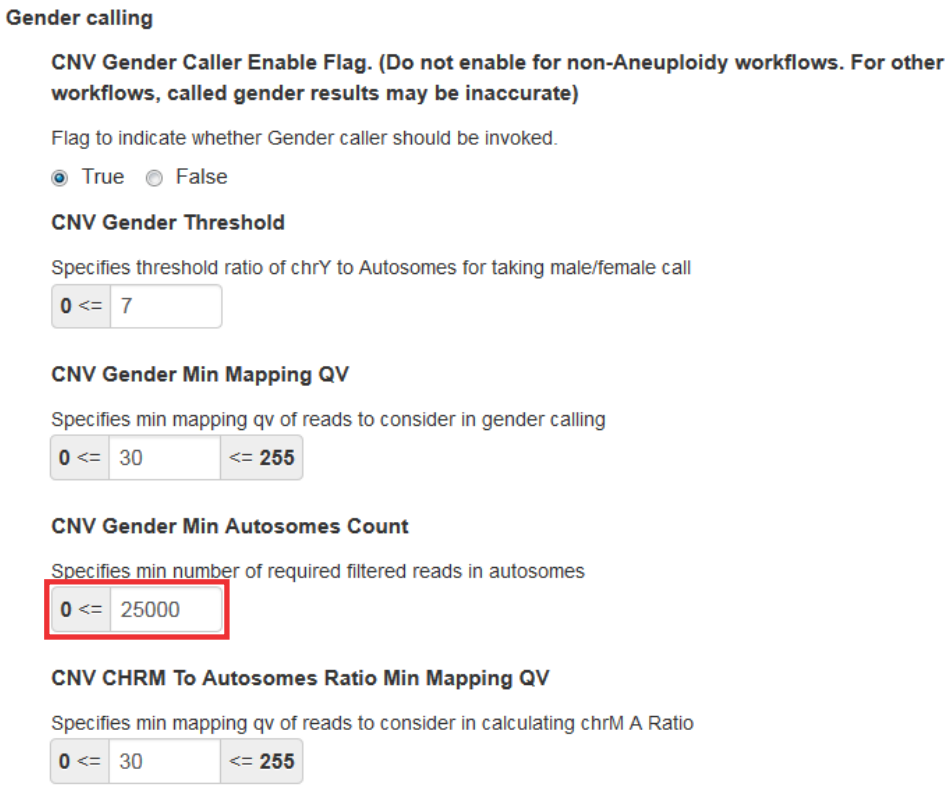


Make any other edits to the parameters in the Preferences dialog box similarly. Click **Reset Preferences** to return settings to the original default values.

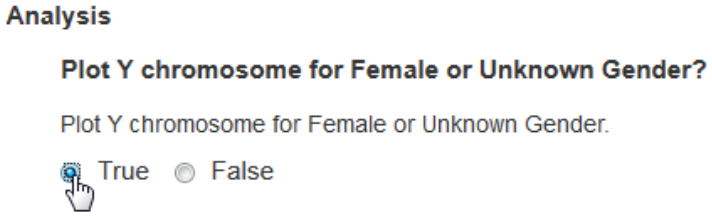
View X and Y chromosome data in low-read samples

Aneuploidy samples with low total read counts can yield analysis results where X and Y chromosome ploidies are not displayed and gender is not called. There are two options you can try to visualize X and Y chromosome data in this situation, although we recommend that you interpret with caution any analysis results showing high MAPD values.

- A low-read sample that results in no display of X and Y chromosome data can be analyzed in a custom aneuploidy workflow with a lower value entered for the parameter "CNV Gender Min Autosomes Count" (default = 25000). Copy your existing ReproSeq PGS workflow, then edit this parameter by navigating to **Parameters ▸ Cnv Finding ▸ Advanced**.



- Create a custom aneuploidy workflow with the CNV parameter "Plot Y chromosome for Female or Unknown Gender" enabled. Copy your existing ReproSeq PGS workflow, then enable this parameter by navigating to **Parameters ▸ Cnv Finding ▸ Advanced** and selecting **True** for this parameter.



Note: With this parameter enabled, the analysis displays X and Y chromosome data, but does not attempt to call the gender. Also, the software calls X and Y chromosome reads as CNV gains by default, regardless of read count.

See "Copy and edit a workflow" for further information on how to copy and edit an Ion Reporter™ aneuploidy workflow.

Download additional examples of Ion ReproSeq™ PGS data

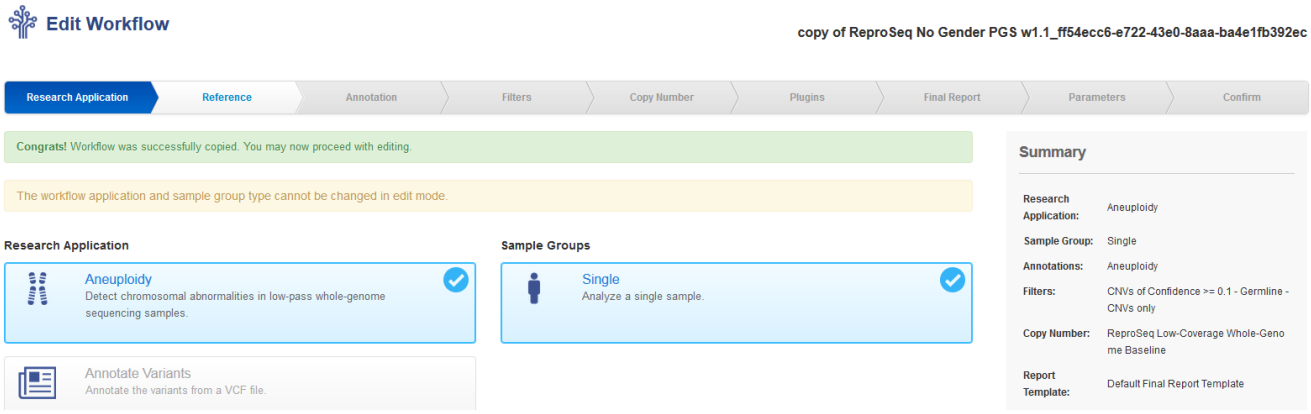
Additional examples of Ion ReproSeq™ PGS datasets and analyses can be downloaded at **https://apps.thermofisher.com/apps/publiclib/#/datasets**. Sign in is required.

Copy and edit a workflow

You can create a new workflow from an existing Ion Reporter™ aneuploidy workflow by copying and editing it. For example, after copying the ReproSeq No Gender PGS w1.1 workflow, you can enable mosaicism detection (which reports a CNV event as a decimal ploidy value instead of an integer value), change the CNV sensitivity, or disable smoothing, by modifying appropriate workflow settings in the **Parameters** tab, and saving the changes. The following steps describe how to copy and edit an existing Ion Reporter™ workflow to create a new workflow.

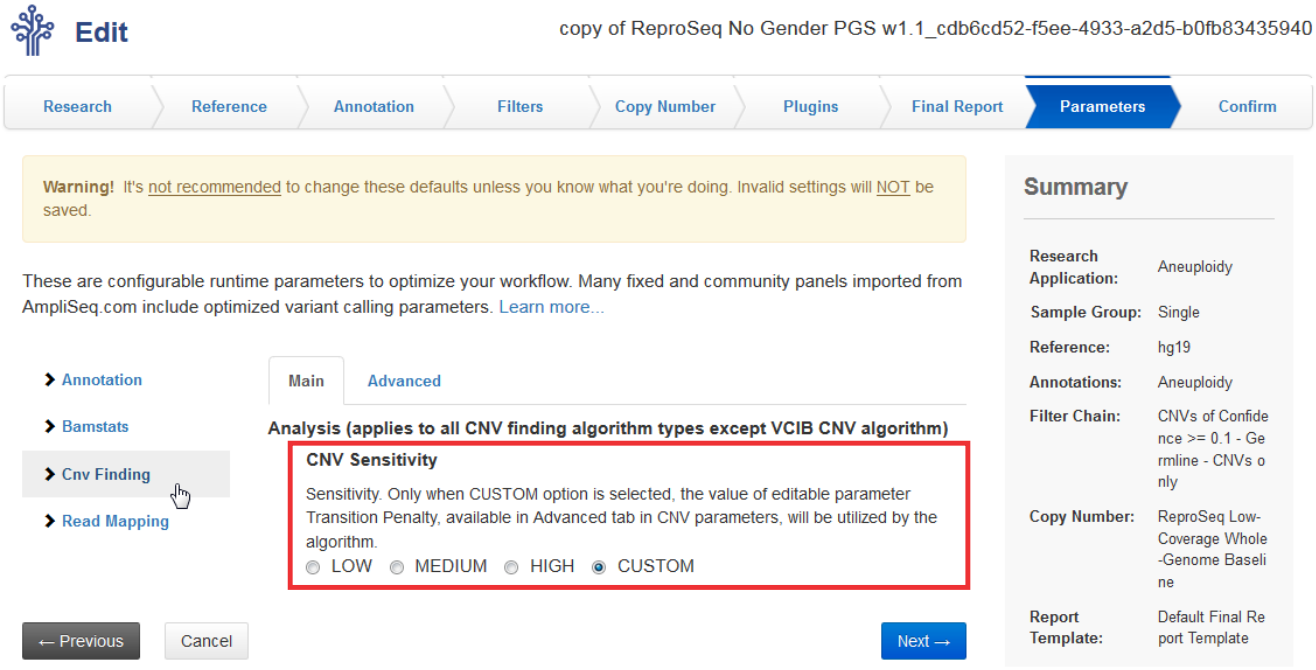
1. On the Ion Reporter™ home page, click **View workflows**, select **Reproductive** from the **Research Category** dropdown list, then select **Aneuploidy** from the **Research Application** list. Select the workflow of interest, then click **Copy** from the **Actions** dropdown list.

2. Click **Next ->** on each page, making any necessary changes, to proceed to **Parameters**.

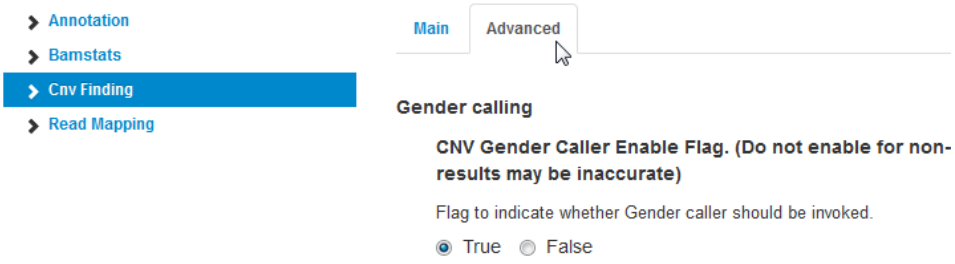


3. Click **Cnv Finding**, then change the **CNV Sensitivity** setting.

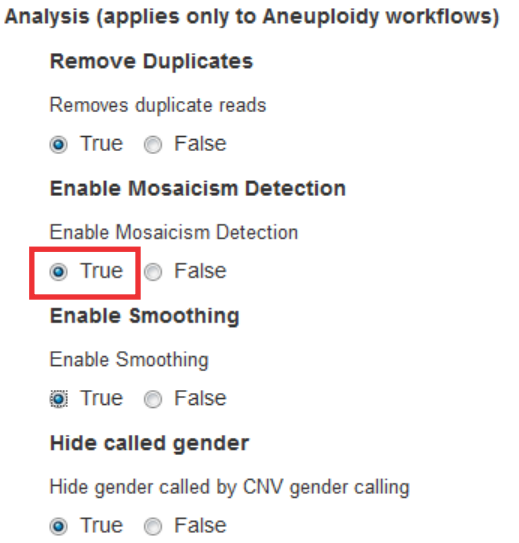
   Note: Low sensitivity results in fewer false positives, but more false negatives. High sensitivity results in fewer false negatives, but more false positives. High sensitivity is required to make segmental aneuploidy calls of ~20 Mbp. Custom sensitivity allows you to change the CNV Transition Penalty parameter to detect even smaller segmental CNV events more sensitively. See "Adjust the CNV Transition Penalty" on page 92 for further information.



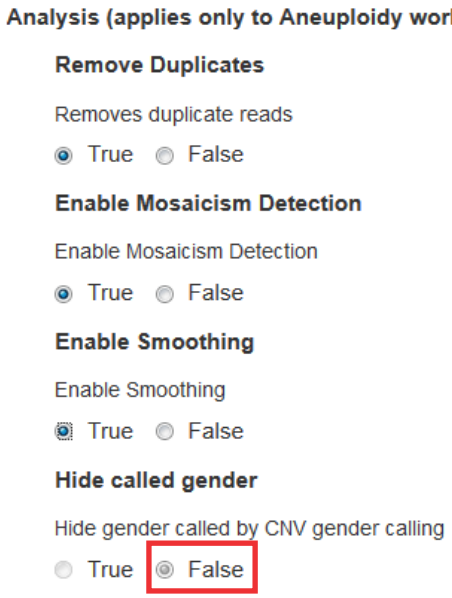
4. Click the **Advanced** tab to access more analysis parameter settings.



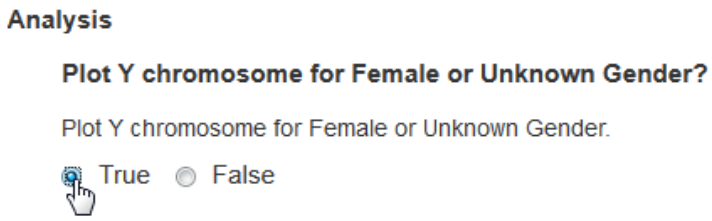
5. Scroll to the **Analysis (applies only to Aneuploidy workflows)** section, then make desired changes.
   In this example, select **True** under **Enable Mosacism Detection** to make this change.



To enable mosaicism detection and/or smoothing in a No Gender workflow, copy and edit the ReproSeq No Gender w1.1 workflow as described. You cannot enable gender hiding in the other ReproSeq workflows because the **Hide called gender** setting is locked as **False** (gender is called), and cannot be set to **True**.



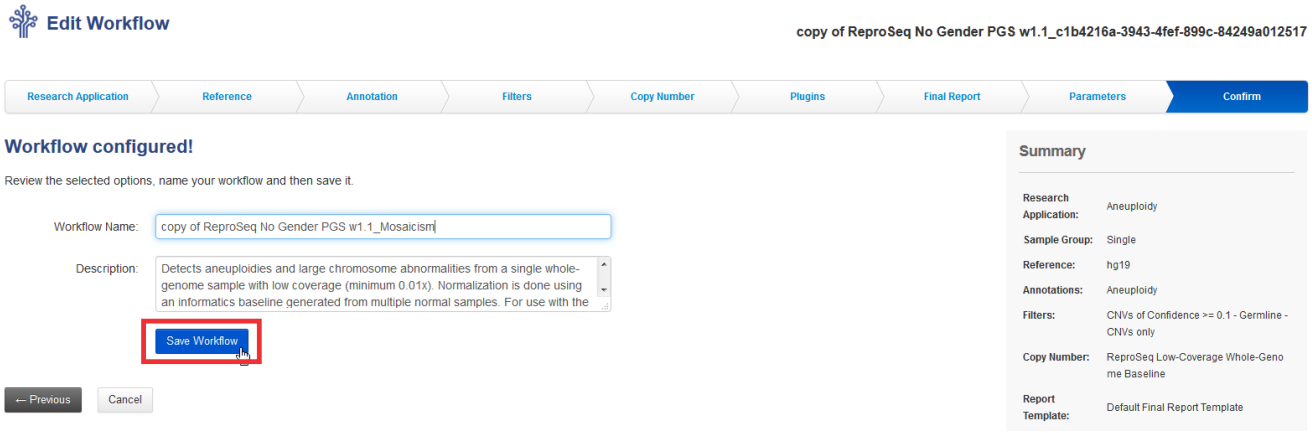
To show Y chromosome data for every sample, whether it is female, male, or unknown, set **Plot Y chromosome for Female or Unknown Gender** to **True**. The default value for this setting is **False**, which shows Y chromosome data only if the sample is male.



6. There are additional workflow parameters if you click the Annotation, Bamstats, and Read Mapping links at the left of the screen. When you have completed your edits, click **Next ->** at the bottom of the page to proceed to **Confirm**.

   Note: Do not change parameters from the default settings unless you understand how the change can affect your analysis.

7. Rename the workflow, then click **Save Workflow**.



The workflow appears in the list of available Ion Reporter™ workflows.

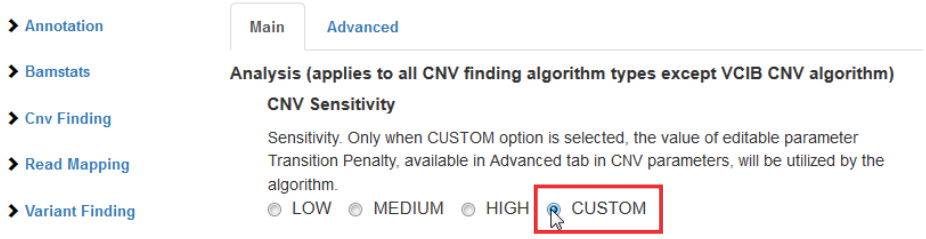
Adjust the CNV Transition Penalty

In the **Parameters** tab, the CNV Transition Penalty parameter accessed in **Advanced** settings can be adjusted to improve the detection of small segmental CNV events by your Ion Reporter™ aneuploidy workflow. The value of this parameter in mosaic and non-mosaic workflows is set at high sensitivity is –3. The highest allowable value for this parameter in the custom CNV sensitivity setting is:

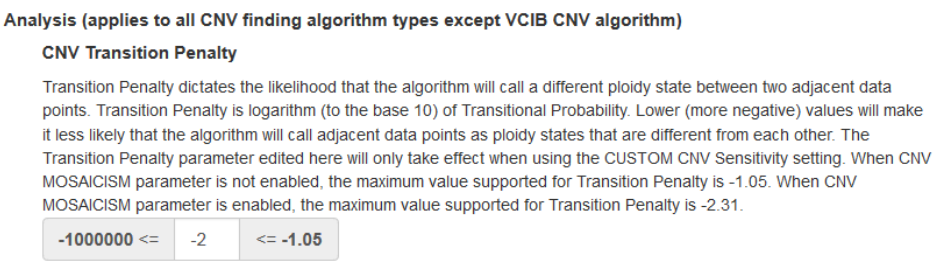
- –1.05 for non-mosaic workflows
- –2.31 for mosaic workflows

Follow these steps to adjust the CNV Transition Penalty parameter for an aneuploidy workflow.

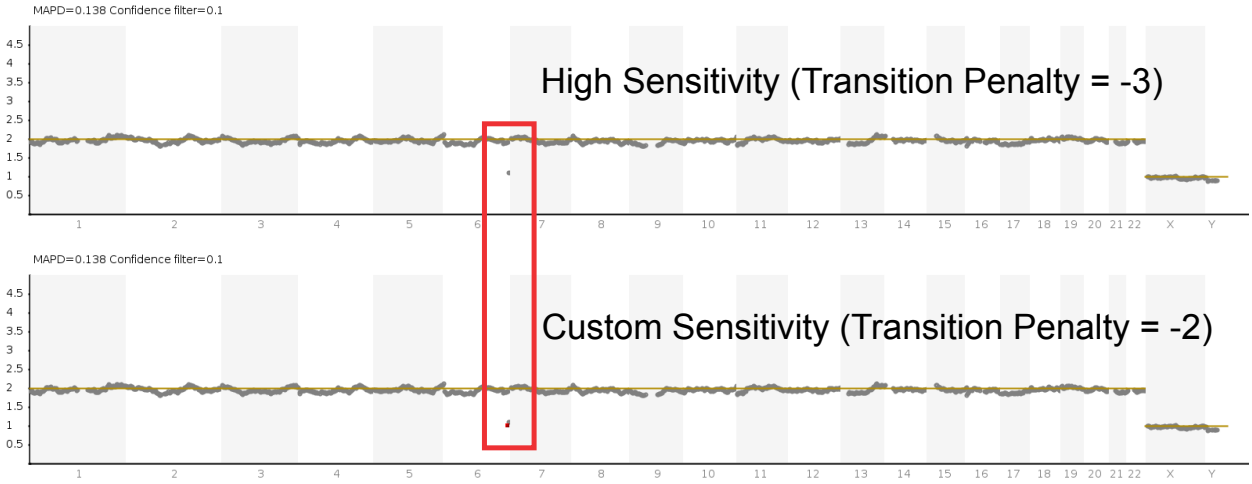
1. In the **Create Workflow** or **Edit** workflow screen, navigate to **Parameters** ▸ **CNV Finding**. Under the **Main** tab, select **Custom** for **CNV Sensitivity**.



2. Click **Advanced**, scroll to the CNV Transition Penalty parameter, then enter the desired value.



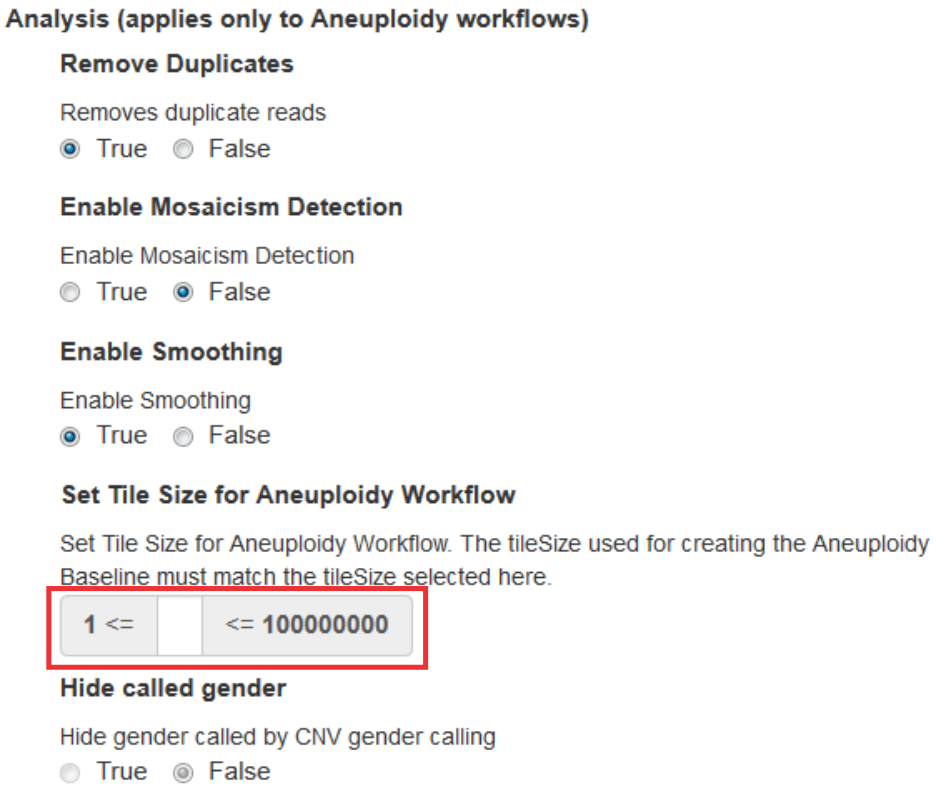
3. In the **Confirm** step, click **Save Workflow**.
   Example of increased sensitivity: a 7.9-Mb deletion is called on chromosome 6 with Transition Penalty set to −2.



Adjust the tile size

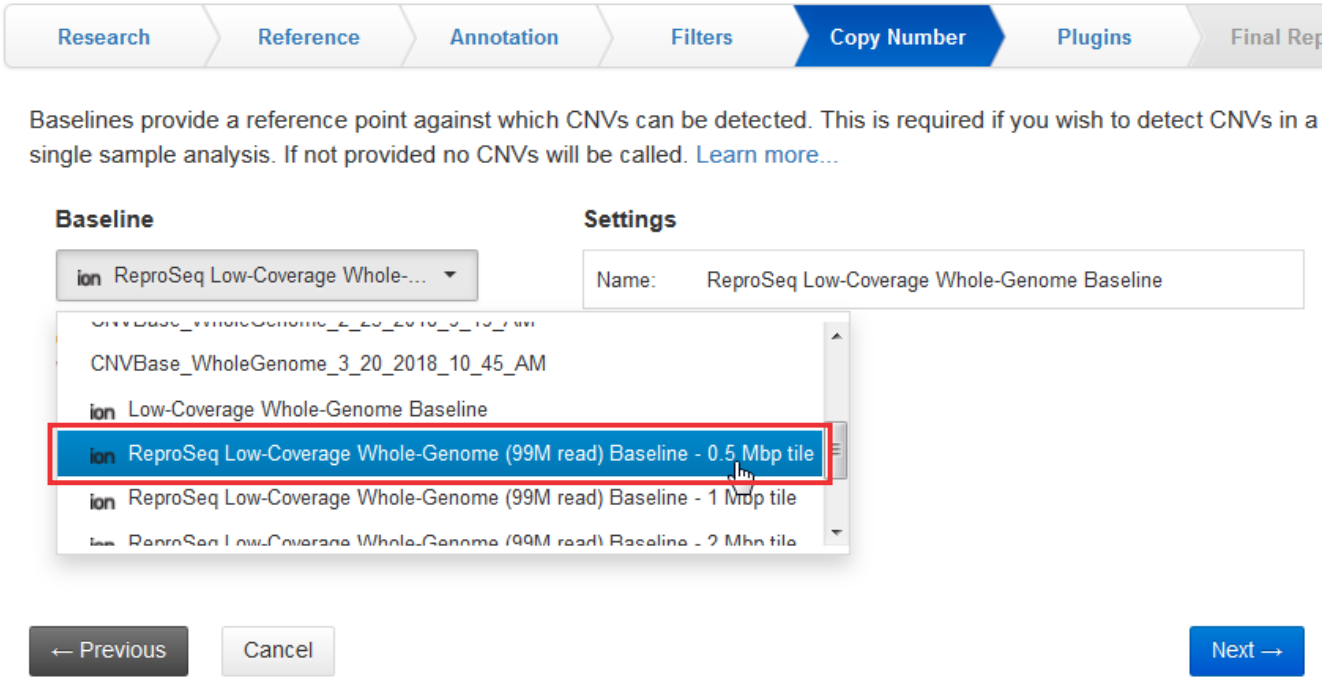
Further improvement in sensitivity to detect small segmental CNV events can be obtained by decreasing the tile size. This adjustment must be accompanied by selection or creation of a CNV Baseline with a corresponding smaller tile size. Pre-built CNV baselines corresponding to smaller tile sizes are included in Ion Reporter™ Software and are ready to use in customized aneuploidy analysis workflows. Follow these steps to adjust the tile size parameter in an aneuploidy workflow.

1. In the **Create Workflow** or **Edit** workflow screen, navigate to **Parameters** ▸ **CNV Finding**. Under the **Advanced** tab, scroll to the **Analysis (applies only to Aneuploidy workflows)** section.

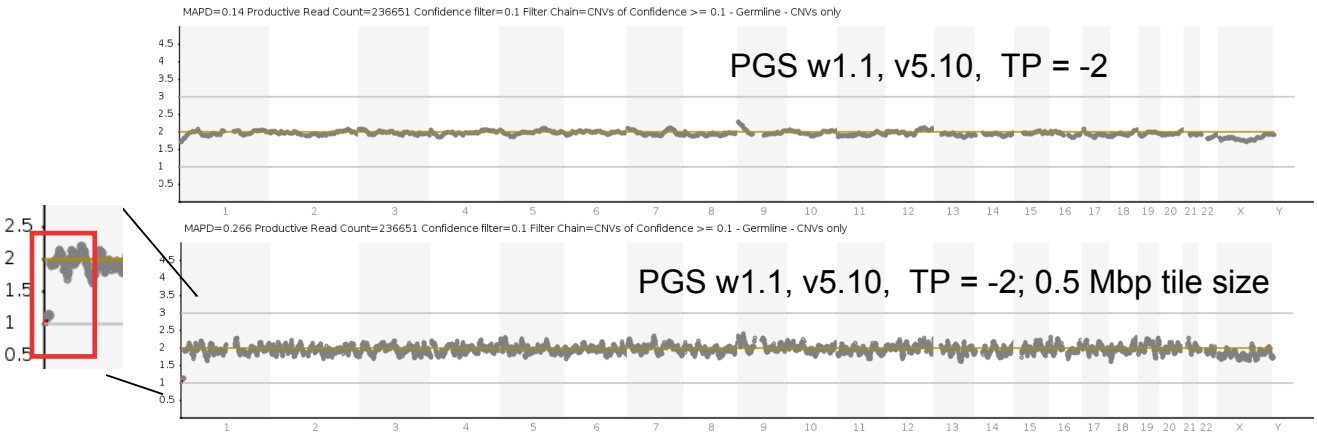


2. For a tile size of 0.5 Mbp, enter 500000 in the **Set Tile Size for Aneuploidy Workflow** field.

3. Navigate back to the **Copy Number** step of the workflow screen, then select **ReproSeq Low-Coverage Whole-Genome (99M read) Baseline - 0.5 Mbp tile** from the **Baseline** dropdown list.



4. In the **Confirm** step, click **Save Workflow**.
   Example of increased sensitivity: a 4.5-Mb deletion is called on chromosome 1 with tile size set to 0.5 Mbp.

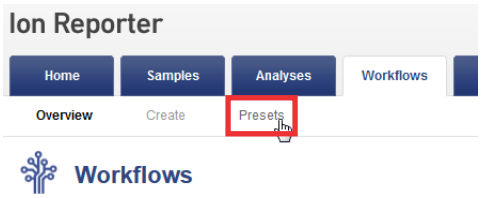


Create and add a new filter chain

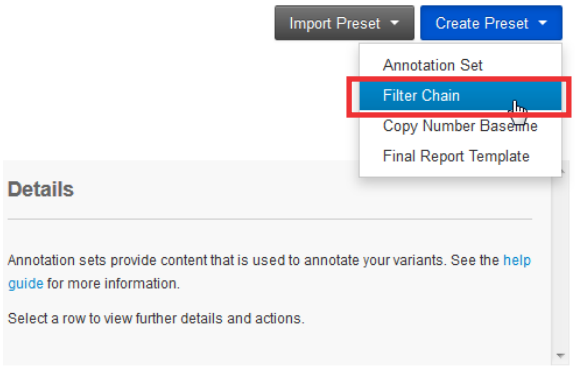
Modify the CNV Confidence Range

ReproSeq aneuploidy workflows have the CNV Confidence Range set at 0.1 to 1.0E7 as default. To specify another range for more stringent (1.0 to 1.0E7) or less stringent (0.01 to 1.0E7) CNV filtering, first create a new filter chain, then add it to your workflow.

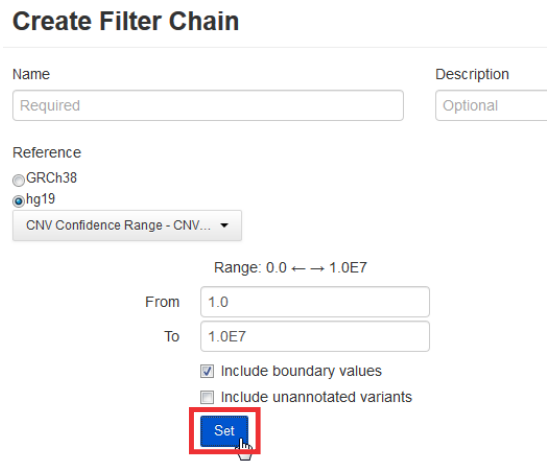
1. In the Ion Reporter™ **Workflows** tab, click **Presets**.



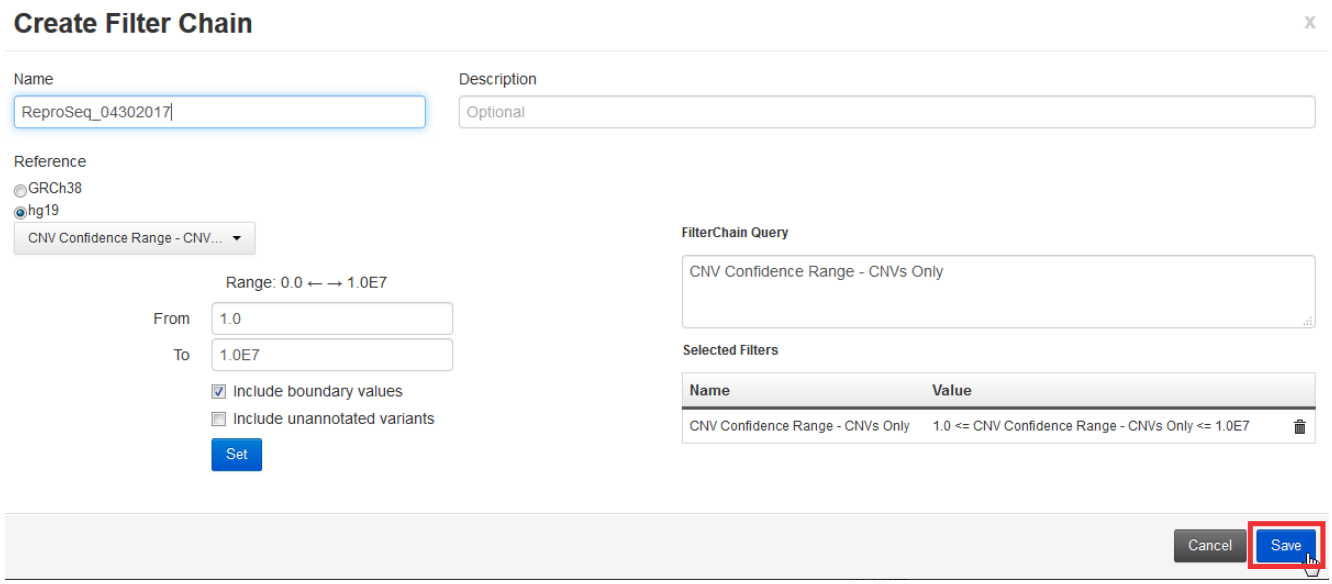
2. In the **Create Preset** dropdown list, select **Filter Chain**.



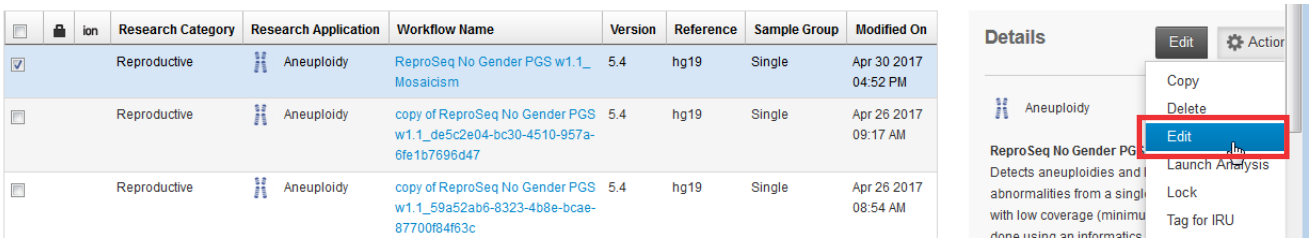
3. In the dialog, select **CNV Confidence Range - CNVs Only** from the dropdown list on the left, enter the confidence range, then click **Set**.



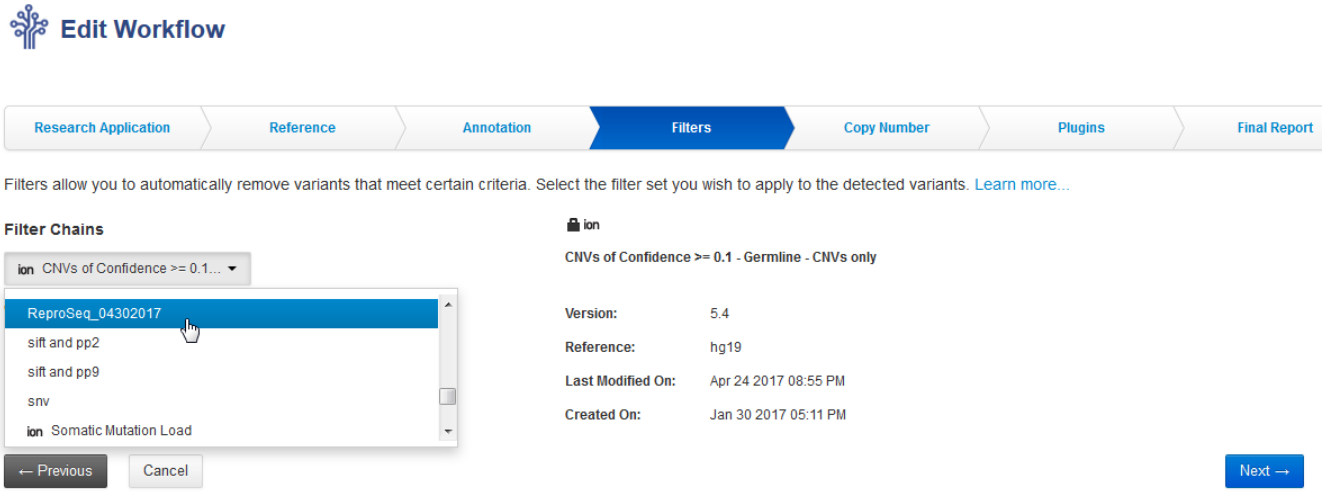
4. Enter a name for the filter chain, then click **Save**.



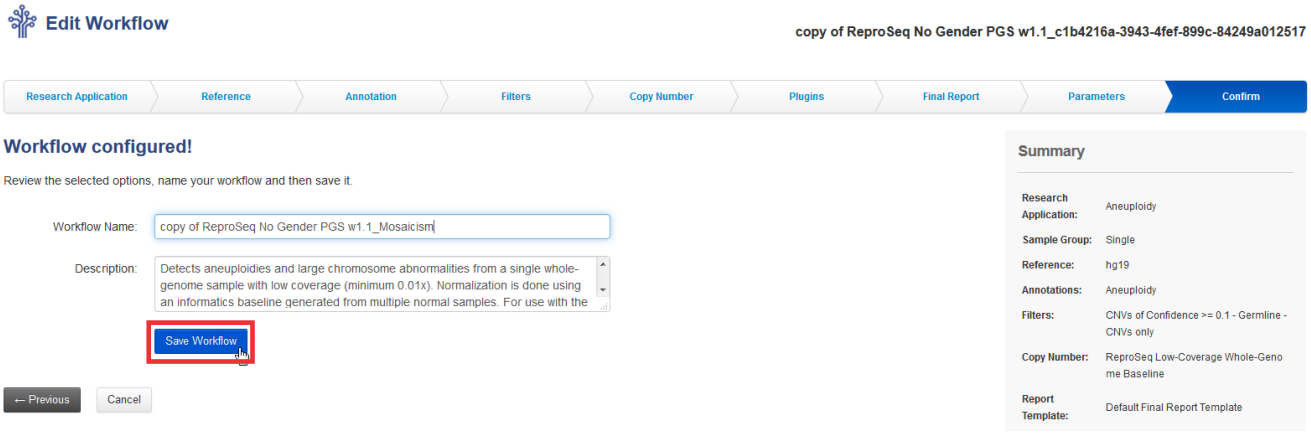
5. Select the workflow that you want to edit with a new filter chain from the list of workflows in the Ion Reporter™ **Workflows** tab, then click **Edit** in the **Actions▼** dropdown list.



6. In the Filters step of the **Edit Workflow** wizard, select your filter chain from the dropdown list, then click **Next**.



7. Advance through the remaining steps of the wizard to make any additional edits, name your edited workflow in the **Confirm** step, then click **Save Workflow**.



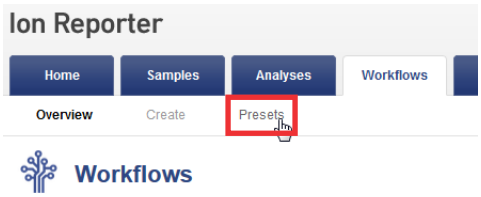
Note: See *Ion Reporter™ Software Help* for more details.

Create an Expected Normal Ploidy Buffer filter chain

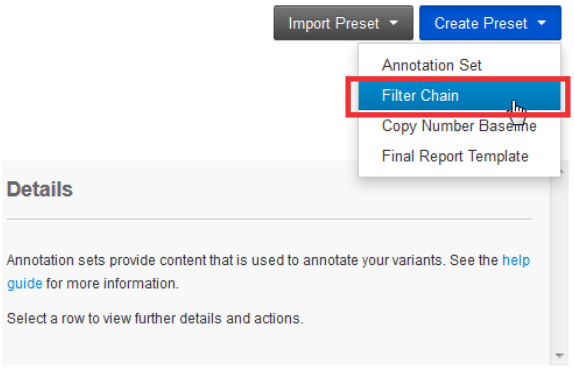
Changing the transition penalty parameter can increase sensitivity of the analysis to detect segmental mosaic CNV events. However, a side effect of this enhanced sensitivity is increased frequency of false positives. To filter false positives, you can create an Expected Normal Ploidy Buffer (ENPB) filter chain and add it to your Ion Reporter™ workflow.

By default, the ENPB filter is set to filter out all copy number variant segments of gain or loss within 0.2 ploidy value of expected normal. With this setting, ploidy changes between 2.2 and 1.8 on autosomes and female X chromosomes are filtered, as are changes between 1.2 and 0.8 on male X and Y chromosomes. You can customize the amount of buffer by adjusting the range values when you configure the filter chain. The confidence parameter in the ENPB filter overrides any other confidence filtering in effect within the buffer zone. ENPB filter chains can be selected in the **Filter Chain** dropdown list in the IRGV visualization tab.

1. In the Ion Reporter™ **Workflows** tab, click **Presets**.

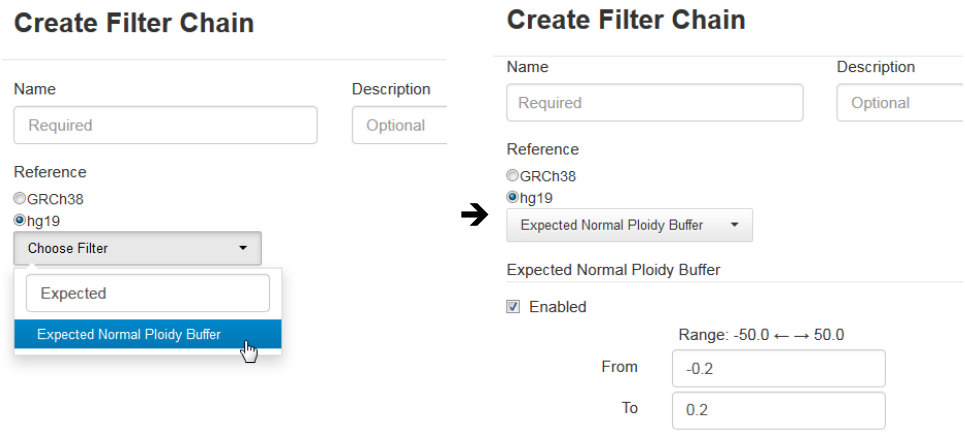


2. In the **Create Preset** dropdown list, select **Filter Chain**.

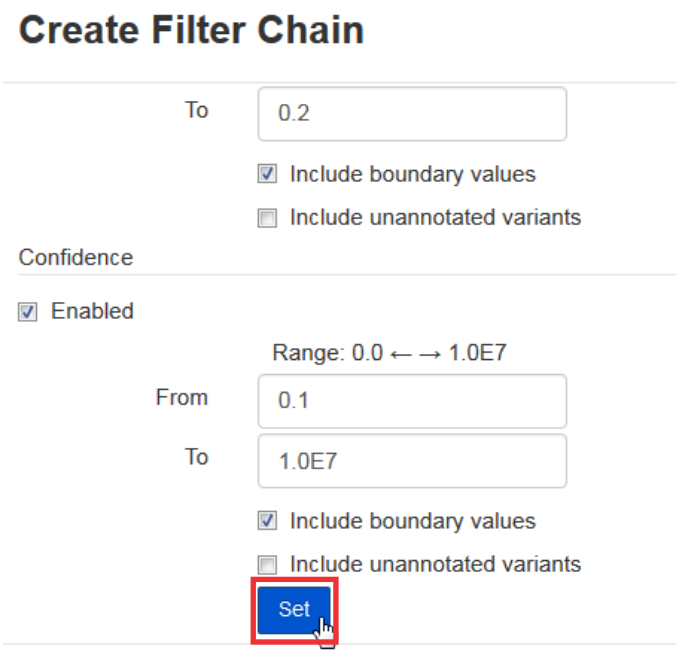


3. In the dialog, select **Expected Normal Ploidy Buffer** from the dropdown list on the left, then adjust the values in the **From** and **To** fields, if needed.

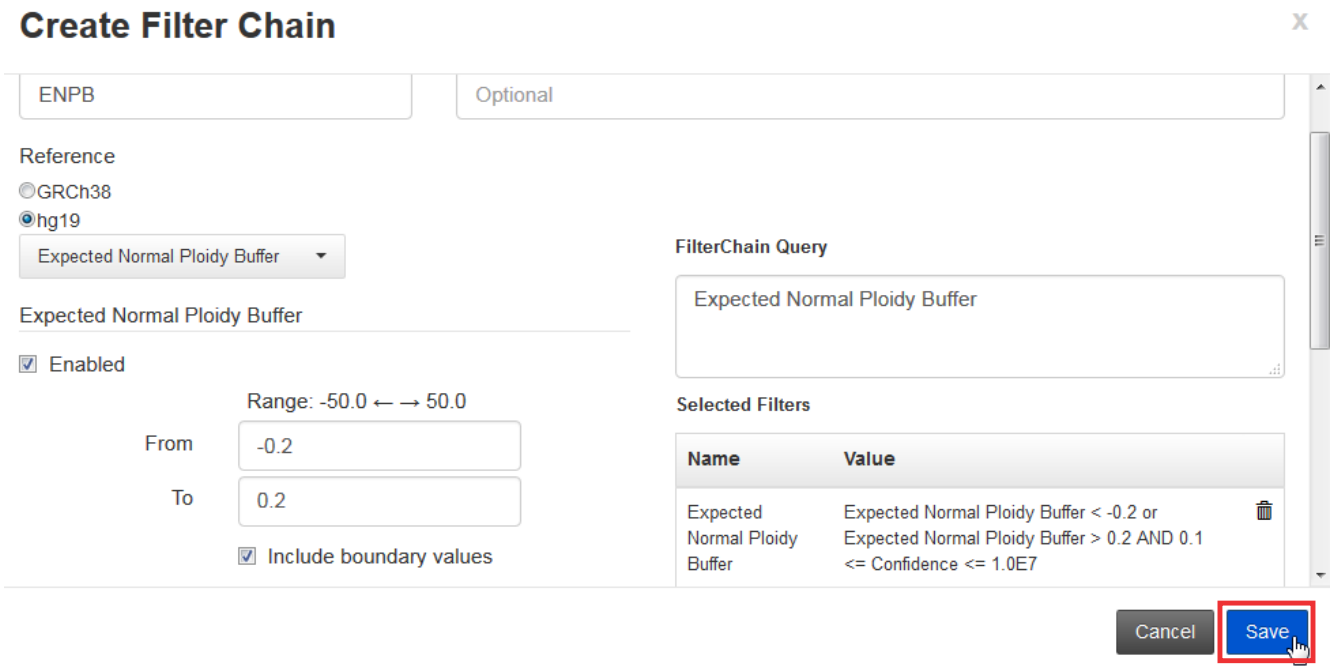
   Note: You can type "Expected" in the **Choose Filter** field to do a quick search of the dropdown list.



4. Scroll to the **Confidence** section of the dialog, adjust the confidence range parameters, if needed, then click **Set**.



5. Enter a name for the filter chain, click **Save**.



6. Go to step 5 on page 97 in "Modify the CNV Confidence Range" to add the filter chain to your workflow.

A

Troubleshooting

Ion SingleSeq™ library preparation

Observation	Possible cause	Recommended action
Library yield is low	Genomic DNA was amplified inefficiently.	Use the Human CEPH Genomic DNA Control at 15–60 pg input as a positive control in pre-amplification and amplification reactions to determine whether DNA is amplifiable in your system.
		Use real-time PCR to monitor amplification.
		Check the quality and quantity of positive control and sample libraries after amplification by running 10 µL aliquots on 2% agarose gels.
	Amount of starting material was insufficient due to loss or mishandling of cells.	Increase the number of cells in your sample.
		Keep the cells on ice.
		Do not insert pipette tip into sample – cell(s) may adhere to the tip after addition of reagent.
	Agencourt™ AMPure™ XP Reagent was over-dried during cleanup.	Do not dry the Agencourt™ AMPure™ XP Reagent more than 4 minutes.
	Percentage of ethanol in wash of Agencourt™ AMPure™ XP Reagent was less than 70%.	Prepare 70% ethanol from newly-opened ethanol reagent bottle.
		If preparing from an ethanol reagent bottle that has been frequently used and has possibly absorbed water from air, increase the percentage of ethanol in the wash from 70% to 75%.
Barcode balance in a pool is poor.	Variation in gDNA input in a pool of samples was too high.	Avoid pooling single cell samples with multiple cell samples; i.e. pool single-cell samples together and pool multiple-cell samples in a second pool if possible.

Ion OneTouch™ ES

For Ion OneTouch™ ES vertical and horizontal axis calibration and residual volume test procedures, see Chapter 3 of the *Ion OneTouch™ 2 System User Guide* (Pub. No. MAN0014388).

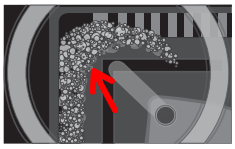
Observation	Possible cause	Recommended action
Excessive foaming occurs	• Instrument was improperly calibrated resulting in inadequate volume in one or more wells. • Fitting was loose. • Pipette tip was cracked.	1. Use the recommended volumes for all wells. 2. Ensure that fittings are tight, especially at the elbow fitting, and the pipette tip is not cracked. 3. If necessary, perform the residual volume test. If the residual volume test fails, then calibrate the instrument.
Brown pellet is present in centrifuged tube of enriched ISPs	Residual Dynabeads™ MyOne™ Streptavidin C1 Beads were present.	1. Pipet the suspension with the brown pellet up and down 10 times to resuspend the pellet. 2. Place the 0.2-mL PCR tube against a magnet such as a DynaMag™-2 magnet for 4 minutes. 3. Transfer the supernatant with the enriched ISPs to a new 0.2-mL PCR tube without disturbing the pellet of Dynabeads™ MyOne™ Streptavidin C1 Beads. 4. Sequence or store the enriched ISPs.
E12, E22, or E23 errors display during the run or during calibration	Calibration values were out of range.	1. Power OFF the instrument and wait 3 seconds. 2. While holding down **Vert. Adjust**, power ON the instrument. This step restores the factory default settings. 3. Recalibrate the vertical axis: **Note:** The default setting for the vertical axis is 310. If the setting is <310, the instrument will likely display an error, because the Tip Arm position is too high. a. Press the ▼ (minus) button to lower the Tip Arm until the tip touches the shelf. b. Press the ▼ (minus) button *8* more times. Typical vertical axis settings are ~340–370. 4. Recalibrate the horizontal axis: Press the ▲ (plus) button to move the Tip Arm to the right until the tip touches the left tab of the strip. **Note:** The default setting for the horizontal axis is 625. Typical horizontal axis settings are ~640–670.

Observation	Possible cause	Recommended action
E12, E22, or E23 errors display during the run or during calibration	AC line voltage module was installed incorrectly.	1. Determine the voltage of the electrical outlet to plug in the Ion OneTouch™ ES. 2. Align the arrow by the correct voltage on the AC line voltage module with the adjacent white arrow in the lower-right corner of the fuse socket. If the AC line voltage module is installed incorrectly: 1. Gently remove the module with your fingernail or a small flathead screwdriver. 2. Rotate the module so that the correct voltage on the module is aligned and adjacent to the white arrow in the lower right-hand corner of the fuse socket. 3. Insert the AC line voltage module into the fuse socket.
E12 or E22 error is displayed when the unit is initializing	• Fuse was installed incorrectly. • Unit was below operating temperature. • Program or calibration setting was bad, *or* • Tip Arm was not moving.	1. Ensure that the fuse module is installed correctly and that the unit is at its recommended operating temperature. 2. Reboot the instrument: Power OFF the instrument, wait 3 seconds, then power ON the instrument. 3. If the error persists, restore the factory defaults, then re-calibrate the instrument: a. Power OFF the instrument and wait 3 seconds. b. While holding down **Vert. Adjust**, power ON the instrument. This step restores the factory default settings. c. Repeat 3a–3b as needed to restore the factory defaults. d. Calibrate the vertical and horizontal axes.

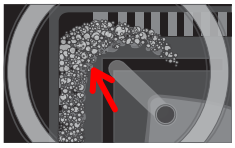
Observation	Possible cause	Recommended action
Either of the following: • E12 or E22 errors are displayed. • Tip Arm does not move or moves slightly.	AC line voltage module was installed incorrectly.	1. Determine the voltage of the electrical outlet serving the Ion OneTouch™ ES. 2. Align the arrow by the correct voltage on the AC line voltage fuse module with the adjacent white arrow in the lower-right corner of the fuse socket. If the AC line voltage fuse module is installed incorrectly: 1. Gently remove the module with your fingernail or a small flathead screwdriver. 2. Rotate the module so that the correct voltage on the module is aligned and adjacent to the white arrow in the lower right-hand corner of the fuse socket. 3. Insert the AC line voltage fuse module into the fuse socket.
	Instrument was not operated at the recommended temperature.	Ensure that the Ion OneTouch™ ES is at an operating temperature of 60°F to 77°F (15°C to 25°C).
Solution overflows during run	Reagent volumes were overloaded.	Repeat with reagent volumes described in enrichment procedure.
Tip is causing 8-well strip to lift out of tray slot during run	Tip was not aligned vertically.	Perform the vertical calibration procedure.
Percent template-positive ISPs after enrichment is <50% as measured by flow cytometry	Multiple causes are possible.	Contact Technical Support.
Problems with the strip position • Strip lifts up during strip push. • Strip lifts up when tip is raised from well. • Immediately after strip push, the strip is not in contact with the magnet.	Instrument was not calibrated properly.	• Perform horizontal calibration. • Perform vertical calibration.
Tip grinds into base of instrument and Code "1999" displays	• Unit was not calibrated properly. • Vertical calibration setting was too low or out-of-range.	1. Restore the factory default settings on the instrument: Hold down the vertical adjust button while powering ON the instrument. The instrument beeps several times. 2. Re-calibrate the instrument. 3. Perform a residual volume test.
Tip hits the top of the tray at start of run	Tray was not properly seated in the instrument.	Check for debris between the tray and the instrument, then reinstall the tray. Press down firmly to ensure that tray is fully seated in the instrument.

Observation	Possible cause	Recommended action
Error messages display	Various causes are possible.	1. Power the instrument OFF, then ON. 2. If the error continues to display, restore the factory default settings on the instrument. Hold down the vertical adjust button while powering ON the instrument. The instrument beeps several times. 3. Re-calibrate the instrument. 4. Perform a residual volume test.
Instrument does not aspirate or dispense liquids	Fitting(s) were loose.	• Ensure that the Luer-Lok™ connections at the elbow on the Tip Arm and at the tubing on the rear syringe pump are finger-tight. • Ensure that the metal tip adapter fitting on the Tip Arm is finger-tight. **IMPORTANT!** After any adjustments to the metal tip adapter, recalibrate the Ion OneTouch™ ES.

Chip Check

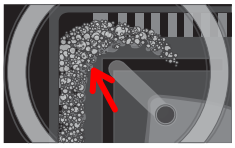
Observation	Possible cause	Recommended action
Chip Check fails	• Clamp was not closed. • Chip was not properly seated. • Debris was present on the chip socket. • Chip was damaged.	1. Open the chip clamp, remove the chip, and look for signs of water outside the flow cell:  2. If the chip appears damaged, replace it with a new one. 3. Look for debris on the chip socket. Remove any debris by rinsing with 18-MΩ water and gently dabbing the socket with a lab wipe tissue. **IMPORTANT!** Never rub or wipe the socket. Rubbing the socket can damage it and cause it to fail. 4. Close the clamp and repeat the Chip Check. 5. If the chip passes, click **Next**. If the chip fails, replace it with a new chip, scan the barcode of the new chip, then press **Chip Check**. 6. If Chip Check continues to fail, there could be a problem with the chip socket. Contact Technical Support.

Chip calibration (before loading sample)

Observation	Possible cause	Recommended action
Leak of unknown origin occurs	• Chip was leaking. • Chip clamp was not closed properly. • Problem existed with the chip clamp or socket.	1. Press **Main Menu**. 2. Open the chip clamp, remove the chip, and gently dab the chip socket with a lab wipe tissue to absorb any fluid. **IMPORTANT!** Never rub or wipe the socket. Rubbing the socket can damage it and cause it to fail. 3. Rinse the socket with 18-MΩ water and gently absorb most of the water with the lab wipe. 4. Repeat the rinse, then gently dab the chip socket until dry. 5. Place a lab wipe on the grounding plate and dampen it with 18-MΩ water. Wipe the bottom of the chip on this wipe to remove salts from the chip contacts. 6. Remove the wipe, dry the grounding plate, and place chip on grounding plate. Ensure that there is no condensation outside the flow cell:  7. Replace the chip with a new (unused) one if needed. **Note:** The new chip can be used for sequencing after initialization completes. 8. Press **Run** to restart the experiment. 9. When prompted to install the new chip, ensure that the chip clamp is fully closed. 10. If the chip leaks again, clean the chip socket as described above. Continued leaking, even with new chips, can indicate a chip clamp or socket problem. Contact Technical support.

Observation	Possible cause	Recommended action
Error message: Calibration FAILED	• Chip was not seated in socket correctly. • Chip was damaged.	1. Remove the chip and confirm that there is no leakage or debris on the chip socket. If leaking or debris is seen, follow the procedure for inspecting the chip and clearing debris as described under "Chip Check fails" and/or "Leak of unknown origin" above. If no leaking or debris is seen, reseat the chip in the socket. 2. Press **Calibrate** to repeat the calibration. 3. If the chip passes, press **Next**. If the chip still fails return to the main menu and restart the experiment with a new chip. 4. If you continue to have chip calibration issues, there may be an issue with the chip socket. Contact Technical Support.

Chip calibration (after loading sample)

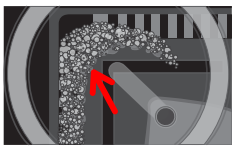
Observation	Possible cause	Recommended action
Leak of unknown origin occurs	• Chip was leaking. • Chip clamp was not closed properly.	1. Press the **Abort** button. 2. Open the chip clamp, remove the chip, and gently dab the chip socket with a lab wipe tissue to absorb any fluid. Do not rub or wipe the chip socket. 3. Rinse the socket with 18 MΩ water and gently absorb most of the water with the lab wipe tissue. 4. Repeat the rinse, then gently dab the chip socket until dry. 5. Place a lab wipe tissue on the grounding plate and dampen it with 18 MΩ water. Wipe the bottom of the chip on this wipe to remove salts from the chip contacts. 6. Remove the wipe, dry the grounding plate, and place the chip on the grounding plate. Check for condensation outside the flow cell:  7. If there is condensation or fluid, the chip is damaged and cannot be run. 8. If there is no condensation or fluid, press **Calibrate** to restart the calibration procedure. 9. If calibration passes and no leaks are visible, press **Next** to start the experiment. 10. If the chip leaks again, clean the chip and chip socket as described above. Continued leaking can indicate a chip clamp or socket problem. Contact Technical Support.

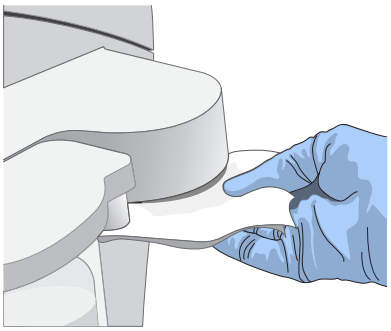
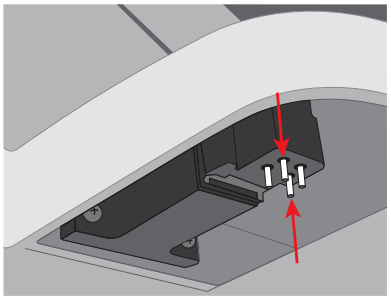
Observation	Possible cause	Recommended action
Error message: Calibration FAILED	• Chip was not seated in socket correctly. • Chip was damaged.	1. Remove the chip and check for leaks and/or debris on the chip socket, following the procedures described in "Chip Check fails" and/or "Leak of unknown origin," above. If no leaks or debris are visible, reseat the chip in the socket. 2. Press **Calibrate**. 3. If the chip passes, press **Next** to start the experiment. If the chip still fails, you can try reseating the chip multiple times and pressing **Calibrate**. If you are still unable to pass calibration, press **Next** to start the run anyhow-you may still get some data on your sample. 4. If you continue to have chip calibration issues, there may be an issue with the chip or chip socket. Contact Technical Support.

Initialization—General errors

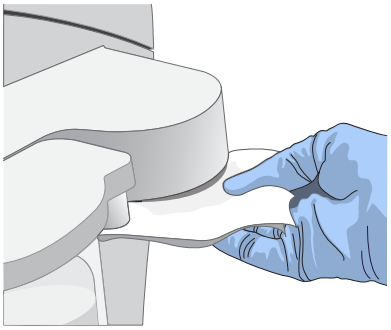
Observation	Possible cause	Recommended action
Error message: Confirm instrument has gas pressure	Gas cylinder may be turned off or empty.	1. Verify that the cylinder has at least 500 PSI and 30 PSI at the outlet of the regulator. Confirm that all valves between the cylinder and the Ion PGM™ Sequencer are open. 2. Once you confirm gas pressure leading into the instrument, press **Yes** to retry verification of gas pressure. If the test continues to fail, contact Technical Support.
Bottle leak check fails	• Bottle seal was not tight. • Bottle was damaged or defective.	1. Finger-tighten the bottles. 2. If the bottle continues to leak, replace the bottle. 3. If leak check continues to fail, contact Technical Support.

Initialization—Auto pH errors

Observation	Possible cause	Recommended action
Error message: Please insert a chip and press **Start**	Instrument could not detect the chip in chip socket.	1. Open the chip clamp and remove the chip. 2. Check for debris under the chip or in the chip socket. Remove any debris by rinsing with 18-MΩ water and gently dabbing the socket with a lab wipe tissue. **IMPORTANT!** Never rub or wipe the socket. Rubbing the socket can damage it and cause it to fail. 3. Look for liquid outside the flow cell of the chip:  4. If you see liquid, replace the chip with a new (unused) one. Wash the new chip once with 100% isopropanol and twice with SEQ Sample Buffer before using. **Note:** The new chip can be used for sequencing after initialization completes. 5. Close the clamp, then press **Start** to restart the process. 6. If the new chip also fails, there could be a problem with the chip socket. Contact Technical Support.
Error message: Chip calibration failed	• Chip was not seated in socket correctly. • Chip was damaged. • Sipper was loose.	Follow the procedure for "Error message: Please insert a chip and press Start." Follow the procedure for "Error message: Wash 2 average not stable."

Observation	Possible cause	Recommended action
Error message: The system did not reach the target W2 pH and/or has a clog	The waste lines were clogged.	1. Press the **Troubleshoot** button. **Note:** You can skip the **Troubleshoot** button and change the chip to restart the Auto-pH routine. 2. Remove the waste bottle. 3. Place lab wipes under the waste arm. 4. Gently wipe the waste arm with a lab wipe to clear liquid near the waste line.  5. Press **Next** to start buffer flow. Observe flow rates from both waste lines. One line should drip slightly faster than the other. Following the flow rate check, one of 3 results is possible:  a. If flow rate appears normal, press **Cancel** and test another chip. If Auto pH failure persists, contact Technical Support. b. If flow is blocked, press **Line Clear** to run the standard Line Clear procedure. If the line is unable to clear, contact Technical Support. c. If the result of the flow rate check is uncertain, press **Re-flow** to re-flow the buffer and re-test the flow.

Observation	Possible cause	Recommended action
Error message: The system did not reach the target W2 pH (*continued*)	Wash 1 or Wash 2 sipper was loose.	1. Loosen the Wash 1 cap and re-tighten the sipper. Since the gas flows when the cap is loose, tighten the sipper as quickly as possible. (The gas is not harmful to the NaOH solution and is not a hazard.) 2. Loosen the Wash 2 cap and re-tighten the sipper. Since the gas flows when the cap is loose, tighten the sipper as quickly as possible. (The gas is not harmful to the W2 Solution and is not a hazard.) 3. Press **Start** to re-start the auto-pH process.
	NaOH was not added to the Wash 1 Bottle.	1. If there is no NaOH in the Wash 1 Bottle, loosen the cap and add 350 µL of 100 mM NaOH to the Wash 1 Bottle. (The flowing gas is not harmful to the NaOH solution and is not a hazard.) 2. Recap the bottle and shake gently to mix. 3. Press **Start** to restart auto-pH.
	Chip is damaged.	1. Replace the chip with a new (unused) one. Insert the chip in the socket, then press **Start**. **Note:** The new chip can be used for sequencing after initialization completes. 2. If the error persists, there could be a problem with the chip clamp. Contact Technical Support.

Observation	Possible cause	Recommended action
Error message: W2 average not stable. Try reseating/replacing chip	Reading for W2 solution did not stabilize quickly enough.	1. Remove the waste bottle and gently wipe excess fluid from the waste lines with a lab wipe.  2. Check for leaks and reseat the chip (see troubleshooting for "Chip Check" and "Chip calibration" above). Replace the chip with a new (unused) one if needed. **Note:** The new chip can be used for sequencing after initialization completes. 3. Loosen the cap in the W2 position and re-tighten the sipper. Because the gas flows when the cap is loose, tighten the sipper as quickly as possible. (The gas is not harmful and not a hazard.) 4. After performing one or more above steps, press **Start** to re-start auto-pH. If auto-pH fails even after replacing the chip, contact Technical Support and manually adjust the pH of the Wash 2 Bottle as described in "Manually adjust W2 pH" in the *Ion PGM™ System Reference Guide* (Pub. No. MAN0009783).
Error message: W2 out of range	• Chip measurements were very unstable. • Chip was damaged.	See troubleshooting tips for "W2 average not stable" above.
Error message: Chip reading inconsistent. Please replace chip and try again	• pH response of the chip was not uniform or reliable. • Ran out of W3 Solution, or volume was too low.	1. Verify that there is enough W3 Solution (>25 mL) in the Wash 3 Bottle and that the sipper is secure. 2. If necessary, loosen the Wash 3 Bottle cap, tighten the sipper, and add more W3 Solution to fill to 50 mL. Since the gas flows when the cap is loose, perform these operations as quickly as possible. (The gas is not harmful to the W3 Solution and is not a hazard.) 3. If there is enough W3 Solution, replace the chip with a new (unused) one. Insert the chip in the socket, then press **Start**. **Note:** The new chip can be used for sequencing after initialization completes.

Observation	Possible cause	Recommended action
Error message: Added too much W1 to W2	• Water quality was poor. • 18 MΩ water was exposed to air for too long. • Incorrect solution was added to the Wash 2 Bottle. • Too little NaOH was added to the Wash 1 Bottle. • Chip was damaged.	1. Check whether the water meets the 18 MΩ specification and 100 mM NaOH and W2 Solution were added correctly. 2. If solutions are not correct or water does not meet specifications, correctly prepare the solution(s) and/or use high-quality water. Abort the initialization and restart using correct solutions/water. 3. If solutions are correct and water meets specifications, abort the initialization, return to the main menu, and proceed to the next steps. 4. Leave the Wash 2 Bottle on the instrument. 5. Remove the Wash 1 Bottle, leaving the sipper on the W1 port. Empty the bottle, and rinse the bottle twice with 18 MΩ water. 6. Add 350 µL of 100 mM NaOH to the Wash 1 Bottle and reinstall on the instrument. 7. Press **Initialize**, select the kit type, and keep pressing the **Next** button to skip all bottle prep steps until the instrument starts purging air from the bottle. Then proceed through the touchscreens as normal to complete the initialization. 8. The next time that you initialize the instrument, add 140 µL of 100 mM NaOH to the Wash 2 Bottle instead of 70 µL. Continue to use this larger volume for subsequent initializations until you receive an "Overshot Target" error message at the first auto-pH iteration, at which point follow the troubleshooting steps in "Error message: The system overshot the target W2 pH." on page 115, then return to adding 70 µL of 100 mM NaOH. 9. If you still receive the same initialization error ("Added too much W1 to W2"), contact Technical Support.
Error message: UNDERSHOT TARGET PH: W2 pH = n.nn Failed	Auto-pH couldn't add enough Wash 1 to the Wash 2 before the maximum iterations, 10, occurred.	1. A blockage may have occurred. Follow the procedure for "Error message: There may be a blockage or no NaOH in W1. Please check W1 and run line clear then try again." 2. Press **Start** to re-start auto-pH. If you still get the "Undershot target pH" error, try replacing the chip with a new (unused) chip and restarting auto-pH. **Note:** The new chip can be used for sequencing after initialization completes.

Observation	Possible cause	Recommended action
Error message: The system overshot the target W2 pH.	Auto-pH added more NaOH from the Wash 1 Bottle to the Wash 2 Bottle than was needed, and reports the pH value.	1. Press the **Overshoot** button to proceed with W2 pH adjustment. 2. Unscrew the cap of the Wash 2 Bottle. Without removing the sipper from the bottle, lift the cap high enough to pipette 15 µL of 100 mM HCl into the Wash 2 Bottle, close and tighten cap.  3. Press **Next** to re-pressurize the Wash 2 Bottle and mix the W2 solution. 4. Press **Start** to retry auto-pH.

Initialization—Reagent pH verification

Observation	Possible cause	Recommended action
Red failure screen, reagent pH is displayed	One or more reagents were not within the target pH.	1. Press **Start** to repeat the pH measurements to confirm the measurement. 2. If any reagents still fail, try replacing the chip with a new (unused) chip and repeating. **Note:** The new chip can be used for sequencing after initialization completes. 3. If any reagents still fail, clean and reinitialize the instrument with fresh reagents and a new chip.
	A possible line clog exists which persisted through the Auto pH process.	1. From the **Tools** menu, perform a W1 line clear. 2. Press **Start** to repeat the pH check.

Observation	Possible cause	Recommended action
Red failure screen, reagent pH is *not* displayed	Chip did not calibrate.	1. Replace the chip with a new (unused) one. **Note:** The new chip can be used for sequencing after initialization completes. 2. Press **Start** to restart the pH measurement. 3. If the second test fails, contact Technical Support.

Ion Reporter™ results

Observation	Possible cause	Recommended action
High MAPD value (>0.3) is observed	Low library read representation (<100,000 reads per sample) occurred.	Sequence poorly represented samples to a higher depth by increasing their proportional concentration in the sample library pool.
		Reduce the number of samples run per chip.
	Library read representation was normal (>200,000 reads per sample), but library quality was poor.	Repeat preparation of sample libraries.
Chromosome X and Y data are missing in IRGV plots, preventing gender call These samples also tend to show high MAPD values. See "View X and Y chromosome data in low-read samples" on page 87 for further information.	The sample exhibited very low total read counts, resulting in no display of X and Y chromosome data.	Sequence poorly represented samples to a higher depth.
		Reduce the number of samples run per chip.
		Navigate to **Parameters** ▸ **CNV Finding** ▸ **Advanced** ▸ **Gender section** and lower the value of the "CNV Gender Min Autosomes Count" parameter.
		Navigate to **Parameters** ▸ **CNV Finding** ▸ **Advanced** ▸ **Analysis** and set the "Plot Y chromosome for Female or Unknown Gender" parameter to **True**.

B Additional instrument information

See the *Ion PGM™ System Reference Guide* (Pub. No. MAN0009783) for additional information on general instrument operation and maintenance, including:

- Touchscreen reference
- How to update instrument software
- How to manually adjust W2 Solution pH
- Ion Chip™ Minifuge and Barcode Scanner operation
- Sequencing run times
- Site requirements
- Instrument safety

C

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.

Instrument safety

For detailed information on instrument safety symbols and alerts, safety and electromagnetic compatibility standards, and general instrument safety, see the Safety appendix of the *Ion PGM™ System Reference Guide* (Pub. No. MAN0009783), available at **thermofisher.com**.

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. 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.

Biological hazard safety

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. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

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- 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.

Limited product warranty

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