Ion Sphere™ Assay on the Qubit™ 3.0 Fluorometer

for use with: Ion Sphere™ Quality Control Kit (Cat. No. 4468656)

Publication Number MAN0016388 **Revision** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

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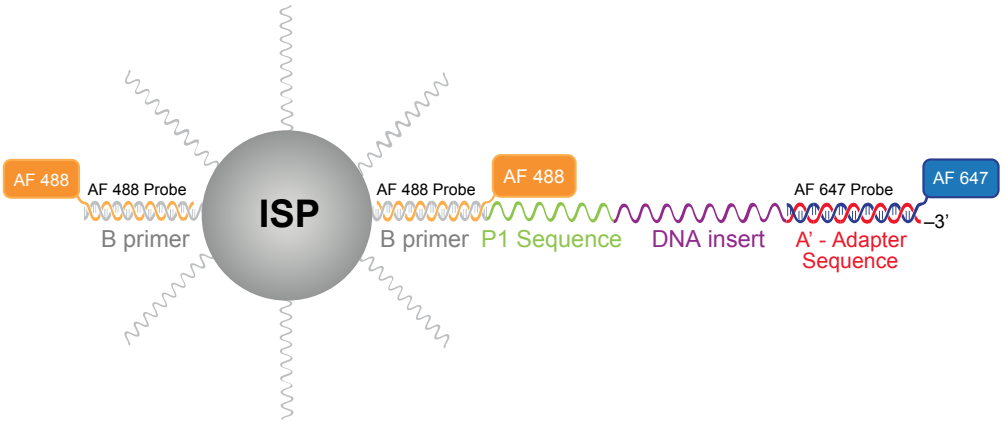
Ion Sphere™ Assay overview

The Ion Sphere™ Assay on the Qubit™ 3.0 Fluorometer measures the fluorescence of template-positive Ion Sphere™ Particles (ISPs) labeled with two fluorophores: Alexa Fluor™ 488 and Alexa Fluor™ 647.

- A probe labeled with Alexa Fluor™ 488 anneals to primer B sites, or all of the ISPs present.
- A probe labeled with Alexa Fluor™ 647 anneals to primer A sites, or only the ISPs with extended templates.

The ratio of the Alexa Fluor™ 647 fluorescence (templated ISPs) to the Alexa Fluor™ 488 fluorescence (all ISPs present) yields the % templated ISPs.



Alexa Fluor™ 488- and Alexa Fluor™ 647-labeled probes annealed to an ISP

Materials required

Ion Sphere™ Quality Control Kit

The Ion Sphere™ Quality Control Kit (Cat. No. 4468656) provides reagents for performing templated ISP quality control using the Qubit™ 3.0 Fluorometer.

Component	Amount	Storage
Ion Probes (blue cap)	20 µL	–30°C to –10°C
Alexa Fluor™ 488 Calibration Standard (green cap)	400 µL	
Alexa Fluor™ 647 Calibration Standard (red cap)	400 µL	
Annealing Buffer (white cap)	400 µL	
Quality Control Wash Buffer	20 mL	–30°C to –10°C or 2°C to 8°C

Other materials

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Qubit™ 3.0 Fluorometer	Q33216
Qubit™ Assay Tubes	Q32856
PCR tubes, 0.2 mL (Axygen™ Maxymum Recovery PCR Tube, or equivalent)	Fisher Scientific 14-222-283, or MLS
Qubit™ Easy Calculator Microsoft™ Excel™ Spreadsheet file	Download at Ion Sphere™ Quality Control Kit product page (thermofisher.com/4468656)
SimpliAmp™ Thermal Cycler, or equivalent	A24811
Microcentrifuge	MLS
Unenriched ISPs	Provided by user

Update the software

We recommend updating the instrument software of the Qubit™ 3.0 Fluorometer to the latest available version.

1. To verify the version of software currently on your instrument, touch **Settings** on the Qubit™ 3.0 Fluorometer home screen, then touch **About instrument**.
2. If your instrument requires a software update, download the latest software at **thermofisher.com/qubit/qubit-technical-resources**, then transfer the file to a USB drive.
3. Insert the USB drive into the Qubit™ 3.0 Fluorometer, touch **Settings**, then **Update software**. When the new software is detected, touch **Update**.
4. When prompted, touch **Restart** to complete the update.

   Note: For more detailed instructions, see the *Qubit™ 3.0 Fluorometer User Guide* (Pub. No. MAN0010866).

Calculate the Qubit™ 3.0 Fluorometer Calibration Factor

The Qubit™ 3.0 Fluorometer features a pre-loaded quality control Ion Sphere™ Assay for determining the percentage of templated ISPs. A unique instrument-specific Calibration Factor must be calculated and applied to all percent templated ISP calculations.

Note: It is only necessary to calculate the Calibration Factor once for a particular instrument, unless a problem is suspected.

Download the Qubit™ Easy Calculator

Download the Qubit™ Easy Calculator, a Microsoft™ Excel™ spreadsheet file, from: **http://tools.thermofisher.com/content/sfs/brochures/qubit-calibration-ion-sphere-calculator.xlsx**, and save the file to the computer used for Qubit™ 3.0 Fluorometer data analysis.

Prepare the calibration standard

1. From the Ion Sphere™ Quality Control Kit, thaw the Alexa Fluor™ 488 and Alexa Fluor™ 647 Calibration Standard reagents.

   Note: Both the Alexa Fluor™ 488 and Alexa Fluor™ 647 molecules are photosensitive, so avoid exposure to light for long periods of time, and to direct sunlight.

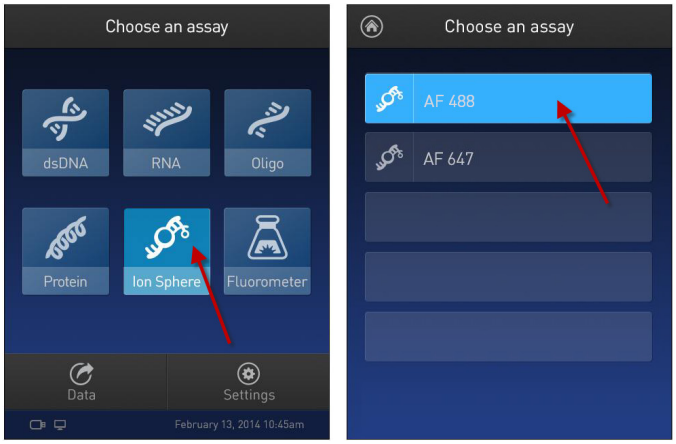
2. Vortex well to mix, then pulse-centrifuge the tube to remove any liquid that is trapped in the cap.

3. Transfer 200 µL of each standard into two separate Qubit™ assay tubes. Pulse-centrifuge to bring all the liquid to the bottom of the tube.

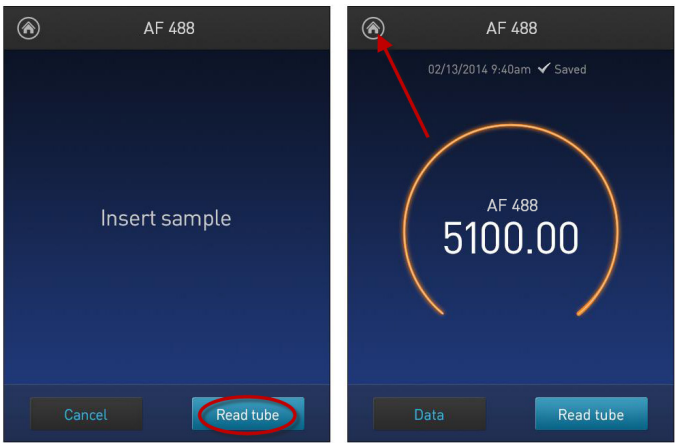
Measure the calibration standard

1. Power on the Qubit™ 3.0 Fluorometer.

2. Touch **Ion Sphere** on the Qubit™ 3.0 Fluorometer home screen open the Ion Sphere™ Assay. Touch **AF 488**.

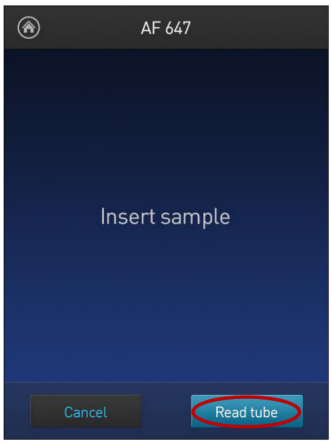


3. Insert the Alexa Fluor™ 488 Calibration Standard reagent into the Qubit™ 3.0 Fluorometer, close the lid, and touch **Read tube** (Read tube).

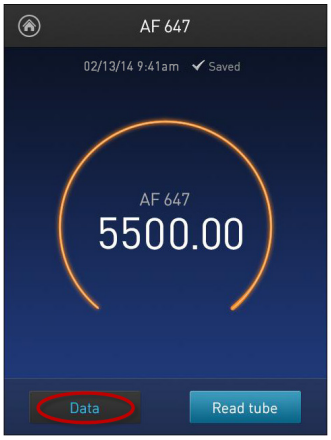


4. Record the RFU value, remove the assay tube from the Qubit™ 3.0 Fluorometer and touch the **Home** icon in the upper left corner of the screen.

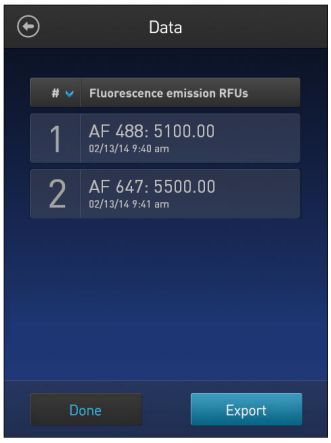
5. On the Home screen, touch **Ion Sphere**, and then touch **AF 647**. Insert the Alexa Fluor™ 647 Calibration Standard into the Qubit™ 3.0 Fluorometer, close the lid, and touch **Read tube** (Read tube).



6. Record the RFU value, remove the assay tube from the Qubit™ 3.0 Fluorometer. Touch **Data** (Data).



7. Touch **Export** (Export) to export data to a USB storage drive or to a USB-connected computer. Touch **Done** (Done) to return to the Home screen.



Calculate the Calibration Factor

1. In the Qubit™ Easy Calculator, enter each recorded RFU value in the appropriately labeled green cell to display the Calibration Factor specific for the Qubit™ 3.0 Fluorometer.

2. Save a copy of the Qubit™ Easy Calculator containing the Calibration Factor for use as a template for future Percent Templated ISPs calculations:

   Note: Affix a sticker with the instrument-specific Calibration Factor to the Qubit™ 3.0 Fluorometer.

A	B	C	D	E	F	G	H	I	J
Qubit Calibration Factor Calculation									
Calibration Standard	**RFU**	**Calibration Factor**							
Alexa Fluor® 488 Calibration Standard		#DIV/0!							
Alexa Fluor® 647 Calibration Standard									
Percent Templated ISPs									
	Raw RFU Value		**Background RFU (Negative Control Tube)**						
Sample ID	**AF 488**	**AF 647**	**AF 488**	**AF 647**	**Conversion Factor***	**Percent Templated ISPs**			
						#DIV/0!			
						#DIV/0!			
						#DIV/0!			
						#DIV/0!			
						#DIV/0!			
						#DIV/0!			
						#DIV/0!			
Green Cells = Raw RFU values of Alexa Fluor® 488 and Alexa Fluor® 647 Calibration Standards supplied in the Ion Sphere Quality Control Kit									
Red Cells = Raw RFU values measured in "Measure the templated unenriched sample"									
Purple Cells = Raw RFU values measured for negative control in "Measure the templated unenriched sample"									
Blue Cells= Template kit lot specific conversion factor									

IMPORTANT! For each Qubit™ 3.0 Fluorometer used, save a separate Qubit™ Easy Calculator Microsoft™ Excel™ Spreadsheet file containing the Calibration Factor specifically calculated for that particular instrument.

Measure the templated unenriched sample

This section describes the procedure for determining the percent templated ISPs for unenriched Ion Sphere™ Particles.

Prepare the sample: Ion OneTouch™ 2 users

1. From the Ion Sphere™ Quality Control Kit, thaw the Ion Probes tube, Annealing Buffer, and Quality Control Wash Buffer.
2. Adjust the sample volume of unenriched ISPs to 100 µL, if needed, with Ion OneTouch™ Wash Solution (Ion PGM™ users) or ISP Resuspension Solution (Ion Proton™ and Ion S5™ users) from the OT2 Solutions box, then transfer 2 µL to a 0.2-mL PCR tube.
3. Add Ion Probes to the sample.
   - If processing one sample, add 19 µL Annealing Buffer and 1 µL Ion Probes directly to the 0.2-mL PCR tube containing the ISPs and mix well by pipetting up and down.
   - If processing more than one sample, make an Ion Probe Master Mix:
     a. (19 µL Annealing Buffer * # samples) + (1 µL Ion Probes * # samples) = total volume required

        Note: To compensate for pipetting error, prepare an extra 5–10% overage.

     b. Add 20 µL of Ion Probe Master Mix to the 0.2-mL PCR tubes containing the ISPs, then mix well by pipetting up and down.
4. Load the tube into a thermal cycler, then perform the following protocol to anneal the Ion Probes:

Stage	Temperature	Time
Hold	95 ºC	2 minutes
Hold	37 ºC	2 minutes

5. Remove unbound probes by washing the samples 3 times with 200 µL of Quality Control Wash Buffer.
   a. Add 200 µL of Quality Control Wash Buffer to the 0.2-mL tube.
   b. Vortex to mix, then centrifuge at:
      - 15,500 × *g* for 1.5 minutes (Ion PGM™ users), *or*
      - Maximum speed for 3 minutes (Ion Proton™ and Ion S5™ users)
   c. Being careful not to disturb the pelleted ISPs, remove the supernatant leaving 10 µL behind.

      Note: Compare to a 10-µL standard for reference.

   d. Repeat steps a – c two times for a total of 3 washes.

6. After the final wash, add 190 µL of Quality Control Wash Buffer for a total volume of 200 µL, mix by pipetting up and down 5 times, then transfer the entire sample to a Qubit™ Assay Tube.

   IMPORTANT! Ensure that you measure the volumes accurately.

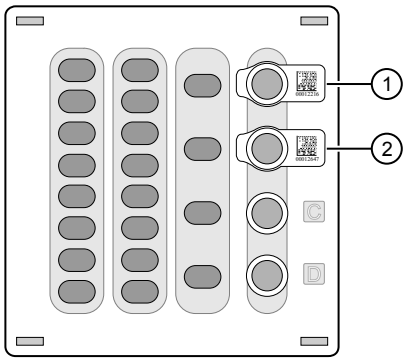
7. To generate a negative control, add 200 µL of Quality Control Wash Buffer to a new Qubit™ Assay Tube.

Proceed to "Measure the sample" on page 10.

Prepare the samples: Ion Chef™ users

1. From the Ion Sphere™ Quality Control Kit, thaw the Ion Probes tube, Annealing Buffer, and Quality Control Wash Buffer.

2. Centrifuge the ISP samples taken from Positions A and B on the Reagents cartridge at:
   - 15,000 × *g* for 2 minutes (Ion PGM™ users), *or*
   - Maximum speed for 3 minutes (Ion Proton™ and Ion S5™ users)



① Position A (QC sample)
② Position B (QC sample)

Note:
- If you want use both the Guava™ easyCyte™ 5 Flow Cytometer and Qubit™ fluorometry for quality assessment, remove 1 µL of the sample before centrifugation for flow cytometric analysis before processing the remainder for Qubit™ analysis.
- If you are evaluating quality after completion of the Ion Chef™ run, you can remove the Library Sample Tubes from the Reagents cartridge and centrifuge the QC samples in these tubes.

3. Remove supernatant to reduce the total volume per sample to approximately 10 µL.

4. Pipet each sample up and down to mix, then transfer each sample to a new labeled 0.2-mL PCR tube.

5. Add 10 µL Annealing Buffer and 1 µL Ion Probes directly to each 0.2-mL PCR tube containing the ISPs, then mix well by pipetting up and down.

   Note: If processing multiple samples, make an Ion Probe Master Mix:

(10 µL Annealing Buffer × # samples) + (1 µL Ion Probes × # samples) = total volume required

6. Load the tubes into a thermal cycler, then perform the following protocol to anneal the Ion Probes:

Stage	Temperature	Time
Hold	95 ºC	2 minutes
Hold	37 ºC	2 minutes

7. Remove unbound probes by washing the samples 3 times with 200 µL of Quality Control Wash Buffer.
   a. Add 200 µL of Quality Control Wash Buffer to the 0.2-mL tubes.
   b. Vortex properly to mix, then centrifuge at:
      - 15,000 × *g* for 2 minutes (Ion PGM™ users), *or*
      - Maximum speed for 3 minutes (Ion Proton™ and Ion S5™ users)
   c. Being careful not to disturb the pelleted ISPs, remove the supernatant leaving behind 10 µL.

      Note: Compare to a 10-µL standard for reference.
   d. Repeat steps a – c two times for a total of 3 Quality Control Wash Buffer washes.
8. After the final wash, add 190 µL of Quality Control Wash Buffer for a total volume of 200 µL, mix by pipetting up and down 5 times, then transfer the entire sample to a Qubit™ Assay Tube.

   IMPORTANT! Ensure that you measure the volumes accurately.
9. To generate a negative control, add 200 µL of Quality Control Wash Buffer to a new Qubit™ Assay Tube.

Proceed to "Measure the sample".

Measure the sample

1. Power on the Qubit™ 3.0 Fluorometer.
2. Touch **Ion Sphere** to access Alexa Fluor™ 488 and Alexa Fluor™ 647 measurement options.
3. Touch **AF 488**, insert the sample into the Qubit™ 3.0 Fluorometer, close the lid, then touch **Read tube**.

   Note: If more than one sample is being processed, all samples can be read with the AF 488 setting before moving on to the AF 647 setting.

4. Record the value.

   Note: The data retained on the Qubit™ 3.0 Fluorometer can be transferred to a USB drive. See the "(Optional) Transfer the data to a USB Drive" for details. If more than one sample is being processed, all samples can be read with the AF 488 setting before moving on to the AF 647 setting.

5. Touch **Home**, touch **Ion Sphere**, then touch **AF 647**. Insert the sample into the Qubit™ 3.0 Fluorometer, close the lid, then touch **Read tube**.

6. Record the value.

   IMPORTANT! Ensure that you read the negative control (Quality Control Wash Buffer only) in both the Alexa Fluor™ 488 and Alexa Fluor™ 647 settings and record the RFU values.

(Optional) Transfer the data to a USB Drive

1. Ensure that the USB drive is inserted in the instrument, or a computer is connected by USB cable.
2. On the Home screen, touch **Data** (at the bottom-left of the screen).
3. On the Data screen, touch **Export**, then wait for the instrument to download the data to the USB drive or computer.

   Note: The download creates a ".csv" file that can be opened on your computer using any spreadsheet software, such as Microsoft™ Excel™ software.

Evaluate the templated ISPs

1. Open the saved Qubit™ Easy Calculator containing the Calibration Factor specifically calculated for the Qubit™ 2.0 Fluorometer used.
2. Enter the raw RFU values from Alexa Fluor™ 488 and Alexa Fluor™ 647 Calibration Standards measurements in the appropriate fields for both the ISPs containing samples (red cells) and negative control sample (purple cells).

   IMPORTANT! The Alexa Fluor™ 488 value must be >100 counts to produce a valid % Templated ISPs value.

Fluorophore	Acceptable RFU Range
Alexa Fluor™ 488	>100 counts; no upper limit Samples with <100 counts usually correlate with no or very few ISPs in the assay.
Alexa Fluor™ 647	Any value, with the condition that the Alexa Fluor™ 488 RFU value is >100 counts.

3. In the appropriate field (blue cells), enter the template kit- and lot-specific conversion factor for unenriched ISPs, available at: **thermofisher.com/qubit-conversion-factors-guide**, or downloaded from the Ion Sphere™ Quality Control Kit product page (**thermofisher.com/4468656**).

   Note: Ion PGM™ and Ion S5™ users: Use the 200-base-read library conversion factor for libraries <300 bp in length. Use the 400-base-read library conversion factor for libraries ≥300 bp in length.

4. The Percent Templated ISPs calculates automatically and is displayed for each sample:

Qubit Calibration Factor Calculation

Calibration Standard	RFU	Calibration Factor
Alexa Fluor® 488 Calibration Standard	6548	0.58 ①
Alexa Fluor® 647 Calibration Standard	10265	

Percent Templated ISPs

	Raw RFU Value		Background RFU (Negative Control Tube)			
Sample ID	AF 488	AF 647	AF 488	AF 647	Conversion Factor*	Percent Templated ISPs
						#DIV/0!
						#DIV/0!
	②		③		④	#DIV/0! ⑤
						#DIV/0!
						#DIV/0!
						#DIV/0!
						#DIV/0!

Green Cells = Raw RFU values of Alexa Fluor® 488 and Alexa Fluor® 647 Calibration Standards supplied in the Ion Sphere Quality Control Kit
Red Cells = Raw RFU values measured in "Measure the templated unenriched sample"
Purple Cells = Raw RFU values measured for negative control in "Measure the templated unenriched sample"
Blue Cells= Template kit lot specific conversion factor

① Previously calculated instrument-specific Calibration Factor.
② Enter raw RFU values for the ISP-containing samples in the red cells.
③ Enter raw RFU values for negative control sample in the purple cells. Values will be the same for all samples measured at the same time.
④ Enter template kit- and lot-specific value in the blue cells. Go to **thermofisher.com/qubit-conversion-factors-guide**.
⑤ Automatically calculated Percent Templated ISPs value displayed in this column.

Acceptance criteria for unenriched ISPs

The optimal amount of library corresponds to the library dilution point that gives Percent Templated ISPs between 10–25%, or 10–30%, depending on the Ion templating and sequencing systems used (see the following tables).

Samples that fall within the recommended range generally produce the most data; however, samples that fall outside of the recommended range can still meet the throughput specifications on the Ion chips.

The recommended optimal range is not intended to be a pass/fail criteria. The range provides guidance for the quality of the sample.

Note: If the results are outside the desired Percent Templated ISPs range, then increase or decrease the library input appropriately. See the "Ion Sphere™ Assay troubleshooting table" on page 14 for more information.

Template preparation on the Ion OneTouch™ 2 System for the specified sequencing system.

Percent Templated ISPs			Description
Ion PGM™ System	**Ion Proton™ System**	**Ion S5™/Ion S5™ XL System**	
<10%	<10%	<10%	Sample contains an insufficient number of templated ISPs to achieve optimal loading density on the Ion Chip.
10–30%	10–25%	10–25%	Optimal amount of library.
>30%	>25%	>25%	Sample will yield multi-templated ISPs (mixed reads).

Template preparation on the Ion Chef™ System for the specified sequencing system.

Percent Templated ISPs			Description
Ion PGM™ System	**Ion Proton™ System**	**Ion S5™/Ion S5™ XL System**	
<10%	<10%	<10%	Sample contains an insufficient number of templated ISPs to achieve optimal loading density on the Ion Chip.
10–30%	10–30%	10–30%	Optimal amount of library.
>30%	>30%	>30%	Sample will yield multi-templated ISPs (mixed reads).

Ion Sphere™ Assay troubleshooting table

The following table provides troubleshooting information for unenriched ISPs tested with the Ion Sphere™ Assay on the Qubit™ 3.0 Fluorometer.

Qubit™ Fluorometer observation	Sequencing system observation	Possible cause	Recommended action
<10% Templated ISPs	• Lower loading • Lower % enriched • Lower key signal • Lower throughput	Too little library input into template preparation	• Increase library input to target 20–25% templated ISPs. *or* • Continue with sequencing; expect lower throughput.
>30% (Ion Chef™ or Ion PGM™ Systems), or >25% (Ion OneTouch™ 2 and Ion Proton™/Ion S5™ Systems) Templated ISPs, but <70%	• Increased number of filtered reads	Too much library input into template preparation	• Decrease library input to target 20–25% templated ISPs. *or* • Continue with sequencing; expect lower throughput.
>70% Templated ISPs	• Increased % primer dimer filtered reads • Lower throughput	Adapter dimer contaminating library, more likely in short amplicon, Ion AmpliSeq™ or miRNA libraries	• Check Agilent™ 2100 Bioanalyzer™ traces for adapter dimer peak (Amplicon library or Ion AmpliSeq™ library peak around 70 bp; miRNA library peak around 60bp). • Re-purify library using Agencourt™ AMPure™ XP Kit clean-up steps as outlined in the appropriate user guides.
	• Low loading • Low % enriched • Lower throughput • High % filtered reads	Ion OneTouch™ 2 or Ion Chef™ System underperformance	• Troubleshoot with Technical Support or a Field Application Scientist.

Customer and technical support

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- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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Revision history: Pub. No. MAN0016388

Revision	Date	Description
A.0	11 January 2017	New user guide

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