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Best Practices: Workflow QC

The world leader in serving science

Best Practices

Focus on General Ion Workflow and Importance of QC Checkpoints:

- Starting material
  - Quality and Quantity
- Library Construction
  - Post-Shear Profile
  - Final Library Profile
  - Quantification
- Templated Bead Preparation
  - Qubit® → *Pre*-enrichment
- Sequencing
  - Run Reports

“Best Practices”, Ion Community

Check for updates!

PRODUCT OVERVIEWS AND BEST PRACTICES (ION PGM SEQUENCER AND TORRENT SUITE)

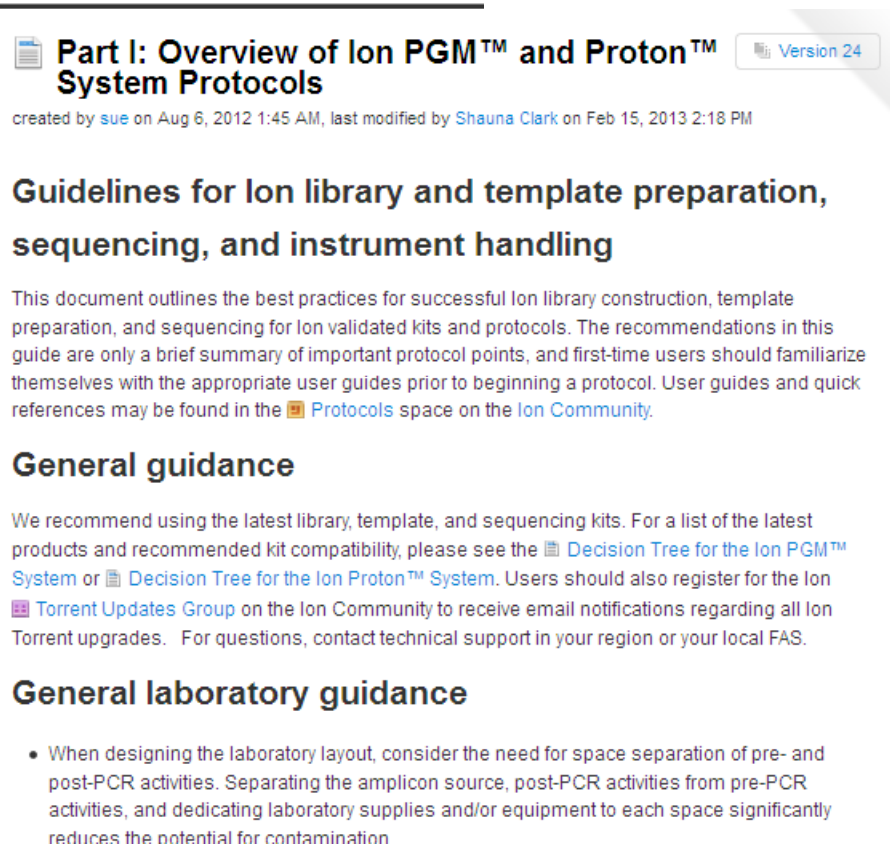
Part I: Overview of Ion PGM™ System Protocols
Guidelines for library and template preparation, sequencing, a

Part II: Overview of Torrent Sequencing and Alignment (v2.
Sequence generation and alignment for Ion semiconductor da

Part III: Advanced Topics in Filtering of Ion Sequencing Da
Options to reanalyze Ion sequencing data for improved data q

Part IV: Overview of Variant Calling in Ion Sequencing Data
Calling SNPs and indels in Ion semiconductor sequencing da

Part V: Advanced Topics in Variant Calling (v3.4.x)
Optimal sample-specific performance with the Torrent Variant



Part I: Overview of Ion PGM™ and Proton™ System Protocols Version 24

created by sue on Aug 6, 2012 1:45 AM, last modified by Shauna Clark on Feb 15, 2013 2:18 PM

Guidelines for Ion library and template preparation, sequencing, and instrument handling

This document outlines the best practices for successful Ion library construction, template preparation, and sequencing for Ion validated kits and protocols. The recommendations in this guide are only a brief summary of important protocol points, and first-time users should familiarize themselves with the appropriate user guides prior to beginning a protocol. User guides and quick references may be found in the Protocols space on the Ion Community.

General guidance

We recommend using the latest library, template, and sequencing kits. For a list of the latest products and recommended kit compatibility, please see the Decision Tree for the Ion PGM™ System or Decision Tree for the Ion Proton™ System. Users should also register for the Ion Torrent Updates Group on the Ion Community to receive email notifications regarding all Ion Torrent upgrades. For questions, contact technical support in your region or your local FAS.

General laboratory guidance

- When designing the laboratory layout, consider the need for space separation of pre- and post-PCR activities. Separating the amplicon source, post-PCR activities from pre-PCR activities, and dedicating laboratory supplies and/or equipment to each space significantly reduces the potential for contamination

http://ioncommunity.lifetechnologies.com/community/products/torrent_suite/best_practices

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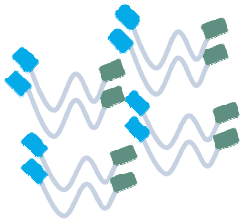
Ion Workflow Overview

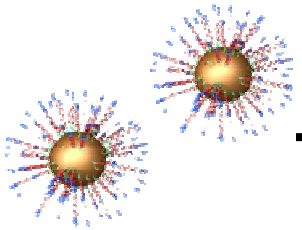
Starting Material
DNA/RNA

Library Construction → Templated Bead Preparation → Sequence + Run Evaluation → Data Analysis



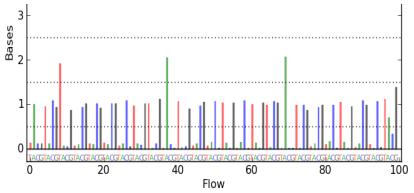
Prepare Library

Clonal Amplification



Isolate Positive Ion Sphere™ Particles

Load Chip and Sequence



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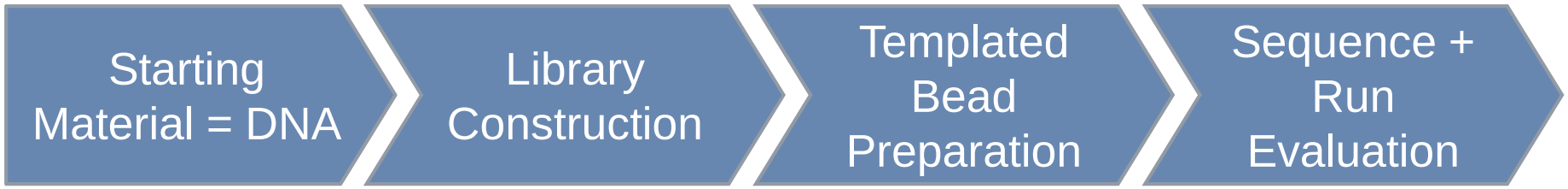
How QC Fits in Workflow

Reminder: All examples herein are for gDNA as starting material unless otherwise noted



Starting Material = DNA	Library Construction	Templated Bead Preparation	Sequence + Run Evaluation
❑Quantity ❑Quality	❑Post-Shearing Profile ❑Check size selection ❑Quantify library	❑Calculate TDF ❑Qubit® Assay: post-amplification, unenriched sample	❑Check Run Report

Last but not least….
Do not mix kit components and align with the most current protocol

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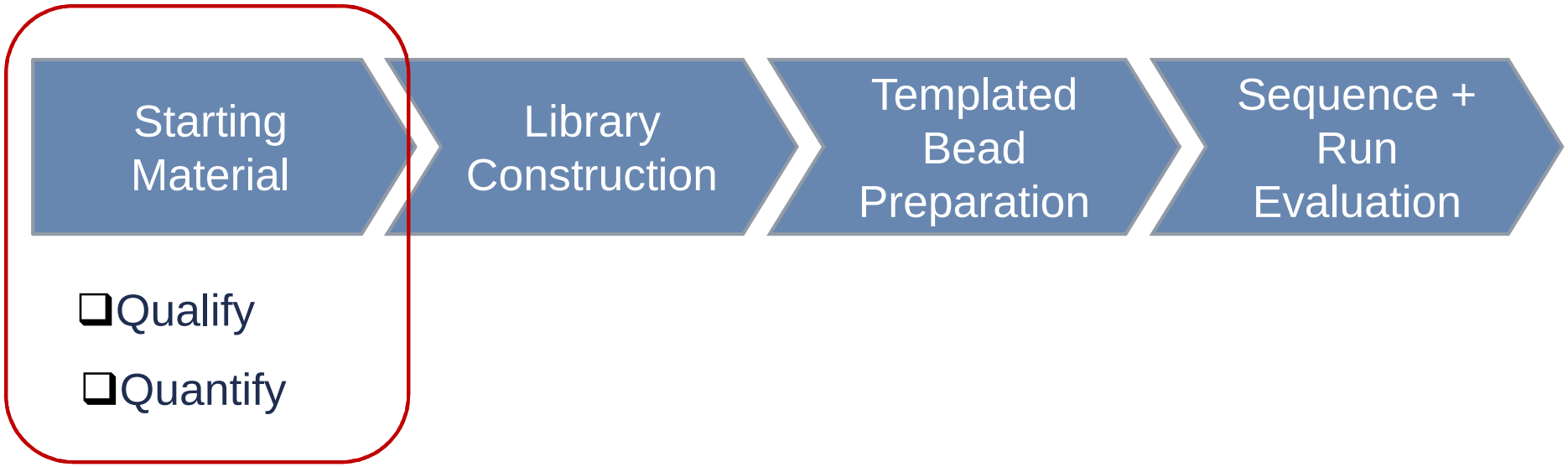
A Closer Examination



Starting Material: Qualify

- **Ion Xpress™ Plus gDNA Fragment Library Preparation**
- Begin with pure and intact starting material

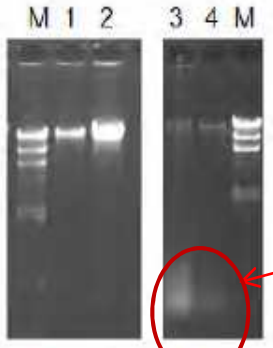
Appendix A. Evaluate the quality of the genomic DNA

Assess the integrity and size by gel electrophoresis

We recommend checking the integrity and size of your DNA preparation by gel electrophoresis. Use of a spectrophotometer to assess DNA quality can be misleading, because many molecules absorb in ultraviolet light.

Figure 2 Examples of genomic DNA preparations

Examples of high-quality DNA with no contaminating RNA (lanes 1 and 2), compared to lower quality samples containing RNA contamination (lanes 3 and 4). The RNA runs as a diffuse smear at the bottom of the gel. M is a lambda *Hind*III molecular weight marker.



RNA Contamination

If your DNA preparation shows RNA contamination, treat it with RNase I, as described in the following section.

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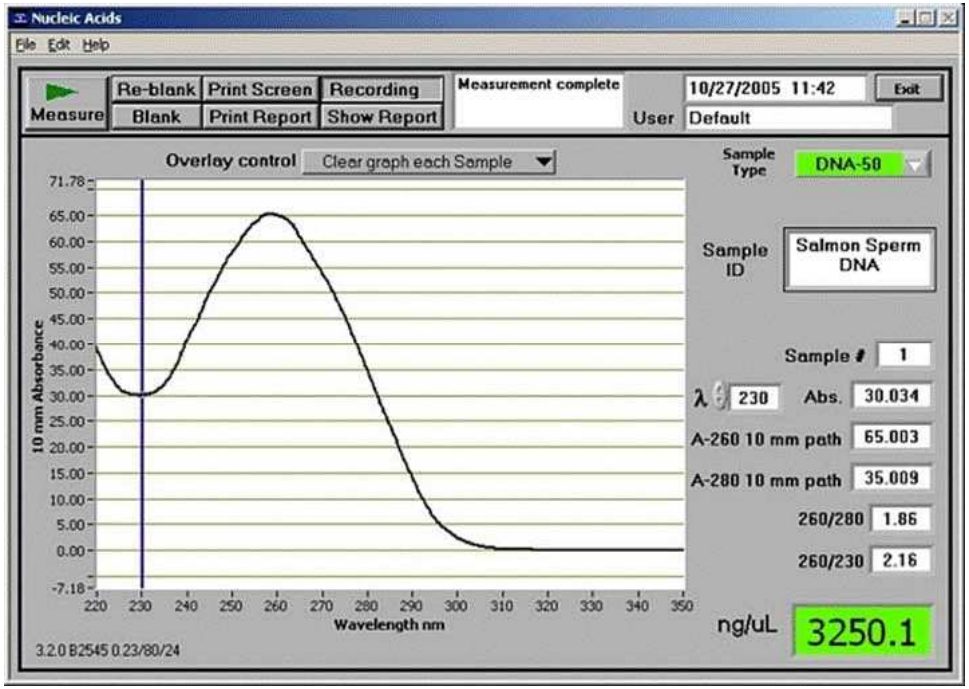
Starting Material: NanoDrop® Considerations

Example: optimal NanoDrop® curve



- **260/230 ratio** = Organic chemicals and solvent contamination
  Important to have this ratio above 1.6 for QPCR

- **260/280 ratio** = Protein Contamination
  Ratios between 1.8 – 2.0 are considered pure

DNA has no ethanol contamination, curve is normal shape with OD260 having higher absorbance than OD230

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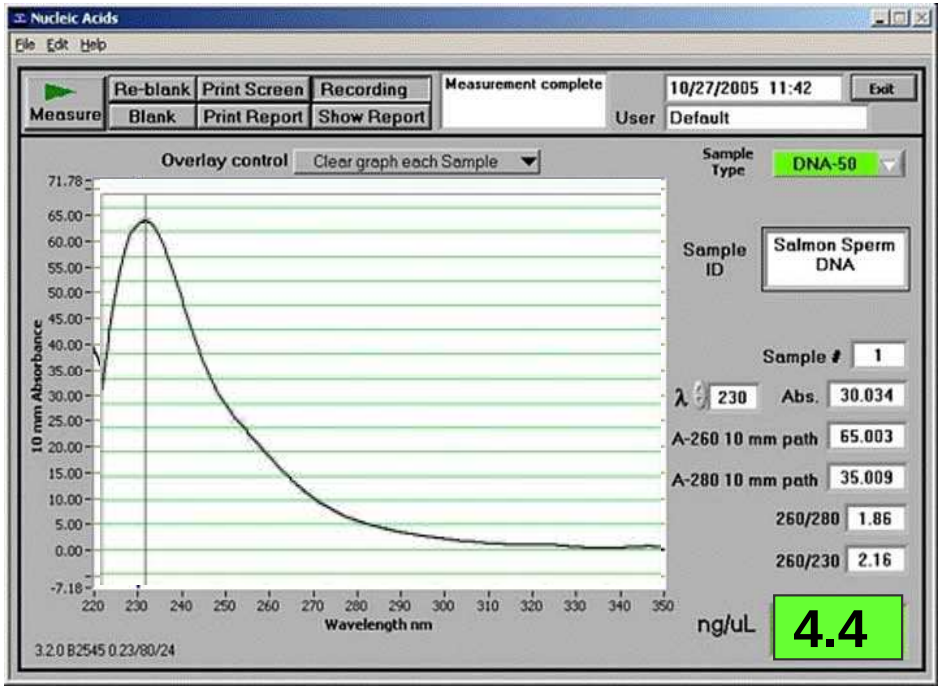
Starting Material: NanoDrop® Considerations

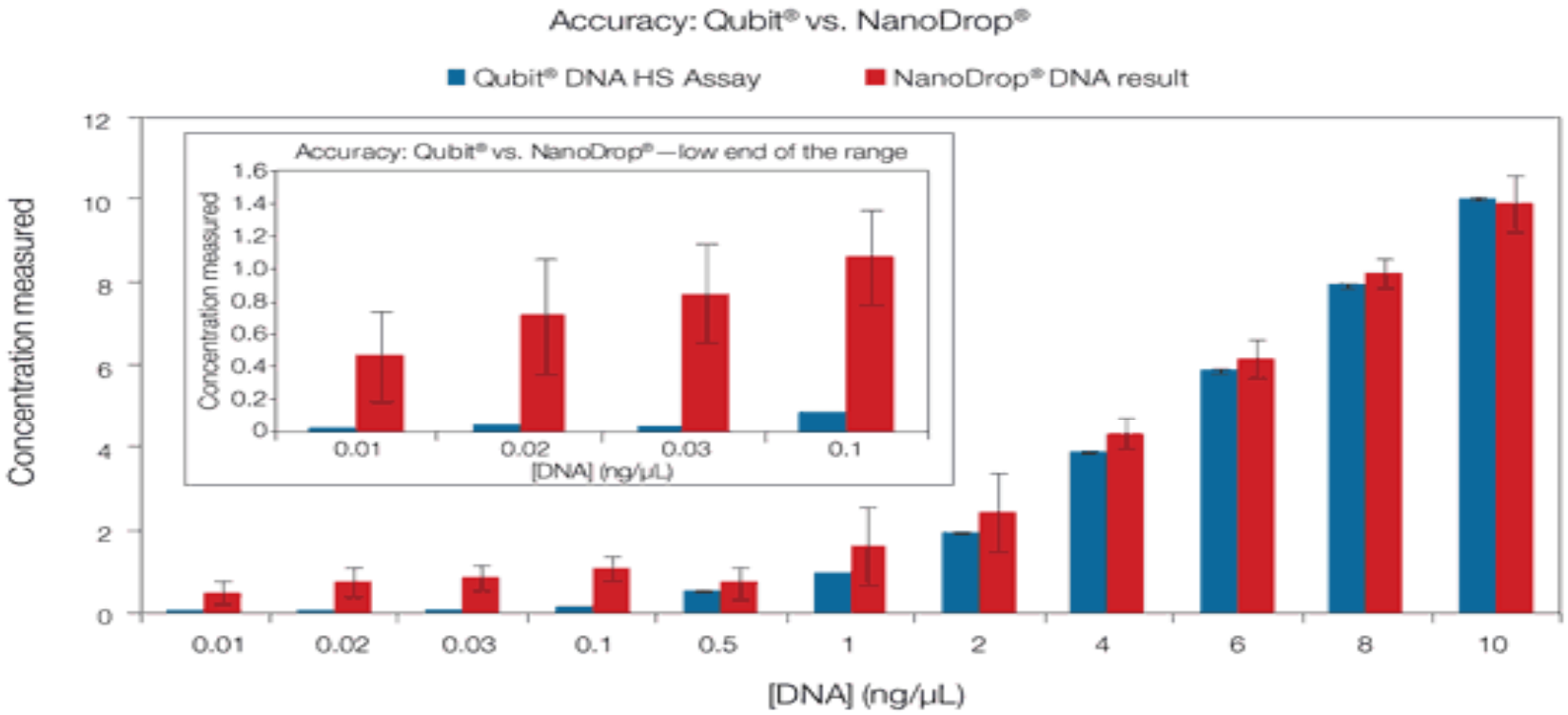
Example: Residual Ethanol



- OD 230 reading is much higher than OD 260

- This may result in inaccurate concentration readings and affect downstream enzymatic steps, especially when DNA concentration is low

Qubit® 2.0 Fluorometer vs. NanoDrop® Spectrophotometer



Qubit® Instrument: effective range 10pg/µL to 1µg/µL DNA

NanoDrop® Instrument : effective range 2ng/µL to 15µg/µL

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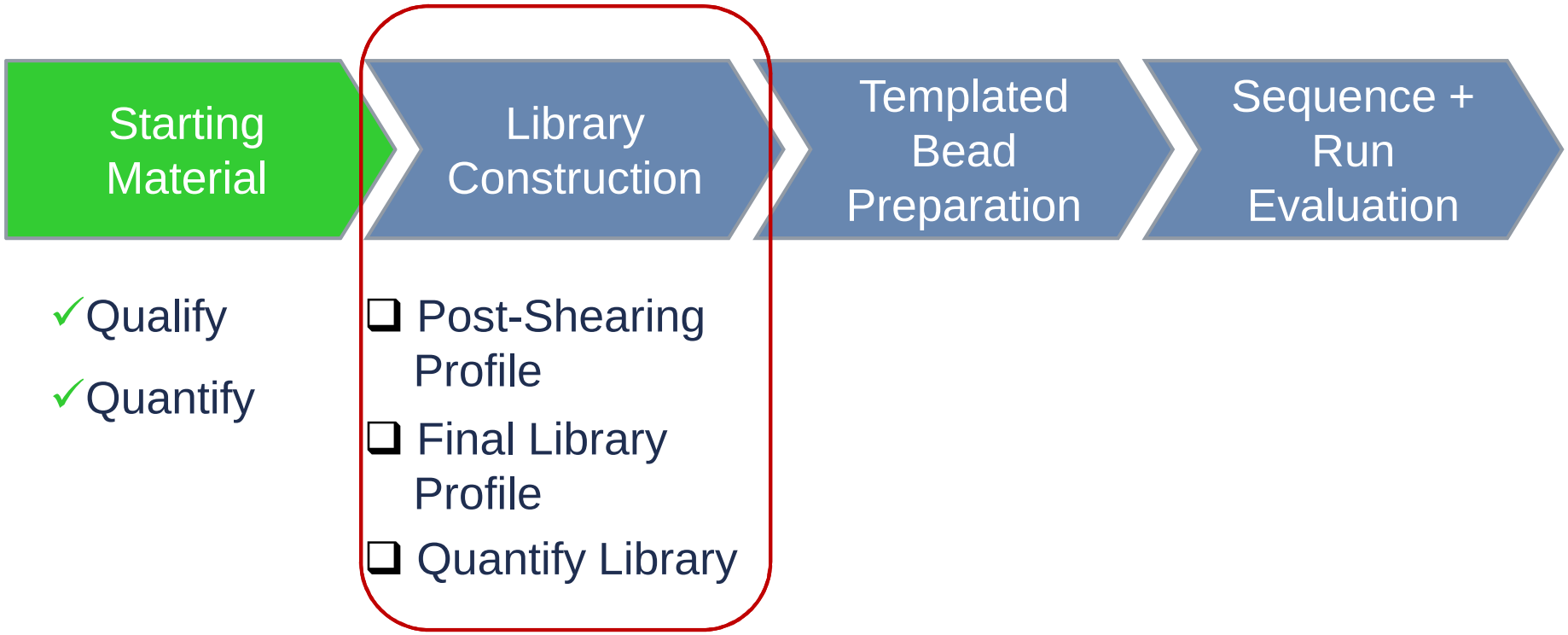
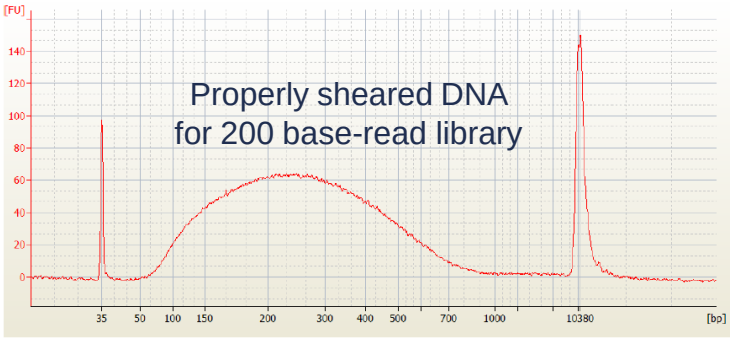
A Closer Examination

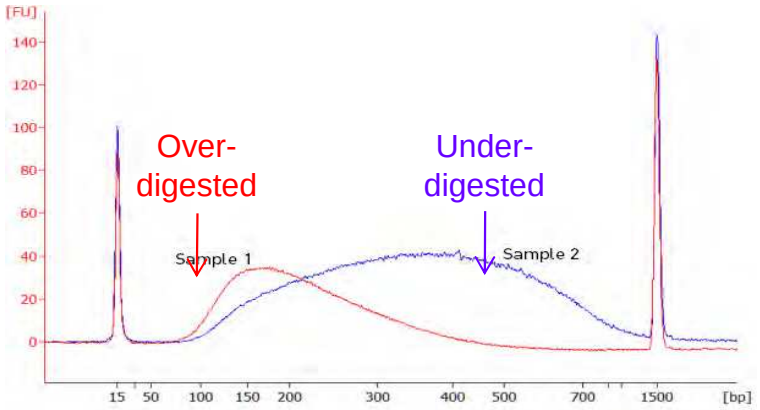


Post Shearing Profile

Shearing: Example of Ion Shear™ Plus Reagent digestion

- Applications (starting material) may drive profile of sheared DNA
- Shearing target size is read length dependent
- Shearing conditions may need to be optimized (e.g., incubation time and temp. with enzymatic methods)
- Input amount → expected recovery after size selection





Caution!

Library size	Median fragment size
200 base-read	200–300 bp
100 base-read	100–200 bp

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Final Library Profile

Analyze size distribution

What are we looking for?

- Does observed size match expected size?
- How much non-specific product exists relative to desired library?
  - Little or no remaining primer, primer-dimer, artifacts, by-products

- Options
  - Bioanalyzer® (strongly recommended) or equivalent instrument
    - Qualitative and quantitative measure of library
    - Define regions for proper sizing and quantitation
    - Important to follow maintenance recommendations
  - Gels
    - Requires more material
    - Gives a qualitative output of size distribution

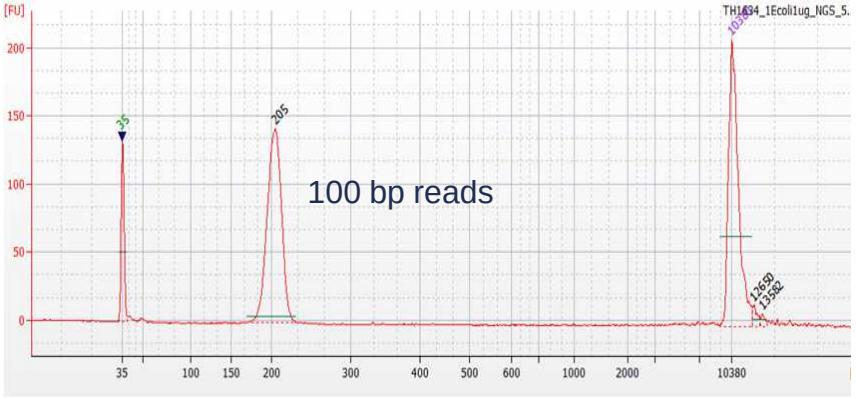
Final Library Profile: Bioanalyzer® Traces

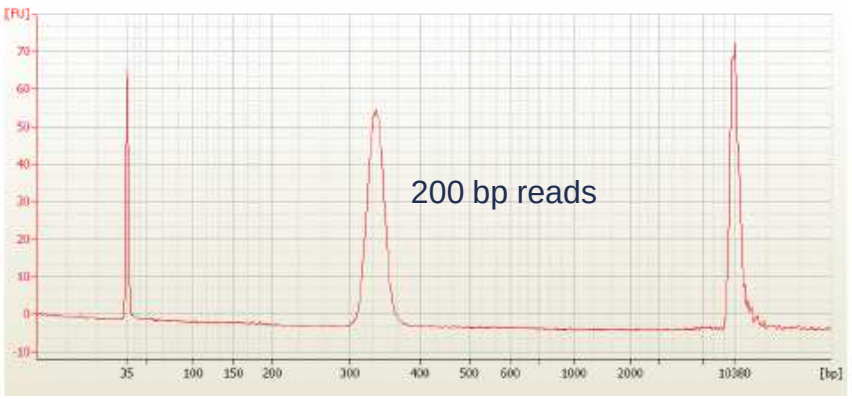
For optimal sequencing performance, libraries need to be appropriately sized before template preparation

- 100 bp template prep = ~130 bp insert, ~200 bp library
- 200 bp template prep = ~260 bp insert, ~330 bp library

Case Study 1

Fragment DNA libraries





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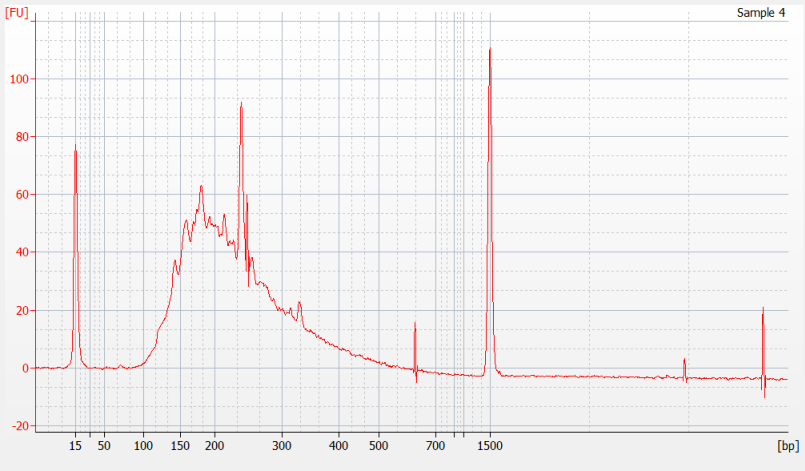
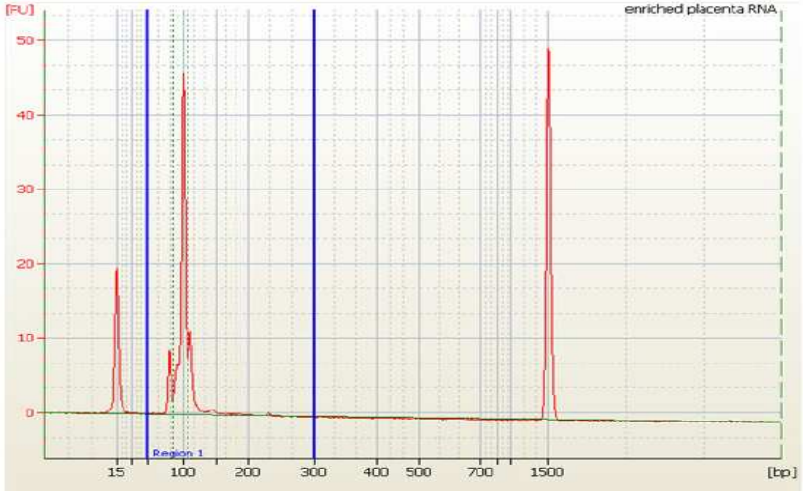
Final Library Profile: Bioanalyzer® Traces

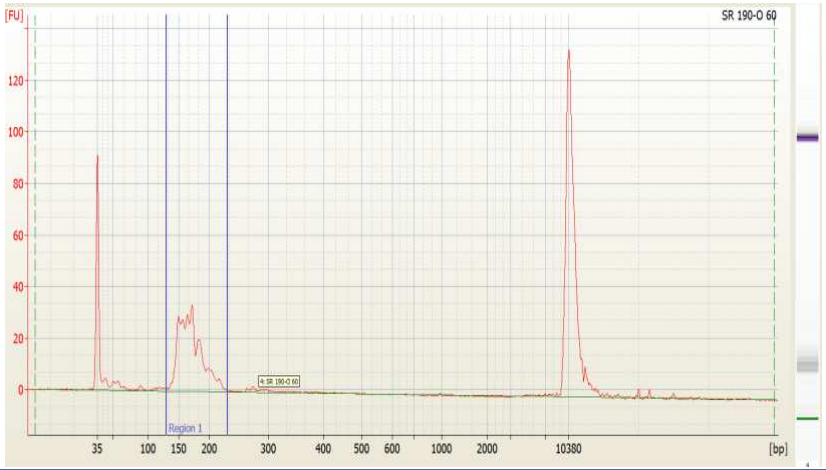
Whole transcriptome library (RNA)



smRNA library (RNA)



Ion AmpliSeq™ DNA library (Cancer Primer Pool)



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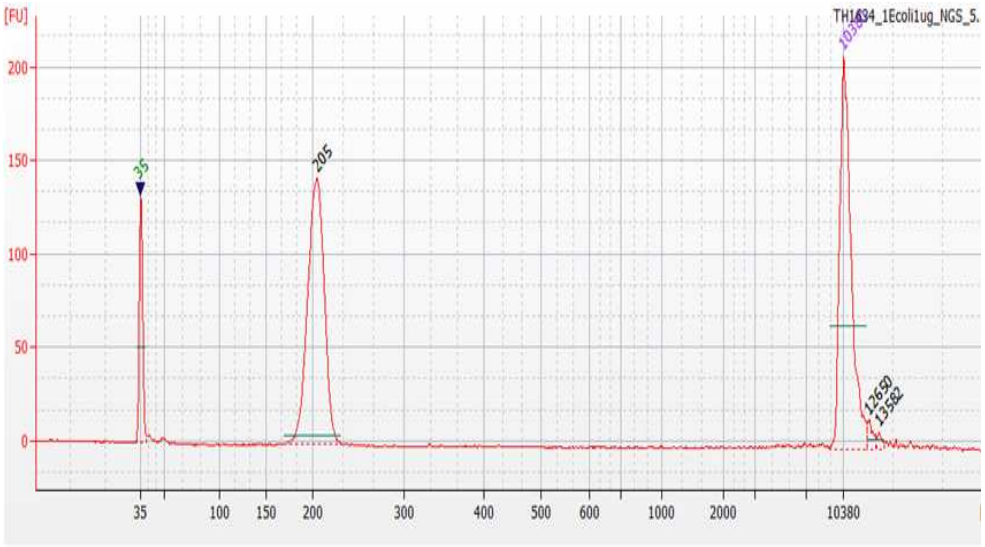
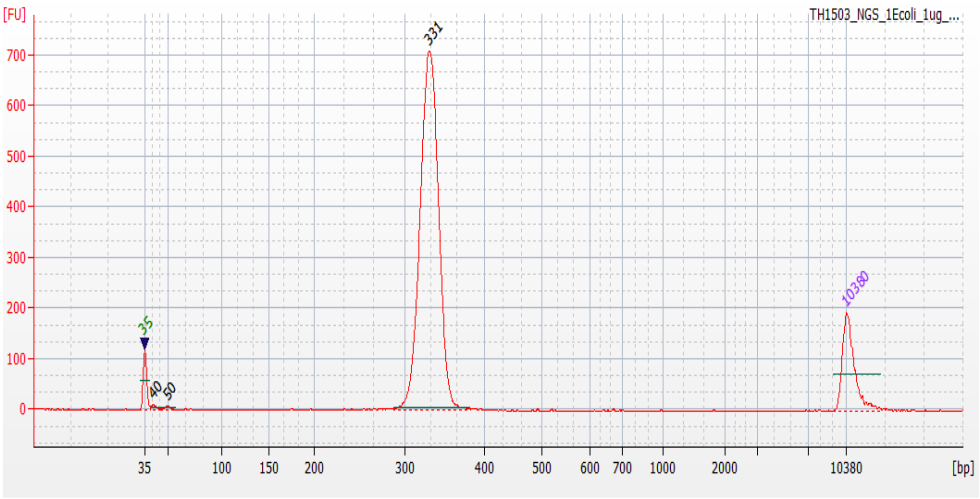
Final Library: Quantification prior to Template Preparation

- ***Quantification*** – critical to success of template prep
  - To properly determine the molar concentration and library yield, consider the following:
    - Precision and accuracy of the measurement system
    - Sensitivity and specificity of the detection method
    - Stay within the quantitative range of the assay
- Measurement systems
  - Real-time PCR
  - Bioanalyzer® analysis (also gives library size distribution)
  - Qubit® fluorometer
  - Nanodrop® Instrument– *Not recommended*
    - "total nucleic acid" is measured: Detects anything at A260 (DNA, RNA, primers, dNTPs, etc)

Final Library: Quantitative Range



- Quantitative range varies by assay
  - Note RFUs for marker, ladder, and sample peaks



- Overloaded

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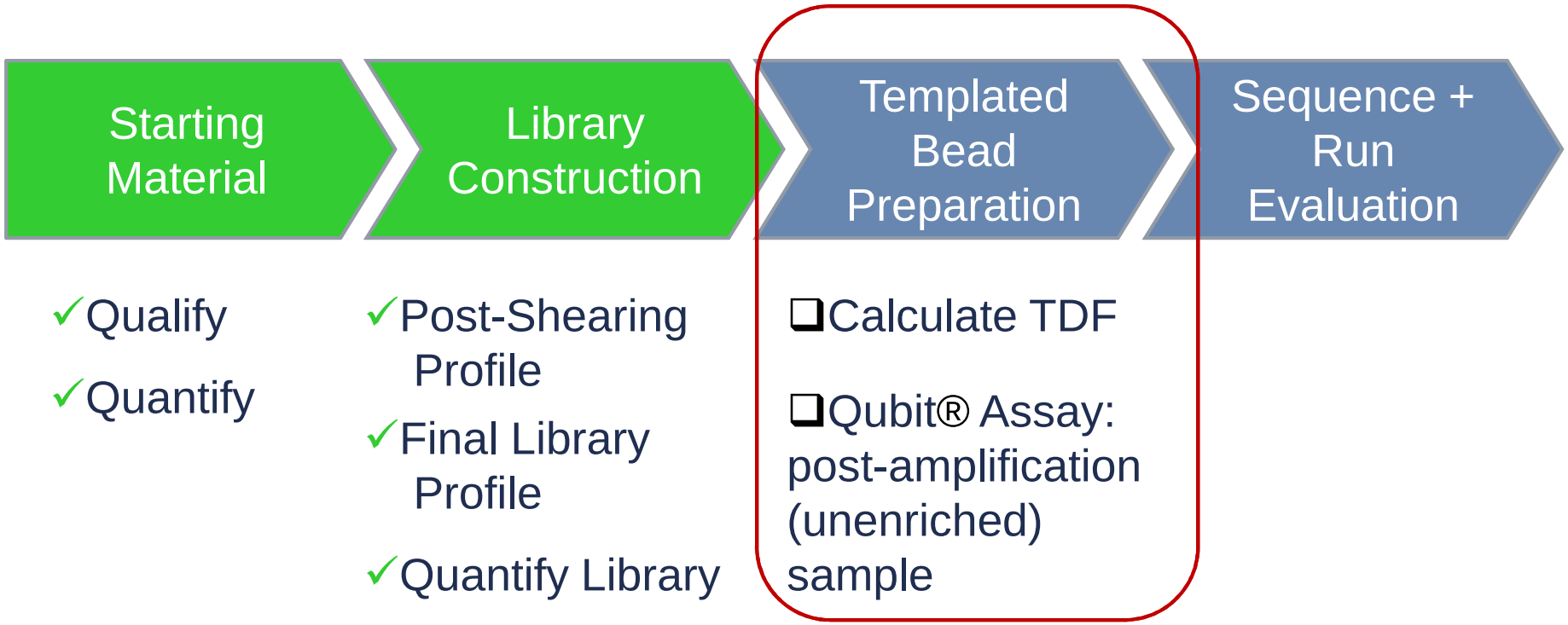
A Closer Examination



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Templated Bead Prep QC

- To maximize system throughput:
  - Properly qualify and quantify your library
  - Calculate appropriate template dilution factor for your library type and template prep kit
  - Run the Qubit® assay (Ion Sphere Quality Control Kit) after the amplification, prior to enrichment.
    - Save 2 uL of your sample and store at 4°C if you skip this step. You can choose to run the assay later, if needed.
    - Use the correct Conversion Factor for each kit lot, available on the Ion Community
  - Following guidance in the Template Prep User Guide, proceed to enrichment OR re-do step(s) as needed

Templated Bead Prep QC: Steps 1 & 2

✓1. Properly qualify and quantify your library

2. Calculate appropriate template dilution factor for your library type and template prep kit

Case Study 2

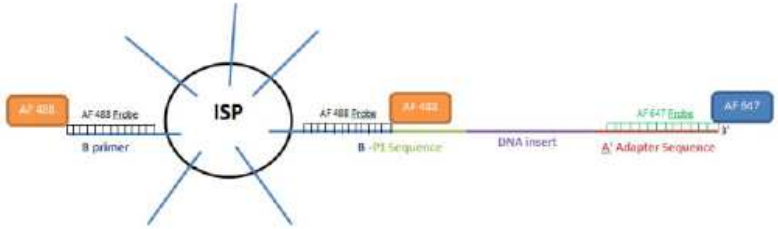
Library Dilution Guidelines:

Starting material	Input type	1x TDF
DNA	gDNA - whole genome	~26 pM
	amplicon – long	
	amplicon – short	
	Ion AmpliSeq v2.0	16-20 pM
	fusion PCR	~26 pM
RNA	WT: polyA-selected RNA	~32 pM
	WT: rRNA$^-$	
	small RNA	~32 pM

Note: Depending on version of Ion OneTouch™ kit, volume of dilution added to aqueous phase varies

Templated Bead Prep QC: Step 3

3. Run the Qubit® assay (Ion Sphere™ Quality Control Kit) after the template amplification, prior to enrichment



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
Alexa Fluor® 647 Calibration Standard	10266	

Previously calculated calibration factor. Different for each Qubit® 2.0 Fluorometer.

Enter raw RFU values for negative control sample, here. Values will be the same for all samples measured at the same time.

Percent Templated ISPs

Enter raw RFU values for the ISPs containing samples, here.

Calibration Standard	Raw RFU Value		Background RFU (Negative Control Tube)			
Sample ID	Alexa Fluor® 488	Alexa Fluor® 647	Alexa Fluor® 488	Alexa Fluor® 647	Conversion Factor*	Percent Templated ISPs
						#DIV/0!
						#DIV/0!
						#DIV/0!
						#DIV/0!

* Conversion factor can be found on the Ion Community website (www.iontorrent.com/qubit_files/) and is Templated Kit LOT specific.

Automatically calculated Percent Template ISPs value.

Enter template kit LOT specific value, here.

Legend

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 Section 3: Measure the Ion Sphere™ Particles (ISPs) 200 sample.

Purple cells = Raw RFU values measured for negative control in Section 3: Measure the Ion Sphere™ Particles (ISPs) 200 sample.

Blue cells = Template kit LOT specific conversion factor.

Templated Bead Prep QC: Step 4

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

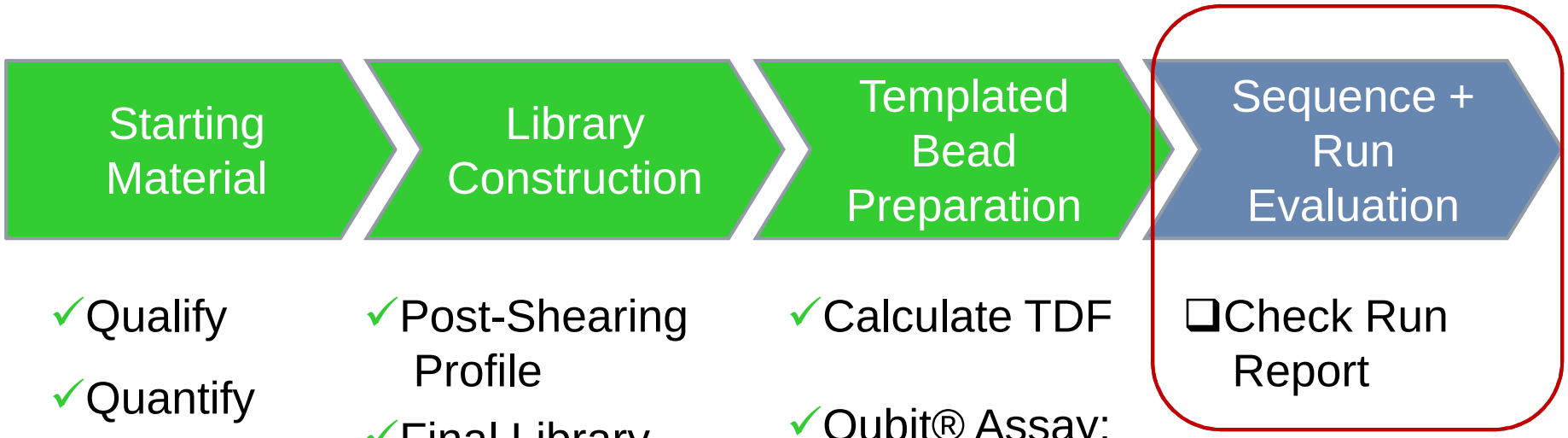
Note: If the results are outside the desired Percent Templated ISPs range, then increase or decrease the library input appropriately.

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

Templated Bead Prep QC Considerations

Sample	Qubit® 2.0 Fluorometer Phenotype	Ion Personal Genome Machine™ (PGM™) Possible Observation	Possible Root Cause	Recommended Action
Unenriched	<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% 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	• Low loading • Low % enriched • Lower throughput • High % filtered reads	Ion OneTouch™ Instrument underperformance	Troubleshoot with Life Technologies Technical Support or a Field Application Scientist
		• Increased % primer dimer filtered reads • Lower throughput	Adapter dimer contaminating library, more likely in short amplicon, AmpliSeq™ or miRNA libraries	• Check 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

A Closer Examination



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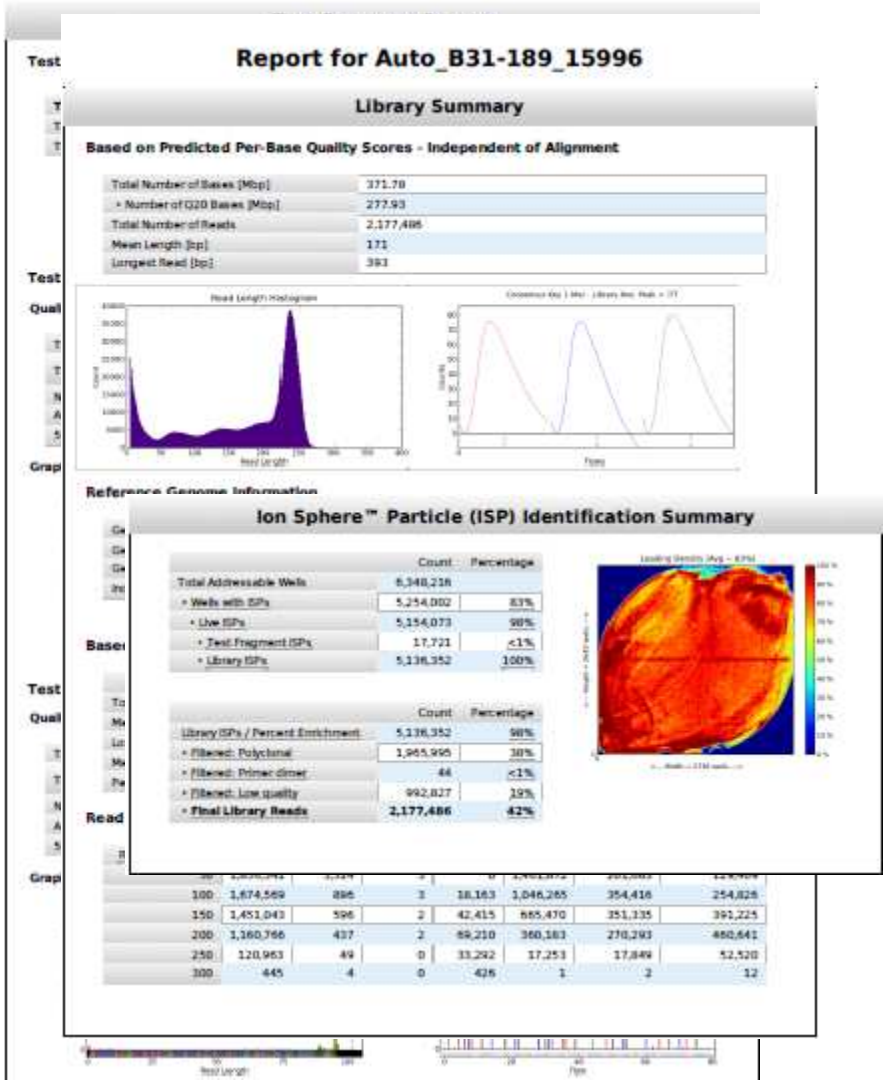
Check Run Report



- ❑ Library Summary
- ❑ Test Fragments
- ❑ ISP Summary
- ❑ Software Version

The Run Report is a .pdf document that is *included* in the Customer Support Archive (CSA) file

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Run Report: Library

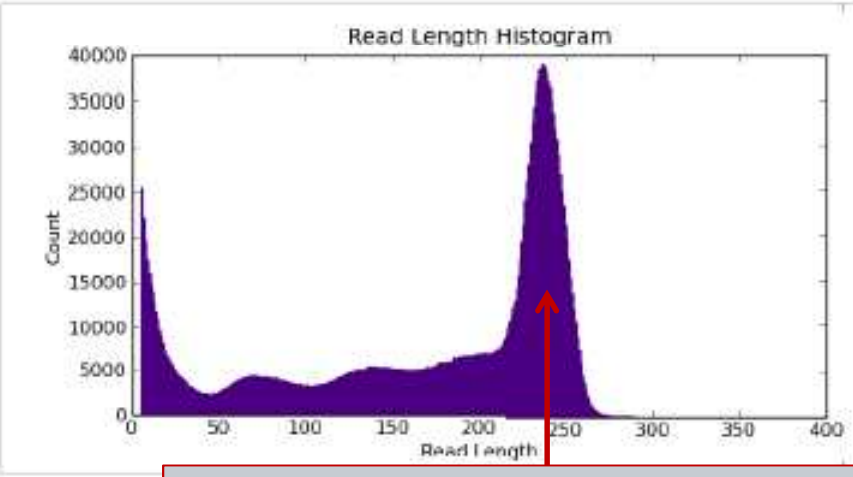
Library Summary

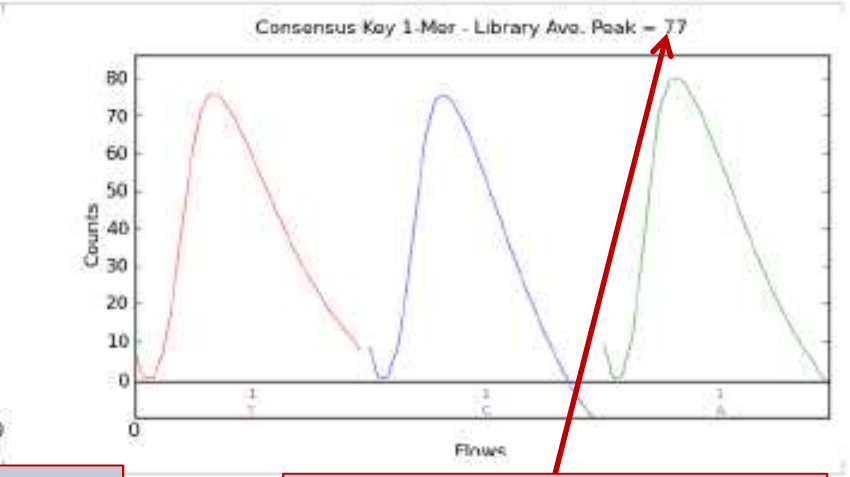
Based on Predicted Per-Base Quality Scores - Independ

Total Number of Bases [Mbp]	371.78
▸ Number of Q20 Bases [Mbp]	277.93
Total Number of Reads	2,177,486
Mean Length [bp]	171
Longest Read [bp]	393

Is Q20 value within expected range?

Do the number of reads look reasonable for your chip?





Does read length histogram show distribution centered at the correct size for your library?

How high is the key signal for library?

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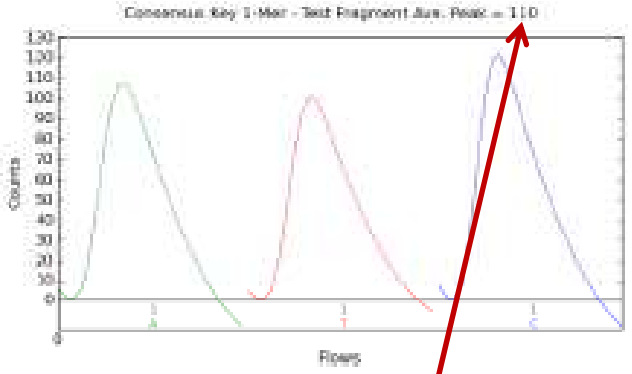
Run Report: Test Fragments

Test Fragment Report

Test Fragment Summary

Test Fragment	Percent (50AQ17 / Num)
TF_A	87%
TF_D	96%



What % of TFs are at least 50 bases with AQ17 (no more than 1 error after base 50)?

How high is key signal for TFs?

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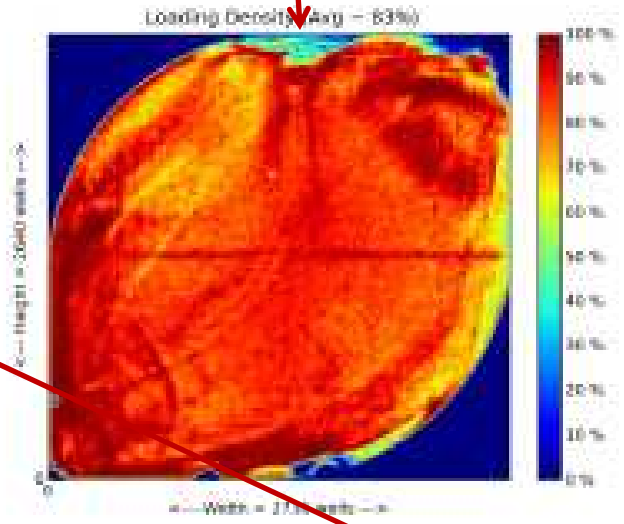
Run Report: ISP Summary

What is the loading %? Are the ISPs "live"?

Review loading density (heat map)

	Count	Percentage
Total Addressable Wells	6,348,216	
• Wells with ISPs	5,254,002	83%
• Live ISPs	5,154,073	98%
• Test Fragment ISPs	17,721	<1%
• Library ISPs	5,136,352	100%

	Count	Percentage
Library ISPs / Percent Enrichment	5,136,352	98%
• Filtered: Polyclonal	1,965,995	38%
• Filtered: Primer dimer	44	<1%
• Filtered: Low quality	992,827	19%
• **Final Library Reads**	**2,177,486**	**42%**



Are TFs present?

What % of reads pass the filters? Which filters rejected the reads?

What is the enrichment %?

Run Report: Software Version

Is the software version current?

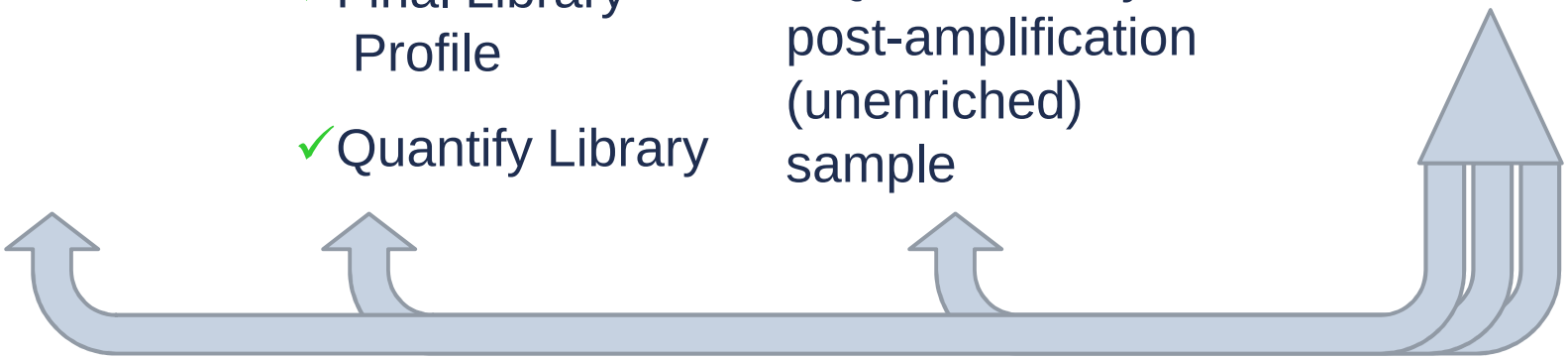
Software Version

Torrent_Suite	2.2
Datacollect	210
Graphics	18
LiveView	345
OS	19
Script	18.1.6
host	tahiti19
ion-alignment	2.2.4-1
ion-analysis	2.2.12-1
ion-gpu	1.2-1
ion-pipeline	2.2.12-1
ion-torrentR	2.2.8-1

- Different software versions produce different “expected” outputs
- Some software versions are not compatible with certain applications…make sure you are using the most recent version

Bringing it all together

Starting Material	Library Construction	Templated Bead Preparation	Sequence + Run Evaluation
✓Qualify ✓Quantify	✓Post-Shearing Profile ✓Final Library Profile ✓Quantify Library	✓Calculate TDF ✓Qubit® Assay: post-amplification (unenriched) sample	Run Report



PGM™ System Troubleshooting: Case Study I

✓Post-Shearing Profile

✓Final Library Profile

❑ **Quantify Library**

STOP!

Qubit®, qPCR, Bioanalyzer®:
Always *inspect results*

Example: Bioanalyzer® → ladder and markers are integral to accurate sizing and quantification

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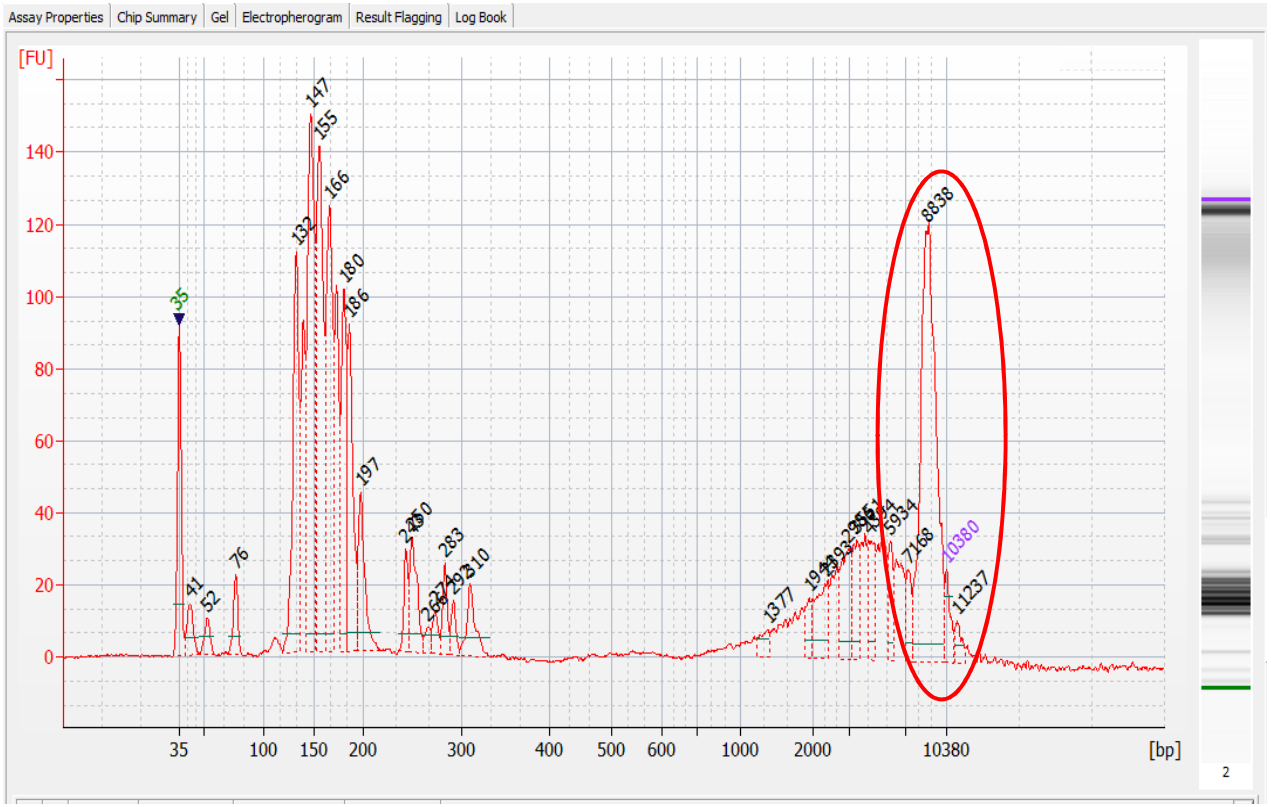
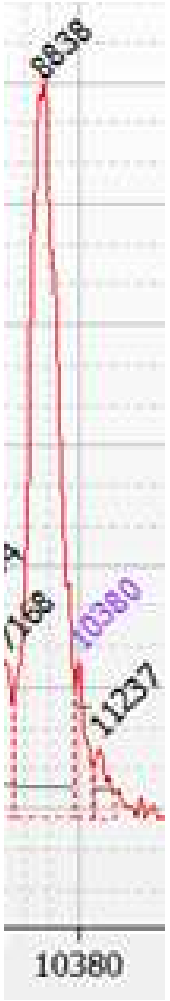
Bioanalyzer® Instrument: Importance of Ladder & Markers





Original [library] = 114.8 nM

After Correction: [library] = **8.2 nM** (14 fold difference)

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PGM™ System Troubleshooting: Case Study II

	Count	Percentage
Library ISPs / Percent Enrichment	162843	52%
▸ Filtered: Too short	678	<1%
▸ Filtered: Keypass failure	7905	5%
▸ Filtered: Mixed / Polyclonal	149905	92%
▸ Filtered: Poor Signal Profile	4264	3%
▸ **Final Library Reads**	**91**	**<1%**

Note: Screen captures are from Torrent Suite™ v.1.4 software.
Later versions of Torrent Suite™ software may look different

PGM™ System Troubleshooting: Case Study II

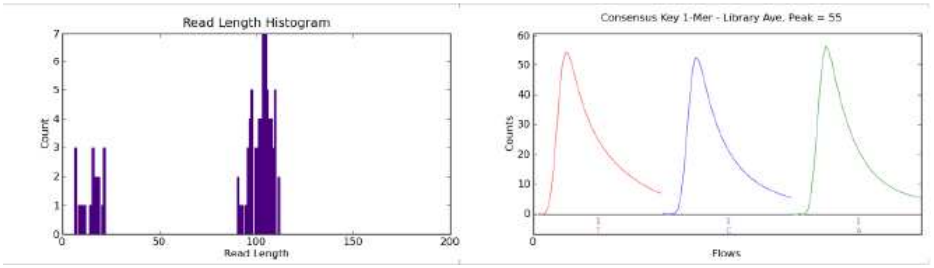
Library Summary

Based on Predicted Per-Base Quality Scores - Independent of Alignment

Total Number of Bases [Mbp]	0.01
▸ In Predicted Q17 Read Stretches [Mbp]	0.00
▸ In Predicted Q20 Read Stretches [Mbp]	0.00
Total Number of Reads	89
▸ At Least 50 bp Long	68
▸ At Least 100 bp Long	48
Mean Length [bp]	81
Longest Read [bp]	111

Based on Full Library Alignment to Provided Reference

	AQ17	AQ20
Total Number of Bases [Mbp]	0.00	0.00
Total Number of Reads	62	61
▸ At Least 50 bp Long	40	23
▸ At Least 100 bp Long	4	1
Mean Length [bp]	56	48
Longest Alignment [bp]	110	109
Mean Coverage Depth	0.00×	0.00×
Percentage of Library Covered	0%	0%



- Very low throughput: only 62 reads aligned
- Key signal is very good, both intensity and shape

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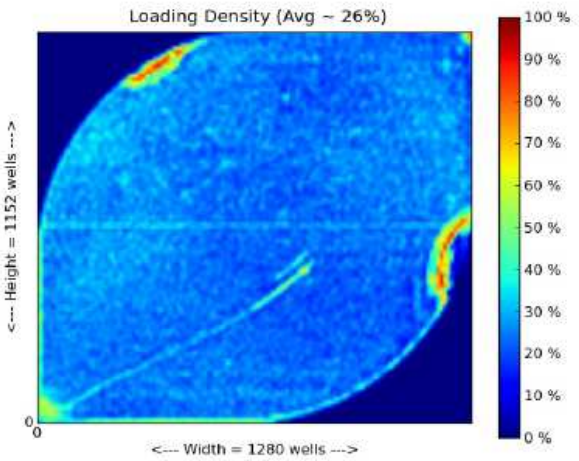
PGM™ System Troubleshooting: Case Study II

Ion Sphere™ Particle (ISP) Identification Summary

	Count	Percentage
Total Addressable Wells	1262519	
▸ Wells with ISPs	333985	26%
▸ Live ISPs	182178	55%
▸ Test Fragment ISPs	19335	11%
▸ Library ISPs	162843	89%

	Count	Percentage
Library ISPs / Percent Enrichment	162843	52%
▸ Filtered: Too short	678	<1%
▸ Filtered: Keypass failure	7905	5%
▸ Filtered: Mixed / Polyclonal	149905	92%
▸ Filtered: Poor Signal Profile	4264	3%
▸ **Final Library Reads**	**91**	**<1%**



- Low loading density
- Extremely high % of reads rejected by mixed filter → **Possible reasons?**

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PGM™ System Troubleshooting: Case Study II

Calculate TDF

Caution!

Be cognizant of your dilution scheme
(Follow Template Prep User Guide for guidance):

- Typically, high polyclonality indicates too much library was added into Templated Bead Preparation

- **In this example, undiluted stock library was added into the amplification mix**

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PGM™ System Troubleshooting: Case Study lll

- "Polyclonality happens"
  - Qubit % template-positive ISPs indirectly measures polyclonality
  - Higher %positive on Qubit means higher % polyclonal
  - Can change polyclonality by re-doing template prep with different amount of library

	Count	Percentage
Library ISPs / Percent Enrichment	630,569	84%
▸ Filtered: Polyclonal	173,663	28%
▸ Filtered: Primer dimer	75	<1%
▸ Filtered: Low quality	90,938	14%
▸ **Final Library Reads**	**365,893**	**58%**

	Count	Percentage
Library ISPs / Percent Enrichment	987,953	95%
▸ Filtered: Polyclonal	228,637	23%
▸ Filtered: Primer dimer	223	<1%
▸ Filtered: Low quality	77,111	8%
▸ **Final Library Reads**	**681,982**	**69%**

- Consider % final library reads

Remember…

- QC Checkpoints are there for a reason
- Each is integral to optimizing PGM™ performance

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