Library QC and Troubleshooting with the BioAnalyzer

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By the end of this webinar you should…

- Know how to use the BioAnalyzer as a tool for library QC
- Be able to identify features of an ideal final library trace
- Recognize possible issues
- Learn fixes and prevention for future preparations

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Troubleshooting Topics

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The Perfect Prep

What does an ideal trace look like?

[FU]
100
50
0

Y-axis: flourescence intensity

Lower marker

Flat baseline

Upper marker

15 150 300 500 1500 [bp]

X-axis: should be in base pairs [bp]

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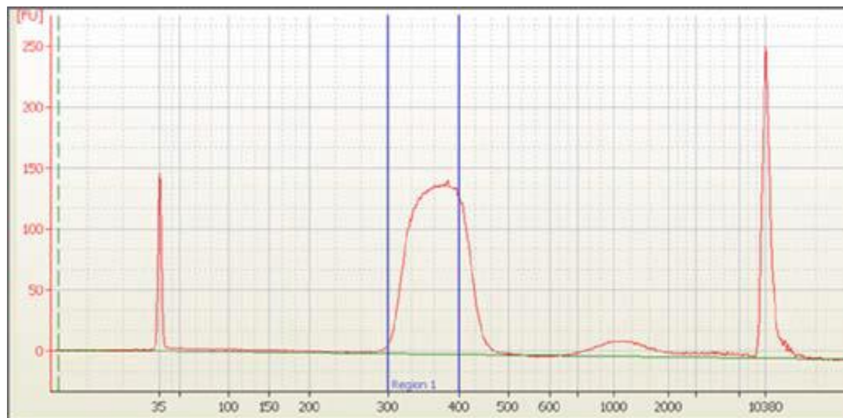
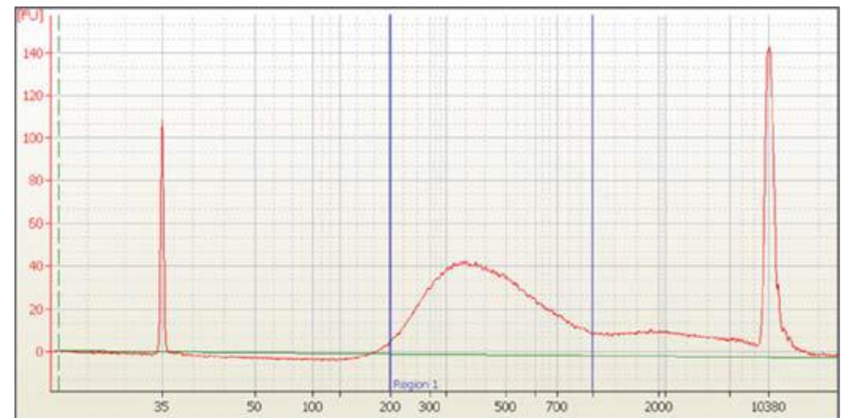
The Perfect Prep: TruSeq

What does an ideal library look like?

Gel Library on High Sensitivity DNA Chip



Gel-free Library on High Sensitivity DNA Chip



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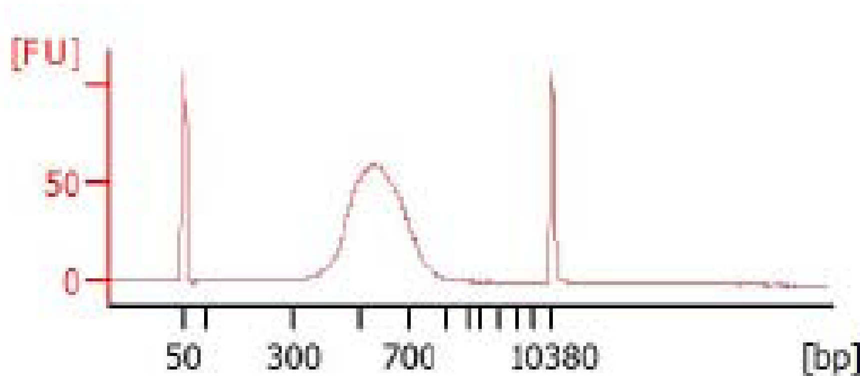
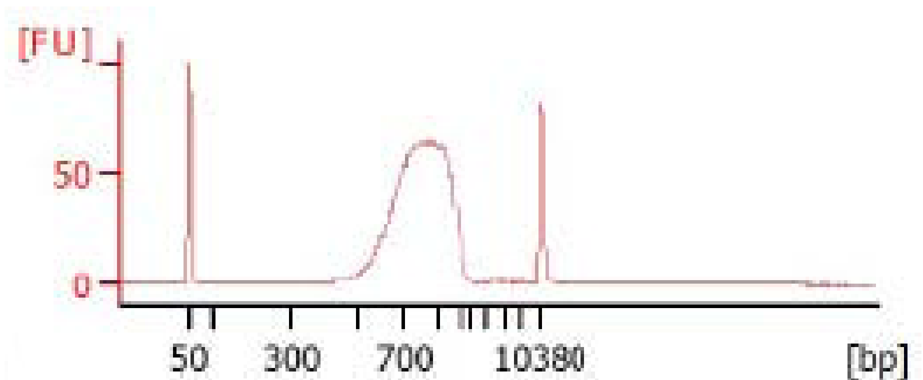
The Perfect Prep: TruSeq

What does an ideal library look like?

TruSeq Nano 350 bp insert



TruSeq Nano 550 bp insert



TruSeq Nano kits use beads for size selection. The final library will include the insert and ~120 bp (LT) or ~135 (HT) of adapters.

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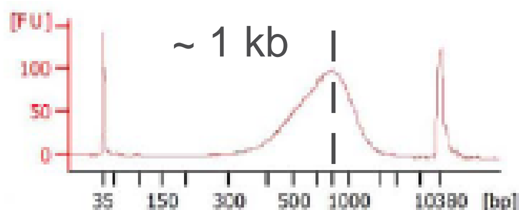
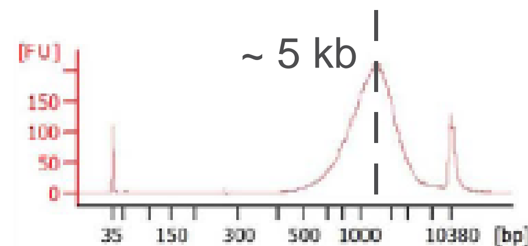
The Perfect Prep: TruSeq
What does an ideal library look like?

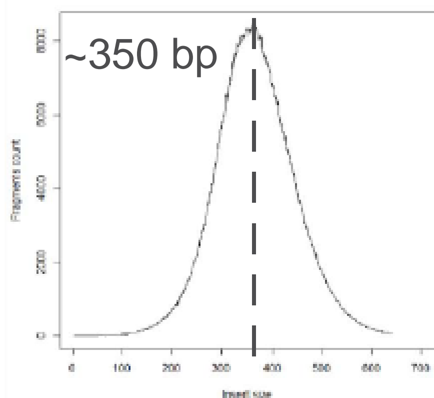
TruSeq PCR-Free 350 bp insert

TruSeq PCR-Free 550 bp insert

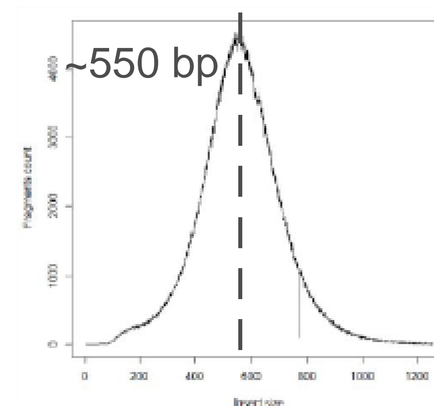


Final library
Bioanalyzer traces





Post-run analysis
average insert sizes



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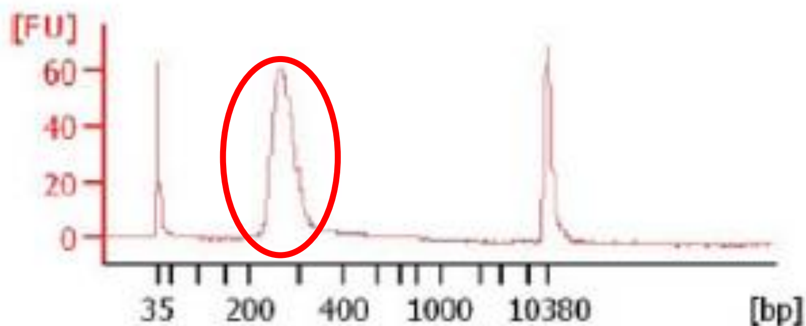
The Perfect Prep: TruSeq

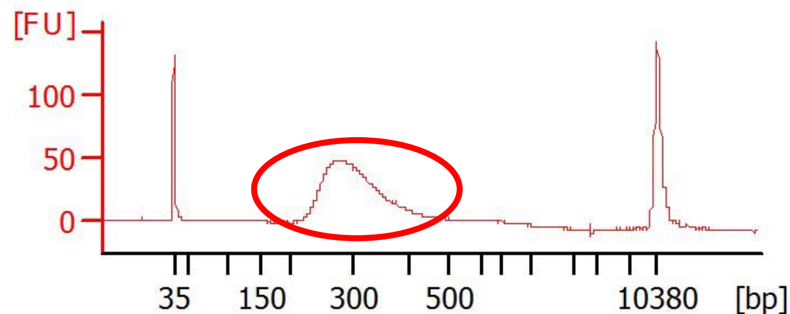
What does an ideal library look like?

TruSeq ChIP



Gel Size Selection

TruSeq RNA



Heat and Cations

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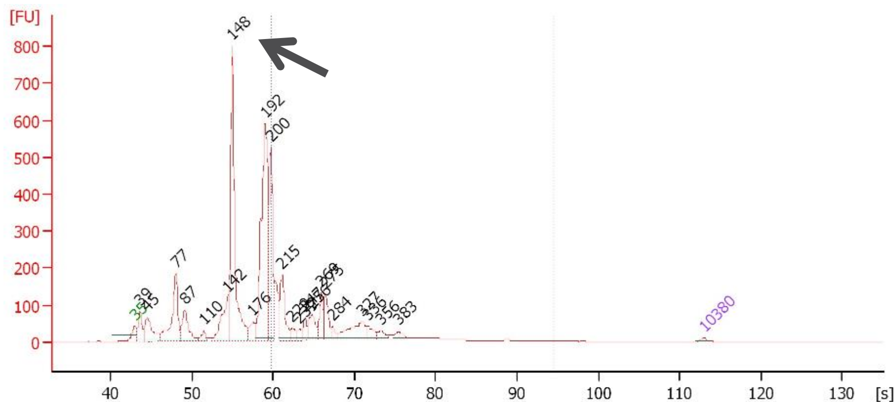
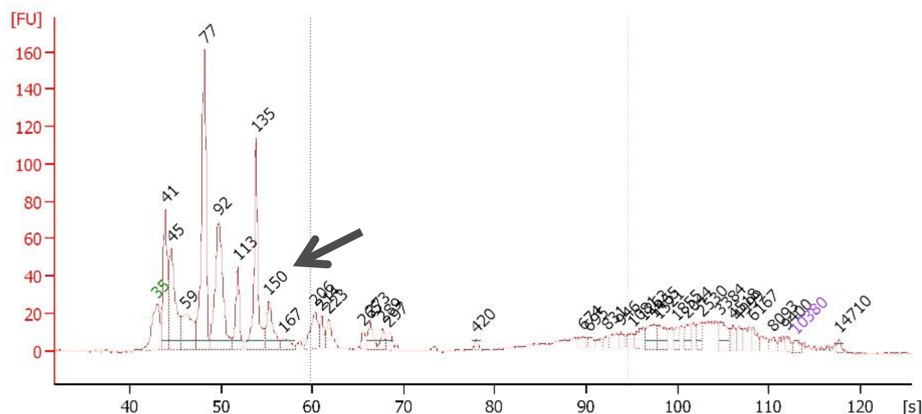
The Perfect Prep: TruSeq Small RNA
What does an ideal library look like?

Pre gel-selection:

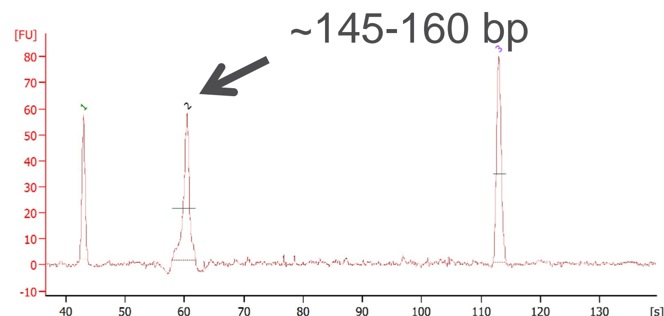
Look for peaks in the gel excision range: ~145-160bp





Final library

~145-160 bp



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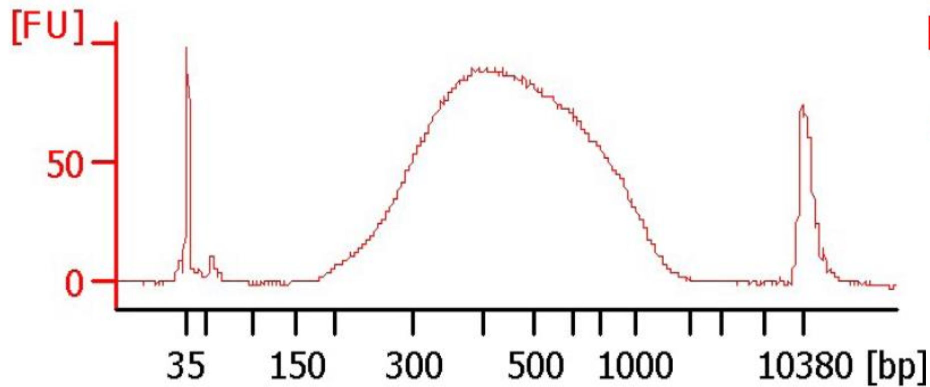
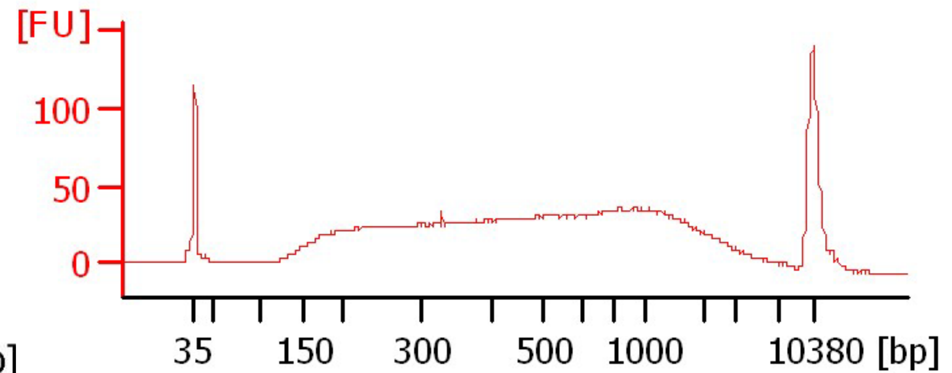
The Perfect Prep: Nextera

What does an ideal library look like?



Rounded peak



Flat topped

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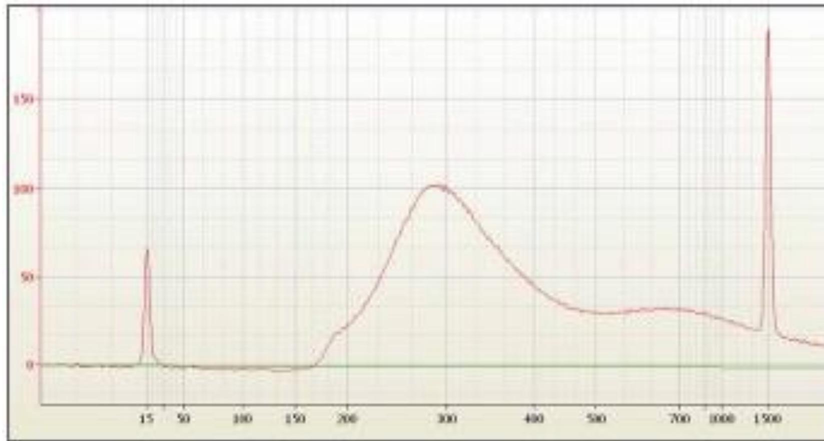
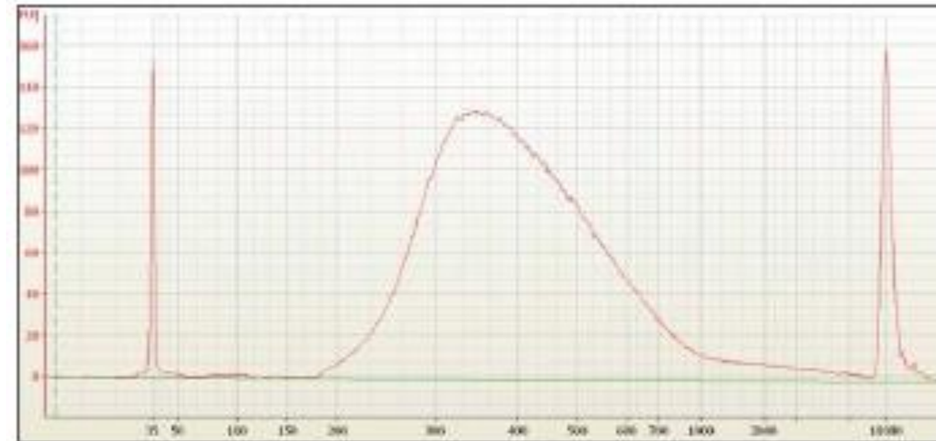
The Perfect Prep: Nextera Rapid Capture Enrichment and TruSight Rapid Capture Enrichment

What does an ideal library look like?



Pre-enrichment

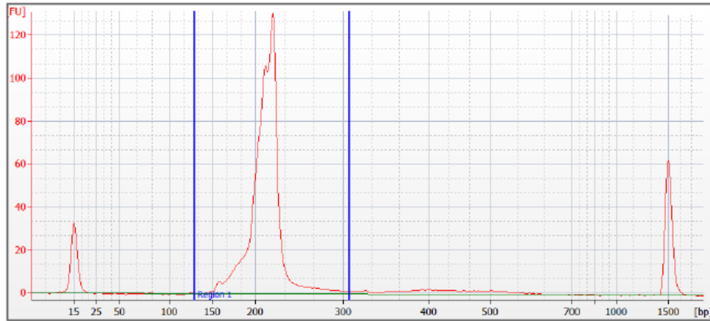


Post-enrichment

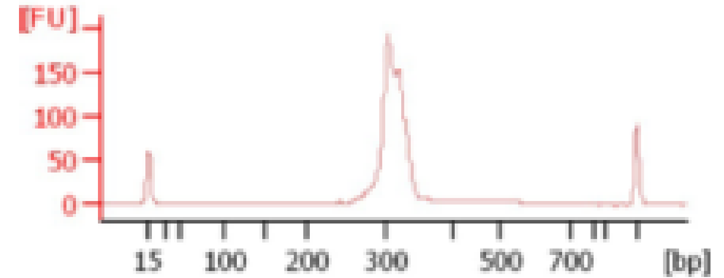
The Perfect Prep: Amplicon Kits

What does an ideal library look like?

TruSeq Targeted RNA Expression



TruSight Tumor



Amplicon based libraries will have a sharp peak at expected final library size.

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Troubleshooting with a Bioanalyzer

- Unless your sample peak is missing, the majority of cases discussed here can be sequenced.
- Missing Library Peaks will need to be re-prepped.

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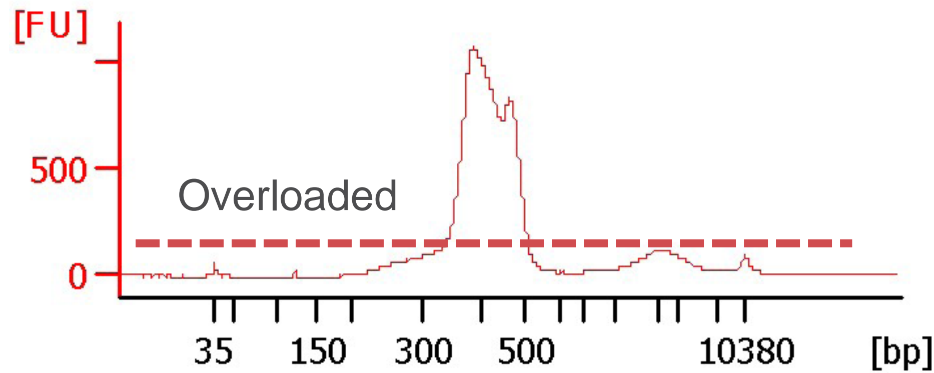
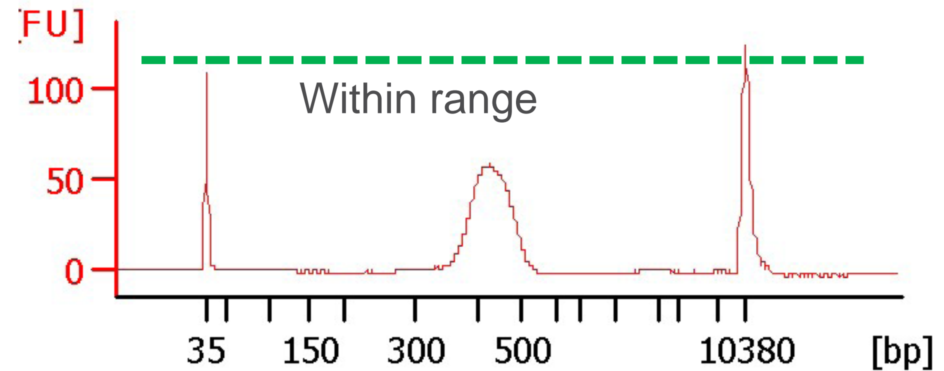
The Perfect Prep
The Wrong Size
Missing Library Peak
Additional Big Peaks
Additional Small Peaks

The Wrong Size

Overloaded Bioanalyzer Trace

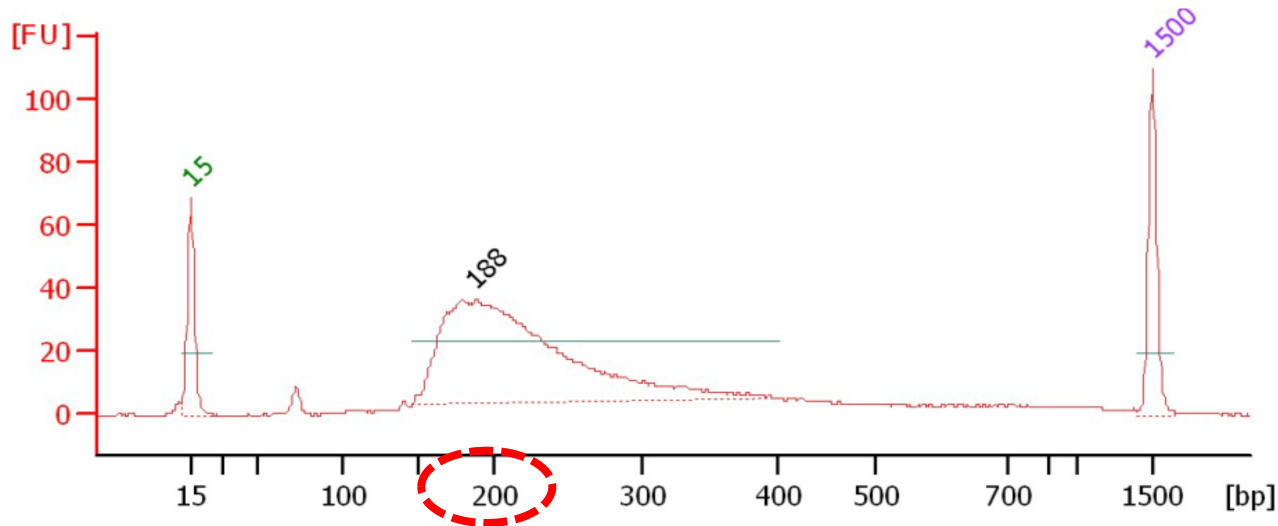




To Fix: Further dilute the samples and rerun the trace

The Wrong Size: Nextera

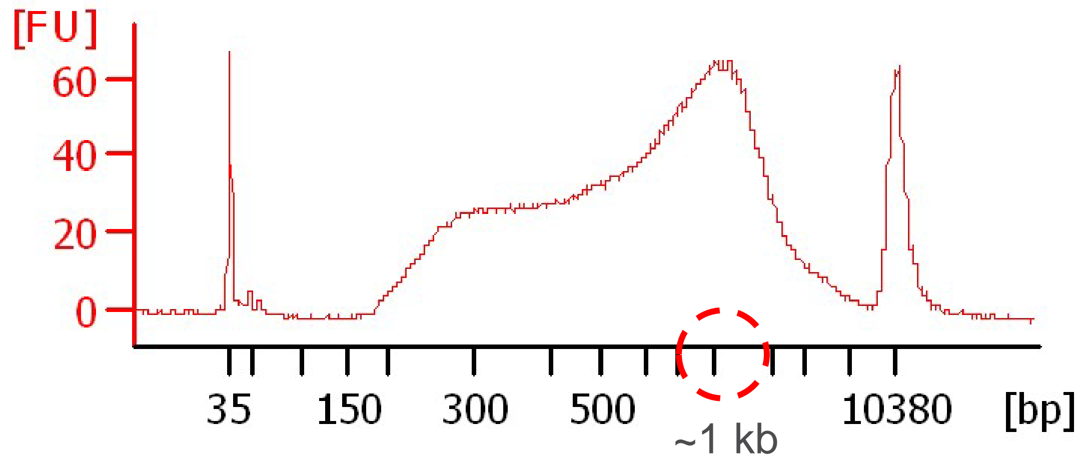
Library is too small



Possible Cause	Solution
Low quality starting material	QC staring material to ensure high quality DNA
Not enough starting material	Quantitate starting material by fluorescent dye
Tagmentation incubation is too long or too warm	Calibrate thermocycler and proceed to stop tagmentation step immediately

The Wrong Size: Nextera

Library is too large



Possible Cause	Solution
Too much starting material	Quantitate starting material by fluorescent dye
Tagmentation incubation too short or not warm enough	Calibrate thermocycler and incubate for the recommended time
Tagmentation enzyme is inhibited	Check DNA purification kit for inhibitors

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The Wrong Size: Nextera
Library is too large

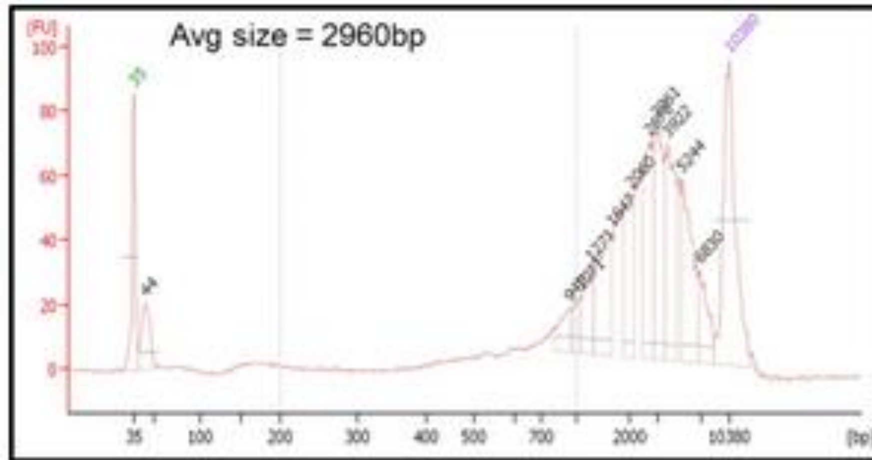
Despite differences in final bioanalyzer trace, Nextera libraries show little difference in post-run analysis

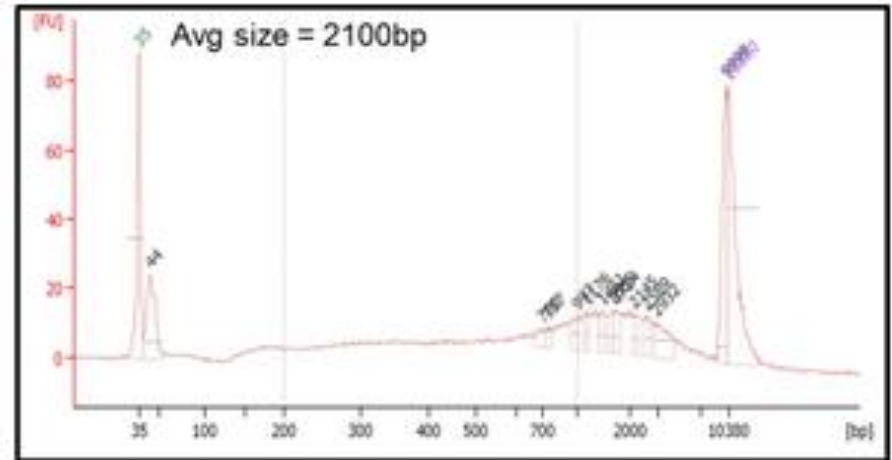
Use the bioanalyzer as validation of successful tagmentation of the sample.

An unexpected size range will likely not result in poor sequence data.

The Wrong Size: Nextera
Library is too large

Inhibited Tagmentation Enzyme





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The Wrong Size: TruSeq Nano and PCR-Free

Smaller Peak

Possible Cause	Solution
Low quality or insufficient starting material	QC high quality starting material with Fluorometric-based method
Sample lost during bead size selection	Follow best practices for bead handling

Larger Peak

Possible Cause	Solution
Too much input DNA	QC starting material with Fluorometric-based method
Supernatant discarded during removal of large fragments	Transfer the supernatant to the next step instead of discarding it

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The Wrong Size: TruSeq RNA v2, TruSeq Stranded mRNA, TruSeq Stranded Total RNA

Smaller Peak

Possible Cause	Solution
Low quality or insufficient starting material	QC starting material with Fluorometric-based method and bioanalyzer
Thermocycler is overheated	Calibrate thermocycler and set to the appropriate temperature
Elute, Prime, Frag incubation longer than 8 minutes	Repeat prep with 8 minute Elute, Prime, Frag incubation

Larger Peak

Possible Cause	Solution
Too much input RNA	Repeat prep with correct amount of input RNA
Thermocycler under heated	Calibrate thermocycler and set to the appropriate temperature
Elute, Prime, Frag incubation shorter than 8 minutes	Repeat prep with 8 minute Elute, Prime, Frag incubation

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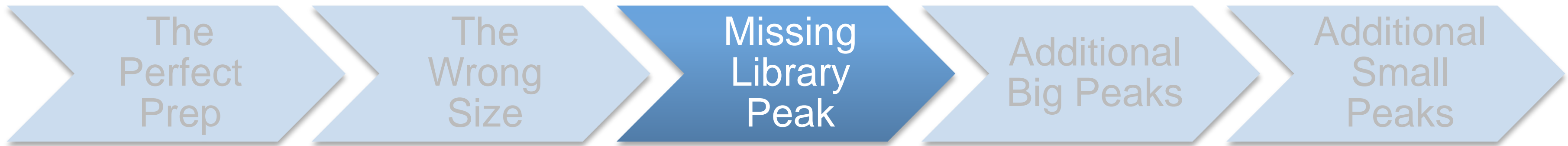

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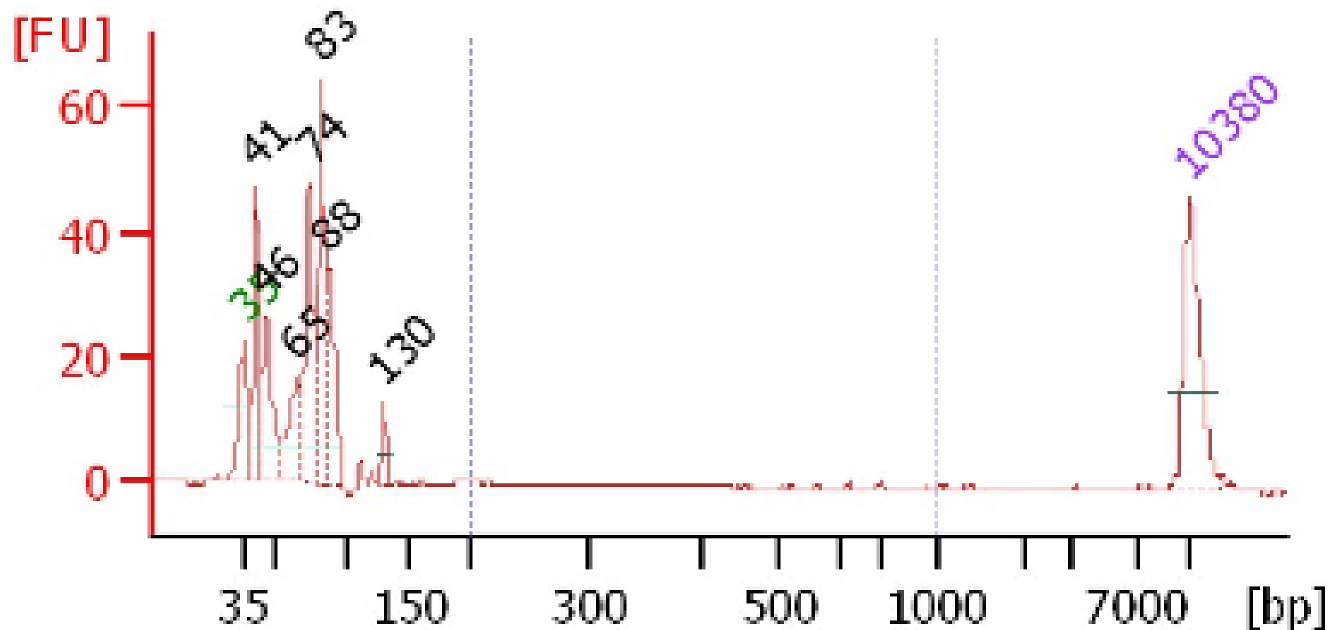
Missing Library Peak: TruSeq small RNA

Trace is missing the 145-160bp peaks for small RNA
Expected small RNA fraction was not captured by the kit



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Missing Library Peak: TruSeq small RNA

Trace is missing the 145-160bp peaks for small RNA
Expected small RNA fraction was not captured by the kit

Possible Cause	Solution
Low quality starting material	QC starting material to ensure high quality RNA
Secondary structure formation in the adapter oligos	Ensure 70˚C incubation moves *immediately* to ice
Small RNAs not preserved in initial sample preparation	RNA isolation kit should be specific for small RNAs

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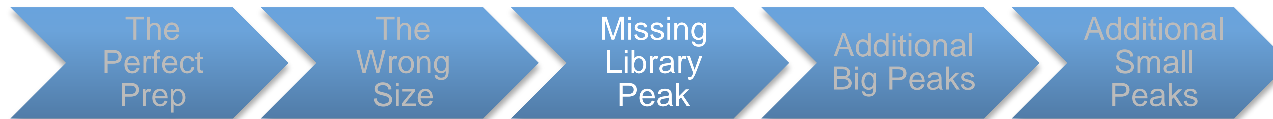
Missing Library Peak: TruSeq small RNA

Expected Peaks

Peak Size	Product
144bp – 150bp	Primary product
150bp – 155bp	Secondary product

Unexpected Peaks

Peak Size	Product
≤ 100bp	Primer or primer dimer
120bp – 125bp	Amplified adapter dimer
130bp – 138bp	Adapter concatamer
≥ 200bp	tRNA or rRNA with ligated adapters



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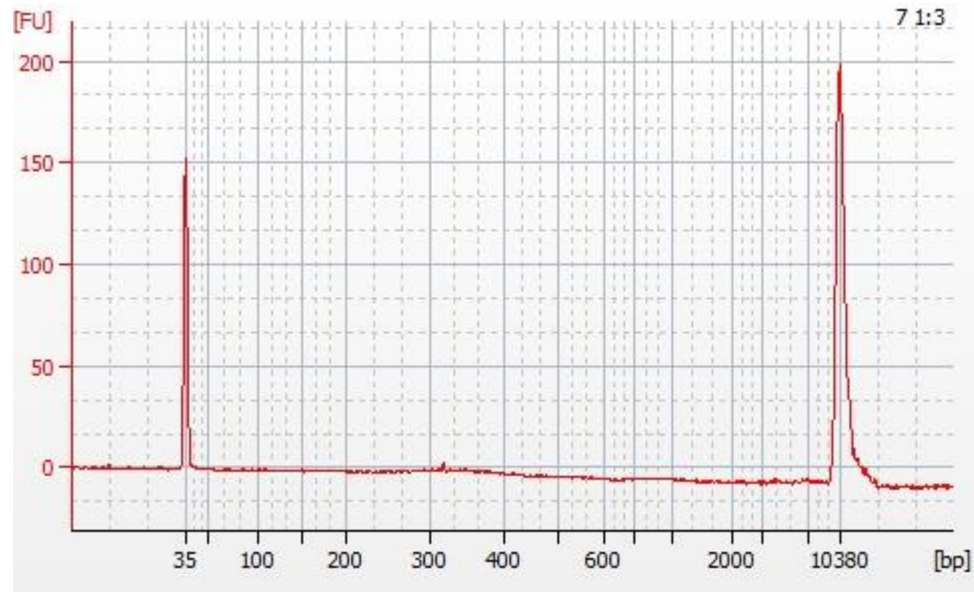
Missing Library Peak: TruSeq and Nextera

Final bioanalyzer trace shows no library peak or only dimer peaks. This library should not be sequenced.



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Missing Library Peak: TruSeq DNA or RNA

Possible Cause	Solution
Low quality or insufficient starting material	QC starting material with Picogreen or Qubit
Sample lost during bead clean up	Follow best practices for bead handling
Not using a heated lid on the thermocycler	The thermocycler lid should be heated to 100C for any incubation step above room temperature
Incomplete fragmentation	Check the size distribution after fragmentation
Poor enzyme quality	Make sure the enzyme is not expired and has not gone through multiple freeze thaws

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Missing Library Peak: Nextera

Possible Cause	Solution
Low quality or insufficient starting material	QC starting material with Picogreen or Qubit
Sample lost during bead clean up	Follow best practices for bead handling
Incorrect primers used during PCR	Make sure the indexed adapters that are used are for the correct kit
Poor enzyme quality	Make sure the enzyme is not expired and has not gone through multiple freeze thaws

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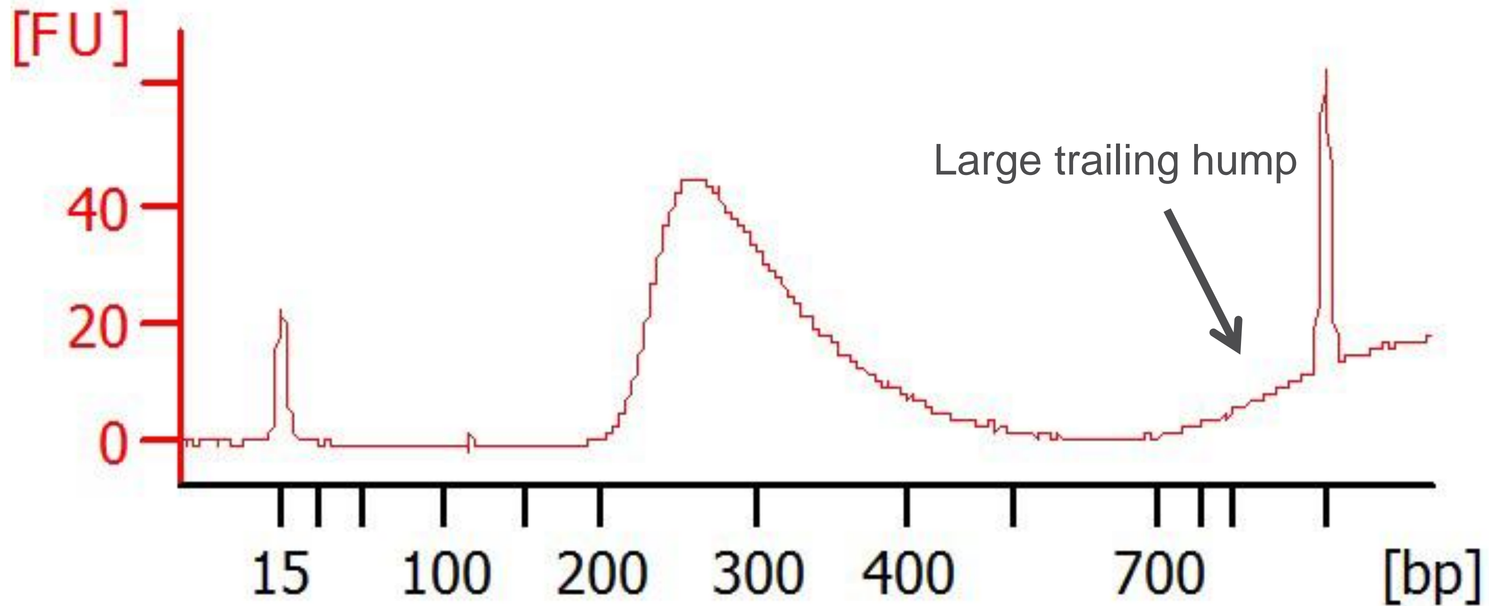
Additional Big Peaks
AMPure bead carryover



Possible Cause	Solution
Magnetic AMPure beads were carried over from clean up steps	Place samples on magnet and re-extract Use best practices when handling beads

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Additional Big Peaks

PCR artifacts – Bubble Products

Overamplified “bubble”

Primary product

Primary product

Secondary artifact peak

[FU] 60 40 20 0

35 150 300 500 10380 [bp]

Possible cause: too much input, too manny PCR products
To Prevent: decrease input, minimize number of PCR cycles
To Check: Re-heat sample to 95C for 5 minutes, allow to cool slowly to RT in heatblock, re-run BA and look for a size shift of all product

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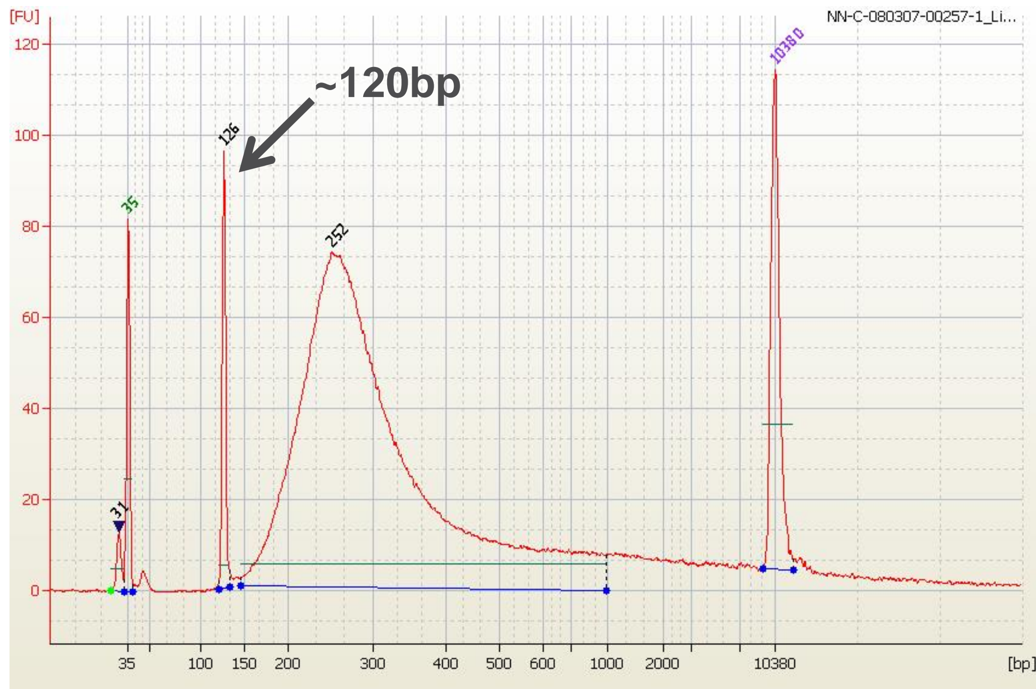
Additional Small Peaks: TruSeq

Adapter Dimers



Possible Cause	Solution
Insufficient starting material	QC starting material with Fluorometric-based method
Poor AMPure bead clean up	Use best practices when handling beads
End Repair or A-Tailing failure	Check reagent quality and thermocycler calibration
Heat inactivation step not performed	Perform 70C incubation after A-tailing

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Additional Small Peaks: TruSeq

Adapter Dimers

c0almacxx Lane 1

A C G T

All Bases % Base

Cycle

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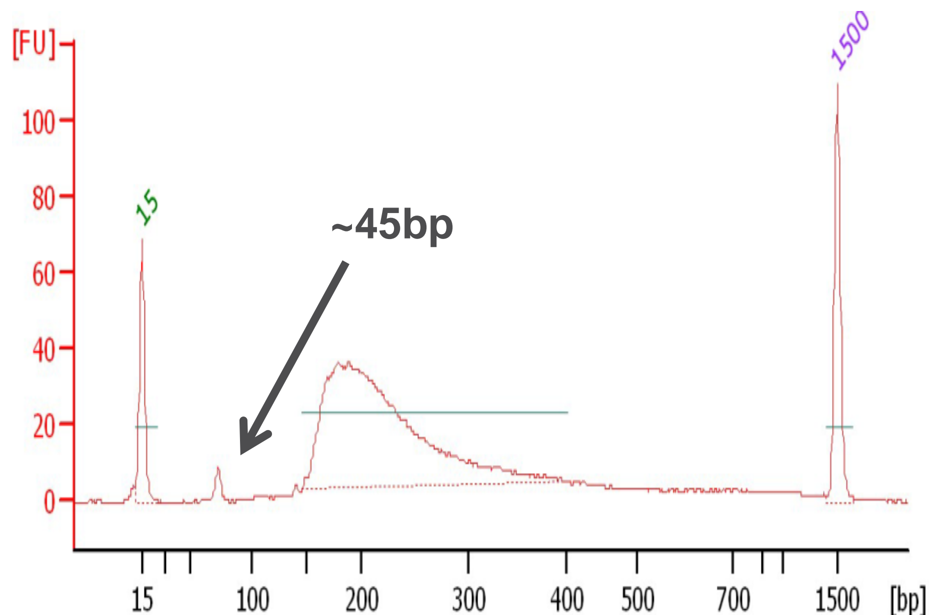
Additional Small Peaks: Nextera

Primer Dimers



Possible Cause	Solution
Insufficient starting material	QC starting material with Fluorometric-based method
Poor AMPure bead clean up	Use best practices when handling beads
Insufficient tagmentation	Confirm that tagmentation step was performed at the correct temperature and time

Review

The Perfect Prep

- Ideal trace: flat baseline, upper/lower markers, x axis in base pairs

The Wrong Size

- Overloaded Bioanalyzer: sample larger than markers. Re-dilute.
- Library is too small: poor bead handling (T), overtagmentation (N)
- Library is too big: poor bead handling (T), undertagmentation (N), should not interfere with analysis
- Libraries can be sequenced

Missing Library Peak

- Desired product not captured by kit. Ensure isolation kit preserves desired product.
- Product was lost during sample preparation. Ensure that sample is obtained throughout each step.
- Library must be re-prepped

Additional Big Peaks

- AMPure carryover: large trailing hump, clean up and re-run sample
- PCR artifacts: overamplification,
- Libraries can be sequenced

Additional Small Peaks

- Adapter dimers: peak at ~120bp (T)
- Primer dimers: peak at ~45bp (N)
- Libraries can be sequenced

(T) TruSeq (N) Nextera

Additional Resources

- Recorded Webinars
- Upcoming Webinars
  - Introduction to the HiSeq 3000/4000 (06/30/2015 10:30 AM GMT)
  - Introduction to MiSeq Reporter Software (MSR) (07/07/2015 10:30 AM GMT)
  - Nextera XT - Sample Preparation and Usage (07/21/2015 10:30 AM GMT)
- Other resources
  - DNA/RNA Isolation Considerations When Using TruSeq Library Prep Kits
  - Considerations for DNA isolation when using Nextera kits
  - Nextera XT Library Prep: Tips and Troubleshooting
  - TruSeq Sample Preparation Best Practices and Troubleshooting Guide
  - Nextera Bioanalyzer traces and sequenced insert size: a comparison
- Illumina General Support

Questions?

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