# ① Integrity of starting material

▪ **When performing RNA-seq, it is necessary to assess the integrity of total RNA prior to mRNA or small RNA isolation using the RNA 6000 Nano Assay.**

▪ **A RNA Integrity Number (RIN) of at least 8 is the recommended threshold.**

▪ **The quality of chromatin immunoprecipitated (ChIP) DNA can be assessed using the High Sensitivity Assay.**
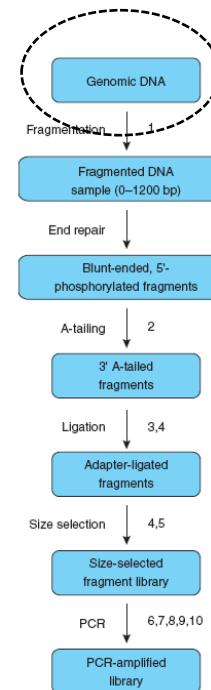
Total RNA Integrity

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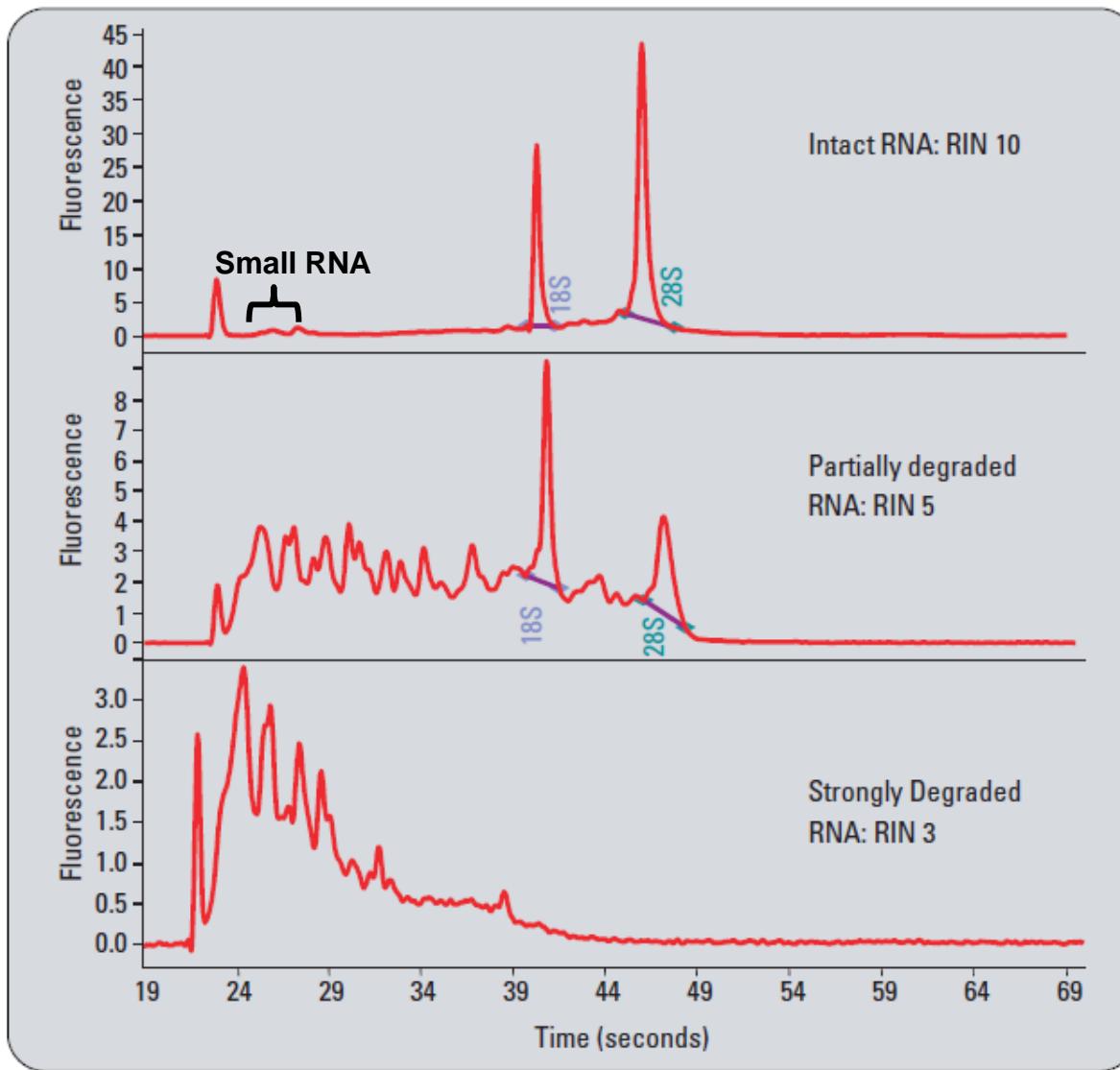
Analyzing small amounts of ChIP DNA

High Sensitivity DNA Assay : concentration range of 100 – 10000 pg/µl
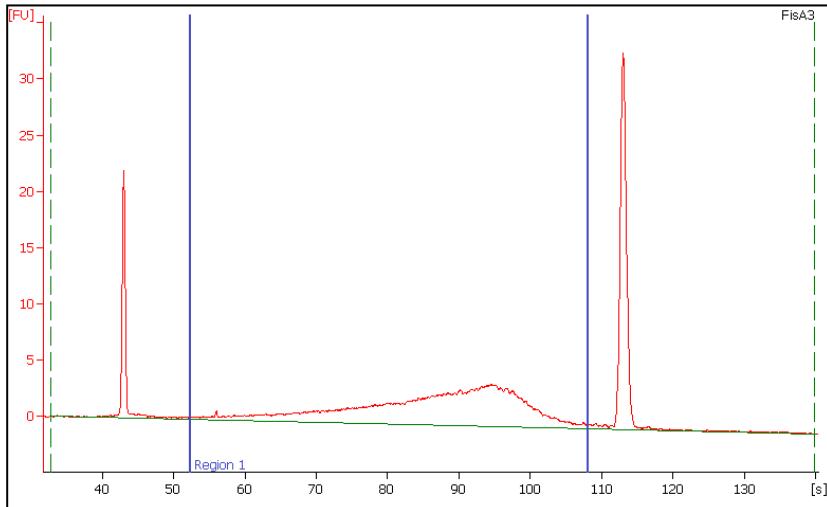
**ChIP Post-IP**

Average Size: 827 bp

Conc: 335 pg/µl
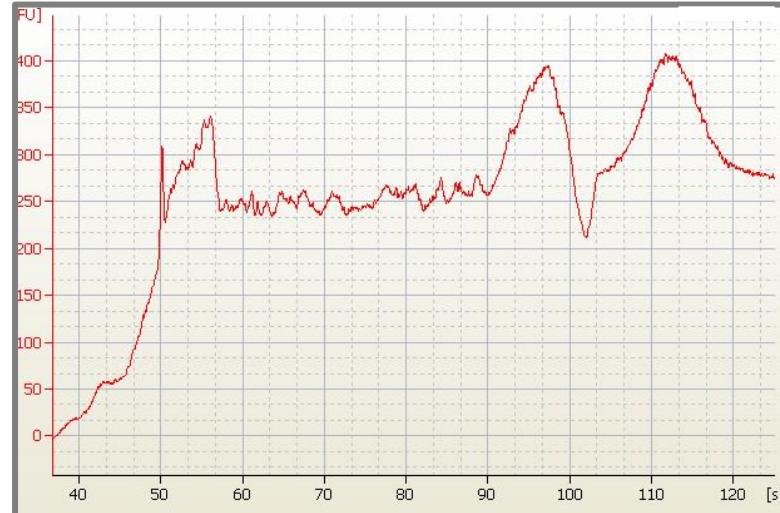
ChIP Post-IP

High molecular weight DNA contamination

🔶 **Fragment DNA prior to IP using Covaris, sonication or enzymatic digestion.**

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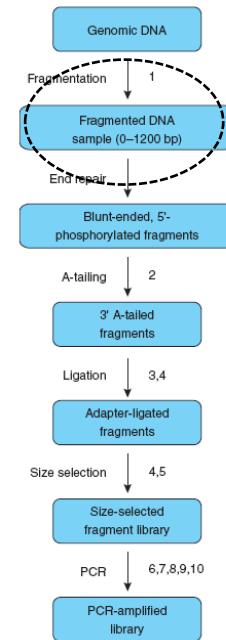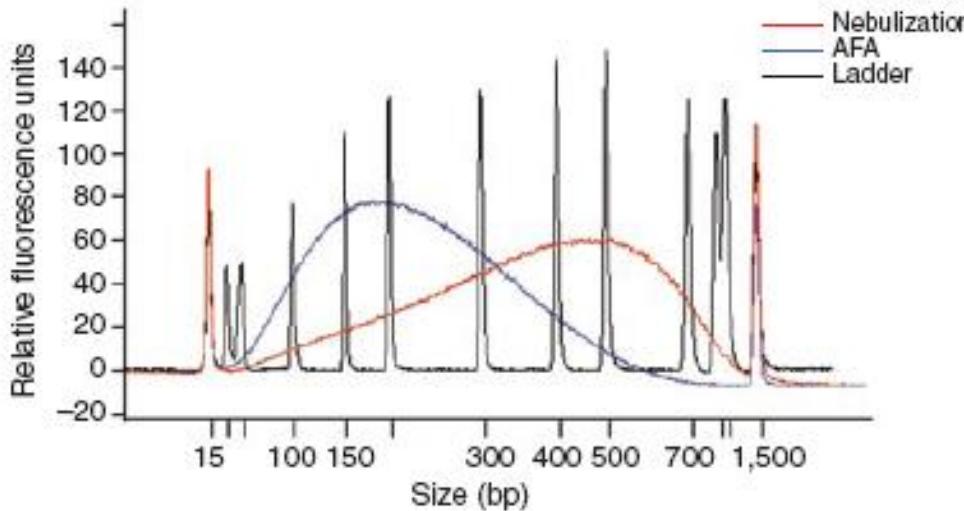
▪ **Comparison of fragmentation by nebulization with AFA technology (Covaris).**

▪ **For a 200-bp (±20 bp) library, the yield produced by AFA was four-to fivefold greater than that produced by nebulization.**

# The importance of monitoring size distribution

- **Choice of fragmentation can significantly affect the recovery of desired fragments and hence the amount of starting material required.**

- **For example, the Covaris typically produces narrower fragment distributions than nebulization, resulting in 4-fold greater recovery.**

- **Sequencing fragments that do not fall within the recommended size distribution may lead to low read depth or even a lack of read coverage for specific portions of the sequence.**

- **When performing SureSelect target enrichment, fragment size distribution can affect final % on-target capture.**

- **Probe-based sonication methods routinely introduce sample-to-sample variability and are not recommended.**

RNA Fragmentation

SOLiD
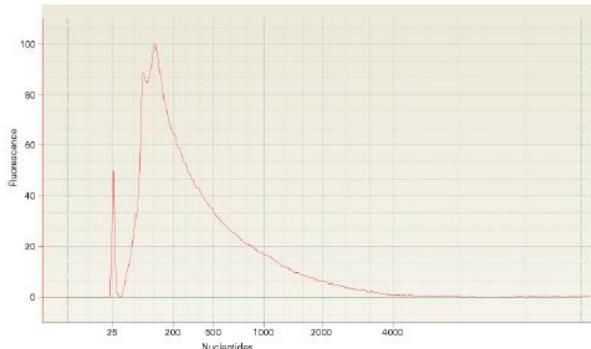
Poly(A) RNA fragmented by RNAse III
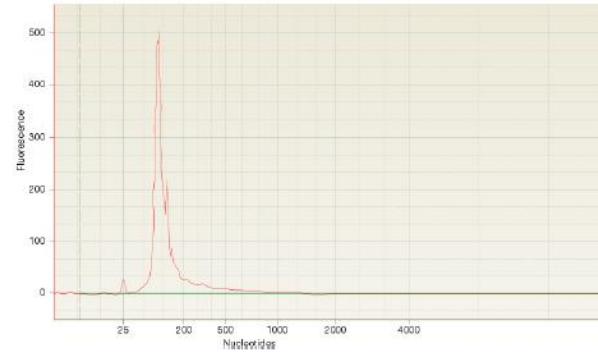
rRNA-depleted RNA fragmented by RNAse III

Roche/454
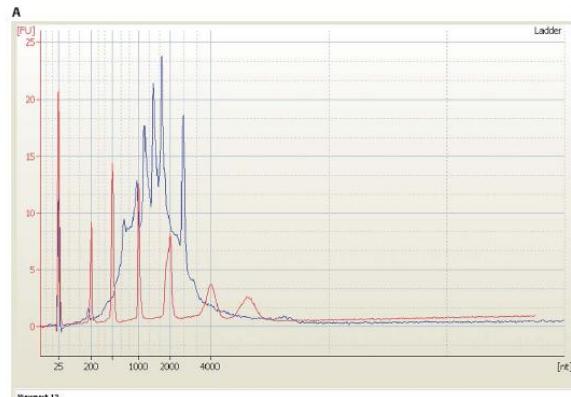
Non-fragmented mRNA

Fragmented mRNA

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Applied Biosystems – *Whole Transcriptome Library Preparation for SOLiD Sequencing*
Roche - *cDNA Rapid Library Preparation Method*
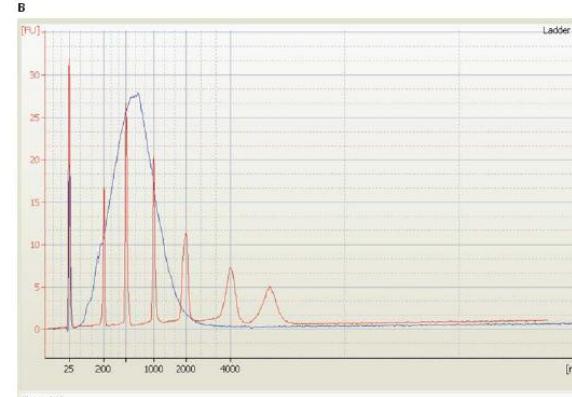
DNA Fragmentation

DNA sheared using the Covaris in the SureSelect^{XT} Illumina Paired-End sequencing protocol.
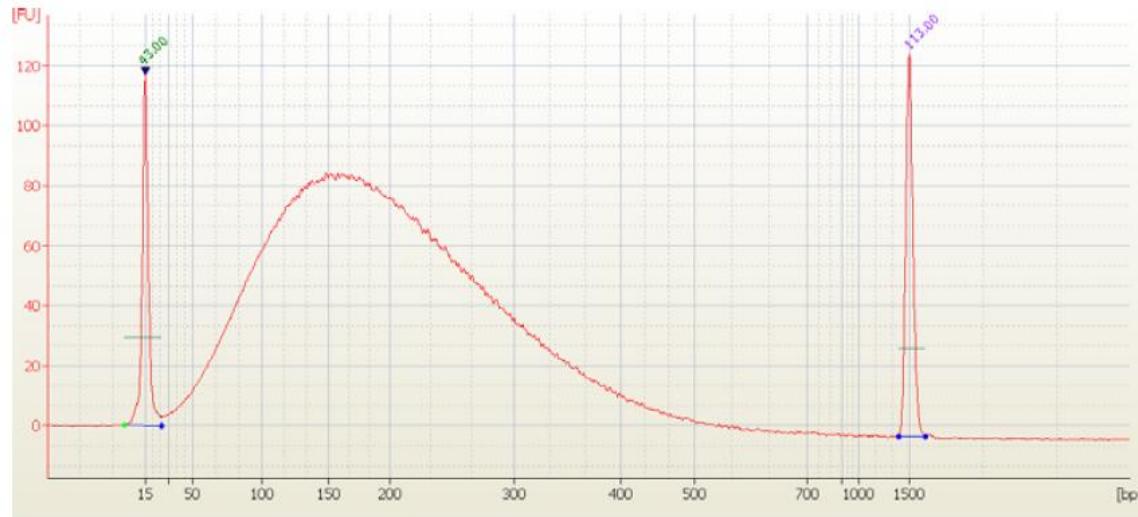
Size distribution with peak height between 150-200nt.

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# DNA Fragmentation - Tailing
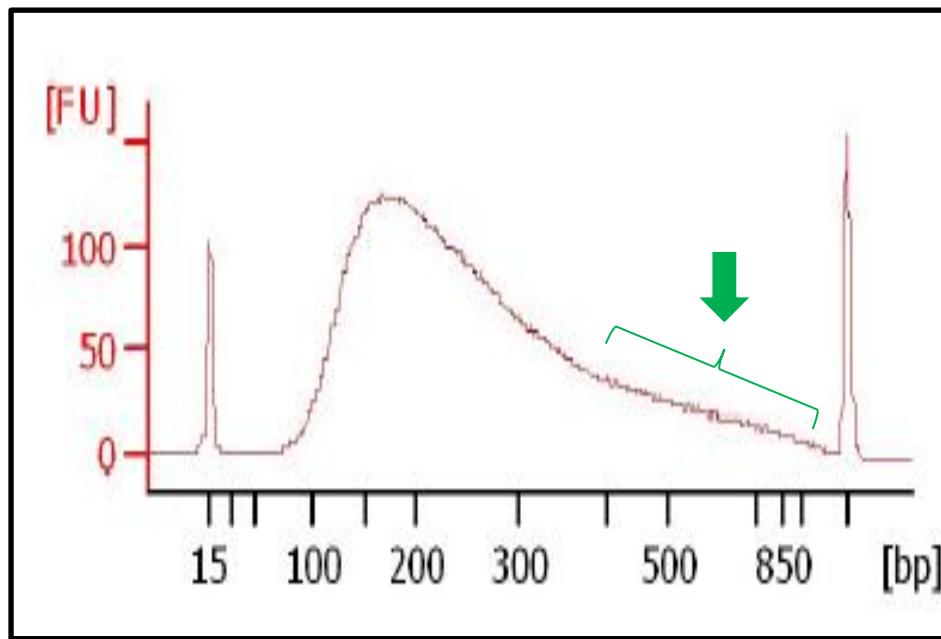
Cause : Too much DNA was used for shearing on Covaris, resulting in incomplete shearing. For example, Covaris settings in the SureSelect protocol are optimized to yield a peak size range of 150-200bp when starting with a maximum of 3μg gDNA. Adhere to recommended amounts of starting material.

Maria-Celeste Ramirez, PhD
Agilent Technologies

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DNA Fragmentation – Uneven shearing
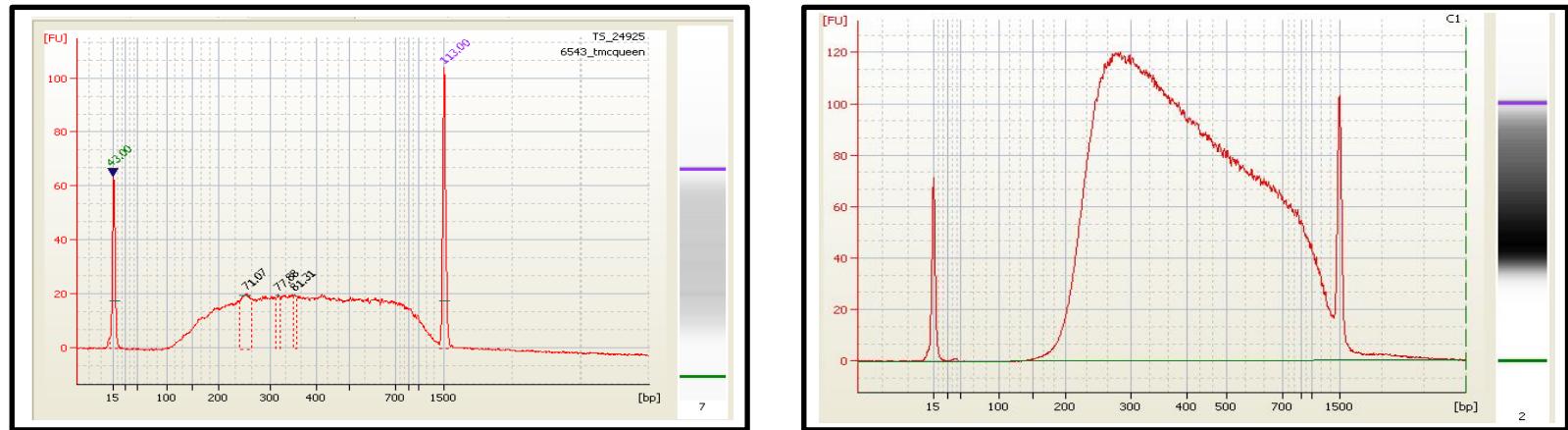
Causes :

🔶 **Poor DNA quality.**

🔶 **DNA eluted in wrong buffer (not TE).**

🔶 **Covaris issues**

- **Sample volume less than 120µl. This allows an air gap, resulting in inconsistent fragmentation.**
- **Water level is too high/low.**
- **Temperature of waterbath not between 6-8ºC.**
- **Insufficient degassing.**

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DNA Fragmentation – Increased size range
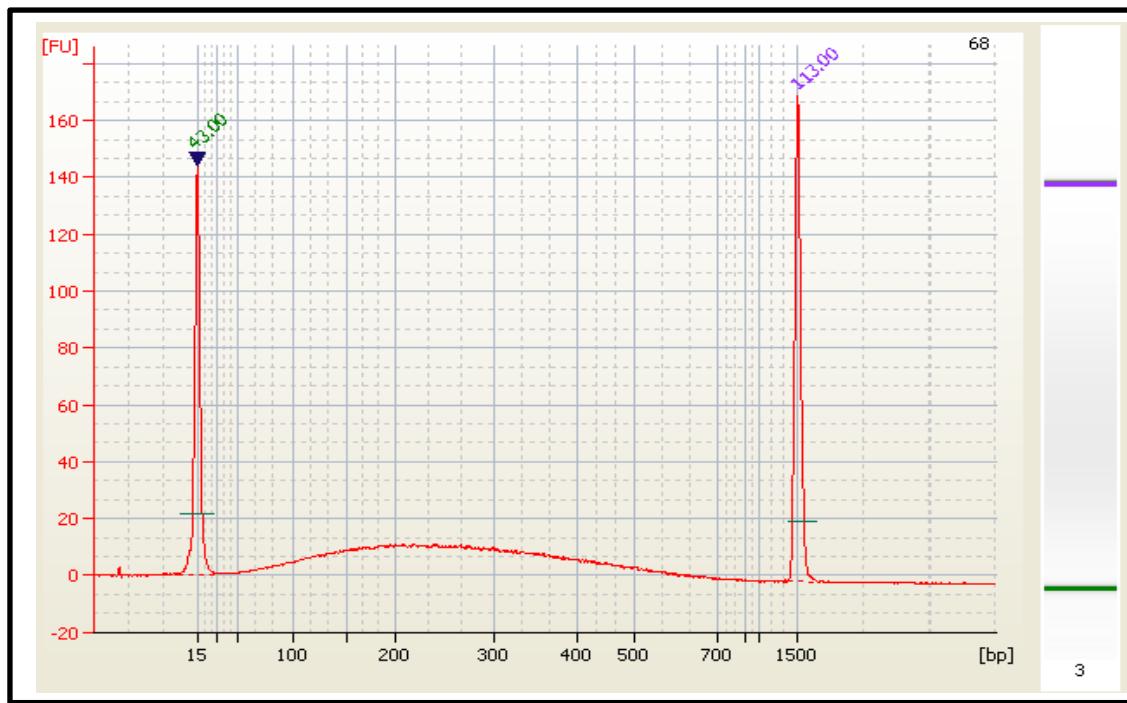
Cause **: Covaris issues. Perform a control experiment using DNA of known quality, such as commercially available lambda DNA.**

3 **Monitoring size distribution after adapter-ligation**

Post - fragmentation
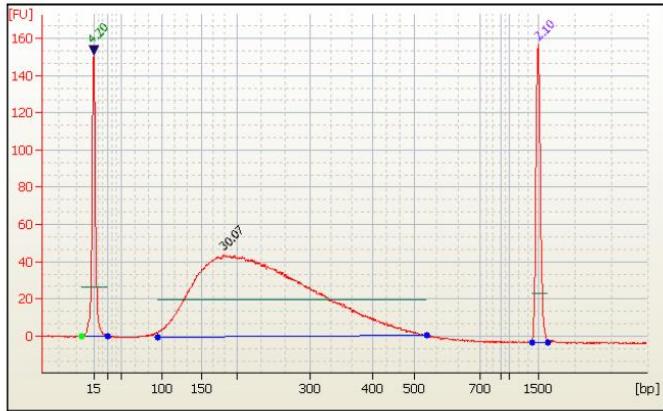
Peak size of 190bp

After adaptor ligation
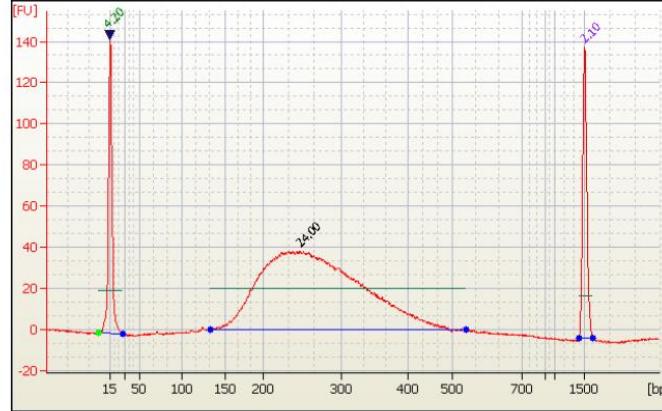
Peak size of 250± 10bp
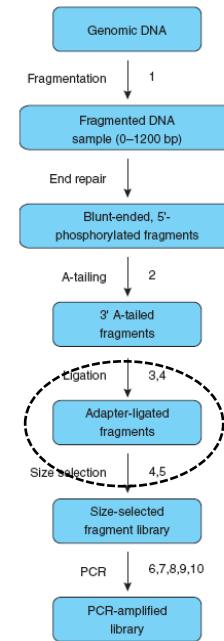
- Adaptor ligation is not 100% efficient so the DNA 1000 assay can be used to visualize the size shift after successful ligation of adaptors.

- Inefficient adapter ligation will result in reduced library complexity after PCR.

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Adapter ligation – Excess adapters
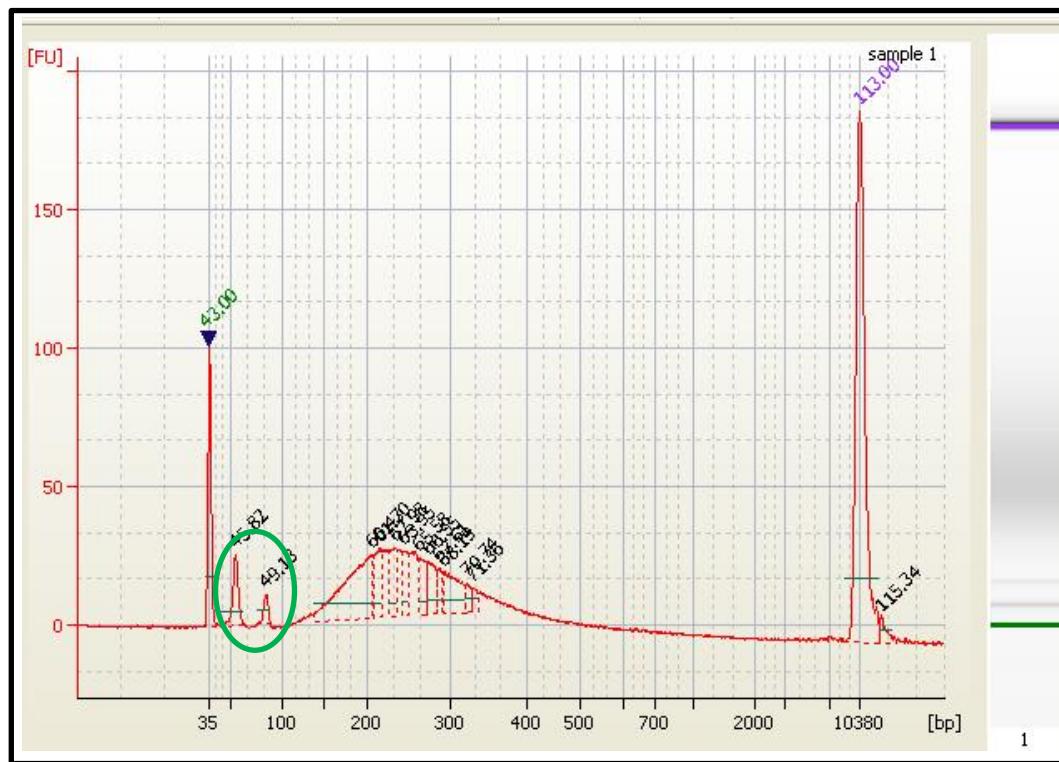
Cause : **Inefficient ligation due to too much input DNA or the use of incorrect ligation temperature (ligation is performed at 20-25ᵒC. When using a PCR machine, make sure the lid is not heated).**

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Automated size selection after adapter ligation

In whole genome sequencing, small-RNA seq and ChIP-seq protocols, a gel-based size selection step is performed after adapter ligation (SureSelect™ uses SPRI beads).

This can be automated using the Pippin Prep™ DNA size selection system, which uses pre-cast agarose gel cassettes and elutes size-selected fragments in buffer.

Size selection using Pippin Prep™

Quality control of fractions using the Bioanalyzer

Sequencing

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http://www.sagescience.com/

Automated size selection after adapter ligation

Update

- **Resolving Illumina ligation reactions directly on gel have been observed to be affected by the DNA-bound ligase, resulting in extraction of incorrectly-sized DNA.**

- **A simple protocol was developed that includes an additional proteinase K digestion for 10min at 37ºC prior to gel electrophoresis.**

- **Samples were then loaded on a 2% Pippin Prep cassette using settings to collect DNA fragments of 335bp (range: 308-362bp). Extracted samples analyzed on Bioanalyzer.**
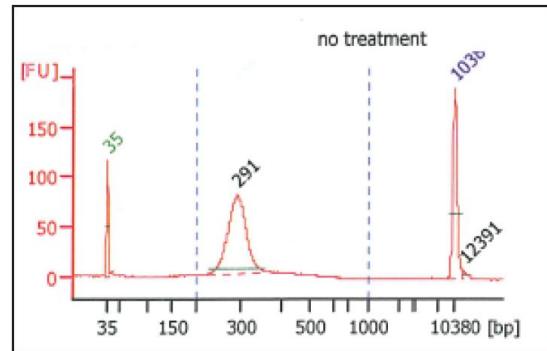
Pıppın Prep™ DNA Size Selection System

A Rapid Proteinase K Cleanup Method for Illumina Adapter Ligation Reactions Prior to Pippin Prep Fractionation

Matthew Mayho and Michael Quail. The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK.

No treatment

Proteinase K treatment

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http://www.sagescience.com/
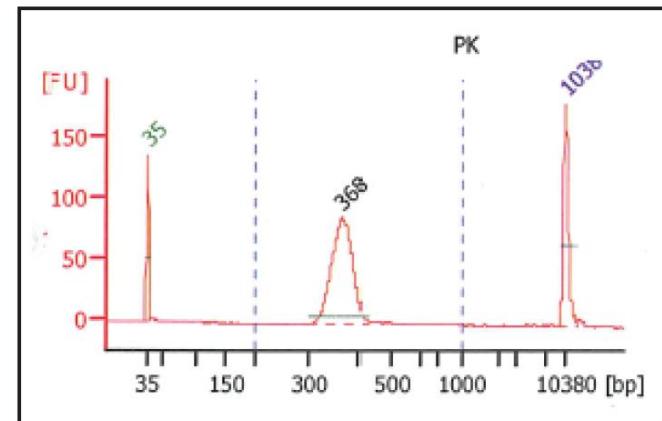
4 **Quality control after PCR amplification**

▪ **PCR amplification is necessary in library preparation to enrich for adapter-ligated fragments, as well as to add indexes.**

▪ **In the SureSelect protocol, PCR is also used to amplify captured DNA for final QC and quantification.**

▪ **PCR can create bias since some regions of template DNA amplify more poorly than others (eg, GC-rich regions). PCR artifacts caused by overamplification or primer dimers can also affect sequencing coverage and accuracy.**

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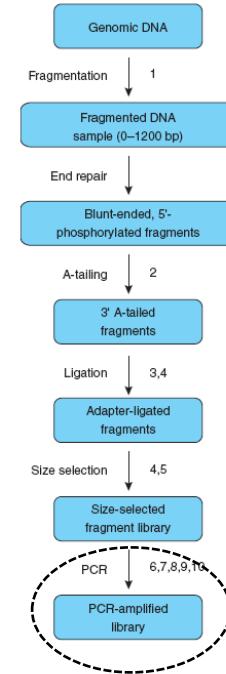
PCR amplification – detecting artifacts

High Quality DNA library
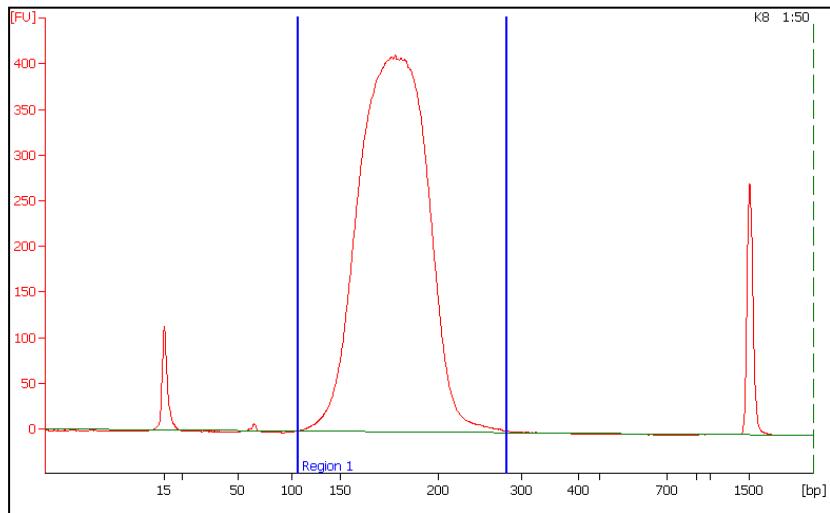
Primer dimers and PCR artifact
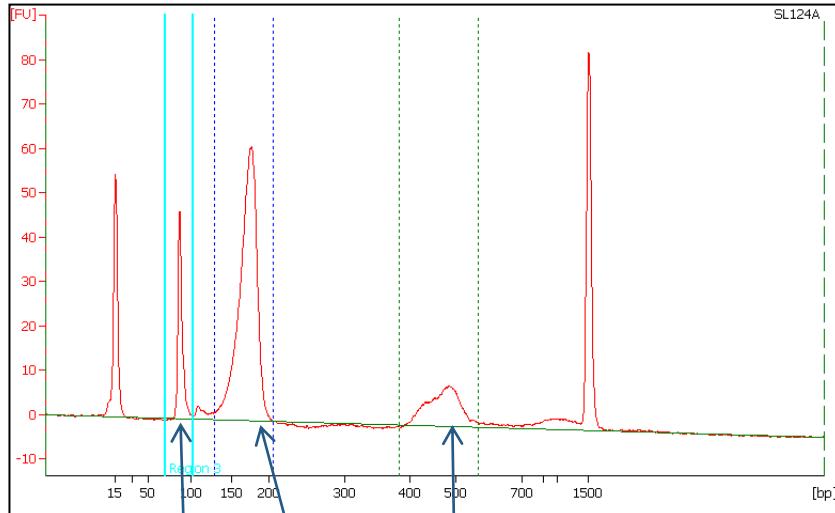
Primer dimers

PCR artifact

DNA library (50% of total DNA)

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Dealing with primer dimers and artifacts

- **Primer dimers present in the library will be sequenced, resulting in wastage of sequencing capacity and increased duplicate reads.**

- **When working with small amounts of DNA, such as in ChIP-seq or SureSelect™ target enrichment, primer dimers can be removed by performing additional SPRI bead clean-up steps.**

- **Gel-based size selection can also be performed to remove primer dimers.**

- **Repeat PCR with fewer cycles to prevent formation of artifacts using remaining library.**

Reducing number of amplification cycles

Illumina GAIIx library enriched using SureSelect™.
Analyzed using DNA High Sensitivity Kit.

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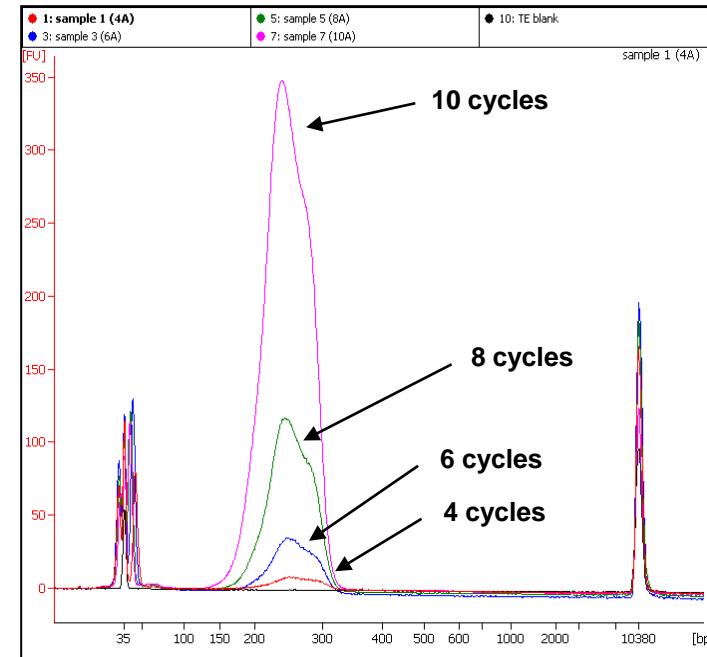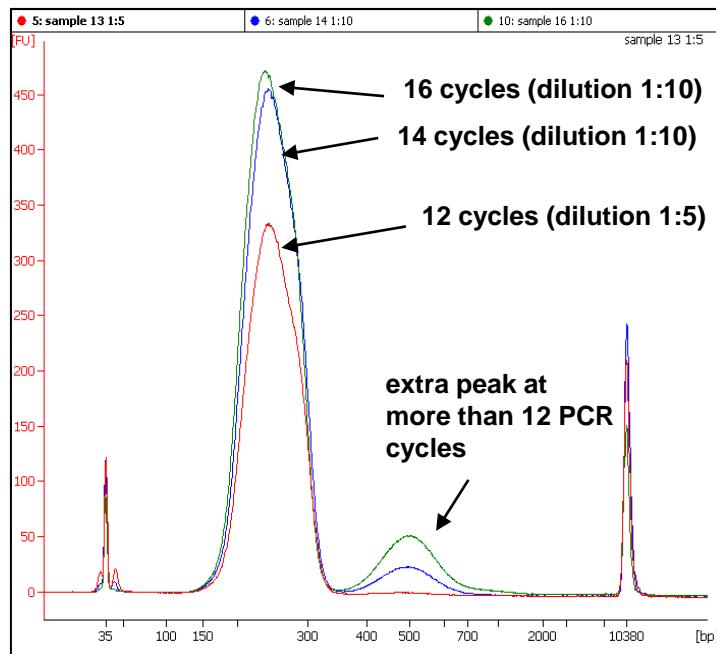
PCR amplification – Bead carryover
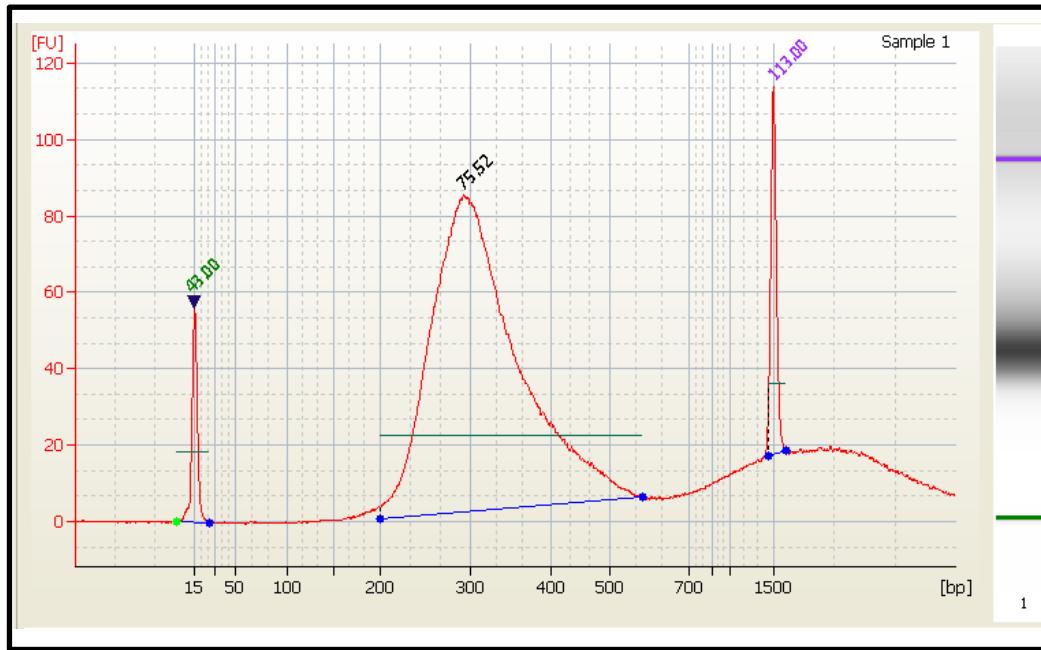
Cause : **SPRI bead carryover from post-PCR clean-up step. Use a strong magnet for bead separation and pipette carefully during elution to avoid disturbing beads.**

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PCR amplification – Residual Buffer
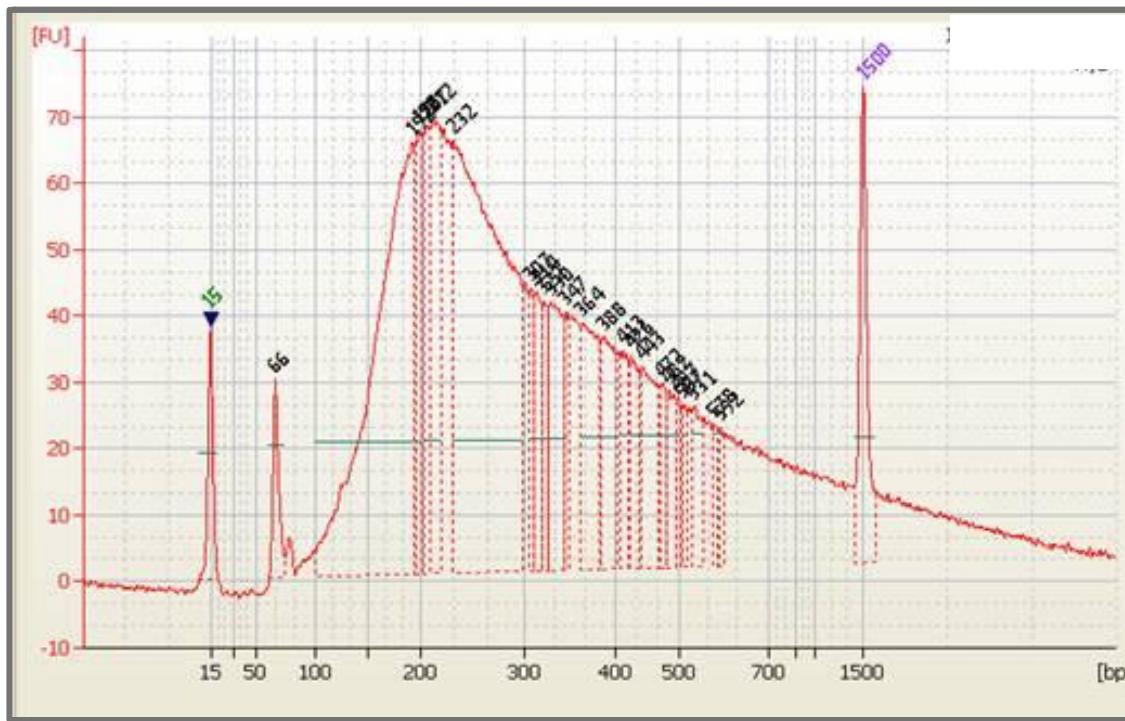
Cause : Likely due to buffer carryover from post-PCR clean-up using columns.

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PCR amplification – Low PCR yield
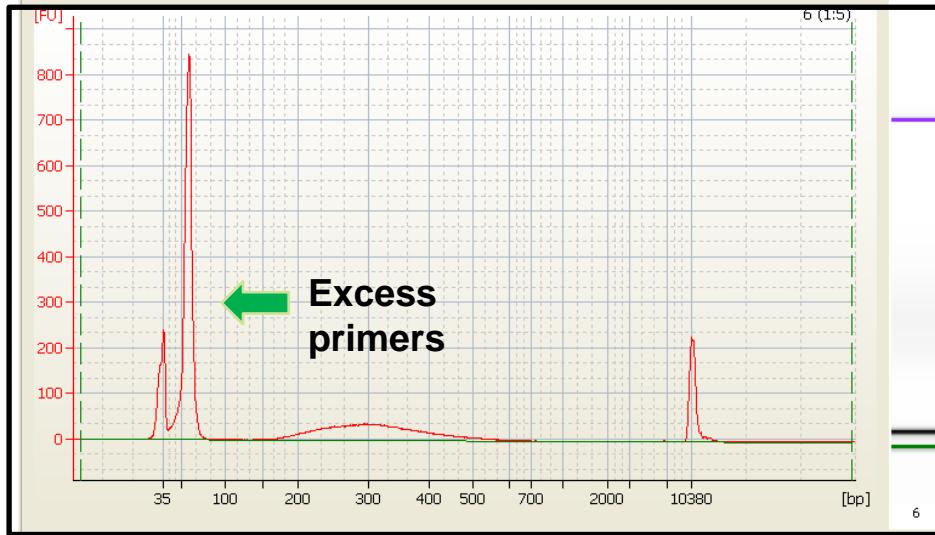
Cause : Inefficient PCR cycling results in low yield and an excess of primers. This can be a result of poor adapter ligation, low DNA quality, inefficient bead clean-up, use of too few cycles or PCR instrument is not well-calibrated. When performing target capture, low yields after post-capture PCR can also indicate suboptimal hybridization.

Based on previous Bioanalyzer runs, it is possible to identify the cause. Ensure that excessive evaporation has not occurred during hybridization (at least 21µl left). If there is sufficient DNA, proceed with sequencing. If not, repeat PCR with remaining library.

PCR amplification – Chip overloading and spurious peaks
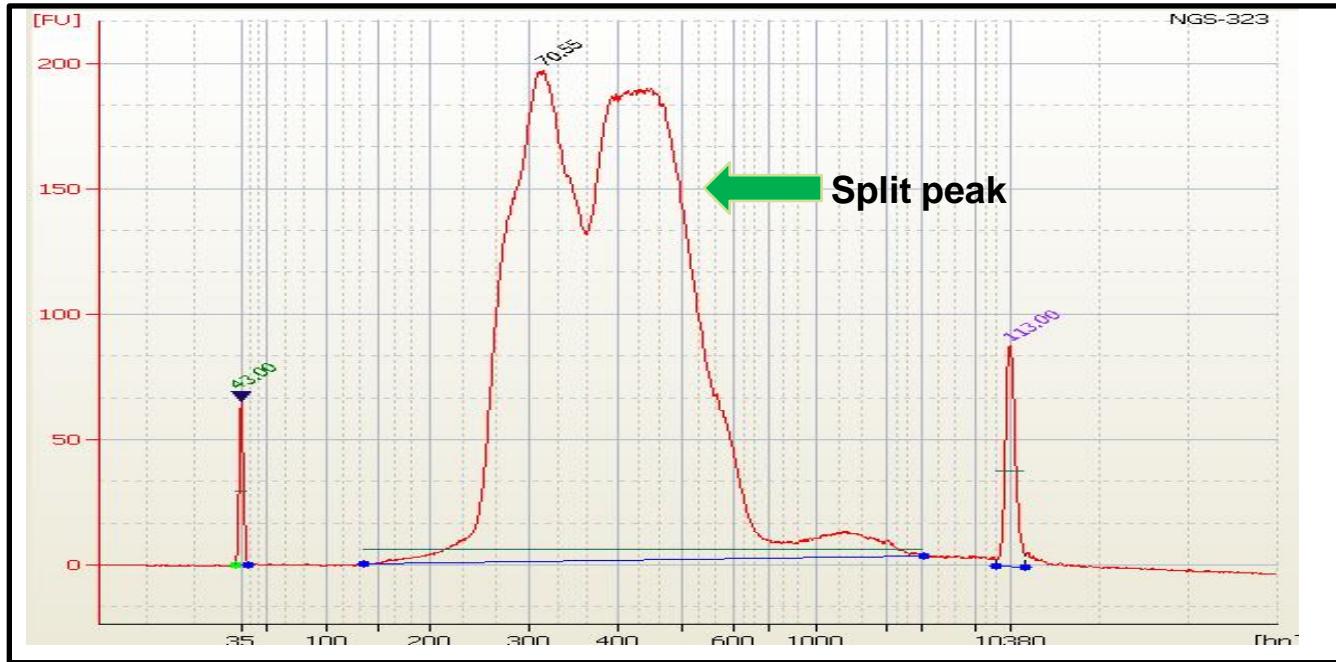
Cause : **Too much DNA loaded on chip for analysis.**

DNA 1000/7500	**0.1-50ng/µl**
High Sensitivity DNA	**100-10000pg/µl**

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