Bioanalyzer Applications for Next-Gen Sequencing: Updates and Tips

March 1st, 2011

Charmian Cher, Ph.D
Field Applications Scientist

Agilent Technologies

Agenda

1. **Next-gen sequencing library preparation workflow and important quality control steps.**

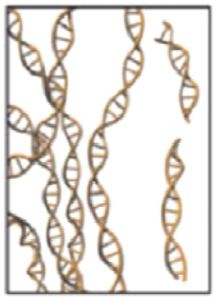
2. **Introduction to the 2100 Bioanalyzer.**

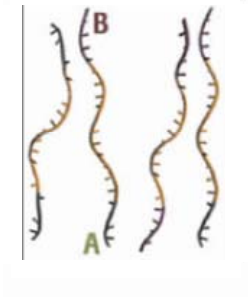
3. **The use of Bioanalyzer assays for:**

   - **assessing quality of starting material**
   - **monitoring size distribution after fragmentation and adapter ligation**
   - **quantifying yield and detection of artifacts post-PCR amplification**
   - **detecting small quantities of DNA in amplification-free protocols**

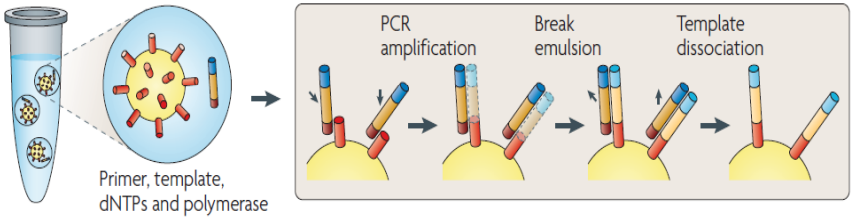
4. **Best practice tips to always obtaining accurate and reproducible data when using the Bioanalyzer.**

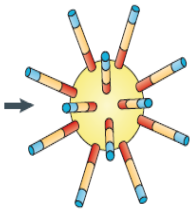
Next-Gen Sequencing





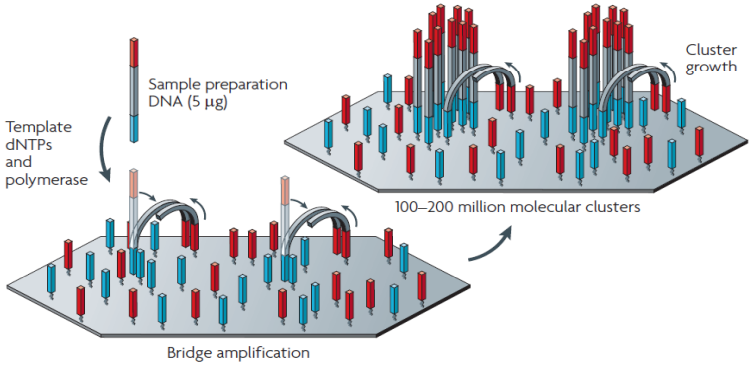
Roche/454 and Life Technologies





GS FLX Titanium

Illumina



SOLiD 4

HiSeq 2000

Adapted from Metzker, M. (2009) Nature Reviews

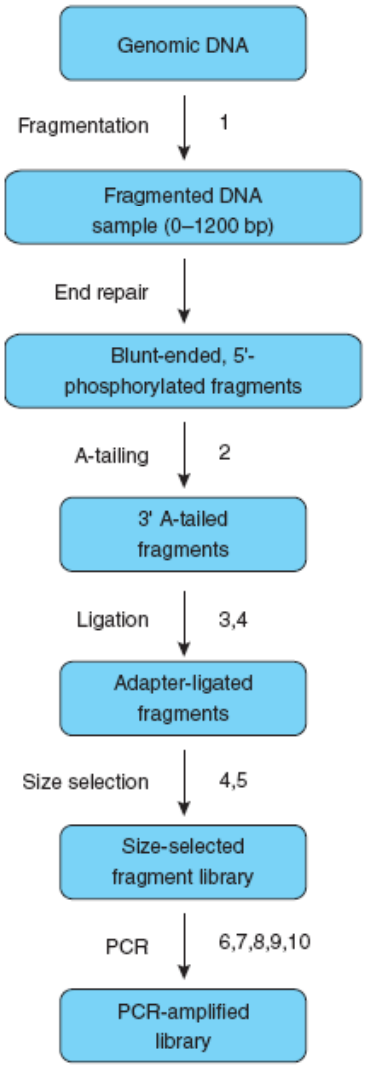
Quality of DNA libraries is critical for sequencing success

For every application of next-gen sequencing eg. genome sequencing, transcriptome sequencing (RNA-seq), chromatin immunoprecipitation sequencing (ChIP-seq) or targeted resequencing, there is a specific protocol to convert the source nucleic acid to standard DNA libraries.

The aim of recent developments in library preparation methods is to produce a high-quality representative, non-biased DNA library from small amounts of starting material.

Quality Control during Library Preparation

Genome sequencing



Integrity of starting material

Size distribution after fragmentation

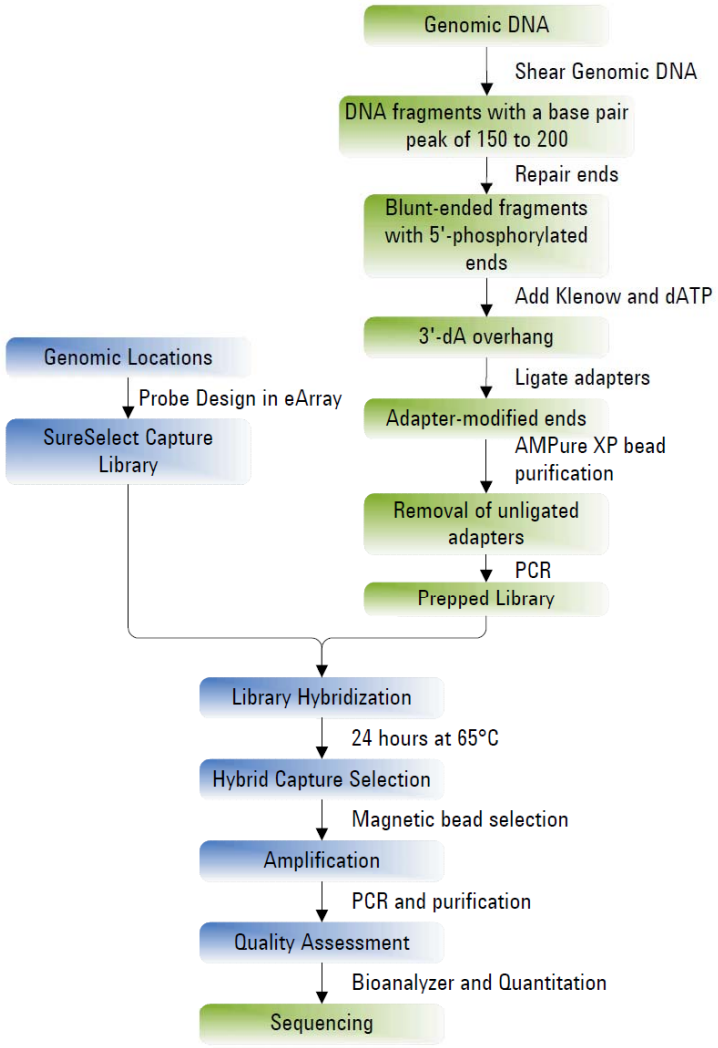
Size distribution of adapter-ligated fragment library

Quality control and quantification after PCR

Adapted from Quail et al (2008) Nature Methods)

Quality Control during Library Preparation

SureSelect™ Target Enrichment System



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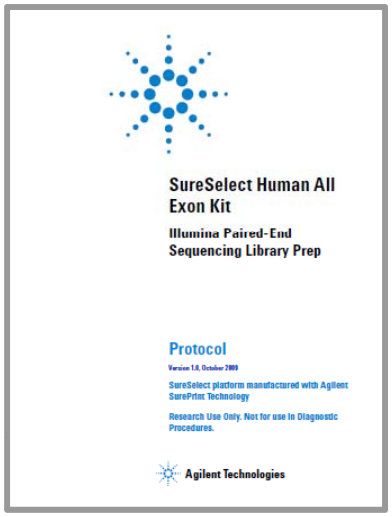
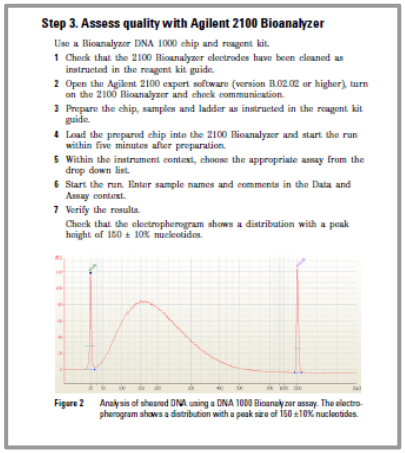
Size distribution of adapter-ligated fragment library

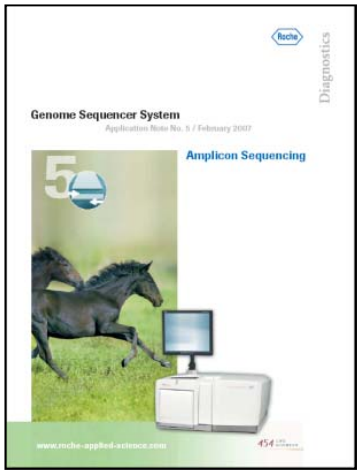
Quality control and quantification after PCR

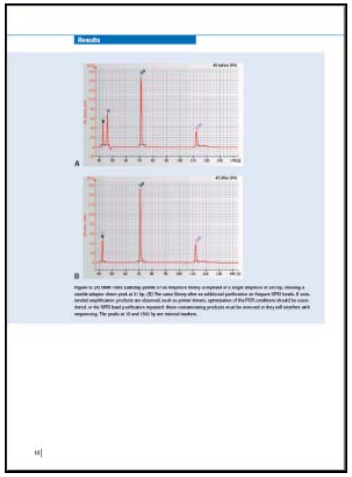
Implementing quality control with the 2100 Bioanalyzer

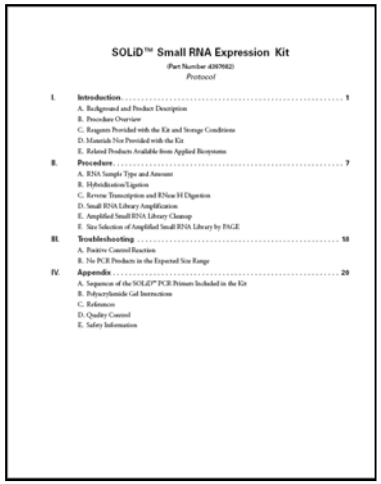


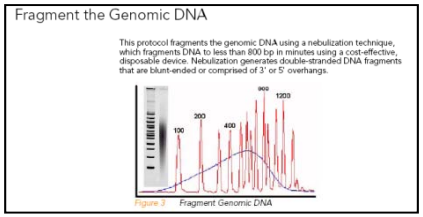


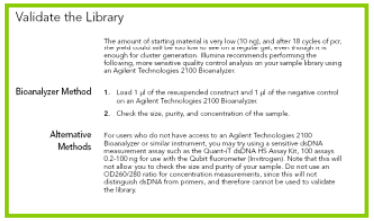
All major next-gen sequencing providers recommend using the Bioanalyzer at various steps in the library preparation workflow.











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4. **Best practice tips to always obtaining accurate and reproducible data when using the Bioanalyzer.**

2100 Bioanalyzer

Sample volumes 1 - 5 µl

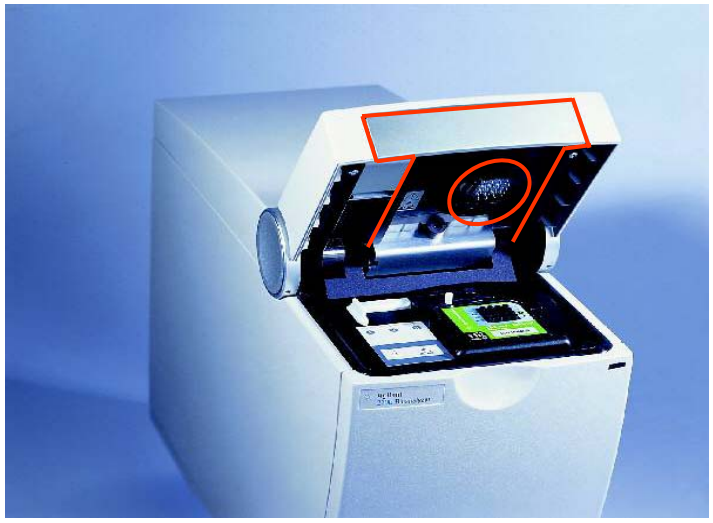
10 -12 samples depending on Assay

Separation, staining, detection of samples

Results in 5-30 minutes available

No extra waste removal needed

Disposable Chip, no cross-contamination



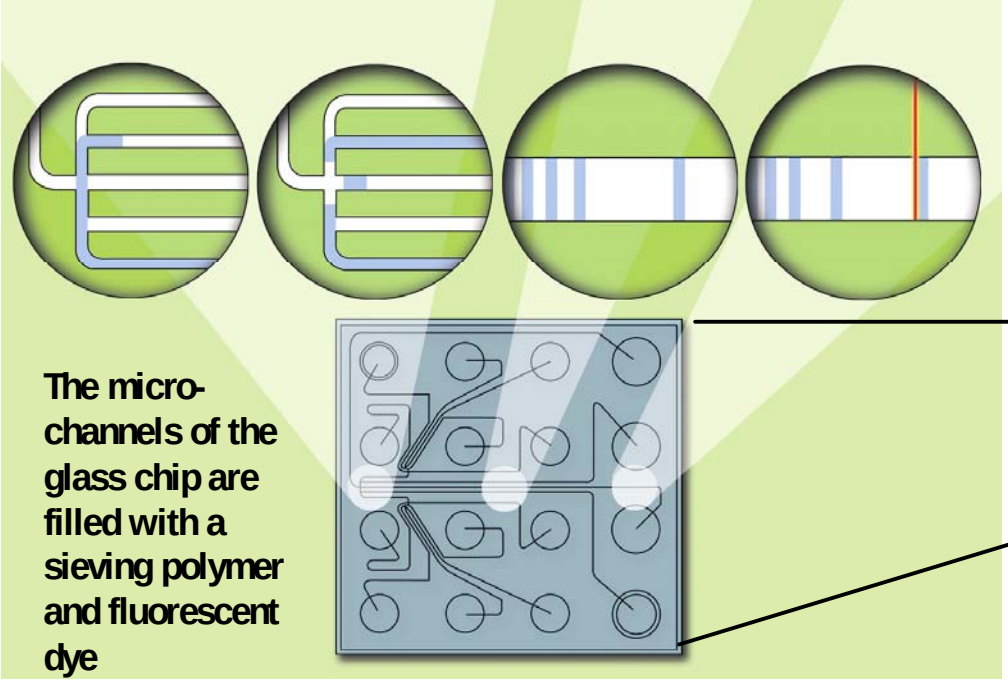
Principle of Electrodriven Flow

The sample moves from the sample well through the micro-channels

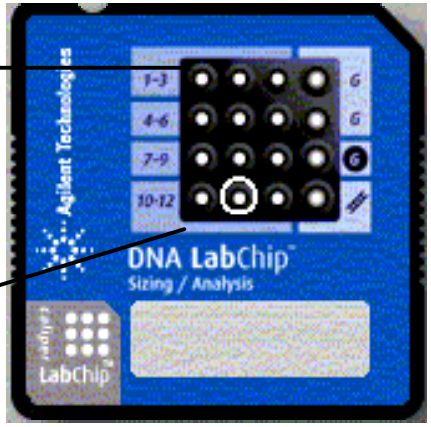
The sample is injected into the separation channel

Sample components are electro-phoretically separated

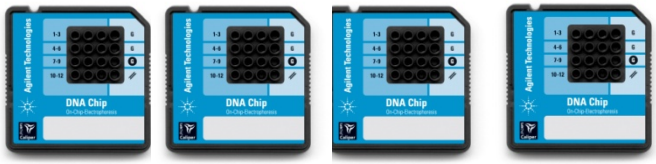
Components are detected by their fluorescence and translated into gel-like images (bands) and electropherograms (peaks)



The micro-channels of the glass chip are filled with a sieving polymer and fluorescent dye



Bioanalyzer Kit Portfolio



DNA1000 DNA7500 DNA12000 High Sensitivity DNA

DNA Assays:

- Sizing
- Quantitation
- PCR products, digests, larger DNA fragments
- 12 samples in 30 min.

6000 Nano 6000 Pico Small RNA

RNA Assays:

- Quantitation (Sizing in Small RNA)
- total RNA, mRNA
- purity & integrity determination
- 10 samples in 30 min.

Flow Cytometry

Cell Assays:

- Analysis of 6 samples
- Two color detection
- Analysis of protein expression in cells

P230 P80 HSP 250

Protein Assays:

- Sizing
- Quantitation
- cell lysates, column fractions, purified proteins, antibodies etc.
- 10 samples in 40 min.

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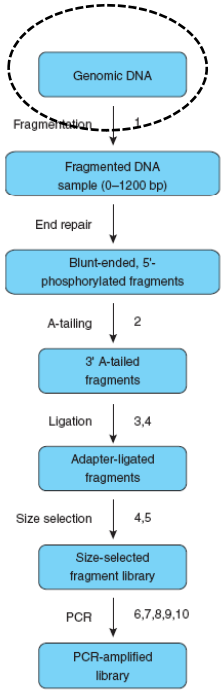
1 Integrity of starting material

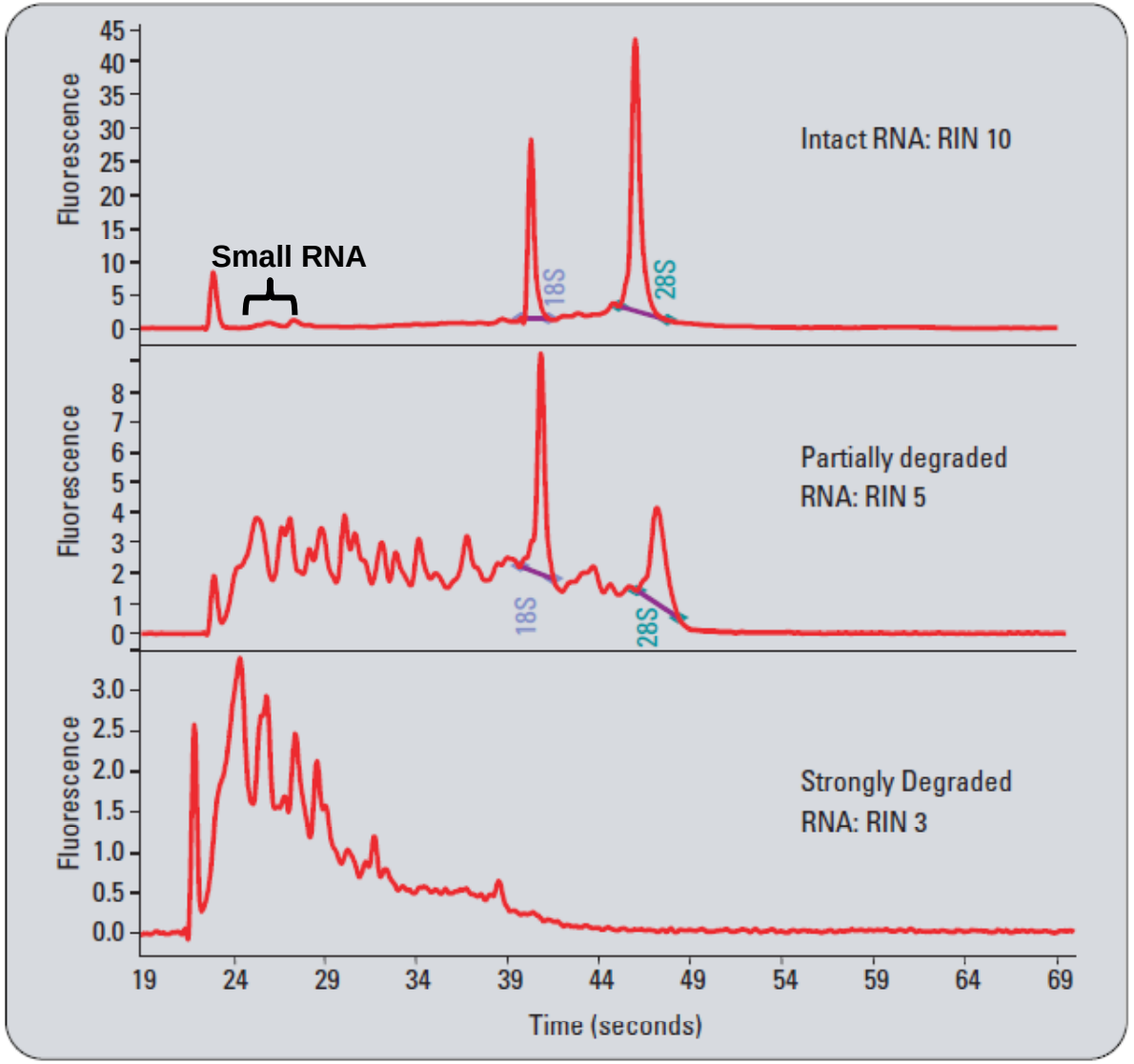


- **When performing RNA-seq, it is necessary to assess the integrity of total RNA prior to mRNA or small RNA isolation using the RNA 6000 Nano Assay.**

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

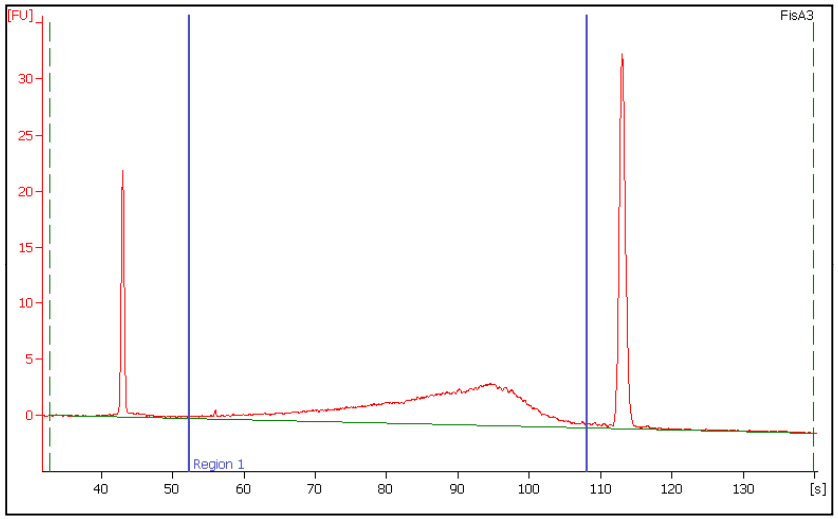
- **The quality of chromatin immunoprecipitated (ChIP) DNA can be assessed using the High Sensitivity Assay.**

Total RNA Integrity



Analyzing small amounts of ChIP DNA

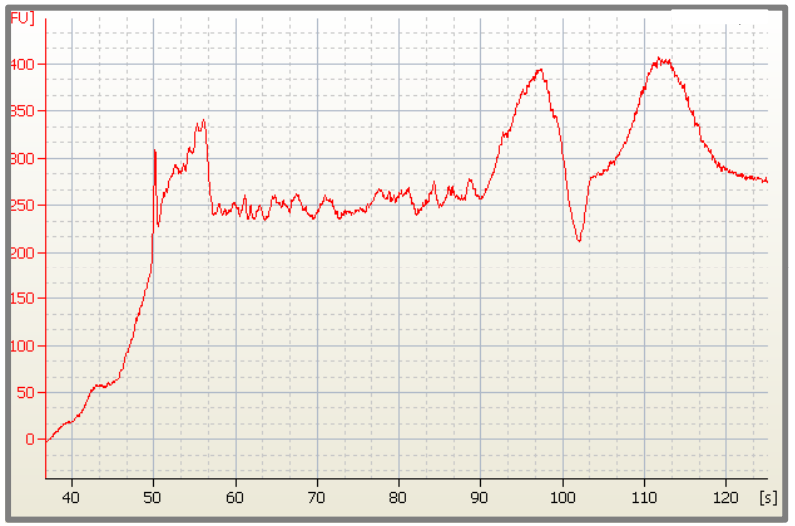
High Sensitivity DNA Assay : concentration range of 100 – 10000 pg/µl



ChIP Post-IP

Average Size: 827 bp

Conc: 335 pg/µl

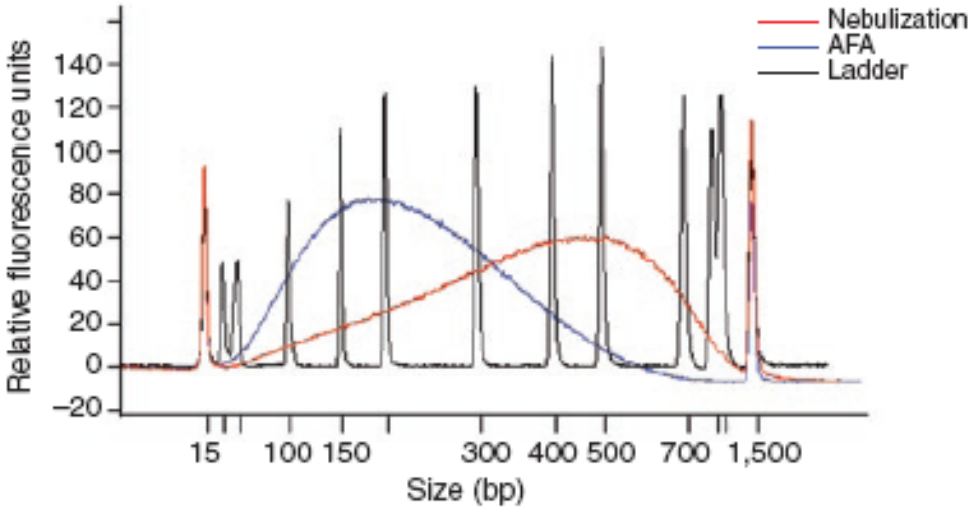


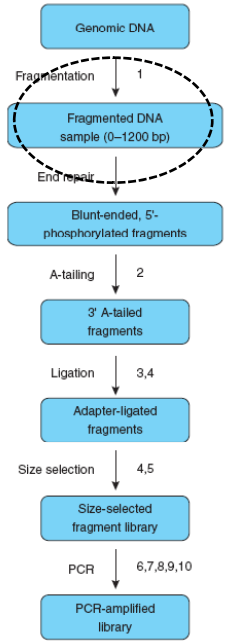
ChIP Post-IP

High molecular weight DNA contamination

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

2 Monitoring size distribution after fragmentation





- **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.**

A large genome center's improvements to the Illumina sequencing system

Michael A Quail, Iwanka Kozarewa, Frances Smith, Aylwyn Scally, Philip J Stephens, Richard Durbin, Harold Swerdlow & Daniel J Turner

NATURE METHODS | VOL.5 NO.12 | DECEMBER 2008 | 1005

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.**

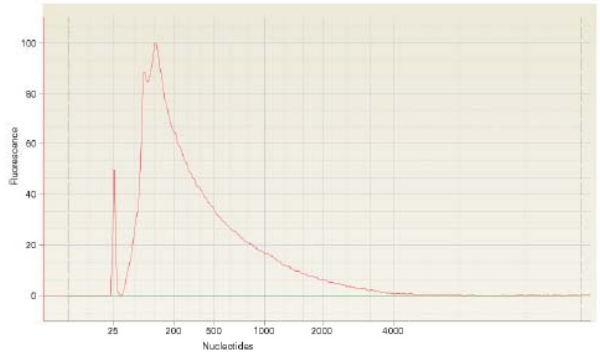
- **When performing SureSelect target enrichment, fragment size distribution can affect final % on-target capture.**

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

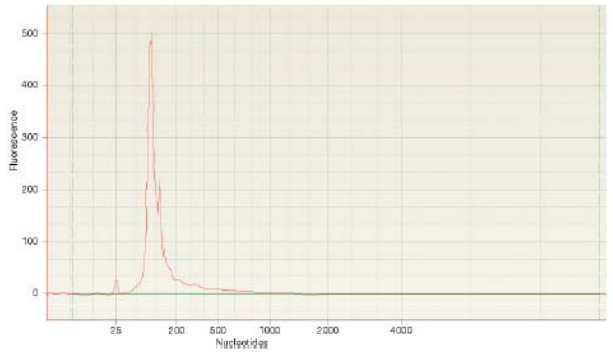
RNA Fragmentation

Update

SOLiD

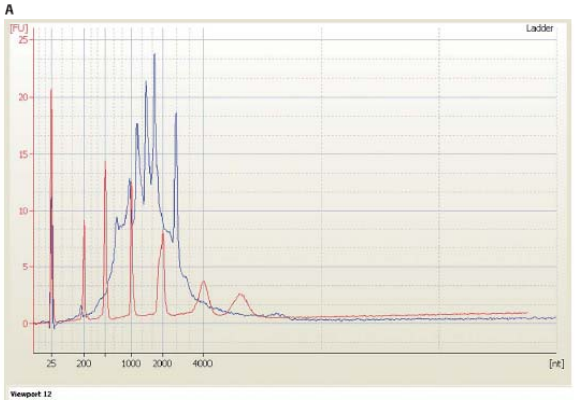


Poly(A) RNA fragmented by RNAse III

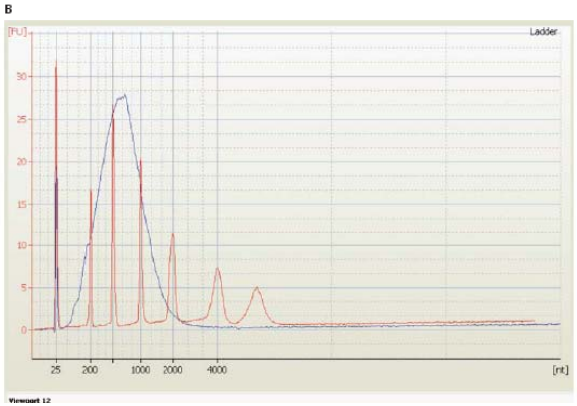


rRNA-depleted RNA fragmented by RNAse III

Roche/454



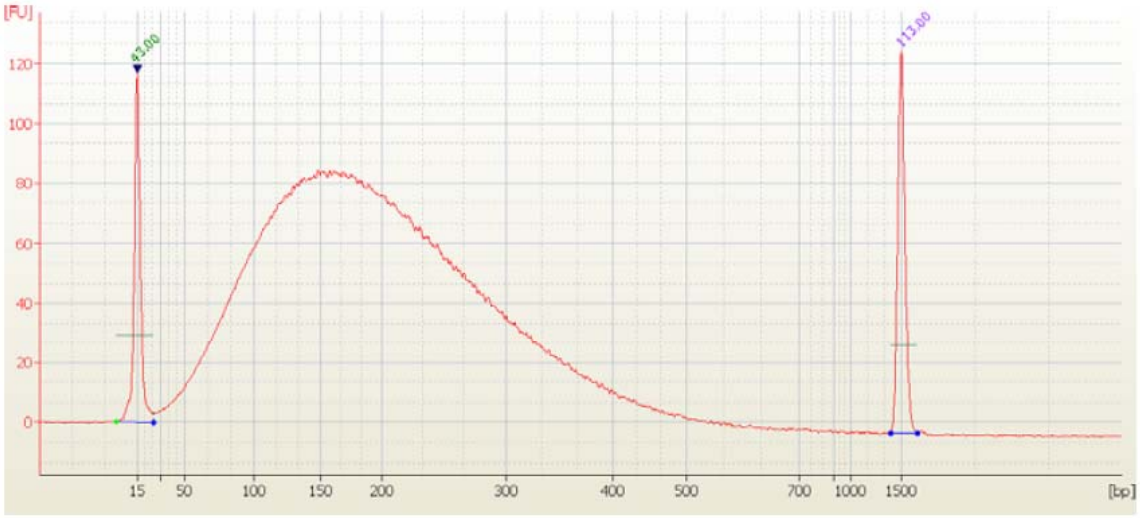
Non-fragmented mRNA



Fragmented mRNA

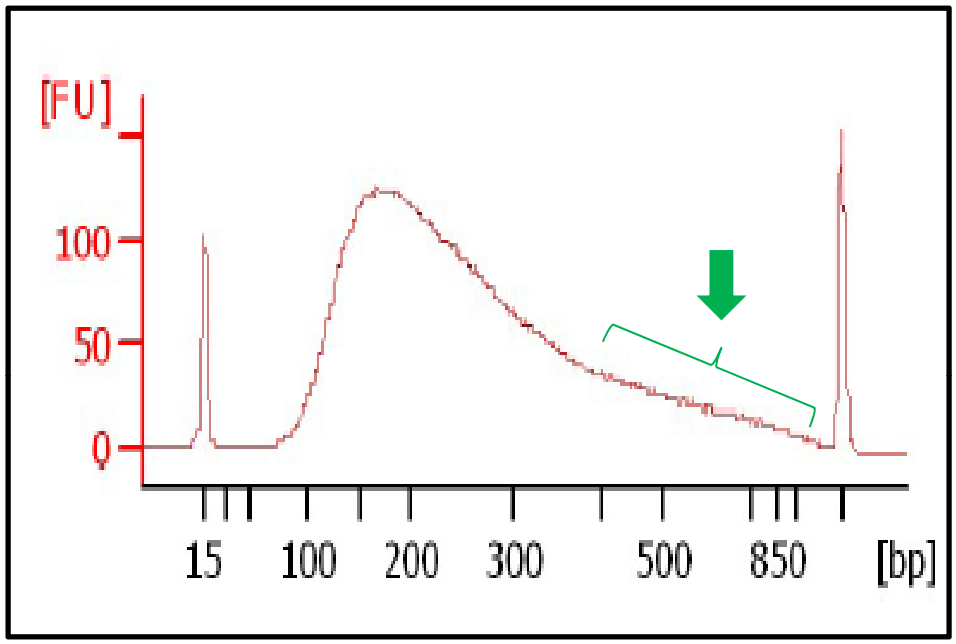
DNA Fragmentation

DNA sheared using the Covaris in the SureSelect^{XT} Illumina Paired-End sequencing protocol.



Size distribution with peak height between 150-200nt.

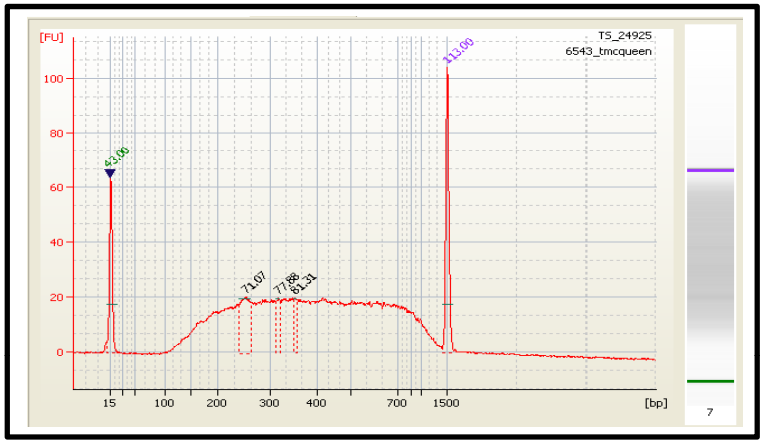
DNA Fragmentation - Tailing

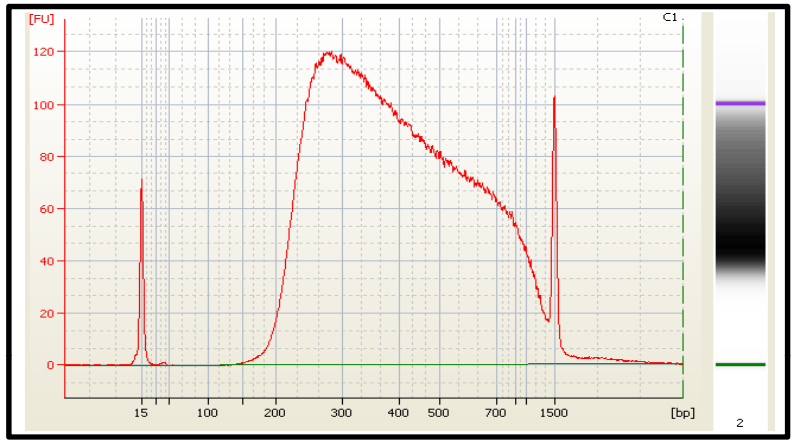


Cause : Too much DNA was used for shearing on Covaris, resulting in incomplete shearing. For example, Covaris settings in the SureSelect protocol are optimized to yield a peak size range of 150-200bp when starting with a maximum of 3µg gDNA. Adhere to recommended amounts of starting material.

Maria-Celeste Ramirez, PhD
Agilent Technologies

DNA Fragmentation – Uneven shearing

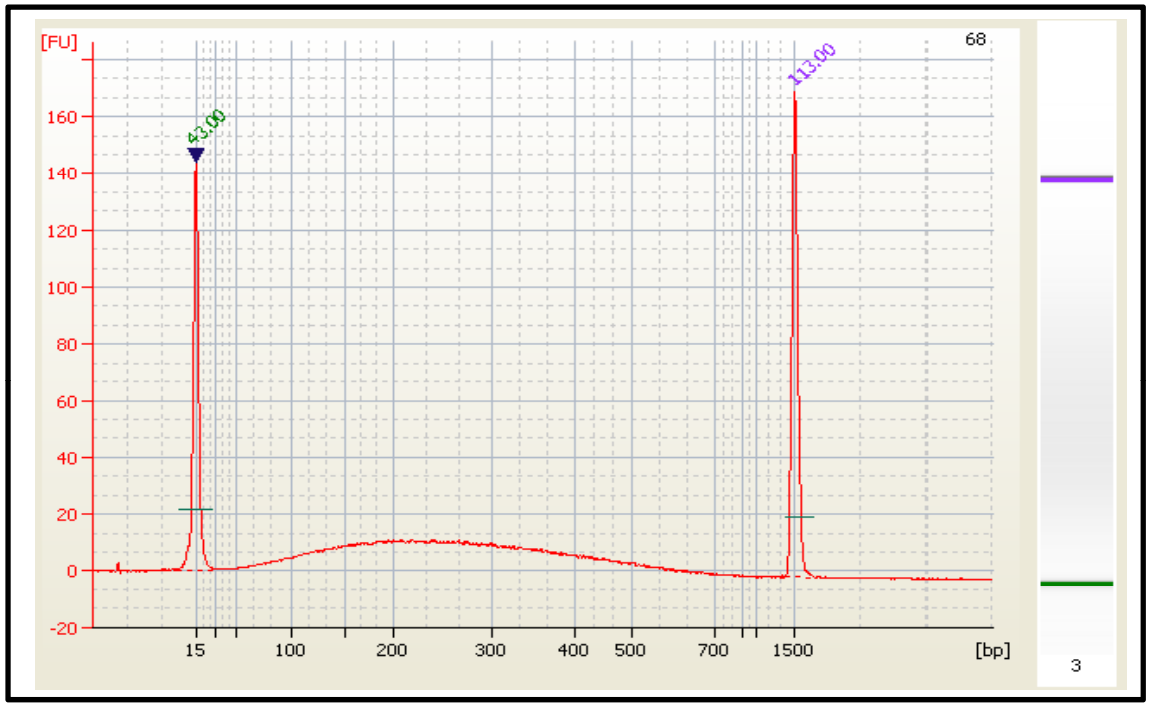




Causes :

- **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 6-8^{o}C.**
  - **Insufficient degassing.**

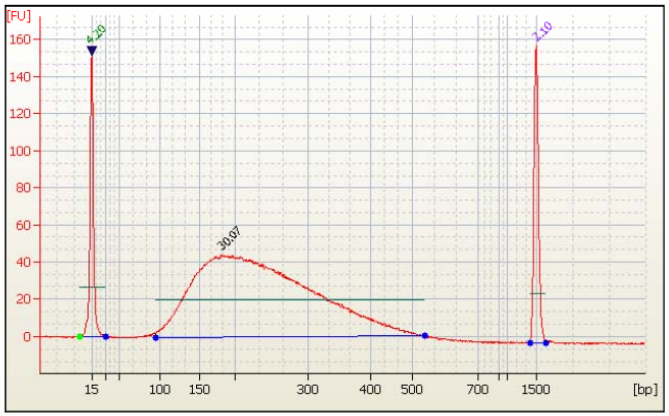
DNA Fragmentation – Increased size range



Cause : Covaris issues. Perform a control experiment using DNA of known quality, such as commercially available lambda DNA.

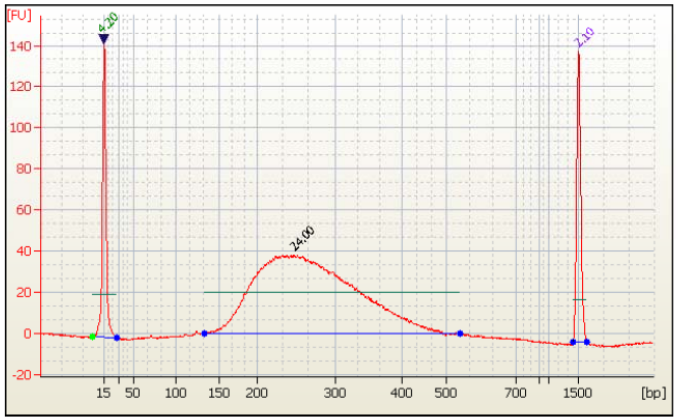
3 Monitoring size distribution after adapter-ligation

Post - fragmentation

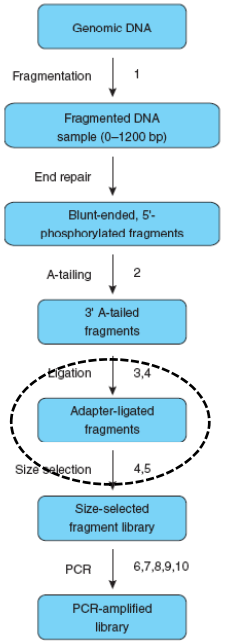


Peak size of 190bp

After adaptor ligation

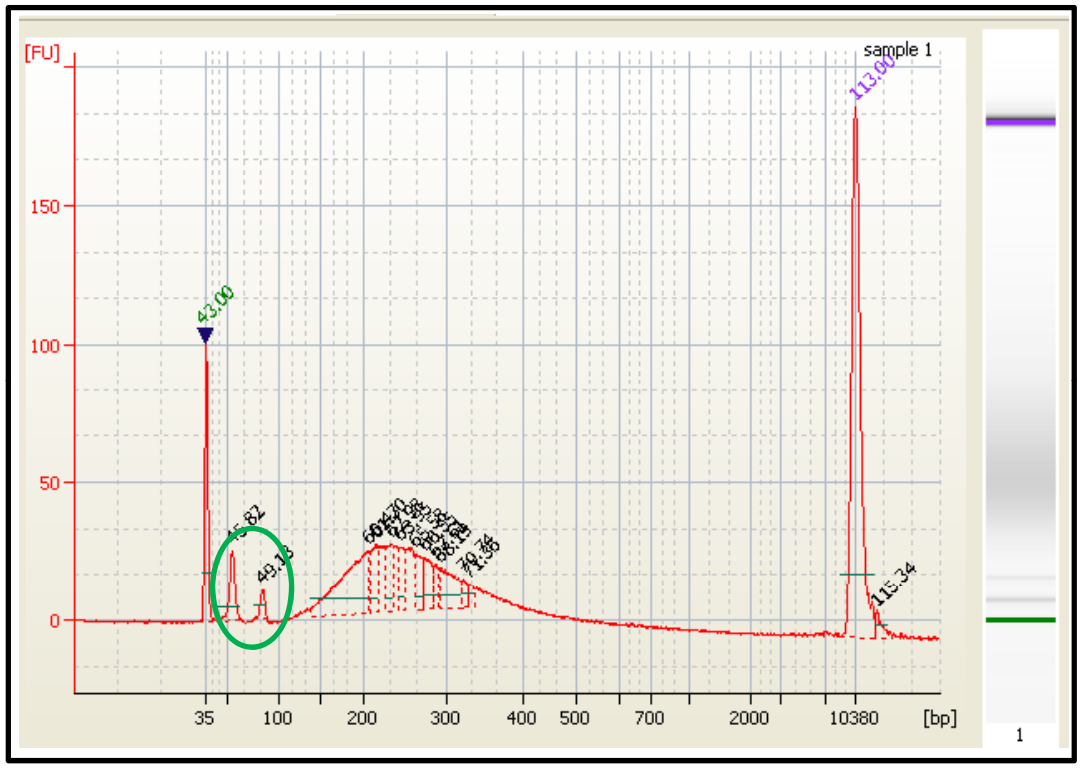


Peak size of 250± 10bp



- **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.**

Adapter ligation – Excess adapters



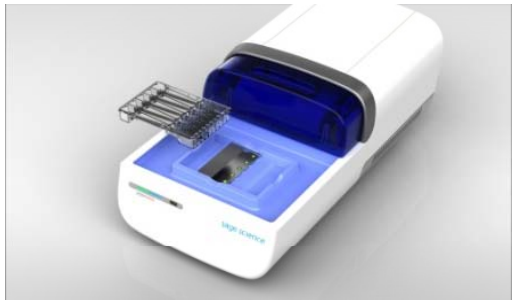
Cause : Inefficient ligation due to too much input DNA or the use of incorrect ligation temperature (ligation is performed at 20-25°C. When using a PCR machine, make sure the lid is not heated).

Automated size selection after adapter ligation

Update

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

Automated size selection after adapter ligation

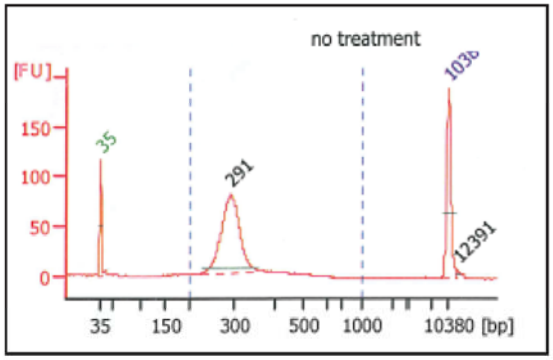
Update

- **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 37°C 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.**

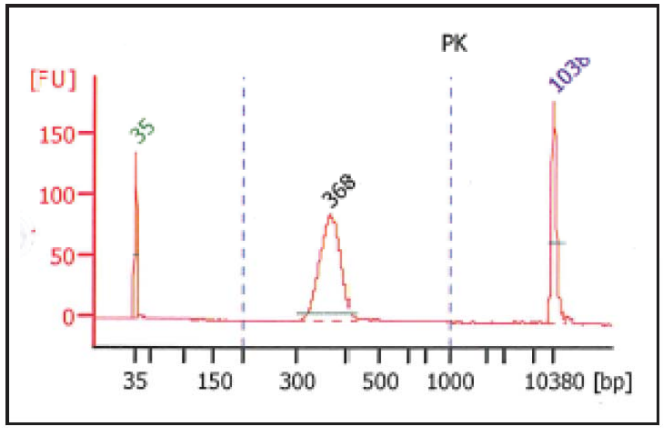
Pippin Prep™ DNA Size Selection System

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.

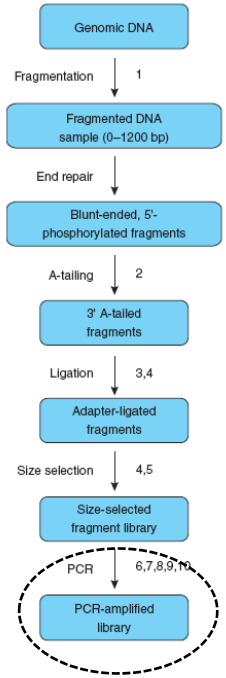


No treatment



Proteinase K treatment

4 Quality control after PCR amplification



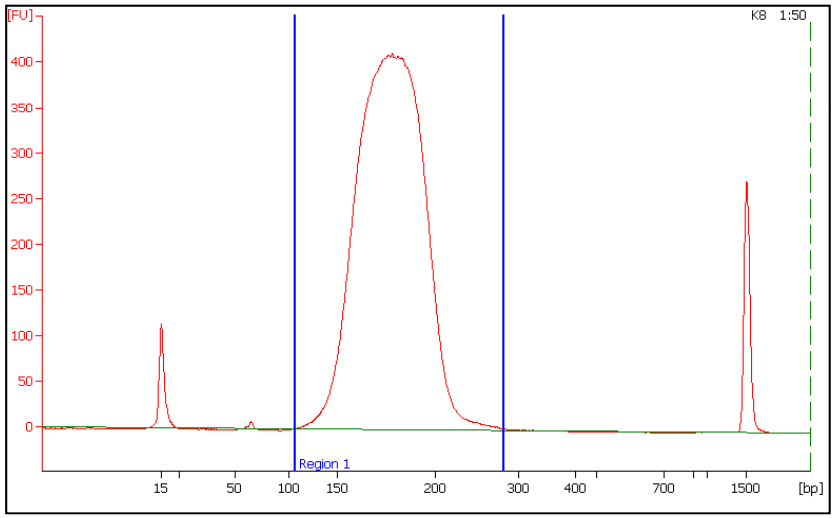
- **PCR amplification is necessary in library preparation to enrich for adapter-ligated fragments, as well as to add indexes.**

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

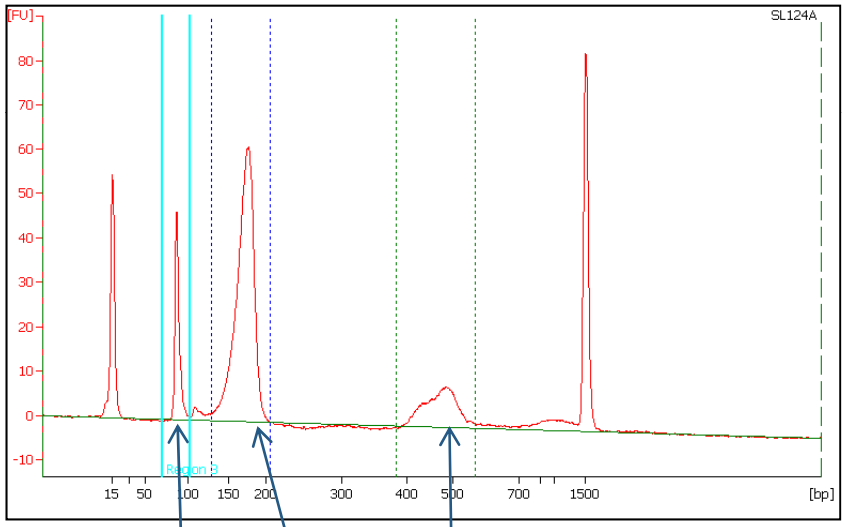
- **PCR can create bias since some regions of template DNA amplify more poorly than others (eg, GC-rich regions). PCR artifacts caused by overamplification or primer dimers can also affect sequencing coverage and accuracy.**

PCR amplification – detecting artifacts

High Quality DNA library



Primer dimers and PCR artifact



Primer dimers

PCR artifact

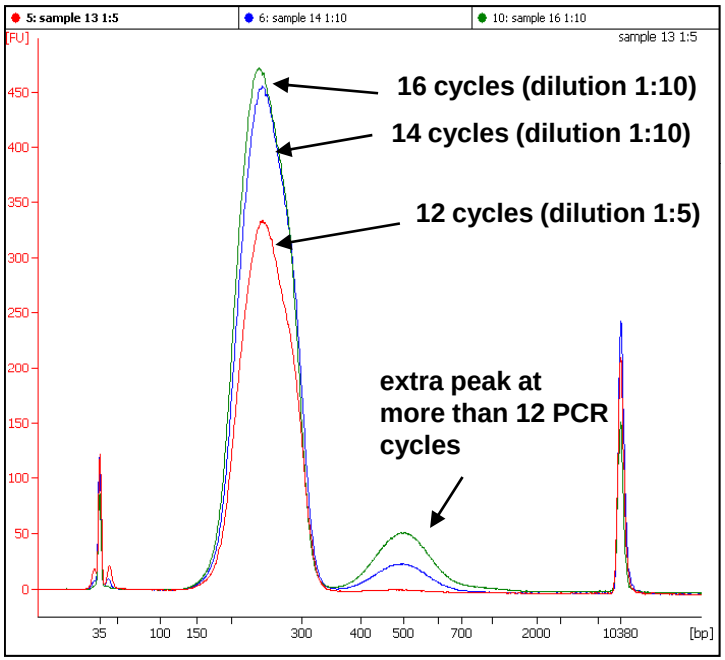
DNA library (50% of total DNA)

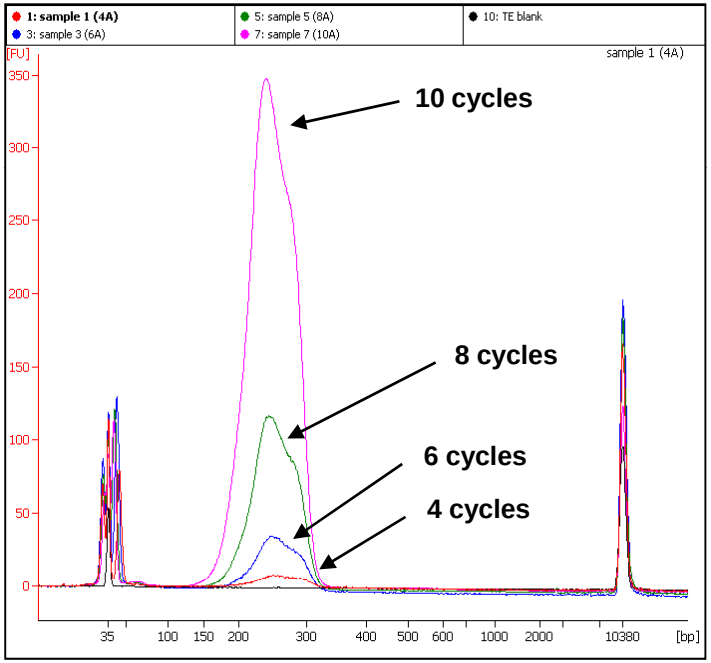
Dealing with primer dimers and artifacts

- **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.**

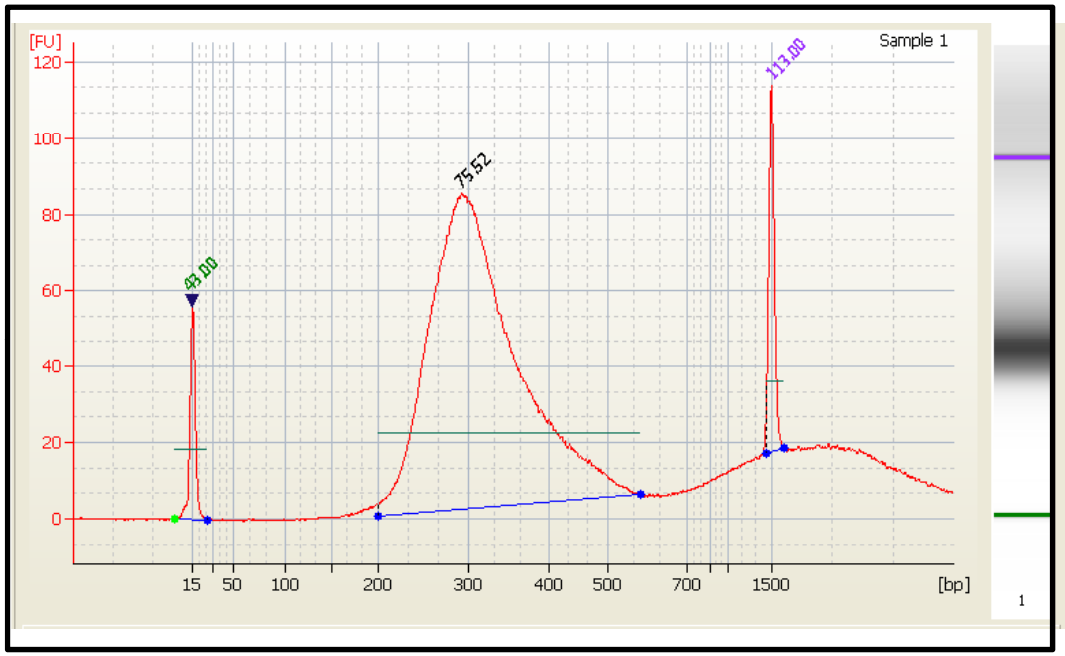
Reducing number of amplification cycles

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



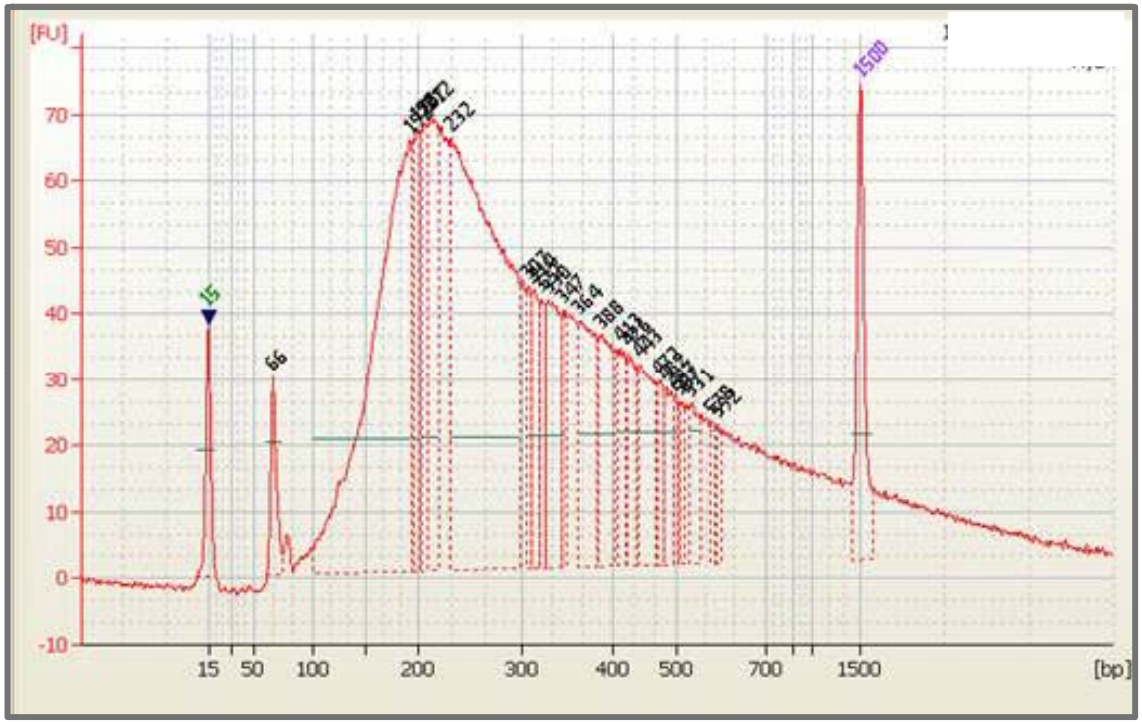


PCR amplification – Bead carryover



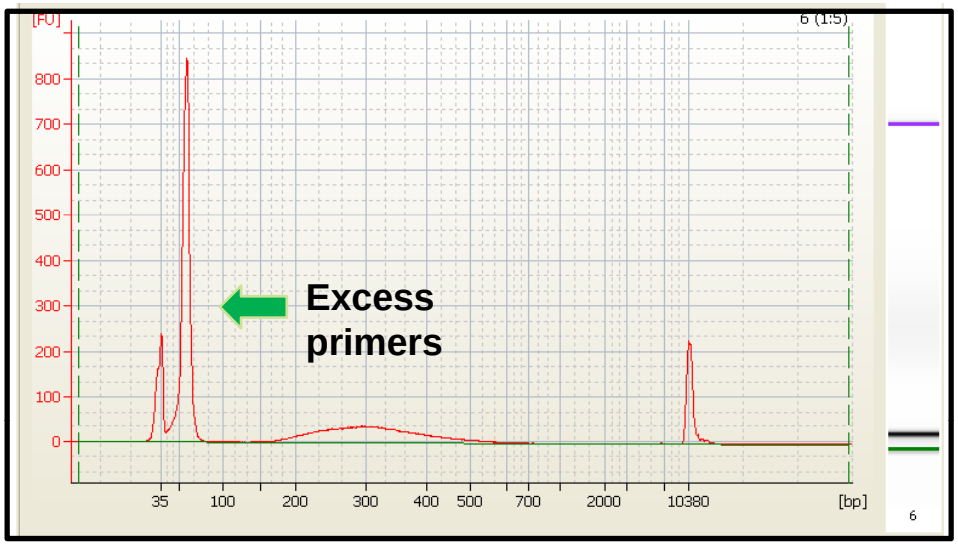
Cause : SPRI bead carryover from post-PCR clean-up step. Use a strong magnet for bead separation and pipette carefully during elution to avoid disturbing beads.

PCR amplification – Residual Buffer



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

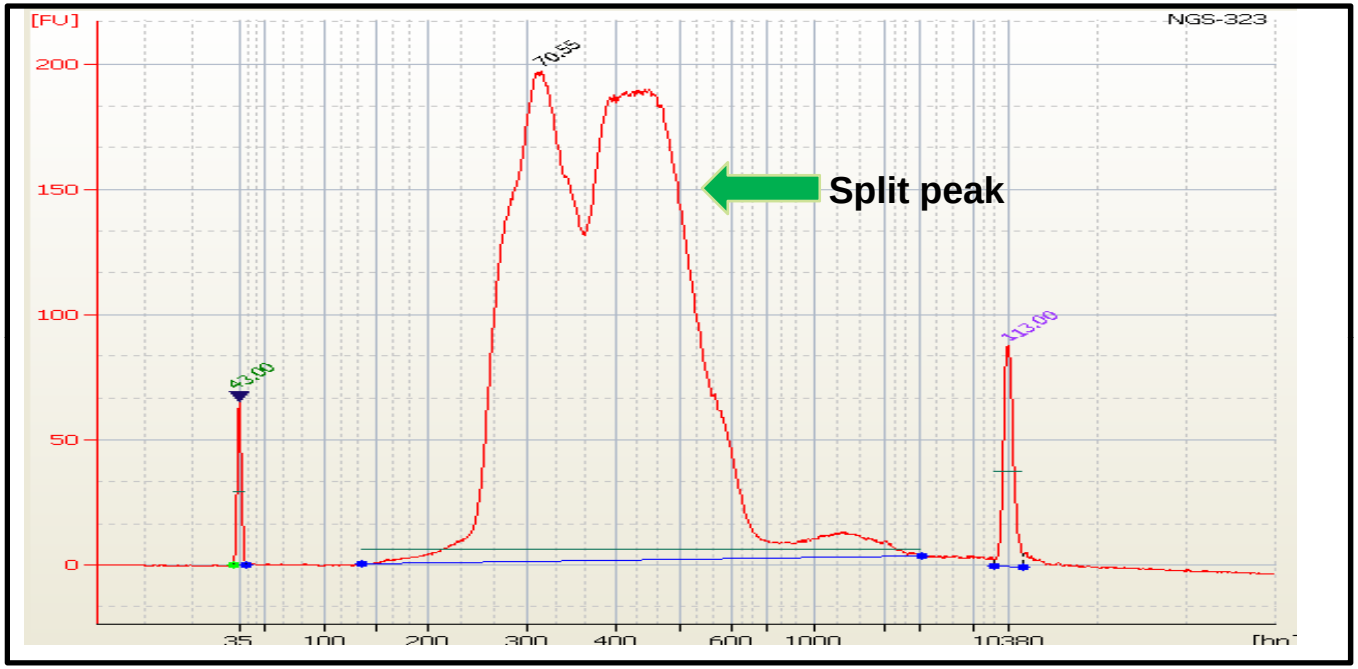
PCR amplification – Low PCR yield



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**

Amplification-free Library Preparation

Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes

Iwanka Kozarewa[1,2], Zemin Ning[1,2], Michael A Quail[1], Mandy J Sanders[1], Matthew Berriman[1] & Daniel J Turner[1]

- **Publication from the Sanger Institute on sequencing of GC-biased genomes using an amplification-free protocol for library construction.**
- ***P.falciparum*** **(malaria) and** ***B.pertussis*** **genomes are 85% AT-rich and 68% GC-rich, respectively.**
- **Any PCR-based amplification steps will create significant bias.**
- **Special adapters were designed to contain complementary sequences to oligos on surface of flow cell.**
- **Due to the absence of amplification, they used the High Sensitivity DNA Kit to quality control their libraries and QPCR to quantitate.**

Library[a]	Organism	Genome size (Mb)	Insert size (bp)	Read length (bp)	Number of reads	Fold raw read coverage	Number of assembled bases
NP-3D7-S	*P. falciparum* 3D7	23	200	36	28,009,122	43	19,025,823
NP-3D7-L	*P. falciparum* 3D7	23	200	76	19,556,224	64	21,092,855
STD-PF88	*P. falciparum* 3D7	23	200	37	110,939,984	174	NA[b]
STD-PF3	*P. falciparum* 3D7	23	200	37	75,083,768	114	NA[b]
STD-PF2	*P. falciparum* 3D7	23	200	37	62,802,164	96	NA[b]
STD-PF85	*P. falciparum* 3D7	23	200	37	13,530,194	21	NA[b]

Kozarewa *et al*, Nat Methods. 2009 Apr;6(4):291-5.

Agenda

1. **Next-gen sequencing library preparation workflow and important quality control steps.**

2. **Introduction to the 2100 Bioanalyzer.**

3. **The use of Bioanalyzer assays for:**

   - **assessing quality of starting material**
   - **monitoring size distribution after fragmentation and adapter ligation**
   - **quantifying yield and detection of artifacts post-PCR amplification**
   - **detecting small quantities of DNA in amplification-free protocols**

4. **Best practice tips to always obtaining accurate and reproducible data when using the Bioanalyzer.**

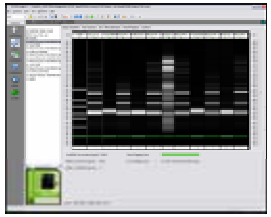
Bioanalyzer RNA/DNA Assay Workflow

Prepare gel-dye mix

Prime chip and load samples

Load chip and run

Quality, Sizing and Quantification data



Critical steps to success - Reagents

- **All reagents need to be warmed up to room temperature (~ 25°C) for at least 30min prior to preparation. Using cold reagents will affect the run.**

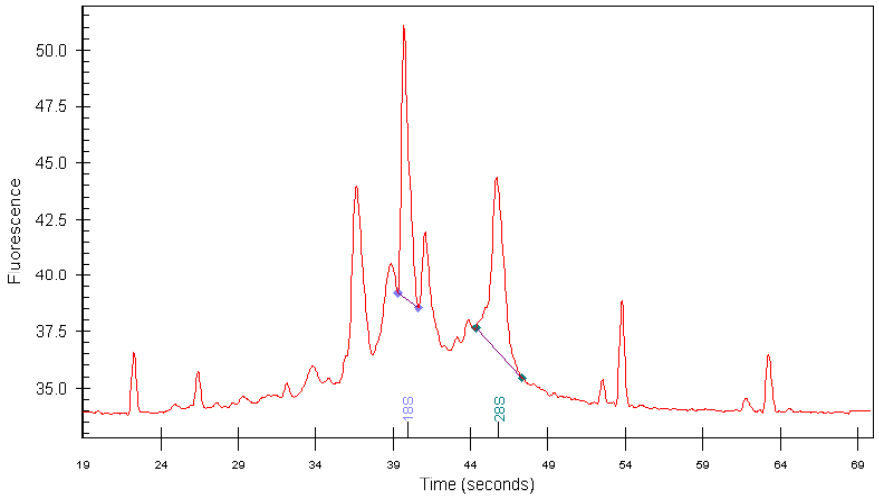
- **Vortex and spin down gel matrix, dye, marker and ladder to mix well.**

- **For the RNA assay, both the ladder and RNA samples have to be denatured at 70°C for 2-5min before loading on chip.**

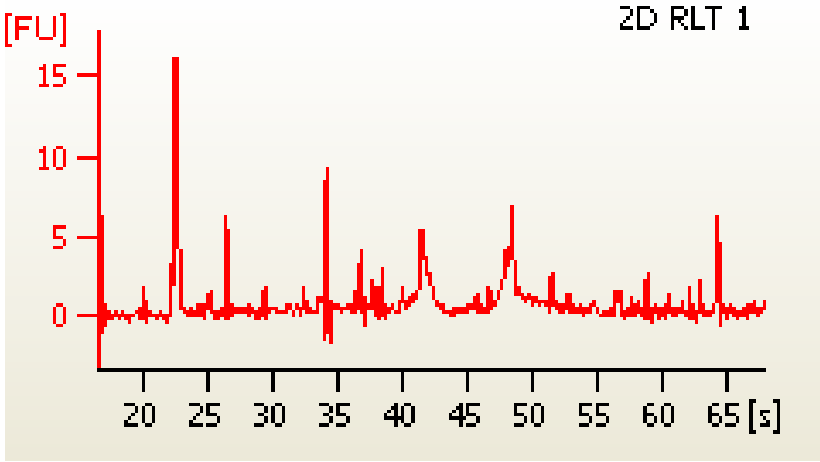
- **After centrifuging the gel/dye mix, avoid pipetting from the bottom of tube to prevent picking up gel particles. These particles can cause spikes.**

DO NOT use expired reagents!

Effects of suboptimal reagent preparation



‘Ghost peaks’ due to use of cold reagents.



Spikes due to presence of gel particles or use of expired reagents

Critical steps to success - Priming

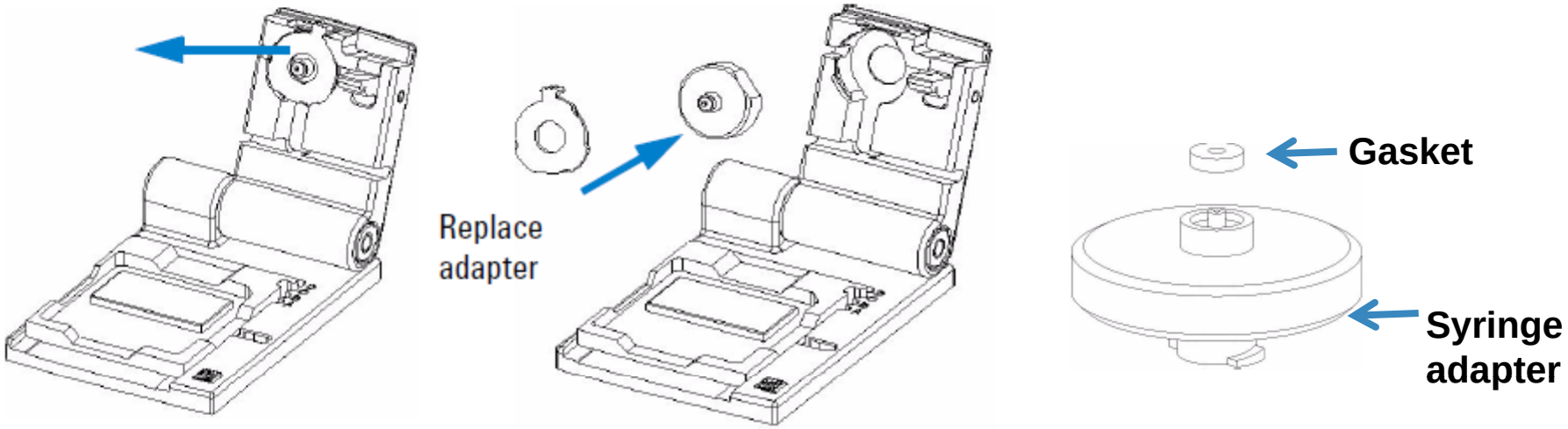
- **Ensure that the syringe clip is in the correct position for your assay eg. highest position for RNA Nano assay and lowest for DNA 1000 assay. Refer to Quick Guides.**

- **Retract plunger to 1ml mark before locking the priming station. Press the silver metal down till you hear a 'Click'. If the plunger is not at 1ml mark, unlock priming station before retracting to prevent drawing up gel/dye.**

- **Upon releasing the syringe clip, the plunger will retract quickly and come to a stop. Count 5sec before pulling plunger back up to 1ml mark.**

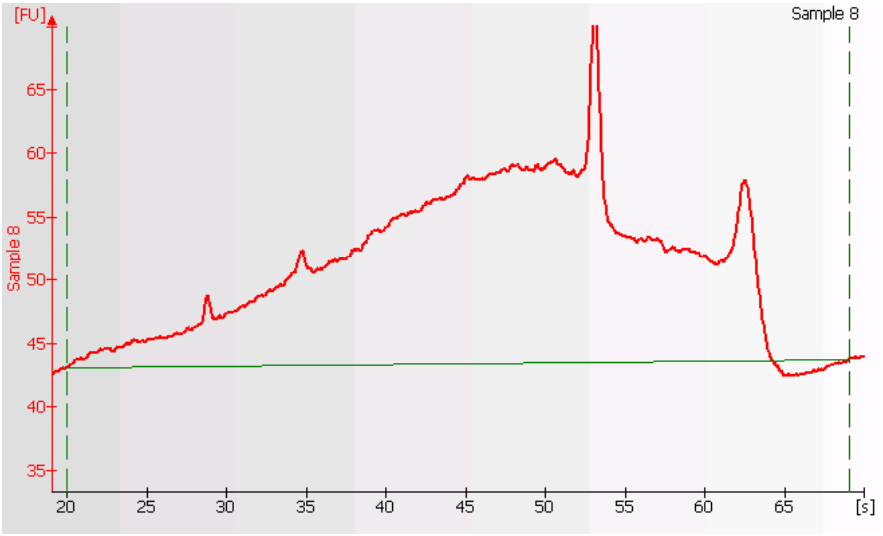
- **Do not over- or underprime. Load samples and run chip within 5-10min of priming.**

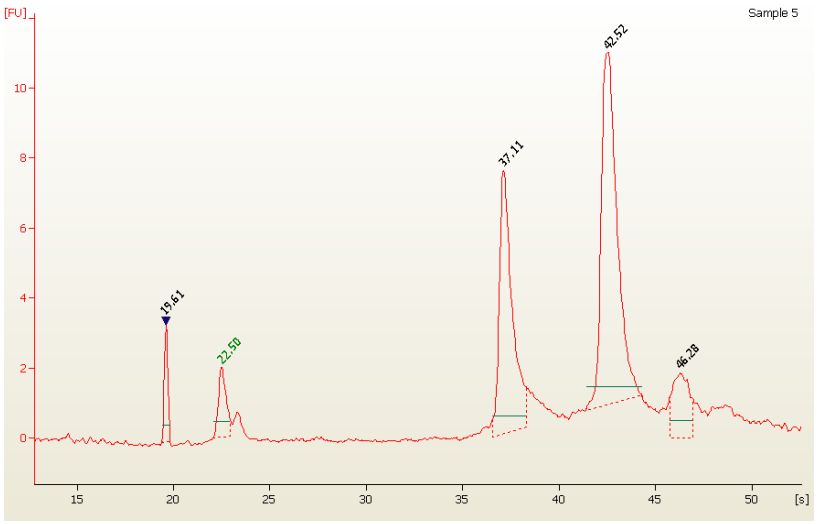
Critical steps to success – Maintaining your priming station



- **Replace gasket and clean syringe adapter every 3 months or as needed. The pores should not be clogged with dried gel.**
- **Replace syringe with every new kit.**
- **Perform a pressure test to check condition of priming station. With an unused chip, prime, count 5 sec and release. The plunger should retract to the 0.3ml mark in 1-2 sec.**

Effects of mispriming





Wavy and elevated baseline

Late migration

Critical steps to success – Electrode Cartridge

- **The electrode cartridge needs to be completely dry and clean before a run. Liquid or salts on the pins will cause leak currents.**

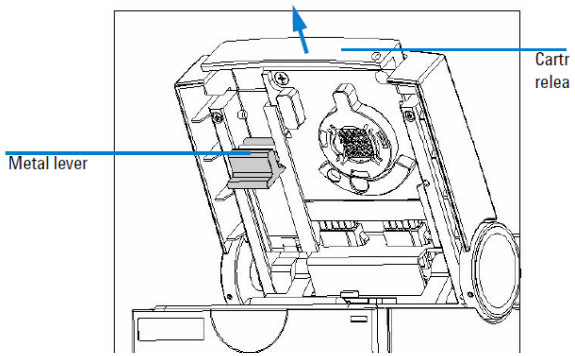
- **Prior to running the RNA Nano assay, fill electrode cleaner with 350µl of RNaseZap and soak pins for 30 sec. Clean three times each with 350µl fresh sterile water for 1min and let pins dry. Wash with sterile water once after run. Do not use RNaseZap with the RNA Pico assay unless there is suspected RNase contamination.**

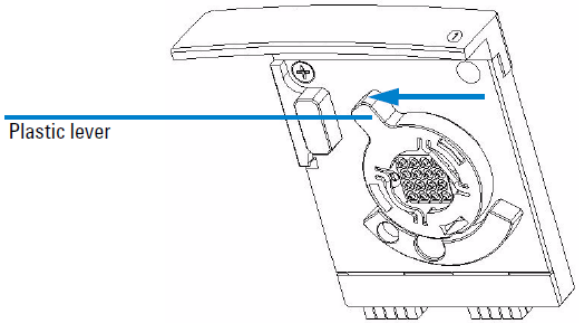
- **For DNA assays, wash once before and after run with 350µl of sterile water.**

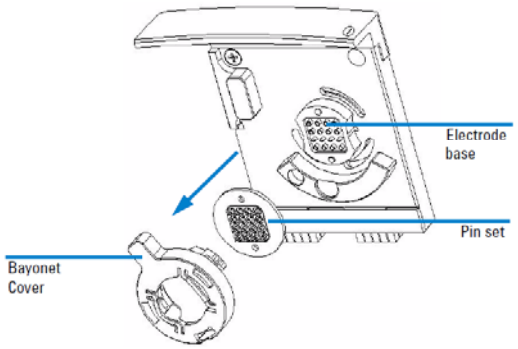
- **Remove chip within 30 min of run. DO NOT leave chips in Bioanalyzer overnight.**

- **Do not vortex chips for more than 1min. Check for spills on surface of chip.**

Critical steps to success – Maintaining the electrode cartridge





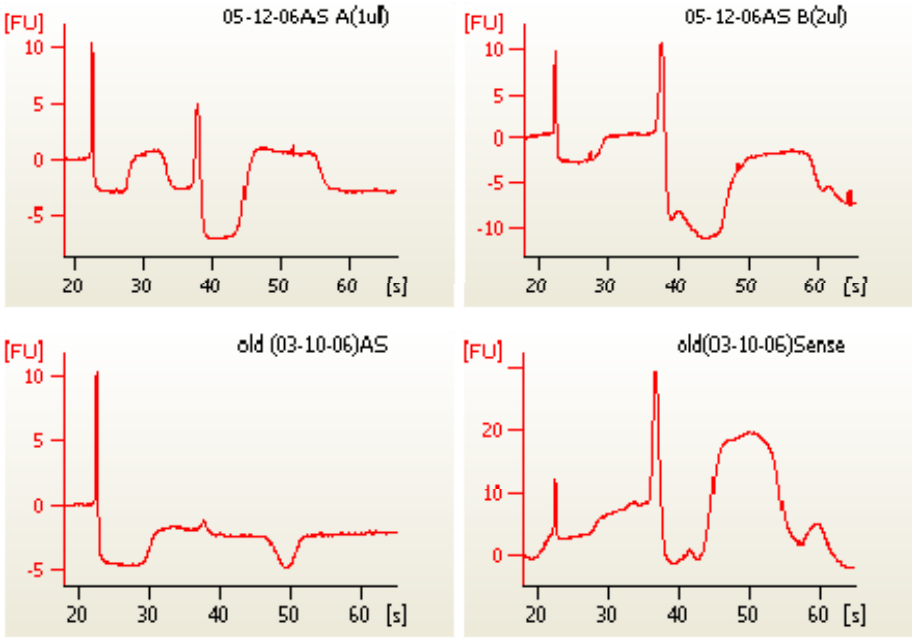


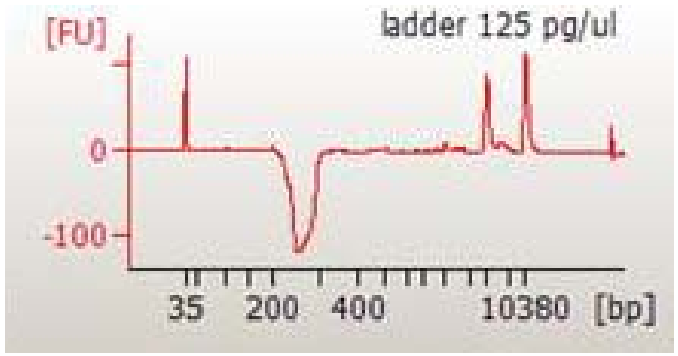
- **Remove pins and clean with sterile water and a soft toothbrush every 3 months or as needed.**

- **Let pins dry completely before re-assembling the cartridge.**

- **To check dryness and cleanliness of pins, run a Short Circuit Test using an unused chip. Go to ‘Instrument Context’, select ‘Diagnostics’ tab and check ‘Short Circuit Test’.**

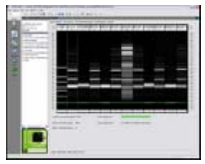
Effects of a wet or dirty pinset

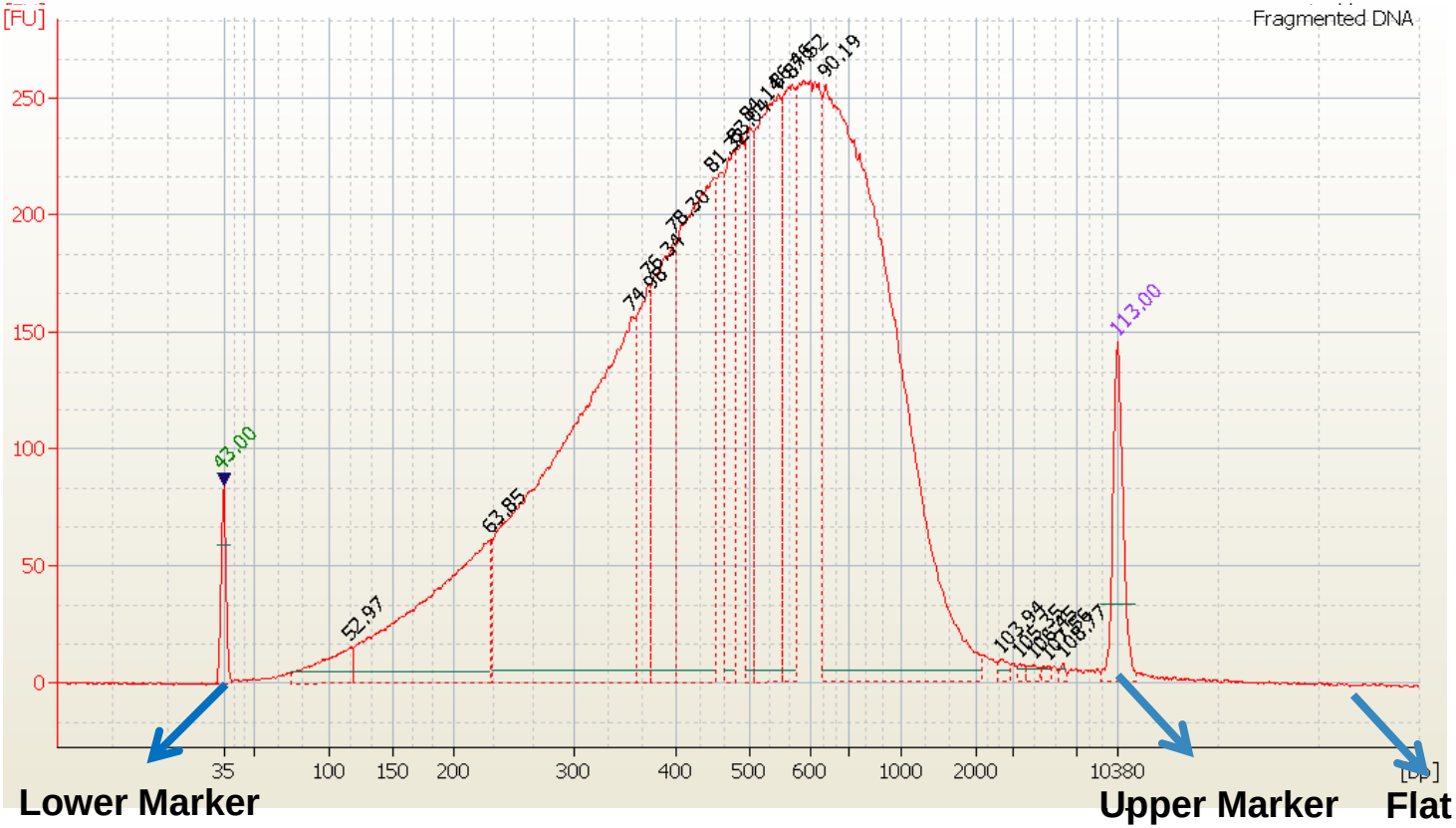




Wavy baselines and dips caused by leak currents.

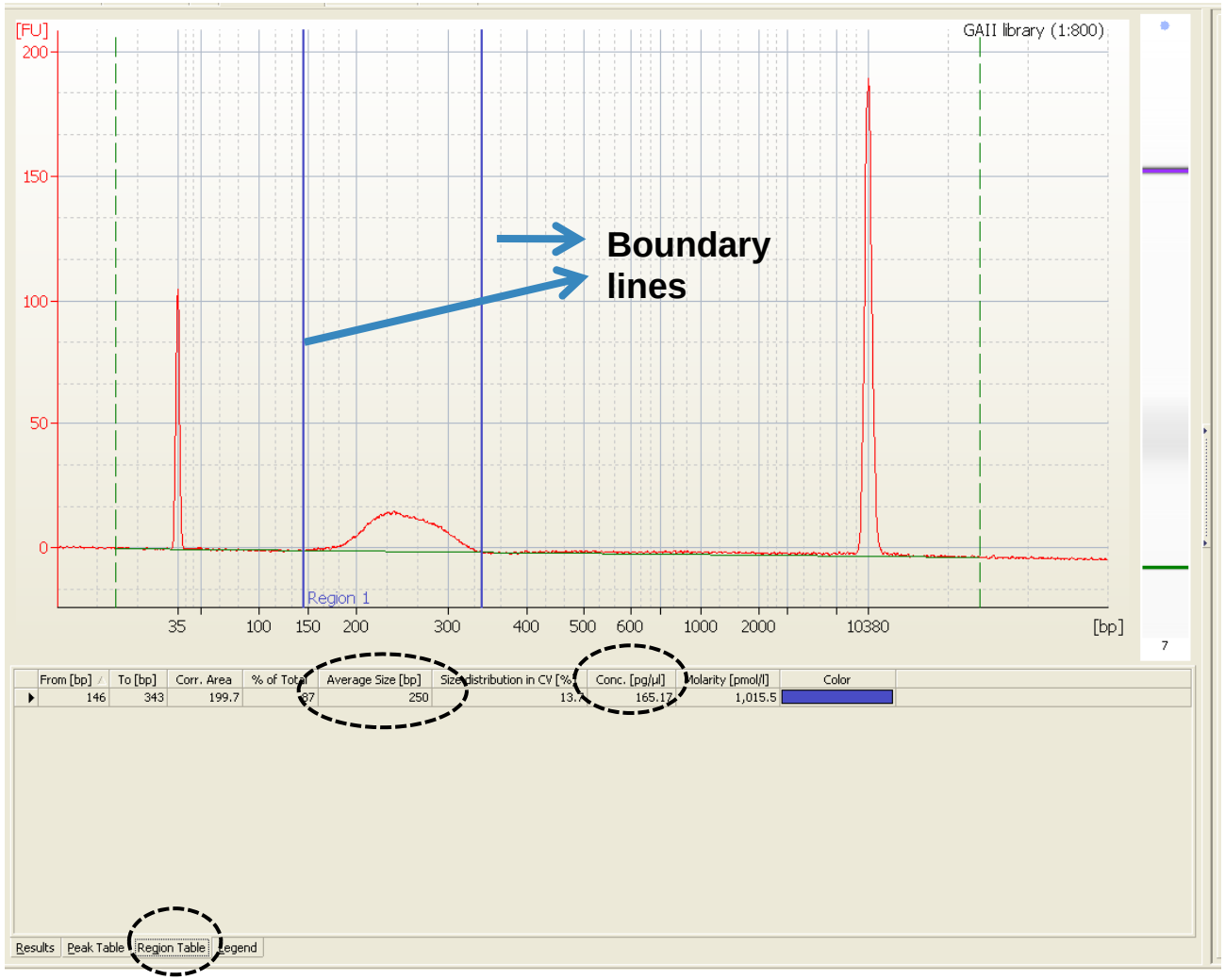
Critical steps to success – Data Analysis



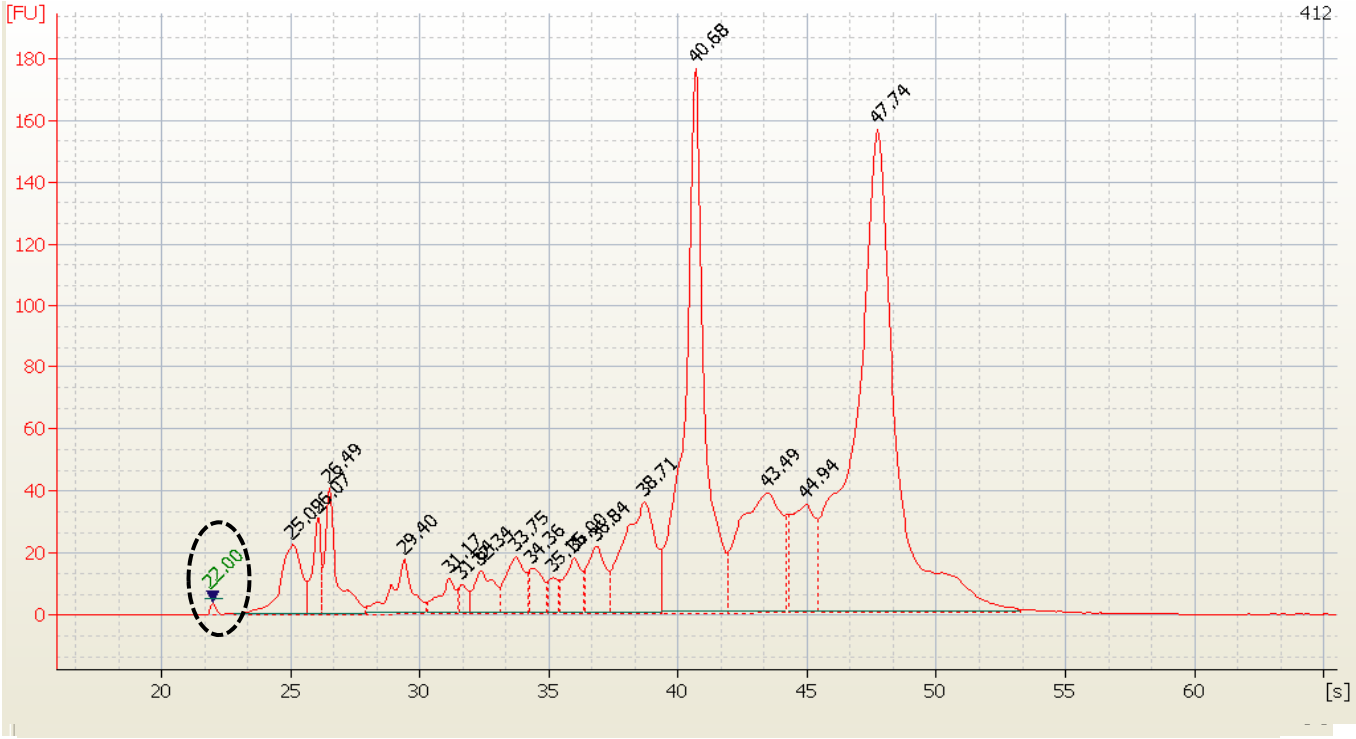


- **Check that all ladder peaks are present.**
- **Ensure that Lower and Upper Markers have been assigned correctly.**
- **Ensure that baselines are flat**

Critical steps to success – Data Analysis



Critical steps to success – Manually assigning markers



The Lower Marker is now correctly assigned. Sizing, fragment recognition and quantification are now accurate.

Summary

- The importance of starting a next-gen sequencing experiment with a high-quality library of DNA fragments cannot be over-emphasized.
- All major next-gen sequencing and SureSelect ™ protocols incorporate multiple quality control steps during library preparation, utilizing Bioanalyzer assays.
- RNA and DNA assays are used to :

  - Assess quality of starting material
  - Monitor size distribution after fragmentation
  - Determine success of adapter-ligation
  - Determine yield and quality of PCR-amplified libraries
  - Detect very small amounts of RNA/DNA in minimally-amplified or amplification-free protocols

- It is necessary to adhere to Bioanalyzer assay protocols, as well as to maintain the priming station and electrode cartridge regularly to ensure succesful sample analysis and obtain accurate data.