TruSeq™ Sample Preparation Best Practices and Troubleshooting Guide

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Introduction

When preparing libraries for sequencing, you should always adhere to good molecular biology practices. This document explains Illumina® recommended best practices when performing TruSeq™ sample preparation and enrichment protocols. It is a supplement to the *TruSeq DNA Sample Preparation Guide*, *TruSeq RNA Sample Preparation Guide*, and *TruSeq Enrichment Guide*, which also contain best practices information, and assumes that you have reviewed those documents for details on performing the procedures.

Liquid Handling

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes give rise to very large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent BioAnalyzer.
- If small volumes are unavoidable, then use due diligence to ensure that pipettes are correctly calibrated.
- Ensure that pipettes are not used at the volume extremes of their performance specifications.
- Care should be taken with solutions of high molecular weight double-stranded DNA (dsDNA). These can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.
- To minimize pipetting errors, especially with small volume enzyme additions, prepare the reagents for multiple samples simultaneously. As a result, you pipette once from the reagent tubes with a larger volume, rather than many times with 1 µl volumes. This will allow you to aliquot in a single pipetting movement to individual samples and standardize across multiple samples.

RNA Handling

When preparing mRNA libraries for sequencing, you should always adhere to good molecular biology practices. RNA is highly susceptible to degradation by RNase enzymes. RNase enzymes are present in cells and tissues, and carried on hands, labware, and dust. They are very stable and difficult to inactivate. For these reasons, it is important to follow best laboratory practices while preparing and handling RNA samples.

- When harvesting total RNA, use a method that quickly disrupts tissue and isolates and stabilizes RNA.
- Wear gloves and use sterile technique at all times.
- Reserve a set of pipettes for RNA work. Use sterile RNase-free filter pipette tips to prevent cross-contamination.
- Use disposable plasticware that is certified to be RNase-free. Illumina recommends the use of non-sticky sterile RNase-free microfuge tubes. A set of these tubes should be designated for this protocol and should not be used for other lab work.
- All reagents should be prepared from RNase-free components, including ultra pure water.
- Store RNA samples by freezing. Avoid extended pauses in the protocol until the RNA is in the form of double-stranded (ds) DNA.
- Use a RNase/DNAse decontamination solution to decontaminate work surfaces and equipment prior to starting this protocol.

Master Mix Reagent Handling

When handling the master mix reagents:

- Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze-thaw cycles. However, if you aliquot, you may not have enough reagents for 48 reactions over multiple uses.
- Add reagents in the order indicated in the *TruSeq DNA Sample Preparation Guide* and *TruSeq RNA Sample Preparation Guide* and avoid making master-mixes containing the in-line controls.
- Take care while adding the A-Tailing Mix (ATL) and Ligation Mix (LIG) due to the viscosity of the reagents.

AMPure XP Handling

Follow appropriate handling methods when working with Agencourt AMPure XP Beads:

NOTE

Cleanup procedures have only been verified using a 300 µl 96-well PCR or MIDI plate. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats.

NOTE

Cleanup procedures have only been tested and validated using the magnetic stand specified in the *TruSeq DNA Sample Preparation Guide*, *TruSeq RNA Sample Preparation Guide*, and *TruSeq Enrichment Guide*. Comparable performance is not guaranteed when using other magnets.

- Prior to use, allow the beads to come to room temperature.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- **When performing the LT protocol**, after adding the beads to the reaction, mix the solution gently and thoroughly by pipetting up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.
- **When performing the HT protocol**, after adding the beads to the reaction, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes. Repeat, if necessary, until the color of the mixture appears homogeneous after mixing.
- Take care to minimize bead loss which can impact final yields.
- Change the tips for each sample.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- For the wash steps, prepare fresh 80% ethanol. Ethanol tends to absorb water from the air, therefore, fresh 80% ethanol should be prepared for optimal results.

- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, as the presence of ethanol will impact the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time may be required.
- Use the Resuspension Buffer (RSB) for DNA elution.
- To maximize elution of the DNA, incubate the DNA/bead mixture for 2 minutes at room temperature before placing the samples onto the magnet.

Avoid Cross-Contamination

Follow these practices to avoid cross contamination:

- Open only one adapter at a time.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Potential DNA Contaminants

Avoid potential DNA contaminants:

- Incorrect DNA quantitation may result from DNA contamination, for example, by interference from superfluous nucleic acids in a sample (e.g., RNA, small nucleic acid fragments, nucleotides, single-stranded DNA), excess proteins, or other contaminating materials.
- DNA quality may also affect the quantity of usable DNA in a sample. For example, if the DNA is damaged (e.g., heavily nicked or containing extensive apurinic/apyrimidinic sites), then many of these fragments may fail during library preparation.
- High molecular weight dsDNA derived from host genomes can also interfere with accurate quantitation. For example, bacterial artificial chromosomes (BACs) and other bacterially-derived plasmids usually contain a small percentage of the chromosomal DNA from the host cells, despite the best purification efforts. These sequences may ultimately give rise to unwanted clusters on a flow cell lane. However, this contamination can be accurately quantified by analyzing aligned reads generated during sequencing against known bacterial sequences and subtracting these out. High molecular weight contamination may also be estimated prior to library preparation using qPCR assays designed to target unique chromosomal markers.

Temperature Considerations

Temperature is an important consideration for making gDNA libraries:

- Keep libraries at temperatures ≤37°C.
- Avoid elevated temperatures, particularly in the steps preceding the adapter ligation.
- DNA fragments that have a high AT content are more likely to denature into single strands than GC-rich fragments, which can result in an increased probability of creating a bias in the sequencing coverage.
- Take care not to denature the library prior to the agarose gel electrophoresis process, because single-stranded DNA has a different migration rate.
- When performing the TruSeq RNA protocol, temperature is less of an issue after the adapters have been ligated onto the ends of the ds cDNA.

Input Recommendations

DNA Input

When performing the TruSeq DNA Sample Preparation or TruSeq Enrichment protocols, follow these gDNA input recommendations:

Input DNA Quantitation

- Correct quantification of genomic DNA is essential.
- Illumina recommends 1 µg input DNA.
- The ultimate success or failure of a library preparation strongly depends on using an accurately quantified amount of input DNA.
- Illumina recommends using fluorometric based methods for quantification including Qubit or PicoGreen to provide accurate quantification for dsDNA. UV-spec based methods, such as the Nanodrop, will measure any nucleotides present in the sample including RNA, dsDNA, ssDNA, and free nucleotides which can give an inaccurate measurement of gDNA.
- DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to excess nucleic acids.
  - These methods require the preparation of calibration curves and are highly sensitive to pipetting error.
  - Ensure that pipettes are correctly calibrated and are not used at the volume extremes of their performance specifications.

Assessing DNA Quality

- Absorbance measurements at 260 nm are commonly used to assess DNA quality.
  - The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity, and values of 1.8–2.0 are considered indicative of relatively pure DNA.
  - Both absorbance measurements can be compromised by the presence of RNA or small nucleic acid fragments such as nucleotides.
  - Genomic DNA samples should be carefully collected to ensure that they are free of contaminants.

- Gel electrophoresis is a powerful means for revealing the condition (even the presence or absence) of DNA in a sample.
  - Impurities, such as detergents or proteins, can be revealed by smearing of DNA bands.
  - RNA, which interferes with 260 nm readings, is often visible at the bottom of a gel.
  - A ladder or smear below a band of interest may indicate nicking or other damage to DNA.
  - Where possible, or necessary, a gel should be run to assess the condition of the DNA sample.

RNA Input

When performing the TruSeq RNA Sample Preparation protocol, follow these mRNA input recommendations:

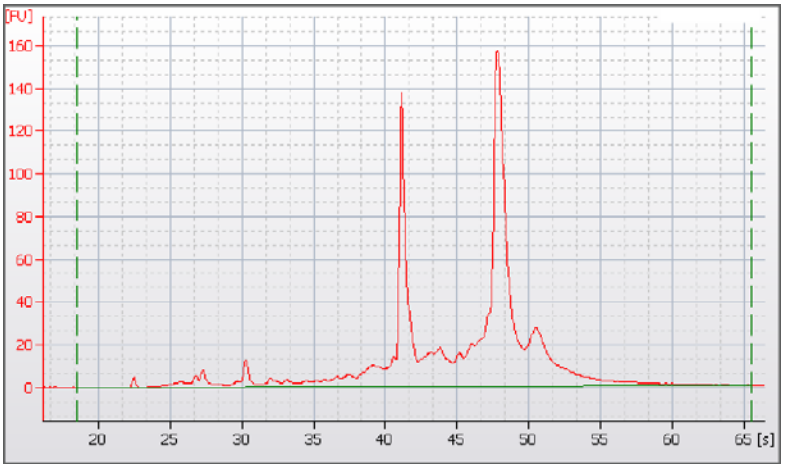
Total RNA Input

- Protocol is optimized for 0.1–4 µg of total RNA.
- Lower amounts may result in inefficient ligation and low yield.
- The protocol has been tested using 0.1–10 µg of high-quality universal human reference total RNA as input.
- Use of RNA from other species, tissues, or qualities may require further optimization with regard to the initial input amount.
- The protocol recommends diluting the in-line controls for tracking the steps involved in converting dsDNA into libraries.
  - The dilution is optimized for 0.1–4 µg of high quality input RNA.
  - When using less RNA or RNA with very low mRNA content, these controls may need further dilution.
  - If no controls are added, use Resuspension Buffer (RSB) in place of the controls in the protocol.
- It is very important to use high-quality RNA as the starting material.
  - Use of degraded RNA can result in low yield, over-representation of the 3' ends of the RNA molecules, or failure of the protocol.
  - Illumina recommends that you check total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value greater than or equal to 8. RNA that has DNA contamination will result in an underestimation of the amount of RNA used.

- Illumina recommends including a DNase step with the RNA isolation method. However, contaminant DNA will be removed during mRNA purification.

The following figure shows a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 1 Starting RNA Bioanalyzer Trace



Alternatively, you can run a formaldehyde 1% agarose gel and judge the integrity of RNA upon staining with ethidium bromide. High quality RNA shows a 28S rRNA band at 4.5 kb that should be twice the intensity of the 18S rRNA band at 1.9 kb. Both kb determinations are relative to a RNA 6000 ladder. The mRNA will appear as a smear from 0.5–12 kb.

Purified mRNA Input

You can also use previously isolated mRNA as starting material:

- Use the entire fraction of mRNA purified from 0.1–4 µg of total RNA.
- If you start with isolated mRNA, follow the Illumina recommendations for isolated mRNA specified in the introduction of the *Purify and Fragment mRNA* procedures in the *TruSeq RNA Sample Preparation Guide*. Begin mRNA fragmentation with the Make RFP step that adds the Elute, Prime, Fragment Mix to each well of the RBP plate.

Enrichment Input

When performing the TruSeq Enrichment protocol, follow these input recommendations:

- To assess the library quality, ensure that your DNA library is of the proper size by running a small aliquot on an agarose gel or running the sample on a Agilent BioAnalyzer.
  - If running samples on a Agilent BioAnalyzer High Sensitivity DNA chip, Illumina recommends first diluting the final library 1:50 followed by running 1 µl on the chip.
  - The BioAnalyzer should only be used for qualitative purposes as sample quantification can easily be skewed by improper loading of sample onto the BioAnalyzer chip.
- Determining library quantity using a fluorometric based system such as the Qubit dsDNA BR Assay System.
  - Ensure all solutions are at room temperature.
  - Illumina recommends using 2 µl of each DNA sample with 198 µl of the Qubit working solution for sample quantification.
  - Vortex the sample, taking care not to introduce any bubbles, and incubate for at least 2 minutes prior to taking any measurements.

User-Supplied Consumables and Equipment

Refer to the *TruSeq DNA Sample Preparation Guide, TruSeq RNA Sample Preparation Guide,* and *TruSeq Enrichment Guide* for complete lists of user-supplied consumables and equipment and their suppliers. Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation. Important items to note when ordering and collecting equipment and consumables:

- Different plates are required, depending upon if you are performing LT and HT processing:
  - LT processing: 300 µl 96-well skirtless PCR plates or Twin.Tec 96-well PCR plates
  - HT processing: Microseal 96-well PCR plates ("HSP" plate)
- 96-well magnetic stand
- Microseal 'A' film
- Microseal 'B' adhesive seals
- Freshly prepared 80% Ethanol
- 96-well thermal cycler with programmable heated lid
- To purify ligation products using the gel method for TruSeq DNA sample preparation, the following user-supplied consumables and equipment are required:

Consumable or Equipment	Supplier
6X gel loading dye	BioLabs, catalog # B7021S
BenchTop 100 bp DNA ladder	Promega, part # G829B
Certified low-range ultra agarose	BIO-RAD, part # 161-3107
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
MinElute Gel Extraction Kit	QIAGEN, part# 28604
SyBr Gold Nucleic acid gel stain	Invitrogen, part # S11494
Thermo Scientific Owl B2 EasyCast Mini Gel System	(US) Thermo Scientific, part # B2, or Fisher Scientific, part # 09-528-110B (Other Regions) Fisher Scientific, part # OWL-130-101J B

Covaris Shearing

The TruSeq DNA Sample Preparation fragmentation process was optimized to obtain final libraries with the following differences:

	Whole-genome Resequencing	TruSeq Enrichment	
	Gel Method	Gel-free Method	Gel Method
Covaris Shearing Duration	40 seconds	120 seconds	
Insert Size	300–400 bp	100–900 bp	200–300 bp

- Covaris recommended water fill volume:
  - S Series: fill line level 12
  - E Series: fill line level 6
- Fragment the DNA using the following settings:

	Whole-genome Resequencing	TruSeq Enrichment
Duty cycle	10%	10%
Intensity	5.0	5.0
Bursts per second	200	200
Duration	40 seconds	120 seconds
Mode	Frequency sweeping	Frequency sweeping
Power	23W	23W
Temperature	5.5° to 6°C	5.5° to 6°C

NOTE

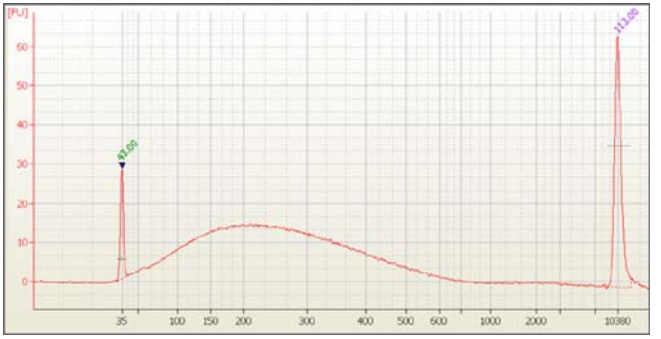
These settings are optimized for creating library inserts of 200–400 bp and may need to be modified for other insert sizes.

- Low input requirements for TruSeq DNA Sample Prep requires shearing of 1 µg of gDNA in 52.5 µl of Resuspension Buffer
- The formation of excessive bubbles or an air gap can lead to incomplete shearing.
  - Load the DNA into the Covaris tube very slowly to avoid creating air bubbles.
  - Centrifuge the Covaris tube to collect the sample at the bottom of the tube prior to shearing.

The following are examples of traces of the DNA sample at intermediate stages of the TruSeq DNA sample preparation process.

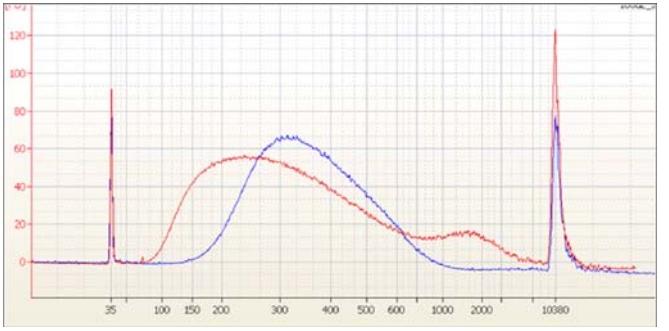
gDNA post Covaris shearing run on Agilent BioAnalyzer High-Sensitivity DNA chip with a 1:5 dilution.

Figure 2 gDNA Post Covaris Shearing



DNA library post Clean Up IMP run on an Agilent BioAnalyzer High-Sensitivity DNA chip with a 1:20 dilution. Libraries prepared with the gel method are shown in red and those prepared with the gel-free method are shown in blue.

Figure 3 gDNA Post Clean Up IMP



Purify Ligation Products (Gel Size Selection)

Follow these recommendations when performing the TruSeq DNA Sample Preparation protocol and purifying ligation products using the gel method:

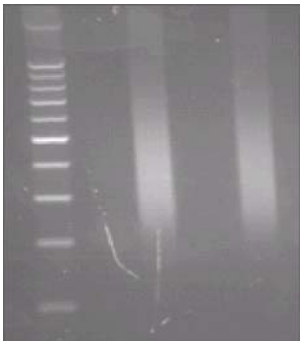
NOTE

These recommendations only apply to the TruSeq DNA Sample Preparation protocol when using the gel method.

- See *User-Supplied Consumables and Equipment* on page 16 of this guide for required user-supplied consumables and equipment.
- Do not overload your DNA ladder, which can lead to skewed migration patterns and incorrect size excision.
- DNA migration patterns can also be impacted by staining procedures. Illumina strongly recommends pre-staining your gel with SyBr Gold Nucleic acid gel stain.
- To reduce the likelihood of having to re-prep samples, you may opt to gel extract multiple bands from the gel (+/- 100 bp region from desired size range).
- Do not use smaller size gels or E-gels. DNA will not migrate as expected and can result in the purification of libraries in an unintended size range.

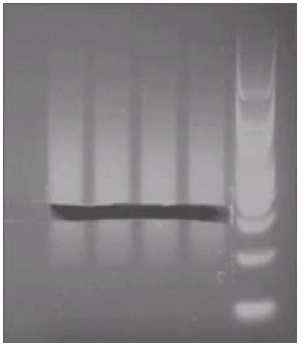
- Take care not to overload the ladder:
  - A correctly loaded ladder will show:
    - Clearly distinct and crisp ladder bands.
    - Good size separatio.n

Figure 4 Correctly Loaded Ladder



  - An incorrectly loaded ladder will show:
    - A “smile” which can lead to inaccurate size excision.
    - Poor size separation, which will lead to a broad library size distribution.

Figure 5 Incorrectly Loaded Ladder

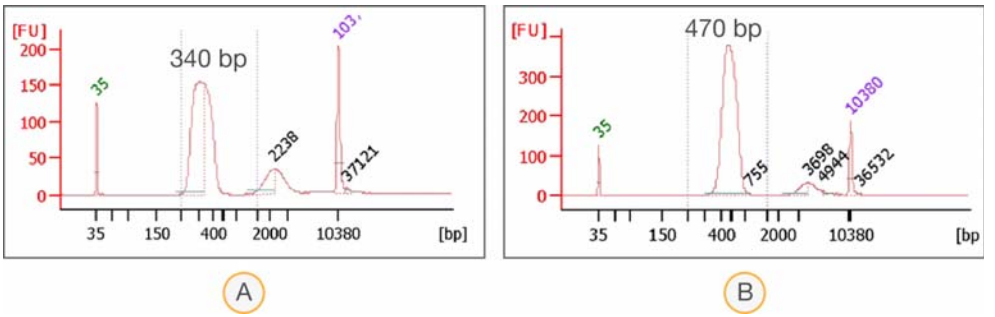


Pre and Post Staining

To help ensure accurate size-selection it is very important to pre-stain your gel with SyBr Gold. If the gel is stained post-electrophoresis, the DNA library can migrate differently with respect to your ladder. This can result in cutting out the wrong size fragments.

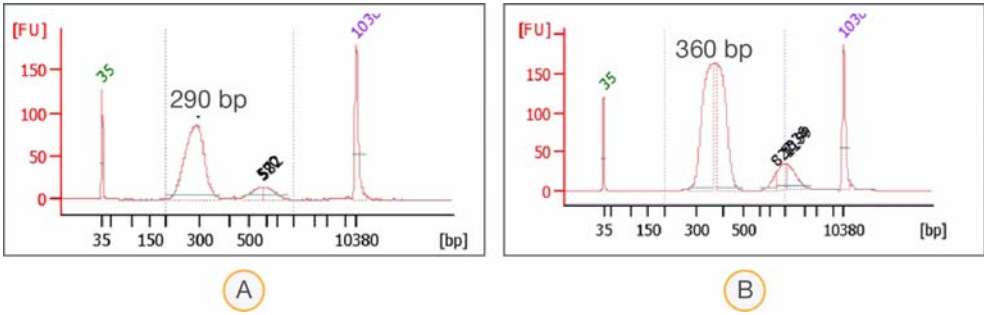
In the examples below, the same DNA sample was run on an agarose gel that was stained with SyBr Gold either pre- or post-electrophoresis (Figure 6 and Figure 7 respectively). Both the 300–400 bp and 400–500 bp fragments were gel-excised and taken through the remaining steps of TruSeq DNA Sample Prep. An aliquot of the library was then run on a high-sensitivity BioAnalyzer chip to examine size distribution. As shown in Figure 7, staining of the gel post-electrophoresis resulted in a final library distribution sized smaller than expected.

Figure 6 SyBr Gold at 1x, Pre-electrophoresis



A Selected 300–400 bp
B Selected 400–500 bp

Figure 7 SyBr Gold at 1x, Post-electrophoresis



A Selected 300–400 bp
B Selected 400–500 bp

Assessing Library Quality and Quantity

The ultimate success of enrichment strongly depends on using an accurately quantified amount of input DNA library and also ensuring that the size distribution of your library is as expected. Confirming the size distribution of your library is especially important when performing gel size selection, to ensure the fragment that was excised from the gel is of the expected size.

To assess the size distribution of your library, Illumina recommends running an aliquot on a gel or an Agilent Technologies 2100 Bioanalyzer.

High Sensitivity DNA chip

When running samples on an Agilent High Sensitivity DNA chip, load 1µl of a 1:50 dilution of your library. The following are example traces of a final DNA library made through either the gel (Figure 8) or gel-free (Figure 9) method when run on a High Sensitivity DNA chip.

Figure 8 Gel Library on High Sensitivity DNA Chip

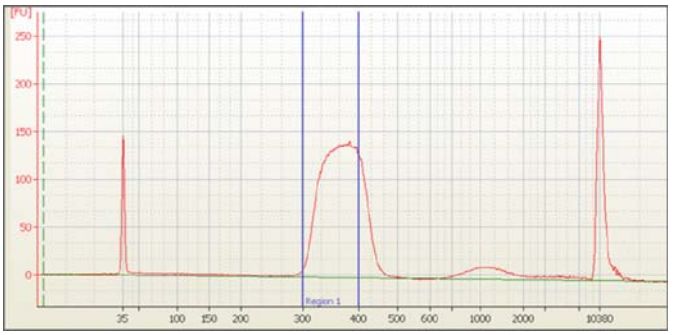
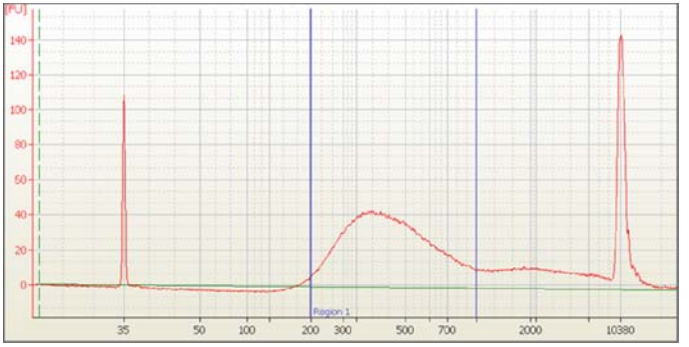


Figure 9 Gel-free Library on High Sensitivity DNA Chip



DNA 1000 Chip

When running samples on an Agilent DNA1000 chip, no dilution is required. The following are example traces of a final DNA library made through either the gel (Figure 10) or gel-free (Figure 11) when run on a DNA1000 chip.

Figure 10 Gel Library on DNA 1000 Chip

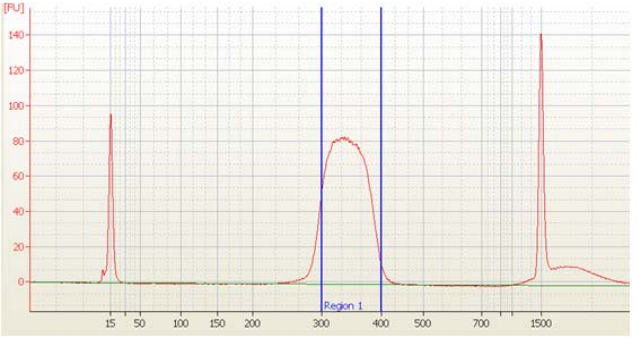
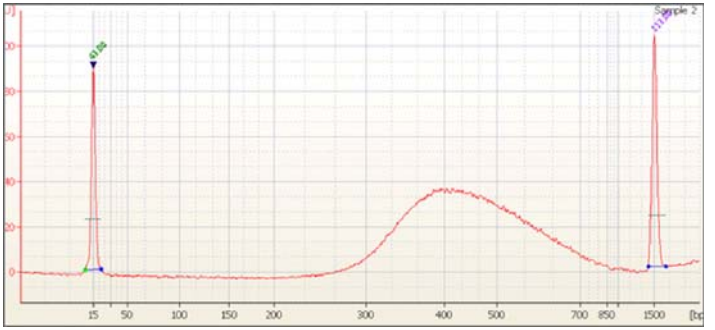


Figure 11 Gel-free Library on DNA 1000 Chip



Quantitation

For accurate quantitation of your DNA library, Illumina recommends using a fluorometric based system such as the Qubit dsDNA BR Assay System (see *Assessing DNA Quality* on page 12).

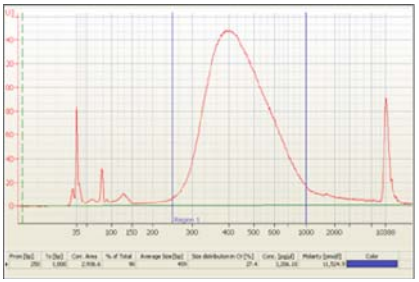
Illumina does not recommend using the Agilent Bioanalyzer for quantitative purposes as this method can report inaccurate yields due to the sensitive nature of this quantification method.

Gel-free Libraries with Sample Overloading

The following demonstrates the importance of not relying on the BioAnalyzer as a method for sample quantification. These figures show the same gel-free library run on a High Sensitive DNA chip after diluting the sample either 1:5 or 1:20.

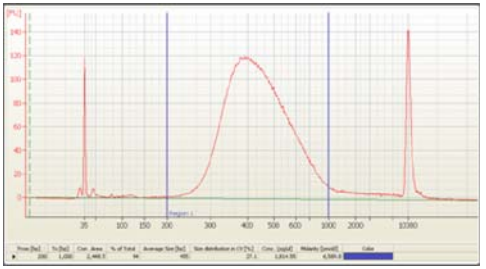
A 1:5 dilution results in overloading of the sample and a calculated concentration of 16 ng/µl. Note that the height of the sample peak is well above the standards.

Figure 12 DNA Library Diluted 1:5 Prior to Loading



However, a 1:20 dilution of the sample results in a calculated concentration of 36 ng/µl. Note that the sample peak height is within the range of the standards.

Figure 13 DNA Library Diluted 1:20 Prior to Loading



Enriched Library Validation

Illumina recommends using qPCR to quantify the final library post enrichment.

- In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the *Illumina Sequencing Library qPCR Quantification Guide*.
- Due to potential remnants of non-specific blocking DNA post PCR, Illumina does not recommend using other UV-spec or fluorometric-based methods for quantification.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 1 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 2 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at http://www.illumina.com/msds.

Product Documentation

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to http://www.illumina.com/support/documentation.ilmn. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF. To register for an iCom account, please visit https://icom.illumina.com/Account/Register.

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