



Nov 25, 2022

Library clean up and quality control for Illumina sequencing

DOI

<https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1>



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Protocol Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe 2022. Library clean up and quality control for Illumina sequencing. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1>



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Protocol status: Working

We use this protocol and it's working

Created: October 26, 2022

Last Modified: November 25, 2022

Protocol Integer ID: 71839

Keywords: Next generation sequencing, Illumina, Ampure clean up, Daisy chains, Adapter dimer removal, reliable sequencing result, quality control for illumina, sequencing, sequencing result, libraries for illumina, preparing library, quality control, illumina, standard library, library

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Abstract

There are many different ways of preparing libraries for Illumina sequencing but when it comes to the post-library preparation clean up and quality control (QC) there are common rules to apply. For reliable sequencing results it is essential that certain criteria are reached and so accurate QC is crucial. Here we describe a standard library clean up and QC with tips for how to rectify common problems including low yield, adapter dimers and daisy chains.

Materials

Reagents:

⊗ Agencourt AmPure XP beads **Catalog #A63880**

⊗ KAPA HiFi HotStart Library Amplification Kit Illumina® Platforms **Roche Catalog #KK2620**

⊗ Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

⊗ High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

⊗ High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

Additional reagents:

10 mM Tris pH8

Nuclease-free water

Absolute Ethanol

QC Equipment:

Equipment

Qubit

NAME

Flurometer

TYPE

Invitrogen

BRAND

Q33228

SKU

<https://www.thermofisher.com/order/catalog/product/Q33228>^{LINK}



Equipment

4200 TapeStation System

NAME

Electrophoresis tool for DNA and RNA sample quality control.

TYPE

TapeStation Instruments

BRAND

G2991AA

SKU

<https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263>

LINK

Protocol materials

⊗ Agencourt AmPure XP beads **Catalog #A63880**

⊗ KAPA HiFi HotStart Library Amplification Kit Illumina® Platforms **Roche Catalog #KK2620**

⊗ Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

⊗ High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

⊗ High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

Safety warnings

⚠ Qubit reagent is known to bind nucleic acid and is provided as a solution in DMSO. Treat the Qubit dsDNA HS Reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

Before start

Library clean-ups should be done in a separate lab or area to the pre-PCR stage of the library preparation to prevent contamination.

Library clean up

16m 30s

- 1 Equilibrate Ampure XP beads to Room temperature for 00:30:00 and vortex well to mix.

Agencourt AmPure XP beads **Catalog #A63880**

30m

- 2 If needed make libraries up to 50 μL with 10mM Tris pH8.

- 3 Add 45 μL AmpureXP to the samples (ratio 0.9:1) and mix well.

Note

We recommend a ratio of 0.9:1 Ampure:library to remove small unwanted fragments such as primer/adaptor dimers from the library. Depending on the expected library size, the ratio can be increased for small libraries and decreased for large libraries. See Figure 1.

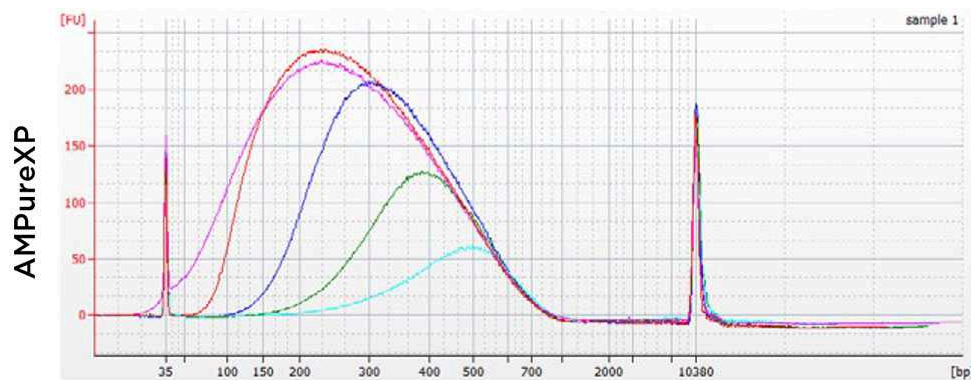


Figure 1. Size selection using different ratios of Ampure XP beads. Pink line shows the input sample with relative sizes of recovered DNA after incubation with 1.8X (red), 0.9X (blue), 0.7X (green) or 0.6X (cyan) volume of Ampure XP. Image taken from <https://www.mybeckman.uk/reagents/genomic/cleanup-and-size-selection/pcr/performance>.

- 4 Incubate at Room temperature room temperature for 00:05:00 .

5m

**Note**

This time can be increased to improve recovery if yield is expected to be low.

- 5 Place on a magnetic rack for  00:05:00 until beads and solution have fully separated.

5m

Note

It is important to wait until the beads have fully separated, the time it takes will vary depending on the ratio of Ampure beads:sample, the level of DNA and the magnetic rack used.

- 6 Carefully remove supernatant without disturbing the beads.

Note

The supernatant contains the small unwanted DNA fragments including primer/adaptor dimers.

- 7 Carefully add  200 μ L 80% Ethanol (freshly prepared) without disturbing the beads.

- 8 Incubate at  Room temperature for  00:00:30 .

30s

- 9 Place on magnetic rack for  00:01:00 until the beads and solution have fully separated.

1m

- 10 Keeping on the magnet and carefully remove supernatant without disturbing the beads.

- 11 Repeat wash with  200 μ L 80% Ethanol (freshly prepared) .

12 Remove all traces of Ethanol.

Note

Sample can be briefly centrifuged and placed back on the magnet to remove residual ethanol.

13 Keeping on the magnet air dry for up to  00:03:00 .

3m

Note

It is important not to over dry the beads as this can interfere with DNA recovery, see Figure 2.

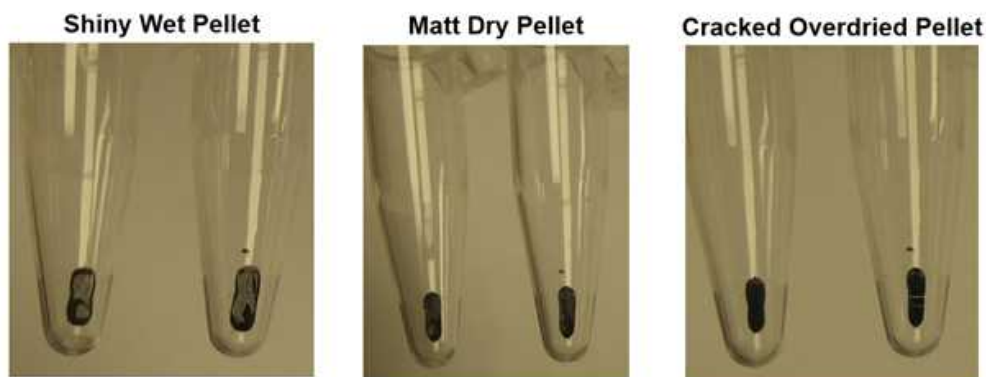


Figure 2. Examples of wet, dry and over dry beads. Image taken from <https://www.ogt.com/resources/ngs-resources-support/ngs-tips-troubleshooting/ngs-success/purification-procedures/>

14 Add  18 μ L 10mM Tris pH8 , remove from the magnet and mix well to fully suspend the beads.

Note

The elution volume can be adjusted as needed.

Note

If you plan on using the libraries for targeted enrichment with concentration by speedy vac then suspend the beads with nuclease-free water instead of 10 mM Tris pH8. For targeted enrichment with concentration by Ampure XP either nuclease-free water or 10 mM Tris pH8 can be used.

15 Incubate at  Room temperature for at least  00:02:00 to elute the DNA.

2m

16 Place back on magnet until the beads and solution have fully separated.

17 Transfer the supernatant containing the library DNA to a fresh tube.

Library quantification

2m

18 It is recommended that library quantification is performed using a fluorometric method (e.g. Qubit) rather than an absorbance based method (e.g. Nanodrop) as it is more specific, sensitive and accurate.

Equipment

Qubit NAME

Flurometer TYPE

Invitrogen BRAND

Q33228 SKU

<https://www.thermofisher.com/order/catalog/product/Q33228> ^{LINK}





- 19 Prepare a 1:5 dilution of each sample by adding  2 μ L library to  8 μ L 10 mM Tris pH8 in fresh tubes.

Note

The dilution will depend on the expected yield of library. For accurate results the concentration in the diluted sample should vary between 1-50 ng/ μ L.

- 20 Prepare 0.5 mL thin-walled PCR tubes, including 2 tubes for the standard solution and 1 tube per sample. Label the tube lids and not the sides.
- 21 Prepare the Qubit dsDNA High Sensitivity master mix for the total number of samples and standards with an excess as follows:

A	B
Component	Volume (μ L)
Qubit dsDNA HS Buffer	199
Qubit dsDNA HS Reagent	1
Total	200

 Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Safety information

Qubit reagent is known to bind nucleic acid and is provided as a solution in DMSO. Treat the Qubit dsDNA HS Reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

Note

Ensure that the Qubit standards are equilibrated to room temperature for 30 min before use.



22 Aliquot the Qubit dsDNA High Sensitivity master mix into the assay tubes as follows:

Standards  190 μ L Qubit master mix

Samples  199 μ L Qubit master mix

23 Add  10 μ L Qubit standard to each standard assay tube.

24 Add  1 μ L diluted sample to each sample assay tube.

25 Vortex assay tubes and briefly centrifuge.

26 Incubate at  Room temperature for  00:02:00 .

2m

27 Select dsDNA high Sensitivity assay on the Qubit Fluorimeter and press "Read Standards".

28 Insert Standard 1 and 2 into the sample chamber when prompted, close the lid and and press "Read Standard".

Note

It is recommended that you record the fluorescence values for the standards and monitor them to determine if there are any problems with your assay mix. The exact values will vary between machines and assay types.

29 Select "Run samples" and select the sample volume as  1 μ L .

30 Insert a sample tube into the sample chamber, close the lid and press "Read tube".

31 Record the concentration of the Qubit sample in ng/ μ L.



Note

You may need to repeat the Qubit reading with a different sample dilution to be within range.

- 32 Calculate the concentration of the original sample by multiplying the Qubit concentration (see Step 31) by the dilution factor (see Step 19).

$$Sample(ng/\mu l) = Qubit(ng/\mu l) * dilution\ factor$$

Library visualisation

30m

- 33 It is recommended that the libraries are visualised using capillary electrophoresis, we describe visualisation with a TapeStation and High Sensitivity D5000 ScreenTape. Alternatives such as the BioAnalyzer or Fragment Analyzer can also be used.

The purpose is to provide a size for the library fragments to give accurate molar quantification and to determine the quality of the library.

Equipment

4200 TapeStation System

NAME

Electrophoresis tool for DNA and RNA sample quality control.

TYPE

TapeStation Instruments

BRAND

G2991AA

SKU

<https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263>

LINK

- 34 Ensure that the D5000 ScreenTape and Reagents are equilibrated to 🌡️ Room temperature for at least 🕒 00:30:00 before use, vortex and briefly centrifuge.

30m



 High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

 High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

Note

D5000 ScreenTape has a sizing range of 100-5000 bp. The D1000 ScreenTape and Reagents could be used as an alternative which has a sizing range of 35-1000 bp.

35 Dilute each sample to approximately 1 ng/ μ L.

36 In fresh PCR strip tubes prepare the ladder assay tube as follows:

	A	B
	Component	Volume (μL)
	D5000 sample buffer	2
	D5000 ladder	2
	Total	4

37 Prepare the sample assay tubes as follows:

	A	B
	Component	Volume (μL)
	D5000 sample buffer	2
	Diluted library	2
	Total	4

38 Spin down, using IKA vortexer mix at  2000 rpm, 00:01:00 then spin down again.

1m

- 39 Load the assay tubes and ScreenTape into the TapeStation instrument.
- 40 Select the required sample/ladder positions in the TapeStation software and click "start".

Note

The TapeStation analysis software will open automatically at the end of the run to display the results.

- 41 Analyse the results a typical library should give a single peak of 200-500 bp depending on the library type.

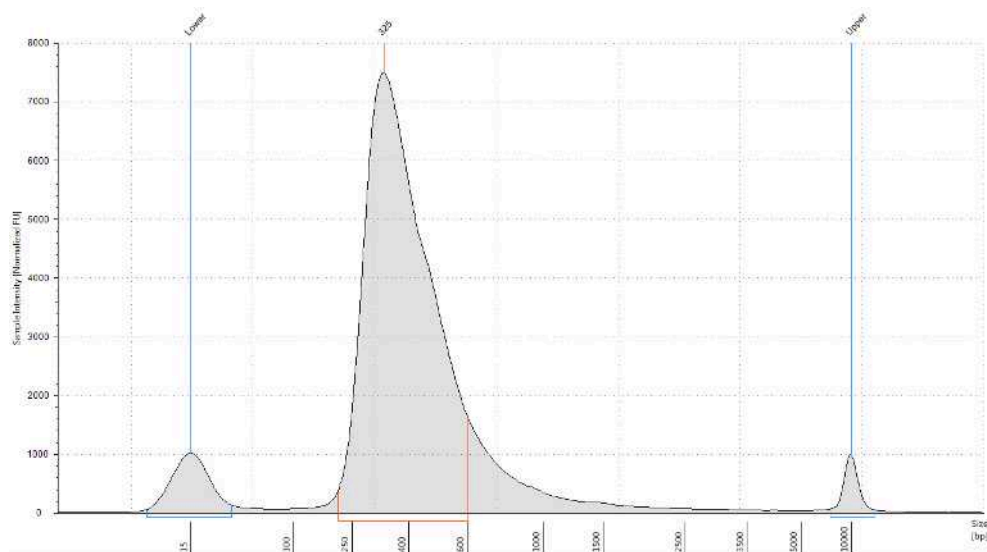


Figure 3. Example profile for a typical Illumina library.

Note

See Troubleshooting section for examples of common problems.

- 42 Determine the library peak size in bp.

**Note**

If your library doesn't give a single sharp peak then a region size can be used instead.

- 43 Calculate the library molar concentration using the fragment size (Step 42) and sample mass concentration determined by Qubit (Step 32).

$$Conc(nM) = \frac{sample(ng/\mu l)}{size(bp) * 660(g/mol)} * 1000000$$

Library troubleshooting

3m

- 44 Below we list a number of common problems associated with Illumina sequencing libraries and steps you can take to rectify them.

45 **Low yield**

If your library yield is very low (e.g. less than 1 ng/μL) then the library can be amplified using the KAPA HiFi Library Amplification kit which uses primers directed against the Illumina adapter sequences.



KAPA HiFi HotStart Library Amplification Kit Illumina®
Platforms **Roche Catalog #KK2620**

Note

Be aware that excessive PCR cycles can lead to over amplification of reads and PCR duplicates.

- 45.1 Prepare the PCR mix (for multiple reactions a master mix with 10% excess is recommended):

	A	B
	Component	Volume (μl)
	2X KAPA HiFi Ready Mix	25
	10X Universal primer mix	5



	A	B
	Total	30

45.2 Add  30 μ L PCR mix to fresh PCR tubes.

45.3 Add  20 μ L sample .

Note

If necessary make sample up to 20 μ L with 10 mM Tris pH8.

45.4 Incubate as follows on PCR machine:

 98 °C for  00:00:45

1-6 cycles of

 98 °C for  00:00:15

 65 °C for  00:00:30

 72 °C for  00:00:30

Final cycle of

 72 °C for  00:01:00

 4 °C hold .

3m

45.5 Repeat library clean up and QC (Steps 1-43).

 [go to step #1](#)

46 Adapter dimers

Adapter dimers are small fragments (typically ~120-170 bp) that contain full length adapter sequences meaning that they are able to bind to the Illumina flow cell and be clustered. Due to the small size they cluster more efficiently than the full-length libraries meaning that it is very important that they are removed before sequencing.

Adapter dimers cause a number of problems:

- Reduce the number of library specific reads.
- Over clustering, reducing the data quality for all the samples.
- Shorter length than run length results in adapter dimer cluster giving no signal in later run cycles reducing quality.
- Free adapter can be incorporated into library clusters causing index hopping.

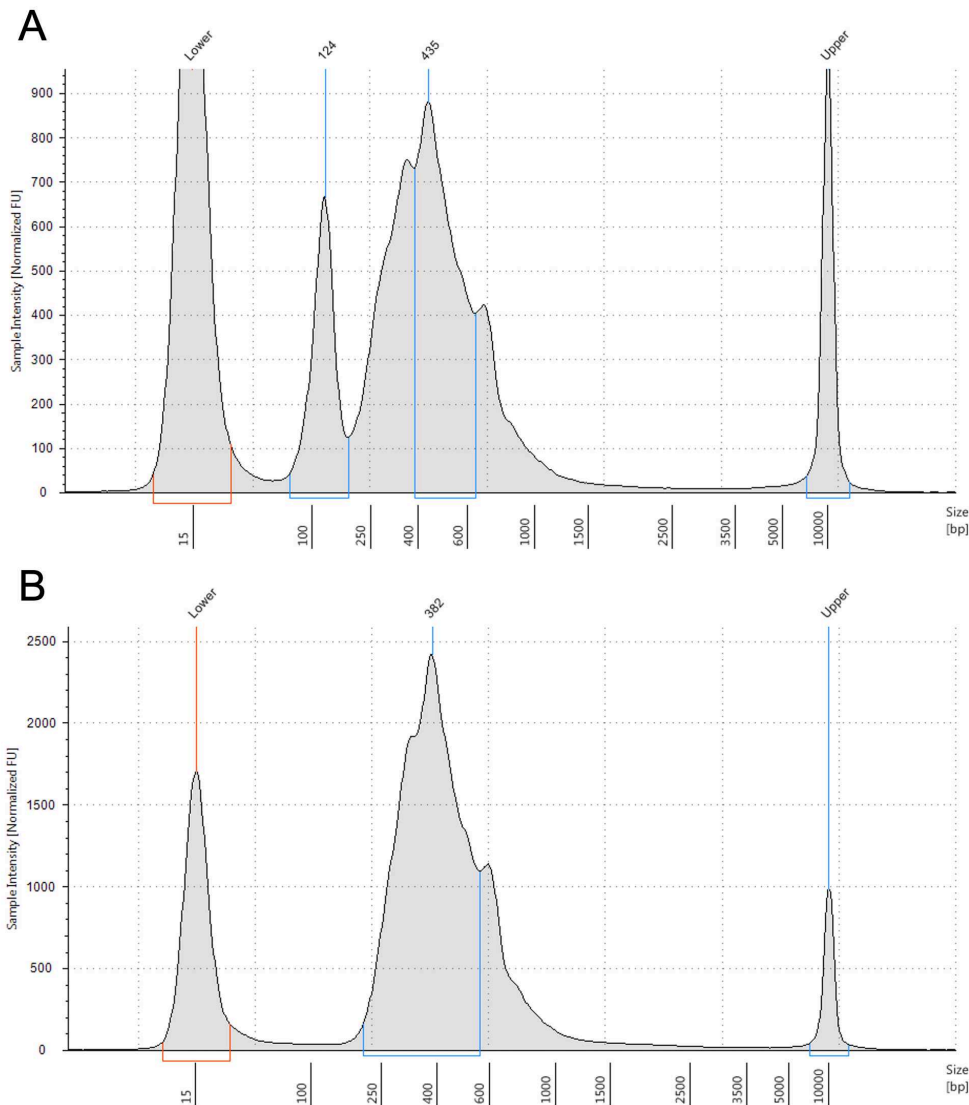


Figure 4. Example profile showing an Illumina sequencing library with prominent adapter dimer peak at 124 bp (A) and the same library after additional round of Ampure XP clean up (B).

46.1 Adapter dimers are removed by repeating the Ampure XP clean up (Steps 1-17)

go to step #1



If your specific library fragment size permits a more stringent ratio of Ampure:sample (e.g. 0.8X or 0.7X) can be used.

Depending on the extent of the adapter dimer, multiple rounds of clean up can be performed.

Note

An alternative to Ampure clean up is gel excision.

46.2 After you have completed your rounds of clean up repeat the QC (Steps 18-43)

 [go to step #18](#)

47 **Daisy chains**

Assemblies of improperly annealed, partially double-stranded, hetero-duplex DNA called daisy chains can result from over amplification causing the depletion of primers/dNTP's in later cycles, or contaminants such as ethanol carried over from incomplete Ampure bead clean up into the final PCR reaction.

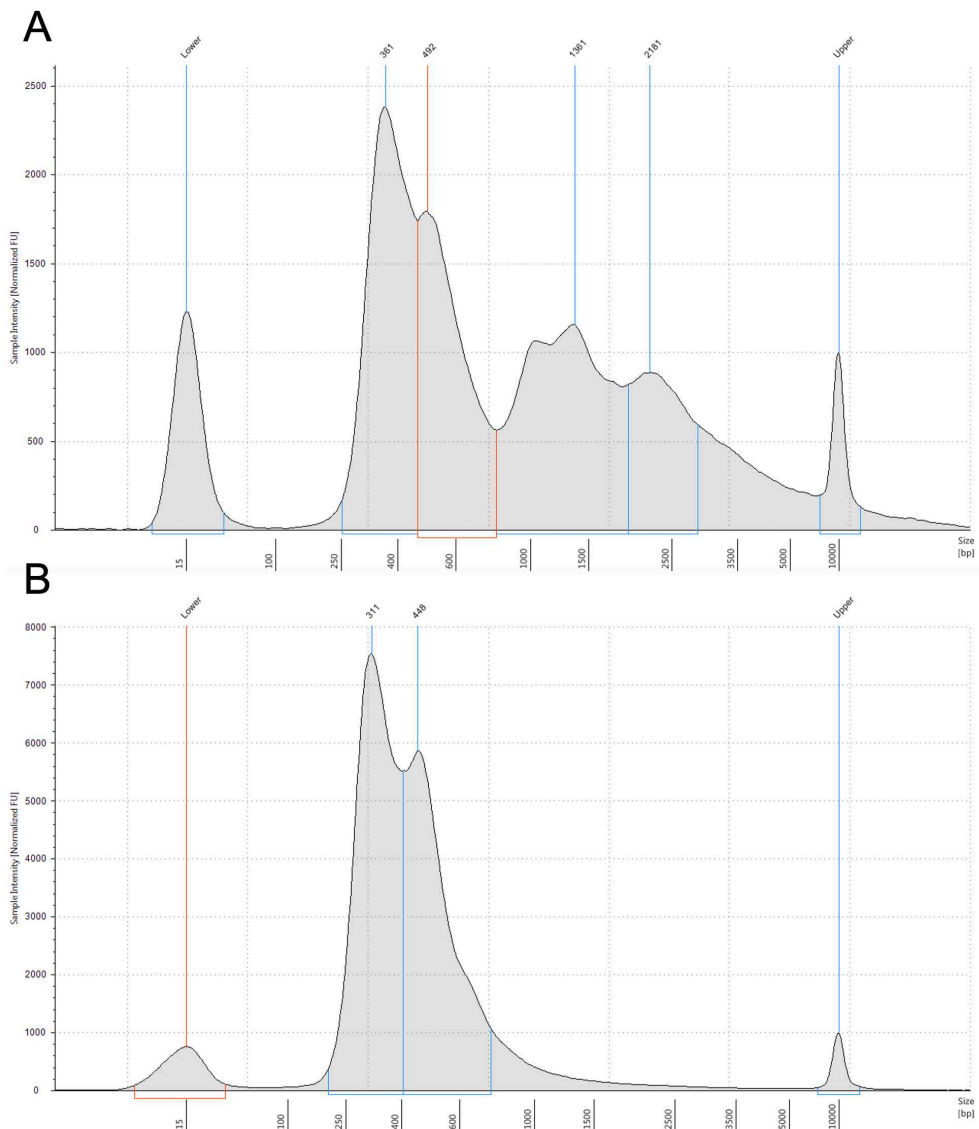


Figure 5. Example profile showing an Illumina sequencing library with larger daisy chain peaks (A) and the same library after additional PCR cycle (B).

- 47.1 Daisy chains can be broken up by performing 1-2 additional PCR cycles (Steps 45.1 - 45.5).

[go to step #45.1](#)

48 Large fragment contamination

Contamination with larger fragments are less serious than small fragment contamination and adapter dimers, as they cluster less efficiently than the smaller, specific libraries.

They do interfere with accurate quantification and can be removed by reverse-ampure clean up.

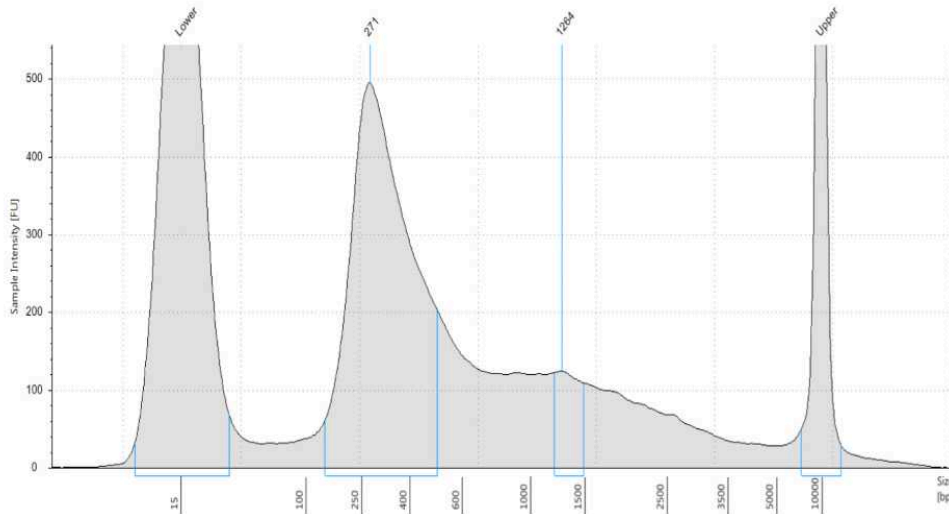


Figure 6. Example profile showing an Illumina sequencing library showing larger fragment contamination.

Note

Reverse Ampure to remove larger fragments is less efficient than small fragment removal and will likely result in some loss of library.

48.1 If needed make libraries up to  50 μL with 10mM Tris pH8.

48.2 Add  30 μL AmpureXP to the samples (ratio 0.6:1).

 Agencourt AmPure XP beads **Catalog #A63880**

Note

Depending on the target library size this ratio can be reduced to 0.52:1 for more efficient large fragment removal.

48.3 Incubate at  Room temperature room temperature for  00:05:00 .

5m

**Note**

This time can be increased to ensure recovery if yield is expected to be low.

- 48.4 Place on a magnetic rack for  00:05:00 until beads and solution have fully separated.

5m

Note

It is important to wait until the beads have fully separated, the time it takes will vary depending on the ratio of Ampure beads:sample and the level of DNA.

- 48.5 Carefully transfer the supernatant containing the smaller DNA fragments to fresh tubes.

Note

IMPORTANT: Unlike the previous clean ups it is the supernatant containing the smaller fragments NOT the beads containing the larger fragments that is retained.

- 48.6 Recover the DNA fragments from the supernatant by adding  60 μ L AmpureXP (including the solution from step 48.2 this makes a ratio 1.8:1).

- 48.7 Incubate at  Room temperature room temperature for  00:05:00 .

5m

Note

This time can be increased to ensure recovery if yield is expected to be low.

- 48.8 Continue with library clean up and QC as above (Steps 7-43).

 [go to step #7](#)