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WORKS FOR ME 2

Library clean up and quality control for Illumina sequencing

COMMENTS 0

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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.

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KEYWORDS

Next generation sequencing, Illumina, Ampure clean up, Daisy chains, Adapter dimer removal

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MATERIALS TEXT

Reagents:

Agencourt AmPure XP beads **Contributed by users Catalog #A63880** In 2 steps

KAPA HiFi HotStart Library Amplification Kit Illumina® Platforms **Roche Catalog #KK2620** Step 45

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** Step 21

High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592** Step 34

High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593** Step 34

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

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

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

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.

DISCLAIMER

BEFORE STARTING

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

16m 30s

Library clean up

30m

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

Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

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

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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/adapter 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.

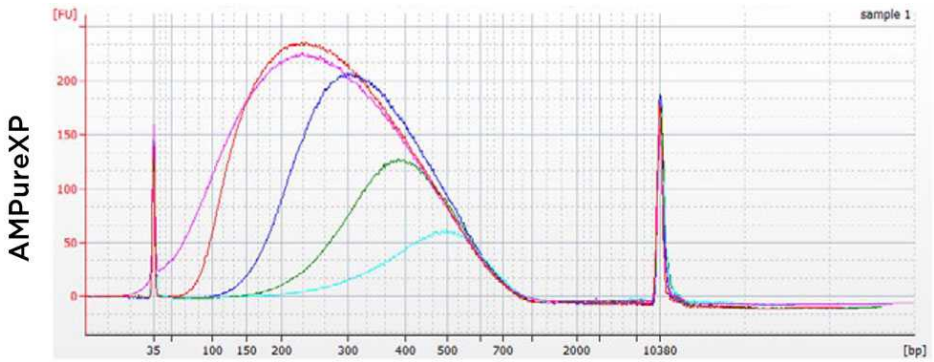

Figure 1. Size selection using different ratios of Ampure XP beads. Pink line shows the input sample with relative sizes of recovered DNA after incubation with 1.8X (red), 0.9X (blue), 0.7X (green) or 0.6X (cyan) volume of Ampure XP. Image taken from https://www.mybeckman.uk/reagents/genomic/cleanup-and-size-selection/pcr/performance.

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

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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/adapter dimers.

7 Carefully add 200 µL 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 µL 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.

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3m

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

Note

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

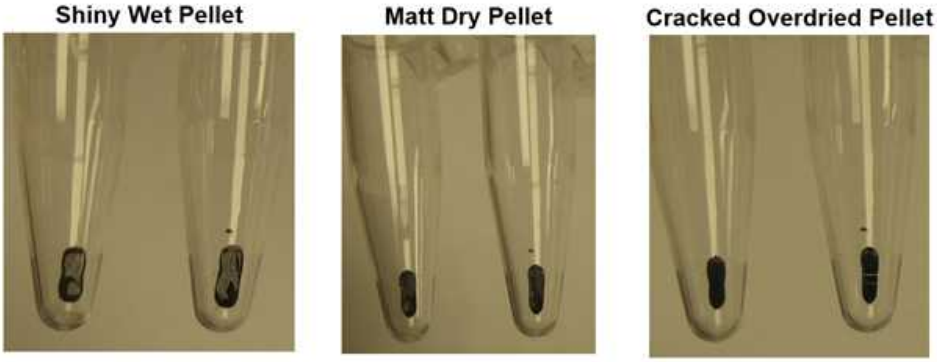

Figure 2. Examples of wet, dry and over dry beads. Image taken from https://www.ogt.com/resources/ngs-resources-support/ngs-tips-troubleshooting/ngs-success/purification-procedures/

14 Add 18 µL 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.

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

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 µL library to 8 µL 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/µL.

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

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 (µl)
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 µL Qubit master mix
Samples 199 µL Qubit master mix

23 Add 10 µL Qubit standard to each standard assay tube.

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

24 Add 1 µL 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 µL .

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/µL.

Note

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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/µl) = Qubit (ng/µl) * dilution factor

30m

Library visualisation

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 Fragent 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

30m

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.

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.

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35 Dilute each sample to approximately 1 ng/µL.

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.

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

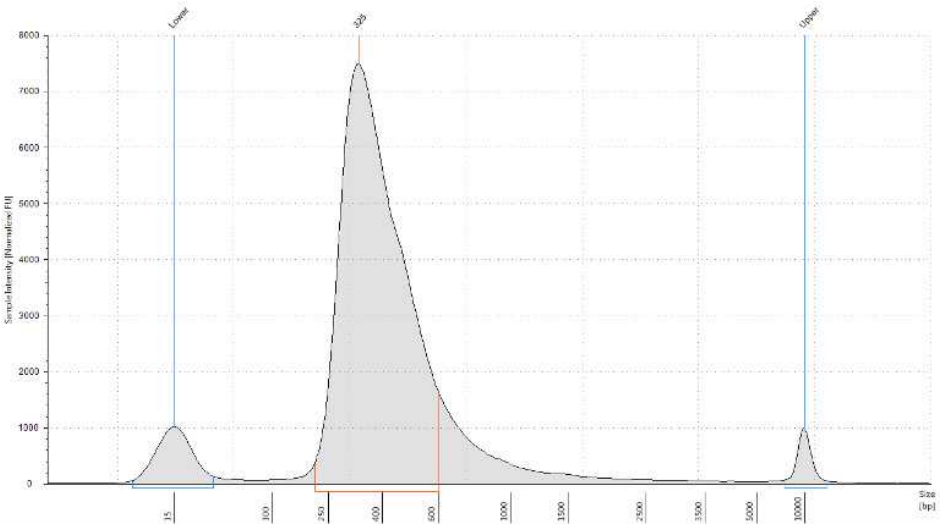

Figure 3. Example profile for a typical Illumina library.

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/µl)}{size (bp) * 660 (g/mol)}*1000000

3m

Library troubleshooting

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

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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
Total	30

45.2 Add 30 µL PCR mix to fresh PCR tubes.

45.3 Add 20 µL sample.

> Note
>
> If necessary make sample up to 20 µL with 10 mM Tris pH8.

3m

45.4 Incubate as follows on PCR machine:

98 °C for 00:00:45

1-6 cycles of

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

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 .

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.

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

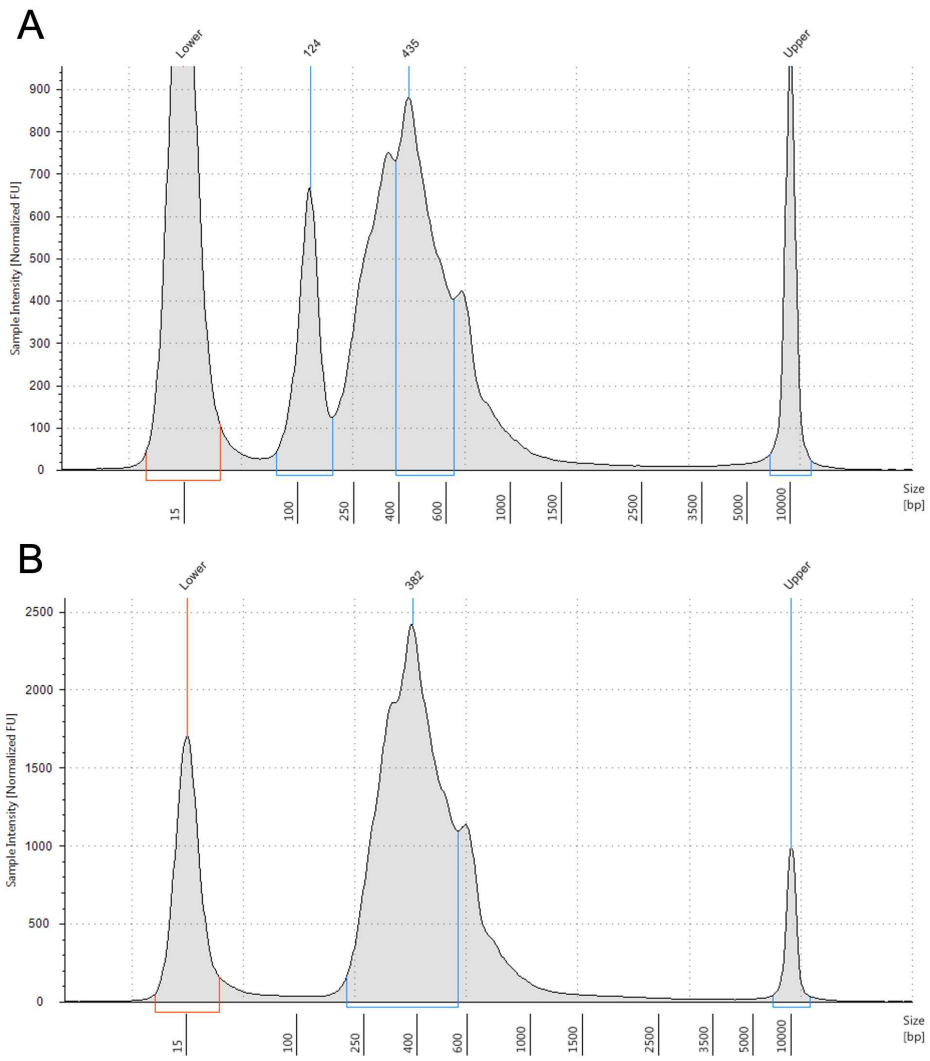

Figure 4. Example profile showing an Illumina sequencing library with prominant adapter dimer peak at 124 bp (A) and the same library after additional round of Ampure XP clean up (B).

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.

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

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.

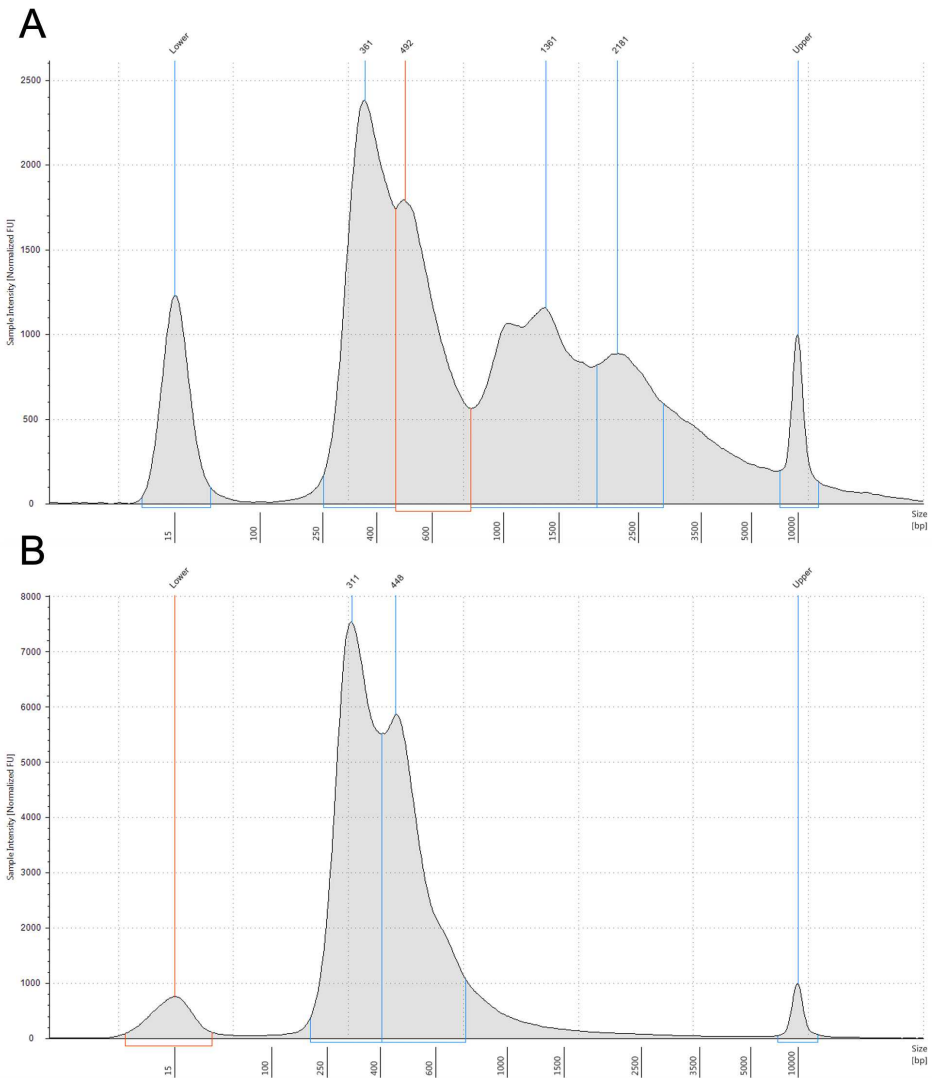

Figure 5. Example profile showing an Illumina sequencing library with larger daisy chain peaks (A) and the same library after additional PCR cycle (B).

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

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1

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.

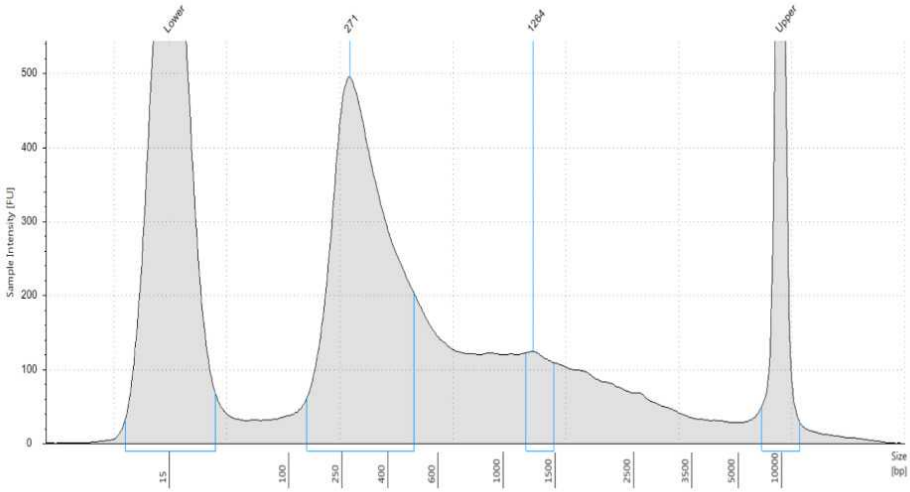

Figure 6. Example profile showing an Illumina sequencing library showing larger fragment contamination.

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 **Contributed by users Catalog #A63880**

Note

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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 form the supernatant by adding 60 µL 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.

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

go to step #7

Citation: Katherine Smollett, Lily Tong, Kyriaki Nomikou, Jenna Nichols, Kirsty Kwok, Ma. Jowina Galarion, Daniel Mair, Ana Filipe Library clean up and quality control for Illumina sequencing https://dx.doi.org/10.17504/protocols.io.n2bvj861ngk5/v1