Applications of the Sage Science Pippin Fractionator in Life Technologies/Ambions RNA-SEQ Protocols

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Amplicon DNA size-selection is a critical aspect of sequencing library preparation. Failure to produce a regularly sized amplicon library reduces the efficiency of the sequencing process itself and may result in substantial losses of potential data. Life Technologies / Ambion's Personal Genome Machine™ RNA-SEQ protocols size select using Agencourt AMPure® XP beads in a 2-step post-reverse transcription procedure. This application note describes an alternative means of size selection utilizing the Sage Science Pippin Fractionator, with some minor modifications to the RNA-SEQ protocols.

The Sage Science Pippin Fractionator size selects through an automated gel extraction process. Each electrophoretic gel lane splits into two termini: an elution and a waste chamber. The user designates a desired sample size (or range of sizes), and based on an external DNA ladder calibration the machine switches anodes accordingly. Desired amplicons are ultimately eluted into a TE/TAPS buffer. Following elution the sample is immediately moved downstream in the experimental pipeline – no intervening purification step was necessary. The pippin fractionator is capable of running up to four samples at once. Run time is dependent on the size of target amplicons. For a target size of 195bp, our run times averaged 70 minutes.

The Pippin Fractionator

Protocol Considerations

Protocol changes are required in order to size-select using the pippin fractionator. Figure 1 shows original and modified RNA-SEQ protocol flowcharts. The original protocols call for amplicon library size-selection immediately following reverse transcription using 2 rounds of Agencourt AMPure® XP bead purification. The initial reaction utilizes a 1.8X bead-to-sample volume ratio to remove fragments less than 100bp in size, and a subsequent reaction at a 1.6X ratio removes fragments smaller than 150bp. Also removed are enzymes, salts, unincorporated nucleotides, and various residual impurities from previous reactions. Only one round of purification using the Agencourt AMPure® XP beads was performed, at the ascribed

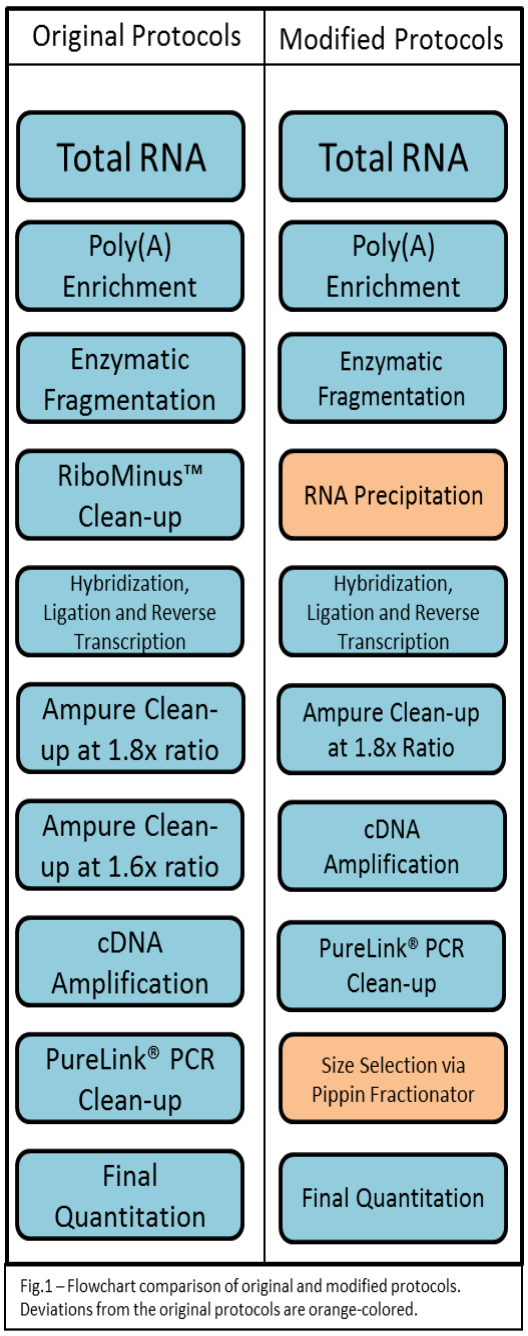


Fig.1 – Flowchart comparison of original and modified protocols. Deviations from the original protocols are orange-colored.

1.8X ratio. This step was retained for purposes of sample purification, not size selection. At this point in the procedure ligase, reverse transcriptase, enzymatic cofactors and unincorporated nucleotides are all present with the sample in the same reaction vessel. For this reason its best to retain a single AMPure clean-up.

Size selection from the amplicon pool occurred immediately before template IonSphere™ preparation, for two reasons: (1) higher post-PCR concentrations mitigate expected sample loss during size selection, and (2) prior PureLink® PCR clean-up removes any impurities which may interfere with pippin function.

Ribominus™ clean-up kits – previously unavailable – were replaced with an RNA precipitation reaction, conducted using 20uL ammonium acetate, 1uL glycogen, and 550uL 100% ethanol. Precipitated samples were reconstituted in purified water or Ambions MicroPoly(A)Purist™ THE RNA storage solution. Replacing RiboMinus™ clean-up with an RNA precipitation reaction had no apparent effects on downstream results.

Relative Performance

Figure 2 Agilent Bioanalyzer profiles shows the performance of Agencourt AMPure® XP facilitated size-selection as described by the original protocols. The material used was cDNA derived from poly(A) enriched total-RNA isolated from two pancreatic cell lines (Htert121 and CFPAC-1, samples 1 and 2 respectively). The first column shows the size distribution of samples directly after reverse transcription. The second column after two rounds of AMPure size selection. The last column ("Post-PCR amplification") shows the size distribution of our samples immediately prior to Ion Torrent ISP preparation when the original protocols were followed. Figure 3, by contrast, shows a size distribution profile of sample 1 and 2 aliquots run concurrently through our

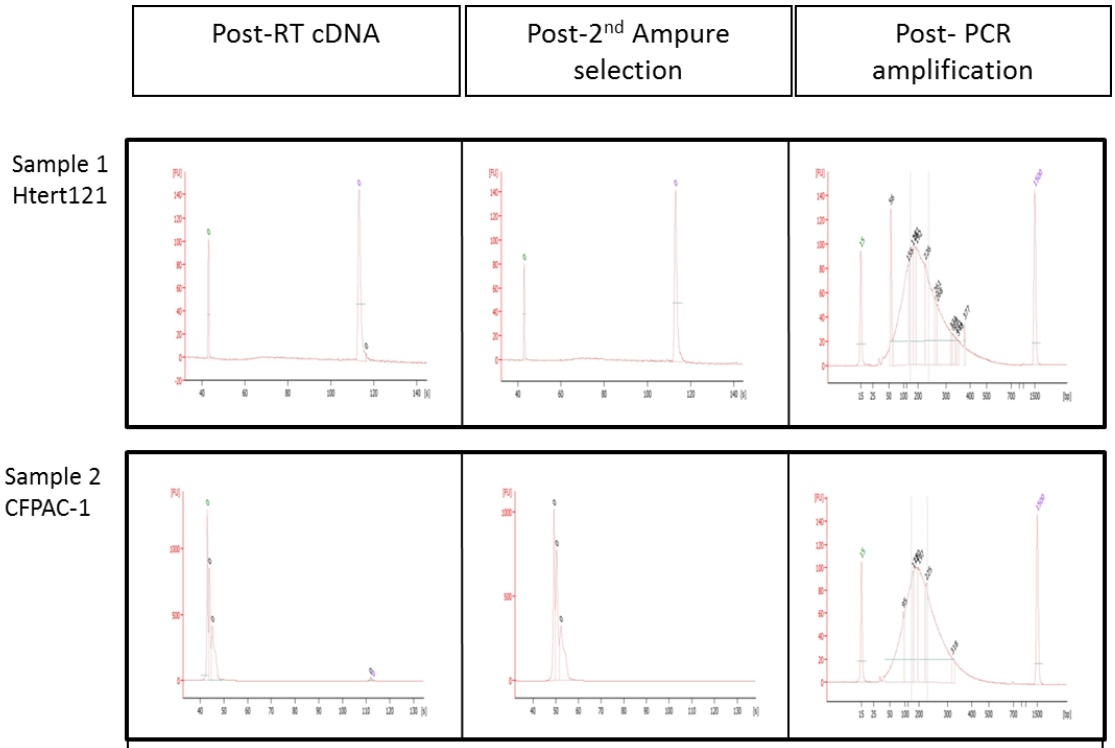


Figure 2 – Progression of samples through RNA-SEQ protocols proceeds from right to left. The first two columns depict the size distributions of samples 1 and 2 immediately after reverse transcription (column 1) and following the second round of AMPure size selection (column 2). The 3rd column shows the final size distributions after cDNA PCR.

modified protocols. We used a 2% agarose gel-cassette (100-600bp DNA size range collection) with ladder marker B(1). Each aliquot was run through the pippen at a "range" setting of 195+/-20bp. The size distributions of our pippen eluents have been overlaid on the fig.2 post-PCR amplification column agilent traces to emphasize the difference in the relative methods of size selection. This level of performance was remarkably consistent across samples (additional sample electropherograms are provided in the supplementary figure section). After the pippin run, samples 1 and 2 exhibit amplicon library sizes with a range of 167-238 and 166-228, respectively. By comparison, the same samples run through the original protocols exhibit amplicon size ranges of 32-698 and 53-567bp. This magnitude of difference in amplicon sizes may

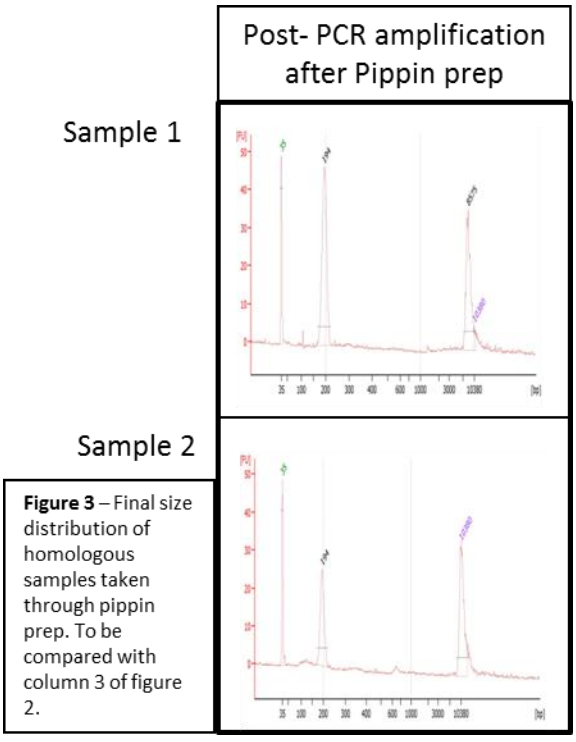


Figure 3 – Final size distribution of homologous samples taken through pippin prep. To be compared with column 3 of figure 2.

Size-Selection Platform	Sample	Average Amplicon Size	Amplicon Size Range	Estimated % library above 220bp in length	Estimated % library between 185-210bp in length
AMPure XP beads	Sample 1	214	32-698	19.33%	9.97%
Pippin Fractionator	Sample 1	200	167-238	2.82%	71.02%
AMPure XP beads	Sample 2	206	35-567	19.79%	11.68%
Pippin Fractionator	Sample 2	194	166-228	1.12%	46.83%

Figure 4: Amplicon information was collected using Agilent Bioanalyzer electropherograms. Percentages were calculated using region-specific molarites of the described samples and spectra regions.

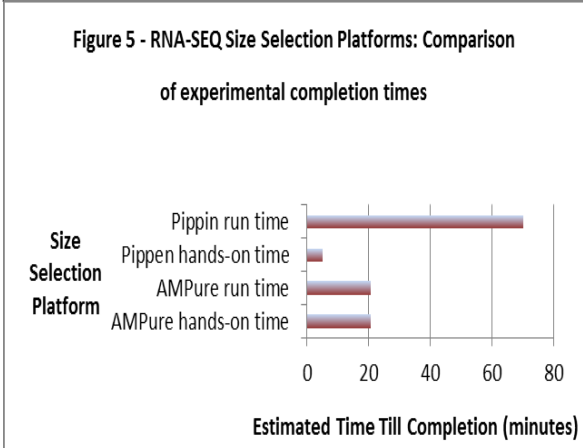


Figure 5 – Pippin time estimates made based on elution of 195+/- 20bp amplicons. AMPure time estimates made assuming 5 minute incubation times (protocol recommendations: 2-5 mins) to ensure maximal sample recovery.

contribute to large discrepancies in library quality and subsequent sequencing data quality.

For optimal sequencing results on the Personal Genome Machine™, Life Technologies recommends a library of amplicons ranging in size from 185-210bp (1). Additionally, it is stated in their Ion Fragment Library Kit User Guide that amplicon sizes above 220bp in length produce lower quality data (1).

Figure 4 provides tabulated data from two size-selected samples with respect to these conditions.

Other Considerations

While size selection is a critical element in preparing libraries, other factors such as time, complexity, cost and throughput are also important. Figure 5 summarizes the experimental process time for each size-selection procedure. Since the total run time required for each procedure may be misleading, two "hands-on" time categories have been added to reflect the amount of time the researcher must actually spend manually performing the experiment. Thus, although pippin fractionation has a three times the run time per sample, in terms of manual operations the time commitment drops to 25% of that required for AMPure bead purification. This reduces opportunities for human error and/or handler based variability in experimental efficacy.

For large scale projects there is also a question of sample throughput. The pippin fractionator is equipped to handle only four samples per run. AMPure based size selection is a scalable process,

however, and with multi-channel pipets can be performed on 96-well plates to accommodate a larger number of samples. Finally, a certain amount of sample loss is expected during size-selection procedures. Sage Science estimates that 50-80% of sample material, including material in the designed elution range, is lost during separation. Our sample loss was in the upper range of this percentage, although this was not sufficient to interfere with downstream applications, expected sample loss should be considered by those working with minute and/or precious samples.

Conclusion

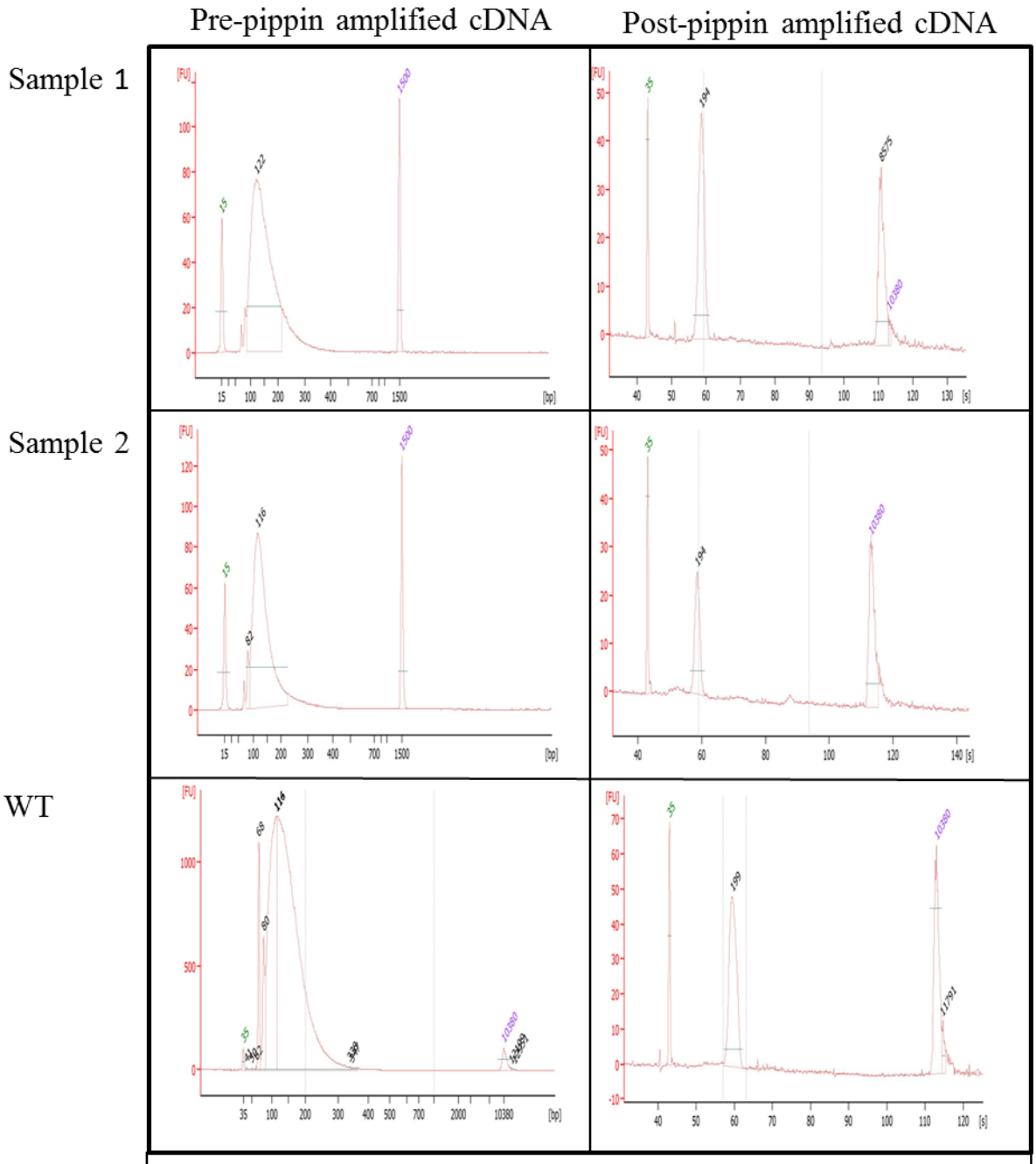
The Sage Science Pippin Fractionator is an excellent automated implementation in the RNA-SEQ library preparation protocols. In terms of size-selection, it is superior to its AMPure bead based alternative. This is an important factor in optimizing the quality of sequencing data. In terms of the incorporation of pippin prep™ into RNA-SEQ PGM™ standard operating protocols there are only two limitations. The presence of only four wells per cassette reduces throughput capacity, and obligate sample loss during electrophoresis is a potential issue for those working with extremely small amounts of sample.

References

1. "Pippin PreP™ DNA Size Selection System Operations Manual", Sage Science. 2011.

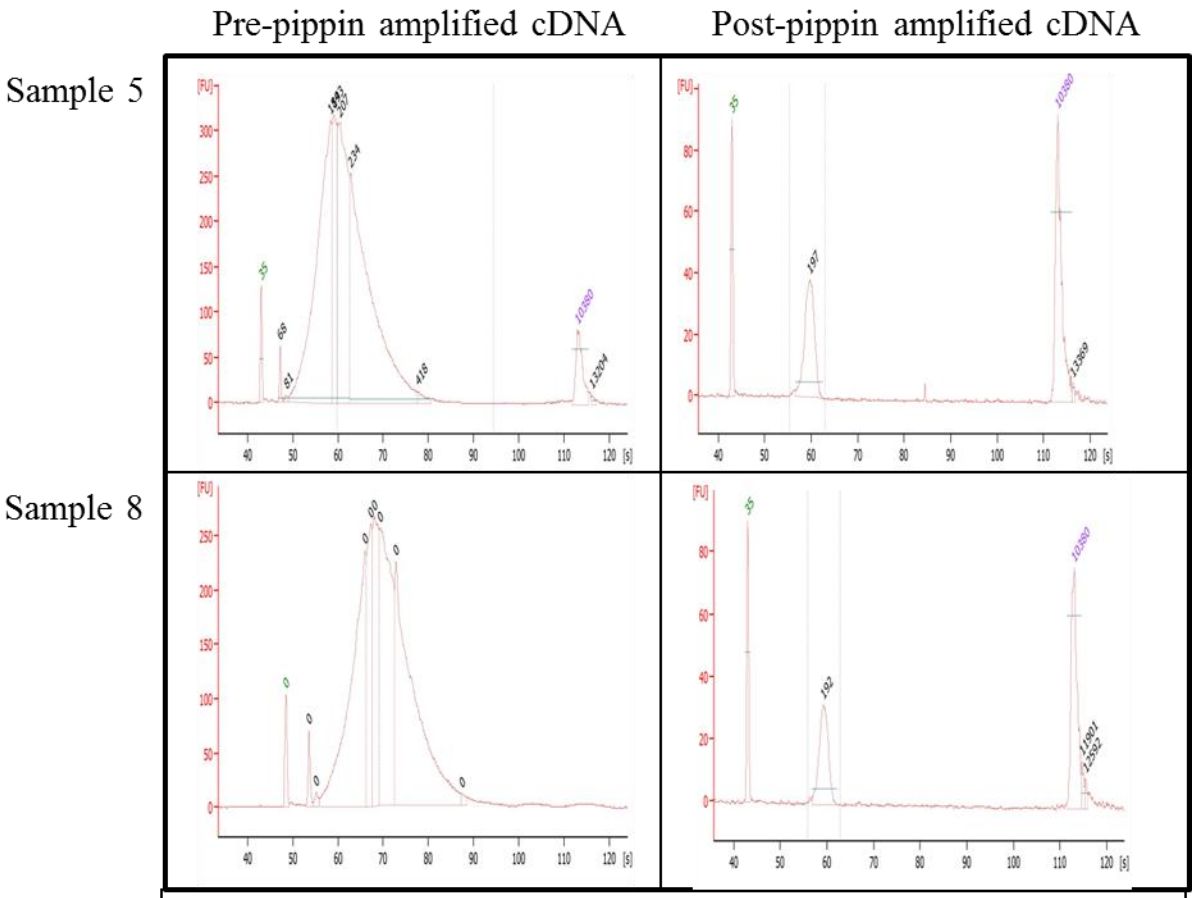
1. "Ion Fragment Library Kit User Guide", Ion Torrent by Life Technologies. 2001, Life technologies Corporation.

Supplementary Figures



Electropherograms taken from Agilent Bioanalyzer DNA 1000 (pre-pippin) and High-sensitivity DNA assays (post-pippin). Samples are human pancreatic cell lines 121Htert (sample 1), CFPAC-1 (sample 2), and Whole Transcriptome Control (WT)

Supplementary Figures



Electropherograms taken from Agilent Bioanalyzer DNA 1000 (pre-pippin) and High-sensitivity DNA assays (post-pippin). Samples are human pancreatic cell lines Sw1990 (sample 5) and 131MIAPACA2 (sample 8).

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