

Comparison of ExoSAP-IT and ExoSAP-IT *Express* reagents to alternative PCR cleanup methods

Abstract

Here we present superior workflow advantages of enzymatic PCR purification technology, specifically Applied Biosystems™ ExoSAP-IT™ and ExoSAP-IT™ *Express* reagents, over magnetic beads and spin columns (Figure 1). A novel exonuclease was developed with increased heat sensitivity for PCR amplicon purification in preparation for cycle sequencing. This novel enzyme was the basis for development of the ExoSAP-IT *Express* PCR Product Cleanup Reagent. PCR amplicons of 151, 403, and 634 bp purified enzymatically, by spin column, or by magnetic bead separation all showed similar percent recovery as quantified by fluorescence. Subsequent sequencing using the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit and capillary electrophoresis (CE) all gave similar results with respect to contiguous read length (CRL) and trace quality. An in-depth sequencing study of only the 634 bp amplicon was carried out with 24 replicate samples purified using ExoSAP-IT *Express* reagent, ExoSAP-IT reagent, or Agencourt™

AMPure™ XP magnetic beads (Beckman Coulter). Statistical analysis of quality scores showed that out of 4,000 reads, >90% of treated samples had quality values (QVs) above 58 whereas mock-treated samples had >90% of QVs between 30 and 50. Measurement of the fluorescence signal beneath each fragment peak showed that purified samples had a 4- to 5-fold increase in signal-to-noise ratio. Here we demonstrate that ExoSAP-IT and ExoSAP-IT *Express* PCR cleanup reagents offer significant workflow advantages compared to magnetic beads or spin columns, while achieving similar results.

Introduction

Direct sequencing of PCR products is highly desirable, since it avoids the time-consuming and costly need for cloning and plasmid preparation in *E. coli*. To facilitate direct sequencing of PCR products, it is essential to remove the excess primers and nucleotides prior to sequencing. This step can be performed in several ways, including separation by magnetic beads, gel filtration by spin column, or enzymatic degradation. ExoSAP-IT and

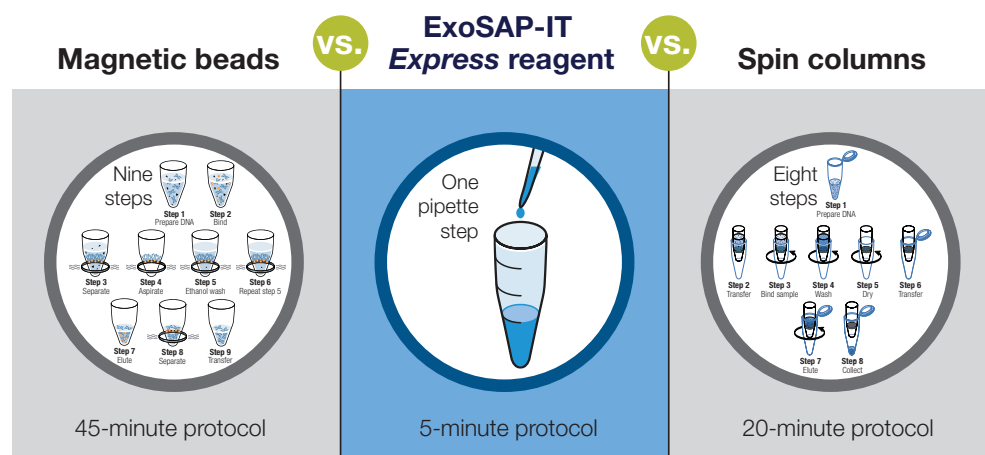


Figure 1. ExoSAP-IT *Express* reagent compared to spin columns and magnetic beads. The novel technology used for ExoSAP-IT *Express* reagent allows for a significant reduction in sample cleanup time with minimal steps, providing the simplest workflow.

ExoSAP-IT *Express* reagents achieve enzymatic purification using patented mixtures of recombinant shrimp alkaline phosphatase (rSAP) and exonuclease I (Exo I) in specially formulated buffers (Figure 2). Exonuclease I degrades single-stranded DNA in the 3' to 5' direction, releasing deoxyribonucleoside 5'-monophosphates in a stepwise manner, and leaves 5'-terminal dinucleotides. Alkaline phosphatase dephosphorylates all dNTPs to nucleosides and inorganic phosphate. While these breakdown products have been extensively shown to not interfere with downstream sequencing, they do interfere with quantitation of duplex DNA by UV spectroscopy. To mitigate this issue, our customers routinely implement an Invitrogen™ PicoGreen™ fluorescence assay.

ExoSAP-IT *Express* reagent contains a newly developed exonuclease with increased heat lability, which enables a 1-step, 5-minute purification protocol. The original ExoSAP-IT reagent enables a 1-step, 30-minute purification protocol. In comparison, QIAquick™ PCR spin columns (Qiagen) and Agencourt AMPure XP magnetic beads require multiple pipetting and dispensing steps over the course of approximately 20 minutes and 45 minutes, respectively.* Enzymatic cleanup is designed to minimize errors by reducing the protocol to a single pipetting step, allowing for automated or manual processing from a single tube or microtiter well. In this study we show that each method can produce similar high-quality sequencing results, with ExoSAP-IT and ExoSAP-IT *Express* reagents offering the additional advantage of a simplified workflow.

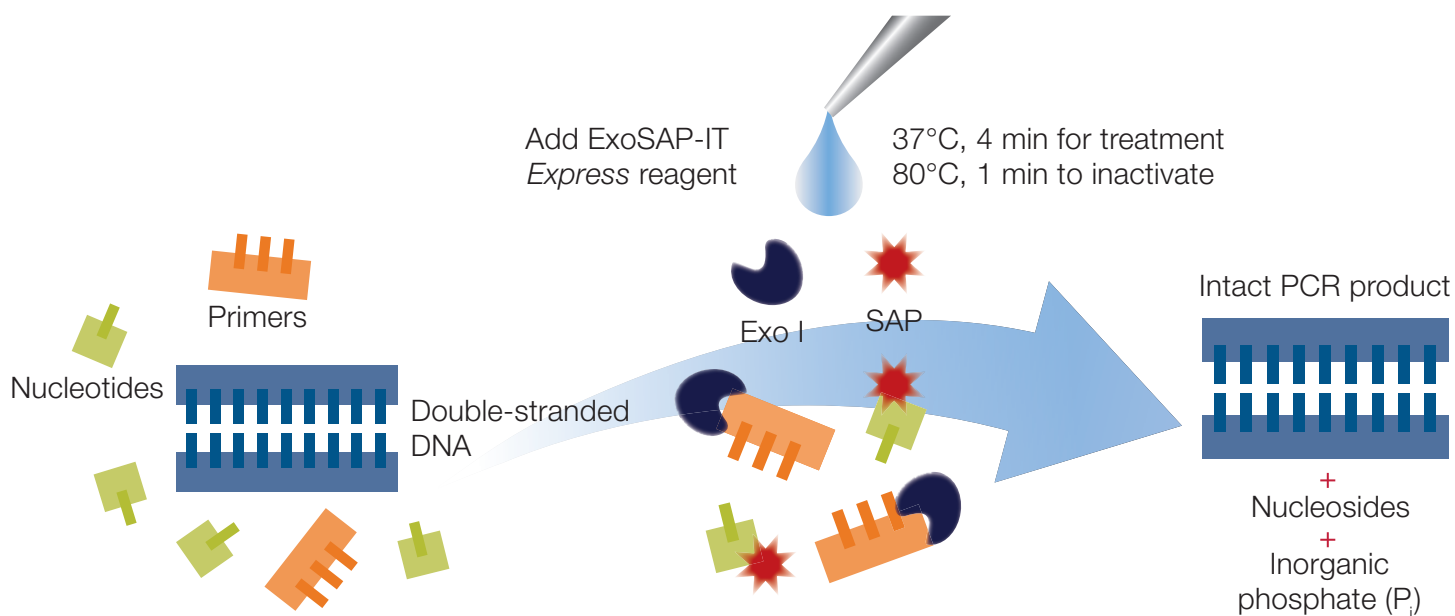


Figure 2. Enzymatic cleanup of PCR reaction with ExoSAP-IT *Express* reagent.

* Workflow times are estimates based on the associated product manuals.

Materials and methods

Primer digestion assay for Exo I activity

Activity of Exo I was assessed by incubating ExoSAP-IT or ExoSAP-IT *Express* reagent at 80°C for up to 15 minutes. A control sample was not subjected to heat denaturation. A 5 µL, 1X PCR mix was prepared containing 20 pmol of 24-mer primer labeled with Applied Biosystems™ HEX™ dye. For the digestion assay, 2 µL of buffer containing no enzyme, or 2 µL of ExoSAP-IT reagent, or 2 µL of ExoSAP-IT *Express* reagent (incubated for different times at 80°C) was added to the reaction mix containing labeled primer and incubated at 37°C for 5 minutes. The reaction was then diluted with electrophoresis loading dye and run on a 15% denaturing polyacrylamide gel. The integrity and quantity of the primer was assessed using densitometry.

Preparation of amplicon for sequencing

PCR amplicons were prepared using Applied Biosystems™ AmpliTaq Gold™ 360 Master Mix. M13-tailed primers were used at a final concentration of 480 nM each. Applied Biosystems™ Control Genomic DNA (Human) was used as a template at 1 ng/µL. PCR was performed in a 96-well plate at a final volume of 10 µL per reaction. The thermal cycling profile was as follows: 95°C for 10 min; 35 cycles of 95°C for 30 sec, 62°C for 30 sec, and 68°C for

45 sec; 72°C for 2 min; 4°C hold. The fragment size and purity was verified by 2% agarose gel electrophoresis. For enzymatic PCR purification, 10 µL of PCR product was treated with 4 µL buffer, or ExoSAP-IT reagent, or ExoSAP-IT *Express* reagent using two different heat incubation procedures: for ExoSAP-IT reagent and the buffer control, 37°C for 15 min, then 80°C for 15 min; for ExoSAP-IT *Express* reagent, 37°C for 4 min, then 80°C for 1 min.

CE sequencing

From each PCR reaction, 3 µL was transferred to a 96-well plate containing 7 µL per well of Applied Biosystems™ BigDye™ Terminator v3.1 sequencing reagent. HPLC-purified M13 sequencing primer was at a final concentration of 320 nM. Cycle sequencing reactions were purified using the Applied Biosystems™ BigDye XTerminator™ Purification Kit. Capillary electrophoresis was performed on an Applied Biosystems™ 3730x/ or 3500xL Genetic Analyzer configured with 50 cm arrays and Applied Biosystems™ POP-7™ Polymer. Two injections were performed with each sample to check instrument reproducibility. Raw QV data for each base call was extracted from PHD files and imported to JMP software for histogram analysis. Median peak under peak (PUP) was calculated as the integrated area of off-target signal under each target peak between 100 and 500 bp.

Results and discussion

Inactivation time of wild-type and mutant Exo I

The ExoSAP-IT *Express* reagent for enzymatic purification of PCR products offers a time-saving advantage (5-minute protocol) over the original product (30-minute protocol). This was achieved by replacing wild-type Exo I (ExoSAP-IT reagent) with an engineered Exo I mutant (ExoSAP-IT *Express* reagent), the latter which retains single-stranded primer digestion ability but now has increased heat sensitivity compared to the wild type. The increased heat sensitivity allows for complete inactivation in 1 minute (Figure 3).

Primer digestion was used to evaluate both wild-type and engineered Exo I for residual activity following a time

course of inactivation at 80°C. Both forms of Exo I were evaluated in the context of the final product formulation and included rSAP in the mixture. The electrophoresis results in Figure 3A show complete digestion of primer by wild-type Exo I that was not subjected to 80°C incubation. Similarly, in Figure 3B, complete digestion of primer by mutant Exo I is demonstrated. These results show that both forms of Exo I have the ability to rapidly digest single-stranded primers under these conditions (37°C, 5 min). In Figures 3A and 3B, the results over a series of 80°C incubations revealed slower inactivation of wild-type Exo I than of the engineered form used in ExoSAP-IT *Express* reagent.

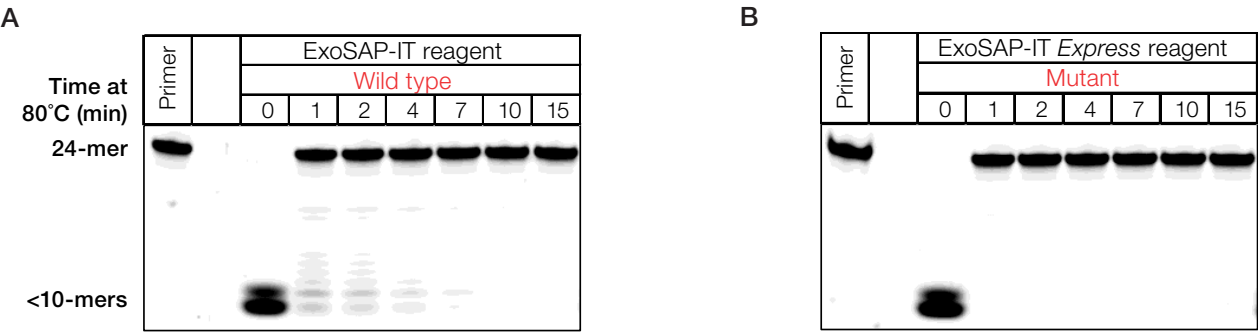


Figure 3. Faster inactivation of mutant Exo I compared to wild-type Exo I. The polyacrylamide gels show intact 24-mer primer, completely digested 24-mer primer (time = 0), and digestion of 24-mer primer to various degrees after incubation with (A) ExoSAP-IT reagent and (B) ExoSAP-IT *Express* reagent that were inactivated at 80°C for the indicated times.

Sanger sequencing results using different PCR purification methods

BigDye Terminator chemistry is designed for use in combination with Applied Biosystems™ CE instruments for what is considered to be the gold-standard technology in *de novo* sequencing. We investigated how amplicon size impacts the results from BigDye sequencing when 3 amplicons are purified by 4 different methods. Targets *CHRNA7* (151 bp), *POLR2A* (403 bp), and *ABLIM* (634 bp) were amplified from human genomic DNA with AmpliTaq Gold 360 Master Mix. Amplicons were purified by ExoSAP-IT reagent, ExoSAP-IT *Express* reagent, Agencourt

AMPure XP magnetic beads, or QIAquick spin columns. Amplicons were quantified by the PicoGreen fluorescence assay, and all purification methods demonstrated similar recovery (data not shown). BigDye Terminator v3.1 cycle sequencing reactions were ethanol-precipitated, and CE was performed on an Applied Biosystems™ 3730xI DNA Analyzer. Data were analyzed by Applied Biosystems™ Sequence Analysis Software 6, and the averages from 3 replicates were plotted (Figure 4). High-quality sequencing data were obtained with all purification methods, with no significant differences in the sample score and length of read values for all amplicons tested.

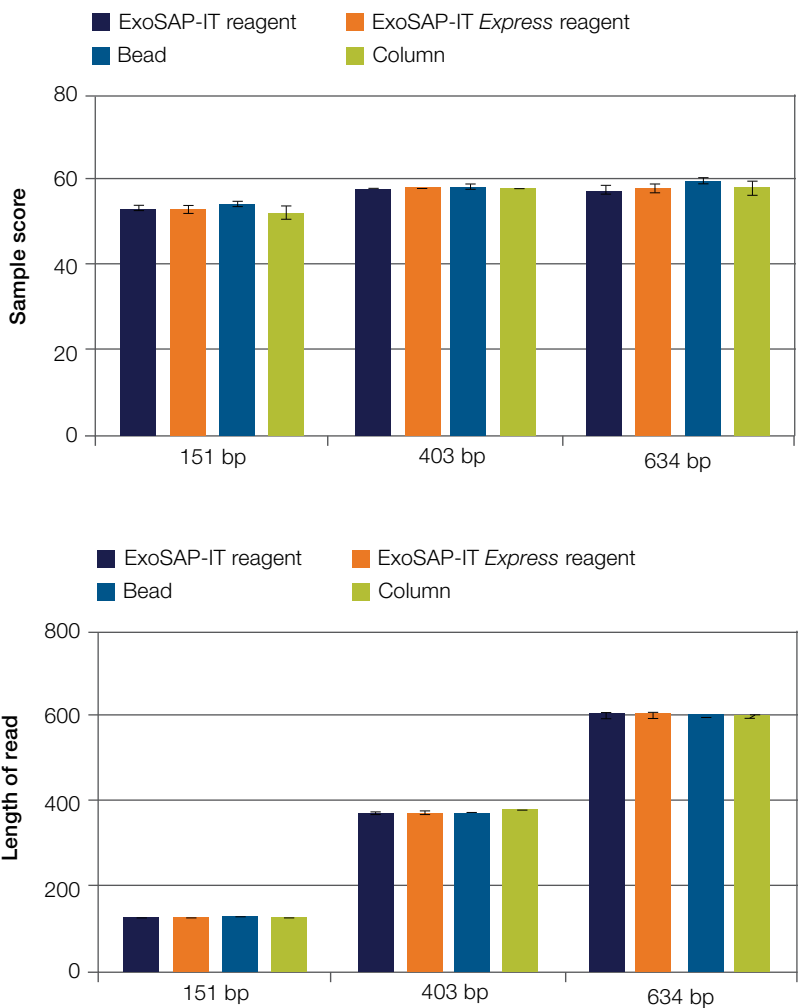


Figure 4. Different PCR sample purification methods yield comparable Sanger sequencing results. Targets of 151, 403, and 634 bp in length were amplified from human genomic DNA and purified using the indicated methods in preparation for Sanger sequencing. There are no significant differences in the sample score and length of read values for all amplicons tested.

To determine whether or not a larger sample size could impact these results, 24 replicates of the 634 bp amplicon were compared using 3 different purification methods. We excluded the QIAQuick product from this experiment. The impact of ExoSAP-IT and ExoSAP-IT *Express* reagents on Sanger sequencing is directly observable in the fluorescence electropherograms. In Figure 5, single traces produced by each method are shown. When PCR products are left unpurified, there is observable baseline noise and the majority of peaks do not resolve to baseline. For example, between 280 bp and 300 bp, two cytosine triplets in the unpurified sample do not resolve to baseline. In comparison, these same triplets clearly resolve to baseline when the sample is purified with ExoSAP-IT reagent or ExoSAP-IT *Express* reagent. When magnetic beads are used to purify the duplex, the same triplets resolve to baseline; however, the starting position is offset (250–270 bp) (Figure 5).

In Figure 6, the quantitative impact of PCR product purification is demonstrated by the PUP metric. When no purification is performed, the observed median PUP is higher than the observed PUP when ExoSAP-IT products are used for purification. ExoSAP-IT products are comparable in observed PUP to magnetic bead purification. With measurement of the fluorescence signal beneath each fragment peak, purified samples demonstrated a 4- to 5-fold increase in signal-to-noise ratio.

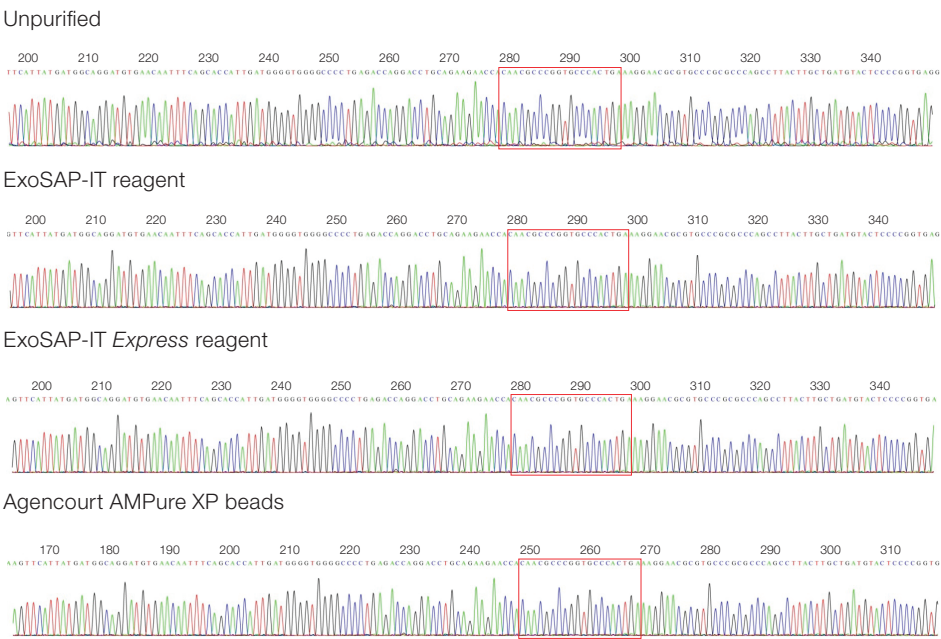


Figure 5. Electropherograms from 3 different purification methods. Replicate samples were either mock-treated with buffer (unpurified), or purified with ExoSAP-IT reagent, ExoSAP-IT *Express* reagent, or Agencourt AMPure XP magnetic beads per manufacturers' recommendations.

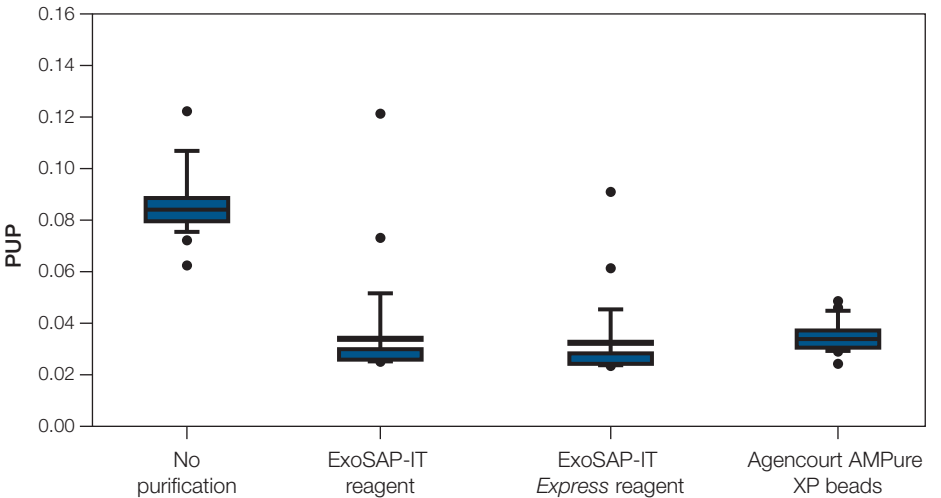


Figure 6. Comparison of sequencing signal-to-noise ratio of PCR products. Box plots represent 24 replicates of the conditions shown from the 24-capillary array of the 3500xL Genetic Analyzer. The median PUP is the integrated area of off-target signal under each target peak between 100 and 500 bp.

In Figure 7, for each of the PCR purification methods tested, 24 replicates were performed to comprise a single injection on the 3500xL Genetic Analyzer. The histograms show the number of base calls with a given QV (quality value or PHRED score) from all 24 replicate sequencing reactions (roughly 13,000 base calls for each purification category). The effect of enzymatic purification compared to a no-enzyme treatment is dramatically improved quality of sequencing data. Similar results are shown with magnetic bead purification of the amplicon. With purified samples, the majority of the base calls score a QV between 54 and 56. With unpurified samples, poor-quality base calls ranging from 34 to 56 in QV were obtained. Where the PCR amplicon was mock-treated, most of the base calls have a QV of 30 or above (1/1,000 probability of miscall). In contrast, the majority of base calls of purified samples have a QV of >50 (1/100,000 probability of miscall, 100-fold improvement). Statistical analysis of quality scores showed that out of 4,000 reads, treated samples had >90% of QVs above 58, whereas mock-treated samples had >90% of QVs between 30 and 50.

Conclusion

Thermo Fisher Scientific now offers a complete portfolio for the Sanger sequencing workflow. ExoSAP-IT reagents for PCR cleanup are recommended for all PCR-based Sanger sequencing workflows because of their simplicity. The utility of ExoSAP-IT reagent in Sanger sequencing is referenced in over 10,000 publications. Compared to alternative products such as magnetic beads and spin columns, ExoSAP-IT reagents offer significant workflow advantages while enabling similar high-quality sequencing results.

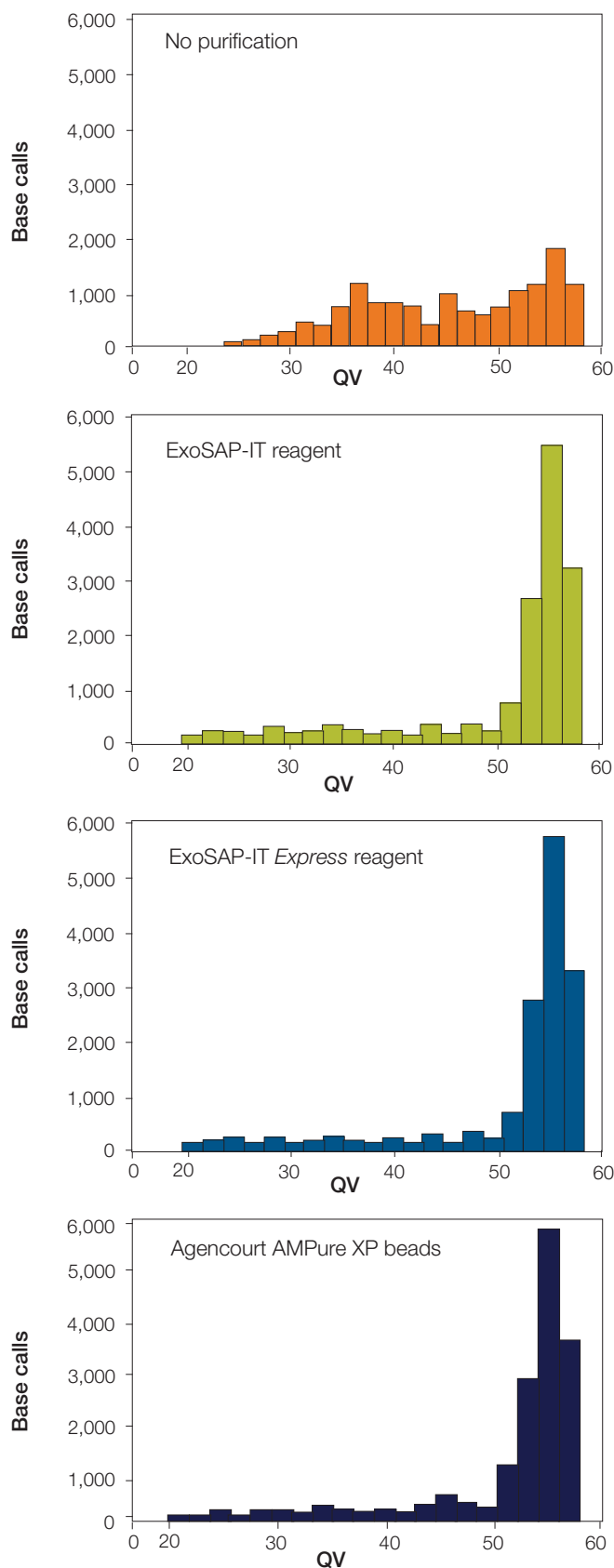


Figure 7. Different PCR purification methods yield comparable Sanger sequencing results. A 634 bp PCR amplicon was purified and sequenced in 24 replicates, allowing comparison of data quality and consistency across an entire capillary array on the 3500xL Genetic Analyzer. Replicate samples were either mock-treated with buffer or purified using ExoSAP-IT reagent, ExoSAP-IT *Express* reagent, or Agencourt AMPure XP magnetic beads.

Ordering information

Product	Quantity	Cat. No.
ExoSAP-IT <i>Express</i> PCR Product Cleanup Reagent	100 reactions	75001.200.UL
	500 reactions	75001.1.ML
	2,000 reactions	75001.4X.1.ML
	5,000 reactions	75001.10.ML
	480 reactions x 8-tube strip	75001.1.EA
ExoSAP-IT PCR Product Cleanup Reagent	100 reactions	78200.200.UL
	500 reactions	78201.1.ML
	2,000 reactions	78202.4X.1.ML
	5,000 reactions	78205.10.ML

Find out more at thermofisher.com/exosapit

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