Comparison of CleanSweep and ExoSAP-IT PCR clean-up reagents

Abstract

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*. In order to facilitate direct sequencing of PCR products, it is essential to remove the excess primers and nucleotides from the PCR products prior to sequencing. Enzymatic PCR clean-up enables an optimal workflow for generating accurate and consistent sequencing results compared to column or magnetic-bead purification. This study compares our two enzymatic PCR clean-up reagents, the Applied Biosystems™ CleanSweep™ PCR Purification Reagent and the original, single-tube, Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup Reagent. Both CleanSweep and ExoSAP-IT reagents contain Thermo Scientific™ Exonuclease I (Exo I) and shrimp alkaline phosphatase (SAP) for degrading primers and nucleotides, and follow the same protocol. Primer digestion and *p*-nitrophenol phosphate (pNPP) assays using Exo I and SAP, along with functional Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit were used to compare the two products. Based on these data, we conclude that CleanSweep and ExoSAP-IT reagents are equivalent in functionalities.

Introduction

The Applied Biosystems™ portfolio of Sanger sequencing products is the industry standard for accurate *de novo* sequence determination. Since we offer an expansive array of PCR enzymes, Sanger sequencing can be performed by many investigators using PCR protocols already established in their labs. The incorporation of an enzymatic PCR clean-up step in targeted sequencing of PCR amplicons enables easy methodology for improved sequencing results and better utility of data.

In PCR sequencing workflows, a PCR clean-up step is required to remove leftover primers and unincorporated dNTPs that would otherwise interfere with the downstream sequencing reaction. PCR clean-up can be performed enzymatically using Exo I to hydrolyze single-stranded DNA (ssDNA) primers, and SAP to dephosphorylate nucleotides. Ethanol precipitation and solid-phase extraction can also be used, but both methods include several more steps and can result in low yields. We offer two enzymatic PCR clean-up products, CleanSweep and ExoSAP-IT reagents, both of which include a mixture of Exo I and SAP in a specialized buffer to enable rapid PCR clean-up for sequence-ready products. Both CleanSweep and ExoSAP-IT reagents follow an identical one-step procedure (Figure 1) that can be integrated directly into the Sanger sequencing workflow using Applied Biosystems™ BigDye™ Terminator sequencing kits. This study details the sequencing results from a side-by-side comparison of CleanSweep and ExoSAP-IT reagents, and demonstrates their effectiveness and functional equivalency.

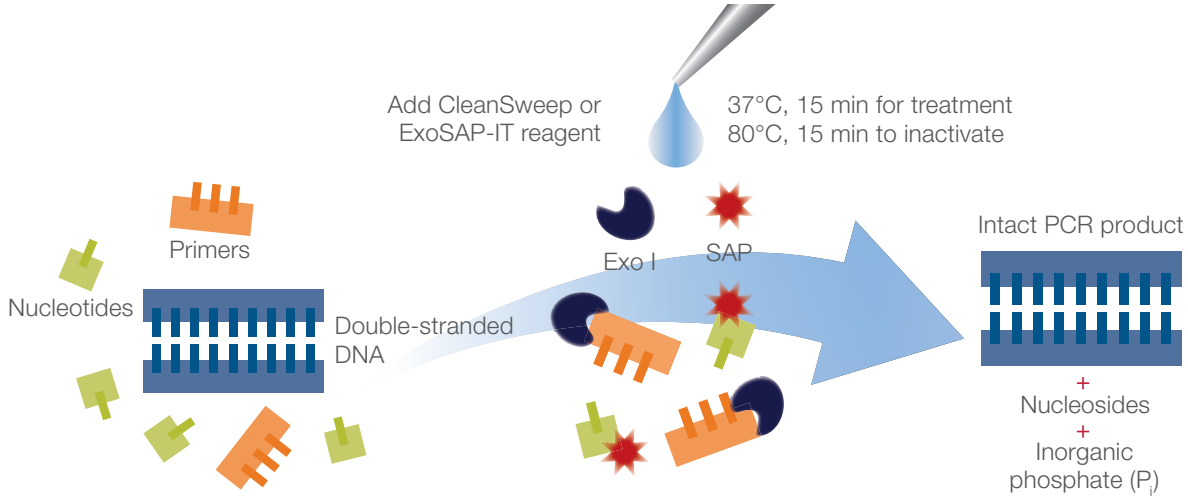

Figure 1. Workflow diagram for enzymatic PCR clean-up using CleanSweep or ExoSAP-IT reagent.

Materials and methods

Primer digestion assay

This assay is designed to assess Exo I performance in the hydrolysis of an Applied Biosystems™ HEX™ phosphoramidite dye-labeled, 24-mer ssDNA primer. Briefly, a 5 µL 1X PCR mix was prepared containing 20 picomoles of HEX dye-labeled primer. An amount of 2 µL of buffer (for mock treatment with enzyme storage buffer without enzymes), CleanSweep, or ExoSAP-IT reagent was added to the PCR mix and incubated per the common protocol (15 min at 37°C; 15 min at 80°C). The reaction was then diluted with loading dye and electrophoresis was performed on a 15% denaturing polyacrylamide gel. The primer integrity was visualized on a laser scanner equipped with a HEX filter. Densitometry was used to quantify gel-band intensity.

pNPP assay

This assay is designed to assess SAP performance in dephosphorylation of a pNPP substrate. Briefly, a 5 µL mix containing PCR buffer and 50 nmol pNPP was prepared. Then, 2 µL buffer, CleanSweep, or ExoSAP-IT reagent was added to the pNPP mix, and incubated according to the common protocol (15 min at 37°C; 15 min at 80°C). Samples were diluted in glycine buffer (pH 10.4) and dephosphorylated product was measured spectrophotometrically at 405 nm using the extinction coefficient of 18,200 $M^{-1}cm^{-1}$.

PCR

The PCR products from two sizes (1 kb and 640 bp) used in this analysis were prepared by standard methods. The 1 kb PCR products contained excess PCR primers for assessing functional performance of CleanSweep and ExoSAP-IT reagents. The 640 bp PCR products were prepared using M13-tailed primers in 72 individual reactions in a 96-well plate for clean-up of 24 replicates with each enzymatic clean-up reagent to access assay precision.

Enzymatic clean-up using CleanSweep or ExoSAP-IT reagent

Prior to sequencing, PCR products were treated with buffer, CleanSweep, or ExoSAP-IT reagent in a 2:5 (enzyme:PCR) ratio and incubated according to the common protocol (15 min at 37°C; 15 min at 80°C).

Sanger sequencing

After enzymatic PCR clean-up, amplicons were analyzed by Sanger sequencing using the BigDye Terminator sequencing kit to assess functional equivalency of the two clean-up reagents. The 1 kb PCR products were sequenced in replicates of 12 using Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit and Applied Biosystems™ 3730 DNA Analyzer according to the manufacturer's recommendations, in order to compare the treatment efficacy of buffer, CleanSweep, and ExoSAP-IT reagents in the presence of disrupting background primer. Data were analyzed using Applied Biosystems™ Sequence Scanner Software v1.0 and electropherograms were extracted from DNA Sequence Assembler v4 (2013), Heracle BioSoft, **DnaBaser.com**

The 640 bp PCR products were sequenced in replicates of 24 using the BigDye v3.1 kit and the Applied Biosystems™ 3500xL Genetic Analyzer according to the manufacturer's recommendations to compare buffer, CleanSweep, and ExoSAP-IT reagents for data quality and consistency across an entire capillary array. Multiple injections were performed on each set of 24 samples to insure instrument reproducibility. Raw quality value (QV) score for each base call was extracted from .PHD files and imported to JMP software for statistical analysis. Peak under peak (PUP) and contiguous read length (CRL) were extracted from .ab1 files using customized MATLAB script used in house for product development.

Results

Primer digestion and pNPP assays revealed equivalent performance of the Exo I and SAP, respectively, in both CleanSweep and ExoSAP-IT reagents (Figure 2). A 24-mer ssDNA primer was completely digested by Exo I when treated with either clean-up reagent, but no digestion was observed in the presence of buffer (Figures 2A and 2B). pNPP substrate was also completely dephosphorylated by SAP when treated with either clean-up reagent (Figure 2C).

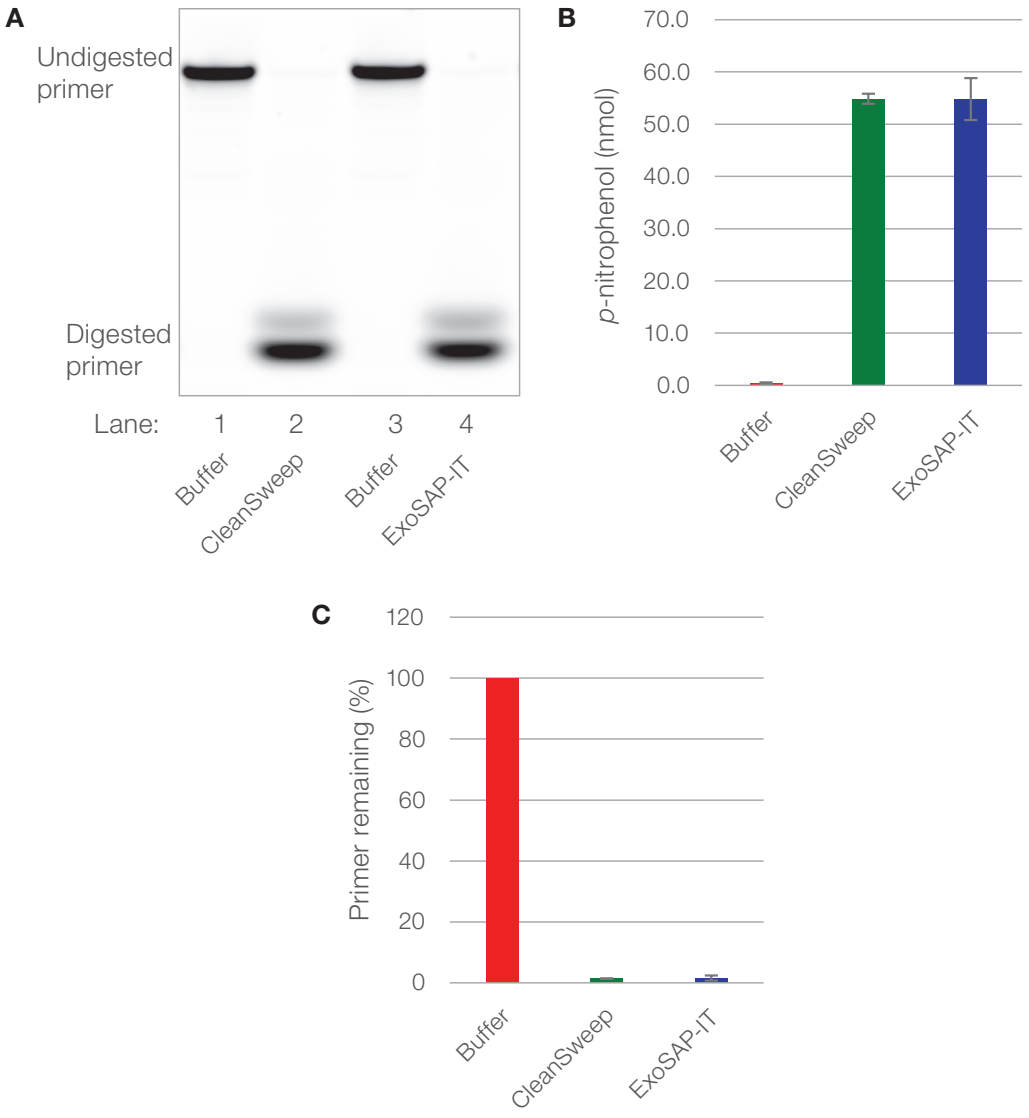

Figure 2. Exo I and SAP performance. Primer digestion assays were used to assess Exo I performance using CleanSweep or ExoSAP-IT reagent. **(A)** Gel electrophoresis of a 24-mer ssDNA primer treated with buffer (lanes 1 and 3), CleanSweep (lane 2), or ExoSAP-IT reagent (lane 4). **(B)** pNPP assays were used to assess SAP performance from CleanSweep and ExoSAP-IT reagent. The extinction coefficient of *p*-nitrophenol was used to calculate nanomoles of dephosporylated product (end point ≥50 nmol). **(C)** Densitometry plots from triplicate primer digestion assays quantifying percent of primer remaining relative to that in buffer controls.

The benefit of enzymatic PCR clean-up prior to Sanger sequencing is directly observable in the sequencing electropherograms (Figures 3A, 3B, and 3C). Following PCR synthesis of a 1 kb amplicon, excess background primers were added to the PCR products followed by treatment with CleanSweep reagent (Figure 3A), ExoSAP-IT reagent (Figure 3B), or buffer (Figure 3C). When PCR products were treated with enzyme, baseline resolution of each peak was clearly visible and baseline noise level was much lower compared to the samples mock-treated with buffer. The effectiveness of CleanSweep and ExoSAP-IT PCR clean-up reagents was also seen when plotting QV (Figure 3D) and CRL (Figure 3E) data. This analysis further supports equivalent functional performance by CleanSweep and ExoSAP-IT reagents in the presence of interfering levels of background primer.

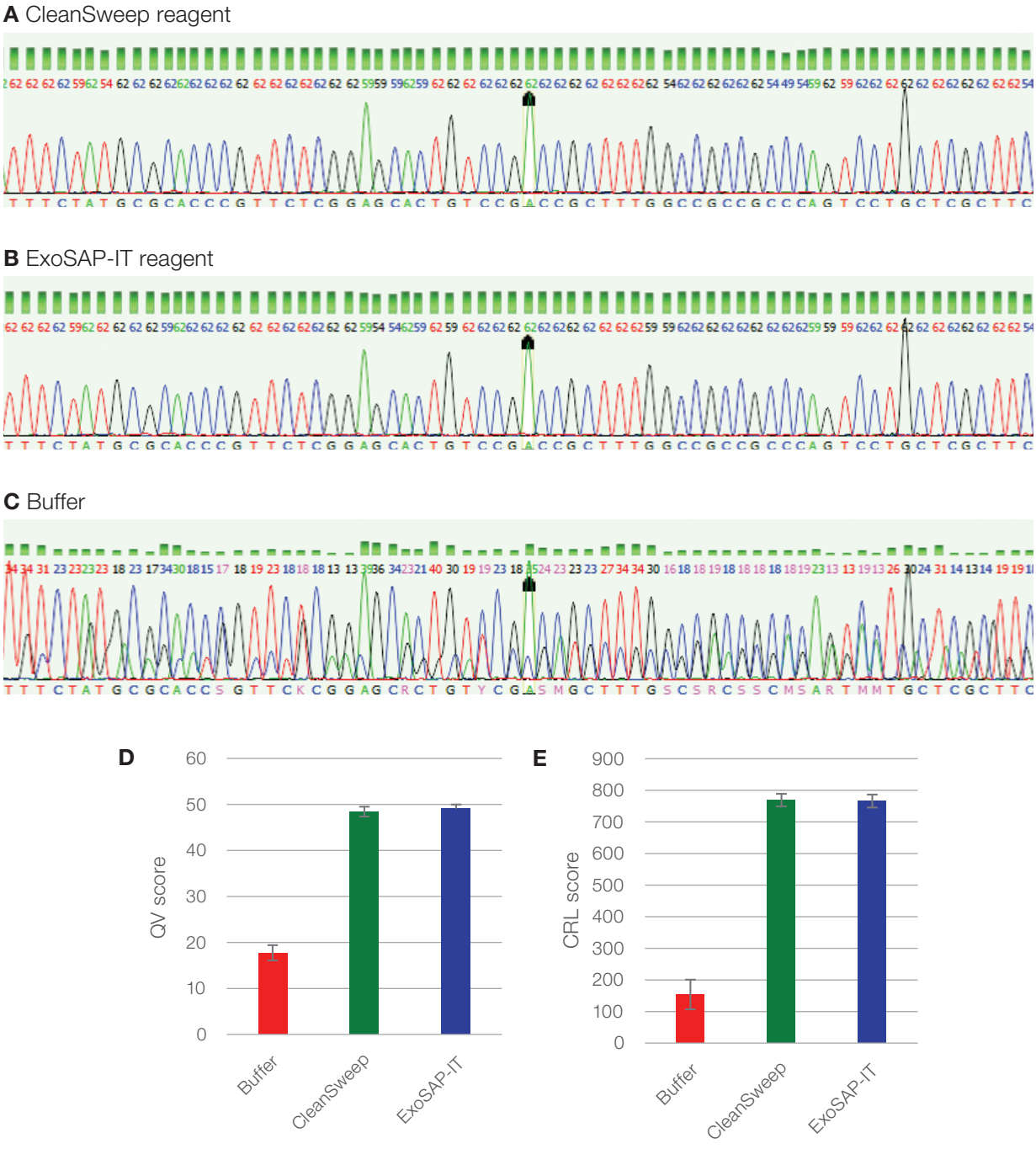

Figure 3. Sequencing electropherograms comparing treatment with (A) CleanSweep reagent, (B) ExoSAP-IT reagent, and (C) Buffer (miscalls coded per IUPAC). Quality values and bars are noted above the traces, and base calls below. **(D)** QV and **(E)** CRL averages across replicates (buffer n = 12; CleanSweep reagent n = 9; ExoSAP-IT reagent n = 12).

To determine if functional equivalency can be supported statistically, 24 replicates of a 640 bp PCR amplicon were treated with buffer, CleanSweep, or ExoSAP-IT reagent and were sequenced to determine data consistency across an entire capillary array on the 3500xL Genetic Analyzer. Following cycle sequencing, ethanol precipitation, and capillary electrophoresis (CE), 72 samples were analyzed in Sequence Scanner and SAS™ JMP™ software. Figure 4 results are histogram plots showing the number of base calls with a given QV. Each histogram represents QV score of each base call from 24 capillaries of sequencing. The number of base calls above QV 50 is ~11,000 for both CleanSweep (Figure 4A) and ExoSAP-IT reagents (Figure 4B). For the samples mock-treated with buffer, most of the QV scores fall in the 30–40 range (Figure 4C), and the number of base calls above QV 50 is only ~1,500.

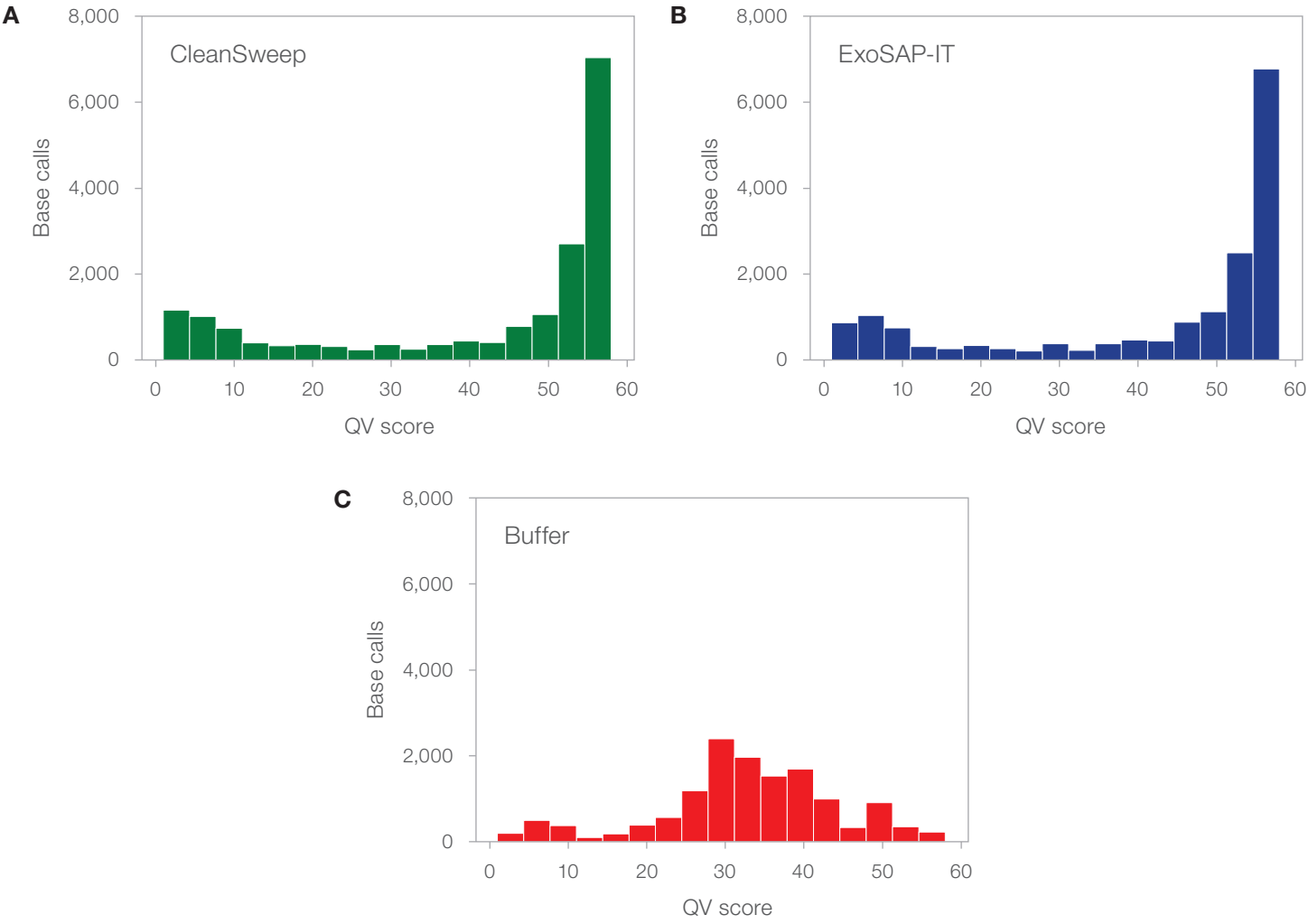

Figure 4. Histogram data of base calls vs. QV score comparing enzymatic clean-up versus mock-treated PCR product. Each graph represents 24 replicates, or one injection on 3500xL Genetic Analyzer. Each histogram correlates the number of base calls with a given QV score. Data was compiled in JMP software from .phd files. (**A**) CleanSweep reagent, (**B**) ExoSAP-IT reagent, and (**C**) Buffer.

The box plot results (Figures 5 and 6) show the distribution of numerical data from 24 capillaries (one injection). Each injection includes 24 capillaries containing the same sample treated with either CleanSweep reagent (A in Figures 5 and 6), ExoSAP-IT reagent (B in Figures 5 and 6), or buffer (C in Figures 5 and 6). Figure 5 shows the distribution of CRL scores for the three experimental groups. For the enzyme-treated samples (A and B in Figure 5) the distribution is much smaller compared to the samples mock-treated with buffer (C in Figure 5). Furthermore, the average CRL score is comparably higher for the enzyme-treated samples. Figure 6 shows the distribution of PUP scores for the three experimental groups. The same correlation is observed for PUP score in terms of improved average and distribution for the enzyme-treated samples (A and B in Figure 6) compared to samples mock-treated with buffer (C in Figure 6).

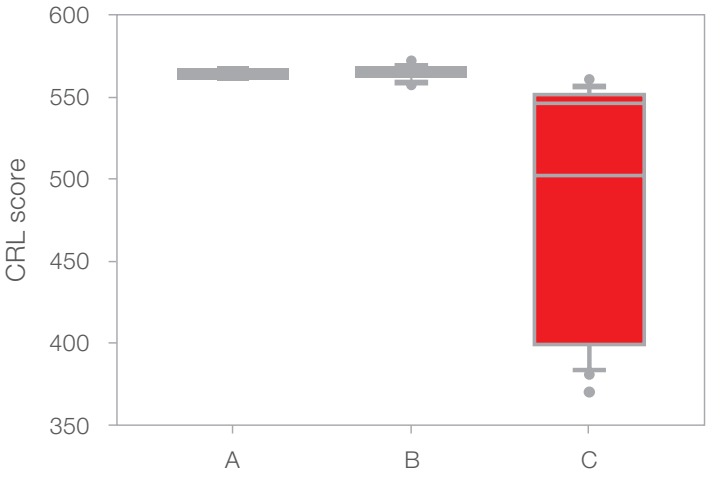

Figure 5. CRL scores comparing enzymatic clean-up by CleanSweep reagent, ExoSAP-IT reagent, or buffer (A, B, and C on x-axis, respectively). Each box plot represents 24 replicates, or one injection on a 3500xL Genetic Analyzer.

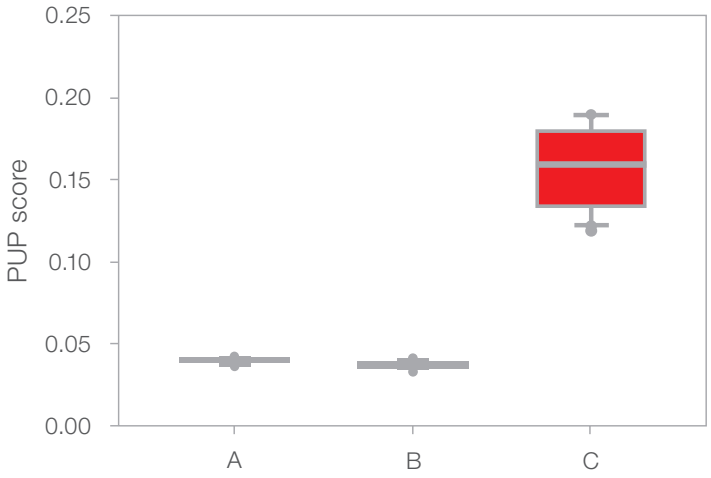

Figure 6. PUP scores comparing enzymatic clean-up by CleanSweep reagent, ExoSAP-IT reagent, or buffer (A, B, and C on x-axis, respectively). Each box plot represents 24 replicates, or one injection on a 3500xL Genetic Analyzer.

Conclusion

In this white paper, we demonstrate functional equivalence between CleanSweep and ExoSAP-IT reagents in assays evaluating enzyme performance, and by Sanger sequencing analysis. An effective workflow for targeted PCR amplification, enzymatic PCR clean-up, and Sanger sequencing using BigDye Terminator kits is described. In addition, the benefits of cleaning up PCR products with CleanSweep or ExoSAP-IT reagent are revealed.

With ExoSAP-IT reagent, we are now offering a robust enzymatic PCR clean-up. It is recommended for all PCR-based, Sanger sequencing workflows. The utility of ExoSAP-IT reagent in Sanger sequencing is referenced in over 10,000 publications and is reflective of its ease of use and ability to increase workflow efficiency and data quality. With ExoSAP-IT PCR clean-up reagent, the power of sequencing can be applied using a short protocol and very little development time.

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Latest development

We recently introduced the new Applied Biosystems™ ExoSAP-IT™ *Express* PCR Product Cleanup Reagent, which provides the same high-resolution sequencing results as CleanSweep reagent and the original ExoSAP-IT reagent (Figure 7). ExoSAP-IT *Express* reagent contains a novel version of Exo I, which enables complete enzyme inactivation in only 1 min. With a time-saving protocol of 4 min at 37°C for treatment, followed by 1 min at 80°C for inactivation, PCR clean-up can be performed without having to walk away from the bench.

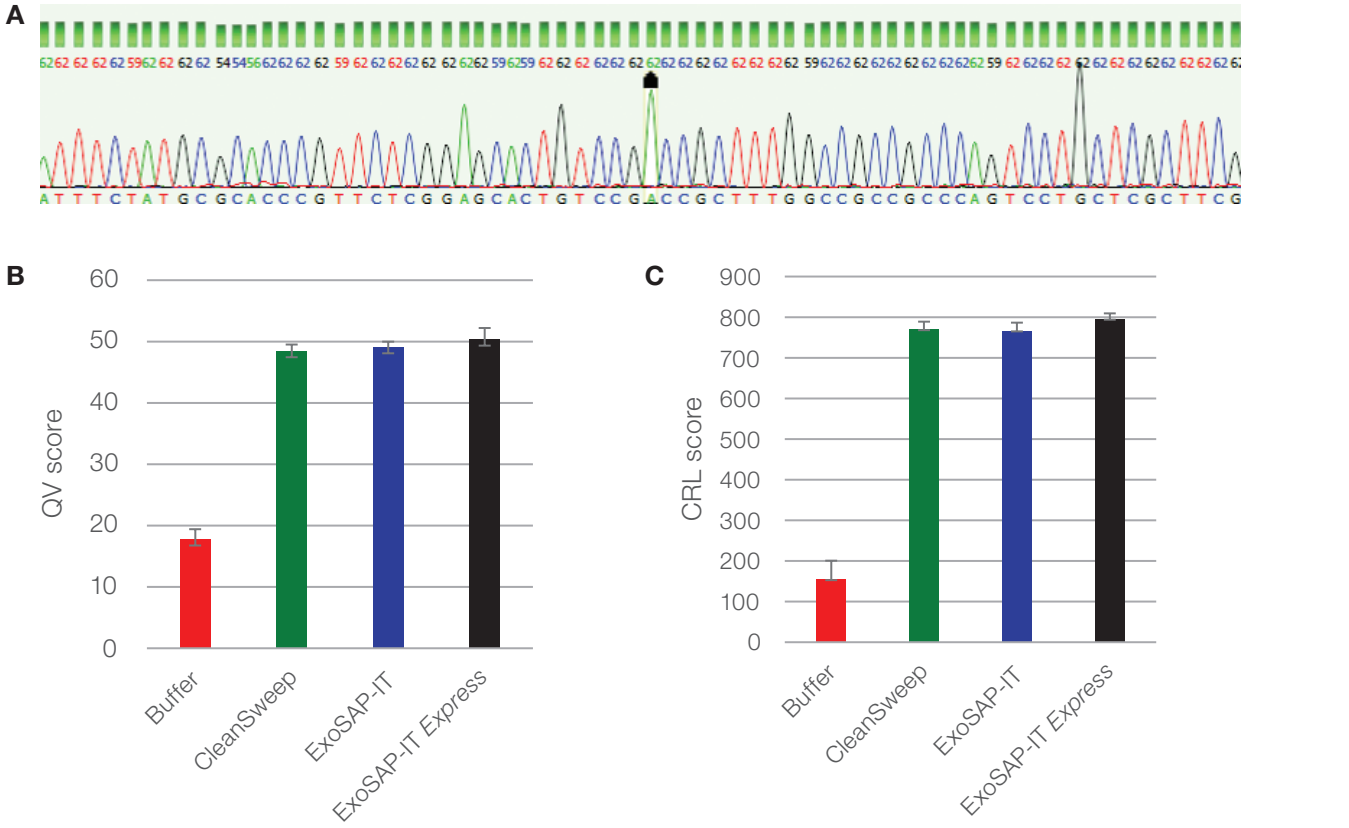

Figure 7. Functional equivalence of ExoSAP-IT *Express* reagent compared to CleanSweep and ExoSAP-IT reagents shown by (A) sequencing electropherograms (compared to Figures 3A and 3B), (B) QV, and (C) CRL average scores across replicates (Buffer n = 12; CleanSweep reagent n = 9; ExoSAP-IT reagent n = 12; ExoSAP-IT *Express* reagent n = 12).

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