ApplicationNote

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Use of ExoSAP-IT® PCR Product Cleanup Reagent in Next Generation Sequencing (NGS)

Cited in over 10,000 publications, ExoSAP-IT reagent is considered the gold standard for enzymatic PCR cleanup. The one-tube, one-step ExoSAP-IT method has many advantages over using spin columns or magnetic beads for PCR cleanup. With its simple protocol and 100% recovery of both short and long amplicons, ExoSAP-IT reagent enables researchers to conserve limited samples and improve workflow efficiency. While Sanger-based sequencing methods remain popular for validation and long contiguous DNA sequence reads (>500 nucleotides), many genomic analysis applications are transitioning to Next Generation Sequencing (NGS) technology for its scalability and affordability when sequencing a large number of targets[1].

In addition to its routine use in Sanger-based NGS validation, ExoSAP-IT reagent is proving beneficial in library preparation workflows across a broad range of NGS applications and platforms including: Thermo Fisher Scientific Ion Torrent, Illumina, and PacBio (Table 1). This Application Note reviews NGS applications by platform and highlights the utility of ExoSAP-IT reagent and its benefits for each workflow.

Table 1: ExoSAP-IT reagent is improving NGS workflows across a broad range of applications and platforms.

Application	Platform	Workflow	ExoSAP-IT reagent benefits	Clinical relevance	Ref.
Species ID	Ion Torrent PGM™	Mitochondrial DNA PCR → ExoSAP-IT reagent → Ion Xpress™	Efficient PCR cleanup prior to Ion Xpress	ID species population in meat	(2)
	Ion Torrent PGM	16S PCR → ExoSAP-IT reagent → PCR2 adds adapters	PCR2 efficiency	ID bacteria population in water	(3)
	Ion Torrent PGM	16S PCR → ExoSAP-IT reagent → Ion Xpress	Efficient PCR cleanup prior to Ion Xpress	ID bacteria population in water	(4, 5)
Mutation analysis	Illumina® MiSeq®	Long Range (LR)-PCR → ExoSAP-IT reagent → Nextera®	Efficient PCR cleanup prior to Nextera	Detect markers for chronic fatigue	(6)
	Illumina MiSeq	LR-PCR → ExoSAP-IT reagent → column purification → TruSeq®	Improve purification efficiency	Validate INDELs from WGS	(7)
	Illumina MiSeq	LR-PCR → ExoSAP-IT reagent → column purification → TruSeq	Improve purification efficiency	Validate INDELs from WGS	(8)
	Illumina MiSeq	Multiplex PCR → ExoSAP-IT reagent → PCR2 adds adapters	PCR2 efficiency	Tumor profiling for cancer mutations	(9)
	ABI 3730	PCR → ExoSAP-IT reagent → BigDye Terminator sequencing	High-resolution Sanger sequence data	Confirm deletions vs. allele dropouts	(10)
Genotyping	Ion Torrent PGM	HLA PCR → ExoSAP-IT reagent → Ion Xpress	Remove ssDNA for Ion Shear™ efficiency	HLA genotyping	(11)
	Illumina HiSeq®	Shear gDNA → ExoSAP-IT reagent → PCR2 adds adapters	Increase % on target reads	Genotyping in Thousands (GT)-seq	(12)
	Illumina GAIIx	Add adapters with PCR → ExoSAP-IT reagent → Direct seq	Remove primers that would bind flow cell	Antenatal SNP genotyping	(13)
	454	HLA PCR → ExoSAP-IT reagent → 454 library prep	Efficient PCR cleanup prior to library prep	HLA genotyping	(14)

Application	Platform	Workflow	ExoSAP-IT reagent benefits	Clinical relevance	Ref.
Targeted DNA/RNA sequencing	Ion Torrent PGM	PCR → ExoSAP-IT reagent → Ion Xpress	Remove ssDNA for Ion Shear efficiency	Growth rate genes during development	(15)
	Ion Torrent PGM	RT-PCR → ExoSAP-IT reagent → Ion Xpress	Efficient PCR cleanup prior to Ion Xpress	Rotavirus in children presenting diarrhea	(16)
	Illumina HiSeq	RT → ExoSAP-IT reagent → polyA tailing → PCR → TruSeq	PolyA tailing efficiency	Circular (circ)RNAs during development	(17)
	Illumina HiSeq	IP → Adapter ligation → RT → ExoSAP-IT reagent → RNase H → Adapter ligation → PCR	Downstream ligation and PCR efficiency	ID RNA-binding protein binding sites	(18)
	Illumina MiSeq	PCR → ExoSAP-IT reagent → PCR2 adds adapters	PCR2 efficiency	Nitrogenase gene diversity in trees	(19)
	Illumina MiSeq	PCR → ExoSAP-IT reagent → PCR2/3 adds indices/adapters	PCR2/3 efficiency	Leaf mycobiome	(20, 21)
Epigenetics	PacBio®	SMRTbell™ prep → Exo III/VII → Direct SMRT® sequencing	Remove incomplete SMRTbell templates	Directly sequence intergenic mod sites	(22)

Thermo Fisher Scientific Ion Torrent

Ion Torrent's Personal Genome Machine (PGM) uses semiconductor technology to sequence Ion Torrent DNA libraries like those prepared using the Ion Xpress Plus Fragment Library Kit. This technology works by detecting the positively charged hydrogen ion (H+) that is released when a nucleotide is incorporated. By stepwise addition of one particular nucleotide after another, each position of a DNA template can be determined. The Ion Torrent libraries consist of DNA fragments (~200 bp) ligated to blunt-ended adapters that enable sequencing on the PGM platform.

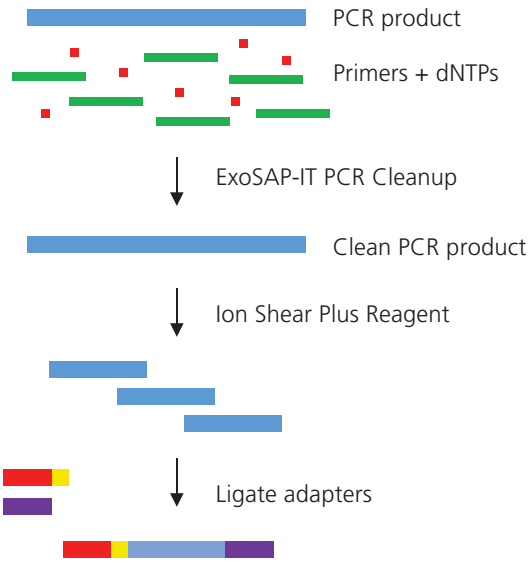
ExoSAP-IT reagent applications:
As displayed in Table 1, ExoSAP-IT reagent for PCR cleanup is used in Ion Torrent workflows across a range of NGS applications, including species identification, genotyping, and targeted sequencing.

In a study by Bertolini, *et al.*[2], mitochondrial (mt)DNA extracted from meat samples were amplified by PCR to enrich the species specific mtDNA loci. PCR products were then cleaned with ExoSAP-IT reagent prior to Ion Xpress Plus library prep and sequenced to identify the species population in the meat samples. Similar workflows were applied for 16S sequencing to identify bacteria populations in water [3, 4, 5] and in HLA genotyping assays[11]. The utility of ExoSAP-IT PCR cleanup has also been demonstrated in targeted sequencing workflows studying growth rate genes in pigs[15] and RNA Rotavirus in South African children presenting diarrhea[16]. Several of these workflows benefit from the ability of ExoSAP-IT PCR Cleanup reagent to efficiently remove excess primers that can interfere with the enzymatic shearing step by Ion Shear Plus Reagent within the Ion Xpress Plus Fragment Library Kit (Fig. 1).

Illumina instruments

Illumina offers a range of instruments that vary in read length, sequencing depth, and throughput capacity. Illumina NGS technology is based on Sequencing By Synthesis (SBS) chemistry that incorporates fluorescent nucleotides stepwise for base calling at each position of a DNA template. There are several methods available for preparing Illumina libraries including: a tagmentation approach with Nextera, ligating T-tailed adapters with TruSeq, and amplicon-based methods to add sequencing adapters.

Fig. 1. Ion Xpress Plus Fragment Library prep workflow



Example of Ion Xpress Plus Fragment Library prep workflow starting with PCR amplified DNA. ExoSAP-IT PCR Cleanup removes ssDNA and dNTPs, which enables more efficient fragmentation by Ion Shear Plus Reagent.

ExoSAP-IT reagent applications:
As displayed in Table 1, ExoSAP-IT reagent for PCR product cleanup is being used in Illumina workflows across a range of NGS applications including mutation analysis, genotyping, and targeted sequencing.

In a study by Billing, *et al.*[6], Long Range (LR)-PCR was used to amplify mtDNA to assess markers associated with Chronic Fatigue Syndrome. LR-PCR products were cleaned with ExoSAP-IT reagent prior to Nextera library prep and sequenced for mutation analysis. This study demonstrated an efficient workflow for quantifying ExoSAP-IT cleaned PCR products with Quant-IT™ PicoGreen® prior to pooling at equal molar ratios for Nextera library prep.

ExoSAP-IT reagent has been cited in several publications involving amplification steps prior to TruSeq library prep. Studies by Fang, *et al.*[7] and Narzisi, *et al.*[8], describe a similar workflow where LR-PCR products were pooled, cleaned with ExoSAP-IT reagent, and then passed through a purification column prior to TruSeq library prep and NGS confirmation of INDELs. In this workflow, ExoSAP-IT reagent removes the large population of primers and nucleotides in the PCR pool, which is important for spin column efficiency and yield. In a study by Fan, *et al.*[17], RNA from single cells were reverse transcribed and cleaned with ExoSAP-IT reagent to enable polyA tailing in a sample prep workflow upstream of TruSeq. Targeted NGS was then performed to sequence circular (circ)RNAs to investigate their regulatory function during development. A recent article by Van Nostrand, *et al.*[18] describes another example of using ExoSAP-IT reagent to clean up after reverse transcription (RT) in a targeted NGS workflow for identifying RNA-binding protein binding sites with enhanced UV crosslinking and immunoprecipitation (eCLIP).

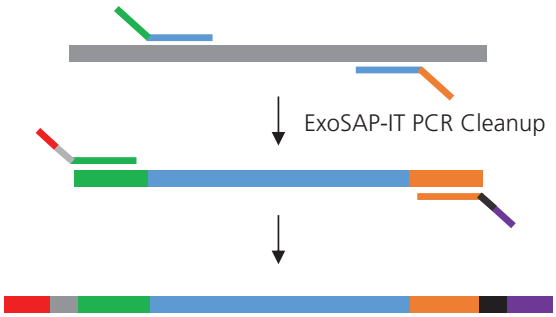
Based on recent publications, ExoSAP-IT reagent is proving to be very useful in workflows that add sequencing adapters using amplicon-based methods (Fig. 2). Examples include a study by Doty, *et al.*[19] that describes an amplicon-based library prep workflow incorporating ExoSAP-IT reagent in experiments investigating the plant microbiome, and in similar studies by Siddique, *et al.*[20] and Unterseher, *et al.*[21] investigating the mycobiome. ExoSAP-IT reagent was also used to clean up multiplex PCR products in tumor profiling assays[9], and sheared DNA in Genotyping in Thousands (GT)-seq[12] prior to amplicon-based library prep. In a study by Rieneck, *et al.*[13], adapter sequences were included in PCR primers to amplify a single nucleotide polymorphism (SNP) position, enabling direct sequencing of the SNP-containing PCR product in an antenatal genotyping assay. In this workflow, ExoSAP-IT PCR cleanup effectively removes unincorporated primers that would otherwise bind to the Illumina flow cell and decrease NGS efficiency.

PacBio platform

The Ion Torrent and Illumina platforms are similar in that the sequencing workflows include a clonal amplification step to augment the library to be sequenced. PacBio offers a third generation sequencing platform that uses the novel SMRT sequencing technology to directly sequence single molecules in Real-Time (SMRT) using uniquely colored nucleotides. PacBio library preparation is performed using the SMRTbell Template Prep Kit to construct large DNA fragments (500 bp to greater than 20 kb) flanked by SMRTbell hairpin adapters. During this process, Exonuclease treatment is necessary to remove incomplete SMRTbell templates that cause inefficiencies in the direct sequencing reaction.

ExoSAP-IT reagent applications:
Although only Exonuclease is used in this workflow, the PacBio technology is another example of how enzymatic cleanup is being applied in NGS. In a study by Seib, *et al.*[22], the PacBio workflow is described using USB® Exonuclease VII for SMRTbell template purification in order to accurately characterize intergenic modulation sites involved in gene regulation.

Fig. 2. Amplicon-based library prep



Amplicon-based methods use PCR to target specific genes and add sequencing adapters. ExoSAP-IT PCR Product Cleanup can be used in between PCR steps to increase workflow efficiency.

Summary

The prevalence of ExoSAP-IT reagent usage in Sanger-based sequencing methods is reflective of its ease of use and ability to increase the efficiency and quality of traditional DNA sequencing results. As genomic research moves toward NGS applications run on platforms developed by Thermo Fisher Scientific Ion Torrent, Illumina, and PacBio, the utility of ExoSAP-IT reagent in NGS library preparation protocols is becoming increasingly evident. Recent additions to the ExoSAP-IT reagent family (Table 2), including the fastest cleanup reagent on the market, ExoSAP-IT *Express* PCR Product Cleanup, are making it easier than ever to incorporate ExoSAP-IT PCR cleanup steps in NGS workflows for improved efficiency and more consistent results.

A product FAQ, including questions about ExoSAP-IT reagents in NGS, is available online. To speak with a member of our technical team regarding your specific NGS workflow and how ExoSAP-IT reagents can be beneficial, please contact USBtechsupport@affymetrix.com.

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Table 2: ExoSAP-IT PCR Product Cleanup reagent family.

	ExoSAP-IT *Express* ***NEW***	**ExoSAP-IT reagent** Our original formulation	**HT ExoSAP-IT *Fast* High-Throughput** For automated liquid handlers
Protocol time	5 minutes	30 minutes	14 minutes
Format	Single tube 8-tube strips 96-well plate	Single tube	Single tube 8-tube strips 96-well plate
Throughput level	Low to high; Recommended for processing any sample size	Low to mid; Recommended for processing 1-96 samples at a time	High; Recommended for processing ≥ 96 samples at a time
Platform	Single- or multi-channel pipette, automated liquid handling platforms	Single-channel pipette	Automated liquid handling platforms
Freezes at -20°C	No	No	Yes
Stability	-20°C for up to 2 years	-20°C for up to 2 years	-20°C for up to 2 years; Once thawed, stable at 4°C for 1 month and RT for 2 days

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