Sequencing NEBNext® RNA Libraries with the Element Cloudbreak Freestyle™ Workflow

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INTRODUCTION

Advances in sequencing chemistries have resulted in the release of new instrumentation with a focus on lowering costs and reducing run times while also improving the quality of sequencing data. The Element Biosciences AVITI™ benchtop sequencer utilizes a novel Avidite Base Chemistry™ (ABC™) coupled with rolling circle amplification (RCA) to achieve cost-effective, high-accuracy, massively parallel sequencing.

What is Cloudbreak Freestyle?

The Element Cloudbreak Freestyle workflow expands the compatibility of the Element Biosciences AVITI sequencer to a breadth of applications by enabling the sequencing of a wide range of third-party native libraries, without the need for a conversion step. Accordingly, existing library prep workflows can be directly sequenced on the AVITI, including the range of NEBNext® Library Preparation product workflows.

Here, we demonstrate the compatibility and performance of libraries generated using the NEBNext RNA Library Prep Kits and associated Multiplex Oligos, with the Element Cloudbreak Freestyle workflow — including the NEBNext UltraExpress® RNA and Ultra™ II Directional RNA library prep kits.

NEBNEXT ULTRAEXPRESS RNA

For RNA workflows, NEBNext UltraExpress RNA Library Prep, when combined with either NEBNext Poly(A) mRNA Enrichment or NEBNext rRNA Depletion modules, delivers strand-specific RNA library prep in as little as 3.5 hours, greatly reducing the time from sample to sequencer-ready library. Pairing this workflow with the Element Cloudbreak Freestyle represents a breakthrough advance in the speed, simplicity and throughput for a wide range of applications including full-length transcript profiling, novel transcript discovery, and quantitative expression profiling.

MATERIALS

- Universal Human Reference RNA (Agilent® #740000)
- ERCC RNA Spike-In Mix (Thermo Fisher Scientific #4456740)
- NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)
- NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400)
- NEBNext UltraExpress RNA Library Prep Kit (NEB #E3330)
- NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760)
- NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 1–5) (NEB #E6440, #E6442, #E6444, #E6446, #E6448)
- AVITI 2x75 Sequencing Kit Cloudbreak FS High Output (Element Biosciences #860-00015)
- Cloudbreak FS PhiX Control, 3rd Party (Element Biosciences #830-00023)

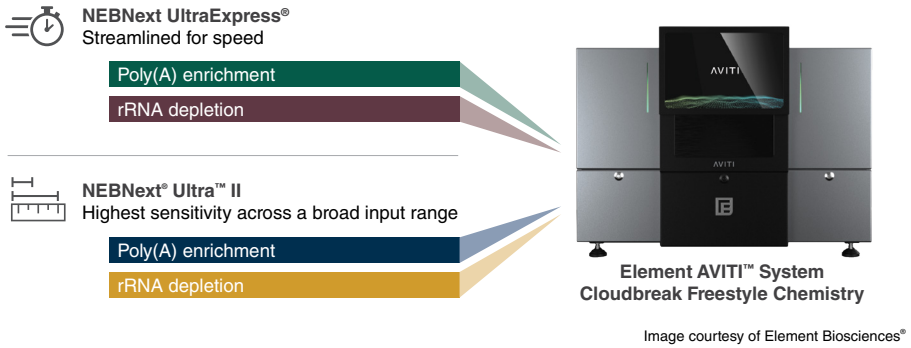


Image courtesy of Element Biosciences®

NEBNEXT ULTRA II DIRECTIONAL RNA

For samples where RNA input is extremely limited, the NEBNext Ultra II Directional RNA Library Prep Kit provides the highest sensitivity. When combined with the NEBNext Poly(A) mRNA Enrichment or NEBNext rRNA Depletion modules, the kit generates high-yield, high-quality, strand-specific libraries from a broad input range, spanning as little as 10 ng up to 1 µg of total RNA. Robust performance is maintained across a wide range of RNA qualities, including challenging samples such as FFPE. Pairing this workflow with the Element Cloudbreak Freestyle platform represents a sensitive and streamlined solution for the most demanding RNA sequencing applications.

METHODS & RESULTS

To demonstrate the compatibility between NEBNext RNA Library Prep Kits and the Element Cloudbreak Freestyle workflow, libraries were prepared using NEBNext UltraExpress RNA and Ultra II Directional RNA kits, in combination with mRNA enrichment and rRNA depletion. The resulting libraries were then sequenced using the Element Cloudbreak Freestyle reagents. The resulting high-quality sequencing data demonstrates high mapping rates, low duplication, low error rates, and uniform transcript coverage.

NEBNext UltraExpress RNA with mRNA Enrichment

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent®), using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490), and libraries were prepared using the NEBNext UltraExpress RNA Library Prep Kit (NEB #E3330) with NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 4) (NEB #E6446). The NEBNext UltraExpress RNA Library Prep Kit was used with a single adaptor dilution (50X) and 12 PCR cycles for all inputs.

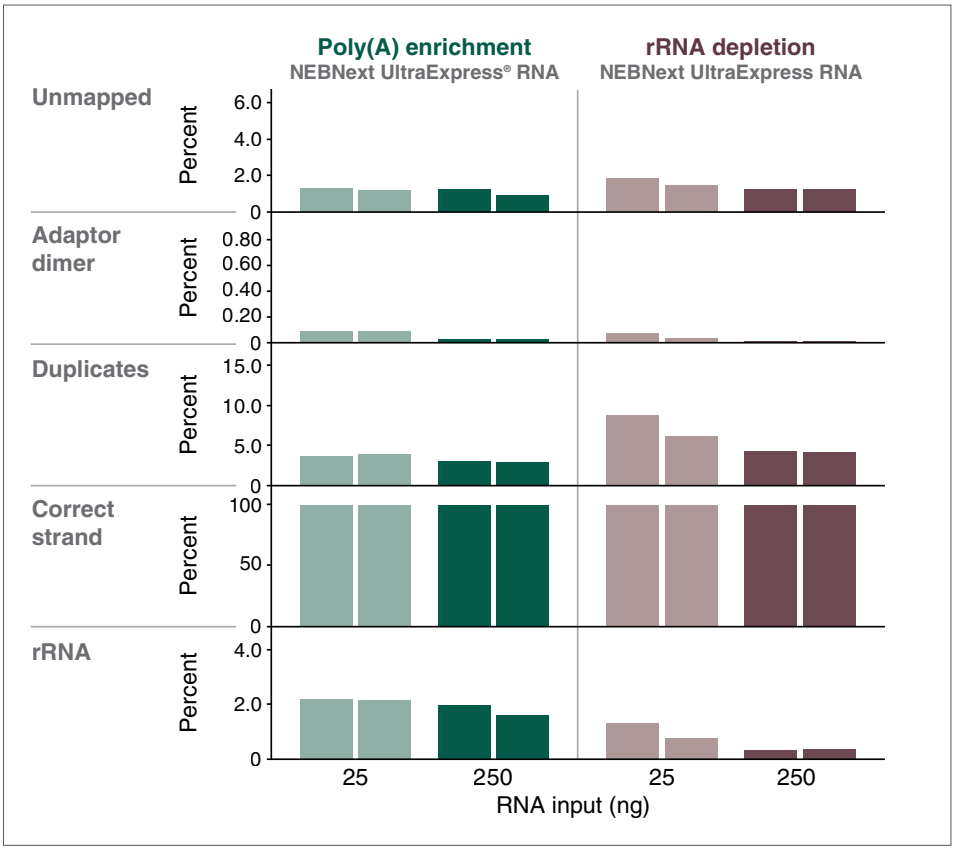
NEBNext UltraExpress RNA with Ribosomal RNA Depletion

Ribosomal RNA (rRNA) was depleted from Universal Human Reference RNA (Agilent) using the NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat – NEB #E7400), and libraries were prepared using the NEBNext UltraExpress RNA Library Prep Kit (NEB #E3330) with NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 5) (NEB #E6448). The NEBNext UltraExpress RNA Library Prep Kit was used with a single adaptor dilution (50X) and 12 PCR cycles for all inputs.

NEBNext Ultra II Directional RNA with mRNA Enrichment

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent), using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490), and libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760) with NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 5) (NEB #E6448).

FIGURE 1: **NEBNext Ultra Express RNA libraries sequenced using the Element Cloudbreak Freestyle chemistry produce high quality sequencing data**



NEBNext UltraExpress RNA libraries were prepared from 25 ng and 250 ng of Universal Human Reference RNA using mRNA enrichment or rRNA depletion, with a single adaptor dilution (50X) and 12 PCR cycles. Libraries were prepared in duplicate, sequenced (2 × 75 bp) on the Element AVITI instrument and downsampled to 3 million read pairs for analysis.

NEBNext Ultra II Directional RNA with Ribosomal Depletion

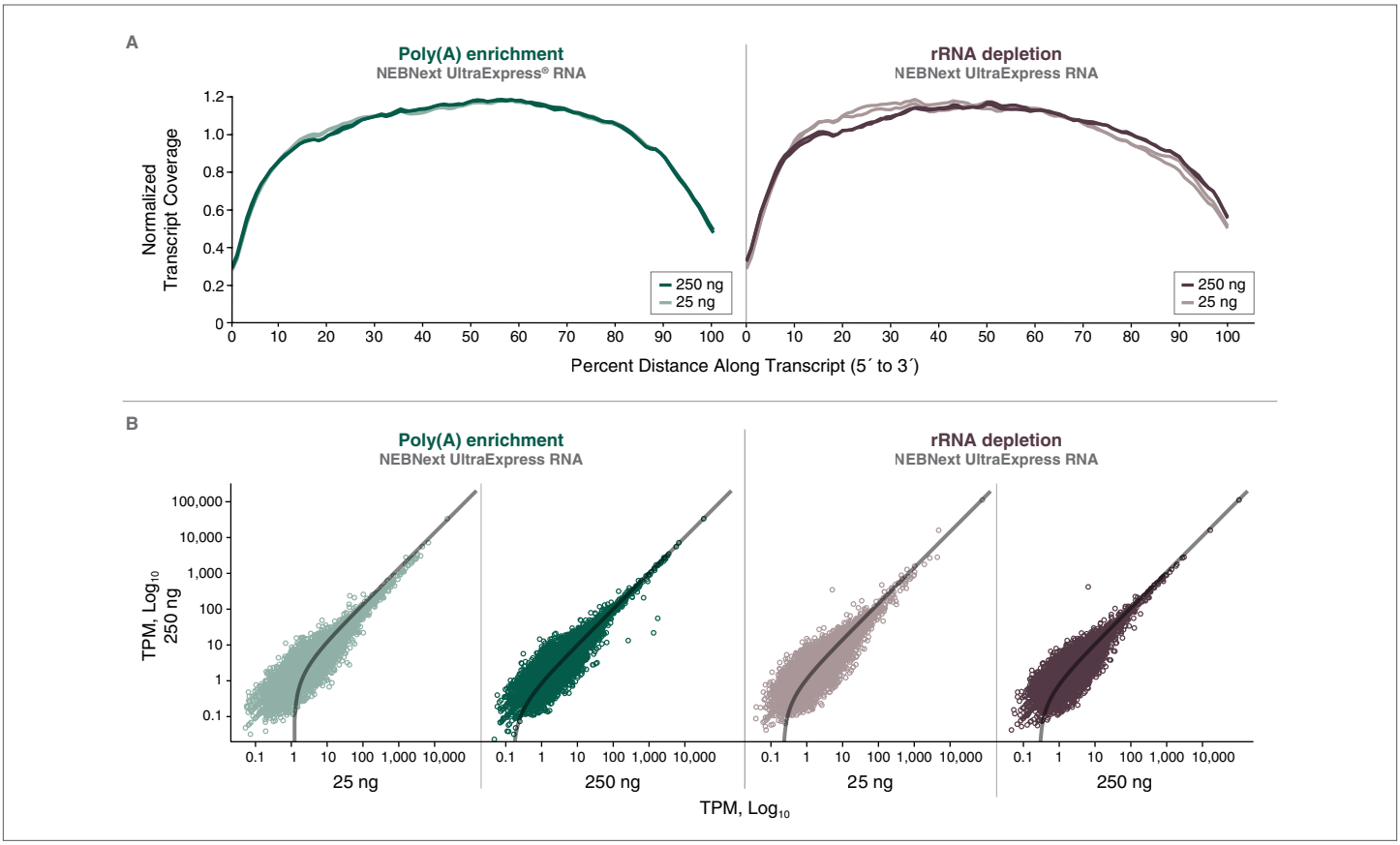
Ribosomal RNA (rRNA) was depleted from Universal Human Reference RNA (Agilent) using the NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat) (NEB #E7400), and libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760) with NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 1) (NEB #E6440).

Cloudbreak Freestyle Sequencing and Data Analysis

Libraries were sequenced on an Element AVITI using Cloudbreak Freestyle reagents (AVITI 2x75 Sequencing Kit Cloudbreak FS High Output, Element Biosciences #860-00015) and 3 million read pairs were sampled from each library. Reads were adaptor-trimmed using Flexbar v3.5.0 and reads shorter than 2 bp or containing uncalled bases were filtered.

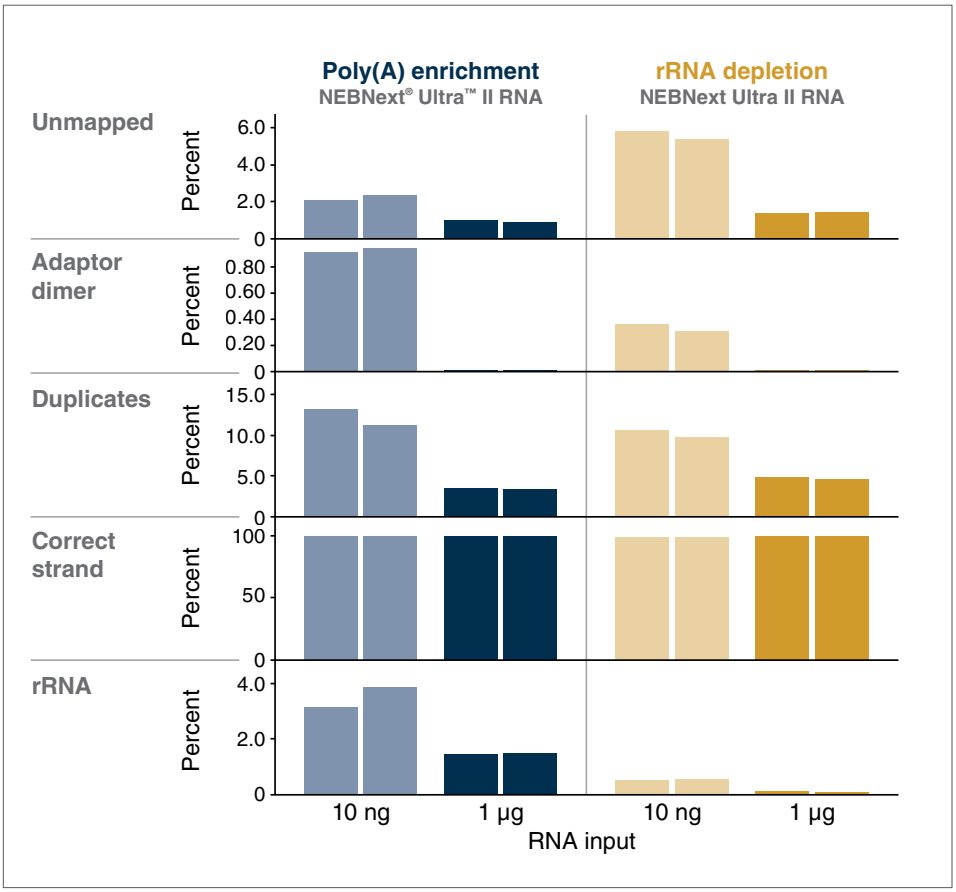
The fraction of mapped reads was determined using samtools FlagStat v1.9. The fraction of duplicate reads was calculated using picard MarkDuplicates v3.1.1. The fraction of reads identified as adaptor dimer was calculated using Flexbar v3.5.0. The fraction of reads mapping to the correct strand was calculated using picard CollectRNASeqMetrics v3.1.1. The percentage of ribosomal RNA (rRNA) reads was calculated using BBDuk v39.01 by identifying reads containing at least six kmers (k=25) from rRNA sequences. Results shown in Figure 1 and Figure 3. Reads were mapped to the hg38 reference genome using RNA STAR v2.7.8a, and 5′ to 3′ Transcript coverage was calculated from the top 1,000 transcripts using the CollectRnaSeqMetrics (Picard) tool v3.1.1. Transcript abundance, reported as TPM (Transcripts Per Kilobase Million), was correlated across inputs using Salmon v1.10.1 quantification of all gencode v38 and ERCC transcripts. Results shown in Figure 2 and Figure 4.

FIGURE 2: **NEBNext UltraExpress RNA libraries sequenced using the Element Cloudbreak Freestyle chemistry result in even transcript coverage and high transcript abundance correlation across input amounts**



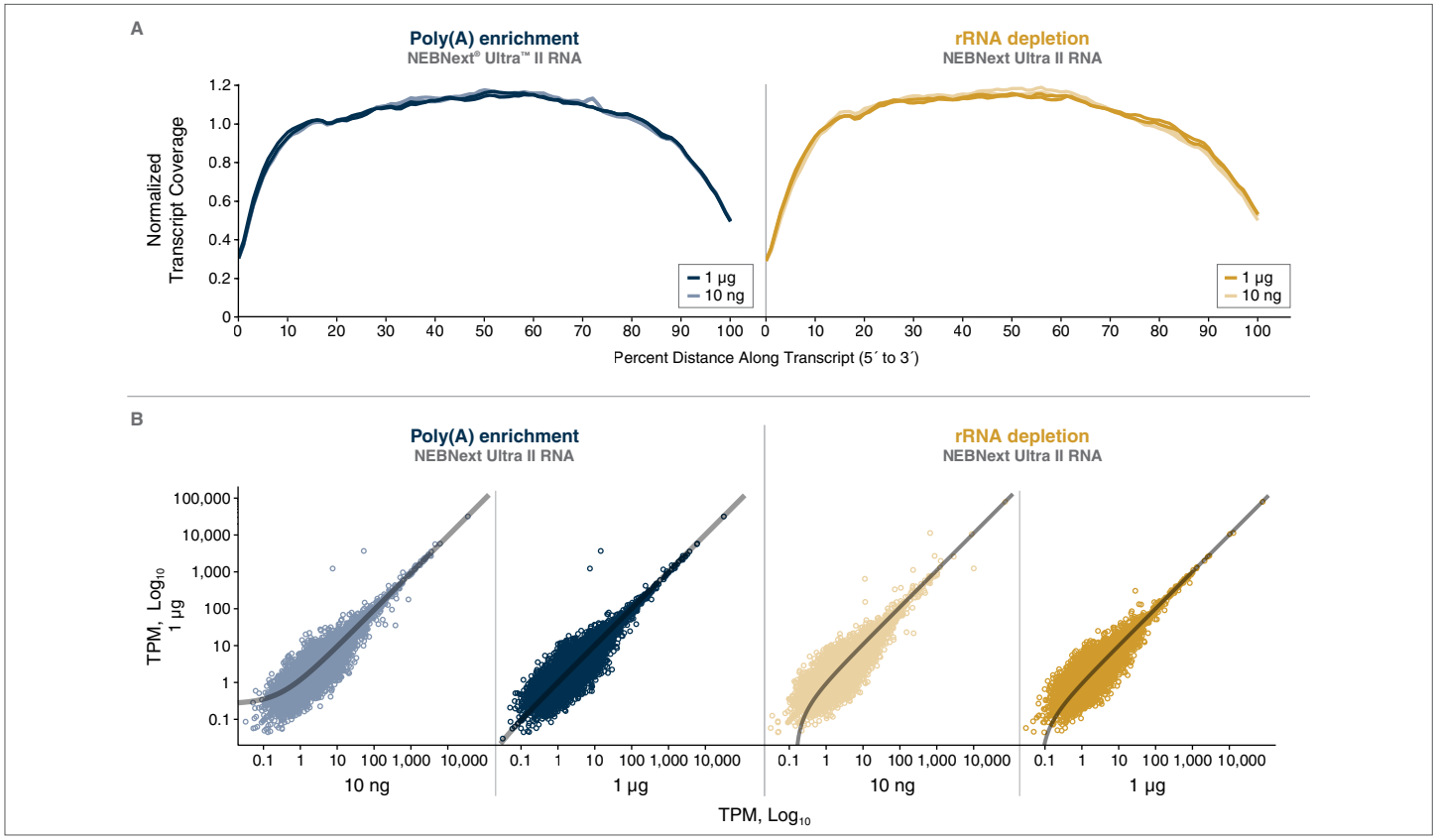
NEBNext UltraExpress RNA libraries were prepared from 25 ng and 250 ng of Universal Human Reference RNA using mRNA enrichment or rRNA depletion, with a single adaptor dilution (50X) and 12 PCR cycles. Libraries were prepared in duplicate, sequenced (2 × 75 bp) on the Element AVITI instrument and downsampled to 3 million read pairs for analysis. (A) 5´to 3´ transcript coverage for the top 1,000 transcripts; (B) Transcript abundance correlation of all gencode v38 and ERCC transcripts across inputs, reported as TPM (Transcripts Per Kilobase Million).

FIGURE 3: **NEBNext Ultra II Directional RNA libraries sequenced using the Element Cloudbreak Freestyle chemistry produce high quality sequencing data**



NEBNext Ultra II Directional RNA libraries were prepared from 10 ng and 1 µg of Universal Human Reference RNA using mRNA enrichment or rRNA depletion. Libraries were prepared in duplicate, sequenced (2 × 75 bp) on the Element AVITI instrument and downsampled to 3 million read pairs for analysis.

FIGURE 4: **NEBNext Ultra II Directional RNA libraries sequenced using the Element Cloudbreak Freestyle chemistry produce even transcript coverage and high transcript abundance correlations across inputs**



NEBNext Ultra II Directional RNA libraries were prepared from 10 ng and 1 µg of Universal Human Reference RNA using mRNA enrichment or rRNA depletion. Libraries were prepared in duplicate, sequenced (2 × 75 bp) on the Element AVITI instrument and downsampled to 3 million read pairs for analysis. (A) 5´ to 3´ transcript coverage for the top 1,000 transcripts; (B) Transcript abundance correlation of all gencode v38 and ERCC transcripts across inputs, reported as TPM (Transcripts Per Kilobase Million).

TABLE 1: **NEBNext RNA Products with Demonstrated Element Cloudbreak Freestyle Workflow Compatability**

RNA LIBRARY PREP PRODUCTS	CATALOG #	INPUT RANGE	WORKFLOW TIME	CLOUDBREAK FREESTYLE COMPATIBLE?
NEBNext UltraExpress RNA Library Prep Kit	E3330	25 ng – 250 ng	3 hrs	Yes
NEBNext Ultra II Directional RNA Library Prep Kit	E7760/E7765	10 ng – 1 ug	5.5 hrs – 6.5 hrs	Yes

CONCLUSION

NEBNext RNA library preparation kits are fully compatible with sequencing on the Element AVITI platform using Cloudbreak Freestyle chemistry, requiring no workflow adjustments. Sequencing results deliver high-quality metrics aligned with expected performance standards.

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