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A Differential Depth Sequencing Method, SPRE-Seq, for Enhancing Targeted Region Coverage in Hybridization Capture-Based NGS

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ABSTRACT

Sequencing depth is a crucial parameter for variant calling accuracy and sensitivity. The trade-off between sequencing breadth and depth is a well-known limitation in capture-based targeted next-generation sequencing (NGS). Herein, we propose a differential depth sequencing method, SPRE-Seq, to acquire different sequencing depths for different targeted regions in an NGS panel. The SPRE-Seq performance was evaluated using a panel of reference standards and clinical samples based on our custom-designed homologous recombination deficiency (HRD) assay. By applying SPRE-Seq, the effective sequencing depths of the homologous recombination repair (HRR) and HRD regions of all seven HRD reference standards met the required thresholds with only half the sequencing data volume (reduced from 12 to 6 GB). The results for the HRR genes and HRD showed 100% consistency with the expected results. In clinical samples, the effective sequencing depth of the HRR regions was significantly higher, with a sequencing data volume of 6 GB using the SPRE-Seq approach compared with 6 GB using a regular capture approach. However, there was no significant difference between a data volume of 6 GB using SPRE-Seq and 12 GB using a regular capture method. The SPRE-Seq approach was feasible and reliable for determining the HRD status and HRR somatic variants in reference standards and clinical samples at a low sequencing volume. SPRE-Seq is a reliable, feasible, and cost-effective method that can acquire an adequate sequencing depth of an NGS panel at a low sequencing data volume.

1 | Introduction

In recent years, the continuous, rapid progress of next-generation sequencing (NGS) technology has provided a novel avenue for answering complicated biological questions. NGS now plays an important role in human genomic research and clinical applications. As a powerful technology, NGS can reveal large-scale genomic variations contributing to various complex human diseases through whole genome sequencing (WGS), whole exome sequencing (WES), and targeted gene sequencing panels [1–4].

Abbreviations: HRD, homologous recombination deficiency; HRR, homologous recombination repair; NGS, next-generation sequencing; SPRE-Seq, for Specific-Regions-Enriched sequencing via streptavidin pre-blocked partly oligonucleotide probes.; WES, whole exome sequencing; WGS, whole genome sequencing.

The power and accuracy of these NGS-based methods depend substantially on sequencing depth and read coverage. Sequencing depth, or the number of reads sequenced, is a major determinant of the quality and detection of genetic event calls from NGS data, particularly when identifying rare genetic variants in Mendelian diseases [5–7], or low-frequency genomic alterations in cancers [8–10]. Increasing the sequencing depth typically increases the ability of NGS methods to detect genetic events. For example, in RNA sequencing, an increase in read depth results in an increase in the accuracy of transcript detection, expression estimates, and gene expression profiles [11, 12]. Genomic variation calling performance, particularly the accuracy of low-frequency mutation detection, has been shown to improve dramatically by increasing sequencing depth [13–15].

Although most current NGS approaches generate a large number of genomic alterations per sample, only a minority of these are high-impact, low-frequency loci that determine the overall performance of the method, particularly when testing large cancer gene panels for precision oncology [16]. Increasing the sequencing depth directly by increasing the sequencing data volume may appear to be the simplest and best method for improving the performance of NGS methods. However, these approaches often involve intrinsic trade-offs in breadth versus depth, particularly when funding is limited. High depth can be used to detect low-abundance alterations with great accuracy, but this severely limits the coverage breadth (the number of genomic loci or samples that can be tested simultaneously). Despite the dramatic reductions in NGS costs, it is still prohibitively expensive to analyze a large number of genomic alterations using high-depth sequencing [7, 17]. Moreover, in hybridization capture-based panels, it is common to waste a large majority of reads by redundantly sequencing regions with a low minimum desired depth to provide sufficient information for regions with a high minimum desired depth.

Here, we proposed specific region-enriched sequencing (SPRE-Seq) using oligonucleotide probes partially pre-blocked with streptavidin, a differential depth sequencing method that can acquire different sequencing depths for different targeted regions in an NGS panel. In this study, we applied SPRE-Seq to our custom homologous recombination deficiency (HRD) assay, which consisted of a 60-gene homologous recombination repair (HRR) panel, termed the HRR panel, and a homologous recombination (HR) genomic scar assay, termed the HRD panel.

2 | Materials and Methods

2.1 | Samples

Formalin-fixed, paraffin-embedded (FFPE) samples were collected from patients with ovarian cancer who underwent clinical NGS testing at our lab from March 2023 to February 2024. The tumor cellularity was evaluated by two board-certified pathologists. FFPE ovarian tissue with a minimum tumor content of 30% was selected for this study. Written informed consent following approved guidelines was obtained from each participant. This study was approved by the Ethics Committee of Xuanwu Hospital (No. [2019] 081-R1). HRD reference standards were purchased from GeneWell Tech (Shenzhen, China) and Cobier Tech (Nanjing, China).

2.2 | Streptavidin Pre-Blocked DNA Oligonucleotide Probe Preparation

The HRR panel was 245,760 bp in size and consisted of 2048 probes covering the entire exonic regions of the 60 HRR genes in the panel. The HRD panel was 4.44 Mbp in size and consisted of 37,000 probes against approximately 38,000 single-nucleotide polymorphisms (SNPs) in the whole human genome. To prepare the HRD assay control probes, 4 μL of HRR panel probes (0.4 fmol/μL) was mixed with 4 μL of HRD panel probes (0.4 fmol/μL).

To assess the optimal streptavidin volume, the HRD panel probes (0.4 fmol/μL) were blocked with ready-to-use streptavidin (0.1 mg/mL) by adding 0.5, 1, 2.5, 5, or 10 μL of streptavidin to 4 μL of HRD panel probes. Then, the mixtures were pipetted 20 times to mix and incubated at 25°C for 1 h. Next, 4 μL of each pre-blocked HRD panel probe was mixed with 4 μL of HRR panel probes to generate a series of ready-to-use pre-blocked HRD assay probes.

2.3 | Nucleic Acid Preparation and Library Construction

Genomic DNA was purified from the FFPE tissue sections (after microdissection if necessary) using a Concert FFPE DNA kit (ConcertBio, Xiamen, China) following the manufacturer's recommendations. A total of 100 ng of purified genomic DNA was fragmented using a 5 × WGS fragmentation mix kit (QIAGEN, MA, USA) for 20 min at 32°C to generate an average fragment length of 150–200 bp. The fragmented DNA then underwent pre-capture library construction using a 5 × WGS fragmentation mix kit according to the manufacturer's protocol. In brief, this included end repair, ligation, and low-cycle PCR amplification. The pre-capture library was hybridized and captured using the control HRD assay probes and pre-blocked HRD assay probes using a Twist Fast hybridization and wash kit (Twist Bioscience, CA, USA) according to the manufacturer's instructions. The captured DNA fragments were amplified with index primers and pooled before sequencing.

2.4 | Sequencing and Data Analysis

The indexed sequencing libraries were pooled and sequenced using a MGISEQ-2000 platform (BGI, Shenzhen, China) with 100-bp paired-end cycles. A mean sequencing depth of 1000 × was desired for HRR panel target regions, while 200 × was adequate for HRD panel target regions. Raw sequence reads were trimmed to remove adapters, low-quality sequence data, and short reads using Fastp (v0.22) to generate clean sequence data. The clean sequence data were mapped to the human reference genome Hg19/GRCh37 using Burrows–Wheeler Aligner (BWA, v0.7.17)-mem [18] to create binary alignment map (BAM) files. The HRD scores were calculated using the Sequenza software

package (V.3.0.0) [19] and the scarHRD R package (https://github.com /Rong-Zh/scarHRD) [20]. The HRD score was calculated as follows: HRD score = loss of heterozygosity score + telomeric allelic imbalance score + large-scale state transitions score. HRD was considered positive if the HRD score was ≥ 42.

2.5 | Additional Data Analysis and Statistics

All the data were analyzed using GraphPad Prism 8.0 (GraphPad Software, CA, USA) and SPSS (v22.0, IBM Corporation, Armonk, NY, USA) statistical software. $p < 0.05$ was considered statistically significant. The somatic variants were further verified using the Integrative Genomics Viewer (IGV, version 2.5.14), if necessary.

3 | Results

3.1 | Specific Region-Enriched Sequencing via Oligonucleotide Probes Partially Pre-Blocked With Streptavidin

SPRE-Seq is a differential depth sequencing approach that can increase the sequencing depth of specific regions of interest and disease-related variant regions by partially and randomly pre-blocking DNA oligonucleotide probes of regions with a low minimum desired depth of a targeted NGS panel with streptavidin (Figure 1). This allows SPRE-Seq to acquire different read depths among different target regions in a targeted NGS panel.

To determine how the amount of streptavidin affected the sequencing depths of the HRD and HRR regions and the optimal streptavidin volume (0.1 mg/mL streptavidin) for our HRD assay, a series of streptavidin volumes was used to generate pre-blocked HRD assay probes using different volumes of streptavidin-treated HRD panel probes (see Section 2). Seven HRD reference standards were analyzed using the pre-blocked HRD assay probes and the control HRD assay probes. At the same sequencing data volume, both the raw and effective sequencing depths of the HRR regions and the sequencing depth ratios of the HRR and HRD regions demonstrated a positive correlation with the volumes of streptavidin added to the HRD probes. The sequencing depth of the HRD regions was not affected by the volumes of streptavidin (Figure 2A,B).

For our HRD assay, the optimal sequencing depth ratio of the HRR and HRD regions was 5:1. Both the raw and effective sequencing depth ratios of the HRR and HRD regions were around 5:1 (5.5:1 and 4.39:1, respectively) using a streptavidin volume of 2.5 µL (0.1 mg/mL) (Figure 2A,B). Thus, we determined that the optimal streptavidin volume for our HRD assay was 2.5 µL. HRD assay probes pre-blocked with 2.5 µL of 0.1 mg/mL streptavidin were determined to be the optimal HRD assay probes. All seven HRD reference standards were recaptured using the optimal HRD assay probes, and the sequencing data volume was reduced from 12 to 6 GB. Both the effective sequencing depths of the HRR and HRD regions met the requirements of the HRD assay (Figure 2C, 200 × for HRD regions and 1000 × for HRR regions). The status of the HRR genes and HRD showed 100% consistency with the expected results (Table 1, Figure 2D).

3.2 | Performance of SPRE-Seq Using Real-World Clinical Samples

FFPE samples from 29 patients with ovarian cancer were analyzed using the control HRD assay probe with a sequencing data volume of 12 GB (control-12G group) or 6 GB (control-6G group), or the pre-blocked HRD assay probe with a sequencing data volume of 6 GB (pre-blocked-6G group). The average sequencing data volume of the samples in the control-12G group was 11.53 GB, while an average sequencing data volume of 6.35 GB was achieved in the pre-blocked-6G group. The average effective sequencing depths of the HRD and HRR regions in the control-12G group were 699.79 and 1141.66, respectively, and in the pre-blocked-6G group were 410.31 and 1052, respectively. The effective sequencing depth and capture efficiency of the HRD regions were significantly higher in samples in the control-12G group than in the pre-blocked-6G group (Figure 3A,B). However, the effective sequencing depth of the HRR regions showed no significant difference between the control-12G and pre-blocked-6G groups (Figure 3A). The capture efficiency of the HRR regions was significantly higher in samples in the pre-blocked-6G group than in the control-12G group (Figure 3B). The sequencing uniformity of the HRD and HRR regions showed no difference between the control-12G and pre-blocked-6G groups (Figure 3C).

The average sequencing data volume of samples in the control-6G group was 6.36 G, and the average effective sequencing depths of the HRD and HRR regions in the control-6G group were 441.24 and 737.13, respectively. The sequencing depth of the HRR regions in 28/29 (96.55%) of the samples was less than 1000, which did not meet the minimum desired depth of the HRR panel. The effective sequencing depth and capture efficiency of the HRR regions were significantly higher in samples in the pre-blocked-6G group than in the control-6G group (Figure 3D,E). The effective sequencing depth of the HRD regions showed no difference between the pre-blocked-6G and control-6G groups. The capture efficiency of the HRR regions was significantly higher in samples in the pre-blocked-6G group than in the control-6G group (Figure 3D,E). The sequencing uniformity of the HRD and HRR regions showed no difference between the pre-blocked-6G and control-6G groups (Figure 3F).

The HRD status of all 29 samples showed 100% concordance between the control-12G and pre-blocked-6G groups (Figure 3G) and a 93.13% (27/29) concordance between the pre-blocked-6G and control-6G groups (Figure 3H). In the HRR regions, there were 89 somatic variants detected in all three groups, with 92 somatic variants detected in the control-12G and pre-blocked-6G groups. Concordance rates of 96.84% (92/95) and 95.69% (89/93) were observed between the control-12G and pre-blocked-6G groups and between the pre-blocked-6G and control-6G groups, respectively (Figure 3I). Among the three discordant variants detected between the control-12G and pre-blocked-6G groups, two variants were found only in the control-12G group, and one variant was found only in the pre-blocked-6G group. The variant allele frequencies (VAFs) of all three variants were near the cut-off value. All three variants were confirmed as true variants using Integrative Genomics Viewer (Figure S1).

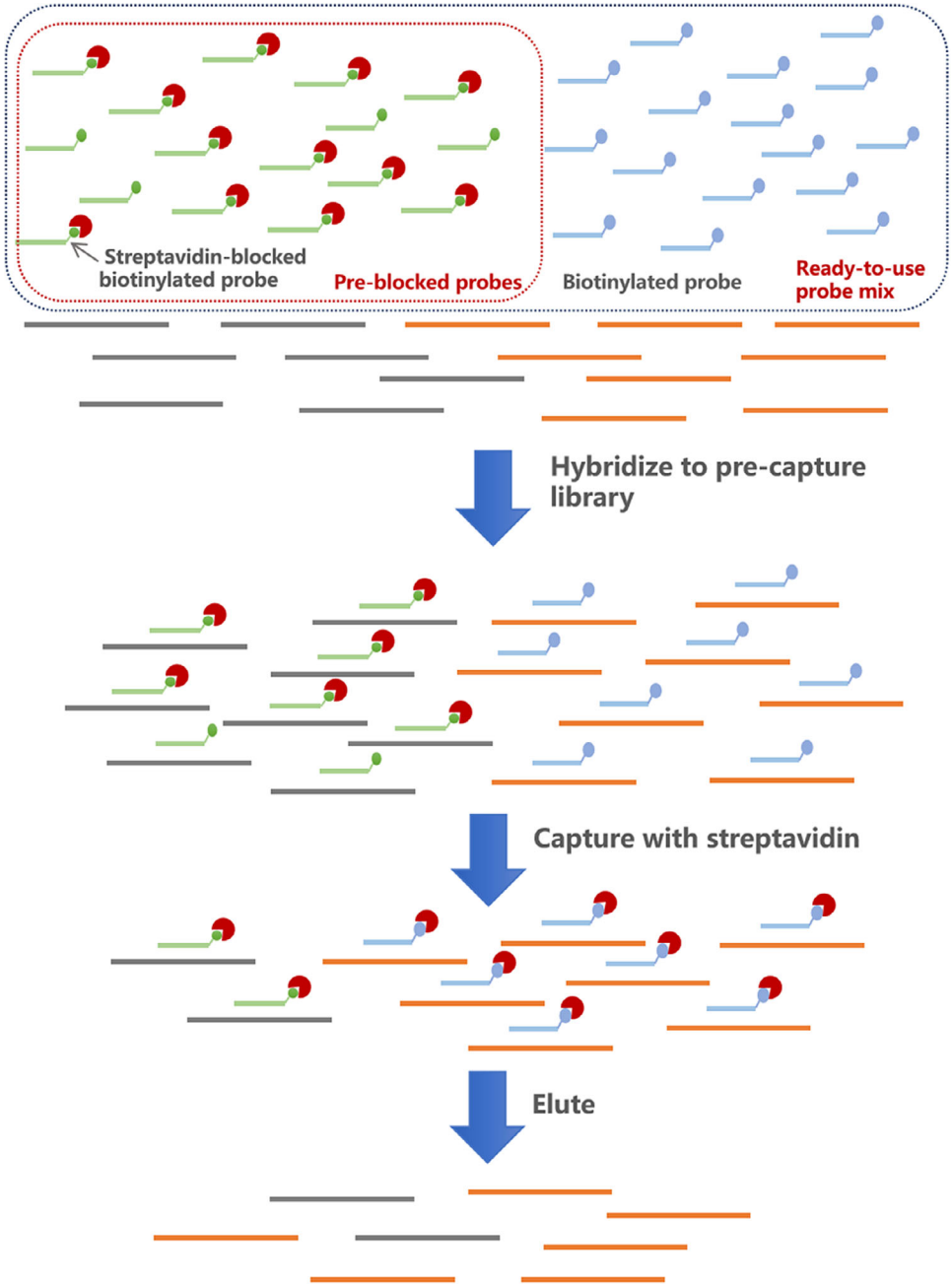

FIGURE 1 | Principal diagram of SPRE-Seq. The biotinylated DNA oligonucleotide probes (green), designed to target regions with low minimal desired depth, were partially and randomly blocked by streptavidin at a predetermined concentration, generating pre-blocked probes (orange box). Those pre-blocked probes were then combined with probes targeting regions requiring high minimal desired depth (blue) to prepare the ready-to-use probe mixture (gray box). Following hybridization with the pre-capture library, the pre-blocked probes—unable to bind streptavidin—ensured selective enrichment of molecules from high-depth regions in the final library.

4 | Discussion and Conclusion

Hybridization capture-based targeted sequencing, in which a set of DNA hybridization probes is designed to bind and enrich DNA regions of interest [21, 22], is becoming a dominant technique in research and clinical applications. Sequencing depth is a crucial parameter for variant calling accuracy and sequencing cost. In this study, we presented SPRE-Seq, a novel differential depth sequencing approach aimed at partially overcoming the inherent trade-off between breadth and depth in capture-based NGS. Notably, this method is compatible with any biotinylated probe-based NGS panel, provided it comprises at least two independent probe panels.

In this study, biotinylated DNA oligonucleotide probes were pre-blocked using a universal biotin-streptavidin method. The streptavidin volume (at a fixed concentration) used for blocking the targeted probes was of utmost importance for the successful implementation of this method. Several critical factors, including probe panel size (the number of probes), %GC content, and sample variability, should be carefully considered when determining the optimal streptavidin volume for custom assay

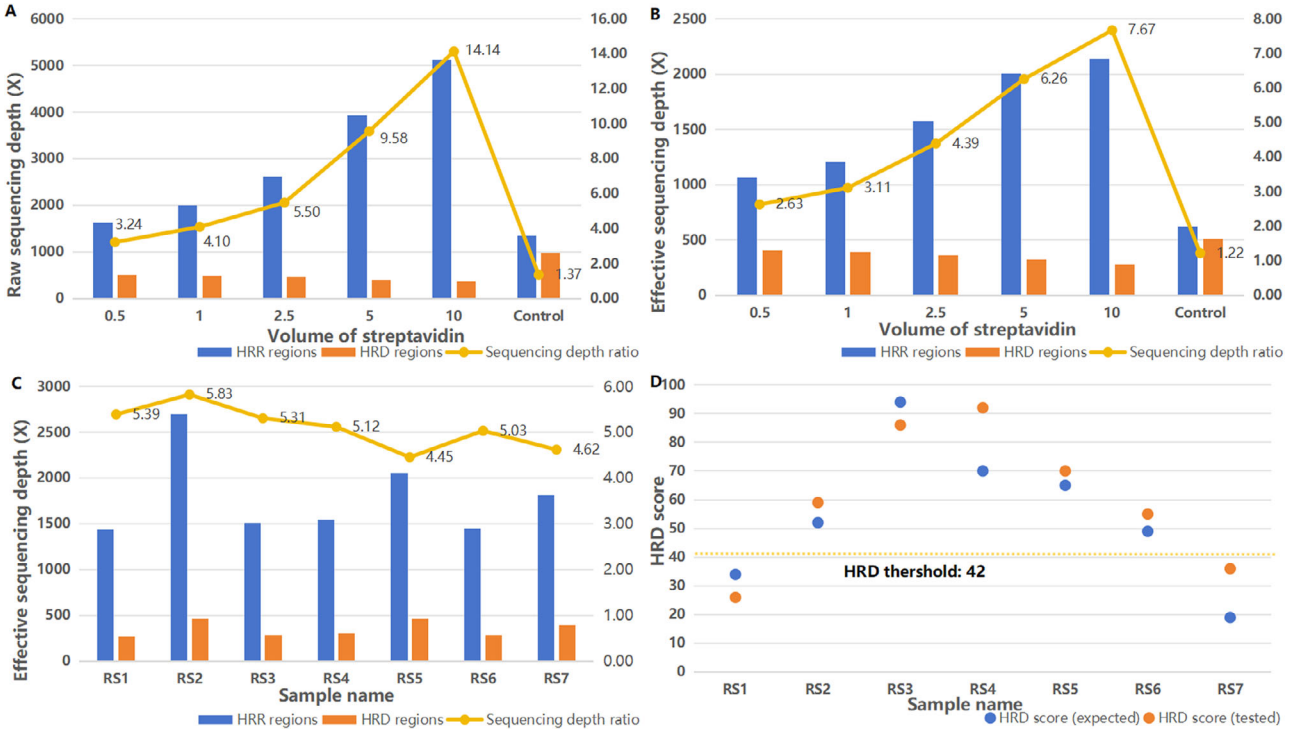

FIGURE 2 | Impact of streptavidin pre-blocking on sequencing depth. At a fixed sequencing data volume (12 GB), both the raw sequencing depth (A) and effective sequencing depth (B) of HRR regions increased progressively with higher streptavidin volumes added to the HRD probes. In contrast, the raw and effective sequencing depths of HRD regions remained unaffected. Using the optimized HRD assay probes (HRD probes pre-treated with 2.5 μL streptavidin and combined with HRR panel probes), the effective sequencing depths of HRR and HRD regions for all seven HRD reference standards met the required thresholds at half the original sequencing data volume (reduced from 12 GB to 6 GB) (C). Furthermore, the HRD status calls for all seven reference standards demonstrated 100% concordance with expected results at the designated HRD cut-off value of 42 (D). Raw sequencing depth: estimated coverage before filtering; Effective sequencing depth: practical coverage after filtering; Sequencing depth ratio: Average depth of HRR region/Average depth of HRD region.

TABLE 1 | HRR gene status of the reference standards.

Sample name	Expected results	Test results	Correspondence
RS1	/	/	100%
RS2	BRCA1: c.5425_5426del:p.V1809fs	BRCA1: c.5425_5426del:p.V1809fs	100%
RS3	BRCA1: c.5266dup:p.Q1756fs	BRCA1: c.5266dup:p.Q1756fs	100%
RS4	/	/	100%
RS5	/	/	100%
RS6	/	/	100%
RS7	/	/	100%

optimization and real-world implementation. The concentrations of the designed biotinylated probes and the streptavidin solution, along with the binding capacity of streptavidin (each streptavidin molecule can bind up to four biotin molecules), were the critical parameters required to calculate the theoretically optimal streptavidin volume. We recommend preparing a series of streptavidin volumes based on the calculated optimal streptavidin volume to ensure precise optimization. The optimal streptavidin volume should be determined by users based on the sequencing depth ratio between the high and low minimum desired sequencing depth regions, tailored to their specific assay requirements.

As a key molecular characteristic of probes, GC content substantially impacts sequencing depth [23, 24]. High GC content frequently corresponds to regions with low sequencing coverage [25]. Theoretically, GC content within the 40%–60% range (typically considered GC-neutral) has a negligible impact on determining optimal streptavidin volume. Even with thorough GC-content optimization during probe selection and assay design, biologically essential high-GC regions may occasionally be unavoidable in the panel. In such cases, we do not recommend employing this method. In this study, we tested a series of streptavidin volumes from 0.5 to 10 μL (0.1 mg/mL) based on our pre-blocked

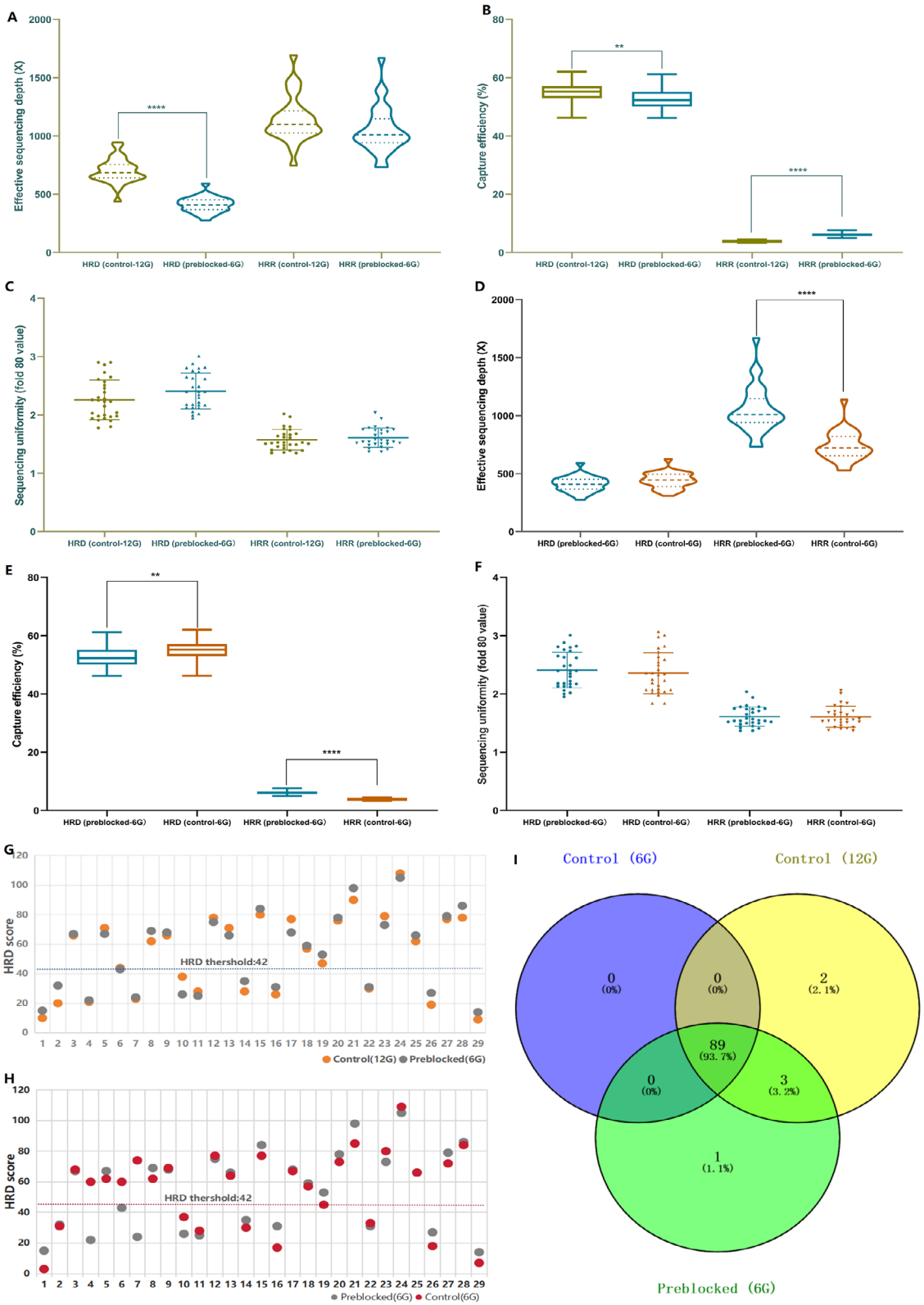

FIGURE 3 | Performance comparison between pre-blocked probes and control probes. C: Comparison between control-12G group and pre-blocked-6G group for: (A) Effective sequencing depth, (B) Capture efficiency, and (C) sequencing uniformity across HRD and HRR regions. HRR regions showed comparable effective depth (A) and uniformity (C) ($p > 0.05$), significantly improved capture efficiency for HRR regions with pre-blocked-6G (B, $p < 0.05$); D–F: Comparison between pre-blocked-6G group and control-6G group. Pre-blocked-6G demonstrated superior effective depth (D) and HRR capture efficiency (E) ($p < 0.05$); No significant difference in sequencing uniformity (F, $p > 0.05$). G–H: HRD score concordance: 100% agreement between control-12G and pre-blocked-6G (G); 93.13% (27/29) concordance between pre-blocked-6G and control-6G (H). I: Somatic variant detection overlap among groups: 96.84% (92/95) shared variants: control-12G group versus pre-blocked-6G group; 95.69% (89/93) shared variants: pre-blocked-6G group versus control-6G group. (Venn diagram: control-12G group = yellow; control-6G group = blue; pre-blocked-6G group = green).

HRD panel probes 4.44 Mbp in size, with an average 42% GC content (no high-GC regions). For our HRD assay, the optimal streptavidin volume was 2.5 μL, according to the sequencing depth ratio of the HRR and HRD regions.

Sample variability is another critical factor influencing the successful real-world application of the SPRE-Seq method, particularly due to variations in nucleic acid quality, as commonly observed in FFPE samples [26, 27]. Our validation studies using seven reference standards demonstrated perfect concordance (100%) between SPRE-Seq and expected results for both HRD status and HRR gene alterations, while achieving these results with only 50% of the standard sequencing data requirement. In clinical evaluation across 29 samples, SPRE-Seq (6 GB data volume) showed complete concordance with the control-12G group for HRD classification and the majority of HRR alterations. However, we observed three discordant HRR gene alterations: two were exclusively identified in the control-12G group, while one additional variant was uniquely detected in the SPRE-Seq pre-blocked-6G group. These minor discordances are consistent with expected stochastic variation in low-VAF variant detection, wherein all three discrepant variants approached the assay's established sensitivity threshold. Notably, compared with the standard method, the SPRE-Seq method demonstrated significantly higher capture efficiencies for HRR regions and significantly lower capture efficiencies for HRD regions. This was attributed to the partial blocking of HRD probes, which was consistent with the theoretical prediction. Importantly, sequencing uniformity was not affected.

This study has several limitations. First, although we validated the feasibility of SPRE-Seq using our self-designed HRD assay, the sample size was limited. Further validation with a larger number of samples is required to confirm the applicability of this method in real-world clinical testing. Second, due to the dynamic binding between streptavidin and biotin, it was challenging to theoretically determine the optimal streptavidin volume. Researchers must empirically establish this through extensive experimentation tailored to their specific panel. Further studies are needed to refine the process and reduce the experimental workload to achieve a more precise volume of streptavidin for probe blocking.

In conclusion, our study presented a reliable, feasible, and cost-effective method, SPRE-Seq, that acquired different sequencing depths for different targeted regions in an NGS panel. This method holds further clinical and investigational potential and can be used in the majority of hybridization capture-based targeted sequencing panels.

Author Contributions

Hui-Juan Chen designed the study, prepared the figures, wrote the original, and revised the manuscript. Bing Wang, Yi-Ran Zhang, Xue-NaYao, Chun-Yan Yang, and He-Nan Dong acquired resources, collected the samples, and conducted the analysis. Li-Li Cai conducted the bioinformatics analysis. Dong-Jie Fan and Qi-Ming Zhou supervised the whole study, acquired the funding, supervised the study, reviewed, and revised the manuscript. All authors reviewed and approved the manuscript.

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The authors have nothing to report.

Ethics Statement

This study was approved by the Ethics Committee of Xuanwu Hospital (No. [2019]081-R1). Written informed consent was obtained from each participant, and the methods were carried out in accordance with approved guidelines.

Conflicts of Interest

The authors declare no conflicts of interest

Data Availability Statement

Sequence data that support the findings of this study have been deposited in the Genome Sequence Archive (GSA) repository with the primary accession code PRJCA036580 (https://ngdc.cncb.ac.cn/gsa-human/browse/HRA012077).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: elsc70047-sup-0001 figureS1.tif