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A rapid and efficient zirconia bead-mediated ultrasonic strategy for DNA fragmentation up to 10 kbp†

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Single-molecule sequencing (SMS), a long-read DNA sequencing technology, plays a crucial role in genomics research. However, traditional ultrasonic shearing techniques struggle to efficiently produce DNA fragments ≥10 kbp, limiting the efficiency of SMS library preparation. Here, we developed a zirconia bead-mediated ultrasonic shearing method that enables precise DNA fragmentation through zirconia bead mechanical agitation induced by sonication cavitation. By optimizing parameters such as zirconia bead size, quantity, ultrasonic probe distance, ultrasonic time, water bath temperature, DNA sample volume, and DNA concentration, we obtained target fragments in the 10–20 kbp range. The results demonstrated that this method sheared purified λDNA (48.5 kbp) into fragments averaging 15 kbp within 20 seconds, achieving performance comparable to commercial g-TUBE methods. The method was also successfully applied to human genomic DNA. This simple, rapid and reliable DNA fragmentation method provides an effective solution for SMS library preparation with great potential for molecular detection and diagnostic applications.

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1. Introduction

Recent advancements in DNA sequencing technologies have revolutionized our ability to decode complex genomes.[1–4] Among these, single-molecule sequencing (SMS) represents a transformative leap as the third generation of sequencing technologies. Unlike traditional short-read sequencing methods, SMS enables direct sequencing of individual DNA molecules up to 10 kbp or longer without PCR amplification, offering unprecedented advantages in genomics research. This technology provides long read lengths, high accuracy, and uniform genome coverage, making it widely applicable in the detection of genomic structural variants, highly repetitive regions, and clinical diagnostics.[5–7] Platforms such as the Single Molecule Real-Time (SMRT) Sequencing from Pacific Biosciences (PacBio) and the Nanopore Sequencing from Oxford Nanopore Technologies (ONT) have demonstrated the potential of SMS in diverse applications, from genome assembly to clinical diagnostics and personalized medicine.[8,9] To take full advantage of the advancements in SMS long-read DNA sequencing technology, researchers require technologies that can efficiently generate extensive libraries of DNA fragments. The construction of long-read sequencing libraries depends on several critical parameters, including the control of DNA shearing and fragment size distribution.[10,11] The fragment size of long fragment SMS libraries is set to ≥10 kbp compared to next generation sequencing (NGS) libraries (300–500 bp).[12] However, achieving a concentrated distribution of long DNA fragments (≥10 kbp) remains a major challenge for SMS.[13]

DNA fragmentation methods mainly include enzymatic digestion and various physical shearing methods. Enzymatic digestion involves transposases and endonuclease, which effectively reduces the time for NGS library construction.[14,15] However, these enzymes exhibit site preferences, leading to incomplete fragmentation of certain DNA types, such as those with high G–C content or tightly packed structures.[16–18] The physical shearing methods include nebulization, hydrodynamic shearing, and sonication.[19,20] Nebulization generates random DNA fragments by forcing DNA solutions through a narrow aperture under high pressure,[21] but it is prone to cross-contamination and operational instability.[15] Hydrodynamic shearing, including methods like the Diagenode Megaruptor and Covaris g-TUBE,[22] produces small, uniform fragments but is limited by high costs, complexity, and poor reproducibility, particularly for generating long fragments suitable for SMS.[23,24] Sonication is a low-cost, user-friendly method for DNA fragmentation that effectively avoids cross-contamination.[25–27]

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Conventional sonication is suitable for generating short fragments (300–500 bp) to meet NGS requirements,[20] but its inhomogeneous fragment distribution, excessive shearing, and thermal damage severely limit its application in SMS. Therefore, the development of a new method that can rapidly, efficiently and reliably generate DNA fragments larger than 10 kbp in length is essential for the construction of high-quality SMS libraries.

Here, we introduce a novel zirconia bead-mediated ultrasonic fragmentation method that addresses these challenges. Zirconia beads,[28,29] known for their high hardness, chemical inertness, and efficiency,[30,31] are combined with ultrasonic cavitation to achieve precise and controlled DNA shearing. As illustrated in Scheme 1, ultrasonic cavitation induces high-frequency mechanical collisions of zirconia beads to act directly on DNA molecules to improve the shearing efficiency, and the acoustic flow generated by ultrasonic vibration produces a localised high shear force field to further promote DNA fracture. The synergistic effect of ultrasonic cavitation and zirconia bead motion enables precise shearing and avoids over-shearing or uneven fragment distribution. A video illustrating the fragmentation process is provided in the ESI materials (ESI, Video S1†). By optimizing parameters such as zirconia bead size, quantity, ultrasonic probe distance, ultrasonic time, water bath temperature, DNA sample volume, and DNA concentration, we obtained target fragments in the 10–20 kbp range. The zirconia bead-mediated ultrasonic method has been successfully applied to shear both purified λDNA (48.5 kbp) and human genomic DNA to produce fragments with a concentrated size distribution, well suited for SMS library construction. This method offers the advantages of rapid fragmentation (20 seconds), high reproducibility and minimal DNA damage. By accurately generating long DNA segments, our method improves the quality of SMS libraries, thereby increasing the accuracy and reliability of genomic analyses in clinical studies.

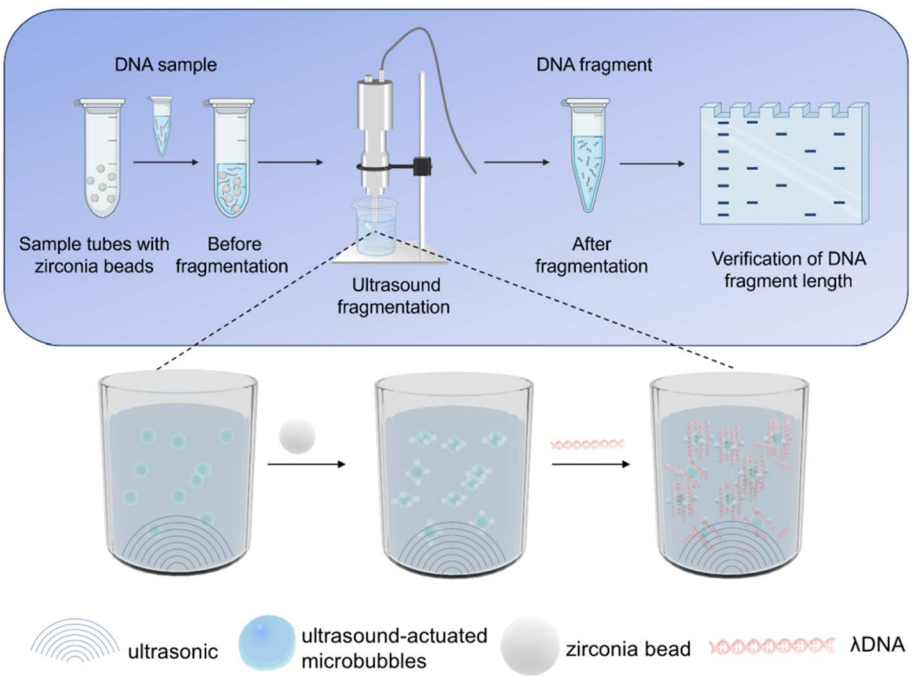
2. Experimental section

2.1 Materials

The λDNA (Thermo Fisher Scientific, US) samples were used in the optimization of fragmentation experiment parameters. The λDNA samples (48 502 bp, double-stranded linear state) were diluted with 0.1× TE Buffer (1 mM Tris–HCl, pH 8.0; 0.1 mM EDTA) to a final concentration of 2 ng μl^{-1}. Human gDNA was extracted from MCF-7 cells by Nanobind CBB kit (Pacific Biosciences, US), diluted with 0.1× TE Buffer to a final concentration of 2 ng μl^{-1} and was used only for fragmentation. Genomic DNA Screen Tape and genomic DNA reagents (Agilent, US) were used in automated electrophoresis analysis. Agarose, DNA Marker, 6× DNA Loading Dye, TRIZOL reagents and 10 000× GelRed were obtained from Sangon Biotech (Shanghai, China). 1.5 mL and 2 mL centrifuge tubes, 1.5 mL grinding tubes are conventional consumables.

2.2 Zirconia beads and ultrasonic equipment

Zirconia beads (Shandong Hanhe, China) were sequentially cleaned with 50% ethanol and deionized water using ultrasonic cleaning, followed by high-temperature sterilization and drying for subsequent use. The standing wave suspension high energy ultrasonic probe equipment used was from the high-energy ultrasonic technology team (Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences).



Scheme 1 The flowchart of zirconia bead-mediated ultrasonic fragmentation of long-stranded DNA.

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2.3 DNA fragmentation with zirconia bead-mediated ultrasonic method

First, a quantity of 20 zirconia beads of 1 mm were added to the sample tube, and then 100 μL of 2 ng μl^{-1} λDNA was added and mixed well. The sample tubes were loaded into a sample tube rack, which was placed in a beaker equipped with an ultrasonic water bath, and the ultrasonic time was set in the range of 20–180 s. After ultrasonication, the sample tubes were removed and the DNA samples were transferred to new centrifuge tubes and stored at 4 °C for subsequent analysis. To avoid cross-contamination, each DNA sample was sheared in a separate sample tube. The main parameters that can be adjusted during DNA fragmentation were as follows: ultrasonic power, ultrasonic time, distance between ultrasonic probe and sample, type of sample tube, size of zirconia beads, number of zirconia beads, DNA sample concentration, DNA sample volume and temperature of ultrasonic water bath. In this study, we explored the effect of these parameters on the length distribution of DNA fragments and applied the method to the fragmentation of human gDNA samples.

2.4 Analysis of DNA fragmentation results

All DNA fragment length distributions were analysed by 1% agarose gel electrophoresis. Each DNA fragment sample (10 μL) was combined with loading dye (2 μL) and characterized using 1% (w/w) agarose gel electrophoresis (AGE) at 4 °C (110 V) for about 70 min in 1× TAE buffer (Tris, HCl, EDTA) buffer. The bands were stained with GelRed and photographed by chem-iDoc MP (Bio-Rad, US). In addition, we used the automated electrophoresis 4150 TapeStation System (Agilent, US) to further analyse the range of length distributions of the DNA fragments.

2.5 λDNA samples fragmentation with other methods

In this experiment, five control groups were set up: (1) under the protocol of the methods, no zirconia beads were added to the sample tube, and other conditions were consistent for fragmentation; (2) glass beads combined with ultrasound. (3) Zirconia beads combined with Vortexer. (4) Fragmentation by automatic sample grinding instrument (Shanghai Jingxin, China); (5) using the commercial g-TUBE shearing tube (Covaris, US) to achieve fragmentation. According to the Covaris manufacturer's instructions, 150 μL of λDNA sample was centrifuged at 8000 rpm for 60 s for both cycles in a bench-top centrifuge (Thermo Fisher Scientific, US).

3. Results and discussion

3.1 Characterisation of zirconia bead-mediated ultrasonic DNA fragmentation method

To evaluate the potential of zirconia bead-mediated ultrasonication for long DNA fragmentation, we used λDNA (48 502 bp), a linear double-stranded DNA extracted from a mild *E. coli* phage, as a standard sample. Zirconia beads were introduced into λDNA samples and ultrasonic treatment was performed for 20 s. 1% agarose electrophoresis showed that a low-mobility band with a distribution of 10–20 kbp was clearly visible in comparison to the control λDNA bands, indicating that the combination of the DNA zirconia beads and ultrasonication resulted in the λDNA being efficiently sheared (Fig. 1). In contrast, ultrasonication alone, even when extended to 120 s, failed to produce significant DNA fragmentation, highlighting the critical role of zirconia beads in enhancing shearing efficiency A). To optimize the method for generating DNA fragments within the desired 10–20 kbp range, we evaluated ultrasonication power and centrifuge tube conditions. We found that 100 W of ultrasonication power and a 1.5 mL tip-bottomed centrifuge tube were the optimal parameters for obtaining λDNA fragments that met expectations (Fig. S1 and S2†). Under these optimized conditions, the zirconia bead-

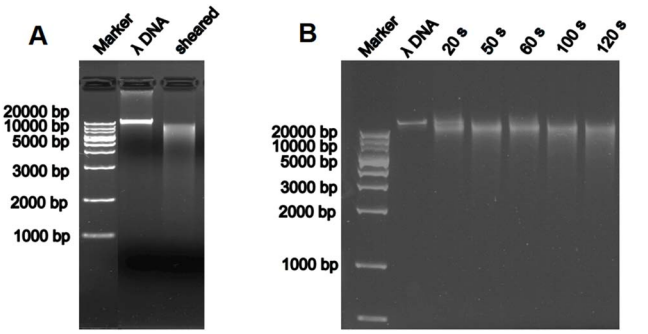

Fig. 1 1% agarose gel electrophoresis images of DNA fragments by (A) zirconia bead-mediated ultrasonic fragmentation strategy; (B) ultrasonic fragmentation only.

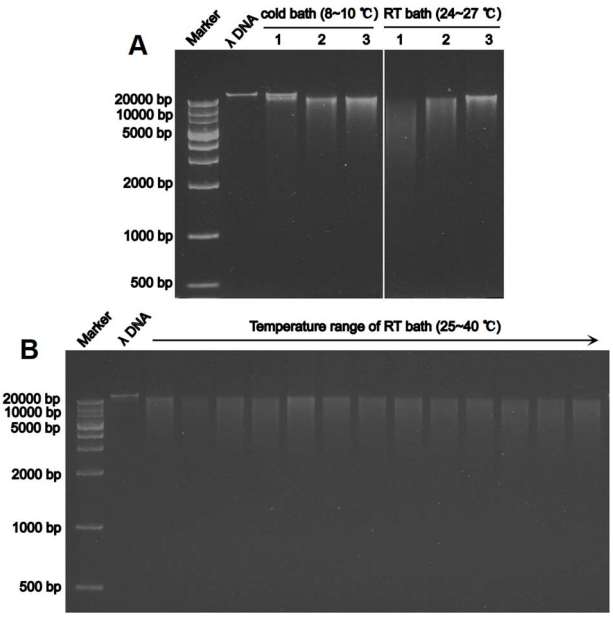

Fig. 2 1% agarose gel electrophoresis images of DNA fragments after fragmentation with various parameters. (A) The influence of the temperature of the ultrasonic bath water and the distance between the ultrasonic probe and the sample. The temperature range of the cold bath measured by the thermometer was 8–10 °C, and the room temperature bath (RT bath) was 24–27 °C. (1) The distance was 0.5 cm, (2) the distance was 1 cm and (3) the distance was 2 cm. (B) The influence of RT bath temperature increasing on fragments.

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mediated ultrasonic method produced λDNA fragments of the desired size. Thus, the zirconia bead-mediated ultrasonic method can efficiently shear long fragments of DNA.

3.2 Influence of the ultrasonic water bath temperature and the distance between ultrasonic probe and sample

To optimize the zirconia bead-mediated ultrasonic fragmentation method, we investigated the effects of two critical parameters: the distance between the ultrasonic probe and the sample, and the water bath temperature. The distance between the probe and the sample significantly influenced the trajectory of the zirconia beads, which directly affects DNA fragmentation efficiency. We evaluated three distances: far (2 cm), medium (1 cm), and near (0.5 cm), with a fixed ultrasonication duration of 20 s. At a far distance (2 cm), λDNA remained largely unfragmented, while a closer distance (0.5 cm) resulted in shorter DNA fragments concentrated around 5000 bp (Fig. 2A). The medium distance (1 cm) provided a balance, enabling efficient fragmentation without over-shearing, making it the optimal choice for generating longer DNA fragments suitable for SMS library preparation.

We also examined the impact of water bath temperature on DNA fragmentation by comparing cold (8–10 °C) and room temperature (24–27 °C) conditions. Agarose gel analysis revealed that cold water bath conditions yielded fragment distributions similar to untreated λDNA, indicating minimal fragmentation, whereas room temperature conditions significantly enhanced fragmentation efficiency (Fig. 2A). Furthermore, we assessed the effect of a slight temperature increase (25–40 °C) during ultrasonication and found no significant impact on DNA fragment size distribution (Fig. 2B). This suggests that the method is robust and stable under moderate temperature variations.

The reproducibility of the method was confirmed by iterative experiments under room temperature and medium distance (1

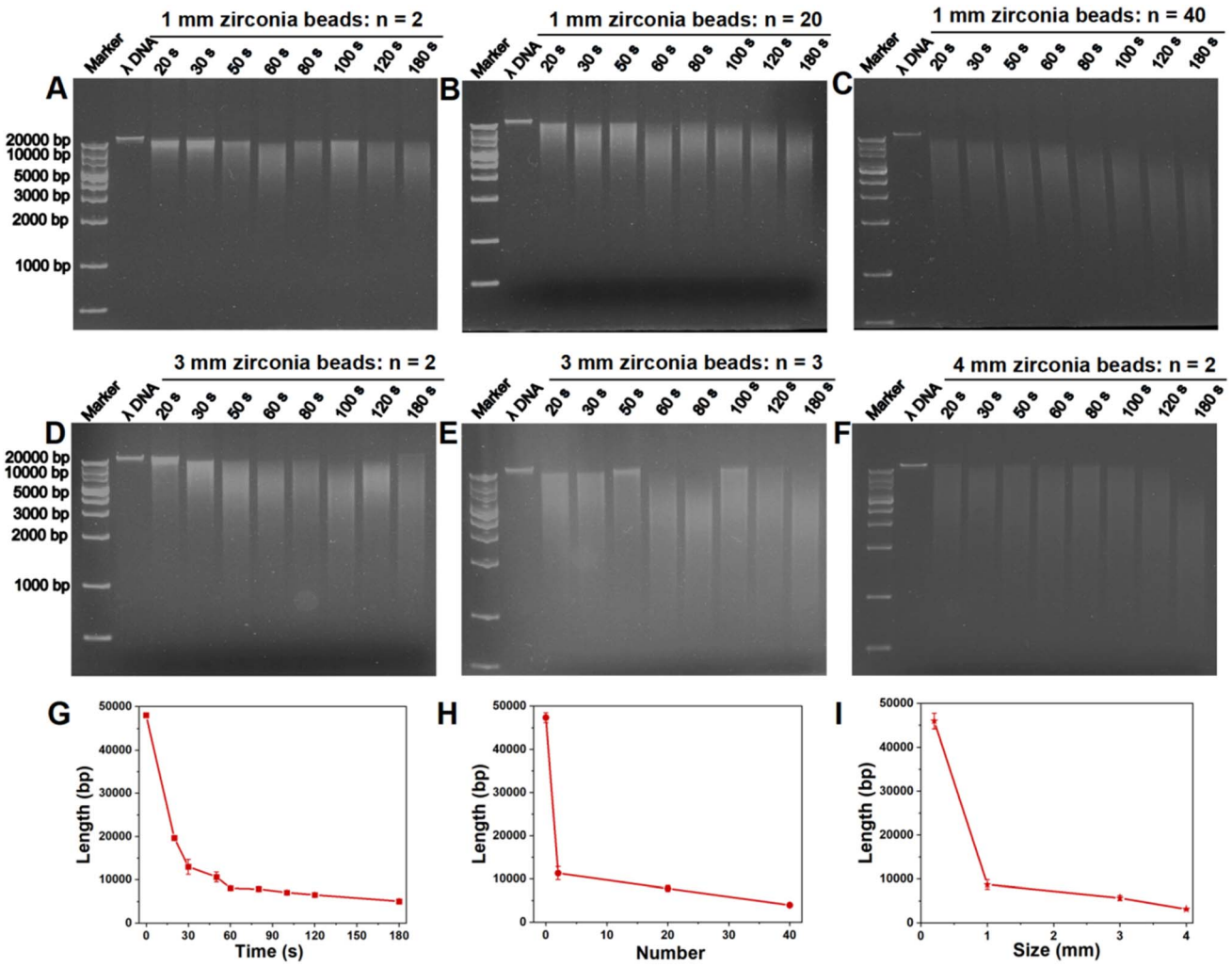

Fig. 3 The influence of zirconia beads and ultrasonic time on λDNA fragmentation. The length distribution of λDNA fragments based on different numbers of 1 mm zirconia beads: (A) $n = 2$, (B) $n = 20$ and (C) $n = 40$, respectively. The length distribution of λDNA fragments based on different numbers of 3 mm zirconia beads: (D) $n = 2$ and (E) $n = 3$, respectively. (F) The length distribution of λDNA fragments based on 2 numbers of 4 mm zirconia beads. The relationship between the average fragment length of λDNA and (G) the ultrasonic time, (H) the number of 1 mm zirconia beads, (I) the sizes of zirconia beads.

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cm) conditions, which consistently produced reliable and reproducible results (Fig. S3†). These findings demonstrate that room temperature water bath and a medium probe-sample distance (1 cm) are optimal for inducing effective zirconia bead movement and achieving controlled DNA fragmentation.

3.3 Influence of zirconia beads (size and number) and ultrasonic time

To systematically investigate the relationship between zirconia bead size, quantity, and ultrasonication time on DNA fragmentation, we evaluated a range of parameters. Zirconia bead sizes of 0.2 mm, 1 mm, 3 mm, 4 mm, and 6 mm were tested, along with quantities ranging from 1 to 40 beads per tube.[32] Our results revealed that excessively small beads (0.2 mm) failed to fragment λDNA even after prolonged ultrasonication (180 s), while excessively large beads (≥6 mm) could not be loaded into the sample tube (Fig. S4†). Therefore, we focused on 1 mm, 3 mm, and 4 mm beads for further analysis.

Using 1 mm zirconia beads, we observed that DNA fragmentation efficiency was highly dependent on bead quantity and ultrasonication time. With 2 beads, DNA fragments longer than 5000 bp were obtained only after 50 s of ultrasonication, while shorter durations left most λDNA unfragmented (Fig. 3A). Increasing the bead quantity to 20 significantly improved fragmentation efficiency, with optimal results achieved at 20 s, yielding fragments predominantly concentrated around 15 kbp, ideal for SMS library preparation (Fig. 3B). However, further increasing the bead quantity to 40 led to over-fragmentation, producing shorter fragments (<5000 bp) unsuitable for long-read sequencing (Fig. 3C). In contrast, larger beads (3 mm and 4 mm) resulted in a wider distribution of fragment lengths and a higher proportion of short fragments, failing to meet the requirements for long-read sequencing platforms (Fig. 3D–F).

Taken together, the increase of ultrasonic time resulted in more effective DNA fragmentation, with a decreasing trend in the length of DNA fragments with increasing ultrasonic time, which was consistent with previously reported results[20] (Fig. 3G). Fig. 3H showed the length of DNA fragments became shorter with increasing the number of 1 mm zirconia beads. Fig. 3I demonstrates that the length of DNA fragments decreased with increasing size of zirconia beads at the same ultrasonic time. Under optimized conditions in Table 1 (room temperature, 1 cm probe-sample distance, consistent sample volume and concentration), ultrasonication with 20 beads of 1 mm diameter for 20 s yielded DNA fragments predominantly within the 10–20 kbp range, making it the preferred method for SMS library preparation.

3.4 Influence of sample volume and DNA concentration

Under conditions of equal sonication time and amplitude, the density of sonication (*i.e.*, processing efficiency) is expected to decrease as the sample volume increases. To investigate the effect of sample volume on the zirconia bead-mediated sonication fragmentation method, we increased the volume of treated samples from 50 μL to 200 μL for the study while maintaining consistent sonication parameters and DNA concentration. As shown in Fig. 4A, smaller sample volumes (50 μL and 100 μL) yielded longer λDNA fragments, with the 100 μL volume producing the most concentrated and reproducible fragment distribution. Therefore, a sample volume of 100 μL was chosen as optimal for further experiments.

Next, we explored the impact of DNA concentration on fragmentation efficiency, testing concentrations ranging from 2 ng μl^{-1} to 20 ng μl^{-1}. Consistent with previous studies on hydrodynamic shearing-based methods,[33] we found that DNA concentration had no significant effect on fragment length distribution (Fig. 4B). This indicates that the zirconia bead-mediated ultrasonic method is robust and not limited by DNA concentration, making it suitable for a wide range of sample inputs.

3.5 Comparison with other fragmentation methods and practical sample application

The zirconia bead-mediated ultrasonic fragmentation method was systematically compared with several alternative approaches,

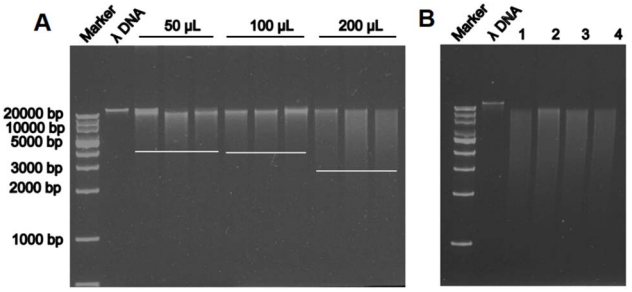

Fig. 4 1% agarose gel electrophoretic images characterize (A) the effect of sample volume on DNA fragmentation. From left to right, there are three parallel experiments at 50 μL, 100 μL and 200 μL; (B) the effect of λDNA concentration on DNA fragmentation. Lane 1 : 2 ng μl^{-1}, 2 : 5 ng μl^{-1}, 3 : 10 ng μl^{-1}, 4 : 20 ng μl^{-1}.

Table 1 Results of λDNA fragmentation of 1 mm zirconia beads at different sonication times and different number of zirconia beads

Devices	Zirconia beads		Time (s)	Distance (cm)	RT bath (°C)	Volume (μL)	[λDNA] (ng μl^{-1})	Peak (bp)	Homogeneity (bp)	Figure
	Size (mm)	Number								
—	—	—	—	—	—	—	2	48 000	48 000	—
Ultrasound	—	—	60	1	28	100		40 000	20 000–48 000	Fig. 1B
	1	2	30					20 000	20 000–23 000	Fig. 3A
	1	20	20					15 000	10 000–20 000	Fig. 3B
	1	20	50					10 000	5000–20 000	Fig. 3B

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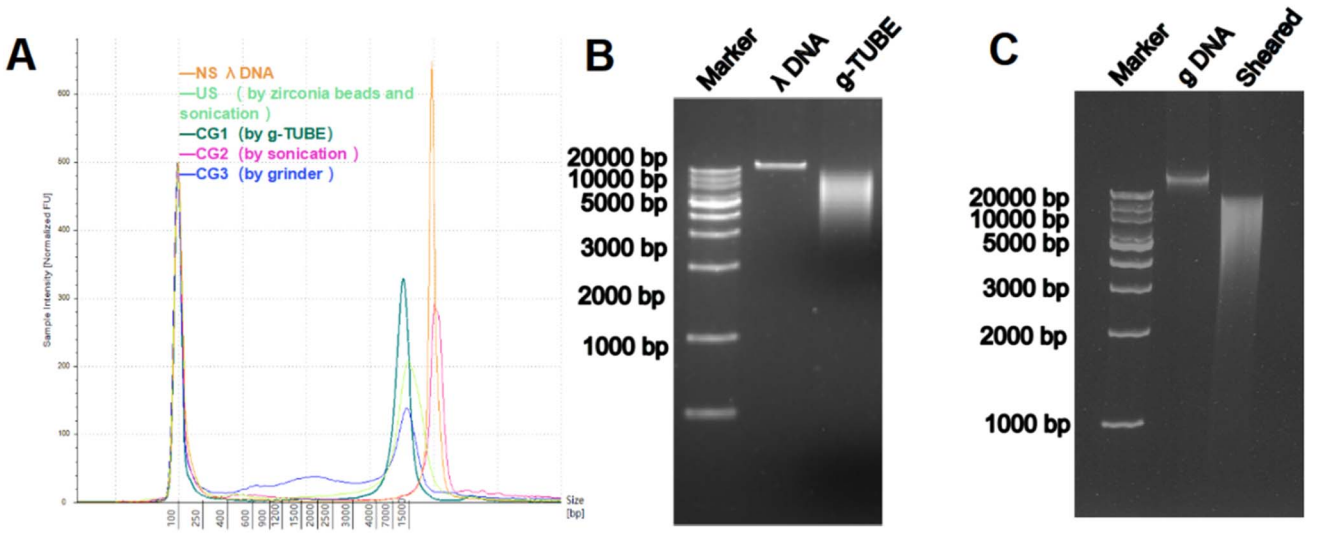

Fig. 5 (A) Bioanalyzer images of λDNA fragment length distribution under different fragmentation methods. (B) Agarose gel (1%) electrophoresis image demonstrating the performance of the commercial g-TUBE fragmentation method for λDNA shearing. (C) Agarose gel (1%) electrophoresis image illustrating the application of the zirconia bead-mediated ultrasonic fragmentation method to human genomic DNA (gDNA) shearing.

Table 2 Comparison with other recently developed DNA fragmentation methods

Methods	Sample type	Sample volume (μL)	DNA concentration (ng μl^{-1})	Shearing time (s)	Fragmentation range (bp)	Reference
Ultrasonic microfluidic chip	λDNA	30	1–10	30	2000	27
Cyclical hydrodynamic shearing	Salmon DNA	100	40	60	6800–8700	23
Ultrasonic-based strategy	Genomic DNA	100	100	120	100–1200	20
Sonication with glass beads	Genomic DNA	100	10–20	50	250	32
Zirconia bead mediated ultrasonic strategy	λDNA	100	2–20	20	10 000–20 000	This study

including simple ultrasonication, the combination of ultrasound and glass beads, the combination of zirconia beads and vortexer, direct grinding of zirconia beads, and the commercial g-TUBE method. The results demonstrated that simple ultrasonication without zirconia beads was significantly less effective in fragmenting λDNA (Fig. 5A and S5A†). Similarly, the combination of ultrasonication with glass beads or zirconia beads with vortexing showed low genome fragmentation efficiency (Fig. S5B and S5C†). While direct grinding of zirconia beads produced fragments comparable to our method, it required higher frequencies (50 Hz), leading to an increased proportion of short fragments and non-uniform distribution, whereas low frequencies (30 Hz) failed to break λDNA effectively (Fig. S6†). Notably, the zirconia bead-mediated ultrasonic method achieved a shearing efficiency comparable to the commercial g-TUBE method (Fig. 5B), while offering additional advantages such as rapid processing (20 s), cost-effectiveness, and ease of operation.

The proposed zirconia bead-mediated ultrasonic fragmentation method demonstrates significant advantages over other recently developed DNA fragmentation techniques, as summarized in Table 2. Notably, our method achieves shorter shearing times (20 s) while producing longer DNA fragments (10–20 kbp), making it highly suitable for SMS applications. Unlike other methods that rely on expensive instrumentation or complex chip designs, our approach utilizes cost-effective and readily available zirconia beads, significantly reducing operational costs. Overall, the method provides an easy-to-operate, fast, efficient, and inexpensive method for DNA fragmentation, which is expected to be widely used in three-generation gene sequencing.

To assess the practical applicability of the zirconia bead-mediated ultrasonic fragmentation method, we applied it to the shearing of human genomic DNA (gDNA). Fig. 5C showed that the shearing of gDNA was successfully achieved, and the resulting gene fragments were concentrated in the range of 10–20 kbp. This result underscored the suitability of the zirconia bead-mediated ultrasonic fragmentation method for the fragmentation of third-generation SMS libraries, which have specific fragment length requirements. The successful application to human gDNA further validates the method's efficiency, reliability, and potential for use in complex genomic studies and clinical diagnostics.

4. Conclusion

In conclusion, we have developed a zirconia bead-mediated ultrasonic fragmentation method for preparing SMS libraries, leveraging the synergistic effects of mechanical collisions from zirconia beads and ultrasonic shearing to efficiently fragment

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high molecular weight DNA. Through systematic optimization, we identified key experimental parameters, including 100 W ultrasonic power, a 100 μL sample volume, and a 1 cm distance between the ultrasonic probe and the sample, as optimal conditions for achieving consistent and reproducible results. Notably, the method demonstrated robustness to small variations in ultrasonic bath temperature (25–40 °C) and DNA concentration (2–20 ng μl^{-1}), with minimal impact on fragmentation efficiency. We observed that fragment length decreased with increasing ultrasonication time, zirconia bead quantity, and bead size, and under optimized conditions (20 zirconia beads of 1 mm diameter, 20 s ultrasonication), λDNA was efficiently fragmented into 10–20 kbp fragments, meeting the requirements for SMS library preparation. The performance of this method was comparable to the commercial Covaris g-TUBE system, while offering additional advantages such as rapid processing, cost-effectiveness, and ease of operation. The zirconia bead-mediated ultrasonic fragmentation method provides a rapid, reliable and inexpensive solution for generating DNA fragments suitable for SMS libraries, enabling downstream biomolecular analyses and diagnostic applications without the need for further purification.

Data availability

The data for this article, including all figures and tables in both the manuscript and ESI,† are available upon request from the authors.

Author contributions

Z. J. conceived the idea. Z. J., Y. L. and W. Y. supervised the project. F. P., Z. T. and W. P. performed experiments and corresponding data processing. K. T. and Q. S. conducted the schematic drawing and editing. F. P. and Z. T. wrote the paper. All authors discussed and contributed to the work.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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