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Development of a fast and efficient ultrasonic-based strategy for DNA fragmentation

Miguel Larguinho [a], Hugo M. Santos [b], Gonçalo Doria [a,b], H. Scholz [b], Pedro V. Baptista [a], José L. Capelo [b,c,*]

[a] CIGMH/DCV, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, 2829-516 Caparica, Portugal
[b] REQUIMTE/DQ, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, 2829-516 Caparica, Portugal
[c] BioScope Group, Nutrition and Bromatology Area, Faculty of Science, University of Vigo at Ourense Campus, 32004 Ourense, Spain

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ABSTRACT

Several ultrasound-based platforms for DNA sample preparation were evaluated in terms of effective fragmentation of DNA (plasmid and genomic DNA)—ultrasonic probe, sonoreactor, ultrasonic bath and the newest Vialtweeter device. The sonoreactor showed the best efficiency of DNA fragmentation while simultaneously assuring no cross-contamination of samples, and was considered the best ultrasonic tool to achieve effective fragmentation of DNA at high-throughput and avoid sample overheating. Several operation variables were studied—ultrasonication time and amplitude, DNA concentration, sample volume and sample pre-treatment—that allowed optimisation of a sonoreactor-based strategy for effective DNA fragmentation. Optimal operating conditions to achieve DNA fragmentation were set to 100% ultrasonic amplitude, 100 μL sample volume, 8 min ultrasonic treatment (2 min/sample) for a DNA concentration of 100 μg mL^{-1}. The proposed ultrasonication strategy can be easily implemented in any laboratory setup, providing fast, simple and reliable means for effective DNA sample preparation when fragmentation is critical for downstream molecular detection and diagnostics protocols.

1. Introduction

The development of biomolecular assays and diagnostics based on nucleic acid recognition requires suitable procedures for optimal sample preparation. This is particularly relevant when the recognition step of the assay depends on the specific hybridisation of oligonucleotide probes [1–4], where small DNA fragments with known molecular weight and concentration are required. Among the most commonly used methods, PCR and restriction enzyme digestion produce compatible DNA fragments but require addition of specific enzymes and optimisation of pH and ionic strength conditions. These reagents may sometimes hamper the detection step [5] and more often involve an additional purification step, with concomitant loss of valuable sample [6]. The use of ultrasounds as fragmentation procedure for DNA samples has been gradually gathering momentum as a robust approach to attain DNA fragments susceptible of use in downstream detection protocols without further purification.

Ultrasounds are described as high frequency acoustic waves (20 kHz or higher) requiring a medium in order to propagate [7,8]. When passing through an aqueous solution, acoustic waves cause the formation of microbubbles filled with gas (cavities), a process known as cavitation. Two types of cavitation have been described: stable cavitation (gas body activation) and inertial cavitation (transient or vaporous). Stable cavitation occurs during low intensities of ultrasound, but inertial cavitation requires high intensities of ultrasound. The latter is responsible for high energy events, considered to be extremely destructive to biological molecules [9]. During inertial sonication, the size of microbubbles varies: first, there is a rapid increase in size, followed by a decrease, until it implodes. The high temperatures and high pressure inside the bubble are sufficient to cause hydrolysis, sonoluminescence phenomena [10] and shearing of biological molecules [11]. Besides cavitation, two additional phenomena may lead to the damage of biological molecules: mechanical and thermal degradation; and reaction with radicals generated from water [12–16]. Also, the use of ultrasonic waves directly in cells was shown to induce DNA fragmentation and damage [11,17].

The study of the influence of acoustic shear waves in the behaviour of oligonucleotides [18] has prompted the development of methodologies for DNA sample treatment relying on the use of ultrasonic probes resulting in efficient *in vitro* fragmentation of purified DNA, which can then be used in a multitude of biosensing applications [19,20]. Several ultrasound platforms can be used towards DNA sample preparation, such as probes, sonoreactors, baths, but no data have yet been produced comparing these systems

* Corresponding author at: BioScope Group, Nutrition and Bromatology Area, Analytical & Food Chemistry Department, University of Vigo at Ourense Campus, 32004, Ourense, Spain.
E-mail address: jlcapelo@uvigo.es (J.L. Capelo).

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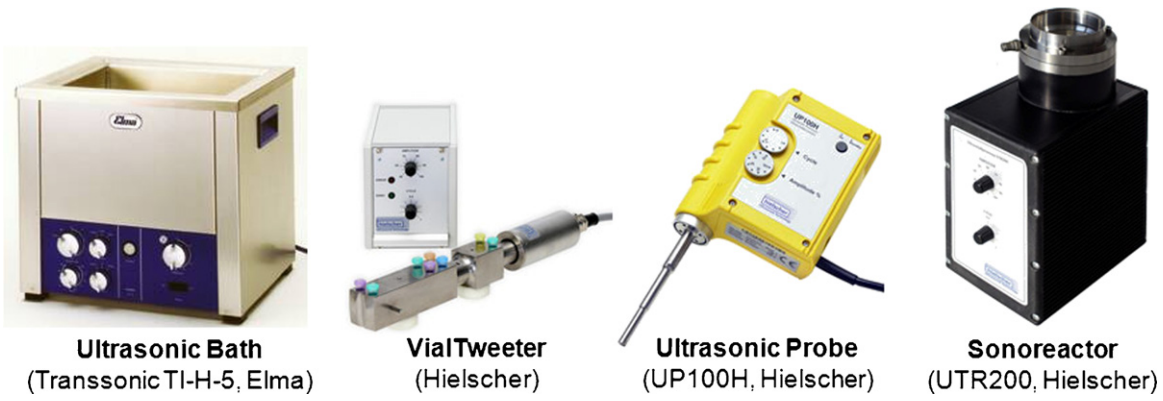

Fig. 1. Image of the four ultrasonic devices used in the present study.

in terms of optimisation for subsequent biomolecular recognition assays. Moreover, to the best of our knowledge only ultrasonic probes have been described as a practical tool for DNA fragmentation [20].

Here we present a comparative analysis of available ultrasonic platforms towards optimisation of DNA fragmentation. We also explore the advantages and disadvantages of ultrasound versus enzymatic digestion, as a means to generate DNA fragments.

2. Experimental

2.1. Materials

All thiol-modified oligonucleotides were purchased from STAB_Vida (Portugal). RNase A, restriction enzymes (Eco47I, MvaI) and digestion buffers were purchased from Fermentas (Canada). Trizma® base *BioChemika*, EDTA *BioUltra* and boric acid *BioUltra* were purchased from Fluka (USA). UltraPure™ Agarose was purchased from Invitrogen (Spain). Ethanol absolute was purchased from Panreac Quimica S.A.U. (Spain). Phenol (molecular biology grade) and chlorophorm (biotechnology grade), were purchased from Sigma–Aldrich (USA). DNA samples were prepared with ultrapure water from a Millipore Milli-Q system.

2.2. Apparatus

Several ultrasonic systems were assessed in this study: probe (UP100H: 30 KHz/100 W, Heilscher Ultrasound Tech, 1 mm tip diameter), sonoreactor (UTR200: 24 KHz/200 W, Heilscher Ultrasound Tech), VialTweeter: 24 KHz/250 W (Heilscher Ultrasound Tech) and ultrasonic bath (Transsonic TI-H-5: 130 KHz, Elma Ultrasonic Tech). The four devices studied are presented in Fig. 1.

2.3. DNA purification

Total genomic DNA was purified from peripheral blood. For plasmid DNA, a plasmid harbouring a fragment of the human β-globin gene was used. DNA was purified following modified alkaline lysis protocol [21]. Further purification included a RNase digestion step followed by a phenol:chlorophorm extraction and ethanol precipitation. The resulting purified DNA was resuspended in water and stored at 4 °C until further use.

2.4. Ultrasonic procedure and agarose gel electrophoresis

Standard ultrasonication was performed in 1.5 mL tubes with a total volume of 100 μL and final DNA concentration of 100 μg mL^{-1}. After sonication, all samples were stored at 4 °C and analysed by agarose gel electrophoresis (1% agarose; 1× TBE (Tris, boric acid, EDTA); 2 V/cm; 300 min; post-staining with EtBr 0.5 μg mL^{-1}) using a GeneRuler DNA Mix Ladder (Fermentas) 0.1 μg mL^{-1}.

An initial assay was performed to compare DNA fragmentation through several ultrasonic systems: probe, sonoreactor, VialTweeter and ultrasonic bath. When using an ultrasonic probe for ultrasonication, the tip of the probe needs to be inserted into the sample, whilst the other three methods allow for indirect sonication, thus the sample can be treated in sealed cups. The differences in performance between these devices have been recently reviewed [22].

2.5. Restriction enzyme digestion

Eco47I and MvaI were carefully chosen from a vast number of restriction enzymes, to produce a considerable amount of DNA fragments ranging from 100 to 1000 bp. Enzymatic digestion was carried out at 37 °C, for 2 h, with a final concentration of 1 enzyme unit/μg of DNA. Inactivation was performed at 65 °C, for 20 min. The digestion products were left at room temperature, to cool down, and stored at 4 °C for subsequent analysis.

3. Results and discussion

3.1. Ultrasonic devices

The outcome and efficiency of ultrasonication depends on the chosen ultrasonic device. Parameters such as frequency and intensity of ultrasonication must be carefully analysed in the implementation of this type of energy in any sample treatment workflow. To assess the differences between three ultrasonic devices, 1 mL of water was submitted to ultrasonication using 50% of the nominal amplitude in different times and every 5 s the temperature was recorded. Fig. 2 plots the increment in time versus the increment in *Q*. The slope of the linear relation obtained increases according to the following: sonoreator (0.626) > UP100 (0.311) > UB. Since the increment in *Q* can be associated to the cavitation effects, especially for short operation times, one may speculate that the best performance is obtained with the UP100. This was later confirmed with experimental data (see below). The Vialtweeter was not included in this study.

3.2. Finding out the best device for DNA ultrasonic fragmentation

For effective hybridisation protocols, the average length of target DNA fragments should be reduced to decrease secondary structure formation [2–4]. The efficiency of four different ultrasonic-based platforms to yield suitable DNA fragments was

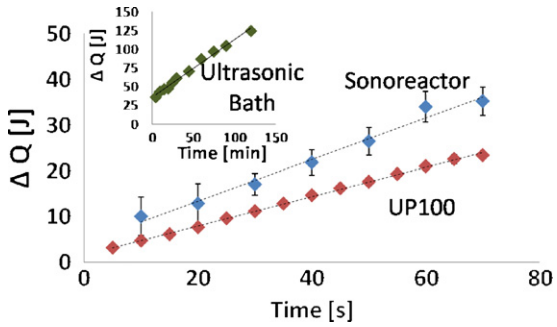

Fig. 2. ΔQ [J] versus time of sonication for three of the four ultrasonic devices assessed in this work. The slopes of the lines (J/s) are as follows: sonoreactor, 0.453; ultrasonic probe UP100, 0.319; ultrasonic bath 0.013. The Vialtweeter was not analysed in this study due to its low performance.

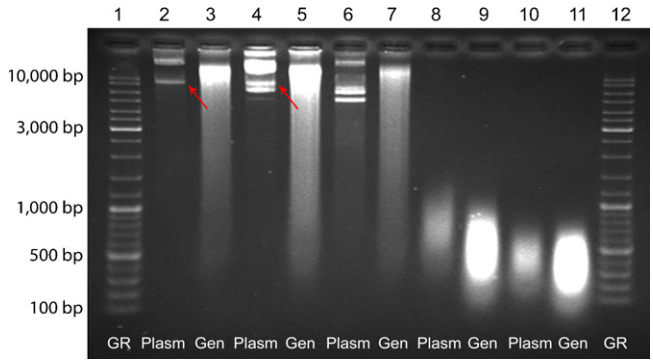

Fig. 3. DNA fragmentation potential for different sonication setups. 1% Agarose gel (in 1× TBE) electrophoresis of plasmid and genomic DNA, respectively: lanes 2 and 3: non-sonicated; lanes 4 and 5: ultrasonic bath; lanes 6 and 7: VialTweeter; lanes 8 and 9: sonoreactor; lanes 10 and 11: UP100H probe (1 mm diameter tip). All samples were sonicated for 2 min at maximum amplitude. Lanes 1 and 12: GeneRuler DNA Mix Ladder (Fermentas). Red arrows show intact plasmid DNA conformations. (For interpretation of the references to color in the citation of this figure, the reader is referred to the web version of the article.)

initially assessed setting the amplitude of ultrasonication at its maximum value (100%) and the time of treatment at 2 min.

Fig. 3 shows the electrophoretic profile of fragments originated from plasmid and genomic DNA submitted to ultrasonication. Results show that the ultrasonic bath and the VialTweeter have little effect in DNA integrity, causing a slight disruption of some plasmid conformations—see lanes 4 and 5 for ultrasonic bath and lanes 6 and 7 for the VialTweeter. Other native conformations of the plasmid are still present after the ultrasonic treatment (red arrows in Fig. 3 showing the intact conformations). This can be easily explained since both systems have low intensity of sonication, and therefore cavitation efficiency is not enough to promote considerable DNA fragmentation. Conversely, the ultrasonic probe and the sonoreactor originated smaller fragments in short operation times, yielding low molecular weight fragments with total disruption of native DNA conformations (see Fig. 3 lanes 8–11). The best performance was obtained for the ultrasonic probe, where 2 min ultrasonic time and 100% ultrasonic amplitude yielded a DNA fragment distribution comprised between 600 and 100 bp—Table 1. The sonoreactor also provided good results for the same experimental conditions as no native DNA conformations were observed and DNA fragments' size dispersion within the 1500–100 bp range—see Table 1 and Fig. 3 lanes 8 and 9. What is more, is that, when the time of ultrasonication was increased to 8 min, the DNA fragments' size homogeneity was better, being comprised between 1000 and 100 bp. These results are in agreement with previously reported data by Bankier and by Fukudome et al. [23,24], that observed that the molecular weights of DNA fragments after ultrasound exposure were remarkably decreased when ultrasonic power was increased from 25 to 105 W. The use of an UP to fragment DNA and the evaluation of the influence of ultrasonic power and ionic strength on DNA fragmentation has already been reported [20]. More recently, it was observed that the plasmid DNA condensation affects its resistance to fragmentation caused by ultrasonic waves [25].

Despite the UP probe's potential for DNA fragmentation, this device presents two major disadvantages: aerosol formation when ultrasonication is applied to the sample, which causes loss of sample; and risk of carry over contamination, as the tip of the probe is inserted into the sample. Also, as the tip vibrates to generate ultrasonic waves, it is subjected to micro-degradation [26], potentially leading to sample contamination with metal fragments that may hamper downstream applications (e.g. enzymatic amplification). On the other hand, the sonoreactor does not enter in direct contact with the sample, therefore presenting the advantage of reducing the contamination risk and possible losses of sample. In addition, the formation of an aerosol is not observed. Also, since the sonoreactor inputs 50 times less energy into the sample than the ultrasonic probe [22,27], the risk of thermal degradation derived from temperature is also reduced. In addition, although 2 min was enough to complete the treatment with the UP (1 sample), 4 samples can be treated at once using the sonoreactor. Therefore sample throughput is equivalent. Last but not least, the minimum sample volume that can be treated with a regular UP is 100 μL, whilst with the sonoreactor is 1 μL. Based on these data, the sonoreactor-based ultrasonic treatment was selected for further studies and optimisation.

3.3. Sample treatment optimisation

3.3.1. Influence of ultrasonication time

The effect of the time in ultrasonication was evaluated by ultrasonication of samples at different time intervals comprised between 2 and 6 min—see Fig. 4 in Section 3.3.1. The ultrasonication amplitude was set at 100%, since previous data had shown that lower amplitudes (<80%) led to lower efficiency in the fragmentation of DNA (data not shown). As may be seen in Fig. 4, the sample treatment is clearly time-dependent. For plasmid DNA, 4 min of sample treatment was enough to reach a fragment distribution comprised between 1000 and 100 bp, whilst for genomic DNA a distribution comprised between 800 and 100 bp was obtained with 6 or 8 min of sample treatment. To guarantee the robustness of the procedure, the duration of treatment in further experiments was set to 8 min.

Table 1
Comparison of DNA fragmentation potential for different sonication setups.

Devices	Conditions				Plasmid DNA		Genomic DNA	
	Time (min)	Amplitude (%)	Volume (μL)	[DNA] (μg mL^{-1})	Peak	Homogeneity	Peak	Homogeneity
Non-sonicated	2	–	100	100	8500 bp	13,000–8500 bp	12,000 bp	12,000-500 bp
Bath		Max			8500 bp	13,000–8500 bp	12,000 bp	12,000-500 bp
VialTweeter					8500 bp	8500 bp	12,000 bp	12,000-500 bp
Sonoreactor					800 bp	1500-200 bp	700 bp	1200-100 bp
Probe					500 bp	600-100 bp	400 bp	600-100 bp

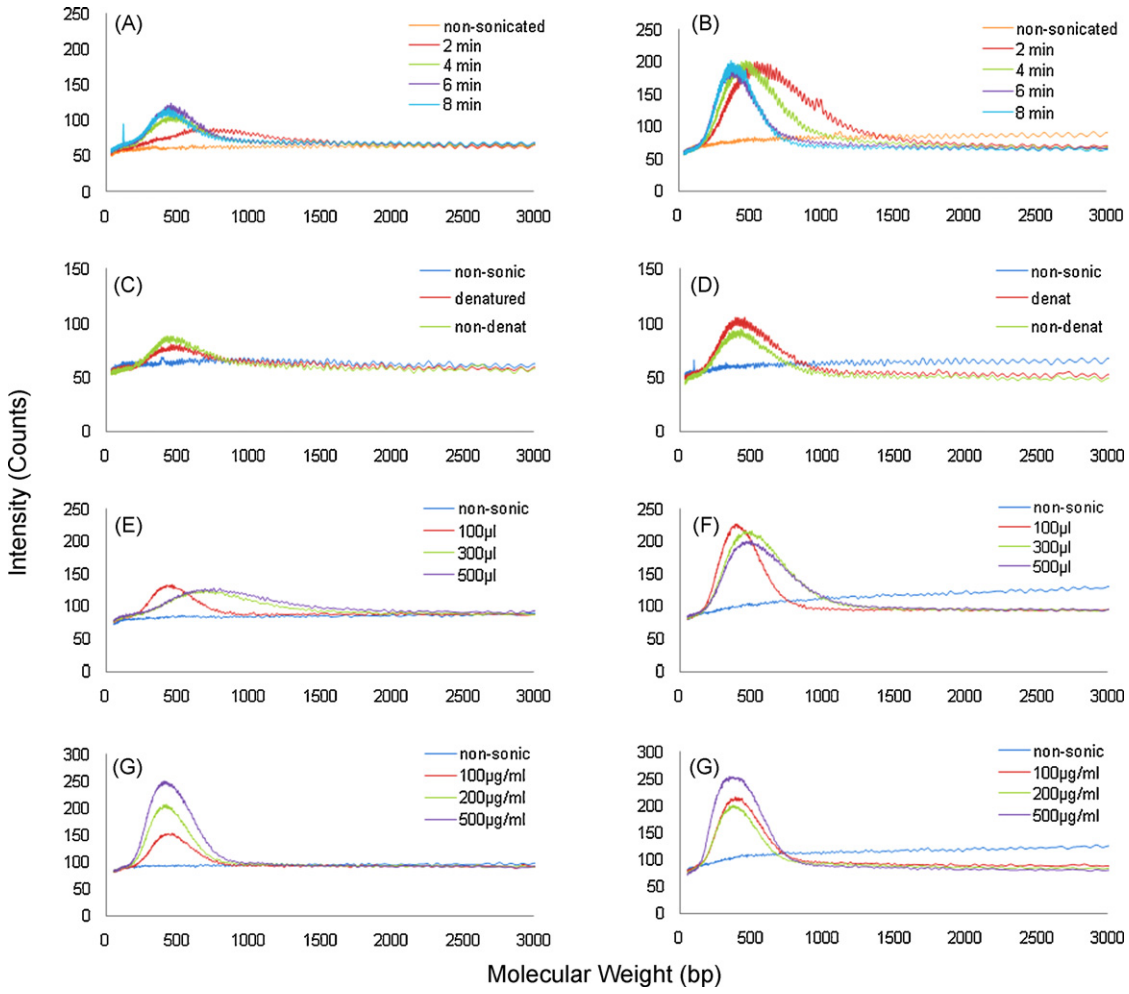

Fig. 4. Optimisation of the sonoreactor method for plasmid (A, C, E, G) and genomic (B, D, F, H) DNA. Variation of DNA size distribution: (A and B) influence of ultrasonication time; (C and D) influence of sample pre-treatment; (E and F) influence of volume of sample sonicated; (G and H) influence of DNA concentration. Amplitude of sonication was always 100%.

3.3.2. *Influence of sample pre-treatment*

To study the influence of the DNA conformation in the efficiency of the ultrasonic induced fragmentation, previously denatured and non-denatured DNA were submitted to the same conditions of ultrasonication. Fig. 4 in Section 3.3.1 shows that there was no improvement in the efficiency of the treatment due to previous denaturation. This suggests that the fragmentation process caused by cavitation is mainly a physical process and not chemical, i.e. caused by the creation of radicals during the cavitation process. Ultrasonication may also cause denaturation of proteins and DNA and therefore previous DNA denaturation may be of little help in the fragmentation process. These findings suggest that the main contribution to DNA strands' fragmentation is due to hydrodynamic forces as consequence of cavitation bubble collapse. This has been proposed by Grokhovsky [19] to explain the influence of temperature in the breaking of DNA, where hydrodynamic forces are the

Table 2
Comparison of different conditions using the sonoreactor system and its influence on DNA fragmentation potential.

Devices	Conditions				Plasmid DNA		Genomic DNA	
	Time (min)	Amplitude (%)	Volume (µL)	[DNA] (µg mL^{-1})	Peak	Homogeneity	Peak	Homogeneity
Non-sonicated	–	–	100	100	8500 bp	13,000–8500 bp	12,000 bp	12,000–500 bp
Sonoreactor	2	100%	100	100	800 bp	1500–200 bp	700 bp	1200–100 bp
	4				450 bp	1000–100 bp	500 bp	1100–100 bp
	6				420 bp	1000–100 bp	380 bp	800–100 bp
	8				420 bp	1000–100 bp	380 bp	800–100 bp
			300		700 bp	1500–100 bp	500 bp	1200–100 bp
			500		900 bp	2100––100 bp	550 bp	1200–100 bp
	8			200	500	1200–100 bp	480 bp	1000–100 bp
				500	500	1500–100 bp	480 bp	1000–100 bp
			100					
Sonoreactor (sample denatured beforehand)				100	420 bp	1000–100 bp	380 bp	800–100 bp

main causes of ultrasonic DNA fragmentation, and that chemical processes generating radicals during cavitation play only a minor role, if any (Table 2).

3.3.3. Influence of sample volume

For equal conditions of time and amplitude of ultrasonication, as the sample volume increases, the ultrasonication density (i.e. efficiency of treatment) is expected to decrease. This was confirmed in a set of experiments in which the ultrasonic conditions and DNA concentration were maintained, but the sample volume treated was incremented from 100 to 500 μL. As shown in Fig. 4 (in Section 3.3.1), the average size of the plasmid DNA fragments was 900, 700 and 420 bp whilst for genomic DNA was 550, 500 and 380 bp for sample volumes of 500, 300 and 100 μL, respectively. A volume of 100 μL was chosen as optimal for further experiments.

3.3.4. Influence of DNA concentration

The influence of DNA concentration was also assessed. Results suggest that for the same ultrasonication time and amplitude, the fragmentation efficiency is slightly diminished as the DNA concentration increases (Fig. 4 in Section 3.3.1). The size distribution of DNA fragments obtained incremented from 1000–100 to 1500–100 bp for plasmid DNA and from 800–100 to 1000–100 bp for genomic DNA. However, if the time of treatment is increased, the average size of the fragments and the size distribution can be the same, regardless of the DNA concentration (range 100–500 $\mu g\,mL^{-1}$).

After exhaustive analysis of all different conditions and setups, an optimised protocol for DNA fragmentation was set out in which the sample was sonicated by means of a sonoreactor for 6 min at 100% ultrasonication amplitude in a volume of 100 μL.

3.4. Ultrasonic fragmentation versus restriction enzyme digestion

The optimised ultrasonic protocol was compared to restriction enzyme digestion for fragmentation of DNA samples. To assure digestion, a double restriction was performed with two frequent cutters—Eco47I and MvaI. Fig. 5 displays the electrophoretic patterns for sonicated and digested DNA samples (plasmid and total genomic DNA). The patterns corresponding to plasmid DNA samples sonicated (lane 3) and digested (lane 4) show considerable differences. The ultrasonicated sample exhibits a fragment distribution (100–500 bp), whilst the digested sample consists of numerous DNA fragments with discrete molecular weight (100–3000 bp). This is not observed for the genomic DNA samples, where both the sonicated and digested samples (Fig. 5—lanes 6 and 7, respectively) exhibit a smear corresponding to extensive fragmentation. It should be noted that, whilst ultrasonication yields fragments with low size dispersion (<500 bp), the digested sample shows a wider range of fragment distribution from high to low molecular weight fragments (10,000–<100 bp). The restriction enzyme treatment of genomic DNA results in a fragment profile which can be compared to that attained by simple mechanic fragmentation occurring during DNA extraction and purification (untreated sample). When compared to the untreated sample, the restriction enzyme treatment can be considered negligible as it shows a much lower capability of fragmentation than that of ultrasonication.

Restriction enzyme digestion, due to the specific sequence recognition, may allow a more discrete cleavage but it is time consuming, relies on expensive reagents (e.g. enzymes, buffers) for the reaction to occur at optimum activity conditions, and requires subsequent sample purification. Contrary to what it might seem, DNA fragmentation through ultrasounds does not occur totally at random, as 5′-CpG-3′ dinucleotides have been shown to be primary hotspots for double-strand breaks [19]. What is more, the ultrasonication approach here proposed avoids any purification step, is significantly faster and less expensive than restriction enzyme methods, generating fragments of equivalent molecular weight and suitable for downstream detection applications.

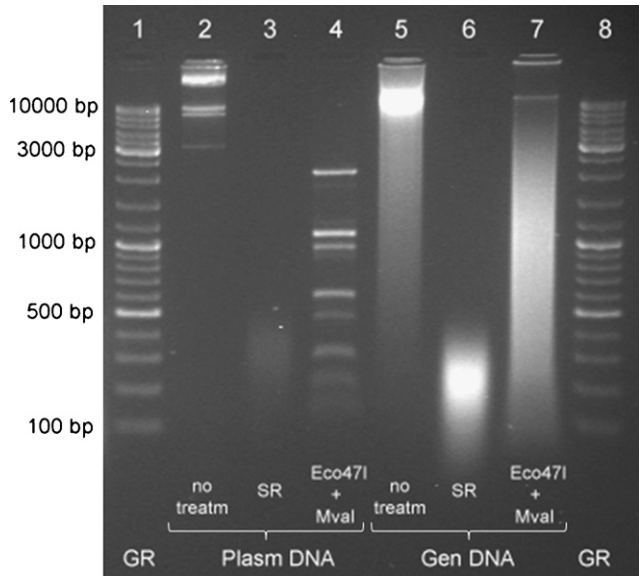

Fig. 5. Comparison between the sonoreactor system and restriction enzyme digestion in generating DNA fragments. 1% Agarose gel (in 1× TBE) electrophoresis of plasmid DNA samples, respectively: lane 2: non-sonicated; lane 3: sonicated with the sonoreactor; lane 4: digested with restriction enzymes; and genomic DNA samples, respectively: lane 5: non-sonicated; lane 6: sonicated with the sonoreactor; lane 7: digested with restriction enzymes. Lanes 1 and 8: GeneRuler DNA Mix Ladder (Fermentas). Conditions of ultrasonication: ultrasonication time 8 min, ultrasonication amplitude 100%.

4. Conclusions

We have presented a simple, fast, high-throughput and inexpensive sonoreator-based method for DNA fragmentation. Four ultrasonic devices were compared in terms of fragmentation efficiency, and only the sonoreactor and the ultrasonic probe were found suitable for efficient DNA fragmentation. However, the sonoreactor shows several advantages when compared to the ultrasonic probe: (i) allows for processing of smaller sample volumes; (ii) sample overheating is greatly diminished (no degradation of analyte); and (iii) avoids cross-contamination between samples.

Despite both being noteworthy methodologies for DNA fragmentation, the proposed sonoreactor methodology shows several advantages when compared to commonly used restriction enzyme digestion, namely a larger extent of DNA fragmentation with smaller size dispersion is attained in less time at smaller costs. Last but not least, all this is achieved without the need to further sample purification, greatly diminishing the risk of loss of sample or contamination while increasing the reliability of the approach.

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