Biochemical and Biophysical Research Communications 448 (2014) 45–49

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Soni-removal of nucleic acids from inclusion bodies

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ARTICLE INFO

Article history:
Received 28 March 2014
Available online 18 April 2014

Keywords:
Nucleic acid contamination
Soni-Removal
Sonication
Economic
Inclusion bodies purification

ABSTRACT

Inclusion bodies (IBs) are commonly formed in *Escherichia coli* due to over expression of recombinant proteins in non-native state. Isolation, denaturation and refolding of these IBs is generally performed to obtain functional protein. However, during this process IBs tend to form non-specific interactions with sheared nucleic acids from the genome, thus getting carried over into downstream processes. This may hinder the refolding of IBs into their native state. To circumvent this, we demonstrate a methodology termed soni-removal which involves disruption of nucleic acid–inclusion body interaction using sonication; followed by solvent based separation. As opposed to conventional techniques that use enzymes and column-based separations, soni-removal is a cost effective alternative for complete elimination of buried and/or strongly bound short nucleic acid contaminants from IBs.

1. Introduction

Inclusion bodies (IBs) are cellular insoluble aggregates that are commonly formed during over expression of heterologous proteins in *Escherichia coli*. This occurs mainly due to the formation of partially folded aggregates in the cytoplasm of *E. coli*, where post-translational modifications are absent [1]. To obtain soluble protein, these inclusion bodies are processed by a series of steps like cell lysis, denaturation and refolding *in vitro*. However, many instances show that, IBs can form complexes with contaminants like lipids, nucleic acids and other proteins along the process [1]. The elimination of such contamination is inevitable for protein–nucleic acid interaction studies and therapeutic applications [2–4].

During cell lysis endogenous nucleic acids may get sheared resulting in shorter to longer fragments. These sheared nucleic acids interact differently forming weak to stronger non-specific interactions with IBs depending on their affinity. Moreover, with steps that follow like denaturation and refolding, IBs and nucleic acids in soluble state are more susceptible to form complexes as the contamination is carried over. Thus, nucleic acids are the major contaminants during the expression of recombinant proteins as IBs [5]. To remove nucleic acids from IBs, mechanical, chemical and enzymatic techniques are commonly employed. However, the procedures used for such applications results in loss of sample as they require multiple steps to remove nucleic acids [6–8]. Enzymatic methods should be avoided as downstream applications maybe hindered; for example, the use of nucleases should be avoided for protein–nucleic acid interaction studies, selection of aptamers for a specific protein target, etc. Also, in many cases, enzymes like benzonases fail to completely remove nucleic acids from complexes due to inaccessibility of shorter nucleic acids buried in the refolded protein as demonstrated in this study. The failure of conventional techniques in eliminating nucleic acid contamination creates a need for the development of more effective yet simple methodologies.

It is a common practice to solubilize IBs using chemical denaturants like Urea, Guanidine-HCl and Lauryl glutamate before refolding them. However, these denaturants would solubilize both IBs and nucleic acids, thus making them inseparable [1]. Thus, by maintaining IBs in their insoluble state and nucleic acids in solution; appropriate aqueous buffers would allow separation by high-speed centrifugation. This scenario can be simulated prior to the denaturation step. However, conventional methods do not target this critical step, thus could result in carryover of contamination [6–8].

Soni-removal aids in disruption of interaction between IBs and nucleic acids by simple sonication steps without the use of enzymes or chromatographic procedures. Sonication breaks the weak ionic, hydrogen and hydrophobic interactions but not the covalent peptide bonds therefore leaving IBs unaffected [6]. Interestingly, as the interaction between nucleic acid contaminants and IBs are not covalent in nature, we identified sonication as a key technique in achieving the above objectives prior to denaturation

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http://dx.doi.org/10.1016/j.bbrc.2014.04.049
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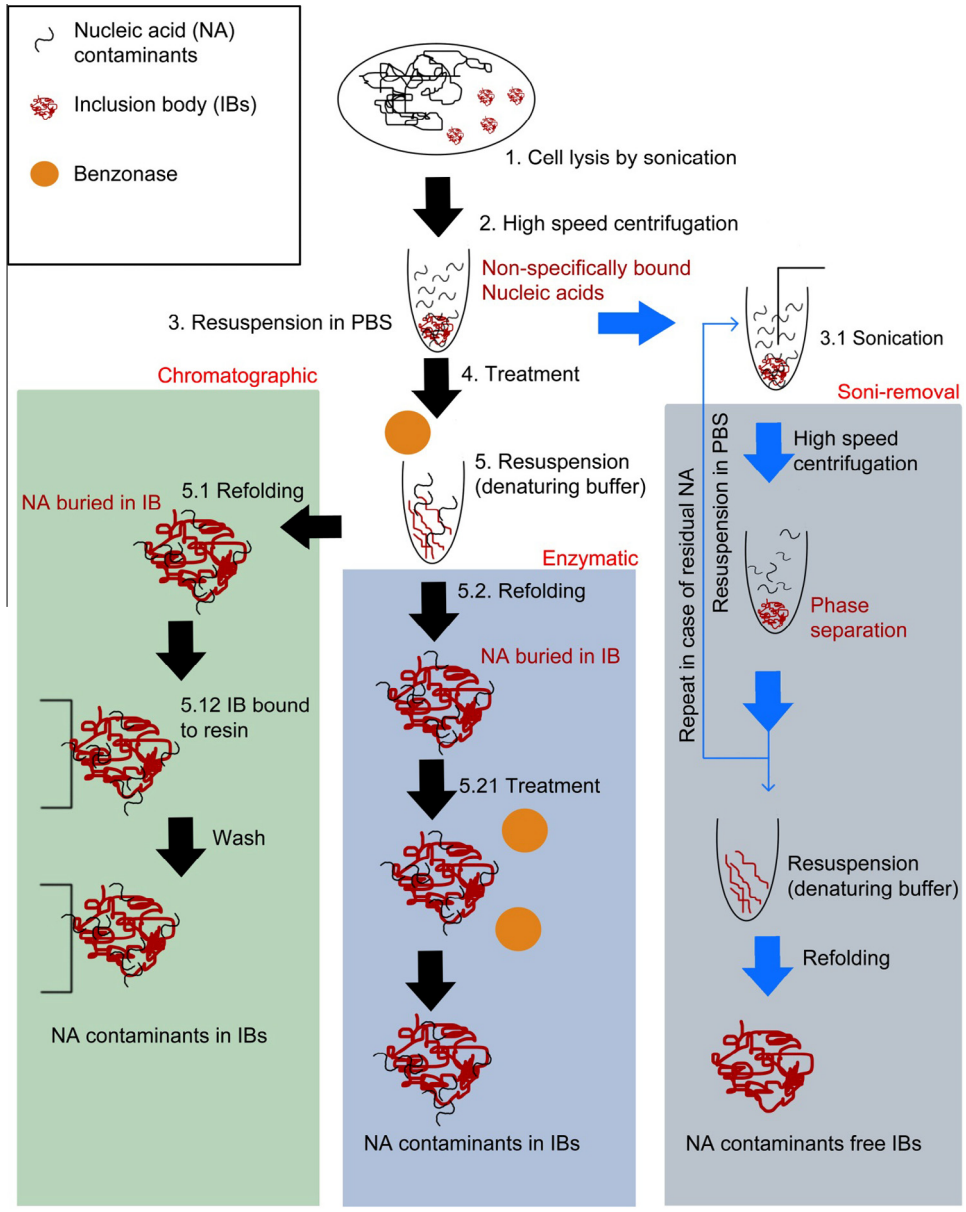

Fig. 1. Schematic representation for the removal of nucleic acid contaminants from IBs.

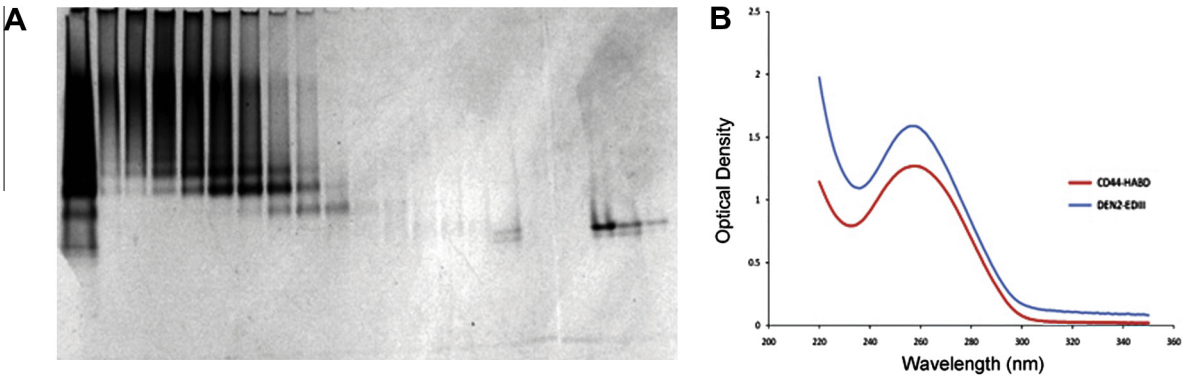

Fig. 2. Determination of nucleic acid contamination in purified protein: (A) CD44 was refolded and monomeric proteins were separated by gel filtration on Superdex-75. The fractions were collected and run on a 12% Native PAGE. (B) UV analysis shows sheared nucleic acid contaminants strongly bound or buried in the refolded proteins of CD44-HABD and DENV2-EDIII.

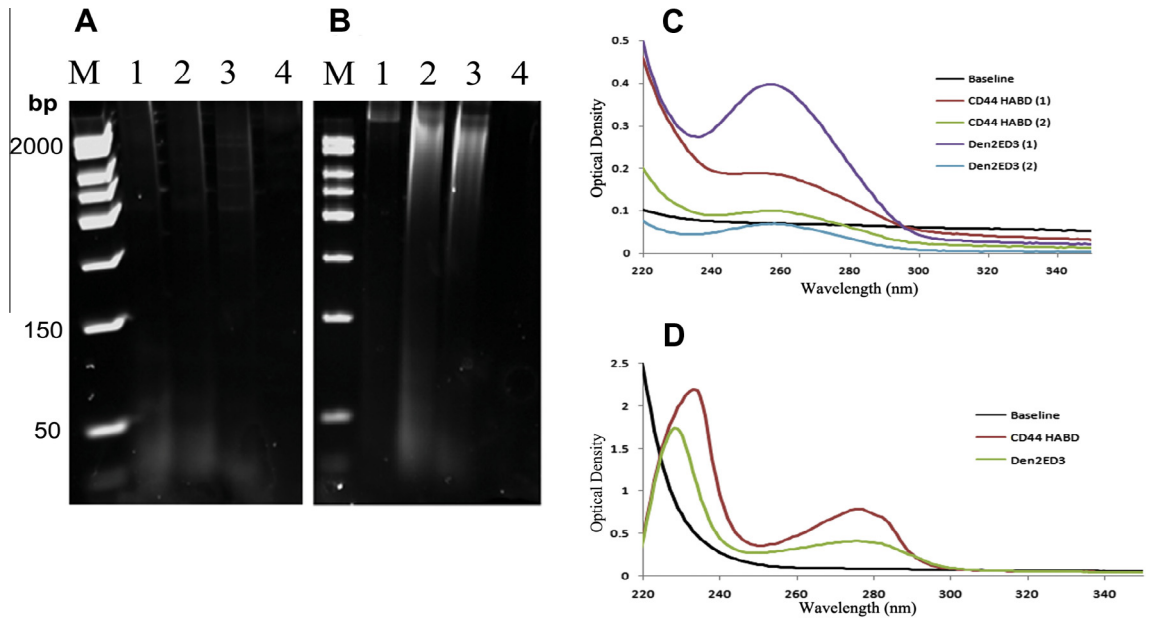

Fig. 3. Removal of nucleic acid contaminants: SYBR green stained NuPAGE 3–8% Tris–Acetate gel showing removal of nucleic acid contamination by sonication treatment for CD44HABD (A) and DENV2-EDIII (B), respectively. Lane M – exATCgene Low range DNA marker (Fisher Scientific). Lanes 1 and 2 – Insoluble pellet from cell lysate in PBS showing nucleic acid contamination before and after sonication treatment, respectively. Lane 3 – Supernatant from centrifugation after sonication treatment showing presence of nucleic acids in PBS. Lane 4 – Absence of nucleic acid contamination in the pellet containing the protein, resuspended in denaturing buffer. (C) The UV scans of CD44-HABD (1) and DENV2-EDIII (1), represents the diluted supernatants of CD44-HABD (10 times) and DENV2-EDIII (2 times) that were obtained from the first spin (16,000*g*) and the scans of CD44-HABD (2) and DENV2-EDIII (2) represents the supernatants of undiluted samples after the second spin (16,000*g*). (D) UV scan showing for the protein sample free of nucleic acids after the sonication treatment. The pellet was resuspended (after centrifugation 16,000*g*) in the denaturing buffer (8 M Urea, 50 mM MES pH 6.5, 0.1 mM EDTA).

(Fig. 1). When this disruption is performed prior to the critical denaturation step in aqueous buffer, insoluble IBs are easily separated by high-speed centrifugation as mentioned earlier. This sonication has a different effect as compared to the one applied during cell lysis, which disrupts the cell wall and shears endogenous nucleic acids. Here, we demonstrate soni-removal for two proteins, namely CD44-Hyaluronan binding domain (CD44-HABD) and Dengue Virus type-2 envelope domain III (DENV2-EDIII). Of which, both IBs display nucleic acid contamination after refolding by using conventional purification steps including benzonase treatment. After demonstrating the failure of conventional methods, we elucidate how the application of soni-removal at the critical step can fulfill the need for complete elimination of nucleic acid contaminants from IBs.

2. Material and methods

2.1. Expression of IBs of CD44-HABD and DENV-2 EDIII

Human CD44 DNA construct coding 20–178 of the final residue after the signal sequence and first 158 residues from the N-terminus of the mature protein was synthesized from GeneScript® with NcoI and BglII sites for ligation into the expression vector pET19b (Novagen). The DENV2-EDIII protein was expressed in CR2566 strain of *E. coli* containing a plasmid encoding for DENV2-EDIII. The protein expression was checked on SDS–PAGE. The previously published protocol for purification of Flavivirus ED3s was followed [9,10].

In case of both proteins, cells were lysed by sonication. Where, both samples were subjected to 6 cycles of 30 s ON and OFF at 50% power (Misonix sonicator) until the pellets were homogeneous. The sonicated cells were centrifuged at 4 °C for 1 h at 10000 rpm to pellet the IBs. For DENV2-EDIII, the supernatant was discarded and pellet was resuspended in 40 ml denaturing buffer [20 mM Tris–HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 6 M Guanidine HCl and 5% (v/v) glycerol] using a rotating mixer or slow rocking at 4 °C for about 12–24 h until pellet is homogenous. The resuspended solution was centrifuged at 8000 rpm for 1 h (at 4 °C) to pellet the insoluble fraction and the supernatant contains the denatured IBs. CD44-HABD protein was expressed as IBs and purified as described by Banerji et al. [11].

2.2. Purification of IBs of DENV-2 EDIII and CD44-HABD

DENV-2 EDIII and CD44-HABD were purified as described by Volk et al. [9,10] and Banerji et al. [11], respectively. In case of DENV-2EDIII, the dialyzed protein was passed through 30 kDa cut-off in Amicon Ultra centrifugal filters (15 ml volume – from Millipore) for removing higher molecular weight protein and nucleic acid contaminants that might have co-eluted with protein (checked for nucleic acid contamination using UV-spec 260/280 ratio). The protein was then concentrated and buffer exchanged, using 3 kDa cut-off in Amicon Ultra centrifugal filters (Millipore) and purity was confirmed by UV scan.

2.3. Sonication treatment for nucleic acid removal

IBs of both proteins obtained from 1 L culture were processed as described earlier, resuspended in the 30 ml phosphate buffer saline (1 × PBS) and homogenized. A portion (15 ml) of the pellet was centrifuged and supernatant was discarded. The pellet was resuspended in denaturing buffer (8 M Urea containing 50 mM MES (pH6.5), 0.1 mM EDTA and 0.1 mM DTT) for gel electrophoresis and UV scan.

The second portion of both pellets was employed for sonication treatment. The sonication treatment was performed for 2–10 cycles of 30 s pulse with 30 s interval on ice. Further, the treated samples were centrifuged for 10 min at 16,000*g*. A small aliquot

of both samples were preserved prior and after centrifugation for confirming the presence or absence of the nucleic acids contamination.

2.4. Detecting nucleic acid contamination on PAGE

Presence or absence of nucleic acid contamination in both IBs was checked by loading 10 µl on PAGE (NuPAGE 3–8% Tris–acetate). The PAGE gel was further subjected to SYBR Green staining (Molecular Probes, OR, USA) and observed at OD260.

3. Results and discussion

3.1. Detection of nucleic acid contamination in refolded protein

CD44-HABD and DENV2-EDIII IBs were expressed to validate our strategy (Fig. 1). Soluble CD44-HABD IBs were obtained by cytoplasmic expression as three intra-molecular sulfide bonds are required to bring it to soluble form [11,12]. On the other hand, DENV2-EDIII inclusion bodies were generated by expression at 37 °C [13]. Next, the cells were lysed by sonication and centrifugation was employed to isolate the IBs. The IBs being insoluble were easily separated and refolded *in vitro*. However, after purification, UV scan showed nucleic acid contamination in both refolded proteins (Fig. 2A and B). Even after repeated washing, centrifugation, denaturing and gel-permeation, the contamination was prevalent. We also treated the refolded monomeric CD44-HABD protein (Fig. 2A) with benzonase that cleaves both DNA and RNA. However, it did not prove to be effective. We propose that this maybe due to short nucleic acids getting buried inside the protein molecule or strongly bound to the IBs at the denaturation–refolding step.

3.2. Sonication treatment for removal of nucleic acid contaminants

Conventional techniques fail to completely remove nucleic acid contaminants in case of refolded proteins; such contamination could be carried over from the denaturation–refolding step. To overcome this problem, we employed sonication prior to denaturation for breaking the weak ionic, hydrogen and hydrophobic interactions between IBs and nucleic acid contaminants. Here, both IBs were prepared as described in the materials and methods section

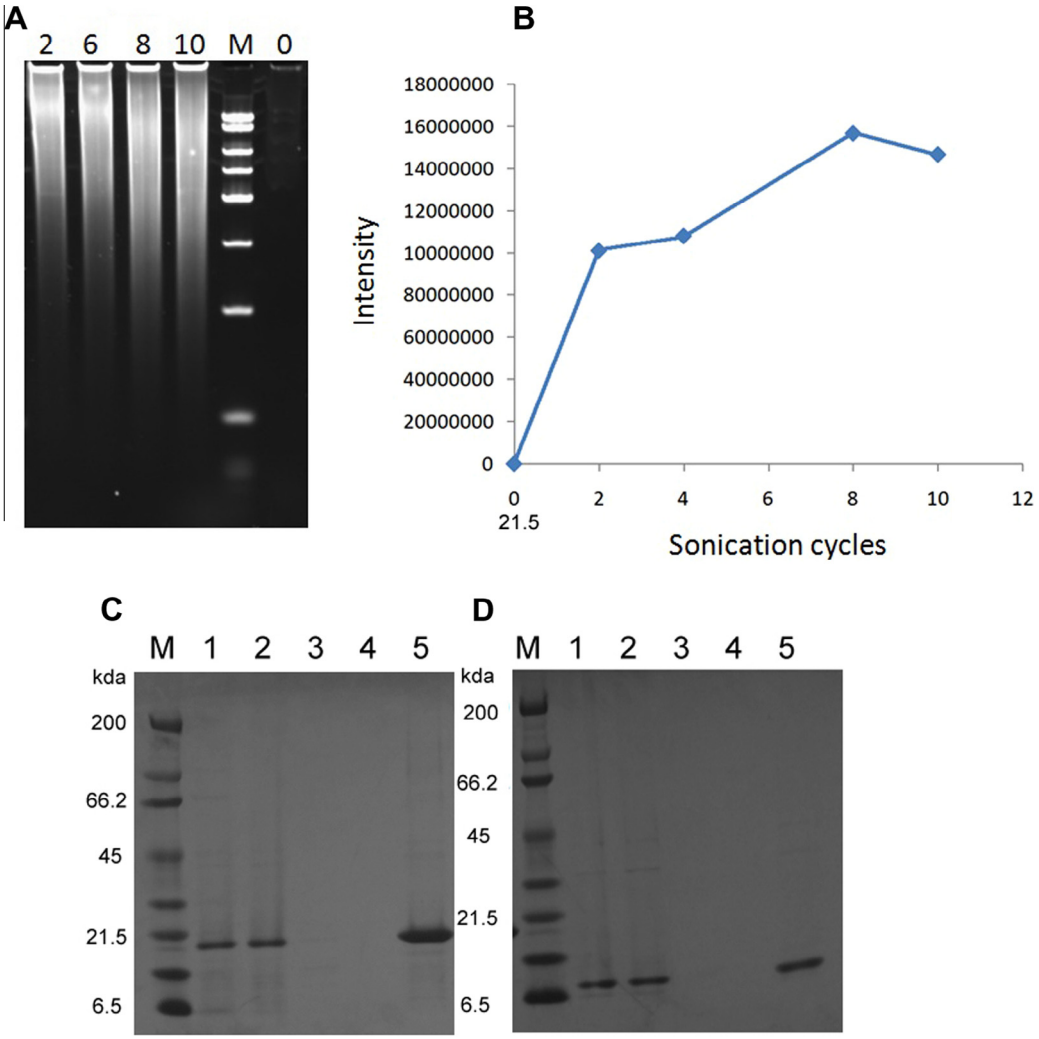

Fig. 4. Optimization of sonication cycles for optimal disruption and SDS–PAGE: (A) 3–8% Tris–acetate gel with SYBR green stain was run to determine the amount of nucleic acid contaminants removed by the sonication procedure at each cycle (0, 2, 6, 8, 10 represents the number of sonication cycles, M is the marker). (B) Using densitometric analysis data generated by FlurochemQ, a graph of intensity against number of sonication cycles was plotted. Maximum removal of nucleic acid contamination from the target protein occurred between 8 and 10 sonication cycles. (C) CD44-HABD and (D) DENV2-EDIII were run on SDS–PAGE, Lane M – Protein marker; Lanes 1 and 2 – Insoluble pellet from cell lysate containing intact protein in PBS before and after sonication treatment, respectively; Lanes 3 and 4 – Supernatant from first and second centrifugation after sonication treatment, respectively; Lane 5 – Pellet after sonication treatment in denaturing buffer showing presence of intact protein.

and nucleic acid contamination was confirmed by PAGE (Fig. 3A and B, lane 1). To break the interaction and release nucleic acids from IBs, optimization by varying the number of sonication cycles was carried out. This optimization was performed upto10 cycles (i.e. 30 s pulse with 30 s intervals each) for both IBs containing nucleic contamination (Fig. 4A and B). The number of cycles at which maximum contamination is eliminated (10 cycles) was applied for both IBs and the sonicated sample was centrifuged. As insoluble IBs were present in aqueous buffer, they could be pelleted down easily as opposed to nucleic acids. The presence of nucleic acids in the supernatant (Fig. 3A, see lane 3 and B, see lane 3) of both IBs (CD44-HABD (OD260 nm ~20) and DENV2-EDIII (OD260 nm ~0.8)) indicated that nucleic acids were successfully separated by sonication. We suspected that one sonication treatment would not be sufficient to eliminate strongly bound nucleic acids as trace amounts of contamination may be present. Therefore, the above sample was again resuspended in PBS and a second sonication treatment (10 cycles) was employed. As nucleic acid contaminants were observed in the first sonication step, an identical second sonication was performed. After the second sonication treatment, UV scan at OD 260 nm showed the release of some nucleic acid in supernatant of both IBs (Fig. 3C). This highlights the probable importance of the second sonication treatment to release nucleic acid contaminants (Fig. 3C). A third sonication was not applied as no more nucleic acid contamination was detected by both UV scan and PAGE (Fig. 3A, B and D, lanes 4). This additional sonication step varies from IB to IB; hence optimization will have to be carried out for each IB of interest. Absence of nucleic acid contaminants shows that this methodology is effective. Even the short nucleic acid fragments (less than 50 bp) observed in the CD44-HABD IBs (Fig. 3A and B, lanes 2) were eliminated by our methodology. We propose that repeated sonication cycles cause shearing and release of nucleic acids whereas; the IBs remain unaffected (Fig. 4C and D). The use of our methodology with minimal steps for complete removal of nucleic acids at the critical step was successful.

The conventional procedures involving multiple steps to remove nucleic acid contamination can be time consuming, costly and less effective. Also, with increase in the number of steps involved, there will be sample loss at each step during the process as discussed earlier. However, our technique is simple, employs minimal steps and therefore avoids the loss of sample. The unique feature of our methodology lies in the separation of short nucleic acid fragments that might be trapped in the protein during refolding. As seen in case of CD44-HABD, such contamination cannot be removed by using benzonase because of two reasons, (1) either the benzonase cannot access the contaminants as they may be buried inside the IB or (2) the cleavage of nucleic acids could lead to shorter fragments that get rebound to the IB [14]. As such contaminants were successfully eliminated using our methodology, soni-removal is more advantageous being employed at the critical step resulting in complete elimination of nucleic acid contamination from IBs.

Author contribution

M.N. and S.G. conceptualized, developed, and executed the experiments. M.N., S.G. and S.M. were involved in the writing, editing and data analysis/interpretation of the manuscript.

Funding

This work was supported by the Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, under Prof. David G. Gorenstein.

Acknowledgment

The authors wish to thank Dr. Gorenstein for providing the constructs of CD44-HABD and DENV2-EDIII.

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