[Letter to the Editor]

NaOH concentration and streptavidin bead type are key factors for optimal DNA aptamer strand separation and isolation

Single-stranded DNA (ssDNA) aptamer preparations eluted from biotin–streptavidin beads using NaOH during systematic evolution of ligands by exponential enrichment (SELEX) commonly suffer from streptavidin and double-stranded DNA (dsDNA) contamination. Based on an extensive literature review, most protocols describe elution with NaOH concentrations >100 mM. Here, we present data that call into question the validity of using such high molarity NaOH melting solutions and further suggest that care should be taken to empirically determine the optimal NaOH concentrations applicable for the specific type of streptavidin-coated magnetic bead being used.

Conversion of dsDNA into ssDNA is a critical step in SELEX-based DNA aptamer selection that is repeated in each selection cycle of the SELEX procedure. In *Current Protocols in Molecular Biology*, Wakimoto et al. describe a protocol for biotin–streptavidin ssDNA isolation using Dynabeads M-270 Streptavidin and 125 mM NaOH (1). Multiple references were found that compared thermal denaturation, lambda exonuclease digestion, or denaturing PAGE with biotin–streptavidin magnetic beads utilizing 150–200 mM NaOH alkaline denaturation (2–6). Most found NaOH methods unsatisfactory because, in addition to liberating the non-biotinylated strand, the undesired biotinylated strand and streptavidin were also found in the ssDNA (2,4) eluates. Sheng and Bowser (7) described a microfluidic, flow-through dialysis device for isolating ssDNA from biotinylated dsDNA bound to streptavidin polystyrene beads. While 25 mM NaOH was found to be optimal, this approach may not be readily available to many

laboratories. Some studies (3,6) have suggested that NaOH pre-conditioning of the streptavidin beads can prevent contamination of the eluate by both streptavidin and the biotinylated strand. However, extended exposure of streptavidin to NaOH can affect its 3-D structure and, thus, its biotin binding efficiency. Gel purification and other complex approaches have been used to improve the quality of ssDNA preparations for SELEX. Careful consideration and the incorporation of our results can help researchers circumvent these extended, complex, time-consuming procedures and improve the quality of their ssDNA preparations.

In an effort to optimize the quality of our ssDNA preparation, levels of dsDNA and streptavidin co-eluted with NaOH ssDNA preparations from Sera-Mag magnetic streptavidin-coated beads (GE Healthcare, Pittsburgh, PA) were compared with elutions from Dynabeads MyOne Streptavidin C1 (Invitrogen, Grand Island, NY). The initial biotinylated dsDNA aptamer was generated by PCR, followed by incubation with various types of streptavidin beads. After washing, the desired non-biotinylated strand was eluted by separately resuspending each pellet in 50 µl of 10 mM, 20 mM, 75 mM, or 150 mM NaOH buffer. The supernatant was transferred to a clean tube, briefly spun, and 45 µl was carefully transferred to a new tube containing 6 µl 10× TE buffer, then neutralized to pH 7.5 and normalized to a final volume of 60 µl. Fifty percent of the sample was subjected to electrophoresis on a TAE-buffered 3% agarose gel (Figure 1, A and C). Additionally, 25% of the eluate was mixed 1:1 with 2× Laemmli buffer supplemented with 2% β-mercaptoethanol, and boiled for 5 min. This preparation was then analyzed on

4%–20% Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) in Tris/glycine/SDS buffer (Bio-Rad), western blotted, and incubated with anti-streptavidin antibody. (Figure 1B).

We first compared the quality of ssDNA eluted from Dynabeads MyOne Streptavidin C1 (Figure 1A, Lanes 2–5) with ssDNA eluted from Sera-Mag beads (Figure 1A, Lanes 6–9) using NaOH concentrations of 10 mM, 20 mM, 75 mM, and 150 mM. After agarose gel electrophoresis, ssDNA and dsDNA bands from three independent experiments were quantified using ImageJ software (<https://imagej.nih.gov/ij/>). The ssDNA/dsDNA ratio for each data point was then calculated and used as an indirect measure of the quality of ssDNA eluted at each NaOH concentration (Figure 1D). The quantity of ssDNA recovered at each concentration of NaOH was calculated based on the densitometry band intensity of the 0.625 µg ssDNA aptamer library input (Figure 1A, Lane 10). Elutions using 10 mM and 20 mM NaOH (Figure 1A, Lanes 2 and 3 for Dynabeads and Lanes 6 and 7 for Sera-Mag beads) had approximately 4× (for Dynabeads) and 2× (for Sera-Mag beads) less dsDNA contamination compared with 75 mM and 150 mM NaOH elutions (Figure 1A, Lanes 4 and 5 for Dynabeads and Lanes 8 and 9 for Sera-Mag beads). Overall, 10 mM and 20 mM NaOH elutions from Dynabeads MyOne Streptavidin C1 had 1.6× less dsDNA contamination and a slightly higher ssDNA yield (Figure 1A, bottom panel) compared with the corresponding elutions from Sera-Mag beads (Figure 1A, Lanes 2 and 3 compared with Lanes 6 and 7; and Figure 1D, Bars 1 and 2 compared with Bars 5 and 6). Denaturing SDS-PAGE and western blotting for NaOH elutions from Dynabeads with anti-strepta-

vidin antibody (Figure 1B) showed no detectable streptavidin contamination at 10 mM and 20 mM NaOH and only minor contamination at 75 mM and 150 mM (Figure 1B, Lanes 1, 2, 3, and 4, respectively). Note that streptavidin exists as a 60 kDa homotetramer complex that can dissociate into trimers (~45 kDa), dimers (~30 kDa), or monomers (~15 kDa) under denaturing conditions (Figure 1B, streptavidin bands marked *, *1, and *2, respectively). Interestingly, streptavidin contamination in Sera-Mag bead NaOH elutions (Figure 1B, Lanes 5–8) was clearly detectable even at 10 mM and increased significantly with increasing NaOH concentrations, suggesting a possible difference in NaOH tolerance between the 2 types of streptavidin-coated magnetic beads. At 20 mM NaOH, elutions from all types of Dynabeads tested (MyOne C1, M-270, M-280, and MyOne T1) generated clean ssDNA free of any detectable dsDNA (Figure 1C). NaOH is a known protein denaturant (8,9), and high concentrations can interfere with the 3-D structure of streptavidin, including monomer–monomer interactions and rupture of the critical biotin binding loop3–4 (9,10). The fact that dsDNA and streptavidin contamination are higher in eluates from Sera-Mag beads indicates that the destabilizing effect of NaOH on streptavidin 3-D structure is more pronounced in Sera-Mag beads compared with Dynabeads. In SELEX experiments, the generation of high-quality ssDNA is central to the selection of aptamers with high target affinity and specificity. Our close scrutiny of the quality of ssDNA eluted at different NaOH concentrations was prompted by the persistent presence of dsDNA contaminants in our ssDNA aptamer elutions when 150 mM NaOH was used, as per most published reports (2–4,6,11–14). Using various types of streptavidin magnetic beads, we show that an 82-mer ssDNA eluted with low molarity NaOH (10–20 mM) is significantly free of detectable dsDNA and streptavidin contamination. However, 75–150 mM eluates are heavily contaminated with both dsDNA and streptavidin. Importantly, different types of streptavidin magnetic beads appear to have different NaOH tolerance limits. Whether differences in attachment chemistries can account for

Figure 1. Comparison of the quality of ssDNA eluted from different streptavidin magnetic beads using different NaOH concentrations. For each data point, approximately 0.1 μM of ssDNA aptamer was subjected to 25 cycles of PCR amplification in a final volume of 50 μl per reaction. PCR products were then incubated 1:1 (v/v) with the indicated type of streptavidin magnetic beads for 30 min at room temperature, washed, and eluted with 50 μl of the indicated concentration of NaOH. (A) For comparison of the purity of ssDNA generated from Dynabeads MyOne Streptavidin C1 and Sera-Mag beads, 50% of the eluate was electrophoresed on a 3% agarose gel in cold 1× TAE buffer. Lane 1: 50 bp molecular weight marker; Lanes 2–5: Dynabeads MyOne Streptavidin C1 eluates; Lanes 6–9: Sera-Mag bead eluates, (10 mM, 20 mM, 75 mM, and 150 mM NaOH, respectively); Lane 10: 0.625 μg of unamplified ssDNA aptamer library; Lane 11: 0.5 μg of PCR-amplified dsDNA aptamer. The quantity (μg) of ssDNA recovered (bottom panel) was calculated by dividing the densitometry band intensity of each concentration of NaOH by the band intensity of the 0.625 μg ssDNA aptamer library input. (B) Twenty-five percent of the eluate was mixed 1:1 with 2× Laemmli buffer supplemented with 2% β-mercaptoethanol, boiled for 5 min. These samples were then analyzed on 4%–20% Mini-PROTEAN TGX Precast Gels (Bio-Rad) in Tris/glycine/SDS buffer (Bio-Rad), western blotted, and incubated with anti-streptavidin antibody. Lanes 1–4: Dynabeads MyOne Streptavidin C1 eluates; Lanes 5–8: Sera-Mag bead eluates (10 mM, 20 mM, 75 mM, and 150 mM NaOH, respectively); Lane 9: purified streptavidin [exists as a tetramer with a combined molecular weight of approximately 60 kDa that can break into trimers (~45 kDa, *), dimers (~30 kDa, *1) or monomers (~15 kDa, *2) under denaturing conditions]. (C) Comparison of the purity of ssDNA eluted from different types of streptavidin-conjugated Dynabeads using 20 mM NaOH. All of the eluate was electrophoresed on a 3% agarose gel in 1× TAE buffer. Lane 1: 50 bp molecular weight marker; Lanes 2–5: MyOne C1, MyOne T1, M-270, and M-280 Dynabeads, respectively; Lane 6: 0.625 μg of unamplified ssDNA aptamer library; Lane 7: 0.5 μg of PCR-amplified dsDNA aptamer. Results are representative of two independent experiments. (D) Quantitative comparison of dsDNA contaminants in ssDNA eluates at different NaOH concentrations: 10 mM, 20 mM, 75 mM, and 150 mM for Dynabeads and Sera-Mag beads using the ssDNA/dsDNA band densitometric intensity ratio. Each data point represents the average of three independent experiments ± standard error of the means (SEM). A detailed experimental protocol is included in the Supplementary Material.

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the differences in NaOH effects remains to be determined. While an 82-mer aptamer was used in this study, it is important to note that results can differ based on variables such as the size of the study molecule and biotinylation procedure. Thus, the optimal NaOH concentration required for the generation of quality ssDNA should be empirically determined for each experimental setup. We hope our observations will serve as a caution and guide for other researchers in their search for a streptavidin magnetic bead elution protocol that produces high-quality ssDNA.

Author contributions

G.K.K. conceived the idea for the study. G.K.K. and L.T. performed the experiments. C.K. supervised the project. G.K.K. and C.K. analyzed data and wrote the manuscript.

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Competing interests

The authors declare no competing financial interests. Reference to any commercial materials, equipment, or process does not, in any way, constitute approval, endorsement, or recommendation by the U.S. Food and Drug Administration.

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