A Simple One-Pot Method for Generating Circular Single-Stranded DNA (cssDNA) from Plasmid DNA

Optimization of nicking endonuclease selection, site orientation, and exonuclease reaction conditions

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INTRODUCTION

Circular single-stranded DNA (cssDNA) has emerged as a valuable reagent across a broad range of biotechnology applications, including structural nanotechnology, synthetic biology, gene delivery and genome engineering, and molecular diagnostics. The absence of free DNA ends, coupled with enhanced nuclease resistance and structural flexibility, makes cssDNA an attractive substrate for both in vitro and in vivo technologies. Peer-reviewed studies have demonstrated the utility of cssDNA as scaffolds for DNA origami and nanostructure assembly, switchable gene-expression vectors, templates for rolling circle amplification (RCA)-based bioanalysis, and homology-directed repair (HDR) templates for CRISPR-mediated genome editing [1–4]. As these applications expand, there is growing demand for robust and scalable methods capable of producing high-quality cssDNA across a wide range of sequence lengths.

Several approaches have been described for generating single-stranded DNA, including chemical oligonucleotide synthesis, asymmetric PCR (aPCR), phagemid-based production, and rolling circle amplification (RCA). While effective in specific contexts, these methods can be limited by sequence length, scalability, cost, product heterogeneity, or the requirement for specialized reagents and workflows. These limitations become increasingly significant when producing multi-kilobase cssDNA molecules, which are often required for nanotechnology and genome engineering applications [5,6]. Consequently, enzymatic conversion of readily accessible double-stranded plasmid DNA into cssDNA has become an attractive alternative.

A particularly straightforward strategy employs site-specific nicking endonucleases in combination with exonucleases. In this approach, a nicking enzyme introduces a single-strand break at a defined recognition site, generating a free DNA end while preserving the circular topology of the plasmid. Subsequent treatment with a double-stranded DNA-directed exonuclease degrades the nicked strand while leaving the complementary circular strand intact. Depending on the orientation of the nicking site, either the top or bottom strand can be selectively removed to generate the desired cssDNA molecule [7,8].

MATERIALS

Substrate DNA:

- pUC19 (NEB #N3041)
- pBR322 (NEB #N3033)
- ΦX174 RF I (NEB #N3021)

Nicking enzymes:

- Nb.BbvCI (NEB #R0631)
- Nt.BbvCI (NEB #R0632)
- Nb.BsmI (NEB #R0706)
- Nb.BtsI (NEB #R0707)
- Nt.BspQI (NEB #R0644)
- Nb.BssSI (NEB #R0681)

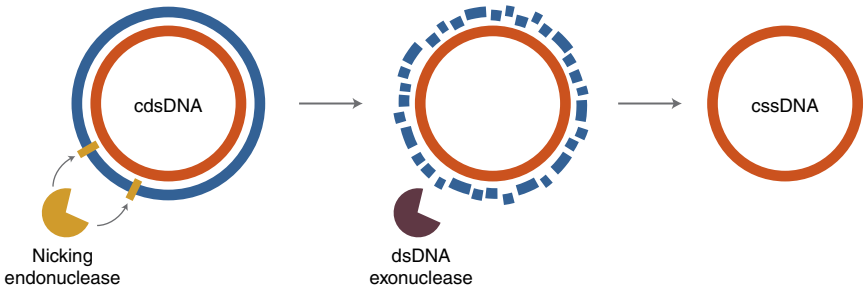
Exonucleases:

- Exonuclease III (NEB #M0206)
- T7 Exonuclease (NEB #M0263)
- Exonuclease I (NEB #M0293)

Reaction buffers:

- NEBuffer™ r3.1 (NEB #B6003)
- rCutSmart™ (NEB #B6004)

FIGURE 1: **Overview of nicking endonuclease and exonuclease-mediated generation of cssDNA**



A nicking endonuclease introduces a strand-specific nick into circular double-stranded DNA, after which a double-stranded DNA-directed exonuclease selectively degrades the nicked strand to generate circular single-stranded DNA.

Several exonucleases have been reported for this workflow, including Exonuclease III (NEB #M0206), a 3′→5′ exonuclease that digests duplex DNA from nicks, blunt ends, and recessed 3′ termini, and T7 Exonuclease (NEB #M0263), a highly processive 5′→3′ exonuclease with activity on double-stranded DNA substrates [7,9,10]. Although the general approach has been described previously, systematic comparisons of nicking enzymes, exonuclease selection, substrate design, and reaction conditions remain limited.

In this application note, we establish an efficient workflow for cssDNA generation from circular double-stranded plasmid DNA using nicking enzymes and exonucleases. We systematically evaluated three plasmid substrates, six nicking enzymes, and two exonucleases across a range of

reaction conditions to identify optimal enzyme pairings and incubation parameters for efficient conversion of plasmid dsDNA to cssDNA. The resulting workflow provides a simple, scalable, method for producing cssDNA suitable for downstream research applications.

METHODS

To establish a robust workflow for generating circular single-stranded DNA (cssDNA) from circular double-stranded plasmid DNA, we systematically evaluated key factors influencing nicking endonuclease-dependent strand degradation. Specifically, we examined (1) the performance of multiple nicking enzymes across different plasmid substrates, (2) the effect of nicking-site copy number, (3) the impact of plasmid size ranging from 2.7 to 5.4 kb, (4) optimal nicking endonuclease-to-exonuclease ratios, and (5) the effect of enzyme addition order on cssDNA production. This experimental framework was designed to identify conditions that maximize conversion efficiency while minimizing unwanted double-stranded DNA degradation and was informed by previously reported nicking endonuclease-initiated strand-removal strategies [7,8].

Because successful cssDNA generation depends on selective degradation of only one DNA strand, careful nicking endonuclease selection and substrate design were critical considerations. The engineering and application of strand-specific nicking endonuclease derived from BbvCI and BspQI have previously been well described [7,8], and an expanded panel of nicking enzymes was evaluated. Plasmid substrates were analyzed in silico using Benchling to confirm nicking-site orientation and predict the strand targeted by each enzyme. This analysis helped ensure single strand degradation and avoid nicking events on opposite strands, which can result in unintended double-strand cleavage and loss of the desired circular substrate [7].

To identify the most effective strand-removal strategy, multiple exonucleases with distinct substrate preferences and directionalities were tested. Exonuclease III (NEB #M0206), a 3′→5′ exonuclease active on double-stranded DNA, and T7 Exonuclease (NEB #M0263), a highly processive 5′→3′ exonuclease, were evaluated for their ability to degrade the nicked strand following nicking endonuclease treatment [9,10]. Exonuclease I (NEB #M0293), which specifically degrades single-stranded DNA from free 3′ termini, was used as a control to assess product circularity [11].

Reaction products were analyzed by agarose gel electrophoresis to monitor conversion of plasmid dsDNA to cssDNA and to evaluate product purity. Putative cssDNA products were subsequently challenged with Exonuclease I. Because circular ssDNA lacks a free 3′ end, it is resistant to Exonuclease I digestion, whereas linear ssDNA is rapidly degraded. Resistance to Exonuclease I treatment therefore served as an additional confirmation of successful cssDNA formation and maintenance of product circularity [11].

RESULTS

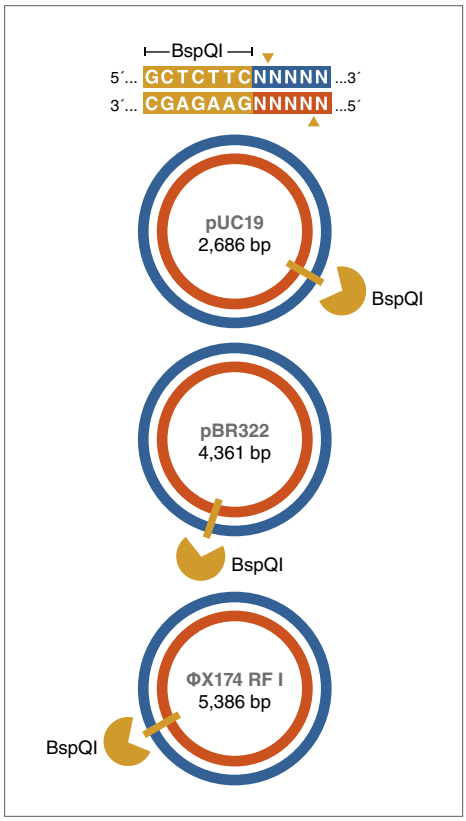
Nt.BspQI Enables Efficient cssDNA Generation Across Multiple Plasmid Substrates

To evaluate the feasibility of generating circular single-stranded DNA (cssDNA) from plasmid DNA using a nicking endonuclease/exonuclease workflow, three plasmid substrates containing a single BspQI recognition site were tested (Figure 2). Nt.BspQI (NEB #R0644) was selected as an initial nicking enzyme because it exhibits well-characterized strand specificity and because each plasmid contained a single appropriately oriented recognition site, simplifying downstream strand-selective degradation [8]. Because all three substrates contain a single Nt.BspQI site, we used Nt.BspQI and Exonuclease III as a model enzyme pair to demonstrate the principles of one-pot cssDNA generation using a nicking enzyme and exonuclease.

Following nicking with Nt.BspQI and treatment with Exonuclease III (NEB #M0206), a distinct DNA species consistent with cssDNA was observed for all three plasmids (Figure 3, page 3). Conversion of supercoiled plasmid DNA to nicked intermediates and subsequently to cssDNA was readily monitored by agarose gel electrophoresis. Restriction digestion with BspQI generated linearized products, while treatment with Nt.BspQI alone produced the expected nicked circular intermediates. Addition of Exonuclease III resulted in efficient removal of the nicked strand and accumulation of the cssDNA product.

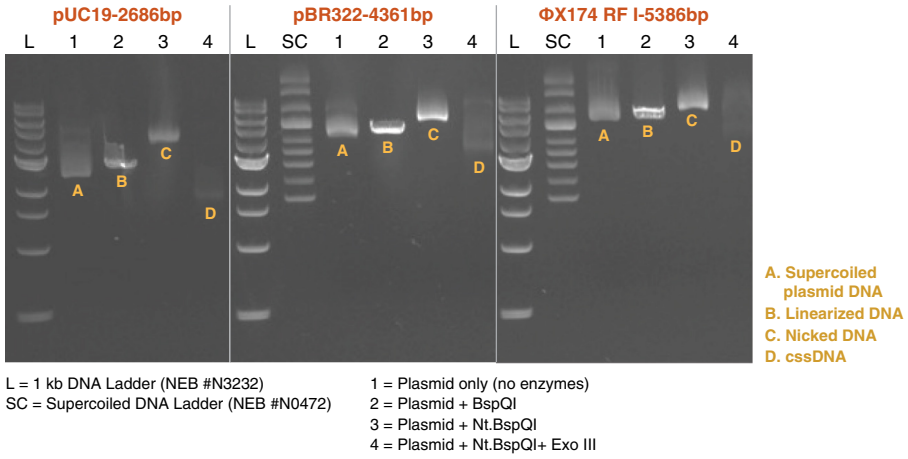
These results demonstrate that Nt.BspQI is broadly compatible with enzymatic cssDNA generation and can be applied to multiple plasmid substrates without substantial protocol modification.

FIGURE 2: **Plasmid maps showing the location and orientation of the single BspQI recognition site within each tested plasmid**



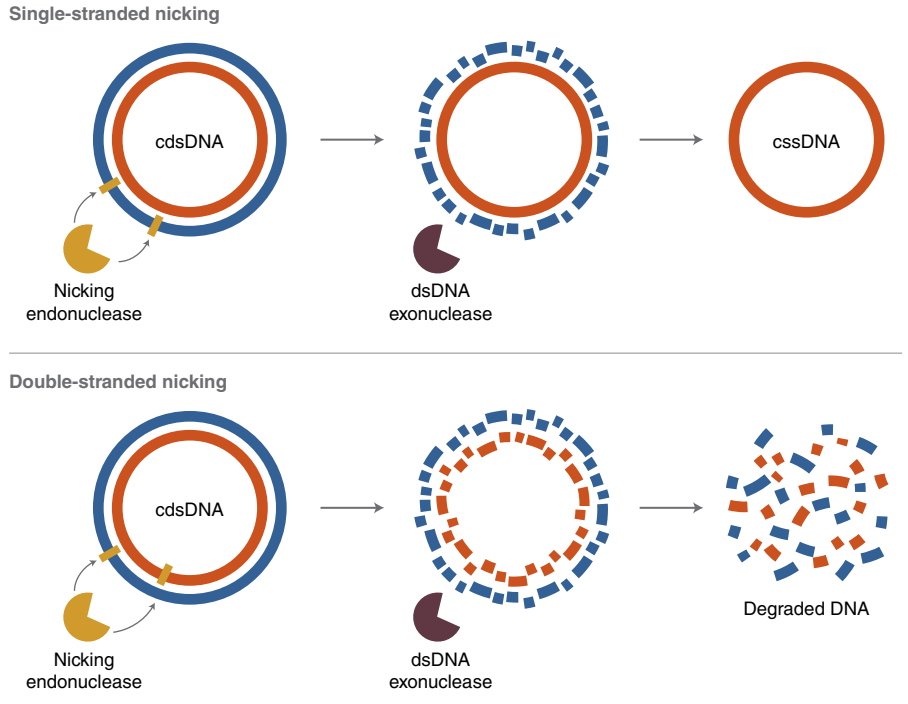
Plasmid maps showing the location and orientation of the single BspQI recognition site in pUC19, pBR322, and ΦX174 RF I.

FIGURE 3: **Generation of cssDNA from three plasmid substrates using Nt.BspQI and Exonuclease III**



pUC19, pBR322, and ΦX174 RF I were treated with Nt.BspQI and Exonuclease III, and reaction products were analyzed by agarose gel electrophoresis. Lane 1, plasmid only; lane 2, plasmid treated with BspQI; lane 3, plasmid treated with Nt.BspQI; lane 4, plasmid treated with Nt.BspQI and Exonuclease III. L, 1 kb DNA Ladder; SC, Supercoiled DNA Ladder. A, supercoiled plasmid DNA; B, linearized DNA; C, nicked plasmid DNA; D, putative cssDNA.

FIGURE 4: **Effect of nicking-site orientation on cssDNA generation**



Nicking sites oriented to target the same DNA strand (top) create a substrate that directs strand-specific nicking of a single DNA strand, creating a substrate for dsDNA-specific exonuclease digestion and efficient production of circular single-stranded DNA (cssDNA). In contrast, nicking sites that target opposite strands (bottom) generate nicks on both DNA strands, resulting in double-stranded DNA cleavage. Subsequent exonuclease treatment degrades the resulting DNA fragments rather than producing the desired cssDNA product.

Nicking Site Orientation Is More Important Than Nicking Site Number

Because exonuclease-mediated strand degradation requires a defined nicked strand, the orientation of nicking sites was evaluated as a critical design parameter. Previous reports have shown that nicking sites that target opposite strands can lead to nicking of both strands and unintended double-stranded DNA cleavage (Figure 4) [7].

Consistent with these observations, in-silico analysis was used to verify nicking-site strand orientation for all plasmid constructs prior to experimentation. Within the plasmids evaluated, the presence of multiple nicking sites did not improve cssDNA generation efficiency relative to substrates containing a single site. Instead, the primary consideration was ensuring that all recognition sites directed degradation of the same DNA strand. Constructs containing correctly oriented nick sites generated cssDNA efficiently, whereas additional nicking sites provided little measurable benefit (data not shown).

These findings suggest that plasmid design should prioritize correct nick orientation over increasing nick-site copy number.

Plasmid Length Has Minimal Impact on cssDNA Production

To determine whether plasmid size influences enzymatic cssDNA generation, substrates ranging from approximately 2.7 to 5.4 kb were examined.

Across this size range, comparable conversion of plasmid DNA to cssDNA was observed (Figure 3). No substantial differences in reaction efficiency or product formation were detected, indicating that modest differences in plasmid length do not significantly affect strand degradation under these conditions within the confines of the qualitative methods used in this study.

These observations are consistent with previous reports suggesting that exonuclease access to the nicked strand and proper nicking-site strand placement are more important determinants of cssDNA production than plasmid size itself [5].

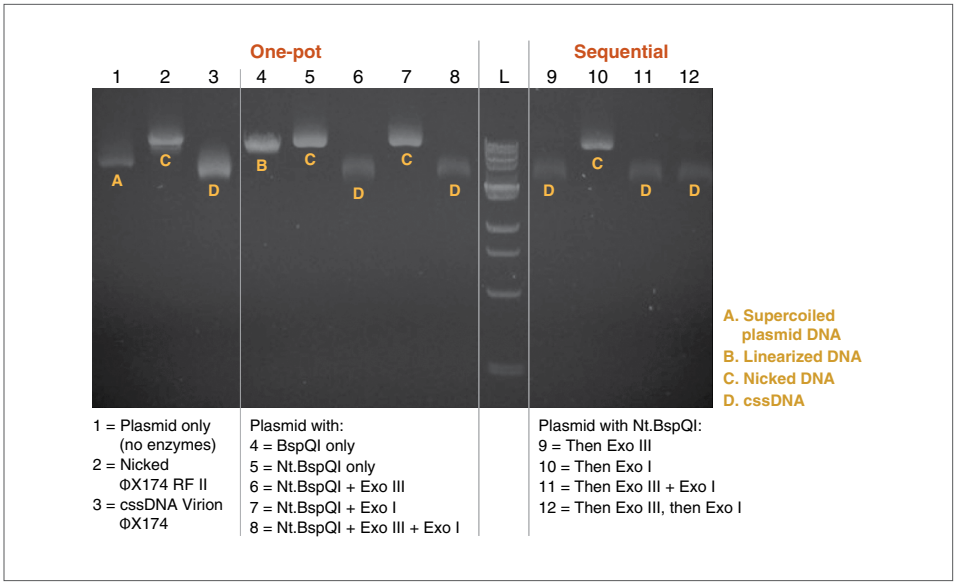
Exonuclease Requirements Depend on Reaction Buffer Conditions

Efficient strand degradation required optimization of exonuclease concentration relative to the reaction buffer used for nicking endonuclease activity. Because nicking enzymes possess different buffer preferences, exonuclease performance was evaluated under multiple buffer conditions.

Exonuclease III demonstrated robust activity in both rCutSmart Buffer (NEB #B6004) and NEBuffer r3.1 (NEB #B6003), although substantially different enzyme concentrations were required to achieve equivalent strand degradation. Reactions performed in rCutSmart Buffer required only 10 units of Exonuclease III, whereas reactions in NEBuffer r3.1 required more units for comparable conversion to cssDNA.

These results highlight the importance of considering reaction buffer composition when selecting exonuclease loading and emphasize that optimal exonuclease concentrations should be determined relative to the nicking endonuclease reaction conditions.

FIGURE 5: **Comparison of one-pot and sequential cssDNA generation workflows using Nt.BspQI and Exonuclease III**



Reaction products generated by simultaneous or sequential treatment with Nt.BspQI and Exonuclease III were analysed by agarose gel electrophoresis alongside the indicated controls. cssDNA was observed with both reaction formats. Lane identities and band assignments are indicated below the gel.

One-Pot and Sequential Workflows Produce Comparable Results

To simplify workflow design, simultaneous enzyme addition was compared with sequential nicking endonuclease and exonuclease treatments using Nt.BspQI and Exonuclease III. Reactions were analyzed alongside control substrates, including nicked ΦX174 RF II DNA (NEB #N3022L) and cssDNA ΦX174 virion DNA (NEB #N3023L) (Figure 5).

Comparable cssDNA products were observed whether enzymes were added sequentially or together in a one-pot reaction format. No substantial improvement in yield or product quality was observed with sequential processing. These findings suggest that exonuclease activity does not significantly interfere with nicking and that both reaction formats can successfully generate cssDNA.

The ability to perform the reaction in a single tube simplifies the workflow and reduces hands-on time while maintaining efficient cssDNA production.

Exonuclease I Confirms Product Circularity

To verify that the reaction products were circular rather than linear single-stranded DNA, samples were challenged with Exonuclease I (NEB #M0293). Exonuclease I degrades single-stranded DNA possessing a free 3′ terminus but does not digest circular ssDNA molecules lacking accessible ends [11].

The cssDNA products generated by the nicking endonuclease/exonuclease workflow remained resistant to Exonuclease I treatment (Figure 5), while appropriate linear ssDNA controls were degraded (data not shown). This resistance confirms that the products retained a circular topology and demonstrates successful conversion of double-stranded plasmid DNA into circular single-stranded DNA.

CONCLUSION

The results presented here demonstrate that circular single-stranded DNA (cssDNA) can be generated efficiently from circular double-stranded plasmid DNA using a simple workflow that combines strand-specific nicking enzymes with double-stranded DNA-directed exonucleases. Across multiple plasmid substrates and reaction conditions, the approach consistently produced cssDNA with minimal workflow complexity, highlighting its utility as an accessible alternative to methods such as asymmetric PCR, rolling circle amplification, or phage-based production systems.

Among the parameters evaluated, nicking-site orientation emerged as the single most important determinant of successful cssDNA production. Because exonuclease-mediated strand degradation depends on generating a defined entry point on only one DNA strand, improper orientation of multiple nicking sites can result in nicking of both strands and subsequent double-strand DNA cleavage, eliminating the desired circular substrate. These observations are consistent with previous reports and underscore the importance of verifying nicking endonuclease, recognition site orientation during plasmid design [7,8]. In contrast, increasing the number of nicking sites provided little benefit when all sites were properly oriented (data not shown), suggesting that careful construct design is more important than maximizing nick frequency.

Nt.BspQI (NEB #R0644) proved convenient for cssDNA generation with the chosen substrates, since each substrate had only one BspQI recognition site. The enzyme demonstrated consistent performance across all plasmids tested and enabled efficient strand-selective degradation when paired with Exonuclease III (NEB #M0206).

Reaction conditions also influenced cssDNA generation efficiency. In particular, optimal exonuclease loading depended on the reaction buffer selected for nicking endonuclease, endonuclease, activity. Exonuclease III remained effective under multiple buffer conditions, although substantially different enzyme concentrations were required to achieve comparable strand degradation. These findings are consistent with the known effects of buffer composition, ionic strength, and pH on exonuclease activity and emphasize the importance of optimizing exonuclease concentration within the context of the chosen nicking endonuclease, reaction conditions [9,10].

Notably, one-pot reactions produced cssDNA with efficiency comparable to sequential nicking endonuclease, and exonuclease additions. The ability to combine enzymatic steps into a single reaction simplifies the workflow, reduces hands-on time, and minimizes sample handling without compromising performance. For most applications, a one-pot approach represents the most practical implementation of the method.

Finally, Exonuclease I resistance provided an additional layer of confidence that the resulting products retained their circular topology. Because Exonuclease I degrades linear ssDNA but not circular ssDNA lacking a free 3′ terminus, the observed resistance supports successful conversion from circular dsDNA to cssDNA.

Collectively, these results establish strand-specific nicking followed by exonuclease digestion as a rapid, robust, and scalable method for cssDNA production. By selecting an appropriate nicking enzyme, verifying recognition-site orientation in silico, and optimizing exonuclease loading for the chosen reaction conditions, researchers can readily generate cssDNA from standard plasmid templates for a variety of downstream applications. Given the growing use of cssDNA in DNA nanotechnology, genome engineering, synthetic biology, and RCA-based diagnostics, this workflow provides a practical route for generating custom cssDNA molecules from readily available plasmid DNA.

Future Directions

While this study focused on plasmids ranging from approximately 2.7 to 5.4 kb, future work should explore performance across larger constructs, where processivity and reaction kinetics may become increasingly important. Additional studies examining scalability, including larger reaction volumes and higher DNA inputs, would further establish the utility of this approach for applications requiring substantial quantities of cssDNA.

Future optimization efforts could also evaluate post-reaction cleanup strategies and product purification methods to maximize cssDNA recovery and purity. Finally, while Exonuclease I resistance provides a convenient confirmation of circularity, complementary characterization approaches such as Nuclease P1/Duplex DNase analysis, atomic force microscopy (AFM), electron microscopy (EM), or next-generation sequencing-based assessments could provide additional confirmation of product identity and structural integrity. Such studies would further expand the utility of this workflow and support its adoption across emerging cssDNA applications.

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