SureVector Cloning and Protein Expression

Application Note

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Abstract

SureVector is a state-of-the-art NextGen cloning system to rapidly design and build your own custom plasmids. The SureVector cloning method allows multiple DNA modules to be combined together into a recombinant plasmid that contains your target gene of interest (GOI) and any additional features you want to express. Our main goal was to clone and overexpress a protein of interest. First, we used the SureVector system to create a plasmid containing our gene of interest (pSV). Second, we transformed the plasmid into *E. coli* BL21(DE3) cells, and produced our recombinant protein of interest fused to a 6xHis tag. Finally, we purified our protein of interest with a His-Trap column. In addition, we were also interested in removing the 6xHis tag. To achieve this goal, we quickly created another plasmid, SureVectorThrombin (pSVT) using the SureVector system. To do this, we introduced a thrombin cleavage site between the 6xHis sequence and the gene of interest by modifying one of the original PCR primers, to enable us to enzymatically remove the 6xHis tag after protein purification. Our plasmids were created with the following SureVector DNA modules: a promoter-tag fusion (T7-His6), a selection marker (Kanamycin resistance), an origin of replication (pUC), an XP1 fragment (XP1), an XP2 fragment (lactose inhibitor) and our gene of interest.

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Gene Amplification with Overlapping Sequence

The gene of interest was amplified by PCR to append the appropriate overlapping sequences of the adjacent DNA modules. We performed a PCR amplification with the designed downstream and upstream primers which included 30 nucleotides of overlapping sequence as recommended by the SureVector User Manual, and an additional 16 nucleotides of the gene of interest (Table 1). The first three nucleotides of the upstream primer that complement the gene of interest encode the first amino acid codon for the gene of interest. In the downstream primer, the end of the region that complements the gene of interest includes a stop codon.

In order to create a second unique plasmid, containing a thrombin cleavage site, we designed a different upstream primer, replacing the last 13 nucleotides of the 30 nucleotides of SureVector overlapping sequence that codify glycine residues with the thrombin cleavage site sequence (CTGGTGCCGCGCGGCAGC), while keeping the remaining 17 nucleotides overlapping the gene of interest (Table 1).

Thus, we performed two different PCR reactions: one to amplify the gene of interest with overlapping sequences that the SureVector manual recommended (1), and a second to amplify the gene of interest with a thrombin cleavage site (2). The PCR product was purified and the DNA checked for the correct size using an agarose gel (Fig.1).

Assembly Reaction and Transformation

We next carried out the SureVector assembly reaction, creating our two plasmids: pSV and pSVT. We followed the SureVector protocol as recommended in the User Manual, using a thermal cycler. Each reaction was incubated with DpnI for 5 minutes at 37°C. Subsequently, we performed transformation reactions with 45µL of XL10-Gold Supercompetent Cells, and plated samples of each transformation onto LB-agar plates containing kanamycin, which were then incubated at 37°C overnight.

After 16 hours, we examined the plates for colonies. A lot of colonies grew on the pSV plate (Fig. 2), and a smaller number grew on the pSVT plate where the gene of interest included the thrombin cleavage site (Fig.3). One possible reason for this could be the decreased length of overlapping sequence in the upstream primer. In the future,

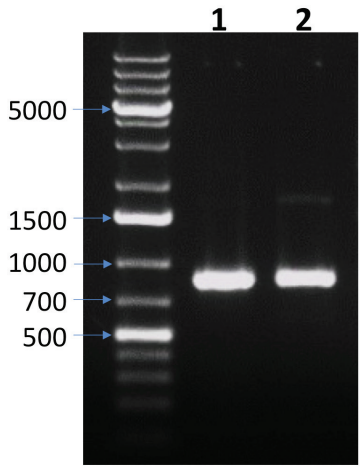

Figure 1. Agarose gel analysis of PCR amplification products. Lane 0: molecular weight markers; lane 1: PCR product of the GOI for pSV; lane 2: PCR product of the GOI with thrombin cleavage site for pSVT.

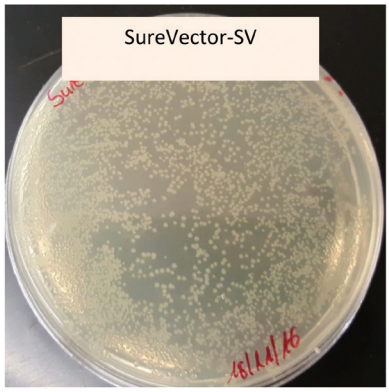

Figure 2. Transformation plate containing pSV (SureVector with gene of interest) colonies.

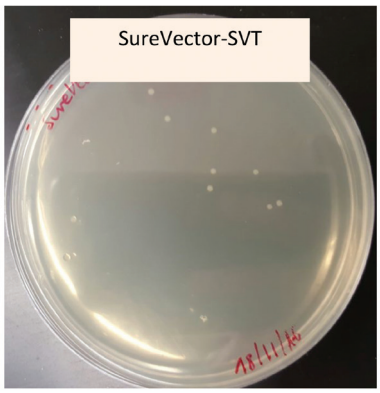

Figure 3. Transformation plate containing pSVT (SureVector with gene of interest and thrombin cleavage site) colonies.

SureVector (pSV)	Upstream primer	5′ GGTGGCGGAGGTTCTGGAGGCGGTGGAAGT-GOI 3′
	Downstream primer	5′ CTCGAGGAGATATTGTACACTAAACCAAATG-GOI 3′
SureVectorThrombin modified (pSVT)	Upstream primer	5′ GGTGGCGGAGGTTCTGGCCTGGTGCCGCGCGGCAGC-GOI 3′
	Downstream primer	5′ CTCGAGGAGATATTGTACACTAAACCAAATG-GOI 3′

Table 1. Gene of interest PCR Primer Overlap Sequences

higher amounts of DNA could be used in the assembly reaction in order to get more colonies. Nevertheless, the colonies obtained in this experiment were all positive.

We next randomly selected colonies from each assembly for testing and verification by restriction digest. Three single colonies from the pSV plate and another three single colonies from the pSVT plate were picked and expanded in 5ml of LB medium supplemented with 100ng/µL of Kanamycin. Plasmid DNA was purified by miniprep, and 100ng/µl of each sample, SureVector plasmid (pSV) and SureVector Thrombin plasmid (pSVT), were separated on a 0.7% agarose gel to check their quality (Fig.4).

Plasmid Verification

To verify that each vector contained our gene of interest, plasmid DNA was cut with a single site restriction enzyme, AsiSI. The anticipated vector digest would result in a single band of 5198bp. A second restriction analysis was performed using two restriction enzymes simultaneously, BamHI and HindIII. This would result in three bands of 1250bp, 1489bp, and 2459bp. We used both BamHI and HindIII because BamHI cuts inside our gene of interest and HindIII cuts in the vector backbone, generating 3 specific DNA fragments (Fig. 5). We ran 200ng of each DNA product on an agarose gel, and the sizes of the fragments obtained were as expected (Fig. 6). Therefore, all six DNA plasmids were correct and the efficiency of the system was very good. Lastly, pSV-3 and pSVT-3 composition were further confirmed by Sanger DNA sequencing.

Protein Expression

Both plasmids, pSV and pSVT, were transformed into *E.coli* BL21(DE3) cells to express the protein of interest. The expression of the protein of interest is controlled by IPTG induction, and the pilot assay included further purification using Ni-NTA, a nickel-charged affinity resin that specifically recognizes the 6XHis-tag. Transformed cells were grown in 100 ml LB medium at 37ºC with 180 rpm shaking. When the OD = 0.8, the cells were induced with IPTG (final concentration 0.5 mM) and the cells further incubated at 25ºC and 140 rpm for 6 hours. To check the production of protein, samples were taken at 0 hours (control, no IPTG induction), 2 hours, 4 hours and 6 hours (20 ml of cells were harvested by centrifugation at 4500 rpm for 15 minutes and washed with PBS). These cells were resuspended in lysis buffer containing protease inhibitors (10 mM benzamidine, 1 mM PMSF, 10 µg/ml trypsin inhibitor). The cells were lysed and centrifuged at 14,000 rpm for 30 min at 4 ºC. The supernatants were incubated with Ni-NTA resin, and the resin was washed two times. Finally, the bound protein was

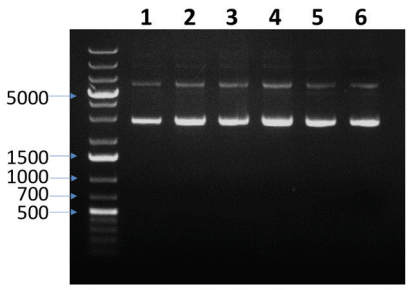

Figure 4. Agarose gel analysis of plasmids. Lane 0: molecular weight markers; lane 1: pSV-1; lane 2: pSV-2; lane 3: pSV-3; lane 4: pSVT-1; lane 5: pSVT-2; lane 6: pSVT-3.

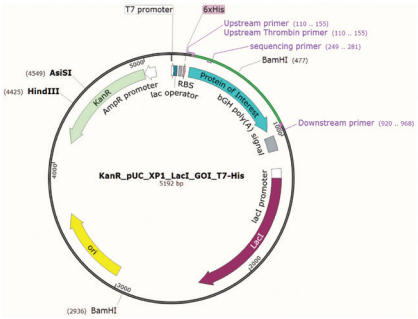

Figure 5. Plasmid map showing upstream and downstream PCR primer sites, sequencing primer sites, and restriction sites. Vector map made with SnapGene Viewer.

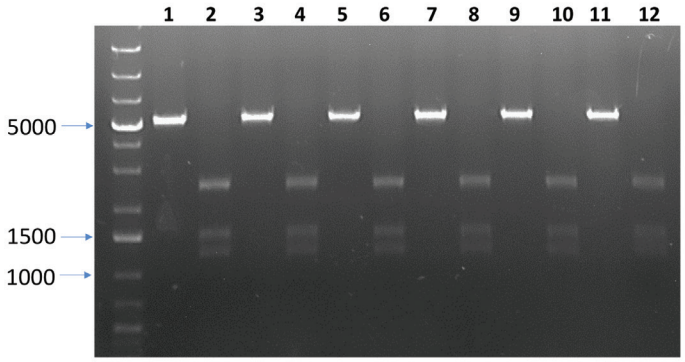

Figure 6. Agarose gel analysis of plasmids digested with restriction enzymes. Lane 0: molecular weight markers; Lane 1: pSV-1 digested with AsiSI; lane 2: pSV-1 digested with BamHI and HindIII; lane 3: pSV-2 digested with AsiSI; lane 4: pSV-2 digested with BamHI and HindIII; lane 5: pSV-3 digested with AsiSI; lane 6: pSV-3 digested with BamHI and HindIII; lane 7: pSVT-1 digested with AsiSI; lane 8: pSVT-1 digested with BamHI and HindIII; lane 9: pSVT-2 digested with AsiSI; lane 10: pSVT-2 digested with BamHI and HindIII; lane 11: pSVT-3 digested with AsiSI; lane 12: pSVT-3 digested with BamHI and HindIII.

eluted with elution buffer containing 500 mM imidazole. Our protein of interest with the thrombin cleavage site was further processed by a 1h thrombin incubation to remove the 6xHis tag. The expected sizes of the target proteins are 32.51kDa with the 6xHis tag, and 30.85kDa after thrombin cleavage to remove the 6xHis tag. Equal amounts of each protein sample was loaded onto a 17% SDS-PAGE. Our results show a highly purified protein of the expected molecular weight, with maximal expression 6h after IPTG induction (Fig. 7). Furthermore, the pSVT plasmid containing the thrombin cleavage site was shown to work very well, as the amount of protein was slightly higher and the tag can be completely removed with thrombin incubation (Fig. 8).

Conclusions

We can conclude that the SureVector system is a good choice to make your own custom plasmids in an easy and quick way. Furthermore, it can be adapted to individual needs as demonstrated by the inclusion of a thrombin cleavage site in one of our vector assemblies. In addition, we have obtained a significant amount of our protein of interest enabling us to directly continue our research.

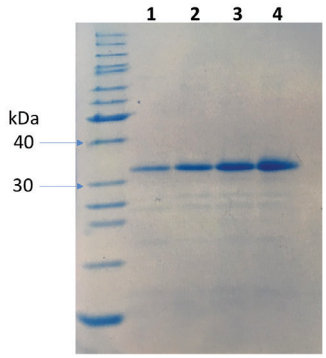

Figure 7. SDS-PAGE analysis of pSV protein expression. Lane 0: molecular weight markers; lane1: pSV-3 (Control, 0 hours IPTG induction); lane 2: pSV-3 (2 hours IPTG induction); lane 3 pSV-3 (4 hours IPTG induction); lane 4: pSV-3 (6 hours IPTG induction). Note that the control (no IPTG, time 0 hours) normally expresses a small amount of the protein of interest due to the high basal activity levels of the T7 RNA polymerase of the bacterial strain BL21(DE3). Expected size of target protein: 32.51kDa with 6xHis tag.

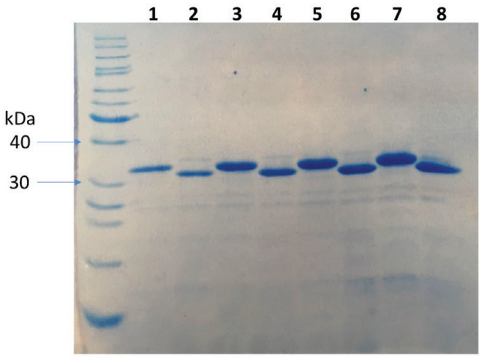

Figure 8. SDS-PAGE analysis of pSVT protein expression. Lane 0: molecular weight markers; lane1: pSVT-3 (Control, 0 hours IPTG induction, before thrombin cleavage); lane 2: pSVT-3 (Control, 0 hours IPTG induction, after thrombin cleavage); lane 3: pSVT-3 (2 hours IPTG induction, before thrombin cleavage); lane 4: pSVT-3 (2 hours IPTG induction, after thrombin cleavage); lane 5: pSVT-3 (4 hours IPTG induction, before thrombin cleavage); lane 6: pSVT-3 (4 hours IPTG induction, after thrombin cleavage); lane 7: pSVT-3 (6 hours IPTG induction, before thrombin cleavage); lane 8: pSVT-3 (6 hours IPTG induction, after thrombin cleavage). Note that the control (no IPTG, time 0 hours) normally expresses a small amount of the protein of interest due to the high basal activity levels of the T7 RNA polymerase of the bacterial strain BL21(DE3). Expected sizes of target proteins: 32.51kDa with 6xHis tag, 30.85kDa without 6xHis tag after thrombin cleavage.

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