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RESEARCH ON THE PRODUCTION OF MRNA ENCODING SLEEPING BEAUTY TRANSPOSASE IN CAR-T CELL ENGINEERING

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ABSTRACT

Objectives: *To produce mRNA Encoding Sleeping Beauty Transposase from the DNA Template Plasmid pSB100X.*

Subjects and methods: *The gene encoding the Sleeping Beauty (SB) transposase protein was amplified from the DNA template plasmid pSB100X using PCR. The PCR product was then directly purified using the QIAquick PCR Purification Kit (Qiagen). In vitro transcription of mRNA was performed using T7 polymerase (New England Biolabs) with optimized incubation times of 2 hours, 6 hours, and 15 hours, and varying enzyme concentrations of 10 U, 30 U, and 100 U to determine optimal conditions. The final mRNA product was purified and subjected to Sanger sequencing at Macrogen (Seoul, South Korea).*

Results: *In vitro transcription for 2 hours produced a significantly higher yield of mRNA encoding the SB100X protein compared to reactions conducted for 30 minutes or 1 hour ($p < 0.05$). The resulting mRNA appeared as a distinct, specific band corresponding to an approximate size of 1,200 bp. The optimal amount of T7 polymerase for each transcription reaction was determined to be 30 U. After purification, the transcribed mRNA was subjected to Sanger sequencing at Macrogen (Seoul, South Korea), yielding a specific band of approximately 1,200 bp with a sequence that was completely accurate.*

Conclusion: *mRNA encoding the SB transposase was successfully produced with high purity and correct sequence, using 30 U of enzyme after 15 hours of in vitro transcription.*

Keywords: mRNA, Sleeping Beauty transposase, plasmid pSB100X.

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1. INTRODUCTIONS

The use of T lymphocytes engineered to express chimeric antigen receptors (CARs) to direct the immune response against cancer cells, known as CAR T cell immunotherapy, has shown remarkable efficacy in patients with refractory B-cell malignancies [1]. In this therapy, T cells are isolated from the patient and genetically modified to express anti-CD19 CARs, enabling targeted treatment of pediatric patients with acute B-cell lymphoblastic leukemia and B-cell lymphomas.

Recently, regulatory agencies in the United States, the European Union, and Japan have approved and commercialized CAR-T cell therapies targeting CD19, marking a significant milestone in the application of cell and gene therapies in human healthcare [2]. CAR-T cell manufacturing processes are being designed to meet increasing demand while reducing production time and cost. Among these approaches, the Sleeping Beauty (SB) transposon system represents a promising method for genetic modification of T lymphocytes. In this context, plasmid DNA is commonly used as a substrate for gene transfer into cells, either via viral vectors or transposon-transposase systems [3].

Transposons are dual genetic elements consisting of one plasmid carrying the gene of interest and another plasmid encoding the transposase enzyme. However, the persistence of the plasmid expressing transposase in transfected cells can lead to undesired homologous recombination events, potentially resulting in physiological alterations of the cells [4]. To mitigate this issue, mRNA encoding the transposase can be used in transfection procedures. In addition to eliminating the need for transcription-thus enabling rapid protein expression following transfection-mRNA does not enter the cell nucleus. Monjezi and

colleagues (2017) were pioneers in applying mRNA and minicircle DNA for the generation of CAR-T cells [5].

Based on the above rationale, we conducted this study with the objective of producing mRNA encoding SB transposase as an alternative to plasmid DNA substrates in transfection procedures for CAR-T cell generation.

2. SUBJECTS AND METHODS

2.1. Subjects

- Subjects: the pSB100X vector; primer pairs synthesized by Macrogen (South Korea), including primers for gene amplification, clone screening, and sequencing (Table 1).

Table 1. Sequences of primer pairs used in the study

No.	Primer Name	Sequence (5'-3')	Purpose
1	T7Pro-SB100-V40pAN-F	CGGAATTAATACGACTCACTATAGG	Amplification of SB100 gene
2	T7Pro-SB100-V40pAN-R	TGTTGTTAACTTGTTTATTGCAGC	Amplification and sequencing of SB100 gene
3	SB100-seq-F	GGGACTAGTACCATGGGAAA	Sequencing of SB100 gene
4	ybbW-401-F	TGATTGGCAAAATCTGGCCG	Amplification of *E. coli* ybbW gene
5	ybbW-611-R	GAAATCGCCCAAATCGCCAT	

- Equipment: thermal cycler for 96 samples (Eppendorf, Germany); gel imaging system (Gensnap, USA); horizontal mini-DNA electrophoresis apparatus (CBS Scientific, USA); NanoDrop 2000 spectrophotometer (Thermo Fisher, USA); thermoshaker (Eppendorf, USA); centrifuge (Eppendorf, Germany); vortex mixer (Rotolab, OSI); various micropipettes (Gilson, France; Biohit, Finland).

- Reagents and chemicals: PCR product purification kits: QIAquick PCR Purification Kit (Qiagen); RNA purification kit: RNeasy Mini Kit (Qiagen). Electrophoresis reagents: agarose, Tris-base, acetic acid, EDTA, High Ranger 1kb DNA Ladder (Norgen). Enzymes: GoTaq® DNA Polymerase, SuperScript III RT/Platinum Taq Mix, T7 RNA Polymerase, RNase Inhibitor.

2.2. Methods

- Method for amplifying the gene encoding SB100 protein: the gene encoding the SB100 protein was amplified from the DNA template plasmid pSB100X, following the PCR reagent composition and protocol recommended by the manufacturer. The pSB100X vector and primer pairs were provided by Macrogen (South Korea), including primers for gene amplification, clone screening, and sequencing.

- Direct purification of PCR products: The PCR product was directly purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The purified product was subsequently used as the template for in vitro transcription.

- In vitro transcription method for mRNA synthesis:

In vitro transcription of mRNA was carried out using T7 polymerase according to the manufacturer's instructions (New England Biolabs). The reaction time was optimized at 2 hours, 6 hours, and 15 hours. Additionally, the amount of T7 polymerase used was optimized at 10 U, 30 U, and 100 U. The incubation durations and enzyme concentrations for each transcription reaction were evaluated and optimized using RT-PCR, with reaction components listed in Table 2, following the manufacturer's protocol.

Table 2. RT-PCR reaction components

Component	Volume
2X Reaction Mix	25 µl
Forward primer SB100-seq-F 10 µM	1 µl
Reverse primer T7Pro-SB100-SV40pAN-R 10 µM	1 µl
SuperScript III RT/Platinum Taq Mix	1 µl
RNA	5 µl
Water	Up to 25 µl

- Data analysis: statistical analyses were performed using SPSS version 26.0. Variables are presented as mean and standard deviation (SD). The Kruskal–Wallis test and Mann–Whitney U test were used to assess differences between two or more independent groups with non-normal distributions. $p < 0.05$ was considered statistically significant.

Table 3. PCR primer reaction mix for experimental use

Component	Volume
5X Colorless GoTaq Reaction Buffer	5 µl
MgCl2 25 mM	2 µl
dNTPs 10 mM	0.5 µl
GoTaq 5 U/µl	0.125 µl
Forward primer T7Pro-SB100-SV40pAN-F 10 µM	0.5 µl
Reverse primer T7Pro-SB100-SV40pAN-R 10 µM	0.5 µl
DNA	5 µl
Water	Up to 25 µl

3. RESULTS

- PCR amplification results of the gene encoding SB100 protein:

The gene region encoding the SB100 protein was amplified by PCR as described in the Methods section. The results showed a clear, distinct band of approximately 1,200 bp, consistent with the theoretical size. No nonspecific products were observed (Figure 1). This indicates that the PCR product was of high purity and suitable for direct use in subsequent *in vitro* transcription reactions.

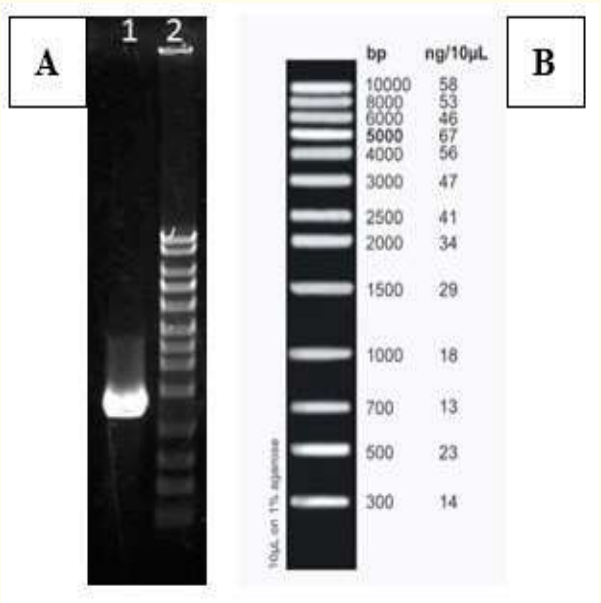

Figure 1. A: PCR amplification result of the gene encoding SB100 protein (1: PCR product of the SB100 coding gene; 2: HighRanger 1 kb DNA Ladder – Norgen). B: Size reference scale of the HighRanger 1 kb DNA Ladder (Norgen).

- *In vitro* transcription results of SB100 mRNA:

mRNA was synthesized through an in vitro transcription reaction using T7 polymerase. The RNA electrophoresis results after the reaction showed that, following 2 hours of *in vitro* transcription, a specific product band was observed, corresponding to the theoretical size of approximately 1,200 bp.

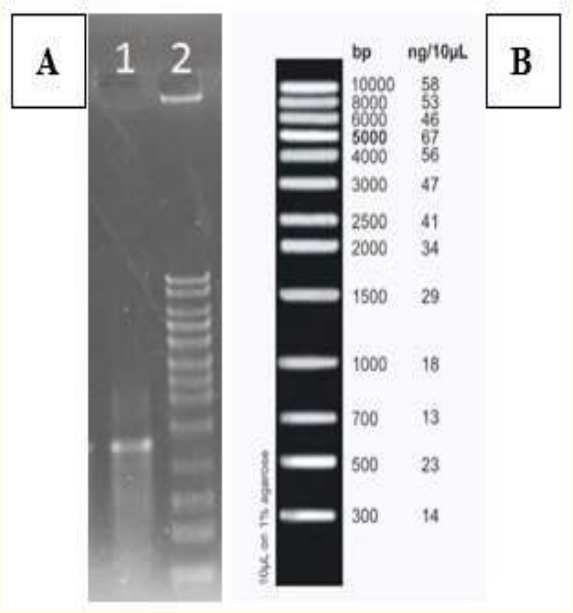

Figure 2. A: In vitro transcription result of SB100 mRNA after 15 hours (1: In vitro transcribed SB100 mRNA; 2: HighRanger 1 kb DNA Ladder - Norgen). B: Size reference scale of the HighRanger 1 kb DNA Ladder (Norgen).

Table 4. Optimization results of *in vitro* transcription duration for mRNA expressing SB100X protein

RNA/DNA Yield (µg/µg)	Time		
	0.5 hour(1)	1 hour(2)	2 hour(3)
Trial 1	14.4	22.0	31.2
Trial 2	15.1	22.9	31.1
Trial 3	15.0	21.3	30.6
± SD	14.83 ± 0.38	22.07 ± 0.80	30.97 ± 0.32
p	$p_{1-3} < 0.05$; $p_{2-3} < 0.05$		

The results of the transcription time optimization experiment showed that the 2-hour in vitro transcription produced a significantly higher yield compared to the 30-minute and 1-hour reactions ($p < 0.05$).

The amount of T7 polymerase used per reaction was optimized. The results indicated that transcription efficiency reached saturation at an enzyme concentration of 30 U per reaction.

Table 5. Optimization results of T7 polymerase enzyme amount used for 1 *in vitro* transcription reaction

RNA/DNA Yield (µg/µg)	T7 Polymerase amount (µg/µg)		
	10U	30U	100U
Trial 1	22.4	31.2	31.2
Trial 2	24.1	32.5	31.4
Trial 3	23.1	31.7	32.5
± SD	23.20 ± 0.85	31.80 ± 0.66	31.7 ± 0.14

- RT-PCR analysis results of *in vitro* transcribed SB100 mRNA:

After purification, the in vitro transcribed product was used as a template for RT-PCR using the specific primer pair SB100-seq-F and T7Pro-SB100-SV40pAN-R. The result showed a distinct, specific band of approximately 1,200 bp.

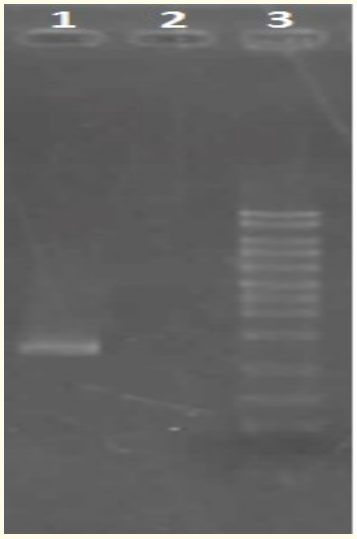

Figure 3. RT-PCR analysis of in vitro transcribed SB100 mRNA. (1: RT-PCR product of in vitro transcribed SB100 mRNA; 2: Negative RT-PCR control; 3: HighRanger 1 kb DNA Ladder -Norgen).

- Sequencing results of the SB100 RT-PCR product:

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T7Pro-SB100-SV40pAN-R ←  ------------------------------------------------------------ 60
SB100-seq-F           →  GGGACTAGTACCATGGGAAAATCAAAAGAAATCAGCCAAGACCTCAGAAAAAGAATTGTA 60
SB100                    GGGACTAGTACCATGGGAAAATCAAAAGAAATCAGCCAAGACCTCAGAAAAAGAATTGTA 60
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ------------------------------------------------------------ 120
SB100-seq-F           →  GACCTCCACAAGTCTGGTTCATCCTTGGGAGCAATTTCCAAACGCCTGGCGGTACCACGT 120
SB100                    GACCTCCACAAGTCTGGTTCATCCTTGGGAGCAATTTCCAAACGCCTGGCGGTACCACGT 120
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ------------------------------------------------------------ 180
SB100-seq-F           →  TCATCTGTACAAACAATAGTACGCAAGTATAAACACCATGGGACCACGCAGCCGTCATAC 180
SB100                    TCATCTGTACAAACAATAGTACGCAAGTATAAACACCATGGGACCACGCAGCCGTCATAC 180
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ------------------------------------------------------------ 240
SB100-seq-F           →  CGCTCAGGAAGGAGACGCGTTCTGTCTCCTAGAGATGAACGTACTTTGGTGCGAAAAGTG 240
SB100                    CGCTCAGGAAGGAGACGCGTTCTGTCTCCTAGAGATGAACGTACTTTGGTGCGAAAAGTG 240
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ------------------------------------------------------------ 300
SB100-seq-F           →  CAAATCAATCCCAGAACAACAGCAAAGGACCTTGTGAAGATGCTGGAGGAAACAGGTACA 300
SB100                    CAAATCAATCCCAGAACAACAGCAAAGGACCTTGTGAAGATGCTGGAGGAAACAGGTACA 300
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ------------------------------------------------------------ 360
SB100-seq-F           →  AAAGTATCTATATCCACAGTAAAACGAGTCCTATATCGACATAACCTGAAAGGCCACTCA 360
SB100                    AAAGTATCTATATCCACAGTAAAACGAGTCCTATATCGACATAACCTGAAAGGCCACTCA 360
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ------------------------------------------CCAGACTACGGTTTGCAACT 420
SB100-seq-F           →  GCAAGGAAGAAGCCACTGCTCCAAAACCGACATAAGAAAGCCAGACTACGGTTTGCAACT 420
SB100                    GCAAGGAAGAAGCCACTGCTCCAAAACCGACATAAGAAAGCCAGACTACGGTTTGCAACT 420
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  GCACATGGGGACAAAGATCGTACTTTTTGGAGAAATGTCCTCTGGTCTGATGAAACAAAA 480
SB100-seq-F           →  GCACATGGGGACAAAGATCGTACTTTTTGGAGAAATGTCCTCTGGTCTGATGAAACAAAA 480
SB100                    GCACATGGGGACAAAGATCGTACTTTTTGGAGAAATGTCCTCTGGTCTGATGAAACAAAA 480
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  ATAGAACTGTTTGGCCATAATGACCATCGTTATGTTTGGAGGAAGAAGGGGGAGGCTTGC 540
SB100-seq-F           →  ATAGAACTGTTTGGCCATAATGACCATCGTTATGTTTGGAGGAAGAAGGGGGAGGCTTGC 540
SB100                    ATAGAACTGTTTGGCCATAATGACCATCGTTATGTTTGGAGGAAGAAGGGGGAGGCTTGC 540
                         ************************************************************

T7Pro-SB100-SV40pAN-R ←  AAGCCGAAGAACACCATCCCAACCGTGAAGCACGGGGGTGGCAGCATCATGTTGTGGGGG 600
SB100-seq-F           →  AAGCCGAAGAACACCATCCCAACCGTGAAGCACGGGGGTGGCAGCATCATGTTGTGGGGG 600
SB100                    AAGCCGAAGAACACCATCCCAACCGTGAAGCACGGGGGTGGCAGCATCATGTTGTGGGGG 600
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Figure 4. Sequencing results of the SB100 RT-PCR product.

After direct purification, the PCR product was sequenced at Macrogen (Seoul, South Korea). The sequencing results confirmed that the obtained mRNA had a completely accurate sequence (Figure 4).

4. DISCUSSIONS

The transposition process in the Sleeping Beauty (SB) system involves a cut-and-paste mechanism, in which the transposon element moves from one DNA molecule to another. Integration of the transposon into the host chromosome forms the basis for the stable maintenance of transgenes in genetically modified cells and organisms. In 2009, Mátés et al. described an mRNA encoding a hyperactive variant of the SB transposase [6]. In 2011, Jin Z et al. referenced SB100 mRNA in a study, suggesting that it is responsible for encoding the SB transposase [7].

The study results showed that the gene region encoding the SB100 protein, amplified by PCR, produced a clear and distinct band of approximately 1,200 bp, consistent with the theoretical size, with no nonspecific products observed. This indicates that the PCR product was of high purity and suitable for direct use in in vitro transcription reactions. mRNA was synthesized through in vitro transcription using T7 polymerase. RNA electrophoresis results revealed a specific product band consistent with the theoretical size (approximately 1,200 bp). Optimization of transcription duration showed that the 2-hour reaction produced a significantly higher yield compared to the 30-minute and 1-hour reactions ($p < 0.05$). Furthermore, the amount of T7

polymerase used per reaction was also optimized. The results indicated that transcription efficiency plateaued at an enzyme concentration of 30 U per reaction. However, the in vitro transcription efficiency observed in this study did not reach the level reported by the manufacturer (New England Biolabs, E2040S). This discrepancy may be due to the nature of the DNA template, which can significantly affect the reverse transcription efficiency of T7 RNA polymerase [8, 9]. After direct purification, the PCR product was sequenced at Macrogen (Seoul, South Korea), and the sequencing results confirmed that the obtained mRNA had a completely accurate sequence.

Multiple studies have demonstrated that mRNA can serve as an effective alternative to DNA as a source of SB transposase targeting cells and tissues in the body. Although RNA may undergo reverse transcription followed by cDNA integration, this occurrence is rare, making RNA a safer alternative to DNA as a transposase source for gene therapy applications. These findings are consistent with the study by Wilber A et al. (2006), which proposed the laboratory production of mRNA as a potential future source of transposase [10].

5. CONCLUSIONS

The study successfully produced mRNA encoding SB transposase using 30 U of enzyme after 15 hours of in vitro transcription. The final mRNA preparation was free of contaminating DNA and was verified by RT-PCR and Sanger sequencing, confirming that the sequence was accurate and consistent with the original design.

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