3.2. DNA-Template Construction for Transcription

Cap and poly A (pA), found on almost all eukaryotic mRNAs, stimulate translation initiation and stabilize mRNA by their synergistic action. However, for in vitro translation the use of cap and pA can be a major problem *(4)*. This has been effectively overcome by inserting optimized 5'- and 3'-untranslated regions (UTRs) upstream and downstream from the coding regions *(4)*. Optimized 5'- and 3'-UTRs can be fused to the gene of interest in two different ways: (1) by cloning the same gene in a plasmid vector carrying the proper UTRs and then amplifying the gene attached to 5'- and 3'-UTRs from the plasmid, and (2) by fusing the 5'- and 3'-UTRs during the amplification reaction from cDNAs. The optimized elements are assembled into a pSP65-derived vector called pEU **(Fig. 1A,B)**. By using this assembled vector, the desired genes can be expressed efficiently without cap and poly(dA/dT) *(4)*. Cloning of the desired gene into the pEU3b expression system is achieved by using standard methods. After confirming the DNA sequence, the plasmid containing the desired ORF is prepared by either the CsCl method *(5)* or by using small-scale plasmid preparation kits. Prior to mRNA synthesis, recombinant plasmid should be treated with phenol-chloroform to eliminate any RNases activity.

3.2.1. Template Preparation From pEU Carrying Optimized 5'- and 3'- UTRs

We have optimized a PCR-based linear template DNA preparation by designing a set of universal primers for pEU vector. The designed primers are SPu (5'- GCGTAGCATTTAGGTGACACT) and AODA2303 (5'- GTCAGACCCCGTAGAAAAGA). By using these primers, the desired DNA template can be easily generated and used directly for the preparation of mRNA without further purification as follows.

1. Prepare 100 µL of PCR reaction mixture as per the manufacturer's instructions (Takara) by mixing 100 pg/µL of plasmid and 200 n*M* of each primer, 200 µ*M* of each dNTP, and 1.25 U of ExTaq DNA polymerase.
2. Set the PCR thermo-cycler (Takara) on 1 min denaturation at 96°C followed by 25 cycles of amplification: 98°C for 10 s, 55°C for 30 s, and 72°C for 5 min depending on the length of the ORF (1 kb per min).

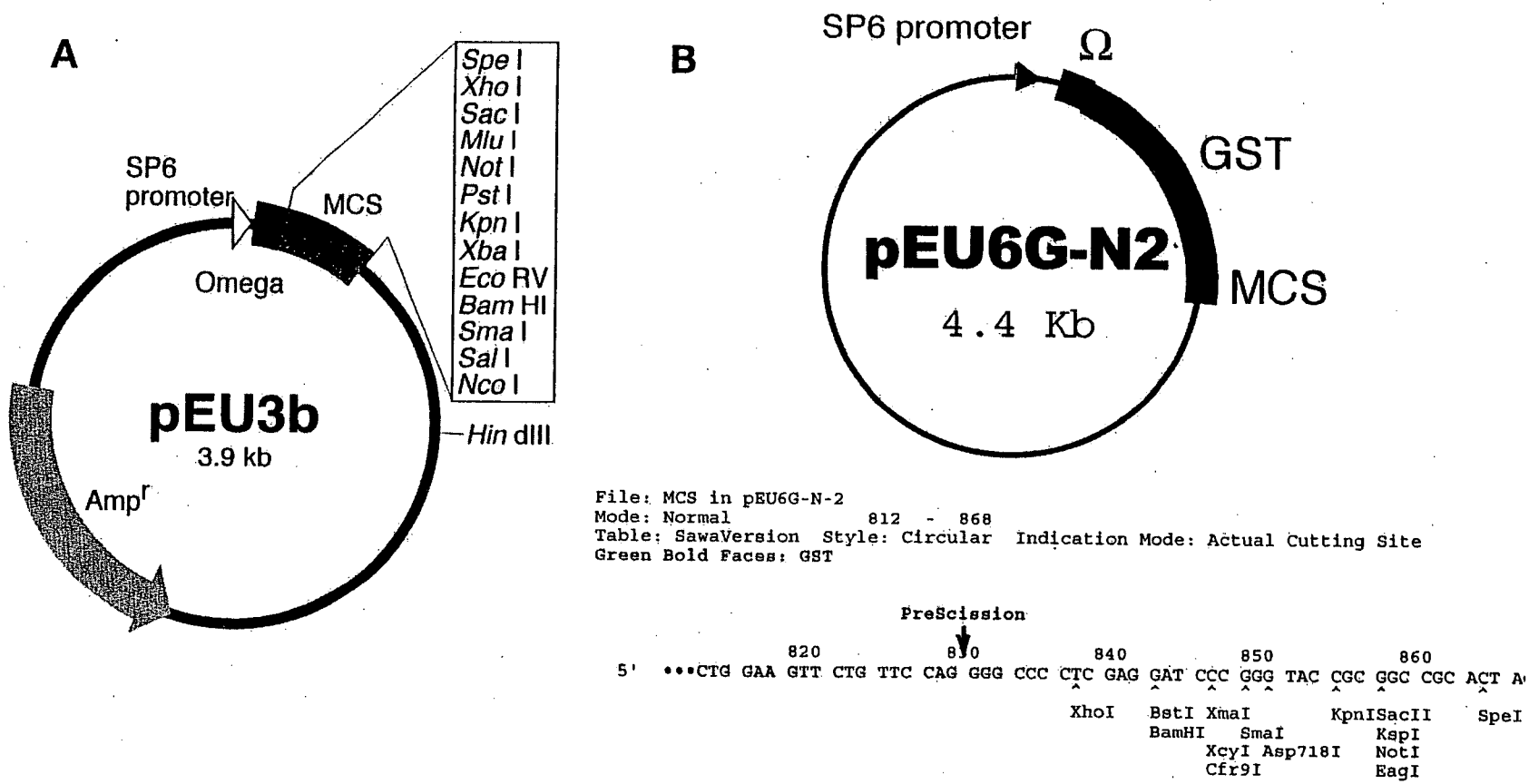

Fig. 1. (**A**) Schematic diagram of the pEU expression vector. (**B**) Map and sequence in multicloning site of pEU6G-N2 for GST-tag purification.

3. Concentrate the PCR product to approx 20 µL (~5 times) using Microcon according to the manufacturer's instructions.
4. After analyzing the integrity of DNA template by agarose gel electrophoresis, the template DNA is used for the transcription.

3.2.2. Direct Fusion of 5'- and 3-UTRs by Split-Primers PCR Technique

Because cloning of cDNA into an expression vector is one of the most laborious and time-consuming steps of the entire process, direct synthesis of the template by PCR is preferred *(6,7)*. This is achieved by the incorporation of transcriptional and translational start and stop signals into primers used for the amplification of the gene of interest. In this section, we focus on the construction of template DNA starting from cDNA clones by using the split-primers PCR technique *(4)*. The strategy is illustrated in **Fig. 2**.

3.2.2.1. Design of Primers

As shown in **Fig. 2**, split-primers PCR technique makes use of four primers:

1. The target specific primer (primer 3) is designed in such a way that its 3'-end can anneal to the 5'-end of the target gene and its 5'-end has part of the omega sequence *(4)*.
2. Primer 2 has the full-length omega sequence, thus allowing the annealing to the primer 3-derived amplification product and a part of the SP6 promoter sequence.
3. Primer 1 has the remaining part of the SP6 promoter and can anneal to primer 2-derived amplification product.
4. Primer 4 is specific for the gene-carrying vector and can anneal several hundreds of basepairs downstream from the 3'-end of the gene of interest.

Therefore, for each clone of cDNA, primer 3 is the only specific primer and the remaining primers are common for all cDNAs. The sequences of the promers used are reported below:

Primer 1: 5'-GCGTAGCATTTAGGTGACACT (the underlined sequence is the 5' half of the promoter).

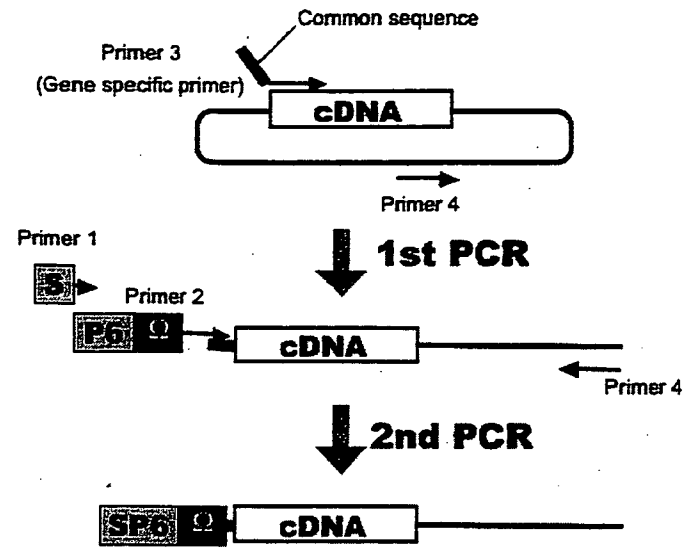

Fig. 2. Direct transcriptional template generation from cDNA library by using the split-primers PCR technique. A schematic representation of the split-primers design for equipping the cDNA sequences with the required UTRs.

Primer 2: 5'-*GGTGACACT*ATAGAAGTATTTTTACAACAATTAC CAACAACAACAACAAACAACAACAACATTACATTTTACATTCT ACAACTA*CCACCCACCACCACCAATG* (underlined is the 3'-half sequence of the promoter and the sequences in italic denote the region annealing to primer 1 and primer 3).

Primer 3: 5'-CCACCCACCACCACCAatgnnnnnnnnnnnnnnnn (the stretch of "ns" indicates the nucleotide sequence which is specific for the gene of interest).

Primer 4: 5'-AGCGTCAGACCCCGTAGAAA (based on the sequence of the vector plasmid described in **refs. *9–11***).

3.2.2.2. First PCR

1. Prepare 60 µL of a PCR reaction by mixing 3 ng of plasmid or 3 µL of an overnight culture of *Escherichia coli* carrying the plasmid, of dNTPs (200 µ*M* each), 1.5 U of ExTaq DNA polymerase, 10 n*M* of primer 3 and primer 4, and the polymerase buffer supplied by the manufacturer.

2. Set the PCR thermo-cycler on 4-min denaturation at 94°C followed by 30 cycles of amplification: 98°C for 10 s, 55°C for 30 s, and 72°C for 4–5 min, depending on the length of the gene (1 kb per min).

3.2.2.3. Second PCR

To attach the omega and SP6 promoter sequences to the first PCR product, a second 30 µL PCR was carried out by mixing 3 µL of the first PCR product (without any purification), 100 n*M* of primer 1 and 4, and 1 n*M* of primer 2 using the same amplification conditions as for the first PCR.

3.3. Sequential Transcription-Translation Reactions

Cell-free translation of proteins can be achieved mainly by three different modes, batch mode translation *(3,7)*, bilayer system *(8)*, and continuous-flow cell-free protein synthesis method *(4,9)*. Their basic concepts are described in our previous paper *(10)*. In this section, the bilayer mode of protein synthesis using unpurified mRNA will be explained in detail. **Table 1** summarizes the composition of the mixture, which varies depending on the amount of protein needed.

1. Prepare transcription reaction as indicated in **Table 1** except for wheat embryo extract and creatine kinase.
2. Incubate the reaction mixture at 37°C for 6 h.
3. Add 25 µL (250 µL for large scale [LS]) of wheat embryo extract, and 1 µL of 2 mg/mL creatine kinase (20 mg/mL creatine kinase for LS), and mix well (reaction mixture).
4. Prepare 550 µL (5.5 mL for LS) of TSB in the U-shaped titer plate well (six-well plate for LS).
5. Carefully transfer 50 µL (500 µL for LS) of the reaction mixture to the titer plate well by inserting the pipet tip down to the bottom of the well. Because of the higher density of the **step 3** mix compared with the **step 4** mix, one can clearly see two layers.
6. Place a sealing film and then a cover on the plate to avoid evaporation.
7. Keep the plate in an incubator at 26°C for 18 h without shaking.

Table 1
Reaction Mixture for Transcription and Translation

Reagen	Small scale (SS)	Large scale (LS)	Final concentration
Template DNA	*x* µL	*x* µL	1/5 vol (HC PCR)[a] 100 ng/µL (CP)[b]
5X TB	5	50	1X
25 m*M* NTP mix	2.5	25	2.5 m*M*
80 U/µL SP6 RNApolymerase	0.3	3	1 U/µL
80 U/µL RNasin	0.3	3	1 U/µL
Milli-Q	up to 24	up to 249	
Wheat embryo extract	25	250	120 A_{260}/mL
2 mg/mL creatine kinase	1	–	40 µg/mL
20 mg/mL creatine kinase	–	1	40 µg/mL

[a]Highly concentrated PCR product (>50 ng/mL).
[b]High quality circular plasmid.

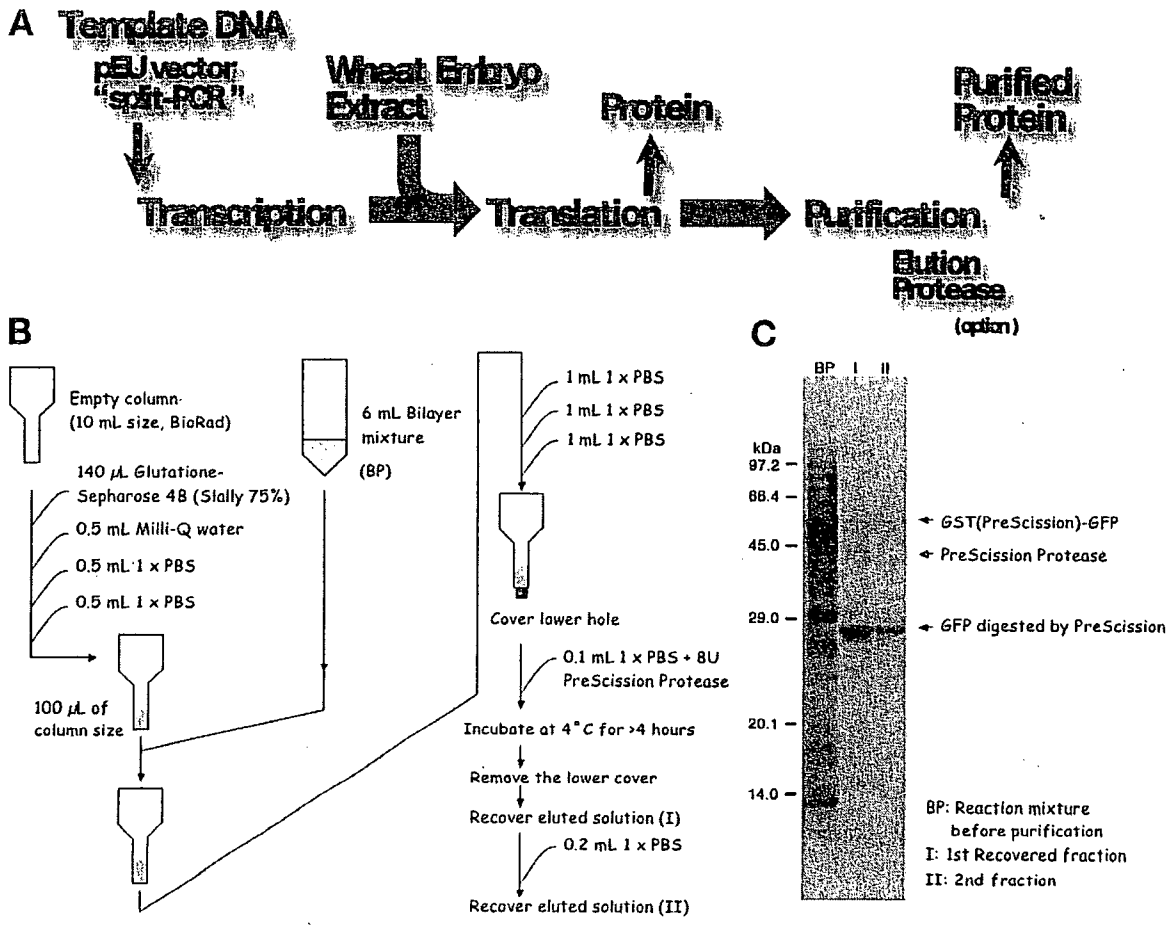

Fig. 3. (**A**) Flowchart of the cell-free production and purification process based on sequential transcription-translation reaction and purification. (**B**) Flowchart of protein purification by using GST-tag and on-column digestion with PreScission. (**C**) An example of purified protein.

3.4. Purification of Synthesized Protein by On-Column Digestion

This section describes the methods for (1) isolation of a GST-fused protein as obtained when a pEU-derived transcript is used (**Subheading 3.**), (2) removal of the GST moiety by PreScission protease, and (3) purification of the protein (*see* **Fig. 3B**,C).

1. Prepare the column (10 mL size) by adding 140 µL of glutathione-sepharose 4B resin.
2. Wash the column with 0.5 mL of Milli-Q water followed by 1 mL of 1X PBS.
3. Load 6 mL of the synthesized protein from **Subheading 3.3.** into the column.
4. Wash off the unbound proteins with 3 mL of 1X PBS.
5. Cap the bottom of the column.
6. Treat the column with 8 U PreScission protease in 0.1 mL of 1X PBS and then incubate at 4°C for 4 h.
7. Remove the cap and then elute the target protein without GST tag
8. Recover the target protein with 0.1 mL of 1X PBS one more time.

4. Notes

1. A possible procedure for the preparation of proteins can be:
   a. Selection and design of suitable genes from the data bank.
   b. Generation of template for the transcription by PCR, or by cloning into pEU.
   c. Transcription of mRNA.
   d. Translation in the wheat germ cell-free system.
   e. Fine tuning of translation conditions such as ion concentrations and incubation temperature for large-scale protein production.
   f. Purification of the product.

   Because the translation machinery prepared from wheat embryos are efficient and robust, we could in fact succeeded in the robotic automation of the processes from **steps c** through **f**.

References

1. Sawasaki, T., Hasegawa, Y., Morishita, R., Seki, M., Shinozaki, K., and Endo, Y. (2004) Genome-scale, biochemical annotation method based on the wheat germ cell-free protein synthesis system. *Phytochemistry* **65,** 1549–1555.
2. Vinarov, D. A., Lytle, B. L., Peterson, F. C., Tyler, E. M., Volkman, B. F., and Markley, J. L. (2004) Cell-free protein production and labeling protocol for NMR-based structural proteomics. *Nature Methods* **2,** 1–5.
3. Madin, K., Sawasaki, T., Ogasawara, T., and Endo, Y. (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. USA* **97,** 559–564.
4. Sawasaki, T., Ogasawara, T, Morishita, R., and Endo, Y. (2002) A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA* **99,** 14,652–14,657.
5. Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual, 3rd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6. Gurevich, V. V. (1996) Use of bacteriophage RNA polymerase in RNA synthesis. *Methods Enzymol.* **275,** 382–397.
7. Hanes, J. and Pluckthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA* **94,** 4937–4942.
8. Sawasaki, T., Hasegawa, Y., Tsuchimochi, M., et al. (2002) A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett.* **514,** 102–105.
9. Spirin, A. S., Baranov, V. I., Ryabova, L. A., Ovodov, S. Y., and Alakhov, Y. B. (1988) A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242,** 1162–1164.
10. Endo, Y. and Sawasaki, T. (2004) High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *J. Struct. Funct. Genomics* **5,** 45–57.