Taiyo Nippon Sanso Corporation
DN: SIIC-DN-0009
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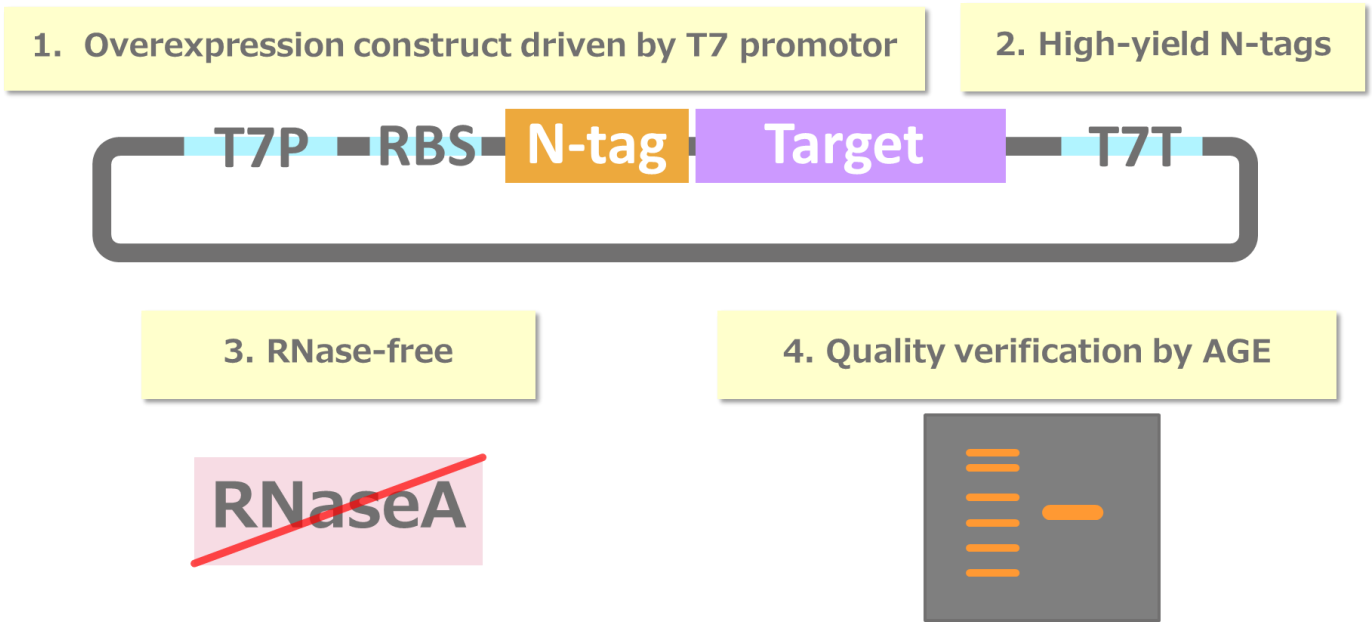
# How to prepare plasmid template DNA for Musaibo kun cell-free protein synthesis kit?

## Requirements for Plasmid Template DNA




The plasmid template DNA appropriate for Musaibo kun cell-free protein synthesis (CFPS) should possess the following characteristics:

**1. Overexpression construct driven by the T7 promotor**

Constructs engineered for the production of recombinant proteins within E. coli cells, regulated by the T7 RNA polymerase, can be employed. The design of the construct should encompass the T7 promoter, a ribosome binding sequence (RBS), the open reading frame (ORF) consisting of an N-terminal tag and the target protein, alongside the T7 terminator.

**2. Starting with high-yield N-terminal tag**

It is highly advisable to incorporate an N-terminal tag sequence known to enhance protein yield, as codons proximal to the translational start (spanning a few to several amino acids) significantly influence the overall protein yield.

**3. RNase-free**

The plasmid deployed for protein synthesis must be devoid of RNase contamination. Plasmid DNA extracted using commercially available preparation kits often retains traces of RNaseA, sufficient to impede protein synthesis reactions. A thorough elimination of residual RNaseA through re-purification is imperative.

**4. Quality Verification through Agarose Gel Electrophoresis (AGE)**

The quantification of plasmid concentration may frequently lead to overestimations due to impurities. It is recommended to confirm the appropriate intensity of the plasmid band through AGE.

# Protocol - Plasmid Construction, Preparation and CFPS Trial

This protocol outlines plasmid template DNA construction and protein expression trial.

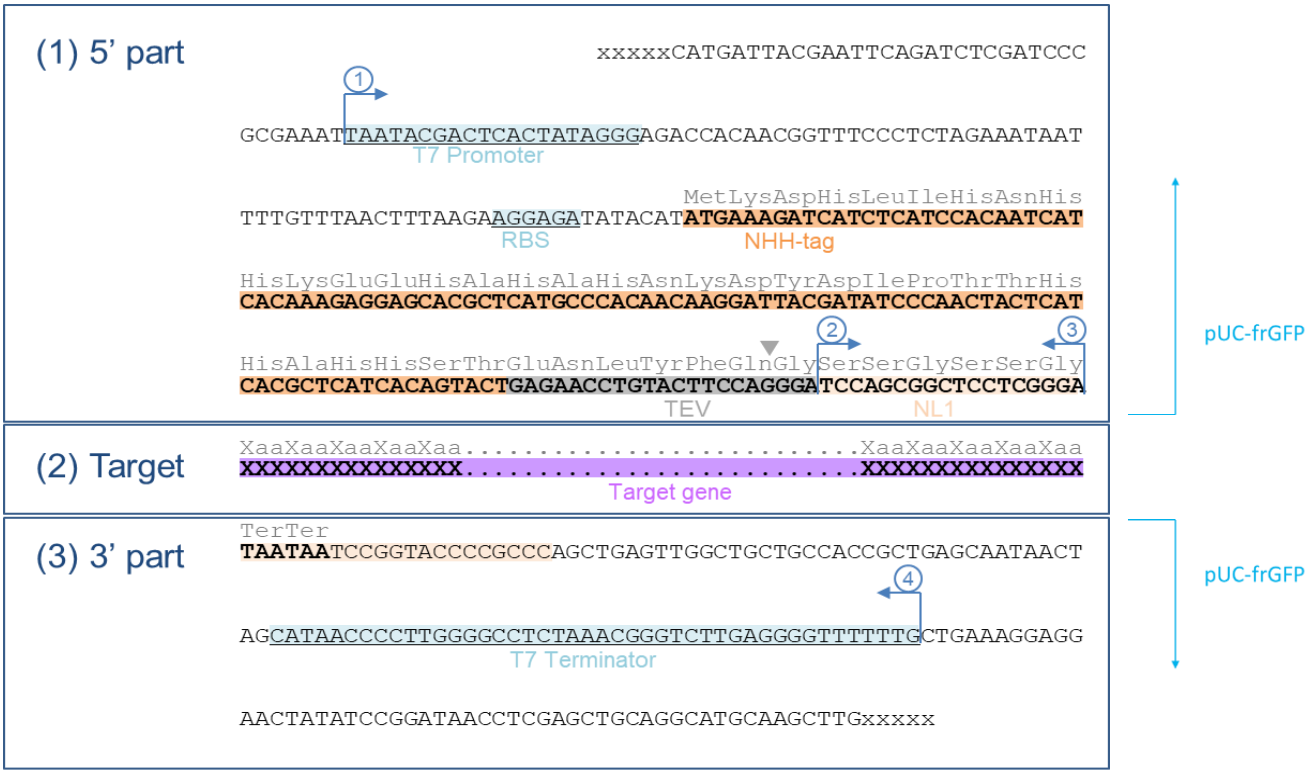
## 1. Design DNA Sequence and Prepare Plasmid

Plasmid template DNA can be prepared using either (a) kit control plasmid pUC-frGFP or (b) gene synthesis services.

### (a) Construction from control plasmid pUC-frGFP

This protocol generates an expression construct using pUC-frGFP (a control template DNA plasmid included in the Musaibo kun N series kits).

- Prepare gene* for the target protein.
  **Codon optimization of the gene is not always necessary, but codon optimization to E. coli strains can sometimes increase yield of the protein.*
- Replace frGFP coding sequence in the pUC-frGFP with the target gene sequence designed above using In-Fusion® HD cloning kit (Takara Bio) or similar to obtain the sequence below:




*Expression plasmid construct should contain the sequence from T7 promotor to T7 terminator (region 1–4).*

*NHH-tag: A High-yield N-terminal tag capable of His-tag affinity purification.*

*TEV: Recognition sequence by TEV protease to cleave the tag. Cleavage site is indicated by triangle.*

*NL1: Spacer sequence to improve tag cleavage efficiency. The spacer sequence (region 2–3) can be omitted but it sometimes lowers cleavage efficiency.*

– Confirm sequence.

– Prepare plasmid by commercially available Midiprep* kits.
**Preparation scale is on your choice. As a general guideline, 5 µg of plasmid sufficient for 1 mL of cell-free protein synthesis reaction, typically yielding 1 mg of protein, although actual protein yield can vary by proteins.*

**(b) Construction using whole gene synthesis services**

This protocol generates the complete DNA sequence spanning from the T7 promoter to the T7 terminator using gene synthesis services.

– Prepare the amino acid sequence for the target protein.
– Reverse-transcribe the amino acid sequence into an ORF DNA sequence, incorporating codon optimization to *E. coli* strains*.
**Codon optimization services, available through gene synthesis service enterprises, can be employed.*
– Append the subsequent 5'- and 3'-control sequences to the respective 5' and 3' ends of the ORF to achieve the complete construct. The complete construct corresponds to the sequence region 1–4 of 1(a) (region 2–3 omitted).

**5'-Control sequence:**
TAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAGATCATCTCATCCACAATCATCACAAAGAGGAGCACGCTCATGCCCACAACAAGGATTACGATATCCCAACTACTCATCACGCTCATCACAGTACTGAGAACCTGTACTTCCAGGGA (203 nt)
*This sequence consists of the T7 promotor, RBS, N-terminal high yield NHH tag (purifiable by His-tag affinity resins), TEV protease cleavage site. Underlined region is translated. Translation is* MKDHLIHNHHKEEHAHAHNKDYDIPTTHHAHHST***ENLYFQ/G***.
*Bold indicates TEV protease recognition site. Slash represents cleavage site by TEV protease.*

**3'-Control sequence:**
TAATAATCCGGTACCCCGCCCAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATAACCTCGAGCTGCAGGCATGCAAGCTTG (156 nt)
*This sequence consists of termination codons (underlined), a spacer and the T7 terminator.*
– Order gene synthesis of the complete construct prepared above. Optimal preparation scale can be midiprep*.
**Preparation scale is on your choice. As a general guideline, 5 µg of plasmid sufficient for 1 mL of cell-free protein synthesis reaction, typically yielding 1 mg of protein, although actual protein yield can vary by proteins.*

**2. Plasmid Re-purification**

To remove residual RNases, plasmid prepared by either 1(a) or 1(b) above should be re-purified prior to CFPS reaction.

– Purify obtained plasmid through phenol-chloroform-isoamyl alcohol (25:24:1) extraction, followed by chloroform extraction and ethanol precipitation.
– Quantify the concentration of the plasmid DNA through UV absorbance at 260 nm*.
** The yield after re-purification may sometimes be reduced to 50%, as the concentration data provided by gene synthesis manufacturers might be overestimated due to impurities.*
– Validate the integrity of the plasmid through AGE, ensuring a clearly visible band with consistent intensity.

## 3. Cell-Free Protein Synthesis Trial

– Employ Musaibo kun N100 or SI Start kit to conduct 100 µL-scale CFPS reaction following the instructions in the kit manual.

**Typical reaction conditions:**
Concentration of plasmid template DNA: 5 ng/µL*
** Template DNA concentration can be about 1 to 10 ng/µL. Protein yield somewhat varies by template concentration.*
Incubation: 30°C for 6 hours*
** Shorter incubation (2-4 hours) or lower incubation temperature (25°C) may improve quality (solubility or activity) of fragile proteins. Longer incubation (up to 16 hours) may increase yield for durable proteins.*

– Collect CFPS reaction solution (Total Fraction).

– Centrifuge at 15,000 x *g* for 1 min at 4°C to obtain supernatant (Supernatant Fraction).

– Prepare SDS-PAGE sample for the Total and Supernatant fractions with a 10–20 times dilution.

– Analyze the sample using PAGE, loading 10 µL per lane*.
**Usually, using 0.5–1 µL equivalent of reaction mixture per lane provides optimal visibility.*