Cat. # 6143

For Research Use

TaKaRa

Cloning Kit for mRNA Template

Product Manual

v202504Da

Table of Contents

I. Description

Cloning Kit for mRNA Template (Cat. #6143) is used for the construction of the template plasmid for *in vitro* transcription (IVT) using cap analogs [CleanCap Reagent AG or CleanCap Reagent AG (3′ OMe)] from TriLink BioTechnologies. The pre-linearized vector in the kit contains a T7 promoter, transcription start sequence (AGG), 5′-UTR (untranslated region), 3′-UTR, and a 105-base Poly(A) sequence, which make it possible to construct a IVT template plasmid for IVT using CleanCap Reagent AG for efficient translation in human, murine, or other mammalian cells. CleanCap Reagent AG is a co-transcriptional capping reagent for the capping of mRNA, creating a Cap 1 structure. This kit can be used to construct IVT template plasmid containing the desired gene (coding sequence; CDS) by In-Fusion® cloning (Fig. 1, Fig. 2).

The template plasmid constructed using the kit can then be used to produce the desired mRNA by performing high-yield *in vitro* transcription using Takara IVTpro™ T7 mRNA Synthesis Kit (Cat. #6144).

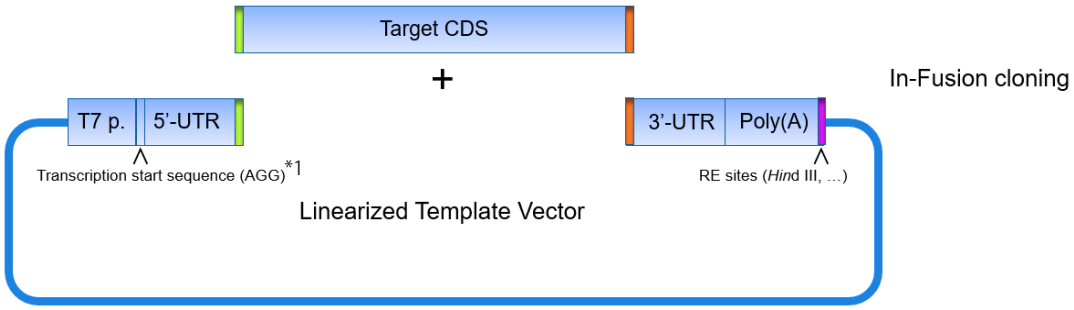

Fig. 1. IVT template Plasmid construction by In-Fusion cloning*2.

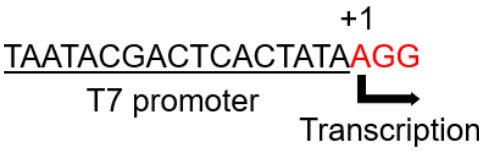

Fig. 2. Transcription start sequence (AGG) of IVT template when CleanCap Reagent AG is used.

*1 The "transcription start sequence" (AGG) is needed to efficiently prepare capped RNA with CleanCap Reagent AG.

*2 For more information on In-Fusion Snap Assembly Master Mix (Cat. #638943/638944/638947 to 638949), please see our website:
https://www.takarabio.com

II. Components (10 reactions)

LTV Linearized Template Vector (50 ng/μl)	10 μl
FLuc FLuc Control Fragment (100 ng/μl)*1	10 μl
In-Fusion 5X In-Fusion Snap Assembly Master Mix*2	20 μl

*1 Control DNA fragment for In-Fusion cloning, which contains *Photinus pyralis* luciferase CDS, optimized for use in human cells.

*2 Same as that in In-Fusion Snap Assembly Master Mix (Cat. #638943/638944/638947 - 638949)

Note: This product is also sold as part of Takara IVTpro mRNA Synthesis System (Cat. #6141).

III. Storage

-20℃

IV. Materials Required but not Provided

A. Reagents
- Competent cells
  - Stellar™ Competent Cells (Cat. #636763)*, etc.
- SOC medium
- Luria-Bertani (LB) medium
- LB /kanamycin (50 μg/ml) plate

B. Equipment
- Constant temperature bath or thermal cycler
- Reaction tubes
- Micropipettes, and tips

* Not available in all geographic locations. Check for availability in your area.

V. General Considerations

RNase contamination of the double-stranded DNA template, reagents, tubes, micropipette tips, or other materials used in the reaction can significantly decrease or digest RNA obtained with the kit. Use dedicated tubes and micropipette tips in the reaction and wear new disposable gloves to prevent RNase contamination.

VI. Construction of IVT Template Plasmid

A) Coding sequence (CDS) design of desired gene

1. Obtain the CDS sequence for the desired gene.
   - In order to express a certain gene, you need its CDS (DNA sequence from the start codon to the stop codon). Even to express a part of a gene, the start and stop codons must be added (See "VI-B, PCR amplification of the desired CDS fragment").
   - Even though the vector also contains a stop codon, prepare a CDS containing the stop codon to complete translation of the desired protein reliably.

2. Optimize the CDS codons for the cell types into which the mRNA will be transfected.
   - Use online tools or commercially available software.
   - When RNA is synthesized by *in vitro* transcription, a pseudo-UTP is often used instead of UTP to reduce the immunogenicity in mammalian cells (Karikó *et al.*, 2008). However, reduction of uridine (U) usage in the sequence is also important for immunogenicity reduction (Vidyanathan *et al*., 2018 and Xia 2021). Design the CDS considering both specific codon optimization and the frequency of uridines (U).

3. Confirm that the restriction site (*Hin*d III; recommended) used for linearizing the IVT template plasmid is not present in the CDS of the desired gene. If the restriction site *Hin*d III is present in the CDS, change the DNA sequence by changing the codon in the restriction sequence while keeping the amino acid sequence the same (e.g., switch from serine codon "UCU" to "UCC).

   Note: We strongly recommend using *Hin*d III to linearize the plasmid containing the CDS.

   - To synthesize IVT transcripts of uniform length, linearize the template plasmid with a restriction enzyme. Cutting the template plasmid using restriction enzymes that produce a 3' overhang may produce undesired RNA transcripts that correspond to the antisense strand or to the vector DNA (Schenborn *et al*., 1985). Therefore, we recommend plasmid linealization with a restriction enzyme that will produce a 5' overhang or a blunt end. Moreover, addition of extra bases after the Poly(A) sequence in the mRNA may cause a decrease in the translation efficiency in some cases. Whenever possible, use a restriction enzyme site that will not leave any extra.

4. Prepare the CDS of the desired gene, using DNA synthesis and cDNA cloning, etc.

B) PCR amplification of the desired CDS fragment

1. As shown below, the 15-base sequence (red) for In-Fusion cloning is added to the 5′ end of the Forward and Reverse primers that can amplify the CDS of the desired gene.
   - Start codon (blue) and stop codon (green: complementary strand) of the CDS
   - When the CDS containing the In-Fusion sequence is prepared by DNA synthesis, proceed to "V-2-C) In-Fusion cloning".

   Example: FLuc

   Forward primer:
   5′-AGAGAACCCGCCACCATGGAGGACGCCAAGAACATCAA-3′

   Reverse primer:
   5′-CGAGGCTCCAGCTCATCACACGGCGATCTTGCCGC-3′

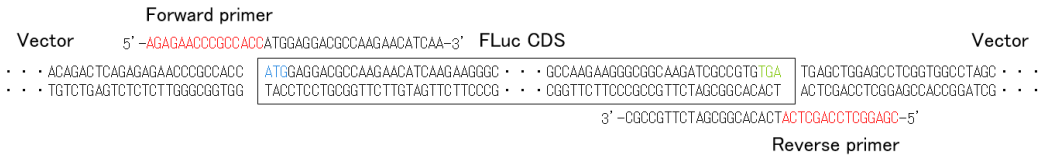

Fig. 3. PCR primer for FLuc CDS fragment

2. Perform PCR amplification of the CDS with the primers above. The resulting amplicon has a region of 15-base overlap with the ends of the LTV Linearized Template Vector.
   - For PCR amplification, we recommend using PrimeSTAR® Max DNA Polymerase (Cat. #R045A/B), which has the highest level of accuracy, or TaKaRa Ex Premier™ DNA Polymerase (Cat. #RR370S/A/B, RR371S/A/B), which has a high PCR success rate in addition to high accuracy.

3. Perform agarose gel electrophoresis with 5 μl of the PCR solution and confirm the amplified PCR product and its quantity.
   - About 50 to 100 ng/μl of the purified PCR product is needed for In-Fusion cloning (VI-2-C). Confirm that you have a sufficient amount.

4. Purify the PCR product with spin column purification kit (NucleoSpin Gel and PCR Clean-up; Cat. #740609.10/.50/.250).
   - If not using the DNA immediately after purification, store it at -20℃.
   - If there are multiple amplification products, purify the DNA from the desired PCR band, optimize the PCR conditions, or redesign the primer.

C) In-Fusion Cloning

1. Thaw the LTV Linearized Template Vector and CDS fragment from VI-B-4 at room temperature and spin down after mixing briefly.

2. Prepare the reaction solution as follows.

< Per reaction >

Reagent	Volume
LTV Linearized Template Vector (50 ng/μl)	1 μl
CDS fragment with In-Fusion sequence*	100 ng
Nuclease-Free Water	x μl
In-Fusion 5X In-Fusion Snap Assembly Master Mix	2 μl
Total	10 μl

* If using FLuc FLuc Control Fragment, use 1 μl (100 ng).

3. Incubate at 50℃ for 15 minutes.

Note: If not proceeding with transformation immediately, store the reaction solution on ice or at -20℃ for longer storage.

D) Transformation

Follow the protocol below for the transformation of Stellar Competent Cells (Cat. #636763). Use the recommended competent cells or ones with a transformation efficiency of at least 1 x 10^8 cfu/μg.

1. Thaw the competent cells on ice.
2. After light mixing, transfer 50 μl of the competent cells to a fresh tube.
3. Add 2.5 μl of reaction solution prepared in Step C, mix lightly, and let stand on ice for 30 minutes.
4. Heat shock the competent cells at 42℃ for 45 seconds.
5. Cool them on ice for 1 to 2 minutes.
6. Add 450 μl of SOC medium and shake-culture at 37℃ for 1 hour.
7. Spread 50 μl of the culture medium from Step 6, and a 10-fold dilution with SOC medium on LB plates containing kanamycin and culture overnight at 37℃.

E) Expected results

When FLuc FLuc Control Fragment is used, 100 or more colonies will ordinarily be obtained from plating 50 μl of the undiluted medium. Make liquid cultures of the individual colonies and purify the plasmid by the standard method (using NucleoSpin Plasmid, Cat. #740588.10, etc.). Confirm the sequence of the purified plasmids. See the product page for sequence information for the LTV Linearized Template Vector and the FLuc FLuc Control Fragment cloned in Linearized Template Vector.

- The Poly(A) sequence may be truncated depending on the *E. coli* strain or culturing method. We recommend confirming the sequence of the colony-derived plasmid and then preparing several glycerol stocks of the verified *E. coli* clone.

  Use the following sequencing primers to confirm the presence of the Poly(A) sequence.
  Poly(A) Forward primer: 5′-CCTCGGTGGCCTAGCTTCTT-3′
  Poly(A) Reverse primer: 5′-CAGGGCTTCCCAACCTTACC-3′

VII. Troubleshooting

Problems obtaining the desired IVT template plasmid

See the troubleshooting guide for In-Fusion cloning and "In-Fusion cloning tips and FAQs" in the user manual for In-Fusion Snap Assembly Master Mix (Cat. #638943/638944/638947 - 638949).

See our website: https://www.takarabio.com

VIII. References

1) Karikó, K. *et al.* Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol Ther J Am Soc Gene Ther.* (2008) **16:** 1833-1840.
2) Vaidyanathan, S. *et al.* Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification. *Mol Ther Nucleic Acids.* (2018) **12:** 530-542.
3) Xia, X. Detailed Dissection and Critical Evaluation of the Pfizer/BioNTech and Moderna mRNA Vaccines. *Vaccines (Basel).* (2021) **9:** 734.
4) Schenborn, E. T. and Mierindorf, R. C. A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. *Nucleic Acids Res.* (1985) **13:** 6223-6236.

IX. Related Products

Takara IVTpro™ mRNA Synthesis System (Cat. #6141)
Takara IVTpro™ T7 mRNA Synthesis Kit (Cat. #6144)
Takara IVTpro™ mRNA Synthesis System (low dsRNA) (Cat. #6131)
Cloning Kit for mRNA Template (BspQ I) (Cat. #6133)
Takara IVTpro™ T7 mRNA Synthesis Kit (low dsRNA) (Cat. #6134)
PrimeSTAR® Max DNA Polymerase (Cat. #R045A/B)
PrimeSTAR® Max DNA Polymerase Ver.2 (Cat. #R047S/A/B)
TaKaRa Ex Premier™ DNA Polymerase (Cat. #RR370S/A/B)
TaKaRa Ex Premier™ DNA Polymerase Dye plus (Cat. #RR371S/A/B)
Takara Stable Competent Cells (Cat. #9132)
Stellar™ Competent Cells (Cat. #636763)*
NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250)*
NucleoSpin Plasmid (Cat. #740588.10/.50/.250)*
In-Fusion® Snap Assembly Master Mix (Cat. #638943/638944/638947 - 638949)

* Not available in all geographic locations. Check for availability in your area.

NOTE : This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

Takara products may not be resold or transferred, modified for resale or transfer, or used to manufacture commercial products without written approval from Takara Bio Inc.

If you require licenses for other use, please contact us from our website at www.takarabio.com.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product web page. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.