Cat. # 6144

For Research Use

TaKaRa

Takara IVTpro™ T7 mRNA Synthesis Kit

Product Manual

v202204Da

Table of Contents

I. Description

The Takara IVTpro T7 mRNA Synthesis Kit (Cat. #6144) is designed for synthesizing mRNA through an *in vitro* transcription (IVT) using double-stranded DNA (containing the T7 promoter) as a template. It is essential to add a cap structure to the 5′ end of the mRNA, for efficient protein translation in eukaryotes. This kit is useful for preparing high-yield capped mRNA through *in vitro* transcription (IVT) using cap analogs, such as CleanCap (TriLink BioTechnologies) and Anti-Reverse Capping Analog (ARCA).

Moreover, modified NTPs, such as pseudo-UTP, etc., can be used instead of UTP to reduce the innate immune response (Kariko *et al.*, 2008) of mRNA in the transfected cells without affecting mRNA yield. In the case that 1.9 kb mRNA containing the CleanCap structure and pseudo-uridine is synthesized using 1 μg of (PCT) Positive Control Template (FLuc*) in the kit, about 180 μg of mRNA will be obtained in a 20 μl reaction.

*FLuc: *Photinus pyralis* luciferase CDS optimized for use in human cells.

II. Compotents (20 reactions, 20 μl volume)

(TB) 10X Transcription Buffer	40 μl
(ATP) 10X ATP	40 μl
(CTP) 10X CTP	40 μl
(GTP) 10X GTP	40 μl
(UTP) 10X UTP	40 μl
(EM) 10X Enzyme Mix	40 μl
(H_2O) Nuclease-Free Water	1 ml x 3
(DNase) DNase I	80 μl
(LiCl) Lithium Chloride Precipitation Solution	600 μl
(PCT) Positive Control Template (FLuc) (0.5 μg/μl)*	10 μl

* Linearized plasmid template containing T7 promoter + 5′UTR + FLuc-CDS + 3′UTR + Poly(A)

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

- Cap analog
  - CleanCap Reagent AG (TriLink BioTechnologies, No. N-7113-1/5/10)
  - CleanCap Reagent AG (3′ OMe) (TriLink BioTechnologies, No. N-7413-1/5/10)
  - ARCA (Anti-Reverse Cap Analog)
  - m^7G-Cap (N7-Methyl-Guanosine-5′-Triphosphate-5′-Guanosine), etc.
- Modified NTP
  - N^1-Methylpseudouridine-5′-Triphosphate
  - Pseudouridine-5′-Triphosphate
  - 5-Methoxyuridine-5′-Triphosphate
  - 5-Methylcytidine-5′-Triphosphate, etc.
- Ethanol
- 3M sodium acetate (pH 5.2)
- TE buffer (containing 0.1 mM EDTA)

B. Equipment

- Reaction tubes
- Micropipettes, and tips
- Constant temperature bath or thermal cycler
- Refrigerated micro centrifuge
- Spectrophotometer
  - NanoDrop (Thermo Fisher Scientific), etc.

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. Preparation of IVT Template DNA

A linearized plasmid or PCR product containing a T7 promoter can be used as the template DNA for IVT synthesis of RNA using this kit. For "Preparation of linearized plasmid template," see VI-B, for "Preparation of PCR template," see VI-C. Cloning Kit for mRNA Template (Cat. #6143) can be used for efficient construction of template DNA for IVT synthesis of RNA using CleanCap Reagent AG [or CleanCap Reagent AG (3' OMe)] as the cap analog. When using the Cloning Kit for mRNA Template to construct the plasmid, proceed to "VI-B, Preparation of linearized plasmid template", for "Preparation of PCR template," see VI-C.

A) Structure of IVT template DNA

a) Template structure

Refer to the following example to prepare the necessary sequence downstream of the T7 promoter (T7 p.).

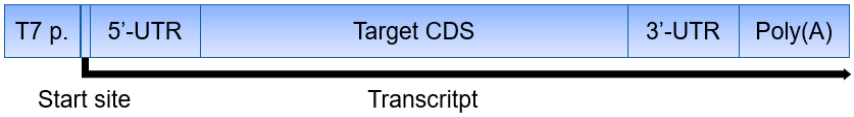


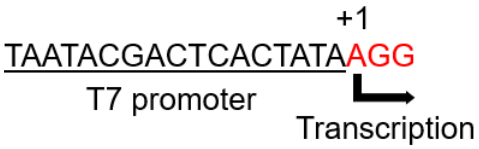
Fig. 1. Structure of IVT template DNA used in mRNA synthesis for protein expression.

- If Cloning Kit for mRNA Template is not used, prepare 5' UTR, 3' UTR, Poly(A), and other sequences as needed depending on the purpose of each experiment (Mignone *et al*., 2002 and Orlandini *et al*., 2019). If the template does not contain the Poly(A) sequence, you can also add poly(A) with Poly(A) Polymerase to the IVT transcript.

b) T7 promoter and transcription start sequence

The transcription start sequence depends on the cap analog used.

1. For using CleanCap Reagent AG [or CleanCap Reagent AG (3' OMe)], use the AGG transcription start sequence.



2. For using ARCA or m^{7}G-Cap, use the GGG transcription start sequence.

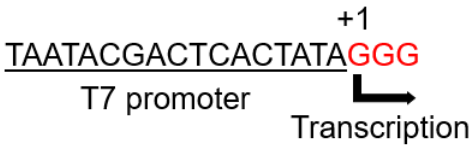


Fig. 2. T7 promoter and transcription start sequence.

- Template DNA with these transcription start sequences can also be used in the preparation of RNA that does not require a cap structure.

c) Codon optimization

Optimize the codons of the coding sequence (CDS) for the cell types that will be transfected with IVT transcript.

- 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 (Kariko *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).

B) Preparation of linearized plasmid template

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 produces 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 bases.

a) Restriction enzyme treatment

A plasmid template DNA concentration of 0.5 to 1.0 μg/μl is needed for IVT. Refer to the following example for restriction enzyme treatment. Account for recovery loss following ethanol precipitation or column purification after restriction enzyme treatment.

Example

< Per reaction >

Reagent	Volume
Template Plasmid	50 μg
10X M Buffer	20 μl
Nuclease-Free Water	X μl
*Hin*d III (15 U/μl)	10 μl
Total	200 μl

Incubate at 37℃ for 3 hours.

Note: If the restriction digestion is incomplete and some uncut circular template plasmid remains, some of RNA synthesized by IVT will be at a size larger than desired. Confirm complete linearization of the plasmid by agarose gel electrophoresis using 5 μl of the reaction solution.

b) Ethanol precipitation

1. Add 1/10 amount of 3M sodium acetate (pH 5.2) and twice the amount of ethanol to the restriction digestion solution.
2. Mix well and cool at -20℃ for at least 15 minutes.
3. Centrifuge for 15 minutes at maximum speed at 4℃.
4. Carefully remove the supernatant using a pipette, add 1 ml of 70% ethanol, and centrifuge again under the same conditions.
5. Carefully remove the supernatant and dry the pellet.
6. Dissolve the DNA in H2O Nuclease-Free Water or TE buffer (containing 0.1 mM EDTA) and measure the concentration of the DNA. If necessary, adjust the concentration to between 0.5 to 1.0 μg/μl. Store the IVT plasmid template solution at -20℃ until use.

   Note: Fragmentation of the mRNA synthesized using the linearized plasmid template above may indicate RNase contamination. To prevent RNA degradation, perform phenol-chloroform extraction of the plasmid template after restriction digestion and then purify it by ethanol precipitation.

C) Preparation of the PCR template

When using a PCR product as IVT template, perform PCR amplification of the sequence from T7 promoter to the Poly(A). Prepare IVT template following the same purification method described above.

VII. IVT Reaction

1. Preparation of reagents
   - Thaw components other than (EM) 10X Enzyme Mix in Takara IVTpro mRNA Synthesis Kit at room temperature. Mix gently, and spin down.
   - Spin down the (EM) 10X Enzyme Mix briefly and keep it on ice until use (do not vortex).

2. Prepare the reaction solution as shown below at room temperature.
   * Be sure to add each component in the **order shown**.
   Calculate the amount of Nuclease-Free Water needed beforehand.

   a) mRNA synthesis reaction using CleanCap Reagent AG or CleanCap Reagent AG (3′ OMe)

   < Per reaction >

Reagent	Volume
(H_2O) Nuclease-Free Water	X μl
(TB) 10X Transcription Buffer*1	2 μl
(ATP) 10X ATP*2	2 μl
(CTP) 10X CTP*2	2 μl
(GTP) 10X GTP*2	2 μl
(UTP) 10X UTP*2	2 μl
CleanCap Reagent AG*3	1.6 μl
Template DNA*4	1 μg
(EM) 10X Enzyme Mix	2 μl
Total*5	20 μl

*1 (TB) 10X Transcription Buffer contains spermidine, which forms a complex with nucleic acid and in some cases may precipitate out as an insoluble material. Be sure to add the components in the **order shown**.

*2 **The concentration of each NTP is 100 mM.** When using a modified NTP, replace the corresponding NTP with an equivalent amount.

*3 Use CleanCap Reagent AG or CleanCap Reagent AG (3′ OMe) at a 4:5 molar ratio with NTP (final concentration 8 mM) (See Fig. 3 A/B).

*4 Use a template that has the AGG transcription start sequence. The optimal amount of template differs depending on the size and type of template used, but you can ordinary use a range between 0.5 to 2 μg (See Fig. 4). For (PCT) Positive Control Template (FLuc), use 2 μl (1 μg).

*5 Scale-up as necessary (See Fig. 5A/B).

b) mRNA synthesis reaction using ARCA or m7G-Cap

< Per reaction >

Reagent	Volume
(H2O) Nuclease-Free Water	X μl
(TB) 10X Transcription Buffer*1	2 μl
(ATP) 10X ATP*2	2 μl
(CTP) 10X CTP*2	2 μl
(GTP) 10X GTP*2	0.4 μl
(UTP) 10X UTP*2	2 μl
ARCA or m7G-Cap (100 mM)*3	1.6 μl
Template DNA*4	1 μg
(EM) 10X Enzyme Mix	2 μl
Total*5	20 μl

*1 (TB) 10X Transcription Buffer contains spermidine, which forms a complex with nucleic acid and in some cases may precipitate out as an insoluble material. Be sure to add the components in the **order shown**.

*2 **The concentration of each NTP is 100 mM.** When using a modified NTP, replace the corresponding NTP with an equivalent amount.

*3 At first, add ARCA or m7G-Cap at a 4:1 molar ratio with the GTP (a final concentration of cap analog is 8 mM. Total of the cap analog and GTP is 10 mM). Adjust the optimal molar ratio with GTP, depending on the capping efficiency or the size and yield of the transcript (See Fig. 6).

*4 Use a template that has the GGG transcription start sequence. The optimal amount of template differs depending on the size and type of template used, but you can ordinary use a range between 0.5 to 2 μg. (PCT) Positive Control Template (FLuc) cannot be used in this case because it is for use with CleanCap Reagent AG or CleanCap Reagent AG (3′ OMe), which have the transcription start sequence of "AGG".

*5 Scale-up as necessary.

c) mRNA synthesis reaction without a cap analog

< Per reaction >

Reagent	Volume
(H2O) Nuclease-Free Water	X μl
(TB) 10X Transcription Buffer*1	2 μl
(ATP) 10X ATP*2	2 μl
(CTP) 10X CTP*2	2 μl
(GTP) 10X GTP*2	2 μl
(UTP) 10X UTP*2	2 μl
Template DNA*3	1 μg
(EM) 10X Enzyme Mix	2 μl
Total*4	20 μl

*1 10X Transcription Buffer contains spermidine. Spermidine forms a complex with nucleic acid and in some cases may precipitate out as an insoluble material, so be sure to add the components in the **order shown**.

*2 **The concentration of each NTP is 100 mM.** When using a modified NTP, replace the corresponding NTP with an equivalent amount.

*3 Use a template that has the GGG or AGG transcription start sequence. The optimal amount of template differs depending on the size and type of the template, but you can ordinary use a range between 0.5 to 2 μg.

*4 Scale-up as necessary.

3. Mix well and incubate at 37℃ for 2 hours.
   - Adjust the reaction time depending on the target length and the desired RNA yield (See Fig. 7 A/B).
   - A white precipitate may form at the end of the reaction. This will likely be magnesium pyrophosphate produced when the pyrophosphate released by the reaction reacts with the magnesium in the solution. This precipitate does not affect subsequent operations, so proceed to the next step, "VIII. DNase I Treatment."

VIII. DNase I Treatment

After the reaction in VIII-3, add 4 μl of (RNase-free DNase I) DNase I, mix briefly and incubate at 37℃ for 15 minutes.

IX. Purification by LiCl Precipitation

The LiCl precipitation can effectively remove uncaptured NTPs and proteins. However, RNA cannot be efficiently recovered if the RNA size is less than 300 bases or the RNA concentration is less than 0.1 μg/μl. If RNA are a smaller size or concentration less than 0.1 μg/μl, purify the RNA by spin column (NucleoSpin RNA Clean-up, Cat. #740948.10/.50/.250) or ethanol precipitation after phenol-chloroform extraction. RNA purified by these methods can be used in transfection, electroporation, and microinjection experiments (See Fig. 8 A/B).

1. Thaw LiCl Lithium Chloride Precipitation Solution at room temperature before use. Mix well, and if a precipitate is observed, warm the solution at 37℃. If the precipitate persists, use as is. The precipitate does not affect the subsequent steps.
2. Add 30 μl of Ⓦ Nuclease-Free Water and 30 μl of Ⓛ Lithium Chloride Precipitation Solution to the IVT reaction solution (about 24 μl) treated with DNase I in Step VIII to stop the reaction.
3. Mix well, then cool at -20℃ for at least 30 minutes.
4. Centrifuge at 4℃ for 15 minutes at maximum speed.
5. Carefully remove the supernatant and wash the pellet with 1 ml of 70% ethanol.
6. Centrifuge again at 4℃ for 15 minutes at maximum speed.
7. Carefully remove the supernatant.
8. Air-dry the pellet and dissolve in 100 μl of Ⓦ Nuclease-Free Water.
   Note: Excessive air drying will make resuspension in Ⓦ Nuclease-Free Water difficult.
   Note: Depending on the RNA yield, the pellet may take time to dissolve. Let stand at room temperature or 4℃, and mix as needed.
9. After dissolving, measure the RNA concentration with NanoDrop, etc. If not using the RNA sample immediately, store at -20℃.
   Note: Presence of residual unused NTP, cap analogs, or the template DNA will affect the OD measurement. Measure a sample that has been purified by the method described above.
   Note: As needed, confirm the length and purity of the RNA by denaturing agarose/acrylamide gel or Bioanalyzer (Agilent).

X. Experimental Examples

Example 1-A

< Method >
FLuc mRNA was synthesized in IVT reaction solution with or without CleanCap Reagent AG (3′ OMe).

< Results >
Use of CleanCap had almost no effect on RNA yield.

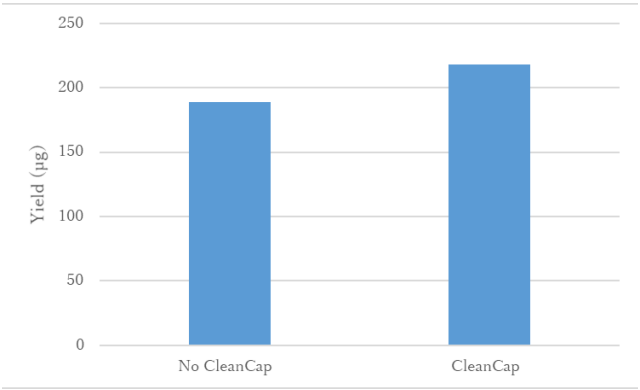


Fig. 3A. Affect of CleanCap Reagent AG (3′ OMe) on RNA yield.

Example 1-B

< Method >
HEK293T cells were transfected with 0.5 µg of the RNA obtained in Example 1-A, using *Trans*IT-mRNA Transfection Kit (Mirus Bio, No. MIR2225).

< Results >
The cells were recovered 24 hours after transfection and FLuc luciferase activity was measured. The FLuc mRNA synthesized in IVT reaction with CleanCap showed activity equivalent to that of the commercially available FLuc mRNA positive control. By contrast, no activity was observed in the FLuc RNA synthesized in the IVT reaction without CleanCap. This shows that addition of a cap is essential for protein expression by the resulting mRNA.

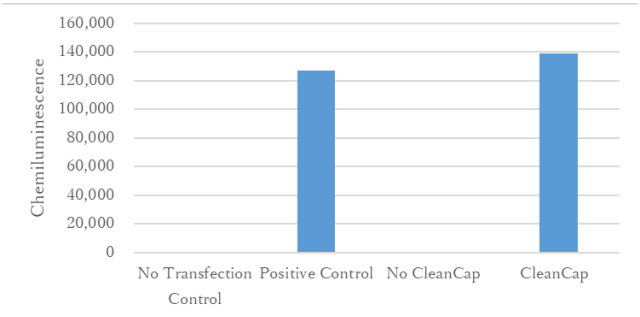


Fig. 3B. Expression of FLuc mRNA synthesized using CleanCap Reagent AG (3′ OMe) in HEK293T cells.

Example 2

< Method >
IVT reaction (20 μl) was performed using various amounts of Positive Control Template (FLuc) with CleanCap Reagent AG (3′ OMe) and N^1-methyl pseudo UTP.

< Results >
The RNA yield peaked when the amount of DNA was 0.5 μg, and no change in yield was observed over 0.5 μg (0.5 to 2 μg).

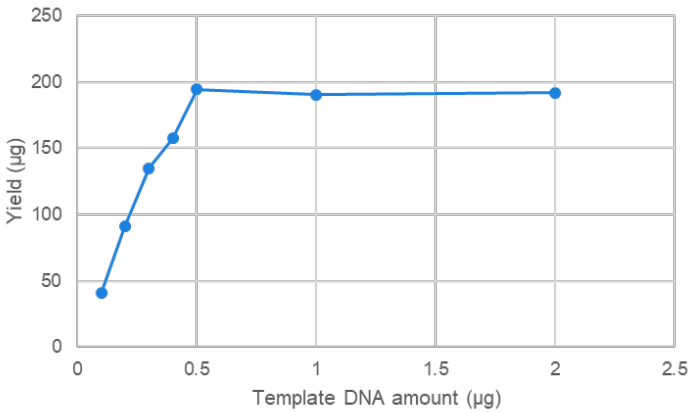


Fig. 4. Correlation between amount of template DNA and RNA yield.

Example 3-A

< Method >
IVT reactions were performed at various reaction volume (20 - 200 μl) with CleanCap Reagent AG (3′ OMe) using the Positive Control Template (FLuc). Details other than reaction volume are as described in this Manual.

< Results >
Total amount of RNA obtained was proportional to the reaction volume. However, the concentration and the yield per 20 μl reaction was not affected by scaling up the reaction volume.

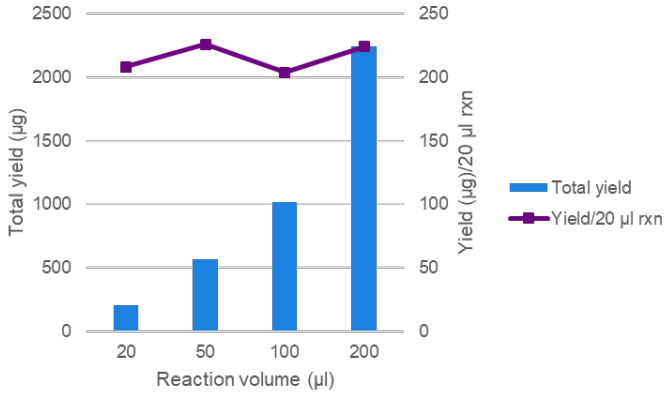


Fig. 5A. Correlation between IVT reaction volume and RNA yield.

Example 3-B

< Method >
1 ng of the RNA product obtained in Example 3-A was examined by Bioanalyzer.

< Results >
The IVT reaction volume did not affect the quality of the RNA product.

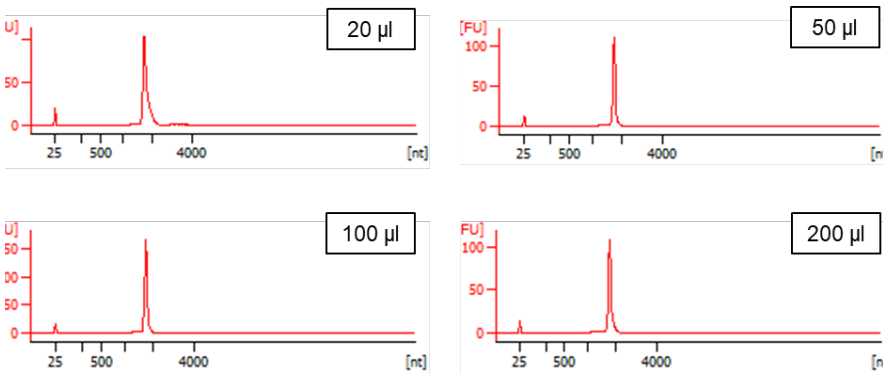
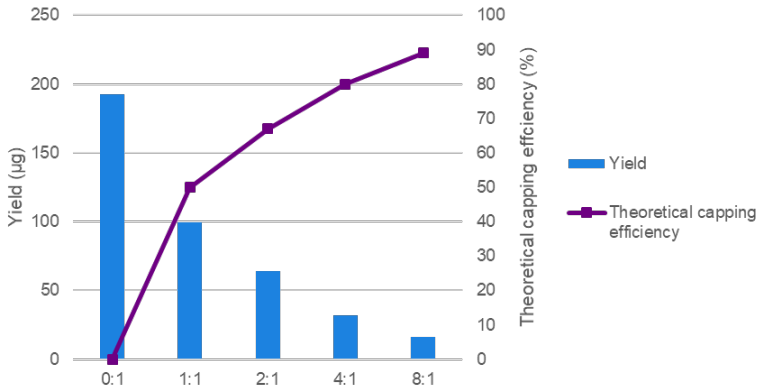


Fig. 5B. Analysis of the RNA product with Bioanalyzer.

Example 4

< Method >
2 kb of RNA transcript was synthesized by IVT reaction while changing the molar concentration ratio of ARCA and GTP. The sum of the molar concentrations of ARCA and GTP was 10 mM.

< Results >
The RNA yield decreased significantly as the ARCA concentration increased. Since there is no difference in the incorporation efficiency of ARCA and GTP in the first base, theoretical capping efficiency is 50% at ARCA:GTP=1:1. The RNA yield will be greatly reduced when mRNA capped at high efficiency is required by increasing the ARCA concentration.



No.	Sample	RNA yield (µg/20 µl rxn)	Theoretical capping efficiency
1	ARCA:GTP=0:1	192.4	0%
2	ARCA:GTP=1:1	99.3	50%
3	ARCA:GTP=2:1	64.1	67%
4	ARCA:GTP=4:1	31.8	80%
5	ARCA:GTP=8:1	16.2	89%

Fig. 6. RNA yield and theoretical capping efficiency with ARCA.

Example 5-A

< Method >
IVT reactions were performed with various reaction times using the Positive Control Template (FLuc) with CleanCap Reagent AG (3′ OMe) and N^1-methyl pseudo UTP.

< Results >
In the case of 1.9 kb long synthesized mRNA, the RNA yield peaked in about 1 hour, after which no change was observed (for 1 to 16 hours).

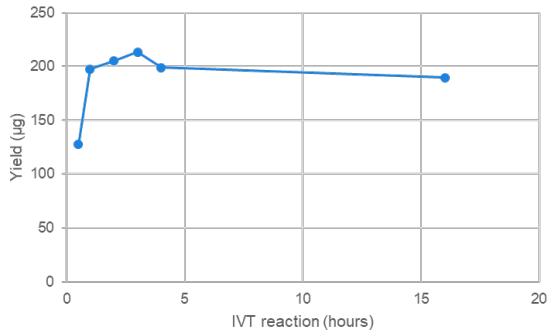


Fig. 7A. Correlation between IVT reaction time and RNA yield.

Example 5-B

< Method >
1 ng of the RNA product obtained in Example 5-A was analyzed by Bioanalyzer.

< Results >
No changes were observed for different reaction times.

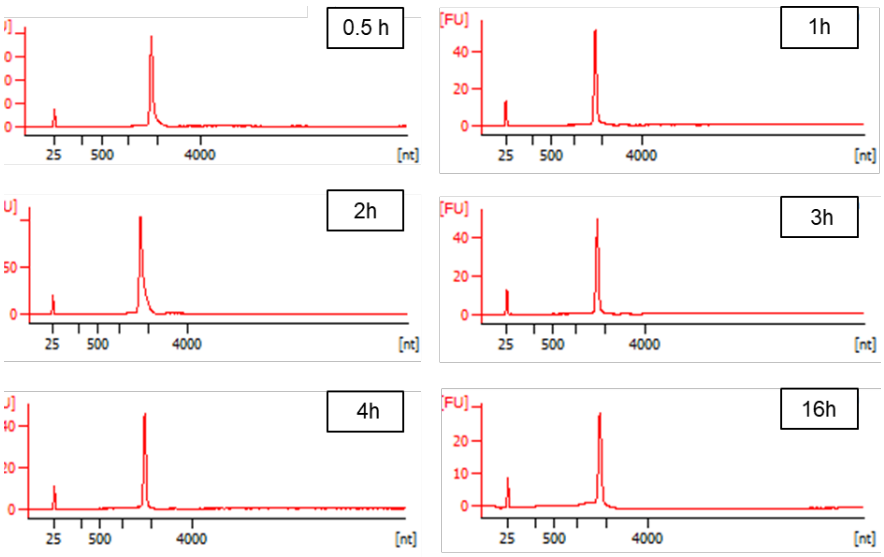


Fig. 7B. Comparison of RNA quality over reaction time using Bioanalyzer.

Example 6-A

< Method >
FLuc mRNA was synthesized in an IVT reaction with CleanCap Reagent AG (3′ OMe) and N^1-methyl pseudo UTP, and was purified by LiCl precipitation or spin column method using NucleoSpin kit.

< Results >
The amount of RNA obtained was less for one elution with the spin column method (Elution x1) compared to the LiCl precipitation method, but the amount of RNA with two elutions (Elution x2) with the spin column was approximately same as that with LiCl method. We strongly recommended performing elution twice when using the spin column method.

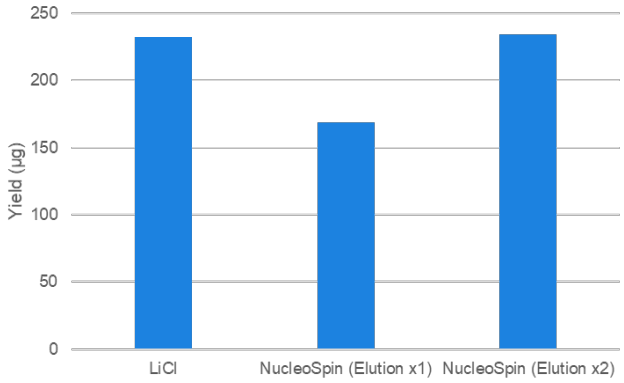


Fig. 8A. Comparison of RNA purification between LiCl precipitation and spin column methods.

Example 6-B

< Method >
HEK293T cells were transfected with 0.5 µg of the RNA obtained in Example 6-A, using the *Trans*IT-mRNA Transfection Kit.

< Results >
The FLuc activity in cells recovered after 24 hours was equivalent to that of the commercially available FLuc mRNA positive control.

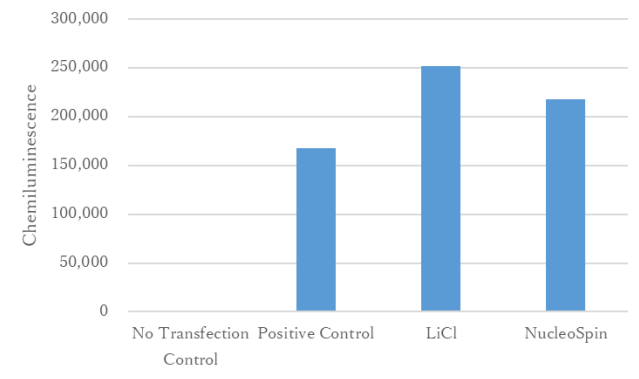


Fig. 8B. Expression of FLuc mRNA purified by different methods in HEK293T cells.

XI. Troubleshooting

Problem	Cause	Solution
RNA yield is less	RNase contamination of template DNA	Perform phenol-chloroform extraction of template DNA after restriction digestion and then purify by ethanol precipitation.
	Amount of template DNA is too little	Check the amount of template DNA by agarose gel electrophoresis. If there is a discrepancy with the OD measured value, try purifying the template DNA again.
	Reaction time is insufficient	Lengthen the IVT reaction time.
	RNA length is less than 300 bases or RNA concentration is less than 0.1 μg/μl	Instead of purifying by the LiCl purification method, try using the spin column method or ethanol precipitation method after phenol-chloroform extraction.
	RNA pellet loss	Remove the supernatant carefully using a micropipette with as small a tip as possible.
	Insufficient RNA dissolution/elution	Dissolution may take time depending on the RNA yield. Allow the solution to stand at room temperature or 4℃, mix as needed, and then measure the RNA after it has completely dissolved. If it still does not dissolve, add some more solution. When the spin column method is used for purification, a single round of elution may not be sufficient. It is strongly recommended that you perform elution twice (e.g., 50 μl x 2).
	RNase contamination of reagent, equipment, or during processing	Take precautions against RNase contamination by using dedicated tubes and micropipette tips for the reaction and wearing new disposable gloves.
	Reagent deterioration	The enzymes should be stored at -20℃ and kept on ice. Avoid excessive agitation and freezing/thawing. If at least 100 μg of RNA cannot be obtained with (PCT) Positive Control Template (FLuc), it is time to repurchase.
The RNA obtained is larger than the desired size	Linearization of template plasmid is insufficient	Repeat restriction enzyme treatment of the template plasmid and confirm complete plasmid linearization with agarose gel electrophoresis before using the template.
	Denaturation of RNA is insufficient	Perform electrophoresis with denaturing agarose or acrylamide gel.
RNA that is smaller than the desired size	The CDS contains a sequence similar to the transcription termination signal of T7 RNA Polymerase	If possible, change the sequence. When changing the coding sequence, change the codons keeping the amino acid sequence the same.
Fragmented RNA smaller than the desired size is observed	RNase contamination	Take precautions against RNase contamination by using dedicated tubes and micropipette tips in the reaction and wearing new disposable gloves.

XII. References

1) Mignone, F. *et al.* Untranslated regions of mRNAs. *Genome Biol.* (2002) **3:** reviews0004.1-0004.10.
2) Orlandini von Niessen, A. G. *et al.* Improving mRNA-based therapeutic gene delivery by expression-augmenting 3' UTRs identified by cellular library screening. *Mol Ther.* (2019) **27:** 824-836.
3) Karikó, K. *et al.* Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol Ther.* (2008) **16:** 1833-1840.
4) 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.
5) Xia, X. Detailed Dissection and Critical Evaluation of the Pfizer/BioNTech and Moderna mRNA Vaccines. *Vaccines (Basel).* (2021) **9:** 734.
6) 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.

XIII. Related Products

Takara IVTpro™ mRNA Synthesis System (Cat. #6141)
Cloning Kit for mRNA Template (Cat. #6143)
*Hin*d III (Cat. #1060A/B)
NucleoSpin RNA Clean-up (Cat. #740948.10/.50/.250)*

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