**In vitro synthesis of capped Cas9 mRNA**

The full length of zebrafish-codon-optimized Cas9 cDNAs with double NLS were cloned into pCS2 vector (Amp-resistant) and linearized by NotI. Capped Cas9 mRNA was synthesized and purified using mMESSAGE mMACHINE mRNA transcription synthesis kit (Ambion; cat#AM1344). Detailed information is as follows.

1. pT3TS-nzCas9n lineared by NotI

|  |  |
| --- | --- |
| component | Volume |
| DNA (10 ug) | —— |
| 10 X buffer | 10 ul |
| NotI | 40 u |
| H2O | —— |
| Total | 100 ul |

37 oC for 3-5 hrs, purified using Fermentas GeneJETTM PCR Purification Kit.

1) Add isopropanol and binding buffer to the DNA-enzyme solution (isopropanol: binding buffer: DNA-enzyme solution=1 volume: 1 volume: 1 volume), mix well.

2) Transfer the solution to the GeneJETTM Purification column, centrifuge for 60s

at 10000 rcf, discard the flow-through.

3) Add 700 ul of wash buffer and centrifuge for 1 min at 12000 rpm. Discard the flow-through. Centrifuge empty column for 2 min at 12000rpm.

4) Place the column into a fresh 1.5 ml RNase-free EP tubes. Add 40-50 ul RNase-free water to the column (using RNase-free Pipette Tips), centrifuge for 1.5 min at 12000 rpm, collect the flow-through.

Note: purified lineared DNA > 100 ng/ul.

2. In vitro mRNA transcription (Ambion mMESSAGE mMACHINE kit)

component Volume

2X NTP/CAP 10 ul

10X Reaction Buffer 2 ul linear template DNA 0.5-1 ug Enzyme Mix 2 ul

Nuclease-free Water ——

Total 20 ul

37 oC for 2-2.5 hrs.

3. mRNA purification (Ambion mMESSAGE mMACHINE kit)

DNase I treatment: add 2 ul TURBO DNase I, 37 oC for 15 min, purified as follows:

**Protocol 1**

Phenol: chloroform extraction and isopropanol precipitation: This is the most rigorous method for purifying transcripts. It will remove all enzyme and most of the free nucleotides from mMESSAGE mMACHINE® Kit reactions. Since the RNA is precipitated, this method can also be used for buffer exchange.

1) Add 430 ul RNase-free water and 50 ul Ammonium Acetate Stop Solution, mix well.

2) Extract with an equal volume of phenol/chloroform (it can be water-saturated, buffer-saturated, or acidic), mix well for 15s, RT for 3 min, then centrifuge for 15 min at 12000g at 4 oC. Recover aqueous phase and transfer to new tube.

3) Extract the supernatant with an equal volume of chloroform, mix well, centrifuge for 10 min at 12000g at 4 oC. Recover aqueous phase and transfer to new tube. Repeat it.

4) Precipitate the RNA by adding 1 volume of isopropanol and mixing well. Chill the mixture for 1 hrs (at least 15 min) at –20°C, centrifuge for 30 min at

12000g at 4 oC to pellet the RNA.

5) Carefully remove the supernatant solution and resuspend the RNA in 20 ul

RAase-free water.

6) Detection: add 0.5 ul RNA solution to 9.5 ul RAase-free water, mix well and determine the RNA concentration, then the rest sample is electrophoresed through the gel.

7) Store frozen at –80°C. Note: “DO NOT add GTP”.

using RNase-free Pipette Tips and RNase-free PCR tubes.

**Protocol 2**

Ammonium Acetate precipitation: Ammonium Acetate precipitation is a convenient and effective way to remove unincorporated nucleotides and most proteins.

1) Stop the reaction and precipitate the RNA by adding 20 μL Ammonium

Acetate (5M) Solution. Mix thoroughly. Chill on ice for 15 min.

2) Centrifuge at 4°C for 15 min at maximum speed to pellet the RNA.

3) Carefully remove the supernatant. Wash the pellet once with ~1 mL 70% ethanol (RNase-free water diluted), and centrifuge at 4°C for 5 min at 12000 rpm to maximize removal of unincorporated nucleotides.

4) Carefully remove the 70% ethanol, RT X 10 min to dry the sample.

5) Resuspend the RNA in 20 ul RAase-free water.

6) Detection: add 0.5 ul RNA solution to 9.5 ul RAase-free water, mix well and determine the RNA concentration, then the rest sample is electrophoresed through the gel.

7) Store frozen at –80°C.