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In vitro synthesis of PE2 (nCas9-MMLV RT fusion) polyA mRNA using T7 RNA polymerase

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Donald Rio[1]

[1]Dept. of Molecular and Cell Biology, University of California, Berkeley

Donald Rio: don_rio@berkeley.edu

Ezgi Booth
University of California, Berkeley

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We use this protocol and it's working

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Abstract

We describe the preparation of synthetic capped and polyadenylated messenger RNA (mRNA) encoding a Cas9 nickase-reverse transcriptase fusion protein (PE2) for use in genome editing with a method called prime editing.

Attachments

DOCX

336-739.docx
18KB

Materials

Materials

HiScribe T7 ARCA mRNA Kit (with Tailing) - 20 rxns **New England Biolabs Catalog #**E2060S

Monarch RNA Cleanup Kit (50 µg) - 100 preps **New England Biolabs Catalog #**T2040L

PmeI - 2,500 units **New England Biolabs Catalog #**R0560L

DNase I (RNase-free) - 5,000 units **New England Biolabs Catalog #**M0303L

E.coli Poly (A) Polymerase - 500 units **New England Biolabs Catalog #**M0276L

- CutSmart buffer
- 5M NaCl
- 100% ethanol
- 10mM Tris-HCl
- 1mM EDTA
- TE buffer
- New England Biolabs Monarch RNA cleanup column
- Nanodrop1000c spectrophotometer

Protocol Overview

1 Preparation of linearized plasmid DNA encoding the PE2 protein for use as a template for in vitro transcription by T7 RNA polymerase.

2 Synthesis of mRNA by T7 RNA polymerase, using a 5′ cap nucleotide to initiate the mRNA and using E. coli poly A polymerase to add a synthetic poly A tail after transcription.

3 Column purification of the final synthetic, capped and polyadenylated mRNA.

Part 1: Linearization of pCMV-PE2 plasmid

4 Digest 100 µg plasmid with 200 units Pme I restriction endonuclease in a 1 mL reaction using CutSmart buffer for 04:00:00 . at 37 °C . 4h

5 Purify the cleaved DNA by phenol-chloroform extraction, collect the upper (aqueous) phase and transfer to fresh 1.5 mL Eppendorf tubes.

6 Add [M] 5 Mass Percent NaCl to a final concentration of [M] 0.1 Mass Percent (20 µL) and 3 volumes of 100% ethanol.

7 Leave at -80 °C for 02:00:00 to Overnight to precipitate. 4h

8 Spin 16000 x g, 4°C, 00:10:00 . 10m

9 Remove the ethanol and air dry for 00:30:00 . 30m

10 Resuspend at 500 µg/ml in TE ([M] 10 millimolar (mM) Tris-HCl, pH 7.5 , [M] 1 millimolar (mM) EDTA) buffer. Store the DNA sample at -20 °C .

Part 2: In vitro transcription and polyA tailing

11 In order get a high yield of the 6500nt long PE2 mRNA by T7 RNA polymerase transcription, set up 8 x 20 µL in vitro transcription reactions using 1 µg of cleaved template DNA in each reaction and the New England Biolabs HiScribe T7 ARCA kit with tailing (E2060S), according to the manufacturer's instructions.

12 Incubate at 37 °C for 02:00:00 in an incubator (not a temp block). 2h

13 For each 20 µL reaction, add 2 µL DNase I (stock is 2 U/uL).

14 Incubate at 37 °C for 00:15:00 . 15m

15 Dilute the reaction to 50 µL with 20 µL RNase-free water, 5 µL 10X polyA polymerase buffer and 5 µL E. coli polyA polymerase.

16 Incubate at 37 °C for 00:30:00 . 30m

Part 3: Purification of IVT product 5m

17 Purify the polyadenylated mRNA on 50 µg New England Biolabs Monarch RNA cleanup columns (2 of 20 µL reactions per column) (T2040L).

18 Dilute each 20 µL reaction with 100 µL binding buffer and 150 µL 100% ethanol.

19 Add the two diluted reactions to one column and spin at 16000 x g, 00:01:00 . 1m

20 Wash each column with wash buffer and spin twice (see below).

20.1 Wash with 500 µL wash buffer (spin at 16000 x g, 00:01:00). (1/2) 1m

20.2 Wash with 500 µL wash buffer (spin at 16000 x g, 00:01:00). (2/2) 1m

21 Elute in 25 µL of RNase-free H_2O per column, incubate for 00:01:00 at Room temperature. 1m

22 Spin at 16000 x g, 00:01:00 into fresh 1.5 mL tubes and pool elution together into one tube. 1m

23 Measure the RNA concentration using a Nanodrop1000c spectrophotometer.

Note

The yield from the total of 8 reactions is usually ~ 200 µg.

24 Store the mRNA at -80 °C and avoid repeated freezing and thawing.