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

Donald Rio[1]

[1]Dept. of Molecular and Cell Biology, University of California, Berkeley
Donald Rio: don_rio@berkeley.edu

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Devin E Snyder

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

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KEYWORDS

Linearization of plasmid, In vitro transcription, Purification of IVT product, polyA tailing, ASAPCRN

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MATERIALS TEXT

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.

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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 4h

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** .

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 **5 Molarity (M)** NaCl to a final concentration of **0.1 Molarity (M)** (**20 µL**) and 3 volumes of 100% ethanol.

7 4h

Leave at **-80 °C** for **02:00:00** to **Overnight** to precipitate.

8 10m

Spin **16000 x g, 4°C, 00:10:00** .

9 30m

Remove the ethanol and air dry for **00:30:00** .

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10 

Resuspend at **500 undetermined** in TE (**10 millimolar (mM)** Tris-HCl, **7.5**, **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 

2h

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

13 

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

14 

15m

Incubate at **37 °C** for **00:15:00** .

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 

30m

Incubate at **37 °C** for **00:30:00** .

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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  1m

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

20 

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

20.1  1m

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

20.2  1m

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

21  1m

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

22  1m

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

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23 Measure the RNA concentration using a Nanodrop1000c spectrophotometer.

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.

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