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CasX GFP-Targeting gRNA IVT

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1 Works for me dx.doi.org/10.17504/protocols.io.8uwhwxe

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ABSTRACT

This protocol explains how to transcribe gRNA in vitro.

CasX_IVT_Protocol (2).docx

GUIDELINES

It's recommended to perform 8x25µL PCR reactions per gRNA, which results in 3x1mL IVT reactions.

MATERIALS

NAME	CATALOG #	VENDOR
Q5 High-Fidelity DNA Polymerase - 100 units	M0491S	New England Biolabs
APS	BP179-25	Fisher Scientific
100g EDTA, Disodium Salt (Ethylenediaminetetraacetic acid, disodium dihydrate)	786-050	G-Biosciences
10g Xylene Cyanol	RC-115	G-Biosciences
Bisacrylamide, 25g (N,N'-Methylenebisacrylamide)	V3141	Promega
Bromophenol blue	BB2230.SIZE.25g	Bio Basic Inc.
Sodium acetate	1.06268.1000	Merck Millipore
Acrylamide	A9099	Sigma
Diethyl pyrocarbonate	D5758	Sigma Aldrich
Urea	U5378	Sigma Aldrich
200 Proof Ethanol pure	E7023	Sigma Aldrich
Formamide	17899	Thermo Fisher
TEMED	17919	Thermo Fisher
dNTP Mix (10 mM each)	R0191	Thermo Fisher
T7 RNA Polymerase	2540B	Takara
NTP Mix (10mM)	18109017	Thermo Fisher Scientific
DNase I recombinant RNase-free	04716728001	Sigma Aldrich
UltraPure™ TBE Buffer 10X	15581044	Thermo Fisher Scientific
Corning syringe filters regenerated cellulose membrane diam. 4 mm pore size 0.2 µm	CLS431212	Sigma Aldrich

NAME	CATALOG #	VENDOR
Amicon® Ultra-15 Centrifugal Filter Unit (3kDa NMWL)	UFC900308	Millipore Sigma

SAFETY WARNINGS

For safety warnings and hazard information please refer to the SDS (Safety Data Sheet).

Q5 PCR Amplification

1

Prepare amplification reactions (x8) (plus 1.1x for pipette error) for each sgRNA.

- **8.8 µl Oligo 1 Primer (10µM)**
- **8.8 µl Oligo 2 Primer (10µM)**
- **8.8 µl Oligo 3 Primer (10µM)**
- **4.4 µl dNTPs (10mM)**
- **44 µl Q5 Buffer**
- **2.2 µl Q5 Polymerase**
- **143 µl DEPC**

Amplification reactions of 8.8x are recommended, but the 1x Q5 PCR reaction is as follows (able to scale up or down):

1µL (10µM) Oligo 1 Primer
1µL (10µM) Oligo 2 Primer
1µL (10µM) Oligo 3 Primer
0.5µL (10mM) dNTPs
5µL Q5 Buffer
0.25µL Q5 Polymerase
16.25µL DEPC

2

Run using PCR conditions as follows:

1 cycle:
00:00:30 , **98 °C** (initial denaturation)

35 cycles of:
00:00:10 , **98 °C** (denaturation)
00:00:20 , **51 °C** (annealing)
00:00:20 , **72 °C** (extension)

1 cycle:
00:02:00 , **72 °C** (final extension)

Hold at **4 °C** .

Reaction can be frozen at -20°C.

3

Run some reaction mix on an agarose gel to check PCR product size.

In Vitro Transcription (IVT)

4

From the 200µL PCR reactions (25µL x 8 tubes), split into 3 tubes of 66.6µL. Each tube will have a final volume of 1mL after IVT.

The PCR product doesn't need to be cleaned before IVT.

5

For each 1mL IVT reaction, add the following:
100 µl 10x IVT buffer
200 µl 5x NTPs
100 µl T7 Polymerase
40 µl RNase Inhibitor
66.6 µl DNA template (15ug) (from previous step)
493.4 µl DEPC

6

Incubate in RNase free heat block at **37 °C** , **Overnight** .

DNase Treatment

7

Take 10µL sample **pre**-DNase, mixed with **10 µl 2x formamide dye** for analytical gel.

2x Formamide Dye can be made as follows:
20mL formamide
2.2mL of 100mM (10mM final concentration) EDTA
A spatula tip-ful of powdered xylenecyanol
A spatula tip-ful of powderedbromophenol blue

8

Add **100 µl 10x DNase Buffer** to each 1mL reaction.

9

Add **50 µl RNase-Free DNase** to each 1 ml reaction.

10

Return samples to RNase-Free heat block and incubate at **37 °C** for **00:30:00** .

11

Spin down **4 °C** , **00:08:00** , **15000 x g** to pellet pyrophosphate.

12

Move 3mL to a new tube. Add **3 ml 2x formamide dye** .

13

Keep **On ice** until Gel Purification.

Reaction can be frozen at -80°C.

14

Take 10µL sample **post**-DNase, mixed with **10 µl 2x formamide dye** for analytical gel.

Gel Purification

15 

Cast large 15% Urea-PAGE gel:

- **250 ml 15% Urea-PAGE**
- **250 µl TEMED**
- **833 µl 30% APS**

16 Pre-run, 25 W for about **00:30:00** or until metal plate is warm.

17 

Heat denature samples at **65 °C** , **00:10:00** .

18 Load each sample into separate wells.

19 Run at 45W for **03:00:00** to **04:00:00** .

20 Take gel out and sandwich between saran wrap.

21 Use UV shadowing to identify RNA bands.

22 Highlight bands with sharpie.

23 With clean razor blade, cut out bands and place in 50 ml Falcon tube.

24 Crush gel pieces with RNase Free serological pipette.

25 

Add **14.5 ml DEPC** , **500 µl NaOAc** .

Want: 5mL of DEPC/NaOAc per 1mL of IVT reaction and 1/30 NaOAc.

26 

Centrifuge **4 °C** , **00:10:00** , **20000 x g** .

27 Rock at **4 °C** , **Overnight** .

RNA Elution

28 Filter sample through 0.22µm Corning Filter into 50mL tubes and keep **On ice**

29 Move samples to 15mL Amicon 3kDa MWCO Concentrators.

30 

Centrifuge **4 °C** , **4000 rpm** , ~ **01:00:00** .

Want 1mL each reaction.

31 

Split each into 3 Eppendorf tubes of **333 µl** .

32 

Add 0.3 volumes **3 Molarity (M) NaOAc** (100uL) to each sample.

33 

Add **1 ml Chilled 100% EtOH** (3 volumes) to each sample.

34 Chill in **-80 °C** at least **02:00:00** .

35 

Centrifuge samples **4 °C** , **00:30:00** , **20000 x g** .

Do Fast Temp before to make sure it's at **4 °C** as soon as samples go in.

36 

Pipette supernatant off.

37 

Add **200 µl** **70% EtOH** to wash pellet.

38 Combine with other pellets of the same sample.

39 

Centrifuge **4 °C** , **00:10:00** , **20000 x g** .

40 

Pipette off supernatant.

41 

Repeat wash step: add **200 µl** **70% EtOH** per pellet in sample.

42 

Centrifuge **4 °C** , **00:10:00** , **20000 x g** .

43 

Pipette off supernatant.

44 Place eppendorf with pellet on **37 °C** heat block with the lid open and a kim-wipe on top for **00:30:00** - **01:00:00** .

45 

Resuspend each sample pellet in **200 µl** - **300 µl** **DEPC total** .

46 

Nanodrop RNA.

47 Label and put RNA at **-80 °C** .

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