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DDO in vitro transcription V.1

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol for in vitro transcription of DDO and QC

Materials

REAGENT	SOURCE	IDENTIFIER
EcoRI-HF Buffer CutSmart	BioLabs	Cat#R3101S
MEGAscript® T7 Transcription Kit	Ambion Thermo Fisher Technologies	Cat#1334
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen	Cat#10777-019
EDTA	Invitrogen	Cat# 15575-038

Perform all steps with RNase-free reagents in RNase-free tubes in the hood after surface decontamination with RNase Zap.

I. Plasmid Preparation

1 2h 30m

A. Linearization: in a PCR microtube

- 5 µg of rDVG plasmid
- 5 µL CutSmart Buffer BioLabs
- 5 µL EcoRI-HF BioLabs
- H_2O up to 50 µL

Incubation time: overnight in 37 °C using a PCR machine.

B. Plasmid DNA Precipitation: Transfer to 1.5 mL RNase free tube and add:

- 4 µL Linear acrylamide
- 2.5 µL [M] 0.5 Molarity (M) EDTA pH 8 (1/20 volume)
- 5 µL [M] 3 Molarity (M) ammonium acetate (1/10 volume)
- 100 µL COLD 100% ethanol (2 volume)

- Mix well and incubate -20 °C for 02:00:00 to ensure plasmid precipitation
- Centrifuge 00:20:00 at maximum speed at 4 °C
- Remove supernatant and add 500 µL 70% COLD ethanol
- Centrifuge 00:05:00 at maximum speed at 4 °C
- Remove supernatant; resuspend in 10-20 µL RNase free H_2O (based on the size of the pellet)
- Place the tube 00:05:00 at 65 °C on heating block
- Measure DNA concentration

II. In vitro transcription with NEB Standart RNA Synthesis kit (E2040S):

2 6h 15m

- Spin-down all reagents
- Keep the 10X Reaction Buffer at room temperature while assembling the reaction.
- Vortex all solutions until they are completely in solution.
- Assemble dNTP mix. (Amount per sample) ***Store the ribonucleotides on ice!***

- dATP ([M] 75 micromolar (µM)) 2 µL
- dCTP ([M] 75 micromolar (µM)) 2 µL
- dGTP ([M] 75 micromolar (µM)) 2 µL

- dUTP ([M] 75 micromolar (µM)) 2 µL

• Assemble the reaction in an appropriate RNase free PCR tube:

1) 1 µg DNA plasmid (digested)
2) 8 µL NTP Mix
3) 2 µL 10X Reaction Buffer
4) 1 µL DTT
5) 2 µL T7 enzyme mix
6) RNase Free water up to 20 µL

- Pipette the mixture up and down gently and microfuge
- Incubate Overnight at 37 °C using a PCR machine. The shorter the ivtRNA, the longer the reaction time needed.
- Template plasmid Digestion: add 70 µL nuclease free water, 10 µL of 10X DNase I buffer and 2 µL DNase I. Mix well, and incubate 00:15:00 at 37 °C

III. RNA precipitation:

3 Transfer to 1.5 mL RNase free tube and add: 2h 5m

- 4 µL Linear acrylamide
- 30 µL LiCl

Mix well, incubate 02:00:00 at -20 °C

• Centrifuge at 4°C/20 min/max
• Remove supernatant
• Add 1 mL 70% COLD ethanol
• Centrifuge at 4°C/5 min/max
• Remove supernatant and allow the pellet to dry
• Resuspend in 15-20 µL RNase-free H_2O
• Place the tube 00:05:00 at 65 °C water bath
• Dilute 1 µL in 9 µL H_2O to measure concentration

III. RNA precipitation:

4 **Alternative to RNA purification: Monarch RNA Cleanup Kit. NEB #T2030**

Before to start

- The standard protocol outlined below will purify RNA ≥ 25 nt. A simple modification in Step 2 can allow for the purification of RNA as small as 15 nt.
- Centrifugation should be carried out at 16,000 x g in a standard laboratory microcentrifuge at room temperature.
- Add 4 volumes of ethanol (>95%) to one volume of RNA Cleanup Wash Buffer

1. Add 100 µL Cleanup Binding Buffer to the 50 µL sample. A starting sample volume of 50 µL is recommended. For smaller samples, nuclease-free water can be used to adjust the volume. For samples larger than 50 µL, scale buffer volumes accordingly. Samples with a starting volume > 150 µL will require reloading of the column during Step 3.
2. Add 150 µL (1 volume) of ethanol (≥ 95%) to your sample and mix by pipetting or flicking the tube. Do not vortex. This will enable the binding of RNA ≥ 25 nt. If you wish to bind RNA as small as 15 nt, add 2 volumes (300 µL) of ethanol to your sample instead of 1 volume (150 µL). The addition of 2 volumes of ethanol shifts the cutoff size of RNA binding from 25 nt down to 15 nt.
3. Insert column into collection tube, load sample onto column and close the cap. Spin for 00:01:00, then discard flow-through. For diluted samples > 900 µL, load a portion of the sample, spin, and then repeat as necessary.
4. Re-insert column into collection tube. Add 500 µL Cleanup Wash Buffer and spin for 00:01:00. Discard the flow-through.
5. Repeat wash (Step 4).
6. Transfer column to an RNase-free 1.5 mL microfuge tube (not provided). Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-spin for 00:01:00 to ensure traces of salt and ethanol are not carried over to the next step.
7. Elute in 20 µL nuclease-free water.
8. Place the tube 00:05:00 at 65 °C water bath
9. The eluted RNA can be used immediately or stored at -80 °C. Care should be used to ensure the elution buffer is delivered onto the matrix and not the wall of the column to maximize elution efficiency.

Lopez Lab Specifics: Characterization: To check QC (size and degradation) and endotoxins.

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- For QC

https://research.wustl.edu/core-facilities/genome-technology-access-center/

Person in charge: Michael Heinz **mheinz@wustl.edu**
4444 Forest Park Ave. 04140
Online submission form: https://gtac-old.wustl.edu/request/service/index.php
Quantity: 30 ng in 10 µL nuclease free H_2O.
Shipment: 1.7-2.0 mL microcentrifuge tubes / dry ice

- For Endotoxins

https://research.wustl.edu/core-facilities/biologic-therapy-core-facility-siteman-cancer-center/

Person in charge: Kelsi Rotter **krotter@wustl.edu**
Southwest Tower 00719
Quantity: 500 ng in 150 µL nuclease free H_2O.
Shipment: 1.5 mL microcentrifuge (new plasticware certified to be pyrogen free) / dry ice

Protocol references

From plasmid DNA obtained by Promega PureYield Plasmid Miniprep System

Modified by Victoria Gnazzo from: IVT (in vitro transcription) STAR method_DF