

Jan 05, 2025

Version 2

DDO in vitro transcription V.2

DOI

<https://dx.doi.org/10.17504/protocols.io.3byl49qprgo5/v2>

Carolina Lopez¹

¹Washington University



Carolina Lopez

Washington University

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.3byl49qprgo5/v2>

Protocol Citation: Carolina Lopez 2025. DDO in vitro transcription. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.3byl49qprgo5/v2> Version created by **Carolina Lopez**

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: December 20, 2024



Last Modified: January 05, 2025

Protocol Integer ID: 116764

Keywords: transcription protocol for in vitro transcription, vitro transcription, transcription protocol, ddo

Abstract

Protocol for in vitro transcription of DDO and QC

Materials

Plasmid Linearization with EcoRI

- The DNA template containing the promoter sequence (e.g., T7) upstream of the target sequence.
- EcoRI Restriction Enzyme
- Buffer for EcoRI
- Nuclease-free Water
- Thermal Cycler/Water Bath/Incubator
- Gel Electrophoresis Kit

In Vitro Transcription

- NEB HiScribe IVT Kit Components: NTP Mix: ATP, CTP, GTP, UTP; T7 RNA Polymerase Mix; Reaction buffer
- Linearized DNA Template
- Nuclease-free Water
- Incubator or Thermocycler
- DNase I
- RNA Cleanup Kit
- Nanodrop
- Bioanalyzer

Additional Materials

- Pipettes and Tips
- RNase-free Environment



I. Plasmid Preparation

1 A. Linearization: in a PCR microtube

2h 30m

- 5 µg of rDVG plasmid
- 5 µL CutSmart Buffer BioLabs
- 5 µL EcoRI-HF BioLabs
- H₂O 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 0.5 Molarity (M) EDTA pH 8 (1/20 volume)
- 5 µL 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 H₂O (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 • Spin-down all reagents

6h 15m

- 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 (75 micromolar (µM)) 2 µL
- dCTP (75 micromolar (µM)) 2 µL
- dGTP (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 H₂O
- Place the tube 00:05:00 at 65 °C water bath
- Dilute 1 μL in 9 μL H₂O 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 $\geq$ 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 μ L Cleanup Binding Buffer to the  50 μ L sample. A starting sample volume of  50 μ L is recommended. For smaller samples, nuclease-free water can be used to adjust the volume. For samples larger than 50 μ L, scale buffer volumes accordingly. Samples with a starting volume > 150 μ L will require reloading of the column during Step 3.
2. Add  150 μ L (1 volume) of ethanol ($\geq$ 95%) to your sample and mix by pipetting or flicking the tube. Do not vortex. This will enable the binding of RNA $\geq$ 25 nt. If you wish to bind RNA as small as 15 nt, add 2 volumes (300 μ L) of ethanol to your sample instead of 1 volume (150 μ L). 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 μ L, load a portion of the sample, spin, and then repeat as necessary.
4. Re-insert column into collection tube. Add  500 μ L 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 μ L 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.

- 5
 - 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 H₂O.
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 H₂O.
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