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How to make Tol2 mRNA

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Abstract

These are instructions to make highly concentrated (> 1000 ng/µL) Tol2 mRNA. Note, the in-vitro transcription kit (mMESSAGE mMACHINE) is not cheap. 5–6 reactions like the protocol suggests come to ~ 100–120£, so please be thrifty with it.

Linearise the Tol2 plasmid

1 Find the Tol2 plasmid from the Wilson lab freezer. It is #1151 and is labelled Tol2.

The concentration (measured on Qubit) is ~ 690 ng/µL (as of the Eppendorf in 2022).

Thaw it.

2 We linearise the plasmid using the restriction enzyme NotI HiFi.

On ice, prepare 6 reactions in 0.5 mL Eppendorfs.

Each reaction is (pipet in this order):
- Tol2 plasmid 2.9 µL
- NotI HiFi 2 µL
- CutSmart buffer 10× 10 µL
- nuclease-free H2O 91.1 µL

Total = 100 µL

(Add the enzyme last).

3 Place the Eppendorfs in the 37˚C water bath.

Incubate for at least 1 hour. Digesting for more than 1 hour may be beneficial (cf. https://international.neb.com/tools-and-resources/usage-guidelines/restriction-endonucleases-survival-in-a-reaction).

4 Clean (remove the enzyme etc.) the reaction using the QIAquick PCR Purification Kit. Similar column-based kits are probably OK.

Follow the protocol in the box or online.

Use a single column.

For the first step (adding 5 volumes of PB buffer): transfer each 3 reactions in a 2 mL Eppendorf to obtain two 2 mL Eppendorfs, each containing 300 µL of reaction.

Add 1500 µL (5 volumes) of PB buffer. Vortex and spin down (with the benchtop centrifuge).

Add ~ 700 µL on the column and spin down following the kit's instructions (big centrifuge). Discard flow-through. Repeat until you got all 600 µL of reaction (the two 2 mL Eppendorfs) through the column.

5 At the last step of the kit's protocol, elute in 30 µL nuclease-free H2O.

Measure the concentration on the Qubit (dsDNA BR or HS kit). We expect the concentration to be 200–400 ng/µL *.

Note

* Logic is: each reaction had ~ 2,000 ng of plasmid (2.9 µL of 690 ng/µL) and we did 6 reactions, so 12,000 ng total. If you lost 0% on the column, you will get 12,000 ng in 30 µL, i.e. 400 ng/µL. If you lost 50% on the column, you will get 6,000 ng in 30 µL, i.e. 200 ng/µL.

In-vitro transcription

6 Next step is to turn the linearised Tol2 plasmid (DNA) into mRNA. Find the SP6 mMESSAGE mMACHINE kit.

! Make sure you are using the SP6 version.

In my experience, each reaction generates 3,000–4,000 ng mRNA. Therefore, to get ~ 12 µL of > 1,000 ng/µL, you will need at least 3 reactions. This is assuming a good output and losing nothing on the column during clean-up, so I try to do 5–6 reactions for critical injections where I need high integration rate directly in F0 injected embryos.

Prepare each reaction on ice in a small 0.5 mL Eppendorf.

Each reaction is:

- 2× NTP/CAP 10 µL
- 10× reaction buffer 2 µL
- linearised Tol2 plasmid ***
- enzyme mix 2 µL
- nuclease-free H2O *to 20 µL*

*** Calculate from the concentration found in the previous step so that you use 1 µg.
e.g. I found the concentration to be 343 ng/µL, so I use 2.91 µL here.

7 Place the Eppendorfs in the 37˚C water bath.

Incubate for 2 hours.

There is no benefit in incubating for more than 2 hours (cf. reaction time course: https://www.thermofisher.com/order/catalog/product/AM1340#:~:text=mMESSAGE%20mMACHINE%E2%84%A2%20kits%20are,structure%20at%20the%205'%20end.). Should be OK if reaction lasts a bit longer if for whatever reason you cannot stop it in time.

8 Add 1 µL TURBO DNase in each reaction (included in the kit). This is removes the DNA, i.e. the linearised plasmid.

Incubate in the 37˚C water bath for 15 min.

9 Clean-up the mRNA using the ZYMO RNA Clean & Concentrator kit.

Follow the kit's instructions. Use a single column.

At the end, elute in 13 µL nuclease-free H2O. Add the 13 µL directly on the column's filter and wait for a few minutes at room temperature.

Heating the nuclease-free H2O to 70˚C prior to adding it to the water may also help with retrieving as much as possible mRNA from the column, but I have never tried.

(You can always dilute later if you find that the Tol2 mRNA is highly concentrated, so best to elute in a small volume.)

10 Measure the concentration on Qubit (BR or HS RNA kit). Qubit can measure low concentrations, so no need to waste precious Tol2 mRNA. You can e.g. dilute 10×:

- 0.4 µL Tol2 mRNA
- 3.6 µL nuclease-free H2O

and measure that concentration. Just remember to multiply the concentration you find by 10.

You are expecting (hoping) > 1,000 ng/µL. For reference, if each of 6 reactions produced 3000 ng and nothing is lost on the column, you would expect ~ 1384 ng/µL (6 × 3000 ng, eluted in 13 µL).

11 Aliquot the ~ 13 µL in 3–4 small 0.5 mL Eppendorfs and store in the –80˚C freezer.