Tol2 mRNA preparation protocol

1. Linearized Tol2 plasmid preparation
   a) pSP6-Tol2 (From He Jie' lab) miniprep, **Concentration > 200 ng/μL**;
   b) NotI digestion:
      i. 50μL reaction mixture, 2ug of template plasmid DNA
      ii. Digestion rate check via agarose gel electrophoresis[1]
      iii. Column purification, recovery concentration >250ng/μL[2];
   c) PCR isolation
      i. …
2. *In vitro* transcription
   a) Thaw the frozen reagents
      i. Enzyme Mix: not be frozen, brief centrifugation, then **place on ice**;
      ii. 10x Reaction mix: vortex till thawed, then keep it **at room temperature**[3].
      iii. 2x NTP/CAP: vortex till thawed, then **place on ice**.
   b) Assemble transcription reaction at room temp.
      i. Add solutions to PCR tube following order in the table bellow

Amount	Component
to 20 μL	Nuclease-free Water
10 μL	2X NTP/CAP
2 μL	10X Reaction Buffer
(1 μL)	(optional) [α-32P]UTP as a tracer
0.1–1 μg	linear template DNA†
2 μL	Enzyme Mix [4]

      ii. Mix thoroughly by pipetting the mixture up and down gently, and then microfuge tube briefly.
   c) Incubate at 37℃, **2hr**
   *d) Add **1μL** TURBO DNase, mix well and incubate 15min at 37°C.*
3. Recovery of RNA -- Lithium chloride precipitation[5]
   a) Add 30uL LiCl precipitation solution and 30uL Nuclease-free water;
   b) Mix thoroughly, chill for ⩾30 min at -20℃;
   c) Centrifuge at 4℃ for 15min at max speed to pellet the RNA;
   d) Carefully remove the supernatant. Wash the pellet once with 1mL 70% ethanol, and re-centrifuge to maximize removal of unincorporated nucleotides.
   e) Carefully remove the 70% ethanol[6], air dry in clean hood. Resuspend the RNA in 50uL of Nuclease-free water. Determine the RNA concentration via Nanodrop and AGE (agarose gel electrophoresis)
   f) Aliquot Tol2 mRNA into 100ng/uL, 1uL per tube. Store at -80℃.

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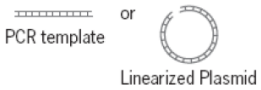
[1] 必须过夜酶切完全，少量残存的未酶切质粒将很大程度影响体外转录产物。
[2] The suggested template concentration is 0.5ug/uL in water or TE (AM1340 kit user guide).
[3] The spermidine in the 10X Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.
[4] Use 0.1-0.2ug PCR-product or ~1ug linearized plasmid template.
[5] LiCl precipitation for RNAs >300nt, >0.1ug/uL.
[6] 超净台吹干至透明.

Preparation of template DNA



Capped transcription reaction assembly

1. "Thaw the frozen reagents" on page 11
2. "Assemble transcription reaction at room temp" on page 11
3. "Mix thoroughly" on page 11
4. "Incubate at 37°C, 1 hr" on page 11
5. "(optional) Add 1 µL TURBO DNase, mix well and incubate 15 min at 37°C" on page 12

Recovery of the RNA

1. "MEGAclear™ Kit" on page 12
2. "Lithium chloride precipitation" on page 12
3. "Spin column chromatography" on page 12
4. "Phenol:chloroform extraction and isopropanol precipitation" on page 13