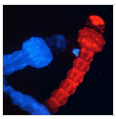

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Transposase injection mix protocol

Ben Matthews[1]

[1]University of British Columbia

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Ben Matthews
University of British Columbia

ABSTRACT

This protocol details the steps necessary to generate injection mixes (comprised of plasmid DNA for integration and transposase mRNA) for transposon-mediated integration into *Aedes aegypti* and other insects. It has been empirically tested in *Ae. aegypti* embryos with piggyBac transposase. In our hands, using mRNA as a source of transposase instead of a helper plasmid increases transformation rates substantially.

Transposase injection mix protocol.pdf

GUIDELINES

This protocol includes the following sections:

1. Purifying injection-ready integration plasmid (Steps 1-5)
2. Generating transposase mRNA by *in vitro* transcription: template PCR (Steps 6-12)
3. Generating transposase mRNA by *in vitro* transcription: IVT (Steps 13-21)
4. mRNA cleanup (Steps 22-25)
5. mRNA quantitation and sizing verification (Steps 26-29)
6. Injection mix preparation (Steps 30-33)

MATERIALS

NAME	CATALOG #	VENDOR
HiScribe T7 ARCA mRNA Kit (with Tailing) - 20 rxns	E2060S	New England Biolabs
Agencourt RNAClean XP SPRI beads	A63987	Beckman Coulter
NucleoBond Xtra Midi EF plasmid purification kit	740420.10	Macherey and Nagel

MATERIALS TEXT

- NucleoBond Xtra Midi EF plasmid purification kit - Machery Nagel item # 740420.10
  Protocol: Machery Nagel Nucleobond EF
- HiScribeTM T7 ARCA mRNA Kit (with tailing) - New England Biolabs (NEB) item # E2060S
  Protocol: HiScribe T7 ARCA mRNA kit
- Standard PCR reagents
- Magnet stand compatible with 1.5mL Eppendorf tubes
- Nuclease free water

Kits can almost certainly be substituted for similar versions from other manufacturers, but these have been empirically tested in *Ae. aegypti* and generates plasmid DNA and mRNA that require no further cleanup beyond the steps outlined here.

SAFETY WARNINGS

For Safety Warnings and Hazard Information please refer to the SDS (Safety Data Sheet) for each kit.

BEFORE STARTING

All steps should be performed under RNAse-free conditions, with special care taken to do the final resuspension of plasmid and all steps of *in vitro* transcription with a separate set of RNAse- free pipettes/tips/tubes, and ideally on a separate bench from any bacterial work.

Purifying injection-ready integration plasmid

1 Perform a midiprep of the integration plasmid from a **50 ml** overnight culture of your transposon integration plasmid.

2 Follow the kit protocol (if using recommended kit: Machery Nagel Nucleobond EF)

Be sure to follow the instructions precisely for all endotoxin-removal steps.

3

Fully dry the final pellet, and re-suspend in **50 µl nuclease free water**, with nuclease free pipettes and tips.

4

Quantitate by NanoDrop or Qubit and record final concentration.

Should be > 1 µg/µL - if much higher, should be diluted with nuclease free water to ~2 µg/µL and re-quantified.

5 Aliquot and store at **-80 °C** (unless preparing injection mix same-day, in which case, plasmid can be left on ice).

If using the kit recommended here, plasmid is injection ready and needs no additional cleanup (at least for *Ae. aegypti*).

Generating transposase mRNA by *in vitro* transcription: template PCR

6

PCR:

To generate DNA template for IVT, perform PCR from a plasmid containing transposase cDNA. The primer sequences below represent an amplification strategy for wild-type or a hyperactive form of piggyBac transposase. To generate template for a different transposase (eg Mos), add the T7 polymerase initiation sequences and a linker to the 5' end of a forward primer corresponding to the first 20-30 bp of the transposase sequence. Use an appropriate primer to match the reverse complement of the last 20-30 bp of the transposase sequence.

pBac forward primer (***bold/italic*** represents T7 initiation sequence and necessary linkers):

GAAACTAATACGACTCACTATAGGGAGAGCCGCCACATGGGTAGTTCTTTAGACGATG

pBac reverse primer (wild-type PBac, our plasmid obtained from ITF/Rob Harrell):
CTTATTAGTCAGTCAGAAACAAC

Alternate pBac reverse primer:
TCAGAAACAACTTTGGCACATATCA

This reverse primer can be used when amplifying cDNA for a hyperactive PBac, described in Otte et al., 2018.

M. Otte, O. Netschitailo, O. Kaftanoglu, Y. Wang, R. E. Page Jr. & M. Beye (2018). Improving genetic transformation rates in honeybees.. Scientific Reports.
http://10.1038/s41598-018-34724-w

6.1 Perform a PCR reaction (scaled to 100 µL), using ~1 ng plasmid as template.

I use KOD, but any polymerase should work. Adjust time and temperature accordingly. For KOD, I used an annealing temperature of 56° and extension time of 40s.

6.2 Run an agarose gel to verify the presence and appropriate size of the template.

7 To cleanup product, perform magnetic bead purification with RNAClean XP beads as described in the following sub-steps.

All following steps should be performed with nuclease free water/tips/tubes/pipettes!!

7.1 Combine 1.5x volume of RNAClean XP beads to the PCR reaction in a 1.5 ml eppendorf tube.

7.2 

Vortex to mix well.

7.3 

Incubate for **00:05:00** at **Room temperature**.

7.4 Place on magnet stand until solution is clear; approximately **00:05:00** .

7.5 Remove supernatant.

7.6 

Rinse beads with **300 µl freshly made 80% EtOH**. (1/2)

Use nuclease-free alcohol and water!

7.7 Let stand for **00:00:30** - **00:01:00** . (1/2)

7.8 Remove supernatant. (1/2)

7.9

Rinse beads with **300 µl freshly made 80% EtOH**. (2/2)

Use nuclease-free alcohol and water!

7.10 Let stand for **00:00:30** - **00:01:00** . (2/2)

7.11 Remove all remaining ethanol - switch to 10 µL tip! - and air dry for **00:10:00** until pellet is completely dry.

8 Remove from magnet stand and re-suspend bead pellet in **20 µl nuclease free water**.

9

Incubate **00:10:00** at **Room temperature** and then return to magnet stand.

10 Once solution has fully cleared (~ **00:05:00**), carefully transfer **18 µl supernatant** with a 10 µL tip to a separate tube.

11

Check concentration with a spectrophotometer (Nanodrop) or Qubit.

Hopefully >250 ng/µL!

12 Proceed to *in vitro* transcription (IVT) - any leftover template can be stored at **-20 °C** and used for subsequent IVT reactions.

Generating transposase mRNA by *in vitro* transcription: IVT

13

All following steps should be performed with nuclease free water/tips/tubes/pipettes!!

Perform 20 µL IVT reaction per NEB protocol (briefly descipbed in the following steps).

14

Add reagents in the following order.

14.1 Nuclease free water (to 20 µL total reaction volume)

14.2 **10 µl 2X ARCA/NTP Mix**

14.3 **2 µl PCR template**

2 µL - should be 500-1000 ng

14.4 **2 µl T7 RNA Polymerase Mix**

15

Mix thoroughly and pulse-spin to collect.

16

Incubate for **00:30:00** at **37 °C** .

17

Add **2 µl DNAse I**.

18

Mix thoroughly by pipetting up and down.

19

Incubate for **00:15:00** at **37 °C**.

20 Remove **1 µl** and save on ice for sizing and integrity analysis.

21 

Add the following reagents.

21.1 **66 µl nuclease free water**

21.2 **10 µl 10x Poly(A) polymerase reaction buffer**

21.3 **5 µl Poly(A) polymerase**

22 

Incubate for **00:30:00** at **37 °C** .

23 Proceed immediately to cleanup and validation.

mRNA cleanup

24 Cleanup *in vitro* transcription reaction with RNAClean XP beads as described in the following substeps.

24.1 

Combine 1.5x volume of RNAClean XP beads to the PCR reaction in a 1.5 ml eppendorf tube.

24.2 

Vortex to mix well.

24.3 

Incubate for **00:05:00** at **Room temperature**.

24.4 Place on magnet stand until solution is clear; approximately **00:05:00** .

24.5 Remove supernatant.

24.6 

Rinse beads with **300 µl freshly made 80% EtOH**. (1/2)

Use nuclease-free alcohol and water!

24.7 Let stand for **00:00:30** - **00:01:00** . (1/2)

24.8 Remove supernatant.

24.9

Rinse beads with **300 µl freshly made 80% EtOH**. (2/2)

Use nuclease-free alcohol and water!

24.10 Let stand for **00:00:30** - **00:01:00** . (2/2)

24.11 Remove all remaining ethanol - switch to 10 µL tip! - and air dry for **00:10:00** until pellet is completely dry.

25 Remove from magnet stand and re-suspend bead pellet in **55 µl nuclease free water**.

26

Incubate **00:10:00** at **Room temperature** and then return to magnet stand.

27 Once solution has fully cleared (~ **00:05:00**), carefully transfer **50 µl supernatant** with a 10 µL tip to a separate tube.

mRNA quantitation and sizing verification

28

Quantitate using spectrophotometer (Nanodrop) or Qubit.

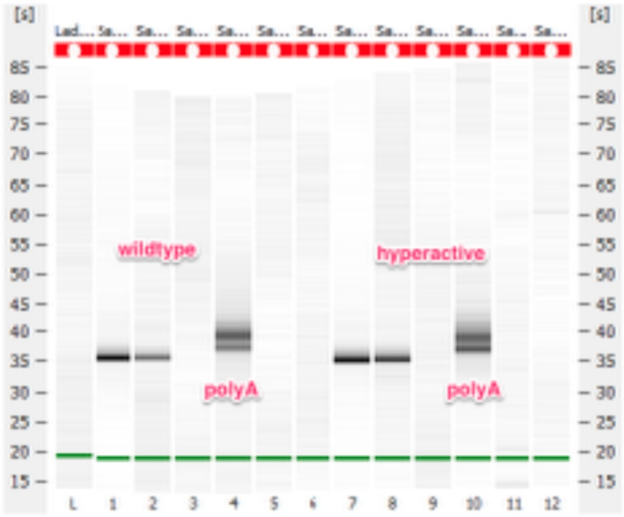
A successful IVT reaction should generate 50 µL of purified product at >400 ng/µL.

29 Run **1 µl final mix** (alongside **1 µl reserved pre-poly(A)-tailing**) on a Bioanalyzer, Tapestation, or agarose gel with an RNA ladder.

30 Things to look for:

- Pre-poly(A)-tailing: a single, bright band with no sign of degradation products

- Post-poly(A)-tailing: one or multiple bands, broader in size distribution but all product should be longer than the pre-tailing product



This image is a Bioanalyzer gel representing wild-type (lanes 1, 2, and 4) and hyperactive PBac transposase (lanes 7, 8, and 10). Lanes 4 and 10 are post-poly(A)-tailing procedure.

Note the increased size of tailed product and the single coherent band in the pre-tailed product, with no evidence in either lane of degradation products.

This procedure should generate >20 µg of injection-ready mRNA.

31 Proceed immediately to final injection mix preparation. Any leftover mRNA should be aliquoted and stored at **-80 °C**.

Final injection mix preparation

32 Combine mRNA and donor plasmid at final concentrations of 300 ng/µL each (or any other desired concentration). Any necessary dilution must be done with nuclease free water or nuclease free injection buffer, as appropriate. For *Ae. aegypti*, we use water.

33

Aliquot injection mix into **5 µl** aliquots in small nuclease-free tubes.

34 Freeze at **-80 °C**.

35 Keep injection mixes frozen until immediately before injection. Thaw a single aliquot on ice for an injection session, and do not re-freeze.