Overview:

This protocol details the steps necessary to generate injection mixes for transposon-mediated integration into *Aedes aegypti* and other insects. It has been empirically tested in *Ae. aegypti* embryos with piggyBac transposase.

Specific materials:

NucleoBond Xtra Midi EF plasmid purification kit - Machery Nagel item # 740420.10
Protocol: Machery Nagel Nucleobond EF
HiScribe™ T7 ARCA mRNA Kit (with tailing) - New England Biolabs (NEB) item # E2060S
Protocol: HiScribe T7 ARCA mRNA kit
RNAclean SPRI XPbeads - Beckman Coulter item # A63987
Magnet stand compatible with 1.5mL Eppendorf tubes

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.

Notes:

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.

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Purifying injection-ready integration plasmid

- Perform a midiprep of the integration plasmid from a 50mL overnight culture of your transposon integration plasmid.
- Follow the kit protocol (if using recommended kit: Machery Nagel Nucleobond EF)
  - Be sure to follow the instructions precisely for all endotoxin-removal steps.

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

- 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 ~2μg/μL and re-quantified).

- 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:

PCR:

To generate DNA template for IVT, perform PCR from a plasmid containing transposase. 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 20-30bp of the transposase sequence. Use an appropriate primer to match the reverse complement of the last 20-30bp of the transposase sequence.

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

pBac reverse primer (wild-type PBac, from ITF/Rob Harrell):
CTTATTAGTCAGTCAGAAACAAC
Alternate pBac reverse primer (hyperactive PBac, obtained from Martin Beye):
TCAGAAACAACTTTGGCACATATCA

- Perform a PCR reaction (scaled to 100μL), 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.

- An agarose gel should be run to verify the presence and appropriate size of the template

- To cleanup product, perform magnetic bead purification with RNAClean XP beads as follows:

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

- Combine 1.5x volume of RNAClean XP beads to the PCR reaction in a 1.5ml eppendorf tube
- Vortex to mix well
- Incubate for 5min at room temperature
- Place on magnet stand until solution is clear; approximately 5 minutes
  - Remove supernatant
  - Rinse beads with 300μL freshly made 80% EtOH (use nuclease-free alcohol and water!)
  - Let stand for 30-60s
  - Remove supernatant and repeat rinse step (for a total of two rinses)
  - Remove all remaining ethanol - switch to 10μL tip! - and air dry for 10 min until pellet is completely dry

- Remove from magnet stand and re-suspend bead pellet in 20μL of nuclease free water
- Incubate 10 minutes at room temperature and then return to magnet stand
- Once solution has fully cleared (~5 min), carefully transfer 18μL supernatant with a 10μL tip to a separate tube
- Check concentration with a spectrophotometer (Nanodrop) or Qubit (hopefully >250ng/μL!)

Proceed to *in vitro* transcription - any leftover template can be stored at -20°C.

In vitro transcription (IVT):

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

Perform 20μL IVT reaction per NEB protocol (briefly reprinted here):

- Add in the following order:
  - Nuclease free water (to 20μL total reaction volume)
  - 2X ARCA/NTP Mix (10μL)
  - PCR template (2μL - should be 500-1000ng)
  - T7 RNA Polymerase Mix (2μL)
- Mix thoroughly and pulse-spin to collect.
- Incubate for 30min @ 37°
- Add 2μL of DNAse I, mix thoroughly by pipetting up and down, and incubate for 15min at 37°
- Remove 1μL and save on ice for sizing and integrity analysis
- Add the following:
  - nuclease free water (66μL)
  - 10x Poly(A) polymerase reaction buffer (10μL)
  - Poly(A) polymerase (5μL)
- Incubate for 30min @ 37°
- Proceed immediately to cleanup and validation

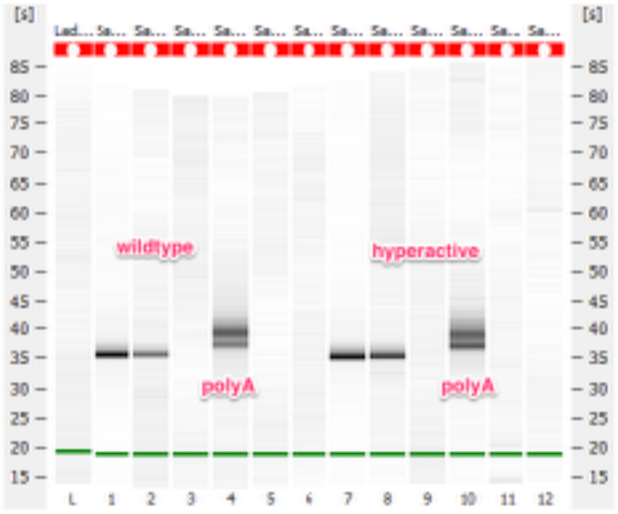
mRNA cleanup:

Cleanup in vitro transcription reaction with RNAClean XP beads as follows:

- Combine 1.5x volume of RNAClean XP beads to the PCR reaction in a 1.5ml eppendorf tube
- Vortex to mix well
- Incubate for 5min at room temperature
- Place on magnet stand until solution is clear; approximately 5 minutes
  - Remove supernatant
  - Rinse beads with 300μL freshly made 80% EtOH (use nuclease-free alcohol and water!)
  - Let stand for 30-60s
  - Remove supernatant and repeat rinse step (for a total of two rinses)
  - Remove all remaining ethanol - switch to 10μL tip! - and air dry for 10 min until pellet is completely dry
- Remove from magnet stand and re-suspend bead pellet in 55μL of nuclease free water
- Incubate 10 minutes at room temperature and then return to magnet stand
- Once solution has fully cleared (~5 min), carefully transfer 50μL supernatant with a 10μL tip to a separate tube

mRNA quantitation and sizing verification:

- Quantitate using spectrophotometer (Nanodrop) or Qubit. A successful IVT reaction should generate 50μL of purified product at >400ng/μL
- Run 1μL of final mix (alongside 1μL reserved pre-poly(A)-tailing) on a Bioanalyzer, Tapestation, or agarose gel with an RNA ladder.
- 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 >20μg of injection-ready mRNA.
- Proceed immediately to final injection mix preparation. Any leftover mRNA should be aliquoted and stored at -80°C.

Final injection mix preparation:

- Combine mRNA and donor plasmid at final concentrations of 300ng/μL each or any other desired combination. Any necessary dilution must be done with nuclease free water.
- Aliquot injection mix into 5μL aliquots in small nuclease-free tubes
- Freeze at -80° and ship on dry ice.
- Keep frozen until immediately before injection.