Construction of mRNA synthesis vectors
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There are two major vectors for *in vitro* mRNA synthesis for Ciona: pBS-RN3 and pBS-HTB. Both plasmids have a 5'UTR and a 3'UTR of a gene and a multicloning site flanked by the UTRs (Figure 1). ORFs can be subcloned into the multicloning sites of the vectors. In the mRNAs synthesized from the constructs, the UTRs sandwich the ORF. The function of the UTRs is suspected to be the enhancement of translation by increasing the translation efficiency and stability of mRNAs.

pBS-RN3 was originally created by Dr. Patrick Lemaire for the synthesis of RNAs in *Xenopus laevis* (Lemaire et al., 1995, Cell 81, 85-94). The vector therefore uses the UTRs of Xenopus globin. pBS-HTB was created by Dr. Hiroki Nishida for use in *Halocynthia roretzi* (Akanuma et al., Dev Genes Evol 212, 459-472). The vector uses the UTRs of *Halocynthia* beta tubulin.

In our experience, mRNAs created from pBS-HTB have better translation efficiency than pBS-RN3 in *Ciona intestinalis* Type A/*Ciona robusta*. Because the difference is significant, we recommend using pBS-HTB for this ascidian (Sasakura et al., 2010, Mol Genet Genomics 283, 99-110).

Dr. Nishida mentioned that translation efficiency seems to be the same between pBS-RN3 and pBS-HTB in *Halocynthia*. This suggests that you must test the appropriate vector for use in another ascidian. We can provide pRN3-eGFP, pHTB-nEGFP, and pHTB-nKaede for the test.

The following explanation is based on pBS-HTB. You can subclone your ORF in the MCS of this vector (Figure 1) by an In-fusion reaction. The MCS includes BamHI, EcoRI, PstI, SmaI, and EcoRV restriction sites. To synthesize mRNAs, you must linearize the vector with XhoI or KpnI/Asp3I. If your ORF has an XhoI or KpnI site, you can subclone the ORF in the MCS of pBS-HTB-N. The vector has a NotI site at the 3' end of the 3'UTR, and you can linearize the vector with NotI. NotI is an 8-base cutter and the recognition site is gcggccgc, which is rarely present in the Ciona genome since the Ciona genome is rich in A and T.

mRNAs can be synthesized with T3 RNA polymerase of the mMessage mMachine transcription kit or with the Megascript T3 kit (Ambion). When using the latter, you may have to use a cap structure analog (S1404L or S1404S from New England Biolabs), as none is supplied in the kit. The use of a poly A tailing kit is optional, but it may enhance translational efficiency (although we did not test its effectiveness). The following protocol is for the Megascript T3 kit.

Linearize 1-2 ug of template DNA with XhoI, Asp3I, or NotI.
Check linearization by electrophoresis.
Extract the DNA twice with phenol−chloroform.
Extract the DNA with chloroform once.
Ethanol precipitation.
Dissolve the linearized template DNA in an appropriate amount of RNase-free water.

Reaction buffer	2 ul
A solution	2 ul
U solution	2 ul
C solution	2 ul
G solution	0.4 ul
Cap structure analog	1.6 ul
1 ug Template DNA + water	8 ul
T3 RNA polymerase	2 ul
Total	20 ul

Incubate for 5 hr at 37 ºC.

Add 1 ul of DNase I (supplied in the kit).
Incubate for 15 min at 37 ºC.

Add the following solutions of Poly A tailing kit (Ambion):

Water	36 ul
5x reaction buffer	20 ul
MnCl2	10 ul
ATP	10 ul

Store 0.5 ul (RNA-A) for later comparison of the length.

Add 4 ul E-PAP, then incubate for 1 hr at 37 ºC.

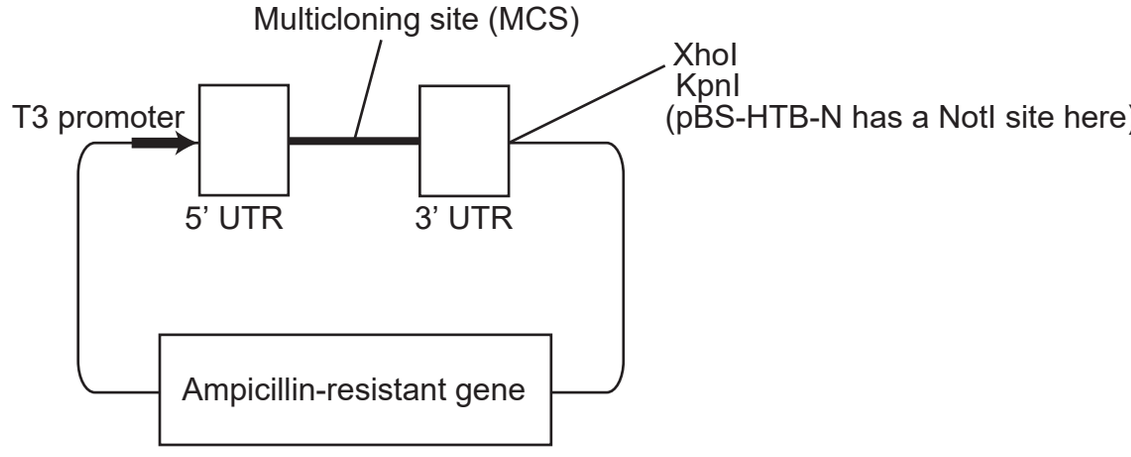
Take 0.5 ul of reacted solution (RNA-B) for electrophoresis. The success of the poly A addition will be seen by the longer RNA in RNA-B relative to that in RNA-A.

Add 50 ul of LiCl precipitation solution supplied in the Megascript kit.

Centrifuge.
Wash three times with 75% ethanol to remove Li^+.
Dry (Caution: never dry for too long).

Dissolve RNA in 10-20 ul water.
Vortex and put on ice.
Repeat this step several times to dissolve the RNA completely.
Measure OD 260.
Divide into aliquots and store in a deep freezer until use.

Figure 1 pBS-HTB and pBS-HTB-N



MCS of pBS-HTB and pBS-HTB-N: