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

pBS-RN3 was originally created by Dr. Patrick Lemaire for synthesizing 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 experiences, mRNAs created from pBS-HTB has better efficiency of translation than pBS-RN3 in Ciona intestinalis Type A/Ciona robusta. The difference is significant and therefore we recommend to use pBS-HTB for this ascidian (Sasakura et al., 2010, Mol Genet Genomics 283, 99-110).

Hiroki mentioned that the efficiency of translation seems to be the same between pBS-RN3 and pBS-HTB in *Halocynthia*. This suggests that you must test the appropriate vector for the 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 In-fusion reaction. The MCS includes BamHI, EcoRI, PstI, SmaI and EcoRV restriction sites. For synthesizing mRNAs, you must linearize the vector with XhoI or KpnI/Asp3I. If your ORF has XhoI or KpnI site, you can subclone the ORF in the MCS of pBS-HTB-N. The vector has NotI site at the 3' end of 3'UTR, and you can linearize with NotI. Because NotI is 8-base cutter and the recognition site is gcggccgc, which is rarely present in the Ciona genome because Ciona genome is rich in A and T.

The synthesis of mRNAs can be done with T3 RNA polymerase of mMessage

machine, or Megascript T3 kit (Ambion). When using latter kit, you may have to use cap structure analog (S1404L or S1404S of New England Biolabs) that is not supplied in the kit. The use of poly A tailing kit is optional but this may enhance translational efficiency (although we did not test the effectiveness). The following protocol is for Megascript T3 kit.

Linearize 1-2 ug of template DNA with XhoI, Asp3I or NotI.
Check linearization by electrophoresis
Twice extraction with Phenol-chloroform
Chloroform extraction once
Ethanol precipitation
Dissolve linearized template DNA in 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

(Tips: Cap structure analog will be supplied in dried form. Add 17.3 ul water to the tube for ready-to-use).

Incubate 5 hr at 37 ºC.

Add 1 ul of DNase I (supplied in the kit)
Incubate 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 comparison of the length afterward.

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

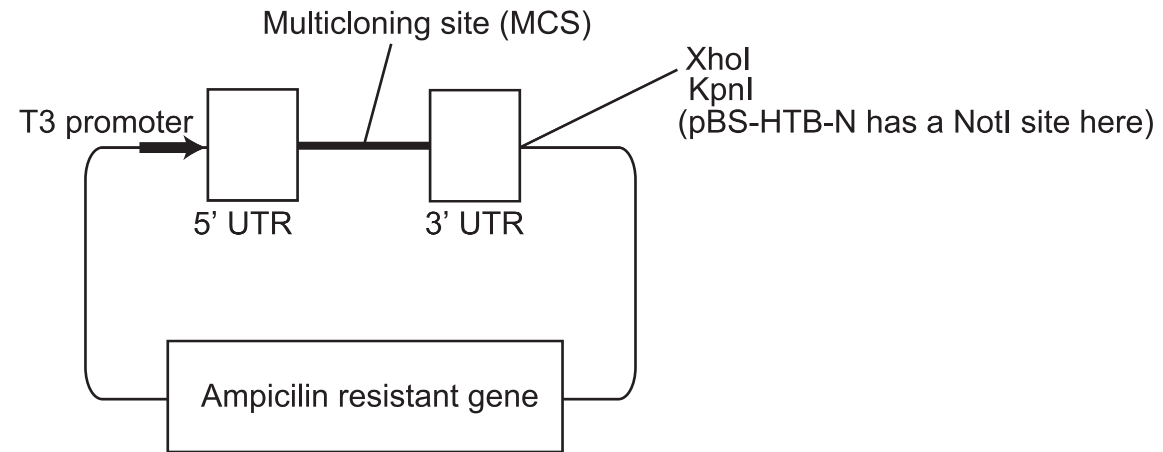
Take 0.5 ul of reacted solution (RNA-B) for electrophoresis. The success of poly A addition will be seen by the longer RNA in RNA-B than RNA-A.

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

Incubate 1 hr or longer in freezer, then Centrifugation.
Wash three times with 75 % Ethanol to remove Li^+.
Dry up (Caution: never dry up too long).

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

Figure 1 pBS-HTB and pBS-HTB-N



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