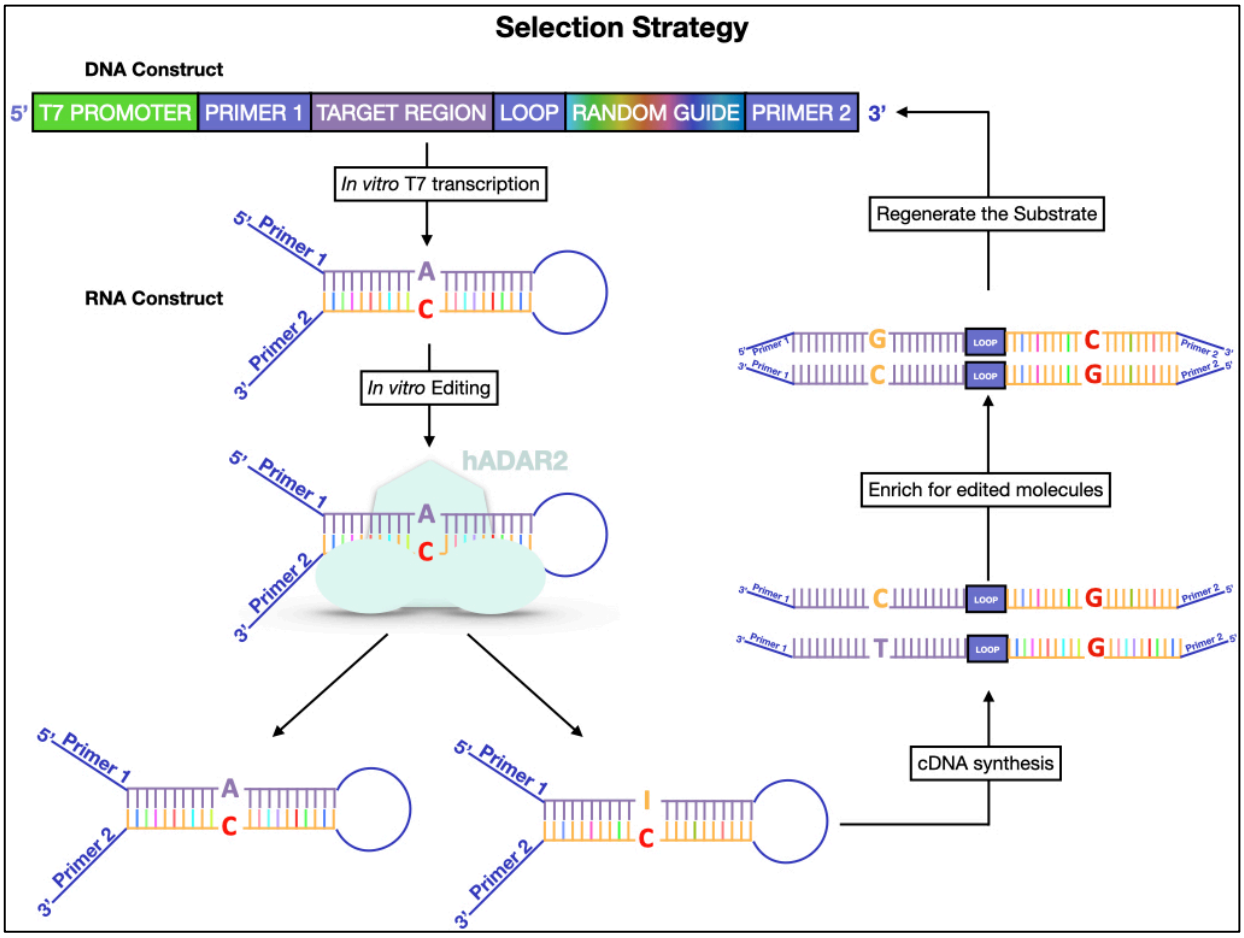
THE UNIVERSITY OF CHICAGO
MARINE BIOLOGICAL LABORATORY

GUIDE SELECTION ASSAY

I. Graphical Abstract



DNA construct design:
The template sequences as well as the primers used are shown in the DNA files.
The original construct was synthetized from two DNA Oligos (Table 1), one containing the target region and another containing the random guide region. The synthesis was done following the *Guide Annealing (step 6.2)* and *Full Length Amplification (step 6.3)* steps later described in the Substrate regeneration section (step 6).

The obtained DNA construct is used as template for RNA synthesis.

II. Protocol

1. RNA synthesis (T7 flash kit CellSCRIPT):

1.1 Set up reaction as follows:

10X T7 Flash Buffer	2.0 µl
100mM ATP	1.8 µl
100mM CTP	1.8 µl
100mM GTP	1.8 µl
100mM UTP	1.8 µl
RNase inhibitor	0.5 µl
T7 Flash enzyme	2.0 µl
100mM DTT	2.0 µl
DNA Template	100-1000 ng
H_2O	up to 20 µl
Total volume	20.0

Incubate at 37˚C x 2 hr

1.2 DNase treatment:

- After the incubation, add 2 µl of DNase I enzyme (from the kit or from NEB) and incubate at 37˚C x 1 hr.
- After the incubation, bring volume to 176 µl by adding 155 µl of nuclease-free water
- Add 20 µl of 10X DNase I Buffer and 4 µl of DNase I enzyme (NEB).
- Incubate at 37˚C for 2 hr.
- Add another 4 µl of DNase I and incubate for another 2 hours at 37˚C.
- Add 200µl of Phenol:Chloroform:Isoamyl Alcohol in a fume hood.
- Vortex for 30 sec and centrifuge for 5 min at 10,000g.
- Carefully collect the aqueous (top) layer in a new tube.
- Add 100µl of 7.5M LiCl solution to the collected aqueous layer to precipitate RNA
- Incubate at -20˚C overnight.

1.3 EtOH precipitation:

- Next day, centrifuge at 10,000 g for 15 min at 4˚C
- Remove supernatant, wash pellet twice with ice-cold 70% EtOH (10,000g for 10 min at 4˚C).

- Air-dry pellet at RT for 15 min.
- Resuspend the pellet in 30 µl of water and incubate at 50˚C for 10 min.
- Measure concentration of the template RNA using a Qubit fluorometer

1.4 Confirm DNA elimination:

- Set up a Non-Selective PCR (Step 4) using 1-1.5 ng of the RNA as template and run on a 3% agarose gel. No band should be visible.
- If the RNA is free of DNA contamination it can now be used as substrate in a round of editing assay. Otherwise, repeat DNase treatment.

2. In vitro editing assay:

- Supplement buffer Q200 [50mM Tris pH 7, 200mM Potasium Glutamate, 20% Glycerol] with PMSF [5mM], DTT [5mM], tRNA [0.5 µg/µl], RNase Inhibitor [1 U/µl], cOmplete™ EDTA-free Protease Inhibitor [1x].
- Add 45.5 µl of supplemented Q200 to a tube for each reaction.
- Dilute RNA to 45 nM concentration in water.
- Heat up the diluted RNA to 95˚C for 1 minute and place immediately back on ice.
- Add 2.5 µl of diluted RNA to the supplemented Q200 buffer (2.25 nM final concentration).
- Dilute the enzyme to 2 µM concentration in Q200 buffer supplemented with PMSF and Protease Inhibitor.
- Do a 1:1 serial dilution of the enzyme down to 15.625 nM.
- Add 2 µl of the enzyme to the Q200 buffer + RNA (80 to 0.625 nM final concentration).
- Mix and incubate at 32.5˚C for 2 hrs.

3. RNA Recovery

3.1 Phenol:Chloroform:Isoamyl Alcohol extraction:

- Bring the volume to 200 µl by adding 150 µl of nuclease-free water.
- Add 200 µl of Phenol:Chloroform:Isoamyl Alcohol in a fume hood.
- Vortex for 30 sec and centrifuge for 5 min at 10,000g.
- Carefully collect the aqueous (top) layer in a new tube.
- Add 200µl of 5M Ammonium Acetate.
- Incubate at -20˚C overnight.
- Next day, do EtOH precipitation.

3.2 EtOH precipitation:

- Next day, centrifuge at 10,000g for 15 min at 4°C.
- Remove supernatant, wash pellet once with ice-cold 70% EtOH (10,000g for 10 min at 4°C).
- Air-dry pellet at RT for 15 min.
- To resuspend the pellet, add 30µl of water and incubate at 50°C for 10 min.

4. cDNA synthesis:

- Using the AccuScript High Fidelity RT-PCR kit (Agilent) set up the following reaction:

10X AccuScript RT Buffer	2.0 µl
25mM dNTPs (NEB)	0.8 µ
10µM RT-PCR-Rev prime	2.0 µl
Edited RNA template	11.7 µl
Total volume	16.5 µl

- Denature the RNA and anneal the primer by incubating at 72˚C for 5 min followed by a 5 min incubation at 25˚C

- Then add the following reagents:

100mM DTT	2.0 µl
RNase Inhibitor (NEB)	0.5 µl
AccuScript High Fidelity RT	1.0 µl
Total volume	3.5 µl

- Incubate at 42˚C for 1 hr

3. Non-selective PCR:

3.1 Set up reaction as follows:

cDNA	3.0 µl
10µM RT-PCR-Fwd primerq	0.75 µl
10µM RT-PCR-Rev primer	0.75 µl
2.5mM dNTP (NEB)	0.5 µl
10X ThermoPol Buffer	2.5 µl
Vent Polymerase	0.25 µl
H_2O	17.25 µl
Total volume	25.0 µl

- Cycling Conditions:

Step Number	STAGE 1	STAGE 2		STAGE 3	STAGE 4
Temperature	98 ˚C	98 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	30 sec	10 sec	30 sec	10 sec	∞
Number of Cycles	1X	25x		1X	1 X

- Run the samples on an 3% agarose gel and cut the appropriate bands (163 bp). Gel-purify the PCR product using Monarch gel purification kit (NEB) and send for Sanger Sequencing using the RT-PCR-Rev primer, to determine the editing efficiency.

Based on the sequencing result, select the cDNA of one of the samples for ARMS PCR and subsequent regeneration of the substrate.

4. ARMS PCR:

4.1 For 31nt set up reactions as follows:

Selected sample's cDNA	4.0µl
10µM 31_ARMS_-1G_Fwd primer	5.0µl
10µM RT-PCR-Rev primer	1.0µl
2.5mM dNTPS (NEB)	1.0µl
10X ThermoPol Buffer	5.0µl
Vent Polymerase	0.5µl
100mM MgSO4	0.25µl
H_2O	33.25µl
Total volume	50.0µl

- Cycling Conditions:

Step Number	STAGE 1	STAGE 2		STAGE 3	STAGE 4
Temperature	95 ˚C	95 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	2 min	30 sec	1 min	5 min	∞
Number of Cycles	1X	22x		1X	1 X

- The product of the ARMS PCR is the starting template for the Substrate Regeneration process.

5. Substrate Regeneration:

- The regeneration process requires three steps:

1) Amplify the selected guide from the ARMS PCR product.
2) Facilitate annealing between the original target sequence and the amplified guide sequence.
3) A final PCR to amplify full-length product.

5.1 Guide Amplification:

- Set up reaction as follows:

10X ThermoPol Buffer	4.0 µl
2.5mM dNTPs	0.8 µl
10µM Reg-Fwd primer	1.5 µl
10µM RT-PCR-Rev primer	1.5 µl
ARMS PCR product (1:10 dilution)	1 µl
Vent Polymerase	0.5 µl
H_2O	30.7 µl
Total volume	40.0

- Cycling Conditions:

Step Number	STAGE 1	STAGE 2			STAGE 3	STAGE 4
Temperature	98 ˚C	98 ˚C	60 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	30 sec	10 sec	30 sec	45 sec	5 min	∞
Number of Cycles	1X	30X			1X	1 X

- Run the samples on an 3% agarose gel and cut the appropriate bands (96 bp). Gel-purify the PCR product using Monarch gel purification kit (NEB). Check the concentration using Qubit.
- Use the product (Guide Amplicon) as the template in the guide annealing PCR.

5.2 Guide Annealing:

- Set up reaction as follows:

10X ThermoPol Buffer	2.0 µl
25mM dNTPs	0.8 µl
10µM Target Oligo	0.5 µl
Guide Amplicon	Variable
Vent Polymerase	0.4 µl
H_2O	up to 20 µl
Total volume	20.0 µl

- The volume of Guide amplicon should be calculated based on the obtained concentration to give a 1:10 Guide:Target molar concentration ratio.

- Cycling Conditions:

Step Number	STAGE 1	STAGE 2			STAGE 3	STAGE 4
Temperature	98 ˚C	98 ˚C	50 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	30 sec	10 sec	30 sec	45 sec	5 min	∞
Number of Cycles	1X	30X			1X	1 X

- Run the samples on an 3% agarose gel and cut the appropriate bands (202 bp). Gel-purify the PCR product using Monarch gel purification kit (NEB). Check the concentration using Qubit.
- Use the product (Annealed Template) as template for the full-length amplification PCR.

5.3 Full length amplification:

- Set up reaction as follows:

10X ThermoPol Buffer	4.0 µl
2.5mM dNTPs	0.8 µl
10µM Synthesis-Fwd primer	1.5 µl
10µM RT-PCR-Rev primer	1.5 µl
Annealed template	0.8 ng
Vent polymerase	0.4 µl
H_2O	up to 40 µl
Total volume	40.0 µl

- Cycling Conditions:

Step Number	STAGE 1	STAGE 2		STAGE 3	STAGE 4
Temperature	98 ˚C	98 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	30 sec	10 sec	30 sec	10 min	∞
Number of Cycles	1X	45x		1X	1 X

- For each sample set up 5 reactions. After cycling, pool the 5 reactions together and do PCR cleanup (Monarch kit) and check the concentration using Qubit.
- To confirm that the target A was replaced, send the product for Sanger Sequencing using the RT-PCR-Rev primer.
- The same product is also sent for MiSeq sequencing at Genewiz after adding Sequencing adaptors by PCR.
- Once the replacement of the target A has been confirmed by Sanger, the product can now be used as template for RNA synthesis for the next round of editing assay.