Protocol Number:	Application: RNA Guide Selection Assay	Date:

In vitro and in cellula site-directed RNA editing using the λDD-BoxB system for ADAR.

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I. Objective: The cell-free editing system can be used as a tool for ASO selection.

II. Required Materials and Reagents

All reagents should be RNase, DNase free. All tubes and tips should be RNase, DNase free and low retention.

Materials and Reagents	Vendor (Catalog Number)	Storage
96-well sample plates	Thermo Fisher, 10387491	Ambient
Plate foil seal	Thermo Fisher, 11570274	Ambient
Target RNA, 50 nM	In-house	-20˚C
Guide RNA, 0.5 uM	In-house	-20˚C
ADAR protein	In-house	-80˚C
BM buffer (50mM Tris, pH 7.0, 200mM Potassium Glutamate and 20% (w/v) Glycerol, 0.1 mg/ml tRNA, acetylated BSA 0.1 mg/ml, 5 mM DTT)	In-house	-20˚C
100x cOmplete™ EDTA-free Protease Inhibitor	Roche, 78425	4˚C
DNase/RNase Free Distilled Water	Boston BioProducts	Ambient
Murine RNase inhibitor, 40000 U/ml	New England Biolabs, M0314L	-20˚C
1M tris-HCL, pH 7.0	Invitrogen, AM9851	4˚C
1 M Potassium Glutamate	Teknova, P2000	Ambient
100% Glycerol	Invitrogen, 15514011	Ambient
Acetylated BSA	Promega, R3961	-20˚C
tRNA	Invitrogen, AM7119	-20˚C
1M DTT	Invitrogen, P2325	-20˚C

To prepare 10 ml of BM buffer:

- 50 mM tris-HCl pH 7.0 – 0.5 mL of 1M stock solution
- 200 mM Potassium Glutamate – 2 mL of 1M stock solution
- 20% glycerol – 2 mL of 100% stock solution
- 100ug/ml aBSA – 100ul of 10mg/ml stock solution

- 100ug/ml tRNA – 100ul of 10mg/ml stock solution
- 5mM DTT – 50 ul of 1M stock solution
- 1x proteinase inhibitors – 100 ul of 100x stock solution
- RNase, DNase free water – 5.15 mL

III. Equipment

Equipment	Vendor (Catalog Number)	Notes
96-wells PCR thermocycler	-	-
ThermoCycler MasterCycler X508	Eppendorf	-
Plate Vortexer MixMate	Eppendorf	-
Vortex	-	-
96-wells dry bath	-	-

IV. Sample Preparations and Protocol

1. Allow BM buffer, target RNA and gRNA to thaw in ice.

2. Calculate the volumes required to set up the following 20 ul reaction (for 1 guide RNA at 1 time point):
   - 2.25nM Target RNA – 0.9ul (of 50 nM solution)
   - 20nM gRNA - 0.8ul (of 0.5 uM solution)
   - 1U/µL RNase inhibitor – 0.5 ul
   - BM buffer up to 20µL
   - 38 nM pure ADAR protein**: ADAR2 ThermoFisher - 0.18mg/ml**, MW of ADAR2 is 77 kDa

     0.008ug/ul = 100 nM

     0.003ug/ul = 38 nM

     For 20ul of reaction you will need to have 0.061 ug of the protein or 0.34 ul. For 1120 ul (112 reactions) you will need 1082 ul of BM buffer + 38.1 ul of ADAR2. Gently mix it, spin it and then add by 10 ul to your Target/ASO solution in 10 ul at 37˚C.

   - 38nM p110 protein: **p110 ThermoFisher – 0.14mg/ml,** MW of p110 is 104 kDa

0.01ug/ul – 100 nM

0.004ug/ul – 38 nM

For 20 ul of reaction you will need to have 0.08 ug of the protein or 0.57 ul. For 1120 ul (112 reactions) you will need 1056 ul of BM buffer + 63.8 ul of p110. Gently mix it, spin it and then add by 10 ul to your Target/ASO solution in 10 ul at 37˚C.

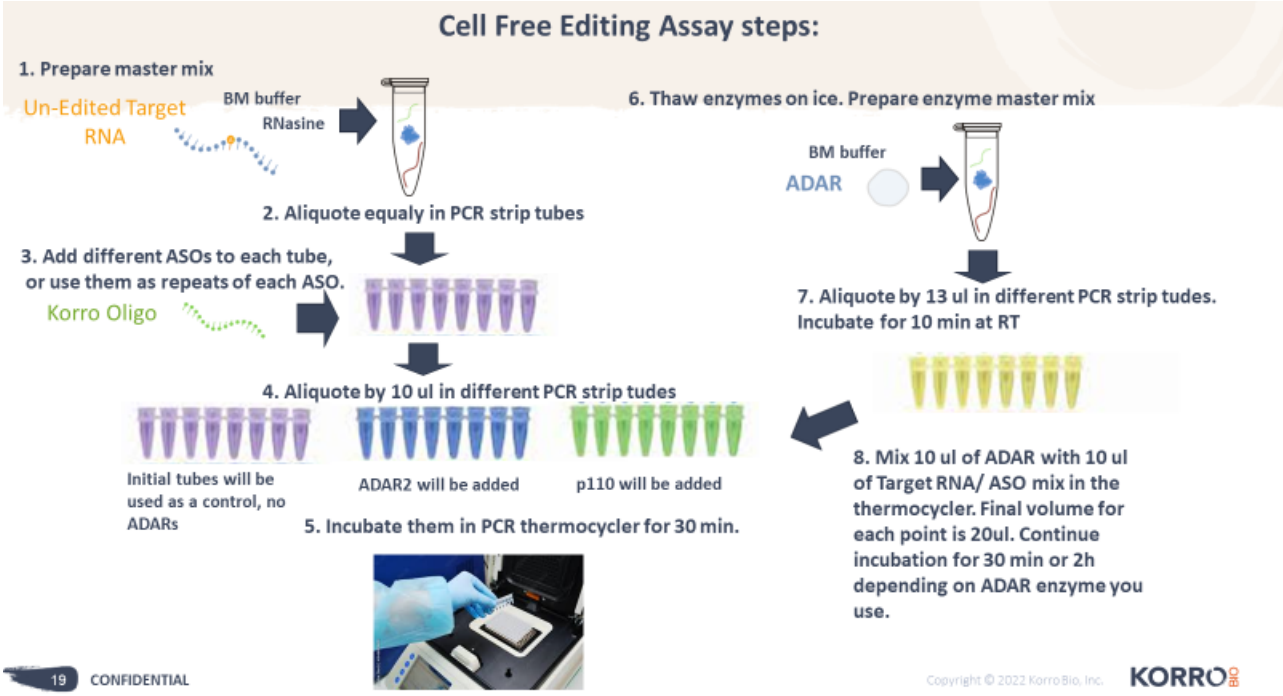
How to calculate the concentration of a Target RNA:

Measure a purified RNA using Nanodrop (choose ssRNA). MW of 409nt ssRNA is about 135kDa. For example, if RNA concentration = 4 mg/ml (4ug/ul), then 1uM = 0.135 ug/ul, 50 nM = 0.007ug/ul. You will need to dilute your RNA in 571 times (4 divided by 0.007) to have 50 nM RNA in water.

3. At ambient temperature, in a 0.2mL PCR strip tubes mix the calculated amount of target RNA, gRNA, and add BM buffer till **10ul** (add 7.8 ul). If you have multiple gRNAs and one target RNA you can prepare a Master Mix:
    - (7.8 ul BM buffer +0.5 ul RNAsine +0.9 ul Target RNA) x amount of gRNAs, then distribute the 9.2 ul in each tube and add 0.8 ul of ASO (**Please never add 0.8 ul of ASO**, either calculate for several repeats or dilute small amount of ASOs in BM buffer with RNasine to add more than 1ul, at least 2-3 ul. **Please look at the example of calculations on how to set up an experiment on MBLP002**).
    - Incubate a gRNA and a target mRNA in a PCR thermocycler at 37°C for 30 min.
    - After the incubation, open the thermocycler cover, open the tube, and add **10ul** of the calculated amount of ADAR protein in BM buffer to the mix, pipette it, start your timer, and incubate at 37°C for desired time.

IMPORTANT: ADAR proteins should be thawed in ice just before adding to the reaction mix (15 min before adding). Then ADARs in BM mixture should be prepared at ambient temperature (If added protein solution is cold it will lower editing efficiency, especially for ADAR2). For a short incubation of up to 2h other incubators can be used.

4. To stop a reaction, remove the tube from the PCR thermocycler and place it in a dry bath at 95°C. Bring the volume to 80uL by adding 60 ul of hot DNase/RNase Free Distilled Water (95C). Incubate it for 2 min.
5. Cold down the sample and proceed to RT-PCR protocol. For RT-PCR reaction use 10 ul of the diluted mixture (or freeze the samples at -20C).
6. After RT-PCR dilute the sample 1:10000 and proceed to dsPCR protocol. Do not keep diluted sample for a longer time, cDNA can be degraded.



*The cell-free editing protocol is not the final version and will be adjusted on request.