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Polyclonal Allelic Expression Assay Protocol V.1

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1 Works for me dx.doi.org/10.17504/protocols.io.2jigcke

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ABSTRACT

Polyclonal allelic expression assay for detecting the regulatory effects of genetic variants within transcripts using CRISPR/Cas9 genome editing and amplicon sequencing.

MATERIALS

NAME	CATALOG #	VENDOR
Q5 High-Fidelity 2X Master Mix - 500 rxns	M0492L	New England Biolabs
NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns	M0541L	New England Biolabs
Epicentre QuickExtract™ DNA Extraction Solution	QE09050	Epicentre
Opti-MEM (Reduced Serum Medium)	31985062	Thermo Fisher Scientific
Doxycycline (Hyclate) 1 g	72742	Stemcell Technologies
Nextera XT Index Kit, 96 indices, 384 samples	FC-131-1002	illumina
TrypLE™ Express Enzyme	12604013	Thermo Fisher Scientific
AmpureXP beads	A63880	Beckman Coulter
TRIzol™ LS Reagent	10296028	Thermo Fisher
SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme	11766500	Thermo Fisher
Lipofectamine™ MessengerMAX™ Transfection Reagent	LMRNA001	Thermo Fisher
gBlock gene fragments	View	IDT

1 Resuspend 500 ng of IDT gBlock gene fragment in 100 uL to make ~100 uM solution.

2 Resuspend 5 nmol of ssDNA IDT ultramer oligos in 50 uL to make 100 uM solution.

3 Perform gBlock PCR in order to amplify sufficient gblock for transfection (2X50ul reactions per gblock):

5 uL gBlock F primer (10 uM)
5 uL gBlock R primer (10 uM)
25 uL Q5 2X master mix
2 uL gBlock (100 uM)
18 uL H2O

4 Pool duplicate gBlock PCRs and perform 1X Ampure bead clean-up, resuspending in 25 uL.

5 Run a gel to confirm gBlock amplification and quantify using nanodrop or qubit.

6 24 hours before transfection: plate 293T iCas9 cells into 24 well plates with 120K cells/well in 0.5 mL 5 ug/mL doxycycline-supplemented media.

7 1. Transfect using Lipofectamine Messenger Max and 500ng gBlock and 0.5 uL 100 uM ssDNA template:

7.1 Make LF master mix with 25 uL opti-MEM and 1.5 uL LF per sample. Mix.

7.2 For each sample, add nucleic acids to 25 uL opti-mem. Mix.

7.3 Let sit for 5 min.

7.4 Add 25 uL LF master mix to each sample's nucleic acid mix.

7.5 Let sit for 20 min.

7.6 Drop mixture into each cell culture well gently.

8 24 hours later, change to regular media.

9 Let cultures grow for nine days post-transfection, splitting as needed to prevent confluency (e.g. day 4 and 6) with 100 uL tryplE.

10 Nine days post-transfection, harvest mRNA and gDNA:

10.1 Aspirate media from transfected cultures.

10.2 Add 100 uL tryplE to each well. Let sit for 5 min, tapping gently occasionally to dissociate.

10.3 Add 500 uL media to each well.

10.4 For each well, pipette up and down 400 uL (x5) and move 400 uL to an Eppendorf tube (for RNA).

10.5 Spin Eppendorf tubes at 1100 rpm at 4 degrees for 7 minutes.

10.6 In the meantime, take 50 uL of remaining cell mixture from each well and deposit into a 96-well PCR plate (for gDNA).

10.7 Spin down gDNA plate at 1100 rpm at 4 degrees for 7 minutes.

10.8 Remove RNA tubes from centrifuge. Aspirate supernatant. Add 500 uL TriZol. Let sit for 5 minutes.

10.9 Pipette up and down to dissociate cell pellets in RNA tubes. Either store samples in TriZol at -80 or proceed immediately with phenol-chloroform extraction as per TriZol documentation, resuspending in 20 uL nuclease-free water.

10.10 Aspirate supernatant from gDNA plate and add 10 uL Quick Extract buffer to each well. Shake plate to mix samples. Run Quick Extract program on thermalcycler. When program is done, store at -20.

11 Measure RNA concentration and quality using nanodrop.

12 Synthesize cDNA using Vilo IV superscript 2x master mix with EZ DNase. Can use up to 2.5 ug of RNA. Pick a volume of RNA where most are around 1 ug and none exceed the 2.5 ug limit.

13 Use custom nextera primers specific to the cDNA sequence of the locus of interest to amplify cDNA. Separately, amplify quick extract gDNA using primers specific to gDNA. Use Q5 master mix 62 degree protocol to amplify 1 uL of cDNA or gDNA PCR reaction:

5 uL Q5 2x master mix
0.5 uL F&R primer mix (10 uM)
1 uL cDNA/gDNA
3.5 uL H2O

14 Perform 1.2X ampure bead clean up (adjust based on size of amplified product).

15 Perform indexing PCR using Nextera indexing primers:

2.5 uL NEB Next master mix
0.5 uL Nextera indexing F&R primer mix (10 uM)
0.5 uL Clean Q5 PCR reaction
1.5 uL H2O

16 Pool samples equally and perform 1X ampure bead clean up on pooled library.

17 Quantify using qubit/tapestation and dilute for sequencing on Miseq.