Graveley Lab CRISPR Genome Editing followed by RNA-seq Biosample Preparation and Characterization Document

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Project: ENCODE3
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Sample Description: CRISPR genome editing of DROSHA in HepG2 cells
Cell Line: HepG2
RNA ID: DROSHA_BGHcLV05-13
ENCODE BIOSAMPLE ACCESSION: ENCBS047KDJ

This document contains the protocols used to clone target guid sequence, to generate lentiCRISPRv2-gRNA particles, transduction of HepG2 cells, harvesting of RNA, characterization of the RNA integrity and measurement of target knockdown efficiency by both qRT-PCR and Western blotting.

Target guide sequence cloning protocol - Adopted from ZhangLab's

In this portion of the protocol we will clone the target sequence into the lenti-CRISPRv2.

Item	Info
Target	DROSHA-human
gRNA ID	BGC#0000032
gRNA sequence	CTCGCCACCGCAGCTACGAA

In order to clone the target sequence into the lentiCRISPRv2 , synthesize two oligos of the following form. All plasmids have the same overhangs after BsmBI digestion.

Lentiviral vector digestion, oligo annealing and cloning into digested vector

1. Digest and dephosphorylate 5ug of the lentiviral CRISPR plasmid with BsmBI for 30 min at 37 °C:

Reagent	Quantity
lentiCRISPRv2	5 μg
FastDigest BsmBI (Fermentas)	3 μl
FastAP (Fermentas)	3 μl
10X FastDigest Buffer	6 μl
100 mM DTT	0.6 μl
ddH_2O (freshly prepared)	X μl
Total volume	60 μl

2. Gel purify digested plasmid using QIAquick Gel Extraction Kit and elute in EB.

   If BsmBI digested, a ∼2kb filler piece should be present on the gel. Only gel purify the larger band. Leave the 2kb band.

3. Phosphorylate and anneal each pair of oligos:

Reagent	Quantity
Oligo 1 (100 μM)	1 μl
Oligo 2 (100 μM)	1 μl
10X T4 Ligation Buffer (NEB)	1 μl
T4 PNK (NEB M0201S)	0.5 μl
ddH_2O	6.5 μl
Total volume	10 μl

Please use the T4 Ligation Buffer since the buffer supplied with the T4 PNK enzyme does not include ATP (or supplement to 1mM ATP).

Put the phosphorylation/annealing reaction in a thermocycler using the following parameters:

37 °C	30 min
95 °C	5 min and then ramp down to 25 °C at 5 °C/min

4. Dilute annealed oligos from Step 3 at a 1:200 dilution into sterile water or EB.

5. Set up ligation reaction and incubate at room temperature for 10 min:

Reagent	Quantity
BsmBI digested plasmid from Step 2 (50μg)	X μl
diluted oligo duplex from Step 4	1 μl
2X Quick Ligase Buffer (NEB)	5 μl
ddH_2O	X μl
Subotal volume	10 μl
Quick Ligase	1 μl
Total volume	11 μl

Also perform a negative control ligation (vector-only with water in place of oligos) and transformation.

6. Transformation into Stbl3 bacteria.

Lentiviral transfer plasmids contain Long-Terminal Repeats (LTRs) and must be transformed into recombination-deficient bacteria. We use home-made Stbl3 (propagated from Invitrogen C7373-03) and get excellent plasmid yields. Although other RecA-strains may work, we have found the most consistent transformations and yields using Stbl3.

Protocol for producing lentiCRISPRV2-gRNA particles

Day 1

1. Plate 0.8-1x10^6 293 T cells (catalog number: CRL-11268, ATCC) in each well of 6-well plate with 10 % FBS (catalog number: 30-2020, ATCC) DMEM (catalog number: 11995-065, Life technologies) medium without penicillin and streptomycin.

2. Incubate overnight. Cells should be 70-80% confluent.

Day 2

1. In polypropylene tubes, make a cocktail for each transfection as follows:

Reagent	Quantity
lentiCRISPRv2-gRNA	1 μg
psPAX2 Packaging DNA	750 ng
pCMV-VSV-G DNA	250 ng
serum-free OPTI-MEM	to 100 μl

2. Add 6 μl of FuGENE HD Transfection reagent (Catalog number: E2311, Promega) to the tube (FuGENE:DNA=3:1)
3. Incubate for 20 minutes at room temperature.
4. Gently add the DNA mix dropwise to cells.
5. Incubate the cells at 37 °C for 12-15 hr.

Day 3

1. In the morning, change the media to remove the transfection reagent, wash with PBS once and add 1.5 ml fresh media +10% FBS + penicillin/streptomycin.

Day 4

1. Harvest media from cells, store at 4 °C.
2. Add 1.5 ml fresh media.

Day 5

1. Harvest the media from the cells and pool with the media collected on Day 4.
2. Spin the media at 1250 rpm for 5 min to remove cells.
3. Freeze the virus stock at −40 °C.

Lentiviral CRISPR Protocol

For HepG2 cells

Source: ATCC HBâĂŘ8065 (lot 59635738)

Growth Media for HepG2

500 ml DMEM (HyClone, SH30022.01)
50 ml Fetal Bovine Serum (FBS) (10% Final Concentration) (Hyclone, SH30071.03)
5 ml Pen-Strep (1% Final Concentration) (Life Technologies,15140122)

Culturing

1. Thaw a frozen stock vial of HepG2 cells by gentle agitation in a 37 °C water bath.
2. Remove the vial from the water bath as soon as the contents are thawed.
3. Transfer the cells into the growth medium and centrifuge at 1000rpm for 5 minutes.
4. Resuspend the cell pellet in an appropriate amount of fresh growth medium.
5. Incubate the cells at 37 °C in a 5% CO_2 in air atmosphere incubator.
6. Change the fresh growth medium every 2 to 3 days.
7. Cells are ready to split when the cell density reaches 70−80% confluence.
8. Remove culture medium.
9. Wash cells with 1X PBS.
10. Add 2 to 3 ml of 0.25% TrysinâĂŘEDTA and return to incubator for 5 minutes.
11. Add 4.0 to 6.0 mL of complete growth medium and aspirate cells by gently pipetting.
12. Remove cells and pellet at 1000 rpm for 5 min.
13. Gently reâĂŘsuspend cell pellet in warm fresh growth medium.
14. Perform 1:8 to 1:16 cell split as needed.

Prepare cells for transduction

Day 0

1. Plate 0.8-1ml of lentiCRISPRv2-gRNA particles in each well of 12-well plates.
2. Add 5-7x10ˆ5 cells to appropriate wells with 8 μg/ml of polybrene (Catalog Number H9268, Sigma-Aldrich), incubate at 37 °C for 2 hours, and then add 1 ml of complete medium.

Day 1

1. After 24 hrs, change to fresh media (2 ml) with 3 μg/ml of puromycin.

Day 3

1. Change to fresh media with 3 μg/ml of puromycin.

Day 5

1. Change to fresh media with 3 μg/ml of puromycin.

Day 6

1. Detach the cells, harvest half of the cells to prepare RNA and half of the cells to prepare a protein lysate for western blotting.

RNA Isolation

RNA isolation is performed using a Promega Maxwell®16 Instrument and the Maxwell®16 LEV simplyRNA Cells Kits (Catlog Number AS1270).

1. Pellet cells at 300 x g for 3 minutes and remove medium.
2. Add 200 μl of chilled 1-Thioglycerol/Homogenization solution to the cell pellet and vortex until the pellet is dispersed.
3. Add 200 μl of lysis buffer and votex vigorously for 15 sec to mix.
4. Transfer all 400 μl lysate to well 1 of the Maxwell 16 LEV cartridge.
5. Add 5 μl of DNase I solution to well 4 of the cartridge.
6. Put elution tubes with 40-50 μl of nuclease-free water and LEV plungers in the cartridge.
7. Transfer the Maxwell 16 LEV cartridge rack containing prepared cartridges on the Maxwell 16 Instrument.
8. Push Run/Stop button to start run.

RNA Quality Control

The quality of the RNA is measured using an Agilent TapeStation Instrument with the RNA screen tape (Catlog Number 5067-5576).

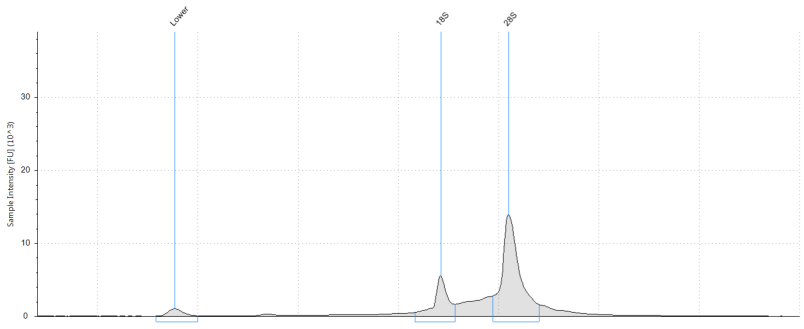


Figure 1: Agilent TapeStation image of 1 μl of total RNA sample.

qRT-PCR Assay to Monitor mRNA Target Knockdown Efficiency

cDNA Synthesis

This assay uses the iScript cDNA Synthsis Kit from BIO-RAD (Catalog number: 170-8891)

1. Reaction Setup:

Reagent	Quantity
5x iScript reaction mix	2 μl
iScript reverse transcriptase	0.5 μl
Nuclease-free water	x μl
RNA template (200 ng)	x μl
Total volume	10 μl

2. Reaction Protocol:

Time	Temperature
5 minutes	25 °C
30 minutes	42 °C
5 minutes	85 °C
Hold	4 °C

qPCR Assay

This assay uses Phusion High-Fidelity DNA Polymerase from NEB (Catalog number: M0530L) and SYBR Green from Invitrogen (Catalog number: S7563)

1. Reaction setup:

Reagent	Quantity
5X Phusion HF Buffer	4 μl
10 mM dNTPs	0.4 μl
10 μM Forward Primer	1 μl
10 μM Reverse Primer	1 μl
Template (1:20 of cDNA reaction)	1 μl
Phusion DNA Polymerase	0.2 μl
SYBR Green (10,000 X)	0.1 μl
Nuclease-free water	to 20 μl
Total volume	20 μl

2. Reaction Protocol:

STEP	TEMP	TIME
Initial Denaturation	98 °C	30 seconds
35 Cycles	98 °C	10 seconds
	58 – 66 °C	15 seconds

3. Data Analysis:

Data analysis is perfomed using the $2 - \Delta\Delta$Ct Method.

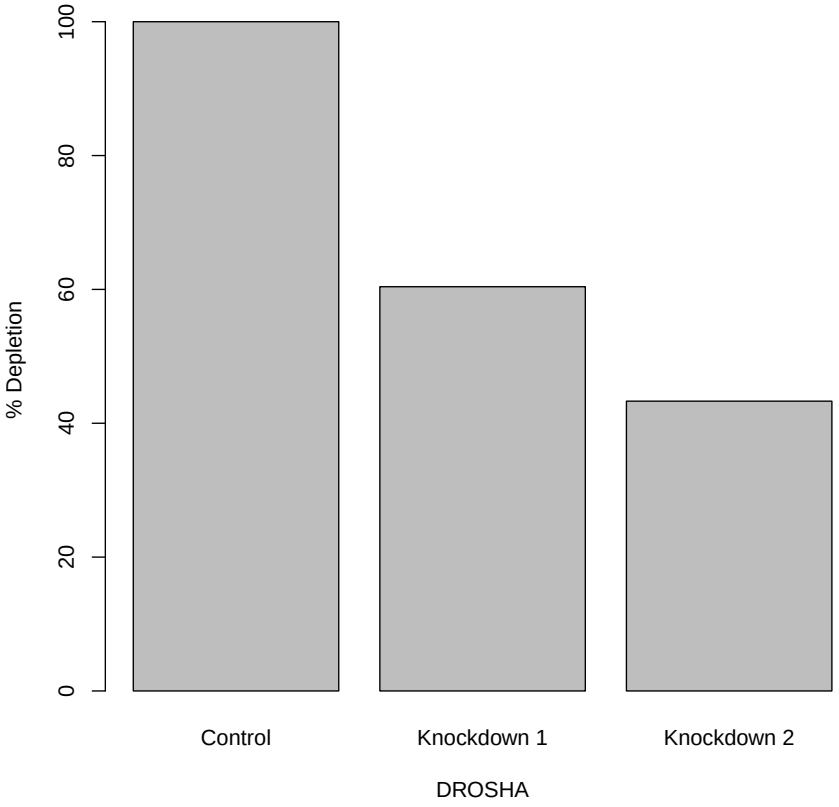


Figure 2: qRT-PCR analysis of depletion level of the target RNA binding protein in control and knockdown cells. The percent depletion was calculated in the RNA sample isolated from HepG2s transduced with an shRNA targeting DROSHA (ENCODE Biosample ENCBS047KDJ) in comparison to HepG2 cells transduced with a control non-target shRNA (ENCODE Biosample ENCBS332SEB). The efficiency of depletion is normalized using GAPDH as a control.

Western Blot Assay to Monitor Protein Target Knockdown Efficiency

A western blot is performed to determine the knockdown efficiency of the target RNA binding protein. For this biosample, the following antibodies were used:

RNA binding protein primary antibody: DROSHA

Loading control primary antibody: GAPDH

Wes Protocol

12-230 kDa Master kit with split Running Buffer from Proteinsimple

A. PREPARE STANDARD PACK REAGENTS

1. Add 40 μL deionized water to make a 400 mM solution of the DTT.
2. Add 20 μL 10X Sample buffer and 20 μL prepared 400 mM DTT to make 5X Fluorescent Master Mix.
3. Add 16 μL deionized water, 2 μL 10X Sample Buffer and 2 μL prepared 400 mM DTT solution to make Biotinylated Ladder.

B. PREPARE YOUR SAMPLES

1. Combine 1 part 5X Fluorescent Master Mix with 4 parts lysate in a microcentrifuge tube (If needed, dilute the lysate with 0.1X Sample Buffer).
2. Denature the samples and biotinylated ladder at 70 °C for 10 min.

C. PREPARE REAGENTS FROM DETECTION MODULE

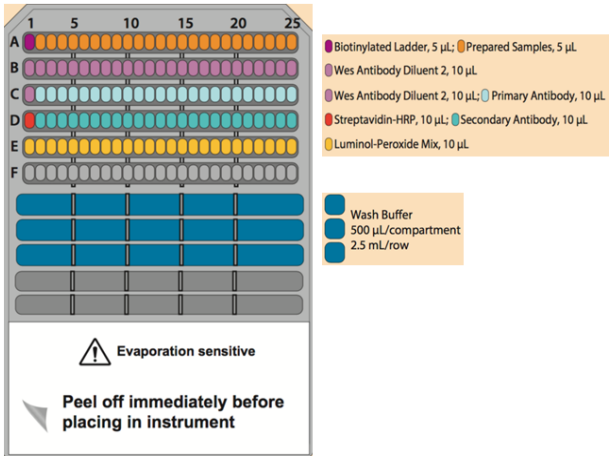
1. Dilute primary antibody with antibody diluent 2.
2. The supplied secondary antibody is ready to use without dilution.
3. Combine 150 μL Luminol-S and 150 μL Peroxid in a microcentrifuge tube. Gently pipette up and down to mix and store on ice.

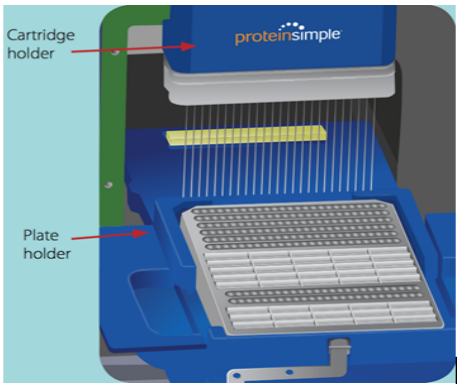
D. PIPETTE YOUR PLATE (IMMUNOASSAY)

For more consistent results, keep the lid on between reagent additions. Centrifuge the plate for 5 minutes at 2500 rpm at room tempereature.

E. START WES

1. Load the desired assay in Compass software v2.7 or higher.
2. Open Wes' door.
3. Insert a capillary cartridge into the cartridge holder. The interior light will change from orange to blue.





4. Remove the assay plate lid. Hold plate firmly on bench and carefully peel off evaporation seal. Pop any bubbles observed in the Separation Matrix wells with a pipette tip.
5. Place the assay plate on the plate holder
6. Close Wes' door.
7. Click the Start button in compass.
8. When the run is complete, discard the plate and cartridge.

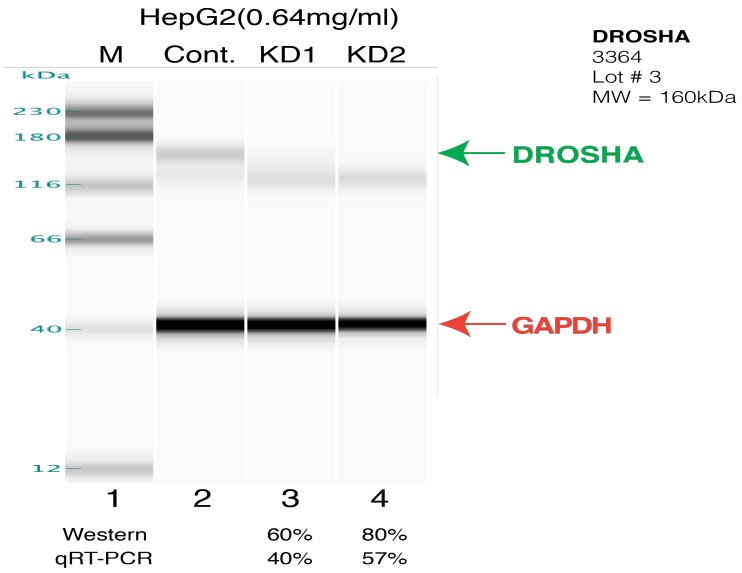


Figure 3: Western Blot Analysis of depletion level of the target RNA binding protein in control and knockdown cells. Lane 1: Molecular weight marker. Lane 2: 30 μg of protein from HepG2 transduced with a control, non-target shRNA (ENCODE Biosample ENCBS332SEB and ENCBS901AHP). Lane 3: 30 μg of protein from HepG2 transduced with an shRNA targeting DROSHA (ENCODE Biosample ENCBS047KDJ). Lane 4: 30 μg of protein from HepG2 transduced with an shRNA targeting DROSHA (ENCODE Biosample ENCBS076OJD). Samples were separated by SDS-PAGE, transferred to a membrane and blotted using antibodies against DROSHA (ENCODE Antibody ENCAB953TNR) and GAPDH as controls.