

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

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**Project:** ENCORE

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**Sample Description:** CRISPR genome editing of CARS in HepG2 cells

**Cell Line:** HepG2

**RNA ID:** CARS-BGHcLV27-40

**ENCODE BIOSAMPLE ACCESSION:** ENCBS104KVQ

This document contains the protocols used to clone target guide 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 lentiCRISPRv2.

Item	Info
Target	CARS-human
gRNA ID	BGC0000666
gRNA sequence	TGAGATGCGTCATAGACGGT

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 $\mu$ g
FastDigest BsmBI (Fermentas)	3 $\mu$ l
FastAP (Fermentas)	3 $\mu$ l
10X FastDigest Buffer	6 $\mu$ l
100 mM DTT	0.6 $\mu$ l
ddH <sub>2</sub> O (freshly prepared)	X $\mu$ l
Total volume	60 $\mu$ l

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 $\mu$ M)	1 $\mu$ l
Oligo 2 (100 $\mu$ M)	1 $\mu$ l
10X T4 Ligation Buffer (NEB)	1 $\mu$ l
T4 PNK (NEB M0201S)	0.5 $\mu$ l
ddH <sub>2</sub> O	6.5 $\mu$ l
Total volume	10 $\mu$ l

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 (50 $\mu$ g)	X $\mu$ l
diluted oligo duplex from Step 4	1 $\mu$ l
2X Quick Ligase Buffer (NEB)	5 $\mu$ l
ddH <sub>2</sub> O	X $\mu$ l
Subtotal volume	10 $\mu$ l
Quick Ligase	1 $\mu$ l
Total volume	11 $\mu$ l

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<sup>6</sup> 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 $\mu$ g
psPAX2 Packaging DNA	750 ng
pCMV-VSV-G DNA	250 ng
serum-free OPTI-MEM	to 100 $\mu$ l

2. Add 6  $\mu$ l 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  $-40^{\circ}\text{C}$ .

## Lentiviral CRISPR Protocol

### For HepG2 cells

Source: ATCC HB-8065 (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<sub>2</sub> 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% Trysin-EDTA 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 re-suspend 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  $5-7 \times 10^5$  cells to appropriate wells with 8  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}/\text{ml}$  of puromycin.

### **Day 3**

1. Change to fresh media with 3  $\mu\text{g}/\text{ml}$  of puromycin.

### **Day 5**

1. Change to fresh media with 3  $\mu\text{g}/\text{ml}$  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  $\mu$ l of chilled 1-Thioglycerol/Homogenization solution to the cell pellet and vortex until the pellet is dispersed.
3. Add 200  $\mu$ l of lysis buffer and vortex vigorously for 15 sec to mix.
4. Transfer all 400  $\mu$ l lysate to well 1 of the Maxwell 16 LEV cartridge.
5. Add 5  $\mu$ l of DNase I solution to well 4 of the cartridge.
6. Put elution tubes with 40-50  $\mu$ l 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).

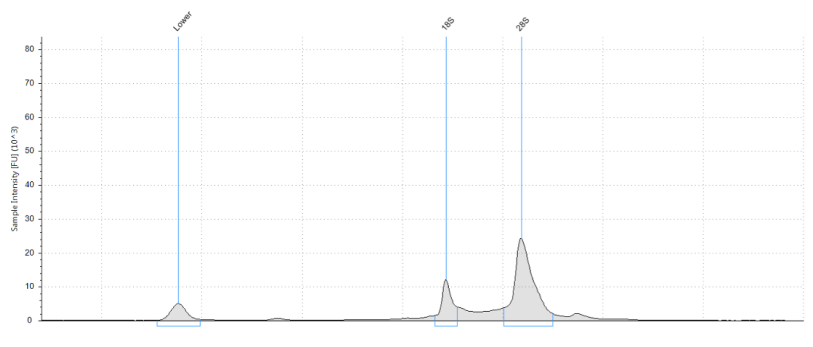


Figure 1: Agilent TapeStation image of 1  $\mu$ l of total RNA sample.

# qRT-PCR Assay to Monitor mRNA Target Knock-down Efficiency

## cDNA Synthesis

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

### 1. Reaction Setup:

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

### 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 $\mu$ l
10 mM dNTPs	0.4 $\mu$ l
10 $\mu$ M Forward Primer	1 $\mu$ l
10 $\mu$ M Reverse Primer	1 $\mu$ l
Template (1:20 of cDNA reaction)	1 $\mu$ l
Phusion DNA Polymerase	0.2 $\mu$ l
SYBR Green (10,000 X)	0.1 $\mu$ l
Nuclease-free water	to 20 $\mu$ l
Total volume	20 $\mu$ l

### 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 performed using the  $2^{-\Delta\Delta C_t}$  Method.

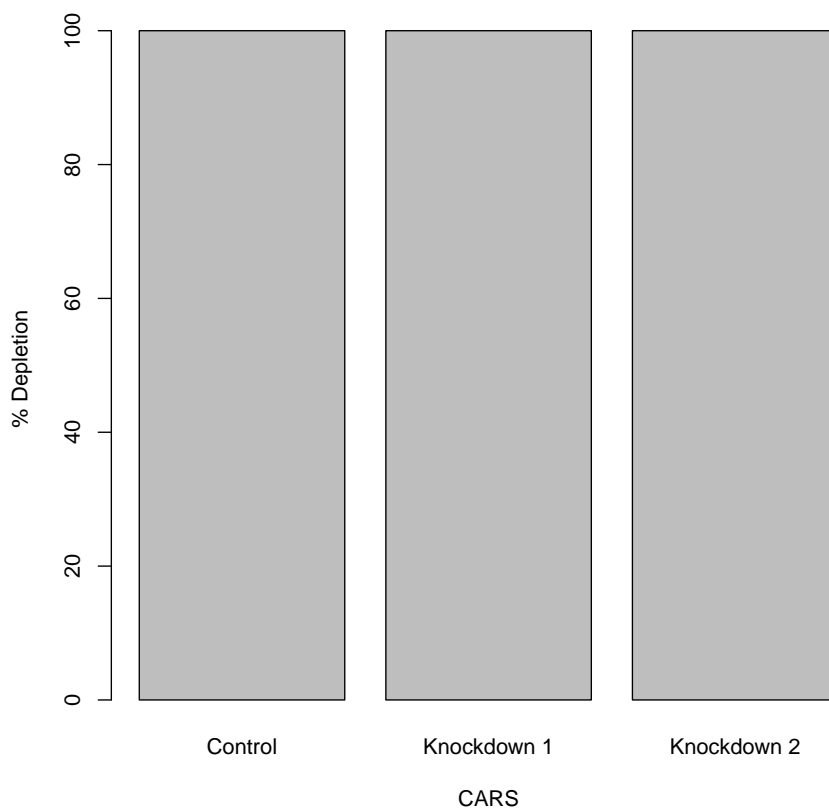


Figure 2: qRT-PCR analysis of depletion level of the target RNA binding protein in control and knockdown cells. The percent depletion was calculated in the RNA sample isolated from HepG2s transduced with a gRNA targeting CARS (ENCODE Biosample ENCBS916HYF and ENCBS104KVQ) in comparison to HepG2 cells transduced with a control non-target gRNA (ENCODE Biosample ENCBS622KXD and ENCBS462HZR). 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: CARS**

**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  $\mu\text{L}$  deionized water to make a 400 mM solution of the DTT.
2. Add 20  $\mu\text{L}$  10X Sample buffer and 20  $\mu\text{L}$  prepared 400 mM DTT to make 5X Fluorescent Master Mix.
3. Add 16  $\mu\text{L}$  deionized water, 2  $\mu\text{L}$  10X Sample Buffer and 2  $\mu\text{L}$  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  $\mu\text{L}$  Luminol-S and 150  $\mu\text{L}$  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 temperature.

### 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.



- 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.
- Place the assay plate on the plate holder
- Close Wes' door.
- Click the Start button in compass.
- When the run is complete, discard the plate and cartridge.

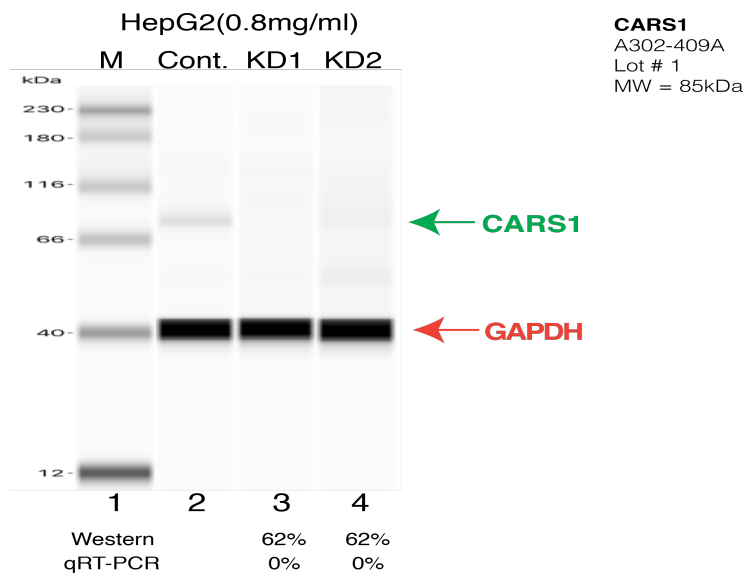


Figure 3: Western Blot Analysis of depletion level of the target RNA binding protein in control and knockdown cells. Lane 1: Molecular weight marker. Lane 2: 30  $\mu$ g of protein from HepG2 transduced with a control, non-target gRNA (ENCODE Biosample ENCBS622KXD and ENCBS462HZR). Lane 3: 30  $\mu$ g of protein from HepG2 transduced with a gRNA targeting CARS (ENCODE Biosample ENCBS916HYF). Lane 4: 30  $\mu$ g of protein from HepG2 transduced with a gRNA targeting CARS (ENCODE Biosample ENCBS104KVQ). Samples were separated by SDS-PAGE, transferred to a membrane and blotted using antibodies against CARS (ENCODE Antibody ENCAB833ALL) and GAPDH as controls.