Graveley Lab shRNA knockdown followed by RNA-seq Biosample Preparation and Characterization Document

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Project: ENCODE3
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Sample Description: shRNA Knockdown of RBM39 in HepG2 cells
Cell Line: HepG2
RNA ID: RBM39_BGHLV26_39
ENCODE BIOSAMPLE ACCESSION: ENCBS285EHM

This document contains the protocols used to generate shRNA expressing lentiviral 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.

Protocol for producing shRNA lentiviral particles

In this portion of the protocol we will generate lentiviral particles expressing either an shRNA targeting an RNA binding protein mRNA or a non-target control shRNA.

Item	Info
Target	RBM39-human
shRNA Source	The RNAi Consortium
Product ID	TRCN0000021770
Target sequence	GCTGGACCTATGAGGCTTTAT
Vector backbone	pLKO.1

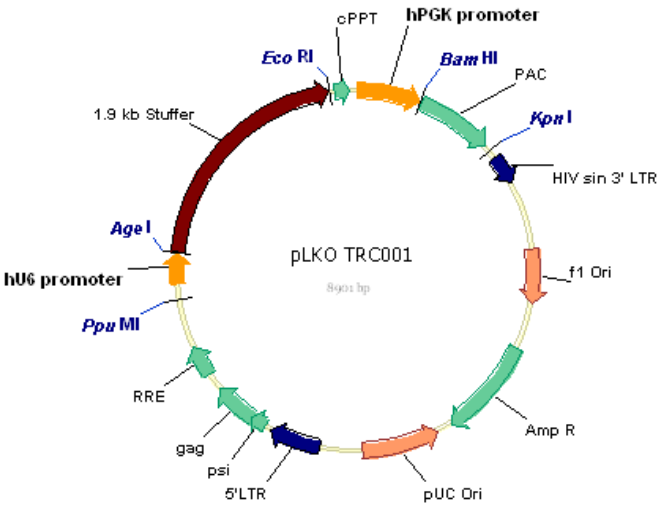

Figure 1: Schematic depiction of backbone of the pLKO.1 plasmid encoding the shRNA.

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
pLKO-shRNA	500 ng
psPAX2 Packaging DNA	500 ng
PMD2.G Envelope DNA	50 ng
serum-free OPTI-MEM	to 100 μl

2. Add 3.1 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.

qPCR Lentivirus Titration Assay

Lentiviral titrations are performed using the qPCR Lentivirus Titration kit from Applied Biological Materals Inc. (Catalog Number LV900).

1. Add 2 μl of the viral supernatant to 18 μl of Virus Lysis buffer and incubate at RT for 3 mins. This is now referred to as viral lysate.
2. qRT-PCR set up:

Component	Viral lysate	Positive Control (STD1)	Positive Control (STD2)	Negative control (NTC)
2x qPCR Mastermix	12.5 μl	12.5 μl	12.5 μl	12.5 μl
Viral Lysate	2.5 μl			
STD1		2.5 μl		
STD2			2.5 μl	
Reagent-mix	10 μl	10 μl	10 μl	10 μl
Final vol.	25 μl	25 μl	25 μl	25 μl

3. qRT-PCR program:

STEP	TEMP	TIME
Reverse Transcription	42 °C	20 minutes
Enzyme Activation	95 °C	10 minutes
40 Cycles	95 °C	15 seconds
	60 °C	1 minute

4. Calculate the titer from Ct values by using abm's on-line lentiviral titer calculator at http://www.abmgood.com/High-Titer-Lentivirus-Calculation.html or by using the formula: IU/ml=Dilution factor x 5x10ˆ7/2ˆ3(Ct sample-Ct STD1)/(CtSTD2-CtSTD1)

Lentiviral Transduction 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 37ÂřC 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

1. Plate 5x10ˆ5 cells in each well of 6-well plates.
2. Incubate for overnight, cells should be 50−60% confluent.

Day 0

1. Change the fresh media with 8 μg/ml of polybrene (Catalog Number H9268, Sigma-Aldrich) to the cells.
2. Add lentiviral particles (MOI $\sim$ 10) to appropriate wells.

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).

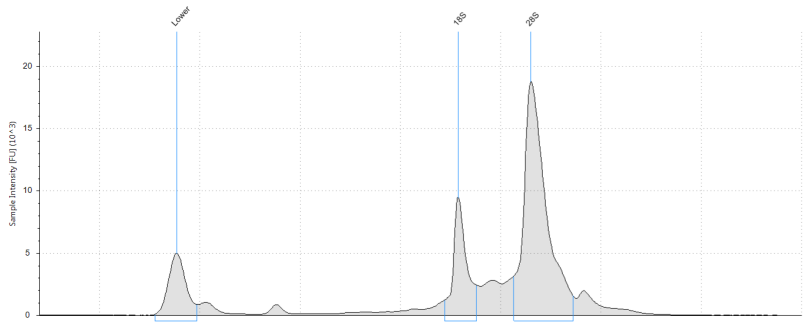

Figure 2: Agilent TapeStation image of 1 μl of total RNA sample.

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
	72 °C	10 seconds

3. Data Analysis:

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

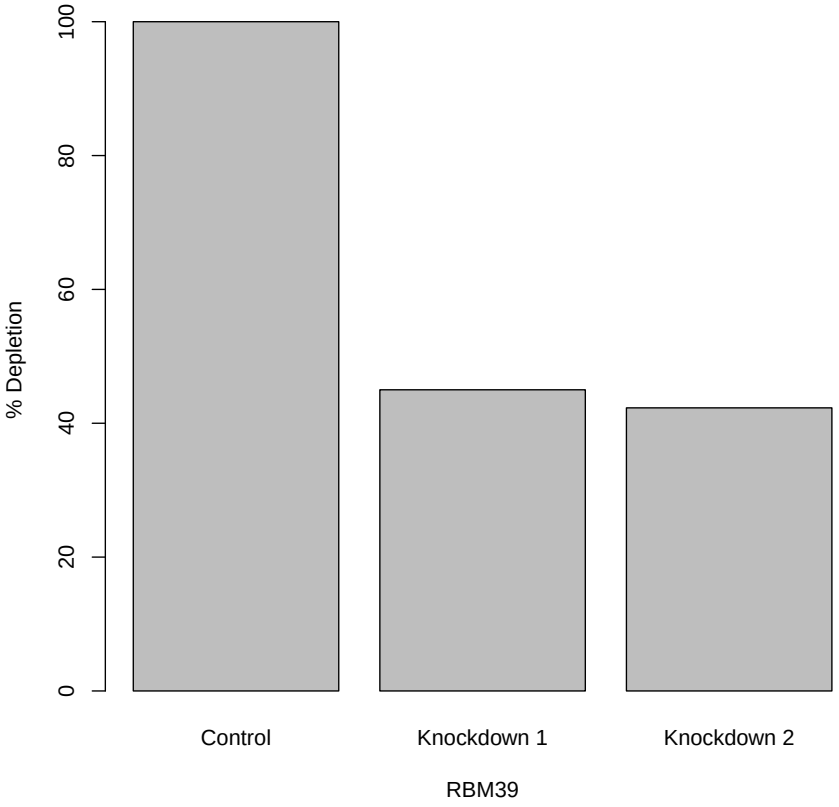

Figure 3: qRT-PCR analysis of depletion level of the target RNA binding protein in control and knockdown cells. The percent depletion was calculated in the RNA sample isolated from HepG2s transduced with an shRNA targeting RBM39 (ENCODE Biosample ENCBS285EHM) in comparison to HepG2 cells transduced with a control non-target shRNA (ENCODE Biosample ENCBS356MOV). 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: RBM39

Loading control primary antibody: GAPDH

1. Purify protein by resuspending cell pellets in 100 μl Lysis buffer (50 mM Tris−HCl, pH 7.4; 100 mM NaCl; 1% NP−40; 0.1% SDS; 0.5% sodium deoxycholate) with protease inhibitor (Roche cocktail).
2. Vortex vigorously and leave tubes on ice at least 30 min.
3. Spin 18,000g, 20 min, 4 °C. Recover the supernatant in a new tube on ice. Discard the pellet.
4. Quantitate the protein concentration using the Pierce BCA kit.
5. Load 30−60 μg of protein per sample depending on expression levels of the protein of interest in the cell type.
6. Dilute the protein samples with 4X sample buffer and 10X reducing agent (Invitrogen NuPage reagents) and heat at 70 °C for 10 min.
7. While samples are heating, prepare the gel: 4−12% Bis−Tris gel for proteins 10−220 kDa, or 8% Tris−glycine for proteins over 220 kDa. Dilute MOPS running buffer to 1X and pour into buffer tank. Add 500 μl of antioxidant to the inner chamber of the buffer tank. Wash out all wells of the gel with buffer.
8. Load 3 μl of Licor Odyssey prestained molecular weight marker (928−4000) and protein samples. Run the gel at 200 V for about an hour, until the dye just runs off the gel (less for small molecular weight proteins).
9. Transfer to PVDF for 30 min in transfer buffer, with methanol and antioxidant, using BioRad Semi−dry transfer apparatus.
10. After transfer, block membrane in 5−10 mls of Licor Blocking buffer for 1 hour at room temperature.
11. Incubate with the RNA binding protein primary antibody (0.2 μg/ml) and the loading control primary antibody (mfg recommended dilution) diluted in Licor block with 0.1% tween 20 on a rocker at 4 °C overnight.
12. The next day, wash the blot 4 x 5 min each in TBST.
13. Prepare the secondary antibody (Rockland Fluorescent TrueBlot anti-rabbit IgG IRDye800 (Catalog number 18−3216−32) for the RBP and Licor IRDye680 secondary antibody for the loading control). Dilute the antibodies according to the mfg instructions in Licor blocking buffer with 0.1% Tween 20 and 0.01% SDS. Incubate on a rocker for 30−60 min, at room temperature.

14. Wash 4 x 5 min each in TBST. Rinse 1x in TBS (no T) and scan on the Licor Odyssey instrument.

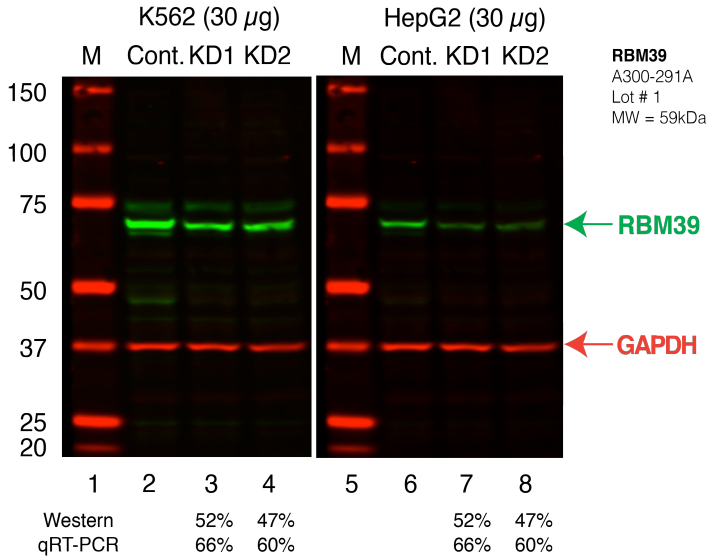

Figure 4: Western Blot Analysis of depletion level of the target RNA binding protein in control and knockdown cells. Lane 1 and 5: Molecular weight marker. Lane 2: 30 μg of protein from K562 transduced with a control, non-target shRNA (ENCODE Biosample ENCBS136ZNW and ENCBS221YFO). Lane 3: 30 μg of protein from K562 transduced with an shRNA targeting RBM39 (ENCODE Biosample ENCBS023ECI). Lane 4: 30 μg of protein from K562 transduced with an shRNA targeting RBM39 (ENCODE Biosample ENCBS192SEZ). Lane 6: 30 μg of protein from HepG2 transduced with a control, non-target shRNA (ENCODE Biosample ENCBS356MOV and ENCBS967UNG). Lane 7: 30 μg of protein from HepG2 transduced with an shRNA targeting RBM39 (ENCODE Biosample ENCBS285EHM). Lane 8: 30 μg of protein from HepG2 transduced with an shRNA targeting RBM39 (ENCODE Biosample ENCBS144MRF). Samples were separated by SDS-PAGE, transferred to a membrane and blotted using antibodies against RBM39 (ENCODE Antibody ENCAB975CWB) and GAPDH as controls.