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

Wet Lab: Sara Olson and Lijun Zhan
Computational Lab: Xintao Wei and Michael Duff
PI: Brenton Graveley

Department of Genetics and Genome Sciences
UConn Institute for Systems Genomics
UConn Health
400 Farmington Avenue
Farmington, CT 06030 USA

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Project: ENCODE3
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Sample Description: shRNA Knockdown of non-target in K562 cells
Cell Line: K562
RNA ID: NT_BGKLV32-2
ENCODE BIOSAMPLE ACCESSION: ENCBS619KUS

This document contains the protocols used to generate shRNA expressing lentiviral particles, transduction of K562 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	Non-specific target control-human
shRNA Source	The RNAi Consortium
Product ID	SHC002
Target sequence	non-mammalian target
Vector backbone	pLKO.1

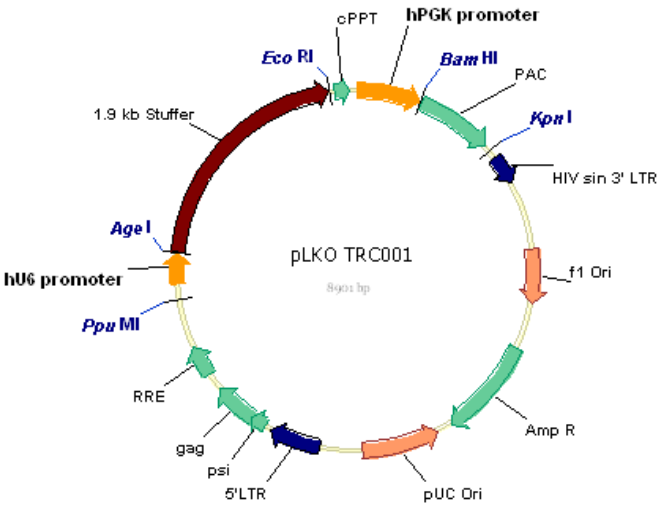


Figure 1: Schematic depiction of backbone of the pLKO.1 plasmid encoding the shRNA.

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 K562 cells

Source: ATCC CCL-243 (lot 59300853)

Growth Media for K562 cells:

500 ml RPMI-1640 with glutamine medium (Hyclone, SH30027.01)
50 ml Fetal Bovine Serum (FBS) (10% Final Concentration) (Hyclone, SH30071.03)
5 ml Pen-Strep (1% Final Concentration) (Invitrogen, 15140-163)

Culturing

1. Remove a frozen stock vial of K562 cells from liquid nitrogen.
2. Thaw it in a 37 °C water bath.
3. As soon as it thawed, transfer the cells into the growth medium.
4. Centrifuge at 1000 rpm for 5 minutes.
5. Resuspend the cells in the growth medium such that the final cell density is about 1x10ˆ5 cells/ml.
6. Split the cells every 2 to 3 days.
7. Grow the cells to required numbers.

Prepare cells for transduction

Day 0

1. Plate 5-7x10^5 cells in each well of 12-well plates.
2. Add 8 μg/ml of polybrene (Catalog Number H9268, Sigma-Aldrich) to the cells.
3. 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 (2 ml) with 3 μg/ml of puromycin.

Day 5

1. Change to fresh media (2 ml) with 3 μg/ml of puromycin.

Day 6

1. 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).

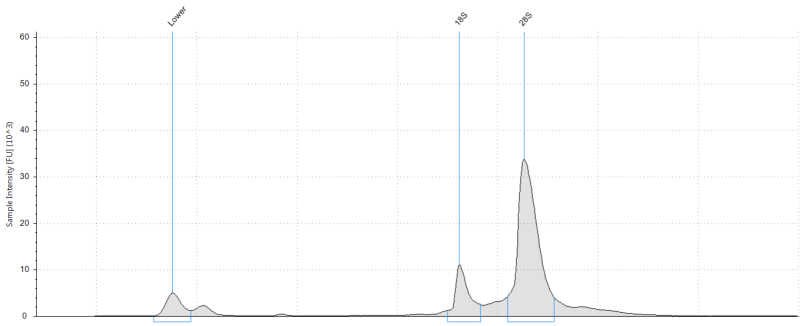


Figure 2: Agilent TapeStation image of 1 μl of total RNA sample.

qRT-PCR Assay to Monitor mRNA Target Knock-down 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.

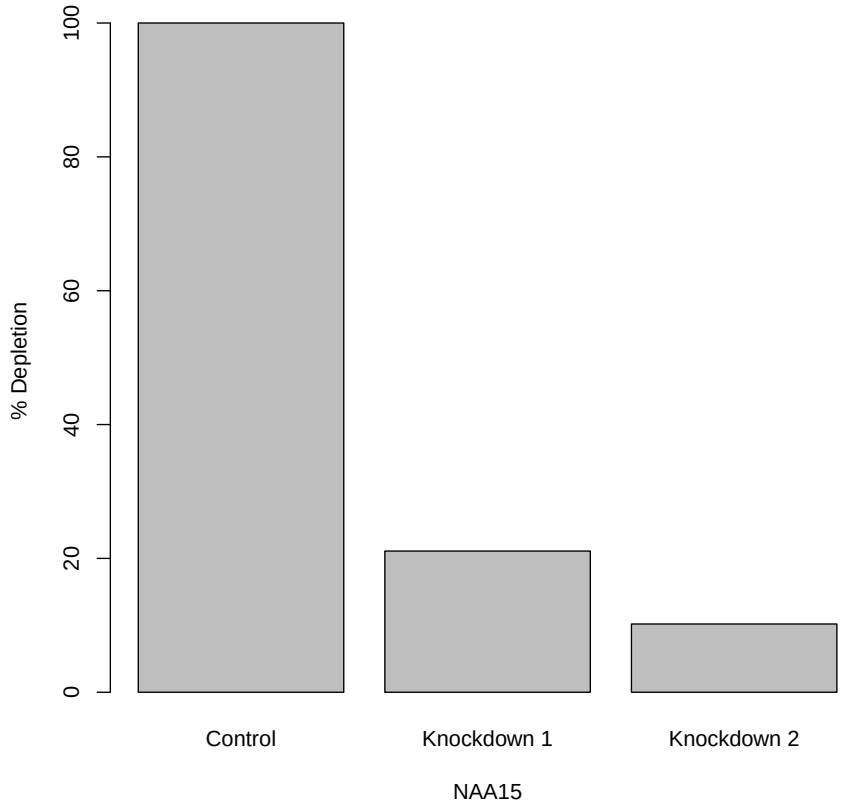


Figure 3: qRT-PCR analysis of depletion level of the target RNA binding protein in control and knockdown cells. The percent depletion was calculated in the RNA sample isolated from K562s transduced with an shRNA targeting NAA15 (ENCODE Biosample ENCBS171PLV) in comparison to K562 cells transduced with a control non-target shRNA (ENCODE Biosample ENCBS061XBB). 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: NAA15

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.

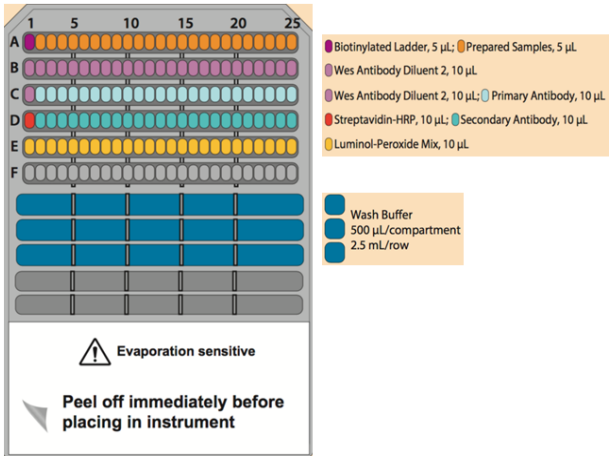
B. PREPARE YOUR SAMPLES

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.

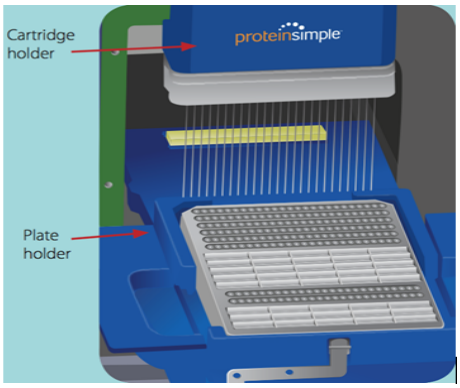
C. PREPARE REAGENTS FROM DETECTION MODULE

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.

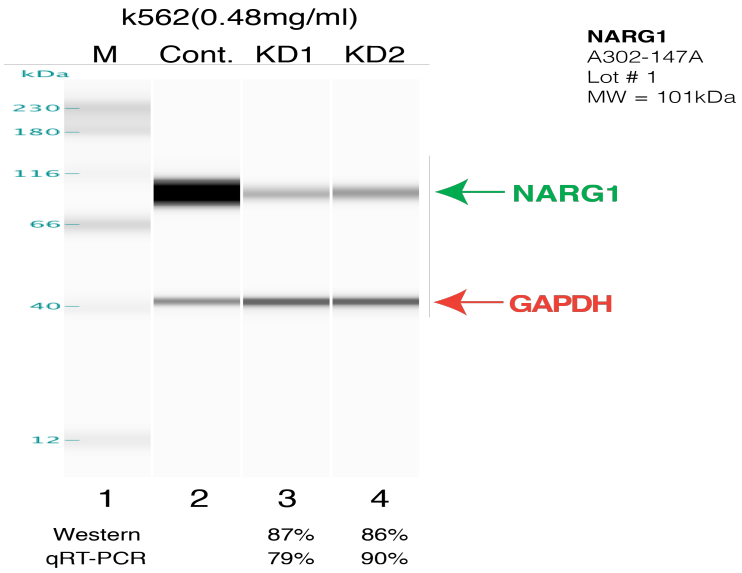


Figure 4: Western Blot Analysis of depletion level of the target RNA binding protein in control and knockdown cells. Lane 1: Molecular weight marker. Lane 2: 30 μg of protein from K562 transduced with a control, non-target shRNA (ENCODE Biosample ENCBS061XBB and ENCBS619KUS). Lane 3: 30 μg of protein from K562 transduced with an shRNA targeting NAA15 (ENCODE Biosample ENCBS171PLV). Lane 4: 30 μg of protein from K562 transduced with an shRNA targeting NAA15 (ENCODE Biosample ENCBS331OZE). Samples were separated by SDS-PAGE, transferred to a membrane and blotted using antibodies against NAA15 (ENCODE Antibody ENCAB161WRM) and GAPDH as controls.