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

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Sample Description: CRISPR genome editing of PSMA1 in K562 cells
Cell Line: K562
RNA ID: PSMA1-BGKcLV13-61
ENCODE BIOSAMPLE ACCESSION: ENCBS662MSX

This document contains the protocols used to clone target guide sequence, to generate lentiCRISPRv2-gRNA 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.

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	PSMA1-human
gRNA ID	BGC#0000334
gRNA sequence	CCGCAATTGAGATACCAATA

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 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 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 (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).

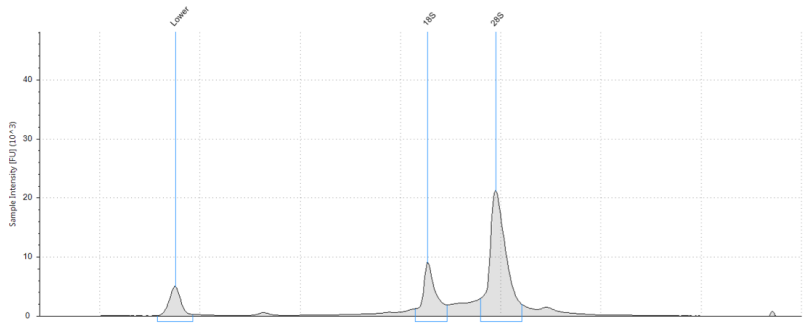

Figure 1: Agilent TapeStation image of 1 μl of total RNA sample.

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.

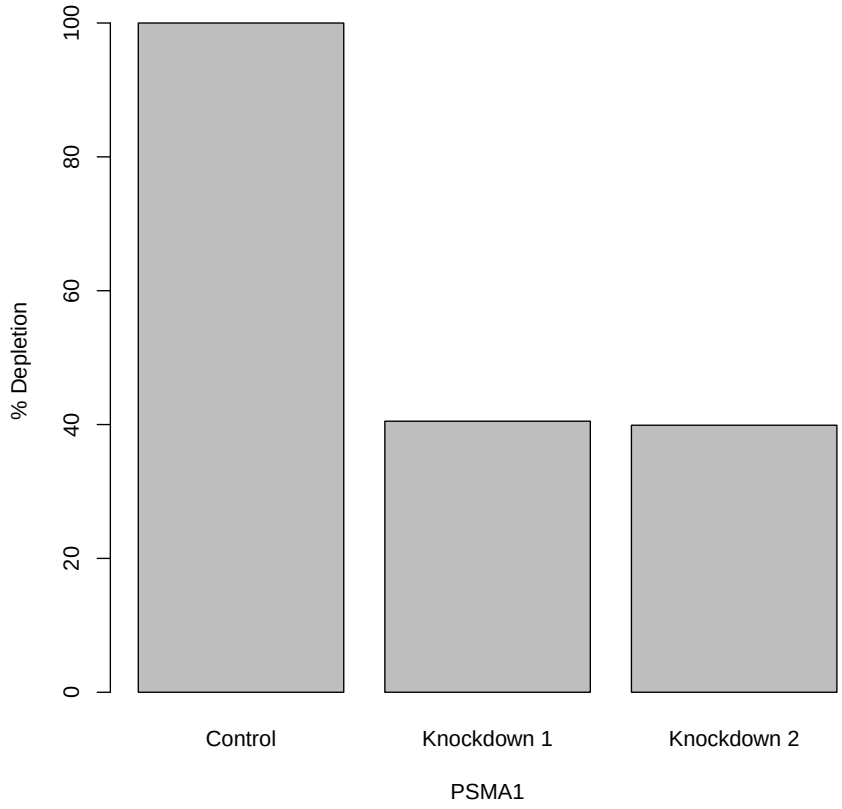

Figure 2: qRT-PCR analysis of depletion level of the target RNA binding protein in control and knockdown cells. The percent depletion was calculated in the RNA sample isolated from K562s transduced with a gRNA targeting PSMA1 (ENCODE Biosample ENCBS662MSX) in comparison to K562 cells transduced with a control non-target gRNA (ENCODE Biosample ENCBS323JXU). 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: PSMA1

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.

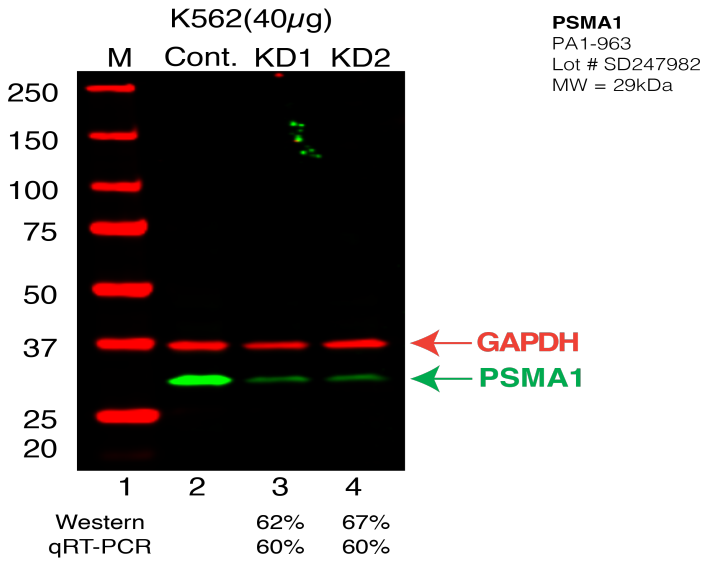

Figure 3: Western Blot Analysis of depletion level of the target RNA binding protein in control and knockdown cells. Lane 1: Molecular weight marker. Lane 2: 30 μg of protein from K562 transduced with a control, non-target gRNA (ENCODE Biosample ENCBS323JXU and ENCBS160IFA). Lane 3: 30 μg of protein from K562 transduced with a gRNA targeting PSMA1 (ENCODE Biosample ENCBS662MSX). Lane 4: 30 μg of protein from K562 transduced with a gRNA targeting PSMA1 (ENCODE Biosample ENCBS903RNW). Samples were separated by SDS-PAGE, transferred to a membrane and blotted using antibodies against PSMA1 (ENCODE Antibody ENCAB320WSD) and GAPDH as controls.