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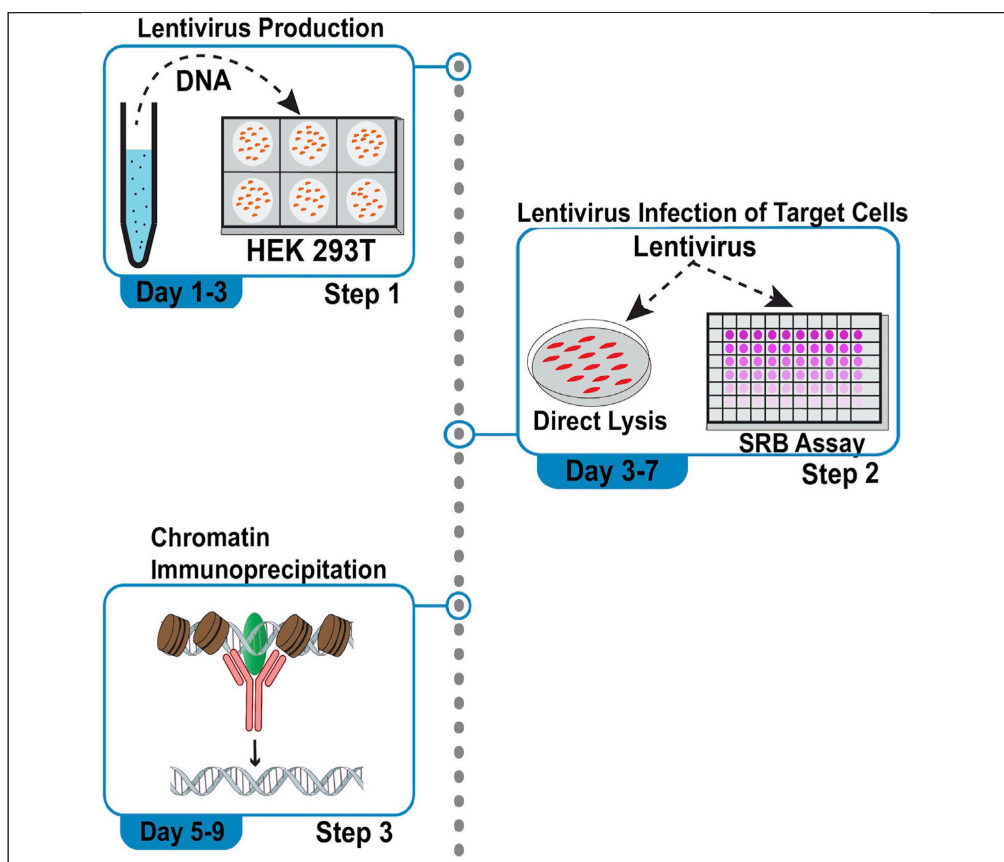
Protocol

Optimized lentiviral vector transduction of adherent cells and analysis in sulforhodamine B proliferation and chromatin immunoprecipitation assays



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Highlights

A high-efficiency calcium phosphate/ BES approach to lentivirus transduction

Allows fast analysis of gene expression by direct lysis

Reproducible measurements of growth over several days in SRB assays

Includes an optimized protocol for ChIP and PCR validation

Transduction with lentiviral vectors is a useful approach to study the molecular function of specific genes in mammalian cells. Here, we present a calcium phosphate-based transfection protocol that guarantees highly efficient production and delivery of lentiviral vectors in adherent cultured cells. We also describe in detail a direct lysis technique to measure protein expression, an optimized sulforhodamine B proliferation assay, and a step-by-step chromatin immunoprecipitation procedure to verify the binding of ETV5 to *E2F1* first intron in SYO-1 sarcoma cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Li et al., STAR Protocols 4, 102109
March 17, 2023 © 2023 The Author(s).
https://doi.org/10.1016/j.xpro.2023.102109

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https://doi.org/10.1016/j.xpro.2023.102109

SUMMARY

Transduction with lentiviral vectors is a useful approach to study the molecular function of specific genes in mammalian cells. Here, we present a calcium phosphate-based transfection protocol that guarantees highly efficient production and delivery of lentiviral vectors in adherent cultured cells. We also describe in detail a direct lysis technique to measure protein expression, an optimized sulforhodamine B proliferation assay, and a step-by-step chromatin immunoprecipitation procedure to verify the binding of ETV5 to *E2F1* first intron in SYO-1 sarcoma cells.

For complete details on the use and execution of this protocol, please refer to Kingston et al. (2003),[1] Ireton et al. (2002),[2] Brown et al. (2009),[3] DeSalvo et al. (2021),[4] Vichai and Kirtikara (2006),[5] and Boyer et al. (2005).[6]

BEFORE YOU BEGIN

The protocol below describes a step-by-step transduction of HEK 293T cells using calcium phosphate/DNA precipitates in bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; BES) buffer. The main advantages of this transduction method are its low cost, simplicity, efficiency, and the speed at which it yields results. After two days of lentivirus production by the 293T packaging cells, the viral supernatant is directly applied to the target cells, no selection required. The cells can be analyzed as early as one day post-infection. An additional advantage is the ability to scale-up the experiment and simultaneously infect enough cells (~10^8 range) to implant in animals on the following day.[4]

We also have successfully used this transfection method with non-viral expression vectors in a variety of mammalian cells, including NIH3T3, HCT116 (colon cancer cells), and U2OS (osteosarcoma cells), for protein expression or in reporter activity assays.

In this protocol, we use the round cell sarcoma CDS-X1 cells as targets for infection with the lentiviral vector pCW57.1-DUX4-CA to show efficient overexpression of the DUX4 cDNA, and the synovial sarcoma SYO-1 cells for transduction with three short hairpin (sh) vectors -PLKO.1-TRC2 (non-target control), PLKO.1-shSS18-1, PLKO.1-shSSX2-2- to demonstrate efficient depletion of the SS18-SSX2 fusion oncoprotein in the sarcoma cells. However, this infection method has proven to be highly efficient in other mammalian cells such as human and mouse mesenchymal stem cells (h/mMSCs), C2C12 myoblasts, C28 human chondrocytes, TC-71 Ewing's sarcoma cells, and round cell sarcoma CDS-X1 and CDS-S2 cells. Moreover, we have successfully transduced these target cells with

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numerous other lentiviral expression or depletion vectors. DUX4 overexpression in SYO-1 cells with pCW57.1-DUX4-CA was previously reported in DeSalvo et al.[4]

Institutional permissions

All experiments involving the use of lentiviral vectors conform to the relevant regulatory standards of the Institutional Biosafety Committee (IBC) at University of Miami, under approved protocol 22-121. You will need to acquire permissions from the relevant institutions.

Preparation one: HEK 293T packaging cells

Timing: 3 days

1. Stock preparation:
   a. Expand early passage 293T cells.
   b. Freeze in multiple aliquots in liquid nitrogen for long term storage. Cells are frozen in 10% DMSO and 90% heat-inactivated fetal bovine serum (FBS) or 90% growth medium.

Note: For consistent high efficiency transfection, it is essential to thaw and use a new vial of 293T cells every 8–10 weeks.

2. 293T passaging and maintenance:
   a. Grow cells in DMEM supplemented with 10% heat-inactivated FBS and 1× penicillin/streptomycin.
   b. Split cells 1:5 to 1:6 every 2–3 days or when they reach 80%–90% confluency.
      i. Aspirate the growth medium from 100 mm dish.
      ii. Add 3 mL of trypsin, gently swirl and aspirate.
      iii. Add 2 mL of trypsin and incubate at 37°C for 1–5 min to detach the cells.
      iv. Add 6 mL of growth medium (3× trypsin volume) to neutralize the trypsin.
      v. Add the 8 mL cell suspension to a 15 mL centrifuge tube.
      vi. Pellet cells in a tabletop centrifuge at 500 × *g* for 5 min at 20°C–22°C.
      vii. Aspirate the supernatant and resuspend the cell pellet in 6 mL of growth medium.
      viii. Add 1 mL of cells to a 100 mm tissue culture (TC) dish containing 9 mL growth medium.
      ix. Swirl dish in four directions several times to spread cells evenly and incubate at 37°C.

Note: 293T cells adhere poorly, they should therefore be gently handled upon passaging. Do not let them reach full confluency, grow in clumps, or allow their growth media to turn yellow, as this will significantly decrease their transfection efficiency.

Preparation two: Lentiviral DNA vectors

Timing: 5 days

Note: High purity DNA is necessary to achieve high transfection efficiency.

3. Transform ultracompetent bacteria with DNA, unless plasmids are provided as glycerol stocks to be directly plated on bacterial growth agar.
4. Grow bacterial colonies on Terrific Broth (TB)/agar medium supplemented with selection antibiotic (e.g., ampicillin-100 μg/mL).

Note: We found that TB growth medium is superior to Luria-Bertani (LB) broth for low-copy number plasmids such as some short hairpin RNA (shRNA) vectors (e.g., TRC2 backbone sh-lentiviral vectors from Mission-Sigma used in this protocol). TB medium (#T9179; Sigma-Aldrich) is prepared by dissolving 20.6 g of TB powder in 1 l tap water. It is distributed in

200 mL aliquots/flask, sealed with aluminum foil and autoclave tape, then autoclaved. The 2 mL mini culture media are taken from a separate TB stock autoclaved in a glass bottle and stored at 4°C for multiple uses. To avoid contamination of the TB stock with atmospheric bacteria, open the bottle on the bench and aspirate the total TB volume needed for all mini cultures with a sterile plastic pipette, transfer it to a plastic (50 mL) tube, then close the lid quickly and return the bottle to the fridge. Add antibiotics to the TB in the plastic tube and distribute as 2 mL aliquots in bacterial culture tubes for a 16–18 h growth.

5. Mini culture: pick and inoculate a bacterial colony in a 14 mL bacterial culture tube containing 2 mL of TB/antibiotic medium. Grow for 16–18 h in a shaker incubator (220–250 rpm) at 37°C.
6. Maxi culture: inoculate 200 μL of the bacterial mini culture in a 1-liter flask (covered with aluminum foil) containing 200 mL of TB/antibiotic medium. Grow in a shaker incubator (220–250 rpm) for 16–18 h at 37°C.
7. Next day: pour the 200 mL of bacterial culture in a 250 mL plastic centrifuge bottle and pellet the bacteria in a cold (4°C) centrifuge at 5,000 × *g* for 15 min.

Note: For maximum DNA yield, it is critical to grow bacteria for no longer than 16–18 h to achieve an OD600 of 0.5–1 and the bacteria are in a log phase of growth. Bacterial pellets (without supernatant) can be stored frozen at −20°C (up to one month) until ready for DNA isolation.

8. Purify DNA from the bacterial pellets using a commercial DNA maxiprep kit (e.g., GenElute #NA0300; Sigma-Aldrich), according to the manufacturer's instructions; https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/116/626/na0310bul.pdf.

Note: Helpful tips to increase DNA yield: in the last step of the maxiprep protocol, elute DNA with molecular biology grade H_2O prewarmed in a 37°C water bath and distribute as 500 μL aliquots in 1.5 mL microcentrifuge tubes. To precipitate and sterilize DNA, add 56 μL of 3 M sodium acetate (NaOAc) pH 5.2 to achieve a final 0.3 M concentration, then add 1.1 mL of molecular biology grade pure ethanol (70% final concentration). Mix and store the DNA in alcohol at −80°C for 16–18 h. Next day, pellet DNA in a cold microcentrifuge (4°C) at 20,000 × *g* for 30–40 min. Move the tubes to the TC hood and decant the ethanol supernatants without disturbing the DNA pellets. Wash the pellets with addition of chilled 70% ethanol (500 μL/tube) and spin at 20,000 × *g* for 3 min at 4°C. Repeat the wash one more time. Carefully decant the ethanol wash, then do a pulse spin to remove the last remaining ethanol with a thin pipette tip without disturbing the DNA pellets. Let the DNA pellets dry in the TC hood for a few minutes. Add sterile molecular biology H_2O to pellets (60 μL–100 μL/tube) and let the DNA dissolve for 16–18 h at 4°C (leave the tubes in the fridge). On the following day, mix the dissolved DNA pellets of each prep by pipetting and combine them in one tube; save a few microliters for measuring DNA concentration and purity.

⚠ CRITICAL: Always run what is left of the aliquot on an agarose gel next to a DNA marker ladder to confirm the integrity and identity of the purified DNA.

9. Measure DNA concentration and purity in a nanodrop spectrophotometer. The 260 nm/280 nm absorbance ratio determines DNA purity, and it should be $\geq$ 1.8.
10. DNA stocks are stored at −20°C and can be repeatedly thawed for transfection.

Alternatives: To minimize time, the DNA pellets may be resuspended in sterile 10 mM Tris pH 8.0 (same volumes as above) and dissolved immediately by pipetting, a brief vortex, followed by a pulse spin. Also, the above method has been optimized for the use of GenElute SIGMA kits. For other commercial DNA purification kits (e.g., Qiagen, Thermo Fisher, Zymo Research), follow manufacturer's instructions.

pH	0.1 M Na_2HPO_4 dibasic (mL)	0.1 M NaH_2PO_4 monobasic (mL)
5.8	7.9	92.1
6.0	12.0	88.0
6.2	17.8	82.2
6.4	25.5	74.5
6.6	35.2	64.8
6.8	46.3	53.7
7.0	57.7	42.3
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
7.8	89.6	10.4
8.0	93.2	6.8

Table 1. Preparation of 0.1 M sodium phosphate buffer at 25°C

0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 are stable for 1 year at 20°C–25°C.

Preparation three: 2× BES, pH 6.95

Timing: 3 h

CRITICAL: The BES buffer optimal pH for transfections falls within a very narrow range: 6.95–6.98. It is extremely critical to adjust the pH to the exact range (we routinely aim for 6.95).

11. Prepare 10 mL of 0.1 M sodium phosphate pH 6.95 as follows:
    a. Make a 0.1 M NaH_2PO_4 (monobasic sodium phosphate) solution.
    b. Make a 0.1 M Na_2HPO_4 (dibasic sodium phosphate) solution.
    c. Following the sodium phosphate buffer volumes listed in Table 1, mix the two solutions at the ratio that generates a 0.1 M sodium phosphate solution pH 6.95: for 10 mL add 4.27 mL of 0.1 M NaH_2PO_4 and 5.73 mL of 0.1 M Na_2HPO_4.
12. Prepare 5 M sodium hydroxide (NaOH) in H_2O.
13. Prepare 2× BES buffer in a 500 mL batch.
    a. Calibrate the pH meter.
    b. Add 400 mL molecular biology grade H_2O.
    c. Add BES powder to final 50 mM (5.33 g).
    d. Add sodium chloride (NaCl) to final 280 mM (8.18 g).
    e. Add 0.1 M sodium phosphate pH 6.95 stock to final 1.5 mM (7.5 mL).
        i. Adjust the pH to 6.95 with a few drops of 5 M NaOH (carefully added).
    f. Add H_2O to 500 mL.
14. Sterilize in the TC hood through a 0.45 μm filter membrane (Corning-500 mL unit).
15. Distribute in 50 mL aliquots, store at −20°C.

Note: 2× BES can be thawed repeatedly. The BES buffers maintain their pH over long periods of time (6 months-1 year) when stored properly.

Preparation four: 2.5 M calcium chloride ($CaCl_2$)

Timing: 2 h

16. Add 80 mL molecular biology grade H_2O.
17. Add 36.75 g $CaCl_2$ dihydrate.
18. Adjust with H_2O to 100 mL.
19. Sterilize through a 0.45 μm filter membrane (Corning-150 mL unit), in the TC hood.

20. Distribute in 25 mL aliquots, store at $-20^\circ C$.

Note: Frequent freeze-thaws of 2.5 M $CaCl_2$ do not affect transfection efficiency.

Pretest 2× BES/$CaCl_2$ precipitate formation before filtering and storage

Timing: 1 h

⚠ CRITICAL: Success of the calcium phosphate transfection method depends on the formation and size of the calcium phosphate/DNA precipitates. To save precious time and reagents, and to guarantee high efficiency transfections, it is critical to test the freshly made solutions for the quality of the calcium phosphate precipitates before filtering and storage. The pretest can be performed on the bench.

21. Make a 0.25 M $CaCl_2$ solution by diluting the 2.5 M stock 1:10 in H_2O.
22. Add equal volumes (500 μL) of 2× BES and 0.25 M $CaCl_2$.
23. Mix by a very brief vortex (1–2 full swirls only; do not over-vortex).
24. Allow precipitate formation; 15–20 min.
25. Add a drop of the $CaCl_2$/BES mix onto a slide.
26. Examine the calcium phosphate precipitate formed under a light microscope. Ideally, the precipitate should be the size of sand granules covering the surface.

Preparation five: Chloroquine

Timing: 1 h

27. Prepare a 25 mM stock solution in 1× PBS.
28. In the TC hood, sterilize through a 0.2 μm syringe filter and distribute 1 mL aliquots in sterile 1.5 mL tubes.
29. Store at $-20^\circ C$.

Preparation six: Polybrene

Timing: 1 h

30. Prepare a 4 mg/mL stock solution in 1× PBS.
31. In the TC hood, sterilize through a 0.2 μm syringe filter and distribute 1 mL aliquots in sterile 1.5 mL tubes.
32. Store at $-20^\circ C$.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-DUX4	EMD Millipore	CAT#MABD116
Rabbit polyclonal anti-SSX2	University of Miami- J. Eid	N/A
Rabbit polyclonal anti-E2F1	Cell Signaling Technology	CAT#3742S
Rabbit polyclonal anti-ETV5 for immunoblot	Abcam	CAT#ab102010
Rabbit polyclonal anti-ETV5 for ChIP	ProteinTech Group	CAT#13011-1-AP
Rabbit polyclonal anti-beta-actin	Cell Signaling Technology	CAT#4967
Rabbit normal IgG	Cell Signaling Technology	CAT# 2729

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
MAX Efficiency DH10B bacteria	Invitrogen	CAT#18297-010
Chemicals, peptides, and recombinant proteins		
Acetic acid- Glacial	VWR Chemicals	CAT#BDH3096
Ampicillin	Sigma-Millipore	CAT#A9518
BES Free Acid- ULTROL grade	Millipore	CAT#391334
BSA (20 mg/mL) molecular biology grade	New England Biolabs	CAT#B9000
Calcium chloride dihydrate	Sigma-Aldrich	CAT#C7902
Chloroform	Sigma-Aldrich	CAT#C2432
Chloroquine	Sigma-Aldrich	CAT#C6628
cOmplete Protease Inhibitor Cocktail	Roche	CAT#04693132001
DMEM 4.5 g/L glucose	Corning	CAT#10-013-CV
DPBS-1×-calcium and magnesium free	VWR	CAT#02-0119-0500
EDTA disodium salt dihydrate	EMD-Millipore	CAT#324503
EGTA	Sigma-Aldrich	CAT#3889
Ethyl alcohol, pure	Sigma-Aldrich	CAT#459836
FBS- Benchmark, heat-inactivated	GeminiBio	CAT#100-106
Formaldehyde- 16% methanol-free	Thermo Scientific	CAT#28908
Glycine	Alfa Aesar	CAT#43497
Glycerol	Sigma-Aldrich	CAT#G5516
Glycogen	Thermo Scientific	CAT#R0561
HEPES	Sigma-Aldrich	CAT#H3375
Hydrochloric acid (HCl)	EMD-Millipore	CAT#HX0603-3
Lithium chloride (LiCl)	Sigma-Aldrich	CAT#62476
N-lauroylsarcosine	Sigma-Aldrich	CAT#L9150
NP40 (IGEPAL)	Sigma-Aldrich	CAT#I8896
Penicillin:streptomycin (100×)	GeminiBio	CAT#400-109
Phenol:chloroform:isoamyl alcohol	Thermo Scientific	CAT#17908
Polybrene- hexadimethrine bromide	Sigma-Millipore	CAT#H9268
Potassium hydroxide (KOH)	EMD-Millipore	CAT#1050330500
Protein A/G magnetic beads	PIERCE	CAT#88802
Proteinase K (20 mg/mL) RNA grade	Thermo Scientific	CAT#EO0491
RNAse A (10 mg/mL) DNAse Protease-free	Thermo Scientific	CAT#EN0531
SDS	Sigma-Aldrich	CAT#71725
Sodium chloride (NaCl)	Sigma-Aldrich	CAT#71376
Sodium deoxycholate	Sigma-Aldrich	CAT#D6750
Sodium phosphate- monobasic dihydrate	Sigma-Aldrich	CAT#71505
Sodium phosphate- dibasic dihydrate	Sigma-Aldrich	CAT#71643
Sodium hydroxide (NaOH)	Sigma-Aldrich	CAT#S8045
Sodium acetate (NaOAc) trihydrate	Sigma-Aldrich	CAT#32318
Sulforhodamine B (SRB)	Sigma-Aldrich	CAT#S1402
Terrific Broth (TB)	Sigma-Aldrich	CAT#T9179
Trichloroacetic acid (TCA)	Sigma-Aldrich	CAT#T0699
Tris base	Bio-Rad	CAT#161-0719
Triton X-100	Sigma-Aldrich	CAT#T8787
Trypsin 0.25%-EDTA	Corning	CAT#25-053-CI
Water-molecular biology grade H2O	Corning	CAT#46-000-CV
Cell lifter	CELLTREAT	CAT#229305
Cellulose acetate filter unit- 0.22 μm-150 mL	Corning	CAT#431154
Cellulose acetate filter unit- 0.45 μm-500 mL	Corning	CAT#431206
Millex-HA syringe filter- 0.45 μm	Millipore	CAT#SLHAR33SB
Syringe- Slip Tip- 20 mL	BD Biosciences	CAT#302831
Critical commercial assays		
GenElute HP Plasmid Maxiprep kit	Sigma-Aldrich	CAT#NA0300
Accustart II PCR supermix	Quantabio	CAT#95137

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
HEK 293T cells	University of Miami-J Eid	N/A
SYO-1 synovial sarcoma cells	Memorial Sloan-Kettering Cancer Center- Marc Ladanyi	N/A
CDS-X1 round cell sarcoma cells	NCCRI-Tokyo-Tadashi Kondo	N/A
Oligonucleotides		
E2F1-E12-Forward: 5′- AGGTAGAAGCTGCCTAACTG-3′	Sigma	N/A
E2F1-E12-Reverse: 5′- TGCTGCTGATGGGGTTAAATG-3′	Sigma	N/A
Recombinant DNA		
PLKO.1-TRC2 (non-target control)	MISSION-Sigma	CAT#SHC202
PLKO.1-shSS18-1	MISSION-Sigma	CAT#TRCN0000108564
PLKO.1-shSSX2-2	MISSION-Sigma	CAT#TRCN0000021689
pCW57.1-DUX4-CA	Addgene	CAT#99281
Software and algorithms		
Microsoft Excel	Microsoft	N/A
R/RStudio	Open Source	N/A
ImageJ	National Institutes of Health (NIH)	N/A
Other		
37°C bacterial incubator	N/A	N/A
37°C shaker platform	N/A	N/A
37°C – 5% CO2 tissue culture incubator	N/A	N/A
Benchtop shaker platform	N/A	N/A
Benchtop centrifuge	N/A	N/A
Cell counter	N/A	N/A
End-to-end rotator	N/A	N/A
Heating block	N/A	N/A
Hemocytometer	N/A	N/A
Inverted microscope	N/A	N/A
Microcentrifuge	N/A	N/A
Microplate reader iMARK	Bio-Rad	N/A
Multichannel pipette	N/A	N/A
Nanodrop spectrophotometer	Thermo Scientific	NANODROP 2000
PCR cycler	N/A	N/A
pH meter	N/A	N/A
Sonicator	MISONIX	XL-2000
Standing magnet	Thermo Fisher	DYNAL MPC-S
Stir plate	N/A	N/A
Tissue culture hood - Biosafety Level 2	N/A	N/A
Vortex mixer	N/A	N/A
Water baths- 37°C, 55°C, 65°C	N/A	N/A

MATERIALS AND EQUIPMENT

2× SDS-PAGE sample buffer

Reagent	Final concentration	Amount
1 M Tris-HCl 6.8	0.125 M	2.5 mL
10% SDS	4%	8.0 mL
Glycerol	20%	4.0 mL
2-Bromomercaptoethanol	10%	2.0 mL
Bromophenol blue	0.02% (w/v)	4 mg
H_2O mol biol grade	N/A	3.5 mL

Note: The 2× sample buffer is stable for up to 6 months when stored at −20°C.

⚠ CRITICAL: SDS (sodium dodecyl sulfate) is an irritant, harmful if inhaled. Face masks should be worn upon handling SDS powder.

- 1% Acetic acid: add 10 mL glacial acetic acid (GAA) to 990 mL milli-Q H_2O.

Note: The 1% acetic acid solution is prepared fresh before each use at 20°C–22°C.

⚠ CRITICAL: GAA is corrosive to tissues, use in well ventilated area and wear protective clothing (face mask, goggles, gloves) during preparation. Never add H_2O to GAA, always add GAA to H_2O to prepare the 1% solution.

- 0.057% SRB in 1% acetic acid: dissolve 5.7 mg SRB B in 10 mL 1% acetic acid.

Note: The 0.057% SRB in 1% acetic acid solution is prepared fresh before each use at 20°C–22°C.

- 10% tricholoroacetic acid (TCA): add 10 mL TCA to 90 mL milli-Q H_2O.

Note: The 10% TCA solution is stable for 6 months when stored at 4°C in a glass bottle.

⚠ CRITICAL: TCA is corrosive and a strong irritant to skin and eyes. Protective clothing (face mask, goggles, gloves) is highly recommended.

- 10 mM Tris (unbuffered): dissolve 12.11 mg Tris base in 10 mL milli-Q H_2O.

Note: The Tris solution is prepared fresh before each use at 20°C–22°C.

- 2.5 M glycine: dissolve 1.87 g glycine powder in 10 mL mol biol grade H_2O.

Note: Place solution in a 37°C water bath to help dissolve glycine. This solution is prepared fresh at 20°C–22°C before use.

- 50× Protease inhibitors (PI): dissolve 10 tablets of Complete Protease Inhibitor Cocktail in 10 mL mol biol grade H_2O. Aliquot in 1.5 mL centrifuge tubes.

Note: PI aliquots are stable at −20°C. Avoid frequent freeze-thaws; consider the expiration date indicated by the vendor.

Tris-EDTA (TE) 8.0

Reagent	Final concentration	Amount
1 M Tris-HCl 8.0	10 mM	0.1 mL
0.5 M EDTA 8.0	1 mM	0.02 mL
H_2O mol biol grade	N/A	9.88 mL

Note: TE buffer is stable for one year, stored at 20°C–22°C.

TE-50 mM NaCl

Reagent	Final concentration	Amount
1 M Tris-HCl 8.0	10 mM	0.1 mL

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Continued		
Reagent	Final concentration	Amount
0.5 M EDTA 8.0	1 mM	0.02 mL
5 M NaCl	50 mM	0.1 mL
H_2O mol biol grade	N/A	9.78 mL

Note: TE-NaCl is prepared fresh for each use.

Lysis Buffer 1 (LB1)		
Reagent	Final concentration	Amount
1 M HEPES-KOH 7.5	50 mM	0.5 mL
5 M NaCl	140 mM	0.28 mL
0.5 M EDTA 8.0	1 mM	0.02 mL
50% glycerol	10%	2.0 mL
10% NP-40 (IGEPAL)	0.5%	0.5 mL
10% Triton X-100	0.25%	0.25 mL
50× PI	1×	0.2 mL
H_2O mol biol grade	N/A	6.25 mL

Note: LB1 buffer is prepared fresh for each use.

Lysis Buffer 2 (LB2)		
Reagent	Final concentration	Amount
1 M Tris-HCl 8.0	10 mM	0.1 mL
5 M NaCl	200 mM	0.4 mL
0.5 M EDTA 8.0	1 mM	0.02 mL
0.5 M EGTA 8.0	0.5 mM	0.01 mL
50× PI	1× PI	0.2 mL
H_2O mol biol grade	N/A	9.27 mL

Note: LB2 buffer is prepared fresh for each use.

Lysis Buffer 3 (LB3		
Reagent	Final concentration	Amount
1 M Tris-HCl 8.0	10 mM	0.1 mL
5 M NaCl	100 mM	0.2 mL
0.5 M EDTA 8.0	1 mM	0.02 mL
0.5 M EGTA 8.0	0.5 mM	0.01 mL
10% Na-Deoxycholate	0.1%	0.1 mL
20% N-lauroylsarcosine	0.5%	0.25 mL
50× PI	1× PI	0.2 mL
H_2O mol biol grade	N/A	9.12 mL

Note: LB3 buffer is prepared fresh for each use.

RIPA wash buffer		
Reagent	Final concentration	Amount
1 M HEPES-KOH 7.5	50 mM	1.25 mL

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Continued		
Reagent	Final concentration	Amount
5 M LiCl	500 mM	2.5 mL
0.5 M EDTA 8.0	1 mM	0.05 mL
10% NP-40 (IGEPAL)	1%	2.5 mL
10% Na-Deoxycholate	0.7%	1.75 mL
H_2O mol biol grade	N/A	16.95 mL

Note: RIPA buffer is prepared fresh for each use.

Elution Buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl 8.0	50 mM	0.5 mL
5 M NaCl	50 mM	0.1 mL
0.5 M EDTA 8.0	10 mM	0.2 mL
10% SDS	1%	1.0 mL
H_2O mol biol grade	N/A	8.2 mL

Note: Elution buffer is prepared as a 10 mL batch and stored at 4°C for 2 months.

STEP-BY-STEP METHOD DETAILS

Step 1. HEK 293T transfection and lentivirus production

Timing: 3 days

In this step we describe in detail the calcium phosphate/BES buffer method used for high efficiency transfection of 293T packaging cells with lentiviral vectors. After transfection on Day 1, virus production by 293T cells is allowed to proceed for two days (Day 1-Day 3). On Day 2, the target cells (SYO-1 sarcoma cells) are plated for infection with the produced virus on the following day (Day 3).

All procedures are performed in TC hood.

293T cells were transfected with the pCW57.1-DUX4-CA, PLKO.1-TRC2, PLKO.1-shSS18-1, and PLKO.1-shSSX2-2 lentiviral vectors, one 6-well plate for each vector.

The following details are provided for the transfection of one 6-well plate/ one vector.

Day 0

Timing: 1 h

1. 293T plating in 6-well plate, one day before transfection.
   a. Add 2 mL of fresh growth medium to each well of a 6-well plate.
   b. Detach with 0.25% trypsin and pellet 293T cells from a confluent (80%–90%; ~1.5×10^7 cells) 100 mm plate in a 15 mL centrifuge tube, as described above.
   c. 1:6 dilution: resuspend the cell pellet in 6 mL of growth medium, mix well, then add 1 mL (~2.5×10^6 cells) of the suspension to a centrifuge tube containing 5 mL of fresh growth medium.
   d. Mix cells thoroughly by pipetting, then add 1 mL to each of the 6 wells.
   e. Return cells to 37°C TC incubator for 16–18 h.

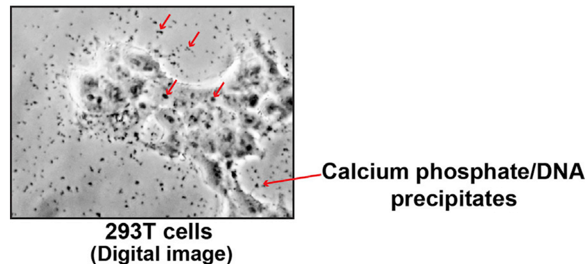


Figure 1. Calcium phosphate transfection of 293T cells with lentiviral vectors
Digital image of calcium phosphate/DNA precipitates (red arrows) that form in BES buffer and cover the surface of the transduced 293T cells. Image was obtained with a Canon PowerShot SD600 digital camera and an inverted microscope (Vista Vision) 40× objective.

Day 1

Timing: 8 h

2. Transfection of 1× 6-well plate.
   a. In a 15 mL centrifuge tube combine in sequence:
      i. 750 µL of 0.25 M $CaCl_2$; dilute the 2.5 M $CaCl_2$ stock 1:10 in molecular biology grade H_2O.
      ii. 15 µg of packaging plasmid- pCMV-dR7.74psPAX2.
      iii. 6 µg of envelope plasmid- pMD2.G.
      iv. 20 µg of one of the lentiviral vectors mentioned above.
      v. 750 µL of 2× BES buffer.
   b. Close the tube and mix with a brief vortex (1–2 full swirls only).
   c. Let sit in TC hood for 20–30 min to allow formation of DNA/calcium phosphate precipitate.
   d. During this incubation period, 10 min prior to transfection, add 5 µL of 25 mM chloroquine to each of the 6-wells, swirl to mix, and return the culture plate to the 37°C incubator.
   e. At time of transfection, add dropwise 250 µL of the DNA/BES transfection mix to each well.
   f. Gently swirl the plate to mix and return it to the 37°C incubator for 6–7 h.
   g. Replace the transfection medium in each well with 3 mL of growth medium.

Note: At the end of the transfection period, it is advisable to examine the 293T cells for overlying sand-like precipitates, as shown in Figure 1.

Day 2

Timing: 1 h

3. Plate sarcoma cells for next day infection.
   a. For infections performed in 100 mm TC dishes: split cells in their regular growth media so they are 30%–40% confluent the next day.

Note: It is critical that the passaged cells are not confluent and are actively growing before plating for infection. Quiescent cells are poorly transduced.

   b. For infections performed in 96-well plates- the sulforhodamine B (SRB) assay:
      i. Detach cells with 0.25% trypsin from an actively growing culture.
      ii. Pellet cells and resuspend in growth medium.
      iii. Count cells in the suspension.
      iv. Dilute the cell suspension in growth medium to achieve a final concentration of 2–3 × 10^4 cells/mL.

v. Thoroughly mix the cell suspension, transfer it into a sterile trough, and add 100 μL to each well (2–3 × 10^3 cells/well) with a multichannel pipette.
vi. For consistent results, plate cells in the "inner" wells of the plate and reserve the "outer-most" wells to fill with fluid (plain growth medium or PBS) in order to prevent drying of the inner wells that contain the plated cells.

Note: For reproducible results, it is critical to maintain even distribution of cells among dishes and wells during plating. Therefore, we strongly advise repeated mixing of cell suspensions in-between plates and prompt plating, especially when dealing with multiple plates, to prevent cell sedimentation at the bottom of the trough or in the pipette. 2,000–3,000 cells/well is a good starting average number that provides room for SYO-1 cell division over several days. However, this number can be optimized (1,000–5,000 cells/well range), depending on the specific growth rate of the tested cell line.

Alternatives: Lipid-based transfection reagents such as lipofectamine, have been successful alternates to the long-established calcium phosphate method. However, their cost has prohibited their use in large scale transfections. By contrast, the Polyethylenimine (PEI) polymer, with a significantly higher cost-effectiveness is considered a good substitute as a transfection reagent.

Step 2. Lentiviral infection of sarcoma cells for protein and SRB growth analyses

In this section we provide a step-by-step description of lentivirus transduction in target cells on Day 3. We also include a direct lysis method for protein analysis and the details of the SRB assay to measure the effect of lentivirus expression on cell growth over three days.

Infection plus protein analysis in SYO-1 and CDS-X1 cells (days 3-5)

Infection plus growth analysis in SYO-1 cells (days 3-7)

In this protocol 293T cells are transfected with the lentivirus vector pCW57.1-DUX4-CA for overexpression, and the sh-vectors PLKO.1-TRC2, PLKO.1-shSS18-1, and PLKO.1-shSSX2-2 for depletion. The viral supernatant of one transfected 293T 6-well plate (~18 mL yield) dedicated to each vector is sufficient for the infection of 2 × 100 mm dishes or 180 wells of 96-well plates.

Day 3

Timing: 1 h for step 4

Timing: 6 h for step 5

Timing: 6 h for step 6

Timing: 6–10 h for step 7

Timing: 8 h and 40 min for step 8

Timing: 2 days 4 h for step 9

Timing: 2 h/plate/graph for step 10

Timing: 2 h/graph for step 11

4. Lentivirus collection.
   a. After two days of virus production, collect the 293T medium containing the lentivirus and pass it through a 0.45 μm syringe filter (mixed cellulose esters; Millex-HA).

   Note: Alternatively, the virus can be harvested by centrifugation of the 293T supernatant at 500 × *g* for 5 min at 20°C–22°C.

   b. Add 36 μL of polybrene to the 18 mL viral supernatant collected from the 6-well plate (3 mL/well) and mix.
   c. For best results, immediately infect SYO-1 cells with the virus thus produced.

   Note: Do not be alarmed if the transfected 293T cells appear to have detached from the plate after two days. Floating 293T cells do produce virus.

   Alternatives: The virus supernatant may be stored at 4°C for 2–3 days without losing potency. It may also be aliquoted and stored at −80°C for 2–3 weeks. However, it loses titer, hence efficiency overtime. It is critical to avoid frequent freeze/thaw cycles of the supernatant. If long term storage is necessary or desired without loss of titer, the virus can be concentrated up to 100-fold as follows: 1) ultracentrifugation, or 2) mixing the virus with polyethylene glycol (PEG; TAKARA Lenti-X concentrator) followed by a slower spin cycle. The concentrated virus is aliquoted and stored at −80°C.

5. Lentivirus infection of SYO-1 and CDS-X1 cells plated in 100 mm TC dishes.

   Note: One 6-well plate of transfected 293T produces enough virus for 2 × 100 mm dishes of target cells.

   a. Add 8–8.5 mL of the filtered virus supernatant/polybrene to each 100 mm dish.
   b. Place dish in the TC incubator on a flat shelf for even spread of the virus.
   c. Allow infection to proceed for 5–6 h.
   d. Aspirate the infection medium and add to the dish 10 mL of growth medium.
   e. Allow cells to grow in TC incubator until time of analysis (direct lysis or ChIP).

6. Lentivirus infection of SYO-1 cells in 96-well plates- SRB assay.
   a. Aspirate the growth medium of the cells plated on Day 2.
   b. Add 100 μL/well of the filtered virus/polybrene supernatant with a multichannel pipette.
   c. Allow infection to proceed for 5–6 h.
   d. Replace the infection medium with 100 μL/well of fresh growth medium.
   e. Allow cells to grow in TC incubator until time of SRB assay (Day 5–7).

   Note: To build a growth curve over several days, remember to dedicate a separate 96-well plate for each consecutive day.

7. Sequential infections.

   Note: Sequential infections are beneficial when improved efficiency is needed, for transduction of different vectors, or when dealing with transduction-resistant cells.

   a. Allow the first infection to proceed for 3–5 h.
   b. During this time, store the virus supernatant destined for the second infection at 4°C.
   c. Aspirate the first virus medium and replace it with the second virus.
   d. Incubate cells for additional 3–5 h.
   e. Replace the infection medium with fresh growth medium.

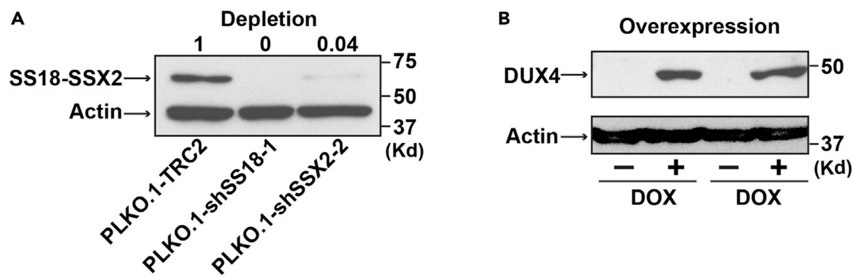


Figure 2. Efficient lentiviral SS18-SSX2 depletion and DUX4 overexpression in sarcoma cells with one round of infection following the calcium phosphate/BES method

(A) Immunoblot shows SS18-SSX2 depletion with sh-lentiviral vectors PLKO.1-shSS18-1 and PLKO.1-shSSX2-2 in SYO-1 cells. PLKO.1-TRC2 is the non-target control vector. SS18-SSX2 band intensities were quantified after subtracting background values in ImageJ software. Each value was then measured as a ratio relative to the intensity of the corresponding actin in the panel below. Finally, the relative SS18-SSX2 intensity ratios of PLKO.1-shSS18-1 and PLKO.1-shSSX2-2 were compared to the relative ratio of the control PLKO.1-TRC2 that was designated as 1.

(B) Immunoblot shows inducible expression of lentiviral DUX4 in CDS-X1 sarcoma cells. DOX is doxycycline. β-actin (Actin) served as loading control.

Note: The results presented in this protocol- protein analysis, SRB assays, and ChIP assay -were all derived from single-round infections.

Day 5

8. Testing SYO-1 and CDS-X1 cells infection efficiency by direct lysis and western blotting.

   Note: We normally assess infection efficiency and protein expression by western blotting. DUX4 overexpression with pCW57.1-DUX4-CA in CDS-X1 cells, and SS18-SSX2 depletion with PLKO.1-shSS18-1 and PLKO.1-shSSX2-2 vectors in SYO-1 cells, are shown in Figure 2.

   a. Place the 2× SDS-PAGE sample buffer at 37°C to thaw.

      Note: Return the sample buffer to the −20°C freezer immediately after use.

   b. Take the infected SYO-1 and CDS-X1 cells out of the 37°C incubator.
   c. Under light microscope, thoroughly examine every dish and estimate the density of adherent/surviving cells in every dish; write this percentage on the lid of the dish with a magic marker (e.g., 30%, 50%, 80%, etc.).
   d. Make a mental or written note of the extent of dead/floating cells in every dish.

      Note: This visual test is very beneficial as it provides first clues of a growth phenotype caused by the transduced vectors. Recording percentage of cell confluency helps determine the appropriate volume of 2× SDS-PAGE buffer later used for direct cell lysis.

   e. Place cells on working bench and aspirate growth medium.
   f. Wash the cells three times with ice-cold 1× PBS.
   g. Decant the last PBS wash.
   h. Tilt the dish and let it stand on the bench to drain the remaining PBS.
   i. Aspirate the last of PBS.
   j. Add to each dish the quantity of 2× SDS sample buffer adjusted according to cell density written on the lid. For example, if a 100% confluent dish that would count 8×10^6–10^7 cells is lysed in 1 mL 2× SDS-PAGE buffer, a 50% confluent dish is lysed in 0.5 mL SDS-PAGE buffer.

k. Scrape the lysed cells with a cell lifter.
l. Place cell lysate in a 1.5 mL centrifuge tube.
m. Boil lysate at 95°C for 5 min, pulse spin tubes to set the condensation at the tube lids and store the lysates at −20°C until time of loading on SDS-PAGE gel for western transfer.

Note: Frozen protein lysates are stable, they can be used in several immunoblots and withstand re-freezing. However, it is best to avoid re-boiling and instead warm the lysates at 50°C–60°C for 3–5 min in subsequent western blots to protect some labile proteins (e.g., ETV4) that degrade upon frequent boiling.

n. Load and run on SDS-PAGE gel equal volumes of lysates which, according to the lysis method described above (i.e., adjusting lysate volumes to cell density), should contain equal protein concentrations. Assess protein expression levels by immunoblotting. Include a loading control (β-actin) for quantification of relative band intensities.
o. Quantify changes in protein expression by comparing band intensities in ImageJ imaging analysis software.

Note: For protein content, we estimate that on average, lysis of a fully confluent 100 mm dish that contains $\sim10^7$ adherent (non-round) cells yields ~1 mg protein. Thus, 50 μL of ~1 mg/mL lysate are used to load ~50 μg of protein in a lane. We often study labile proteins that include transcription factors with short half-lives. Direct lysis and boiling in the protein sample buffer that contains 4% SDS and the reducing agent 2-mercaptoethanol protect and allow detection of unstable proteins that would otherwise be rapidly degraded in standard RIPA buffer, despite protective supplements such as protease and phosphatase inhibitors. Therefore, determining the volume of lysis buffer for every dish based on cell density estimate is a reliable method for equal protein loading. It does however require a practiced eye and a longstanding habit of observing cells under a microscope.

Alternatives: Lysis with RIPA buffer and the Bradford assay for protein quantification are excellent alternatives to the direct lysis method, provided the tested cellular proteins are stable.

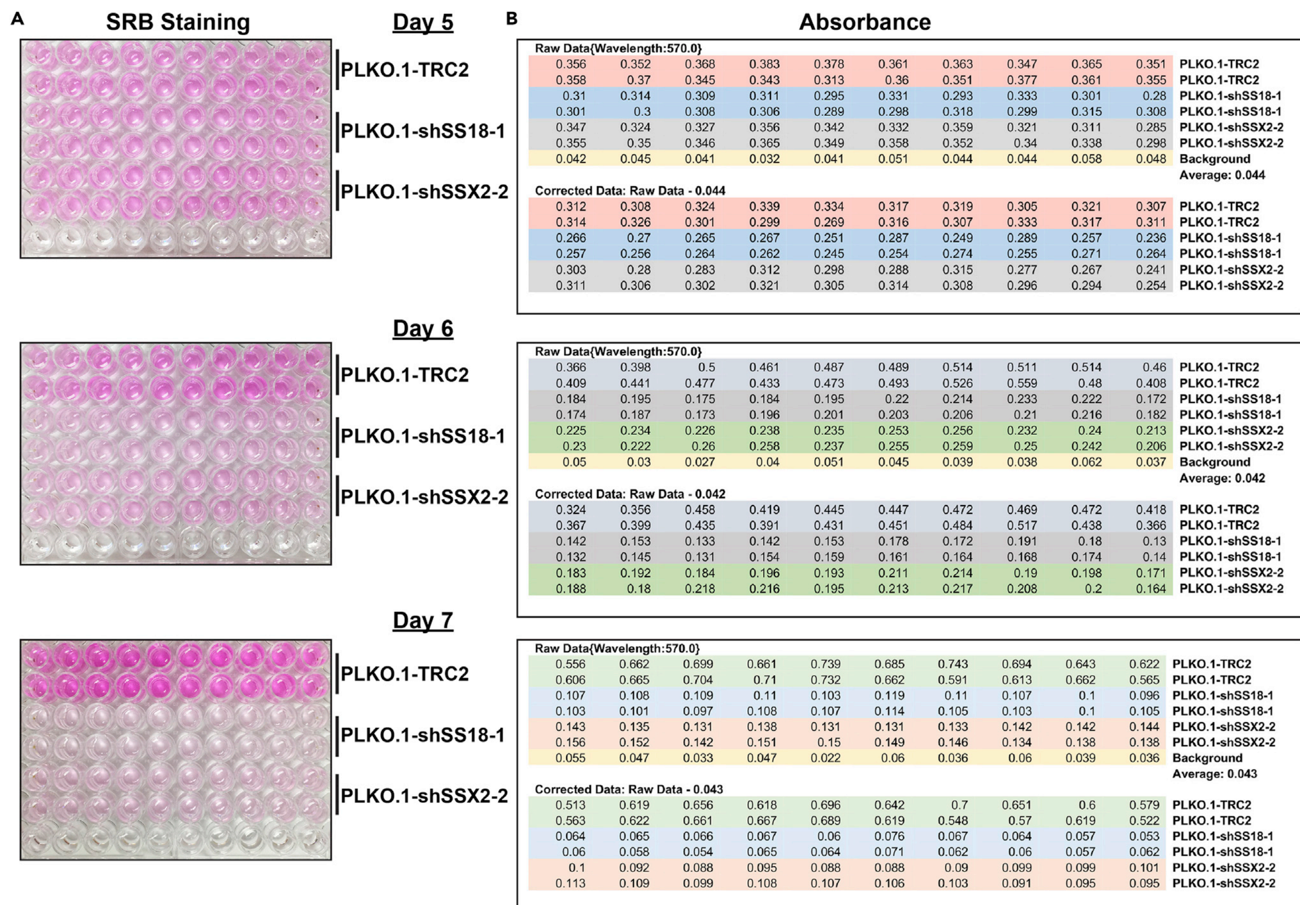
Day 5–7

The SRB assay was developed by Vichai and Kirtikara[5] for cytotoxicity screening. We have been successfully applying this method to measure the effects of a variety of lentiviral vectors on cell proliferation. We also use this assay to build drug dose-response curves.

One main advantage of the SRB assay is its performance in 96-well plates, which allows the inclusion of several experimental groups and acquisition of multiple data points for each group in one plate, two important criteria for reproducibility and statistical significance. SRB is a bright pink dye that binds basic amino acid residues under acidic conditions. It measures cellular protein content, and the dye intensity is proportional to cell mass. It is a highly sensitive assay and gives linear readings for cell densities ranging from 1,000 cells to 180,000 cells/well. With this wide room for growth, we can measure cell proliferation over several days under diverse conditions.

Figures 3 and 4A show the effect of SS18-SSX2 depletion by lentiviral PLKO.1-shSS18-1 and PLKO.1-shSSX2-2 on SYO-1 growth. Cell growth was measured in SRB assays over three days (Days 5, 6, and 7), as detailed below.

9. SRB staining performed on SYO-1 cells on designated days post-infection (Figure 3A).
   a. Cell fixation.
      i. Take the 96-well plate out of the 37°C incubator and transfer to the working bench.
      ii. Add 100 μL of cold 10% TCA to each well with a multichannel pipette.



Day 5

Raw Data{Wavelength:570.0}										
0.356	0.352	0.368	0.383	0.378	0.361	0.363	0.347	0.365	0.351	PLKO.1-TRC2
0.358	0.37	0.345	0.343	0.313	0.36	0.351	0.377	0.361	0.355	PLKO.1-TRC2
0.31	0.314	0.309	0.311	0.295	0.331	0.293	0.333	0.301	0.28	PLKO.1-shSS18-1
0.301	0.3	0.308	0.306	0.289	0.298	0.318	0.299	0.315	0.308	PLKO.1-shSS18-1
0.347	0.324	0.327	0.356	0.342	0.332	0.359	0.321	0.311	0.285	PLKO.1-shSSX2-2
0.355	0.35	0.346	0.365	0.349	0.358	0.352	0.34	0.338	0.298	PLKO.1-shSSX2-2
0.042	0.045	0.041	0.032	0.041	0.051	0.044	0.044	0.058	0.048	Background
										Average: 0.044

Corrected Data: Raw Data - 0.044										
0.312	0.308	0.324	0.339	0.334	0.317	0.319	0.305	0.321	0.307	PLKO.1-TRC2
0.314	0.326	0.301	0.299	0.269	0.316	0.307	0.333	0.317	0.311	PLKO.1-TRC2
0.266	0.27	0.265	0.267	0.251	0.287	0.249	0.289	0.257	0.236	PLKO.1-shSS18-1
0.257	0.256	0.264	0.262	0.245	0.254	0.274	0.255	0.271	0.264	PLKO.1-shSS18-1
0.303	0.28	0.283	0.312	0.298	0.288	0.315	0.277	0.267	0.241	PLKO.1-shSSX2-2
0.311	0.306	0.302	0.321	0.305	0.314	0.308	0.296	0.294	0.254	PLKO.1-shSSX2-2

Day 6

Raw Data{Wavelength:570.0}										
0.366	0.398	0.5	0.461	0.487	0.489	0.514	0.511	0.514	0.46	PLKO.1-TRC2
0.409	0.441	0.477	0.433	0.473	0.493	0.526	0.559	0.48	0.408	PLKO.1-TRC2
0.184	0.195	0.175	0.184	0.195	0.22	0.214	0.233	0.222	0.172	PLKO.1-shSS18-1
0.174	0.187	0.173	0.196	0.201	0.203	0.206	0.21	0.216	0.182	PLKO.1-shSS18-1
0.225	0.234	0.226	0.238	0.235	0.253	0.256	0.232	0.24	0.213	PLKO.1-shSSX2-2
0.23	0.222	0.26	0.258	0.237	0.255	0.259	0.25	0.242	0.206	PLKO.1-shSSX2-2
0.05	0.03	0.027	0.04	0.051	0.045	0.039	0.038	0.062	0.037	Background
										Average: 0.042

Corrected Data: Raw Data - 0.042										
0.324	0.356	0.458	0.419	0.445	0.447	0.472	0.469	0.472	0.418	PLKO.1-TRC2
0.367	0.399	0.435	0.391	0.431	0.451	0.484	0.517	0.438	0.366	PLKO.1-TRC2
0.142	0.153	0.133	0.142	0.153	0.178	0.172	0.191	0.18	0.13	PLKO.1-shSS18-1
0.132	0.145	0.131	0.154	0.159	0.161	0.164	0.168	0.174	0.14	PLKO.1-shSS18-1
0.183	0.192	0.184	0.196	0.193	0.211	0.214	0.19	0.198	0.171	PLKO.1-shSSX2-2
0.188	0.18	0.218	0.216	0.195	0.213	0.217	0.208	0.2	0.164	PLKO.1-shSSX2-2

Day 7

Raw Data{Wavelength:570.0}										
0.556	0.662	0.699	0.661	0.739	0.685	0.743	0.694	0.643	0.622	PLKO.1-TRC2
0.606	0.665	0.704	0.71	0.732	0.662	0.591	0.613	0.662	0.565	PLKO.1-TRC2
0.107	0.108	0.109	0.11	0.103	0.119	0.11	0.107	0.1	0.096	PLKO.1-shSS18-1
0.103	0.101	0.097	0.108	0.107	0.114	0.105	0.103	0.1	0.105	PLKO.1-shSS18-1
0.143	0.135	0.131	0.138	0.131	0.131	0.133	0.142	0.142	0.144	PLKO.1-shSSX2-2
0.156	0.152	0.142	0.151	0.15	0.149	0.146	0.134	0.138	0.138	PLKO.1-shSSX2-2
0.055	0.047	0.033	0.047	0.022	0.06	0.036	0.06	0.039	0.036	Background
										Average: 0.043

Corrected Data: Raw Data - 0.043										
0.513	0.619	0.656	0.618	0.696	0.642	0.7	0.651	0.6	0.579	PLKO.1-TRC2
0.563	0.622	0.661	0.667	0.689	0.619	0.548	0.57	0.619	0.522	PLKO.1-TRC2
0.064	0.065	0.066	0.067	0.06	0.076	0.067	0.064	0.057	0.053	PLKO.1-shSS18-1
0.06	0.058	0.054	0.065	0.064	0.071	0.062	0.06	0.057	0.062	PLKO.1-shSS18-1
0.1	0.092	0.088	0.095	0.088	0.088	0.09	0.099	0.099	0.101	PLKO.1-shSSX2-2
0.113	0.109	0.099	0.108	0.107	0.106	0.103	0.091	0.095	0.095	PLKO.1-shSSX2-2

Figure 3. SRB assay to measure effect of SS18-SSX2 depletion on SYO-1 cell growth

Growth was measured 2 days (Day 5), 3 days (Day 6), and 4 days (Day 7) after infection of SYO-1 cells with lentiviral vectors PLKO.1-shSS18-1, PLKO.1-shSSX2-2, and PLKO.1-TRC2 (control) on Day 3, as described in the text.

(A and B) (A) Shows digital images of SRB-stained 96-well plates of infected SYO-1, and (B) displays corresponding absorbance reads at 570 nm on the respective days. The raw data (upper tables) were corrected (lower tables) by subtracting the average value of the background noise (blank wells) in each plate. Twenty wells were allocated to each vector in one plate.

   iii. Transfer plate to a flat surface at 4°C (cold room or fridge).
   iv. Allow TCA fixation to proceed for 1 h.
b. Wash after fixation.
   i. After 1 h of TCA fixation, bring the plate to a lab sink.
   ii. Place a stack of folded paper towels on the edge of the sink.
   iii. Open the distilled water tap and regulate the water to a smooth jet.
   iv. Remove the lid and tilt the 96-well plate at an angle.
   v. Allow the water to fill the 96 wells from one side of the plate.

Note: Do not allow the water jet to hit the bottom of the wells as it can cause cells to detach.

   vi. Empty the wells by inverting the plate.
   vii. Tap the inverted plate on the stack of paper towels to remove remaining water.
   viii. Repeat the wash three more times.
   ix. Let the open plate air dry for 16–18 h, at 20°C–22°C.

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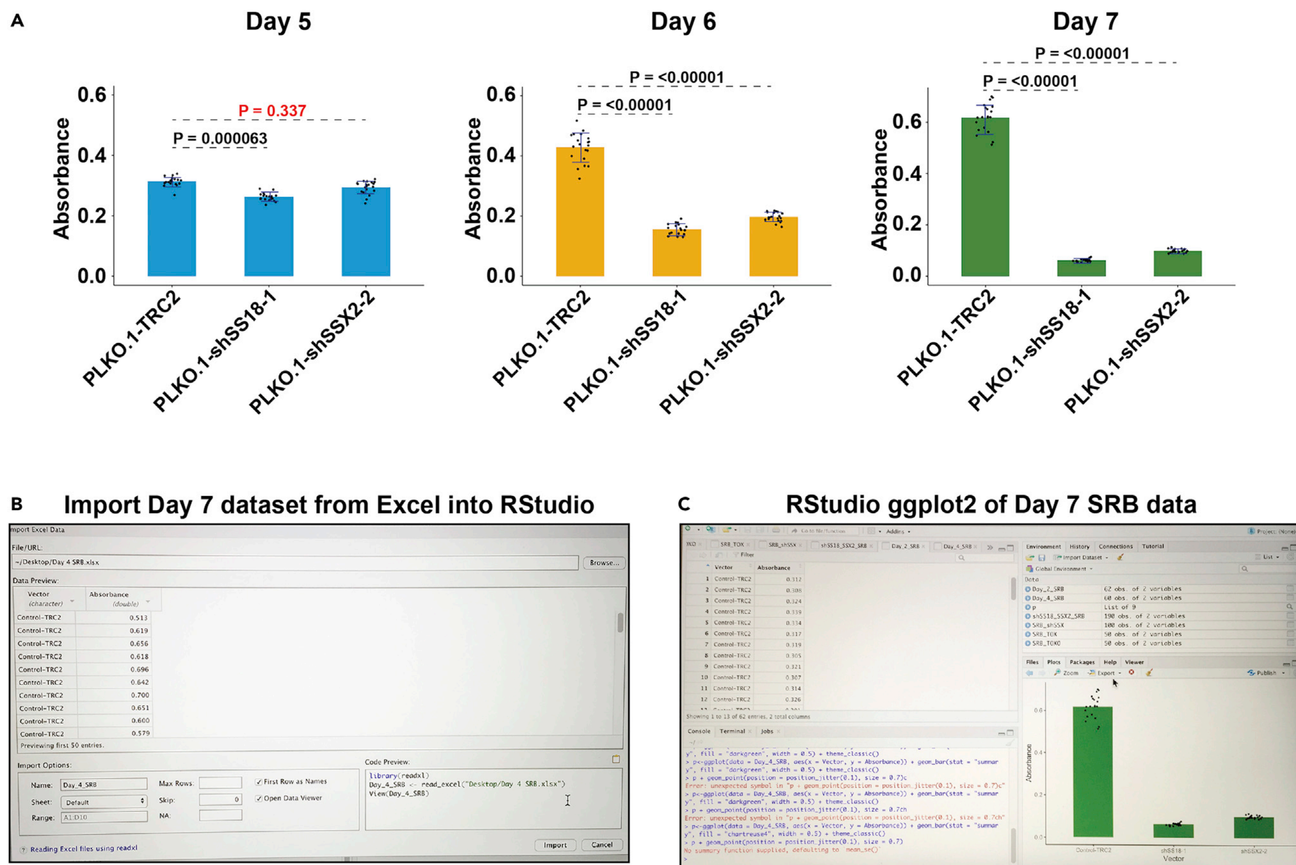

Figure 4. SS18-SSX2 knockdown inhibits SYO-1 cell growth

(A) Bar graphs (R) of the SRB results obtained on days 5, 6, and 7 and presented in Figure 3. Note the continued proliferation of SYO-1 cells transduced with control vector PLKO.1-TRC2 versus the apparent cell growth arrest and eventual death with the SS18-SSX2-depleting vectors, PLKO.1-shSSX8-1 and PLKO.1-SSX2-2. The plots were built in RStudio. Error bars represent standard deviation. P values were calculated from Z scores of PLKO.1-shSSX8-1 and PLKO.1-SSX2-2 relative to control PLKO.1-TRC2 and two-tailed hypothesis. $P \leq 0.05$ are considered significant. $P = 0.337$ (in red) in Day 5 graph of PLKO.1-SSX2-2 indicates non-significant growth effect.

(B) Screenshot shows imported Excel dataset of Day 7 SRB values into RStudio.

(C) Screenshot displays the commands written to generate the bar graph of Day 7 SRB data.

Note: The dry plate can be stored indefinitely at 20°C–22°C after fixation.

c. Cell staining.
   i. Add 100 μL of 0.057% SRB solution to each well.
   ii. Let the plate stain flat on the bench for 30 min at 20°C–22°C.
d. Washes.
   i. Bring to the sink: the stained plate, 500 mL of 1% acetic acid, and a stack of paper towels.
   ii. Invert the plate in the sink to empty the SRB solution.
   iii. Wash the plate four times with 1% acetic acid, as described for the water washes.
   iv. Remember to fill the wells by pouring the acid solution from the side, by tilting the plate.
   v. Let the plate air dry for 16–18 h on the bench at 20°C–22°C.

Note: The dry plate can be stored indefinitely at 20°C–22°C.

e. Absorbance measurement (Figure 3B).
   i. Add 200 μL of 10 mM Tris to each well.
   ii. Agitate the plate on a bench shaker at 20°C–22°C for 5 min to dissolve the SRB stain.

iii. Place the plate in a microplate reader, measure absorbance at wavelength 490 nm–570 nm.

Note: We routinely measure our assays at 570 nm wavelength, with consistent results. In the event the reads at 570 nm appear to be outside the linear range, a lower wavelength such as 490 nm is a good alternative.

Alternatives: The MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) assay has commonly been used to measure changes in cell viability/proliferation. This assay in general represents a good alternative to the SRB assay. However, its high sensitivity to labile metabolic conditions could be a source of false apparent fluctuations in viability/growth.

10. Data analysis and graphing (Figures 3B and 4).
    a. Save the absorbance data in Excel.
    b. In Excel, calculate the average of reads in one row at the edge of the plate that did not contain cells. This average will serve as "background" noise.
    c. Adjust reads in the experimental wells by subtracting the average background value in b).
    d. Transfer the "corrected" values of each experimental group to RStudio to draw a bar graph with overlaid dots that represent individual values.
11. Build a basic bar graph in RStudio from an Excel sheet (Figure 4).

Note: RStudio is an excellent medium for building all sorts of graphs; bar graphs, box plots, dot plots etc.., for clear data presentation.

Figure 4 illustrates the steps for SRB data (Figure 3B) retrieval and R wording to generate the bar graphs shown in Figure 4A.
    a. Watch a tutorial on the use of RStudio to learn R language. Several good tutorials exist online. b. Download and install R software (open source).
    c. Download and install RStudio for data plotting.
    d. Create a new excel sheet. Name the data of the Y axis (Absorbance) and under the name organize the SRB values in a continuous column according to sequential groups (control-TRC2, PLKO.1-SS18-1, PLKO.1-SSX2-2). In the adjacent column, give the X axis a name (Vector), and in the X axis column insert the name of each data point (control-TRC2, PLKO.1-SS18-1, PLKO.1-SSX2-2).
    e. Open RStudio and import the Excel dataset in the Environment box, as depicted in Figures 4B and 4C.
    f. Select the Excel data to be viewed in the Base box (top left, Figure 4C).
    g. To build the SRB graph of Absorbance/growth values on Day 7 (Figure 4A), the following commands were written in the Console (lower left box, Figure 4C):

```
library(ggplot2)
   p<-ggplot(data=Day_4_SRB, aes(x=Vector, y=Absorbance))
   + geom_bar(stat=''summary'', fill=''darkgreen'', width=0.5)
   + theme_classic()
   p+geom_point(position=position_jitter(0.1, size=0.7)) + ylim(0,0.6)
```

    h. The graph image will appear in the plots box and saved as a TIFF file.
12. Statistics.

a. Calculate the mean, SEM (Standard Error of the Mean), and SD (Standard Deviation) in R or Excel.
b. Calculate P values to compare means between 2 groups using 2-tailed tests.
c. For 3 or more experimental groups, use 1-way ANOVA F ratios and P values to compare multiple means.

Step 3. Chip assay in SYO-1 cells transduced with lentiviral sh-SS18-SSX2 vectors

Timing: 4 days 13 h; Day 5–9

Timing: 1 h for step 13

Timing: 4 h 15 min for step 14

Timing: 45 min for step 15

Timing: 1 h for step 16

Timing: 1 day 8 h for step 17

Timing: 1 day 1 h for step 18

Timing: 1 day 4 h 45 min for step 19

Timing: 1 day 2 h for step 20

Timing: 5–6 h for step 21

Chromatin immunoprecipitation (ChIP) is a widely used technique to measure the binding of transcription and chromatin regulators to genomic sequences. In this protocol we describe detailed steps of a CHIP experiment performed in SYO-1 cells transduced with the lentiviruses PLKO.1-TRC2 (non-target control), PLKO.1-shSS18-1, PLKO.1-shSSX2-2, to study the effect of SS18-SSX2 depletion on ETV5 binding to a recently discovered specific site located in intron 1 of the *E2F1* gene locus (Figure 7B). This question arose when we discovered that E2F1 expression (Figure 7A) was significantly reduced upon SS18-SSX2 depletion (Figure 2A), whereas ETV5 levels were increased in the same SYO-1 cells.

Note: We have used this method to determine cell cycle genes occupancy by transcription factors such as Capicua (CIC), ETV5 and E2F1 in naïve and lentivirus transduced sarcoma cells. The method is a modified version of the original protocol published by the Young laboratory.[6] It was adjusted to fit a ChIP experiment with 1.5×10^7–2.5×10^7 cells or the equivalent of five 100 mm dishes at 40%–50% confluency. This cell number is sufficient for four individual IP reactions. It is critical to conduct ChIP experiments with actively growing cells at ~ 40%–50% density unless it is specifically intended for analysis during quiescence (full confluency).

CRITICAL: Cleaning of work surfaces and instruments: it is imperative to conduct any experiment that involves an extremely sensitive assay such as PCR in a clean, uncontaminated environment. Before the start of the ChIP experiment, thoroughly wipe the working surfaces and pipet aids with a 10% bleach solution (100 mL Clorox bleach/900 mL milli-Q water in a spray bottle). In addition, use RNAse-free and DNAse-free plasticware such as plastic pipettes, filtered (aerosol barrier) pipette tips, gloves (rub gloved hands every now and then with a spray of 10% bleach).

Day 5

Note: The quantities suggested throughout were fitted for 1.5×10^7–2.5×10^7 cells or the equivalent of 4–5× 100 mm dishes at 40%–50% confluency. This cell number is sufficient for four individual IP reactions.

13. SYO-1 cell crosslinking and harvest - 2 days post-infection with lentiviral vectors.
    a. Have ready: ice-cold 1× PBS, 16% formaldehyde (20°C–22°C), and 2.5 M glycine (20°C–22°C).
    b. Remove the cells from 37°C incubator and place them on a working bench (20°C–22°C).
    c. With a 10 mL plastic pipette, adjust the volume of growth medium to 7.5 mL in each dish.
    d. Add to each dish 0.5 mL 16% formaldehyde to obtain a 1% final concentration.
    e. Place the dish on a benchtop shaker platform and slowly agitate for 10 min at 20°C–22°C.
    f. Quench with 1/20th volume of 2.5 M glycine (0.42 mL/dish) and agitate for 5 min at 20°C–22°C on a benchtop shaker platform.
    g. Wash cells with cold 1× PBS, repeat wash twice.
    h. After washes, tilt the dishes on the bench and let stand for 1–2 min, then aspirate the last of PBS with vacuum.
    i. Add 1 mL of cold PBS to each dish, collect cells with a cell scraper, and place cells in a 15 mL centrifuge tube kept on ice.

    Note: We typically combine the contents of 4–5× 100 mm dishes in one 15 mL tube.

    j. Pellet cells in a benchtop (4°C or 20°C–22°C) centrifuge at 1,350 × *g* for 5 min.
    k. Aspirate the PBS supernatant.
    l. If not used immediately, flash freeze the cell pellet in a dry ice-ethanol bath or liquid nitrogen for a few minutes and store at −80°C until IP.
14. Blocking magnetic beads: conducted at 4°C with solutions and tubes on ice.
    a. Place amounts of magnetic beads (protein A/G in this experiment) to be used for lysate pre-clearing or IP in two separate tubes.

    Note: We routinely use 15–25 μL of the bead slurry for every IP reaction. The choice of appropriate beads- protein G versus protein A/G -depends on the optimal binding affinity of the IP antibody.

    b. Wash the beads 3 times with 1 mL cold PBS, by inverting the tubes 25 times.
    c. Collect the beads on a standing magnet and remove the PBS with a pipette tip.
    d. Resuspend the beads in 1 mL cold PBS containing 0.1% BSA (mol biol grade).
    e. Block beads for 2–4 h on an end-to-end rotator at 4°C.
    f. Collect blocked beads on a standing magnet and remove supernatants.
    g. Keep one tube of beads on ice for lysate preclearing. Store the second tube with blocked beads at 4°C for use in IP the next day.
15. Cell lysis while blocking beads: conducted at 4°C with solutions and tubes on ice.
    a. Resuspend cell pellet in 3 mL cold lysis buffer 1 (LB1) and transfer 1.5 mL to two chilled 1.5 mL tubes. Rock tubes while rotating at 4°C for 10 min.
    b. Spin tubes at 1,350 × *g* in a cold (4°C) microcentrifuge for 5 min.
    c. Carefully remove supernatant and resuspend each pellet in 1.5 mL cold lysis buffer 2 (LB2). Rock tubes at 4°C for 10 min.
    d. Spin tubes at 1,350 × *g* in a cold (4°C) microcentrifuge for 5 min.
    e. Resuspend each pellet in 1.0 mL cold lysis buffer 3 (LB3).
    f. Keep the two tubes on ice and prepare for sonication.

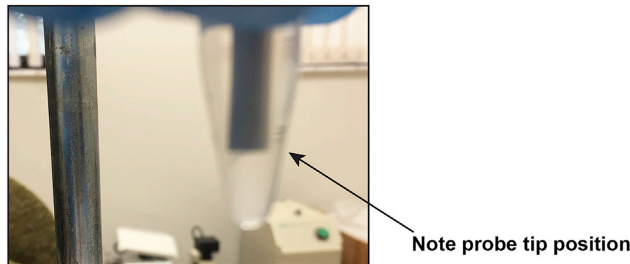

Figure 5. Sonicator probe positioning
Digital image shows how to prevent foam formation and protein denaturation with the probe held centered, close to the bottom of the tube, and away from the tube walls.

16. Sonication to obtain DNA fragments between 200 bp and 600 bp.

Note: Below details describe chromatin sonication using a Misonix XL-2000 sonicator. Refer to Figures 5 and 6 for illustration.

a. Adjust the sonicator gauge to middle power- level 5.
b. Place the ice bucket containing the two tubes under the sonicator probe.
c. Open one tube and with one hand hold it steady, centered (avoid touching the sides), with the probe tip near the bottom of the tube to prevent foam formation, as shown in Figure 5.
d. Pulse for 10 s.
e. Return the tube to ice immediately and pulse the second tube for 10 s.
f. Set the timer for a pause/cooling period of 3 min.

Note: Pause time can be prolonged beyond 3 min to allow a larger number of tubes sufficient time to cool between pulses.

g. Repeat this cycle nine more times, for a total of 10 × 10 s pulses.

Note: The suggested sonication program is adequate for our ChIP-PCR or ChIP-qPCR assays as it reproducibly generates DNA fragments in the required size range (200 bp-600 bp; Figure 6). Sonication conditions must be optimized if different sonicators or a bioruptor are used for shearing chromatin.

⚠ CRITICAL: Chromatin sonication conditions are optimized before beginning the ChIP project. For this, 50 μL–100 μL aliquots of lysates representing various sonication conditions are processed following steps 16 (h-l), 19, and 20 (below, with volumes adjusted accordingly), to obtain purified DNA. The size range of the sonicated DNAs is determined by electrophoresis on 1%–1.5% agarose gels. Once the optimal sonication conditions are established, the size of sheared DNA can be confirmed at the end of the ChIP assay by running 5 μL–10 μL of the input (whole cell extract; WCE) sample on an agarose gel.

h. After sonication, add to each tube 1/10th volume of 10% Triton-X (100 μL).
i. Rotate tubes for 10 min at 4°C.
j. Spin the lysates for 10 min at 20,000 × *g* in cold microcentrifuge.
k. Pool the supernatants in a 15 mL conical tube on ice.
l. Save 50 μL of the lysate as WCE input control and store at −80°C.

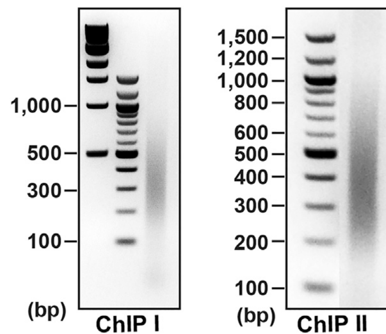

Figure 6. Chromatin shearing with MISONIX XL 2000

Images show sonicated DNA in two independent ChIP experiments with 10 s × 10 pulse cycles and 3-min pauses between cycles. DNAs were run next to a 100 bp DNA ladder and a 1 kb DNA ladder (left image).

Days 5 and 6

17. Immunoprecipitation of shSS18-SSX2 transduced SYO-1 lysates with ETV5 and rabbit IgG (R-IgG) antibodies.
    a. Transfer the combined lysate to two 1.5 mL tubes (1 mL each).
    b. Preclearing: resuspend the blocked beads (see above) in 100 μL of LB3, add 50 μL of the slurry to each tube and rock on rotator for 2 h at 4°C.
    c. Capture beads on a magnet and combine the precleared lysates in a 15 mL tube.
    d. Divide the lysate into individual 1.5 mL tubes. The lysate thus prepared is sufficient for up to four IP reactions.
    e. Add antibodies to the tubes and incubate while rotating for 16–18 h at 4°C.

    Note: We generally use 3–5 μg of antibody for 1 mL lysate.

    f. Next day: resuspend the BSA-blocked magnetic beads prepared on the previous day in cold LB3 (kept in the fridge from the day before), add equal amounts to the lysate/antibody IP tubes, and rock for 4 h at 4°C.
    g. Collect bead-bound chromatin complexes on a magnet and remove supernatant.
    h. Wash beads with 1 mL cold RIPA buffer by rocking for 10 min at 4°C and repeat wash 3 times.
    i. Wash beads with 1 mL cold TE (Tris/EDTA), containing 50 mM NaCl by inverting tubes 25 times. Capture beads on magnet and remove supernatant.

Day 6 to Day 7

18. Elution of ETV5-ChIP complexes and reverse crosslinking.
    a. Resuspend beads in each tube with 70 μL elution buffer prewarmed at 65°C.
    b. Incubate tubes in a 65°C water bath. Set a timer for 20 min, and every 2 min briefly vortex tubes and return to 65°C water bath.
    c. At the end of the 20-min elution, spin tubes in a microcentrifuge at full speed (20,000 × *g*) for 1 min at 20°C–22°C.
    d. Transfer 65 μL of the supernatant/eluate to a new tube and discard the beads. Incubate the eluates at 65°C for 16–18 h to reverse crosslinking.
    e. Thaw the WCE/input tube (stored at −80°C), add 3× volume (150 μL) elution buffer, and incubate at 65°C for 16–18 h to reverse crosslinking.

Day 7 to Day 8

19. ETV5-bound DNA cleaning and precipitation.
    a. Next day: add an equal volume TE; 65 μL to IP tubes and 200 μL to WCE.

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b. RNA digestion: add RNAse A (10 mg/mL) to 200 μg/mL final concentration to all tubes and incubate at 37°C for 2 h.
c. Protein digestion: add proteinase K (20 mg/mL) to 200 μg/mL final concentration to all tubes and incubate at 55°C for 2 h.
d. DNA cleaning: add 5 M NaCl to 200 mM final concentration to all tubes. Add an equal volume of phenol:chloroform:isoamyl alcohol and mix by vigorous vortexing for 20 s. Spin tubes at maximum speed (20,000 × *g*) for 5 min at 20°C–22°C and carefully transfer the top aqueous phase to a new tube. Add an equal volume of chloroform and repeat the same procedure, then carefully transfer the top aqueous phase to a new tube.
e. DNA precipitation: add to each tube 35 μg of glycogen (7 μL of a 5 mg/mL solution) and ≥ 2× volume of absolute ethanol. Thoroughly mix by inverting tubes several times and store overnight at −80°C.

Day 8–9

20. DNA reconstitution.
    a. Next day: pellet DNA at maximum speed (20,000 × *g*) for 40 min at 4°C, carefully decant the supernatant without disturbing DNA pellet.
    b. Add 500 μL of ice-cold 70% ethanol to DNA pellet and spin at maximum speed for 3 min at 4°C. Repeat the ethanol wash once and sediment the last of the remaining ethanol with a pulse spin, then carefully remove the extra alcohol with a thin tip.
    c. Allow the DNA pellet to air dry for a few minutes on the bench.
    d. Add to each tube 30 μL–50 μL of molecular biology grade H_2O (without disturbing the DNA pellet) and allow the DNA pellets to dissolve for 16–18 h at 4°C.

    Note: 10 mM Tris pH 8.0 may be used in place of H_2O to immediately dissolve DNA, with pipetting, a brief vortex, then a pulse spin.

    e. Next day: mix dissolved DNA by pipetting, a brief vortex, followed by a pulse spin.
    f. Measure DNA concentration in a NanoDrop spectrophotometer.

    Note: Glycogen may lower the 260/280 ratio; however, phenol/chloroform cleaning should ensure DNA purity.

21. Pretesting the ChIP DNA with PCR before qPCR.

    Note: To save time and costly resources, we routinely test the quality of ChIP DNA in a semi-quantitative PCR reaction before the qPCR assay; we consider it as a consolidation of the results obtained in the accompanying qPCR.

    a. We present as an example the PCR experiment in Figure 7B that we conducted on the ETV5-ChIP in SYO-1 cells. The results show an evident decrease in ETV5 specific binding to *E2F1* intron 1 region upon SS18-SSX2 depletion with transduced vectors PLKO.1-shSS18-1, PLKO.1-shSSX2-2, compared to control PLKO.1- TRC2 vector.

PCR reaction master mix

Reagent	Amount
DNA - SYO-1 ChIP DNA	25 ng
E2F1-Forward primer-10 μM	1 μL
E2F1-Reverse primer-10 μM	1 μL
2× Accustart II PCR supermix	12.5 μL
H_2O mol biol grade	To 25 μL total

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	35
Annealing	56°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	3 min	1
Hold	4°C	Forever	

b. PCR products were resolved on a 2.5% agarose gel.
c. Relative DNA band intensities were measured with ImageJ software.

EXPECTED OUTCOMES

The detailed protocol for high efficiency lentivirus expression in cultured cells is well established in our laboratory. We have successfully applied this method to overexpress or knockdown proteins using diverse lentiviral vectors in several mammalian cell lines. As depicted in Figure 2, we anticipate a ~100% success rate with any lentivirus tested.

The SRB assay to measure the effect of lentivirus expression on cell growth is easy to perform and reliable. As illustrated in Figures 3 and 4, we expect reproducible results with statistical significance with any adherent cell line tested. Moreover, the SRB assay is a good method to measure drug effects in cytotoxicity screens.

Figure 7 data illustrate how the ChIP protocol described in detail can help uncover previously unknown genomic sites for transcription regulators, such as ETV5 and the *E2F1* locus. We anticipate that this protocol will repeatedly yield robust ChIP data to investigate other transcription factors binding to and regulating different genes.

LIMITATIONS

The limitation with the lentiviral transduction calcium phosphate/BES method is the ability to analyze acute transcriptional and translational cellular events, build growth curves, or study chromatin regulation for transduction effects lasting up to 10–14 days. For permanent effects, methods for genomic integration of vectors such as the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system are more fitting.

TROUBLESHOOTING

Problem 1

293T transfection efficiency is poor (related to step 1).

Potential solution

There are several reasons why the 293T transfection efficiency is low, thus leading to low virus titer and poor transduction of adherent cells: old or overcrowded 293T cells, omitting FBS heat inactivation, BES pH outside the optimal range (prevents formation of optimal calcium phosphate/DNA precipitates), impurities in DNA.

- Thaw a new vial of 293T, split cells 1:5–1:6 every other day, keep cells in proliferative phase, avoid overgrowth. Use a new 293T batch every 8–10 weeks.
- Heat inactivation of FBS is necessary for 293T transduction and virus production. If commercial heat-inactivated serum is not available, this can be performed manually by incubating the FBS bottle in a 56°C water bath for 30 min.
- Calibrate the pH meter for every new BES batch and adjust the pH to an exact 6.95.

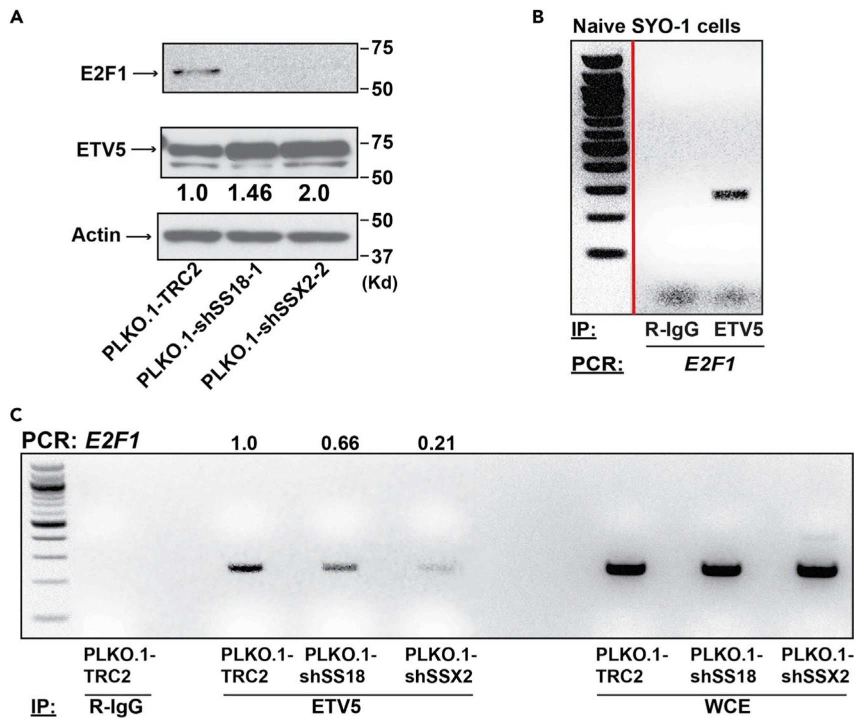

Figure 7. SS18-SSX2 depletion decreases ETV5 specific binding to *E2F1* intron 1

(A) immunoblots show decreased E2F1 levels and increased ETV5 levels in SYO-1 transduced with the SS18-SSX2-depleting lentiviral vectors, PLKO.1-shSSX8-1 and PLKO.1-SSX2-2. PLKO.1-TRC2 is non target control. Numbers under ETV5 image represent relative protein band intensities. ETV5 band intensities were quantified after subtracting background values in ImageJ software. Each value was then determined as a ratio relative to the intensity of the corresponding actin intensity in the panel below. Finally, the relative ratios of intensities of ETV5 in PLKO.1-shSS18-1 and PLKO.1-shSSX2-2 cells were compared to that of the relative ratio of ETV5 intensity in control PLKO.1-TRC2 that was designated as 1.

(B) semi-quantitative PCR that shows ETV5 specific binding to *E2F1* first intron, performed on DNA purified from ETV5/R-IgG ChIP in naïve SYO-1 cells. The IP lanes are designated as R (rabbit)-IgG control and ETV5 (specific rabbit antibody. The DNA band represents a PCR-amplified intron 1 region of the *E2F1* gene that contains an ETV5 binding site. The primers used were: *E2F1*-E12-Forward: 5′- AGGTAGAAGCTGCCTAACTG-3′, and *E2F1*-E12-Reverse: 5′-TGCTGCTGATGGGGTTAAATG-3′. The red line indicates that the markers lane in the DNA gel was not consecutive with the R-IgG and ETV5 lanes.

(C) semi-quantitative PCR performed on DNA purified from ETV5/R-IgG ChIP performed in SYO-1 cells expressing the designated lentiviral vectors. The *E2F1* primers used were the ones described in B). WCE is input DNA. Numbers above the PCR image represent relative intensities. *E2F1* band intensities that reflect ETV5 binding were quantified after subtracting background values in ImageJ software. Each value was then determined as a ratio relative to the intensity of the corresponding WCE (input DNA) intensity. The relative ratios of intensities of *E2F1* band in PLKO.1-shSS18-1 and PLKO.1-shSSX2-2 cells were compared to that of the relative ratio of *E2F1* band intensity in control PLKO.1-TRC2 that was designated as 1. 25 ng of DNA were used in every PCR reaction.

- Although commercial DNA purification kit manuals claim that the DNA eluted directly from the columns is clean enough for experiments, it is advisable to remove potential impurities with ethanol precipitation. If this is not enough, then a good solution is to clean the DNA with phenol/chloroform followed by ethanol precipitation.

Problem 2

Poor infection efficiency (related to step 2).

Potential solution

Dense or quiescent cells are not receptive to viruses, whether it is because of lack of available surface, or their transcription/translation machineries are turned off. Moreover, mammalian cells differ

in their capacity to integrate and express lentiviral vectors, one factor being variations in cell surface composition. More specifically, some cell types such as primary cells or stem cells display low transduction efficiency.

- Plate target cells so they are sparse (~ 30% density) on day of infection.
- To enhance infection rate, subject the cells to repeated infection cycles.
- Alternatively, if the vector carries a selection cassette, positive cells may be selected and expanded.

Problem 3

ChIP assay: high background- strong non-specific signal in IgG controls (related to step 3).

Potential solution

Several factors can generate a high background signal in a ChIP assay, including prolonged crosslinking that induces non-specific interactions, insufficient bead blocking, excessive antibody concentration, and excessive quantity of beads.

- Shorten formaldehyde crosslinking to 5–7 min.
- Increase BSA concentration to 0.5%–1% in the blocking solution or prolong blocking of magnetic beads with 0.1% BSA from 4 h to 16–18 h, or both.
- Avoid using more than 5 μg of antibody in 1 mL lysate.
- 15 μL of bead slurry are often sufficient for IP of 1 mL lysate.

Problem 4

Low or no specific signal in positive control IP (related to step 3).

Potential solution

If no specific signal is detected in the ChIP-PCR reaction, it may be due to low efficiency crosslinking, specific binding sites blocked by BSA, suboptimal sonication conditions (fragments are too small from excessive sonication, too large from insufficient sonication, or chromatin-associated proteins are denatured because of foaming or overheating during sonication), insufficient incubation of chromatin-bound complexes with magnetic beads, wrong choice of magnetic beads, or low affinity/poor-quality antibody.

- Use fresh formaldehyde.
- Skip the bead blocking step and preclear lysate with magnetic beads washed three times with 1 mL cold LB3 buffer.
- Adjust the length or number of pulses to obtain an appropriate DNA size range of 200–600 bp.
- Keep the lysates on ice between pulses, avoid long pulses to prevent overheating and allow enough time (≥ 2 min) for tubes to cool between pulses. Hold the probe tip near the bottom of the tube to avoid foaming, as shown in Figure 5.
- Incubate the beads with the antibody/lysate mix for 16–18 h instead of 4 h.
- Identify the appropriate magnetic beads for the tested IP antibody– e.g., A/G beads have a broader binding range compared to protein G dynabeads.
- Change to a higher affinity IP antibody.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be addressed to the lead contact, Josiane E Eid (jee64@med.miami.edu).

Materials availability

This study did not generate new unique reagents.

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Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

This work was supported by the National Cancer Institute of the National Institutes of Health, United States, under award number P30CA240139.

AUTHOR CONTRIBUTIONS

Conceptualization, J.E.E., J.C.T.; investigation, L.L., J.E.E.; writing, J.E.E., L.L.; funding acquisition, J.C.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Kingston, R.E., Chen, C.A., and Okayama, H. (2003). Calcium phosphate transfection. Curr. Protoc. Cell Biol. *Chapter 20*, Unit 20.3. https://doi.org/10.1002/0471143030.cb2003s19.

2. Ireton, R.C., Davis, M.A., van Hengel, J., Mariner, D.J., Barnes, K., Thoreson, M.A., Anastasiadis, P.Z., Matrisian, L., Bundy, L.M., Sealy, L., et al. (2002). A novel role for p120 catenin in E-cadherin function. J. Cell Biol. *159*, 465–476. https://doi.org/10.1083/jcb.200205115.

3. Brown, M.V., Burnett, P.E., Denning, M.F., and Reynolds, A.B. (2009). PDGF receptor activation induces p120-catenin phosphorylation at serine 879 via a PKCalpha-dependent pathway. Exp. Cell Res. *315*, 39–49. https://doi.org/10.1016/j.yexcr.2008.09.025.

4. DeSalvo, J., Ban, Y., Li, L., Sun, X., Jiang, Z., Kerr, D.A., Khanlari, M., Boulina, M., Capecchi, M.R., Partanen, J.M., et al. (2021). ETV4 and ETV5 drive synovial sarcoma through cell cycle and DUX4 embryonic pathway control. J. Clin. Invest. *131*, e141908. https://doi.org/10.1172/JCI141908.

5. Vichai, V., and Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat. Protoc. *1*, 1112–1116. https://doi.org/10.1038/nprot.2006.179.

6. Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947–956. https://doi.org/10.1016/j.cell.2005.08.020.