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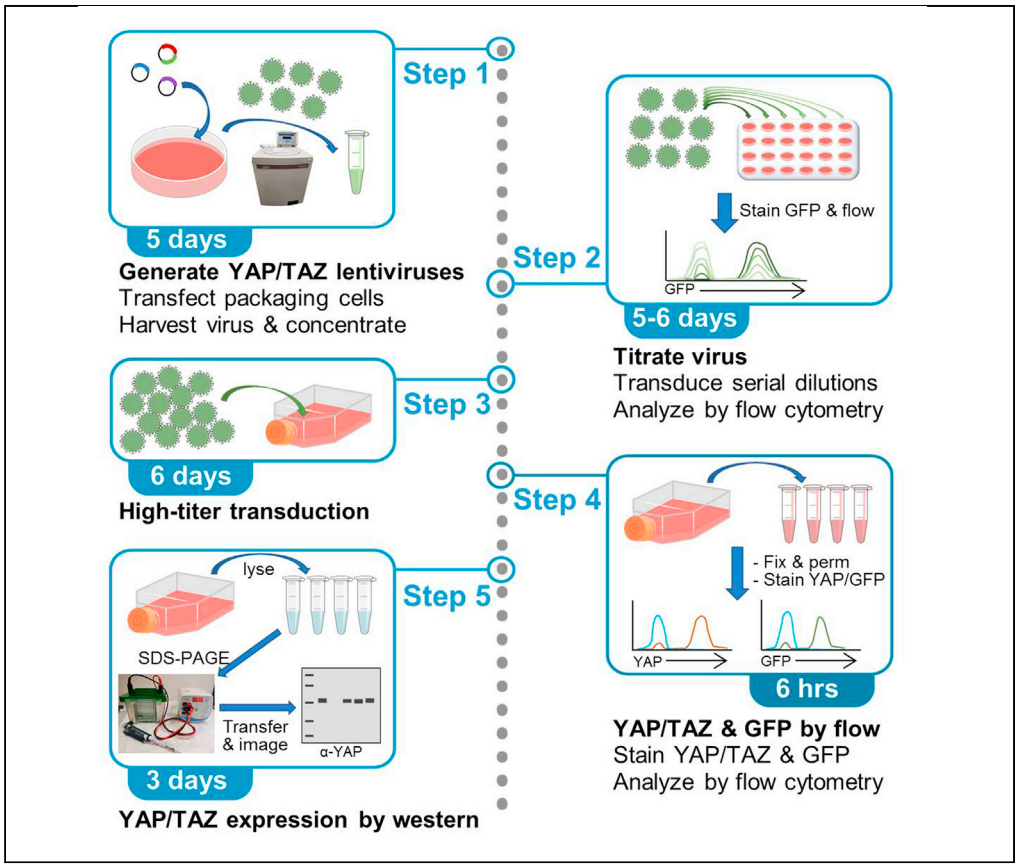
Protocol

Lentiviral-mediated ectopic expression of YAP and TAZ in YAP^{off} cancer cell lines



Ectopic/overexpression systems are important for studying protein function, but care must be taken to avoid artifacts due to excessively high levels of overexpression. To study the function of YAP/TAZ in YAP/TAZ-deficient (YAP^{off}) cancers, we developed a lentiviral system using weak, constitutive promoters to ectopically express YAP/TAZ to physiologically relevant levels. We detail this system along with protocols to assess YAP/TAZ expression by flow cytometry and quantitative western blotting. This system can also be easily adapted for the study of other proteins.

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Highlights

Protocol for ectopic expression of YAP/TAZ in YAP/TAZ-deficient (YAP^{off}) cancers

Quantitative methods to accurately assess ectopic YAP/TAZ expression

Expression of YAP/TAZ to physiological levels avoids over-expression artifacts

Flexible lentiviral vector system can be modified for study of other genes

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Lentiviral-mediated ectopic expression of YAP and TAZ in YAP^{off} cancer cell lines

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SUMMARY

Ectopic/overexpression systems are important for studying protein function, but care must be taken to avoid artifacts due to excessively high levels of overexpression. To study the function of YAP/TAZ in YAP/TAZ-deficient (YAP^{off}) cancers, we developed a lentiviral system using weak, constitutive promoters to ectopically express YAP/TAZ to physiologically relevant levels. We detail this system along with protocols to assess YAP/TAZ expression by flow cytometry and quantitative western blotting. This system can also be easily adapted for the study of other proteins.

For complete details on the use and execution of this protocol, please refer to Pearson et al. (2021).

BEFORE YOU BEGIN

This protocol describes the use of a lentiviral system using weak, constitutive promoters to ectopically express YAP or TAZ (WWTR1) in YAP/TAZ-deficient (herein referred to as "YAP^{off}") cancer cell lines to physiologically relevant levels observed in YAP/TAZ-positive ("YAP^{on}") cancers. We outline the steps to prepare high-titer lentivirus and transduce cells to ectopically express YAP or TAZ along with a GFP reporter (linked through an IRES; e.g., YAP-IRES-GFP) in the YAP^{off} NCI-H209 small cell lung cancer (SCLC) cell line using this system. Plasmids expressing wild type YAP, a TEAD-binding mutant (Serine 94 mutated to Alanine; YAP^{S94A}) (Zhao et al., 2008) and an overactive YAP mutant (all five LATS Serine phosphorylation sites mutated to Alanine; YAP^{5SA}) (Zhao et al., 2007, 2010) are described and can be obtained from Addgene. It further details the steps to assess the expression of YAP/TAZ/GFP by flow cytometry and quantitative western blotting to ensure expression to physiologically-relevant levels.

We have applied these same protocols to express YAP/TAZ in many other YAP^{off} cell lines, although not all lines are appropriate for flow cytometry due to their clumpy growth characteristics. We used a modified pLKO.1-puro vector backbone driving YAP-IRES-GFP expression from an EF-1 alpha short promoter (EFSp) in NCI-H209 cells, as well as other SCLC and neuroendocrine cells, while a similar set of plasmids using the original phosphoglycerate kinase promoter (PGKp-GFP) is also available and was used in retinoblastoma cell lines. TAZ is expressed using the PGKp-GFP vector. We have also used these plasmids to express other genes by first digesting the YAP plasmid with EcoRI+BamHI restriction enzymes to remove the YAP cDNA and then cloning in a different expression construct.

⚠ CRITICAL: This protocol makes use of a 2nd generation lentiviral system. Although they are engineered to be replication defective, these agents pose a potential risk of infection through accidental injection or contact with mucous membranes. Use of these agents should have biosafety approval according to institutional guidelines and appropriate biosafety level 2 procedures should be followed.

Preparing plasmids

Timing: 3 days for plasmid preps, variable time to obtain the plasmids

1. Obtain the psPax2 and pMD2.G lentiviral packaging plasmids and the YAP/TAZ transfer plasmids described here from Addgene (see key resources table).
2. Prepare sufficient quantities of each plasmid. We recommend the EndoFree Plasmid Maxi Kit from Qiagen according to the manufacturer's protocol, although other plasmid purification kits should also be appropriate. Resuspend the plasmid DNA in sterile TE buffer at a concentration of 0.5–1.0 μg/μL.

⚠ CRITICAL: The pLKO-based YAP and TAZ plasmids should be propagated using a recombination-deficient *E. coli* strain, such as NEB Stable. We also recommend growing the *E. coli* in Terrific Broth, as opposed to LB media, for higher plasmid yields.

Culturing Lenti-X 293T lentiviral packaging cells

Timing: About 1 week

3. Lenti-X 293T virus packaging cells are cultured in DMEM supplemented with 10% FBS and are maintained at 37°C in a humidified atmosphere containing 5% CO_2. Always allow media to warm to at least 20°C–25°C prior to use.

Note: Lenti-X 293T cells are weakly adherent, so it is important to gently dispense media/buffer onto cell monolayers.

Culturing NCI-H209 (YAP^{off}) cells for viral transduction

Timing: At least 1 week

4. YAP^{off} NCI-H209 SCLC cells are grown in RPMI-1640 with 7.5% FBS and maintained at 37°C in a humidified atmosphere containing 5% CO_2. NCI-H209 cells grow as floating clusters of cells and can be harvested by pipetting the cells from the tissue culture flask or dish.

Note: If substantial numbers of dead cells are observed in the culture, you can perform a simple clean-up to remove most dead cells. Gently (ensuring not to break up the clusters) transfer cells to a conical tube, and then place the tube upright in a tissue culture incubator for ~1hr until the clusters of cells settle to the bottom while single, dead cells stay suspended. Approximately 75% of the media can then be carefully removed ensuring the clusters of cells are not disturbed, and the live cells then passaged. Sometimes cells might be weakly "stuck" to the surface of the culture dish, but these can be easily dislodged by pipetting the culture media against the bottom of the dish 2–3 times.

Culturing control NCI-H661 (YAP^{on}) cells

Timing: Several days to weeks

5. YAP^{on} NCI-H661 non-SCLC cells are cultured in RPMI-1640 with 10 mM HEPES and 10% FBS and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-YAP/TAZ (clone 63.7) (flow cytometry use 1:100; western blotting use 1:500)	Santa Cruz Biotechnology	Cat# sc-101199; RRID: AB_1131430
Rabbit monoclonal anti-YAP (clone D8H1X) (flow cytometry use 1:100; western blotting use 1:1000)	Cell Signaling Technology	Cat# 14074; RRID: AB_2650491
Goat polyclonal anti-GFP (flow cytometry use 1:1000)	Abcam	Cat# ab6673; RRID: AB_305643
Mouse monoclonal anti-α tubulin (clone DM1A) (western blotting use 1:2000)	Santa Cruz Biotechnology	Cat# sc-32293; RRID: AB_628412
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (use 1:750)	Thermo Fisher	Cat# A-31571
Donkey Anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (use 1:750)	Thermo Fisher	Cat# A-31573
Donkey Anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (use 1:750)	Thermo Fisher	Cat# A-11057
Goat Anti-Mouse IgG IRDye 680RD Secondary Antibody (use 1:5000)	LI-COR Biosciences	Cat# 926-68070
Bacterial and virus strains		
NEB stable *E. coli*	New England Biolabs	Cat# C3040
Chemicals, peptides, and recombinant proteins		
Terrific broth	Wisent Bioproducts	Cat# 800-067
Dulbecco's Modified Eagle Medium (DMEM)	Wisent Bioproducts	Cat# 319-005-CL
Roswell Park Memorial Institute (RPMI) 1640 Medium	Wisent Bioproducts	Cat# 350-000-CL
RPMI 1640 + 10 mM HEPES	Wisent Bioproducts	Cat# 350-007-CL
Fetal bovine serum (FBS)	Wisent Bioproducts	Cat# 080-150
Opti-MEM I	Thermo Fisher	Cat# 31985070
Phosphate-buffered saline (PBS)	Wisent Bioproducts	Cat# 311-010-CL
Tris buffered saline (TBS), 10× concentrate	Wisent Bioproducts	Cat# 811-030-FL
TE buffer	Thermo Fisher	Cat# 12090015
Sucrose, ultrapure, >99.5%	BioShop	Cat# SUC507.2
Tween-20	BioShop	Cat# TWN510.500
Triton X-100	Sigma	Cat# T9284
FuGENE HD	Promega	Cat# E2311
Polybrene solution	Santa Cruz Biotechnology	Cat# sc-134220
16% Paraformaldehyde	Electron Microscopy Sciences	Cat# 15710
Bovine serum albumin (BSA), Fraction V	BioShop	Cat# ALB001.500
Tris-Glycine-SDS running buffer, 10× concentrate	Wisent Bioproducts	Cat# 811-570-FL
RIPA Lysis Buffer System (includes PMSF)	Santa Cruz Biotechnology	Cat# sc-24948A
Protease/Phosphatase Inhibitor Cocktail	Cell Signaling Technology	Cat# 5872S
SDS (20% solution)	Wisent Bioproducts	Cat# 880-552-LL
Glycerol	BioShop	Cat# GLY001.4
2-Mercaptoethanol	Bio Basic	Cat# MB0338
Bromophenol blue	Sigma	Cat# B8026
Critical commercial assays		
EndoFree Plasmid Maxi Kit	QIAGEN	Cat# 12362
Bio-Rad Protein Assay Kit II	Bio-Rad	Cat# 5000002
Trans-Blot Turbo RTA Mini 0.2 μm Nitrocellulose Transfer Kit	Bio-Rad	Cat# 1704270
Trans-Blot Turbo RTA Midi 0.2 μm Nitrocellulose Transfer Kit	Bio-Rad	Cat# 1704271
Experimental models: Cell lines		
Lenti-X 293T (human, embryonic)	Takara/Clontech	Cat# 632180
NCI-H209 (human, adult, male)	Susan P.C. Cole's Lab	RRID: CVCL_1525
NCI-H661 (human, adult, male)	ATCC	Cat# HTB-183; RRID: CVCL_1577

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
psPax2	Didier Trono	Addgene plasmid #12260; RRID: Addgene_12260
pMD2.G	Didier Trono	Addgene plasmid #12259; RRID: Addgene_12259
PGKp-GFP-TAZ	(Pearson et al., 2021)	Addgene plasmid #174176
PGKp-GFP-Empty	(Pearson et al., 2021)	Addgene plasmid #174175
PGKp-GFP-YAP (5SA)	(Pearson et al., 2021)	Addgene plasmid #174174
PGKp-GFP-YAP (S94A)	(Pearson et al., 2021)	Addgene plasmid #174173
PGKp-GFP-YAP	(Pearson et al., 2021)	Addgene plasmid #174172
EFSp-GFP-Empty	(Pearson et al., 2021)	Addgene plasmid #174171
EFSp-GFP-YAP (5SA)	(Pearson et al., 2021)	Addgene plasmid #174170
EFSp-GFP-YAP (S94A)	(Pearson et al., 2021)	Addgene plasmid #174169
EFSp-GFP-YAP	(Pearson et al., 2021)	Addgene plasmid #174168
Software and algorithms		
Kaluza, flow cytometry analysis software	Beckman Coulter	https://www.mybeckman.ca/flow-cytometry/software/kaluza
Odyssey Software version 3.0	LI-COR	N/A
Other		
Open-top thinwall ultra-clear round bottom 25 × 89 mm tube	Beckman Coulter	Cat# 344058
Filtropur S Syringe Filter, 0.45 µm pore size	SARSTEDT	Cat# 83.1826
30 cc Luer-Lok syringe	Becton Dickinson	Cat# 302833
FACS tube with cell strainer cap	Fisher Scientific	Cat# 352235
PageRuler Prestained Protein Ladder	Thermo Fisher	Cat# 26616
4%–20% Mini-PROTEAN TGX Stain-Free Protein Gels, 15 well	Bio-Rad	Cat# 4568096
4%–20% Criterion TGX Stain-Free Protein Gel, 26 well	Bio-Rad	Cat# 5678095
Avanti J-30I high-performance centrifuge	Beckman Coulter	N/A
JS-24.38 swinging bucket rotor	Beckman Coulter	Product No:360743
Gallios flow cytometer	Beckman Coulter	N/A
Model 100 Sonic Dismembrator	Fisher Scientific	N/A
Mini-PROTEAN Electrophoresis Cell	Bio-Rad	Cat# 1658005
Trans-Blot Turbo Transfer System	Bio-Rad	Cat# 1704150EDU
Odyssey Classic Infrared Imaging System	LI-COR	N/A
5810R Refrigerated Centrifuge	Eppendorf	Product# 022627023
Sorvall Legend Micro 21R Microcentrifuge	Thermo Fisher	Product# 75002446

MATERIALS AND EQUIPMENT

Below are recipes to prepare solutions needed for this protocol:

20% sucrose

Reagent	Final concentration	Amount
1× PBS	1×	100 mL
Sucrose	20% (w/v)	20 g
Total	**n/a**	**100 mL**

After the sucrose is dissolved, sterilize through a 0.2 µm filter and store up to 6 months at 4°C.

4% paraformaldehyde (PFA)

Reagent	Final concentration	Amount
16% PFA	4%	1 mL
1× PBS	n/a	3 mL
Total	**n/a**	**4 mL**

Prepare fresh daily as needed.

CAUTION: PFA is toxic, corrosive and a potential mutagen. PFA solutions should be handled in a chemical fume hood with proper personal protective equipment (lab coat and gloves).

10% (w/v) Triton X-100		
Reagent	Final concentration	Amount
Triton X-100	10%	5 g
ddH_2O	n/a	50 mL
Total	**n/a**	**50 mL**

Weigh Triton X-100 into a beaker then add ddH_2O. Add a magnetic stir bar and stir on a magnetic stir plate until fully dissolved. Store up to 6 months at 4°C.

Flow cytometry perm/block buffer		
Reagent	Final concentration	Amount
1× PBS	n/a	9.9 mL
BSA	2% (w/v)	0.2 g
Triton X-100 (10% stock)	0.1%	100 µL
Total	**n/a**	**10 mL**

Dissolve BSA in 1× PBS, then add Triton X-100. Prepare fresh daily as needed.

Flow cytometry stain/wash buffer		
Reagent	Final concentration	Amount
1× PBS	n/a	49.75 mL
BSA	2% (w/v)	1 g
Triton X-100 (10% solution)	0.05%	250 µL
Total	**n/a**	**50 mL**

Dissolve BSA in 1× PBS, then add Triton X-100. Prepare fresh daily as needed.

FACS buffer		
Reagent	Final concentration	Amount
1× PBS (sterile)	1×	490 mL
FBS	2%	10 mL
Total	**n/a**	**500 mL**

If kept sterile, FACS buffer can be stored at 4°C for 6 months.

2× Laemmli sample buffer		
Reagent	Final concentration	Amount
SDS (20% solution)	4%	6 mL
Glycerol	20%	6 mL
1M Tris-HCl, pH 6.8	125 mM	3.75 mL
ddH_2O	n/a	11.25 mL
2-mercaptoethanol	10%	3 mL
Bromophenol blue	0.1%	30 mg
Total	**n/a**	**30 mL**

Aliquot and store at −20°C for at least 1 year. Once defrosted, store 4°C for several months. If precipitate is observed, briefly warm buffer at 37°C prior to use.

Tris-Glycine-SDS running buffer		
Reagent	Final concentration	Amount
Tris-Glycine-SDS running buffer, 10× concentrate	1×	100 mL
ddH_2O	n/a	900 mL
Total	**n/a**	**1000 mL**
Store at 20°C–25°C for several months.		

Western blotting transfer buffer		
Reagent	Final concentration	Amount
5× Trans-Blot Turbo buffer (Bio-Rad, part of Cat# 1704270)	1×	100 mL
Ethanol	20%	100 mL
ddH_2O	n/a	300 mL
Total	**n/a**	**500 mL**
Store at 20°C–25°C for 1 month.		

10% (w/v) Tween-20		
Reagent	Final concentration	Amount
Tween	10%	10 g
ddH_2O	n/a	100 mL
Total	**n/a**	**100 mL**
Weigh Tween-20 into a beaker then add ddH_2O. Add a magnetic stir bar and stir on a magnetic stir plate until fully dissolved. Store for several months at 4°C.		

0.1% TBST		
Reagent	Final concentration	Amount
10× TBS	1×	100 mL
Tween-20 (10% solution)	0.1%	10 mL
ddH_2O	n/a	890 mL
Total	**n/a**	**1000 mL**
Store at 20°C–25°C for 1 month.		

Alternatives:

Alternative centrifuges and microfuges capable of the speeds listed are acceptable.

Other flow cytometers capable of multicolor analysis are appropriate alternatives for the Beckman Coulter Gallios flow cytometer.

Sonicators equipped with probes for small volumes are appropriate alternatives for the Fisher Scientific Model 100 Sonic Dismembrator, although settings may need to be optimized

Other protein gel electrophoresis and transfer systems can be used in place of the Bio-Rad systems described here. Follow standard/manufacturer's protocols for use of alternative systems.

The LI-COR Odyssey Classic Infrared Imaging System used in these studies has been discontinued, but several new imaging systems are now available from LI-COR (https://www.licor.com/bio/imaging-systems). Fluorescent or chemiluminescent imaging systems with a large linear range are also appropriate, although these may require additional modifications or steps not detailed here. Quantification using chemiluminescence and film is not recommended due to its limited linear range.

Table 1. Amounts for preparing transfection mixtures

	1 × 10 cm plate		3 × 10 cm plates (+ 10% excess)	
	Tube 1	Tube 2	Tube 1	Tube 2
psPax2 plasmid	4.5 μg	-	14.85 μg	-
pMD2.G plasmid	3.0 μg	-	9.9 μg	-
Transfer plasmid (e.g., YAP or TAZ)	7.5 μg	-	24.75 μg	-
FuGENE HD	-	45 μL	-	148.5 μL
Opti-MEM	to 250 μL	to 250 μL	to 825 μL	to 825 μL

STEP-BY-STEP METHOD DETAILS

Generation of YAP, TAZ, and control lentiviruses

Timing: 5 days to complete with hand-on time of ~2 h for steps 1–3 and ~4 h for step 4

This step describes the generation of YAP and TAZ-encoding lentiviruses, including transfection of packaging cells, viral collection, and concentrating the virus by high-speed centrifugation. Typically, 3 × 10 cm plates are transfected for each transfer plasmid (e.g., YAP or TAZ), but this can be increased or decreased if more or less virus is required for a given construct. Alternatively, an individual 15 cm culture dish can be used and is roughly equivalent to 3 × 10 cm plates. Each 10 cm plate will yield ~10 mL of viral supernatant. The Avanti J-30I high-performance centrifuge with JS-24.38 rotor described here has six buckets, each of which holds ~30 mL of supernatant, so it is ideal to generate a total of 180 mL (18 plates) of viral supernatant in multiples of 30 mL for each virus. For example, in a single batch you can prepare:

- 30 mL (3 plates) of the EFSp-GFP-Empty virus (control for YAP viruses)
- 30 mL (3 plates) of the EFSp-GFP-YAP virus
- 30 mL (3 plates) of the EFSp-GFP-YAP^{S94A} virus
- 30 mL (3 plates) of the EFSp-GFP-YAP^{5SA} virus
- 30 mL (3 plates) of the PGKp-GFP-Empty virus (control for TAZ)
- 30 mL (3 plates) of the PGKp-GFP-TAZ virus

⚠ CRITICAL: For transfection and subsequent steps use freshly prepared culture media (within ~2 weeks) that is not supplemented with antibiotics (Pen/Strep).

Day 1: Seed packaging cells

1. The day prior to transfection, seed ~4 × 10^6 Lenti-X 293T cells per plate into 18 × 10 cm plates so that they will be 85%–95% confluent for transfection. Usually a 1:1 split of cells that are 85%–95% confluent is sufficient to achieve this.

Day 2: Transfect packaging cells

2. Transfect lentiviral packaging cells.
   a. Aspirate media from Lenti-X 293T cells and replace with 8 mL of DMEM + 10% FBS (without antibiotics).
   b. Prepare transfection mixture using two sterile tubes per transfection (1.5 mL for 1 × 10 cm plate; 2 mL for 3 × 10 cm plates) according to Table 1, consisting of:
      - Tube 1: plasmid DNA mixture
      - Tube 2: FuGENE HD transfection reagent mixture

⚠ CRITICAL: Minimize contact time of the FuGENE HD reagent with plastic ware (e.g., pipette tips and tubes) when in its undiluted form and do not use siliconized pipette tips or tubes. Add the FuGENE HD reagent directly to media without contacting the side of the tube. Once the FuGENE HD is diluted in media, contact with plastic surfaces is no longer a concern.

i. Incubate mixtures ~5 min at 20°C–25°C.
ii. Add the entire volume of Tube 1 to Tube 2, pipette to mix and incubate 10–15 min at 20°C–25°C.

c. Add 500 µL of transfection mixture dropwise to each 10 cm plate of Lenti-X 293T cells and gently swirl. Incubate cells 12–18 h.

Day 3: Replace media on packaging cells

3. Remove media from cells and replace with 10 mL of DMEM + 10% FBS (without antibiotics). Incubate cells for 36–48 h prior to collecting virus-containing supernatant.

⚠ CRITICAL: The day following transfection, waste media contains infectious lentiviral particles. Waste should be bleached and disposed of appropriately according to institutional biosafety guidelines.

Note: The day following transfection, GFP fluorescence should be clearly visible using a standard fluorescent microscope. If GFP fluorescence is not readily visible it indicates there is likely a problem with the plasmids or the transfection (Troubleshooting 1).

Day 5: Collect and concentrate lentivirus

4. Harvest and concentrate lentivirus by high-speed centrifugation.
   a. Inside a BSC, place an open-top thin wall ultra-clear round bottom 25 × 89 mm tube into each bucket of a JS-24.38 swinging bucket rotor.
   b. Collect lentivirus-containing media from Lenti-X 293T cells and combine up to 30 mL from each virus in a 50 mL conical tube. Centrifuge at 500 *g* for 5 min to remove large debris.
   c. Filter up to 30 mL of supernatant directly into the centrifuge bucket (containing a round bottom tube) using a 0.45 µm, low protein binding syringe filter attached to a 30 cc Luer-Lok syringe.

⚠ CRITICAL: This filtering step is essential to ensure that all of the Lenti-X packaging cells are removed from the viral preparation. It is also critical to use 0.45 µm, low protein binding filters as other materials or the use of 0.2 µm filters can result in reduced viral recovery.

   d. Carefully underlay 4 mL of 20% sucrose (in PBS) underneath the virus-containing media to create a sucrose cushion and tightly seal the rotor bucket.
   e. Weigh each bucket and adjust volumes to balance the buckets. We recommend keeping the buckets within 0.05 g of one another. DMEM + 10% FBS can be added to "underweight" buckets if needed; 100 µL is ≈ 0.1 g.
   f. Tightly seal the rotor buckets and load the buckets onto the JS-24.38 rotor.
   g. Tightly secure the rotor into the Avanti J-30I high-performance centrifuge. Centrifuge for ≥ 2 h at 4°C and max speed of 24,000 rpm (~103,000 *g*). Set centrifuge to "SLOW" for ACCEL and DECEL.
   h. Once the centrifuge has stopped, immediately remove the buckets and transport to a biological safety cabinet. Unscrew the bucket lids and carefully remove the round bottom tubes with sterile forceps.

i. Decant the supernatant and place the tube opening down on a sterile wipe to allow excess media to drain down. After ~5 min, wipe any excess media from the edge of the tube and place the tube in a rack with the opening facing up.
j. Add 300 μL of sterile, cold PBS to the bottom of each tube. Adjust the pipette volume to ~200 μL to avoid bubbles/frothing, and pipette up and down ~2 min to resuspend the virus. Resuspending each tube in 300 μL gives a 100-fold concentration, although this volume can be adjusted if necessary. If multiple tubes were used for the same virus, these can now be combined.
k. Prepare appropriately sized aliquots of the virus into sterile tubes and store at −80°C. We typically prepare aliquots ranging from 10–100 μL in 200 μL strip tubes, depending on the desired downstream experiments. Avoid multiple freeze-thaw cycles as this will result in decreased titer. We have stored virus at −80°C for over 1 year without a noticeable drop in titer.

Virus titration

Timing: 5–6 days total, with hands-on time of ~1 h for steps 5 and 6, and ~6 h for step 7

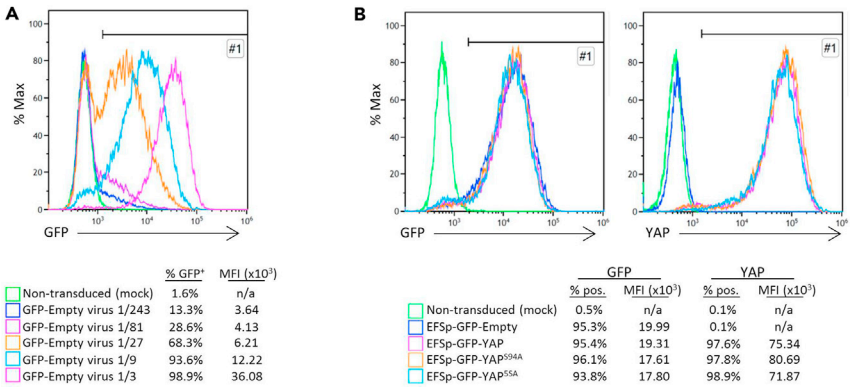
This section describes the steps to titrate the lentiviruses generated in previous steps. Here, we describe the procedure using the NCI-H209 SCLC cell line, but we use the same protocol for other YAP^{off} cell lines. Titrations do not need to be performed using the exact cell numbers described here, but we recommend keeping the ratio of cell number:media volume consistent with what will be used in subsequent experiments. We also suggest initially titrating viruses separately for each different cell line to be used, as we have observed differences in transduction efficiency of some lines.

Days 1 and 2: Transduce NCI-H209 cells

5. Prepare NCI-H209 cells for viral titration. Approximately 1.5 × 10^6 cells are required for each different virus plus 2.5 × 10^5 cells for a non-transduced (mock) control.
   a. Transfer "confluent" NCI-H209 cells (typically at a density of 0.5–1.0 × 10^6 cells/mL) to a conical tube and centrifuge at 300 *g* for 5 min.
   b. Aspirate the supernatant and resuspend cells back to their original volume in culture media. Pipette the cells vigorously to ensure a single cell suspension.
   c. Count the cells using a hemocytometer (or other means) and dilute to a concentration of 5 × 10^5 cells/mL.
   d. Add Polybrene to a final concentration of 4 μg/mL and transfer 500 μL of cell suspension (2.5 × 10^5 cells) to separate wells of a 24-well tissue culture plate. We recommend starting with six wells per virus, plus one additional well as a non-transduced control. This may be decreased as experience is gained with each vector system.
6. Transduce H209 cells with lentivirus.
   a. Thaw an aliquot of each virus stock and prepare a series of five, 3-fold serial dilutions of each virus into PBS (i.e., 1/3, 1/9, 1/27, 1/81 and 1/243).
   b. Add 2.5 μL from each dilution, as well as undiluted virus, to separate wells of the 24-well plate and incubate 14–18 h.
   c. Collect cells using a P1000 pipette. Pipette up and down 2–3 times to remove any cells loosely attached to the plate and transfer to a sterile microfuge tube.
   d. Centrifuge the cells 5min at 800 *g*, carefully remove the supernatant and resuspend in 750 μL of media. Transfer the cells to a new 24-well plate and incubate for 3–4 days.

Day 5 or 6: Calculate viral titer

7. Calculate viral titer



	% GFP+	MFI (x10³)
Non-transduced (mock)	1.6%	n/a
GFP-Empty virus 1/243	13.3%	3.64
GFP-Empty virus 1/81	28.6%	4.13
GFP-Empty virus 1/27	68.3%	6.21
GFP-Empty virus 1/9	93.6%	12.22
GFP-Empty virus 1/3	98.9%	36.08

	GFP		YAP	
	% pos.	MFI (x10³)	% pos.	MFI (x10³)
Non-transduced (mock)	0.5%	n/a	0.1%	n/a
EFSp-GFP-Empty	95.3%	19.99	0.1%	n/a
EFSp-GFP-YAP	95.4%	19.31	97.6%	75.34
EFSp-GFP-YAP^{S94A}	96.1%	17.61	97.8%	80.69
EFSp-GFP-YAP^{S5A}	93.8%	17.80	98.9%	71.87

Figure 1. Analysis of YAP and GFP by flow cytometry in NCI-H209 cells

(A) GFP staining and flow cytometry analysis of non-transduced or NCI-H209 cells transduced with a titration curve of EFPs-GFP-Empty virus.

(B) Flow cytometry analysis of GFP (left) and YAP (right) expression in non-transduced or NCI-H209 cells transduced with high titer EFPs-Empty-GFP or the indicated YAP viruses. MFI: mean fluorescence intensity; n/a: not applicable.

a. After 3–4 days in culture (4–5 days after viral transduction), stain the cells for GFP expression using flow cytometry (see steps 10–11 below for a detailed protocol) (Figure 1A). GFP fluorescence can also be assessed using a fluorescent microscope, although accurately calculating the titer using this method is challenging due to the relatively weak GFP fluorescence and because NCI-H209 cells grow in large clusters.

b. Viral titer (in infectious particles/μL) can be calculated using dilutions that produce <30% YAP/TAZ/GFP-positive cells. Use the following formula to calculate viral titer:

$$\text{- particles/}\mu\text{L} = (F \times N \times D)/V$$

where: F = the fraction of GFP-positive cells
N = number of cells initially transduced (e.g., 2.5×10^5) D = virus dilution factor that produced F
V = volume of virus added (e.g., 2.5 μL)

Note: Using these vectors we have found that YAP/TAZ/GFP expression plateaus 4–5 days after lentiviral transduction in most YAP^{off} cell line we have tested, so we typically examine expression at this point. For new cell lines we recommend following GFP expression over time using a fluorescent microscope to determine at which point GFP expression plateaus. Since YAP and TAZ suppress the growth of YAP^{off} cells we recommend assessing YAP/TAZ/GFP expression as soon as it plateaus when titrating the virus.

High titer viral transduction for functional experiments

Timing: 6 days total, with hands-on time of ~2 h

This section describes the steps to transduce NCI-H209 cells for downstream biological assays and molecular analysis. The number of cells initially transduced may vary depending on the downstream assays to be performed and the specific cell line being used, but for most assays we transduce 1–10 × 10^6 cells. Here, we describe the protocol for transduction of 2 × 10^6 NCI-H209 cells, which will typically yield ~5 × 10^6 cells five days later. Amounts can be scaled up or down to accommodate specific experiments. We transduce cells so that >90% are YAP/TAZ/GFP-positive, and have found that a MOI of ~4 is sufficient to achieve this.

8. Prepare NCI-H209 cells for transduction.
   a. Harvest and count cells as described in steps 5a–5c.
   b. Add Polybrene to a final concentration of 4 μg/mL and transfer 4 mL of cell suspension (2×10^6 cells) to separate wells of a 6-well tissue culture plate. Prepare 1 well for each virus to be used plus one additional well for a non-transduced (mock) control.
9. Transduce NCI-H209 with high titer lentiviruses.
   a. Thaw viral aliquots and add the appropriate volume of the Empty vector control or each of the individual YAP/TAZ viruses to the cells to achieve MOI of ~4 (e.g., 8×10^6 viral particles) and gently swirl plate to mix. Incubate cells 14–18 h.
   b. Collect cells and transfer to a 15 mL conical tube. Centrifuge 300 *g* for 5 min, discard the supernatant and wash cells once in culture media.
   c. Resuspend the cells in 6 mL of fresh culture media, transfer to a T25 tissue culture flask and incubate for 4 days. Monitor density of the cells and passage or supplement with additional media if necessary.

Assessing YAP/TAZ/GFP expression by flow cytometry

Timing: ~6 h

This section describes the protocol for examining YAP/TAZ and GFP expression in transduced NCI-H209 cells using flow cytometry. This is a key step in accurately determining the proportion of cells transduced with the YAP/TAZ lentiviruses and in assessing the level of ectopic YAP/TAZ expression, although comparison between different cell lines can be misleading due to differences in instrument settings or background fluorescence of the different cell lines. We use a Beckman Coulter Gallios flow cytometer and Kaluza analysis software, although other flow cytometers capable of multicolor acquisition are also appropriate.

⚠ CRITICAL: Non-transduced NCI-H209 cells function as an important negative control for both GFP and YAP/TAZ expression since they are negative for both, and the Empty vector cells function as a single-stained (GFP+/YAP-) control. However, we recommend initially including additional unstained and single antibody-stained controls for each cell line being tested until experience is gained with the techniques (Table 2). Additional controls may also be necessary to perform compensation depending on the combination of fluorophores and instrument used.

Note: We suggest including a YAP/TAZ-positive (YAP^{on}) cell line as a control. We routinely use NCI-H661, A549 or NCI-H1650 non-SCLC cell lines, as they perform well using the same staining conditions, but other lines are also likely appropriate.

YAP and GFP expression is usually very similar between the YAP, YAP^{S94A} and YAP^{5SA} samples, so the unstained and single stained controls for YAP should also be sufficient as controls for all three.

10. Harvest and fix cells.
    a. Collect a control, YAP^{on}, cell line (e.g., NCI-H661) by trypsination and transfer $0.5–1.0 \times 10^6$ cells to each of two different 1.5 mL microfuge tubes. One tube will be stained for YAP/TAZ and the other will be used as an unstained control.
    b. Transfer $0.5–1.0 \times 10^6$ transduced or control NCI-H209 cells to separate 1.5 mL microfuge tubes. Prepare separate tubes for each unstained or single stained control.
    c. Centrifuge cells at 800 *g* for 5 min in a table-top microfuge, discard the supernatant and wash the cells once with 1 mL of PBS.
    d. Resuspend the cells in 250 μL of 4% PFA, and incubate 10–15 min at 20°C–25°C.
    e. Centrifuge cells at 800 *g* for 5 min at 4°C, discard the supernatant and wash the cells twice with 1 mL of PBS.

Table 2. Proposed experimental set-up for flow cytometry analysis

	No 1° Ab (unstained)	α-GFP alone	α-YAP/TAZ alone	α-GFP + α-YAP/TAZ
non-transduced	(+)	(–)	(–)	(+)
GFP-Empty	(+)	(+)	(+)	(+)
GFP-YAP	(+)	(+)	(+)	(+)
GFP-YAP^{S94A}	(–)	(–)	(–)	(+)
GFP-YAP^{5SA}	(–)	(–)	(–)	(+)
NCI-H661 (YAP^{on})	(+)	(–)	(+)	(–)

Pause point: After fixation and washes, cells can be stored in 500 μl of PBS at 4°C for up to 1 week. After storage, cells should be pelleted as before and old PBS removed prior to continuing to step 11.

11. Stain and analyze cells by flow cytometry.
    a. Resuspend cells in 500 μL of perm/block buffer and transfer to a new 1.5mL microfuge tube. Incubate 30 min at 20°C–25°C. After ~15min, invert the tubes 2–3 times to mix.

⚠ CRITICAL: After permeabilization, YAP^{off} cells do not pellet very well. Transferring the cells to a fresh tube in step 11a helps with this problem and reduces cell loss (Troubleshooting 2).

    b. Centrifuge cells at 800 *g* for 5 min at 4°C. Very carefully remove the supernatant using a P1000 pipette (Troubleshooting 2) and resuspend cells in 100–200 μL of primary antibody diluted in stain/wash buffer, or stain/wash buffer alone for unstained controls. The YAP or YAP/TAZ antibodies are diluted 1:100, whereas the GFP antibody is diluted 1:1000 (Troubleshooting 3).
    c. Incubate the cells for 1 h at 20°C–25°C. Every ~15 min gently flick the tube to resuspend the cells.
    d. Add 1 mL of stain/wash buffer to each sample, centrifuge as before, and carefully remove the supernatant. Wash the cells once more with 1 mL stain/wash buffer.
    e. Resuspend the cells in 100–200 μL of secondary antibodies diluted 1:750 in stain/wash buffer. Unstained (no 1° Ab) and single stained controls should also be incubated with both secondary antibodies.
    f. Incubate the cells for 30 min at 20°C–25°C protected from light. After ~15 min gently flick the tube to resuspend the cells.
    g. Add 1 mL of stain/wash buffer to each sample, centrifuge as before, and carefully remove the supernatant. Wash the cells once more with 1 mL stain/wash buffer.
    h. Resuspend cells in 300 μL of FACS buffer and filter through a cell strainer cap affixed to a FACS tube.

Pause point: Samples can be analyzed immediately or stored at 4°C for up to 24 hrs prior to analysis.

    i. Analyze on Beckman Coulter Gallios flow cytometer using Kaluza analysis software and display data as a histogram (Figure 1B).

Assessing YAP/TAZ expression by quantitative western blotting

Timing: 3 days

This section describes the protocol for examining YAP/TAZ expression by quantitative western blotting using an Odyssey LI-COR Infrared imaging system. Gel electrophoresis and western blotting protocols have been described in detail elsewhere (Burckhardt et al., 2021;

Eslami and Lujan, 2010; Mahmood and Yang, 2012). We utilize pre-cast SDS-PAGE gradient gels (Bio-Rad) for electrophoresis and the Bio-Rad Trans-Blot Turbo Transfer System for protein transfer, but alternative electrophoresis and transfer protocols should also acceptable.

⚠ CRITICAL: During cell lysis, ensure that lysis buffer is "ice cold," maintain cells and protein lysates on ice and perform all centrifugation steps at 4°C.

12. Prepare protein lysates.
    a. Harvest a portion of the transduced NCI-H209 cells as well as control YAP^{on} cells (e.g., NCI-H661) similarly to steps 10a and 10b. We recommend starting with at least 2.5 × 10^6 cells.
    b. Pellet cells by centrifugation, aspirate the supernatant, resuspend in 1 mL of PBS and transfer to a 1.5 mL microfuge tube.
    c. Prepare sufficient RIPA lysis buffer for all samples, plus some extra for step 13, by adding Protease/Phosphatase inhibitor cocktail and PMSF at a 1:100 dilution.
    d. Add 100 μL of cold lysis buffer to each cell pellet and pipette to fully resuspend the pellet. For larger cell numbers the volume of lysis buffer may be increased.
    e. Incubate samples on ice for 5 min, then sonicate 2 × 3 s (with a 3 s pause between) using a Fisher Scientific Model 100 Sonic Dismembrator at a setting of "4" (power output ~12 W).

    Note: If an appropriate sonicator is not available, sonication can be omitted without a major reduction in YAP signal, but we suggest increasing the incubation time to 10 min.

    f. Centrifuge samples in a table-top microfuge for 10 min at 4°C and ≥ 16,000 *g*.
    g. Transfer supernatants (protein lysate) to pre-chilled microfuge tubes.

    ⏸ **Pause point:** Protein lysates can be stored long term at −80°C. Frozen lysates should be thawed on ice.

13. Determine protein concentration using the Bio-Rad Protein Assay Kit II essentially as outlined in the manufacturer's "microtiter plate protocol".
    a. Dilute 5 μL of lysate or lysis buffer alone 1:10 in dH_2O.
    b. Prepare BSA standards as outline in the manufacturer's protocol.
    c. Dilute Dye Reagent Concentrate 1:4 in dH_2O.
    d. Add 10 μL of standard or sample to separate wells of a 96-well plate in at least duplicate (triplicate preferred).
    e. Add 200 μL of diluted reagent and incubate 5 min at 20°C–25°C.
    f. Read absorbance at 595 nm using a standard microplate reader.
    g. Prepare a standard curve plotting the absorbance vs. concentration of BSA standards. Determine the concentration of each sample by linear regression and multiply by 10 to account for the initial dilution in step 13a to obtain the protein concentration/μL of sample.
    h. Dilute a portion of each sample using RIPA lysis buffer so that samples are of equal concentration. For example, if the most dilute sample is 2 μg/μL, dilute all other samples to 2 μg/μL by adding the appropriate amount of RIPA lysis buffer.

14. Gel electrophoresis and protein transfer.

    Note: The amounts/volumes suggested below are for a gel with "small" (15 lanes, 15 μl) wells. For larger wells (e.g., 10 lane, 30–50 μl), amounts can be increased accordingly.

    a. Dilute protein lysates 1:1 with 2× Laemmli sample buffer (Laemmli, 1970), then heat at 95°C for 5 min. Allow samples to cool to 20°C–25°C and briefly centrifuge.

b. Remove pre-cast SDS-PAGE gel from packaging and assemble electrophoresis apparatus. Fill the chambers with Tris-Glycine-SDS running buffer, remove comb from the gel and flush wells with a P200 pipette set to ~30 μL.
c. Mix 3 μL of PageRuler Prestained protein marker with 7 μL of 1× Laemmli sample buffer (2× buffer diluted 1:1 with water) and load into lane 1 of the gel. Load 10–25 μg of protein lysate from each sample onto subsequent wells of the gel. For any empty wells, load 10 μL of 1× Laemmli sample buffer.
d. Attach lid onto the electrophoresis unit and connect to power supply.
e. Run at constant amperage of 30 mA/gel (for mini gels) or 70 mA/gel (for midi gels) with a maximum voltage of 200 V for ~1 h until the blue dye front runs off the end of the gel.
f. Turn power unit off, remove gel(s) and separate plates. Gently cut off the top of the gel where the wells were and carefully transfer the gel(s) to a reservoir containing 1× transfer buffer.
g. Briefly equilibrate one nitrocellulose membrane per gel in transfer buffer and wet two transfer stacks per gel (all from the Bio-Rad Trans-Blot Turbo RTA Mini or Midi Transfer Kit).
h. Assemble "transfer sandwich" (Figure 2A) by placing one transfer stack in the bottom of the Trans-Blot Turbo cassette. Carefully place the nitrocellulose membrane on the transfer stack and align sides, followed by the gel and finally the last transfer stack on the top. Gently roll out to remove bubbles and carefully "dab" away excess buffer.
i. Place the cassette lid on the top and turn dial to lock in place.
j. Turn on the Trans-Blot Turbo instrument using the switch located on the right hand side of the instrument near the back and slide the assembled cassette into either slot "A" or "B" of the instrument.
k. Using the control panel (Figure 2B), select:
    i. "LIST" using the bottom set of buttons on the display, then
    ii. "BIO-RAD" using the buttons on the right hand side of the display, then
    iii. Either "1 MINI GEL" or "2 MINI OR 1 MIDI GEL" as appropriate using the buttons on the right and side of the display, then
    iv. Scroll down using the buttons on the right hand side of the display until "MIXED MW" is highlighted and select "RUN" using the bottom buttons, then
    v. Either "A:RUN" or "B:RUN" using the bottom set of buttons on the display depending whether the cassette is located in slot A or B.
l. When the transfer is complete, turn of the instrument, remove the cassette and then remove the cassette lid.
m. Discard the top transfer stack from the transfer sandwich and slowly begin to lift the gel from the side containing the protein marker, ensuring the marker has properly transferred to the membrane (Figure 2C). Fully remove the gel and place the membrane in a container containing 20–30 mL of 5% milk (the bottom half of a P20/P200 tip box works well as a container for mini gels). Incubate 1–2 h at 20°C–25°C or 4°C for 14–24 h.

15. Quantitative immunoblotting.
    a. Briefly rinse the membrane in 0.1% TBS-T and add enough YAP/TAZ primary antibody (Santa Cruz Biotechnology, clone 63.7 diluted 1:500 in 1× TBS) to cover the membrane (~10 mL for a mini gel). Incubate 1 h at 20°C–25°C then 4°C for 14–24 h.
    b. Remove primary antibody (this can be stored at 4°C and reused several times) and wash membrane 3 × 10 min with ~15 mL of 0.1% TBS-T.
    c. Add 10–15 mL of goat anti-mouse IRDye 680RD (diluted 1:5000 in 0.1% TBS-T), cover to protect from light and incubate 1 h at 20°C–25°C.
    d. Wash membrane 3 × 10 min with ~15 mL of 0.1% TBS-T, then image using a LI-COR Odyssey Infrared imaging system according to manufacturer's protocols.
    e. Using the LI-COR Odyssey software, quantify band intensities for YAP and/or TAZ.

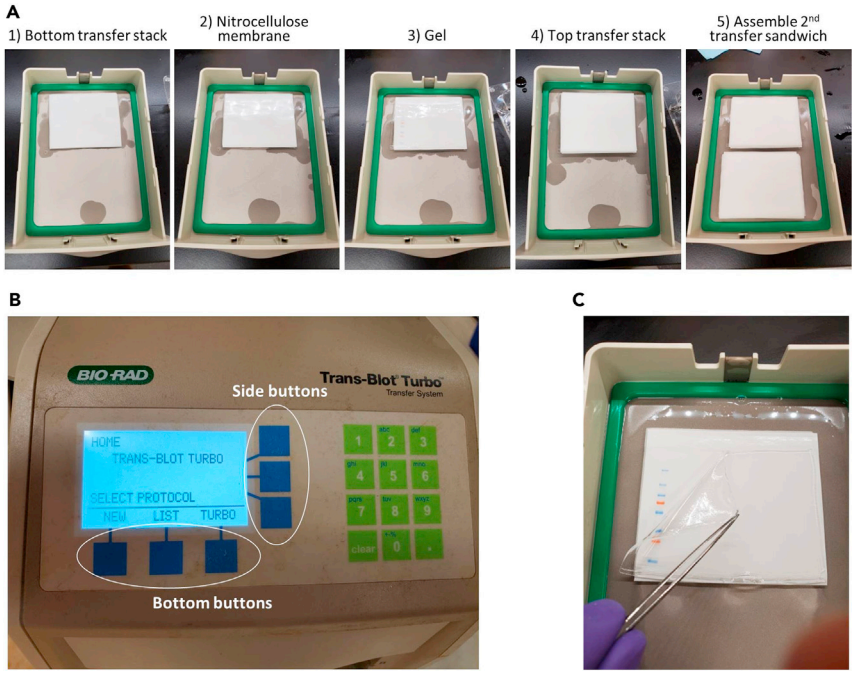


Figure 2. Use of the Trans-Blot Turbo transfer system

(A) Order of assembly to prepare the "transfer sandwiches" for protein transfer.

(B) Display layout of the Trans-Blot Turbo instrument.

(C) Disassembly of "transfer sandwich" ensuring proper transfer to the nitrocellulose membrane.

⚠ CRITICAL: Ensure bands are not saturated after scanning (indicated by a blue color). If they are, re-scan at a lower "Intensity".

f. Strip membrane in 15–20 mL of TBS, pH 2.0 for 1–2 h at 20°C–25°C.

g. Wash membrane 3 × 10 min with ~15 mL of 0.1% TBS-T and add primary anti-TUBULIN antibody (Santa Cruz Biotechnology, clone DM1A diluted 1:2000 in TBS).

h. Incubate at 4°C for 14–24 h, then repeat steps 15b–15e to image and quantify TUBULIN band intensities (Figure 3).

Optional: If the desired expression of YAP/TAZ was observed (by flow cytometry and western blotting), remaining cells can be used for biological assays or additional molecular analysis (see Pearson et al., 2021 for examples).

EXPECTED OUTCOMES

Steps 5–7: For virus titration, the proportion of transduced cells and GFP expression should increase as the amount of virus is increased, giving a roughly linear response until ~30% of cells are transduced and then increasing the proportion and fluorescence intensity after that (Figure 1A). We typically obtain titers of 0.2–2.0 × 10^6 particles/μL when the virus is concentrated 100-fold, with the GFP-Empty vector producing 2–5 fold higher titers compared to the YAP or TAZ viruses (Troubleshooting 5).

Steps 10–11: If the virus was titrated correctly, at least 90% of the YAP-transduced NCI-H209 cells should be GFP and YAP/TAZ-positive 5 days after transduction (Figure 1B). GFP alone transduced cells (e.g., EFSp-GFP-Empty) should be positive for GFP (>90% of cells), but negative for YAP/TAZ, while non-transduced cells should be negative for both GFP and YAP/TAZ (Figure 1B).

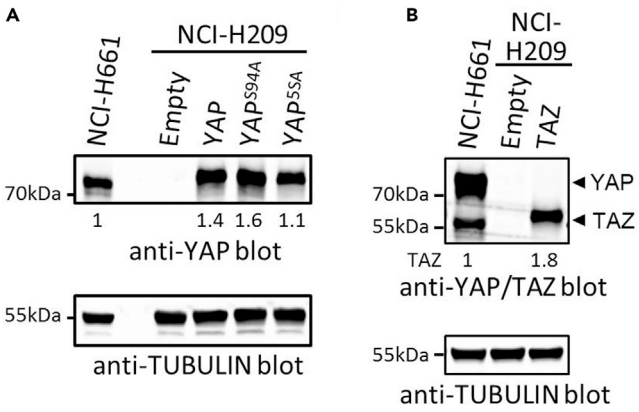


Figure 3. Quantitative western blotting of YAP and TAZ

YAP/TAZ western blots from YAP-transduced (A) or TAZ-transduced (B) YAP^{off} NCI-H209 cells. Relative YAP/TAZ expression is shown below the blots and is presented relative to YAP^{on} NCI-H661 cells.

Steps 12–15: For western blots, the band for YAP should be visible at ~75 kDa, whereas TAZ and TUBULIN both run at ~55 kDa (Figure 3). Normalized expression of YAP or TAZ should be within approximately 2-fold of the level observed in the NCI-H661 YAP^{on} cell line (Figure 3).

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry

The proportion of YAP/TAZ/GFP-positive cells is determined by creating a histogram plot with YAP, TAZ or GFP as the value of the x-axis. A horizontal linear gate/marker is then added to the plot so that the left bound is placed where the non-transduced (mock) histogram ends (Figure 1). The percentage of cells that fall within this gate as well as their mean fluorescence intensity (MFI) can then be calculated using the analysis software.

Quantitative western blotting

Band intensities are quantified using the Li-Cor Odyssey software and then YAP or TAZ expression is normalized for protein loading by dividing the YAP/TAZ band intensity by the TUBULIN band intensity. The relative intensity of YAP/TAZ in the transduced YAP^{off} cells is then compared relative to the expression in YAP^{on} cells (Figure 3 and Table 3).

LIMITATIONS

We have used these vector systems successfully in 15 different YAP^{off} cell lines and four YAP^{on} cell lines. However, we tested two additional YAP^{off} cell lines (NCI-H510A SCLC cells, and RB3823 retinoblastoma cells) in which the transduction efficiency and/or promoter activity was too low to drive significant YAP expression. Thus, these vectors may not be suitable for use in all cell lines.

Expression of YAP and TAZ causes cytostasis in YAP^{off} cells, and we have observed that some cell lines strongly downregulate the ectopic YAP over a period of 2–3 weeks. This largely precludes using this system to generate stable YAP-expressing YAP^{off} cell lines and they must be used for transient, shorter-term assays (<2–3 weeks) in most cell lines.

YAP^{off} cell lines often grow in large clumps. For some cells, these are easily dissociated to single cells by centrifugation followed by pipetting (e.g., NCI-H209 cells). However, some lines are much more difficult to dissociate to single cell, and still remain in small clumps of 3–4 cells, which are not suitable for flow cytometry analysis.

Table 3. Example calculations for quantifying YAP expression by western blotting

	YAP intensity	TUBULIN intensity	YAP/ TUBULIN	Relative YAP
NCI-H661	197.97	91.77	2.16	1
H209-Empty	0	103.33	0	0
H209-YAP	338.87	113.83	2.98	1.38
H209-YAP^{S94A}	356.78	105.89	3.37	1.56
H209-YAP^{5SA}	248.71	106.59	2.33	1.08

TROUBLESHOOTING

Problem 1

No GFP fluorescence is observed in transfected 293T cells (step 3).

Potential solution

Ensure plasmids were propagated in recombination-deficient *E. coli*. Confirm the identity and integrity of the transfer plasmids using restriction digest and DNA sequencing. Repeat future transfections with a different GFP-encoding plasmid to ensure all reagents are functioning properly.

Problem 2

Cells (especially YAP^{off} cells) are gradually lost at each centrifugation step during flow cytometry staining (step 11).

Potential solution

Ensure the cells were transferred to a fresh tube (after fixation) for permeabilization. Try increasing the speed of the microfuge by 100–200 *g* and remove supernatant from the cell pellet using a P1000 pipette (as opposed to aspiration) leaving 30–50 μL of liquid behind. If available, centrifuge the samples in a centrifuge equipped with a swinging bucket rotor. If this does not resolve the problem, start with more cells.

Problem 3

High background staining in non-transduced cells during flow cytometry (Figure 4) (step 11).

Potential solution

Try titrating the primary antibody to find a concentration that gives low background and the largest separation between non-transduced and YAP/GFP-transduced cells (Figure 4). Increase the number of washes after antibody staining can also help reduce background.

Problem 4

YAP expression is well below or above the expression in YAP^{on} cells (steps 11 and 15).

Potential solution

Increase or decrease the amount of virus used. If this is unsuccessful, using the alternative YAP plasmids (e.g., PGKp *vs.* EFSp) is another option.

Problem 5

Viral titers are well below expected yields of 0.2–2.0 × 10^6 particles/μL when the virus is concentrated 100-fold (Expected Outcomes, steps 5–7).

Potential solution

Collect a small amount of the viral supernatant before and after concentration and titrate these to test whether virus is being lost during the ultracentrifugation step.

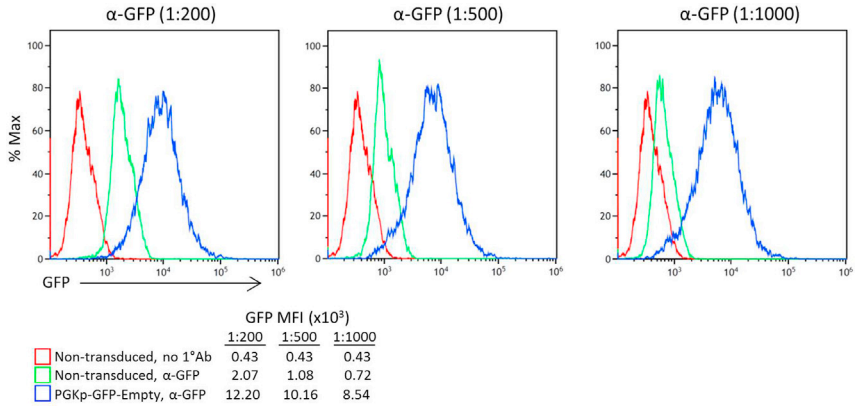


Figure 4. Example of α-GFP antibody titration for flow cytometry

Staining of control (non-transduced) or GFP-expressing (PGKp-GFP-Empty) YAP^{off} Y79 retinoblastoma cells with the anti-GFP primary antibody at a dilution of 1:200, 1:500 or 1:1000. The same, "no 1°Ab" sample is shown on each plot for reference. Note how the separation between non-transduced and GFP-expressing cells increases as the antibody concentration is reduced.

If significant amounts of virus are found in the supernatant after concentration, increase centrifugation time. If significant viral titer is "lost" during the centrifugation, ensure that the centrifuge is pre-cooled to 4°C prior to beginning spin, and increase the amount of time for resuspending the virus. For example, after addition of PBS to the viral pellet, the tube can be sealed with Parafilm and incubated on ice with gentle agitation (e.g., on an orbital shaker) for 1–2 h.

If low titers are observed, but this does not appear to be a problem at the concentration step (i.e., the initial viral supernatant has low titers), confirm the identity and integrity of the packaging and transfer plasmids by DNA sequencing and restriction digest. One can also try using fresh Lenti-X packaging cells and confirm that these are free of mycoplasma contamination.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rod Bremner (bremner@lunenfeld.ca).

Materials availability

Plasmids generated in this study have been deposited to Addgene (plasmid #174168–174176).

Data and code availability

This study did not generate any new datasets or code.

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AUTHOR CONTRIBUTIONS

J.D.P. generated reagents, conducted experiments, and performed data analysis. J.D.P. and R.B. designed the study and drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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