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TITLE:

Identification of Transcription Factor Regulators using Medium-Throughput Screening of Arrayed Libraries and a Dual Luciferase-Based Reporter

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Transcription factor, arrayed RNAi screen, YAP, TAZ, TEAD, dual luciferase reporter, transcriptional reporter assay, cancer

SUMMARY:

To identify novel regulators of transcription factors, we developed an approach to screen arrayed lentiviral or retroviral RNAi libraries using a dual luciferase-based transcriptional reporter assay. This approach offers a quick and relatively inexpensive way to screen hundreds of candidates in a single experiment.

ABSTRACT:

Transcription factors can alter the expression of numerous target genes that influence a variety of downstream processes making them good targets for anti-cancer therapies. However, directly targeting transcription factors is often difficult and can cause adverse side effects if the transcription factor is necessary in one or more adult tissues. Identifying upstream regulators that aberrantly activate transcription factors in cancer cells offers a more feasible alternative, particularly if these proteins are easy to drug. Here, we describe a protocol that can be used to combine arrayed medium-scale lentiviral libraries and a dual luciferase-based transcriptional reporter assay to identify novel regulators of transcription factors in cancer cells. Our approach offers a quick, easy, and inexpensive way to test hundreds of genes in a single experiment. To demonstrate the use of this approach, we performed a screen of an arrayed lentiviral RNAi library containing several regulators of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), two transcriptional co-activators that are the downstream effectors of the Hippo pathway. However, this approach could be modified to screen for regulators of virtually any transcription factor or co-factor and could also be used to screen CRISPR/CAS9, cDNA, or ORF libraries.

INTRODUCTION:

The purpose of this assay is to use viral libraries to identify regulators of transcription factors in

a relatively quick and inexpensive manner. Aberrant transcriptional activity is associated with cancer and metastasis[1-6], so targeting transcription factors in cancer cells is a promising therapeutic approach. However, transcription factors are often difficult to target pharmacologically[7] and many are required for normal cellular function in adult tissues[8-10]. Targeting the cancer-associated pathways that aberrantly activate transcription factors to drive disease is a more feasible approach with the potential to have less severe side effects. The commercial availability of arrayed lentiviral and retroviral RNAi, CRISPR/CAS9, cDNA, or ORF libraries allows researchers to test the importance of numerous genes in a single experiment. However, a reliable readout for altered transcriptional activity is required.

Here, we describe the use of a dual luciferase-based transcriptional reporter assay and arrayed lentiviral libraries to identify proteins that regulate transcription factors in cancer cells. In this assay, shRNAs that target cancer-associated genes are delivered to mammalian cancer cells via lentiviral transduction and cells are selected for stable integration using puromycin. The cells are next transfected with a reporter construct that expresses firefly luciferase driven by a promoter specific to the transcription factor that is being investigated and a control construct that expresses *Renilla* luciferase from a constitutively active promoter that is not responsive to the transcription factor being investigated. We demonstrate this approach, with a proof-of-concept screen for regulators of YAP and TAZ, the critical downstream effectors of the Hippo pathway[8,10,11]. Abnormal activity of YAP and TAZ promotes several steps of the metastatic cascade[11] and is observed in many cancers[11-13]. However, how YAP and TAZ become aberrantly activated in some cancer cells is not yet fully understood. YAP and TAZ do not bind DNA, but instead are recruited to promoters by other transcription factors. Members of the TEA domain (TEAD) family of transcription factors are the major binding partners for YAP and TAZ, and are critical for most YAP and TAZ-dependent functions. Our reporter construct expresses firefly luciferase from a YAP/TAZ-TEAD-responsive promoter and previous studies have demonstrated that it faithfully detects changes in YAP-TEAD and TAZ-TEAD transcriptional activity[2,14,15].

Our approach is rapid, medium throughput, and requires neither screening facilities, automated robots, nor deep sequencing of pooled libraries. The costs are relatively low and there are numerous commercially available libraries to choose from. The required equipment and reagents are also relatively standard in most laboratories. It can be used to screen for regulators of virtually any transcription factor if a luciferase-based reporter exists or is generated. We use this approach to screen shRNAs in cancer cells, but any cell line that can be transfected with reasonable efficiency could be used with any type of arrayed library.

PROTOCOL:

NOTE: A schematic summary of this protocol is shown in **Figure 1**.

1. Lentiviral vector library preparation

NOTE: The demonstrated screen used an arrayed shRNA library purchased as glycerol stocks in

96-well plates, but libraries can also be assembled manually based on a list of candidates. Appropriate controls should be considered and included in any library. This includes a non-targeting control shRNA (shNTC), a control shRNA targeting the transcription factor being investigated, and if possible, an shRNA targeting firefly luciferase.

1.1. Add 1.3 mL of Luria Broth (LB) (1% bacto-trypton, 0.5% yeast extract, 1% NaCl, pH 7.5) containing 100 µg/mL of ampicillin to each well of a 96-well deep well plate. Inoculate each well with 2 µL of glycerol stock and grow at 37 °C overnight with constant agitation at 225 rpm.

1.2. Transfer each bacterial culture into a 1.5 mL centrifuge tube and pellet the bacteria by centrifugation at 21,000 x *g* at 4 °C for 10 min.

1.3. Purify each vector using a bacterial mini-prep kit by following the manufacturer's protocol.

1.4. Determine the concentration of each vector using a spectrophotometer.

1.5. Store the plasmids at -20 °C.

NOTE: The protocol can be paused here.

2. Packaging of the arrayed lentiviral library

NOTE: All work involving lentivirus, including packaging, infection, and subsequent culturing of infected cells should strictly follow the institutional biosafety rules and regulations.

2.1. Expand the 293FT cells using complete growth media (Dulbecco's modified Eagle medium (DMEM) containing 4 mM L-glutamine, 4,500 mg/L glucose and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, 100 µg/mL streptomycin antibiotic and 2 mM L-glutamine).

2.2. For each vector in the library from step 1.4, seed one 24-well with 1 x 10^5 293FT cells.

NOTE: It is recommended that some extra wells of a control viral vector be packaged and used to test the titer of the virus prior to proceeding to step 3 (see below).

2.3. Incubate the cells at 37 °C with 5% CO_2 for 24 h.

NOTE: A general protocol for packaging lentivirus that was described previously[14] has been scaled down to 24 wells for this protocol. It uses psPAX2 for lentiviral packaging and VSVG as a coat protein. If ectropic virus is desired, a vector delivering Eco can be used instead of VSVG. It is highly recommended that this protocol is optimized to achieve a viral titer that gives between 30%-70% infection efficiency of the target cells (see Discussion). See **Supplemental Table 1** for a list of all vectors used.

2.4. Set up a transfection mixture for each viral vector from Step 1.4 as described below. Each transfection should contain 250 ng of the viral vector, 125 ng of psPAX2, 125 ng of VSVG, 1.25 µL of transfection reagent 1, AND 23.75 µL of transfection buffer (see **Table of Materials**).

2.4.1. Dilute each lentiviral vector to 50 ng/µL with nuclease-free water, and then transfer 5 µL (250 ng) into a well of a 96-well PCR plate.

2.4.2. Make the transfection super mix by mixing 1.25 µL * X of transfection reagent 1 and 23.75 µL * X of pre-warmed transfection buffer where "X" is the total number of transfections plus several extra to account for volume loss during pipetting.

2.4.3. Incubate the transfection super mix at room temperature for 5 min.

2.4.4. Add 125 ng * X of psPAX2 and 125ng * X of VSVG to the tube of transfection super mix from Step 2.4.3 and gently pipet up and down to mix. Rapidly proceed to Step 2.4.5.

2.4.5. Immediately aliquot the mixture from Step 2.4.4 into each tube of a PCR strip, and then use multi-channel pipettor to transfer 25 µL the mixture into each well containing viral vector from Step 2.4.1.

2.4.6. Incubate at room temperature for 20 min.

2.4.7. Transfer all 30 µLs from each 96-well from Step 2.4.6 into a well of the 24-well containing 293 FT cells from Step 2.3.

2.5. Incubate the cells at 37 °C with 5% CO_2 for 24 h and then replace the media in every well of the 24-well plate with 500 µL of fresh complete growth media. Incubate the cells at 37 °C with 5% CO_2 for another 24 h.

2.6. Using a multi-channel pipettor, collect the viral supernatant from each well and aliquot 220 µL (enough for 1 infection in Step 3 plus some extra volume) into two 96-wells each. These are the arrayed viral supernatant plates.

2.7. Store the arrayed viral supernatant plates at -80 °C.

NOTE: The protocol can be paused here. It is also recommended that before proceeding to Step 3 that some of the extra control virus that was packaged (see above) is tested on the cells to be infected to ensure that the titer is sufficient to achieve at least 30% infection efficiency.

3. Infection of the cells for the screen

NOTE: Human melanoma cells (A375) were used to demonstrate this approach, but this method can be applied to any adherent cells that infect with lentivirus. However, cell culture

and plating conditions should be optimized for each cell line (see Discussion).

3.1. Expand the cells to be infected in complete growth media.

3.2. Seed 24-well plates with 1 x 10^5 cells per well in complete growth media. Seed one well for each viral vector to be tested (including controls) and include an extra well that will not be infected and will serve as a control for drug selection in Step 3.7.

3.3. Incubate the cells at 37 °C with 5% CO_2 for 24 h.

3.4. Infect each well from Step 3.2 with a different viral supernatant from the frozen arrayed lentiviral supernatant as follows.

3.4.1. Prepare complete growth media that contains 20 µg/mL polybrene.

3.4.2. Thaw the arrayed lentiviral library supernatants from Step 2.7 to room temperature.

3.4.3. Aspirate the growth media from the 24-well plates from Step 3.2 and immediately add 200 µL of polybrene-containing growth media to each well.

3.4.4. Using a multi-channel pipettor, transfer 200 µL of viral supernatant from each 96-well from Step 3.4.2 to the 24 wells from Step 3.4.3.

3.5. Incubate the cells at 37 °C with 5% CO_2 for 24 - 48 h.

NOTE: Some cell lines may require longer than 24 h to express the shRNAs and become puromycin resistant. The viral vector used here delivers a Turbo-GFP-IRES-puroR with a miR30-based shRNA in the 3'UTR of puroR (**Figure 1**). Infection efficiency and expression of the puromycin resistance gene and the shRNA were monitored by green fluorescent protein.

3.6. Prepare complete growth media that contains 2.5 µg/mL puromycin.

NOTE: The selection concentration of puromycin varies between cell lines. It is recommended that an antibiotic kill curve be performed for each cell line to be assayed prior to the screen.

3.7. Aspirate the media from each well and replace with 500 µL of puromycin-containing complete growth media.

NOTE: Be sure to also add puromycin to a control noninfected well that can be used in subsequent steps to ensure the puromycin selection is complete.

3.8. Incubate the cells at 37 °C with 5% CO_2 for 48 h.

NOTE: It is best to select for 48 h, so plating density and viral titer should be optimized so that

the cells are not overconfluent prior to 48 h.

3.9. Ensure that the infected cells are green under the fluorescent microscope, and that cells on a control non-infected well treated with puromycin are all dead before proceeding to Step 4.

4. Seeding cells for dual-luciferase reporter assay

NOTE: A test transfection should be done to determine the optimal seeding density for each new cell line.

4.1. Add 400 µL of puromycin-containing complete growth media to empty wells on 24-well plates.

4.2. Trypsinize each well from Step 3.9 and transfer roughly 1 x 10^5 cells into wells at the corresponding position on the new 24-well plate as follows.

NOTE: This protocol is designed for the screening of libraries with hundreds of shRNAs so it is not feasible to count every well of infected cells. Therefore, the steps below were used to estimate cell numbers in each well to help ensure roughly equal plating density.

4.2.1. Group the wells from Step 3.9 into 3 - 4 groups based on the confluency of the well.

4.2.2. Trypsinize 1 representative well from each group with 200 µL of trypsin-EDTA (1x PBS supplemented with 0.5 mM EDTA and 0.1% trypsin) for 5 min at 37 °C. Then neutralize the trypsin-EDTA by adding 400 µL of puromycin containing complete growth media.

4.2.3. Count each representative well to determine the total cell number and dilute the cell suspension from each representative well to 2 x 10^5 cells/mL of using complete growth media.

4.2.4. Seed 0.5 mL (1 x 10^5 cells) of each well from Step 4.2.3 into the corresponding position on the 24-well plates prepared in Step 4.1.

4.2.5. For each group from Step 4.2.1, use the total cell number determined in Step 4.2.3 to calculate the volume of trypsin-EDTA to add to each well so that the resulting cell suspension will be 1 x 10^6 cells/mL.

4.2.6. Add the appropriate volume of trypsin-EDTA to each well and incubate for 5 min at 37 °C.

4.2.7. Transfer 100 µL of cell suspension (approximately 1 x 10^5 cells) from each well to the corresponding position on the new 24-well plates prepared in Step 4.1.

4.3. Incubate the cells at 37 °C with 5% CO_2 for 24 h.

5. Transfection of dual-luciferase reporter

5.1. Transfect each well from Step 4.3 with the dual luciferase reporter constructs as follows.

NOTE: The total amount of DNA and the optimal ratio of firefly luciferase reporter vector to control *Renilla* luciferase vector should be determined prior to starting this assay. Here, 400 ng of a DNA mixture that contains 20 parts firefly luciferase reporter and 1 part control *Renilla* luciferase was used.

5.1.1. Make the transfection dilution mixture (Tube A) and the reporter dilution mixture (Tube B) by mixing the indicated volumes of each reagent (**Table 1**) multiplied by the total number of transfections (plus several extra).

NOTE: This protocol is optimized for transfection reagent 2 (see **Table of Materials**). If a different transfection reagent is used, the transfection should be optimized prior to this step.

5.1.2. Mix the transfection dilution mixture (Tube A) with reporter dilution mixture (Tube B) and incubate at room temperature for 15 min to produce transfection mixture.

5.1.3. During the above incubation, rinse each 24-well from Step 4.3 with 0.25 mL of phosphate buffer saline (PBS), and add 447 µL of complete growth media to each well.

5.1.4. After the 15 min incubation, use a multi-channel pipettor to distribute 53 µL of transfection mix to each well of the 24-well plates.

5.2. Incubate the cells at 37 °C with 5% CO_2 for 24 h.

6. Quantification of dual luciferase activity

6.1. Measure luciferase activity using a plate reader and a dual luciferase reporter assay kit as described below.

NOTE: This protocol is optimized for the indicated reporter assay kit (see **Table of Materials**) and follows the manufacturer's recommended protocol.

6.1.1. Prepare enough 1x passive lysis buffer for all wells plus several extra (75 µL is needed per well) by diluting 5x passive lysis buffer (provided in kit) 1 to 5 with deionized water. Also thaw reagent A and reagent B Buffer (provided in kit, 100 µL of each is needed for each well).

6.1.2. Aspirate the media from each well of the 24-well plate from Step 5.2.

6.1.3. Add 75 µL of 1x passive lysis buffer to each well and incubate at room temperature for 30 min with occasionally shaking.

6.1.4. Prepare reagent B by diluting 50x reagent B substrate (provided in kit) 1:50 with thawed reagent B buffer.

6.1.5. Add 30 µL of 1x passive lysis buffer to 4 wells for blanking (see **Supplemental Table 2**).

6.1.6. Transfer 30 µL of lysate from Step 6.1.3 into duplicate wells of a 96-well flat bottom white assay plate.

6.1.7. Use a multi-channel pipettor to add 50 µL of reagent A to each well and read the firefly luciferase signal with a plate reader.

6.1.8. Use a multi-channel pipettor to add 50 µL of reagent B from Step 6.1.4 to each well and read the *Renilla* luciferase signal with a plate reader.

6.2. Process the raw data as follows (for a detailed description, see **Supplemental Table 2**).

6.2.1. Exclude samples with very low *Renilla* luciferase signal as low values indicate that the viral construct was toxic or that too few transfected cells were assayed.

NOTE: As explained in the Discussion, significantly "low" *Renilla* luciferase signal can result in anomalous results. Here, wells in which the *Renilla* luciferase signal was more than 1 standard deviation below the mean were excluded (see **Supplemental Table 2**). This was based on previous studies performed using this reporter system in these cells[14], but may differ in other cell lines.

6.2.2. Normalize the raw firefly luciferase value of each well to the raw *Renilla* luciferase value of the same well to obtain the firefly/*Renilla* ratio.

6.2.3. Average the firefly/*Renilla* ratios of all replicate control wells and then divide the firefly/*Renilla* ratio of every other well by that number to get a fold change.

6.2.4. Assign the control sample and set its firefly/*Renilla* ratio value to 1.

6.2.5. Average the firefly/*Renilla* ratios of the duplicate wells and plot with the standard deviation..

NOTE: The standard deviation is not used for statistical analysis, but instead as a means to identify wells where the replicates differ significantly.

REPRESENTATIVE RESULTS:

Our YAP/TAZ-TEAD reporter construct (pGL3-5xMCAT (SV)-49[2,14,15]) contains a minimal SV-49 promoter with 5 repeats of the canonical TEAD binding element (MCAT)[15] driving the firefly luciferase gene (**Figure 1**). It is co-transfected into cells along with the PRL-TK control vector (Promega), which expresses *Renilla* luciferase from the constitutively active HSV TK promoter

(**Figure 1**). It is critical to ensure that the YAP/TAZ-TEAD reporter construct is behaving as expected in the cell line(s) being tested. Therefore, we first co-transfected the PRL-TK and pGL3-5xMCAT (SV)-49 constructs into A375 cells stably expressing either a control vector, a highly active LATS-insensitive mutant of YAP (YAP ^{2SA}), or a mutant of YAP^{2SA} unable to bind TEADs (YAP $^{2SA, S94A}$). As expected, firefly luciferase activity was significantly increased by YAP^{2SA}, but not the control vector or the YAP$^{2SA, S94A}$ (**Figure 2A**). This suggests that YAP increases the activity of the YAP/TAZ-TEAD reporter in a TEAD-dependent manner. Importantly, YAP^{2SA} did not significantly alter the levels of the *Renilla* luciferase. As an additional control, we also confirmed that the YAP^{2SA} does not alter the activity of a minimal promoter construct that lacks the MCAT TEAD binding elements (**Figure 2B**).

A second control experiment was also done in which A375 cells were infected with viral constructs that deliver either a non-targeting control shRNA (shNTC) or a construct with tandem YAP and TAZ shRNAs. After stable selection, the cells were co-transfected with the PRL-TK construct and either the YAP/TAZ-TEAD reporter construct or the minimal promoter construct. In cells transfected with the YAP/TAZ-TEAD reporter construct, the YAP/TAZ shRNA significantly reduced firefly luciferase levels, but the control shNTC did not (**Figure 2C**). Firefly luciferase levels were not changed by either the shNTC or the YAP/TAZ shRNA in cells transfected with the minimal promoter (**Figure 2C**). Consistent with the results above, the *Renilla* signal in each well was also not significantly altered by the control shNTC or the YAP/TAZ shRNA (**Fig 2C**), further indicating that the PRL-TK control construct is not responsive to YAP or TAZ. Collectively, these experiments show that the reporter system is behaving as expected in A375 cells, which is consistent with previous studies using these vectors[2,14].

As a proof of concept experiment, we screened a small lentiviral shRNA library in A375 cells. Our test library contained shRNAs targeting several genes that were previously shown to regulate YAP/TAZ function in other cell types. However, the role of several of these genes in the regulation of YAP and TAZ in melanoma is unknown. Our library included genes that are required for YAP/TAZ activity, such as RAF1, MDM2, PIK3CA, MAPK8, PAK4, EZH2, PDK1, ERBB4, and CCNE2[16-38], and genes predicted to inhibit YAP/TAZ activity, such as CSK, ERBB2, ATM, CDH1, GSN, PTEN, ATR, and RB1[39-54]. As controls, we included a tandem YAP/TAZ shRNA[14], a non-targeting control shRNA (shNTC), and an shRNA targeting Src, which we previously showed was required for maximal YAP/TAZ activity in A375 cells[14].

The raw data and analysis for this screen is shown in **Supplemental Table 2**. Several shRNAs (shPDK-1, shMDM2-1, shMDM2-2, shPAK4, shMAPK8-1 and shMAPK8-2) significantly reduced *Renilla* luciferase signal relative to the mean *Renilla* signal for all wells, suggesting that these shRNAs were causing cytotoxicity in A375 cells. Indeed these wells had significantly fewer cells at the time of the assay (not shown). This makes it very difficult to distinguish candidates that may regulate YAP/TAZ from the ones that are essential for cell survival. Therefore, the results from these samples are excluded from further data analysis. As expected, shRNAs targeting YAP and TAZ or Src significantly reduced YAP/TAZ-TEAD activity (**Figure 3**). Consistent with published work showing that PIK3CA promotes YAP and TAZ activity[21,26,31], we found that shRNAs targeting PIK3CA reduced normalized firefly luciferase levels. shRNAs targeting ATM, CDH1,

CSK, ERBB2, GSN each increased normalized firefly luciferase levels, which is consistent with published studies showing that these proteins repress YAP and/or TAZ[39,41-43,45-48,50,51,53,54]. Despite established roles for ATR, CCNE2, and ERBB4 in other cell types[27,28,35,36,38,49], shRNAs targeting these genes did not significantly change normalized firefly luciferase levels in A375 cells. shRNAs targeting EZH2 and RB1 showed an effect on firefly luciferase levels that was opposite of what was expected based on studies in other cell types[32,34,40,52]. Both PTEN and RAF1 were targeted by two distinct shRNAs that had opposite effects on luciferase activity. Results inconsistent with previous studies could indicate that the influence of these proteins on YAP/TAZ-TEAD function is cell type-dependent; however, it may also be due to poor knockdown efficiency or off-target effects by some shRNAs. As an n=1 "blind" screen, some false positives and false negatives would also be expected. As discussed in more detail in the Discussion, additional validation experiments would need to be done using other assays such as qPCR for genes that are regulated by YAP and TAZ and western blots for post translational modifications that influence YAP/TAZ function. This validation would also include confirming which shRNAs effectively knocked down the protein of interest. It is also important to note that since our reporter is TEAD responsive, any genes identified by our screen could be influencing TEADs but not YAP and TAZ directly. Follow-up mechanistic studies would be required to determine whether it was YAP and/or TAZ or the TEADs that the identified protein is regulating. However, YAP and TAZ are the major drivers of TEAD-mediated transcription, so it is likely that most of these genes are regulating YAP and TAZ. Indeed, as stated above, most of the shRNAs that hit in the screen target proteins that are known to regulate YAP and/or TAZ directly.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the workflow. The critical steps of this protocol are summarized with step numbers corresponding to the numbers in the protocol.

Figure 2: Optimization of the YAP/TAZ-TEAD dual luciferase transcriptional reporter system. (**A**) A375 cells stably expressing control vector (MSCV-IRES-Hygro), LATS-insensitive YAP (YAP^{2SA}), or LATS-insensitive YAP unable to bind TEADs ($YAP^{2SA,S94A}$) were co-transfected with the PRL-TK (*Renilla*) and pGL3-5xMCAT (SV)-49 (YAP/TAZ-TEAD reporter) constructs and luciferase signal was measured 24 hours later. n= 7 independent experiments. (**B**) A375 cells were co-transfected with either YAP^{2SA} or control vector, the PRL-TK construct, and either the pGL3-5xMCAT (SV)-49 construct or the pGL3 (SV)-49 construct (minimal promoter) and luciferase signal was measured 24 hours later. n=1 experiment transfected in quadruplicate. (**C**) A375 cells were infected with retrovirus encoding a non-targeting control shRNA (shNTC) or tandem YAP and TAZ shRNAs (shYAP/TAZ). After stable selection, cells were co-transfected with the PRL-TK construct and either the pGL3-5xMCAT (SV)-49 construct or the pGL3 (SV)-49 construct and luciferase signal was measured 24 hours later. n= 4 independent infections; Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test; n.s. = $p>0.05$, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Figure 3: Results of representative screen. The result from each knock down vector is compared with shNTC and presented as fold difference. The bar graph shows the average of firefly luciferase/*Renilla* luciferase ratio ± standard deviation. n = 1 experiment.

Table 1: Preparation of transfection mix. For the transfection in each well of the 24-well plates, 1.5 µL of transfection reagent 2 is diluted into 25 µL of transfection buffer to produce the transfection dilution (tube A); while 400 ng of 20:1 firefly luciferase reporter: control *Renilla* mix and 1 µL of transfection reagent 3 is diluted into 25 µL of Transfection Buffer to produce the reporter dilution (tube B).

Supplemental Table 1: Table of vectors. All vectors used are listed with new vectors described. Standard molecular biology techniques were used to generate new vectors.

Supplemental Table 2: Luciferase assay data analysis. The arrangement of the shRNA library is shown in the first table. The second and third tables show the raw firefly and *Renilla* luciferase signals, respectively. The mean and standard deviation for the *Renilla* luciferase signal for all wells are indicated in the yellow boxes. Wells with a *Renilla* luciferase signal more than 1 standard deviation below the mean are highlighted with red text and excluded from further analysis. The firefly/*Renilla* ratio of every well was obtained by dividing the raw firefly luciferase signal by the raw *Renilla* luciferase signal (fourth table). The firefly/*Renilla* ratio of each well was then normalized to the average of firefly/*Renilla* ratio of the control (shNTC) wells (fifth table). The duplicate wells were next averaged for each construct and the standard deviation was calculated (sixth table).

DISCUSSION:

In this study, we demonstrate an approach for medium throughput screening of arrayed viral libraries in combination with a dual luciferase-based transcriptional reporter assay that can be used to identify and test novel regulators of transcription factors. It is critical to characterize and optimize the reporter system for each cell line prior to any screen. Experiments should be done to confirm that the reporter is responsive to altered activity of the transcription factor being investigated and the magnitude of change in activity should be tested relative to control vectors. Co-transfection of the PRL-TK construct along with the reporter construct is important because it helps control for the number of cells transfected with the reporter and the copy number of the reporter construct, both of which can alter the magnitude of the luciferase signal. Optimization experiments should also ensure that the transcription factor does not influence the activity of the constitutive *Renilla* construct or a minimal promoter construct as this could complicate the interpretation of the results. Controls to be included in the library should also be carefully considered. This protocol is designed for a “blind screen” of libraries with hundreds of shRNAs, where it is not be feasible to confirm effective knockdown by each shRNA. Therefore, some false positives and negatives are likely. Increasing the number of technical and/or biological replicates in the screen could help reduce the number of false positives and negatives. However, this will also significantly increase the number of wells to culture and assay so care should be taken to ensure this does not result in suboptimal culture conditions (see below). Another approach to reduce false positives and negatives would be to repeat the entire screen in the same cell line or additional cell lines, or to screen a smaller library including only the “hits” using 3 biological replicates. In all cases, any hits should be validated using readouts besides the reporter, such as qPCR for known target genes. Effective

knockdown of the targeted gene should also be confirmed in these validation steps. Conclusions should not be made about shRNAs that do not alter activity unless effective knockdown by the shRNA is confirmed. Validation experiments should also ensure that the transcription factor being investigated is what is regulated by the targeted gene(s) and that these genes do not influence the *Renilla* or minimal promoter constructs.

It is also important to optimize the cell culture conditions for each cell line to avoid suboptimal conditions such as over-confluence, too few cells, poor cell viability, or variable proliferative capacity. Cell seeding density is particularly important at the time of the transfection of dual-luciferase reporter construct (Step 5) and when the reporter activity is measured (Step 6). Transfection efficiency can vary significantly with cell density and poor transfection efficiency can result in anomalous results if only a small fraction of the cell population is being assayed. Most cell lines tested show the highest transfection efficiency when at 40 – 60% confluence, but it is recommended that transfection conditions and seeding density are optimized for a given cell line using a vector that delivers a fluorescent protein. Cell lines that are difficult to infect and/or transfect, such as primary cells may not be suitable for this screen. Poor reporter transfection efficiency or poor cell viability typically result in very low *Renilla* luciferase signal. However, a pilot experiment should be performed to determine the range of *Renilla* luciferase signal for a cell line. It is important that the titer of the virus produced from the arrayed library is high enough to give an infection efficiency of at least 30% in the cell line being assayed. Infection efficiency can vary greatly and several factors can influence viral titer. The amount of viral supernatant used should be optimized for each cell line and if necessary, Step 2 can be optimized further to improve viral titers.

Cell density and cell viability can also influence the activity of cellular pathways that regulate transcription factors, so it is critical to seed the cells for the reporter transfection so that they are all at a similar density on the day the dual luciferase assay is read (Step 6). It is also important to ensure that the cells adhere uniformly across the well rather than clustering in a dense patch in the center. As described above, significant variation in the *Renilla* luciferase signal from well to well may result in data that is difficult to interpret. It is best to exclude wells that show a significant reduction in *Renilla* luciferase signal when compared to all other wells as this suggests that either the viral vector is reducing cell viability or the transfection efficiency for that well was very low. Here we excluded wells greater than 1 standard deviation from the mean *Renilla* luciferase signal, but optimization experiments should be done to determine appropriate cutoffs for each cell line. It is also possible that shRNAs that reduce cell viability could be doing so by altering the activity of the transcription factor being assayed. If this is a concern, shRNAs that reduce *Renilla* luciferase signal could be re-tested using inducible shRNAs or other assays. It is also critical to limit the amount of time that adherent cells are in suspension following trypsinization. Use a multi-channel pipettor for most steps during the trypsinization and seeding of cells, and for larger screens, work with the plates in batches. In addition, it is best to limit the time between the infection of the cells and the reporter assay. This is particularly important if the transcription factor being assayed regulates cell proliferation or survival. In this case, cells with effective knockdown would be outcompeted by cells with less efficient knockdown.

Several modifications of the described protocol are feasible. This approach can be used effectively to identify regulators of any transcription factor if a luciferase-based reporter is generated, and for many of the most common transcription factors reporter constructs already exist. This protocol can be modified for the use of a wide variety commercially available or manually-assembled libraries, including RNAi, CRISPR/CAS9, ORF, or cDNA. Indeed, we previously used a similar strategy to test a small cDNA library for YAP/TAZ regulators[14]. Libraries can be delivered using retrovirus, lentivirus, adenovirus, or transient transfection. The viral coat protein used here (VSVG) generates lentivirus that is human-infectious. If rodent cells are being used and non-human infectious ecotropic virus is desired, a vector delivering the Eco coat protein can be used instead of VSVG.

Other methods can be used to screen libraries for regulators of a transcription factor. Access to a high throughput screening facility would allow for the screening of much larger libraries in an automated manner. Likewise, genome-wide screens can be done using pooled libraries with deep sequencing as a means to identify the "hits". However, both of these approaches require equipment that is not accessible to many researchers, and the fees for high throughput screening or deep sequencing can be prohibitively high. In addition, pooled screens would require sorting of cells using a fluorescent reporter or another way to enrich the cells that show the desired changes in transcriptional activity. Screening of large arrayed expression libraries using a dual luciferase-based transcriptional reporter assay has been demonstrated previously using a benchtop robot[55], but this is not commonly available for all researchers. In contrast, the method described here is rapid, medium throughput, relatively inexpensive, and uses equipment and reagents that are likely accessible to most investigators. This method can reveal multiple regulators in a single experiment, which is a cost effective and convenient way to discover potential regulatory pathways upstream of a transcription factor.

In the described approach, candidate genes were stably knocked down and then after selection, the reporter constructs were transiently transfected into the cells. However, transfection efficiency is poor in many cell lines and can introduce variability and cause cellular toxicity. An improved alternative approach would be to stably integrate the reporter construct into the genome of the cell line of interest and then infect the reporter-expressing cells with the viral libraries for screening. This improvement would reduce the variability introduced by the transient transfection and prevent any potential changes in transcription factor activity due to prolonged post-infection culture. As mentioned above, the use of a small benchtop robot would greatly streamline the process and increase the size of the libraries that could be screened. Another considerable alteration is to use arrayed inducible vector libraries, which would reduce the likelihood of selection against cells with more effective knockdown and reduce variability caused by changes in cell viability.

A significant challenge that prevents the effective treatment of many cancers is that tumor cells acquire resistance to even the most effective targeted therapies. The identification of proteins that are required for this therapeutic resistance is necessary to improve patient outcome. The described approach could easily be used in combination with targeted therapeutics to reveal

genes that cause increased sensitivity or resistance to these compounds. Another potential future application of this approach would be to use this arrayed screening to test candidate therapeutic targets in combination. For this, cells would be infected with two viral vectors each with the goal of identifying proteins that have a synergistic effect on transcription factor activity.

ACKNOWLEDGMENTS:

We would like to thank Emily Norton and Mikaelan Cucciarre-Stuligross for assisting in the preparation of shRNA vectors. This work was supported in part by a Susan G. Komen Career Catalyst Grant that awarded to J.M.L. (#CCR17477184).

DISCLOSURES:

The authors have nothing to disclose.

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719

Figure 1

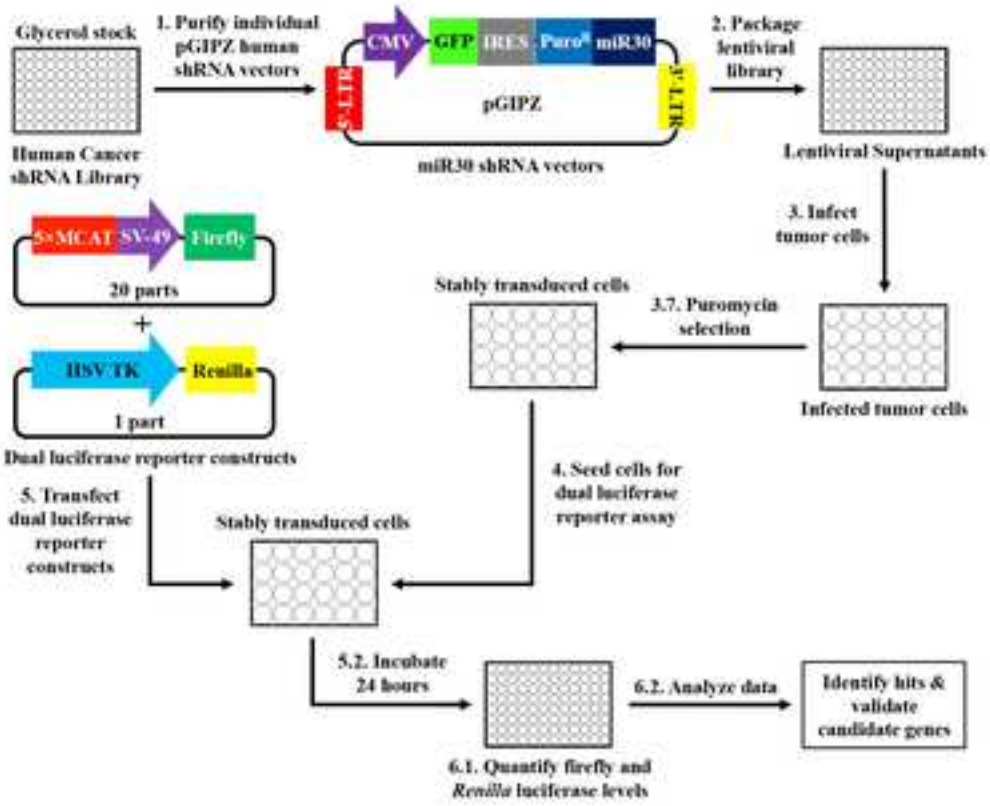


Figure 2

Click here to access/download;Figure;Figure 2.tif

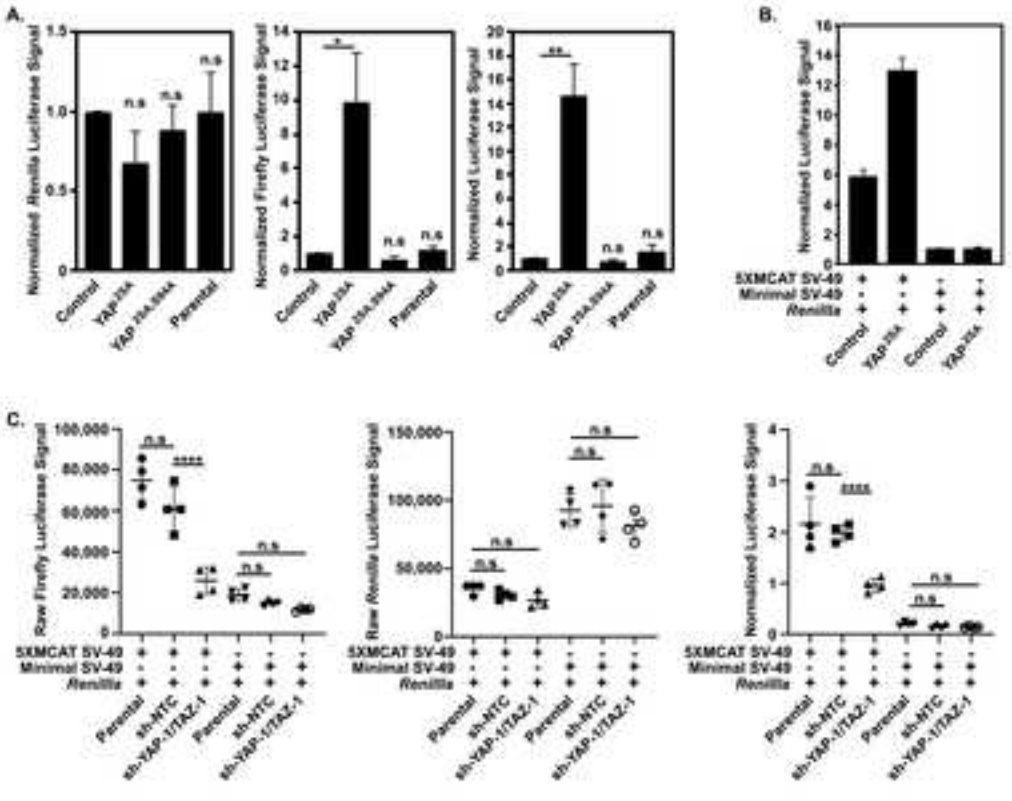
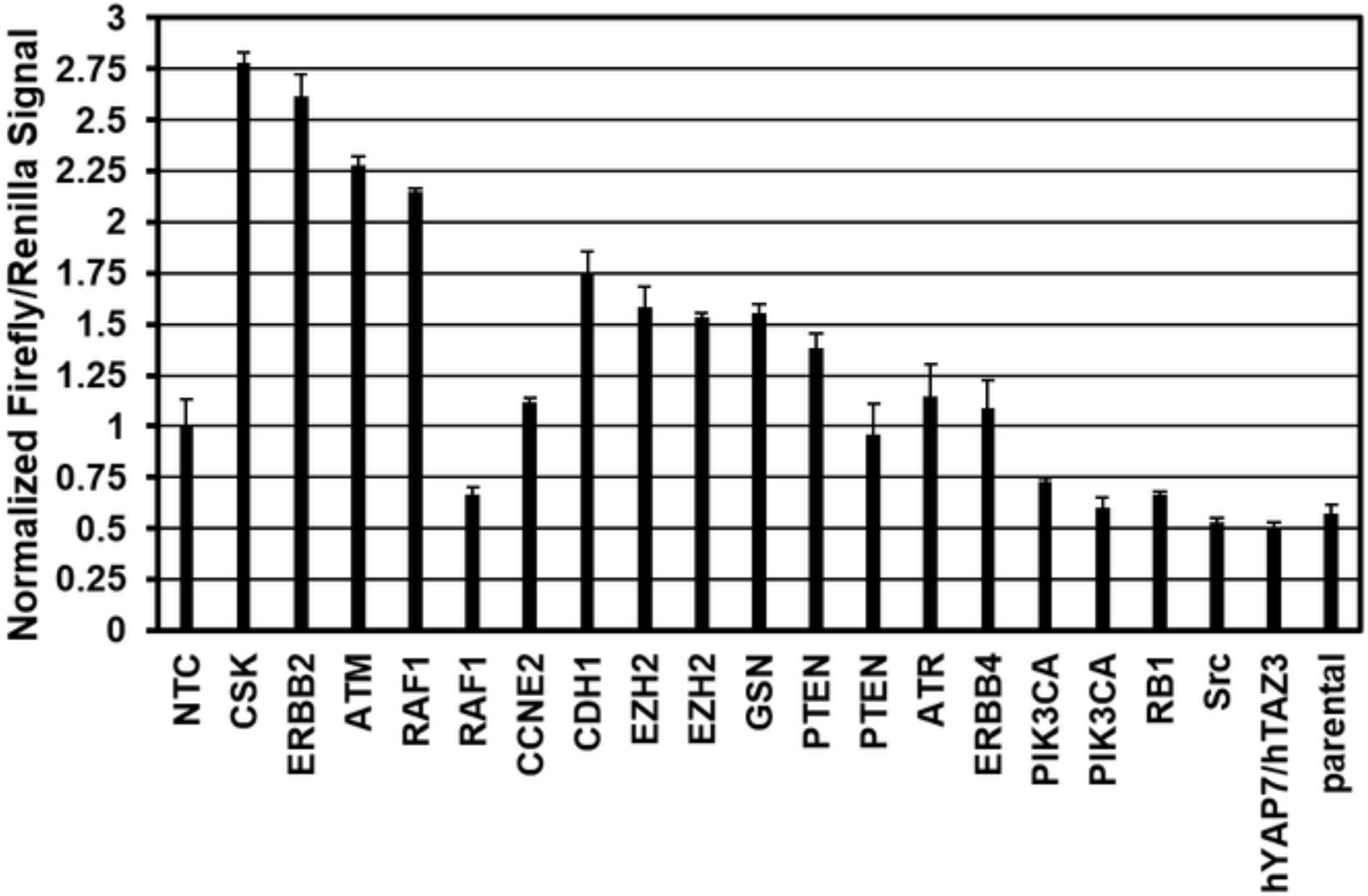


Figure 3



Tube A – Transfection Dilution

Reagent	**Volume**
Transfection Buffer	25 µl per reaction
Transfection Reagent 2 (add second)	1.5 µl per reaction
	Total: 26.5 µl per reaction

Tube B – Reporter Dilution

Reagent	**Volume**
Transfection Buffer	25 µl per reaction
20:1 Firefly luciferase reporter: control Renilla mix (add second)	400 ng per reaction
Transfection Reagent 3 (add third)	1 µl per reaction
	Total: 26.5 µl per reaction

Name of Material/ Equipment
2.0 ml 96-well deep well polypropylene plate
Trypsin
96 well flat bottom white assay plate
Ampicillin
Bacto-tryptone
Dual-luciferase reporter assay system, which include LAR II reagent (reagent A), Stop & Glo substrate (reagent B substrate) and Stop & Glo buffer (reagent B buffer)
Dulbecco's phosphate buffered saline w/o calcium, magnesium and phenol red
EDTA
Ethanol
Foetal Bovine Serum
Hexadimethrine bromide (Polybrene)
HyClone DMEM/High glucose
I3-P/i3 Multi-Mode Microplate/EA
L-Glutamine
Lipofectamine 3000 (Transfection Reagent 2)
Molecular Biology Water
NaCl
NanoDrop One Microvolume UV-Vis Spectrophotometer
Opti-MEM (Transfection Buffer)
Penicillin Streptomycin
PureLink Quick Plasmid Miniprep Kit
Puromycin
TC20 automated cell counter
X-tremeGENE 9 DNA transfection reagent (Transfection Reagent 1)
Yeast extract
P3000 (Transfection Reagent 3)

Concentration	Company	Catalog Number
	USA Scientific	1896-2000
2.50%	Gibco	15090-046
	Corning	3922
100 mg/ml	Sigma-Aldrich	45-10835242001-EA
powder	Sigma-Aldrich	95039
Kit	Promega	E1960
9.6 g/L	Himedia	TS1006
0.5 M	VWR	97061-406
100%	Pharmco-AAPER	111000200
100%	VWR	97068-085
8 mg/ml	Sigma-Aldrich	45-H9268
4 mM L-Glutamine; 4500 mg/L glucose; sodium pyruvate	GE Healthcare life sciences	SH30243.01
	Molecular devices	
200 mM	Gibco	25030-081
100%	Life technologies	L3000008
100%	VWR	02-0201-0500
powder	BDH	BDH9286
	Thermo scientific	
100%	Gibco	31985-062
10,000 Unit/ml (Penicillin); 10,000 μg/ml (Streptomycin)	Gibco	15140-122
Kit	Thermo Fisher Scientific	K210010
2.5 mg/ml	Sigma-Aldrich	45-P7255
	Bio-Rad	
100%	Roche	6365787001
powder	VWR	J850
100%	Life technologies	L3000008

Comments/Description
For bacterial mini-prep
Componenet of trypsin-EDTA
For dual luciferase assay
For bacterial mini-prep
Component of LB broth
For dual luciferase assay
For PBS
Componenet of trypsin-EDTA
For bacterial mini-prep
Component of complete growth media
For virus infection
Component of complete growth media
For dual luciferase assay
Component of complete growth media
For transfections
For dilutionof shRNA vector for virus packaging
Component of LB broth
For measuring vector DNA concentration
For transfections
Component of complete growth media
For bacterial mini-prep
For antibiotic selection after infection
For cell counting
For virus packaging
Component of LB broth
For transfections

Rebuttal:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done
2. Please do not use more than one note for each step. Corrected
3. Step 2.9: Please ensure that all text is written in the imperative tense. Corrected
4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Stop & Glo, Milli Q, etc. Corrected
5. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Corrected
6. Figure 2: Please add a title for the whole figure in Figure Legend. Done
7. Please specify the composition of all buffer or media used in the protocol. Corrected

Reviewers' comments:
Reviewer #1:
The authors have addressed all of the concerns raised. This manuscript can now be accepted.
We thank the reviewer for their time and their helpful comments.

Reviewer #2:
Manuscript Summary:
In the 2nd version manuscript, the authors addressed most of the concerns. Protocol workflow is provided in the new manuscript that makes it easy to understand. Two control experiments are added in the demonstration to prove the specificity of the method. Advantages of proposed method compared to other alternative methods are highlighted in discussion section. Detailed notes are provided throughout the protocols, and critical steps and limitations are fully discussed in the new manuscript, which enable other researchers to follow and utilize the proposed method for their own research.

Major Concerns:
None
We thank the reviewer for their time and their helpful comments.

Minor Concerns:
However, there remains some minor comments which the authors could consider addressing to increase the convenience of the readers and as well promote the reproducibility of the protocol. We have addressed the remaining concerns as described below.

1. In figure 2A, the authors could consider showing firefly Luc data (3rd graph) in front of firefly/Renilla Luc ratio data (2nd graph). We have altered the figure as suggested.

2. In figure 2C, is there any explanation as to why cells transfected with minimal promoter construct without TEAD binding elements have nearly two-fold higher Renilla Luc signal than cells with TEAD reporter construct? The ratio of TEAD reporter to Renilla is the same as minimal reporter to Renilla and the constructs are not drastically different in size so we would expect a similar number of Renilla vectors to be transfected into the cells in each case. So we do not know why the Renilla signal is higher in the minimal promoter transfected cells, but this is very reproducible. We have observed this across numerous experiments with A375 cells. Interestingly, this seems to be cell line specific since in other cell lines we reproducibly see the opposite (Renilla signal is lower in the minimal promoter transfected cells than the TEAD reporter cells). Though we cannot explain this, we do not feel it influences the interpretation of the results since we do not typically make quantitative comparisons between cells transfected with the TEAD Reporter/Renilla mix and cells transfected with the minimal reporter/Renilla mix. Instead, we use the minimal promoter construct to ensure a candidate that regulates the TEAD reporter does not regulate a promoter that lacks TEAD binding elements.

3. In protocol 6.2.1, the authors do not specify how to exclude 'very low Renilla luciferase signal'. The authors define 'below 1000' as significant reduction in the legend of supplemental table 2, which is bit confusing. In supplemental table 2, it looks like there is still large variance of Renilla signal between groups after excluding the groups with 'below 1000' signal. The authors may consider performing statistical analysis (also related to the 5th point) to exclude groups with significant lower Renilla signal. We chose 1000 as our cutoff because numerous previous experiments in A375 cells showed that Renilla luciferase signal lower than 1000 typically indicates either too few viable cells or poor transfection efficiency. However, we agree this cutoff is arbitrary so we have changed our cutoff to exclude any sample greater than 1 standard deviation below the mean Renilla signal for all wells. Based on this we had to exclude an additional shRNA from our analysis and have altered Supplemental Table 2, Figure 3 and the results section accordingly. We also now suggest cutoff criteria in the Discussion section.

4. In supplemental table 2, there is large variance in terms of the firefly signal between replicates, as you can see in the readout of ERBB4 knock-down and ATR knock-down. Will the authors consider three replicates instead of two? We do not typically see that much variation between technical replicates so are not sure why these samples showed such variation. Three replicates could help and we have suggested it in the Discussion. We also suggest other methods for false positives and negatives (see comment number 5)

5. The authors may consider including biological replicates. Biological replicates are

necessary for statistical analysis. More false positive hits will be identified in the screening without performing statistical analysis, which requires more efforts to exclude in the following validation experiments. In the Discussion, we suggest several modifications to reduce false positives and negatives, including increasing the number of biological replicates (see pages 10 and 11). It should be noted that we elect to use two technical replicates only because doing 2-3 biological replicates would drastically increase (2-3 fold) the size and scale of the assay and potentially introduce additional variability at other steps. Therefor we suggest a smaller library of only the "hits" be re-screened and in this smaller screen 3 biological replicates are included. We discuss this option in the Discussion on pages 10 and 11.

Reviewer #3:

The author have answered my main concerns in the new work. We thank the reviewer for their time and their helpful comments.

Supplemental Table 1

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Existing/Purchased/Gift Vectors	
Vector	**Source**
GIPZ human shRNA lentiviral vectors	GE Healthcare
VSVG	Hynes Lab [2]
psPAX2	Addgene (#12260)
gag/pol	Addgene (#14887)
pGL3-(SV)-49	Iain Farrance [15]
pGL3-5xMCAT(SV)-49	Iain Farrance [15]
PRL-TK	Promega
MSCV-IRES-Hygro	LAMAR LAB [2]
MSCV-YAP-S127A,S381A-IRES-Hygro	LAMAR LAB [2]
MSCV-ZSGreen-2A-Puro-hYAP7/hTAZ3	LAMAR LAB [14]

New Vectors	
Vector	**Source Backbone**
MSCV-YAP-S94A,S127A,S381A-IRES-Hygro	MSCV-IRES-Hygro

Supplemental Table 2

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PlateLayout	1	2	3	4	5	6	7
A	NTC	MDM2-1	Src	MDM2-2	RB1	PIK3CA-1	RAF1-1
B	NTC	MDM2-1	Src	MDM2-2	RB1	PIK3CA-1	RAF1-1
C	NTC	PAK4	EZH2-1	PDK1	RAF1-2	CDH1	MAPK8-1
D	NTC	PAK4	EZH2-1	PDK1	RAF1-2	CDH1	MAPK8-1
E	NTC	ERBB4	PTEN-2	ATR	CSK	ATM	PTEN-1
F	NTC	ERBB4	PTEN-2	ATR	CSK	ATM	PTEN-1
G	EZH2-2	PIK3CA-2	CCNE2	GSN	ERBB2	MAPK8-2	Blank
H	EZH2-2	PIK3CA-2	CCNE2	GSN	ERBB2	MAPK8-2	Blank

Firefly signal

	1	2	3	4	5	6	7
A	14975.75	6678.75	15826.75	1210.75	22491.75	21399.75	57072.75
B	23345.75	8669.75	19191.75	1303.75	22242.75	30358.75	61053.75
C	27356.75	634.75	15310.75	12747.75	19545.75	32499.75	2694.75
D	30642.75	634.75	17174.75	13958.75	21779.75	40147.75	2326.75
E	20655.75	8929.75	19724.75	17518.75	62879.75	22218.75	42731.75
F	27726.75	14291.75	32452.75	32129.75	71657.75	24278.75	49835.75
G	11305.75	25095.75	24581.75	52531.75	25933.75	3212.75	178.75
H	13384.75	26281.75	26686.75	57037.75	26608.75	3218.75	114.75

Renilla signal

	1	2	3	4	5	6	7
A	6481	807	7693	183	7930	9884	6517
B	7222	886	8304	147	8398	10993	6844
C	4941	20	2466	1374	7657	4913	177
D	5195	31	2677	1581	7394	5141	265
E	6481	2434	6431	4632	5636	2436	8125
F	6173	2714	6747	5707	6113	2529	8183
G	1920	8147	5511	7917	2284	338	20
H	1884	8966	5640	9312	2615	314	5

Firefly/Renilla Ratio

	1	2	3	4	5	6	7
A	2.310715939		2.057292		2.836286	2.16509	8.757518797
B	3.232587926		2.311145		2.648577	2.761644	8.920770018
C	5.536682858		6.208739		2.552664	6.615052	
D	5.898508181		6.415671		2.945598	7.809327	
E	3.187123901	3.668755	3.067136	3.782114	11.1568	9.120998	5.259292308
F	4.491616718	5.265936	4.809953	5.629884	11.72219	9.600138	6.090156422
G	5.888411458	3.080367	4.460488	6.63531	11.35453		
H	7.104432059	2.931268	4.731693	6.125188	10.17543		

Fold Change Firefly/Renilla relative to Control Sample Average **Control Sample (NTC) Average**

	1	2	3	4	5	6	7
A	0.562281024		0.500614		0.690171	0.526845	2.131022058
B	0.786605925		0.562385		0.644495	0.672008	2.170747003

C	1.347275818		1.510811		0.621156	1.609682	
D	1.435321046		1.561165		0.716771	1.900293	
E	0.775542878	0.892741	0.746345	0.920325	2.714855	2.21947	1.279776633
F	1.092973309	1.281393	1.170436	1.369955	2.852434	2.336062	1.481956016
G	1.432864147	0.749565	1.085399	1.614612	2.762969		
H	1.728766078	0.713284	1.151393	1.49048	2.476051		

Fold Change

shRNA target	Average	S.D.
NTC	1	0.317874
EZH2-2	1.580815113	0.147951
MDM2-1		
PAK4		
ERBB4	1.087067244	0.194326
PIK3CA-2	0.731424469	0.018141
Src	0.531499677	0.030886
EZH2-1	1.535988416	0.025177
PTEN-2	0.95839069	0.212045
CCNE2	1.118395617	0.032997
MDM2-2		
PDK1		
ATR	1.145140286	0.224815
GSN	1.552546068	0.062066
RB1	0.667333119	0.022838
RAF1-2	0.668963316	0.047807
CSK	2.783644768	0.068789
ERBB2	2.619510413	0.143459
PIK3CA-1	0.599426543	0.072582
CDH1	1.754987359	0.145305
ATM	2.2777658	0.058296
MAPK8-2		
RAF1-1	2.15088453	0.019862
MAPK8-12		
PTEN-1	1.380866325	0.10109
shYAP/TAZ	0.509708718	0.042262

8
shYAP/TAZ
shYAP/TAZ
shYAP/TAZ
shYAP/TAZ
shYAP/TAZ
shYAP/TAZ
Blank
Blank

8
10255.75
12459.75
12177.75
13652.75
9400.75
11719.75
-115.25
-178.25

Renilla mean	4810.383
Renilla SD	2978.072

Number of Standard Deviations above or beow the mean Renilla Signal

8	1	2	3	4	5	6	7
5575	0.560972555	-1.34429	0.967947	-1.55382	1.047529	1.703658	0.573061
5928	0.809791253	-1.31776	1.173114	-1.56591	1.204678	2.076047	0.682863
6238	0.043859472	-1.60855	-0.78722	-1.1539	0.955859	0.034457	-1.55583
5986	0.129149552	-1.60486	-0.71636	-1.08439	0.867547	0.111017	-1.52628
4574	0.560972555	-0.79796	0.544183	-0.0599	0.277232	-0.79729	1.113008
5012	0.457549938	-0.70394	0.650292	0.301073	0.437403	-0.76606	1.132483
-10	-0.970555218	1.120395	0.235258	1.043164	-0.84833	-1.50177	
-15	-0.982643576	1.395405	0.278575	1.511588	-0.73718	-1.50983	

8
1.8395964
2.1018472
1.9521882
2.2807802
2.055258
2.338338

4.1095393
8
0.4476406
0.5114557

0.4750382
0.5549966
0.5001188
0.5690025

8
0.256749
0.375282
0.479376
0.394758
-0.07937
0.0677