Promoter Selection Kit –pZIP lentiviral vectors

TLN0005

Introduction

Successful RNAi using short hairpin RNAs requires not only potent RNA designs, but also sufficient expression of the shRNA and processing to its mature form. Poor knockdown of target gene expression is often the result of low expression of the shRNA, which can be directly attributable to the activity of the promoter controlling its expression. Cell types differ greatly with respect to the activity of any one particular promoter; making it critically important before conducting an RNAi experiments to first evaluate the cell line's promoter activity. This is especially true for pooled shRNA screens, where potency at single-copy representation is required.

The transOMIC Promoter Selection Kit is designed for quick assessment of the relative promoter activity in a cell line of interest. It includes viral particles for five pZIP lentiviral vectors with different promoters (hCMV, mCMV, hEF1a, mEF1a, and SFFV) (Fig. 1). See Appendix B for vector schematic of each or see the **Documents Page** for detailed sequence information.

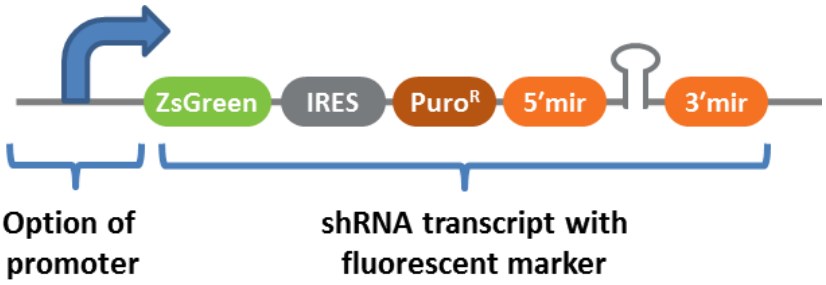


Figure 1. Vector schematic of the ZIP vector. The promoter (hCMV, mCMV, hEF1a, mEF1a, or SFFV) expresses the fluorescent marker and the shRNA on the same transcript.

Each vector in the panel expresses the fluorescent marker and the shRNA from the same transcript (Fig. 1). ZsGreen expression directly correlates with shRNA expression allowing a quick visual assessment of promoter activity using a fluorescent microscope.

Once the optimal promoter is determined shRNA can be transferred to the vector of choice. The workflow is shown in Figure 2.

Basic workflow

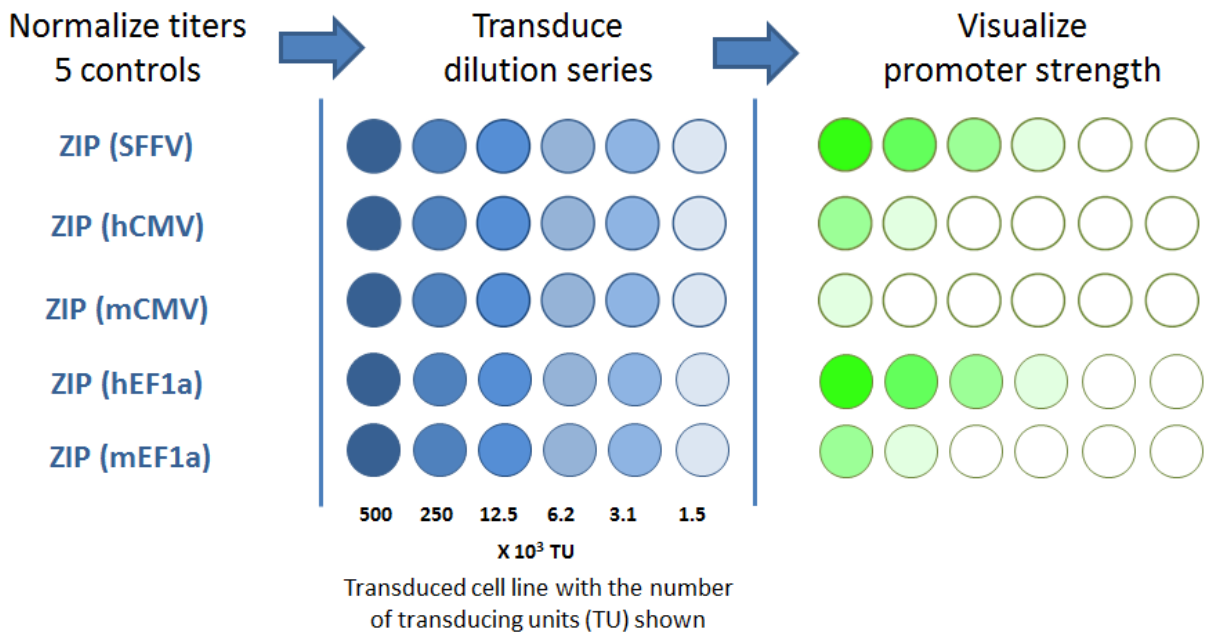


Figure 2. Depiction of the general workflow for promoter selection kit. Normalize viral particles for the five control vectors (Left). In a 96-well plate, transduce cells with equal number of transducing units (TU) from each of the vectors using a two-fold dilution series (Center). Determine the optimal promoter by comparing the fluorescence intensity of each well (Right).

Safety

See Appendix A for safety considerations.

Promoter Selection Assay

A cell line must be efficiently transduced to compare promoter activity. The Promoter Selection Kit is designed to provide a sufficient number of viral particles to transduce most cell lines. If there is insufficient fluorescence to identify an active promoter it may be necessary to optimize transduction. A number of variables may be optimized for increased transduction efficiency including components of the media, duration of transduction, cell type, cell health and plating density. Below is brief description of how to optimize transduction.

- Serum is a known inhibitor of transduction and should be minimized (0 - 2%) in transduction media. For cells sensitive to low serum conditions either reduce the transduction time in low serum media or increase the transduction time in complete media.
- Hexadimethrine bromide (Polybrene) is a cationic lipid known to enhance viral particle binding to the surface of many cells types. A range of concentration (0 - 10 µg/ml) should be tested to determine the highest transduction efficiency that can be achieved without cell toxicity.

- Extending transduction incubation times may increase efficiency. However, it may be necessary to increase the volume of media applied to the cells for transduction to limit the effects of evaporation.
- Cell density may influence transduction efficiency. Plate cells at a range of densities to determine its effect on your cell line. Rapidly dividing cells are often transduced more efficiently.

Transduction

Required Materials

- Experimental cells
- Complete media for experimental cell line
- Serum free media for each cell line
- 96-well tissue culture plate
- 96-well round bottom plate (for viral particle dilutions)
- Lentiviral particles (25 µl/tube; > 1 x 10^8 TU/ml)

  (five different promoters)
  - ZIP (SFFV) -NT#4
  - ZIP (hCMV)-NT#4
  - ZIP (mCMV)-NT#4
  - ZIP (hEF1a)-NT#4
  - ZIP (mEF1a)-NT#4
- Sterile Microcentrifuge tubes
- Polybrene

Equipment

- Automatic pipettor /Pipette-aid (for tissue culture)
- Multichannel Pipettor (for seeding cells, and handling/dilutions of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO2 cell culture incubator at 37°C
- Fluorescent microscope

Protocol

1. Plate your target cells 18-24 hours prior to transduction in a 96 well plate. Seed at a cell density per well so that the cell confluency ranges between 40 and 50% at the time of transduction. Seed cells in all wells of the 96-well plate in 100 µl of complete media. Incubate overnight in the appropriate culture conditions (*e.g.* 5% CO_2 at 37°C).

2. The following day, thaw each of the five viral particles by placing tubes in 37°C water bath and quickly placing on ice. Before use, quick-spin tubes in microcentrifuge to ensure all viral particles are brought to the bottom of the tube.

3. Prepare a round-bottom 96-well plate for viral particle dilution series. There will be six serial dilutions for each virus. Label the plate accordingly (see Figure 3 below, dilutions are depicted in duplicate). Add 100 µl of medium containing 1% serum and 6 µg/ml Polybrene to each well.

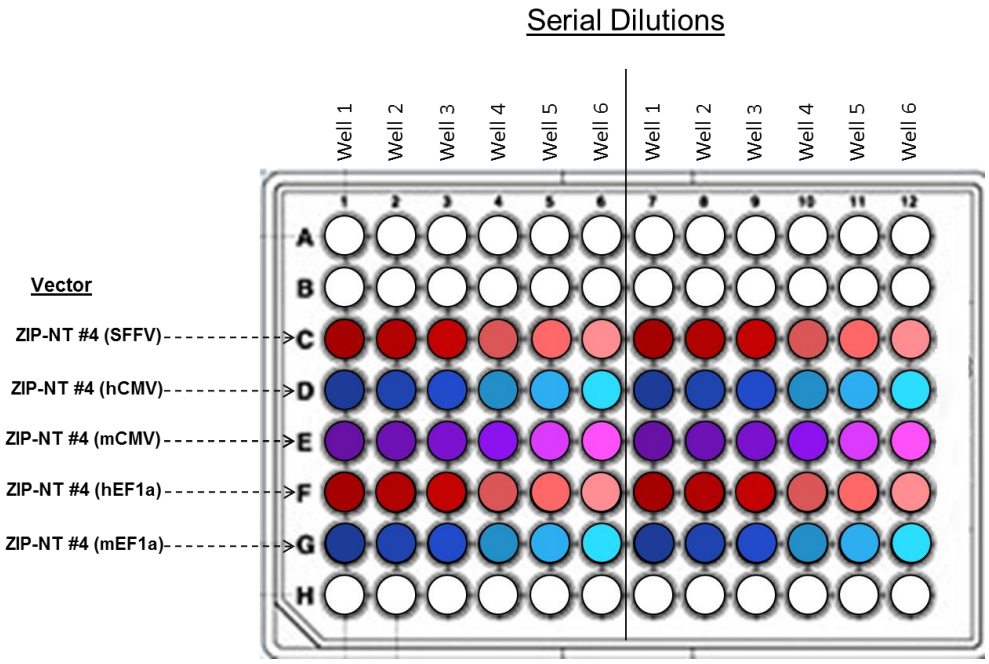


Figure 3. Serial dilution setup. 96-well round bottom plate is used for 2-fold serial dilutions of the five vectors. Dilutions are done in duplicate.

4. Normalize each of the five viral particles to 2 x 10^7 TU/ml. Dilute the viral particles accordingly with medium containing 1% serum and 6 µg/ml Polybrene. Normalization can be performed in the tube that the viral particles are shipped in by diluting the entire 25 µl volume of viral particles. The amount of media for normalization will depend on the titers that are provided in the C of A (Formula and example are provide below).

Normalization formulas

$$Total\ volume\ (\mu l\ ) = \frac{25\ \mu l\ x\ Viral\ Particle\ Concentration\ \frac{TU}{ml}}{(Desired\ Concentration{:}\ 2\ x\ 10^7\ \frac{TU}{ml})}$$

$$Volume\ to\ add\ to\ stock\ tube\ (\mu l\ ) = Total\ volume - 25\ \mu l$$

Example: *If the titer for ZIP-NTC#4 (SFFV) is 1.4 x 10^8 TU/ml, then the calculations will be as follows:*

$$\textbf{\textit{Total volume}}\ (\mu\textbf{\textit{l}}) = \frac{25\ \mu l\ x\ 1.4\ x\ 10^8\ \frac{TU}{ml}}{\left(2\ x\ 10^7\ \frac{TU}{ml}\right)} = \mathbf{175}\ \mu\textbf{\textit{l}}$$

$$\textbf{\textit{Volume to add to stock tube}}(\mu\textbf{\textit{l}}) = 175\ \mu l - 25\mu l = \mathbf{150}\ \mu\textbf{\textit{l}}$$

150 µl of medium containing 1% serum and 6 µg/ml Polybrene will be added *to the 25 µl of viral particles.*

5. Prepare a series of two-fold dilutions with media and viral particles (see Figure 3 below):
   a. Transfer 100 µl of each of the normalized viral particles (Step 4) to the corresponding wells labeled "Well 1". The final concentration of viral particles in Well 1 will be 1 x 10^7 TU/ml.
   b. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
   c. Transfer 100 µl from the first well to the second well. Mix well and discard the tip.
   d. Serially dilute the viral particles by repeating the procedure for the remaining wells.
   e. Allow viral dilutions with Polybrene to incubate for 10 minutes at room temperature.

6. Using multichannel pipettor, remove all of the media from the cells to be transduced. Dispense and discard tips appropriately.

7. Using multichannel pipettor again, transfer 50 µl of viral particles to the corresponding wells. Dispense the viral particles slowly into the wells, being careful not to dislodge the cells. The remaining wells (without viral particles) should be evaluated as negative controls. The total number of transducing units (TU) per well are as follows:

Well	Transducing units
1	500,000 TU
2	250,000 TU
3	125,000 TU
4	62,500 TU
5	31,250 TU
6	15,625 TU

Table 1. Transducing units applied to each well.

8. Rock plate gently a few times to distribute the viral particles across the well.

9. Incubate overnight with 5% CO_2 at 37°C (12-16 hours).

10. Replace the viral supernatant with complete media and allow cells to grow for 48-72 hours. Be careful not to dislodge cells.

11. Visually inspect cultures in each of the wells daily using fluorescence microscopy. It is generally not necessary to quantify fluorescent intensity, as differences in promoter activity are usually easy to distinguish.

12. Identify the optimal promoter from brightest well.

13. Select this promoter when ordering pooled shRNA screening libraries (or other shRNA products).

Note: All available vector options may not be shown on the web. Contact support@transomic.com if the optimal promoter is not shown for a product of interest.

Appendix

Appendix A. *Safety*

Recombinant lentivirus is considered a Biosafety Level 2 organism by the National Institutes of Health and the Center for Disease Control and Prevention. However, local health and safety regulations should be determined for each institution.

For more information on Biosafety Level 2 agents and practices, download Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (Revised December 2009) published by the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. The publication can be found here: http://www.cdc.gov/biosafety/publications/bmbl5/.

If additional measures are needed, review biosafety guidance documents such as the NIH's "Biosafety Considerations for Research with Lentiviral Vectors" which refers to "enhanced BL2 containment". More information can be found through the NIH Office of Biotechnology Activities web site (http://oba.od.nih.gov/rdna_rac/rac_guidance_lentivirus.html)

Summary of Biosafety Level 2 Practices
The following is meant to be a summary of Biosafety Level 2 practices and should not be considered comprehensive. A full account of required practices should be determined for each institute and/or department.

Standard microbiological practices

- Limit access to work area
- Post biohazard warning signs
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Use precautions with sharps (e.g., syringes, blades)
- Review biosafety manual defining any needed waste decontamination or medical surveillance policies

Safety equipment

- Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is not recirculated
- Protective personal equipment includes: protective laboratory coats, gloves, face protection if needed

Facilities

- Autoclave available for waste decontamination
- Chemical disinfectants available for spills

Appendix B. ***Vector maps***

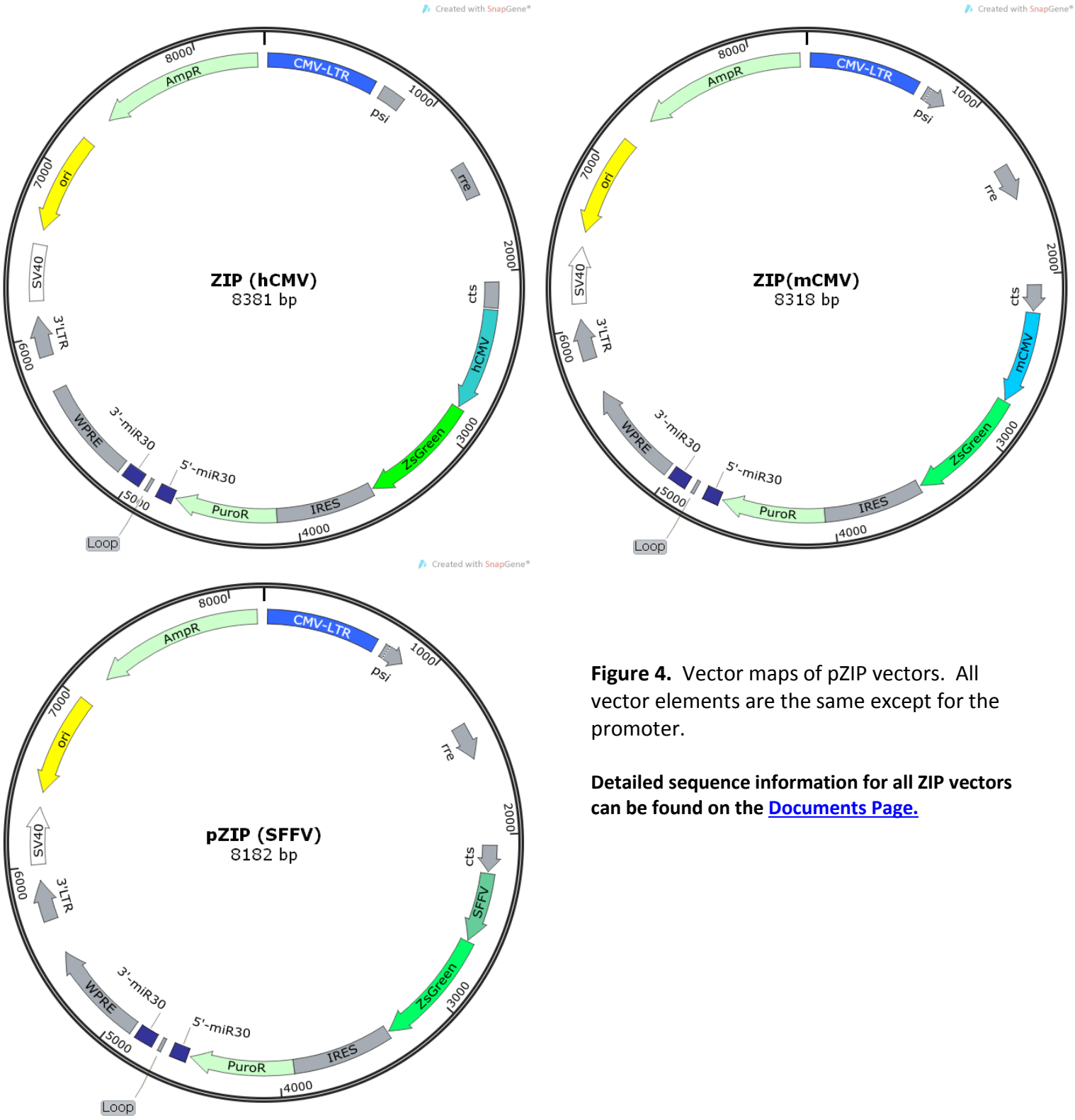
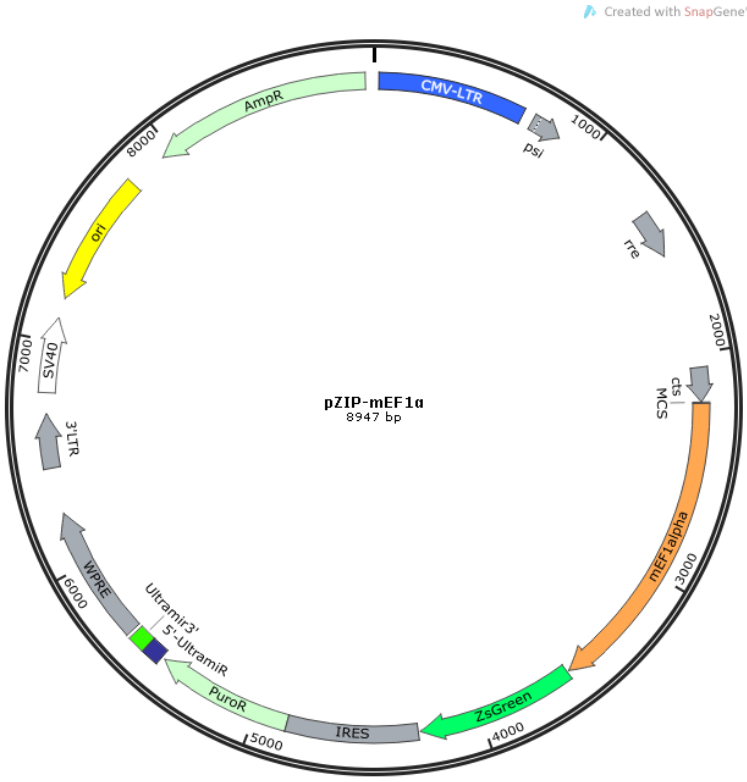
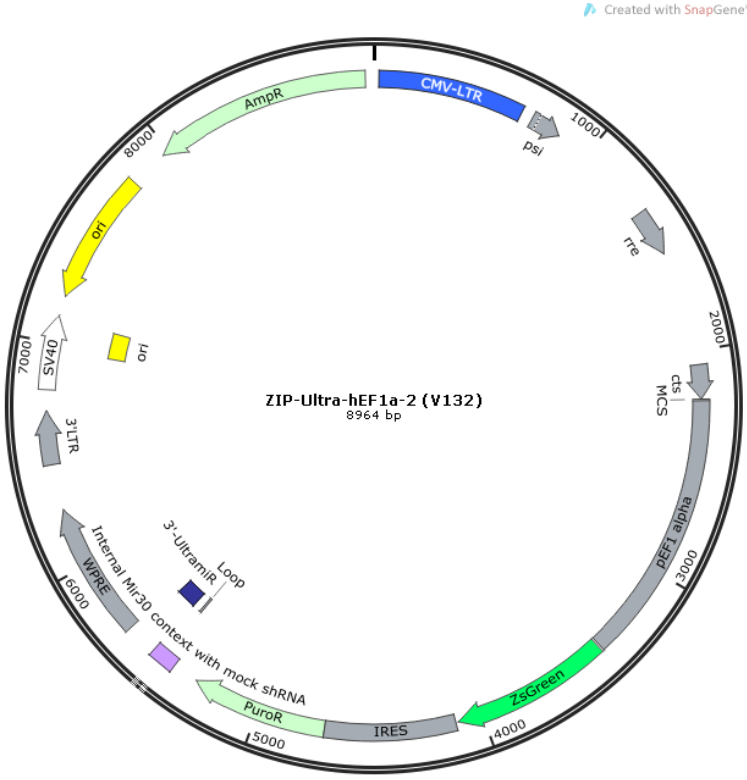


Figure 4. Vector maps of pZIP vectors. All vector elements are the same except for the promoter.

Detailed sequence information for all ZIP vectors can be found on the Documents Page.





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