Chapter 16

Dual-Luciferase Reporter Assay for Prescreening CRISPR (d)Cas9-Mediated Epigenetic Editing on a Plant Promoter Using Human Cells

Ann-Kathrin Hinrichs, Aline Koch, and Antje M. Richter

Abstract

Epigenetic editing, also known as EpiEdit, offers an exciting way to control gene expression without altering the DNA sequence. In this study, we evaluate the application of EpiEdit to plant promoters, specifically the *MLO* (mildew locus o) gene promoter. We use a modified CRISPR-(d)Cas9 system, in which the nuclease-deficient Cas9 (dCas9) is fused to an epigenetic modifier, to experimentally demonstrate the utility of this tool for optimizing epigenetic engineering of a plant promoter prior to in vivo plant epigenome editing. Guide RNAs are used to deliver the dCas9-epigenetic modifier fusion protein to the target gene sequence, where it induces modification of MLO gene expression. We perform preliminary experiments using a plant promoter cloned into the luciferase reporter system, which is transfected into a human system and analyzed using the dual-luciferase reporter assay. The results suggest that this approach may be useful in the early stages of plant epigenome editing, as it can aid in the selection of appropriate modifications to the plant promoter prior to conducting in vivo experiments under plant system conditions. Overall, the results demonstrate the potential of CRISPR (d)Cas9-based EpiEdit for precise and controlled regulation of gene expression.

Key words Epigenetic editing, CRISPR (d)Cas9 System, Dual-luciferase reporter assay, Cell transfection

1 Introduction

The dual-luciferase assay employs two co-reporters, *Renilla* and firefly luciferase, to enable the specific investigation and characterization of the plant promoter of the mildew locus o gene (*MLO*) using epigenetic editing (EpiEdit). In this experimental setup, the *Renilla* luciferase measures the activity of the promotor of interest, while the firefly luciferase serves as a control to normalize for transfection efficiency and cell number variation [1].

Fatemeh Maghuly (ed.), *Plant Functional Genomics: Methods and Protocols, Volume 2*, Methods in Molecular Biology, vol. 2788, https://doi.org/10.1007/978-1-0716-3782-1_16,

To perform the dual-luciferase assay, the plant promoter sequence is cloned in advance into the pRLnull plasmid, which also contains the *Renilla* luciferase gene. In addition, predesigned guide RNAs targeting the *MLO* promoter are cloned into a corresponding U6 promoter-driven vector (px459). A human cell line is then transfected with both plasmids as well as a plasmid containing the firefly luciferase gene, which serves as a transfection control in our study.

At the same time, an epigenetic effector or transcriptional modifier is fused to a nuclease-deficient Cas9 (dCas9) is co-transfected with the *MLO* promoter-luciferase reporters. In the cell line, the modifier fused to dCas9 is delivered to its target sequence on the *MLO* promoter by the guide RNAs. Depending on whether the plant promoter driving luciferase gene expression is activated or inhibited, the cell produces corresponding amounts of luciferase [2]. This assay is optimized for human target promoters earlier [3, 4]. After an appropriate incubation period, typically 24 h (except for time-dependent assays), cells are lysed and luciferase activity is measured using the Dual-Glo® Assay in a luminometer. The Dual-Glo® luciferase assay is advantageous because it provides a highly sensitive and accurate measurement of gene expression and promoter activity, while allowing normalization of the data to correct for experimental variation [5]. In this chapter, we describe our experimental approach using EpiEdit on plant promoters in human cells with the Promega's Dual-Glo® Luciferase Reporter Assay System. It has proven to be a powerful tool for prescreening plant promoters (Fig. 1).

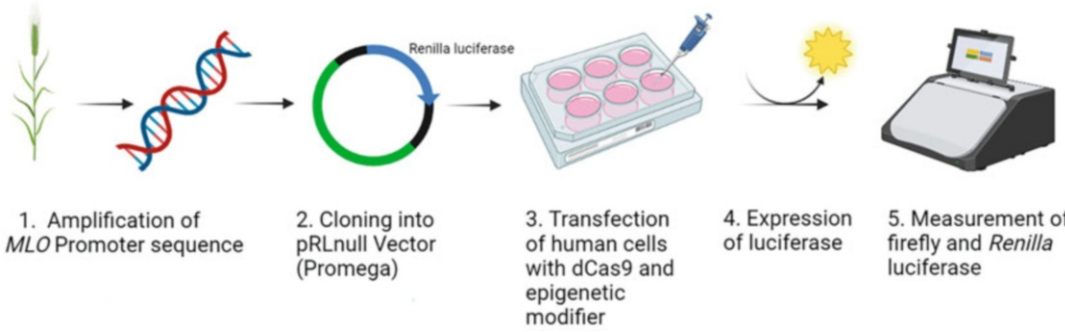

Fig. 1 Overview of the EpiEdit plant promoter prescreening experimental procedure. The MLO promoter sequence was amplified from *Hordeum vulgare* genomic DNA and cloned into the pRLnull vector containing the reporter gene for *Renilla* luciferase. Human HEK cells were transfected with (d)SpCas9 fused to an epigenetic effector, together with the plant promoter sequence (MLO) and with guide RNAs (promoter and SpCas9 specific). 24 h post transfection cells were lysed and lysates were analyzed by luciferase assay. The amount of *Renilla* luciferase is proportional to the promoter activity and firefly luciferase is used as an internal control. (Figure was created with biorender.com)

We first outline the cloning strategy of the *MLO* promoter construct into the pRL null vector and the cloning of guide RNAs into the U6 promoter-driven px459 vector backbone. We then describe the performance of the Dual-Glo® luciferase assay, including a detailed description of the transfection of promoter-luciferase reporter vectors and control luciferase reporter vector into a human cell line.

2 Materials

In the following, we describe the experimental workflow with preparatory cloning of the target promoter and the required EpiEdit constructs, transfection of EpiEdit with the plant promoter in cell culture, and subsequent measurement of the EpiEdit-modified promoter activity using the luciferase assay.

2.1 Plasmid Constructions

1. *MLO* promoter sequence from barley (cv. Ingrid)) for amplification using PCR: 2.5 μL $NH4^+$, 1.75 μL $MgCl_2$, 2 μL dNTP mix (10 mM), 5 μL Betaine (1X), 1 μL Primer forward (10 μM, *AGATCT*AAGACGCCACGCCTGTTTTGCT), 1 μL Primer reverse (10 μM, *GAATTC*GTGTGTCTGGTTTGCTGGCG GA), 0.5 μL *Taq* polymerase, 11.25 μL ddH2O. Add 50 ng of genomic DNA from barley (cv. Ingrid).
2. Agarose, high melting point: for 1% agarose-gel dilute 1 g agarose in 100 mL TBE buffer (1X, tris-borate-EDTA, 10x stock at 89 mM Tris–HCl, 89 mM boric acid, 2 mM EDTA, pH 8,5) and heat up in the microwave until the agarose is completely dissolved. Let agarose solution cool down to about 50 °C, add 2.5 μL ethidium bromide, and pour the agarose into a gel tray with the well comb in place.
3. CloneJET PCR Cloning Kit (Thermo Fisher Scientific): cloning vector pJET1.2 blunt, T4 DNA ligase, 2X reaction buffer, DNA blunting enzyme.
4. DH5a competent cells.
5. pRL *Renilla* luciferase control reporter vector (Promega).
6. SOC medium: Weight 20 g Trypton, 5 g yeast extract, 0.5 g NaCl, 10 mL 250 mM KCl, and transfer to 1 L volumetric flask. Make up to 1 L with ddH2O. Autoclave and add under sterile filtration 5 mL 2 M $MgCl_2$ and 10 mL 2 M Glucose.
7. LB medium: Weight 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose, and transfer to 1 L volumetric flask. Make up to 1 L with ddH_2O, mix, autoclave, and store at 4 °C.
8. LB Plates: Add agar to final concentration of 1.5% (15 g for 1 L LB medium) and the selective antibiotic ampicillin with a working concentration of 60 μg/mL.

9. Ampicillin (stock 50 mg/mL).
10. Restriction enzymes: *Bbs*I, *Bam*HI, *Eco*RI, *Bgl*II.
11. Buffer O (1X): 50 mM Tris–HCl (pH 7.5 at 37 °C), 10 mM MgCl2, 100 mM NaCl, 0.1 mg/mL BSA.
12. Buffer Tango (1X): 33 mM Tris-acetate (pH 7.9 at 37 °C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.
13. Plasmid preparation kit for high and low copy plasmid DNA purification (Macherey and Nagel).
14. NucleoSpin Gel and PCR Clean-up, mini kit for gel extraction and PCR clean up (Macherey and Nagel).
15. pRL *Renilla* luciferase control reporter vector (Promega).
16. px459 pdelSpCas9(BB)-2A-Puro V2.0 (modified Addgene #62988, delCas9, available on request).
17. 5′-Phosphorylation of oligonucleotides: 0.5 μL T4 Polynucleotide Kinase (10 U/μL), 5 μL reaction buffer (10X), 0.5 μL ATP (10 mM), 2 μL Oligo 1 forward (10 μM), 2 μL Oligo 2 reverse (10 μM). Incubate for 1 h at 37 °C.
18. Dephosphorylation reaction for px459 pdelSpCas9(BB)-2A-Puro V2.0: 1 μg px459 pdelSpCas9(BB)-2A-Puro V2.0 (Addgene #62988), 5 μL FastAP thermosensitive alkaline phosphatase (1 U/μL), 5 μL FastAP buffer (10X). Fill up to 50 μL with ddH_2O. Mix thoroughly, spin briefly, and incubate at 37 °C for 10 min. Stop the reaction by heating at 75 °C for 5 min.
19. Ligation of oligonucleotides into the px459 pdelSpCas9(BB)-2A-Puro V2.00 plasmid: 1 μL annealed oligonucleotides, 1 μL (linearized and dephosphorylated) vector, 1 μL T4 DNA Ligase, 1 μL T4 DNA Ligase buffer (10X). Fill up to 10 μL with ddH_2O and incubate overnight at room temperature.
20. pAC154-dual-dCas9VP160-sgExpression (Addgene #48240).
21. DNMT3A-dCas9 (Addgene #100090).
22. pcDNA3/Myc-DNMT3L (Addgene #35523).

2.2 Cell Culture and Transfection

1. Human Embryonic Kidney 293 T (HEK) cells (Cat# CRL-3216; ATCC, RRID: CVCL_0063).
2. Culture medium: 90% Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin/streptomycin (1000 U/mL penicillin, 10,000 μg/mL streptomycin).
3. Phosphate-buffered saline (PBS, 10X): 81.82 g NaCl, 2 g KCl, 14.19 g Na_2HPO_4, 2.45 g KH_2PO_4, pH 7.3.
4. Trypsin (TrypLE Express, Gibco, Thermo Fisher Scientific).

5. Branched polyethylenimine (Sigma-Aldrich, 6.14 g in 1 L H_2O (29.5 mM), adjust pH to 7.14 with HCl. Sterile filter if necessary. Dilute 1:6).
6. Opti-MEM (Gibco, Thermo Fisher Scientific).

2.3 Luciferase Assay

1. Dual-Glo® Luciferase Assay System (Promega).
2. Passive lysis buffer (Promega).
3. Tissue plates.
4. Reaction tubes for preparation of lysates.
5. White 96-well plate for measurement of luciferases.
6. Luminometer (Orion L Microplate Luminometer, Berthold Detection systems).

3 Methods

3.1 Plasmid Constructions

3.1.1 MLO Promoter Sequence Amplification

To clone the *MLO* promoter into the pRLnull vector, specific primers are necessary to amplify the promoter sequence of the *MLO* gene from genomic DNA (barley, cv. Ingrid) by PCR (Table 1). The *MLO* promoter sequence should then be cloned into the pRLnull vector containing the *Renilla* luciferase reporter gene via the *Bgl*II cleavage site. It is important to note that the cloning strategy may vary depending on the specific promoter and should be adapted to the appropriate cleavage sites (*see* **Note 1**).

1. Add 50 ng genomic DNA (barley, cv. Ingrid). Set the PCR conditions according to the *Taq* polymerase used and run the PCR for 50 cycles (endpoint PCR) (*see* **Note 2**).

Table 1
Composition of PCR reaction for a total volume of 25 μL

Volume (in μL)	Reagent
2.5	NH_4^+
1.75	$MgCl_2$
2	dNTP mix (10 mM)
5	Betaine (1X)
1	Primer forward (10 μM)
1	Primer reverse (10 μM)
0.5	*Taq* polymerase
11.25	ddH_2O

2. Run the PCR product (mixed with loading dye) on the 1% agarose gel with the according DNA ladder. Verify expected and correct size under UV light.
3. To set up the reaction for sticky-end cloning in the pJET1.2/blunt cloning vector, follow the manufacturer's instructions (Thermo Fisher Scientific).
   (a) Mix 10 μL reaction buffer (2X), 1 μL PCR product and up to 17 μL nuclease-free H_2O. Add 1 μL DNA blunting enzyme and vortex briefly. Centrifuge for 3–5 s. Incubate the mixture at 70 °C for 5 min. and chill on ice.
   (b) To set up the ligation reaction, keep the reaction mixture on ice and add the following components to the blunting reaction: 1 μL pJET1.2/blunt cloning vector (50 ng/μL) and 1 μL T4 DNA Ligase (*see* **Note 3**).

       Vortex briefly and centrifuge for 3–5 s to collect any droplets. Incubate the ligation mixture for 5 min. at room temperature (22 °C).
4. Transformation of (in-house produced) DH5a competent *E. coli* cells with the ligation mixture (for other cells to be used, see manufacturer's protocol).
   (a) Use 50 μL DH5a competent cells and add 1 μL of ligation product.
   (b) Incubate for 30 min on ice. Place reaction tube in water bath at 42 °C for 1 min (*see* **Note 4**).
   (c) Place the reaction tube back on ice and add 250 μL SOC full medium. SOC full medium will increase the transformation efficiency.
   (d) Place the reaction tube on a shaker at 150 rpm for 1 h at 37 °C for cell generation.
   (e) Transfer an appropriate volume of cells to a selective LB plate containing ampicillin for pJET1.2 selection (60 μg/mL). Incubate overnight at 37 °C.
   (f) Pick one colony with a toothpick/pipette tip into 3 mL LB medium containing ampicillin (60 μg/mL). Allow bacteria to grow on a shaker at 37 °C overnight.
   (g) Perform small-scale plasmid preparation.
5. Confirm the results by digestion with restriction enzyme *Bgl*II for 1 h at 37 °C.
6. Set up the opening reaction of pRLnull vector (10 ng) with *Bgl*II for 3 h at 37 °C (*see* **Note 1**).
7. Run both the *MLO* promoter and the opened pRLnull vector on 1% TBE gel and perform gel extraction.

8. Dephosphorylate the opened pRLnull vector with FastAP thermosensitive alkaline phosphatase for 10 min at 37 °C followed by an inactivation step at 75 °C for 5 min (*see* **Note 5**).
9. Ligation of the *MLO* promoter into the pRLnull luciferase reporter vector.
   (a) Ligate the insert DNA into the vector overnight at room temperature using T4 DNA Ligase. Combine the following in a microfuge tube: 0.1 μg insert, 50 ng pRLnull vector,1 μL T4 DNA Ligase, 1 μL buffer (10X).
   (b) Add 6 μL ddH_2O to make a total volume of 10 μL. (Use a cloning control: set up the above reaction without insert and add ddH_2O instead, no or less clones should grow in comparison with promoter-pRLnull ligation).
10. Transform into DH5a competent *E.coli* cells as above, plate on selective LB overnight, pick according clones in selective LB).
11. Perform small-scale plasmid preparation for isolating plasmid DNA (Macherey and Nagel, for high and low copy plasmid DNA purification).
12. Measurement of DNA concentration occurs in a spectrophotometer by measuring the absorbance at 260 nm (A_{260}).

3.1.2 Oligonucleotides/ RNA Guides for Epigenetic Editing Based on CRISPR-dCas

The guide RNAs should be designed to direct the epigenetic modifier fused to the (d)Cas9 to the *MLO* target gene promoter in EpiEdit. Four guide RNAs (20 nt length) specific for (d)SpCas9 are generated in, e.g., *benchling.com*, each targeting a different position within the target *MLO* promoter (positioned approximately 200 bp apart). The oligonucleotides should be individually cloned into px459 pdelSpCas9(BB)-2A-Puro.

1. Guide RNAs can be generated using *benchling.com* targeting different positions on the *MLO* promoter (Fig. 3a).
2. Set up the reaction for 5′-Phosphorylation of oligonucleotides (*see* **Note 6**).
3. Annealing of oligonucleotides by first heating the reaction tube to 95 °C for 5 min (denaturation) and then cooling the reaction tube by turning off the heating block (renaturation).
4. Digest 10 μg of px459 pdelSpCas9(BB)-2A-Puro) V2.0 with 2.5 μL *Bbs*I restriction enzyme and add 5 μL Buffer G (10X, commercially available through Fermentas). Add ddH_2O to make the total volume 50 μL. Incubate the reaction tube overnight at 37 °C.
5. Run the digested plasmid on the gel. Check for correct size under UV light.

6. Purify the linearized plasmid using gel extraction kit according to the manufacturer's instructions (Macherey and Nagel, NucleoSpin Gel and PCR Clean-up).
7. Set up the dephosphorylation reaction for px459 pdelSpCas9 (BB)-2A-Puro) V2.0 (*see* **Note 5**).
8. Ligation of the oligonucleotides into the px459 pdelSpCas9 (BB)-2A-Puro V2.0 plasmid (*see* **Note 3**).
9. Transform the mixture into competent DH5a cells as described above.
10. Incubate the transformed cells in a suitable medium and perform plasmid preparation of plasmid DNA from the bacterial culture according to the manufacturer's instructions (Macherey and Nagel).
11. Send the purified plasmid DNA for sequencing to confirm successful cloning.

3.1.3 Epigenetic Modifiers and Transcriptional Effectors

In this experimental setup, several epigenetic modifiers will be used to modify gene expression (Fig. 2). These modifiers need to be fused to (d)SpCas, a Cas protein commonly used in CRISPR-based gene editing. To demonstrate activation of the target gene promoter, you can select the CRISPR transcriptional activator from herpesvirus protein 16 (VP16) and choose a modified activator containing ten repeats of the transcriptional activation domain of VP16 [6]. It is recommended to use the pAC154-dual-dCas9VP160-sgExpression plasmid (Addgene #48240), which contains (d)SpCas fused to VP160 (Fig. 3c, e, f).

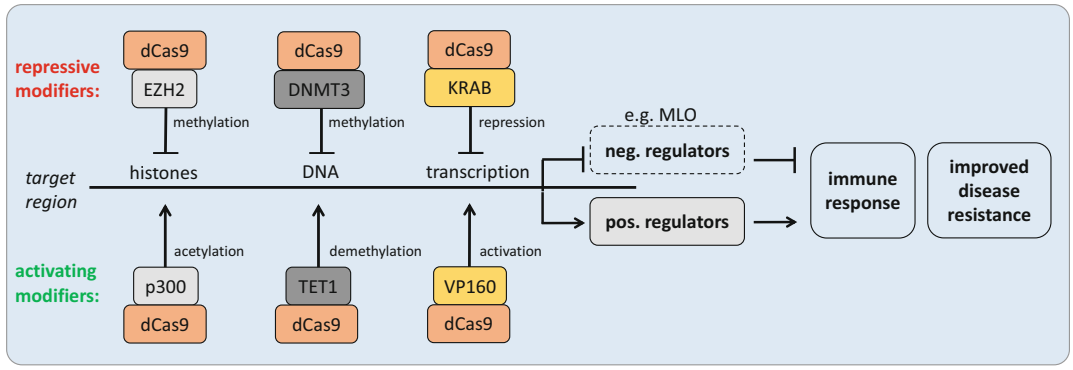

Fig. 2 Epigenetic modification of target genes using CRISPR-dCas. The DNA target region (promoter) is targeted by CRISPR-dCas9-mediated RNA guides specific for the intended site of the promoter of the *MLO* (mildew locus o) gene. These RNA guides are incorporated into the nuclease-deficient (d)Cas9 (orange), which is fused to an epigenetic modifier/transcriptional modifier (EZH2, DNMT3, KRAB, p300, TET1, or VP160) and recruited to the site of action. The modifiers act on the promoter by methylation/acetylation of histones or methylation/demethylation of DNA or by transcriptional regulation. This modification then modulates *MLO* gene expression

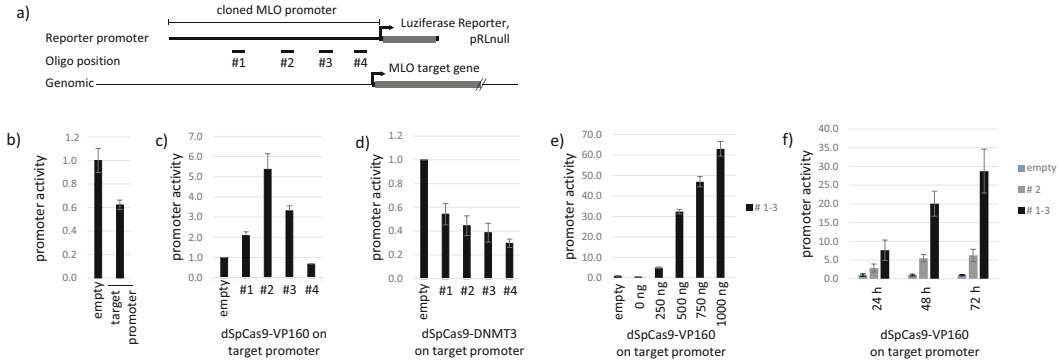

Fig. 3 Prescreening and verification of RNA oligonucleotide function on target promotor activity using EpiEdit. Using both *Renilla* and firefly luciferase co-reporters in the Dual-Glo® luciferase assay, the efficiency of guide RNAs on the *MLO* promoter was investigated. Eukaryotic HEK cells were transfected with the reporter construct, RNA oligos and the dCas9 coupled epigenetic modifier. For the dual-luciferase assay, the *MLO* promoter was examined using *Renilla* luciferase in the pRLnull vector to measure promoter activity, while co-transfection with the pGL 3.1 vector (Promega) allows for normalization of transfection efficiency in the same experimental setup. (**a**) Overview of genomic promoter organization, cloned reporter promoter and relative RNA guide (#) position. The *MLO* promoter was cloned into the pRLnull vector, which contains the *Renilla* luciferase reporter. Four SpCas9-specific guide RNAs (#1–4), each 20 nt in length, were generated, each targeting a different position on the target gene promoter. To prescreen the efficacy of EpiEdit on the target promoter, we transfected HEK cells with EpiEdit components for 24 h and analyzed promoter activity using a Dual-Glo® luciferase assay. (**b**) Basal target promoter activity is shown relative to its empty pRLnull vector control (set to 1). (**c**) EpiEdit viral activator VP160 stimulates *MLO* promoter activity in a guide dependent manner and d) guide-dependent inhibition of promoter activity by EpiEdit inhibitor DNMT3A is shown. (**e**) Determination of the optimal effector dCas dose using EpiEdit activator VP160 and (**f**) determination of time kinetics for EpiEdit using VP160 together with different guides/combinations is shown

In order to induce inhibition on activity of MLO promotor, the repressive epigenetic modifier DNMT3A with DNMT3L should be combined. The activity of the DNA methyltransferase DNMT3A will be stimulated by the use of the catalytically inactive DNMT3L [7]. For repression, you should transfect both, the epigenetic modifier DNMT3a fused to (d)SpCas (Addgene #100090) and the catalytic stimulator DNMT3L from the pcDNA3/Myc-DNMT3L plasmid (Addgene #35523) (Fig. 3d) (*see* **Note 7**).

3.1.4 Transfection of HEK 293 T Cells

To evaluate the activity of EpiEdit in modifying the target gene promoter, a human cell line is transfected with plasmids containing the necessary components. The transfection setup should include the target gene promoter within the *Renilla* luciferase reporter plasmid (pRLnull), the firefly luciferase reporter plasmid (pGL3.1) as a control, and (d)Cas9 fused to the appropriate epigenetic modifier (DNMT3/L, VP160-dCas9). After transfection, RNA guides are expressed and the EpiEdit dCas-modifier complex will be recruited to the target sequence on the promoter construct

encoding *Renilla* luciferase. Twenty-four hours after transfection, cells need to be lysed and the Dual-Glo® Luciferase Assay can be performed to measure promoter activity.

1. Grow HEK 293 T cells in cell culture dishes/flasks using DMEM medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (1000 U/mL penicillin, 10,000 µg/mL streptomycin) (*see* **Note 8**).
2. One day before transfection, plate the cells in a 6-well plate so that they reach 80% confluence on the day of transfection. Add 1 mL of DMEM medium to each well and incubate the cells overnight at 37 °C in a humidified 5% CO_2 incubator. Note that other cell lines and different transfection reagents may require different confluency levels and doubling times for optimal transfection.
3. Prepare plasmids for transfection using a total of 4000 ng per transfection. Mix in a 2 mL tube and vortex briefly. They can be stored overnight at +4 °C. Also prepare a fluorescence control to evaluate the transfection efficiency after transfection under the microscope. Prior to transfection, add 100 µL Opti-MEM to each tube containing plasmids.
4. On the day of transfection, mix 100 µL Opti-MEM and 6 µL polyethylenimin in a separate 2 mL tube. Vortex briefly and spin for 3–5 s (*see* **Note 9**).
5. Gently add the Opti-MEM/ polyethylenimin mix to the 2 mL tube containing the 4 µg plasmid-Opti-MEM mix. Vortex briefly and spin again for 3–5 s. Incubate for 15 min under a laminar flow hood (*see* **Note 10**).
6. Meanwhile, remove the DMEM medium from the 6-well plate and carefully add 600 µL Opti-MEM (*see* **Note 11**).
7. Slowly add the plasmid mix containing polyethylenimin to the cells. Mix gently and place the 6-well plate in a 5% CO_2 incubator at 37 °C for 6 h.
8. After 6 h, remove Opti-MEM from each well and add 1000 µL DMEM medium instead. Incubate the cells overnight at 37 °C in a 5% CO_2 incubator. The total incubation time after transfection should at least be 24 h.
9. Finally, evaluate the transfection efficiency by fluorescence microscopy. Evaluation occurs by calculating the number of GFP-expressing cells divided by the total number of cells. Transfection efficiency should be calculated for each experiment to ensure ideal transfection conditions and comparable plasmid uptake.

3.2 Luciferase Reporter Assay

Prior to performing the Dual-Glo® Luciferase Assay, the reagents for measuring both luciferases must be prepared. To measure firefly luciferase, prepare Dual-Glo® Luciferase Assay Buffer II, which consists of Dual-Glo® Luciferase Buffer II and lyophilized substrate. *Renilla* luciferase is assayed using Dual-Glo® Stop & Glo Reagent and appropriate buffer.

3.2.1 Preparation of Reagents

1. To prepare Dual-Glo® Luciferase Assay Reagent II (LAR II), add 10 mL of Dual-Glo® Luciferase Buffer II to the vial containing the lyophilized Dual-Glo® Luciferase Substrate. Mix well until the substrate is completely dissolved. Divide the reagent into appropriate aliquots and store at −20 °C to prevent repeated freeze-thaw cycles.
2. Calculate the amount of Dual-Glo® Stop & Glo Reagent needed. Transfer a 1:100 ratio of Dual-Glo® Stop & Glo Substrate to an appropriate volume of Dual-Glo® Stop & Glo Buffer in a glass or polypropylene tube (*see* **Note 12**).
3. To prepare 1X passive lysis buffer, add 1 volume of 5X passive lysis buffer to 4 volumes of ddH_2O and mix by inverting the tube.

3.2.2 Luciferase Assay: Measuring Activity of *Firefly and* Renilla *Luciferases*

To perform the Dual-Glo® luciferase reporter assay, the previously transfected cells should be lysed and then be loaded onto a white 96-well plate with LAR II to measure firefly luciferase luminescence as a control. Subsequent addition of Dual-Glo® Stop & Glo Reagent to the same well allows measurement of *Renilla* luciferase promoter activity (*see* **Note13**).

1. Harvest and lyse the transfected cells by removing the DMEM media from each well. Add 200 μL of passive lysis buffer to each well. Place the plate on a shaker for 15 min.
2. Transfer the cell lysate into 1.5 mL reaction tubes and place the reaction tubes on ice (*see* **Note 14**). Centrifuge the non-lysed cell debris for 30 s at 13000 rpm. Return the tubes to ice.
3. Transfer the supernatant to a new 1.5 mL tube.
4. Lysates can be used immediately for luciferase activity assay or stored at −80 °C for later use.
5. Pipet 10 μL lysate into each well of a white 96-well plate and add 33 μL LAR II (*see* **Note 15**). Place the 96-well plate in the luminometer to measure the firefly luminescence and record the light units of firefly luciferase activity.
6. Remove the 96-well plate from the luminometer, immediately add 33 μL of Dual-Glo® Stop & Glo Reagent, and replace the plate in the luminometer to measure *Renilla* luminescence.
7. Record the light in units of *Renilla* luciferase activity.
8. Remove the plate and calculate the ratio of firefly:*Renilla* luminescence for each well. Normalize the ratio of the sample wells

to the ratio of a control well for subtraction of background luminescence (Fig. 3b). Luminescence data for both measurements for each sample should be recorded in a spreadsheet. For each sample, the calculation of the relative firefly:*Renilla* luciferase value is necessary. Then, the relative firefly/*Renilla* luciferase value needs to be compared to the vector control. Therefore, the mean value of experimental sample is divided by the mean value of the vector control.

4 Notes

1. The restriction enzymes are selected based on the selected promoter sequence and vector sequence. Make sure that the restriction sites are not in the promoter sequence, but within the multiple cloning sites of the vector.
2. PCR conditions vary depending on the DNA sequence to be amplified and the polymerase used.
3. Ligation will transfer the DNA fragment (insert) to the vector by connecting the appropriate overhangs by esterifying the 3′hydroxyl ends with the 5′-phosphate ends.
4. It is recommended to put the reaction tube directly from ice into the water bath and from the water bath directly into the ice to obtain best transformation results.
5. Dephosphorylation by thermosensitive alkaline phosphatase removes 5′ and 3′-phosphate groups from DNA.
6. Phosphorylation occurs by T4 Polynucleotide Kinase, which causes transfer of phosphate from ATP to 5′-OH group of DNA.
7. Cloning strategies vary depending on the effectors to be used. For example, other effectors can be used to target DNA methylation if TET1 oxygenases are used to demethylate the artificially methylated target promoter [8] or Krüppel-associated box (KRAB) for transcriptional repression [9].
8. Cultivation and transfection of the human cell line should be performed under a laminar flow hood.
9. It is recommended to prepare a master mix of Opti-MEM and polyethylenimin to ensure that all plasmids receive the same volume of transfection reagent. This will eliminate inter-sample variability in this assay.
10. If multiple plasmids are to be transfected at the same time, it is helpful to dispense the Opti-MEM/polyethylenimin mix into the respective reaction tubes using a reverse pipetting technique to ensure accuracy.
11. Carefully remove the medium from the side of the well, taking care not to disturb the cell lawn with the pipette tip.

12. Prepare the required volume of Stop & Glo Master Mix just prior to use and mix well.
13. The protocol and use of experimental reagents have been minimized and optimized for maximum performance.
14. Cells may not detach properly. In this case, it may be helpful to gently pipette up and down.
15. Avoid air bubbles when pipetting the reagents into the wells as this may affect the luciferase luminescence readings.

Acknowledgments

We thank Prof. Dr. Ralph Panstruga from RWTH Aachen, Germany, for providing *MLO* promoter sequence from *Hordeum vulgare* (cv. Ingrid).

We thank *Prof. Reinhard Dammann*, University Giessen, Institute for Genetics, for his project support with laboratory space and equipment.

This work was supported in the program *PLANT 2030* (project "BarEpiEdit") funded by the *Federal Ministry of Education and Research, Germany (BMBF),* and due to the LGFG-scholarship of the Ministry of Science, Research and the Arts Baden-Württemberg, Germany. Additionally, we are thankful for the invitation for contributing our method in the lab protocol series *Methods in Molecular Biology published by Springer Nature.*

References

1. Yun C, DasGupta R (2014) Luciferase reporter assay in Drosophila and mammalian tissue culture cells. Curr Protoc Chem Biol 6:7–23
2. Richter AM, Küster MM, Woods ML, Walesch SK, Gökyildirim MY et al (2019) RASSF10 is a TGFβ-target that regulates ASPP2 and E-cadherin expression and acts as tumor suppressor that is epigenetically downregulated in advanced cancer. Cancers 11(12):1976
3. Deutschmeyer V, Breuer J, Walesch SK et al (2019) Epigenetic therapy of novel tumour suppressor ZAR1 and its cancer biomarker function. Clin Epigenet 11:182
4. Nilles J, Weiss J, Haefeli WE et al (2022) How to avoid misinterpretation of dual reporter gene assay data affected by cell damage. Arch Toxicol 96:2501–2510
5. Hart RC, Stempel KE, Boyer PD, Cormier MJ (1978) Mechanism of the enzyme-catalyzed bioluminscent oxidation of coelenterate-type luciferin. Biochem Biophys Res Commun 81:980–986
6. Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW et al (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Res 23:1163–1171
7. Siddique AN, Nunna S, Rajavelu A, Zhang Y, Jurkowska RZ, Reinhardt R et al (2013) Targeted methylation and gene silencing of VEGF-A in human cells by using a designed Dnmt3a-Dnmt3L single-chain fusion protein with increased DNA methylation activity. J Mol Biol 425:479–491
8. Morita S, Noguchi H, Horii T, Nakabayashi K, Kimura M, Okamura K, Sakai A et al (2016) Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions. Nat Biotechnol 34(10): 1060–1065
9. Amabile A, Migliara A, Capasso P, Biffi M, Cittaro D, Naldini L, Lombardo A (2016) Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. Cell 167(1):219–232