pMIR-REPORT™ System

miRNA Expression Reporter Vector
Part Number AM5795

Applied Biosystems

A. Product Description

The pMIR-REPORT™ miRNA Expression Reporter Vector System consists of an experimental firefly luciferase reporter vector and an associated β-gal reporter control plasmid. By inserting predicted miRNA target sequences in the multiple cloning site, pMIR-REPORT Luciferase miRNA Expression Reporter Vector can be used to conduct accurate, quantitative evaluations of miRNA function. It can also be used to evaluate siRNA target sites and to analyze the influence of 3' UTR sequences on gene expression. pMIR-REPORT Luciferase contains a firefly luciferase reporter gene under the control of a CMV promoter/termination system (Figure 1). The 3' UTR of the luciferase gene contains a multiple cloning site for insertion of predicted miRNA binding targets or other nucleotide sequences. By cloning a predicted miRNA target sequence into pMIR-REPORT, the luciferase reporter is subjected to regulation that mimics the miRNA target.

pMIR-REPORT β-gal is a beta-galactosidase reporter plasmid that is designed for transfection normalization (Figure 2). β-gal expression from this control plasmid can be used to normalize variability due to differences in cell viability and transfection efficiency.

As an additional feature, pMIR-REPORT Luciferase and pMIR-REPORT β-gal include puromycin and neomycin resistance genes, respectively, to enable antibiotic selection of transfected mammalian cells. Antibiotic selection can be used to generate stable cell lines that express pMIR-REPORT if this is required for your research. For most applications, transient expression is sufficient, and antibiotic selection is not needed.

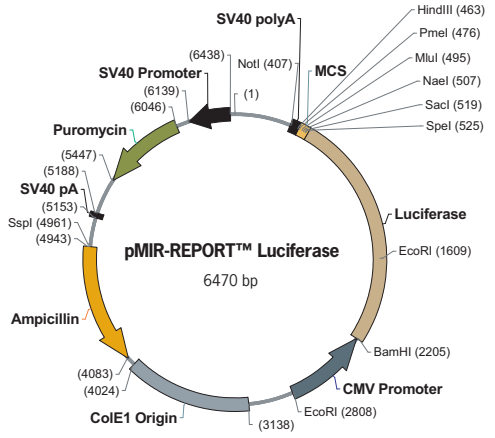

Figure 1. pMIR-REPORT Luciferase

CMV Promoter: 2210–2813
Firefly luciferase: 540–2210
MCS: 467–539
SV40 Poly(A): 404–467
SV40 Promoter: 6139–6438
Puromycin: 5447–6046
SV40 pA signal: 5153–5188
Ampicillin: 4083–4943
ColE1 Origin: 3138–4024

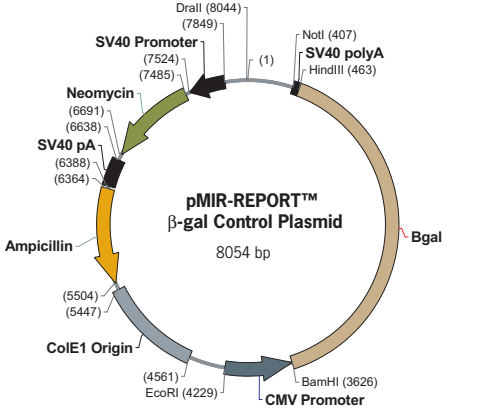

Figure 2. pMIR-REPORT β-gal Control Plasmid

CMV Promoter: 3627–4232
β-gal: 463–3627
SV40 Poly(A): 404–463
SV40 Promoter: 7524–7849
Neomycin: 6691–7485
SV40 pA signal: 6388–6638
Ampicillin: 5504–6364
ColE1 Origin: 4561–5447

B. Components Supplied With pMIR-REPORT

Amount	Component	Storage
500 µL	pMIR-REPORT Luciferase in *E. coli*	–70°C
500 µL	pMIR-REPORT β-gal Control Plasmid in *E. coli*	–70°C

The pMIR-REPORT plasmids are supplied as *E. coli* glycerol stocks which can be used directly to inoculate overnight cultures for plasmid growth. Links to vector maps, sequences, and licensing terms and conditions are available at:

www.ambion.com/catalog/CatNum.php?5795

C. Required Material Not Supplied

Bacterial culture reagents

The pMIR-REPORT plasmids are supplied as *E. coli* glycerol stock cultures; you must grow them up using routine laboratory procedures. Luria Broth (LB) plates and liquid LB media containing ampicillin (100 µg/mL) or carbenicillin (100 µg/mL) are needed to propagate the plasmids.

Plasmid purification and restriction enzyme digestion

After expanding the pMIR-REPORT plasmids in *E. coli*, you will need to purify the plasmid and digest it with the restriction enzymes of or flanking your insert(s). We typically clone inserts between the *Spe* I and *Hin*d III sites of the MCS if possible.

Insert(s) to be cloned

Inserts can consist of annealed synthetic oligonucleotides or gel purified PCR fragments. To facilitate directional cloning into pMIR-REPORT, we recommend designing insert DNA with flanking restriction nuclease sites. See section E on page 5 for information of insert design, and see section F.3 on page 7 for information on annealing and purifying insert DNA.

Ligation and transformation reagents

DNA ligase, ligase reaction buffer and competent *E. coli* cells are needed to clone inserts and propagate experimental constructs. *E. coli* to grow the pMIR-REPORT miRNA Expression Reporter Vector System plasmids

Mammalian cell transfection reagents, and cell culture facility and supplies

The optimal mammalian cell transfection conditions including transfection agent and pMIR-REPORT plasmid amount must be determined empirically. Routine cell culture equipment and supplies are needed for mammalian cell culture.

Luciferase and β-gal assay supplies and equipment

Luciferase and β-galactosidase assays are used to evaluate experiments with the pMIR-REPORT miRNA Expression Reporter Vector System.

D. Related Products Available from Applied Biosystems

T4 DNA ligase

P/N AM2134

T4 DNA Ligase (E.C. 6.5.1.1) catalyzes the formation of phosphodiester bonds between adjacent 3' hydroxyl and 5' phosphate groups in double-stranded DNA. T4 DNA ligase will join both blunt-ended and cohesive-ended DNA and can also be used to repair nicks in duplex DNA. Includes 10X Ligase Reaction Buffer.

siPORT™ *XP-1* Transfection Agent

P/N AM4507

siPORT *XP-1* is an easy-to-use transfection reagent that efficiently delivers both plasmid DNA and PCR products into a variety of mammalian cell types. Comprised of a proprietary formulation of polyamines, siPORT *XP-1* exhibits low toxicity and can be used either in the presence or absence of serum.

Pre-miR™ miRNA Precursor Molecules

P/N AM17100, AM17101, AM17103

*mir*Vana™ Pre-miR™ miRNA Precursors are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNA molecules. These ready-to-use miRNA mimics can be introduced into cells using transfection or electroporation parameters identical to those used for siRNAs and enable detailed study of miRNA biological effects via gain-of-function experiments. Pre-miR miRNA Precursors are available for all miRNAs listed in the miRBase database and custom design is available.

Anti-miR™ miRNA Inhibitors

P/N AM17000, AM17001

*mir*Vana™ Anti-miR™ miRNA Inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous microRNA (miRNA) molecules. These ready-to-use inhibitors can be introduced into cells using transfection or electroporation parameters similar to those used for siRNAs, and enable miRNA functional analysis by down-regulation of miRNA activity.

E. pMIR-REPORT Applications and Insert Design

The primary application of pMIR-REPORT is to analyze miRNA function on predicted miRNA binding sites

Synthesize insert chemically

For analysis of predicted miRNA binding sites, synthesize complementary 50–60 mer DNA oligonucleotides consisting of the test sequence flanked by single-stranded overhangs encoding restriction enzyme sites in the MCS.

For internal testing and evaluation, we cloned inserts into *Spe* I and *Hin*d III sites of the MCS. We also added a unique restriction enzyme site into the inserts to facilitate screening for positive clones.

Amplify insert using PCR

Alternatively, predicted miRNA binding sites can be amplified from genomic DNA or cDNA. To facilitate directional cloning, we recommend using primers with 5' restriction enzyme sites. Sometimes more than one round of PCR is needed to amplify enough product for ligation.

A secondary application is testing for effective siRNA target sites

Very similar to analyzing predicted miRNA binding sites, pMIR-REPORT Luciferase can be used to test predicted siRNA binding sites. Using the same strategies as described in the section above, prepare insert that corresponds to the putative siRNA binding site that you want to analyze.

Third, pMIR-REPORT can be used to analyze the effects of 3' UTR sequences on gene expression

3' UTR regulatory sequences have been shown to be important for mRNA stability, translation, and transport. Using pMIR-REPORT Luciferase, these sequences can be cloned into the 3' UTR of the luciferase reporter gene to analyze their effect on gene expression.

F. Using pMIR-REPORT miRNA Expression Reporter Vector

1. Grow *E. coli* from the glycerol stocks provided, and isolate plasmid DNA

a. Prepare two flasks containing ~100 mL of LB containing 100 µg/mL ampicillin or carbenicillin.

b. Inoculate each flask with ~50–100 µL of one of the supplied glycerol stocks, and incubate overnight in a shaking 37°C incubator.

Alternatively, you can follow the classical method of streaking out the glycerol stock and inoculating the flask with a single colony.

c. Purify plasmid DNA from each culture. We routinely purify plasmids using commercially available plasmid purification products.

2. Linearize pMIR-REPORT Luciferase with the restriction enzymes that will be used for cloning

Linearize purified pMIR-REPORT Luciferase plasmid with the restriction enzymes represented on your insert.

We typically retrieve vector from the restriction digestion by agarose gel electrophoresis and purification of the band corresponding to digested pMIR-REPORT Luciferase. Alternatively, you can prepare vector according to routine subcloning procedures used in your lab.

3. Prepare inserts for ligation

PCR-generated inserts

We recommend gel purifying PCR products to prepare them for ligation into pMIR-REPORT Luciferase.

Synthetic oligonucleotide inserts

Anneal synthetic oligonucleotides as described below to prepare them for ligation into pMIR-REPORT Luciferase.

a. Prepare 1X DNA annealing buffer

Table 1. 1X DNA Annealing Buffer

Concentration	Component
30 mM	HEPES pH 7.4
100 mM	Potassium Acetate
2 mM	Magnesium Acetate

b. Assemble the annealing mixture in a nuclease-free tube as follows:

Amount	Component
2 µL	sense oligonucleotide (1 µg/µL)
2 µL	antisense oligonucleotide (1 µg/µL)
46 µL	1X DNA annealing buffer

c. Heat the annealing mixture to 90°C for 3 min, then place in a 37°C incubator for 1 hr.

d. The annealed insert can either be ligated into pMIR-REPORT Luciferase immediately, or it can be stored at –20°C for at least 1 year.

4. Ligate insert into pMIR-REPORT Luciferase

We suggest ligating a 3–10 fold molar excess of insert into prepared pMIR-REPORT Luciferase. It is also a good idea to include a no-insert ligation negative control ligation in the experiment.

5. Transform *E. coli* with the ligation products

a. Transform *E. coli* with the ligation products.

b. Plate the transformed cells on LB plates containing 100 µg/mL ampicillin or carbenicillin and grow overnight at 37°C. Plate 2–3 different amounts of transformed cells so that at least one of the plates will have distinct colonies. Also include a non-transformed competent cell control.

c. Examine each plate and evaluate the number of colonies promptly after overnight growth at 37°C.

6. Expected results

Non-transformed control culture:

The non-transformed control culture should yield no colonies (indicating that the antibiotic in the culture medium effectively inhibits growth of *E. coli* that do not contain the pMIR-REPORT plasmid).

Plus- and minus-insert ligation transformations

Identify the dilution of plus- and minus-insert ligation transformations that yield well-spaced (countable) colonies, and count the colonies on those plates. The minus-insert ligation may yield some ampicillin resistant colonies (background), but ***the plus-insert ligation should yield 2–10 fold more colonies than the minus-insert ligation.*** (Remember to take the dilution into account when calculating the proportion of background colonies.)

7. Identify clones with the insert

Pick clones, isolate plasmid DNA, and sequence through the promoter/insert to confirm there are no unwanted mutations. We suggest using the following sequencing primers:

1. Forward 5'–AGGCGATTAAGTTGGGTA–3'
2. Forward 5'–CTCGGGTGTAATCAGAAT–3'
3. Forward 5'–GAGGTAGATGAGATGTGA–3'
4. Forward 5'–CACCGTACACGCCTACCG–3'
5. Reverse 5'–ATTGCAACGATTTAGGTG–3'

Complete sequence information for pMIR-REPORT Luciferase is available on our website at the following address:
www.ambion.com/catalog/CatNum.php?5795

8. Purify plasmid for transfections

pMIR-REPORT plasmid preparations must be free of salts, proteins, and other contaminants for efficient transfection. We routinely purify using commercially available plasmid purification products.

G. Transfecting pMIR-REPORT into Mammalian Cells

Include appropriate controls

In order to get meaningful results from your experiment, it is important to include appropriate positive and negative controls when possible. The pMIR-REPORT β-gal Control Plasmid provides a means for normalizing variability due to transfection differences. You should also plan to include a non-transfected cell control, and cells transfected individually with pMIR-REPORT Luciferase, pMIR-REPORT β-gal, and pMIR-REPORT Luciferase with no insert.

Transfection suggestions

Aside from controls, cotransfect equal amounts of the purified pMIR-REPORT plasmids. The optimal conditions for transfecting plasmid DNA into cells is highly dependent on the identity and condition of the DNA and on the cells. To identify the optimal transfection procedure for your cells and DNA, you will need to test different amounts of transfection agent, DNA, and cells. We recommend using Ambion siPORT™ *XP-1* transfection agent (P/N AM4507) to deliver plasmids into mammalian cells with high efficiency and minimal toxicity. Follow the instructions for using siPORT *XP-1* provided with the product.

Evaluate firefly luciferase and β-gal expression 24–48 hr after transfection

Assay samples for firefly luciferase and β-galactosidase expression 24–48 hr after transfection. Luciferase expression may be affected by the sequence cloned into the MCS of pMIR-REPORT Luciferase (located in the 3' UTR of the luciferase gene), whereas β-gal expression from pMIR-REPORT

β-gal provides an internal control that serves as the baseline response. Normalizing luciferase expression according to β-gal expression minimizes experimental variability.

H. Quality Control

Functional Testing

Both plasmids must give bands of the correct size on an agarose gel when analyzed by restriction digestion. Key regions of the plasmids undergo sequence analysis; this includes the MCS of pMIR-REPORT Luciferase.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity

Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

I. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.

- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

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