BBF RFC 72: miTuner - a kit for microRNA based gene expression tuning in mammalian cells

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1   Purpose

The purpose of this RFC is to introduce a modular expression tuning kit for use in mammalian cells. The kit enables the regulation of the gene expression of any gene of interest (GOI) based on synthetic microRNAs, endogenous microRNAs or a combination of both.

2   Relation to other RFCs

All parts provided in the miTuner Kit are constructed in BB-2 standard (RFC 12, RFC 45). Some parts (microRNA binding site patterns) were constructed by the RA-PCR method described in RFC 42. RFC 73 provides complementary measurement instructions for the applications described in this RFC.

3   Copyright Notice

4   Rationale

MicroRNAs (miRNAs) are small, single stranded RNA molecules that regulate gene expression by binding to target sites, preferably in the 3' UTR of a gene (Fig. 1).[1] Exploiting this property of miRNAs, we introduce the miTuner Kit, enabling the user to control gene expression of any GOI according to the following three modes:

Figure 1: Interaction of an shRNA-miR (synthetic microRNA) with its' binding site in the 3' UTR of an mRNA

1) Tuning: adjusting the co-expression of the GOI by expressing a synthetic miRNA in the target cell/tissue and a binding site for the synthetic miRNA in the 3' UTR of the GOI (Fig. 2a)

2) Off-targeting: switching OFF the expression of the GOI in case a certain endogenous miRNA is present in the target cell/tissue (Fig. 2b)

3) On-Targeting: switching ON the expression of the GOI in case a certain endogenous miRNA is present in the target cell/tissue (Fig. 2c)
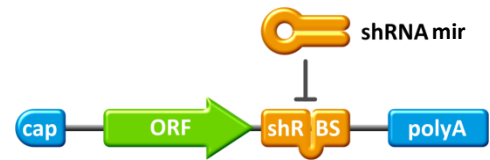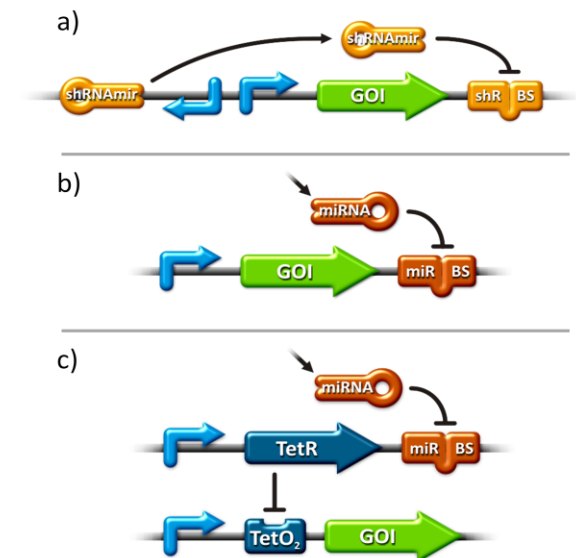

Figure 2: Different regulation modes enabled with the miTuner kit: a) expression tuning based on a synthetic miRNA (shRNAmir) expressed in the target cell b) Off-targeting based on specific endogenous miRNAs c) On-targeting based on a tetR feedback loop coupled to endogenous off-targeting

5   Introduction and Application of the miTuner Kit

The miTuner Kit consists of three basic components:

a) a modular dual-luciferase measurement and expression construct

b) synthetic miRNAs designed to have different, orthogonal target sites

c) synthetic miRNA binding sites for either synthetic or endogenous microRNAs
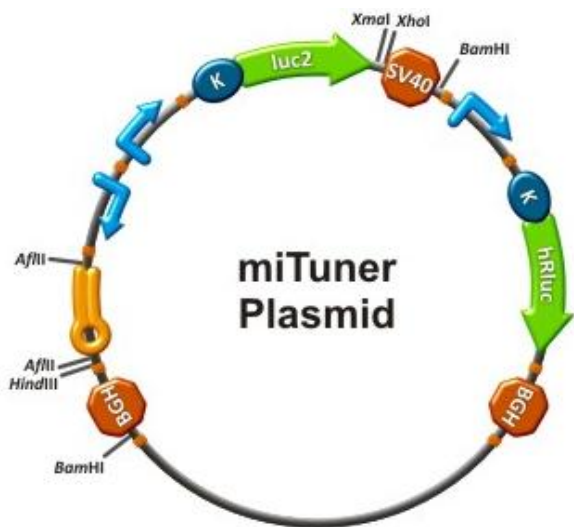

Figure 3: Basic scheme of the miTuner plasmid.

All basic parts (promoters, miRNAs, miRNA-binding sites, terminators, firefly luciferase *luc2*, *Renilla* luciferase *hRluc*) are available in the registry (parts BBa_K337000 - BBa_K337051). Many composite parts (containing different combinations of basic parts) and construction intermediates are also available, enabling maximum flexibility of the constructs and easy combination with other parts in the registry (i.e. other cDNAs) that the user would like to regulate. Here, we introduce the miTuner standard tuning construct (Fig. 3) enabling precise gene regulation based on synthetic miRNAs. The miTuner construct can be applied by the user for his or her individual purpose by exchanging parts of choice.

5.1   Tuning Mode

The dual-luciferase miTuner construct is the basic measurement and expression construct (Fig. 3). It contains three expression cassettes: the synthetic miRNA expression cassette in reverse complementary orientation for tuning of gene expression, as well as a measurement firefly luciferase and a reference *Renilla* luciferase in forward direction. *Hin*dIII and *Afl*II sites allow for the exchange of the synthetic miRNA, *Xma*I and *Xho*I sites allow for introducing synthetic miRNA binding sites into the 3' UTR of the firefly luciferase gene. *BamH*I sites enable

an easy cloning of the microRNA_cDNA (*luc2*) fragment into a pTRUF3 single stranded AAV context for production of AAVs and efficient infection of target cells with the miTuner construct. As synthetic miRNA we define the adaption of the guiding strand and passenger strand sequence of hsa-mir122 to a target sequence by applying a fusion PCR based protocol (find method below). Synthetic miRNAs MAY be expressed from Pol-II promoters, such as RSV, CMV or SV40. Alternatively, shRNAs constructs (we refer to as shRNA-mir) driven from an H1 or U6 Pol-III promoter MAY also be used but may behave differently. The synthetic miRNAs / shRNA-mirs are finally chosen for down-regulating the expression of the GOI *luc2*. Furthermore, the synthetic miRNA or shRNA-mir expression cassette MAY also be located on a separate plasmid in order to enable further adjustment of miRNA expression strength via co-transfection (or infection) with the miTuner construct.

The synthetic miRNA binding sites are introduced into the 3' UTR of the GOI. Thereby an internal knockdown loop is created via the synthetic miRNA and its corresponding target binding site in the 3' UTR of *luc2*, enabling down-regulation of the reporter gene expression.

5.2   Synthesis of artificial miRNAs

The miRNAs we propose here MAY be used in the miTuner construct, but can also be applied in other contexts. If miRNAs with costumized targets are required, online tools, such as siRNA Wizard v3.1 SHOULD be applied.[2] siRNA Wizard allows the creation of a list of siRNAs that can down-regulate your gene of interest without the problem of off-targeting within the organism you selected.

For our purposes the *lac-Z* gene from the *lac* operon (E. coli) was used in order to create a list of siRNAs that were then adapted to the hsa-miR122 sequence. Default settings were used and the above mentioned siRNA did not have off targets either in humans and mice.

MicroRNAs SHOULD be constructed using the hsa-mir122 template (Part Nr. BBa_K337016), in order to bring the synthetic miRNA into an appropriate context and enable driving the expression with normal Pol-II promoters. Customized guiding and passenger strand can be introduced via the following miR fusion PCR protocol (shown by the example of a synthetic miRNA).

Desired customized miRNA sequence (guiding strand, loop, passenger strand):

5'…GGAGGTGAAGTTAACACCTTCGTGGCTACAGAGTTTCCTTAGCAGAGCTGGACA
CCACGGCCACCGATATTATGTCTAAACTATTAATATCGGTGACCGTGGTACCAGCTA
CTGCTAGGCAATCCTTCCCTCGATAAATGTCTTGGCATCGTTTGCTT …3'

Oligos needed:

miRNA_fusion_fw:

ATTATGTCTAAACTATTAATATCGGTGACCGTGGTACCTAGCTACTGCTAGGC

miRNA_fusion_rev:

ATTAATAGTTTAGACATAATATCGGTGGCCGTGGTGTCCAGCTCTGCTAAGG

miRNA_AflII_fw: ttttctgcagcggccgcgcgctagccttaagTGGAGGTGAAGTTAACACCTTCGTG

miRNA_HindIII_rev:

ttttGAATTCGCGGCCGCACTAGTaagcttAAGCAAACGATGCCAAGACATTTATCG

1) Two separate PCR reactions MUST be performed, using 50 ng of part BBa_K337016 as template with primer pairs miRNA_fusion_fw/miRNA_HindIII_rev and miRNA_fusion_rev/miRNA_AflII_fw. PCR SHOULD be performed in a total volume of 50 µl, using Phusion HF (high fidelity) PCR Mastermix. Touchdown PCR SHOULD be performed according to the following protocol:

95 °C/5 min

---------------------------- **1x**

95 °C/ 30 s

68 °C (- 0.5 °C/cycle)/ 30 s

72 °C/ 15 s

---------------------------- **16x**

95 °C/ 30 s

60 °C/ 30 s

72 °C/ 15 s

--------------------------- **19x**

72 °C/ 5 min

--------------------------- **1x**

4 °C/ forever

Alternatively, a PCR protocol with constant annealing temperature at 60 °C over thirty cycles MAY also be applied.

2) A nucleotide removal kit SHOULD be applied for purifying the PCR products (~ 100 bp in size). 3-5 µl of the first PCR reaction MAY be analyzed on a 2 % agarose gel. 50 ng of each first PCR product SHOULD be applied in a second PCR reaction with the primers miRNA_fusion_fw and miRNA_fusion_rev for obtaining the whole miRNA sequence (200 bp band).

3) The PCR product SHOULD be purified by applying a PCR purification kit and be analyzed on a 1.5 % agarose gel. The purified product SHOULD be digested with *Hin*dIII first and subsequently with *Afl*II and cloned into the destination miTuner plasmid (BBa_K337036) precut with the same enzymes.

4) Selection of the synthetic miRNA via colony PCR MAY be performed by using primers miRNA_fusion_rev and standard primer VF2 at 60 °C annealing temperature.

One of the following synthetic miRNAs MAY be used:

miR-hAAT (targets human hAAT): CCTTAGCAGAGCTG TGTTAAACATGCCTAAACGCTT TGTCTAAACTAT AAGCGTTTAGGAATGTTTACAA TAGCTACTGCTAGGC

miR-sAg (no human target): CCTTAGCAGAGCTG ACAAATGGCACTAGTAAACTGA TGTCTAAACTAT TCAGTTTACTAATGCCATTAAT TAGCTACTGCTAGGC

5.3   Designing synthetic miRNA binding sites

Synthetic miRNA binding sites can be constructed by taking the reverse complementary of the guiding strand of the according miRNA that shall target the binding site specifically. Therefore, binding sites SHOULD be 21-23 nucleotides long, having nucleotides 2-7 at the 5' UTR of the miRNA as seed region and the remaining ones as proximate region. The seed region SHOULD offer a perfect target for the miRNA, whereas the proximate region MAY contain mismatch sites for creating bulges and thereby weakening the binding site strength which influences the resulting GOI knockdown percentage.

We propose an automatic designing tool for engineering miRNA binding sites of certain strength for any desired synthetic or endogenous miRNA in a customized user context on an oligo basis.[3]

5.4   Tuning Mode Operation

Part BBa_K337036 SHOULD be used in a test experiment in order to estimate the knockdown percentage with the desired binding sites. Depending on the application, the miTuner construct SHOULD be used for expression of the synthetic miRNA or a second plasmid such as pBS_U6 or pBS_H1 (Fig. 4) MAY be used for co-expression of either the required synthetic miRNA or shRNA-mir. A standard dual-luciferase assay SHOULD be performed by either single transfection of the miTuner plasmid which expresses a synthetic miRNA or co-transfection of the miTuner plasmid with a miRNA/shRNA-mir

Figure 4: pBS with H1 promoter driving a synthetic shRNA; GFP serves as transfection control marker.

expression construct. The knockdown percentage MUST be calculated in comparison to a reference construct, containing a synthetic miRNA/shRNA-miR not targeting the measurement binding site. The miTuner system MAY be used in a virus context, i.e. for *in vivo* studies and applications. Therefore, the pBS_H1_shR construct expressing the GOI MAY be packaged into any AAV (adeno-associated virus) capsid of the users choice.
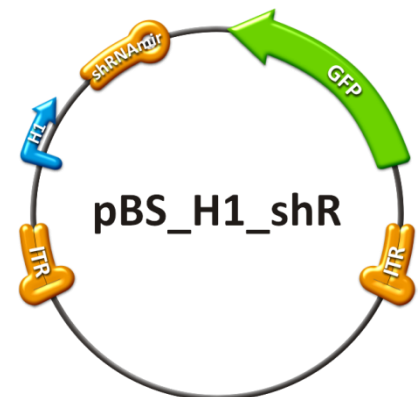
5.5   Off-targeting

Off-targeting addresses expression of the GOI in all cells except the target cells/tissue. The required construct to achieve this is rather simple. It MAY contain any promoter of choice driving the user's GOI that MUST be tagged with a miRNA binding site in its 3' UTR. Therefore a binding site for a miRNA that is exclusively up-regulated in the target cell MUST be used, e. g. has-miR122 in hepatic cells. Thus, expression of the GOI is affected exclusively within the target cells. To enhance the efficiency of the specific knockdown even further, multiple binding sites MAY be put right behind each other (e. g. see: BBa_K337003). RA-PCR (RFC 42) is a method that allows for concatenation of miRNA binding sites and may be applied in these situations.

Standard cloning MAY be applied as an alternative method as well. As a huge variety of tissue or temporal specific endogenous miRNAs exists, profiles of miRNA patterns for different cell types SHOULD be used to designate target cells.[4] Besides that, the Off-targeting construct MAY be delivered into the cells or organisms of choice by employing a virus shuttle (e. g. adeno-associated viruses). Thereby, a sufficient amount of cells will be infected with the plasmid and cell type specific gene expression can be quantified easily.
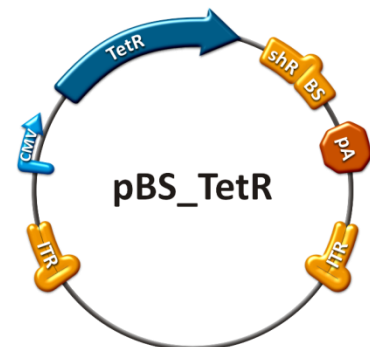

Figure 5: Basic scheme of pBS_TetR

5.6   On-targeting

On-targeting is based on cell/tissue specific endogenous miRNAs, but it includes an inverter. Thereby, endogenous miRNAs expressed in a certain target tissue are used for specifically switching on the GOI.

The system consists of two components that MUST be co-expressed: a Tet repressor (TetR) construct (pBS_TetR, Fig. 5) tagged with perfect binding sites for a cell type/tissue specific miRNA and a promoter with a Tet Operator (TetO$_2$) driving the GOI (pBS_SV40_TetO$_2$_Luc2, Fig.6).
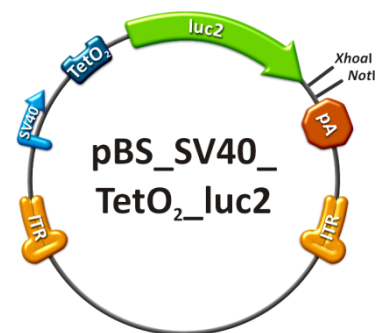

Figure 6: Basic scheme of pBS_SV40_TetO$_2$_luc2.

Multiple binding sites cloned behind the TetR (about 2-4 binding sites in a row) MAY be used to increase the knockdown of Tet repressor. Thereby the rescue of expression of the GOI in the cell type/tissue of interest can be increased. Different ratios of the TetR construct compared to the GOI expression construct MAY be used to increase the level of specificity and expression rescue in the target cells/tissue. The system MAY be used in a virus context, i.e. for *in vivo* studies and applications. For this purpose, the pBS_H1_shR construct expressing the GOI MAY be packaged into any AAV capsid.

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7   References

1. Brown, B.D. & Naldini, L.: Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. *Nat Rev Genet* **10**, 578-85 (2009).

2. http://www.sirnawizard.com/, 25/10/2010, 04.51 pm

3. http://2010.igem.org/Team:Heidelberg/Notebook/Methods, 25.10.2010, 04.01 pm

4. http://www.microrna.org/microrna/getDownloads.do, 25/10/2010, 03.54 pm