BBF RFC XX: 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 Biobrick-2 standard (RFC12, RFC45). Some parts (microRNA binding site patterns) were constructed by the raPCR method described in RFC42. RFCXX provides complementary measurement instructions for the applications described in this RFC.

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4 Rationale

MicroRNAs 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 microRNAs, we introduce the miTuner Kit, enabling the user to control gene expression of any gene of interest according to the following three modes:

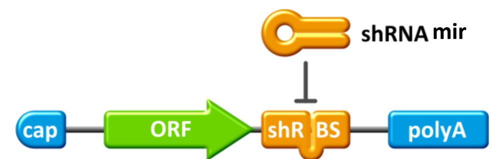


Figure 1: Interaction of an shRNA-miR (synthetic microRNA) with its' binding site in the 3' UTR of an mRNA

1) Tuning: adjusting the co-expression of the GOI by expressing a synthetic microRNA in the target cell/tissue and a binding site for the synthetic microRNA in the 3'UTR of the GOI (Fig. 2a)
2) Off-Targeting: switching OFF the expression of the GOI in case a certain endogenous microRNA is present in the target cell/tissue (Fig. 2b)
3) On-Targeting: switching ON the expression of the GOI in case a certain endogenous microRNA is present in the target cell/tissue (Fig. 2c)

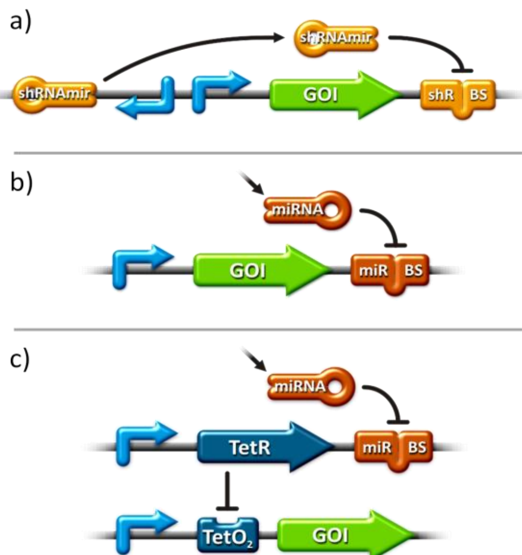


Figure 2: Different regulation modes enabled with the miTuner kit. a) expression tuning based on a synthetic microRNA expressed in the target cell b) off-targeting based on specific endogenous microRNAs c) On-targeting based on a tetR feedback loop coupled to endogenous off-targeting

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 microRNAs designed to have different, orthogonal target sites
c) synthetic microRNA binding sites for either synthetic or endogenous microRNAs

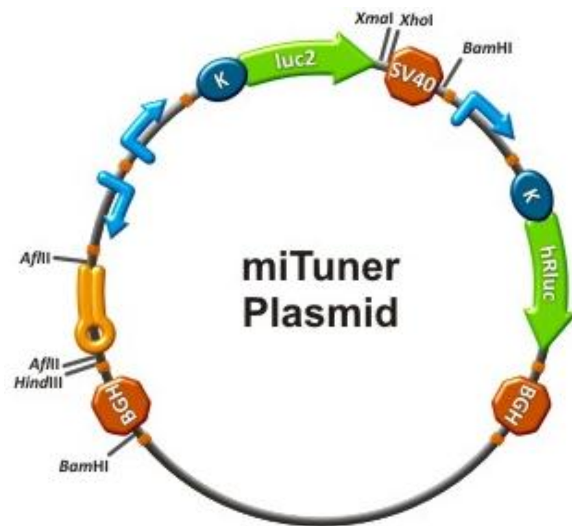


Figure 3: Basic scheme of the miTuner Plasmid. The constructs enables tuning of gene expression by expression of a synthetic microRNA

All basic parts (promoters, microRNAs, 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 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 microRNAs. 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. 2). It contains three expression cassettes: the synthetic microRNA expression cassette in reverse complementary orientation for tuning of gene expression, as well as a measurement firefly luciferase and a reference *Renilla* luciferease in forward direction. *Hind*III and *Afl*II sites allow for the exchange of the synthetic microRNA, *Xma*I and *Xho*I sites allow for introducing synthetic microRNA 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 microRNA we define the adaption of the guiding strand and passanger strand sequence of hsa-mir122 to a target sequence by applying a fusion PCR based protocol (find method below). Synthetic microRNAs 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 microRNAs/shRNA-mirs' are finally chosen for down-regulating the expression of the GOI Luc2. Furthermore, the synthetic microRNA or shRNA-miR expression cassette MAY also be located on a separate plasmid in order to enable further adjustment of microRNA expression strength via co-transfection (or infection) with the miTuner construct.

The synthetic microRNA binding sites are introduced into the 3'UTR of the GOI. Thereby an internal negative feedback loop is created via the synthetic microRNA 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 microRNAs

The microRNAs we propose here MAY be used in the miTuner constructs, but can also applied in other contexts. If microRNAs with costumized targets are required, online tools, such siRNA Wizard v3.1 SHOULD be applied. [2] siRNA Wizare 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 l*ac-Z* gene from the lac operon (E. coli) was used in order to create a list of siRNAs that were then adopted 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 has-mir122 template (Part Nr. BBa_K337016), in order to bring the synthetic microRNA 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 microRNA):

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

5'…GGAGGTGAAGTTAACACCTTCGTGGCTACAGAGTTTCCTTAGCAGAGCTGGACACCACGGCCACCGATATTATGTCTAAACTATTAATATCGGTGACCGTGGTACCAGCTACTGCTAGGCAATCCTTCCCTCGATAAATGTCTTGGCATCGTTTGCTT …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 primers miRNA_fusion_fw and miRNA_fusion_rev for obtaining the whole microRNA 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 microRNA 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 microRNAs MAY be used:

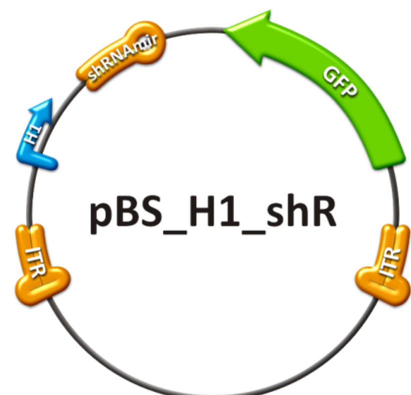
miR-hAAT (targets human hAAT): CCTTAGCAGAGCTG TGTTAAACATGCCTAAACGCTT TGTCTAAACTAT AAGCGTTTAGGAATGTTTACAA TAGCTACTGCTAGGC

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

5.3 Designing Synthetic MicroRNA Binding Sites

Synthetic microRNA binding sites can be constructed by taking the reverse complementary of the guiding strand of the according microRNA 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 microRNA, 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 microRNA binding sites of certain strength for any desired synthetic or endogenous microRNA in a customized user context on an oligo basis.[3]



5.4 Tuning and Expression Measurement

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 process, the Tuning Construct SHOULD be used for expression of the synthetic microRNAs or a second plasmid such as pBSU6 or pBSH1 (Fig. 4) MAY be used for co-expression of either the required synthetic microRNA or shRNA. A standard dual luciferase assay SHOULD be performed by either single transfection of the miTuner plasmid which expresses a synthetic microRNA or co-transfection of the miTuner plasmid with a microRNA/shRNA-miR expression construct. The knockdown percentage MUST be calculated in comparison to a reference construct, containing an synthetic microRNA/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 pBSH1_shR construct expressing the GOI MAY be packaged into any AAV (adeno-associated virus) capsid of user's choice.

Figure 4: pBSU6 with H1 promoter driving a synthetic shRNA; GFP serves as transfection control marker

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 miR-122 in hepatic cells. Thus, expression of the GOI is affected exclusively within 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 microRNA binding sites and MAY be applied to assess this matter. 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 and a referring GOI.[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 visualized easily.

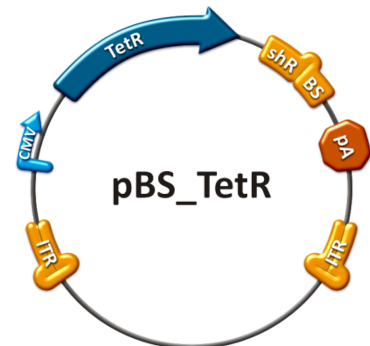


Figure 5: Basic scheme of pBS_TetR

5.6 On-targeting

On-Targeting is based on cell/tissue specific endogenous microRNA, but it includes a negative feedback loop. Thereby, endogenous microRNAs overexpressed in a tissue are used for specifically switching on the users GOI.

The system consists of two components that MUST be coexpressed: a Tet Repressor (TetR) construct (i.e. pBS_TetR, Fig. 5) tagged with perfect binding sites for a cell type/tissue specific microRNA and a promoter with a Tet Operator ($TetO_2$) driving the GOI (i.e. pBS_SV40_$TetO_2$_Luc2, Fig.6). Multiple binding sites cloned behind the TetR (about 2-4 binding sites in a row) MAY increase the knockdown of Tet Repressor. Thereby the rescue of expression of the GOI in the cell type/tissue of interest can also be increased. Different ratios of the TetR construct compared to the GOI expression construct MAY increase the level of specificity or expression rescue in the target cells/tissue. The system MAY be used in a virus context, i.e. for *in vivo* studies and applications. Therefore, the pBSH1_shR construct expressing the GOI MAY be used and packaged into any AAV capsid of the user's choice.

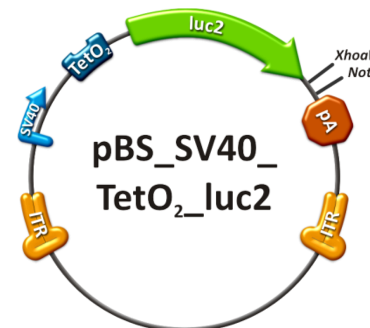


Figure 6: Basic scheme of pBS_sv40_$TetO_2$_Luc2

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