

BBF RFC 73: miMeasure – a standard for miRNA binding site characterization in mammalian cells

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1 Purpose

This RFC proposes a standard for the quantitative characterization of miRNA binding sites (miRNA-BS) in mammalian cells. The miMeasure standard introduces a ready-to-use standard measurement plasmid (pSMB_miMeasure, BBa_K337049) enabling rapid experimental characterization of any miRNA-BS of choice. We recommend a new standard unit, RKDU (*relative knock-down unit*) to describe the knock-down efficiency of a miRNA-BS in a specific cell type. pSMB_miMeasure allows for an easy and fast measurement of RKDU while providing effective normalization against variance stemming from differences in transfection efficiency and from other sources.

2 Relation to other RFCs

This RFC represents an extension of the idea of ensuring measurement comparability via *in vivo* reference standards introduced in RFC 19 by JR Kelly and D Endy. The unit RKDU is closely related to the REU standard unit proposed for the characterization of promoters in mammalian cells (RFC 41).

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4 Motivation

A standard for the quantitative characterization of miRNA-BS is a prerequisite for the systematic exploitation of the potential of the cellular miRNA machinery in synthetic biology. An exemplary, highly promising application is miTuner - a miRNA based gene expression tuning kit for mammalian cells introduced in RCF 72. MiTuner allows fine-tuning of gene expression from arbitrary promoters with the help of synthetic miRNAs as well as on/off-targeting based on the presence of tissue specific endogenous miRNAs. The potential of miTuner is complemented by the quantitative description of miRNA-BS proposed in this RFC.

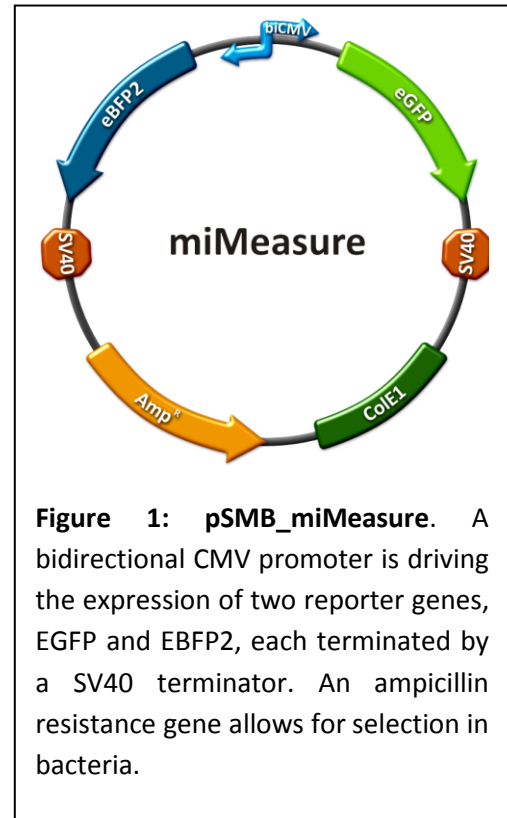
Moreover, a method for the quantitative read-out of miRNA-BS properties will facilitate the investigation of and the communication about the fundamental processes underlying the miRNA machinery in the cell.

Finally, while many computational approaches have been used to identify miRNA targets (Bentwich 2005; Rajewsky 2006; Griffiths-Jones *et al.* 2008), experimental identification, validation and quantitative description of miRNA targets is still a necessary complement for *in silico* approaches. Experimental miRNA-BS characterization will help validate and improve current *in silico* methods.

5 Rationale

5.1 Design of pSMB_miMeasure

The miMeasure standard is based on the pSMB_miMeasure plasmid (BBa_K337049, Fig 1): Two fluorescent proteins EGFP and EBFP2 are driven by a bidirectional CMV promoter to ensure the same expression strength for both reporters. This allows for an exceptionally effective normalization of varying transfection efficiencies and for a highly reproducible determination of the RKDU of the binding site of interest, as will be explained in further detail below. The two fluorescent proteins are destabilized by fusion to a MODC degradation domain which leads to a protein half life of two hours. The shortened half life of the destabilized fluorescent proteins allows for time-laps experiments. Furthermore the plasmid contains a BB-2 (RFC12, RFC41) standard site in the 3' UTR of the EGFP to enable user friendly insertion and exchange of the binding site to be measured. The plasmid further contains an ampicillin resistance for selection in bacteria. For more details of pSMB_miMeasure, refer to http://partsregistry.org/Part:BBa_K337049. The pSMB_miMeasure plasmid was submitted to the Registry of Standard Biology Parts with no miRNA-BS insert.



5.2 RKDU – a standard unit for miRNA-BS characterization

We introduce the new measurement unit RKDU (*relative knock-down unit*) as an extension to the principle of *in vivo* reference standard-based part characterization introduced in RFC 19 by JR Kelly and D Endy. The RKDU unit is furthermore closely related to the REU unit for the description of promoter activity (RFC 41). pSMB_Measure MUST be used for the measurement of RKDU.

We denote the amount of EGFP mRNA transcribed from pSMB_Measure as M_{EGFP} if no miRNA-BS is inserted, and as M_{EGFP_miRNA} if the miRNA-BS of interest is inserted.

Analogously, we denote the amount of totally folded EGFP as P_{EGFP} respectively P_{EGFP_miRNA} . Finally, we describe the amount of EBFP2 mRNA as M_{EBFP2} and define P_{EBFP2} as described above.

P_{EGFP} and P_{EGFP_miRNA} MUST be normalized by division through P_{EBFP2} . The normalized protein amounts are denoted as P_{EGFP}^* and $P_{EGFP_miRNA}^*$:

$$P_{EGFP}^* = \frac{P_{EGFP}}{P_{EBFP2}}$$

$$P_{EGFP_miRNA}^* = \frac{P_{EGFP_miRNA}}{P_{EBFP2}}$$

We have introduced three factors to guarantee maximal reproducibility of the measurements and optimal elimination of transfection variance with the help of this normalization: (1) EGFP and EBFP2 are expressed from the same (bidirectional) promoter (2) EGFP and EBFP2 are expressed from the same plasmid (3) EGFP and EBFP2 have very similar properties (97 % sequence identity on DNA level).

Finally, we can define:

$$RKDU = \frac{P_{EGFP_miRNA}^*}{P_{EGFP}^*}$$

Please note that RKDU describes the down-regulation mediated by the miRNA-BS only at the protein level, but not at the level of mRNA. A proportional relationship between the abundance of unblocked mRNA and the measured abundance of EGFP cannot be assumed (Vogel *et al.* 2010). RKDU is nevertheless a very useful measure, as for most miRNA applications (e.g. gene expression tuning) control of protein levels, not of mRNA levels, is decisive. Please note furthermore that the RKDU for a certain miRNA-BS must be measured and designated separately for every cell line of interest, as miRNA expression varies between different cell types.

5.3 Transfection

Cells SHOULD be prepared in 96-well format with 5×10^3 cells/well. Transfection SHOULD be carried out with 100 ng DNA/well. pSMB_miMeasure containing the binding site of interest SHOULD be transfected in at least 4 replicates. On the same plate, cells transfected with pSMB_Measure without inserted miRNA binding site MUST be prepared to allow for RKDU calculation (see section 5.2) – at least 4 replicates are RECOMMENDED.

5.4 Measuring RKDU by microscopy

Cells MUST be grown in black 96-well plates. The microscope MUST be equipped with a laser to excite at 405 nm (EBFP2) and one to excite at 488 nm (EGFP) to allow for quantitative measurement of EGFP and EBFP2 fluorescence. The cells SHOULD be grown in medium not containing phenolred. Otherwise the phenolred medium SHOULD be removed before starting microscopy measurements. Measurement MUST be carried out after 48 h.

5.5 Measuring RKDU by FACS

The flow cytometer MUST be equipped with a 488 nm and 405 nm excitation laser. This allows for simultaneous and quantitative measurement of EGFP and EBFP2 fluorescence. Before measurement, medium MUST be removed, cells MUST be washed with 1xPBS and trypsinized with 60 μ l of trypsin per well. After 10 minutes incubation at 37 °C in 1x PBS, 1 % BSA MUST be added up to a volume of 200 μ l per well. Measurement MUST be carried out after 48 h. Samples with low cell numbers (1000-3000 cells/well) or transfection efficiencies MUST be excluded.

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