BBF RFC 41: Units for Promoter Measurement in Mammalian Cells

Lars Velten, Nao Iwamoto, Corinna Hiller, Hannah Uckelmann, Chenchen Zhu, Bingqing Zhao, Daniela Richter, Phillip Hundeshagen, Michaela Reichenzeller, Jens Keienburg and Roland Eils

October 19, 2009

1 Purpose

The purpose of this RFC is to provide units for the characterization of promoter strength for use in mammalian cells. RMPU is mRNA based and directly proportional to PoPS, whereas REU is protein based and not proportional to PoPS.

2 Relation to other BBF RFCs

BBF RFC 41 is an extension of RFC 19. RMPU is defined in analogy to RFC 19. REU is a new standard unit.

3 Copyright Notice

4 A promoter meaurement kit for use in mammalian cells

pSMB_MEASURE (BBa_K203100) MUST be used for promoter characterization in mammalian cells. pSMB_MEASURE contains a reference promoter, JeT (BBa_K203112), which is flanked by BBb_2 (RFC 12) sites and can therefore be replaced by the promoter to be measured, if such promoter is flanked by BBb_2 (RFC 12) sites. The plasmid further contains an ampicillin resistance for selection in bacteria and a hygromycin resistence for selection in mammalian cells. For more details of pSMB_MEASURE, refer to http://partsregistry.org/Part:BBa_K203100. A promoter of interest SHALL

only be compared with said reference promoter under the same experimental condition. Measurements SHOULD be done with cell lines stably transfected with the pSMB_MEASURE plasmid (e.g. via a FRT recombination site, which is included in pSMB_MEASURE). We generated also the plasmid pSMB_REFERENCE (BBa K203099), which is equal to pSMB_MEASURE, but contains mCherry instead of GFP. If no stable cell line is used (*i.e.* a transient cell line), the pSMB_REFERENCE plasmid SHOULD be used to analyze differences in transfection efficiency. In order to obtain reliable results and reduce the variance during the measurement procedure, measurement for each promoter MUST be done with at least three replicates, but SHOULD be done with 6-12 replicates for high statistical confidence.

5 RMPU

1 relative mammalian promoter unit (RMPU) is defined in analogy to [1], but mRNA based. It is the amount of total mRNA generated by a promoter of interest (denoted with x) relative to the amount of total mRNA generated by the JeT (BBa_K203112) promoter (denoted with j) in a steady state. This unit is directly proportional to PoPS, as we show. In steady state, change of mRNA levels is 0, thus:

$$\dot{M}_x = n_x \cdot PoPS_x - \gamma_x \cdot M_x = 0 \tag{1}$$
$$\dot{M}_j = n_j \cdot PoPS_j - \gamma_j \cdot M_j = 0 \tag{2}$$

$\dot{M}$ is the change in total mRNA level, γ is the mRNA degradation constant and n is the number of promoters per cell (adapted from [1]). Therefore

$$M_x \cdot \gamma_j = n_j \cdot PoPS_j \tag{3}$$
$$M_j \cdot \gamma_j = n_j \cdot PoPS_j \tag{4}$$

Since both promoters are cloned separately into an identical backbone, they generate identical mRNA. As the most important determinant for mRNA stability is the 3' untranslated region, and mRNA stability is generally not affected by promoter structure [2], we are able to assume the same mRNA degradation constant γ for both promoters.

$$\gamma_x = \gamma_j \text{ and } n_x = n_j \tag{5}$$

We also expect the same n for both promoters. We define RMPU as

$$\text{Relative activity of promoter (RMPU)} := \frac{PoPS_x}{PoPS_{JeT}} = \frac{M_x}{M_j} \tag{6}$$

6 REU

1 relative expression unit (REU) is the amount of total folded protein generated by a promoter, relative to the amount of folded protein generated by the JeT promoter (Bba_K203112). It is not directly proportional to PoPS as too many levels of regulation, such as post-transcriptional modifications, enhanced splicing and nuclear shuttling/transport lie between transcription and protein synthesis [3], and REUs would strongly depend on conditions which affect RNA. Still, it is a very useful measure, as for most applications such as metabolic pathway engineering, protein levels, not mRNA levels, are of importance. If no stable cell line is used, REU SHOULD be measured with a setup which allows for normalization of transfection efficiency, such as flow cytometry [4] using double transfection with pSMB_REFERENCE. If a stable cell line is used, pSMB_REFERENCE SHOULD NOT be used.

7 Measuring RMPU by qRT-PCR

The extraction SHOULD be done using a pipetting robot to avoid possible mistake and variance. The measurement of GFP from pSMB_MEASURE MUST at least be accompanied with the following two house keeping genes: glucose-6-phosphate dehydrogenase and β-actin. It SHOULD be measured with the five house keeping genes: β-actin, glycerol-aldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, γ-tubulin, 18S rRNA. For transient cell lines pSMB_REFERENCE SHOULD be used in parallel. For stable cell lines pSMB_REFERENCE SHOULD NOT be used. The primers in Table 1 SHOULD be used in conjunction with the promoter measurement kit described in section 4. Probes SHOULD be tagged with 5'-6FAM-NNN-TAMRA-3'.

pri_gapdh_fw	GGTATCGTGGAAGGACTCATGACC
pri_gapdh_rv	GATGACCTTGCCCACAGCCTTG
probe_gapdh	CTCTCCAGAACATCATCCCTGCCTCTAC
pri_g6pd_fw	GAGTCCTGCATGAGCCAGATAGG
pri_g6pd_rv	GCACCATGAGGTTCTGCACCATC
probe_g6pd	ACCAGATCTACCGCATCGACCACTACCT
pri_18srrna_fw	GGCCCTGTAATTGGAATGAGTCCAC
pri_18srrna_rv	GCTCCCAAGATCCAACTACGAGCTT
probe_18srrna	CAGCAGCCGCGGTAATTCCAGCTCC
pri_g2tub_fw	TTCTACCAGGCAGACGATGAGCAC
pri_g2tub_rv	CCCAGTTGTTGCCAGCTCCTC
probe_g2tub	CTGCTGGACTTGGAACCCCGGGTG
pri_bact_fw	CAGGCACCAGGGCGTGATGG
pri_bact_rv	CTCCATGTCGTCCCAGTTGGTG
probe_bact	AGGATTCCTATGTGGGCGACGAGGC
pri_egfp_fw	CGACCACATGAAGCAGCACGAC
pri_egfp_rv	CGATGCCCTTCAGCTCGATGC
probe_egfp	AGGCTACGTCCAGGAGCGCACCATC
pri_mcherry_fw	CAAGTGGGAGCGCGTGATGAAC
pri_mcherry_rv	CCAGCCCATGGTCTTCTTCTGC
probe_mcherry	TGACCGTGACCCAGGACTCCTCCC

Table 1: Primer and probe sequences for qRT-PCR.

8 Measuring REU by flow cytometry

In the case of transient cell lines, a flow cytometer SHOULD be equipped with a 488 nm and 561 nm excitation laser for REU measurements, thereby allowing simultaneous and quantitative measurement of GFP and mCherry fluorescence. In the case of stable cell lines, a 561 nm excitation laser is not needed. Cells SHOULD be prepared in 96-well format with 10^4 cells/well and transfected with the promoter of interest. Before measurement, medium MUST be removed, cells MUST be washed with 1xPBS and trypsinized with 60 μl of trypsin per well. After 10 minutes incubation at 37°C in 1xPBS + 1% BSA MUST be added up to a volume of 200 μl per well. If transient cell lines are used the gates and the background signal MUST be adjusted to the negative control to select only cells containing the plasmid. Transfection (% gated)/expression levels of mCherry MUST be compared to GFP levels if using transient transfection. Samples with low cell numbers or transfection efficiencies MUST be excluded.

9 Authors' Contact Information

Lars Velten: velten@stud.uni-heidelberg.de
Nao Iwamoto: n.iwamoto@dkfz-heidelberg.de
Corinna Hiller: corinna.hiller@arcor.de
Hannah Uckelmann: hannahuckelmann@yahoo.com
Chenchen Zhu: jimmyzcc@googlemail.com
Bingqing Zao: bingqing.zhao@googlemail.com
Daniela Richter: d.richter@dkfz.de
Phillip Hundeshagen: p.hundeshagen@dkfz.de
Michaela Reichenzeller: m.reichenzeller@dkfz.de
Jens Keienburg: jens.keienburg@bioquant.uni-heidelberg.de
Roland Eils: r.eils@dkfz.de

References

[1] Kelly, JR et al in Journal of Biological Engineering 3 (2009): "Measuring the activity of BioBrick promoters using an in vivo reference standard"

[2] Alberts, B. et al: Molecular Biology of the Cell (Book. 5th edition, 2008. Garland Science) Chapter 7, p. 492

[3] Alberts, B. et al: Molecular Biology of the Cell (Book. 5th edition, 2008. Garland Science) Chapter 6

[4] Ducrest, A-L et al. in Nucleic Acids Res. 30 (2002), p. e65: "Detection of promoter activity by flow cytometric analysis of GFP reporter expression"

Supplementary information can also be found at
http://2009.igem.org/Team:Heidelberg/Project_Measurement