BBF RFC 58: Measurement of bacterial promoter absolute activity and determination of dynamic performance in cell-free expression system by qPCR

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1. Purpose

The purpose of this RFC is to provide standard methodology for the measurement of the absolute strength of bacterial promoters. The absolute activity can be experimentally measured in Moles of RNA per Moles of DNA in time, and therefore it is can be expressed in PoPS [1]

2. Relation to other BBF RFCs

This RFC extends RFC 19 and is related to RFC 41.

3. Copyright Notice

4. Standard promoter absolute activity measurement kit for use in cell-free system.

Abslolute promoter activity measurement kit MUST include ribosome binding site, reporter gene, terminator and vector backbone. Standard absolute promoter activity measurement kit MUST comprise of ribosome binding site BBa_B0034, reporter gene GFPmut3b BBa_E0040, terminator BBa_B0015, it MUST comprise of high-copy number ampicilin-resistance encoding vector pSB1A2. Those requirements are fulfilled by part BBa_I13504,

that is distributed in pSB1A2 plasmid and SHOULD be used as a base for building measurement devices.

Use of strong, well characterized RBS and fluorescent reporter gene allows visual tracking of the experiment progress and preliminary relative estimation of the results. Since cell free expression systems do not facilitate DNA replication the high copy number vector was chosen only to facilitate growth and DNA purification step in the experiment. Cell free systems that comprise of S30 fraction or the PURE systems comprising of defined factors MUST allow expression from measured promoter.

5. Experimental procedure

Desired promoter MUST be cloned before BBa_I13504 to obtain suitable construct. After this step all reagents and substrates MUST be RNase free. Experiments MUST be conducted in RNase-free environment. RNAse free isolation and quantification of the DNA MUST be performed. Known amount of DNA encoding tested construct MUST be added to of cell-free expression system. RNase inhibitor MUST be added to the reaction. Reaction mixture has to be incubated according to call-free system manufacturer instructions. In constant time intervals constant amount of the reaction MUST be collected for analysis. Samples SHOULD be collected until reaction reaches steady state. In cell free system with no RNA degradation it means that the reaction has run out of substrates. Collected samples MUST be DNAseI treated. Reverse transcription on those samples MUST be performed and suitable RT- controls MUST be included in the experimental setup. Reverse transcription gene specific primer GFPr (TCGAAAGGGCAGATTGTG) SHOULD be used. The amount of mRNA MUST be determined using realtime PCR (qRT-PCR). cDNA Standard curve that allows to convert delta-Ct units to RNA concentration MUST be used. In real time PCR following primers: GFPf (GATGACGGGAACTACAAGAC) and GFPr (TCGAAAGGGCAGATTGTG) SHOULD be used. PCR program for suggested primers SHOULD be as follows: 95oC for 10 minutes followed with 40 cycles of 95oC for 15s, 55oC for 30 s, 72oC for 40s. Reaction specificity MUST be confirmed using melting curve analysis.

6. ngRNA per ngDNA in time to PoPS conversion

Promoters MUST NOT be measured at steady state, the dynamic performance MUST be measured.

moles of RNA produced per mole of DNA per second can be calculated from measured value of ng of RNA per ng of DNA in time for a standard measurement kit as follows:

- the relation between ng of RNA and time of sample collection in seconds SHOULD be plotted
- the parameters of best linear regression curve function SHOULD be obtained in form: ng of RNA=a*time in seconds+b
- the a coefficient, which is equal number of ng of RNA produced per second, MUST be divided by the amount of ng of DNA used as a protein synthesis template to obtain amount of ng of RNA produced per ng of DNA per second
- that value MUST be converted to moles of RNA produced per mole of DNA per second which is equal PoPS (Polymerases per second) units assuming that polymerase passes the last base of the transcript.

To convert amount of ng of RNA produced per ng of DNA per second to PoPS following procedure SHOULD be used:

- molar weight of DNA used as a template SHOULD be calculated from equation: DNA MW = construct length x 607.4 gram/mole
- molar weight of RNA SHOULD be calculated from equation: mRNA transcript MW = number of nucleotides x 320.5 + 159.0 and for the experimental setup described in this document mRNA transcript MW=283160,5g/ mole
- PoPS SHOULD be calculated as follows: PoPS = amount of ng of RNA produced per ng of DNA per second / mRNA transcript MW / DNA MW

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8. References

1. PoPS definition http://partsregistry.org/wiki/index.php/ Part_Types:Measurement_Systems