DOI: 10.1002/cbic.200500490

High-Throughput-Compatible Assay for glmS Riboswitch Metabolite Dependence

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The increasing appearance of antibiotic-resistant strains of microbial pathogens seriously threatens our ability to control most of the major diseases.[1] Coping with this threat will require continuous efforts to identify, explore and validate novel targets for antibiotic intervention. With the recent discovery that many bacteria utilize certain RNA domains, namely riboswitches and metabolite-dependent ribozymes, to regulate gene expression, a completely unexplored potential target class for new antibiotics has emerged.[2] A broad-scale exploration of these targets requires the development of assay formats that are compatible with high-throughput screening (HTS) for modulatory small molecules. Here we report a HTS-compliant fluorescence-based assay format that allows direct monitoring of the *cis*-cleaving reaction catalyzed by the glmS ribozyme.

The glmS ribozyme is a *cis*-cleaving catalytic riboswitch located in the 5′-UTR of bacterial mRNA that codes for glucosamine-6-phosphate synthetase.[3] The ribozyme can be specifically activated for glmS–mRNA cleavage by the metabolite glucosamine-6-phosphate (GlcN6P), that is, the metabolic product of the *glmS*-encoded protein itself. This complex regulation thus relies on a feedback-inhibition mechanism that senses the presence of metabolites that serve as cell-wall precursors. The ribozyme exhibits remarkable sensitivity and specificity for GlcN6P; related metabolites, such as glucose, glucose-6-phosphate or glucosamine (GlcN), cannot activate it.[3] On the other hand, if small molecules can be found that activate the ribozyme in an analogous fashion to GlcN6P, they are likely to exhibit antibiotic activity because they trigger destruction of the mRNA that encodes for a protein required for the synthesis of a bacterial cell-wall precursor molecule. The *glmS* element resides upstream of the monocistronic *glmS* gene in 18 Gram-positive organisms, including severely pathogenic ones like *Staphylococcus aureus*.[4]

We used the glmS ribozyme from *Bacillus subtilis* to test its suitability for the development of a HTS-compatible assay as a prerequisite for the search for ribozyme-activating small molecules. Because the natural glmS ribozyme is *cis*-cleaving, we sought to monitor this reaction as a function of GlcN6P concentration by fluorescence polarization (FP). FP can quantify dynamic binding events by measuring the amount of depolarization after excitation of fluorescent molecules with polarized light. This relates to the molecular weight of the excited molecule: if its molecular weight is high, it rotates and tumbles more slowly in space, and FP is preserved. If it is small, rotation and tumbling occur faster, and FP is reduced.[5] The FP phenomenon has been used for analyzing binding events involving nucleic acids,[6] such as in DNA– or RNA–protein interactions,[7–9] or DNA detection by strand-displacement amplification.[10] To apply FP to the detection of distinct, metabolite-dependent activation states of a catalytic riboswitch, we used an 81-nucleotide minimal ribozyme variant (Figure 1 A).[3] Fluorescence labelling at the 5′-end was achieved by in vitro transcription in the presence of guanosine monophosphothioate (GMPS) to introduce a single 5′-thiophosphate,[11] followed by derivatization with 5-(iodoacetamido)fluorescein.

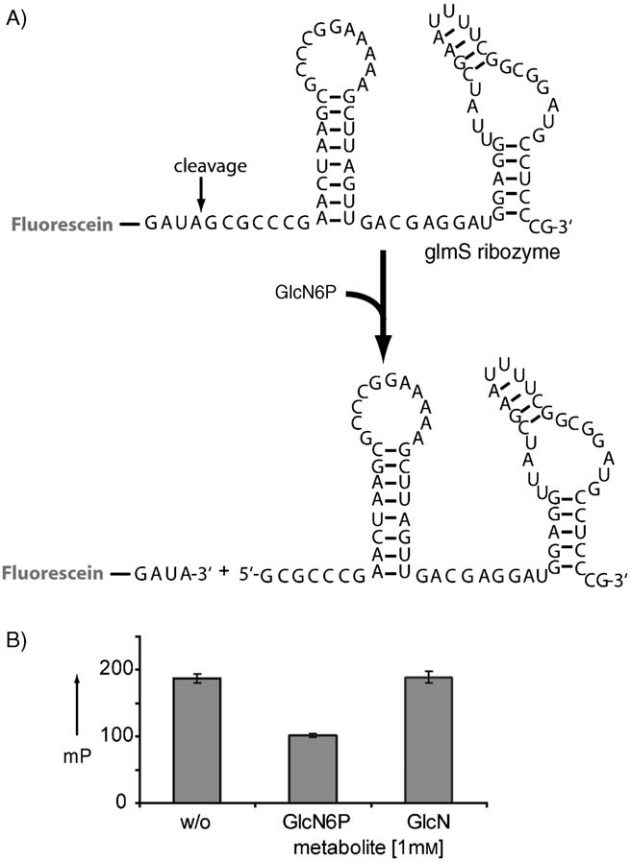

Figure 1. A) Schematic representation of the fluorescent-labelled glmS mini ribozyme and *in cis* cleavage induced by GlcN6P. B) Fluorescence polarization (mP) of the fluorescein-labelled glmS mini ribozyme in the presence of GlcN6P and GlcN or without (w/o) metabolites.

The GlcN6P-triggered *cis*-cleaving reaction generates a fluorescent-labelled tetranucleotide, transforming the fluorescent molecule from a high-molecular-weight 81-mer to a low-molecular-weight 4-mer, which should be well within the range of FP sensitivity. Indeed, as shown in Figure 1 B, the GlcN6P-initiated cleavage reaction results in a significantly reduced FP signal compared to the glucosamine (GlcN) negative-control

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reaction. This activity was confirmed by assays with radiolabeled glmS ribozyme (see Supporting Information).

We next investigated whether changes in the FP signal are proportional to the concentration of GlcN6P used. Figure 2A shows that the intensity of the FP signal strictly depends on the concentration of GlcN6P. From the obtained curve, the half-maximal activity was determined to be 28.3 μM. Figure 2B shows that the absolute change in the fluorescence polarization (ΔmP) gradually increases with the amount of GlcN6P. As shown in Figure 2C and D, decreasing concentrations of the glmS ribozyme in the FP assay resulted in equalization of the ΔmP values with and without GlcN6P. The most marked differences between the signals corresponding to the cleaved and the uncleaved ribozyme were obtained at ribozyme concentrations exceeding 50 nM. The ability to find active substances ("hits") from any random collection is largely determined by the quality of the assay and screening conditions. To assess the quality of our FP-based riboswitch cleavage assay with respect to an application in a HTS setting, it is important to determine the *z′* factor. A *z′* factor between 0.5 and 1.0 characterizes an excellent assay with a high dynamic range and tiny standard deviations.[12] To determine the *z′* factor and its HTS compatibility, we integrated our assay into an automated pipetting system (see Supporting Information). 10% DMSO was added to allow for the screening of "drug-like" molecules, many of which require the addition of organic solvents under in vitro screening conditions. Each row on a 96-well microtiter plate contained 200 μM of either GlcN6P or GlcN. The standard deviation of the automated pipetting was 3.8%, based on the mean fluorescence intensity averaged over each well. The obtained *z′* factor of 0.61 matches very well with industrial standards, and shows that the assay is suitable for identifying active compounds.

The reliability of the FP assay can be further increased by performing the cleavage reaction in the presence of a "blocking oligodeoxynucleotide" that masks the cleavage site. This allows for a post-screening validation of the mode of action of identified and potentially active compounds regarding their ability to induce cleavage at position A4 (Figure 1A). Compounds that alter the mP value of the riboswitch in the presence of the blocking oligodeoxynucleotide are probably false positives. The assay also tolerates various buffer conditions (see Supporting Information).

To investigate whether the assay can detect GlcN6P in complex mixtures such as bacterial extracts, we prepared water-soluble metabolite extracts from *E. coli* cultures. Contaminant nucleases were removed from the extracts with phenol/chloroform, and aliquots were directly used in cleavage assays. To induce the cleavage reaction, bacterial extracts were supplemented with GlcN6P (Figure 3).

To obtain further evidence for the robustness of the established assay under actual screening conditions, we performed an exemplary model screen using 88 commercially available compounds (Figure 4) in addition to positive (GlcN6P) and negative (GlcN) controls on a 96-well plate. An active "hit" is

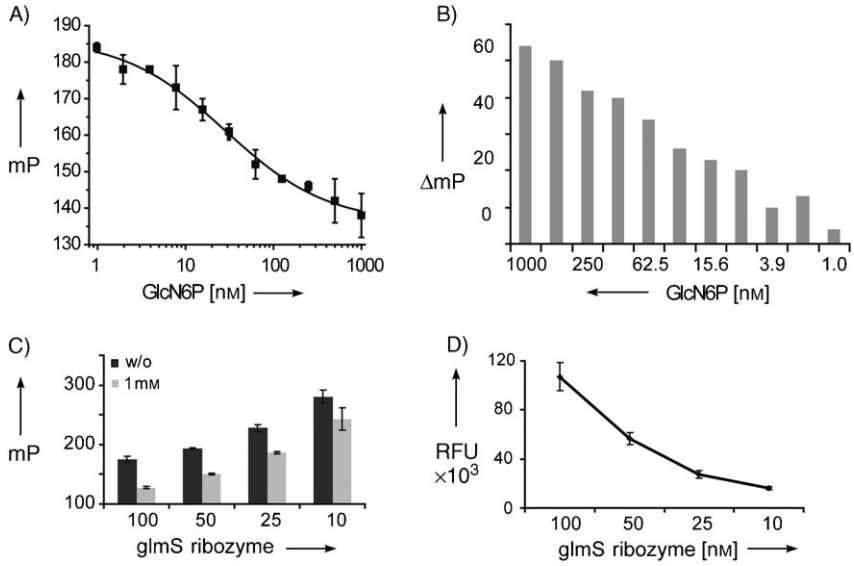

Figure 2. Concentration-dependent cleavage reaction of the glmS mini ribozyme [100 nM] A) induced by GlcN6P. B) Difference mP value of the uncleaved [100 nM] and the cleaved ribozymes at different GlcN6P concentrations. C) Dependence of the fluorescence-polarization signal on the glmS ribozyme concentration in the presence (grey) and absence (black) of GlcN6P. D) Relative fluorescence intensities (RFU) of the different glmS ribozyme concentrations used in (C).

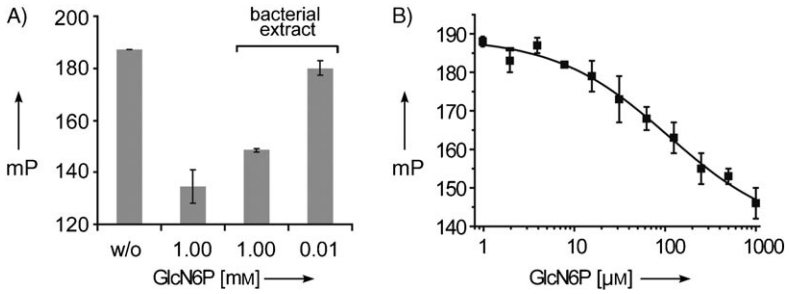

Figure 3. A) Activation of the glmS ribozyme in bacterial extracts. Cleavage can activated in bacterial extracts supplemented with GlcN6P in a concentration-dependent manner. For comparison, the GlcN6P-induced activation in cleavage buffer is also shown. B) GlcN6P-concentration-dependent activation of the glmS ribozyme in bacterial extracts.

only obtained in the positive-control wells containing the natural inducer GlcN6P. These data further support the stability of our format, although, as expected, our model screen did not yet reveal any inducer of glmS activity among the compounds tested.

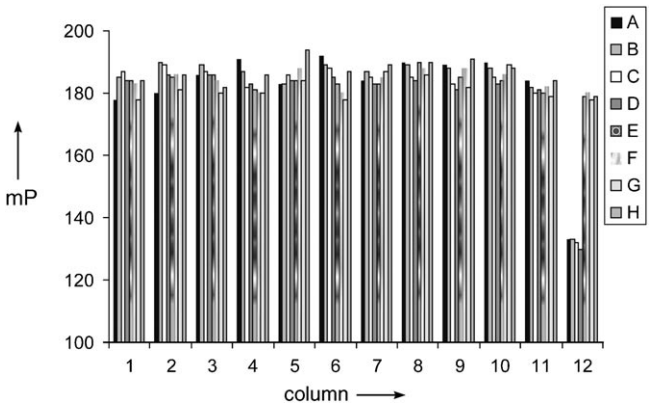

Figure 4. Automated 96-well plate format. Simulation of a "real" screening situation. Columns 1–11 (rows A–H) contain 88 structurally different, commercially available small organic compounds (Comgenex; final concentration 100 μM). Column 12 contains either 200 μM GlcN6P (rows A–D) or 200 μM GlcN (rows E–H).

In summary, we have developed a HTS-compatible assay that can now be readily used to screen compound libraries for small molecules that modulate bacterial riboswitches or allosteric ribozymes. Our findings have implications for the practical development of new antimicrobial drugs by showing that RNA molecules, like proteins, are amenable to highly parallel screening formats, providing a means to systematically search within an essentially unexplored target group for completely novel classes of antibiotics. Two precedents for riboswitches as targets for antibiotic intervention exist. First, *S*-(2-aminoethyl)-L-cysteine (AEC) binds to the lysine riboswitch with only a 30-fold reduced affinity compared to lysine.[13] Second, the antimicrobial action of the thiamine analogue pyrithiamine is mediated by interaction with TPP riboswitches in bacteria and fungi.[14] We are currently assembling large chemical libraries to start a screen for glmS ribozyme modulators.

Acknowledgements

We thank Christian Wingen for assistance and acknowledge support from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

Keywords: fluorescence polarization · riboswitches · ribozymes · screening

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Received: November 25, 2005
Published online on February 17, 2006

