Published in final edited form as:

Chembiochem. 2015 March 23; 16(5): 725–730. doi:10.1002/cbic.201402615.

A Unified Sensor Architecture for Isothermal Detection of Double-Stranded DNA, Oligonucleotides, and Small Molecules

Carl W. Brown III^a, Matthew R. Lakin^c, Aurora Fabry-Wood^a, Eli K. Horwitz^a, Nicholas A. Baker^a, Darko Stefanovic^{*,a,c}, and Steven W. Graves^{*,a,b}

^aCenter for Biomedical Engineering University of New Mexico, Albuquerque, NM 87131, USA

^b Department of Chemical and Biological Engineering University of New Mexico, Albuquerque, NM 87131, USA

^c Department of Computer Science University of New Mexico, Albuquerque, NM 87131, USA

Abstract

Pathogen detection is an important problem in many areas of medicine and agriculture, which may involve genomic or transcriptomic signatures, or small molecule metabolites. We report a unified, DNA-based sensor architecture capable of isothermal detection of double-stranded DNA targets, single-stranded oligonucleotides, and small molecules. Each sensor contains independent target detection and reporter modules, enabling rapid design. We detected gene variants on plasmids via a straightforward isothermal denaturation protocol. The sensors were highly specific, even with a randomized DNA background. We achieved a limit of detection of ~15 pM for single-stranded targets and ~5 nM for targets on denatured plasmids. By incorporating a blocked aptamer sequence, we also detected small molecules using the same sensor architecture. This work provides a starting point for multiplexed detection of multi-strain pathogens, and disease states caused by genetic variants (e.g., sickle cell anemia).

Keywords

pathogen detection; DNAzymes; deoxyribozymes; strand displacement; amplification

Recent advances in DNA nanotechnology have enabled the construction of nucleic acid devices that use the predictable nature of Watson-Crick hybridization to perform computations,^[1] and to detect targets with high specificity^[2]. These properties, together with inherent biocompatibility and rapidly decreasing synthesis costs, make DNA an attractive material for the development of low-cost bioassays^[3]. These requirements call for a simple, flexible design, for which DNA is ideally suited. However, nearly all bacterial and viral pathogens have double-stranded genomes, and the inaccessibility of gene targets in their natural, double-stranded state makes direct, isothermal detection using hybridization-based biosensors challenging.

*graves@unm.edu, darko@cs.unm.edu.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.20xxxxxxx>.

Here we present a unified architecture and protocol for modular DNA-based sensors capable of specific detection of single- and double-stranded genetic targets, as well as small molecules. Our sensors combine toehold-mediated strand displacement (TMSD) and DNAzyme-catalyzed substrate cleavage to enable amplified target detection^[4]. TMSD^[5] is a versatile and powerful DNA computing technique that has been used to implement digital logic circuits^[1a, 6], neural networks^[7], enzyme-free catalytic networks^[8], hairpin assembly systems^[9] and molecular walkers^[10]. TMSD reactions can be thermodynamically biased towards activation based on the lengths of the toeholds that nucleate the binding reactions^[11]; this makes them ideal for detecting targets at low concentrations. By combining TMSD with inherently catalytic DNA strands (known as DNAzymes or deoxyribozymes), we can achieve an amplified signal output via multiple-turnover substrate cleavage. We have previously reported logic gates^[12] and multi-layer DNAzyme signaling cascades^[13] using these mechanisms.

Inspired by our previous work^[12], in this paper we extend the hybrid TMSD-DNAzyme logic gate approach to engineer a unified sensor architecture capable of detecting a range of target types through a multi-step binding pathway. Figure 1 depicts our unified sensor architecture, which consists of a catalytically inactive DNAzyme-inhibitor complex comprising two independent modules, a *detection module* (DM) that is responsible for target detection and a *reporter module* (RM) that produces the output signal, as well as an auxiliary fuel strand. The mechanism of activation for various target types is also shown in Figure 1. When the correct target is present, it binds to the DM and causes unbinding of the DNAzyme strand up to the beginning of the RM. DNA targets bind to the complementary DM toehold t_1^* and trigger branch migration across the x domain, whereas small molecule targets displace the x domain via aptamer binding, causing a conformational shift in the DM. Either of these reactions partially displace the DNAzyme strand and open up the RM toehold t_2^* , previously sequestered in a small, 5 or 8 nucleotide (nt) bulge between the two modules. The fuel strand then hybridizes to the free RM toehold t_2^* and displaces the remainder of the DNAzyme strand via the s_1 and cc_1 domains. The cc_1 domain contains enough of the catalytic core of the DNAzyme to prevent the core from spontaneously attaining a catalytically active conformation when the DNAzyme is bound to the inhibitor. Once displaced, the free DNAzyme is able to fold into a catalytically active conformation as the entire catalytic core (cc) is now single-stranded. This allows it to cleave its complementary FRET-labeled substrate to produce a fluorescent signal. If substrate is present in excess, each DNAzyme is capable of catalyzing the cleavage of many substrate molecules in a multiple-turnover kinetic regime, providing the potential for isothermal signal amplification in the readout module. All of our designs were based on the 8-17 DNAzyme motif^[14] because of its compact size and high catalytic efficiency^[15].

The separation of target detection and reporter modules in our unified sensor architecture allowed the sequences of the detection and reporter modules to be varied independently. In particular, we varied the target module while keeping the reporter module fixed, enabling detection of multiple targets with a single fluorescent readout. We used this approach for DNA detection by designing five sensors that target corresponding sequences from two plasmids that encode GFP-fusion protein variants, a commercially available Emerald GFP

plasmid (referred to as emGFP) and a Pinpoint Xa plasmid containing a SNAP25-GFP fusion protein (referred to as SNAP25) previously developed in our lab^[16]. All five sensors used a common reporter module. The locations of the sensor targets on the plasmids are illustrated in Figure 2. We chose three targets common to both plasmids, including a conserved region of the GFP sequence (C1), the gene coding for antibiotic resistance (C2), and the origin of replication (C3). We also chose one target specific to the emGFP variant (named E) and one target specific to the SNAP25 GFP variant (named S), enabling discrimination between different GFP-fusion proteins. Data from the initial characterization of the five sensors using synthetic, single-stranded oligonucleotides corresponding to the five detection targets is presented in Figure 3, including detection of individual targets using single sensors, multiplexed detection of targets using multiple sensors, and the demonstration of a single sensor limit of detection ~15 pM. Error bars indicate one standard deviation from the mean. The performance of the C1-3 and E sensors was comparable, whereas the S sensor was slower to activate. This difference may be due to unwanted secondary structure formation or input sequestration in the S target strand.

Our goal was to use the unified sensor architecture to detect gene targets on the emGFP and SNAP25 plasmids. Detection of double-stranded DNA (dsDNA) is particularly challenging because of its inherent stability. Because toehold-mediated strand displacement relies on the availability of single-stranded toeholds to initiate branch migration, additional treatment steps are necessary to expose the target toeholds. We addressed the challenge of double-stranded DNA detection using an isothermal chemical denaturation protocol based on pH cycling. Briefly, we used a strongly basic solution (1M NaOH) to increase the pH of the plasmid solution, which denatured the duplexes due to the disruption of the hydrogen bonding of DNA base pairs. We then used a strong acid (1M HCl) to bring the solution back down to physiological pH, so that the strand displacement and DNase reactions underlying our sensor architecture could proceed. The denaturation step was essential to expose the single-stranded target domains from double-stranded complexes; in all cases, the control in which the plasmid was not denatured produced no fluorescent response (Figure S1). Data illustrating the detection of targets on chemically denatured emGFP and SNAP25 plasmids using the five sensors individually are presented in Figures 4a and 4b, respectively. In each case, we observed a fluorescent response from a given sensor if and only if the corresponding target was present on the plasmid in question. Additional experiments in the presence of a randomized herring sperm DNA background yielded similarly positive results (Figure S2), which showed that the sensors are highly sequence-specific.

To boost the observed signal intensity from positive target detection assays, we used all five sensors in a single assay with the emGFP or SNAP25 plasmids. The effective concentration of accessible binding sites on the plasmids may be a kinetically limiting factor, but additional sensors for different areas of the plasmid can increase the effective concentration of binding sites without increasing the plasmid concentration itself. The multiplexed sensor responses in the presence of emGFP and SNAP25 plasmids are presented in Figure 4c. Using multiple sensors simultaneously provides a significant kinetic advantage, seen in Figure 5. The use of all five sensors provides a significant increase in response compared with a single sensor, and it is notable that the use of five sensors to detect plasmid generates

a signal comparable to that of a single-stranded target. Thus, the multiplexed sensor approach works well for the detection of low analyte concentrations.

Detection of low input concentrations is vital in many pathogen detection applications. We investigated the limit of detection (LOD) for the combination of sensors in a plasmid detection assay. The left-hand axis of Figure 4d shows kinetic traces of all five sensor outputs in the presence of decreasing concentrations of the emGFP plasmid. Lowering the sensor concentrations reduced non-specific signal generation in the absence of input while slowing down the reactions. We determined the optimal LOD to be ~5 nM after 15 hours when all five sensors were used. A single sensor showed no response at these concentrations, demonstrating the benefit of the multiple sensor approach.

While the detection of genetic targets is critical for pathogen identification and typing, small molecule sensors also have important applications, e.g., in drug or metabolite detection^[17]. DNA aptamers can bind to small molecules, resulting in a conformational change^[18]. We used this capability to replace the detection module of our sensor design with a partially blocked ATP aptamer sequence^[19], as shown in Figure 1, to produce an ATP sensor. As shown in Figure 6, we observed significant signal over background in the presence of 1 mM ATP but no response in the presence of GTP. These data demonstrate the specificity of our small molecular sensor and show that our unified sensor architecture can detect a range of target types. Constraints imposed by the kinetics of the ATP aptamer (Figures S3-S5) required us to use a 5 nt loop toehold rather than an 8 nt toehold, and the incorporation of other aptamers may require similar minor sensor modifications. The wide variety of available aptamer sequences, in conjunction with our modular sensor design, provides great flexibility that may be leveraged in future work to detect metabolites in complex biological mixtures. This may serve as an alternative or supplemental means of detecting pathogenic bacteria.

A key aspect of our sensor design is the use of a bulge to sequester the secondary toehold where the fuel strand binds. This is a non-standard approach to the design of multi-step DNA strand displacement processes, which typically rely on spontaneous dissociation of the secondary toehold following incomplete strand displacement. Our approach enabled us to implement a two-step strand displacement reaction using a two-strand complex, as opposed to the three-strand complex that is typically required^[1a, 20]. Control experiments (Figure S2) showed that the DNAzyme-inhibitor complexes are highly stable in the absence of the fuel strand, even in the presence of the target sequence. However, this was a potential source of leakage (unwanted DNAzyme activation) due to unwanted fuel binding to the single-stranded toehold in the bulge in the absence of target binding. This effect was exacerbated by imperfections in the sensor complex and breathing of the duplexes near to the secondary toehold bulge. We minimized leakage in our designs by introducing a number of mismatched bases into the toehold and branch migration domain on the fuel strand^[21]. A comparison of the performance of sensor variants with different toehold loop lengths and patterns of mismatches is presented in Figures S6 and S7. Our chosen design employed an 8 nt toehold loop, with two mismatches in the loop adjacent to the beginning of the branch migration domain and a partial (G-T) mismatch in the first base of the migration domain. We used this toehold design in all experiments reported in this paper except for the ATP

sensing experiments, as discussed above. Each sensor toehold used a universal sequence chosen from a restricted three-base alphabet (A,C,T) to prevent unwanted binding interactions. Alternative toehold sequences can be used for each of the sensors to obtain similar results (Figures S8-S11).

Our approach to pathogen detection has the advantage of simplicity compared with approaches based on protein enzymes, such as the polymerase chain reaction (PCR), strand displacement amplification^[22], rolling circle amplification^[23], or other isothermal nucleic acid amplification approaches^[24]. Our rational engineering approach to sensor design is distinct from approaches based on selecting DNazymes for affinity for the various products excreted by bacteria using directed evolution^[25]. However, the methods we used for preparing dsDNA samples prior to detection could also be integrated with other groups' work on DNzyme-based pathogen sensing^[26]. While PCR remains the gold standard for nucleic acid detection in terms of sensitivity, it is protein-based and is not isothermal, which may hinder application in resource-poor areas. Although our sensor architecture addresses such limitations, our aim is not to compete directly with PCR; rather it is to provide an assay format that is more specific, flexible and cost-effective than alternative approaches, such as the enzyme-linked immunosorbent assay (ELISA).

In summary, this work explores the concepts required to create nucleic acid-only detection systems for the isothermal detection of a range of targets. We have designed a unified sensor architecture and applied it to the detection of oligonucleotides, double-stranded plasmid DNA, and small molecules. The modularity of the unified sensor architecture allowed new sensors to be easily designed to target new genetic variants, enabling swift detection of emerging and evolving pathogen strains. Furthermore, our design requires only simple modification of the reporter module to cleave a different substrate sequence. This enables the use of different readout substrates to multiplex the detection of different targets in a single assay. In the solution phase, each substrate would be monitored in a separate fluorescent channel. In a flow cytometry assay format, substrates labeled with a single fluorophore could be monitored via a multiplex set of fluorescently dyed microspheres.

These sensors achieved direct detection of single-stranded targets at a limit of detection of ~15 pM and ~5 nM for isothermally denatured double-stranded targets. The LOD obtained from the single-stranded targets was nearly 250 times lower than for denatured plasmid targets, which may be due to various factors including diffusion rates, the presence of competing sequences, and steric hindrance and/or secondary structure occluding the target site. However, prediction of the secondary structure of plasmids is particularly challenging due to the lack of computational algorithms for modeling large, circular DNA strands. The response times and limits of detection for double-stranded DNA targets may be improved by a deeper understanding of the underlying biophysical principles, which would enable the optimization of preparation protocols. The incorporation of additional molecular signal processing motifs, such as signal amplification via input recycling,^[6, 8a] may also improve limits of detection by increasing the rate of signal generation.

Experimental Section

Materials

All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Substrate molecules (DNA-RNA chimeras) were purified by RNase-free HPLC by the manufacturer. The Emerald GFP (pRSET-emGFP) plasmid was purchased from Life Technologies (Grand Island, NY). The PinPoint Xa plasmid was purchased from Promega (Madison, WI). Sequences for all oligonucleotides used herein are presented in Tables S1-S6 in the Supporting Information.

Sensor preparation

DNAzymes and inhibitors were heated together at 95 °C for 3 minutes on a heat block, and subsequently annealed by cooling to room temperature over a minimum of 90 minutes. Typical reaction concentrations were as follows, unless otherwise noted: 5 nM DNAzyme, 5.75 nM inhibitor, 5 nM fuel, 5 nM target DNA oligomer, 25 nM plasmid DNA, and 250 nM substrate, except in experiments to investigate individual sensor performance and LOD, which were run at 50 nM substrate.

Plasmid preparation

Plasmids were transformed into SCS110 cells (Aligent Technologies, Santa Clara, CA) and grown in LB media (Becton-Dickenson, Franklin Lakes, NJ). Plasmid DNA was extracted with a Qiagen Maxiprep kit (Valencia, CA) into H₂O. The plasmid denaturation used a previously established protocol^[27]. Briefly, 2 parts 1M NaOH was added to 9 parts plasmid in H₂O. After 10 min, 2 parts 1M HCl was added.

Limit of Detection calculation

For these experiments (Figures 3c and 4d), the system contained 1 nM/sensor concentration with 150 pM excess inhibitor and 1 nM fuel for each sensor. The background signal in the absence of input has been subtracted from all traces. Thick lines are mean fluorescence values from 5 replicates, and thin broken lines are one standard deviation above and below the mean in each case. Data points plotted as a cross (right axis) indicates the limit of detection using the IUPAC definition: 3 standard deviations over background^[28].

Assay conditions and instrumentation

All assays were performed at room temperature (23 °C) in a buffer of 1M NaCl, 50 mM HEPES, 1 mM ZnCl₂, pH 7.0. Fluorescence was read on a Spectramax M2e fluorescent plate reader (Molecular Devices, Sunnyvale, CA) in a 200 µL reaction volume (492 nm excitation, 518 nm emission).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This material is based upon work supported by the National Science Foundation under grants 1027877, 1028238 and 1318833. C.W.B. gratefully acknowledges support from INCBN IGERT DGE-0549500. M.R.L. gratefully acknowledges support from the New Mexico Cancer Nanoscience and Microsystems Training Center (NIH/NCI grant 5R25CA153825).

References

1. a Seelig G, Soloveichik D, Zhang DY, Winfree E. *Science*. 2006; 314:1585. [PubMed: 17158324] b Stojanovic MN, Stefanovic D. *Nat Biotechnol*. 2003; 21:1069. [PubMed: 12923549]
2. a Chen SX, Zhang DY, Seelig G. *Nature Chemistry*. 2013; 5:782. b Zhang DY, Chen SX, Yin P. *Nat Chem*. 2012; 4:208. [PubMed: 22354435]
3. Carlson R. *Nat Biotechnol*. 2009; 27:1091. [PubMed: 20010582]
4. Li Y, Breaker RR. *Curr Opin Struct Biol*. 1999; 9:315. [PubMed: 10361095]
5. Zhang DY, Seelig G. *Nat Chem*. 2011; 3:103. [PubMed: 21258382]
6. Qian L, Winfree E. *Science*. 2011; 332:1196. [PubMed: 21636773]
7. Qian L, Winfree E, Bruck J. *Nature*. 2011; 475:368. [PubMed: 21776082]
8. a Zhang DY, Turberfield AJ, Yurke B, Winfree E. *Science*. 2007; 318:1121. [PubMed: 18006742] b Zhang DY, Winfree E. *J Am Chem Soc*. 2008; 130:13921. [PubMed: 18823118]
9. a Yin P, Choi HMT, Calvert CR, Pierce NA. *Nature*. 2008; 451:318. [PubMed: 18202654] b Li B, Ellington AD, Chen X. *Nucleic Acids Res*. 2011; 39:e110. [PubMed: 21693555] c Chen X, Briggs N, McLain JR, Ellington AD. *Proc Natl Acad Sci U S A*. 2013; 110:5386. [PubMed: 23509255] d Li B, Jiang Y, Chen X, Ellington AD. *J Am Chem Soc*. 2012; 134:13918. [PubMed: 22894754]
10. a Bath J, Green SJ, Allen KE, Turberfield AJ. *Small*. 2009; 5:1513. [PubMed: 19296556] b Yin P, Yan H, Daniell XG, Turberfield AJ, Reif JH. *Angew Chem Int Ed Engl*. 2004; 43:4906. [PubMed: 15372637]
11. Zhang DY, Winfree E. *J Am Chem Soc*. 2009; 131:17303. [PubMed: 19894722]
12. Brown CW III, Lakin MR, Stefanovic D, Graves SW. *ChemBioChem*. 2014; 15:950. [PubMed: 24692254]
13. Brown CW III, Lakin MR, Horwitz EK, Fanning ML, West HE, Stefanovic D, Graves SW. *Angew Chem Int Ed*. 2014; 53:7183.
14. Santoro SW, Joyce GF. *Proc Natl Acad Sci U S A*. 1997; 94:4262. [PubMed: 9113977]
15. Li J, Zheng W, Kwon AH, Lu Y. *Nucleic Acids Res*. 2000; 28:481. [PubMed: 10606646]
16. Saunders MJ, Edwards BS, Zhu J, Sklar LA, Graves SW. *Curr Protoc Cytom*. 2010; 54:13.12.1.
17. a Stojanovic MN, de Prada P, Landry DW. *J Am Chem Soc*. 2001; 123:4928. [PubMed: 11457319] b Stojanovic MN, Landry DW. *J Am Chem Soc*. 2002; 124:9678. [PubMed: 12175205]
18. Achenbach JC, Nutiu R, Li Y. *Analytica Chimica Acta*. 2005; 534:41.
19. Huizenga DE, Szostak JW. *Biochemistry*. 1995; 34:656. [PubMed: 7819261]
20. Soloveichik D, Seelig G, Winfree E. *Proc Natl Acad Sci U S A*. 2010; 107:5393. [PubMed: 20203007]
21. Jiang YS, Bhadra S, Li B, Ellington AD. *Angew Chem Int Ed*. 2014; 53:1845.
22. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP. *Nucleic Acids Res*. 1992; 20:1691. [PubMed: 1579461]
23. a Fire A, Xu SQ. *Proc Natl Acad Sci U S A*. 1995; 92:4641. [PubMed: 7753856] b Zhao W, Ali MM, Brook MA, Li Y. *Angew Chem Int Ed Engl*. 2008; 47:6330. [PubMed: 18680110]
24. a Kim J, Easley CJ. *Bioanalysis*. 2011; 3:227. [PubMed: 21250850] b Vincent M, Xu Y, Kong H. *EMBO Rep*. 2004; 5:795. [PubMed: 15247927]
25. a Aguirre SD, Ali M, Monsur, Kanda P, Li Y. *Journal of Visualized Experiments*. 2012; 63:e3961. b Aguirre SD, Ali M, Monsur, Salena BJ, Li Y. *Biomolecules*. 2013; 3:563. [PubMed: 24970181] c Huang P-JJ, Liu M, Liu J. *Reviews in Analytical Chemistry*. 2013; 32:77.
26. a Gerasimova YV, Cornett EM, Edwards E, Su X, Rohde KH, Kolpashchikov DM. *ChemBioChem*. 2013; 14:2087. [PubMed: 24106198] b Gerasimova YV, Kolpashchikov DM.

Angew Chem Int Ed Engl. 2013; 52:10586. [PubMed: 24038733] c Gerasimova YV, Kolpashchikov DM. Biosens Bioelectron. 2013; 41:386. [PubMed: 23021850] d Lu CH, Wang F, Willner I. J Am Chem Soc. 2012; 134:10651. [PubMed: 22612395] e Wang F, Elbaz J, Orbach R, Magen N, Willner I. J Am Chem Soc. 2011; 133:17149. [PubMed: 21954996] f Wang F, Elbaz J, Teller C, Willner I. Angew Chem Int Ed Engl. 2011; 50:295. [PubMed: 21082638] g Wang F, Elbaz J, Willner I. J Am Chem Soc. 2012; 134:5504. [PubMed: 22404383]

27. Oh SH, Chater KF. J Bacteriol. 1997; 179:122. [PubMed: 8981988]

28. Long GL, Winefordner JD. Analytical Chemistry. 1983:55.

Figure 1.

Unified sensor architecture and activation mechanism in the presence of various target types. Binding of nucleic acid targets (oligonucleotides or denatured dsDNA) to the detection module by toehold-mediated DNA strand displacement exposed the reporter module toehold. Small molecule targets bound to a structure-switching aptamer in the detection module, similarly exposing the reporter module toehold. In both cases, this allowed the fuel strand to bind and complete displacement of the DNAzyme strand from the complex. The free DNAzyme strand then folded into a catalytically active conformation and generated an amplified fluorescent output by cleaving multiple substrate molecules labeled with a FRET pair.

Figure 2.

Locations of genetic elements and targets on the studied plasmids. Black arrows represent the open reading frame for each GFP protein variant. Locations of target sequences are denoted by white bars. a) Emerald GFP (emGFP) plasmid. b) SNAP25 GFP plasmid.

Figure 3.

Detection of single-stranded DNA target oligonucleotides. a) Individual sensor performance in the absence of target (white), other sensor targets (black), and the correct sensor target (gray). b) Multiplex sensor performance with all five sensors present. c) Limit of detection decreased over 15 hours to ~15 pM.

Figure 4. Biosensor detection of denatured plasmid target sequences. a) Results from emGFP plasmid detection using the five sensors individually. b) Results from SNAP25 plasmid detection using the five sensors individually. c) Results from emGFP and SNAP25 plasmid detection using all five sensors simultaneously in a combined assay. d) Investigating the limit of detection of the five sensors for emGFP plasmid detection. Lines (left axis) show the response of the five sensors with various concentrations of denatured emGFP plasmid, for an LOD (right axis) of ~5nM after 14-15 hours. Data without error bars indicate representative traces.

Figure 5.

Comparison of responses for the E sensor and the combination of all five (C1-3,E,S) sensors, for oligonucleotide targets (solid lines) and emGFP plasmid targets (broken lines). We observed that the plasmid and oligonucleotide targets generated a similar response in the presence of all five sensors after 15 hours.

Figure 6.

Aptamer sensing using modular DNAzyme sensors. Replacing the detection module with an ATP aptamer enabled small molecule sensing. The ATP sensor used a 5 nt loop instead of the typical 8 nt loop. Kinetic traces showed a highly specific response to 1 mM ATP concentration, with no activation in the presence of a related small molecule (GTP) at 1mM. Sensor concentrations were 100 nM with 15 nM excess inhibitor.