

Active Analyte Import Improves the Dynamic Range and Sensitivity of a Vitamin B₁₂ Biosensor

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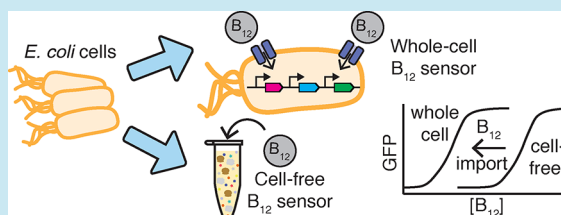
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ABSTRACT: Cell-free systems provide a versatile platform for the development of low-cost, easy-to-use sensors for diverse analytes. However, sensor affinity dictates response sensitivity, and improving binding affinity can be challenging. Here, we describe efforts to address this problem while developing a biosensor for vitamin B₁₂, a critical micronutrient. We first use a B₁₂-responsive transcription factor to enable B₁₂-dependent output in a cell-free reaction, but the resulting sensor responds to B₁₂ far above clinically relevant concentrations. Surprisingly, when expressed in cells, the same sensor mediates a much more sensitive response to B₁₂. The sensitivity difference is partly due to regulated import that accumulates cytoplasmic B₁₂. Overexpression of importers further improves sensitivity, demonstrating an inherent advantage of whole-cell sensors. The resulting cells can respond to B₁₂ in serum, can be lyophilized, and are functional in a minimal-equipment environment, showing the potential utility of whole-cell sensors as sensitive, field-deployable diagnostics.

KEYWORDS: diagnostics, cell-free sensors, whole-cell sensors, circuit tuning



Biosensors have shown great promise for use as low-cost, point-of-care diagnostic tools, as they can harness nature's diverse repertoire of sensing molecules to detect clinically relevant biomarkers and transduce the signal into an easily detectable output. Early biosensors used whole cells as the chassis for sensing and responding,¹ but in recent years, these have been overshadowed by "cell-free" sensor systems, which require small volumes of sample, can be freeze-dried and stored at ambient temperature, have no cell membranes to interfere with the transport of the target molecule, and can produce distinct colored outputs. These sensors use cell-free expression (CFE) systems consisting of a cytoplasmic protein extract, supplemented reagents, and a DNA template to perform transcription and translation of regulator and reporter genes.² They have been used to detect the presence of pathogens such as Ebola and Zika viruses,^{3,4} water contaminants,^{5–7} and small molecules indicative of disease.^{8,9}

Because of the extreme field-friendliness of CFE diagnostics, they hold promise as particularly useful tools in public health surveillance programs, whose capabilities are often limited to assays that can be easily and cheaply performed in the field.¹⁰ Levels of micronutrients, specifically of vitamins A and B₁₂, folate, iodine, iron, and zinc, are a critical concern for public health agencies,¹¹ but a lack of field-deployable diagnostic assays has limited their assessment at the scale necessary to inform public policy. To meet the need for better micronutrient diagnostic tools, our group recently developed a cell-free sensor for serum zinc levels,⁹ and here, we sought to create a similar sensor to quantify clinically relevant B₁₂ levels.

B₁₂, also referred to as cobalamin, is a micronutrient critical to many physiological processes including red blood cell

synthesis and preservation of cognitive function; B₁₂ deficiency can cause anemia, dementia, and other neurological disorders.¹² B₁₂ exists in multiple forms: cyanocobalamin is most commonly used in vitamin supplements, and adenosylcobalamin and methylcobalamin are the forms of B₁₂ that are active cofactors in cells. Since B₁₂ is primarily found in animal products, B₁₂ deficiency is most common among people who eat a vegetarian or vegan diet, but it is also prevalent among elderly populations and in parts of the developing world, with potential genetic causes as well. It can be treated through supplementation or food-fortification programs, but an assessment of the deficiency is first required. Assessment requires a measurement of the total amount of cobalamin present in the serum. Clinically relevant serum concentrations range from 75 to 400 pM, with thresholds for B₁₂ deficiency between 75 and 200 pM.¹² Other biomarkers, such as serum homocysteine and methylmalonic acid, are secondary indicators of B₁₂ deficiency, but total serum B₁₂ is used in initial screens and is considered the best biomarker for population-level assessment of B₁₂ levels.¹² Current methods for evaluating serum B₁₂ require immunoassays or microbiological assays, and while these can be readily performed in a laboratory, they are not amenable to in-field testing.

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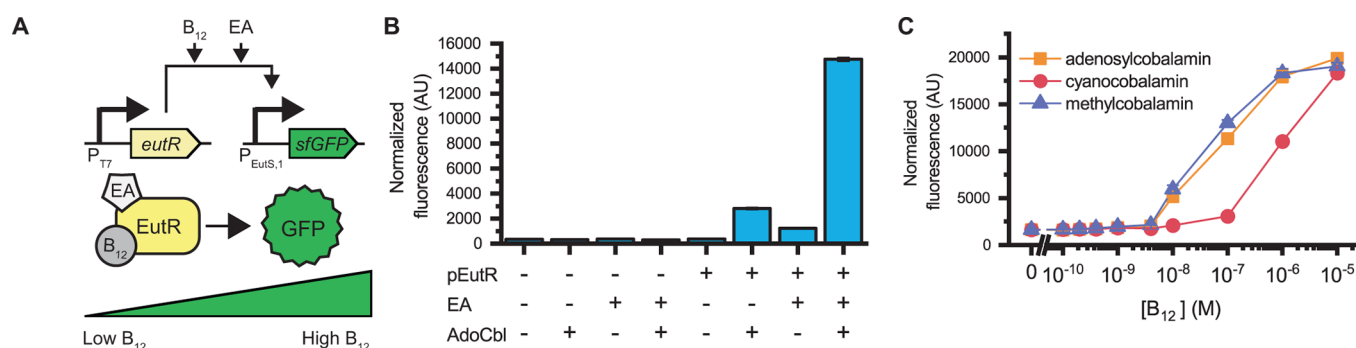


Figure 1. Assessment of B₁₂ levels with cell-free expression systems. (A) Circuit diagram and schematic depicting the design of a cell-free sensor for B₁₂ levels. The first plasmid (pEutR) contains the transcription factor EutR expressed constitutively from the T7 consensus promoter. The second plasmid (pEutSGFP1) contains GFP expressed from P_{EutS,1}, containing EutR's cognate promoter. The addition of adenosylcobalamin (AdoCbl) and ethanolamine (EA) should activate transcription from P_{EutS,1} and lead to high levels of GFP expression. (B) Characterization of the sensor system. The effect of individual and combinatorial addition of the plasmid pEutR (5 nM), EA (5 mM), and AdoCbl (10 μM) was determined via GFP measurements. When the plasmid pEutR is present, the system shows a response to both EA and AdoCbl addition, with the strongest increase in fluorescence when both are added. (C) Response of the CFE sensor to different variants of B₁₂ in the presence of 5 mM EA. The responses to added adenosylcobalamin and methylcobalamin are similar, but much higher concentrations of cyanocobalamin are required to see a comparable increase in fluorescence. The response to all three types of B₁₂ is above clinically relevant concentrations. In all subpanels, data were collected after 16 h of incubation at 37 °C, and error bars indicate the standard deviation of three biological replicates.

Because bacteria have extensive regulatory networks that control import and metabolism of B₁₂, a bacterial-based sensor is a potentially viable option for a field-deployable B₁₂ assay. *E. coli*, as well as over 100 other bacterial species, control B₁₂ import with an adenosylcobalamin (AdoCbl) responsive riboswitch:¹³ in the presence of B₁₂, the riboswitch turns off, preventing additional import of B₁₂. *E. coli* can import multiple types of B₁₂ and has enzymes that convert B₁₂ precursors into active AdoCbl. AdoCbl is a critical cofactor for multiple cellular processes, including the breakdown of ethanolamine (EA), a compound present at high levels in the gut.^{14,15} This process is metabolically intensive and is tightly regulated by the transcription factor EutR, which activates expression of this pathway when both EA and AdoCbl are present.¹⁶

Here, we use *E. coli*'s B₁₂-regulatory elements to create and improve a bacteria-based B₁₂ biosensor. We first characterize a B₁₂-responsive transcription factor in CFE systems and show that it can mediate a response to added B₁₂. However, the resulting CFE sensor shows high variability in response based on the type of B₁₂ added and responds to concentrations far above clinical thresholds. When we use the same sensing framework in a whole-cell sensor, the cells respond to much lower B₁₂ concentrations, counter to the common expectation that CFE sensors typically have better sensitivity due to the lack of a cell membrane to interfere with transport. We demonstrate that active import of B₁₂ into cells is partially responsible for the difference in sensitivity between the two sensor types and that overexpressing these importers can further increase response sensitivity. We then show that the resulting whole-cell sensor can respond to B₁₂ added to human serum, can be lyophilized, and can be incubated in a field-friendly setting, demonstrating the potential utility of whole-cell sensors as a field-deployable platform. Combined, this work illustrates a fundamental limitation in current CFE diagnostic platforms and demonstrates the power of harnessing cellular importers to create novel sensors that meet application-dictated requirements.

RESULTS AND DISCUSSION

Development and Evaluation of a Cell-Free Biosensor for B₁₂

Because of their demonstrated ease-of-use and field deployability, we first aimed to create a B₁₂ sensor using CFE systems. We worked with a B₁₂-responsive transcription factor rather than a previously used *E. coli* B₁₂-responsive riboswitch¹⁷ since it was easier to express with complete functionality in our CFE system (data not shown). We used EutR, a transcriptional activator that responds to AdoCbl: when bound to both ethanolamine (EA) and AdoCbl, EutR binds to its cognate promoter (P_{EutS})¹⁶ and activates the transcription of downstream genes. While EutR has been studied in *Salmonella* and *Escherichia* species, its regulatory elements have not been thoroughly characterized. As the first step in building a EutR-based CFE sensor, we constitutively expressed EutR from a T7 promoter and used the sequence upstream of the *E. coli* *eutS* gene (which is controlled by EutR¹⁶) to control GFP expression (Figure 1A). We designed the promoter sequence (termed P_{EutS,1}) based on the sequence of the arabinose-responsive promoter pBad, since EutR is an AraC-family transcriptional regulator. Because AraC has a dual repressor-activator role that requires the interaction with an operator site over 200 nucleotides upstream of the RNA polymerase-binding domain,¹⁸ we added an additional sequence upstream of the consensus EutR binding site (Supplementary Table 1). We used this promoter for all subsequent experiments but ultimately validated that the addition of the upstream sequence does not confer statistically significant additional benefits (Supplementary Figure 1). The resulting CFE sensor responds to added EA and to added AdoCbl (Figure 1B). Though tests were evaluated after an overnight incubation for convenience, cell-free reactions saturate after 4 h of incubation,¹⁹ so tests could be interpreted at an earlier time point that is more amenable to field testing.

Because the assessment of B₁₂ status requires the measurement of all active B₁₂ in the serum (not just AdoCbl), we tested whether this sensor could respond to other variants of B₁₂, specifically cyanocobalamin and methylcobalamin. While the addition of all three types of B₁₂ increases GFP expression, much higher concentrations of cyanocobalamin are required

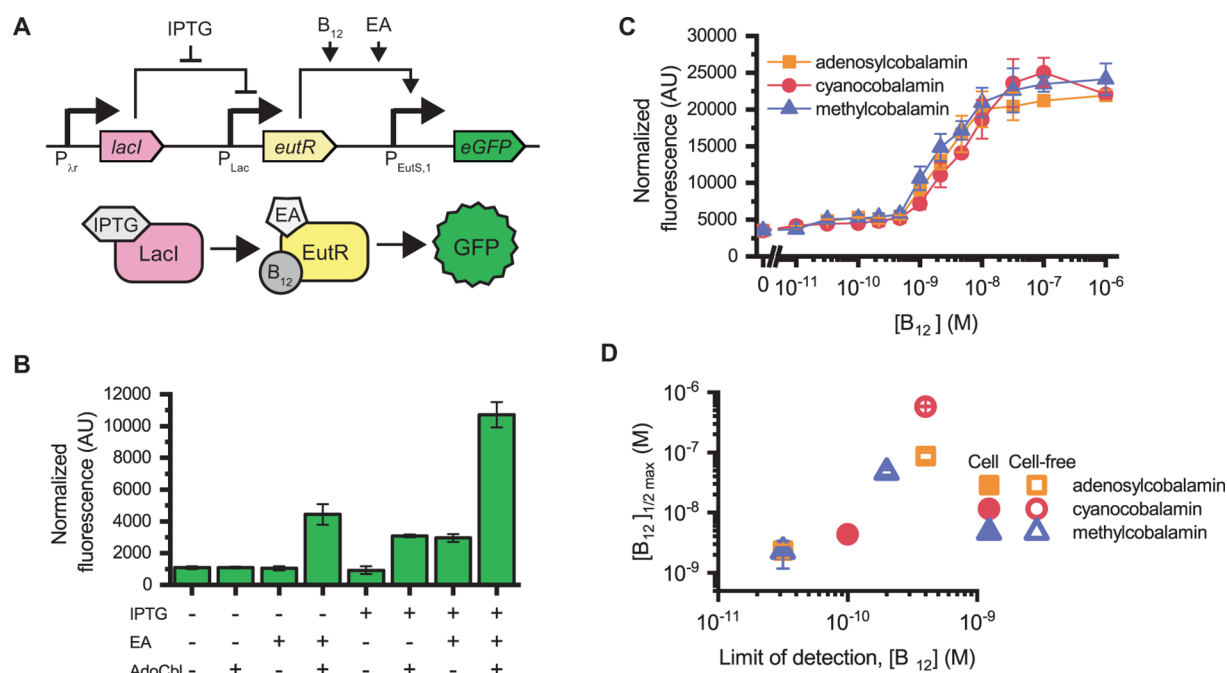


Figure 2. Assessment of B_{12} levels with *E. coli* whole-cell sensor systems. (A) Circuit diagram and schematic depicting the design of a whole-cell sensor for B_{12} levels. The transcription factor EutR is expressed from an IPTG-inducible promoter. The $P_{EutS,1}$ promoter controls the expression of GFP. Maximal GFP expression should occur when IPTG, B_{12} , and EA are all present. (B) Characterization of whole-cell sensor system. Combinations of IPTG, EA, and AdoCbl were added to the growth media. The strongest increase in fluorescence occurs when all three are present. (C) Dose response of whole-cell sensor to different variants of B_{12} in the presence of 5 mM EA. The response to all three is similar, and relative to the cell-free sensor, smaller concentrations of B_{12} induce GFP expression. (D) Sensitivities of the cell-free and whole-cell sensor systems. The whole-cell sensor (filled shapes) has a lower limit of detection and lower $[B_{12}]_{1/2max}$ for all types of B_{12} compared to the cell-free sensor (open shapes). In all subpanels, data were collected after 16 h of incubation at 37 °C, and error bars indicate the standard deviation of three biological replicates.

for the GFP production elicited by lower concentrations of both adenosylcobalamin and methylcobalamin (Figure 1C), suggesting that EutR has different affinities for the different types of B_{12} . An ideal sensor would have a nearly equivalent response to all three types of B_{12} , as clinicians perform assessments by measuring the total serum B_{12} .¹²

To more quantitatively assess the sensor response, we calculated the limit of detection and a representative B_{12} concentration that indicates the on/off threshold. We determined the limit of detection by assessing the statistical significance of fluorescence at a given B_{12} concentration relative to the fluorescence of the reactions with no added B_{12} . We determined the on/off threshold through a calculation of the $[B_{12}]_{1/2max}$ value, which is the concentration of B_{12} that corresponds to half of the maximum fluorescence. Of the two metrics, we considered the $[B_{12}]_{1/2max}$ to be more important for a field-based application, as it represents a threshold between “low” and “high” B_{12} levels that could be easily distinguished with a low-cost, battery-powered blue light device.⁵ The CFE sensor most sensitively detects AdoCbl but with a $[B_{12}]_{1/2max}$ of around 100 nM, far above the target concentration of about 100 pM; variation in the reaction setup or the amount of EutR added does not improve the sensitivity (Supplementary Figure 2). While protein engineering is a potentially viable strategy to alter the sensor’s sensitivity,²⁰ an improvement in the sensitivity by 3 orders of magnitude is a daunting task.

Whole-Cell Sensors Respond to Lower Concentrations of Added B_{12} . Because previous characterization of a B_{12} riboswitch in *E. coli* cells showed $[B_{12}]_{1/2max}$ values of 1

nM,²¹ we hypothesized that the poor sensitivity we observed was a result of the use of a CFE reaction, so we next characterized a EutR-based sensor system in *E. coli* cells (rather than in an *E. coli* CFE lysate). We constructed a plasmid in which the transcription factor EutR is expressed from an IPTG-inducible promoter and EutR controls expression of GFP (Figure 2A). The highest expression should occur when IPTG, EA, and AdoCbl are all added to the system. Sensor cells show the expected behavior: when EutR expression is induced with IPTG, the addition of either EA or AdoCbl increases GFP, but the addition of both most dramatically increases GFP expression (Figure 2B). To assess sensor sensitivity, we next characterized the response to added B_{12} when both IPTG and ethanolamine were added to the media, taking measurements after 16 h of culture. In contrast to the cell-free system, the system responds similarly to all types of B_{12} , and the response occurs at lower B_{12} concentrations (Figure 2C).

Using the calculations of both limit of detection and $[B_{12}]_{1/2max}$, we more quantitatively compared the whole-cell sensor to the cell-free sensor. For all three types of B_{12} , the limit of detection and the $[B_{12}]_{1/2max}$ are lower in the cell-based system (Figure 2D), indicating a more sensitive response in cells. Additionally, in the cell-based sensor system, the differences in $[B_{12}]_{1/2max}$ for the three types of B_{12} are statistically indistinguishable (perhaps because of upregulation of the enzymes that convert different variants of B_{12} into AdoCbl^{22,23}), demonstrating the utility of a whole-cell sensor for the assessment of total B_{12} . Because the identical transcriptional regulator and promoter sequence were used

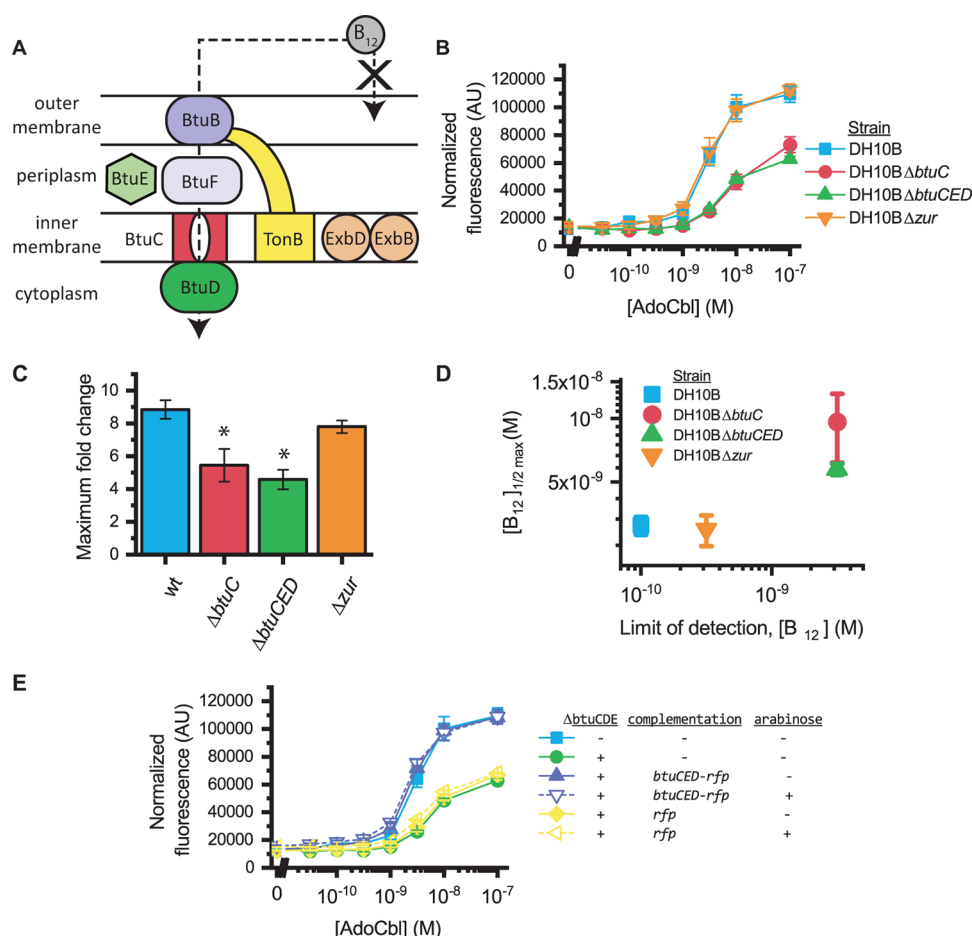


Figure 3. Effect of B₁₂ importers on cellular response to added B₁₂. (A) Schematic of the B₁₂ import system, adapted from Perez *et al.*²⁶ BtuB imports extracellular B₁₂ across the outer membrane, and its activity is driven by a protomotive force generated from TonB, ExbD, and ExbB. In the periplasm, BtuF binds B₁₂ and transports it to the BtuCD complex, which transports B₁₂ into the cytoplasm. BtuE is a periplasmic peroxidase with no known role in B₁₂ import. (B) Response of different strains to added B₁₂. The Δ*btuC* and Δ*btuCED* strains have shallower induction curves relative to both the wild-type strain and the Δ*zur* strain that was used to control for potential confounding effects of gene knockouts. (C) Maximum fold change of the sensor cells. Relative to the wild-type and Δ*zur* strains, the Δ*btuC* and Δ*btuCED* strains have significantly lower fold changes. Asterisks indicate a *p*-value < 0.05 relative to the wild-type strain. (D) Quantitative comparison of the sensitivities of the sensors in different strains. Relative to the wild-type and Δ*zur* strains, the Δ*btuC* and Δ*btuCED* strains have higher limits of detection and higher [B₁₂]_{1/2max} values. (E) Effect of *btuCED* complementation in response to B₁₂ in the Δ*btuCED* strain. The expression of *btuCED*, both when uninduced on a very repressible promoter and when induced with arabinose, restores a response to B₁₂ that is similar to that of the wild-type strain. Complementation with *rfp* has no effect. In all subpanels, data were collected after 16 h of incubation at 37 °C, reactions contained 5 mM EA, and error bars indicate the standard deviation of three biological replicates.

in both the whole-cell and cell-free sensors, the observed differences in specificity and sensitivity suggest that the use of cells provides some unique and substantial advantage relative to CFE reactions, likely related to some aspect of bacterial regulation.

Active Import of B₁₂ into Cells Increases Sensor Sensitivity. Because CFE systems very closely mimic the environment of the *E. coli* cytoplasm, we hypothesized that the difference in sensitivity between the two systems could be because CFE systems do not have the membrane proteins that import B₁₂ from the extracellular environment into the cytoplasm and effectively concentrate it. In *E. coli* cells, BtuB, an outer membrane protein, binds extracellular B₁₂ and imports it into the periplasm with a protomotive force generated by TonB, ExbD, and ExbB.²⁴ BtuF binds B₁₂ in the periplasm, and a complex of BtuC and BtuD transfer it across the inner membrane and into the cytoplasm (Figure 3A). Despite its location in the *btuCED* operon, BtuE has no known function in B₁₂ transport.²⁵

To test whether the importers are critical for a sensitive response, we constructed strains of *E. coli* in which either *btuC* alone or the *btuCED* genes are knocked out (DH10BΔ*btuC* and DH10BΔ*btuCED*, respectively) and then characterized their response to added B₁₂. Because of the similarity in response to all types of B₁₂ (Figure 2C), we only used AdoCbl for these and all subsequent evaluations. To control for the effects of the knockout process, we simultaneously characterized a previously engineered strain with a zinc-responsive transcription factor knocked out (DH10BΔ*zur*). Though all strains grow similarly (Supplementary Figure 3), cells in both *btu* knockout strains have much shallower induction curves than the wild-type and Δ*zur* strains (Figure 3B), resulting in lower fold changes (Figure 3C), higher limits of detection, and higher [B₁₂]_{1/2max} values (Figure 3D), indicating that wild-type strains have a higher detection sensitivity, dynamic range, and test interpretability. By all metrics used for comparison, DH10BΔ*btuC* and DH10BΔ*btuCED* sensor cells are statistically indistinguishable, which is consistent with previous work

showing that the removal of BtuC from the importer complex is sufficient to decrease the response to added B₁₂.²⁶ Notably, Δ *btuC* and Δ *btuCED* strains still show some response to added B₁₂, consistent with previous work demonstrating detectable B₁₂ uptake in a Δ *btuC* strain.²⁷ High levels of periplasmic B₁₂ (resulting from active BtuB-mediated import across the outer membrane) likely drive passive B₁₂ diffusion across the inner membrane.

To confirm that the sensitivity decrease is caused by the removal of B₁₂ importers, we performed complementation experiments by expressing *btuCED* from an arabinose-inducible promoter (termed P_{Bad,weak}) that we had previously engineered to have very low basal activity.²⁸ To confirm the expression of the *btuCED* genes, we expressed *rfp* in the same operon and used an identical plasmid with all elements except the *btuCED* genes as a negative control. As expected, complementation of *rfp* alone has no effect on the overall response to B₁₂, and induction of the *btuCED* genes with arabinose restores the response to that of the wild-type strain (Figure 3E). Interestingly, *btuCED* complementation, even without added arabinose, also restores the wild-type response. These cells have no detectable RFP production (Supplementary Figure 4), indicating that very low levels of B₁₂ importers are sufficient to increase the intracellular B₁₂ concentration to that present in wild-type *E. coli* cells.

To test whether the importance of cellular importers is specific to our selected B₁₂ sensor (EutR), we also characterized a B₁₂ riboswitch construct in the wild-type and Δ *btuCED* strains. The riboswitch is located in the 5' untranslated region of *btuB* and naturally controls the expression of the *btuB* gene in *E. coli* at both the transcriptional and translational levels.¹³ It is an "off" riboswitch such that the addition of B₁₂ turns off the expression of this B₁₂ importer, so increased B₁₂ concentrations should lead to decreased GFP expression (Figure 4A). In wild-type and Δ *zur* strains, the riboswitch responds as expected, and in Δ *btuC* and Δ *btuCED* strains, the response to added B₁₂ is much weaker (Figure 4B). Relative to the wild-type and Δ *zur* strains, in Δ *btuC* and Δ *btuCED* strains, cells have lower degrees of repression and higher limits of detection (Supplementary Figure 5), indicating that active B₁₂ import is also critical for sensitive detection with a riboswitch.

Importer Overexpression Can Improve Sensor Response. Because we demonstrated that removing active B₁₂ importers adversely affects sensor response, we next tested whether overexpression of these importers could improve sensor sensitivity. We hypothesized that excess importers would increase the cytoplasmic concentration of B₁₂, which could increase the overall sensor sensitivity and move the response range toward clinically relevant B₁₂ concentrations. To test this theory, we expressed either *btuCED* or *rfp* (as a negative control) from P_{Bad,weak} in wild-type cells, expecting the induction of *btuCED*, but not *rfp*, to increase sensor sensitivity (Figure 5A). Because of the higher dynamic range of the EutR-mediated sensor, we focused on modulating the activity of this sensor rather than the riboswitch-mediated sensor. Contrary to an expected obvious shift toward lower B₁₂ concentrations, sensor cells with overexpressed *btuCED* show similar induction profiles to cells that have either no supplementation or *rfp* supplementation (Figure 5B). However, more quantitative analysis reveals that induction of the *btuCED* genes slightly lowers the [B₁₂]_{1/2max} (Figure 5C), partially supporting the

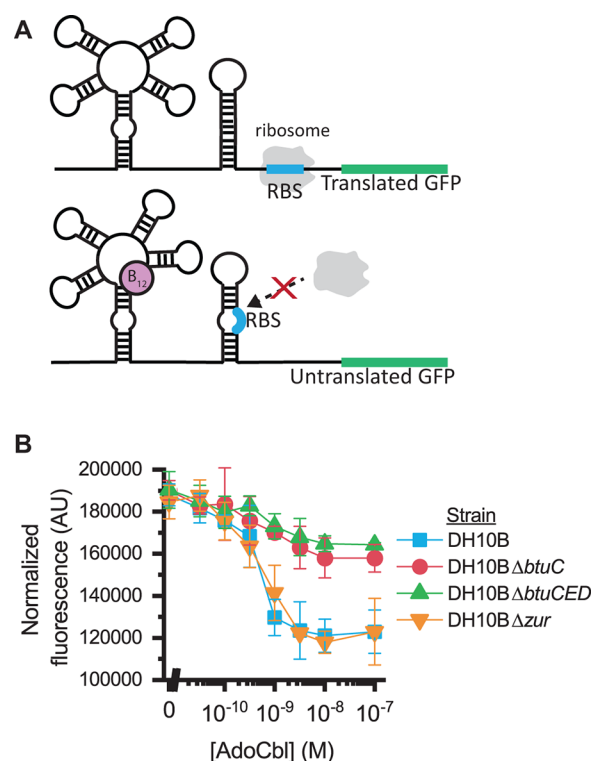


Figure 4. Importers are critical for sensitive B₁₂ detection with a riboswitch. (A) Schematic of B₁₂-responsive riboswitch controlling translation of GFP. In the absence of B₁₂, RNA folds so that the ribosomal binding site (RBS) is accessible to the ribosome, enabling translation of GFP. In the presence of B₁₂, the riboswitch binds to B₁₂, leading to different folding of the RNA and occlusion of the RBS in a hairpin, preventing translation of GFP. Riboswitch regulation also occurs at the transcriptional level (not shown). (B) Riboswitch-mediated response of different strains to added B₁₂. The Δ *btuC* and Δ *btuCED* strains have shallower induction curves relative to both the wild-type strain and the Δ *zur* strain, resulting in less repression upon addition of B₁₂ and higher limits of detection. Data were collected after 16 h of incubation at 37 °C, reactions contained 5 mM EA, and error bars indicate the standard deviation of three biological replicates.

feasibility of using importer overexpression to improve sensor response.

Toward a Field-Deployable B₁₂ Sensor. We next tested whether a whole-cell sensor for B₁₂ could meet some of the requirements for field-friendly test assessment, which is a primary reported advantage of cell-free sensors. We envisioned being able to lyophilize sensor cells, send them to the testing site, incubate cells with just body heat, and interpret the results in the field (Figure 6A). Recently developed low-cost, portable devices such as a hand-operable centrifuge²⁹ and a 3D-printed fluorescent illuminator⁵ could make parts of this testing pipeline possible, but implementation requires that the sensor system responds to B₁₂ (1) in the matrix of unprocessed human serum, (2) following lyophilization, and (3) when incubated with only body heat.

We have previously shown that cells can survive and be metabolically active in untreated human serum if added at a large enough inoculation density.^{30,31} With this as a starting point, we used a large culture of uninduced sensor cells to densely inoculate smaller cultures containing high serum concentrations and the appropriate inducers. Interestingly, cells grown in 25% and 50% serum show dramatically higher

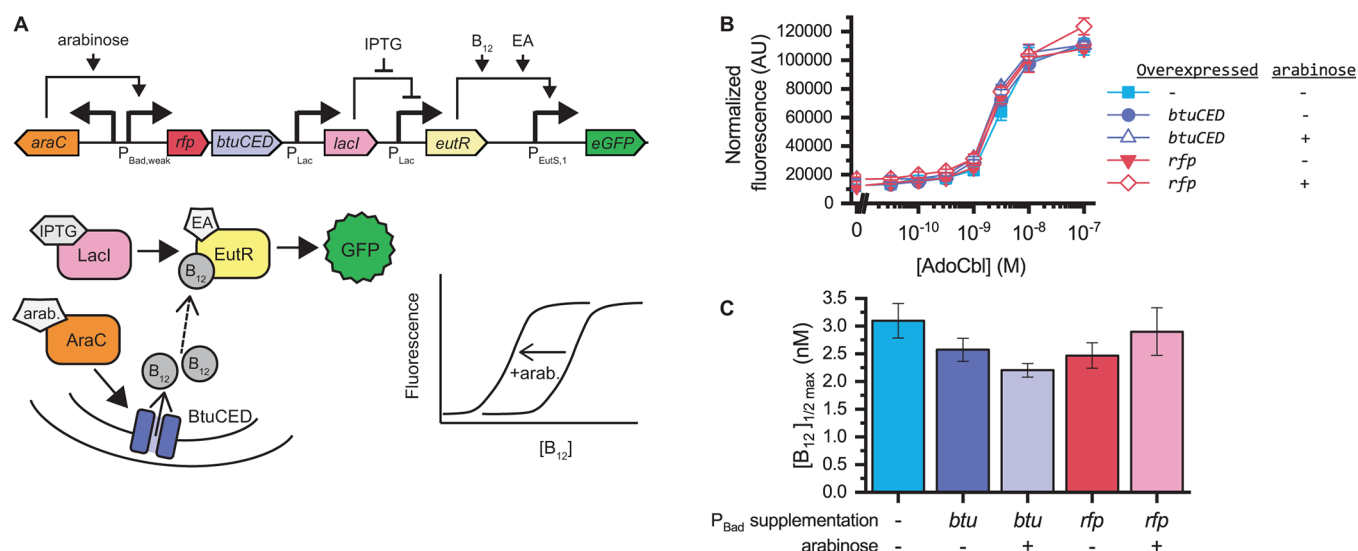


Figure 5. Effect of importer overexpression on the sensitivity of whole-cell B_{12} sensors. (A) Circuit diagram and schematic depicting the design to improve the sensitivity of a B_{12} sensor. Arabinose induces the expression of the reporter *rfp* and *btuCED* genes, ideally leading to an increased import of B_{12} into the cytoplasm and a response to lower concentrations of added B_{12} (inset schematic graph). The identical construct without *btuCED* was used as a control. (B) Effect of importer overexpression in response to added B_{12} . Qualitatively, the behaviors of the different strains and conditions seem similar. (C) Effect of importer overexpression on $[B_{12}]_{1/2max}$. Of the strains tested, the one with the lowest value relative to the base strain and relative to the strain with arabinose-induced *rfp* is the strain with arabinose-induced *btuCED*. In all subpanels, data were collected after 16 h of incubation at 37 °C, reactions contained 5 mM EA, and error bars indicate the standard deviation of three biological replicates.

GFP production at all B_{12} concentrations (Figure 6B). Cells grown in 25% serum have slightly lower fold changes than cells grown in M9 alone, but the $[B_{12}]_{1/2max}$ values and the limits of detection are indistinguishable (Supplementary Figure 6), demonstrating the feasibility of using a whole-cell sensor to measure biomarkers in human serum.

We next showed that simple sensor cells could function following lyophilization and storage at ambient temperature. Sensor cells expressing a simple genetic circuit (Supplementary Figure 7A) were lyophilized using a 10% sucrose solution as a lyoprotectant. Lyophilized cells are viable and retain their ability to respond to added inducer, showing a response to added arabinose that is indistinguishable from control cells that had been stored at 4 °C (Supplementary Figure 7B,C). Interestingly, the induction of GFP prior to lyophilization reduced cell viability by over 2 orders of magnitude, demonstrating the importance of freeze-drying cells in the “off” state.

Finally, we lyophilized cells containing the B_{12} -responsive circuit, stored them at room temperature, and rehydrated them in 50% human serum containing a range of added B_{12} concentrations. We simultaneously evaluated a field-friendly incubation technique by incubating cells either in ventilated round-bottom culture tubes in a standard shaking incubator or in sealed microcentrifuge tubes taped to the user’s stomach. Lyophilized cells grown in a standard shaking incubator or incubated with just body heat and agitation through standard daily motion show a response to B_{12} similar to untreated cells cultured under normal conditions (Figure 6C). In fact, lyophilized cells that were incubated on the body have a lower limit of detection and a significantly higher fold change (Figure 6D,E). Together, this demonstrates that whole-cell sensors can be engineered to be the basis of field-deployable assays with similar ease to that of cell-free sensors.

CONCLUSION

Here, we demonstrate the development of a biosensor for B_{12} levels and show the importance of active B_{12} import for improved response sensitivity. The CFE-based sensor responded to added B_{12} , but its sensitivity limit was far above the clinically relevant range. Partially because of the presence of B_{12} importers, a whole-cell sensor using the same sensing machinery responded to lower concentrations of B_{12} , and overexpressing the importers further increased sensitivity. Despite these successes, the sensitivity of the presented whole-cell sensor is still just above clinically relevant B_{12} concentrations, likely because the engineered sensor cells harness just a small fraction of the B_{12} import machinery (Figure 3A). Overexpression of the other proteins involved in B_{12} import (such as *BtuB*) or engineering strategies that reduce intracellular B_{12} metabolism and storage could increase the cytoplasmic pool of free B_{12} and thus further improve response sensitivity. Alternatively, the naturally occurring B_{12} riboswitch (with an inverter) could be used in combination with the transcription factor-based sensor, which together, might move the limit of detection closer to the clinically relevant thresholds. Additionally, while the 16 h incubation time required for the whole-cell assay is longer than ideal for an in-field test, nutritional epidemiologists have indicated that results in this time frame would still be useful to them (personal communications): the results of in-field B_{12} assays would be used on an aggregate population scale rather than for the diagnosis of individuals, which means that an overnight incubation would still provide information in a useful time frame.

CFE systems are well-poised to become a cornerstone of diverse point-of-care diagnostics,³² but here, we have shown that their easy-to-use, open reaction setup inherently precludes them from harnessing membrane transporters that could concentrate target biomarkers and thus increase detection sensitivity. The incorporation of importers into CFE sensor

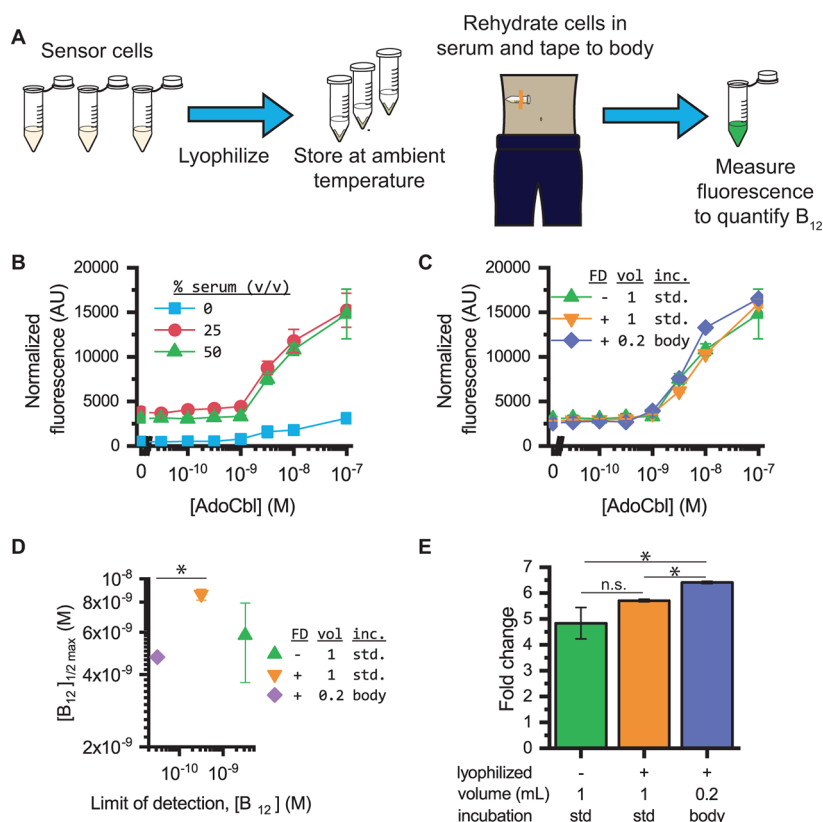


Figure 6. Whole-cell sensors have the potential for use as field-deployable diagnostics. (A) Schematic of workflow for evaluating the in-field potential of whole-cell sensors. Sensor cells are aliquoted into individual test tubes, and cells are lyophilized. Lyophilized cells are stored at ambient temperature and then rehydrated with a volume of serum corresponding to what could be taken from a fingerpick of blood. Sensor cells are incubated at roughly 37 °C by taping a sealed tube to the abdomen. After overnight incubation, fluorescence is assessed with a plate reader, although a field-friendly illuminator could alternatively be used. (B) Response to added B₁₂ of cultures that contain unprocessed human serum. Cultures of uninduced sensor cells were used to inoculate smaller cultures containing 0%, 25%, or 50% serum. The addition of serum increases fluorescence at all B₁₂ concentrations, and cultures in serum show a response to the same B₁₂ concentrations as cultures without added serum. (C) Effect of lyophilization and incubation conditions in response to B₁₂ in 50% unprocessed human serum. Induction curves are similar across all conditions tested. (D) Quantitative assessment of the effect of lyophilization and incubation conditions. Lyophilized (FD) cells have a lower limit of detection than untreated cells. Lyophilized cells incubated in standard culture conditions have a significantly higher [B₁₂]_{1/2max}. (E) Maximum fold change of the sensor cells. Relative to both untreated cells and lyophilized cells incubated in standard conditions, lyophilized cells incubated on the body have a significantly higher maximum fold change. For panels (C), (D), and (E), “FD” indicates whether cells were lyophilized; “vol” indicates the volume (in mL) of serum added to the test; “inc.” indicates the method of incubation, with “std.” indicating incubation in a standard shaking incubator and “body” indicating the incubation taped to the user’s stomach. Asterisks indicate a *p*-value < 0.05. In all subpanels, data were collected after 16 h of incubation at 37 °C, reactions contained 5 mM EA, and error bars indicate the standard deviation of three biological replicates.

systems is theoretically possible, since CFE systems can be encapsulated in membrane-like compartments that can be used to express functional membrane-bound proteins.^{33–35} Much of the work in this area has been toward the development of artificial cells, but CFE diagnostics could benefit from the same advances. While designing and optimizing whole-cell sensors is more time-consuming than for CFE sensors because of longer design–build–test cycles and complications that can arise from disrupting native cell metabolism, we have demonstrated here that whole-cell sensors, based on their potential for enhanced sensitivity and for field-friendly operation, can in fact serve as the basis for field-deployable diagnostics that enable sensitive assessment of serum biomarkers in a minimal-equipment setting.

METHODS

Materials. T4 DNA ligase, T5 exonuclease, Taq ligase, Phusion polymerase, Q5 polymerase, and restriction endonucleases were purchased from New England Biolabs (Ipswich,

MA, USA). E.N.Z.A. Plasmid Mini and Midi Kits were purchased from Omega Biotek (Norcross, GA, USA), and QIAquick PCR Purification Kits and QIAquick Gel Extraction Kits were purchased from QIAGEN (Valencia, CA, USA). Pooled human serum was purchased from Corning (Corning, NY).

Strains and Plasmids. *Escherichia coli* K12 DH10B (New England Biolabs, Ipswich, MA) was used for plasmid assembly and as the wild-type *E. coli* host strain. Lambda red recombination³⁶ was used to make all knockout strains. The kanamycin resistance cassette was amplified from the plasmid pKD4 using primers that contained the P1 and P2 priming sequences specified in Datsenko and Wanner³⁶ and an additional 50 nucleotides of the appropriate upstream (P1) or downstream (P2) homologous sequence for the specific knockout. The PCR product was gel-purified and transformed via electroporation into DH10B cells that contained the plasmid pKD46, which expresses the lambda red recombinase under control of the P_{Bad} promoter. Two knockout strains were

constructed: a strain with the *btuC* gene deleted to make DH10B Δ *btuC* and a strain with the *btuC*, *butE*, and *btuD* genes deleted to make DH10B Δ *btuCED*. Successful knockouts were selected for on kanamycin plates. Replacement of the target gene with the kanamycin cassette was confirmed via PCR with primers specific to upstream and downstream genomic sequences and primers within the kanamycin resistance cassette and the *btuC* gene. The kanamycin selection marker was not excised.

The plasmid pJL1, with a *ColE1* origin and kanamycin resistance cassette, was used as the backbone vector for all plasmids used in cell-free experiments. The plasmid pSB3T5, with a *p15A* origin and tetracycline resistance cassette, was taken from the Standard Registry of Biological Parts and used as the backbone vector for all plasmids used in the whole-cell experiments.

Cloning and Construct Assembly. All constructs were assembled with either Gibson assembly³⁷ or restriction endonuclease digestion of components and subsequent ligation and transformation following the BioBricks idempotent standard assembly.³⁸ LB medium composed of 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone was used for all cell growth during the cloning steps. The following antibiotics were used for the appropriate selection: tetracycline (15 μ g/mL) and kanamycin (30 μ g/mL). The coding sequences for the *eutR*, *btuC*, *butE*, and *btuD* genes, for the EutR-responsive promoters, and for the *btuB* riboswitch were isolated from DH10B genomic DNA; *sfGFP* was amplified from the plasmid pJL1, and $P_{\text{Bad,weak}}$, P_{Lac} , *araC*, *lacI*, and *eGFP* were amplified from previously constructed plasmids.²⁸ All plasmid sequences are described in the [Supporting Information](#), and the annotated sequences of all plasmids are available as [Supplementary Files](#).

Preparation of Cellular Lysate. Cellular lysate for all experiments was prepared as previously described.^{39,40} BL21-(DE3) cells were grown in 2 $\times$ YTP medium at 37 $^{\circ}$ C and 180 rpm to an OD of 2.0, which corresponded with the midexponential growth phase. Cells were then centrifuged at 2700 rcf and washed three times with S30 buffer. S30 buffer contains 10 mM Tris-acetate (pH 8.2), 14 mM magnesium acetate, 60 mM potassium acetate, and 2 mM dithiothreitol. After the final centrifugation, the wet cell mass was determined, and cells were resuspended in 1 mL of S30 buffer per 1 g of wet cell mass. The cellular resuspension was divided into 1 mL aliquots. Cells were lysed using a Q125 Sonicator (Qsonica, Newton, CT) at a frequency of 20 kHz and at 50% amplitude. Cells were sonicated on ice with three cycles of 10 s on, 10 s off, delivering approximately 180 J, at which point the cells appeared visibly lysed. An additional 4 mM of dithiothreitol was added to each tube, and the sonicated mixture was then centrifuged at 12 000 rcf and 4 $^{\circ}$ C for 10 min. The supernatant was removed, divided into 0.5 mL aliquots, and incubated at 37 $^{\circ}$ C and 220 rpm for 80 min. After this runoff reaction, the cellular lysate was centrifuged at 12 000 rcf and 4 $^{\circ}$ C for 10 min. The supernatant was removed and loaded into a 10 kDa MWCO dialysis cassette (Thermo Fisher). Lysate was dialyzed in 1 L of S30B buffer (14 mM magnesium glutamate, 60 mM potassium glutamate, 1 mM dithiothreitol, pH-corrected to 8.2 with Tris) at 4 $^{\circ}$ C for 3 h. Dialyzed lysate was removed and centrifuged at 12 000 rcf and 4 $^{\circ}$ C for 10 min. The supernatant was removed, aliquoted, and stored at -80 $^{\circ}$ C for future use.

Cell-Free Reactions. Cell-free reactions for all experiments were run as previously described.⁴¹ Each cell-free reaction contained 0.85 mM each of GTP, UTP, and CTP, in addition to 1.2 mM ATP, 34 μ g/mL folinic acid, 170 μ g/mL *E. coli* tRNA mixture, 130 mM potassium glutamate, 10 mM ammonium glutamate, 12 mM magnesium glutamate, 2 mM each of the 20 standard amino acids, 0.33 mM nicotinic adenine dinucleotide (NAD), 0.27 mM coenzyme-A (CoA), 1.5 mM spermidine, 1 mM putrescine, 4 mM sodium oxalate, 33 mM phosphoenol pyruvate (PEP), 27% cell extract, 10 nM of the GFP reporter plasmid, and 5 nM of the regulator plasmid pEutR, unless otherwise specified. Unless otherwise specified, reactions contained 5 mM ethanolamine and 10 μ M adenosylcobalamin.

In experiments in which EutR was pre-expressed and added to the reaction ([Supplementary Figure 2](#)), EutR was first produced overnight in a cell-free reaction. Reactions were carried out in 1.5 mL microcentrifuge tubes; the reaction volume in each tube was 50 μ L, with the regulator plasmid pEutR added at 20 nM. Reactions were incubated at 30 $^{\circ}$ C for 16 h. Reactions were then centrifuged at 12 000 rcf and 4 $^{\circ}$ C for 10 min, and the supernatant was removed and subsequently used. The overnight EutR reaction was added to fresh reactions at the specified concentration.

CFE reactions were run in 8 μ L volumes in 384-well small volume plates (Greiner Bio-One), and a clear adhesive film was used to cover the plate and prevent evaporation. Plates were incubated for 16 h at 37 $^{\circ}$ C, and fluorescence was measured with a plate reader (Synergy4, BioTek). Excitation and emission for sfGFP were 485 and 510 nm, respectively.

Cell Culture. For expression experiments performed in cells without serum, M9 media contained 1 $\times$ concentration of 5 $\times$ M9 salts (Difco), 0.01% (w/v) thiamine hydrochloride, 2 mM magnesium sulfate, 100 μ M calcium chloride, 1.92 g/L SC-Ura (Sunrise Scientific), and 0.56% (v/v) glycerol. When indicated, 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), 5 mM ethanolamine, 1 mM arabinose, and 200 nM adenosylcobalamin were added. For GFP expression, freshly transformed colonies were inoculated in triplicate into 3 mL of M9 medium with tetracycline and grown at 37 $^{\circ}$ C and 180 rpm overnight. Then 10 μ L of each starter culture was then used to inoculate 1 mL of M9 media containing tetracycline, the appropriate inducers, and the specified concentration of B₁₂. Cultures were grown at 37 $^{\circ}$ C and 180 rpm for 18 h. Cultures were vortexed, and 150 μ L was removed for OD₆₀₀ and fluorescent analysis.

For expression experiments performed with added serum, 3 mL starter cultures were inoculated in triplicate and grown as previously described. After overnight growth, 1 mL of each starter culture was used to inoculate 150 mL of M9 media containing tetracycline, and this culture was grown at 37 $^{\circ}$ C and 180 rpm overnight. Cells were concentrated through centrifugation and resuspended in M9 without inducers to an OD of 80. This mixture was used to inoculate cultures of M9 containing the specified amount of serum, tetracycline, the appropriate inducers, and the specified concentration of adenosylcobalamin. Cultures were inoculated to a final OD of 2.0 and grown at 37 $^{\circ}$ C and 180 rpm for 18 h. Cultures were vortexed, and 150 μ L was removed for OD₆₀₀ and fluorescent analysis.

Lyophilization of Bacterial Cells. To prepare cells for lyophilization, saturated 150 mL cultures of sensor cells were centrifuged; the supernatant was removed, and cells were resuspended in M9 media to an OD of approximately 50. At

this point, the dense culture was divided into two groups: a control group and a lyophilization group. The control cells were stored at 4 °C. The cells to be lyophilized were subject to further processing: cells were resuspended in water to an OD of 2.0; then, the culture was recentrifuged, the supernatant was removed, and the cells were resuspended in an identical volume of a 10% sucrose solution. 200 μ L of the solution was aliquoted into 2 mL microcentrifuge tubes, and a needle was used to poke a hole in the lid. Tubes were flash-frozen in liquid nitrogen and transferred to a prechilled Labconco Fast-freeze flask that contained a small amount of liquid nitrogen. Care was taken to transfer samples quickly to prevent thawing. Flasks were connected to a LabConco benchtop freeze-drier and lyophilized at -50 °C and 0.05 mbar for 12 h. Tubes were then removed and immediately recapped with a new lid. Tubes were stored in a sealed bag containing desiccant packets at room temperature for 2 days prior to testing. At the time of testing, lyophilized cells were resuspended in a 200 μ L solution of M9 that contained 100 μ L of untreated human serum, the appropriate inducers, and the specified concentration of adenosylcobalamin. Control cells were removed from storage at 4 °C and used to inoculate a 200 μ L solution of M9 containing 100 μ L of untreated human serum to an OD of 2.0.

Measurement of Optical Density and Fluorescence.

Optical density, GFP production, and RFP production of cells were quantified with a Biotek Synergy H4 plate reader. 150 μ L of culture was placed in a clear flat-bottom 96-well plate. Optical density was quantified by measuring the absorbance at 600 nm. For GFP quantification, fluorescence at 485 nm excitation and 510 nm emission was measured. For RFP quantification, fluorescence at 585 nm excitation and 610 nm emission was measured.

Data Processing and Statistical Analysis. For CFE experiments, all reported fluorescence values are background-subtracted using the fluorescence of a CFE reaction containing no plasmid. For whole-cell experiments, all reported fluorescence values are OD₆₀₀-normalized. Both fluorescence and OD₆₀₀ values are background subtracted using the corresponding readings of either M9 or M9 containing the specified percentage of human serum.

To determine the limit of detection, the normalized fluorescence values of the cultures with a specified B₁₂ concentration were compared to the values of cultures that had no added B₁₂ with a two-tailed student's *t*-test, assuming equal variance. The lowest concentration of B₁₂ that yielded a *p*-value < 0.05 was considered the limit of detection. To determine the concentration of B₁₂ that corresponds with the half-maximum fluorescence ($[B_{12}]_{1/2max}$), OD-normalized fluorescence and the log₁₀ concentrations of B₁₂ were first fit to a sigmoidal curve using MATLAB. The half-maximum fluorescence was calculated as the average between the maximum and minimum value of the fitted curve, with $[B_{12}]_{1/2max}$ being the corresponding B₁₂ value. All presented values except the limit of detection are the average of three biological replicates. A two-tailed student's *t*-test assuming equal variance was used to evaluate statistical significance.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.9b00429>.

Additional sequence minimally affects behavior of EutR-regulated promoter; altering reaction conditions to improve sensitivity of a cell-free B₁₂ sensor; strain and B₁₂ concentration minimally affect cell growth; RFP expression from cells with either *rfp* or *rfp* and *btuCED* expressed from P_{Bad,weak}; quantitative assessment of riboswitch-mediated response to added B₁₂; quantitative assessment of the response to added B₁₂ in media containing different concentrations of serum; evaluation of the lyophilization method; annotated EutR-responsive promoter sequences; primers used to construct and validate knockout strains; names and descriptions of plasmids used in this study (PDF)

Annotated sequences of all plasmids (ZIP)

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Notes

The authors declare no competing financial interest.

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