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An improved genetically modified *Escherichia coli* biosensor for amperometric tetracycline measurement

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Abstract The bacterial respiratory gene, *nuoA*, was previously used as a reporter gene in an amperometric, whole cell biosensor for tetracycline (Tet) detection. While the *nuoA*-based bioassay responded sensitively to Tet, the signal declined at high Tet concentrations, probably partly due to transgene over-expression. Also, at zero concentration of Tet, the assay registered a relatively high background signal when compared to the *nuoA* knockout *Escherichia coli* strain without the biosensor transgene construct. This was probably due to incomplete repression of *nuoA* expression. In order to reduce gene over-expression, the sensor cells were incubated with Tet at a relatively low temperature (15 °C). Also, a low-copy number plasmid pBR322 was used to carry the transgene, instead of the high-copy number plasmid pBluescript in order to reduce over-expression and to reduce background expression. Both assays improved the biosensor response. By using a low-copy number plasmid and tetracycline resistance, the sensor was less inhibited at higher Tet concentrations; but, this did not significantly increase the linear range of the sensor. The low temperature *nuoA* assay could detect Tet at a range of 0.001–1 μg ml^{-1}. In contrast, the low-copy number *nuoA* assay was able to detect Tet at a range of 0.0001–1 μg ml^{-1}. The detection limit of Tet determined by the low-copy number *nuoA* assay was 0.00023 μg ml^{-1}, which is one order of magnitude more sensitive than in the previous *nuoA* assay.

Keywords Biosensor · *nuoA* · Tetracycline · pBR332 · KFCIII

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Introduction

The SciTOX™ toxicity assay measures the toxicity of compounds to cells through inhibition of bacterial respiration (Tizzard et al. 2004). In this assay, the natural co-substrate, oxygen, is substituted by the redox mediator potassium ferricyanide (KFCIII) (Pasco et al. 2005). Electrons derived from substrate oxidation are released into the electron transport chain and ultimately, to the mediator. The process leads to the accumulation of reduced mediator in solution. The measurable current generated by the transfer of electrons from the mediator to a microelectrode during current-limited amperometry, is used to quantify the magnitude of respiration inhibition and therefore toxicity. The SciTOX™ assay therefore measures general toxicity and does not identify specific compounds. However, by placing a respiration gene under the control of specific inducible promoters, it is possible to make the assay analyte-specific.

In a previous study (Song et al. 2012), genetically modified *Escherichia coli* (*E. coli*) bioassays based on substrate-specific induction of the *nuoA* gene were developed to detect and quantify the antibiotic tetracycline (Tet) in the SciTOX™ assay. The *nuoA* gene is part of the *nuoA-N* operon which encodes the enzyme NADH dehydrogenase I, which catalyses the transfer of electrons from NADH into the start of the respiratory chain (Wackwitz et al. 1999), and functions in both aerobic and anaerobic conditions (Calhoun et al. 1993).

The *nuoA*-based bioassay is as sensitive as visual reporter gene-based (*lacZ*, *gfp* and *lux*) whole cell biosensors (Hansen and Sorensen 2000, 2001; Pikkemaat et al. 2010; Scaria et al. 2009; Shen et al. 2011; Virolainen et al. 2008). Additionally,

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nuoA could also be used as a reporter gene to specifically and sensitively detect other environmental compounds in the SciTOX™ assay (e.g., copper [Cu] and silver [Ag]) rather than Tet, simply by changing the specific inducible promoter (Song et al. 2012).

Two aspects of the assay need to be improved. Firstly, the SciTOX™ current increases as Tet concentrations increase; but beyond a certain concentration, the SciTOX™ current declines rapidly, dropping below the current detected at zero Tet concentration. This limitation reduces the effectiveness of the assay. Secondly, the *nuoA*-based assay has a relatively high background current. In the absence of Tet, the *nuoA* assay showed a significantly higher SciTOX™ signal than the *nuoA* knockout *E. coli* strain without the biosensor transgene construct. This limitation might play a role in lowering the Tet detection sensitivity (detection limit [DL]). Both these limitations were also observed in *lacZ*-based blue-white colour assay. Therefore, this study was directed at overcoming these two limitations, thereby improving the SciTOX™ response (DL and detection range) of the *nuoA* bioassay to Tet.

Tet is known as a protein synthesis inhibitor, which binds to the 30S ribosome subunit (Sambrook and Russell 2001). Tet toxicity might partially cause the decline of the SciTOX™ signal at high Tet concentrations. To overcome Tet toxicity and thereby improve the effectiveness of *nuoA* assay, the f' plasmid which carries the *Tn10* tetracycline resistant gene was introduced into the biosensor .

Proteins over-expressed in *E. coli* often accumulate in the form of inclusion bodies, which are insoluble, inactive aggregates of misfolded proteins (Buchanan 1999; Kiefer 2003). Many factors influence the formation of protein inclusion bodies, including culture media, growth temperature, protein production rate (e.g., gene dosage, promoter strength, mRNA stability, and codon usage), and the availability of heat-shock chaperones (Chen et al. 2003; Ventura and Villaverde 2006). Newly synthesised proteins readily form inclusion bodies at high-growth temperature (37 °C) due to a high-protein synthesis rate (Chen et al. 2003). By lowering the growth temperature to 15 °C, the formation of protein inclusion bodies can be significantly reduced (Lethanh et al. 2005). Similarly, it should be possible to reduce over-expression by reducing the transgene copy number. In a previous study (Song et al. 2012), transgene constructs were carried on the high-copy number, pBluescript-derived, plasmids. In this work, a low-copy number plasmid pBR322 (with 15–20 copies/chromosome) (Mayer 1995) was used as the vector to carry the transgene (fusion of *tetA* promoter with *nuoA* gene [*PtetA*::*nuoA*]), instead of pBluescript.

The high background SciTOX™ current detected at zero Tet concentration from the biosensor strain might be caused by reporter gene basal expression due to incomplete turning off of the *tetA* promoter. The high background reporter gene expression is also likely to be exacerbated by the many transgene copies present in each cell on the high-copy number plasmid. Therefore, we also investigated the possibility of reducing background SciTOX™ signal by placing the transgene on a low-copy number plasmid.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. All bacteria were *E. coli* strains cultured in shaking flask culture in Luria broth (LB) medium at 200 rpm, 37 °C, and supplemented, where appropriate, with: ampicillin 100 μg ml^{-1}, kanamycin (Kan) 50 μg ml^{-1} and Tet 10 μg ml^{-1}.

Construction of plasmids

(pLBRSong10): the low-copy number plasmid pBR322 was digested by *SalI* and *BamHI*, and ligated to the Tet-inducible expression casette *PtetA*::*nuoA* amplified from plasmid pSong10 by polymerase chain reaction (PCR) using primers 5′ – ATCA GTCGAC AAT GGG AAT TGA CGT TCC TTC – 3′ and 5' – ATAG GGATCC TCA ACC GGG ATG AAT TTA TCG – 3' and also digested with *SalI* and *BamHI* restriction enzymes.

PCR and DNA sequencing

PCR for construction of plasmids was by High Fidelity DNA polymerase (Invitrogen, Carlsbad, USA) as per manufacturer's instructions. The PCR reactions were: 94 °C for 3 min

Table 1 Bacterial strains and plasmids

Strain/ plasmid	Genotype/characteristics	Source
BW25113	rrnB DElacZ4787 HsdR514 DE(araBAD)567 DE(rhaBAD)5 68 rph-1	Keio
JW2283	BW25113 Δ*nuoA*	Keio
DH5α	endA1 hsdr17 supE44 thi-1 recA1 gyrA relA1Δ(lacZYA-argF) U169 deoR (phi 80lacZ Δ M15)	Invitrogen
Top10f'	F′{*lac*Iq Tn*10* (TetR)} *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
pBR322	Vector with 299 multi-cloning sites; [Amp^r]; [Tet^r];	Invitrogen
pSong10	pBluescript with *lacZ* replaced by *nuoA* coding region fused to *tetA* promoter	Song et al. 2012
pLBRSong10	pBR332 with *nuoA* coding region fused to *tetA* promoter	This study

followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min followed by one step at 72 °C for 7 min. All plasmids were verified by sequencing the insert and bordering sequences. PCR-amplified DNA fragments were purified with PCR purification kit (Purelink™, Invitrogen, Carlsbad, USA), quantified by nano-drop ND-1000 (100 W. Rockland RD, Montchanin, DE, USA) by measuring the absorbance at 260 nm (A_{260}) and sequenced using ABI Prism 3130xl Genetic analyzer at the Lincoln University Bio-Protection Centre.

Conjugation (transfer Tet-resistant f' plasmid from Top10f' to *nuoA* knockout *E. coli* strains)

Both *E. coli* Top10f' (Tet resistant) and *nuoA* knockout *E. coli* (Kan-resistant) strains were grown in separate flasks with 50 ml of LB broth and appropriate antibiotic, at 37 °C, 200 rpm overnight. 10 ml each of overnight culture was transferred into a new flask. The mixture of the two overnight cultures was then incubated together at 37 °C, 200 rpm for 4–5 h. 100 μl of the mixture was spread onto a LB agar plate which was coated by both Tet/Kan. The plate was then incubated at 37 °C overnight (Raya and Jabri 2008).

SciTOX™ assay

E. coli was grown until late-exponential growth phase (OD_{600} was around 0.5–0.8). The cells were maintained in culture media. The SciTOX™ assay was performed in 24-well microtest plates. 1 ml of cultured cells was incubated with 1 μl of variable concentrations of Tet. The plates were incubated at 37 °C, 200 rpm for 2 h; then, 300 μl of 250-mM KFCIII was added to each well.

Low temperature assay

E. coli was grown until late-exponential growth phase (OD_{600} was around 0.5–0.8). The cells were maintained in culture media. The SciTOX™ assay was performed in 24-well microtest plates. One milliliter of cultured cells was incubated with 1 μl of variable concentrations of Tet. The plates were incubated at 15 °C, 200 rpm for 6 h; then 300 μl of 250-mM KFCIII was added to each well.

For both methods, oxygen was removed from the headspace by nitrogen flushing and excluded from the plates by sealing with an adhesive acetate lid. The microtest plates were wrapped in aluminium foil to exclude light, and incubated on an orbital shaker at 37 °C, 200 rpm for 1 h. Assays were terminated by transferring the contents of each well into 1-ml Eppendorf tubes and centrifuging at 13 K rcf for 4 min to pellet cells. The supernatants were transferred into clean Eppendorf tubes. The samples were stored at 4 °C until microelectrode amperometry analysis could be performed.

Microelectrode amperometry signal detection

For limiting current microelectrode amperometry, a 3-electrode system comprising a working reference and auxiliary electrode, was used to measure the quantity of reduced mediator produced during the microbial incubation. The reference and auxiliary electrode were directly immersed in phosphate saline buffer supporting electrolyte, contained within a cell vial (BAS MF-1082) and chemically connected to the 1-ml sample which was within a working-electrode compartment (BAS MF-2031, 2-ml mini cell). 200-μl samples were injected into BAS MF-2031, 2-ml mini cells. The 25-μm platinum working-electrode was poised at +450 mV relative to the Ag/AgCl reference electrode and anodic current obtained 10 s after imposition of the applied potential was taken as the limiting current value. After each analysis, the working-electrode was re-polished and the BAS MF-2031 mini cell was rinsed in distilled water and dried using a lint-free tissue. The limiting current measured in microelectrode amperometry is proportional to the amount of KFCIII reduced during the 1-h incubation with the induced cells.

Statistical analysis (*P* value)

All the statistical significances (*P* value) were calculated by two-tail *t* test in this paper.

Calculation of detection limit

The DL was calculated as previously described (Group 2005).

$$DL = 3.3\sigma/S \quad (1)$$

Where σ = the standard deviation of the response
S = the slope of the calibration curve

Results

Tet toxicity assay

Part of the reduction in SciTOX™ signal at high analyte concentrations could be due to a toxic effect since some of the transgene biosensor strains developed were not specifically resistant to the analyte. Therefore, the Tet-resistant f' plasmid was transferred into the *nuoA* knockout *E. coli* strain (to create *E. coli* f'). The *E. coli* f' strain was then exposed to a broad concentration range of Tet in the SciTOX™ assay to confirm its Tet resistance. Without the f' plasmid, the strains were affected by toxicity at Tet above 0.1 μg ml^{-1}. In contrast, there was no observed toxic effect detected in either *E. coli* Top10f' or *E. coli* f' assays at Tet up to 10 μg ml^{-1} (Fig. 1).

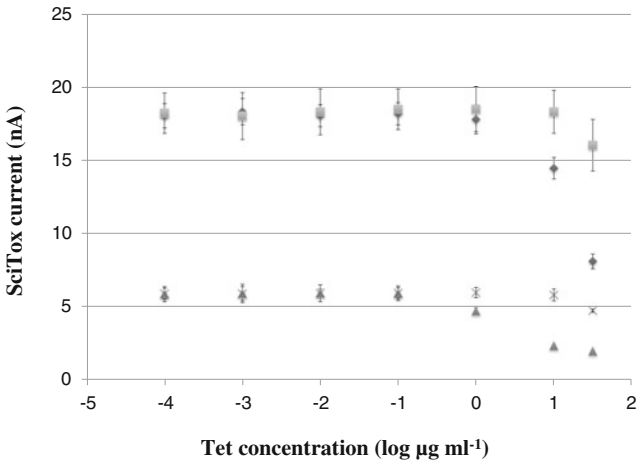

Fig. 1 The comparison of SciTOX™ response (mean ± SD) between unmodified *E. coli* and *nuoA* knockout *E. coli* strains, in the presence and absence of the Tet-resistant encoding f' plasmid. Tet concentration is in log scale. ♦ unmodified *E. coli* assay. ■ *E. coli* Top10f' (Tet resistance) assay. ▲ *nuoA* knockout *E. coli* assay. × *E. coli* f' (*nuoA* knockout and Tet resistance) assay. The nuoA knockout strains lack a key respiration gene and have a lower SciTOX™ current

Tet-resistant *nuoA* assay (*E. coli* f'pSong10 assay)

The response of *E. coli* f'pSong10 sensor to a broad range of concentrations of Tet was tested in the SciTOX™ assay. The assay responded to Tet in a dose-dependent manner from 0.001 to 0.1 µg ml^{-1} Tet and the SciTOX™ signal declined at Tet above 0.1 µg ml^{-1} (Fig. 2a). The DL of the assay was 0.0017 µg ml^{-1} (Fig. 2b). As this strain carried the f' plasmid encoding Tet resistance, the loss of SciTOX™ signal (at Tet from 0.1–10 µg ml^{-1}) was probably not due to Tet toxicity (Fig. 1) but possibly due to over-expression of the transgene.

Low temperature *nuoA* assay (*E. coli* f'pSong10)

If gene over-expression was limiting the optimal induction concentration of Tet, it might be possible to improve the response at high Tet concentrations by using a low temperature assay as low temperatures tend to reduce the loss of function caused by over-expression. Therefore, a low temperature assay was tested using Tet-resistant *nuoA* biosensor (*E. coli* f'pSong10). For this assay, the maximum induction Tet concentration increased to 1 µg ml^{-1} (Fig. 2). The Tet detection range and DL was 0.001 to 1 and 0.0028 µg ml^{-1}, respectively (Fig. 2). However, the SciTOX™ response still declined at non-toxic Tet levels (Fig. 2).

Low-copy number *nuoA* assay (*E. coli* f'pLBRSong10 assay)

Whether the decline in the SciTOX™ response could be reduced by lowering the copy number of the reporter gene was then tested. A low-copy number plasmid, pBR322, was used to carry the transgene (*PtetA*::*nuoA*) to reduce gene over-expression in a Tet-resistant *nuoA* strain carrying the f' plasmid. The response of the sensor to a broad range of Tet concentrations was then tested in the SciTOX™ assay. The sensitivity of this assay to Tet was higher (Fig. 2) than that in other *nuoA* bioassays (Fig. 2). The DL of Tet determined by this assay was 0.00023 µg ml^{-1} (Fig. 2b). This result might indicate that the sensitivity of *nuoA* bioassay could be improved by using a low-copy number plasmid. For this assay, the optimal Tet induction concentration was 1 µg ml^{-1}, and the detection range was from 0.0001–1 µg ml^{-1} (Fig. 2a). However, similar to low temperature *E. coli* f'pSong10 assay (Fig. 2), the SciTOX™ signal declined at Tet above 1 µg ml^{-1} (Fig. 2). A combination of a low temperature assay with the Tet-resistant low-copy number strain was tested but that did not offer a better response. When the low-copy number assay was performed with strains that were not Tet resistant, the signal declined after 0.1 µg ml^{-1} Tet indicating that toxicity could also affect the reporter gene function (Fig. 2).

Tet and mediator (KFCIII) interaction assay

In order to confirm that the decline in SciTOX™ signal at high concentrations of Tet was not due to the interaction between Tet and the mediator (KFCIII), the response of KFCIII to a broad range of concentrations of Tet was tested in the SciTOX™ assay. KFCIII did not respond to Tet in a dose–response manner (data not shown). This assay confirmed that there was no interaction between the mediator (KFCIII) and the analyte (Tet).

Comparison of gene basal expression between high-copy number and low-copy number *nuoA* assays

In order to confirm that gene basal expression could be reduced by using a low-copy number plasmid, the SciTOX™ currents of four different *E. coli* assays were measured and compared at two different concentrations of Tet. Both unmodified *E. coli* and *nuoA* knockout *E. coli* assays were used as controls. At zero Tet, there was still a significant background current (P <0.05) observed in the low-copy number *nuoA* assay (Fig. 3). Despite this, the SciTOX™ background reading was significantly (P <0.005) reduced, in contrast to the high-copy number *nuoA* assay (Fig. 3). At a Tet concentration of 0.1 µg ml^{-1}, the low-copy number *nuoA* assay showed a significantly higher induced SciTOX™ current (P <0.05) than in the high-copy number *nuoA* assay (Fig. 3). This result indicated that the effectiveness of *nuoA* assay was improved by using low-copy number plasmid.

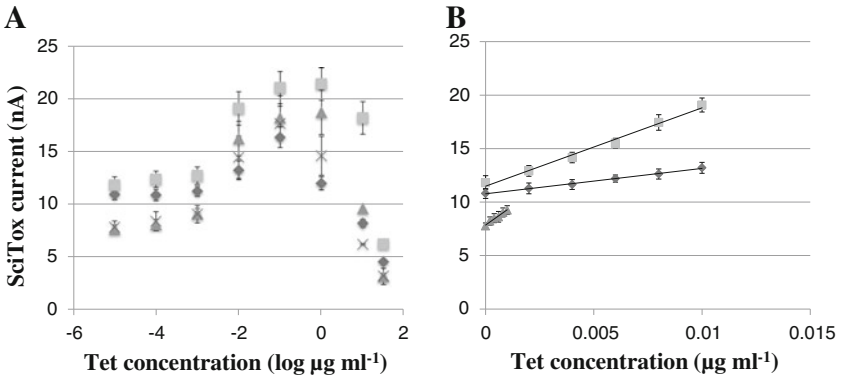

Fig. 2 SciTOX^{TM} response (mean ± SD) of different *E. coli* sensors, exposed to different Tet concentrations in water sample. **a** broad concentration range in log scale. **b** dose-dependent range in linear scale. ♦: represents *E. coli* f' pSong10 (high-copy number, Tet resistance) assay; the fitted linear regression line is y=237.21x+10.78, R^2=0.99 for the dose-dependent data range. ■ 15 °C *E. coli* f'pSong10 (low incubation temperature, high-copy number, Tet resistance) assay; the fitted linear regression line is y=732.57x+11.49, R^2=0.99 for the dose-dependent data range. ▲ *E. coli* f' pLBRSong10 (low-copy number, Tet resistance) assay; the fitted linear regression line is y=1,424.30x+7.90, R^2=0.98 for the dose-dependent data range. × *E. coli* pLBRSong10 (low-copy number) assay

Discussion

The decrease in SciTOX^{TM} signal at high analyte concentrations makes the transgenic SciTOX^{TM} assay ambiguous since at high analyte concentrations, the assay showed a value that is lower than the signal produced at lower analyte concentrations. In practical applications, the ambiguity could be removed by testing samples at more than one dilution, though ideally, that would not be necessary. The mechanism of signal inhibition at higher concentrations might also reduce the magnitude of the response at lower concentrations and that may limit the assay maximal response, reducing the effectiveness of the assay. Consequently, we tested strategies to overcome this limitation.

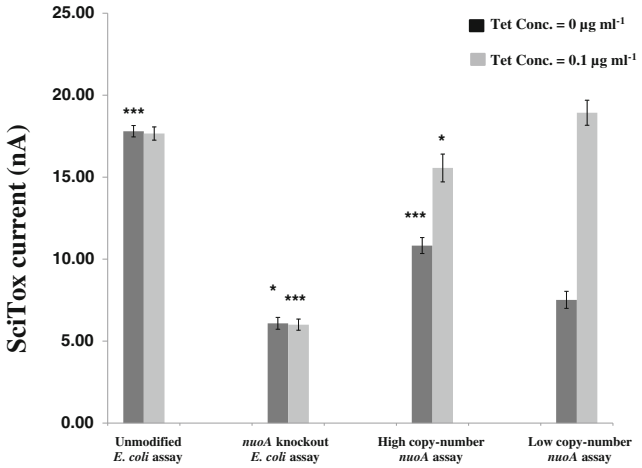

Fig. 3 Amperometric currents comparison between four different *E. coli* assays, when exposed to two different concentrations of Tet in SciTOX^{TM} assay. The name of different *E. coli* strains is given below the corresponding data bar. * significantly different from low-copy number *nuoA* assay. The number of *stars* (*) indicates the significance level. * P <0.05; ** P <0.01; *** P <0.005. The significant difference was determined in paired test. n=6, *error bars* standard error

The test analyte, Tet, is an antibiotic that is toxic to bacterial cells. The *Tn10* Tet-resistant gene carried on the f' plasmid conferred resistance to Tet in our strains to more than 10 µg ml^{-1}. On its own, however, Tet resistance did not significantly improve the assay. Another factor that might decrease the signal at high analyte concentrations is transgene over-expression, leading to the formation of inclusion bodies which is a common limitation of recombinant protein production in bacteria (Ventura and Villaverde 2006), especially in *E. coli* (Mayer and Buchner 2004). Two strategies were tried to reduce over-expression: gene induction at low temperature, and placing the inducible transgene in a low-copy number plasmid. By combining the two strategies to reduce over-expression with the provision of Tet resistance, it was possible to increase the maximum SciTOX^{TM} response and to significantly increase the SciTOX^{TM} signal at higher analyte concentrations. The loss of signal at high analyte concentrations was likely at least in part due to a combination of toxicity of the analyte and loss of active *nuoA* protein due to over-expression of *nuoA* at high concentrations of the analyte.

Clearly, it is advantageous if the assay is to be used to measure toxic analytes at high concentrations, to provide the biosensor bacteria with a mechanism for resistance to the analyte. The *Tn10* encoded the Tet-resistant gene *tetA* and the repressor gene *tetR* (Beck et al. 1982; Gatz and Quail 1988; Postle et al. 1984). *TetA* encodes an inner membrane transport protein TetA, which facilitates the active efflux of Tet from the cytosol (Speer et al. 1992; Teo et al. 2002). The transcription of TetA protein is controlled by an inducible *tetA* promoter, which is regulated by the repressor TetR protein (Oehmichen et al. 1984).

It is surprising that expression of an efflux pump was not detrimental to the assay, as it should lead to a lower Tet concentration inside the cell and consequently, lower *nuoA* gene induction. Presumably, there was not enough time available during the induction period for the efflux pump to be expressed and to reduce the intracellular Tet concentration sufficiently to noticeably reduce *nuoA* induction. This suggests that efflux pumps, which are quite a common mode of toxin resistance in bacteria, are compatible with gene induction, whole-cell biosensors.

The background SciTOX^{TM} signal from uninduced transgenic cells was significantly higher than the background SciTOX^{TM} signal obtained from the parent *nuoA* strain without the inducible transgene construct. This suggests that the inducible transgene was not fully repressed even when there was no target analyte present. The high background transgene expression was probably exacerbated by the fact that the construct was carried on a high-copy number plasmid. Once the transgene was transferred to a low-copy number plasmid, the background expression was reduced to a level very similar to that of the parent strain.

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