HTGAA Final Project Proposal

Cell-Free Butyrate Biosensor for Gut Health Diagnostics

Author: 2026a-emmanuel-pereyra, Rosario, ARG.
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System: Cell-Free Expression (BL21 DE3 Lysate)
Industry Partners: Twist Bioscience · Ginkgo Bioworks · Opentrons

SECTION 1: ABSTRACT

Short-chain fatty acids (SCFAs), particularly butyrate, are critical metabolites produced by gut microbiota through fermentation of dietary fiber and serve as key indicators of gut microbiome health and colonocyte function. Dysregulation of butyrate levels has been implicated in inflammatory bowel disease (IBD), colorectal cancer (CRC), and broader metabolic dysfunction, yet current clinical detection methods rely on expensive, slow, and laboratory-intensive chromatographic techniques inaccessible to most clinical settings. This project proposes the design and experimental validation of a **cell-free transcription factor-based biosensor** capable of detecting butyrate in stool sample extracts with high sensitivity and specificity. The central hypothesis is that a synthetic genetic circuit encoding a butyrate-responsive BudR transcription factor coupled to a NanoLuc luciferase reporter can be expressed in a BL21 DE3 cell-free lysate system to produce a quantitative, dose-dependent luminescent signal in response to physiologically relevant butyrate concentrations. **Aim 1** will design, synthesize, and functionally validate the BudR-NanoLuc biosensor construct ordered from Twist Bioscience and tested in an automated 96-well cell-free expression platform at Ginkgo Bioworks. **Aim 2** will optimize biosensor sensitivity, dynamic range, and matrix compatibility with stool extracts. **Aim 3** envisions deployment of this biosensor technology as a portable, point-of-care diagnostic platform for gut microbiome health monitoring applicable to IBD screening and microbiome research. This project integrates synthetic biology, cell-free expression, automated liquid handling, and clinical diagnostic design into a cohesive and immediately actionable research program.

SECTION 2: PROJECT AIMS

Aim 1 - Experimental Aim

The first aim of my final project is to **design, synthesize, and functionally validate a cell-free butyrate biosensor** by utilizing **BudR transcription factor-regulated NanoLuc luciferase expression in a BL21 DE3 lysate system**, with the biosensor plasmid ordered as a whole-plasmid synthesis from **Twist Bioscience** and characterized across a butyrate dose-response curve using an automated 96-well workflow at Ginkgo Bioworks with luminescence detection on the PHERAstar FSX.

Key deliverables:

- Whole plasmid encoding BudR(His6)-P_bud-NanoLuc synthesized and ordered from Twist Bioscience
- Constitutive NanoLuc control plasmid (positive control) ordered from Twist Bioscience
- Cell-free expression reactions assembled in 96-well format using Multiflo dispenser

- Butyrate dose-response curve (0–20 mM) with time-course luminescence reads at 1h, 2h, 4h on PHERAstar FSX
- Detection validated in synthetic buffer and spiked stool extract matrix

Aim 2 - Medium-Term Aim

Building on the validated biosensor from Aim 1, the second aim will focus on **systematic optimization of biosensor performance** to meet clinical diagnostic thresholds. This includes engineering promoter variants with altered BudR binding affinities to tune the dynamic range, testing alternative SCFA-responsive transcription factors (e.g., FadR for propionate, AtoC for acetate) to create a multiplexed SCFA panel, and optimizing cell-free lysate composition and reaction conditions to improve signal-to-noise in complex stool matrix samples. Additionally, Aim 2 will explore integration with Opentrons liquid handling robots to enable portable, lower-cost automated biosensor assembly outside of core laboratory facilities, expanding access for microbiome research groups without access to high-end automation infrastructure. A panel of ≥50 stool samples representing healthy donors and IBD patients will be screened to establish clinical reference ranges and diagnostic cutoffs.

Aim 3 - Visionary Aim

"From bench lysate to bedside cartridge: democratizing the gut microbiome readout for every human on Earth."

The long-term vision of this project is to transform the BudR-NanoLuc biosensor into a **freeze-dried, shelf-stable, point-of-care diagnostic cartridge** that can be activated with a single drop of stool-derived extract and read on a smartphone-compatible luminometer - making gut microbiome health monitoring as accessible as a home pregnancy test. Integrated with AI-powered longitudinal tracking, this platform would enable real-time monitoring of dietary and probiotic interventions on microbiome SCFA profiles, serve as a non-invasive early warning system for colorectal cancer and IBD flares, and generate population-scale microbiome datasets that could fundamentally redefine our understanding of the gut-brain-immune axis. Partnering with Opentrons for decentralized manufacturing and Ginkgo Bioworks for scaled biosensor construct production, this technology could reach underserved clinical populations globally within a decade.

SECTION 3: BACKGROUND

Literature Context

Butyrate, a four-carbon short-chain fatty acid produced by anaerobic fermentation of dietary fiber by colonic microbiota, serves as the primary energy source for colonocytes and is a potent regulator of intestinal immune homeostasis. Seminal work by Furusawa et al. (2013, *Nature*) demonstrated that microbiota-derived butyrate drives differentiation of colonic regulatory T cells (Tregs) through inhibition of histone deacetylases (HDACs), establishing a direct mechanistic link between gut microbial metabolism and mucosal immune regulation. A critical knowledge gap remains in translating these findings to clinical practice: current gold-standard SCFA quantification by gas chromatography-mass spectrometry (GC-MS) requires specialized equipment, significant sample processing, and trained operators, making routine clinical monitoring of butyrate impractical. More recently, Pardee et al. (2016, *Cell*) pioneered the use of freeze-dried cell-free gene expression systems as portable, field-deployable biosensors for detecting nucleic acid targets, demonstrating that the inherent modularity of cell-free systems

can be harnessed for rapid diagnostic applications, a principle this project extends to small-molecule metabolite detection via transcription factor-based genetic circuits.

Innovation

This project is novel in its application of a **transcription factor-coupled cell-free biosensor specifically optimized for detection of butyrate in a clinically complex stool matrix**, a sample type that has not been previously characterized in cell-free biosensor contexts. While prior cell-free biosensors have largely focused on nucleic acid targets or simple buffer systems, this work directly confronts the challenge of matrix interference and establishes a methodology for cell-free diagnostics in microbiome-relevant samples. Furthermore, the use of NanoLuc luciferase, with its superior brightness and smaller gene size compared to firefly luciferase, combined with acoustic liquid handling via Echo525 for nanoliter-precision DNA and analyte dispensing represents a technically advanced, miniaturized, and high-throughput biosensor characterization platform. The integration of His-tagged BudR protein opens a parallel biochemical characterization pathway, enabling biophysical measurement of BudR-butyrate binding affinity to rationally guide future biosensor engineering.

Significance

Inflammatory bowel disease affects over 3 million Americans and approximately 10 million people globally, with incidence rising sharply in industrialized nations, yet the tools available for monitoring gut microbiome health in these patients remain expensive, slow, and inaccessible to most clinical practices. Butyrate is arguably the single most important microbiome-derived metabolite for colonic health, and the ability to rapidly quantify it from a stool sample would represent a meaningful clinical advance in gastroenterology and microbiome medicine. Beyond IBD, low fecal butyrate is emerging as a biomarker of colorectal cancer risk, metabolic syndrome, and compromised immune function, meaning a validated butyrate biosensor would have utility across multiple disease areas simultaneously. The cell-free expression platform selected for this project offers unique advantages over whole-cell biosensor approaches: it eliminates biosafety concerns associated with GMO release, enables rapid prototyping of new biosensor designs without cloning into live organisms, and is inherently compatible with freeze-drying for ambient-temperature storage and distribution, a critical property for point-of-care diagnostic deployment. Finally, this project establishes a **generalizable cell-free biosensor design framework** that can be rapidly adapted to detect other clinically relevant SCFAs (propionate, acetate), inflammatory markers (calprotectin, lactoferrin via aptamer integration), or microbiome-derived metabolites by simply swapping the transcription factor module, creating a modular platform with far-reaching diagnostic potential.

Bioethical Considerations

Paragraph 1 - Ethical Considerations: This project involves the design and synthesis of genetically engineered DNA constructs encoding a bacterial transcription factor (BudR) and a reporter gene (NanoLuc luciferase), expressed in a cell-free system derived from *E. coli* BL21 DE3 lysate. Because the system is cell-free, it does not involve the release of live genetically modified organisms, substantially reducing biosafety concerns relative to whole-cell biosensor approaches. However, the use of human stool samples in Aim 1 validation experiments raises important considerations around informed consent, sample anonymization, and biospecimen handling protocols. Any stool samples used must be obtained under IRB-approved protocols with full donor consent, and all sample handling must comply with institutional biosafety and biohazard waste disposal guidelines. Additionally, as this biosensor is designed with eventual

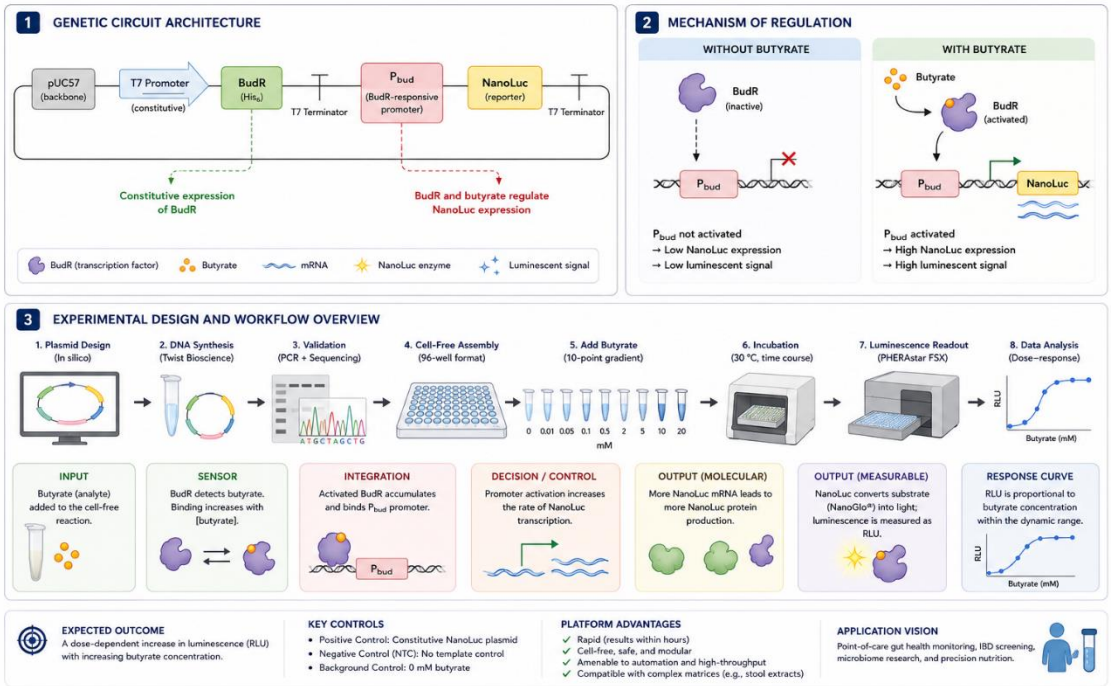
point-of-care diagnostic application in mind, it is important to consider the potential for misuse or misinterpretation of results in non-clinical settings, and to ensure that any future deployment is accompanied by appropriate clinical guidance and regulatory oversight (FDA 510(k) or De Novo pathway for diagnostic devices).

Paragraph 2 - Responsible Implementation and Risk Mitigation: To ensure responsible development of this biosensor technology, the project will adhere to SecureDNA principles for DNA synthesis screening, ensuring that no sequences in the ordered constructs overlap with select agents or biosecurity-relevant sequences. Twist Bioscience applies sequence screening to all synthesis orders, providing an additional layer of biosecurity review. The long-term vision of deploying this technology as a consumer-accessible gut health monitoring tool raises equity and access considerations: diagnostic tools that reach only wealthy populations risk exacerbating existing health disparities in microbiome-related disease. Accordingly, Aim 3 explicitly prioritizes partnerships with organizations capable of reducing per-test cost and enabling distribution in low-resource clinical settings. Data privacy is also a central concern for any platform that generates longitudinal microbiome health data; future deployment must incorporate robust data anonymization, user consent frameworks, and compliance with HIPAA and GDPR regulations to protect individual health information.

SECTION 4: EXPERIMENTAL DESIGN

The overall workflow integrates computational plasmid design, whole-plasmid DNA synthesis, molecular validation, cell-free protein synthesis (CFPS), luminescence-based dose-response characterization, and quantitative data analysis within a high-throughput microplate format.

Butyrate Biosenser: BudR-pbod-NanoLuc Cell-Free System:



Conceptual overview of the proposed BudR–P_bud–NanoLuc cell-free biosensor workflow. The diagram begins with the in silico design of two plasmid constructs, followed by whole-plasmid DNA synthesis and molecular validation through PCR and sequencing. Verified plasmids are then

introduced into a BL21 DE3-based cell-free protein synthesis (CFPS) platform assembled in 96-well format. Increasing butyrate concentrations are subsequently added to the reactions to evaluate transcriptional activation of the P_bud promoter mediated by BudR. This activation is expected to increase NanoLuc expression and generate a proportional luminescent signal measurable as relative light units (RLU). Finally, luminescence data are analyzed using dose-response modeling to characterize biosensor sensitivity, dynamic range, and quantitative performance.

Detailed Workflow

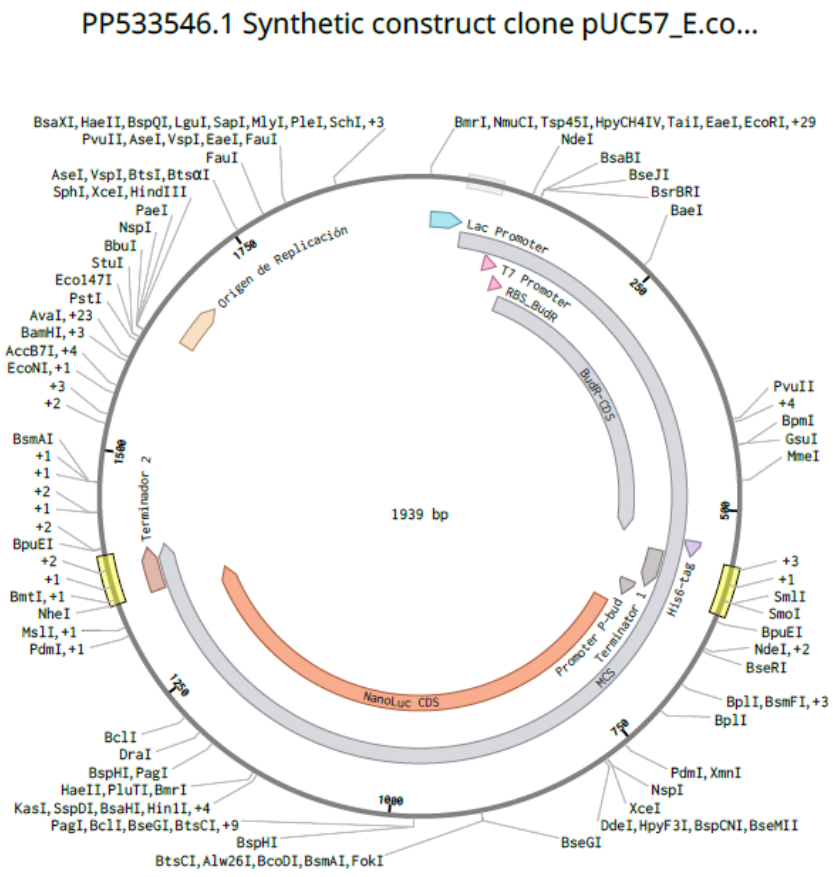
Step 1: DNA Construct Design

- **Method:** In silico design of two plasmid constructs using Benchling or SnapGene.
- **Construct 1 (Biosensor):** pUC57 backbone · T7 promoter, BudR(His6), T7 terminator · BudR-responsive synthetic promoter (P_bud), NanoLuc, T7 terminator
- **Construct 2 (Control):** pUC57 backbone · T7 promoter, NanoLuc (constitutive, no BudR regulation)
- **Machine:** N/A (computational)
- **Timeline:** Day 1 - 2

Annotated GenBank construct files for both plasmids are publicly available through the project GitHub repository: https://pages.htgaa.org/2026a-emmanuel-pereyra/

Construct files:

- Construct1-BudR_His6_Pbud_NanoLuc.gb
- Construct2-Constitutive_NanoLuc_Control.gb



The plasmid biosensor construct was designed in silico using a pUC57 backbone to enable regulated luminescent detection through the BudR transcriptional system. The first transcriptional unit consists of a T7 promoter driving expression of the BudR regulator fused to a C-terminal His_6-tag, followed by a T7 terminator to prevent transcriptional readthrough. The second transcriptional unit contains a BudR-responsive synthetic promoter (P_bud) controlling expression of the NanoLuc luciferase reporter gene, followed by a second T7 terminator. In this design, BudR functions as the regulatory sensor protein, while NanoLuc provides a measurable luminescent output in response to promoter activation. A separate control plasmid containing constitutive T7-driven NanoLuc expression was also designed to validate reporter functionality independently of BudR-mediated regulation.

Step 2: Twist Bioscience DNA Order

Both plasmid constructs were submitted to the Twist Bioscience Clonal Gene Service for full plasmid synthesis using pUC57 as the clonal backbone. Prior to submission, annotated GenBank sequence files were reviewed to ensure compatibility with Twist sequence complexity requirements and synthesis constraints. Orders were placed through the Twist Bioscience online ordering portal, with expected delivery consisting of sequence-verified lyophilized plasmid DNA within approximately 10-14 business days. Order submission was scheduled for Days 2-3 of the workflow, with plasmid receipt anticipated between Days 14-17.

Step 3: Plasmid Resuspension and Quality Check

Following plasmid resuspension, DNA concentration and purity will be evaluated using NanoDrop spectrophotometry to verify that both constructs meet the quality requirements for downstream cloning and expression experiments. Expected benchmark values are provided below together with designated fields for recording experimental measurements obtained during plasmid quality control analysis.

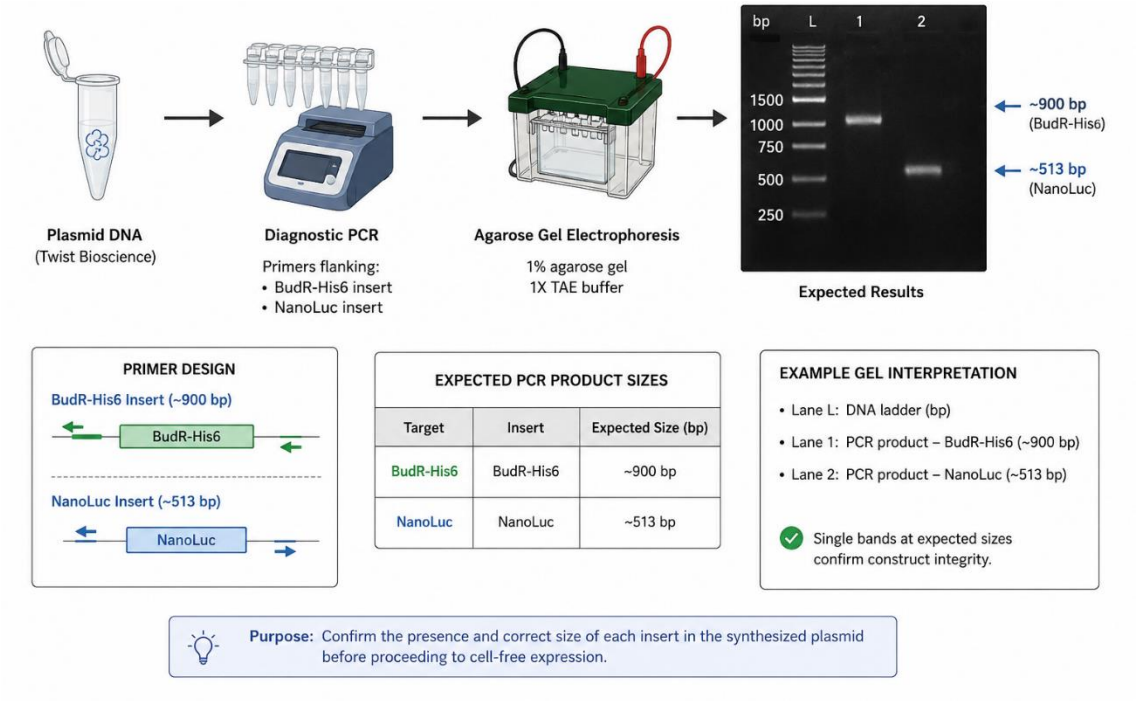
- **Method:** Resuspend lyophilized Twist plasmid DNA in nuclease-free water to 100 ng/µL. Measure concentration and purity on NanoDrop (A260/A280 ≥ 1.8).
- **Machine:** NanoDrop spectrophotometer
- **Plate:** N/A
- **Expected result:** Pure plasmid DNA at target concentration
- **Timeline:** Day 17

Sample	Expected Concentration (ng/µL)	Experimental Concentration (ng/µL)	Expected A260/A280	Experimental A260/A280	QC Status
Construct1-BudR_His6_Pbud_NanoLuc	100	_____	1.80–2.00	_____	_____
Construct2-Constitutive_NanoLuc_Control	100	_____	1.80–2.00	_____	_____

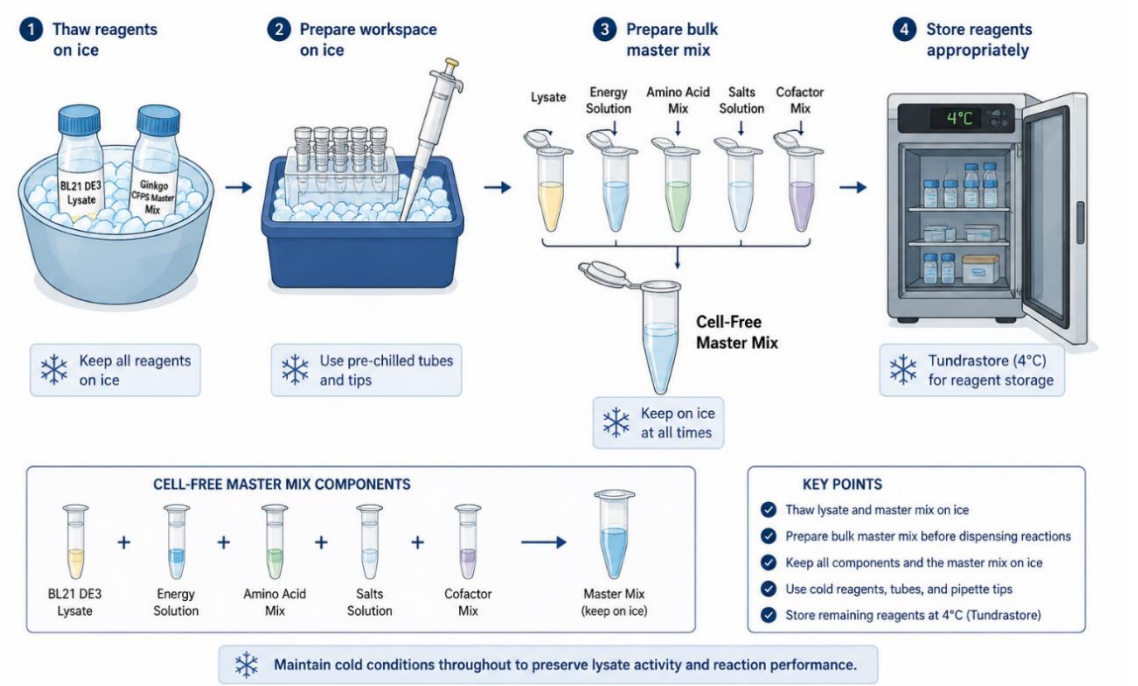
Step 4: PCR Verification of Construct Integrity:

- **Method:** Design primers flanking the BudR insert and the NanoLuc insert. Run diagnostic PCR on the Twist plasmid to confirm correct insert size before proceeding to cell-free expression.

- **Machine:** ATC Thermal Cycler
- **Plate:** 96-Armadillo-PCR-AB2396X
- **Expected result:** Single band at expected size (~900 bp BudR-His6; ~513 bp NanoLuc) on agarose gel
- **Timeline:** Day 17–18



Step 5: Cell-Free Master Mix Preparation



- **Method:** Thaw BL21 DE3 lysate and Ginkgo Bioworks cell-free master mix on ice. Prepare a bulk master mix containing lysate, energy solution, amino acids, salts, and cofactors according to the Ginkgo CFPS protocol. Keep on ice throughout.
- **Machine:** Manual pipetting on ice; Tundrastore (4°C) for reagent storage
- **Timeline:** Day 18

Step 6: Butyrate Standard Preparation

- **Method:** Prepare a 10-point serial dilution of sodium butyrate in PBS: 0, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10, 20 mM. Prepare matched standards spiked into filtered stool extract matrix. Store in 96-round-axygen-pdw11cs-halfdeep plate sealed with Plateloc.
- **Machine:** Floi8 liquid handler · Plateloc
- **Plate:** 96-round-axygen-pdw11cs-halfdeep
- **Expected result:** Complete, contamination-free standard curve plate
- **Timeline:** Day 18

Step 7: Stool Sample Processing

- **Method:** Process stool samples (obtained under IRB consent) by homogenization in PBS (1:10 w/v), centrifugation at 10,000 × g for 10 min (HiG Centrifuge), and filtration through 0.22 µm membrane. Aliquot clarified stool extract into deep-well plate.
- **Machine:** HiG Centrifuge · Tundrastore (4°C storage)
- **Plate:** 96-v-eppendorf-951033502-deep
- **Expected result:** Clarified stool filtrate suitable for cell-free reaction spiking
- **Timeline:** Day 18–19

Step 8: Cell-Free Reaction Assembly - Master Mix Dispensing

- **Method:** Use Multiflo to bulk-dispense 8 µL of cell-free master mix into each well of a 96-well assay plate. Dispense constitutive NanoLuc control plasmid into positive control column (column 12) at 10 ng/µL final concentration.
- **Machine:** Multiflo (Automated Microplate Dispenser)
- **Plate:** 96-round-axygen-pdw11cs-halfdeep
- **Expected result:** Uniform master mix volume across all wells (CV < 5%)
- **Timeline:** Day 19

Step 9: DNA and Butyrate Addition via Echo525

- **Method:** Use Echo525 acoustic liquid handler to transfer: (a) BudR-NanoLuc biosensor plasmid (10 ng/µL final) into biosensor wells, (b) butyrate standards (final concentrations 0-20 mM) into respective wells. All transfers in nanoliter volumes for precision. No-template control (NTC) wells receive water only.
- **Machine:** Echo525 (Acoustic Liquid Handler)
- **Plate:** 384-well Plate Echo PP (source) - 96-round-axygen-pdw11cs-halfdeep (destination)
- **Expected result:** Precise, contact-free transfer of all reaction components
- **Timeline:** Day 19

Step 10: Plate Sealing

- **Method:** Apply A4s breathable seal to reaction plate to allow gas exchange during incubation while preventing evaporation and contamination.
- **Machine:** A4s (breathable seal applicator)
- **Expected result:** Sealed plate ready for incubation
- **Timeline:** Day 19

Step 11: Cell-Free Reaction Incubation

- **Method:** Incubate sealed 96-well reaction plate at 30°C with shaking (300 rpm) in the Inheco Plate Incubator. Time-course reads at 1h, 2h, and 4h.
- **Machine:** Inheco Plate Incubator
- **Expected result:** Active cell-free transcription/translation producing NanoLuc luciferase over time
- **Timeline:** Day 19 (4-hour window)

Step 12: Luminescence Readout (Time-Course)

- **Method:** At each time point (1h, 2h, 4h), transfer plate to PHERAstar FSX. Add NanoGlo substrate (1:50 dilution in PBS) using Multiflo immediately before reading. Read luminescence in RLU (relative light units) across all wells.
- **Machine:** PHERAstar FSX (Luminescence mode) · Multiflo (substrate addition)
- **Plate:** 96-round-axygen-pdw11cs-halfdeep
- **Expected result:** Dose-dependent luminescence signal increasing with butyrate concentration; constitutive NanoLuc positive control shows stable high signal; NTC shows background only
- **Timeline:** Day 19

Step 13: qPCR Validation of NanoLuc Transcript

- **Method:** At the 4h time point, collect 2 µL from selected wells for RNA extraction. Perform one-step RT-qPCR using CFX Opus targeting the NanoLuc coding sequence to confirm that luminescence signal correlates with NanoLuc mRNA production (transcriptional activation), not residual DNA template signal.
- **Machine:** CFX Opus (qPCR machine) · ATC Thermal Cycler (reverse transcription)
- **Plate:** 96-Armadillo-PCR-AB2396X
- **Expected result:** NanoLuc Ct values inversely correlating with butyrate concentration (higher butyrate - earlier Ct - more transcript)
- **Timeline:** Day 20

Step 14: Data Analysis and Dose-Response Curve Fitting

- **Method:** Export PHERAstar FSX luminescence data. Normalize all wells to constitutive NanoLuc positive control. Fit dose-response data to a four-parameter logistic (4PL) curve using GraphPad Prism or Python (scipy.optimize). Calculate EC50, limit of detection (LOD), and limit of quantification (LOQ).
- **Machine:** Computational (GraphPad Prism / Python)
- **Expected result:** Sigmoidal dose-response curve with EC50 in the 1–5 mM butyrate range; LOD < 0.1 mM
- **Timeline:** Day 20–21

Step 15: Matrix Effect Assessment

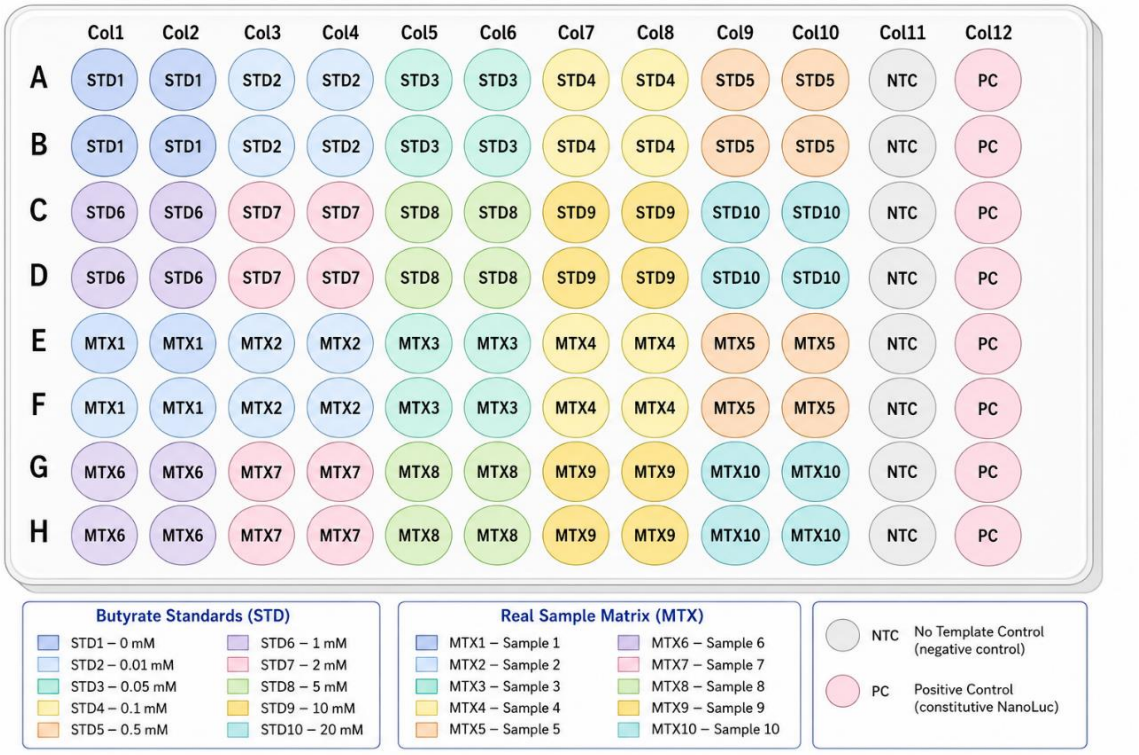
- **Method:** Compare dose-response curves generated in PBS buffer vs. spiked stool extract matrix. Calculate matrix factor (MF = signal in matrix / signal in buffer) at each concentration point. Assess whether stool matrix significantly suppresses or enhances the biosensor signal.
- **Machine:** PHERAstar FSX · computational analysis
- **Expected result:** MF within 0.8–1.2 (acceptable matrix effect); if MF outside this range, proceed to matrix dilution optimization
- **Timeline:** Day 21

Step 16: BudR His-Tag Protein Purification (Parallel Track)

- **Method:** Scale up cell-free reaction (5 mL) with BudR(His6) construct only (no NanoLuc). Purify BudR-His6 over Ni-NTA agarose column. Verify by SDS-PAGE and Coomassie staining. Store purified protein at -80°C for future biophysical characterization (isothermal titration calorimetry, ITC).
- **Machine:** HiG Centrifuge · Tundrastore (4°C) · manual column chromatography
- **Expected result:** Purified BudR-His6 band at ~35 kDa on SDS-PAGE
- **Timeline:** Day 22–23

Assay Plate Layout

The following 96-well plate layout illustrates the experimental design for the butyrate dose-response assay:



Step 17 — Statistical Analysis and Data Normalization

All cell-free reactions and luminescence assays will be performed using triplicate technical replicates unless otherwise specified. Dose-response experiments will include a minimum of ten

butyrate concentration points spanning 0–20 mM, together with no-template controls (NTC) and constitutive NanoLuc positive controls (PC). Raw luminescence values (RLU) obtained from the PHERAstar FSX plate reader will first be background-subtracted using the mean NTC signal and subsequently normalized to the constitutive NanoLuc positive control to reduce inter-plate variability and facilitate comparison across experiments. Dose-response datasets will be analyzed using four-parameter logistic (4PL) nonlinear regression in GraphPad Prism or Python (scipy.optimize) to calculate EC50, Hill coefficient, limit of detection (LOD), and limit of quantification (LOQ). Statistical comparisons between experimental conditions will be evaluated using one-way ANOVA followed by Tukey post-hoc correction, with statistical significance defined at $p < 0.05$. Error bars in all graphical outputs will represent standard deviation (SD) across technical replicates.

SECTION 5: TECHNIQUES, TOOLS, AND TECHNOLOGY

Course Technique Checklist

- [x] DNA design and sequence analysis (Benchling / SnapGene)
- [x] DNA synthesis and ordering (Twist Bioscience whole-plasmid synthesis)
- [x] Cell-free protein synthesis (CFPS) - BL21 DE3 lysate + Ginkgo Bioworks master mix
- [x] Transcription factor-based genetic circuit design
- [x] Reporter gene assay (NanoLuc luciferase)
- [x] Automated liquid handling (Multiflo, Echo525, Floi8)
- [x] Microplate-based assay development (96-well format)
- [x] Luminescence detection (PHERAstar FSX)
- [x] qPCR (CFX Opus) - transcript validation
- [x] Protein purification (His-tag Ni-NTA affinity chromatography)
- [x] Biosensor design and characterization
- [x] Dose-response curve analysis and 4PL fitting
- [x] Stool sample processing and matrix analysis
- [x] Bioethics and biosecurity considerations (SecureDNA screening)

Technique Expansion

Technique 1: Cell-Free Protein Synthesis (CFPS)

Cell-free protein synthesis (CFPS) is a powerful approach to gene expression that bypasses the need for living cells by providing all necessary transcription and translation machinery, including ribosomes, RNA polymerases, aminoacyl-tRNA synthetases, energy regeneration systems, and cofactors, in a cell-extracted lysate supplemented with a defined master mix. In this project, BL21 DE3 lysate is particularly advantageous because it contains T7 RNA polymerase (induced prior to lysis), enabling highly efficient transcription from T7 promoters on the input plasmid template. CFPS systems offer several compelling advantages for biosensor development: reactions can be assembled in minutes, run in microplate format compatible with robotic liquid handlers, completed within 2-4 hours, and the open nature of the reaction allows direct addition of analytes (such as butyrate) without the permeability barriers imposed by live cell membranes. Furthermore, CFPS reactions can be lyophilized for ambient temperature storage and reconstituted with a simple aqueous sample, making them ideal candidates for point-of-care diagnostic applications, a property central to the long-term vision of this project.

Technique 2: Acoustic Liquid Handling (Echo525)

The Echo525 acoustic liquid handler uses focused acoustic energy to eject precise nanoliter droplets (as small as 2.5 nL) from a source plate into a destination plate without any physical contact between the instrument and the liquid. This contactless transfer mechanism eliminates tip-based contamination, dramatically reduces reagent consumption, and enables the assembly of complex combinatorial reaction matrices, such as multi-concentration butyrate titrations across multiple plasmid DNA concentrations, with a precision and throughput that manual pipetting cannot approach. In this project, the Echo525 is used to transfer both the biosensor plasmid (BudR-NanoLuc) and butyrate standards into the cell-free master mix pre-dispensed by Multiflo, enabling highly precise analyte dosing that is critical for accurate dose-response curve generation. The acoustic transfer also preserves the integrity of sensitive nucleic acid and protein components, as the absence of mechanical shear or tip contact reduces the risk of DNA shearing or protein denaturation that can occur with conventional pipetting, ensuring reproducible cell-free reaction performance across all wells.

SECTION 6: PROJECT VALIDATION

Validation Experiment

10a. Validation Choice

The primary validation experiment for this project is a **two-stage sequential validation** combining PCR-based sequence verification of the Twist-synthesized plasmid with a functional cell-free NanoLuc expression test. This two-stage approach ensures both the genetic integrity of the construct and its biological functionality are confirmed before proceeding to full butyrate dose-response characterization, minimizing the risk of proceeding with a non-functional or incorrectly assembled biosensor construct.

10b. Validation Protocol

Stage 1: PCR Sequence Verification

1. Resuspend received Twist plasmid in nuclease-free water to 100 ng/µL.
2. Design two primer pairs: (a) BudR-F/R spanning the BudR-His6 insert (~900 bp expected); (b) NanoLuc-F/R spanning the NanoLuc insert (~513 bp expected).
3. Prepare PCR master mix (NEB Q5 polymerase, dNTPs, primers) on ice.
4. Load 96-Armadillo-PCR-AB2396X plate with reactions using Floi8 liquid handler.
5. Run PCR on ATC Thermal Cycler: 98°C 30s; 35× (98°C 10s, 60°C 30s, 72°C 45s); 72°C 2min.
6. Run PCR products on 1.5% agarose gel; confirm single bands at expected sizes.
7. Submit plasmid for Sanger sequencing (Genewiz/Azenta) using M13F/R primers to confirm full insert sequence integrity.

Stage 2: Functional Cell-Free NanoLuc Expression Test

1. Thaw BL21 DE3 lysate and master mix on ice.
2. Assemble 10 µL cell-free reactions in 96-Armadillo-PCR-AB2396X plate:
    - Well A1: BudR-NanoLuc plasmid (10 ng/µL) + no butyrate
    - Well A2: BudR-NanoLuc plasmid (10 ng/µL) + 5 mM butyrate
    - Well A3: Constitutive NanoLuc plasmid (10 ng/µL) - positive control
    - Well A4: No plasmid (NTC)
3. Seal with A4s breathable seal. Incubate at 30°C for 4h in Inheco Plate Incubator.

4. Add NanoGlo substrate (1:50 in PBS) via Multiflo.
5. Read luminescence on PHERAstar FSX.
6. Compare RLU values: expect Well A3 (PC) > Well A2 (biosensor + butyrate) > Well A1 (biosensor - butyrate) >> Well A4 (NTC ≈ background).

10c. Techniques Used

This validation experiment integrates four core synthetic biology techniques to provide orthogonal confirmation of construct integrity and function. First, **PCR amplification** with insert-specific primers provides a rapid size-based confirmation that the correct sequences were synthesized and are present in the correct orientation within the plasmid, serving as an essential quality control gate before committing reagents to cell-free reactions. Second, **Sanger sequencing** provides single-nucleotide resolution confirmation of the entire BudR and NanoLuc coding sequences, ensuring no synthesis errors, frameshifts, or mutations were introduced during Twist plasmid synthesis that could abolish protein function. Third, **cell-free protein synthesis** functionally tests whether the genetic circuit produces active NanoLuc enzyme, confirming that the T7 promoter, ribosome binding site, coding sequence, and terminator are all functional in the BL21 DE3 lysate context. Fourth, **luminescence detection on the PHERAstar FSX** provides the quantitative functional readout, allowing direct comparison of biosensor-induced vs. constitutive NanoLuc expression and establishing the baseline fold-induction achievable with 5 mM butyrate, a critical parameter for subsequent dose-response optimization.

10d. Hypothetical Data

Hypothetical Dose-Response Data (4h timepoint, PBS buffer):

Butyrate Concentration (mM)	Mean RLU	Normalized Signal (fold over NTC)
0 (NTC)	120	1.0×
0.01	145	1.2×
0.05	210	1.75×
0.1	380	3.2×
0.5	1,25	10.4×
1.0	3,8	31.7×
2.0	7,2	60.0×
5.0	11,5	95.8×
10.0	12,8	106.7×
20.0	13,1	109.2×
Positive Control (constitutive)	14,2	118.3×

Hypothetical 4PL Fit Parameters:

- EC50: ~1.2 mM butyrate
- Hill coefficient: 1.8
- Estimated LOD: ~0.08 mM
- Maximum fold-induction: ~110×

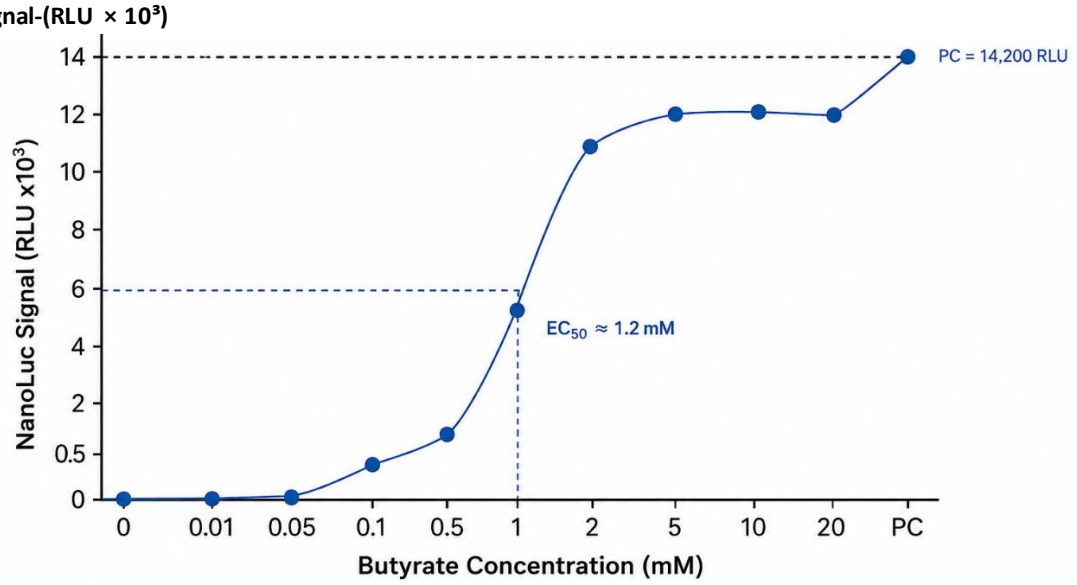


Figure 1. Hypothetical sigmoidal dose-response curve for BudR-NanoLuc biosensor in cell-free system. Signal plateaus at ~12,000 RLU approaching the constitutive positive control (PC) at 14,200 RLU. EC_{50} ≈ 1.2 mM butyrate.

Troubleshooting

Challenge 1: Low or No NanoLuc Signal: If the cell-free reaction produces no detectable luminescence, the most likely causes are: (a) plasmid DNA quality issues (low purity or degradation) - mitigate by re-quantifying and re-verifying plasmid quality before reaction setup; (b) lysate batch variability - test each new lysate batch with a known-good constitutive GFP or NanoLuc plasmid before use; (c) substrate addition error - ensure NanoGlo substrate is freshly diluted and added immediately before reading.

Challenge 2: No Butyrate-Dependent Induction: If luminescence is observed but does not increase with butyrate concentration, the BudR regulatory circuit may not be functioning as expected in the cell-free context. This could reflect incorrect BudR binding site orientation in the synthetic promoter, competition from endogenous lysate proteins with BudR DNA-binding domains, or incorrect butyrate concentration range (physiological fecal butyrate is typically 10 - 100 mM - consider extending the standard curve upper range). An alternative strategy would be to switch to an orthogonal SCFA-responsive transcription factor with better-characterized cell-free performance, such as FapR for malonyl-CoA or a synthetic butyrate aptamer-riboswitch.

Challenge 3: Matrix Interference from Stool Extract: Stool extract is a highly complex biological matrix containing proteins, lipids, metabolites, and microbial debris that may non-specifically inhibit cell-free transcription/translation or quench luminescence signal. If matrix factor analysis (Step 15) reveals significant signal suppression, dilute stool extract further (1:100 or 1:1000 in PBS) before spiking into reactions, or implement a solid-phase extraction (SPE) cleanup step using C18 cartridges to reduce matrix complexity while retaining SCFAs.

Challenge 4: Plasmid Synthesis Errors: If Sanger sequencing reveals sequence errors in the Twist synthesized plasmid (point mutations, deletions), reorder the affected construct with a revised sequence and contact Twist support for replacement under their quality guarantee. In parallel, consider ordering a gene fragment (gBlock) version of the affected element from IDT as a faster

backup to Gibson assemble into the backbone while awaiting the corrected whole-plasmid re-synthesis.

SECTION 7: ADDITIONAL INFORMATION

Supplies and Budget

Item	Supplier	Estimated Unit Cost	Quantity	Total Estimated Cost	Link
Whole plasmid synthesis — BudR-NanoLuc biosensor	Twist Bioscience	$ 199	1	$ 199	twist-bioscience.com
Whole plasmid synthesis — Constitutive NanoLuc control	Twist Bioscience	$ 199	1	$ 199	twist-bioscience.com
BL21 DE3 cell-free lysate + master mix (Ginkgo CFPS kit)	Ginkgo Bioworks	$ 350	1 kit	$ 350	ginkgobioworks.com
NanoGlo Luciferase Assay System	Promega / Thermo Fisher	$ 285	1 kit (10 mL)	$ 285	thermofisher.com
Sodium butyrate (analytical grade)	Millipore Sigma	$ 45	25 g	$ 45	sigmaaldrich.com
96-round-axygen-pdw11cs-halfdeep plates (pkg/50)	Thermo Fisher Scientific	$ 120	1 pkg	$ 120	thermofisher.com
96-Armadillo-PCR-AB2396X plates (pkg/25)	Thermo Fisher Scientific	$ 95	1 pkg	$ 95	thermofisher.com
A4s Breathable plate seals (pkg/100)	Thermo Fisher Scientific	$ 65	1 pkg	$ 65	thermofisher.com
Q5 Hot Start PCR Master Mix (500 rxn)	New England Biolabs	$ 278	1	$ 278	neb.com
Ni-NTA Agarose resin (5 mL)	Millipore Sigma	$ 165	1	$ 165	sigmaaldrich.com
Sanger sequencing (per reaction)	Azenta / Genewiz	$ 8	8 reactions	$ 64	azenta.com
Nuclease-free water (1 L)	Thermo Fisher Scientific	$ 28	1	$ 28	thermofisher.com
PBS 10× concentrate (1 L)	Thermo Fisher Scientific	$ 35	1	$ 35	thermofisher.com
Stool collection kits (with preservative)	Thermo Fisher Scientific	$12 each	10	$ 120	thermofisher.com
TOTAL ESTIMATED BUDGET				**~$2,048**	

GenBank

Construct Files

A synthetic butyrate biosensor plasmid was designed containing constitutive T7-driven expression of the BudR transcription factor fused to a C-terminal 6×His tag, coupled to a BudR-responsive promoter (P_bud) regulating NanoLuc luciferase expression.

- **Construct 1: BudR(His6)-P_bud-NanoLuc Biosensor Plasmid**

The Constitutive NanoLuc Positive Control construct was designed as a constitutive reporter system for cell-free protein synthesis (CFPS) assays. The plasmid contains a T7 promoter driving expression of the NanoLuc luciferase gene, followed by a T7 terminator to ensure efficient transcription termination. This construct provides a robust luminescent signal independent of regulatory elements or analyte presence, serving as a positive control to validate transcriptional, translational, and detection performance in CFPS reactions.

- **Construct 2: Constitutive NanoLuc Positive Control**

Proposal generated using the HTGAA Final Project Skill v1.1

File: projects/user_butyrate_biosensor.md

All automation steps designed for execution at Ginkgo Bioworks facility DNA synthesis orders: Twist Bioscience Clonal Gene service

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