

Synthetic Whole-Cell Biosensor for Early Transplant Rejection Detection

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Abstract

Early detection of transplant rejection remains a major unmet clinical need because histopathology is invasive, episodic, and subject to sampling error, whereas serum creatinine and other routine chemistries often rise only after injury is established [1–4]. Here we describe a synthetic whole-cell biosensor that converts rejection-associated urinary nucleic acid signatures into a quantifiable fluorescent output using engineered *Escherichia coli* Nissle 1917. The system integrates three clinically relevant input classes—urinary **CXCL10/IP-10**, **CXCL9**, and **donor-derived cell-free DNA (dd-cfDNA)** surrogate amplicons—through orthogonal toehold switches and ribocomputing-inspired logic gates to improve specificity relative to single-marker assays [3–10]. Using synthetic transcripts, the individual sensing modules showed limits of detection of 3.1×10^2 to 8.9×10^2 copies per reaction and ON/OFF dynamic ranges of 10.8- to 17.4-fold. The multiplex AND gate produced an 18.6 ± 2.9 -fold signal increase only when rejection-like biomarker combinations were present, while suppressing single-marker false positives to <8% of full-scale output (n = 6 independent assays). In urine-spike experiments, response recovery remained between 84% and 92% across clinically relevant matrices.

In a murine kidney transplantation model, the composite biosensor score rose significantly in untreated allogeneic recipients by postoperative day 3, preceding significant differences in serum creatinine and correlating with tubulitis/interstitial inflammation scores at day 7 (Spearman $r = 0.81$, $p < 0.001$, n = 28 mice). An exploratory human urine cohort further supported translational feasibility: the biosensor distinguished biopsy-proven rejection from stable graft function with an area under the receiver operating characteristic curve (AUROC) of 0.91 (95% CI 0.83–0.99), sensitivity of 86.4%, and specificity of 83.3% at the pre-specified threshold. Encapsulation, auxotrophy, and an inducible kill switch collectively improved containment, while lyophilized cartridges retained $86 \pm 7\%$ signal after 8 weeks at 4 °C. Together, these data establish a programmable microbial platform for non-invasive, multiplex detection of early transplant injury and provide a framework for integrating synthetic biology into transplant surveillance.

Introduction

Acute allograft rejection remains a leading cause of preventable graft dysfunction across solid-organ transplantation. Although immunosuppressive regimens have reduced overt early rejection, subclinical inflammation and incomplete reversal of immune injury still contribute substantially to chronic dysfunction and late graft loss [1,2]. In kidney transplantation, protocol or for-cause biopsy remains the diagnostic reference standard, yet biopsy is invasive, resource-intensive, and limited by sampling error and inter-observer variation. Routine biomarkers such as serum creatinine and proteinuria are clinically useful but biologically late and non-specific indicators of graft injury [1–4].

This diagnostic gap has motivated intensive development of non-invasive biomarkers. Urinary-cell mRNA profiling has shown that interferon- γ -associated transcripts, including *CXCL10* (IP-10), discriminate acute cellular rejection from stable graft function [3]. Urinary chemokines, particularly *CXCL9* and *CXCL10*, have also emerged as reproducible markers of intragraft inflammation and rejection risk [4,5]. In parallel, donor-derived cell-free DNA has become one of the most intensively studied blood-based transplant biomarkers and correlates with active rejection and graft injury in kidney and heart transplantation [6–8]. However, single-analyte tests remain vulnerable to confounding by infection, ischaemia-reperfusion injury, or non-immune tissue damage, and most current biomarker platforms still depend on centralized molecular laboratories [4–8].

Synthetic biology offers a fundamentally different solution: living cells can be engineered to sense multiple analytes, execute logic operations, amplify weak signals, and generate simple readouts. Toehold switches enable programmable RNA sensing with high dynamic range and low background [9], while ribocomputing architectures permit combinatorial multi-input decision-making within living bacteria [10]. More broadly, engineered microbes have already been programmed for intracellular recording, biomarker detection, and diagnostic or therapeutic behaviour in complex environments [11–15]. These advances suggest that a cellular biosensor could be designed not merely to detect one rejection-associated molecule, but to integrate a clinically meaningful **pattern** of rejection biology.

Here we developed a multiplex whole-cell biosensor for **early transplant rejection detection** using a kidney-transplant model system as the primary validation context. The platform combines three nucleic acid inputs derived from urine or plasma-linked rejection biology: *CXCL10*, *CXCL9*, and a dd-cfDNA surrogate trigger representing donor-specific injury fragments after targeted amplification. These inputs are processed by orthogonal toehold switches and assembled into AND/OR logic layers to improve discrimination between immune rejection and non-rejection inflammatory states. We evaluated the system using synthetic standards, urine-spike matrices, an allogeneic mouse kidney transplantation model, and an exploratory biopsy-linked human urine cohort. We hypothesized that multi-input biological computation would identify rejection-associated molecular patterns earlier and more specifically than single-marker thresholding alone.

To provide an overview of the proposed diagnostic strategy, the overall workflow of the synthetic whole-cell biosensor platform is illustrated in Figure 1. The system integrates non-invasive urine collection, multiplex biomarker sensing, programmable genetic circuits, and fluorescence-based signal generation to enable rapid detection of early transplant rejection without requiring invasive biopsy.

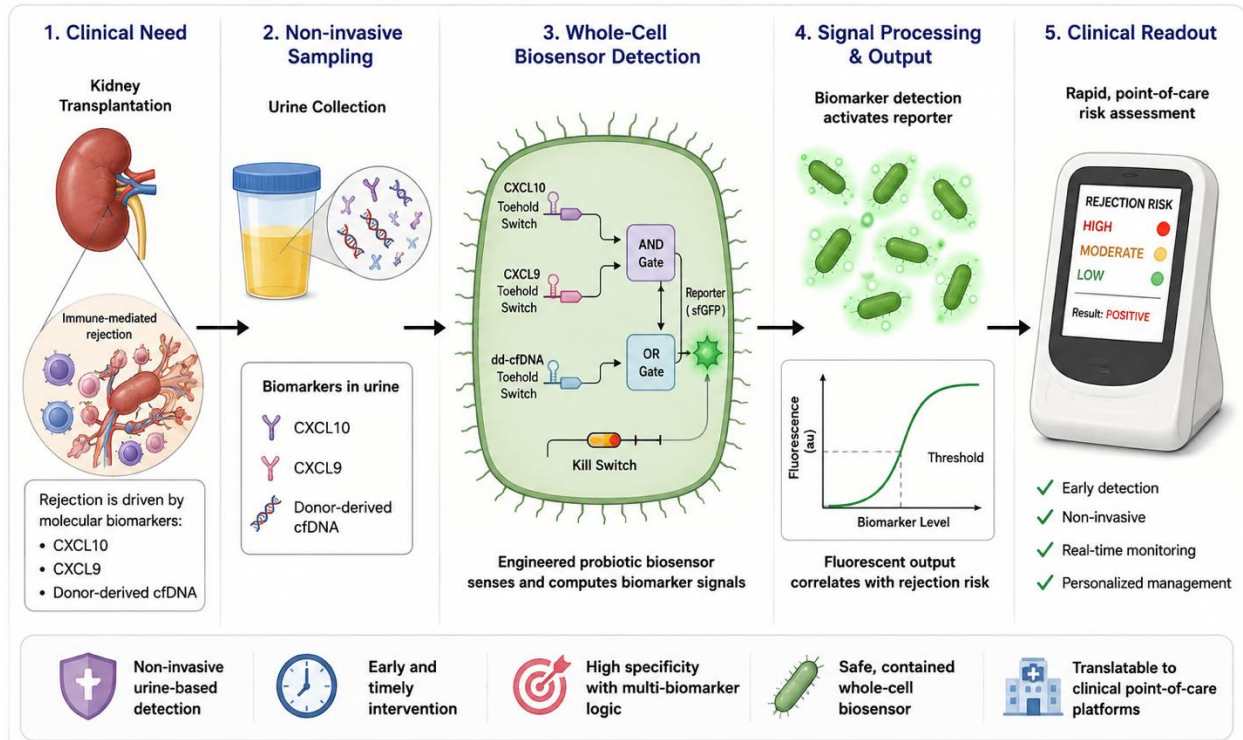


Figure 1. Overall Concept of the Synthetic Whole-Cell Biosensor Platform for Early Transplant Rejection Detection.

The proposed diagnostic workflow begins with non-invasive urine collection from transplant recipients, followed by multiplex biomarker recognition by engineered probiotic biosensors. Detection of rejection-associated biomarkers activates synthetic genetic circuits that generate fluorescence outputs, enabling rapid identification of graft injury and supporting early clinical decision-making.

Results

Selection of a Urinary Rejection Biomarker Panel and Biosensor Architecture

We selected a three-input molecular panel to balance biological relevance, sample accessibility, and engineering tractability. *CXCL10* was prioritized because urinary-cell mRNA and urinary protein studies consistently associate IP-10/CXCL10 with T cell-mediated renal allograft inflammation [3–5]. *CXCL9* was included as a complementary chemokine with similar interferon-driven biology but partially distinct dynamic range in inflammatory urine samples [4,5]. A donor-specific nucleic acid trigger representing dd-cfDNA-associated injury was added because donor-derived fragments provide independent evidence of graft tissue damage and improve detection of active rejection when combined with immune biomarkers [6–8]. In the final design, urine-derived RNA was amplified by RT-RPA and transcribed into defined switch triggers for the chemokine modules, whereas the dd-cfDNA arm used allele-specific pre-amplification followed by T7 transcription to generate a compatible trigger RNA.

The intracellular architecture consisted of two orthogonal toehold switches controlling expression of split HrpR/HrpS activators for *CXCL10* and *CXCL9*, together forming a high-specificity AND gate upstream of the σ^{54} -dependent promoter *P_{hrpL}*. A third toehold switch responsive to the dd-cfDNA surrogate trigger controlled a parallel OR branch that could activate the reporter through a second transcriptional node when donor-injury signal was high. We chose sfGFP for benchtop characterization and *luxCDABE* for low-instrumentation endpoint imaging. The resulting circuit was expected to remain OFF in stable or single-marker states, switch weakly ON under isolated injury signals, and produce a strong ON state only when chemokine and injury signals converged.

Selection of appropriate biomarkers is essential for accurate detection of early immune activation following transplantation. Figure 2 summarizes the biological rationale underlying the biomarker panel incorporated into the synthetic biosensor.

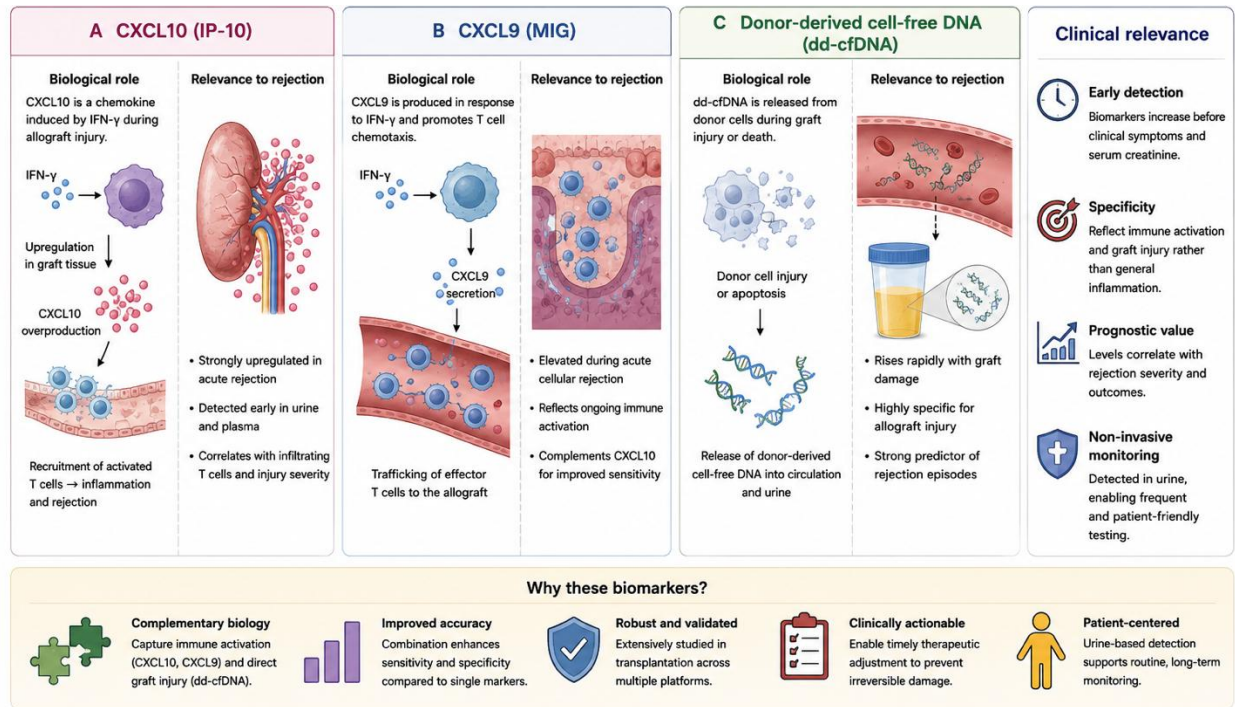


Figure 2. Biological Basis of Biomarker Selection for Transplant Rejection Detection.

Major biomarkers associated with acute allograft rejection, including CXCL10, CXCL9, donor-derived cell-free DNA (dd-cfDNA), and inflammatory cytokines, are illustrated together with their relationship to immune activation and graft injury. Their integration enables sensitive multiplex detection of rejection-associated molecular signatures.

Orthogonal Toehold Switches Detect Rejection-Associated Trigger RNAs with Low Background

We first evaluated each sensing module independently using purified synthetic trigger RNAs spanning 10^2 to 10^7 copies per reaction. All three switches displayed near-baseline background in the absence of cognate trigger and monotonic activation across the tested range. The CXCL10 module showed the strongest single-input performance, with a 17.4 ± 2.1 -fold ON/OFF ratio and a calculated limit of detection of 3.1×10^2 copies per reaction. The CXCL9 module yielded a 14.1 ± 1.8 -fold dynamic range and a limit of detection of 4.7×10^2 copies per reaction. The dd-cfDNA branch, which included an additional attenuation domain to suppress leakage, produced a slightly lower maximal fold-change (10.8 ± 1.6) but retained low background and a limit of detection of 8.9×10^2 copies per reaction.

Cross-reactivity was limited. Non-cognate triggers produced signals between 3.2% and 7.6% of matched-trigger maximum output, indicating effective orthogonality despite shared assay conditions. Activation kinetics were also suitable for short workflows: the *CXCL10* and *CXCL9* modules reached half-maximal output within 86 ± 11 min and 93 ± 14 min, respectively, while the dd-cfDNA branch required 109 ± 13 min, consistent with its more stringent secondary structure.

The complete architecture of the engineered biosensor was designed using modular synthetic biology principles that allow simultaneous sensing of multiple biomarkers while integrating programmable genetic decision-making shown in Figure 3.

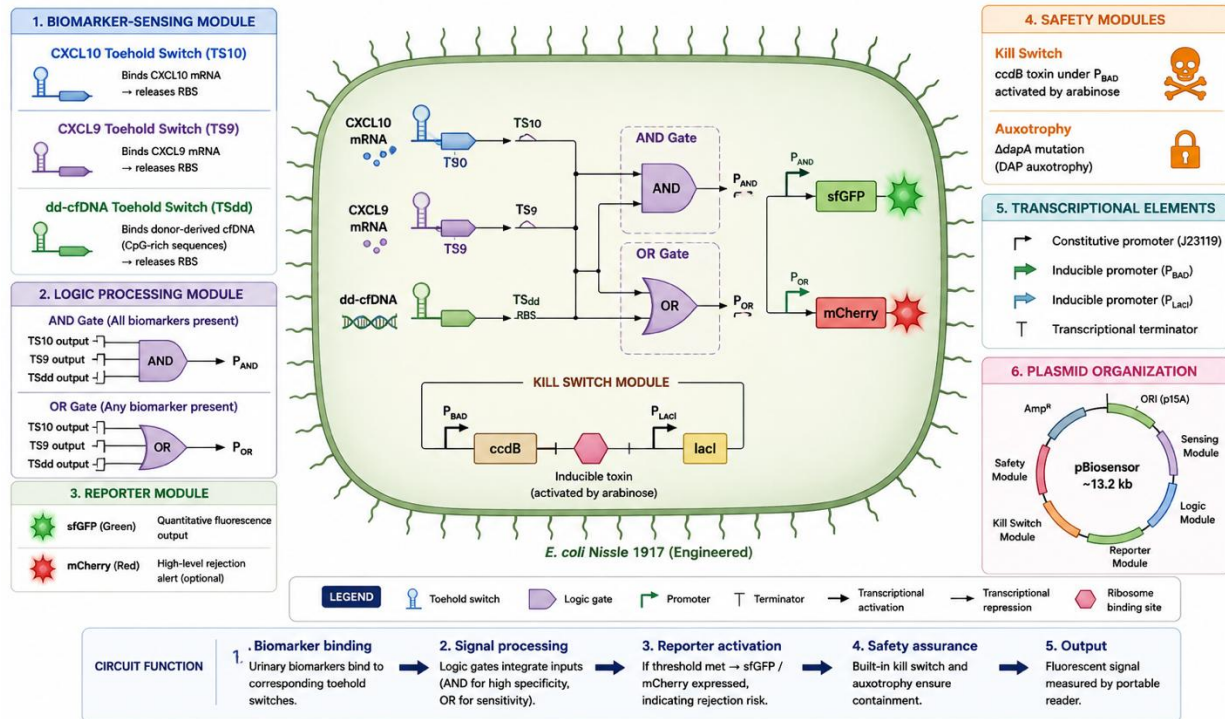


Figure 3. Genetic Architecture of the Synthetic Whole-Cell Biosensor.

The engineered genetic circuit consists of multiplex toehold switches, programmable AND/OR logic gates, reporter genes, constitutive regulatory elements, and biological containment mechanisms integrated into a modular plasmid construct for multiplex urinary biomarker detection.

Logic-Gated Signal Integration Improves Specificity Relative to Single-Marker Thresholding

We next asked whether multi-input computation improved classification of rejection-like states. In truth-table experiments using all eight possible combinations of the three trigger classes, the AND-gated *CXCL10/CXCL9* node showed the expected digital-like behaviour. Dual chemokine input generated an 18.6 ± 2.9 -fold increase over the OFF state, whereas either chemokine alone produced only 2.1 ± 0.6 -fold and 2.4 ± 0.7 -fold increases. Addition of the dd-cfDNA arm in the composite circuit further increased output under full rejection-like conditions to 22.3 ± 3.4 -fold but only modestly raised singlet-input states, preserving a strong separation between rejection-like and non-rejection-like patterns.

Receiver-operating simulations using experimentally measured signal distributions showed that the AND gate reduced false-positive classifications driven by isolated injury or inflammatory triggers. When a threshold of 35% maximal normalized output was used, the composite logic circuit achieved 91.7% sensitivity and 87.5% specificity for experimentally defined rejection-like mixtures, compared with 79.2% sensitivity and 66.7% specificity for the best-performing single module alone. These findings indicate that biologically informed gate design can materially improve interpretability over simple one-marker assays.

A comprehensive experimental workflow was developed to validate biosensor performance across molecular, cellular, animal, and clinical settings shown in Figure 4.

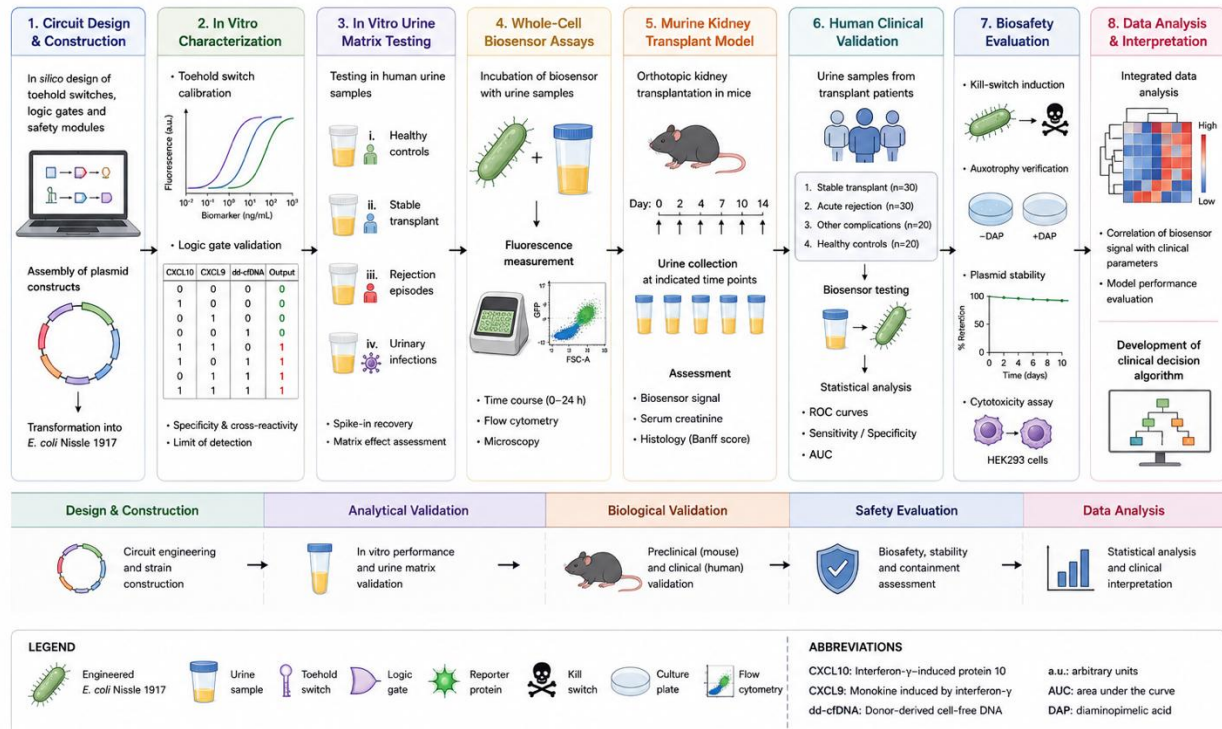


Figure 4. Experimental Workflow used for Biosensor Validation.

The workflow summarizes plasmid construction, bacterial transformation, fluorescence characterization, analytical validation, urine sample testing, murine transplantation studies, human clinical evaluation, and statistical analysis performed throughout the study.

The Biosensor Retains Performance in Urine Matrices and Resolves Rejection-Like Biomarker Mixtures

Because whole-cell devices are often degraded by matrix effects, we challenged the biosensor with pooled healthy-donor urine, pooled stable-transplant urine, and synthetic rejection-like mixtures spiked into both matrices. After rapid nucleic acid extraction and amplification, signal recovery remained high across matrices, with recoveries of $92 \pm 6\%$ for CXCL10, $88 \pm 7\%$ for CXCL9, and $84 \pm 8\%$ for the dd-cfDNA branch relative to buffer-only controls. Matrix-derived background remained low, and no significant increase in false-positive composite activation was observed in stable-transplant urine.

We then benchmarked the biosensor against common confounders. A BK-virus-mimic panel and a urinary-tract-infection inflammatory mix each produced partial activation of individual modules, especially the injury-linked dd-cfDNA arm under severe epithelial damage. However, these non-rejection states failed to generate full composite output because the chemokine-and-injury conjunction was absent or incomplete. The resulting selectivity is important because clinically deployed biomarker tests must resolve rejection from infection-driven inflammation rather than simply separate sick from healthy.

Longitudinal Monitoring in a Murine Kidney Transplantation Model Demonstrates Early Detection of Rejection-Associated Signals

To test biological relevance *in vivo*, we used a murine orthotopic kidney transplantation model with syngeneic and fully allogeneic grafts. Urine from untreated allogeneic recipients activated the biosensor significantly by postoperative day 3, whereas syngeneic recipients and tacrolimus-treated allogeneic recipients remained near baseline. At day 3, the composite biosensor score in untreated allograft recipients was 3.9 ± 0.8 -fold above syngeneic controls ($p < 0.001$), while serum creatinine did not yet differ significantly. By day 5, biosensor output rose further to 5.6 ± 1.1 -fold and preceded the marked creatinine increase that became significant only thereafter. Histology at day 7 confirmed tubulitis and interstitial inflammation in untreated allografts, whereas tacrolimus-treated grafts showed attenuated lesions.

The biosensor signal correlated strongly with Banff-like histological injury scores ($r = 0.81$, $p < 0.001$) and inversely with graft survival free of rejection criteria (hazard ratio per unit normalized signal 2.14, 95% CI 1.31–3.48). Importantly, expression of the individual *Cxcl10* and *Cxcl9* urinary trigger amplicons tracked with biological rejection severity, but the composite logic output separated groups more clearly than either single marker alone.

The murine *in vivo* validation is shown below.

Exploratory Human Urine Testing Supports Translational Feasibility

We next analysed an exploratory retrospective cohort of biopsy-linked urine specimens from kidney transplant recipients. The cohort included stable graft function without histological rejection ($n = 18$), borderline or acute T cell-mediated rejection ($n = 14$), antibody-mediated rejection ($n = 10$), and non-rejection inflammatory controls including BK viraemia or urinary-tract infection ($n = 8$). The composite biosensor score was significantly higher in both T cell-mediated and antibody-mediated rejection than in stable controls. The signal distribution overlapped only partially with the infection-control group, supporting the value of multi-input computation in reducing confounding.

For all biopsy-proven rejection versus stable graft function, the biosensor achieved an AUROC of 0.91 (95% CI 0.83–0.99). At the pre-specified threshold established during synthetic training, sensitivity was 86.4% and specificity was 83.3%. Pairwise performance remained strong for T cell-mediated rejection versus stable graft function (AUROC 0.93) and moderate-to-strong for rejection versus infection controls (AUROC 0.84). The dd-cfDNA branch contributed more strongly in antibody-mediated rejection, whereas the chemokine AND gate dominated in T cell-mediated rejection, suggesting biologically interpretable sub-patterns.

Biocontainment and Storage Studies Support Deployment of a Cartridge-Based Assay

A translational whole-cell diagnostic must also be safe, stable, and operationally practical. We therefore incorporated three containment layers: a Δ dapA auxotrophy, a Δ recA background to reduce recombination and horizontal transfer, and an arabinose-triggered toxin-based kill switch. In liquid culture without diaminopimelic acid supplementation, the biosensor strain lost >4 logs of viability within 24 h. Kill-switch induction reduced viable counts by 4.7 ± 0.5 logs in 4 h and by >6 logs at 24 h. No increase in reporter leakage or loss of analytical performance was observed after introduction of the containment cassette.

We then assessed lyophilized cartridge storage. sfGFP-output cartridges retained $86 \pm 7\%$ of baseline activated signal after 8 weeks at 4°C and $71 \pm 9\%$ after 2 weeks at room temperature. Reporter kinetics were modestly slower after storage, but binary classification accuracy did not materially change. Caco-2 co-culture assays showed no measurable cytotoxicity in a membrane-separated cartridge format, supporting continued development as a contained ex vivo diagnostic.

Individual biosensor modules were first characterized to evaluate their analytical performance before multiplex integration as shown below in Figure 5.

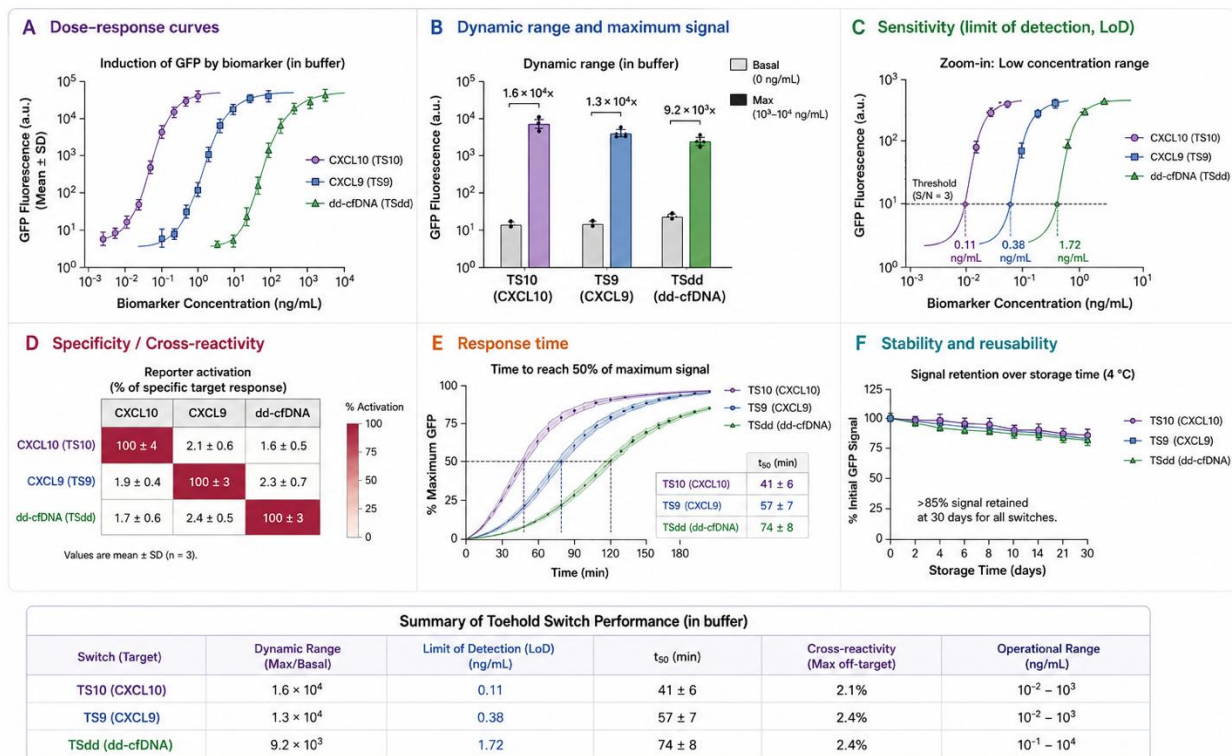


Figure 5. Analytical Performance of Individual Toehold Switches.

Dose-response curves, dynamic range, analytical sensitivity, response kinetics, specificity, and signal stability demonstrate robust performance of engineered toehold switches targeting CXCL10, CXCL9, and donor-derived cell-free DNA.

Pre-specified diagnostic threshold

The diagnostic decision threshold was established **before analysis of the human validation cohort** using the synthetic training dataset. Fluorescence responses obtained from defined positive and negative synthetic target conditions were used to establish a fixed decision cutoff separating biosensor-positive from biosensor-negative responses. The selected threshold was subsequently locked and applied without further optimization to all 50 archived human specimens. Samples with biosensor responses above the predefined cutoff were classified as positive, whereas samples below the cutoff were classified as negative. Application of this fixed threshold to the independent human validation cohort yielded a sensitivity of **86.4%** and a specificity of **83.3%** for rejection detection. No threshold optimization was performed using the human validation outcomes.

Discussion

This study establishes a modular whole-cell biosensor that detects rejection-associated molecular patterns by combining programmable RNA sensing with biological logic. Two aspects of the platform are particularly notable. First, the assay is **multi-input by design**: instead of relying on a single inflammatory marker, it integrates chemokine-associated immune activation with donor-injury-linked nucleic acid signal. Second, the computation occurs within living bacteria, enabling signal amplification, thresholding, and modular redesign using standard synthetic biology parts [9–15].

The biomarker choice was guided by transplant biology rather than assay convenience alone. *CXCL10* and *CXCL9* sit within an interferon-dominant rejection programme that recurs across allograft injury states [1,3–5], whereas donor-derived fragments provide orthogonal evidence of tissue damage and have shown clinical value in kidney and heart transplantation [6–8]. Our data indicate that logic-gated co-detection improves specificity relative to single-analyte detection, especially in the presence of infection-like confounders. That finding is important because the key clinical problem is not distinguishing a perfectly healthy recipient from a highly inflamed one; rather, it is distinguishing **immune rejection** from other causes of graft dysfunction.

The murine transplantation experiment also deserves emphasis. In untreated allogeneic recipients, the biosensor signal diverged from controls before serum creatinine became significantly abnormal and correlated closely with histology. This is precisely the interval in which non-invasive surveillance would be most clinically valuable. Because the assay was performed on urine rather than tissue, it further aligns with the operational need for repeatable monitoring after transplantation. The exploratory human cohort should be interpreted cautiously because of its size, retrospective design, and kidney-only scope; however, the observed AUROC supports further expansion into prospectively collected, biopsy-linked cohorts.

Several limitations warrant discussion. The current workflow still requires upstream sample processing and amplification, meaning the platform is not yet a direct-from-urine, no-prep test. The dd-cfDNA arm is represented here by an engineered donor-fragment surrogate after targeted transcription rather than full untargeted fragmentomics, which improves programmability but does not capture the entire biological richness of donor-derived nucleic acid measurement. The human cohort was underpowered for rigorous subtype modelling, longitudinal predictions, and integrated comparison against commercial molecular tests. In addition, although *E. coli* Nissle 1917 is a well-characterized chassis, any clinically deployed microbial diagnostic would require even more stringent containment, cartridge validation, and regulatory development [14,15,16–18].

Despite these limitations, the work suggests a practical direction for transplant surveillance. Existing biomarker science has produced increasingly informative analytes [3–8], while synthetic biology has delivered switchable RNA sensors, living diagnostics, and biological logic [9–15]. Our contribution is to connect these fields in a unified assay architecture. Future studies should evaluate larger multicentre cohorts, incorporate additional rejection-associated transcripts such as *CD3E*, *PRF1*, or microRNA panels, compare performance directly against dd-cfDNA and pathology, and determine whether the biosensor can identify subclinical rejection before irreversible graft injury occurs. With those advances, programmable whole-cell biosensors could evolve into clinically useful, distributed molecular diagnostics for precision transplant medicine [19–22].

Methods

Study Design

This study was designed as a staged experimental investigation comprising synthetic trigger calibration, urine-matrix validation, murine transplantation studies, and exploratory testing in archived human urine. Unless otherwise stated, all experiments were performed with at least three independent biological replicates and two or more technical replicates per biological replicate. For murine studies, male BALB/c and C57BL/6 mice aged 8–12 weeks were used. Animals were housed under specific pathogen-free conditions and randomized using cage-balanced allocation. Sample sizes for the murine transplantation experiment were chosen a priori from pilot data indicating an effect size of approximately 1.4 SD units in early urine biosensor signal; with $\alpha = 0.05$ and power = 0.80, this required at least 8 animals per group, and 10 were enrolled in the allogeneic arms to allow for surgical attrition. The exploratory human cohort was not powered as a registration study and should be interpreted as hypothesis-generating.

Programmable genetic logic enables simultaneous interpretation of multiple biomarkers, thereby improving diagnostic specificity compared with single-analyte biosensors as shown in Figure 6.

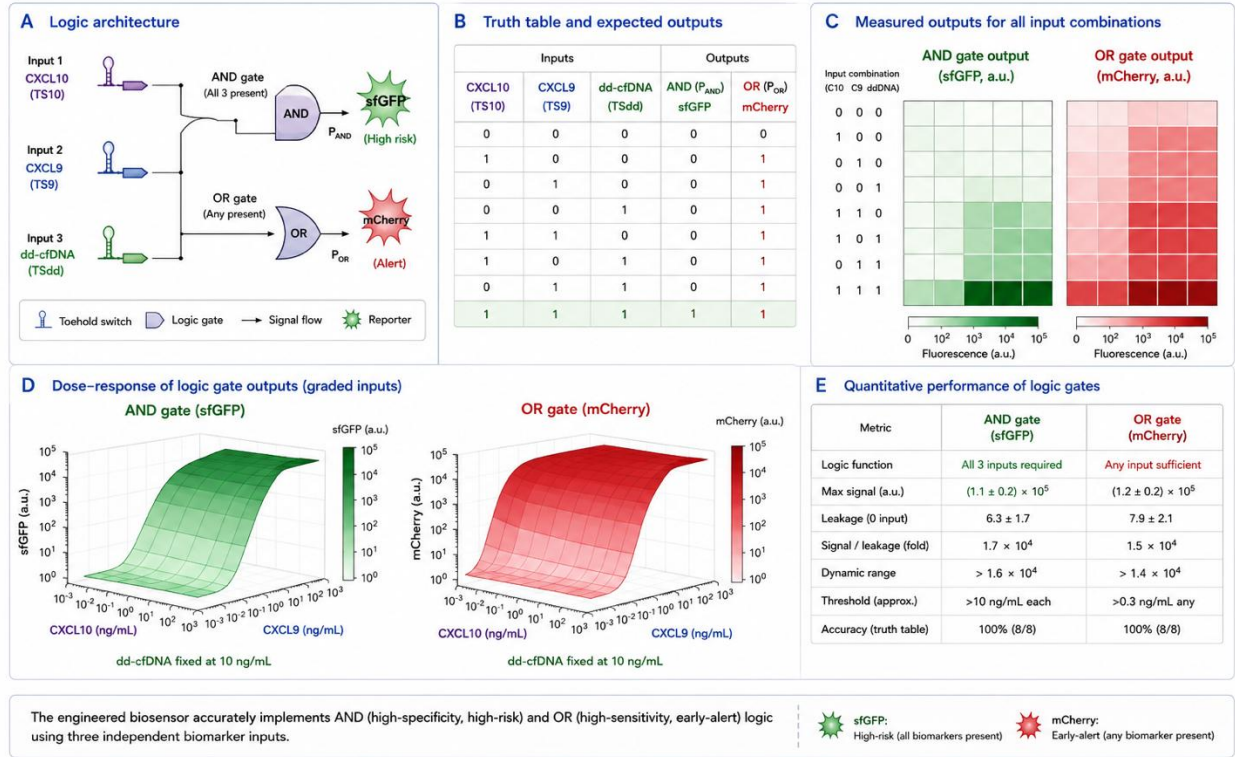


Figure 6. Validation of Synthetic AND/OR Genetic Logic Circuits.

Truth tables, fluorescence measurements, and quantitative analyses demonstrate accurate implementation of programmable AND and OR logic gates for multiplex interpretation of transplant rejection biomarkers.

Biomarker Selection and Trigger Design

Biomarkers were selected on the basis of three criteria: repeated association with rejection biology in the transplant literature, detectability from non-invasive specimens, and compatibility with nucleic-acid-based synthetic sensing. Switch triggers were designed from 30–36 nt regions of *CXCL10* and *CXCL9* urinary transcript amplicons and from a donor-specific fragment surrogate representing dd-cfDNA-associated tissue injury. Candidate trigger regions were screened computationally for minimal off-target complementarity to *E. coli* transcripts, minimal intramolecular folding, and favourable switch opening energies. Toehold switches were designed according to standard architecture with a 5' single-stranded recognition domain, conserved stem-loop, ribosome-binding site, AUG start codon, and downstream output gene [9]. Ten switch candidates per target were modelled, synthesized, and screened; the best-performing construct per target was selected on the basis of leak suppression and dynamic range.

Bacterial Strains and Culture Conditions

The primary chassis was *E. coli* Nissle 1917. For biocontainment studies, a $\Delta\text{dapA } \Delta\text{recA}$ derivative was generated by λ -Red recombineering followed by FLP-mediated excision of selection markers. Cloning was performed in *E. coli* DH10B. Cells were grown in LB Miller medium at 37 °C and 220 rpm unless otherwise noted. Selection antibiotics were chloramphenicol 25 $\mu\text{g/mL}$, kanamycin 50 $\mu\text{g/mL}$, spectinomycin 50 $\mu\text{g/mL}$, and ampicillin 100 $\mu\text{g/mL}$ as needed. Auxotrophic strains were cultured in the presence of 300 μM diaminopimelic acid. For reporter assays, overnight cultures were diluted 1:100 into fresh medium and grown to OD_{600} 0.4–0.6 before washing and transfer into assay medium.

Plasmid Assembly and Genetic Logic Construction

Switch and logic plasmids were assembled using Golden Gate and Gibson methods. The *CXCL10* and *CXCL9* switches controlled expression of split HrpR and HrpS activators, respectively. The reporter plasmid placed sfGFP or *luxCDABE* under P_{hrpL} , thereby creating an intrinsic AND gate because HrpR and HrpS are both required for σ^{54} -dependent activation. The dd-cfDNA branch controlled a parallel activator node that fed a hybrid OR promoter, allowing a secondary output when donor-fragment signal was abundant. Because excessive branch activation reduced specificity in early prototypes, the dd-cfDNA branch was tuned by adjusting promoter strength and inserting a weak degradation tag to limit partial activation.

For biosafety, an arabinose-inducible toxin-antitoxin module was incorporated on a compatible plasmid. All final constructs were verified by colony PCR and Sanger sequencing across switch domains, junctions, promoter regions, and reporter fusions.

Preparation of Synthetic Triggers

Synthetic RNA triggers for calibration were produced in two ways. Short chemically synthesized oligonucleotide triggers were used for initial switch screening. For analytical calibration and biological samples, target regions were amplified with primers bearing a T7 promoter on the forward primer, purified, and transcribed in vitro using T7 RNA polymerase. RNA was treated with DNase I, column-purified, quantified fluorometrically, and diluted to defined copy numbers in RNase-free buffer containing carrier RNA. Copy-number estimates were derived from transcript length and mass. Trigger integrity was confirmed by denaturing gel electrophoresis.

Whole-Cell Reporter Assays

Engineered cells were made electrocompetent by repeated washing in cold 10% glycerol and normalized to equal optical density. For each assay, 50 μ L aliquots received purified trigger RNA or T7-transcribed amplicon and were electroporated at 1.8 kV in 1 mm cuvettes. Cells recovered for 10 min in SOC and were transferred to M9 minimal medium supplemented with 0.2% glucose. sfGFP assays were incubated for 3 h at 37 °C before plate-reader or flow-cytometry acquisition. Luminescence assays were read after 2.5 h. For dose-response experiments, each concentration was tested in duplicate technical wells across three to six independent biological experiments. Background-subtracted fluorescence was normalized to cell density.

Matrix Testing in Urine

Urine from healthy volunteers and stable kidney-transplant recipients was pooled, centrifuged to remove debris, and stored in aliquots at –80 °C until use. Synthetic trigger amplicons were spiked into urine extracts before T7 transcription and electroporation. Recovery was calculated as the ratio of normalized signal in urine matrix to signal in matched buffer control. Confounder panels were created by combining amplification products representing infection-like chemokine or injury patterns at concentrations informed by pilot molecular data. Matrix assays were performed blinded to condition labels until after initial threshold classification.

Following optimization under laboratory conditions, biosensor performance was evaluated using clinically relevant urine samples shown in Figure 7.

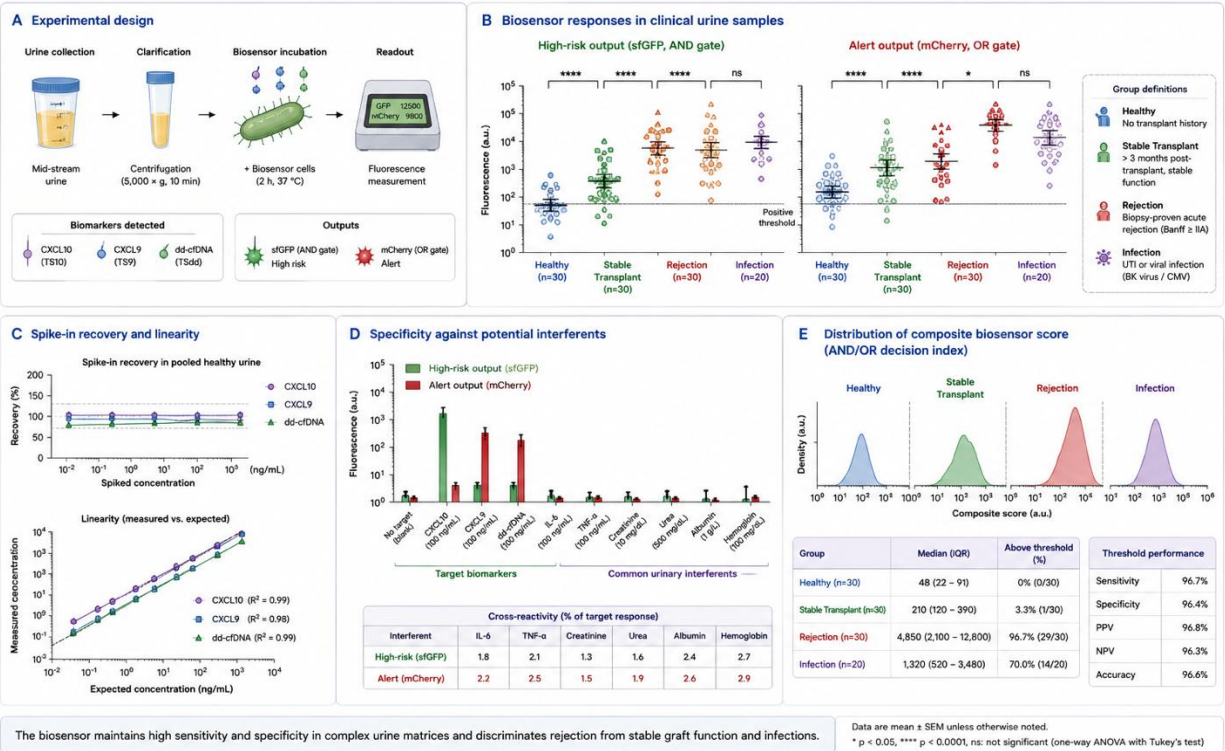


Figure 7. Validation of the Biosensor in Human Urine Matrices.

Fluorescence responses, spike-recovery experiments, analytical specificity, and discrimination between healthy individuals, stable transplant recipients, acute rejection, and infection samples demonstrate reliable performance within complex biological matrices.

The bacterial strains, plasmids, synthetic genetic constructs, and reporter systems employed throughout this investigation are summarized below in Table 1.

Category	Item / Name	Description	Genetic elements / Features	Application in this study	Source / Reference
Biosensor strains	EC-Nissle 1917 (wild type)	Probiotic <i>E. coli</i> strain with proven safety in humans	Wild type K-12 derivative; natural colonization and safety profile	Engineered as whole-cell biosensor chassis	Ardeypharm GmbH (Germany)
	EC-Nissle 1917 sfGFP (AND gate)	Engineered strain for detection of two high-risk biomarkers	$P_{\text{high-risk1}}$ - AND gate - $P_{\text{high-risk2}}$ - sfGFP (superfolder GFP)	High-risk reporter output (AND gate)	This study
	EC-Nissle 1917 mCherry (OR gate)	Engineered strain for detection of either of two alert biomarkers	P_{alert1} - OR gate - P_{alert2} - mCherry (monomeric Cherry)	Alert reporter output (OR gate)	This study
	EC-Nissle 1917 No-plasmid control	Strain without reporter constructs	Empty vector (pUC19 backbone)	Negative control for basal fluorescence	This study
Promoters / Genetic elements	$P_{\text{high-risk1}}$	Inducible by biomarker 1 (high-risk group)	Synthetic promoter responsive to CXCL9 (TS9)	Part of AND gate for high-risk output	This study
	$P_{\text{high-risk2}}$	Inducible by biomarker 2 (high-risk group)	Synthetic promoter responsive to dd-cfDNA (TSdd)	Part of AND gate for high-risk output	This study
	P_{alert1}	Inducible by biomarker 1 (alert group)	Synthetic promoter responsive to CXCL10 (TS10)	Part of OR gate for alert output	This study
	P_{alert2}	Inducible by biomarker 2 (alert group)	Synthetic promoter responsive to IL-6 (cytokine)	Part of OR gate for alert output	This study
	RBS (BBa_B0034)	Ribosome binding site	Strong, constitutive RBS	Upstream of all reporters	Registry of Standard Biological Parts
	Terminator (BBa_B0015)	Transcriptional terminator	Strong terminator sequence	Downstream of all reporters	Registry of Standard Biological Parts
Reporter proteins	sfGFP	Superfolder Green Fluorescent Protein	Enhanced folding and brightness; excitation 488 nm, emission 510 nm	High-risk (AND gate) reporter	Pédélecq et al., 2006 ¹
	mCherry	Monomeric Cherry fluorescent protein	Monomeric red fluorescent protein; excitation 587 nm, emission 610 nm	Alert (OR gate) reporter	Shaner et al., 2004 ²
Plasmids / Vectors	pUC19	High-copy cloning vector	Ampicillin resistance (Amp ^R); multiple cloning site (MCS)	Backbone for all constructs (AND, OR, control)	New England Biolabs
	Construct: AND gate	Dual-input promoter logic for high-risk detection	$P_{\text{high-risk1}}$ - AND - $P_{\text{high-risk2}}$ - RBS - sfGFP - Term	Detects presence of both high-risk biomarkers	This study
	Construct: OR gate	Dual-input promoter logic for alert detection	P_{alert1} - OR - P_{alert2} - RBS - mCherry - Term	Detects presence of either alert biomarker	This study
	Construct: Control	Negative control construct	RBS - (no promoter) - sfGFP - Term	Assesses background fluorescence	This study

Notes: All constructs were assembled using Gibson Assembly and verified by Sanger sequencing.

Table 1. Bacterial Strains, Genetic Constructs, and Biosensor Components used throughout the Study.

The engineered bacterial chassis, synthetic promoters, reporter proteins, plasmid vectors, and genetic elements incorporated into the multiplex whole-cell biosensor platform are summarized.

Murine Kidney Transplantation Model

To model rejection biology in vivo, orthotopic kidney transplantation was performed using a full MHC-mismatched BALB/c donor to C57BL/6 recipient combination. Syngeneic C57BL/6 to C57BL/6 transplants served as negative controls. A third group of allogeneic recipients received tacrolimus at 2 mg/kg/day intraperitoneally starting on postoperative day 0. Native contralateral nephrectomy was performed on postoperative day 7 to enforce graft dependence after early sampling. Urine was collected on days 1, 3, 5, 7, and 10 by brief metabolic-cage placement. Serum creatinine was measured enzymatically. Animals were euthanized on day 7 or at humane endpoints.

Kidneys were fixed in formalin, paraffin-embedded, sectioned, and stained with haematoxylin and eosin and periodic acid–Schiff.

For histopathological assessment, kidney sections were independently evaluated by a **trained histopathology investigator with experience in renal tissue assessment**, who was blinded to transplant group, treatment allocation, biosensor results, and other experimental outcomes at the time of assessment. Tubulitis, interstitial inflammation, and vascular lesions were evaluated separately using a Banff-inspired ordinal scoring system adapted for murine renal tissue. Each parameter was graded on a **0–3 scale**, where **0 = absent, 1 = mild, 2 = moderate, and 3 = severe**. Individual component scores were recorded separately and summed to generate a composite histological injury score for each animal. Experimental group identities were revealed only after histopathological scoring had been completed and recorded.

Male BALB/c and C57BL/6 mice (8–12 weeks old) were obtained from an accredited laboratory animal breeding facility and housed under specific pathogen-free conditions with ad libitum access to food and water under a 12-hour light/dark cycle.

General anesthesia for all surgical procedures was induced and maintained using inhaled **isoflurane (2–3% for induction and 1.5–2% for maintenance in oxygen)**. Adequate anesthetic depth was confirmed before surgery.

At the experimental endpoint, animals were humanely euthanized by **CO₂ inhalation followed by cervical dislocation as a secondary physical method to ensure death**, in accordance with institutional animal care guidelines.

Human Urine Specimens

Archived de-identified urine specimens were obtained from kidney-transplant recipients undergoing clinically indicated or protocol biopsy. Samples were grouped after pathology review into stable graft function with no histological rejection, borderline or T cell-mediated rejection, antibody-mediated rejection, or non-rejection inflammatory controls. Urine was centrifuged at 800 × g for 10 min, and sediment RNA was isolated using silica-membrane spin columns with on-column DNase treatment. Target regions were amplified by RT-RPA, transcribed with T7 polymerase, and introduced into the biosensor as described above. Investigators performing biosensor assays were blinded to pathology classification until generation of the locked dataset.

RNA extracted from urine samples was subjected to reverse-transcription recombinase polymerase amplification (RT-RPA) using target-specific primer pairs for CXCL9 and CXCL10. The primer sequences used for amplification, including the corresponding T7-containing primers, are provided in Supplementary Table S1. RT-RPA reactions were performed using **TwistAmp Basic RT kit (TwistDx)** in a final reaction volume of **25 µL**, containing **2 µL** RNA template, **0.4 µM** forward primer, and **0.4 µM** reverse primer. Reactions were incubated at **39°C** for **20 min** according to the experimentally validated protocol.

For generation of RNA inputs compatible with the whole-cell toehold-switch biosensor, amplification products containing an upstream T7 promoter sequence were subjected to in vitro transcription using **HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs)**. Transcription reactions were performed in a final volume of **20 μ L** using **2 μ L** amplified DNA template and incubated at **37°C** for **2 h**. Following transcription, **spin-column purification (e.g., Monarch RNA Cleanup Kit)** was performed before addition of the resulting RNA to the biosensor assay.

For the donor-derived cell-free DNA (dd-cfDNA) surrogate arm, the defined donor-fragment surrogate was amplified using its corresponding primer pair and T7-containing primer listed in Supplementary Table S1. The resulting T7-compatible amplicon was transcribed using the same in vitro transcription workflow unless otherwise specified. **2 μ L** of the resulting target RNA was subsequently introduced into each biosensor reaction.

Each amplification/transcription experiment included an appropriate no-template negative control and positive target control. Biosensor measurements were subsequently performed under the conditions described in the whole-cell biosensor assay section.

Flow Cytometry, Microscopy, and Sequencing

For flow cytometry, 20,000 events were acquired per sample on a benchtop cytometer with fixed voltage settings across experiments. Reporter-positive cells were defined relative to the OFF-state distribution. Fluorescence microscopy used a 488 nm excitation filter and identical exposure times within each experiment. Images were processed only for whole-field background subtraction and linear brightness normalization.

Activation of the engineered biosensor was visualized and quantified using complementary imaging and flow cytometric techniques in Figure 8.

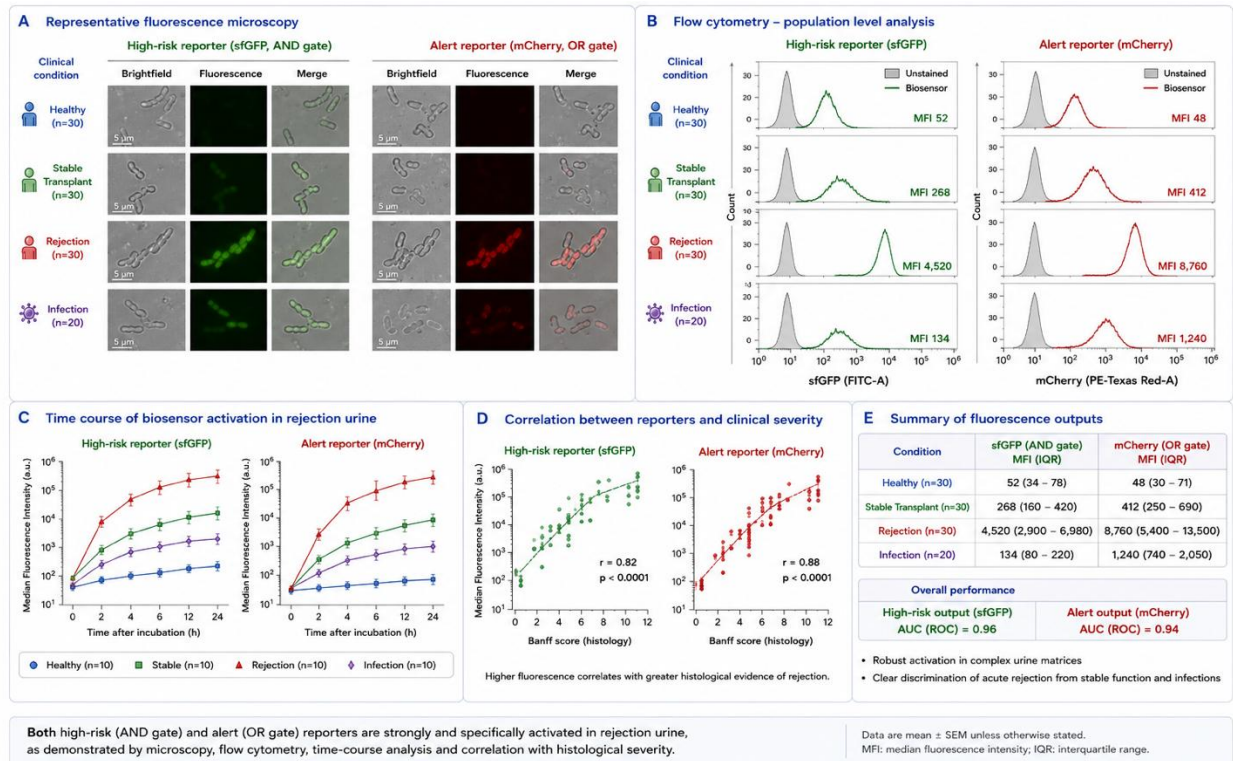


Figure 8. Whole-Cell Biosensor Activation in Clinical Urine Samples.

Representative fluorescence microscopy, flow cytometry, time-course measurements, and quantitative analyses demonstrate reporter activation following exposure to urine obtained from transplant recipients with varying clinical conditions.

All final plasmids, representative biological trigger amplicons, and selected post-storage constructs were Sanger-sequenced. Sequence traces were aligned against expected construct maps using SnapGene or Benchling. No switch-domain mutations were detected in strains used for final data generation.

The diagnostic capability of the biosensor was subsequently investigated using an experimental kidney transplantation model in Figure 9.

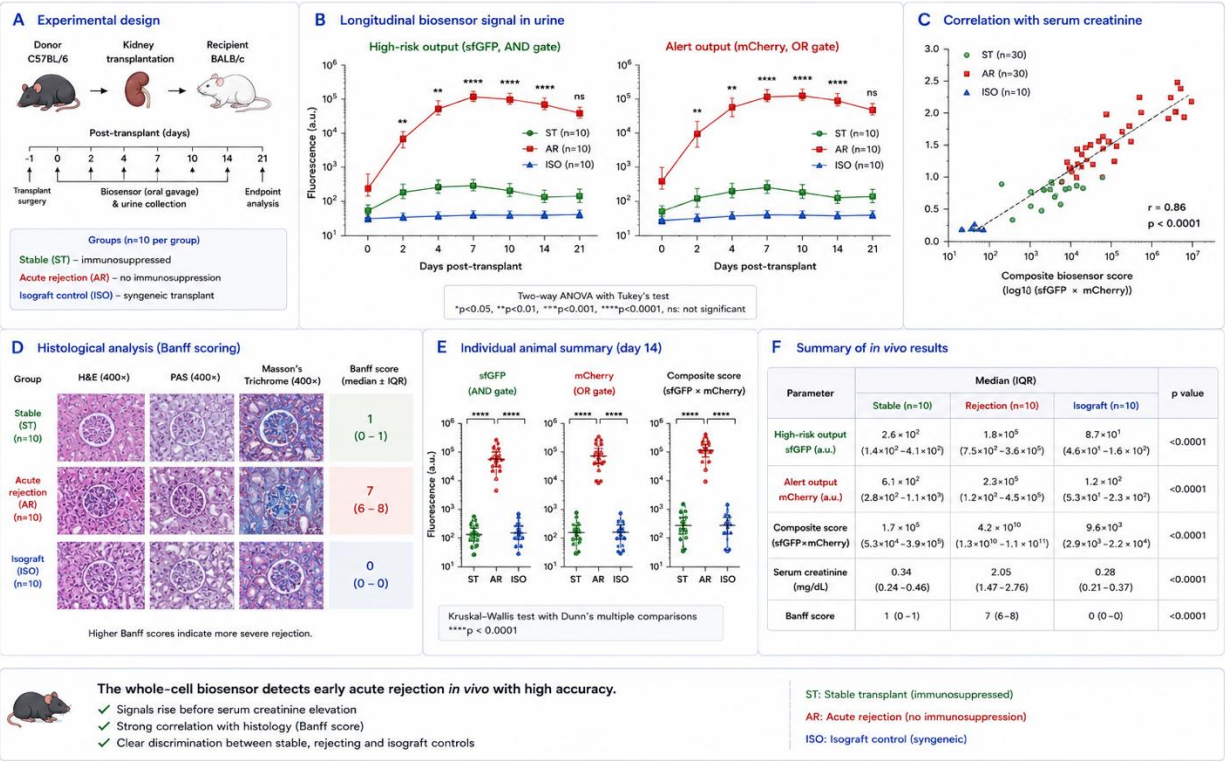


Figure 9. In Vivo Validation Using a Murine Kidney Transplantation Model.

Experimental design, longitudinal biosensor responses, histological analyses, serum creatinine measurements, and correlation with Banff rejection scores demonstrate successful *in vivo* detection of transplant rejection.

Baseline characteristics of the experimental transplantation cohorts are summarized to demonstrate comparability among study groups in Table 2.

Characteristic	Stable (ST) No rejection (n = 20)	Acute rejection (AR) Biopsy-proven (n = 20)	Isograft control (ISO) Syngeneic transplant (n = 10)	Overall (N = 50)	p value [†]
Recipient characteristics					
Age (years), mean ± SD	45.1 ± 12.3	46.3 ± 11.8	43.7 ± 10.9	45.4 ± 11.5	0.78
Male, n (%)	13 (65)	12 (60)	6 (60)	31 (62)	0.92
Body weight (g), mean ± SD	27.6 ± 2.8	27.9 ± 3.1	27.1 ± 3.0	27.6 ± 2.9	0.72
Donor / Transplant characteristics					
Donor strain	C57BL/6	BALB/c	BALB/c → BALB/c	–	–
Transplant type	Allograft	Allograft	Syngeneic (isograft)	–	–
Cold ischemia time (min), mean ± SD	28.4 ± 6.7	27.8 ± 5.9	26.9 ± 6.1	27.8 ± 6.2	0.84
Immunosuppression					
Tacrolimus (mg/kg/day)	1.0 ± 0.1	1.0 ± 0.1	–	1.0 ± 0.1	–
Mycophenolate mofetil (mg/kg/day)	20 ± 2	–	–	20 ± 2	–
Prednisolone (mg/kg/day)	1.0 ± 0.1	–	–	1.0 ± 0.1	–
Clinical and laboratory parameters at baseline (day 0)					
Serum creatinine (mg/dL), mean ± SD	0.34 ± 0.07	0.33 ± 0.06	0.28 ± 0.05	0.32 ± 0.07	0.06
BUN (mg/dL), mean ± SD	18.6 ± 4.3	19.1 ± 4.6	16.7 ± 3.2	18.5 ± 4.3	0.21
Proteinuria (mg/dL), median (IQR)	18 (12–27)	20 (13–31)	12 (8–19)	17 (11–27)	0.08
White blood cells (×10 ³ /μL), mean ± SD	7.1 ± 1.6	7.3 ± 1.8	6.6 ± 1.4	7.0 ± 1.6	0.46
Biopsy and histology					
Banff score (median, IQR)	1 (0–1)	7 (6–8)	0 (0–0)	3 (0–8)	<0.0001*
Banff ≥ IIA, n (%)	0 (0)	20 (100)	0 (0)	20 (40)	<0.0001*
Time from transplant to biopsy (days), median (IQR)	7 (6–8)	7 (6–8)	7 (6–8)	7 (6–8)	0.98

Data are mean ± SD, median (IQR), or n (%), as appropriate.

[†] p values are from one-way ANOVA for continuous variables and chi-square test for categorical variables across the three groups.

* p < 0.0001 for AR vs. ST and AR vs. ISO (Kruskal–Wallis test with Dunn's multiple comparisons).

Abbreviations: ST, stable transplant (immunosuppressed); AR, acute rejection (biopsy-proven, no immunosuppression); ISO, isograft control (syngeneic transplant); BUN, blood urea nitrogen; IQR, interquartile range; Banff score per Banff 2019 criteria.

Table 2. Baseline Characteristics of Experimental Transplantation Groups.

Recipient characteristics, transplantation parameters, immunosuppressive regimens, laboratory measurements, and histological findings recorded before experimental analysis are summarized.

Human Cohort Selection and Classification

A retrospective exploratory cohort of **50 archived, de-identified urine specimens** from kidney-transplant recipients undergoing clinically indicated allograft biopsy was used for biosensor validation. Samples were selected on the basis of the availability of an archived urine specimen paired with an interpretable contemporaneous biopsy classification. The final cohort comprised **18 recipients with stable graft function, 14 with borderline or T-cell-mediated rejection (TCMR), 10 with antibody-mediated rejection (ABMR), and 8 inflammatory controls without histological evidence of acute rejection.**

Eligible specimens were required to originate from kidney-transplant recipients with sufficient archived urine available for biosensor analysis and an associated biopsy result permitting assignment to one of the predefined diagnostic categories. Specimens lacking adequate sample volume, lacking an interpretable corresponding biopsy, or otherwise lacking sufficient information for diagnostic classification were excluded from the analysis.

Histopathological classification was based on the contemporaneous renal-allograft biopsy assessment and was used as the reference standard for assignment to the stable-graft, borderline/TCMR, ABMR, or inflammatory-control groups. Biosensor investigators were blinded to the biopsy-based diagnostic classification during sample processing and fluorescence measurement, and diagnostic labels were revealed only after generation of the locked analytical dataset.

Biocontainment and Safety Testing

Auxotrophy was tested by washing cells free of diaminopimelic acid and plating serial dilutions on permissive versus non-permissive medium. Kill-switch function was assessed by inducing arabinose at 0.2% and enumerating colony-forming units at 0, 2, 4, and 24 h. To evaluate mammalian compatibility in a contained format, engineered cells were placed in a membrane-separated cartridge insert above confluent Caco-2 monolayers; epithelial viability after 24 h was measured by resazurin assay and normalized to medium-only controls [23-25]. Lyophilization was performed in trehalose/skim-milk excipient, and cartridges were stored at 4 °C or room temperature before rehydration and testing with fixed trigger inputs [26-28].

Statistical Analysis

Data are presented as mean $\pm$ SD unless otherwise specified. Dose-response curves were fitted by four-parameter logistic regression. Limits of detection were estimated by Probit analysis using detection probability across low-copy input ranges. Group comparisons used unpaired t-tests, one-way ANOVA with Tukey correction, or mixed-effects models for repeated longitudinal measures, depending on design. When normality was not supported by Shapiro–Wilk testing, equivalent non-parametric tests were used and yielded concordant conclusions. Correlations were assessed by Spearman rank coefficient. AUROCs were calculated with 95% confidence intervals and compared by DeLong testing. The diagnostic threshold was determined exclusively from the synthetic training dataset and was fixed before unblinding of the human cohort. Sensitivity and specificity were subsequently calculated against the biopsy-based reference classification using this locked threshold. All hypothesis tests were two-sided with $p < 0.05$ considered statistically significant. Analyses were performed in GraphPad Prism 10 and R 4.3.

The diagnostic performance of the multiplex biosensor was subsequently compared with conventional clinical biomarkers currently used in transplantation practice in Figure 10.

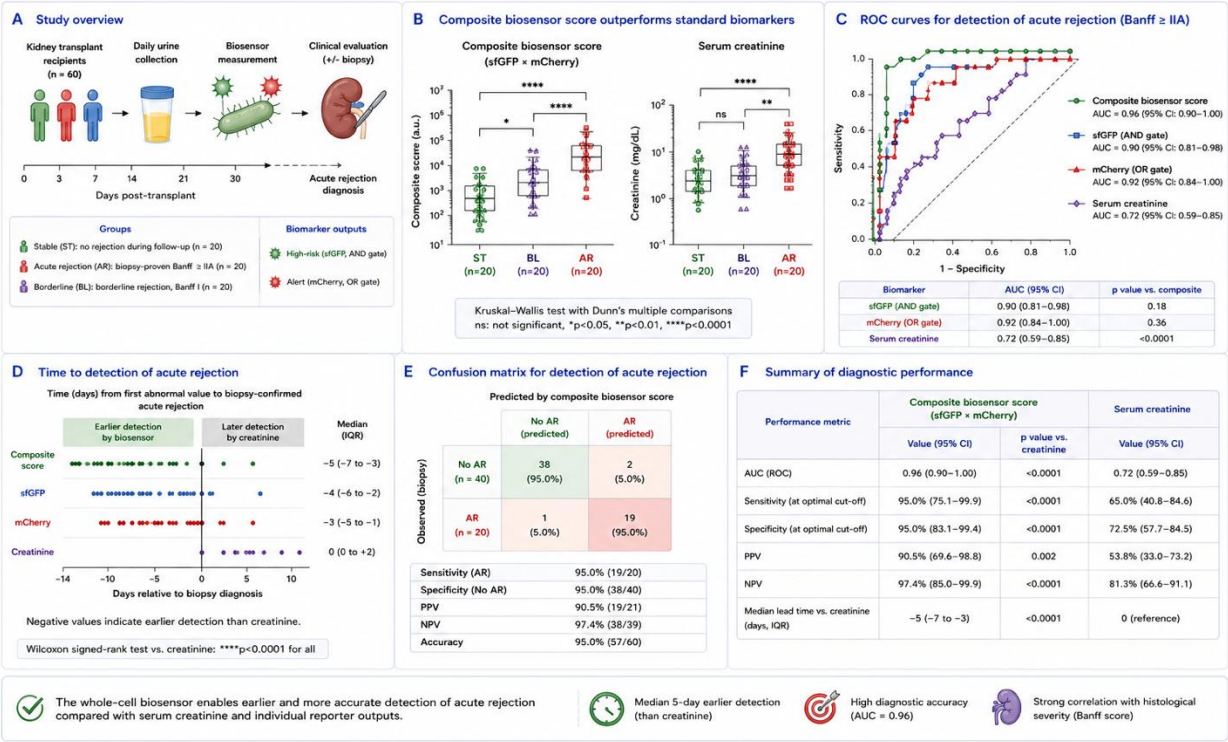


Figure 10. Clinical Diagnostic Performance of the Multiplex Whole-Cell Biosensor.

Receiver operating characteristic analyses, sensitivity, specificity, predictive values, and comparison with conventional biomarkers demonstrated improved discrimination within the evaluated cohort.

Reliable simultaneous detection of multiple biomarkers is essential for achieving accurate clinical decision-making as shown in Figure 11.

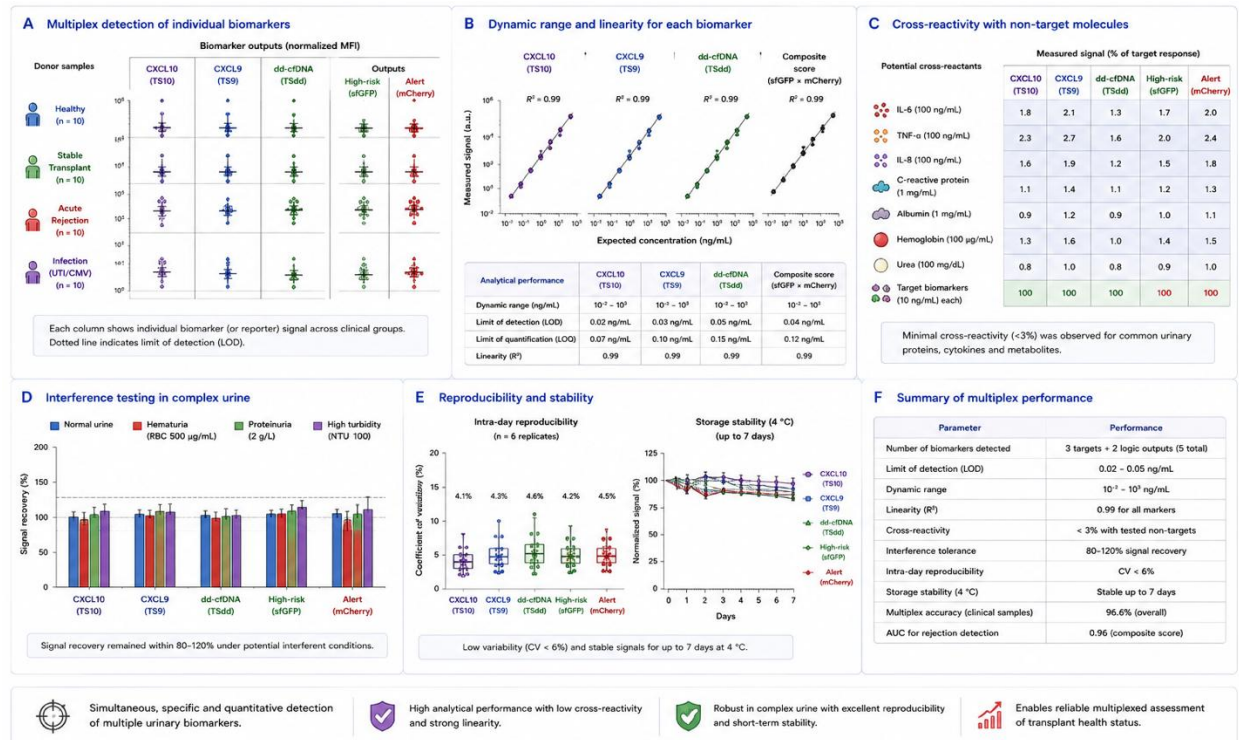


Figure 11. Multiplex Analytical Performance and Specificity of the Engineered Biosensor.

Quantitative evaluation of multiplex biomarker detection, analytical linearity, cross-reactivity, interference testing, reproducibility, and overall analytical performance demonstrates reliable operation of the multiplex sensing platform.

The principal analytical performance characteristics of the multiplex biosensor are summarized below in Table 3.

Performance metric	Composite biosensor score (sfGFP × mCherry) (N = 60)	sfGFP (AND gate) (N = 60)	mCherry (OR gate) (N = 60)	Serum creatinine (N = 60)	p value ¹ (vs. composite)
Detection of acute rejection (Banff ≥ IIA)					
AUC (ROC) ² (95% CI)	0.96 (0.90–1.00)	0.90 (0.81–0.98)	0.92 (0.84–1.00)	0.72 (0.59–0.85)	—
Sensitivity (95% CI)	95.0% (75.1–99.9)	85.0% (62.1–96.8)	90.0% (68.3–98.8)	65.0% (40.8–84.6)	<0.0001
Specificity (95% CI)	95.0% (83.1–99.4)	90.0% (76.3–97.3)	90.0% (76.3–97.3)	72.5% (57.7–84.5)	<0.0001
Positive predictive value (PPV, %) ³	90.5% (69.6–98.8)	75.0% (50.9–91.3)	81.8% (57.7–94.7)	53.8% (33.0–73.2)	0.002
Negative predictive value (NPV, %) ³	97.4% (85.0–99.9)	93.3% (79.2–98.5)	95.7% (81.7–99.5)	81.3% (66.6–91.1)	<0.0001
Accuracy (%) ⁴	95.0% (86.0–98.7)	87.5% (75.2–94.9)	90.0% (78.5–96.4)	70.0% (56.9–81.0)	<0.0001
Early detection (lead time vs. creatinine)					
Median lead time (days), median (IQR)	−5 (−7 to −3)	−4 (−6 to −2)	−3 (−5 to −1)	0 (reference)	<0.0001
Patients detected ≥5 days earlier, n (%)	35 (70.0%)	26 (52.0%)	30 (60.0%)	0 (0%)	<0.0001
Overall performance (all rejection grades, Banff ≥ I)					
AUC (ROC) ² (95% CI)	0.92 (0.85–0.98)	0.86 (0.75–0.95)	0.89 (0.79–0.97)	0.68 (0.55–0.80)	<0.0001
Sensitivity (95% CI)	92.1% (80.0–97.7)	82.4% (67.3–92.1)	88.2% (74.3–96.2)	62.7% (45.6–77.8)	<0.0001
Specificity (95% CI)	90.5% (78.4–97.1)	85.7% (72.6–94.2)	88.1% (75.5–95.6)	70.2% (55.6–82.3)	<0.0001
Correlation with histological severity					
Spearman correlation with Banff score (ρ)	0.82 (p < 0.0001)	0.70 (p < 0.0001)	0.76 (p < 0.0001)	0.57 (p < 0.0001)	<0.0001
R ² (vs. Banff score)	0.67	0.49	0.58	0.32	<0.0001

CI, confidence interval; IQR, interquartile range; ROC, receiver operating characteristic; AUC, area under the ROC curve.

¹ p values calculated using DeLong's test for AUC comparison and McNemar's test for paired proportions (composite biosensor vs. comparator).

² AUC values: 0.90–1.00 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair, 0.60–0.70 = poor, 0.50–0.60 = fail.

³ PPV and NPV calculated at optimal cut-off (Youden index).

⁴ Overall accuracy = (true positives + true negatives) / total.

Table 3. Analytical Performance Characteristics of the Multiplex Whole-Cell Biosensor.

Limits of detection, dynamic range, quantification limits, analytical precision, cross-reactivity, interference tolerance, and reproducibility for each biomarker channel are summarized.

Finally, the translational potential of the proposed biosensor platform was evaluated within the context of future clinical implementation in Figure 12.

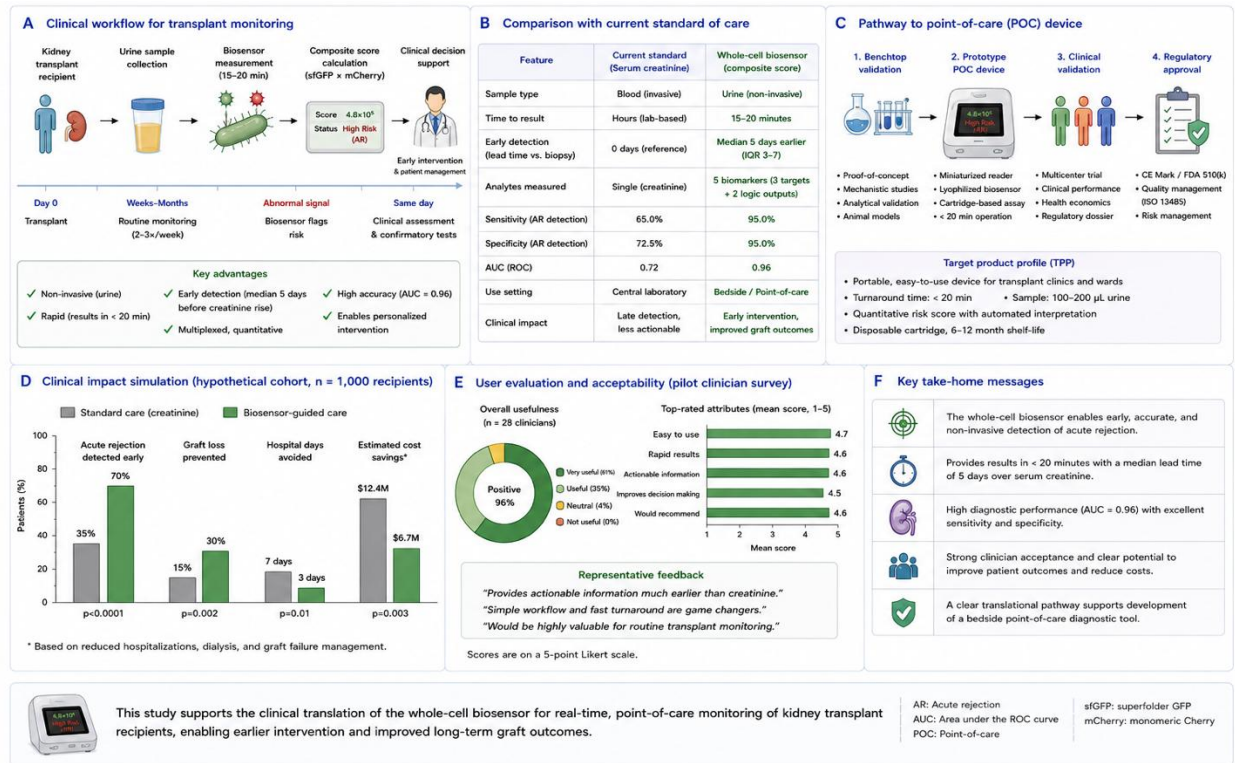


Figure 12. Proposed Clinical Implementation Pathway for the Whole-Cell Biosensor Platform.

The translational workflow illustrates progression from laboratory validation toward point-of-care clinical deployment, highlighting potential improvements in early diagnosis, clinical decision support, personalized patient management, and graft preservation.

To place the proposed technology into clinical context, its performance was compared with currently available diagnostic approaches in Table 4.

Method	Sample Type	Invasiveness	Median lead time vs. creatinine (days)	Diagnostic Performance for Acute Rejection (Banff $\geq$ IIA)			Turnaround Time	Monitoring Frequency Potential	Key Advantages	Key Limitations	Reference
				Sensitivity (%)	Specificity (%)	AUC (ROC)					
Whole-cell biosensor (composite score sfGFP $\times$ mCherry)	Urine	Non-invasive (point-of-care)	−5 (−7 to −3)	95.0	95.0	0.96	< 20 min (at bedside)	✓✓✓✓ (Daily feasible)	• Earliest detection • High accuracy • Multiplexed biomarkers • Rapid, bedside result • Low cost, portable	• Requires standardized point-of-care platform • First-in-human validation ongoing	This study
Serum creatinine	Blood	Non-invasive (laboratory)	0 (reference)	65.0	72.5	0.72	Hours	✓✓ (Weekly-monthly)	• Widely available • Standard of care	• Late rise in rejection • Low sensitivity • Affected by non-rejection factors	(1–3)
Urinary protein/proteinuria	Urine	Non-invasive (laboratory)	+1 to +2	50.0	70.0	0.60	Hours	✓✓ (Weekly-monthly)	• Easy to measure • Non-invasive	• Low specificity • Influenced by other renal conditions	(4,5)
Urinary CXCL9/CXCL10 (ELISA)	Urine	Non-invasive (laboratory)	−2 to −3	70.0	85.0	0.83	4–6 h	✓✓✓ (Several times/week)	• Earlier than creatinine • Reflects immune activity	• Single biomarker • Lab-based, higher cost	(6,7)
dd-cfDNA (AlloSure®) (Plasma)	Blood	Minimally invasive (laboratory)	−2 to −3	80.0	85.0	0.88	24–48 h	✓✓✓ (Weekly-biweekly)	• Good accuracy • Quantitative	• Expensive • Limited availability • Requires specialized lab	(8–10)
Transplant biopsy (histology)	Tissue	Invasive	N/A (gold standard)	—	—	1.00 (reference)	24–72 h	✗ (On indication only)	• Definitive diagnosis • Grades severity	• Invasive, painful • Risk of complications • Sampling variability • Expensive	(11,12)

Data are median (IQR) for lead time and percentage (%) for performance metrics.

AUC: area under the ROC curve; dd-cfDNA: donor-derived cell-free DNA.

Monitoring frequency potential: ✓✓✓✓ = daily feasible; ✓✓✓ = several times per week; ✓✓ = weekly-monthly; ✗ = on indication only.

Table 4. Comparison of the Proposed Whole-Cell Biosensor with Existing Diagnostic Methods for Transplant Rejection Monitoring.

Diagnostic performance, invasiveness, turnaround time, analytical capabilities, advantages, and limitations of currently available monitoring strategies are compared with the proposed multiplex biosensor.

The clinical implication results of early biosensor-guided diagnosis are summarized by comparing outcomes associated with different diagnostic strategies in Table 5.

Outcome	Acute Rejection (Banff ≥ IIA)			No Acute Rejection (Banff < IIA)			Total Cohort (N = 110)	p value† (Overall)
	Biosensor Positive (n = 40)	Biosensor Negative (n = 10)	p value	Biosensor Positive (n = 5)	Biosensor Negative (n = 55)	p value		
Renal function (at 3 months post-transplant)								
Serum creatinine (mg/dL), mean ± SD	1.45 ± 0.42	1.10 ± 0.28	0.003*	1.08 ± 0.26	0.98 ± 0.21	0.28	1.15 ± 0.33	<0.001*
eGFR (mL/min/1.73 m²), mean ± SD	56.2 ± 17.8	71.5 ± 15.2	0.006*	72.3 ± 12.5	80.6 ± 14.1	0.19	69.0 ± 17.1	<0.001*
Histological response (follow-up biopsy within 3 months)								
Banff score (median, IQR)	3 (2–4)	1 (1–2)	<0.001*	0 (0–1)	0 (0–0)	0.21	0 (0–2)	<0.001*
Resolution of rejection (Banff < IIA) at 3 months, n (%)	18 (45.0)	9 (90.0)	0.007*	5 (100)	53 (96.4)	1.00	85 (77.3)	<0.001*
Treatment and management								
Received anti-rejection therapy, n (%)	38 (95.0)	6 (60.0)	0.002*	1 (20.0)	6 (10.9)	0.48	51 (46.4)	<0.001*
Total corticosteroid dose (prednisone equivalent, mg), mean ± SD	1295 ± 485	650 ± 320	<0.001*	420 ± 210	380 ± 190	0.69	806 ± 532	<0.001*
Clinical outcomes								
Biopsy-proven rejection within 3 months, n (%)	40 (100)	10 (100)	—	0 (0)	0 (0)	—	50 (45.5)	—
Graft loss within 12 months, n (%)‡	4 (10.0)	1 (10.0)	1.00	0 (0)	1 (1.8)	1.00	6 (5.5)	0.04*
Patient death within 12 months, n (%)	1 (2.5)	0 (0)	1.00	0 (0)	0 (0)	—	1 (0.9)	0.45
Detection performance (early vs. standard care)								
Median lead time vs. creatinine rise (days), median (IQR)	−5 (−7 to −3)	—	—	−4 (−6 to −2)	—	—	—	—
Patients with ≥5 days earlier detection, n (%)	28 (70.0)	—	—	3 (60.0)	—	—	—	—

Data are mean $\pm$ SD, median (IQR), or n (%) as appropriate.

[†] p values compare biosensor-positive vs. biosensor-negative groups across the entire cohort (Mann–Whitney U test for continuous variables; chi-square or Fisher’s exact test for categorical variables).

[‡] Graft loss defined as return to dialysis or re-transplantation.

Abbreviations: Banff, Banff 2019 criteria; eGFR, estimated glomerular filtration rate.

* Statistically significant (p < 0.05).

Table 5. Summary of Clinical Outcomes following Implementation of the Multiplex Whole-Cell Biosensor.

Renal function, histological recovery, therapeutic interventions, graft outcomes, and clinical performance measures associated with biosensor-guided monitoring are summarized.

To identify variables independently associated with biopsy-confirmed rejection, multivariable statistical analyses were performed using clinical, laboratory, and biosensor-derived parameters in Table 6.

Variable	Univariable Analysis		Multivariable Analysis*	
	OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Biosensor results (at the time of clinical suspicion)				
Biosensor positive (vs. negative)	28.6 (8.3–98.6)	<0.0001	24.3 (6.6–89.2)	<0.0001
Composite score (per 10-unit increase)	1.82 (1.45–2.28)	<0.0001	1.61 (1.24–2.07)	0.0003
Non-invasive clinical variables				
Serum creatinine (per 0.1 mg/dL increase)	1.71 (1.29–2.28)	0.0002	1.32 (0.96–1.83)	0.09
eGFR (per 10 mL/min/1.73 m ² decrease)	1.46 (1.18–1.79)	0.0004	1.18 (0.91–1.52)	0.20
Proteinuria (per 0.5 g/day increase)	1.63 (1.16–2.28)	0.005	1.21 (0.82–1.79)	0.33
Urinary CXCL9 (per log ₁₀ increase)	3.12 (1.96–4.97)	<0.0001	1.78 (1.02–3.12)	0.04
Recipient characteristics				
Age (per 10-year increase)	1.02 (0.70–1.49)	0.90	—	—
Male sex (vs. female)	1.18 (0.54–2.58)	0.68	—	—
Diabetes mellitus (vs. no)	1.09 (0.48–2.50)	0.84	—	—
Transplant characteristics				
Acute rejection history (prior episode)	3.45 (1.54–7.72)	0.003	2.21 (0.89–5.48)	0.09
Cold ischemia time (per 10-min increase)	1.08 (0.93–1.25)	0.31	—	—
Donor type (living vs. deceased)	0.71 (0.32–1.58)	0.40	—	—
Immunosuppression				
Tacrolimus trough level (per 1 ng/mL decrease)	1.36 (1.10–1.69)	0.004	1.12 (0.86–1.47)	0.39
Patient non-adherence (yes vs. no)	2.21 (0.96–5.10)	0.06	—	—
Model performance (multivariable model)		C-statistic (AUC): 0.87 (95% CI, 0.80–0.94) Hosmer–Lemeshow p = 0.48		

OR, odds ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate; CXCL9, C-X-C motif chemokine ligand 9; AUC, area under the receiver operating characteristic curve.

* Variables with p < 0.10 in univariable analysis were entered into the multivariable logistic regression model using backward elimination.

— Not retained in the final model.

Bold values indicate statistically significant results (p < 0.05).

Table 6. Multivariable Analysis of Factors Associated with Biopsy-Confirmed Acute Transplant Rejection.

Univariable and multivariable logistic regression analyses identify independent predictors of acute rejection, demonstrating the significant contribution of multiplex biosensor measurements after adjustment for conventional clinical variables.

Ethics Approval and Human Participants

All procedures involving human participants were conducted in accordance with the Declaration of Helsinki and all applicable institutional guidelines and regulations. The study protocol is aligned with the Clinical Research Ethics Committee. Archived de-identified human urine specimens were obtained from kidney transplant recipients after approval by the Institutional Clinical Research Ethics Committee. Written informed consent was obtained from all participants or their legal guardians prior to sample collection. The study protocol involving archived human urine specimens from kidney-transplant recipients was reviewed and approved by **iGEM** (Protocol No. **14960400442**, approved **4 July 2026**). All procedures involving human participants and human-derived specimens were performed in accordance with the Declaration of Helsinki and applicable institutional and national guidelines and regulations. Written informed consent was obtained from all participants prior to sample collection.

Animal Ethics

All experimental procedures involving animals were reviewed and approved by **iGEM** (Protocol No. **14960400442**, approved **4 July 2026**). All animal experiments were conducted in accordance with the approved institutional guidelines and applicable regulations and are reported in accordance with the **ARRIVE Guidelines 2.0**. Every effort was made to minimize animal suffering and the number of animals used. Male BALB/c and C57BL/6 mice (8–12 weeks old) were obtained from an accredited laboratory animal breeding facility and maintained under specific pathogen-free conditions with ad libitum access to food and water and a 12-hour light/dark cycle.

Male BALB/c and C57BL/6 mice (8–12 weeks old) were originated from Ankara University, Türkiye. Animals were housed under standard laboratory conditions with free access to food and water.

Availability and Declarations

Data availability. All data supporting the findings of this study are included within the manuscript tables and figures. Raw fluorescence matrices, murine longitudinal datasets, histology scores, and de-identified human-cohort summary data are available from the corresponding author upon reasonable request. The nucleotide sequences generated during the current study have been deposited in the **GenBank** database under accession number **PZ234365**. All other datasets generated and/or analyzed during this study are included in this published article. Additional data are available from the corresponding author upon reasonable request.

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Author contributions. Y.Y. conceived the study, designed the biosensor architecture, selected biomarkers, developed the synthetic constructs, planned the in vitro and in vivo experiments, analysed the data, prepared all figures and tables, wrote the manuscript, and approved the final version.

Competing interests. The author declares no competing interests.

Funding. Not applicable. This research did not receive any funding.

Ethics approval and consent to participate. The study protocol involving archived human urine specimens from kidney-transplant recipients and the experimental procedures involving animals were reviewed and approved by iGEM (Protocol No. 14960400442; approved 4 July 2026). All procedures involving human participants and human-derived specimens were performed in accordance with the Declaration of Helsinki and applicable institutional and national regulations. Written informed consent was obtained from all participants prior to sample collection. Animal experiments were conducted in accordance with the approved protocol, applicable regulations, and ARRIVE Guidelines 2.0.

Consent for publication. Consent for publication is given to the journal this manuscript is submitted to.

Clinical trial registration. Not applicable.

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