OVERCOMING LYSATE INTERFERENCE:

A RESPIRATION-BASED MIC ASSAY FOR RELIABLE PHAGE SUSCEPTIBILITY TESTING

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INTRODUCTION

The rapid emergence and global dissemination of multidrug-resistant (MDR) bacterial pathogens constitute a severe and escalating threat to global public health and military medical readiness. This crisis has renewed intense interest in bacteriophage (phage) therapy as a precision, targeted antimicrobial strategy. However, the transition of phage therapy from compassionate-use exemptions to widespread, standardized clinical implementation remains severely hindered. Specifically, the field lacks robust, universally accepted methodologies for accurately measuring phage susceptibility, defining standardized phage Minimum Inhibitory Concentration (MIC) values, and rapidly selecting optimal therapeutic phages for individual patients (Figure 1).

Traditional liquid-culture phage susceptibility assays frequently rely on continuous optical density (OD) measurements to monitor bacterial growth inhibition. While these assays are amenable to high-throughput screening, they suffer from inherent optical limitations. Phage-mediated bacteriolysis generates substantial extracellular debris, including intact "ghost" cells and membrane fragments, which remain suspended in the culture. This lysate often scatters light, resulting in artificially elevated turbidity measurements. Consequently, this lysate interference can mask actual bactericidal activity, leading to false-negative interpretations of phage efficacy and inaccurate estimates of bacterial viability (Figure 2).

Metabolic assays based on tetrazolium dye reduction, such as those utilized by the legacy Biolog OmniLog system, successfully overcome this turbidity limitation by measuring cellular respiration rather than physical mass. Active bacterial electron transport chains reduce the colorless tetrazolium dye to a visible, purple-red formazan product; conversely, phage-mediated killing suppresses metabolic activity, halting color development. Although this approach provides a highly reliable measure of true bacterial viability, the colorimetric reaction is cumulative and chemically irreversible. Once the dye is reduced, the optical signal remains permanently elevated even if the bacterial population subsequently lyses. This "signal memory" effectively blinds the assay to delayed pharmacodynamic events, limiting the detection of late-onset lysis, the emergence of phage-resistant bacterial subpopulations, or precise culture collapse timing (Figure 3).

To address these critical limitations, we used a novel, respiration-based phage MIC assay utilizing the Odin platform. Unlike legacy systems restricted to single-channel metabolic tracking, Odin incorporates a multiplexed dual-readout architecture that simultaneously measures tetrazolium-based respiration at 590 nm and optical density at 740 nm. The 740 nm wavelength was specifically selected because it falls strictly outside the absorption spectrum of the red formazan reaction product, thereby eliminating signal crossover while still providing highly accurate measurements of culture turbidity. This dual-readout approach synergizes the advantages of metabolic viability tracking with the capacity to monitor physical growth dynamics. As a result, it allows for the precise detection of late lysis events, the emergence of resistance, and complex phage-antibiotic interactions (PAI) in real-time Figure 4).

Beyond precision susceptibility testing, the Odin platform represents a massively scalable phenotyping engine capable of capturing approximately 5,000 discrete phage-bacterial interactions simultaneously. The resulting high-density, multi-parametric kinetic datasets are crucial for mapping complex biological interactions at an unprecedented scale. Ultimately, this data provides the foundational training architecture necessary for developing artificial intelligence (AI)-driven predictive models. By linking genomic data with Odin-derived phenotypic kinetics, these AI systems will be capable of rapidly identifying optimal therapeutic phages from expansive, globally distributed phage biobanks to treat newly acquired clinical isolates, significantly accelerating the delivery of personalized antimicrobial therapeutics (Figure 9).

MATERIALS & METHODS

Development and Validation of the Dual-Readout Microdilution Assay
Phage susceptibility testing was successfully executed using a standardized, high-throughput microdilution assay engineered specifically for the Odin platform. Standardized inocula of clinical bacterial isolates were dispensed into 96-well microtiter plates containing a proprietary tetrazolium redox dye formulation. These cultures were subsequently challenged with standardized serial dilutions of bacteriophages to establish precise Minimum Inhibitory Concentration (MIC) profiles. Throughout the incubation period, the Odin platform continuously captured high-density kinetic data by simultaneously acquiring two independent optical measurements:

Metabolic Viability (590 nm): Cellular respiration was tracked via the reduction of the tetrazolium dye. Metabolically active and proliferating bacterial cells efficiently reduced the colorless dye into a purple-red formazan product, yielding an optical signal directly proportional to cellular respiration. Successful phage infection and subsequent cellular hijacking rapidly arrested this metabolic activity, resulting in a quantifiable suppression of color formation.

Physical Turbidity (740 nm): Simultaneously, optical density was continuously monitored at 740 nm. This specific near-infrared wavelength successfully decoupled physical turbidity from the colorimetric assay, entirely avoiding optical interference from the red formazan reaction product. This provided an uninterrupted real-time profile of bacterial mass, culture turbidity, and physical lysis kinetics.

Synergistic Insights from Multiplexed Data
The integration of these two distinct data streams provided a comprehensive physiological profile of the phage-host interaction that cannot be achieved with single-readout systems. By correlating metabolic suppression (590 nm) with physical lysis (740 nm), the assay could reliably distinguish between active bacterial growth, bacteriostatic states, and true bactericidal (lytic) events.

Assay Optimization and Benchmarking To ensure clinical reproducibility, assay performance was rigorously optimized across several critical parameters. Comprehensive evaluations of the Multiplicity of Infection (MOI), initial phage titer concentrations, and variable incubation durations were conducted across a diverse panel of bacterial strains. When benchmarked against traditional, respiration-only datasets generated by the legacy Biolog platform, the Odin dual-readout data demonstrated superior analytical resolution. Notably, the Odin system bypassed the "signal memory" limitations of the Biolog platform, allowing for precise detection of post-metabolic lytic events that would otherwise remain masked.

Longitudinal Monitoring of Resistance and Synergy
The continuous kinetic monitoring capabilities of the Odin platform proved highly effective for extended longitudinal tracking. The 740 nm turbidity data successfully captured delayed secondary outgrowth events, providing a reliable early-warning indicator for the emergence of phage-resistant bacterial subpopulations. Furthermore, the platform was leveraged to evaluate Phage-Antibiotic Synergy (PAS). By testing multidimensional matrices of phage-antibiotic combinations, the dual-readout system accurately mapped synergistic, additive, and antagonistic interactions, highlighting optimal therapeutic pairings for multidrug-resistant isolates.

Scalability and AI-Driven Predictive Modeling
Beyond individual susceptibility testing, the platform successfully executed high-throughput screening campaigns, capturing simultaneous kinetic profiles for thousands of distinct phage-host interactions. These large-scale, multidimensional datasets provide a high-fidelity quantitative foundation for the development of machine-learning and artificial intelligence (AI) algorithms. Ultimately, this robust training data will drive the creation of predictive models capable of rapidly identifying optimal therapeutic phage candidates from global biobanks for newly emerging clinical isolates.

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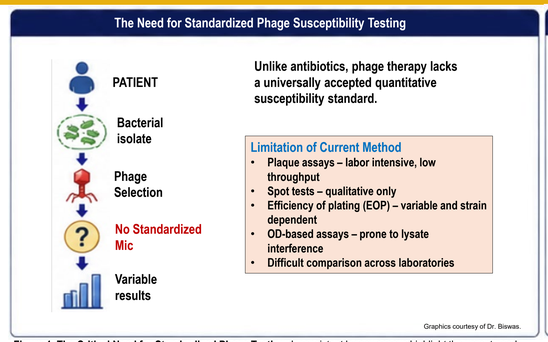

Figure 1. The Critical Need for Standardized Phage Testing: Inconsistent legacy assays highlight the urgent need for a reproducible MIC platform to guide clinical phage selection.

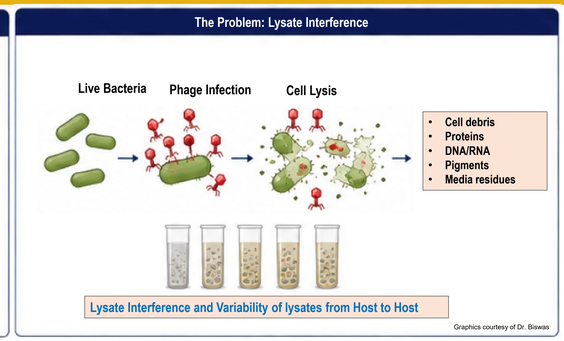

Figure 2. The Optical Artifact: Phage-lysed cellular debris acts as colloidal particles that scatter light (Mie scattering), causing false-positive OD600 turbidity readings that mask bactericidal activity and lead to inaccurate MIC determinations.

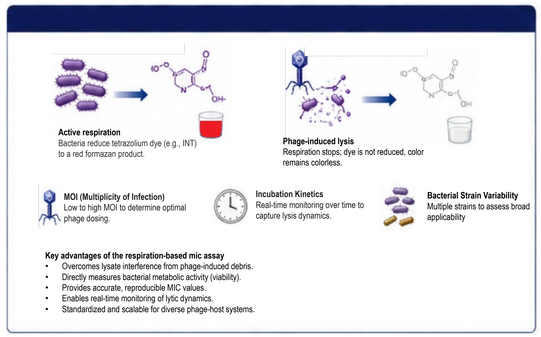

Figure 3. Respiration-Based Phage MIC Assay: This optimized assay measures tetrazolium reduction (respiration) rather than turbidity, bypassing conventional optical artifacts to deliver highly accurate susceptibility profiles (Ref. 2).

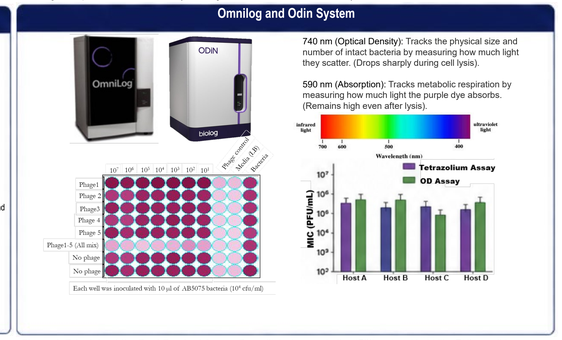

Figure 4. Principle of the Dual-Readout Assay: By multiplexing respiration (590 nm) and turbidity (740 nm), this assay eliminates false-positive signals from light-scattering lysate debris while successfully detecting late physical lysis (Ref. 2)

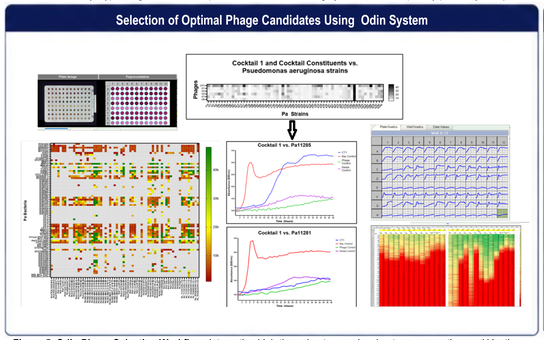

Figure 5. Odin Phage Selection Workflow: Integrating high-throughput screening, heatmap generation, and kinetic analysis accelerates broad-host-range phage selection while tracking emerging bacterial resistance.

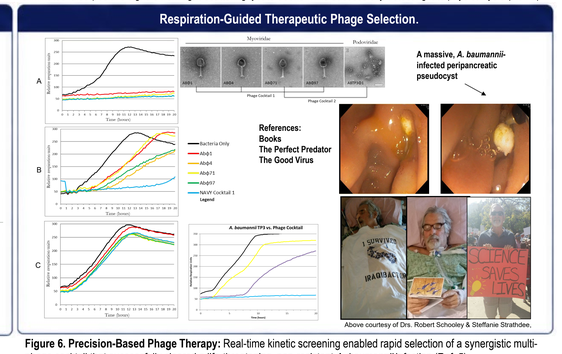

Figure 6. Precision-Based Phage Therapy: Real-time kinetic screening enabled rapid selection of a synergistic multi-phage cocktail that successfully cleared a life-threatening, pan-resistant *A. baumannii* infection (Ref. 5)

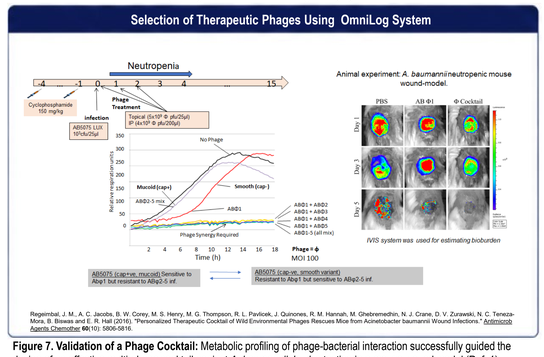

Figure 7. Validation of a Phage Cocktail: Metabolic profiling of phage-bacterial interaction successfully guided the design of an effective multi-phage cocktail against *A. baumannii*. *In vivo* testing in a mouse wound model (Ref. 1).

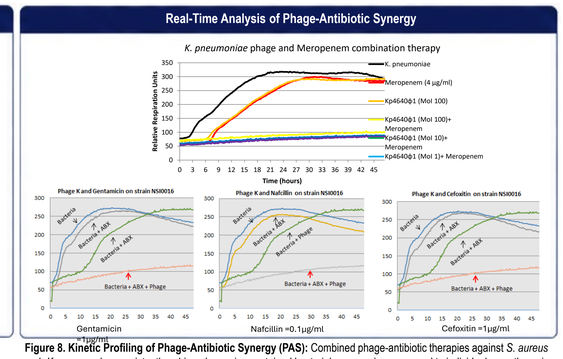

Figure 8. Kinetic Profiling of Phage-Antibiotic Synergy (PAS): Combined phage-antibiotic therapies against *S. aureus* and *K. pneumoniae* consistently achieved superior, sustained bacterial suppression compared to individual monotherapies.

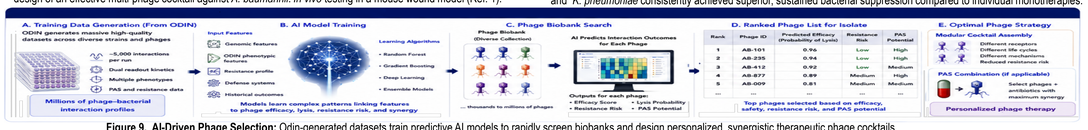

Figure 9. AI-Driven Phage Selection: Odin-generated datasets train predictive AI models to rapidly screen biobanks and design personalized, synergistic therapeutic phage cocktails.

RESULTS

Superior Accuracy of Respiration-Based Viability Tracking
The respiration-based assay effectively decoupled bacterial viability from physical culture mass, allowing for the accurate quantification of phage-mediated bacterial inhibition. Measurements collected at 590 nm directly correlated with active cellular respiration, bypassing the optical artifacts caused by lysate-derived turbidity. Consequently, this metabolic readout generated highly reproducible Minimum Inhibitory Concentration (MIC) values across highly diverse phage-host systems, establishing a rigorous standard for susceptibility testing.
Overcoming the Artifacts of Conventional
Turbidity Direct comparisons between the two independent optical readouts explicitly demonstrated the certain limitations of legacy assays. Conventional turbidity measurements using spectrophotometer alone consistently overestimated bacterial survival (data not shown). This discrepancy is driven by phage-generated cellular debris, intact "ghost" cells, and membrane fragments that remain in suspension and violently scatter light. By contrasting these false-positive turbidity spikes with the 590 nm metabolic data, the respiration readout proved to be a vastly more accurate indicator of true bacterial viability and actual phage efficacy (Figure 4)..
The Dual-Readout Advantage: Resolving Late Lysis Events
Crucially, the Odin dual-readout platform captured complex pharmacodynamic data that metabolic tracking alone would miss. Because the reduction of the tetrazolium dye is a chemically irreversible process, bacterial cultures that exhibited robust initial growth developed substantial color *prior* to phage-mediated killing. This metabolic "signal memory" meant the culture remained visually colored even after complete cellular lysis occurred. By incorporating continuous optical density measurements at 740 nm, the system successfully identified these late-onset lysis events. While the 590 nm signal remained elevated, a sharp reduction in 740 nm turbidity confirmed the physical destruction of the cells, providing indispensable, complementary data regarding infection kinetics.
Real-Time Detection of Phage Resistance
Both the Odin and Biolog platforms successfully captured the emergence of phage-resistant bacterial subpopulations. These escape mutants were easily identifiable through biphasic kinetic profiles, presenting as renewed spikes in respiration (590 nm) and/or secondary outgrowths in turbidity (740 nm) following an initial period of suppression. This real-time, longitudinal tracking provides a critical early-warning mechanism for resistance development during therapeutic screening (Figure 5 & 6). Subsequent evaluation of diverse phage-bacterial interactions confirmed the high reproducibility and robustness of the system. (Ref. 3 and 4).
High-Resolution Mapping of Phage-Antibiotic Synergy (PAS)
Furthermore, the assay successfully quantified Phage-Antibiotic Synergy (PAS) across comprehensive, multi-dose matrices. The dual-readout architecture proved uniquely capable of distinguishing between various therapeutic outcomes. By analyzing both metabolism and physical mass simultaneously, the platform accurately differentiated between bacteriostatic effects (metabolic and physical arrest without cell death), true bactericidal activity (concurrent metabolic collapse and physical lysis), delayed lysis, and highly synergistic interactions between phages and conventional antibiotics (Figure 8).
High-Throughput Scalability for AI-Driven Therapeutics
Leveraging the platform's massively parallel architecture, the Odin system enabled the simultaneous kinetic monitoring of hundreds of individual phage-bacterial interactions. These resulting high-dimensional datasets captured a comprehensive phenotypic footprint—including growth rates, respiration kinetics, lysis dynamics, resistance emergence, and PAS outcomes. Ultimately, generating these large-scale, standardized phenotypic datasets provides the essential foundational training architecture required for artificial intelligence (AI) and machine learning models. Once trained, these AI systems will be capable of rapidly predicting phage-host compatibility, accelerating the identification of optimal therapeutic phages from expansive global libraries (Figure 9).

CONCLUSION

The Odin platform overcomes optical artifacts of cell debris by simultaneously measuring metabolic viability at 590 nm (via tetrazolium dye reduction) and physical culture turbidity at 740 nm. Because near-infrared light at 740 nm falls outside the absorbance spectrum of the red tetrazolium dye, the system can measure physical light scattering (cell mass) completely independently of metabolic color changes. This multiplexed approach eliminates the interference of phage-generated cellular debris while retaining the ability to detect delayed physical lysis events. This dual Readout of Odin system establishes a standardized respiration-based phage MIC assay that overcomes the limitations of traditional turbidity-based susceptibility testing. By integrating respiration measurements at 590 nm with optical density measurements at 740 nm, the Odin platform provides a comprehensive assessment of phage-host interactions while eliminating artifacts caused by lysate interference. Unlike conventional OD assays that are confounded by phage-generated debris, and unlike respiration-only systems that may fail to detect late lysis events, Odin combines viability and turbidity measurements within a single assay framework. This dual-readout capability improves accuracy, reproducibility, and mechanistic interpretation of phage susceptibility data.
In addition to MIC determination, Odin enables detection of phage-resistant bacterial populations, assessment of phage-antibiotic synergy, and large-scale phenotypic screening of phage-host interactions. The platform's ability to simultaneously monitor approximately 5,000 individual phage-bacterial interactions creates unprecedented opportunities for generating AI-ready datasets that can support the development of machine-learning systems for rapid phage selection from phage biobanks against clinical bacterial isolates. The adoption of standardized dual-readout methodologies and AI-assisted phage selection strategies may significantly accelerate the clinical translation of phage therapeutics, improve therapeutic precision, and facilitate regulatory standardization for the treatment of multidrug-resistant bacterial infections.

FUTURE WORK

Future investigations will focus on leveraging the high-throughput capabilities of the Odin platform to drive the clinical translation of phage therapy. Having established a robust method for generating highly accurate, artifact-free kinetic data, our immediate goal is to utilize these large-scale phenotypic datasets to train artificial intelligence (AI) and machine-learning models. Once validated, these AI systems will be designed to predict phage-host compatibility and rapidly identify optimal therapeutic candidates from expansive global phage biobanks for newly acquired clinical isolates. Additionally, we plan to expand our evaluation of Phage-Antibiotic Synergy (PAS) across wider panels of MDR pathogens and correlate these *in vitro* dual-readout metrics with *in vivo* treatment outcomes. Ultimately, this standardized framework will be instrumental in establishing official clinical breakpoints and regulatory standards for phage therapeutics.

DISCLAIMER

Drs. Biswas, Poly and Bishop-Lilly are federal employees of the United States government. This work was prepared as part of his official duties. Title 17 U.S.C. 105 provides that 'copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. 101 defines a U.S. Government work as work prepared by a military service member or employee of the U.S. Government as part of that person's official duties. The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.
The study protocol was reviewed and approved by the NMRC/WRAIR Institutional Animal Care and Use Committee in compliance with all applicable federal regulations governing the protection of animals and research.

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