Two Assays, One Sample: Validation of Sequential Analysis in Biofilms Under Simulated Microgravity

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*Staphylococcus aureus* is a relevant clinical pathogen, frequently implicated in nosocomial infections and known for robust biofilm formation. Studying its phenotype under extreme stress, like microgravity, provides insights into resilience and virulence mechanisms; however, such experiments face severe sample-volume constraints. Sequential assays, assessing metabolic viability with resazurin followed by biomass quantification with crystal violet on the same sample, can optimize these protocols. Nevertheless, validating whether stressed biofilms undergo alterations during the resazurin assay that compromise crystal violet readings is essential. The objective of this study was to validate the sequential resazurin and crystal violet assay in *S. aureus* biofilms under simulated microgravity, comparing it to the direct biomass assay. *S. aureus* biofilms were grown in 25 cm² flasks. The experimental group was incubated for 24h in a 3D clinostat (simulated microgravity, µG), and the control under static conditions (Earth gravity, 1G). Four groups were established: G1-Direct/1G; G2-Direct/µG; G3-Sequential/1G; and G4-Sequential/µG. "Direct" indicates the isolated crystal violet assay; "Sequential" refers to resazurin followed by crystal violet on the same sample. The design included 3 biological replicates per condition, with 3 technical replicates per biological replicate (n=9 readings/group). Absorbance was quantified at 570 nm. Welch's t-tests and a two-way factorial ANOVA with HC3 variance estimates were used to evaluate the environment-by-method interaction, adopting $p<0.05$ as the significance level. Microgravity markedly reduced biomass compared to the terrestrial control (0.39±0.07 vs. 2.12±0.05 in direct method; $p<0.0001$). In 1G, a statistically significant difference was observed between direct and sequential assays (2.12±0.05 vs. 1.97±0.18; $p=0.04$), corresponding to a 7.2% reduction in measured biomass between assays. At µG, the 11% reduction (0.39±0.07 vs. 0.35±0.06) was not statistically significant ($p>0.05$). The two-way ANOVA (HC3) indicated no interaction between environment and method ($p=0.15$), with equivalent inhibition proportions: 81.6% (direct) and 82.4% (sequential). Although simulation significantly reduces biomass, the adhered matrix maintains structural integrity to withstand sequential handling. The observed mechanical losses (7.2% in 1G; 11% in µG) do not alter the main environmental effect, captured equivalently by both

methods, with reductions above 81% in either approach. This protocol allows the extraction of metabolic and structural data from the same sample, reducing biological variability and circumventing volume limitations. In conclusion, the sequential resazurin/crystal violet assay is a statistically validated approach for analyzing *S. aureus* biofilms under stress, enabling sample optimization without compromising reliability. This standardization favors screening studies by saving time and biological samples, expanding the analytical capacity of microbiology laboratories under sample restriction.

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