Improving Robustness of Rapid ATP-based Microbial Enumeration in Complex Dairy & Food Matrices

Brandon Katz, Paul Meighan, Lukas Weise
Hygiena Diagnostics GmbH, Hermannswerder 17, Potsdam, Germany
Hygiena®, 2 Boulden Circle, New Castle, DE 19720

EnSURE Touch SuperSnap UltraSnap KLEANZ

INTRODUCTION:

Enumeration of total viable microorganisms is essential for dairy quality control. It supports timely assessment of raw materials, processing conditions, and finished product quality. Rapid microbiological methods can help this workflow by providing actionable results in an expedited manner. MicroSnap® Total is an ATP-based bioluminescence method that can provide enumeration within a single shift, providing a faster alternative to conventional culture-based methods(1).

However, the biochemical composition of dairy products presents challenges for accurate bioluminescent measurements. Differences in fat content, protein composition, pH, and other matrix characteristics can influence bioluminescent signal generation through optical quenching and interference in luciferase-luciferin reactions. These matrix-dependent effects can reduce signal intensity and introduce variability in the relationship between relative light units (RLUs) and colony forming units (CFUs).

Ultimately, this reduces test accuracy, requiring increased sample preparation in the workflow to overcome matrix effects. Improving the robustness of the chemistry is therefore important for a consistent enumeration across diverse dairy products. This study aims to optimize the MicroSnap Total ATP-dependent bioluminescent reaction by increasing buffer capacity, luciferin concentration, and luciferase concentration.

By improving bioluminescence robustness, this approach has the potential to provide more consistent and reliable microbial enumeration across a broad range of products. Reducing the need for excessive sample preparation or method adjustments could simplify laboratory workflows, increase confidence in results and provide faster, more actionable outcomes. These advancements aim to provide manufacturers with a more practical and dependable tool for maintaining food safety.



PURPOSE:

This study was designed to provide improvements to MicroSnap Total ATP-dependent bioluminescence technology (1). The improvements are focused on robustness and quantitative accuracy of rapid ATP-based microbial enumeration across diverse dairy matrices. Improvements focused on optimizing the luciferase-luciferin bioluminescent reaction, with emphasis on reducing matrix-induced interference. Ultimately, this improves the consistency of RLU-to-CFU measurements, allowing for a potentially more accurate test with minimal sample preparation.

REFERENCES:

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2. ISO 4833-2:2013. *Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 2: Colony count at 30 °C by the surface plating technique*. International Organization for Standardization.
3. Hygiena. *MicroSnap Total Enrichment and Detection Device Instructions for Use*. Document INS-MSTOTAL-REVC. www.hygiena.com

REGISTERED TRADEMARKS / GLOBAL CERTIFICATIONS:

Hygiena® is a registered trademark of Hygiena®
MicroSnap® is a registered trademark of Hygiena®
MicroSnap Total is AOAC PTM certified #031501

METHODS: Bioluminescence Robustness

Experiment 1- Evaluation of Bioluminescent Reagent Buffering Capacity

Buffering capacity was evaluated using increasing concentrations of BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid). Four reagent formulations containing 5, 20, 100, and 200 mM BES were prepared with an initial pH of 6.8. To evaluate resistance to pH shifts induced by acidic matrices, working mixtures were prepared containing 6 parts bioluminescent reagent and 1 part sample. Three acidic matrices were evaluated: Cola (pH 2.63), green tea (pH 3.16), and cold-pressed juice (pH 4.22). The final pH of each working mixture was measured with a pH meter to assess the effects of increased BES concentrations on the stability of the bioluminescence reaction pH.

Experiment 2- Evaluation of Matrix-Induced Bioluminescent Inhibition

Each bioluminescent buffer formulation was converted to a bioluminescent reagent by adding Firefly luciferase at 5 mg/L and D-luciferin potassium salt at 3 mg/L. For each BES formulation, each matrix was measured for bioluminescent inhibition using a pi-102 tube luminometer. Three independent measurements were performed to calculate the percent inhibition. 1) **Matrix background**: 2 µL of sample, 1 µL of sterile water, and 100 µL of bioluminescent reagent were added to a cuvette and measured in the luminometer. 2) **ATP-spiked matrix**: 2 µL of sample, 1 µL of 100nM ATP solution, and 100 µL of bioluminescent reagent were added to a cuvette and measured in the luminometer. 3) **ATP control**: 2 µL of sterile water, 1 µL of 100nM ATP solution, and 100 µL of bioluminescent reagent were added to a cuvette and measured in the luminometer. Matrix-induced inhibition was calculated by comparing the measured ATP-spiked matrix with the theoretical uninhibited signal, determined by summing the matrix background and ATP control RLUs:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{RLU}_{\text{matrix+ATP}}}{\text{RLU}_{\text{matrix}} + \text{RLU}_{\text{ATP control}}}\right) \times 100$$

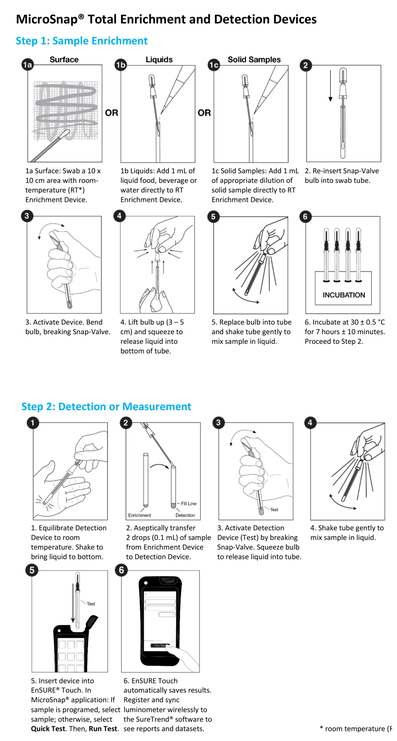
Experiment 3- Luciferase-Luciferin Titrations

Three bioluminescent reagents were created using 100mM BES bioluminescent buffer. **Reagent 1**) 5 mg/L Firefly luciferase and 3 mg/L D-luciferin. **Reagent 2**) 10 mg/L Firefly luciferase and 6 mg/L D-luciferin. **Reagent 3**) 30 mg/L Firefly luciferase and 6 mg/L D-Luciferin. Next, 0.1mL of whole milk, containing natural ATP, was added to a cuvette, followed by 0.45mL of bioluminescent reagent. The bioluminescent reaction was measured using a pi-102 tube luminometer with a 90-second kinetic read.

METHODS: MicroSnap Total System Evaluation

Experiment 4- Optimized Bioluminescent Reagent in MicroSnap Total System

Optimized bioluminescent reagent was evaluated against the current MicroSnap Total system using naturally contaminated 1% and fat-free milk. Milk samples were serially diluted 1:10 in buffered peptone water (BPW), generating concentrations ranging from neat undiluted milk to 0.0001%. The MicroSnap Total procedures were followed as described in the instructions. Optimized bioluminescent was used in place of the MS2-Detection reagent. Microbial concentrations were independently determined using ISO 4833-2 spread plating (2). Both RLUs and CFU measurements were $\log_{10}$-transformed. The relationship between $\log_{10}$(RLU) and $\log_{10}$(CFU) was evaluated for each reagent formulation using linear regression.



RESULTS:

Table 1. Effect of Increasing BES Buffer Concentrations on Bioluminescent Reaction pH and Matrix-induced Inhibition

Matrix (Acid)	Matrix pH	BES	5 mM	20 mM	100 mM	200 mM
Cola (Phosphoric)	2.63	Working pH	5.86	6.14	6.64	6.72
		Bioluminescent Inhibition (%)	37	24	15	12
Green Tea (Citric)	3.16	Working pH	6.53	6.71	6.77	6.84
		Bioluminescent Inhibition (%)	14	3	1	1
Pressed Juice (Ascorbic)	4.22	Working pH	5.8	6.64	6.73	6.76
		Bioluminescent Inhibition (%)	56	29	25	24

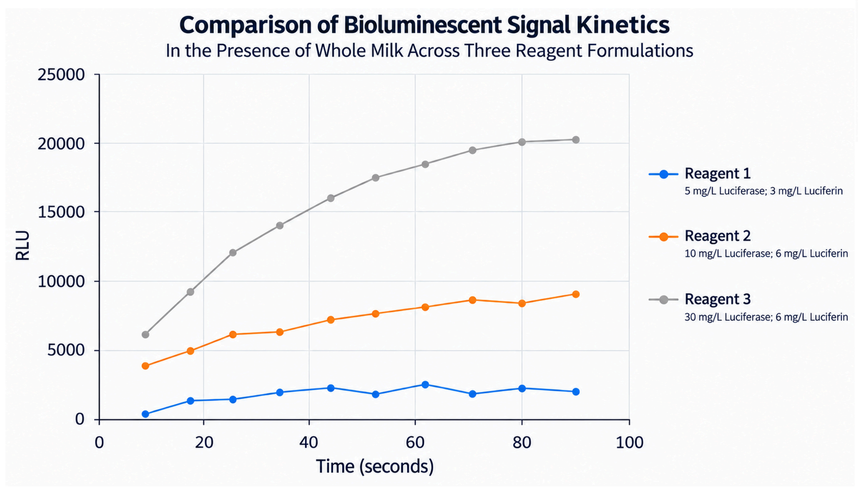


Figure 1. Bioluminescent Signal Kinetics of Three Formulations Containing Different Concentrations of Firefly Luciferase and D-luciferin.

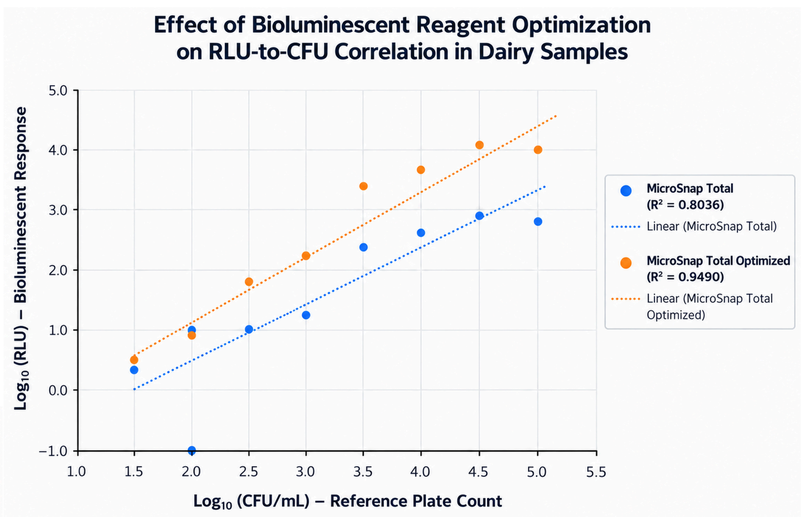


Figure 2. Effect of bioluminescence optimization on the RLU-to-CFU relationship in naturally contaminated dairy samples

SIGNIFICANCE:

Optimized ATP-dependent bioluminescent chemistry improved assay robustness, signal intensity, and quantitative performance. Increasing BES buffer capacity reduced pH shifts from non-neutral matrices. This allowed the bioluminescent reaction to work within a defined pH range regardless of matrix. This ultimately reduces bioluminescent inhibition, providing the end user a more consistent result matrix-to-matrix. Increasing the luciferase-luciferin concentrations yields a larger dose response to ATP, thereby enhancing the RLU signal in a shorter time. This reduces the need for long-reading architectures in luminometers and shortens the time required to obtain an actionable answer. Combining these improvements and integrating them into the MicroSnap Total system, an optimized bioluminescent reagent demonstrated a stronger relationship between traditional CFU plate counts and the bioluminescent RLU response, improving the coefficient of deamination (R^2) from 0.8036 to 0.9490.

These findings demonstrate the potential benefits of increasing robustness in the bioluminescent chemistry. Greater resistance to matrix interference could reduce the need for sample dilution, additional preparation, or matrix-specific RLU-to-CFU adjustments for quantification when using the MicroSnap Total System. Continued development and validation across a broader range of dairy products, including fermented and acidic matrices, may further expand the applications of a more robust MicroSnap Total System.