Determination of Incubation Time to Detect Microbial Contamination in Compliance with USP <73> Guidelines

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charles river

1 ABSTRACT AND INTRODUCTION

ABSTRACT

- USP <73> outlines a risk-based approach for the use of ATP-bioluminescence for rapid detection of microorganisms in short-life products.
- Generation time of the slowest-growing bacteria, yeast, and fungi in the product matrix is used to establish the incubation time of the assay for microbial detection.
- Foundational data were generated for 4 microorganisms *(A. brasiliensis, B. spizizenii, P. paraeruginosa*, and *C. acnes*) in 3 product matrices (CAR-T cells, Adeno-Associated Viruses, and Plasmids) to evaluate the adoption of the USP <73> guidelines.
- When background ATP is present in the product (e.g., CAR-T cells), a concentration step using hollow fiber tips was used to capture microbial ATP only.
- Our study demonstrates the successful application of USP <73> guidelines for determining incubation time and provides insights into resolving challenges associated with implementation.

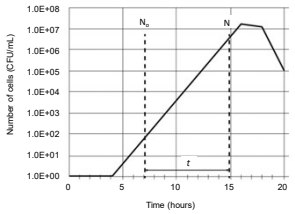
INTRODUCTION

- ATP-bioluminescence (Celsis®) provides detection of viable microorganisms via Luciferin–Luciferase reaction emitting light in the presence of ATP (universal to all living cells).
- The Celsis® technology measures bioluminescence in relative light units (RLUs) and provides a presence/absence call relative to a background media calibrator.
- When background ATP was present due to mammalian cells in the product, a concentrating tip was used to remove non-microbial ATP.
- USP <73> in conjunction with USP <1071> and USP <71> provides guidelines for the product volumes, incubation conditions, and inoculum levels (<10 CFU) to be used.
- USP <73> specifically provides guidelines regarding data collection to capture microbial growth through the exponential phase so that the appropriate interpretation of metrics can be derived to calculate generation and incubation time.
- Incubation time in the product to be examined includes a safety margin, which is the time needed for a 10-fold increase in the amount of the slowest-growing microorganism.

2 METHODS

- Microorganisms tested at <10 CFU: *A. brasiliensis* ATCC 16404, *B. spizizenii* ATCC 6633, *P. paraeruginosa* ATCC 9027, and *C. acnes* ATCC 11827.
- Products tested: CAR-T cells (2x10^6 cells/mL), Adeno-Associated Viruses (100 μL at 5.72 x 1011 GC/mL), and Plasmids (50 μL at 28 to 1030 ng/μL).
- CFU and RLU data were collected for each organism in each product over a series of time points to capture the growth profile.
- Incubation Time (T) for microbial detection in product was determined as per USP <73>.

Figure 1. Growth phase curve



Generation Time (G):

$$G = \frac{t}{3.3 \times \log_{10}(\frac{N}{N_o})}$$

t = the time interval in hours for the calculation that falls within the exponential growth phase.
$\mathbf{N_o}$ = the average CFU (or RLU) observed at the start of the chosen time interval (t).
N = the average CFU (or RLU) observed at the end of the chosen time interval (t).

Incubation Time (T):

$$T = tttd + log_2(10) \times G$$

$\mathbf{t_{td}}$ = the longest time to detection in the method suitability test, *e.g.*, number of hours needed to observe Celsis® positive using the slowest growing microorganism.
G = the generation time in hours of the slowest grower.

3 METHODS (continued)

Figure 2. Testing workflow for product *with* background ATP (CAR-T cells)

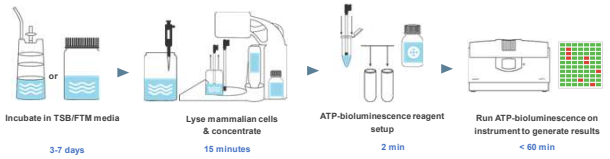
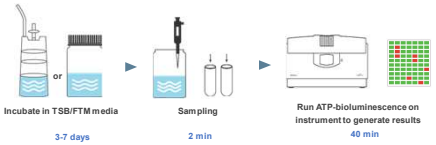


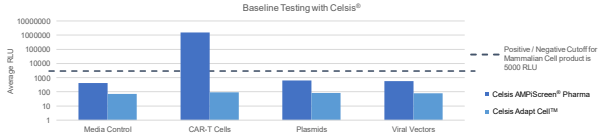
Figure 3. Testing workflow for product *without* background ATP (Plasmids and Adeno-Associated Viruses)



4 RESULTS

Understanding which Products need Elimination of Background ATP

Figure 4. Sample Interference Testing

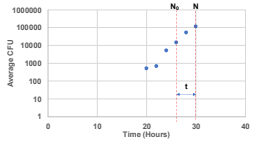


- Products containing mammalian cells (CAR-T) need a workflow that eliminates background ATP.

Determination of Generation Time (G) and Incubation Time (T)

Fig 5a. Measurements based on CFU (Colony Forming Units)

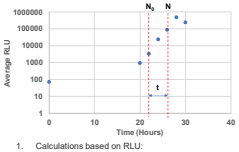
Product: CAR-T Cells | Microorganism: *Bacillus spizizenii* (<10 CFU/mL)



1. Calculations based on CFU:
   N = 52,200
   N_o = 675
   t = 6
2. Generation Time (G) = 0.96 hours
3. Time point of ATP detection (t_{td}) = 24 hours
4. **Incubation Time T = 27.2 hours (or 1.13 days)**

Fig 5b. Measurement: RLU (Relative Light Units)

Product: CAR-T Cells | Microorganism: *Bacillus spizizenii* (<10 CFU/mL)

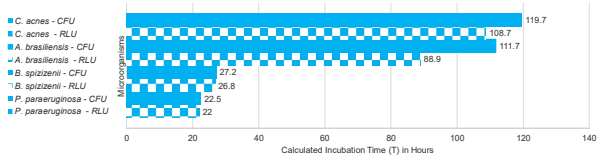


1. Calculations based on RLU:
   N = 491,968
   N_o = 3,277
   t = 6
2. Generation Time (G) = 0.84 hours
3. Time point of ATP detection (t_{td}) = 24 hours
4. **Incubation Time T = 26.8 hours (or 1.12 days)**

- CFU were found by plating the culture intermittently during incubation, and alternatively RLU data from similar time points could be used to calculate the Generation and Incubation times.

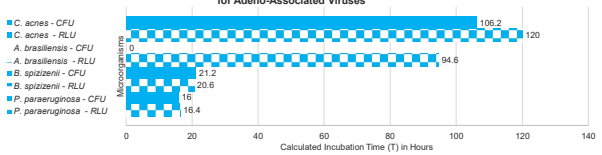
5 RESULTS (continued)

Fig 6a. Calculated Incubation Time (T) vs Microorganisms - RLU and CFU for CAR-T Cells



- *C. acnes* is the slowest growing organism tested using CAR-T cells with an Incubation time of 119.7 hours (or 4.99 days).

Fig 6b. Calculated Incubation Time (T) vs Microorganisms - RLU and CFU for Adeno-Associated Viruses



- *C. acnes* is the slowest growing organism tested using Adeno-Associated Viruses with an Incubation time of 106.2 hours (or 4.43 days).

6 RESULTS (continued) and CONCLUSIONS

Observations about Data Collection for Generating Growth Curves

- Potential challenges with strict anaerobic cultures:
  - When not using continuous monitoring.
  - Slower growth observed.
- Localized colony growth of molds in enrichment broth.
- Challenges with achieving less than 10 CFU for inoculum.
- Need to consider 8-hour shift and data collection requirements for robust growth curves.
- Delayed results when needing to wait for colony growth versus collecting real time data with rapid method.

CONCLUSIONS

- The Incubation Time across a cohort of microorganisms consisting of bacteria, yeasts and filamentous fungi in products could be successfully determined using USP <73> guidelines.
- Both RLU (Relative Light Units) or CFU (Colony Forming Units) can be used to generate the growth curves to determine Incubation Time.
- Removal of background ATP is required when testing mammalian cell products (CAR-T cells).
- This study highlighted unique challenges in collecting data to generate growth curves, however, the results provide valuable guidance for developing protocols to evaluate microorganisms using ATP-bioluminescence (Celsis®).
- USP <73> provides a clear compendial pathway for the use of ATP-bioluminescence for rapid sterility testing of short-life products.
- This new general chapter will help pave the way for implementation of rapid microbial detection methods for sterile manufacturing.