Validation of Rapid Microbiological Testing Methods Using Cryopreserved and Live Cell Therapeutics Mimic Samples

Hyo-kyung Jang1, Tae-jin Shin[1], Eunjee Lee[1], Da-Hye Lee[1, 2]

[1]Bio-metrology Group, Korea Research Institute of Standards and Science
[2]Department of Precision Measurement, University of Science and Technology, Daejeon, Republic of Korea

Abstract

Compendial microbiological testing methods are time-consuming, prompting the need for rapid microbiological testing (RMT) methods. This study focuses on validating RMT methods using both cryopreserved cell therapeutics and live cell therapeutics mimic samples. The samples were prepared under controlled conditions and proper preservatives. Respiration based and ATP bioluminescence based rapid microbiological testing methods were selected for validation. The samples were inoculated with known concentrations of microbial contaminants, including bacteria, yeast, and fungi. The inoculated samples were subjected to RMT, and the results were compared with traditional culture-based methods. Key parameters such as specificity, limit of detection, and time to result were evaluated. Data were statistically analyzed to determine the accuracy, precision, and robustness of the RMT methods. Two methods were The RMT methods offer a faster and equally reliable alternative to compendial microbiological testing, enhancing the efficiency of quality control in cell therapeutic production. These methods were found to differ in terms of convenience, detection sensitivity for different strains, and validation design. Nevertheless, the RMT methods offer a faster and equally reliable alternative to compendial microbiological testing, enhancing the efficiency of quality control in cell therapeutic production.

Introduction

In the rapidly advancing field of cell and gene therapies, ensuring the sterility of products is critical for patient safety. Contamination in these therapies, even at minute levels, can lead to severe clinical consequences. Compendial microbiological testing methods, while highly reliable, are often too slow, potentially delaying the release of life-saving treatments. To address this, there is a growing need for Rapid Microbiological Methods (RMMs), which offer faster, yet accurate, detection of microbial contamination.

However, the validation of these rapid methods is of utmost importance. Without proper validation, RMMs may yield unreliable results, leading to either false assurances of sterility or unnecessary rejection of viable therapies. Validation ensures that these rapid methods perform consistently, accurately, and are fit-for-purpose, especially when testing complex products such as cryopreserved and live cell therapeutics, which present unique challenges.

are used for sample preparation.

Materials & Methods

Sample preparation

K562 cells were produced according to an established production protocol in a cGMP-compliant facility. The cells were aliquoted in a cryopreservation solution composed of human Plasma-Lyte A injection, human albumin, and 10% of DMSO. The cell suspension was slowly cooled using a controlled-rate freezer to -80 °C, followed by long-term storage in liquid nitrogen (-196°C). Live cell therapeutics mimic samples were prepared using Jurkat. After cell culture, the cells were suspended in a Lactate Ringer's solution and stored at 4 °C.

Microorganisms

The each microorganism for tests was purchased from Microbiologics Inc. (EZ-Accu Shot Micoorganism set for growth promotion Test or EZ-CFU Microorganism set for media promotion Test). For determination of CFU concentration, TSA or SDA plate (Synergy Innovation Co., Ltd) were used.

ATP bioluminescence based RMTM (Celsis Adapt, Charles River Laboratories International Inc.)

This method detects microbial contamination by measuring adenosine triphosphate (ATP) levels, an indicator of living organisms. When cryopreserved samples were directly inoculated into TSB or FTM, they showed positive results despite negative outcomes from the compendial sterility test. As a result, the membrane filtration Steritest™ method was implemented. A Canister Set was prepared, and 70 mL of Treatment Solution along with 1 mL of K562 Cell Suspension were added to each bottle, allowing them to dwell for at least 15 minutes. For validation with live cell samples, the direct inoculation method was available. The samples were inoculated into TSB or FTM, then incubated at proper temperatures. After a 7-day incubation, 5 mL of the cultured samples were lysed, concentrated, and treated with a background ATP removal reagent. The luminescence signal was then measured using a luminometer to detect microbial contamination.

Respiration based RMTM (BacT/Alert 3D, Biomerieux UK Ltd)

The respiration-based RMTM detects microbial contamination by measuring the metabolic activity of microorganisms. This is done by detecting carbon dioxide (CO_2) production or oxygen (O_2) consumption as microbes metabolize nutrients in the sample. The rise in CO_2 levels or depletion of O_2 correlates directly with microbial growth. 1 mL of thawed cryopreserved samples or 1 mL of live cell samples was inoculated into sterile, closed vials containing a culture medium designed to promote microbial growth.

Acknowledgement

This research was conducted as part of a project commissioned by the Ministry of Food and Drug Safety (MFDS, 22202MFDS125). Also, this work was supported by Establishment of measurement standards for Chemistry and Radiation funded by Korea Research Institute of Standards and Science (KRISS – 2023– GP2023-0003).

Results and discussion

The validation parameters for the Rapid Microbiological Testing Method (RMTM) include specificity, limit of detection (LOD), ruggedness, robustness, and equivalency testing. Among these, our primary focus was on conducting the LOD test. The following results were obtained from the ATP bioluminescence-based RMTM performed on cryopreserved cell mimic samples.

Table 1. LOD test results of cryopreserved cell mimic samples

	ATP bioluminescence based RMTM			Compendial sterility test		
Microorganisms	0.1CFU	1CFU	10CFU	0.1CFU	1CFU	10CFU
A. Brasiliensis (ATCC 16404)	0/4	5/6	4/4	0/4	5/6	4/4
B. Subtilis (ATCC 6633)	3/4	4/6	3/4	2/4	4/6	3/4
C. acnes (ATCC 11827)	0/4	2/6	4/4	0/4	2/6	4/4
C. acnes (10^6 cells)	1/4	4/6	4/4	1/4	4/6	4/4
C. Albicans (ATCC 10231)	0/4	4/6	4/4	0/4	4/6	4/4
C. Sporogenes (ATCC 19404)	0/4	3/6	4/4	0/4	3/6	4/4
M. Luteus (ATCC 4698)	0/4	2/6	4/4	0/4	2/6	4/4
M. luteus (10^6 cells)	0/4	3/6	4/4	0/4	4/6	4/4
P. Aeruginosa (ATCC 9027)	1/4	4/6	4/4	1/4	4/6	4/4
P. Chrysogenum (ATCC 10106)	0/4	3/6	4/4	0/4	3/6	4/4
S. Aureus (ATCC 6538)	0/4	3/6	4/4	0/4	3/6	4/4
S. Pyogenes (ATCC 19615)	1/4	4/6	4/4	1/4	4/6	4/4
All microorganisms	**6/48**	**41/72**	**47/48**	**5/43**	**42/72**	**47/48**

Table 2. Inoculated concentration of each microorganism

Microorganisms	0.1 CFU	1 CFU	10 CFU
A. brasiliensis	0.12	1.2	12
B. subtilis	0.18	1.8	18
C. acnes	0.24	2.4	4
C. acnes (10^6 cells)	0.24	2.4	4
C. albicans	0.12	1.2	9
C. sporogenes	0.09	0.9	9
M. luteus	0.04	0.4	4
M. luteus (10^6 cells)	0.04	0.4	4
P. aeruginosa	0.08	0.8	8
P. chrysogenum	0.11	1.1	11
S. aureus	0.12	1.2	12
S. pyogenes	0.1	1.0	10

Table 3. statistical analysis of LOD and equivalency

		ATP bioluminescence	Traditional Sterility test
LOD	LOD	0.074 CFU	0.078 CFU
	95% CI	0.028 CFU, 0.132 CFU	0.03 CFU, 0.137 CFU
Equivalency test	1 CFU detection probability	0.5694	0.5833
	difference in detection probability	-0.0139 (Δ > -0.2 → non-inferiority margin)	
	lower 95% Confidence interval	-0.04174	

We also performed both respiration based RMTM and bioluminescence-based RMTM on live cell samples. The *Cutibacterium acnes,* known for its slow growth, showed almost no positive results within our experimental setup over a 7-day incubation period, but it was confirmed positive after 10 days of cultivation. According to USP 71, *Pseudomonas aeruginosa* should be cultured in FTM; however, under our experimental conditions, it exhibited better growth in TSB compared to FTM.

Table 4. LOD test results of live cell mimic samples

Microorganism	Respiration based RMTM			ATP bioluminescence based RMTM			Compendial sterility test		
	0.1CFU	1CFU	10CFU	0.1CFU	1CFU	10CFU	0.1CFU	1CFU	10CFU
A. Brasiliensis (1)	0/0	6/10	1/10				0/10	6/10	10/10
A. Brasiliensis (2)				0/10	9/10	10/10	0/10	9/10	10/10
B. subtilis	1/10	6/10	10/10	1/10	6/10	10/10	1/10	6/10	10/10
C. acnes	0/10 (3/10)*	0/10 (10/10)*	1/10 (10/10)*	2/10	8/10	10/10	2/10	9/10	10/10
C. albicans	2/10	6/10	10/10	0/10	1/10	10/10	0/10	1/10	10/10
C. sporogenes	2/10	10/10	10/10	1/10	10/10	10/10	1/10	10/10	10/10
P. Aeruginosa (TSB)	2/10	10/10	10/10	2/10	10/10	10/10	2/10	10/10	10/10
S. aureus	0/10	6/10	10/10	0/10	8/10	10/10	0/10	9/10	10/10
S. pyogenes	1/10	4/10	10/10	1/10	7/10	10/10	1/10	7/10	10/10
All microorganisms	**8/80**	**48/80**	**70/80**	**7/80**	**59/80**	**80/80**	**7/80**	**61/80**	**80/80**

** Indicates 10 day-incubation results*

Table 5. LOD test results of live cell mimic samples

Microorganism	Respiration based RMTM			ATP bioluminescence based RMTM			Compendial sterility test		
	RealCFU	MPNCFU	95%CI	RealCFU	MPNCFU	95%CI	RealCFU	MPNCFU	95%CI
A. Brasiliensis (1)	10.4	**0.8**	0.4 1.8				10.4	**0.8**	0.4 1.8
A. Brasiliensis (2)					**3.4**	1.6 7.1	4.6	**3.4**	1.6 7.1
B. subtilis	7.5	**1.2**	0.6 2.5	7.5	**1.2**	0.6 2.5	7.5	**1.2**	0.6 2.5
C. acnes	8.4	**0.01**	0 0.1	8.4	**2.2**	1.1 4.3	8.4	**2.9**	1.4 5.7
C. albicans	7.8	**1.4**	0.7 2.8	7.8	**0.3**	0.2 0.7	7.8	**0.3**	0.2 0.7
C. sporogenes	19.6	**1.7**	0.8 3.9	19.6	**1.4**	0.7 3.1	19.6	**1.4**	0.7 3.1
P. Aeruginosa (TSB)	19.6	**1.7**	0.8 3.9	19.6	**1.7**	0.8 3.9	19.6	**1.7**	0.8 3.9
S. aureus	11.5	**0.7**	0.3 1.5	11.5	**1.1**	0.5 2.4	11.5	**1.5**	0.7 3.1
S. pyogenes	9.4	**0.7**	0.3 1.5	9.4	**1.3**	0.6 2.7	9.4	**1.3**	0.6 2.7

The study demonstrated that both cryopreserved cell mimic samples and live cell mimic samples showed no inferior microbial detection capability using rapid microbial detection methods compared to compendial pharmacopeial sterility tests. However, it is important to note that for anaerobic media and slow-growing microbial strains, positive results in the respiration-based rapid detection method were observed only after more than 7 days of incubation. The quantity of samples required for each rapid detection method varies, with ATP bioluminescence-based methods requiring fewer samples when using the same media as traditional pharmacopeial sterility tests. However, the feasibility of direct inoculation with ATP bioluminescence-based methods may be hindered by ATP background interference, depending on the composition of the preservation solution. Therefore, feasibility testing is essential before using this approach.

Reference

1. **PDA Technical Report No. 33 (Revised 2013). Evaluation, Validation, and Implementation of Alternative and Rapid Microbiological Methods. Parenteral Drug Association (PDA), 2013.**
2. **United States Pharmacopeia (USP) <1223>. Validation of Alternative Microbiological Methods. United States Pharmacopeial Convention, 2022.**
3. **United States Pharmacopeia (USP) <1071>. Rapid Microbial Tests for Release of Sterile Short-Life Products: A Risk-Based Approach. United States Pharmacopeial Convention, 2022.**
4. **European Pharmacopoeia (EP) 2.6.27. Microbiological Control of Cellular Products. European Directorate for the Quality of Medicines & HealthCare (EDQM), 2023.**
5. **European Pharmacopoeia (EP) 5.1.6. Alternative Methods for Sterility Testing. European Directorate for the Quality of Medicines & HealthCare (EDQM), 2023.**