

# Polymerase Chain Reaction Detection Sensitivity of Gamma Irradiated Bacillus Spores

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## Abstract

This study investigated the relationship between gamma irradiation levels and bacterial growth, polymerase chain reaction (PCR), and spore scanning electron microscopy of various preparations of *Bacillus thuringiensis* subsp. *kurstaki* spores. Four spore suspensions (clean, washed, crude, and dirty) at 10<sup>8</sup> spores/mL, and one dry powder preparation at 10<sup>9</sup> spores/gram were used, all of which were irradiated at eight different doses. PCR analysis was performed to determine if there were changes in sensitivity of the target sequence while enumeration and scanning electron microscopy (SEM) were used for determining viability and observing spore morphology, respectively.

## Introduction

Gamma irradiated material is used by the defense community to challenge systems as a safer and less costly alternative to using live agent. However, comparisons of inactivated and viable pathogen data suggest that there may be variations in the results. When testing systems that are intended to be fielded, it is important to be able to produce reliable and repeatable challenges in order to accurately assess detection technologies under realistic test conditions and make an informed decision on system performance and system readiness.

Exposure to an adequate amount of gamma irradiation will inactivate bacterial spores and render them non-pathogenic, but it is unknown whether inactivation is due to destruction of the DNA, organelles, or essential proteins. There is also conflicting information in the literature as to what level of dosage is required for inactivation of spores and whether irradiation has an effect on the sensitivity of nucleic acid-based detection assays such as PCR.

Like *Bacillus anthracis*, the causative agent of anthrax, *Bacillus thuringiensis* subsp. *kurstaki* is a gram-positive, spore forming bacterium belonging to group I of the *Bacillus* species. This bacterium is commonly used as a surrogate for *Bacillus anthracis* because it is non-pathogenic to humans and the spores behave similarly.

## Methods

### Spore Preparation

Five different preparations were made as follows:

- 1) Dirty: spores were suspended in deionized sterile water.
- 2) Washed: spores were washed 6 times by centrifugation using water and then re-suspended in water.
- 3) Crude: spores were grown on agar plates until sporulation and harvested.
- 4) Clean: spores were grown on agar plates until sporulation, harvested, and washed 6 times with deionized sterile water.

- 1) Powder: spores were kept dry.

All liquid preparations were brought to 10<sup>8</sup> CFU/mL and stored at 4°C. Spore viability in each preparation was calculated following plating and incubation at 37°C.

### Gamma Irradiation

One mL (or 1g for powder) samples were subjected to 8 levels of gamma irradiation: (1) no radiation, (2) 1.5 x 10<sup>6</sup> rads, (3) 2.0 x 10<sup>6</sup> rads, (4) 2.5 x 10<sup>6</sup> rads, (5) 4.15 x 10<sup>6</sup> rads (ECBC minimum), (6) 5.43 x 10<sup>6</sup> rads (ECBC Standard), (7) 8.2 x 10<sup>6</sup> rads, and (8) 10.86 x 10<sup>6</sup> rads. Samples were stored at 4°C following irradiation. Serial ten-fold dilutions were performed in phosphate buffered saline, spread plated onto trypticase soy agar, and incubated for 24 hours at 37°C to determine the survival ratio and generate inactivation curves.

### Real-time PCR Analysis

PCR amplification reactions were carried out on the 7500 Fast Dx Real-time PCR instrument (Applied Biosystems). *Btk* specific PCR master mixture was purchased from Critical Reagent Program (CRP) in Frederick, MD. Data acquisition and analysis were automatically driven using auto threshold mode and used to establish the threshold PCR cycle (Ct).

### Electron Microscopy Scanning of Spores

Irradiated and non-irradiated spores were fixed, post-fixed and embedded in resin. Ultrathin sections were observed with a scanning electron microscope.

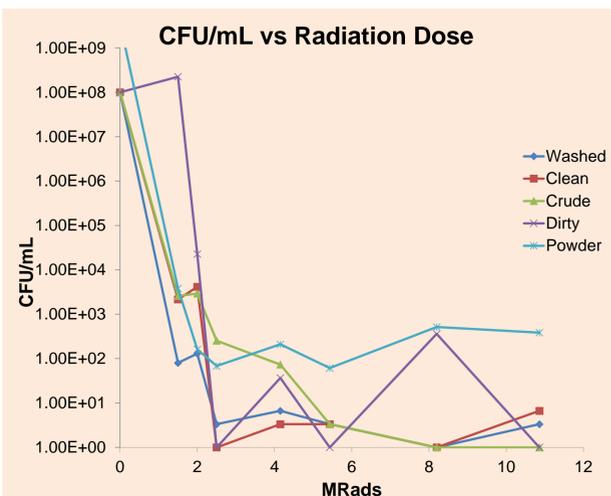


Figure 1. Effect of gamma irradiation on the viability of various preparations of *Bacillus thuringiensis* spores.

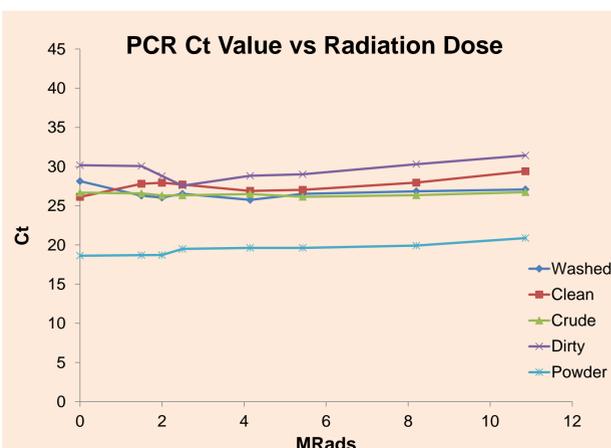


Figure 2. Results depicting the effect of gamma radiation on PCR detection sensitivity. For practical reasons, it was necessary to dilute the spore preparations by 1/10 to overcome PCR inhibition.

## Results

### Effect of gamma irradiation on viability of *B. thuringiensis* spores.

The results of gamma inactivation of *B. thuringiensis* spores are shown in Figure 1. After gamma irradiation, viability of spores was evaluated using spore germination and colony counting. With the exception of some variation in the dirty preparation, all inactivation curves had an initial swift decay rate followed by tail-off. The viable colonies counted at 5.43 and 8.2 Mrads for the dirty preparation could have been a contamination or technique issue. At the range of 1.5 to 2.5 Mrad, a 6-log reduction of viable spores was achieved for all spore suspensions.

### Effect of gamma irradiation on PCR analysis of *B. thuringiensis* spores.

The data were plotted as the average PCR cycle (Ct) versus gamma irradiation doses (Mrads) for each spore preparation (Figure 2). All samples were detectable by PCR after gamma irradiation and Ct values of each preparation remained consistent regardless of irradiation dose. When spores were prepared as crude, dirty, cleaned, or washed; the average Ct value for untreated spores was 27.77±1.46 versus 27.52±1.46 for irradiated spores. For powder spores, the average Ct value for untreated spores was 18.60±0.00 versus 19.82±1.16 for irradiated spores.

The maximum increase in PCR Ct was 3.28 cycles and the lowest decline was 2.60 cycles across spores that were subject to irradiation doses ranging from 1.5-10.86 Mrad (Table 1). This variation of 3.28 cycles is equivalent to less than one logarithmic unit in genomic DNA. Based upon these data, gamma irradiation had no substantial effect on the sensitivity of PCR on the target sequences.

### Effect of gamma irradiation on spore morphology.

Microscopic scanning of gamma treated spores revealed differences in spore morphology. Compared to non-irradiated spores, most of the gamma-irradiated spores showed irregular deformed shapes (Figure 3). The changes in spore morphology were revealed within 1.5-2.5 Mrad treatment which is the same dosage that caused a 6-log reduction in viability.

Spore Preparations	Gamma radiation [Mrad]								Mean (Ct)	SDV (Ct)
	[1.5]	[2]	[2.5]	[4.15]	[5.43]	[8.2]	[10.86]			
Washed	-1.85	-2.09	-1.61	-2.39	-1.62	-1.29	-1.05	-1.70	0.46	
Dirty	-0.09	-1.37	-2.60	-1.34	-1.15	0.14	1.26	-0.74	1.26	
Crude	-0.08	-0.35	-0.33	-0.16	-0.52	-0.31	0.08	-0.24	0.20	
Powder	0.08	0.09	0.87	1.00	1.00	1.29	2.25	0.94	0.74	
Cleaned	1.67	1.81	1.58	0.77	0.90	1.82	3.28	1.69	0.82	

Table 1. Effect of gamma irradiation on real time PCR assay detecting DNA on spores of *B. thuringiensis*. The rows and columns indicate the differences of PCR threshold cycle (Ct) between treated [1-10.86 Mrad] and non treated spores. The analysis showed that gamma radiation decreased Ct value by as low as 2.60 cycles or increased the Ct by up to 3.28 cycles. This variation equates less than 1-log difference in genomic DNA. Therefore, gamma radiation has no substantial effect on PCR.

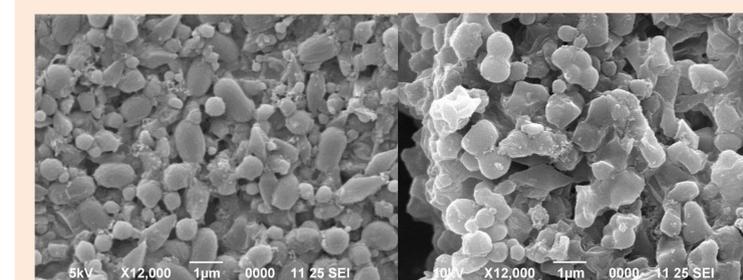


Figure 3. SEM image of *B. thuringiensis* at no radiation (left) and 10.86 Mrads radiation (right).

Acknowledgements: The authors thank Edgewood Chemical Biological Center for their assistance in this research and the U.S. Army for funding of this work through the 2013 In-House Laboratory Independent Research Program. We also thank Erin Durke, Karen Pongranch, and Jerold Bottiger for their contributions. The views expressed in this presentation are those of the authors and do not necessarily reflect the official policy or position of the Department of Defense or the US Government.



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