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Evaluation and microbiological investigations of radiation sterilization of disposable blood tubing set

[1]Mahmoud Abd El-Naby Radwan; [2]Abd El-Monaem Sayed Bashandy; [1]Abbas El-Ghamry; [1]Amr Fouda

[1]Faculty of Science, Al Azhar University; [2] NCRRT, Atomic Energy Authority, Cairo, Egypt
*Corresponding author: *mahmoud.radwan@allmedgroup.com*

Abstract

Ionizing radiation is widely used for sterilization of single-use medical devices, and it is for its lethal effect on microbial life that radiation is employed for this purpose. One of the most important points in the manufacture of healthcare products is the production of a sterile product with specified (SAL). Healthcare product manufacturers strive to provide safe and sterile products by validating and controlling manufacturing procedures. At present, (SAL) of 10^{-6} is generally accepted for pharmacopoeia and (FDA). Sterility is free from viable microorganisms "Sterile" can be used to describe a packaged products that was prepared using a terminal sterilization process validated according to the methods of the applicable pharmacopoeia, EN ISO standards and FDA regulations. Delivering sterile product is responsibility of manufacturer of aseptically processed device or a manufacturer of a terminally sterilized device. The current regulatory expectation of the term "sterile" for blood-contacting medical devices and implants is to produce only one non-sterile device out of one million.. For microbiological tests, culture condition should be selected in both cases of the bioburden and sterility testing. Bioburden is used to describe the population of viable microorganisms present on or in a product and/or a sterile barrier system. Bioburden estimations are used to indicate possible problems in the production process that can lead to inadequate sterilization, calculate the necessary dose for effective sterilization, and to monitor product to ensure adequate dosing. This study presents some results and practical solutions chosen to perform a sterilization validation, compliant with EN ISO Standards, Pharmacopeia and FDA regulations. In this study gamma radiation was selected to sterilize Biocompatible Blood tubing set of Allmed Group. Gamma sterilization validation was performed using $VD_{max}25$ in accordance to EN ISO 11137-1, and EN ISO 11137-2 to achieve (SAL) 10^{-6}. Protocol of gamma sterilization validation was achieved.

Keywords: *Bioburden/Recovery factor/Sterilization/ Radiation/ Bloodtubing/ $VD_{max}25$.*

Introduction

A terminal sterilization process is commonly defined as one that achieves a sterility assurance level (SAL) of 10^{-6}, assurance of less than one chance in a million that viable microorganisms are present in the sterilized article. Alternatively, "sterile" can be used to describe a solution prepared for immediate use by a continuous process, such as filtration, that has been validated according to the methods of the applicable pharmacopoeia to produce a solution free from microorganisms for the validated life of the filter[(1)].

In the world of medical devices, "sterilization" is defined as a validated process used to render product free from viable microorganisms. Terminal sterilization is defined as the "process whereby product is sterilized within its sterile barrier system[(2)].Terminal sterilization is a safe and effective approach to manufacture sterile combination products. Combination products have unique material compatibility challenges that must be addressed to ensure successful validation of the sterilization process at a reasonable cost[(3)].

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Sterilization is an essential step in the process of producing sterile medical devices. To guarantee sterility, the process of sterilization must be validated. Because there is no direct way to measure sterility, the techniques applied to validate the sterilization process are based on statistical principles[4]. Regulatory authorities like EMA and FDA have published guidelines relating to process validation. The purpose of process validation is to ensure varied inputs lead to consistent and high quality outputs. Process validation is an ongoing process that must be frequently adapted as manufacturing feedback is gathered. End-to-end validation of production processes is essential in determining product quality because quality cannot always be determined by finished-product inspection. Process validation can be broken down into 3 steps: process design, process qualification, and continued process verification[5].

The number of agents capable of sterilizing product or material without adversely or deleteriously affecting product quality or material integrity is few. There is no singular sterilization method that is compatible with all healthcare products including drugs, polymers, devices, and materials, because of the severity of a process to meet the sterilization criteria and definition[1]. Polyvinyl chloride (PVC), is a polymer widely used for radio-sterilizable of medical devices. However when the polymer systems are submitted to sterilization by gamma radiation (25 kGy dose), their molecular structures undergo modification mainly as a result of main chain scission and cross linking effects [7]. It had been recommended to use PVC material (Anti-gamma material) to Minimize, eliminate effect of Gamma radiation. Physical test including tensile strength force, chemical characterization, integrity of tubes, durability of pump segment, and particulate test should be performed to confirm the applicability of radiation dose.

Radiation sterilization has now become a commonly used method for sterilization of several active ingredients in drugs or drug delivery systems containing these substances. In this context, many applications have been performed on the human products that are required to be sterile, as well as on pharmaceutical products prepared to be developed. The new drug delivery systems designed to deliver the medication to the target tissue or organ, such as microspheres, nanospheres, microemulsion, and liposomal systems, have been sterilized by gamma (γ) and beta (β) rays, and more recently, by e-beam sterilization[8].

Bioburden is defined as the number of bacteria living on a surface that has not been sterilized. Bioburden testing is the enumeration and microbial characterization of the population of viable microorganisms on or in a medical device, component, raw material, or package. The term is most often used in the context of bioburden testing, also known as microbial limit testing, which is performed on pharmaceutical products and medical products for quality control purposes[9].An extensive study of the radiation resistance of microbial species constituting the bioburden of a number of different medical devices obtained. A standard protocol for determining radiation resistance was used and validated. The overall distribution of radiation resistance among the isolates was considered to be similar to that forming the "Standard Distribution of Resistance" (SDR) included in the EN ISO 11137- 2. For a number of years, the establishment of an appropriate radiation sterilization dose required to be usedfor a large range of the medical devices sterilized by ionizing radiation has been based on verification that the radiation resistance of the natural bioburden found on the device does not exceed that of a standard distribution of radiation resistance. Appropriate tables for both the verification dose and sterilization dose, based on bioburden numbers, have been supplied in the EN ISO Standard 11137[10].

Bulk material sterilization is a process in which a treatment is applied to entire batch in order to decrease its bioburden until a sterility assurance level (SAL) for that specific product is obtained. Validation of the irradiation sterilization of medical devices is regulated [11]. Sterilization is a term referring to any process that eliminates (removes) or kills all forms of microbial life, including transmissible agents such as fungi, bacteria, viruses, and spore forms [12].

The international and European standards for the validation and routine control of sterilization using ionizing radiation require that the effectiveness of a sterilization dose of 25 kGy be demonstrated (substantiated). Use of existing documented methods for substantiation has led to the observation of unexpected failures in the verification part of the procedures. Examination of the radiation response of microbial populations comprising the reference Standard Distribution of Resistances has revealed a potential reason for these unexpected failures and led to the development of a new approach for choosing the dose to be used in the verification experiment for substantiation of 25 kGy. This approach, which involves the calculation of a maximal acceptable

verification dose (VD_{max}) for a given bioburden and verification sample size, provides a direct link between the outcome of the verification dose experiment and the attainment of an SAL of 10^{-6} at a 25 kGy sterilization dose. Use of a maximal verification dose would minimize the probability of unexpected and unwarranted failure in the verification procedure [13].

The recent publication of the international and European standards for the validation and routine control of sterilization using ionizing radiation (ISO 11137 and EN 552 respectively) is the culmination of a significant effort in preparing and agreeing their content. Although not identical, the standards have essentially the same content in their normative sections. Both standards allow one of two possible approaches to be used for the selection of a minimum radiation dose to achieve sterility. The first of these approaches is what may be described as rational. It is based on knowledge of the number and radiation resistance of contaminating microorganisms that occur naturally, in/on product, and a prediction of the dose needed to achieve a predetermined standard of sterility or sterility assurance level (SAL). To reduce the rational approach to practice, Informative Annex B of ISO 11137 describes two procedures of dose selection, designated Method 1 and Method 2[13].

Materials and Methods

Materials

Media used in this study were, Thioglycollate Broth, Tryptic Soy Agar (TSA), Tryptic Soya Broth (TSB), supplied by Oxoid, USP. Analytical profile index was used, supplied by bioMérieux.

Objective and product definition

The objective of this study is the determination of the Sterilization Dose (D_{min}) of Blood tubing set of Allmed Group. Blood tubing sets was classified in accordance to Medical Device directive as class IIa Satisfying rule 3 non-invasive, and blood channelling devices[14].

Establishment of the sterilization dose for the productD_{min}

The blood tubing set is considered an individual pharmaceutical product in its packing system to be used independently in clinical practice.

Methodology

The establishment of the Sterilization Dose, i.e. gamma radiation dose necessary to achieve a Sterility Assurance Level (SAL) of 10^{-6} was based on Method VD_{max} substantiation of 25 kGy described in the International Standard EN ISO 11137-2[15].

The experimental methodology is divided in three phases:

A) Validation of bioburden determination method.
B) Determination of bioburden frequency.
C) Establishment of the Sterilization Dose.

Sample item portion and sampling

The Sample Item Portion (SIP) is the portion of the product to be tested. As blood tubing set is composed mainly from PVC tubes, and rigid components. It is expected to have a low number of bioburden. Based on this hypothesis and in accordance with the guide lines expressed on the International Standard EN ISO 11137-2, an entire product (SIP = 1.0) was used for testing[16].The product sampling plan for establishing the sterilization dose was representative of that subjected to routine processing and conditions. Sample product items were selected from final normal product ensuring that they had been subjected to the same processing and conditions as the remainder of production and that they had undergone the packing process.The sampling plan was composed by a total of 50 product items divided in:

- 10 items for the validation of bioburden determination method (n = 10)
- 10 randomly items from three independent one production batch (n = 30) for Bioburden frequency
- 10 randomly items from one production batch (n = 10) for the verification dose experiment).

Experimental procedure

A)Validation of Bioburden determination method

Since the blood tubing set is considered a low bioburden product, the validation of bioburden determination method was performed by artificial contamination of product samples with known

concentrations (100 CFU/ sample) of a bacterial pure culture. This procedure was made to evaluate the efficiency of the method to recover and quantify the microorganisms present in/on the product. Inoculation method was used and artificial bioburden was created [16] by inoculating the sterile product by a known number of spores of (*Bacillus subtilis* ATCC 9372) [17].

Bacterial strains: *Bacillus subtilis* ATCC 9372

Testing protocol:

All the following described steps were performed using aseptic procedures and inside a vertical laminar flow cabinet. The experimental procedures used were based on conventional bacteriologic techniques and mentioned in EN ISO 11737-1[11].

- A pure culture suspension of *Bacillus subtilis* ATCC 9372 was preparedin 5 ml of saline solution (0.9% NaCl).
- Initially the culture suspension's concentration was quantified using the Neubauer counting chamber and the microscope.
- The concentration of *Bacillus subtilis* culture suspensions was determined by spreading aliquots (0.1 ml) of two decimal dilutions in TSA medium(three replicates per dilution). Incubated at 32.5 ± 2.5 ºC and counted after 24 and 48 hours.
- Aseptically the samples (n=10) from their package were placed individually in sterile stomacher bags.
- Based on the Neubauer counting method, estimation of the *Bacillus subtilis* culture suspension's concentration were made, the inside of three samples (three replicates) were spiked with a syringe with a *Bacillus subtilis* concentration 100 CFU/sample
- The samples were leftto dry approximately 15 minutes and the samples were sealeded in the samples' original bags.
- The opened samples (n = 10) were taken each aseptically from their package and transferred into sterile stomacher bags.
- The vacuum pump was connected to the vacuum flask. The holder was installed into a sterile 10 litre vacuum flask ISO 11737 to rinse the blood tubing set with isotonic normal saline 0.9%. Near the flame of a bunsen burner sterile scissors and sterile forceps were used to open and to remove the filter from the packaging and the filter was placed into the sterile filter holder. The diluents were poured from the pooling vessel through the filter paper 0.45µm EN ISO 11737-1the entire filter paper was transferred to an appropriately labelled sterile Tryptic Soy Agar (TSA) growth medium, the plates were incubated inverted at 32.5 ± 2.5°C for 3-5 days [18].
- Three TSA Petri dishes were opened inside the laminar flow chamber during all the experimental procedure (environmental control). A sterile membrane (Ø 45 µm) was placed into a TSA plate (membrane control). Before the processing of the samples, 50 ml of the saline solution were filtrated with the tensoactive used to homogenise the samples (saline solution control). This procedure was repeated (saline solution control) in the middle and in the end of the filtration process of the samples (filtration system control).
- The number of colonies forming units (CFU) was counted after 24h, 78 h, 72h and 5thday of incubation.
- The number of CFU for each sample was calculated and for the initial *Bacillus subtilis* culture suspension. The average, standard deviation and standard error estimated.
- Validation of recovery factor was calculated as follows:

Recovery factor= No. of inoculated organisms ÷ Mean No. of spores removed.

Interpretation of results:

The interpretation of results was made following this hypothesis:

- **H0:** The average number of microorganisms in blood tubing samples is equivalent to the artificial inoculation concentration → The bioburden determination method is validated.
- **H1**: The number of microorganisms in the blood tubing samples is higher than the artificial inoculation concentration→ the bioburden determination method should be investigated and validated.
- **H2**: The number of microorganisms in the blood tubing samples is lower than the artificial inoculation concentration.
- If the difference is > 70%, calculate a correction factor → the bioburden determination method is validated.

- If the difference is < 70% → adjust the bioburden determination method (volume of eluent, flushing method)

B) Bioburden determination

This phase was performed after the validation of the method in order to compensate incomplete removal of microorganisms (if any).

Protocol:

All the following described steps were performed using aseptic procedures and inside a vertical laminar flow cabinet. The experimental procedures used were based on conventional bacteriologic techniques and mentioned in EN ISO 11737-1[(11)].

- The samples were carefully opened in the original package (tube).
- The opened samples (n = 10/batch → 3 different batches) were aseptically transferred from their package into individual sterile stomacher bags.
- The vacuum pump was connected to the vacuum flask. The holder was installed into a sterile 10 litre vacuum flask ISO 11737 to rinse the blood tubing set. Near the flame of a bunsen burner sterile scissors and sterile forceps were used to open and to remove the blood tubing set from the packaging and the filter was placed into the sterile filter holder. The diluents were poured from the pooling vessel through the filter paper 0.45µm EN ISO 11737-1the entire filter paper was transferred to an appropriately labelled sterile Tryptic Soy Agar (TSA) growth medium, the plates were incubated inverted at 32.5 ± 2.5°C for 3-5 days.
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- The number of colonies forming units (CFU) was countedat 24h, 78 h, 72h and 5th day of incubation.

Interpretation of results:

- The obtained samples bioburden values (CFU) were divided into contamination classes (e.g. <1; 1-3; 3-30; 30- 300; etc.). For each contamination class a bioburden frequency was determinate based on the following equation:

$$\text{Bioburden frequency}_{i}\ (\%) = (n^{o}\ \text{samples}_{I} / n^{o}\ \text{total samples}) \times 100$$

Where i corresponds to each contamination class.
Verify if bioburden frequency follows a normal distribution.

- **H0:** The bioburden frequency follows a normal distribution → estimate the product bioburden.
- **H1:** The bioburden frequency does not follow the normal distribution → characterize and identify the natural microbiota of the product and study the production line →identify critical control points and apply corrective actions.
- Average bioburden per batch was estimate. ANOVA test was appliedto verify the equality of the bioburden average values between batches.
- **H0:** The bioburden average per batch values are not significantly different (P>0.05 (→assume as the product bioburden, the overall average (n = 30).
- **H1:** The bioburden average per batch values are significantly different (P<0.05→ (assume as the product bioburden, the higher bioburden average per batch value (n = 10).
- **Note:** Bioburden frequency determination is valid, only if the controls are not contaminated.

Identification of isolated colony by gram staining and analytical profile index (API)

Colonies were examined morphologically as shape, size color and microscopically as Gram stain reaction, and the presence or absence of spores. Identification was performed in accordance to the Keys of Bergey's Manual Determinative Bacteriology[(19)], and Bergey's

Manual of Systematic Bacteriology[20], and Cowan and Steel's Manual for the Identification of Medical Bacteria[21].

Catalase test was performed for Gram positive bacteria (*Cocci*). Identification was performed according to schematic diagram. Catalase test was performed by using 3% hydrogen peroxide H_2O_2[22] and[23].

Bacterial isolate was identified according to the aforementioned schematic diagram using API. Analytical profile index API STAPH (ID 32 STAPH) was used. API ID 32 Staph (BioMérieux) system was used for Gram-positive *cocci*

C) Establishment of Sterilization Dose

The establishment of Sterilization Dose, i.e. gamma radiation dose necessary to achieve a Sterility Assurance Level (SAL) of 10^{-6}, is based on the Method $VD_{max}25$ - substantiation of 25 kGy described in the International Standard EN ISO 11137-2. In this particular case, the verification was conducted with a SAL of 10^{-1}, using 10 product items from one batch (n= 10) irradiated during the verification dose experiment.

The dose corresponding to this SAL is the maximal verification dose (VD_{max}) and it is the dose at which the verification dose experiment was carried out. Based on the product's estimated bioburden, the VD_{max} dose was read from Table 9 of the EN ISO 11137-2. After which, 10 product items were exposed to the VD_{max} dose and each item was submitted individually to a sterility test.

The irradiation of blood tubing set® product in its package was performed in a licensed semi-industrial gamma facility located in the NCRRT. The product's absorbed dose was monitored by calibrated routine dosimeters (Perspex, Harwell) to identify the highest and lowest doses absorbed by the product EN ISO 11137-3 [24].

The irradiation was performed in a calibrated place that is comparable to the whole irradiation process in the irradiation chamber. The irradiation geometry was planned in a way that minimized the Dose Uniformity (D max/D min).

The sterility test was performed in an aseptic chamber room by placing the product in the inner bag (direct contact with the product) and in a sterile stomacher bag. To verify the presence/ absence of microorganisms, 250 ml of a nutrient rich liquid medium (Tryptic Soy Broth, TSB) was added to each sample bag. The samples were incubated at 30ºC during 14 days.

Interpretation of results:

If the arithmetic mean of the highest and lowest doses delivered to product items is less than 90% of the established VD max, the verification dose experiment would be repeated.A product positive/ negative sterility test was defined by the presence/ absence of microorganisms growth (turbidity of liquid culture medium) confirmed by isolation in solid culture medium (TSA), after the 14 days of incubation.The verification dose experiment would be accepted if no more than one single positive test of sterility were found.

- **H0:** The highest delivered dose to the product does not exceed 10% of the established verification dose and sterility tests results are acceptable (≤ 1 positive test) → substantiate 25 kGy as the sterilization dose (1 sterility positive test).
- **H1:** Two positive tests of sterility → perform a confirmatory verification dose experiment.
- **H2:** More than two positive tests of sterility → do not accept the verification dose experiment → repeat the bioburden determination and establish the sterilization dose by the Method 2 of EN ISO 11137-2.

Results

Recovery factor is numerical value applied to compensate incomplete removal from product and/or culture of microorganisms, in this study recovery factor determination was conducted in to compensate in complete removal of microorganisms from the product during filtration and extraction in accordance with EN ISO 11737-1[11].

Recovery Factor = 100/100=1

The average number of microorganisms in blood tubing samples is equivalent to the artificial inoculation concentration → The bioburden determination method was validated.

The average bioburden observed in 30 samples (collected from three batches) of blood tubing set was found as 1.63 CFU. The maximum Bioburden level of these 30 samples was 4 CFU/device. The minimum bioburden level was (0). Standard division was 1.1, bioburden results of three batches were normally distributed.

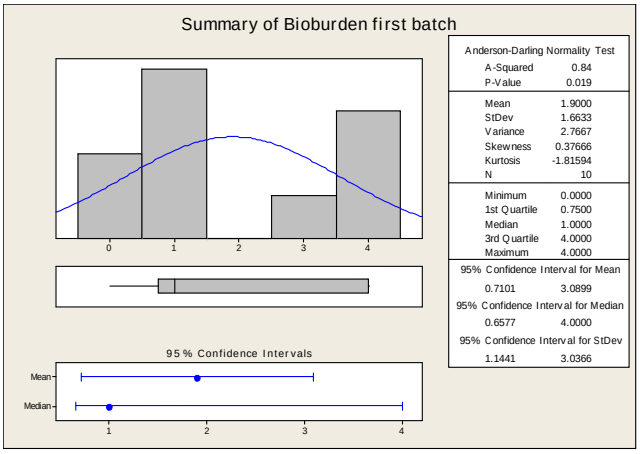

Figure (1) Summary of statistical analysis Bioburden first batch.

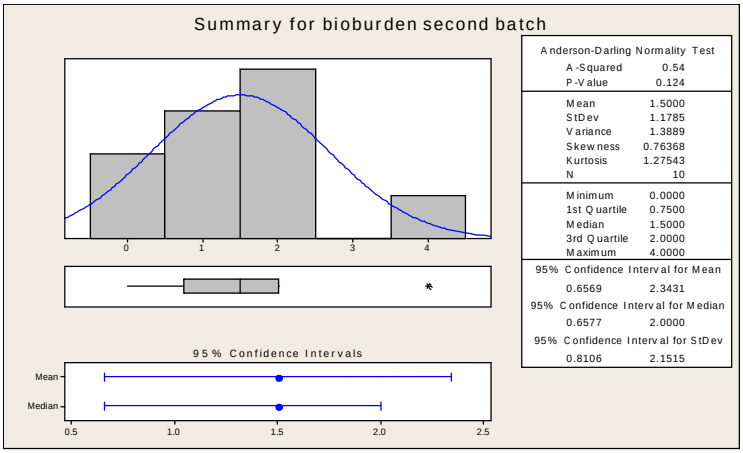

Figure (2) Summary of statistical analysis Bioburden second batch.

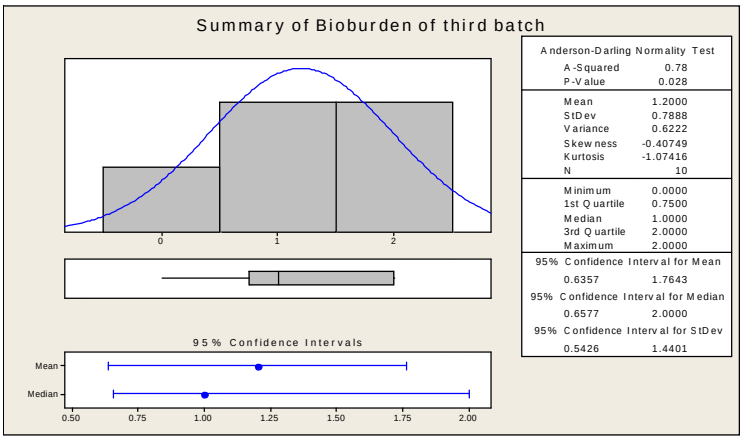

Figure (3) Summary of statistical analysis Bioburden third batch.

One colony was isolated and identified by gram staining and API. Isolated colony was identified as gram positive. Taxon was gram positive *cocci*; Cataase test was perfomred by using 3% hydrogen peroxide H_2O_2, the isolated taxon was catalase positive. Isoalted taxonwas identifed by using API STAPH,Identifcatio of isolated colony obtained from Table 1.

Table (1): identification of isolated taxon by API *Staphylococcus aureus*

GOOD IDENTIFICATION	
Strip	API STAPH V4.1
Profile	6 7 3 6 1 5 3
Note	POSSIBILITY OF Staph.intermedius IF OF VETERINARY ORIGIN

Significant taxa	% ID	T	Tests against			
Staphylococcus aureus	97.8	1.0				

Next taxon	% ID	T	Tests against			
Staphylococcus simulans	1.0	0.74	MAL 11%			

Complementary test(s)	YELLOW	dTURANOSE		
Staphylococcus aureus	+(-)	+(-)		
Staphylococcus intermedius	-	-		

For estimation of the verefication dose, 10 samples of blood tubing set were selected randomly from batch used in Bioburden experiment and sterilized at 3.4 kGy (Verification dose 10^{-1}) to have final sterility assurance level 10^{-6} (SAL 10^{-6}). Sterility test of 10 irradiated samples were performed. Results showed that non of the examined blood tubing set was non sterile. The maximum and minimum dose was recorded and showed in Table, the data were obtained from 18 film of dosimeter. The minimum and maximum doses were reported to 4.22 and 5.22 respectively within the specified limits (4.8 kGy +10% = 5.28 kGy). Process was control and capable (Cpk greater than one). If no more than one positive tests of sterility are obtained from the 10 tests carried out, verefication dose is accepted, The verifcation dose was accepted.[8]. Results of sterility test was accepted, 10 samples were sterile.

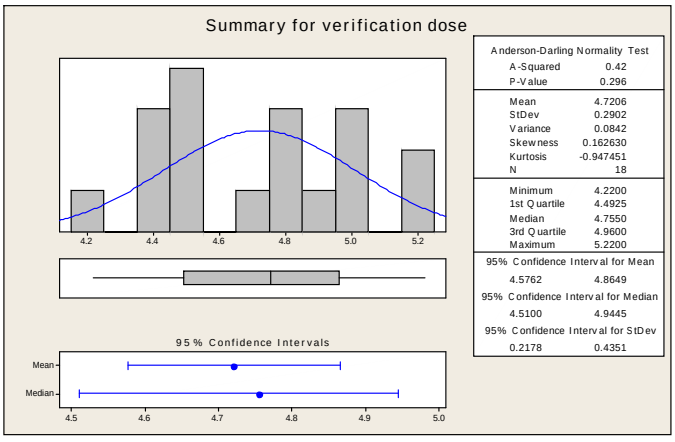

Figure (4) summary of statistical analysis of verification dose (dosimetry)

Table (02): Readings of 18 dosimeters distributed for verification dose

Film No.	Dose kGy	Film No.	Dose kGy
1.	4.93	10.	4.81
2.	4.81	11.	4.96
3.	5.22	12.	4.44
4.	4.51	13.	4.52
5.	5.02	14.	5.20
6.	4.77	15.	4.74
7.	4.43	16.	4.51
8.	4.40	17.	4.96
9.	4.22	18.	4.52
Average	4.72	**Minimum**	4.22
Maximum	5.22	**STDEV**	0.29

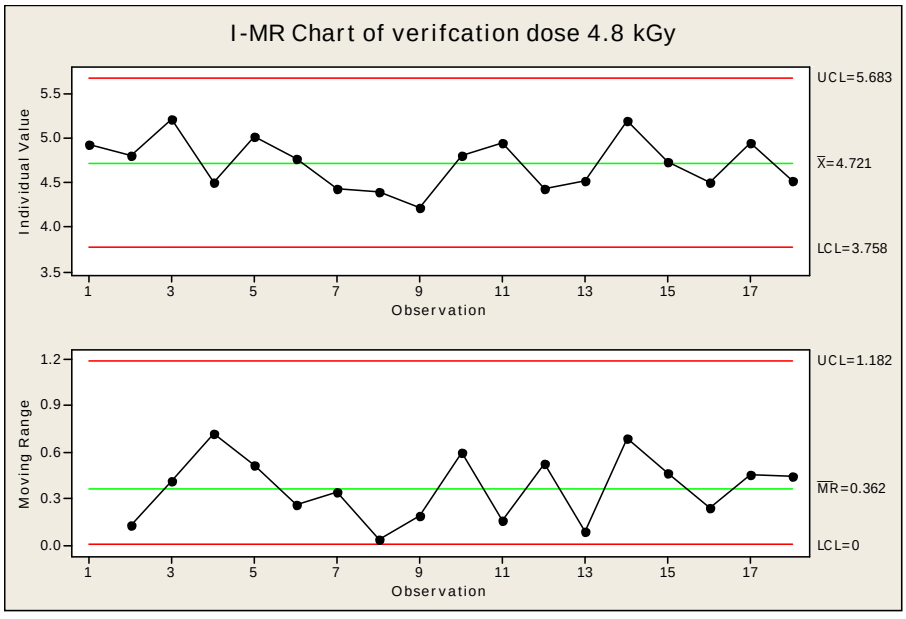

Figure (5) control chart of verification dose (dosimetry)

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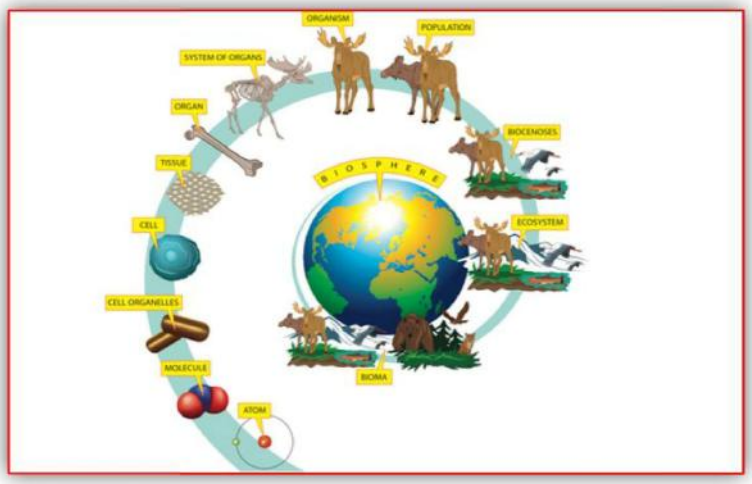

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