

Assessment of bioburden on human and animal tissues: Part 1—Results of method development and validation studies

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Abstract Recovered human and animal tissues are used extensively in surgery for wound repair and reconstruction. In preparation for the validation of chemical disinfection and radiation sterilization processes, studies were performed on the development and validation of quantitative bioburden recovery methods for human bone and soft tissue and also for porcine dermis. The use of a swab-based method was not considered due to the known poor efficiency of recovery for this technique. The “exhaustive extraction” and “inoculated product” approaches to validation of a bioburden recovery efficiency factor have inherent strengths and weaknesses; in this study, tissues were inoculated and also subjected to a series of extractions to determine if/when “exhaustion” occurred. Femoral and tibial shaft rings, iliac crest wedges, sections of Achilles tendon, a soft tissue

composite sample, and porcine dermis, were inoculated at several sites with *Bacillus atrophaeus* spores, and then subjected to either shaking by hand, mechanical shaking, or sonication plus mechanical shaking. Each of these methods of agitation were performed in combination with three rinse (extraction) fluids: phosphate buffer (Butterfield’s buffer), phosphate buffer with 0.2% polysorbate 80 (a surfactant), and water with 1% peptone and 1% polysorbate 80 (Fluid D). The highest recovery efficiencies were observed with sonication plus mechanical shaking; of the three extraction media, Fluid D gave the highest first-rinse recovery efficiency (65%) and Butterfield’s buffer gave the lowest (39%). Each of the three recovery methods, however appeared to reach “exhaustion”, a subsequent rinse giving less than 10% of the recovery found in the first rinse. The results demonstrated the importance of performing bioburden method development and validation studies. The method validation strategy described here, using a combination of tissue inoculation and repetitive extraction, showed the superiority of sonication plus mechanical shaking using Fluid D as the rinse medium. In addition, the use of only the exhaustive extraction approach could have resulted in the development of a methodology that consistently underestimated the bioburden present on/in recovered tissue.

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Introduction

Over the past 20 years, the use of transplanted human and animal tissue in surgical procedures has increased significantly. Human tissues, allografts, are generally recovered using aseptic techniques and they are disinfected in some manner and/or exposed to a validated sterilization process prior to distribution. The processing of the recovered human tissue, up to and including the packaging step, is performed in a controlled cleanroom-type environment. Animal tissues, however, are often recovered in an abattoir environment which is significantly less sanitary when compared to that used for recovery of human tissue. Consequently, collected animal tissues tend to possess a much greater diversity of microbial species and higher microbial counts than those seen on recovered human tissues.

Studies (Forsell and Liesman 2000) have shown that a number of factors can contribute to bacterial contamination of recovered human cadaveric tissue: location of recovery, length of time taken for a recovery, size of the recovery team, and whether the recovery was performed after an autopsy. Swab cultures are known to have poor sensitivity (Vehmeyer et al. 2001; Dennis et al. 2009); the results of blood culture testing might be helpful (Vehmeyer et al. 2002) but such results, as with swab cultures, are not quantitative. The results of testing recovered human tissue for *Clostridium* species showed that in eight cases tissue samples were positive but not blood or marrow (Malinin et al. 2003).

Irrespective of the process(es) used for disinfection/sterilization, it is critical to understand the microbiological challenge to these processes, the bioburden (ISO 11135-1:2007; ISO 11137-1:2006; ISO 17665-1:2006). Processes used for disinfection/sterilization can have limitations that are either inherent in the process itself or related to the microbial challenge used in the process development and validation studies. For example, exposure to alcohol cannot be validated as a sterilization process due to its poor or lack of antimicrobial activity against bacterial spores. A process developed and validated for low levels of readily-inactivated microorganisms can lead to an undesirable outcome when challenged with high levels of more resistant microorganisms. “Bioburden”, therefore, has two dimensions, the numbers and types of contaminating microorganisms.

For a quantitative bioburden determination method to be valid, there must be knowledge of the recovery efficiency of the method (ISO 11737-1:2006; Bryans and Alexander 2002; Technical Report No. 21 1990). One cannot assume that a given method when applied to a bone allograft, for example, will be 100% efficient in rinsing the bioburden into the extraction medium. Method validation studies were performed for the determination of bioburden on bone recovered from human donors and also on processed dermis tissue of porcine origin. Due to the low expected bioburden on aseptically recovered tissue, the “inoculated product” approach was used for both tissue types; additional rinse steps (repetitive extractions) were added to determine when “exhaustion” was reached. This strategy gave a more complete understanding of the recovery efficiency of the methodology.

Three agitation methods were used in combination with three rinse fluids for human bone; two agitation methods were used for porcine tissue. The agitation methods included shaking by hand, mechanical shaking, and sonication plus mechanical shaking. The rinse fluids were Butterfield’s phosphate buffer, phosphate buffer plus the surfactant polysorbate 80 (possibly improve recovery efficiency), and Fluid D (water, peptone, polysorbate 80), commonly used in pharmacopoeial methods for rinsing of medical products for microbial limits and sterility testing (higher level of surfactant, 0.1% versus 0.02%).

Materials and methods

Tissue samples

The human tissue samples that were used in these studies were from donors where swab cultures were taken at the time of recovery and the results of this testing indicated the absence of contamination that would interfere with subsequent testing. Recovered human femurs and tibia were debrided, rinsed with purified water, and cut into allograft-sized sections ~10 mm in length. The cut sections were again rinsed with purified water, aseptically packaged in plastic bags, and frozen at -20°C . Wedges, ~10 mm thick, were cut from the iliac crest, rinsed, packaged and stored as above. Three sections, ~10 × 50 mm, were cut from a recovered Achilles tendon and rinsed, packaged, and stored as above. The composite

soft tissue sample included a section of Achilles tendon, a section of several lower extremity tendons (anterior and posterior tibialis, semitendinosus, gracilis), a section of fascia, and a section of bone-tendon-bone. Porcine dermis tissue was processed to remove cells and hydrated in a proprietary aqueous solution.

Recovery efficiency studies

Tissue samples were inoculated with ~200 colony-forming units (CFU) of *Bacillus atrophaeus* spores (SGM Biotech). The spores were suspended in 40% (v/v) ethanol and the total inoculation volume was 100 μ l; the inoculation volume was generally distributed between four inoculation sites. For bone, the inoculum was distributed between uncut and cut surfaces; inoculated tissue was dried in a laminar-flow hood for approximately 1 h. A longer drying time was not used, particularly for porcine tissue, because the routine manufacturing process maintains the tissue in a hydrated state; prolonged drying might have created an artifact with respect to spore/tissue adhesion.

Inoculated tissue was aseptically transferred into either a sterile Whirl-Pak bag (Nasco, 24 oz) for manual shaking (Wehr and Frank 2004) or a sterile 250-ml glass jar (I-CHEM short) for mechanical shaking and for sonication followed by mechanical shaking. Each tissue-containing jar, negative control jars without tissue, and positive control jars (inoculated with 100 μ l of the spore suspension used above) received 100 ml of sterile rinse medium. Three rinse media were tested: Butterfield's phosphate buffer (0.31 mM potassium phosphate), buffered water with Tween (0.32 mM potassium phosphate + 0.02% polysorbate 80), and Fluid D (USP, 0.1% peptone + 0.1% polysorbate 80). For the initial recovery efficiency study with femoral rings, three agitation methods were tested: manual shaking (25 shakes in ~7 s followed by 30 min of static soak time), mechanical shaking on a reciprocal shaker (Eberbach, 1" stroke, 200 strokes/minute), and 5 min of sonication (Branson 5210, 47 kHz) followed by 30 min of mechanical shaking as above (4, 6, 12). To ensure consistency, the shaking by hand procedure was performed by the same technician. Subsequent first-rinse recovery efficiency studies with human tissue were all performed with Fluid D in combination with 5 min of sonication followed by 30 min of mechanical shaking.

Assay for recovered CFU

For the initial recovery efficiency study with femoral rings and for the porcine tissue studies, all of the rinse medium was removed from the jar and filtered through a 0.45- μ m pore size nitrocellulose filter. Each filter was transferred onto the surface of an individual TSA agar plate that was then incubated at 30–35°C for 48–72 h in a conventional table-top type incubator; previous studies have shown that there is increase in colony diameter but no discernable change in colony count between 48 and 72 h of incubation. Colonies on the surface of the filter were counted and the results recorded.

For the recovery efficiency studies, performed with iliac crest wedges, tibial shaft rings, Achilles tendon, and composite soft tissue samples, recovered CFU were assayed using pour plating. It was found that lipids and tissue debris would occasionally clog the membrane filters used in the initial method development studies. An aliquot (10 ml) of the rinse medium was added to each of ten large Petri dishes and mixed with tempered TSA agar and incubated as above.

Evaluation of spore clumping as the basis for poor recovery efficiency

To investigate the cause of the relatively poor recovery efficiency of the shaking by hand method compared to sonication plus mechanical shaking, an experiment was performed to determine if the spore inoculum was being removed in small clumps rather than predominantly single spores. To test this possibility, three inoculated bone samples (femoral rings) were placed into separate Whirl-Pak bags and 100 ml of Fluid D was added to each bag. The bags were hand shaken and held in a static condition at room temperature for 30 min. An aliquot from each bag (50 ml) was placed into each of two jars; one was assayed for CFU immediately and the other was assayed after 5 min of sonication followed by 30 min of mechanical shaking. The bone samples from each bag were transferred into separate jars containing 100 ml of Fluid D, subjected to 5 min of sonication followed by 30 min of mechanical shaking, and the Fluid D was then assayed for CFU by membrane filtration.

Results

Recovery method development/validation

The results of the method development/method validation studies for the human bone allograft samples are shown in Tables 1, 2 and 3; rinse media tested were Butterfield's phosphate buffer, sterile water with Tween, and Fluid D, respectively. The results with Fluid D as the rinse medium are also shown in Fig. 1. Each of the three rinse media were tested with hand shaking, mechanical shaking alone, or sonication followed by mechanical shaking. The number of CFU recovered for each of the four rinses is shown along with the efficiency of recovery for the first rinse and for the four rinses in total.

The following observations can be made from these results:

1. The poorest recovery efficiencies were found with Butterfield's phosphate buffer, the extraction fluid that did not contain a surfactant.
2. Of the three rinse media, Fluid D gave markedly higher recovery efficiencies. It is interesting to note here that, unexpectedly, relatively low recovery efficiencies, compared to the Fluid D outcomes, were observed in combination with

test conditions where "exhaustion" was observed: one or more subsequent rinses yielded less than 10% of the CFU recovered in the first rinse.

3. For all three rinse media, the highest first rinse and overall recovery efficiency were observed when sonication plus mechanical shaking was used.
4. All of the test conditions displayed exhaustion but the total recovered CFU were significantly different. The first rinse and overall recovery efficiency values for the combination of Fluid D with sonication plus mechanical shaking, were 65 and 82%, respectively. These values are similar to those observed with many types of medical devices that are exposed to similar bioburden recovery methods.

The results of the study investigating the possible role of spore clumping as a cause of the low recovery efficiencies with the shake by hand method are shown in Table 4. The aliquot of the first rinse that was sonicated did not yield a higher CFU count result suggesting that spore clumping was not the underlying basis for the relatively low efficiency of recovery for the hand shake recovery method. Taking the bone samples that had been

Table 1 Results of the recovery efficiency study with inoculated femoral shaft rings using Butterfield's phosphate buffer in combination with three agitation methods

Butterfield's phosphate buffer											
Method	Inoculation control	Sample	Rinse 1	Rinse 2	Rinse 3	Rinse 4	Total	Rinse 1 Total	First rinse recovery efficiency ^a (%)	Four rinse recovery efficiency (%)	
Hand shake	Ave. CFU		CFU								
		1	27	9	3	3	42	0.64	18	28	
		2	30	12	4	2	48	0.63			
		3	31	6	5	3	45	0.69			
		Average	29.3	9.0	4.0	2.7	45	0.65			
Mechanical shake	160		CFU								
		1	23	2	2	0	27	0.85	13	18	
		2	23	3	0	0	26	0.89			
		3	18	2	2	9	31	0.58			
		Average	21.3	2.3	1.3	3.0	28	0.77			
Sonication + mechanical shake			CFU								
		1	69	17	15	3	104	0.66	39	55	
		2	54	9	9	7	79	0.68			
		3	66	11	5	0	82	0.81			
		Average	63.0	12.3	9.7	3.3	88.3	0.72			

^a Rinse 1 CFU/inoculation control CFU

Table 2 Results of the recovery efficiency study with inoculated femoral shaft rings using sterile water with Tween in combination with three agitation methods

Sterile water with tween										
Method	Inoculation control	Sample	Rinse 1	Rinse 2	Rinse 3	Rinse 4	Total	Rinse 1 Total	First rinse recovery efficiency ^a (%)	Four rinse recovery efficiency (%)
Hand shake	Ave CFU	CFU								
		1	38	20	9	12	79	0.48	22	43
		2	44	29	6	12	91	0.48		
		3	37	18	6	5	66	0.56		
		Average	39.7	22.3	7.0	9.7	78.7	0.51		
Mechanical shake	183	CFU								
		1	52	17	2	1	72	0.72	35	44
		2	54	11	5	5	75	0.72		
		3	84	8	2	2	96	0.88		
		Average	63.3	12.0	3.0	2.7	81	0.77		
Sonication + mechanical shake		CFU								
		1	107	20	9	2	138	0.78	55	75
		2	91	25	12	16	144	0.63		
		3	102	19	4	4	129	0.79		
		Average	100.0	21.3	8.3	7.3	137	0.73		

^a Rinse 1 CFU/inoculation control CFU

Table 3 Results of the recovery efficiency study with inoculated femoral shaft rings using Fluid D in combination with three agitation methods

Fluid D										
Method	Inoculation control	Sample	Rinse 1	Rinse 2	Rinse 3	Rinse 4	Total	Rinse 1 Total	First rinse recovery efficiency ^a (%)	Four rinse recovery efficiency (%)
Hand shake	Ave. CFU	CFU								
		1	47	22	16	5	90	0.52	30	53
		2	58	24	8	6	96	0.60		
		3	61	31	11	4	107	0.57		
		Average	55.3	25.7	11.7	5.0	97.7	0.57		
Mechanical shake	185	CFU								
		1	69	37	12	0	118	0.59	32	47
		2	43	7	5	7	62	0.69		
		3	67	4	5	2	78	0.86		
		Average	59.7	16.0	7.3	3.0	86.0	0.71		
Sonication + mechanical shake		CFU								
		1	131	11	9	6	157	0.83	65	82
		2	111	13	18	5	147	0.76		
		3	119	21	6	7	153	0.78		
		Average	120.3	15.0	11.0	6.0	152.3	0.79		

^a Rinse 1 CFU/inoculation control CFU

exposed to hand shaking and the static soak and exposing them to a second rinse with Fluid D and sonication, followed by mechanical shaking, resulted in recovery of a higher number of CFU,

approximately double that observed with the first rinse. Sonication followed by mechanical shaking is clearly more effective in removing spores inoculated onto bone samples.

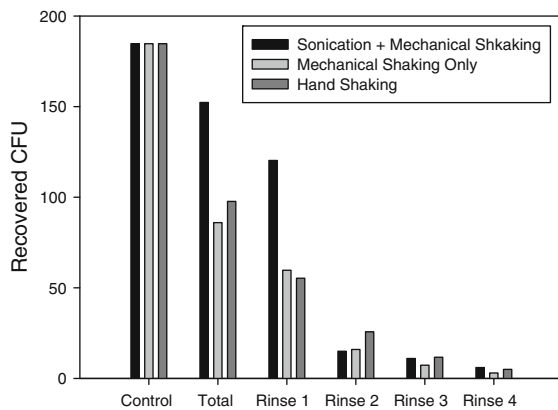


Fig. 1 Results of the recovery efficiency study with inoculated femoral shaft rings using Fluid D in combination with three agitation methods

Testing of sonication/mechanical shaking with Fluid D on additional tissue forms

To further test the applicability of the Fluid D/sonication/mechanical shaking methodology, recovery efficiency studies were performed with additional bone samples and also human soft tissue samples. The results of these studies are shown in Table 5. As had been found previously, relatively high first rinse and overall recovery efficiencies were observed demonstrating the efficiency and reproducibility of the method.

The results of the method development/method validation studies for porcine dermis tissue are shown in Tables 6, 7 and 8. For these studies, the three rinse media tested above were used in combination with mechanical shaking and sonication plus mechanical shaking; shaking by hand was not tested in this study. The results are in agreement with the previous findings; all of the combinations of test conditions exhibited exhaustion but different levels of total CFU

were observed. Also, the combination of Fluid D along with sonication plus mechanical shaking yielded the highest first rinse and overall recovery efficiencies.

Discussion/conclusions

Proper validation of a bioburden recovery method is essential to accurately estimate the number of contaminating microorganisms on a medical product whether it is tissue-based or composed of other natural and/or synthetic materials. The numbers (and types) of microorganisms present on a medical product can have a significant effect on the extent of treatment required in a disinfection/sterilization process to achieve the desired probability of a surviving microorganism. Swab-based methods have a low efficiency of recovery, generally <20%; the first rinse efficiency of recovery for Fluid D using sonication plus mechanical shaking ranged from 61 to 84% in the studies reported here. Also, direct testing of allograft-like samples gives bioburden results that are most relevant to the tissue that is ultimately implanted.

For these method development/validation studies, extraction fluids containing a surfactant were intentionally chosen as was the use of sonication and a reciprocal shaker. It was felt that the use of an extraction fluid with a surfactant might allow for a higher recovery efficiency due to the porous nature of cut bone samples and the likely presence of lipids on tissue samples. Sonication was chosen to possibly aid in the removal of microorganisms from the tissue samples. The mass of the tissue sample, the volume of the extraction fluid, the size and shape of the extraction container, and the method of agitation can also affect recovery efficiency. Early in the method development process, simulated testing showed that

Table 4 Results of studies to investigate role of clumping as the cause of low recovery efficiency

Test condition	Sample number			Average
	1	2	3	
First rinse, hand shake only (50 ml)	22	24	23	23.0
First rinse, hand shake - sonicated (50 ml)	12	20	26	19.3
Second rinse, sonication + mechanical shaking (100 ml)	85	75	93	84.3
Total CFU recovered	119	119	142	126.7

Table 5 Results of recovery efficiency studies with inoculated human bone and soft tissue forms

Tissue form	Replicate	Inoculation control	First rinse recovery	Average first rinse recovery	First rinse recovery efficiency ^a (%)
		Ave. CFU	CFU		
Iliac crest wedge	1	267	179	212	79
	2		256		
	3		200		
Tibial shaft rings	1	267	184	163	61
	2		144		
	3		161		
Achilles tendon	1	232	192	177	76
	2		171		
	3		169		
Soft tissue composite	1	230	203	193	84
	2		191		
	3		184		

^a Rinse 1 CFU/inoculation control CFU

Table 6 Results of the recovery efficiency study with inoculated porcine dermis using Butterfield's Phosphate Buffer in combination with two agitation methods

Butterfield's phosphate buffer											
Method	Inoculation control	Sample	Rinse 1	Rinse 2	Rinse 3	Rinse 4	Total	Rinse 1 Total	First rinse recovery efficiency ^a (%)	Four rinse recovery efficiency (%)	
	Ave. CFU		CFU								
Mechanical shake	123	1	66	8	3	2	79	0.84	59	71	
		2	70	9	3	1	83	0.84			
		3	83	13	4	1	101	0.82			
		Average	73.0	10.0	3.3	1.3	87.6	0.83			
Sonication + mechanical shake	127	1	120	27	7	7	161	0.75	88	114	
		2	89	17	15	2	123	0.72			
		3	126	3	10	9	148	0.85			
		Average	111.7	15.6	10.6	6.0	144.0	0.77			

^a Rinse 1 CFU/inoculation control CFU

the combination of the mass of the tissue sample, 100 ml of extraction fluid, the 250-ml I-CHEM short jar, and reciprocal shaking gave a level of agitation which appeared consistent with efficient removal of microorganisms from the tissue samples. The

combination of the surfactant-containing extraction fluid, Fluid D, with sonication plus mechanical shaking, gave the highest overall recovery efficiencies.

In the studies reported here, some combinations of recovery methods and rinse fluids gave a low first

Table 7 Results of the recovery efficiency study with inoculated porcine dermis using sterile water with Tween in combination with two agitation methods

Sterile water with tween											
Method	Inoculation control	Sample	Rinse 1	Rinse 2	Rinse 3	Rinse 4	Total	Rinse 1 Total	First rinse recovery efficiency ^a (%)	Four rinse recovery efficiency (%)	
	Ave. CFU		CFU								
Mechanical shake	123	1	63	7	3	2	75	0.84	63	75	
		2	66	11	0	4	81	0.82			
		3	105	9	4	3	121	0.87			
		Average	78.0	9.0	2.3	3.0	92.3	0.84			
Sonication + mechanical shake	127	1	45	17	10	4	76	0.59	47	75	
		2	68	22	13	0	103	0.66			
		3	66	26	10	3	105	0.63			
		Average	59.7	21.7	11.0	2.3	94.7	0.63			

^a Rinse 1 CFU/inoculation control CFU

Table 8 Results of the recovery efficiency study with inoculated porcine dermis using Fluid D in combination with two agitation methods

Fluid D											
Method	Inoculation control	Sample	Rinse 1	Rinse 2	Rinse 3	Rinse 4	Total	Rinse 1 Total	First rinse recovery efficiency ^a (%)	Four rinse recovery efficiency (%)	
	Ave. CFU		CFU								
Mechanical shake	123	1	52	7	0	1	60	0.87	59	67	
		2	66	6	3	2	77	0.86			
		3	100	9	2	0	111	0.90			
		Average	72.67	7.3	1.7	1.0	82.7	0.88			
Sonication + mechanical shake	127	1	89	20	16	6	125	0.71	68	95	
		2	81	19	5	2	107	0.76			
		3	87	27	14	1	129	0.67			
		Average	85.7	22.3	11.7	3.0	120.3	0.71			

^a Rinse 1 CFU/inoculation control CFU

rinse recovery efficiency and a low overall recovery efficiency but, interestingly, exhaustion was observed. In view of the results found with Fluid D and sonication plus mechanical shaking, it is clear that the inoculum was not killed in some manner or irreversibly bound to the tissue matrix in the cases where a low recovery efficiency was observed. Another possibility that should be considered when a low recovery efficiency is observed is loss of inoculum by wicking through or sloughing off the product matrix prior to transfer to the rinse container.

If the inoculated product approach is used for the validation of a bioburden recovery method, serious consideration should be given to the performance of multiple sequential rinses, four are recommended, to more fully understand the kinetics of removal and the fate of the inoculated microorganisms. In the ideal case, the efficiency of the first rinse will be very high, exhaustion will be observed after a low number of rinses, and the overall recovery efficiency of recovery will approach 100%. More typically, the first rinse recovery efficiency will be between 50 and 70%,

exhaustion will be observed at some point, and the overall efficiency of recovery will be >80%.

The use of the inoculated product method in combination with repetitive extractions can help confirm whether or not the entire inoculum or at least most of it has remained on the product. If the recovery efficiency is low, the product sample can be extracted by a more rigorous method to confirm whether unremoved organisms remain or removed organisms are clumped in the extract suspension. During the comparison of methods in these studies, those that yielded low CFU recoveries were shown to have poorer removal of the inoculum from the bone tissue rather than having unresolved clumps of spores eluted into the rinse medium.

An unexpected outcome of this study was the observation that the exhaustive recovery results do not fit the expected model. This finding has such significance for bioburden validations in general, that it will be reviewed in a separate manuscript, Part 2 of this series. The repetitive extraction method relies on the premise that when exhaustion has been demonstrated most/all microorganisms present have been removed. This premise was shown to be unreliable and therefore the method could provide an inaccurate recovery factor.

The following steps should be considered for approaching bioburden method validation:

1. Begin with the inoculated product method using samples that are sterile or have low bioburden that does not interfere with the analysis of the test results.
2. If most (>50%) of the inoculum is recovered in the first rinse, consider the method initially acceptable. If <50% is recovered in the first rinse but >50% is recovered in the four rinses, the method can be initially acceptable but the use a more rigorous method and/or different rinse medium should be considered. If <50% is recovered in the four rinses, the method should not be considered initially acceptable; the use a more rigorous method and/or different rinse medium should be considered. If the same low percent recovery occurs, inoculate the product in the rinse container, allow to dry, and repeat. If a higher recovery is obtained, it indicates that the inoculum is sloughing off the product prior to or during transfer to the rinse container. The loss of
3. Test the method, product, and solution with battery of known microorganisms (species identified in the USP or EP Sterility Test for growth promotion or in the USP Anti-Microbial Effectiveness Test) to confirm that no inhibition occurs in the bioburden recovery method.
4. Test the method on the native bioburden and hold the extraction medium for 0, 1, 2, and 3 h to determine whether the viable count is increasing, decreasing or neither. If changes in viable count occur, assess whether the magnitude of the change is significant to either affirm the validity of the method or require the use of an alternate method. The purpose of using hold times that are longer than the extraction time is to aid in obtaining statistically significant results when the product bioburden is low and/or variable.

Tissue products that are dried during processing might require a very rigorous mechanical method and a solution conducive to microorganism removal such as USP Fluid D. Tissue products that remain moist during all phases of processing do not appear to require more rigorous methods for bioburden removal.

The sonication/mechanical shaking method with Fluid D as the extraction medium has been evaluated with allograft-like samples from several hundred human donors; the samples were taken prior to exposure to antibiotics and/or disinfectants. While a majority of samples had no or a very low level of recovered CFU, others had bioburden levels that were >20,000. To date, ~100 different species of microorganisms have been identified in this testing.

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