**Title:**
**The use of Adenosine Triphosphate bioluminescence for assessing the cleanliness of additive-manufacturing materials used in medical applications.**

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**Abstract**
Additive Manufacturing (AM) is widely gaining popularity as an alternative manufacturing technique for complex and customized parts. AM materials are used for various medical applications in both metal and polymer options. Adenosine Triphosphate (ATP) bioluminescence technology is a rapid, user-friendly method of quantifying surface cleanliness and was used in this study to gather quantitative data on levels of contamination on AM materials at three different stage processes: post build, post cleaning and post sterilization. The surface cleanliness of eleven AM materials, three metals and eight polymers, was tested. ATP bioluminescence provided the sensitivity to evaluate different material surface characteristics, and specifically the impact of surface finishing techniques on overall cleanliness.

**Keywords**

- Additive Manufacturing;
- Medical Applications;
- Adenosine Triphosphate Bioluminescence;
- Surface Cleanliness;
- Metals vs. Polymers

## 1. Introduction

There is a clear synergy between the benefits of additive manufacturing (AM) technology and the requirements of patient-specific medical devices. AM parts are best suited to high-value applications that require rapid fabrication of complex geometry. Some of the most challenging medical applications demand bespoke anatomical features to be accurately replicated and delivered in a compressed timescale to meet the needs of trauma surgery. As the field of AM continues to expand then the list of AM-based medical devices is equally likely to grow.

A classification of medical applications of AM by Tuomi *et al*. [1] divides these applications into five areas: (1) medical models; (2) external aids; (3) surgical guides; (4) surgical implants and (5) biomanufacturing. The range of applications covers the relatively simple task of providing insight to the surgeon/patient (medical models) through to biologically-active tissue implants (biomanufacturing). The area of surgical guides covers patient-specific custom-designed drilling, cutting and repositioning devices, and this area provides an ideal fit with AM technology. Typical guides used in maxillofacial and orthopaedic applications are hand-held (small build volumes), incorporate patient-specific features that engage appropriate internal anatomical structures and can be easily cleaned and sterilised [2, 3].

Surgical guides have been fabricated by AM in a range of polymers and metals [2, 3]. Recent research within the field of maxillofacial surgery [4] has evaluated the use of AM surgical guides by a range of surgeons. The results show that surgical teams are keen to engage with AM technology but they have a number of pre-conceived perceptions as to the types of materials that are appropriate. It may be that material choice (specifically metal versus polymer) is strongly influenced by experience of previous conventional manufacturing processes, and there is little quantitative data to guide the clinical team for new AM applications. Three areas have emerged that need more empirical evidence to guide surgical decisions in the use of AM materials for surgical guides: geometrical accuracy, surface

roughness and cleanliness/sterility. Patient safety is the primary consideration when implementing any new medical intervention, therefore quantifying the cleanliness/sterility of AM materials is the main focus of this research paper.

AM technology and material vendors are continuing to develop a wide range of materials that have the potential for medical applications. For invasive surgical devices and implants, there are a series of ISO 10993 standards for the biological evaluation of medical devices that are in permanent (or prolonged) contact with the patient. In these cases criteria on biocompatibility and toxicity take precedent over other material issues. For medical devices that are single-use, disposable items that have limited contact with biological tissue (as in the case of surgical guides) there is a wider choice of potential materials. A typical surgical guide will arrive at the operating theatre within a sterile package, and labelled for a specific patient. The whole medical intervention could last hours but the AM material may only be in contact with the patient for a matter of minutes. In this scenario there are no clear guidelines or specifications to help define cleanliness and sterility.

The whole AM process, in terms of build orientation, cellular elements, removal of support structures and post-processing, provides a number of opportunities to introduce potential contamination into a medical device that could provide a hazard for the end user. Many AM manufacturing processes have fully-prescribed methods for post processing, but there are significant opportunities to detrimentally impact part cleanliness, especially when dealing with complex anatomical-based structures that include small voids that are difficult to fully access with fluids and cleaning implements. Techniques that enable contamination levels to be quantified during the various clinical delivery stages (post-build, post-cleaning and post-sterilisation) of AM medical parts is therefore highly desirable.

Adenosine triphosphate (ATP) bioluminescence technology is a rapid, user-friendly method of quantifying surface cleanliness that has been employed to evaluate contamination of a wide range of instruments and surfaces. Recent studies have used ATP to assess invasive medical devices [5], hospital surfaces [6] and environmental hygiene monitoring [7]. The bioluminescence test utilises the light-producing reaction between ATP, luciferin and luciferase to measure the amount of ATP present on a surface. ATP is the basic source of energy for all animal and microbial cells; its presence on a surface provides an estimate of all viable and non-viable organic residues, including microbiological contamination. The use of ATP bioluminescence tests is growing within healthcare, pharmaceuticals and food science industries. The ATP technology has two key advantages over traditional microbiological testing. Firstly, the technique provides results within minutes (as opposed to days) and effectively gives a real-time evaluation of surface cleanliness. Secondly, the test apparatus is highly portable and does not need specialist training or dedicated controlled facilities. ATP testing is therefore a very practical technique that can be adopted by non-specialists. The source of ATP can be anything that the sample comes into contact with, for example the way it is handled or where the sample was stored. The ATP method cannot identify the exact source of the contamination.

In the context of medical applications, a measure of residual organic matter is an indicator of surface cleanliness, but also quantifies the potential for surface reservoirs to harbour bacteria, fungi and viruses. Therefore ATP bioluminescence may be employed to give a dual estimate of: (1) the cleanliness of a surface at a fixed point in time; (2) the likelihood that a surface is susceptible to microbiological contamination over a longer period of time.

To date, the use of ATP bioluminescence to measure the cleanliness of AM materials intended for medical use has not been reported. The aim of this paper is to demonstrate that ATP bioluminescence testing is an appropriate technique for quantifying the cleanliness of a range of polymeric and metallic AM samples. It is hoped that the results can be used to highlight which AM materials (and associated surface modifications) have the greatest potential to be used in single-use, disposable medical applications, specifically materials that maintain levels of surface cleanliness that are appropriate for patient-specific surgical guides.

## 2. Materials and Methods

The aim of this study was to evaluate the ATP bioluminescence test in terms of its application to a range of representative AM materials to quantify their surface cleanliness. In this context, material properties are of more concern than geometrical features. The test sample geometry was therefore kept relatively simple, and is shown in Figure 1. The two 25x25mm square areas were the surfaces of interest for cleanliness/sterility testing, and the majority of samples were fabricated with the (x, y) plane as the up-facing surface. The surface area of the samples needed to be a minimum of 10x10mm to order gain a good enough reading,

Eleven AM materials were chosen to provide a representative sample of polymers and metals that have been employed in a range of medical applications. Details of the AM materials used in this research study are provided in Table 1. Each material category had 12 test samples manufactured. The three metals were all manufactured using Laser Melting (LM) technology, with one of the cobalt chrome set of samples having additional electro-polishing finishing. The eight polymer categories can be divided into: three Stereolithography (SLA, 3D-Systems, USA) resins; three polyjet (Objet, Statasys Ltd., Israel) materials; and two Selective Laser Sintering (SLS, EOS GmBH, Germany) materials.

The ATP bioluminescence test employed in this study was the 3M Clean-Trace system (www.3M.com/infectionprevention). The procedure starts by taking the test swab and applying it to the surfaces to be evaluated. The swab is gently rotated as it is swept across the test area. The swab is then immediately placed in a cylindrical vial, which brings it into contact with the enzyme solution (luciferin-luciferase) and the enzyme reacts with any ATP residue on the swab. The cylindrical vial is then placed in a hand-held 3M luminometer, and the light generated from the bioluminescence reaction is captured, and the measurement is expressed in Relative Light Units (RLUs). The greater the level of ATP present on the swab, the higher the RLU reading produced. The test can be performed in less than 30s, providing a real-time indication of the cleanliness of the surface tested. The swab and enzyme solution are disposed of after each test reading.

The 3M instrument manufacturers recommend a pass/fail threshold of 250 RLUs to indicate part cleanliness [8]. In addition, a literature review by Amodio and Dino [6], covering the period 1990-2012, has shown that the <250 RLUs threshold is the most widely used benchmark for indicating clinical surface cleanliness. In addition a recent Danish standard DS 2451 – 10 has been monitoring hospital cleanliness with standardised ATP measurements using a hygiene 5 level, the cleanest of the levels, which is set at 250 RLU’s [9].

Here a pilot study was undertaken to test three stages of production, cleaning and sterilization to evaluate which procedures gave AM parts with ATP readings in the region of the pass/fail threshold (250 RLUs). An overview of each stage is given below:

- Stage 1: Post Build. The AM parts were removed from the build platforms, support structures were removed, and the parts finished for standard delivery to a medical customer. In this scenario the parts were packaged and sealed for delivery, but all post-production handling was in a non-clean/non-sterile environment.

- Stage 2: Post Cleaning. The sealed post-production AM parts were taken through a series of cleaning steps; the standard operating procedure for this is given in figure 2. The key element was soaking each test sample in 250ml of Isopropanol for 60 minutes. This was achieved by placing four or five test samples in a one-litre beaker, and rotating the sample after 30 minutes to ensure an even contact time on both the main 25x25mm surfaces. After 60 minutes, the samples were removed individually, dried and packaged.

- Stage 3: Post Sterilization. Post-production parts were dispatched directly to a clinical partner (Morriston Hospital, UK), and individual samples were placed in labelled autoclave bags and sterilised on the standard 134$^{o}$C autoclave cycle specified by BS EN ISO 17665-1:2006 standard on the sterilisation of health care products: moist heat. This sterilisation process was chosen as it is standard across all UK NHS hospitals in HSDU departments.

The three different stages produced a wide range of RLU readings; the data for each set of samples was compiled into a series of spread-sheets for analysis. Each set of sample readings were characterised in terms of mean and standard deviation values. Statistical analysis of the data employed the t-test for hypothesis testing, and highlighting differences between discreet sample sets (assuming pseudo-normal distributions).

In addition to the ATP testing, the surface properties of the AM materials were further analysed using the Talysurf surface roughness apparatus (Renishaw Plc, UK) in order to measure the arithmetic average roughness (Ra) of selected surfaces.

## 3. Results

### 3.1 Pilot Study Results

The preliminary investigation into the feasibility of using ATP measurements to evaluate the cleanliness of AM materials took a small sample size of representative polymers and metals through the three stages of cleaning/sterilization. Two samples of SLA Clear (Accura ClearVue, 3D-systems) SLA Grey (Accura Xtreme, 3D-Systems), LM Cobalt Chrome (F75) and LM Titanium (6/4 alloy) were tested post-build, post-cleaning and post-sterilisation. The n=2x4 RLU readings at the three stages were pooled to give overall mean values, and the results are shown in Figure 3. There was an order of magnitude difference in RLU values between the three stages; post-build readings were order thousands, post-cleaning were order hundreds and post-sterilisation were order tens. There were no outliers from the small samples size, and the polymers and metals gave a similar range of values at each cleaning stage.

The pilot study results show that the cleaning protocol established for stage 2 gave ATP readings in the region of the threshold value of 250 RLUs. The post-build samples were well in excess of the threshold value (mean±SD=2651±606 RLUs). The autoclave sterilization gave results well below the threshold value (25±12 RLUs), as would be expected. The data from the post-cleaning samples (424±165 RLUs) are of most interest because they span the threshold region. The pilot data indicates that it may be possible to process and clean AM materials for clinical use in the absence of sterilization. The full set of AM samples from Table 1 were taken through the cleaning protocol (stage 2), and this data set gives the core sets of results for this research study.

### 3.2 Cleanliness Threshold Value

The aim of the initial analysis of the ATP results was to evaluate whether each AM material could be labelled as 'clean' relative to the 250 RLU threshold value following a relative simple cleaning process. The full data set from the eleven materials gave a wide range of RLU readings (from thousands to tens), and the results have been divided into three groupings to aid analysis: (a) Objet materials; (b) remaining polymers; and (c) metallic materials.

The three Objet materials are shown in Figure 4. The highest readings were for the Objet Tango Plus (6325±1429 RLUs) – this data is comparable to the post-build values obtained during the pilot study. The lowest readings were for the Objet Vero Blue (861±606 RLUs), whilst the Objet Vero White gave an intermediary range (1805±278 RLUs). Given the low sample size (n=12), a t-test statistic was employed to compare the data relative to the threshold value. Based on a one-tailed test at the 99% significance level, all three Objet material samples gave mean values significantly higher than 250 RLUs.

The remaining five polymeric material results are shown in Figure 5. In contrast to the Objet readings, all five samples gave a mean value significantly lower than the 250 RLU threshold value (t-test 99% level: $p<0.01$). This indicates that the Projet, SLA and SLS materials used in this study can be cleaned for clinical use. Across this grouping there are a range of readings. The highest values are given by SLA Clear at 181±59 RLUs, whilst the lowest readings were given by the glass-filled SLS Nylon at 41±8 RLUs.

The final grouping of three metallic materials is given in Figure 6. In this set of readings, the main difference is between the Cobalt Chrome and the Titanium. The two cobalt chrome samples give mean values significantly below 250 RLUs (t-test 99% level: $p<0.01$), whilst the titanium sample is not significantly different ($p>0.05$).

In summary, across the eleven AM materials tested there are two key findings from the post-cleaning analysis: (a) the three Objet materials are significantly above the 250 RLU threshold; and (b) the Projet, SLA, SLS and Cobalt Chrome materials are significantly below the 250 RLU threshold.

### 3.3 Inter-Sample Comparisons

For inter-sample analysis, the maximum number of pair-wise comparisons was 55. This was used as the Bonferroni correction factor for further t-test statistical analysis across the sample sets. It was assumed that a conservative correction factor would militate against inflated false positives for multiple comparisons ($p<0.01/55$ now required for 99% significance level).

Within the Objet grouping, the Vero Blue samples gave a mean RLU value significantly lower than both the Tango Plus and Vero White samples ($p<0.0005$). It is worth noting that the Objet Vero Blue samples were sourced direct from a UK NHS hospital (Southern General, Glasgow), and it is their material of choice for anatomical medical models – these models are not used for direct patient contact. For the polymers shown in Figure 5, the notable pair-wise comparisons are between the two SLA materials and the two SLS materials. There is a significant difference between the SLA Clear and the SLA Grey ($p<0.0005$). In this context, the Accura ClearVue resin tends to be employed in medical applications then the Accura Xtreme Grey resin since it has undergone prerequisite testing to USP Class 23/6. There is also a significant difference between the non-glass-filled SLS Nylon and the glass-filled SLS Nylon ($p<0.0005$). These samples were sourced from different suppliers so more data is required in order to draw a firm conclusion as to the reason for this difference.

The interesting result from the metallic materials in Figure 6 is the fact that the electro-polished Cobalt Chrome samples gave a mean RLU value that was significantly higher than the non-electro-polished Cobalt Chrome samples ($p<0.0005$). Electro polishing is employed (particularly in the dental industry) to inhibit contaminants adhering to the surface. However, the ATP results indicate that the non-electro polished surface is 'cleaner'. The greatest variability was exhibited by the Titanium samples (207±108 RLUs), and the mean value for the Titanium was not significantly different to the two Cobalt Chrome samples.

## 4. Discussion

The results have shown that the ATP methodology is a practical technique for providing real-time measurements of surface cleanliness across a range of AM materials. The use of AM materials for medical applications with direct patient contact is growing, therefore tests to verify cleanliness and sterility at various stages of the AM process are of significant importance. This study is the first to report the application of the ATP technique to a range of AM sample materials. The work has been undertaken within the context of an AM/RP research centre, specialist biomedical/clinical facilities were not required. The results show that it is feasible to use ATP as a screening technique to highlight AM materials that may be more suited to medical devices and the clinical environment.

The data from the initial pilot study has shown that ATP testing can identify and highlight the differences between three nominal delivery stages: post-build, post-cleaning and post-sterilisation. Across the various materials, there was an order of magnitude change in RLU measurements associated with each of these stages. There is the potential to establish benchmark RLU values for each key phase of AM in order to provide the process validation required for any medical product.

The key <250 RLU threshold reported in this paper is the nominal cut-off for surface cleanliness. A relatively simple cleaning protocol was used to generate ATP readings in the region of this threshold value. The main finding was that the Objet materials could not be

cleaned or disinfected – all three Objet AM materials were significantly above the threshold value. In contrast, the majority of the remaining polymers and metallic AM materials were significantly below the 250 RLU threshold value (post cleaning). It would appear that the Objet polyjet process adheres more surface contaminants when compared to the other AM processes. More research is required in order to identify the cause of Objet's higher RLU readings.

For the AM materials below the 250 RLU threshold an interesting feature was the influence of surface finishing. Metallic medical parts are electro-polished for aesthetic and functional purposes; however, the results from this study question whether this is beneficial. The electro-polished Cobalt Chrome samples had significantly higher RLU readings compared to the non-electro-polished samples. Contaminants from the finishing process may have been impregnated into the electro-polished surface.

The surface characteristics of a selection of the AM materials were further investigated through surface roughness measurements. Build orientation naturally has an impact on surface roughness, therefore only the top build surface measurements are given as representative values. The electro-polished Cobalt Chrome gave Ra=0.10µm, in comparison to Ra=2.82µm for the non-electro-polished samples. This latter reading was comparable to the SLA Clear (Ra=2.32µm) and SLA Grey (Ra=2.31µm) measurements. The Objet materials gave values of Ra=0.19µm for Vero White and Ra=0.47µm for Vero Blue. These surface roughness readings are in line with expectations, but they do not highlight surface discrepancies that could account for differences in RLU readings.

This research has used a single ATP reading (post cleaning) in order to evaluate the suitability of various AM materials for medical applications. More research is required in order to show how a fixed time-point ATP reading correlates to the likelihood that a surface is susceptible to microbial contamination over a longer period of time.

## 5. Conclusions

ATP bioluminescence is an appropriate test method for measuring the surface cleanliness of AM materials intended for medical applications. The experimental technique is a user-friendly, quick method for quantifying cleanliness levels during the various stages of AM production, preparation and clinical delivery.

The three Objet materials produced consistent elevated RLU readings (post cleaning), and this indicates that they may not be appropriate for patient-contact medical devices. Following the cleaning protocol, a number of the polymeric and metallic samples were significantly below the 250 RLU threshold value, and this shows that a number of standard AM materials have the potential for a wide range of medical applications.

The ATP test method appears to have the sensitivity to evaluate different material surface characteristics, specifically the impact of surface finishing techniques on overall cleanliness.