Monitoring of Bacterial-contamination of Dental Unit Water Lines using ATP-bioluminescence

Running title: **Bacterial-contamination Monitoring**

Akari Watanabe[1]*, Naofumi Tamaki[2], Kenji Yokota[3], Miwa Matsuyama[1] and Susumu Kokeguchi[4]

[1]Department of Oral Health Care and Rehabilitation, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan.

[2]Department of Preventive Dentistry, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan.

[3] Graduate School of Health Sciences, Okayama University, Okayama, Japan

[4]Department of Oral Microbiology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan.

* Corresponding author

Akari Watanabe

Department of Oral Health Care and Rehabilitation, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan.

3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan

TEL/FAX: 81-88-633-7898

E-mail: akari.watanabe@tokushima-u.ac.jp

Summary

Bacterial contamination of dental unit waterlines (DUWLs) was evaluated by using ATP-bioluminescence analysis and conventional culture method. Water samples (n=44) from DUWLs were investigated for heterotrophic bacteria by culture on R2A agar, which ranged from 1.4×10^3 to 2.7×10^5 CFU/mL. The ATP-bioluminescence results for DUWL samples were ranged from 6 to 1189 RLU and obtained within one minutes. These results were well correlated with the culture results (r=0.727-0.855). We conclude that differences in the bacterial contamination of each water supply were confirmed by the ATP-bioluminescence assay. This method would be potentially useful for rapid and simple monitoring of DUWL bacterial contamination.

Keywords:

ATP-bioluminescence analysis, Dental unit water line, Bacteria contamination, Infection control

Introduction

Dental chair units are equipped with narrow-bore, flexible, plastic tubes called dental unit waterlines (DUWLs) that supply water to all dental instruments (air/water syringe, High-speed turbine, rinsing equipment) [1]. Bacterial contamination is often observed in these complex dental units, and biofilms containing multiple bacterial species easily form within the DUWLs [2]. Bacterial numbers per millilitre in DUWLs can reach several million overnight [3]. Heavy bacterial contamination of DUWLs is thought to be the result of biofilm formation within the small-bore plastic tubes [4].

Adenosine triphosphate (ATP) is present in all living microorganisms (bacteria, fungi, and protozoa) and cells and is a good marker of viability and contamination of microorganisms. The ATP-bioluminescence-based luciferine/luciferase reaction has been used in the field for rapid detection in sanitary surveys of hospitals and in the food and beverage industries [5, 6].

It is reported that ATP bioluminescence is a sensitive and rapid system of bacterial detection. However, the relationships among ATP bioluminescence assessment and conventional culture methods for DUWLs are unclear. In this study, we analysed the relationship between total heterotrophic bacterial counts in DUWLs and ATP levels, and evaluated the usefulness with bacterial-contamination monitoring in DUWLs.

Methods

Sample collection

Bacterial contamination of the DUWLs of 11 dental units (J. Morita. Mfg. Corp,

Tokyo, Japan, Model: SIGNO Type G40), which have been usually used according to the maintenance management manual of our university was investigated by using ATP bioluminescence in addition to conventional culture.

Fifty-millilitre water samples (n = 44) as the initial residual water in DUWLs were collected into the sterilized Falcon® 50mL plastic tube (Product #352070) without neutralizer, sodium thiosulfate from the water supplies to the dental instruments (air/water syringe, high-speed turbine, micromotor and cup filler) by the routine clinical use at the beginning of a working day; the dental units had last been used 3.5 days previously. The water samples after water flushing for 2 min were also collected from the dental instruments (air/water syringe, high-speed turbine, micromotor and cup filler).

ATP-bioluminescence assessment

We used a Kikkoman Lumitester PD-20 rapid hygiene monitoring system with a LuciPac Pen-AQUA (Kikkoman Biochemifa Co., Tokyo, Japan) for ATP-bioluminescence measurement. The LuciPac Pen-AQUA sampling stick which can collect up to 0.15-millilitre of water was soaked in the sample water and then placed back into the LuciPac Pen-AQUA. ATP-bioluminescence values in relative light units (RLU) were measured by using the Kikkoman Lumitester PD-20 in accordance with the manufacturer's instructions.

Method of culture for heterotrophic plate counts (HPCs)

After 10-fold dilution, 100 microlitre of the water samples were plated on R2A agar (DAIGO®, Nihon Pharmaceutical Co. Ltd, Tokyo, Japan) selective for

heterotrophic bacteria and incubated at 25 °C for 7 days to obtain total HPCs in terms of colony forming units (CFU) per millilitre. The CFU values were compared with the RLU values obtained by the Kikkoman Lumitester PD-20 on the basis of the ATP-bioluminescence reaction.

Identification of bacterial species by cultural method

Bacteria in a selected representative colony on the R2A agar plate were Gram stained and then examined under a light microscope.

The NF-18 ID test (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), which is commercially available bacterial identification microplate kit including several sugar fermentation, nitrate reduction, urease and indole formation, and gelatin liquefaction tests was used for bacterial species identification.

Statistics analysis

Correlations between ATP (RLU) and HPCs (Log CFU) were determined by using Spearman's rank correlation analysis. Mann-Whitney *U*-test was used to assess the differences between before and after water flushing in each dental instrument. The JMP software program (version 12, SAS Institute. Cary, USA) was used to conduct the statistical analysis.

Results

ATP measurement and heterotrophic plate counts (HPCs)

The results of ATP measurements and HPCs (Log CFU) of samples of the water supplied to the dental instruments (air/water syringe, high-speed turbine,

micromotor and cup filler) were shown in Fig 1. ATP measurements and HPCs of air/water syringe ranged at 22 to 356, and at 6.4×10^3 to 1.4×10^5 CFU/mL, respectively. ATP measurements and HPCs of high-speed turbine ranged at 7 to 248, and at 1.4×10^3 to 1.1×10^5 CFU/mL, respectively. ATP measurements and HPCs of micromotor ranged at 15 to 1189, and at 5.3×10^3 to 2.7×10^5 CFU/mL, respectively. ATP measurements and HPCs of cup filler ranged at 6 to 281, and at 5.6×10^3 to 1.0×10^5 CFU/mL, respectively.

Furthermore, we conducted simple correlation analyses for each dental instrument (air/water syringe, high-speed turbine, micromotor and cup filler) between ATP measurements and HPCs (Log CFU). ATP measurements were positively correlated with HPCs to air/water syringe ($r = 0.852$, $p < 0.001$; Fig. 1-A), high-speed turbine ($r = 0.727$, $p < 0.01$; Fig. 1-B), micromotor ($r = 0.747$, $p < 0.001$; Fig. 1-C), and cup filler ($r = 0.855$, $p < 0.001$; Fig. 1-D).

ATP measurement values before and after water flushing

Comparisons of ATP measurement before and after water flushing were shown in Fig. 2. ATP measurement values of each dental instrument (34-420 RLU) were significantly reduced at 4 to 30 RLU after water flushing for 2min (Fig 2). Water flushing played an important role in reducing bacterial contamination of DUWLs.

Identification of bacterial species

Bacterial contamination of the water samples from each of the dental instruments was confirmed by culture method. Bacteria isolated from R2A agar

plates were confirmed by Gram staining and microscopic analysis to be Gram-negative rods. *Novosphingobium* sp., *Methylobacterium mesophilicum*, *Burkholderia* spp., *Sphingomonas paucimobilis, Sphingomonas parapaucimobilis* and *Sphingobacterium mizutae* were identified as the most prevalent bacterial species in the DUWLs by Gram-stain and the NF-18 ID test.

Discussion

A complex network of interconnected plastic tubes, which is called DUWLs, supplies water to all dental instruments (air/water syringe, high-speed turbine, micromotor and cup filler) in a dental unit. In general, DUWLs are prone to form microbial biofilm, and their output water are invariably contaminated by heterotrophic bacteria and Gram-negative environmental bacteria, including high densities *of Pseudomonas aeruginosa* and *Legionella* species [7]. Although only limited numbers of cases of infection resulting from exposure to water from contaminated DUWLs have been reported [7].The presence of high levels of microbial contamination may be a health problem for dentists and patients, especially those who are immunocompromised. Therefore, it is important to monitor the levels of bacterial contamination in DUWLs and to maintain the water quality.

The US Center for Disease Control and Prevention (CDC) recommends that the water for non-surgical dental treatment from DUWLs should be always maintained at the regulatory standards for drinking water, namely heterotrophic bacterial levels are no more than 500 CFU/mL. The American Dental Association (ADA) also recommends that heterotrophic bacterial levels in the water from

DUWLs used for dental treatment should not exceed 200 CFU/mL. Water Supply Division, Health Service Bureau, Ministry of Health, Labour and Welfare in Japan recommends that heterotrophic bacterial levels in the drinking water would not exceed 2000 CFU/mL as the target value for complementary items (April 2010 ～), not drinking water quality standards[8].

However, reported levels of microorganisms in untreated DUWLs are often greater than 500 CFU/mL, thus exceeding the CDC and ADA water standards [2, 3, 9]. In this study, we also detected HPCs at 1.4×10^3 to 2.7×10^5 CFU/mL in water samples from DUWLs. Thus, the quality of water from the DUWLs sampled did not fulfilling the water standards criteria set by the CDC and ADA. Several reports have demonstrated that bacterial counts and bacterial flora differ among the DUWLs supplying different dental instruments within each unit (air/water syringe, high-speed turbine, micromotor and cup filler) [9, 10]. We also confirmed the differences in the HPCs of water supplying different dental units and supplying different dental instruments within each unit. The results of ATP-bioluminescence method in water samples, the HPCs and ATP values of the DUWL water samples were strongly correlated. Therefore, this method seems suitable for measuring high concentrations of heterotrophic bacteria samples from DUWLs.

The CDC also recommends that DUWLs should be flushed at the beginning of the clinic day to reduce microbial loads. In this study, the effectiveness of water flushing for 2 min to reduce bacterial loads in DUWLs was also investigated by ATP-bioluminescence assay. The results of ATP values of each dental instrument were significantly reduced after water flushing for 2 min. The water

quality test in DUWLs by using conventional cultural methods need a long period (several days or a week). Simple, rapid and inexpensive methods are necessary to monitor water quality with estimating the numbers of heterotrophic bacteria in DUWLs. Although ATP bioluminescence could not identify bacterial species, the importance of this method is that it can determine the bacterial quality of DUWLs within less than a few minutes, without the need for special instruments and techniques. We compared ATP measurements between before and after flushing, and showed the significance. Consequently, the ATP-bioluminescence method would be suitable for daily monitoring the water quality in DUWLs for a shorttime.

In conclusion, our data suggest that the ATP-bioluminescence method is potentially useful for rapid and simple monitoring of DUWL bacterial contamination.

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Conflict of interest statement

The authors state that they have no conflict of interest.

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Figure 1 Correlation between ATP meter and Log CFU prior to water flushing.

The y-axis represents Log CFU to air/water syringe (A), high-speed turbine (B), micromotor (C) and, cup filler (D). The solid line represents regression line. Spearman's rank correlation coefficient (*r*) and its probability (*p*) were represented.

Figure 2 ATP measurement values before and after water flushing.

Data represent means ± standard error of the mean (SEM). **: $p<0.01$, significantly different from before water flushing.

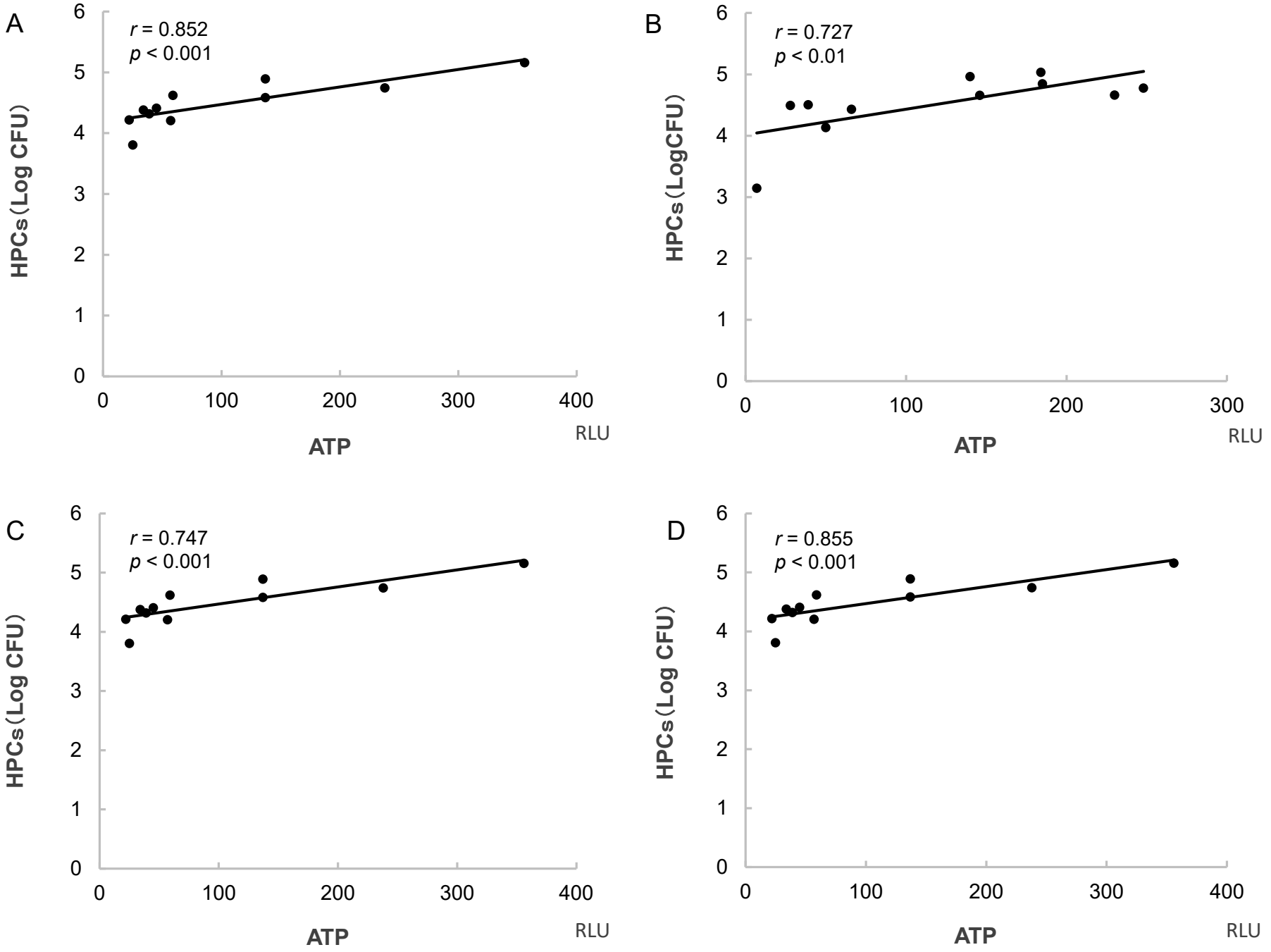
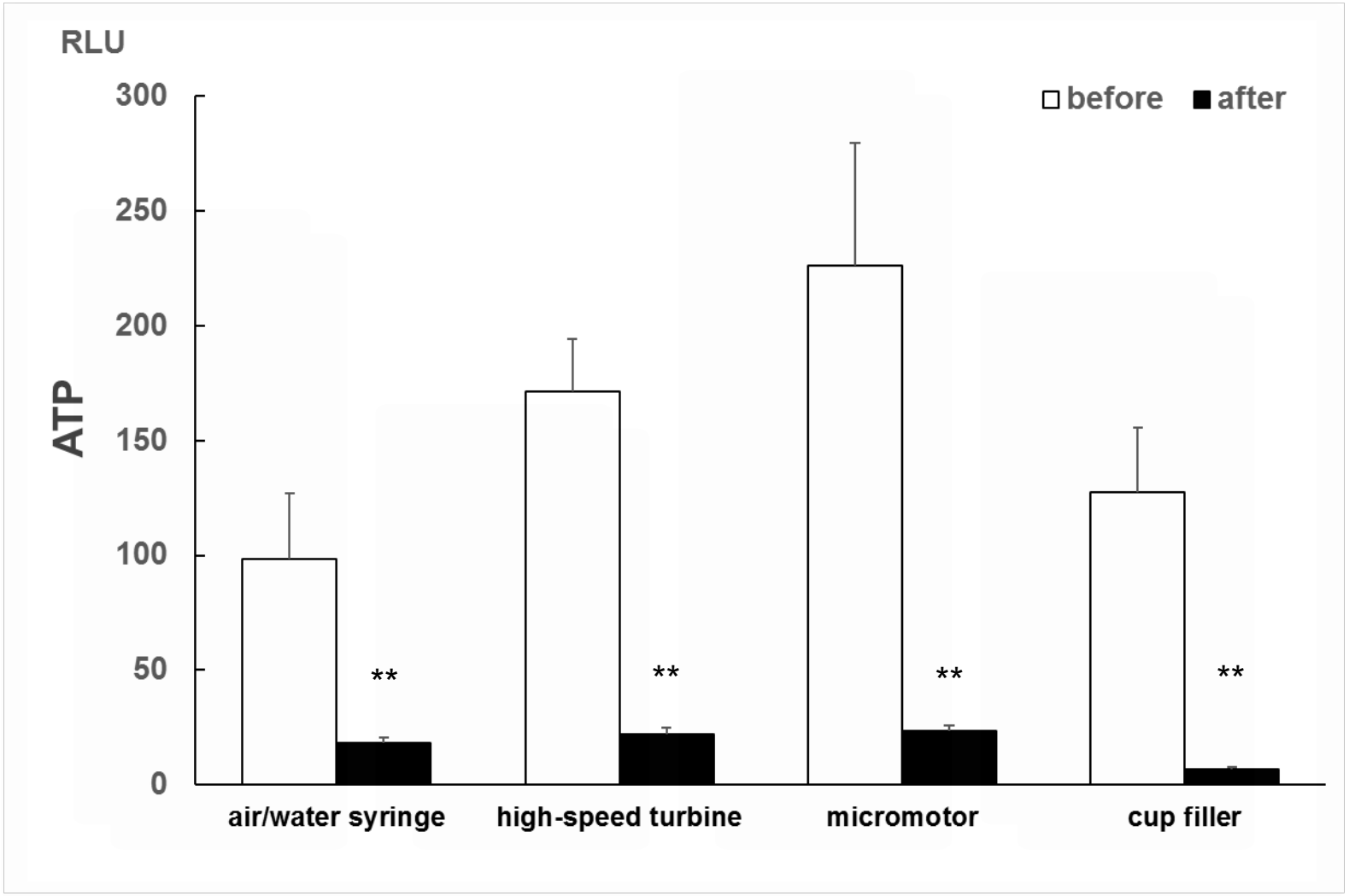

A
r = 0.852
p < 0.001
HPCs（Log CFU）
ATP
RLU
B
r = 0.727
p < 0.01
HPCs（LogCFU）
ATP
RLU
C
r = 0.747
p < 0.001
HPCs（Log CFU）
ATP
RLU
D
r = 0.855
p < 0.001
HPCs（Log CFU）
ATP
RLU

Figure 1


RLU
300
250
200
150
100
50
0
ATP
before
after
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air/water syringe
high-speed turbine
micromotor
cup filler

Figure 2