

# Investigation of Appropriate Sanitization Frequency for Rodent Caging Accessories: Evidence Supporting Less-frequent Cleaning

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The *Guide for the Care and Use of Laboratory Animals* states that sanitization of caging accessories (for example, filter tops and wire-bar lids) should be done every 2 wk. In this study we tested the hypothesis that organic contamination measured by the presence of ATP associated with organic material (measured with luciferase test swabs) and the number of bacterial colony-forming units (as determined by use of replicate organism detection and counting plates) on caging accessories did not differ significantly at 2 wk versus several months of use. The study evaluated 4 groups: mouse and rat ventilated and static wire-bar cages with or without filter tops (n = 10 per group). The cages were evaluated at several time points from 2 wk to 6 mo. For every cage type, ATP levels did not differ significantly between 14 and 90 d and, in most cases, between 14 and 180 d. In addition the number of bacterial colonies did not differ significantly between 14 and 120 d (and, in some cases, between 14 and 180 d). This study provides data relevant to establishing a validated frequency for sanitization of rodent caging accessories while controlling, and potentially decreasing, costs associated with sanitization.

**Abbreviations:** CFU, colony-forming units; RLU, relative light units; RODAC, replicate organism detection and counting

The use of rodents as models in biomedical research has grown dramatically in the past decade with the advent of genetically engineered mutant mice. Because of the tremendous expense associated with maintaining large numbers of rodents, institutions have been forced to reevaluate how they care for and house these animals. Frequent sanitization of wire bar lids and filter tops is an expensive part of rodent use. *The Guide for the Care and Use of Laboratory Animals* states "In general, enclosures and accessories, such as tops, should be sanitized at least once every 2 wk."<sup>6</sup> However, there are no published scientific data justifying this recommendation; further, it is unclear whether these guidelines are meant to minimize organic contamination, bacterial contamination, or both. This study helped to determine the necessary frequency for sanitization of rodent caging accessories. Supporting less frequent cleaning than that recommended in the *Guide*, several other studies addressing rodent pheromones provide evidence that rodents are stressed when moved from a dirty cage to a clean one.<sup>2,7,8</sup> The Jackson Laboratory examined the effects on breeding mice whose ventilated cage wire bar lids were not sanitized for up to 6 mo and showed that the number of bacterial colony-forming units (CFU) on replicate organism detection and counting (RODAC) plates reached a plateau within 1 wk and did not change significantly over time.<sup>4</sup> The scope of that study is limited, however, because it evaluated only mouse ventilated cages.

The measurement of ATP is a relatively new method for evaluating sanitization. This method gives a more accurate measurement of cleanliness because it measures ATP from all organic matter instead of only replicating bacteria and fungi. The amount of ATP is expressed in relative light units (RLU).

Recently, this method has been compared against the conventional RODAC technique for evaluating cage sanitization and has been found to be a more efficient and less expensive alternative.<sup>3</sup> The ATP method is now used frequently in food production facilities, state health laboratories, drug companies, and as a method of verifying cage washer efficacy in laboratory animal facilities.<sup>3</sup> Traditional microbial evaluation programs advocate evaluating cages for sanitation by monitoring for different morphologic groups of bacteria, the most important and useful being gram-negative morphology, instead of testing for specific organisms.<sup>9</sup> In addition, the Institute of Laboratory Animal Resources recommended that sanitized equipment at barrier facilities should be free of gram-negative organisms.<sup>5</sup> As such, the historically recommended practice was to use qualitative assessments of caging to show freedom from gram-negative organisms as documentation of effective sanitation.<sup>9</sup>

Currently there are no standards stating the acceptable levels of RLU and CFU consistent with adequate cleanliness of caging accessories. Our study did not attempt to define such standards; rather, we sought to demonstrate the lack of significant difference between cage accessory contamination at 2 wk and that of several months. Mouse and rat ventilated and static cages were evaluated for a period of 6 mo. These cages were evaluated for bacterial contamination in the form of CFU of gram-negative organisms and organic contamination in the form of ATP expressed as RLU. We hypothesized that the levels of organic contamination and CFU of gram-negative bacteria would not differ significantly between 14 d and several months of use, thus conflicting with the requirement for biweekly sanitation of caging accessories.

## Materials and Methods

**Animals.** Each cage contained 2 female outbred rats (Carl's [SD] IGSBR, Charles River Laboratories, Wilmington, MA) or 5

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female outbred mice (CrI:CD-1 [ICR]BR, Charles River Laboratories) to achieve maximal stocking density and thus maximal potential contamination. Upon purchase from the vendor, the rats were known to be free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus–rat coronavirus, Kilham rat virus, Toolan H-1 virus, rat parvovirus, reovirus, rat enterovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, Hantaan virus, mouse adenovirus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Salmonella* spp., *Helicobacter hepaticus*, *Helicobacter bilis*, *Helicobacter* spp., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pasturella multocida*, *Pasturella pneumotropica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*,  $\beta$ -hemolytic *Streptococcus* spp. (groups B and G), and all ecto- and endoparasites. Upon purchase from the vendor, the mice were known to be free of Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, reovirus, rat and mouse parvovirus, mouse poliovirus, epizootic diarrhea of infant mice virus, polyoma virus, mouse pneumonitis virus, mouse cytomegalovirus, mouse thymic virus, ectromelia virus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, Hantaan virus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium bovis*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Salmonella* spp., *Streptobacillus moniliformis*, *Helicobacter hepaticus*, *Helicobacter bilis*, *Helicobacter* spp., *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pasturella pneumotropica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*,  $\beta$ -hemolytic *Streptococcus* spp., *Pneumocystis* spp., *Bacillus piliformis*, and all ecto- and endoparasites. All animals were fed nonautoclaved, nonirradiated standard laboratory rodent chow ad libitum (Lab Diet 5001, Purina Mills International, Richmond, IN). The feed hopper was filled completely when the cage was occupied, and was refilled every week. The feed hopper was dumped of all food every 2 wk and completely refilled. Water was given ad libitum and was treated by reverse osmosis in a recirculating system. The rat and mouse static cages were given water bottles that were changed out once weekly. The ventilated cages were placed on automatic watering systems. The room temperature was 68 to 72 °F and was on a 12:12-h light:dark cycle.

**Housing.** We evaluated 4 groups ( $n = 10$  per group) of thermoplastic cages: rat ventilated (model 4, Maxi-Miser System, Thoren Caging Systems, Hazleton, PA), rat open-top (Rat Shoebox Cage, Lab Products, Seaford, DE), mouse ventilated filter-top, and mouse static filter-top (Super Mouse 750 Micro-Isolator, Lab Products). The cages were lined with 1/8-in. corncob bedding (Anderson's Bed-o'-Cobs, Maumee, OH) that was heat-treated. The cage bottoms were changed according to our regular schedule, once weekly for static cages and once every 2 wk for ventilated cages. The filter tops and wire-bar lids were moved to the clean cage bottom, and the food was refilled every week and changed completely every 2 wk during these changes. All rooms were negatively pressurized with regard to the hallway. The relative humidity was kept between 45% and 75%, and there were 8 to 10 air changes hourly. All personnel handling the mouse cages wore latex gloves and disposable gowns, and all work was done under a biosafety cabinet with a high-efficiency particulate air filter. All rat cages were handled by personnel wearing latex gloves and disposable gowns. The study was approved by Emory University's Institutional Animal Care and Use Committee and was conducted in compliance with local and federal guidelines governing laboratory animal care and housing at facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

**Contamination testing.** We swabbed a 4-cm  $\times$  4-cm area on each cage and evaluated the swab for organic contamination in the form of ATP (expressed as RLU) by using luciferase test swabs (Firefly swabs, Charm Sciences, Malden, MA). Gram-negative CFU were counted on RODAC plates (BBL Trypticase Soy Agar with Lecithin and Polysorbate 80, Becton Dickinson, Franklin Lakes, NJ) that had been pressed to target surfaces. The cages were monitored at 0, 14, 30, 60, 90, 120, 150, and 180 d. The sampling areas for the swabs and RODAC plates for filter-top lids included the filter material and associated plastic supporting material. The sampling areas of the rat cage wire-bar lids were the 2 sides of the feeder for the swabs and the wire bars for the RODAC plates. For wire-bar lids, RODAC plates were touched to the surface, removed, rotated 90°, and touched to the surface a second time. The mouse cage wire-bar lids were sampled in the area surrounding the grommet for the swabs and the wire bars for the RODAC plates. All CFU on RODAC plates were counted by a veterinarian. All colonies were sampled and Gram-stained according to standard methods by trained clinical laboratory personnel, to determine whether the organisms were gram-positive or -negative. All Gram stains were examined microscopically by a veterinarian or veterinary pathologist.

**Statistical methods.** The cages were evaluated for significant differences in RLU and gram-negative CFU between 14 and 180 d by use of 1-way analysis of variance with Bonferroni correction (Graph Pad Software, San Diego, CA). When cages showed a significant difference ( $P < 0.05$ ) between 14 and 180 d, data were recalculated to find the longest interval at which the differences were not significant.

## Results

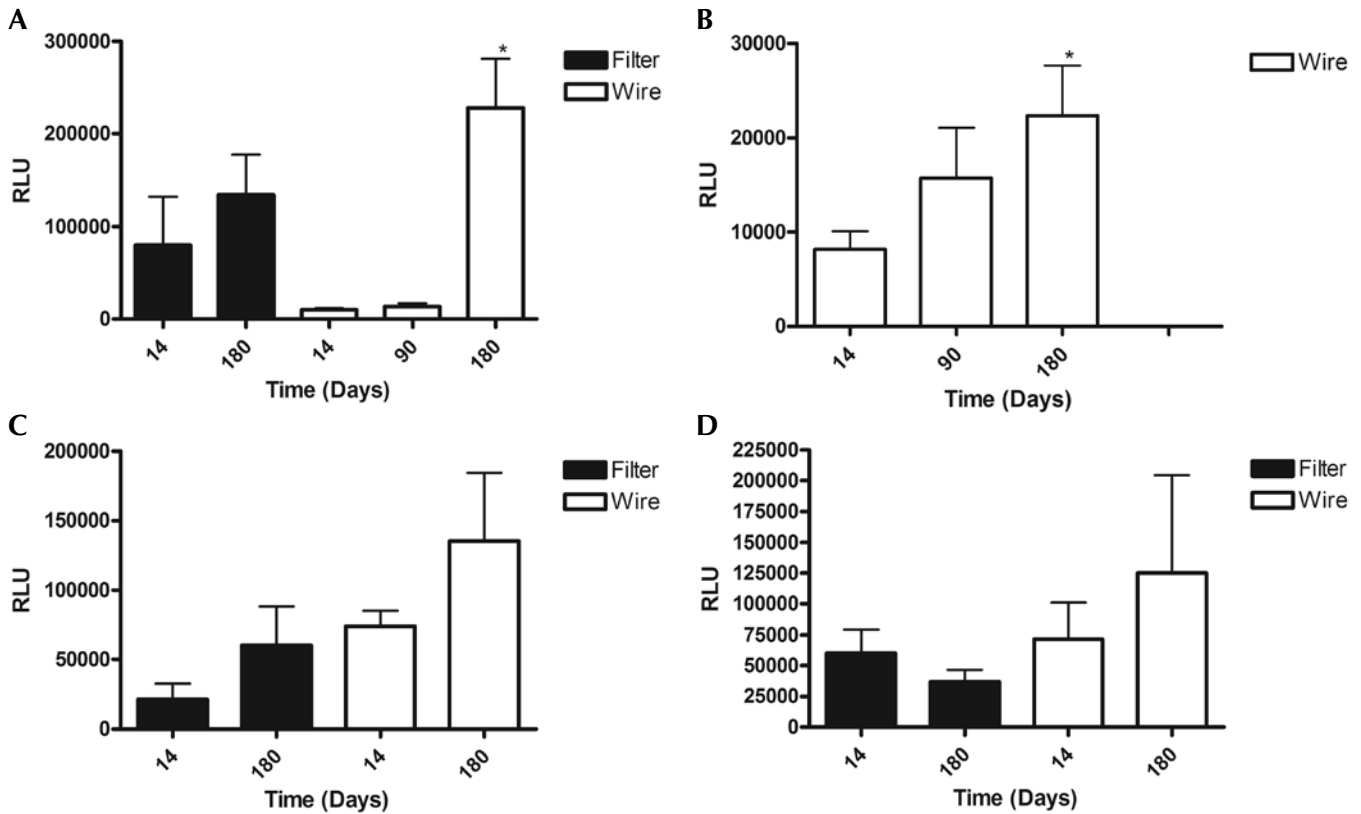
The organic material data demonstrated no significant difference in RLU between 14 d and 180 d in all groups except the mouse ventilated and rat static wire-bar lids (Figure 1 A, D). There was no significant difference between 14 and 180 d for mouse ventilated filter tops ( $P = 0.2579$ ; Figure 1 A), mouse static filter tops ( $P = 0.1675$ ; Figure 1 D), mouse static wire-bar lids ( $P = 0.2878$ ; Figure 1 D), rat ventilated filter tops ( $P = 0.1210$ ; Figure 1 C), and rat ventilated wire-bar lids ( $P = 0.1334$ ; Figure 1 C). The mouse ventilated wire-bar lids showed no significant difference ( $P = 0.2021$ ) between 14 and 90 d, but data differed significantly ( $P = 0.0014$ ) between 14 and 180 d (Figure 1 A). Rat static wire bars showed no significant difference ( $P = 0.0552$ ) between 14 and 90 d, but data differed significantly ( $P = 0.0100$ ) between 14 and 180 d (Figure 1 B).

The numbers of gram-negative CFU did not differ for 3 of the 7 test categories (Figure 2): mouse ventilated wire-bar lids ( $P = 0.2240$ ; Figure 2 A), mouse static filter tops ( $P = 0.5886$ ; Figure 2 D), and mouse static wire-bar lids ( $P = 0.4916$ ; Figure 2 D). Compared with those at 14 d, CFU for the mouse ventilated filter tops were significantly different ( $P = 0.0014$ ) at 180 d but not at 120 d (Figure 2 A). Similarly CFU for rat ventilated filter tops and wire-bar lids were significantly different ( $P < 0.0001$ ) at 180 d but not at 150 d (Figure 2 C), and those on the rat static wire-bar lids were significantly different at 180 d ( $P < 0.0001$ ) but not at 150 d (Figure 2 B).

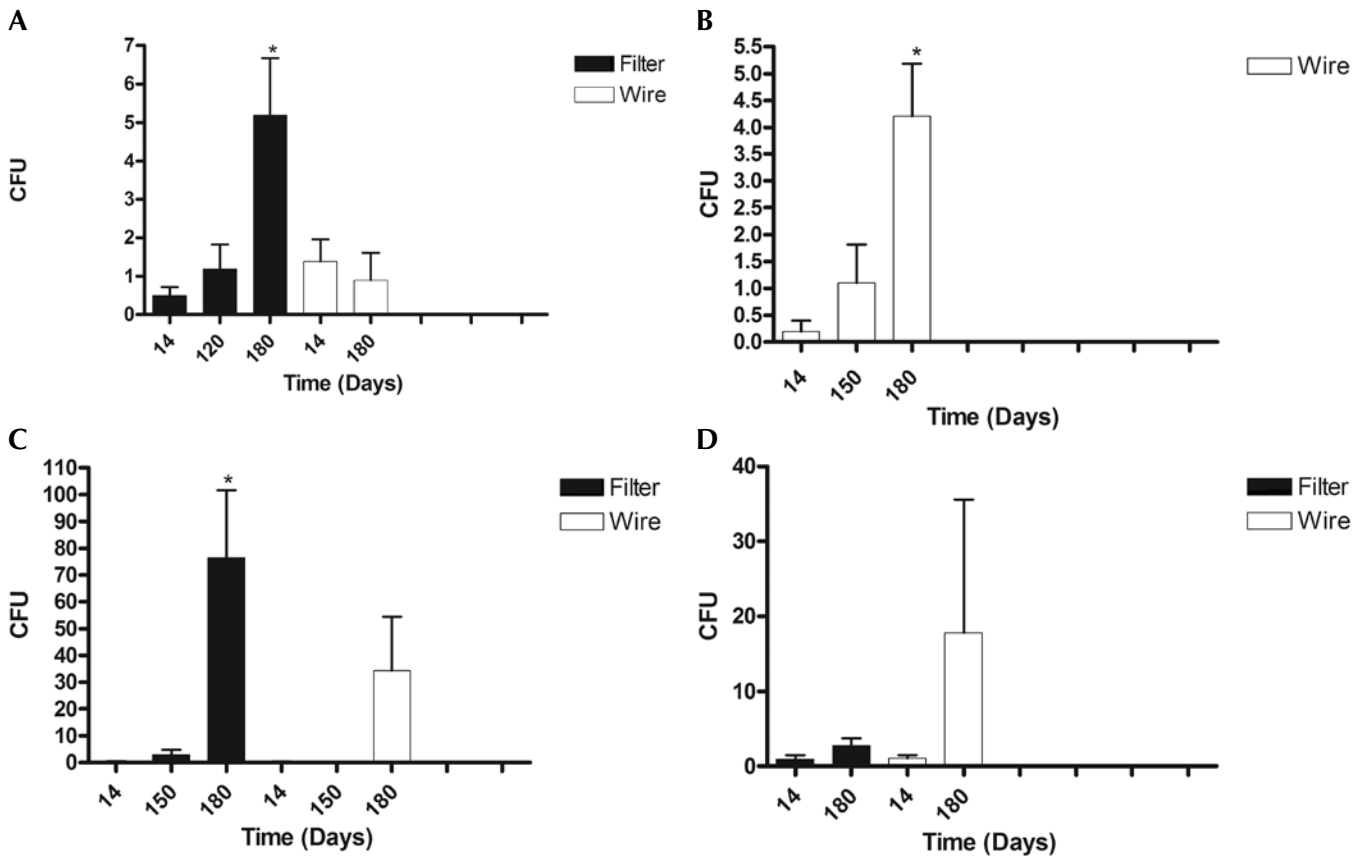
The data can be summarized by showing the time points at which differences in the RLU and CFU counts became significant compared with those on day 14 (Table 1).

## Discussion

Because of the tremendous expense associated with maintaining large and growing colonies of rodents, institutions



**Figure 1.** Number of relative light units (RLU) on specific caging accessories at 14 and 180 d. When cages did not have a significant difference ( $P < 0.05$ ) between 14 and 180 d, data were recalculated to find the latest time point at which the differences were not significant. \*,  $P < 0.05$  versus day 14 value. (A) Mouse ventilated cages. (B) Rat static cages. (C) Rat ventilated cages. (D) Mouse static cages.



**Figure 2.** Number of colony forming units (CFU) on specific caging accessories at 14 and 180 d. When cages did not have a significant difference ( $P < 0.05$ ) between 14 and 180 d, data were recalculated to find the latest time point at which the differences were not significant. \*,  $P < 0.05$  versus day 14 value. (A) Mouse ventilated cages. (B) Rat static cages. (C) Rat ventilated cages. (D) Mouse static cages.

**Table 1.** Latest time point at which relative light units and colony forming units showed no significant difference from that on day 14

	90 d	120 d	150 d	180 d
Mouse vent wire bar	R			C
Mouse vent filter top		C		R
Mouse static wire bar				RC
Mouse static filter top				RC
Rat vent wire bar			C	R
Rat vent filter top			C	R
Rat static wire bar	R		C	

C, colony-forming units; R, relative light units.

have been forced to reevaluate how they care and house such animals. Less intensive sanitation, providing that environmental conditions do not deteriorate to the extent that the quality of the research or animal well-being is compromised, can have a direct diminishing effect on labor and operating costs for laboratory animal facilities, including a decrease in the number of caging accessories needed on-hand, storage space needed for housing accessories, and time spent by personnel at change-out. All of these factors have the ability to control and potentially reduce costs associated with cage accessory sanitization. It should be noted that if sanitization frequency is extended to such time points at which environmental conditions deteriorate, to limits at which animal health and well-being and the data derived from affected animals are compromised, the effects could be detrimental and cost-prohibitive.

When we examined the amount of organic material (RLU) that accumulated on caging accessories over a 180-d period, the majority of the equipment demonstrated no significant difference between 14 and 180 d (Table 1). It is important to keep in mind that the cleanliness of equipment is not linked to just replicating bacteria and fungi. The total amount of organic material present (RLU) is important because it is a measure of uncleanness and includes food residue and biofilm from humans and animals as well as microorganisms.<sup>3</sup> The elimination of all of these factors is vital to equipment sanitization. The data clearly demonstrate that it is possible to not sanitize caging accessories for at least 90 d without compromising cleanliness. In the majority of cases, the 180-d time point still did not show any significant difference in the amount of RLU and CFU and suggests that it may be possible, in some cases, to extend sanitization frequency beyond 180 d.

It is difficult to determine why some of the RLU and CFU counts became significant at earlier time points, as in the mouse ventilated wire bars (Table 1). Possible explanations include contamination by care staff on regular change out of the cage bottom, contamination upon gathering samples, and the difference in sampling location between swabs and RODAC plates. Cross-contamination could take place through the gloves of the animal care staff. These personnel handle the cage bottoms during regular change-out and then handle the caging accessories as they put the cage back together. The care staff also handles the animals directly on change-out. Contamination with bio-film or bacteria could take place as the care staff transfer the animals from the dirty to the clean cage. How the animal care staff handles the accessories as they change the cage could explain why there are differences in the rates of contamination between different accessories. In addition, it was not physically possible to fit the RODAC plates near the grommets in the ventilated mouse cages or the feeders in the

rat static cages. The swabs detect ATP in all organic material, including food residue. The presence of food residue in these areas could explain the 2 RLU time points, mouse ventilated wire bars and rat static wire bars (Figure 1 A, B), that showed significant difference at only 90 d versus the nonsignificant CFU counts obtained at that time point.

The early time points with regard to the CFU could have been due to the general handling by the care staff. When the CFU data are examined more closely, it is seen that the overall numbers of gram-negative bacterial CFU are quite low. In all but 1 case, there are fewer than 50 gram-negative CFU per RODAC plate. Current American Public Health Association standards for judging RODAC plate counts for human patient room floors describes CFU counts of 0 to 25 as good and 25 to 50 as fair.<sup>1</sup> Although these guidelines refer to CFU from gram-positive and -negative bacteria, gram-negative organisms are considered to be most clinically important.<sup>9</sup> It is also noteworthy that the animals studied were specific pathogen-free vendor mice and rats and that caging accessories from conventionally raised, breeding animals and experimental manipulated animals may have different levels of contamination, which could potentially affect the time points at which levels of organic and bacterial matter become significant.

In conclusion, the results of this study indicate that biweekly sanitization of filter tops and wire-bar lids for laboratory mice and rats is not justified based on measures of RLU or CFU. Under the conditions of our study, all filter tops and wire bars remain in place on cages for at least 90 d with no significant change in the amount of organic material or gram-negative bacterial contamination. Although gram-negative bacterial contamination significantly increases between 90 and 180 d, the level of contamination standards in the majority of cases remains below that allowed by American Public Health Association and thus may be clinically and biologically insignificant.

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