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Mini-Review

Implications of Adenylate Metabolism in Hygiene Assessment: A Review

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ABSTRACT

The assessment of a hygienic state or cleanliness of contact surfaces has significant implications for food and medical industries seeking to monitor sanitation and exert improved control over a host of operations affecting human health. Methods used to make such assessments commonly involve visual inspections, standard microbial plating practices, and the application of ATP-based assays. Visual methods for inspection of hygienic states are inherently subjective and limited in efficacy by the accuracy of human senses, the degree of task-specific work experience, and various sources of human bias. Standard microbial swabbing and plating techniques are limited in that they require hours or even days of incubation to generate results, with such steps as enrichment and colony outgrowth resulting in delays that are often incompatible with manufacturing or usage schedules. Rapid in conduct and considered more objective in operation than visual or tactile inspection techniques, swabbing surfaces using ATP-based assessments are relied on as routine, even standard, methods of hygienic assessment alone or in complement with microbial and visual inspection methods. Still, current ATP methods remain indirect methods of total hygiene assessment and have limitations that must be understood and considered if such methods are to be applied judiciously, especially under increasingly strict demands for the verification of hygiene state. Here, we present current methods of ATP-based bioluminescence assays and describe the limitations of such methods when applied to general food manufacturing or health care facilities.

Key words: ATP bioluminescence; Food residue detection; Hygiene monitoring; Rapid detection

The assessment of the hygienic state or cleanliness of contact surfaces in the food and medical industries is a major task requiring significant diligence, time, and expense. Traditionally, visual and tactile inspections, complemented by standard microbial swabbing or plating practices, were the sole means of making such assessments. However, beginning in the 1990s, bioluminescence assays based on ATP have been increasingly used in food industries and more recently in medical industries as key methods of hygienic assessment alone or in complement with conventional microbial and inspection methods. Initially adopted in the 1960s both by scientists at the National Aeronautics and Space Administration (NASA) looking to determine levels of biological material *(27, 62, 63, 70)* and marine biologists looking at ocean biomass *(51),* the basis of the ATP bioluminescence assay has been studied over several decades *(4, 33, 37, 74–76, 112, 114, 115)*. ATP bioluminescence is centered on the firefly luciferin-luciferase enzyme reaction, in which the luciferase enzyme is exposed to ATP and oxygen, converting luciferin to oxyluciferin with the emission of light. This resulting light can be detected using a luminometer, and its intensity corresponds to the amount of ATP present in the sample. A nearly ubiquitous energy-bearing molecule in biology, the resulting light intensity is interpreted as a measure of the degree of biological surface contamination. Rapid in conduct and considered more objective in operation, ATP-based assessments measure ATP levels of a surface, liquid or substance, that are indicative of the presence of biological matter, including food residues, bodily fluids, and microorganisms.

ATP-BASED ASSAYS IN APPLICATION

ATP-based assessments were first used to test concentrations of ATP in food products in the 1970s *(99)* and on surfaces in the 1980s *(73, 100, 114)*; however, reagents were still expensive and testing was still lengthy *(114)*. Over time, the introduction of rapid handheld luminometers and less expensive and better reagents that could go longer before decay postreaction improved the time, reproducibility, and cost efficiency. By the 1990s, ATP bioluminescence-based assays became more commonplace as a key component in response to regulatory and industry demands to better assure surface sanitation in the food industry *(13, 15, 40, 42, 43, 89)*. ATP-based assessments allowed rapid assessment of hygienic state or cleanliness of contact surfaces, including as a means of process verification in the

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greater context of food manufacturing systems based on hazard analysis and critical control point *(32)*. Since the early 1990s, ATP bioluminescence assays have gained substantial value in the food industry for dairy *(13, 15, 43),* meat *(14, 89),* brewing *(103),* and other processed food production as a means of hygiene monitoring. Currently, ATP-based assays are typically conducted using a specialized swab applied to the desired test area and read using a handheld luminometer, which provides results in relative light units (RLUs). Surfaces such as stainless steel, Teflon, tiles, plastics and polymers, gaskets, and milk processing machinery are commonly tested for various nonvisual soils, such as processing residues, microbiological matter, allergens, and other bioburdens *(89, 124)*. The rapid assessment of surface contamination by microorganisms, both culturable and nonculturable; residual allergens; or biological material that can support growth of microorganisms is a key goal for manufacturing industries.

In more recent years, medical industries have adopted ATP bioluminescence testing, first to test the cleanliness of various surfaces in hospital kitchens or canteens *(10, 39, 96),* testing sinks, cutting boards *(60),* meat cutters, and knives for biological matter, especially where pathogen control was critical. Starting in the early 2000s, several initiatives were launched in the United Kingdom to combat nosocomial or hospital-acquired infections within the National Health System, which saw the introduction over the next decade of ATP bioluminescence hygiene testing of surgical equipment, intensive care areas, surgical operating theaters, nurse stations, and patient areas, especially high-touch objects such as bed controls, phones, call buttons, light switches, intravenous (i.v.) poles, doorknobs, and other machine buttons for microbial contamination or contamination from bodily fluids and other biological soils *(10, 29, 39)*. However, although some surfaces tend to be hygienically designed, many surfaces in either food manufacturing or hospital settings are often porous or otherwise improperly designed with regard to sanitation *(98)*. In addition, because immediate recontamination is all but guaranteed in most food manufacturing and medical areas due to constant use, ATP bioluminescence is often used more as an indicator of effective cleaning and sanitization or to validate new cleaning methods *(65)*.

CURRENT KNOWLEDGE OF ADENYLATE METABOLISM

The underlying basis of the ATP-based bioluminescence assay is rooted in the understanding that all living organisms generate and maintain a pool of ATP, which in general is used as an energy-imparting molecule for a host of metabolic reactions *(9, 26, 67)*. It is acknowledged that there are exceptions, such as bacterial spores that have shown to have little to no ATP levels (approximately 10^{-21} mol ATP per spore versus 10^{-17} mol ATP per bacterium *(58, 120)*) and thus may not display sufficient ATP for ATP-based hygiene assays *(97)*. However, studies by NASA scientists on the degradation of both intracellular and extracellular ATP on the surface of spacecraft have contradicted the typically accepted idea of immediate degradation of ATP at time of cell death and indicated that free ATP from lysed cells may remain present longer than previously thought *(94, 120)*. The ability of some residual ATP to remain present, as well as trends demonstrating that most ATP decays into ADP and AMP, are supported by subsequent work on adenylate homologue profiles over the course of significant metabolic changes in various organisms *(105, 106)*.

A basis of ATP bioluminescence carries assumptions of reasonably high and stable ATP concentrations from a cell, tissue, or biological material so as to allow a correlation between the ATP concentration levels and the bioburden on a particular surface. Although some studies have demonstrated that the concentrations of ATP within a given organism are reasonably constant under a given condition *(42, 111, 112),* different bacterial species have been shown to have as much as a 30-fold difference among ATP concentrations per cell of bacterial species, ranging from 0.28 to 8.90 μg of ATP per bacterial cell *(27),* with differences in cellular mass serving as a significant source of variation. ATP concentrations in yeast and mammalian cells are around 100-fold higher than in bacterial cells *(99)*. On the average, there are 10^{-15} g of ATP per bacterial cell compared with 10^{-12} g of ATP per yeast or mammalian cell *(112)* or, as reported in moles, an average of 10^{-18} mol of ATP per bacterial cell and 10^{-15} mol of ATP per mammalian cell *(67)*. It has also been shown that the age of the cells, stage in the growth cycle *(61, 106),* nutritional status, oxygen levels *(72),* pH *(6, 44, 104),* temperature *(80),* and even specific organism can influence ATP content *(9, 44, 104, 111, 131)* through ATP synthesis, utilization, or decay.

LIMITATIONS OF ATP-BASED ASSAYS

Although ATP bioluminescence has many uses and benefits, it is important to recognize and consider the limitations of current ATP-based methods. One of the major limitations is the inability to directly compare RLU values or results from different methods or ATP test systems. Brands of luminometers can read differently when compared, even differing sometimes from model to model of same manufacturer *(5, 79)*. RLUs cannot be used in direct cross-comparison; instead, the luminometer values must be converted back to ATP concentration from a standard curve or some other internal comparison. Even then, comparisons of different luminometers *(5, 86, 95, 127, 128)* have been shown to have differing minimum detection limits, differing levels of quenching or enhancement effects from disinfectants, or differing abilities to detect ATP from various environments. Furthermore, various surfaces swab differently; smooth surfaces may be more easily swabbed than rough ones *(19),* and various surfaces can be difficult to accurately swab because of surface porosity *(88)* or accessibility. Differing brands of luminometers have variations in swab tips or swabbing methods, which could also contribute to these brand-to-brand effects *(95)*. Collectively, these variables place a responsibility on the user to carefully select the best luminometer for the application.

Although ATP bioluminescence methods are valuable tools for real-time detection, bioluminescence methods are generally less suited to applications in which an especially

low bioburden is present or spore contamination is the specific target for assessment. Furthermore, ATP-based assays cannot differentiate ATP-generating microorganisms from organic debris containing ATP and thus should not be considered replacements for routine, microbiological testing as a means of verification. Similarly, RLUs do not correspond directly with CFUs, although the trends are similar (i.e., high CFUs usually have high RLUs, but not necessarily vice versa).

Although many of these problems are minimized or eliminated with proper internal controls, most assay methods do not seem to routinely incorporate sufficient internal controls or when developing or adjusting testing methods, including controls to verify upper and lower limits of the luminometer for each protocol, which are necessary for proper use. This partly results from the current lack of a generally accepted standard for internal controls, because the bioluminescence reaction of a luminometer does not process and detect purified stock ATP applied directly onto a swab in the same manner as free ATP found on a swabbed surface or even ATP from bacteria swabbed from a surface. It has been shown that there is some difference in certain studies between detection of gram-positive and gram-negative bacteria, theoretically because of difficultly lysing of the cell in the reaction *(118)*. One recommendation established upper limits based on swabs before cleaning and sanitation and established lower limits from swabs after proper cleaning and sanitation *(48)* for every tested environment, with the additional recommendation of completion of 10 consecutive cleaning and sanitation cycles to establish an obtainable lower limit *(50)*.

Lastly, specific chemicals, sanitizers, disinfectants, or even residual chemicals found in cleaning cloths can quench results or create errors if residual chemicals come in contact with the proprietary ATP test reagents. Chemicals shown to exhibit this quenching effect include trisodium phosphate, lactic acid, hydrogen peroxide, triclosan, and sodium hypochlorite *(22, 38, 59, 119)*. Although beyond the scope of this article, additional factors within luminometer design, such as system software or selection of photodiode versus photomultiplier tubes, may manifest variations in performance parameters including test sensitivity, initial and maintenance costs, and unit longevity.

BENCHMARKS IN ATP BIOLUMINESCENCE

Another major limiting factor of ATP-based assays is the lack of a recognized standard of cleanliness, both in method of testing and in standards for pass-or-fail indicators. Although it seems to be generally accepted that an area 10 by 10 cm (or 100 cm^2) is appropriate for swabbing *(18, 32),* other test sizes are often seen (Table 1), especially with irregularly shaped objects, smaller buttons, and touchpads.

A benchmark of what is considered clean varies with the industry and instrument and can range anywhere from $<$100 to $<$500 RLU (Table 1). The food industry seems to generally adhere to more stringent levels of cleanliness, partly because surfaces can be more contained when not in use or even during use, whereas the medical industry tends to accept higher pass levels, with the exception of surgical equipment. ATP testing methods for medical settings proposed by Griffith et al. *(39),* seem to be the most cited reference standard with a target level of cleanliness of $<$500 RLU, although these recommendations were later modified to a target level of $<$250 RLU with the proviso that this target RLU was specifically for the luminometer used in the study *(65)*. This RLU-based circumstance was noted by Boyce et al. *(18)* when they observed lower median RLU values than those previously reported *(39, 65)* using the same brand of luminometer. For all these reasons, the establishment of a user-based, validated protocol for establishing a pass-or-fail benchmark may be more appropriate than having a universal benchmark RLU benchmark.

In addition, differing assay protocols can potentially minimize or maximize the results, leading Ho et al. *(49)* to recommend the use of RLU per square centimeter as the standard unit, similar to units used in microbial swabbing. As they discussed *(49),* and as shown more fully in Table 1, a lower RLU benchmark will seem more stringent, but differing the surface area tested can make a higher RLU benchmark more stringent (i.e., 500 RLU from 100 cm^2 is 5 RLU/cm^2 versus 100 RLU from 10 cm^2 is 10 RLU/cm^2). Accordingly, common brands of luminometers have different settings for pass or fail and even have various settings for a machine for different methods or testing conditions. Recommended pass or clean RLUs given for common brands of luminometers are as follows: The Clean-Trace/Bio trace (3M, St. Paul, MN) recommends cleanliness with $\leq$250 RLU for environmental surfaces with 100-cm^2 test areas *(1),* with $\leq$200 RLU for surgical endoscopes (before final sterilization) *(2),* and with $\leq$150 RLU for surgical equipment *(3)*. The AccuPoint Advanced ATP Reader (Neogen, Lansing, MI) also suggests a test area of 10 by 10 cm with preprogrammed default threshold settings of $\leq$149 RLU as pass or clean and $\geq$300 RLU as fail *(84)*. The Lumitester PD-30 (Kikkoman, Tokyo, Japan) has three preprogrammed modes with pass-or-fail limits set as pass or clean with RLU $\leq$ 1,500, 500, and 200 and corresponding failure with RLU $>$ 3,000, 1,000, and 400 *(57)*. The SystemSURE Plus (Hygiena, Camarillo, CA) defaults to 10 and 30 RLU for pass and fail thresholds, respectively *(54)*.

ALTERNATIVE ADENYLATE ASSAYS

Although the sensitivity of the traditional ATP-based bioluminescence assays is generally accepted for many assessment applications, greater sensitivity or additional consideration may be advised, including the need to detect low microbial contamination levels or testing for the presence of especially immunogenic ATP-containing allergens *(124)*. To that end, various alternative methods of adenylate testing have been proposed. Cumulative adenylate levels (total adenylate [AXP]) tend to undergo less of an overall concentration change in comparison to ATP concentrations alone as dictated by growth or environmental changes *(113)*; such changes would most likely influence the accuracy and precision of bioluminescence testing.

Squirrell and Murphy *(109, 110)* proposed the use of the intracellular enzyme adenylate kinase as a bacterial cell marker in place of ATP, as depicted in Figure 1. Using ADP

TABLE 1. *Literature survey of basic methods used for surface ATP bioluminescence testing in various industries*

Year	Source	Area swabbed	Benchmark for cleanliness used	Benchmark per area	Luminometer[a]	Surface type
1992	Bautista et al. *(13)*	10 cm^2	<50 RLU	5 RLU/cm^2	Biotrace HMK/100 M	Milk processing: tanker truck, silo, shelves, milk crate
1993	Poulis et al. *(89)*	100 cm^2 (10 × 10 cm)	Not listed (comparison)	NA	Lumac M 1500 P	Snack production, chicken slaughter line, turkey processing operation, butchery, butchery linked to meat processing: stainless steel, Teflon, tiles, plastic conveyor belts
1994	Bell et al. *(15)*	100 cm^2 (10 × 10 cm) (adapted from *(23)*)	<50 RLU	0.5 RLU/cm^2	Biotrace	Milk tanker (inside of vessel, lid, air elimination vessel [AEV], hose), rinse water residue
			<500 RLU	<5 RLU/cm^2	Sonoco	
1994	Seeger and Griffiths *(96)*	25 cm^2	<24 RLU (<3× control reading)	~1 RLU/cm^2	Biotrace M3	Hospitals and nursing homes: Meat slicer feeder tray, slicer blade
1998	Murphy et al. *(82)*	50 cm^2	<100 RLU	2 RLU/cm^2	Inspector Hygiene Monitoring System Lumac Hygiene Monitoring QM	Milk factory: pipeline gaskets, pipe fittings, plug valves, filler valves, tanks, hand-washed items
1999	Davidson et al. *(32)*	100 cm^2	NA	NA	Clean-Trace (Biotrace)	Food-grade stainless steel (inoculated)
1999	Tebbutt *(116)*	100 cm^2	≤400 RLU	4 RLU/cm^2	Uni-lite (Biotrace)	Hotel kitchens: cutting boards from used for raw fish, meat, or ready-to-eat foods
2000	Griffith et al. *(39)*	Not listed	<500 RLU	NA	Clean-Trace (Biotrace)	Operating room, surgical ward, kitchen, toilet areas
2001	Oulahal-Lagsir et al. *(88)*	10 cm^2	Not listed (comparison)	NA	Checkmate (Celsis Lumac)	Dairy industry: testing swabbing versus sonication to remove biofilms from fouled stainless and polypropylene sheets
2001	Miettinen et al. *(77)*	100 cm^2	≤1 RLU	0.01 RLU/cm^2	1253 Luminotetri (BioOrbit)	Fish processing factory surfaces: knives, cutting boards, conveyor belt, scales, brine basins, control panels, doors, door handles, aprons
2002	Moore and Griffith *(78)*	100 cm^2	<500 RLU	5 RLU/cm^2	Uni-lite (Biotrace)	Food contact surfaces from 4 food production locations: frozen ready production plant, bakery, cheese manufacturer, cook meat processor
2003	Malik et al. *(71)*	100 cm^2 (cited in *(32)*)	<500 RLU	5 RLU/cm^2	Clean-Trace (Biotrace)	Kitchen, bath, sluice room, treatment room, patient rooms
2005	Obee et al. *(85)*	100 cm^2 (where possible) 30 cm^2 for distal tip of endoscope	<500 RLU	5 RLU/cm^2	Uni-lite (Biotrace)	8 locations important to the success of the decontamination of endoscopes
		Liquid samples	<500 RLU	NA	Aqua-Trace Uni-lite (Biotrace)	Rinse water from endoscope channels after disinfection
2004	Hansen et al. *(46)*	Not listed	<30–<100 RLU	NA	Luminester PD-10 (Kikkoman)	Flexible endoscopes (gastroscopes, colposcopes, duodenoscopes, bronchoscopes)
2006	Aycicek et al. *(10)*	10 cm^2 (2 × 5 cm)	Not listed	NA	Luminester PD-10 (Kikkoman)	Hospital kitchen surfaces: steel, polyethylene plastic, wooden and marble workbenches, meat grinder, mash machine, polyethylene plastic cutting boards, knives, gastronome basin, tap head, oven handle

TABLE 1. *Continued*

Year	Source	Area swabbed	Benchmark for cleanliness used	Benchmark per area	Luminometer[a]	Surface type
2007	Cooper et al. *(29)*	Not listed	<500 RLU	NA	Clean-Trace (Biotrace)	Mostly stainless and laminate plastic (in patient rooms, sluice room, kitchen, bathroom)
2007	Griffith et al. *(41)*	100 cm^2	<500 RLU	5 RLU/cm^2	Clean-Trace Uni-lite (Biotrace)	Floors, toilet handle, sluice handle, phone, trolleys, bed rail
2007	Tebbutt et al. *(117)*	100 cm^2 (10 × 10 cm)	No specific limit applied	NA	Uni-lite NG (Biotrace)	Food service areas, including cutting boards, plastic storage containers, basin faucets, refrigerator door handles, microwave controls, lids of bins
2007	Willis et al. *(130)*	10 cm^2	<100 RLU	10 RLU/cm^2	SystemSURE II (Hygeina)	Toilet seat, patient equipment, clinical workstations
			<300 RLU	30 RLU/cm^2		Hospital floors
2008	Lewis et al. *(65)*	100 cm^2 or entire handle	<250 RLU	2.5 RLU/cm^2	Clean-Trace Uni-lite NG (Biotrace)	Toilets, patient rooms, trolley, tap handle
2008	Vilar et al. *(122)*	1 cm^2	<152 RLU	152 RLU/cm^2	Hy-LITE (Merck)	Milking equipment: teat cup rubbers
			<242 RLU	242 RLU/cm^2		Milking equipment: teat dip containers
			<282 RLU	282 RLU/cm^2		Milking equipment: milk receivers
			<1,821 RLU	1,821 RLU/cm^2		Milking equipment: pipeline joints
2008	Whitehead et al. *(126)*	16 cm^2 (4 × 4 cm)	<10 RLU	0.625 RLU/cm^2	Hygiena (Herts)	Soiled stainless steel from meat, fish, dairy
2009	Boyce et al. *(18)*	Not listed	<250 RLU	NA	Clean-Trace	High-touch surfaces, including bed rails, bed tables, TV remote, toilet seat, toilet grab bar
2009	Sherlock et al. *(101)*	100 cm^2	<500 RLU	5 RLU/cm^2	Clean-Trace Uni-lite NG (Biotrace)	High-touch surfaces, including bed tables, door handles, nurse desk, toilet floor
2010	Boyce et al. *(20)*	Did not use specific size template	<250 RLU	NA	Clean-Trace (3M)	High-touch surfaces in hospital, including bed side rails, overbed tables, TV remote, bathroom grab bar, toilet seats
2010	Moore et al. *(79)*	Not listed	<250 RLU	NA	Clean-Trace NG (Biotrace)	Bed rails, storage trolleys, chart tables, patient equipment in intensive care unit (ICU)
2010	Turner et al. *(118)*	NA	Comparison	NA	novaLUM (Charm)	None—various cultures (prokaryotic, eukaryotic) or contaminants (feces, urine) directly onto swab
2010	Wang et al. *(124)*	100 cm^2 (10 × 10 cm)	Not listed	NA	Biotrace	Chicken processing plant: conveyor belt
2011	Aiken et al. *(5)*	NA	Not listed (compared with CFU)	NA	BioProbe Clean-Trace Junior Lumat	None—bacterial cultures directly onto swab
2011	Anderson et al. *(8)*	100 cm^2 (10 × 10 cm)	<100 RLU	1 RLU/cm^2	SystemSure Plus (Hygiena)	40 high-touch areas in hospital ward
2011	Boyce et al. *(19)*	10.16 cm^2 (4 in^2)	<250 RLU	24.6 RLU/cm^2	Clean-Trace	High-touch surfaces in hospital
2011	Cunningham et al. *(31)*	100 cm^2	<200 RLU	2 RLU/cm^2	Clean-Trace NG	Areas in food service kitchen: dining tables, milk dispenser, slicers, plates, food prep counter, knives
			<1,000 RLU	10 RLU/cm^2		Food service kitchen: cutting boards, bathroom door handle

TABLE 1. *Continued*

Year	Source	Area swabbed	Benchmark for cleanliness used	Benchmark per area	Luminometer[a]	Surface type
2011	Ferreira et al. *(35)*	None listed	<500 RLU	NA	Clean-Trace (3M)	ICU ward of hospital: bed rails, crank, bedside table, buttons of infusion pump
2011	Mulvey et al. *(81)*	2 × 5 cm	<100 RLU	10 RLU/cm^2	Hygiena	Areas in medical and surgical wards: locker, bedframe, bedtable, floor, bedside curtain, patient notes, computer keyboard, nurses desk, toilet door pushplate
2012	Bellamy *(16)*	Not listed	<100 RLU	NA	System Sure Plus (Hygiena)	High-touch surfaces in hospital ward, including bed rails, mattress, tables, nurse call bells, phone, computer, blood pressure equipment, chair arms, computer key boards, sluice door handle, bathroom door handle, toilet, toilet flush handle, toilet roll holder
2012	Carrascosa et al. *(24)*	20 cm^2	≤150 RLU	7.5 RLU/cm^2	Uni-Lite EXCEL (Biotrace)	Stainless steel or polyethylene surfaces at cheesemaking factories: curd vats, fillers, cheese molds, table, milk reception tanks
2012	Sciortino and Giles *(95)*	11.2 cm^2 (2 × 5.6 cm)	NA	NA	Clean-Trace (3M) System Sure II (Hygiena) NovaLUM (Charm) SURE II (Hygiena)	Stainless steel plenums (inoculated)
2013	Alfa et al. *(7)*	0.122 mL	<200 RLU	NA	Clean-Trace water kit (3M)	Liquid samples taken from duodenoscope channels
2013	Snyder et al. *(107)*	25.81 cm^2 (2 × 2 in)	<250 RLU	9.7 RLU/cm^2	Clean-Trace (3M)	High-touch surfaces in hospital: bed rail, tray table, call button, bedside phone, bedside table, chair, room sink, room light switch, bathroom handrail, toilet handle, toilet seat, bathroom sink, bedpan cleaner
2014	Branch-Elliman et al. *(21)*	Not listed	<300 RLU	NA	Clean-Trace NG (3M)	Hospital high-touch surfaces (bedside rails, tops of overbed tables, toilet seats) and low-touch surfaces (bottoms of overbed tables)
2014	Choi et al. *(28)*	100 cm^2 (10 × 10 cm)	<500 RLU	5 RLU/cm^2	Not listed	Restaurant menus (before and after cleaning)
2014	Edmiston et al. *(34)*	Not listed	≤45 RLU	NA	Not listed	5 surfaces in hospital operating rooms
2014	Gibbs et al. *(36)*	100 cm^2 (exceptions noted at 50–112 cm^2)	Not listed (compared with CFU)	NA	Clean-Trace NGi (3M)	Inoculated hospital-related surfaces
2013	Neal *(83)*	100 cm^2 (10 × 10 cm)	<200 RLU	2 RLU/cm^2	SystemSURE plus (Hygiena)	Deli meat slicers
2014	Omidbakhsh et al. *(86)*	NA	Not listed (compared with CFU)	NA	Clean-Trace NG (3M) EnSURE Hygiene (Hygiena) Lumitester PD-20 (Kikkoman) novaLUM (Charm)	None—bacterial cultures directly onto swab

TABLE 1. *Continued*

Year	Source	Area swabbed	Benchmark for cleanliness used	Benchmark per area	Luminometer[a]	Surface type
2014	Osimani et al. *(87)*	100 cm^2	<150 RLU/100 cm^2	1.5 RLU/cm^2	Clean-Trace NG (3M)	Canteen surfaces made of stainless steel, nylon, polyvinyl chloride (PVC), including prep tables, knives, slicing machine
			<100 RLU/100 cm^2	1 RLU/cm^2		Vegetable washer and canteen tables
			<400 RLU/100 cm^2	4 RLU/cm^2		PVC cutting boards
2014	Watanabe et al. *(125)*	100 cm^2	100 RLU/100 cm^2	1 RLU/cm^2	Clean-Trace (3M)	Hospital areas in internal medicine, surgical, obstetrics, and gynecology wards, including nurse stations, nurse wagon, nurse station doorknob, corridor guard rail, entrance floor, inpatient lockers, bed table, windowsill
2014	Zambrano et al. *(132)*	Not listed	<3 RLU	NA	Lightning MVP (Arquimed)	Surfaces in hospital patient units and operating rooms
2015	Chan et al. *(25)*	100 cm^2	≤250 RLU	2.5 RLU/cm^2	Clean-Trace Ngi (3M)	Cardiology ICU and medical ICU areas: bed rails, bed tables, vital monitors or electronic devices near bed, i.v. injection sets, nursing care cart handles
2015	Hammons et al. *(45)*	100 cm^2	Not listed (compared with ACC of Listeria)	NA	AccuPoint2 (Neogen)	Deli surfaces, including cold room drain, deli drain, deli case handle, slicer knob, squeegee, cutting board, sink interior, scale, cold room handle, deli case
2015	Huang et al. *(53)*	100 cm^2 (or actual size for smaller objects)	<127 RLU/cm^2 (detection limit)	1.27 RLU/cm^2	Uni-Lite NG (3M)	High-touch surfaces in hospital, including doorknobs, light switches, windowsills, bed rails, bedside cabinets, couches, toilet seats, toilet hand rails, refrigerators, kettles, closet handles, blood pressure cuffs, working cart tables, bed control buttons, vacuum switches for sputum suction
2015	Kajigaya et al. *(55)*	12.25 cm^2 (3.5 × 3.5 cm)	Not listed (compared with CFU and between data)		Lumitester PD-20 (Kikkoman)	Doorknobs throughout hospital, including staff bathrooms, staff breakrooms, linen closets, dirty utility rooms, newborn care unit, clinic bathrooms, clinic exam rooms
2015	Lewis et al. *(64)*	2 cm^2	≤45 RLU	22.5 cm^2	Getinge SafeStep (Getinge)	Surfaces from 4 operating rooms
2015	Shimoda et al. *(102)*	10 × 10 cm^2	<250 RLU	2.5 RLU/cm^2	Clean-Trace (3M)	Tested materials commonly found in hospitals, including vinyl chloride, stainless steel, wood, and materials coated in acrylonitrile-butadiene styrene resin
2015	Whiteley et al. *(128)*	NA	Not listed (compared with ATP standards)	NA	Clean-Trace (3M) Lucipac-Pen (Kikkoman) Pocketswab Plus (Charm) Ultrasnap (Hygiena)	Direct application to swab of known ATP concentrations and bacterial cultures

TABLE 1. *Continued*

Year	Source	Area swabbed	Benchmark for cleanliness used	Benchmark per area	Luminometer[a]	Surface type
2017	Bilici et al. *(17)*	100 cm^2 (10 × 10 cm)	<200 RLU	2 RLU/cm^2	Lumitester PD-20 (Kikkoman)	Laminated restaurant menus
2016	Ho et al. *(49)*	25 cm^2 (5 × 5 cm) or actual size for smaller surfaces	7.34 RLU/cm^2 (also used 5 RLU/cm^2, 10 RLU/cm^2, <250 RLU, <500 RLU for comparison)	7.34 RLU/cm^2	Clean-Trace (3M)	Surfaces in patient rooms, including bedside rail, bedside table, chair, doorknob, drawer handle, emergency button, light switch, hand sanitizer pump, toilet flush handle, toilet safety rail, wardrobe handle
2016	Villanueva and Guanche *(123)*	100 cm^2	<200 RLU (ICU, OR) <250 RLU (ER, inpatient areas)	2 RLU/cm^2 2.5 RLU/cm^2	Clean-Trace NGI (3M)	Phone, touch screens, keyboards, oximeter power, call button, suction regulator, patient bed rail, mattress, bedside table handle, light switch, control panels, door handle, entrance door plate, room inner door (knob and plate), light switch and sink, lamps, patient care cart, incubator alarm button, tray table, i.v. control button
2017	Machado and Cutter *(69)*	100 cm^2 (10 × 10 cm)	Not listed (comparison before and after training)	NA	Clean-Trace (3M)	Surfaces in dairy farm cheesemaking rooms, including floors, drains, hoses, cheese hoops, doorknobs, vats, mixing utensils, cheesemaking table
2017	Richard and Bowen *(90)*	Not listed	<500 RLU (also compared with <400, <250 RLU benchmarks)	NA	Clean-Trace NGi (3M)	Orthopedic surgery operating rooms, including prep table, light handles, machine buttons, supply closet countertops, hose inside, operating room table headboard, socket attachment, patient positioners, computer keyboard
2017	Viator et al. *(121)*	~100 cm^2 (4 × 4 in)	<150 RLU	1.5 RLU/cm^2	AccuPoint Advanced (Neogen)	Stainless steel coupons inoculated with bacterial or yeast cultures or residues of various types of food
2018	Lindell et al. *(66)*	16.5–80 cm^2 (depending on equipment size)	<300 RLU	3.75–18.2 RLU/cm^2	Clean-Trace (3M)	Aging rubber materials (teat cup liners, tubes) from milking equipment
2018	Salsgiver et al. *(93)*	103.2 cm^2 (16 in^2)	<200 RLU	1.9 RLU/cm^2	Clean-Trace (3M)	High-touch surfaces in a hospital, including overbed tables, mobile workstations, visitor chairs, toilet seats, nursing station countertops, glucometers
2018	Whiteley et al. *(129)*	10 cm^2 (2 × 5 cm)	≤100 RLU	10 RLU/cm^2	Hygiena	Food preparation surfaces: cutlery, tongs, plates, cups, jugs, bowls, pots, pans, cutting boards, machinery, benches, shelves, handles, taps, soft materials, touchpads

[a] Some luminometers were listed specifically by model, and some were listed by the luminometry system. If available, the specific luminometer model was listed. Otherwise, the information is included as provided by the methods in the manuscript. Pass or fail by area was calculated from information provided in the method section of the cited articles (because most studies did not specify the RLU per square centimeter limit).

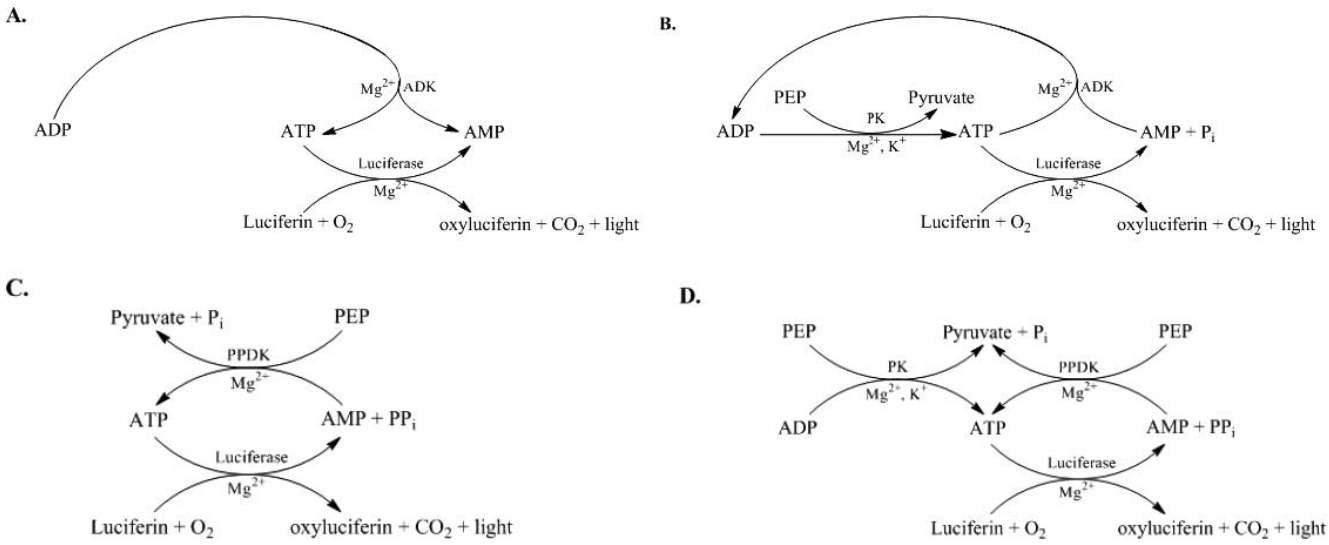

FIGURE 1. *Alternate adenylate assays. (A) Squirrell and Murphy (109) used adenylate kinase (ADK), along with ADP as the substrate to drive the reaction to ATP. (B) That same year, Hawronskyj et al. (47) proposed an ATP recycling system that in theory would amplify all AMP into ATP and allow detection of low levels of microbes using both ADK and pyruvate kinase (PK). (C) Sakakibara et al. (91) used pyruvate orthophosphate dikinase (PPDK) in an alternative ATP recycling method. (D) Bakke et al. (12) used a combination of previous methods to obtain an updated ATP recycling system that could measure AXP. For each panel, coefficients involving stoichiometry have been removed for simplicity.*

as the substrate to drive the reaction in the direction of ATP synthesis, the standard firefly luciferase-luciferin reaction was then used to detect the ATP generated from adenylate kinase activity. In theory, this would make 40 to 50 times as much ATP available for bioluminescence. Further experimentation demonstrated a lower limit detection of 10^1 to 10^2 cells versus 10^4 cells with standard methodology *(109, 110)*. In 2000, Corbitt et al. *(30)* compared the Squirrell and Murphy method with standard ATP bioluminescence and found that adenylate kinase bioluminescence was more sensitive than traditional ATP bioluminescence for certain foods, especially meat tissues because of the high levels of adenylate kinase in muscle tissue. However, no advantage in sensitivity was seen in testing of pure bacterial cultures with the Squirrell and Murphy adenylate kinase bioluminescence method over standard ATP bioluminescence *(30)*.

In 1994, Hawronskyj et al. *(47, 48)* used an ATP recycling system to amplify all AMP into ATP with the use of adenylate kinase and pyruvate kinase (Fig. 1), where the ATP concentration was the rate-limiting factor for adenylate kinase. This system used updated bioluminescence technology but was related to methods proposed in prior years *(52, 56, 68, 108)*. In 1978, Holm-Hansen and Karl *(52)* used the luciferase-luciferin reaction to determine ATP concentrations, using adenylate kinase, pyruvate kinase, and phosphoenolpyruvate (PEP) to measure AXP levels. ATP+ADP levels were determined with just pyruvate kinase and PEP, and AMP levels were calculated from calculations of the differences *(52)*. In 1986, Lundin et al. *(68)* measured AXP levels in a single aliquot using adenylate kinase, pyruvate kinase, and PEP to convert all AMP and ADP into ATP and measured with luciferase reaction, although cytidine triphosphate was also used to help the reaction to an endpoint.

More recently, another ATP recycling method was proposed that used ATP+AMP monitoring in which the AMP and pyrophosphate produced from ATP by firefly luciferase were converted back into ATP by pyruvate orthophosphate dikinase (PPDK) *(91, 92)* (see Fig. 1). The signal strength was ATP dependent, but the system allowed long-lived RLU signals even with low numbers of swabbed ATP molecules. Later, Bakke and Suzuki *(11)* suggested a combination of this PPDK recycling method and pyruvate kinase recycling to allow measurement of AXP, eliminating changes from ATP degradation by measuring the AXP pool. This difference was seen recently in studies that used this method to test ATP, AMP, ADP, and AXP levels of animal carcasses postslaughter *(106),* test bacterial growth over extended periods *(106),* and assess the hygienic state in health care settings *(12)*.

Although rapid in conduct and considered more objective in application than previous methods of hygiene assessment, ATP-based assessments are relied on as a routine method of choice. Under the ever-increasing regulatory need to use validated methods for analyses affecting health and safety, several technologies considered in this report have obtained certification under AOAC International's Performance Tested Methods Program. Yet current ATP methods remain indirect methods of total hygiene assessment and have limitations that must be understood if such methods are to be applied judiciously. In our attempt to survey current methods of ATP-based bioluminescence assays and the limitations of such methods, it has become readily apparent that the benefits of these ATP-based assays may not be yet fully realized or fully refined as a technology. A better use of positive and negative controls, standardization of units compared with areas swabbed, calibration with ATP standards, and more concrete definitions of what is clean in various industries or

medical settings would allow better utilization of the full advantages of these ATP-based bioluminescence assays, including increased sensitivity and lower rates of false-negative outcomes. In addition, to accurately apply ATP-based technologies, an understanding of the reality of ATP variations over time and environment is necessary. Alternative or refined methods of ATP-based assays may advance ATP-based technologies and improve rapid surface hygiene assessments.

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