APPENDIX A6.1.3.20 URINE MUTAGENICITY- FACTSHEET

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Principle of the bacterial reverse mutation test (Ames assay)

Urinary mutagenicity can be considered as a biomarker of exposure, as the mutagen load of urine samples indicates exposure to mutagenic compounds. It is, however, not specific to one specific mutagenic compound, but reflects the exposure to all mutagenic compounds that have been absorbed and excreted in urine. Such activity can best be tested using the Ames test [1].

The principle of the test is well defined by the Organisation for Economic Co-operation and Development (OECD) guideline for testing of chemicals no 471 (adopted in 1997): "*The bacterial reverse mutation test uses amino acid-requiring strains of Salmonella typhimurium and Escherichia coli to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs […]. The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain*." [2].

Different tester strains of bacteria can be used, some of which are more responsive than other to specific mutagens. This test is commonly used as an initial screen for genotoxic activity, particularly for point-mutation inducing activity. One of the limitation of this assay is that compounds with bactericidal properties, or compounds that interfere specifically with the mammalian cell replication system might not appear as positive [2]. Also, even though many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute [2]. Finally, because prokaryotic cells differ from mammalian cells, the Ames test generally requires an exogenous source of metabolic activation, which often consists of a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents previously treated with enzyme-inducing agents such as Aroclor 1254 [2].

Several criteria need to be considered to determine whether a result is positive or not, such as a concentration-dependent increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system [3].

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity.

Ames assay applied to tobacco smoke

After the publication of the Ames method [1], the mutagenicity of tobacco smoke condensate was evaluated [4], and the assay proved to be sensitive for the detection of mutagenic activity of the total particulate matter phases of the tobacco smoke [5, 6]. Tester strain TA98 in presence of an S9 metabolic activation system is particularly sensitive to the cigarette smoke mutagenicity [7]. Proteins and amino acids are known precursors in tobacco leaves for a number of toxicants in smoke, including aromatic amines, such as 1-NA, 2-NA, 4-ABP or *o*-toluidine [8] that contribute to mutagenicity. Polycyclic aromatic hydrocarbons, such as benzo[a]pyrene are also well described mutagens [9], The mutagenicity of cigarette smoke condensate can be influenced by

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several factors, such as tobacco type, stalk position, age, sugar and nitrogen content of tobacco leaves [10]. Mutagenicity may also be affected by the "draw resistance', filters, and other physical characteristics [11]. Finally, the effect of pyrolysis temperature has been shown to greatly affect the mutagenicity of tobacco smoke condensate, with lower temperatures producing much less mutagens than higher temperatures [12].

The Ames assay has largely been used to characterize tobacco smoke of conventional cigarettes, and is now also used for the assessment of candidate modified risk tobacco products [8, 13]. The assay went through many protocol adaptations through the years, making it more sensitive to certain mutagens, including those of cigarette smoke. For example, the Ames test has been adapted to assess the genotoxicity of the gas/vapor phase of tobacco smoke by carefully characterizing the tester strains and using an efficient exposure system [14].

Because cigarette smoke is a very complex matrix, with more than 7000 identified compounds [15], the Ames results remain, however, difficult to interpret, as different mutagens will contribute differently to the overall mutagenicity. It was described, for example, that the heterocyclic aromatic amines found in cigarette smoke condensate and mainstream smoke (twenty of which have been identified as potent mutagens by the Ames assay) can contribute from as little as 2% to as much as 15% of the observed mutagenicity [16]. It is not even clear whether the specific mutagenic activity of the individual compounds is preserved in the complex chemical mixture produced in the cigarette smoke, and thus potential synergistic and/or antagonistic effects that may occur in the complex mixtures cannot be accounted for by this assay [16].

Ames assay applied to urine

The Ames assay has since its publication been adapted to be used as non-invasive way of evaluating human exposure to mutagens, including tobacco smoke [17]. Typically, the Salmonella strain YG1024, an O-acetyltransferase-overproducing derivative of TA98, has been shown to be more sensitive to the presence of mutagens in urine than the TA98, TA100, and TA1537 (with S9 activation) strains recommended by OECD [18-20]. Because only small amounts of liquids can be added to the test system, urine samples are concentrated, usually through adsorption by a non-polar resin [17]. By concentrating these samples, mutagenic activity can be detected even at relatively low levels [17]. *S. typhimurium* strain YG1024 can be used as a test system for mutagenicity, with metabolic activation with liver S9 fraction from Sprague Dawley rats treated with Aroclor. The method has been improved throughout the years to increase the sensitivity of the response of urine [21, 22].

Urinary mutagenicity studies have been used to assess environmental, dietary and occupational exposures; for example, methods of food preparation can significantly affect urinary mutagenicity [23].

The output of urinary mutagenicity testing is a combination between the exposure dose to the mutagenic agent and the metabolism of those toxicants within the exposed subject. The half-life of the smoking-related urine mutagenic activity is reported to be approximately 7–23h [24], but this will also depend on the half-life of the mutagens

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absorbed and metabolic interindividual differences. In the case of smoking cessation, the urine mutagenic activity should have reached non-smoker levels after 8 days [25]. However, because of the increase of mutagenic urine activity with an intake of fried or broiled meal, or other environmental factors, such activity is unlikely to become zero [23].

Levels of urine mutagenicity in smokers

It is known since the adaptation of the Ames assay for measurement of urine mutagenicity that urine of smokers has higher mutagenic activity than for non-smokers [17]. This was shown to be true even when both groups were maintained on a low-mutagenic, boiled food diet [26]. Aromatic amines and heterocyclic aromatic amines have particularly high activities in the Ames assay (*S. typhimurium* strain YG1024), so the results obtained from studies of smokers' urine may mainly reflect the concentrations of these compounds, but this remains speculative [27]. This assay has been commonly used to compare the mutagenicity of different cigarette brands, with different tar levels or with new flavors [6]. It is, for example, part of the tobacco reporting regulations issued by Health Canada [28] Urinary mutagenicity studies have also been used to compare cigarettes which burn or primarily heat tobacco [22, 29].

Studies have shown that urinary mutagenicity increases with the number of cigarettes smoked. Tuomisto et al. for example, showed that there is a dose-dependent response to the number of cigarettes smoked (0, 5, 10 or 20 cigarettes per day), but no difference between the smokers of low-tar and medium-tar cigarettes [30]. Furthermore, reductions in urine mutagenicity occurred in smokers who decreased their cigarette consumption per day, in a controlled manner, from 20 cigarettes per day to 20, 10, 5 or 0 (with values of 23'000, 17'300, 9'900, and 5'400 rev/24h, respectively, at the end of the study) [31]. Similarly to what had been demonstrated with tobacco smoke extracts, the type of tobacco also influences the urine mutagenicity. For example, after taking into account the level of daily tobacco consumption, it has been shown that smokers of black tobacco have a higher urinary genotoxicity than smokers of blond tobacco [32-34].

The level of urine mutagenicity was evaluated in a population without a known mutagenic exposure. The study showed that diet and indoor exposure to environmental tobacco smoke, fireplace and cooking fume exposure seemed to play a major role among the lifestyle behaviors investigated [35]. Females showed a mutagenicity level twice as high as men, possibly in consequence of their greater cooking fumes exposure. In a study comparing smokers and non-smokers over a week, the levels of urine mutagenicity in non-smokers was of about 7% of those in smokers [22]. In this study, the diet was considered representative of that consumed by a US family, except that broiled or pan-fried meat was not allowed.

The metabolism of several harmful and potentially harmful constituents has been shown to be affected by the genotype of enzymes involved in their degradation pathway, thus the urine mutagenic activity of carriers of certain variant alleles is also likely to be affected, as shown in a study on workers exposed to polycyclic aromatic hydrocarbons with different predicted microsomal epoxide hydrolase activities [36]. Because the output of the urinary Ames test is a combination between the exposure dose and the metabolism

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of this toxicant within the exposed subject, there is a high source of assay variation [37]. No apparent gender effect was observed, the mutagenicity of smokers' urine being generally similar between males and females, except when exposure to environmental sources, such as cooking fumes, occurs. Also, the pH of the urine samples does not seem to affect the mutagenic effect of urine [38], even though a study by Sarkar et al. showed that an acidic pH seems to increase the mutagenic activity of buffered solutions spiked with synthetic 4-NOHABP (4-N-hydroxyl aminobiphenyl), an intermediate metabolite of 4-aminobiphenyl (4-ABP), which can convert to a reactive electrophile, that in turn can form covalent adducts with urothelial DNA [39].

Despite multiple sources of variability, this urine mutagenicity ''biomarker'' has been claimed to be highly relevant because: (1) it reflects exposure to potential mutagens, (2) it goes beyond measurement of individual chemicals to capture an integrative toxicological response in an integrative biological matrix (24-hour urine), and (3) it follows a clear dose–response with the number of cigarettes per day [31]. On the other hand, the urinary mutagenic activity is the net effect of the exposure dose and the metabolic conversion taking place [37]. Furthermore, the assay is not based on chemical standards and its characteristics as bioassay (such as linearity, precision, accuracy, limits of detection, etc.) clearly differ from the methods used for more classical biomarkers of exposure. It provides a relative assessment of the mutagenicity of samples made in a direct comparison test and its use within a regulatory standard would be problematic, requiring reference cultures, standards and frequent inter-laboratory comparisons to ensure robust data [37].

Table 1 presents values of urine mutagenicity in smokers, and quitters, or smokers who switch from smoking conventional cigarette to potentially reduced risk products. Only the publications identified that report values with tester strain *S. typhimurium* strain YG1024 ± S9 metabolic activation in 24-hour urine collections (i.e., number of revertants per 24h) are reported. Urine of non-smokers, apparently non-exposed subjects, was assessed over a period of 4 weeks and ranged from 3706 to 7890 revertants per 24h (with strain YG1024 and S9 metabolic activation) [22]. In a controlled diet study, the number or revertants per 24h was shown to go from as low as 100 up to 9594, in nonsmoking subjects ingesting low or high mutagen diets, respectively (using the Salmonella typhimurium TA98 mammalian microsome assay and S9 metabolic activation). A diet rich in mutagenic compounds was composed of foods such as charcoal-broiled meat, toasted bread, smoked sausage and leafy vegetables which accumulate polycyclic aromatic hydrocarbons (PAH), while these were avoided in the diet poor in mutagens. Urinary mutagenicity determined with the YGI024 strain showed no statistically significant difference between nonsmokers exposed to environmental tobacco smoke (ETS) and nonsmokers with minimal or no ETS exposure at all.

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Table 1 Representative urine mutagenicity data (revertants/24h) in smokers and quitters (*S. typhimurium* strain YG1024 ± S9 metabolic activation)

Author (year)	Smokers	Levels in quitters (or "switchers")	Length of cessation (or switch)	Percent decrease
Sarkar (2010) [40]	32118	1249 [d]	mean	-97%
Tricker (2012) [41]	21231 ± 11424	5879 ± 2941 [a]	mean ± SD	-70% e
Tricker (2012) 376	36228 ± 16848	6131 ± 6121 [d]	mean ± SD	-81%
Tricker (2012) 625	17139 ± 6258	8420 ± 3681 [d]	mean ± SD	-45%
Tricker (2012) 555	85882 ± 73332	9913 ± 13211 [g]	mean ± SD	-86%
Theophilus (2015) [31]	34100 ± 14600	2700 ± 1700 [g]	mean ± SD	-92%
Frost-Pineda (2008) [42]	22200 ± 10300	5250 ± 4440 [d]	mean ± SD	-61% h
Roethig (2007) 371	Not reported	Not reported [d]	mean ± SD	-75% ± 32%

[c] LS means

[d] After 8 days of smoking cessation

[e] Square-root transformed data

[f] Non-smokers

[g] After 5 days of smoking cessation

[h] Square-root transformed median values

List of abbreviations

1-NA	1-aminonaphthalene
2-NA	2-aminonaphthalene
4-ABP	4-aminobiphenyl
ETS	Environmental tobacco smoke
LS	Least square
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbons
SD	Standard deviation

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