Validation of an *In Vitro* Mutagenicity Assay Based on Pulmonary Epithelial Cells from the Transgenic MutaMouse: Intra-Laboratory Variability and Metabolic Competence

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Abstract:

Genetic toxicity tests used for regulatory screening must be rigorously validated to ensure accuracy, reliability and relevance. Hence, prior to establishment of an internationally-accepted test guideline, a new assay must undergo multi-stage validation. An *in vitro* transgene mutagenicity assay based on an immortalized cell line derived from MutaMouse lung (i.e., FE1 cells) is currently undergoing formal validation. FE1 cells retain a *lacZ* transgene in a λgt10 shuttle vector that can be retrieved for scoring of chemically-induced mutations. This work contributes to validation of the *in vitro* transgene (*lacZ*) mutagenicity assay in MutaMouse FE1 cells. More specifically, the work includes an intra-laboratory variability study, and a follow-up study to assess the endogenous metabolic capacity of FE1 cells. The former is essential to determine assay reliability, the latter to define the range of chemicals that can be reliably screened without an exogenous metabolic activation mixture (i.e., rat liver S9). The intra-laboratory variability assessment revealed minimal variability; thus, assay reproducibility can be deemed acceptable. Assessment of metabolic capacity involved exposure of FE1 cells to 5 known mutagens, and subsequent assessment of changes in the expression of genes involved in xenobiotic metabolism; induced transgene mutant frequency (±S9) was assessed in parallel. The results revealed that the FE1 cell line is capable of mobilising several Phase I and Phase II gene products known to be involved in the bioactivation of mutagens. Collectively, the results presented support the contention that the FE1 cell mutagenicity assay can be deemed reliable and reproducible. Consequently, the assay is an excellent candidate for continued validation, and eventual establishment of an OECD (Organization for Economic Cooperation and Development) Test Guideline.

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List of Abbreviations:

1-HMP	1-Hydroxymethylpyrene
1-MP	1-Methylpyrene
2-AAF	2-Acetylaminofluorene
3R's	Reduction, refinement and replacement of animal use in research
7,12-DMBA	7,12-Dimethylbenz[a]anthracene
AA	Amino acid transferases
AFB1	Aflatoxin B1
AHH-1	Human B lymphoblastoid cell line
AhR	Aryl hydrocarbon receptor
Aldh	Aldehyde dehydrogenases
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATM	ATM serine/threonine kinase Signalling
ATP	Adenosine triphosphate
BaP	Benzo[*a*]pyrene
BER	Base Excision Repair
BMD	Benchmark Dose
BMDL	Benchmark Dose Lower 90% Confidence Limit
BMDU	Benchmark Dose Upper 90% Confidence Limit
BMR	Benchmark Response
BPDE	Benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide
CAR	Constitutive Androstane Receptor
cDNA	Complimentary DNA
CEPA	Canadian Environmental Protection Act 1999
CHL	Chinese hamster lung cells
CHO	Chinese hamster ovary cells
CMP	Chemicals Management Plan
CoQ	Ubiquinol-10
Cq	Quantification cycle
CV	Coefficient of variation
CYP	Cytochrome P450 isozyme
DDI	DNA damage inducing
Dh	Dehydrogenases
DHEW	United States Department of Health Education and Welfare
DMN	*N*-nitrosodimethylamine
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic acid
DSB	Double Strand Break repair
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ENU	*N*-ethyl-*N*-nitrosourea
Ep	Epoxidases
EPA	United States Environmental Protection Agency
Est	Esterases
EU	European Union
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FBS	Fetal bovine serum
FE1	Flat Epithelial Isolate #1
FISH	Fluorescent in situ hybridization
FMO	Flavin containing monooxygenases
G-6-P	Glucose-6-phosphate
galE	UDP-galactose epimerase
GFP	Green Fluorescence Protein
GST	Glutathione-*S*-transferases
GTTC	HSEI Genetic Toxicology Technical Committee
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human liver carcinoma cells
Hprt	Hypoxanthine-guanine phosphoribosyltransferase gene
hrs	Hours
HSEI	Health and Environmental Sciences Institute
HuLy	Primary human lymphocytes
IARC	International Agency of Research in Cancer
IARC	International Agency for Research on Cancer
ICATM	International Cooperation on Alternative Methods
ICCR	International Cooperation on Cosmetics Regulation
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IL-1	Interlukin-1
IWGT	International Workshop on Genotoxicity Testing
JaCVAM	The Japanese Center for the Validation of Alternative Methods
L5178Y	Mouse lymphoma cell line
lacZ	Bacterial gene (transgene in FE1 and MutaMouse scored for mutations)
LCL	Lower confidence limit

LPS	Lipopolysaccharides
MAD	Mutual Acceptance of Data
MCL-5	Human B lymphoblastoid cell line
MLA	Mouse lymphoma assay
MMR	Mismatch Repair
MN	Micronucleus
MOA	Mode of Action
mRNA	Messenger RNA
Mt	Methyltransferase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	*N*-acetyltransferase
NER	Nucleotide Excision Repair
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NOAEL	No observed adverse effect level
NOGEL	No observed genotoxic effect level
NQO1	NAD(P)H quinone oxidoreductase 1
NRF2	Nuclear factor-like 2
NSERC	National Society and Research Council of Canada
NSNR	New Substances Notification Regulations
OECD	Organization for Economic Cooperation and Development
PAH	Polycyclic aromatic hydrocarbons
PAPS	3'-phosphoadenosine- 5'-phosphosulfate
PBS	Phosphate buffered saline
PETA	People for the Ethical Treatment of Animals
pfu	Plaque forming units
P-Gal	phenyl-β-D-galactopyranoside
PhIP	2-Amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine
PoD	Point of departure
PPAR	Peroxisome Proliferator Activated Receptors
PXR	Pregnane X receptor
qPCR	Real-time Quantitative PCR (polymerase chain reaction)
RICC	Relative Increase in Cell Count
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RSMN	*in vitro* 3D EpiDerm™ human reconstructed skin MN assay
RXR	Retinoid X Receptors
S9	Aroclor 1254-induced rat liver S9

SD	Standard Deviation of the mean
SDS	Sodium dodecyl sulfate
SKY	Spectral Karyotyping
SOP	Standardised Operating Procedure
SPSF	Standardised Project Submission Form
SULT	Sulfotransferases
TSCA	Toxic Substances Control Act
TG	Test Guideline
TGR	Transgenic rodent
Tk	Thymidine kinase gene
TK6	Human lymphoblastoid cells
Ub	Ubiquitin
UCL	Upper confidence limit
UDP	Uridine Triphosphate
UGT	UDP-Glucuronosyltransferases
U.S.	United Stated of America
V79	Chinese hamster fibroblast cells
Xprt	Xanthine phosphoribosyltransferase
XRE	Xenobiotic Response Element

Statement of Contributions:

Chapter 2: Intra-laboratory Variability in *lacZ* Mutant Frequency Values Generated Using the MutaMouse FE1 Cell *in Vitro* Transgene Mutation

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Statement of Contributions:

Chapter 3: Xenobiotic-induced Gene Expression Changes in MutaMouse FE1 Pulmonary Epithelial Cells.

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Chapter 1: Introduction

1.1 Brief History of Genotoxicity Assessment:

Testing of a chemical substance for genetic toxicity assessment began in 1941 when Auerbach et al. determined that mustard gas (i.e., dichloroethyl sulphide), an alkylating agent, induced a 7-24% mutation rate in *Drosophila melanogaster* (Auerbach et al. 1947; Auerbach 1967). Auerbach concluded that the mustard gas induced chromosome rearrangements, including 7 translocations in 816 treated nuclei; however, this work was classified for several years since it involved experimentation with military warfare gases (Auerbach et al. 1967). The realm of genetic testing at this time focused primarily on the study of gene and chromosome function, but by the 1970's the exponential expansion of the chemical industry stimulated major efforts to screen chemicals for genetic toxicity (i.e., the ability to damage genetic material).

Early genetic toxicology research (i.e., 1950's) discovered that mammalian metabolism is able to either alter a chemical to produce metabolites that are less toxic, which are conjugated to endogenous metabolites for easy excretion from the body, or generate increasingly toxic metabolites able to react with, and potentially damage, macromolecules such as DNA (Miller and Miller 1966). For instance, xenobiotics acting as environmental carcinogens (i.e. polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, aflatoxins, etc.) are frequently only mutagenic following metabolic conversion to reactive metabolites (Glatt et al. 2004). To "activate" potential mutagens (i.e., promutagens), xenobiotic metabolism pathways generally require processes collectively referred to as Phase I and Phase II metabolism. The main route of cellular exposure involves the xenobiotic binding to cellular receptors, which allows for binding

of the xenobiotic-receptor complex to proteins that permit translocation into the cell nucleus and induced transcription of Phase I and II metabolic genes. Phase I metabolism often involves oxidation, which is generally carried out by isozymes of the cytochrome P450 enzyme family. This is followed by Phase II conjugation of the metabolite to an endogenous compound (i.e. glutathione). The latter is often achieved by sulfotransferases, glucuronosyltransferases or glutathione-*S*-transferases (Guengerich 2008; Glatt and Meinl 2005). Phase I metabolism generally leads to the production of polar compounds that can more readily be conjugated to endogenous metabolites via Phase II detoxification in preparation for clearance via urine or bile (Klaassen 2008).

Conversely, both Phase I and Phase II reactions can also generate metabolites that can readily react with endogenous macromolecules such as DNA. For example, benzo[*a*]pyrene (BaP), a prototypical polycyclic aromatic hydrocarbon formed during incomplete combustion of organic matter, requires a three step bioactivation process that begins with catalysis by cytochrome P450 CYP1A1 that, oxidises BaP into metabolites such as benzo[*a*]pyrene-7, 8-epoxide. This compound can subsequently be metabolised by an epoxide hydrolase, which "opens up" the epoxide forming benzo[*a*]pyrene-7, 8-dihydrodiol. Finally, the dihydrodiol can be oxidised by CYP1A1 to form benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, a highly reactive metabolite that is capable of covalently binding to DNA (Figure 1.3) (Klaassen 2008). Heterocyclic amines, like the amino acid pyrolysis product 2-Amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP), which are comprised of one or more closed rings containing carbon and nitrogen, require both Phase I and II metabolism to generate DNA-reactive metabolites (Schut and Snyderwine 1999). For example, PhIP will undergo Phase I oxidation catalyzed by CYP1A2

with resultant hydroxyl compounds that are esterified by Phase II sulfotransferase catalysis. The latter contains an excellent leaving group (i.e., sulfate) that can generate nitrenium or carbenium ions that can readily react with DNA to form covalently-linked products known as DNA adducts (Figure 3.4) (Glatt et al. 2000). Thus, mammalian metabolism of xenobiotic compounds can alternatively diminish or augment genetic toxicity.

Early microbial models for mutagenicity testing included several yeast and fungal assays that employed *Saccharomyces cerevisiae* or *Neurospora crassa*, as well as bacterial assays (Flamm et al. 1977; Zeiger 2004). The first bacterial mutagenicity assays arose in 1950 when G. Bertani, et al. 1951 developed a method for detecting *Escherichia coli* mutants based on the induced ability to resist the antibacterial effects of streptomycin. In 1971, Bruce Ames and colleagues developed multiple histidine-dependent mutants of *Salmonella typhimurium* (i.e., histidine auxotrophs), and an assay that examined reversion to histidine prototrophy (Ames 1971). However, none of the aforementioned systems could simulate the endogenous metabolic capacity of mammalian cells, which as noted, is essential for the conversion of some chemicals into genotoxic metabolites. Thus, these assays were unable to correctly identify many potent promutagens.

In vitro metabolic activation systems able to simulate *in vivo* metabolic capacity were first established by Gabridge and Legator in 1969. These researchers determined that indictor organisms (i.e., Salmonella*, E. coli*) could be injected in the peritoneal cavity of a "host" mouse, and following chemical treatment, recovered and screened for mutation induction (Gabridge and Legator 1969). The metabolic systems possessed by the host are able to convert the test compounds into DNA reactive metabolites, thereby permitting the induction of detectable

mutagenic activity in the indicator organisms (Flamm et al. 1977; Gabridge and Legator 1969). Simpler activation systems were developed in 1971 when H. Malling and R. Garner showed that mouse liver homogenate is able to provide *in vitro* activation of DMN and AFB1, with the metabolically-activated mixture inducing mutations in the aforementioned histidine auxotrophs of *Salmonella typhimurium* (Malling 1971; Garner et al. 1971). Since the preparation of the *in vitro* metabolic activation system requires centrifugation of the hepatic homogenates at 9000 x g, Bruce Ames named this liver homogenate "S9" (Ames et al. 1973). In 1975 Ames optimized S9 by injecting the animal (i.e., male Sprague-Dawley rats) with phenobarbital, and later with Aroclor 1254 (i.e., a commercial mixture of polychlorinated biphenyls) (Ames et al. 1975). These agents are aryl hydrocarbon receptor (AhR) agonists that augment the production of Phase I isozymes such as CYP1A1 (Cox et al. 2016). The establishment of exogenous metabolic activation mixtures based on Aroclor 1254-induced rat liver S9 was a major advancement in the development of convenient *in vitro* genetic toxicity assessment assays that reliably identify genotoxic hazards.

In response to concerns about pesticides, regulatory policies requiring the screening of chemicals for mutagenic potential were initiated by the U.S. Department of Health, Education and Welfare (DHEW) in 1969 (Zeiger 2004). The U.S. Environmental Protection Agency (EPA) subsequently implemented a regulatory framework in the 1976 Toxic Substance Control Act (TSCA), which requires the assessment and regulation of all chemicals that pose an "unreasonable risk" to human health or the environment (Krewski et al. 2010). The increased regulatory need for mutagenicity testing, and the numerous assays available, encouraged a U.S. DHEW committee to evaluate the suitability and utility of available test methods for detection

of genotoxic hazard (Flamm et al. 1977). The EPA utilized the DHEW 1977 recommendations, which were based on performance and reliability of screening numerous chemical classes, in the implementation of a 'tiered' testing system for genetic toxicity assessment (Zeiger 2010). The tiered testing battery included *in vitro* and *in vivo* tests that reduce redundancies, costs and the need for animal studies. Subsequently, the Organisation for Economic Cooperation and Development (OECD) employed the DHEW document in the foundation of their Test Guideline (TG) program (Zeiger 2004; Zeiger 2010). The 1970's burst in assay development also encouraged validation studies, which evaluate efficiency, reliably, reproducibility and utility of standardized genetic toxicity test methods (Zeiger 2004; Zeiger 2010). Although the Ames test quickly became the preferred genetic toxicity assay, the regulatory requirements in Canada, the USA, and several OECD member countries now include a minimum test battery including bacterial and mammalian assays for the detection of gene mutation as well as chromosome damage and aneuploidy, ensuring that all relevant genotoxicity endpoints are monitored (Pfuhler et al. 2007; U.S. Department of Health and Human Services 2012).

Toxicological evaluations of chemicals in commerce in Canada are required under the Canadian Environmental Protection Act 1999 (CEPA), which dictates the required assessments and management of toxic chemicals that permit pollution prevention and sustainable development (Government of Canada 2005). Under CEPA, the New Substances Notification Regulations (NSNR) require routine screening of all new compounds; many new chemicals must be tested using both *in vitro* gene mutation and mammalian chromosomal aberrations assays, with and without the addition of exogenous metabolic activation (Minister of Justice 2015). For elevated production levels (i.e., 10,000kg/calendar year), an *in vivo* mammalian assay assessing

gene mutation, chromosomal aberrations or other relevant “indicators of mutagenicity” must be carried out (Minister of Justice 2015). The most common internationally utilized regulatory battery includes the aforementioned Salmonella reverse mutation or “Ames test”, the *in vitro* micronucleus assay for detection of chromosome damage, and the mouse lymphoma assay (MLA) for detection of gene mutation in mammalian cells (Pfuhler et al. 2007). However, there is ongoing debate about reducing the test battery via elimination of the MLA, since it is thought to offer little improvement in sensitivity and can increase the likelihood of false positives (Kirkland et al. 2011). CEPA stimulated the development of the Chemicals Management Plan, which is currently screening 4300 chemicals in commerce (Government of Canada 2006). Canadian legislation additionally includes the Food and Drugs Act that ensures food additives and therapeutic agents are judiciously evaluated for human safety (Government of Canada 2017). Analogously, the Pest Control Products Act requires testing and labelling of pesticides that appropriately document human health and the environmental hazard (Government of Canada 2016). Thus, the internationally recognized need to screen chemicals for the ability to mutate and/or alter DNA is recognized in Canada; moreover, viewed as essential for the adequate protection of human health and the environment.

1.2 The Organisation for Economic Cooperation and Development (OECD) Test Guideline Program:

The OECD administers international guidelines for the use of both *in vivo* and *in vitro* toxicity test methods for regulatory use, to ensure harmonized human and environmental health protection. The organisation was established in 1960 with 20 original member countries, including the European Union (i.e., United Kingdom, France, Germany, etc.), the U.S. and Canada (Eskes and Whelan 2016). This was increased to 34 members in 2015 (Eskes and Whelan 2016). To ensure that chemicals are fairly, economically and safely traded, and that trade-barriers are minimized among member countries that may have differing chemical safety legislation, the OECD developed its Test Guideline (TG) Program in 1981 (Eskes and Whelan 2016). The OECD has approved a range of reliable and effective toxicological test procedures to identify chemicals that have the potential to induce undesirable effects such as mutations or chromosome damage, *in vitro* and/or *in vivo* (Table 1.1) (OECD 2005); details about assay validation and international acceptance are addressed in a succeeding section. The TG program aims to provide state of the art, internationally accepted and standardized test protocols for regulatory assessments that keep pace with the progression of technology and animal welfare needs. As such, the TG program permits the generation of reliable and robust results such that the "Mutual Acceptance of Data" (MAD) principle can be implemented; allowing data to be reliably transferred between international jurisdictions (OECD 2005). Therefore, member countries are able to share the burden of work for assessing the thousands of new chemicals making their way into the marketplace; moreover by avoiding duplicate testing, costs and the use of laboratory animals is minimized (OECD 2008; Eskes and Whelan 2016). The OECD TG

Program is constantly evolving, thereby continuing to increase the efficiency and effectiveness of human health and environmental hazard identification and assessment.

1.3 Detection of Mutagenicity:

Determination of potential human and environmental hazards requires reliable and relevant toxicity information, such as that generated using the OECD's internationally-accepted TGs that assess the potential hazards of chemicals in commerce. *In vitro* assays are preferable due to the cost-savings and higher through-put of screening compared to *in vivo* protocols. There are currently 2 internationally accepted *in vitro* mammalian mutagenicity assays, the *in vitro* mammalian cell gene mutation assay that employs the *Hprt* and *xprt* loci (i.e., Test No. 476), and the *in vitro* mammalian cell gene mutation assay that uses the thymidine kinase (TK) locus (i.e., Test No. 490) (OECD 2015a; OECD 2015c). Both assays have proven to be useful and reliable for genotoxicity screening; however, the assays have been criticized for the time and labour required for the clonal selection and isolation that is necessary for enumeration of mutations (Kirkland et al. 2007). In addition, the cell systems employed generally lack cytogenetic stability and cannot produce xenobiotic metabolizing enzymes. Specifically, cell lines that are recommended for tests 476 and 490 (i.e., TK6, CHO, V79, L5178Y) are unable to produce the cytochrome P450 isozymes that are known to be involved in the *in vivo* production of reactive metabolites capable of interacting with DNA. Consequently, addition of exogenous enzymatic activation mixtures, such as the aforementioned Aroclor 1254-induced S9, is required for metabolic activation, which, when combined with the necessity for clonal isolation, is rather laborious and fastidious. Further criticisms of the established cell lines employed for Tests 476 and 490 relate to "hypersensitivity" (i.e., frequency of false positives). False positive rates have been shown to be unacceptably high among these mammalian cell tests; a major case study by Kirkland, et al. 2007 established that 80% of non-carcinogens tested positive with

at least one end-point of a 2-3 assay battery that includes Tests 476 and/or 490 (Kirkland et al. 2007). Since regulatory agencies have implemented a tiered-testing approach, the high rate of false positives can lead to high rates of unnecessary follow-up *in vivo* tests; and therefore unnecessary costs and animal use (Kirkland et al. 2007). Alternatively, it could result in a non-hazardous compound being pulled from development and/or production and use (Kirkland et al. 2007). This problem is considerable given the thousands of chemicals in commerce that must be screened to ensure human health and environmental safety standards are met. The aforementioned study by Kirkland, as well as several follow-up studies by Fowler and colleagues, revealed that the metabolic and p53 deficiencies associated with the cell lines employed for these *in vitro* assays are influencing the high rate of false positives (Kirkland et al. 2007; Fowler et al. 2014; Fowler et al. 2012a; Fowler et al. 2012b). For instance, the p53 deficient CHO (Chinese Hamster ovary) cells consistently showed greater sensitivity to cytotoxicity and micronucleus induction, and therefore greater susceptibility to false positive results in comparison with p53 competent cells lines such as HepG2, (human liver) (Fowler et al. 2012). Furthermore, the lack of metabolic competency, and the universal necessity for Aroclor 1254-induced S9 in *in vitro* genotoxicity tests, can contribute to a high frequency of false positives due to the overrepresentation of CYP1A and CYP2B isozymes, and/or absence of Phase II enzymes in S9 (Kirkland et al. 2007). As an alternative, *in vitro* genotoxicity assays could employ cells derived from the transgenic rodents that are used for the *in vivo* detection of mutations in somatic and germ cells (i.e., TG No. 488) (OECD 2011).

1.3.1 Transgenic Rodent (TGR) Mutagenicity Detection Systems:

Transgenic rat and mouse models for scoring *in vivo* mutations in somatic and germ cells employ stably-integrated, genomic shuttle vectors that contain transgenic targets for detecting chemically-induced mutations (Lambert et al. 2005). The assays utilize whole animal models, and thus are able to incorporate metabolic activation and DNA repair pathways. Furthermore, the assays have been shown to be sensitive to a variety of chemical mutagens, and the effects can be assessed in virtually any tissue via retrieval of transgenes and *in vitro* scoring of chemically-induced mutant frequencies. The transgenic rodents (TGR) used for *in vivo* mutagenicity assessment include the Big Blue® rat and mouse, the *lacZ* plasmid mouse, the *gpt* delta rat and mouse, and the MutaMouse (Lambert et al. 2005). The MutaMouse, which is routinely employed at Health Canada (i.e., the Environmental Health Science Research Bureau) for mutagenicity assessment, was developed by microinjecting fertilized eggs with λgt10 shuttle vectors containing a bacterial *lacZ* transgene (Blakey et al. 1995). Cells contain 29 ± 4 concatenated copies of the shuttle vector stably integrated on chromosome 3 (Shwed et al. 2010); the genetic structure of the integrated λgt10*lacZ* shuttle vector is shown in Figure 1.1. The shuttle vector, which is 47kb in length, can be recovered from genomic DNA using a commercial lambda bacteriophage *in vitro* packaging system, and the recovered shuttle vectors in lambda bacteriophage particles can subsequently be absorbed to a suitable *E.coli* C host (*lacZ*$^-$, galE$^-$, RecA$^-$, Kan^r, pAA119) for enumeration of induced *lacZ* mutations (Lambert et al. 2005; Gossen et al. 1989).

Mutations within the *λgt10lacZ* construct can be scored using one of three approaches:

I. A positive selection method for identifying *lacZ* mutations utilizing the selective agent phenyl-β-D-galactopyranoside (P-Gal).

II. A method for identifying *cII* mutations via temperature-mediated positive selection.

III. A colorimetric method for identifying *lacZ* mutations (see Figure 1.2) (Lambert et al. 2005).

The work described in this thesis employed the P-Gal positive selection for scoring induced *lacZ* mutations. When the *lacZ* gene is functional (i.e., wild-type), β-galactosidase can cleave the P-Gal, releasing galactose. In the galE$^-$ *E. coli* strain employed, galactose is converted to UDP-galactose, which will accumulate in the absence of galE (UDP-galactose epimerase), eventually leading to the activation of cell death responses (Ning et al. 2008; Mientjes et al. 1996; Schulz et al. 2005). Thus, since P-Gal is toxic to galE$^-$ *E. coli* when *lacZ* in the reporter construct is still functional, the P-Gal-containing selection medium allows for the enumeration of recovered shuttle vectors that contain mutant copies of *lacZ*. The response metric, *lacZ* mutant frequency, is expressed as the ratio of the number of *lacZ* mutants detected relative to the total number of recovered shuttle vectors (i.e., plaque-forming units enumerated in the absence of the selective agent), (Figure 1.2). The results obtained, which are generally expressed as *lacZ* mutant frequency per 10^5 recovered plaque-forming units (pfus), permit an assessment of a substances ability to induce mutations in the tissue examined. The principles of TGR systems can be employed for *in vitro* mutagenicity assessment in TGR-derived cell lines. Such *in vitro* systems, which constitute *in vitro* complements to existing *in vivo* systems, are significantly faster and cheaper; moreover, compliant with the 3R's of animal usage for toxicity assessment (i.e., replace, reduce and refine).

1.3.2 The MutaMouse FE1 Cell Line:

White et al. 2003, established an alternative *in vitro* method for mutagenicity assessment that is based on cells isolated from pulmonary tissue of the transgenic MutaMouse. The cell line, denoted FE1 for Flat Epithelial Isolate #1, retains the same transgenic reporter system, i.e., *λgt10lacZ* construct, as that of the parent MutaMouse (Shwed et al. 2010; White et al. 2003). As such, the cells can readily be used for *in vitro* assessment of mutagenicity. The FE1 cell line, which is spontaneously immortalized and cytogenetically stable (i.e., mode of 78 chromosomes), has been implemented as the cornerstone of an *in vitro* mutagenicity assessment system that can detect both direct- and indirect-acting mutagens with differing metabolic requirements and mechanisms of action (White et al. 2003; Maertens et al. 2017). The assay makes use of the well-validated methods for scoring mutations at the *lacZ* and lambda *cII* transgenic loci, and consequently, does not necessitate the laborious clonal isolation required by other *in vitro* mammalian mutagenicity assays (OECD 2011). Since the *in vivo* transgenic mutation assays have already been validated (i.e., TG No. 488), an assay based on MutaMouse FE1 cells constitutes an attractive *in vitro* alternative that can generate results for hazard identification and regulatory decision-making. Moreover, it can rationally be used for *in vitro* assessment and extrapolation prior to follow-up *in vivo* testing using TG 488.

1.3.2.1 Morphological Features and Growth Characteristics of MutaMouse FE1 Cells:

FE1 cells are polygonal in shape at low densities, with visible subcellular inclusions (White et al. 2003). At higher confluence they form tight, uniform monolayers that make them ideal for visualization under substrate-adherent culture conditions (White et al. 2003). The cell line has been characterized, and the results obtained to date indicate that the cells are epithelial in

origin (i.e., presence of pan-epithelial cytokeratins), and retain type II alveolar cell markers (i.e., surfactant proteins A, B, C), as well as some type I and Clara cell properties (Berndt-Weis et al. 2009) (White et al. 2003). Robustness and ease of culture are represented by FE1's steady growth, with a doubling time of 18.7 ±1.2 hours and mitotic index of 14.1 ± 2.4% under sub confluent culture conditions (White et al. 2003).

1.3.2.2 Genetic and Biochemical Features:

FE1 cells are pseudo-tetraploid, with a modal chromosome frequency of 78 (White et al. 2003). SKY karyotyping and G-banding analysis showed cytogenetic irregularities such as the duplication and deletion of chromosomes with respect to tetraploid (i.e., gain of chromosomes 2, 8, 19 and Y, and loss of 3, 4, 7, 14, 17 and 18) (data not published). Additionally, FISH (Fluorescent *in situ* Hybridization) analysis revealed that FE1 retain 3 transgenic loci per cell (i.e., sites with the *λgt10lacZ* sequence), which is critical to employment of the *in vitro* transgene mutagenicity assay (White et al. 2003).

The FE1 cell line retains p53 functionality, which enhances genomic stability via activation of DNA repair or apoptosis and/or stalling of cell cycle progression upon detection of DNA damage (White et al. 2003; Klaassen 2008). FE1's also retain activities of CYP1A1 and GST isozymes, and thus are at least partially competent in the catalysis of Phase I and II metabolic activation reactions (White et al. 2003; Berndt-Weis et al. 2009). As such, the cells have been shown to be sensitive to both direct-acting chemical mutagens (i.e., the alkylating agent *N*-ethyl-*N*-nitrosourea or ENU) and indirect-acting mutagens that require metabolic transformation into DNA-reactive metabolites (i.e., BaP), without the addition of exogenous Aroclor 1254-induced S9. This contrasts with the aforementioned need to use exogenous

metabolic activation mixtures to carry out the OCED-compliant mammalian cell gene mutation assays (OECD 2015a; OECD 2015c). The established role of xenobiotic metabolism in the production of DNA-reactive compounds emphasises the utility of an *in vitro* assay based on tools such as the FE1 cells, which possess an endogenous capacity to generate DNA-reactive metabolites that are known to be produced *in vivo*.

1.3.2.3 Performance of the MutaMouse FE1 Cell Mutagenicity Assay:

The frequency of spontaneous *lacZ* mutations in FE1 cells is known to be low and stable (i.e., 39.8 ± 11.74 $x10^{-5}$, 5^{th} to 95^{th} percentile, N=114), thus allowing effective detection of elevated mutant frequencies resulting from chemical exposure (White et al. 2003). Assessment of BaP can yield mutant frequency values up to 32-fold above background (Berndt-Weis et al. 2009). Furthermore, 9 substances that frequently elicit "false positives" (i.e., false for *in vivo* effects) when using OECD-compliant mammalian cell gene mutation assays (i.e., *tk* mutation assay in Mouse Lymphoma Cells), all failed to elicit a significant positive response in FE1 cells (Maertens et al. 2017). More specifically, FE1 cells were able to correctly classify false positive compounds such as *tert*-butylhydroquinone and eugenol, even when tested using exogenous metabolic activation or extended sampling times (Maertens et al. 2017). This demonstrates excellent specificity relative to other *in vitro* mammalian cell systems currently used for regulatory mutagenicity assessment (Maertens et al. 2017). Despite encouraging performance, the utility of the FE1 cell mutagenicity assay will also depend on cost and throughput relative to existing assays. Table 1.2 compares the cost and through-put (i.e., number of compounds that can be tested per month) of the FE1 cell assay with that of OECD Tests 476 and 490. The data show that the FE1 cell assay is generally cheaper and faster, and thus, constitutes an effective,

efficient and reliable alternative to those currently employed for *in vitro* mammalian cell mutagenicity assessment.

1.4 Organisation for Economic Cooperation and Development (OECD) Test Guideline Validation:

In order for a test procedure to be accepted by the OECD, and utilized by regulatory authorities for the protection of human health and the environment, it must first be "validated". Ultimately, validation demonstrates that the test method is reliable and relevant, thus permitting governments, industry and academia to be confident in the accuracy of the generated data. Reliability refers to the reproducibility of the assay independent of date, laboratory or operator, whereas relevance refers to the utility of the assay for the specified purpose (Eskes and Whelan 2016). Validation procedures for new tests encourage a process that is unbiased, and ensures that all regulatory performance standards and principles are strictly upheld. OECD has developed criteria for the evaluation and endorsement of new test methods. These relate to regulatory necessity, time and cost effectiveness, improvements in scientific progression, transferability among laboratories, spectrum of test chemicals examined to date, and functionality for hazard assessment (OECD 2005; Eskes and Whelan 2016). An OECD Guideline Document (i.e., Document No. 34) has been established to assist in assay validation and TG development. The criteria outlined in Document 34 must be fulfilled as a prerequisite to determining the validity and utility of new or revised assessment methods (OECD 2005). To increase flexibility and ease of the process, the validation protocol has been segregated into the following components:

I. *Test definition* – define the relevance of the relationship between the pathophysiologic effect(s) of interest and the assay's endpoint, as well as a rational for regulatory usage.

II. *Intra-laboratory variability* – assessment of the extent to which different operators on different test dates can reproduce the same results within the same laboratory.

III. *Transferability* – assessment of reproducibility of results in a second laboratory.

IV. *Inter-laboratory variability* – assessment of the reproducibility of results among 2-5 different laboratories, operators and test dates.

V. *Predictive capacity* – the ability of a test method to accurately predict the intended *in vivo* endpoint.

VI. *Applicability domain* – assessment of the range and/or classes of chemicals that can reliably be assessed using the assay.

VII. *Performance Standards* – establishment of protocol standards that ensure the test operator is utilizing the methodology correctly and efficiently, and that generated data is usable, (OECD 2005; Hartung et al. 2004; Eskes and Whelan 2016).

Once all modules have been completed, and the reliability and relevance confidently established, the new or revised TG undergoes critical review by several OECD committees, with particular emphasis on the scientific basis and regulatory needs (OECD 2005). If approved by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT), the TG is passed along to the OECD Joint Committee who determine any policy implications that could potentially be associated with acceptance, before it is finally sanctioned by the OECD Secretariat for regulatory use (OECD 2005; Eskes and Whelan 2016). Once the TG has been accepted by regulatory authorities, the data are valid, and can be utilised for regulatory decisions according to the aforementioned MAD principle (Eskes and Whelan 2016). It is equally important that the OECD delete or archive TG deemed no longer relevant or unfit for purpose

if/when they are superseded by new or more refined technologies. This eliminates redundancy and ensures that the most appropriate information is utilized for hazard identification and assessment.

1.5 Requirement for the Development of *In Vitro* Alternatives:

Russell and Burch (1959) presented the "3R's" concept for refining, reducing and replacing laboratory animals in scientific research. With the advancement of the animal rights movement, the 3R's concept has since become increasingly relevant and important during the development and/or review of strategies for toxicity assessment. For example, EU Directive 86/609/EEC, implemented in 1986, states "*An animal experiment shall not be performed if another scientifically satisfactory method of obtaining the result is sought, not entailing the use of an animal, is reasonably and practically available*" (Council Directive 86/609/EEC 1986). Subsequently, Directive 2010/63/EU focused on the protection and welfare of animals who's use is necessary for scientific purposes (i.e., pharmaceutical development) (Council Directive 2010/63/EU 2010). The 3R's are also encouraged within the OECD TG's, and the organisation has developed an agency of "invited experts" committed to the task - The International Council for Animal Protection in OECD Guidelines (Eskes and Whelan 2016). This has been reflected in the increased movement towards *in vitro* high throughput assays, and the reduction of large *in vivo* studies; moreover, implementation of *in vitro* testing options wherever possible.

In vitro assays such as those described earlier (i.e., the Ames/Salmonella mutagenicity test) offer cost-effective and reliable alternatives to animal testing. Indeed, regulatory agencies, including Health Canada and the EPA, have implemented tiered testing systems whereby *in vitro* tests are utilized first, and *in vivo* tests are only rationalised if clear positives are obtained (Krewski et al. 2010). In comparison with the average cost of running an animal toxicity assessment study (i.e., $200 000 and up), which can be prohibitive, the complete cost of an *in vitro* study is more manageable (i.e., approximately $50,000), (P.A. White, personal

communication). Moreover, the advent of high-throughput genomic technologies may permit further reductions in the cost of toxicity assessment via the use of surrogate endpoints that can be efficiently assessed in exposed cells or animals (Szymański et al. 2012). Thus, clear financial savings and animal welfare benefits rationalise the development of reliable *in vitro* genotoxicity test methods for human and environmental health hazard identification and assessment.

1.6 International Organizations Promoting the Development of *In Vitro* Assays:

Increased public apprehension regarding the use of animals in research has encouraged the development of many animal welfare groups (i.e., PETA), as well as international committees specifically concerned with the promotion, development, validation and general movement towards alternative methods. Notably, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) was created in 1991 to promote the development and use of test methodologies that are congruent with the 3R's principle, and the use of methodologies in the EU's REACH program for the regulatory registration and testing of chemicals (Eskes and Whelan 2016). EURL-ECVAM also works closely with the OECD on the validation of TG's for the purpose of chemical hazard identification and assessment. Relatedly, in 2005, Japan established a committee called JaCVAM (Japanese Centre for the Validation of Alternative Methods), which is also focused on the 3R's and the development and validation of alternative methods. Korea followed in 2009 with KoCVAM, the Korean Centre for the Validation of Alternative Methods, France and Norway in 2007 with FRANCOPA and NORECOPA, respectively, and Finland in 2008 with the Finish Center for Alternative Methods (Eskes and Whelan 2016). Similarly, Australia and India have adopted alternative methods committees within specific universities, and with the steady growth of Brazil's and Romania's chemicals industries over the last decade, BraCVAM (the Brazilian Center for Validation of Alternative Methods) was developed in 2011, and the RoCVAM (the Romanian Center for Validation of Alternative Methods) in 2015 (Eskes and Whelan 2016). Germany initiated the formation of these alternative methods committees with the creation of ZEBET (Center for the Assessment and Evaluation of Substitute and Supplementary Methods for Animal Testing) in

1989 (Eskes and Whelan 2016). Although Canada does not have an official validation committee, Health Canada is a signatory of ICATM, the tripartite commission of International Cooperation on Alternative Test Methods (i.e., Canada, U.S., and Japan) that supports the timely validation of alternative methods, and the Canadian Council on Animal Welfare is responsible for ensuring the national standard of care of animals in science. Lastly, non-governmental organizations such as the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use), and the ICCR committee for International Cooperation on Cosmetics Regulation, encourage the development of alternative methods (Eskes and Whelan 2016).

1.7 Thesis Objectives:

As an ICATM signatory, Health Canada is committed to the validation and promotion of alternative toxicity assessment methods. As such, Health Canada researchers are currently pursuing validation of the aforementioned *in vitro* transgene mutagenicity assay in MutaMouse FE1 cells. The overall purpose of this project is to contribute to that validation. In the context of this thesis, validation involves examination of intra-laboratory variability and reproducibility, and investigation of the FE1 cell metabolic capacity that pertains to the assay's applicability domain. Specifically, via quantitative comparisons of dose-response data, the thesis research assesses variability in responses across operators and/or dates (i.e., intra-laboratory variability). Contributions to the establishment of the applicability domain are realised by studying the induced metabolic competency of the FE1 cells following exposures to 5 genotoxicants known to require mammalian metabolism. More specifically, qPCR arrays are utilized to determine the profile of chemically-induced gene expression across 84 Phase I and 84 Phase II genes associated with xenobiotic metabolism. Overall, the results obtained will contribute to fulfillment of the OECD's aforementioned validation criteria for critically evaluating test methods under development. Once validated, the mammalian cell gene mutation assay in MutaMouse FE1 cells, which already boasts reliability, sensitivity, and specificity, can be confidently added to the battery of test methods currently used for regulatory assessments of chemically-induced genotoxic hazard.

1.8 Tables and Figures:

Table 1.1 OECD Test Guidelines (TG) for genetic toxicity assessment, showing the date of original adoption for standardized testing. TG currently accepted for regulatory use are included, with dates of revisions in blue, as well as those deleted/archived with date of deletion in red (OECD 2015b).

Current Status	TG No	Test Method	Date of Adoption
Retained/Revised	471	Bacterial Reverse Mutation Test (Ames)	1983 (1997)
	485	Mouse Heritable Translocation Assay	1986
	486	*in vivo* Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells	1997
	473	*in vitro* Mammalian Chromosome Aberration Test	1983 (1997 & 2014)
	474	*in vivo* Mammalian Erythrocyte Micronucleus Test	1983 (1997 & 2014)
	475	*in vivo* Mammalian Bone Marrow Chromosome Aberration Test	1984 (1997 & 2014)
	476	*in vitro* Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes	1984 (1997 & 2014)
	478	Rodent Dominant Lethal Test	1984 (2015)
	483	Mammalian Spermatogonial Chromosomal Aberration Test	1986 (1997 & 2015)
Recently Adopted	487	*in vitro* Mammalian Cell Micronucleus Test	2010 (2014)
	488	Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays	2011 (2013)
	489	*in vivo* Mammalian Alkaline Comet Assay	2014
	490	*in vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	2015
Deleted / Archived	472	Escherichia coli Reverse Mutation Assay	1983 (1997)
	477	Sex-linked Recessive Lethal Test in *Drosophila melanogaster*	1984 (2014)
	479	*in vitro* Sister Chromatid Exchange assay in Mammalian Cells	1986 (2014)
	480	*Saccharomyces cerevisiae*, Gene Mutation Assay	1986 (2014)
	481	*Saccharomyces cerevisiae*, Mitotic Recombination Assay	1986 (2014)
	482	*in vitro* Unscheduled DNA Synthesis with mammalian liver cells	1986 (2014)
	484	Mouse Spot Test	1986 (2014)

Table 1.2 Cost and through-put of OECD Test 476, OECD Test 490, and the FE1 cell mutagenicity assay. Abbreviations are as follows: CHO-Chinese Hamster ovary cells, CHL-Chinese Hamster lung cells, TK6-human TK6 lymphoblastoid cells, V79-V79 Chinese Hamster cells, FE1 – Flat Epithelial Isolate 1 (Transgenic rodent cells). Labour, infrastructure and overhead costs not included.

TG No.	Test Method	Cell Line(s)	Approx. Cost per Compound[1] ($CAD)	Throughput (Compounds tested/month)	References
476	*in vitro* Mammalian Cell Gene Mutation Tests using the *Hprt* and *xprt* genes	CHO, CHL, V79, L5178Y, TK6	$1250	1-3	OECD TG No. 476
490	*in vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	L5178Y, TK6	$1250	1-3	OECD TG No. 490
Currently under validation	*in vitro* transgene mutagenicity assay	FE1	$600 - $1200	4-8	White et al. 2003; Berndt-Weis et al., 2009

[1]Costs for consumables only

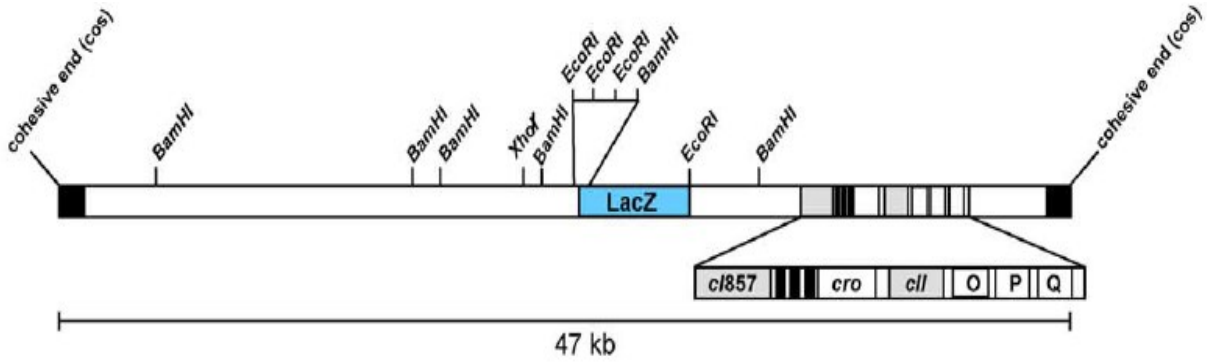

Figure 1.1 The *λgt10lacZ* construct integrated in the MutaMouse and FE1 cell line genome (reproduced from Lambert, et al. 2005).

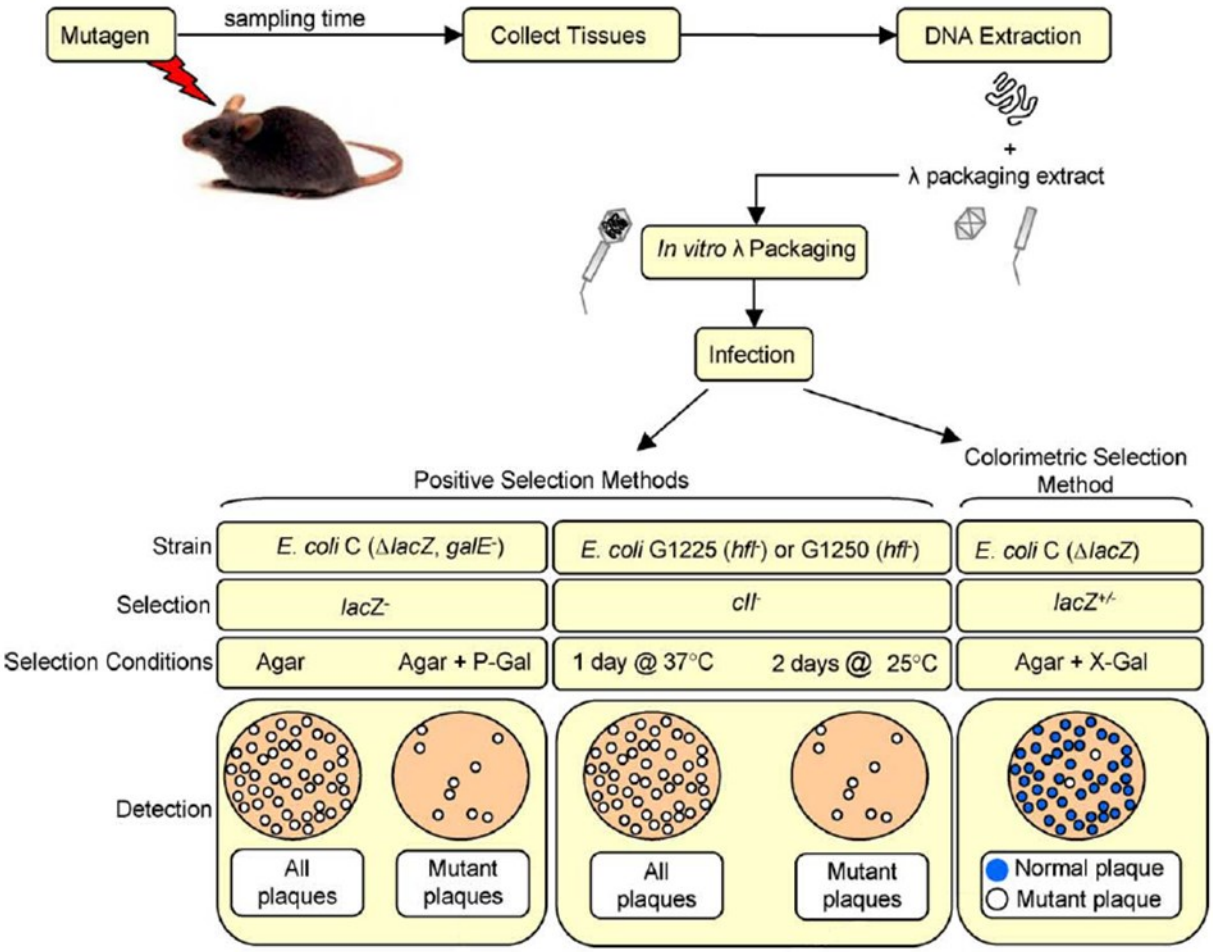

Figure 1.2 The MutaMouse transgenic mutation scoring system employing an *in vitro* bacterial method for positive selection of *cII* or *lacZ* mutants (reproduced from Lambert, et al. 2005).

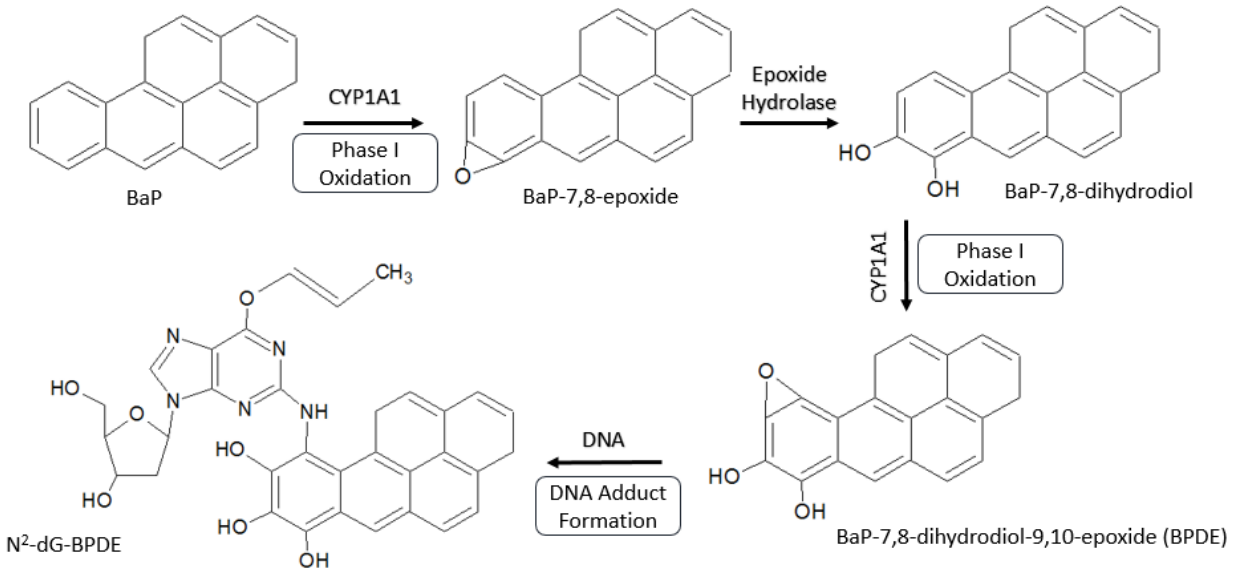

Figure 1.3 Bioactivation of BaP (benzo[*a*]pyrene) illustrating DNA adduct formation (i.e., N^2-dG-BPDE).

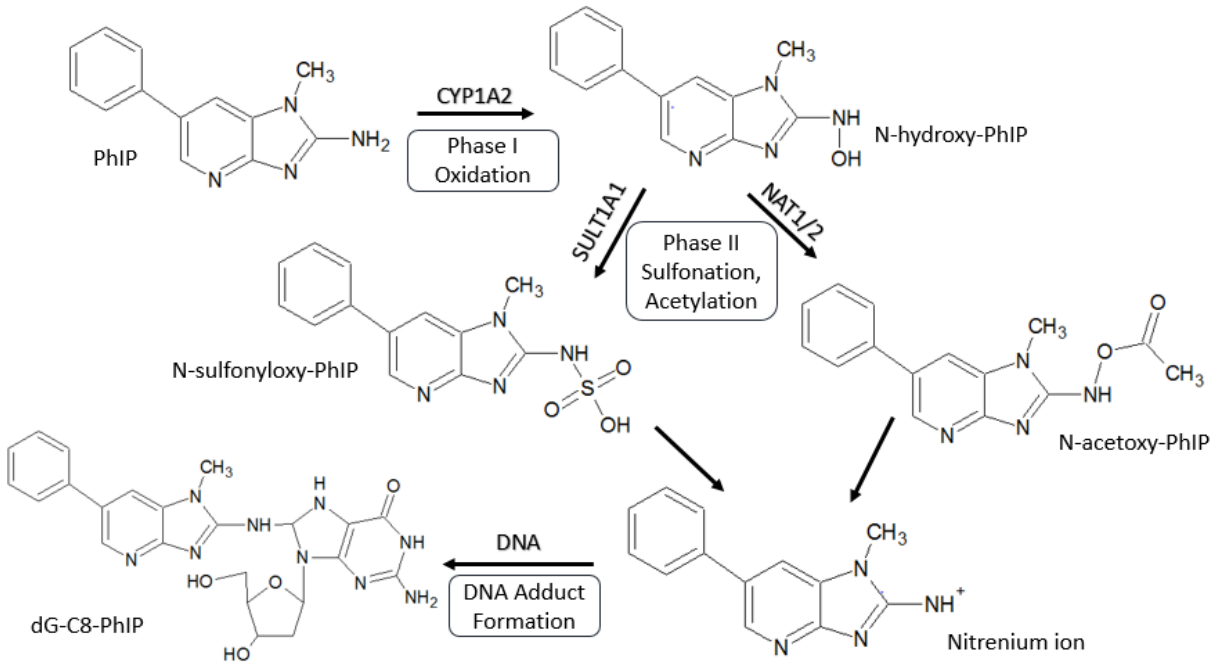

Figure 1.4 Bioactivation of PhIP (2-Amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine) illustrating DNA adduct formation (i.e., dG-C8-PhIP).

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Chapter 2: Intra-laboratory Variability in *lacZ* Mutant Frequency Values Generated Using the MutaMouse FE1 Cell *in Vitro* Transgene Mutation Assay.

Abstract:

Toxicological evaluations of chemicals in commerce are required under Canadian legislation (i.e., Canadian Environmental Protection Act or CEPA); the tests employed for regulatory assessments require validation prior to standardized use. A validation study is underway to evaluate the utility and reliability of an *in vitro* assay for mutagenicity assessment in MutaMouse FE1 cells. Validation requires assessment of the intra-laboratory variability of assay results, such that 8 reference chemicals were tested by a minimum of 2 operators on different dates. Test chemicals included "true positive" mutagens screened with or without the addition of exogenous metabolic activation by Aroclor 1254-induced S9 as well as "true negative" and "false positive" compounds tested without S9. The FE1 cell transgene mutagenicity assay was able to correctly classify negative and false positive compounds, as well as benzo[*a*]pyrene (BaP, -S9), *N*-ethyl-*N*-nitrosourea (ENU, -S9) and 2-amino-1-methyl-6-phenylimidazo (4, 5-*b*) pyridine (PhIP, +S9). However, the assay was not able to correctly classify the known mutagen *N*-nitrosodimethylamine (DMN, +S9). Benchmark dose analysis demonstrated that with respect to BaP and PhIP, intra-laboratory reproducibility is remarkably stable. In contrast, ENU potency showed considerable variability between Operator 1 and Operators 2 and 3 that is likely due to compound instability. Based on sensitivity, specificity, reproducibility and reliability, and the ability to metabolise and activate some mutagens without exogenous S9, the FE1 cell *in vitro* mutagenicity assay can be considered a suitable, alternative to currently validated *in vitro* assays. Next steps in validation include definition of

the assay's applicability domain, and completion of transferability and inter-laboratory variability studies to determine reproducibility across different labs.

2.1 Introduction:

In order to ensure adequate protection of human health and the environment, Canadian regulations, such as the New Substance Notification Regulations (NSNR) specified in CEPA (Canadian Environmental Protection Act), necessitate toxicological evaluations of chemicals in commerce. This evaluation must include an assessment of the potential to induce genetic damage, i.e., genetic toxicity (Minister of Justice 2015). Importantly, the tests employed for regulatory assessment of genetic toxicity must be properly validated to ensure the reliability of the results. For example, genetic toxicity endpoints specified in the NSNR must be assessed using assays that have been validated and approved by the OECD (Organisation for Economic Cooperation and Development). Such assays, which form the cornerstone of regulatory evaluations, are carried out according to official test guidelines (TG) that have been scrutinised and approved by OECD-member countries.

Prior to preparation and approval of an OECD TG, assays to be used for regulatory assessments must be validated with respect to performance, relevance, reliability and reproducibility (Eskes and Whelan 2016). With respect to new tests that are under development and/or undergoing validation, policies and public concern over animal usage, as well as concerns about testing efficiency and costs, have stimulated the expansion and improvement of dependable *in vitro* assays (Eskes and Whelan 2016). Although the OECD has already established TGs for *in vitro* detection of mutagenicity in cultured mammalian cells, the commonly-used tests (i.e., the *tk* and *hprt* gene mutation assays specified in TG 476 and TG 490) employ cell lines that have been criticized for cytogenetic instability, hypersensitivity, and lack of the metabolic capacity required to transform chemicals (i.e., promutagens) into DNA-

reactive metabolites (i.e., CHO, TK6, V79, L5178Y Mouse Lymphoma Cells) (OECD 2015a; OECD 2015b; Kirkland et al. 2007). To overcome the latter issue, assays specified in TG 476 and 490 require the use of exogenous metabolic activation mixtures containing rodent liver homogenates (i.e., Aroclor-1254-induced rat liver S9). In addition to drawbacks and problems associated with use of rat liver S9 (e.g., cytotoxicity), the assays require labour intensive clonal selection and enumeration. Thus, their throughput is decidedly low (i.e., approximately 1-3 chemicals can to be tested per month) (OECD 2015a; OECD 2015b).

White et al. 2003, isolated a stable epithelial cell line from the lung tissue of an adult male MutaMouse, a transgenic animal employed for *in vivo* assessment of mutagenicity (i.e., OECD TG 488). The cell line, denoted FE1 for Flat Epithelial Isolate #1, can be used for *in vitro* assessment of mammalian cell mutagenicity; indeed, an *in vitro* transgene mutagenicity assay based on the FE1 cells is currently undergoing validation. The FE1 cell line is immortal and cytogenetically stable, retains P53 functionality, and endogenously expresses metabolic enzymes such as cytochrome P450 monooxygenase isozymes CYP1A1, 1A2 and 1B1. The latter attribute confers the ability to transform promutagenic substances into DNA-reactive metabolites without the addition of an exogenous metabolic activation mixture (White et al. 2003; Berndt-Weis et al. 2009). The most noteworthy attribute of the cell line is the retention of the integrated, retrievable λgt10*lacZ* shuttle vector that can be used to score the frequency of induced mutations without labour intensive clonal selection and enumeration (Lambert et al. 2005). The target *lacZ* transgene is rescued from genomic DNA and packaged into λ-bacteriophage that are used to "shuttle" the vector to an appropriate strain of *Escherichia coli* for *in vitro* scoring. The *E. coli* host employed is *galE*$^-$ and *lacZ*$^-$, thus permitting enumeration of

lacZ mutant frequency using the P-Gal (phenyl-β-D-galactoside) positive selection system (Lambert et al. 2005; Gossen et al. 1989).

To date, the FE1 cell transgene mutation assay has been used to successfully assess the genetic toxicity of numerous xenobiotic substances, including polycyclic aromatic hydrocarbons (PAHs), nitroarenes, multi-walled carbon nanotubes, and complex mixtures such as cigarette smoke condensate (Lemieux et al. 2015; Chen et al. 2008; Poulsen et al. 2013). Moreover, recent analyses showed that the assay can correctly classify 9 problematic substances that were previously shown to elicit false positive responses in other *in vitro* mammalian cell gene mutation assays (e.g., *tk* assay in Mouse Lymphoma Cells) (Maertens et al. 2017). Thus, the aforementioned cellular characteristics, coupled with the relative ease, higher throughput, and reliability of the assay, make the FE1 cell *in vitro* mutagenicity assay a superb candidate for regulatory assessment of chemically-induced genetic toxicity. Moreover, an effective alternative to currently-employed assays, and a logical candidate for validation and eventual establishment of an OECD Test Guideline.

The assay validation process recommended by the OECD requires pre-validation experimentation that provides evidence of an assay's performance, utility and overall reliability. A major component of validation involves the completion of an intra-laboratory variability study for determination of assay reproducibility. Essentially, an intra-laboratory study assesses the extent to which different operators on different test dates are able to generate the same results within the same laboratory (Eskes and Whelan 2016). More specifically, in order to demonstrate acceptable reproducibility of the assay, numerous operators must achieve similar test outcomes (i.e., positive, negative or equivocal responses) when testing the same chemicals

with the same protocol (OECD 2005). This and subsequent validation procedures, including assessment of inter-laboratory reproducibility, are extremely important for ensuring that only the most scientifically sound and reliable tests are employed for standardized testing and generation of results for regulatory decision-making. Examples of *in vitro* genotoxicity assays that have recently undergone and/or are currently undergoing validation exercises for the development of OECD test guidelines are summarized in Table 2.1. Such validation eliminates costs attributable to unnecessary redundancies and re-testing; and more importantly, ensures that regulatory decisions adequately safeguard human and environmental health.

Traditionally, evaluations of genetic toxicity data have been restricted to hazard identification, whereby a dichotomous screen-and-bin approach is used to merely identify genotoxic agents (i.e., positive or negative). More recently, quantitative assessment of dose-response data has been recommended, with point-of-departure (PoD) potency metrics being used for regulatory evaluations and decision-making (White and Johnson 2016; MacGregor et al. 2015a; MacGregor et al. 2015b). For example, the International Workshop on Genotoxicity Testing (IWGT) (MacGregor et al. 2015, MacGregor et al. 2015a,) and the HSEI Genetic Toxicology Technical Committee (GTTC) (White and Johnson 2016), are now encouraging dose-response analysis and use of potency metrics for regulatory evaluations. Specifically, use of the benchmark dose (BMD) approach to analyse dose-response data, and determine the concentration or dose required to elicit a pre-defined increase in response above control (e.g., 10%) (White and Johnson 2016; Crump 1984). Analyses conducted by the GTTC showed that the BMD approach provides a potency metric that is more useful, accurate and reliable in comparison with the traditional no observed adverse effect level (NOAEL). NOAEL, or NOGEL for

genetic toxicity, identifies the tested concentration at which there is no observable genotoxic effect. It is restricted to the selected doses, and thus subjective and potentially biased (i.e., if only one low dose is tested, the NOGEL may be more conservative than necessary) (Slob 2002).

The BMD method fits several mathematical models to the dose-response data in order to determine the concentration or dose, with confidence intervals, that is required to elicit a predetermined response level known as the benchmark response (BMR) (Slob 2002). For instance, BMD at an effect level, or BMR, of 10%, will determine the concentration at which there is a 10% increase in response above the control, with associated upper and lower confidence intervals denoted the BMDU and BMDL, respectively. More recently, to additionally assess the effects of selected covariates, the BMD approach has been employed to simultaneously analyse multiple dose-response data sets for a given endpoint. Data sets are compared across a covariate(s) of interest (i.e., substance, laboratory, operator) to determine if the covariate has a significant influence on the BMD (Slob and Setzer 2014; Wills et al. 2016a; Wills et al. 2016b). For example, Johnson et al. 2016 recently employed the BMD covariate approach to investigate the effect of test laboratory on *Pig-A in vivo* gene mutation data generated by 13 different labs. Thus, the BMD approach not only provides a robust potency metric for regulatory decision-making, it permits analyses across covariates that can be used for compound potency ranking and/or robust scrutiny of response variations attributable to a wide range of variables such as laboratory, operator, sex, tissue, cell type, etc. (White and Johnson 2016; Wills et al. 2016).

This work pertains to intra-laboratory validation of the *in vitro* MutaMouse FE1 cell mutagenicity assay that is currently being conducted at Health Canada (i.e., the Environmental

Health Science and Research Bureau). The work involves a minimum of 2 operators, and screening of 8 EURL-ECVAM (European Union Reference Laboratory for Validation of Alternatives to Animal Testing) recommended reference chemicals. EURL-ECVAM, an agency dedicated to the validation of alternative test methods, has developed a recommended list of reference chemicals from which the test chemicals have been selected (Kirkland et al. 2008). The selected chemicals include 4 known positives (i.e., known mutagens), 2 known negatives (i.e., known non-mutagens), and 2 compounds known to elicit false positives in other *in vitro* mammalian cell gene mutation assays. *LacZ* mutagenicity results are qualitatively and quantitatively compared to determine assay consistency and reproducibility; quantitative comparisons across operators and/or test dates employed the BMD approach.

2.2 Materials & Methods:

2.2.1 Chemicals and Reagents:

FE1 cells were exposed to 8 reference chemicals recommended for the evaluation of new or revised mammalian genetic toxicity assays. Known positives benzo[*a*]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP) were purchased from Moltox Inc. (Boone, North Carolina), and *N*-ethyl-*N*-nitrosourea (ENU) and *N*-nitrosodimethylamine (DMN) purchased from Sigma Aldrich (Oakville, Ontario). Known negative compounds, D-mannitol and ampicillin trihydrate were also purchased from Sigma Aldrich; the false positive compounds *tert*-butylhydroquinone and phthalic anhydride were graciously provided by Dr. Paul Fowler (formerly Covance, UK). All compounds were dissolved in high-purity DMSO from Sigma Aldrich. Cell culture reagents including DMEM/F12 culture medium, fetal bovine serum (FBS), trypsin, penicillin/streptomycin mix, sterile phosphate-buffered saline (PBS), and murine epidermal growth factor were purchased from Thermo Fisher Scientific (Burlington, Ontario). Where required, an exogenous metabolic activation mixture was prepared using Aroclor 1254-induced S9 rat liver extract procured from Moltox Inc. The S9 cofactor reagents, HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NADP (nicotinamide adenine dinucleotide phosphate), G-6-P (glucose-6-phosphate), $MgCl_2$ (magnesium chloride) and KCl (potassium chloride) were obtained from Thermo Fisher, Roche (Laval, Quebec), and Sigma Aldrich. EDTA (ethylenediaminetetraacetic acid), Tris (2-Amino-2-(hydroxymethyl) propane-1, 3-diol) and SDS (sodium dodecyl sulfate) were purchased from Sigma Aldrich. Proteinase K was purchased from Thermo Fisher, and P-Gal (phenyl-β-D-galactoside) was acquired from G-Biosciences (St. Louis, Missouri).

2.2.2 Cell Culture:

FE1 cells were cultured in standard DMEM/F12 (1:1) medium supplemented with 2% v/v fetal bovine serum (FBS), 1% v/v penicillin/streptomycin and 0.02% v/v murine epidermal growth factor, and incubated at 37°C, 95% humidity, 5% CO_2. FE1 cell stocks were stored in liquid nitrogen, thawed and cultured on 10cm polystyrene plates with 10mL of medium until 70% confluency. Cells were passaged at least 2 more times before experimental use. To reduce variability, all experimentation in this study was performed on cells at passage #25.

Routine sub-culturing or passaging was performed by removing existing media, rinsing the cells with approximately 5mL PBS, and then treating with 0.5mL 0.25% v/v trypsin for approximately 1 minute. Once the cells had lifted from the culture surface they were collected in 4 mL culture medium. Plates were then rinsed with another 2mL of medium to ensure collection of as many cells as possible. Trypan blue staining in a 1:1 cell solution to stain ratio was used for determination of cell density using a Countess Automated Cell Counter (haemocytometer; Invitrogen, Burlington, Ontario). 300,000 cells were diluted in 10mL of media, deposited on a 10cm culture plate, and incubated for 3-4 days until the desired confluency was attained (i.e., 70%).

2.2.3 FE1 Cell Transgene Mutation Assay:

FE1 cells were passaged as described, with 300,000 cells deposited on a 10cm plate and incubated overnight (approximately 18 hours). The following morning the cells were exposed to test chemicals for 6 hours in FBS-free culture medium. Positive and negative (i.e., vehicle/solvent only or medium only) controls were run concurrently. The concentration of the vehicle solvent remained constant at 1% v/v. Each assessment included duplicate, single dose

positive controls consisting of 0.1µg/mL BaP if testing without exogenous metabolic activation (i.e., S9), or 1µg/mL PhIP if testing with exogenous metabolic activation. An exogenous activation mixture, including Aroclor 1254-induced rat liver S9, was only added for mutagens previously shown to require exogenous activation (i.e., PhIP). Final S9 concentration was 0.5% v/v, and, as required, the S9 was added with a co-factor mixture containing NADP, G-6-P, $MgCl_2$ and KCl in a HEPES buffer. The final concentrations of each co-factor in the exposure medium were 1mM, 1.26mM, 1.26mM, and 8.3mM, respectively. The final HEPES concentration was 1mM.

Following the exposure, cells were rinsed twice with PBS and incubated in 10mL of the complete culture media for a 72-hour sampling period that permits mutation fixation. Following the sampling period cells were lysed and digested overnight using 3mL of freshly-prepared proteinase K lysis buffer (i.e., 10mM Tris pH 7.6, 10mM EDTA, 150mM NaCl, 1% SDS, 1mg/mL Proteinase K). The following morning the lysate was poured into a 15mL polystyrene Falcon tube, and high molecular weight DNA extracted and purified using the phenol:chloroform protocol described by Gingerich et al. 2014. Briefly, lysates are first extracted with (24:24:1) Tris-buffered phenol: chloroform: isoamyl alcohol, then again with (24:1) chloroform: isoamyl alcohol and 200mM of sodium chloride, before the DNA is precipitated in 2 volumes of ethanol. DNA was spooled out of the ethanol using a sealed Pasteur pipette, and dried and dissolved in TE^{-4} buffer (i.e., 10mM Tris pH 7.6 and 0.1mM EDTA) at 4°C for a minimum of 48 hours before further analysis.

LacZ mutant frequency was determined using the P-Gal positive selection method (See Chapter 1, Section 1.3.1) (Lambert et al. 2005; Gossen et al. 1989). Basically, FE1 cell DNA

contains concatenated copies of the λgt10*lacZ* shuttle vector that can be excised and packaged into λ-bacteriophage particles using the commercially available Transpack system from Agilent Technologies (Mississauga, Ontario). The bacteriophage particles were combined with *E coli* strain C (*lacZ* $^{-}$, *galE* $^{-}$, *recA* $^{-}$, *Kan*^{r}, pAA119) and agar, and then added to Petri plates in one of 2 groups: the positive selection group containing P-Gal, and the reference (or titer) group without P-Gal. Plates were incubated overnight at 37°C, and the frequency of mutant (i.e., positive selection) and titre plaques manually scored using a light table. The ratios of mutant plaque forming units (pfu) to total pfu were calculated, and the results expressed as *lacZ* mutant frequency x 10^{-5} (i.e., per 100 000).

2.2.4 Study Design:

Intra-laboratory trials were run to ensure that the FE1 cell mutagenicity assay is able to generate reproducible and reliable results across different operators and test dates within the same laboratory. 8 EURL-ECVAM reference chemicals, including 4 known positives, 2 known negatives and 2 false positives, were screened at 4 concentrations in duplicate on 3 different test dates a minimum of one week apart. Since it was previously shown that FE1 cells cannot convert some mutagens (i.e., PhIP) into their DNA-reactive metabolites, it was necessary for the intra-laboratory validation to be carried out both with and without exogenous S9.

Concentration selection was based on previous cytotoxicity determinations that employed the relative increase in cell count (RICC) method, which measures relative cell survival under each chemical exposure condition (Maertens et al.2017). BaP test concentrations were 0.05, 0.1, 0.25 and 0.5µg/mL, and ENU was tested at 100, 150, 250 and 500µg/mL, both without S9. PhIP was tested at 0.1, 0.5, 1 and 5µg/mL, and DMN at 1000, 2000, 3500 and 5000µg/mL, both

with S9. Note that the highest test concentration of DMN is consistent with OECD recommendations, i.e., screening chemicals up to a limit of 10mM or 5000µg/mL to avoid false positives associated with extreme cytotoxicity and/or testing artifacts (OECD 2015b). DMN did not induce a significant increase in *lacZ* mutant frequency at 10mM (i.e., approximately 750µg/mL); and therefore, was tested up to the recommended 5000µg/mL. FE1 cells were exposed to two 'true negatives': ampicillin trihydrate at concentrations of 100, 500, 1000 and 3500µg/mL (-S9), and D-mannitol at 0.1, 1, 10 and 100µg/mL (-S9). The 'false positives' *tert*-butylhydroquinone and phthalic anhydride were tested at concentrations of 0.1, 0.5, 1 and 2µg/mL (-S9), and 100, 500, 1000 and 1700µg/mL (-S9), respectively. Using the BMD approach, *lacZ* mutant frequency results were compared across dates; the same approach was used to compare the results obtained across operators.

2.2.5 Data Analysis:

Statistical analysis of the *lacZ* mutant frequency dose-response data was conducted using R version 3.1.1 (The R Project for Statistical Computing Software). Briefly, Poisson regression analysis was employed to compare the number of mutant plaques at a given concentration with the number in the concurrent solvent control. Data were fit to the model $\log(E(Y_i)) = \log t_i + \acute{\imath}\mathbf{x}_i$, where E(Yi) is the expected value for the *i*th observation, ί is the vector of regressions coefficients, $\mathbf{x}_i$ is a vector of covariates for the *i*th observation, and t_i is the offset variable used to account for differences in the size of the count window (i.e., total pfus scored) (Lemieux et al. 2011). Statistical comparison of responses at any test concentration to the concurrent control were corrected for multiple comparisons using the Bonferonni method. A response was considered to be positive, and therefore the test article mutagenic, when any test

concentration elicited a significant *lacZ* mutant frequency response compared to the concurrent DMSO solvent control, and concurrent positive and negative controls met previously-defined acceptance criteria. Results are therefore negative if no significant increase in mutant frequency was observed in comparison with the concurrent DMSO control. The standard deviation (SD) was calculated for each control group mutant frequency, with the acceptable control range estimated as the mean ± 3SD, which represents the upper (UCL) and lower control limits (LCL) recommended by the International Workshop on Genotoxicity Testing (Hayashi et al. 2011).

Assay performance was qualitatively evaluated using sensitivity and specificity metrics. Sensitivity assesses the frequency of correctly-detected known mutagens (i.e., the frequency of known mutagens yielding positive responses in the assay). Specificity assesses the frequency of correctly-detected non-mutagens (i.e., the frequency of known non-mutagens yielding negative responses in the assay). These metrics have frequently been used to assess the regulatory utility of novel genetic toxicity assays (Lambert et al. 2005; Bryce et al. 2014; Hu et al. 2009).

BMD analysis was performed on all the positive *lacZ* mutant frequency data using the online PROAST software version 64.0 (https://proastweb.rivm.nl). PROAST employs nested models of both the Hill (i.e., $y=a\{1+(c-1)x^d/(b^d+x^d)\}$) and exponential families (i.e., $y=a\{(c-(c-1))\exp(-bx^d)\}$) containing various numbers of parameters to determine the best fit for dose-response data sets. Parameters *a* and *b* are the scaling parameters representing the mean response of the controls and potency of the test chemical, respectively, while *c* and *d* correspond to the shape parameters of the maximum response level, and the log-steepness of the response curve (Slob 2002; Slob and Setzer 2014). The distribution of the response across

the doses examined was assumed to be log-normal; conveniently, PROAST analyses are conducted using log transformed data and the software automatically back-transforms the resultant BMD values. Initial analyses employed PROAST to examine individual dose-response data sets from each operator and/or test date, and determine the BMD_{10} values, as well as the 90% confidence interval represented by the BMDL and BMDU. Subsequently, the data sets were combined by test chemical, and analysed using either operator or test date as a covariate. In this approach parameters *a* and *b* are examined for covariate dependence, while shape parameters *c* and *d* are assumed to be constant across the datasets. For a detailed description of the computational models employed for PROAST analyses, the reader is referred to Slob and Setzer 2014.

2.3 Results:

To determine the ability of an assay to successfully classify chemicals, intra-laboratory variability studies should examine several compounds with different modes of action, and/or compounds that belong to different chemical classes. As noted, this study examined 8 compounds, including 4 known mutagens, 2 known non-mutagens, and 2 compounds described as *in vitro* false positives. In addition, the study examined variability in responses across operators and test dates. For operational reasons, not all operators examined all test articles; a maximum of 5 operators examined the same test chemical across multiple dates.

2.3.1 Analysis of Controls:

2.3.1.1 Negative Control Groups:

An effective intra-laboratory validation study must include vehicle (i.e., DMSO) controls for all assessments. Media controls are also desirable and generally recommended; however, they were only regularly included in testing by Operators 1 and 2, and occasionally in testing by Operator 3. The vehicle and media control groups are included in tests both with and without exogenous S9; however, S9 was only included for test agents known to require exogenous metabolic activation (see below). Figure 2.1 shows the *lacZ* mutant frequency values yielded by each operator for all intra-laboratory validation data sets for the vehicle (Panel A) and media only (Panel B) negative controls, both with and without S9. The DMSO vehicle control groups generated average mutant frequency values (± standard deviation) of 37.3×10^{-5} (± 13.3, N=48), with LCL and UCL values of -2.7×10^{-5} and 77.3×10^{-5}, respectively, when tested without S9. With S9, mutant frequency values averaged 39.0×10^{-5} (± 25.1, N=15), exhibiting an acceptable control range of 36.1 to 114.2×10^{-5} (Figure 2.1A). The overall variability of vehicle control

mutant frequency data across operators appears quite low; however, variations in the -S9 results from Operator 4, and the +S9 results from Operator 3 are large compared to other operators. The media-only control groups generated average mutant frequency values of 31.2 x 10^{-5} (± 10.1, N=35), with LCL and UCL values of 0.9 x 10^{-5} and 61.5 x 10^{-5}, respectively, when tested without S9, and 28.2 x 10^{-5} (± 5.8, N=13) with an acceptable control range of 10.7 to 45.6 x10^{-5}, when tested with S9 (Figure 2.1B). The variability of the mutant frequency yielded by media controls across operators also appears quite low, however variability of the -S9 results from Operator 2 are large compared to the rest of the data sets. The background *lacZ* mutant frequency was lower for the media control compared to the solvent/vehicle, thus, it appears that DMSO may induce a small increase in mutant frequency. Importantly, although more variable, only 1 DMSO control replicate was outside the ±3SD acceptance range. Interestingly, Operator 2 generated quite deviant mutant frequency values for media-only controls without S9; however, the values did not exceed the ±3SD control limits, and other data generated from this operator are comparable to those associated with other operators. Operator 1 alone tested media-only controls +S9; thus, little variability was observed.

2.3.1.2 Positive Control Groups:

To ensure that the assay functioned as expected, duplicate, single-dose, positive controls were included in all experiments. More specifically, 0.1µg/mL BaP was employed as the positive control in experiments conducted without S9, and 1µg/mL PhIP was included as the positive control for experiments conducted with S9 metabolic activation. The BaP control group yielded an average mutant frequency of 561.4 x 10^{-5} (± 171.8, N=45), with an acceptable control range of 46.1 to 1076.8 x 10^{-5}. PhIP yielded an average of 67.1 x 10^{-5} (± 32.3, N=15), with an

acceptable range of -29.7 to 164.0 x 10^{-5} (Figure 2.2). There is visibly more variability across the positive controls in comparison with the medium or vehicle controls, this is not surprising since the magnitude of variability is expected to increase with increasing response level; the variance across assessments is still low. There is variability seen with duplicate samples of BaP generated by Operators 2 and 4, compared to the rest of the BaP control sample cohort; however, both data points are well within the control limit range, as are all other positive control data generated by Operator 2 (Figure 2.2A). Note that only one data set was generated by Operator 4. Upon writing this thesis, only 2 operators had generated data on PhIP positive controls. Although there is little variability across samples generated from the same operator, there is high variability across assessments by Operator 3, such that some values achieved *lacZ* mutant frequency values approximately 3x higher than other PhIP controls values.

2.3.2 Analysis of Test Chemicals:

The intra-laboratory variability study included screening of reference chemicals recommended by EURL-ECVAM for the evaluation of new mammalian genotoxicity assays (Kirkland et al. 2008). The first compound screened was the potent mutagen BaP without the addition of S9, providing the largest data cohort (i.e., 5 operators, 8 different test dates). ENU was also screened in the absence of S9, generating a data set from 3 operators on 5 different test dates. Known mutagens PhIP and DMN were tested in the presence of S9. PhIP was screened by 2 operators across 4 test dates; DMN by only Operator 1 on 3 different test dates. True negative compounds D-mannitol and ampicillin trihydrate, as well as false positive compounds phthalic anhydride and t-hydroquinone, were screened in the absence of S9 by 2 operators on 5 test dates.

To evaluate cross-operator and cross-date response variability, FE1 cells were exposed to BaP. When tested across 3 different laboratory trials in duplicate, the latter 3 doses were able to induce a significant increase in *lacZ* mutant frequency without the addition of exogenous metabolic activation (i.e., N=3 with 2 technical replicates). When ENU was screened across 3 assessments, all test concentrations elicited a significant increase in *lacZ* mutant frequency without the addition of exogenous metabolic activation. Summarised *lacZ* mutant frequency dose response results for compounds that do not require exogenous S9 are presented in Figure 2.3A.

Although FE1 cells have been shown to be metabolically competent in some respects, White et al. 2003 indicated that the cells are unable to convert PhIP into a DNA reactive metabolite; thus necessitating the addition of an exogenous metabolic activation mixture containing Aroclor 1254-induced rat liver S9. When FE1 were exposed to PhIP +S9 in duplicate, all tested concentrations induced a significant increase in *lacZ* mutant frequency. Summarised *lacZ* mutant frequency dose response results for compounds tested with exogenous S9 are illustrated in Figure 2.3B. DMN also requires addition of S9 for metabolic transformation of the compound into mutagenic metabolites. Testing of DMN (+S9) on 3 different test dates yielded negative responses at all test concentrations (Figure 2.3B). In an effort to screen DMN with elevated levels of S9 (i.e., >0.5% v/v), additional testing was conducted to assess the cytotoxicity of different S9 concentrations between 1 and 10% v/v. The additional assessments at S9 concentrations of 1, 2.5, 5, 7.5 and 10% v/v failed to elicit a positive response, presumably due to the high cytotoxicity of S9 at concentrations above 0.5% (data not shown). In other

words, it was not possible to evaluate responses to DMN, a compound that is known to induce mutations both *in vitro* and *in vivo* (Souliotis et al. 1998; Bean et al. 1994).

The non-mutagens ampicillin trihydrate and D-mannitol are EURL-ECVAM-recommended "true negative" test compounds screened to determine assay specificity (Kirkland et al. 2008). The compounds were screened in 3 different assessments in duplicate, and both compounds failed to elicit an increase in *lacZ* mutant frequency compared to concurrent controls at all tested concentrations (Figure 2.3C).

In vitro assays are often criticized for the high potential of generating false positives, i.e., positive responses that cannot be confirmed *in vivo*. Therefore, EURL-ECVAM recommends the testing of "false positive" compounds that have been shown to elicit a positive response *in vitro* despite a lack of ability to react with DNA (Kirkland et al. 2007; Kirkland et al. 2008). None of the concentrations elicited significant increases in *lacZ* mutant frequency compared to the concurrent control (Figure 2.3D).

2.3.3 Comparisons of Responses across Operator and/or Test Date:

Intra-laboratory variability analyses for the true negatives and the false positives revealed that both Operators 1 and 2 yielded negative responses, such that specificity of the assay is 100% (Table 2.2).

The intra-laboratory variability study of BaP without S9 involved comparison of 5 different operators and 8 different test dates. All assessments yielded significant positive responses (Table 2.2). The OECD validation guidelines only require this type of qualitative response comparison (i.e., dichotomous +/- classification); however, this study also employed the BMD approach to analyse the dose-response data, thereby assessing intra-laboratory

potency variability. Figure 2.4A illustrates the BMD_{10} values and associated confidence intervals (i.e., $BMDL_{10}$ and $BMDU_{10}$) for each individual BaP dose-response. Reproducibility of BaP-induced mutant frequency is illustrated as overlapping BMD confidence intervals. BMD analysis employing operator or test date as the covariates was employed to examine variability of chemically-induced mutant frequency dose responses attributable to operator or test date. Covariate analysis examining all 3 data sets from Operator 1 shows little variability in BMD across the data sets (Supplementary Figure 2.1A). Similarly, analysis across test dates for Operator 2 also shows little variability in BMD across the data sets (Supplementary Figure 2.1B). BMD covariate analysis on all BaP exposure data sets, employing operator (Supplementary Figure 2.1C) or test date (Supplementary Figure 2.1D) as a covariate shows that there is little variability in response across either variable.

The intra-laboratory variability study of PhIP with S9 is based on data from 2 different operators and 4 different test dates. All assessments yielded significant positive responses (Table 2.2). Quantitative dose-response analysis of individual data sets yielded results that revealed relatively little variation in BMD across operator and test date such that all BMD confidence limits overlap (Figure 2.4B). When the covariate approach was applied to data sets generated by Operator 1, BMD_{10} analysis shows little variability in response (Supplementary Figure 2.2A). However, when the data from Operators 1 and 2 are combined and analyzed using either operator (Supplementary Figure 2.2B) or test date (Supplementary Figure 2.2C) as covariates, the analyses show that the values generated by one operator are elevated relative to the other. Nevertheless, Figure 2.4B does not show any appreciable difference in BMD (i.e., mutagenic potency) across operators and/or test dates.

The intra-laboratory variability study of ENU without S9 is based on data from 3 different operators and 5 different test dates. All assessments yielded significant positive responses (Table 2.2). Quantitative dose-response analysis of individual data sets yielded BMD_{10} and confidence intervals (i.e., $BMDL_{10}$ and $BMDU_{10}$) that permitted assessment of cross-operator and/or test date variability. BMD analysis demonstrated that Operator 1 and 2/3 display marked differences in BMD, as indicated by the laterally distinct BMD confidence intervals (Figure 2.4C). Nevertheless, BMD conference intervals overlap for data generated by Operator 1, and similarly across Operators 2 and 3. When the covariate approach was applied to the 3 data sets from Operator 1, the results showed little variability in mutagenic potency (i.e., BMD) (Supplementary Figure 2.3A). Covariate analyses across all ENU data sets examining the effect of operator (Supplementary Figure 2.3B) or test date (Supplementary Figure 2.3C), improved the precision of the analysis, thus reducing the noteworthy cross-operator differences in BMD shown in Figure 2.4C. Similarly, the covariate analyses does not reveal any appreciable cross-date variability in mutagenic potency (i.e., BMD) (Supplementary Figure 2.3C).

2.4 Discussion:

Results published to date indicate that the *lacZ* transgene mutagenicity assay in MutaMouse FE1 cells is a promising new tool for routine regulatory assessments of chemically-induced genetic toxicity (White et al. 2003; Berndt-Weis et al. 2009; Shwed et al. 2010; Poulsen et al. 2013; Maertens et al. 2017). The cells are easily cultured and handled, cytogenetically stable, maintain functional P53, and retain sufficient metabolic capacity to convert noteworthy pro-mutagens (i.e., BaP) into DNA-reactive metabolites. Moreover, enumeration of chemically-induced transgene mutations in an exposed population of cells does not require laborious clonal selection and isolation. Nevertheless, the assay cannot be employed for routine regulatory assessments of genetic toxicity until it has been sufficiently validated. This validation, which is currently in its early stages, must include critical examinations of intra-laboratory variability, applicability domain, protocol transferability, and inter-laboratory variability. This study, which is contributing to the ongoing validation process, employed intra-laboratory variability analysis to assess reproducibility and reliability. More specifically, the study examined variability in responses to selected test articles across a series of operators and/or test dates. Variability in *lacZ* mutant frequency values for vehicle and untreated controls were also examined.

The OECD validation guidelines require compilation and scrutiny of all historical controls collected to date, including those that may be considered irregular, unless there is a "scientifically justified" reason for their omission (Hayashi et al. 2011). Consequently, Maertens et al. 2017 examined all controls collected at the time of publication (N=460); since then additional data have been compiled such that 653 observations are now available for

examination. Scrutiny of these data indicate that the values are log-normally distributed with a geometric mean of 47.8 x 10^{-5}, and variation between the 5th and 95th percentiles ranging from 23.5 x10^{-5} to 95.5 x10^{-5} (P.A. White, personal communication).

This study also examined variability in negative and positive control results across all operators and dates included in the intra-laboratory validation exercise. The purpose of the intra-laboratory validation is to scrutinise the reproducibility of the assay across operators and dates; thus, inclusion of all samples is especially important. The negative and positive control data (Figures 2.1 and 2.2) shows low overall variability across both dates (i.e., operator replicates) and operators (i.e., date replicates).

The geometric mean (± geometric standard deviation) of the negative control *lacZ* mutant frequency values generated as part of this intra-laboratory study is 35.4 x 10^{-5} (± 1.4, N=47) for DMSO solvent controls without S9, 34.0 x 10^{-5} (± 1.7, N=14) with S9, and 29.7 x 10^{-5} (± 1.4, N=34) for media-only controls without S9, and finally 27.6 x 10^{-5} (± 1.2, N=13) for media-only controls with S9 (see Figure 2.1). These values are all within the aforementioned, historical range (i.e., the 5th and 95th percentiles). The arithmetic central tendency (i.e., mean values shown in Figure 2.1) are also in line with the aforementioned historical control values that encompass all FE1 mutagenicity assessments conducted to date. The coefficient of variation (CV) for the historical control data was 52%; for the intra-lab DMSO control data it is 36% (-S9) and 64% (+S9), and for media-only control data it is 32% (-S9) and 21% (+S9). Note that the use of control limits expressed as ±3SD encompasses (i.e., as recommended in the literature) the range between approximately the 1st and 99th percentiles, and is less conservative than the historical control range examined herein (Hayashi et al. 2011). Furthermore, although 6

observations are outside the extremes of the aforementioned historical controls values, all but one fit within the ±3SD range. Thus, all negative controls can be deemed acceptable.

It is interesting to compare the aforementioned CV values to those published in the scientific literature. For example, intra-laboratory negative control values across 4 labs, which were generated as part of the validation of the *in vitro* flow cytometric micronucleus (MN) assay that assesses induced MN frequency in TK6 cells, revealed CV values ranging from 42.8% to 186% (Bryce et al. 2014). Similarly, the new *in vitro* 3D EpiDerm™ reconstructed skin MN assay (RSMN) (Table 1), that also employs flow cytometric detection of MN frequency changes, generated negative control (i.e., acetone) CV values of 21.5% (N=43), 24% (N=67) and 21% (N=47) within each of 3 labs (Hu et al. 2009). A comparison of control CV values are presented in Table 2.3. Additionally, Zeller et al. 2017 recently examined the CV of negative control data for several *in vivo* genotoxicity assays; values ranged from 9% to 174% across the different endpoints, with the lowest value corresponding to the *in vivo* flow cytometric MN assay. This exceptionally low variability has been attributed to the automated, high-throughput flow cytometry-based methods (Zeller et al. 2017). The average CV associated with *in vivo* Comet assay (i.e., DNA strand breaks) assessments across 5 tissues was 33.6%, the CV for the Pig-A gene mutation assay in red blood cells was 144% and 174% in reticulocytes, the CV associated with the microscopic MN assay was 39%, and the CV associated with the transgenic rodent assay in liver was 50% (Zeller et al. 2017). Thus, it is readily apparent that variability in negative control values observed within this intra-lab validation study are comparable to that observed for a range of *in vitro* and *in vivo* genotoxicity endpoints. In other words, variability in the negative control mutant frequency values for the FE1 cell *in vitro* transgene mutagenicity assay

(i.e., 21-64%) are comparable or better, relative to those associated with *in vivo* and *in vitro* genotoxicity assays already used for regulatory evaluations. Nevertheless, it should be noted that the FE1 cell assay sample size is relatively small; therefore, screening by more operators and test dates will be required to thoroughly assess variability in control responses.

The background *lacZ* mutant frequencies were essentially unaffected by the addition of exogenous S9; however, mutant frequencies values were lower for the media control compared to the solvent control. Thus, DMSO appears to induce a slight increase of *lacZ* mutant frequency in FE1 cells; moreover, an increase that has been shown to be statistically significant at $p<0.0001$ (N=359, increase in geometric mean response = 24%), (P.A. White, personal communication). Importantly, the solvent concentration was kept at 1% v/v in order to avoid cytotoxicity associated with the generation of reactive oxygen species (Timm et al. 2013). Interestingly, Timm et al. determined that different cell types respond differently to different concentrations of DMSO, such that some cell lines showed toxic effects, and others showed increased cellular activation at concentrations as low as 0.25 and 0.5% DMSO (Timm et al. 2013). Although the variability of the spontaneous mutant frequency values associated with the media-only and DMSO solvent controls are low (i.e., $<10 \times 10^{-5}$) and stable, it is plausible that DMSO has a slightly genotoxic effect in FE1 that can be deemed acceptable.

The geometric mean (± geometric standard deviation) of the *lacZ* mutant frequency generated by the positive controls examined in this intra-laboratory study were 537.1×10^{-5} (± 171.8, N=44) for BaP without S9 and 62.5×10^{-5} (± 32.3, N=14) for PhIP with exogenous S9 (Figure 2.2). All mutant frequency values generated by positive control samples are within the acceptable control limit of ±3SD. The CV for the BaP control was 31% (-S9) and 48% for PhIP

(+S9). More variability is expected across PhIP controls, in comparison with BaP controls, due to the requirement for S9 and cofactors (see below) (Cox et al. 2016). Pre-validation exercises for the *in vivo* MN assay in TK6 cells, that employed 500µM cyclophosphamide as the positive control, revealed a CV of 31%, such that the positive controls responses observed herein can be considered comparable and acceptable (Hégarat et al. 2014). A comparison of control CV values is presented in Table 2.3. Furthermore, the intra-laboratory positive controls were much less variable than those generated within different labs validating the RSMN assay; among which positive control CV values (i.e., 3µg/mL Mitomycin C) were 62% (N=32), 77% (N=35) and 84% (N=45). Note that the RSMN assay employs technologically-advanced flow cytometric scoring, yet the FE1 cell transgene mutagenicity positive control data were still less variable. The variation in response to a single dose positive control is therefore comparable to that of the positive controls examined for other OECD-endorsed *in vivo* and *in vitro* genotoxicity assays.

The intra-laboratory variability study subsequently investigated the cross-operator and date variability in responses to selected chemicals that have a known response profiles (i.e., known mutagens, known non-mutagens and false positives). Although not all operators tested all compounds, all compound classifications were consistent across all operators. Moreover, with the exception of DMN, all compounds were appropriately classified (Table 2.2). With respect to 3 of the 4 known mutagens, all operators produced results that showed significant elevation of *lacZ* mutant frequency values (i.e., BaP, PhIP and ENU, Figure 2.3); moreover, using the aforementioned criteria, the responses could clearly be classified as positive. Additionally, all operators appropriately yielded negative responses (i.e., no significant change in *lacZ* mutant frequency) for the known non-mutagens and the false positive compounds (Figure 2.3). More

specifically, none of the responses met the criteria for designation of a positive response (i.e., responses cannot be classified as positive).

Interestingly, the FE1 cell transgene mutagenicity assay was not able to detect elevated *lacZ* mutations in response to DMN exposure, even in the presence of Aroclor-induced rat liver S9. Although the compound was not expected to yield negative results, the response was consistent across operator such that the deficiency is likely due to the assay-compound combination. DMN is known to be a potent mutagen, and it is widely employed as a reference chemical for genotoxicity assays (Kirkland et al. 2008). The biotransformation of DMN into DNA reactive metabolites is known to require CYP2E1 monooxygenase (Awogbindin et al. 2014); thus, it may not be surprising that activation cannot be accomplished by FE1 cells. FE1 cells originated from murine lung, and CYP2E1 is generally not present in murine lung tissue (Renaud et al. 2011). Consequently, studies by Souliotis et al., which exposed MutaMouse adults to DMN, found significantly increased *lacZ* mutant frequencies in liver and spleen tissue, but no elevation in mutant frequency in bone marrow or lung (Souliotis et al. 1998). DMN is able to induce mutations in Salmonella (i.e., Ames test), and at both the *hprt* and *tk* loci in L5178Y Mouse Lymphoma Cells in the presence of S9 (Prival and Mitchell 1981; Clive et al. 1979). Thus, the addition of S9 should be sufficient to provide the enzyme activity necessary to biotransform the compound into a metabolite that is capable of inducing mutations in FE1 cells. Nevertheless, it should be noted that DMN can act via a clastogenic mode of action, such that it is able to induce chromosome aberrations (i.e., breaks, deletions, translocations, etc.) and chromatid exchanges in cells exposed in the presence of exogenous S9 (Natarajan et al. 1976). Extensive testing of the *lacZ* mutation scoring protocol employed for the MutaMouse

transgenic rodent assay noted that “bacteriophage-based systems are not efficient for detection of mutants containing deletions, particularly if these deletions extend into, or through, sequences necessary for phage propagation” (Lambert et al. 2005). Thus, given the metabolic requirements of DMN, and it’s propensity to cause chromosome breakage and large deletions, it is perhaps not surprising that the FE1 *in vitro* assay failed to detect a positive response. Consequently, future selection of compounds for intra- and inter-laboratory validation of the FE1 cell assay should pay careful attention to mode-of-action, so as to exclude known clastogens that would not be expected to yield a positive response in any assay that requires recovery of the λgt10 shuttle vector.

The FE1 cell transgene mutagenicity assay was able to detect ENU mutagenicity across all operators. ENU is a highly potent mutagen, able to induce both MN formation and significant increases in mutant frequency at the *HPRT* locus of human lymphoblastoid cells (i.e., AHH1) and at the *tk* locus in Mouse Lymphoma Cells (Doak et al. 2007). The compound is an alkylating agent, which is able to transfer its ethyl group to a ring-nitrogen or exocyclic oxygen on a DNA base, thereby creating covalent bonds that generate stable DNA adducts (Fu et al. 2012). For instance, ENU can react with the N^7- and O^6-positions on nucleophilic guanine; N^7-methylguanine can spontaneously form apurinic sites that are toxic and mutagenic, and O^6-methylgunanine can easily mispair with thymine eliciting mutations during DNA replication (Doak et al. 2007; Fu et al. 2012). Therefore, it can be assumed that ENU is able to induce alkylation of DNA in FE1 cells without the addition of exogenous metabolic activation; moreover, that this alkylation is responsible for the observed elevation in *lacZ* mutant frequency.

The FE1 cell assay was able to detect the 2 mutagenic test compounds that are known to require metabolic conversion to DNA-reactive metabolites (i.e., BaP and PhIP). While BaP elicited a clear positive response without exogenous activation, PhIP required the addition of exogenous activation (i.e., Aroclor-induced rat liver S9). The positive BaP responses were expected since White et al. 2003 have already shown a strong response in FE1 cells without S9, and demonstrated that the response is dependent on intracellular cytochrome P450 isozymes 1A1 and/or 1A2. Berndt-Weiss et al. 2009 further demonstrated that FE1 cells express both *Cyp1a1* and *1a2*; moreover, the expression of these genes is inducible by exposure to Ah receptor agonists (i.e., BaP). The addition of S9 is known to be required for the bioactivation of PhIP in both bacteria (i.e., Salmonella) and mammalian cells (i.e., V79 and CHO) (Holme et al. 1989; Thompson et al. 1987), and indeed it was previously shown to be required to elicit a positive response in FE1 cells (White et al. 2003). The metabolic activation of PhIP and similar heterocyclic amines are known to be dependent on both Phase I (i.e., CYP1A2) and Phase II (i.e., NAT1/2, SULT1A1) isozymes (Kaderlik et al. 1994). The Phase I reactions convert the parent compound into *N*-hydroxy-PhIP that can then be acetylated or sulfated by enzymes such as *N*-acetyltransferases (NAT) or sulfotransferases (SULT) (Hein et al. 1994; Glatt et al. 2004). Acetate and sulfate ions are excellent leaving groups that can readily generate highly reactive, electrophilic nitrenium or carbenium ions that rapidly react with nucleophilic DNA. Since Berndt-Weiss et al. 2009 noted that FE1 cells express *Cyp1a2*, it seems likely that the lack of conversion of PhIP to reactive mutagenic metabolites can be attributed to a deficiency in Phase II enzymes such as NAT and/or SULT (Kaderlik et al. 1994; Glatt et al. 2004). Moreover, since studies by Glatt et al., have shown that amino acid pyrolysis products such as PhIP generally

require SULT in order to generate DNA-reactive metabolites, it seems that FE1 cells may lack the capacity to express SULT isozymes (Glatt and Meinl 2005; Glatt et al. 2004). Given the interest in defining the applicability domain of the FE1 cell mutagenicity assay, further studies should examine the presence and levels of Phase II isozymes such as SULT. Nevertheless, it is important to emphasise that the ability to biotransform compounds such as BaP into DNA-reactive metabolites can be termed unique since all other mammalian and bacterial cell gene mutation assays require the addition of S9 for all compounds that are benign until metabolic activation by Phase I and/or Phase II enzymes. Thus, the FE1 cells can be viewed as superior in terms of their endogenous metabolic capacity and consequent ability to generate DNA-reactive metabolites that cannot be generated by the cells lines frequently employed for *in vitro* regulatory assessment of mammalian cell mutagenicity (i.e., TK6, V79, CHO, and L5178Y Mouse Lymphoma Cells).

True negative compounds D-mannitol and ampicillin trihydrate were classified as negative for induction of *lacZ* mutants (i.e., compared to concurrent negative controls) by all operators; the assay showed no qualitative variability across operator or test date. Thus, the assay shows good specificity for the analysis of non-mutagenic compounds.

False positive compounds phthalic anhydride and *tert*-buytlhydroquinone were classified as non-mutagenic in all cases; indicating high reproducibility across operator and test dates. Previous studies have found that despite a non-DNA reactive mode of action, phthalic anhydride is able to induce MN formation in L5178Y cells (-S9), and chromosome aberrations in CHO cells at high doses that generate a precipitate (-S9). Similarly, MN formation in TK6 cells was positive in some studies, but negative in others (Hilliard et al. 1998; Fowler et al. 2012;

Whitwell et al. 2015). Note that the highest concentrations of phthalic anhydride tested herein formed a precipitate, but the results still revealed a negative response. Exposure of V79 and CHO cells to *tert*-butylhydroquinone induces a significant increase in MN frequency, and similar responses were observed for TK6 cells exposed at high concentrations (Fowler et al. 2012); however, the compound did not induce a positive response in FE1 cells. *In vitro* assays have been criticized for the frequency of false positive classifications, and the ability of FE1 to successfully classify acknowledged false positive compounds indicates improved specificity of the assay compared to those currently used for regulatory purposes. Relatedly, the recent study by Maertens et al 2017 showed that the FE1 cell assay correctly classified an additional 7 compounds as *in vitro* false positives.

Overall, the aforementioned results are comparable, if not better, than those obtained during the validation of other *in vitro* genetic toxicity assays. Across the 8 compounds tested in this intra-laboratory variability study, the sensitivity of the FE1 cell transgene mutation assay is 75%, and the specificity is 100%. Note, that although 75% sensitivity may be regarded as low, the value is attributable to a single compound that can be termed an FE1 cell false negative (i.e., DMN). Consequently, examination of 8 compounds is not sufficient for precise estimation of sensitivity and specificity. FE1 assay sensitivity values are likely much higher than estimated herein, and it will be necessary to determine sensitivity across a considerably larger number of compounds. Nevertheless, FE1 results obtained to date are comparable to those generated during the validation of the high-throughput MN assay in primary human lymphocytes, which yielded sensitivity and specificity values ranging from 82-98% and 86-97%, respectively, across 32 compounds (Bryce et al. 2014). The results are also comparable to the GreenScreen

GADD45a-GFP genotoxicity assay that is currently under validation (Table 2.1), which yielded a sensitivity and specificity of 90% and 100%, respectively, across 75 compounds (Hastwell et al. 2006). Furthermore, during validation of the *tk* locus gene mutation assay in L5178Y Mouse Lymphoma Cells, the sensitivity and specificity were 77% and 83%, respectively (Amacher et al. 1980). Overall, as the FE1 cell *in vitro* transgene mutagenicity assay demonstrated comparable qualitative reproducibility (i.e., 100%) relative to other mammalian cell *in vitro* assays currently undergoing validation; it constitutes a reliable alternative to assays currently in use for regulatory screening.

In general, the FE1 cell assay results showed little potency variability for BaP (-S9), such that the range from the highest $BMDU_{10}$ and lowest $BMDL_{10}$ was 3-fold, and the majority of this variability is due to a single data set generated by Operator 4 (Figure 2.4A). The other 7 data sets only vary by 2-fold between the highest $BMDU_{10}$ and lowest $BMDL_{10.}$ Furthermore, all plotted BMDL to BMDU confidence intervals overlap to some extent. Since the true BMD could theoretically lie at any point on the line, overlapping intervals represent equipotency. Therefore, quantitative analysis of the BaP responses established high reproducibility, with response potencies that are independent of operator and/or test date.

When comparing the BMD for each PhIP data set (+S9), it is apparent that there is more variability across BMD confidence intervals in comparison with BaP, with larger confidence intervals, ranging 3-fold. Variability in the PhIP results are likely attributable to the necessary addition of S9 and the NADPH-generating cofactor mixture. S9 supplementation of the exposure media likely introduces variability due to enzymatic differences across lots of Sprague-Dawley rat liver S9. A recent study by Cox et al. 2016 described the variability of CYP1A1 and

CYP1A2 enzyme activity across 100 different lots of Aroclor 1254-induced S9; the 5th to 95th percentiles of EROD (ethoxyresorufin-O-deethylase) activity extended over an order of magnitude from 1000 to 12000 pmoles/min/mg protein. Thus, cross-lot variability of CYP1A2 enzymatic activity could contribute to the variability in PhIP-induced mutagenicity observed in this intra-lab study. Cox et al. also investigated variability in the Salmonella mutagenic potency of PhIP across 23 different S9 lots. The results showed that the potency values, which averaged 220.3 revertants/μg, showed a difference between the 25th and 75th percentiles that ranged over three orders of magnitude; thus confirming that the mutagenic potency of S9-activated PhIP can show considerable cross-study variability (Cox et al. 2016). In general, the PhIP BMD_{10} confidence intervals overlap, extending only 3-fold from highest to lowest (Figure 2.4B). Hence, with respect to a compound that requires exogenous metabolic activation, the intra-laboratory variability of the FE1 cell responses appears relatively low, providing further evidence of assay reproducibility. Note that Operator 3 carried out the PhIP assessments in 2001, prior to development of standardized cell culture and exposure protocols; it is notable that the assay has been able to generate reproducible results across 15 years of analyses. Moreover, variability across operators, even those 15 years apart, is less than the variability yielded by a single operator (i.e., Operator 4); again demonstrating the robustness of the assay.

The screening of ENU (-S9) revealed greater BMD variability in comparison with other tested chemicals. There appears to be 2 groupings of data sets, with Operator 1 achieving a BMD_{10} approximately 2-fold lower than those yielded by Operators 2 and 3 (Figure 2.4C). Among each grouping the variability is quite low; however, the overall range in BMD_{10} confidence limits was large, ranging approximately 5 orders of magnitude across operators and

test dates. This is mainly due to the large confidence interval associated with Operator 2. The range in BMD values, especially the discrepancy between Operator 1 and Operators 2 and 3, can likely be attributed to variability in the time it takes to complete the cellular exposures. ENU is sensitive to light, humidity and pH such that the compound has a half-life of only 34 minutes at pH 7.0, 37°C (Soewarto et al. 2003). Consequently, ENU is not stable in DMSO, and the length of time taken for preparation of chemical dilutions (i.e., prior to exposure) will impact the BMD. Operator 1 ensured that all ENU dilutions were completed immediately before addition to the exposure medium, while Operators 2 and 3 may have diluted ENU in DMSO prior to aliquoting the media preparation in Falcon tubes and/or adding the exposure medium to the culture plates. Thus, the Operator 1 results likely show a smaller (i.e., more potent) and less variable response (i.e., lower and more consistent BMD) because the unstable compound was applied to FE1 cell cultures more quickly relative to Operators 2 and 3. Overall, the BMD_{10} values associated with each individual BaP and PhIP data set demonstrate low intra-laboratory variability, and thus high reproducibility of the assay. In contrast, ENU induced mutant frequencies showed 2 cross-operator groupings of BMD_{10}; however, the variability within each grouping was low.

To further investigate intra-laboratory variability, the BMD covariate approach was employed to simultaneously analyse multiple data sets. The covariate approach increases the precision of the BMD values, since all dose-response data sets included in the analysis contribute information towards the shape of the function; reducing BMD confidence intervals and permitting robust comparisons across selected covariates (Slob and Setzer 2014). The approach is well suited to comparisons across variables such as compound, test date, operator,

sex, and tissue. For example, a recent publication by Wills. et al. utilized the covariate approach to compare BMD values for *in vitro* MN data across cell type (i.e., MCL-5 and AHH-1 human lymphoblastoid cells) and compound (Wills et al. 2016a). Employing compound or cell line as covariates, the results revealed distinct (i.e., non-overlapping) confidence intervals reflecting valid differences in potency. This showed that potency was greater for the MCL-5 cell line, likely due the metabolic competency of the cell line; the compound-potency pattern suggested different mutagenic modes of action for the different dyes tested (Wills et al. 2016a). The aforementioned study by Johnson et al. employed laboratory as the covariate in their analysis of Pig-A mutant frequency data generated across 13 labs that screened ENU (Johnson et al. 2016). The results showed that the BMD confidence intervals overlap one another to some extent, thus establishing that mutant frequency (i.e., potency) was reproducible across labs (Johnson et al. 2016). These study results are analogous to those presented herein whereby operator or test date were used as covariates in the analyses of FE1 cell mutagenic potency values (i.e., BMD_{10}). The covariate approach increased the precision of the BMD values assessed across all operators and test dates, with the range of BMD_{10} reduced to approximately 2.5-fold for the BaP exposures, and 3-fold for ENU exposures. Consequent BMD confidence intervals overlap; thus demonstrating reproducibility of the assay. The PhIP-induced mutant frequency dose-response data also showed increased BMD precision when employing operator as the covariate (i.e., 2-fold range from lowest BMDL to highest BMDU). However, utilizing test date as a covariate increased the range of the PhIP potency values (i.e., 4.5-fold range); nevertheless, all confidence intervals overlap. The PhIP results suggest that the higher BMD values yielded by Operator 3 (see Supplemental Figure 2.2) are due to higher mutant frequency values obtained

throughout the entire assay, including the controls. This may be due to variations in the enzymatic activity of the Aroclor 1254-induced S9 lots employed across the 15 year date differences. As more operators within the laboratory complete the assay, the data set generated by Operator 3 may be deemed more or less acceptable based on control limit values. Essentially, employing operator or test date as a covariate generally improved BMD precision and cross-BMD comparisons showed that operator and test date have no (i.e., BaP) or minimal (i.e., PhIP) effect on the reproducibility of the assay. More specifically, overlapping compound-specific *lacZ* mutant frequency across operators in the same laboratory supports the assertion that intra-laboratory reproducibility of the FE1 cell assay is acceptable.

In summary, intra-laboratory evaluation of the *in vitro* transgene mutagenicity assay in MutaMouse FE1 cells showed that responses to negative and positive control samples were consistent across operator and test date; moreover, variability of control values is similar to that observed for other OECD-endorsed *in vivo* and *in vitro* genotoxicity tests. The FE1 cell assay was able to correctly classify EURL-ECVAM recommended true positive compounds BaP (-S9), ENU (-S9) and PhIP (+S9) as mutagenic, and all true negative and false positive compounds as non-mutagenic. The ability of the FE1 cell line to correctly classify compounds without the addition of exogenous metabolic activation is unique, and superior to any of the mammalian cell lines currently specified in the relevant OECD Test Guidelines. The assay was not able to correctly classify DMN (+S9); thus, the ability to screen compounds with known clastogenic activity can be considered a limitation of the assay. An intra-laboratory variability comparison determined that operators testing on different dates all achieved the same qualitative positive or negative *lacZ* mutant frequency results for all compounds, with a specificity of 100% and

sensitivity of 75%. The OECD only requires qualitative analysis of intra-laboratory results; therefore, as all operators achieved the same positive or negative results when testing recommended compounds, the *in vitro* transgene mutagenicity assay can be denoted as ready to progress to inter-laboratory variability analyses. BMD analysis of individual data sets showed little variability of dose-response across operators and dates for BaP and PhIP, with considerable cross-operator variability in BMD (i.e., approximately 5 orders of magnitude) for ENU. Implementation of the BMD covariate approach increased the precision of the analysis such that it can be determined that the operator had no effect, while test date did have a small effect on the variability of the assay. However, all confidence intervals were overlapping, thus demonstrating equipotency and reproducibility. Overall, the results show adequate reproducibility and reliability, suggesting that the assay may be a suitable alternative to currently-employed regulatory assays; and moreover, is a worthy candidate for continued validation. Nevertheless, the study also revealed that it will be necessary to expand the current intra-laboratory analyses to include additional operators and test dates, as well as a wider range of compounds such as those analysed in Chapter 3. Analyses of a wider range of compounds will contribute to definition of the applicability domain.

2.5 Tables and Figures:

Table 2.1 Summary of *in vitro* genotoxicity assays that have recently undergone, or are currently undergoing, validation for the development of an OECD test guideline. Abbreviations are as follows: TK6 - human lymphoblastoid cells, CHO - Chinese Hamster ovary cells, HepG2 - human hepatoblastoma cells, HuLy - primary human lymphocytes, MN-micronucleus, mES - mouse embryonic stem cells, HCT116 - human colon carcinoma cell line, GFP - green fluorescence protein.

Assay Name	End-point Expressed	Cell Types	Validation Status	References
***In vitro* Micronucleus Assay (Flow Cytometric)**	Chromosome damage, measured as MN induction	Several, including TK6, CHO, HepG2, HuLy	Intra-laboratory, Transferability, & Inter-laboratory, OECD TG No. 487	(Bryce et al. 2010; Thougaard et al. 2014; Sobol et al. 2012; Westerink et al. 2011; Lukamowicz et al. 2011; Lukamowicz-Rajska et al. 2012)
ToxTracker	DNA damage, general cell stress, oxidative stress, and protein stress (i.e., protein unfolding), measured as induced fluorescence of genetic biomarkers	mES used to generate novel GFP-based reporter cell lines	Intra-laboratory and Transferability. Inter-laboratory validation study is underway	(Hendriks et al. 2016; Hendriks 2017)
Anthems Genotoxicity Screen	DNA damage, measured by changes in expression of DNA damage genes p21, GADD153 and p53.	HCT116	Intra-laboratory	(Rajakrishna et al. 2014)
3D EpiDerm™ Human Reconstructed Skin MN Assay	Chromosome damage, measured as MN induction	Reconstructed skin models generated from primary human keratinocytes	Intra-laboratory, Transferability, and Inter-laboratory	(Chapman et al. 2014; Aardema et al. 2010; Hu et al. 2009)

Hen's Egg MN Test (HET-MN)	Chromosome damage, measured as MN induction	Exposure of inner shell membrane of hen's egg, MN assessed in nucleated peripheral erythrocytes	Intra-laboratory & Transferability. Inter-laboratory validation study underway.	(Hothorn et al. 2013; Greywe et al. 2012)
γH2AX In-cell Western Assay	DNA damage, measures H2AX phosphorylation that occurs following a DNA double strand break	HepG2	Intra-laboratory	(Khoury, Laure, Zalko, Daniel and Audebert, Marc 2013)
GreenScreen	DNA damage, measures GFP fluorescence associated with the human reporter gene GADD45a involved in growth arrest and DNA damage response	TK6	Intra-laboratory, Transferability, & Inter-laboratory	(Hastwell et al. 2006; Billinton et al. 2010; Billinton et al. 2008; Birrell et al. 2010)
***In vitro* Alkaline Elution Assay**	DNA damage, the fraction of DNA remaining on a filter after elution. Measures DNA strand breaks	Primary rat hepatocytes	Intra-laboratory	(Gealy et al. 2007)
***In vitro* Comet Assay**	DNA damage, via cell lysis and gel electrophoresis with fluorescence detection. Intensity of comet head relative to comet tail after electrophoresis measures the number of DNA breaks.	HepaRG L5178Y, CHO, CHL	Intra-laboratory, & Inter-laboratory (Not officially under OECD validation)	(Tice et al. 2000; Hégarat et al. 2014)
Vitotox™	DNA damage, luminescent quantification of luciferase measures the free ends of DNA breaks and DNA mismatches	*Salmonella typhimurium*	Intra-laboratory	(Westerink et al. 2009)

RadarScreen	DNA damage, luminescent quantification of luciferase and galactose expression associated with repair of double strand breaks by homologous recombination	Yeast (SKAM4)	Intra-laboratory	(Westerink et al. 2009)
Luciferase Reporter Assays	DNA damage and oxidative stress, quantification of luciferase gene expression controlled by promoter regions of RAD51C (DNA damage) and Cystatin A (DNA repair), as well as response elements for p53 (DNA damage) and Nrf2 (oxidative stress).	HepG2	Intra-laboratory	(Westerink et al. 2010)

Table 2.2 Qualitative summary of intra-laboratory validation results obtained to date. A positive result is indicated by (+) and a negative result by (-). Not all operators tested all compounds.

Reference Test Chemicals:		**Induced LacZ Mutant Frequency Response in FE1 Cells**							
		Operator 1			Operator 2		Operator 3	Operator 4	Operator 5
Group 1: True Positives	BaP	+	+	+	+	+	+	+	+
	PhIP	+	+	+			+		
	ENU	+	+	+	+		+		
	DMN	-	-	-					
Group 2: True Negatives	D-mannitol	-	-	-	-	-			
	Ampicillin trihydrate	-	-	-	-	-			
Group 3: False Positives	*Tert*-butylhydroquinone	-	-	-	-	-			
	Phthalic anhydride	-	-	-	-	-			

Table 2.3 Summary of coefficient of variation values associated with the testing of negative and positive control compounds. Abbreviations are as follows: CV- coefficient of variation, MN- micronucleus, FE1- Flat Epithelial Isolate 1, TK6- human lymphoblastoid cells, BaP- benzo[*a*]pyrene, PhIP- 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, RBC- red blood cells, RET- reticulocytes, DMSO- dimethyl sulfoxide, N/A- not available.

Assay Name	CV	Control Substance	Reference
Negative Controls			
***In Vitro* Flow Cytometric MN Assay**	42.8% - 186%	DMSO (1%)	Bryce et al. 2014
***In Vitro* 3D EpiDerm™ Reconstructed Skin MN Assay**	21% - 24%	Acetone	Hu et al. 2009
***In Vivo* Flow Cytometric MN Assay**	9%	N/A	Zeller et al. 2017
***In Vivo* Comet Assay**	33.6% (average of liver, blood, stomach, jejunum, duodenum)	N/A	Zeller et al. 2017
Pig-A Gene Mutation Assay	144% in RBC 174% in RET	N/A	Zeller et al. 2017
***In Vivo* Microscopic MN assay**	39% (bone marrow)	N/A	Zeller et al. 2017
Transgenic Rodent Assay	50% (liver)	N/A	Zeller et al. 2017
FE1 Cell Transgene Mutagenicity Assay	21-64%	DMSO (1%)	Present Study
Positive Controls			
***in vivo* MN assay in TK6 cells**	31%	500µM cyclophosphamide	Hégarat et al. 2014
***In Vitro* 3D EpiDerm™ Reconstructed Skin MN Assay**	62% - 84%	3µg/mL Mitomycin C	Hu et al. 2009
FE1 Cell Transgene Mutagenicity Assay	31%	BaP 0.1µg/mL (-S9)	Present Study
FE1 Cell Transgene Mutagenicity Assay	48%	PhIP 1 µg/mL (+S9)	Present Study

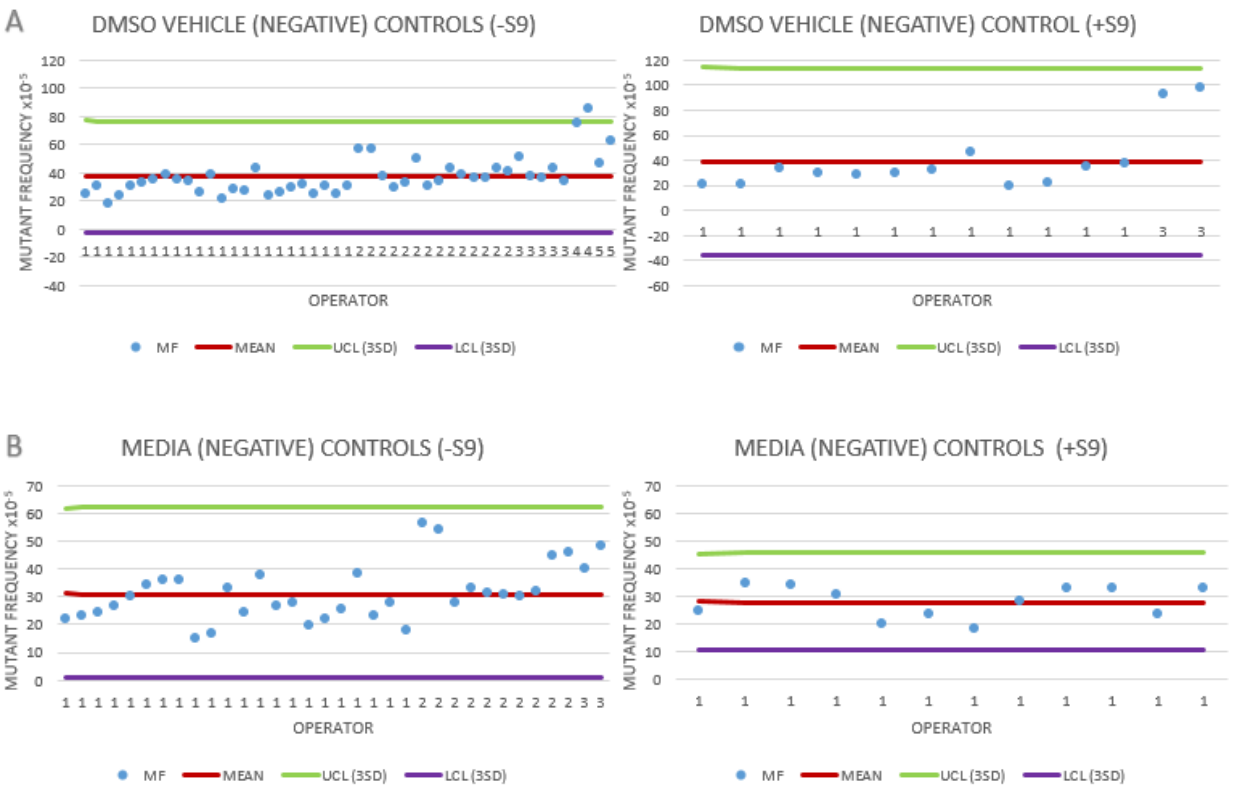


Figure 2.1 Comparison of negative control *lacZ* mutant frequency values across operators and test dates. Negative controls generated during the intra-laboratory validation study include the DMSO vehicle control group and media-only controls tested both with and without the addition of Aroclor 1254-induced S9 rat liver extract. The blue dots indicate measured mutant frequency (MF) on the y-axis achieved by each operator on each test day (x-axis). Red lines indicate the mean value across all experiments, green and purple lines show the UCL (mean + 3SD) and LCL (mean - 3SD) values, respectively.

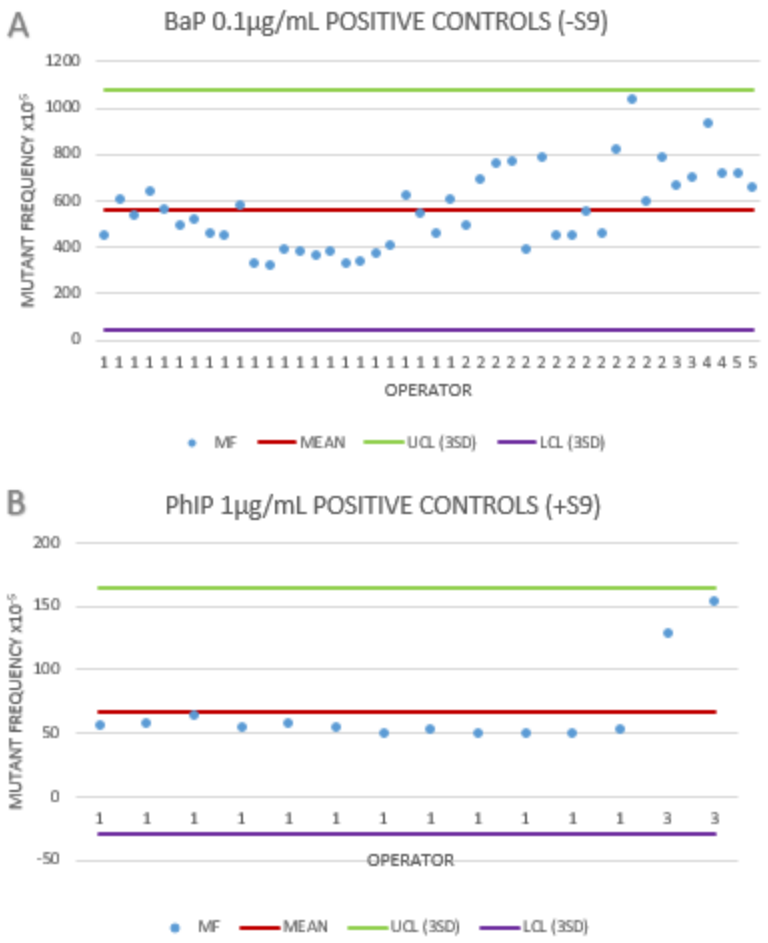


Figure 2.2 Cross-operator and/or test day comparisons of the *lacZ* mutant frequency values for the positive controls. BaP (i.e., 0.1µg/mL) was employed as the positive control without S9, and PhIP (1µg/mL) was employed with S9. Blue dots indicate the measured mutant frequency (MF) for each test. Red lines indicate the mean, green and purple lines show the UCL (mean + 3SD) and LCL (mean - 3SD) values, respectively.

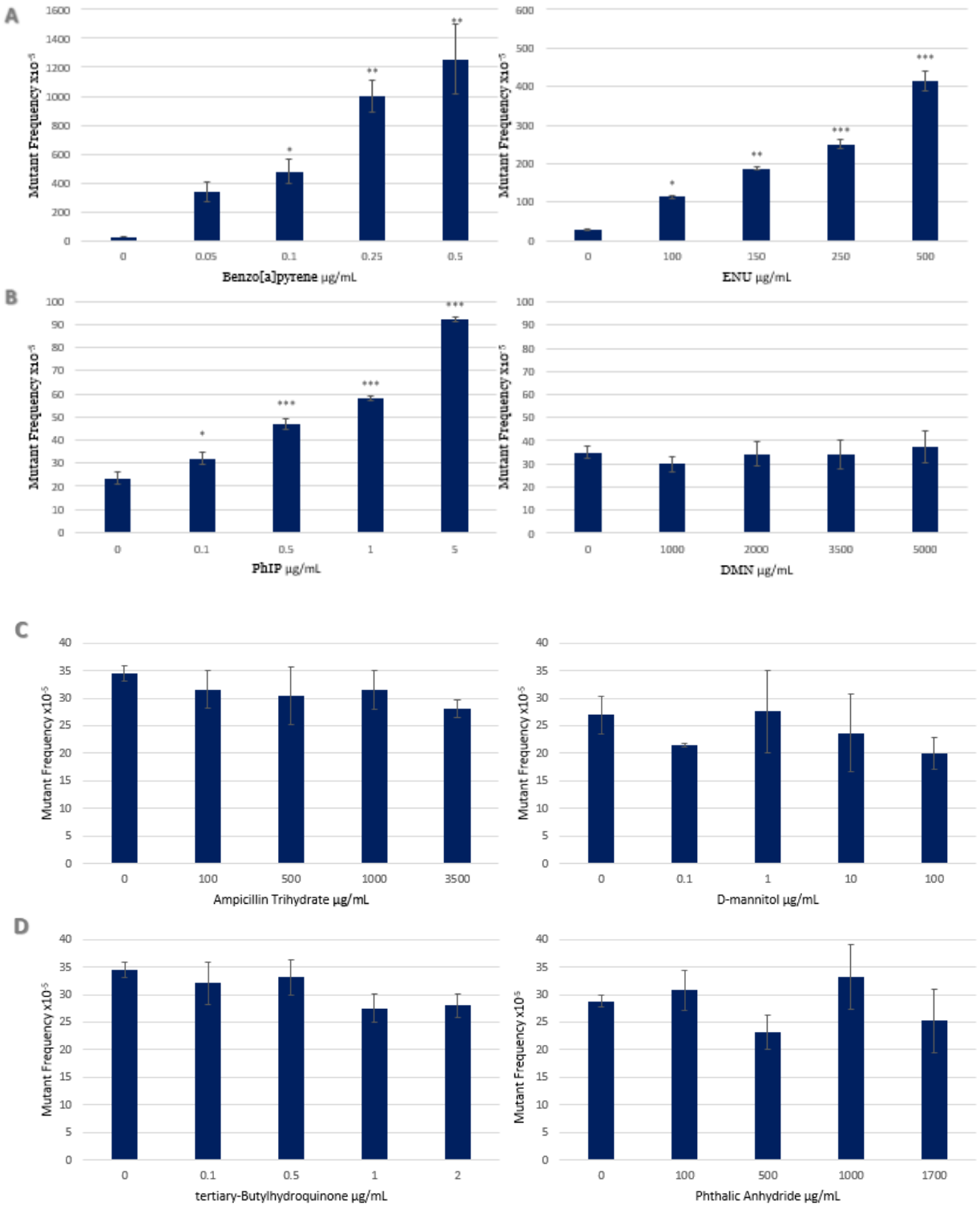
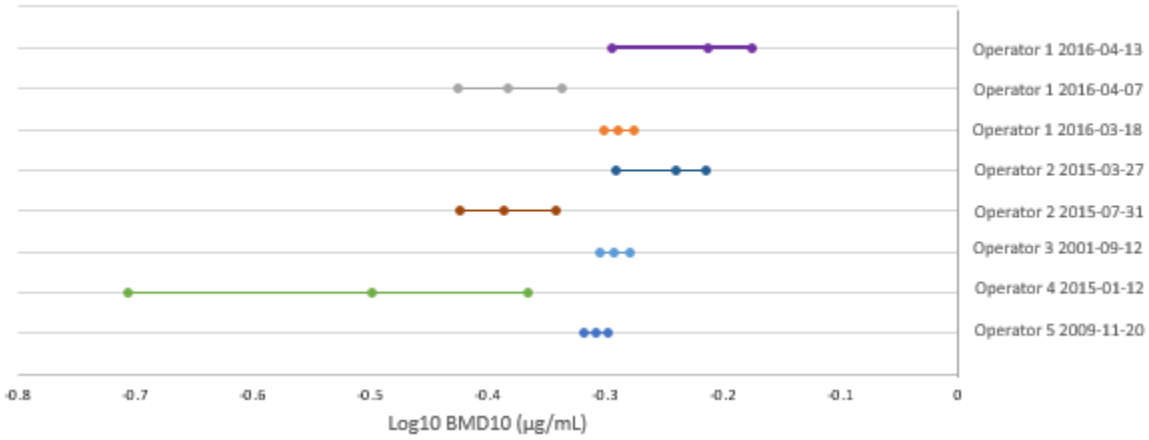


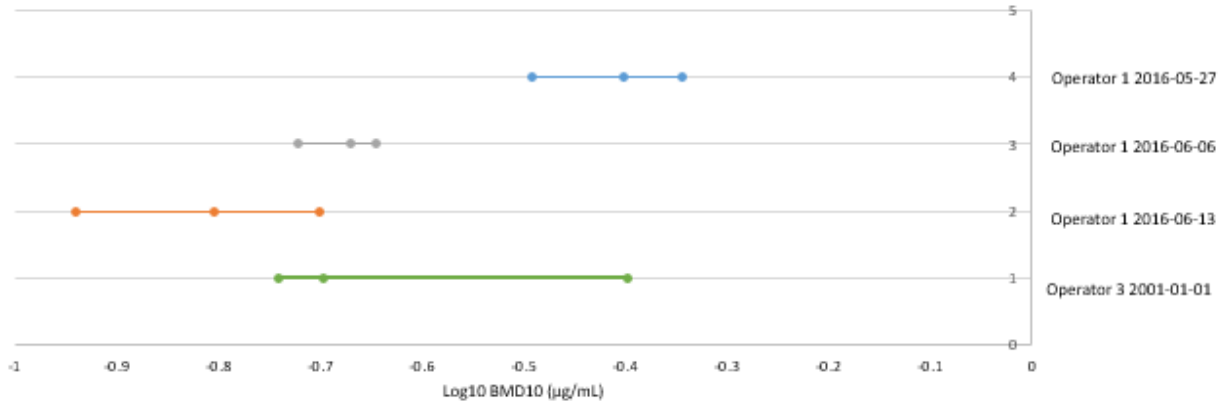
Figure 2.3 Intra-laboratory validation of 8 ECVAM reference chemicals tested by Operator 1, using the MutaMouse FE1 cell *in vitro* transgene mutation assay. Panels A and B show the responses to two "true positive" compounds tested without S9 (i.e., BaP and ENU), and two

'true positive' compounds tested with S9 (i.e., PhIP and DMN), respectively. Panel C shows the responses to two "true negative" compounds tested without S9 (i.e., ampicillin trihydrate and D-mannitol). Panel D shows the responses to two '"false positive" compounds tested without S9 (i.e., *tert*-butylhydroquinone and phthalic anhydride). Each chemical was tested in duplicate in three separate experiments (i.e., 3 biological replicates). Error bars show the standard error of the mean. Symbols above the bars show the results of statistical comparisons with concurrent vehicle (solvent) control. *P<0.05, **P<0.01, and ***P<0.001.

A Cross-operator and Test Date Comparisons of BaP Mutagenic Potency



B Cross-operator and Test Date Comparisons of PhIP Mutagenic Potency



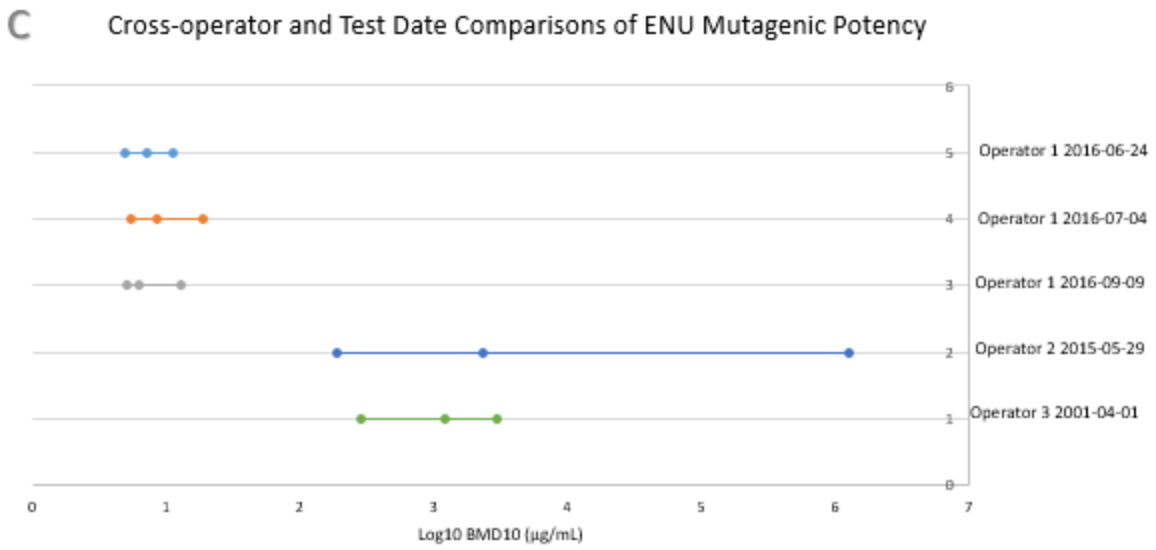
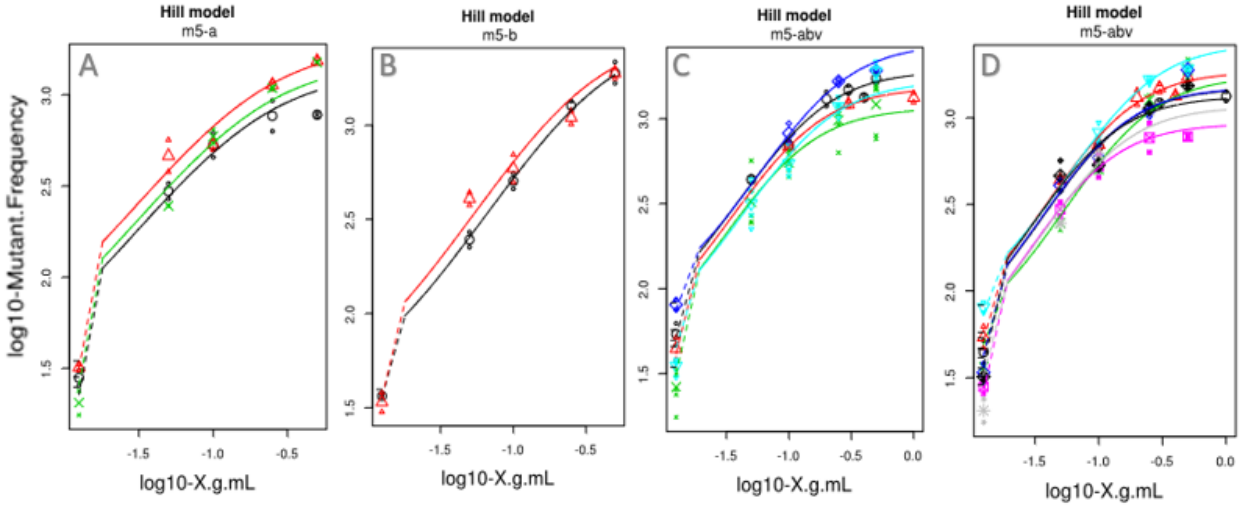
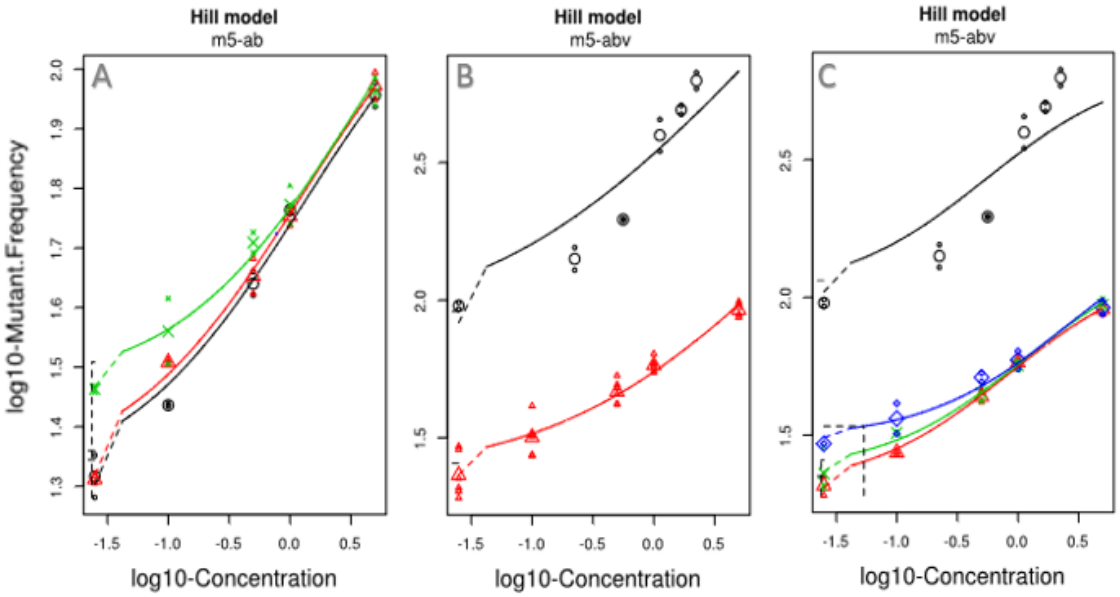


Figure 2.4 Intra-laboratory variability and reproducibility of the *in vitro* FE1 cell mutagenicity assay. The BMD approach was used to determine mutagenic potency, and potency values expressed as BMD_{10} were compared across operators and test dates. Results are shown for (A) BaP without activation, (B) PhIP with activation (i.e., 0.5% S9 v/v), and (C) ENU without activation. The central point shows the BMD, with the right and left extremes showing the BMDU and BMDL values, respectively.

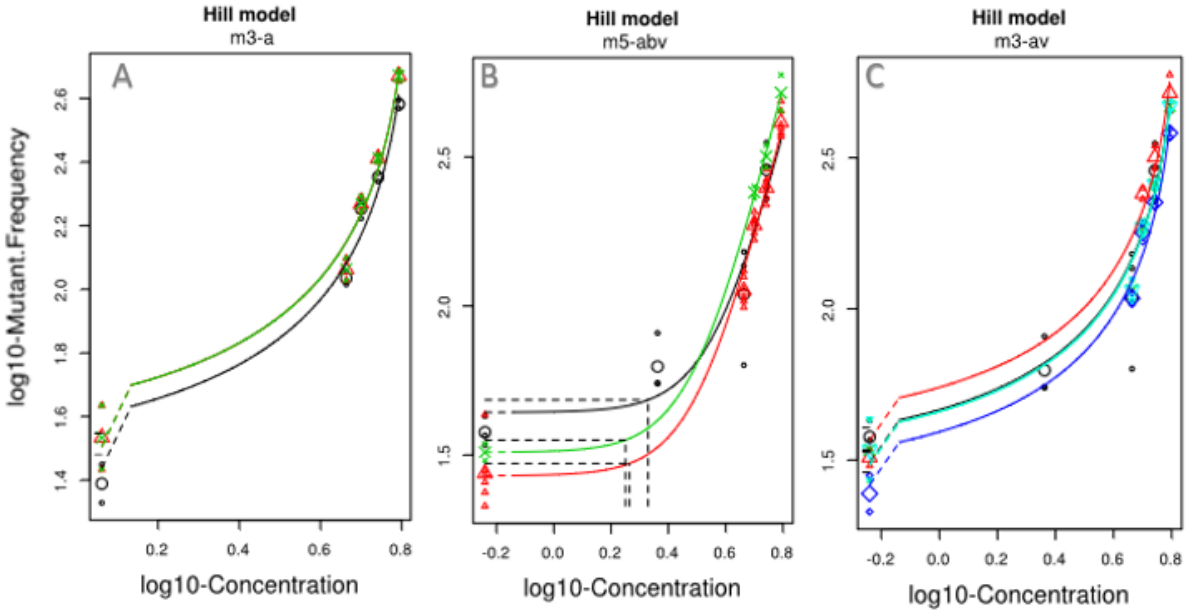
2.6 Supplementary Data:



Supplementary Figure 2.1. BMD covariate analysis of *lacZ* mutant frequency changes induced by BaP exposure. (A) Dose-response relationship variability across test dates for Operator 1 only, and (B) Operator 2 only. (C) Comparison of dose-response relationship across all operators, and (D) across all test dates. PROAST was employed to determine BMD_{10} values, and the Hill model was selected as the most appropriate for each data set (i.e., model m5-a in panel A, m5-b in panel B, and m5-abv in panels C and D).



Supplementary Figure 2.2 BMD covariate analysis of the *lacZ* mutant frequency changes induced by PhIP exposure. (A) Dose-response relationship variability for Operator 1 only. (B) Dose-response relationship variability across operators, and (C) across test dates. PROAST was employed to determine BMD_{10} values, and the Hill model was selected as the most appropriate model for each data set (model m5-ab for panel A and m5-abv for panels B and C).



Supplementary Figure 2.3 BMD covariate analysis of the *lacZ* mutant frequency induced by ENU exposure. (A) Dose-response relationship variability for Operator 1 only. (B) Dose-response relationship variability across operators, and (C) across test dates. PROAST was employed to determine BMD_{10} values, and the Hill model was selected as the most appropriate model for each data set (model m5-ab for panel A and m5-abv for panels B and C (model m3-a in panel A, m5-abv in panel B, and m3-av in panel C).

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Chapter 3: Xenobiotic-induced Gene Expression Changes in MutaMouse FE1 Pulmonary Epithelial Cells.

Abstract

In vitro genetic toxicity assays are often criticised for using cultured mammalian cells that lack the endogenous metabolic capacity to transform compounds into DNA-reactive metabolites. In contrast, the MutaMouse FE1 cell line, which is the cornerstone of a novel *in vitro* assay to assess chemically-induced mutations, has sufficient endogenous capacity to metabolise and activate the mutagenic carcinogen benzo[*a*]pyrene. The performance of the FE1 cell transgene mutagenicity assay is currently being scrutinised and validated. To characterize the ability of FE1 cells to endogenously metabolize several chemically-distinct mutagens, the expression of 168 genes involved in Phase I and Phase II xenobiotic metabolism was assessed following exposures to 1-methylpyrene (1-MP), 2-acetylaminofluorene (2-AAF), aflatoxin B1 (AFB1), 7,12-dimethylbenz[*a*]anthracene (DMBA) and 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone (NNK). Phase I cytochrome P450 isozymes are required for metabolic activation of DMBA, NNK and AFB1; Phase II sulfotransferase and UDP-glucuronosyltransferases are required for activation of 1-MP and 2-AAF. DMBA, AFB1 and 2-AAF induced a significant increase in *lacZ* transgene mutations both with and without exogenous metabolic activation; 1-MP and NNK yielded negative results. The companion gene expression analysis confirmed that FE1 cells possess the Phase I and Phase II metabolic capacity required to generate DNA-reactive metabolites of compounds such as DMBA, AFB1 and 2-AAF. More specifically, they retain significant, chemically-inducible cytochrome P450 isozymes, sulfotransferases, UDP-glucuronosyltransferases and glutathione-*S*-transferases. In contrast, FE1 cells do not possess the metabolic capacity to generate DNA-reactive metabolites of 1-MP and NNK (i.e.,

cytochrome P450 isozymes 2E1, 2A4 and 2A5). Although complete characterization of the FE1 assay's applicability domain will require similar screening of additional compounds, the data obtained to date confirms that the cell line is metabolically superior to those employed in mammalian cell-based assays routinely used for regulatory screening (e.g., CHO, V79, L5178Y Mouse Lymphoma, and TK6).

3.1 Introduction:

Toxicological evaluations of chemicals in commerce in Canada are required under existing legislation (i.e., the New Substance Notification Regulations specified in the Canadian Environmental Protection Act (CEPA) (Minister of Justice 2015)). Toxicological assessment routinely involves determination of a compound's ability to adversely interact with genetic material (i.e., genetic toxicity). In some cases, adverse interaction with DNA causes permanent sequence changes (i.e., mutation), and/or permanent changes in chromosome structure (i.e., chromosomal aberrations), and/or permanent changes in chromosome number (i.e., aneuploidy). The potential of compounds to cause mutation and/or chromosomal alterations requires toxicological assessment using one of several *in vitro* and/or *in vivo* bioassays that have been approved and standardized by international organizations such as the OECD (Organisation for Economic Cooperation and Development) (Eskes and Whelan 2016). Guidelines such as the *OECD Guidelines for the Testing of Chemicals* describe approved tests; moreover, provide detailed instructions for the proper execution of tests used to evaluate the likelihood that chemicals will adversely affect human and/or environmental health (Eskes and Whelan 2016).

Chemical safety assessments, such as those that employ OECD-approved tests recommended under CEPA, can be laborious and expensive. Accordingly, OECD guidelines only include assays that have been sufficiently validated to ensure reliability, utility, performance and relevance, thus ensuring that investments of time and funding are judicious. Accordingly, assay developers must provide evidence that assays for regulatory evaluations have been adequately validated, and this validation must include determination of an assay's applicability domain, i.e., the range and/or classes of chemicals the assay can reliably detect. The greater the

range of chemicals that can be successfully screened and detected, the greater the *applicability domain* (Eskes and Whelan 2016), and, by extension, the greater the utility for regulatory assessment of toxicological hazard.

During assay development and validation, cost and complexity are also considered, as is the ability of the assay to contribute to the replacement, refinement or reduction of animal usage (3R's) (Eskes and Whelan 2016). The latter is in response to international initiatives and directives such as European Union Directive 86/609/EEC, which prohibits the use of animal tests for toxicity assessment when suitable alternatives are available. Numerous agencies/organisations currently advocate the adoption of novel alternatives to animal testing; these include the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), the OECD, the International Cooperation on Alternative Test Methods (ICATM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM), and numerous others. The organisations and related initiatives officially acknowledge concerns regarding animal testing, and the need for *in vitro* alternatives for effective and efficient genetic toxicity assessment. Consequently, there is great interest in the development and validation of effective and affordable *in vitro* alternatives for genetic toxicity assessment.

The use of cultured cells, including immortalised cell lines and/or primary cells, is an accessible way to reduce or replace animals. However, replacement and reduction via the use of cultured cells is often challenging since cultured animal cells, especially immortalised or transformed cells, undergo substantial physiological and morphological changes for adaptation to survival in culture plates. These changes commonly include genetic aberrations (i.e.,

polyploidy), as well as structural, biochemical and metabolic changes (Berndt-Weis et al. 2009). The latter can include adaptations that result in loss of the ability to metabolise xenobiotic chemicals. Some potent mutagens are benign until they are metabolically converted into DNA-reactive substances (i.e., promutagens), and, as noted in Chapter 1, compounds such as the mutagenic carcinogen benzo[*a*]pyrene (BaP) cannot interact with DNA until enzymatically converted into one of several reactive metabolites (i.e., benzo(*a*)pyrene-diol-epoxide or BPDE) (Klaassen 2008). Therefore, sound *in vitro* alternatives based on mammalian cell lines or primary cells should have the capability to manifest mammalian enzymatic processes that would be expected to occur *in vivo* in whole animal models.

Mammalian metabolism of xenobiotic organic chemicals is generally divided into two stages, typically referred to as Phase I and Phase II metabolism. Phase I metabolism includes a variety of enzymatic processes that prepare a compound for Phase II conjugation reactions that generally precede excretion via the urine or bile (Klaassen 2008). The principle Phase I metabolic pathways involve oxidation of the xenobiotic compound, a reaction often catalysed by isozymes of the cytochrome P450 monooxygenase family (i.e., CYP1A1, CYP2C, CYP3A4; Table 3.1). Generally, Phase I oxidation produces a more polar compound that is prepared for Phase II conjugation to an endogenous substrate such as glutathione, sulfate or UDP-glucuronic acid (uridine diphosphate-glucuronic acid) (Guengerich 2008; Glatt and Meinl 2005). Table 3.1 lists representative types of metabolic reactions, and the common groups of genes and enzymes implicated. Phase II conjugation, which produces soluble metabolites that can be excreted via the bile or urine, involve enzymes such as sulfotransferases (i.e., SULT), glutathione-*S*- transferases (i.e., GST), or UDP-glucuronosyltransferases (i.e., UGT) (Table 3.1)

(Klaassen 2008; Luch 2005). Importantly, although the primary purpose of Phase I and Phase II reactions is detoxification and excretion; these reactions can generate metabolites that can interact with endogenous macromolecules such as DNA and protein. In fact, as suggested above and noted Chapter 1, many xenobiotic compounds, including polycyclic aromatic hydrocarbons (PAH's), aromatic amines, nitrosamines, and aflatoxins, are mutagenic only following metabolic conversion into reactive metabolites that can form stable DNA adducts (i.e., DNA bases covalently linked to metabolite). Adducts can contribute to the establishment and accumulation of mutations; moreover, contribute to tumour establishment and progression (i.e., carcinogenesis) (Glatt et al. 2004). Thus, metabolic processing in mammalian cells is essential to generate highly reactive intermediates that are known mutagenic carcinogens.

Cellular exposures to genotoxic substances can induce a range of physiological changes related to compound metabolism and detoxification, as well as changes related to DNA repair and cell cycle control. Interestingly, several of the Phase I metabolic pathways can be chemically augmented via ligand binding with receptors such the Aryl hydrocarbon receptor (AhR), the Pregnane X receptor (PXR), and the Constitutive Androstane receptor (CAR), with binding up-regulating the expression of an array of metabolism-related genes. For example, Berndt-Weis et al. 2009 showed that exposure of MutaMouse FE1 cells to BaP increases expression of *Cyp1a1.* Furthermore, *in vitro* exposure to agents such as AFB1 and BaP induce changes in the expression of genes involved in DNA damage repair, including *Atm* (Ataxia Telangiectasia Mutated serine/threonine kinase), *Bax* (Bcl-2-like protein 4) and *Cdkn1a* (Cyclin-dependent kinase inhibitor 1A or p21), *Exo1* (Exonuclease 1) and *Rad51* (RAD51 Recombinase); genes involved in cell cycle control and the repair of DNA adducts single- and double-strand

breaks (Josse et al. 2012; Hrubá et al. 2011). Changes in the expression of genes involved in DNA damage detection and repair are generally accompanied by changes in the expression of genes that control the progression of the cell cycle (i.e., transition between phases), and/or apoptosis and the initiation of cell death processes. The latter is manifested if the damage is too extensive for effective repair and recovery (Klaassen 2008).

In an effort to establish an effective *in vitro* tool for genetic toxicity assessment, a spontaneously immortalized epithelial cell line, denoted FE1, was isolated from the pulmonary tissue of an adult male MutaMouse (White et al. 2003). The MutaMouse contains concatenated copies of a *lacZ* transgene within a stably-integrated λgt10*lacZ* shuttle vector. The vector is retained in the FE1 cell line, such that the cells can be used to assess chemically-induced mutations at target genes within the shuttle vector (i.e., *lacZ* or *cII*) (White et al. 2003; Lambert et al. 2005). More specifically, the shuttle vector can be recovered from genomic DNA using a commercial λ-bacteriophage packaging system. The bacteriophage particles containing the packaged shuttle vector can then be absorbed to a suitable *Escherichia coli* host for enumeration of *lacZ* mutations, thus permitting assessment of exposure-induced mutagenicity (Lambert et al. 2005). The FE1 cell line possesses many traits that makes it suitable for standardized mutagenicity testing, including cytogenetic stability, sensitivity to noteworthy mutagens, and steady growth in uniform monolayers (White et al. 2003).

The initial FE1 cell characterization study by White et al. (2003), as well as the later DNA microarray study by Berndt-Weis et al. (2009), determined that FE1 cells retain the P53 DNA damage signalling protein, as well as the xenobiotic metabolism isozymes CYP1A1, CYP1A2 and CYP1B1, and several glutathione-*S*-transferase isozymes. These characteristics allow the cell line

to metabolically convert compounds such BaP into metabolites capable of reacting with DNA and inducing mutations without the addition of an exogenous metabolic activation mixture such as that described in Chapter 1. In contrast, the cell lines commonly recommended for OECD-approved *in vitro* mutagenicity assays (i.e., Test Guidelines 476 and 490), including CHO (Chinese Hamster Ovary) cells, L5178Y Mouse Lymphoma Cells and human TK6 (Thymidine Kinase 6) cells, cannot metabolically activate promutagens without addition of an exogenous metabolic activation mixture containing, for example, Aroclor 1254-induced rat liver S9 (OECD 2015a; OECD 2015b). Thus, *in vitro* chemical assessments that employ these cells (i.e., CHO, etc.) must be conducted in the presence of exogenous S9, which has proved to be problematic. For example, exogenous metabolic activation mixtures are often highly cytotoxic, potentially masking responses to the chemical under investigation (Cox et al. 2016). Furthermore, these cell lines are P53 deficient, cytogenetically unstable, and the aforementioned requirement for exogenous activation has been associated with low specificity (i.e., high frequency of false positives) (Kirkland et al. 2007). Although further examination of FE1 cells is required to clearly delineate the metabolic capacity of the cell line, the metabolic capabilities that have been observed to date already indicate that for routine, standardised *in vitro* assessment of mutagenic activity, they can be considered more suitable than the cell lines such as CHO, L5178Y and TK6.

An *in vitro* transgene mutagenicity assay based on MutaMouse FE1 cells is currently undergoing validation for eventual international acceptance by organizations such as the OECD and EURL-ECVAM. As noted, establishment of an internationally-accepted test guideline requires examination of the assay's reliability, reproducibility and applicability domain. The

work presented in Chapter 2 examined intra-laboratory reproducibility across different operators and test dates, and its performance regarding successful assessment of selected substances (i.e., known non-mutagens, known mutagens, and false positives). However, additional investigation is required to delineate metabolic capacity, and by extension, the applicability domain. Collectively, this information will contribute to detailed characterization of the cell line; moreover, the validation of an *in vitro* mutagenicity assay based on the cell line.

Further scrutiny of the metabolic characteristics of FE1 cells can employ targeted gene expression profiling. Although the aforementioned study by Berndt-Weis et al. (2009) employed DNA microarrays to examine gene expression across the entire FE1 cell genome, more targeted analyses are warranted to more carefully assess metabolic capacity. This type of analysis can readily be carried out using targeted quantitative real-time PCR (polymerase chain reaction), or simply qPCR. Due to its capacity to measure extremely small amounts of nucleic acids quickly and cost-effectively, with excellent sensitivity and specificity, qPCR has become the gold-standard for rapid, multiplexed gene expression profiling (Nolan et al. 2006). The technique employs reverse transcription to generate cDNA from total RNA, which can be quantified in real-time as the PCR amplification reaction proceeds (Nolan et al. 2006). More specifically, fluorescent DNA-binding dyes (i.e., SYBR green) are added to each sample such that as the amount of DNA product is amplified, the fluorescence signal increases; where the total amount of amplicon or DNA product is proportional to the level of fluorescence detected. qPCR methods detect the quantification cycle (Cq) or cycle number at which the amplicon accumulates enough product to generate a fluorescence signal above background level (Nolan et al. 2006). For example, if there is a small amount of mRNA template and resultant cDNA in a

given sample, several amplification cycles will be required to accumulate enough product to generate a fluorescence signal above background. In contrast, samples containing abundant RNA template will require fewer amplification cycles to detect fluorescence, thus generating lower Cq values. The simplicity and utility of qPCR has encouraged its use in gene expression analysis, detection of genetically modified organisms in foods, and pathogen detection for clinical analysis (Bustin et al. 2009). qPCR methodologies have also been shown to be proficient for the analysis of metabolic gene expression induced by chemical exposures. As noted, xenobiotic substances can interact with one of several binding proteins (e.g., AhR), and the resultant receptor agonism can increase transcription of genes necessary for chemical metabolism, DNA damage and stress responses. The increases in expression can readily be detected as amplicon copy number via Cq analysis. Thus, targeted use of qPCR, which has been shown to be an accurate and efficient means of gene expression profiling, can be effectively employed to metabolically profile FE1 cells.

The work presented herein employed quantitative real-time PCR (qPCR) to assess the gene expression of FE1 cells exposed to mutagens with different metabolic requirements. The results presented pertain to chemically-induced changes in the expression of genes associated with xenobiotic metabolism. Initial analysis also examined changes in the expression of genes involved in DNA damage and cell cycle control. More specifically, qPCR arrays were employed to assess the expression of 84 Phase I and 84 Phase II xenobiotic metabolism genes following exposure to the known mutagens 2-acetylaminofluroene (2-AAF), aflatoxin B1 (AFB1), 4-(methylnitrosamino)- 1-(3- pyridyl)-1-butanone (NNK), 1-methylpyrene (1-MP), and 7,12-dimethylbenz[*a*]anthracene (DMBA). 2-AAF is an aromatic amine originally produced in 1940 as

an insecticide; however, the compound was found to induce liver tumors in mice and was too toxic to ever make it to the marketplace (Heflich and Neft 1994). Aflatoxins are mycotoxins produced by the fungus *Aspergillus flavus.* They are noteworthy food crop (i.e., corn and nut) contaminants; several have been classified by IARC (International Agency of Research in Cancer) as a known human carcinogens (i.e., Group 1). AFB1 is generally regarded as the most toxic aflatoxin (IARC 2002). NNK is a tobacco-specific nitrosamine found in cigarette smoke and smokeless tobacco. It causes tumors in rodents, and IARC has classified the compound as a known human carcinogen (i.e., Group 1) (IARC 2007a; IARC 2007b). 1-MP is an alkylated PAH found in cigarette smoke, coal tar, diesel engine exhaust, and smoked cheeses; it bioaccumulates in the tissues of some marine animals (e.g., molluscs), and it has been shown to induce hepatic tumours in rats and mice (Bendadani et al. 2014; Bendadani et al. 2016; Pancirov and Brown 1977; Gullen et al. 2004). DMBA is a PAH found in grilled foods, cigarette smoke and diesel exhaust. It is listed as an EURL-ECVAM "true positive" reference chemical for evaluating *in vitro* genetic toxicity assays (Luch 2005; Kirkland et al. 2008). These compounds collectively represent chemical mutagens requiring differing metabolic enzymes for generation of DNA-reactive metabolites. More specifically, Phase I cytochrome P450 isozymes are known to be required for the metabolic activation of DMBA, NNK and AFB1; while the Phase II sulfotransferases and glucuronosyltransferases are known to be additionally necessary for activation of 1-MP and 2-AAF (Figure 3.1). In conjunction with the gene expression analyses, the FE1 cell mutagenicity assay was employed to assess the ability of each compound to induce an increase in *lacZ* mutant frequency.

3.2 Methods:

3.2.1 Chemicals and Reagents:

Test compounds DMBA, AFB1, 1-MP and 2-AAF were purchased from Sigma Aldrich (Oakville, Ontario). NNK was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario). BaP and Aroclor 1254-induced rat liver S9 were purchased from Moltox Inc. (Boone, NC). All chemicals were dissolved in DMSO (dimethyl sulfoxide) from Sigma Aldrich. The sources of biochemical reagents and culture medium are outlined in Chapter 2.

3.2.2 Cell Culture:

See Chapter 2, section 2.22.

3.2.3 Cytotoxicity and Concentration Selection:

Cytotoxicity studies were carried out to determine appropriate concentrations of each test chemical for the mutagenicity and gene expression experiments. Using the protocol outlined in Chapter 2 exponentially growing FE1 cells, at passage 25, were exposed to test chemicals in 6-well plates for 6 hours and allowed to recover for a sampling time of 72 hours. Negative controls included a media-only control and a DMSO solvent control. The positive control was 0.1µg/mL BaP. A minimum of 4 chemical concentrations were tested in duplicate as follows: AFB1 0 – 10µg/mL, 1-MP 0 - 40µg/mL, 2-AAF 0 - 200µg/mL, NNK 0 - 2250µg/mL and DMBA 0 - 50µg/mL. For cytotoxicity assessment, all chemicals were tested without exogenous metabolic activation. Note that the OECD recommends testing to a maximal concentration of 10mM when a chemical is not able to induce cytotoxicity and does not precipitate in the exposure medium (OECD 2015a; Galloway et al. 2011); therefore, no tested concentrations exceeded 10mM.

Cytotoxicity was scored using the relative increase in cell count (RICC) approach. Following a 1-2 minute 0.5mL trypsin (0.25%) exposure, and collection of the cells in 0.25 – 1 mL media, initial cell counts from duplicate plates were taken prior to exposure. Trypan blue staining using a 1:1 collection mixture to stain ratio was conducted to enumerate live cells on glass slides. Cell enumeration employed a Countess Automated Cell Counter (Invitrogen, Burlington, Ontario). The same collection procedure and Countess Cell counter were utilized for final counts conducted following the chemical exposure and a 72 hour recovery period. The DMSO vehicle control was considered the point of 100% relative cell survival, and all test concentration results were compared to this reference. RICC was determined by dividing the difference between live cells before and after exposure to the test chemical, by the difference between live cells before and after exposure to the solvent control, as outlined below:

$$\text{RICC} = \frac{[\text{Cell counts after sampling period - Initial cell counts}]\ \text{Treated Cells}}{[\text{Cell counts after sampling period - Initial cell counts}]\ \text{Control Cells}} \times 100\%$$

The results obtained were employed to generate a cytotoxicity curve (i.e., plot of relative increase in cell survival versus test chemical concentration), from which concentrations were selected that correspond to interpolated relative survival of 100%, 70% and 40%.

3.2.4 *In Vitro* FE1 Cell Transgene Mutation Assay:

For details see Chapter 2, section 2.23.

Utilizing the previously described mutagenicity assessment protocol, FE1 cells were exposed to 3 test concentrations, with 3 biological replicates, and 2 technical replicates of each concentration (i.e., N=3). Each compound was screened both with and without the addition of an exogenous activation mixture containing Aroclor 1254-induced rat liver S9.

3.2.5 Time-Point Determination:

To determine the appropriate time for gene expression profiling, a small time-series study was conducted for all 5 chemicals, alongside DMSO and media-only control. Phase I xenobiotic metabolism and DNA damage signalling RT^2 Profiler Arrays (Qiagen, Toronto, Ontario) were employed for qPCR analysis of gene expression changes at a single concentration corresponding to 40% relative cell survival (see Appendix 1 for complete list of the genes on each array). FE1 cells were exposed to the test substances for 6 hours according to the standard protocol, rinsed with PBS, and the cells either immediately lysed, homogenized and frozen (i.e., 6+0 hour samples); or alternatively, fresh media was added and the cells allowed to recover for 4 hours prior to cell lysis (i.e., 6+4 hour samples). Total RNA was then extracted from frozen cell lysates.

3.2.6 Total RNA Extraction:

Isolation and purification of total RNA employed the spin column approach (i.e., RNeasy Mini Kit, Qiagen; Toronto, Ontario). A proprietary lysis buffer (i.e., Buffer RLT) that contains high concentrations of guanidine isothiocycanate, which promotes RNA binding to the silica membrane of the spin column, was added directly to cell culture dishes. The lysate was pipetted into QIAShredder spin columns (Qiagen, Toronto, Ontario) and centrifuged for 2 minutes at 20,000g to homogenize the lysate. 1 volume of 70% ethanol was added to homogenates, mixed by pipetting, and the mixture transferred to a spin column and centrifuged for 15 seconds at $\geq$8000g. The mixture was then washed with RW1 Buffer, which washes membrane-bound RNA, and then with RPE Buffer, which primarily removes salts. Centrifugation for 15 seconds at $\geq$8000g followed the addition of each buffer. A second RPE

Buffer wash was carried out, and the column centrifuged for 2 minutes at ≥8000g. Finally, the spin column was placed inside a collection tube, 50µL of RNase-free water added, and the column centrifuged for 1 minute at ≥8000g to elute the RNA. A NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Burlington, Ontario) was employed to determine total RNA concentration, purity and integrity. Samples used for gene expression analyses all showed A260/280 absorbance ratios ≥2.0.

3.2.7 Quantitative Real-Time PCR:

First Strand Kits (Qiagen, Toronto, Ontario) were employed to synthesize cDNA from 800ng of total RNA. Kits include a proprietary buffer (i.e., Buffer GE) that is used to remove any residual DNA contamination. The kit also includes buffers containing reverse transcriptase enzymes and primers, nucleoside triphosphates and random hexamers to prime cDNA synthesis. Murine RT^2 Profiler Arrays including 84 Phase I or 84 Phase II xenobiotic metabolism genes were utilized for gene expression analysis (see Appendix I for complete gene list). SYBR Green Mastermix, containing the SYBR green fluorescent dye, was added to cDNA samples with RNase-free water, and 25µL of the mixture was aliquoted into each well of 96-well plates containing the commercial primers. qPCR was conducted using a Bio-Rad CFX96™ thermocycler (Mississauga, Ontario) according to the following program: an initial 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Expression of Phase I and Phase II xenobiotic genes were assessed utilizing the fluorescence detection approach, where Cq values were determined using the proprietary thermocycler software. Cq values represent the PCR quantification cycle at which a sample fluoresces above the threshold limit. For all arrays, the threshold cut-off limit was set at 35 relative fluorescence units. qPCR analyses

examined all 5 test chemicals, at 3 concentrations corresponding to 100%, 70% and 40% relative survival, in triplicate, at the 6+0 hour time-point. DMSO solvent exposure was employed as the control point of reference (N=3).

3.2.8 Data Analysis:

LacZ mutant frequency values were calculated and analysed according to the methods described in Chapter 2, section 2.24.

Normalized gene expression was calculated using the Delta Delta Cq (ΔΔCq) method that describes the change in Cq values between treated and control samples (Livak and Schmittgen 2001). The ΔΔCq method involves use of the equations presented below (Livak and Schmittgen 2001):

[1] ΔΔCq = ΔCq of exposed sample – ΔCq of the unexposed sample.

[2] ΔΔCq = [(Cq of gene of interest in DMSO solvent) - (Cq of reference gene in DMSO solvent)] - [(Cq of gene of interest exposed to test compound) - (Cq of reference gene exposed to test compound)]

[3] $2^{\Delta\Delta Cq}$ = fold change compared to reference.

B2m (Beta-2 microglobin), an endogenous housekeeping gene, exhibited stable expression independent of chemical exposure, and was thus employed as the reference gene for normalization of relative gene expression. qPCR results are presented as the up- and down-fold 'regulation' compared to the control. An upregulation of gene expression is represented as a positive fold increase; for example, an upregulation of expression 4x above the control is

deemed a 4-fold change. Down-regulation of gene expression is represented as a negative fold regulation. For example, a decrease of expression 4x below the control would result in a fold change of 0.25; this can alternatively be presented as a -4-fold, (i.e., - (1/*x*), where - (1/0.25) =-4). Importantly, the gene expression change is not biologically negative; however, the representation of down-regulation as a negative fold visually demonstrates the equal strength and relevance of up- and down-fold regulation. A significant gene expression change was defined as a minimum of a 2-fold change, and $p<0.05$ (using the Student's t-test) compared to the control. Note that the gene names in the results correspond to the murine homolog of each studied human gene.

Benchmark dose modeling of the Cq responses associated with each gene employed BMDExpress software as described by Yang et al. 2007. BMDExpress output files were uploaded into the online BMDExpress DataViewer after filtering using a p-value threshold of $p<0.05$ (Student's t-test) (Kuo et al. 2016). The software was used to identify pathways that show a concentration-related response to the chemicals of interest. In accordance with the criteria of Thomas et al. 2007, pathways containing a minimum of 5 significant genes were termed relevant.

3.3 Results:

3.3.1 Time-Point Determination Analyses:

Time-point determination studies involved 6 hour exposures of cells to a single dose of each test chemical, as well as media and solvent controls. Subsequently, cells were lysed immediately (i.e., 6+0 hour), or allowed to recover for an additional 4 hours before lysis (i.e., 6+4 hour). qPCR analysis of Phase I xenobiotic metabolism genes showed significant changes in the gene expression of many more genes, as well as greater fold changes relative to the solvent, for the 6+0 hour time-point in comparison with the 6+4 hour time-point for all 5 test chemicals (data not shown). For example, analysis of responses elicited by FE1 exposure to DMBA at 6+0 hours showed an increased expression of 10 different Cyp monooxygenases (i.e., *Cyp1b1, Cyp21a1*), with *Cyp1a1* increasing 96-fold relative to the solvent control, as well as a 2-fold increased expression of Flavin containing monooxygenases (i.e., *Fmo2* and *Fmo3*). In contrast, the 6+4 hour time-point revealed downregulation of *Cyp1a1* and *Cyp1a2*, showing decreases of 3.5- to 7.7-fold below the control, respectively; *Cyp2c55* showed a modest 2-fold upregulation. Responses elicited by AFB1 exposure showed an upregulation of 6 Cyp's for the 6+0 hour time point, ranging 2.2- to 12.2-fold above the control. However, when allowed to recover for 4 hours, the response revealed increased expression of only a single monooxygenase (i.e., *Cyp4b1*, approximately 19-fold above control). The metabolic responses to 1-MP exposure were much more erratic, yielding expression results that show both up- and down-regulation of 28 different Cyp's, with changes ranging from approximately 11-fold down to 50-fold up for *Cyp1a2* and *Cyp1a1*, respectively, at 6+0 hours. In contrast, at the 6+4 hour

time-point only 6 Cyp's were expressed, with fold changes from approximately 3.5-fold down to 9-fold up (-S9) (i.e., *Cyp1a2* and *Cyp3a57*).

Although the work presented in this Chapter focused primarily on the expression of genes associated with Phase I and II metabolism, initial analysis also investigated changes in the expression of genes involved in DNA damage signalling. The analyses, which are not presented in detail, show that qPCR analysis of DNA damage signalling genes for the 6+0 hour time-point resulted in noteworthy changes in the expression of genes associated with cell cycle arrest (i.e., *Cdkn1a, Gadd45a*) (Table 3.2). Moreover, the responses were similar across all test chemicals. In contrast, the 6+4 hour time-point showed significant increases in genes associated with double-stand break DNA repair (i.e., *Brca1, Brca2*), DNA mismatch repair (i.e., *Exo1*) and cell cycle arrest (i.e., *Cdkn1a*), (Table 3.2). AFB1 additionally showed evidence of changes in the expression of genes involved in nucleotide excision (i.e., *Dclre1a*) and base excision repair (i.e., *Mbd4*) (Table 3.2). Interestingly, NNK did not induce significant changes in the expression any of the 84 DNA damage genes tested following the 6+4 hour exposure. Table 3.2 provides a summary of the DNA damage signalling genes that responded to the chemicals investigated. Collectively, the results revealed maximal gene expression changes for the 6+0 hour time point. Thus, the 6+0 hour exposure was selected for all further analyses (i.e., experimentation to assess chemically-induced changes in the expression of xenobiotic metabolism genes).

3.3.2 Cytotoxic, Mutagenic and Genomic Effects of Examined Chemicals:

The described methods were employed to assess cytotoxic, mutagenic and genomic (i.e., gene expression alterations) effects elicited by exposures to DMBA, 2-AAF, 1-MP, AFB1 and NNK. The following sections describe the results obtained for each compound.

3.3.2.1 7, 12-Dimethylbenz[a]anthracene:

Cytotoxicity studies involved exposure of FE1 cells to 10 concentrations of DMBA (0-50µg/mL) alongside positive, media and solvent controls. Significant cytotoxicity was observed at concentrations of approximately 0.1 µg/mL and higher. Concentrations corresponding to 100%, 70% and 40% relative cell survival, i.e., 0.0001, 0.001, 0.008µg/mL, were chosen for subsequent gene expression and mutagenicity testing (Figure 3.2A).

The results obtained revealed that DMBA induced significant increases in *lacZ* mutant frequency at both the high and intermediate concentrations. Cytotoxicity and induced *lacZ* mutant frequency (-S9) results are presented in Figure 3.2A. The intermediate and high test concentrations also showed significantly increased mutant frequency when tested in the presence of Aroclor 1254-induced rat liver S9. These results are shown in Figure 3.3A.

RT^2 Profiler qPCR arrays corresponding to 84 Phase I or Phase II xenobiotic metabolism genes were employed to assess changes in expression elicited by the chemical exposures Gene expression changes were considered significant if the exposure induced a change at least 2-fold above or below the DMSO solvent control and $p<0.05$; gene expression changes observed following treatments with DMBA, 2-AAF, AFB1, NNK or 1-MP are illustrated in Figure 3.11. The results show qualitative differences between the different treatments, as well as some evidence of concentration-related trends in gene expression (see below). Additionally, the results revealed that the gene expression changes are empirically and mechanistically consistent with the mutagenicity results (see below). With respect to DMBA, the high concentration exposure elicited significant 38.6- and 11.66-fold increases in the expression of *Cyp1a1* and *Cyp1b1* isozymes, respectively, above the control (Figure 3.4A). High

concentrations of DMBA also increased expression of *Aldh7,* decreased the expression of *Cyp21a1*, and both the low and high concentrations significantly decreased the expression of *Aldh1b1* (Figure 3.4A, see Table 3 for description of primary gene functions). With respect to genes involved in Phase II metabolism, the high concentration of DMBA significantly induced expression of *Nqo1*, an NADPH dependent quinone dehydrogenase (Table 3.3), as well as Ugt's such as *Ugt1a1, Ugt1a2*, *Ugt1a6a* and *A4galt* (Figure 3.4B). The low and high tested concentrations were able to induce increased expression of *Sult6b1*, as well as *Acsm3*, a methyltransferase (Mt) (Figure 3.4B). The lowest concentration decreased the expression of many genes involved in both Phase I and II metabolism, including *Sult1e1, Sult5a1* and *Ugt2b37*, as well as *A3galt2* (another Ugt), *Ephx2* (an epoxide hydrolase), and *Glyat* (an amino acid transferase) (Figure 3.4B). Overall, DMBA was able to mobilise the Phase I metabolic enzymes necessary for biotransformation and activation, as demonstrated by induced *lacZ* mutagenicity.

3.3.2.2 2-Acetylaminofluroene:

Cytotoxicity range finder studies examined FE1 cell exposure to 5 concentrations of 2-AAF (0-200µg/mL), as well as a positive, media and solvent controls. The cytotoxicity curve reached a plateau at approximately 50µg/mL, a concentration that corresponds to 15% relative cell survival. The concentrations achieving 100%, 70% and 40% relative cell survival (i.e., 0.26, 1.18 and 5.5µg/mL, Figure 3.2C) were selected for further testing. Mutagenicity scoring using the standardized *in vitro* transgene mutation assay revealed a significant increase in mutant frequency following exposure to the highest concentration of test chemical without S9 (Figure 3.2C). Addition of exogenous S9 activation resulted in a significant increase in *lacZ* mutant frequency for all tested concentrations (Figure 3.3C).

Gene expression analysis using qPCR revealed a significant decrease in *Cyp1a2* at the intermediate concentration (Figure 3.5A). *Cyp11b2, Cyp2c38* and *Gzma* (an esterase involved in stress/apoptosis responses, Table 3.3) were significantly decreased at all concentrations, and *Cyp26b1* expression was reduced at the low and intermediate concentrations (Figure 3.5A). The low concentration additionally reduced the expression of *Cyp2s1* and *Aldh1a7*, while transcription of *Ascm3* increased at the low and intermediate concentrations. Phase II arrays revealed increased expression in genes *Ugt1a2* and *Ugt1a6a* at the intermediate and high concentrations, as well as *Ugcg* (a Ugt), and *Ptges* (a Gst, Table 3.3), at the intermediate concentration (Figure 3.5B). Notably, *Sult6b1* increased in expression across all tested concentrations in a dose-dependent manner (Figure 3.5B). Overall, a 6 hour 2-AAF exposure induced a significant *lacZ* mutant frequency, and yielded decreases in the expression of Phase I genes and increases in the expression of Phase II genes.

3.3.2.3 1-Methylpyrene:

The cytotoxicity range-finder study for 1-MP involved exposure of FE1 cells to 16 different concentrations (0-40µg/mL), alongside positive, media and solvent controls. The testing of numerous concentrations was required in order to find the extremely small cytotoxicity range (i.e., between approximately 1.5 and 3µg/mL). Concentrations corresponding to 100%, 70% and 40% relative cell survival (i.e., 1.66, 1.84 and 2µg/mL) were selected for the subsequent gene expression and mutagenicity analyses (Figure 3.2D). Mutagenicity testing of 1-MP without the addition of S9 yielded a negative mutagenicity result (Figure 3.2D). There was no significant induction of mutant frequency above the solvent control; however, a slight dose-response was noted. Exposure with exogenous S9 did not result in any significant increase in

mutant frequency, and therefore, the overall mutagenicity response to 1-MP was labelled negative (Figure 3.3D).

qPCR analysis was employed to investigate changes in the expression of Phase I and II metabolism genes in response to the 1-MP exposure. The results showed a dose-dependent increase in the expression of *Cyp3a57* at all tested concentrations, and *Cyp27b1* at the intermediate and high concentrations (Figure 3.6A). Exposure to the intermediate concentration also increased the expression of *Cyp1a1* and *Cyp2r1* (i.e., Vitamin D 25-hydroxylase; Table 3.3), while *Cyp4b1* was increased at the higher concentration (Figure 3.6A). *Cyp21a1* expression was decreased at both the low and high concentrations, and the high concentration also reduced the expression of *Aldh1b1*. Increased transcription of an esterase, *Uchl1* (see Table 3.3), was noted for the low and intermediate concentrations, as well as *Xdh* at the intermediate concentration (Figure 3.6A). The Phase II array showed increased *Sult4a1* expression at all concentrations, as well as *Sult1a1* and *Sult6b1* at the intermediate concentration (Figure 3.6B). All tested concentrations elicited an increased expression of the detoxification gene *Gsta3*; the low and intermediate increased *Ugt1a1* expression, and the intermediate concentration alone increased expression of *Ugcg, Ugt1a2* and *Ugt1a6a* (Figure 3.6B). Additional significant changes in gene expression related to increased *Acsm3* expression at the low concentration, *Ptges* at low and intermediate concentrations, and *Pomgnt1* at the intermediate and high concentrations (Figure 3.6B). Overall, gene expression analysis illustrated increased transcription of both Phase I and II xenobiotic metabolism genes; however, 1-MP was not mutagenic in FE1 cells even in the presence of exogenous activation.

3.3.2.4 Aflatoxin B1:

The AFB1 cytotoxicity range-finder experiment involved exposures of FE1 cells to 7 different test concentrations (0-10µg/mL), in addition to positive, media and solvent controls. Based upon the levels of relative cell survival, the concentrations selected for the subsequent gene expression and mutagenicity studies were 0.00015, 0.01, 0.7µg/mL (Figure 3.2B). Mutagenicity testing revealed a significant increase in *lacZ* mutant frequency at the highest test concentration, both with and without the addition of exogenous S9 activation (Figures 3.2B & 3B).

qPCR analysis of Phase I and II xenobiotic metabolism genes demonstrated reduced expression of *Cyp11b1, Cyp11b2, Cyp2c8* and *Gzma* following exposures to the lowest concentration (Figure 3.7A). The low and intermediate concentrations reduced *Cyp1a2* expression, and the low concentration additionally increased the expression of *UchL1* (Figure 3.7A). All expression changes occurred in a dose-dependent manner. Phase II arrays showed an increased expression of *Sult1a1* and *Sult4a1* at the low and intermediate concentrations, and *Sult6b1* at the intermediate concentration (Figure 3.7B). Results also indicated increased expression of genes such as *Gsta3* and *Ptges* at the low concentration, and *Ugt1a1* and *Ugt1a2* at the low and intermediate concentrations (Figure 3.7B). AFB1 additionally increased the expression of *Acsm3* at the intermediate and high test concentrations (Figure 3.7B). Overall, the AFB1 exposures induced an increase in expression of both Phase I and Phase II metabolism genes necessary to facilitate the observed FE1 cell mutagenic activity.

3.3.2.5 4-(Methylnitrosamino)-1- (3-pyridinyl) - 1-butanone:

Cytotoxicity range-finder studies for NNK involved exposures to 12 test concentrations (0-2250µg/mL), alongside positive, media and solvent controls. Significant cytotoxicity was not

observed at any of the tested concentrations. 10mM for NNK is equivalent to 2072.3µg/mL; therefore, 2000µg/mL was selected as the highest test concentration. No decrease in relative cell survival was observed at test concentrations lower than 1250µg/mL; thus, 1000, 1500 and 2000µg/mL were ultimately selected for subsequent analyses. Mutagenicity testing following exposure to NNK showed no significant induction of *lacZ* mutant frequency at any tested concentration, with or without exogenous metabolic activation (S9). Therefore, the overall mutagenicity response was termed negative (Figures 3.2E-3.3E).

As with the aforementioned substances, qPCR arrays were used to examine exposure-related changes in the expression of Phase I and Phase II genes. The results showed that NNK increased the expression of *Aldh1b1, Cyp4b1, Cel* and *Fmo2* at the intermediate concentration (Figure 3.8A). Reduction in the expression of *Aldh1a7* was observed at the intermediate concentration, and increased expression of *Xdh* was observed at the high concentration (Figure 3.8A). Several Phase II genes increased in expression following NNK exposure, including *Sult1a1, Sult4a1, Tst* (thiosulfate sulfotransferase, see Table 3.3) at the high concentration, and *Sult2b1* and *Sult6b1* at the intermediate concentration (Figure 3.8B). Many genes associated with xenobiotic metabolism conjointly increased in expression, including *Nqo1, Gsta3, Gstt1* and *Mgst2* at the high concentration, *Gstm5, Ugt2b37, Ugt1a1, Ugt1a2* and *A3galt2* at the intermediate concentration, *Acsm3* and *Gcnt1* (member of the N-acetyltransferase family, Table 3.3) at the intermediate and high concentrations, and *Ephx2* at the high concentration, as well as *Ugt1a6a* at the low and high concentrations (Figure 3.8B). Overall, NNK was not mutagenic in FE1 cells, yielded a reduction of Phase I gene expression, with little representation

from the P450 mono-oxygenase family, and showed increased expression of Phase II genes corresponding to sulfo-, glucuronosyl- and glutathione-*S*-transferase enzymes.

3.3.3 Benchmark Concentration Modelling of Signalling Pathways:

BMD modelling utilizing BMDExpress and the online BMDExpress DataViewer was employed to scrutinise the chemically-induced gene expression profiles. BMDExpress DataViewer has several noteworthy features, particularly the ability to map the transcriptional BMD data to biological pathways, thus determining the BMD values at which entire pathways are altered by a chemical exposure (Chauhan et al. 2016). Determination of activated pathways can potentially provide information for the determination of the mode of action (MOA) associated with the test chemical.

Exposures to all tested mutagens induced biological signalling pathways related to xenobiotic metabolism, aryl hydrocarbon exposure, acetone degradation, ubiquinol-10 biosynthesis, and LPS/IL-1Mediated inhibition of RXR function pathways. Biological signalling pathways altered by FE1 cell exposure to test chemicals 1-MP, 2-AAF, DMBA, AFB1 and NNK, and the BMD at which the pathway was triggered are presented in Table 3.4. 1-MP, 2-AAF, DMBA and NNK each initiated induction of NRF2-mediated oxidative stress responses, nicotine degradation II and III pathways, and melatonin and serotonin degradation pathways, as well as the super-pathway of melatonin degradation (Table 3.4). Additional groupings of signalling pathways included stearate biosynthesis signalling induction by 1-MP, AFB1, DMBA and NNK, glutathione-mediated detoxification by 1-MP, DMBA and NNK, and noradrenaline and adrenaline degradation by 1-MP, DMBA and AFB1. 1-MP and 2-AAF individually activated biological signalling of PXR/RXR pathways, as well as oxidative ethanol and bupropion

degradation. 1-MP and AFB1 each induced histidine degradation and pregnenolone biosynthesis, while 1-MP and DMBA induced signalling pathways for dopamine degradation. The final pathway induction chemical grouping relates to initiation of thyroid hormone metabolism following DMBA or NNK exposures. Note that 1-MP and DMBA were able to initiate the activation of several additional biological signalling pathways, i.e., estrogen biosynthesis and ethanol degradation, and heparin sulfate biosynthesis and methionine degradation, respectively (Table 3.4).

In addition to defining altered cellular pathways, the BMDExpress DataViewer also proposes a BMD distribution profile that provides insight into pathway sensitivity across tested concentrations (Chauhan et al. 2016). The software generates mean BMD_{10} values for biological pathways based on the mean BMD_{10} of the associated gene expression profiles induced by chemical exposure; such that it can be determined whether a compound activates a gene or pathway at a low or high concentrations. BMD analysis following FE1 exposures to DMBA, NNK and 1-MP showed that different genes are sensitive to different concentrations, and that the distribution profile of gene expression is fairly consistent across increasing concentrations (i.e., one mode). Exposure to 2-AAF and AFB1 exhibited 2 distinct modes of differentially expressed genes; providing a clear indication that the cell is employing primary Phase I at low concentrations, with Phase II and secondary Phase I xenobiotic metabolism being manifested at higher concentrations (i.e., Phase II genes are only found in the second BMD mode) (Table 3.5; Figure 3.9). BMD mode analysis also determined that different Cyp genes showed altered expression at different concentrations. For instance, AFB1 increased *Cyp1a1* at a BMD value of 0.01µg/mL in the first mode, while the *Cyp1a2* isozyme was not upregulated until the second

mode at a BMD value of 0.68µg/mL (Table 3.5; Figure 3.9). Table 3.5 summarises the different gene sensitivities across tested concentrations of 2-AAF and AFB1. BMD mode analysis at the pathway level indicates that major signalling pathways such as xenobiotic metabolism (i.e., for AFB1) are activated at lower mean BMD values, while minor pathways such as ubiquinol-10 biosynthesis signalling require higher concentrations (i.e., higher BMDs) to trigger pathway activation (Figure 3.10, Table 3.4). Overall, BMD analysis of qPCR generated Cq values determined that the FE1 cell line retains the capacity of many signal transduction cascades involved in xenobiotic metabolism, cellular stress responses, and tertiary pathways involved in maintenance of cellular homeostasis.

3.4 Discussion

Defining the metabolic capacity of a mammalian cell line used for genetic toxicity assessment is a fundamental part of assay validation; specifically, for defining the applicability domain. Moreover, the nature and identity of the genes in the mobilised pathways provide information on the mechanisms underlying metabolism of the putative chemical under investigation (Figures 3.4, 3.5, 3.6, 3.7, 3.8, 3.10 and 3.11). Exposure of MutaMouse FE1 cells to selected mutagens induced the expression of Phase I enzyme genes such as *Cyp1a1, Cyp1b1* and *Cyp1a2* (e.g., 3.6-fold down to 38.9-fold up) such that it can be asserted that the cell line is competent to mobilize the ligand-dependent AhR (aryl hydrocarbon receptor) signalling cascade. AhR remains in an inactive state in the cytoplasm bound by chaperone proteins (i.e., heat shock protein 90, immunophilin-like X-associated protein 2 and prostaglandin E synthase 3) until ligand binding to an exogenous chemical in the cytoplasm allows translocation into the nucleus via dimerization with ARNT (aryl hydrocarbon receptor nuclear translocator) (Singhal et al. 2007). The AhR-ARNT heterodimers can then bind to DNA at Xenobiotic response elements (XREs), which are located within the promoter region of several Cyp monooxygenases (i.e., *Cyp1a1, Cyp1b1, Cyp1a2),* aldehyde dehydrogenases (i.e., hALDH1), and *Ugt1a1*; thus regulating the transcription of these genes (Fujii-Kuriyama and Mimura 2005; Stanford et al. 2016; Beischlag et al. 2008). In addition to AhR signalling, the FE1 cells retained the capacity to employ other members of the nuclear receptor family including the PXR (Pregnane X Receptor), and most likely the CAR (Constitutive Androstane Receptor). PXR and CAR both reside in the cytoplasm in an inactive state; they translocate to the nucleus forming dimers with RXR (9-cis Retinoid X Receptors) once activated. PXR activation is ligand-dependent (i.e., xenobiotics,

drugs and steroids); upon ligand binding the PXR/RXR heterodimer complex translocates to the nucleus and binds to an XRE in the 5' region of several target xenobiotic genes, most notably *Cyp3a4* (Ayed-Boussema et al. 2012; Lehmann et al. 1998). PXR induces a wide range of Phase I metabolism genes (i.e., *Cyp3a*'*s*, *Cyp2b6, Cyp2c9*, *Aldh1a 1, Aldh1a7*), several Sult's (i.e., *Sult1b1, Sult1d1, Sult2, Sult3a1, Sult5a1*) and Ugt's (i.e., *Ugt1a1, Ugt1a5, Ugt1a6, Ugt1a9*), as well as *Gsta3* (Knight et al. 2008; Alnouti and Klaassen 2008; Sugatani et al. 2005; Xie et al. 2003; Drocourt et al. 2001; Chen et al. 2004; Ferguson et al. 2005; Maglich et al. 2002; Buckley and Klaassen 2009). In addition to the induced expression of several PXR regulated genes in FE1 cells (i.e., *Cyp3a*, Sult's, Ugt's), BMD analysis specifically indicated that PXR signalling pathways were activated following exposure to 1-MP and 2-AAF (Table 3.4).

The CAR receptor is another member of the nuclear receptor family that acts via sensor of several xenobiotic and endogenous compounds. CAR is constitutively expressed and much less promiscuous to chemical activation compared to PXR. CAR dimerizes with RXR upon translocation to the nucleus following activation by ligand binding, and the CAR-RXR complex then binds to XREM (Xenobiotic responsive enhancer module) or PBREM (Phenobarbital-responsive enhancer module) promoter sites inducing transcription of genes such *Cyp2b6* (Honkakoski et al. 1998; H. Wang et al. 2003). CAR/RXR heterodimers are also able to induce transcription of Sult's (i.e., *Sult1d1, Sult2, Sult2a*), Ugt's (i.e., *Ugt1a1, Ugt1a9, Ugt2a3, Ugt2b36*), Gst's (i.e., *Gsta1, Gsta2, Gsta3, Gsta4*), and Phase I genes *Cyp2c8*, *Cyp3a* and *Aldh1a7*, many of which are mobilised in mutagen-exposed FE1 cells (Maglich et al. 2002; Buckley and Klaassen 2009; Ferguson et al. 2005; Sugatani et al. 2005; Xie et al. 2003; Alnouti and Klaassen 2008; Knight et al. 2008). Overall, the differential gene expression in FE1 cells

exposed to different chemical stimuli (i.e., Cyp1, Cyp2, Cyp3) exemplifies the cell line's ability to mobilize signalling cascades controlled by the AhR, PXR and CAR nuclear receptor families. The subsequent sections contain detailed discussions of the gene expression patterns induced by the chemical mutagens investigated.

DMBA is an alkyl-PAH derived from the incomplete combustion of organic material. Most PAH's, including DMBA, become DNA-reactive once bound to the AhR and translocated to the nucleus via AhR-ARNT heterodimers that can bind to promoter regions and increase transcription of Cyp monooxygenases such as *Cyp1b1* and *Cyp1a1* (Singhal et al. 2007). Bioactivation of DMBA into a reactive metabolite specifically requires Phase I oxidation by CYP1B1 isozymes, followed by an epoxidation reaction catalyzed by an epoxide hydrolase (Ephx), and a second oxidation again catalyzed by CYP1B1 or CYP1A1 monooxygenases (Figure 3.1A) (Igawa et al. 2009). The *in vitro* transgene mutation assay results showed that DMBA exposure augmented *lacZ* mutant frequency in the absence of exogenous S9; not surprisingly the Phase I metabolism qPCR results confirmed large and significant increases (i.e., 11.7- to 38.9-fold) of both *Cyp1b1* and *Cyp1a1* expression. Therefore, the FE1 cell line retains the capacity to induce transcription of *Cyp1a1* and *Cyp1b1* monooxygenases that transform the parent compound into DNA reactive metabolites able to induce the formation of stable, bulky adducts and consequent mutations at loci such as the *lacZ* transgene. Gene expression analysis also showed significant augmentation of Phase I aldehyde dehydrogenase genes *Aldh7* and *Aldh1b1* (e.g., 3.2 up- to 4.3 down-fold); this is likely indicative of a secondary metabolic pathway whereby hydroxyl groups are oxidized to aldehydes (Luch 2005). Since hydroxyl groups are more polar and water-soluble than aldehydes, the induction of this secondary metabolic

pathway likely makes a minor contribution to transformation in preparation for excretion. DMBA exposure of FE1 cells additionally induced a repression of *Cyp21a1*, steroid 21-hydroxylase, which assists in systemic steroid metabolism. This is consistent with studies showing that PAH exposures can interfere with steroid metabolism (Zhao et al. 2012; Naiki et al. 2016).

Phase II conjugation is not required for the conversion of DMBA into DNA-reactive metabolites. Nevertheless, DMBA exposure induced significant differential expression of Sult's (i.e., *Sult6b1*), several Ugt's (i.e., *A3galt2, Ugcg, Ugt1a1, Ugt1a2, Ugt1a6a*), quinione dehydrogenase *Nqo1*, a methyltransferase (Mt) (i.e., *Acsm3*), and an amino acid transferase (i.e., *Glyat*), which are all indicative of cellular pathways involved in clearance/excretion of the chemical (Luch 2005; R. Li 1995; Ritter 2000; Glatt 2000). *Acsm3*, an acyl-CoA synthase member of the methyltransferase family, which is involved in the activation of medium-chain length fatty acids, was differentially expressed in response to all 5 test chemicals (Table 3.4). This could be indicative of FE1's ability to implement fatty acid metabolism as an alternative detoxification pathway following xenobiotic exposure (Watkins et al. 2007; Lambert et al. 2005). Phase II arrays also showed a decrease in epoxide hydrolase, *Ephx2*; this could possibly indicate that the Phase I epoxidation reaction necessary for the biotransformation of DMBA was induced such that the Phase II epoxide hydrolase was down-regulated to compensate. This is plausible since *Ephx1* is primarily involved in detoxification of xenobiotics, and *Ephx2* more so in the detoxification of fatty acids (Decker et al. 2009). Alternatively, the compound may have induced *Ephx2* within lipid metabolism pathways to the point where rate limiting factors were depleted and the gene expression down-regulated at the time-point of cell lysis. Overall, the

gene expression results show that the FE1 cell line possesses the metabolic capacity to bind the appropriate cellular receptor (i.e., AhR) to mobilise the isozymes (e.g., *Cyp1a1* and Cyp1b1) that transform DMBA into DNA-reactive metabolites. This was confirmed by a significant increase in *lacZ* mutant frequency.

2-AAF is an aromatic amine that acts as a PXR agonist that is translocated to the nucleus via PXR binding and dimerization with RXR, which stimulates the transcription of *Cyp1a2* and *Cyp3a11* (Anapolsky et al. 2006); employment of CYP1A2 also implies implementation of AhR. Metabolic transformation of 2-AAF into metabolites able to generate DNA adducts requires Phase I oxidation primarily by CYP1A2, and subsequent Phase II acetylation by NAT1/2 or sulfonation by SULT1A2 isozymes (Figure 3.1B). CYP3A11 has also been implicated in 2-AAF metabolism (Wasalathanthri et al. 2015; Hein et al. 1993; Anapolsky et al. 2006).

The *in vitro* transgene mutation assay yielded positive mutagenicity results for 2-AAF without the addition of exogenous metabolic activation. The attendant gene expression study showed differential expression of several Cyp monooxygenases (i.e., *Cyp11b2, Cyp26b1, Cyp2c38, Cyp2s1*), including *Cyp1a2*. This indicates that the cell line can utilize available monooxygenases, including *Cyp1a2*, to carry out the oxidation reaction necessary to transform 2-AAF into *N*-hydroxy-AAF. Interestingly, the BMDExpress pathway analysis showed that the 2-AAF exposure elicits both AhR and PXR/RXR pathway activation (Table 3.4), indicating that the compound was able to bind both receptors and induce transcription of the corresponding genes. Although PXR pathways indicate induction of isozyme genes such as *Cyp3a11*, these are generally minimally expressed in lung tissue; FE1 cells are likely congruent (Renaud et al. 2011).

Observed down-regulation of genes such as *Aldh1a7* suggest minimal Phase I oxidation of hydroxyl groups to their corresponding aldehydes. To reduce energy expenditure associated with cellular detoxification, cells efficiently coordinate regulation of Phase I and II xenobiotic metabolism gene transcription, as well as the transcription of genes coding for transport enzymes involved in receptor activation (Aleksunes and Klaassen 2012). Since the genotoxicity results showed that FE1 cells can mobilise the enzymes required to transform 2-AAF into DNA-reactive metabolites that induce transgene mutations, decreased expression of some Phase I metabolism genes may be attributable to experimental timing. More specifically, upregulation of Phase I metabolism genes may have occurred early within the 6 hour exposure, and expression was ultimately depleted or down-regulated to conserve energy during up-regulation of Phase II enzyme genes. Induced expression of Cyp1 and Cyp2 gene families is known to decrease with time after induction, and the observed reduction in the expression of some Cyp's is likely due to cellular recovery taking place by the time of observation (Baker et al. 2001). Observed qPCR results showed a post-exposure increase in the expression of several Phase II genes including *Sult6b1*, several Ugt's (i.e., *Ugcg, Ugt1a2, Ugt1a6a*), a Gst's (i.e., *Ptges*), and an Mt (i.e., *Acsm3*). Little is known about the tissue distribution of the *Sult1a2* isozyme that can metabolically transform compounds such as 2-AAF; however, it has been implicated in liver and bladder cancers (Meinl and Glatt 2001). Thus, low inducibility in a lung-derived cell line could explain the cell line's preferential mobilization of *Sult6b1*.

The demonstrated capacity of 2-AAF to induce expression changes of several genes corresponding to Phase II isozymes collectively indicates that the cell line is competent to conjugate intermediate 2-AAF metabolites (i.e., N-hydroxy- AAF). For example, increased

expression of Sult's, when interpreted alongside the augmentation of *lacZ* mutant frequency, implies that FE1 cells are capable of generating PAPS (3'-phosphoadenosine- 5'-phosphosulfate), an essential cofactor required for Sult's to carry out metabolic transfer of a sulfonate group to a xenobiotic metabolite (Klaassen 2008). It should be noted that *in vivo* transformation of 2-AAF is additionally able to employ *N*-acetyltransferases (*Nat1* and *Nat2*) for Phase II conjugation (Wasalathanthri et al. 2015; Hein et al. 1993); however, the FE1 cell line either does not possess the capacity to alter the expression of Nat isozymes or preferentially mobilises Sult's. Further analyses is required to characterize the capacity of FE1 cells to mobilise Nat's.

The augmentation of *lacZ* mutant frequency observed at high concentrations of 2-AAF (-S9) suggests dose-dependent expression of Phase I metabolism genes. For example, upregulation of *Cyp1a2* appears to be manifested only at elevated test concentrations (Table 3.5). The distribution of induced gene expression BMD values support this hypothesis since the results show that various Cyp's and other Phase I enzymes are differentially expressed across increasing BMD values (Table 3.5; Figure 3.9). Furthermore, pathway analysis indicates that xenobiotic metabolism signalling pathways only respond to 2-AAF exposure at higher BMD values, and thus FE1 appears to transform 2-AAF when exposure levels are relatively elevated (Table 3.4). Overall, gene expression analysis of 2-AAF-exposed FE1 confirmed that the cells possess the capacity to bind xenobiotics that require PXR; moreover, transcribe the metabolic genes required for the biotransformation of 2-AAF into genotoxic metabolites.

Metabolic transformation of 1-MP requires both Phase I and II enzymes, following binding to the AhR (Machala et al. 2001). The primary oxidation reaction can be catalyzed by

several AhR-regulated Cyp monooxygenases, but it primarily utilizes CYP2E1 or CYP3A4, with the resulting metabolite conjugated by SULT1A1/2-catalysed sulfonation. The ultimate metabolites can generate DNA-reactive agents that form stable, bulky DNA adducts and induce mutations (Figure 3.1C) (Bendadani et al. 2016; Bendadani et al. 2014; Jiang et al. 2015; Engst et al. 1999). However, 1MP exposure did not elicit a positive *lacZ* mutagenicity response, even in the presence of exogenous S9 activation.

The qPCR results corresponding to Phase I metabolism displayed a significant increase in the expression of several Cyp monooxygenases (i.e., *Cyp1a1, Cyp27b1, Cyp3a57, Cyp2r1* and *Cyp4b1*), which are potentially capable of methyl group oxidation, thus generating promutagenic metabolites. However, the results showed no differential expression of *Cyp2e1* or *Cyp3a11* and *Cyp3a41b*. This is not surprising since *Cyp2e1* is constitutively expressed in mammalian liver, and cellular activity levels are regulated by post-translational modification (i.e., *Cyp2e1*-substrate binding blocks ubiquitin conjugation associated with gene degradation) (Kocarek et al. 2000). Thus, since the gene's transcription cannot be induced by xenobiotic exposure, and the expression in exposed cells should not be expected to be altered relative to controls. Furthermore, *Cyp2e1, Cyp3a11* and *Cyp3a41b* are all minimally expressed and not inducible in murine lung tissue *in vivo,* and as such, are not likely to be inducible in lung-derived cells *in vitro* (Renaud et al. 2011; Hart et al. 2009). Interestingly, the BMDExpress DataViewer results revealed 1-MP-induced signalling of PXR/RXR pathways (Table 3.4), suggesting PXR-driven *Cyp3a* transcription.

The expression of a host of other Phase I and II genes were also affected by 1-MP. Similar to DMBA, another alkylated PAH, 1-MP exposure reduced the expression of *Cyp21a1*;

providing further evidence of PAH interference in steroid metabolism (Luch 2005). Phase I arrays also showed decreased transcription of *Aldh1b1* indicating that the cell may be reducing expression to ensure that the monooxygenase generated hydroxyls are not converted to aldehydes, thus conserving cellular capacity to transcribe Phase II genes. Interestingly, *UchL1*, an ubiquitin carboxyl-terminal hydrolase, expression was increased. As the name suggests, *UchL1* hydrolyzes ubiquitin (Ub) proteins at the carboxyl terminus, such that Ub is cleaved and *UchL1* participates in the generation of free Ub for several cellular signalling processes (Ovaa et al. 2004). Increased *UchL1* generation of free ubiquitin may in turn be contributing to the degradation of *Cyp2e1* mRNA. This outcome would be accentuated by lack of interaction between 1-MP and *Cyp2e1* mRNA, since substrate-mRNA binding sterically hinders or prevents binding of Ub targets (Roberts et al. 1995). Alternatively, the free Ub may be utilized for secondary signalling processes (i.e., cell proliferation, apoptosis, immunity) (Ovaa et al. 2004). In either case, it appears likely that the lack of 1-MP mutagenicity may be a combined consequence of a lack of CYP2E1 activity in a lung cell line and the inability to mobilise CYP2E1 via ligand-receptor binding, as well as the 1-MP-induced increase in *UchL1* and the consequent increase in free ubiquitin. Lastly, expression of *Xdh,* which preferentially oxidizes purines and heterocyclic compounds, also increased, suggesting that Phase I metabolism in FE1 cells may be employing secondary pathways to detoxify 1-MP (Kitamura et al. 2006).

The Phase II gene expression array results indicate that 1-MP exposed FE1s induce the expression of several Ugt's (i.e., *Ugt1a1, Ugt1a2, Ugt1a6a*), Gst's (i.e., *Gsta3, Ptges*) and Sult's (i.e., *Sult4a1, Sult6b1*), including *Sult1a1* that is essential for bioactivation of the chemical. Although the results discussed above indicate that the monooxygenases required for 1-MP

bioactivation (e.g., CYP2E1) are not present in murine lung tissue, and do not appear to be present in FE1s cells, the Phase II array results indicate the cell line retains the capacity to conjugate 1-hydroxymethylpyrene (1-HMP), a necessary step for the generation of a DNA-reactive metabolite. This discrepancy between the aforementioned deficiency in the Cyp isozymes required to generate 1-HMP, and the availability of the Phase II isozymes required to generate the ultimate DNA-reactive metabolite (i.e., Sult's), (see Figure 3.6) is in general agreement with the observed lack of mutagenic activity in the FE1 cell assay. Nevertheless, it is interesting to note that the lack of FE1 mutagenicity may also be due to low permeation of substrate into the target cell. This contention is supported by Glatt (2000) who noted that cell membranes can constitute a difficult barrier for sulfuric acid esters such as 1-sulfooxymethylpyrene. Thus, even in the presence of exogenous metabolic activation, which would be expected to generate extracellular 1-sulfooxymethylpyrene, inability to cross the cell membrane may preclude formation of DNA damage and mutation. Furthermore, studies of the 1-HMP metabolite have also shown that the reactive metabolite 1-sulfooxymethylpyrene, which is generated following sulfonation by Sult's, possesses an aqueous half-life of less than 5 minutes (Bendadani et al. 2014). Overall, the observed increase in expression of several Cyp isozymes suggests that 1-MP was able to bind the AhR and PXR. While the increased expression of Phase II genes encoding Sult's and Ugt's indicate that FE1 cell can potentially carry out the conjugation required for generation of a DNA-reactive metabolite, the results indicate that the cell line does not possess the Phase I metabolic capacity necessary for conversion of 1-MP into the metabolite that serves as the substrate for the Phase II reaction. It is unfortunate that screening of 1-MP in FE1 cells did not provide an accurate portrayal of mutagenic potential. The

incongruence with known mutagenicity appears to be due to a combination of insufficient intracellular Phase I metabolic capacity, and/or inability of stable extracellular metabolites to enter the cell, and/or the instability of highly reactive extracellular metabolites. Thus, testing of a more soluble compound such as the aromatic amine, 1-aminopyrene, a PAH-derivative requiring somewhat analogous Phase I and II transformation, may be necessary.

Aflatoxins are mycotoxins able to act as AhR, PXR and CAR agonists. AFB1-receptor binding allows translocation to the nucleus and transcription of *Cyp1a2* or *Cyp1a1* if bound to AhR-ARNT dimers, and *Cyp3a11* if activated via PXR-RXR or CAR-RXR dimers (Ayed-Boussema et al. 2012). Bioactivation of AFB1 (i.e., generation of DNA-reactive metabolites) only requires Phase I oxidation catalyzed by CYP1A2 at lower doses, and/or CYP3A11/CYP3A41B monooxygenases at higher doses (Figure 3.1D) (Luch 2005; Bbosa et al. 2013). As previously mentioned, murine lung tissue does not generally possess the human CYP3A4 homologs *Cyp3A11* and *Cyp3a41b* (Renaud et al. 2011).

The mutagenicity results showed a significant increase in *lacZ* mutant frequency following exposure to AFB1 without the addition of S9. Gene expression analysis confirmed that exposure to AFB1 significantly altered the expression of several Cyp monooxygenases including *Cyp1a2*, *Cyp11b1, Cyp11b2, Cyp2c38* and *Cyp4f18*. CYP1A2 is known to be required for bioactivation of AFB1, and the other available Cyp's may facilitate and/or increase Cyp-catalysed oxidation of AFB1. Interestingly, the low and medium concentrations elicited a dose-dependent decrease in Cyp expression with congruent absence of mutagenicity; at the higher concentration Cyp expression was increased with an attendant increase in *lacZ* mutant frequency. Indeed, the BMD distribution profile revealed 2 distinct modes of gene expression,

those genes that are activated at low concentrations (i.e., *Cyp1a1*) and those do not respond until higher concentrations are reached (i.e., *Cyp1a2*) (Figure 3.9, Table 3.5). Phase I arrays also indicated differential expression in *Gzma,* a gene involved in apoptosis signalling (see Table 3.3), as well as *UchL1*; induced expression of these genes suggests that exposed cells are toxicologically stressed and preparing for cell cycle alterations and/or programmed cell death. Although Phase II metabolism is not explicitly required for the biotransformation of AFB1, the gene expression results showed an increase in the expression of many genes involved in chemical detoxification and excretion, including Sult's (i.e., *Sult1a1, Sult4a1*, *Sult6b1*), Ugt's (i.e., *Ugt1a1*, *Ugt1a2*), and Gst's (i.e., *Ptges, Gsta3*), in addition to the Mt *Acsm3*. Overall, the observed AFB1 mutagenicity is congruent with the observed gene expression changes. Moreover, FE1 cells are metabolically competent to generate mutagenic AFB1 metabolites, presumably via ligand binding to the appropriate nuclear receptors (i.e., AhR) and mobilisation of Cyp isozymes. Similar to what was hypothesised for 2-AAF, the observed Cyp down-regulation in AFB1-exposed cells may simply be a result of experimental timing, i.e., Cyp's are upregulated, but their expression is ephemeral and reduced by the time of observation. Evaluation of this hypothesis would require subsequent analyses of temporal changes in toxicant-exposed FE1 cells. The cost of rigorous time-series analyses would likely be prohibitive.

NNK is a tobacco-specific bioactivated into DNA reactive metabolites requires Phase I oxidation, primarily catalyzed by CYP2A4 and/or CYP2A5 isozymes (Wasalathanthri et al. 2015). The ligand binding activity of NNK has not been as extensively studied as the aforementioned test compounds; however, a recent *in vivo* study confirmed that mouse lung tumours induced by NNK were generated via a CAR-mediated mechanism (Fukumasu et al. 2015). Interestingly,

in vitro studies with human hepatocyte cells show that induced *CYP2A6* and *CYP2A13* gene expression involves binding and activation of PXR and/or CAR (Itoh et al. 2006; Smith et al. 2014), and transformation of NNK into a DNA-reactive metabolite requires Phase I oxidation by CYP2A4 or CYP2A5 isozymes (i.e., human homologs CYP2A6 and CYP2A13) (Figure 3.1E) (Wasalathanthri et al. 2015; Chiang et al. 2011). An earlier study noted that CYP2E1 in human liver microsomes can also transform NNK into reactive metabolites (Yamazaki et al. 1992)

Despite the aforementioned carcinogenicity and receptor binding likelihood, NNK exposure of FE1 cells did not induce a cytotoxic or mutagenic response with or without S9. The lack of mutagenicity may be linked to the fact that expression of *Cyp2a4/2a5* in lung tissue is known to be negligible (Renaud et al. 2011). In addition, as noted earlier CYP2E1 activity in FE1 cells is likely very constrained. The gene expression analysis confirmed that there was no exposure-induced up-regulation of the Cyp genes that are essential for Phase I oxidation of NNK. However, the results showed differential expression of aldehyde and xanthine dehydrogenases, Flavin-containing monooxygenase and esterases. This may indicate that NNK-exposed cells are mobilising detoxification enzymes other than the aforementioned Cyp monooxygenases. The transcription of *Cyp4b1* was significantly up-regulated at the intermediate test concentration; this isozyme is an omega hydrolyse known to be highly expressed in the lung and involved in the hydrolysis of short chain fatty acids (Hardwick 2008). Therefore, *Cyp4b1* may employ lipid metabolism pathways to detoxify the xenobiotic. Of the 5 chemicals tested, NNK induced the largest increase in Phase II genes, including large increases in the expression of acetyltransferases, Gst's, Sult's and Ugt's. This is consistent with *in vivo* studies where murine NNK exposure was shown to elicit major spikes, and inevitable depletion,

of Phase II enzymes such as Gst's and Ugt's (IARC 2007a). Overall, the results reveal that the FE1 cell line is able to mobilise the Phase II enzymes involved in NNK detoxification; however, the cells do not possess the Phase I capacity to convert NNK into mutagenic metabolites. This is consistent with the known metabolic requirements of NNK bioactivation, and the deficiency of the required Cyp isozymes (e.g., CYP2A4 and/or 2A5) in lung tissue *in vivo*.

qPCR results indicate that the FE1 cell line retains the capacity to regulate endogenous expression of several Phase I (i.e., *Cyp1a1, Cyp1b1, Cyp2c38, Cyp3a57, Cyp4b1 Fmo2, Aldh1a7*) and Phase II genes (i.e., *Sult1a1, Sult6b1, Ugt1a1, Gsta3, Acsm3, Nqo1*) (Table 3.3). Induced expression of these metabolism genes implies that the cells retain expression of the AhR and PXR nuclear receptors; potentially the CAR nuclear receptor as well. Generally, FE1's appear to be able to bind xenobiotics, presumably via the appropriate nuclear receptors, translocate the receptor-bound xenobiotic into the nucleus, therein affecting the transcription of genes that are capable of controlling the generation of genotoxic metabolites. More specifically, the results obtained indicate that DMBA, 2-AAF and AFB1 appear to be able to enter the cell nucleus via binding of AhR and/or PXR, with activated dimers binding to DNA promoter sequences thereby activating transcription of genes such as *Cyp1a1, Cyp1b1, Cyp1a2, Cyp2c38, Ugt1a1* and *Sult6b1*. Increased transcription presumably results in increased translation and increased enzymatic activity, and these enzymes where presumably mobilised to catalyze the oxidation and conjugation reactions necessary for bioactivation of the test agents. The bioactivation (i.e., conversion into DNA-reactive metabolites) was confirmed by positive *lacZ* mutagenicity results. The FE1 applicability domain therefore includes compounds with similar metabolic requirements for bioactivation via Phase I Cyp monooxygenases and aldehyde

dehydrogenases, and Phase II sulfo- and glucuronyltransferases. In contrast, 1-MP and NNK did not elicit a positive mutagenicity response, even in the presence Aroclor 1254-induced S9, indicating that compounds (i.e., nitrosamines) requiring similar oxidation by CYP2E1, CYP3A11 and/or CYP2A4/2A5 monooxygenases for generation of DNA-reactive metabolites likely lie outside the assay's applicability domain.

The results obtained clearly indicate that the applicability domain of the FE1 cell transgene mutagenicity assay conducted in the absence of exogenous S9 activation is far more extensive than that of the mouse lymphoma assay (MLA), the *hprt* gene mutation assay, and the *in vitro* MN assay, all of which require exogenous S9 to yield positive responses for AFB1 and DMBA, or similar compounds (Table 3.6) (Preisler et al. 2000; Shi et al. 1995; Corvi et al. 2008; OECD 2015a; OECD 2015b). The FE1 cell assay also demonstrated greater applicability range in reference to 2-AAF, since the MLA and *in vitro* MN testing of 2-AAF require S9 (Mitchell et al. 1988; Corvi et al. 2008). Interestingly, the *hprt* gene mutation assay in human lymphoblast cells (i.e., AHH-1 and MCL-5) yielded a positive 2-AAF response without S9 (Heflich and Neft 1994). With respect to the 5 compounds investigated, Table 3.6 provides an overall comparison of results obtained for the MLA, the *hprt* gene mutation assay, the *in vitro* MN assay, and the MutaMouse FE1 cell transgene mutagenicity assay. Screening of NNK revealed a limitation of the FE1 cell mutagenicity assay; positive results have been reported for the *hprt* gene mutation assay without S9 (Krause et al. 1999), the MLA with S9, and the *in vitro* MN assay with S9 (Table 3.6), (Guo et al. 2016; Ates et al. 2016). 1-MP has not been as extensively tested *in vitro*. It did not elicit positive responses in the *in vitro hprt* gene mutation and MN assays for most cell lines; however, a positive result for both endpoints was obtained using a (Chinese hamster) V79-

derived cell line expressing human CYP2E1 and SULT1A1 (Jiang et al. 2015). Therefore, although the inability to bioactivate 1-MP limits the applicability domain of the FE1 assay, the response obtained is comparable to that observed using OECD-recommend test methods. Importantly, the range of compounds reliably assessed by the FE1 cell assay also includes direct-acting mutagens (i.e., ENU, ICR-191), nitroarenes (i.e., 3-NBA), (nano)particulate test articles (i.e., carbon black, diesel exhaust particulates, multi-walled carbon nanotubes, and complex matrices such as cigarette smoke condensate and coal tar extract as well as EURL-ECVAM-recommended false positive compounds (Lemieux et al. 2015; Poulsen et al. 2013; Chen et al. 2008; Maertens et al. 2017; White et al. 2003; Jacobsen et al. 2008). Clearly, rigorous characterization of the FE1 cell assay applicability domain requires screening of more compounds; nevertheless, results obtained to date indicate that the assay's applicability domain is expansive when compared to the *in vitro* systems currently endorsed by the OECD.

The aforementioned BMDExpress DataViewer analysis mapped the mean gene expression BMD to noteworthy cellular signalling pathways altered by the chemical exposure (Kuo et al. 2016). Since the commercial RT^2 Profiler arrays employed in this study specifically targeted Phase I and II genes involved in 'drug metabolism', it is not surprising that BMDExpress analysis revealed activation of xenobiotic metabolism pathways. Biological signalling pathways activated by FE1 cell exposures to 1-MP, 2-AAF, DMBA, AFB1 and NNK, and the BMD at which the pathways were triggered, are presented in Table 3.4. Since all compounds altered the expression of Cyp monooxygenases, it is not surprising that the results show alterations of AhR-related pathways.

BMD analysis also showed that LPS/IL-1 Mediated inhibition of RXR function and Ubiquinol-10 (CoQ) biosynthesis pathways were activated by all tested compounds. Lipopolysaccharides (LPS) and Interlukin-1 (IL-1) can inhibit RXR function by ensuring that RXR cannot dimerize with receptor proteins, including the PXR, CAR and PPAR (Peroxisome Proliferator Activated Receptor), thus restricting the binding to promoters of genes associated with regulation of lipid metabolism (Wang et al. 2005). This cascade will likely interfere with upregulation of xenobiotic metabolism gene targets. It is interesting to note that exposures in FE1 to multi-walled carbon nanotubes also altered LPS/IL-1 mediated inhibition of the RXR signalling (Poulsen et al. 2013). CoQ, which is thought to have antioxidant properties, is a component of the electron transport chain that is specifically involved in the transfer of electrons across membranes (Kawamukai 2009). The induction of CoQ synthesis by all test chemicals likely indicates that CoQ dependent transfer of electrons is essential for redox processing of xenobiotics (Table 3.4). CoQ metabolic regulation involves RXR dimerization, which in turn activates the transcription of genes involved in fatty acid metabolism and transport, as well as bile acid synthesis (Turunen et al. 2004). As mentioned, xenobiotic are often conjugated to endogenous sulfate or glutathione; however, xenobiotics and their metabolites can also conjugate to endogenous lipids such as cholesterol, bile acids, triacylglycerol's and phospholipids (Ansari et al. 1995).

Interestingly, PPAR, RXR, and several other receptors (e.g., liver X receptor, retinoic acid receptors, and thyroid receptors) are able to metabolically alter xenobiotics and endogenous fatty acids via ligand-activated transcription factors that interact with DNA response elements that regulate the expression of genes coding for lipid metabolising enzymes (Vanden Heuvel

1999; Karagianni and Talianidis 2015). Fatty acid metabolism can donate electrons for ATP production, and FE1 cells may use the source of energy for cellular homeostasis and/or xenobiotic metabolism (Karagianni and Talianidis 2015). Consequently, the results imply that fatty acid oxidation pathways are sensitive to *in vitro* xenobiotic exposures.

BMD analysis also demonstrated that all chemicals activated acetone degradation pathways that involve methylglyoxal signalling and lipid metabolism (Kalapos 2008); 1-MP additionally induced methylglyoxal degradation signalling (Table 3.4). During periods of glucose-depletion (i.e., exercise or stress), acetone is formed by metabolic processing that transports energy from the liver to remote tissues *in vivo* (Laffel 1999). Thus, during periods of chemically-induced stress, FE1 cells may be activating acetone degradation pathways to maintain homeostasis via lipid metabolism. Alterations of the aforementioned biological signalling pathways indicate that FE1's manifested chemically-induced stress via alterations of pathways related to metabolism of xenobiotics and endogenous compounds (i.e., lipids). These alterations will modulate conversion of test chemicals into a variety of metabolites and/or mobilise energy and endogenous substrates to support metabolism.

Mutagen exposures are known to generate reactive oxygen species and/or free radicals, such that oxidative stress is an inevitable consequence (Deavall et al. 2012). Accordingly, NRF2-mediated oxidative stress responses were altered following FE1 exposures to all test chemicals except AFB1 (Table 3.4). NRF2 is a transcription factor that acts on the ARE enhancer (Antioxidant Response Element), which in turn regulates genes that encode enzymes associated with oxidative stress responses. Specifically, genes involved in defense against oxidative damage and maintenance of cellular redox homeostasis (Nguyen et al. 2003). *In vivo* rat studies

have shown that NRF2-stimulated transcription of ARE can also affect transcription of GSTA2 and NQO1 (Nguyen et al. 2003). FE1 cell signalling of NRF2-mediated processes signifies that the xenobiotic exposures investigated augmented cellular defenses against oxidative stress, including increased Gst's and *Nqo1* expression in response to PAH and nitrosamine (i.e., NNK) exposures. PAH exposure (i.e., DMBA and 1-MP) also activated signal transduction of heparan sulfate and stearate biosynthesis pathways, both fatty acids that have been found to possess antioxidant properties (Table 3.4) (Kliment et al. 2015). Increased expression of Gst's, characterised by activated glutathione-mediated detoxification and cysteine biosynthesis pathways, were revealed in the BMDExpress analysis of NNK, 1-MP and DMBA exposed cells (Table 3.4) (Lu 2009). Overall, in addition to the experimentally confirmed xenobiotic metabolism, the results reflect induction of several pathways involved in the transcriptional activation of factors associated with endogenous antioxidant pathways.

BMD analysis of the gene expression results also identified alterations of more minor pathways. For example, the results suggest that FE1 cells may have employed a tertiary cascade of biological signalling pathways related to oxidative and non-oxidative ethanol degradation (i.e., 1-MP, 2-AAF; Table 3.4). Indeed, the qPCR results show differential expression of genes transcribing aldehyde dehydrogenases, esterase's, Flavin-containing monooxygenases, etc. (see Table 3.3). It is known that cells often compensate for increased fatty acid biosynthesis by up-regulating ethanol degradation, since both essentially employ NADPH as a cofactor (de Jong et al. 2014). Therefore, it is reasonable to assert that FE1 cells are employing the ethanol degradation pathway to preserve NADPH for more critical pathways. Similarly, tryptophan (Trp) degradation has immunosuppressive properties that have been linked to increased protection

against inflammation (Opitz et al. 2007). FE1 cells showed induced sensitivity to Trp degradation (i.e., 1-MP exposure; Table 3.4) that may be reciprocally activating responses to combat chemically-induced inflammation and oxidative stress. In addition, to relieve energy and NADPH expenditures for xenobiotic metabolism, FE1 cells appear to be concomitantly downregulating pathways associated with growth and angiogenesis (i.e., methionine and histidine degradation, both amino acids involved in protein synthesis; Table 3.4) (Klaassen 2008; Ravanel et al. 1998). It is important to note that these relatively minor pathways are only modified at higher test concentrations (i.e., higher BMD values, Table 3.4, Figure 3.10), wherein the cells are presumably under stress (i.e., only 40% of the cells survive the exposure conditions). Figure 3.10 provides an example of pathway sensitivities (i.e., BMD) to increasing concentrations of AFB1. The summarised results (i.e., Figure 3.10) are consistent with the assertion that responses to extreme chemical and oxidative stresses are manifested as pathway alterations related to a basic need for cell survival. At high(er) concentrations cell recruit these pathways to boost cellular defences and preserve energy.

It is interesting to note that there is a wide range of overlap between pathways that maintain cellular homeostasis under 'normal' conditions, and pathway responses to xenobiotic stress. Such pathway overlap is exhibited in FE1 via activation of neurotransmitter and steroid degradation pathways. Four of the five test chemicals (i.e., excluding AFB1) activated signalling pathways related to nicotine, serotonin and melatonin degradation (Table 3.4). For example, NNK, a nicotine derivative, induced nicotine degradation pathways that are involved in metabolic transformation via break-down of the pyridine and pyrrolidine rings that characterise nicotine and steroid structures (Gurusamy and Natarajan 2013). Interestingly, the literature

provides evidence of many links between steroid and xenobiotic metabolism. A notable link is the transcriptional regulation of Cyp monooxygenases via various co-activation mechanisms involving SRC (steroid receptor coactivators). For example, SRC-1 co-binds to *Cyp1a1, Cyp1a2, Cyp2c9* and *Cyp7a1* promoters with PXR/RXR dimers, and SRC-3 activates *Cyp2b1* and *Cyp3a11* in conjunction with CAR and PXR (Stashi et al. 2014; McKenna et al. 2015; Li and Chiang 2005). The FE1 cells may therefore activate steroid and neurotransmitter degradation pathways in conjunction with stress response and metabolic pathways associated with the xenobiotic exposure. The BMD analysis showed that in addition to pathways related to direct metabolism of xenobiotic compounds, the FE1 cell line is also proficient in secondary signalling pathways that offer protection against oxidative stress and/or augment cellular xenobiotic processing.

Future studies addressing the applicability domain should screen compounds in a high-throughput manner utilizing technologies such as RNA-seq to determine the chemically-induced expression changes of selected metabolism genes. Basically, RNA-seq utilizes next generation sequencing tools, where RNA converted to cDNA are amplified with adaptors that are used to 'map' generated reads to a reference genome; in this manner gene expression levels can be quantified via counting the number of sequence reads per gene of interest (Wang et al. 2009). Ideally, 25 compounds or more should be tested, including many known mutagens that require different MOA's such as the aforementioned aromatic amine, 1-aminopyrene, a heterocyclic amine (e.g., 2-amino-3-methylimidazo[4,5*f*]quinolone (IQ)), nitrosamines (e.g., *N*-Nitrosodiethylamine or *N*-Nitrosoanabasine), a nitrogen mustard (e.g., mechlorethamine hydrochloride), and 2,4-diaminotoluene, or cadmium chloride, an organic azo dye precursor and inorganic pigment precursor, respectively. A few aneugenic agents (e.g., carbendazim,

diethylstilbestrol) and clastogens (e.g., methyl methanesulfonate, etoposide) should also be tested to further investigate the assay limitation associated with these types of compounds. Although the are no specific OECD recommendations regarding the number of compounds required for testing in pre-validation studies, most employ as many as possible (e.g., validation of the Mouse Lymphoma assay screened 43 compounds and the retrospective validation of the *in vitro* MN assay screened 142 test articles (Clive et al. 1979; Corvi et al. 2008)). In order to increase the number of compounds that can be screened, the number of genes of interest can be reduced from 84 Phase I and 84 Phase II genes to 15-20 primary genes of interest (i.e., *Cyp1a1, Cyp1a2, Cyp1b1, Cyp2e1, Cyp3a41, Sult1a1, Ugt1a1, Nat1, Gsta3*). Although it is impossible to test all compounds, and to achieve a complete determination of an assay's applicability domain, it is important that an extensive range of compounds requiring differing MOA's are screened.

The different metabolic requirements associated with the test articles screened in this study, as well as within the intra-laboratory study (Chapter 2; i.e., benzo[*a*]pyrene, *N*-ethyl-*N*-nitrosourea, 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and *N*-Nitrosodimethylamine), provide a suitable range of compounds that provide representation of the *in vitro* transgene mutagenicity assay applicability domain. The results of this assessment show that the assay is competent in the bioactivation of several PAH's, aflatoxins and aromatic amines (-S9) as well as heterocyclic amines (+S9); however, they also showed limitation in the screening of a clastogen, nitroso-compound and an alkyl-PAH. Although the range of compounds tested to date imply that the FE1 cell assay has a more expansive, and thus more useful, applicability domain than

assays currently employed for regulatory assessments, several more compounds will need to be screened in order to confirm these conclusions.

In summary, gene expression profiling of FE1 cells exposed to DMBA, AFB1 and 2-AAF confirmed endogenous Phase I metabolic capacity. More specifically, the results showed an endogenous capacity to express several Cyp monooxygenase genes (i.e., *Cyp1a1, Cyp1a2, Cyp1b1*), as well as numerous genes associated with minor detoxification pathways (e.g., aldehyde dehydrogenases, Flavin-containing monooxygenases). The cell line was not able to transform 1-MP or NNK into mutagenic metabolites, likely due to the fact that the Cyp isozymes required for Phase I oxidation of these compounds are absent from murine lung, or cells derived from murine lung. In addition, the results also verify the cell line's retention of AhR, PXR and CAR nuclear receptor signalling pathways. The results also confirmed endogenous Phase II metabolic capacity; more specifically, the ability to mobilise Sult's, Gst's, and Ugt's. However, further investigation is necessary to characterize the capacity of chemically-regulated NAT's transcription; this would involve testing of compounds that only employ acetyl group conjugation during Phase II bioactivation (e.g., nitroarenes). Furthermore, BMD analysis illustrated the concentration dependency of the observed transcriptional changes, and allowed identification of several biological signalling pathways induced by the chemical exposures. The primary pathways included genes involved in xenobiotic metabolism and oxidative stress responses at low concentrations, and, at higher concentrations, a variety of pathways involved in cellular energy preservation and processing of lipids, steroids and neurotransmitters. These results clearly demonstrate that the FE1 cell line has the endogenous metabolic capacity necessary to detect noteworthy mutagens that require Phase I and/or Phase II processes to

generate DNA-reactive metabolites. Thus, the applicability domain of an *in vitro* transgene mutagenicity assay based on the FE1 cells is more comprehensive than that associated with the aforementioned test methods currently used for regulatory evaluations. Correspondingly, the FE1 cell line can be termed metabolically superior to the cells currently used for routine regulatory assessments of genetic toxicity (i.e., Mouse Lymphoma, TK6, CHO,). Nevertheless, it is also clear that FE1 cells, by virtue of being derived from murine lung, do not possess the complete complement of metabolic attributes that would permit complete abandonment of exogenous S9 activation. Therefore, effective use of the FE1 cell *in vitro* mutagenicity assay will necessitate use of exogenous S9 when a negative response is obtained in the absence of S9. In conclusion, the MutaMouse FE1 cell mutagenicity assay constitutes a practical and effective alternative to current mammalian cell mutagenicity tests. Since the cells retain the metabolic capacity to bioactivate some promutagens, the assay is a logical candidate for continued validation and an internationally-accepted test guideline.

3.5 Tables and Figures:

Table 3.1 Xenobiotic metabolizing enzyme types, typical genes, and the general function of each enzyme type. Abbreviations are as follows: C- carbon, O- oxygen, N- nitrogen, S- sulfur, P – phosphate. Based on (Klaassen 2008).

	Enzymes	Typical Genes	Reactions
Phase I oxygenases	Cytochrome P450s (CYP)	*Cyp1a1, Cyp1a2, Cyp1b1, Cyp2c, Cyp2e1, Cyp3a4*	C and O oxidation, De-alkylation, others
	Flavin-containing monooxygenases (FMO)	*Fmo1, Fmo2*	N, S and P oxidation
	Epoxide hydrolases (mEH, sEH)	*Ephx1, Ephx2*	Hydrolysis of epoxides
Phase II transferases	Sulfotransferases (SULT)	*Sult1a1, Sult1b1*	Addition of sulfate
	UDP-glucuronosyltransferases (UGT)	*Ugt1a1, Ugt1a2*	Addition of glucuronic acid
	Glutathione-*S*-transferases (GST)	*Gsta3, Mgst1*	Addition of glutathione
	N-acetyltransferases (NAT)	*Nat1, Nat2*	Addition of acetyl group
	Methyltransferases (MT)	*As3mt, Comt*	Addition of methyl group
Other	Alcohol dehydrogenases	*Adh1, Adh7*	Reduction of alcohols
	Aldehyde dehydrogenases	*Aldh1a1, Aldh1a2, Aldh1a7*	Reduction of aldehydes
	NADPH-quinone oxidoreductase (NQO)	*Nqo1, Nqo2*	Reduction of quinones

Table 3.2 Summary of DNA damage signalling gene expression changes observed following chemical exposures of FE1 cells. Upregulated genes are marked with ↑, and downregulated genes with ↓. All presented genes showed a minimum 2-fold change in expression. Abbreviations are as follows: ATM – ATM/ATR signalling, MMR- mismatch repair, BER- base excision repair, DSB- double strand break repair, NER- nucleotide excision repair.

Gene Symbol	Fold Change	Gene Function	Gene Symbol	Fold Change	Gene Function
	DMBA 6+0 hours			**DMBA 6+4 hours**	
Cdkn1a	↑2.4	Apoptosis/ Cell cycle stall	*Brac1*	↑3.0	ATM/ Apoptosis/ DSB
Rad9a	↓88.4	ATM/ Apoptosis	*Brac2*	↑2.7	DSB/ NER
			Cdkn1a	↑2.4	Apoptosis/ Cell cycle stall
			Exo1	↑2.0	MMR
			Mbd4	↑2.8	BER
	AFB1 6+0 hours			**AFB1 6+4 hours**	
Cdkn1a	↑3.9	Apoptosis/ Cell cycle stall	*Bax*	↑2.0	Apoptosis
Fancd2	↑2.1	ATM	*Blm*	↑3.0	DSB
Rad9a	↓100.0	ATM/ Apoptosis	*Brac1*	↑3.4	ATM/ Apoptosis/ DSB
Xrcc3	↓6.3	Homologous recombination repair	*Brac2*	↑3.7	DSB/ NER
			Brip1	↑3.1	DSB
			Cdc25a	↑2.1	Cell cycle stall
			Cdkn1a	↑9.1	Apoptosis/ Cell cycle stall
			Dclre1a	↑2.2	NER
			Exo1	↑2.8	MMR
			Fancg	↑2.5	Other
			Lig1	↑2.0	NER/ DSB/ BER
			Mbd4	↑2.3	BER
			Mdc1	↑2.0	DSB
			Ppm1d	↑2.5	Cell Cycle
			Rad51	↑2.4	DSB
	2-AAF 6+0 hours			**2-AAF 6+4 hours**	
Ddit3	↑2.6	Cell cycle stall	*Brac1*	↑2.1	ATM/ Apoptosis/ DSB
Gadd45a	↑2.6	Cell cycle stall/ Apoptosis/ ATM	*Brac2*	↑2.5	DSB/ NER
Ppp1r15a	↑2.6	Cell cycle stall/ Apoptosis			
Rad9a	↓3.0	ATM/ Apoptosis			
	1-MP 6+0 hours			**1-MP 6+4 hours**	
Ddit3	↑2.4	Cell cycle stall	*Brac1*	↑2.0	ATM/ Apoptosis/

					DSB
Gadd45a	↑2.7	Cell cycle stall/ Apoptosis/ ATM	*Brac2*	↑2.2	DSB/ NER
Gadd45g	↓2.1	Cell cycle stall/ Apoptosis/ ATM			
Ppp1r15a	↑2.9	Cell cycle stall/ Apoptosis			
Rad9a	↓69.0	ATM/ Apoptosis			
	NNK 6+0 hours			**NNK 6+4 hours**	
Apex1	↓2.2	BER	No change above 2-fold		
Cdc25a	↓2.2	Cell cycle stall			
Rad9a	↓3.0	ATM/ Apoptosis			

Table 3.3 Summary of Phase I and II xenobiotic metabolism gene expression changes observed following chemical exposures of FE1 cells, with a description of gene function. All genes presented showed a minimum 2-fold change in expression. Abbreviations are as follows: Aldh - Aldehyde Dehydrogenases, Cyp - Cytochrome P450, Nat - *N*-acetyltransferases, Sult – Sulfotransferases, Dh – Dehydrogenases, Ugt - UDP-glycosyltransferases, AA - Amino Acid Transferases, Gst – Glutathione-*S*-transferase, Fmo - Flavin containing monooxygenases, Mt – methyltransferase, Ep – epoxidases, Est – esterase, PAH's – polycyclic aromatic hydrocarbons, CoA – Acetyl coenzyme A.

Gene Name	Family	Function of Enzyme Activity	Reference
		DMBA, Genes Upregulated	
Aldh7	Aldh	Oxidation of alcohols into aldehydes	(Klaassen 2008)
Cyp1a1	Cyp	Catalyzes hydroxylation and epoxidation, to activate PAHs	(Hussain and Nasser 2014)
Cyp1b1	Cyp	Catalyzes oxidation of PAHs and steroid hormones	(Hussain and Nasser 2014)
Acsm3	Nat	Catalyze esterification to CoA Activation of medium-chain length fatty acids and carboxylic acids	(Watkins et al. 2007)
Sult6b1	Sult	Catalyzes sulfonation of thyroxine and some xenobiotics	(S. Takahashi et al. 2009)
Nqo1	Dh	Catalyzes the 2 electron reduction of quinones	(R. Li 1995)
Ugcg	Ugt	Catalyses transfer of glucose to ceramide to generate glucosylceramide the primary structure for more than 300 glycosphingolipids (lipid synthesis)	(Ichikawa et al. 1996)
Ugt1a1	Ugt	Glucuronidation of phenols and quinols	(Ritter 2000)
Ugt1a2	Ugt	Glucuronidation of bile acids, estrogen and xenobiotics	(Ritter 2000)
Ugt1a6a	Ugt	"Bulky phenol Ugt" N/O-glucuronidation of hydroxamic acids	(Ritter 2000)
		DMBA, Genes Downregulated	
Aldh1b1	Aldh	Catalyzes the oxidation of alcohols into aldehydes	(Ying Chen et al. 2011)
Cyp21a1	Cyp	Catalyzes hydroxylation of progesterone into 11-deoxycorticosterone (steroid metabolism)	(Zhao et al. 2012)
A3galt2	Ugt	Catalyzes transfer of galactose from UDP-galactose to acceptor in lipid synthesis (i.e., Galβ-1,4-glucosylceramide)	(Jamaluddin et al. 2007)
A4galt	Ugt	Catalyzes transfer of galactose to lactosylceramide to form globotriaosylceramide (lipid metabolism and membrane receptor synthesis)	(Thuresson et al. 2011)
Ephx2	Ep	Catalyzes the hydrolysis of epoxy fatty acids to the corresponding dihydrodiols at C-terminus	(Newman et al. 2003)
Glyat	AA	Catalyses transfer of an acyl group off of acyl-CoA to glycine amino groups (detoxification)	(Badenhorst, et al. 2013)
Sult1e1	Sult	Catalyzes sulfonation of estrogen (17β-estradiol) and	(Cole et al. 2010)

		some xenobiotics	
Sult5a1	Sult	Catalyzes sulfonation (little known about substrate affinity and function)	(Nagata and Yamazoe 2000)
Ugt2b37	Ugt	Catalyzes glucuronidation of several endogenous compounds (i.e., bile and fatty acids, steroid hormones, fat soluble vitamins)	(Zhou et al. 2016)
		2-AAF, Genes Upregulated	
Acsm3	Nat	Catalyze esterification to CoA Activation of medium-chain length fatty acids and carboxylic acids	(Watkins et al. 2007)
Ptges	Gst	Converts prostaglandin (PG)H2 to PGE2 (inflammation, immunity, stress)	(Williams et al. 1999)
Ugcg	Ugt	Catalyses transfer of glucose to ceramide to generate glucosylceramide (lipid synthesis)	(Ichikawa et al. 1996)
Sult6b1	Sult	Catalyzes sulfonation of thyroxine and some xenobiotics	(Takahashi et al. 2009)
Ugt1a2	Ugt	Catalyzes glucuronidation of bile acids, estrogen and xenobiotics	(Ritter 2000)
Ugt1a6a	Ugt	"Bulky phenol UGT" Catalyzes *N*/*O*-glucuronidation of hydroxamic acids	(Ritter 2000)
		2-AAF, Genes Downregulated	
Aldh1a7	Aldh	Catalyzes the oxidation of alcohols into aldehydes	(Luch 2005)
Cyp11b2	Cyp	Catalyzes 1β-hydroxylation of 1-deoxycarticosterone into aldosterone	(Provost and Tremblay 2005)
Cyp1a2	Cyp	Primarily catalyzes *N*- hydroxylation of aromatic amines	(Hussain and Nasser 2014)
Cyp26b1	Cyp	Inactivation of all-trans-retinoic acid (all-trans-RA) by hydroxylation into metabolites 4-hydroxy-RA, 4-oxo-RA and 18-hydroxy-RA. Metabolism of 1 xenobiotic substrate to date (i.e., tazarotenic acid)	(Foti et al. 2016)
Cyp2c38	Cyp	Catalyzes synthesis of 11,12- epoxyeicosatrienoic acid (EET) and 8,9-EET from arachidonic acids (lipid metabolism)	(Tsao et al. 2001)
Cyp2s1	Cyp	Metabolizes retinoic acids into epoxyeicosatrienoic acids (lipid metabolism)	(Fromel et al. 2013)
Gzma	Est	Catalyzes single strand nicks in DNA and disruption of repair and stability mechanisms (apoptosis)	(Martinvalet et al. 2005)
		1-MP, Genes Upregulated	
Cyp1a1	Cyp	Catalyzes hydroxylation and epoxidation, to activate PAH's	(Hussain and Nasser 2014)
Cyp27b1	Cyp	Catalyzes hydroxylation of Vitamin D to active form 25-hydroxyvitamin D	(Seifert et al. 2009)
Cyp3a57	Cyp	Hydroxylation of 6β- or 16α- testosterone	(MGI Mouse Gene Detail 2017)
Cyp2r1	Cyp	Catalyzes hydroxylation of Vitamin D to active form 25-hydroxyvitamin D	(Zhu et al. 2013)
Cyp4b1	Cyp	Catalyzes hydroxylation of xenobiotics and fatty acids	(Imaoka et al.

		(i.e., 2-aminofluroine)	2001)
Uchl1	Est	De-ubiquitinating enzyme that cleaves bound ubiquitin (Ub) to generate free Ub	(Ovaa et al. 2004)
Xdh	Dh	Catalyze oxidation of *N*-heterocycles	(Kitamura et al. 2006)
Acsm3	Nat	Catalyze esterification to CoA Activation of medium-chain length fatty acids and carboxylic acids	(Watkins et al. 2007)
Gsta3	Gst	Catalyzes transfer of glutathione, that is conjugated to xenobiotic substrates for detoxification	(Ilic et al. 2010)
Ptges	Gst	Converts prostaglandin (PG)H2 to PGE2 Initiates cox2 pathway (inflammation, immunity, stress)	(Williams et al. 1999)
Pomgnt1	Nat	Catalyzes the transfer of N-acetylglucosamine(GluNac) from UDP- GluNac to O-mannosyl glycoproteins (cell signalling)	(Yoshida et al. 2001)
Nqo1	Dh	Catalyzes the 2 electron reduction of quinones	(R. Li 1995)
Sult1a1	Sult	Catalyzes sulfonation of xenobiotics, steroids and other endogenous substrates	(Gamage et al. 2006)
Sult4a1	Sult	Catalyzes sulfonation of xenobiotics and neurotransmitters	(Sidharthan et al. 2014)
Sult6b1	Sult	Catalyzes sulfonation of thyroxine (thyroid hormone T4) and some xenobiotics	(S. Takahashi et al. 2009)
Ugcg	Ugt	Catalyses transfer of glucose to ceramide to generate glucosylceramide (lipid synthesis)	(Ichikawa et al. 1996)
Ugt1a1	Ugt	Glucuronidation of phenols and quinols	(Ritter 2000)
Ugt1a2	Ugt	Glucuronidation of bile acids, estrogen and xenobiotics	(Ritter 2000)
Ugt1a6a	Ugt	"Bulky phenol UGT" N/O-glucuronidation of hydroxamic acids	(Ritter 2000)
		1-MP, Genes Downregulated	
Aldh1b1	Aldh	Oxidizes alcohols into aldehydes	(Ying Chen et al. 2011)
Cyp21a1	Cyp	Catalyzes hydroxylation of progesterone into 11-deoxycorticosterone (steroid metabolism)	(Zhao et al. 2012)
		AFB1, Genes Upregulated	
Uchl1	Est	De-ubiquitinating enzyme that cleaves bound ubiquitin (Ub) to generate free Ub	(Ovaa et al. 2004)
Acsm3	Nat	Catalyze esterification to CoA Activation of medium-chain length fatty acids and carboxylic acids	(Watkins et al. 2007)
Ptges	Gst	Converts prostaglandin (PG)H2 to PGE2 Initiates cox2 pathway (inflammation, immunity, stress)	(Williams, Mann, and DuBois 1999)
Gsta3	Gst	Catalyzes transfer of glutathione, that is conjugated to xenobiotic substrates for detoxification	(Ilic et al. 2010)
Sult1a1	Sult	Catalyzes sulfonation of xenobiotics, steroids and other endogenous substrates	(Gamage et al. 2006)
Sult4a1	Sult	Catalyzes sulfonation of xenobiotics and neurotransmitters	(Sidharthan et al. 2014)
Sult6b1	Sult	Catalyzes sulfonation of thyroxine (thyroid hormone T4) and some xenobiotics	(S. Takahashi et al. 2009)

Ugt1a1	Ugt	Glucuronidation of phenols and quinols	(Ritter 2000)
Ugt1a2	Ugt	Glucuronidation of bile acids, estrogen and xenobiotics	(Ritter 2000)
		AFB1, Genes Downregulated	
Cyp11b1	Cyp	Catalyzes hydroxylation of 11-deoxycorticosterone into corticosterone (steroid synthesis)	(Provost and Tremblay 2005)
Cyp11b2	Cyp	Catalyzes 1β-hydroxylation of corticosterone into aldosterone (steroid synthesis)	(Provost and Tremblay 2005)
Cyp1a2	Cyp	Primarily catalyzes *N*- hydroxylation of aromatic amines	(Hussain and Nasser 2014)
Cyp2c38	Cyp	Catalyzes synthesis of 11,12- epoxyeicosatrienoic acid (EET) and 8,9-EET from arachidonic acids (lipid metabolism)	(Tsao et al. 2001)
Cyp4f18	Cyp	Catalyzes hydroxylation of Leukotriene B_4 (inflammatory response)	(Vaivoda et al. 2015)
Gzma	Est	Catalyzes single strand nicks in DNA and disruption of repair and stability mechanisms (induce apoptosis)	(Martinvalet et al. 2005)
		NNK, Genes Upregulated	
Aldh1b1	Aldh	Oxidizes alcohols into aldehydes	(Ying Chen et al. 2011)
Cel	Est	Catalyzes ester hydrolysis cholesterol and hydrolysis of glycerol's, phospholipids and lysophospholipids (lipid metabolism)	(Hui and Howles 2002)
Cyp4b1	Cyp	Catalyzes hydroxylation of xenobiotics and fatty acids (i.e., 2-aminofluroine)	(Imaoka et al. 2001)
Fmo2	Fmo	Catalyzes oxidation of xenobiotics containing nitrogen or sulfur compounds	(Krueger and Williams 2005)
Xdh	Dh	Catalyze oxidation of N-heterocycles	(Kitamura et al. 2006)
A3galt2	Ugt	Catalyzes transfer of galactose from UDP-galactose to acceptor in lipid synthesis (i.e., Galβ-1,4-glucosylceramide)	(Jamaluddin et al. 2007)
Acsm3	Nat	Catalyze esterification to CoA Activation of medium-chain length fatty acids and carboxylic acids	(Watkins et al. 2007)
Ephx2	Ep	Epoxy fatty acids hydrolyzed to the corresponding dihydrodiols at C-terminus	(Newman et al. 2003)
Gcnt1	Nat	Catalyzes the transfer of N-acetylglucosamine to O-glycan to form a 2 branched O-glycan (synthesis of oligosaccarides)	(Sato et al. 2016)
Gsta3	Gst	Catalyzes transfer of glutathione, that is conjugated to xenobiotic substrates for detoxification	(Ilic et al. 2010)
Gstm5	Gst	Catalyzes conjugation of glutathione to several endogenous and exogenous electrophilic substrates	(Takahashi et al. 1993)
Gstt1	Gst	Catalyzes conjugation of glutathione to several xenobiotics (i.e., dichloromethane)	(Whittington et al. 1999)
Mgst2	Gst	Catalyzes conjugation of glutathione to Leukotriene A4 to produce Leukotriene C4 (inflammatory response)	(Jakobsson et al. 1997)
Nqo1	Dh	Catalyzes the 2 electron reduction of quinones	(R. Li 1995)

Pnmt	Mt	Forms epinephrine via *N*-methylation of norepinephrine	(Klaassen 2008)
Sult1a1	Sult	Catalyzes sulfonation of xenobiotics, steroids and other endogenous substrates	(Gamage et al. 2006)
Sult4a1	Sult	Catalyzes sulfonation of xenobiotics and neurotransmitters	(Sidharthan et al. 2014)
Sult2b1	Sult	Catalyzes sulfonation of hydroxy-steroids	(Sakakibara et al. 1998)
Sult6b1	Sult	Catalyzes sulfonation of thyroxine (thyroid hormone T4) and some xenobiotics	(S. Takahashi et al. 2009)
Ugt1a1	Ugt	Glucuronidation of phenols and quinols	(Ritter 2000)
Ugt1a2	Ugt	Glucuronidation of bile acids, estrogen and xenobiotics	(Ritter 2000)
Ugt1a6a	Ugt	"Bulky phenol UGT" N/O-glucuronidation of hydroxamic acids	(Ritter 2000)
Ugt2b37	Ugt	Catalyzes glucuronidation of several endogenous compounds (i.e., bile and fatty acids, steroid hormones, fat soluble vitamins)	(Zhou et al. 2016)
Tst	Sult	Transfer sulfur atom from an anionic donor to acceptor, primarily for detoxification of cyanide to thiocyanide	(Krueger et al. 2010)
		NNK, Genes Downregulated	
Aldh1a7	Aldh	Oxidizes alcohols into aldehydes	(Luch 2005)

Table 3.4 Biological signalling pathways activated by FE1 cell exposures to 1-MP, 2-AAF, DMBA, AFB1 and NNK. The BMD mean is the mean dose (µg/mL), at which each pathway was triggered. The number of significantly differentially-expressed genes in each pathway, as well as the total number of genes in each pathway (i.e., pathway size), are provided.

Pathway	Compound	BMD Mean	# of Significant Genes	Pathway Size
Xenobiotic Metabolism Signaling	1-MP	1.5	31	63
	2-AAF	3.1	10	63
	AFB1	0.26	7	63
	DMBA	0.002	23	63
	NNK	1,365.0	19	63
Aryl Hydrocarbon Receptor Signaling	1-MP	1.4	29	43
	2-AAF	2.4	11	43
	AFB1	0.22	5	43
	DMBA	0.002	10	43
	NNK	1,390.3	13	43
Ubiquinol-10 Biosynthesis (Eukaryotic)	1-MP	1.3	32	43
	2-AAF	2.06	10	43
	AFB1	0.36	7	43
	DMBA	0.004	5	43
	NNK	1,226.5	6	43
LPS/IL-1 Mediated Inhibition of RXR Function	1-MP	1.4	37	61
	2-AAF	2.1	12	61
	AFB1	0.27	6	61
	DMBA	0.002	14	61
	NNK	1,390.7	16	61
Acetone Degradation I (to Methylglyoxal)	1-MP	1.4	22	29
	2-AAF	2.2	9	29
	AFB1	0.4	5	29
	DMBA	0.003	6	29
	NNK	1,304.4	5	29
Melatonin Degradation I	2-AAF	1.8	15	38
	1-MP	1.8	15	38
	DMBA	0.002	17	38
	NNK	1,287.9	8	38
Nicotine Degradation II	1-MP	1.4	20	39
	2-AAF	2.3	10	39
	DMBA	0.002	15	39
	NNK	1,296.5	9	39
Nicotine Degradation III	1-MP	1.4	16	33

	2-AAF	2.0	9	33
	DMBA	0.002	14	33
	NNK	1,346.7	7	33
Serotonin Degradation	1-MP	1.3	13	35
	AFB1	0.48	5	35
	DMBA	0.002	16	35
	NNK	1,227.9	8	35
Super pathway of Melatonin Degradation	1-MP	1.4	17	40
	2-AAF	2.0	9	40
	DMBA	0.002	18	40
	NNK	1,287.9	8	40
NRF2-mediated Oxidative Stress Response	1-MP	1.4	26	40
	2-AAF	2.0	11	40
	DMBA	0.002	8	40
	NNK	1,604.5	10	40
Stearate Biosynthesis I (Animals)	1-MP	1.4	13	20
	AFB1	0.39	5	20
	DMBA	0.001	9	20
	NNK	1,566.4	8	20
Noradrenaline and Adrenaline Degradation	1-MP	1.3	13	20
	AFB1	0.48	5	20
	DMBA	0.002	5	20
Glutathione-mediated Detoxification	1-MP	1.6	8	15
	DMBA	0.001	5	15
	NNK	1,620.0	5	15
PXR/RXR Activation	1-MP	1.3	12	18
	2-AAF	1.7	7	18
Oxidative Ethanol Degradation III	1-MP	1.3	18	25
	2-AAF	2.0	7	25
Histidine Degradation VI	1-MP	1.3	12	18
	AFB1	0.27	5	18
Dopamine Degradation	1-MP	1.3	10	21
	DMBA	0.002	7	21
Bupropion Degradation	1-MP	1.4	15	21
	2-AAF	1.9	8	21
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	DMBA	0.001	13	17
	NNK	1,288.1	5	17
Pregnenolone Biosynthesis	1-MP	1.2	12	18

	AFB1	0.27	5	18
Methylglyoxal Degradation I	1-MP	1.4	9	10
Putrescine Degradation III	1-MP	1.4	11	15
Fatty Acid oxidation	1-MP	1.4	9	13
Tryptophan Degradation X (Mammalian, via Tryptamine)	1-MP	1.4	10	14
Estrogen Biosynthesis	1-MP	1.4	16	22
Ethanol Degradation II	1-MP	1.3	11	17
Ethanol Degradation IV	1-MP	1.3	8	13
Histamine Degradation	1-MP	1.3	8	13
Cysteine Biosynthesis III (mammalian)	DMBA	0.001	5	9
Heparan Sulfate Biosynthesis (Late Stages)	DMBA	0.002	8	15
Super pathway of Methionine Degradation	DMBA	0.001	5	9
Methionine Degradation I (to Homocysteine)	DMBA	0.001	5	9
Heparan Sulfate Biosynthesis	DMBA	0.002	8	15

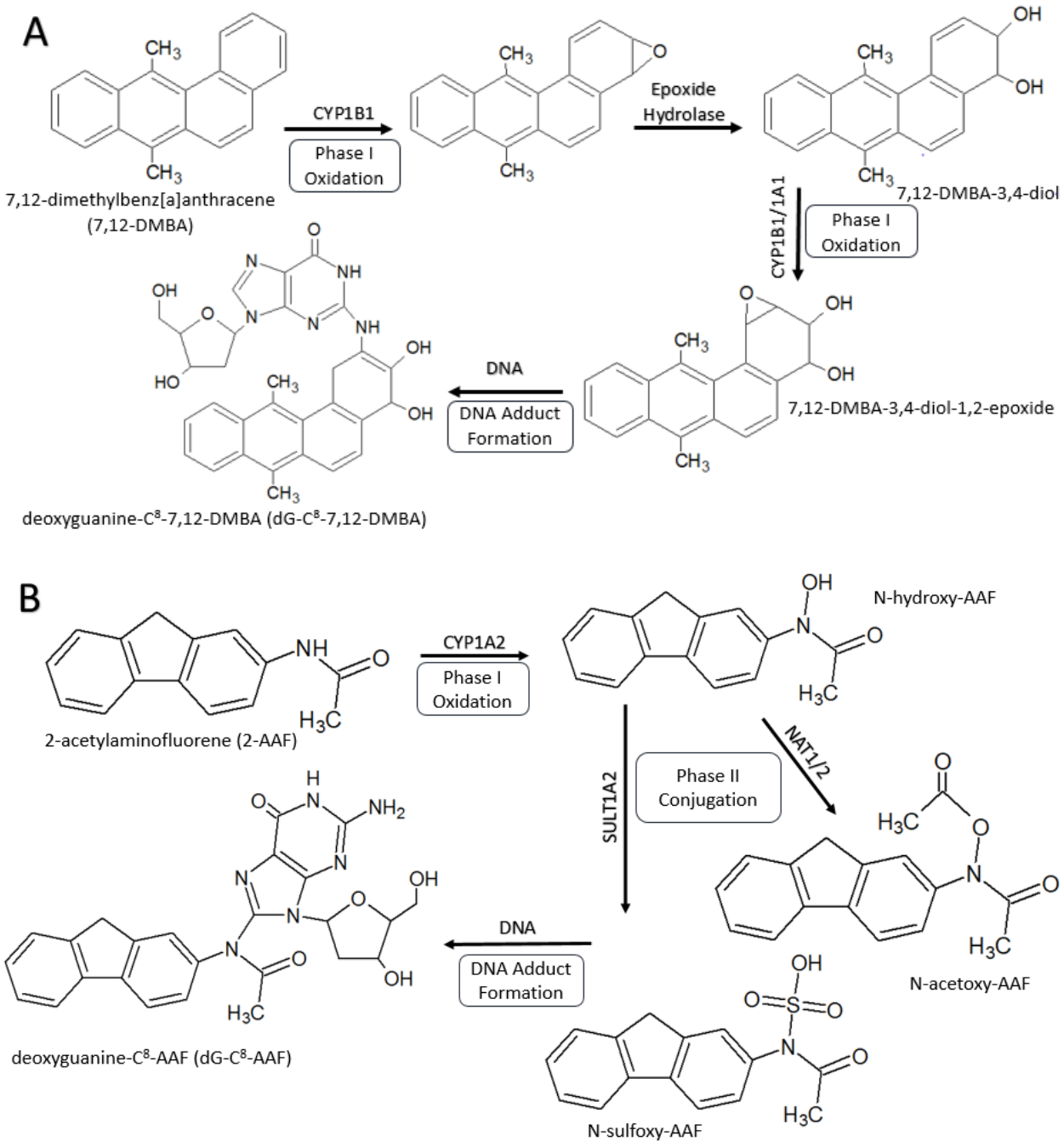
Table 3.5 BMDExpress analysis of the mean BMD at which individual genes become differentially expressed following FE1 exposure to 2-AAF and AFB1. Fold change corresponds to the highest dose tested (5.5µg/mL for 2-AAF and 0.7µg/mL for AFB1). ↑ indicates upregulated; ↓ indicates downregulated. Genes significantly differentially-expressed at $P \leq 0.05$, and fold change ≥ 2 in either direction, are indicated by *.

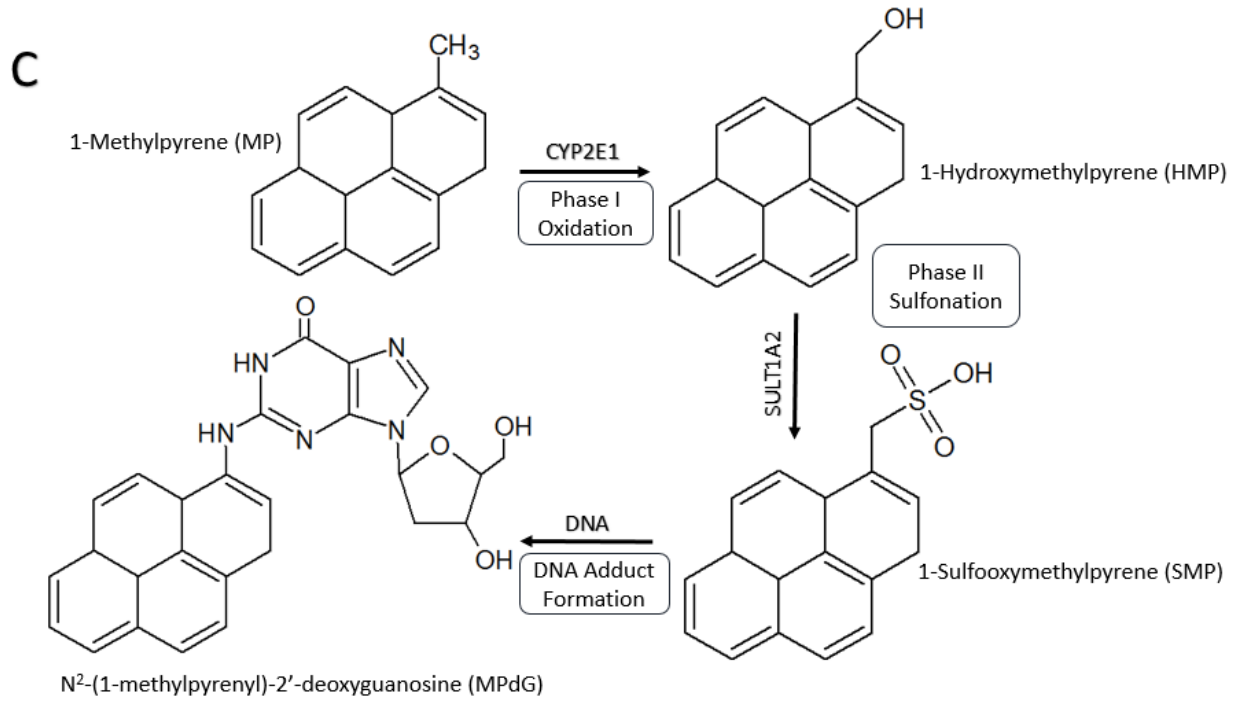
Test Compound	Gene Differentially Expressed	BMD_{10} (µg/mL)	Fold Change
2-AAF	*Acsl4*	0.37	↓1.62
	Cyp2a4	0.86	↓2.05
	Cyp3a44	0.52	↓1.81
	Cyp3a57	0.96	↓3.73
	Cyp2c54	0.86	↓1.31
	Cyp2c39	0.83	↓2.16
	*Cyp2c38**	0.55	↓3.47
	Cyp2c29	0.79	↓2.54
	*Gzma**	0.89	↓3.64
	Dpyd	1.08	↑1.12
	Cyp2c55	1.01	↓1.14
	Cyp26b1	1.06	↓1.66
	Cyp26c1	1.01	↓1.25
	Ugt1a1	3.37	↑7.36
	Ptgs2	4.84	↓20.74
	Xdh	4.88	↓1.32
	Esd	4.92	↓37.02
	Cyp2c65	4.84	↓7.31
	Fmo3	4.76	↓5.89
	Ces2c	5.39	↓1.06
	Cyp1a1	5.41	↓6.19
	Acsm3	5.25	↑4.25
	Gstk1	5.16	↑3.22
	Aldh9a1	5.02	↓7.20
AFB1	*Acsl3*	0.01	↑2.29
	Cyp1a1	0.01	↑1.15
	Cyp26c1	0.01	↑1.16
	Hsd17b10	0.02	↓1.6
	Cyp26b1	0.05	↓1.27
	Fmo4	0.01	↓1.13
	Ptges	0.01	↑2.12
	Aldh1a3	0.01	↑1.7
	Nqo2	0.02	↑9.21
	Aldh9a1	0.41	↑1.72
	Cyp11a1	0.59	↑2.07
	Cyp2d22	0.49	↑1.88
	Aadac	0.59	↑1.94
	Cyp1a2	0.68	↑1.5

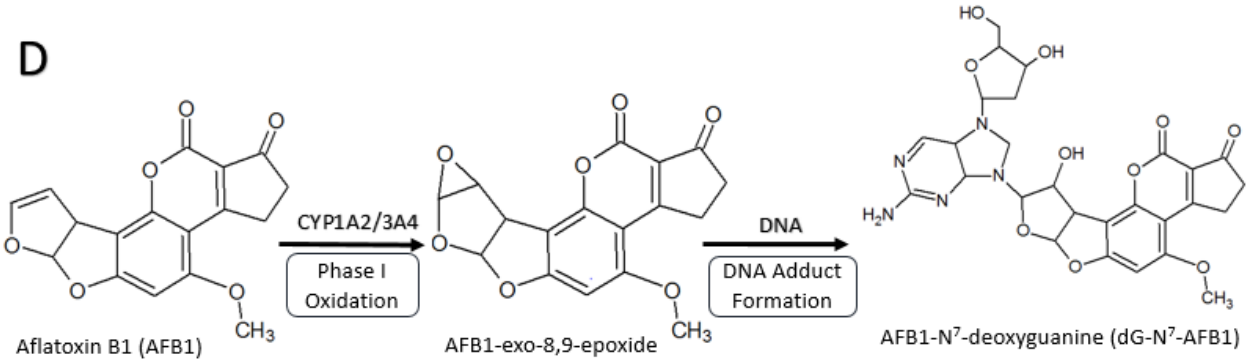
	Dpyd	0.64	↑2.00
	Adh4	0.61	↑1.89
	Adh5	0.67	↑1.48
	Cyp4f18	0.68	↑1.37
	Moab	0.69	↑1.7

Table 3.6 Comparison of responses observed in MutaMouse FE1 cells to results yielded in other, OECD-approved mammalian cell genotoxicity assays. Abbreviations are as follows: MLA- mouse lymphoma assay, MN – micronucleus, S9 – Aroclor 1254-induced S9 rat liver extract.

Test Chemical	Response Profile in Other Mammalian Cell Assays	Response Profile in MutaMouse FE1 Cells	Enzymes Required to Generate DNA-reactive metabolites	Ability of FE1 Cells to Mobilise Required Enzymes
AFB1	Positive - MLA +S9 (Preisler et al. 2000). Positive - *hprt* gene mutation assay +S9 (Shi et al. 1995). Positive – *in vitro* MN assay +S9 (Corvi et al. 2008).	Positive -S9	*Cyp3a4* *Cyp1a2*	Yes
DMBA	Positive - MLA +S9 (OECD 2015b). Positive - *hprt* gene mutation assay +S9 (OECD 2015a). Positive – *in vitro* MN assay +S9 (Corvi et al. 2008).	Positive -S9	*Cyp1b1* *Cyp1a1* Epoxide Hydrolase	Yes
2-AAF	Positive - MLA -S9 (Mitchell et al. 1988). Positive - *hprt* gene mutation assay +S9 (Heflich and Neft 1994). Positive – *in vitro* MN assay +S9 (Corvi et al. 2008).	Positive -S9	*Cyp1a2* *Nat1/2* *Sult1a2*	Yes
NNK	Positive in MLA +S9 (Guo et al. 2016) Positive - *hprt* gene mutation assay -S9 (metabolically competent human lymphoblastoid cell line MCL-5) (Krause et al. 1999). Positive – *in vitro* MN assay +S9 (Ates et al. 2016).	Negative +S9	CYP2A6 CYP2A13 Murine homologs: *Cyp2a4* *Cyp2a5*	No
1-MP	*In vivo* carcinogen rat, mouse, human (Bendadani, Meinl, Monien, Dobbernack, and Glatt 2014). Negative for MN and *hprt* mutation in V79. Positive for MN and *hprt* mutation in V79-hCYP2E1-hSULT1A1 -S9 (Jiang et al. 2015).	Negative +S9	*Cyp2e1* *Sult1a2*	No


A
7,12-dimethylbenz[a]anthracene
(7,12-DMBA)
CYP1B1
Phase I
Oxidation
Epoxide
Hydrolase
7,12-DMBA-3,4-diol
CYP1B1/1A1
Phase I
Oxidation
7,12-DMBA-3,4-diol-1,2-epoxide
DNA
DNA Adduct
Formation
deoxyguanine-C8-7,12-DMBA (dG-C8-7,12-DMBA)
B
2-acetylaminofluorene (2-AAF)
CYP1A2
Phase I
Oxidation
N-hydroxy-AAF
SULT1A2
Phase II
Conjugation
NAT1/2
N-acetoxy-AAF
DNA
DNA Adduct
Formation
deoxyguanine-C8-AAF (dG-C8-AAF)
N-sulfoxy-AAF


C
1-Methylpyrene (MP)
CYP2E1
Phase I Oxidation
1-Hydroxymethylpyrene (HMP)
Phase II Sulfonation
SULT1A2
1-Sulfooxymethylpyrene (SMP)
DNA
DNA Adduct Formation
N2-(1-methylpyrenyl)-2'-deoxyguanosine (MPdG)


D
CYP1A2/3A4
Phase I Oxidation
DNA
DNA Adduct Formation
Aflatoxin B1 (AFB1)
AFB1-exo-8,9-epoxide
AFB1-N7-deoxyguanine (dG-N7-AFB1)

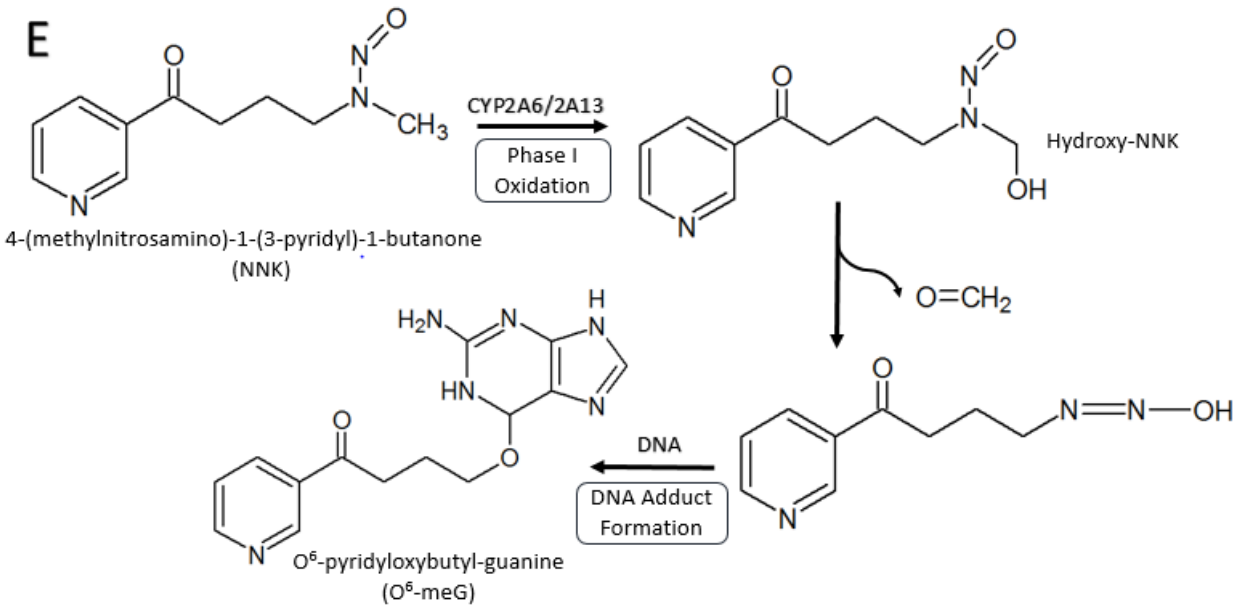


Figure 3.1 Bioactivation of (A) 7,12-dimethylbenz[*a*]anthracene (DMBA), (B) 2-acetylaminofluroene (2-AAF), (C) 1-methylpyrene (1-MP), (D) Aflatoxin B1 (AFB1), and (E) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), illustrating the pathway to DNA adduct formation. In some cases numerous pathways are possible; only the most prevalent pathway is illustrated.

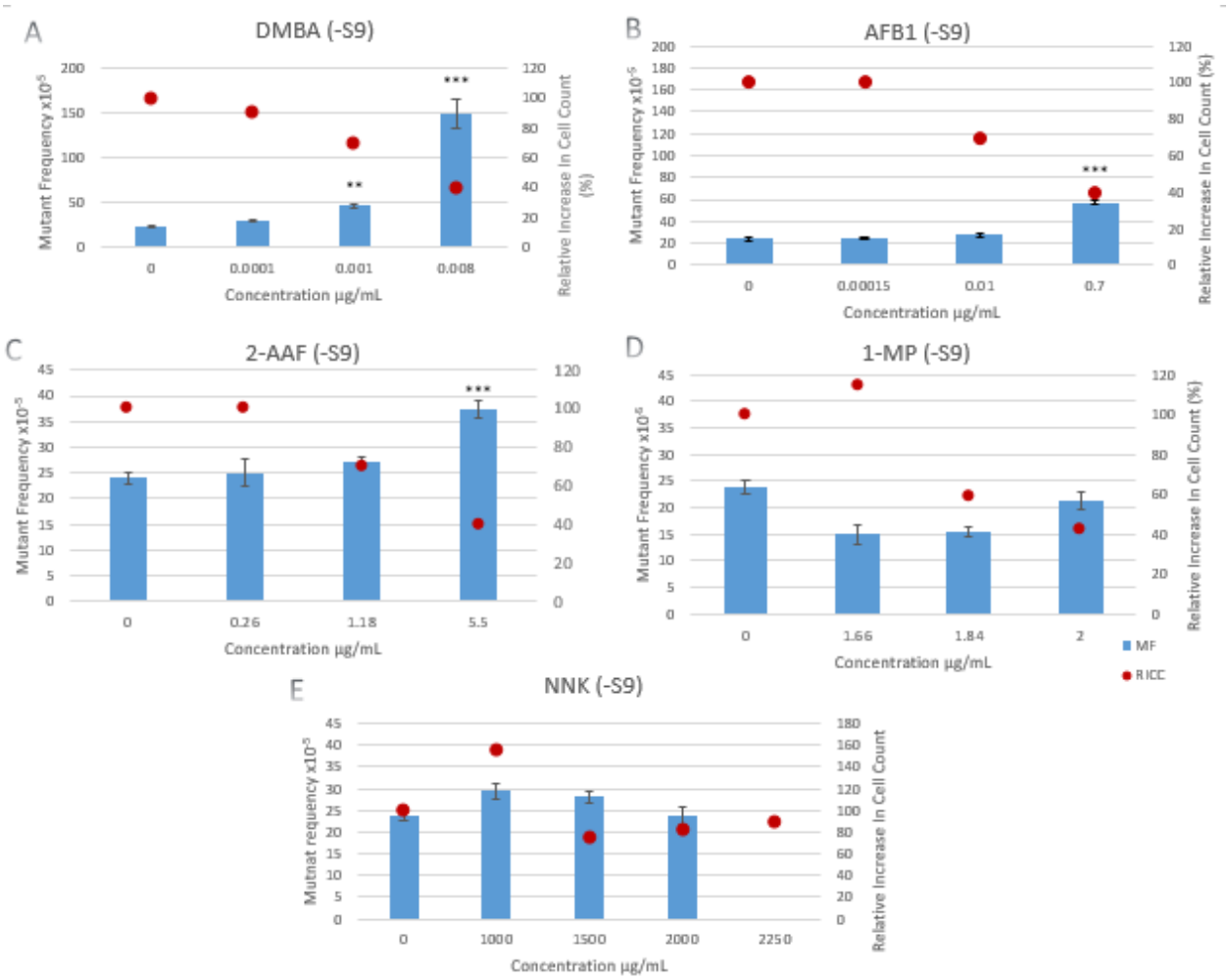


Figure 3.2 Cytotoxicity (red dots) and mutagenicity (blue bars) of selected compounds tested using the MutaMouse FE1 cell assay without exogenous metabolic activation (-S9). Panels represent exposures to the following test articles: (A) DMBA, (B) AFB1, (C) 2-AAF, (D) 1-MP and (E) NNK. Error bars represent one standard error of the mean. Symbols above the bars show the results of the comparisons with concurrent control. *P<0.05, **P<0.01, and ***P<0.001.

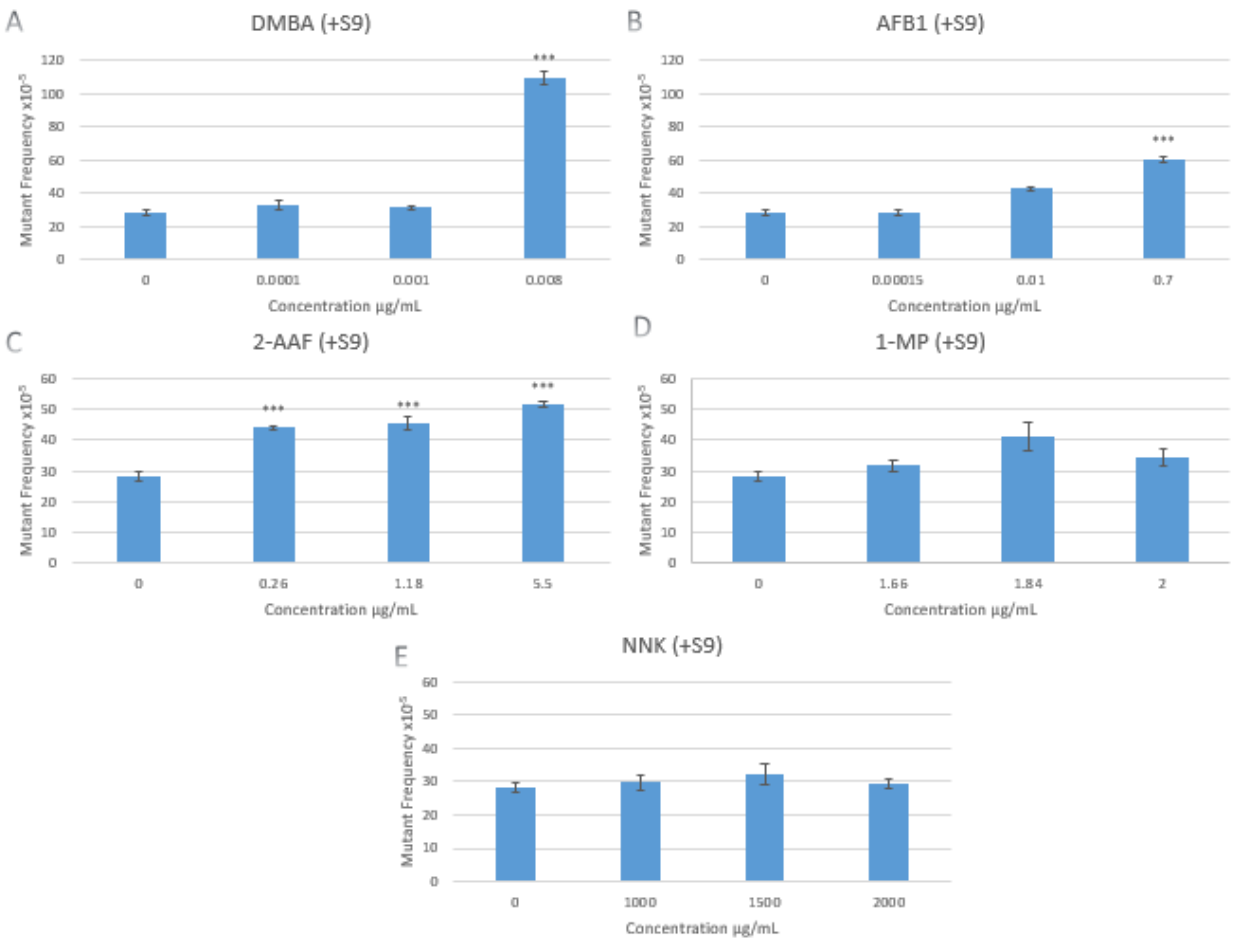


Figure 3.3 Mutagenicity of selected compounds tested using the MutaMouse FE1 cell assay with exogenous metabolic activation (+S9). Panels represent exposures to the following test articles: (A) DMBA, (B) AFB1, (C) 2-AAF, (D) 1-MP and (E) NNK. Error bars represent one standard error of the mean. Symbols above the bars show the results of the comparisons with concurrent control. *P<0.05, **P<0.01, and ***P<0.001.

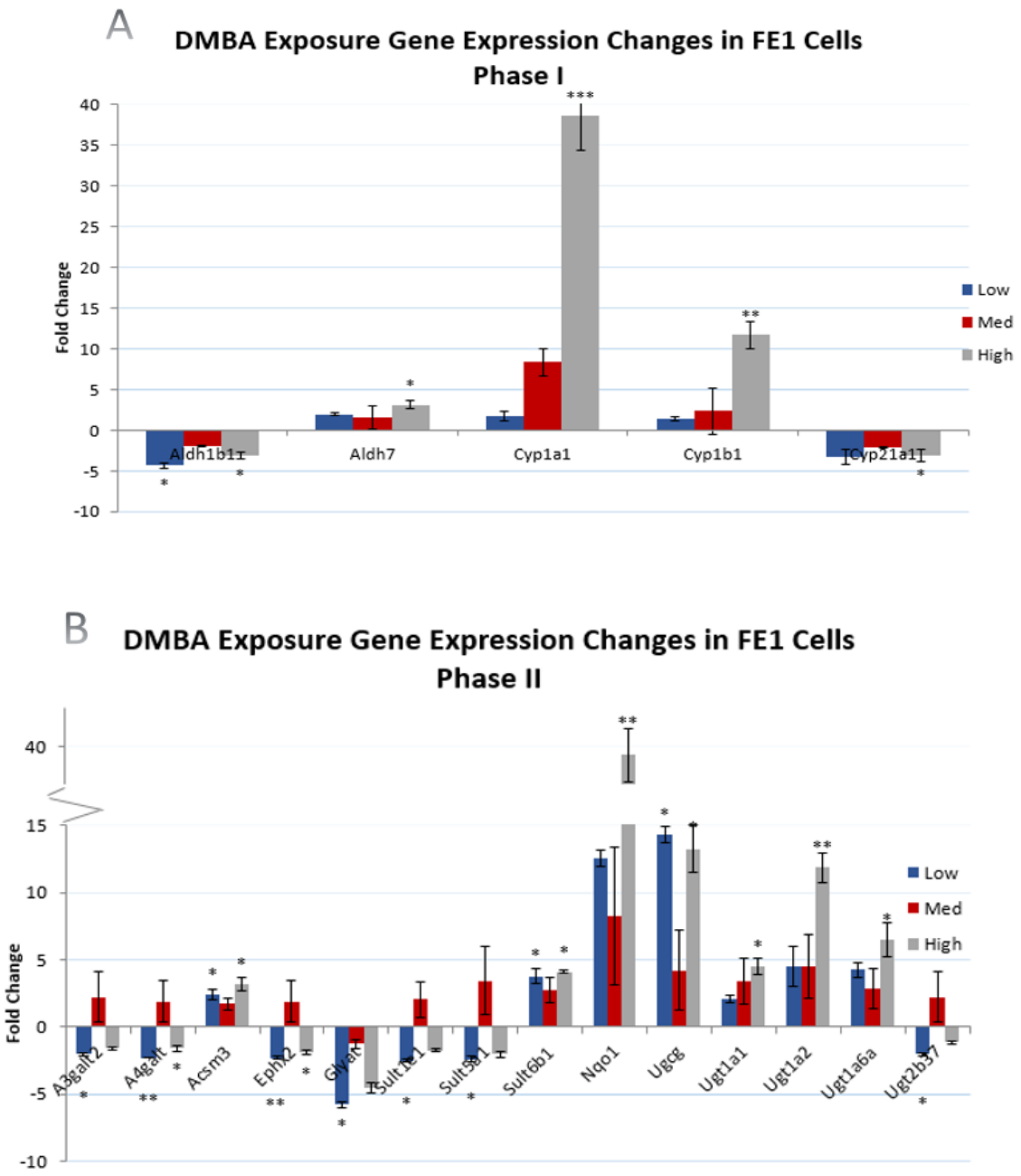


Figure 3.4 Normalized expression of Phase I (A) and II (B) xenobiotic pathway genes assessed in FE1 cells following 6 hour exposures to DMBA (n=3 replicate exposures). *P<0.05, **P<0.01, and ***P<0.001 compared with the solvent control. Error bars show one standard error of the mean. Low, medium and high concentrations of each compound correspond with 100%, 70% and 40% relative cell survival.

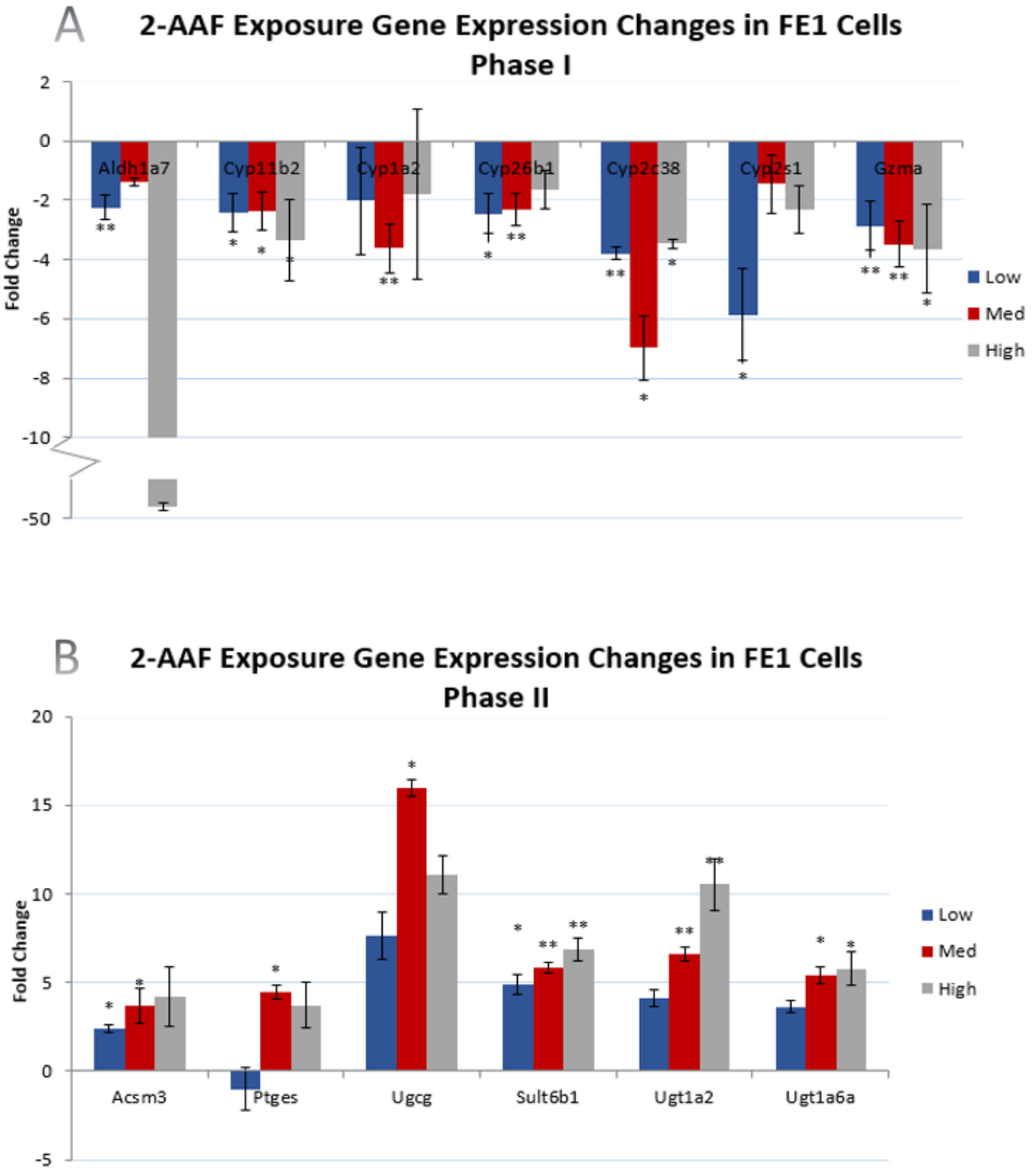


Figure 3.4 Normalized expression of Phase I (A) and II (B) xenobiotic pathway genes assessed in FE1 cells following 6 hour exposures to 2-AAF (n=3 replicate exposures). *P<0.05, **P<0.01, and ***P<0.001 compared with the solvent control. Error bars show one standard error of the mean. Low, medium and high concentrations of each compound correspond with 100%, 70% and 40% relative cell survival.

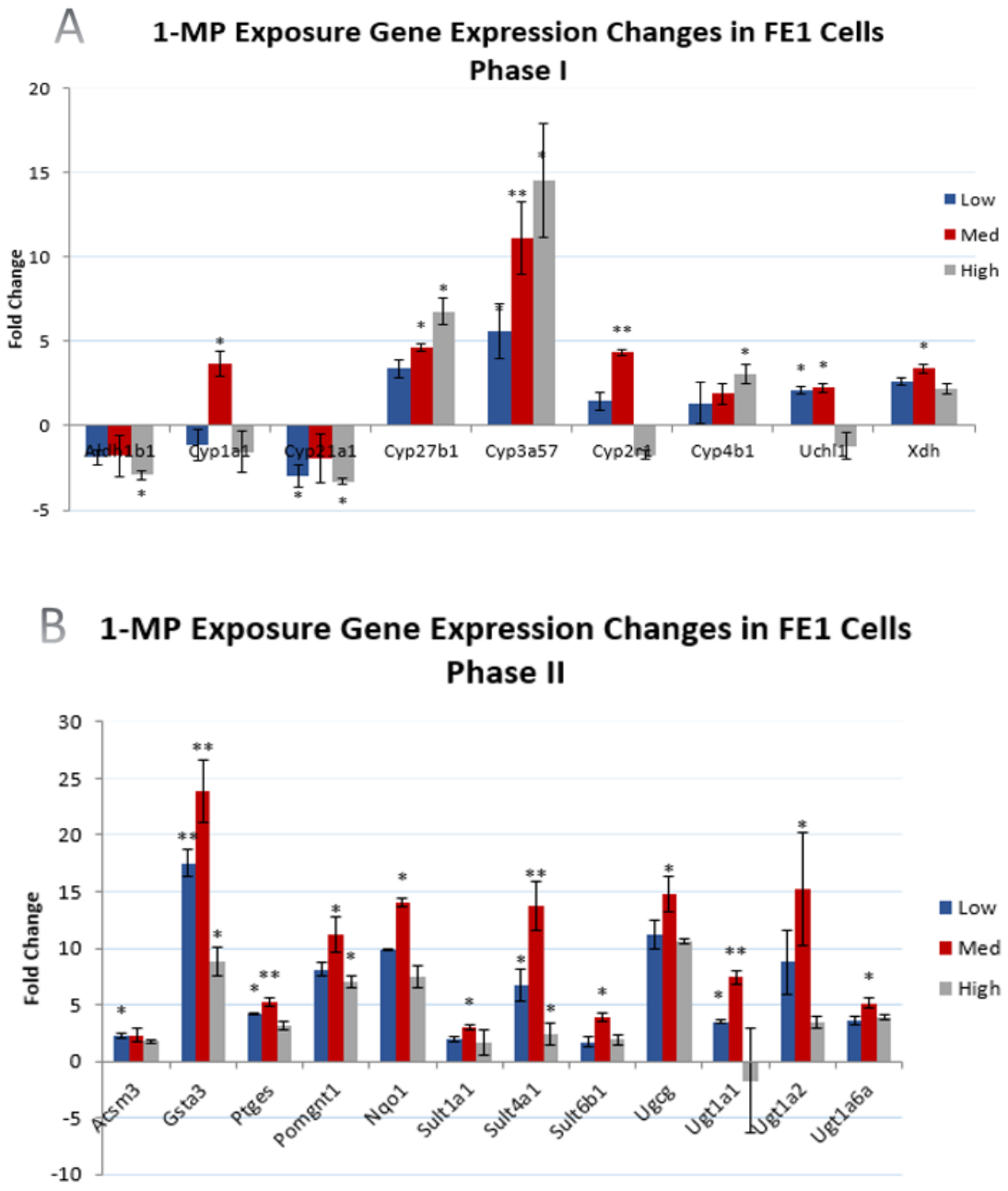


Figure 3.6 Normalized expression of Phase I (A) and II (B) xenobiotic pathway genes assessed in FE1 cells following 6 hour exposures to 1-MP (n=3 replicate exposures). *P<0.05, **P<0.01, and ***P<0.001 compared with the solvent control. Error bars show one standard error of the mean. Low, medium and high concentrations of each compound correspond with 100%, 70% and 40% relative cell survival.

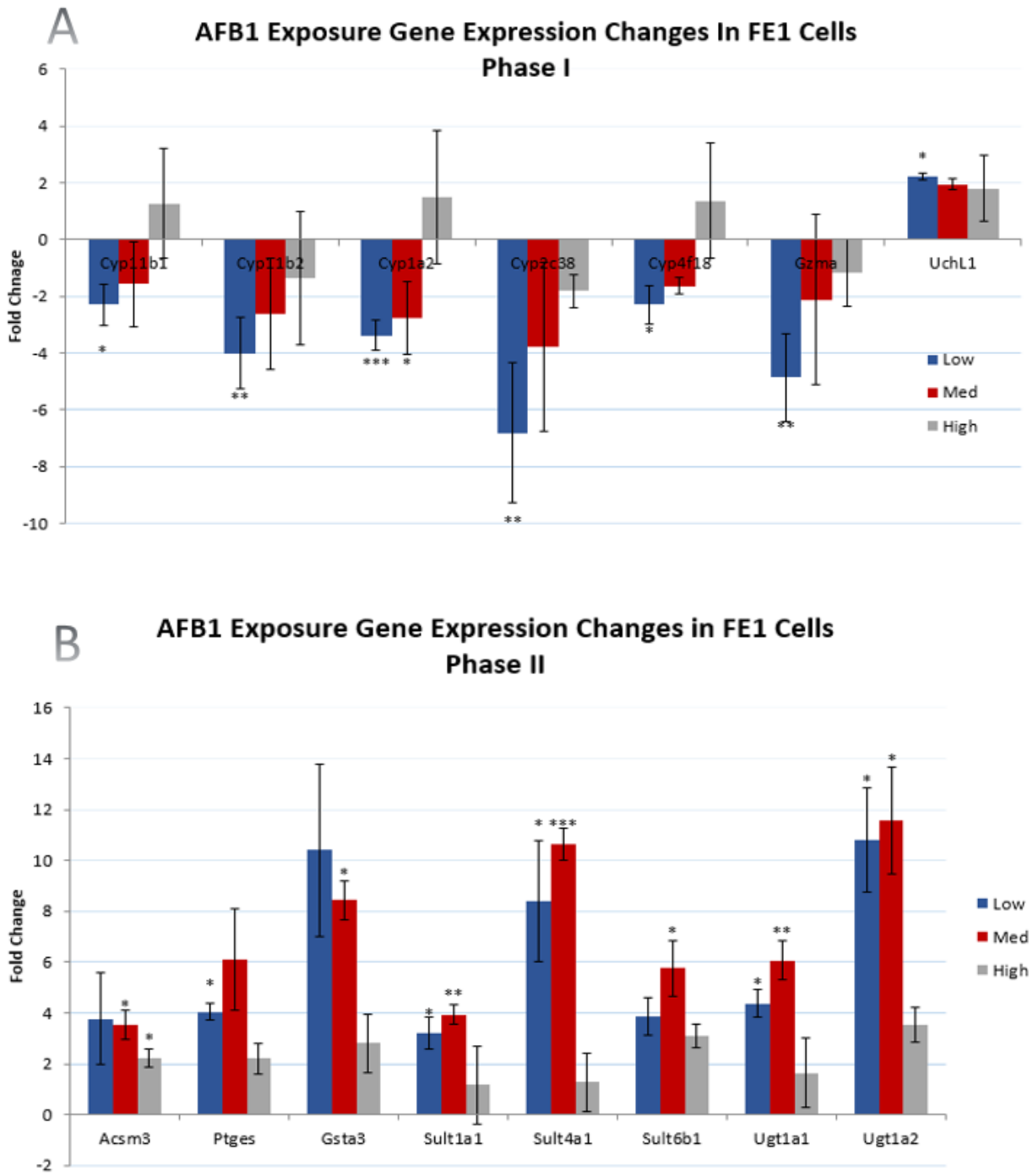


Figure 3.7 Normalized expression of Phase I (A) and II (B) xenobiotic pathway genes assessed in FE1 cells following 6 hour exposures to AFB1 (n=3 replicate exposures). *P<0.05, **P<0.01, and ***P<0.001 compared with the solvent control. Error bars show one standard error of the mean. Low, medium and high concentrations of each compound correspond with 100%, 70% and 40% relative cell survival.

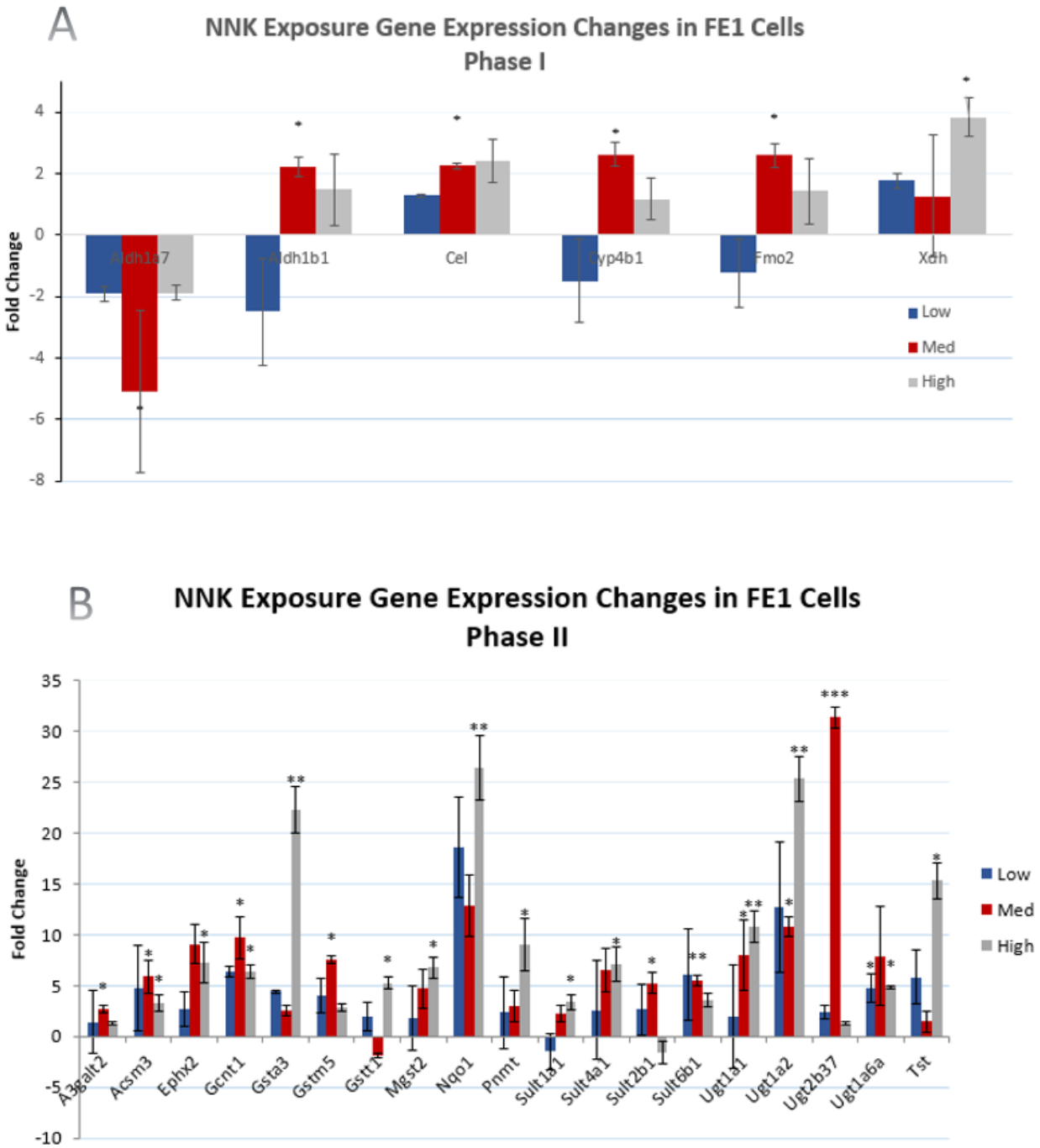


Figure 3.8 Normalized expression of Phase I (A) and II (B) xenobiotic pathway genes assessed in FE1 cells following 6 hour exposures to NNK (n=3 replicate exposures). *P<0.05, **P<0.01, and ***P<0.001 compared with the solvent control. Error bars show one standard error of the mean. Low, medium and high concentrations of each compound correspond with 100%, 70% and 40% relative cell survival.

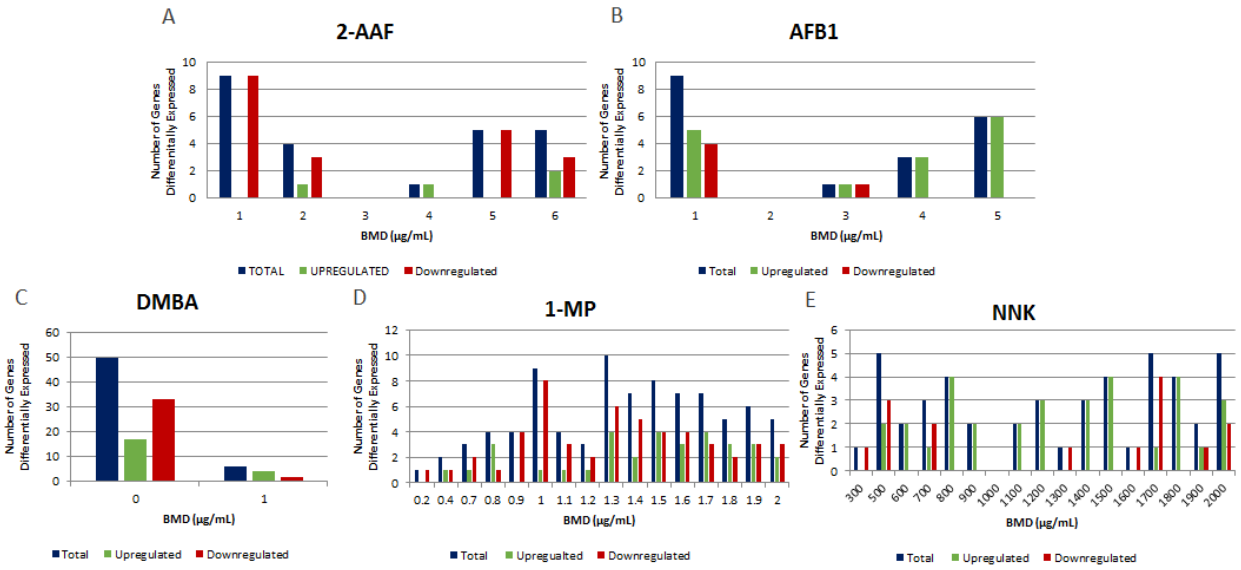


Figure 3.9 Illustration of BMD modes (i.e., distribution profiles) identified using the BMDExpress DataViewer. The DataViewer allows visualization of variations in the sensitivity of genes to selected chemical exposures. Increased BMD corresponds to reduced sensitivity (i.e., expression alterations at higher tested concentrations). (A) shows the 2-AAF BMD profile, (B) the AFB1 BMD profile, (C) the DMBA BMD profile, (D) the 1-MP BMD profile, and (E) the NNK BMD profile. BMD on the x-axis is expressed as test concentration; the y-axis indicates the number of differentially expressed genes at the corresponding BMD. Blue bars indicate the total number of genes differentially expressed, green bars indicate the number of genes upregulated, and red bars the number of genes downregulated.

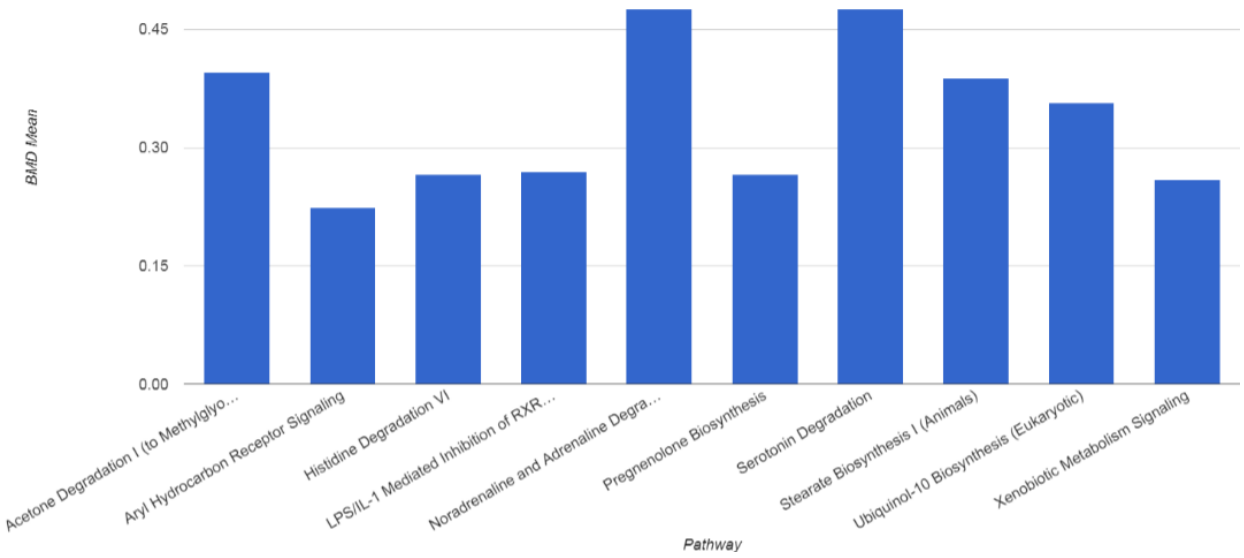


Figure 3.10 Pathway sensitivity across tested concentrations of AFB1 represented as the mean BMD at which each pathway is activated.

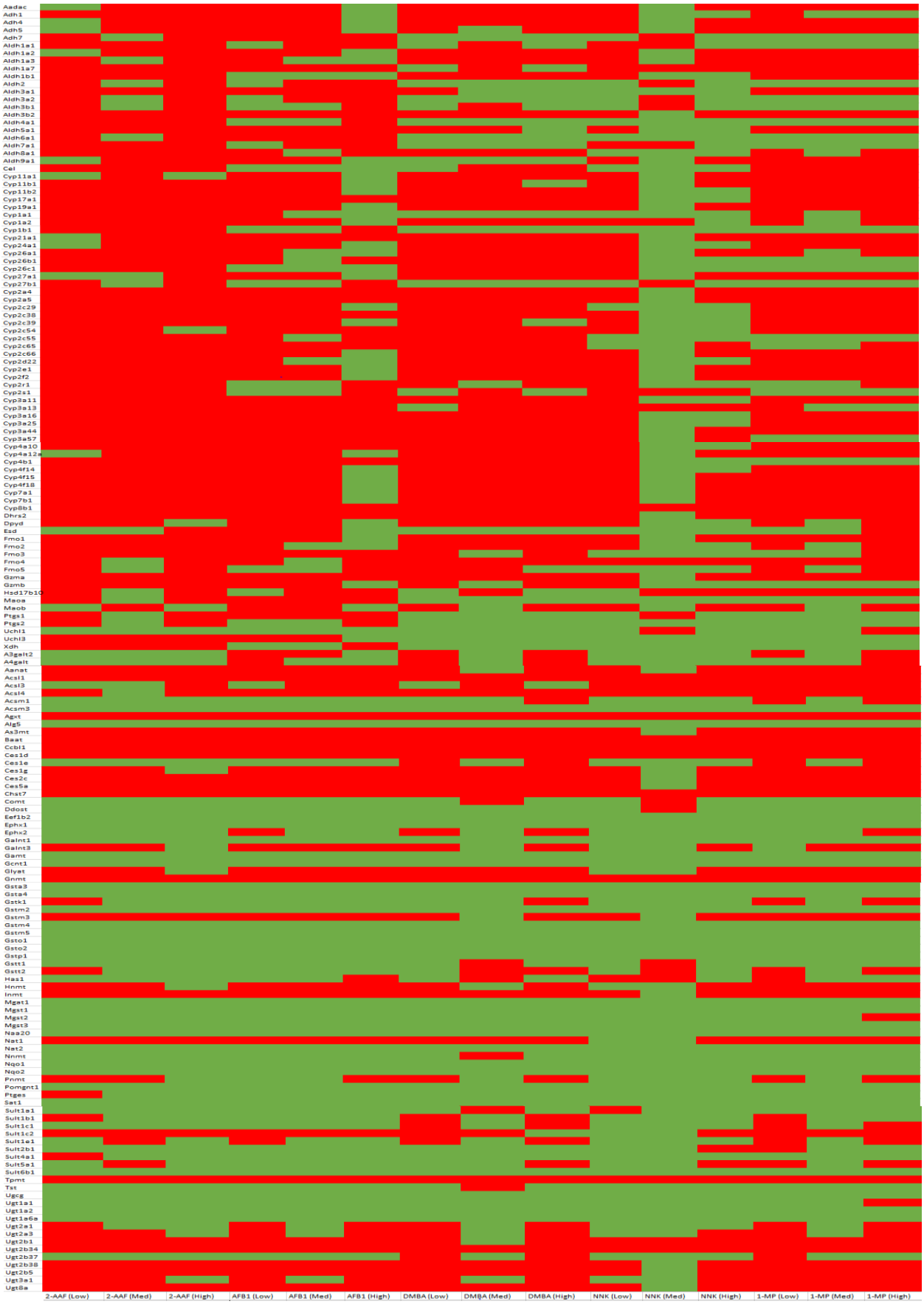

Aadac
Adh1
Adh4
Adh5
Adh7
Aldh1a1
Aldh1a2
Aldh1a3
Aldh1a7
Aldh1b1
Aldh2
Aldh3a1
Aldh3a2
Aldh3b1
Aldh3b2
Aldh4a1
Aldh5a1
Aldh6a1
Aldh7a1
Aldh8a1
Aldh9a1
Cel
Cyp11a1
Cyp11b1
Cyp11b2
Cyp17a1
Cyp19a1
Cyp1a1
Cyp1a2
Cyp1b1
Cyp21a1
Cyp24a1
Cyp26a1
Cyp26b1
Cyp26c1
Cyp27a1
Cyp27b1
Cyp2a4
Cyp2a5
Cyp2c29
Cyp2c38
Cyp2c39
Cyp2c54
Cyp2c55
Cyp2c65
Cyp2c66
Cyp2d22
Cyp2e1
Cyp2f2
Cyp2r1
Cyp2s1
Cyp3a11
Cyp3a13
Cyp3a16
Cyp3a25
Cyp3a44
Cyp3a57
Cyp4a10
Cyp4a12a
Cyp4b1
Cyp4f14
Cyp4f15
Cyp4f18
Cyp7a1
Cyp7b1
CypBb1
Dhrs2
Dpyd
Esd
Fmo1
Fmo2
Fmo3
Fmo4
Fmo5
Gzma
Gzmb
Hsd17b10
Maoa
Maob
Ptgs1
Ptgs2
Uchl1
Uchl3
Xdh
A3galt2
A4galt
Aanat
Acsl1
Acsl3
Acsl4
Acsm1
Acsm3
Agxt
Alg5
As3mt
Baat
Cbl1
Ces1d
Ces1e
Ces1g
Ces2c
Ces5a
Chst7
Comt
Ddost
Eef1b2
Ephx1
Ephx2
Galnt1
Galnt3
Gamt
Gcnt1
Glyat
Gnmt
Gsta3
Gsta4
Gstk1
Gstm2
Gstm3
Gstm4
Gstm5
Gsto1
Gsto2
Gstp1
Gstt1
Gstt2
Has1
Hnmt
Inmt
Mgat1
Mgst1
Mgst2
Mgst3
Naa20
Nat1
Nat2
Nnmt
Nqo1
Nqo2
Prmt
Pomgnt1
Ptges
Sat1
Sult1a1
Sult1b1
Sult1c1
Sult1c2
Sult1e1
Sult2b1
Sult4a1
Sult5a1
Sult6b1
Tpmt
Tst
Ugcg
Ugt1a1
Ugt1a2
Ugt1a6a
Ugt2a1
Ugt2a3
Ugt2b1
Ugt2b34
Ugt2b37
Ugt2b38
Ugt2b5
Ugt3a1
Ugt8a
2-AAF (Low)
2-AAF (Med)
2-AAF (High)
AFB1 (Low)
AFB1 (Med)
AFB1 (High)
DMBA (Low)
DMBA (Med)
DMBA (High)
NNK (Low)
NNK (Med)
NNK (High)
1-MP (Low)
1-MP (Med)
1-MP (High)

Figure 3.11 Changes in the expression of Phase I and Phase II xenobiotic metabolism genes following treatment of FE1 cells with 2-AAF, AFB1, DMBA, NNK or 1-MP. Downregulated genes are represented in red and upregulated genes in green. Low, medium and high refer to tested concentrations that corresponds to 100%, 70% and 40% relative cell survival. Gene abbreviations are indicated vertically on the left, sample abbreviations are indicated horizontally at the bottom. Sample abbreviations, from left to right are: 2-AAF(Low), 2-AAF(Med), 2-AAF(High), AFB1(Low), AFB1(Med), AFB1(High), DMB(Low), DMBA(Med) DMBA(High), NNK(Low), NNK(Med), NNK(High), 1-MP(Low), 1-MP(Med), and 1-MP(High).

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Chapter 4: General Conclusions

Toxicological assessment of mutagenicity and/or carcinogenicity is essential for minimizing the likelihood of adverse human and/or environmental health effects; effective identification of substances with mutagenic and/or carcinogenic properties requires suitably validated bioassays. A recent 'paradigm shift' in regulatory toxicology is moving away from laborious, expensive *in vivo* bioassays towards more focused, cheaper and less labour intensive *in vitro* assays (Krewski et al. 2010). Indeed, it is anticipated that use of *in vitro* assays will be the new norm; however, *in vitro* tools for toxicity assessment have been criticised for the lack of endogenous metabolic capacity associated with many mammalian cell lines, as well as the limited mechanistic information provided by most assays (Eskes and Whelan 2016; Krewski et al. 2010).

Ultimate acceptance of a toxicity assessment bioassay by an international organization such as the OECD necessitates testing to provide evidence of the assay's reliability and reproducibility; by extension, its utility for routine regulatory screening of new and existing chemicals (Eskes and Whelan 2016). The first step in the assay validation process is determination of response reproducibility; this involves completion of an intra-laboratory variability study to determine the consistency of results generated by different operators on different test dates (i.e., Chapter 2) (Eskes and Whelan 2016). A secondary phase involves determination of applicability domain, the range and/or classes of chemicals that can be reliably assessed (Eskes and Whelan 2016). This thesis provides experimental results that contribute to the validation of the MutaMouse FE1 cell *in vitro* transgene mutagenicity assay. More specifically, results related to the intra-laboratory variability of mutagenicity responses,

and results related to the metabolic competency of the FE1 cell line. The former task involved examination of chemically-induced *lacZ* mutagenicity (±S9) across operators and dates; moreover, examination of responses across a series of carefully selected test articles. The latter task relates to definition of the "domain" of chemical types that can be reliably assessed using the FE1 cell assay. The domain reflects the ability of the FE1 cell line to metabolically convert promutagenic compounds into DNA-reactive metabolites that can form DNA adducts which contribute to the establishment of mutations. Metabolic competency was assessed by examining chemically-induced changes in the expression of genes associated with xenobiotic metabolism (i.e., Chapter 3). The gene expression study, which was conducted alongside mutagenicity assessment, provided evidence of the FE1 cell line's overall ability to mobilise the enzymes necessary to create DNA reactive metabolites. Collectively, this thesis' examination of the assay's intra-laboratory variability and applicability domain contribute to the validation required for eventual development of an internationally accepted test guideline for chemical safety assessment.

4.1 Summary of Study Outcomes:

4.1.1 Intra-laboratory Variability Study:

The intra-laboratory variability study included a minimum of 2 (and up to 5) operators screening 8 EURL-ECVAM- (European Union Reference Laboratory for Alternatives to Animal Testing) recommended reference chemicals. All operators who screened the "true negative" compounds ampicillin trihydrate and D-mannitol yielded negative mutagenicity results for all replicates. Similarly, all operators who screened the "false positive" compounds *tert*-butylhydroquinone and phthalic anhydride yielded negative results, demonstrating acceptable assay specificity. All operators who screened the known genotoxicants (i.e., known positives) benzo[*a*]pyrene (BaP) and *N*-ethyl-*N*-nitrosourea (ENU) without S9 yielded positive results, as did operators who screened 2-amino-1-methyl-6-phenyllimidazo(4,5-*b*)pyridine (PhIP) with S9. The testing revealed an assay limitation with respect to its inability to detect *N*-nitrosodimethylamine (DMN) (+S9), a known clastogen (Bean et al. 1994). This result is presumably related to the assay's inability to detect compounds that act via a clastogenic mode of action, since such compounds can induce large deletions that are known to be problematic for assays that employ a bacteriophage-based shuttle vector such as λgt10 (Lambert et al. 2005). Overall, these results, which can be interpreted alongside the results of previous studies (e.g., White et al., 2003; Maertens et al., 2017), indicate that the assay exhibits excellent sensitivity, specificity, and reproducibility.

In addition to the aforementioned qualitative response assessment, the benchmark dose (BMD) approach was employed to quantitatively investigate assay reproducibility. In general, BMD analysis of the BaP, PhIP and ENU data sets revealed little variability in mutagenic

potency (i.e., BMD_{10}) across different operators screening the same test compounds on different test dates. Nevertheless, it should be noted that the quantitative analysis revealed higher variability in BMD_{10} for ENU (-S9). More specifically, 5 orders of magnitude between the highest $BMDU_{10}$ and lowest $BMDL_{10}$, with Operator 1 replicates showing considerably less cross-assay variability than Operators 2 and 3. This variability was attributed to compound stability and variability in operator performance. BMD covariate analysis was employed to simultaneously analyse multiple data sets, with simultaneous analysis improving the precision of the BMD values. Use of operator or test date as a covariate showed little cross-operator or test date variability in chemically-induced mutagenic potency. Some variability was associated with assessment data generated prior to standardization of the assay protocol. With respect to future analyses, deviant potency values can be excluded based on scrutiny of solvent controls. Overall, the intra-laboratory variability study concludes that the MutaMouse FE1 cell transgene mutagenicity assay demonstrates acceptable and consistent reproducibility that is largely independent of operator and test date.

4.1.2 Metabolic Competency Study:

The metabolic competency of the MutaMouse FE1 cell line was investigated following exposure to 5 known mutagens with differing modes of actions and enzymatic requirements. Test compounds 7,12-dimethylbenz[*a*]anthracene (DMBA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and Aflatoxin B1 (AFB1) require Phase I xenobiotic enzymes for oxidation and biotransformation into mutagenic metabolites, while 1-methylpyrene (1-MP), and 2-acetylaminofluorene (2-AAF) require activation by both Phase I and Phase II metabolism (Igawa et al. 2009; Wasalathanthri et al. 2015; Bendadani et al 2014a; Bendadani et al. 2014b; Bbosa et

al. 2013). Mutagenicity screening of test compounds DMBA, 2-AAF and AFB1 without exogenous metabolic activation yielded significant positive responses; responses were also positive in the presence of Aroclor 1254-induced rat liver S9. NNK and 1-MP did not induce significant increases in *lacZ* mutant frequency both with and without S9, and were termed negative. The former results suggest that FE1 cells retain the capacity to generate the Phase I xenobiotic-metabolizing enzymes required to generate DNA-reactive metabolites. In contrast, the latter result indicates that FE1 cells do not retain the capacity to generate the Phase I and/or II xenobiotic-metabolizing enzymes necessary for biotransformation of compounds such as 1-MP and NNK. To confirm suppositions based on the genotoxicity results, chemically-induced gene expression changes of 164 Phase I and II xenobiotic metabolism genes were assessed following exposure to the 5 test mutagens without S9. qPCR analysis revealed that the cell line is able to differentially express several cytochrome P450 monooxygenase genes, including the commonly-required *Cyp1a1, Cyp1b1* and *Cyp1a2*, as well as those corresponding to numerous sulfotransferases, UDP-glucuronosyltransferases and glutathione-*S*-transferases. Thus, the metabolic capacity of FE1 is complete with respect to biotransformation of mutagenic compounds such 2-AAF, AFB1 and DMBA. However, the results indicate that the FE1 cell line cannot mobilise the enzymes required to biotransform mutagens such as 1-MP and NNK (e.g., CYP2A4 and CYP2E1). This is perhaps not surprising since these CYP isozymes are generally not present in murine lung tissue (Renaud et al. 2011). Quantitative analysis of the gene expression results revealed response modes that are consistent with the expression of primary Phase I enzymes (i.e., CYP1A2, CYP1B1, etc.) at lower doses, and secondary Phase I and/or Phase II enzymes at high doses (i.e., ALDH9A1, CYP26A1, FMO3, UGT1A1, GSTK1, etc.). With respect to

secondary metabolism, the results showed that FE1 cells also have the ability to mobilise the enzymes involved in fatty acid, ubiquinol, and steroid and neurotransmitter metabolism. Some of these enzymes have been shown to be involved in the secondary metabolism of xenobiotic chemicals. Overall, the retained metabolic competency of the cell line, which appears to be comparable to the MutaMouse pulmonary tissue from which FE1 cells were derived, is extensive in comparison with the mammalian cell lines currently employed for regulatory screening of genotoxic activity (i.e., CHO, V79, L5178Y Mouse Lymphoma, TK6). Collectively, the accomplishments related to assay reproducibility and applicability domain, which are outlined and discussed in Chapters 2 and 3, fulfil the thesis objective outlined in Chapter 1 (i.e., page 23).

4.2 Contribution to Original Knowledge:

The information available to date, much of which is presented for the first time in this thesis, indicates that the *in vitro* transgene mutagenicity assay in MutaMouse FE1 cells constitutes a suitable alternative to the *in vitro* mammalian cell assays currently employed to detect chemically-induced mutations. Although the aforementioned cell lines specified in OECD Test Guidelines 476 and 490 have proven useful for the detection of chemically-induced mutations, they lack the ability to endogenously mobilise the enzymes necessary for xenobiotic metabolism and bioactivation. Moreover, they are cytogenically unstable, and often yield a high frequency of false positives (Kirkland et al. 2007; OECD 2015a; OECD 2015b). Additionally, the assays entail laborious cloning of induced mutants (OECD 2015a; OECD 2015b). FE1 cells were previously shown to be cytogenetically stable, P53 proficient, and endogenously competent to convert a mutagenic carcinogen such as benzo[*a*]pyrene to its DNA-reactive, mutagenic metabolite. Moreover, previous analyses provided some information regarding assay performance and applicability domain (i.e., proper classification of several *in vitro* false positives, stable spontaneous mutant frequency, etc.), (White et al. 2003; Maertens et al. 2017; Lemieux et al. 2015; Chen et al. 2008; Jacobsen et al. 2008; Poulsen et al. 2013). This work expanded on those analyses and demonstrated, for the first time, that the transgene mutagenicity responses are qualitatively and quantitatively consistent across test article, operator, and test date. Moreover, the work provided new knowledge about the metabolic capacity and applicability domain of the FE1 cell assay. More specifically, that the cell line retains the ability to mobilise many of the Phase I and II enzymes required to endogenously convert selected chemicals into DNA-reactive metabolites that can induce the formation of

mutations (i.e., 2-AAF, AFB1, DMBA). Although two of the five compounds investigated in Chapter 3 could not be endogenously converted into their respective mutagenic metabolites (i.e., 1-MP and NNK), the documented metabolic deficiency of the FE1 cells is entirely consistent with their origin (i.e., lung tissue).

Quantitative investigation of FE1's capacity to activate biological signalling pathways also provides novel insight into the cell line's endogenous ability to maintain cellular stability under chemically-induced stress. In addition to the expected xenobiotic metabolism signalling, pathways activated at lower concentrations included those associated with electron and energy transfer (i.e., ubiquinol-10 biosynthesis and acetone degradation) and activated antioxidant pathways (i.e., NRF2-mediated processes). At the higher concentrations, the cells responded by activating more minor endogenous pathways related cellular survival and growth (i.e., methionine and histidine degradation), and protection against inflammation (i.e., tryptophan degradation). It was also established that the observed responses exhibit an overlap between the activation of xenobiotic, neurotransmitter, and steroid degradation pathways. Thus, although this was not a major objective of the thesis, the work highlighted the unexpected involvement of pathways related to lipid, ubiquinol, steroid, and neurotransmitter metabolism in the endogenous processing of chemical mutagens. Collectively, the new information pertaining to reliability, reproducibility and applicability domain presented in the thesis constitutes a respectable expansion of current knowledge. This new information, when combined with existing data, indicates that the FE1 cell mutagenicity assay is an excellent candidate for continued validation and ultimate international acceptance for routine chemical

safety assessment. Nevertheless, it is also clear that additional studies will be required to further expand the state of knowledge.

4.3 Future Directions:

Overall, the original work presented in this thesis, when combined with the aforementioned existing information, indicates that the *in vitro* transgene mutagenicity assay in MutaMouse FE1 cells displays laudable sensitivity, specificity, reproducibility and reliability. These results collectively justify continued validation of the assay in accordance with OECD and/or EURL-ECVAM guidelines. More specifically, although more work is required to appropriately define applicability domain, sensitivity and specificity, an inter-laboratory variability study should be initiated. This would involve transfer of the protocol to a naïve lab to demonstrate "protocol transferability", as well as analyses of coded compounds across a series of 3-5 laboratories. The former will determine the suitability of the current SOP (standardised operating procedure) for routine assessments of genetic toxicity; the later will assess qualitative and quantitative reproducibility across independent laboratories. Additionally, although not explicitly required for assay validation, follow-up work should investigate the ability to miniaturize the assay, thereby increasing throughput and decreasing test article requirement.

In addition to the OECD and EURL-ECVAM validation guidance documents which were briefly described in Chapter 1, the OECD recently released a draft document entitled *Good in Vitro Method Practices (GIVMP) for the Development and Implementation of in Vitro Methods for Regulatory Use in Human Safety Assessment*. The purpose of the document is additional guidance with respect to improving *in vitro* assay reliability and robustness; moreover, reducing the uncertainties of hazard predictions (Antonelli et al. 2017). Recommendations of particular relevance to the FE1 assay validation exercise include detailed genetic characterization of the cell line, evaluation of the test article limitations (e.g., stability, purity), serum lot variability and

possible replacement in culture medium, detailed characterisation of signal-to-noise ratio and endpoint sensitivity, detailed characterisation of the cell line's growth characteristics, and several assay organizational improvements (e.g., rigorous recording of reagent lot numbers) (Antonelli et al. 2017).

With respect to genetic characterization, it should be noted that the FE1 cell line has been rigorously screened via SKY karyotyping (i.e., multi-colour spectral karyotyping) and FISH (Florescence *in situ* Hybridization) analysis. The former confirmed a modal chromosome number of 78, as well numerous chromosomal rearrangements and duplications (unpublished results). The latter confirmed the presence of three λgt10*lacZ* shuttle vector loci (White et al. 2003). Additional analyses by Shwed et al. (2010), documented 29.0 ± 4.0 transgene copies per *in vivo* locus. The GIVIMP document also recommends the use of SNP (Single Nucleotide Polymorphism) analysis to genotype the cell line and ensure maintenance of genetic integrity over time.

The GIVIMP document, and the aforementioned OECD validation guidance document, (re)emphasise the importance of defining the applicability domain of new assays. Including the test articles examined herein, to date the FE1 cell transgene mutation assay has been used to assess test articles such polycyclic aromatic hydrocarbons (i.e., BaP, 1-MP, DMBA), heterocyclic amines (i.e., PhIP, NNK), aromatic amines (i.e., 2-AAF), aflatoxins (i.e., AFB1), nitroarenes (i.e., 3-NBA), selected false positive compounds (i.e., *tert*-butylhydroquinone, phthalic anhydride, etc.), known negatives (i.e., ampicillin trihydrate, D-mannitol), known direct-acting base-pair, substitution and/or frameshift mutagens (i.e., ENU, ICR-191), (nano)particulate test articles (i.e., carbon black, diesel exhaust particulates, multi-walled carbon nanotubes, etc.), and

complex matrices such as cigarette smoke condensate and coal tar extract (Lemieux et al. 2015; Poulsen et al. 2013; Chen et al. 2008; Maertens et al. 2017; White et al. 2003; Jacobsen et al. 2008). However, while it is not possible to examine the entire world of chemical test articles, the spectrum of tested substances should be expanded in order to more comprehensively define applicability domain. Although an exhaustive list of compounds cannot be tested, the list of compounds tested should be increased to minimum of 25, including additional nitroarenes, aromatic amines, aflatoxins, and complex mixtures. In addition, the assay could be used to examine *in vivo* genotoxicants that fail to elicit a positive response in the Salmonella reverse mutation assay (i.e., Ames test). Recent compilation of "Ames Negative" compounds by EURL-ECVAM revealed 103 compounds that can elicit positive responses *in vivo* (unpublished data). These compounds are ideal for determining the utility and applicability of the FE1 cell assay for effective chemical safety assessment.

Follow-up work should continue to employ gene expression profiling to define the endogenous metabolic capacity of the FE1 cell line; more specifically as it relates to the conversion of selected promutagens into DNA-reactive metabolites. However, to reduce the costs of such analyses, and increase throughput, subsequent studies should not employ commercial qPCR arrays of Phase I and II metabolism genes. Rather, future analyses should employ RNA-seq technology for targeted analyses of chemically-induced gene expression assessment of a comparatively small subset of primary Phase I/II metabolism genes (i.e., *Cyp1a1, Cyp1b1, Sult1a1, Nat1*, etc.). It will also be interesting to expand on the results defining the involvement of secondary metabolic pathways in metabolizing chemical mutagens (e.g., genes associated with oxidative stress NFR2-signalling such as *Gsta4, Mgst1* and *Sat1,* or *Cyp4*

genes associated with acetone degradation pathways, etc.). The results presented in Chapter 3 provide an excellent foundation for selection of both primary and secondary metabolism genes.

It is interesting to note that any assay, including the FE1 cell mutagenicity assay, that employs culture media with animal serum cannot be truly considered "animal-free". This is the primary justification underlying the GIVIMP document's recommendation for serum-free media. Secondarily, the GIVMP document also notes that animal sera are variable from batch to batch, contain unknown factors, and can interfere with cell stability (Antonelli et al. 2017). Thus, it would be a worthwhile exercise to evaluate the effects of serum source on assay results; moreover, the ability to avoid serum altogether. Although a worthy objective, serum-free media often contain a complex mixture of costly biochemical reagents, some of which are obtained from animal sources.

Finally, promotion of the validated *in vitro* transgene mutagenicity assay will require effective distribution of FE1 cells. This will necessitate establishment of an FE1 cell repository; moreover, determination of the effect of pass number and cell lot on assay performance. These analyses will be necessary to ensure that lots distributed worldwide can be reliably used to assess chemical safety.

With respect to the aforementioned validation guidance documents, Table 4.1 provides a summary of tasks to be completed prior to international acceptance of the FE1 cell transgene mutagenicity assay; moreover, proposal of an official Test Guideline. New tests for OECD consideration are proposed via an SPSF (Standard Project Submission Form). Once the SPSF is accepted, the utility, reliability, applicability, and reproducibility of the test is scrutinized by

several OECD committees before eventual acceptance by the member countries as a standardized assay for regulatory screening of human and environmental hazards.

4.4 Concluding Remarks:

The objective of this thesis was to generate new data that contributes to the validation of a novel mammalian cell assay to assess chemically-induced mutagenicity. Moreover, an assay that demonstrates superior performance and utility compared to existing mammalian cell-based assays. However, in light of recent initiatives towards the development of high-throughput reporter-based systems for identification of DNA-damage inducing (DDI) agents, it is worthwhile to objectively consider the necessity of a new *in vitro* mammalian cell mutagenicity assay.

Three major factors justify the development of the FE1 mammalian cell-based *in vitro* mutagenicity assay. First, as mentioned numerous times, the existing assays are laborious and employ cell lines with numerous deficiencies. Second, mutagenicity, in all its shapes and forms (i.e., substitutions, insertions, deletion, chromosome breakage and rearrangements, and aneuploidy), has been definitively linked to human diseases such as cancer and heritable genetic disorders (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011; Meier et al. 2017). Third, and perhaps most importantly, legislated genetic toxicity testing requirements, such as those specified in Canada's New Substance Notification Guidelines (Minister of Justice 2015), explicitly require tests that assess mutation *per se*. Nevertheless, going forward, recently-developed, high-throughput, reporter-based assays for genetic toxicity assessment should be considered. Table 4.2 summarises several of the more prominent assays, some of which are currently undergoing rigorous performance evaluations. Although many of these assays show a great deal of promise, their overall utility for regulatory screening has yet to be determined. In most cases more work is required to determine performance with respect to

effective identification of DNA-damaging agents that are known to cause mutations and/or chromosomal aberrations. In addition, it will ultimately be necessary to adjust/update legislation such that these assays are permitted for regulatory assessments. Until such time, the MutaMouse FE1 cell transgene mutagenicity assay can be regarded as a suitable alternative to the *in vitro* gene mutation assays commonly employed to meet the requirements of existing Canadian legislation.

4.5 Tables:

Table 4.1 Validation tasks to be completed prior to the development of an OECD Test Guideline. Listed tasks adhere to OECD Document 34 and the draft GIVIMP document (Antonelli et al. 2017; OECD 2005). Current status is shown in bold.

Major Tasks (must be completed prior to OECD consideration)	Intra-laboratory variability study - one or more operators screen reference compounds across test dates. **Complete**
	Transferability Study – cells and protocols transferred to naïve lab; feedback obtained. **Partially completed**.
	Inter-laboratory variability study – cells, protocols and coded compounds transferred to several naïve laboratories. Testing results compared. **Not initiated.**
	Applicability Domain Refinement - additional screening of carefully selected test articles. **Not yet initiated**.
	Final drafting of Standard Project Submission Form. **Not yet initiated**.
Additional Tasks to be Considered (recommended, but not explicitly required)	Assay miniaturization – determine the ability to execute the assay in 6- or 12-well plates.
	Improved genetic characterization of FE1 cells – provide criteria for definitive identification of FE1 cells. Allows users to ensure genetic stability over time.
	Evaluate impact of reagent sources (i.e., serum lot).
	Examine options for serum-free medium – to reduce response variability due to serum lot.
	Establish FE1 cell repository; refine information related to cell morphology and growth characteristics.
	Improve records tracking reagents and test articles (i.e., lot numbers).
	Standardize templates for record keeping and data analysis. Improve and standardize methods for secure data storage and distribution.
	Investigate the impact of pass number on cell integrity and mutagenicity response. Develop appropriate quality assurance criteria.
	Examine the signal to noise ratio to determine the limit of detection (e.g., signal window or Z-factors).
	Publish results to facilitate distribution to assay users and ensure appropriate peer review of results.

Table 4.2 Summary of high-throughput, reporter-based assays for genetic toxicity assessment. Some are undergoing performance evaluations in preparation for eventual establishment of an internationally-accepted test guideline.

Assay Name	Endpoint Assessed	References
TGx 28.65 Gene Expression Biomarker	Gene expression – expression of 65 genes mechanistically and empirically linked to DNA damage response pathways.	(Li et al. 2015; Yauk et al. 2016; Buick et al. 2017)
GreenScreen	DNA damage – fluorescence indicative of human reporter gene expression (*GADD45a* growth arrest and DNA damage response).	(Hastwell et al. 2006; Simpson et al. 2013)
MultiFlow™	DNA damage – multiplexed flow cytometric (fluorescence) assessment of γH2AX, phospho-histone H3, nuclear p53 (double strand breaks, DNA damage, polyploidy).	(Bryce et al. 2016)
ToxTracker	DNA damage and cellular stress – Fluorescent reporters in 6 murine cell lines. DNA damage (*Bscl2*, *Rtkn*), oxidative stress (*Srxn1*, *Blvrb*), protein damage (*Ddit3*), cellular stress (*Btg2*).	(Hendriks et al. 2016; Hendriks et al. 2012)
ATAD-5	DNA damage – ATAD5-luciferase reporter assay (translesion synthesis pathway).	(Fox et al. 2012)
p53 Reporter Assays	DNA damage - numerous assays that assess p53-dependant gene expression.	(Duerksen-Hughes et al. 1999; Sohn et al. 2003; Westerink et al. 2010; Zager et al. 2010; Siqian et al. 2016)
DT-40 Differential Survival	DNA damage - differential survival of repair-deficient chicken DT-40 cell lines (e.g., *Ku70*, *Rad54*, *Rev3*).	(Ridpath et al. 2011; Nishihara et al. 2016)
Cytoprotex CellCiphr® Premier (ToxCast™ screening assays)	Cytotoxicity and genotoxicity - multiplexed assays employing labelled antibodies (e.g., DNA damage via fluorescence detection of activated p53).	(Knight et al. 2009)

4.6 References:

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Appendix I.

Table A1. List of genes assessed using RT^2 Profiler PCR arrays: (1) *Drug Metabolism Phase I Enzymes*, (2) *Drug Metabolism Phase II Enzymes.*

Enzyme Family	Gene Symbol	Gene Name
		Phase I
Cytochrome P450s	*Cyp1a1*	Cytochrome P450, family 1, subfamily a, polypeptide 1
	Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2
	Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1
	Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1
	Cyp11b1	Cytochrome P450, family 11, subfamily b, polypeptide 1
	Cyp11b2	Cytochrome P450, family 11, subfamily b, polypeptide 2
	Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1
	Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1
	Cyp21a1	Cytochrome P450, family 21, subfamily a, polypeptide 1
	Cyp24a1	Cytochrome P450, family 24, subfamily a, polypeptide 1
	Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1
	Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1
	Cyp26c1	Cytochrome P450, family 26, subfamily c, polypeptide 1
	Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1
	Cyp27b1	Cytochrome P450, family 27, subfamily b, polypeptide 1
	Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 4
	Cyp2a5	Cytochrome P450, family 2, subfamily a, polypeptide 5
	Cyp2c29	Cytochrome P450, family 2, subfamily c, polypeptide 29
	Cyp2c38	Cytochrome P450, family 2, subfamily c, polypeptide 38
	Cyp2c39	Cytochrome P450, family 2, subfamily c, polypeptide 39
	Cyp2c54	Cytochrome P450, family 2, subfamily c, polypeptide 54
	Cyp2c55	Cytochrome P450, family 2, subfamily c, polypeptide 55
	Cyp2c65	Cytochrome P450, family 2, subfamily c, polypeptide 65
	Cyp2c66	Cytochrome P450, family 2, subfamily c, polypeptide 66
	Cyp2d22	Cytochrome P450, family 2, subfamily d, polypeptide 22
	Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1
	Cyp2f2	Cytochrome P450, family 2, subfamily f, polypeptide 2
	Cyp2r1	Cytochrome P450, family 2, subfamily r, polypeptide 1
	Cyp2s1	Cytochrome P450, family 2, subfamily s, polypeptide 1
	Cyp3a11	Cytochrome P450, family 3, subfamily a, polypeptide 11
	Cyp3a13	Cytochrome P450, family 3, subfamily a, polypeptide 13
	Cyp3a16	Cytochrome P450, family 3, subfamily a, polypeptide 16
	Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25
	Cyp3a44	Cytochrome P450, family 3, subfamily a, polypeptide 44
	Cyp3a57	Cytochrome P450, family 3, subfamily a, polypeptide 57
	Cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10
	Cyp4a12a	Cytochrome P450, family 4, subfamily a, polypeptide 12a
	Cyp4b1	Cytochrome P450, family 4, subfamily b, polypeptide 1

	Cyp4f14	Cytochrome P450, family 4, subfamily f, polypeptide 14
	Cyp4f15	Cytochrome P450, family 4, subfamily f, polypeptide 15
	Cyp4f18	Cytochrome P450, family 4, subfamily f, polypeptide 18
	Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1
	Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1
	Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1
Alcohol Dehydrogenases	*Adh1*	Alcohol dehydrogenase 1 (class I)
	Adh4	Alcohol dehydrogenase 4 (class II)
	Adh5	Alcohol dehydrogenase 5 (class III)
	Adh7	Alcohol dehydrogenase 7 (class IV)
	Dhrs2	Dehydrogenase/reductase member 2
	Hsd17b10	Hydroxysteroid (17-beta) dehydrogenase 10
Esterases	*Aadac*	Arylacetamide deacetylase (esterase)
	Cel	Carboxyl ester lipase
	Esd	Esterase D/formylglutathione hydrolase
	Gzma	Granzyme A
	Gzmb	Granzyme B
	Uchl1	Ubiquitin carboxy-terminal hydrolase L1
	Uchl3	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)
Aldehyde Dehydrogenases	*Aldh1a1*	Aldehyde dehydrogenase family 1, subfamily A1
	Aldh1a2	Aldehyde dehydrogenase family 1, subfamily A2
	Aldh1a3	Aldehyde dehydrogenase family 1, subfamily A3
	Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7
	Aldh1b1	Aldehyde dehydrogenase 1 family, member B1
	Aldh2	Aldehyde dehydrogenase 2, mitochondrial
	Aldh3a1	Aldehyde dehydrogenase family 3, subfamily A1
	Aldh3a2	Aldehyde dehydrogenase family 3, subfamily A2
	Aldh3b1	Aldehyde dehydrogenase 3 family, member B1
	Aldh3b2	Aldehyde dehydrogenase 3 family, member B2
	Aldh4a1	Aldehyde dehydrogenase 4 family, member A1
	Aldh5a1	Aldehyde dehydrogenase family 5, subfamily A1
	Aldh6a1	Aldehyde dehydrogenase family 6, subfamily A1
	Aldh7a1	Aldehyde dehydrogenase family 7, member A1
	Aldh8a1	Aldehyde dehydrogenase family 7, member A1
	Aldh9a1	Aldehyde dehydrogenase 9, subfamily A1
Flavin Containing Monooxygenases	*Fmo1*	Flavin containing monooxygenase 1
	Fmo2	Flavin containing monooxygenase 2
	Fmo3	Flavin containing monooxygenase 3
	Fmo4	Flavin containing monooxygenase 4
	Fmo5	Flavin containing monooxygenase 5
Monoamine Oxidases	*Maoa*	Monoamine oxidase A
	Maob	Monoamine oxidase B
Prostaglandin-Endoperoxide Synthases	*Ptgs1*	Prostaglandin-endoperoxide synthase 1
	Ptgs2	Prostaglandin-endoperoxide synthase 2
Xanthine	*Xdh*	Xanthine dehydrogenase

Dehydrogenases		
Dihydropyrimidine Dehydrogenase	*Dpyd*	Dihydropyrimidine dehydrogena
		Phase II
Amino Acid Transferases	*Agxt*	Alanine-glyoxylate aminotransferas
	Baat	Bile acid-Coenzyme A: amino acid N-acyltransferase
	Ccbl1	Cysteine conjugate-beta lyase 1
	Glyat	Glycine-N-acyltransferase
Dehydrogenases	*Nqo1*	NAD(P) H dehydrogenase, quinone 1
	Nqo2	NAD(P) H dehydrogenase, quinone 2
	Xdh	Xanthine dehydrogenase
Epoxidases	*Ephx1*	Epoxide hydrolase 1, microsomal
	Ephx2	Epoxide hydrolase 2, cytoplasmic
Esterases	*Ces1g*	Carboxylesterase 1G
	Ces2c	Carboxylesterase 2C
	Ces1d	Carboxylesterase 1D
	Ces5a	Carboxylesterase 5A
	Ces1e	Carboxylesterase 1E
Glucuronosyl-transferases	*A3galt2*	Alpha 1,3-galactosyltransferase 2 (isoglobotriaosylceramide synthase)
	A4galt	Alpha 1,4-galactosyltransferase
	Ddost	Dolichyl-di-phosphooligosaccharide-protein glycotransferase
	Ugcg	UDP-glucose ceramide glucosyltransferase
	Ugt1a1	UDP-glucuronosyltransferase 1 family, polypeptide A1
	Ugt1a2	UDP-glucuronosyltransferase 1 family, polypeptide A2
	Ugt1a6a	UDP-glucuronosyltransferase 1 family, polypeptide A6A
	Ugt2a1	UDP-glucuronosyltransferase 2 family, polypeptide A1
	Ugt2a3	UDP-glucuronosyltransferase 2 family, polypeptide A3
	Ugt2b1	UDP-glucuronosyltransferase 2 family, polypeptide B1
	Ugt2b5	UDP-glucuronosyltransferase 2 family, polypeptide B5
	Ugt2b34	UDP-glucuronosyltransferase 2 family, polypeptide B34
	Ugt2b37	UDP-glucuronosyltransferase 2 family, polypeptide B37
	Ugt2b38	UDP-glucuronosyltransferase 2 family, polypeptide B38
	Ugt3a1	UDP-glycosyltransferases 3 family, polypeptide A1
	Ugt8a	UDP galactosyltransferase 8A
Glutathione –*S*-Transferases	*Eef1b2*	Eukaryotic translation elongation factor 1 beta 2
	Gsta3	Glutathione S-transferase, alpha 3
	Gsta4	Glutathione S-transferase, alpha 4
	Gstk1	Glutathione S-transferase kappa 1
	Gstm2	Glutathione S-transferase, mu 2
	Gstm3	Glutathione S-transferase, mu 3
	Gstm4	Glutathione S-transferase, mu 4
	Gstm5	Glutathione S-transferase, mu 5
	Gsto1	Glutathione S-transferase omega 1
	Gsto2	Glutathione S-transferase omega 2

	Gstp1	Glutathione S-transferase, pi 1
	Gstt1	Glutathione S-transferase, theta 1
	Gstt2	Glutathione S-transferase, theta 2
	Hnmt	Histamine N-methyltransferase
	Inmt	Indolethylamine N-methyltransferase
	Mgst1	Microsomal glutathione S-transferase 1
	Mgst2	Microsomal glutathione S-transferase 2
	Mgst3	Microsomal glutathione S-transferase 3
	Ptges	Prostaglandin E synthase
Methyltransferases	*As3mt*	Arsenic (+3 oxidation state) methyltransferase
	Comt	Catechol-O-methyltransferase
	Gamt	Guanidinoacetate methyltransferase
	Gnmt	Glycine-N-acyltransferase
	Nnmt	Nicotinamide N-methyltransferase
	Pnmt	Phenylethanolamine-N-methyltransferase
	Tpmt	Thiopurine methyltransferase
***N*-Acetyltransferases**	*Aanat*	Arylalkylamine N-acetyltransferase
	Acsl1	Acyl-CoA synthetase long-chain family member 1
	Acsl3	Acyl-CoA synthetase long-chain family member 3
	Acsl4	Acyl-CoA synthetase long-chain family member 4
	Acsm1	Acyl-CoA synthetase medium-chain family member 1
	Acsm3	Acyl-CoA synthetase medium-chain family member 3
	Alg5	Asparagine-linked glycosylation 5 homolog (yeast, dolichyl-phosphate beta-glucosyltransferase)
	Galnt1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1
	Galnt3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
	Gcnt1	Glucosaminyl (N-acetyl) transferase 1, core 2
	Has1	Hyaluronan synthase1
	Mgat1	Mannoside acetylglucosaminyltransferase 1
	Nat1	N-acetyl transferase 1
	Nat2	N-acetyl transferase 2
	Naa20	N(alpha)-acetyltransferase 20
	Pomgnt1	Protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase
	Sat1	Spermidine N1-acetyl transferase 1
Sulfotransferases	*Chst7*	Carbohydrate (N-acetylglucosamino) sulfotransferase 7
	Sult1a1	Sulfotransferase family 1A, phenol-preferring, member 1
	Sult1b1	Sulfotransferase family 1B, member 1
	Sult1c1	Sulfotransferase family, cytosolic, 1C, member 1
	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2
	Sult1e1	Sulfotransferase family 1E, member
	Sult2b1	Sulfotransferase family, cytosolic, 2B, member 1
	Sult4a1	Sulfotransferase family 4A, member 1
	Sult5a1	Sulfotransferase family 5A, member 1

	Sult6b1	Sulfotransferase family, cytosolic, 6B, member 1
	Tst	Thiosulfate sulfotransferase, mitochondrial

Table A2. List of genes assessed using RT^2 Profiler PCR array *DNA Damage Signalling Pathway.*

Gene Symbol	Gene Name
Abl1	C-abl oncogene 1, non-receptor tyrosine kinase
Apex1	Apurinic/apyrimidinic endonuclease 1
Atm	Ataxia telangiectasia mutated homolog (human)
Atr	Ataxia telangiectasia and rad3 related
Atrx	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
Bax	Bcl2-associated X protein
Blm	Bloom syndrome, RecQ helicase-like
Brca1	Breast cancer 1
Brca2	Breast cancer 2
Brip1	BRCA1 interacting protein C-terminal helicase 1
Cdc25a	Cell division cycle 25 homolog A (S. pombe)
Cdc25c	Cell division cycle 25 homolog C (S. pombe)
Cdkn1a	Cyclin-dependent kinase inhibitor 1A
Chek1	Checkpoint kinase 1 homolog (S. pombe)
Chek2	CHK2 checkpoint homolog (S. pombe)
Dclre1a	DNA cross-link repair 1A, PSO2 homolog (S. cerevisiae)
Ddb2	Damage specific DNA binding protein
Ddit3	DNA-damage inducible transcript 3
Ercc1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
Ercc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2
Exo1	Exonuclease 1
Fanca	Fanconi anemia, complementation group A
Fancc	Fanconi anemia, complementation group C
Fancd2	Fanconi anemia, complementation group D2
Fancg	Fanconi anemia, complementation group G
Fen1	Flap structure specific endonuclease 1
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma
H2afx	H2A histone family, member X
Hus1	Hus1 homolog (S. pombe)
Lig1	Ligase I, DNA, ATP-dependent
Mdb4	Methyl-CpG binding domain protein 4
Mcph1	Microcephaly, primary autosomal recessive 1
Mdc1	Mediator of DNA damage checkpoint 1
Mgmt	O-6-methylguanine-DNA methyltransferase
Mif	Macrophage migration inhibitory factor
Mlh1	MutL homolog 1 (E. coli)
Mlh3	MutL homolog 3 (E. coli)
Mpg	N-methylpurine-DNA glycosylase
Mre11a	Meiotic recombination 11 homolog A
Msh2	MutS homolog 2 (E. coli)

Msh3	MutS homolog 3 (E. coli)
Nbn	Nibrin
Nthl1	Nth (endonuclease III)-like 1 (E.coli)
Ogg1	8-oxoguanine DNA-glycosylase 1
Parp1	Poly (ADP-ribose) polymerase family, member 1
Parp2	Poly (ADP-ribose) polymerase family, member 2
Pcna	Proliferating cell nuclear antigen
Pms2	Postmeiotic segregation increased 2 (S. cerevisiae)
Pole	Polymerase, epsilon
Polh	Polymerase, eta (RAD 30 related)
Poli	Polymerase, iota
Ppm1d	Protein phosphatase 1D magnesium-dependent, delta isoform
Ppp1r15a	Protein phosphatase 1, regulatory (inhibitor) subunit 15A
Prkdc	Protein kinase, DNA activated, catalytic polypeptide
Pttg1	Pituitary tumor-transforming gene 1
Rad1	RAD1 homolog (S. pombe)
Rad17	RAD17 homolog (S. pombe)
Rad18	RAD18 homolog (S. cerevisiae)
Rad21	RAD21 homolog (S. pombe)
Rad50	RAD50 homolog (S. cerevisiae)
Rad51	RAD51 homolog (S. cerevisiae)
Rad51c	Rad51 homolog c (S. cerevisiae)
Rad5111	RAD51-like 1 (S. cerevisiae)
Rad52	RAD52 homolog (S. cerevisiae)
Rad9	RAD9 homolog (S. pombe)
Rev1	REV1 homolog (S. cerevisiae)
Rnf8	Ring finger protein 8
Rpa1	Replication protein A1
Smc1a	Structural maintenance of chromosomes 1A
Smc3	Structural maintenance of chromosomes 3
Sumo1	SMT3 suppressor of mif two 3 homolog 1 (yeast)
Terf1	Telomeric repeat binding factor 1
Topbp1	Topoisomerase (DNA) II binding protein 1
Trp53	Transformation related protein 53
Trp53bp1	Transformation related protein 53 binding protein 1
Ung	Uracil DNA glycosylase
Wrn	Werner syndrome homolog (human)
Xpa	Xeroderma pigmentosum, complementation group A
Xpc	Xeroderma pigmentosum, complementation group C
Xrcc1	X-ray repair complementing defective repair in Chinese hamster cells 1
Xrcc2	X-ray repair complementing defective repair in Chinese hamster cells 2
Xrcc3	X-ray repair complementing defective repair in Chinese hamster cells 3
Xrcc6	X-ray repair complementing defective repair in Chinese hamster cells 6