11.0 OTHER CONSIDERATIONS

11.1 Test Method Transferability

11.1.1 Facilities and Major Fixed Equipment

As described in **Section 2.1.1**, adults should be kept in an animal room that is isolated from extraneous light that might interfere with a consistent 12-hour photoperiod. Adults can be maintained in large temperature-controlled aquaria or in fiberglass or stainless steel raceways at densities of four to six animals per 1800 cm^2 of water surface area. For conducting the actual FETAX assay, a constant temperature room or a suitable incubator for embryos is required, although a fixed photoperiod is unnecessary. The incubator or room must be capable of maintaining a temperature of 24 ± 2°C.

A binocular dissection microscope capable of magnifications up to 30x is required to count and evaluate embryos for malformations. A simple darkroom enlarger is used to enlarge embryo images two to three times for head to tail length measurements. It is also possible to measure embryo length through the use of a map measurer or an ocular micrometer. However, the process is greatly facilitated by using a digitizer interfaced to a microcomputer. The microcomputer is also used in data analysis.

Such facilities and equipment should be readily available in most laboratories.

11.1.2 Required Level of Personnel Training and Expertise

The estimated amount of technical training required for conducting the in-life portion of a FETAX study is from three to six months (D. Fort, personal communication). This training period may not be much different from that needed for conducting the in-life portion of a corresponding laboratory study using rats, mice, or rabbits. However, as noted in **Section 7.3.3**, concern was expressed during the FETAX validation studies that at least some of the inter-laboratory variability may have been caused by differences among scorers in their ability to

identify malformations in *X. leavis* embryos. Although this concern was not verified when an analysis of performance characteristics was limited to the two most experienced laboratories, expertise in the recognition of malformations in embryos appears to require extensive training. The use of agent-specific characteristic malformations as a method for increasing the performance characteristics of FETAX (see **Section 6.6.2**) or to increase the level of expertise needed for malformation identification.

11.1.3 General Availability of Necessary Equipment and Supplies

The types of equipment and supplies needed to conduct FETAX are readily available from any major supplier.

11.2 Assay Costs

A complete FETAX study, with and without metabolic activation, following the ASTM FETAX Guideline (ASTM, 1998) and conducted in compliance with national/international GLP guidelines, should cost less than $25,000 per test substance (D. Fort, personal communication). In comparison, a complete rat Prenatal Developmental Toxicity Study (screening plus definitive) would cost about $120,000 (G. Jahnke, personal communication).

11.3 Time Needed to Conduct the Test

A complete FETAX study, with and without metabolic activation, following the ASTM FETAX Guideline (1998) and conducted in compliance with national/international GLP guidelines, would require less than two months to complete. This is in contrast to the six to seven months required for a complete rat study (G. Jahnke, personal communication).

11.4 Potential Effect of Tetraploidy on the Use of *X. laevis* in FETAX

The genome of *X. laevis* is tetraploid (Vogel, 1999). The potential impact of tetraploidy on the extrapolation of teratogenic changes in *X. laevis* to laboratory mammals and humans is unknown. Galitski et al. (1999) have shown recently that gene expression is modulated by ploidy in yeast cells. Isogenic strains of yeast were compared at varying ploidy ranging from haploid to tetraploid. The mRNA levels of all genes were measured during exponential growth using oligonucleotide-probe microassays. It was found that the expression of 17 genes was altered due to changes in ploidy, with ten genes being induced and seven genes being repressed with increasing ploidy. With specific regard to developmental response, the investigators monitored the effect of ploidy on invasiveness, a developmental trait in yeast. As ploidy increases, invasiveness decreases due to repression of the FLO11 gene that is responsible for the production of a cell wall protein. This developmental modulation was verified by the restoration of invasiveness with the addition of a $FLO11^{+}$ plasmid.

In view of this finding, it may be useful to consider the potential value of a diploid species of *Xenopus*, such as *X. (Silurana) tropicalis* in FETAX. Although currently limited in availability, this species potentially offers several advantages over *X. laevis*. These advantages include a smaller size, greater ease in housing, more rapid maturation (four or five months as opposed to the one to two years for *X. laevis*), and the ability to be altered transgenetically for developmental mechanistic studies.

11.5 *Xenopus* Microarray Technology

One recent development, which may greatly increase the utility of FETAX for identifying and prioritizing developmental hazards, is in cDNA microarray technology. A cDNA microarray is a glass slide (or other support) containing a large number of genes or expressed sequence tags in a condensed array. Using cDNA microarrays, the expression of thousands of genes can be monitored simultaneously in multiple biological samples of interest, and the expression patterns compared.

This technology may be useful in identifying toxic substances individually or in mixtures; in determining whether toxic effects occur at low doses; in evaluating susceptible tissues and cell types; and in extrapolating effects from one species to another. In FETAX, treatment with a known developmental toxicant may provide a gene expression "signature" on a microarray, which represents the cellular response to this agent. When an unknown substance is tested, the microarray response can then be evaluated to see if one or more of these standard signatures is elicited. This approach might also be used to elucidate an agent's mechanism of action, assess interactions between combinations of agents, or allow for a comparison between altered gene function in *Xenopus* with changes in analogous genes in mammalian systems.

NIEHS has developed a custom "Toxchip" that is a human cDNA clone subarray-oriented toward the expression of genes involved in responses to toxic insult, including xenobiotic metabolizing enzymes, cell cycle components, oncogenes, tumor suppressor genes, DNA repair genes, estrogen-responsive genes, oxidative stress genes, and genes known to be involved in apoptotic cell death. In addition, chips to study responses in mouse, yeast, rat, and Xenopus are available. A Xenopus Chip v 1.0, containing 1000 Xenopus genes from a normalized library, has been developed by Dr. Perry Blackshear's laboratory at NIEHS. In response to increasing interest in this technology, NIEHS has implemented a cDNA Microarray Center to:

- identify toxicants on the basis of tissue-specific patterns of gene expression (molecular signature),
- elucidate mechanisms of action of environmental agents through the identification of gene expression networks,
- use toxicant-induced gene expression as a biomarker to assess human exposure,
- extrapolate effects of toxicants from one species to another,
- study the interactions of mixtures of chemicals,
- examine the effects of low dose exposures versus high dose exposures, and
- develop a public database of expression profiles.

Information on this NIEHS cDNA Microarray Center and progress on the Xenopus microarray chip can be found on the World Wide Web at http://dir.niehs.nih.gov/microarray/.

11.6 Other *In Vitro* Assays for Mammalian Developmental Hazard Identification

A number of *in vitro* systems have been considered as alternatives or screens to *in vivo* mammalian developmental toxicity assays (Kimmel et al, 1982; Smith et al., 1983; Kimmel, 1990; Brown, 1987, Schwetz et al., 1991; Tanumiura and Sakamoto, 1995; Brown et al., 1995; Spielman, 1998). In 1991, Kavlock et al. described a prototype developmental computerized database suitable for comparing the activity profiles of developmental toxicants. The information contained in these profiles could be used to compare qualitative and quantitative results across multiple assay systems, identify data gaps in the literature, evaluate the concordance of the assays, evaluate relative potencies, and examine structure activity relationships. In addition to *in vivo* mammalian assays, eight cellular assays and six *in vitro* embryo systems, including FETAX, being considered at that time were described.

Most recently, the European Centre for the Validation of Alternative Methods (ECVAM) has sponsored a series of validation studies of three *in vitro* assays considered suitable for the detection of substances posing a mammalian developmental hazard (Brown et al., 1995; Scholz et al., 1998; Genschow et al., 1999). The three *in vitro* assays being evaluated are the postimplantation whole rat embryo culture (WEC) assay, the micromass (MM) test, and the embryonic stem cell (EST) test. The WEC assay involves the cultivation of postimplantation whole rat embryos in which both general growth retardation and specific malformations of the cultivated embryo are assessed. This assay is relatively complex, covers only a part of organogenesis, requires high technical skills, and uses mammalian tissue and serum (Spielmann, 1998). In the MM test, primary limb bud cells of rat embryos are cultured and effects on the viability are compared to effects on the differentiation of these cells into chondrocytes. The EST makes use of the differentiation of cultured ES cells into cardiomyocytes. The advantage of this latter test is the use of an established cell line without the need to sacrifice pregnant animals.

In the prevalidation phase, three test chemicals with non- (saccharin), moderate (5,5 diphenylhydantoin), and strong- (cytosine arabinoside) embryotoxicity, along with a negative (Penicillin G) and a positive (5-fluoruracil) control chemical were repeatedly tested in each test in two laboratories (Scholz et al., 1998). The investigators concluded that the *in vitro* tests could be transferred from one laboratory to another and that reproducible results could be obtained. It was also concluded that the three methods were able to discriminate among the test chemicals according to their embryotoxic potential.

In the validation study, each of the tests is being evaluated in four laboratories under blind conditions. In an initial phase of the validation process, six of 30 test chemicals comprising different embryotoxic potential (non, weak, and strong embryotoxic) were tested (Genschow et al., 1999). The results were used to revise and enhance the prediction models for the three assays. The results obtained from evaluating the complete set of 30 chemicals have not yet been published. A list of the names of the 30 chemicals being tested is provided in **Table 38**, along with information on whether the chemical has been tested in FETAX, with or without metabolic activation. The ability of these *in vitro* assays to react to substances that require metabolic activation to be embryotoxic or the potential need for an exogenous MAS in the study protocol is not well defined and may need clarification.

The relative performance, cost-effectiveness, and flexibility of FETAX against other *in vitro* assays in identifying substances with mammalian developmental toxicity was not considered in developing this BRD. Sakamoto et al. (1992) has concluded that the use of *X. laevis* as an *in vitro* model system for the detection of mammalian developmental hazards offers a number of advantages in comparison to other *in vitro* model systems. The most important advantage is that

Table 37. List of Test Chemicals for the ECVAM Validation Study of Three *In Vitro* Embryotoxicity Tests (Genschow et al., 1999)

Chemical	CAS No.	Tested in FETAX	
		Without metabolic activation	With metabolic activation
Strong Embryotoxicity			
5-Bromo-2′-deoxyuridine	59-14-3	No	No
Methyl mercury chloride	115-09-3	No	No
Hydroxyurea	127-07-1	Yes	No
Methotrexate	59-05-2	Yes	No
all-trans-Retinoic acid	302-79-4	Yes	No
6-Aminonicotinamide	329-89-5	Yes	No
Moderate Embryotoxicity			
Boric acid	10043-35-3	Yes	Yes
Pentyl-4-yn-VPA	-	No	No
Valproic acid (VPA)	99-66-1	No	No
Lithium chloride	7447-41-8	No	No
Dimethadione	695-53-4	No	No
Methoxyacetic acid	625-45-6	No	No
Salicylic acid sodium salt	54-21-7	No	No
No Embryotoxicity			
Acrylamide	79-06-1	Yes	Yes
Isobutyl-ethyl-VPA	-	No	No
D-(+)-camphor	464-49-3	No	No
Dimethyl phthalate	131-11-3	No	No
Diphenhydramine hydrochloride	147-24-0	Yes	No
Penicillin G sodium salt	69-57-8	No	No
Saccharin sodium hydrate	82385-42-0	No	No

the development of the *Xenopus* embryo includes a number of developmental events, including cleavage, gastrulation, neurulation, and organogenesis, that are mechanistically comparable to those of mammals. Secondly, this *in vitro* system does not involve the use of any mammals (except sporadically as a source of materials to prepare an MAS).

11.7 Section 11 Conclusions

Sufficient information on facilities and equipment for establishing FETAX as a routine test is provided in the ASTM FETAX Guideline (1991, 1998). The estimated amount of technical training required for conducting the in-life portion of a FETAX study appears to be sufficient. However, based on concerns about differences in expertise in the identification of some of the more subtle malformations induced in *Xenopus* embryos, a more extensive training period may be required for the classification of malformations. The projected cost and study duration for a GLP compliant complete FETAX study, with and without metabolic activation, following the ASTM FETAX Guideline (1998), appears to be reasonable. The potential impact of tetraploidy on the extrapolation of teratogenic changes in *X. laevis* to laboratory mammals and humans needs to be considered. Furthermore, the advantage of using a diploid species of *Xenopus*, such as *X. tropicalis*, in FETAX, should be evaluated.

A number of *in vitro* systems have been proposed as alternatives or screens to *in vivo* mammalian developmental toxicity assays. A brief description of an ECVAM-sponsored validation of three *in vitro* assays considered potentially suitable for the detection of substances posing a mammalian developmental hazard was included. The relative performance, cost-effectiveness, and flexibility of FETAX against other *in vitro* assays in identifying substances with mammalian developmental toxicity was not considered in developing this BRD. However, the most important advantage of FETAX is that the development of the *Xenopus* embryo includes cleavage, gastrulation, neurulation, and organogenesis, and that these developmental events are considered to be mechanistically comparable to those of mammals. Also, this *in vitro* system does not involve the use of any mammals (except sporadically as a source of ingredients for the MAS).