ANNEX 4-1 Summary of Protocol (Tributyltin chloride)

Test Compound	Animals	No. of Animals	Admini-stration	Dosages	Volume	Study Design	Observatios of Dams	Observations of Pups	Remarks
Tributyl tin chloride	Rat Wistar Imami-chi	Males:50 Females:90 (15 females /group) 6 groups	Diet Dis-solved in EtOH	0 0.15 0.45 1.5 4.5 30.0 mg/L Immuno-toxicity at 25 mg/L in diet in 4-week study in rats	To be given freely	To be continuously administered from Day 0 of gestation to Day 21 of lactation On Day 4 of lactation, pups will be randomly selected for study. Each group will contain 4 males and 4 females or 3 males/females and 5 males/females	*General appearance, body weight, food consumption, food efficiency *Delivery and maternal behavior (observation of state and completion of delivery, fertility index, delivery index, gestation length, number of implantation sites, live birth index) *Autopsy on Day 20 of gestation(3 females/group) and on Day 22 of lactation(all live dams except for 3 females/group) organ weight (absolute and relative weights) and storage #Organs to be weighed: brain (cerebrum and cerebellum), pituitary gland, thymus, thyroid (including parathyroid, bilateral), adrenals (bilateral), liver, spleen, kidneys (bilateral), ovaries (bilateral), uterus (bilateral horns and cervix). For autopsy and storage, heart, urinary bladder, vagina, mammary gland (right abdomen in principle) and gross abnormal regions are added to those described above.	*General appearance, body weight, calculation (number of pups born, sex ratio, viability), AGD measurement (on Days 0 and 4 of lactation) of all animals *Reflex response and learning ability (shuttle box) on a male and a female/litter *Estrus cycle (of all females except for one) *Hormone levels and mRNA ($ER\alpha$ $ER\beta$,AR) at 10 weeks of age (a male and a female). *Number, motility and morphology of sperms in epididymis in all live males. *Autopsy, organ weight, histopathological findings and storage of live males and females of 22 days of age (a half of live males and females) and those of 10 weeks of age (remaining half of them). #For measurement of organ weight, testes (bilateral), epididymides (bilateral), seminal vesicle (including coagulating gland and secretion) and prostate (abdominal lobe) are added to the same organs and tissues as dams. For histopathology, vagina, mammary gland, sternum, mesenteric lymph node and submandibular lymph node are added. For autopsy and storage, eyeballs, harderian gland, etc. are added.	

ANNEX 4-2 Summary of Protocol (Di-n-butyl phthalate)

Test Compound	Animals	No. of Animals	Admini-stration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Di-n-butyl phthalate	Wistar Imamichi rat.	males: n=60 females: n=100 Groups(females) 12x7 group	Gavage Solubuled in corn oil	0 0.031 0.063 0.125 0.25 0.5 (mg/kg/day) LOAEL: 5ppm (28-day dosing in the diet)	1 mL/kg	Administration period:From gestation day 0 to Lactation day 21 (every day). On Day 4 post partum, litters were culled to 10 offspring (5 female, 5 male).	Clinical finding Body weight Food intake Water intake Observe delivery and nursing of dam Anatomy (No. of implantations)	*F1 pups Birthday:No. of pups, body weight, AOD Lactation period: Body weight(Lactation day 4, 7, 14 and 21), Physical development(Incisor eruption, Eyelied opning, Residual nipples, Descensus testis) Culled pups onDay 4: gross autopsy *F1 offspring Clinical findings, Body weight Maturation (Vaginal, Preputial separation) *anatomy 3 weeks of age: 1 animal/ dam 6 weeks of age: 1 animal/ dam 10 weeks of age: 1animal/ dam weighed and stored organ Brain, Pituiary, Thyroid, Adrenal, Liver, Kidney, Spleen, testis, Epididymis, Seminal vesicle, Ampullary gland, Prostate, Ductus Deferens, Coagulating gland, M. Levator, Penis, M. bulbospongisus, Ovary, Uterus. Organs which showed significantly increase of organ weights were examined histopathologically. Concentrations of E2, testosterone, FSH and LH in serum at 10 weeks of age. mRNA(Uterus: ER, Prostate: AR, response gene) 10 Copulatory Behavior 10 weeks of age, 1 animal/dam, female:Intact Maunt, Intromission, Ejaculation: Frequency and Latency. Intact female:No. of Implants at gestation day 7.) 10 Mating and fertility 11 weeks of age: 3 dam/group. Male and female were paired in same group. Hysterectomy data: Gestation day 21 Sperm examination: motility, No. of sperm, abnormality	
				Positive control: 250mg/kg/day	1 mL/kg				

ANNEX 4-3 Summary of Protocol (Octachlorostyrene)

Test Compound	Animals	No. of Animals	Admini-stration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Octachloro-styrene	Rat Wistar Imamichi	Purchased: 50 males 86 females Group assigments: 12 females/group x6 groups	Route of admini-stration: Oral gavage Vehicle: Corn oil	Dose: 0(μg/kg/day) 2.4 12 60 300 Maximum detection value in the fish for fiscal 1998: 170μg /kg	1 mL/kg/day	Administration period: From Day 0 of gestation to Day 21 of lactation Pups were adjusted to 4 animals of each sex on Day 4 of lactation.	Clinical observation Body weight Food consumption Observations of delivery and lactation Pathological examinations: Necropsy Organ weight: Adrenals Pituitary Thyroids Thymus Liver Kidneys Uterus Ovaries Brain Histopathology Adrenal Pituitary Thyroid Thymus Liver Kidney Uterus Ovary Vagina Cervix of uterus Other organs showing positive gross findings No. of implantations	(Lactation period) No. of live newborns No. of stillborns, Sex ratio Clinical observation Body weight, AGD Nipple appearance Postnatal differentation Emotional fanction Necropsy(pups excluded on Day 4 of lactation) (On Day 22 of lactation: all pups of each sex from 6 dams/group) Pathological examinations (Necropsy, Organ weight, Histopathology) (From weaning to mating or 10 weeks of age) Clinical observation Body weight Vaginal opening Preputial separation Leaning ability test Estrous cycle, Mating (At 10 weeks of age: all pups of each sex from 3 dams/group) Plasma hormones concentration Pathological examinations (Necropsy, Organ weight, Histopathology) mRNA expression (After mating: all pups of each sex from 3 dams/group) Body weight of dams Pathological examinations (Necropsy, Organ weight, Histopathology) No. of implantations Caesarean section (observations of fetuses) Spermatology	
				Positive control: 50 mg/kg/day	1 mL/kg/day				

ANNEX 4-4 Summary of Protocol(Benzophenone)

Test Compound	Animals	No. of Animals	Administration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Benzophenone	Wistar-Imamichi rat	Purchase males: n=60 females: n=100 Groups(females) 12x6group	Gavage Solubilized in corn oil Administration period: Day 0(G) to day21 (L) (7day/W)	0 2 10 50 (μg /kg/day) Detection limit in environment : 1μg /kg/day Presumable intake: 6.6g/kg/day	1 mL/kg	On Day 4 post partum, litters were culled to 12 offspring (6 females,6males)	Clinical findings Body weight Food intake Water intake Parturition Nursing Pathology	*F1 pups Birthday: No. of pups, body weight, AGD Lactation period: Body weight (day 4, 7, 14 and 21), Physical development(Incisor eruption, Eyelied opning, Residual nipples, Descensus testes) Culled pups onDay 4: gross autopsy *F1 offspring Clinical findings, Body weight, Vaginal opening, Preputial separation *Anatomy (Organs were weighed and fixed) 3 weeks of age: 3 dam/ group 10 weeks of age: 3 dam/ group Weighed and fixed organs : Brain, Pituiary, Thyroid, Adrenasl, Liver, Kidney, Spleen, testes, Epididymides, Seminal vesicles, Ampullary gland, Prostate, Ductus Deferens, Coagulating glands, M. Levatorani, Penis, M. bulbospongisus, Ovary, Uterus. Organs which showed significant difference in organ weights were examined histopathologically. #Concentrations of E2, testosterone, FSH and LH in serum at 10 weeks of age. *Copulatory Behavior 10 weeks of age, 3 dam/group, female : Intact Mount, Intromission, Ejaculation: Frequency and Latency. Intact female:No. of Implants at gestation day 7.) 10 Mating and fertility 11 weeks of age: 3 dam/group. Male and female were paired in same group. Hysterectomy data: On day 21 of gestation	
				20 100 mg/kg/day Positive control Administration for 28 days, Test substance in diet. LOAEL 100 mg/kg/day	1mL/kg/day				

ANNEX 4-5 Summary of Protocol (Di-cyclohexyl phthalate)

Test Compound	Animals	No. of Animals	Admini-stration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Di-cyclohexyl phthalate	Rat Wistar Imamichi	Purchased: 50 males 86 females Group assigments: 12 females/group x6 groups	Route of admini-stration: Oral gavage Vehicle: Corn oil	Dose: 0(μg/kg/day) 1.6 8 40 200 Maximum detection value in the bottom sediment for fiscal 1998-1999: 170μg/kg	1 mL/kg/day	Administration period: From Day 0 of gestation to Day 21 of lactation Pups were adjusted to 4 animals of each sex on Day 4 of lactation.	Clinical observation Body weight Food consumption Observations of delivery and lactation Pathological examinations: Necropsy Organ weight: Adrenals Pituitary Thyroids Thymus Liver Kidneys Uterus Ovaries Brain Histopathology Adrenal Pituitary Thyroid Thymus Liver Kidney Uterus Ovary Vagina Cervix of uterus Other organs showing positive gross findings No. of implantations	(Lactation period) No. of live newborns No. of stillborns Sex ratio, Clinical observation Body weight, AGD Nipple appearance Necropsy(pups excluded on Day 4 of lactation) (On Day 22 of lactation: 2 pups of each sex from a dam/group) Plasma hormones concentration Pathological examinations (Necropsy, Organ weight, Histopathology) (From weaning to mating or 10 weeks of age) Clinical observation Body weight Vaginal opening Preputial separation Estrous cycle, Mating (At 10 weeks of age: 1 pups of each sex from a dam/group) Plasma hormones concentration Pathological examinations (Necropsy, Organ weight, Histopathology) mRNA expression (After mating: 2 pups of each sex from a dam/group) Body weight of dams Pathological examinations (Necropsy, Organ weight, Histopathology) No. of implantations Caesarean section (observations of fetuses) Spermatology	
				Positive control: 500 mg/kg/day	5 mL/kg/day				

ANNEX 4-6 Summary of Protocol (Di-(2-ethylhexyl)phthalate)

Test Compound	Animals	No. of Animals	Admini-stration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Di-(2-ethylhexyl) phthalate	Species: Rat Strain: Wistar-Imamichi	Male:60 Female:100 Female: 15/group x 6 group	Gavage Dissolution in corn oil	0 10 50 250 1,250 100,000 μg/kg/day Reason for selecting dose: Meek ME, Chan PKL. Bis(2-ethylhexyl)phthalate: Evaluation of risks to health from environmental exposure in Canada. J Environ Sci Health Part C 12:179-194 (1994).	1 mL/kg/day	Duration of dosing: From day 0 of gestation to day 21 of lactation Adjustment of litter size: Litters were standardized to 8 pups (4 males and 4 females, in principle) on postnatal day 4.	General condition Body weight Food consumption Delivery and lactation Necropsy Organ weight (Pituitary, Thyroid, Liver, Spleen, Kidney, Adrenal gland, Ovary, Uterus) Fixative organ (Vagina, Uterus, Mammary gland) Histopathological examination(Pituitary, Liver, Ovary, Vagina, Uterus, abnormal organ)	*Pre-weaning: No. of pups (live pups and dead pups), Viability index, morphlogical examination, AGD, nipples/areolas, general condition, body weight, necropsy (dead pup, sacrificed pups) *Weanlings (A half in each litter was necropsied on day postnatal day 21.): Serum concentrations of testosterone, FSH, LH in male, FSH, LH in female, Weighing and fixation of organ (liver, testis, epididymis, prostate + seminal vesicle, ovary, uterus) *Post-weaning (A half in each litter was continued breeding.): General condition, body weight, sexual maturation (vaginal opening, preputial separation), estrous cycle, times of estrus in mating period, copulation index, fertility index *F1 females (pregnant): Body weight, cesarean sectioning (No. of corpora lutea, No. of implantations, intrauterine mortality), Histopathological examination, Weighing and fixation of organs (pituitary gland, thyroid gland, adrenal gland, liver, ovary, uterus, brain, vagina, uterine servix, mammary gland, abnormal organ) *F1 males: Necropsy, Sperm analysis(Sperm counts, sperm motility), Histopathological examination (pituitary gland, testis, abnormal organ, Weighing and fixation of organs (pituitary gland, thyroid gland, adrenal gland, liver, testis, epididymis, coagulating gland, seminal vesicle, ventral prostate, brain, mammary gland, abnormal organ)	

ANNEX 4-7 Summary of Protocol (Butylbenzyl phthalate)

Test Compound	Animals	No. of Animals	Administration	Dosages	Volume	Study Design	Observation of Dams	Observation of Pups		Remarks
Butylbenzyl phthalate	Rats (Iar:Wistar-Imamichi)	45 males 90 females 14 dams/group x6 groups	Oral (dissolved in corn oil)	0 2 12 60 300 μg/kg/day (Occupational exposure: 286 μg kg/workday, NTP)	1 mL/kg/day	Administration: Day 0 of gestation to Day 20 of lactation Litter adjustment: 4 males and 4 females/litter on PND 4	Clinical sign, body weight, food intake, parturition and nursing (fertility index, gestation length, no. of implantations, birth index, gestation index), organ weight (pituitary, thyroid, liver, adrenal, ovary, uterus), necropsy	F_1	Clinical sign, viability (PND 0, 4 and 21), body weight, AGD (PND 0 and 4), nipple development (PND 12), sexual maturation (vaginal opening, preputial separation), estrous cycle, blood hormone concentration (10w, 6 males/group: LH, FSH, testosterone, dihydrotestosterone; 6 females/group: LH, FSH, estradiol-17β, proestrus stage), mRNA (10w, AR, ERα, ERβ; 6 males/group: testis, epididymis, prostate; 6 females/group: ovary, uterus, proestrus stage), reproduction test (days until copulation, no. of estrous stages without copulation, copulation index, fertility index), parturition and nursing (gestation length, no. of corpora lutea, no. of implantations, implantation index, birth index, gestation index), sperm analysis (sperm motility, homogenization-registrant spermatids counts, sperm counts, sperm morphology: abnormal sperm, tailless sperm), organ weight (3w: liver, testis, epididymis, prostate, ovary, uterus; 6w: testis, epididymis, seminal vesicle, prostate, vas deference, levator ani, ovary, uterus; 10w: brain, pituitary, thyroid, thymus, liver, kidney, adrenal, spleen, testis, epididymis, seminal vesicle, prostate, vas deference, levator ani, ovary, uterus), necropsy, histopathology (10w, brain, pituitary, thyroid, thymus, liver, kidney, adrenal, spleen, mammary grand, testis, epididymis, seminal vesicle, coagulate grand, prostate, vas deference, levator ani, ovary, oviduct, vagina, uterus)	
				500 mg/kg/day Positive control (NOAEL for offspring: 250 mg/kg/day, NTP)	1 mL/kg/day					
								F_2	Clinical sign, viability (PND 0 and 4), body weight	

ANNEX 4-8 Summary of Protocol (Diethyl phthalate)

Test Compound	Animals	No. of Animals	Admini-stration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Diethyl phthalate	Rat Wistar Imamichi	Purchased: 60 males 100 females Used: 90 females 15 females/group x6 groups Prepared: >12 pregnant females/group	Route of admini-stration: Oral gavage Vehicle: Corn oil	Dose: 0(μg/kg/day) 0.4 2 10 50 Estimated maximum intake for Japanese: 50μg/kg/day Estimated intake from environment and food: 0.035 μg/kg/day	1 mL/kg/day	Administration period: From Day 0 of gestation to Day 21 of lactation Pups were adjusted to 4 animals of each sex on Day 4 of lactation.	Clinical observation Body weight Food consumption Observations of delivery and lactation Pathological examinations: Necropsy Organ weight: Adrenal Pituitary Thyroid Liver Kidney Uterus Ovary Histopathology Adrenal Pituitary Thyroid Liver Kidney Uterus Ovary Vagina Other organs showing positive gross findings No. of implantations	(Lactation period) No. of fetuses delivered No. of live newborns Clinical observation Body weight, AGD Confirmation of nipple Examinations of behavioral and physical development Necropsy(pups excluded on Day 4 of lactation) (On Day 21 of lactation) Serum hormones concentration Pathological examinations (Necropsy, Organ weight, Histopathology) (From weaning to mating) Clinical observation Body weight, Vaginal opening Preputial separation Estrous cycle Motor activity, Mating (F_1 female after mating) Body weight Pathological examinations (Necropsy, Organ weight, Histopathology) No. of implantations Caesarean section (observations of fetuses) (F_1 male after mating) Serum hormones concentration Pathological examinations (Necropsy, Organ weight, Histopathology) Spermatology	
				Positive control: 2000 mg/kg/day	5 mL/kg/day				

ANNEX 4-9 Summary of Protocol (Di-(2-ethylhexyl) adipate)

Test Compound	Animals	No. of Animals	Administration	Dosages	Volume	Study Design	Observation of Dams	Observation of Pups	Remarks
Di-(2-ethylhexyl) adipate	Rat: Wistar Imamichi	Male: 50 Female: 100 12 females /group x6 groups	P.O. Dissolved in corn oil	0 15 150 1,500 15,000 μg/kg/day 8.2mg/60kg/day (Maximum intake in England)x100(safety margin)= 14,000 μg/kg/day	1 mL/kg/day	Dosing: Day 0 of pregnancy to Day 21 of lactation Selection of pups: 5 males and 5 females /litter on Day 4 of lactation	General signs Body weight Food consumption Delivery and nursing conditions Necropsy Organ weight measurement (pituitary, liver, ovaries and uterus)	*Observation at birth (No. of pups, sex, external abnormalities, and necropsy in stillbirths) *General signs *Body weight (Days 0,4,7,14, and 22) *AGD(Days 0 and 4) *Necropsy on Day 4 of lactation Organ weight measurement (pituitary, thyroids, liver, adrenals, testes, epididymides, seminal vesicle, ventral prostate, ovaries, and uterus) Histopathological examination (liver, ovaries, uterus, and testes) *Estrous cycle *Necropsy on Day 70 Organ weight measurement (pituitary, thyroids, liver, adrenals, testes, epididymides, seminal vesicle, ventral prostate, ovaries, and uterus) Histopathological examination (liver, ovaries, uterus, and testes) Testosterone in male serum Estradiol in female serum AR mRNA in testes ER_{α} and ER_{β} mRNA in ovaries *Reproductive performance (copulation index, fertility index, No. of corpora lutea, No. of implantation sites, and No. of embryos) *Sperm analysis (sperm mortility, viavility, morphology, and No. of sperms)	
				600 mg/kg/day Positive control	1 mL/kg/day				

ANNEX 4-10 Summary of Protocol (Triphenyltin chloride)

Test Compound	Animals	No. of Animals	Administration	Dosages	Volume	Study Design	Observations of Dams	Observations of Pups	Remarks
Triphenyltin chloride	Rat Wistar Imamichi	Males:50 Females: 90 (15 females /group) x 6 groups	Diet Dissolved in EtOH	0 0.015 0.15 1.5 5.0 15.0 mg/L Immunotoxicity at 150 mg/L in 2-week study in rats Detection limit: 0.005 mg/L	To be given freely	To be continuously administered from Day 0 of gestation to Day 21 of lactation On Day 4 of lactation, pups will be randomly selected for study. Each group will contain 4 males and 4 females or 3 males/females and 5 males/females	*General appearance, body weight, food consumption, food efficiency *Delivery and maternal behavior (observation of state and completion of delivery, fertility index, delivery index, gestation length, number of implantation sites, live birth index) *Autopsy on Day 20 of gestation (3 females/group (and on Day 22 of lactation (all live dams except for 3 females/group) organ weight (absolute and relative weights) and storage #Organs to be weighed: brain (cerebrum and cerebellum), pituitary gland, thymus, thyroid (including parathyroid, bilateral), adrenals (bilateral), liver, spleen, kidneys (bilateral), ovaries (bilateral), uterus (bilateral horns and cervix). For autopsy and storage, heart, urinary bladder, vagina, mammary gland (right abdomen in principle) and gross abnormal regions are added to those described above.	*General appearance, body weight, calculation (number of pups born, sex ratio, viability), AGD measurement (on Days 0 and 4 of lactation) of all animals *Reflex response and learning ability (shuttle box) on a male and a female/litter *Estrus cycle (of all females except for one) *Hormone levels and mRNA ($ER\alpha$, $ER\beta$,AR) at 10 weeks of age (a male and a female). *Number, motility and morphology of sperms in epididymis in all live males. *Autopsy, organ weight, histopathological findings and storage of live males and females of 22 days of age (a half of live males and females) and those of 10 weeks of age (remaining half of them). #For measurement of organ weight, testes (bilateral), epididymides (bilateral), seminal vesicle (including coagulating gland and secretion) and prostate (abdominal lobe) are added to the same organs and tissues as dams. For histopathology, vagina, mammary gland, sternum, mesenteric lymph node and submandibular lymph node are added. For autopsy and storage, eyeballs, harderian gland, etc. are added.	