

Appendixes

Appendix 8. References of immunotoxicological information of the chemicals used in Phase I and II studies	315
Appendix 9. References for toxicological information of 60 chemicals	317
Appendix 10. The Multi-Immuno Tox Assay Data sheet	328
Appendix 11. The summary of the study by the independent biostatistician	342
Appendix 12. Study plan	347
Appendix 13. MITA QC confirmation table.....	361
Appendix 14. MITA coded chemical list.....	365
Appendix 15. The list of proficiency chemicals.....	367
Appendix 16. The list of performance standard chemicals	368

Appendix 8. References of immunotoxicological information of the chemicals used in Phase I and II studies

- Chen, S., Golemboski, K., Piepenbrink, M., et al., 2004. Developmental immunotoxicity of lead in the rat: influence of maternal diet. *J Toxicol Environ Health A* 67, 495-511.
- Chikanza, L.C., Panayi, G.S., 1993. The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. *Eur J Clin Invest* 23, 845-850.
- Demenesku, J., Mirkov, I., Ninkov, M., et al., 2014. Acute cadmium administration to rats exerts both immunosuppressive and proinflammatory effects in spleen. *Toxicology* 326, 96-108.
- Fernandez-Cabezudo, M.J., Ali, S.A., Ullah, A., et al., 2007. Pronounced susceptibility to infection by *Salmonella enterica* serovar Typhimurium in mice chronically exposed to lead correlates with a shift to Th2-type immune responses. *Toxicol Appl Pharmacol* 218, 215-226.
- Goodwin, J.S., Atluru, D., Sierakowski, S., et al., 1986. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B4. *J Clin Invest* 77, 1244-1250.
- Goutet, M., Ban, M., Binet, S., 2000. Effects of nickel sulfate on pulmonary natural immunity in Wistar rats. *Toxicology* 145, 15-26.
- Hansen, J.F., Nielsen, C.H., Brorson, M.M., et al., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. *PLoS One* 10, e0131168.
- Hemdan, N.Y., Emmrich, F., Adham, K., et al., 2005. Dose-dependent modulation of the in vitro cytokine production of human immune competent cells by lead salts. *Toxicol Sci* 86, 75-83.
- Iavicoli, I., Marinaccio, A., Castellino, N., et al., 2004. Altered cytokine production in mice exposed to lead acetate. *Int J Immunopathol Pharmacol* 17, 97-102.

- Kim, J.Y., Huh, K., Lee, K.Y., et al., 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. *Exp Mol Med* 41, 288-295.
- Kooijman, R., Devos, S., Hooghe-Peters, E., 2010. Inhibition of in vitro cytokine production by human peripheral blood mononuclear cells treated with xenobiotics: implications for the prediction of general toxicity and immunotoxicity. *Toxicol In Vitro* 24, 1782-1789.
- Metushi, I.G., Uetrecht, J., 2014. Isoniazid-induced liver injury and immune response in mice. *J Immunotoxicol* 11, 383-392.
- Pathak, N., Khandelwal, S., 2008. Comparative efficacy of piperine, curcumin and picroliv against Cd immunotoxicity in mice. *Biometals* 21, 649-661.
- Ringerike, T., Ulleras, E., Volker, R., et al., 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). *Toxicology* 206, 257-272.
- Thomas, P., Barnstorf, S., Summer, B., et al., 2003. Immuno-allergological properties of aluminium oxide (Al₂O₃) ceramics and nickel sulfate in humans. *Biomaterials* 24, 959-966.
- Tsuboi, I., Tanaka, H., Nakao, M., et al., 1995. Nonsteroidal anti-inflammatory drugs differentially regulate cytokine production in human lymphocytes: up-regulation of TNF, IFN-gamma and IL-2, in contrast to down-regulation of IL-6 production. *Cytokine* 7, 372-379.
- Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. *Toxicol Lett* 162, 55-70.
- Wang, P., Wang, J., Sun, Y.J., et al., 2017. Cadmium and chlorpyrifos inhibit cellular immune response in spleen of rats. *Environ Toxicol* 32, 1927-1936.

Appendix 9. References for toxicological information of 60 chemicals

- 1993a. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser 394, 1-274.
- 1993b. NTP Toxicology and Carcinogenesis Studies of p-Nitroaniline (CAS No. 100-01-6) in B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 418, 1-203.
1995. NTP Toxicology and Carcinogenesis Studies of Benzethonium Chloride (CAS No. 121-54-0) in F344/N Rats and B6C3F1 Mice (Dermal Studies). Natl Toxicol Program Tech Rep Ser 438, 1-220.
1996. NTP Toxicology and Carcinogenesis Studies of Nickel Sulfate Hexahydrate (CAS No. 10101-97-0) in F344 Rats and B6C3F1 Mice (Inhalation Studies). Natl Toxicol Program Tech Rep Ser 454, 1-380.
1997. NTP Toxicology and Carcinogenesis Studies of Salicylazosulfapyridine (CAS No. 599-79-1) in F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 457, 1-327.
2003. NTP toxicology and carcinogenesis studies of citral (microencapsulated) (CAS No. 5392-40-5) in F344/N rats and B6C3F1 mice (feed studies). Natl Toxicol Program Tech Rep Ser, 1-268.
- Almoussa, L.A., Salter, A.M., Langley-Evans, S.C., 2018. Magnesium deficiency heightens lipopolysaccharide-induced inflammation and enhances monocyte adhesion in human umbilical vein endothelial cells. *Magn Res* 31, 39-48.
- Auli, M., Domenech, A., Andres, A., et al., 2012. Multiparametric immunotoxicity screening in mice during early drug development. *Toxicol Lett* 214, 200-208.
- Beschorner, W.E., Namnoum, J.D., Hess, A.D., et al., 1987. Cyclosporin A and the thymus. *Immunopathology. Am J Pathol* 126, 487-496.
- Bessler, H., Straussberg, R., Gurary, N., et al., 1996. Effect of dexamethasone on IL-2 and IL-3 production by mononuclear cells in neonates and adults. *Arch Dis Child Fetal Neonatal Ed* 75, F197-201.
- Blanke, T.J., Little, J.R., Shirley, S.F., et al., 1977. Augmentation of murine immune

- responses by amphotericin B. *Cell Immunol* 33, 180-190.
- Bruserud, O., Lundin, K., 1987. The effect of drugs used in anticoagulation therapy on T lymphocyte activation in vitro. II. Warfarin inhibits T lymphocyte activation. *J Clin Lab Immunol* 23, 169-173.
- Bunn, T.L., Parsons, P.J., Kao, E., et al., 2001. Exposure to lead during critical windows of embryonic development: differential immunotoxic outcome based on stage of exposure and gender. *Toxicol Sci* 64, 57-66.
- Bygbjerg, I.C., Svenson, M., Theander, T.G., et al., 1987. Effect of antimalarial drugs on stimulation and interleukin 2 production of human lymphocytes. *Int J Immunopharmacol* 9, 513-519.
- Caren, L.D., Oven, H.M., Mandel, A.D., 1985. Dimethyl sulfoxide: lack of suppression of the humoral immune response in mice. *Toxicol Lett* 26, 193-197.
- Cesario, T.C., Slater, L.M., Kaplan, H.S., et al., 1984. Effect of antineoplastic agents on gamma-interferon production in human peripheral blood mononuclear cells. *Cancer Res* 44, 4962-4966.
- Chetty, K.N., Subba Rao, D.S., Drummond, L., et al., 1979. Cobalt induced changes in immune response and adenosine triphosphatase activities in rats. *J Environ Sci Health B* 14, 525-544.
- Chikanza, L.C., Panayi, G.S., 1993. The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. *Eur J Clin Invest* 23, 845-850.
- de Abreu Costa, L., Henrique Fernandes Ottoni, M., Dos Santos, M.G., et al., 2017. Dimethyl Sulfoxide (DMSO) Decreases Cell Proliferation and TNF-alpha, IFN-gamma, and IL-2 Cytokines Production in Cultures of Peripheral Blood Lymphocytes. *Molecules* 22.
- De Waal, E.J., Timmerman, H.H., Dortant, P.M., et al., 1995. Investigation of a screening battery for immunotoxicity of pharmaceuticals within a 28-day oral toxicity study using azathioprine and cyclosporin A as model compounds. *Regul Toxicol Pharmacol* 21, 327-338.
- Dieter, M.P., Luster, M.I., Boorman, G.A., et al., 1983. Immunological and biochemical responses in mice treated with mercuric chloride. *Toxicol Appl Pharmacol* 68, 218-228.

- Dupont, E., Huygen, K., Schandene, L., et al., 1985. Influence of in vivo immunosuppressive drugs on production of lymphokines. *Transplantation* 39, 143-147.
- Dupuis, G., Martel, J., Bastin, B., et al., 1993. Microtubules are not an essential component of phytohemagglutinin-dependent signal transduction in Jurkat T lymphocytes. *Cell Immunol* 146, 38-51.
- el Fouhil, A.F., Iskander, F.A., Turkall, R.M., 1993a. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. II: Changes in T- and B-cell areas in spleen. *Toxicol Pathol* 21, 383-390.
- el Fouhil, A.F., Iskander, F.A., Turkall, R.M., 1993b. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. III: Changes in T- and B-cell areas in lymph nodes. *Toxicol Pathol* 21, 391-396.
- el Fouhil, A.F., Turkall, R.M., 1993. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. I: Effect on blood cell count, immunoglobulin concentrations, and body and organ weights. *Toxicol Pathol* 21, 377-382.
- Exon, J.H., Koller, L.D., Talcott, P.A., et al., 1986. Immunotoxicity testing: an economical multiple-assay approach. *Fundam Appl Toxicol* 7, 387-397.
- Ezendam, J., Hassing, I., Bleumink, R., et al., 2004. Hexachlorobenzene-induced Immunopathology in Brown Norway rats is partly mediated by T cells. *Toxicol Sci* 78, 88-95.
- Freed, B.M., Lempert, N., Lawrence, D.A., 1989. The inhibitory effects of N-ethylmaleimide, colchicine and cytochalasins on human T-cell functions. *Int J Immunopharmacol* 11, 459-465.
- Freed, B.M., Rapoport, R., Lempert, N., 1987. Inhibition of early events in the human T-lymphocyte response to mitogens and alloantigens by hydrogen peroxide. *Arch Surg* 122, 99-104.
- Fujiwara, M., Mitsui, K., Yamamoto, I., 1990. Inhibition of proliferative responses and interleukin 2 productions by salazosulfapyridine and its metabolites. *Jpn J Pharmacol* 54, 121-131.
- Gabryel, B., Labuzek, K., Malecki, A., et al., 2004. Immunophilin ligands decrease release of pro-inflammatory cytokines (IL-1beta, TNF-alpha and IL-2 in rat

- astrocyte cultures exposed to simulated ischemia in vitro. *Pol J Pharmacol* 56, 129-136.
- Garly, M.L., Trautner, S.L., Marx, C., et al., 2008. Thymus size at 6 months of age and subsequent child mortality. *J Pediatr* 153, 683-688, 688.e681-683.
- Gentile, D.A., Henry, J., Katz, A.J., et al., 1997. Inhibition of peripheral blood mononuclear cell proliferation by cardiac glycosides. *Ann Allergy Asthma Immunol* 78, 466-472.
- Ghare, S., Patil, M., Hote, P., et al., 2011. Ethanol inhibits lipid raft-mediated TCR signaling and IL-2 expression: potential mechanism of alcohol-induced immune suppression. *Alcohol Clin Exp Res* 35, 1435-1444.
- Goodwin, J.S., Atluru, D., Sierakowski, S., et al., 1986. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B4. *J Clin Invest* 77, 1244-1250.
- Haley, P.J., Shopp, G.M., Benson, J.M., et al., 1990. The immunotoxicity of three nickel compounds following 13-week inhalation exposure in the mouse. *Fundam Appl Toxicol* 15, 476-487.
- Hanke, J.H., Nichols, L.N., Coon, M.E., 1992. FK506 and rapamycin selectively enhance degradation of IL-2 and GM-CSF mRNA. *Lymphokine Cytokine Res* 11, 221-231.
- Hansen, J.F., Nielsen, C.H., Brorson, M.M., et al., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. *PLoS One* 10, e0131168.
- Hattori, A., Kunz, H.W., Gill, T.J., 3rd, et al., 1987. Thymic and lymphoid changes and serum immunoglobulin abnormalities in mice receiving cyclosporine. *Am J Pathol* 128, 111-120.
- He, Y.W., Deftos, M.L., Ojala, E.W., et al., 1998. RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* 9, 797-806.
- Henderson, D.J., Naya, I., Bundick, R.V., et al., 1991. Comparison of the effects of FK-506, cyclosporin A and rapamycin on IL-2 production. *Immunology* 73, 316-321.
- Himmerich, H., Schonherr, J., Fulda, S., et al., 2011. Impact of antipsychotics on cytokine production in-vitro. *J Psychiatr Res* 45, 1358-1365.

- Hu, H., Abedi-Valugerdi, M., Moller, G., 1997. Pretreatment of lymphocytes with mercury in vitro induces a response in T cells from genetically determined low-responders and a shift of the interleukin profile. *Immunology* 90, 198-204.
- Huchet, R., Grandjon, D., 1988. Histamine-induced regulation of IL-2 synthesis in man: characterization of two pathways of inhibition. *Ann Inst Pasteur Immunol* 139, 485-499.
- Iatropoulos, M.J., Hobson, W., Knauf, V., et al., 1976. Morphological effects of hexachlorobenzene toxicity in female rhesus monkeys. *Toxicol Appl Pharmacol* 37, 433-444.
- Kanariou, M., Huby, R., Ladyman, H., et al., 1989. Immunosuppression with cyclosporin A alters the thymic microenvironment. *Clin Exp Immunol* 78, 263-270.
- Karas, K., Salkowska, A., Sobalska-Kwapis, M., et al., 2018. Digoxin, an Overlooked Agonist of RORgamma/RORgammaT. *Front Pharmacol* 9, 1460.
- Khan, M.M., Melmon, K.L., Fathman, C.G., et al., 1985. The effects of autacoids on cloned murine lymphoid cells: modulation of IL 2 secretion and the activity of natural suppressor cells. *J Immunol* 134, 4100-4106.
- Kim, J.H., Park, J.S., 2002. Potentiation of the immunotoxicity of ethanol by acetaminophen in mice. *Int Immunopharmacol* 2, 15-24.
- Kim, J.Y., Huh, K., Lee, K.Y., et al., 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. *Exp Mol Med* 41, 288-295.
- Kim, S.K., Kwon, D.A., Lee, H.S., et al., 2019. Preventive Effect of the Herbal Preparation, HemoHIM, on Cisplatin-Induced Immune Suppression. *Evid Based Complement Alternat Med* 2019, 3494806.
- Kloppenburger, M., Verweij, C.L., Miltenburg, A.M., et al., 1995. The influence of tetracyclines on T cell activation. *Clin Exp Immunol* 102, 635-641.
- Knight, J.A., Plowman, M.R., Hopfer, S.M., et al., 1991. Pathological reactions in lung, liver, thymus, and spleen of rats after subacute parenteral administration of nickel sulfate. *Ann Clin Lab Sci* 21, 275-283.
- Kouchi, Y., Maeda, Y., Ohuchida, A., et al., 1996. Immunotoxic effect of low dose cisplatin in mice. *J Toxicol Sci* 21, 227-233.
- Kucharz, E.J., Sierakowski, S.J., 1990. Studies on immunomodulatory properties of

- isoniazid. II. Effect of isoniazid on interleukin 2 production and interleukin 2-receptor expression. *J Hyg Epidemiol Microbiol Immunol* 34, 207-211.
- Labuzek, K., Kowalski, J., Gabryel, B., et al., 2005. Chlorpromazine and loxapine reduce interleukin-1beta and interleukin-2 release by rat mixed glial and microglial cell cultures. *Eur Neuropsychopharmacol* 15, 23-30.
- Landewe, R.B., Miltenburg, A.M., Verdonk, M.J., et al., 1995. Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. *Clin Exp Immunol* 102, 144-151.
- Lee, J., Lim, K.T., 2012. SJSZ glycoprotein (38 kDa) modulates expression of IL-2, IL-12, and IFN-gamma in cyclophosphamide-induced Balb/c. *Inflamm Res* 61, 1319-1328.
- Lehmann, D.M., Williams, W.C., 2018. Development and utilization of a unique in vitro antigen presentation co-culture model for detection of immunomodulating substances. *Toxicol In Vitro* 53, 20-28.
- Lemster, B., Woo, J., Strednak, J., et al., 1992. Cytokine gene expression in murine lymphocytes activated in the presence of FK 506, bredinin, mycophenolic acid, or brequinar sodium. *Transplant Proc* 24, 2845-2846.
- Loose, L.D., Silkworth, J.B., Pittman, K.A., et al., 1978. Impaired host resistance to endotoxin and malaria in polychlorinated biphenyl- and hexachlorobenzene-treated mice. *Infect Immun* 20, 30-35.
- Lu, Z., Liu, F., Chen, L., et al., 2015. Effect of Chronic Administration of Low Dose Rapamycin on Development and Immunity in Young Rats. *PLoS One* 10, e0135256.
- Maeda, M., Ishii, H., Tanaka, S., et al., 2010. Suppressive efficacies of antimicrobial agents against human peripheral-blood mononuclear cells stimulated with T cell mitogen and bacterial superantigen. *Arzneimittelforschung* 60, 760-768.
- Meredith, C., Scott, M.P., 1994. Altered gene expression in immunotoxicology screening in vitro: Comparison with ex vivo analysis. *Toxicol In Vitro* 8, 751-753.
- Miller, L.C., Kaplan, M.M., 1992. Serum interleukin-2 and tumor necrosis factor-alpha in primary biliary cirrhosis: decrease by colchicine and relationship to HLA-DR4. *Am J Gastroenterol* 87, 465-470.
- Miller, T.E., Golemboski, K.A., Ha, R.S., et al., 1998. Developmental exposure to

- lead causes persistent immunotoxicity in Fischer 344 rats. *Toxicol Sci* 42, 129-135.
- Munson, A.E., Sanders, V.M., Douglas, K.A., et al., 1982. In vivo assessment of immunotoxicity. *Environ Health Perspect* 43, 41-52.
- Nalesnik, M.A., Todo, S., Murase, N., et al., 1987. Toxicology of FK-506 in the Lewis rat. *Transplant Proc* 19, 89-92.
- Northoff, H., Carter, C., Oppenheim, J.J., 1980. Inhibition of concanavalin A-induced human lymphocyte mitogenic factor (Interleukin-2) production by suppressor T lymphocytes. *J Immunol* 125, 1823-1828.
- Palacios, R., Sugawara, I., 1982. Hydrocortisone abrogates proliferation of T cells in autologous mixed lymphocyte reaction by rendering the interleukin-2 Producer T cells unresponsive to interleukin-1 and unable to synthesize the T-cell growth factor. *Scand J Immunol* 15, 25-31.
- Pally, C., Tanner, M., Rizvi, H., et al., 2001. Tolerability profile of sodium mycophenolate (ERL080) and mycophenolate mofetil with and without cyclosporine (Neoral) in the rat. *Toxicology* 157, 207-215.
- Parenti, D.M., Simon, G.L., Scheib, R.G., et al., 1988. Effect of lithium carbonate in HIV-infected patients with immune dysfunction. *J Acquir Immune Defic Syndr* 1, 119-124.
- Parthasarathy, N.J., Kumar, R.S., Devi, R.S., 2005. Effect of methanol intoxication on rat neutrophil functions. *J Immunotoxicol* 2, 115-121.
- Peterson, K.P., Van Hirtum, M., Peterson, C.M., 1997. Dapsone decreases the cumulative incidence of diabetes in non-obese diabetic female mice. *Proc Soc Exp Biol Med* 215, 264-268.
- Poluektova, L.Y., Huggler, G.K., Patterson, E.B., et al., 1999. Involvement of protein kinase A in histamine-mediated inhibition of IL-2 mRNA expression in mouse splenocytes. *Immunopharmacology* 41, 77-87.
- Quemeneur, L., Flacher, M., Gerland, L.M., et al., 2002. Mycophenolic acid inhibits IL-2-dependent T cell proliferation, but not IL-2-dependent survival and sensitization to apoptosis. *J Immunol* 169, 2747-2755.
- Ress, N.B., Hailey, J.R., Maronpot, R.R., et al., 2003. Toxicology and carcinogenesis studies of microencapsulated citral in rats and mice. *Toxicol Sci* 71, 198-206.

- Riesbeck, K., 1999. Cisplatin at clinically relevant concentrations enhances interleukin-2 synthesis by human primary blood lymphocytes. *Anticancer Drugs* 10, 219-227.
- Ringerike, T., Ulleras, E., Volker, R., et al., 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). *Toxicology* 206, 257-272.
- Roche, Y., Fay, M., Gougerot-Pocidallo, M.A., 1988. Enhancement of interleukin 2 production by quinolone-treated human mononuclear leukocytes. *Int J Immunopharmacol* 10, 161-167.
- Saito, R., Hirakawa, S., Ohara, H., et al., 2011. Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. *Toxicol Appl Pharmacol* 254, 245-255.
- Salazar, V., Castillo, C., Ariznavarreta, C., et al., 2004. Effect of oral intake of dibutyl phthalate on reproductive parameters of Long Evans rats and pre-pubertal development of their offspring. *Toxicology* 205, 131-137.
- Santarelli, L., Bracci, M., Mocchegiani, E., 2006. In vitro and in vivo effects of mercuric chloride on thymic endocrine activity, NK and NKT cell cytotoxicity, cytokine profiles (IL-2, IFN-gamma, IL-6): role of the nitric oxide-L-arginine pathway. *Int Immunopharmacol* 6, 376-389.
- Schleuning, M.J., Duggan, A., Reem, G.H., 1989. Inhibition by chlorpromazine of lymphokine-specific mRNA expression in human thymocytes. *Eur J Immunol* 19, 1491-1495.
- Sfikakis, P.P., Souliotis, V.L., Katsilambros, N., et al., 1996. Downregulation of interleukin-2 and alpha-chain interleukin-2 receptor biosynthesis by cisplatin in human peripheral lymphocytes. *Clin Immunol Immunopathol* 79, 43-49.
- She, Y., Wang, N., Chen, C., et al., 2012. Effects of aluminum on immune functions of cultured splenic T and B lymphocytes in rats. *Biol Trace Elem Res* 147, 246-250.
- Sheikhi, A., Jaber, Y., Esmaeilzadeh, A., et al., 2007. The effect of cardiovascular drugs on pro-inflammatory cytokine secretion and natural killer activity of peripheral blood mononuclear cells of patients with chronic heart failure in vitro. *Pak J Biol Sci* 10, 1580-1587.
- Silvestrini, B., Lisciani, R., Barcellona, P.S., 1967. Anti-granuloma and thymolytic

- activity of certain drugs. *Eur J Pharmacol* 1, 240-246.
- Song, Y., Han, S., Kim, H., et al., 2006. Effects of mizoribine on MHC-restricted exogenous antigen presentation in dendritic cells. *Arch Pharm Res* 29, 1147-1153.
- Sookoian, S., Castano, G., Flichman, D., et al., 2004. Effects of ribavirin on cytokine production of recall antigens and phytohemagglutinin-stimulated peripheral blood mononuclear cells. (Inhibitory effects of ribavirin on cytokine production). *Ann Hepatol* 3, 104-107.
- Sugiyama, K., Ueda, H., Ichio, Y., et al., 1995. Improvement of cisplatin toxicity and lethality by juzen-taiho-to in mice. *Biol Pharm Bull* 18, 53-58.
- Synzynys, B.I., Sharetskii, A.N., Kharlamova, O.V., 2004. [Immunotoxicity of aluminum chloride]. *Gig Sanit*, 70-72.
- Sztejn, M.B., Simon, G.L., Parenti, D.M., et al., 1987. In vitro effects of thymosin and lithium on lymphoproliferative responses of normal donors and HIV seropositive male homosexuals with AIDS-related complex. *Clin Immunol Immunopathol* 44, 51-62.
- Takai, K., Jojima, K., Sakatoku, J., et al., 1990. Effects of FK506 on rat thymus: time-course analysis by immunoperoxidase technique and flow cytofluorometry. *Clin Exp Immunol* 82, 445-449.
- Tam, R.C., Pai, B., Bard, J., et al., 1999. Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile. *J Hepatol* 30, 376-382.
- Tsukue, N., Toda, N., Tsubone, H., et al., 2001. Diesel exhaust (DE) affects the regulation of testicular function in male Fischer 344 rats. *J Toxicol Environ Health A* 63, 115-126.
- Turka, L.A., Dayton, J., Sinclair, G., et al., 1991. Guanine ribonucleotide depletion inhibits T cell activation. Mechanism of action of the immunosuppressive drug mizoribine. *J Clin Invest* 87, 940-948.
- Van Dijk, H., Bloksma, N., Rademaker, P.M., et al., 1979. Differential potencies of corticosterone and hydrocortisone in immune and immune-related processes in the mouse. *Int J Immunopharmacol* 1, 285-292.
- Van Wauwe, J., Aerts, F., Van Genechten, H., et al., 1996. The inhibitory effect of pentamidine on the production of chemotactic cytokines by in vitro stimulated human blood cells. *Inflamm Res* 45, 357-363.

- Vandebriel, R.J., Meredith, C., Scott, M.P., et al., 1998. Effects of in vivo exposure to bis(tri-n-butyltin)oxide, hexachlorobenzene, and benzo(a)pyrene on cytokine (receptor) mRNA levels in cultured rat splenocytes and on IL-2 receptor protein levels. *Toxicol Appl Pharmacol* 148, 126-136.
- Vargova, M., Wagnerova, J., Liskova, A., et al., 1993. Subacute immunotoxicity study of formaldehyde in male rats. *Drug Chem Toxicol* 16, 255-275.
- Vos, J.G., van Logten, M.J., Kreeftenberg, J.G., et al., 1979. Hexachlorobenzene-induced stimulation of the humoral immune response in rats. *Ann N Y Acad Sci* 320, 535-550.
- Vos, J.G., Van Loveren, H., 1994. Developments of immunotoxicology methods in the rat and applications to the study of environmental pollutants. *Toxicol In Vitro* 8, 951-956.
- Wagner, W., Sachrajda, I., Pulaski, L., et al., 2011. Application of cellular biosensors for analysis of bioactivity associated with airborne particulate matter. *Toxicol In Vitro* 25, 1132-1142.
- Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. *Toxicol Lett* 162, 55-70.
- Wang, Y., Walker, C., Stadler, B.M., et al., 1984. Transcription and translation dependent induction of interleukin 2 (IL-2) and IL-2 receptors. *Immunol Lett* 8, 227-231.
- Wilson, R., Fraser, W.D., McKillop, J.H., et al., 1989. The "in vitro" effects of lithium on the immune system. *Autoimmunity* 4, 109-114.
- Yamamoto, N., Sakai, F., Yamazaki, H., et al., 1996. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. *Eur J Pharmacol* 314, 137-142.
- Yoshimura, N., Matsui, S., Hamashima, T., et al., 1989. Effect of a new immunosuppressive agent, FK506, on human lymphocyte responses in vitro. II. Inhibition of the production of IL-2 and gamma-IFN, but not B cell-stimulating factor 2. *Transplantation* 47, 356-359.
- Zhang, W.Z., Yong, L., Jia, X.D., et al., 2013. Combined subchronic toxicity of bisphenol A and dibutyl phthalate on male rats. *Biomed Environ Sci* 26, 63-69.

Appendix 10. The Multi-Immuno Tox Assay Data sheet

Multi-ImmunoTox Assay Datasheet for #2H4 cells					
Ver. 008.2					
Laboratory					Round
Exp.	1st exp.	(Highest soluble conc. In the next exp.s			mg/ml
Date: (YYYY/MM/DD)			Operator:		
Code		Dissolution		mg/ml in	
FlnSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA \geq 0.05		#VALUE!
Comment:					
Exp.	2nd exp.	(Highest soluble conc. In the next exp.s			mg/ml
Date: (YYYY/MM/DD)			Operator:		
Code		Dissolution		mg/ml in	
FlnSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA \geq 0.05		#VALUE!
Comment:					
Exp.	3rd exp.				
Date: (YYYY/MM/DD)			Operator:		
Code		Dissolution		mg/ml in	
FlnSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA \geq 0.05		#VALUE!
Comment:					

Exp.	4th exp.	
Date: (YYYYMMDD)		Operator:
Code		Dissolution mg/ml in
FinSLO-LA	#VALUE!	#VALUE! the number of concentration which satisfy $U-SLR-LA \geq 0.05$ #VALUE!
Comment:		
Exp.	5th exp.	
Date: (YYYYMMDD)		Operator:
Code		Dissolution mg/ml in
FinSLO-LA	#VALUE!	#VALUE! the number of concentration which satisfy $U-SLR-LA \geq 0.05$ #VALUE!
Comment:		
Exp.	6th exp.	
Date: (YYYYMMDD)		Operator:
Code		Dissolution mg/ml in
FinSLO-LA	#VALUE!	#VALUE! the number of concentration which satisfy $U-SLR-LA \geq 0.05$ #VALUE!
Comment:		
1st		
2nd		
3rd		
4th		
5th		
6th		
1st Exp.		
2nd Exp.		
3rd Exp.		
4th Exp.		
5th Exp.		
6th Exp.		

MultiReporter Assay System -Tripluc®- Calculation Sheet													
1st exp.													
Transmittance Data													
		SLG	SLO	SLR									
	T0					#VALUE!	#VALUE!	#VALUE!					
	T1					#VALUE!	#VALUE!	#VALUE!					
	T2					#VALUE!	#VALUE!	#VALUE!					
Filter 0 Data		1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													
Filter 1 Data		1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													
Filter 2 Data		1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													

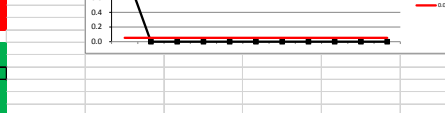
2nd exp.													
Transmittance Data													
		SLG	SLO	SLR									
	T0					#VALUE!	#VALUE!	#VALUE!					
	T1					#VALUE!	#VALUE!	#VALUE!					
	T2					#VALUE!	#VALUE!	#VALUE!					
Filter 0		1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													
Filter 1		1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													
Filter 2		1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													

1st exd.

Line graph showing the change in the number of people in the labor force (in millions) from 1990 to 2010. The Y-axis ranges from 0.2 to 1.2. The X-axis shows years from 1990 to 2010. Two lines are plotted: a black line for the 11-19 age group and a red line for the 20-24 age group. The black line starts at approximately 1.0 in 1990 and decreases to about 0.2 in 2010. The red line starts at approximately 0.8 in 1990 and decreases to about 0.4 in 2010.

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Appendix 11. The summary of the study by the independent biostatistician

1. Results

The concentration-response plot for each experiment is shown in appendix A. We strongly suggest to see the graphs to understand the result of each judgement of experiment.

1.1 Basic results

The judgment of each experiment by chemical is shown in Table 1 for the phase I study and Table 2 for the phase II study. Symbols in column “exp.” means “S” for suppression, “A” for augmentation and “N” for no effect. The column “Judge” lists the final judgment of the assay in each laboratory. The column “Chem. Code” is chemical code.

Table 1. Judgment for 3 independent experiments in Phase I study

(a)					(b)					(c)				
Lab A	exp.				Lab B	exp.				Lab C	exp.			
Chem. Code	1	2	3	Judge	Chem. Code	1	2	3	Judge	Chem. Code	1	2	3	Judge
P101_R1	S	S	S	S	P101_R1	S	S	S	S	P101_R1	S	S	S	S
P101_R2	S	S	S	S	P101_R2	S	S	S	S	P101_R2	S	S	S	S
P101_R3	S	S	S	S	P101_R3	S	S	S	S	P101_R3	S	S	S	S
P102_R1	S	A	S	S	P102_R1	S	S	S	S	P102_R1	N	S	S	S
P102_R2	N	N	N	N	P102_R2	S	S	S	S	P102_R2	S	S	N	S
P102_R3	N	N	N	N	P102_R3	S	S	N	S	P102_R3	N	N	N	N
P103_R1	S	S	S	S	P103_R1	S	S	S	S	P103_R1	S	S	S	S
P103_R2	S	S	S	S	P103_R2	S	S	S	S	P103_R2	S	S	S	S
P103_R3	S	S	S	S	P103_R3	S	S	S	S	P103_R3	S	S	S	S
P104_R1	S	S	S	S	P104_R1	S	S	S	S	P104_R1	S	S	S	S
P104_R2	S	S	S	S	P104_R2	S	S	S	S	P104_R2	S	S	S	S
P104_R3	S	S	S	S	P104_R3	S	S	S	S	P104_R3	S	S	S	S
P105_R1	A	N	N	N	P105_R1	N	N	N	N	P105_R1	N	N	A	N
P105_R2	N	N	N	N	P105_R2	N	S	N	N	P105_R2	N	N	N	N
P105_R3	N	S	N	N	P105_R3	N	N	N	N	P105_R3	N	N	N	N

S : Suppression, A : Augmentation, N : No effect,
A/S : Augmentation/Suppression

Table 2. Judgment for 3 independent experiments in Phase II study.

(a)						(b)						(c)					
Lab A	exp.					Lab B	exp.					Lab C	exp.				
Chem. Code	1	2	3	4	Judge	Chem. Code	1	2	3	4	Judge	Chem. Code	1	2	3	4	Judge
P201	N	N	N		N	P201	N	N	N		N	P201	N	N	N		N
P202	S	S	S		S	P202	A	S	S		S	P202	N	S	S		S
P203	N	N	N		N	P203	N	S	N		N	P203	N	A	N		N
P204	A/S	A/S	A/S		A/S	P204	N	A	A		A	P204	N	N	A		N
P205	S	S	S		S	P205	S	S	S		S	P205	S	S	S		S
P206	N	N	N		N	P206	N	N	N		N	P206	N	N	N		N
P207	N	N	N		N	P207	N	N	N		N	P207	N	N	N		N
P208	A	A	A		A	P208	S	A	A		A	P208	A	N	A		A
P209	A	A	A		A	P209	A	A	A		A	P209	A	A	A		A
P210	S	S	S		S	P210	A	N	N		N	P210	S	S	S		S
P211	N	N	N		N	P211	S	S	S		S	P211	N	N	N		N
P212	A	A	A		A	P212	A	A	A		A	P212	A	A	A		A
P213	S	N	S		S	P213	S	S	S		S	P213	S	S	S		S
P214	A	A	A		A	P214	A	A	A		A	P214	A	A	A		A
P215	A	A	N		A	P215	S	S	S		S	P215	S	S	N		S
P216	N	N	N		N	P216	N	N	N		N	P216	N	N	N		N
P217	N	N	N		N	P217	N	N	N		N	P217	A	N	N		N
P218	S	A	N		N	P218	N	N	N		N	P218	N	N	N		N
P219	N	A	N		N	P219	N	N	N		N	P219	A	N	N		N
P220	S	S	S		S	P220	S	S	S		S	P220	S	S	S		S
S : Immunosuppression, A : Immunoaugmentation, N : No effect, A/S : Immunoaugmentation/suppression																	

1.2 Within-laboratory reproducibility

Table 3 shows the final judgment of each assay by chemical and the concordance based on the results in the phase I study. “R” means round.

Table 3. Judgment for independent 3 rounds and concordance

(a)					(b)					(c)				
Lab A					Lab B					Lab C				
Chem. Code	R1	R2	R3	Concordance	Chem. Code	R1	R2	R3	Concordance	Chem. Code	R1	R2	R3	Concordance
P101	S	S	S	1	P101	S	S	S	1	P101	S	S	S	1
P102	S	N	N	0	P102	S	S	S	1	P102	S	S	N	0
P103	S	S	S	1	P103	S	S	S	1	P103	S	S	S	1
P104	S	S	S	1	P104	S	S	S	1	P104	S	S	S	1
P105	N	N	N	1	P105	N	N	N	1	P105	N	N	N	1

Table 4 shows the concordance rate of the within-laboratory reproducibility which is estimated by data of Table 3.

Table 4. Withing laboratory concordance

Statistics	Lab A	Lab B	Lab C	Average
Within-laboratory concordance rate	80%(4/5)	100%(5/5)	80%(4/5)	86.7%

1.3 Between-laboratory reproducibility

Table 5 shows the final judgment of the assay for each laboratory and the concordance in the phase II study.

Table 5. Final judgment of the assay for each laboratory and concordance

Chem. Code	Lab A	Lab B	Lab C	Concordance
P201	N	N	N	1
P202	S	S	S	1
P203	N	N	N	1
P204	A/S	A	N	0
P205	S	S	S	1
P206	N	N	N	1
P207	N	N	N	1
P208	A	A	A	1
P209	A	A	A	1
P210	S	N	S	0
P211	N	S	N	0
P212	A	A	A	1
P213	S	S	S	1
P214	A	A	A	1
P215	A	S	S	0
P216	N	N	N	1
P217	N	N	N	1
P218	N	N	N	1
P219	N	N	N	1
P220	S	S	S	1

Table 6 shows the concordance rate of the between-laboratory reproducibility which is estimated by data of Table 5.

Table 6. Between laboratory reproducibility in Phase II study

Statistics	
Between-laboratory concordance rate	80%(16/20)

Table 7 is the result from Table 3 and Table 6. The final judgment in Table 3 was summarized with based on the majority.

Statistics		
Between-laboratory concordance rate	80%(20/25)	

Appendix 12. Study plan

Study plan for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc assay) as a test evaluating the immunotoxic potential of chemicals

Version 1.4 February, 2017

Conducted by: IL-2 Luc assay Validation Management Team

INDEX

Background

Objective of the trial

3. Validation Management Team

4. Protocol

5. Chemical

6. Records and archiving

7. Study timeline

1. Background

The multicolor reporter assay using IL-2 Luc in Jurkat cells (IL-2 assay) is important for evaluating the immunotoxic potential of chemicals. This assay forms part of the Multi-ImmunoTox assay (MITA) and has the advantages of technical simplicity and a short test period, and the accuracy of the test result is based on the mechanism underlying immunotoxicity.

The aim of this trial is to (pre)validate the IL-2 Luc assay method to assess its transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals. The IL-2 Luc assay for the validation trial was undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004], iii) according to the concept discussed in the validation trials with the participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of validation trials is described in the context of GLP, and iv) in line with the ISO procedure JRC.I.03.GP.01v.01 (<http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf>).

The studies comprising a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, use of standard operating procedures (SOP) and adequate data recording, reporting and record keeping are essential.

A general conceptual framework [Hartung et al., 2004; OECD, 2005] will be used for documenting the entire study to assess the validation status of the test method. This is called a “modular approach” to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition

Module 2: Within-laboratory repeatability and reproducibility

Module 3: Between-laboratory transferability

Module 4: Between-laboratory reproducibility

Module 5: Predictive capacity

Module 6: Applicability domain

Module 7: Performance standards

The modular approach as introduced by Hartung et al. allows the use of datasets from

various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-2 Luc assay. This IL-2 Luc assay for the validation trial has been performed under GLP principles.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-2 Luc assay with a challenging set of test substances (test items) for which high quality *in vitro* and *in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science, and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-2 Luc assay Validation Management Team

Name	Role and expertise	Affiliation
<u>Trial Coordinator</u> Hajime Kojima	VMT trial coordinator, Chemical supplier and Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
<u>Lead Lab</u> Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
<u>EU liaison</u> Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
<u>EU liaison</u> Erwin L. Roggen	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
<u>ICCVAM liaison</u>	Immunotoxicity expertise	NTP/NIEHS, USA

Dori Germolec		
JSIT liaison Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD): Kohji Yamakage

Test Facility 2: AIST, Tsukuba SD: Yoshihiro Ohmiya

Test Facility 3: AIST, Takamatsu SD: Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-2 Luc assay is transferable and reproducible between experienced laboratories. All laboratories participating in this validation trial will act as unexperienced laboratories to assess between-laboratory transferability, reliability, and relevance of the IL-2 Luc assay method under non-GLP conditions (GLP principle).

Trial management structure

1) Chemical management group

The members of the chemical management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare a tentative list of test chemicals and work with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed in Table 6 and 7 are distributed by the JaCVAM.

2) Data analysis group

The members of the data analysis group are elected by recommendation of the IL-2 Luc assay VMT and check and analyze the data obtained in this validation trial from a third-party standpoint. The members also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of the record management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare the protocol, the test chemical preparation record forms, blank data sheets, etc., and distribute them to the research laboratories participating in this validation trial. The members also collect completed forms and data sheets after completion of the experiments, and point out omissions or flaws in recording, if any, and request corrections of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the necessary data recording or calculation templates. The Trial Coordinator must ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead laboratory and the other participating test facilities might be contacted by the VMT regarding technical issues.

Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- test, reference and control item purchase, coding and distribution to the test facility
- availability of the test systems to the participating laboratories by supporting the lead laboratory with the logistics for delivering the test system to the facility
- independent analysis of data and statistical support (biostatistician) based on the study reports generated
- other costs incurred by the participating laboratories

Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:

- a) Establishment of/support to lead laboratory, including meeting organization
- b) Trial communication and coordination with test facilities
- c) Recording of document and data flow between test facilities
- d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
- e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
- f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
- g) Approval with date and signature of all protocols, Study Plans and Study Reports
- h) The communication of the results of the trial into the public domain

The Trial Coordinator's responsibilities include:

- a) Establishment/support of the lead laboratory, including meeting organization
- b) Trial communication and coordination with the test facilities
- c) Recording of documents and data flow between the test facilities
- d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
- e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
- f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratory and other test facilities involved in the validation trial, and should reflect the overall trial
- g) Approval with date and signature of all protocols, study plans and study reports
- h) Communication of the results of the trial to the public domain

The role of the Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial Coordinator (unless otherwise communicated by the participating test facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate

test method protocols (dated signature by the Trial Coordinator and the lead laboratory) and, if appropriate, validated data recording, data analysis, and data reporting sheets for the test method.

It is the responsibility of the Trial Coordinator to approve the study plans sent for approval by the test facilities, and any amendments to the study plan, by dated signature.

Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to all test facilities, giving details of what training aspects will be covered during the training of the other SDs and study personnel at the lead laboratory. Furthermore, after the training during the Phase 0 study, the lead laboratory will issue to the Trial Coordinator a training report and indicate if critical observations are made by the other test facilities regarding the IL-2 Luc assay protocols. In case any critical observations are made, a new version of the IL-2 Luc assay protocols might need be issued to the other test facilities before initiating the between-laboratory transferability test.

[Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of all test facilities has been done by an independent biostatistical analysis using 5 coded chemicals under the VMT. The concordance should be equal to or greater than 80% as a tentative acceptance criterion for the Phase I study.

3.7 [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3) study is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

Transfer of the IL-2 Luc assay to all test facilities in the Phase 0 study using 5 coded five chemicals was achieved. A few concentrations of each test item were tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.

The 5 test items selected for the Phase I study are coded as A, B, C, D, and E. The facilities will prepare a study according to internal GLP principles. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability study will be reviewed before progressing with module 4 in the between-laboratory reproducibility study. If the transferability data do not meet the test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles).

3.8 [Module 4] Between-laboratory reproducibility

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibilities in the Phase I and II studies. Several concentrations of each test item will be tested in triplicate according to the IL-2 Luc assay method protocol describing the details of the experimental design.

At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles). The concordance for between-laboratory reproducibility should be equal or greater than 80% to meet the acceptance criteria.

[Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data for between-laboratory reproducibility has been assessed. Depending on the statistical analysis, lean design for validation as well as automatisation of the test leading to an increased dataset will be considered.

Protocol

In this validation trial, the protocols ver. 0.08E, Phase I and 0.1E, Phase II will be used. These protocols will be drafted by the lead laboratory and will be finalized by the VMT. The criteria to identify immunotoxicants by the MITA are provisionally fixed in protocol ver. 0.08E prior to the Phase I study. There are 2 temporary criteria to identify immunotoxicants. The VMT adopted these criteria after the Phase I validation study.

A measurement of bioluminescence intensity induced by chemical treatment will be measured by a luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

Chemicals

5.1 Chemical Selection

Test chemicals have been selected from a chemical repository based on published papers on *in vivo* immunotoxicity.

The applied selection criteria were:

information on mode/site of action

coverage of range of relevant chemical classes and product classes

quality and quantity of reference data (*in vivo* and *in vitro*)

high quality data derived from animals and (if available) also humans

knowledge of interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)

coverage of range of toxic effects/potencies

chemicals that do not need metabolic activation

appropriate negative and positive controls

physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)

single chemical entities or formulations of known high purity

availability

cost

In the first phase of the selection procedure, the chemical management group identified and collected several existing lists of potential chemical sensitizers in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes, such as reference compounds for validation studies. An extensive literature research was performed by the chemical management group, insuring that the preselected chemicals fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase of the IL-2 Luc assay validation trial using data generated at the test facilities, 5 chemicals will be tested 3 times for each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of the Phase I results, detailed test planning for Phase II will be established. Currently, it is planned that 20 chemicals will be tested in the Phase II trial to establish predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study	Chemicals	Test Number	Information obtained
Phase I	5 non- coded	1	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II	20 coded	1	Between-lab reproducibility & predictability

5.2 Chemical Acquisition, Coding and Distribution

The within-laboratory reproducibility (Module 2) and between-laboratory transferability (Module 3) in all test facilities have been assessed with coded chemicals. This IL-2 Luc validation trial plan describes generation of the missing data sets under coded test item. If the results obtained are not highly similar to the previously obtained sets, the VMT must assess if coded chemicals need to be tested in all the test facilities.

Coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible for coding and distribution of the test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive safety information concerning hazards identification and exposure controls/personal protection.

Records and archiving

At the end of the trial, the IL-2 Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator, commenting on the

accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3. The duration of this validation trial is around 20 months, from May 2016 to December 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
February, 2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer to know between laboratory transferability
	Data collection of technical transfer (Phase 0 study)
Phase I study	
September 2016	Coding and distribution of five coded test chemicals
September, 2016	Start of Phase I study
December, 2016	End of Phase I study
February, 2017	2nd VMT Meeting / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
April, 2017	Coding and distribution of coded test chemicals and positive chemicals
May, 2017	Start of Phase II study using 20 coded test chemicals
August, 2017	End of Phase II study
November-December, 2017	3rd VMT Meeting /reviewing of Phase II study results
2018	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service

GLP: Good Laboratory Practice

HRI: Hatano Research Institute

FDSC: Food and Drug Safety Center

JaCVAM: Japanese Centre for the Validation of Alternative Methods

NIHS: National Institute of Health Sciences

OECD: Organization for Economic Co-operation and Development

QC: Quality Control

TG: Test Guideline

VMT: Validation Management Team

Appendix 13. MITA QC confirmation table

MITA(P1) confirmation table

	LabB (AIST, Tsukuba)			LabC (FDSC)		LabD (AIST, Takamatsu)	
setA-1	date	2016.9.12	(document 4 - 5)				
(run 1)	Cell culture records	○					
	Weighting records	○					
	Test records	○					
	Datasheet	×					
	Graph	○					
setA-1	date	2016.10.4	(document 4 - 5)				
(run 2)	Cell culture records	○					
	Weighting records	○					
	Test records	○					
	Datasheet	×					
	Graph	○					
setA	Weighting records	○		Weighting records	○	Weighting records	○
	Cell culture records	○		Cell culture records	×	Cell culture records	○
							wake up 2016.8.26~ Last culture 2016.9.29
setA-1	date	2016.10.26		date	2016.10.4	date	2016.9.9
	Cell culture records	○		Cell culture records	○	Cell culture records	○
	Weighting records	○		Weighting records	○	Weighting records	○
	Test records	○		Test records	○	Test records	○
	Datasheet	○		Datasheet	○	Datasheet	○
	Graph	○		Graph		Graph	○
setA-2	date	2016.11.1		date	2016.10.17	date	2016.9.12
	Cell culture records	○		Cell culture records	○	Cell culture records	○
	Weighting records	○		Weighting records	○	Weighting records	×
	Test records	○		Test records	○	Test records	○
	Datasheet	○		Datasheet	○	Datasheet	○
	Graph	○		Graph		Graph	○
setA-3	date	2016.11.4		date	2016.10.21	date	2016.9.15 (document 7 - 8)
	Cell culture records	○		Cell culture records	○	Cell culture records	○
	Weighting records	○		Weighting records	○	Weighting records	×
	Test records	○		Test records	○	Test records	○
	Datasheet	○		Datasheet	○	Datasheet	○
	Graph	○		Graph		Graph	○
setA-4	date			date		date	2016.9.20 (3rd re trial)
	Cell culture records			Cell culture records		Cell culture records	○
	Weighting records			Weighting records		Weighting records	×
	Test records			Test records		Test records	○
	Datasheet			Datasheet		Datasheet	○
	Graph			Graph		Graph	○
setB	Weighting records	○		Weighting records	○	Weighting records	○
	Cell culture records	○		Cell culture records	×	Cell culture records	○
							Newly continue from SetA + starting cell (wake up from 20160923 culture on cell culture till 20161014) the way
setB-1	date	2016.11.8		date	2016.10.27	date	2016.9.23 (document 7 - 8)
	Cell culture records	○		Cell culture records	○	Cell culture records	○
	Weighting records	○		Weighting records	○	Weighting records	○
	Test records	○		Test records	○	Test records	○
	Datasheet	○		Datasheet	○	Datasheet	○
	Graph	○		Graph	○	Graph	○
setB-2	date	2016.11.12		date	2016.10.28	date	2016.9.26 (1st re-trial)
	Cell culture records	○		Cell culture records	○	Cell culture records	○
	Weighting records	○		Weighting records	○	Weighting records	×
	Test records	○		Test records	○	Test records	○
	Datasheet	○		Datasheet	○	Datasheet	○
	Graph	○		Graph	○	Graph	○
setB-3	date	2016.11.16		date	2016.10.31	date	2016.9.29 (2nd trial)
	Cell culture records	○		Cell culture records	○	Cell culture records	○
	Weighting records	○		Weighting records	○	Weighting records	×
	Test records	○		Test records	○	Test records	○
	Datasheet	○		Datasheet	○	Datasheet	○
	Graph	○		Graph	○	Graph	○
setB-4	date			date		date	2016.10.3 (3rd trial)
	Cell culture records			Cell culture records		Cell culture records	○
	Weighting records			Weighting records		Weighting records	×
	Test records			Test records		Test records	○
	Datasheet			Datasheet		Datasheet	○
	Graph			Graph		Graph	○

MITA(P1) confirmation tabale

	LabB (AIST, Tsukuba)		LabC (FDSC)		LabD (AIST, Takamatsu)	
setC	Weighting records	○	Weighting records	○	Weighting records	○
	Cell culture records	○	Cell culture records	×	Cell culture records	○
setC-1	date	2016.11.10	date	2016.11.14	date	2016.10.6 (document 7 - 8)
	Cell culture records	○	Cell culture records	○	Cell culture records	○
	Weighting records	○	Weighting records	○	Weighting records	○
	Test records	○	Test records	○	Test records	○
	Datasheet	○	Datasheet	○	Datasheet	○
	Graph	○	Graph	○	Graph	○
setC-2	date	2016.11.14	date	2016.11.25	date	2016.10.14 (document 7 - 8)
	Cell culture records	○	Cell culture records	○	Cell culture records	○
	Weighting records	○	Weighting records	○	Weighting records	×
	Test records	○	Test records	○	Test records	○
	Datasheet	○	Datasheet	○	Datasheet	○
	Graph	○	Graph	○	Graph	○
setC-3	date	2016.11.18	date	2016.12.09	date	2016.10.17 (document 7 - 8)
	Cell culture records	○	Cell culture records	○	Cell culture records	○
	Weighting records	○	Weighting records	○	Weighting records	×
	Test records	○	Test records	○	Test records	○
	Datasheet	○	Datasheet	○	Datasheet	○
	Graph	○	Graph	○	Graph	○
setC-4	date		date		date	2016.10.20 (document 7 - 8)
	Cell culture records		Cell culture records		Cell culture records	○
	Weighting records		Weighting records		Weighting records	×
	Test records		Test records		Test records	○
	Datasheet		Datasheet		Datasheet	○
	Graph		Graph		Graph	○
	SDS back calibration records	○ 20170322実績	SDS back calibration records	○	SDS back calibration records	○

MITA(P2) Confirmation table

項目	LabB (AIST, Tsukuba)	LabC (FDSC)	LabD (AIST, Shikoku)
Weighing records	○	○	○
Cell culture records	○ 3sets 2017.05.02 2017.05.19 2017.06.12	○ 3sets 2017.05.29 2017.07.03 2017.07.31	○ 3sets 2017.05.08 2017.06.06 2017.07.03
Solubility check records	○ per each samples	○ per each tests	○ per each samples
1 Test date	2017.5.19.	2017.06.30	2017.05.22
Test samples No. (repeat No.)	1-5(1)	6,4,6,7(1)	2,7,8,12(1)
Others records	○	○	○
Datasheets	○	○	○
2 Test date	2017.5.31	2017.07.06	2017.05.23
Test samples No.	1,3-5(2),2(re1)	4,6,7(2)	14,16,17,19,20,01(1)
Others records	○	○	○
Datasheets	○	○	○
3 Test date	2017.6.8	2017.07.07	2017.05.29
Test samples No.	1,3-5(3),2(2)	4,6,7(3)	3,4,10,11(1)
Others records	○	○	○
Datasheets	○	○	○
4 Test date	2017.6.12	2017.07.13	2017.05.30
Test samples No.	2(3),5(re3)	1,3,5,8(1)	5,6,9,13,15,18(1)
Others records	○	○	○
Datasheets	○	○	○
5 Test date	2017.6.5	2017.07.14	2017.06.12
Test samples No.	6-10(1)	1,3,5,8(2),9,10(1)	5,6,9,13,15,18(re1)
Others records	○	○	○
Datasheets	○	○	○
6 Test date	2017.6.6.	2017.07.18	2017.06.19
Test samples No.	6(re1),7-10(2)	1,3,5,8(3),9,10(2)	3,4,10,11(re1)
Others records	○	○	○
Datasheets	○	○	○
7 Test date	2017.6.9.	2017.07.21	2017.06.20
Test samples No.	6(2),7-10(3)	9,10(3),11-14(1)	2,7,8,12(2)
Others records	○	○	○
Datasheets	○	○	○
8 Test date	2017.6.14	2017.07.24	2017.06.26
Test samples No.	11-15(1)	11,12,14(2),13(re1),15,16(1)	14,16,17,19,20,01(re1)
Others records	○	○	○
Datasheets	○	○	○
9 Test date	2017.6.21	2017.07.27	2017.06.27
Test samples No.	11-15(2)	11,12,14(3),13(2)	5,6,10,11,3,4 (2)
Others records	○	○	○
Datasheets	○	○	○
10 Test date	2017.6.22	2017.07.28	2017.07.03
Test samples No.	11-13,15(3)14(re2),	2(re1),13(3),15,16(2),18,20(1)	16,17,19,20,15,18 (2)
Others records	○	○	○
Datasheets	○	○	○
11 Test date	2017.6.29	2017.08.03	2017.07.04
Test samples No.	6,14(3)	15,16(3),17,19(1),18,20(2)	1,9,13,14 (2)
Others records	○	○	○
Datasheets	○	○	○
12 Test date	2017.6.28	2017.08.04	2017.07.10
Test samples No.	16-20(1)	1,3(4),2(3),19(2),18,20(3)	17,19,7,3 (3)
Others records	○	○	○
Datasheets	○	○	○
13 Test date	2017.7.7	2017.08.07	2017.07.11
Test samples No.	16-20(2)	2(4),17(2),19(3)	2,8,12,16,14,20 (3)
Others records	○	○	○
Datasheets	○	○	○
14 Test date	2017.7.11	2017.08.08	2017.07.18
Test samples No.	16-20(3)	5,8,19(4),17(3)	1,4,5,6,10,11 (3)
Others records	○	○	○
Datasheets	○	○	○
15 Test date		2017.08.14	2017.07.24
Test samples No.		13(4)	9,13,15,18(3),3,19(4)
Others records		○	○
Datasheets		○	○
16 Test date			2017.07.25
Test samples No.			10,13,14,9,4(4)
Others records			○
Datasheets			○

Appendix 14. MITA coded chemical list

【Phase I coded list for the MITA validation study in Sep 2016】

No.	Chemical	CASRN	MW	Supplier	Catalog No.	Content	Physical characteristics	Lot	Storage	Purity	LabA TOHOKU univ.	LabB AIST-TSUKUBA	LabC FDSC	LabD AIST-SHIKOKU
1	Dibutyl phthalate	84-74-2	278.34	Wako	021-06936	500mL	Liquid	TLN0112	RT	98.0+ % (Capillary GC)	MIA003A	MIB014A	MIC027A	MID036A
											MIA004B	MIB017B	MIC026B	MID033B
											MIA007C	MIB016C	MIC023C	MID034C
2	Hydrocortisone (for Cell Culture)	50-23-7	362.46	Wako	080-10194	50g	Solid	SAH3714	RT	97% (HPLC)	MIA005A	MIB017A	MIC029A	MID038A
											MIA007B	MIB019B	MIC028B	MID035B
											MIA009C	MIB018C	MIC025C	MID037C
3	Lead(II) acetate trihydrate (Deleterious substances)	6080-56-4	379.33	Sigma-Aldrich	316512-100G	100g	Solid	09901TS	RT	99.999% trace metals basis	MIA007A	MIB018A	MIC021A	MID310A
											MIA008B	MIB011B	MIC210B	MID037B
											MIA001C	MIB110C	MIC027C	MID038C
4	Zinc dimethyl/dithiocarbamate (DMDTC)	137-30-4	305.82	Kanto Chemical	48028-31	25g x2	Solid	403N2204	RT	>95.0% (T)	MIA009A	MIB110A	MIC023A	MID037A
											MIA010B	MIB013B	MIC027B	MID039B
											MIA003C	MIB017C	MIC029C	MID310C
											MIA001A	MIB012A	MIC025A	MID034A
5	Nickel (II) sulfate hexahydrate	10101-97-0	262.85	Wako	146-01171	100g	Solid	LKQ2263	RT	99.0-102.0% (as NiSO4 · 6H2O) (Titration)	MIA002B	MIB015B	MIC024B	MID031B
											MIA005C	MIB014C	MIC021C	MID032C

MITA(phase2) coded chemicals

	Chemical	Cas.no.	LabA TOHOKU univ.	LabB AIST-TSUKUBA	LabC FDSC	LabD AIST-SHIKOKU	Note	State	Storage	Supplier	Lot
1	2,4-Diaminotoluene	95-80-7	MIA401	MIB515	MIC618	MID702	Deleterious	S	RT	Wako	CDF0347
2	Benzo(a)pyrene	50-32-8	MIA413	MIB516	MIC601	MID703		S	RT	TCI	M8DFD
3	Cadmium chloride	10108-64-2	MIA403	MIB502	MIC602	MID714	Deleterious	S	RT	Wako	DEE3332
4	Dibromoacetic acid	631-64-1	MIA406	MIB518	MIC610	MID720		S	RT	ALDRICH	BCBR5175V
5	Diethylstilbestol	56-53-1	MIA420	MIB509	MIC611	MID711		S	RT	SIGMA	BCBR9766V
6	Diphenylhydantoin	630-93-3	MIA412	MIB510	MIC615	MID704		S	RT	SIGMA	SLBB3874
7	Ethylene dibromide	106-93-4	MIA407	MIB507	MIC605	MID705	Deleterious	L	RT	Wako	KWG5479
8	Glycidol	556-52-5	MIA408	MIB505	MIC607	MID712		L	2-8℃	ALDRICH	MKBX5752V
9	Indomethacin	53-86-1	MIA409	MIB508	MIC609	MID715		S	RT	SIGMA	122K0718
10	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	MIA411	MIB517	MIC612	MID707		S	RT	Fluka	SLBF8371V
11	Nitrobenzene	98-95-3	MIA402	MIB519	MIC603	MID701	Deleterious	L	RT	Sigma-Aldrich	SHBG5577V
12	Urethane, Ethyl carbamate	51-79-6	MIA415	MIB520	MIC604	MID719		S	RT	Sigma-Aldrich	WXBC3505V
13	Tributyltin chloride	1461-22-9	MIA404	MIB506	MIC613	MID713	Deleterious	L	RT	TCI	2442A-1Q
14	Perfluorooctanoic acid	335-67-1	MIA414	MIB514	MIC614	MID718		S	RT	TCI	O3U70
15	Dichloroacetic acid	79-43-6	MIA416	MIB511	MIC606	MID716	Deleterious	L	RT	Sigma-Aldrich	SHBH3492V
16	Toluene	108-88-3	MIA417	MIB512	MIC616	MID706	Deleterious	L	RT	Sigma-Aldrich	J5136
17	Acetonitril	75-05-8	MIA405	MIB501	MIC617	MID708	Deleterious	L	RT	Wako	KWH4805
18	Mannitol	69-65-8	MIA418	MIB503	MIC619	MID717		S	RT	Wako	LKP4362
19	Vanadium pentoxide	1314-62-1	MIA419	MIB504	MIC608	MID709	Deleterious	S	RT	Wako	SAE6958
20	o-Benzyl-p-chlorophenol	120-32-1	MIA410	MIB513	MIC620	MID710		S	RT	Wako	KPQ0988

positive
negative

Appendix 15. The list of proficiency chemicals

The list of proficiency chemicals

No.	Chemical name	CAS No.	T cell targeting	Physical state	Phase
1	Dibutyl phthalate	84-74-2	Yes	Liquid	I
2	Lead(II) acetate trihydrate	6080-56-4	Yes	Solid	I
3	Nickel (II) sulfate hexahydrate	10101-97-0	Yes	Solid	I
4	Benzo(a)pyrene	50-32-8	Yes	Solid	II
5	Diethylstilbestrol	56-53-1	Yes	Solid	II
6	Urethane, Ethyl carbamate	51-79-6	Yes	Solid	II
7	Tributyltin chloride	1461-22-9	Yes	Liquid	II
8	2,4-diaminotoluene	95-80-7	NO	Solid	II
9	Acetonitril	75-05-8	NO	Liquid	II
10	Vanadium pentoxide	1314-62-1	NO	Solid	II

Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Appendix 15 in compliance with the Good in vitro Method Practices (1). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 15) and with the positive and solvent/vehicle controls (see paragraphs 21-24), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

1. OECD (2017), Draft Guidance document: Good *In Vitro* *€i0Method Practices (GIVIMP) for the Development and Implementation of In Vitro €i0Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf]*.

Appendix 16. The list of performance standard chemicals

No.	Chemical name	CAS No.	T cell targeting	Physical state	Phase
1	Dexamethasone	50-02-2	Yes	Solid	positive control
2	Cyclosporine	59865-13-3	Yes	Solid	-
3	Indomethacin	53-86-1	Yes	Solid	II
4	Perfluorooctanoic acid	335-67-1	Yes	Solid	II
5	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	No	Solid	I
6	Mannitol	69-65-8	No	Solid	II

Performance standards (PS) (15) are shown to facilitate the validation of modified in vitro IL-2 luciferase test methods similar to the IL-2 Luc assay and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD.