Evaluation and Interpretation of Results

59. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if:

a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control,

b) the increase is dose-related when evaluated with an appropriate trend test,

c) any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations.

When all of these criteria are met, the test chemical is then considered able to induce DNA strand breakage in the tissues studied in this test system. If only one or two of these criteria are satisfied, see paragraph 62.

60. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test

c) all results are inside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations

d) direct or indirect evidence supportive of exposure of, or toxicity to, the target tissue(s) has been demonstrated.

The test chemical is then considered unable to induce DNA strand breakage in the tissues studied in this test system.

61. There is no requirement for verification of a clearly positive or negative response.

62. In case the response is neither clearly negative nor clearly positive (i.e. not all the criteria listed in paragraphs 59 or 60 are met) and in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations conducted, if scientifically justified. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using optimised experimental conditions (e.g. dose spacing, other routes of administration, other sampling times or other tissues) could be useful.

63. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.

64. To assess the biological relevance of a positive or equivocal result, information on cytotoxicity at the target tissue is required (see paragraphs 54-55). Where positive or equivocal findings are observed solely in the presence of clear evidence of cytotoxicity, the study would be concluded as equivocal for genotoxicity unless there is enough information that is supportive of a definitive conclusion. In cases of a negative study outcome where there are signs of toxicity at all doses tested, further study at non-toxic doses may be advisable.

Test Report

65. The test report should include the following information:

Test chemical:
- source, lot number if available;
- stability of the test chemical, limit date for use, or date for re-analysis if known;

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/vehicle:
- justification for choice of solvent/vehicle;
- solubility and stability of the test chemical in the solvent/vehicle, if known;
- preparation of dose formulations;
- analytical determinations on formulations (e.g., stability, homogeneity, nominal concentrations);

Test animals:
- species/strain used and scientific and ethical justifications for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, enrichment, etc.;
- individual weight of the animals at the start and at the end of the test, including body weight range, mean and standard deviation for each group;

Test conditions:
- positive and negative (vehicle/solvent) control data;
- results from the range-finding study (if conducted);
- rationale for dose level selection;
- details of test chemical preparation;
- details of the administration of the test chemical;
- rationale for route of administration;
- site of injection (for subcutaneous or intravenous studies);
- methods for sample preparation, where available, histopathological analyses, especially for a substance giving a positive comet response;
- rationale for tissue selection;
- methods for verifying that the test chemical reached the target tissue, or general circulation, if negative results are obtained;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test chemical concentration (ppm) and consumption, if applicable;
- details of diet and water quality;

- detailed description of treatment and sampling schedules and justifications for the choices (e.g. toxicokinetic data, where available);
- method of pain relief, analgesia;
- method of euthanasia;
- procedures for isolating and preserving tissues;
- methods for preparing single cell/nucleus suspension;
- source and lot numbers of all reagents (where possible);
- methods for evaluating cytotoxicity;
- electrophoresis conditions;
- staining techniques used; and
- methods for scoring and measuring comets;

Results:

- General clinical observations, if any, prior to and throughout the test period for each animal;
- evidence of cytotoxicity if performed;
- for studies longer than one week: Individual body weights during the study, including body weight range, mean and standard deviation for each group; food consumption;
- dose-response relationship, where evident;
- for each tissue/animal, the % tail DNA (or other measures, if chosen) and median values per slide, mean values per animal and mean values per group;
- concurrent and historical negative control data with ranges, means/medians and standard deviations for each tissue evaluated;
- concurrent and historical positive control data;
- for tissues other than liver, a dose-response curve using the positive control. This can be from data collected during the demonstration of proficiency (see paragraphs 16-17) and should be accompanied by a justification, with citations to current literature, for the appropriateness of the magnitude and scatter of the responses to the controls in that tissue;
- statistical analyses and methods applied; and
- criteria for considering a response as positive, negative or equivocal;
- frequency of hedgehogs in each group and per animal;

Discussion of the results

Conclusion

References

LITERATURE

(1) Kirkland, D., G. Speit (2008), Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*, *Mutation Research*, Vol. 654/2, pp. 114-32.

(2) Brendler-Schwaab, S. et al. (2005), The *in vivo* Comet assay: use and status in genotoxicity testing, *Mutagenesis*, Vol. 20/4, pp. 245-54.

(3) Burlinson, B. et al. (2007), Fourth International Workgroup on Genotoxicity Testing: result of the *in vivo* Comet assay workgroup, *Mutation Research*, Vol. 627/1, pp. 31-5.

(4) Burlinson, B. (2012), The *in vitro* and *in vivo* Comet assays, *Methods in Molecular Biology*, Vol. 817, pp. 143-63.

(5) Smith, C.C, et al. (2008), Recommendations for design of the rat Comet assay, *Mutagenesis*, Vol. 23/3, pp. 233-40.

(6) Hartmann, A. et al. (2003), Recommendations for conducting the *in vivo* alkaline Comet assay, *Mutagenesis*, Vol. 18/1, pp. 45-51.

(7) McKelvey-Martin, V.J. et al (1993), The single cell gel electrophoresis assay (Comet assay): a European review, *Mutation Research*, Vol. 288/1, pp. 47-63.

(8) Tice, R.R. et al. (2000), Single cell gel/Comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, *Environmental and Molecular Mutagenesis*, Vol. 35/3, pp. 206-21.

(9) Singh, N.P. et al. (1988), A simple technique for quantitation of low levels of DNA damage in individual cells, *Experimental Cell Research*, Vol. 175/1, pp. 184-91.

(10) Rothfuss, A. et al. (2010), Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies, *Mutation Research*, Vol., 702/1, pp. 40-69.

(11) OECD (2015), "Introduction document to the OECD Test Guidelines on genotoxicity", OECD Publishing, Paris. In preparation.

(12) OECD (2014), *Reports of the JaCVAM initiative international pre-validation and validation studies of the in vivo rodent alkaline comet assay for the detection of genotoxic carcinogens*, Series on Testing and Assessment, Nos. 195 and 196, OECD Publishing, Paris.

(13) Olive, P.L., J.P. Banath, R.E. Durand (1990), Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells using the "Comet" assay, *Radiation Research*, Vol. 122/1, pp. 86-94.

(14) Tice, R.R., G.H. Strauss (1995), The single cell gel electrophoresis/Comet assay: a potential tool for detecting radiation-induced DNA damage in humans, *Stem Cells*, Vol. 13/1, pp. 207-14.

(15) Collins, A.R (2004), The Comet assay for DNA damage and repair: principles, applications, and limitations, *Molecular Biotechnology*, Vol. 26/3, pp. 249-61.

(16) Rothfuss, A. et al. (2011), Improvement of *in vivo* genotoxicity assessment: combination of acute tests and integration into standard toxicity testing, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 723/2, pp. 108-20.

(17) Kushwaha, S. et al. (2010), Evaluation of multi-organ DNA damage by Comet assay from 28 days repeated dose oral toxicity test in mice: A practical approach for test integration in regulatory toxicity testing, *Regulatory Toxicology and Pharmacology*, Vol. 58/1, pp. 145–54.

(18) Vasquez, M.Z. (2010), Combining the *in vivo* Comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation, *Mutagenesis*, Vol. 25/2, pp. 187-99.

(19) Bowen, D.E. (2011), Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the Comet assay and the flow-cytometric peripheral blood micronucleus test, *Mutation Research*, Vol. 722/1, pp. 7-19.

(20) Recio, L. et al. (2010), Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and Comet assay protocol, *The Journal of Toxicological Science*, Vol. 35/2, pp. 149-62.

(21) O'Donovan, M., B. Burlinson (2013), Maximum dose levels for the rodent comet assay to examine damage at the site of contact or to the gastrointestinal tract, *Mutagenesis*, Vol. 28/6, pp. 621-3.

(22) Hartmann, A. (2004), Use of the alkaline *in vivo* Comet assay for mechanistic genotoxicity investigations, *Mutagenesis*, Vol. 19/1, pp. 51-9.

(23) Nesslany, F. (2007), *In vivo* Comet assay on isolated kidney cells to distinguish genotoxic carcinogens from epigenetic carcinogens or cytotoxic compounds, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 630/1, pp. 28-41.

(24) Brendler-Schwaab, S.Y., B.A. Herbold (1997), A new method for the enrichment of single renal proximal tubular cells and their first use in the Comet assay, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*,Vol. 393/1-2, pp. 175-8.

(25) Toyoizumi, T. et al. (2011), Use of the *in vivo* skin Comet assay to evaluate the DNA-damaging potential of chemicals applied to the skin, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 726/2, pp. 175-80.

(26) Struwe, M. et al. (2008), Detection of photogenotoxicity in skin and eye in rat with the photo Comet assay, *Photochemical and Photobiological Sciences*, Vol. 7/2, pp. 240-9.

(27) Wada, K. et al. (2012), A comparison of cell-collecting methods for the Comet assay in urinary bladders of rats, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 742/1-2, pp. 26-30.

(28) Wang, A. et al. (2007), Measurement of DNA damage in rat urinary bladder transitional cells: improved selective harvest of transitional cells and detailed Comet assay protocols, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 634/ 1-2, pp. 51-9.

(29) Burlinson, B. et al. (2007), *In Vivo* Comet Assay Workgroup, part of the Fourth International Workgroup on Genotoxicity Testing. Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 627/1, pp. 31-5.

(30) Jackson, P. et al. (2012), Pulmonary exposure to carbon black by inhalation or instillation in pregnant mice: effects on liver DNA strand breaks in dams and offspring, *Nanotoxicology*, Vol. 6/5, pp. 486-500.

(31) Sasaki, Y.F. et al. (2000), The comet assay with multiple mouse organs: comparison of Comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database, *Critical Reviews in Toxicology*, Vol. 30/6, pp. 629-799.

(32) Sekihashi, K. et al. (2002), Comparative investigations of multiple organs of mice and rats in the Comet assay, *Mutation Research*, Vol. 517/1-2, pp. 53-74.

(33) Speit, G, M. Vasquez, A. Hartmann (2009), The comet assay as an indicator test for germ cell genotoxicity, *Mutation Research*, Vol. 681/1, pp. 3-12.

(34) Zheng, H., P.L. Olive (1997), Influence of oxygen on radiation-induced DNA damage in testicular cells of C3H mice, *International Journal of Radiation Biology*, Vol. 71/3, pp. 275-282.

(35) Cordelli, E. et al. (2003), Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation, *Journal of Radiation Research*, Vol. 160/4, pp. 443-451.

(36) Merk, O., G. Speit (1999), Detection of crosslinks with the Comet assay in relationship to genotoxicity and cytotoxicity, *Environmental and Molecular Mutagenesis*, Vol. 33/2, pp. 167-72.

(37) Pfuhler, S., H.U. Wolf (1996), Detection of DNA-crosslinking agents with the alkaline Comet assay, *Environmental and Molecular Mutagenesis*, Vol. 27/3, pp. 196-201.

(38) Wu, J.H., N.J. Jones (2012), Assessment of DNA interstrand crosslinks using the modified alkaline Comet assay, *Methods in Molecular Biology*, Vol. 817, pp. 165-81.

(39) Spanswick, V.J., J.M. Hartley, J.A. Hartley (2010), Measurement of DNA interstrand crosslinking in individual cells using the Single Cell Gel Electrophoresis (Comet) assay, *Methods in Molecular Biology*, Vol. 613, pp. 267-282.

(40) Kumaravel, T.S., A.N. Jha (2006), Reliable Comet assay measurements for detecting DNA damage induced by ionizing radiation and chemicals, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Vol. 605(1-2), pp. 7-16.

(41) Burlinson, B.et al. (2007), Fourth International Workgroup on Genotoxicity Testing: result of the *in vivo* Comet assay workgroup, *Mutation Research*, Vol.627/1, pp. 31-5.

(42) Kumaravel, T.S. et al. (2009), Comet Assay measurements: a perspective, *Cell Biology and Toxicology*, Vol. 25/1, pp. 53-64.

(43) Ersson, C., L. Möller (2011), The effects on DNA migration of altering parameters in the Comet assay protocol such as agarose density, electrophoresis conditions and durations of the enzyme or the alkaline treatments, *Mutagenesis*, Vol. 26/6, pp. 689-95.

(44) Møller, P. et al. (2010), Assessment and reduction of Comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group, *Mutagenesis*, Vol. 25/2, pp. 109-11.

(45) Forchhammer, L. et al. (2010), Variation in the measurement of DNA damage by Comet assay measured by the ECVAG inter-laboratory validation trial, *Mutagenesis*, Vol. 25/2, pp. 113-23.

(46) Azqueta, A. et al. (2011), Towards a more reliable comet assay: Optimising agarose concentration, unwinding time and electrophoresis conditions, *Mutation Research*, Vol. 724/1-2, pp. 41-45.

(47) Hayashi, M. et al. (2011), Compilation and use of genetic toxicity historical control data, *Mutation Research*, Vol. 723/2, pp. 87-90.

(48) Ryan, T. P. (2000), *Statistical Methods for Quality Improvement,* John Wiley and Sons, New York 2nd ed.

(49) Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123)

(50) OECD (2009), *Test No. 412: Subacute Inhalation Toxicity: 28-Day Study*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

(51) OECD (2009), *Test No. 413: Subchronic Inhalation Toxicity: 90-day Study*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

(52) Blakey, D.H., G.R. Douglas (1984), Transient DNA lesions induced by benzo[a]pyrene in Chinese hamster ovary cells, *Mutation Research*, Vol. 140/2-3, pp. 141-45.

(53) Blakey, D.H., G.R. Douglas (1990), The role of excision repair in the removal of transient benzo[a]pyrene-induced DNA lesions in Chinese hamster ovary cells, *Mutation Research*, Vol. 236/1, pp. 35-41.

(54) OECD (2002), "Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 19, OECD Publishing, Paris.

(55) Nakajima, M. (2012), Tissue sample preparation for *in vivo* rodent alkaline Comet assay, *Genes and Environment*, Vol. 34/1, pp. 50-4.

(56) Hartmann, A. et al. (2003), Recommendations for conducting the in vivo alkaline Comet assay, *Mutagenesis*, Vol.18/1, pp.45–51.

(57) Atlas of Comet Assay Images, Scientist Press Co., Ltd., Tokyo, Japan.

(58) Lovell, D.P., G. Thomas, R. Dubow (1999), Issues related to the experimental design and subsequent statistical analysis of *in vivo* and *in vitro* Comet studies, *Teratogenesis Carcinogenesis Mutagenesis*, Vol. 19/2, pp. 109-19.

(59) Wiklund, S.J., E. Agurell (2003), Aspects of design and statistical analysis in the Comet assay, *Mutagenesis*, Vol. 18/2, pp. 167-75.

(60) Bright, J. et al. (2011), Recommendations on the statistical analysis of the Comet assay, *Pharmaceutical Statistics*, Vol. 10/6, pp. 485-93.

(61) Lovell, D.P., T. Omori (2008), Statistical issues in the use of the Comet assay, *Mutagenesis*, Vol. 23/3, pp. 171-82.

ANNEX 1

Definitions

Alkaline single cell gel electrophoresis: Sensitive technique for the detection of primary DNA damage at the level of individual cell/nucleus

Comet: The shape that nucleoids adopt after submitted to one electrophoretic field, due to its similarity to comets: the head is the nucleus and the tail is constituted by the DNA migrating out of the nucleus in the electric field.

A critical variable/parameter: This is a protocol variable for which a small change can have a large impact on the conclusion of the assay. Critical variables can be tissue-specific. Critical variables should not be altered, especially within a test, without consideration of how the alteration will alter an assay response, for example as indicated by the magnitude and variability in positive and negative controls. The test report should list alterations of critical variables made during the test or compared to the standard protocol for the laboratory and provide a justification for each alteration.

Tail intensity or % tail DNA: This corresponds to the intensity of the comet tail relative to the total intensity (head plus tail). It reflects the amount of DNA breakage, expressed as a percentage.

ANNEX 2

The Factorial Design for Identifying Sex Differences in the *in vivo* Comet Assay

The factorial design and its analysis

1. In this design, a minimum of 5 males and 5 females are tested at each concentration level resulting in a design using a minimum of 40 animals (20 males and 20 females, plus relevant positive controls.)

2. The design, which is one of the simpler factorial designs, is equivalent to a two-way analysis of variance with sex and concentration level as the main effects. The data can be analysed using many standard statistical software packages such as SPSS, SAS, STATA, Genstat as well as using R.

3. The analysis partitions the variability in the dataset into that between the sexes, between the concentrations and that related to the interaction between the sexes and the concentrations. Each of the terms is tested against an estimate of the variability between the replicate animals within the groups of animals of the same sex given the same concentration. Full details of the underlying methodology are available in many standard statistical textbooks (see references) and in the 'help' facilities provided with statistical packages.

4. The analysis proceeds by inspecting the sex x concentration interaction term in the ANOVA table[1]. In the absence of a significant interaction term the combined values across sexes or across concentration levels provide valid statistical tests between the levels based upon the pooled within group variability term of the ANOVA.

5. The analysis continues by partitioning the estimate of the between concentrations variability into contrasts which provide for a test for linear and quadratic contrasts of the responses across the concentration levels. When there is a significant sex x concentration interaction this term can also be partitioned into linear x sex and quadratic x sex interaction contrasts. These terms provide tests of whether the concentration responses are parallel for the two sexes or whether there is a differential response between the two sexes.

6. The estimate of the pooled within group variability can be used to provide pair-wise tests of the difference between means. These comparisons could be made between the means for the two sexes and between the means for the different concentration level such as for comparisons with the negative control levels. In those cases where there is a significant interaction comparisons can be made between the means of different concentrations within a sex or between the means of the sexes at the same concentration.

[1] Statisticians who take a modelling approach such as using General Linear Models (GLMs) may approach the analysis in a different but comparable way but will not necessarily derive the traditional ANOVA table which dates back to algorithmic approaches to calculating the statistics developed in a pre-computer age.

References

There are many statistical textbooks which discuss the theory, design, methodology, analysis and interpretation of factorial designs ranging from the simplest two factor analyses to the more complex forms used in Design of Experiment methodology. The following is a non-exhaustive list. Some books provide worked examples of comparable designs, in some cases with code for running the analyses using various software packages.

Box, G.E.P, Hunter, W.G. and Hunter, J.S. (1978). Statistics for Experimenters. An Introduction to Design, Data Analysis, and Model Building. New York: John Wiley & Sons.

Box G.E.P. & Draper, N.R. (1987) Empirical model-building and response surfaces. John Wiley & Sons Inc.

Doncaster, C.P. & Davey, A.J.H. (2007) Analysis of Variance and Covariance: How to choose and Construct Models for the Life Sciences. Cambridge University Press.

Mead, R. (1990) The Design of Experiments. Statistical principles for practical application. Cambridge University Press.

Montgomery D.C. (1997) Design and Analysis of Experiments. John Wiley & Sons Inc.
Winer, B.J. (1971) Statistical Principles in Experimental Design. McGraw Hill.

Wu, C.F.J & Hamada, M.S. (2009) Experiments: Planning, Analysis and Optimization. John Wiley & Sons Inc.

ANNEX 3

Current Limitations of the Assay

Due to the current status of knowledge, several limitations are associated with the *in vivo* comet assay. It is expected that these limitations will be reduced or more narrowly defined as there is more experience with application of the assay to answer safety issues in a regulatory context.

1. Some types of DNA damage may be short-lived, i.e. may be repaired too quickly to be observed 24 hours or more after the last dose. There is no identifiable list of the types of short-lived damages, nor of the substances which are likely to cause this type of damage, nor is it known over what time period this type of damage can be detected. The optimum sampling time(s) may also be substance- or route-specific and sampling times should be determined from kinetic data (for example the time, T_{max}, at which the peak plasma or tissue concentration is achieved), when such data are available. Most of the validation studies supporting this guideline specified necropsy 2 or 3 hours following administration of the final dose. Most studies in the published literature describe administration of the final dose between 2 and 6 hours prior to sacrifice. Therefore these experiences were used as the basis for the recommendation in the test guideline that, in the absence of data indicating otherwise, the final dose should be administered at a specified time point between 2 and 6 hours prior to necropsy.

2. There are no identifiable study data that examine the sensitivity of the test for the detection of short-lived DNA damage following administration in food or drinking water compared to administration by gavage. DNA damage has been detected following administration in feed and drinking water, but there are relatively few such reports compared to the much greater experience with gavage and i.p. administration. Thus the sensitivity of the assay may be reduced for substances which induce short-lived damage administered through feed or drinking water.

3. No inter-laboratory studies have been conducted in tissues other than liver and stomach, therefore no recommendation has been established for how to achieve a sensitive and reproducible response in tissues other than liver, such as expected positive and negative control ranges. For the liver, agreement on setting a lower limit to the negative control value also could not be reached.

4. Although there are several publications demonstrating the confounding effect of cytotoxicity *in vitro*, very little data have been published *in vivo* and therefore no single measure of cytotoxicity could be recommended. Histopathological changes such as inflammation, cell infiltration, apoptotic or necrotic changes have been associated with increases in DNA migration however, as demonstrated by the JaCVAM validation trial (OECD, 2014), these changes do not always result in positive comet findings and consequently no definitive list of histopathological changes that are always associated with increased DNA migration is available. Hedgehogs (or clouds, ghost cells) have previously been suggested as an indicator of cytotoxicity, however, the etiology of the hedgehogs is uncertain. Data exist which suggest that they can be caused by substance-related cytotoxicity, mechanical/enzyme-induced damage initiated during sample preparation (Guerard et al., 2014) and/or a more extreme effect of test chemical genotoxicity. Other data seem to show they are due to extensive, but perhaps repairable DNA damage (Lorenzo et al., 2013).

5. Tissues or cell nuclei have been successfully frozen for later analysis. This usually results in a measurable effect on the response to the vehicle and positive control (Recio at al., 2010; Recio at al., 2012; Jackson at al., 2013). If used, the laboratory should demonstrate competency in freezing methodologies and confirm acceptable low ranges of % tail DNA in target tissues of vehicle

treated animals, and that positive responses can still be detected. In the literature, the freezing of tissues has been described using different methods. However, currently there is no agreement on how to best freeze and thaw tissues, and how to assess whether a potentially altered response may affect the sensitivity of the test.

6. Recent work demonstrates that the list of critical variables is expected to continue to become shorter and the parameters for critical variables more precisely defined (Guerard et al., 2014).

References

Guerard, M., C. Marchand, U. Plappert-Helbig (2014), Influence of Experimental Conditions on Data Variability in the Liver Comet Assay, *Environmental and Molecular Mutagenesis*, Vol. 55/2, pp. 114-21.

Jackson, P. et al. (2013), Validation of use of frozen tissues in high-throughput comet assay with fully-automatic scoring, *Mutagenesis*, Vol. 28/6, pp. 699-707.

Lorenzo ,Y. et al. (2013), The comet assay, DNA damage, DNA repair and cytotoxicity: hedgehogs are not always dead, *Mutagenesis*, Vol. 28/4, pp. 427-32.

OECD (2014), *Reports of the JaCVAM initiative international pre-validation and validation studies of the in vivo rodent alkaline comet assay for the detection of genotoxic carcinogens*, Series on Testing and Assessment, Nos. 195 and 196, OECD Publishing, Paris.

Recio L, Hobbs C, Caspary W, Witt KL, (2010), Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and Comet assay protocol, *J. Toxicol. Sci.* 35:149-62.

Recio, L. et al. (2012), Comparison of Comet assay dose-response for ethyl methanesulfonate using freshly prepared versus cryopreserved tissues, *Environmental and Molecular Mutagenesis*, Vol. 53/2, pp. 101-13.

厚生科学研究費補助金(化学物質リスク研究事業)
新規の安全性評価試験法を国際的なガイドラインにするための手法に関する研究
平成 26 年度　分担研究報告書

内分泌かく乱化学物質試験法のバリデーション

研究分担者　小野　敦　国立医薬品食品衛生研究所・総合評価研究室

研究要旨

本研究では、OECD-EDTA で提案された化学物質の内分泌かく乱性評価のコンセプチュアルフレームワークのレベル 2 に示されている in vitro スクリーニング試験法として行政的有用性が期待される試験法について、国内外の研究機関との協力により多施設国際バリデーションを実施し、得られた結果より信頼性・再現性の評価を行い OECD ガイドライン提案することを目的とした。我が国で開発された HeLa9903 細胞を用いたエストロジェン受容体α(ERα)転写活性化試験法（ER STTA 法）のアンタゴニスト試験法については、バリデーション試験結果をもとに定性的評価法として OECD にガイドライン（TG455）アップデート案及びアンタゴニスト試験法パフォーマンススタンダード案の提案を行い、平成 26 年度末にガイドラインが成立する見込みである。一方、AR EcoScreen 細胞を用いたアンドロゲン受容体転写活性化法（AR STTA 法）については、OECD ピアレビューにより要求された追加バリデーション試験を実施し、非常に再現性の良い結果を得た。得られた結果をもとに、バリデーションレポート案及び OECD 新規ガイドライン案を作成して OECD に提出した。

A. 研究目的

本研究では、化学物質による内分泌かく乱性評価のための国際的な枠組みとして OECD 内分泌かく乱物質評価タスクフォース(EDTA:Task Force on Endocrine Disrupters Testing and Assessment)により示された 5 段階からなるコンセプチュアルフレームワークのレベル 2 に分類される in vitro 試験法である転写活性化試験法のうち、行政的有用性が期待される試験法として、我が国で開発された HeLa 細胞をベースにしたエストロジェン受容体α(ERα)に対する試験法（ER STTA 法）及び AR EcoScreen 細胞を用いたアンドロゲン受容体転写活性化試験法（AR STTA 法）について、国内外の研究機関と協力してバリデーション試験を実施し、得られた結果から信頼性や再現性が確認された試験法を OECD ガイドラインとして提案することを目的として研究を進めた。

B. 研究方法

1）ER STTA 法

ER STTA 法によるアゴニスト試験法については、既に OECD ガイドライン（TG455）が成立しており、本研究ではアンタゴニスト試験法のバリデーション試験の取り纏め及びガイドライン案作成を行った。これまでに、試験プロトコールを開発した化学物質評価研究機構（CERI）をリードラボとして、3 タスクからなるバリデーション試験デザインに従ってバリデーション試験を実施し、予定したバリデーション測定を全て終了した。得られた結果の詳細解析を行い、SMT においてクライテリアの変更等について検討を行った。平成 26 年度は、バリデーション

レポート及びガイドライン案、パフォーマンススタンダード案を作成して OECD に提出し、WNT レビューコメントへの対応について OECD VMG-NA 会議において同意された内容を反映した修正稿を OECD に再提出した。

2）AR STTA 法

AR STTA 法については、本研究班開始以前に実施された国内 3 施設によるバリデーション試験結果を基にしたバリデーションリポートのピアレビューにおいて測定化合物数が少ないことから追加バリデーション試験の実施が要求された。これまでに本研究では、OECD VMG-NA メンバーの協力を得て追加測定化合物の選定、バリデーション試験計画を策定するとともに、試験管理グループ（SMT）を組織して追加バリデーション試験を実施してきた。平成 26 年度は、昨年度終了したバリデーション試験結果の解析を行い、得られた結果をもとにバリデーションリポート及び OECD ガイドライン案の作成を行い、OECD に提出した。

倫理面への配慮

本研究は動物実験に替わる新しい *in vitro* 安全性試験法の開発を主とするものである。本研究では動物を用いる試験、ヒト臨床試験やヒト由来試料を利用した試験は行っていない。

C. 研究結果

1）ER STTA 法

ER STTA アンタゴニスト試験法バリデーションは、当初、国内 3 施設、海外 2 施設の参加により開始されたが、海外 2 施設においては全てのタスクが終了する前に試験継続が困難となった。また国内 1 施設では予定した測定を終了したものの、リファレンスクライテリアを完全に満たすデータの取得は出来なかった。そこで、さらに国内の別の 1 施設の参加を得て試験を継続したものの、追加参加した施設においても、コード化化合物の作用判定においては再現性を示す結果を得たものの、リファレンスクライテリアを完全に満たすデータを取得することは出来なかった（表 1）。バリデーション試験結果の詳細解析から、リファレンスクライテリアは定量評価の信頼性を保証するために設定されたもので、リファレンスクライテリアを逸脱した結果であっても、プレート採用基準を満たしていれば定性的評価においては信頼性が示されたと判断された（表 2）。このことからアンタゴニスト試験法については、当初設定したクライテリアの変更等を行ったうえで定性的評価を行う系としてガイドライン化する方針について SMT 及び OECD VMG-NA 会議で合意された。合意内容に従い、バリデーションレポート案及びガイドライン（TG）案、パフォーマンススタンダード（PS）案を作成した。ER STTA アンタゴニスト試験法については、既に成立しているアゴニスト試験法ガイドライン(TG455)提案時のレビューにおいて追加が要求されたものであることから、バリデーションレポートについては、既に提出済みのアゴニスト試験法バリデーションレポートを part A として、本研究班で作成したアンタゴニスト試験法バリデーションレポートを part B として、TG 案について TG455 のアップデートとして、PS 案については、アゴニスト試験法の PS とは独立した案として提案を行った。OECD 提出後、WNT レビューにより TG 案、PS 案に対して米国、日本及び英国よりコメントが出された。いずれのコメントも主に記載の修正等であったため、コメントを反映した TG、PS 修正案について OECD VMG-NA 会議では、更なるコメント募集を行わず、次回の WNT で承認を得る方針で合意された。また、現在の TG455 は、ER STTA 法と TG457 として成立している BG1luc 法のアゴニスト試験法のみの PBTG（パフォーマンスベーステストガイドライン）であり、アンタゴニスト試験法を追加した TG455 アップデートの成

立後、TG457を廃止することがVMG-NA会議で合意された（図1）。

2）AR STTA法

AR STTA試験法については、過去に実施されたバリデーション試験報告に対してOECDピアレビューによりリファレンス化合物となる化合物数が少ないことから追加バリデーションが要求された。そこで、追加バリデーションに先立って、OECD VMG-NAメンバーの協力により被験物質（アゴニスト・アンタゴニスト各5物質）及びリファレンスコントロールとして設定されていたR1881が入手困難であることから代替物質の選定を行った。次に、本研究班が中心となってVMG-NAメンバーから構成されるSMTを組織して試験計画を策定し、国内3施設、海外1施設（韓国）の参加により2フェーズからなるバリデーション試験を実施し、ほぼ計画通り終了した。バリデーション試験結果の解析により、いずれの参加施設とも非常に再現性（施設内、施設間とも）の良い結果が得られたことにより、本試験系の信頼性・再現性が確認された（図2, 3）。バリデーション試験で明らかになった問題点として、フェーズ2で実施したコード化化合物のアンタゴニスト測定結果（図3）において、溶解性試験により施設ごとに設定した最高測定濃度が低かったため、陽性判定されるべき被験物質について陰性の結果となった施設があった。同施設でより高濃度での追加測定を実施した結果、他施設と同じ結果を得た（図4）ことから、明らかに測定濃度設定の問題であり、最高濃度設定の注意点として、多少の析出が認められても出来るだけ高い濃度での測定実施を行うようプロトコールに記載することとなった。得られた結果をもとにバリデーションレポート案を作成し、VMG-NA会議において報告を行ったが、レポート提出が会議直前であったため、レポートそのものについては詳細な議論は行われなかったが、バリデーション試験結果については、非常に良い結果を得ていることから、早急にガイドライン案を作成してOECDへ提出することが合意された。なお、昨年度のVMG-NA会議において事務局より作成が要求されたパフォーマンススタンダードについては、バリデーションが終了している系が他にないことから、現時点では作成する必要はないこととなったため、バリデーションレポート案及びガイドライン案を作成してOECD事務局に提出した。参考資料としてOECDに提出したバリデーションリポート案及びテストガイドライン案を添付した（参考資料1, 2）。

D. 考察

本研究では、OECDにおいて化学物質による内分泌かく乱性評価のための試験法として必要とされているin vitro試験法である転写活性化試験法として、いずれも我が国で開発された、エストロゲン受容体（ER）、アンドロゲン受容体（AR）の転写活性化試験法（STTA法）について、バリデーション試験の取り纏めを行い、得られた結果をもとに信頼性・再現性が示された手法についてOECDガイドライン化するための研究を進めてきた。

ER-STTAアンタゴニスト法については、一部の参加施設でクライテリアを満たす結果を得ることが出来ず、バリデーション試験終了は当初の計画より遅れたが、結果としてバリデーション試験実施によりアンタゴニスト試験法の再現性の問題が明らかとなったことは、本研究の成果である。最終的に定性的評価法としてプロトコールの修正を行った上でのガイドライン提案にSMT及びVMG-NAの同意が得られた。熟練した特定の施設でしかデータ取得出来ないクライテリアが設定された当初のプロトコールより、むしろ、試験実施の基本条件を満たす施設であれば実施可能な試験法である定性的評価のプロトコールは、OECDガイドラインとしてより適切であると考察された。本試験法については、

OECD にガイドライン（TG455）アップデート案及びアンタゴニスト試験法パフォーマンススタンダード案の提案を行い、平成 26 年度末にガイドラインが成立する見込みである。

一方、AR STTA 法については、OECD レビューにより要求された追加バリデーションを実施した。AR STTA 追加バリデーションにおいては、被験物質選定から SMT への参画など OECD VMG-NA メンバーからの多くの協力により実施された。参加施設の測定技術や試験法そのものの安定性から、非常に再現性の良い結果が得られバリデーション計画は、順調に進捗、終了することが出来た。参加施設の中には初めて本試験法を実施する施設も含まれており、結果は本系の安定性を示すものである。本系は培地交換無しにルシフェラーゼ活性測定が実施出来るようデザインされていることも本系の安定性に寄与しているものと思われる。本試験法についても、SMT メンバーの協力により、バリデーションレポート、OECD ガイドライン案を作成し、OECD へ提出した。バリデーションレポート案及びガイドライン案については、来年度、WNT コメント募集を行い、その結果をもとに、次回の VMG-NA 会議で最終化に向けた議論を行う予定をしている。

E. 結論

本研究で対象とした 2 つの試験系は、OECD において化学物質の内分泌かく乱性評価法としてガイドラインの整備が必要な試験法として取り上げられた試験法であるとともに、OECD としては初めてのレポーターアッセイ系をベースとしたガイドライン試験法である。新たな試験法のバリデーションやガイドライン化には、バリデーションの方法論だけではなく、当該試験系をよく理解した専門家の協力体制が不可欠であり、さらに OECD ガイドラインを始めとした国際ガイドライン化においては、関係各国の専門家を交えた議論が重要である。本研究で対象とした ER STTA 法、AR STTA 法はいずれも我が国で開発された試験法であるが、そのバリデーション試験実施やガイドライン化においては、内分泌かく乱性の in vitro 評価法のバリデーションについて議論するため設置された OECD 加盟各国の専門家会合である VMG-NA メンバーから助言・提案や SMT 参画等による非常に多くの協力を得ることが出来た。今後、新たな試験法のガイドライン化にあたっては、試験法そのもののリスク評価における有用性や信頼性が示されることはもとより、バリデーションからガイドライン化にいたるプロセスにおいて、各試験法に対応した協力体制の整備が重要である。

F. 研究発表

F-1. 論文発表

小野 敦 ; 効能の高い化粧品原料の安全性リスク評価に対する考え方; *Cosmetic stage,* 9,(1) 21–26 (2014)

H. Kato, S. Fujii, M. Takahashi, M. Matsumoto, M. Hirata-Koizumi, A. Ono and A. Hirose ; Repeated dose and reproductive/developmental toxicity of perfluorododecanoic acid in rats.; *Environ Toxicol,* (2014)

M. Ema, K. Endoh, R. Fukushima, S. Fujii, H. Hara, M. Hirata-Koizumi, A. Hirose, H. Hojo, M. Horimotoet.al. ; Historical control data on developmental toxicity studies in rodents.; *Congenit Anom,* 54, 150-161 (2014)

M. Takahashi, S. Ishida, M. Hirata-Koizumi, A. Ono and A. Hirose ; Repeated dose and reproductive/developmental toxicity of perfluoroundecanoic acid in rats.; *J Toxicol Sci,* 39,(1) 97–108 (2014)

F-2. 学会発表

A. Ono :Toxicogenomics as alternative of traditional toxicological endpoints. *Korean Society for*

Alternatives to Animal Experiments 11th Annual Meeting (2014.11, Korea)

A. Ono, M. Takeyoshi, Y. Maeda, N. Suzuki, H. Kojima, S. Takeuchi, J. Hong, H. Lee, A. Vinggaard, J. Odum, S. Laws and H. Kojima :Inter-laboratory validation study of Androgen receptor stably transfected transcriptional activation (STTA) assay for new OECD test guideline. *The 9th World Congress on Alternatives and Animal Use in the Life Sciences* (2014.8, プラハ、チェコ)

小野 敦 :トキシコゲノミクスによる肝毒性バイオマーカー. *第41回日本毒性学会学術年会* (2014.7, 神戸)

G．知的所有権の取得状況

G-１．特許取得

特になし

G-２．実用新案登録

特になし

G-３．その他

特になし

添付資料

参考資料 1 : 2nd Validation Study For Androgen Receptor (AR) Mediated Stably Transfected Transcriptional Activation (AR-STTA) Assay to Detect Androgenic and Anti-androgenic Activities: AR EcoScreen™

参考資料 2 : Draft OECD GUIDELINE FOR THE TESTING OF CHEMICALS: Stably Transfected Human Androgen Receptor- α Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals

表 1　ER STTA アンタゴニストバリデーション Task3 におけるリファレンスクライテリア測定値
（赤字はクライテリアを逸脱した項目）

lab	Task	ID	4OH-Tamoxifen		Tamoxifen		RU-486		Flutamide	
			IC30	IC50	IC30	IC50	IC30	IC50	IC30	IC50
			-9.58 ~ -8.63	-9.36 ~ -8.09	-7.68 ~ -6.37	-7.14 ~ -5.90	-6.10 ~ -5.41	-5.57 ~ -5.10		
CERI	Task3a	1	-9.542	-9.277	-7.573	-7.21	-6.115	-5.608	ND	ND
	Task3a	2	-8.958	-8.494	-7.003	-6.445	-5.761	-5.359	ND	ND
	Task3a	3	-9.038	-8.563	-6.982	-6.382	-5.721	-5.397	ND	ND
	Task3a	4	-9.129	-8.683	-7.358	-6.764	-6.095	-5.583	ND	ND
	Task3b	2	-9.058	-8.604	-6.655	-6.273	ND	ND	ND	ND
	Task3b	3	-9.128	-8.678	-7.036	-6.466	-5.815	-5.271	ND	ND
	Task3b	4	-9.16	-8.683	-7.055	-6.46	-5.907	-5.416	ND	ND
	Task3b	5	-8.895	-8.355	-6.777	-6.175	-5.691	-5.28	ND	ND
	Task3b	6	-9.05	-8.508	-7.149	-6.383	-5.843	-5.415	ND	ND
	Task3b	7	-8.515	-8.085	-6.685	ND	-5.895	-5.405	ND	ND
	Task3b	8	-9.178	-8.832	-7.318	-6.833	-5.862	-5.446	ND	ND
	Task3b	9	-9.027	-8.6	-7.193	-6.68	-5.893	-5.347	ND	ND
	Task3b	10	-8.917	-8.431	-6.893	-6.322	-5.903	-5.28	ND	ND
	Mean		-9.046	-8.599	-7.052	-6.533	-5.875	-5.401		
	SD		0.227	0.275	0.269	0.290	0.129	0.108		
OTSUKA	Task3a	1	-8.743	-8.453	-6.922	-6.598	-5.726	-5.36	ND	ND
	Task3a	2	-8.798	-8.535	-6.898	-6.627	-5.477	-5.202	ND	ND
	Task3a	3	-8.818	-8.558	-6.942	-6.566	-5.467	-5.185	ND	ND
	Task3b	1	-8.928	-8.352	-6.867	-6.376	-5.696	-5.276	ND	ND
	Task3b	2	-8.736	-8.369	-6.849	-6.439	-5.811	-5.192	ND	ND
	Task3b	3	-8.905	-8.402	-7.064	-6.454	-5.767	-5.324	ND	ND
	Mean		-8.821	-8.445	-6.924	-6.510	-5.657	-5.257		
	SD		0.080	0.086	0.077	0.101	0.149	0.075		
KANEKA	Task3a	1	-9.465	-9.3	-7.501	-7.221	-5.395	-5.097	ND	ND
	Task2a	2	-8.51	-8.277	-6.426	-6.047	-5.342	ND	ND	ND
	Task3a	3	-8.32	-8.054	-6.188	ND	-5.264	ND	ND	ND
	Task3a	4	-8.495	-8.105	-6.586	-6.075	-5.716	-5.378	ND	ND
	Task3a	5	-8.361	-8.106	-6.13	ND	-5.441	ND	-4.7	ND
	Mean		-8.630	-8.368	-6.566	-6.448	-5.432	-5.238		
	SD		0.474	0.528	0.554	0.670	0.172	0.199		
HIYOSHI	Task3a	1	-8.548	-8.307	-6.548	-6.256	-5.338	ND	ND	ND
	Task3a	2	-8.415	-8.199	-6.422	-6.096	-5.359	ND	ND	ND
	Task3a	3	-8.472	-8.225	-6.417	-6.146	-5.173	ND	ND	ND
	Task3a	4	-8.6	-8.206	-6.361	-5.506	-5.504	ND	ND	ND
	Task3b	1	-8.544	-8.292	-6.373	-6.009	-5.713	ND	ND	ND
	Task3b	2	-8.483	-8.167	-6.126	-5.556	-5.341	ND	ND	ND
	Task3b	3	-8.633	-8.239	-6.266	-5.788	-5.359	ND	ND	ND
	Task3b	5	-8.343	-8.051	-6.059	ND	-5.197	ND	ND	ND
	Mean		-8.505	-8.211	-6.322	-5.908	-5.373	NA		
	SD		0.096	0.080	0.163	0.295	0.172	NA		
	No. of values		32	32	32	28	31	20		
	Total mean		-8.803	-8.437	-6.769	-6.363	-5.632	-5.341		
	Total SD		0.324	0.305	0.414	0.401	0.263	0.126		
	Minimum		-9.542	-9.300	-7.573	-7.221	-6.115	-5.608		
	Maximum		-8.320	-8.051	-6.059	-5.506	-5.173	-5.097		
	Mean+2SD		**-8.156**	**-7.828**	**-5.942**	**-5.561**	**-5.107**	**-5.088**		
	Mean−2SD		-9.450	-9.046	-7.596	-7.165	-6.157	-5.594		

表２　ER STTA アンタゴニストバリデーション Task3 におけるコード化被験物質のアンタゴニスト活性評価結果

Code	Chemical name	Candidate effect	CERI	OTSUKA	KANEKA	HIYOSHI	Total
ATG001	ICI 182,780	Strong	Positive	Positive	Positive	Positive	Positive (4/4)
ATG002	Mifepristone(Mifeprex)＝RU-486	mild	Positive	Positive	Positive	Positive	Positive (4/4)
ATG003	4,4'-(Hexafluoroisopropylidene)diphenol	Negative	Negative	Negative	Negative	Negative	Negative (4/4)
ATG004	Methylpiperdinylpyrazole dihydrochloride	mild	Positive	Positive		Positive	Positive (3/3)*
ATG005	4-Hydroxytamoxifen	moderate	Positive	Positive	Positive	Positive	Positive (4/4)
ATG006	Raloxifene HCl	moderate	Positive	Positive		Positive	Positive (3/3)
ATG007	Clomiphene citrate(cis and trans mixture)	moderate, mild	Positive	Positive	Positive	Positive	Positive (4/4)
ATG008	Dibutyl phthalate	Negative	Negative	Negative	Negative	Negative	Negative (4/4)
ATG009	Atrazine	Negative	Negative	Negative		Positive	Negative (2/3)
ATG010	Flutamide	Negative	Negative	Negative	Negative	Negative	Negative (4/4)
ATG011	4,4'-Cyclohexylidenebisphenol	Negative	Negative	Negative	Negative	Negative	Negative (4/4)
ATG012	4,4'-[1-[4-[1-(4-Hydroxyphenyl)-1-methylethyl]phenyl]ethylidene]bis[phenol]	mild	Positive	Positive		Positive	Positive (3/3)
ATG013	Apigenin	Negative	Negative	Negative		Negative	Negative (3/3)
ATG014	Genistein	to be negative	Negative	Negative	Negative	Negative	Negative (4/4)
ATG015	Dibenzo[a,h]anthracene	positive	Negative	Positive		Positive	Positive (3/4)
ATG016	p-n-nonylphenol	not tested	Negative	Negative	Negative	Negative	Negative (4/4)
ATG017	Flavone	to be negative	Negative	Negative		Negative	Negative (3/3)
ATG018	Resveratrol	to be negative	Negative	Negative	Negative	Negative	Negative (4/4)
ATG019	Fenarimol	not tested	Negative			Negative	Negative (2/2)
ATG020	17b-estradiol	to be negative	Negative	Negative	Negative	Negative	Negative (4/4)
2x2 table analysis compared with candidate effects		Accuracy:	94%	100%	100%	94%	100% (97%)*
		Sensitivity:	88%	100%	100%	100%	100% (97%)*
		Specificity :	100%	100%	100%	88%	100% (97%)*

*: Values in parenthesis are calculated with all individual data derived in 4 laboratories (N=65).

図 1A　OECD VMG-NA で合意された今後の予定

Project 4. 34 TG STTA for anti-estrogenic activity of chemicals [JP]

- Validation completed in 2013 and report and draft TG available in spring 2014.
- TG 455 and TG 457 merged into a new TG 455 that includes both the ER agonist and ER antagonist components
- 1st WNT commenting round in July 2014 for:
  - The draft TG 455, updated to include test methods for the detection of anti-estrogenic activity of chemicals
  - The validation report of the human ER mediated reporter gene assay to detect antagonist activity using hERα-HeLa-9903 cell line,
  - The draft updated Performance Standards for STTA in vitro assays to detect ER antagonists
- Few comments received, will be addressed during autumn. Any remaining issues will be discussed at the VMG NA meeting (Paris, 2-4 December 2014)
- Draft updated TG expected to be submitted for approval to WNT in April 2015.

図 1B　TG455 アップデート成立後のガイドライン及びパフォーマンススタンダードの整理