Appendix-1

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

The treatment of serum with Dextran-coated charcoal (DCC) is a generally used methodology for the removal of estrogenic compounds from serum. It is added to the cell medium in order to exclude the biased response associated with residual estrogens in serum.

The following materials and equipments will be required;

Materials

- Activated charcoal (Sigma, Catalog# C9157)
- Dextran (MW 64,000~76,000, Sigma, Catalog# D4751)
- Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, Wako, Catalog# 135-00165, >=98% or its equivalent)

  Prepare 1 M $MgCl_2$ aq. by dissolving 20.3 g of $MgCl_2 \cdot 6H_2O$ in 100 mL of Milli-Q and filtering it with sterile filter.
- Sucrose (Wako, Catalog# 196-00015 or its equivalent)
- 1 M HEPES buffer solution (pH 7.4) (Gibco, Catalog# 15630)
- Ultrapure water produced from a filter system

Equipment

- Autoclaved glass container (size should be adjusted as appropriate)
- General Laboratory Centrifuge (that can set temperature at 4°C.)

The following procedure is adjusted for the use of 50 mL centrifuge tubes.

[Day-1] Prepare 1 litre of dextran coated charcoal suspension by adding the following reagents in the autoclaved glass container and stir it at 4°C, overnight.

• 1 M $MgCl_2$ aq.	1.5 mL
• Sucrose	85.5 g
• Activated charcoal	2.5 g
• Dextrane	0.25 g
• 1 M HEPES	5 mL

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10.000 rpm at 4°C for 10 minutes. Remove the supernatant and store half of the charcoal sediment at 4°C for the use on Day-3. Suspend the other half of the charcoal with fetal bovine serum (FBS) that is thawed at 42°C and left for 30 minutes at 56°C for heat inactivation, and transfer into the autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at 10.000 rpm at 4°C for 10 minutes. Collect FBS and transfer into the new charcoal sediment prepared and stored on Day-2. Suspend the charcoal sediment and stir this suspension gently at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10.000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.22 μm sterile filter. This DCC treated FBS should be stored at -20°C.

添付資料 2

HeLa ATG Validation Study
Test Plate Quality Criteria and Assay Performance Criteria

Amendment:

Reason: The prior criteria were provisionally defined based solely upon the preliminary assay data from the lead laboratory (CERI), and it was expected that these might need minor modification following data analyses from the participating laboratories.
This amendment modifies the Quality Criteria and the Performance Criteria for the HeLa ATG assay based on the Task2 results from three Japanese laboratories. These modifications were agreed upon with the biostatistician at the data analysis meeting of HeLa ATG validation study held on 15 Dec 2008 at CERI.

Quality Criteria for each plate for anti-estrogenic assay:

The newly defined quality criteria and background calculated data are shown in Table 1.
Modified sections are indicated in red characters.
Detailed individual data were shown in Appendix. Table 1 ~4.

Table 1. Quality Criteria for each plate for anti-estrogenic assay

	Prior	New	mean±2SD	mean±2.5SD	mean±3SD
Fold-induction of Spike-in Control (25 pM of E2)	> 6	>= 4	6.0 ~ 14.7	4.9 ~ 15.8	3.9 ~ 16.8
		(>6 recommend)	7.5 ~ 12.8	6.8 ~ 13.4	6.2 ~ 14.1
RTA of 1 nM E2	> 100%	>= 100%	114.9 ~ 204.5	103.7 ~ 215.7	92.5 ~ 226.9
			130.4 ~ 185.5	123.6 ~ 192.3	116.7 ~ 199.2
RTA of 1 μM OHT	< 16.9%	=< 39.4%	0.5 ~ 39.4	-4.4 ~ 44.3	-9.3 ~ 49.1
			-0.2 ~ 38.7	-5.0 ~ 43.6	-9.9 ~ 48.4
RTA of 100 μM Dig.	< 0%	=< 0%	-14.2 ~ -5.9	-15.2 ~ -4.8	-16.3 ~ -3.8
			-13.8 ~ -6.9	-14.6 ~ -6.1	-15.5 ~ -5.2

RTA : Relative transcriptional activation

Rationale for definition of Quality Criteria:

Fold induction:

While all data from three Japanese laboratories met the prior provisional criteria, two additional laboratories have so far had difficulty in achieving >6 fold induction (FI). This suggests that the prior provisional criteria may be too stringent, and that for practical reasons the range could be extended a little, whilst also maintaining adequate performance, thereby accommodating all participating laboratories. It is expected that sufficient performance will be achieved if the FI is >=4, based upon the experience of the lead laboratory with the agonist assay (the FI Criteria of the agonist assay is >=4).

However, it is still recommended that the FI >6 remains as the preferred FI recommended value to obtain optimal results

RTA of 1 μM OHT:

One laboratory (Kaneka) could not meet this criteria within five assay runs (see Appendix Table 3). However, their data was reproducible and concordant results (i.e. IC30, IC50) were observed for the reference chemicals. We therefore concluded that this was inter-laboratory variation. New criteria was defined by mean+2SD (39.4%) of all data (n=13).

RTA of 1 nM E2 and RTA of 100 μM Dig.:

There was no change in prior criteria.

Performance Criteria for anti-estrogenic assay (Acceptable range for reference chemicals):

The newly defined criteria range for reference chemicals are shown in Table 2. Modifications are indicated in red characters.

Detailed individual data are shown in Appendix. Table 5 ~7.

Table.2 Performance Criteria for anti-estrogenic assay

		Prior	New	n*
OHT	log [lin.IC30]	-9.86 ~ -8.76	-9.62 ~ -8.73	13
	log [lin.IC50]	-9.79 ~ -8.28	-9.46 ~ -8.16	13
	log [var.IC50]	-9.15 ~ -8.94	-9.32 ~ -8.20	13
TAM	log [lin.IC30]	-7.88 ~ -6.99	-7.55 ~ -6.84	13
	log [lin.IC50]	-7.48 ~ -6.50	-7.08 ~ -6.26	13
	log [var.IC50]	-7.17 ~ -6.77	-7.02 ~ -6.32	13
RU486	log [lin.IC30]	-6.20 ~ -5.32	-6.18 ~ -5.41	10
	log [lin.IC50]	-5.70 ~ -5.09	-5.61 ~ -5.08	10
	log [var.IC50]	-6.22 ~ -5.32	-5.53 ~ -4.86	10

n*: Number of data used for calculation.

lin.IC30: 30% inhibition concentration by linear fitting.

lin.IC50: 50% inhibition concentration by linear fitting.

var.IC50: 50% inhibition concentration by non-linear fitting to Hill equation using Prism ver.4.

Rationale for definition of Performance Criteria:

The criteria ranges are defined based on mean+-2SD as calculated from the results obtained from 13 assay runs during Task 2 from three Japanese laboratories (CERI: 5 assays, Otsuka: 3 assays, Kaneka: 5 assays). There were no large differences between the prior provisional range and the newly proposed provisional ranges on each parameter for each compound. For the parameter of RU486, three assay data sets (CERI: #5, Kaneka: #2, #5) were excluded from the calculation due to cytotoxicity, and at the recommendation of the biostatistician (see follows).

Exclusion of data based upon chemical-induced cytotoxicity:

The limit for cytotoxicity was set at <20% in the protocol for this STTA. Thus, no data were used for the calculation of the criteria parameters from any chemical concentration for which cell viability was less than 80% (e.g., cell cytoxicity > 20%). In addition, non-linear curve fitting was not performed in cases where there were no data points were observed for more than 50% inhibition of ER-mediated gene transcriptional activation, after exclusion of the data points for cytotoxicity.

添付資料3

VALIDATION OF THE LUMI-CELL® ER ASSAY FOR THE DETECTION OF ESTROGEN RECEPTOR AGONISTS AND ANTAGONISTS

STUDY DESIGN and WORK PLAN

17 August 2007

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STUDY DESIGN and WORK PLAN

Validation of the LUMI-CELL® ER for the Detection of Estrogen Agonists and Antagonists

1.0 PROJECT OBJECTIVES AND GENERAL REQUIREMENTS

1.1 Project Objectives

This document specifies the procedures that participating laboratories will use to conduct the international validation study of an estrogen receptor (ER) transcriptional activation (TA) assay (LUMI-CELL® ER assay) for the detection of ER agonists and antagonists. The list of 78 ICCVAM recommended substances, which possess varying degrees of ER agonist and/or antagonist activity (ICCVAM 2002; ICCVAM 2003; Federal Register, Vol. 71, No. 51, pp. 13597-13598, March 16, 2006;), will be used in this validation study to characterize the reliability and relevance of the LUMI-CELL® ER assay.

1.2 General Capabilities

Participating laboratories will be capable of the following:

1. Providing Standard Operating Procedures (SOPs, see **Section 1.4**) for the performance of the LUMI-CELL® ER agonist and antagonist assays
2. Conducting the study in accordance with or in the spirit of Good Laboratory Practices (GLP)
3. Providing study reports and all associated data from studies outlined in this document to the Study Management Team (SMT) through the designated contacts listed in **Section 2.2**.

1.3 Guidelines

The Project Officer and designated members of the SMT may inspect participating laboratory testing facilities and audit any procedures. participating laboratories should notify the SMT of any changes in Key Personnel (see **Section 3.1.1**)

1.4 Definitions

Good Laboratory Practices (GLPs): Regulations governing the conduct, procedures, and operations of toxicology laboratories developed to assure the quality and integrity of the data and to address such matters as organization and personnel, facilities, equipment, facility operations, and study conduct (OECD, 1998).

Standard Operating Procedures (SOPs): Written documents that describe in sufficient detail the routine procedures to be followed for a specific operation, analysis, or action. Consistent use of an approved SOP ensures conformance with organizational practices; reduced work effort; reduction in error occurrences; and improved data comparability, credibility, and defensibility, SOPs also serve as resources for training and for ready reference and documentation of proper procedures.

Study Design and Work Plan: A description of all phases of the validation study and the purpose of the procedures; also provides guidance for the preparation of reports.

Test Method Protocols: Specific and detailed guides for performing the LUMI-CELL® ER assay for the detection of ER agonists and antagonists.

Test Substances: Chemicals supplied to participating laboratories that are coded and distributed such that only the Project Officer, the SMT, and the Substance Inventory and Distribution Management (identified in **Section 2.2.2**) have knowledge of the identity of each test substance. The test substances will be purchased, aliquoted, coded, and distributed by the Substance Inventory and Distribution Management, under the guidance of the Project Officer and the SMT.

2.0 ORGANIZATION

2.1 Validation Study Sponsors

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

The European Centre for the Validation of Alternative Methods (ECVAM)

The Japanese Center for the Validation of Alternative Methods (JaCVAM)

2.2 Study Management

2.2.1 International Study Management Team

2.2.1.1 *NICEATM*

Dr. William Stokes (NICEATM/NIEHS) – Co-Chair/Project Officer

Dr. Raymond Tice (NICEATM/NIEHS) – Co-Chair

Dr. David Allen (NICEATM/ILS) – NICEATM Principal Investigator

Mr. Frank Deal (NICEATM/ILS) – Project Coordinator

Ms. Patricia Ceger (NICEATM/ILS) – Assistant Project Coordinator

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2.2.1.2 *ECVAM*

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2.2.1.3 *JaCVAM*

Dr. Hajime Kojima

Dr. Atsushi Ono

Mailing Address:

National Institute of Health Sciences

Kamiyouga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

2.2.2 Substance Inventory and Distribution Management

Dr. Cynthia Smith

Chemistry Resources Group Leader

Mailing Address:

NIEHS

111 Alexander Dr.

Research Triangle Park, NC 27709

3.0 TESTING FACILITY AND KEY PERSONNEL

3.1 Competence and Capabilities

Participating laboratories should be competent in the conduct of the LUMI-CELL® ER assay and will provide competent personnel, adequate facilities, equipment, supplies, proper health and safety guidelines, and quality assurance procedures.

3.1.1 Personnel

3.1.1.1 *Facility Management*

Participating laboratory facility management is responsible for establishing scientific guidelines and procedures, training and supervision of technical staff, and evaluation of results. The facility manager must maintain training files that include qualifications, experience, and a job description for each individual involved in the LUMI-CELL® ER assay validation study.

3.1.1.2 *Study Director*

The Study Director has the overall responsibility for the LUMI-CELL® ER assay validation study conducted at each participating laboratory. The Study Director should be responsible for providing Standard Operating Procedures (SOPs) for use during the validation study.

3.1.1.3 *Quality Assurance (QA) Personnel*

Participating laboratory QA personnel or SMT sponsored reviewer (independent reviewer) should monitor the validation study to assure compliance with good laboratory practices for all aspects of the validation study.

3.1.1.4 *Consultant(s)*

Consultants are scientists or other professionals of appropriate education, training, and experience with the LUMI-CELL® ER assay who provide scientific guidance to the SMT or participating laboratories.

3.1.1.5 *Laboratory Technician(s)*

Each individual engaged in the conduct of or responsible for the supervision of the assay should have education, training, and experience, or combination thereof, to enable that individual to perform the assigned duties.

3.1.1.6 *Safety Officer*

A designated Safety Officer (someone not involved in the actual conduct of the validation study) will receive the blinded (coded) test substances from Substance Inventory and Distribution Management and transfer the substances to the Study Director. A sealed health and safety information package will accompany the coded test substances and the Safety Officer should retain the package until the completion of the validation study. The Safety Officer will promptly notify the SMT Project Coordinator if this is opened at any time during the validation study.

3.1.2 Facilities, Equipment, and Supplies

3.1.2.1 *Cell Culture Laboratory*

A designated cell culture laboratory should be available to ensure that the LUMI-CELL® ER assay can be performed using good cell culture practice (Coecke et al. 2005). Access to the validation study assays and reference substances should be restricted to appropriate personnel as determined by participating laboratory management.

3.1.2.2 *Equipment*

Equipment that is required for conducting the LUMI-CELL® ER agonist and antagonist assays, as specified in the LUMI-CELL® ER assay ER agonist and antagonist protocols. All equipment maintenance and calibration should be routinely performed and documented.

3.1.3 Health and Safety

Participating laboratories should conform to all relevant health and safety regulations in the conduct of the validation study. The designated Safety Officer should be the point of contact for health and safety issues.

3.1.4 Quality Assurance

Participating should conduct this validation study in compliance with or in the spirit of Good Laboratory Practice (GLP) Standards (OECD 1998). QA personnel from the participating or representing the SMT should review the protocol and audit the in-life phase, study workbook, and final report data.

The final reports for all phases of the validation study should be audited by QA personnel for GLP compliance and a QA Statement should be provided with each final report. Each final report should identify: 1) the phases and data inspected, 2) the dates of inspection, and 3) the dates findings were reported to the Study Director and participating laboratory management. The QA Statement should identify whether the methods and results described in the final report accurately reflect the raw data produced during the validation study.

4.0 TEST PHASES AND SCHEDULE

4.1 Study Timeline and Deliverables

4.1.1 Study Timeline

TASK	ACTIVITIES	TIMELINE
Phase I	• Development of automated testing procedures (XDS) • Qualification/protocol refinement by testing reference standards and controls • Establish historical database for standards and controls by conducting independent experiments (10 each for the agonist and antagonist protocols) • Submission of draft report and review by SMT	Mar. 07 – Jul. 07
Phase IIa	• Four substances from ER minimum list tested independently three times for agonism and antagonism (24 total experiments) • Submission of draft report and review by SMT	Aug. 07 – Sep. 07
Phase IIb	• Eight substances from ER minimum list tested independently three times (48 total experiments) • Submission of draft report and review by SMT	Oct. 07 – Dec. 07
Phase III	• Remaining 41 substances from ER minimum list tested once for agonism and antagonism (82 total experiments) • Submission of draft report and review by SMT	Jan. 08 – Feb. 08
Phase IV	• Testing of remaining 25 substances from ER list for agonism and antagonism (XDS only), (50 total experiments) • Submission of draft report and review by SMT	Mar. 08

4.1.2 Study Deliverables

4.1.2.1 *Test Results (Phases I-IV)*

Participating laboratories will provide raw and quality control data in electronic format (i.e., email with attachments) to the SMT Project Coordinator on a weekly basis during *in-life* (i.e.,

during those weeks when LUMI-CELL® ER assay data is being collected and/or analyzed) portions of the study.

4.1.2.2 *Study Status Reports (Phases I-IV)*

Participating laboratories will provide study status reports during each phase of the study to the SMT Project Coordinator on a biweekly basis. These reports will be provided in electronic format (i.e., email with attachments) and will include raw and quality control data as the study progresses. Reports should contain the information outlined in **Appendix A**.

4.1.2.3 *Draft Reports (Phases I-IV)*

At the conclusion of each phase of the study, a draft report will be provided by the Study Director to the SMT Project Coordinator. The draft report will be provided electronically in Word®. Reports should contain the information outlined in **Appendix A** and should follow the recommended formats and styles provided in the "Style Guide for LUMI-CELL® ER Validation Study Laboratory Reports and Documents" (**Appendix B**).

4.1.2.4 *Final Reports (Phases I-IV)*

Each draft report that is approved by the SMT will be followed by a final report, which has been reviewed by the QA for GLP compliance, for each phase of the study. The final report will be provided electronically in Word® by the Study Director to the SMT Project Coordinator. Copies of the audited Study Workbook pages should submitted in electronic format (i.e., pdf files) as an attachment to the report. However, completion of the final report is not required prior to initiation of the next phase of the validation study.

4.1.3 Estimated Due Dates for Reports

ESTIMATED DUE DATES					
REPORTS	**PHASE I**	**PHASE IIa**	**PHASE IIb**	**PHASE III**	**PHASE IV**
Study Status	*	*	*	*	*
Draft	Jul., 2007	Sep., 2007	Dec., 2007	Feb, 2008	Mar., 2008
Final	Aug., 2007	Oct., 2007	Jan., 2008	Mar, 2008	Apr., 2008

*Study status reports will be provided biweekly during each phase of the study.

4.2 Phase I

This phase will be used for initial laboratory qualification/protocol refinement by all participating laboratories and is limited to the testing of reference standards, positive controls,

and the solvent control. The results will be used to establish an historical database in each laboratory for reference standards and controls.

4.2.1 Initial Laboratory Qualification/Protocol Refinement

Repetitive testing of agonist and antagonist reference standards and positive/solvent controls will be used to demonstrate proficiency with the LUMI-CELL® ER assay, demonstrate intralaboratory repeatability and intra- and inter-laboratory reproducibility, and establish an historical database. Results will be compared to historical control data established during the LUMI-CELL® ER Protocol Standardization Study. If there is excessive variation of reference standard and control data within or among the participating laboratories, the SMT (through the designated contacts) will work with the laboratories to determine cause and recommend appropriate actions needed to reduce variation. Statements of Work, Test Method Protocols, and SOPs will be revised, if necessary, and testing repeated until acceptable proficiency is demonstrated (i.e., acceptable intralaboratory repeatability and intra- and inter-laboratory reproducibility). The SMT may convene a teleconference with appropriate participants of the validation study to discuss information concerning the progression of the study.

4.2.2 Criteria for Advancing to Phase II

The SMT will decide when all laboratories will advance to Phase II of the validation study, based on the following criteria:

- Data, reviewed by the participating laboratory QA personnel (or independent reviewer), has been received by the SMT
- All participating laboratories have submitted acceptable draft reports as outlined in **Section 4.1.2.2**.
- Acceptable intralaboratory repeatability and intra- and inter-laboratory reproducibility has been demonstrated by the participating laboratories
- A suitable historical negative and positive control database has been established

4.3 Phase II

Phase II provides for initial laboratory qualification using procedures that have been refined in Phase I, but is also the initial phase for testing substances from the ICCVAM list of 78 reference substances recommended for validation of ER TA assays. In this phase, four coded test substances (Phase IIa) and then eight coded test substances (Phase IIb) will be tested in all three

participating laboratories. Acceptance criteria for experimental data for Phase IIa will be based on the historical database established in Phase I for reference standards and controls. Reference standard and control data collected during Phase IIa will also be included in the historical database, which will then be used to establish acceptance criteria for Phase IIb.

4.3.1 Phase IIa Limited Testing of Protocol and Protocol Refinement

After a range-finding assay is completed for each of the four coded test substances in Phase IIa, recommended starting concentrations for the comprehensive concentration-response experiment and the rationale for their selection are to be sent to the SMT for review and approval. The comprehensive concentration-response experiment for each test substance should not begin until the starting concentrations have been approved, and they should not be modified without approval from the SMT. The comprehensive concentration-response experiment should be performed three times, once on each of three different days. Laboratories will calculate EC_{50} values for the agonist reference standard or IC_{50} values for the antagonist reference standard (in µg/mL). Laboratories will also calculate EC_{50} or IC_{50} values (in µg/mL), when possible, for coded test substances. These data, along with all quality control, raw, derived and supporting data, will be reported to the SMT through the designated contacts. If there is excessive variation within or among participating laboratories, the SMT will work with the laboratories to determine the cause and recommend appropriate actions needed to reduce variation. Statements of Work, Test Method Protocols, and SOPs will be revised, if necessary, and testing repeated until acceptable proficiency is demonstrated (i.e., acceptable intralaboratory repeatability and intra- and inter-laboratory reproducibility). The SMT may convene a teleconference with appropriate participants of the validation study to discuss information concerning the progression of the study.

4.3.2 Criteria for Advancing to Phase IIb

The SMT will decide when all laboratories will advance to the Phase IIb of the validation study, based on the following criteria:

- Data, reviewed by the QA Officer (or independent reviewer), has been received by the SMT
- All participating laboratories have submitted acceptable draft reports as outlined in **Section 4.1.2.2**.

- Acceptable intralaboratory repeatability and intra- and inter-laboratory reproducibility has been demonstrated by the participating laboratories

4.3.3 Phase IIb Testing of Protocol and Protocol Refinement

Phase IIb includes the testing of eight coded substances and is the last phase for evaluating any protocol refinements from Phase I or IIA.

After a range-finding assay is completed for each of the eight coded test substances in Phase IIb, recommended starting concentrations for the comprehensive concentration-response experiment and the rationale for their selection are to be sent to the SMT for review and approval. The comprehensive concentration-response experiment for each test substance should not begin until the starting concentrations have been approved and should not be modified without approval of the SMT. The comprehensive concentration-response experiment should be performed three times, once on each of three different days. Laboratories will calculate EC_{50} values for the agonist reference standard or IC_{50} values for the antagonist reference standard (in µg/mL). Laboratories will also calculate EC_{50} or IC_{50} values (in µg/mL), when possible, for coded test substances. These data, along with all quality control, raw, derived and supporting data, will be reported to the SMT through the designated contacts. If there is excessive variation within or among participating laboratories, the SMT will work with the laboratories to determine the cause and recommend appropriate actions needed to reduce variation. Statements of Work, Test Method Protocols, and SOPs will be revised, if necessary, and testing repeated until acceptable proficiency is demonstrated (i.e., acceptable intralaboratory repeatability and intra- and inter-laboratory reproducibility). The SMT may convene a teleconference with appropriate participants of the validation study to discuss information concerning the progression of the validation study.

4.3.4 Criteria for Advancing to Phase III

The SMT will decide when all laboratories will advance to the Phase III of the validation study, based on the following criteria:

- Data, reviewed by participating laboratory QA personnel (or independent reviewer), has been received by the SMT
- All participating laboratories have submitted acceptable draft reports as outlined in **Section 4.1.2.2**.