内分泌かく乱物質スクリーニングの国際バリデーション研究

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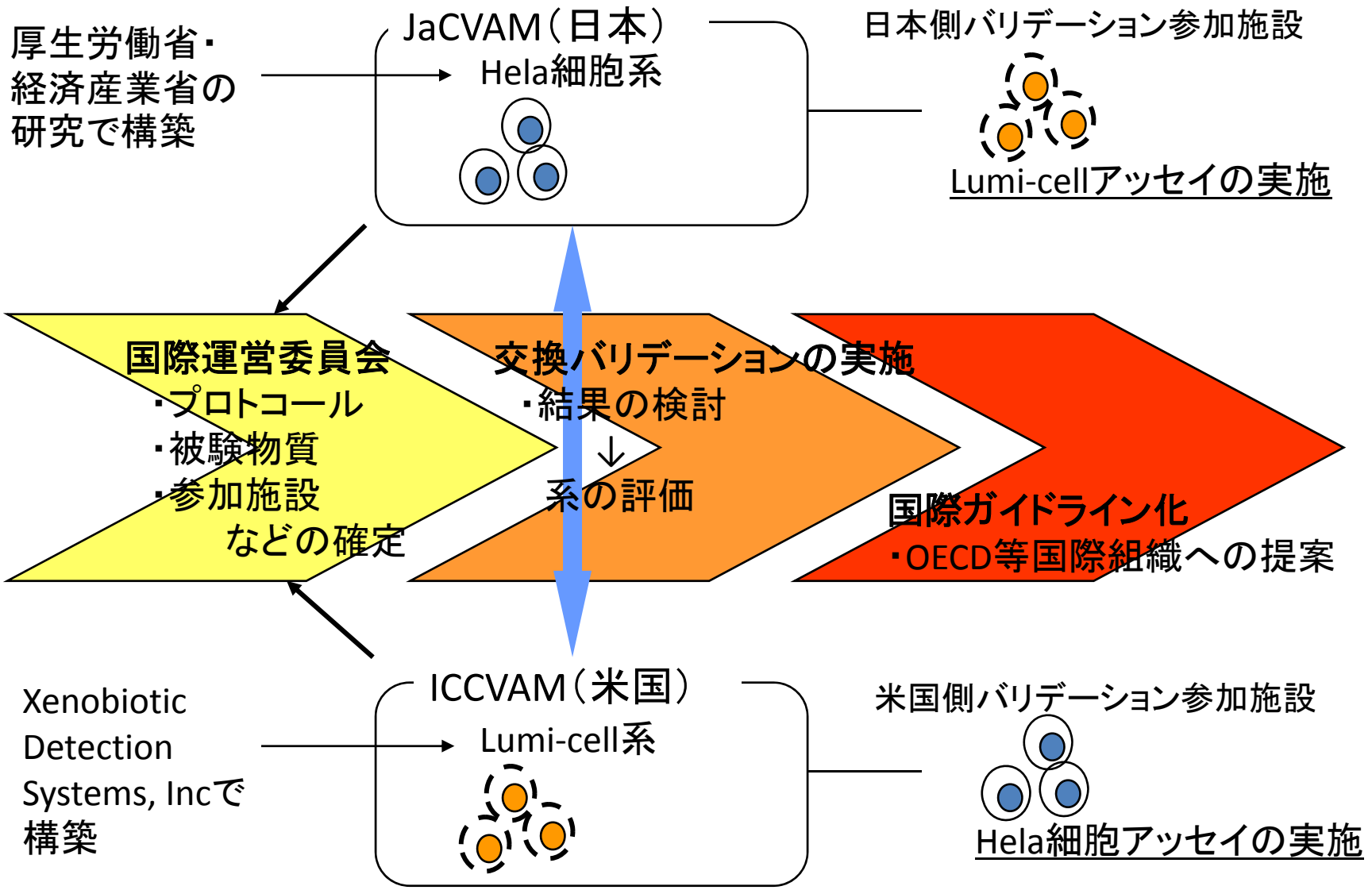
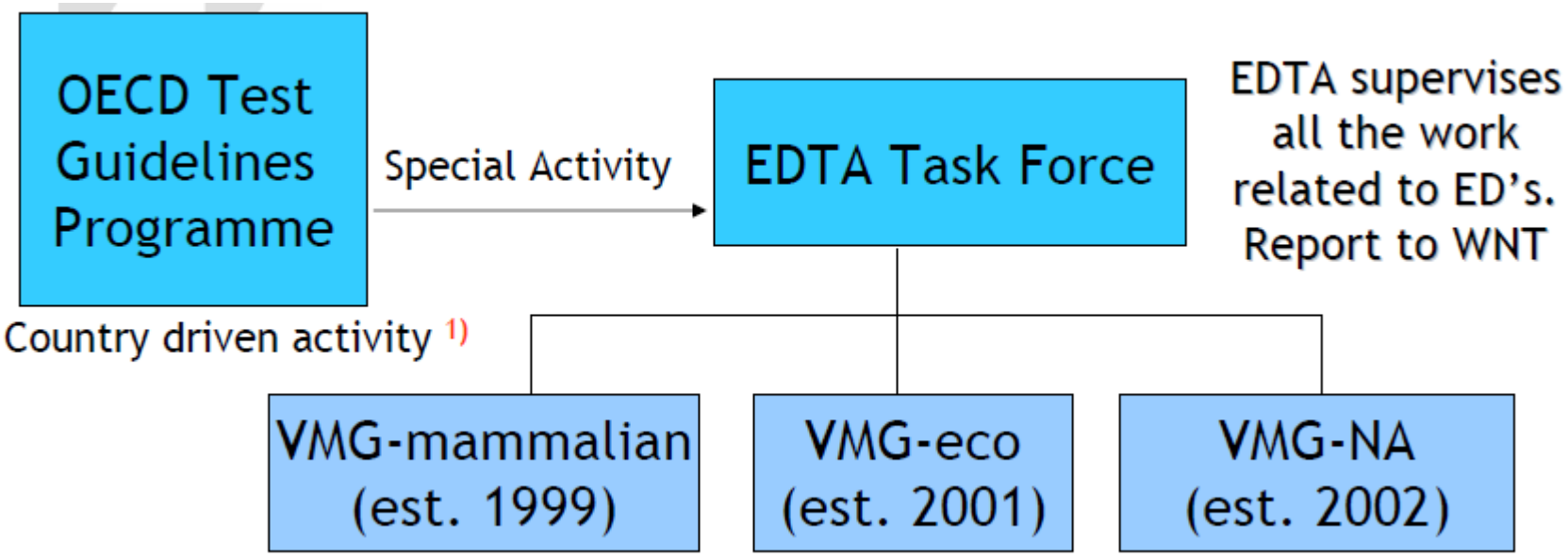
内分泌かく乱物質高速スクリーニング系の国際交換バリデーション



The OECD Work on ED: A Special Activity of the Test Guidelines Programme

* Basic goal and scope of test guidelines programme is mutual acceptance of data.



1) OECD Member countries make proposals to develop new or update existing TG; proposals are prioritized by countries (WNT) and a lead is designated for the work.

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters as agreed by the EDTA6 (2002)

Level 1
Sorting & prioritization based upon existing information

- physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability,
- human & environmental exposure, e.g., production volume, release, use patterns
- hazard, e.g., available toxicological data

Level 2
In vitro assays providing mechanistic data

- ER, AR, TR receptor binding affinity
- Transcriptional activation
- Aromatase and steroidogenesis *in vitro assays*
- Aryl hydrocarbon receptor recognition/binding
- QSARs
- High Through Put Prescreens (HTPS)
- Thyroid function
- Fish hepatocyte VTG assay
- Others (as appropriate)

Level 3
In vivo assays providing data about single endocrine mechanisms and effects

- Uterotrophic assay (estrogenic related)
- Hershberger assay (androgenic related)
- Non -receptor mediated hormone function
- Others (e.g. thyroid)

- Fish VTG (vitellogenin) assay (estrogenic related)

Level 4
In vivo assays providing data about multiple endocrine mechanisms and effects

- enhanced OECD 407 (endpoints based on endocrine mechanisms)
- male and female pubertal assays
- adult intact male assay

- Fish gonadal histopathology assay
- Frog metamorphosis assay

Level 5
In vivo assays providing adverse effects data from endocrine & other mechanisms

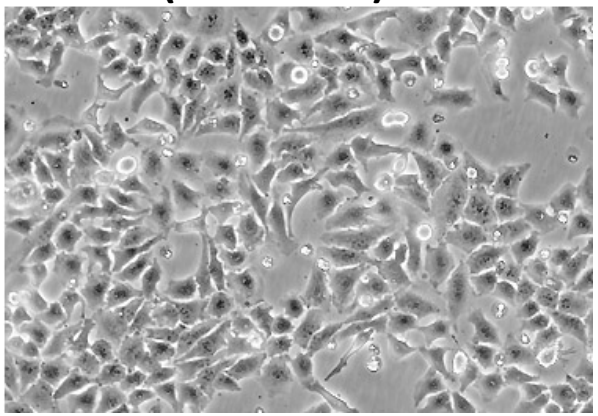
- 1-generation assay (TG415 enhanced)[1]
- 2-generation assay (TG416 enhanced)[1]
- reproductive screening test (TG421 enhanced)[1]
- combined 28 day/reproduction screening test (TG 422 enhanced)[1]

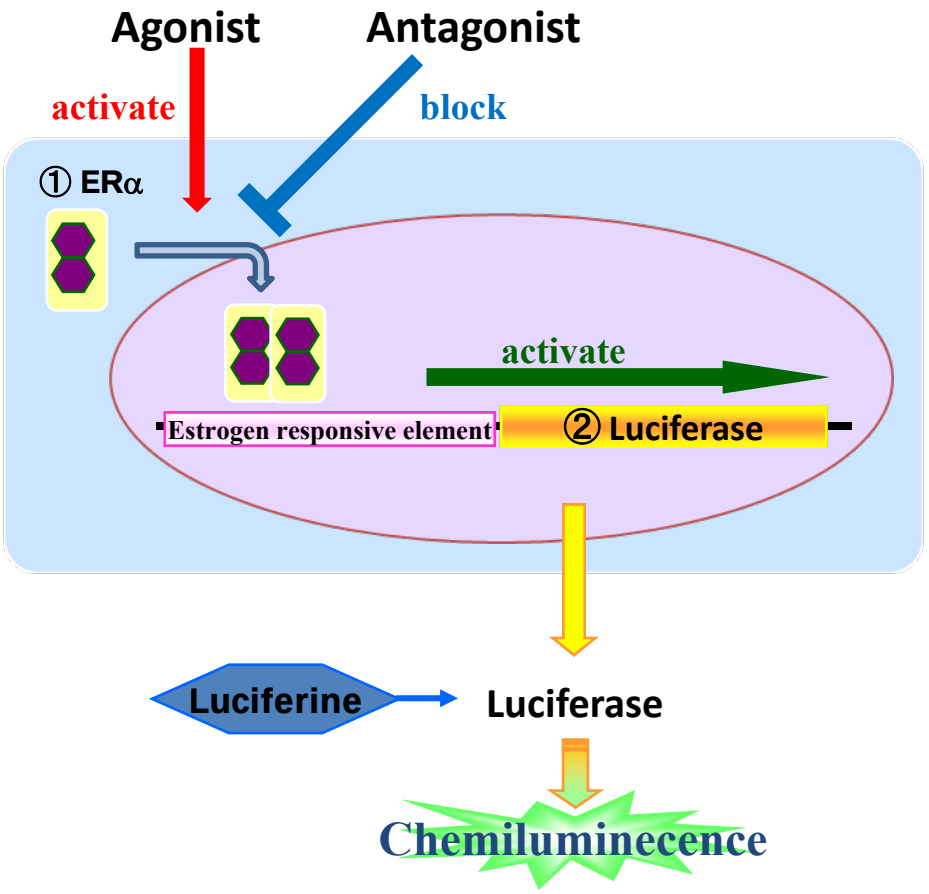
- Partial and full life cycle assays in fish, birds, amphibians & invertebrates (developmental and reproduction)

International validation study of ERα STTA antagonist assay using HeLa9903.

ERα STTA assay using HeLa9903

hERα-HeLa-9903
(HeLa9903)





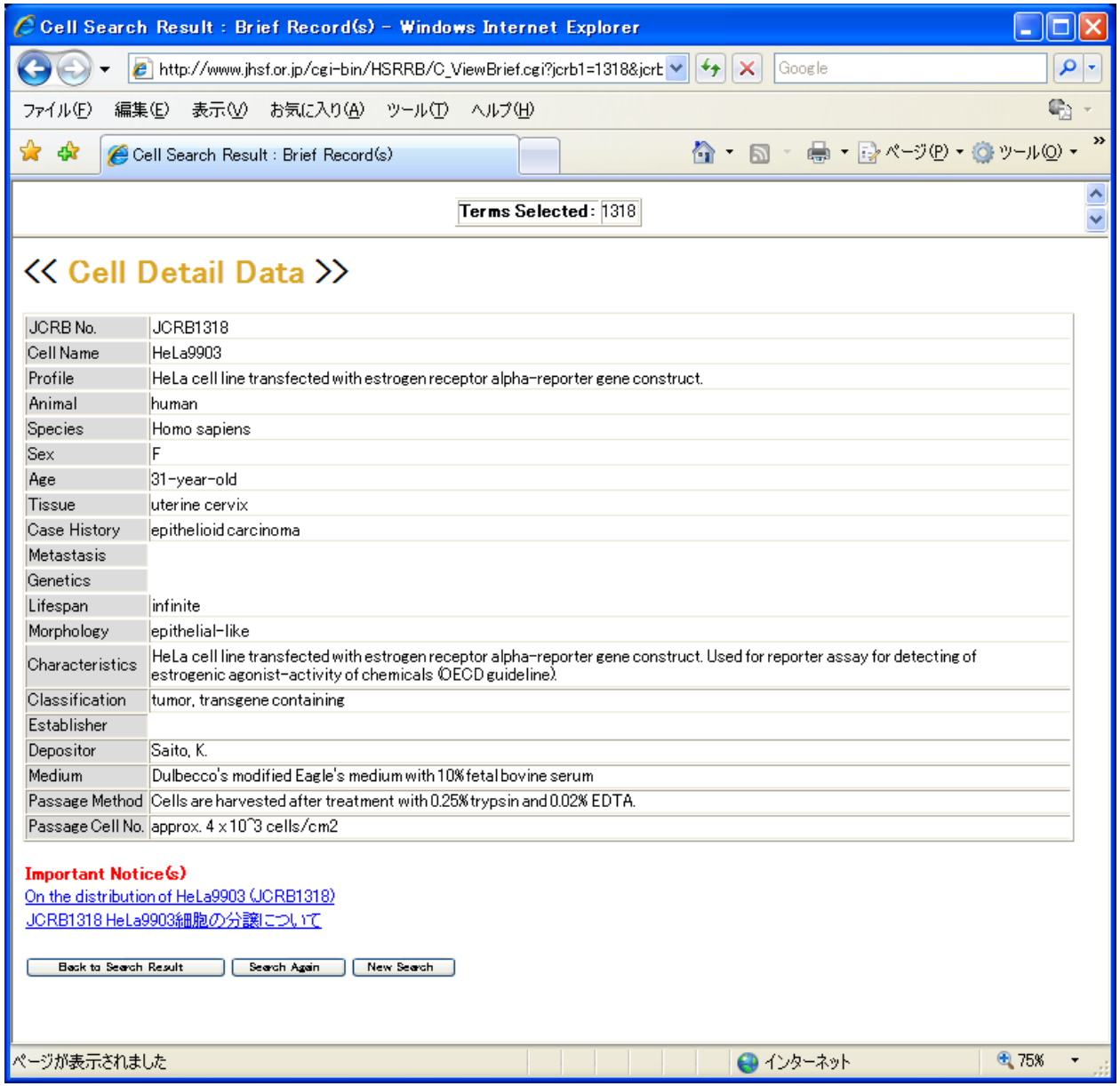
- **Developed by Sumitomo Chemical Co.**
- **Host Cell: HeLa cell line (human cervical tumor cells)**
- **Inserted construct:**
  - **① Human ERα expression vector (full-length)**
  - **② Firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element (ERE) driven by a mouse metallothionein promoter TATA element**
- **Expression of other nuclear receptor**

  No functional ERα, ERβ, AR, TRα and TRβ in host cell
- **Available from JCRB (NIBIO)**

Availability of HeLa9903

JCRB1318:

HeLa9903 is available from JCRB (NIBIO) – HSRRB



Status of ERα STTA assay

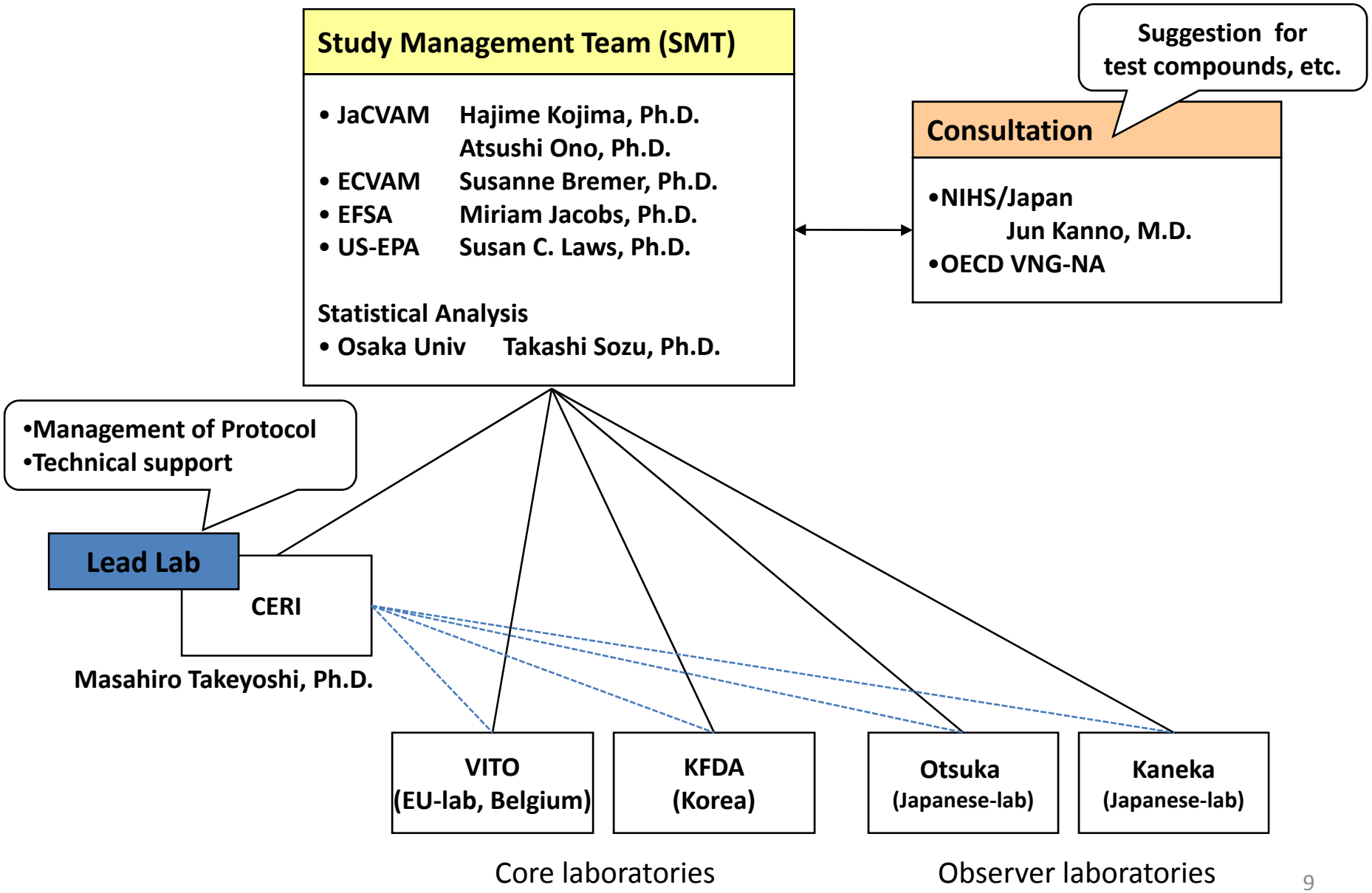
Validation of agonist assay was already finished.

Draft test guideline for "*the Stably Transfected Human Estrogen Receptor Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (TG 455)*", was presented to the WNT20 in April 2008 for approval.

Validation of antagonist assay was requested.

Comment by Peer Review Panel (PRP) included:
The STTA assay can at this point only be used for estrogen agonist testing and further studies would be needed if also **estrogen antagonists** could be tested.

Validation Organization



Study Design

Tasks	Purpose	Note
Start chemical distribution from JaCVAM.		
Start cell culturing at each lab and prepare cell stocks.		
Task-1	Confirm the edge effects (establish the plate layout)	no edge effects→ use 96-well edge effects are expected →not use edge wells
	Confirm if the test system is properly setup and the participating lab can provide the basic assay performance.	Test un-coded 3 chemicals in "Agonist" Assay.
Task-2	Confirm lab performance for "Antagonist" (ATG) assay (including range finding test, cytotoxicity (cytotox.) test)	Test un-coded 4 chemicals in "Antagonist" Assay.
Task-3	Test coded chemicals in "Antagonist" (ATG) assay	•Test "anti-estrogenic" activities of coded 12 chemicals/lab.

Task1

[Task-1] Procedure of ER agonist assay

[Assay Procedure]

Culture cells with 10% FBS-EMEM

↓

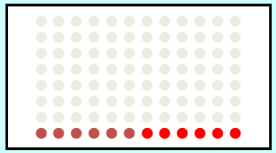
Seeding cells at $1x10^4$/100 µL/wells in a 96-well plate

↓

Incubate for 2-3 hours in CO_2 incubator at 37° C

↓

Expose chemicals, vehicle control and positive controls to cells



●: Vehicle Control (VC)
●: 1 nM of E2

↓

Incubate for 20-24 hours in CO2 incubator at 37° C

↓

Measure luciferase activity

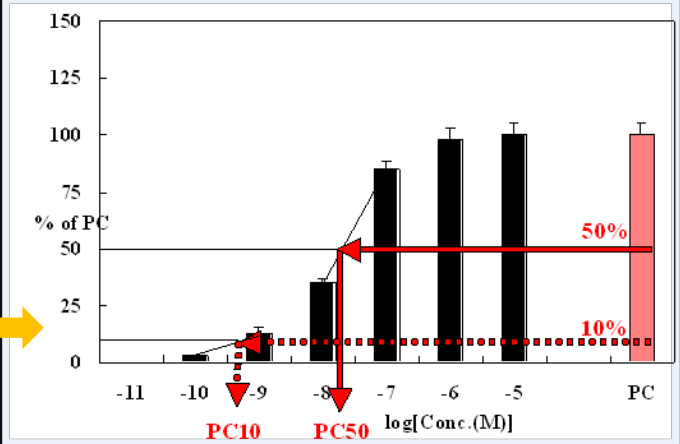
[Test Chemicals]

	1	2	3	4	5	6	7	8	9	10	11	12
A	17b -Estradiol			17b -Estradiol			17a -Estradiol			Cortico -sterone		
B												
C												
D												
E												
F												
G												
H	Vehicle Control (DMSO (0.1%))						Positive Control (1nM of E2)					

[Data Analysis]

Endpoints

- PC10
- PC50
- EC50 (Hill's Equation)



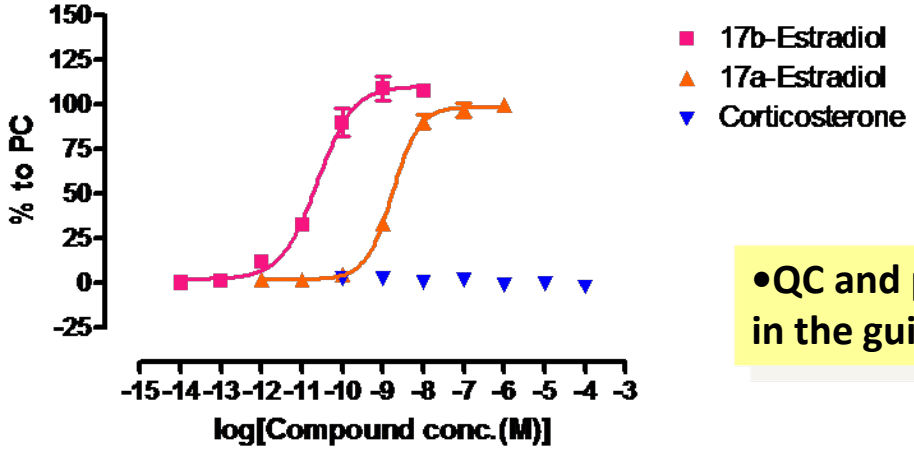
Task-1
[Quality Control and Performance Standard]

Quality Controls

Fold-induction of Positive Control (1 nM of E2) [=(AVG of PC)/(AVG of VC)]	>=4
10% fold-induction of 1 nM E2	> 1 ± 2SD of fold-induction of VC
CV of the raw data triplicates (i.e. luminescence intensity) of the data points that are used for the calculation of PC10	within 20%

Performance Standard

	log [PC50 (M)]	log [PC10 (M)]	log [EC50 (M)]	Hill Slope
17beta-Estradiol	-11.4 ~ -10.1	<-11	-11.3 ~ -10.1	0.7 ~ 1.5
17alpha-Estradiol	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0
Corticosterone	-	-	-	-



- **QC and performance criteria is shown in the guideline for agonist assay.**

Summary of Task1

- **The results exhibited that there was no edge effect for the HeLa STTA assay in all participant laboratory.**

- **Results of all laboratories indicated the proficiency for the agonist assay with their results which met the criteria of the quality control and performance standard.**

Task2

[Task-2] Procedure of ER antagonist assay

[Assay Procedure]

Culture cells with 10% FBS-EMEM

↓

Seeding cells at 1x10^4/100 µL/wells in a 96-well plate
(prepare 2 plates for ATG and cytotox. assay each)

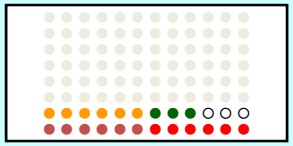
↓

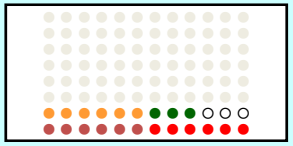
Incubate for 2-3 hours in CO_2 incubator at 37° C

↓

Expose chemicals, vehicle control and positive controls to cells
(plate format for ATG and Cytotox. assays are same)

[ATG assay] **[Cytotx. assay]**





●: Vehicle Control (VC)
●: 1 nM of E2
●: 25 pM of E2
●:10 µM of OHT
○: Cytotox. Control

↓

Incubate for 20-24 hours in CO2 incubator at 37° C

↓

Measure luciferase activity

Measure cell viability

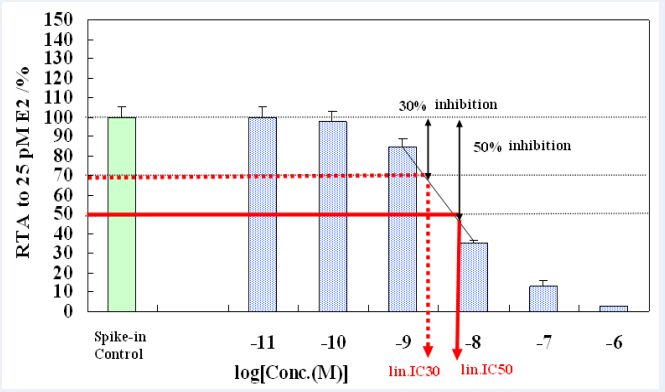
[Test Chemicals]

	1	2	3	4	5	6	7	8	9	10	11	12
A	4-Hydroxy tamoxifen			Tamoxifen			RU-486			Negative		
B												
C												
D												
E												
F												
G	Spike-in Control (25 pM of E2)						10uM of OHT			Cytotox. Ctrl.		
H	Vehicle Control (DMSO (0.1%))						Positive Control (1nM of E2)					

[Data Analysis]

Endpoints

- **linIC30**
- **linIC50**
- **IC50 (Hill's Equation)**



Task-2
[Quality Control and Performance Standard]

- **The tentative quality control and performance standard values were preliminary defined by the result of pre-validation study at lead laboratory and should be updated by the Task2 &3 results.**

Task3

[Task-3] Study design

All lab will participate task-1 and task-2.
Core and Observer lab will test each 12 chemicals (5 chemicals are overlap).
Lead lab (CERI) test all 20 chemicals.

CERI	Core	Observer
X	X	X
X	X	X
X	X	X
X	X	X
X	X	X
X	X	
X	X	
X	X	
X	X	
X	X	
X	X	
X	X	
X		X
X		X
X		X
X		X
X		X
X		X
X		X
X		

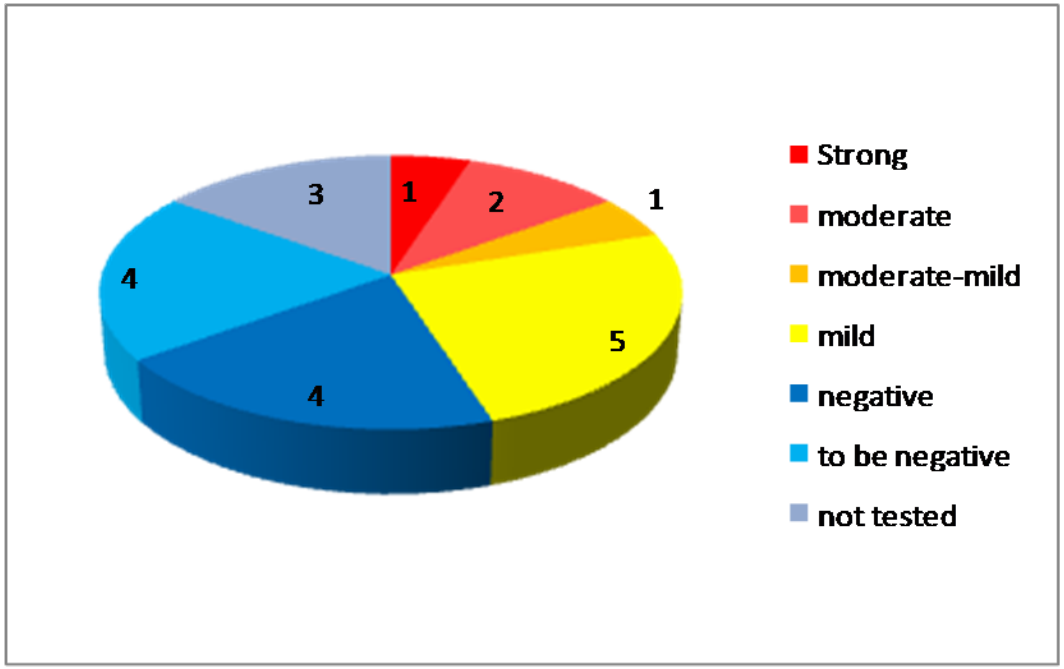
Test at 5 labs
5chemicals

Test at 3 labs
14chemicals

Test at 1 lab
1 chemical

Chemical Selection for Task-3

- Chemicals for Task 3 are selected based on ICCVAM and ECVAM (ReproTect) lists.
- Cytotoxic chemicals (antagonist negatives and positives) are included to strength the sensitivity of the assay.
- "Additional" chemicals are selected from the ER binding (and uterotrophic) assay(s).
- Proposed distribution of Positives and Negatives is as below,



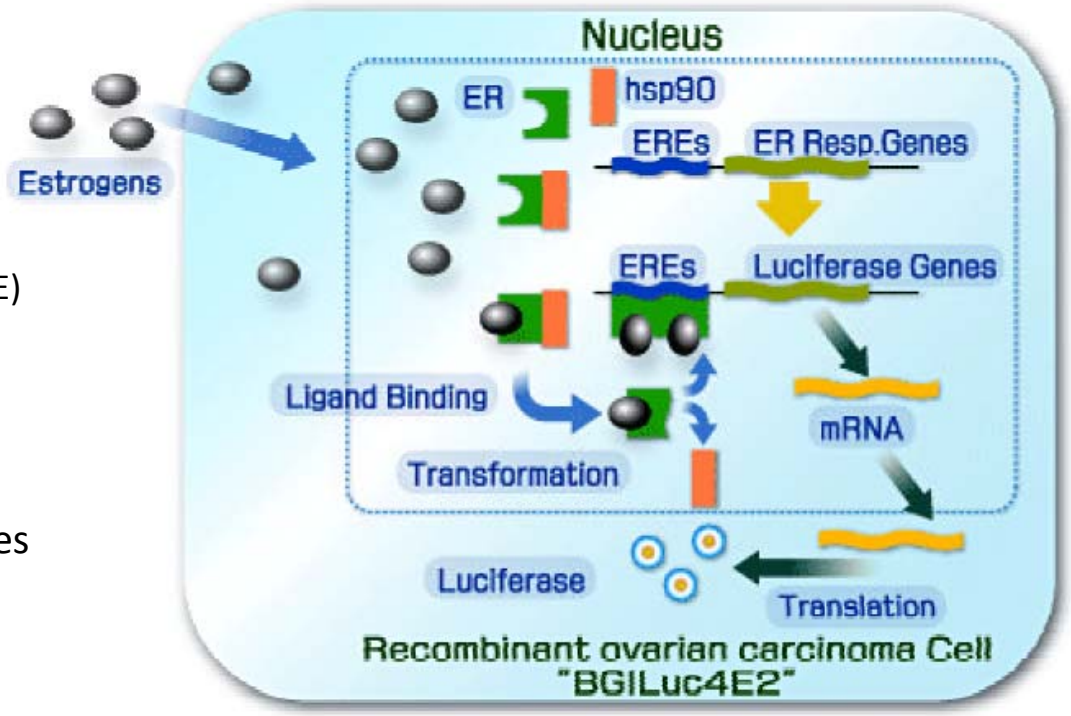
Status of the HeLa ATG validation

- All five laboratory succeeded in Task1 with the results which met quality criteria of the agonist assay in Task1.
- Japanese three labs. were finished Task2, successfully. However, European and Korean labs failed in antagonist assay in Task2. Additional experiments to clear the cause of fail is on-going.
- By the Task2 results of Japanese three labs, the quality criteria was updated to cover inter-laboratory variations.
- Updated criteria will be re-evaluated by the Task3 of Japanese labs and the Task2 &3 of European and Korean labs.
- Task3 experiment is on-going in Japanese labs to evaluate the consistency of the results for coded compounds.
- The validation report will be available by summer 2009.

International Validation Study of LUMI-CELL® ER Assay

ERα STTA assay using LUMI-CELL

- The LUMI-CELL® ER assay is based on a stable recombinant cell line (BG1Luc4E2)
  - BG1 - human ovarian carcinoma cell that expresses endogenous alpha (95%) and beta (5%) estrogen receptors
  - Plasmid pGudLUC7.ERE used to transfect cell line
    - Contains 4 copies of synthetic oligonucleotide containing estrogen response element (ERE)
    - Mouse mammary tumor promoter
    - Firefly luciferase gene
  - Exposure to estrogenic substances causes activation of ERE, which drives transcription of luciferase
  - Luminometer is used to quantify luciferase expression



Validation Organization

Study Management Team:

NICEATM

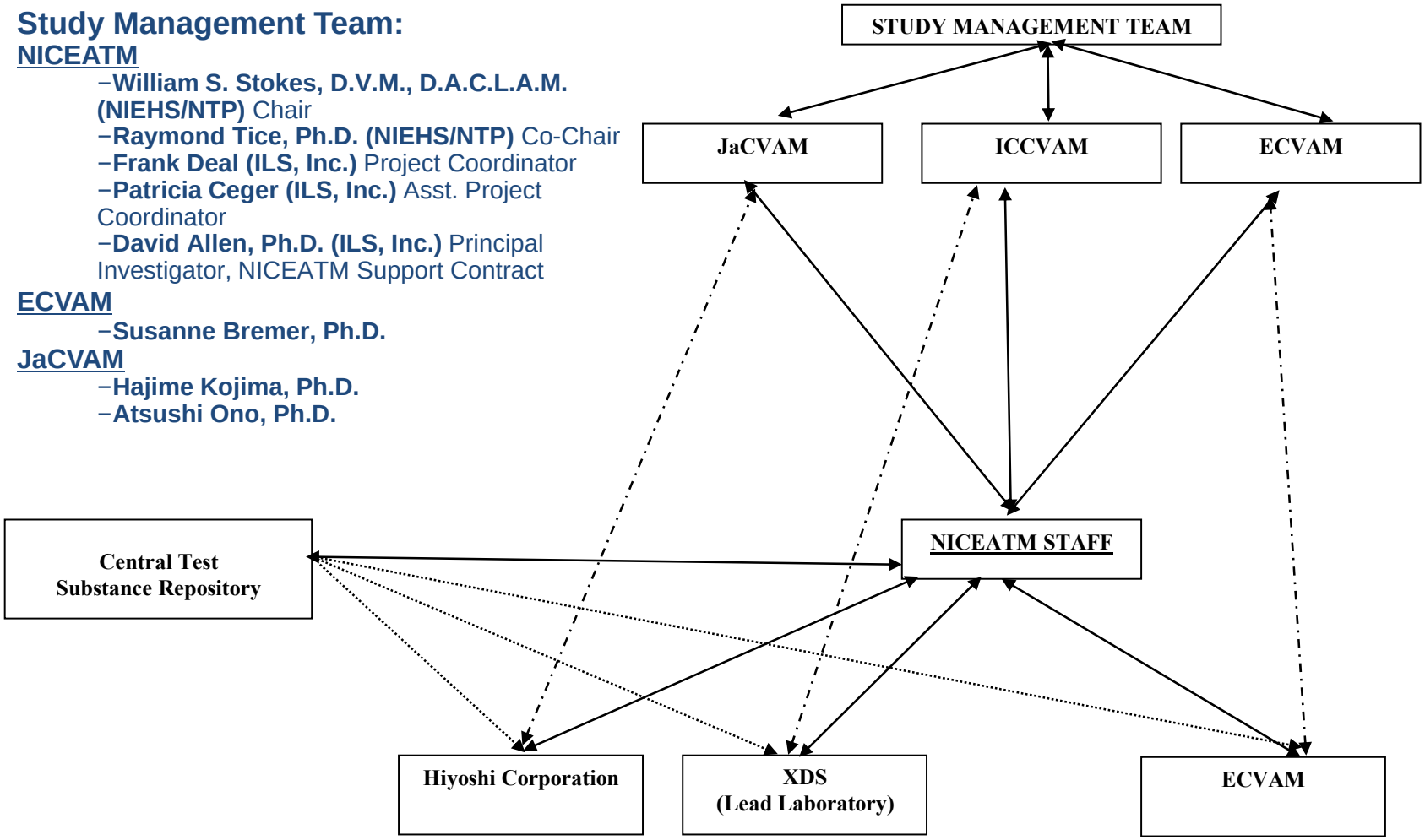
- **William S. Stokes, D.V.M., D.A.C.L.A.M. (NIEHS/NTP)** Chair
- **Raymond Tice, Ph.D. (NIEHS/NTP)** Co-Chair
- **Frank Deal (ILS, Inc.)** Project Coordinator
- **Patricia Ceger (ILS, Inc.)** Asst. Project Coordinator
- **David Allen, Ph.D. (ILS, Inc.)** Principal Investigator, NICEATM Support Contract

ECVAM

- **Susanne Bremer, Ph.D.**

JaCVAM

- **Hajime Kojima, Ph.D.**
- **Atsushi Ono, Ph.D.**



Solid lines indicate direct communication.
Dotted lines indicate shipment of test substances.
Dashed lines indicate indirect communication (e.g., CC'ed e-mails).

Study Design

STUDY PHASE	ACTIVITY
Phase I	Each laboratory conducts multiple testing of reference standards and controls to demonstrate proficiency with agonist and antagonist protocols, establish historical databases to be used to develop acceptability criteria for tests conducted in Phase IIa, and to provide measured for calculated reference standard and control data for an evaluation of intra- and inter-laboratory reproducibility.
Phase IIa	Four substances each from the ICCVAM recommended ER minimum list tested independently by each laboratory three times for agonist and antagonist activity.
Phase IIb	Eight substances each from the ICCVAM recommended ER minimum list tested independently by each laboratory three times for agonist and antagonist activity.
Phase III	Remaining 41 substances from ICCVAM recommended ER minimum list tested once by each laboratory for agonist and antagonist activity.
Phase IV	Remaining 25 substances from ICCVAM recommended ER list tested once each by the lead laboratory only for agonist and antagonist activity.

All participant Lab

Lead lab only

Phase I

Summary of Phase I

- No edge effects were detectable.
- Statistically significant differences were observed in intra- and inter-laboratory reference standard and control values.
- It was not possible to identify the causes for these differences but some of the contributing factors may be:
  - Lot-to-lot differences in cell culture media and tissue culture supplies (for intra- and inter-lab differences)
  - Differences in luminometers (for inter-lab differences)
- This underscores the importance of developing an historical control database for each individual laboratory.

Phase IIa

Four coded test substances covering a range of ER agonist and antagonist activities were each tested in at least three independent experiments for both agonist and antagonist protocols at each of the participating laboratories

Summary of Phase IIa

- **Agonist and Antagonist reference standard, control, and coded test substance data demonstrated the intra- and inter-laboratory reproducibility**
  - Reference standard and control data were reproducible within laboratories.
  - Classification of coded substances were almost consistent for all laboratory.
  - Statistically significant differences were observed in intra- and inter-laboratory reference standard and control values.
  - It was not possible to identify the causes for these differences but some of the contributing factors may be:
    - ✓ Lot-to-lot differences in cell culture media and tissue culture supplies (for intra- and inter-lab differences)
    - ✓ Differences in luminometers (for inter-lab differences)
  - This underscores the importance of developing and maintaining an historical control database for each individual laboratory
- **A large number of test plates failed one or more study acceptance criteria during Phase IIa.**
  - Based on the results of the qualitative and quantitative comparisons, the acceptance criteria was modified to improve failure rates without compromising the ability of the assay to detect and quantify test substance agonist or antagonist activity.

Phase IIb

Eight coded test substances covering a range of ER agonist and antagonist activities were tested in at least three independent experiments at each of the participating laboratories

Summary of Phase IIb

- Eight coded test substances covering a range of ER antagonist activities were tested in at least three independent experiments at each of the participating laboratories
- Classification of test substances as positive or negative for antagonism varied across the laboratories.
  - Results indicated interlaboratory differences in the maximum concentration selected for evaluation, based on differences in perceived solubility and/or cytotoxicity.
  - The SMT recommended to conduct additional assay for protocol modifications to better standardize these steps in the assay.
- Retest of compounds which was classified inconsistently within three laboratory is ongoing.