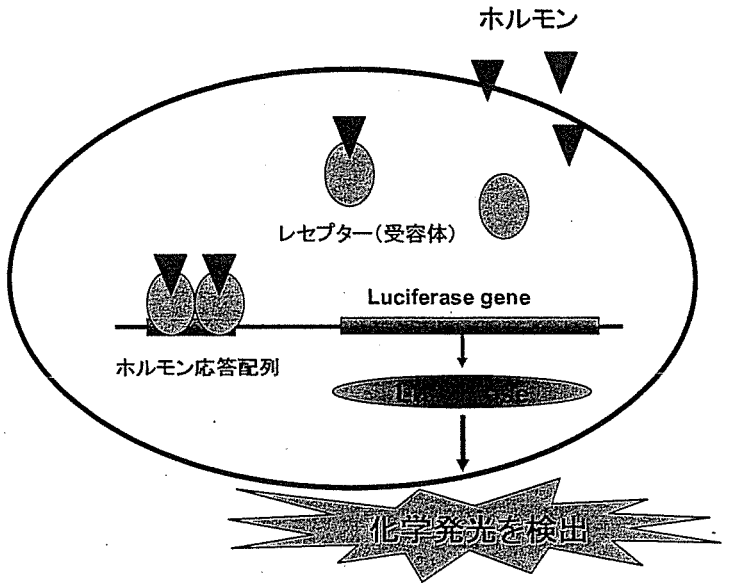
1. HeLa-9903細胞を用いたレポーター遺伝子アッセイ

原理



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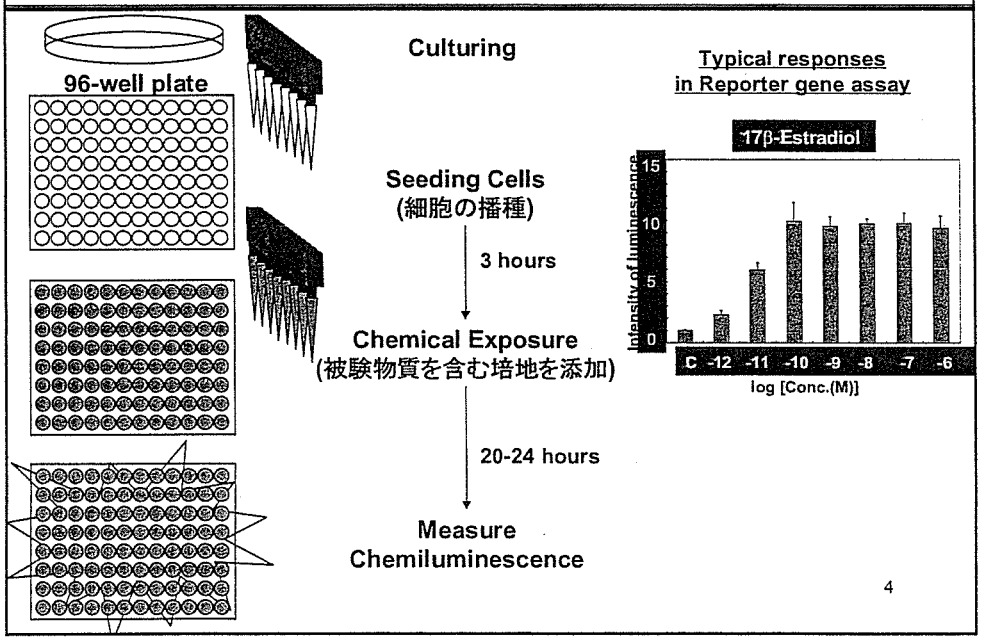
1. HeLa-9903細胞を用いたレポーター遺伝子アッセイ

アッセイ方法



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1. HeLa-9903細胞を用いたレポーター遺伝子アッセイ

HeLa-9903細胞について

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

   No functional ERα, ERβ, AR, TRα and TRβ in host cell

   (Confirmed by Mock transfection assay with each hormone responsive reporter construct)
5. **Infections**

   No mycoplasma infection was confirmed.

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1. HeLa-9903細胞を用いたレポーター遺伝子アッセイ

測定実績

試験名	試験系			プレスクリーニング物質数	バリデーション
レポーター遺伝子アッセイ	ERα	安定株	アゴニスト	約1600物質	マルチラボ実験済
			アンタゴニスト	約250物質	−
		一過性	アゴニスト	約300物質	マルチラボ実験済
	AR	安定株	アゴニスト	約900物質	マルチラボ実験済
			アンタゴニスト	約650物質	マルチラボ実験済
		一過性	アゴニスト	約250物質	マルチラボ実験済
			アンタゴニスト	−	マルチラボ実験済

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3. HeLa-9903細胞を用いた レポーター遺伝子アッセイの バリデーションについて

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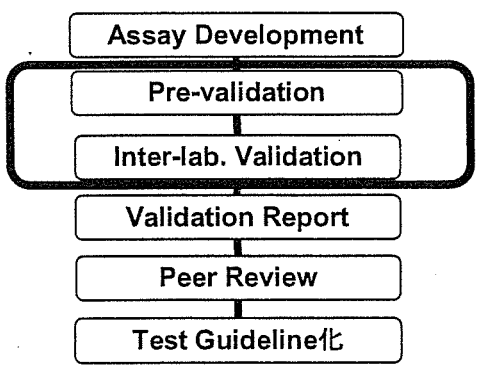
国際的な標準化のための手順 （OECDテストガイドライン化に向けて）

- OECDでは2003年に非動物試験法検証管理グループ会議 Non-animal Validation management group (VMG-NA)が組織され、種々の*in vitro*試験法について、検証試験 (バリデーション)を開始することが決定された。
- OECDテストガイドライン化に向けた作業の流れ



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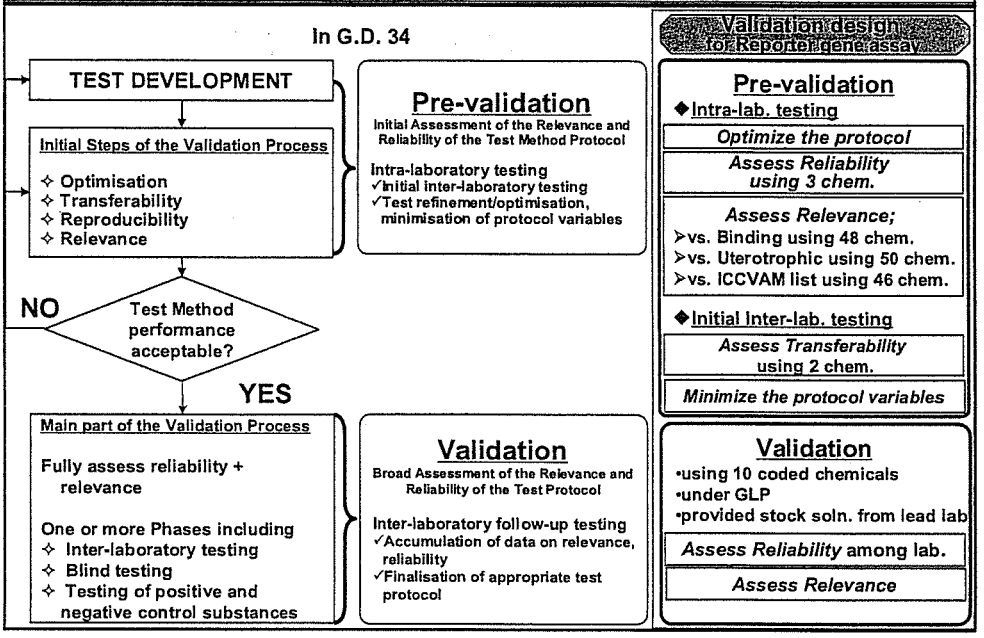
3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

バリデーションの概要



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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Intra-lab. testing [Response Stability of HeLa-9903]

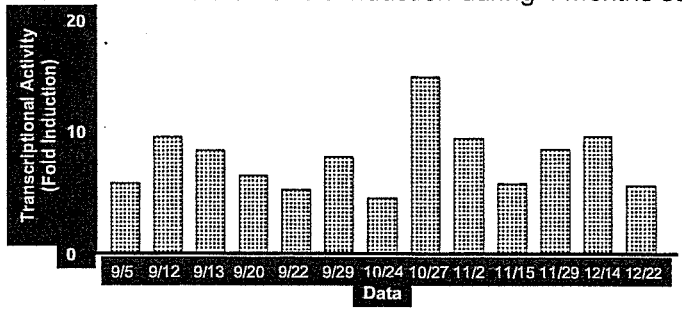
◆**Stability of the response using E2**

➢**Method;** Cells from 1-stock vial were continuously cultured by passage through 4-month (more than 30 passages) and measured fold-induction at 100 pM of E2.

➢**Results;**

✧No time dependent variation of fold-induction at 100 pM of E2.

✧Showed more than 5-fold induction during 4 months culturing.



→ **Sufficient response to E2 during continuous culturing.**

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Intra-lab. testing [Pre-Val. Reproducibility]

◆Reproducibility (13-run) using 3 chemicals

	CVs of Log[EC50(M)]	CVs of log[PC50(M)]	CVs of Log[PC10(M)]
17b-Estradiol	2.26	1.94	*
Bisphenol A	-	-	1.10
Testosterone	-	-	9.77

*:PC10 for E2 was not calculable in the concentration range 10^{-12} to 10^{-6} M.

→ Highly Reliable [Reproducible] !

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

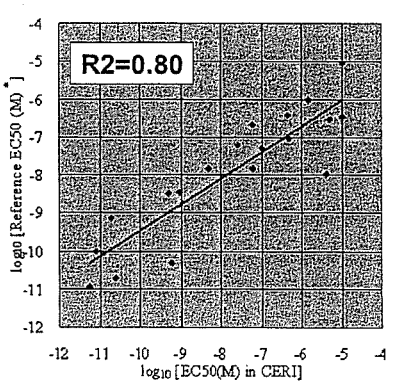
Accuracy (qualitative)

◆ vs. ICCVAM list using 22 chemicals

➢ **Chemical Selection;** From ICCVAM list (2002) which provide median EC50 from other *in vitro* mammalian cell reporter gene studies. Some chemicals in that list were excluded due to its availability or regulation problems.

Chemical	EC50(M) in CERI	Reference EC50 (M)*
Ethynyl Estradiol	5.68E-12	1.10E-11
Diethylstilbestrol	2.40E-11	1.89E-11
Alpha-Estradiol	6.04E-10	4.60E-11
Beta-Estradiol	8.17E-12	1.00E-10
Estriol	1.91E-11	7.10E-10
Estrone	4.89E-10	3.20E-09
Zearalenone	9.05E-10	3.43E-09
17alpha-Methyltestosterone	(4.11E-06)	1.08E-08
Beta-Zearalenol	4.79E-09	1.50E-08
Coumestrol	6.05E-08	1.50E-08
4-Tert-Octylphenol	1.01E-07	5.00E-08
Genistein	(2.45E-08)	6.20E-08
4-Nonylphenol	4.91E-07	9.45E-08
Testosterone,19-Nor	5.91E-08	2.12E-07
Daidzein	4.99E-06	2.90E-07
Phloretin	(4.95E-06)	3.00E-07
Levonorgestrel	(ca. 1.00E-05)	3.30E-07
Bisphenol A	4.55E-07	3.99E-07
Naringenin	(1.48E-06)	1.00E-06
Methoxychlor	(ca. 1.00E-05)	8.85E-06
Progesterone	-	-
Atrazine	-	-

*:http://ecvam.jrc.it/publications/publications_methods_endocrine_final_erta_brd_ERTA034505.pdf]



Good consistency with the reference EC50 reported in ICCVAM list (2002)

→Highly Accurate

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Note:EC50s of 6/22 chemicals were not calculated. The value in parenthesis are PC50.

3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Accuracy (quantitative)

◆ vs. ICCVAM list (2003) using 46 (Pos./Neg.=24/22) chemicals

➢ **Chemical Selection;** Selected from ICCVAM list (2003) which provide Pos./Neg. information.

		Reporter (PC50)		Total
		P	N	
Reference data	P	19	5	24
	N	4	18	22
Total		23	23	46

		Reporter (PC10)		Total
		P	N	
Reference data	P	23	1	24
	N	6	16	22
Total		29	17	46

	PC50	PC10
Concordance	**80%**	**85%**
Negative predictivity	78%	94%
Positive predictivity	83%	79%
False positive rate	18%	27%
False negative rate	21%	4%

→More than 80% concordance.

Note: The results using PC50 and PC10 were compared because EC50s of some chemicals were not calculated.

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Summary Results under Pre-validation [intra-lab. testing]

"Can provide information of mechanism of action ?" (Relevance)

◆ vs. Receptor binding assay using 48 chemicals

➢ **Chemical Selection;** From the list of EPA core chemical

➢ **Source of binding data;** Conducted at CERI using hERa-LBD

		Reporter (PC50)		Total
		P	N	
Binding	P	17	7	24
	N	4	20	24
Total		21	27	48

Concordance	**77%**
Negative predictivity	74%
Positive predictivity	81%
Sensitivity	71%
Specify	83%
False positive rate	17%
False negative rate	29%

→YES. Suitable tool to detect ER mediated effects.

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて
Summary Results under Pre-validation [intra-lab. testing]
"Can predict biological effects ?" (Relevance)

◆ **vs. immature rat Uterotrophic assay (s.c.) using 50 chemicals**

- **Chemical Selection;** Available results conducted at CERI at that time
- **Source of Uterotorophic assay Data;** Conducted at CERI according to the draft OCED test guideline under compliance with GLP

		Reporter(PC50)		Total
		P	N	
Utero.	P	30	3	33
	N	2	15	17
Total		32	18	50

Concordance	**90%**
Negative predictivity	83%
Positive predictivity	94%
Sensitivity	91%
Specify	88%
False positive rate	12%
False negative rate	**9%**

High concordance and Low false negative rate

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→ YES. Satisfactory performance to predict biological effects

3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて
Summary Results under Pre-validation (Transferability)

1. Distributed CERI protocol to all participating lab.

2. Run assay using E2 and BPA at each lab.

3. Checked the results

All participating lab. used Glo-type reagent to detect luciferase activity.
2/4 participating lab. could not provide sufficient fold-induction (x5).
→The problem was due to the difference of luminometer
→By changing the reagent type to flash-type, fold-induction at the participating lab. where failed to detect sufficient fold-induction was improved.

4. Minimized the protocol variable

CERI protocol were used at all participating lab. But the combination of luminometer and luciferase reagent was adjusted based on height of fold-induction.

Name of Participating lab.	CERI	Sumitomo	Otsuka Pharm.	Kaneka
Luminometer	Lumister [BMG]	Top-count [Packerd]	ARVO [PerkinElmer]	Top-count [Packerd]
Type of Luciferase Reagent	Flash [Promega]	Glo [Promega]	Flash [Promega]	Glo [Promega]

5. Started inter-lab. validation

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて
Design for Inter-lab. Validation

- 4 Japanese participating laboratories
- 3 repeated trials in triplicate for agonist assay using coded 10 chemicals
- Conducted under GLP

Chemical Name	CAS	RBA	Utero.	Category	Chemical Class
17b-Estradiol	50-28-2	100	P	Strong ER and AR agonist; AR antagonist	Steroid, phenolic Estrene
17a-Estradiol	57-91-0	80.1	P	ER agonist	Steroid, phenolic Estrene
Genistein	446-72-0	0.12	P	Weak ER agonist and antagonist	Flavonoid; Isoflavone; Phenol
Bisphenol A	80-05-7	0.195	P	ER agonist	Diphenylalkane; Bisphenol; Phenol
17a-Methyltestosterone	58-18-4	N.D.	P	ER and AR agonist	Steroid, non-phenolic; Androstene
4-*tert*-Octylphenol	140-66-9	0.124	P	ER agonist	Alkylphenol; Phenol
p-tert-Pentylphenol	80-46-6	0.0173	P		Alkylphenol; Phenol
Hematoxylin	517-28-2	0.0301	N		
Di(2-ethylhexyl)phthalate	117-81-7	0.071	N	Negative. ER binder	Phthalate
Benzophenone	119-61-9	N.B.	N		Benzophenone17

3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて
GLP Compliance

<u>This inter-lab. validation study was conducted in compliance with OECD GLP standard controlled by CERI QAU system.</u>

1. Inspection prior to start the study by CERI QAU
CERI QAU visited each lab prior to start the study and inspected items below;
- ✓ Implementation system
- ✓ Status of SOPs
- ✓ Management status of equipments, test substances, cells and reagents used in the study.

2. Quality assurance of data in each lab
- ✓ Conduct the study compliance with GLP standard
- ✓ All data obtained in the study were gathered and analyzed by CERI.
- ✓ Data Quality check by the responsible person in each lab and CERI.

3. Records
- ✓ All records are retained in each participating laboratory and CERI.

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Summary Results under Inter-lab. Validation ([Reproducibility])

Chemical Name	Log[PC10(M)]		Log[PC50(M)]		Log[EC50(M)]		ICCVAM		Utero.
	AVG.	CV	AVG.	CV	AVG.	CV	Class	Log[EC]50	
17b-Estradiol	-11.82	1.7	-10.79	1.8	-10.78	2.1	Pos.	-10.96	P
17a-Estradiol	-8.6	1.6	-8.93	3.9	-8.98	1.6	Pos.	-10.72	P
Genistein	-7.94	1.6	-7.34	1.5	-5.2	8.0	Pos.	-7.21	P
Bisphenol A	-7.14	3.7	-6.41	2.4	-6.13	2.2	Pos.	-6.40	P
17a-Methyltestosterone	-7.35	5.4	-5.79	5.8	-5.11*4	21.7	Pos.	-7.96	P
4-*tert*-Octylphenol	-7.98	2.3	-6.94	2.8	-6.82	3.5	Pos.		P
p-*tert*-Pentylphenol	-7.42	4.7	-6.13	3.5	-5.86	5.0			P
Hematoxylin	-8.23*1	-	Neg.		Neg	-			N
Di(2-ethylhexyl)phthalate	-6.26*3	-	Neg	-	Neg	-	Neg.		N
Benzophenone	-6.54*2	6.1	Neg	-	Neg	-			N

AVG. and CVs were among lab. from average of inter-day.
*1: detected once at one lab
*2: detected once at three labs
*3: detected once at one lab
*4: not calculate once at one lab and twice at another one lab

→ Highly reproducible among 4-lab.

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Multi-lab.Validation(実験系)後の作業

	内容
第1回VMG-NA (2003年3月)	・ Pre-Validationが終了し、Multi-Lab Validationを進める旨、報告。 ・ CERIがLeadすることが決まった。
第2回VMG-NA (2004年11月)	・ Multi-lab Validation結果を報告→Validation report及び標準化ﾌﾟﾛﾄｺｰﾙをOECDに提出することとなる。
第3回VMG-NA (2005年12月)	・ "Validated"といえる試験系かを審議・ｱﾄﾞﾊﾞｲｽする目的で、Preliminary validation assessment panel (PVAP) を実施することとなった。 ・ OECD (Dr. Miriam Jacobs) が事務局をすることで合意。
第3回VMG-NA後 (2006年1月)	・ 第3回VMG-NAの決定を受けてvalidation report案をPVAPメンバーに送付。 ・ 以後、 PVAPによる3回にわたる電話会議により試験法の事前評価が行われた。

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Preliminary validation assessment panel (PVAP)

- Dr. Miriam Jacobs (OECD call leader)
- Dr Jun Kanno (NIHS)
- Dr Hajime Kojima (JaCVAM)
- Prof. Daniel Dietrich (on behalf of ECVAM)
- Dr. Susan Laws (US EPA)
- Mr. Gary Timm (US EPA)
- Dr. Yutaka Aoki (ASPH Fellow at US EPA)
- Dr.Tim Schrader (Health Canada)
- Dr. William Stokes (NIEHS/NICEATM, ICCVAM)
- Dr. Ray Tice (NIEHS/NICEATM, ICCVAM)
- Ms. Patricia Ceger (ILS. Inc./NICEATM, ICCVAM)
- Dr. Frank Deal (ILS. Inc./NICEATM, ICCVAM)
- Dr. Masahiro Takeyoshi (CERI)
- Ms. Yumi Akahori (CERI)

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

Preliminary validation assessment panel(PVAP)による電話会議及びEDTA9の経過

回	内容
第1回電話会議（2006年2月）	・CERIよりPre-Validation及びMulti-lab. validationの概要及び結果を報告。
第2回電話会議（2006年3月）	・概ねOECD GD 34'の要求に沿ったValidationであったことで合意。 ・但し、ICCVAM及びECVAMのValidation criteriaには合致しない。
EDTA 9（2006年4月）	・PVAPでは「GD34に合致している」旨、OECD事務局から報告。 [EDTAでの合意事項] ・PVAPからのreport作成。 ・PVAP reportを用いて、Scientific Peer Reviewに向けたvalidation report作成（改訂）を日本で実施。 ・Scientific Peer Reviewの方法について日本が決定する。
第3回電話会議（2006年5月）	・改訂内容について概ね了承された。
以降の電子メール上でのやり取り	・ICCVAMは78物質の試験を要求。 ・一方、US-EPAは78物質の試験は不要との考えを示した。 ・ICAPOはECVAM/ICCVAMのCriteriaから外れている点についてもう少し審議する必要があるとの意見を表明。

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3. HeLa-9903細胞を用いたレポーター遺伝子アッセイのバリデーションについて

レポーター遺伝子アッセイのガイドライン化の進め方

- Standard Project Submission Form（SPSF）提出
- PVAP報告書の作成（Dr. Miriam Jacobs）
- Validation report of ER TA validation（CERI）
- Validation reportの国内レビュー
- Scientific Peer-reviewの進め方の決定
- OECDに『Validation report』+『Scientific Peer-reviewの進め方』を提出
- Scientific Peer-review実施
- Scientific peer-review報告書のWNTへの提出
- VMG-NA / EDTAにおけるガイドライン化への合意
- WNTでの承認

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1. HeLa-9903細胞を用いたレポーター遺伝子アッセイ

OECDのけるバリデーション

Level 1 Sorting and prioritizing based upon existing information	-Physical & chemical properties, e.g., M.W., 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 assay -Transcriptional activation -Aromatase and steroidogenesis in vitro -Aryl hydrocarbon receptor recognition/binding	-QSARs -High Through Put Screening -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 TG407 (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 data on effects from endocrine & other mechanisms	-1-gen assays (TG415 enhanced) -2-gen assays (TG416 enhanced) -reproductive screening (TG421 enhanced) -combined 28days/reproduction screening test (TG422 enhanced)	-Partical and full life cycle assays in fish, birds, amphobians & invertebrates (developmental and reproduction)

Draft summary minutes of teleconference meeting held on 17 March 2006: 1.30 pm Paris; 7.30 am USA/Canada; 9.30 pm Japan

The preliminary validation assessment panel of the 'Japanese multi-laboratories validation study of a stably transfected ERα mediated reporter gene assay in Japan'.

Second teleconference call.

Call participants:

1. Dr. Yumi Akahori (CERI)
2. Dr. Masahiro Takeyoshi (CERI)
3. Dr Jun Kanno (NIHS)
4. Dr Hashime Kojima (JaCVAM)
5. Prof. Daniel Dietrich (on behalf of ECVAM)
6. Dr. Susan Laws (US EPA)
7. Mr. Gary Timm (US EPA)
8. Dr. Yutaka Aoki (ASPH Fellow at US EPA)
9. Dr.Tim Schrader (Health Canada)
10. Dr. Bill Stokes (NIEHS/NICEATM)
11. Dr. Ray Tice (NIEHS/NICEATM)
12. Ms. Patricia Ceger (ILS. Inc./NICEATM)
13. Mr. Frank Deal (ILS. Inc./NICEATM)
14. Dr. Miriam Jacobs (OECD call leader)

Opening of teleconference

1. Apologies: Prof. Bob Combes sent his apologies.

2. Introductions: Dr Jun Kanno (NIHS) and Dr Hashime Kojima (JaCVAM) were welcomed as full participants in the preliminary validation assessment panel.

3. Draft agenda approved: but with item 5. Discussion of 'Issues regarding log EC 50' moved after item 6. Presentation regarding updates from 1st teleconference.

4. Draft summary of first teleconference on 6 February 2006: approved.

5. Update regarding outstanding action points from first teleconference: Drs Yumi Akahori and Masahiro Takeyoshi: presentation 'Updates from 1st teleconference' Dr. Yumi Akahori gave the first half of the presentation, and Dr Masahiro Takayoshi followed with the second half.
The presentation was supported by the following submitted documents (to be attached as appendices for final agreed telecon summary):

- 5.1. Checked list
- 5.2 Monitoring of cytotoxic effect of chemicals in reporter gene assay by Dr Takeyoshi (15/3/06),
- 5.3. Outline of ER α antagonist assay using hER-HeLa-9903 by Dr Takeyoshi (15/3/06),
- 5.4. Characterisation of hER-HeLa-9903.

It was emphasised that although there might be further applications, the assay was primarily designed to provide mechanistic information. There was also concern expressed by CERI with respect to the number of chemicals tested, that this should probably be more extensive, and that the triplicates were not always repeated.

Following the presentation, general queries were addressed following the action items addressed by CERI during the presentation, and also outstanding from the first teleconference.

6a Fold induction.

Raw fold induction data is available for the positive controls and also for the chemicals assessed under the pre validation stage. Dr Takeyoshi can provide this.

Action item: CERI will provide raw fold induction data for the positive controls and for the chemicals assessed under the (pre) validation stage from data generated by the CERI laboratory and the other laboratories where possible.

This was not provided at this teleconference, so will be provided for the next teleconference. It was explained that the panel required this information to assess the extent of the variation in fold induction over time.

6b Action Item: CERI is to compare the information presented in their submission with the guidelines set forth by ICCVAM in *ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors* (NIH Pub. No. 03-4503), and OECD GD 34, and state rationale for deviations from these guidelines.

6c Action Item: CERI to provide further information on cell line characterisation, methods of cytotoxicity evaluation, and antagonist testing.

6d Action Item: CERI to submit antagonist assay protocol and data to OECD for circulation

Discussion began first with concerns regarding the number of chemicals tested. For statistical purposes it was recommended that in order to a get good grasp of assay reliability for each class of chemicals, a minimum of three chemicals is required for each class. Also that 'difficult' chemicals should be included (but were not), to address for example solubility and cytotoxicity responses. A total of 10 chemicals were tested, therefore with 2 to 3 chemicals for each *class* of chemicals for 4 classes. Dr. Akahori pointed out that 5 chemicals classified as "weak" were tested and so were 3 "negatives." While for these particular classes the number of chemicals satisfies the minimum requirement, for the remaining classes, the chemical class-specific information on reliability is somewhat limited. It is therefore suggested that the best course of action at this point is to assess the repeatability of each chemical class by obtaining the chemical class-specific estimation of between- and within-laboratory variations, and examining any statistical evidence that they differ across chemical classes. If they do not, then estimate the variations common to all applicable chemical classes, assuming the true levels of variations are comparable across chemical classes.

Before doing this, the intra (within-lab) and inter (between-lab) laboratory variations require reassessment as currently they are overestimated in the draft report.

(A further suggestion is the use of the terms 'inter' and 'intra' be replaced by 'within-lab' and 'between-lab' only to reduce aural misunderstandings during teleconferences).

Action Item: Yutaka Aoki (US EPA) will provide information on proposed methods for between- and within-variation estimation to the whole group and consult with CERI on how to proceed.

Discussion followed concerning the use of the concordance, sensitivity and specificity (and which was updated after the teleconference). It was recommended that sensitivity and specificity be the primary endpoints, and that the use of concordance as a summary measure of the sensitivity and specificity should be avoided unless a caveat is included stating that here the term concordance is used to mean the weighted average of sensitivity and specificity, with weights being the prevalence of the other two, and that prevalence is not a well defined concept in this example. The reason for this is that here true positives and true negatives have been chosen arbitrarily, so prevalence does not have real meaning. As such, the

concordance here is a function of an arbitrary number (prevalence). Sensitivity and specificity values are far more accurate terms to use, as they are not influenced by an arbitrary level of prevalence.

While previous discussion indicated that concordance, sensitivity, and specificity be used (hence the provision of this information by CERI), after due consideration, it is recommended that sensitivity and specificity be used primarily. The panel extend their apologies to CERI on this matter.

A discussion followed concerning maximum concentrations that can be realistically tested. ICCVAM's expert panel recommend a maximum concentration of 1mM which is extremely high for cellular systems, and it was agreed that for practicality, one does not really need such a high response if a full dose response curve is obtained at a lower dose, or depending upon the reasons for conducting the assay or if practical reasons such as solubility/cytotoxicity preclude it.
However it was agreed that for the negative substances used here (where negative is defined as no observed transcription in this particular assay system), information/justification should be provided on solubility and the maximum concentration used. This is because in some instances, higher doses have been shown to be positive in other cellular assay systems. This should therefore help explain where negative data is discordant with that published in the literature (as seen with nonylphenol for example, which is positive at higher concentrations) and identify limitations of the test, or possibly the literature.

Was ICI 182 780 used to inhibit effects seen to verify if the result was ER mediated?

Action Item: For the negative substances used here, information and justification should be provided on solubility and the maximum concentration used.

Cytoxicity.
Questions were raised about the cytotoxicity tests, and whether the control cells were the same as those used for assay purposes. It was explained that the same basal cell line had been used to develop both the experimental cell line and one for monitoring and testing cytotoxicity, and that the tests did not need to be run at the same time.

From the summary information provided on the ERα antagonist TA assay (document 5.3) it was noted that the vehicle and positive controls were placed on the far edge of the plate. The question was therefore raised about assessment of edge effects, by dosing test plates with all vehicle controls and another with all positive controls to assess any variation for both controls. CERI informed the participants that the plate layout was different for the ERα agonist TA assay. CERI confirmed that they had assessed edge effects and it was not a concern. The panel asked to see the raw data, and CERI said that this could be provided for their laboratory, but not necessarily the other participating laboratories.

Action Item: CERI to provide raw data on edge effects, and to request, where possible such data from the other participating laboratories.

Action Item: CERI to submit antagonist assay protocol (SOP) and raw data to OECD for circulation.

GLP. Although preferable, GLP and GCCP were not considered to be an issue of major concern, so long as the laboratory practice was clear and transparent. However an internal audit was requested, such that all data transcription is double checked by an additional operator (as with QA in GLP), to ensure error reduction. CERI said that this had not yet been conducted.

Action Item: CERI to conduct an internal audit of data transcribed.

Discussion with respect to the Prediction Model (PM) and the Data Interpretation Procedure (DIP).

The applicability of the Prediction Model (PM) concept, that is the relevance of the assay to the applicability domain(s) of the chemical universe that the test can be applied to, was not planned for this domestic validation at the outset, and does not appear to be possible on the basis of the chemicals selected for testing in this reporter gene assay domestic validation. This may have implications for similar reporter gene assays that may be taken forward for validation assessment at the OECD level. Concern was expressed at setting a precedent that did not comply with the more stringent PM validation requirements considered by both ICCVAM and ECVAM to be an essential component of a successful validation exercise. Further, that this means the current assay does not meet ICCVAM and ECVAM regulatory requirements in the formal sense.
Relevance with respect to the DIP, can however be established on the mechanistic knowledge of the broadly-defined "estrogenic effects", as proposed by CERI, although the panel felt that some supplemental analyses on relevance based on comparison of this assay to other "semi-gold standard" assays would be useful, particulary with reference to sensitivity and specificity.

6. Dr. Yutaka Aori: presented a paper describing 'Issues regarding log EC50' for information and discussion (paper forwarded by email 8 March 2006 and will be an appendix to the agreed summary of this telecon).
It was agreed that there was a need to continue the discussion on the potential usefulness of logPC10 and logPC50 over logEC50, relative potency measures and definition of a positive chemical based on these or other measures including a traditional LOAEL. Preliminary suggestions on the best approach for the relative induction potency, is to use difference between logPC10 for estradiol and logPC10 for a test chemical, which is similar to logIC50-based RBA for the estrogen receptor (ER).
It was suggested that a face-to face meeting attached to the next OECD VMG-NA meeting would be useful to discuss this. However this would be difficult to accommodate with respect to attendance and timeliness. Instead it was requested that as the participating statistician, Yutaka Aoki might expand the two page document presented into a more comprehensive document describing these issues and potential solutions, such that this group as a whole can have a detailed discussion based on it in the future. While such a document is already intended for US EPA and peer reviewed literature purposes, producing a paper specifically for circulation to this panel will require further consideration by Dr Aoki, with later confirmation of an appropriate timescale.
Further statistical concerns included the size of error bars and classification of positives and magnitude of response. CERI confirmed a positive to be a PC 10 value (three fold increase above vehicle control).

It was agreed that this required further exploration, to achieve consensus on an agreed statistical uniformity/consistency with particular respect to reporter gene assays (beyond this study) between countries and individual regulatory bodies, and that this should be the sole subject of a future teleconference, date as yet unconfirmed.

7. A.O.B

7.1. The panel were informed that the secretariat will give a summary presentation of the progress of this panel to the EDTA meeting in April and WNT meeting in May. The presentation will be based on the summaries of the first two teleconferences and a draft ppt will be circulated prior to the EDTA meeting to the participants, for input. The presentation will be prepared in the beginning of the week of the 18 April and sent out by mid week. Please note that comments on the slides will need to be received by 21 April 2006 if they are to be included in the presentation.

7.2. Further to the AOB item included in the first teleconference, ICCVAM indicated that 78 chemicals were reviewed in the revised ICCVAM Endocrine Disruptor Reference Substances list, and of these, 6 chemicals had limited or no availability so had been replaced.

Comments from the participants of the teleconference and colleagues are invited by 1 May 2006 on proposed revisions to the ICCVAM list of recommended substances for the validation of in vitro estrogen and androgen receptor binding and transcriptional activation originally published in May of 2003 (ICCVAM Evaluation on In Vitro Methods for Detecting Potential Endocrine Disruptors, NIH Publication No. 03-4503, available at http://iccvam.niehs.nih.gov/methods/endodocs/edfinrpt/edreport.htm). An addendum to this NIH publication describes the rationale for proposed revisions to the recommended substance list and is available at http://iccvam.niehs.nih.gov/methods/endodocs/EDAddendum.pdf.

ICCVAM would also appreciate receipt of any data from completed in vitro studies (2002 - present) using or evaluating ER and AR binding or TA assays, or information about ongoing or planned studies using or evaluating these test methods. They would also appreciate receipt of data from in vivo studies that have evaluated ED activity of these substances (e.g., uterotrophic, Hershberger, intact male or male/female pubertal assays).

"Official" notice of this request for comments and data was published on 16 March in Federal Register Notice Vol. 71, No. 51, pp. 13597-8, and is available at http://iccvam.niehs.nih.gov/docs/FR/7113597.pdf.

Comments or data should be sent to:
Email to niceatm@niehs.nih.gov,
Fax to 919-541-0947, or mail to:
Dr. William Stokes
NICEATM, NIEHS
79 T.W. Alexander Drive
PO Box 12233
MD EC-17
Research Triangle Park, NC 27709
US

The secretariat suggested that ICCVAM might like to put this information forward to the US National Coordinator so that the latter can inform the EDTA and WNT meetings of this request for comments and data.

8. Next Meeting:

The call leader set the data of the next teleconference for Friday, May 19th, 2006 from 1330 to 1530 (Central European Time), 12:30 to 14:30 (UK time), 7:30 am to 9:30 am (USA/Canada), and 9:30 pm to 11:30 pm (Japan).

9. Adjournment

The call leader adjourned the meeting at 3.40pm (CET).

10. Subsequent developments

10.1 The membership of this panel has grown rather large, and a little unbalanced with respect to numbers of persons with validation expertise representing different bodies. The secretariat therefore recommends that the numbers of such persons for each of the different participating bodies, during the teleconferences, is reduced and/or maintained at two persons. This will improve the balance in representation across the participating bodies and improve manageability of the teleconference procedures.

10.2 Overall, the feeling from the Japanese participants for the domestic validation of this ERα reporter gene assay is that they consider that the current status of the assay is sufficient to be taken forward for official independent scientific peer review with respect to pre-screening for ERα mediated ED effects, and that this recommendation could be made to the next WNT meeting in May 2006.
Queries with respect to protocol optimisation, chemical selection, data analyses with sufficient statistical power for the assay, and relatively minor and non essential questions regarding inter (or between) laboratory assessment of making up the chemicals in stock solution are in the process of being addressed as far as reasonably possible. From a retrospective point of view, taking the validation data generated together with the extensive data set conducted by CERI in-house using this assay (which is generally in concordance with that from other published ERα mediated in vitro assays), they consider that this assay is robust.
However it must be noted that major deviation from the ICCVAM and ECVAM validation requirements will mean that the assay will not be considered by these validation bodies as correctly and formally validated for regulatory use.

Addressing the action items identified so far described by the teleconference participants, together with the creation of a smaller steering panel (membership to be discussed with the Japanese participants) may help facilitate a successful outcome of the independent scientific peer review, within a given time frame.

Draft summary minutes of the third teleconference meeting held on 19 May 2006: 1.30 pm Paris; 7.30 am USA/Canada; 8.30 pm Japan

The preliminary validation assessment panel of the 'Japanese multi-laboratories validation study of a stably transfected ERα mediated reporter gene assay in Japan'.

Third teleconference call.

Call participants:

1. Dr. Yumi Akahori (CERI)
2. Dr. Masahiro Takeyoshi (CERI)
3. Dr Jun Kanno (NIHS)
4. Prof. Daniel Dietrich (on behalf of ECVAM)
5. Dr. Susan Laws (US EPA)
6. Dr. Yutaka Aoki (ASPH Fellow at US EPA)
7. Dr.Tim Schrader (Health Canada)
8. Dr. Bill Stokes (NIEHS/NICEATM)
9. Dr. Ray Tice (NIEHS/NICEATM)
10. Dr Patric Amcoff (OECD Secretariat)
11. Dr. Miriam Jacobs (OECD call leader)

1. Welcome: Opening of teleconference

2. Apologies: Dr Hashime Kojima, Prof. Bob Combes and Gary Timm sent their apologies.

3. Draft agenda approved.

4. Draft summary of second teleconference on 17 March 2006: approved.

5. Update from the 9th EDTA and 18th WNT regarding the activities of this panel and the preparation of the preliminary validation assessment report: Patric Amcoff

Dr Patric Amcoff began by thanking all the participants of the panel for participating in this important activity of the Test Guideline programme work plan. He then gave a brief overview of the inception of this preliminary assessment panel at the VMG-NA last December. He emphasised that the panel is informal and unofficial, as member countries did not make official nominations for panel membership.
Using GD 34 criteria as a basis, the primary tasks or charges of the panel are to assist the Japanese in a transparent manner in assessing whether there is sufficient information on the domestic validation to submit a report for independent scientific review, with the independent review procedure to be agreed by the Japanese.
The completion of the ensuing official report from this panel will provide the first step in the process. This report will outline our discussions, the steps taken in the validation assessment, and include summary statements from participants (Appendices 1 and 2 attached give the draft views of NICEATM.) This initial report will help in the further assessment and validation development work for this assay.
It will be under the auspices of the individual expertise of the participants, and will therefore reflect their expert opinion, and not that of the organisations in which the experts are employed.
The second step will include writing of a comprehensive validation report, or peer charge, using the preliminary peer review report as a basis.
There is a possibility that the Secretariat (Dr Miriam Jacobs) will be able to assist with this at the end of June, depending on the successful outcome of a visiting scientist Japanese travel funding application.

For the third step, the Japanese will need to decide how to go ahead with the formal peer review process. Three routes for the organisation of independent peer review were identified and discussed at the 9th EDTA and 18th WNT:

1. via a contract house
2. via the OECD secretariat
3. by member country competent authority (e.g. ICCVAM or ECVAM).

With the fourth step, the Japanese will need to submit a standard submission form (SPSF) to the Secretariat for consideration by the WNT, should the assay be ultimately considered by the Japanese to be appropriate for submission to the test guideline process.

6. Update regarding outstanding action points from second teleconference: Dr Yumi Akahori: presentation 'Updates from 2nd teleconference'. The presentation was supported by the following submitted documents (to be attached as appendices for final agreed telecon summary):

6.1 Action item: CERI will provide raw fold induction data for the positive controls and for the chemicals assessed under the (pre) validation stage from data generated by the CERI laboratory and the other laboratories where possible.
Files provided by CERI: Pre-validation fold-induction.zip; Multi-lab fold-induction.zip; ERalpha antagonist Prevalidation to OECD.zip.

6.2 Action Item: For the negative substances used in the validation study, information and justification should be provided on solubility and the maximum concentration used.

The following data for three compounds were provided.

	Hematoxylin	Di(2-ethylhexyl)phthalate	Benzophenone
Preparable max. conc. in DMSO	>1 M	100 mM	>1 M
Solubility in assay medium	>1 mM	Not soluble at 100 uM	>1 mM
Control cell assay	Decrease of luminescence more than 100 uM.	-	Not cytotoxic at 1 mM
Overall conclusion of testable conc.	10 uM	10 uM	Could be tested up to 1 mM

Discussion followed with concern again expressed that for some substances classified as negative; they had not been tested at 1mM so that very weak agonists might not be detected. A counter argument was that such doses may be unrealistic for physiological purposes, even in an extreme exposure situation. The medium is considered to be equivalent to the *in vivo* situation.
The question was asked to what extent does one need to identify very weak agonists or antagonists. By testing at higher doses, the EC50 can be measured, and is particulary appropriate for prioritizing for testing. However it was pointed out that the US EPA (and other regulatory authorities and agencies) would never prioritize based on just one assay, rather on the basis of a battery of tests.
It was further pointed out that one cannot control what the test might be used for, and that it would be constructive to consider more long term planning, particularly with the 3R's in mind. A robust and broad testing strategy would be ideal. A short discussion on the US EPA tier 1 and tier 2 ED screening program followed. While this assay falls under tier 1 screening, identifying substances for further testing, provision of data evaluating the ability of the test method to predict *in vivo* ED effects would be of great prospective

value particularly, by allowing better characterization of the ability of this test method to reduce animal use in ED testing.
In the protocol it could be indicated that it may be possible go higher than 10mM, but some substances could also be inducing other effects that might impact upon and increase the chemical luminescence. Dr Susan Laws mentioned that the EPA had collected such QA data on a contract basis and that she might be able to provide further information. Prof Dan Dietrich also mentioned that Dr Jean-Claude Nicholas of INSERM had conducted similar work, and the Secretariat agreed to contact him to explore this issue further.
New Action item: Dr Laws and the Secretariat to pursue data sources providing information on possible substance impacts upon chemical luminescence that are not transcription related.

6.3 Action Item: CERI to provide raw data on edge effects, and to request, where possible such data from the other participating laboratories. *Files provided by CERI: Raw data on edge effects: Edge effect to OECD.pzf (including raw data and analyzed data)*

CERI provided CERI laboratory data on edge effects conducted after the 2nd teleconference. CERI considered that there was no edge effect affecting the final results. Data from the other participating laboratories was not available.
This was discussed further, both during and after the teleconference.
At the edge of the plate the wells may suffer from humidity effects and evaporative loss, and the conditions of incubation at the CERI lab are the same as that generally found in other laboratories.
Dr Yutaka Aoki assessed the data and noted higher signals of the edge wells, compared with the central wells, although it was agreed with CERI that these would not affect the final result.
For this reason it would be preferable to document the overall CV, and even conduct a formal analysis to see that these results do not affect the final data. It was agreed that as long as the CV for the whole plate is less than 3% with clear dose response, then the edge effects could be considered not to affect the final data for practical purposes, and that this should be clearly stated in the protocol and monitored by individual laboratories. Criteria for rejecting a plate above 3% should be stated in the protocol.

6.4 Action Item: CERI to submit antagonist assay protocol (SOP) and raw data to OECD (for circulation prior to the conference call).
This was submitted and with panel agreement will be attached as an appendix to the final agreed minutes.
It was noted that no positive control was present for antagonistic activity, although a large data set is available for the antagonists, and the data can be released. It was not conducted by three laboratories, however it was proposed that extrapolation might be possible by consideration of the validated agonist protocol as the assay is almost the same. The data is currently available on the CERI website in Japanese but can be prepared and sent out to the participants.
New Action item: Provision of antagonist data by CERI to panel participants.
Concern was expressed that from a regulatory standpoint not having the antagonist data would mean that a binding assay would need to be used also to check whether there was transcription or not for an adequate ER alpha screening tool.
A compromise was suggested such that at a later time point the currently validated protocol could be updated and extended in a catch-up manner, with the validated antagonistic protocol as and when such a protocol might be supported and made available (within a year or so). However for the present, the progression of this test should continue, as other similar assays are not so close to being validated and independently scientifically reviewed. It was generally preferred that there is no delay with moving the assay forward now.
Provision of the range of antagonistic data in the report for independent peer review submission, which shows that the antagonistic assay is working well, will be of great value to the reviewers.