

# ***In vitro* assays for endocrine disruptors: test performance and steps to validation.**

*Witters Hilda*



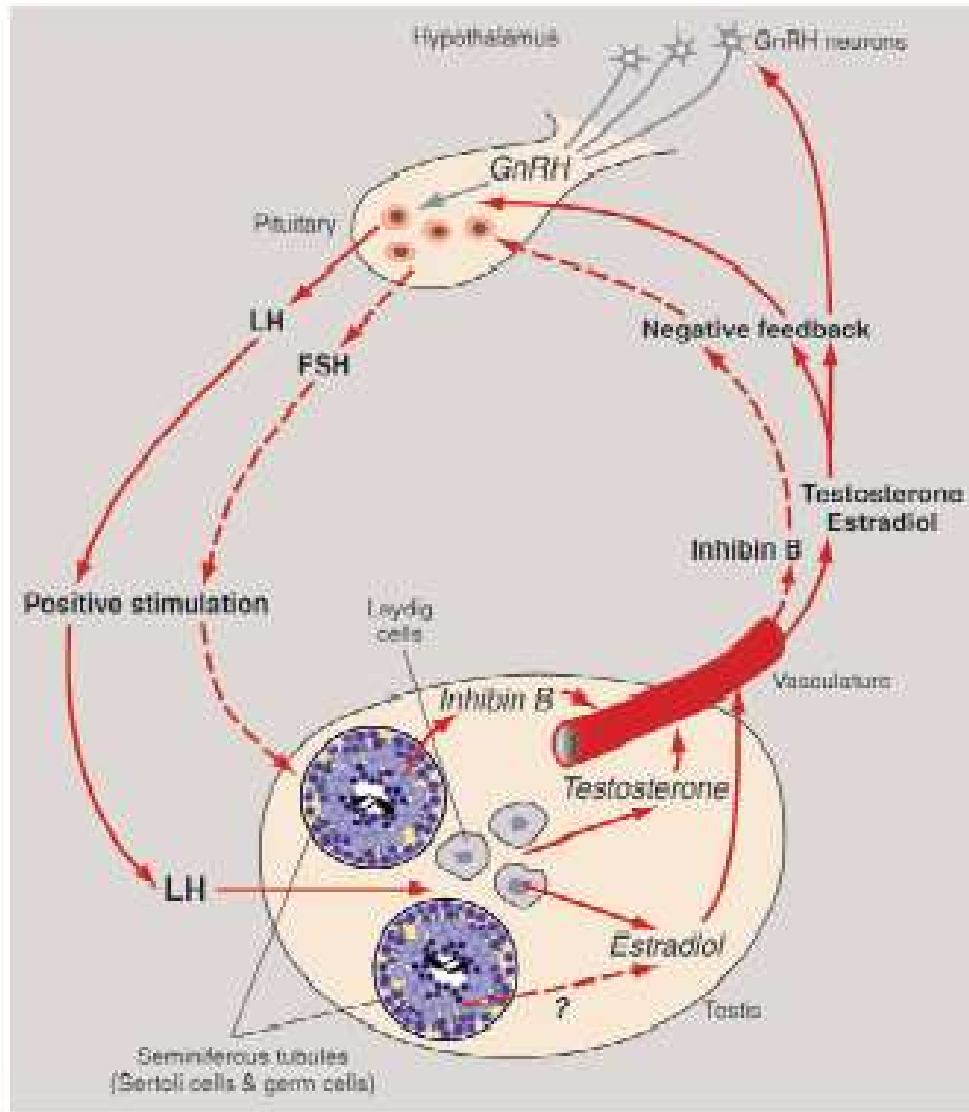
- Background ED
- Validation of *in vitro* tests
- ER transactivation assay
- Conclusions and next steps



- **Man-made chemicals & natural compounds**
  - Interfere with endocrine system
  - Adverse effects on wildlife and humans



*The endocrine system*



Source: WHO/IPCS,2002



- Man-made chemicals & natural compounds
- **Testing needs**
  - US EPA → EDSP (1998)
  - OECD → conceptual framework for testing (2002)
  - tiered testing programme



**Note:** Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

## OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

<p><b>Level 1</b> Sorting &amp; prioritization based upon existing information</p>	<ul style="list-style-type: none"> <li>- physical &amp; chemical properties, e.g., MW, reactivity, volatility, biodegradability,</li> <li>- human &amp; environmental exposure, e.g., production volume, release, use patterns</li> <li>- hazard, e.g., available toxicological data</li> </ul>	
<p><b>Level 2</b> <i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> <li>- ER, AR, TR receptor binding affinity</li> <li>- Transcriptional activation</li> <li>- Aromatase and steroidogenesis <i>in vitro</i></li> <li>- Aryl hydrocarbon receptor recognition/binding</li> <li>- QSARs</li> <li>- High Through Put Prescreens</li> <li>- Thyroid function</li> <li>- Fish hepatocyte VTG assay</li> <li>- Others (as appropriate)</li> </ul>	
<p><b>Level 3</b> <i>In vivo</i> assays providing data about single endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> <li>- Uterotrophic assay (estrogenic related)</li> <li>- Hershberger assay (androgenic related)</li> <li>- Non-receptor mediated hormone function</li> <li>- Others (e.g. thyroid)</li> </ul>	<ul style="list-style-type: none"> <li>- Fish VTG (vitellogenin) assay (estrogenic related)</li> </ul>
<p><b>Level 4</b> <i>In vivo</i> assays providing data about multiple endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> <li>- enhanced OECD 407 (endpoints based on endocrine mechanisms)</li> <li>- male and female pubertal assays</li> <li>- adult intact male assay</li> </ul>	<ul style="list-style-type: none"> <li>- Fish gonadal histopathology assay</li> <li>- Frog metamorphosis assay</li> </ul>
<p><b>Level 5</b> <i>In vivo</i> assays providing data on effects from endocrine &amp; other mechanisms</p>	<ul style="list-style-type: none"> <li>- 1-generation assay (TG415 enhanced)<sup>1</sup></li> <li>- 2-generation assay (TG416 enhanced)<sup>1</sup></li> <li>- reproductive screening test (TG421 enhanced)<sup>1</sup></li> <li>- combined 28 day/reproduction screening test (TG 422 enhanced)<sup>1</sup></li> </ul> <p><small><sup>1</sup> Potential enhancements will be considered by VMG mamm</small></p>	<ul style="list-style-type: none"> <li>- Partial and full life cycle assays in fish, birds, amphibians &amp; invertebrates (developmental and reproduction)</li> </ul>

- Man-made chemicals & natural compounds
- Testing needs
- ***In vitro* mechanistic studies**
  - Extended panel of tests, reviewed (ICCVAM; OECD)
  - Receptor based tests → recommended for validation



*Study design: ED*

**Management team**

B. Stokes (ICCVAM/US)  
P. Pazos/M. Jacobs (ECVAM/EU)  
A. Mantovani (ISS/IT)  
C. McArdle (Univ. of Bristol, UK)

**Chemical Selection**

K. Aschberger (ECB)

**Protein binding assays**

**ER binding assay (Pan Vera)**

1. lab: Research Triangle Park/US EPA
2. lab: Bayer/ Germany
3. lab: CERI/Japan

**AR binding assay (Pan Vera)**

1. lab: Bayer/ Germany
2. lab: Research Triangle Park/US EPA
3. lab: Institute of Applied Microbiology /A

**Transcriptional activation assays**

**MELN (MCF-7 cells)**

1. lab: **Vito/B**
2. lab: Bayer/D
3. lab: NN

**ER-Calux (USO2 cells)**

1. lab: Bio Detection System/NL
2. lab: **Vito/ B**
3. lab: Bayer

**PALM (PC-3 cells)**

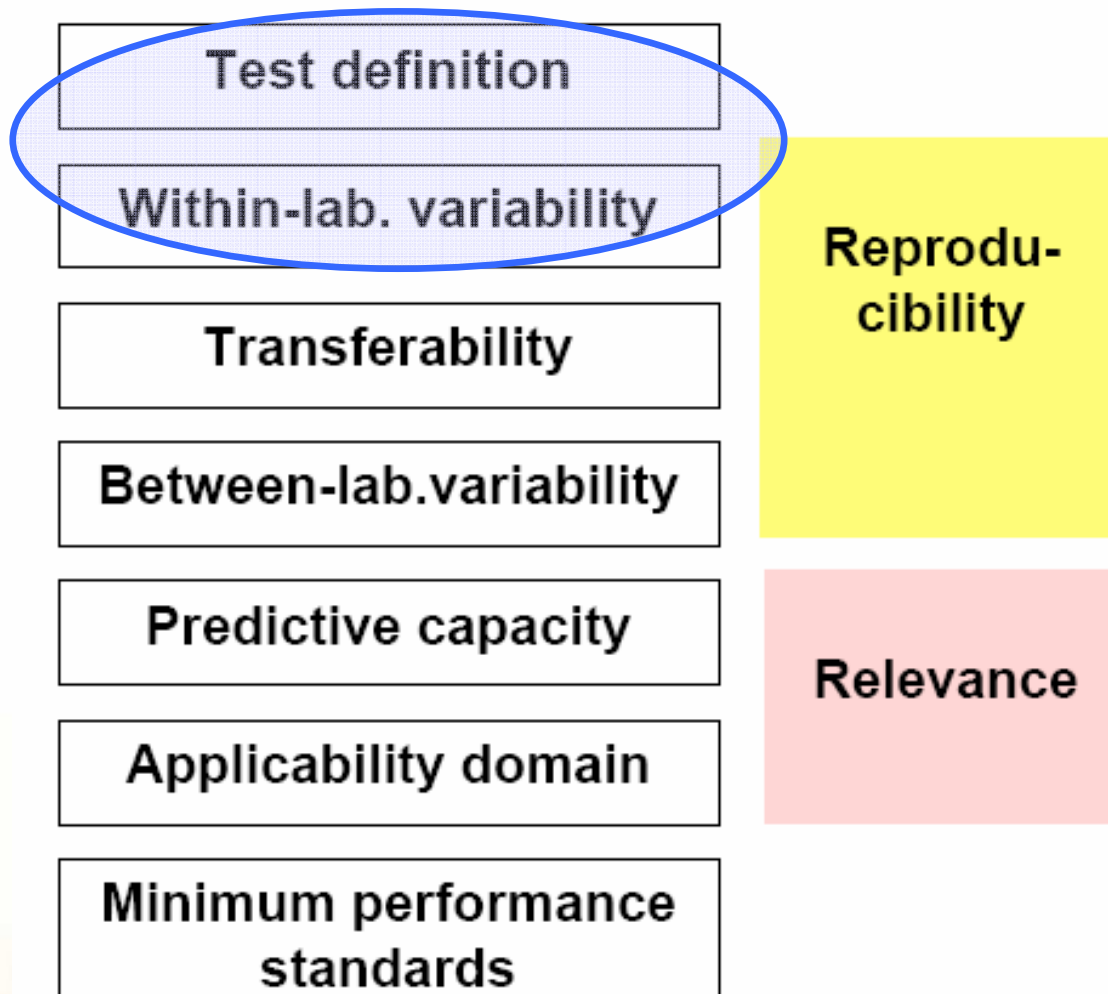
1. lab: Bayer/D
2. lab: **Vito/B**
3. lab: NN

**AR-Calux (USO2 cells)**

1. lab: Bio Detection System/NL
2. lab: **Vito/ B**
3. lab: Bayer/D

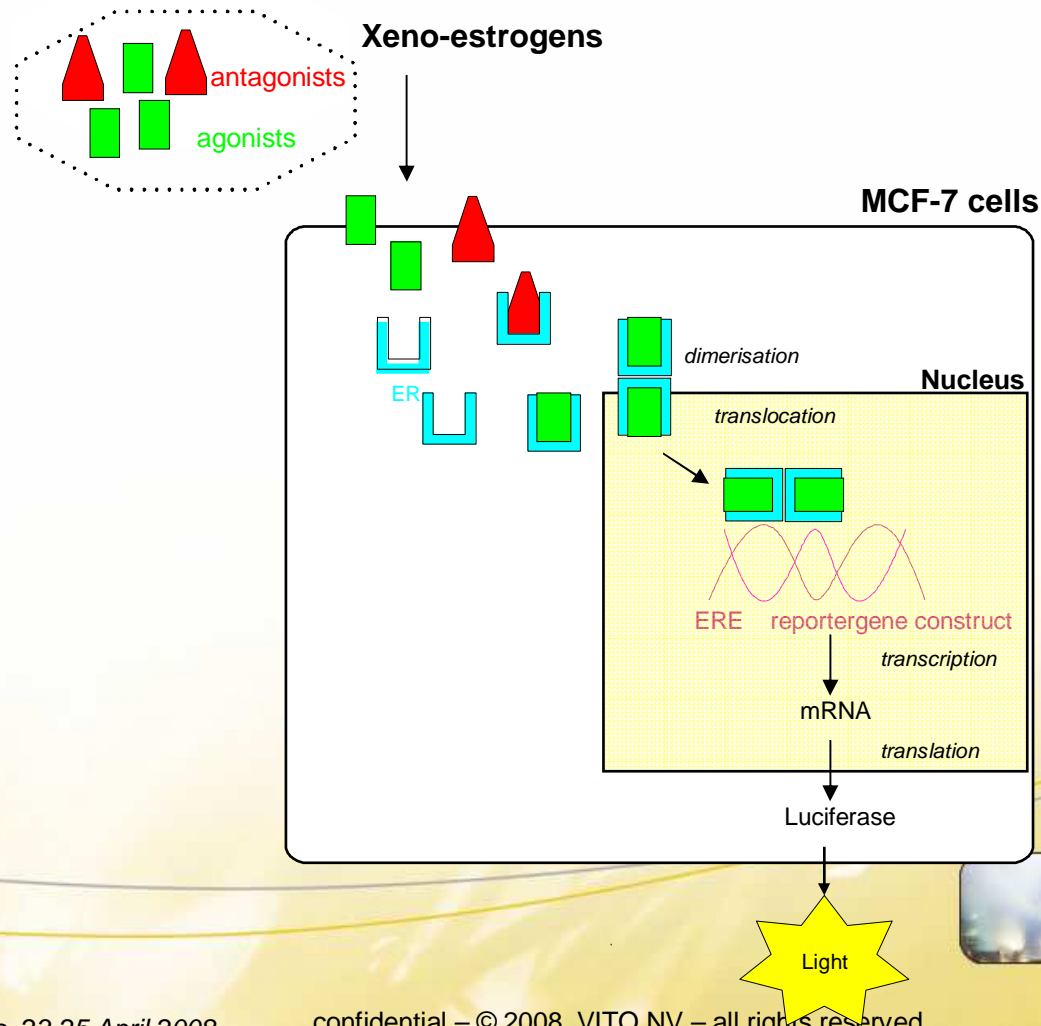






Source: Hartung et al., 2004

- Stably transfected human breast cancer cell line, developed by INSERM (Balaguer et al, 1999) → **MELN**



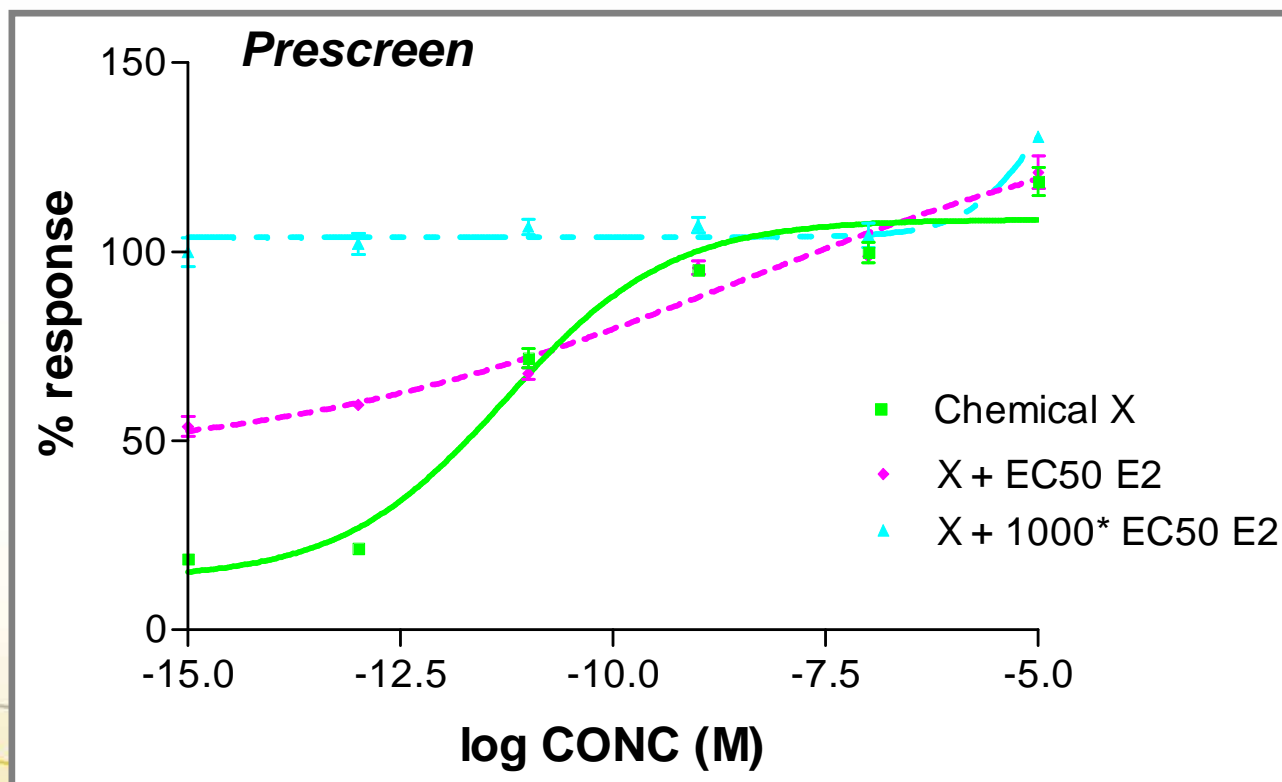
- **Test definition**

- Mode of action : ER – mediated response
- Optimise test procedures → SOPs
- Define acceptance criteria
  - Agonist:
    - ⇒ min. induction ratio; EC50 range for 17 $\beta$ -estradiol (PC)
  - Antagonist:
    - ⇒ min. inhibition ratio; IC50 range for 4OH-tamoxifen (PC)
- Include cytotoxic assay: CytoTox-ONE™

- Screening set up



- Test definition
- **Screening set up**

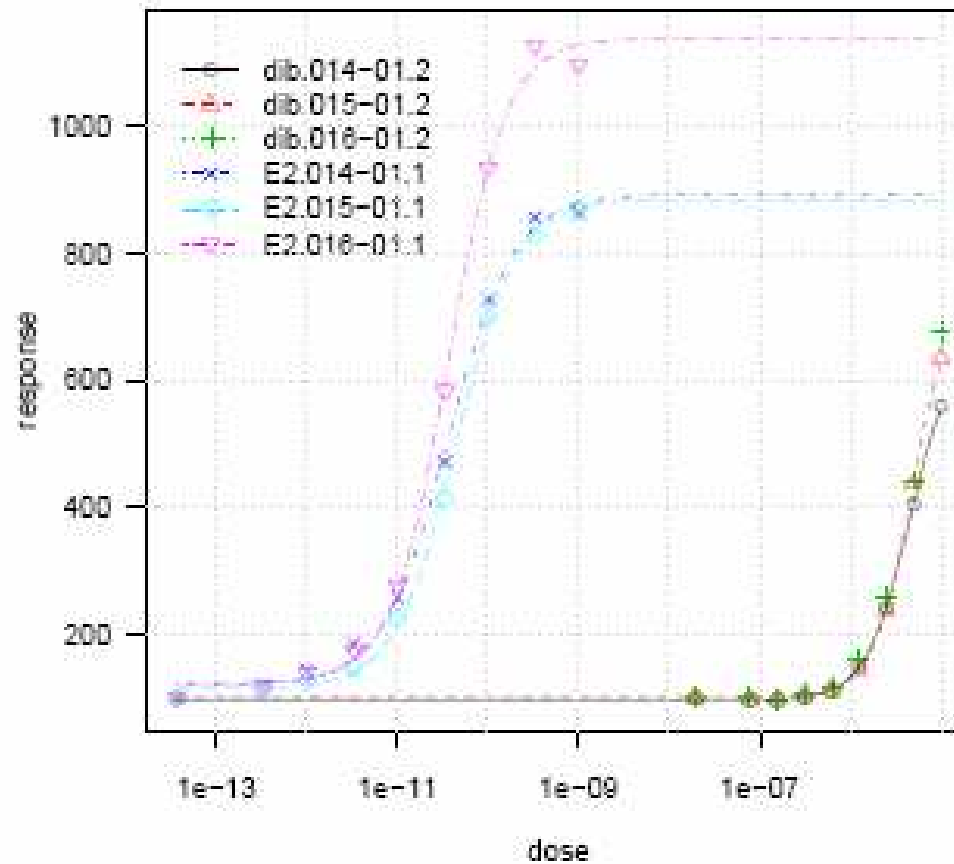


- Test definition
- Screening set up
- **Test chemicals:** selected by EDC task force

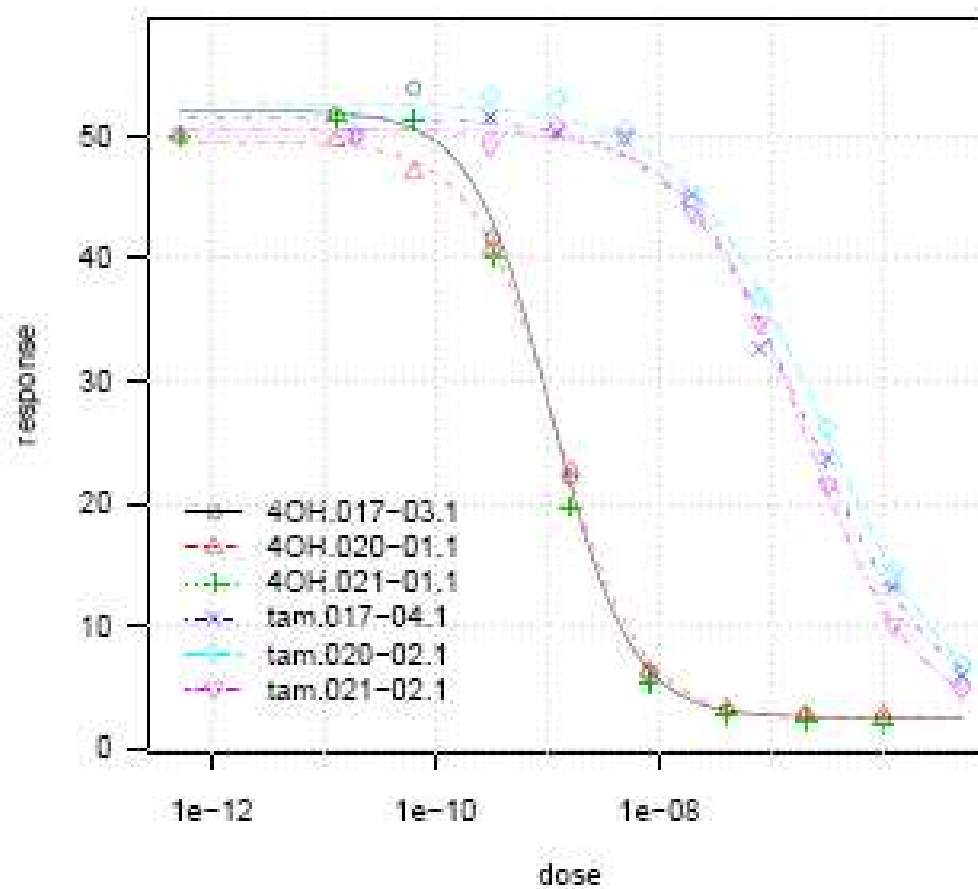
Indicative potency	ER agonist (12)		ER-antagonist (10)	
<b>Strong - moderate</b>	17 $\beta$ -Estradiol (E <sub>2</sub> ) Diethylstilbesterol (DES)	17 $\alpha$ -Ethinylestradiol (EE <sub>2</sub> ) Hexestrol	ICI 182,780 <b>4OH-Tamoxifen</b>	Tamoxifen
<b>Mild - weak</b>	Genistein Equol Norethynodrel	di-Butyl paraben Nonylphenol o,p-DDT	Norethynodrel Raloxifene-HCl Genistein	Nonylphenol o,p-DDT
<b>Negative</b>	Corticosterone	Dibutylphtalate (DBP)	Corticosterone	DBP



- Agonist assay:** dibutylparaben (3 exp.) vs E2



- Antagonist assay:** tamoxifen (3 exp.) vs OH-Tam



Test chemicals	Agonist assay	
	Er-activation	Mean EC50 (M)
17 $\beta$ -Estradiol (E <sub>2</sub> )	+	4.2 10 <sup>-11</sup>
17 $\alpha$ -Ethinylestradiol	+	2.4 10 <sup>-11</sup>
Diethylstilbesterol	+	1.7 10 <sup>-10</sup>
Hexestrol	+	1.0 10 <sup>-10</sup>
Norethynodrel	+	7.5 10 <sup>-9</sup>
Genistein	+	8.2 10 <sup>-7</sup>
Nonylphenol	+	7.4 10 <sup>-7</sup>
opDDT	+	1.7 10 <sup>-6</sup>
Equol	+	1.9 10 <sup>-6</sup>
Dibutylparaben	+	5.4 10 <sup>-6</sup>
Corticosterone	negative	
Dibutylphtalate(DBP)	negative	

Test chemicals	Antagonist assay	
	ER-suppression	Mean IC50 (M)
ICI 182780	+	6.6 10 <sup>-10</sup>
Raloxifene	+	2.4 10 <sup>-10</sup>
4OH-Tamoxifen	+	1.0 10 <sup>-9</sup>
Tamoxifen	+	2.2 10 <sup>-7</sup>
Norethynodrel	-, activation	
Genistein	-, activation	
Nonylphenol	-, activation	
opDDT	-, activation	
Corticosterone	negative	
Dibutylphtalate(DBP)	negative	



- Test performance MELN:
  - OK, only 4/80 tests rejected
  - intralab reproducibility : mean CV  $\pm$  20% , but limits?
  - classify chemicals on EC50/IC50  $\rightarrow$  potency class
- Transfer & Interlab reproducibility : under investigation
- Next:
  - predictive value + applicability  $\rightarrow$  relevance
  - comparison to other ER-transactivation tests
  - validation report  $\rightarrow$  OECD



- VITO N.V.

- Pascale Berckmans
- Clea Vangenechten
- Katrien Smits

- ECVAM

- Miriam Jacobs
- Susanne Bremer



- Partners *in vitro* TA assays

- Bayer
- BDS

- Biostatistical evaluation

- German Cancer Research Center

