Bioassays voor de detectie van stoffen met endocriene effecten

AOAC-LL LowLands Symposium: Ons voedsel: Veilig verpakt?

21 November, Breda, Nederland

Toine Bovee

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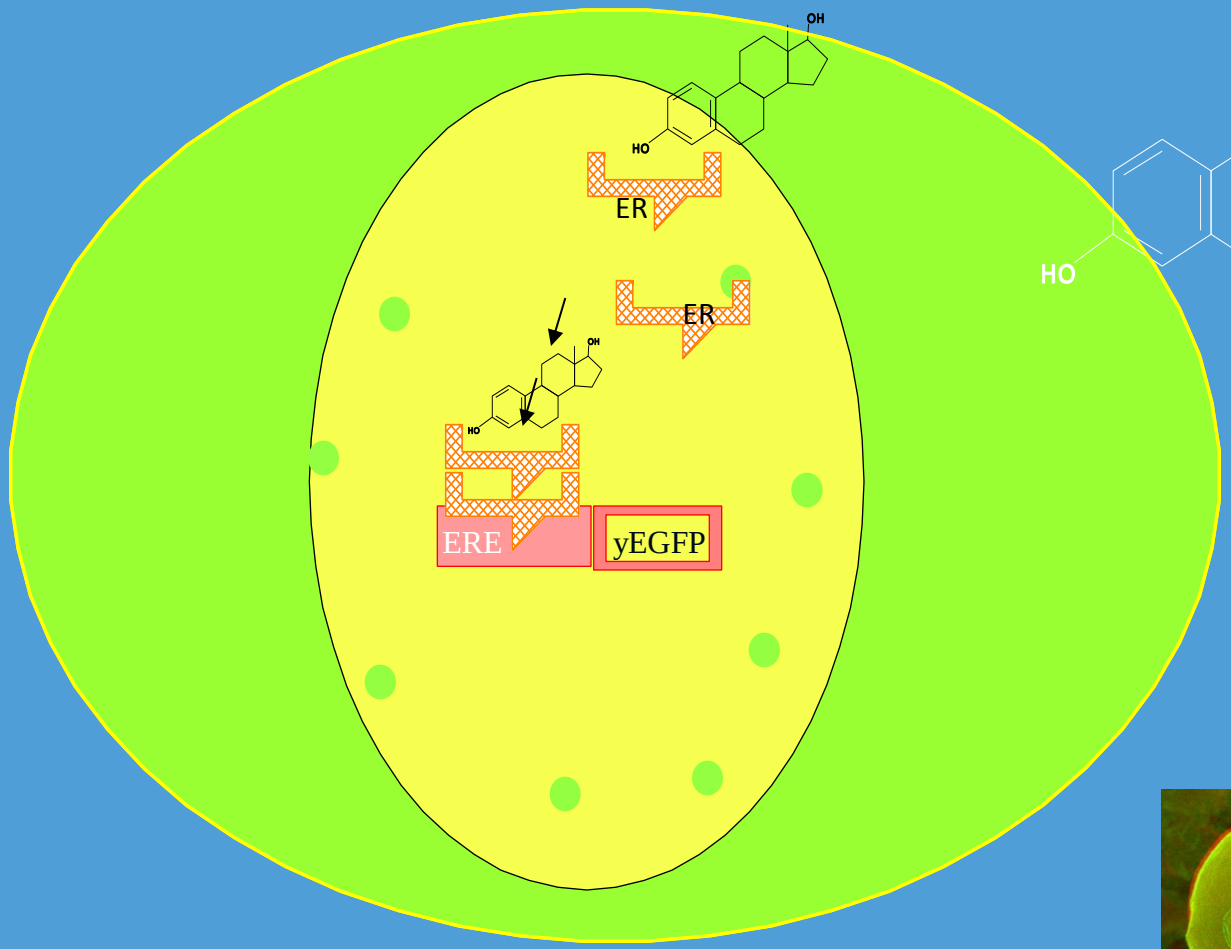
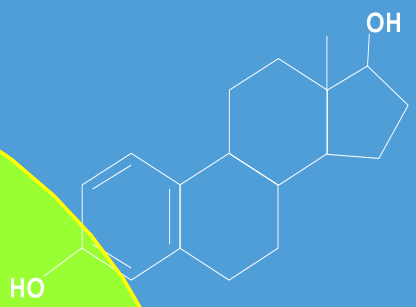
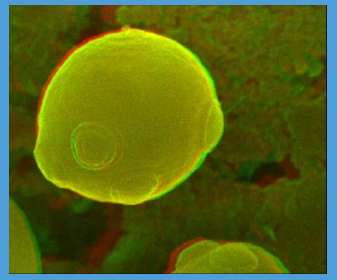
Ontwikkeling van de gist oestrogene bioassay





17β-estradiol



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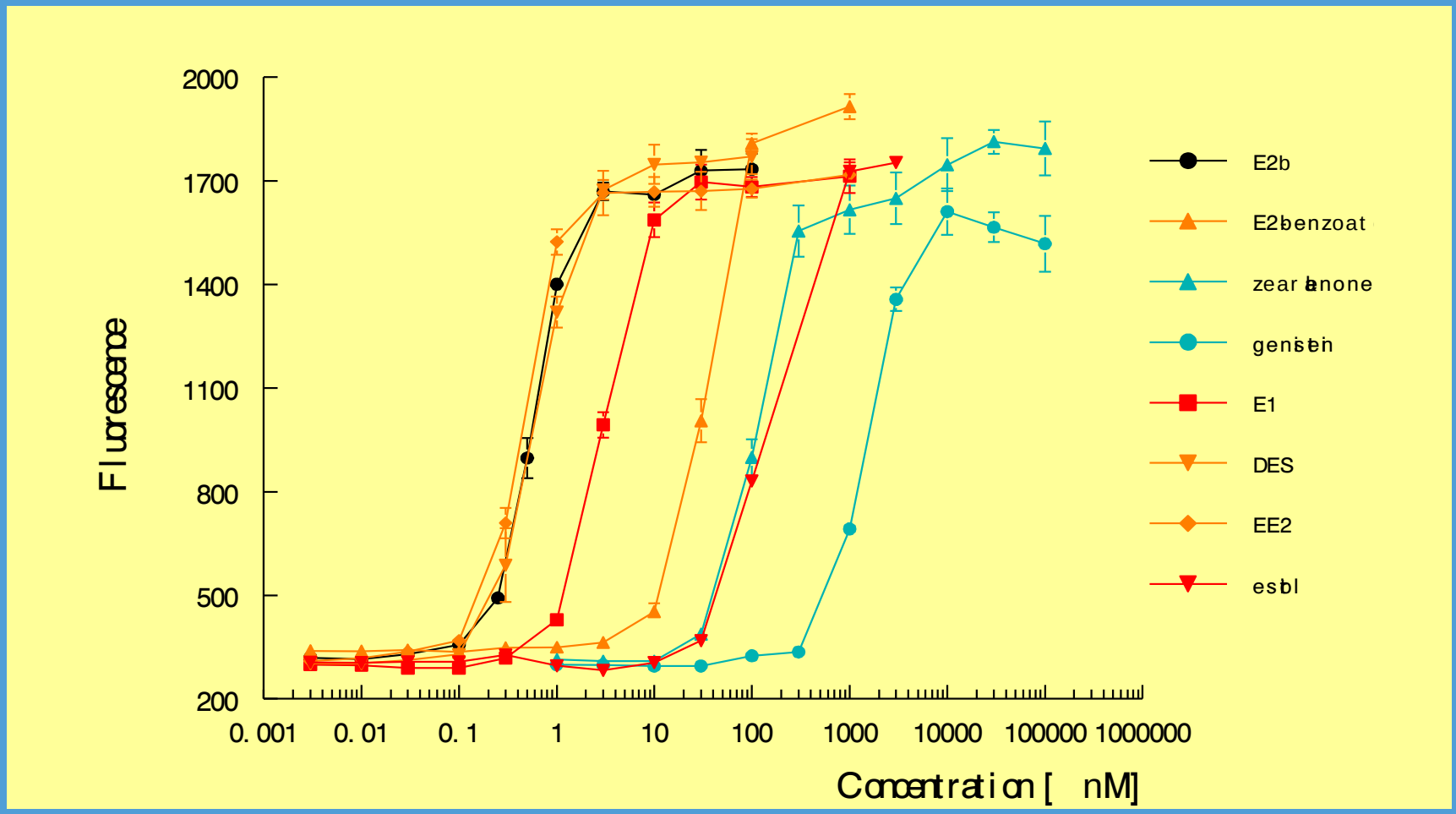
De gist oestrogene bioassay



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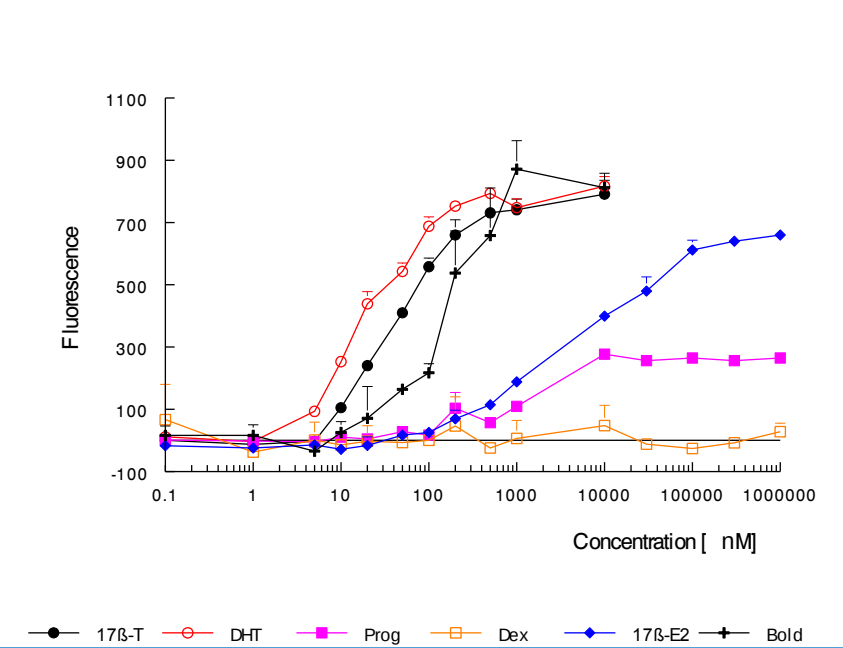
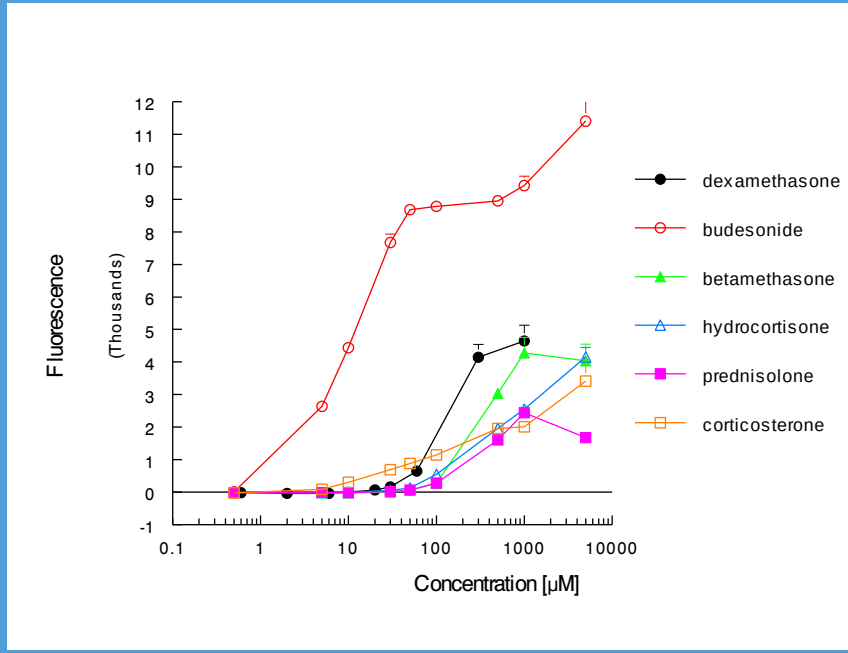
Bovee et al., *Gene* **325** (2004) 187-200
Bovee et al., *JSBMB* **91** (2004) 99-109

RIKILT heeft ook op gist gebaseerde bioassays ontwikkeld voor androgenen en corticosteroïden





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Bovee et al., *ABC* **389** (2007) 1549-1558
Bovee et al., *ABC* **401** (2011) 873-882

Bioactivity measurements

Transcriptional Activation (TA) bioassays (yeast or mammalian cell based)

- Detect all compounds (structures) that are able to activate the receptor, e.g. the estrogen, androgen, progesterone, glucocorticoid or thyroid receptor. As the main mode of action of all active hormones is by activating their cognate receptor, they fulfil Directive 96/22/EC that prohibits all substances having hormonal action

- Moreover, they are:
  - Sensitive and specific
  - Quick, simple and robust
  - Applicable to urine, feed and preparations

Ontwikkeling en/of validatie van bioassays op het RIKILT-Instituut voor Voedselveiligheid

- The DR-CALUX® for dioxins - feed, fat, oil
- The yeast estrogen bioassay - calf urine and feed
- The yeast androgen bioassay - calf urine and feed
- The yeast corticoid bioassay
- The GR-CALUX® for corticosteroids - feed
- Receptor-bindingassay β-agonists - feed
- PPARδ bioassay
- The extended steroidogenesis assay - chemicals (OECD)

- PR-CALUX® for progestagens - validation feed is on-going

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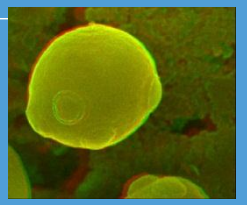
Validatie kalf urine, bepaling CCα REA-Rikilt Estrogenen bioAssay



				Day 1								Day 2								Day 3			
Urine sample #	Mean	SD	CCα	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Blank urine	29	50	179	5	45	48	-29	-32	33	5	64	45	76	23	54	39	18	49	79	-53	83	-85	121
17-βE2 1 ng ml^{-1}	389	70		497	489	456	334	338	410	472	481	342	381	373	278	334	317	285	350	469	391	443	346
DES 1 ng ml^{-1}	353	59		369	387	347	340	316	367	376	409	342	341	334	302	301	307	381	332	241	453	374	439
EE2 1 ng ml^{-1}	437	114		608	524	471	338	372	413	705	474	366	364	378	338	340	336	352	358	369	659	536	442
ZEA 50 ng ml^{-1}	357	55		315	298	425	408	301	265	324	421	364	355	320	349	301	350	384	269	367	304	312	315
MES 10 ng ml^{-1}	432	119		180	590	629	375	410	393	253	594	469	363	407	409	395	379	510	413	343	395	486	648

- Decision limit: CCα = Mean + (3 x SD)
  - Samples giving a signal below CCα are declared **negative**
  - Samples giving a signal above CCα are declared **suspect**

Bovee et al., *ACA* **529** (2005) 57-64

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Ring test REA met kalf urine

Sample number	Sample type	RIKILT	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
1	Blank urine	-35	4.00	24	-2657	-266	-122	-0.050	-77
2	Blank urine	-25	2.33	56	-991	-354	-111	0.013	-109
3	Blank urine	35	5.33	1686	4589	890	682	-0.007	-136
4	Blank urine	-8	3.00	472	160	118	69	0.056	-159
5	Blank urine	26	3.00	-134	-9871	-682	4	0.011	-207
6	Blank urine	237	6.00	2940	9368	3605	1061	0.111	-76
7	Blank urine	122	4.67	2377	13235	2085	542	0.090	63
CCα		343	8.11	4837	25283	5459	1685	0.201	156
8	Urine 1 ppb E2	768	13.00	10153	72899	15375	2227	0.338	566
9	Urine 5 ppb E2	897	14.00	11585	87469	17047	2058	0.475	1689
10	Urine 1 ppb EE2	692	14.33	10122	87127	13678	1570	0.302	622
11	Urine 5 ppb EE2	852	10.33	12184	74531	16796	1764	0.398	1554
12	Urine 10 ppb Mes	398	8.33	5719	37334	8216	1505	0.218	18
13	Urine 50 ppb Mes	708	9.33	9023	52651	13757	1902	0.369	109
14	Urine 100 ppb T	67	2.00	1391	11554	1157	440	0.075	40
15	Urine 100 ppb P	73	1.33	1965	542	894	348	0.078	9
16	Blank urine	56	1.67	1231	4652	971	199	0.054	153
17	Reagent blank	0	0	0	0	0	0	0	0
18	DMSO blank	23	2.00	1189	-8006	355	-153	0.030	-50

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Bovee et al., *ACA* **637** (2009) 265-272

The added value - Dietary supplements

- Dietary supplements → analysed by LC-MS/MS for 49 steroids.
  - 18 supplements - 11 positive and 7 negative

11 positive → also positive in the yeast **androgen** bioassay

7 negative → 2 supplements show androgenic activity in the yeast androgen bioassay

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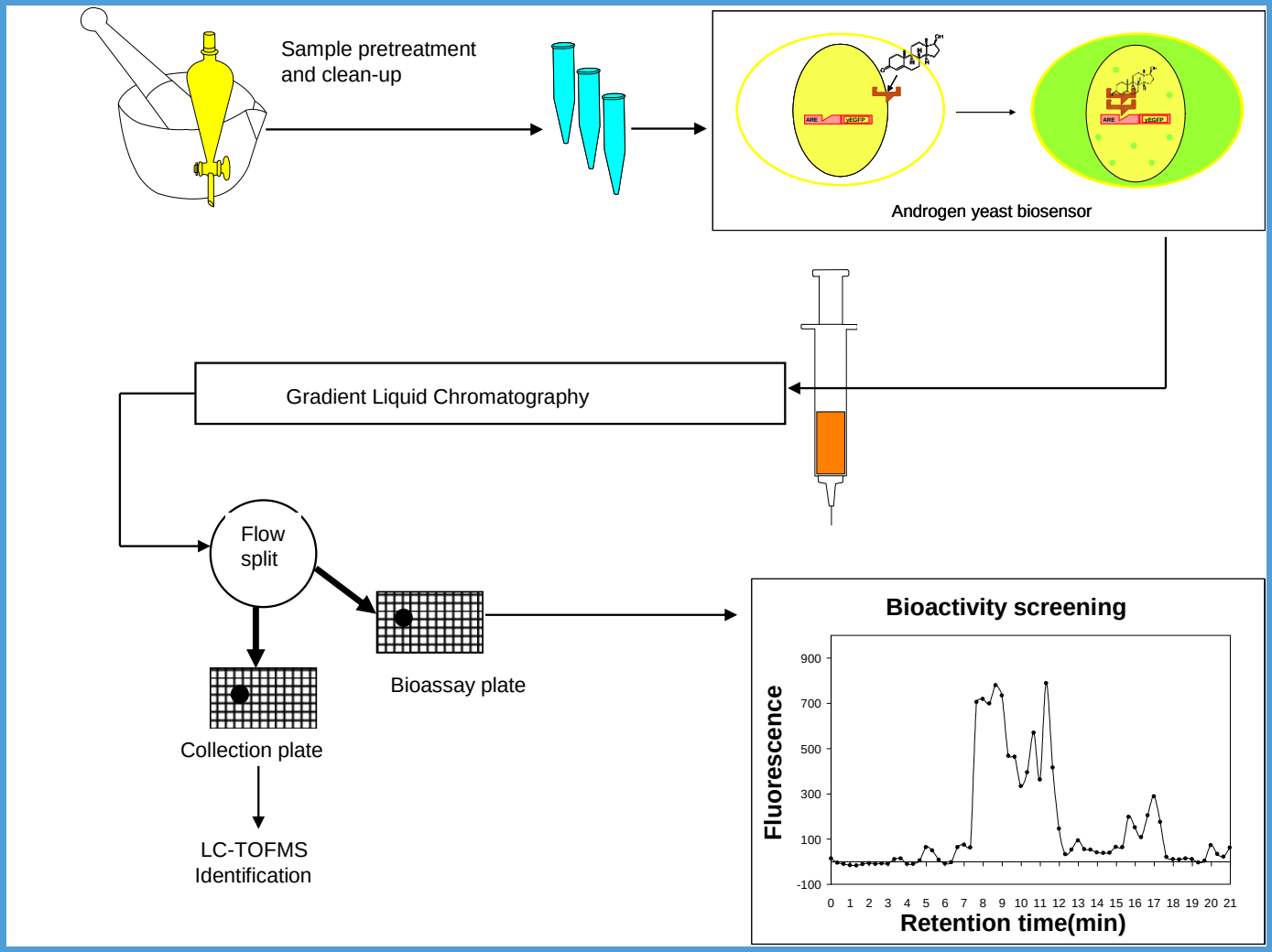
Van Poucke et al., *ACA* **586** (2007) 35-42

Bioassay directed identification of unknowns



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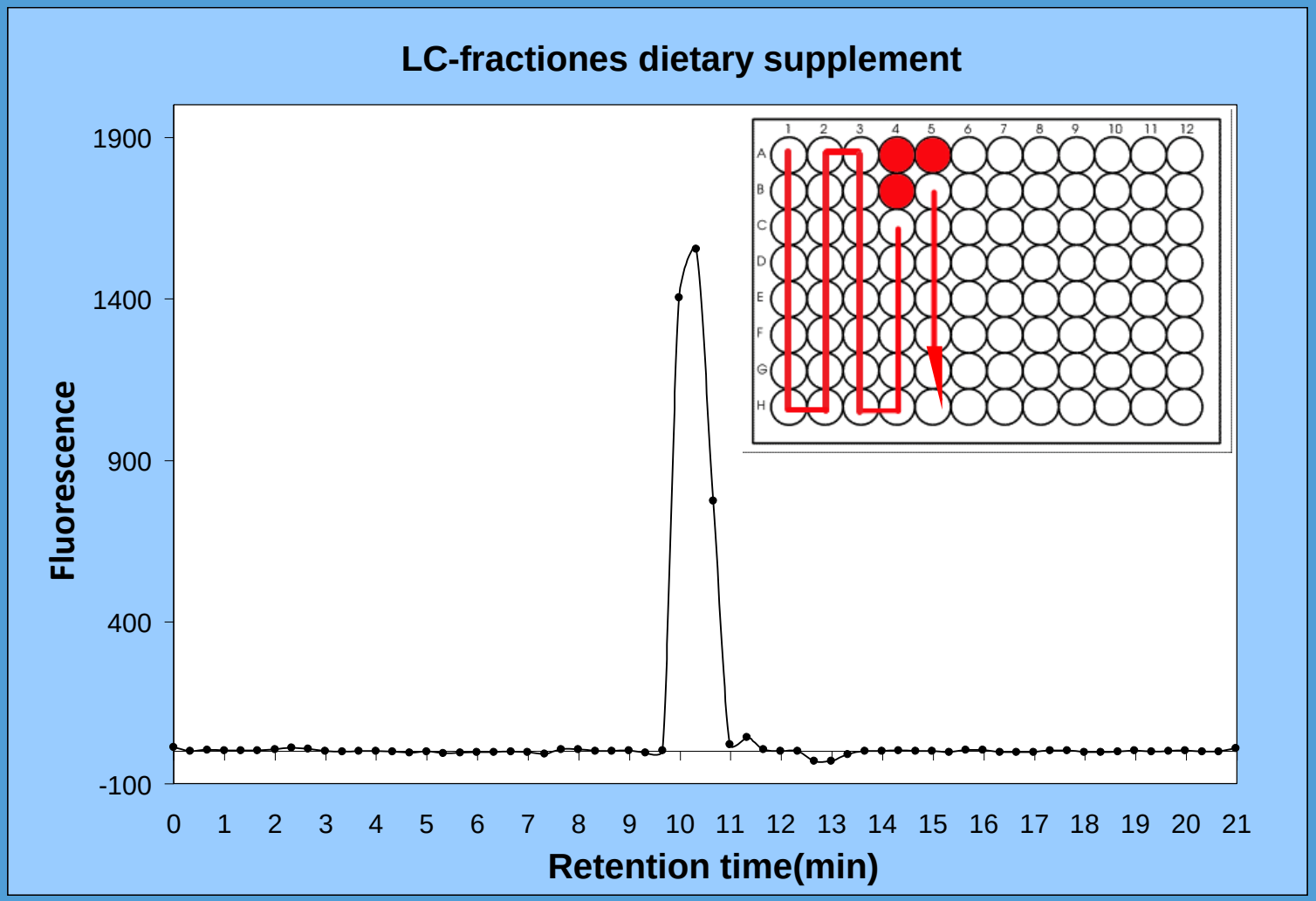
Bioassay directed identification of unknowns



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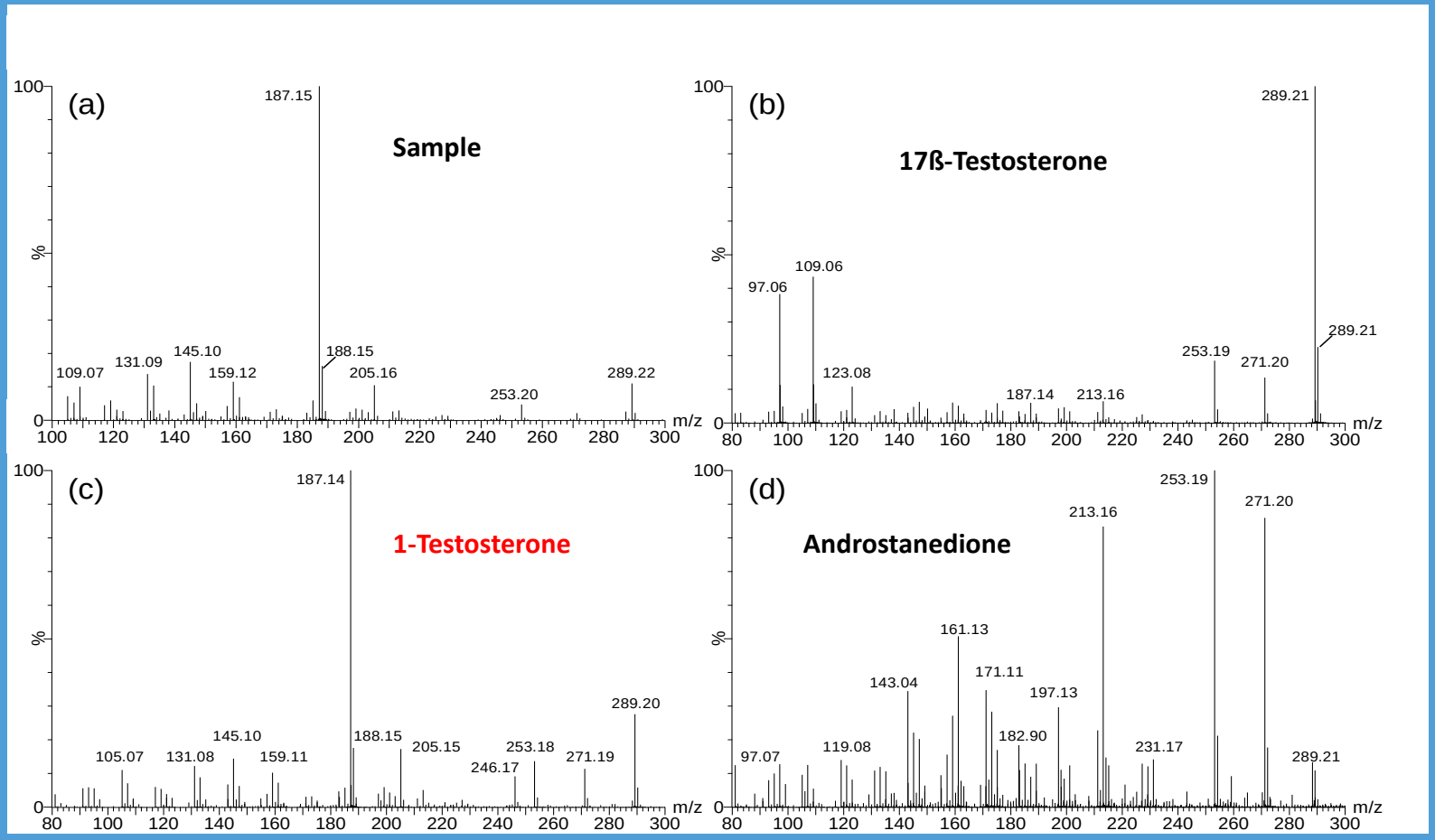
Dietary supplements in yeast androgen bioassay & LC-MS/MS



The other one contained 4-androstene-3β,17β-diol and 5-androstane-3β,17β-diol

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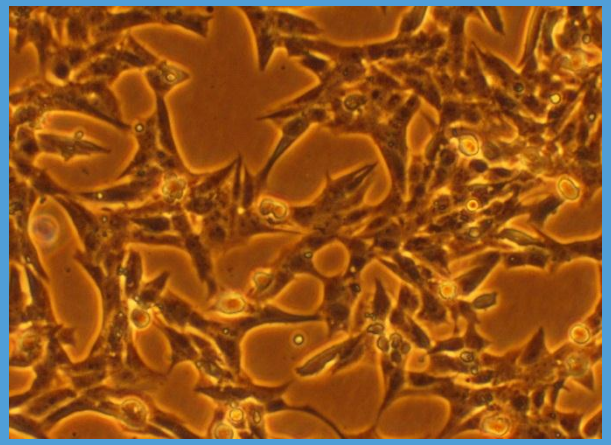
Rijk et al., *ACA* **637** (2009) 305-314

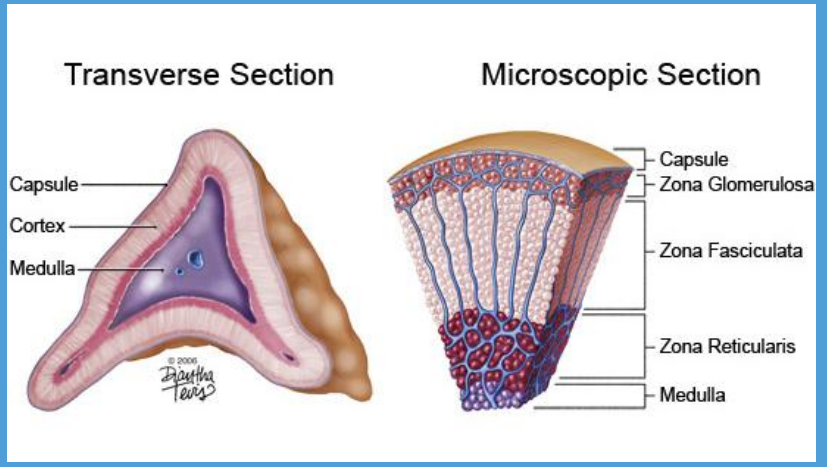
The extended H295R steroidogenesis assay

- Zonally undifferentiated fetal adrenal cells originating from a human adrenocarcinoma



- Express all genes and enzymes involved in steroidogenesis

- OECD validated (TG 456)



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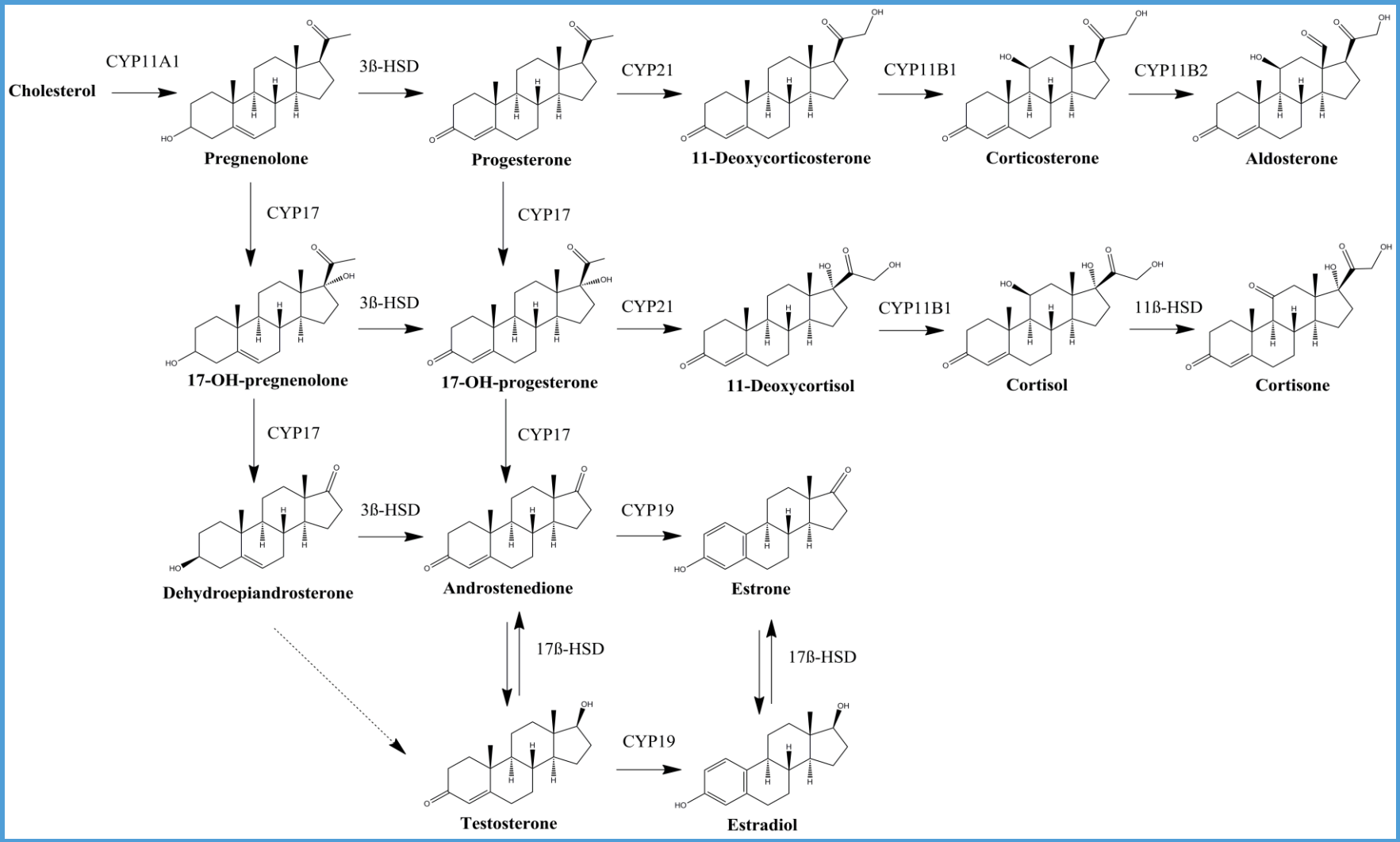
The extended H295R steroidogenesis assay



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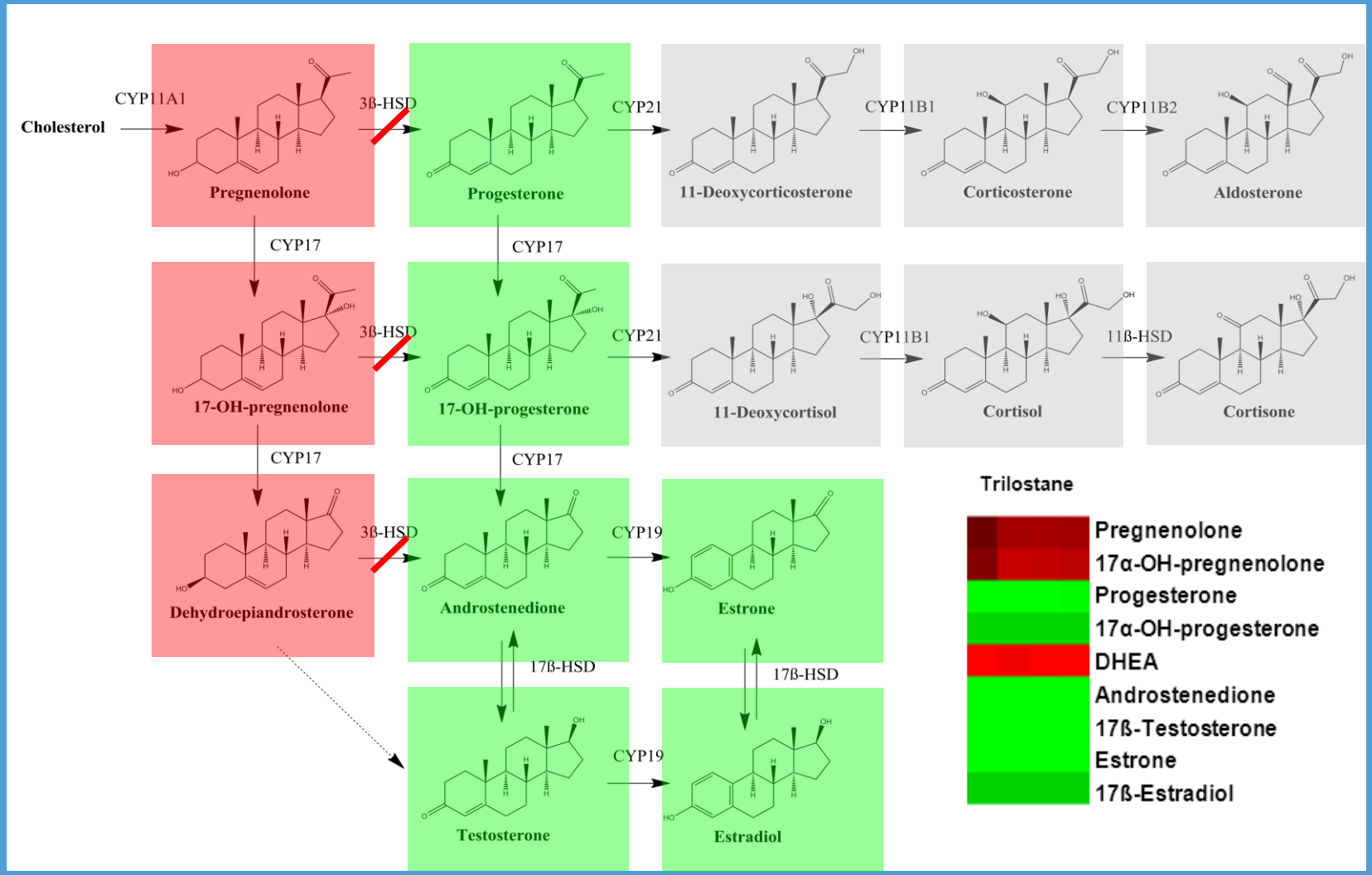
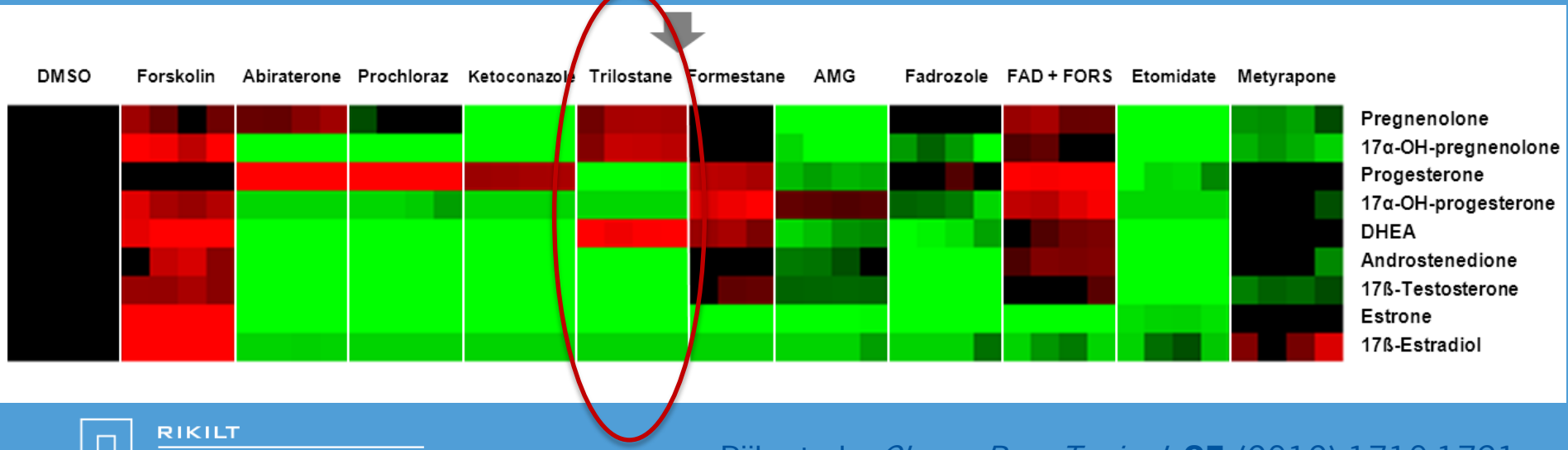
Trilostane effects in the H295R assay – GC-MS/MS



Effects in the H295R assay – GC-MS/MS

DMSO	Forskolin	Abiraterone	Prochloraz	Ketoconazole	Trilostane	Formestane	AMG	Fadrozole	FORS + FAD	Etomidate	Metyrapone	Hormone
1.00±0.14	1.79±0.45*	1.97±0.31**	0.87±0.39	0.1±0.00***	1.15±0.16	0.78±0.27	0.17±0.03***	1.00±0.12	2.06±0.37**	0.07±0.02***	0.49±0.11**	Pregnenolone
1.00±0.11	4.53±2.02*	>0.01***	0.04±0.03***	>0.01***	2.65±0.41***	0.85±0.33	0.15±0.05***	0.40±0.17**	1.52±0.13**	0.05±0.00***	0.38±0.05***	17α-OH-pregnenolone
1.00±0.10	1.20±0.19	135±17.2***	6.12±0.34***	2.41±0.09***	0.22±0.04***	2.30±0.71*	0.38±0.03***	1.45±0.10**	4.32±0.31***	0.33±0.10***	0.97±0.06	Progesterone
1.00±0.09	2.70±0.47***	>0.07***	0.34±0.06***	>0.07***	>0.07***	3.21±1.00**	1.62±0.07***	0.49±0.00**	3.20±0.54**	0.07±0.00***	0.77±0.14	17α-OH-progesterone
1.00±0.10	5.15±1.30**	>0.05***	>0.05***	>0.05***	3.97±0.21***	1.98±0.57**	0.40±0.08***	0.30±0.07***	1.68±0.28*	0.10±0.01***	0.95±0.14	DHEA
1.00±0.15	2.40±0.80***	0.01±0.00***	0.01±0.00***	0.00±0.00***	0.01±0.00***	0.82±0.30	0.61±0.12*	0.11±0.03***	1.86±0.25**	0.04±0.01***	0.79±0.23	Androstenedione
1.00±0.06	2.29±0.16***	0.08±0.01***	0.07±0.01***	0.05±0.00***	0.07±0.00***	1.35±0.44	0.57±0.01***	0.19±0.03***	1.40±0.14*	0.05±0.01***	0.58±0.07***	17ß-Testosterone
1.00±0.15	21.9±2.74***	0.09±0.01***	>0.08***	>0.08***	>0.08***	>0.08***	0.22±0.03***	>0.08***	>0.08***	0.30±0.01***	0.09±0.25	Estrone
1.00±0.34	39.8±2.20***	>0.30*	>0.30*	>0.30**	>0.30***	>0.30***	>0.30*	>0.30*	>0.30*	0.35±0.29	2.07±0.74	17ß-Estradiol



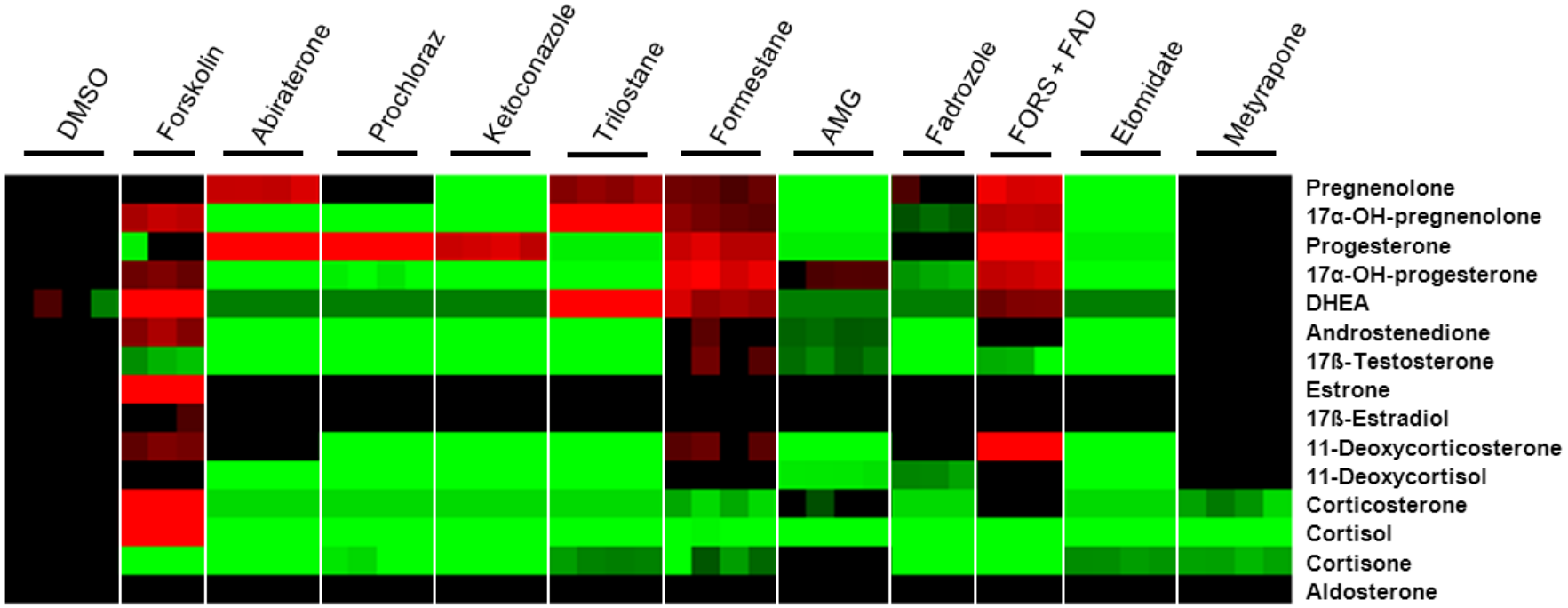
RIKILT WAGENINGEN UR

Rijk et al., *Chem. Res. Toxicol.* **25** (2012) 1710-1731

Effects in the H295R assay

– Hightroughput UPLC-MS/MS

- UPLC-ToF-MS based Metabolomics (targeted search)



RIKILT WAGENINGEN UR

Rijk et al., *Chem. Res. Toxicol.* **25** (2012) 1710-1731

The extended H295R steroidogenesis assay

- Monitoring changes in steroid profiles that also unravels the mechanisms of action (predictive value!)

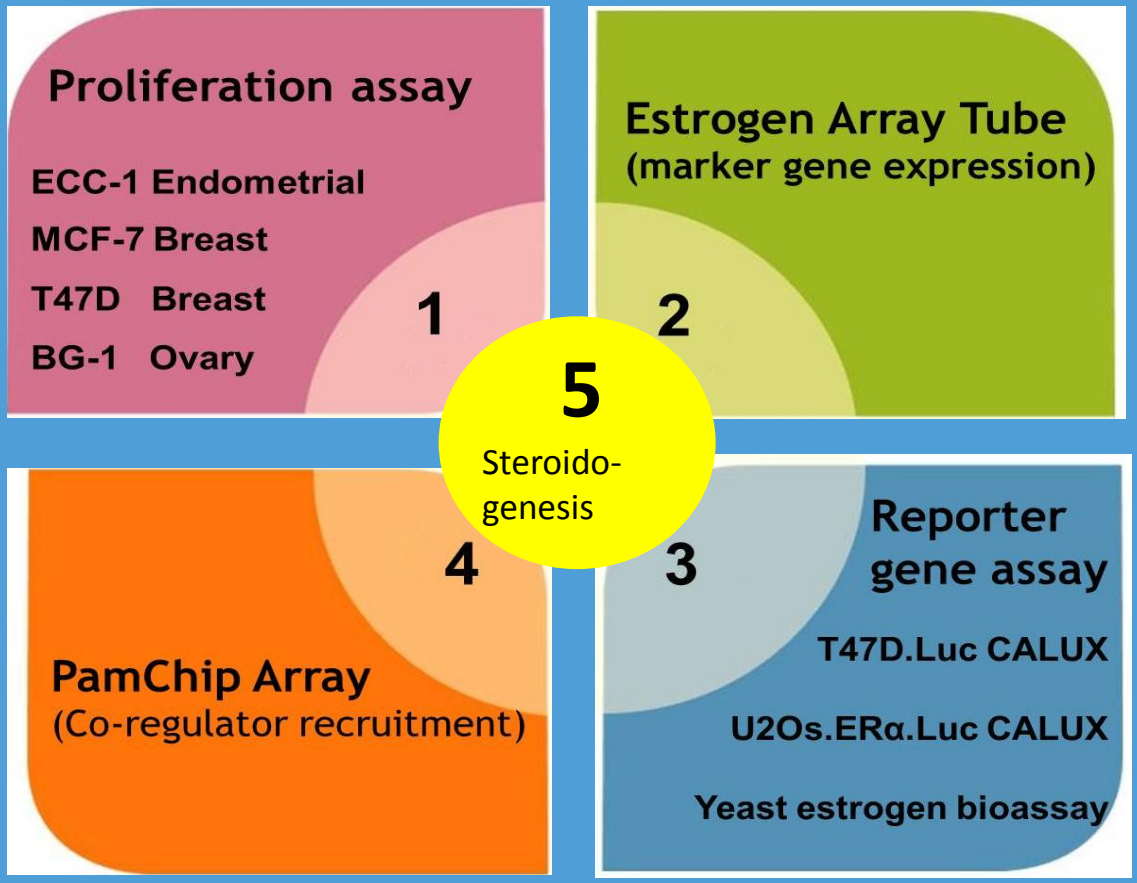
Bioassays for Endocrine Disrupting Chemicals (EDCs)

- The yeast estrogen bioassay (agonists, SERMs and antagonists)
- The yeast androgen bioassay (agonists, SARMs and antagonists)
- The yeast (gluco)corticoid assay (agonists, antagonists)
- The DR CALUX bioassay for dioxins and dl-PCBs
- The U2OS GR CALUX bioassay (for (gluco)corticosteroids)
- The U2OS PR CALUX bioassay (for progestagenes like progesterone)
- The extended H295R steroidogenesis assay
- PPARδ assay
- The receptor-bindingassay for β-agonists

- A LBD-ERalpha bindingassay in combination with MS (BioMS)
- Thyroid transport disruption: TBG and TTR binding assays
- T3 and T4 synthesis: TPO enzyme assay
- Hormone transport disruption: SHBG binding assay
- ELISAs and Luminex methods, e.g. for (gluco)corticosteroids

Combining different assays: an *in vitro* testing strategy (ITS) for estrogenicity

1 x
2 x
3 ERα CALUX & yeast assay
4 PamChip Array
5 Extended steroidogenesis



Si Wang, PhD thesis 2013 on alternatives for estrogenicity testing

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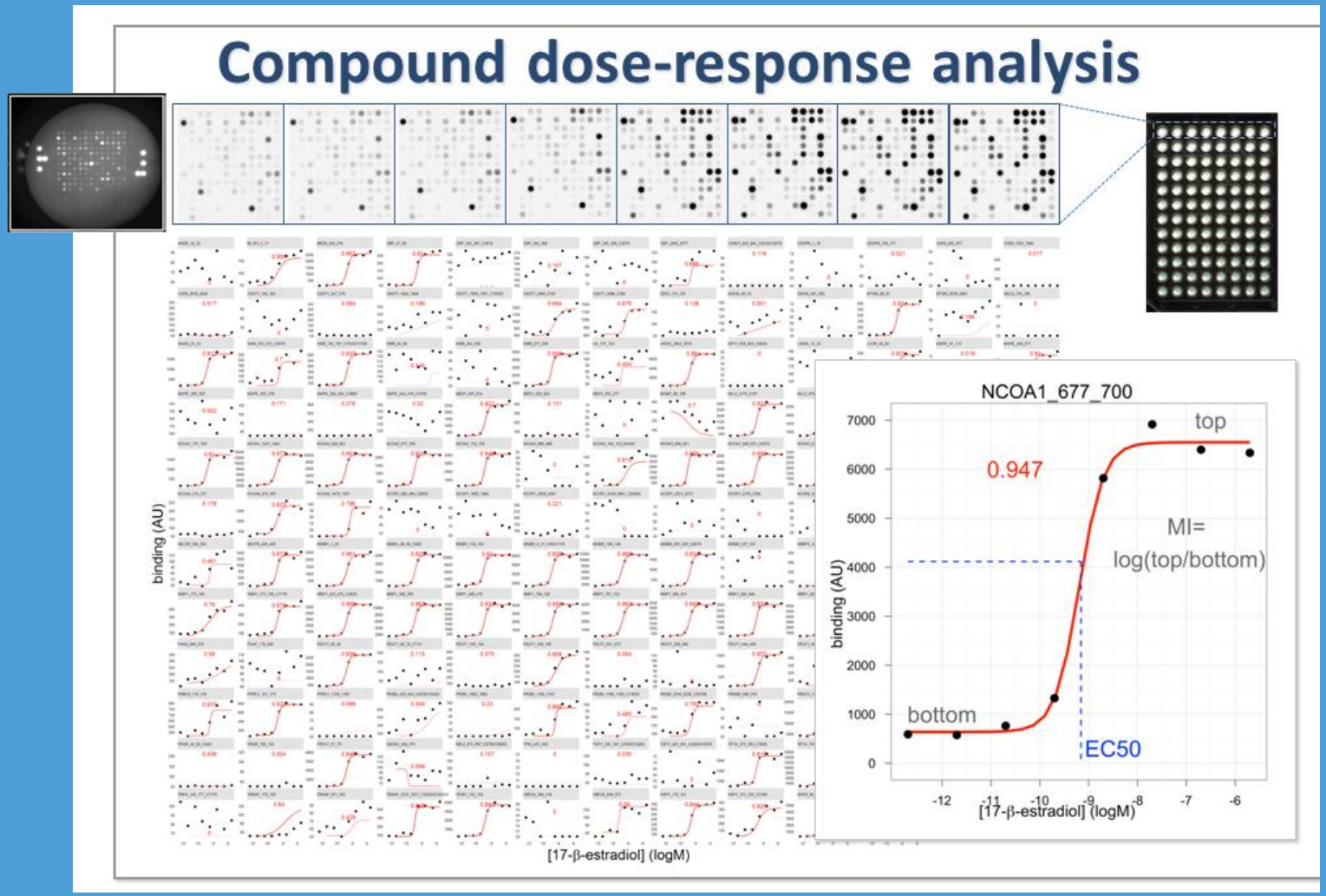
The PamChip® peptide array



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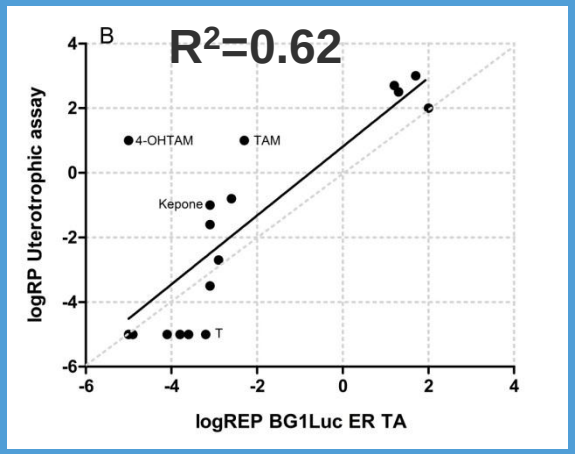
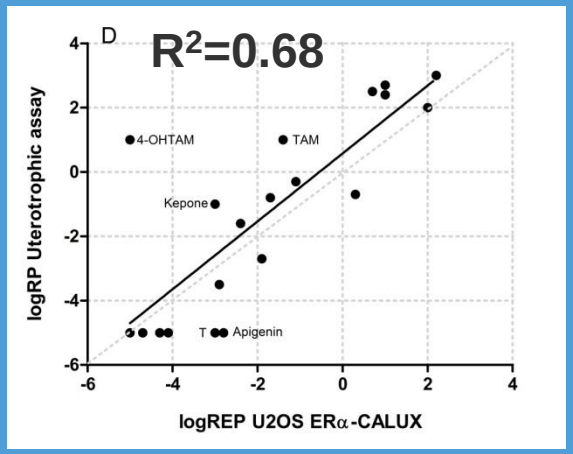
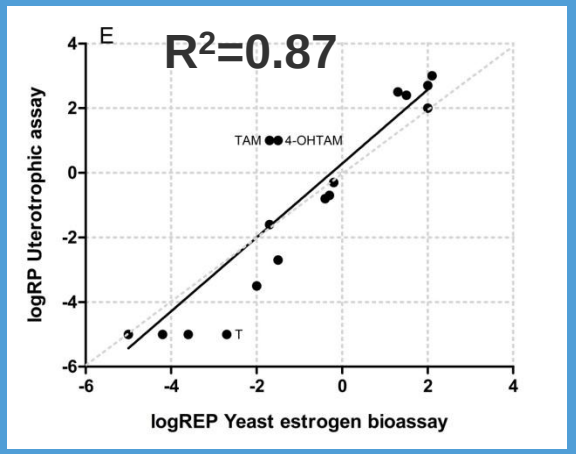
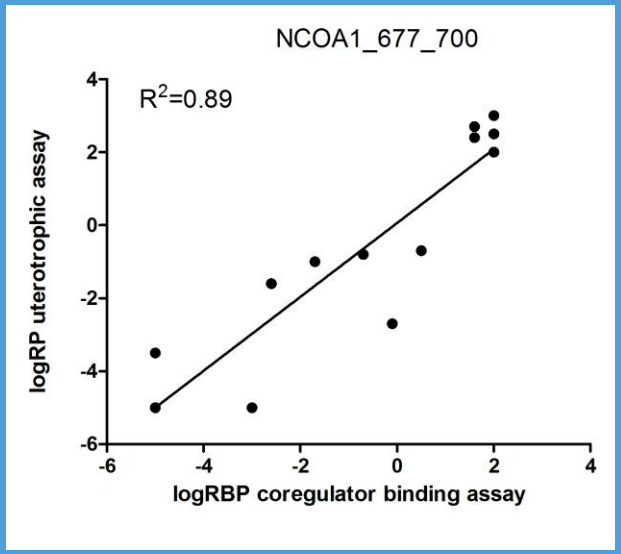
An ITS for estrogenicity







BG-1Luc ER TA
OECD TG457



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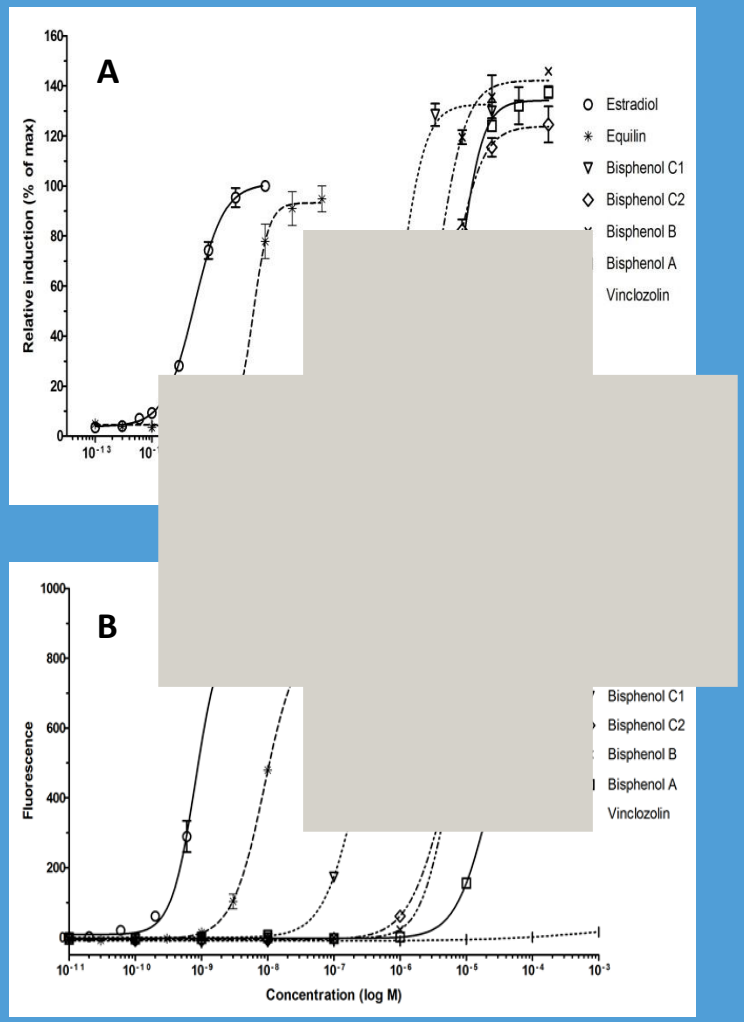
Wang et al., *ALTEX* **30** (2013) 145-157
Wang et al., *J. Appl. Toxicol.* (2013) in press

Combining different assays: an *in vitro* testing strategy (ITS)

- The example of Bisphenol A
  - ITS estrogenicity: BDS U2OS-ERα-CALUX®, RIKILT yeast estrogen bioassay, PamChip® peptide array, extended H295 steroidogenesis assy
  - Extended with: the BDS U2OS-AR-CALUX® and RIKILT yeast androgen bioassay

- This goes beyond the ITS for estrogenicity testing (replace the *in vivo* uterotrophic assay: OECD TG440)

An extended ITS for the Bisphenols



A: BDS U2OS ERα-CALUX®

B: RIKILT Yeast Estrogen Bioassay

Wang et al., in preparation

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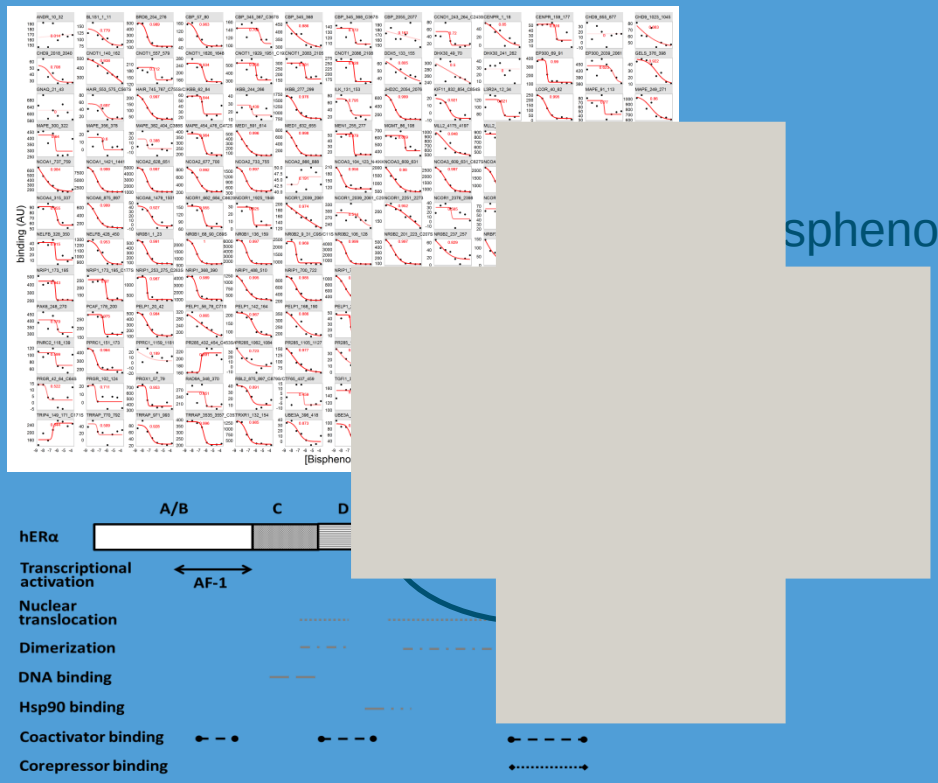
An extended ITS for the Bisphenols

Bisphenol B, C1 and C2 are AF2 antagonists



Wang et al., in preparation

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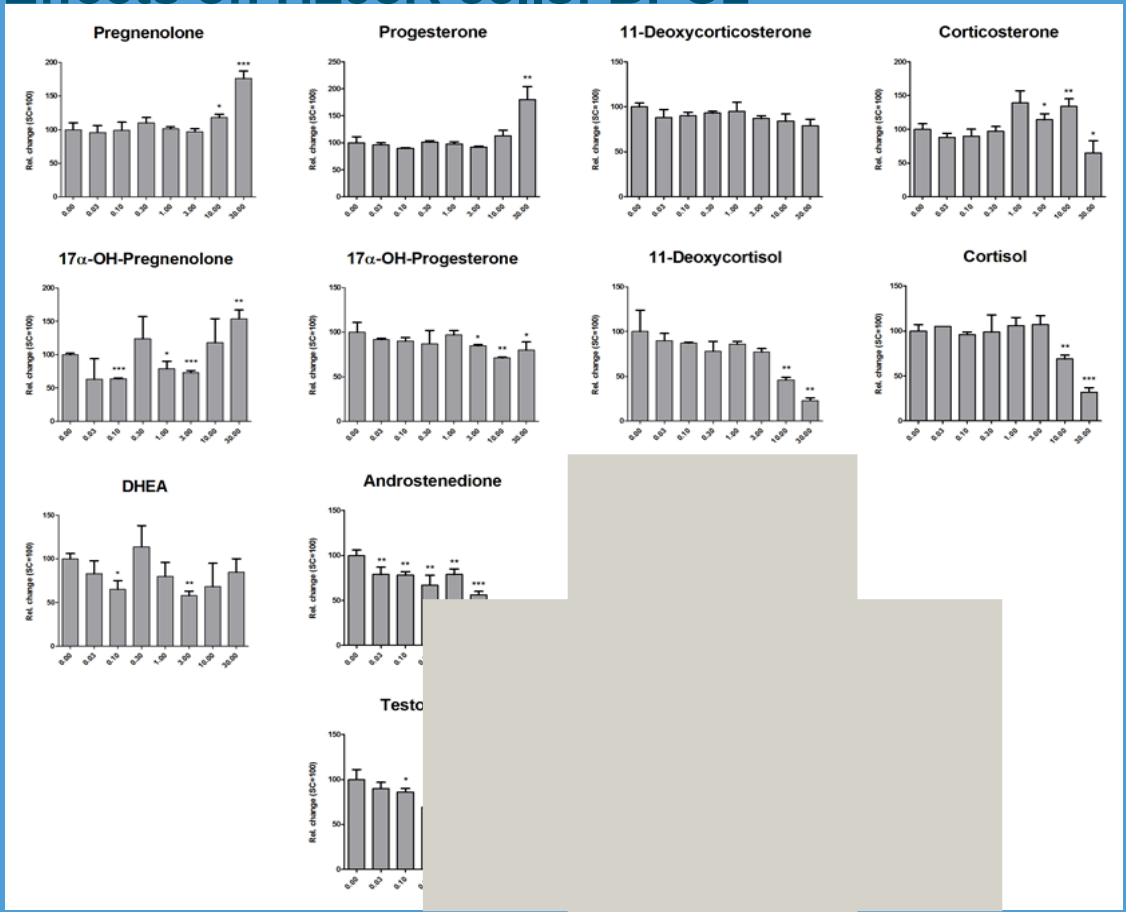
An extended ITS for the Bisphenols

Effects on H295R cells: BPC1



Wang et al., in preparation

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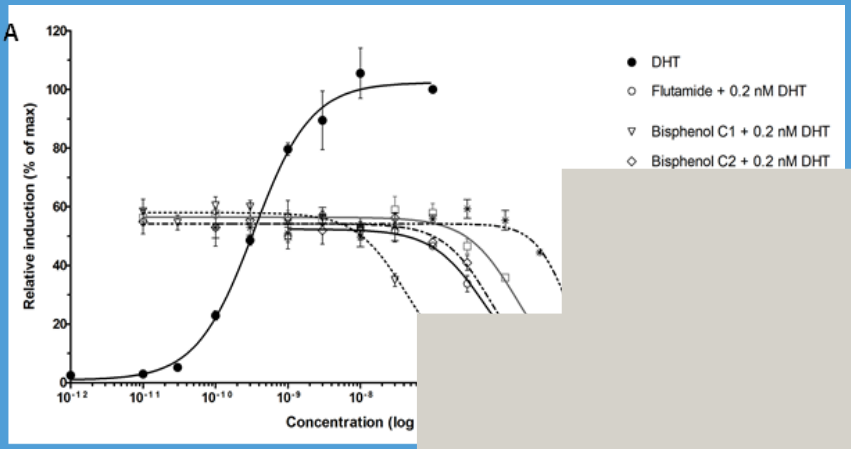
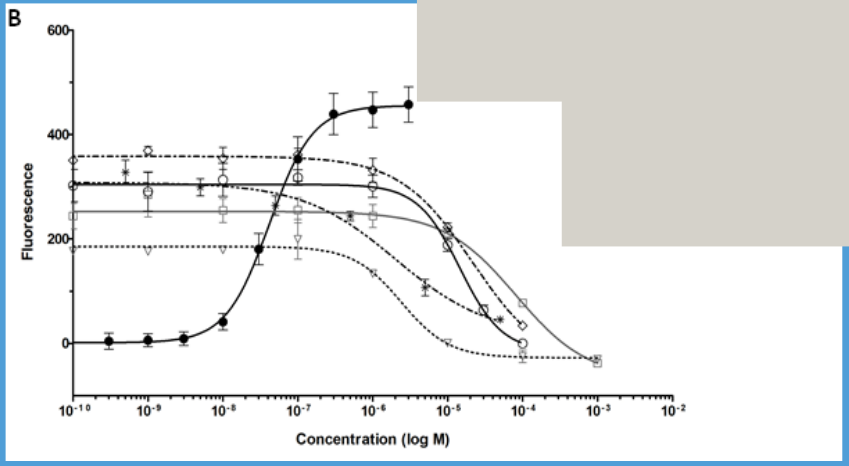
An extended ITS for the Bisphenols



A: BDS U2OS AR-CALUX®



B: RIKILT Yeast Androgen Bioassay

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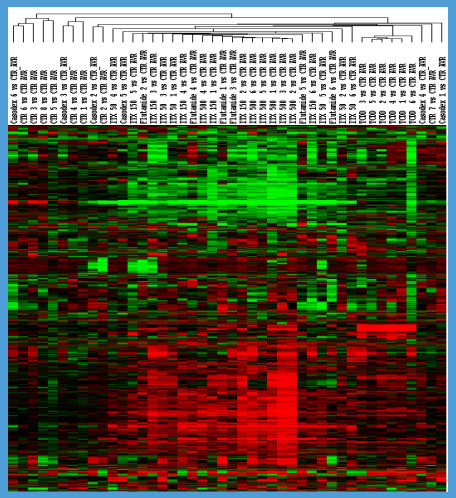
Wang et al., in preparation

ITX

An example were bioassays are used and results are compared with omics based methods and in vivo



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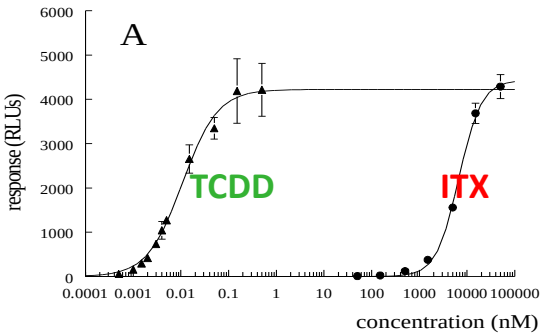
Regarding ITX

- 2-Isopropylthioxanthone (2-ITX) is a photo initiator in printing ink of packaging materials (e.g. found in milk).
- Little is known about possible effects of ITX on human health.
- Here we test the *in vitro* AhR agonist activity and both the (anti-)estrogenic and (anti-)androgenic properties of ITX with bioassays first.

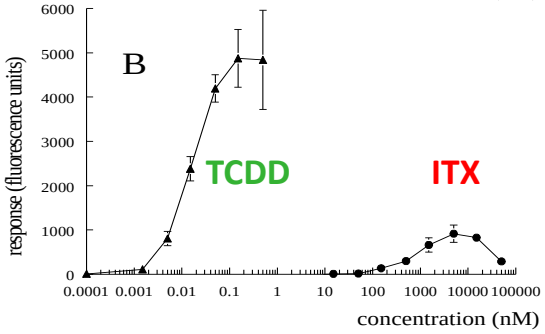
ITX in the DR-CALUX and yeast estrogen and androgen bioassays

Rat liver H4IIE cells

CALUX

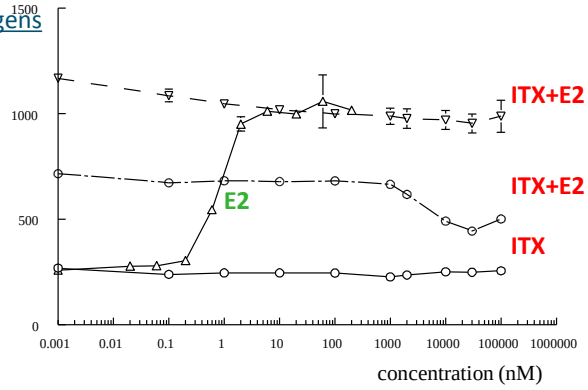


EROD

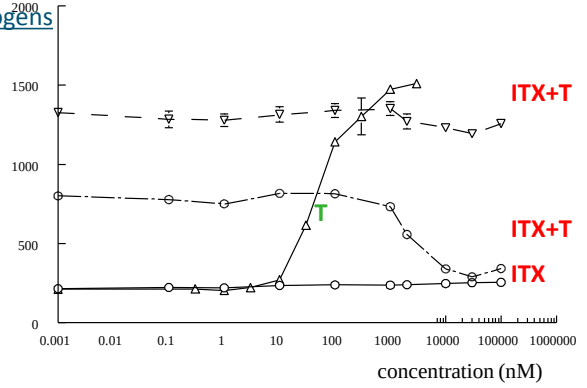


Yeast cells

Estrogens



Androgens



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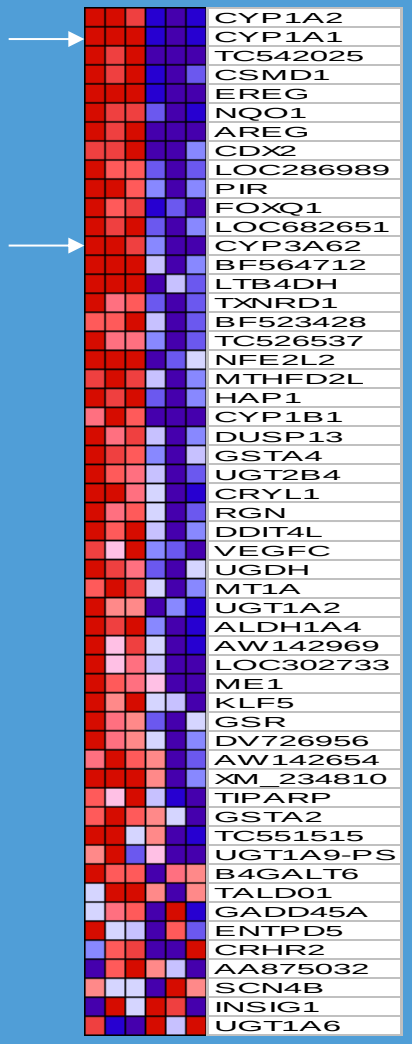
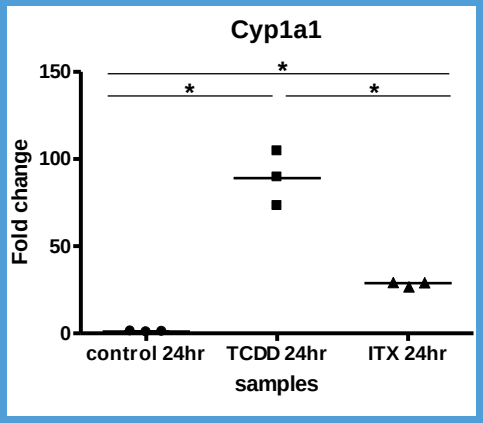
In vitro gene expression profiles in H4IIE cells: ITX vs TCDD

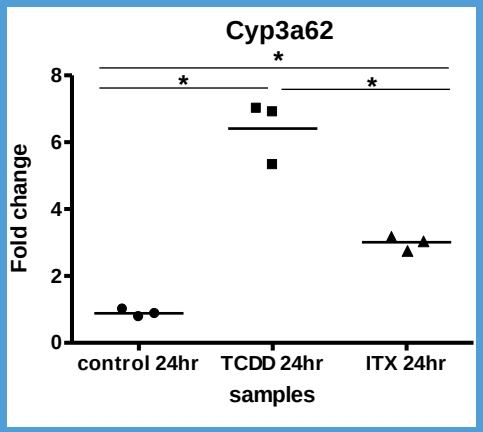
ITX/DMSO



Genes differentially expressed by TCDD were selected on an average up-regulation in rat H4IIE hepatoma cells by ≥1.5-fold at 24 h in combination with a P-value of <0.01 (students t-test). The heat map shows the relative expression levels of these TCDD-affected genes in hepatoma cells treated for 24 h either with 5 µM ITX or with the vehicle DMSO (CTR). Red: relatively high expression; blue: relatively low expression.

⇒ The vast majority of TCDD-up-regulated genes are also up-regulated by ITX.





RT-PCR confirmation of microarray data. Quantitative real-time PCR was performed for Cyp1a1 and Cyp3a62. The expression levels of the genes are relative to tubulin alpha 1C (Tuba1c). The mean gene expression value within each group is indicated with —. Significant difference between the groups: *P < 0.005, **P < 0.05.

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Summary of all *in vivo* results

	Body weight	Preputial separation	Sem vesicles + Coag gland	Ventral prostate	Testes	Epididymi	Liver	Kidneys	Sperm count caput	Sperm count cauda	Sperm motility
ITX-50				↓			↑				
ITX-150			↓	↓			↑↑				
ITX-500	↓		↓↓	↓↓			↑↑↑			↓	
TCDD							↑↑				
Flutamide	↓	↑↑↑	↓↓↓	↓↓↓			???		↓↓↓	↓↓↓	↓↓↓
Casodex		↑↑↑	↓↓↓						↓↓	↓↓	↓↓

Anti-ar **ivity** **Anti-androgenic**

Flutamide & Casodex only

Flutamide & Casodex only

Flutamide & Casodex only

Flutamide & Casodex only

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Hendriksen et al., in preparation

Questions?

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