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1 **Interlaboratory prevalidation of a new *in vitro* transcriptional activation assay for the**  
2 **screening of (anti-)androgenic activity of chemicals using the UALH-hAR cell line**

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24 *Keywords: androgen receptor; transactivation assay; stable luciferase reporter cell line;*  
25 *interlaboratory study.*

26 **Abstract**

27 We report an interlaboratory evaluation of a recently developed androgen receptor (AR)  
28 transactivation assay using the UALH-hAR reporter cell line that stably expresses the luciferase  
29 gene under the transcriptional control of androgen receptor elements (AREs) with no  
30 glucocorticoid receptor (GR) crosstalk. Herein, a two-step prevalidation study involving three  
31 laboratories was conducted to assess performance criteria of the method such as transferability  
32 as well as robustness, sensitivity, and specificity. The first step consisted in the validation of  
33 the transfer of the cell line to participant laboratories through the testing of three reference  
34 chemicals: the AR agonist dihydrotestosterone, the AR antagonist hydroxyflutamide and the  
35 glucocorticoid dexamethasone. Secondly, a blinded study was conducted by screening a  
36 selection of ten chemicals, including four AR agonists, five AR antagonists, and one non-active  
37 chemical. All test compounds yielded the same activity profiles in all laboratories. The logEC<sub>50</sub>  
38 (agonist assay) or logIC<sub>50</sub> (antagonist assay) were in the same range, with intra-laboratory  
39 coefficients of variation (CVs) of 0.1-3.4% and interlaboratory CVs of 1-4%, indicating very  
40 good within- and between-laboratory reproducibility. Our results were consistent with literature  
41 and regulatory data (OECD TG458). Overall, this interlaboratory study demonstrated that the  
42 UALH-hAR assay is transferable, produces reliable, accurate and specific (anti)androgenic  
43 activity of chemicals, and can be considered for further regulatory validation.

44

## 45        **1. Introduction**

46        Androgens are steroid hormones that play pivotal roles in the development and reproduction of  
47        vertebrates, notably in the male reproductive system. Most of their biological effects are  
48        mediated by their action on nuclear androgen receptor (AR), a ligand-dependent transcriptional  
49        factor that regulates many downstream androgen-dependent signalling pathways. Disrupting  
50        androgen signalling alters normal reproductive functions and can also account for several  
51        pathophysiological functions, such as prostate cancer. The AR signalling pathway is for  
52        instance an important target of anti-cancer drugs, with an active search for new AR inhibitors  
53        in the context of prostate cancer therapies (Nigro et al., 2021). It is also now well documented  
54        that AR signalling can be disrupted by chemicals exerting either androgenic or antiandrogenic  
55        activities (Gray et al., 2001; Scott et al., 2009). Environmental chemicals such as  
56        pharmaceuticals (Runnalls et al., 2010), pesticides (Kojima et al., 2004; Lemaire et al., 2004;  
57        Raun Andersen et al., 2002), or flame retardants (Hamers et al., n.d.) that can be found in air,  
58        surface waters or food (Biedermann and Grob, 1998; Creusot et al., 2014; Haith and Rossi,  
59        2003; Rodriguez-Mozaz et al., 2004; Russo et al., 2019; Sumpter, 2005), might pose risks to  
60        wildlife and humans through the disruption of the AR-signalling pathway. Therefore, there is a  
61        need for reliable tools to assess the (anti)androgenic activities of chemicals and their mixtures.  
62        As such, several established stable reporter cell lines such as PALM (Molina-Molina et al.,  
63        2013, 2006; Térouanne et al., 2000), HELN-AR (ER $\alpha$ -DBD) (Delfosse et al., 2012; Grimaldi  
64        et al., 2019), or MDA-kb2 (Aït-Aïssa et al., 2010; Wilson et al., 2002) or AR-EcoScreen (Sato  
65        et al., 2004) have been developed and successfully used to screen (anti)-androgenic activities  
66        of chemicals or environmental mixtures. However, these reporter gene assays present a limit  
67        regarding AR-specificity of their response because of the use of promoter constructs that  
68        possess response elements to other endogenously expressed nuclear receptors such the  
69        glucocorticoid (GR) and progesterone (PR) receptors. Indeed, GR is naturally expressed in

70 many cell lines and, once activated by its ligands, has been shown to activate (PALM, MDA-  
71 kb2, AR-Ecoscreen) or repress (HELN-AR-(ER $\alpha$ -DBD)) AR-responsive luciferase constructs  
72 in these AR-reporter cell lines. To overcome this limit, other *in vitro* cell lines have been  
73 developed for more specific assessment of androgenic activity of chemicals, either by knocking  
74 out endogenous GR expression, such as in the AR-EcoScreen GR KO (Zwart et al., 2017) or  
75 22Rv1/MMTV\_GR-KO (Park et al., 2021; Sun et al., 2016) cell lines, or by using a cell model  
76 that naturally expresses very low levels of GR, such as in the U2OS AR-CALUX assay  
77 (Milcamps et al., 2021, 2020; Sonneveld et al., 2006, 2005; van der Burg et al., 2015, 2010).  
78 All three methods are included in the OECD Guideline TG 458 (OECD, 2020).  
79 To address the current needs of AR specific *in vitro* tools with as minimal GR-crosstalk as  
80 possible, a new U2OS-derived luciferase reporter cell line, UALH-hAR, has been recently  
81 developed (Dellal et al., 2020). This cell line expresses high levels of AR but not GR, as well  
82 as the luciferase gene under the control of an ARE selective promoter (contains 6 AREs), which  
83 makes the luciferase response very sensitive and selective to AR ligands in this cell model.  
84 Such model could represent a relevant tool for screening (anti)androgenic activity of test  
85 substances notably within a regulatory context. In the present study, we conducted a two-step  
86 interlaboratory exercise to assess the robustness of UALH-hAR cell line and test method as a  
87 potential transferable and reliable assay for the specific detection of androgenic and  
88 antiandrogenic activity of chemicals, using a selection of ten substances, including AR agonists  
89 (4) AR antagonists (5) and one inactive chemical.

## 90 **2. Material and Methods**

### 91 **2.1. Design of the interlaboratory prevalidation study**

92 The prevalidation study involved three laboratories: laboratory A coordinated the  
93 prevalidation, prepared and sent around test chemicals, collected, analysed and reported the

94 data (INERIS, Unité Ecotoxicologie des Substances et Milieux, UMR-I 02 SEBIO, Parc  
95 ALATA, 60550 Verneuil-en-Halatte, France); laboratory B created the cell line, developed the  
96 method and distributed the cell line (IRCM, Inserm U1194, 34290 Montpellier, France); finally  
97 laboratory C served as a naive laboratory (Tame-Water, 85000 La Roche-sur-Yon, France). All  
98 three laboratories performed the experiments as described thereafter.

99 The prevalidation was done in two phases. For phase I, each laboratory tested three reference  
100 compounds in both agonist (exposure to test chemical alone) and antagonist (co-exposure to  
101 test chemical with reference agonist dihydrotestosterone DHT  $3.16 \times 10^{-10}$  M) modes in three  
102 independent runs, *i.e.*, separate experiments using cells from a different pool, freshly diluted  
103 chemicals, and conducted on different days. Selected compounds for the phase I were the  
104 androgen receptor (AR) agonist dihydrotestosterone (DHT), the AR antagonist  
105 hydroxyflutamide (HF) and dexamethasone (DEX), a synthetic GR agonist known to exert no  
106 agonistic activity towards AR. These compounds were all sent as powders by the laboratory A  
107 to laboratories B and C and each laboratory then proceeded to the preparation of stock solutions  
108 in dimethyl sulfoxide (DMSO). Results of each independent run and of each laboratory were  
109 then compared qualitatively (*i.e.*, active or non-active compound) and quantitatively (based on  
110 comparison of  $\log EC_{50}$  and  $\log IC_{50}$  obtained after modeling of concentration-response curves)  
111 to assess within laboratory reproducibility (WLR) and between laboratory reproducibility  
112 (BLR).

113 For phase II, WLR and BLR were assessed in real testing conditions by screening a selection  
114 of ten chemical substances in a blind study in agonist and antagonist modes in three independent  
115 runs. Tested compounds included the three reference chemicals used in phase I, as well as three  
116 AR agonists ligands: testosterone (T), medroxyprogesterone acetate (MPA), and  
117 methyltrienolone (R1881); three AR antagonists: bisphenol A (BPA), bisphenol C (BPC), and  
118 vinclozolin M2 (M2V); and one compound with no expected AR effect: mono(2-

119 ethylhexyl)phthalate (MEHP), a metabolite of di(2-ethylhexyl)phthalate (DEHP). All stock  
120 solutions in DMSO were prepared by laboratory A, then anonymized (coded) and sent to  
121 laboratories B and C. At laboratory A, the anonymization of stock solutions was performed by  
122 a person external to the study to ensure blind testing conditions in this lab. Sample codes were  
123 different for each laboratory. Results were analysed as in phase I to evaluate the WLR and BLR  
124 of the test method.

## 125 **2.2. Test chemicals**

126 Chemical structures and main properties of the ten selected substances are presented in Table  
127 1. Dihydrotestosterone (DHT), hydroxyflutamide (HF), dexamethasone (DEX),  
128 methyltrienolone (R1881), testosterone (T), medroxyprogesterone acetate (MPA), bisphenol A  
129 (BPA), bisphenol C (BPC) and mono(2-ethylhexyl)phthalate (MEHP) were obtained from  
130 Sigma-Aldrich Chemical Co. (Saint-Quentin Fallavier, France), and vinclozolin M2 (M2V) was  
131 obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Stock solutions of  
132 chemicals were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. Fresh dilutions of  
133 test chemicals in cell culture medium were prepared before each run and the final DMSO  
134 concentrations did not exceed 0.1% (v/v) or 0.2% (v/v) of the culture medium in agonist and  
135 antagonist modes, respectively. All three laboratories used the same batch of powders (Phase  
136 I) or stock solutions (Phase II), prepared and sent by the lead laboratory (Laboratory A).

## 137 **2.3. The UALH-hAR cell line**

138 The previously established UALH-hAR cell line (Dellal et al., 2020) was derived from human  
139 osteosarcoma cells U2OS using a two-step stable transfection with three plasmids (Fig. 1). To  
140 establish the UALH cell line, U2OS cells were first stably transfected with the  
141 6xARE(RAD9)Luc+/Hygromycin plasmid containing the firefly luciferase gene under the  
142 control of six androgen response elements (AREs) from the AR and PR selective RAD9 gene

143 promoter and placed upstream of the collagenase promoter and containing the resistance gene  
144 to hygromycin under the control of the SV40 promoter. Then, UALH cells were co-transfected  
145 with (i) the expression vector pSG5-AR-Puromycin containing the gene coding for hAR under  
146 the control of the simian virus promoter (SV40) and resistance gene to puromycin under the  
147 control of the phosphoglycerate kinase promoter (PGK) and (ii) the expression vector pSG5-  
148 AR-Renilla-Neomycin containing the gene coding for hAR under the control of the SV40  
149 promoter, the Renilla luciferase reporter gene under the control of the constitutive human  
150 cytomegalovirus (CMV), the firefly luciferase gene under the control of six androgen response  
151 elements (ARE) from the RAD9 gene promoter and placed upstream of the collagenase  
152 promoter, and the resistance gene to neomycin under the control of the SV40 promoter. The  
153 third plasmid was included to overexpress both AR and luciferase expression. The AR sequence  
154 was chosen from the NCBI database under the ID NM\_000044.3. Androgen responses elements  
155 of from the RAD9 gene promoter were chosen due to their high AR selectivity versus GR  
156 (Moehren et al., 2008). Cells were incubated in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.  
157 Selection of UALH-hAR cells was made with 0.5 µg/mL puromycin, 1 mg/L neomycin G418  
158 and 0.25 mg/mL hygromycin. Cells are passaged after reaching 75-90% confluence and the  
159 number of passages did not exceed 50. The cells were cultured using an established protocol,  
160 with slight differences between the three laboratories, as detailed in Supplementary Data S1.

161 **2.4. *Standard operating protocol (SOP) for the in vitro transcriptional activation assay***  
162 ***using the UALH-hAR cell line***

163 The following generic SOP was provided to participants laboratories as recommendations to  
164 run the assays. Due to specificities in participant laboratory facilities and uses, some slight  
165 differences between labs were noted regarding cell culture, cell exposure and luciferase readout  
166 protocols, which are detailed in Supplementary Data S1. Overall, cells were seeded in 96-wells  
167 white opaque culture plates at a density of 50,000 cells per well in culture medium. After 24 h,



168 cells were exposed in test medium with chemicals in a range of concentrations done in serial  
169 dilutions with a factor 3.16 in DMSO (laboratories A and C) or in DMSO-containing culture  
170 medium (laboratory B). In any case, the final DMSO content was always set at 0.1% v/v  
171 (agonist mode) and 0.2% v/v (antagonist mode). In the antagonist mode, tested compounds  
172 were exposed in presence of DHT at a concentration yielding 50-80% of the maximum  
173 luciferase activity, *i.e.*, DHT  $3.16 \times 10^{-10}$  M. Each concentration was tested in technical  
174 quadruplicates. Each plate contained the following controls: culture medium only, solvent  
175 control DMSO 0.1% v/v for agonist assays and 0.2% v/v for antagonist assays as negative  
176 controls; positive control DHT  $10^{-7}$  M leading to the maximal luciferase activity; in antagonist  
177 assays only, the reference compound DHT  $3.16 \times 10^{-10}$  M alone yielding 50-80% of the  
178 maximum luciferase activity. Plates were then incubated at 5% CO<sub>2</sub> ± 37°C for 24 h. At the end  
179 of the incubation, test medium was replaced with 50 µL per well of medium containing 0.5 mM  
180 D-luciferin (laboratory A) or 0.3 mM D-luciferin (laboratories B and C). Luminescence signal  
181 is measured in living cells for 2s per well using a luminometer. Each compound was tested in  
182 at least three independent runs.

### 183 **2.5. Cytotoxicity assay**

184 The viability of the cells was checked with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl  
185 tetrazolium bromide, Sigma M2128) assay to ensure that there was no cytotoxic effect at the  
186 concentrations tested. After exposure, cells were incubated in 100 µL test medium containing  
187 0.5 mg/mL MTT for 3h at 5% CO<sub>2</sub> and ± 37°C. Wells were then emptied, the cells lysed in 100  
188 µL DMSO/well and plates shaken for 10 minutes. The optical density (OD) was measured by  
189 spectroscopy at 570 nm and 640 nm and OD<sub>640</sub> values subtracted from OD<sub>570</sub>. Values obtained  
190 for the solvent control were considered as 100% viability. Concentrations leading to less than  
191 80% viability were excluded from the analysis.

### 192 **2.6. Data analysis**

193 To assess the risk of overlap between negative and positive controls, we calculated the Z-factor,  
194 considering the basal activity in DMSO-treated cells as a negative control and the maximal  
195 activity in DHT  $10^{-7}$  M-treated cells as a positive control, as follows:

$$196 \quad Z - factor = \frac{3(standard\ deviation_{DHT\ 10^{-7}M} + standard\ deviation_{DMSO\ control})}{|mean_{DHT\ 10^{-7}M} - mean_{DMSO\ control}|}$$

197 To express luciferase activity as % of maximal effect exerted by the positive control, relative  
198 light units (RLUs) were normalized between 0% (baseline luciferase activity in DMSO control)  
199 and 100% (luciferase activity in DHT  $10^{-7}$  M positive control) by using the following equation:

$$200 \quad \%effect = \frac{RLU_{test\ chemical} - RLU_{DMSO\ control}}{RLU_{DHT\ 10^{-7}M} - RLU_{DMSO\ control}}$$

201 A chemical is considered to have an agonistic effect when it increases luciferase activity above  
202 10% of the maximum effect induced by positive control for at least two consecutive  
203 concentrations. If not, the chemical is considered as non-agonist. A chemical is considered to  
204 have an antagonistic effect when it decreases luciferase activity by more than 30% in antagonist  
205 mode. If not, the chemical is considered as non-antagonist. For active chemicals, individual  
206 (ant-)agonist concentration-response curves are fitted with the Hill equation model, using the  
207 dose-response function of a graphics and statistics software program (GraphPad Prism 6,  
208 GraphPad Software Inc.). Effective concentrations (EC) and inhibitory concentrations (IC) as  
209 well the slope factor (Hill coefficient) were derived from the Hill equation. For a given  
210 chemical,  $EC_{50}$  is defined as the concentration inducing 50% of its maximal effect and  $IC_{50}$   
211 represents the concentration required for 50% inhibition of the effect induced by DHT  $3.16 \times 10^{-10}$   
212 M. Statistical analysis are performed on  $\log EC_{50}$  and  $\log IC_{50}$  values using Kruskal-Wallis  
213 test and Dunn's multiple comparison test ( $p < 0.05$ ) for the analysis of data from phases I and  
214 II, and t-test (Mann-Whitney) ( $p < 0.05$ ) for the comparison of phase I with phase II.

215 To assess the WLR of the method, the CV between the three runs of a given laboratory was  
216 determined by using the formula:  $CV = (Standard\ Deviation / Mean\ \log EC_{50}\ or\ \log IC_{50}) \times 100$ .

217 To assess the BLR of the method, we calculated the CV between the three laboratories using  
218 the formula:  $CV = (\text{Standard Deviation}/\text{Mean logEC}_{50} \text{ or logIC}_{50}) \times 100$ .

### 219 **3. Results and discussion**

#### 220 **3.1. Phase I: Transferability of the UALH-hAR method**

221 The phase I aimed at assessing the correct transfer of the cell line by comparing the  
222 quantification of the AR bioactivity of three reference chemicals, i.e., DHT, hydroxyflutamide  
223 and dexamethasone, chosen as reference AR agonist, AR antagonists and GR agonist,  
224 respectively.

225 For all three laboratories, the data produced were considered as valid. Z-factors were calculated  
226 in each laboratory and ranged from 0.7 to 0.9, meaning that the risk of overlap between negative  
227 and positive controls was negligible, as a good Z-factor should be comprised between 0.5 and  
228 1. For all runs, the induction factor of the positive control was always more than 5 times the  
229 solvent control (from 5.6- to 31-times over all data), and the mortality never exceeded 20%  
230 (data not shown). Based on this observation, we defined a maximal basal luciferase activity of  
231 20% and a minimal cell viability of 80% as validity criteria for the phase 2.

232 For all three laboratories, DHT increased luciferase activity in a concentration-dependent  
233 manner in the three independent runs as expected, with a mean logEC<sub>50</sub> of -9.8 (EC<sub>50</sub> of 1.6 x  
234 10<sup>-10</sup> M) (Fig. 2). Intra-laboratory CVs of logEC<sub>50</sub> ranged from 0.4 to 0.5% (Table 2), indicating  
235 good WLR. Regarding interlaboratory comparison, logEC<sub>50</sub> for DHT were in the same order of  
236 magnitude with a CV of 1.7%, suggesting good BLR, although there was a significant  
237 difference between laboratory A and laboratory C logEC<sub>50</sub> (Fig. 3).

238 In the three independent runs in all participating laboratories, HF did not induce any luciferase  
239 activity in agonist runs (data not shown) but decreased it in antagonist runs in a concentration-  
240 dependent manner, as expected (Fig. 2). The mean logIC<sub>50</sub> for HF was -7.2 (IC<sub>50</sub> of 6.3 x 10<sup>-8</sup>

241 M). CVs in each laboratory ranged from 0.3% to 2.4%, suggesting good WLR (Table 2).  
242 Interlaboratory CV was 2.0%, suggesting good BLR.

243 DEX did not display any AR-agonistic activity in any laboratory (data not shown). This lack of  
244 agonistic effect of DEX in UALH-hAR cells confirmed the non-detectable expression of  
245 functional GR and/or the inability of GR to induce luciferase expression from the  
246 6xARE(RAD9)Luc plasmids, unlike other established models (Delfosse et al., 2012; Térouanne  
247 et al., 2000; Wilson et al., 2002). However, in antagonist mode, DEX inhibited luciferase  
248 activity in a concentration-dependent manner in the three laboratories (Fig. 2). The mean  
249  $\log IC_{50}$  was -5.6 ( $IC_{50}$  of  $2.5 \times 10^{-6}$  M) and CVs ranged from 1.0% to 3.4%, suggesting  
250 acceptable WLR (Table 2). The mean  $\log IC_{50}$ s for each laboratory were very close, with a 1.4%  
251 CV, suggesting good BLR. The DEX antagonist activity observed in our study was already  
252 reported in literature in AR-CALUX, AR-EcoScreen and the mutant AR-EcoScreen GR-KO  
253 cell lines (Araki et al., 2005; Sonneveld et al., 2005; Zwart et al., 2017). The fact that the UALH-  
254 hAR line expresses GR at very low level suggests that this effect is not due to transcriptional  
255 interference with GR but rather to a direct antagonistic action of DEX on AR. Moreover, the  
256  $IC_{50}$  of DEX on UALH-hAR was relatively high – in the  $\mu$ M range – while its ability to bind  
257 to and activate human GR is reported in the nanomolar range (Molina-Molina et al., 2006). In  
258 addition, binding experiments on isolated receptors have shown that DEX can bind AR at high  
259 concentrations ( $IC_{50}$  of  $1.9 \times 10^{-4}$  M) (Féau et al., 2009). Further binding experiments could be  
260 conducted on UALH-hAR cells using radiolabelled R1881 to assess the binding of DEX to AR  
261 in these cells.

262 For all three substances, the CVs of the other Hill parameters also reflect good WLR and BLR  
263 (Supplementary Data S2).

264 Altogether, the three laboratories obtained perfectly concordant results from a qualitative point  
265 of view for the three substances tested. From a quantitative point of view, the  $\log EC_{50}$  and

266 logIC<sub>50</sub> values in the independent runs are close within and between each laboratory which  
267 indicates good WLR and BLR (Fig. 3). It is worth noting that the slight differences in the  
268 protocols between the three laboratories did not affect the comparability of the data. Main  
269 differences were related to the culture medium used (DMEM vs DMEM-F-12) and the  
270 preparation of serial dilution of chemicals (DMSO or culture medium) (Supplementary Data  
271 S1). These results show the good transferability of the method as well as its robustness when  
272 testing the three reference chemicals.

### 273 **3.2. Phase II: Assessing (anti)androgenic activity of ten substances in a blind study**

274 The robustness of the method was then assessed in a blind study using ten chemicals presenting  
275 different activity profiles. From a qualitative point of view, the activity profiles were perfectly  
276 consistent between the three laboratories and were all as expected for the ten substances tested  
277 (Fig. 4).

278 For DHT, HF and DEX, comparing logEC<sub>50s</sub> and logIC<sub>50s</sub> from phase I and phase II showed no  
279 statistical difference, which highlights a good robustness of the assay in quantifying the activity  
280 of these chemicals in a blinded manner within each participating laboratory.

281 Overall, logEC<sub>50</sub> and logIC<sub>50</sub> for active chemical substances were in the same range in the three  
282 laboratories, despite some minor but statistically significant differences between laboratories in  
283 the logEC<sub>50</sub> or logIC<sub>50</sub> of some chemicals (Fig. 5). Nevertheless, our results indicated good  
284 WLR with CVs ranging from 0.1% to 3.4%, and good BLR with CVs ranging from 1.0% to  
285 4.0% (Table 2). The CVs of the other Hill parameters also reflect good WLR and BLR  
286 (Supplementary Data S3).

287 In both agonist and antagonist runs, the phase II of the study revealed good WLR and BLR,  
288 thus indicating a good robustness of the assay.

### 289 3.3. Performance of the assay

290 The activity profiles for the ten chemicals on UALH-hAR cell line were overall very  
291 comparable with the ones described in the OECD Guideline 458 that uses AR-EcoScreen, AR-  
292 CALUX and 22Rv1/MMTV\_GR-KO (OECD, 2020), as well as with other similar cell models  
293 (Table 3). This highlights the very good sensitivity of our method (*i.e.*, the proportion of active  
294 substances that are correctly classified by the test), as activity profiles obtained for DHT,  
295 R1881, testosterone, MPA, HF, BPA, M2V and BPC in UALH-hAR cells proved similar to  
296 those described in the literature. For DEX, the lack of GR interference in our assay revealed a  
297 direct antagonistic activity, as also reported in AR-CALUX and AR-EcoScreen-GR-KO assays.  
298 Regarding MEHP, the lack of AR activity was previously described in similar *in vitro* models  
299 (Stroheker et al., 2005; Takeuchi et al., 2005), although this compound alone is insufficient to  
300 conclude about specificity (the proportion of all inactive substances that are correctly classified  
301 by the test). The antagonist effect of vinclozolin M2 is consistent with previous report in PALM  
302 cells (Molina-Molina et al., 2006) and MDA-kb2 cells (Wilson et al., 2002). In both studies, a  
303 weak agonist activity of this chemical was reported. Such a weak agonistic activity was also  
304 observed in our study, with a slight increase of luciferase activity by 5% at  $10^{-5}$  M (data not  
305 shown). However, we considered this chemical to be a full antagonist as this result does not  
306 meet the criteria for agonism, *i.e.*, increase of luciferase activity above 10% for at least two  
307 consecutive concentrations.

308 Beyond the qualitative aspect of our results, the  $EC_{50}$  and  $IC_{50}$  values derived from the modelled  
309 concentration-responses curves were within the ranges of acceptable values for OECD  
310 Guideline 458 for reference compounds and similar to the values found in literature (Table 3).  
311 Regarding the WLR and BLR, the CVs of  $\log EC_{50}$  and  $\log IC_{50}$  in our study were comparable  
312 to other published interlaboratory studies (Milcamps et al., 2021; Park et al., 2021).

313 Overall, our study suggests that the method we developed for the evaluation of AR  
314 (ant)agonistic activity of chemicals is well suited to the OECD criteria. It would be interesting  
315 to extend the screening to a higher number of chemical substances, notably regarding non active  
316 ones, to better assess the accuracy and specificity of the UALH-hAR cell line in a regulatory  
317 context.

318 Furthermore, a main advantage of the current assay relies on the possibility to assess  
319 nonspecific effect on luciferase by using the parental UALH cell line (i.e. which expresses  
320 luciferase but no functional AR) as a control for nonspecific effect on luciferase activity (Dellal  
321 et al. 2020). Such a control assay is most often missing in *in vitro* screens based on luminescent  
322 cell lines. Notably, it is of high relevance in the antagonist assay to distinguish between true  
323 AR antagonists and chemicals that negatively interfere with luciferase activity in an AR-  
324 independent manner, e.g. through direct interference with luciferase enzyme or due to early  
325 cytotoxic events. The occurrence of such nonspecific modulation of luciferase activity can also  
326 be critical when assessing environmental complex mixtures such as environmental waters,  
327 which anti-androgenicity is frequently detected but often at relatively high sample  
328 concentrations, close to cytotoxic ones. The use of such cell control without receptor has proven  
329 useful as quality control in environmental bioanalysis, as previously demonstrated by using the  
330 UALH/UALH-hAR (Neale et al. 2020) or HG5LN/HG5LN-hPXR (Creusot et al. 2010) cell  
331 lines.

#### 332 **4. Conclusion**

333 This interlaboratory study demonstrated the validity of the UALH-hAR-based method for the  
334 specific detection of chemicals exerting either androgenic or antiandrogenic activity. The first  
335 phase of the study demonstrated the transferability of the cell line and of the standard operating  
336 protocol to different laboratories, which appear robust as some adaptations to specific lab  
337 conditions (*e.g.*, culture medium, preparation of test chemicals) did not influence the outcome

338 of the assay, both qualitatively and quantitatively. In addition, the tests carried out under blind  
339 test conditions during the second phase produced reliable and accurate results both qualitatively  
340 and quantitatively with satisfying within- and between-laboratory reproducibility of the data,  
341 showing the robustness of the assay as a screening method. Comparison of the results with the  
342 OECD Guideline 458 and literature data showed the performance of the assay and its relevance  
343 and reliability as compared to validated AR-cell lines. Altogether, the UALH-hAR-based assay  
344 represents a promising test method for the hazard assessment of chemicals potentially acting on  
345 AR-signalling pathway and can be further considered for regulatory validation and international  
346 acceptance.

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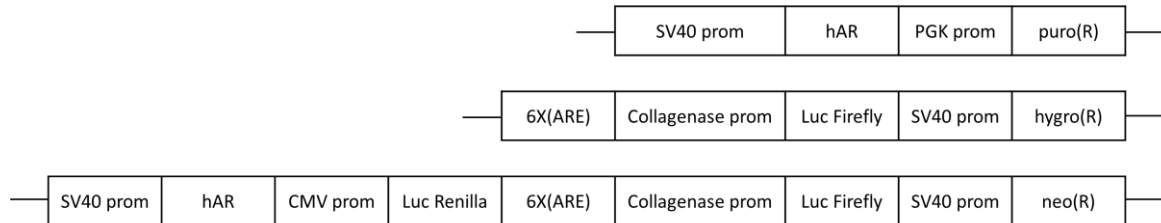
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## Figures and tables

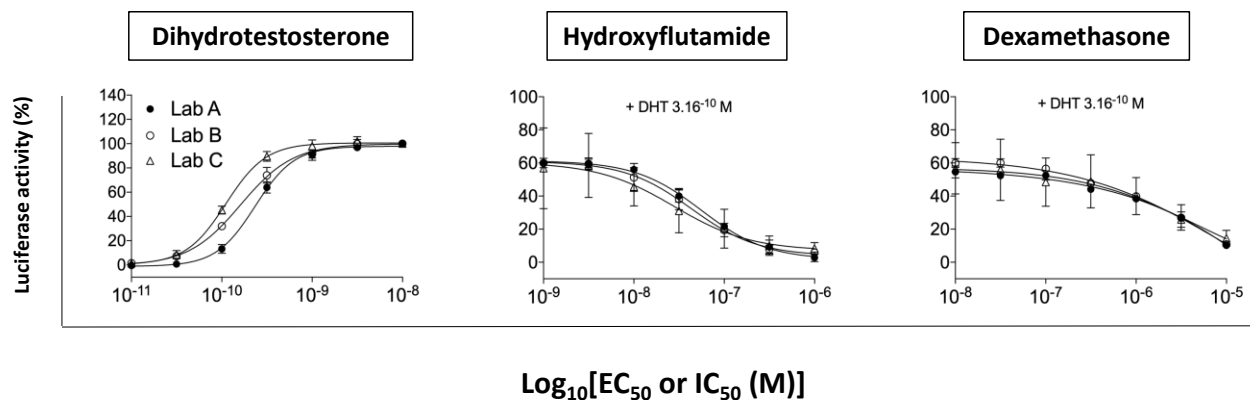
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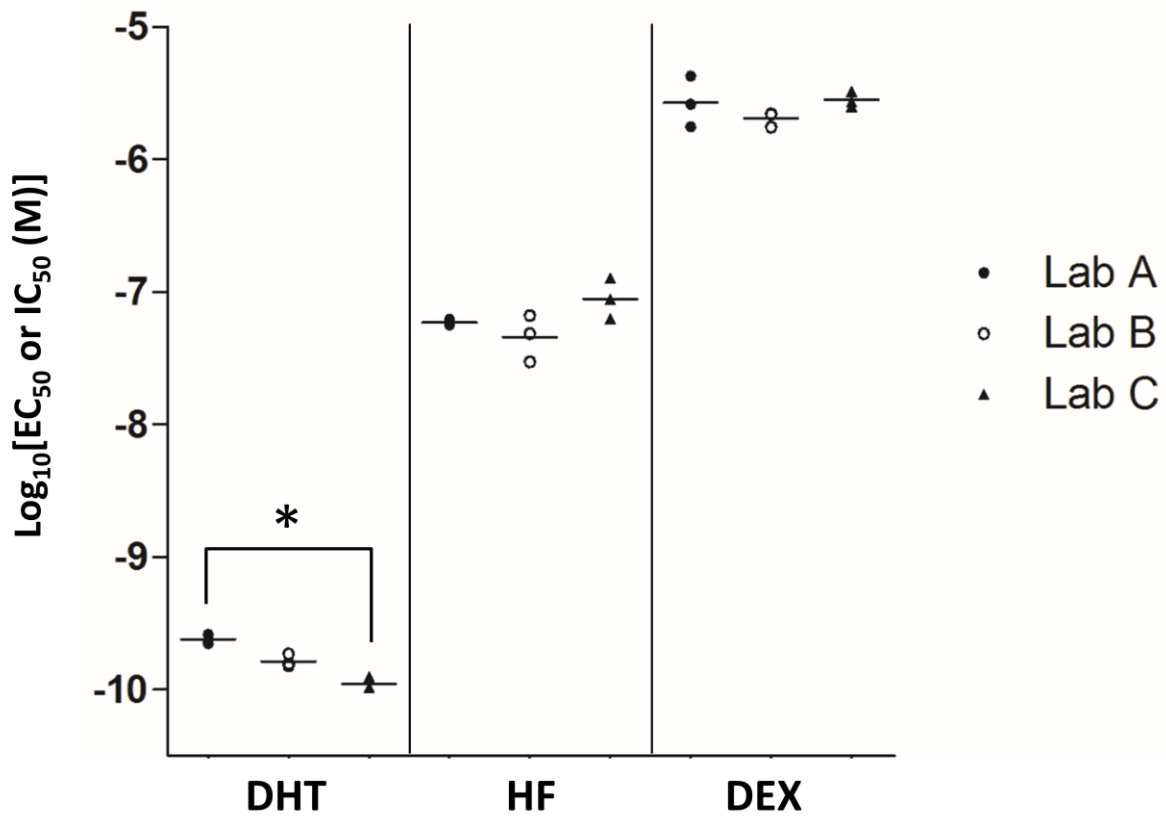
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500 **Figure 1.** The three plasmid constructs transfected into U2OS to create the UALH-hAR cell  
501 line. SV40: simian virus 40; prom: promoter; hAR: human androgen receptor; PGK:  
502 phosphoglycerate kinase; luc: luciferase; ARE: androgen response elements from the RAD9  
503 gene promoter; puro(R): puromycin resistance gene; hygro(R): hygromycin resistance gene;  
504 neo(R): neomycin resistance gene; CMV: human cytomegalovirus.

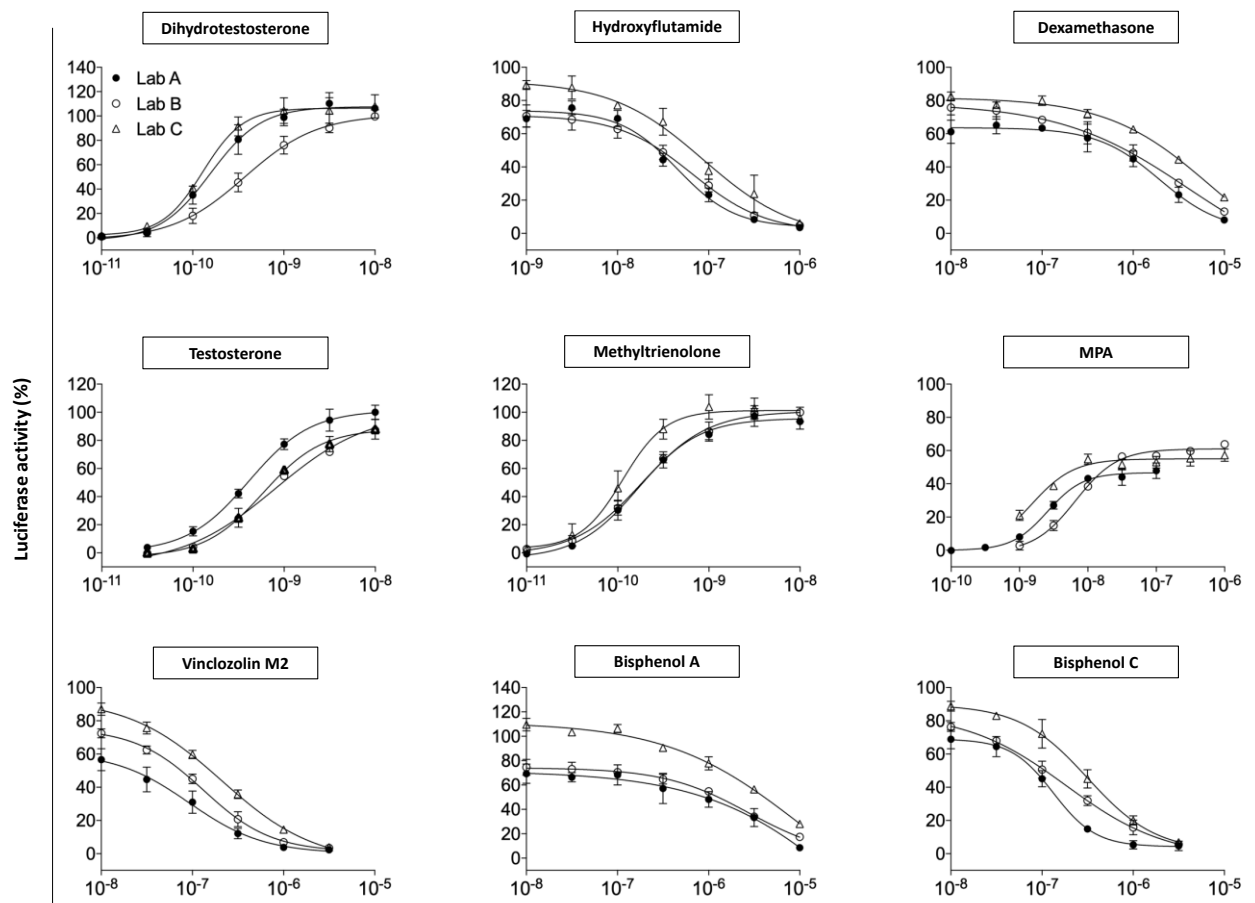


505  
 506 **Figure 2. Phase I transactivation of human nuclear androgen receptor (hAR) by reference**  
 507 **ligands** in UALH-hAR cells in the three laboratories. Data are means of  $n = 3$  independent  
 508 experiments per laboratory; error bars represent standard deviation. Results are expressed as  
 509 the percentage of the maximum luciferase activity induced by  $10^{-7}$  M DHT. Antagonist assays  
 510 are done in coexposure with  $3.16 \times 10^{-10}$  M DHT. Between experiment intra-laboratory  
 511 variability is illustrated in supplementary section (Figure S1).



513

514 **Figure 3. Phase I comparison of  $\text{logEC}_{50}$  and  $\text{logIC}_{50}$  between laboratories** for the testing  
 515 in triplicates of dihydrotestosterone (DHT), hydroxyflutamide (HF) and dexamethasone (DEX).  
 516 Bars represent means of 3 independent determinations. Lab: laboratory; \*:  $p < 0.05$  using  
 517 Kruskal-Wallis test and Dunn's multiple comparison test.



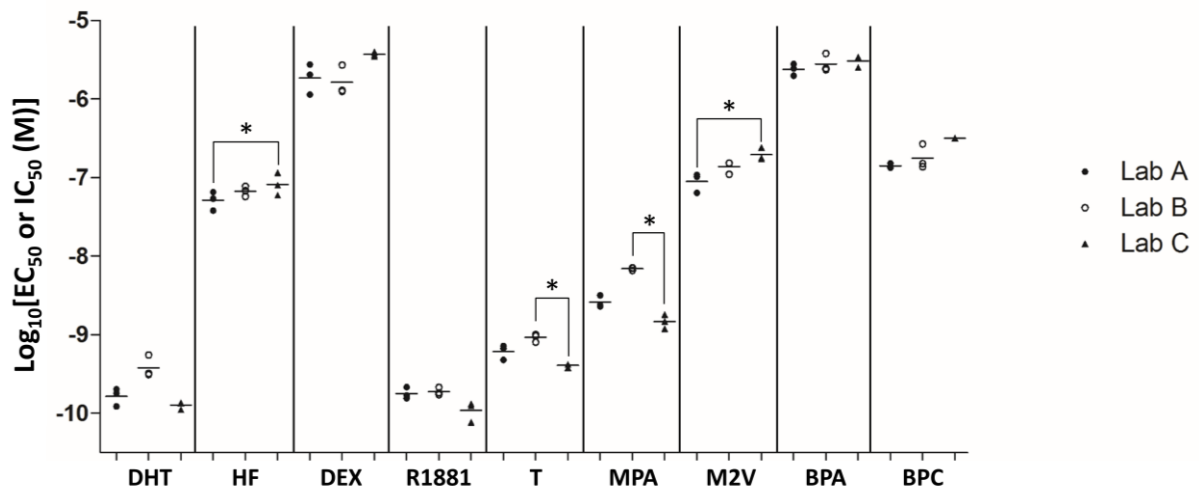
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519 **Figure 4. Phase II transactivation of human nuclear androgen receptor (hAR) by agonistic**  
 520 **and antagonistic ligands in UALH-hAR cells in the three laboratories.** Data are means of n =  
 521 3 independent experiments per laboratory; error bars represent standard deviation. Results are  
 522 expressed as the percentage of the maximum luciferase activity induced by  $10^{-7}$  M DHT.  
 523 Between experiment intra-laboratory variability is illustrated in supplementary section (Figures  
 524 S2 and S3).



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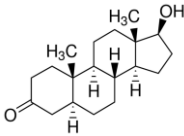
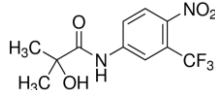
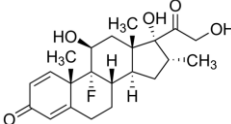
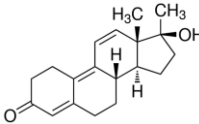
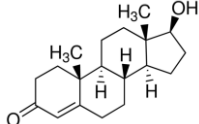
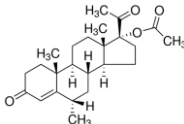
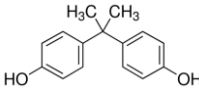
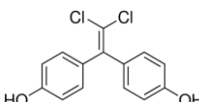
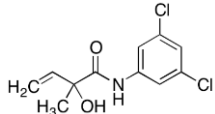
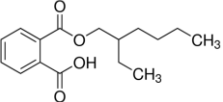
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527

528 **Figure 5. Phase II comparison of logEC<sub>50</sub> and logIC<sub>50</sub> between laboratories** for the blind  
529 testing of dihydrotestosterone (DHT), hydroxyflutamide (HF), dexamethasone (DEX),  
530 methyltrienolone (R1881), testosterone (T), medroxyprogesterone acetate (MPA), vinclozolin  
531 M2 (M2V), bisphenol A (BPA), bisphenol C (BPC). Bars represent means of three independent  
532 determinations. Lab: laboratory; \*: p < 0.05 using Kruskal-Wallis test and Dunn's multiple  
533 comparison test.

534 **Table 1. Test chemicals evaluated in the interlaboratory study.**

| Test chemical                      | Abbreviation | CAS        | Chemical structure  | Molecular weight (g/mol) | Purity |
|------------------------------------|--------------|------------|---|--------------------------|--------|
| <b>Dihydrotestosterone</b>         | DHT          | 521-18-6   |    | 290.44                   | ≥97.5% |
| <b>Hydroxyflutamide</b>            | HF           | 52806-53-8 |     | 292.21                   | ≥98%   |
| <b>Dexamethasone</b>               | DEX          | 50-02-2    |     | 392.46                   | ≥98%   |
| <b>Methyltrienolone</b>            | R1881        | 965-93-5   |    | 284.39                   | ≥98%   |
| <b>Testosterone</b>                | T            | 58-22-0    |  | 288.42                   | ≥98%   |
| <b>Medroxyprogesterone acetate</b> | MPA          | 71-58-9    |  | 386.52                   | ≥97%   |
| <b>Bisphenol A</b>                 | BPA          | 80-05-7    |  | 228.29                   | ≥99%   |
| <b>Bisphenol C</b>                 | BPC          | 14868-03-2 |  | 281.13                   | ≥98%   |
| <b>Vinclozolin M2</b>              | M2V          | 83792-61-4 |   | 260.12                   | ≥98%   |
| <b>Mono(2-ethylhexyl)phthalate</b> | MEHP         | 4376-20-9  |   | 277.34                   | ≥97%   |

536 **Table 2. Activity profiles of the 10 tested chemical substances in the three laboratories in**  
 537 **Phase I and Phase II, represented as mean logEC<sub>50</sub> (agonist assays) and mean logIC<sub>50</sub>**  
 538 **(antagonist assays).**

|                |                             |      | Agonist assays (logEC <sub>50</sub> ) |        |         |             | Antagonist assays (logIC <sub>50</sub> ) |        |             |             |
|----------------|-----------------------------|------|---------------------------------------|--------|---------|-------------|--|--------|-------------|-------------|
|                |                             |      | Lab. A                                | Lab. B | Lab. C. | Interlab.   | Lab. A                                   | Lab. B | Lab. C.     | Interlab.   |
| PHASE I        | Dihydrotestosterone         | mean | -9.6                                  | -9.8   | -10.0   | <b>-9.8</b> |  |        |             |             |
|                |                             | sd   | 0.04                                  | 0.05   | 0.05    | 0.17        | ne                                       | ne     | ne          |             |
|                |                             | CV   | 0.4%                                  | 0.5%   | 0.5%    | 1.7%        |  |        |             |             |
|                | Hydroxyflutamide            | mean |                                       |        |         |             | -7.2                                     | -7.3   | -7.1        | <b>-7.2</b> |
|                |                             | sd   | ne                                    | ne     | ne      |             | 0.02                                     | 0.18   | 0.15        | 0.15        |
|                |                             | CV   |                                       |        |         |             | 0.3%                                     | 2.4%   | 2.2%        | 2.0%        |
|                | Dexamethasone               | mean |                                       |        |         |             | -5.6                                     | -5.7   | -5.6        | <b>-5.6</b> |
|                |                             | sd   | ne                                    | ne     | ne      |             | 0.19                                     | 0.06   | 0.06        | 0.08        |
|                |                             | CV   |                                       |        |         |             | 3.4%                                     | 1.0%   | 1.1%        | 1.4%        |
| PHASE II       | Dihydrotestosterone         | mean | -9.8                                  | -9.4   | -9.9    | <b>-9.7</b> |  |        |             |             |
|                |                             | sd   | 0.11                                  | 0.14   | 0.05    | 0.25        | ne                                       | ne     | ne          |             |
|                |                             | CV   | 1.2%                                  | 1.5%   | 0.5%    | 2.6%        |  |        |             |             |
|                | Hydroxyflutamide            | mean |                                       |        |         |             | -7.3                                     | -7.2   | -7.1        | <b>-7.2</b> |
|                |                             | sd   | ne                                    | ne     | ne      |             | 0.12                                     | 0.06   | 0.14        | 0.10        |
|                |                             | CV   |                                       |        |         |             | 1.6%                                     | 0.9%   | 2.0%        | 1.4%        |
|                | Dexamethasone               | mean |                                       |        |         |             | -5.7                                     | -5.8   | -5.4        | <b>-5.6</b> |
|                |                             | sd   | ne                                    | ne     | ne      |             | 0.19                                     | 0.19   | 0.03        | 0.19        |
|                |                             | CV   |                                       |        |         |             | 3.4%                                     | 3.3%   | 0.5%        | 3.4%        |
|                | Methyltrienolone            | mean | -9.8                                  | -9.7   | -10.0   | <b>-9.8</b> |  |        |             |             |
|                |                             | sd   | 0.07                                  | 0.05   | 0.13    | 0.13        | ne                                       | ne     | ne          |             |
|                |                             | CV   | 0.8%                                  | 0.5%   | 1.3%    | 1.4%        |  |        |             |             |
|                | Testosterone                | mean | -9.2                                  | -9.0   | -9.4    | <b>-9.2</b> |  |        |             |             |
|                |                             | sd   | 0.09                                  | 0.05   | 0.03    | 0.18        | ne                                       | ne     | ne          |             |
|                |                             | CV   | 1.0%                                  | 0.6%   | 0.3%    | 1.9%        |  |        |             |             |
|                | Medroxyprogesterone acetate | mean | -8.6                                  | -8.2   | -8.8    | <b>-8.5</b> |  |        |             |             |
|                |                             | sd   | 0.08                                  | 0.02   | 0.09    | 0.34        | ne                                       | ne     | ne          |             |
|                |                             | CV   | 0.9%                                  | 0.2%   | 1.0%    | 4.0%        |  |        |             |             |
| MEHP           | mean                        |      |                                       |        |         |             |  |        |             |             |
|                | sd                          | ne   | ne                                    | ne     |         | ne          | ne                                       | ne     |             |             |
|                | CV                          |      |                                       |        |         |             |  |        |             |             |
| Vinclozolin M2 | mean                        |      |                                       |        |         | -7.0        | -6.9                                     | -6.7   | <b>-6.9</b> |             |
|                | sd                          | ne   | ne                                    | ne     |         | 0.13        | 0.07                                     | 0.08   | 0.17        |             |
|                | CV                          |      |                                       |        |         | 1.8%        | 1.0%                                     | 1.2%   | 2.5%        |             |
| Bisphenol A    | mean                        |      |                                       |        |         | -5.6        | -5.6                                     | -5.5   | <b>-5.6</b> |             |
|                | sd                          | ne   | ne                                    | ne     |         | 0.08        | 0.11                                     | 0.07   | 0.06        |             |
|                | CV                          |      |                                       |        |         | 1.3%        | 2.1%                                     | 1.3%   | 1.0%        |             |
| Bisphenol C    | mean                        |      |                                       |        |         | -6.9        | -6.8                                     | -6.5   | <b>-6.7</b> |             |
|                | sd                          | ne   | ne                                    | ne     |         | 0.03        | 0.16                                     | 0.00   | 0.18        |             |
|                | CV                          |      |                                       |        |         | 0.4%        | 2.3%                                     | 0.1%   | 2.7%        |             |

539 Lab.: laboratory; Interlab.: interlaboratory comparison; ne: no effect; sd: standard deviation;  
 540 CV: coefficient of variation.

541 **Table 3. Comparison of log effective concentrations logEC<sub>50</sub> and log inhibitory concentrations logIC<sub>50</sub> (log[M]) in UALH-hAR cells with**  
 542 **values reported in other *in vitro* reporter cell models. Bibliographic references are indicated as superscripts. \*AR-EcoScreen GR-KO;**  
 543 **\*\*22Rv1/MMTV**

|             |                             | UALH-hAR (present study) | AR-EcoScreen  | AR-CALUX                            | 22Rv1/MMTV_GR-KO           | PALM  | MDA-kb2  | HELN-AR(ERα-DBD)             | MDA-MB453-pMMTVneo-Luc                     | CHO-K1 pZeoSV2AR-pIND-ARE-pCMVβ-Gal         |
|-------------|-----------------------------|--------------------------|---|-------------------------------------|----------------------------|---|--|------------------------------|--|---|
| Agonists    | Dihydrotestosterone         | -9.7                     | -11.03 ~ -9.00 (OECD, 2020)   | -9.98 ~ -9.42 (OECD, 2020)          | -9.73 ~ -8.95 (OECD, 2020) | -9.5 (Térouanne et al., 2000)   | Agonist (Wilson et al., 2002)<br>-9.8 (Aït-Aïssa et al., 2010)         |                              |  |   |
|             | Methyltrienolone            | -9.8                     | -9.6 (Araki et al., 2005)   | -9.9 (Sonneveld et al., 2005)       | -9.97 (Sun et al., 2016)** | -10.3 (Térouanne et al., 2000)<br>-10.0 (Molina-Molina et al., 2006)                      |  |                              |  |   |
|             | Testosterone                | -9.2                     | -9.46 ~ -8.96 (OECD, 2020)  | -9.25 ~ -8.8 (OECD, 2020)           | -9.67 ~ -8.66 (OECD, 2020) | -7.7 (Térouanne et al., 2000)   | -9.4 (Aït-Aïssa et al., 2010)  |                              |  |   |
|             | Medroxyprogesterone acetate | -8.5                     | -8.77 ~ -8.37 (OECD, 2020)  | -9.23 ~ -7.75 (OECD, 2020)          | -7.64 ~ -6.01 (OECD, 2020) |   | Agonist (Wilson et al., 2002)  |                              |  |   |
| No effect   | MEHP                        | no effect                |   |                                     |                            |   |  |                              | No anti-AR effect (Stroheker et al., 2005) | No (anti-)AR effect (Takeuchi et al., 2005) |
| Antagonists | Hydroxyflutamide            | -7.2                     | -7.80 ~ -6.17 (OECD, 2020)  | -7.80 ~ -7.54 (OECD, 2020)          | -7.79 ~ -7.11 (OECD, 2020) | Antagonist (Térouanne et al., 2000)   | Antagonist (Wilson et al., 2002)                                       |                              |  |   |
|             | Dexamethasone               | -5.6                     | Agonist -8.8 (Zwart et al., 2017)<br>Agonist -8.6 (Araki et al., 2005)<br>Antagonist -5.6 (Zwart et al., 2017)*<br>Antagonist -4.4 (Araki et al., 2005) | Antagonist (Sonneveld et al., 2005) |                            | Agonist (Térouanne et al., 2000)  | Agonist (Wilson et al., 2002)<br>Agonist -8.4 (Aït-Aïssa et al., 2010) |                              |  |   |
|             | Vinclozolin M2              | -6.9                     |   |                                     |                            | Agonist -4.1 (Molina-Molina et al., 2006)<br>Antagonist -6.8 (Molina-Molina et al., 2006) | Agonist/antagonist (Wilson et al., 2002)                               |                              |  |   |
|             | Bisphenol A                 | -5.6                     | -7.05 ~ -4.29 (OECD, 2020)  | -5.93 ~ -5.81 (OECD, 2020)          | -5.68 ~ -5.29 (OECD, 2020) | Agonist -4.3 (Molina-Molina et al., 2013)<br>Antagonist -6.0 (Molina-Molina et al., 2013) |  | -5.6 (Delfosse et al., 2012) |  |   |
|             | Bisphenol C                 | -6.7                     |   |                                     |                            |   |  | -6.7 (Delfosse et al., 2012) |  |   |

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## Supplementary Data

### **Interlaboratory prevalidation of a new *in vitro* transcriptional activation assay for the screening of (anti-)androgenic chemicals using the UALH-hAR cell line**

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*Keywords: androgen receptor; assay; stable luciferase reporter cell line; interlaboratory study.*

570 **Supplementary Table S1. Protocol specificities in the three laboratories.**

|                       |                       | Laboratory A  | Laboratory B  | Laboratory C  |
|-----------------------|-----------------------|---|---|---|
| Culture medium        |                       | - DMEM with phenol red (Gibco 21885-025)<br>- 5% FBS (Sigma F7524)<br>- 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL)<br>- 1% v/v nonessential amino acids (Gibco 11140050)<br>- Puromycin 0.25-0.5 µg/mL (Sigma P8833)<br>- G418 0.5-1 mg/mL (Invivogen ant-gn 100 mg/mL)<br>- Hygromycin 0.125-0.25 mg/mL | - DMEM/F-12 with phenol red (Gibco 31331-028)<br>- 5% FBS (Eurobio CVFSVF00)<br>- 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL)<br><br>- Puromycin 0.25-0.5 µg/mL (Sigma P8833)<br>- G418 0.5-1 mg/mL (Invivogen ant-gn 100 mg/mL)<br>- Hygromycin 0.125-0.25 mg/mL | - DMEM/F-12 with phenol red (Gibco 31331-028)<br>- 5% FBS (Eurobio CVFSVF00)<br>- 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL)<br><br>- Puromycin 0.25-0.5 µg/mL (Sigma P8833)<br>- G418 0.5-1 mg/mL (Invivogen ant-gn 100 mg/mL)<br>- Hygromycin 0.125-0.25 mg/mL |
| Test medium           |                       | - DMEM without phenol red (Gibco 11880)<br>- 2% DCC-treated FBS<br>- 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL)<br>- 1% v/v L-Glutamine<br>- 1% v/v nonessential amino acids (Gibco 11140050)  | - DMEM/F-12 without phenol red (Gibco 21041-025)<br>- 2% DCC-treated FBS<br>- 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL)<br>- 1% v/v L-Glutamine   | - DMEM/F-12 without phenol red (Dutscher P04-41650)<br>- 2% DCC-treated FBS<br>- 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL)<br>- 1% v/v L-Glutamine  |
| Luminescence medium   |                       | - Test medium with 0.5 mM D-luciferin Firefly in Tris buffer pH 8.0 (Caliper)   | - Test medium with 0.3 mM D-luciferin Firefly in Tris buffer pH 8.0 (Perkin Elmer 122799)   | - Test medium with 0.3 mM D-luciferin Firefly in Tris buffer pH 8.0 (Roth)  |
| Plates reference      |                       | Greiner bio-one 655098  | Greiner bio-one 655083-905  | Greiner bio-one 655098  |
| Luminometer           |                       | Synergy H4  | Wallax MicroBeta TriLux   | Victor3 Perkin Elmer  |
| Transactivation assay | Cell seeding          | Cells are seeded in 96-wells white opaque culture plates at a density of 50,000 cells per well in 100 µL test medium.   | Cells are seeded in 96-wells white opaque culture plates at a density of 50,000 cells per well in 150 µL test medium.   | Cells are seeded in 96-wells white opaque culture plates at a density of 50,000 cells per well in 100 µL test medium.   |
|                       | Chemicals preparation | Serial dilutions of stock solutions are prepared at 1000X concentrations in DMSO and then diluted in test medium at 3X concentration.   | 1000X stock solution in DMSO is diluted at 4X in test medium; then, a range of 4X concentrations is done by serial dilutions in DMSO-containing test medium   | Serial dilutions of stock solutions are prepared at 1000X concentrations in DMSO and then diluted in test medium at 3X concentration.   |
|                       | Exposure              | 50 µL of the 3X diluted compounds are distributed to the wells containing 100 µL test medium for a final concentration of 1X, and for a final DMSO content set at 0.1% v/v for agonist assays and 0.2% v/v for antagonist assays.   | Culture medium in the plates is replaced by 150 µL test medium then 50 µL of the 4X concentrated compounds are added, for a final concentration of 1X, and for a final DMSO content set at 0.1% v/v for agonist assays and 0.2% v/v for antagonist assays.                      | 50 µL of the 3X diluted compounds are distributed to the wells containing 100 µL test medium for a final concentration of 1X, and for a final DMSO content set at 0.1% v/v for agonist assays and 0.2% v/v for antagonist assays.   |
|                       | Automation            | Preparation of chemical dilutions in DMSO and their transfer into the microplates containing the cells are performed using a liquid handling workstation (Evo75, TECAN)   | Cell-seeding was automated (Multidrop, ThermoFisher), and preparation of chemical dilutions and their transfer into the microplates containing the cells are performed using a liquid handling workstation (Biomek 3000, Beckman).  | All the steps are performed manually.   |

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572 **Supplementary Table S2. Phase I intra- and interlaboratory comparisons of the effects of dihydrotestosterone, hydroxyflutamide and**  
 573 **dexamethasone** based on the Hill model parameters: Hill slope, logEC<sub>10</sub>, logEC<sub>50</sub> and maximum or logIC<sub>30</sub> and logIC<sub>50</sub>.

|                      |       | Dihydrotestosterone |                     |                     |              | Hydroxyflutamide |                     |                     | Dexamethasone |                     |                     |
|----------------------|-------|---------------------|---------------------|---------------------|--------------|------------------|---------------------|---------------------|---------------|---------------------|---------------------|
|                      |       | Hill slope          | logEC <sub>10</sub> | logEC <sub>50</sub> | Max (%)      | Hill slope       | logIC <sub>30</sub> | logIC <sub>50</sub> | Hill slope    | logIC <sub>30</sub> | logIC <sub>50</sub> |
| Laboratory A         | Run 1 | 1.7                 | -10.2               | -9.6                | 99.1         | -0.9             | -7.6                | -7.2                | -0.9          | -6.2                | -5.8                |
|                      | Run 2 | 2.0                 | -10.1               | -9.6                | 97.2         | -1.1             | -7.6                | -7.2                | -1.0          | -5.7                | -5.4                |
|                      | Run 3 | 2.5                 | -10.0               | -9.7                | 97.7         | -1.3             | -7.5                | -7.2                | -0.9          | -6.0                | -5.6                |
|                      | mean  | <b>2.1</b>          | <b>-10.1</b>        | <b>-9.6</b>         | <b>98.0</b>  | <b>-1.1</b>      | <b>-7.6</b>         | <b>-7.2</b>         | <b>-0.9</b>   | <b>-6.0</b>         | <b>-5.6</b>         |
|                      | sd    | 0.37                | 0.08                | 0.04                | 1.03         | 0.17             | 0.08                | 0.02                | 0.05          | 0.21                | 0.19                |
|                      | CV    | 17.9%               | 0.8%                | 0.4%                | 1.1%         | 15.7%            | 1.0%                | 0.3%                | 5.7%          | 3.6%                | 3.4%                |
| Laboratory B         | Run 1 | 1.7                 | -10.4               | -9.8                | 101.4        | -1.1             | -7.5                | -7.2                | -0.8          | -6.1                | -5.7                |
|                      | Run 2 | 1.4                 | -10.5               | -9.8                | 99.6         | -0.8             | -8.0                | -7.5                | -0.9          | -6.1                | -5.7                |
|                      | Run 3 | 1.4                 | -10.4               | -9.7                | 98.9         | -1.0             | -7.7                | -7.3                | -0.8          | -6.2                | -5.8                |
|                      | mean  | <b>1.5</b>          | <b>-10.4</b>        | <b>-9.8</b>         | <b>99.9</b>  | <b>-1.0</b>      | <b>-7.7</b>         | <b>-7.3</b>         | <b>-0.8</b>   | <b>-6.1</b>         | <b>-5.7</b>         |
|                      | sd    | 0.19                | 0.06                | 0.05                | 1.31         | 0.14             | 0.23                | 0.18                | 0.04          | 0.08                | 0.06                |
|                      | CV    | 12.8%               | 0.6%                | 0.5%                | 1.3%         | 14.1%            | 3.0%                | 2.4%                | 4.9%          | 1.2%                | 1.0%                |
| Laboratory C         | Run 1 | 1.7                 | -10.4               | -10.0               | 98.8         | -0.9             | -7.3                | -6.9                | -0.8          | -6.0                | -5.5                |
|                      | Run 2 | 1.9                 | -10.5               | -9.9                | 103.4        | -1.1             | -7.4                | -7.1                | -0.8          | -6.0                | -5.6                |
|                      | Run 3 | 2.4                 | -10.4               | -10.0               | 99.5         | -0.8             | -7.7                | -7.2                | -0.8          | -6.0                | -5.6                |
|                      | mean  | <b>2.0</b>          | <b>-10.4</b>        | <b>-10.0</b>        | <b>100.6</b> | <b>-0.9</b>      | <b>-7.5</b>         | <b>-7.1</b>         | <b>-0.8</b>   | <b>-6.0</b>         | <b>-5.6</b>         |
|                      | sd    | 0.35                | 0.06                | 0.05                | 2.47         | 0.15             | 0.18                | 0.15                | 0.04          | 0.04                | 0.06                |
|                      | CV    | 17.6%               | 0.6%                | 0.5%                | 2.5%         | 16.3%            | 2.5%                | 2.2%                | 4.8%          | 0.6%                | 1.1%                |
| Interlaboratory mean | mean  | <b>1.9</b>          | <b>-10.3</b>        | <b>-9.8</b>         | <b>99.5</b>  | <b>-1.0</b>      | <b>-7.6</b>         | <b>-7.2</b>         | <b>-0.9</b>   | <b>-6.0</b>         | <b>-5.6</b>         |
|                      | sd    | <b>0.30</b>         | <b>0.19</b>         | <b>0.17</b>         | <b>1.36</b>  | <b>0.11</b>      | <b>0.13</b>         | <b>0.15</b>         | <b>0.08</b>   | <b>0.09</b>         | <b>0.08</b>         |
|                      | CV    | <b>16.2%</b>        | <b>1.8%</b>         | <b>1.7%</b>         | <b>1.4%</b>  | <b>11.1%</b>     | <b>1.7%</b>         | <b>2.0%</b>         | <b>9.0%</b>   | <b>1.5%</b>         | <b>1.4%</b>         |

574 Sd: standard deviation; CV: coefficient of variation; Max: maximum luminescence reached by the tested compound (% of max induced by DHT  
 575 10<sup>-7</sup> M).

576 **Supplementary Table S3. Phase II intra- and interlaboratory comparisons of the effects of dihydrotestosterone, hydroxyflutamide and**  
 577 **dexamethasone** based on the Hill model parameters: Hill slope, logEC<sub>10</sub>, logEC<sub>50</sub> and maximum or logIC<sub>30</sub> and logIC<sub>50</sub>.

|                      |       | Dihydrotestosterone |                     |                     |              | Hydroxyflutamide |                     |                     | Dexamethasone |                     |                     |
|----------------------|-------|---------------------|---------------------|---------------------|--------------|------------------|---------------------|---------------------|---------------|---------------------|---------------------|
|                      |       | Hill slope          | logEC <sub>10</sub> | logEC <sub>50</sub> | Max (%)      | Hill slope       | logIC <sub>30</sub> | logIC <sub>50</sub> | Hill slope    | logIC <sub>30</sub> | logIC <sub>50</sub> |
| Laboratory A         | Run 1 | 1.6                 | -10.3               | -9.7                | 111.5        | -1.3             | -7.5                | -7.2                | -1.6          | -5.8                | -5.6                |
|                      | Run 2 | 2.1                 | -10.4               | -9.9                | 107.4        | -1.3             | -7.7                | -7.4                | -1.3          | -6.0                | -5.7                |
|                      | Run 3 | 1.4                 | -10.4               | -9.7                | 104.6        | -1.1             | -7.6                | -7.3                | -0.9          | -6.4                | -5.9                |
|                      | mean  | <b>1.7</b>          | <b>-10.4</b>        | <b>-9.8</b>         | <b>107.8</b> | <b>-1.3</b>      | <b>-7.6</b>         | <b>-7.3</b>         | <b>-1.2</b>   | <b>-6.0</b>         | <b>-5.7</b>         |
|                      | sd    | 0.39                | 0.07                | 0.11                | 3.47         | 0.14             | 0.11                | 0.12                | 0.35          | 0.29                | 0.19                |
|                      | CV    | 22.7%               | 0.6%                | 1.2%                | 3.2%         | 10.8%            | 1.5%                | 1.6%                | 28.5%         | 4.8%                | 3.4%                |
| Laboratory B         | Run 1 | 1.2                 | -10.3               | -9.5                | 99.1         | -1.1             | -7.4                | -7.1                | -0.7          | -6.4                | -5.9                |
|                      | Run 2 | 1.1                 | -10.1               | -9.3                | 101.1        | -1.0             | -7.5                | -7.2                | -0.7          | -6.4                | -5.9                |
|                      | Run 3 | 1.1                 | -10.4               | -9.5                | 101.8        | -1.0             | -7.6                | -7.2                | -1.3          | -5.9                | -5.6                |
|                      | mean  | <b>1.2</b>          | <b>-10.2</b>        | <b>-9.4</b>         | <b>100.7</b> | <b>-1.1</b>      | <b>-7.5</b>         | <b>-7.2</b>         | <b>-0.9</b>   | <b>-6.2</b>         | <b>-5.8</b>         |
|                      | sd    | 0.06                | 0.14                | 0.14                | 1.38         | 0.05             | 0.08                | 0.06                | 0.34          | 0.34                | 0.19                |
|                      | CV    | 5.0%                | 1.4%                | 1.5%                | 1.4%         | 4.7%             | 1.0%                | 0.9%                | 38.9%         | 5.4%                | 3.3%                |
| Laboratory C         | Run 1 | 2.3                 | -10.4               | -10.0               | 99.2         | -0.9             | -7.4                | -6.9                | -1.0          | -5.8                | -5.5                |
|                      | Run 2 | 1.7                 | -10.4               | -9.9                | 103.1        | -1.0             | -7.5                | -7.1                | -1.0          | -5.8                | -5.4                |
|                      | Run 3 | 1.7                 | -10.4               | -9.9                | 118.6        | -1.0             | -7.6                | -7.2                | -0.9          | -5.8                | -5.4                |
|                      | mean  | <b>1.9</b>          | <b>-10.4</b>        | <b>-9.9</b>         | <b>107.0</b> | <b>-0.9</b>      | <b>-7.5</b>         | <b>-7.1</b>         | <b>-1.0</b>   | <b>-5.8</b>         | <b>-5.4</b>         |
|                      | sd    | 0.36                | 0.04                | 0.05                | 10.25        | 0.05             | 0.12                | 0.14                | 0.04          | 0.03                | 0.03                |
|                      | CV    | 18.7%               | 0.3%                | 0.5%                | 9.6%         | 5.3%             | 1.7%                | 2.0%                | 3.7%          | 0.6%                | 0.5%                |
| Interlaboratory mean | mean  | <b>1.6</b>          | <b>-10.3</b>        | <b>-9.7</b>         | <b>105.2</b> | <b>-1.1</b>      | <b>-7.5</b>         | <b>-7.2</b>         | <b>-1.0</b>   | <b>-6.0</b>         | <b>-5.6</b>         |
|                      | sd    | <b>0.38</b>         | <b>0.08</b>         | <b>0.25</b>         | <b>3.91</b>  | <b>0.16</b>      | <b>0.05</b>         | <b>0.10</b>         | <b>0.19</b>   | <b>0.21</b>         | <b>0.19</b>         |
|                      | CV    | <b>24.3%</b>        | <b>0.8%</b>         | <b>2.6%</b>         | <b>3.7%</b>  | <b>14.8%</b>     | <b>0.7%</b>         | <b>1.4%</b>         | <b>18.8%</b>  | <b>3.5%</b>         | <b>3.4%</b>         |

578 Sd: standard deviation; CV: coefficient of variation; Max: maximum luminescence reached by the tested compound (% of max induced by DHT  
 579 10<sup>-7</sup> M).



580 **Supplementary Table S4. Phase II intra- and interlaboratory comparisons of the effects of methyltrienolone, testosterone and**  
 581 **medroxyprogesterone acetate based on the Hill model parameters: Hill slope, logEC<sub>10</sub>, logEC<sub>50</sub> and maximum.**

|                      |       | Methyltrienolone |                     |                     |              | Testosterone |                     |                     |              | Medroxyprogesterone acetate |                     |                     |              |
|----------------------|-------|------------------|---------------------|---------------------|--------------|--------------|---------------------|---------------------|--------------|-----------------------------|---------------------|---------------------|--------------|
|                      |       | Hill slope       | logEC <sub>10</sub> | logEC <sub>50</sub> | Max (%)      | Hill slope   | logEC <sub>10</sub> | logEC <sub>50</sub> | Max (%)      | Hill slope                  | logEC <sub>10</sub> | logEC <sub>50</sub> | Max (%)      |
| Laboratory A         | Run 1 | 1.5              | -10.4               | -9.8                | 98.6         | 1.5          | -9.8                | -9.2                | 87.3         | 1.9                         | -9.1                | -8.6                | 46.6         |
|                      | Run 2 | 1.5              | -10.3               | -9.7                | 94.4         | 1.4          | -10.0               | -9.3                | 84.1         | 1.9                         | -9.1                | -8.6                | 42.2         |
|                      | Run 3 | 1.3              | -10.5               | -9.8                | 91.4         | 1.6          | -9.7                | -9.1                | 91.2         | 1.4                         | -9.2                | -8.5                | 51.8         |
|                      | mean  | <b>1.5</b>       | <b>-10.4</b>        | <b>-9.8</b>         | <b>94.8</b>  | <b>1.5</b>   | <b>-9.9</b>         | <b>-9.2</b>         | <b>87.5</b>  | <b>1.7</b>                  | <b>-9.2</b>         | <b>-8.6</b>         | <b>46.9</b>  |
|                      | sd    | 0.14             | 0.13                | 0.07                | 3.61         | 0.10         | 0.13                | 0.09                | 3.55         | 0.33                        | 0.04                | 0.08                | 4.80         |
|                      | CV    | 9.5%             | 1.2%                | 0.8%                | 3.8%         | 6.7%         | 1.4%                | 1.0%                | 4.1%         | 18.8%                       | 0.5%                | 0.9%                | 10.2%        |
| Laboratory B         | Run 1 | 1.0              | -10.7               | -9.8                | 101.3        | 1.2          | -9.9                | -9.1                | 95.5         | 1.9                         | -8.7                | -8.2                | 58.9         |
|                      | Run 2 | 1.2              | -10.4               | -9.7                | 98.1         | 1.1          | -9.9                | -9.0                | 96.6         | 1.3                         | -8.9                | -8.1                | 62.2         |
|                      | Run 3 | 1.4              | -10.4               | -9.7                | 102.3        | 1.0          | -10.0               | -9.0                | 97.8         | 1.3                         | -8.9                | -8.2                | 62.1         |
|                      | mean  | <b>1.2</b>       | <b>-10.5</b>        | <b>-9.7</b>         | <b>100.6</b> | <b>1.1</b>   | <b>-9.9</b>         | <b>-9.0</b>         | <b>96.6</b>  | <b>1.5</b>                  | <b>-8.8</b>         | <b>-8.2</b>         | <b>61.1</b>  |
|                      | sd    | 0.18             | 0.14                | 0.05                | 2.21         | 0.09         | 0.03                | 0.05                | 1.13         | 0.34                        | 0.14                | 0.02                | 1.91         |
|                      | CV    | 14.8%            | 1.4%                | 0.5%                | 2.2%         | 8.1%         | 0.3%                | 0.6%                | 1.2%         | 22.6%                       | 1.6%                | 0.2%                | 3.1%         |
| Laboratory C         | Run 1 | 2.0              | -10.4               | -9.9                | 93.9         | 1.4          | -10.0               | -9.4                | 104.9        | 1.3                         | -9.5                | -8.7                | 58.9         |
|                      | Run 2 | 1.6              | -10.7               | -10.1               | 103.0        | 1.2          | -10.2               | -9.4                | 96.4         | 1.1                         | -9.8                | -8.9                | 52.2         |
|                      | Run 3 | 1.6              | -10.5               | -9.9                | 108.1        | 1.1          | -10.3               | -9.4                | 103.3        | 1.5                         | -9.5                | -8.8                | 54.7         |
|                      | mean  | <b>1.8</b>       | <b>-10.5</b>        | <b>-10.0</b>        | <b>101.7</b> | <b>1.3</b>   | <b>-10.2</b>        | <b>-9.4</b>         | <b>101.5</b> | <b>1.3</b>                  | <b>-9.6</b>         | <b>-8.8</b>         | <b>55.3</b>  |
|                      | sd    | 0.22             | 0.16                | 0.13                | 7.17         | 0.16         | 0.11                | 0.03                | 4.54         | 0.20                        | 0.17                | 0.09                | 3.39         |
|                      | CV    | 12.6%            | 1.5%                | 1.3%                | 7.1%         | 12.3%        | 1.0%                | 0.3%                | 4.5%         | 14.9%                       | 1.8%                | 1.0%                | 6.1%         |
| Interlaboratory mean | mean  | <b>1.5</b>       | <b>-10.5</b>        | <b>-9.8</b>         | <b>99.0</b>  | <b>1.3</b>   | <b>-10.0</b>        | <b>-9.2</b>         | <b>95.2</b>  | <b>1.5</b>                  | <b>-9.2</b>         | <b>-8.5</b>         | <b>54.4</b>  |
|                      | sd    | <b>0.27</b>      | <b>0.06</b>         | <b>0.13</b>         | <b>3.68</b>  | <b>0.21</b>  | <b>0.15</b>         | <b>0.18</b>         | <b>7.10</b>  | <b>0.20</b>                 | <b>0.37</b>         | <b>0.34</b>         | <b>7.14</b>  |
|                      | CV    | <b>18.0%</b>     | <b>0.6%</b>         | <b>1.4%</b>         | <b>3.7%</b>  | <b>16.2%</b> | <b>1.5%</b>         | <b>1.9%</b>         | <b>7.5%</b>  | <b>13.4%</b>                | <b>4.1%</b>         | <b>4.0%</b>         | <b>13.1%</b> |

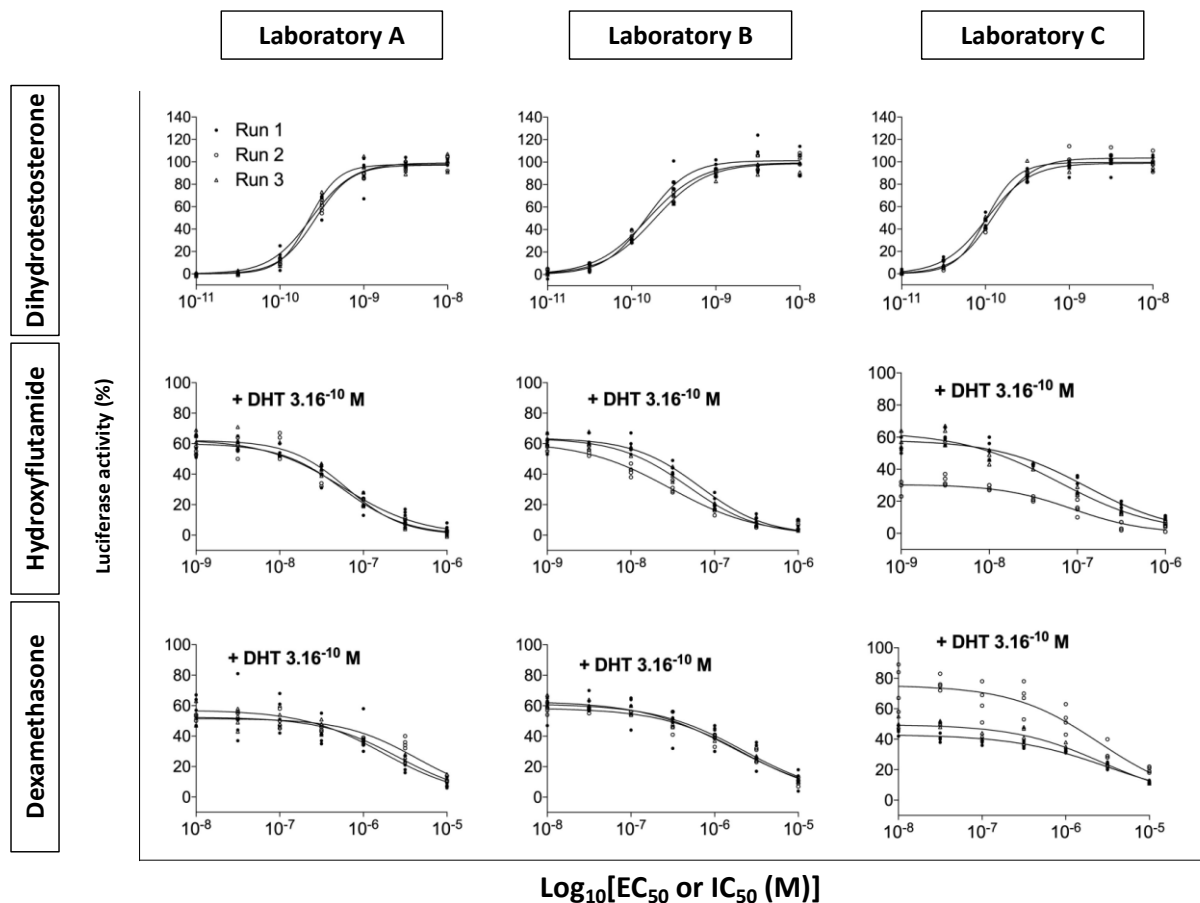
582 Sd: standard deviation; CV: coefficient of variation; Max: maximum luminescence reached by the tested compound (% of max induced by DHT  
 583 10<sup>-7</sup> M).

584 **Supplementary Table S5. Phase II intra- and interlaboratory comparisons of the effects of vinclozolin M2, bisphenol A and bisphenol C**  
 585 based on the Hill model parameters: Hill slope, logIC<sub>30</sub> and logIC<sub>50</sub>.

|                      |       | Vinclozolin M2 |                     |                     | Bisphenol A  |                     |                     | Bisphenol C  |                     |                     |
|----------------------|-------|----------------|---------------------|---------------------|--------------|---------------------|---------------------|--------------|---------------------|---------------------|
|                      |       | Hill slope     | logIC <sub>30</sub> | logIC <sub>50</sub> | Hill slope   | logIC <sub>30</sub> | logIC <sub>50</sub> | Hill slope   | logIC <sub>30</sub> | logIC <sub>50</sub> |
| Laboratory A         | Run 1 | -1.0           | -7.4                | -7.0                | -1.0         | -5.9                | -5.6                | -1.8         | -7.0                | -6.8                |
|                      | Run 2 | -1.2           | -7.3                | -7.0                | -1.4         | -6.0                | -5.7                | -1.3         | -7.1                | -6.9                |
|                      | Run 3 | -1.0           | -7.6                | -7.2                | -0.8         | -6.1                | -5.6                | -1.4         | -7.1                | -6.9                |
|                      | mean  | <b>-1.1</b>    | <b>-7.4</b>         | <b>-7.0</b>         | <b>-1.1</b>  | <b>-6.0</b>         | <b>-5.6</b>         | <b>-1.5</b>  | <b>-7.1</b>         | <b>-6.9</b>         |
|                      | sd    | 0.15           | 0.16                | 0.13                | 0.32         | 0.07                | 0.08                | 0.27         | 0.07                | 0.03                |
|                      | CV    | 13.9%          | 2.2%                | 1.8%                | 29.6%        | 1.2%                | 1.3%                | 17.5%        | 1.0%                | 0.4%                |
| Laboratory B         | Run 1 | -1.0           | -7.3                | -7.0                | -1.0         | -6.0                | -5.6                | -0.9         | -7.3                | -6.9                |
|                      | Run 2 | -1.1           | -7.1                | -6.8                | -0.9         | -6.1                | -5.6                | -0.8         | -7.3                | -6.8                |
|                      | Run 3 | -1.3           | -7.1                | -6.9                | -1.0         | -5.8                | -5.4                | -0.9         | -7.0                | -6.6                |
|                      | mean  | <b>-1.1</b>    | <b>-7.2</b>         | <b>-6.9</b>         | <b>-0.9</b>  | <b>-5.9</b>         | <b>-5.6</b>         | <b>-0.9</b>  | <b>-7.2</b>         | <b>-6.8</b>         |
|                      | sd    | 0.20           | 0.12                | 0.07                | 0.07         | 0.13                | 0.11                | 0.04         | 0.16                | 0.16                |
|                      | CV    | 17.4%          | 1.6%                | 1.0%                | 7.1%         | 2.3%                | 2.1%                | 4.4%         | 2.3%                | 2.3%                |
| Laboratory C         | Run 1 | -1.0           | -7.1                | -6.7                | -0.8         | -6.1                | -5.6                | -1.0         | -6.9                | -6.5                |
|                      | Run 2 | -0.9           | -7.2                | -6.8                | -0.8         | -5.9                | -5.5                | -1.0         | -6.9                | -6.5                |
|                      | Run 3 | -1.1           | -7.0                | -6.6                | -0.9         | -5.9                | -5.5                | -1.5         | -6.8                | -6.5                |
|                      | mean  | <b>-1.0</b>    | <b>-7.1</b>         | <b>-6.7</b>         | <b>-0.8</b>  | <b>-6.0</b>         | <b>-5.5</b>         | <b>-1.2</b>  | <b>-6.8</b>         | <b>-6.5</b>         |
|                      | sd    | 0.06           | 0.10                | 0.08                | 0.07         | 0.10                | 0.07                | 0.28         | 0.06                | 0.005               |
|                      | CV    | 6.4%           | 1.4%                | 1.2%                | 8.5%         | 1.6%                | 1.3%                | 23.8%        | 0.9%                | 0.1%                |
| Interlaboratory mean | mean  | <b>-1.1</b>    | <b>-7.2</b>         | <b>-6.9</b>         | <b>-0.9</b>  | <b>-6.0</b>         | <b>-5.6</b>         | <b>-1.2</b>  | <b>-7.0</b>         | <b>-6.7</b>         |
|                      | sd    | <b>0.07</b>    | <b>0.16</b>         | <b>0.17</b>         | <b>0.13</b>  | <b>0.02</b>         | <b>0.06</b>         | <b>0.34</b>  | <b>0.18</b>         | <b>0.18</b>         |
|                      | CV    | <b>6.4%</b>    | <b>2.2%</b>         | <b>2.5%</b>         | <b>13.4%</b> | <b>0.4%</b>         | <b>1.0%</b>         | <b>28.5%</b> | <b>2.6%</b>         | <b>2.7%</b>         |

586 Sd: standard deviation; CV: coefficient of variation.

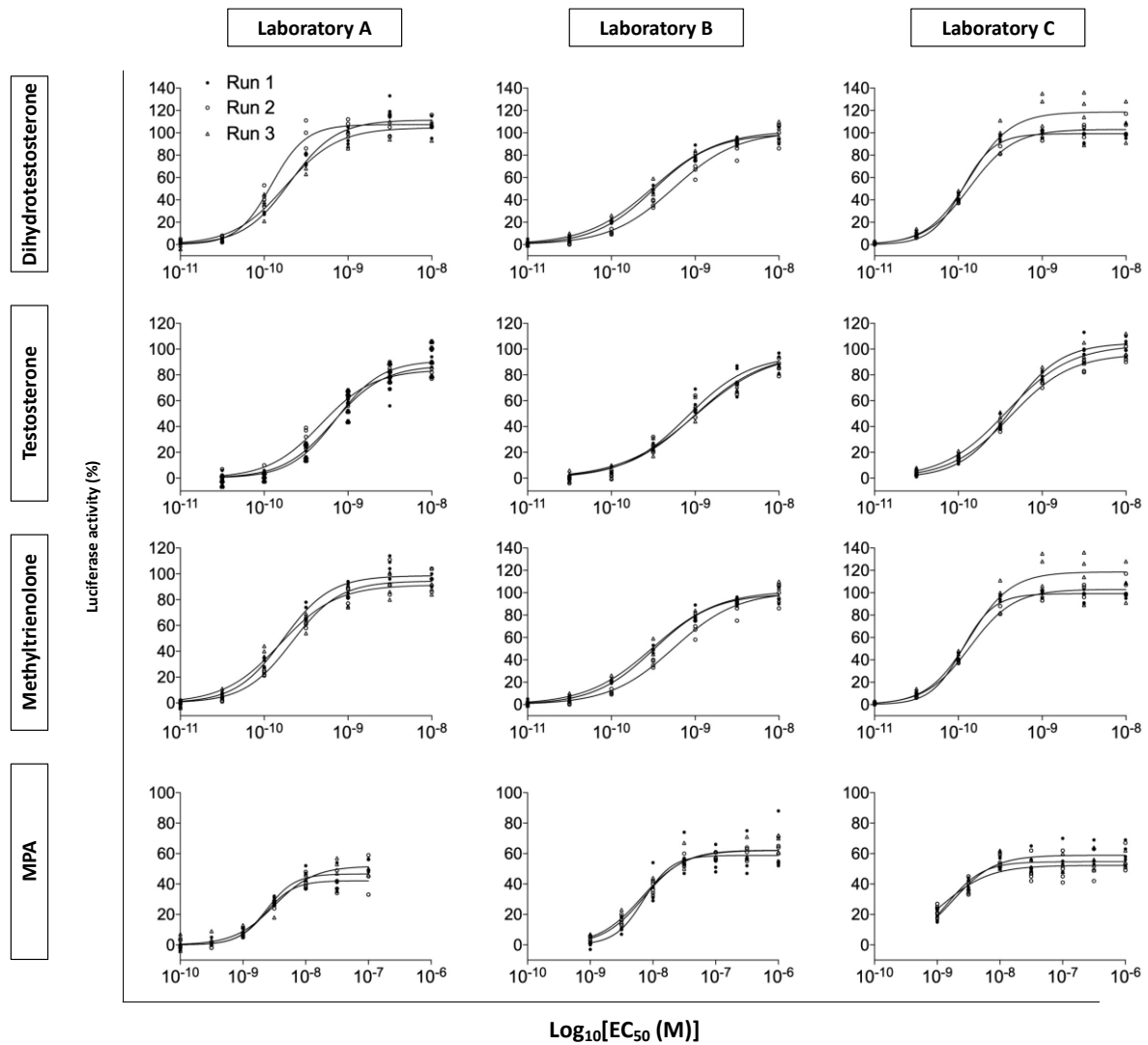
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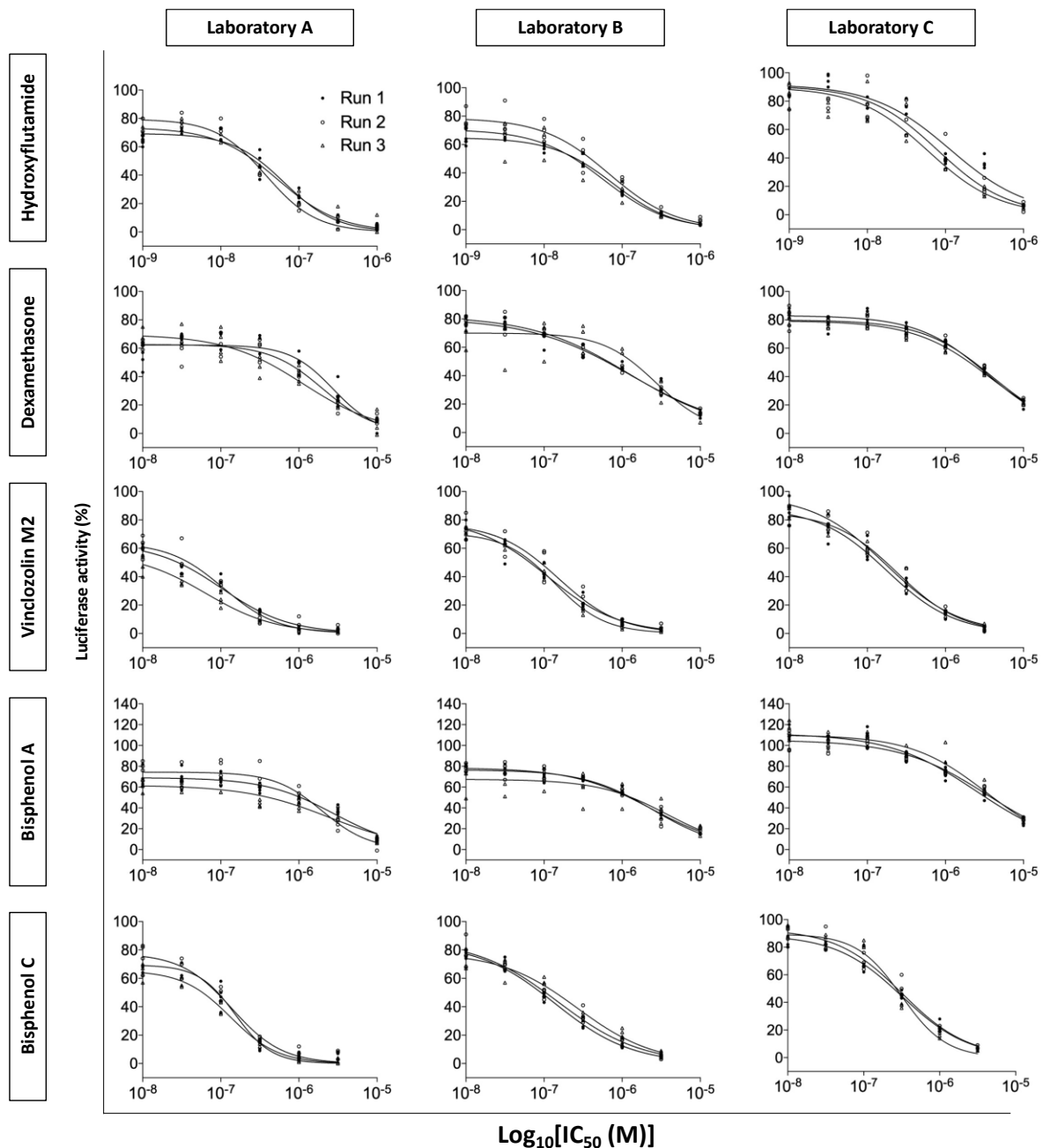
589 **Figure S1. Phase I transactivation of human nuclear androgen receptor (hAR) by**  
 590 **reference ligands** in UALH-hAR cells : intra-laboratory variability. n = 3 independent  
 591 experiments. Results are expressed as the percentage of the maximum luciferase activity  
 592 induced by  $10^{-7}$  M DHT. Antagonist assays are done in coexposure with  $3.16 \times 10^{-10}$  M DHT.

593



594

595 **Figure S2. Phase II transactivation of human nuclear androgen receptor (hAR) by**  
 596 **agonistic ligands in UALH-hAR cells: intra-laboratory variability in the three laboratories.**  
 597 n = 3 independent experiments per laboratory. Results are expressed as the percentage of the  
 598 maximum luciferase activity induced by  $10^{-7}$  M DHT.



599

600 **Figure S3. Phase II transactivation of human nuclear androgen receptor (hAR) by**  
 601 **antagonistic ligands** in UALH-hAR cells: **intra-laboratory variability** in the three  
 602 laboratories. n = 3 independent experiments. Results are expressed as the percentage of the  
 603 maximum luciferase activity induced by  $10^{-7}$  M DHT. Antagonist assays are done in co-  
 604 exposure with  $3.16 \times 10^{-10}$  M DHT.