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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 logEC₅₀
38 (agonist assay) or logIC₅₀ (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 α -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 α -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 3.16×10^{-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% CO₂ 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 3.16×10^{-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 3.16×10^{-10} M alone yielding 50-80% of the
178 maximum luciferase activity. Plates were then incubated at 5% CO₂ ± 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% CO₂ 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 OD₆₄₀ values subtracted from OD₅₇₀. 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 3.16×10^{-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: $CV = (\text{Standard Deviation}/\text{Mean } \log EC_{50} \text{ or } \log IC_{50}) \times 100$.

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 $\log EC_{50}$ of -9.8 (EC_{50} of $1.6 \times$
234 10^{-10} M) (Fig. 2). Intra-laboratory CVs of $\log EC_{50}$ ranged from 0.4 to 0.5% (Table 2), indicating
235 good WLR. Regarding interlaboratory comparison, $\log EC_{50}$ 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 $\log EC_{50}$ (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 $\log IC_{50}$ for HF was -7.2 (IC_{50} of 6.3×10^{-8}

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 2.5×10^{-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 μ 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 1.9×10^{-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 logIC₅₀ 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_{50s} and logIC_{50s} 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, logEC₅₀ and logIC₅₀ 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 logEC₅₀ or logIC₅₀ 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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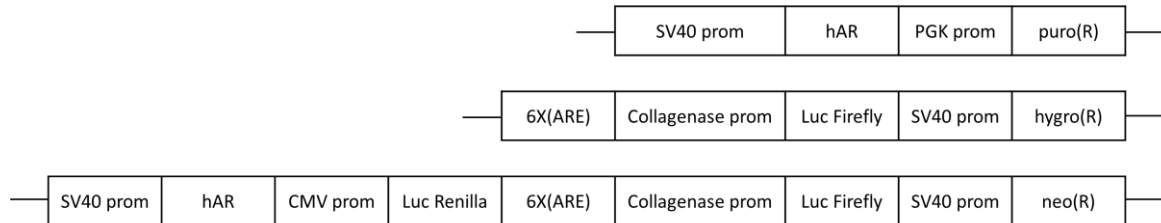
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Figures and tables

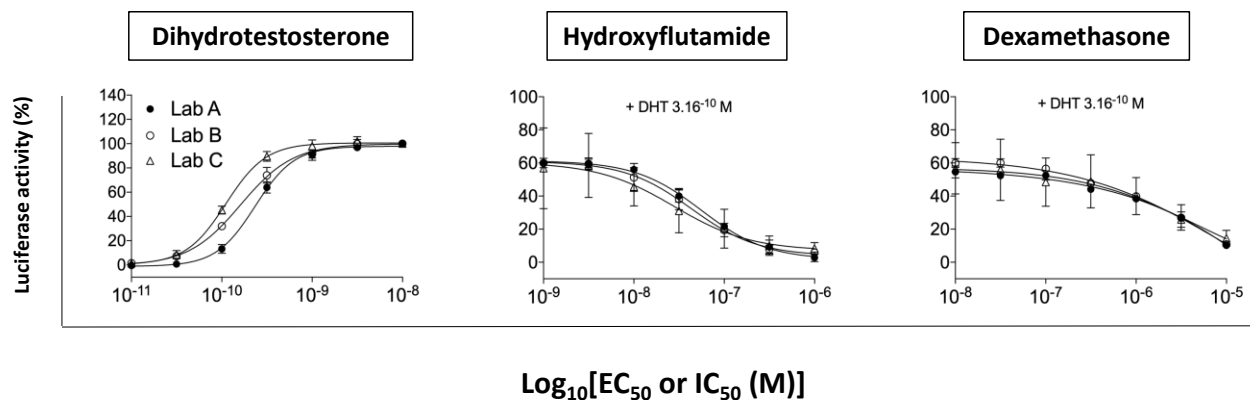
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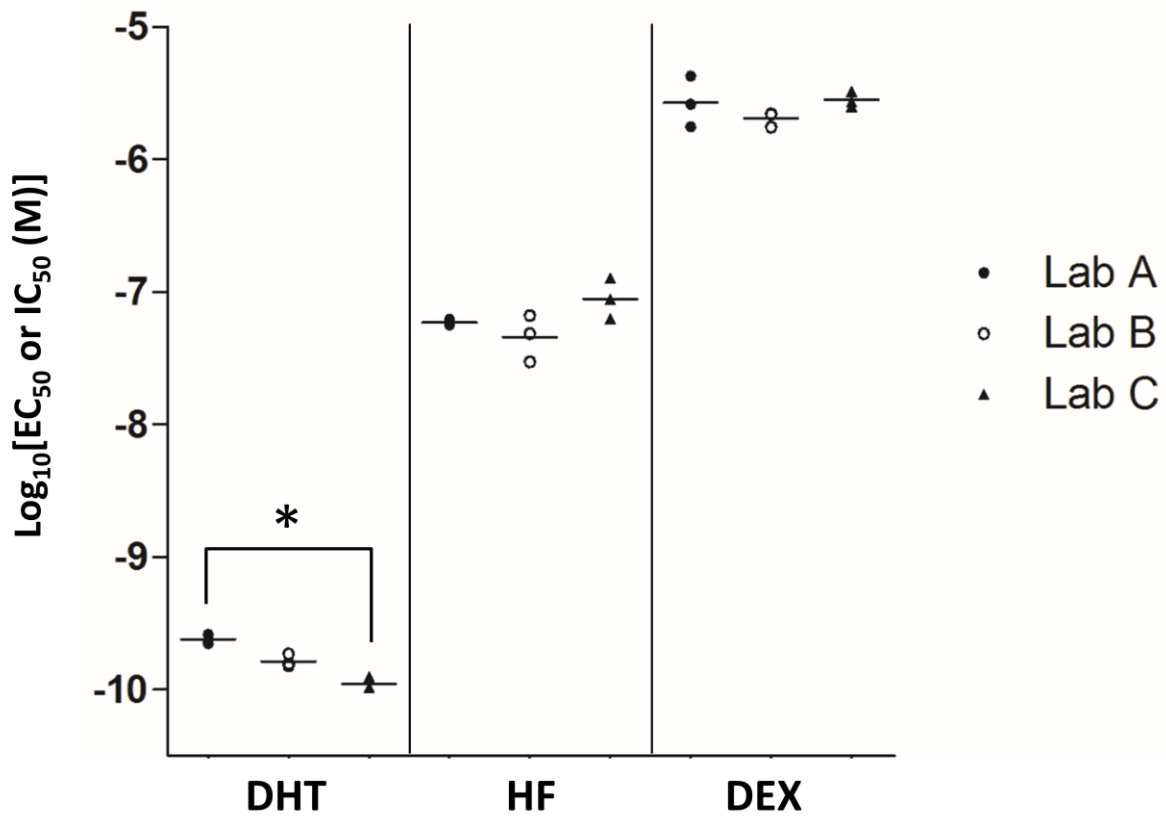
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500 **Figure 1.** The three plasmid constructs transfected into U2OS to create the UALH-hAR cell
501 line. SV40: simian virus 40; prom: promoter; hAR: human androgen receptor; PGK:
502 phosphoglycerate kinase; luc: luciferase; ARE: androgen response 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.

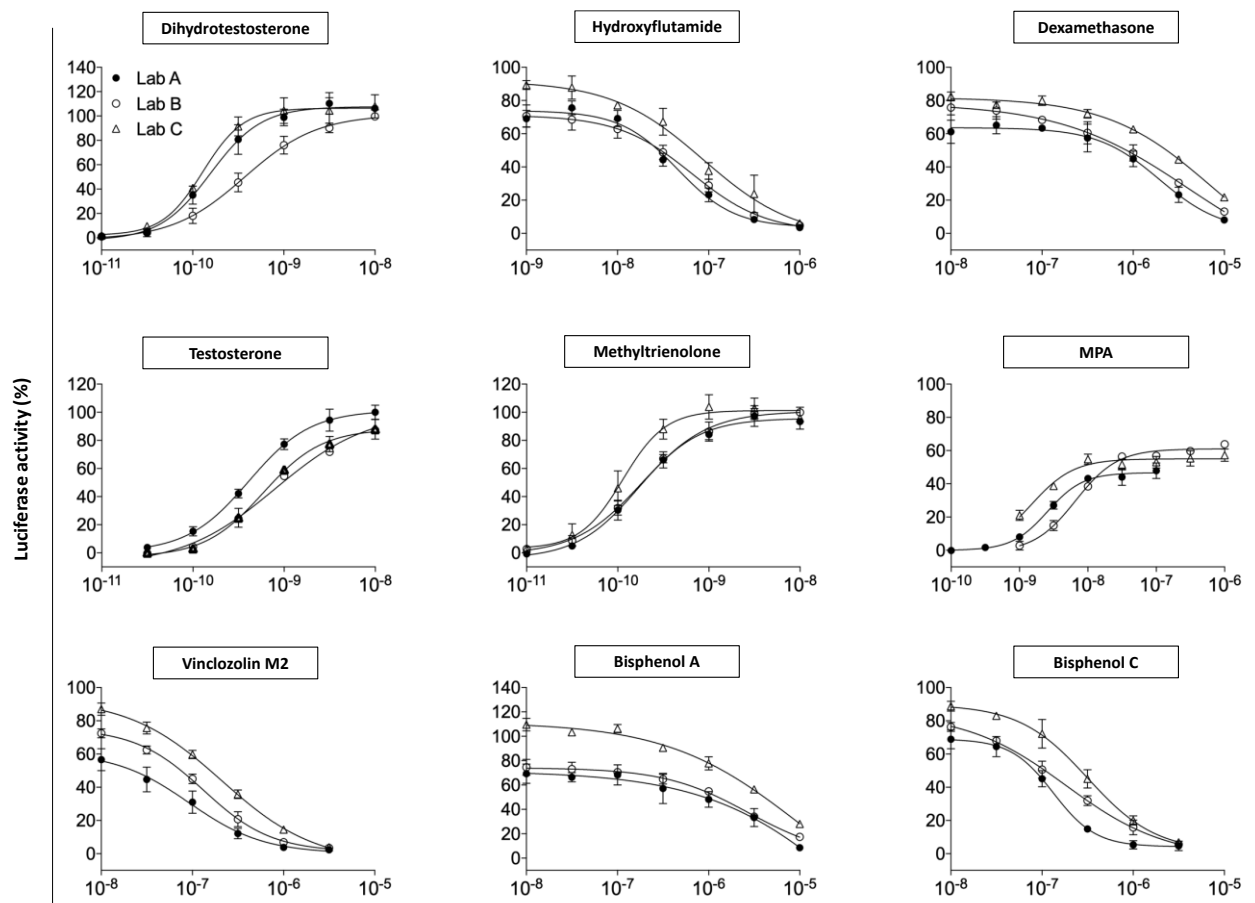


505
 506 **Figure 2. Phase I transactivation of human nuclear androgen receptor (hAR) by reference**
 507 **ligands** in UALH-hAR cells in the three laboratories. Data are means of $n = 3$ independent
 508 experiments per laboratory; error bars represent standard deviation. Results are expressed as
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



513

514 **Figure 3. Phase I comparison of logEC_{50} and 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.

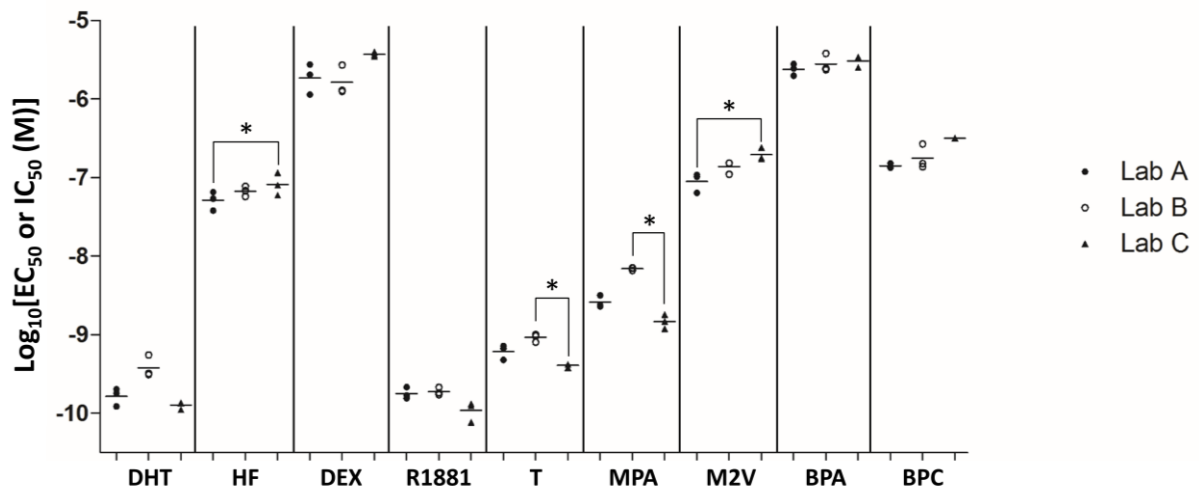


518

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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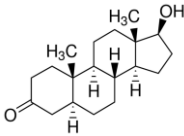
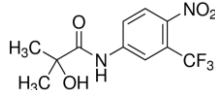
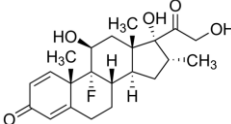
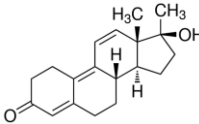
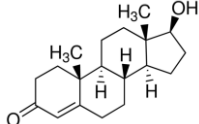
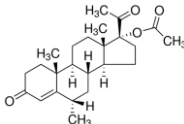
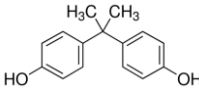
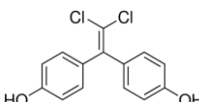
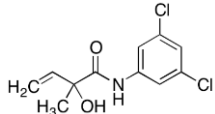
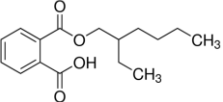
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527

528 **Figure 5. Phase II comparison of logEC₅₀ and logIC₅₀ 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
Dihydrotestosterone	DHT	521-18-6		290.44	≥97.5%
Hydroxyflutamide	HF	52806-53-8		292.21	≥98%
Dexamethasone	DEX	50-02-2		392.46	≥98%
Methyltrienolone	R1881	965-93-5		284.39	≥98%
Testosterone	T	58-22-0		288.42	≥98%
Medroxyprogesterone acetate	MPA	71-58-9		386.52	≥97%
Bisphenol A	BPA	80-05-7		228.29	≥99%
Bisphenol C	BPC	14868-03-2		281.13	≥98%
Vinclozolin M2	M2V	83792-61-4		260.12	≥98%
Mono(2-ethylhexyl)phthalate	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 logEC₅₀ (agonist assays) and mean logIC₅₀**
 538 **(antagonist assays).**

			Agonist assays (logEC ₅₀)				Antagonist assays (logIC ₅₀)			
			Lab. A	Lab. B	Lab. C.	Interlab.	Lab. A	Lab. B	Lab. C.	Interlab.
PHASE I	Dihydrotestosterone	mean	-9.6	-9.8	-10.0	-9.8				
		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	-7.2
		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	-5.6
		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	-9.7				
		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	-7.2
		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	-5.6
		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	-9.8				
		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	-9.2				
		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	-8.5				
		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	-6.9	
	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	-5.6	
	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	-6.7	
	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 logEC₅₀ and log inhibitory concentrations logIC₅₀ (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) -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) -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) Agonist -8.6 (Araki et al., 2005) Antagonist -5.6 (Zwart et al., 2017)* Antagonist -4.4 (Araki et al., 2005)	Antagonist (Sonneveld et al., 2005)		Agonist (Térouanne et al., 2000)	Agonist (Wilson et al., 2002) Agonist -8.4 (Aït-Aïssa et al., 2010)			
	Vinclozolin M2	-6.9				Agonist -4.1 (Molina-Molina et al., 2006) 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) 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) - 5% FBS (Sigma F7524) - 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) - 1% v/v nonessential amino acids (Gibco 11140050) - Puromycin 0.25-0.5 µg/mL (Sigma P8833) - G418 0.5-1 mg/mL (Invivogen ant-gn 100 mg/mL) - Hygromycin 0.125-0.25 mg/mL	- DMEM/F-12 with phenol red (Gibco 31331-028) - 5% FBS (Eurobio CVFSVF00) - 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) - Puromycin 0.25-0.5 µg/mL (Sigma P8833) - G418 0.5-1 mg/mL (Invivogen ant-gn 100 mg/mL) - Hygromycin 0.125-0.25 mg/mL	- DMEM/F-12 with phenol red (Gibco 31331-028) - 5% FBS (Eurobio CVFSVF00) - 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) - Puromycin 0.25-0.5 µg/mL (Sigma P8833) - G418 0.5-1 mg/mL (Invivogen ant-gn 100 mg/mL) - Hygromycin 0.125-0.25 mg/mL
	Test medium	- DMEM without phenol red (Gibco 11880) - 2% DCC-treated FBS - 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) - 1% v/v L-Glutamine - 1% v/v nonessential amino acids (Gibco 11140050)	- DMEM/F-12 without phenol red (Gibco 21041-025) - 2% DCC-treated FBS - 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) - 1% v/v L-Glutamine	- DMEM/F-12 without phenol red (Dutscher P04-41650) - 2% DCC-treated FBS - 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) - 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, logEC₁₀, logEC₅₀ and maximum or logIC₃₀ and logIC₅₀.

		Dihydrotestosterone				Hydroxyflutamide			Dexamethasone		
		Hill slope	logEC ₁₀	logEC ₅₀	Max (%)	Hill slope	logIC ₃₀	logIC ₅₀	Hill slope	logIC ₃₀	logIC ₅₀
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	2.1	-10.1	-9.6	98.0	-1.1	-7.6	-7.2	-0.9	-6.0	-5.6
	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	1.5	-10.4	-9.8	99.9	-1.0	-7.7	-7.3	-0.8	-6.1	-5.7
	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	2.0	-10.4	-10.0	100.6	-0.9	-7.5	-7.1	-0.8	-6.0	-5.6
	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	1.9	-10.3	-9.8	99.5	-1.0	-7.6	-7.2	-0.9	-6.0	-5.6
	sd	0.30	0.19	0.17	1.36	0.11	0.13	0.15	0.08	0.09	0.08
	CV	16.2%	1.8%	1.7%	1.4%	11.1%	1.7%	2.0%	9.0%	1.5%	1.4%

574 Sd: standard deviation; CV: coefficient of variation; Max: maximum luminescence reached by the tested compound (% of max induced by DHT
 575 10⁻⁷ 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, logEC₁₀, logEC₅₀ and maximum or logIC₃₀ and logIC₅₀.

		Dihydrotestosterone				Hydroxyflutamide			Dexamethasone		
		Hill slope	logEC ₁₀	logEC ₅₀	Max (%)	Hill slope	logIC ₃₀	logIC ₅₀	Hill slope	logIC ₃₀	logIC ₅₀
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	1.7	-10.4	-9.8	107.8	-1.3	-7.6	-7.3	-1.2	-6.0	-5.7
	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	1.2	-10.2	-9.4	100.7	-1.1	-7.5	-7.2	-0.9	-6.2	-5.8
	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	1.9	-10.4	-9.9	107.0	-0.9	-7.5	-7.1	-1.0	-5.8	-5.4
	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	1.6	-10.3	-9.7	105.2	-1.1	-7.5	-7.2	-1.0	-6.0	-5.6
	sd	0.38	0.08	0.25	3.91	0.16	0.05	0.10	0.19	0.21	0.19
	CV	24.3%	0.8%	2.6%	3.7%	14.8%	0.7%	1.4%	18.8%	3.5%	3.4%

578 Sd: standard deviation; CV: coefficient of variation; Max: maximum luminescence reached by the tested compound (% of max induced by DHT
 579 10⁻⁷ 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, logEC₁₀, logEC₅₀ and maximum.

		Methyltrienolone				Testosterone				Medroxyprogesterone acetate			
		Hill slope	logEC ₁₀	logEC ₅₀	Max (%)	Hill slope	logEC ₁₀	logEC ₅₀	Max (%)	Hill slope	logEC ₁₀	logEC ₅₀	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	1.5	-10.4	-9.8	94.8	1.5	-9.9	-9.2	87.5	1.7	-9.2	-8.6	46.9
	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	1.2	-10.5	-9.7	100.6	1.1	-9.9	-9.0	96.6	1.5	-8.8	-8.2	61.1
	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	1.8	-10.5	-10.0	101.7	1.3	-10.2	-9.4	101.5	1.3	-9.6	-8.8	55.3
	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	1.5	-10.5	-9.8	99.0	1.3	-10.0	-9.2	95.2	1.5	-9.2	-8.5	54.4
	sd	0.27	0.06	0.13	3.68	0.21	0.15	0.18	7.10	0.20	0.37	0.34	7.14
	CV	18.0%	0.6%	1.4%	3.7%	16.2%	1.5%	1.9%	7.5%	13.4%	4.1%	4.0%	13.1%

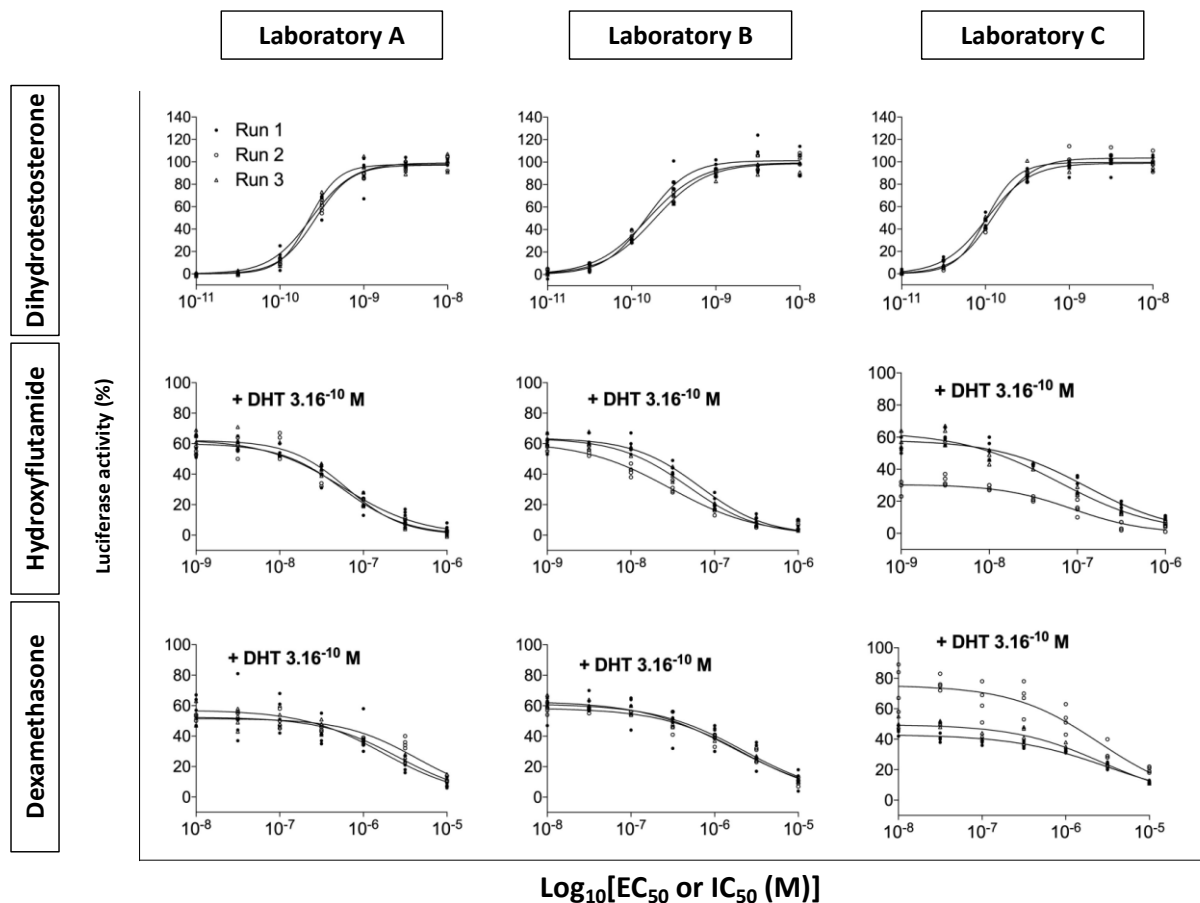
582 Sd: standard deviation; CV: coefficient of variation; Max: maximum luminescence reached by the tested compound (% of max induced by DHT
 583 10⁻⁷ M).

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, logIC₃₀ and logIC₅₀.

		Vinclozolin M2			Bisphenol A			Bisphenol C		
		Hill slope	logIC ₃₀	logIC ₅₀	Hill slope	logIC ₃₀	logIC ₅₀	Hill slope	logIC ₃₀	logIC ₅₀
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	-1.1	-7.4	-7.0	-1.1	-6.0	-5.6	-1.5	-7.1	-6.9
	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	-1.1	-7.2	-6.9	-0.9	-5.9	-5.6	-0.9	-7.2	-6.8
	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	-1.0	-7.1	-6.7	-0.8	-6.0	-5.5	-1.2	-6.8	-6.5
	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	-1.1	-7.2	-6.9	-0.9	-6.0	-5.6	-1.2	-7.0	-6.7
	sd	0.07	0.16	0.17	0.13	0.02	0.06	0.34	0.18	0.18
	CV	6.4%	2.2%	2.5%	13.4%	0.4%	1.0%	28.5%	2.6%	2.7%

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

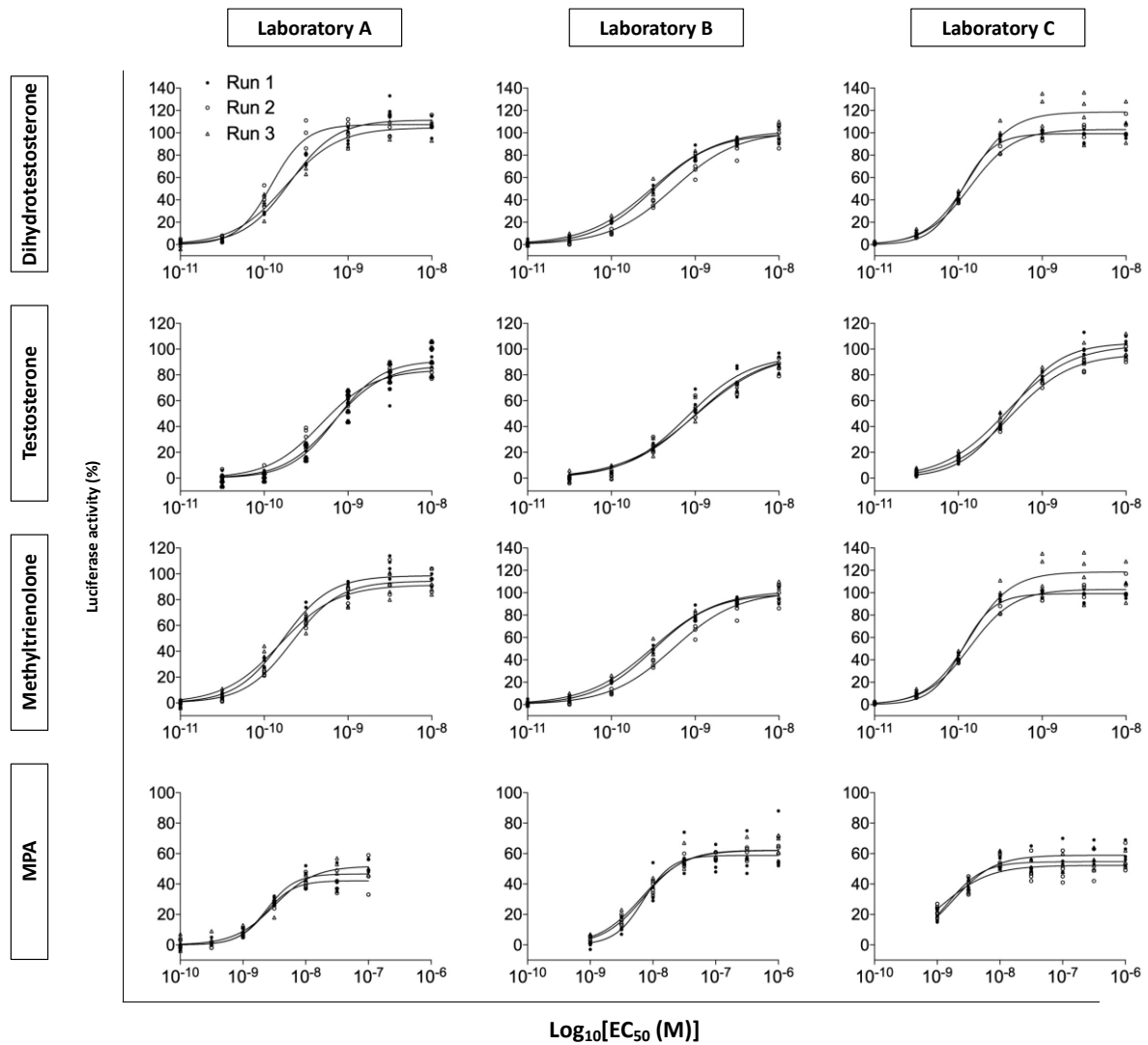
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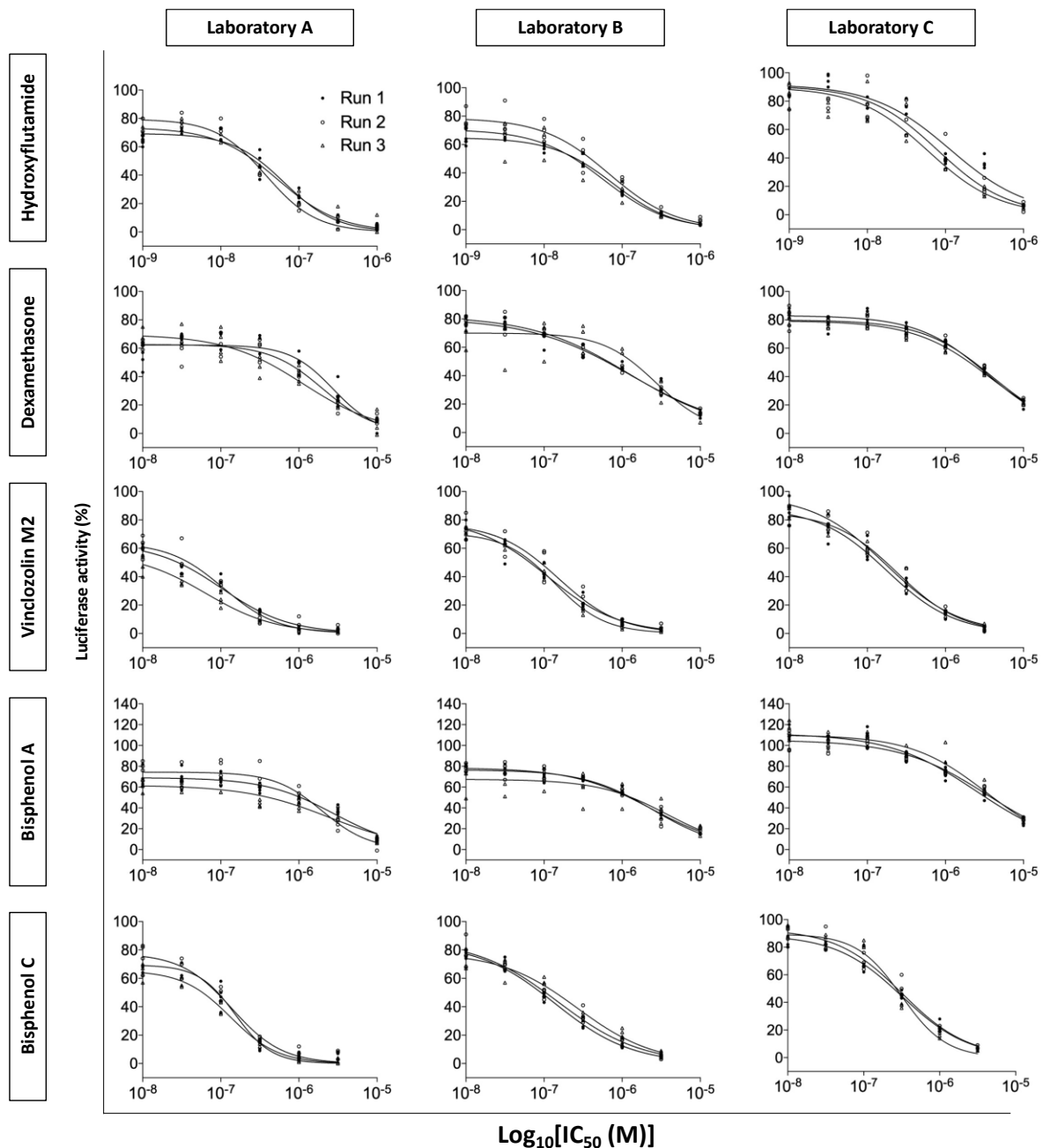
589 **Figure S1. Phase I transactivation of human nuclear androgen receptor (hAR) by**
 590 **reference ligands** in UALH-hAR cells : intra-laboratory variability. n = 3 independent
 591 experiments. Results are expressed as the percentage of the maximum luciferase activity
 592 induced by 10^{-7} M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

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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 3.16×10^{-10} M DHT.