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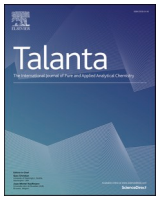

Fast and sustainable planar yeast-based bioassay for endocrine disruptors in complex mixtures: Start of cell cultivation to result within one day

Alisa Ronzheimer [a], Anne E. Ringelmann [a], Gertrud E. Morlock [a,b,*,1,2]

[a] Justus Liebig University Giessen, Institute of Nutritional Science, Chair of Food Science, Heinrich-Buff-Ring 26–32, 35392, Giessen, Germany
[b] Center for Sustainable Food Systems, Justus Liebig University Giessen, Senckenbergstr. 3, 35390, Giessen, Germany

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ABSTRACT

High-performance thin-layer chromatography hyphenated with planar multiplex bioassays and high-resolution tandem mass spectrometry contributes to the non-target detection or even identification of active compounds in complex mixtures such as food, feed, cosmetics, commodities, and environmental samples. It can be used to discover previously unknown harmful or active substances in complex samples and to tentatively assign molecular formulas. This method is already faster than the commonly used *in vitro* assays along with liquid chromatographic separations, but overnight cell cultivation still prevents a planar bioassay from being performed within one day. There is also still potential for optimization in terms of sustainability. To achieve this, the planar bioassay protocols for the detection of androgen-like and estrogen-like compounds were harmonized. The successful minimization of the cell culture volume enabled accelerated cell cultivation, which allowed the bioassay to be performed within one day. This was considered a milestone achieved, as up to 23 samples per plate can now be analyzed from the start of cultivation to the biological endpoint on the same day. Doubling the substrate amount and increasing the pH of the silica gel layer led to a more sensitive and selective bioassay due to the enhanced fluorescence of the formed end-product. The faster and more sustainable bioassay protocol was applied to complex samples such as sunscreen and red wine to detect estrogen-like compounds. The developed method was validated by comparison with a standard method.

1. Introduction

Endocrine-disrupting chemicals are known to cause various diseases [1,2]. Since there may be previously unknown hormone-like active substances in complex sample mixtures, an untargeted, effect-directed screening is required. According to the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation [3], only pure substances are assessed for adverse effects on human health or the environment. Samples of Unknown or Variable composition, Complex reaction product or Biological material (UVCB), and More than One Constituent Substances (MOCS) are regarded as single substances and regulated as such, but there is the desire to consider them as a mixture and test the individual constituents [4]. For a complex mixture, the current *in vitro* assays are not suited without generating high costs for tedious fractionation for chromatographic separation and application of different assay types. Differentiation of the various biological endpoints within a complex mixture to properly understand and assess their impact is essential. Unfortunately, *in vitro* assays produce only a mixed result (sum value) for all different compound responses including opposing effects and false-negative/positive responses. In addition, the commonly used microtiter plates are made of plastic such as polysterol, on which the traces of highly active lipophilic endocrine disruptors can be adsorbed and are therefore undetectable.

Using high-performance thin-layer chromatography (HPTLC), sample mixtures can be separated into individual components. By applying cell suspensions on the chromatograms, HPTLC can be coupled to bioassays such as yeast estrogen (YES) [5] or androgen screen (YAS) [6], used to investigate the effects of a substance on the human estrogen or androgen receptor (hER or hAR). This led to the respective planar bioassays (pYES/pYAS), in which an endocrine effect can tentatively be assigned to an individual substance [7–11]. The glass-backed plates are

* Corresponding author. Justus Liebig University Giessen, Institute of Nutritional Science, Chair of Food Science, Heinrich-Buff-Ring 26–32, 35392, Giessen, Germany.
E-mail address: Gertrud.Morlock@uni-giessen.de (G.E. Morlock).
[1] Dedicated to the 85th birthday of Prof. Dr. David Nurok, Indiana University–Purdue University, USA.
[2] Member of the More than One Constituent Substances (MOCS) Initiative, www.vielstoffgemische.de.

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Abbreviation list

CPB	citrate phosphate buffer
CPRG	chlorophenol red-β-D-galactopyranoside
DMSO	dimethyl sulfoxide
E2	17β-estradiol
EE	ethyl 4-hydroxybenzoate
EHS	ethylhexyl salicylate
FDG	fluorescein di-β-D-galactopyranoside
hAR	human androgen receptor
hER	human estrogen receptor
HPTLC	high-performance thin-layer chromatography
HR	4-*n*-hexylresorcinol
KOH	potassium hydroxide
MOCS	more than one constituent substances
MU	4-methyl umbelliferone
MUG	4-methyl umbelliferyl-β-D-galactopyranoside
ONPG	2-nitrophenyl β-D-galactopyranoside
PEG	polyethylene glycol
(p)YAS	(planar) yeast androgen screen
(p)YES	(planar) yeast estrogen screen
pYAVAS	planar yeast antagonist verification androgen screen
pYAVES	planar yeast antagonist verification estrogen screen
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
RG	resorufin-β-D-galactopyranoside
T	testosterone

plastic-free and enable the detection of the entire spectrum of endocrine-effective compounds. Transferring the bioassay to the planar format also opened up the possibility of simultaneous detection of antagonistic and agonistic effects on the same adsorbent plate (duplex pYAAS and pYAES bioassays) [12]. The recently developed multiplex pYAVAS and pYAVES bioassays incorporated the antagonist verification (V) and thus exclusion of false positive results. They are a perfect tool for effect-directed analysis of complex samples, as several effects can be observed side by side and yet separately from each other. Interesting zones can be further analyzed by automated zone elution and direct coupling to high-resolution mass spectrometry [13,14].

The workflows of the pYES and the pYAS bioassays detecting estrogens or androgens, respectively, are very similar since both use genetically modified *Saccharomyces cerevisiae* cells but the conditions to cultivate the yeast cells differed to some extent. There was a strong desire for utmost efficiency, for harmonization, and for improvements in terms of time and material consumption. In this study, the relevant aspects for harmonization of both pYES and pYAS protocols and for faster and more sustainable pYES/pYAS bioassay performance were studied such as acceleration of the cell cultivation, minimization of the consumed cell culture volume, response increase of the fluorescence signal and thus bioassay sensitivity. As proof for routine screening of androgens and estrogens in complex samples, the optimized all-in-one-day bioassay workflow was applied to screen sunscreen for children and red wine samples. The results were verified by comparison with the conventional bioassay method.

2. Materials and methods

2.1. Chemicals and materials

Double-distilled water was prepared using a Destamat Bi 18 E, Heraeus, Hanau, Germany. *Saccharomyces cerevisiae* BJ1991, genetically modified to contain the hAR [15], were purchased from Xenometrix, Allschwil, Switzerland. *Saccharomyces cerevisiae* BJ3505 with hERα were generated by McDonnell et al. [16]. Polyethylene glycol 400 (PEG 400, for laboratory use, J.T. Baker) and methanol (HPLC grade, VWR) are available at Avantor, Deventer, Netherlands. L-Phenylalanine (>99%) was delivered by Bachem, Bubendorf, Switzerland. Dimethyl sulfoxide (DMSO, ≥99.8%), disodium hydrogen phosphate (≥99%), L-threonine (≥99%), potassium chloride (≥99.5%), potassium dihydrogen phosphate (≥99%), sodium hydroxide (≥99%), toluene (HPLC grade), 2-nitrophenyl β-D-galactopyranoside (ONPG, for biochemistry), and 4-methyl umbelliferyl-β-D-galactopyranoside (MUG, for biochemistry) were obtained from Carl Roth, Karlsruhe, Germany. 17β-Estradiol (E2, 98.5%) was purchased from Dr. Ehrenstorfer, Augsburg, Germany. Copper (II) sulfate pentahydrate (≥99%), *n*-hexane (HPLC grade), and diethyl ether (≥99.8%, with BHT) were from Honeywell, Seelze, Germany. Silica gel 60 F_{254} MS-grade (batch no. HX27248434, layer thickness 90 μm, Supelco), adenine (99%, Sigma-Aldrich), L-aspartic acid (99.5%, Fluka), L-arginine (>99%), citric acid monohydrate (≥99.5%), D-(+)-glucose (99.5%, Sigma-Aldrich), DL-glutamic acid monohydrate (>99%, Fluka), L-isoleucine (>99.0%, free of *allo* diastomeres, Fluka), L-leucine (>99.0%, Fluka), L-lysine monohydrochloride (≥99%, Fluka), L-methionine (≥99%, Sigma-Aldrich), L-serine (for biochemistry), L-tyrosine (for biochemistry, Fluka), L-valine (>99%, Fluka), magnesium sulfate heptahydrate (≥99.0%, Fluka), sodium chloride (≥99%), potassium hydroxide (KOH, ≥85%), trypan blue (0.4% in water, Sigma-Aldrich), testosterone (T, ≥99%, Sigma-Aldrich), triethylamin (≥99.5%, Fluka), and yeast nitrogen base without amino acids (p. a., Sigma-Aldrich) were available at Merck, Darmstadt, Germany. *n*-Heptane (≥95.0%) was purchased from scienTEST-bioKEMIX, Leese, Germany and L-histidine (research grade) was delivered by Serva, Heidelberg, Germany. Ethylhexyl salicylate (EHS, ≥98.0%) was obtained from TCI Deutschland, Eschborn, Germany and ethyl acetate (HPLC grade) was from Th. Geyer, Renningen, Germany. Ethyl 4-hydroxybenzoate (EE, 99%, Acros Organics) and 4-*n*-hexylresorcinol (HR, 99%, Alfa Aesar) are available at Thermo Fisher Scientific, Dreieich, Germany. Purity grades were listed when available.

2.2. Standard and sample solutions

Methanolic stock solutions of E2 and T (1 mg/mL each) were diluted with methanol to concentrations of 200, 20, and 2 ng/mL E2 and 1 μg/mL T. As additional standard solutions, EE and HR (1 mg/mL each) were prepared and diluted to 100 μg/mL EE and 10 μg/mL HR with methanol, whereas EHS was prepared as a 10-mg/mL solution in ethyl acetate.

To prepare a 100-mg/mL sunscreen extract, 150 mg Sunozon Kids 50+ (batch-no. D1972514, Dirk Rossmann, Burgwedel, Germany) were mixed with 1.5 mL ethyl acetate in 2 mL reaction tubes (Eppendorf, Hamburg, Germany) by shaking for 2 min (vortex genie 2, Scientific Industries, Bohemia, NY, USA) and ultrasonication for 10 min (Sonorex Digiplus, Bandelin, Berlin, Germany). After centrifugation at 17,000×*g* for 10 min (microcentrifuge Pico 17, Thermo Fisher Scientific) the supernatant was transferred into a brown glass autosampler vial.

The wine was liquid–liquid extracted by vortexing 8 mL Dornfelder sweet (Rotwild Weinkeller, bottled by Peter Mertes Weinkeller, Bernkastel-Kues, Germany) with 4 mL *n*-hexane – diethyl ether 4:1 (*V*/*V*) in a 20-mL glass vessel for 1 min, followed by centrifugation at 3000×*g* for 5 min (Labofuge 400, Heraeus, Hanau, Germany) to improve phase separation. The upper organic phase was transferred into a 10-mL glass vessel and evaporated to dryness under nitrogen supply for 10–15 min. The residue was resolved in 100 μL methanol, resulting in a 1:80 concentrated red wine extract (80 mL/mL), which was transferred into a brown glass autosampler vial with a conical insert.

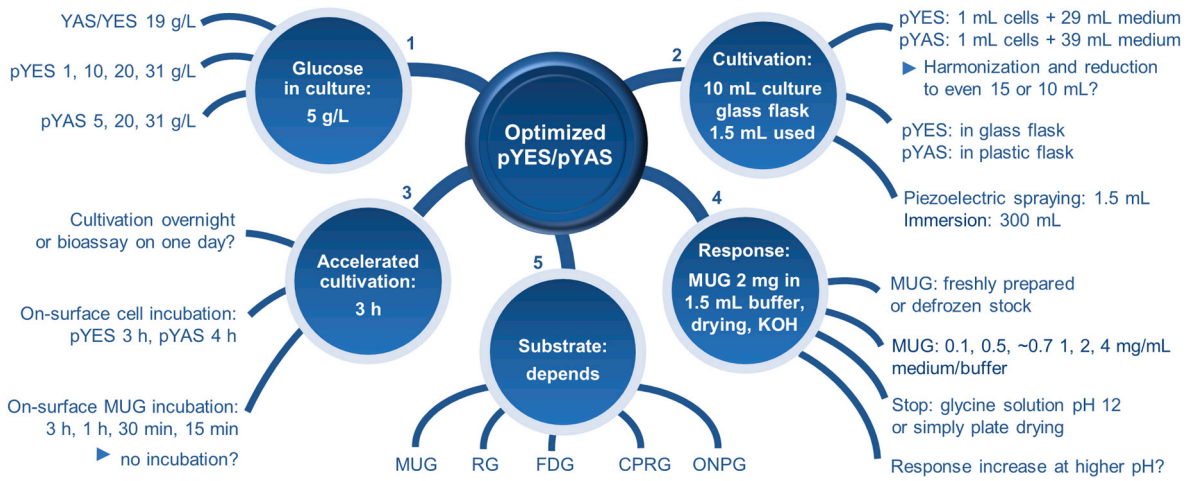

Fig. 1. Overview of the varying parameters and aspects **1**–**5** taken from literature for pYES/pYAS bioassays; after optimization, the recommended parameters are listed in each circle concerning a faster and more sustainable protocol with more sensitive and selective results.

2.3. Plate pre-treatment and preparation of growth medium, buffer, and substrate solution

Before use, all plates were prewashed twice with methanol – water (4:1, *V/V*) in a Simultaneous TLC Developing Chamber (Macherey-Nagel, Düren, Germany) up to approximately 90 mm and dried at 110 °C in an oven (Memmert, Schwabach, Germany) for 20 min. The plates were stored wrapped in aluminum foil in a desiccator until use.

The growth medium was prepared with the amino acids used for the original YES [5] similarly to Ref. [12], following the original pYES preparation [9]. Therefore, 6.8 g/L yeast nitrogen base without amino acids, 5 g/L D-(+)-glucose, 20 mg/L adenine, and 13 amino acids with a total of 870 mg/L (20 mg/L L-arginine, 100 mg/L L-aspartic acid, 100 mg/L DL-glutamic acid, 20 mg/L L-histidine, 30 mg/L L-isoleucine, 100 mg/L L-leucine, 30 mg/L L-lysine, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 400 mg/L L-serine, 200 mg/L L-threonine, 30 mg/L L-tyrosine, and 150 mg/L L-valine) were added to double-distilled water sterilized through sterile filtration (mixed cellulose ester syringe filter, <0.22 µm, Carl Roth).

A citrate phosphate buffer (CPB) was prepared by adding 6 g/L citric acid monohydrate and 10 g/L disodium hydrogen phosphate in double-distilled water, adjusted to pH 12 by solid sodium hydroxide. For the MUG substrate solution, 100 µL of a 20-mg/mL MUG stock solution in DMSO, stored for up to 4 months at −20 °C, were added to 1.5 mL CPB.

2.4. Band pattern application

If not stated otherwise, HPTLC instrumentation was from CAMAG, Muttenz, Switzerland, controlled by visionCATS software (version 3.1.21109.3). Band patterns were created using the Freemode option of

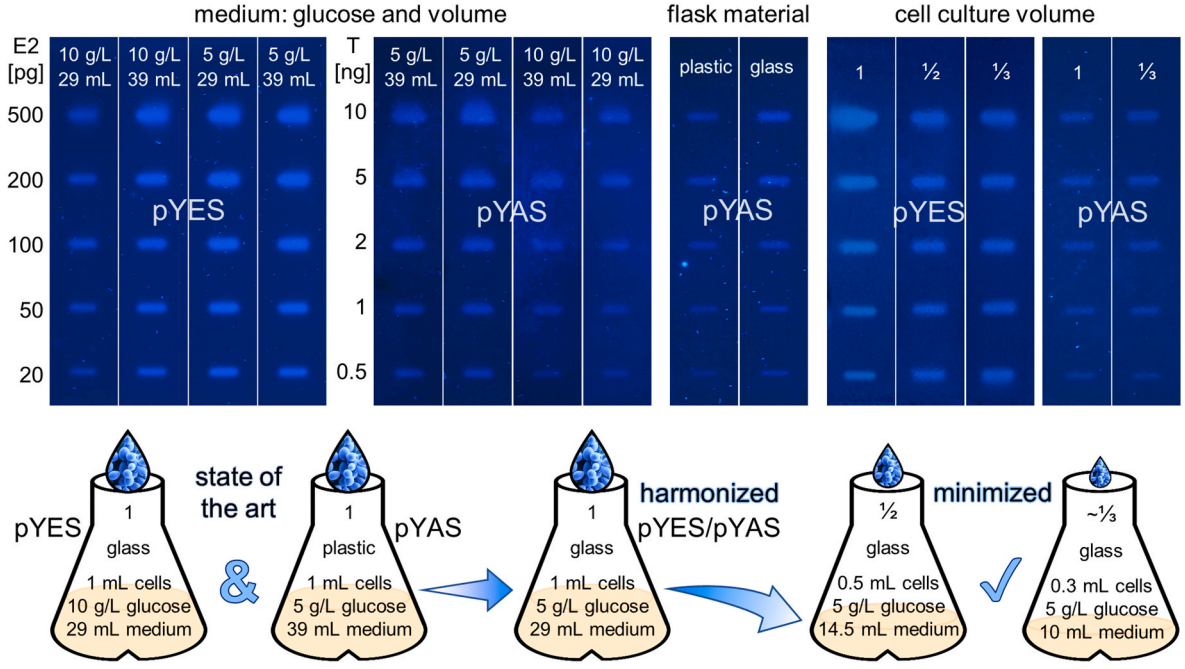

Fig. 2. Harmonization and minimization of the planar yeast estrogen or androgen screen (pYES/pYAS) cultivation using media differing in volume and glucose content (39 mL or 29 mL with 5 g/L or 10 g/L glucose), different flask materials (glass or plastic), and different culture volumes (1, 1/2 or 1/3) applied on HPTLC plates silica gel 60 F_{254} MS-grade with ascending amounts per 6-mm bands of 17β-estradiol (E2) and testosterone (T), followed by substance fixation via Degalan, incubation of cells and substrate (4-methyl umbelliferyl-β-D-galactopyranoside, MUG) and detection at FLD 366 nm.

winCATS software (version 1.4.7.2018) and sprayed on HPTLC plates with an Automated TLC Sampler 4 equipped with a 25-µL syringe. As five stacked 6-mm bands, E2 and T were applied in ascending amounts of E2 (20–500 pg/band; using 1 and 2.5 µL of 20 ng/mL as well as 0.5, 1, and 2.5 µL of 200 ng/mL E2 solution) and T (0.5–10 ng/band; using 0.5, 1, 2, 5 and 10 µL of 1 µg/mL T solution). As indicated, some plates with T and E2 band patterns (proven to have no loss in sensitivity using Degalan fixation [13,14]) were treated with Degalan to fixate the substances in the layer, which avoids diffusion during long incubations. Therefore, plates were placed horizontally in a glass dish filled with Degalan (0.25%, *w/V,* in *n*-hexane) for 10 min [13]. For the other standard substances, also a five-stacked 6-mm-band pattern was used with 10 µg EHS (1 µL, 10 mg/mL) on the lowest band, 200 ng EE (2 µL, 100 µg/mL) on the second band, 40 ng HR (4 µL, 10 µg/mL) on the third band as well as 2 and 12 pg E2 (1 and 6 µL, 2 ng/mL) on the fourth and fifth band, respectively.

2.5. Bioassay variations tested

For modified cell cultivations, cell viability was proven by trypan blue staining (200 µL of a 0.4% solution added to 1 mL of a 1:20 diluted cell suspension, *i.e.* 50 µL in 950 µL sodium chloride 0.9% in double-distilled water, *w/V*). The ratio of colored/uncolored (dead/alive) cells was determined with a hemocytometer (Brand, Wertheim, Germany). For the study of the different MUG substrate concentrations, 50, 100, and 200 µL of the 20-mg/mL MUG in DMSO solution were added to 1.5 mL CPB each, from which 1.5 mL were used. For some experiments (Fig. 2), a PEG 400 solution (5%, *w/V,* in methanol, 1.5 mL red nozzle, level 4) was sprayed onto the dried plate after KOH application. For detection with the ONPG substrate, 2 mg ONPG were dissolved in 1.5 mL CPB (following the MUG concentration), and alternatively, 22.5 mg in 1.5 mL 10 mM aqueous sodium chloride (15 mg/mL, which is the maximum soluble amount of ONPG in water as specified by Sigma Aldrich because supplier Carl Roth did not determine water solubility), followed by its application and incubation for up to 2 h.

2.6. Fast all-in-one-day and sustainable planar pYES/pYAS bioassay

For all-in-one-day bioassay performance, 1 mL of cell cryostock of *Saccharomyces cerevisiae* BJ1991 with hAR (for pYAS bioassay) or *Saccharomyces cerevisiae* BJ3505 with hERα (for pYES bioassay) were added to 9 mL of growth medium in a 100-mL baffled glass flask (DWK Life Sciences, Mainz, Germany). A 3-h cell cultivation was carried out at 30 °C (Cultura M Incubator 70700, Almedica, Giffers, Switzerland) and 110 rpm on an orbital shaker KM CO2 (Edmund Bühler, Hechingen, Germany). After 3 h, the cells grew to an approximate density of 1.2×10^7 cells/mL (resulting in 1.2×10^8 cells in the 10 mL culture). Due to the short cultivation time, there was not much variation in cell number on different days, so cell density was relatively stable and an adjustment was not necessary. The whole cell culture was centrifuged (centrifuge 5702, Eppendorf, Hamburg, Germany) at 2500×*g* for 5 min and the sedimented cells were resuspended in 1.5 mL growth medium, resulting in a cell density of approximately 0.8×10^8 cells/mL. The culture was mixed with 50 µL copper sulfate pentahydrate solution (7 mg/mL in double-distilled water) which resulted in approximately 1 µM.

The cell suspension was sprayed onto the plate (1.5 mL, red nozzle, level 5, Derivatizer [12]). The plate was incubated at 30 °C for 4 h (pYAS) or 3 h (pYES) in polypropylene boxes (KIS 26.5 cm × 16 cm × 10 cm, ABM, Wolframs-Eschenbach, Germany) pre-moistened with water. After plate drying (5 min, 50 °C, plate heater and 1 min stream of cold air, hair dryer), the MUG substrate solution was sprayed onto the plate (1.5 mL, yellow nozzle, level 4) and incubated at 37 °C in the humid box for 1 h, which was reduced to 5 min later or incubation was even completely omitted since the formed end-product 4-methyl umbelliferone (MU) was already present. After plate drying as described, KOH solution (5%, *w/V,* in methanol, 1.5 mL blue nozzle, level 4) was applied and the plate was dried (2 min stream of cold air, hair dryer). Fluorescence was detected at FLD 366 nm and digital evaluation was carried out by generating video densitogram profiles out of the image at FLD 366 nm using visionCATS software.

2.7. Optimized conventional planar pYES/pYAS bioassay

For optimized conventional bioassay performance, 10 mL growth medium were inoculated with 0.3 mL cell cryostock (or existing culture from the previous experiment) in a 100-mL baffled glass flask and incubated overnight (~18 h) as mentioned. Adjustment of cell density is important for the overnight culture, as external influences such as a drop in temperature during the night have an effect and cell numbers vary on the next day. To adjust the cell density of the bioassay cell suspension to 0.8×10^8 cells/mL, the cells from the overnight culture were counted out of a 1:20 dilution using the hemocytometer. The respective volume was centrifuged and the supernatant was discarded. The sedimented cells were resuspended and the planar bioassay was performed as in 2.6.

2.8. Proof of principle and sample analysis

The extracts of the sunscreen for children (2 µL/band) or red wine (6 µL/band) were applied as 6-mm bands at a distance of 10 mm from the lower plate edge and then separated with 3 mL *n*-heptane – toluene 2:1 (*V/V* [17]) or *n*-hexane – ethyl acetate 2:1 (*V/V* [18]), respectively, up to 70 mm migration distance in a Twin-Trough Chamber (10 cm × 10 cm). Plates were dried on a plate heater at 50 °C for 5 min (instead of using a stream of cold air) to reduce diffusion [19] because no Degalan fixation was applied for the samples. The performance of the pYES bioassay followed as in 2.6 (all in one day) and 2.7 (optimized conventional). For proof of principle and validation, both results were compared with the conventional bioassay. Positive control and solvent blank were applied in the upper plate part as described [13–16,20,21].

3. Results and discussion

3.1. Harmonization of cultivation conditions

Glucose concentration in the growth medium for the yeast cells was reported differently, *i.e.* 5 g/L glucose for the pYAS [12] versus 1 g/L [10] or 10 g/L glucose [13,14,22] for the pYES (Fig. 1, **1**). If compared to 19 g/L glucose used for the original longer-lasting *in vitro* bioassay protocols for the YAS (24 h) [6] and YES (72 h) [5], the glucose concentration of the planar bioassays was lower due to the shorter workflow and the associated lower energy nutrient intake. However, such unnecessary high glucose concentrations are still in use for the pYES and pYAS bioassays, *e.g.*, 20 g/L glucose in the growth medium and 31 g/L glucose in a bioassay medium [9,11]. There was also a difference in the volume of medium used to dilute the cell cryostocks (pYES 29 mL medium *versus* pYAS 39 mL medium for 1 mL cryostock each) [12] (Fig. 1, **2**). In addition, YES cells were cultivated in glass flasks, whereas YAS cells were cultivated in plastic flasks as specified by the manufacturer. However, plastic waste should be avoided whereas glassware is reusable. Additionally, plastic may introduce interferences of phthalates when measuring endocrine effects. It was hypothesized that glassware could be used in both cases. In terms of sustainability and reduction of material consumption, it makes sense to reduce the culture volume to the necessary level. In particular, piezoelectric spraying of the cell culture on the MS-grade HPTLC plates (20 cm × 10 cm) with a reduced layer thickness of about 100 µm consumed only 1.5 mL cell suspension [13,14], compared to 300 mL initially prepared for immersion of regular plates (layer thickness of about 200 µm) in the pYES bioassay suspension [7,8].

Different amounts of glucose in the growth medium, *i.e.* 10 g/L glucose (YES) or 5 g/L glucose (YAS), and different volumes of the culture medium added to 1 mL cryostock, *i.e.* 29 mL (YES) or 39 mL

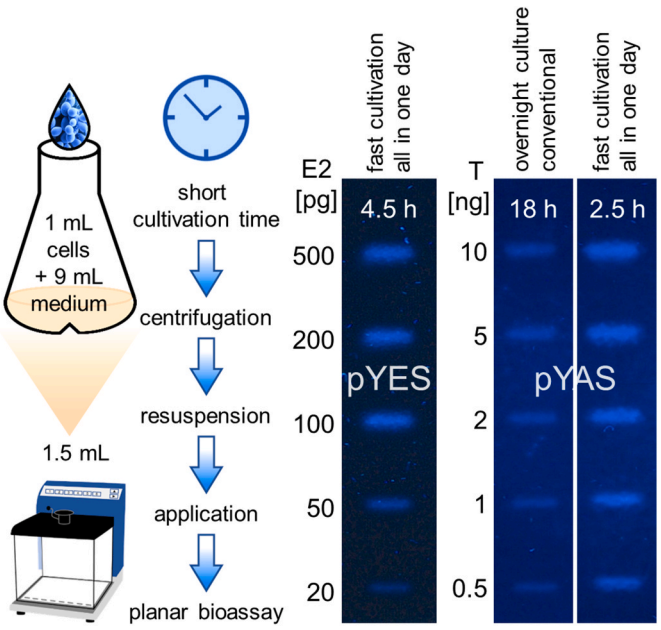

Fig. 3. Fast cultivation for the all-in-one-day planar yeast estrogen or androgen screen (pYES/pYAS), applied on HPTLC plates silica gel 60 F_{254} MS-grade with ascending amounts per 6-mm bands of 17β-estradiol (E2) or testosterone (T), followed by substance fixation via Degalan, incubation of cells and substrate (4-methyl umbelliferyl-β-D-galactopyranoside, MUG) and detection at FLD 366 nm, compared to conventional overnight incubation (pYAS bioassay).

(YAS), different culture flask materials, *i.e.* glass (YES) or plastic (YAS), and due to sustainability reason, even lower volumes of 15 mL or 10 mL, were tested to harmonize the pYAS and pYES cell cultivation protocols (Fig. 1, **1** and **2**). Viability tests via trypan blue staining successfully proved that the cells were not negatively affected by these changes, as there were no deviations from the normal case (<2% dead cells). The planar bioassay images revealed slight differences in the performance of the yeasts under the altered cultivation conditions (Fig. 2). The end-product response formed by the YAS cells was even better for cultivation in the glass flask than in the plastic flask. The cells cultivated with the 10-g/L glucose concentration in the medium generally responded worse to the respective agonist than cells cultivated with the 5-g/L glucose concentration. The cells cultivated with 29 mL medium containing 5 g/L glucose yielded the brightest fluorescent bands in both bioassays. Halving the culture volume (0.5 mL cell suspension plus 14.5 mL medium) or even thirds (0.3 mL cell suspension plus 10 mL medium) provided comparable results, allowing the preparation of only the cell culture volume needed for the day. The reduced material consumption leads to a very sustainable workflow, which affords only 1.5 mL cell suspension per plate using piezoelectric spraying. Thus for screening, six plates with 23 samples each (138 samples) can be handled per day. As a result, the pYES/pYAS cultivation conditions were harmonized to cultivate 0.3 mL cell suspension in 10 mL medium with 5 g/L glucose in a glass flask.

3.2. Achieving start of cell cultivation to result within one day

So far, the cell cultures have been prepared the day before the sample analysis (Fig. 1, **3**), which required planning in advance. Spontaneously deciding on the day to perform the pYES or pYAS bioassay was not possible. Hence, it would be advantageous to obtain the ready-to-use cell culture from the cryostock within a shorter time, so that the bioassay can be performed on the same day. The incubation times of pYES and pYAS differed. A 3-h on-surface cell incubation was sufficient for the pYES bioassay [9,10] but not for the pYAS bioassay because signals were hardly detectable [11], and thus, it was decided for 4 h [12], which difference may be receptor-dependent and difficult for protocol harmonization. The incubation time of the MUG substrate took 3 h at the beginning [7], whereas 1 h was used later [8] which was further reduced to 30 min [13] and even 15 min [9].

Given the smaller culture dimension, the necessity of overnight cultivation was questioned (Fig. 1, **3**). To get as quickly as possible the required cell quantity to perform the bioassay, if possible on the same day, a 1-mL cryostock was added to 9 mL growth medium and cultivated on a first try for 4.5 h (pYES) and in a second try for 2.5 h (pYAS). The whole cultures were centrifuged and resuspended in 1.5 mL growth medium each, which were ready for application on the plate (Fig. 3). For pYES, the background of the bioassay image was slightly darker than usual due to the fast workflow (assumedly, less cell leakage of younger cells). Even the smallest amount of E2 (20 pg/band) was detectable. A harmonized pYES/pYAS cultivation time of 3 h was used henceforth. The HPTLC procedure can easily be performed during this 3-h cell cultivation. With the subsequent 4-h (pYES) or 5-h (pYAS) planar bioassay workflow, the cell cultivation and the planar bioassay can be performed on the same day. The minimized, faster, and more sustainable cell cultivation so that the biological assay could successfully be

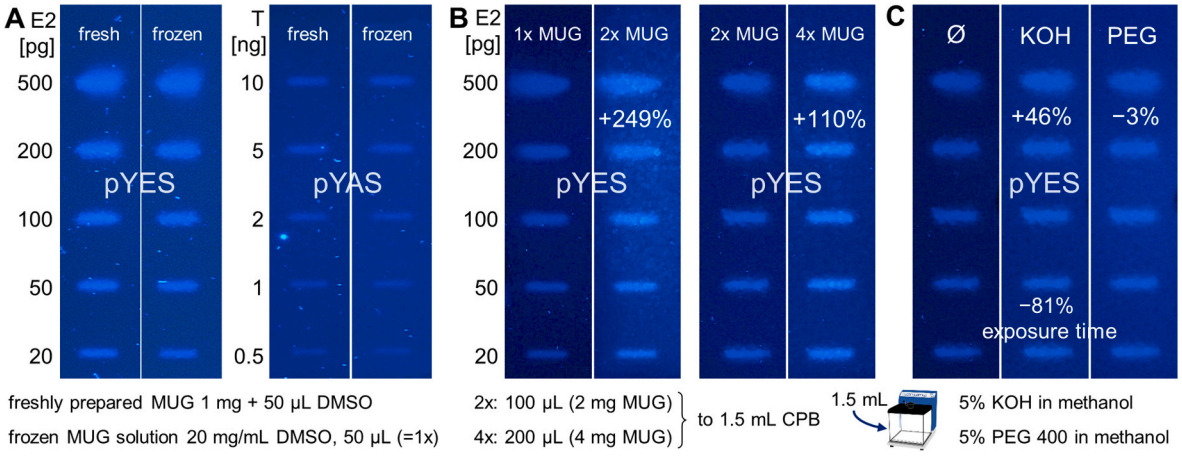

Fig. 4. Handling of the 4-methyl umbelliferyl-β-D-galactopyranoside (MUG) substrate prepared (**A**) freshly in dimethyl sulfoxide (DMSO) or from respective frozen solution (**B**) by adding different MUG concentrations to the citrate phosphate buffer (CPB), and (**C**) by subsequent application of 5% methanolic potassium hydroxide (KOH) solution, followed by 5% methanolic polyethylene glycol 400 (PEG) solution; planar yeast estrogen or androgen screen (pYES/pYAS) on HPTLC plates silica gel 60 F_{254} MS-grade with ascending amounts per 6-mm bands of 17β-estradiol (E2) and testosterone (T), followed by substance fixation via Degalan, incubation of cells and substrate (handled as mentioned), and detection at FLD 366 nm; signal intensities are listed in Table S1/S2.

performed within one working day was considered a milestone achieved.

3.3. MUG substrate handling

Various parameters in the use of the MUG substrate also differed (Fig. 1, **4**). The MUG substrate solution was either prepared freshly (1 mg in 50 μL DMSO) [13,14] or a larger prepared volume was stored at −20 °C which allowed easy reuse [9]. It would be more comfortable to prepare a larger portion stored, which is useable on demand. It was proven that a pre-prepared 20 mg/mL solution in DMSO, which was stored at −20 °C and used for 4 months, showed a comparable fluorescence intensity as the freshly prepared solution (Fig. 4A). It was only important to add the MUG solution to the CPB shortly before use since it precipitates over time.

The first pYES approach used 0.1 mg/mL MUG in a growth medium [7], and thereafter 0.5 mg/mL MUG in buffer were used [9–12]. Since 2021, 1 mg (50 μL, 20 mg/mL) MUG has been added to 1.5 mL buffer [13,14,23]. Doubling the MUG concentration (100 μL, 20 mg/mL) resulted in a substantial increase in the fluorescence intensity by +249% on average (Fig. 4B–Table S1). Using a double-concentrated pre-prepared MUG solution (40 mg/mL) and half the volume (50 μL–1.5 mL CPB) was also tested but precipitated faster. Using the fourfold MUG concentration (200 μL–1.5 mL CPB) even further increased the intensity (+110% on average) but unfortunately, MUG precipitated immediately in the buffer. Hence, the doubled MUG concentration (100 μL of a 20-mg/mL MUG stock solution in DMSO added to 1.5 mL CPB) was used for further analysis, which means that an amount of 2 mg MUG were applied to the plate.

3.4. MUG response enhancement

Initially, a glycine solution of pH 12, usually used for terminating the enzyme-substrate reaction in the *in vitro* bioassay, was also used for the on-surface bioassay [10]. But later, it was skipped since the enzyme–substrate reaction was simply stopped by plate drying [12,23,24]. However, increasing the plate pH value after the bioassay by applying an alkaline solution was still useful because the end-product MU exhibits its optimum fluorescence at pH 10.4 [10,25,26]. Instead of the lacZ buffer (pH 7.0) [8,9], a citrate phosphate buffer (CPB) adjusted to pH 12 was used for the preparation of the MUG substrate solution [10]. Such buffering neutralizes only the acidic adsorbent layer caused by the acidic yeast cell medium (pH optimum of the yeast cells ranges pH 4.5–6.5 [20,27]), which buffering is necessary since the optimum of the activity of the released β-galactosidase is at approximately pH 7.3 [28]. However, this may not raise the plate pH value above 10, however, necessary for the optimal fluorescence of the formed MU end-product to obtain the highest bioassay sensitivity. The formed MU end-product is a coumarin derivative. It was shown that the fluorescence of coumarin can be intensified with potassium hydroxide (KOH) and stabilized with polyethylene glycol (PEG 400) solution for quantification purposes [19,29]. Hence, it was hypothesized whether the application of KOH or triethylamine to ensure a final plate pH > 10 could increase the MU response and thus the sensitivity of the current pYES and pYAS bioassay detection with MUG as substrate.

The fluorescence of the produced MU end-product was enhanced by the additional spraying of KOH solution onto the bioassay image, with the weakest fluorescent zones having the highest intensity increase (Fig. 4C). For the first attempts, single-concentrated MUG (50 μL) was still used. The fluorescence enhancement of a 5% KOH solution (+76% on average) was slightly better than that of a 10% KOH solution (+65% on average), and the 5% KOH solution was selected (Table S2). This increased the pH value of the plate to 10. With the doubled MUG concentration, already exhibiting stronger fluorescent zones, the use of the 5% KOH solution led to an additional fluorescence enhancement (+46% on average, Table S2). Sensitivity can also be evaluated by the time the TLC Visualizer needs to capture an optimally exposed image in automatic exposure mode. The improvement in sensitivity with KOH solution is particularly evident as its image required only 19% of the exposure time for the pYES bioassay image (before KOH application).

Spraying a PEG 400 solution after the KOH solution further improved the MU signal intensity (+58% on average when using the initial MUG amount) and after a 10-min waiting time, even further (+24% on average, Table S2). However, with the doubled MUG amount, the PEG 400 solution slightly worsened the fluorescence intensity (−3% on average, Fig. 4C–Table S2), why it was not used in the optimized workflow, where the doubled MUG amount was applied. Since the bioassay was evaluated directly after the KOH application, fluorescence stabilization with PEG was found not necessary anyway. Instead of KOH treatment, vaporization with triethylamine can alternatively be used to increase the plate pH and thus the fluorescence intensity (Fig. S1).

Due to the achieved intensity enhancement, the long MUG incubation time of 1 h can be reduced or even completely omitted when the formed MU end-product is present at sufficient intensity in a shorter time. After MUG application, the plate was directly dried again, KOH was applied and the fluorescing MU was visible with the automatic image exposure of the Visualizer (Fig. S2). However, the fixed exposure time of 150 ms showed that the response was worse compared to the plate strips with incubation. Already a 15-min incubation at 37 °C tripled the intensity and after that, there was hardly any difference in signal intensity (for both Degalan-fixated and unfixated plates). In addition, bands diffuse over time (although to a lesser extent with Degalan fixation), which also influences the signal intensity (Table S3). Already a 5-min incubation at 37 °C was sufficient to increase the intensity by 124% compared to no incubation. Performing the incubation at room temperature led to worse results than the incubation at 37 °C (data not shown). Since these findings emerged at a later time, the 5-min incubation was not demonstrated using samples, but a shortened incubation period may also be recommended for samples.

To summarize, the fluorescence signal was significantly enhanced by spraying the doubled MUG concentration (2 mg in 1.5 mL CPB), and after the 5-min substrate incubation, the 5% KOH solution. For substances known to have a strong endocrine effect, the incubation with the MUG substrate may even be omitted, but a short incubation (37 °C, 15 min) is recommended for trace analysis.

3.5. Choice of alternative substrates

The substrate used to detect the yeast-produced β-galactosidase also varied (Fig. 1, **5**). The first pYES approach studied the fluorogenic substrate MUG (~0.5 €/mg) due to its higher sensitivity compared to chromogenic substrates [7]. Since the blue fluorescence of its end-product MU can interfere with natively blue fluorescent compounds in samples, especially phenolic acids in botanicals [23], the much more expensive fluorogenic substrates resorufin-β-D-galactopyranoside (RG, ~11 €/mg) [20] and fluorescein di-β-D-galactopyranoside (FDG, >100 €/mg) [30] were used forming an orange and green fluorophore, respectively. While the original YES was performed with the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG, ~1.2 €/mg) [5], the cheapest and most often used chromogenic substrate for β-galactosidase detection is 2-nitrophenyl β-D-galactopyranoside (ONPG, <0.1 €/mg) [21], whose yellow end-product is more difficult to detect on the white layer background (per se not ideal) but could be tested for planar bioassays as well. Chromogenic substrates (e.g., CPRG and ONPG) are inexpensive but less sensitive than fluorogenic substrates. Examples of the latter are MUG, RG, and FDG, which are mentioned as increasing in their sensitivity and unfortunately also in their costs. Hence, this study was made using MUG to save costs.

Otherwise, the selection of the respective substrate depends on the required bioassay sensitivity and the fluorescence hue of the natively fluorescent compound zones. For example, if many natively blue fluorescent compound zones are evident after the separation in the

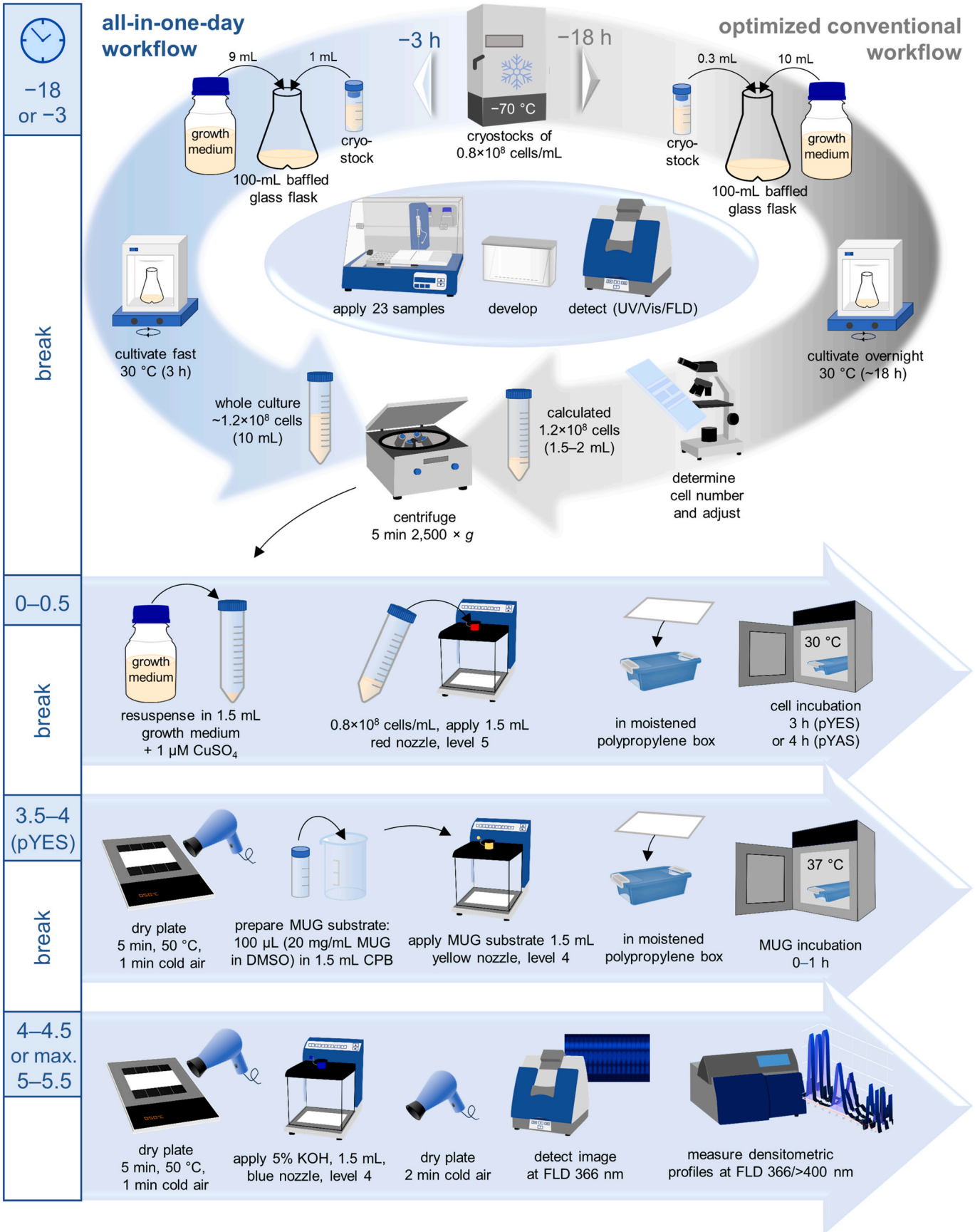

Fig. 5. Workflow scheme of the efficient, fast, and sustainable all-in-one-day planar pYES/pYAS bioassay (start of cultivation and results on the same day, as in 2.6) and optimized conventional bioassay performance (overnight cultivation and results on the next day, as in 2.7). The timeline is exemplarily presented for the pYES bioassay.

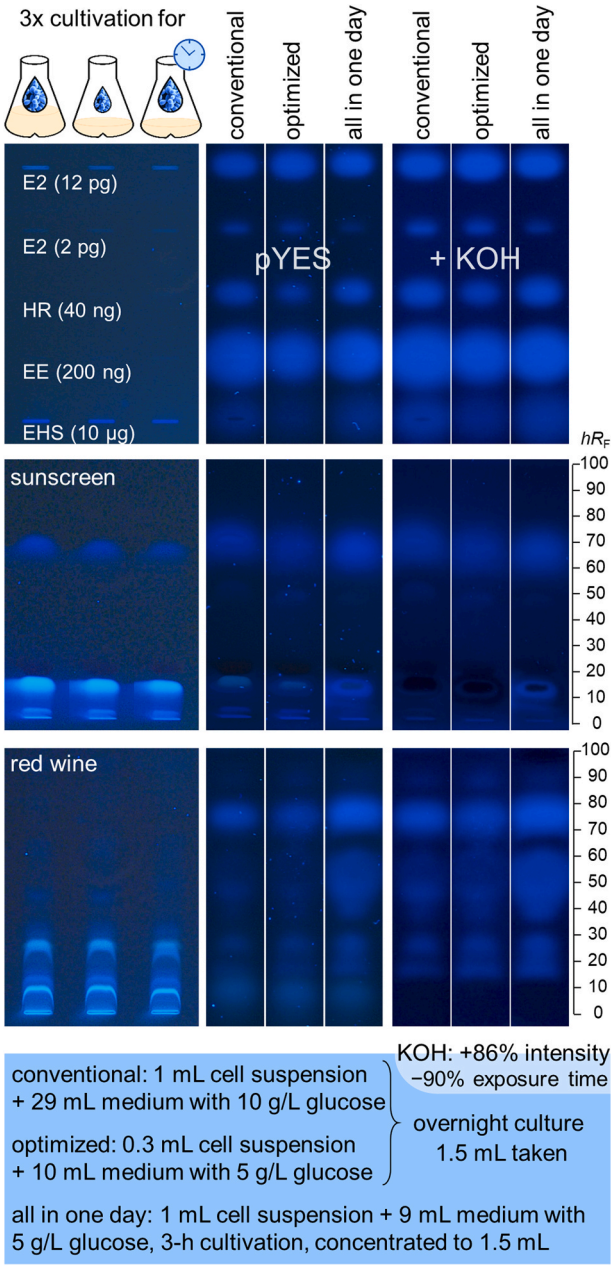

Fig. 6. Planar yeast estrogen screen (pYES) bioassay performed via the established conventional overnight workflow, the optimized conventional overnight workflow (as in 2.7), and the fast and sustainable cultivation workflow (all in one day, as in 2.6) on HPTLC plates silica gel 60 F_{254} MS-grade showing band patterns of different amounts per 6-mm bands of the standards 17*β*-estradiol (E2), hexylresorcinol (HR), ethyl paraben (EE), and ethylhexyl salicylate (EHS) as well as bioautograms of a sunscreen extract (2 µL of 100 mg/mL in ethyl acetate, separated with *n*-heptane – toluene 2:1) and a red wine extract (6 µL of 80 mL/mL extract in methanol, separated with *n*-hexane – ethyl acetate 2:1), followed by incubation of cells (prepared as mentioned) and 4-methyl umbelliferyl-β-D-galactopyranoside substrate, subsequent application of 5% methanolic potassium hydroxide (KOH) solution, and detection at FLD 366 nm; signal intensities are listed in Table S4.

chromatogram at FLD 366 nm and if traces of endocrine-disrupting compounds should be detected, the RG or FDG are useful substrates, generating selective orange or green fluorophores for endocrine-active compounds, respectively. However, the chemical costs are 22-fold (RG) and 200-fold (FDG) higher compared to MUG. Due to their different fluorophore strength and thus the different amounts needed in 1.5 mL buffer, this means for the routine that the real costs per plate are 1 € for MUG (2 mg), 1.7 € for RG (150 µg), and >7.5 € for FDG (75 µg). Hence, the choice is in favor of RG in the case of natively blue fluorescent compound zones.

Except for the cheapest substrate ONPG, all mentioned substrates have already been tested on the planar adsorbent surface. Therefore, the ONPG was tested for the detection of the produced β-galactosidase. However, no yellow response was observed in the pYES bioassay image at white light illumination for the amounts of E2 in the pg range tested in this study (data not shown), even at the maximum soluble amount (15 mg/mL) and an extended incubation time (2 h). As expected, this substrate, though cheapest, was comparatively much worse in sensitivity and thus not recommended as an alternative substrate for the planar screening of unknown samples.

3.6. Sample analysis and proof of principle of the all-in-one-day bioassay as well as the optimized conventional bioassay

The resulting pYES/pYAS bioassay workflows for the newly developed all-in-one-day performance as well as for the optimized

conventional performance are illustrated (Fig. 5). Next, both workflows, using exemplarily the pYES bioassay, were applied to additional control standards as well as sunscreen for children and red wine samples. The results of the new all-in-one-day bioassay (with the fast cultivation, as in 2.6) and of the optimized conventional bioassay (as in 2.7) were verified and validated by comparison with the results of the established conventional pYES bioassay method [12,31]. This three-workflow comparison was performed only once. However, since the cultures were prepared from aliquots of the same cell culture and the three bioassays were performed in parallel, the biological responses were directly comparable in the resulting bioautograms (Fig. 6). The positive control standards showed a comparable response in all three bioassays, while no response was observed in the solvent blanks (not depicted). There were only minor differences between the bioautograms of the three workflows. Regarding the positive control standards, the lowest standard amount (2 pg E2) exhibited a comparatively slightly weaker fluorescent response in the all-in-one-day bioassay. Regarding both samples, brighter fluorescent zones were observed in both all-in-one-day bioautograms. One estrogen-like substance (hR_F 13) in the sunscreen for children was first clearly detected in the all-in-one-day bioautogram. These minor differences were explained by a slightly different sensitivity due to younger cells at the beginning of the exponential growth phase used for the all-in-one-day bioassay. The successful development of a more sustainable and faster sample screening was proven by this comparative evaluation.

Additionally using KOH, the overall fluorescence signal intensities were increased (+86% on average, Table S3) for all tested samples and standards, and consequently, the automatic exposure time was reduced (90% on average). Again, weaker fluorescence signal intensities were more enhanced, *e.g.*, the signal of the weakest fluorescent zone was tripled due to the KOH treatment, which increased the bioassay sensitivity regarding weak signals. Additionally, the KOH treatment made the bioassay detection more selective towards the typical blue fluorescence of the MU end-product, as observed in the lower hR_F region of the red wine (hR_F 0–15) and sunscreen for children (hR_F 13). The latter dark zone or halo zone (dark at the inner part and fluorescent at the outer part) may indicate strong estrogenic or anti-estrogenic or false-positive or cytotoxic effects, which needs further attention. Recently, a six-fold multiplex planar bioassay was developed to clarify such unclear responses [32]. As an alternative to the KOH treatment, treatment with triethylamine resulted also in a higher selectivity and sensitivity (Fig. S1, sunscreen).

4. Conclusions

The successfully developed all-in-one-day bioassay method is considered a milestone. It provides an efficient, fast, and sustainable screening of food, feed, cosmetics, commodities, and environmental samples for endocrine-disrupting chemicals. Further, it provided more sensitive and selective results compared to the conventional planar bioassay protocols. The workflow from the start of cultivation to the biological endpoint can be completed in one day to screen 23 samples in parallel for estrogenic or androgenic substances, together with a co-applied positive control and solvent blank. If more samples a day need to be screened, the optimized conventional planar bioassay protocol allowed for the handling of 138 samples on six plates per day. By avoiding plastic flasks and minimizing cell culture consumption, the sustainability of the bioassay was improved and less waste was produced. The optimized bioassay was successfully applied to different positive control standards as well as wine and sunscreen for children and was successfully verified by comparison with the established conventional method. Pure standards are not always pure due to isomers, degradation, or migration from packaging material, which makes the new all-in-one-day bioassay also interesting for REACH registrations.

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Supplementary information

Supplementary material associated with this article can be found, in the online version, at …

CRediT authorship contribution statement

Alisa Ronzheimer: Formal analysis, Methodology, Visualization, Writing – original draft. **Anne E. Ringelmann:** Investigation. **Gertrud E. Morlock:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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