Miniaturization of a Functional Transcription Assay in Yeast (Human Progesterone Receptor) in the 384- and 1536-Well Plate Format

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ABSTRACT

Miniaturization of high throughput screening assays to high-density microplate formats (384 or 1536 wells) is currently the focus of research activity in modern drug discovery facilities. In this article, we describe the adaptation of a fluorescence-based functional transcription assay in yeast for assessing modulators of human progesterone receptor to the 384- and 1536-well microplate format, comparing the experimental results to those obtained in the well-established 96-well format. The experiences gained from the optimization of the liquid-handling procedures and the miniaturization of an enzyme assay (β-galactosidase) were implemented. Thus optimized pipetting protocols were developed to perform a reporter gene assay in yeast in microplate formats of higher density. In the functional transcription assay in yeast, the reporter gene expression showed the expected dependence on the ligand’s dose and affinity in principle in all three microplate formats. For the first time, this assay system has been established in the 1536-well microplate format using CyBi™-Well 96/384/1536 as the liquid-handling unit. The comparison of the signal:background ratios showed a lower sensitivity of the assay in the microplate formats of higher density. This study is an example of a successful miniaturization of a yeast cell-based assay to high-density plate formats on the basis of a careful adaptation procedure and optimized liquid-handling conditions.

INTRODUCTION

Global competition forces both pharmaceutical companies and research laboratories to develop cost- and time-efficient strategies in the field of drug discovery. Thus laboratory automation was introduced into the drug-screening process, presenting new opportunities to reach the goal of finding high-quality lead compounds. Miniaturization of sophisticated high throughput screening (HTS) assays to high-density microplate formats has been reported to be a promising tool to meet scientific and economic requirements, especially following advances in the instrumentation.1–4

The adaptation of a fluorescence-based enzyme assay (β-galactosidase; β-gal) to the 1536-well microplate format using the CyBi™-Well 96/384/1536 automated micropipettor (CyBio AG, formerly JENOPTIK Bioinstruments GmbH, Jena, Germany) has been reported recently.5 This article reports the performance of an enzyme assay studied in the 1536- and 384-well microplate formats in comparison to the standard 96-well plate format. It was shown that the bioconversion of the substrate fluorescein-di-(β-D-galactopyranoside) (FDG) occurred as a linear function of the enzyme concentration (β-gal) in all three microplate formats. Pushing the envelope of miniaturization to more complex assays (e.g., in vivo assay systems based on cells) has been suggested, but so far, only a very few groups have reported on the adaptation of cell-based assays to 1536-well plates.6

The development of cellular assay systems in yeast has produced numerous advantages versus mammalian cell culture systems. Saccharomyces cerevisiae, in particular, bears the genetic potential to express heterologous genes. These strains are relatively undemanding in handling and grow rather fast, which allows shorter assay running times compared to mammalian cell lines.2,7 The functional transcription assay in yeast used in this study was designed to assess modulators of human progesterone receptor (hPR) and allows a quantitative study of the

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specific binding of ligands to hPR by monitoring the activity of the expressed reporter enzyme (β-gal) via photometric detection.7”

In this study, the reporter gene assay in yeast was adapted to the 1536-well plate format using FDG as fluorescence substrate for the reporter enzyme β-gal. The protocols required an optimization of the liquid-handling parameters for which the experience gained from the adaptation of the enzyme assay was used.7,8 The experiments illustrate the successful implementation steps of a yeast cell-based assay system in the 384- and 1536-well microplate formats.

MATERIALS AND METHODS

Instruments

Liquid distribution into the microplates was performed with the CyBi-Well 96/384/1536 micropipettor. In our experiments, a tip wash station—a sponge for dabbing off small liquid drops from the tops of the tips after washing—and adapters for plates and reservoirs were placed at the track carriage. Pipette tips (polypropylene) with a maximum volume of 250, 25, and 10 μl were used to distribute the liquids into 96-, 384- and 1536-well microplates, respectively. (Results on the accuracy of the distribution of liquids are described in ref. 9). The pipetting routines have been established using the Opal Control Software version 4.24 for Windows 95® (Microsoft Corporation, Redmond, WA). The photometric readout of the microplates was carried out with the POLARstar® microplate reader, Version 4.10.0 (BMG Lab Technologies GmbH, Offenburg, Germany). The arrangement of the instruments is displayed in Figure 1. The temperature-controlled Wesbart IS 89 microplate shaker (Wesart, Billinghamurst, England) was used for cell incubation.

Cells, chemicals, and material

All reagents, solvents, and media components were used in p.A. quality. FDG was obtained from MoBiTec (Göttingen, Germany), DMSO, Na2HPO4·7H2O, NaH2PO4·H2O, and MgSO4·7H2O were supplied by Merck (Darmstadt, Germany). All other chemicals and biochemicals needed were from Sigma (Deisenhofen, Germany). The model steroids were dissolved in neat DMSO and kept refrigerated at 4°C. The recombinant yeast strain S. cerevisiae BY23 was cultured overnight in Yeast Nitrogen Base as described previously.7 The cell suspension was adjusted to OD600 = 1 and pH = 7.5 directly before use. Black microplates in the 96- (F-form—250 μl), 384- (FIA-plate—150 μl), and 1536- (Micro-Assay plate—10 μl) well formats were supplied by Greiner (Frickenhausen, Germany). Plate sealers (Dynex Technologies, Denkendorf, Germany) were used to avoid evaporation from the plates.

Functional transcription assay (hPR) in yeast

Ligands such as progesterone and other progestins bind to hPR, thus inducing conformational changes and dimerization, resulting in a release of the associated proteins. The activated ligand-receptor complex is transported into the nucleus, where it binds to specific DNA promoter sequences and consequently induces the expression of genes regulated by this promoter. In our reporter gene assay, the lacZ gene from Escherichia coli acts as a regulating gene. The expressed β-gal enzyme catalyzes the stepwise bioconversion of the substrate FDG into the fluorescence dye fluorescein by hydrolyzing the two galactosidic bonds of the substrate. The signal transduction cascade is illustrated in Figure 2. Consequently, the transcription-activating properties of the ligand can be determined by monitoring the β-gal activity. This was proven via independent in vitro assays based on progesterone receptor affinity studies.7,8 The activity of this well-characterized enzyme was quantified by fluorescence readout (λexc = 485 nm and λem = 520 nm). This enzymatic reaction was used for comparing kinetics studies on the feasibility of reporter gene assays in yeast in the 96-, 384- and 1536-well microplate formats over an incubation period of 90 min. The final volume in the different plate formats was decreased in the ratio 1:5, resulting in final volumes of 150 μl/well.
FIG. 2. Signal transduction cascade of the human progesterone receptor (hPR) assay in yeast. Ligand and receptor (hPR) form an activated complex that triggers the expression of the enzyme β-gal. Finally, the substrate FDG is converted biocatalytically into fluorescein, which exhibits a fluorescent emission when excited.

in the 96-, 30 µl/well in the 384-, and 6 µl/well in the 1536-well plates; this represented a 25-fold volume reduction from the 96-well to the 1536-well microplates. The assay volume consisted of three equal fractions: the solution of model progestins at different concentrations added to the cell growth medium, the cell suspension, and finally the solution of the substrate FDG dissolved in a lysis buffer. The ligand solutions were adjusted using neat DMSO and Z buffer (16.1 gm/L Na₂HPO₄·7H₂O, 5.5 gm/L NaH₂PO₄·H₂O, 0.75 gm/L KCl, and 0.25 gm/L MgSO₄·7H₂O), respectively. Because of the sensitivity of the living yeast cells to organic solvents, the amount of DMSO needed to be limited to 1% of the total liquid volume.

In a first set of experiments, we analyzed the binding of the natural hormone progesterone and four synthetic derivatives (dieneogest, levonorgestrel, promegestone, and gestodene) varying in their relative binding affinity (RBA) to hPR (see Table 1) in order to check the dependence of the reporter signal on the ligands’ affinity. The most favorable concentration of the ligands was determined to be 50 nM. Each ligand solution was pipetted to the first 6 wells per row of a 96-well motherplate, leaving another 6 wells for a blank control (no ligand). This resulted in 24 replicates in the 384-well assay plate and 96 replicates in the 1536-well assay plate. In a second step, the transcription-activating properties of the synthetic progestin promegestone were studied at different concentrations ranging from 0.5 to 500 nM over 11 dilution steps plus a blank control. The dilution series was pipetted into the first three columns of a 96-well motherplate, resulting in three replicates per concentration in a 96-well assay plate, 12 parallel values in a 384-well assay plate, and 48 parallel values in a 1536-well assay plate.

After an incubation time of 3 h at 30°C, the reporter gene expression was stopped by adding the substrate FDG dissolved in lysis buffer. Because the substrate bioconversion was optimal at 37°C, fluorescence measurements were performed in a temperature-controlled microplate reader. All experiments were repeated three times.

All liquid handling was performed with the automated CyBioWell 96/384/1536 micropipette. The different pipetting protocols have been generated on the basis of optimization experiments concerning cross-contamination, cleansing of the tips, avoiding the formation of foam, mixing of different solutions,

<table>
<thead>
<tr>
<th>Microplate Format</th>
<th>Gestodene (880 ± 160)</th>
<th>Levonorgestrel (670 ± 80)</th>
<th>Promegestone (730 ± 100)</th>
<th>Progesterone (100)</th>
<th>Dienogest (10.5 ± 2.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>27.91</td>
<td>11.40</td>
<td>4.48</td>
<td>2.48</td>
<td>1.08</td>
</tr>
<tr>
<td>384</td>
<td>2.98</td>
<td>2.65</td>
<td>2.80</td>
<td>1.73</td>
<td>1.12</td>
</tr>
<tr>
<td>1536</td>
<td>6.12</td>
<td>5.50</td>
<td>3.77</td>
<td>2.51</td>
<td>0.91</td>
</tr>
</tbody>
</table>

*S:B ratio after 90-min incubation of model steroids at equal concentrations (50 nM). RBA of model steroids to hPR according to Rudakoff et al.⁷
and evaporation. This resulted in a dramatically improved quality of the experiments. Because cell solutions and surfactants were used, special attention was paid to the avoidance of foam and to cleansing the pipette tip after each process to re-establish comparable conditions. The sponge did not represent a major source of cross-contamination because it was sterilized every 3 days and only came into brief contact (~1 s per cleansing routine) with already cleansed pipette tips. Precipitated cells were resuspended by performing an aspiration-and-dispensing cycle at gentle piston speed (40 rpm). The photometric readout was focused on the detection of the fluorescence intensity. In order to maintain the comparability of the absolute readout values, the sensitivity of the reader had to be adjusted by selecting a suitable gain value.

RESULTS AND DISCUSSION

The kinetics of the bioconversion of FDG depend on the affinity of the hPR ligands. Results are shown in Figure 3 for the different microplate formats. The expected relation between fluorescence intensity and both the receptor affinity of the model progestins and the reaction time was confirmed for all three microplate formats. This is manifested in analogous profiles for the test compounds in each case: the higher the affinity of the ligand and the longer the reaction time, the larger the photometric signal. At the selected progestin concentration of 50 nM, dierogest (the compound with the lowest affinity) did not show any transcription-activating properties in any of the microplate formats. The gain values needed to be increased from 5 (96-well plate) to 27 (384-well plate) and 20 (1536-well plate) in order to get comparable photometric signals. The highest gain was required in the 384-well format, because the distance between the liquid and the detector surface (amount of detected photons) was larger than in the 1536- and 96-well formats.

Table 1 shows that the signal/background ratio (S:B = mean signal/mean background) decreased as expected with lower affinity of the ligand, which is in accordance with previous studies. The S:B ratio also decreased when stepping toward higher density of the plate format. The differences in both the fluorescence intensities and S:B values were attributed to physical and biological phenomena. Physical effects such as a changed surface/volume ratio (wall effects) and an increased distance between the surface of the liquid and the detector (see above) become less favorable in higher density microplate formats. In the case of the 384- and 1536-well microplate formats, resuspension of the liquid after the first incubation step perhaps could not stir up cells settled at edges and corners of the well (ratio of the orifice of the pipette tip to the footprint of the well and surface effects). This may lead to a reduced biological interaction as the number of cells floating freely is reduced. The changed surface/volume ratio also influences the oxygen input, an important parameter for cell metabolism. Because of our experimental conditions (geometry of the microplates, liquid volume), physical and biological circumstances were apparently most unfavorable in the 384-well plates with regard to quantity (gain) and quality (S:B) (see Fig. 3 and Table 1). In the case of the 1536-well plates, the optimized prolonged pipetting protocol resulted in a delayed starting point for the photometric measurement of about 6 min.

Figure 4 illustrates that the kinetics of the bioconversion of FDG are dependent on the concentration of the ligands. The gain values were adjusted from 5 (96-well plate) to 60 (384-well plate) and 45 (1536-well plate). The synthetic progestin promegestone was chosen for these studies. In all three microplate formats the kinetics are similar. The fluorescence intensity increased according to time and promegestone concentration, as expected. In order to keep results comparable, the
MINIORIZED FUNCTIONAL TRANSCRIPTION ASSAY IN YEAST

a) 96-well plate format:

![Graph showing fluorescence intensity over time for various concentrations of promegestone in 96-well format.]

b) 384-well plate format:

![Graph showing fluorescence intensity over time for various concentrations of promegestone in 384-well format.]

c) 1536-well plate format:

![Graph showing fluorescence intensity over time for various concentrations of promegestone in 1536-well format.]

FIG. 4. Reporter gene expression is dependent on promegestone concentration in three microplate formats: (a) 96 (gain 5), (b) 384 (gain 60), and (c) 1536 (gain 45). Delay is due to the optimized pipetting protocol.

Gain was adjusted to the same value as in the preceding experiments. The fluorescence signal in the 1536-well microplates reached a considerably higher level at the equivalent concentrations than in the 384-well microplates (by a factor of 2 to 3) even though the gain was already set higher in the latter format. This corresponds qualitatively to the results of promegestone in the affinity study discussed above (column 3 in Fig. 3b and 3c). The S:B values (Table 2) decreased by increasing the density of the plate format from 96 to 1536 wells, and by lowering the concentration of the model progestin. At low promegestone concentrations (Yigals < 5 nM) in the 384- and 1536-well plates, the S:B ratio dropped below a factor of 2, which limits quantitative conclusions. In comparison, in the 96-well plate format the S:B ratio even at a concentration of 0.5 nM was about 33. Both physical and biological effects were suggested to contribute to the behavior of the assay system in analogy to the experiments varying the affinity of the ligands (see above).

CONCLUSION

The transferability of the reporter gene assay in yeast for assessing progesterone receptor modulators from 96- and 384-well formats into the 1536-well microplate format was demonstrated. The reporter gene expression occurred as a function of the ligand’s affinity and concentration in the higher density plate formats as well as in the standard 96-well plate format. With our experimental conditions, the strength of the photometric signal was slightly higher in the 1536- than in the 384-well plate. A decreasing S:B ratio was observed as the density of the microplate format increased, which was attributed to less favorable physical and biological conditions. Consequently, in the routine screening process the detection of weak biological effects (e.g., caused by specific compounds with low receptor affinity or at low concentrations) must be considered problematic using microplate formats of higher density. Because compounds with the mentioned properties can also be interesting lead structures for further drug development, it is necessary to include the results of adaptation experiments into primary screening miniaturization strategies. Miniaturization demands that all physical and biological parameters, the liquid-handling conditions in particular, have to be examined and adjusted carefully. Thus miniaturization using the higher density 384- and 1536-well plate formats becomes advantageous as the next evolutionary step in HTS. Using the CyBi-Well 96/384/1536 micropipettor to perform all liquid-handling procedures, reporter gene assays could be transferred from 96- into 384- and 1536-well microplate formats in sufficient quality.

| Table 2. Dependence of signal: Background (S:B) Ratio on Concentration of Promegestonea |
|-----------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Microplate Format                | 500           | 100           | 50            | 10            | 5             | 1             | 0.5           |
| 96                               | 44.47         | 48.83         | 50.63         | 51.72         | 52.01         | 37.47         | 33.46         |
| 384                              | 40.09         | 28.71         | 24.91         | 11.75         | 9.62          | 1.84          | 1.78          |
| 1536                             | 16.81         | 12.51         | 11.11         | 7.17          | 5.70          | 1.42          | 1.49          |

aS:B ratio after 90-min incubation.
ACKNOWLEDGMENTS

We are grateful to Mary-Ann Gross for excellent technical assistance. Furthermore, we would like to thank Dr. Silke Angersbach from BMG LabTechnologies and Karin Nitsche from Greiner Labortechnik for the support in this project. Special thanks to AJ Kolb with IGEN for his help in preparing the manuscript.

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