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Development of a dual luciferase activity and fluorescamine protein assay adapted to a 384 micro-well plate format: Reducing variability in human luciferase transactivation cell lines aimed at endocrine active substances

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ABSTRACT

There is a need to adapt cell bioassays to 384-well and 1536-well formats instead of the traditional 96-well format as high-throughput screening (HTS) demands increase. However, the sensitivity and performance of the bioassay must be re-verified in these higher micro-well plates, and verification of cell health must also be HT (high-throughput). We have adapted two commonly used human breast luciferase transactivation cell bioassays, the recently re-named estrogen agonist/antagonist screening VM7Luc4E2 cell bioassay (previously designated BG1Luc4E2) and the androgen/glucocorticoid screening MDA-kb2 cell bioassay, to 384-well formats for HTS of endocrine-active substances (EASs). This cost-saving adaptation includes a fast, accurate, and easy measurement of protein amount in each well via the fluorescamine assay with which to normalize luciferase activity of cell lysates without requiring any transfer of the cell lysates. Here we demonstrate that by accounting for protein amount in the cell lysates, antagonistic agents can easily be distinguished from cytotoxic agents in the MDA-kb2 and VM7Luc4E2 cell bioassays. Additionally, we demonstrate via the fluorescamine assay improved interpretation of luciferase activity in wells along the edge of the plate (the so-called "edge effect"), thereby increasing usable wells to the entire plate, not just interior wells.

1. Introduction

Receptor-based, in vitro transactivation cell bioassays have been the most promising tools to effectively screen large numbers of samples for endocrine activity. Federal programs such as the United States Environmental Protection Agency (USEPA) Endocrine Disruptor Screening Program (EDSP) or the Tox21 Program have included such assays for this very purpose (Borgert et al., 2011; Ceger et al., 2015; USEPA, 2009). Major challenges facing programs such as the EDSP include screening tens of thousands of compounds in production and deciding which of those chemicals require more rigorous in vivo assessment. In vitro high throughput screening (HTS) assays are capable of providing data on a large collection of compounds within reasonable time and expense constraints. Indeed, one of the most widely used HTS assays are receptor-dependent, cell based bioassays which utilize luciferase reporter gene expression vectors linked to receptor DNA response elements. Two such assays include the well-known human breast VM7Luc4E2 cell bioassay, a chemically-activated luciferase expression (CALUX) cell bioassay, and the human breast MDA-kb2 cell bioassay (Rogers and Denison, 2000; Wilson et al., 2002). These CALUX cell bioassays have been designed to detect Endocrine active substances (EASs)/endocrine disrupting chemicals (EDCs) which interact with particular hormone signaling pathways (estrogen receptor in the case of the VM7Luc4E2 bioassay or androgen receptor and glucocorticoid receptor in the case of the MDA-kb2 cell bioassay) and function via hormone-inducible, receptor-dependent luciferase reporter gene induction (Rogers and Denison, 2000; Wilson et al., 2002).

Traditionally, cell-based assays such as these have been carried out in 96-well plates. However, the cost and time required to screen large numbers of chemicals or samples (hundreds to thousands) with 96-well plates becomes unfeasible. Both the VM7Luc4E2 and MDA-kb2 cell bioassays have been successfully adapted to the 384 and 1536 micro-well plate formats, for automated high-throughput chemical screens but for smaller or academic laboratories, there is currently no cost-effective, accepted method for evaluating cytotoxicity in these assays without costly plate replication (Ceger et al., 2015; Shi et al., 2016). Assessment of cytotoxicity in cell-based assays is important to help evaluate the cause of non-responsive chemicals/samples. A weak agonist response may be masked if the chemical being screened is cytotoxic at concentrations lower than receptor activation occurs. Additionally, if no

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method of normalization is used for the luciferase assay in a 384-well plate, the luciferase activity can be subject to inconsistent cell seeding (avoided only with an automated cell seeder or an experienced technician) or “edge-effects” (unavoidable in certain cell lines). Thus, the ability to evaluate luciferase induction and protein-based cytotoxicity sequentially in the same plate of a cell-based assay could save time and money. Moreover, normalization of luciferase activity in each well to cellular protein content could help precision and accuracy of these luciferase-based assays. Here, we use these two cell lines as models to describe the luciferase assay adapted to a 384-well plate in combination with a fluorescamine assay for determination of protein of cell lysates in each well. Not only is our method capable of 4-fold greater analysis of chemical treatments per 384-well plate compared to a 96-well plate, we have optimized the dual luciferase/fluorescamine assay in the 384-well plate to be half the cost of the assay in a 96-well plate.

2. Methods and materials

2.1. Chemicals

DMSO was ACS GC-grade DMSO (EMD Millipore, Cat no. MX1458-6). 17 beta-estradiol (E2), ICI-182-780 (fulvestrant), Mifepristone (RU-486), hydroxyflutamide (OHF), Dexamethasone, and methyltrienolone (R1881) were from Sigma-Aldrich with Cat no. E8875, 14409-25MG, H4166-5MG, D1756-100MG, and R0908-10MG, respectively. Doxorubicin hydrochloride (doxorubicin, Cat no. 2252) was from Tocris Biosciences. Triton-X was from Sigma (Cat no. T-6878). Water was Milli-Q-grade.

2.2. Cell bioassays

VM7Luc4E2 cells, graciously provided by Dr. Michael Denison (University of California, Davis), were maintained at 37 °C, 5% CO_2, and approximately 85% humidity in alpha minimal essential medium (α-MEM, Gibco, Cat no. 12000022) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Cat no. S11150) and 400 mg/L G418 (Gemini BioProducts, Cat no. 400-111P) as previously described with slight modifications (Rogers and Denison, 2000). Five days prior to seeding, cells were switched to Dulbecco's Modified Eagles Medium (referred to here as estrogen-stripped medium ‘ESM’, Sigma Aldrich, Cat no. D2902) containing 10% charcoal-stripped FBS (Atlanta Biologicals, Cat no. S11650) with the medium removed and replaced with fresh ESM/charcoal-stripped serum at least three days prior to treatment. Cells were then seeded at a density and volume of 750,000 cells per mL (unless noted otherwise) and 25 µL per well, respectively, onto white, solid low-flange 384-microwell plates (Corning, Cat no. 3570). Wells A1-D1 were left empty at time of seeding to serve as the protein blank control on each plate. Post seeding (24 h), medium/serum was removed and cells were rinsed once with serum-free ESM (rinse volume 25 µL per well) before treatments were applied (25 µL per well) in quadruplet at a 1:1000 dilution for single treatments or 1:2000 dilution for co-treatments in serum-free ESM (0.1% DMSO total). Blank medium was applied to wells A1-D1. Plates were rinsed twice with phosphate-buffered saline 24 h post-treatment, then cells were lysed with 12 µL per well Cell Culture Lysis Buffer (Promega, Cat no. E1531), including wells A1-D1. Micro-well plates were then shaken for 10–15 min and either frozen at − 80 °C for 24–48 h or immediately analyzed with a Synergy 4 Multi-Mode Reader (BioTek, Winooski, VT, USA) for luciferase activity and protein content. The MDA-kb2 cells (American Tissue Culture Collection [ATCC], Cat no. CRL-2713) were cultured in the following conditions: MDA-kb2 cells were maintained in Lebowitz's 15 (L-15) medium (ATCC, Cat no. 30-2008) at atmospheric CO_2 (Wilson et al., 2002) and switched to phenol-red free L-15 medium (Gibco, Cat no. 21-083-027) containing 10% charcoal-stripped FBS 5 days prior to seeding (Tarnow et al., 2013), were seeded at a density of 500,000 cells per mL (unless noted otherwise) in 384-well plates, 25 µL/well, and treatments applied in phenol-red free L-15 medium without serum. Authenticity of both cell lines was verified by American Tissue Culture Collection using Short Tandem Repeat (STR) analysis as described (ANSI/ATCC, 2012; Capes-Davis et al., 2013). All handling and disposal of cell culture materials was in accordance with guidelines set forth by the U.S. Department of Labor Occupational Safety and Health Administration 29 CFR 1910.1030 (OSHA, 1992).

2.3. Analysis of luciferase activity

Luminescence of cell lysates (including wells A1-D1 which consisted of 12 µL per well blank 1 × Cell Culture Lysis Buffer) was analyzed with Gen5 version 3.00 software (BioTek). The protocol for luminescence was set up in well mode with 12 µL injection (dispense rate 225 µL per sec) of luciferase reagent (Promega, Cat no. E1501) followed by 2 s delay and a 10 s integration time with 1 mm probe offset. Luminescence sensitivity was set to 175 for VM7Luc4E2 cell lysates and 255 for MDA-kb2 cell lysates as initially determined by auto-gain and was reported as relative light units (RLUs). Once luminescence (luciferase activity) was analyzed for all wells, well-mode terminated and the protocol proceeded to the fluorescence portion.

2.4. Analysis of protein levels

Fluorescence of cell lysates was analyzed as a continuance of the luciferase protocol described above and is a modification of the original procedure in microplates (Lorenzen and Kennedy, 1993). Fluorescamine (Sigma Aldrich, Cat no. F9015) in acetone (Optima grade, Fisher Scientific, A929-4) was prepared at 1.08 mM and injected into all wells with an injection volume of 8 µL per well (dispense rate 225 µL per sec). This was followed by a 2 min shake at medium speed followed by a 10 min delay to allow the reaction to develop. Fluorescence, set to sensitivity 74 (as initially determined by auto-gain) was then read with a 6 mm offset at 400 nm excitation and 470 nm emission in row-wise fashion starting at Row A. A bovine serum albumin (BSA, Sigma Aldrich, Cat no. 05470) standard curve (0, 0.01, 0.1, 0.125, 0.15, 0.2, 0.3, 0.4, 0.55, 0.7, 0.8, 1.0, 1.25, 1.5, and 2.0 mg/mL) in 1 × Cell Culture Lysis Buffer was prepared in baked glass borosilicate tubes and transferred in quadruplet to empty wells in the micro-well plate containing cell lysates or an identical empty white 384-well plate (12 µL standard per well). To wells containing the BSA standards, 12 µL luciferase reagent was injected (dispense rate 225 µL per sec) immediately followed by 8 µL 1.08 mM fluorescamine in acetone (dispense rate 225 µL per sec) into all wells at which point the protocol proceeded exactly as just described. Un-used BSA standards were stored at − 20 °C in the dark and were never re-utilized more than twice or two weeks from their creation, whichever came first. Un-used fluorescamine in acetone was stored in the dark at 4 °C. Both solubilized fluorescamine and luciferase reagent were allowed to come to room temperature before use. All data for luminescence and fluorescence were exported either as relative light units (RLU, in the case of luminescence) or RFU (relative fluorescence units, in the case of fluorescence) to Microsoft Excel 2013 for processing.

2.5. Determination of protein levels in cell lysates

Significant difference in a BSA standard's relative fluorescence unit (RFU) above that of background RFU (that of the 0 mg/mL BSA control) was determined with Student's *t*-test (2 tailed, Type 2 in Microsoft Excel ($p < 0.01$), and minimal detection limit (MDL) was determined as the lowest BSA standard to exhibit significant fluorescence above background. BSA concentrations were converted to µg protein and plotted as the dependent variable against their respective averaged RFU after subtraction of average background RFU. The resulting curve was fitted with a polynomial cubic equation (Held, 2006) and the resulting parameters from the curve fit were utilized to convert RFUs of cell