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Original article

An optimized lactate dehydrogenase release assay for screening of drug candidates in neuroscience

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ABSTRACT

Introduction: Quantification of lactate dehydrogenase (LDH) release is a widely accepted assay for the quantitative determination of cell viability and late-stage apoptosis. Major disadvantages of commercially available LDH assay kits include proprietary formulations, limited options for optimization and high cost, all resulting in limited reproducibility in research applications. Here, we describe a novel, custom LDH assay suitable in the context of plate reader-based screening of drug candidates for glioprotection, but with wide applicability to other cell types and experimental paradigms. **Methods:** We developed a novel and highly reproducible LDH release assay that is more cost-effective than commercially available assays with comparable performance. The assay was validated by assessing 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid antioxidant protection against *tert*-butylhydroperoxide-induced oxidative stress in C6 astroglioma cells. Assay performance was validated by direct comparison and compatible with other methods of measuring cellular viability, namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 6-carboxy-2′, 7′ dichlorodihydrofluorescein diacetate assays. **Results:** There was no statistically significant difference between results obtained with the novel custom assay and a commercially available assay CytoTox96® (Promega, Madison, WI). **Discussion:** The novel custom LDH release assay allows the reproducible quantification of cell viability and is highly cost-effective when compared to commercially available assays (approximately 25 times cheaper). In addition and in contrast to commercially available assays, the identification and detailed description of all assay components and procedures provide greater control over experimental conditions and design. We provide a detailed standard operating procedure permitting our novel assay to be readily adapted depending on experimental requirements.

1. Introduction

Cell viability assays have become a standard tool in neuroscience drug discovery to quantify the number of cells beginning to undergo apoptotic and necrotic processes, as well as to determine the neuroprotective or glioprotective potential of drug candidates (Stoddart, 2011; Vega-Avila & Pugsley, 2011). L-Lactate dehydrogenase is a cytoplasmic enzyme that catalyzes the interconversion of pyruvate to L-lactate with the concomitant interconversion of NADH to NAD^+ during glycolysis, and catalyzes the reverse reactions during the Cori cycle (Decker & Lohmann-Matthes, 1988; Nachlas, Margulies, Goldberg, & Seligman, 1960). LDH is released from the cytoplasm in response to cell damage or exogenous insults leading to damage of the plasma membrane and, ultimately, cell death. Given its stability in the extracellular environment and in particular in cell culture media, LDH release has been widely used to evaluate the presence of damage and toxicity in tissues and cells (Stoddart, 2011). Traditionally, LDH activity is determined by utilizing a coupled enzymatic reaction, where LDH oxidizes lactate to pyruvate, which subsequently reacts with the iodonitrotetrazolium chloride (INT) to form the colored formazan. Formazan is water-soluble and can be readily detected colorimetrically by measuring absorbance at 490 nm (Decker & Lohmann-Matthes, 1988). The assay relies on the assumption that the increase in the amount of formazan produced in the culture supernatant is directly correlated with cell viability.

Commercially available LDH assays have drawbacks such as proprietary formulation with unknown components and concentrations, thus limiting assay optimization or adaptation to changing experimental needs, and high cost.

Abbreviations: ANOVA, analysis of variance; DCFDA, 6-carboxy-2′, 7′ dichlorodihydrofluorescein diacetate; EtOH, ethanol; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; INT, iodonitrotetrazolium chloride; LDH, lactate dehydrogenase; MPMS, 1-methoxyphenazine methosulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD, beta-nicotinamide adenine dinucleotide sodium salt; PBS, phosphate-buffered saline; ROS, reactive oxygen species; *t*BHP, *tert*-butylhydroperoxide; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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The objective of the present study was to develop a reproducible and at the same time cost-effective LDH assay with known formulation. We have validated the assay for plate reader-based screening of drug candidates for glioprotection utilizing 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a prototypic glioprotectant against *tert*-butylhydroperoxide (*t*BHP)-induced oxidative stress in C6 astroglioma cells. We report here a low variance LDH assay with a reagent cost estimated to be about 25-fold lower than similarly performing commercial assays.

Furthermore, we established a standardized protocol combining multiple assays for cell viability and proliferation as well as for quantification of reactive oxygen species (ROS). To this end, we here report the successful combination of our custom LDH assay with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the 6-carboxy-2′, 7′ dichlorodihydrofluorescein diacetate (DCFDA) assay, using the same sample.

2. Material and methods

2.1. Cell culture

C6 astroglioma cells were obtained under Material Transfer Agreement from the American Type Culture Collection (ATTC® CCL-107™; Manassas, VA) and cultured in F-12 K medium (Cellgro®; Mediatech, Manassas, VA) supplemented with 15% horse serum (heat inactivated, New Zealand origin; Life Technologies, Carlsbad, CA) and 2.5% fetal bovine serum (Gibco® certified, heat inactivated, US origin; Life Technologies) at 37 °C/5% CO_2/95% humidity in standard tissue culture flasks (TPP®; Midwest Scientific, MidSci, St. Louis, MO). For cell viability experiments, cells were seeded into clear, flat bottom 96 well plates (TPP®; Midwest Scientific) at a density of 7500 cells/well.

2.2. Induction of oxidative stress and drug treatment

In order to induce oxidative stress, cells were exposed to various concentrations of *t*BHP (Sigma Aldrich Corp., St. Louis, MO) 16–20 h after seeding. For glioprotection, cells were pre-treated (1 h) with the prototypic antioxidant Trolox (100 μM in 0.1% v/v ethanol; Sigma Aldrich Corp., St. Louis, MO) or ethanol vehicle (0.1%; Sigma Aldrich).

2.3. Custom LDH assay

Following 3 h exposure to *t*BHP, 50 μl of cell culture supernatant was transferred into a non-sterile, clear 96-well multiwell plate (Nunc, Thermo Fisher Scientific, Waltham, MA). 50 μl Assay Buffer (2 mM iodonitrotetrazolium chloride, 3.2 mM β-nicotinamide adenine dinucleotide sodium salt, 160 mM lithium lactate, 15 μM 1-methoxyphenazine methosulfate in 0.2 M Tris–HCl, pH 8.2) was added. Plates were incubated at room temperature in the dark for 1 h. The reaction was stopped by addition of 50 μl 1 M acetic acid. LDH release was quantified by measuring absorbance at 490 nm (A_{490}) using a Synergy H1 plate reader (Biotek, Winooski, VT). Data were exported to Microsoft Excel (Microsoft Corp., Redmond, WA) for processing, normalized to the control condition (0 μM *t*BHP), and analyzed in Prism 5.0 (Graphpad, La Jolla, CA).

Stock solutions for the LDH Assay Buffer were prepared in advance and consisted of Buffer A (2×; 4 mM INT in 0.2 M Tris–HCl, pH 8.2), Buffer B (2×; 6.4 mM NAD, 320 mM lithium lactate in 0.2 M Tris–HCl buffer, pH 8.2), and MPMS supplement (10,000×; 150 mM MPMS in 0.2 M Tris–HCl buffer, pH 8.2). A single 96-well plate required 5 ml Assay Buffer (2.5 ml Buffer A, 2.5 ml Buffer B, 0.5 μl MPMS supplement). Solutions were aliquoted and stored frozen at −20 °C for up to one month. Assay Buffer was prepared and 50 μl was immediately applied to 50 μl samples. A detailed, step-by-step protocol and standard operating procedure is provided in Appendix A.

2.4. CytoTox96® assay

We benchmarked our new custom LDH assay against a commercially available LDH assay (CytoTox96®; Promega, Madison, WI). The assay was performed according to the manufacturer's instructions.

2.5. Carboxy DCFDA assay

In order to quantify the level of oxidative stress we used the fluorescent ROS indicator 6-carboxy-2′, 7′ dichlorodihydrofluorescein diacetate (DCFDA) as described by us previously (Burroughs et al., 2012). Briefly, cells were loaded with 10 μM DCFDA in complete media for 30 min. Subsequently, cells were treated and oxidative stress was induced chemically as described above. At the end of the incubation period and after collection of supernatant samples for LDH release assays, cells were washed twice with 300 μl Hank's Balanced Salt Solution (HBSS; Lonza, Walkersville, MD) supplemented with 2 mM $CaCl_2$. Plates were read in a fluorimetric plate reader (Synergy H1 plate reader; Biotek) at 485/530 nm excitation/emission and data were acquired in Gen5 software (Biotek). Data were exported to Microsoft Excel (Microsoft Corp.) for processing, normalized to the control condition (0 μM *t*BHP), and analyzed in Prism 5.0 (Graphpad).

2.6. MTT assay

The MTT assay was performed essentially as described by us previously for HT-22 cells and primary cortical neuron culture (Burroughs et al., 2012; Kaja et al., 2011). Briefly, media was aspirated from the cells and replaced with 100 μl of 1.2 mM MTT in HBSS with calcium and magnesium (Lonza) supplemented with 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Sigma Aldrich) at pH 7.3. Plates were incubated at 37 °C for 2 h. Media was aspirated and cells were lysed with 100 μl dimethylsulfoxide (DMSO) and gentle shaking. The conversion of MTT was quantified by measuring absorbance at 570 nm (A_{570}) using a Synergy H1 plate reader (Biotek). Absorbance values were corrected for background, normalized to the control condition (0 μM *t*BHP/no chemically induced oxidative stress) and analyzed in Prism 5.0 (Graphpad).

2.7. Data analysis

Data for all assays were acquired in Gen5 software (Biotek) and exported to Microsoft Excel (Microsoft Corp.) for processing. Data was normalized to the untreated, control condition (0 μM *t*BHP). Outlier exclusion was applied to data points (single wells) for each insult (*t*BHP concentration; column of 8 wells). We assumed a Gaussian distribution and excluded any data point 2 standard deviations from the columnar mean. Normalized data were grouped by treatment condition and exported to Prism software (version 5.0; Graphpad) for statistical analysis and plotting. Non-linear regression using a four-parameter logistic equation with variable Hill slope was performed separately for each biological replicate to determine the mean LD_{50} values for *t*BHP under each pre-treatment condition (control, vehicle, and Trolox).

Data was analyzed statistically using 2-Way analysis of variance (ANOVA) and the Bonferroni post-hoc test, with pre-treatment condition (control, vehicle, or Trolox) and insult (*t*BHP concentration) as variables. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Optimization of a custom LDH assay to measure cellular viability, effects of chemically induced oxidative stress, and glioprotection in C6 astroglioma cells

We first determined the optimal LDH assay incubation time using a time course measurement. C6 cells were treated with varying