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Original Article

Development, validation and quantitative assessment of an enzymatic assay suitable for small molecule screening and profiling: A case-study

Vicente Sancenon*, Wei Hau Goh, Aishwarya Sundaram, Kai Shih Er, Nidhi Johal, Svetlana Mukhina, Grant Carr, Saravanakumar Dhakshinamoorthy*

Albany Molecular Research Singapore Research Centre, Pte Ltd, The Galen #05-01, 61 Science Park Road, Singapore 117525, Singapore

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ABSTRACT

The successful discovery and subsequent development of small molecule inhibitors of drug targets relies on the establishment of robust, cost-effective, quantitative, and physiologically relevant *in vitro* assays that can support prolonged screening and optimization campaigns. The current study illustrates the process of developing and validating an enzymatic assay for the discovery of small molecule inhibitors using alkaline phosphatase from bovine intestine as model target. The assay development workflow includes an initial phase of optimization of assay materials, reagents, and conditions, continues with a process of miniaturization and automation, and concludes with validation by quantitative measurement of assay performance and signal variability. The assay is further evaluated for dose–response and mechanism-of-action studies required to support structure–activity-relationship studies. Emphasis is placed on the most critical aspects of assay optimization and other relevant considerations, including the technology, assay materials, buffer constituents, reaction conditions, liquid handling equipment, analytical instrumentation, and quantitative assessments. Examples of bottlenecks encountered during assay development and strategies to address them are provided.

1. Introduction

The discovery and development of small molecule modulators with desired pharmacological properties is a funneled process comprising multiple stages including: (i) identification and validation of druggable targets for specific therapeutic areas; (ii) *in vitro/in silico* screening, identification, and characterization/profiling of small molecules which potently and selectively engage the target of interest, enhancing or inhibiting its molecular function; (iii) toxicology, safety, and efficacy assessments of drug candidates by *in vivo* preclinical and clinical studies. In the early stages of the drug discovery process, the identification and characterization of physiologically relevant small molecule inhibitors markedly relies on the establishment and validation of robust, cost-effective, and scalable cell free and cell based assays that enable to reliably and quantitatively detect and measure variations in the activity of the target of interest or downstream signaling molecules.

The development of such an *in vitro* assay for screening or profiling of small molecule inhibitors is driven by scientific, technical, and budgetary considerations. Scientific considerations include the selection and optimization of materials and conditions that mimic the physiological condition of the target thus enabling the identification of relevant small molecules with desired mechanisms of action. This process may be guided in part by available literature on the target of interest and developed further by the scientific team. Technical considerations include, on one side, the type of technologies and equipment available to measure the desired enzyme activity or receptor-binding affinity, and, on the other side, the throughput, assay format, reaction scale, signal window, and level of automation that such technologies enable. Budget constraints may impose limitations to the type of materials, technologies, and amount of resources invested. Eventually, the suitability of a given assay procedure for a specific screening program must be evaluated by quantitative methods.

Failure to establish and optimize physiologically relevant assay conditions may lead to an excessive rate of false positives or negatives and identification of chemical entities that are inactive *in vivo* or have an undesired mechanism of action. Although some general

Abbreviations: AP, alkaline phosphatase; CV, coefficient of variation; DEA, diethanolamine; DiFMU, 6,8-difluoro-4-methylumbelliferone; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; K_M, Michaelis constant; pNP, p-nitrophenol; pNPP, p-nitrophenol phosphate; SD, standard deviation; V_{max}, maximal reaction velocity; Z', Z prime.

* Corresponding authors. Tel.: +65 6395 3409; fax: +65 6398 5511.
E-mail addresses: VicenteEnrique.Sancenon-Galarza@amriglobal.com (V. Sancenon), Saravanakumar.Dhakshinamoorthy@amriglobal.com (S. Dhakshinamoorthy).

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guidelines on assay development [1] or target specific assay procedures [2,3] can be found in literature, specific examples of assays developed following industry standards with systematic description of the procedures are limited. This study provides a comprehensive description of the development and validation of an enzymatic assay for small molecule screening, emphasizing the most critical parameters, bottlenecks, and the corrective measures to overcome them using alkaline phosphatase from bovine intestine as model target [4–6].

2. Material and methods

2.1. Material

The following reagents were purchased from Sigma-Aldrich: Trizma base (T1503), Hepes (H4034), $MgCl_2$ hexahydrate (M2670), NaCl (S5886), KCl (P9333), $ZnCl_2$ (208086), Tween 20 (F7949), calf intestine alkaline phosphatase (P7923), sodium orthovanadate (450243), 4-nitrophenol (241326), and 4-nitrophenyl phosphate bis(tris) salt (73737).

The following reagents were purchased from Life Technologies: 6,8-difluoro-4-methylumbelliferyl phosphate (D6567) and 6,8-difluoro-4-methylumbelliferone (6,8-difluoro-7-hydroxy-4-methylcoumarin) (D6566).

For the colorimetric assay, 96-well clear non-treated plates were purchased from Cayman Chemical (400014), and 384-well clear non-binding surface plates were purchased from Corning (3640). For the fluorometric assay, 384-well black non-binding standard plates were purchased from Greiner (781900), and 384-well black non-binding low volume plates were purchased from Corning (3676).

Polypropylene reservoirs (Socorex 330.01) and polypropylene 96-well plates (Corning 3363) were used as source container for fresh working solutions prior to their transfer to the assay plate using multichannel pipettes (Gilson and Finntip). Polypropylene 384-well plates (Corning 3657) were used as source container for automated transfers using Hummingbird Plus liquid handler (Digilab). Polypropylene 50 mL Falcon tubes (BD Biosciences 352070) were used as source container for automated transfers using Multidrop Combi dispenser (Thermo Scientific).

2.2. Reagents

The alkaline phosphatase (AP) stock was stored at 4 °C. AP intermediate dilutions were prepared in 1× assay buffer containing 50% glycerol and stored at 4 °C. Working solutions of p-nitrophenol phosphate (pNPP), 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP), Na_3VO_4, and AP were prepared fresh in assay buffer or H_2O as described in the next section and added to reservoirs or 96-well polypropylene plates prior to transfer to the assay plate using multichannel pipettes.

2.3. Alkaline phosphatase assay

2.3.1. Colorimetric assay

Assay buffer containing TRIS was prepared at 2× final concentration and stored at room temperature. pNPP stock solution was prepared at 100 mM in dH_2O and stored at −20 °C. p-Nitrophenol (pNP) stock solution was prepared at 50 mM in dH_2O and stored at −20 °C.

The final reaction conditions were 50 mM Tris–HCl pH 7.5, 135 mM NaCl, 7.5 mM KCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.3 mM Tween 20 or as specified in the text. pNPP and AP concentrations varied as specified in the text. AP was prepared at 2× final concentration in 2× assay buffer, whereas pNPP was prepared at 2× final concentration in dH_2O. Reactions were initiated by adding equal volumes of AP and pNPP to the assay plate (50 μL each to 96-well non-treated plates or 25 μL each to 384-well non-binding plates) using a manual multichannel pipette. Plates were spun down and A_{425} was monitored continuously at room temperature with an Analyst GT microplate reader (Molecular Devices).

2.3.2. Fluorometric assay

Assay buffer containing HEPES was prepared at 1× final concentration and stored at 4 °C. DiFMUP and 6,8-difluoro-4-methylumbelliferone (DiFMU) stock solutions were prepared at 10 mM in DMSO and stored at −20 °C. Na_3VO_4 stock solution was prepared at 50 mM in H_2O and stored at −20 °C.

The final reaction conditions were 50 mM HEPES pH 6.5, 135 mM NaCl, 7.5 mM KCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.3 mM Tween 20 or as specified in the text. DiFMUP and AP concentrations varied as specified in the text. For reactions without inhibitor in standard volume plates, AP was prepared at 2× final concentration in 1× assay buffer, and DiFMUP was prepared at 2× final concentration in 1× assay buffer. Reactions were initiated by adding 25 μL of 2× AP and 25 μL of 2× DiFMUP to the assay plate using a manual multichannel pipette. For reactions with Na_3VO_4 in standard volume plates, AP was prepared at 2.5× final concentration in 1× assay buffer, DiFMUP was prepared at 2× final concentration in 1× assay buffer, and Na_3VO_4 was prepared at 10× final concentration in H_2O. Reactions were initiated by adding 20 μL of 2.5× AP, 5 μL of 10× Na_3VO_4, and 25 μL of 2× DiFMUP to the assay plate using a manual multichannel pipette. For reactions with Na_3VO_4 in low volume plates, AP or a mixture of AP and Na_3VO_4 were prepared at 3× final concentration in 1× assay buffer, and DiFMUP was prepared at 1.5× final concentration in 1× assay buffer. Reactions were initiated by adding 5 μL of 3× AP or AP plus Na_3VO_4, and 10 μL of 1.5× DiFMUP to the assay plate using a Multidrop Combi dispenser (Thermo Scientific). Plates were spun down and incubated at 37 °C. Fluorescence intensity (ex: 358 nm, em: 455 nm) was monitored continuously at 37 °C or at a single end-point as indicated in the text and figure legends with a PHERAstar microplate reader (BMG Labtech).

The concentration of enzyme in each reaction was calculated according to the nominal concentration of the original stock provided by the manufacturer (2000 DEA Units in 15 μL) and expressed as DEA μUnits μL^{-1}. For the validation tests, 0.1 μL of DMSO was transferred to the low volume plates prior to dispensing of the other reagents using Hummingbird Plus liquid handler (Digilab).

For both colorimetric and fluorometric assays, blank reactions contained the same constituents as the test reactions except AP.

2.4. Data analysis

Initial reaction velocities were estimated by converting blank subtracted Absorbance or Fluorescence units from the reaction progress curves into product concentration units using pNP or DiFMU calibration curves, respectively, and calculating the slope of the normalized curves in the initial linearity phase following the equation:

$$v_0 = \frac{\Delta P}{\Delta t} \quad (1)$$

where v_0 is the initial reaction velocity (nmols min^{-1}), ΔP is the increment in amount of product produced in the linear phase (nmols), and Δt is the time window of the linear phase (min).

Enzyme kinetic parameters were calculated by plotting initial reaction velocities against substrate concentration and fitting the data points by non-linear regression to the classical Michaelis Menten steady state model (2) or a variant of the model that