Creating dose-response curves for cell-based and biochemical assays with the HP D300 Digital Dispenser

Direct Digital Dispensing offers advantages compared to manual serial dilution

Introduction

For many years, small molecule drug discovery has relied on the evaluation of one compound against another compound. Measurement of the activity of the small molecule against a target must be standardized, and this is achieved by determining the IC_{50} or EC_{50} value (half maximal inhibitory/ effective concentration respectively) using dose-response curves. To set up dose-response curves, compounds must be dissolved in a solvent to create solutions at various concentrations, and DMSO has become established as the solvent of choice. Today, chemists provide the biologists or compound management team responsible for producing the dose-response curves with compound solutions prepared in DMSO.

The drawbacks of traditional serial dilution

The classical method of generating a dose-response curve is via serial dilution. However, this approach has a number of drawbacks, including:

- limited to dispensing microliter volumes
- high risk of (cross-)contamination
- inter-well dependencies
- edge effects
- limited data accuracy
- poor inter-operator and inter-laboratory reproducibility
- excessive use of consumables
- high consumption of bulk reagents.

Today, there is an alternative solution which eliminates these limitations: the HP D300 Digital Dispenser. This benchtop instrument is designed to produce dose-response curves for small molecules in DMSO by dispensing directly into bioassay plates [1]. The advantages of this technology compared to serial dilution are:

- picoliter to microliter dispensing
- contamination virtually eliminated
- direct dispensing, eliminating inter-well dependencies
- easy plate randomization of concentrations
- targeted dosing
- compound savings
- synergy experiments
- reduced DMSO content
- time savings.

This application note compares the results of dose-response curves set up using the HP D300 Digital Dispenser and a traditional manual serial dilution method in two different assays: the LANCE® cell-based cAMP assay and a biochemical assay for nuclear receptor activation by AlphaScreen®.

Materials & Methods

Manual serial dilution methods

Evaluation of TGR5 activation using the LANCE cell-based cAMP assay

TGR5 is a member of the rhodopsin-like subfamily of G protein-coupled receptors (GPCRs), and is expressed in brown adipose tissue, muscle, liver, intestine and selected areas of the central nervous system, as well as the gallbladder. Originally considered an orphan GPCR, TGR5 has been reclassified as a bile acid (BA) receptor, and is activated by BAs with a different rank order than FXR (farnesoid X receptor, also known as NR1H4), with the secondary BA lithocholic acid and its tauro-conjugate being the most potent natural agonists. It is also suggested that TGR5 may play a protective role in helping to reduce liver injury.

The following homogeneous time-resolved fluorescence resonance energy transfer (HTR-FRET) method [2] has been used to rank TGR5 agonists, assessing activation of TGR5 by measuring the level of cAMP. Briefly, NCI-H716 cells were cultured in 96-well plates using DMEM containing 0.75 mg/ml Matrigel™ (BD Biosciences), 10 % FCS, 100 µg/ml penicillin and 100 µg/ml streptomycin sulfate. After 24 hours, the cells were stimulated with increasing concentrations of test compounds for 60 minutes at 37 °C in Opti-MEM® (Life Technologies) containing 1 mM 3-isobutyl-1-methylxanthine (IBMX). The level of intracellular cAMP was determined using the LANCE cell-based cAMP assay kit (PerkinElmer) according to the manufacturer's protocol [2, 3].

Evaluation of nuclear receptor activation using an AlphaScreen biochemical assay

FXR is highly expressed in the liver, intestines, kidneys, adrenal glands and adipose tissue. Activation of FXR inhibits the synthesis of bile acid from cholesterol, and protects against the toxic accumulation of BAs via their increased conjugation and secretion into bile canaliculi, thereby promoting bile flow. These properties can be beneficial in the prevention of gallstone formation, and in the treatment of cholestatic liver diseases such as primary biliary cirrhosis.

Activation of FXR nuclear receptors was determined using a recruitment co-activator assay based on AlphaScreen technology, according to the method previously described [4]. Briefly, the assay was performed in an OptiPlate™-384 white, low-volume, 384-well microplate (PerkinElmer), with a final assay volume of 25 µl containing 10 nM of GST-tagged FXR-LBD protein and 30 nM biotinylated Src-1 peptide. Stimulation was carried out for 30 minutes at room temperature with ligand solubilized in 100 % DMSO (1 µl). After incubation with detection mix (acceptor and donor beads) for 4 hours at room temperature in the dark, luminescence was measured using a PerkinElmer EnVision® microplate analyzer [2].

HP D300 Digital Dispenser method

Equipment

- HP D300 Digital Dispenser
- HP T8 Cassette
- HP D300 Digital Dispenser software

For the HTR-FRET experiment, three compounds were resuspended in DMSO at an initial concentration of 12.5 mM. Each dose-response curve was created in a 384-well plate with a final volume of 24 µl and a maximum concentration of 2 % DMSO.The end volume of the assay was 24 µl, with a maximum DMSO concentration of 2 %. Dose-response curves were set up in triplicate at concentrations of 0.00677, 0.0104, 0.0203, 0.0406, 0.0812, 0.1625, 0.3182, 0.6432, 1.2797, 5.2083, 10.208, and 100 µM. This wide concentration range covered the EC_{50} response window (Figure 1).

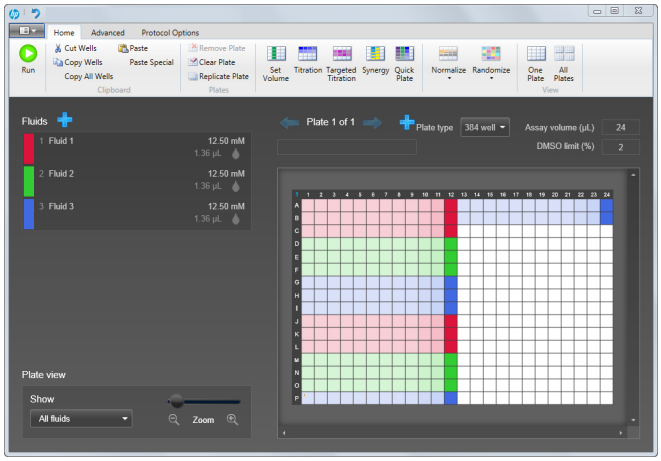

Figure 1 The HP D300 Software set-up for the HTR-FRET experiment.

A total volume of 2 µl of the test compounds was loaded into the HP T8 cassette, dispensing from 0.013 nl to 192 nl per well as specified in the HP D300 software. The total volume dispensed was 1.4 µl.

For the AlphaScreen assay, compounds were resuspended in DMSO at a concentration of 10 mM. The assay was performed in a white, 384-well microplate, with a 25 µl end volume. Dose-response curves of the test compounds were prepared at concentrations of 0.0005, 0.001, 0.002, 0.01, 0.02, 0.1, 0.2, 1, 2, 10, 20, and 100 µM. The volume of test compound dispensed ranged from 0.02 to 0.25 µl per well.

DMSO was not normalized (backfilled) in each well. The positive control was chenodeoxycholic acid (CDCA) at concentrations of 0.01, 0.1, 0.5, 1, 5, 10, 50, and 100 µM.

For both experiments, the HP D300's shaking function was enabled during dispensing of volumes above 100 pl. This feature is used to minimize the localized concentration of DMSO within a well, particularly in cell-based assays, by preventing a bolus of DMSO from forming.

Results

For the HTR-FRET assay, the EC_{50} curves generated using the HP D300 Digital Dispenser were comparable to those set up using traditional manual serial dilution (Figure 2).

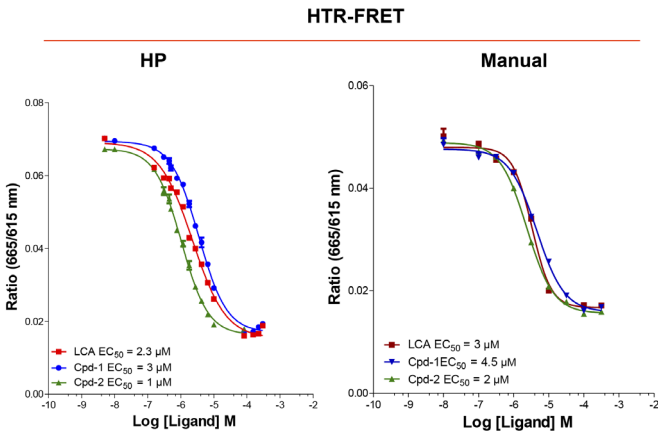

Figure 2 HTR-FRET results for three test compounds; HP D300 Digital Dispenser vs manual serial dilution.

The capability of the HP D300 to dispense more data points along the Hill slope region enabled the creation of a targeted dose-response curve, generating more robust data. This targeted titration would be impractical to set up manually – or even using an automated liquid handler – but can easily be created in the HP D300 software package.

For the AlphaScreen data, the EC_{50} curves for the two compounds tested were also comparable (Figure 3).

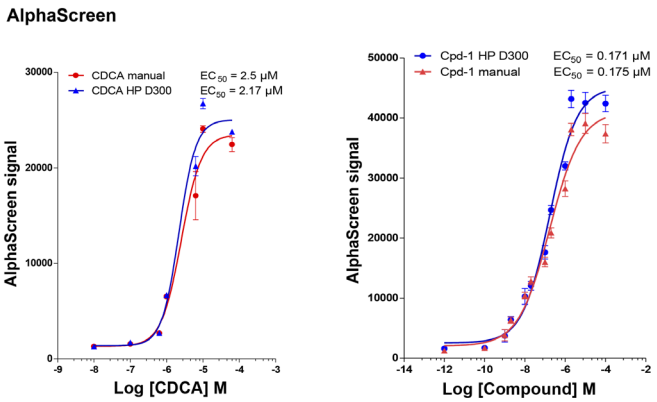

Figure 3 AlphaScreen results for two compounds; HP D300 Digital Dispenser vs manual serial dilution.

The point relative to 100 µM of CDCA in the graph was not displayed because of the so-called 'hooking effect'. This occurs when the signal production is reduced relative to the excess target molecule concentration, all of the binding sites are occupied and the bead association is inhibited.

Preparation of dose-response curves with the HP D300 Digital Dispenser reduced the hands-on time required from approximately two minutes for the manual method to just eight seconds.

Discussion

Dispense any dose in any well

In a typical serial dilution, each dose is equidistant from the others for ease of pipetting, and not necessarily because they are the most scientifically relevant doses. The HP D300 software enables researchers to dispense any dose into any well. In the HTR-FRET assay, this resulted in a custom dose-response curve which could be programmed exactly as desired, allowing more doses to be targeted around the EC_{50} value. This same experimental set-up would be impractical to set up by manual, or even automated, dilution.

Targeted titrations can be defined using the set-up screen (Figure 4), where the user is guided through the creation of a dose-response curve, including a user-defined zone where the doses are densely spaced around the value of interest.

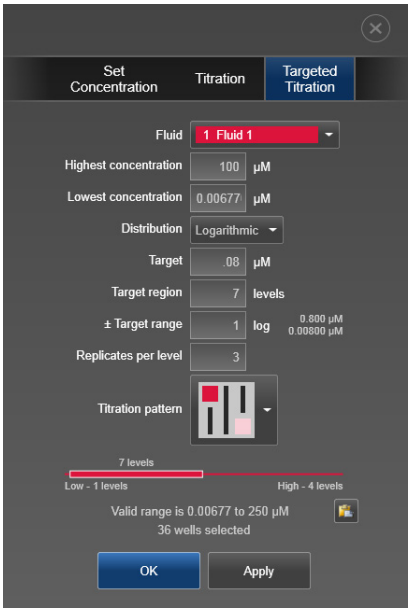

Figure 4 The Targeted Titration window enables users to set up a titration with a specified number of doses around a certain concentration. This allows a high number of doses to be created around the IC_{50}/EC_{50} value.

The software even provides some visual cues to describe the dose-response curve that will be created. Alternatively, the user can define the desired doses in a spreadsheet such as Microsoft Excel®, then copy and paste the values directly into the HP D300 software.

Flexibility for DMSO normalization

High concentrations of DMSO can negatively affect some assays, especially those that are cell-based. Direct dispensing of compounds by the HP D300 allows the scientist to determine on an individual experimental basis whether DMSO should be normalized or not.

Conclusions

The HP D300 Digital Dispenser has enabled the test compounds to be assessed in two different assays – one cell-based and one biochemical. The results are consistent with an expertly performed manual process, but with the benefit of significant time savings, the elimination of serial dilution, higher quality data, and a reduction in compound usage. Moreover, the improved precision offered by the HP D300 enables easier and more rapid generation of data.

References

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Abbreviations

BA	bile acids
CDCA	chenodeoxycholic acid
FXR	farnesoid X receptor
DMEM	Dulbecco`s Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EC_{50}	half maximal effective concentration
FCS	fetal calf serum
GPCR	G protein-coupled receptor
IBMX	1 mM 3-isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitiory concentration
LCA	lithocholic acid
HTR-FRET	homogeneous time-resolved fluorescence resonance energy transfer

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