mosquito® LV: An Assay's Best Friend

introduction

After a successful DNA-encoded library (DEL) screen, it is essential to have access to a high-throughput method of assaying compounds to validate hits. The ability to create up to sixteen serial dilutions in parallel and subsequently plate those dilutions on a nanoscale can vastly speed up the hit finding process, while improving the accuracy and efficiency of the screen. mosquito® LV by SPT Labtech has greatly improved the way we work with our off-DNA compounds synthesized from DELcore and DELflex collaborations. This instrument has strengthened our confidence in assay results with its ability to duplicate our compound-dilution plates with precision. In addition, due to mosquito LV's ability to pipette nanoliter volumes, the ability to miniaturize assays has helped to prolong the use of precious reagents and ultimately create more cost-effective experiments.

mosquito® LV and assay plate setup

mosquito LV instrument is a low-volume positive displacement pipetting solution.

The LV provides a pipetting range of 25 nL-1.2 µL, with an average of less than 3% error throughout the volume range and unrivalled precision with average CVs of 5%. This instrument supports 96-, 384-, and 1536-well plate formats, and has a throughput power of two minutes per 96-well microplate, three minutes per 384-well copy, and five minutes per 4 x 384 plate stamp out. mosquito LV comes either in a 2 or 5 plate position deck, and additional accessories such as an active humidity chamber, spool cover, and plate processor integration (MoPP) can be purchased alongside it.

Figure 1. mosquito LV instrument

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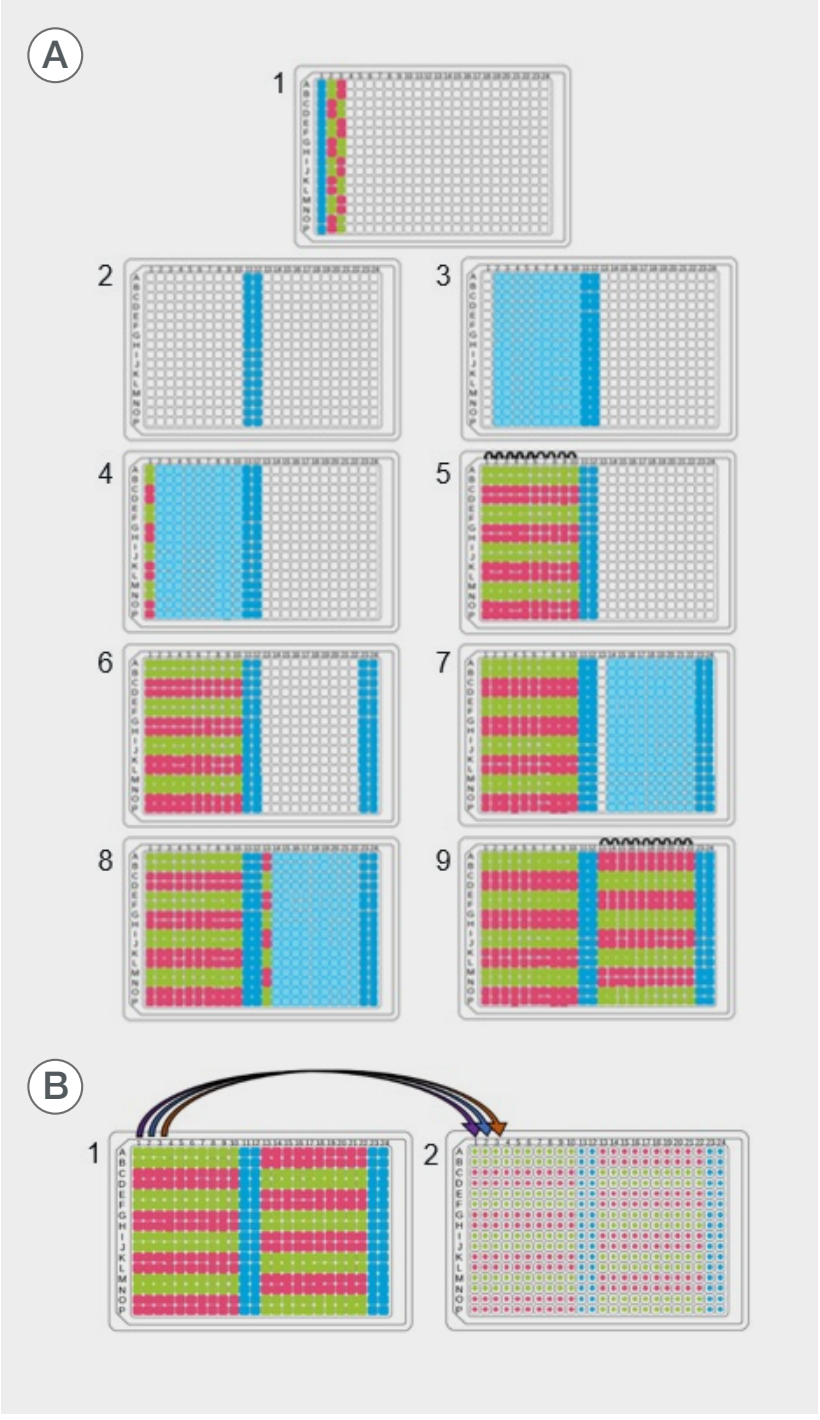
The flexibility of mosquito LV allows for the creation of assay-ready plates in 96-, 384-, and 1536-well formats. X-Chem has found that the 384-well plate format is most suitable for our compound validation workflows.

When creating an assay-ready seral dilution plate in the 384-well format, the initial preparation is to generate the stock plate by pipetting a minimum of 70 µL of DMSO in column 1, and 10 µL of each of your compounds, in vertical duplicate, in columns 2 and 3 (Figure 2A1). The stock plate is spun down for one minute at 10000 RPM, and then loaded onto mosquito LV stage position 1 and an empty plate of the same type is loaded on stage position 2. A predefined protocol is then run. In the first step of the protocol, 2000 nL of DMSO is aspirated from column 1 of the stock plate and 1000 nL each is dispensed into columns 11 and 12 of the destination plate (Figure 2A2). Next, 1000 nL of DMSO is aspirated from column 1 of the stock plate and is dispensed into column 2 of the destination plate; this step is repeated through column 10 (Figure 2A3). Then, 1000 nL is aspirated from the stock plate column 2 and is dispensed into column 1 of the destination plate (Figure 2A4).



Finally, the system completes a 1:3 serial dilution by repeatedly aspirating 500 nL from the previous column into the next column, and mix by pipetting up and down 5 times, i.e., column 1 to column 2, then column 2 to column 3, then column 3 to column 4 and so on (Figure 2A5). Once the first half of the plate is completed, the protocol will repeat this process for columns 13 through 24, but this time pulling compound solution from column 3 of the stock plate (Figure 2A6-2A9).

Once the serial dilution protocol is complete, the destination plate is removed and spun down at 10000 RPM for one minute to remove any air bubbles that may have formed and ensure the solution is at the bottom of the plate. This plate is now loaded back onto the stage in position 1 as the new stock plate. The spotting protocol is loaded and executed. The first step of this protocol is to aspirate 100 nL from column 1 of the new stock plate and dispense into column 1 of the destination plate. The instrument then changes tips and repeats this pattern for the remainder of the columns (Figure 2B). After the spotting protocol is completed, the destination plate should be spun down at 10000 RPM for one minute. The plate is now assay-ready. The spotting protocol can accommodate the creation of up to four assay-ready plates at one time.

Figure 2. Serial Dilution Protocol Steps (A) and Spotting Protocol Steps (B)

case study: HAO1

Since acquiring mosquito LV, this instrument has been used for many high throughput compound validations as well as the development of numerous assays.

One such compound validation was with the protein target hydroxy acid oxidase 1 (HAO1), with the goal of finding an inhibitor of this protein. HAO1 is found in the glyoxylate metabolism pathway and is a key player in primary hyperoxaluria, a disease hallmarked by the buildup of oxalate (1). This causes the repeated growth of kidney stones that eventually leads to the accumulation of oxalate in other organs, progressive kidney deterioration, and eventually end-stage renal disease (1).

Prior to compound validation using the mosquito-enabled workflows, the HAO1 protein and the off-target protein lactate dehydrogenase B (LDHB) were assessed to determine their suitability for DEL selections using quality control techniques such as dynamic light scattering, size exclusion chromatography, and melting temperature. Next, affinity-mediated DNA-encoded library selections were performed using various conditions such as a no-target control, HAO1 at high and low concentrations, off-target, and a competitive condition using a known HAO1 inhibitor in combination with the protein. These selection data were then analyzed, and putative binders were prioritized for off-DNA synthesis and assessment in the in-solution biochemical assay.

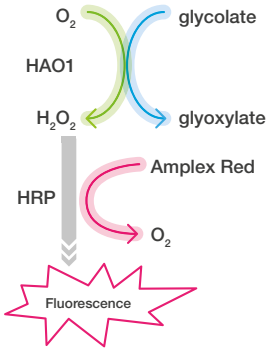


Figure 3. HAO1 HRP Activity Assay Schematic

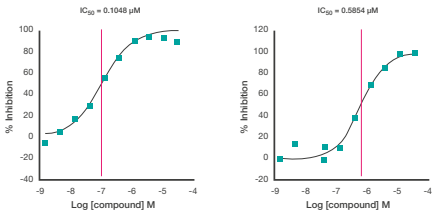
The in-solution, functional assay used to assess these off-DNA compounds was a fluorescence-based assay that determines the inhibition of HAO1 activity through the detection of the product, hydrogen peroxide, via a coupled horseradish peroxidase (HRP) reaction (Figure 3). Compounds that showed inhibition of HAO1 in the HRP activity assay were then tested in an HRP interference assay, which is similar to the previously described format, but lacking the HAO1 protein, to confirm that the inhibitory nature of the compounds was specific to HAO1 and not an artifact.

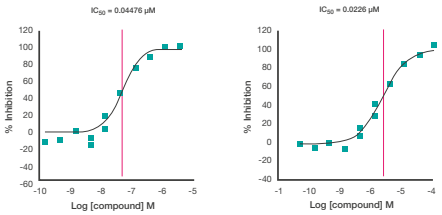
mosquito LV was utilized to prepare the compounds using the serial dilution protocol and the spotting protocol described previously. The most potent compounds identified were compounds 3, 4, 5, and 6 (1). Compound 3 from Series 1 gave an IC_{50} of 110 nM (Figure 4A). From Series 2, compound 4 was slightly less potent, showing an IC_{50} of 510 nM, whereas compounds 5 and 6 were more potent with IC_{50}s of 37 nM and 21 nM, respectively (Figure 4A). The use of mosquito for this assay was crucial to producing accurate and reliable results. As seen in figure 4B, the duplicate data points at each dilution step show nearly equal levels of inhibition with minimal error. This is seen across all replicates for each compound. These results show the consistency of the positive displacement technology.

A

Compound	HAO1 IC_{50} (nM)	Standard Deviation	Replicates (n)
3	107	0.044	>150
4	561	0.024	>1
5	48	0.015	>100
6	21	0.006	>30

B





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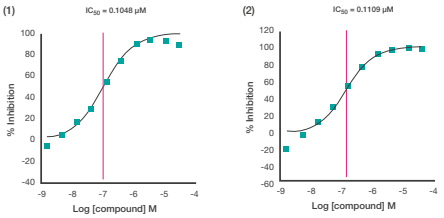

Figure 4. Assay results of the most potent first pass HAO1 compounds (A). Replicate assay results of compound 3 (B). Assay results from July (1) and March (2) of compound 3 (C).

In addition to the standard replicates that are performed during compound testing, the most potent compounds were subsequently used as controls to monitor the quality and reproducibility of the assay in later rounds of testing. In Figure 4C, there is no significant difference in IC_{50}s of assay 1 and 2. Assay 1 was performed in July of 2019 when the average outdoor temperature was 78° Fahrenheit, and the average dew point was 63.07 (2). Assay 2 was performed in March of 2020 when the average outdoor temperature was 42° Fahrenheit, and the average dew point was 27.08 (3). This shows the reliability and reproducibility of mosquito LV across time, temperature, and humidity.

Overall, this project involved the execution of over 300 HAO1 HRP activity assays. Using typical compound dilution techniques, this assay process could have taken over a year to complete. However, the utilization of mosquito LV allowed for the miniaturization of the assay, as well as the efficient creation of assay-ready plates that allowed our scientists to complete the assessment of 330 project compounds in just over nine months.

conclusion

This case study shows the advantages of using mosquito LV to prepare assay plates for high throughput screening activities, including the cost effectiveness of miniaturizing assays in order to reduce the required amounts of precious reagents. mosquito LV's high-throughput capabilities enable testing of numerous hit compounds furnished from a productive DNA-encoded library screen at X-Chem. This story also demonstrates the efficiency, reliability, and high-fidelity nature of mosquito LV across time and varying weather conditions.

mosquito LV may be summarized as:

- Reproducible precision pipetting at low volumes
- Under 5% volume error
- Consistency of pipetting across various weather conditions including temperature and humidity
- Accommodation of up to 5 plates, with the ability to work with 96-well, 384-well, and 1536-well plates
- Easy to navigate software that allows for simple protocol building and executing

About X-Chem

With over 100 libraries comprising more than 250 billion DNA-tagged compounds, we wield a vast index of unique small molecules. This reach enables you to investigate a massively diverse range of chemistry that holds enormous potential. At a success rate of 80% in identifying functional hits, we deliver for our partners and clients. To date we have licensed over 100 discovery programs, comprising over 300 independent chemical series and more than 1000 validated hit compounds.

X-Chem's versatile technology is proven in projects spanning 30 target classes. These include challenging categories such as protein-protein interactions (PPI), bacterial targets, ubiquitin ligases, epigenetic targets, and G protein-coupled receptors (GPCR). Whether you are an investigator looking for quality hit against a novel target or a DEL practitioner, we will help you leverage our platform, technology, and expertise in the best way possible. Find more hits, faster, then transition from hit to candidate with our revolutionary combination of AI, DEL, and medicinal chemistry.

For more information, please visit **www.x-chemrx.com** or contact us at **info@x-chemrx.com**

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