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Compound Inhibitor Profiling using the I.DOT

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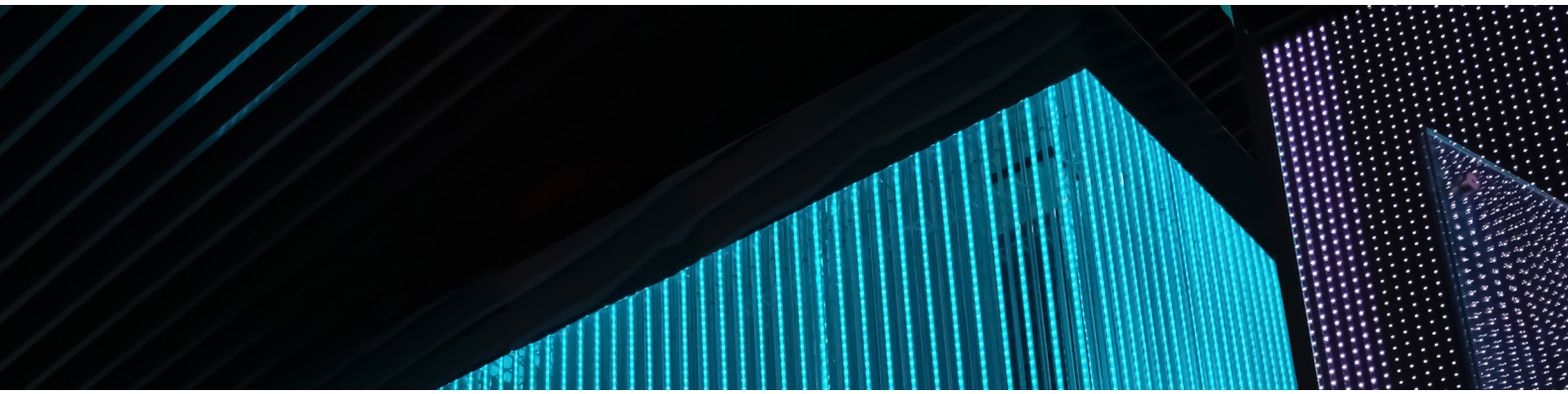


Abstract

In this study the I.DOT, a low-volume, noncontact liquid handling device was used for the performance of the ADP-Glo™ Kinase Assay, a commonly used assay for compound inhibitor profiling. The ADP-Glo™ Kinase Assay measures ADP formed from a kinase reaction, where a reactive phosphate group from ATP is transferred onto a target protein, thereby resulting in a remaining ADP molecule. This ADP is then converted into ATP, which is used to generate light in a luciferase reaction allowing quantification of the amount of phosphate groups transferred by the kinase reaction. Therefore, the assay is well suited for measuring the effects of chemical compounds on the activity of many purified kinases, making it ideal for primary screening as well as kinase selectivity profiling.

In these experiments we used the I.DOT to dispense different reagent addition steps or the whole assay. Two different enzymes and fourteen different compounds were used. The results were then compared to the workflow using another common low volume liquid handler and to hand pipetting. The experiments showed that the I.DOT is dispensing the ADP-Glo™ Kinase Assay with comparable results much faster and with less dead volume compared to the other liquid handler. Furthermore, using the I.DOT reduces the number of laboratory devices used for the assay, as well as reducing consumption of consumables and reagents. This ultimately leads to major cost savings.

Introduction

Kinases belong to the enzymatic family of phosphotransferases, which is one of the largest enzyme families in the cell. They transduce cellular signals by phosphorylating a variety of substrates such as proteins, lipids, or sugars (Hunter, 2000; Manning et al., 2002). Due to their crucial functionality, changes in normal kinase activity can disrupt these signaling pathways. This can lead to the formation of diseases such as cancer, inflammation, and diabetes. Therefore, kinases are among the most prevalent targets of drug discovery research (Cohen, 2002). To develop such drugs, it is necessary to identify selective and potent enzyme inhibitors that have low toxicity and no detrimental effects on other enzymes (Zegzouti et al., 2009). As a result of the large number of kinases with sequence similarities in their catalytic domains, the identification of selective drugs is challenging. Off-target kinase inhibition can be a significant source of side effects including undesirable toxicities (Castoldi et al., 2007; Widakowich et al., 2007). Novel drug candidates should therefore be profiled against various liability targets, such as a wide range of kinases. One such technology for the profiling is the luminescent ADP-Glo™ kinase Assay, which measures kinase activity by quantifying the amount of ADP produced during enzymatic reactions (Zegzouti et al., 2014). The assay is performed in two steps: first, after the kinase reaction, ADP-Glo™ Reagent is added to stop the kinase reaction and deplete the remaining ATP, which was not a substrate during the reaction.

Second, the Kinase Detection Reagent converts the produced ADP to ATP, permitting the newly synthesized ATP to be measured with a luciferase/luciferin reaction. Using an ATP-to-ADP conversion curve, the luminescence can be correlated to ADP concentrations (***Figure 1***). Validated with hundreds of kinases, this assay demonstrates homogeneity, high throughput applicability and robustness. It can cover a broad range of substrate and ATP levels, which makes it an ideal assay for kinase profiling (Davis et al., 2013; Li et al., 2009; Tanega et al., 2009; Zegzouti et al., 2016). With this assay, the compounds which produce positive results can be identified as as "hits", and are distinguished from the effectless compounds. These molecules then go into further testing, refinement and then finally entering clinical testing. Therefore, compound inhibitor profiling is very frequently at the beginning of the extensive development process and crucial to generate a successful drug in the end.

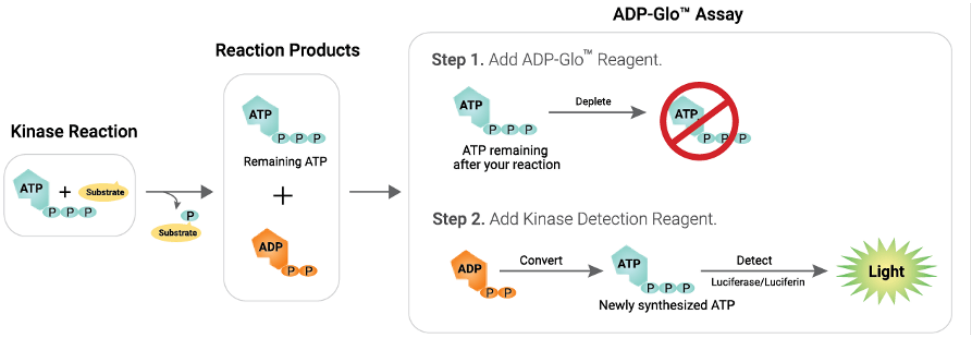


Figure 1. *The assay is performed in two steps; first, after the kinase reaction, an equal volume of ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the remaining ATP. Second, the Kinase Detection Reagent is added to simultaneously convert ADP to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The generated light is measured using a luminometer. Luminescence can be correlated to ADP concentrations by using an ATP-to-ADP conversion curve.*

Materials and methods

Experiment 1: IDOT vs. Echo vs. hand-pipetted plates (compound dilution part)

Material & Methods

- S.100 plates (Dispendix)
- White ProxiPlate™ -384 Plus (PerkinElmer)
- Clear 96-well PP microplate (Greiner)
- ADP-Glo™ Kinase Assay (Promega)
- BMG Labtech microplate reader (Pherastar® FSX)
- Tempest® liquid handling device (Formulatrix)
- Echo® liquid handling device (Beckmann Coulter)
- Pipette tips and other plastic material from various manufacturers

The I.DOT was tested for its ability to support the assay development process, for example by preparing a dilution series of small molecules and adding these dilution series to 384-well plates. Therefore, a hand-pipetted dilution series of a set of small molecules was compared to a dilution series, which was prepared by an Echo® liquid handler or the I. DOT liquid handler.

The aim was to prepare a 12-point half-log unit concentration curve of a set of 14 small molecules in DMSO, starting at the highest concentration of 30 µM, followed up by the transfer of 40 nl of each of these dilutions into a white ProxiPlate™ -384 Plus plate from PerkinElmer in quadruplicates. The dilution series was prepared by hand and by the I.DOT liquid handler in parallel, starting with a 10mM DMSO stock solution of the small molecule solution. In addition, an Echo® liquid handler prepared ready-to-use assay plates containing a dilution series of the 14 small molecules.

For the small molecule dilution prepared by hand, the 10mM stock solution of the small molecules was first prediluted in DMSO, followed by the preparation of a 1:3 dilution series in a 96-well plate and a final 1:12.5 dilution of the whole dilution series in assay buffer. Thereafter, quadruplicates of the dilution series were transferred into white ProxiPlate™ -384 Plus plates from PerkinElmer. All dilution and transfer steps were performed with a multichannel pipette.

For the small molecule dilution prepared by the I.DOT, the 10mM stock solution of the small molecules was first prediluted 1:10 in DMSO. Afterwards, an I.DOT S.100 source plate was filled with the 10mM stock solutions, the 1:10 pre-dilutions, the reference compound, as well as the high and low control substances. An empty S.100 plate was placed as target plate in the I.DOT.

The I.DOT prepared in a first run a 30, 3, 0.3, 0.03 and 0.003 µM solution of the small molecules in the S.100 plate that was used as target plate. In a subsequent step, this target plate was used as source plate to prepare the final dilution series including all controls into a white ProxiPlate™ -384 Plus plate from PerkinElmer.

Afterwards, the hand-made, Echo- and I.DOT-plates were used to perform an ADP-Glo™ Kinase Assay from Promega. Therefore, 2µl enzyme, 2µl substrate, 2µl ADP-Glo reagent and 4µl kinase detection reagent were added to the plates with the help of a Tempest® liquid handling device. All reaction and incubation steps were performed as described in the protocol provided by Promega. After the final 45-minute incubation step the microplates were measured in the Pherastar® FSX and the IC50 of the small molecule kinase inhibitors was calculated from the dose-response curves.

Experiment 2: IDOT vs. Echo vs. hand-pipetted plates (assay part)

Material & Methods

- S.100 plates (Dispendix)
- White ProxiPlate™ -384 Plus (PerkinElmer)
- ADP-Glo™ Kinase Assay (Promega)
- BMG Labtech microplate reader (Pherastar® FSX)
- Tempest® liquid handling device (Formulatrix)
- Echo® liquid handling device (Beckmann Coulter)
- Pipette tips and other plastic material from various manufacturers

The I.DOT was tested for its ability to support the assay development process, for example by adding assay reagents into a 384-well plate. In this experiment, the addition of assay reagents to a 384-well plate, containing a set of small molecules, was performed with the I.DOT, a multichannel-pipette and a Tempest® pipetting station in parallel.

Prior to the assay, three ready-to-use assay plates, containing a 12 point 1:3 dilution series starting at 30µM of a set of 14 possible small molecule kinase inhibitors in DMSO, were prepared by an Echo® liquid handling device. After the addition of 2 µl enzyme solution, the microplate was pre-incubated at room temperature for 15 minutes after which 2 µl of substrate were added. During the following incubation time of 45 minutes, all ADP-Glo™ kinase assay reagents were prepared, and the assay run according to the protocol provided by Promega. The addition of the enzyme, substrate, and assay reagents was done by the I.DOT, a multichannel-pipette, and a Tempest® pipetting station in parallel. After the final 45-minute incubation step the microplates were measured in the Pherastar® FSX and the IC50 of the small molecule kinase inhibitors was calculated from the dose-response curves.

Experiment 3: IDOT vs. hand-pipetted plates (complete assay)

Material & Methods

- S.100 plates (Dispendix)
- White ProxiPlate™ -384 Plus (PerkinElmer)
- Clear 96-well PP microplate (Greiner)
- ADP-Glo™ Kinase Assay (Promega)
- BMG Labtech microplate reader (Pherastar® FSX)
- Echo® liquid handling device (Beckmann Coulter)
- Pipette tips and other plastic material from various manufacturers

The aim was to prepare a 12 point 1:3 dose response series of a set of 14 small molecules in DMSO, starting at the highest concentration of 30µM, followed up by the transfer of 40nl of each of these dilutions into a white ProxiPlate™ -384 Plus plate from PerkinElmer in quadruplicates to enable the conduction of an ADP-Glo™ Kinase Assay from Promega afterwards. The dilution series was prepared by hand and by the I.DOT liquid handler in parallel, starting with a 10mM DMSO stock solution of the small molecule.

For the small molecule dilution prepared by hand, the 10mM stock solution of the compounds was first prediluted in DMSO, followed by the preparation of a 1:3 dilution series in a 96-well plate and a final 1:12.5 dilution of the whole dilution series in assay buffer. Thereafter quadruplicates of the dilution series were transferred into a white ProxiPlate™ -384 Plus plate from PerkinElmer. All dilution and transfer steps were performed with a multichannel pipette.

For the small molecule dilution prepared by the I.DOT, the 10 mM stock solution of the compounds was first prediluted 1:10 in DMSO. Afterwards, an I.DOT S.100 source plate was filled with the 10 mM stock solutions, the 1:10 pre-dilutions as well as the reference compound and the high and low control substances. An empty S.100 plate was placed as target plate in the I.DOT. The I.DOT prepared in a first run a 30, 3, 0.3, 0.03 and 0.003 µM solution of the small molecules in the S.100 target plate.

In the next step, this target plate was used as source plate to prepare the final dilution series including all controls into a white ProxiPlate™ -384 Plus plate from PerkinElmer.

The hand-pipetted plates and I.DOT-dispensed plates were used to perform an ADP-Glo™ Kinase Assay from Promega. Therefore, 2µl enzyme, 2µl substrate, 2µl ADP-Glo reagent and 4µl kinase detection reagent were added to the plates either with a multichannel pipette or the I.DOT liquid handler. All reaction and incubation steps were performed as described in the protocol provided by Promega. After the final 45-minute incubation step the microplates were measured in the Pherastar® FSX and the IC50 of the small molecule kinase inhibitors was calculated from the dose-response curves.

Results and discussion

The Compound Inhibitor Profiling assay is divided in three steps. First, the dilution of the compounds for the final assay concentration, then the addition of the assay components and lastly the ADP-Glo™ Kinase assay performance and readout (see ***Figure 2***). The first two steps, the dilution and the assay preparation, can be performed by the I.DOT. In this experiment we compared the results of the assay performed with the I.DOT in contrast to the workflow performed by the Echo, the Tempest and hand pipetting. Pre-tests with Fluorescein approved well-to-well reproducibility.

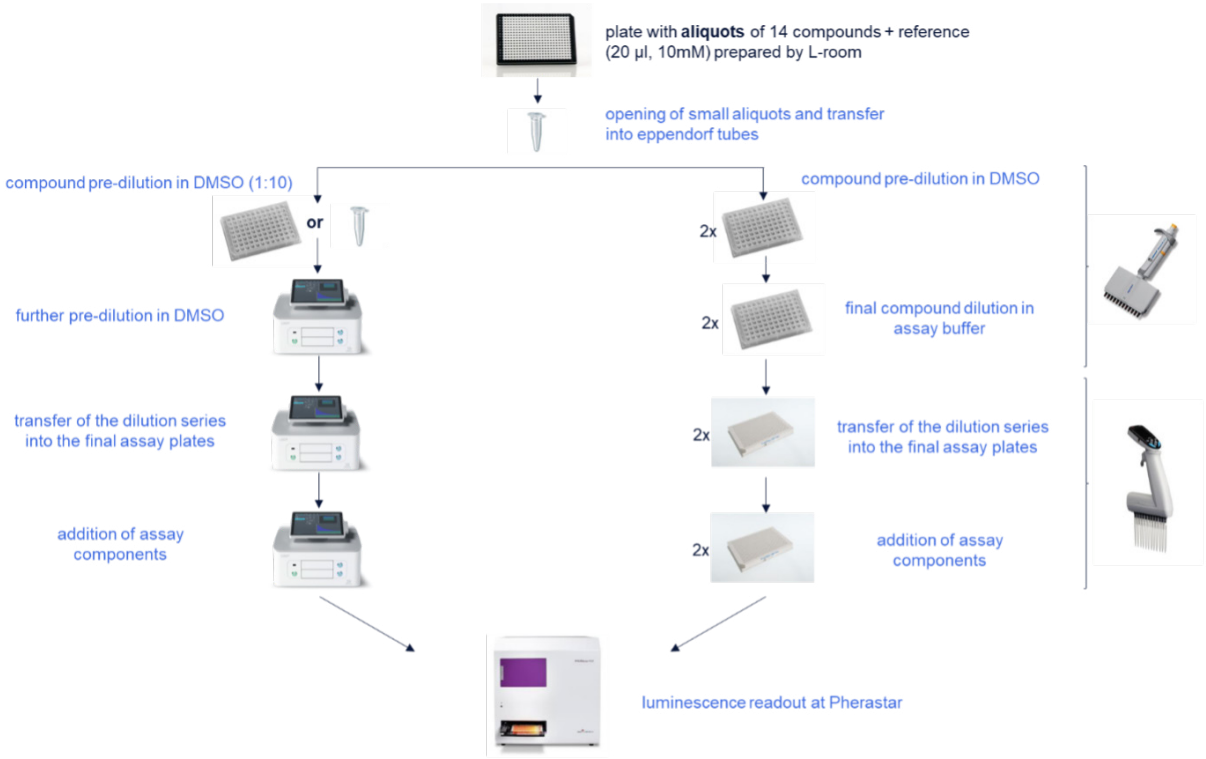


Figure 2. *Workflow steps of the ADP-Glo™ Kinase Assay. The left side shows the assay performing it with the I.DOT. The right side shows the workflow with hand pipetting.*

Comparison of the compound dilution done by the Echo, I.DOT and by hand

In this experiment the dilution of the compounds was done with the I.DOT, with the Echo and by hand pipetting. Afterwards the ADP-Glo™ kinase assay was prepared using the Tempest liquid handler (see ***Figure 3***).

The I.DOT allows fast and fully automated reagent dilution in the sub-microliter range. As displayed in ***Figure 3***, the speed of the I.DOT results in a much faster dispensing time of 14 min, compared to 60 min with the hand pipette, leading to a 4 x faster assay turnaround time.

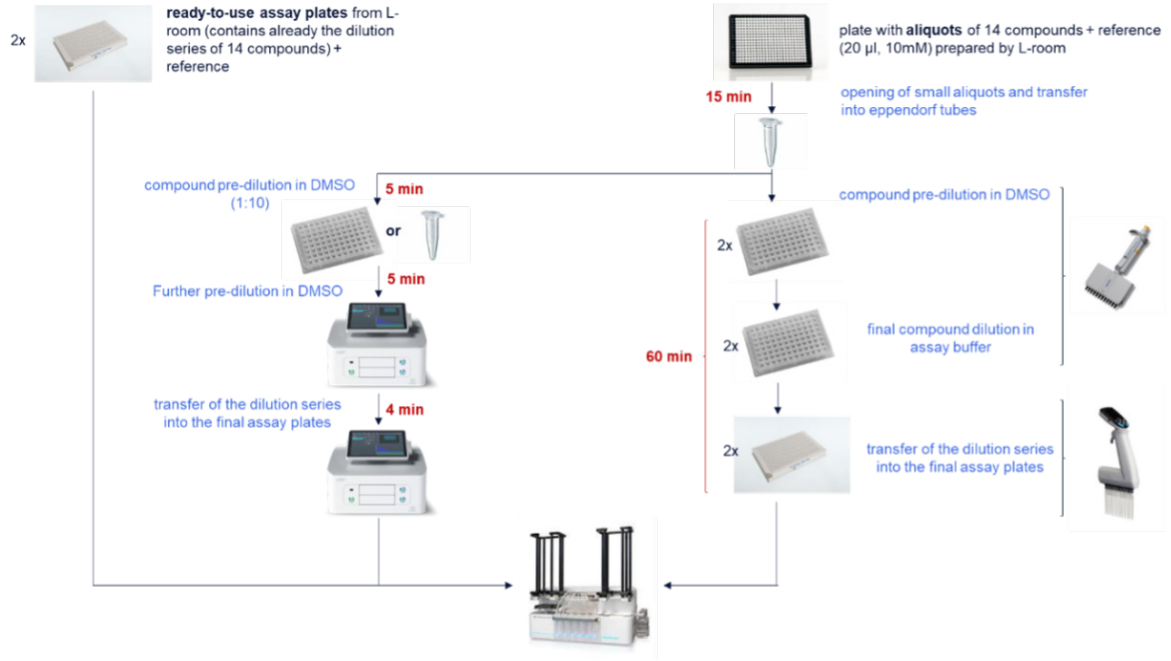
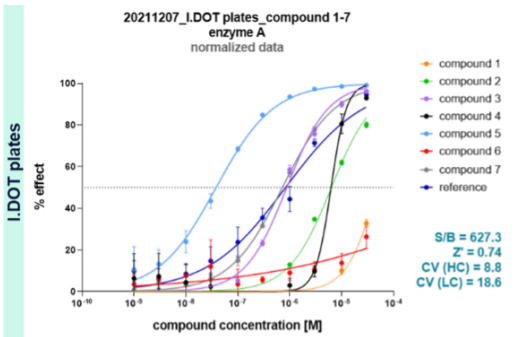
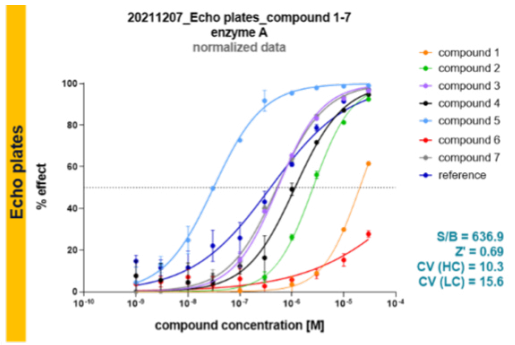


Figure 3. *Setup of the first experiment. The three dilution workflows are shown, once with the Echo [left, not shown], once with the I.DOT [middle] and once with hand pipettes [right].*

The results from the microplate reader show comparable results between the IC 50 determination curves of the compound dilutions made by I.DOT, the Echo and the hand pipetted plates for nearly all compounds (see ***Figure 4***). The outlier at compound 4 (red square) is probably due to a pipetting error or an incompatibility of this compound with the I.DOT.

The Z-factor is a parameter to assess the usefulness of an assay in high throughput environments. This is defined by the mean and standard deviation of the positive and negative controls. The closer the Z factor is to 1, the more robust and reliable the assay. Whereas a Z-factor between 0,5 and 1 describes a very good assay. We see a very good Z-factor of 0,74 in the ADP-Glo™ Kinase Assay performed with the I.DOT, which is comparable with the hand pipetted plates and the assay performed with the Echo.





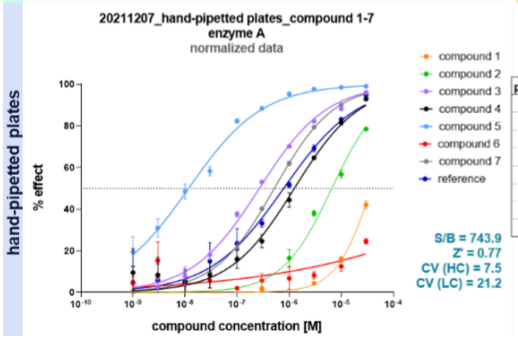


plate set	sample	historical data [µM]	Echo plates IC50 [µM]	I.DOT plates IC50 [µM]	hand-pipetted plates IC50 [µM]
1	compound 1	>30.60	20.31	>30.60	>30.60
1	compound 2	4.2	2.562	6.343	6.595
1	compound 3	0.52800	0.5386	0.8784	0.2678
1	compound 4	1.5000	1.153	6.241	1.259
1	compound 5	0.03460	0.03103	0.03831	0.01169
1	compound 6	>30.60	>30.60	>30.60	>30.60
1	compound 7	0.58300	0.5170	0.7220	0.5196
1	reference	1.16000	0.3876	0.8310	0.7841

Figure 4. *Effect curves and values of the ADP-Glo™ Kinase Assay with Compound 1-7 and enzyme 'A'. The dilution-step was prepared in 1: with the I.DOT, in 2: with the Echo and in 3: with multi-channel pipettes.*

Comparison of the ADP-Glo™ kinase Assay dispensed by the Tempest, I.DOT and by hand

In the following experiment the dilution was done as part of the established workflow using the Echo liquid handler, while the second part of the assay, the addition of the assay components was performed with the I.DOT, with the Tempest and by hand.

As in the previous experiment, the results of the I.DOT dispensed plate are comparable to the assay prepared with the Tempest and by hand (see ***Figure 5***). Again, we assessed the Z-factor, which is with the value of 0,76 comparable to the assay prepared by the Tempest and by hand.

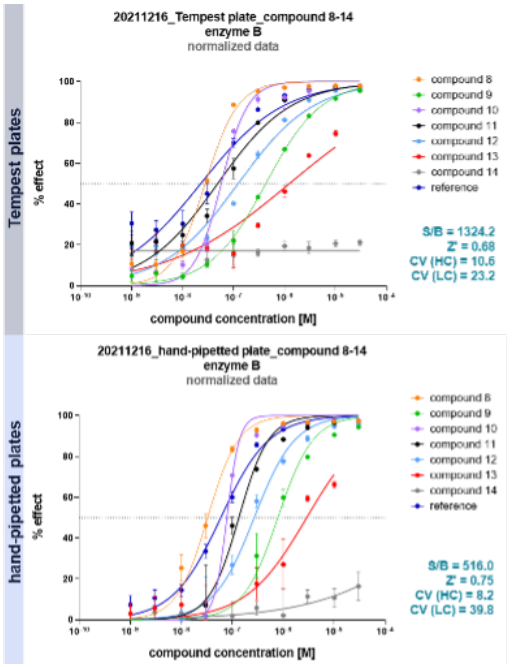


plate set	sample	historical data [µM]	Tempest plates IC50 [µM]	I.DOT plates IC50 [µM]	hand-pipetted plates IC50 [µM]
2	compound 8	0.04374	0.02784	0.04147	0.03283
2	compound 9	0.72416	0.4288	0.7328	0.7844
2	compound 10	0.06777	0.05739	0.06135	0.07850
2	compound 11	0.11553	0.04844	0.1045	0.1345
2	compound 12	0.27885	0.1229	0.2393	0.2694
2	compound 13	0.98711	1.242	2.274	2.884
2	compound 14	>30.60	>30.60	>30.60	>30.60
2	reference	0.06850	0.02259	0.07014	0.05945

Figure 5. *Effect curves and values of the ADP-Glo™ Kinase Assay with Compound 8-14 and the enzyme 'B'. The assay components were prepared 1: with the Tempest, 2: with the I.DOT and 3: with hand pipettes. The rest of the assay was performed with the original workflow.*

As you can see in ***Figure 6***, carrying out the whole experimental workflow from compound dilution to reagent addition of the Promega reaction kit, the I.DOT leads to comparable results to using the tempest or pipetting by hand, but in less time and with significantly lower dead volume. Regarding the time, the I.DOT is comparable to hand pipetting, but is twice as fast as the using the Tempest for dispensing the assay and further, this with just 1/6 of the dead volume compared to hand pipetting or using the Tempest.

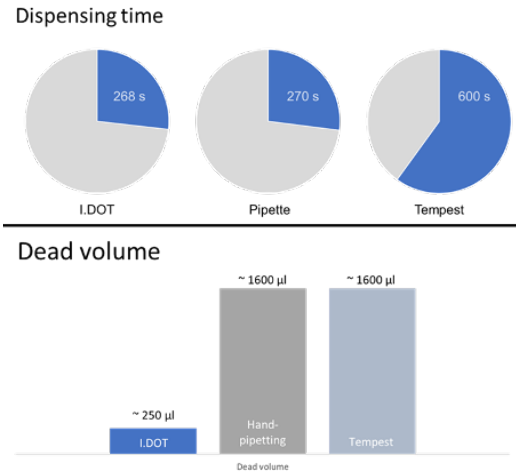


Figure 6. *Comparison of the dispensing time needed and the remaining dead volume when dispensing the assay components with the I.DOT, the hand pipettes and the Tempest.*

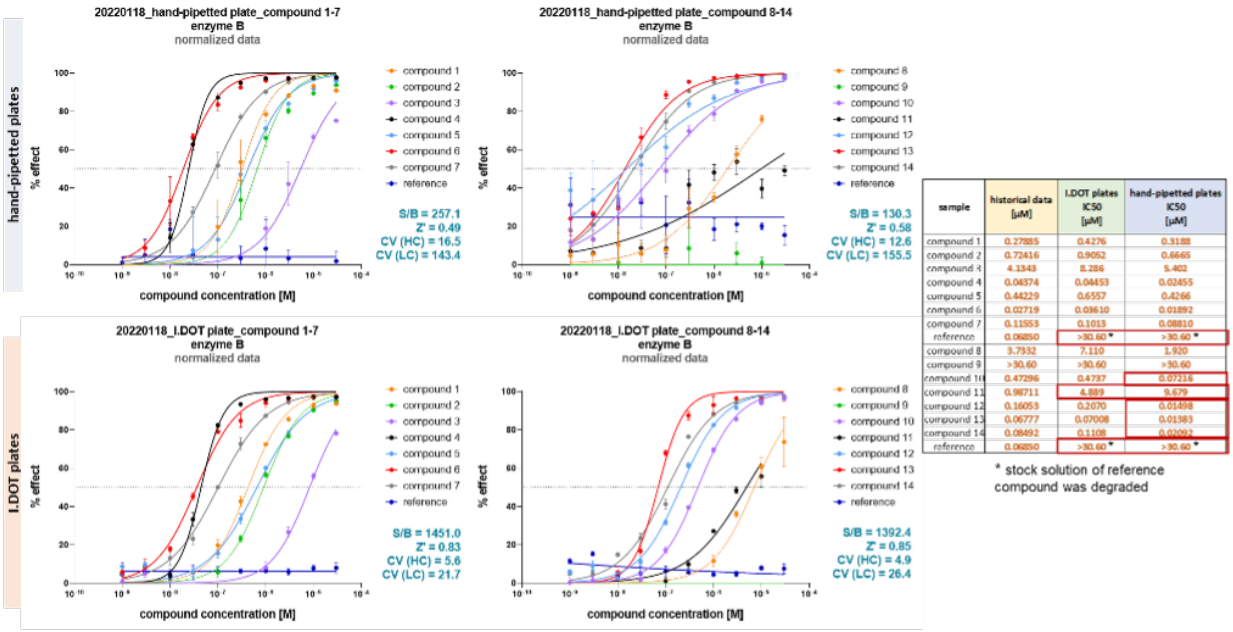
Comparison of the whole assay dispensed by the I.DOT and by hand

In the last experiment, all three steps of the assay were performed using the I.DOT.

As shown in ***Figure 7***, the results of the I.DOT prepared compound dilution plates are comparable to the plates prepared by hand pipetting.

The outliers for some of the compounds are probably due to degraded stock solutions.

The Z-factor for the I.DOT plates show a very good value of ~ 0,84, which is much better compared to the hand-pipetted plates.



sample	historical data [µM]	I.DOT plates IC50 [µM]	hand-pipetted plates IC50 [µM]
compound 1	0.27885	0.4276	0.3188
compound 2	0.72416	0.9052	0.6665
compound 3	4.1343	8.286	5.402
compound 4	0.04374	0.04453	0.02455
compound 5	0.44229	0.6557	0.4266
compound 6	0.02719	0.03610	0.01892
compound 7	0.11553	0.1013	0.08810
reference	0.06250	>30.60 *	>30.60 *
compound 8	5.7532	7.110	1.920
compound 9	>30.60	>30.60	>30.60
compound 10	0.47296	0.4737	0.07216
compound 11	0.98711	4.889	9.679
compound 12	0.16055	0.2070	0.01498
compound 13	0.06777	0.07008	0.01383
compound 14	0.08492	0.1108	0.02092
reference	0.06250	>30.60 *	>30.60 *

* stock solution of reference compound was degraded

Figure 7. *Effect curves and values of the ADP-Glo™ Kinase Assay with Compound 1-14 and enzyme 'B'. The whole workflow was dispensed in 1: with the I.DOT and in 2: with hand pipettes.*

Conclusions

This study provides many insights in the I.DOT's capability of dispensing different steps of the compound inhibitor profiling. In conclusion our data show that:

- The I.DOT is performing low volume dispensing with good reproducibility.
- The I.DOT is dispensing the ADP-Glo™ Kinase Assay much faster and with less dead volume compared to using manual pipetting or the Tempest liquid handler.
- Using the I.DOT for compound screening reduces the consumption of consumables and reagents what leads to high cost savings
- All steps of the assay can be successfully dispensed with the I.DOT which makes other devices redundant

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