High Throughput Secondary Screening—1400 Dose-Response Curves per Hour

Joe Olechno, Jean Shieh, Fereshteh Lesani and Harry Vlahos. Labcyte Inc.

ABSTRACT
We have developed a plate preparation workstation (the POD™ 810 plate assembler) that is controlled by an intellectual scheduler to increase the productivity of secondary screening labs. Dose-response analyses, such as IC_{50} determinations, are among the most important of the secondary screening assays and also among the most time-consuming and difficult. They are prone to poor precision due to serial dilutions, and erroneous results due to absorption of compounds. This workstation coordinates multiple assay plate preparation tools to optimize the throughput of any Echo® acoustic liquid handler. The intellectual scheduler plans multiple tasks simultaneously, according to the availability and need of each device in the workstation. We demonstrate that this plate assembler can set up assay plates for as many as 1400 12-point, six-log, dose-response curves each hour. The throughput and quality of results inherent with the POD plate assembler suggests using a larger number of compounds against a wider range of targets. This can increase the value of the libraries being screened and lead to faster identification of new drug candidates.

Determining Throughput
Throughput, that is the number of multi-point dilution series (**curves**) that can be set up automatically in a specific time period using the POD 810 plate assembler, is specific to the process used. Throughput numbers can change dramatically depending upon:

- the number of points in the curve,
- the concentration range of the assay
- the number of standards or blanks in each curve,
- the number of standard curves in each assay plate
- the number of replicates
- whether replicates are run in the same or different plates
- the density of the source plate
- the density of the assay plate
- the density of an intermediate plate, if used,
- the hit rate when cherry-picking samples directly from library source plates
- whether the DMSO backfill is done with the Echo system or the POD bulk-filler
- OTHERS

Because the exact procedure has such an impact on throughput, we used the POD 810 in a variety of ways that mirror techniques currently used in the pharmaceutical industry. In each case, we developed curves with concentrations at approximately half-log intervals (1, 3, 10, 30, 100, etc.).

Experimental Set-Up
Instrumentation
All experiments were run on a POD (Plates On Demand) 810 plate assembler. The POD was equipped with an Echo 555 acoustic liquid handler, an automated bulk filler, and a plate centrifuge.
The bulk filler added DMSO to each well so that the final volume of DMSO was 75 nL (0, 50, 67.5 and 72.5 nL to the wells with 75, 25, 7.5 and 2.5 nL of sample, respectively).
All intermediate plates were centrifuged for 30 seconds at 2250 RPM before being used as source plates. This ensured a flat meniscus and optimal transfer precision and accuracy.

Microplates and transfer fluids
All 384-well microplates were Echo qualified, polypropylene sources plates (Labcyte catalog number P-05525).
All 1536-well plates were high-base, Echo qualified, COC (cyclic olefin copolymer) sources plates (Labcyte catalog number LP-03730).
All transferred samples were 85% DMSO (dimethylsulfoxide, Sigma, p/n 472301)

Scenario 1
This procedure uses a 1536-well, cyclic olefin copolymer (COC) source plate that is made off-line from the POD plate assembler. The source plate holds three concentrations of each compound submitted for the dose-response experiments. The three concentrations are in ratios of 1:0.01:0.0001 (1:1/100:1/10,000), for example, 10 mM, 100 μM and 1 μM. The final concentrations of compound in the assay will depend upon the final assay volume but for a 5 μL assay, the high concentration is 150 μM and the lowest concentration (not counting the blank DMSO) is 0.15 nM.
The process used to establish the gradients is illustrated in Figure 2.

Scenario 2
This procedure was identical to that used in scenario 1 expect that both the source and the assay plates were 384-well polypropylene plates.

Figure 1 shows a 384-well plate holding 96 compounds at three concentrations each. The fourth component of each tetrad is a blank of DMSO.

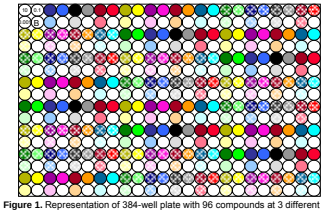


Figure 1. Representation of 384-well plate with 96 compounds at 3 different concentrations each, 10 mM, 0.1 mM and 0.001 mM.

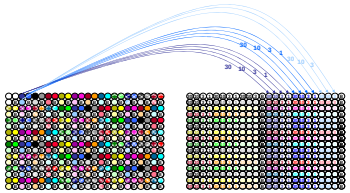


Figure 2. Aliquots of 30, 10, 3 and one droplets (75, 25, 7.5 and 2.5 nL) of each dilution of each sample are transferred from the source plate to the assay plate. The twelfth spot (B) is a blank. This covers almost 6 logs of concentration at half-log intervals. Each assay plate has a the concentrations gradients to develop dose-response curves for 32 different compounds. Each source plate completely fills four assay plates.

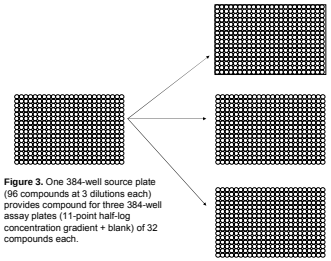


Figure 3. One 384-well source plate (96 compounds at 3 dilutions each) provides compound for three 384-well assay plates (11-point half-log concentration gradient + blank) of 32 compounds each.

Figure 4. The POD 810 plate assembler is designed to optimize the throughput of an Echo liquid handler. The software built into the POD ensures that the Echo system is optimally scheduled so that the highest possible number of plates can be prepared in the shortest time. The POD is designed to optimize workflow for plate replication, plate reformatting, cherry-picking samples, and the set-up of dose-response assays.

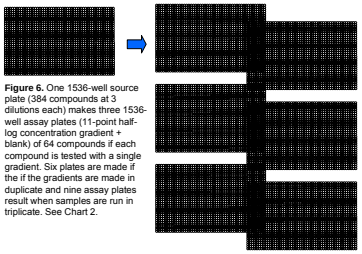


Figure 6. One 1536-well source plate (384 compounds at 3 dilutions each) makes three 1536-well assay plates (11-point half-log concentration gradient + blank) of 64 compounds if each compound is tested with a single gradient. Six plates are made if the if the gradients are made in duplicate and nine assay plates result when samples are run in triplicate. See Chart 2.

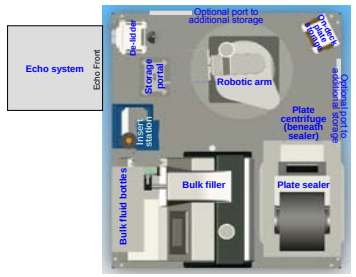


Figure 5. The footprint of the POD is designed to save lab space while providing everything need to transfer samples from source plates to plates for workflow. A multi-purpose POD 810, as used in these experiments, has:

- a robotic arm to move plates,
- a four-fluid, low-volume bulk dispenser for reagents or DMSO backfills,
- a plate heat sealer,
- a plate centrifuge,
- temporary on-deck plate storage
- a vacuum de-lidder
- an insert station (to allow different types of source plates to be used during a single Echo run)
- a port from ambient storage beneath the deck (capable of storing between 135 and 189 plates in a total of nine racks)
- two optional portals to additional external storage

Scenario 3
This procedure automatically makes two intermediate plates for each starting source (library) plate of 320 compounds. Compound from each well of the original source plate is placed into the intermediate plates at two different amounts – 250 nL and 25 nL. Each well of the intermediate plate is diluted with 25 μL of DMSO with the bulk filler to provide solutions that are 1/100 and 1/10,000 that of the original source.
As with Scenarios 1 and 2, 75, 25, 7.5 and 2.5 nL of each compound in the library source plate are transferred to the appropriate wells in the assay plate. When transfers of the high concentrations from the library plate are complete, the library plate is removed from the Echo system and replaced with the first of the intermediate plates. From each of the diluted samples, compounds are transferred to the appropriate wells of the assay plate.
Scenario 3 can be considered a special case of Scenarios 4 and 5 where the hit rate is 100%.

Scenarios 4 and 5
Many labs would like to cherry-pick samples directly from their library source plates into dose-response assays. The POD 810 plate assembler provides that freedom without the extra work of setting up special source plates as in Scenarios 1, 2 and 3.

The results of these five scenarios are described in Table 1 and the throughput of the different experiments is graphically illustrated in Graph 1.

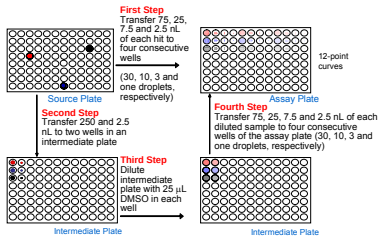


Figure 7. Scenarios 4 and 5 require the least effort from the researcher. The compounds are cherry-picked from the original source plates to four locations in the assay plate and to two locations in the intermediate plate. This is continued for the remaining compounds in the library plate. The next library source plate is loaded and the steps are repeated until the intermediate plate is loaded with two amounts of each sample being analyzed in the dose-response experiment. The intermediate plate is then filled with 25 μL of DMSO in each well. This provides a 1/100 and 1/10,000 dilution of the original library material. The intermediate is then used as a source plate to fill in the remaining wells of the assay plate.

Scenarios 4 and 5 differ only in the hit rate (3% or 12 compounds per 384-well plate vs. 1% or 4 compounds in every 384-well plate. Scenario 3 can be considered a special case of this technique where the hit rate is 100%.

Impact of Replicates on Throughput
The experiment shown as scenario 1 was run singly, in duplicate and in triplicate to determine the impact. The results of these experiments is shown in Graph 2. While the number of curves generated per hour increases as the number of replicates increases, the total number of compounds being tested decreases.

Direct dilutions vs. Serial dilutions
Concentration gradients have traditionally been made with serial dilutions. While this technique is easy to understand, it suffers from a variety of problems.
First, each dilution brings about an increase in the error of the final analysis. Each transfer compounds the error of the previous transfer[1]. The error accumulates and a 5% error in each transfer becomes a 75% error after 11 dilutions (square root of the sum of the squares).
Second, multiple transfers can lead to cross-contaminations through tips that are not completely cleaned or through human error. Automation can eliminate the second but not the first.
Third, multiple transfers require significant amounts of initial sample and lead to diminution of the sample library.
Fourth, serial dilutions use significant amounts of solvents. While the pure grade DMSO used in most cases is relatively inexpensive, the costs of waste management may not be.
Finally, and perhaps most importantly, researchers have shown that many compounds have a tendency to be under-represented in a serial dilution[2]. Compound appears to bind to both the vessel used to make the dilutions (either microplate or tubes) and to the tips used to transfer the samples. Mass spectroscopic analyses have shown that the concentration of the analyte being tested can be as much as 10,000 times less than expected. This leads to falsely high IC_{50} values and false negatives. Analyses of libraries suggest that as many as 10% of typical library compounds are prone to this under representation.

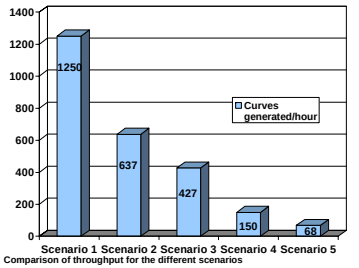
Because of these various problems with traditional serial dilutions, all the concentration gradients made with the POD using an Echo liquid handler go through a maximum of one dilution step.

Reducing [DMSO] in assay
Tjernberg and others[3,4] have shown that even low concentrations of DMSO in the final assay can have strongly deleterious effects on the validity of the assay. In all of these experiments the final amount of DMSO in each assay well is 75 nL.

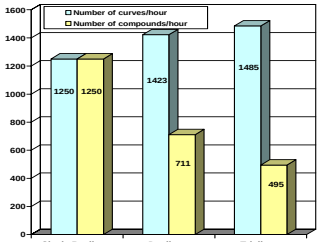
RESULTS
The time required to generate an 11 concentration + 1 blank gradient curve for one compound was calculated for each scenario. A number of other metrics were also determined including an analysis of how long it would take to exhaust the number of plates in storage (189 1536-well plates or 135 384-well plates). This could be considered the "walk-away time" or how long it would be before the researcher needed to go back because the plates were all filled or all the source plates were used and new ones needed to be loaded.
Table 1 shows the ratio of assay plates generated per initial source plate. In the case of pre-processed source plates this could be as high as 10/1Finally, Table 1 also shows the ratio of intermediate plates to source plates needed when intermediate plates are employed.
Two bar graphs illustrate the impact of experimental conditions upon throughput. The second bar graph shows the impact of increasing the number of replicates in the assembly of concentration gradients.

	Scenarios				
	1	2	3	4	5
Process	Pre-formatted source plate		Direct cherry-pick from library source plates		
Hit rate			100%	3%	1%
Source plate	1536 COC	384 polypro	384 polypro	384 polypro	384 polypro
Destination plate	1536 COC	384 polypro	384 polypro	384 polypro	384 polypro
Intermediate plate	NONE	NONE	384 polypro	384 polypro	384 polypro
Sec (min) required to run entire source plate	1105 (18.4)	542 (9.03)	3232 (53.9)		
Wells used in source plate	1536	384	384	12	4
Sec (min) required to generate assay plate	368.3 (6.1)	180.7 (3.0)	269.6 (4.5)	960 (16)	1694 (28.2)
Compounds in source	384 @ 3 conc.	96 @ 3 conc.	384 @ 1 conc.	12/library source plate	4/library source plate
Pts in dose-response curve	11+1	11+1	11+1	11+1	11+1
Replicates	Single	Single	Single	Single	Single
Assay plates/hr	11.1	19.9	13.4	3.75	2.125
11+1 pt curves/ hr., (curves/day)	**1250 (30,000)**	637 (15,303)	427 (10,262)	150 (3,600)	68 (1.632)
Hours before storage is exhausted of plates	14.4	7.1	11.2	12.6	6.9
Ratio assay plates/source plates	3/1	3/1	12/1	0.375/1	0.125/1
Ratio intermediate plates/source plates	0/1	0/1	2/1	1/16	1/48

Table 1. Throughput is extremely dependent upon the exact experiment run. Even when the final dose-response assay is in each case an 11-pt curve + 1 blank over a concentration range of six orders of magnitude, the number of assay concentration gradients developed per hour can vary by more than 20-fold.



Comparison of throughput for the different scenarios
Scenario 1 (1536→1536) transfer from pre-processed source plates provided the largest number of curves per hour, almost 20-fold more than scenario 5. Scenarios 1 and 2 are identical except that scenario 1 used 1536-well plates while scenario 2 used 384→well plates.
Scenarios 3, 4 and 5 are identical except in the hit rate (100%, 3% and 1% for scenarios 3, 4 and 5, respectively).



Comparison of throughput as the number of replicates increases
Scenario 1 (1536→1536) transfers were re-run to produce duplicate and triplicate compound gradients. As the number of replicates increased, the total number of curves produced per hour increased from 1250 to 1486. This leads to a reduction in the number of compounds that are plated out per hour.

CONCLUSIONS
The POD 810 plate assembler is designed to maximize the possible throughput of an Echo acoustic liquid handler. In order to answer the common question "What is the throughput of the system", we compared five different experimental procedures. In each case, the final result was the same—each compound was arrayed into an assay plate at 11 concentrations that varied by half-logs. Each compound was accompanied by a single blank of DMSO. Because a backfill process was used, each assay had the same amount of DMSO in the final assay volume (75 nL).
Despite these similarities, the throughput differed by more than 20-fold.
The highest throughput occurred when source plates were designed with three different concentrations (X, 0.01X and 0.0001X) of each compound. In this case, as many as 1400 gradient arrays (curves) (>700 compounds in duplicate) could be assembled per hour when the transfers were from a 1536-well source plate to a 1536-well assay plate.
However, these high throughputs require setting up special source plates. If a researcher wants to cherry-pick samples directly from a library source plate the throughput will vary with the hit rate—higher hit rates will lead to greater throughput as the number of plate transfers is reduced. In this case, a research can load the POD with the correctly labeled plates and provide a "pick-list" to the system and walk away. Upon return the assembled plates will be ready for assay. Even in these cases with a hit rate of 1%, assembling each concentration gradient takes less than 1 minute, on average, to make all 11 concentrations, and handle all the plates.

- As many as 1400 dose-response experiments can be assembled per hour
- Using pre-assembled source plates with three concentrations of samples dramatically increases throughput
- Using 1536-well plates vs. 384-well plates improves throughput
- Cherry-picking from library source plates to prepare plates has a lower throughput but eliminates the extra steps of assembling special source plates
- A POD 810 plate assembler with an Echo 555 liquid handler can provide assay ready dose-response plates that meet most demands.

References

1. Cesarek, J.; Nie, D. "New Tool for Automating Serial Dilutions for Activity Confirmation Experiments", poster at LabAutomation 2005, January 2005, San Jose, CA
2. Comley, J. "Serial vs Direct Dilution. Time to apply new thinking to IC_{50} determination and dose response analysis?", Drug Discovery World, Spring 2007, 36-50
3. Tjernberg, A.; Markova, N.; Hallén, D. "Is DMSO a friend – or a troublemaker?", poster presented at 11th Anniversary Meeting of the Society for Biomolecular Screening, Geneva, Switzerland, September 2005
4. Tjernberg, A.; Markova, N.; Griffiths, W. J.; Hallén, D. "DMSO-Related Effects in Protein Characterization", *J Biomol Screen*, **2006**, 11(2), 131-137