Acoustic Droplet Ejection Improves Dose-Response Determinations

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Abstract
Dose-response experiments, such as IC_{50} analyses, are time- and labor-intensive. They usually require multiple serial dilutions and large amounts of sample. Aqueous serial dilutions of concentrated stock solutions can precipitate or otherwise become biologically unavailable generating false negatives. Serial dilutions may also accumulate significant error. Cross-contamination of wells is a constant possibility. Finally, even low levels of DMSO in the final assay can dramatically affect the apparent activity of the compound especially in the case of cell-based assays.

A system incorporating acoustic ejection of nanoliter droplets of active compounds dissolved in DMSO improves IC_{50} analyses by eliminating accumulated error (CV%<10% over the entire sample concentration range), eliminating compound precipitation in intermediate dilutions, reducing consumables (plastics and solvents), reducing DMSO concentrations in the final assay to significantly less than 1% and reducing the amount of compound used in the analysis to nanomoles.

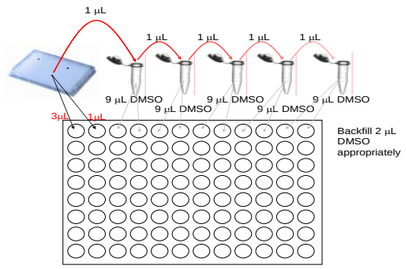

Figure 1. Traditional serial dilution. Five serial dilutions are made to cover a 12-point, half-log concentration curve. A second multi-well plate can be used for the dilutions in place of the microcentrifuge tubes to reduce cost.

Traditional IC_{50} Process
The traditional technique employing serial dilutions, as shown in Figure 1, has its drawbacks. Serial dilutions are time consuming and each successive dilution increases the error. Dilution error for each transfer can propagate causing uncertainty in the compound concentration at each point[1].

Some groups use an aqueous dilution rather than DMSO in order to reduce the amount of DMSO in the assay. While this dramatically reduces the amount of DMSO required per sample or per replicate, many compounds have a very limited solubility in water and the sample may precipitate or "crash out" of solution.

If the sample precipitates or is driven into the plastic of the intermediate dilution vessel because of the partition coefficient, the amount of material in the final assay well is much lower than anticipated. When researchers compared IC_{50} values for 1,000 compounds via an aqueous dilution step vs. maintaining the sample in DMSO up to the addition to the assay well, 110 compounds that were judged active when kept in DMSO were misread as inactive with the aqueous dilution step. Many of the compounds with the lowest IC_{50} values (i.e., most active) showed little activity when an aqueous dilution is used and would have been missed. See Figure 3.

Significant quantities of sample are used in the serial dilution method shown. 5 µL of sample is required for a single analyses and 13 µL if run in triplicate. This method also uses 57 µL of DMSO for a single analysis and 90 µL when run in triplicate.

The final amount of DMSO in each assay well by the manual method is 3 µL. In order to keep the final concentration of DMSO below 1%, the manual method requires an assay volume of 300 µL. Recent publications have shown the potential for significant assay problems when the DMSO concentration is higher[3]. This significantly reduces the potential for assay miniaturization.

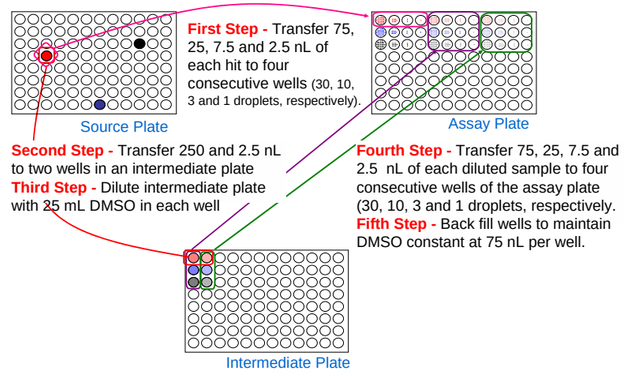

Figure 2. ADE transfers fluids in discrete droplets of 2.5 nL. Both source plate and intermediate plate would typically be 384-well format but a 96-well format is shown for simplicity. Earlier high-throughput screening assays would have found ~1% of the wells to have active compound. Only these compounds would be sampled ("cherry-picked") in the IC_{50} experiment. Material would be transferred from the source plate to the assay plate in four different amounts. Then that same source plate would be used to transfer two volumes of material to the intermediate plate. These wells would be filled with 25 mL DMSO to obtain 100:1 and 10,000:1 solutions. Note that these are not serial dilutions. Fluid from the intermediate plate would be transferred to the assay plate to establish a 12-point, 6-log concentration range

Acoustic Droplet Ejection
Acoustic Droplet Ejection (ADE) uses sound to move liquids and eliminates all physical contact with the solution being transferred. There are no pipette tips, no pin tools and no nozzles. Besides reducing costs of consumables, the elimination of physical contact with the solution dramatically improves both precision and accuracy of transfer to best-in-class. Typical transfers of 2.5 nL have CV <4%.

Because ADE can precisely and accurately transfer nanoliter volumes of DMSO sample directly to assay plates, dilutions can be eliminated or significantly reduced to a single, non-serial step. This eliminates the problem of samples crashing out of solution while maintaining a very low concentration of DMSO in the final assay. The low volumes accessible to ADE transfers also provides a mechanism to miniaturize assays to 1536-well format – a move that saves significantly on assay reagent costs[5].

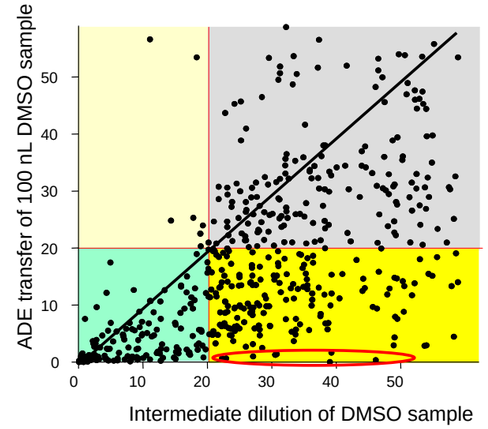

Figure 3. Comparison of IC_{50} values of 1000 compounds produced via direct addition of 100 nL of sample by ADE vs. IC_{50} values obtained using intermediate aqueous dilution. The horizontal axis provides the IC_{50} value determined via aqueous dilution. The cut-off for further testing was set at 20 µM. Any IC_{50} values greater than 20 µM indicated that the compounds were ineffective.
Compounds below the black diagonal line appear less potent using aqueous intermediate dilution than using ADE. Compounds in the grey area have high IC_{50} values by both techniques and are ineffective. Compounds in the green area are hits by both techniques. The 110 compounds (>10% of total) in the dark yellow area were measured as ineffective by the traditional aqueous technique but were active when the intermediate dilution was removed. The compounds in the red oval had IC_{50} values ten to 100 times lower by ADE than the aqueous technique. Seven compounds were judged as ineffective by ADE but were measured as active by the dilution technique. Five of the seven were very close to the cut-off. No compounds showed IC_{50} values of less than 10 µM by dilution that were not also detected by the ADE method[2].

	Serial Dilution	ADE
Amount of sample required for assay in triplicate (µL)	13	**0.5825**
DMSO required for assay in triplicate (µL)	90	**50.99**
DMSO for 192 samples in triplicate (µL)	17,280	**9790**
Final amount of DMSO in assay (nL)	3000	**75**
Amount of consumables for 192 hits in 12-pt assay	960 tubes (or ~2.67 plates) 1152 tips	**1 Echo Qualified 384 PP plate**
Cost for plastic consumables	$240 (tubes) +$60.24= $300.24 OR $8.00 (plates) + $60.24=$68.24	**$3.90**

1. The amount of DMSO used for the process does not assume any DMSO used to wash tips. Current estimates for tip washing suggest that this volume may be several liters for the experiment described
2. Tip usage is based upon a new tip for each fluid not for each transfer. Tip usage can be reduced but only at the cost of extra DMSO purchase and disposal.
3. Dilution plate cost for the serial dilution method is based on a cost of $3.00/plate and making five serial dilutions of 72 compounds in each plate.

Consumables Costs
While the biggest cost to a pharmaceutical company would be the loss of potential drugs due to false negatives in analysis, there are also the immediate costs of consumables to consider. This additional cost for pipette tips or pipette tip washing is incurred at each step as well as the cost of the dilution vessels. The ADE technique illustrated in Figure 2 requires only a single 384-well Echo qualified flat-bottom plate as a consumable. These plates cost $3.90 each. The process shown in Figure 1 requires five intermediate dilution vessels with a typical price of a 0.6 mL microcentrifuge tube at about $0.25. It also requires six pipette tips per sample (regardless of replicate number), one from the original source plate and one from each of the dilution tubes. The number of tips can be reduced by stringently washing them before reuse but the potential for carrying high concentrations of sample in the tip to the next lower dilution remains great. Stringent washes will reduce the waste of pipette tips but significantly increase the generation of liquid waste in the form of contaminated DMSO. Since mL volumes of fluid are used to wash each tip at each washing, DMSO waste can quickly mount to many liters. In HTS analyses, a large pharmaceutical company will generate hundreds of liters of DMSO waste[4].
The microcentrifuge tubes can be replaced with multi-well plates. This reduces the cost of consumables in the serial dilution method but it still remains higher than the ADE method.

Conclusions
ADE improves IC_{50} analyses in the following ways:
- **Improved results with fewer false negatives**. Probably due to elimination of compound precipitation
- Less error, better accuracy per transfer (<5% CV typical)
- Fewer serial transfers – less accumulated error
- Less sample used (save at least 90-95%)
- Less DMSO in final assay (reduction by 97-99%)
- Potential for miniaturization thereby saving assay reagents
- Less DMSO waste is generated (by many liters)
- Significant savings on consumables
- Eliminates manual steps

[1] "New Tool for Automating Serial Dilutions for Activity Confirmation Experiments", J. Cesarek and D. Nie, AstraZeneca, Poster presentation at LabAutomation 2005, San Jose, CA
[2] "Pharmacological Evaluation of Different Compound Dilution and Transfer Paradigms on an Enzyme Assay in Low Volume 384-well Format", T. Spicer, Y. Fitzgerald, N. Burford, S. Matson, M. Chatterjee, M. Gilchrist, J. Myslik and J. O'Connell, Bristol-Myers Squibb, Poster presentation at Drug Discovery Technology August 2005, Boston MA
[3] Tjernberg, A.; Markova, N.; Griffiths, W. J.; Hallén, D. "DMSO-Related Effects in Protein Characterization", J Biomol Screen, 2006, 11(2), 131-137
[4] Olechno, J.; Stearns, R.; Ellson, R.; Heron, E. "Acoustic Droplet Ejection Technology" Innovations in Pharmaceutical Technology, 2006, June, 40-43
[5] "Miniaturized Luminescent Metabolism Profiling Assays", T. Worzella, B. Larson, J. Shieh, and A. Gallagher, Poster at Mid-Atlantic Regional LRIG Show, East Brunswick, NJ, April, 2006