



Contact-free, direct dilution minimizes compound loss in dose-response experiments

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ABSTRACT

Disposable pipette tips can cause errors in dose-response experiments. More accurate dose-response curves can be constructed by eliminating aqueous serial dilution of compounds. Traditional serial dilutions that use aqueous diluents can result in errors in dose-response values of up to four orders of magnitude for a significant percentage of a compound library. When DMSO is used as the diluent, the errors are reduced but not eliminated. We use acoustic droplet ejection (ADE) to transfer different volumes of model library compounds, directly creating a concentration gradient series in the receiver assay plate. Sample losses and contamination associated with compound handling are therefore avoided or minimized, particularly in the case of less water-soluble compounds. ADE is particularly well-suited for assay miniaturization, but gradient volume dispensing (also known as "direct dilution") is not limited to miniaturized applications.

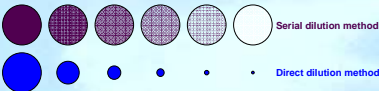


FIG. 1. Traditional serial dilutions transfer the same volume of different concentrations of sample. Different concentrations are made by transferring a fixed volume of the more concentrated fluid into a dilution solution and repeating the dilution in a serial manner. Error is accumulated and sample is lost with each handling step. The direct dilution method, (the bottom series) involves transfer of different volumes of the identical solution. With only one transfer for each new concentration, accumulated error is eliminated. When performed with touchless acoustic transfer, sample adsorption on tips is eliminated.

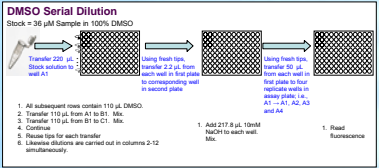
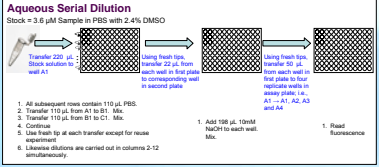


FIG. 2. Comparison of process used to serially dilute compound with an aqueous diluent (top) versus pure DMSO (bottom). DMSO stock solution is more concentrated. Volumes transferred in each plate provide assay plates at the same concentration.

Method

- Experiment 1 – Comparison of serial dilution with phosphate buffered saline to form a concentration gradient vs gradient formation via direct dilution.**
- Sodium fluorescein (Sigma), Fluorescein O,O'-diacrylate (Sigma) and 5-dodecanoylaminofluorescein (DDAF-Invitrogen) were dissolved in anhydrous DMSO to make a 0.15 mM stock solution of each.
 - Serials gradients were prepared as shown in Figure 2 (above) using phosphate buffered saline (PBS) as the diluent. All transfers were made with fresh disposable polypropylene pipette tips (Gilson Pipetman with VWR tips).
 - An aliquot from each well was transferred to a new plate and 10 mM NaOH was added to each well and the fluorescence was read with a SpectraFluor Plus (Tecan) calibrated with a fluorescent standard (Metch).
 - A volume gradient of each dye was made with an Echo® 550 liquid handler (Labcyte) by transferring 120, 60, 30, 15, 10, 7.5, 5, and 2.5 nL directly to a receiver plate which was then filled with 50 μL 10 mM NaOH.
 - ClogP values determined for all three dyes at www.molinspiration.com

- Experiment 2 – Comparison of serial dilution with DMSO to form a concentration gradient vs. PBS.**
- The same procedure was used as in Experiment 1, steps 1-3 except that 100% DMSO was used as the serial diluent as shown in Figure 2 (bottom).

- Experiment 3 – Analysis of the impact of using fresh tips vs. tips exposed to dye.**
- The same procedure as used in Experiment 1, steps 1-3 except that rather than using a fresh tip to transfer each fluid, the tips were reused to determine whether surface adsorption could be saturated.

Dye	ClogP
Fluorescein	0.92
5-Dodecanoylaminofluorescein (DDAF)	5.55
Fluorescein O,O'-diacrylate	4.62

Table 1. Test compound hydrophobicity. The ClogP values for three test compounds were calculated. Both DDAF and fluorescein diacrylate are much more hydrophobic than the extremely water-soluble fluorescein.

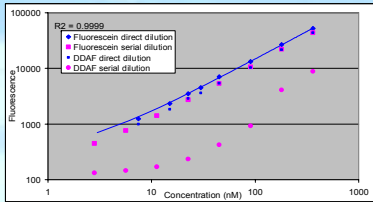


FIG. 3. Results of Experiments 1. The direct dilution technique gave excellent concentrations for both fluorescein (●) and DDAF (■). Serial dilutions worked well for water-soluble fluorescein (●) but failed significantly for DDAF (■). The top three curves had linear R² values between 0.9997 and 0.9999. When plotted linearly, rather than logarithmically, it is difficult to visually resolve the low concentration points.

Acoustic Droplet Ejection: Move Liquids with Sound™

Acoustic Droplet Ejection (ADE) uses focused ultrasonic energy to eject small droplets from a liquid. The technology can be used to eject droplets smaller than one picoliter and as large as 10 μL. Larger volumes can be transferred as multiple drops. ADE requires no tips, pins or nozzles, saving consumables and waste costs. With no contact between the ejection mechanism and the ejected sample there is no chance for cross-contamination. ADE delivers superior precision and accuracy for a wide range of biological applications, including siRNA screening, compound screening with biochemical and cell-based assays, PCR reactions and assay development. See www.labcyte.com for additional information on ADE. Acoustic Droplet (Stroboscopic image of ADE)

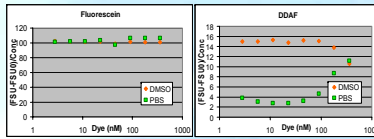


FIG. 4. Results of Experiment 2. When a serial dilution of fluorescein (left) is prepared, the linearity remains consistent when either DMSO or PBS is used as the diluent. When the far more lipophilic DDAF was serially diluted, there were significant deviations from linearity when DMSO was the diluent. These deviations were greater when the diluent was PBS.

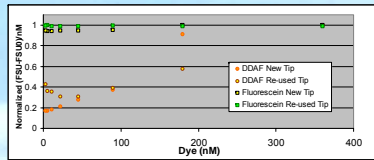


FIG. 5. Results of Experiment 3. As concentration decreases, a linear decrease in fluorescence is expected. To aid in visualization, the fluorescence of the samples were divided by the concentration. If fluorescence scales over the range measured, the results should be a horizontal line. Values were further normalized against the highest fluorescence value for each sample. Very water soluble fluorescein showed only a slight difference between using fresh tips and reused tips during transfer. New tips may have bound a small amount of fluorescein, especially at lower concentrations than used tips, as might be expected if adsorption sites had been filled. The far more hydrophobic DDAF showed significant deviations from a horizontal line at all concentrations. At low concentrations, reused tips gave higher signals than fresh tips. This suggests that fresh tips bind material in solution and keep it from being transferred.

Sources of Assay Error from Liquid Handling

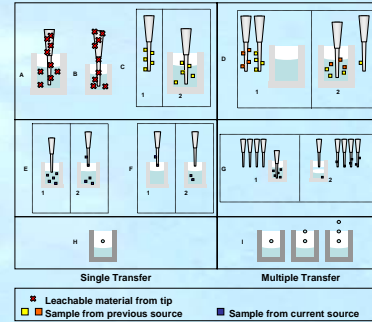


FIG. 8. Pitfalls exist when using tips and may increase as assays are miniaturized or when the source fluid is accessed multiple times. (A) Tips contain materials which can leach out, contaminating both source and assay. (B) Assay miniaturization increases the surface-to-volume ratio, increasing concentrations of contaminants further. Mixing via aspirate/dispense methods (a common process with high density microplates) may further contaminate transfers. (C) Adhesion of previous materials causes cross-contamination. (D) For multiple sample use, each tip entry allows more cross-contamination from different samples (or tip leaching – not shown). (E) Tips remove sample on their exterior surface, depleting the source concentration. (F) Tips carrying adherent sample on their outer surfaces can add more sample than expected. (G) Sample wells revisited many times (e.g., 50 transfers of 100 nL from a source well in a 384-well microplate) can deplete the sample, lowering the expected concentration in the source well. (H) & (I) With no tips, acoustic droplet ejection is free of these errors.

Results

Poor results from serial aqueous dilutions may arise from interactions between the compound being transferred and pipette tip surfaces. Hydrophobic compounds do not yield anticipated concentrations when made by aqueous serial dilutions. The same compounds yield concentration gradients as expected made acoustically by the direct dilution method.

We believe that the deviation from expected seen with high ClogP compounds has multiple causes.

- Compounds with high ClogP values adsorb to the surfaces of the pipette tips and the dilution wells during aqueous dilutions.
- New tips yield lower fluorescence than used tips implying that new tips bind more compound.
- Compounds with high ClogP values often show poor solubility in aqueous solutions. Compounds may precipitate from solution to form solids either on the bottom of the wells or floating on the surface of the solutions reducing their availability to transfer.

While these problems may be ameliorated by using DMSO for serial dilutions rather than water, this greatly increases the volume of DMSO needed and significantly increases waste solution output. Furthermore, we see a smaller yet significant impact on final gradient concentrations even when DMSO is used in the serial dilutions.

For large volume, single-step procedures with soluble, low ClogP compounds, the error induced by surface interactions with the sample may be neglected or minimized with fresh tips and wash procedures. Clearly, with assay miniaturization (with increased surface-to-volume ratios) and more numerous transfers from the same source well, previously neglected mechanisms may have cumulative effects on sample concentration. Our tip re-use study underscores the difference between the first and second use for two model compounds (fluorescein and DDAF).

Concentration gradients for dose-response experiments should not be made with aqueous serial dilutions unless the compound being analyzed is extremely hydrophilic. Researchers in the pharmaceutical industry have reported large deviations in measured C₅₀ values using serial aqueous dilution versus direct transfer¹. The generation of serial dilutions for dose-response experiments using the traditional technique of an aqueous intermediate is prone to significant errors and we have quantified this type of error for compounds in a model system. We believe these mechanisms—surface adsorption, carry-over and poor aqueous solubility—may help explain anomalous measurements seen in industry and that acoustic transfer can help to provide a remedy.

Conclusions

- Compounds are lost when solutions are transferred via disposable pipette tips in the preparation of serial dilutions. This is due, in part, to hydrophobic adsorption of compounds to the tips. This may account for some of the errors seen when tips are used to prepare gradients to determine IC50 values.
- Fluorescein is NOT a good analog to determine whether drug-like compounds are correctly transferred during dilutions. Fluorescein is good for determining if the correct volume of fluid is transferred via pipette tips or acoustic methods.
- Performing serial dilutions with DMSO rather than aqueous solutions does NOT eliminate errors, although it may reduce them.
- Both re-used tips and new tips will bind compounds leading to errors in serial dilutions.
- Direct dilution via ADE eliminates problems associated with serial dilutions of compounds in dose-response experiments.

References

- Gradient, contact-free volume transfers minimize compound loss in dose-response experiments. Harris D, Olechno J, Daiwani S, Ellison R. *J Biomed Screen*. 2010 Jan;15(1):86-94. Epub 2009 Dec 11. (Note many figures in journal are misplaced.)
- Bioactive Contaminants Leach from Disposable Laboratory Plasticware. McDonald GR, Hudson AL, Dunn SM, You H, Baker GB, Whittall RM, Martin JW, Jha A, Edmondson DE, Holt A. *Science* 2008 7 November;322(5903): 917
- www.molinspiration.com
- Assay Sciences: A Model for Improving Efficiency Through Centralization. Wingfield J, Jones D, Clark R, Simpson P. *Assay Design Dev*. April/May 2007 3(3):24-30