

# HIGH-THROUGHPUT DETERMINATION OF AQUEOUS SOLUBILITY, PRECIPITATION OR COLLOIDAL AGGREGATE FORMATION IN SMALL MOLECULE SCREENING SETS

## Introduction:

Determining the aqueous solubility of a compound is an essential parameter in drug discovery. Poor water solubility can interfere with in vitro testing, giving rise to inaccurate and irreproducible data. Compounds with low aqueous solubility tend to be highly bound to plasma proteins with poor systemic tissue distribution and increased potential of CYP-mediated drug inhibition<sup>1</sup>. Another caveat to in vitro testing in aqueous systems is false-positives in bio-assays due to the formation of colloidal aggregates. Compounds that form these aqueous aggregates are found experimentally to be promiscuous inhibitors that act non-competitively and exhibit little to no structure-activity relationships. Aggregate formation occurs when individual molecules group together to form particles 30-1000 nm in diameter, and these aggregates behave as the active inhibitory species<sup>2</sup>. This is a key problem in HTS studies which consumes valuable R&D dollars and must be identified early in the discovery phase to remove them from further testing<sup>3</sup>.

ASDI offers solubility determination in aqueous systems. Linear serial dilutions of each compound to be analyzed are prepared in aqueous buffer at a specified pH. The assay is performed using a commercially available flow cytometer modified to determine particle precipitation via simultaneous analysis of multiple particle size ranges in high-throughput 96-well or 384-well formats<sup>4</sup>.

## Materials & Methods:

1. As little as one micromole (for example diethylstilbestrol (268.4 g/mol)) of each compound to be analyzed is weighed into a 96-deep well high recovery polystyrene plate (mother plate).
2. Using a Tecan Genesis, Tomtec Quadra-3, or other appropriate robotic liquid handling system, an appropriate volume of high-purity, dry DMSO is added to each well to create a 10 mM stock solution and mixed.
3. Using a Tecan Genesis, Tomtec Quadra-3, or other appropriate robotic liquid handling system, each row of twelve compounds from the mother plate is transferred into a secondary 96-well high recovery polystyrene microtiter plate (serial dilution plate). Thus one 96-well mother plate creates eight daughter plates containing 12 unique compounds.
4. Six 2-fold serial dilutions in high-purity, dry DMSO plus a row of blank high-purity dry, DMSO are created in the daughter plate (concentration range of 10 mM to 0.0 mM) for each compound.
5. Using a Tecan Genesis, Tomtec Quadra-3, or other appropriate robotic liquid handling system, 1  $\mu$ l of each well of the daughter plate is transferred to 99  $\mu$ l of filtered aqueous buffer\* in a third high recovery polystyrene microtiter plate.

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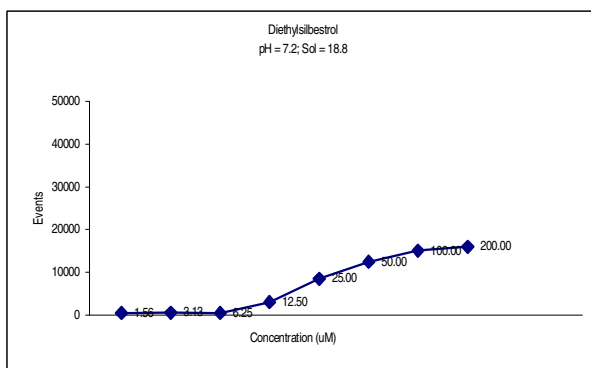
\* Aqueous buffer pH is not limited by the experiment. Typical pH values are 6.5 & 7.4, but the entire pH range can be assessed.

6. Each 96-well analytical plate thus contains twelve compounds over the concentration range 100  $\mu\text{M}$  to 1.56  $\mu\text{M}$  with a constant DMSO concentration of 1% (v/v). Please note that the analytical plate may also be in 384-well format.
7. The compounds are analyzed by an optically modified flow cytometer at a rate of 4 to 5 96-well plates or one 384-well plate per hour.
8. Data is readily evaluated by proprietary software, and the apparent aqueous solubility is determined.
9. Assay<sup>5</sup>
  - Sample is mixed and then injected through a flow cell.
  - The sample is carried through the flow cell by a secondary “carrier” liquid (sheath fluid).
  - The pressurized sheath fluid narrows the sample stream (to less than 6  $\mu\text{m}$ ) so that *individual particles pass through the laser beam one at a time*.
  - The Photo Multiplier tube collects photon signatures for each particle (90 degree scatter). The amount of light scattered is proportional to the size of the particle.
10. Reports are generated in \*.csv, \*.xls, or \*.pdf formats.
11. All raw data is easily stored and archived.

### Experimental Process:

In this study 5  $\mu\text{mol}$  (1.34 mg  $\pm$  0.1 mg) of DES was dissolved in 500  $\mu\text{l}$  high-purity, dry DMSO and mixed well. Six 2-fold serial dilutions of the resulting 10 mM solution were prepared at a concentration range of 0  $\mu\text{M}$  to 10  $\mu\text{M}$  in high-purity, dry DMSO. One microliter of each dilution was transferred to independent wells of a 96-well high recovery polystyrene microtiter plate containing 0.5 M aqueous ammonium acetate (pH = 7.0) filtered through a 0.2  $\mu\text{m}$  membrane. A single analysis set over the dilution is used to construct a curve of frequency of particles through the detection beam vs. concentration. See Figure 2. The same sample plate is measured again about 3-hours later for the colloidal aggregation experiment.

Figure 2. Aqueous solubility ( $S_w$ ) curve generated for DES



Compound Diethylstilbestrol  
 Apparent Solubility ( $\mu\text{M}$ ) 18.8  
 Solubility Range ( $\mu\text{M}$ ) 12.5 - 25.0  
 pH 7.0  
 Time 00:10:00  
 Method Bkgd\*3.0 (Bkgd=411)  
 Breakpoint 1233  
 Plate ID ASDI demo (BD compounds)\_plate 1  
 Filename 0802071.008  
 Plate Location B02; C02; D02; E02; F02; G02; H02;  
 Date Run 8/2/07 3:08 PM  
 Settings PMT= 100, THR= 35

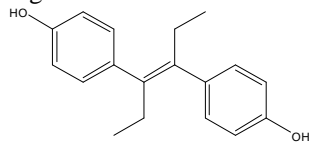
### Results:

#### Part 1: Aqueous Solubility ( $S_w$ )

Diethylstilbestrol (DES) is a synthetic, non-steroidal estrogen originally indicated for estrogen-replacement therapy in the treatment of ovarian dysgenesis, premature ovarian failure, and post-oophorectomy. It was also used in the 1960's for the treatment of advanced breast cancer. Since the late 1990's DES is no longer indicated for humans, since it was

determined to be a teratogen. Low doses of the drug, however, are still indicated for the veterinary treatment of canine incontinence<sup>6</sup>. For our purposes DES is used as a control and to measure system performance on a daily basis. The chemical structure of DES is given in Figure 1.

Figure 1. Chemical structure of DES



*Clarke's Analysis of Drugs and Poisons*<sup>7</sup> states that the salt-free form of DES is “practically insoluble in water; soluble 1 in 5 of ethanol, 1 in 200 of chloroform and 1 in 3 of ether; soluble in acetone and methanol; soluble in fatty oils and alkali hydroxides” and gives its LogP as 5.1. Empirical literature values of the aqueous solubility ( $S_w$ ) at or near pH = 7.4 range from 7.1<sup>1</sup>  $\mu\text{M}$  to 11.2<sup>8</sup>  $\mu\text{M}$ . The calculated values of  $S_w$  for DES range from 13.3  $\mu\text{M}$ <sup>9</sup> to 40.7  $\mu\text{M}$ <sup>10</sup> at pH = 7.2. Thus there is a great deal of variation in the experimentally determined literature values and an even greater range in the theoretical values, depending on the algorithm used in the calculations.

The single analysis set shown in Figure 2 yields an apparent aqueous solubility of 18.8  $\mu\text{M}$  (range 12.5 – 25.0). Two identical subsequent analyses of DES resulted in apparent solubilities of 14.5  $\mu\text{M}$  and 13.9  $\mu\text{M}$  respectively, for an average  $S_w$  of 15.7  $\mu\text{M}$ . Although this value is about 72% (~7  $\mu\text{M}$ ) greater than the average reported literature value (9.2  $\mu\text{M}$ ) from two sources it is within 18% (~3  $\mu\text{M}$ ) of the single theoretical value predicted by the ACD software. Replicate intraday and interday analysis would provide a data set that could be statistically evaluated to determine the mean, range, and variance in the measurement for the ASDI laboratory. Some caveats to the method include<sup>5</sup>:

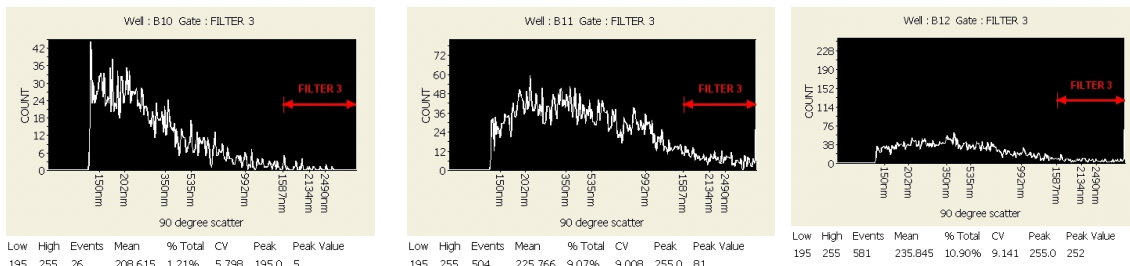
- Solubility is significantly affected by the screening environment: serum, additives, residual solvents, enzyme, and substrates.
- The Solubility of drugs from DMSO stocks in complex biological buffers is not the same as the theoretical maximum aqueous solubility (from a powder).
- The solubility of drugs in assay plates at specific time points is best determined *in those plates* at the *same time points*.

## Part 2: Colloidal Aggregation:

In our study compound an inflection in the aqueous solubility curve is detected at some concentration. In Figure 2 an inflection of about 1233 events is detected around 10  $\mu\text{M}$ . The interpretation of the data results in three possible outcomes for each compound: 1) the compound is soluble over the entire concentration range, 2) the compound is insoluble at some concentration over the range due to precipitation or 3) the compound forms colloidal aggregates at and above a particular concentration. The ability of the assay to simultaneously measure particle size and the frequency of particles passing through the detection beam (events) is key to determining if the apparent insolubility at a given concentration is a true solubility issue (precipitation) or a colloidal aggregate. For compounds that “crash” out of solution due to precipitation, it can be determined that the particle size increases proportionally to compound concentration and that the particles will become larger (ripen)

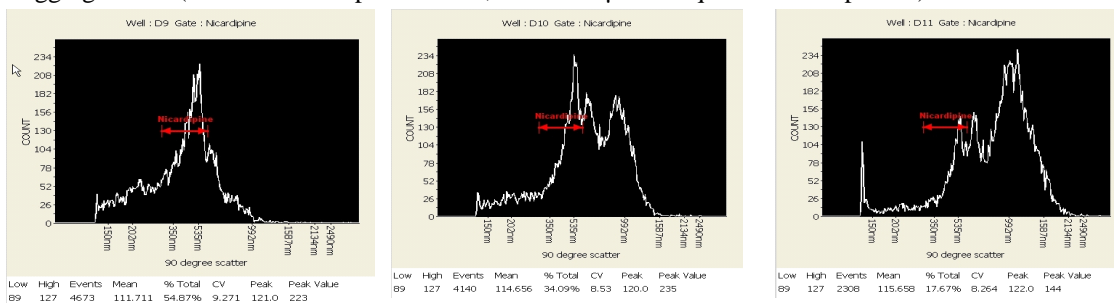
over time. The particle size distribution can be measured on the same experimental plates used for the Sw assay. As seen in Figure 3A<sup>5</sup> precipitation results in the formation of large particles, randomly distributed above 1500nm size range.

Figure 3A. Particle size distributions of DES over increasing concentration indicative of precipitation <sup>5</sup>. (Shown: DES @ 25, 50 & 100  $\mu$ M in aqueous buffer pH 7.4)



In the case of colloidal aggregation, a narrow size distribution is observed generally with particle size greater than 1500nm. See Figure 3B<sup>5</sup>

Figure 3B. Particle size distributions of Nicardipine over increasing concentration indicative of colloidal aggregation <sup>5</sup>. (Shown: Nicardipine @ 37.5, 75 & 150  $\mu$ M in aqueous buffer pH 7.4)



## Conclusions:

Aqueous solubility studies at ASDI provide insight into a critical early drug discovery parameter and enhance the ability to find and remove compounds likely to form colloidal aggregates from HTS screening sets. The assay requires an average of 1 mg to 2 mg of compound and can be formatted into 96-well or 384-well plates. There is no limit to the buffer choice or pH range. The detection method is 90-degree light scattering collected in 256-multiplexed channels. The frequency of particles that pass through the detection cell, as well as the particle size (proportional to the intensity of scattered light), is measured simultaneously. The analysis time for each well is ~ 10 seconds, and the detection limit is less than 0.2  $\mu$ M.

## End Notes

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<sup>1</sup><http://www.cerep.fr/cerep/utilisateurs/pages/Downloads/Documents/Marketing/Pharmacology%20&%20ADME/Application%20notes/Aqueoussolubility.pdf>

<sup>2</sup> Shoichet, BK, et al. A Common Mechanism Underlying Promiscuous Inhibitors from Virtual and High-Throughput Screening. *J. Med. Chem.* **2002**, *45*, 1712-1722

<sup>3</sup> Shoichet, BK, et al. High-throughput assays for promiscuous inhibitors. *Nature Chem Bio Lett* **2005**, August 1:3, 146-148

<sup>4</sup> Crespi, et al. Aqueous Solubility by Flow Cytometry II: New Prototypes Optimized for Drug Solubility Testing. Poster Presentation: *BD Gentest, A BD Biosciences Company, Woburn, MA 01801*

<sup>5</sup> Goodwin, J. Poor Aqueous Solubility and Compound Aggregation: Detection, Differences, and Impact on In-Vitro Screens. *BD Gentest Solubility Scanner. BD Biosciences Company, Woburn, MA 01801*

<sup>6</sup> <http://en.wikipedia.org/wiki/Diethylstilbestrol>

<sup>7</sup> <http://www.medicinescomplete.com/mc/clarke/current/CLK1516.htm>

<sup>8</sup> Internal Document. *BD Biosciences Company, Woburn, MA 01801*

<sup>9</sup> SolDB version 10.0.2. Advanced Chemistry Development, Inc. Toronto, Ontario. Canada

<sup>10</sup> Duffy, EM, et al. Prediction of Drug Solubility from Monte Carlo Simulations. *Bioorg Med Chem Lett.* **2000**, June 5;10(11):1155-1158