# Label-Free Detection of Compound Aggregation Using Corning® Epic® Technology

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**SnAPPShots**

*A brief technical report from the Corning Development Group*

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## Introduction

Small molecule compound aggregation is a major problem encountered in primary screening assays. Once formed, the aggregates can sequester, cause partial unfolding, inhibit or inactivate a protein. The effects can be observed on many structurally and functionally unrelated proteins. It is estimated that up to 19% of compounds in typical pharmacological libraries can form aggregates at concentrations typically used for high-throughput screening (HTS) (1). Considerable time and expense can be wasted pursuing these false hits in hit-to-lead optimization. Several methods have been used to detect formation of aggregates including; a) enzyme activity-based assays with β-lactamase, chymotrypsin, malate dehydrase (2) or kinases (3); b) direct detection of aggregates via static or dynamic light scattering (DLS) (1); c) direct measurement of compound-protein interaction by surface plasmon resonance (4); and d) fluorescence measurements of meniscus shape (5). However, the utility of these methods for rapidly characterizing the aggregation activity of compounds derived from a primary HTS campaign is restricted by either a lack of throughput, reliability or simplicity.

The Corning Epic technology can detect molecular interaction using optical biosensors integrated into the bottom of Epic microplate wells. Binding or settling of compound aggregates to the biosensor can be detected by measuring the wavelength shift of reflected resonance light (Fig. 1). In this study, Epic technology was used to detect aggregate formation using a set of known aggregators and non-aggregators. These data show that the label-free assay format could effectively detect compound aggregation and enabled clear differentiation from a non-aggregating group of compounds. Further, it was demonstrated that aggregation measured using Epic technology shares many features in common with other detection technologies such as steep concentration curves and sensitivity to the presence of non-ionic detergent. These data demonstrate that Epic technology is ideally suited for the rapid identification of aggregators, and can be readily implemented to attenuate false positives from HTS hit pools.

## Materials and Methods

### Compounds

A panel of aggregators and non-aggregators was chosen based on previous characterization with either enzyme inhibition-based assays and/or DLS (1,2). Eight aggregators were chosen for analysis; Clotrimazole (C6019), Sulconazole (S9632), Econazole (E4632), Miconazole (M3512), Nicardipine (N7510), Palatine Chrome Black (45550), Tetraiodophenolphthalein (224987), and Rose Bengal lactone (328960). Six non-aggregators were used as negative controls; Ketoconazole (K1003), Fluconazole (F8929), Sulfadiazine (S8626), Thalidomide (T144), Prednisone (P6254), and Tripelennamine (T7511). All compounds were purchased from Sigma-Aldrich (Saint-Louis, MO) and catalog numbers are shown

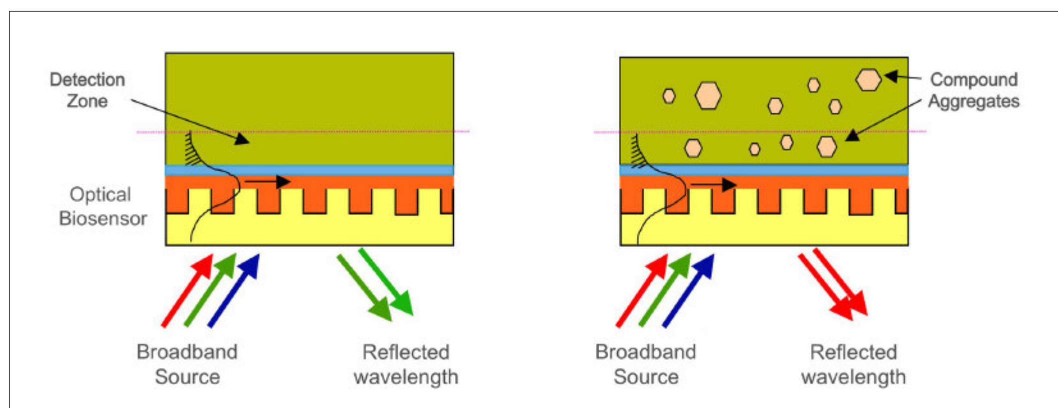


**Figure 1.** Epic Technology can detect compound aggregation using a simple add and read assay format. Uncoated Epic microplates containing integrated optical biosensors in each well are first soaked with assay buffer and a baseline measurement is taken. Compound is added in DMSO and aggregate formation at the surface of the biosensor is detected by measuring the wavelength shift of reflected resonant light.

in parentheses. Compound stock solutions of 50 mM were prepared in DMSO. Dilution series were made for each compound in DMSO to ensure a final 1x assay concentration range of 100 µM to 1.2 µM. For example, if the final DMSO concentration was 1%, compounds were prepared at 100x concentrations ranging from 10 mM to 123 µM.

### Epic® Aggregation Assay Procedure

Epic 384 Well Uncoated Cell Assay Microplates (Cat. No. 5040) were used for detecting aggregation formation. Microplates were first soaked in 50 mM HEPES plus 10 mM $MgCl_2$ (soaking buffer) and temperature-equilibrated in the Epic reader for at least 1 hour. After a baseline measurement was taken for 2 minutes, compound dilution series in DMSO were added with 10 mixes using a CyBio CyBi®-Well pipetting system. Compounds were dispensed in DMSO to avoid predilution prior to addition into the Epic microplate. A range of final DMSO concentrations was tested using the assay formats shown in Table 1. Following compound addition, the microplate was returned immediately to the Epic reader and signal changes were recorded as continuous (kinetic) time traces for at least 1 hour. Sensitivity to non-ionic detergent was tested by including 0.1% Triton X-100 in the soaking buffer. Each of the assays was performed at least three times with excellent reproducibility.

### Data Analysis

Aggregation response profiles were analyzed using the Epic Data Analysis Tool and dose response curves were generated using Graphpad Prism® software. Dose response plots were obtained using the difference in response values between the last baseline read and the aggregation response at 60 minutes. Sensitivity of the aggregation assay was determined using Thalidomide as the negative control and plotting the mean non-aggregator response at each concentration plus 3x the standard deviation of the response (3σ). The 3σ threshold was used to determine the critical aggregation concentration (CAC) below which, no aggregation was observed.

**Table 1. Summary of soak buffer volumes and compound add volumes required to run Epic aggregation assays at different final DMSO concentrations.**

| Final DMSO (%) | Soak Buffer (µL) | Compound Add (µL) |
|---|---|---|
| 1.0 | 49.5 | 0.5 |
| 2.0 | 49.0 | 1.0 |
| 3.0 | 48.5 | 1.5 |
| 4.0 | 48.0 | 2.0 |
| 5.0 | 47.5 | 2.5 |

## Results and Discussion

Current technologies for detecting compound aggregation typically lack throughput capability and involve complex assay formats. A simple add and read assay format was developed to detect aggregation using Epic technology (Fig. 1). Compounds are added directly to assay buffer in uncoated microplates and formation of aggregates in the solution is monitored by changes in reflected wavelength from the biosensor, measured in picometers (pm). Although some aggregation assay formats require the presence of enzyme, which sequesters compound aggregates resulting in inhibition of enzyme function, it was found that little difference in aggregation responses is observed when comparing uncoated microplates with biochemical microplates pre-immobilized with trypsin (data not shown). No compound predilution is required for the Epic aggregation assay; instead, compounds are added directly in DMSO. This results in a DMSO mismatch response which necessitates the inclusion of DMSO alone as a control to establish the background response, which can subsequently be subtracted from the raw data to provide corrected responses.

Representative optical response profiles for a dose series of a known aggregator, Sulconazole and a known non-aggregator, Prednisone are shown in Fig. 2. Both compounds exhibit an

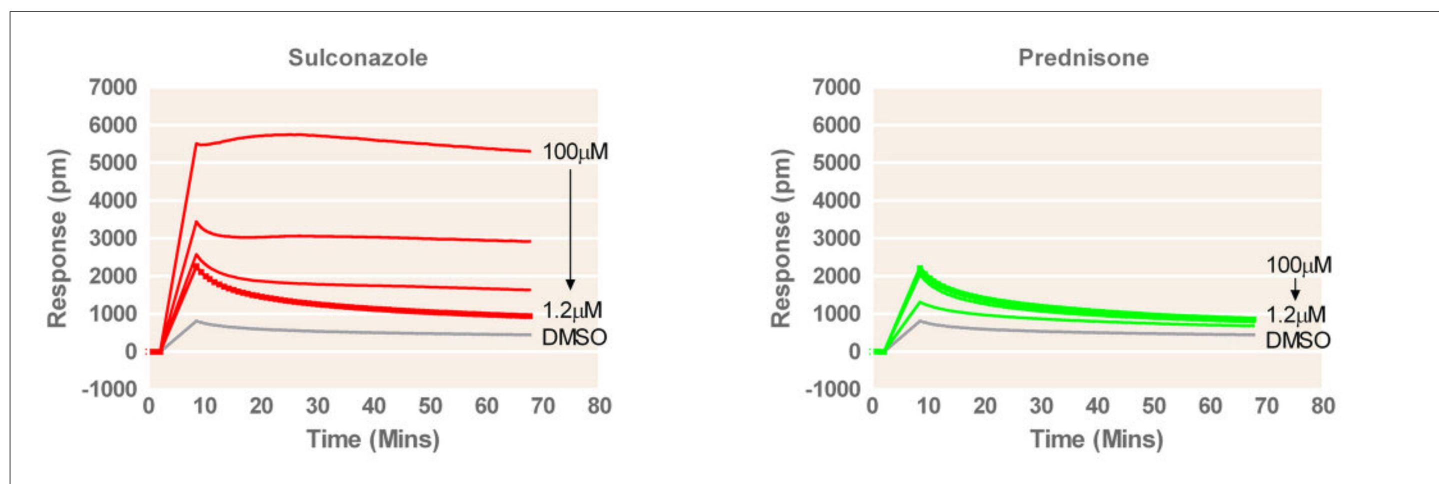


**Figure 2.** Optical response profiles readily distinguish aggregators from non-aggregators. Sulconazole (known aggregator) and Prednisone (known non-aggregator) were added in dose response series from 100 µM to 1.2 µM (1:3 dilutions). DMSO alone was added as control to determine the assay background (grey line). Final DMSO concentration in the assay was 2%.

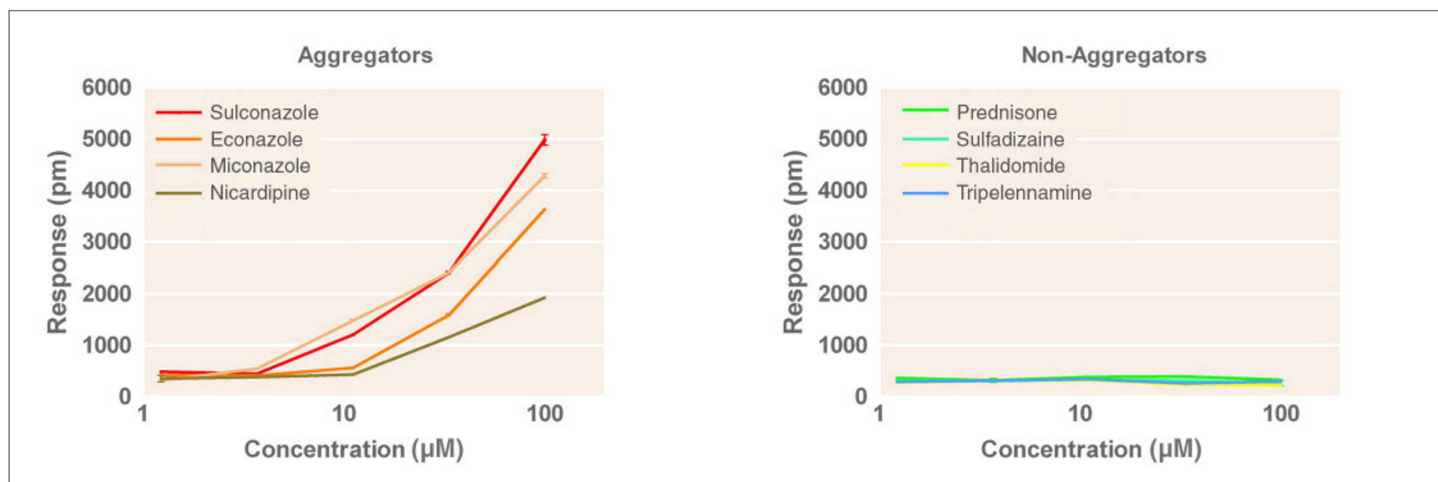


**Figure 3.** Aggregators using Epic technology show characteristic steep dose response curves. A panel of four known aggregators and four known non-aggregators were tested using the same dose response series as in Fig. 2, with a final DMSO concentration of 2%. Plots were generated using the response values at 60 minutes after compound addition and subtracting out the DMSO background response.

initial positive spike over the first 10 to 20 minutes as a result of compound addition, but the response stabilizes between 30 and 60 minutes after compound addition. Sulconazole exhibits a clear dose-dependent response at 60 minutes with responses of several thousand pm at the highest concentrations tested, which is close to the upper limit of detection for Epic® technology. This suggests that the response leads to very large changes in refractive index at the biosensor, as would be expected following the formation of aggregates throughout the solution above the biosensor. Prednisone, on the other hand, exhibits almost no dose-dependency at 60 minutes, and the response is barely above the DMSO background. In this assay, compound was added to leave a final DMSO concentration of 2%, resulting in a DMSO background response at 60 minutes of ~500 pm. The magnitude of the DMSO background response directly correlates with final DMSO concentration, so that at concentrations above 2% the background would be higher whereas at concentrations below 2% it would be lower (data not shown). Figure 3 shows dose response curves for these compounds at 60 minutes, together with additional aggregators and non-aggregators, after the DMSO background has been subtracted. Each of the known aggregators exhibits a characteristic steep dose response curve, one of the hallmarks of aggregation using technologies such as DLS. In contrast, the known non-aggregators show no response at even the highest concentration tested (100 µM), and this observation was extended out to doses as high as 250 µM (data not shown). Thus, Epic technology can readily distinguish between aggregating and non-aggregating compounds in a simple assay format.

Another important characteristic of compound aggregation is sensitivity to non-ionic detergent such as Triton X-100. In order to evaluate whether Epic aggregation assays also demonstrated this attribute, two of the known aggregators were tested in assay buffer containing 0.1% Triton X-100. Figure 4 shows that the aggregation response is fully inhibitable up to 33.3 µM when detergent is included in the assay buffer. At concentrations > 33.3 µM, aggregation responses are observed in the presence of detergent, although they are attenuated in comparison to responses in the absence of detergent (data not shown). In addition to detergent-sensitivity, Figure 5 shows that Epic aggregation assays are sensitive to increasing concentrations of DMSO in the assay. For each of the three aggregators tested, there is a clear reduction in the magnitude of the response with increasing DMSO concentration, particularly at the higher concentrations of 33.3 and 100 µM. These data show that Epic aggregation assays share all of the key features of compound aggregation exhibited by other detection methods.

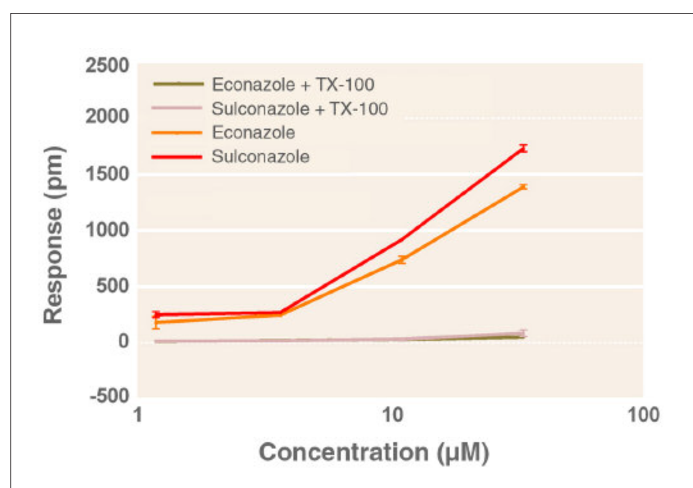


**Figure 4.** Aggregation responses using Epic technology are sensitive to non-ionic detergent. Two known aggregators, Econazole and Sulconazole were tested in assay buffer ± 0.1% Triton X-100 (TX-100) using the same dose response series as Fig. 2, with a final DMSO concentration of 2%.

Shoichet and colleagues have used DLS to define the "critical aggregation concentration" (CAC) of several aggregating compounds, which is the concentration above which a sharp and linear increase in particle numbers is observed (6).

Three of the aggregating compounds were included in that study, so a method was developed to determine the CAC using Epic® technology. A dose response series of each aggregator was run at a final DMSO concentration of 1% and compared to a non-aggregator, Thalidomide, tested at the same concentrations. The aggregation threshold was defined as the average of the Thalidomide response at each concentration plus 3x the standard deviation of the response ($3\sigma$). Figure 6 shows the data for each of the three aggregators with the $3\sigma$ line used to determine the minimum concentration at which an aggregation response is observed. Using this approach, the CAC using Epic technology is shown to be reduced by ~3-fold for Miconazole and Nicardipine, and almost 10-fold for Tetraiodophenolphtalein, when compared to DLS (Table 2). In the DLS assays, these compounds were tested at DMSO concentrations of either 0.1% or 0.5%. Shoichet and colleagues point out that CAC increases from 0.1% to 1% DMSO (6), which indicates that the sensitivity difference using Epic technology could be further enhanced at comparable DMSO concentrations. Finally, the entire panel of compounds was tested and found that all of the known aggregators had a CAC of 11.1 µM or lower (Table 3). While 5/6 of the known non-aggregators showed no response (CAC >100 µM), in three separate experiments it was observed that Ketoconazole gave an unexpected aggregation response with a CAC of 33.3 µM. It is possible that Ketoconazole has been falsely identified as a non-aggregator using other technologies. It is concluded that aggregation assays using Epic technology exhibit greater sensitivity than alternative detection technologies.

**Table 2. Summary of critical aggregation concentrations for three compounds using Epic technology and DLS.**

| | Critical Aggregation Concentration | |
|---|---|---|
| **Compound** | **Epic Technology** | **DLS*** |
| Miconazole | 1.2 µM | 3 µM |
| Tetraiodophenolphtalein | 1.2 µM | 10 µM |
| Nicardipine | 11.1 µM | 32 µM |

*Values for DLS taken from study by Coan and Shoichet (6).

## Conclusions

- Compound aggregation can be detected with Epic technology using a simple add and read assay format.
- Epic technology can readily differentiate aggregators from non-aggregators.

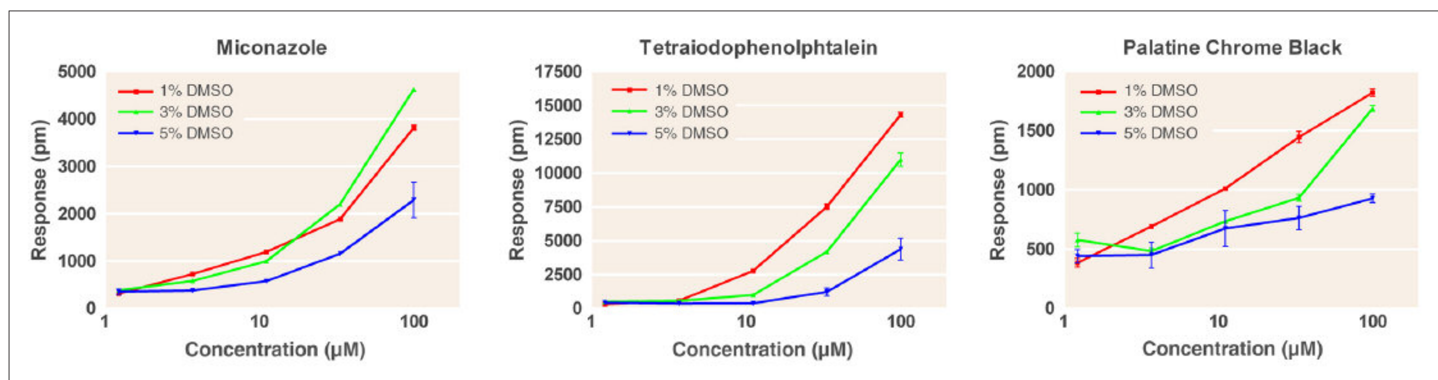


**Figure 5.** Aggregation responses using Epic technology are sensitive to increasing concentrations of DMSO. Three known aggregators, Miconazole, Tetraiodophenolphtalein, and Palatine Chrome Black were tested in assay buffer using the same dose response series as Fig. 2, but with increasing final DMSO concentrations.

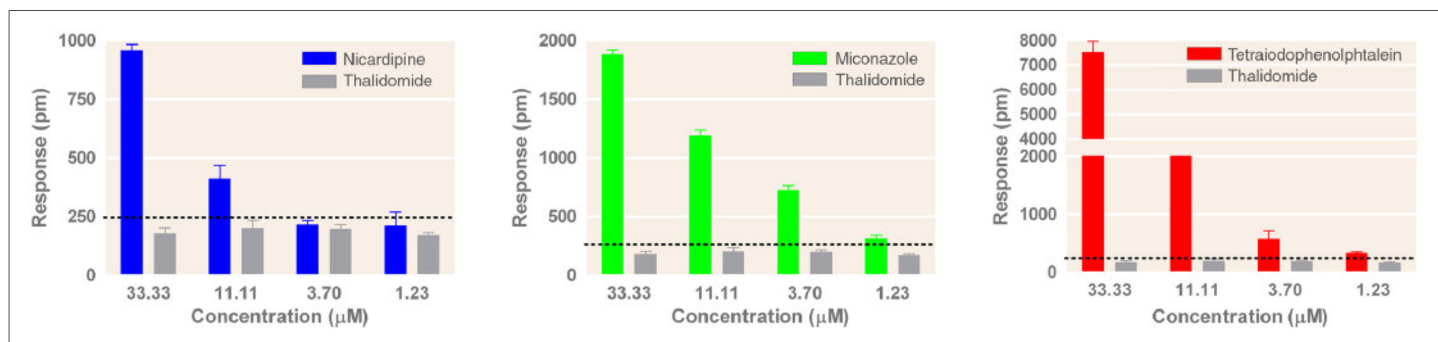


**Figure 6.** Critical aggregation concentration (CAC) can be determined using Epic technology. Dose series of three known aggregators, Nicardipine, Miconazole and Tetraiodophenolphtalein and a known non-aggregator, Thalidomide, was tested from 33.33 µM to 1.23 µM (1:3 dilutions) with a final DMSO concentration of 1%. Aggregation threshold is the average of the Thalidomide response at each concentration plus 3x the standard deviation of the response (dotted line). CAC is defined as the minimum concentration at which the dotted line intersects with the aggregator response.

**Table 3. Summary of CAC for all aggregators (left panel) and non-aggregators (right panel) tested using Epic® Technology.**

| Aggegators | CAC |
|---|---|
| Clotrimazole | 11.1 µM |
| Econazole | 11.1 µM |
| Nicardipine | 11.1 µM |
| Sulconazole | 3.7 µM |
| Palatine Chrome Black Miconazole | 3.7 µM |
| Miconazole | 1.2 µM |
| Tetraiodophenolphtalein | 1.2 µM |
| Rose Bengal lactone | 1.2 µM |

| Non-Aggegators | CAC |
|---|---|
| Fluconazole | >100 µM |
| Prednisone | >100 µM |
| Sulfadiazine | >100 µM |
| Thalidomide | >100 µM |
| Tripelennamine | >100 µM |
| Ketoconazole | 33.3 µM |

- Label-free aggregation shares important characteristics with other detection methods including steep dose response curves and sensitivity to non-ionic detergent.
- Epic aggregation assays are readily amenable to HTS by selecting a single time point for end reads between 30 and 60 minutes after compound addition.

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