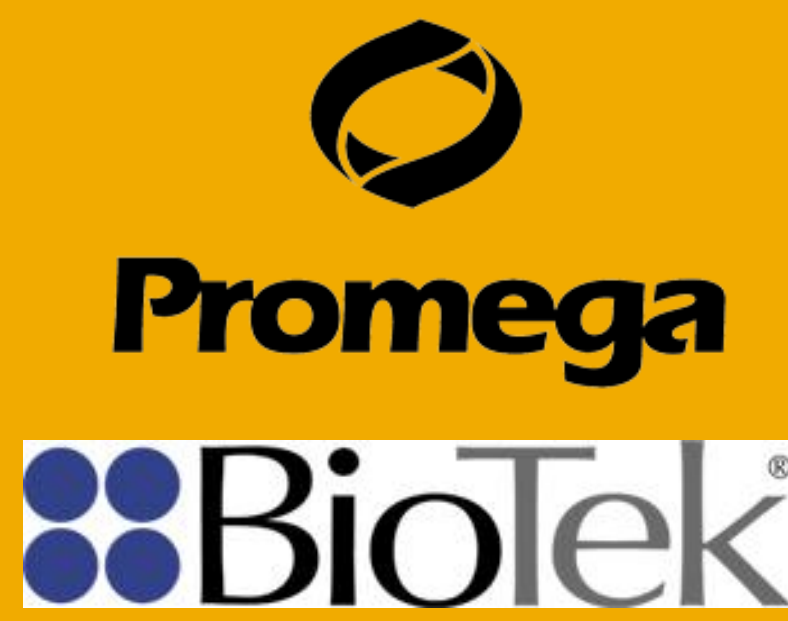


Ruggedness Testing Including an Evaluation of Automation of a Cell-Based Bioluminescent TNF α Blocker Bioassay

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1. Introduction / Purpose

TNF α blocker biopharmaceuticals represent an important and successful class of protein drugs used in the treatment of several autoimmune diseases, including rheumatoid arthritis, psoriasis and Crohn's disease. This success is driving the discovery of new versions of these protein drugs, new indications, and biosimilar development because some of these drugs come off patent protection soon.

Bioassays are indispensable tools in biopharmaceutical drug development and commercialization. They are used to quantify biological activity and stability of drugs or drug candidates. Precision and accuracy of the bioassay are all-important in both drug discovery and development, and in manufactured biopharmaceutical lot release, yet many bioassays suffer from drawbacks such as complexity and variability of results.

We developed a simple, homogeneous and robust bioluminescent TNF α blocker bioassay based on quantification of caspase 3 activity. The bioassay has good precision and accuracy and can be performed in one day. It uses U937 (human) cells which exhibit rapid response to TNF α . By developing and using U937 cells in single-use frozen, thaw-and-use format, we were able to remove variability arising from continuous cell culture.

Part of bioassay development also includes analysis of bioassay ruggedness, in which the influence of external factors on bioassay test results is measured. Our study here describes such an analysis of our TNF α blocker bioassay using a 96-well plate format. We included evaluation of automation in the analysis, as some bioassay laboratories use automation for liquid handling. For this, the assay steps of antibody titration and of cell and reagent dispensing were automated using a simple, yet robust liquid handler. Validation of the use of instrumentation with the assay chemistry was thus part of the study. Ruggedness variables evaluated were (i) manual and automated pipetting, (ii) bioassay plate used, (iii) luminometer / microplate reader used, and (iv) bioassay run (day). Assessment of ruggedness was based on (a) variability around RLUs obtained in plate uniformity tests using a single dose of TNF α blocker antibody, and (b) variability of assay EC₅₀ and Hill Slope obtained between assay runs of full dose-response titrations of TNF α blocker antibody.

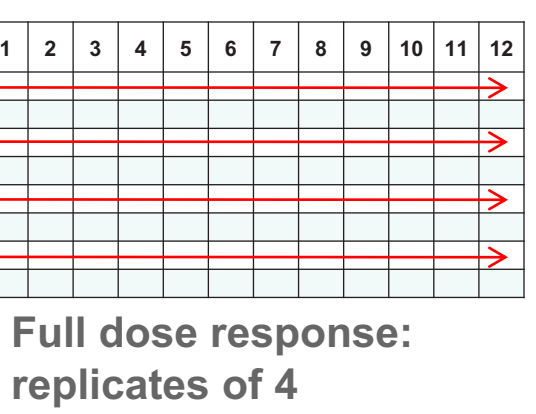
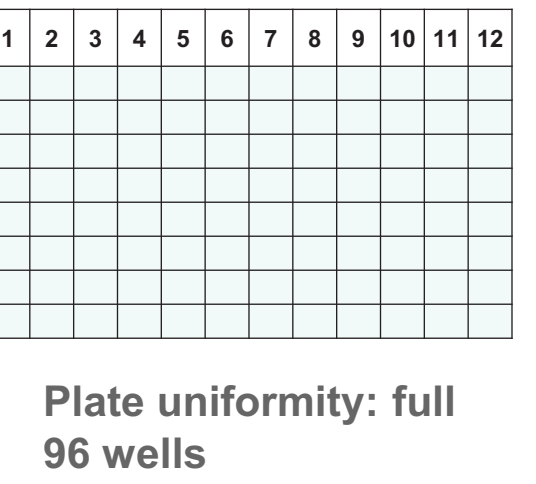
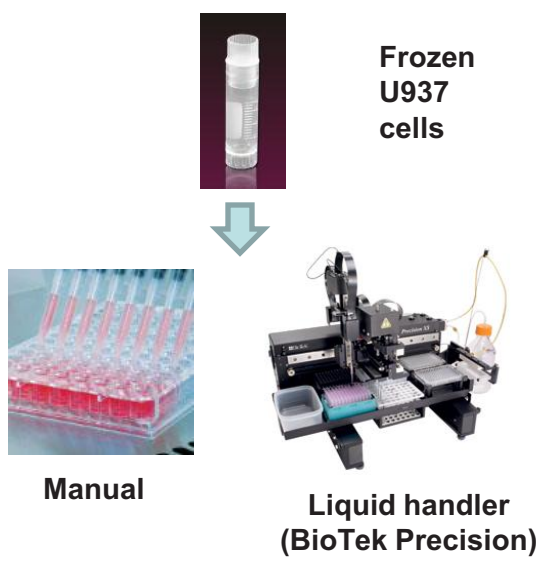
2. Methods

For all cell-based studies, frozen U937 cells (a human cell line which rapidly responds to TNF α with caspase induction) were thawed and diluted directly into warm medium. Cells (15K/well, 50 μ l) were immediately arrayed into several different brands of 96-well plates, manually or automatically using a BioTek Precision™ Microplate Pipetting System. Plates were equilibrated for 40 minutes in a 37°C/5%CO₂ incubator prior to addition of TNF α /Blocker combinations (samples). The same cell suspension was used for both manual and automated processing runs.

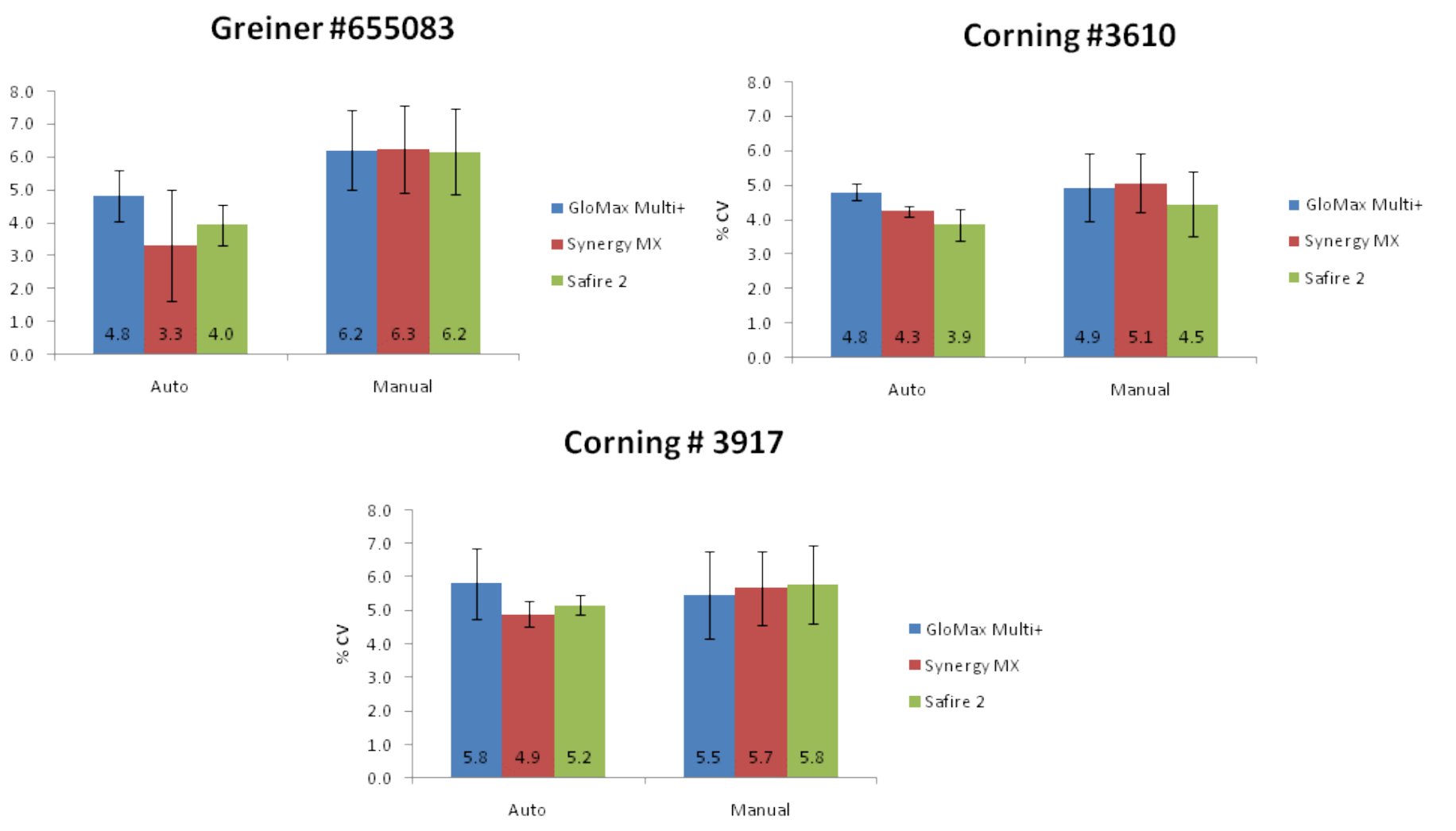
For plate uniformity bioassays, a single sample of TNF α /Blocker antibody, representing a preparation previously characterized to give partial blocking, was prepared (final 2.5ng/ml TNF α + 40ng/ml antibody) and manually dispensed into two identical plates and pre-incubated for 1 hr at 37°C. Samples (50 μ l each) were then transferred to all wells of several different brands of 96-well plates manually or automatically and incubated for 2.5 hr at 37°C. Each series of tests (3 manual, 3 automated) was independently performed 6 times.

For full titration bioassays, a full dose response series of antibody was serially prepared manually or automatically. An equal volume of TNF α (10ng/ml, 4x final) was added to the antibody dilutions (manually or automatically) and incubated for 1hr at 37°C. These samples were then transferred to 4 rows of the cell plates manually or automatically and incubated for 2.5 hr at 37°C. Each series of tests (3 manual, 3 automated) was independently performed 6 times.

To process plates for Caspase-Glo®3/7 detection, assay plates were removed and allowed to cool at room temperature for 30 minutes prior to adding Caspase-Glo® 3/7 reagent (manually or automatically, 100 μ l/well). Plates were shaken and luminescence recorded 1 hour later using 3 different plate luminometers (BioTek Synergy™ MX, Promega GloMax Multi+, and Tecan Safire®).



3. Summary of findings from plate uniformity study



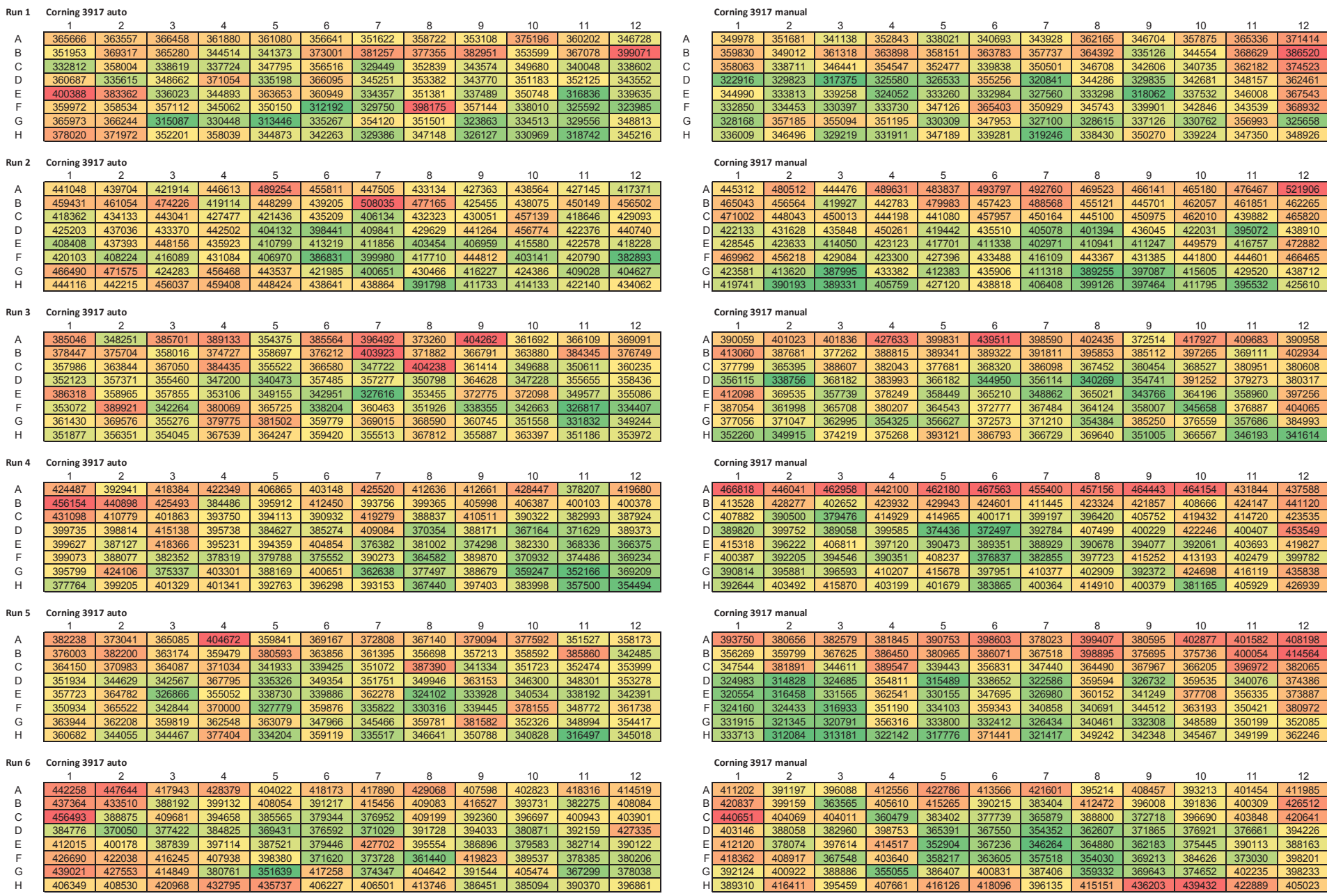
Summary graphs for plate uniformity are shown, arranged according to each of the three plates (vendor and part #) used in this study. Numbers within each bar represent the average % CV for that combination.

Automated vs. manual processing. Automated processing showed slightly better average % CV compared to manual processing. However, the error associated with the manual processing was more consistent across the six runs.

Impact of microplate on plate uniformity. There was no obvious trend in the data that would indicate superiority of one plate over another in this study. Of all the plates tested, the Corning #3610 plate showed the lowest overall full plate CV of those tested, though the other plates performed well.

Impact of microplate reader. In general, all three microplate readers performed equivalently.

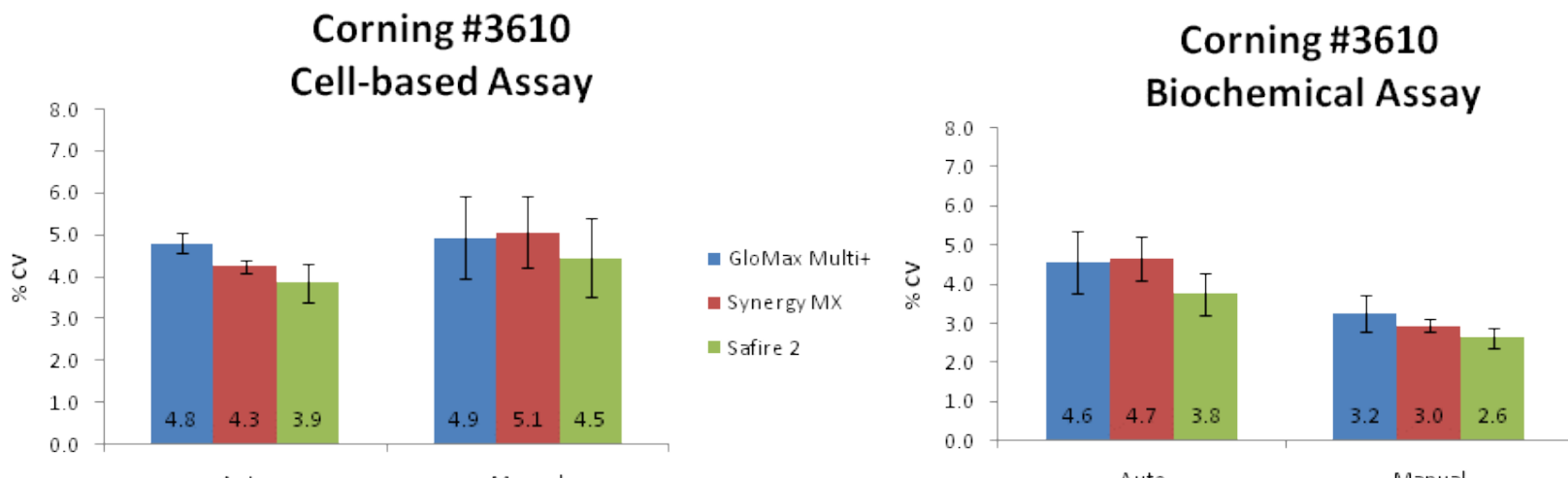
4. Plate uniformity evaluation shows random run-to-run signal variation



Raw RLU values obtained on the Synergy MX microplate reader using the Corning #3917 plate are shown for all six plate uniformity runs for both manual and automated pipetting. Automation processed plates are on the left and manual processed plates are on the right. The conditional formatting function in Excel was used to apply a color scheme to each data set. Yellow represents the 50th percentile signal on each plate, with green to red color gradient scaled accordingly to represent low to high signal from the mean, respectively.

The Synergy MX showed random run-to-run variation across all conditions. The same was true for all other combinations of microplates and readers evaluated.

5. Investigating cell impact on data quality



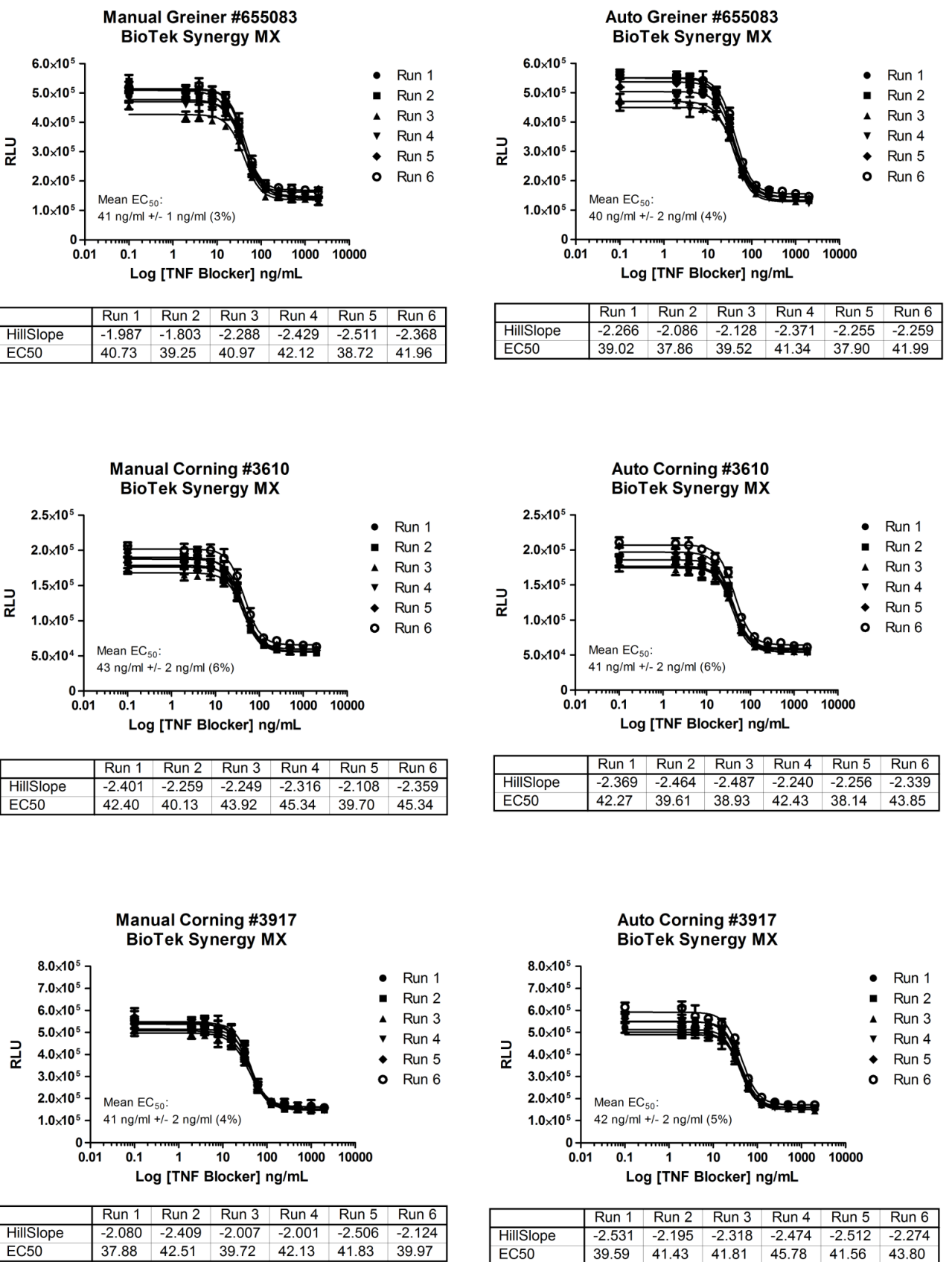
The contribution of cells to assay variability was investigated by comparing results from a cell-based assay to those generated using a biochemical assay to quantify caspase activity. One plate brand, all three plate readers/luminometers, and automation versus manual pipetting were included in the study.

Biochemical assay method. Manual and automated biochemical assays were assembled by adding 100 μ l of recombinant caspase-3 enzyme (Enzo) in 10mM Hepes + 0.1% Prionex to a Corning #3610 plate, followed by 100 μ l addition of Caspase-Glo 3/7 reagent as described previously. The amount of caspase-3 enzyme used was designed to give an assay signal close to that obtained in the cell-based assay. Luminescence was recorded with each microplate reader. This process was repeated six times, and included fresh enzyme preparation for each run.

Automation results. The plate uniformity of the cell-based and biochemical assays is nearly identical on the automated platform with this plate. It is possible that the pipetting precision limitation of the automated platform has been reached, or that additional steps required in the cell-based protocol, such as cell mixing prior to dispensing, are most consistent with the automated program.

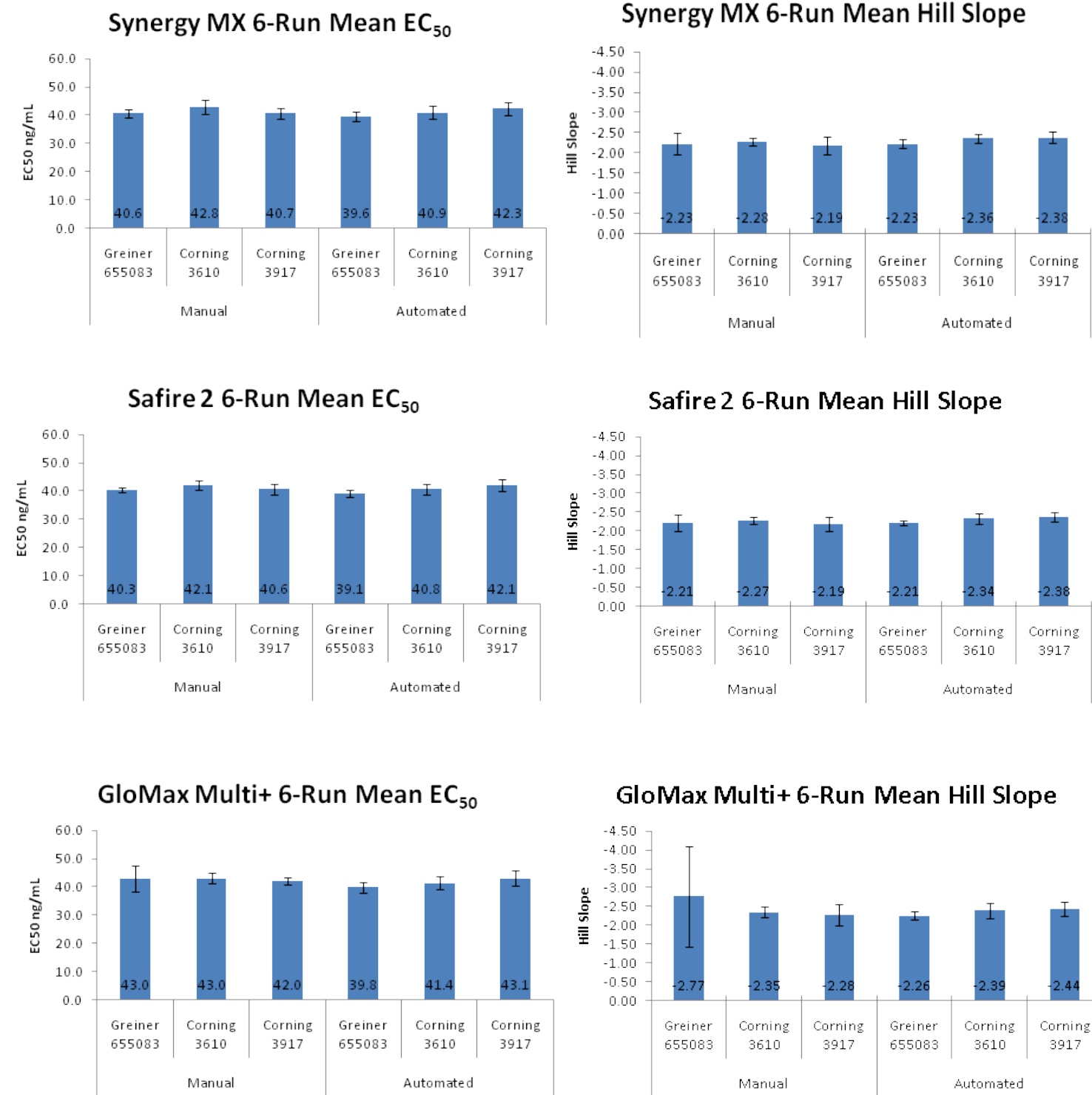
Manual results. Slight differences were observed between the cell-based and biochemical assays performed manually. The higher CV obtained in the cell-based assays could be attributed to inconsistent cell mixing or cell handling from run to run, which, as data suggest, may be improved with an automated platform.

6. Representative TNF α titration curves from the Synergy MX



Representative EC₅₀ titration data are shown from manual and automated processing, with all three brands of microplates, using the BioTek Synergy MX microplate reader for luminescence detection. Data were analyzed with GraphPad Prism v5.03 software, four parameter fit, variable slope curve fit analysis.

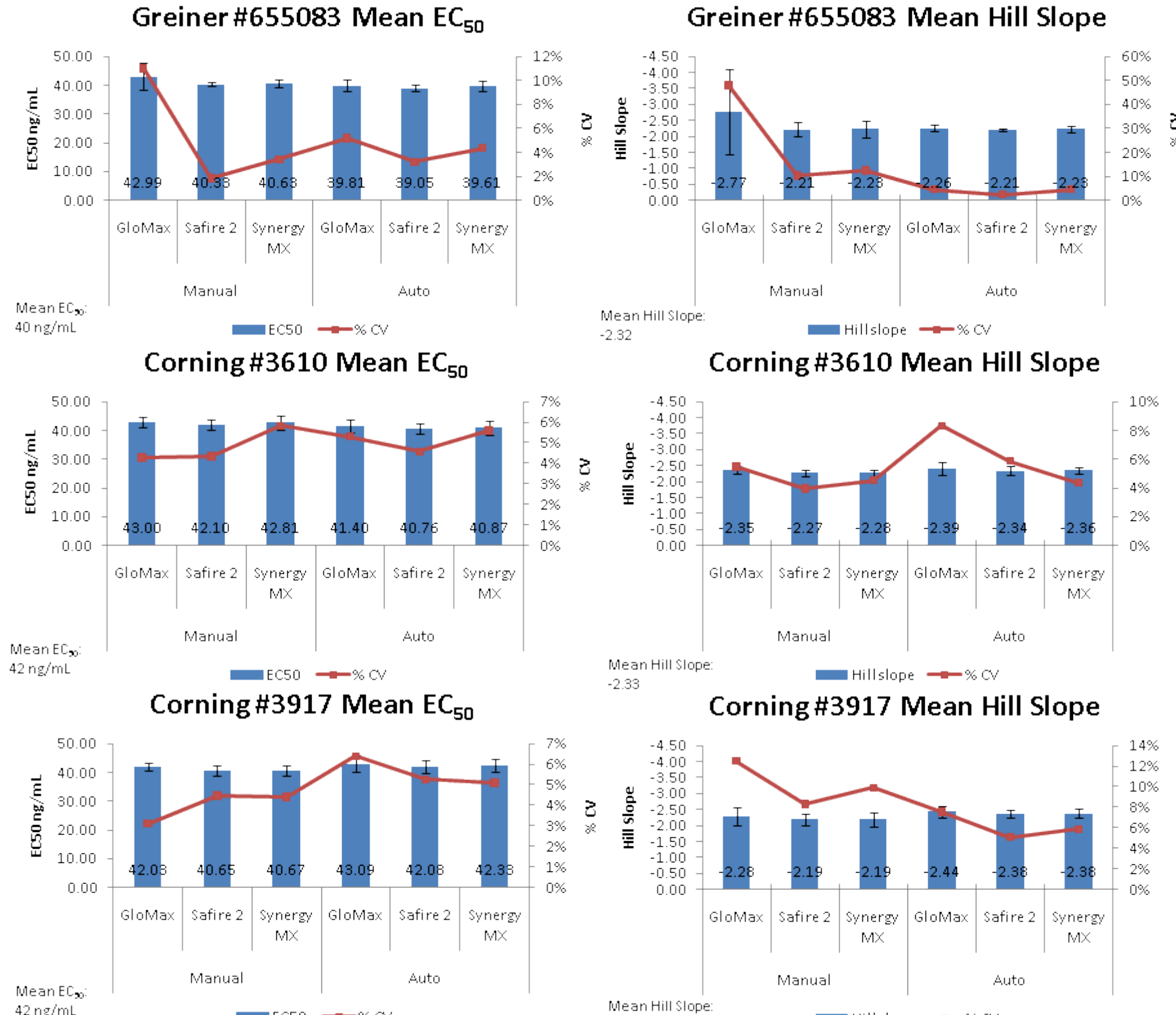
7. Comparison of EC₅₀ and Hill Slope data



Summary graphs of data from six runs for each condition are shown arranged by microplate reader, with EC₅₀ summary on the left and Hill Slope summary on the right. Numbers within each bar represent the average EC₅₀ or Hill Slope for the combination shown.

The average EC₅₀ are equivalent across all conditions, and except for one outlier run, the Hill Slope is also very consistent. The manual Greiner plate read on the GloMax in run 6 generated a more right-shifted EC₅₀ curve compared to previous runs on that reader. Aside from this one anomaly, all runs generated data that suggest equivalent performance across all conditions.

8. Influence of external factors on bioassay variability



Summary graphs of data from six runs for each condition are shown arranged by plate type, with EC₅₀ summary on the left and Hill Slope summary on the right. Numbers within each bar represent the average EC₅₀ or Hill Slope for the combination shown. The red line tracks across % CVs for each condition.

A slight difference in % CVs is noted depending on processing method, with the previously mentioned anomaly in run 6 with the Greiner plate contributing to variability in the GloMax manual condition. Overall the different vendor plates performed equivalently.

9. Summary and conclusions

Automating the liquid and cell pipetting steps of the TNF α blocker bioassay generated results that are comparable to running the assay manually, validating that the bioassay is automatable in 96-well format.

The TNF α blocker bioassay with frozen, thaw-and-use human cells is easy to perform in either manual or automated format.

Automated pipetting systems, such as the Precision, enable hands-free assembly of TNF α blocker bioassays, freeing up technician time for other laboratory activities.

Precision of luminescence signal obtained in the cell-based TNF α blocker bioassay was just as good in automated bioassay and only slightly less in manually set-up bioassay than biochemical assay based on the same detection (caspase activity).

Precision of EC₅₀ and Hill slope results in the cell-based TNF α blocker bioassay was generally good (< 8% CV) for almost all conditions tested.

The TNF α blocker bioassay was rugged, as demonstrated by essentially equivalent results obtained in plate uniformity assays and full titration bioassays using 3 different plate vendor/types, 3 different microtiter plate readers/luminometers and in automated assay assembly versus manual set-up.

The results obtained in these studies demonstrate that the cell-based TNF α blocker bioassay we have developed (i) is suitable for use in different labs using different 96-well assay plates and luminescence plate readers, (ii) can perform equivalently well in manual or automated mode, (iii) has good precision, comparable to that obtained with biochemical assay methods.

The bioassay is suitable for applications where quantification of blocking protein drug action on TNF α activity is needed.