Validation of an Automated Cell-Based Bioluminescent TNFα Blocker Bioassay

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Introduction

TNFα 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 indicators, and biosimilars development due to the fact that some of these drugs will soon lose patent protection.

Bioassays are indispensible tools in biopharmaceutical drug development and commercialization. They are used to quantify biological activity and stability of drugs or drug candidates. The automation of these assays can serve to create an accurate, robust process which can allow the researcher to perform other more important functions. Precision and accuracy of the automated bioassay are all-important in both drug discovery and development, and in manufactured biopharmaceutical lot release.

Here we demonstrate the automation of a 96-well homogeneous bioluminescent TNFα blocker bioassay based on quantification of caspase 3 activity. The bioassay can be performed in a single day, and uses single-use, frozen U937 (human) cells which exhibit rapid response to TNFα. A simple, yet robust liquid handler was used to automate the assay steps of antibody titration and of cell and reagent dispensing.

Part of bioassay development includes analysis of assay ruggedness, in which the influence of external factors on test results is measured. The study described here includes plate uniformity, as well as anti-TNFα blocker antibody titration tests. Variables included microplate used, run-to-run variability, as well as a comparison between manual and automated processing. Assessment of ruggedness was based on (a) variability around RLUs obtained in plate uniformity tests using a single dose of TNFα blocker antibody, and (b) variability of EC_{50} and assay window obtained between runs of full dose-response titrations of TNFα blocker antibody.

TNFα Blocker Bioassay

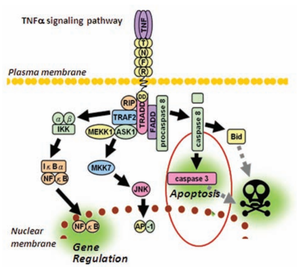

Figure 1 – The TNFα signaling pathway leads to multiple endpoints, including NfkB gene regulation, apoptosis induction, and cell death. The bioassay referenced here monitors caspase 3 activity.

BioTek Instrumentation

BioTek Liquid Handling

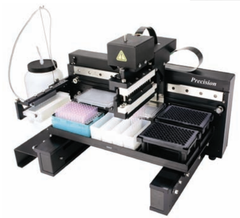

Figure 2 – The Precision™ Microplate Pipetting System combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to dispense U937 cells, serially titrate antibody across a 96-well PP plate, transfer samples from plate to plate, as well as for TNFα and reagent dispensing.

BioTek Detection

Figure 3 – The Synergy™ MX is a monochromator-based multi-mode microplate reader. A dedicated luminescence detection system is used to quantify the luminescent signal from each assay well.

Plate Uniformity Test

The goal of the plate uniformity study was to ensure that the cells, known ligand, blocker antibody, and detection reagent used in the assay could be consistently dispensed across a 96-well assay plate in an automated fashion. Manual dispensing was also performed as a control for comparison purposes. The test was independently performed a total of 6 times, using three different white 96-well plate types (Corning 3610, Corning 3917, and Greiner 655083). Average, standard deviation (SD), and %CV were computed for the 96 replicates tested on each microplate.

Plate Uniformity Test Method

- Manually dispense 200 µL/well of 2.5 ng/mL TNFα + 40 ng/mL anti-TNFα blocker antibody mix into the appropriate wells of a 96-well plate, and preincubate at 37°C/5%CO_2 for 60 minutes.
- Dispense 50 µL/well of U937 cells (15K cells/well) into a separate 96-well plate, and preincubate at 37°C/5%CO_2 for 30-40 minutes.
- Transfer 50 µL of the 2.5 ng/mL TNFα + 40 ng/mL anti-TNFα blocker antibody mix into each well of the cell plate, shake the plate for 30 seconds, and incubate at 37°C/5%CO_2 for 2.5 hours.
- Remove the plate from the incubator and allow to cool to room temperature (RT) for 30 minutes.
- Add 100 µL of Caspase-Glo® 3/7 reagent, shake the plate for 30 seconds, and incubate at RT for 60 minutes.
- Read the luminescent signal from the plate following the incubation period.

All dispensing steps, except for step 1 of the Plate Uniformity Test, were performed manually, and robotically, using the Precision.

Plate Uniformity Test *(continued)*

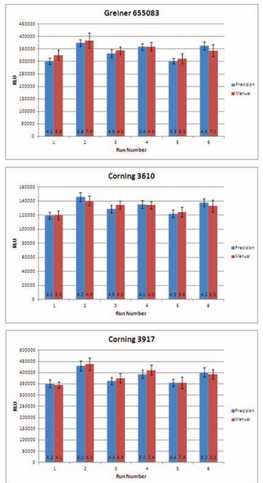

Figure 4 – Manual and automated plate uniformity results. Average ±SD data is shown for the three plates included with each individual performance of the test. Numbers at the bottom of each bar represent the %CV values computed from each average and standard deviation.

Corning 3917 auto

	1	2	3	4	5	6	7	8	9	10	11	12
A	441048	439704	421914	446613	469254	455811	447505	433134	427363	438564	427145	417371
B	459431	461054	474226	419114	448299	439205	508035	477165	425455	438075	450149	456502
C	418362	434133	443041	427477	421436	435209	406134	432323	430051	457139	418646	429093
D	425203	437036	433370	442502	404132	398441	409841	429629	441264	456774	422376	440740
E	408408	437393	448156	435923	410799	413219	411856	403454	406959	415580	422578	418228
F	420103	408224	416080	431084	406970	389831	399560	417710	444812	403141	420796	382893
G	466490	471575	424283	456468	443537	421985	400651	430466	416227	424386	409028	404627
H	444116	442215	456037	459408	448424	438641	438864	391798	411733	414133	422140	434062

Corning 3917 manual

	1	2	3	4	5	6	7	8	9	10	11	12
A	445312	480512	444476	489631	483857	493797	492760	469523	468141	465180	476467	521906
B	465043	456564	419927	442783	479983	457423	488568	455121	445701	462057	481851	462265
C	471002	448043	450013	444198	441080	457957	450164	445100	450975	462010	439882	465820
D	422133	431628	435848	450261	419442	435510	405078	401394	436045	422031	395072	438910
E	428545	423633	414050	423123	417701	411338	402971	410941	411247	449579	416757	472682
F	469962	456218	429084	423300	427396	433488	416109	443367	431385	441800	444601	466465
G	423581	413620	387995	433382	412383	435906	411318	389255	397087	415605	429520	438712
H	419741	390193	389331	405759	427120	438818	406408	399126	397464	411795	395532	425610

Figure 5 – Raw luminescence values from robotically and manually dispensed plates from a single performance of the test, using Corning 3917 plates.

The low %CV values obtained through automated dispensing (from 3.4-5.2%) and the lack of any discernible negative dispensing pattern among all plate types tested, demonstrate the ability of the Precision to consistently and evenly dispense the assay components in 96-well format. Also, compared to the %CV values obtained from manual pipetting (from 4.0-7.9%) by an experienced pipetter familiar with the assay, the %CVs of the automated system show a slight improvement; indicative of a more robust assay procedure. The lower luminescence values seen with the Corning 3610 can be attributed to the clear well bottom of this plate, while the other two plates have solid white well bottoms, which increases the luminescence signal from the wells.

Anti-TNFα Blocker Ab Titration Test

Anti-TNFα blocker antibody titration tests were completed in order to ensure that the Precision could accurately and evenly dilute the blocker antibody across a 96-well plate. Serial 1:2 dilutions were performed to create a 12-point titration series with antibody concentrations ranging from 2000-0 ng/mL. Inhibition curves, EC_{50} values, and assay window were compared between manual and automated processing. The test was once again performed a total of six times using the same three assay plate formats.

Anti-TNFα Blocker Ab Titration Test

1. Serially titrate the anti-TNFα blocker antibody in assay medium using a 100 µL total final volume per well.
2. Dispense 100 µL/well of 10 ng/mL TNFα to each well of the titration series. Shake the 96-well plate for 30 seconds, and incubate at 37°C/5%CO_2 for 60 minutes.
3. Dispense 50 µL/well of U937 cells (15K cells/well) into a separate 96-well plate, and preincubate at 37°C/5%CO_2 for 30-40 minutes.
4. Transfer 50 µL of the TNFα/blocker antibody titration mix into the appropriate wells of the cell plate, shake the plate for 30 seconds, and incubate at 37°C/5%CO_2 for 2.5 hours.
5. Remove the plate from the incubator and allow to cool to room temperature (RT) for 30 minutes.
6. Add 100 µL of Caspase-Glo® 3/7 reagent, shake the plate for 30 seconds, and incubate at RT for 60 minutes.
7. Read the luminescent signal from the plate following the incubation period.

All dispense steps were performed manually and robotically using the Precision.

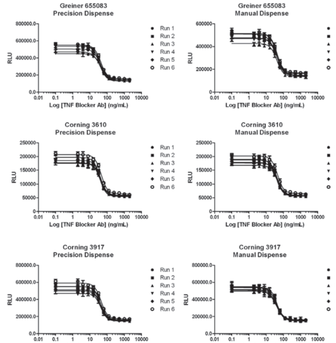

Figure 6 – Representative curves showing increased blocking of the TNFα pathway with increasing antibody concentration.

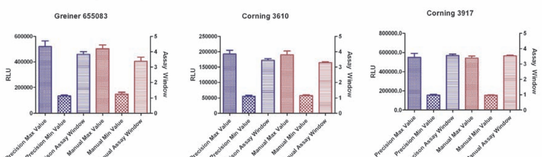

Figure 7 – Average maximum and minimum signal, as well as assay window shown for the six manual and automated runs performed with each assay plate type. Error bars represent the SD for each average value.

Anti-TNFα Blocker Ab Titration Test *(continued)*

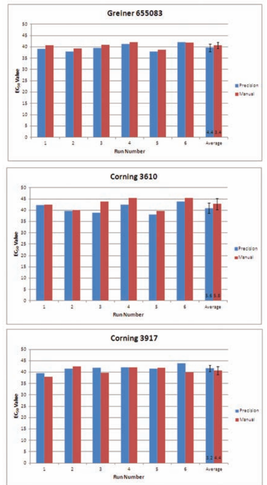

Figure 8 – EC_{50} values generated from anti-TNFα blocker antibody inhibition curves. Individual values from each curve are shown, as well as averages from the six runs for robotic and manual dispensing with each plate type. SD is also shown with each average, in addition to %CV values listed at the bottom of each average bar.

The similarity between the curves generated from each antibody titration, as well as low variation among EC_{50} values, with %CVs ranging from 3.2-5.6%, demonstrate the capabilities of the Precision to consistently titrate the anti-TNFα blocker antibody in 96-well format, using each plate type. In addition, the close agreement in average EC_{50} values between all automated and manual runs completed, 40.73 and 41.37 ng/mL, respectively show that accurate dilutions are achieved with the Precision. Finally, matching average assay window values when compared to those generated using other microplate readers, which all range from 3.2-3.8 (experiment not shown), confirm that no loss in performance is seen when the Synergy Mx is used for signal detection.

Conclusions

1. The cell-based TNFα blocker bioassay provides a simple, efficient process, when compared to other methods, for the quantification of blocking protein drug action on TNFα activity.
2. The assay can be automated in 96-well format, further simplifying the process and freeing the researcher to accomplish other, more important tasks.
3. The Precision can consistently and accurately titrate the anti-TNFα blocker antibody, as well as dispense the other assay components.
4. The Synergy Mx is able to easily and correctly quantify the luminescent signal from assay wells using each plate type tested.
5. The low %CV values seen in the plate uniformity test, as well as similarity in blocking curves and EC_{50} values seen in the antibody titration test, demonstrate that the automated bioassay provides a complete, rugged solution to test potential biosimilars for their effectiveness in blocking TNFα activity.