Expanding Applications of Bioluminescence Technology: Predictive Bioassays for TNFα Biologicals Potency and Dose-Standardization Studies

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Implementing straightforward predictive automation-friendly bioassays as demonstrated in this tutorial allows effective assessment of anti-TNFα and likely other biologicals for quality control.

INTRODUCTION

Tumor Necrosis Factor alpha (TNFα) is a potent cytokine involved in immune surveillance and function. Dysregulated TNFα levels have been implicated in chronic disease states such as rheumatoid arthritis, Crohn's disease and several neurological diseases, making TNFα an attractive drug target. A number of recombinant anti-TNFα biologicals have been developed and licensed for management of human disease, but simple and predictive bioassays for potency and dose-standardization are still sought by the biopharmaceutical industry. Automatable, homogeneous bioassays are desirable because they can help to streamline this evaluative process. Implementing straightforward predictive automation-friendly bioassays as demonstrated in this tutorial allows effective assessment of anti-TNFα and likely other biologicals for quality control.

PREDICTABILITY AND PRODUCTIVITY WITH BIOLUMINESCENCE ASSAYS

Two simple, predictive bioassays from Promega are highlighted in this tutorial. The CellTiter-Glo® Luminescent Cell Viability Assay[a–c] provides a homogeneous bioluminescent assay for quantitation of ATP, an indicator of metabolically active cells, present inside cells in culture. The assay is designed for use in multiwell plate formats, making it ideal for automated cell viability, cell proliferation and cytotoxicity studies. The ONE-Glo™ Luciferase Assay[c–e] provides a homogeneous bioluminescent assay for quantitation of luciferase expression in mammalian cells containing genes for firefly luciferase. The assay contains a new luciferase substrate, resulting in a reagent that is more stable, more tolerant to sample components, and has less odor than standard luciferase assay reagents, which makes it ideally suited for high-throughput applications.

Both assays are rapid, simple and sensitive. The protocols involve adding reagent directly to cells cultured in growth medium (without washing or preconditioning), mixing, and reading the "glowing" luminescent signal on a luminometer. For the CellTiter-Glo® Assay, the luminescent signal is proportional to the amount of ATP present in the sample, which is directly proportional to the number of cells present. For the ONE-Glo™ Assay, luminescence is proportional to the upregulation of a specific genetic element and subsequent expression of firefly luciferase.

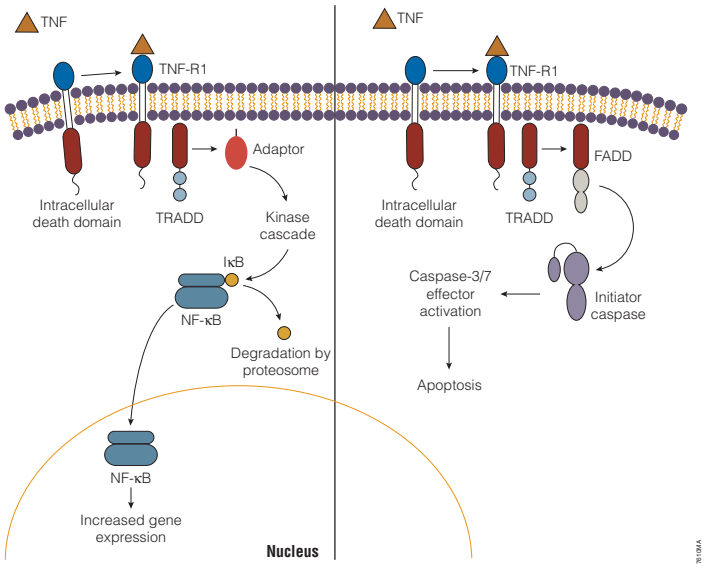

Figure 1. Schematic of two TNFα-mediated pathways inside cells, including survival-based NF-κB activation and apoptosis.

TNFα-MEDIATED ACTIVATION OF NF-κB AND NEUTRALIZATION OF TNFα BIOACTIVITY BY ANTI-HUMAN TNFα MONOCLONAL ANTIBODY IN GLORESPONSE™ CELLS

One of the most important downstream signaling targets activated by TNFα is the NF-κB transcription factor, which is involved in diverse biological processes including inflammatory, anti-apoptotic, and immune responses. In resting cells, the activity of NF-κB is controlled through its cytoplasmic sequestration by inhibitor of kappa B (IκB). Binding of TNFα to its cell surface receptors initiates a series of kinase cascades, which lead to phosphorylation of IκB, ubiquitination and proteasome-dependent degradation. The released NF-κB then undergoes nuclear translocation and activation, which induces the transcriptional expression of target genes (Figure 1).

GloResponse™
NF-κB-RE-*luc*2P HEK293 Cell Line is designed for rapid and convenient analysis of any cellular response that results in modulation of NF-κB activities.

Luciferase reporter assays have been used widely to investigate cellular signaling pathways. In this study, the GloResponse™ NF-κB-RE-*luc*2P HEK293 Cell Line[c,d,f–i] was used. This line is a clonal derivative of Human Embryonic Kidney 293 (HEK293)[h] cells that contains a luciferase gene (*luc*2P) under the control of a minimal TATA promoter with multiple Nuclear Factor-κB Response Elements (NF-κB-REs). This NF-κB reporter cell line is designed for rapid and convenient analysis of any cellular response that results in modulation of NF-κB activities.

To evaluate TNFα-mediated activation of NF-κB, GloResponse™ NF-κB-RE-*luc*2P HEK293 cells were robotically dispensed using an ep*Motion*® 5075 robotic liquid-handling workstation (Eppendorf) at a density of 10,000 cells per well into white-bottom, white opaque 96-well Costar® #3917 microplates (Corning) in 50 µl volumes of DMEM supplemented with 10% fetal bovine serum (FBS). Threefold serial dilution treatments of TNFα in 50 µl medium per well were robotically dispensed across the plate. Treated cells were allowed to incubate for 5 hours at 37 °C, 5% CO_2. Controls were included on each plate, one column of wells containing untreated cells and one cell-free column.

To evaluate anti-TNFα-mediated neutralization of NF-κB, GloResponse™ NF-κB-RE-*luc*2P HEK293 cells were dispensed as described above using a Freedom EVO® Robotic Workstation (Tecan) and treated immediately with 10 ng/ml of TNFα and increasing concentration of anti-human TNFα monoclonal antibody (R&D Systems). Threefold serial dilutions of the anti-TNFα monoclonal antibody plus TNFα in 50 µl medium per well were robotically dispensed across the plate. Treated cells were allowed to incubate for 5 hours at 37 °C, 5% CO_2. Controls also were included on the plate.

Following the treatment, 100 µl of the ONE-Glo™ reagent was robotically added to each well of all plates, and contents were mixed on a rotating platform for 10 minutes. Luminescence associated with luciferase gene expression due to modulation of NF-κB activities was measured using a GloMax®-Multi Plate Reader.

Z´-factor, a statistical measure of reproducibility and robustness, was determined for each assay. A concentration of 10 ng/ml TNFα or 10 ng/ml TNFα plus 1 µg/ml anti-TNFα in 50 µl medium per well was used for induction in part of the plate, whereas the other portion of the plate was left untreated. Cells were robotically prepared and treated similarly to the methods described above.

Dose response curve results using the ONE-Glo™ Assay are shown in Figure 2 for TNFα- and anti-TNFα-treated GloResponse™ NF-κB-RE-*luc*2P HEK293 cells dispensed robotically in 96-well plates. The EC_{50} value, or half maximal effective concentration of TNFα induction, obtained for suspension cell conditions with TNFα-treatment alone was 1.1 ng/ml. The ND_{50} value, or half neutralization dose, obtained for suspension cell conditions for TNFα neutralization was 78 ng/ml. Z´-factor values for both automated treatment conditions were >0.71, indicating excellent assay quality. In addition, both treatment conditions showed a great dynamic response.

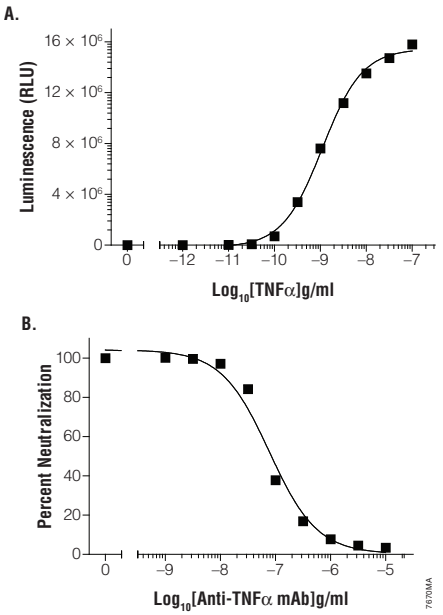

Figure 2. Automated ONE-Glo™ Assay dose-response curve. Dose-response curves were generated using TNFα-treated (**Panel A**) and anti-TNFα-treated (**Panel B**) GloResponse™ NF-κB-RE-*luc*2P HEK293 cells in 96-well plate format. EC_{50} value obtained for TNFα-treatment assay was 1.1 ng/ml. ND_{50} value obtained for TNFα neutralization assay was 78 ng/ml. Z´-factor values for both assays were >0.71.

ANTI-TNFα POTENCY DETERMINATION USING AN L-929 CYTOTOXICITY MODEL

In addition to initiating and augmenting transcriptional activation events, TNFα can promote programmed cell death via the extrinsic apoptosis pathway. Engagement of TNF receptors by the TNFα protein ligand initiates procaspase-8 processing into active caspase-8, whereby active caspase-8 activates procaspase-3 (and -7) to produce cat-

alytically active caspase-3/7. Ultimately, the proteolytic action of caspase-3/7 on various structural elements within a cell leads to apoptotic cell death (Figure 1).

The biological activity of TNFα, or potency of anti-TNFα agents in the presence of a standardized concentration of TNFα, can be measured by determining the number of viable cells remaining after treatment. The CellTiter-Glo® Assay is recognized as a rapid, reliable and robust means of determining viability and is well suited for this application.

The mouse cell line L-929 (ATCC) was selected due to its historical sensitivity to TNFα. L-929 cells were seeded into clear-bottom, white opaque 96-well Costar® #3903 microplates (Corning) in 50 µl volumes of DMEM supplemented with 10% FBS. Cells were allowed to adhere for four hours in a sterile incubator at 37 °C, 5% CO_2. TNFα was diluted to 1 ng/ml in culture medium supplemented with the anti-neoplastic antibiotic, actinomycin D (Sigma). Anti-TNF monoclonal antibody was diluted to 5 µg/ml in the actinomycin D-containing culture medium described above, and twofold serial dilutions were created. The TNFα and antibody dilutions were combined and pre-incubated for 30 minutes at 37 °C, 5% CO_2. Fifty microliters of each dilution was delivered to the assay plate containing cells, mixed briefly using an orbital shaker, and incubated overnight at 37 °C, 5% CO_2. A parallel plate was prepared for Z´-factor analysis using 5 µg/ml anti-TNFα with 1 ng/ml TNFα and medium with TNFα serving as the cytotoxicity control.

After the overnight treatment, 100 µl of CellTiter-Glo® Reagent per well was added to each plate using the Freedom EVO® Robotic Workstation (Tecan), and contents were mixed briefly on an orbital shaker. Luminescence associated with cell viability was measured using a Safire2 Microplate Reader (Tecan).

Dose-response-curve results are shown in Figure 3 for TNFα- and anti-TNFα-treated L-929 cells using the CellTiter-Glo® Assay dispensed robotically in 96-well plates. The ND_{50} value obtained with adherent cell conditions for TNFα neutralization was 45 ng/ml. The Z´-factor value for this automated assay was >0.76, once again indicating excellent assay quality.

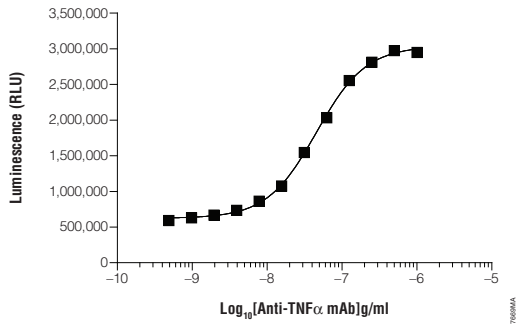

Figure 3. Automated CellTiter-Glo® Assay dose-response curve after TNFα-neutralization in L929 cells in a 96-well plate format. Assay shows ND_{50} to be 45 ng/ml and Z´-factor >0.76.

CONCLUSIONS

The homogeneity and simplicity of both bioluminescent assays presented in this tutorial demonstrates that they are well suited for various applications in assessing cell viability and luciferase expression, including when used on automated platforms (all Z´ factor >0.71). The biological potency of TNFα and anti-TNFα agents can be easily and reproducibly determined using the combination of the GloResponse™ NF-κB-RE-*luc*2P HEK293 Cell Line and the ONE-Glo™ Luciferase Assay or an established cell model for cytotoxicity combined with the CellTiter-Glo® Assay. Both assays deliver functional measures of TNFα bioactivity, which should prove useful for potency characterization in both research and biomanufacturing environments.

ACKNOWLEDGMENT

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ORDERING INFORMATION

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay*	10 ml	G7570
ONE-Glo™ Luciferase Assay System*	10 ml	E6110
Tumor Necrosis Factor-α, Human, Recombinant (rhTNFα)	10 µg	G5241
GloResponse™ NF-κB-RE-*luc*2P HEK293 Cell Line	2 vials	E8520
GloMax®-Multi Detection System		E7031

* For Laboratory Use.

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294 and other patents and patents pending.

[b]U.S. Pat. No. 7,083,911, Australian Pat. No. 2002255553 and other patents pending.

[c]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

[d]Patent Pending.

[e]Certain applications of this product may require licenses from others.

[f]For research use only subject to the purchaser's prior agreement to the terms of the limited use label license. Excluded from such use is imaging living animals. This product and/or its use is subject to one or more of the following Promega patents: U.S. Pat. Appln. Ser. Nos. 09/645,706, 10/664,341, 10/943,508, U.S. Pat. Nos. 5,670,356, 6,387,675 and 6,552,179, Australian Pat. No. 698424 and various corresponding patent applications and issued patents.

[g]Commercial use of this cell line requires a license from AdVec Inc.

[h]HEK293 cells were obtained under license from AdVec Inc.

[i]Use of Genetically Modified Microorganisms (GMM)
Information for European Customers: These products are genetically modified as described in Promega technical literature. As a condition of sale, use of this product must be in accordance with all applicable local guidelines on the contained use of genetically modified microorganisms, including Council Directive 90/219/EEC as amended by Council Directive 98/81/EC.

Potency Characterization: Both the ONE-Glo™ Luciferase Assay in combination with the GloResponse™ NF-κB-RE-*luc*2P HEK293 cell line or the CellTiter-Glo® Assay used with an established cell model for cytotoxicity deliver functional measures of TNFα bioactivity, which should prove useful for potency characterization in both research and biomanufacturing environments.