ESSEN BioScience

APPLICATION NOTE

CellPlayer™ 96-Well Kinetic Reporter Gene Assay

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Introduction

Transcription factors and their associated signaling pathways continue to be promising targets in a variety of different therapeutic areas[1]. Reporter gene assays represent one of the most common ways to investigate these pathways and associated transcription factor activity[2]. The most commonly used reporter assays rely on colorimetric/spectrophotometric (e.g. secreted alkaline phosphatase, or β-galactosidase), fluorometric (using fluorescent proteins), or bioluminescent (luciferase oxidative enzyme) reporter methodologies. However, the widest array of reagents and the most diverse collection of constructs available utilize luciferase as the reporter[3]. More recently, a significant number of reagents using multiple variants of GFP, as well as other specialized varieties of fluorescent proteins as reporter genes, have become commercially available[4]. These reagents include reporter constructs designed to measure transcriptional activation using pre-defined regulatory promoter elements, or empty vectors with multiple cloning sites enabling user-defined customizable promoters. The development of these reagents can be attributed to a number of factors including the use of flow-cytometry as a method to measure reporter activity, the availability of high-content fluorescent imaging platforms containing associated software packages that allow users to quantify multiple parameters using image based analyses, and an increased interest in quantifying reporter activity in a non-perturbing fashion that does not require cell lysis such that temporal morphological observations can be made in addition to quantitative readouts.

This application note describes a proof of concept experiment illustrating the utility of the IncuCyte FLR imaging platform for use in a reporter gene assay. This was accomplished by measuring the activation of the NF-κB transcription factor following stimulation with TNFα as illustrated in Figure 1. This signaling pathway has been extensively studied and is commonly interrogated using reporter gene assays. Our results show that the IncuCyte FLR platform and associated software package can be used to kinetically track the activation of NF-κB following stimulation with TNFα. In comparison to a luciferase assay, this approach has several potential advantages.

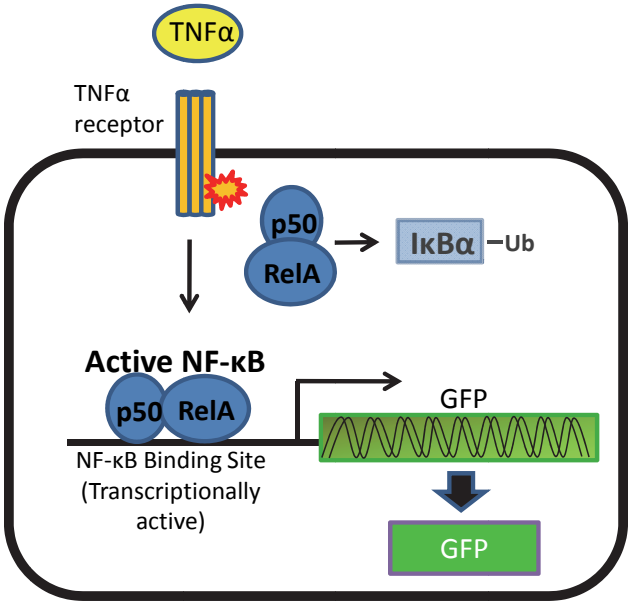

Figure 1: Schematic representation of the NF-κB reporter gene concept. Cells are transfected or infected with a vector containing the NF-κB transcriptional response element just upstream of the GFP reporter gene. Cells are then treated with TNFα which activates the TNFα receptor followed by degradation of IκBα, and translocation of the NF-κB transcription factor subunits to the nucleus where they participate in driving the expression of the GFP transgene. Green fluorescing cells can be kinetically imaged using the IncuCyte FLR microscope and the number of fluorescing cells can be quantified.

Using the IncuCyte FLR for reporter gene assays is:

1) **Data-rich**: providing real-time kinetic data not attainable with end-point assays
2) **Cost-effective**: does not require a terminal reaction substrate such as with luciferase assays saving time and reducing costs
3) **Convenient**: kinetic data enables the user to optimize the signal window within one experiment without making decisions *a priori*, as to when to stop the experiment
4) **Sensitive**: one can utilize multiple temporal data points per condition, enhancing the quantitative robustness of the assay.
5) **Customizable**: the user has the ability to modify the system per their interests (e.g. utilizing custom promoters in multiple cell types).

Approach and Methods

The first challenge in designing a reporter system is delivering the reporter DNA into the cell. In this study, human embryonic kidney (HEK) 293 cells were used based on their ease of transfection. Other strategies, such as viral based infections, could be employed to deliver reporter constructs into difficult to transfect cell types. In this experiment, a reverse transfection strategy was utilized. Briefly, in each well of a poly-D-lysine coated 96-well plate, 320 ng of PathDetect *cis*-Reporter Plasmid pNF-κB-hrGFP (Agilent Technologies, Santa Clara, CA; Cat# 240051) was mixed with 0.4 µl of Lipofectamine 2000 transfection reagent (Life Technologies, Carlsbad, CA) in a total of 50 µl of serum free Opti-MEM reagent consistent with the transfection reagent protocol. Following a 20 minute, room temperature incubation, 60,000 HEK 293 cells were seeded directly on top of the transfection reagent in each well. The plate was placed within a standard tissue culture incubator at 37ºC and 6% CO_2 overnight. The following day, cells were serum starved for 2 hours prior to addition of an 8 point dilution series of rhTNFα (R&D Systems, Minneapolis, MN) in F12-K medium containing 0.5% FBS. The plate was placed into a microplate tray within an IncuCyte FLR contained within a standard tissue culture incubator. The IncuCyte FLR was set to capture both phase and fluorescent images at 15 minute intervals. The following day, the data were analyzed using the object counting algorithm contained within the IncuCyte FLR basic software package. EC50 values were calculated at the 10 hour and 20 hour time points by exporting the data to GraphPad Prism 5.0 for Windows.

Results

As shown in Figure 2, stimulation of HEK 293 cells transiently transfected with pNF-κB-rhGFP with 11 ng/ml rhTNFα resulted in a significant induction of rhGFP expression within 5 hours. Furthermore, we observed a significant increase in the population of rhGFP expressing cells at both 10 and 20 hours post stimulation. In contrast, we did not see a significant increase in the percentage of rhGFP expressing cells in wells that were not treated with rhTNFα. To quantify the percentage of cells expressing rhGFP, we applied an object counting segmentation mask to the fluorescent images. This mask accurately separates fluorescent objects from the background. From this segmentation mask, the IncuCyte FLR software automatically calculates the percentage of the image that is occupied by segmented fluorescent objects. This metric is termed "Object Confluence". Values computed for these images are listed at the bottom of Figure 2.

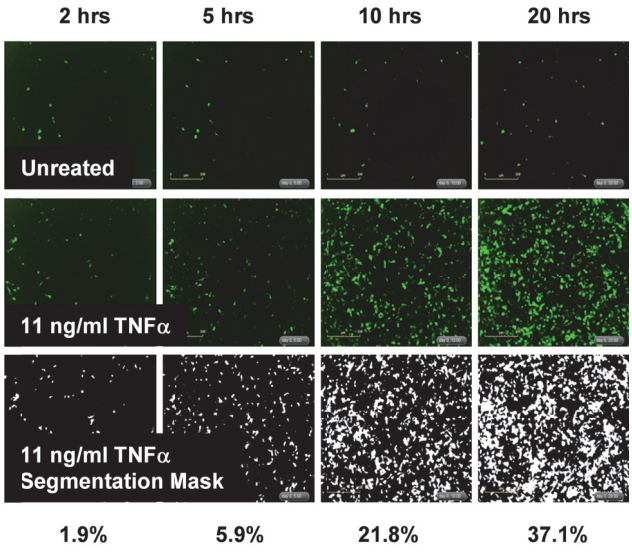

Figure 2: Induction of NF-κB driven rhGFP reporter gene expression following rhTNFα stimulation. HEK 293 cells transiently transfected with the pNF-κB-rhGFP reporter gene construct were treated with 11 ng/ml rhTNFα. Representative images at the indicated time points are shown for untreated control wells and wells treated with rhTNFα. The fluorescent images in the rhTNFα treated group were fitted with an object segmentation mask using the object counting algorithm contained within the IncuCyte basic software package (Bottom row). The percentages represent the "Object Confluence", or the percentage of the image occupied by fluorescent objects.

Using this strategy, all of the images in the time course (8000 in total) were automatically analyzed by the IncuCyte FLR object counting algorithm and graphed within the IncuCyte software (Figure 3). The resulting data illustrate a classic concentration response curve with the induction of reporter gene expression in cells treated with 11.1 ng/ml rhTNFα at 4 hours post stimulation. In contrast cells treated with 0.05 ng/ml rhTNFα did not demonstrate significant expression until approximately 9 hours post stimulation. These data highlight the wide signal window that can be achieved using this assay system; approximately 15-fold difference between untreated controls versus wells treated with 11.1 ng/ml rhTNFα.

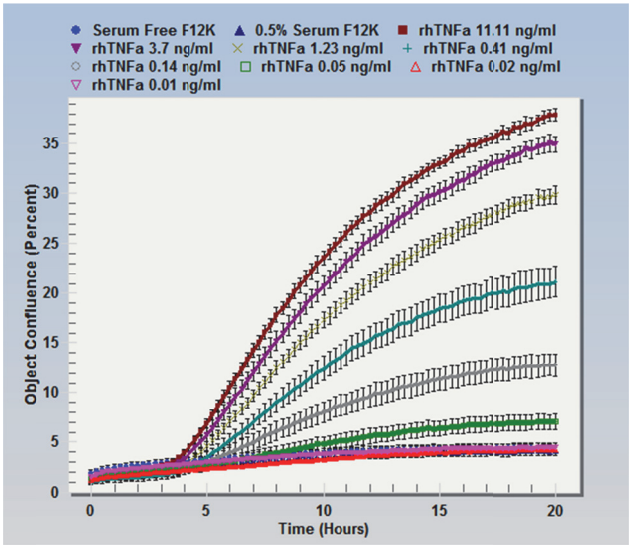

Figure 3: Kinetic induction of NF-κB driven rhGFP reporter gene expression resulting in increased object confluence. HEK 293 cells transiently transfected with the pNF-κB-rhGFP reporter gene construct were treated with 3-fold dilutions of rhTNFα (N=5 wells per treatment; however identical results have been achieved with N=3 wells per treatment). Images were acquired at 15-minute intervals and analyzed using the object counting algorithm within the IncuCyte FLR basic software package. The "Object Confluence", or the percentage of the image occupied by fluorescent objects is plotted as a representation of reporter gene expression and/or promoter activity. Data points represent the mean ± standard error.

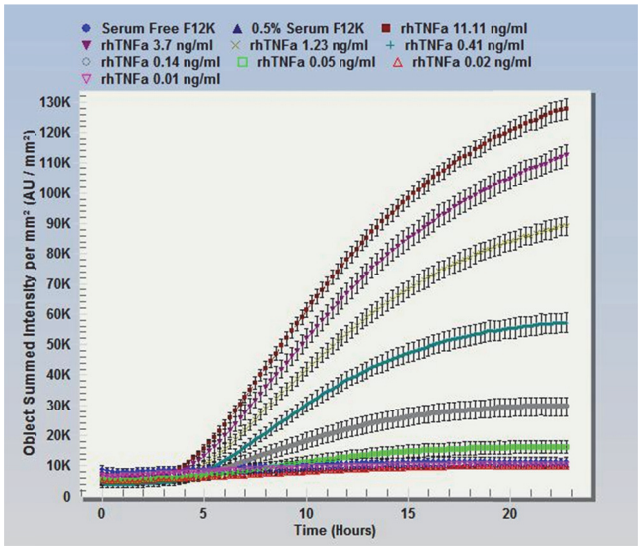

Figure 4: Kinetic induction of NF-κB driven rhGFP reporter gene expression resulting in increased fluorescence intensity. HEK 293 cells transiently transfected with the pNF-κB-rhGFP reporter gene construct were treated with 3-fold dilutions of rhTNFα (N=5 wells per treatment; however identical results have been achieved with N=3 wells per treatment). Images were acquired at 15-minute intervals and analyzed using the object counting algorithm within the IncuCyte FLR basic software package. The intensities of all pixels in the segmentation mask are summed and reported as object summed intensity per mm2. Data points represent the mean ± standard error.

In addition to object confluence, the data can be graphed using the Object Summed Intensity metric contained within the IncuCyte FLR basic package (Figure 4). This metric calculates the total additive brightness of every pixel in the segmentation mask. It is then divided by the total area of the image and the Object Summed Intensity/mm^2 is reported. Graphing this metric clearly shows that increasing concentrations of TNFα results in not only more cells reaching a defined threshold, as defined by object confluence in Figure 3, but it also illustrates that the thresholded cells increase in fluorescence intensity, correlating to an increase in GFP expression/NF-κB activation (Figure 4).

Following data analysis and graphing, we calculated the EC50 value of TNFα at both the 10 hour and 20 hour time points (Figure 5). It is important to note that these evaluation time points were not decided upon *a priori*, but rather were performed during the on-going experiment. This approach allows the user to decide at their convenience if the signal window has been optimized and when to stop the experiment. The EC50 values calculated at both time points illustrate the sensitivity of this assay system. These sensitivities compare favorably to luciferase based NF-κB reporter systems using the same cell type (HEK 293)[5].

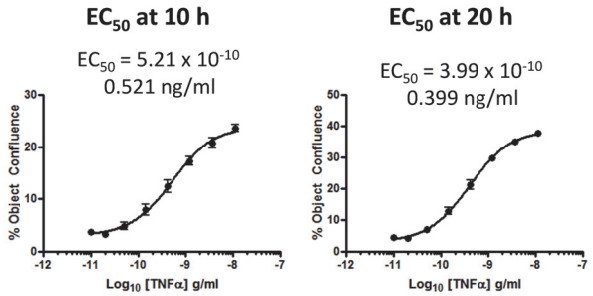

Figure 5: Calculated EC50s of TNFα induced NF-κB activity at two time points. HEK 293 cells transiently transfected with the pNF-κB-rhGFP reporter gene construct were treated with 3-fold dilutions of rhTNFα. Images were acquired at 15-minute intervals and analyzed using the object counting algorithm within the IncuCyte FLR basic software package. The "Object Confluence", or the percentage of the image occupied by fluorescent objects is plotted as a representation of reporter gene expression and/or promoter activity. Data collected at the 10 and 20 hour time points were exported to GraphPad Prism 5.0 and EC50 values were calculated.

Conclusion

The traditional tools used to complete similar reporter assays often require several preliminary time course studies encompassing multiple inconvenient end points in order to characterize the timing of transcription factor activation for each stimulus being tested. In contrast, using the IncuCyte FLR, we were able to evaluate the entire course of activation in one singular experiment that resulted in a robust, quantitative data set from fewer samples than would have been required in an end point time course experiment. Consequently, this assay system not only minimizes the number of preliminary experiments required to validate an assay but also reduces the associated reagent and labor costs while maintaining superior assay sensitivity.

While using this approach provides many advantages, we do not discount the value of other reporter gene assay systems. The breadth of commercially available reagents as well as the intrinsic properties of the reporter protein (e.g. reporter half-life, dynamic range, etc.) requires careful consideration when choosing assay systems. However, there is an ever growing list of companies providing fluorescence (GFP) based reagents compatible with this approach. As but one example, the pGreenFire1 lentivectors available from System Biosciences are especially useful if luciferase based end point data is desired to corroborate kinetic, live-cell based measurements using IncuCyte FLR. These constructs are dual reporter vectors that provide the ability to kinetically monitor and quantify GFP expression in a non-perturbing fashion, while also providing the ability to make an end point assessment with a co-expressed luciferase reporter. Additional companies, such as Clontech (Mountain View, CA) and Evrogen (Moscow, Russia) provide destabilized GFP reporter constructs that could be used to measure oscillations in transcriptional activity. Gasparri and Galvani have recently published an insightful review on the use of fluorescent proteins as reporter genes[6].

In summary, in this application note, we describe a method for using the IncuCyte FLR as a tool for completing kinetic reporter gene assays. This strategy provides rich temporal biological information otherwise not obtainable with end point assays. It also provides the advantages of not requiring the addition of a terminal reaction substrate. This, in-turn has several advantages a.) lower cost b.) convenience to the end-user c.) opportunity to optimize the signal window within one experiment and d.) the potential to enhance the quantitative robustness (signal to noise ratio) using multiple data points collected on the sample. Also, given the range of commercially available reagents, the technique is extendable to a vast array of transcription factors and associated signaling pathways.

References

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