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NLM Citation: Trask OJJr. Nuclear Factor Kappa B (NF-κB) Translocation Assay Development and Validation for High Content Screening. 2012 Oct 1. In: Markossian S, Grossman A, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-.
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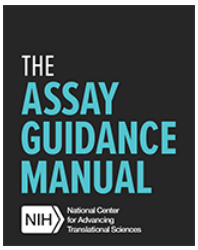

Nuclear Factor Kappa B (NF-κB) Translocation Assay Development and Validation for High Content Screening

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Created: October 1, 2012.

Abstract

In this assay study design, activation through stimulation or inhibition of the Nuclear Factor κB (NF-κB) pathway demonstrates translocation of NF-κB protein from the cytoplasm to nucleus as measured using automated fluorescent microscopy computer-assisted image analysis technology better known as high content screening (HCS), High Content Analysis (HCS), High Content Imaging (HCI), or Image Cytometry (IC). This approach offers a better understanding of novel drug targets by examining the sub-cellular spatial distribution of the target proteins and the effects of target perturbation on cellular processes. Cell imaging provides multi-probe detection and is advantageous over other methods in quantifying spatial measurements such as protein translocation or redistribution, receptor internalization, cell morphology, cell motility, cell cycle, cell signaling, and others. The limitation of traditional microscopy and image analysis has been a log jam of sample throughput, day-to-day variability, poor standardization and of the need for specialized personnel to operate and oversee instrumentation. Automated fluorescent microscopy systems offer one solution for studying cell function in a "high content screening" (HCS) mode that allows the capability of measuring multiple cellular characteristics in a non-biased fashion. This chapter details the assay development, optimization, and validation for running a compound screening campaign and includes descriptions of the cell model, stimuli to activate NF-κB pathway, time course kinetics, effects of serum on assay window, validation of proinflammaory cytokines, reference control inhibitor compound optimization, and examples of nomenclatures used by many manufactures of HCS instruments or software algorithms.

Introduction and background

There is still much that remains unknown within the biology of NF-κB despite the fact that in the year 2011 NF-κB celebrated its 25 year anniversary (1). NF-κB, also referred to as the Rel family of proteins, was first described in 1986 as a protein which binds to specific decameric DNA sequences (ggg ACT TTC C) in mature B-cells nuclear fraction and the immunoglobulin kappa light chain enhancer of plasma cells (2). The subunits of NF-κB or Rel family proteins are part of a structurally related family; currently five different proteins have been identified: p50, p52, p65, Rel-B and c-Rel. The structure of NF-κB resembles a "butterfly" in appearance with DNA binding in a pocket between two domains (Figure 1) (3). All identifiable Rel proteins contain a conserved

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N-terminal region, called the Rel Homology Domain (RHD). The RHD contains the DNA-binding and dimerization domains and the nuclear localization signal of the Rel proteins. p50 (also called NF-κB B1) and p65 (RelA) were the first NF-κB proteins to be identified. In 1988, IκB was described as a specific inhibitor of the NF-κB ability to translocate and turn on transcription factors (4). Seven known IκB's have been identified: IκB-α, IκB-β, IκB-γ, IκB-ε, Bcl-3, p100 and p105. All known IκB's contain ankyrin repeats (30-33aa) that mediate non-covalent interaction between IκK and NF-κB. NF-κB p50 subunit crystal structure was first described by two groups (5, 6). NF-κB has similarities with NF-AT family of proteins with both having interaction with members of FOS and Jun family of proteins (7). Activation of NF-kB signaling is triggered in a series of steps through either the classical canonical pathway, the alternative non-canonical pathway, or the atypical IκK independent pathway (8-11) (Figure 2). NF-κB was initially discovered in B-cells, although B-cells do not express NF-κB until stimulated by LPS or cytokines (12). NF-κB is generally thought to be constitutively active and located in the cytoplasm in most cell types, until induced by a stimulus to migrate to the nucleus. Exceptions are cornel keratinocytes and vascular smooth muscle cells, which have been shown to have nuclear NF-κB in the absence of activation (13). Following activation, translocation, and transcription of NF-κB in the nucleus, re-accumulation of IκBα in the cytoplasm is thought to occur within 60 minutes (14, 15). Furthermore, if IκBα is re-synthesized it may enter the nucleus and then interacts with NF-κB complexes and inhibit DNA binding (16). Dimerization of NF-κB subunits results in the association of NF-κB proteins with DNA. The C-terminal region of the Rel homology domain is where dimerization takes place. The nuclear localization sequence (NLS) that regulates transport of activated NF-κB complexes into the nucleus is located near the end of the C-terminal end of the Rel homology domain. With the exception of Rel B, all Rel protein family members contain a phosphorylation site for PKA approximately 25aa to the N-terminal of the NLS (17).

Translocation of NF-κB is a critical step in the coupling of extracellular stimuli to the transcriptional activation of specific target genes. NF-κB is activated by pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α,); bacterial toxins (LPS, exotoxin B); viral products (HIV-1, HTLV-1, HBV, EBV, Herpes Simplex); and cell death stimuli (O_2-free radicals, UV light, γ-radiation) (17, 18). IκB proteins are rapidly phosphorylated following induction by triggering cytokine receptors. IκK-α and IκK-β are both responsible for modification of IκB complex. NF-κB in response to activation of these cytokines is associated with triggering cellular defense genes (17). Upon cell stimulation, the nuclear localization signal on NF-κB becomes exposed and the protein translocates to the nucleus, where it turns on transcription factors and induces specific gene expression.

The NF-κB activation has direct screening applications for drug discovery for many pharmaceutical companies and in medicine for several therapeutic indications, most notably, inflammatory tissue injury, where NF-κB controls the gene expression of a variety of pro-inflammatory mediators (11, 19, 20). In addition, NF-κB regulates a number of genes that are involved in mediating tumorigenesis, metastasis, proliferation (21, 22) and apoptosis (23-26). Vascular cell adhesion molecule-1 (Vcam-1) found on endothelial cells following exposure to cytokines or LPS is involved in regulation of NF-κB (2, 21), however, it requires an IRF-1 site that is located 3' to the TATA box (27). Other genes that regulate NF-κB and are involved in the immune response and inflammation include peptide transporter (TAP-1), proteasome subunit LMP2 (28), MHC class II variant chain genes (29), and other unknown genes. Inducible nitric oxide synthase (iNOS) is regulated by NF-κB activation, which results in an increase in nitric oxide production (30) and the inducible cyclooxygenase (COX-2), which generates prostanoids (31). Transcriptional regulation of p53, c-myc, and ras gene can be controlled by NF-κB activation (32).

Principle of the Assay

NF-κB is typically present and resides in the cytoplasm of most cells as a complex with members of IκB inhibitor family of proteins. Both the size of this complex and IκB masking of the nuclear localization sequence of NF-κB prevents NF-κB entry through the nuclear membrane. Once IκB is phosphorylated and degrades, the nuclear localization sequences become assessable and NF-κB is free to translocate to the nucleus (Figure 3). Microscopy

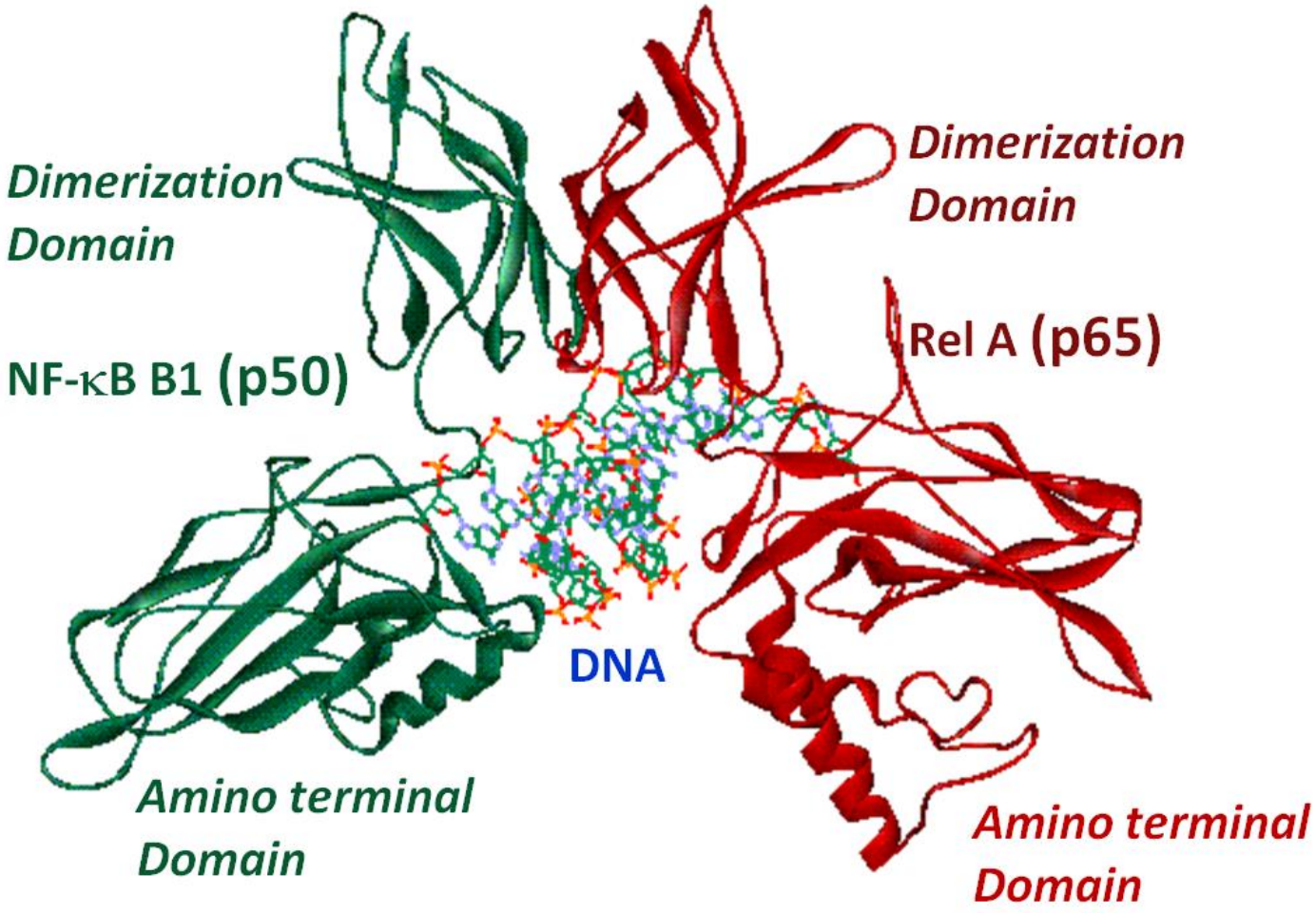


Figure 1: Structure of the NF-κB/DNA complex. There are two subunits of NF-κB, p50 (green) and p65 (red). The representation of the ribbon structure is viewed down the DNA helix axis with hydrophobic core Adapted from Chen F, et al (33).

images reveal strong evidence of the redistribution of NF-κB from the cytoplasm to the nucleus following cytokine stimulation (Figure 4).

Assay Development Considerations

Image Analysis Algorithm to Detect NF-κB Translocation

The basic principle to detect NF-κB translocation is dependent on identifying the nucleus of a cell using one of many nucleic acid probe reporters, typically Hoechst, DAPI, or DRAQ5.

Once the nucleus is identified using image analysis process it is masked, and then a secondary mask overlay is created to either encompass the entire cell boundaries or a subset area of the cytoplasmic area. Within this secondary masked area (nucleus and/or cytoplasm) labeled NF-κB can then be quantified. Most available algorithms report two critical features; (A) NF-κB intensity in the nucleus and (B) NF-κB intensity in the cytoplasm. Once determined, a translocation value is calculated by measuring the average intensity of "difference" of the NF-κB protein between the identified cytoplasmic region and nuclear region (Cyto-Nuc Difference). Additionally, a "ratio" of the average intensity of the nuclear region to average intensity of the cytoplasmic region can be calculated to represent this translocation value; Nuc/Cyt Ratio. Both the "difference" and "ratio" features reflect a biological phenotype of NF-κB translocation. The data feature output from images is commonly reported as a well summary value but the calculation begins at the single cell level as shown in Figure

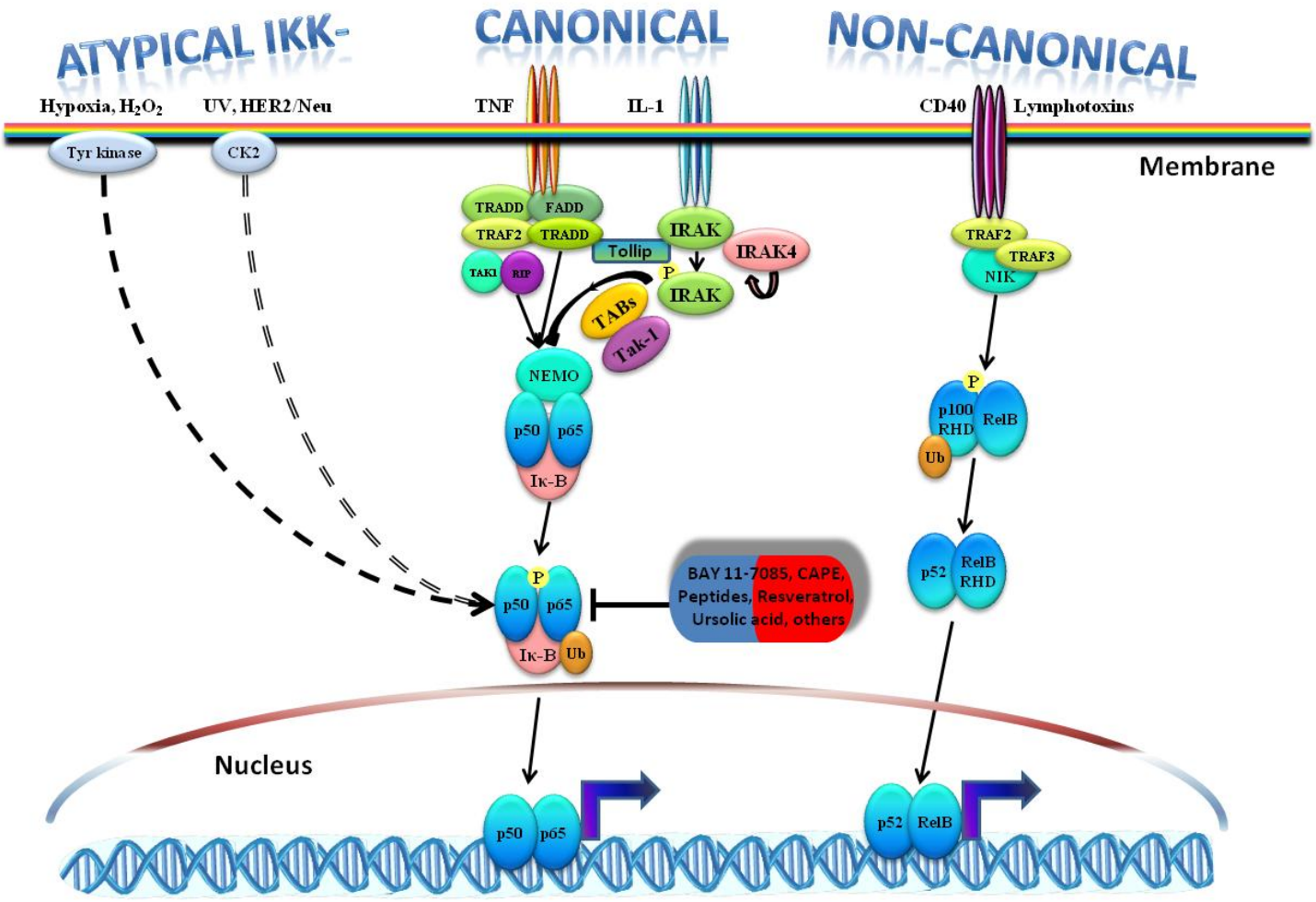


Figure 2: Activation of NF-κB Signaling Pathway. There are currently three recognizable pathways to activate NF-κB, the canonical, the non-canonical, and the atypical IκK independent pathways. NF-κB is naturally inhibited by IκB. Upstream activating signal (e.g., binding of TNF-α, IL-1α, LPS, CD40, Lymphotoxin, UV, HER2/Neu, H_20_2, or other unknown ligands to its receptor) causes phosphorylation of IκB by IκK (IκB kinase). This triggers the degradation of IκB through the ubiquitin system (Ub), where the target molecule is masked by a chain of ubiquitins for degradation by the 26S protesome. The free unbound NF-κB can then translocates to the nucleus and activates transcription (22). NF-kB translocation is reduced by inhibitor compounds such as BAY 11-7085, other IkB kinase inhibitor compounds, and other means.

5. An example of well level data nomenclature describing these key features from different imaging platforms is showcased in Table 1. Representative images and algorithm overlays of HeLa cells in a dose response with IL-1α are shown in Figure 6. Differences at the plate level (96 wells) in cells treated with stimuli (Max) or with DMSO or inhibitor compound (Min) are used to calculate Z-factor values (34) using CytoNuc Difference and Nuc/Cyt Ratio, highlighted in Table 2.

Additional, there are other valuable HCS output features that are useful including identifiable objects (cells), variability of intensity across area within the nucleus or cytoplasm, morphological measurements including the size, shape, length, aspects of individual cells or objects within a cell. For compound screening, the use of nucleus or cytoplasm area intensity may also be helpful to identify nuisance fluorescent compounds that display the same or similar spectral properties as the probes used in the assay design and development. However, keep in mind that these compounds could also be classified as “false positive” activate compounds from the screen. In this case, careful follow ups needs to be employed b measuring basal autofluorescence levels in cells without fluorescent reporters and with compound(s) in question.

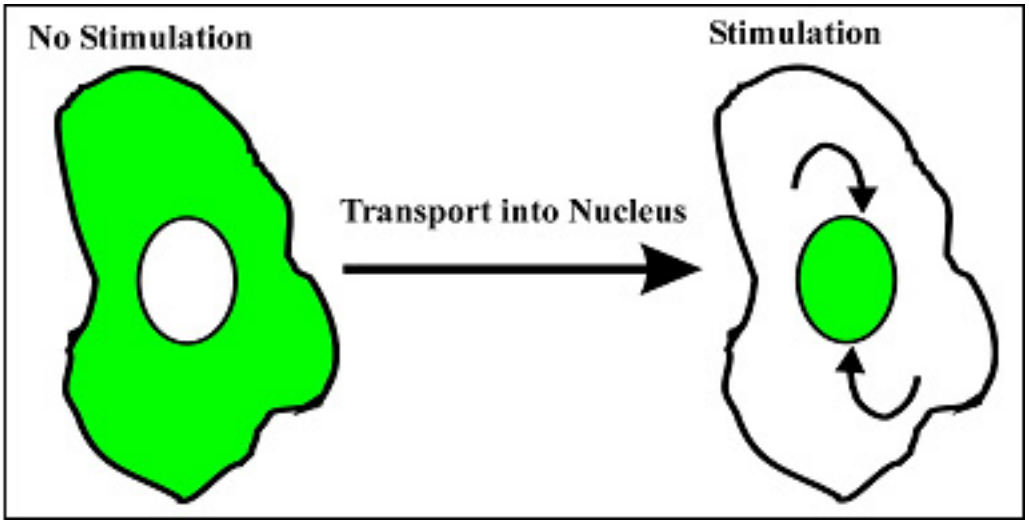


Figure 3: Diagrammatic view of the principle of NF-κB activation. NF-κB (green), normally resides in the cytoplasm, once activated by stimuli (IL-1α, TNF-α ,etc) it translocates to the nucleus.

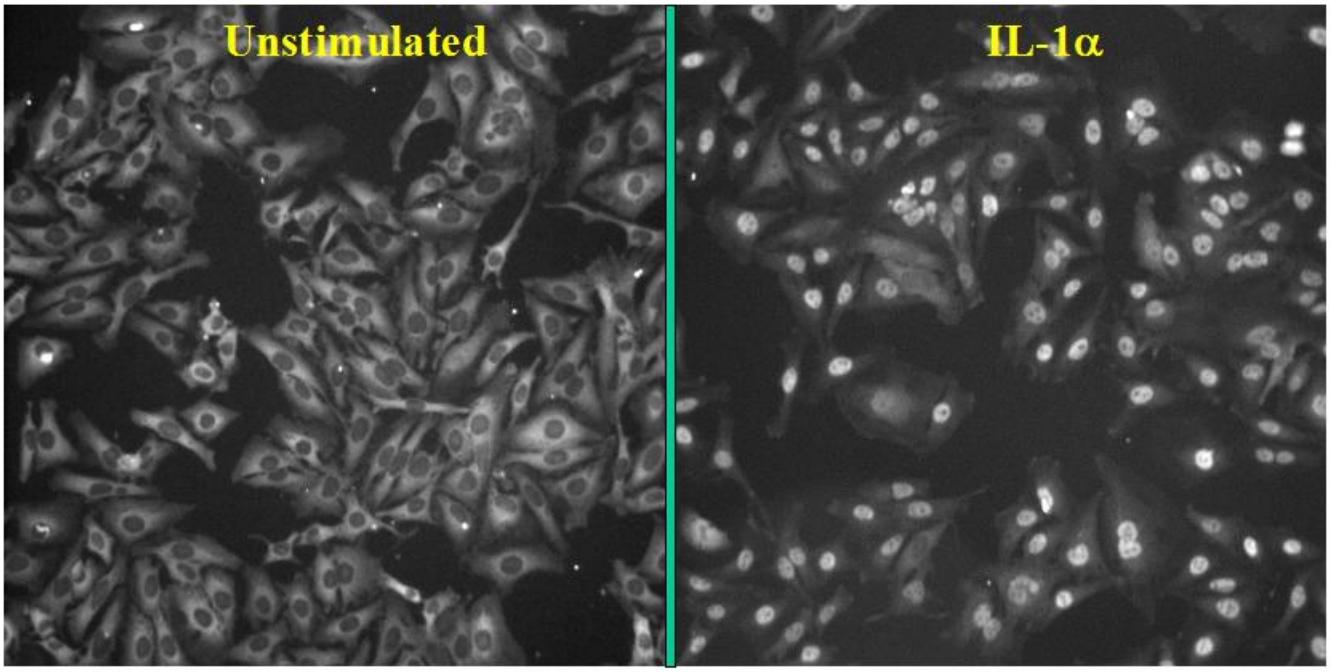


Figure 4: HeLa cells unstimulated or stimulated with 25 ηg/ml IL-1α for ~40 minutes. Cells were then fixed and labeled with NF-κB-p65 polycloncal antibody and secondary AlexaFluor488 with counterstain of Hoechst 33342. (Left). Unstimulated cells showing a shaded cytoplasm containing NF-κB-p65 with a dark nucleus "donut hole" counter stain. (Right). Following activation of NF-κB, the nucleus appears bright as represented in white and the cytoplasm is no longer shaded indicating the protein translocated from the cytoplasm to the nucleus.

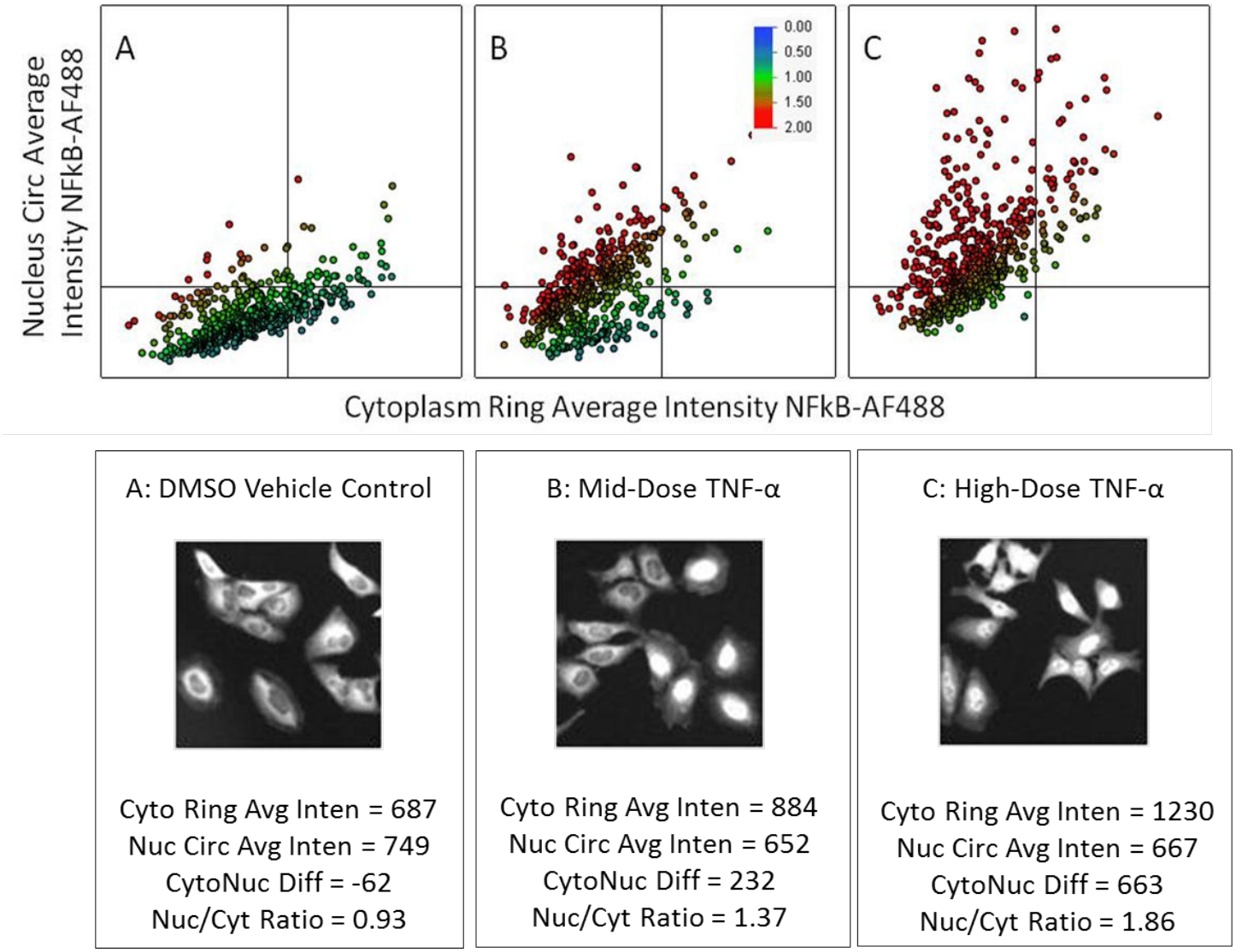


Figure 5: Multiparametric plot (Top) and corresponding images (Bottom) of NF-κB-AF488 average cytoplasm ring and nucleus circ intensities at the single cell level in HeLa cells following TNF-α dosing for 35 minutes. Each "dot" or "bubble" represents a single cell with a total of approximately 550 cells per graph; xy axis crossbar set at grayscale intensity value of 900. The color difference from blue to red is dependent on ratiometric calculation of NF-κB-AF488 Nucleus Circ Average Intensity divided by the Cytoplasm Ring Average Intensity (Nuc/Cyt Ratio); scale is display in graph B for all. (A) Vehicle control (B) Mid dose of TNF-α (C) High dose of TNF-α. Note the cell objects shift from cytoplasm ring average intensity (x-axis) to the nuclear circ average intensity (y-axis) and increase in Nuc/Cyt Ratio (red bubbles) with increasing TNF-α concentration.

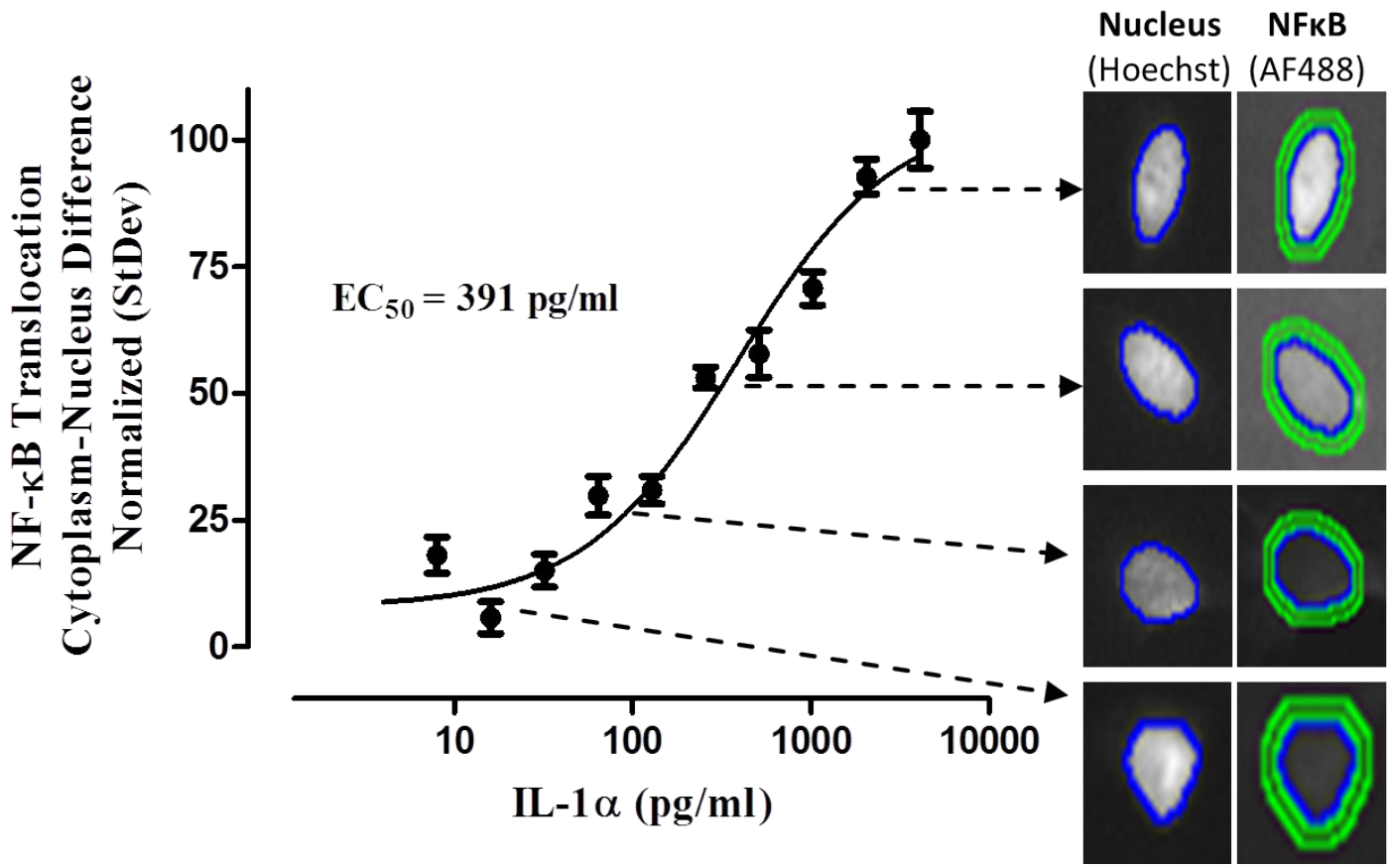


Figure 6: Nuclear Factor κB Translocation in HeLa cells following 30 minute incubation with IL-1α. Cells labeled with Hoechst 33342 are masked with a blue ring outline to identify the nuclear area. Dilation of this mask to identify cytoplasm area is masked and highlighted with two green rings to identify fluorescence of NF-κB-p65 antibody labeling. Note, the first green ring (inner) is dilated away from the outer nuclear boundary; it is then copied and expanded to represent the outer ring. The area between these two rings represents the cytoplasm area measured. The algorithm can output either the difference between the average intensities in the green and blue mask area or output a ratio of between the blue and green mask areas. Although difficult to see in these images NF-κB is expressed in the cytoplasm at low doses of IL-1α.

Table 1: Algorithm nomenclature output comparison of HCS feature data to measure NF-κB autofluorescence only.

Feature	BD AttoVision	Cell Profiler	Definiens Developer	GE HealthCare	MDC MetaXpress	Perkin Elmer	Thermo Scientific
Intensity inside the cytoplasm area	Cyt (Variable)	Variable	Variable	Cell Intensity	Outer Area	Intensity cytoplasm Alexa nnn mean (Variable)	RingAvgInten
Intensity inside the nucleus area	Nuc (Variable)	Variable	Variable	Nuc Intensity	Inner Area (AF488)	Intensity nucleus Alexa nnn mean (Variable)	CircAvgInten
Difference, between intensity in cytoplasm area minus the intensity in nucleus area	Nuc-Cyt (Variable)	Variable	Variable	N/A	N/A	Variable	CytoNuc Diff

Table 1 continued from previous page.

Feature	BD AttoVision	Cell Profiler	Definiens Developer	GE HealthCare	MDC MetaXpress	Perkin Elmer	Thermo Scientific
Ratio, intensity in nucleus area divided by the intensity in the cytoplasm area	Nuc/Cyt (Variable)	Variable	Variable	Nuc/Cell Intensity	Inner/Outer Intensity Ratio	Variable	NucCyt Ratio

Table 2: NF-κB-p65-AF488 HCS feature data output comparison of Nuc/Cyt Ratio and CytoNuc Difference measurements in HeLa cells. The minimum (min) signal is the baseline constitutive expression of NF-κB and the maximum (max) is NF-κB response following ~35 minute incubation with either 25ηg/ml IL-1α or 25ηg/ml TNF-α; data includes the percent coefficient of variation (% CV) from 96-wells. The Z factor is calculated as described in literature (34)

IL-1α	Ratio: Nuc/Cyt	Difference: Cyto-Nuc Diff	TNF-α	Ratio: Nuc/Cyt	Difference: Cyto-Nuc Diff
Signal (Min / Max)	1.0 1.84	-75.2 523	Signal (Min / Max)	0.957 1.86	-38.3 495
% CV (Min / Max)	4% 8.1%	3.5% 4.5%	% CV (Min / Max)	2.4% 6.7%	3.7% 5.4%
Z factor	0.64	0.76	Z factor	0.73	0.73

Assay Detection and Limitation

The assay described below will quantify the redistribution of NF-κB from the cytoplasm to the nucleus upon activation using antibodies to NF-κB p65 subunit in intact cells following fixation. Stimulating cells with proinflammatory cytokines such as IL-1α and TNF-α activates the canonical NF-κB pathway and therefore the noncanoncial pathway (CD40, LT), or the atypical IκK independent pathway (UV, Hypoxia) will likely be non-responsive in this assay format. In addition, this assay can indirectly measure IκB degradation since phosphorylation of IκB is required before degradation of IκBα and subsequent translocation of NF-κB subunit to the nucleus. This assay does not directly measure phosphorlyation of NF-κB protein.

This assay can be multiplexed with other biofluorescent probes. For example, other kinase related bioprobes to measure MAPK kinase pathways such as phospho-JNK-1/2 or viability indicators may provide important information about the biology or selectivity of the compound. The critical consideration for developing this assay is the dose and time using more than one stimulus to triggering multiple signaling pathways.

Assay Development

Cell Model

HeLa cells are endothelial cells from the American Tissue Culture Corporation (ATCC), catalogue number CCL-2, original isolated from the cervix of a 31yo black female with adenocarcinoma, were selected as the cell line as choice for this assay for several reasons. (A) HeLa cells are well documented in the literature to illustrate NF-κB redistribution; (B) HeLa cells are relatively flat once seeded in plates and therefore image well using widefield 2-D bright-field and fluorescent microscopy; (C) there are commercial available validated kits and antibodies to detect redistribution of NF-κB.

Note: *Other cells such as Swiss 3T3, BHK, HepG2-C3A, Rat hepatocytes, LLCPK, HMVEC, CHO-K1, HT1080, HCT-116, U 2-OS, and others are also known to express NF-κB but before adopting one of these cell lines or other unlisted cell models, it is recommended to validate assay performance with a known cell line such as HeLa.*

Cell culturing and harvesting

Maintenance of the HeLa cell line or cell of choice should be carried out following recommendations from the ATCC or the source of the cell line. Typically splitting cells 2-3 times per week is required for optimal growth and performance in the assay. Do not allow cells to become over confluent in tissue culture flasks. If this occurs, it will be necessary to thaw a new vial of cells before proceeding with assay development, validation, or screening. Grow cells in complete media, and then adjust serum concentration prior to cell seeding as necessary (see Effects of Serum for additional information). Upon harvest of cells with trypsin or other cell detachment methodology it is recommended to filter the cells through a cell strainer (40-70 microns) to remove large clumps before cell counting and seeding. If aggregates are not removed this will negatively impact cell object identification and segmentation with image analysis algorithms.

Cell Seeding Number Determination

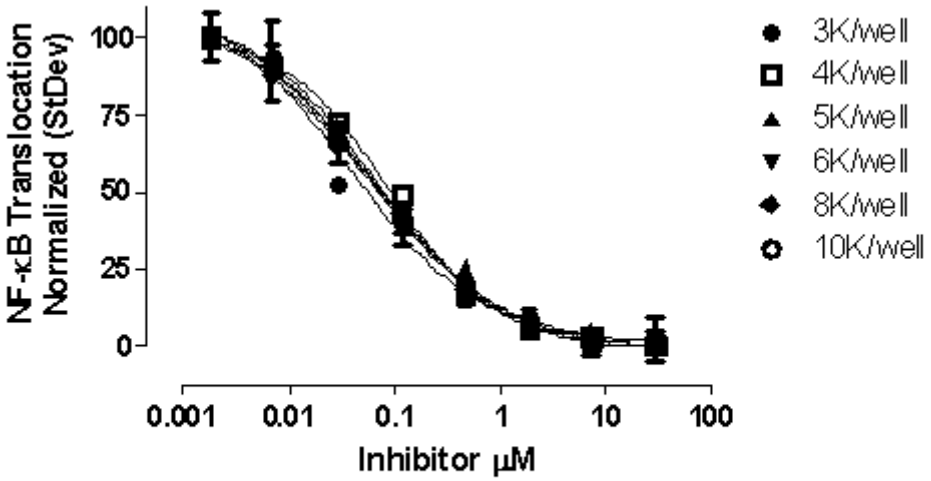
Determine the optimal cell seeding density as described in the assay development HCS chapter by seeding cells at approximately 5,000 cells/well for a 96-well plate as the median starting point; then dilute or increase the cell density by approximately 40-50%.*(Note: In 384-well plates, approximately 2,500 cells/well is recommended).* Incubate cells overnight, and then label as described in subsequent sections with reporter probes. There is some variability but no significant difference in cell number (3,000 to 10,000 cells/well) in 96-well plate format from the NF-κB inhibitor compound IC_{50} curves (Figure 7). However, since identification and segmentation of individual cells in the well is optimal for accurate image analysis measurement of NF-κB, 5,000 cells/well was chosen. An NF-κB reference compound inhibitor, IK202, was used to determine cell number. The number of cells per well was optimized based on reviewing images to observe cell-to-cell contact. Higher concentrations of cells per well resulted in rejection of more cells due to piling of cells, massive cell contact clumps, and poor image analysis segmentation. However, the number of fields required to collect 500 cells, the assay defined threshold, was decreased (Figure 8). The optimal cell seeding density for HeLa cells is 5,000 cells/well for 96-well plate. *{data not shown}).*

Cell Passage Limitations

It is critical to gauge the number of cell passages in the assay before a noticeable decline is observed. Cells with many passages may not survive, become contaminated, or fail to respond in the assay over time. If possible, continue passaging cells at the onset of development with a fresh thaw of cells and maintain a weekly stock over time; then measure NF-κB response in comparison with lower passage cells. HeLa cells with different cell splitting passage numbers up to 40 from ATCC and the same lot were tested to compare sensitivity to TNF-α cytokine to activate translocation of NF-κB and to determine if the response was altered. Even thought the R2 of the curve fitting was similar; there was a significant shift in the NF-κB translocation EC50 response to cytokines in cells with high passage number. A significant loss in responsiveness of NF-κB translocation was observed with an increase in calculated EC_{50} values directly correlated with increasing passage number (Figure 9). Based on these findings it is recommended to use cells with as low passage number as possible and as a general rule never exceed passage of 20.

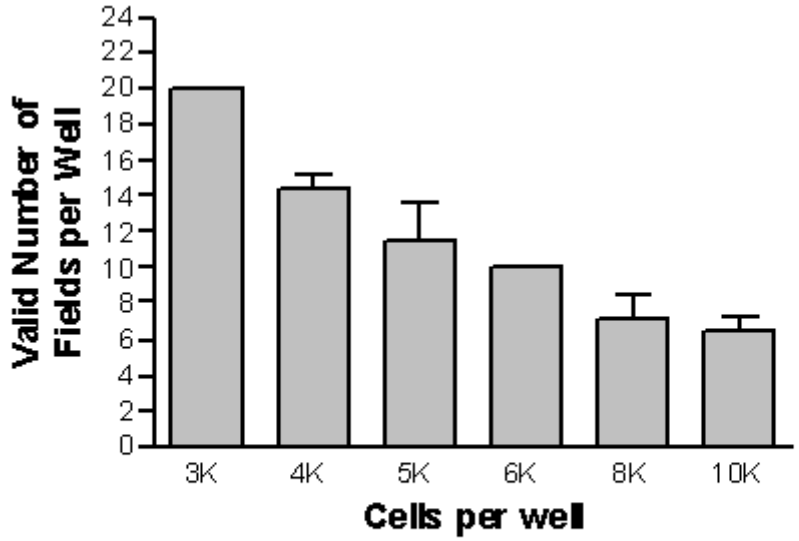
Activators of the NF-κB Signaling Pathway

Activating NF-κB pathway using cellular cytokines as described in the introduction is critical to establish an assay window. Although several stimuli have been identified in activating NF-κB pathway, three cytokines, IL-1α, IL-1β, and TNF-α gave the most robust and best EC_{50} values of all stimuli tested (Figure 10). IL-1α, IL-1β family members and TNF-α cytokines activate NF-κB through different up-stream mechanisms. Platelet-derived growth factor (PDGF) also activates the translocation events of the NF-κB pathway to a lesser extent and is described to work through Ras and PI3 kinase signaling. (33). Lymphotoxin (LT) α1/β2, LT α2/β1, and IFN-γ showed little or no activation of NF-κB (the latter stimuli data is not shown). Additionally, anisomycin and



	3K/well	4K/well	5K/well	6K/well	8K/well	10K/well
EC50	0.03777	0.09116	0.06331	0.06138	0.06414	0.05735

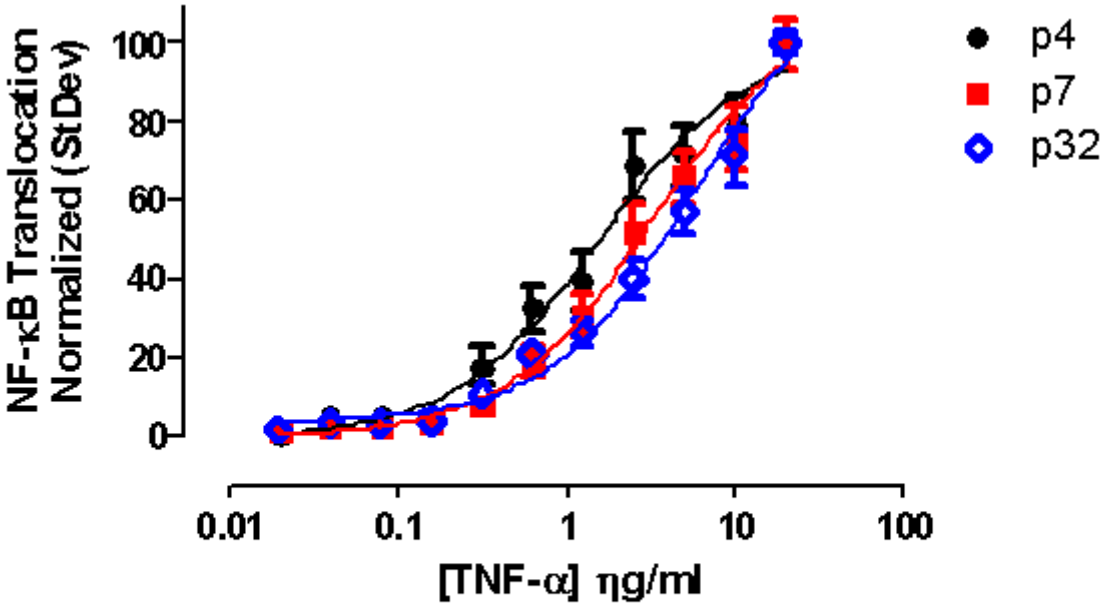
Figure 7: Cell Number Determination. HeLa cells seeded overnight, treated with reference compound inhibitor for 15 minutes and subsequent 25ηg/ml TNF-α for ~35 minutes, and then fixed and stained for NF-κB-p65-AF488. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference; data was normalized to control and plotted in GrapPad Prism using non-linear regression 3-parameter fit.



	3K/well	4K/well	5K/well	6K/well	8K/well	10K/well
Mean	19.75	15.06	11.38	8.250	6.125	5.375

Figure 8: Field numbers required to collect 500 objects (cells) using a 10X/0.3NA objective, no magnifier and a 1392x1024 camera pixel array sensor.

phorbol-12-myristate-13-acetate (PMA) were also tested but did not show NF-κB-p65 activation as robustly as IL-1α, IL-1β, or TNF-α stimulus. This knowledge is important if considering multiplexing NF-κB with other signaling pathways. In this assay design, development, and validation, HeLa cells will be stimulated with both



	p4	p7	p32
EC50	1.556	3.228	5.881

Figure 9: Cell Passage Number Comparison. Different cell splitting passages of HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of TNF-α for ~35 minutes, then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

IL-1α and TNF-α following compound treatment, however, for compound profiling and screening, either stimuli alone will work and choice of cytokine is depended on the upstream or downstream target and biology.

Plate Type

The NF-κB assay is considered a very robust HCS assay using HeLa cells and likely can be adapted to many different SLAS standard plate types including 384-well or even 1536-well formats, however it is recommended to first perform the proof of concept in a validated format such as the 96-well, as discussed within, then move to another well plate format. In this assay sterile 96-well tissue cultured treated Perkin Elmer (Packard) View plates were chosen with no extracellular matrix proteins or PDL substrate coating.

Reagent & Probes

There are a few validated assay kits and antibodies in market that can be used for this assay, or alternatively development of an in-house or "homebrew" assay protocol kit can be developed. In the next section, details of developing and comparing with a commercially available kit are described. The primary advantage of using a commercial kit is it is "ready to use" with "cookbook" instructions included. If just measuring a few plates, then this is a preferable option. The benefit of developing an in-house "homebrew" kit is to know what antibodies, buffers, and reagents are being used in the assay as this may not be disclosed by commercial sources of kits. In addition, the cost for running many plates is reduced once an in-house kit is developed. This chapter should reduce development time and costs. An example comparing a commercial kit with an in-house kit using alternative antibody sources and the process to cross validate their relative performance is described in next section.

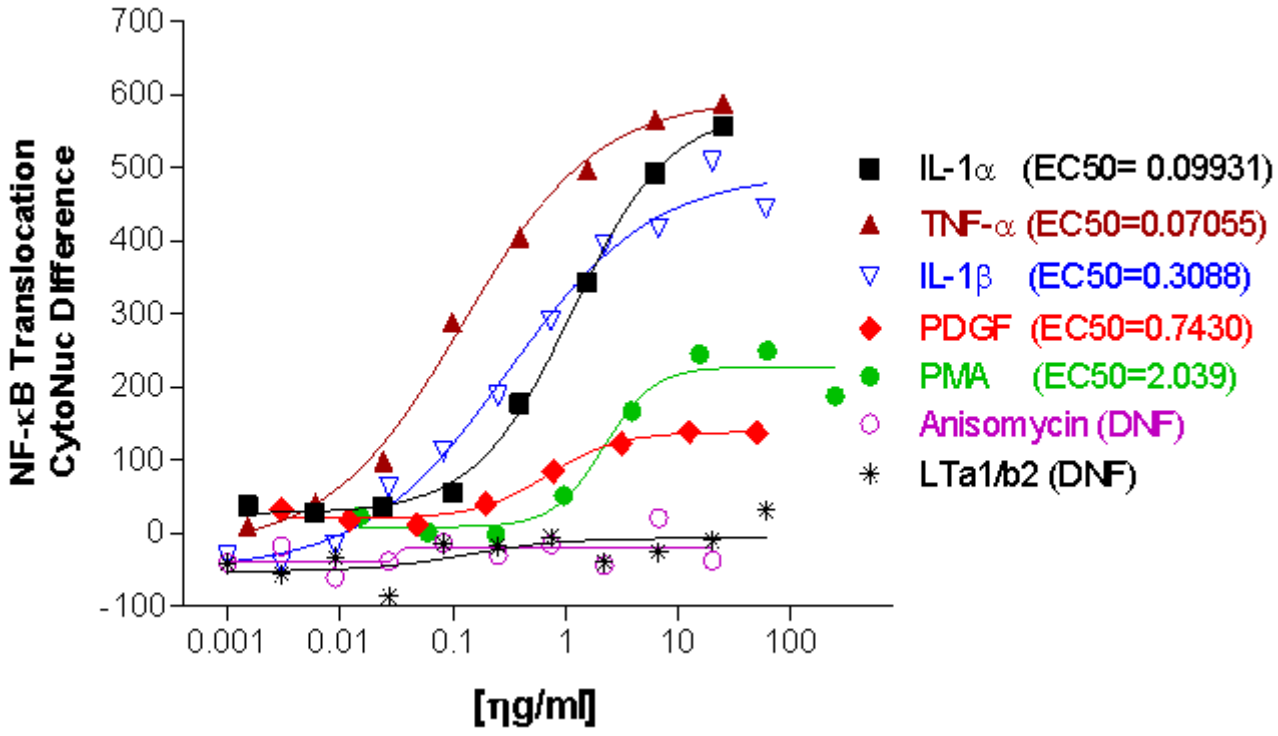


Figure 10: Activation of NF-κB-p65 with Different Stimuli. HeLa cells seeded at 5,000 cells/well overnight and treated with stimuli for 30 minutes, then fixed and labeled with NF-κB-p65-AF488 and Hoechst33342 to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data expressed as raw unit values (y-axis) from algorithm using non-linear regression 3-parameter fit was done in GraphPad Prism; standard deviation error bars (n=3) was removed for visualization.

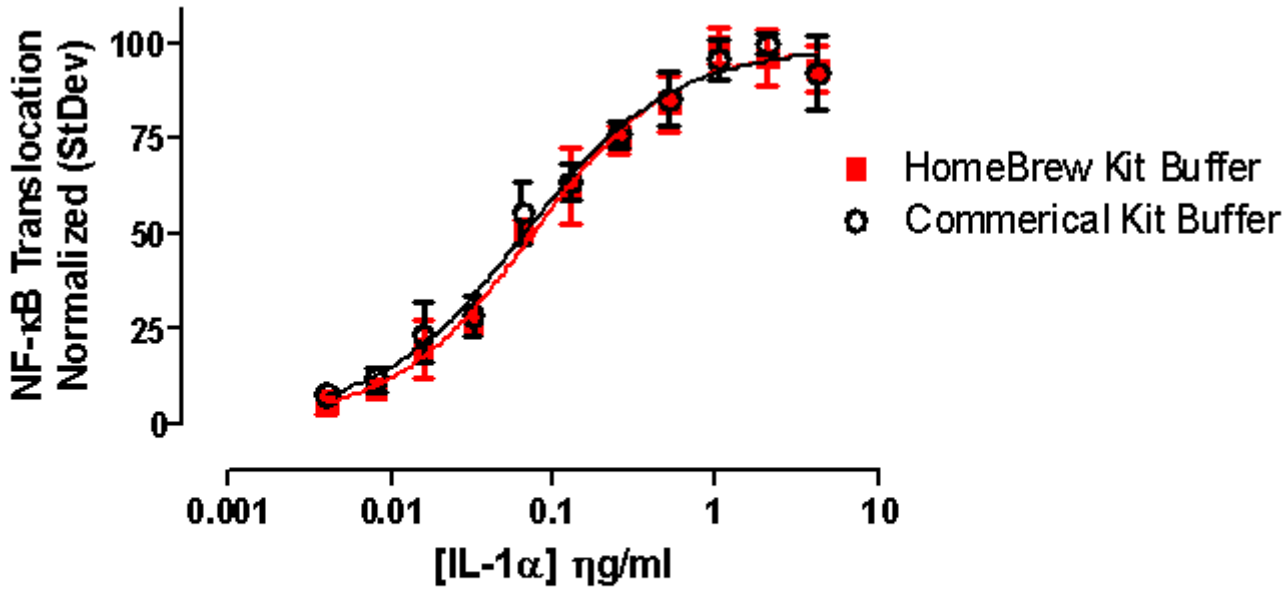
Note: *Before beginning the assay development process determine if a fixed endpoint assay is appropriate for the biological question. Alternatives to the classic antibody recognition and binding approach is the use of a fusion protein of NF-κB with a fluorescent protein reporter such as GFP, mCherry, or HaloTag® (Promega, Madison, WI) are other options to consider for measuring protein function.*

Commercial Kit Validation & Development of In-house "Home-Brew" Kit

The initial assay development was performed using commercial available validated kits for NF-κB. Additional, several different commercial available antibodies were screened for signal-to-noise ratio performance that targeted the p65 subunit of NF-κB (Appendix-1). Although other antibodies worked well in the evaluation, based on performance and comparable EC_{50} calculated values, the rabbit polyclonal IgG NF-κB-p65 antibody from Santa Cruz (SC-372) was used for assay development and validation procedures (see Figure 11).

Since the commercial kit contains other unknown and perhaps proprietary reagents, i.e., antibody, buffers, and reagents including PBS; PBS containing 0.5% Triton X-100; PBS containing 0.01% Tween-20 for testing and comparison with "in-house" buffers and commercial kit; it is always important to verify the signal-to-noise ratio window and insure that assay variability is not sacrificed .

Comparisons of different lots of NF-κB antibody from Santa Cruz were done at three different concentrations of primary antibody following stimulation with IL-1α. There were no significant differences observed (Figure 12). Lot-to-lot variability was evaluated several times with new antibody lot shipments and no significant differences in the calculated EC_{50} values were observed.



	HomeBrew Kit Buffer	Commerical Kit Buffer
EC50	0.07322	0.06533

Figure 11: Comparison of Commercial and HomeBrew Buffers. HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of IL-1α for ~35 minutes, then fixed and stained to measure NF-κB translocation using SC-372 antibody. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

Fixation comparison of two different forms of formaldehyde solution

Formaldehyde, 37% stock solution (Sigma) and Ultra-pure Formaldehyde, methanol-free 10% stock solution (Polysciences) were compared. HeLa cells were treated with IL-1α for about 30 minutes, followed by 1:10 dilution of fixation buffer in PBS for 10 minutes. An approximately 3-fold shift in the EC_{50} values was observed (Figure 13). For this assay, 3.7% formaldehyde from Sigma containing methanol was used. It is possible higher concentrations of Ultrapure formaldehyde may be used but this needs confirmation by testing.

Note: *At the time of testing 16-20% paraformaldehyde solution was not readily available from commercial sources and should be considered as alternative source.*

Please refer to institutional safety guideliens before working with formaldehyde; suggestions are in Safety Consideration Guidelines Precautions. Formaldehyde is specifically regulated by OSHA, so be sure you are in compliance with the OSHA standard (http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=10075&p_table=STANDARDS).

Stability and Lot Variability of Cytokines

Variability of cytokines lots were measured to determine variability of the assay if new reagents were introduced to the assay during the screening campaign or used in subsequent screens. Please note, it is highly recommended to order all reagents before the start of the experiment and/or screen. From this experiment and several other experiments, both IL-1α (data not shown) and TNF-α EC_{50} responses and calculated EC_{50} values indicated very high reproducibility (Figure 14). In addition, the activity and stability of cytokine performance was measured after one or more freeze-thaw cycles (Figure 15).

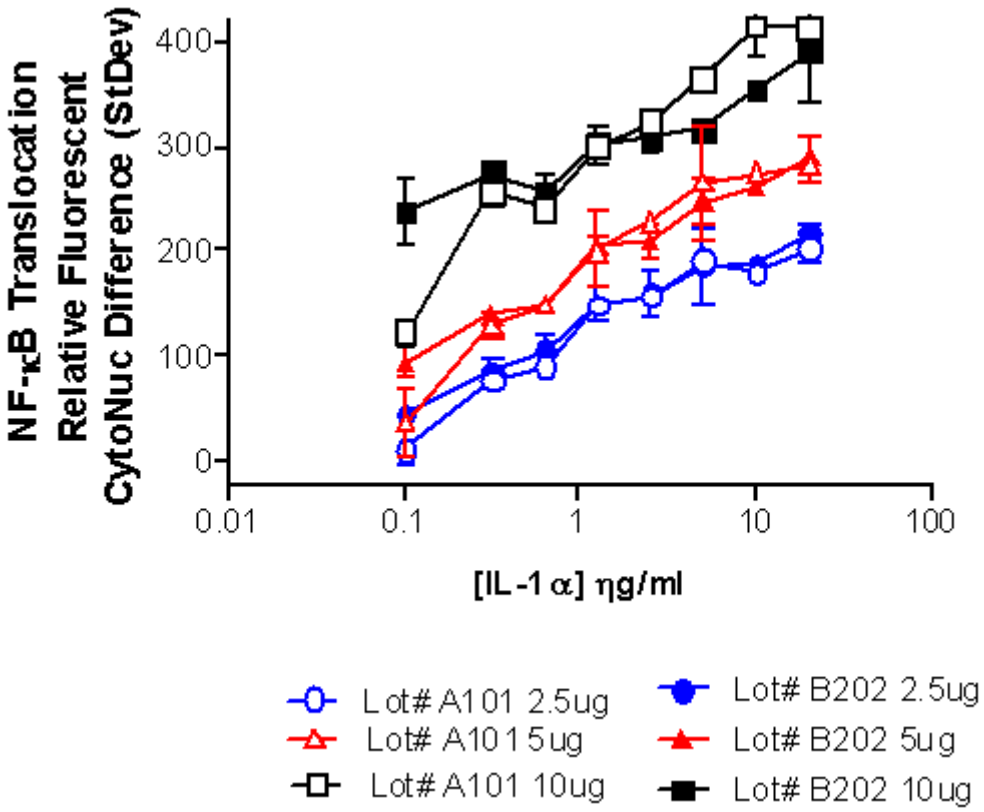


Figure 12: Lot-to-Lot Variability Comparison of Santa Cruz SC-372 NF-κB-p65 Antibody. HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of IL-1α for ~35 minutes, then fixed and stained to measure NF-κB translocation using two different lots of SC-372 antibody and at different concentration dilutions. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was not normalized and plotted with "raw" numbers in GraphPad Prism.

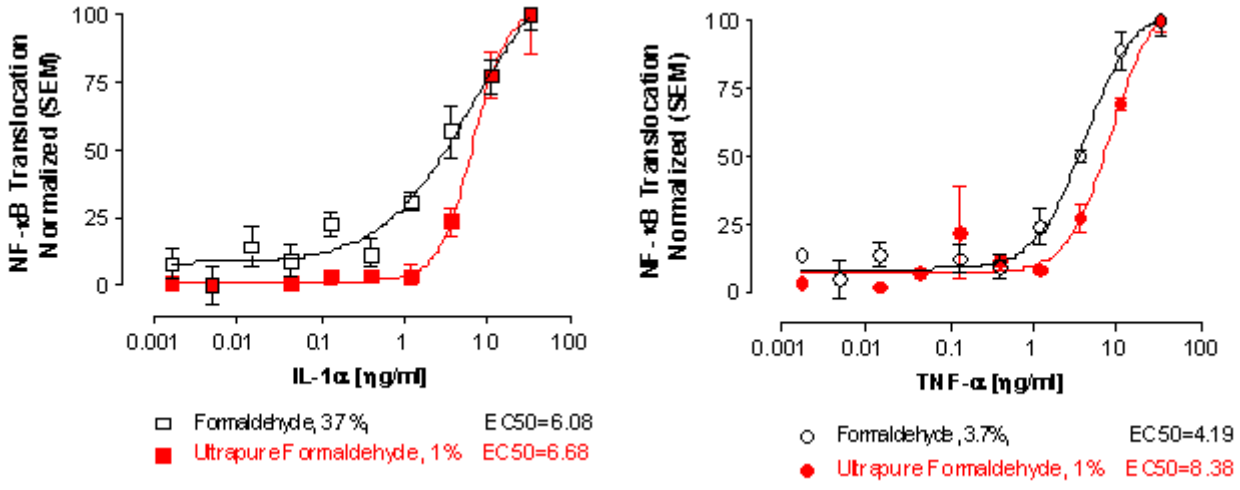
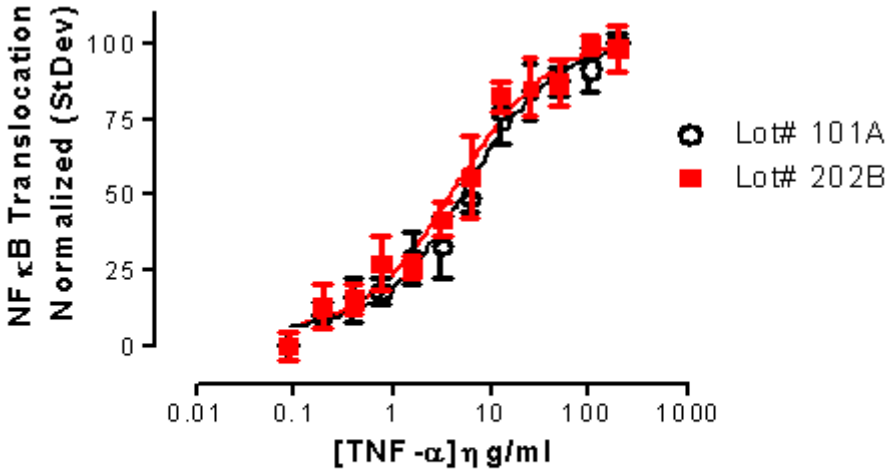
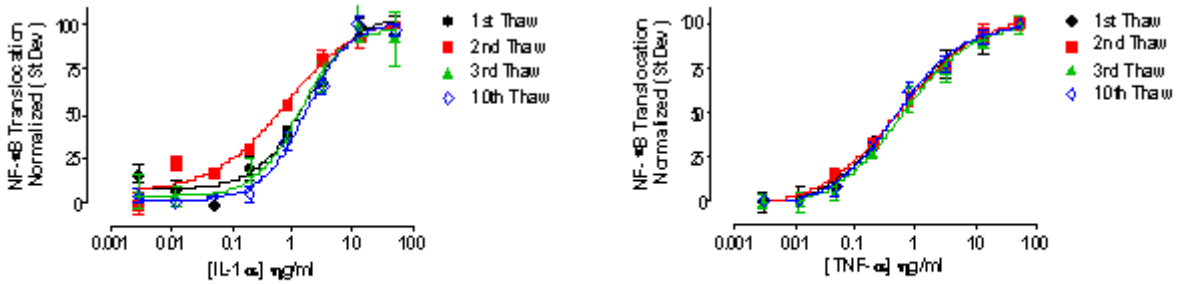


Figure 13: Comparison of Formaldehyde Fixatives. HeLa cells seeded at 5,000 cells/well overnight and treated with either dose response of IL-1α (left) or TNF-α (right) for ~35 minutes, then fixed with either 3.7% formaldehyde or 1% Ultrapure Formaldehyde and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



	Lot# 101A	Lot# 202B
EC50	5.851	4.554

Figure 14: Lot variability of tumor necrosis factor alpha. HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of TNF-α for ~35 minutes, then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



	1st Thaw	2nd Thaw	3rd Thaw	10th Thaw
EC50	1.669	0.7818	1.320	1.677

	1st Thaw	2nd Thaw	3rd Thaw	10th Thaw
EC50	0.4854	0.5408	0.5762	0.4641

Figure 15: Stability of cytokines following multiple freeze-thaw cycles. Following the reconstitution of the cytokine per manufacture suggestion, cytokine reagents were store at –80°C, and then allowed to thaw at room temperature before use. Samples were then re-frozen at –80°C multiple times. Translocation of NF-kB was performed on HeLa cells following treatment with IL-1α (left) or TNF-α (right) as previously described and data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

Stability of NF-κB detection in cells following fixation

The NF-κB protein in HeLa cells is stable for several days at 4°C prior to staining with NF-κB antibody following fixation with 3.7% formaldehyde for 10 minutes, washed and stored in PBS. This provides flexibility in screening operations if a "Stop Point" is required in the workflow. It is recommended that cells stained with NF-κB antibody be imaged as soon as possible, and not to exceed 14-days (Figure 16). For logisitics in screening operations including robotics, liquid handling, and cell plate handling to reduce flutations in pH and temperature, 35 minutes was choosen.

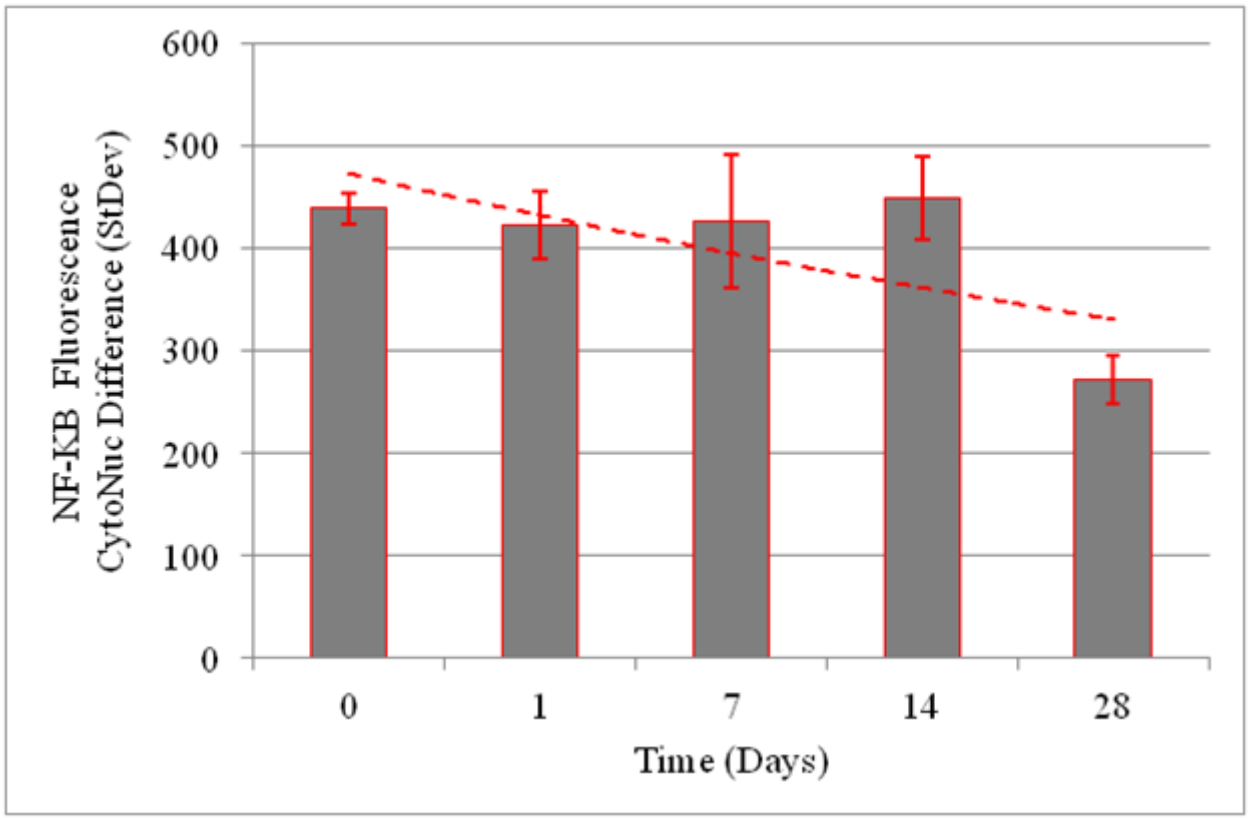


Figure 16: Stability of NF-kB-p65-AF488 complex post staining and fixation. Using several plates, HeLa cells seeded at 5,000 cells/well overnight and treated with 25ηg/ml of TNF-α for ~35 minutes, then fixed and stained to measure NF-κB translocation at the maximum signal. At different time points (days), plates were analyzed on HCS imager to determine NF-κB translocation fluorescent intensity measurements using CytoNuc Difference calculation; raw data was used and plotted for comparison.

Time Course of Cytokine Stimulation

Determine the optimal time window for NF-κB translocation by performing a time dependent stimulation with known stimuli. HeLa cells seeded at 5,000 cells/well in a 96-well plate overnight were then treated with 50 ηg/ml of IL-1α to establish a time course for activation and redistribution of NF-κB. Following incubation with cytokine, cells were fixed at different times and then labeled with NF-κB antibody using an indirect staining method with secondary fluorescent antibody. Images of cells expressing fluorescent antibody are captured using HCS imager and analyzed for NF-κB expression. Time ½ = 24 minutes (Figure 17). Based on these results 30-60 minutes proved to be an excellent window to capture NF-κB translocation.

Effects of Serum

HeLa cells treated with cytokines IL-1α and TNF-α were tested with different concentrations of fetal bovine serum (FBS) to determine if serum affects the assay performance and window. While the assay window did not diminish with concentration of serum, the sensitivity of cytokine stimulation as calculated by EC_{50} values of NF-κB translocation was significantly different; IL-1α sensitivity with 0.5% FBS increased ~3-fold as compared to 10%FBS, while TNF-α sensitivity increased by ~ 2.5-fold using 0.5%FBS as compared to 10%FBS (see Figure 18). Based on this data and subsequent data 0.5% FBS concentration was chosen for validation experiments.

DMSO Tolerance

HeLa cells seeded at 5,000 cells/well in 100µL media containing 0.5%FBS were allowed to attach overnight. Cells were then incubated with DMSO concentrations up to 10% for 30 minutes (2X the dosing time for compound

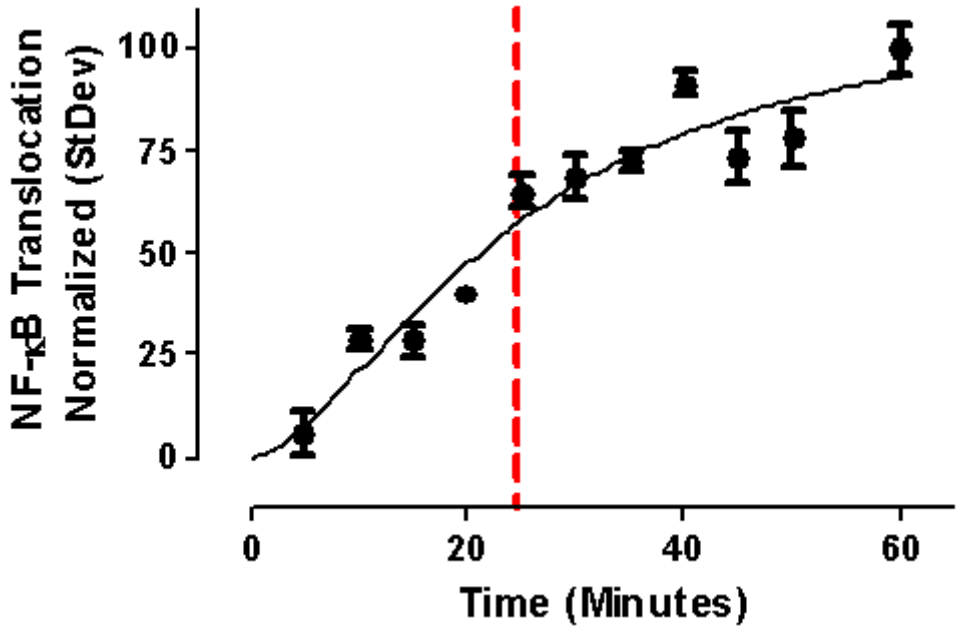
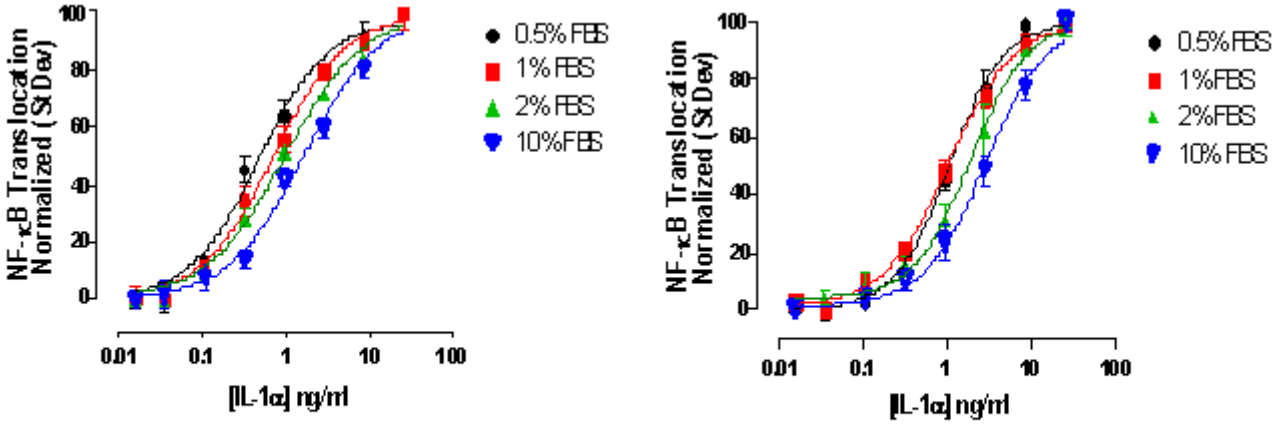


Figure 17: NF-κB Translocation time course kinetics. HeLa cells seeded at 5,000 cells/well overnight and treated with 25ng/ml of IL-1α over time, at 5 or 10 time minute intervals, cells were fixed and then stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression one-site binding to calculate the ½ time response, 24 minutes.



	0.5%FBS	1%FBS	2%FBS	10%FBS
EC50	0.4626	0.6944	0.9515	1.558

	0.5%FBS	1%FBS	2%FBS	10%FBS
EC50	1.060	1.021	1.853	2.784

Figure 18: NF-κB translocation effects from serum concentrations. HeLa cells seeded at 5,000 cells/well overnight in 10% Fetal Bovine Serum (FBS), were then removed from complete serum by washing with serum-free media and replaced with 0.5%, 1%, 2%, or 10% FBS and treated with dose response of either IL-1α (left) or TNF-α (right) for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

treatment) followed by 30-minute incubation of cytokine. The increase and subsequent decrease in signal at DMSO concentrations greater than 1% on the graph is a result in change in cell morphology and cytotoxicity respectively (Figure 19). At 5% DMSO, the cell's cytoplasm area shrunk as a result of increase in nuclear size,

thus the image analysis algorithm measurement is reflective in the data showing an increase in CytoNuc translocation (see figure 19). At 10% DMSO, the cells are fragmented, dissociated, or detached from the plate and therefore not possible to quantify cytoplasm or nuclear fluorescent expression.

Reference Compounds

Dosing Time of compounds on Cells

It is highly recommended to pre-determine the duration of exposure dose of an inhibitor compound prior to stimulation with secondary stimuli such as cytokines or growth factors. Please keep in mind and consider the following:

- Is it possible to co-add the inhibitor compound and stimuli simultaneously?
- How does this affect the S:N window or assay variability?
- Is a pre-incubation required to maximize window and assay variability?
- Is there an advantage for the workflow in screening operations?

For this assay a 15 minute pre-incubation of inhibitor compound was chosen to make certain the compound exposure to the cells was saturated prior to stimulating with cytokines. Comparative data suggests simultaneous co-addition of both the inhibitor compound and stimuli does not affect the performance of the assay window (data not shown). But keep in mind these are reference compounds and unknown chemicals in a compound library may not readily penetrate cells before NF-κB is activated.

Inhibitors of NF-κB Pathway

There are several known inhibitors of NF-κB pathway that have been published such as Bayer's compound, BAY 11-7085, which was initially selected as the control inhibitor reference compound for assay development and validation because it was commercially available and showed IC_{50} activity of about 10 μM (Figure 20). Additionally there were two compounds identified in-house named IK101 and IK202 with improved potency. These compounds were chosen as reference compounds in the assay validation, although BAY 11-7085 and BAY 11-7082 compounds both could serve as reference compounds. The IK101 and IK202 compounds, with IC_{50} values of less than 2 μM and 0.3 μM, respectively, were used for demonstration and performance for assay validation purposes (Figure 21). Inhibitor compounds were pre-incubated with cells for 15 minutes followed by cytokine addition for about 30 minutes.

Reference Compound Stability of Freeze-Thaw Cycles

HeLa cells treated with reference compounds (IK101 or IK202) that underwent multiple freeze-thaw cycles over a course of several weeks were tested for stability and/or loss of activity. Compounds were dissolved in 100% DMSO and aliquoted in cryovials and stored at –80°C for later use. After up to 10 freeze-thaw cycles there is no significant evidence of loss of compound activity (Figure 22).

Performance and Validation Pre-Screen

Following the optimization in the prior steps it is critical to validate the process procedure for screening operations before testing unknown chemicals. In Figure 23 and Figure 24, the reproducibility of cytokine stimuli is determined over a course of three independent assays and at least 3 different days to mimic the time it may take to screen an entire library. This requires independent harvesting of cells from flasks, cell seeding, and so on over 3 or more days. In Figure 25, the reference compound inhibitor IK202 was tested for reproducibility over 3 independent experiments, again to mimic screening operations. The results were acceptable for proceeding with Z-factor determination.

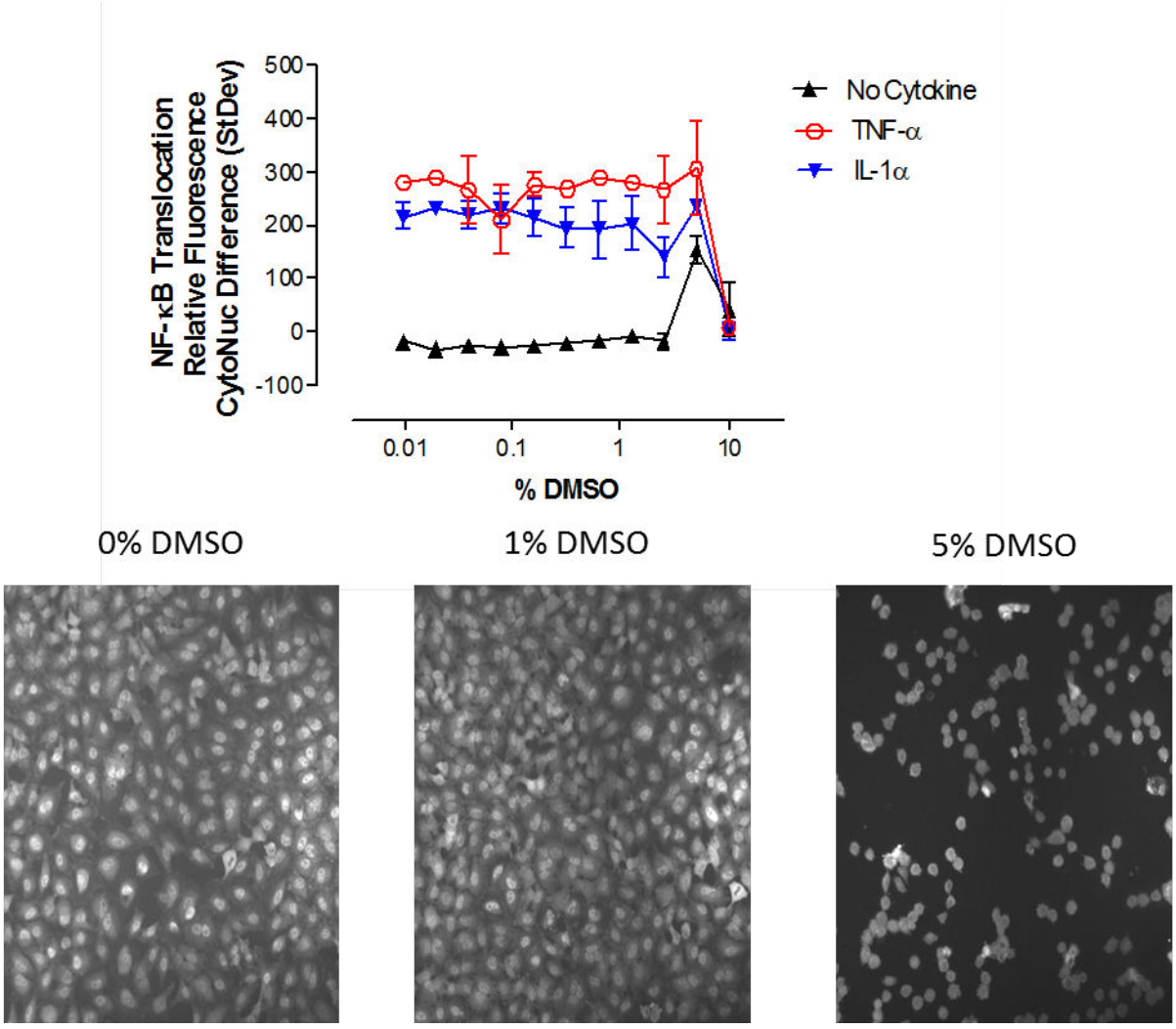
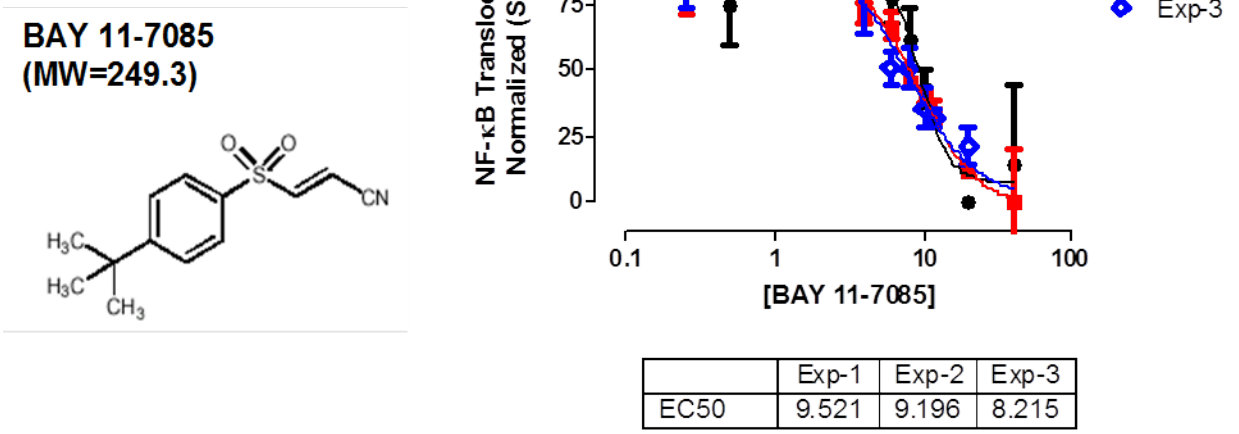


Figure 19: DMSO tolerance. HeLa cells seeded at 5,000 cells/well overnight and treated DMSO for 15 minutes followed by stimulation with either media only containing 0.5% FBS, 25ηg/ml IL-1α, or 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; raw data was used and plotted for comparison (top) and images captured using 10X/0.3NA objective (bottom) show differences in concentration.

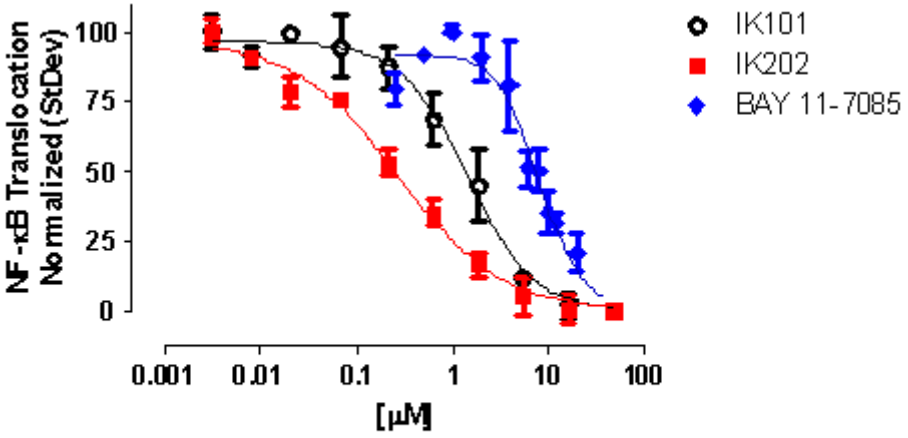
To determine the robustness and variability of single dose addition across the entire plate using liquid handling and robotics, at least 2 full plates with minimum and maximum responses are required to calculate a Z-factor. Z-factor is calculate using the formula 1- [3*(standard deviation of positive control + standard deviation of negative control) / (mean of positive control – mean of negative control)] (33). The values from the Z-factor are indicative of the variability of the data, values of less than 0 is considered too much overlap in the positive and negative signal; 0 – 0.5 is considered a marginal assay and 0.5 – 1 is considered an excellent assay.

For the NF-κB assay, the maximum response (Max or positive) is cytokine addition and the minimum (Min or negative) response is the reference compound inhibitor, i.e., IK202 + cytokine. Additional a Mid response can be added to verify ~50% reduction in the assay window. As mentioned earlier and throughout this chapter measuring translocation of NF-κB with HCS can use one or more HCS data features, namely intensities of the Cytoplasm-Nucleus Difference (CytoNuc Diff) or the Nucleus to Cytoplasm Ratio (Nuc/Cyt Ratio). In Figure 26 (IL-1α) and Figure 27 (TNF-α), both the CytoNuc Diff and Nuc/Cyt Ratio is reported. Interestingly, the TNF-α stimuli showed equivalent Z-factor values, however, the IL-1α stimuli Z-factor was significantly different; the CytoNuc Diff was 0.76 and the Nuc/Cyt Ratio was 0.64. Both are considered excellent screenable assays.



	Exp-1	Exp-2	Exp-3
EC50	9.521	9.196	8.215

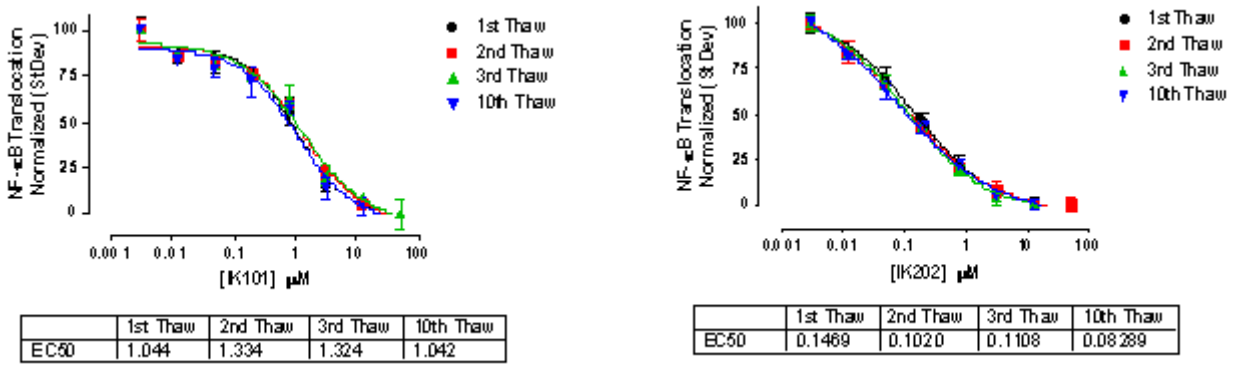
Figure 20: BAY 11-7085 structure and NF-κB translocation inhibition experimental variability. From 3 experiments, HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitor, BAY11-7085 for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



	IK101	IK202	BAY 11-7085
EC50	1.439	0.2682	8.215

Figure 21: Comparison of NF-κB translocation inhibition using reference compound inhibitor compounds BAY 11-7085, IK101 and IK202. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors, BAY11-7085, IK101, or IK202 for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

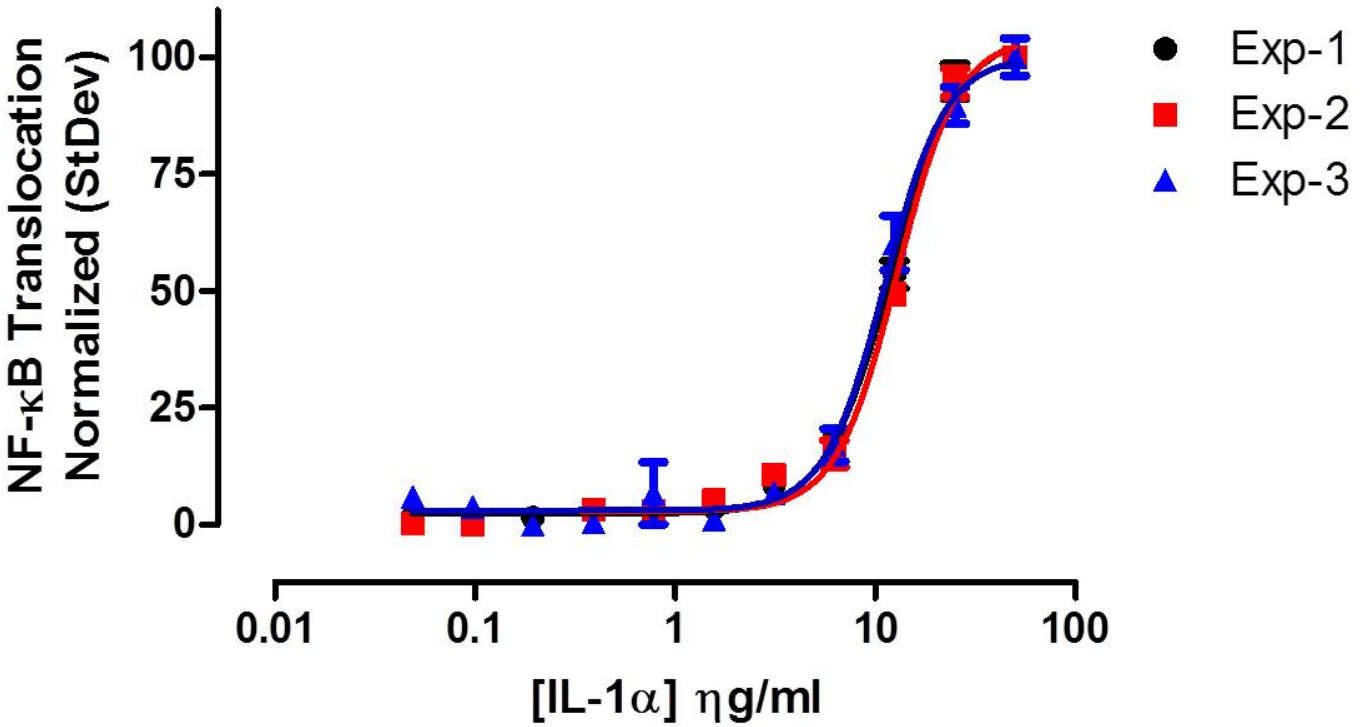
Reagents for assay are listed in Table 3.



	1st Thaw	2nd Thaw	3rd Thaw	10th Thaw
EC50	1.044	1.334	1.324	1.042

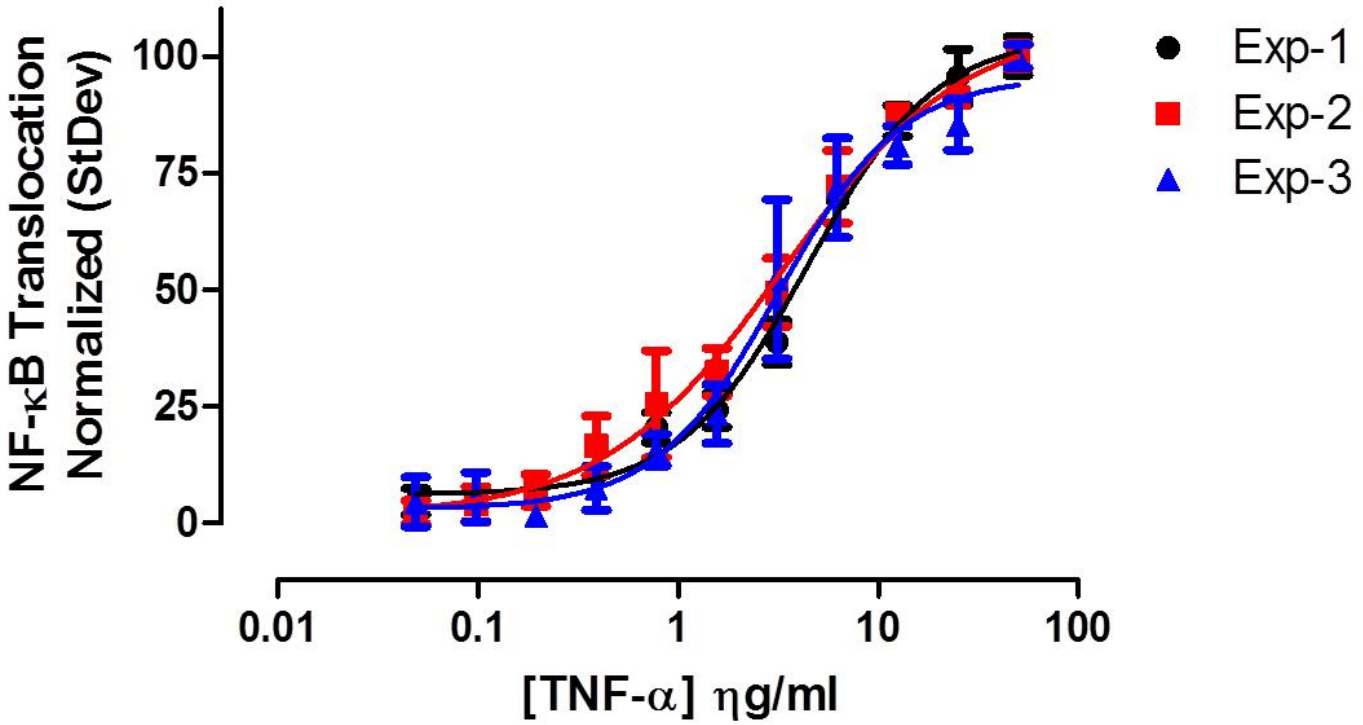
	1st Thaw	2nd Thaw	3rd Thaw	10th Thaw
EC50	0.1469	0.1020	0.1108	0.08289

Figure 22: Freeze-thaw cycle stability of reference compound inhibitors IK101 and IK202. Reference inhibitor compounds were made at 10mM in DMSO and stored at –80°C, and then allowed to thaw at room temperature before use. Compound vial samples were then re-frozen at –80°C multiple times following thaw. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors IK101 (left) or IK202 (right) for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



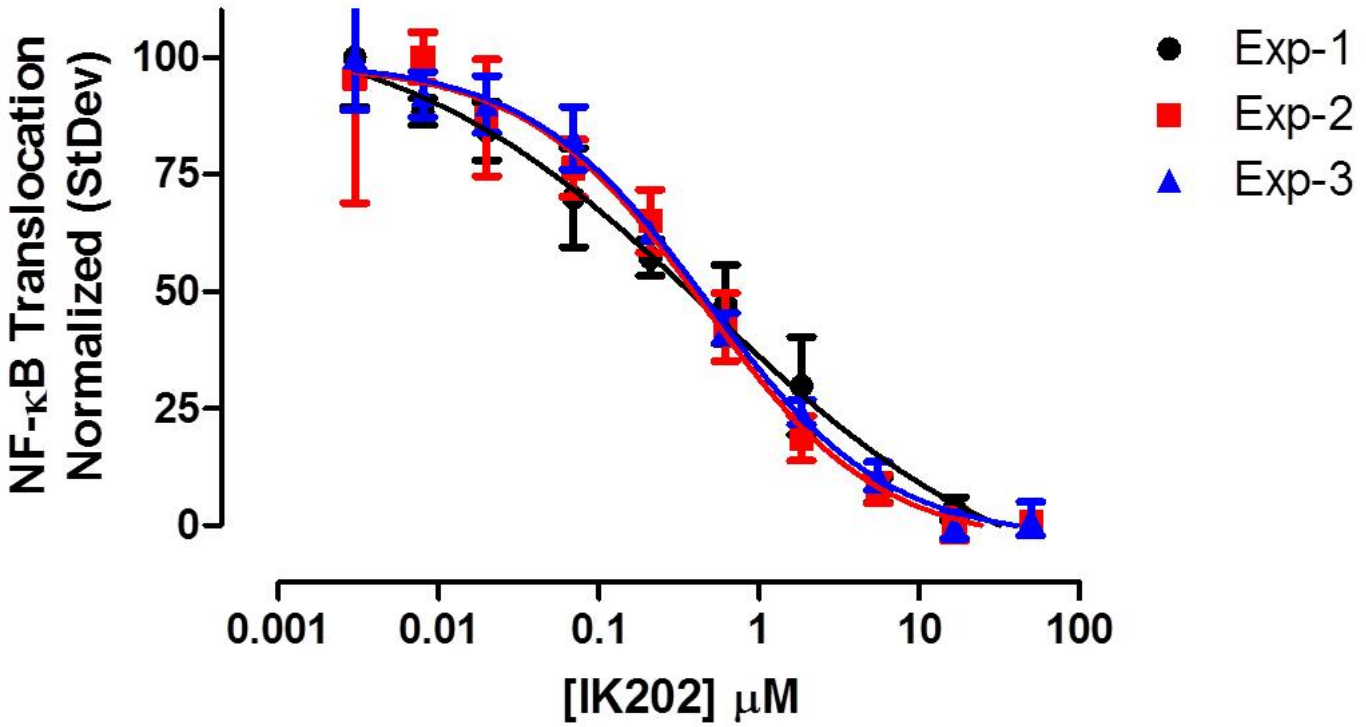
	Exp-1	Exp-2	Exp-3
EC50	12.08	12.86	11.21

Figure 23: IL-1α dose response experimental variability. HeLa cells seeded at 5,000 cells/well overnight and treated with IL-1α in dose response for ~35 minutes were fixed and then stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



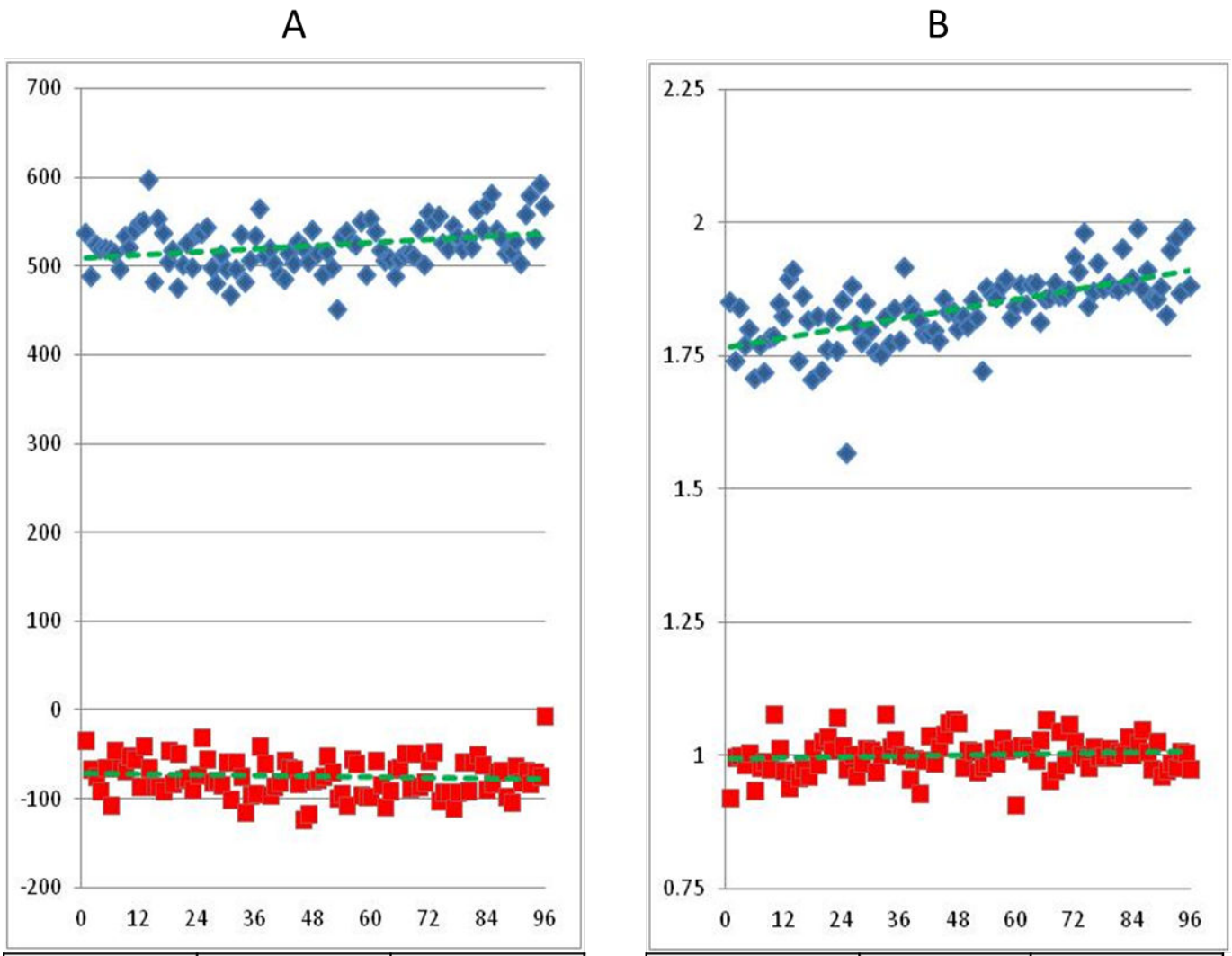
	Exp-1	Exp-2	Exp-3
EC50	4.453	3.357	3.176

Figure 24: TNF-α dose response experimental variability. HeLa cells seeded at 5,000 cells/well overnight and treated with TNF-α in dose response for ~35 minutes were fixed and then stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



	Exp-1	Exp-2	Exp-3
EC50	0.4985	0.4474	0.4717

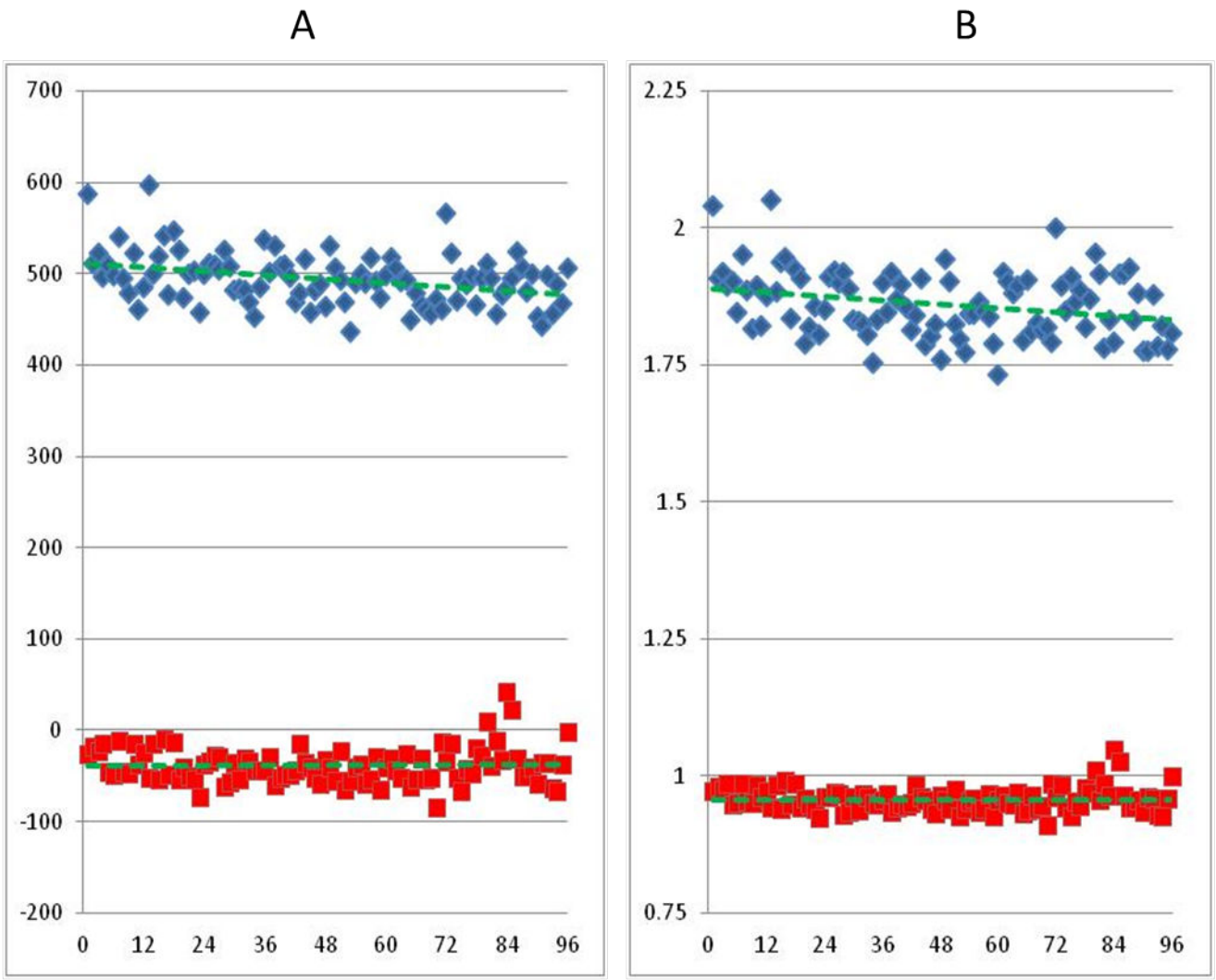
Figure 25: Reference compound inhibitor IK202 dose response 3 day experimental variability. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors IK202 for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



CytoNuc Diff	Max (IL-1α)	Min (Ref Cmpd)
Mean	523.1274	-75.204
% CV	5.2%	3.5%
Z-factor	0.76	

Nuc/Cyt Ratio	Max (IL-1α)	Min (Ref Cmpd)
Mean	1.8389	1.001
% CV	3.7%	3.4%
Z-factor	0.64	

Figure 26: NF-κB translocation IL-1α stimuli Z-factor calculation & graph display. HeLa cells seeded at 5,000 cells/well overnight into 3 different 96-well plates were treated with 0.5% DMSO for maximum response (Red), media for unstimulated (green), and reference compound inhibitors IK202, 50μM for minimum response (blue) for 15 minutes followed by stimulation with 25ηg/ml IL-1α for ~35 minutes. Cells were then fixed and labeled to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference (Panel A) or Nuc/Cyt Ratio (Panel B) calculation. Raw data values from HCS instrument were used to calculate the mean response (left axis) and standard deviation (right axis). Z-factor was calculate using the formula 1- [3*(standard deviation of positive control + standard deviation of negative control) / (mean of positive control – mean of negative control)] (33).



CytoNuc Diff	Max (IL-1α)	Min (Ref Cmpd)
Mean	494.5624	-38.777
% CV	5.8%	3.7%
Z-factor	0.76	

Nuc/Cyt Ratio	Max (IL-1α)	Min (Ref Cmpd)
Mean	1.8606	0.957
% CV	3.7%	2.3%
Z-factor	0.73	

Figure 27: NF-κB translocation TNF-α stimuli Z-factor calculation & graph display. HeLa cells seeded at 5,000 cells/well overnight into 3 different 96-well plates were treated with 0.5% DMSO for maximum response (Red), media for unstimulated (green), and reference compound inhibitors IK202, 50μM for minimum response (blue) for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and labeled to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference (Panel A) or Nuc/Cyt Ratio (Panel B) calculation. Raw data values from HCS instrument were used to calculate the mean response (left axis) and standard deviation (right axis). Z-factor was calculated as previously described.

Table 3: List of materials and reagents

Reagents	Vendor	Cat #
Alexa 488 goat anti-rabbit IgG (H+L, 2 mg/ml	Invitrogen	A-11008
Albumin from Bovine Serum (BSA)	Sigma	A-2153
Dimethylsufloxide (DMSO), 78.13 g/mole	Thermo	20684

Table 3 continued from previous page.

Reagents	Vendor	Cat #
DPBS (PBS), Mg^{2+} and Ca^{2+} free	Lonza	17-512Q
Fetal Bovine Serum, Defined	Thermo	Sh30070
Formaldehyde, 37%	Sigma	F-1268
**Formaldehyde, Ultrapure, MeOH free, 10%*	Polysciences	04018
**Paraformaldehyde, Ultrapure, MeOH free, 16%*	Polysciences	18814-20
HeLa cells	ATCC	CCL-2
Hoehst 33342	Invitrogen	H-21492
L-Glutamine, 200 mM	Lonza	17-605E
Minimum Essential Medium Eagle (EMEM)	Lonza	12-662F
NF-κB p65 (C-20) Rabbit Polyclonal IgG, 200 µg/ml	Santa Cruz	SC-372
Penicillin/Streptomycin, 10000 Units	Lonza	17-602E
Polyoxyethylensorbitan monolaurate (Tween-20) 10%	Roche	1332465
Recombinant Interleukin -1α (rh IL-1α)	R&D Systems	200-LA
Recombinant Tumor Necrosis Factor-α (rh TNF-α)	R&D Systems	210-TA
t-Octylphenoxypoly-ethoxyethanol (Triton X-100), 10%	Roche	1332481
Trypsin-EDTA, 1X (0.05%)	Invitrogen/Gibco	25300-054
Water, Reverse Osmosis (RO-H_2O)	House	
Reference Compound(s)		
BAY 11-7082	Enzo	BML-EI278
BAY 11-7085	Enzo	BML-EI279
Consumables		
12-Place Dilution Reservoir	USA Scientific	1301-1212
150 cm^2 Cell Culture Flask (T-150)	Corning	430825
96-well Packard View Plates	Perkin Elmer	6005182
96-well V-bottom Plates	Nunc	245128
Backing Tape, Black	Perkin Elmer	6005189
Cell Strainer, 70 µM	BD Falcon	352350
Plate Seals	Excel Scientific	100-SEAL-PLT
Reagent Reservoir, 50 ml	Corning (Costar)	4870
Storage Mats, 96-well plate	Corning (Costar)	3092

* Alternative fixative solution; recommend a final solution of 4% paraformaldehyde but test in assay model to verify performance.

Preparation of Stock Reagents:

1. Reference compound (IK202), 10 mM: Make a 10mM stock solution by dissolving 10mg IK202 compound (MW=202.3) into 4.95 ml DMSO. Store at –80°C.
2. Hoechst 33342, 2 mg/ml: Make a 2 mg/ml stock solution by dissolving in 100% DMSO. Protect from light. Store aliquots at -80°C ~indefinitely.

3. IL-1α, 10 µg/ml: Make a 10 µg/ml stock solution by dissolving in PBS containing 0.1% BSA. Store aliquots at -80°C for up to 6 months.
4. TNF-α, 10 µg/ml: Make a 10 µg/ml stock solution by dissolving in PBS containing 0.1% BSA. Store aliquots at -80°C for up to 6 months.

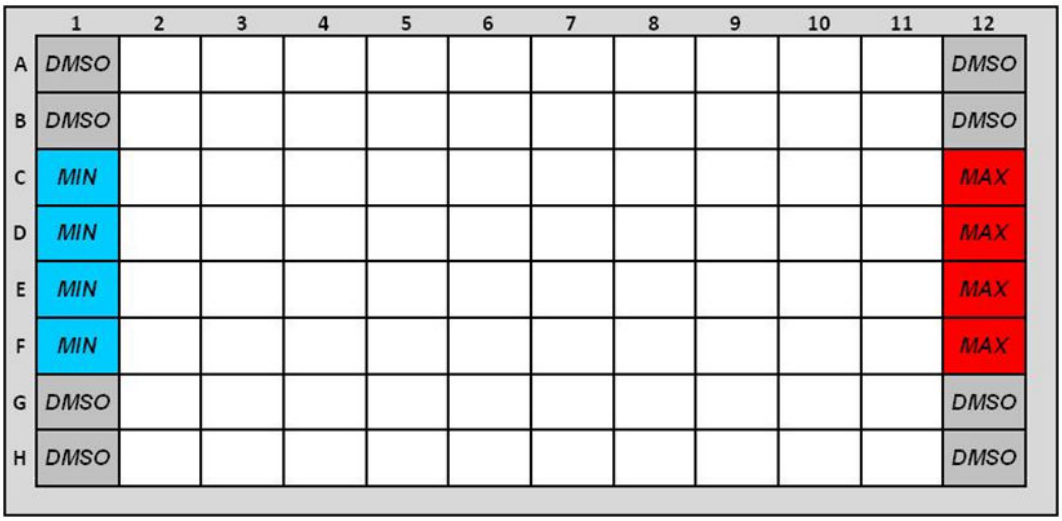
Working Solutions per 96-well Plate (Prepared daily)

1. Inhibitor compound: Dilute in EMEM media without serum at 5X final concentration (50 µM final at 250 µM). For IK202 compound, dilute 25 µl of 10mM stock into 1ml of EMEM media. Note final DMSO concentration should not exceed 1%.
2. IL-1α: Make working solution at 6X (150ηg/ml) the final assay concentration, which is 25ηg/ml. Dilute 15 µl of 10 µg/ml cytokine into every 1ml of EMEM media needed to complete the assay. For one 96-well plate dilute 45 µl of 10 µg/ml cytokine stock in 3ml of EMEM. Note, for EC_{50}, start at 40ηg/ml final and dilute 1:3 in PBS or medium. For time course or kinetics study, use 25 ηg/ml.
3. TNF-α: Make working solution at 6X (150ηg/ml) the final assay concentration, which is 25ηg/ml. Dilute 15 µl of 10 µg/ml cytokine into every 1ml of EMEM media needed to complete the assay. For one 96-well plate dilute 45 µl of 10 µg/ml cytokine stock in 3ml of EMEM. Note, for EC_{50}, start at 40 ηg/ml final and dilute 1:3 in PBS or medium. For time course or kinetics study, use 25 ηg/ml.
4. Formaldehyde: Dilute 37% formaldehyde 1:10 with PBS. Warm to 37°C before use, this must be prepared fresh with each assay run.
5. 0.1% Triton X-100: Dilute 250 µl of 10% Triton X-100 in 24.75 ml of PBS. Prepare fresh daily.
6. 0.01% Tween-20: Dilute 50 µl of 10% Tween-20 in 50ml of PBS. Prepare fresh daily.
7. Primary Rabbit anti-NF-κB-p65 polyclonal antibody: Make 5 µg/ml 1^{o} antibody solution by diluting antibody 1:40 in PBS (137.5 µl of antibody into 5.5ml of PBS). Prepare fresh daily.
8. Goat anti-Rabbit Alexa-488 Secondary Antibody / Hoechst Stain: Make a 10 µg/ml 2^{o} antibody solution by diluting antibody 1:400 in PBS (27.5 µl of 2^{o} antibody into 5.5ml of PBS). Add 5.5 µl of 2 mg/ml Hoechst 33342 stock solution for a 2 µg/ml solution (1:1000 dilution). Prepare fresh daily and protect from light.

Plate Layout

Plate controls used as experimental references can be placed in any wells throughout the plate as long as the "data analysis calculator" is programmed to interrupt the plate layout conditions and concentrations of compound. For simplicity and a guideline use the example plate layout illustrated in Box 1 with controls on the outside columns and all test compounds in the center of the plate. Edge effects for the assay are minimal because of the short incubation period.

Box 1: Example of a Plate Layout.



Protocol for finding Inhibitors (Antagonist) in the NF-κB Pathway

Note: All washes and buffers are at 100µl unless indicated otherwise.Assays can be performed with automated hand-held pipettes for less than 10-plates. For more than 10-plates it is recommended to use automated plate washers and liquid handling devices.

Day-1

1. Seed approximately 5,000 cells/well in 100 µl volume of EMEM media containing 0.5% FBS, 2 mM L-Glutamine in 96-well plate (recommend using automated cell dispensing device for uniformity)
2. Allow cells to attach overnight in complete medium at 37°C, 5% CO_2, 95% relative humidity.

Day-2

3. Transfer 25 µl 37°C pre-warmed compound from v-bottom compound plates to cell plate using automated liquid handling device.

4. Incubate for 15 minutes at 37°C, 5% CO_2, 95% relative humidity.

5. Transfer 25 µl 37°C pre-warmed cytokine (IL-1α or TNF-α) from v-bottom compound plates to cell plate using automated liquid handling device.

6. Incubate for 35 minutes at 37°C, 5% CO_2, 95% relative humidity.

7. Remove all media using hand-held aspirator or with automated liquid handling device.

Note: option to add high concentration of fixative directly to the cells or remove media and add final concentration of fixative buffer.

8. Immediately fix cells by adding 100 µl of "pre-warmed" 3.7% formaldehyde solution in a vented hood or with an automated liquid handling device.

9. Incubate at room temperature for 10 minutes with plate lid.

10. Remove formaldehyde and replace with 100 µl of PBS using an automated liquid handling device. Please read safety precautions before working with formaldehyde.

Note: At this step, you can stop the experiment and store plate at 4°C for several days by filling wells with salt solution and sealing to prevent evaporation.

11. Add 100 µl of 0.1% Triton X-100/PBS working solution and incubate 5 minutes at room temperature (RT). Use automated liquid handling device as needed.

12. Wash plate 2X with 100 µl PBS at RT, leaving PBS on cells.

13. Use 96-head aspirator to remove buffer or use an automated liquid handling device. DO NOT ALLOW WELLS TO DRY.

14. Immediately add 50 µl of 1° antibody using hand-held pipettor or automated liquid handling device. Incubate for 60 min at RT.

15. Remove antibody and discard. Wash plate 1X with 100 µl of 0.01% Tween 20/PBS.

16. Incubate for 15 minutes at RT.

17. Wash 2X with 100 µl PBS at RT, leaving PBS on cells.

18. Use 96-head aspirator or automated liquid handling device to remove buffer. DO NOT ALLOW WELLS TO DRY.

19. Immediately add 50 µl of 2° antibody containing Hoechst dye per well.

20. Incubate 1-hour in the dark at RT. Protect from light by using black tape plate lids or foil.

21. Wash with 100 µl of 0.01% Tween20/PBS. Incubate for 10 minutes at RT.

22. Wash plate with 100 µl PBS at RT

23. Add 200 µl PBS and seal plates.

24. Analyze plates on High Content Imaging device. If required, plates can be store at 4°C in dark for future analysis. Do not freeze plates.

Protocol for Activators (Agonist) of NFκB Pathway

Note: All washes and buffers are at 100 µl unless indicated otherwise.Assays can be performed with automated hand-held pipettes such as Matrix for less than 10-plates. For more than 10-plates, automated plate washers, Multidrop, or MultiMek should be used.

Day-1

1. Plate approximately 5,000 cells/well in 100 µl volume of EMEM media containing 0.5% FBS, 2mM L-Glutamine in 96-well plate (Multidrop)
2. Allow cells to attach overnight in complete medium at 37°C, 5% CO_2, 95% relative humidity.

Day-2

1. Transfer 25 µl of 37°C pre-warmed unknown agonist or known agonist such as cytokines (IL-1α or TNF-α) from v-bottom compound plates to cell plate using automated liquid handling device.
2. Incubate for 35 minutes at 37°C, 5% CO_2, 95% relative humidity.
3. Remove all media using hand-held aspirator or with automated liquid handling device.

Note: option to add high concentration of fixative directly to the cells or remove media and add final concentration of fixative buffer.

4. Immediately fix cells by adding 100 µl of "pre-warmed" 3.7% formaldehyde solution in vented hood or with an automated liquid handling device.

5. Incubate at room temperature for 10 minutes with plate lid.

6. Remove formaldehyde and replace with 100 µl of PBS using an automated liquid handling device. Please read safety precautions before working with formaldehyde.

Note: At this step, you can stop the experiment and store plate at 4°C for several days by filling wells with salt solution and sealing to prevent evaporation.

7. Add 100 µl of 0.1% Triton X-100/PBS working solution and incubate 5 minutes at room temperature (RT). Use automated liquid handling device as needed.

8. Wash plate 2X with 100 µl PBS at RT, leaving PBS on cells.

9. Use 96-head aspirator to remove buffer or use an automated liquid handling device. DO NOT ALLOW WELLS TO DRY.

10. Immediately add 50 µl of 1° antibody using hand-held pipettor or automated liquid handling device. Incubate for 60 minutes at RT.

11. Remove antibody and discard. Wash plate 1X with 100 µl of 0.01% Tween20/PBS.

12. Incubate for 15 minutes at RT.

13. Wash 2X with 100 µl PBS at RT, leaving PBS on cells.

14. Use 96-head aspirator or automated liquid handling device to remove buffer. DO NOT ALLOW WELLS TO DRY.

15. Immediately add 50 µl of 2° antibody containing Hoechst dye per well.

16. Incubate for 1-hour in the dark at RT. Protect from light by using black tape plate lids or foil.

17. Wash with 100 µl of 0.01% Tween20/PBS. Incubate for 10 minutes at RT.

18. Wash plate with 100 µl PBS at RT

19. Add 200 µl PBS and seal plates.

20. Analyze plates on High Content Imaging device. If required plates can be store at 4°C in dark for future analysis. Do not freeze plates.

Note: Cells will respond to cytokines in the presence of serum and NF-κB will translocate to nucleus. For step-3, be sure to adjust either the volume of medium or the amount of cytokine.

Logistics Analysis of Protocol

1. Projected # plates/day: state number of plates that can be processed per day
2. Suggested equipment: automated cell dispensing devices, automated liquid handling devices, 96-well aspirator, HCS imager
3. Timing issues, stop points: After fixation step
4. Stability and process studies: Compound and cytokine freeze-thaw stability acceptable
5. Dealing with multiple lots of reagents: None
6. Reagent and supplies availability: None
7. Flow Chart: None

8. Safety considerations: Example, formaldehyde use, see appendix-6
9. Data Collection Issues: state electronic location of data storage
10. Estimated throughput: state number of plates that can be processed per day
11. Batching Plates/Stop Points:
12. 3.7% formaldehyde fixation – STOP POINT - plates can be stored for several days at 4^{o}C in PBS.
13. Permeabilization with 0.1% Triton X-100 – BATCHING – handle as many plates that can be processed under 15-minute incubation period.
14. Wash steps – BATCHING – do not allow wells to dry and do not introduce air bubbles during wash and aspiration steps. Handle one plate at a time unless automation devices are used.
15. Post staining, after plate is sealed – STOP POINT – store plates at 4^{o}C in the dark and read on HCS Imager within 14-days.

Cell Based Screen

1. Cell Handling for HeLa cells
    a. Cell Bank – l*ocation of cells in the cell bank*
    b. Continuous Passage – Yes, maximum passage is not known.
    c. Transfection – No
    d. Proposed Schedule – continuous passage
    e. Cell Culturing Guidelines:
        i. Subculturing: Remove medium, and rinse with PBS. Remove the solution and rinse cell monolayer with Trypsin-EDTA solution. Remove and allow flask to sit at room temperature for 3-5 minutes or until the cells round up. Lightly tap flask to detach cells. Add fresh culture medium containing at least 0.5%FBS, aspirate and dispense into new culture flasks.
        ii. Split Ratio: A subcultivation ratio from 1:2 to 1:10 is recommended
        iii. Fluid Renewal: 2 to 3 times per week
2. Propagation: Suggested medium: Minimum essential medium Eagle with 2 mM L-glutamine, fetal bovine serum, 10%; 1% (100Units/ml) Penicillin/Streptomycin.
   Temperature: 37°C , 5%CO_2
3. Freeze Medium: culture medium 90%; DMSO, 10%

Compound Libraries

It is recommended to use a library of compounds to test the sensitivity and robustness of the assay for both negative and positive responses. There is a unique NF-κB inhibitor set of 14 -compounds available from Calibiochem (Cat#481487 InhibitorSelect™ NF-κB Signaling Pathway Inhibitor Panel). There are now 4 versions of this library with the latter ones containing IκK inhibitors. Additional there are other larger compound library collections commercially available for purchase including LOPAC. For this assay the Calbiochem kinase Library, otherwise known as the Millipore EMD 539744 InhibitorSelect™ 96-Well Protein Kinase Inhibitor Library-I was the only commercial library available. Interestingly, this 80 chemical compound library contains no NF-κB or IκK inhibitor compounds and this is reflected in the results. For single point determination at 50 μM, two compounds showed an increase in NF-κB CytoNuc difference was a result in changes in the morphological characteristics of the cell in assay both with IL-1α and TNF-α stimulation (Figures 28 and 29). No activates were confirmed with IL-1α or TNF-α stimulation IC_{50} curves follow-ups from 50μM, 3-fold in duplicates.

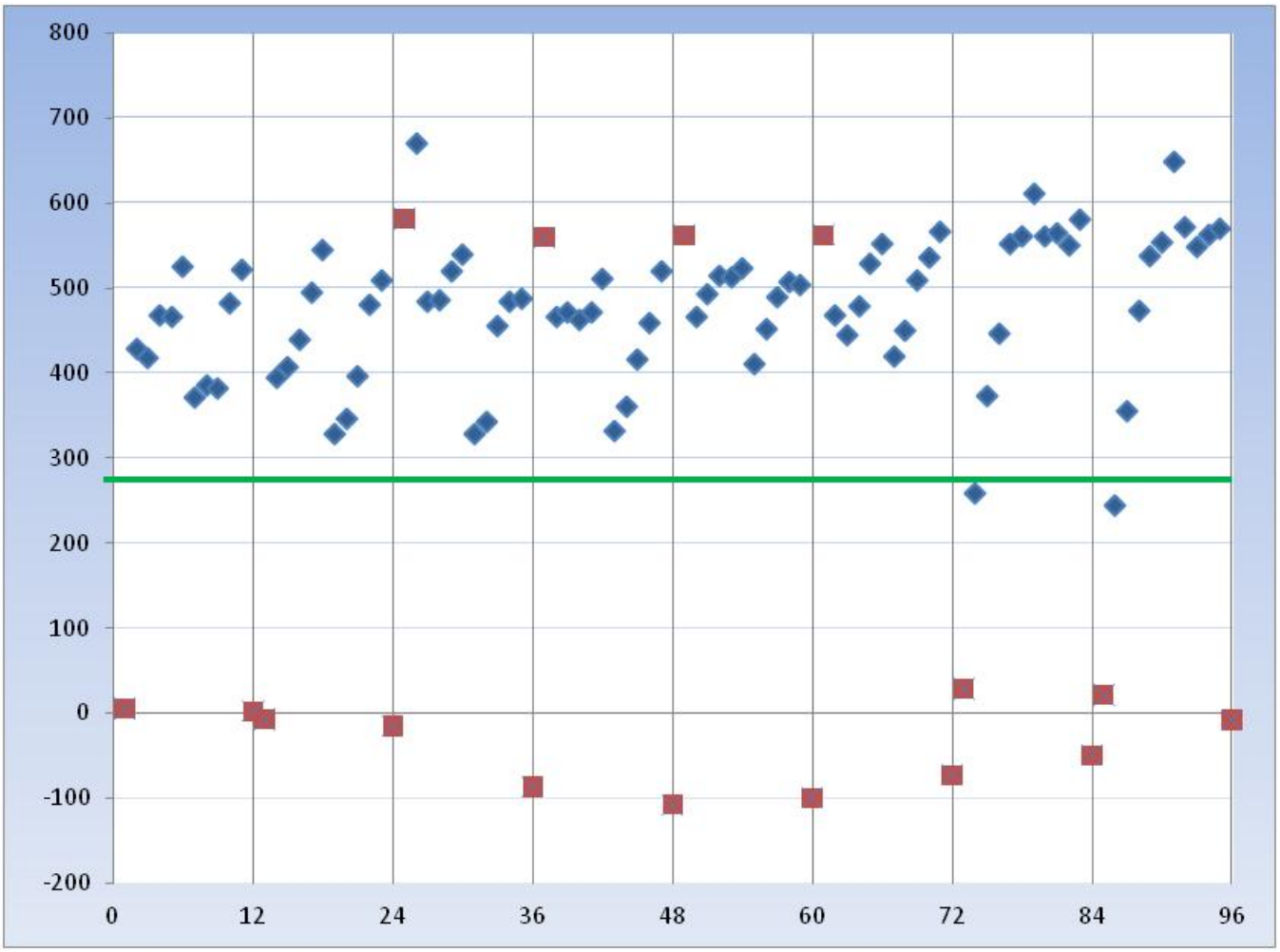


Figure 28: Results from Calbiochem kinase library (EMD 539744 InhibitorSelect™) screen. HeLa cells seeded at 5,000 cells/well overnight and dosed with a single dose of 50μM compound for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and labeled to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation. Raw data values from HCS instrument were used to calculate the mean response. Controls represented as "red squares" and compound tested shown in "blue triangles". Green line represents ~50% activity; points below this line are considered "hits". No inhibitors were confirmed; one compound showed an increase in NF-κB Nuc-Cyt difference that was a result in changes in the morphological characteristics of the cell in assay both with IL-1α and TNF-α stimulation. Images not shown.

Other Considerations

Detection of Cytotoxicity and Fluorescent Compounds in NF-kB Translocation Assay

One of the powers of HCS is providing the capability to measure morpohological and intensities of bioprobe markers at the single cell level. The combination of these capapbilites allows the ability to measure cell populations and subpopulations captured in the image. If a compound dosing concentration is the direct or indirect cause of cytotoxicity it is often measureable with good precision by using population statistics of the number of cell objects detected in the well in comparison to a vehicle control well. Optional, cytotoxic and viable dyes can be used in combination with the biomarker indicator as an reporter. HeLa cells seeded overnight, then pre-incubated with 10-point compound dose response starting at 50μM, ½ log for 15 minutes and susequently treated with TNF-α for 35 minutes, were staiend with NFkB antibody as previously described. The inhibition of NF-κB in the presence of the known reference compound recorded an IC_{50} of 0.2095 μM using 3-parametric fit

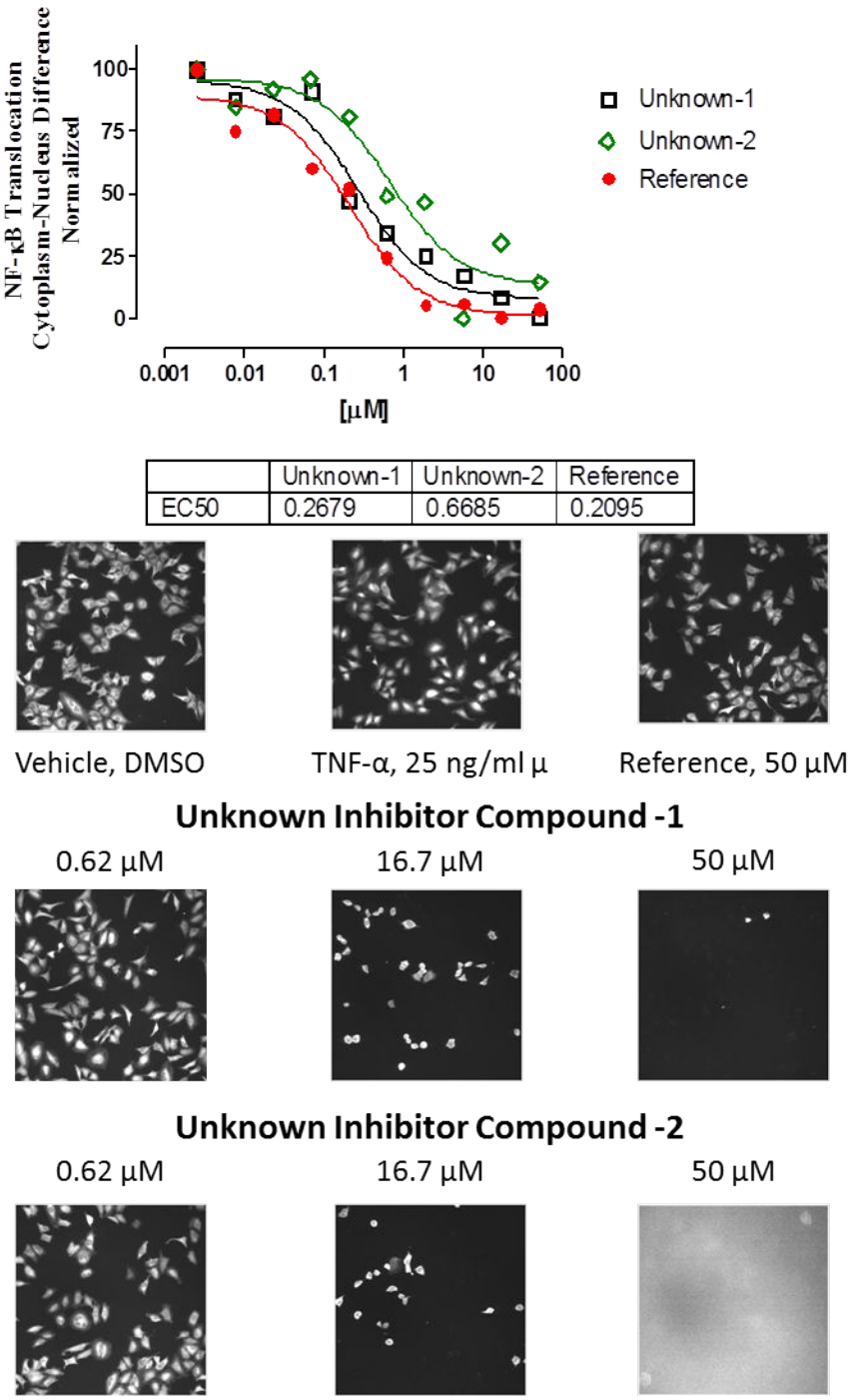


Figure 29: Reference compound and unknown inhibitor dose response with corresponding images. Top panel is a comparison of reference compound inhibitor and two unknown inhibitors identified as actives in screen. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors IK202 or two unknown inhibitor compounds for 15 minutes followed by stimulation with 25ηg/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit. Bottom panel of images: (A) vehicleI DMSO) control; (B) TNF-α, 25ηg/ml control; (C) IK202, 50μM reference compound inhibitor; (D) Unknown inhibitor compound-1, 0.62μM; (E) Unknown inhibitor compound-1, 16.7μM; (F) Unknown inhibitor compound-1, 50μM; (G) Unknown inhibitor compound-2, 0.62μM; (H) Unknown inhibitor compound-2, 16.7μM; (I) Unknown inhibitor compound-2, 50μM.

in GraphPad Prism, whereas the unknown inhibitor compounds recored and IC_{50} of 0.2679 μM and 0.6685 μM. The differences in these values is somewhat subtle in the curve fitting; however, the key differences is observed in

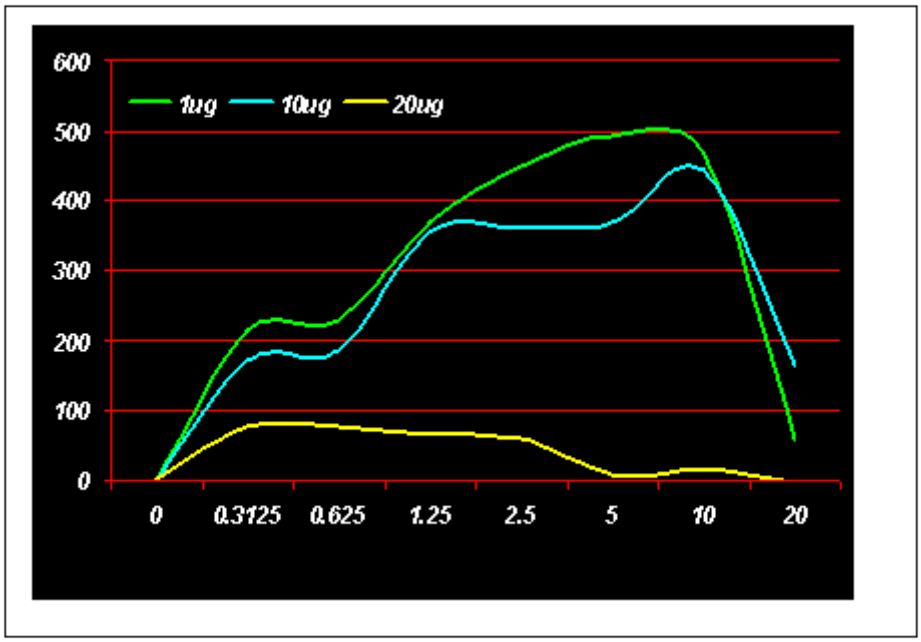
the loss of cells with increasing concentration of the compound above the recorded IC_{50} value. It is important to always review and recored the number of cellular objects, fields, or other HCI feature parameters that provide information about an alteration in the cell seeding density in the well in addition to other key HCS feature values including reviewing the images to validate the results (Figure 30).

To determine if an unknown compound(s) is fluorescent in the visible spectra when screening a chemical compound library using HCS assays can be achived by using intensity measurements in the nucleus or cytoplasm regions of interests (ROI). These compounds can create a false positive and are not commonly identified until follow ups are underway in protein redistrubution assay such as NFkB where fluorescence can occupy both the cytoplasm and nuclear ROI. If a suspect compound is thought to be fluorescent in the HCS assay, this can verify using a high content imager by measuring the baseline autofluorescence of cells using vehicle control, then measure any increase in fluorescence following compound treatment. Evidence of compound fluorescence in the cells is typically observable using fluoresent mcirscopy images not shown.

Screening Commercially Available NF-κB Antibodies (Appendix 1)

NF-κB Antibody Staining (Santa Cruz, SC-372)

Secondary labeling concentration; 1, 10, 20µg/ml.



Graph of commercially available antibodies evaluated

X-axis represents 1^{o}Ab concentration with 10µg/ml 2^{o}Ab;

Y-axis represents average fluorescent intensity of NF-κB difference between the cytoplasm and nucleus subtracted from the 2^{o}Ab control. Values listed below x-axis are the raw data (CytoNuc Diff).

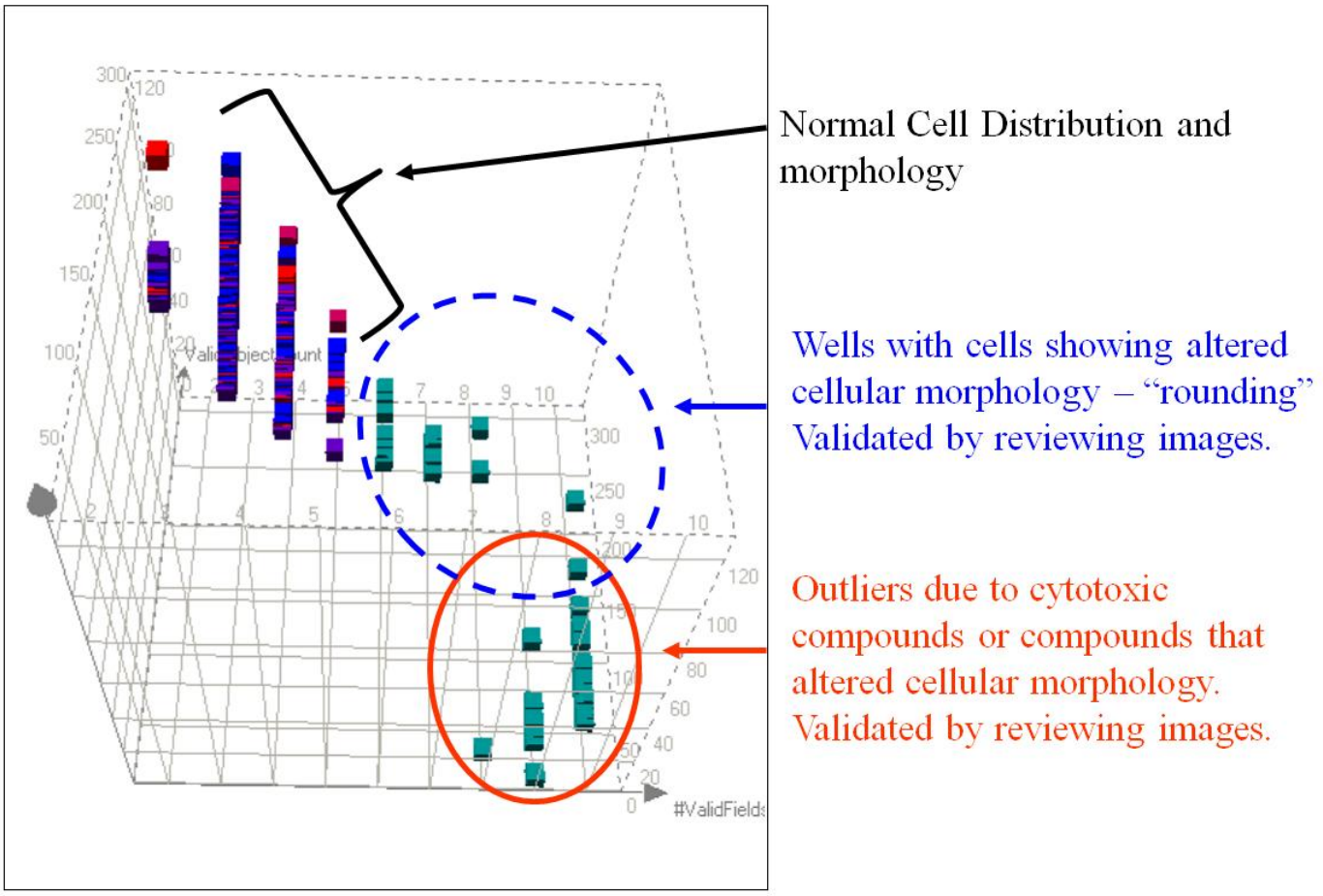
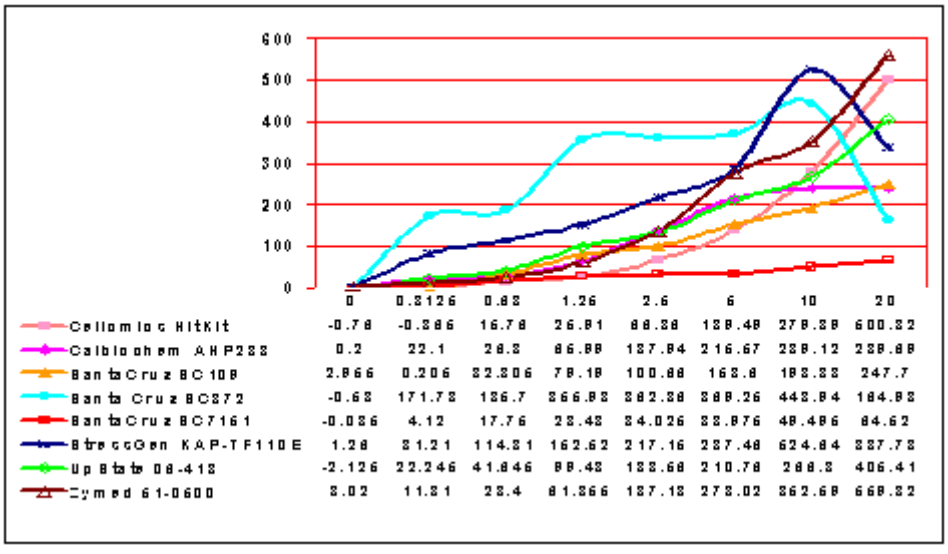


Figure 30: SpotFire 3-D graph visulaization: well level plate HCS features showing separation between normal cell distrubution, altered morhology, or cell loss and/or cytotoxic effects. X axis = number of valid fields; Y axis = valid object count; Z axis = average cell density/field



Safety Considerations: Guidelines & Precautions (Appendix 2)

Please follow institutional laboratory safety considerations and special precautions with regard to formaldehyde and paraformaldehyde use. Training is commonly required for all personnel using formaldehyde and paraformaldehyde

Formaldehyde and Paraformaldehyde Use Guidelines in the Laboratory:

1. All stock solutions greater than 4% must be maintained in a fume hood.
2. Working solution of less than 4% and less than 37°C can be used in the following areas:
    - Fume hood
    - Biosafety cabinet
    - Automation liquid handling robots with standard setup with <4% formaldehyde solution in appropriate reservoir positions. Following addition of formaldehyde, plate lids must cover open plates to minimize formaldehyde exposure during a 10-15 minute incubation period. Transfer of formaldehyde to and from the liquid handling robot reservoir requires a safety container that is larger than the stock reservoir container to collect any spill. It is recommended that the volume of working solution of formaldehyde does not exceed 100ml at any given time. If additional formaldehyde solution volume is required than add as needed.
3. Transport of formaldehyde working solution between laboratories and pre-warming to 37°C requires standard lid cover to prevent potential exposure.
4. Formaldehyde waste should be less than 0.1% before it is discarded; be sure to verify per institution or government ordinance.
5. Formaldehyde mixture with DMSO requires normal DMSO disposal procedures.
6. Stock solutions of 37% formaldehyde contains methanol and disposal of this stock solution should be treated as flammable if appropriate.
7. Report any excessive spill of formaldehyde to your safety department

Acknowledgements

This work began at Sphinx Laboratories, Divison of Eli Lilly in 2001, knowledge and effort gained at Eli Lilly as well as several years at Abbott Laboratories, Duke University Medical Center, and the Hamner Institutes for Health Sciences made it possible to put this manuscript together.

References

1. Celebrating 25 years of NF-κB. Nat Immunol. 2011;12:681. PubMed PMID: 21772274.
2. Sen R, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. Cell. 1986;47:921–928. PubMed PMID: 3096580.
3. Chen FE, Huang DB, Chen YQ and Ghosh G. 1998. Crystal structure of p50/p65 heterodimer of transcription factor NF-B bound to DNA Nature, 391:410- 413.
4. Baeuerle P, Baltimore D. I kappa B: a specific inhibitor of the NF-κB transcription factor. Science. 1988;242:540–46. PubMed PMID: 3140380.
5. Ghosh G, Van Duyne G, Ghosh S, Sigler P. Structure of NF-κBp50 homodimer bound to a κB site. Nature. 1995;373:303–10. PubMed PMID: 7530332.
6. Muller C, Rey F, Sodeoka M, Verdine G, Harrison S. Structure of the NF-κB p50 homodimer bound to DNA. Nature. 1995;373:311–17. PubMed PMID: 7830764.
7. Nolan G. NF-AT-AP-1 and Rel-bZIP: hybrid vigor and binding under the influence. Cell. 1994;77:795–98. PubMed PMID: 8004669.

8. M. Karin. 1999. The beginning of the end: IkappaB kinase (IκK) and NF-kappaB activation. J. Biol. Chem. 274: 27339¯27342
9. Gilmore T. D. Introduction to NFkB: players, pathways, perspectives. Oncogene. 2006;25:6680–6684. PubMed PMID: 17072321.
10. Schreiber S., Nikolaus S., Hampe J. Activation of nuclear factor kappa B inflammatory bowel disease. Gut. 1998;42:477–484. PubMed PMID: 9616307.
11. Tergaonkar V. NFkB Pathway: A good signaling paradigm and therapeutic target. Int J. Biochem. Cell Biol. 2006;38:1647–1653. PubMed PMID: 16766221.
12. Grilli M, Jason JS, Lenardo M. NF-kB and rel–participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. 1993;43:1–62. PubMed PMID: 8449662.
13. Lawrence R, Chang LJ, Siebenlist U, Bressler P, Sonenshein G. Vascular smooth muscle cells express a constitutive NF-κB-like activity. J. Biol. Chem. 1994;269:28,913–18. PubMed PMID: 7961853.
14. Baeuerle P, Henkel T. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. 1994;12:141–79. PubMed PMID: 8011280.
15. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-κB. Annu. Rev. Cell Biol. 1994;10:405–55. PubMed PMID: 7888182.
16. Arenzana-Seisdedos F, Thompson J, Ro-driguez M, Bachelerie F, Thomas D, Hay R. Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activation of NF-κB. Mol. Cell. Biol. 1995;15:2689–96. PubMed PMID: 7739549.
17. A.S. Baldwin, Jr., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol. 14:649¯683.
18. Ding GJ, Fischer PA., Boltz RC., Schmidt JA., Colaianne JJ., Gough A., Rubin RA., Miller DK. Characterization and Quantitation of NF-κB Nuclear Translocation Induced by Interleukin-1α and Tumor Necrosis Factor-α. J Biol Chem. 1998;273:28897–28905. PubMed PMID: 9786892.
19. Ghosh S., May M.J., Kopp E.B. NF-ϰB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 1998;16:225–260. PubMed PMID: 9597130.
20. Heiss E, Herhaus C, Klimo K, et. al. 2001. Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J. Biol. Chem. 276: 32008¯32015.
21. Bharti AC, Aggarwal BB. Nuclear factor-kappa B and cancer: its role in prevention and therapy. Biochemical Pharmacology. 2002;64:883–888. PubMed PMID: 12213582.
22. Baldwin AS, 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. J. Clin. Invest. 107:241¯246
23. Chen F, Castranova V, and Shi X, 2001. New insights into the role of nuclear factor-kappaB in cell growth regulation. Am. J. Pathol. 159: 387¯397.
24. Beg AA and Baltimore D. 1996. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274:782¯784.
25. Wang CY, Mayo MW and Baldwin AS Jr, 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 274: 784¯787.
26. Van Antwerp DJ, Martin SJ, Kafri T, et. al. 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 274: 787¯789.
27. Ahmad M, Marui N, Alexander R, Medford R. Cell type-specific transactivation of the VCAM-1 promoter through an NF-κB enhancer motif. J. Biol. Chem. 1995;270:8976–83. PubMed PMID: 7536741.
28. Wright K, White L, Kelly A, Beck S, Trowsdale J, Ting J. Coordinate regulation of the human TAP1 and LMP2 genes from a shared bidirectional promoter. J. Exp. Med. 1995;81:1459–71. PubMed PMID: 7699330.
29. Brown A, Linhoff M, Stein B, Wright K, Baldwin A, Basta P, Ting J. Function of NF-kB/Rel binding sites in the MHC class II invariant chain promoter is dependent on cell-specific binding of different NF-κB/Rel subunits. Mol. Cell. Biol. 1994;14:2926–35. PubMed PMID: 8164652.
30. Zie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J. Biol. Chem. 1994;269:4705–8. PubMed PMID: 7508926.

31. Pahl H.L. Activators and target genes of Rel/NF- B transcription factors. Oncogene. 1999;18:6853–6866. PubMed PMID: 10602461.
32. Wu H, Lozano G. 1994. NF-_B activation of p53: a potential mechanism for suppressing cell growth in response to stress. J. Biol. Chem. 269:20,067–74
33. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signaling. Nature. 1999;401:86–90. PubMed PMID: 10485711.
34. Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol Screen. 1999;4:67–73. PubMed PMID: 10838414.

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