

Making a CASE for Your Favorite Pathway

Understanding cellular signal transduction is essential for characterizing human diseases and for developing effective therapies. The Pathway Focused DNA Microarrays from SuperArray, known as the GEArrays, have been widely used for analyzing gene expression associated with specific biological pathways or disease states. The company has also recently introduced the Cellular Activation of Signaling ELISA (CASE™) Kits for analyzing the activation of specific biological pathways at the protein phosphorylation level. These two technology platforms work well together to validate or confirm pathway activation at the level of both gene expression and protein function. This article discusses how we have used both a GEArray and a CASE kit together to evaluate how TNF-alpha induces activation of the NFkB pathway.

Introduction:

High-density, genome-wide as well as low-density, pathway-specific DNA microarrays detect changes in the relative expression of genes. If these microarrays demonstrate an increase in the expression of target genes of a specific signal transduction pathway, then the researcher may infer from those results that the experimental conditions under study activate that pathway. However, the results require further validation beyond the typical verification of gene expression changes using RT-PCR. To validate activation of the pathway at the protein level, researchers often examine the activation or phosphorylation state of an upstream activator of the signaling pathway. Typical methods include Western blotting or ELISA-based analyses using antibodies specific for this protein and for its phosphorylated form. Other researchers employ *in vitro* kinase assays with artificial peptide substrates and radioactive nucleotides.

One published example (1) utilizes such an experimental design. Using a microarray specific for the NFkB pathway (GEArray Q Series Human NFkB Signaling Pathway Gene Array, [HS-016.2](#)), this study finds that specific cytokines (IL3, IL5, GM-CSF) increase the expression of several target genes of the NFkB pathway including cell adhesion molecules of interest (ICAM1). After verifying those changes in gene expression by conventional RT-PCR, the study demonstrates that the level of IκBa protein phosphorylation increases by Western blotting under the same conditions. The phosphorylation of IκBa causes its dissociation from the NFkB complex and its degradation by the proteasome. The NFkB p65 protein becomes phosphorylated itself allowing its translocation into the nucleus to activate the expression of target genes. Thus, their cytokine treatment activates NFkB signaling, which in turn activates cell adhesion molecule expression.

However, the Western blot method used in this study and the other protein phosphorylation detection methods have their drawbacks. The generation of cell extracts for traditional ELISA-based methods introduces extra manipulative steps in the experiment that can change the phosphorylation status of the protein under study despite the use of protease and phosphatase inhibitors. Western analyses require separating proteins in that lysate by SDS-PAGE and transferring the proteins to another permeable solid support (such as nitrocellulose or PVDF) before antibody-based detection begins. This process requires at least four hours to complete. *In vitro* kinase assays often require the use of radiolabeled nucleotide generating radioactive waste. However, other assays involve non-radioactive antibody-based methods but still require generating a cell lysate.

More recently, methods have been developed to examine the phosphorylation status of a protein in fixed cells (2) allowing the researcher to perform the antibody-based detection directly in the cell culture wells. These assays include the Cellular Activation of Signaling ELISA (CASE) Kits from SuperArray Bioscience.

What is the CASE Kit?

The Cellular Activation of Signaling ELISA (CASE) Kit is a cell-based protein phosphorylation ELISA kit designed to monitor the activation of a signal transduction pathway by assaying the

extent to which an important upstream regulatory protein is phosphorylated. The CASE Kits include a complete antibody-based detection system for determining the ratio between the amount of the phosphorylated form of a specific protein and the total amount of the same protein. As a signal transduction pathway involving a protein kinase cascade is activated, the extent of phosphorylation of the upstream regulatory proteins increases. Originally developed to follow up DNA microarray gene expression profiling experiments, the CASE kits may also be applied to other gene function studies examining the phosphorylation state of a specific protein. These kits help determine or verify whether a set of experimental conditions activate or inhibit a signal transduction pathway or protein of interest.

How Does the CASE Kit Work?

First, cells are seeded in 96-well plates so that the final density at the time of the CASE assay is roughly 80 percent confluence. Cells attach to the bottom of the well and grow overnight. To enhance the signal transduction response, cells are also starved in serum-free medium overnight (18 hours). Pre-treatment of the cells with a specific inhibitor of the protein's phosphorylation serves as a specificity control for the observed result. (See below.) Finally, the cells are treated with the desired stimulus, and the CASE kit protocol is performed.

After stimulation, cells are immediately fixed to keep the status of activated protein intact. After quenching the fixative and blocking all non-specific protein binding sites, duplicate wells are incubated with primary antibodies specific either for the protein itself or for the phosphorylated form of the protein. A secondary antibody covalently linked with a reporter enzyme, horseradish peroxidase or HRP, binds the primary antibodies. Incubation with a colorimetric substrate for the enzyme generates a signal easily detectable by a standard ELISA plate reader. The color intensity is directly related to the relative amount of the proteins. Finally, readings are normalized to relative cell number as determined by another staining step.

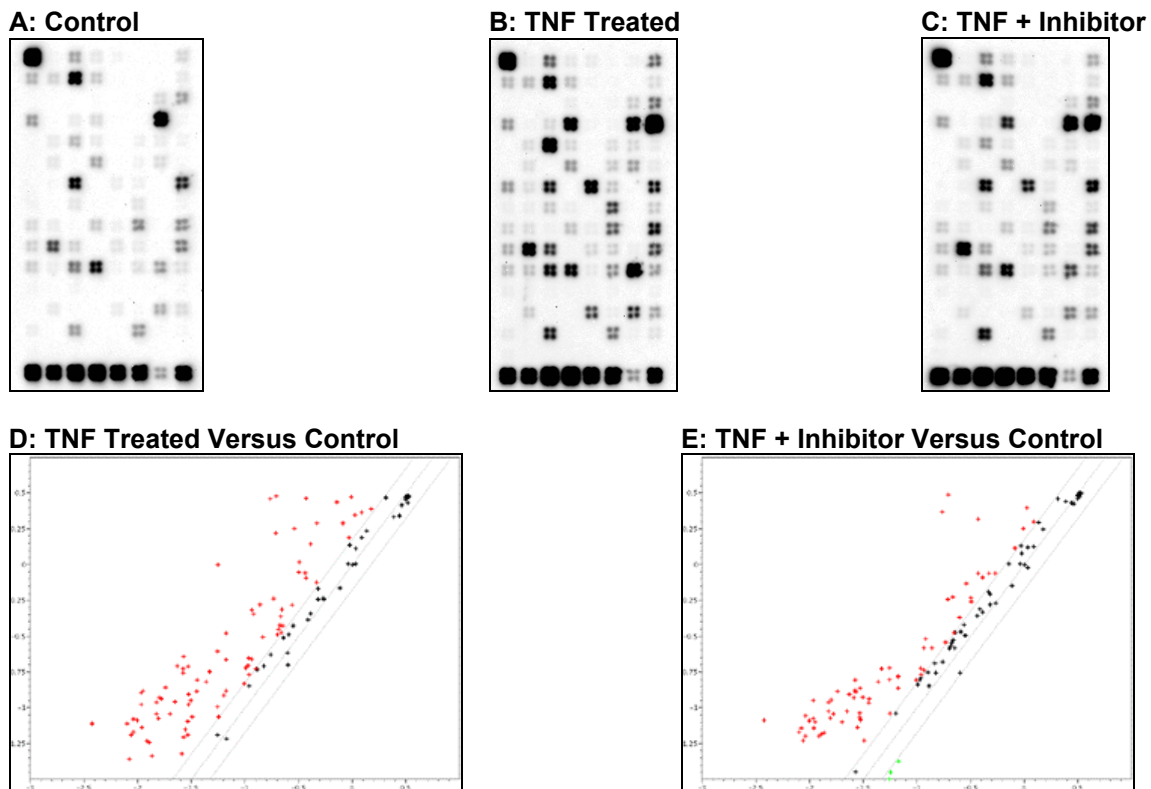


Figure 1: Treatment with TNF-alpha increases expression of NFkB-related genes. Human A431 cells were starved in serum-free medium for 18 hours. One set of wells was left untreated (Control, Panel A), another set was pre-treated with

Bay 11-7085 ([FA-006](#)) at 20 μ M for 1 h (TNF + Inhibitor, Panel **C**) before treating it and another set (TNF, Panel **B**) with 10 nM calyculin A for 30 min followed by 50 ng/ml TNF-alpha for 5 min. Cells were immediately harvested in lysis buffer for RNA isolation (Qiagen RNeasy). The RNA was used with the TrueLabeling-AMP™ Kit ([GA-010](#)) to generate labeled target for characterization on the Oligo GEArray® Human NFkB Signaling Pathway Microarray ([OHS-025](#)). Using the [GEArray Expression Analysis Suite](#) software, genes changing their expression by at least 1.5-fold were identified in a typical scatter plot of the microarray data. Panel D displays a comparison between the expression profiles of TNF-treated cells and control cells. Panel E displays a comparison between the expression profiles of the inhibitor-treated cells and control cells.

Example: Stimulation of the NFkB Pathway by TNF-alpha

Our study examines the signaling downstream of TNF-alpha. We first determine whether TNF-alpha signals through the NFkB pathway at the gene expression level by Pathway-Focused DNA Microarray, or GEArray, analysis. We then verify that TNF-alpha activates this signaling pathway by directly examining the phosphorylation of the NFkB p65 protein using an appropriate CASE kit assay. Using a specific inhibitor of IkbA phosphorylation, we finally ascertain that NFkB may not be the only signaling pathway that TNF-alpha activates.

Figure 1 demonstrates that treatment of A431 cells with TNF-alpha induces the expression of several genes in the NFkB signaling pathway. (Compare the profiles on the arrays in Panels A and B, and see the scatter plot in Panel D.) The expression of a number of genes increases by at least 1.5-fold upon treatment with TNF-alpha. This list of up-regulated genes (Table 1) includes both genes responsible for the signaling itself and genes responsive to the pathway. The results indicate that treatment of cells with TNF-alpha stimulates the NFkB pathway. To verify this notion, the ability of TNF-alpha to increase the phosphorylation status of NFkB p65 was tested with the corresponding CASE kit specific for the most important NFkB p65 residue, serine-536.

Position	Gene Symbol	TNF Versus Control	TNF + Inhibitor Versus Control	TNF Versus TNF + Inhibitor	TNF-Induced Expression Increase Reversed by Inhibitor?
3	AKT1	1.66	1.46	1.1	NO
8	BCL3	3.40	1.99	1.7	YES
28	EGR1	7.97	5.66	1.4	NO
32	FOS	15.52	15.78	1.0	NO
35	HMOX1	3.87	1.43	2.7	YES
46	IL1A	4.21	2.29	1.8	YES
47	IL1B	2.43	1.21	2.0	YES
49	IL6	17.89	1.65	10.8	YES
53	JUN	16.99	13.87	1.2	NO
54	LITAF	3.87	1.94	2.0	YES
62	MAP3K14	6.22	2.58	2.4	YES
64	MAP3K7	3.81	2.54	1.5	YES
72	NFKB1	1.91	1.65	1.2	NO
73	NFKB2	1.64	1.41	1.2	NO
75	PLK2	4.19	1.86	2.3	YES
83	RHOA	1.65	1.19	1.4	NO
86	RIPK2	3.26	1.72	1.9	YES
87	SLC20A1	3.04	1.83	1.7	YES
88	STAT1	2.80	1.86	1.5	YES
101	TNFAIP3	8.60	2.98	2.9	YES
103	TNFRSF10B	2.41	1.61	1.5	YES
104	TNFRSF1A	2.18	2.34	0.9	NO
107	TNFSF10	2.12	2.39	0.9	NO

Table 1: An inhibitor of the NFkB pathway corrects TNF-dependent increases in expression of some NFkB-related genes but not others. The fold-changes in expression of NFkB-related genes increased by at least 1.5-fold upon TNF-alpha treatment relative to control are listed. The fold changes in gene expression for the other pair-wise comparisons between the experimental conditions from Figure 1 are also listed for these same genes. The scatter plots displayed in Figures 1D and 1E from the GEArray Expression Analysis Suite determined the numbers for the **TNF Versus Control** and for the **TNF + Inhibitor Versus Control** comparisons. The **TNF Versus TNF + Inhibitor** comparison was calculated as the ratio between the other two fold-changes in expression. When the expression of a gene decreases by at least 1.5-fold upon pre-treatment with the inhibitor, the inhibitor is deemed to have reversed the stimulation of expression by TNF-alpha (**YES**). The increase in expression of all other genes is not deemed to be reversible by the inhibitor (**NO**).

Figure 2 displays the results of the CASE kit assay. Treatment of cells with TNF-alpha increases the amount of the NFkB p65 protein phosphorylated at serine-536 by at least two-fold; however, the total amount of NFkB p65 protein (the sum of the unphosphorylated and phosphorylated forms of the protein) remains unchanged. Therefore, treatment with TNF-alpha activates the NFkB signaling pathway by inducing the phosphorylation of NFkB thereby activating its ability to signal the increase in the expression of target genes.

To insure that treatments (such as with TNF-alpha) specifically activate a signal transduction pathway (such as NFkB), experiments usually include a control pre-treating the cells with a specific inhibitor of the pathway's activation, such as compounds that inhibit the phosphorylation of its upstream activator. In this case, inhibition of IkbA phosphorylation with the inhibitor Bay 11-7085 protects degradation of this protein by the ubiquitin pathway and allows it to continue to bind NFkB p65. This protein complex with IkbA prevents NFkB p65 activation by phosphorylation, its subsequent translocation into the nucleus, and its ability to stimulate transcription of target genes. Indeed, the results of the CASE Kit assay in Figure 2 demonstrate that pre-treatment with the inhibitor Bay 11-7085 almost completely reverses the TNF-alpha dependent induction of NFkB p65 phosphorylation at serine-536. And again, the total amount of NFkB p65 protein remains unchanged under these conditions.

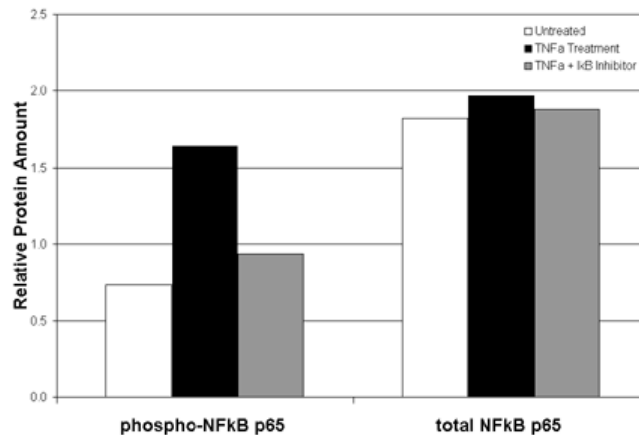


Figure 2: Treatment with TNF-alpha increases but pre-treatment with an inhibitor prevents NFkB phosphorylation. Human A431 cells were starved in serum-free medium for 18 hours. One set of wells was left untreated (Control, white bars), another set was pre-treated with Bay 11-7085 (FA-006) at 20 μ M for 1 h (TNF α + I κ B Inhibitor, gray bars) before treating it and another set (TNF α Treatment, black bars) with 10 nM calyculin A for 30 min followed by 50 ng/ml TNF-alpha for 5 min. Cells were then immediately fixed and used with the CASE Kit for NFkB p65 S536 (FE-005). The relative amount of NFkB p65 phosphorylated at S536 (left) and the relative amount of total NFkB p65 protein (right) are displayed.

As seen in Figure 1, pre-treatment with the inhibitor prevents the increase in the expression of some but not all of these genes induced by TNF-alpha. (Compare the profile on the arrays in Panels B and C, and see the scatter plot in Panel E.) Table 1 includes a summary of this observation by indicating which genes decrease their expression by at least 1.5 fold upon pre-treatment with the inhibitor (YES) and those that do not (NO) relative to TNF-alpha treatment alone. These results suggest that the NFkB pathway itself does not necessarily increase in the expression of at least some of the genes up regulated by TNF-alpha treatment. The genes whose expression the inhibitor does not change may be activated by another signal transduction pathway downstream of TNF-alpha.

Summary:

The work discussed in this article demonstrates the use of gene function assays to validate conclusions made based on gene expression profiles obtained by DNA microarray analyses.

Specifically, monitoring the phosphorylation and thus activation of an upstream regulator of a signal transduction pathway can help verify microarray results indicating an increase in the expression of that pathway's target genes. This notion applies to both high-density genome-wide microarrays as well as the low-density pathway-focused DNA microarrays, the GEArray, from SuperArray Bioscience that highlight individual specific signal transduction pathways or other biological pathways. The Cellular Activation of Signaling ELISA (CASE) Kits from SuperArray Bioscience provide a convenient way to monitor signal transduction pathway activation at the protein level. This cell-based assay determines the amount of the phosphorylated (or activated) form and the total amount of an upstream activator of a specific biological pathway with a simple antibody-based detection system. The use of specific inhibitors of the protein's phosphorylation in conjunction with the CASE Kit assay verifies that the experimental conditions specifically activate the pathway. These inhibitors used in conjunction with the microarray analysis may even yield interesting results as to whether the changes in gene expression actually derive from the activation of this or perhaps another signal transduction pathway.

Related Products from SuperArray Bioscience:

Cellular Activation of Signaling ELISA (CASE) Kits

Useful for validating microarray results implicating the role of a specific signaling pathway
Easy, quantitative, and non-radioactive protocol with minimal hands-on time
No loss of activation state during procedure
Cell-based assay: No extractions, Western blots, or *in vitro* kinase assays required
Detect relative amount of total and activated form of specific protein at the same time

Inhibitors for Functional Assays

Determine the specificity of the pathway activation observed with a Gene Function Assay.
Directly inhibit the enzymatic activation of a pathway's regulator.
Test specificity by simply performing the appropriate control assays.

Ready-to-Use Convenience:

Shipped already dissolved at convenient stock concentrations. Ready to dilute and use.
No need to calculate volumes to re-suspend; just calculate the appropriate dilution factors.
Add directly to cell culture medium to pre-treat cells before adding experimental stimulus.

Pathway-Focused DNA Microarrays

Pathway Focused Design: The design reflects the most current understanding of pathways and functionally related gene families.

Sensitive and Reproducible: Requires as little as 100 ng total RNA, and detects over 3 logs linear dynamic range.

Easy to Use: Designed for use in any molecular biology lab; a thermal cycler, hybridization oven and X-ray film or CCD imaging system are required equipment.

References:

1. Wong CK, Ip WK, Lam CW. Interleukin-3, -5, and granulocyte macrophage colony-stimulating factor-induced adhesion molecule expression on eosinophils by p38 mitogen-activated protein kinase and nuclear factor-[kappa] B. *Am J Respir Cell Mol Biol.* 2003 Jul;29(1):133-47.
2. Versteeg HH, Nijhuis E, van den Brink GR, Evertzen M, Pynaert GN, van Deventer SJ, Coffey PJ, Peppelenbosch MP. A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. *Biochem J.* 2000 Sep 15;350 Pt 3:717-22.