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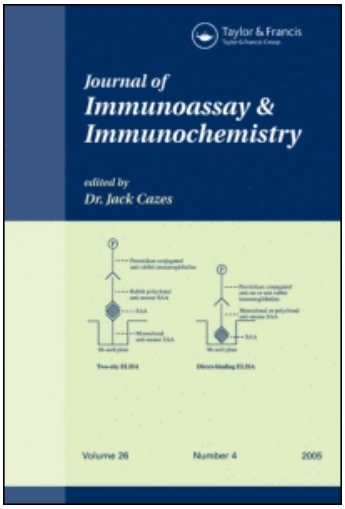

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Quantitative Chemiluminescent Immunoassay for NF-κB-DNA Binding Activity

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Quantitative Chemiluminescent Immunoassay for NF-κB–DNA Binding Activity

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Abstract: Nuclear factor-κB (NF-κB) is a ubiquitous redox-sensitive transcription factor involved in the pro-inflammatory response to several factors, including cytokines and oxidative stress. Upon activation, NF-κB translocates into the nucleus and binds to specific nucleotide sequences. The cellular responses to inflammatory and stress signals have been implicated in disease conditions, such as atherosclerosis, cancer, diabetes, and Alzheimer's disease. The conventional method for detection of NF-κB -DNA binding activity is the electrophoretic mobility shift assay (EMSA), which is time-consuming and non-quantitative. Here, we report (a) development of a rapid, sensitive and quantitative chemiluminescent immunoassay (QCI) for analysis of NF-κB DNA-binding activity, and (b) validation of the QCI with the EMSA using nuclear and cytosolic extracts from cultured prostate cancer cells (PC3), rat liver homogenates and human lymphocytes. The QCI for analysis of NF-κB DNA binding activity has advantages over the EMSA: (1) Higher speed: 3–5 h post sample preparation, (2) Greater sensitivity: 10 pg NF-κB/well, (3) Quantitative: linear range: 10–1000 pg NF-κB; $r^2 = 0.999$ (4) High throughput adaptability: 96-well plate format can analyze up to 40 samples in duplicate, (5) Safety: No

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radioactive isotopes, (6) Simplicity, and (7) Capability of measurement of both activated (free) NF-κB which is translocated into the nucleus and total (bound + unbound) NF-κB present in the cytosol/cell.

Keywords: NF-κB, Oxidative stress, Quantitative, Chemiluminescent immunoassay, Assay, Cell extracts, Human lymphocytes, Rat liver

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INTRODUCTION

NF-κB, Oxidative Stress and Chronic Disease

Cellular oxidative stress is defined as an increase in intracellular reactive oxygen (ROS) and nitrogen (RNS) species such as H_2O_2, superoxide, hydroxyl radical, etc.[1] The state has been linked to normal aging and a variety of chronic diseases, including atherosclerosis, diabetes, pulmonary fibrosis, Alzheimer's disease, and arthritis.[2] ROS, whether produced endogenously as a consequence of normal cell functions or derived from external sources, pose a constant threat to cells as they can cause severe damage to DNA, proteins, and lipids.[3] There is strong evidence that oxidative DNA damage may play a role in the initiation, promotion, and progression of tumors,[4] and that antioxidants may protect cells from carcinogenesis. Increases in ROS generation have been related to a risk for cardiovascular diseases, such as atherosclerosis, angina pectoris, and myocardial infarction.[5] ROS have been believed to play a central role in insulin-producing β-cell death, characteristic of disease progression in Type I or insulin-dependent diabetes.[6]

ROS, generated by redox reactions, have been recognized as important chemical mediators, regulating cell signal transduction pathways.[4] The nuclear factor-κB (NF-κB) pathway is an important redox activated cell signal transduction pathway, playing important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling.[7,8] Oxidative stress activates NF-κB activity, which, in turn, induces the transcription of NF-κB-targeted genes related to immune function, inflammation, apoptosis, and cell proliferation. Conversely, antioxidants such as N-acetyl-L-cysteine (NAC), pyrrolidine-dithiocarbamate (PDTC), and α-tocopherol, diminish or completely inhibit NF-κB activation.[9] Since NF-κB activation is regulated by the cellular redox state, it may be an ideal biomarker for studies on the effects of antioxidant dietary supplements used in chemoprevention studies. Thus, due to its vital role in the regulation of genes related to the pathogenesis of chronic diseases caused by oxidative damage, NF-κB is considered to be a promising target for treatment of ROS-induced development of chronic diseases. A recent report[10] concludes that the large number of major ailments in which aberrant regulation of NF-κB is observed or suspected,

strongly justify NF-κB and the signal pathways that regulate its activity as a focal point for drug discovery and developmental efforts.

Mechanism of NF-κB Activation

The NF-κB family is composed of five proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), each of which may form homo- or hetero-dimers.[11] In almost all cell types, NF-κB is sequestered in the cytoplasm through close association with its inhibitory protein, IκB. NF-κB is activated by a variety of stimuli including cytokines, radiation TNF-α, UV radiation, H_2O_2, free radicals, and oxidative stress. The activation of NF-κB typically occurs through site-specific phosphorylation and ubiquitination of IκB protein. IκB is subsequently degraded by the 26S proteasome. This allows NF-κB to dissociate from IκB and to translocate into the nucleus where it binds to NF-κB-specific DNA-binding sites, thereby regulating the transcription of target genes.[12] Thus, NF-κB controls the expression of many genes that are involved in the cellular physiological processes mentioned above.

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Measurement of Activated NF-κB

Given its pivotal role in physiology and pathophysiology, the ability to accurately determine the level of activated NF-κB concentrations in cells is important. The conventional method for detecting the DNA-binding activity of NF-κB is the electrophoretic mobility shift assay (EMSA). The assay is based on the observation that complexes of nuclear NF-κB protein and oligonucleotides containing the NF-κB concensus sequence migrate through a non-denaturing polyacrylamide gel more slowly than free oligonucleotide fragments. The gel shift assay is typically performed by incubating purified nuclear protein with ^{32}P end-labeled DNA fragment containing NF-κB binding site. The reaction products are then separated on a non-denaturing polyacrylamide gel and exposed to X-ray film. The results can be analyzed by densitometric analysis. Instead of a ^{32}P end-labeled DNA fragment, a biotin-labeled DNA fragment with chemiluminescence detection has now been used for NF-κB EMSA. An antibody against activated NF-κB has been also used for the detection of NF-κB activity by Western blot analysis. However, these methods for measurement of NF-κB are time-consuming, relatively insensitive, and semi-quantitative at best.

Here we report the development of a rapid, sandwich type immunoassay with chemiluminescent detection for the precise quantification of NF-κB DNA binding activity. In contrast to traditional sandwich immunoassays, we utilized a biotin labeled oligonucleotide containing the NF-κB consensus sequence as an affinity capture probe, thereby providing high

sensitivity and specificity. Figure 1 is a diagrammatic representation of the underlying principal of this QCI for NF-κB DNA binding activity. In brief, this sandwich immunoassay employs an oligonucleotide, containing the DNA binding NF-κB consensus sequence, bound to a 96-well microplate to capture active NF-κB in the sample. The DNA bound NF-κB is selectively recognized by the primary antibody (p50 and p105 specific), which, in turn, is detected by the secondary antibody-alkaline phosphatase conjugate. The Relative Light Units (RLU), measured by a chemiluminescence detector after addition of a chemiluminescent alkaline phosphatase substrate, offers greater sensitivity than absorbance detection methods. This higher degree of sensitivity, coupled with a linear correlation (r^2 typically 0.99) between RLUs and the amount of NF-κB bound to the DNA probe, provides precise and quantitative measurement of this transcription factor in biological samples using a standard curve. Aliquots of the same experimental samples were compared for DNA binding activity of NF-κB by this QCI and by the traditional EMSA for validation purposes. The development and validation of an easy to perform high throughput assay for NF-κB containing the

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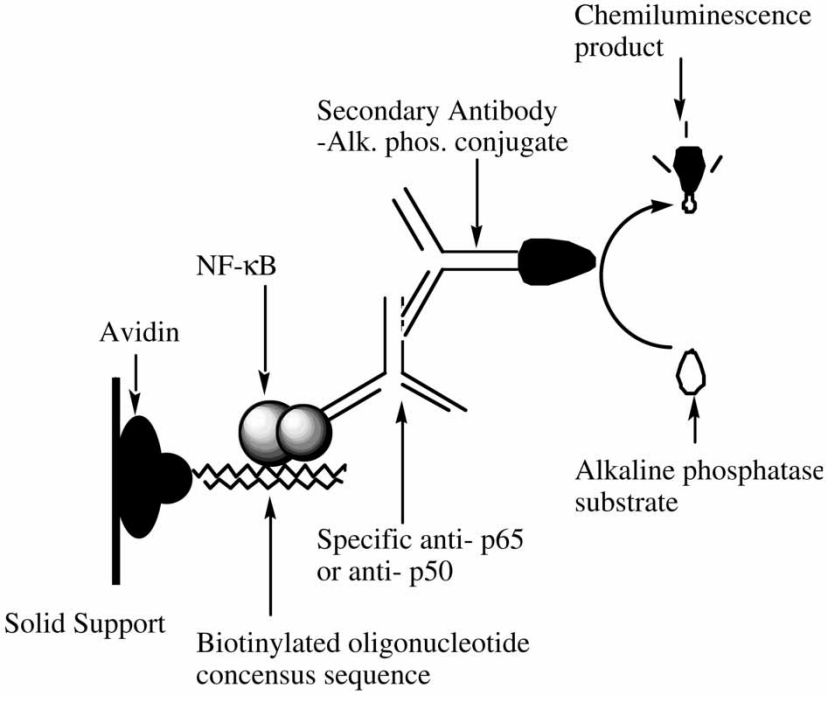

Figure 1. Diagrammatic representation of the underlying principal of the Quantitative Chemiluminescent ImmunoAssay for NF-κB binding activity.

following attractive features: quantitative; cost effective; high sensitivity; high specificity and reproducibility is expected to provide a critically needed and useful surrogate biomarker for many intervention studies, including those for prevention and/or treatment of cancer.

EXPERIMENTAL

PC3 Cell Culture

All cells were cultured in accordance to the procedure described earlier.[13] Briefly, the human prostate cancer cell line, PC3 (ATCC) was cultured in RPMI 1640 medium (Gibco, Rockville, MD), supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. Cells, plated at a density of 1×10^6 cells in 100 mm dishes were stimulated with or without 20 ng/mL of TNF-α for 10 min. The cells were harvested by scraping them from culture dishes, collected by centrifugation and frozen at −80°C until further use. Protein content was determined using the BCA protein assay (Pierce, Rockford, IL).

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Rat Liver Lymphocytes

Eight male Sprague-Dawley rats, 6–8 weeks old weighing 250–300 g (Harlan Sprague-Dawley, Indianapolis, IN) were fasted overnight. The rats were administered intra-peritoneal injections of CCl_4 (Sigma) in canola oil (1.0 mL/kg body weight). The control rats (n = 2) received an equal volume of canola oil. The animals (2 rats/group) were euthanized at various time points (4, 6 and 8 h) with sodium pentobarbital (100 mg/Kg) administered intra-peritonealy. Livers were perfused by adding cold phosphate buffered saline through the portal vein. The perfused liver samples (1 g) were then subjected to ultra-centrifugation, using a sucrose gradient[14] to prepare the cytosolic and nuclear extracts.

Human Lymphocytes

Human peripheral blood lymphocytes were collected and processed as described earlier by Davis et al.[15] A total of 24 (8 × 3) mL of peripheral blood was collected in 3 BD Vacutainer CPT tubes with sodium citrate gradient. After centrifugation at room temperature at 4000 rpm (1650 relative centrifugal force) for 30 minutes, the clear plasma was removed. The whitish buffy coat layer containing mononuclear cells was pipetted into a 15 mL conical centrifuge tube, the volume was brought up to 15 mL by addition of PBS, mixed, and then centrifuged for 15 minutes at 1800 rpm. The process was repeated three times. After the final wash, the pellet was resuspended in

0.5 mL PBS and transferred to a 1 mL vial, followed by microcentrifugation for 10 minutes at 2200 rpm. The pellets thus obtained were stored at -80°C.

Preparation of Cytosolic and Nuclear Extracts

A procedure described earlier[16] was used for the preparation of cytosolic and nuclear extracts from human lymphocytes. In brief, the cell pellet was resuspended in 0.5 mL of 10 mM Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 0.05% (v/v) Triton X-100 and lysed with 20 strokes in a 1 mL Dounce Homogenizer. This was centrifuged at 3,000 × g for 15 min. at 4°C. The supernatant constituting the cytosolic extract was collected and stored at -80°C till further use. The remaining pellet was resuspended in an equal volume of 10 mM Tris-HCl; 5 mM $MgCl_2$. To this was added an equal volume of the lysis buffer (1 mM NaCl; 10 mM Tris-HCl; 4 mM $MgCl_2$; pH 7.4). The nuclei were allowed to lyse for 30 minutes on ice and then centrifuged at 10,000 × g for 15 minutes at 4C. An 80% glycerol solution was added to the supernatant to reach a final glycerol concentration of 20%. This constituted the nuclear extract. Protein content of each prepared cytosolic and nuclear extract was determined by BCA protein assay (Pierce, Rockford, IL). The extracts were stored at -80°C till further use.

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End-Labeling of NF-κB Oligonucleotide with [γ^{32}P]ATP

The following reagents were assembled for the phosphorylation reaction: 3 pmol of NF-κB consensus oligonucleotide (Promega, Madison, WI), 5X T4 polynucleotide kinase buffer, T4 polynucleotide kinase, nuclease-free water and 10 μCi of [γ^{32}P] ATP. The reaction was incubated for 10 min at 37°C and stopped by the addition of 0.5 M EDTA followed by addition of STE buffer. Unicorporated nucleotides were removed by a chromatography column (Stratagene). The probe was diluted with H_2O to 20,000 cpm/μL.[16]

EMSA Procedure

This widely used method for the study of sequence-specific DNA-binding proteins, such as transcription factors, is based on the slower migration of protein-DNA complexes through a nondenaturing polyacrylamide gel compared to free DNA fragments or double-stranded oligonucleotides. The general EMSA protocol, described earlier,[16] is summarized here. Each sample (10 μg protein) is treated with an equal amount (4 μL) of 5X Gel Shift Binding buffer and incubation at R.T for 10 minutes. One μL (20,000 CPM) of ^{32}P-labelled NF-κB oligonucleotide is added followed

by 1 μL of loading dye. The samples are loaded on to a 8% polyacrylamide gel and the electrophoresis is conducted at 30 mA for approximately 2 hours. The gels are dried, exposed to X-ray film overnight at −70°C, and then developed. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest.

RESULTS AND DISCUSSION

Development of the Quantitative Chemiluminescent Immunoassay (QCI)

Our initial attempts to develop the assay system, using microtiter plates coated with streptavidin yielded poor sensitivity (2000 pg/well), with a linear range of 2000–50,000 pg/well (r^2 – 0.86), high background readings and high coefficients of variation. In order to increase sensitivity, expand the useful linear range, and decrease non-specific binding, each step of the sequence (Figure 1) was investigated and the conditions varied to optimize performance. This included evaluation of: (1) Avidin coated solid support (microtiter plate), (2) Oligonucleotide coating, (3) Specific antibody, secondary antibody-alkaline phosphate conjugate and alkaline phosphatase substrates (4) Blocking agents (5) Buffers. Streptavidin and neutravidin® coated 96 well microtiter plates with low or high binding capacities from different vendors (BD, Pierce, Sigma) were evaluated. Although both streptavidin and neutravidin coated plates showed high affinity for the biotinylated NF-κB oligonucleotide and did not pose any problems when using a purified NF-κB p50 peptide, the amino acid sequence of streptavidin contains a segment that is similar to the cell surface recognition motif contained in the sequence of various adhesion molecules, e.g., fibronectin, fibrinogen and hence can be anticipated to bind strongly with integrins and other related cell surface receptors.[17] This may give rise to high background readings when monitoring the DNA binding of NF-κB in extracted biological samples. Since plates with high binding capacities can be coated with greater amounts of the biotinylated oligonucleotide in comparison to those with low-binding capacity, higher signal intensity accompanied with possibly greater sensitivity was anticipated. Contrary to our expectation, no added advantage was observed in terms of signal intensity. Moreover, the low binding plates, with the lower oligonucleotide coating showed a slightly better correlation ($r^2 = 0.9924$ vs. 0.9634) and sensitivity (200 vs. 800 pg NF-κB/well) with respect to increasing NF-κB concentrations in the sample. Hence the low binding neutravidin coated plates, requiring lower concentrations of biotinylated NF-κB oligonucleotide sequence were found to yield better signal intensity and sensitivity in addition to being

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more cost effective. Effects of coating oligonucleotide with concentrations ranging from 5–125 pmoles/well and the degree of biotinylation (1–4) were studied. Optimizing these two factors did not provide substantial improvement in the sensitivity of the assay. Specific antibody, secondary antibody-alkaline phosphate conjugate and alkaline phosphatase substrates from different vendors were evaluated. The selected rabbit anti NF-κB p50 specific antibody (Santa Cruz, CA) was titrated (1:100–1:1000) to select conditions with lowest non-specific binding without compromising sensitivity. Similarly, the anti rabbit IgG-alkaline phosphatase conjugate (Jackson) was evaluated at varying dilutions (1:10,000–1:40,000) for optimum performance. Blocking agents including various concentrations and combinations of Bovine serum albumin (BSA), Fish-gelatin, Goat-serum,and Superblock® (Pierce) were evaluated. Superblock was found to be remarkably superior in its ability to minimize non-specific binding in comparison to the other blocking agents tested. Binding Buffer components were evaluated for optimum operation for example addition of poly dI-dC decreased the signal intensity by approximately 50%. Wash buffer of low ionic strength, pH 7.4 did not disrupt binding of NF-κB with the NF-κB consensus oligonucleotide. In addition, a detergent was required for efficient washing to decrease background noise. Dilution buffer components including salt, blocking agent and detergent concentrations were evaluated. A combination of SuperBlock (Pierce), Tween 20 and a low total salt concentration was determined to be most efficient in reducing non-specific binding and enhancing the usable range for measurement.

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Optimized Quantitative Chemiluminescent Immunoassay (QCI) Procedure for Measurement of NF-κB Binding Activity

Our optimized QCI procedure involves pre-blocking the low binding neutravidin coated 96 well microtiter plate (Pierce, Rockford, IL) with 300 μL/well of SuperBlock® (Pierce) for 1h at room temperature followed by a wash (×3) step using wash buffer (Tris buffered saline; 0.5% Tween 20; pH 7.4). The biotinylated oligonucleotide (custom prepared by Integrated DNA Technologies, Coralville, IA) containing the NF-κB consensus sequence is added to the wells (15 pmoles/well), incubated for 2 h at room temperature and then overnight at 4°C. After thorough washing (×4), the NF-κB p50 standard peptide (Promega, Madison, WI) or the cultured cell/lymphocyte extracts in 40 μL dilution buffer (SuperBlock; 0.5% Tween 20; pH 7.4) are added to the plate containing 60 μl/well of binding buffer (1 mM $MgCl_2$; 0.5 mM EDTA; 0.5 mM DTT; 50 mM NaCl; 10 mM Tris; 4% Glycerol; 0.01 mg/mL sperm DNA; pH 7.4), mixed and allowed to bind for 2 h at room temperature. The rabbit NF-κB p50 specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in dilution buffer (100 μL/well) is then

added, followed by 1h incubation at room temperature. This complex is selectively recognized by donkey anti-rabbit IgG-alkaline phosphatase conjugate (Jackson, West Grove, PA; 100 μL/well), on incubation for 1 h at room temperature. Addition of 100 μL/well of chemiluminescent alkaline phosphatase substrate (Michigan Diagnostics, Troy, MI) ensues a reaction that is monitored by a plate reader equipped with a chemiluminescent detector (Labsystems). The chemiluminescent signal measured as Relative Light Units (RLU) was found to plateau at 90 minutes post addition of enzyme substrate. All determinations were done in triplicate. A thorough wash (×3) between each step was found to be pertinent.

Sensitivity, Specificity and Reproducibility of the Optimized QCI

Meticulous, step-by-step manipulation of the various conditions listed above, allowed us to achieve a reproducible, linear curve using purified NF-κB p50 peptide (Promega) with a correlation coefficient, $r^2 > 0.99$ and a sensitivity of <10 pg NF-κB/well. Figure 2 depicts a typical NF-κB p50 standard curve produced using the optimized conditions. The specificity of binding

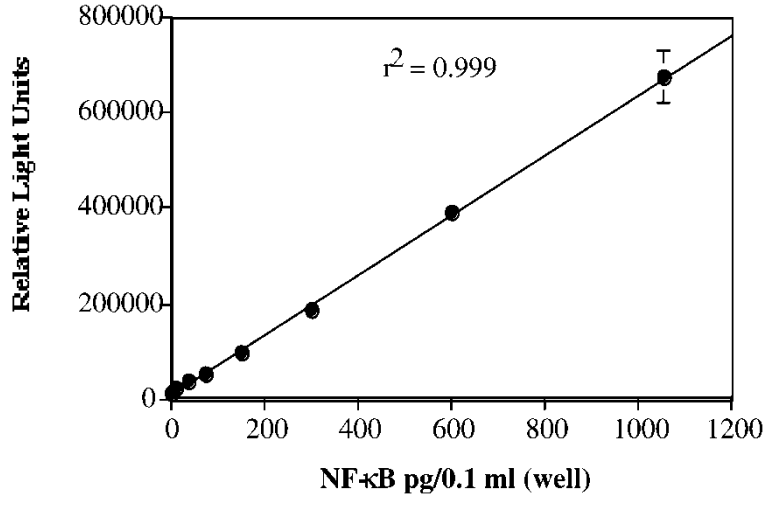

Figure 2. Correlation between RLU and NF-κB concentration (0–1000 pg/well) using a low Binding Neutravidin coated plate coated with 15 pmol/well of biotinylated oligonucleotide. Purified NF-κB peptide, dissolved in 40 μL of dilution buffer was added to 60 μL/well of Binding Buffer in the plate. This was successively incubated with 100 μL/well of specific antibody and secondary antibody-enzyme conjugates. RLU were measured 90 minutes post treatment with alkaline phosphatase substrate with a chemiluminescence microplate reader.

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was determined by conducting parallel experiments with NF-κB p65, BSA, AP1 and TF1 1B. In all cases, binding activity for proteins that do not recognize the NF-κB consensus sequence and/or are not recognized by the primary antibody employed was found to be negligible (<0.001%). The coefficient of variation (CV) for Intra- and Inter-assay variance were <5% and <10 % respectively. Thus, a linear correlation ($r^2 > 0.999$) between Relative Light Units (RLU) and quantity (pg) of purified NF-κB p50 was reproducibly obtained using conditions optimized for this QCI. The quantification is sensitive (10 pg NF-κB/well) and specific for NF-κB p50.

Validation with Electrophoretic Mobility Shift Assay (EMSA)

Quantification of NF-κB DNA Binding Activity in Cultured PC3 Cells

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TNF-α activated and control PC3 cells, used as a model system for analyzing biological samples, were analyzed for NF-κB DNA binding activity using both the optimized QCI procedure, (Figure 3 A) and EMSA (Figure 3 B). A linear increase in RLU was obtained for increasing amounts of protein in nuclear or cytosolic extracts, prepared using the optimized buffers when subjected to analysis by QCI. A 2- to 3-fold increase in the RLU (directly proportional to the amount of NF-κB) was readily observed in the TNF-α activated nuclear protein fraction over the control nuclear extract. The gel scan procured after a 12 hours exposure by EMSA is shown on the right. Although good correlations between the gel shift and QCI data were observed using both the control (Figure 4 A; $r^2 = 0.821$) and TNF-α activated (Figure 4 B; $r^2 = 0.937$) PC3 cells, the correlation using TNF treated cells was better. This can be attributed to the lower sensitivity of the gel shift assay. Determinations for low concentrations of NF-κB (1 μg protein in lane 1, Figure 4B) made by EMSA were unreliable whereas the QCI readily yielded quantitative results for as little as 10 pg NF-κB/well (0.1 μg protein/well). The greater sensitivity and accuracy of the QCI compared to the EMSA is readily apparent when one compares the ratios of NF-κB as measured from signal intensities, in nuclear extracts of activated and control PC3 cells. As shown in Figure 4 panel C, the ratio of NF-κB in activated and control cells remains fairly consistent (ranging from 1.7–2.1), using the QCI for measurement of nuclear extracts with increasing protein concentrations (1–10 μg/well or lane). In contrast, results obtained *via* the EMSA show a wider spread (1.3–2.8). The ratios at the two lower protein concentrations (1, 2.5 μg/lane) were particularly unreliable for the EMSA.

We conclude that the measurement of NF-κB binding activity by QCI shows good correlation ($r^2 > 0.95$) with the conventional electophoretic mobility shift assay (EMSA), using nuclear and cytosolic prostate cancer

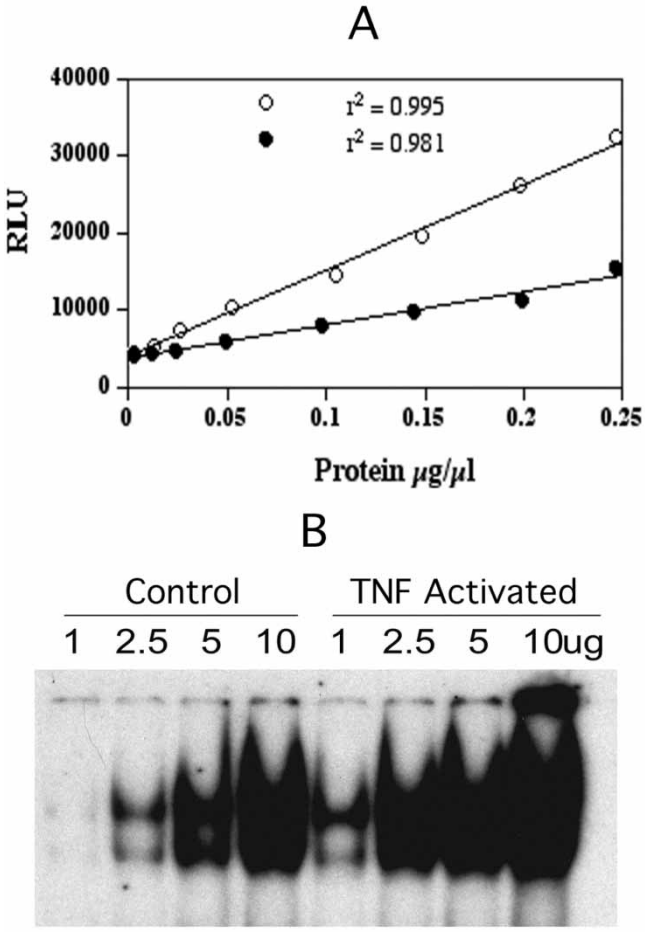

Figure 3. Correlation between RLU and nuclear protein from Control (closed circles) and TNF-α activated (open circles) PC3 cells. A: QCI performed using optimized buffer system. B: EMSA.

cell extracts. In addition, the QCI was found to be at least 50-fold more sensitive than the EMSA for the prostate cancer cell extracts studied.

Quantification of NF-κB DNA Binding Activity in Human Lymphocytes

A pilot study was conducted on human volunteers (n = 20; 10 smokers; 10 non-smokers) to investigate the effect of smoking and hence oxidative stress on NF-κB DNA binding activity. The mean amounts of cellular

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NF-κB (pg NF-κB/μg total protein), as measured by QCI, in individuals with higher oxidative stress (smokers) were higher (although the differences did not reach statistical significance due to the relatively small number of samples examined) than the mean amounts of cellular NF-κB (pg NF-κB/μg

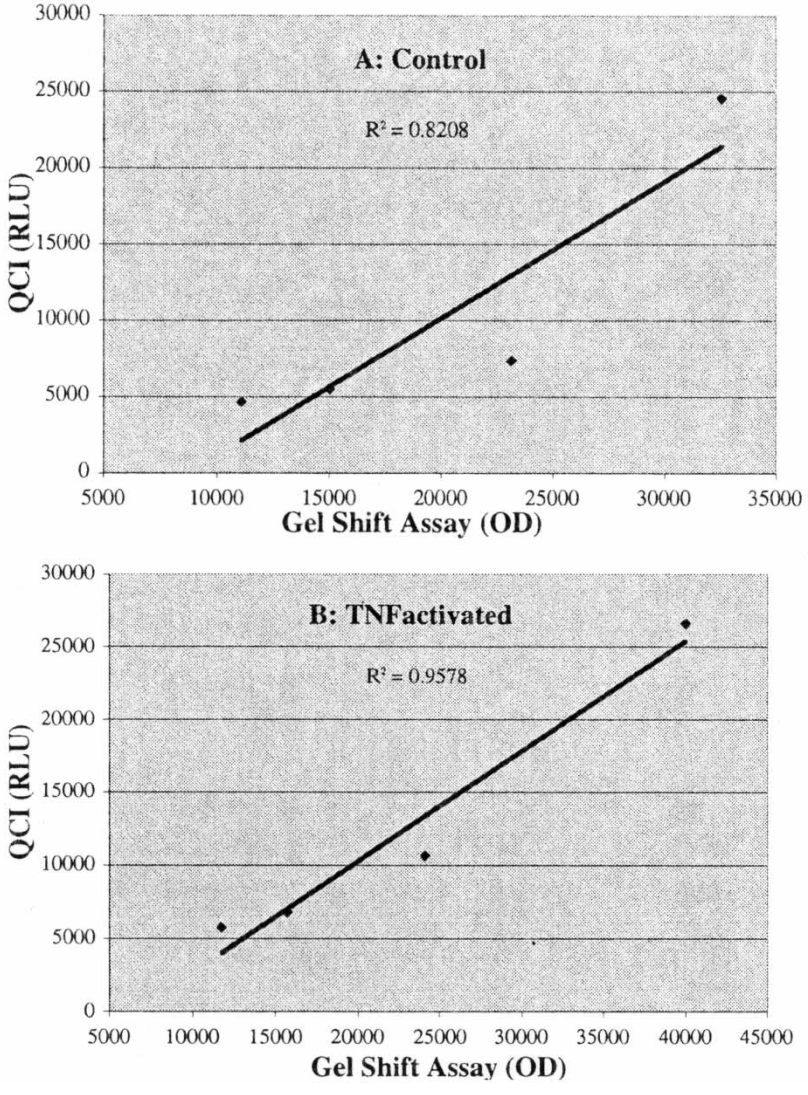

Figure 4. Correlation between OD values (EMSA followed by densitometric measurements) and RLU values (QCI) using PC3 nuclear cell extracts (1–10 μg protein/well). A: Control. B: TNF-α activated. C: Effect of sample size (x-axis) on ratio of NF-κB in activated and control PC3 cells (y-axis) Black bars: EMSA. White bars: QCI.
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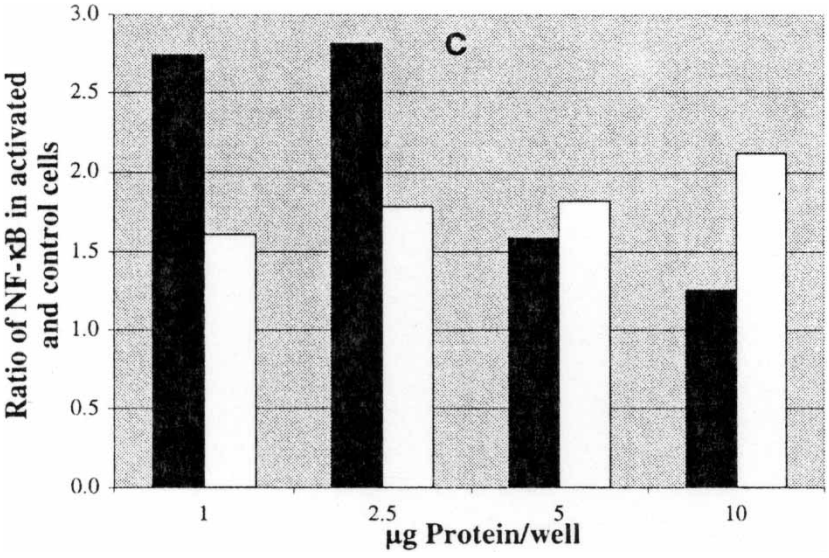

Figure 4. Continued.

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totalprotein) in individuals with lower oxidative stress (non-smokers). Thus determination of cellular NF-κB by QCI may represent a useful method for routine assessment of cellular oxidative stress. Characterization of the NF-κB DNA binding assay, using human lymphocytes was performed on cytosolic and nuclear extracts. Figure 5 depicts effect of increasing amounts of protein in the cytosolic extracts prepared from a sample of human lymphocytes on measured RLU, using the optimized QCI protocol. Cytosolic samples with protein concentrations as little as 0.01 μg/μL could be determined accurately. A comparison of NF-κB response in cytosolic extracts in aliquots of the same sample measured by EMSA is shown in the insert (Figure 5).

In addition to being quantitative, the QCI exhibited significantly greater sensitivity than the EMSA. The correlation between QCI and EMSA assays using cytosolic extracts prepared from human lymphocytes is displayed (Figure 6). A reasonably good correlation ($r^2 = 0.8691$) observed between QCI and EMSA assays in the readable range for the gel shift assay, demonstrates that the QCI may be used instead of the EMSA for determination of NF-κB in nuclear and cytosolic extracts from human lymphocytes. In addition to determination of activated NF-κB in the nuclear extracts from lymphocytes, we were able to determine the total amount of NF-κB present in the cytosol or whole cell extract. The total NF-κB is represented by the free form plus the NF-κB bound to its inhibitor IκB in the cytosol. The quantification of total NF-κB by QCI was achieved by dissociating the interaction between NF-κB and IκB by addition of a combination of detergents (Igepal

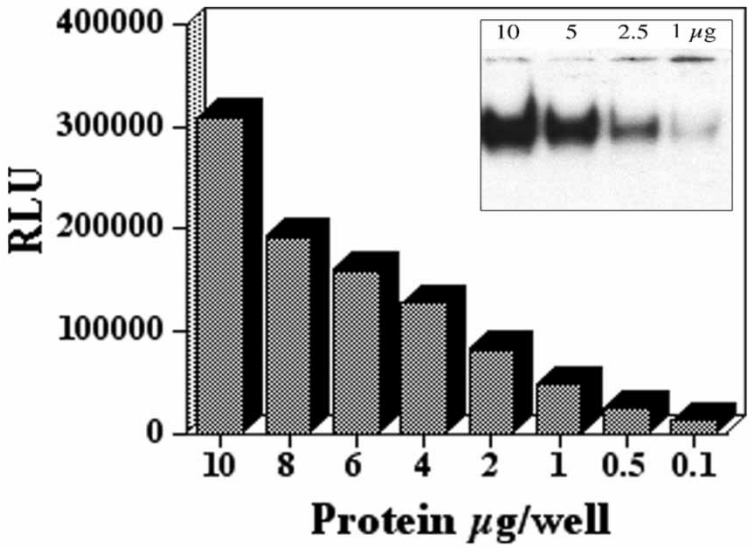

Figure 5. Effect of increasing lymphocyte extracts (protein concentration) on NF-κB signal in cytosolic extract analyzed using the QCI (Bar graph) and using EMSA (insert).

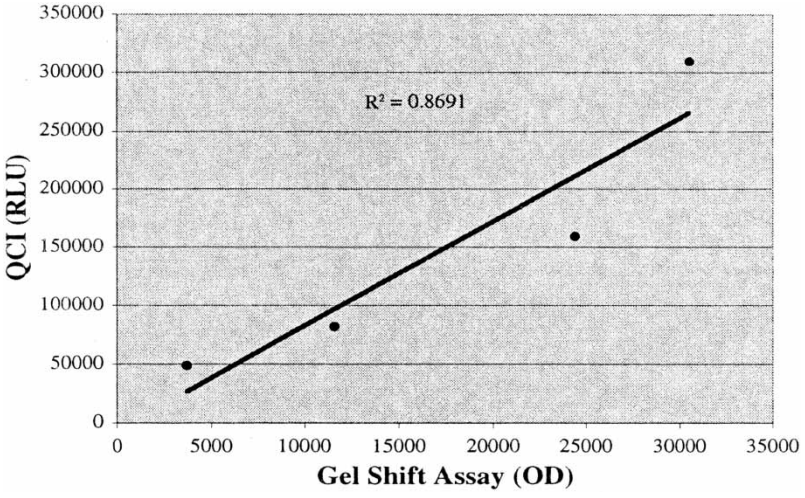

Figure 6. Correlation between RLU (QCI) and OD (EMSA/densitometric measurement) determined for human lymphocytes with increasing protein concentrations (1–10 μg/well) in cytosolic extracts: Selected protein concentration range represents the readable range for gel shift assay.

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and Deoxycholic acid, Sigma) to the binding buffer.[18–20] Thus, depending on the information desired, this QCI method for NF-κB analysis can be used for measurement of either activated and/or total NF-κB. Quantitative analysis of free NF-κB (pg NF-κB/μg protein) in nuclear and cytosolic extracts and the total NF-κB present in the cytosolic extracts prepared from lymphocytes of a human volunteer is shown in Figure 7. The total NF-κB was found to be 2–3 times higher than the free form.

The results obtained using human lymphocyte extracts, demonstrate that this QCI for measurement of NF-κB binding activity shows good correlation ($r^2 > 0.85$) with the conventional gel shift assay (EMSA). In addition to quantification (pg NF-κB/μg total protein) of NF-κB in the nuclear and cytosolic extracts prepared from human lymphocytes, by a simple alteration in the composition of the binding buffer, the QCI allows for measurement of the total amount (bound to IκB + unbound) of NF-κB present in the cell.

Quantification of NF-κB DNA Binding Activity in Animal Tissue

Carbon tetrachloride (CCl_4) has been known to induce oxidative damage in liver tissue.[21] Experiments using an established CCl_4 exposed rat model were undertaken to determine the effect of oxidative stress on NF-κB DNA binding activity. The nuclear extracts prepared from rat livers were subjected to analysis of NF-κB DNA binding activity both by the developed NF-κB QCI (in triplicates) and by EMSA (single determination, as is typical of EMSA). Figure 8 shows the effect of CCl_4 exposure at different time points (0, 4, 6, and 8 h). Both techniques showed a peak in NF-κB

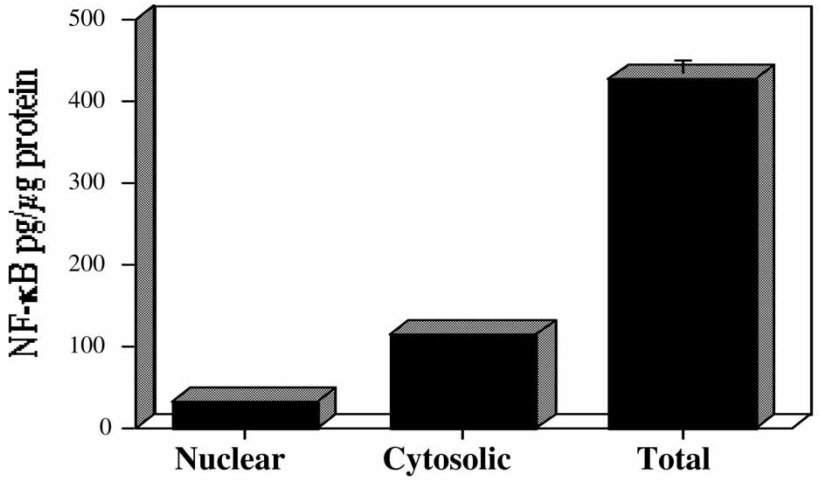

Figure 7. Quantification of NF-κB (pg NF-κB/μg protein) as Nuclear, Cytosolic and Total NF-κB in a sample of human lymphocytes using QCI.

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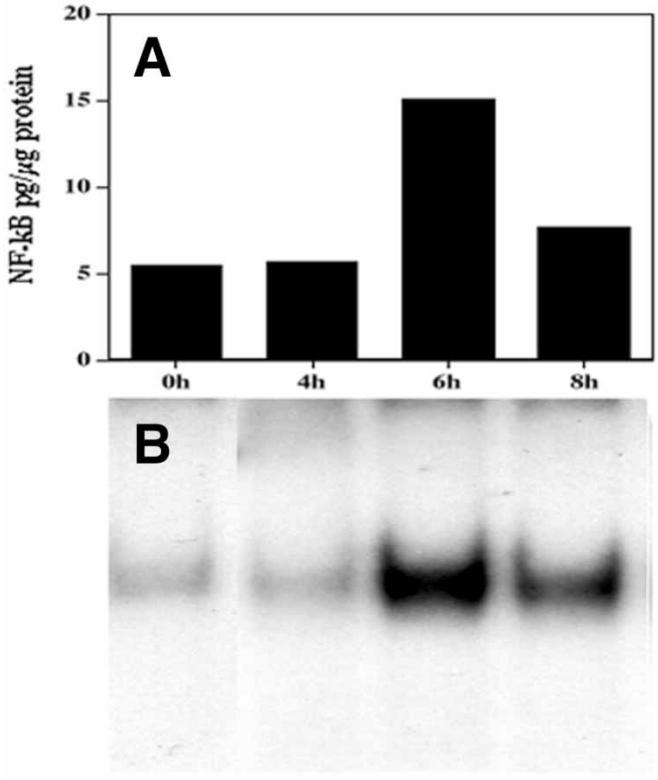

Figure 8. (A): Quantification of NF-κB (pg NF-κB/μg protein) in rat liver samples using QCI. (B): A comparison using EMSA.

binding activity at 6 h. In addition, quantification by our QCI method showed that the increase in NF-κB DNA binding activity at the 6 h time point was $3\times$ that of the control. Thus using CCl_4, an established oxidant, in a controlled rat model, a definite increase in NF-κB DNA binding activity was reproducibly observed.

CONCLUSIONS

NF-κB is a ubiquitous transcription factor which plays central roles in oxidative stress and carcinogenesis. It regulates the expression of genes critically involved in cancers and chronic diseases caused by ROS and may serve as a marker of oxidative stress. Targeting NF-κB may be a novel and important preventive or therapeutic strategy to reduce incidence of human cancers and chronic diseases due to oxidative stress. Thus, accurate

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measurement of activated NF-kB is important for estimation of oxidative stress state and for prevention and treatment of cancers and chronic diseases caused by oxidative stress.

We conclude that this new, rapid QCI can be utilized to replace the traditional EMSA for improved analysis of NF-κB DNA binding activity in cultured cells, in animal tissue extracts as well as in nuclear and cytosolic extracts prepared from human lymphocytes. In addition, the QCI for the DNA binding activity of NF-κB, has the following advantages over the conventional electrophorectic mobility shift procedure: (1) it is quantitative: a two fold difference in protein content is generally required to observe differences in band density with EMSA while the QCI can easily distinguish quantitatively differences in pg amounts of NF-κB. (2) it is highly sensitive: QCI can readily measure the DNA binding activity of NF-κB for samples with protein concentrations as little as 0.1 μg/well while the EMSA generally requires at least 1 μg protein on the gel, with 5–10 μg protein yielding more reliable results (3) it has high speed: The QCI can be completed in 3–5 h post sample preparation while the EMSA requires 12–24 hours (4) it has high throughput adaptability: A single 96 well formatted microtiter plate can be used to quantitatively analyze up to 40 samples in duplicate along with a 8 point standard curve in duplicate. This accompanied with the rapid performance permits adaptation for high throughput operations or routine experiments, (5) it is safe: No radioactive chemicals are employed in QCI, (6) it is simple: the QCI by design is straightforward and safe to perform even by a relatively unskilled technician, (7) it provides options for measurement of both activated (free) and total (bound + unbound) NF-κB: The QCI can be used to measure either the free NF-κB which is translocated into the nucleus or, by using agents which dissociate the dormant NF-κB:IκB complex present in the cytosol, the total amount of NF-κB present in the cytosol/cell.

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ABBREVIATIONS

QCI	quantitative chemiluminescent immunoassay
EMSA	electrophorectic mobility shift assay
NF-κB	nuclear factor-kappa B
PC3	prostate cancer cells
ROS	reactive oxygen species
RNS	reactive nitrogen species
RLU	relative light units
PBS	phosphate buffered saline

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