THE ROLE OF SIRT1 ON THE CROSSTALK OF P65 AND NFAT5 IN U937 MONOCYTES UNDER HYPEROSMOTIC STRESS

By

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Abstract

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Keywords

Hyperosmotic stress, U937, NFAT5, SIRT1, RELA/NF-kappa-B p65, IκBα, regulation

Hyperosmotic stress is the increase in whole extracellular solute concentration in cell causing many disruption that may lead to the physiological disease conditions such as diabetes and hypertension. In order to protect itself cells generated an osmotic stress adaptive mechanism in which intracellular inorganic ion homeostasis is restored by mainly activating NFAT5 (TonEBP) and in return it transactivates the target genes or interact with specific regulatory proteins. NFAT5 and p65 have been previously shown to interact at *IκB α* promoter for regulation of NFκB pathway. In addition to the interaction between NFAT5 and p65, it has been also shown that SIRT1 deacetylates p65 and inhibits its nuclear translocation. However, there is no such study that examines the expression profile of SIRT1, NFAT5 and p65 all together under hyperosmotic stress in U937 cells. Therefore, the aim of this study is to investigate the role of SIRT1 activity on NFAT5 and p65 expression profile under 100mM NaCl induced hyperosmotic stress in U937 monocyte cells. In addition, the aim concerns to understand the scale of contribution of NFAT5 and p65 on NFκB pathway regulation for the cell survival/death under hyperosmotic stress through examining IκB α expression profile. 100mM NaCl induced hyperosmotic stress in U937 monocyte cells indicated high expression levels of NFAT5 and SIRT1 overlapping with the activation of NFκB pathway. It is shown that in U937 cells under 100mM NaCl induced hyperosmotic stress, the activation of NFκB pathway and its regulation may be independent of NFAT5 but highly dependent on translocated p65, and SIRT1 activity may control p65 nuclear translocation, hence NFκB pathway activation.

Abstract - TÜRKÇE

Hiperosmotik stres hücredışı tüm çözünen konsantrasyonunun artışı ile hücrede birçok bozulmaya neden olarak fizyolojik hastalıklardan diyabet ve hipertansiyon ile ilişkilendirilebilir. Hücreler kendini koruma amacı ile hücredışı inorganik iyon dengesini düzeltmek için ozmotik stres adaptasyon mekanizması geliştirmiştir. Bu mekanizmada başlıca aktif olan protein NFAT5 (TonEBP) iken, bu proteinin aktivasyonu hedef genlerin transaktivayonunu sağlar veya NFAT5 çeşitli düzenleyici proteinler ile ilişki kurar. Daha önceki araştırmalarda NFκB yolağını düzenlemek için NFAT5 ve p65 proteinlerinin *IκB α* promotöründe etkileştiği gösterilmiştir. NFAT5 ve p65 etkileşimine ek olarak SIRT1 proteinin p65 proteini ile etkileşip, deacetile edip nükleer translokasyonunu engellediği gösterilmiştir. Fakat, şu ana kadar hiçbir araştırma SIRT1, NFAT5 ve p65 proteinlerinin ekspresyon profillerini birlikte U937 hücrelerinde hiperosmotik stres durumunda incelememiştir. Dolayısıyla, bu araştırmada SIRT1 aktivitesinin NFAT5 ve p65 ekspresyon profilleri üzerindeki rolünün 100mM NaCl ile oluşturulmuş hiperosmotik stres durumda U937 monosit hücrelerinde incelenmesi amaçlanmıştır. Buna ek olarak NFAT5 proteinin hiperosmotik stress durumunda hücre yaşam/ölümünü destekleyici NFκB yolağını düzenlemekte rol oynayan p65 proteinini ne ölçüde desteklediğini IκB α ekspresyon profilini inceleyerek araştırmak da amaçlanmıştır. U937 monosit hücrelerinde 100mM NaCl ile oluşturulmuş hiperosmotik stres yüksek oranda NFAT5 ve SIRT1 protein ekspresyonunu işaret etmekle beraber NFκB yolağının aktivasyonunun da eş zamanlı gerçekleştiğini göstermiştir. Bu araştırmada U937 hücrelerinde 100mM NaCl ile oluşturulmuş hiperosmotik stres durumunda NFκB yolağının aktivasyonunun ve düzenlenmesinin NFAT5 proteininden bağımsız olabileceği ama nükleer translokasyonu gerçekleşmiş p65 proteinine önemli derecede bağlı olduğu ve p65 proteinin nükleer translokasyonunu dolayısıyla NFκB yolağının aktivasyonunu SIRT1 aktivitesinin control edebileceği gösterilmiştir.

Preface

In this study the aim is to investigate the role of SIRT1 activity on NFAT5 an osmoprotective protein and p65 expression profile under 100mM NaCl induced hyperosmotic stress in U937 monocyte cells. In addition, the aim concerns to understand the scale of contribution of NFAT5 and p65 on NFκB pathway regulation for the survival of cell under hyperosmotic stress through examining IκB α expression profile.

DEDICATION PAGE

I dedicate my MSc. thesis to my family, Kaan Mazlumca and my friends who supported me with all their hearts.

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List(s) of Tables and Figures

List(s) of Symbols and Abbreviations,

$CaCl_2$: Calcium chloride

ChIP analysis: chromatin immunoprecipitation

DNA: Deoxyribonucleic acid

DBD: DNA binding domain

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

Ex-527: 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide

FACS: Fluorescence-activated cell sorting

FITC: Fluorescein isothiocyanate

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEPES-KOH: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid- potassium hydroxide

HI FBS: Heat Inactivated Fetal Bovine Serum

HRP: The enzyme horseradish peroxidase

IκB α: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IKK α: IκB kinase α

IKK β: IκB kinase β

KCl: potassium chloride

$MgCl_2$-$6H_2O$: Magnesium Chloride Hexahydrate

NaCl: Sodium Chloride

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT: Nuclear factor of activated T-cells

NFAT5 (TonEBP): Nuclear factor of activated T-cells 5

NAD^+: nicotinamide adenine dinucleotide

Nonidet P-40: octylphenoxypolyethoxyethanol, NP-40

OAADPr: 2′-O-acetyl-ADP-ribose metabolite

PBS: Phosphate buffered saline

PBS-Tween20: PBS- Polysorbate 20

PMSF: phenylmethanesulfonyl fluoride

PVDF: polyvinylidene difluoride

RPMI-1640: Roswell Park Memorial Institute, hence the acronym RPMI

SDS: Sodium dodecyl sulfate

SDS-PAGE: SDS- Polyacrylamide gel electrophoresis

SFM: Serum-Free media for cell cultures

Sir2: Silent Information Regulator Two protein

Sirtuin 1 (SIRT1): sirtuin 1

Tris-HCl: tris (hydroxymethyl) aminomethane- Hydrochloric acid

U937: Human leukemic monocyte lymphoma cell line

WST-1: Water soluble Tetrazolium salt-1

1. INTRODUCTION

1.1 Hyperosmotic Stress and Osmotic Stress Adaptive Mechanism

Hyperosmotic stress is the increase in whole extracellular solute concentration, osmolarity, which may cause many disruptions in cell. In addition to osmotic stress, the increase in extracellular osmolality, hypertonicity, that is caused by increase in only plasma membrane-impermeable solutes such as NaCl may lead to problems in the cellular system [1]. This change in the balance of extracellular osmolality may result in increased DNA strand breaks or DNA damage, cell cycle arrest, increased formation of reactive oxygen species and mitochondrial depolarization leading to apoptosis [2]. Therefore, the balance of extracellular osmolality is highly crucial and cells generated an osmotic stress adaptive mechanism in order to protect itself from such risks. In osmotic stress adaptive mechanism the intracellular inorganic ion homeostasis is restored. First NFAT5 (TonEBP) is activated by osmotic stress and in return it transactivates the target genes for the synthesis of organic osmolytes such as sorbitol, myo-inositol, betaine and taurine [1, 2]. The organic osmolytes may restore the osmotic homeostasis. The physiological condition of hyperosmotic stress can be seen in diabetes and hypertension [3]. However, accumulation of organic osmolytes resulted from the response of adaptive mechanism, may lead to several secondary diseases such as atherosclerosis, a chronic inflammatory disease. In atherosclerosis the high expression of NFAT5 can be seen which transactivates aldose reductase (AR) expression for the sorbitol synthesis leads to the organic osmolyte accumulation. Increased amount of organic osmolytes formed by osmotic stress adaptive mechanism may induce the accumulation of monocytes and lymphocytes in vessels [4]. Therefore, prime molecular members linking to hyperosmotic stress clarifies the reason for related disease condition. In this case, NFAT5 plays a key role in hyperosmotic stress and its downstream actions can be illuminated by further look on molecular link between the structure and function of NFAT5.

1.2 NFAT5, The linking protein of NFAT and NFκB Family

NFAT5 is a member of both NFAT and NFκB family of proteins which play crucial role in variety of biological functions especially immune response and development. NFAT5 is the most ancient member of NFAT family and its DNA binding domain (DBD) shares 43% sequence identity with NFAT family members [5]. When NFAT5 is activated in osmotic stress it translocates from cytoplasm to nucleus and binds to and transactivates target genes involved in the synthesis of transporters and enzymes for the generation of organic osmolytes and heat shock proteins [6]. The dimerization of NFAT5 with itself is essential for its DNA binding and transcriptional activity. By forming homo dimer NFAT5 forms a complete circle around the DNA and it generates an unusual high kinetic stability for the DNA binding and transactivation [5]. On the other hand, NFAT5 is the sole member of the Rel/ family (NFκB family) to be activated by osmotic stress [7]. The C terminal of dimer interface NFAT5 is highly similar with NFκB proteins and it shares similar DNA binding mechanism. The shared features of NFAT5 and NFκB family members proposes that they may form mixed dimers or complexes in cells for crossregulation of gene expression in stress conditions such as hyperosmotic stress [5].

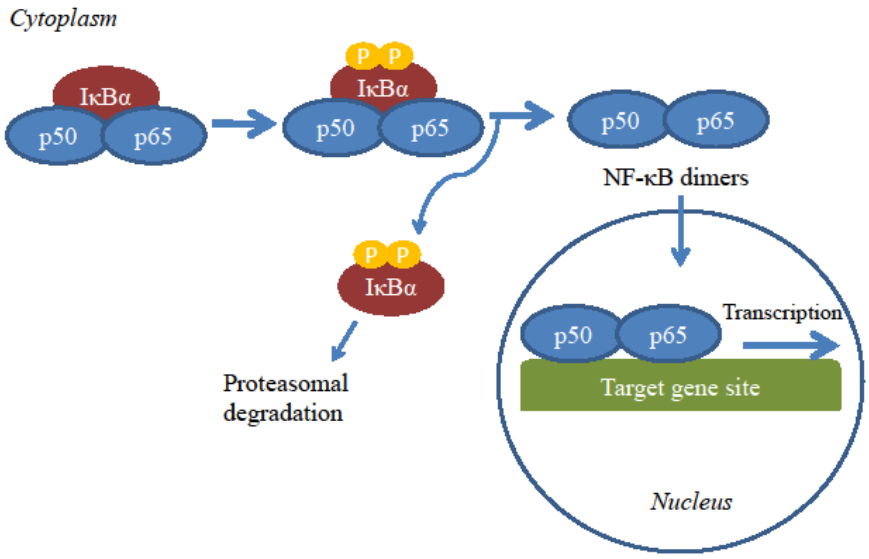
1.3 NFκB Pathway and The Regulatory p65 Protein

NFκB pathway is a fundamental pathway that links many pathway with each other in response to a cellular stimuli and regulates many genes involved in inflammation, immune response, cell survival and cell death [8]. Any dysregulation and unusual activation may result in serious problems leading to a disease. For instance in a chronic inflammatory disease atherosclerosis that stems from organic osmolyte accumulation due to hyperosmotic stress, NFκB pathway is found to be highly active [8, 9]. Besides, this indicates the close relationship and a possible interaction between NFAT5 and NFκB under hyperosmotic conditions. The important members of mammalian NF-κB family are p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). The members have highly conserved Rel homology (RH) domain responsible from dimerization, interaction with IκBs, and binding to DNA [10]. The central member p50/p65 complex localized in cytoplasm with its inhibitor IκB. Activation of NFκB pathway begin with the phosphorylation of inhibitor IκB which releases p50/p65

complex and the free NF-κB dimers translocate to the nucleus. The nuclear NF-κB dimers bind to specific sequences in the promoter or enhancer regions of target genes in order to regulate gene synthesis [10]. The NF-κB pathway can be down-regulated through feedback pathway in which newly synthesized IκBα proteins limits the nuclear translocation of NF-κB dimers. p65 itself may downregulate the NF-κB pathway by binding to *IκBα* promoter region. Thereby, the transitory activation of NF-κB may decrease due to transcriptional increase of *IκBα* [8, 11].

Diagram 1: Simple Representation of The Activation of NF-κB Pathway

(Adapted from the Nature)



1.4 Sirtuin 1

Another important protein that is activated in response to a stress condition and regulates its target genes is Sirtuin 1. It belongs to the family of silent information regulator 2 (Sir2) which is a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase. It involves in the cleavage of NAD^+ and deacetylates the protein substrates in order to form the deacetylated product nicotinamide and 2′-O-acetyl-ADP-ribose (OAADPr) metabolite [12]. The function Sir2 is the regulation of chromatin silencing in Saccharomycescerevisiae. The activation of Sir2 gene depends on several stress signals such as osmotic stress, heat shock and starvation results in regulation of target genes expression in order to regulate cellular homeostasis and survival [13, 14]. Its family member and its mammalian homolog that plays role in transcriptional regulation in accordance with intracellular energetics is Sirtuin 1 (SIRT1), a $NAD^{(+)}$ - dependent protein deacetylase and a metabolic sensor of NAD^+/NADH. SIRT1 can be seen active in cell cycle, response to DNA damage, metabolism, apoptosis and autophagy. It mainly plays role in the transcriptional repression, modulation of chromatin function, deacetylation of histones, and alterations in the methylation of histones and DNA. It functions in cell type-specific manner depending on the disease condition such as cancer, obesity, inflammation and neurodegenerative diseases [13]. The central regulatory mechanism of Sirtuin 1 is deacetylating transcription factors or coregulators of the target genes. It is shown that SIRT1 regulates glucose homeostasis by deacetylating and activating the transcription factor peroxisome proliferator-activated receptor-γ coactivator 1-α [13]. Moreover, SIRT1 is shown to be active in the regulation and deacetylation of the tumor suppressor protein p53 and RELA/NF-kappa-B p65 [15]. The activity of SIRT1 can be increased by resveratrol (3,5,4'-trihydroxy-trans-stilbene) which binds to SIRT1 and induces conformational changes in the deacetylase enzyme allowing tighter binding of the fluorophore required for the covalent attachment on the peptide in activation [12]. The activity of SIRT1 can be inhibited by nicotinamide or Ex-527. Ex-527 which is 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide fills the nicotinamide site and a neighboring pocket, and disrupts the NAD^+ dependent deacetylation mechanism [16]. In addition to the important relation between p65 and SIRT1, p65 is also shown to be interacting with NFAT5 [8].

1.5 NFAT5 and p65 Interaction

As a member of both NFAT and NF-κB, NFAT5 is shown to be active under hyperosmotic stress for transactivation of target genes [8]. p65, the essential member of NF-κB, shares similar DNA binding mechanism with NFAT5. The study of Roth and colleagues examines the binding of p65 to IKBα promoter in order to downregulate NFκB pathway [8]. The relation of NFAT5 to this binding is examined in duct principal cells and macrophages under hypertonic conditions. It is shown that increased p65 nuclear translocation is followed by a complex formation with NFAT5. The ChIP analysis of the study revealed that as a complex, p65 and NFAT5 bind to κB elements of NFκB responsive genes. Thus, under hypertonic conditions NFAT5 can be an additional intracellular component mediating NF-κB activation. This proposes that under hypertonic/hyperosmotic stress NFAT5 may help binding of p65 to IκBα promoter to increase the transcriptional activity of IκBα and downregulate NF-κB activation.

1.6 Objectives and Outcomes

The model of this study based on 100mM NaCl induced hyperosmotic stress condition which mimics the physiological disease states of diabetes and hypertension. The induction of hyperosmotic stress in U937 cells by the 100mM NaCl treatment is parallel with the former studies in literature. The model focuses on the expression profiles of NFAT5, SIRT1 and p65 in accordance for observing the NF-κB pathway regulation for the inflammation/cell survival or death under 100mM NaCl induced hyperosmotic stress. The model is implemented on U937 human leukemic monocyte lymphoma cell line which is perfectly suitable for hyperosmotic stress induction. The objectives of this study seek to examine the role of SIRT1 activity on NFAT5 and p65 crosstalk on *IκB α* synthesis under 100mM NaCl hyperosmotic stress in U937 monocyte cells. Since there is no such study that examines the expression profile of SIRT1, NFAT5 and p65 together under hyperosmotic stress in U937 cells, our secondary goal is to examine them with one accord. Our tertiary goal is to understand the scale of the contribution of NFAT5 on the activity of p65 on the regulation of *IκBα* synthesis in U937 cells under hyperosmotic stress.

We showed that in U937 cells under hyperosmotic stress the activation of NFκB pathway and its regulation is independent of NFAT5 but highly dependent on translocated p65 and SIRT1 activity may control p65 nuclear translocation, hence NFκB pathway activation.

2. EXPERIMENTAL

2.1 Cell Culture and Treatments

U937, human leukemic monocyte lymphoma cells were cultured in RPMI-1640 supplemented with 10% HI FBS, 2mM glutamine [5Mm Glucose, 100 IU/ml penicillin/streptomycin]. Cultures were maintained at 37ºC in a humidified 5% CO2 atmosphere. Cells were collected, quantified in SFM and seeded (~1,500,000 cells/ml) in 12-well, 100mm or 60mm culture plates depending on the experiment. Except the negative control groups the seeded cells were treated with 100mM NaCl to mimic hyperosmotic stress condition. In SIRT1 activity inhibiting and increasing treatment cells were seeded on 100mm well plates and before the addition of 100mM NaCl one group of cells were pretreated with the Ex-527 and the other with resveratrol for 1 hr. The cells collected for analysis at indicated specific time points at each treatment.

2.2 Chemicals

SIRT1 activity inhibiting chemicals are nicotinamide or Ex-527. Ex-527 which is 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide fills the nicotinamide site and a neighboring pocket, and disrupts the NAD^+ dependent deacetylation mechanism [16]. The activity of SIRT1 was increased by resveratrol (3,5,4'-trihydroxy-trans-stilbene) which binds to SIRT1 and induces conformational changes in the deacetylase enzyme allowing tighter binding of the fluorophore required for the covalent attachment on the peptide in activation [12]. In this study 15µM resveratrol and 10µM Ex-527 were used in treatments.

2.3 Cell death and viability assays

Cell death response was evaluated by FITC conjugated Annexin-V (Alexis). Manufacturer's protocols were applied during FITC-Annexin-V staining. Briefly, U937 cells grown in 5mM Glucose seeded in 12-well plates. One group leaved as control group, one well left without dye and the other group treated with 100mM NaCl. Groups that completes indicated treatment duration transferred to flow cytometry tubes and cells were harvested by centrifugation at 300 g for 5minutes. Then the cells were resuspended in 1 ml of cold PBS and centrifuged again at

300 g for 5 minutes. The supernatant was removed and the cells were incubated in Annexin V buffer (140 mM HEPES, 10 mM NaCl, 2,5 mM CaCl2, pH:7.4) containing 1% (v/v) Annexin V (FITC) for 15 minutes in the dark. Cells were analyzed by FACS (FACSCanto, Becton Dickinson) on FlowJo software.

Cell viability or cell proliferation was detected by WST-1 assay (Roche) according to manufacturer's instructions. 5mM Glucose and 100mM NaCl treated group of cells were seeded in 96-well plate. Results are expressed as percentage of cell viability. The absorbance was measured with a microtiter plate reader (Bio-Rad, CA, USA) at a test wavelength of 550 nm and a reference wavelength of 650 nm.

2.4 Protein Extraction and Immunoblotting

Cells were treated as indicated and collected at specific time points with 1-2ml PBS, centrifuged at 300 g for 5 minutes. Following resuspension in 1 ml of ice-cold PBS and transfer to 1.5-ml microfuge tubes, cells were centrifuged at 13200 rpm for 30 seconds. For total protein extraction the pellet was lysed by incubation for 30 minutes in 75-150µl (depending on pellet size) of cold cell lysis buffer containing 50 mM Tris-HCl (pH:8.0), 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), protease and phosphatase inhibitor cocktails (all 20X). After centrifugation at 13200 rpm for 10 minutes, supernatant containing the total protein extract was removed and stored at -80^{o}C. For cytoplasmic-nuclear protein extraction pellet was resuspended in cytoplasmic lysis buffer mix contains 20X protease inhibitor, phosphatase inhibitor, 100mM PMSF, 100mM Dithiothreitol (DTT) and T1 buffer containing 10mM HEPES-KOH, 2mM M $ZgCl_2$-$6H_2O$, 0.1mM KCl and Nonidet P-40 1% (v/v), and incubated on ice for exactly 15 minutes. After brief vortexing, the cells centrifuged at 13200 rpm for 1 minute. The collected supernatant stored as cytoplasmic protein extract. The remaining pellet was washed with PBS without touching it and centrifuged again. The pellet resuspended in nuclear protein extraction buffer mix contains 20X protease inhibitor, phosphatase inhibitor, 100mM PMSF, 100mM Dithiothreitol (DTT) and T2 buffer containing 50mM HEPES-KOH, 2mM $MgCl_2$-$6H_2O$, 0.1mm EDTA, 50mM KCl, 400Mm NaCl and %10 Glycerol, and incubated at least 20 minutes on ice (or overnight at -80^{o}C). After vortexing briefly cells centrifuged at 13200 rpm for 20 minutes. Collected supernatants stored as nuclear proteins at -80^{o}C. Protein concentrations were determined by Quick-Start Bradford protein assay and the absorbance was measured with a microtiter plate

reader at a test wavelength of 595 nm. Proteins (40 µg) were mixed with loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0,004% bromophenol blue, 0,125 M Tris-HCl pH:6,8) and separated on 6% SDS-PAGE (only for NFAT5)-12% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with 5% blocking reagent (non-fat milk) in PBS-Tween20 and incubated with appropriate primary and HRP-conjugated secondary antibodies (Cell Signaling and Santa Cruz-NFAT5) in 5% blocking reagent. After three times washes with PBS-Tween20, proteins were analyzed using an enhanced chemiluminescence detection system (ECL Advance, Amersham Pharmacia Biotech, Freiburg, Germany) and exposed to Hyperfilm- ECL (Amersham Pharmacia Biotech, Freiburg, Germany).

2.5 Statistical Analysis

All the illustrated results represent one of at least three independent experiments with similar outcomes. Statistical significance of responsive differences among differentially treated populations were assessed with unpaired or paired student's t-test, respectively. Values lower than P,0.05 are marked as *.

RESULTS

Under 100mM NaCl induced hyperosmotic stress the effect of SIRT1 expression on NFκB pathway and NFAT5 activity in U937 cell line is examined. Time dependent examination focused on hours 0, 1, 4, 16 and 48 in order to follow the changes in molecular level in response to increasing stress experience. The novel findings in this work revealed that independent of nuclear NFAT5 activity, translocated p65 is essential for regulation of NFκB pathway activity under 100Mm NaCl induced hyperosmotic stress in U937 cells.

3.1 Confirmation of Osmotic Stress by Osmometer

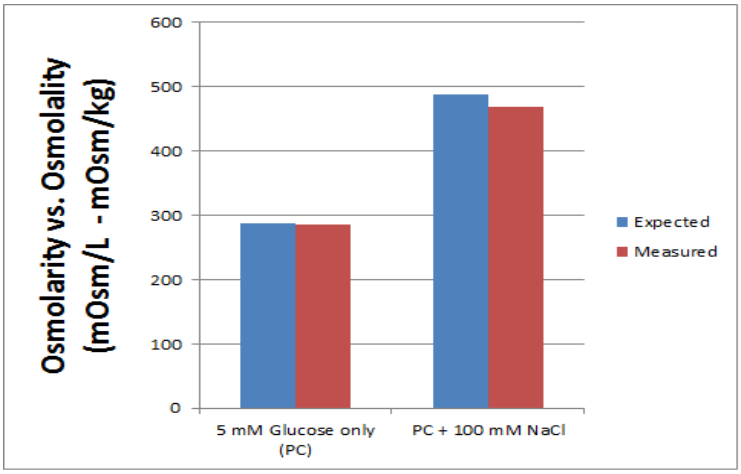


Figure 1: Confirmation of Osmotic Stress

In the preparation of 100mM NaCl treated cell culture the expected osmolarity of solution is shown based on volume, the number of miliosmoles per liter (mOsm/L) of solution. The stress measurement values are given as osmolality, the number of miliosmoles per kg (mOsm/kg) of the solvent which indicates the concentration of particles dissolved in solution. In *figure 1* the expected osmolarity and the measured osmolality by osmometer are highly close to each other indicating that 100mM NaCl induced hyperosmotic stress is confirmed by the measurements of osmometer.

3.2 WST-1 Time Dependent Assay

100mM NaCl induced hyperosmotic stress mimics physiological conditions such as diabetes or hypertension. The effect of hyperosmotic stress on U937 cell viability is observed among samples taken at 0, 1,4,16 and 48 hour respectively (*Figure 2*). WST-1 Time dependent

viability assay is done for U937 cells cultured in 5mM Glucose and 100mM NaCl containing RPMI-SFM medium. The viability of stressed group significantly decreasing compared to control group starting from first hour. The difference of viability between control and stress group is highest at hour 4. Therefore, 100mM NaCl significantly affects the viability of cells. Thereby generation of a hyperosmotic stress condition by 100mM NaCl is confirmed.

3.3 Annexin V-FITC FACS

Alongside, Annexin V-FITC FACS results indicated that compared to control group apoptotic cell portion is significantly higher at hour 16 and 48 (*Figure 3*). In other words, hyperosmotically stressed cells tend to undergo apoptosis earlier than control group in a higher fraction. Viability and Annexin V assay results coincide with each other and indicate the negative effect of 100mM NaCl on U937 cells especially starting from hour 4.

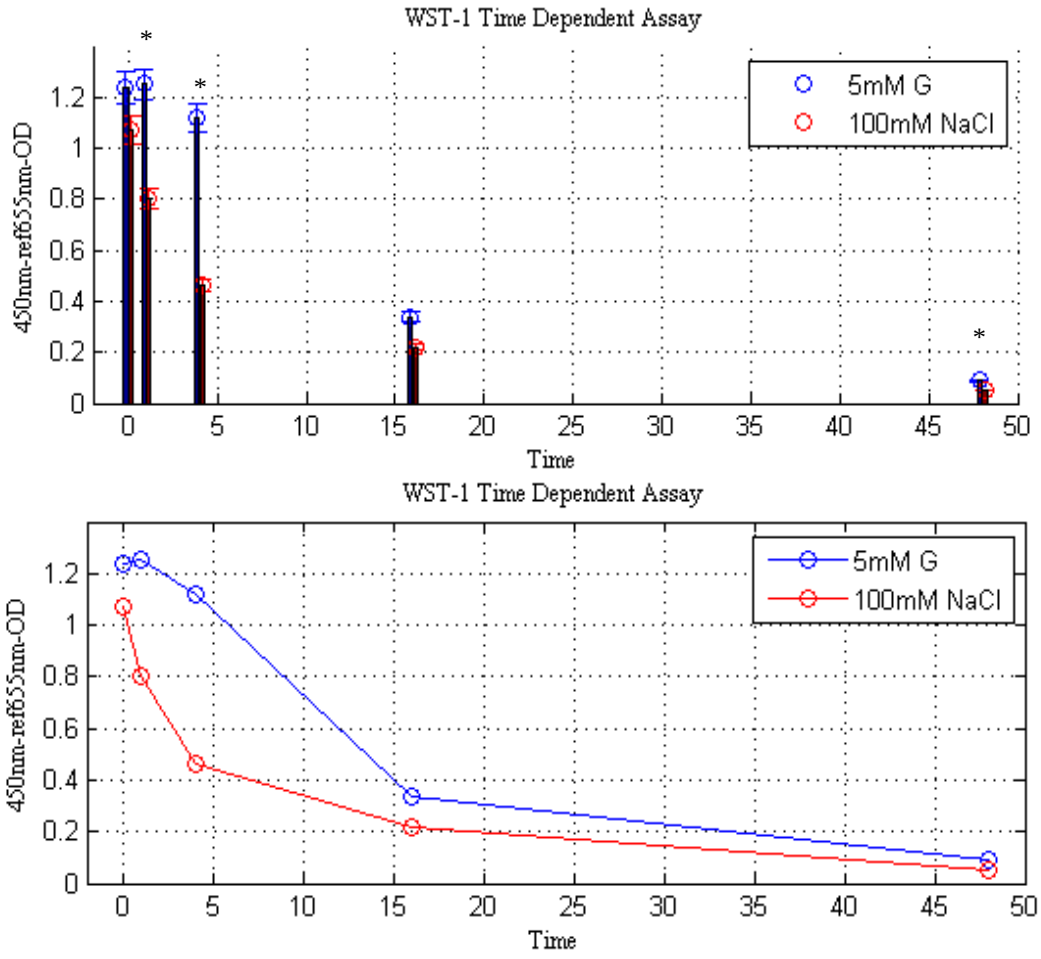


Figure 2: Time Dependent Effect of 100mM NaCl on U937 Cell Viability
WST-1 Time dependent Assay is done for U937 cells in 5mM Glucose and 100mM NaCl. Data is collected from 0, 1, 4, 16, 48 hour cell samples and measured at 450nm-ref655nm OD.

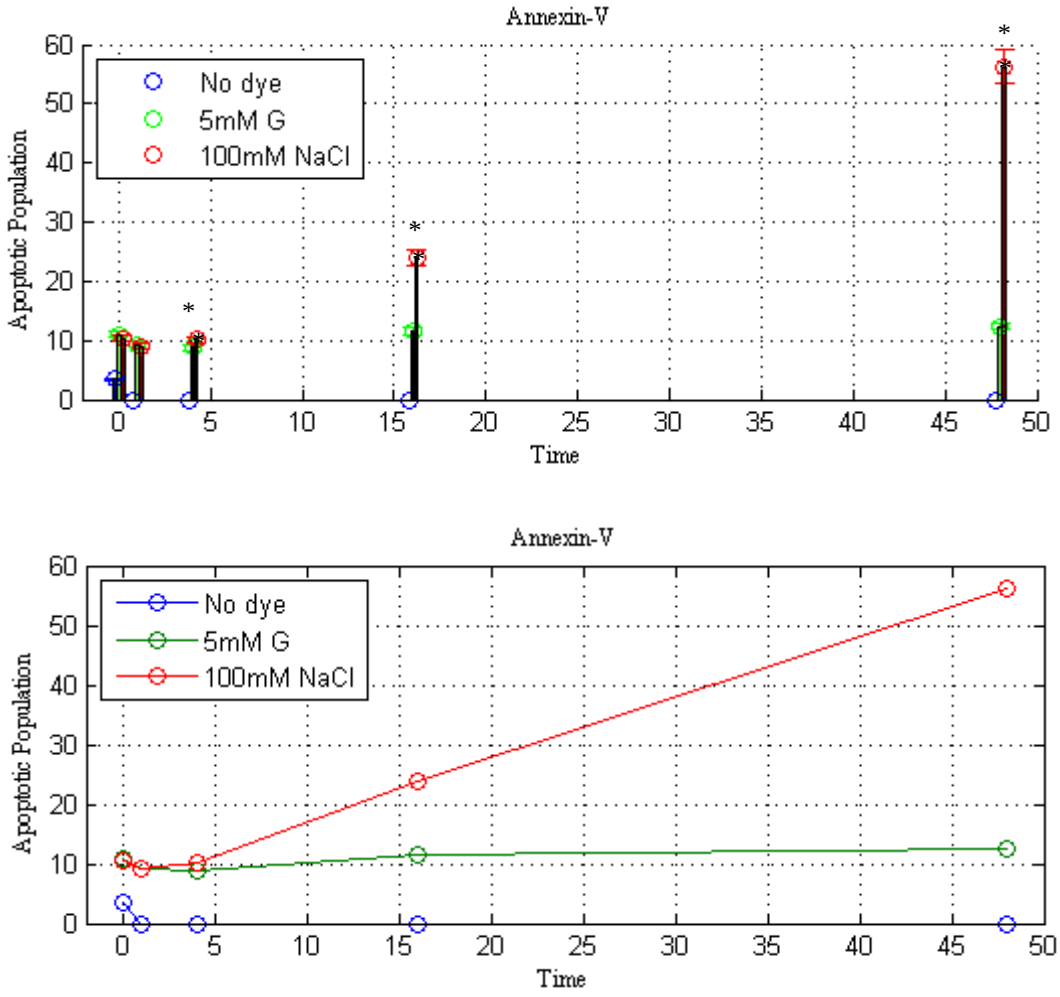


Figure 3: 100mM NaCl Annexin V-FITC FACS
100mM NaCl Annexin V-FITC apoptotic cell portion within U937 cell populaion in 5mM Glucose and 100mM NaCl . Data is collected from 0,1,4,16,48 hour stress cell samples

3.4 100mM NaCl treatment 1

First the protein expression is examined by western blotting on total protein extraction samples. The treatments were done on 60mm well plates and results are obtained (*Figure 4*). Although with low amount of protein concentration the expression level of SIRT1 and NFAT5 is visualized. Since the NFκB pathway is also concerned, p65, phospho IκB α and IκB α expression levels are observed first and foremost. The results indicated that NFAT5 expression is significantly increased at 4th hour due to hyperosmotic stress. Alongside, SIRT1 expression is also starting to increase at 4th hour of hyperosmotic stress compared to initial times. Concurrent with NFAT5 and SIRT1 overexpression, NFκB pathway is active at 16th hour. Since the protein samples are from total protein extraction, p65 result does not indicate a significant point until nuclear portion is analyzed. However, the clear phosphorylation of IκB α and decreased expression of IκB α at 16th hour clearly indicates an activation of NFκB pathway. Therefore, hyperosmotic stress increases NFAT5 and SIRT1 synthesis starting from 4th hour in U937 cells and at 16th hour NFκB pathway become active due to certain cellular responses.

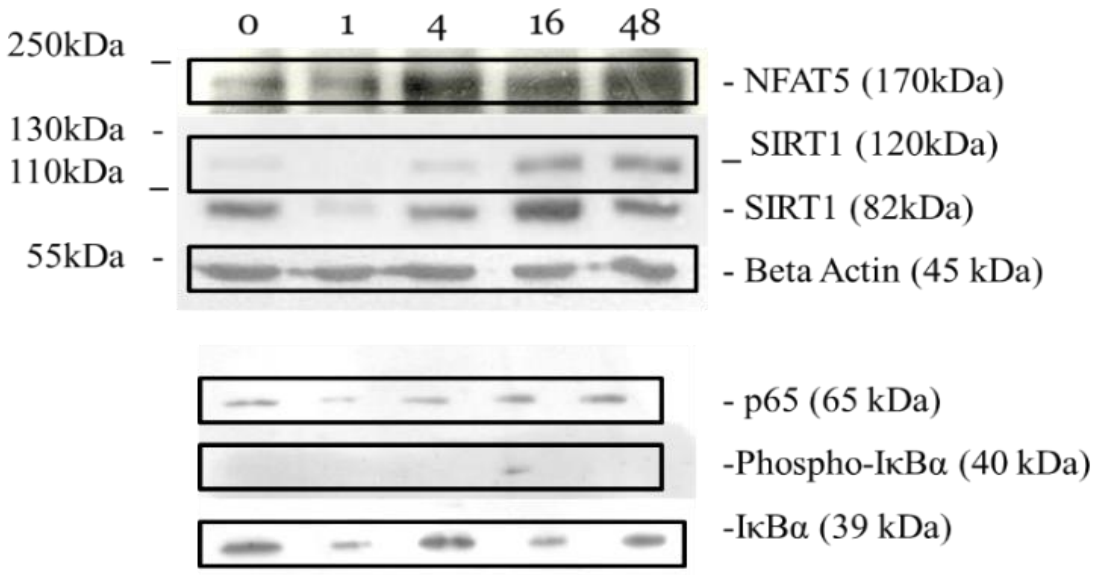


Figure 4: 100mM NaCl treatment 1

Western Blotting of samples obtained by total protein extraction from U937 cell culture in 100mM NaCl containing medium in 60mm cell culture plates. Samples are taken at 0, 1,4,16, 48 hours respectively. (Duplicate result)

3.5 100mM NaCl treatment 2

In order to obtain a higher protein concentration, 100mM NaCl treatment is repeated in 100mm well plates. The second treatment scans more NFκB family member for a better understanding of the response of NFκB pathway due to hyperosmotic stress (*Figure 5*). First, the NFAT5 result revealed that the expression of NFAT5 increases significantly at 16th hour which is correlated with the activation of NFκB pathway at 16th hour. SIRT1 and IκB results are parallel with the first treatment. The phosphorylation pattern become clearer with the second treatment in 100mm cell culture plates with higher concentration of proteins. The phosphorylation is starting at 4th hour but highest phosphorylation present at 16th hour. The upstream of the IκB protein is also examined. IKK α and IKK β expression levels are decreased at 16th hour which is overlapping with the IκB α phosphorylation time. In accordance with IKK α/β, the phosphorylated IKK α/β is higher at 16th hour compared to 4th hour. The p65 result again cannot indicate a significant result until a nuclear form is visualized. Therefore, larger scanning of NFκB family members clearly indicated an activation at 16th hour.

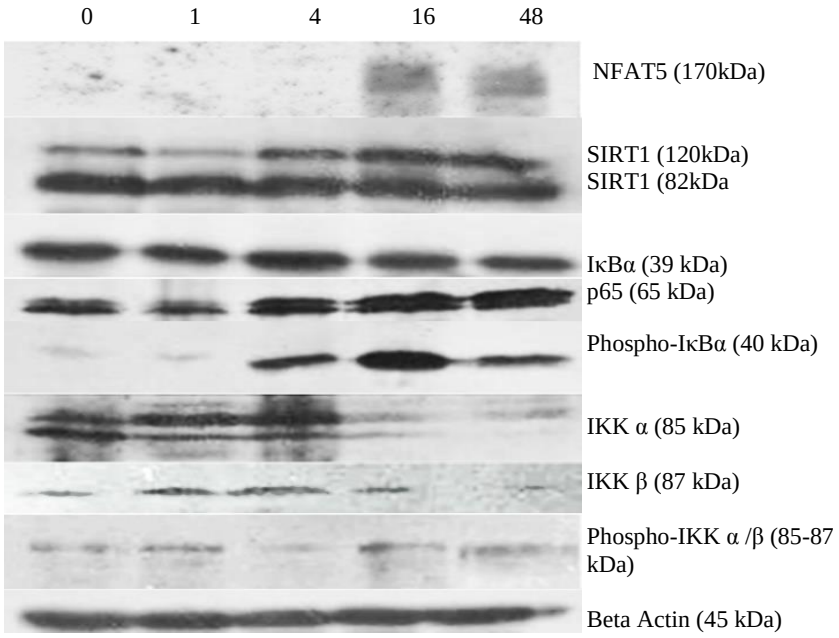


Figure 5: 100mM NaCl treatment 2
Western Blotting of samples obtained by total protein extraction from U937 cell culture in 100mM NaCl containing medium in 100mm cell culture plates. Samples are taken at 0, 1,4,16, 48 hours respectively. (Duplicate result)

3.6 100mM NaCl treatment, Cytoplasmic/ Nuclear proteins WB result

Total protein extraction results do not show a significant outcome on p65 expression and nuclear translocation level. Therefore, nuclear/cytoplasmic protein extraction is performed and results are examined (*Figure 6*). At 16th hour both cytoplasmic and nuclear NFAT5 protein level is increased compared to initial hours. The nuclear translocation of NFAT5 significantly occurs at 16th hour. At 48th hour the protein level of cytoplasmic and nuclear NFAT5 decreases. The SIRT1 and IκB α results correlate with the total protein extraction results. SIRT1 cytoplasmic protein expression increases at 16th hour and IκB α cytoplasmic protein expression displays an oscillating pattern and decreases at 16th hour. The nuclear translocation of p65 is visible at 4th hour and significantly increased at 16th hour. At 48th hour p65 nuclear portion is decreased and the cytoplasmic protein increases. Therefore the nuclear translocation peaks at 16th hour but slows at 48th hour. The Lamin A result is used as nuclear fraction control and β actin result is used as protein loading control.

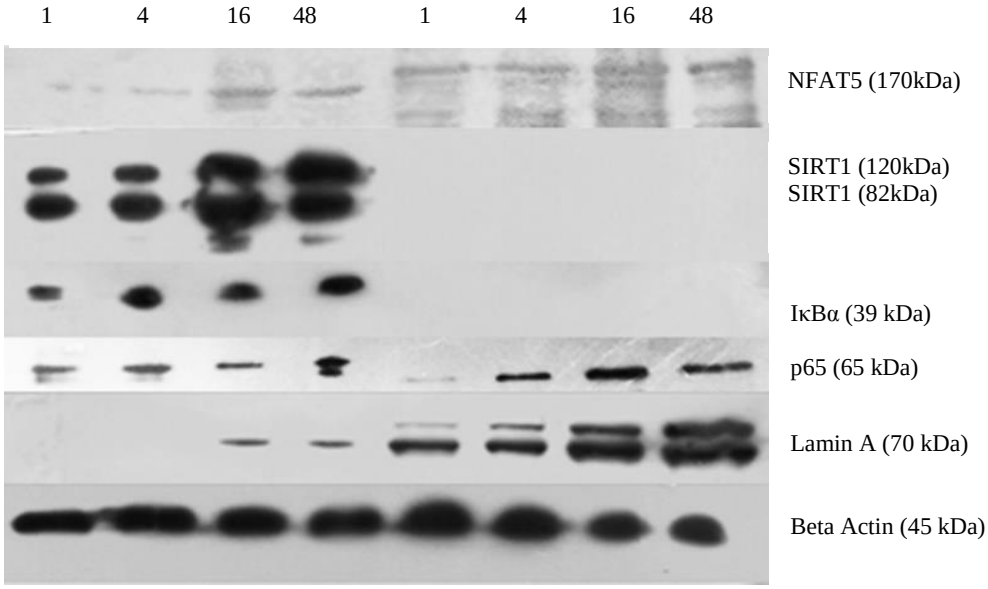


Figure 6: 100mM NaCl treatment, Cytoplasmic/ Nuclear proteins WB result

Western Blotting of samples obtained by both cytoplasmic and nuclear protein extraction from U937 cell culture in 100mM NaCl containing medium in 100mm cell culture plates. Samples are taken at 1, 4, 16, 48 hours respectively. (Duplicate result)

3.7 15uM Resveratrol and 10uM Ex527 Pretreated, and 100mM treated Cytoplasmic/ Nuclear proteins WB result

In order to examine the effect of SIRT1 on NFAT5 and p65 expression and nuclear translocation, the activator and inhibitor of SIRT1 is used in the pretreatment of the U937 cells (*Figure 7)*. The results indicated that in presence of resveratrol, the activator of SIRT1, SIRT1 expression is decreased. In presence of EX-527, inhibitor of SIRT1, SIRT1 expression is increased. The effect of increased SIRT1 activity on NFAT5 is positive. 15uM resveratrol pretreated U937 cells expressed increased NFAT5 and the nuclear translocation of NFAT5 is increased compared to positive control. Whereas, when cells pretreated with 10mM EX-527 NFAT5 expression and nuclear translocation is quite decreased. The resveratrol effect on IκB α is turned out to be negative. The results indicated that when SIRT1 activity increases IκB α expression decreases compared to positive control and when SIRT1 activity decreases IκB α expression increases. Moreover, the effect of SIRT1 activator on p65 nuclear translocation is revealed as quite negative. In cells pretreated with resveratrol nuclear translocation of p65 is lower compared to positive control. When SIRT1 activity is decreased almost all cytoplasmic p65 translocated to nuclei.

	Cytoplasmic				Nuclear			
	Negative Control	Positive Control	15µM Resveratrol	10µM EX527	Negative Control	Positive Control	15µM Resveratrol	10µM EX527
100 mM NaCl	-	+	+	+	-	+	+	+

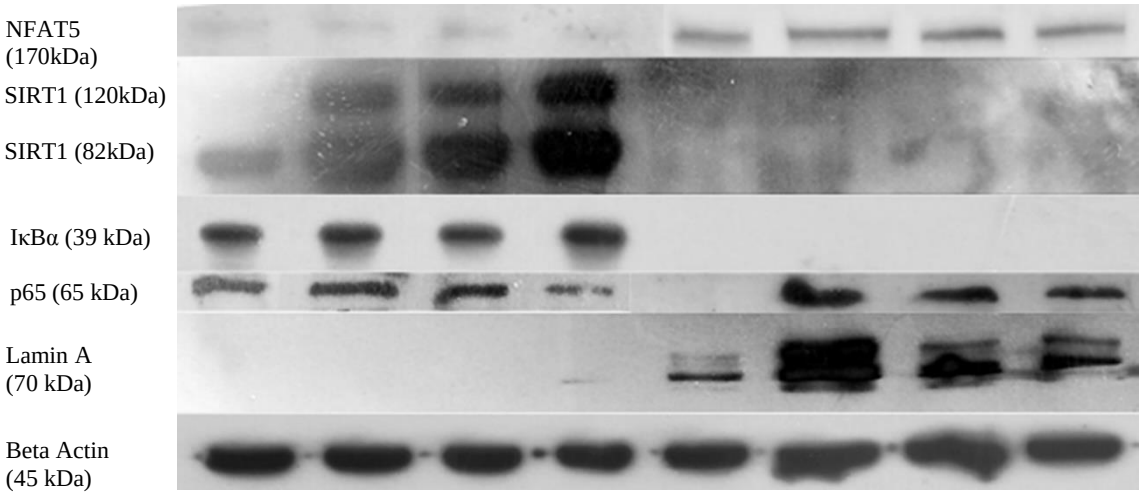


Figure 7: 15uM Resveratrol and 10uM Ex527 Pretreated , and 100mM treated Cytoplasmic/ Nuclear proteins WB result
Western Blotting of samples obtained by both cytoplasmic and nuclear protein extraction from U937 cell culture treated with 100mM NaCl containing medium in 100mm cell culture plates. Cells are pretreated with Sirtuin 1 inhibitor Ex-527 and activator resveratrol. Samples are taken at 16 th hour. (Duplicate result)

3. DISCUSSION

Hyperosmotic stress disrupts cell in many ways leading to changes in signaling. In this study the molecular response of the cell monitored by scanning the NFκB pathway and tonicity-responsive enhancer binding protein (TonEBP/NFAT5) expression level. In addition, the role of SIRT1 on NFκB pathway activation and NFAT5 expression is observed since it regulates target gene expression in response to metabolic changes and stress. The results indicated that at 16th hour of hyperosmotic stress NFAT5 and SIRT1 is overexpressed and NFκB pathway is activated. At the key 16th hour the nuclear translocation of p65 and NFAT5 are seen. The presence of NFAT5 and p65 in the nuclear at the same time points raises a probability of complex formation at target gene *IκB α* promoter for the NFκB pathway regulation under hyperosmotic stress. When the activity of SIRT1 is increased and decreased by resveratrol and Ex-527 respectively, the effect of SIRT1 on the expression and translocation of p65 and NFAT5 is observed. It is revealed that p65 is essential for the regulation of *IκB α* synthesis and NFAT5 may only have a role in tuning of the effect of p65.

In this study first of all, hyperosmotic stress for U937 cells is generated by 100mM NaCl treatment. The purpose is to mimic the physiological disease conditions stem from hyperosmotic stress. As the decrease of viability and increase of apoptotic cell portion are seen, results confirmed the generation of hyperosmotic stress by 100mM NaCl treatment in U937 monocyte cells (*Figure 2, 3*). In a molecular aspect, as it was expected hyperosmotic stress increased NFAT5 synthesis starting from 4^{th} hour in U937 cells and the overexpression is highest at 16th hour (*Figure 4, 5*). Therefore, the hyperosmotic stress condition affects cells highest at 16^{th} hour and cells activated osmotic stress adaptive mechanism to recover the cellular homeostasis through NFAT5 activation. If SIRT1 expression is monitored, the highest expression is also present at 16^{th} hour as it is for NFAT5. The mutual activation of both proteins proposes a probability common regulatory function under 100mM NaCl induced hyperosmotic stress. However, in order to prove that further research should be conducted on their direct or indirect interaction. On the other hand, expression levels of NFκB family members points out a possible activation at 16^{th} hour. These results indicated a significance of 16th hour for NFAT5, SIRT1 and NFκB proteins.

Remaining research in this study focused on 16th hour in order to understand the details of NFκB pathway signaling under hyperosmotic stress. Once nuclear protein level is examined,

increased nuclear p65 confirmed the activation of NFκB pathway at 16th hour due to hyperosmotic stress (*Figure 6*). Stressed cells may have activated the osmotic stress adaptive mechanisms through NFAT5, and NFκB pathway is activated by p65 nuclear translocation which in return may control the NFκB pathway negatively or positively at nuclei which leads to the survival or death of the cell depending on the severity of hyperosmotic stress condition. In addition, at 16th hour the presence of NFAT5 at nuclei is suggesting an interaction with p65 on a target gene for regulation of the activity of NFκB pathway.

In this study SIRT1 activator and inhibitor are used in order to enlighten the possible interaction between NFAT5 and p65 at nuclei, and give an insight on the role of SIRT1 on p65 and NFAT5 nuclear translocation and expression. The results of the treatment with resveratrol and Ex-527 showed in *figure 7* revealed that in presence of Resveratrol or Ex-527, SIRT1 regulates its synthesis depending on its protein level by negative feedback loop in order to manage its activity. The increased activity of SIRT1 increases expression of both nuclear and cytoplasmic NFAT5 whereas decreases p65 translocation. In other words, increased activity of SIRT1 has a positive effect on NFAT5 expression which may indicate a regulatory relation between NFAT5 and SIRT1 once SIRT1 activity increase enough. The studies that have conducted in different cell lines and stress conditions indicated that p65 can be deacetylated and inhibited by SIRT1 [15]. Thus, the decreased p65 nuclear translocation may be due to p65 deacetylation by SIRT1 which may inhibit its activity at U937 cells under 100mM NaCl hyperosmotic stress. On the other hand, p65 nuclear translocation increases and IκB α expression increases when SIRT1 activity decreased by Ex-527 *(Diagram 2)*. In other

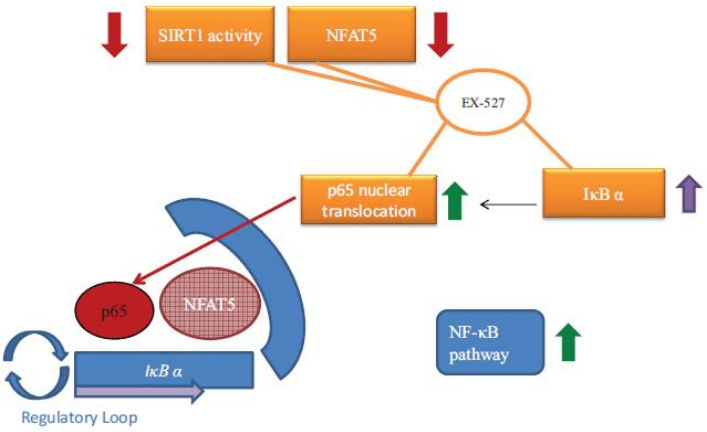


Diagram 2:
Expression profiles when U937 cells pretreated with EX-527 under hyperosmotic stress

words, p65 nuclear translocation is strongly correlated with *IκB α* gene expression increase. As it has been shown in several studies with different cell lines and stress conditions [8, 11], this result proposes that p65 may also have an autoregulatory function on NFκB pathway through *IκB α* in U937 cells under hyperosmotic stress. Therefore, acetylated and translocated p65 is essential for regulation of *IκB α* synthesis, thereby NFκB pathway activity. In addition, since presence of resveratrol decreased p65 translocation, SIRT1 activity may control the nuclear translocation of p65, hence NFκB pathway activity through deacetylation *(Diagram 3)*. Moreover, while p65 nuclear translocation is low during increased SIRT1 activity, increased nuclear NFAT5 expression cannot significantly affect IκB α expression alone. On the contrary of the outcomes of the study of Roth and colleagues [8] this result proposes that NFAT5 may not have a major role but a tuning role on *IκB α* synthesis regulation and it may support nuclear p65 activity on *IκB α*. However, decreased NFAT5 expression via SIRT1 inhibitor alone may not rule out its binding to *IκB α* promoter with p65 because the NFAT5 protein population remaning after SIRT1 inhibition may still act on the *IκB α* promoter. This may be confirmed in future electromobility shift assay or ChIP analysis.

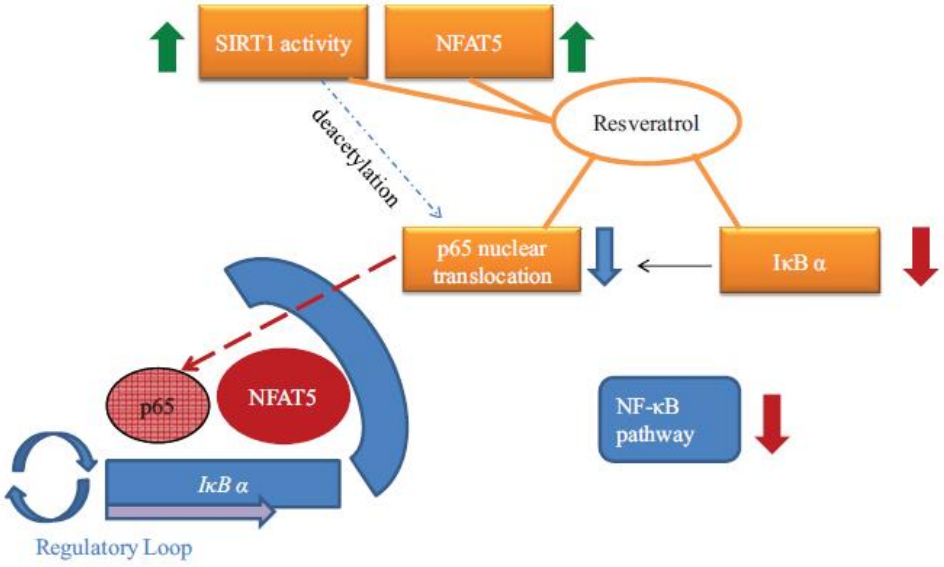


Diagram 3: Expression profiles when U937 cells pretreated with resveratrol under hyperosmotic stress

These results may propose that *IκB α* is the target in NFκB pathway regulation under hyperosmotic stress and p65 is the key regulator in stressed U937 cells. The time dependent expression profiles of proteins of interest and viability assays revealed that this regulation under hyperosmotic stress may result in either inflammation or cell death in a time depending manner due to the level of severity of hyperosmotic stress *(Diagram 4)*.

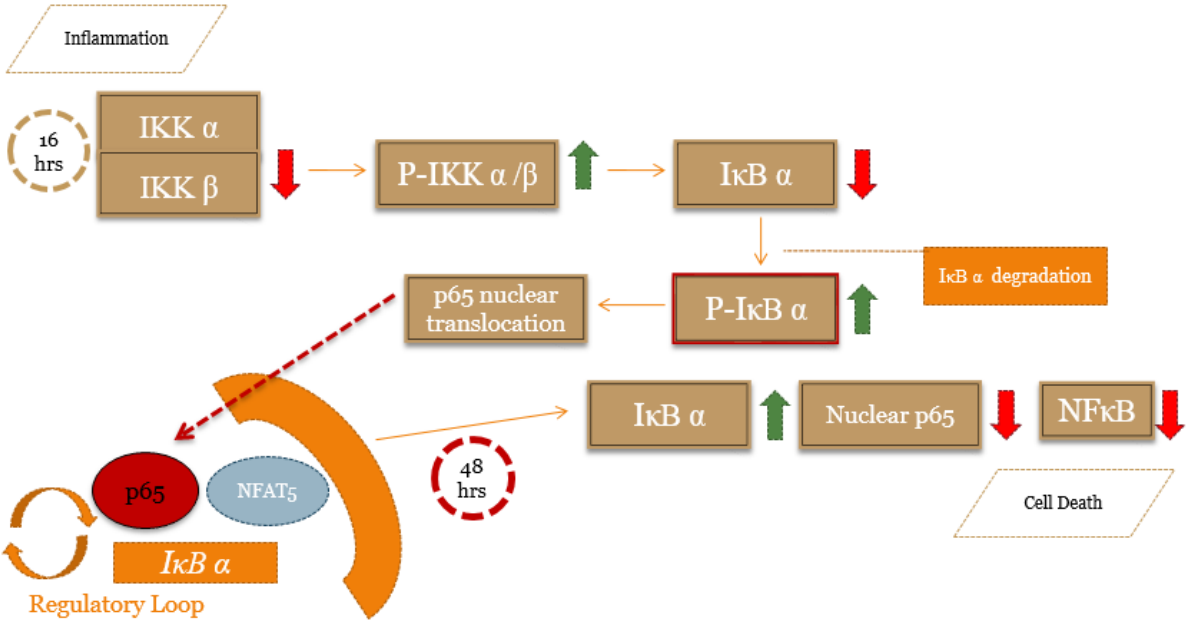


Diagram 4: Time dependent NFκB activation and its relation with inflammation and apoptosis

In future this study can be broadened by repeating it in other cell lines and comparing the expression profiles of p65, NFAT5, SIRT1 all together and examine their effect on *IκB α* regulation. Repeating this study in other cell lines under 100mM NaCl induced hyperosmotic stress may indicate similar results as in Roth and collegues study and in contrast to this current study in U937 cells, NFAT5 may be more influential on *IκB α* regulation when p65 is downregulated with SIRT1 upon resveratrol pretreatment or with another regulator. In addition, although former studies indicating an earlier activation of NFκB pathway, in this study the focused time is 16th hour of hyperosmotic stress. Since the expression of NFAT5 and SIRT1 comes into picture starting from 4th hour, examining earliear timepoints in which NFκB pathway is active was not beneficial in U937 cells. Therefore, maybe other cell lines

can be searched in which both NFAT5 and SIRT1 is active at earlier time points when NFκB pathway is starting to be active. This search may enlighten their effect on NFκB pathway regulation at the initial hours upon hyperosmotic stress. Thus, it can be plainer whether their role on NFκB pathway regulation is typical or time dependent upon hyperosmotic stress.

Moreoever, this research can be broadened by focusing on the interaction studies. As it is mentioned before due to their parallel expression profiles under hyperosmotic stress and EX-527/Resveratrol pretreatments SIRT1 and NFAT5 may have an interaction or a common regulatory purpose. This possible interaction can be examined in future whether they have an indirect or direct interaction under hyperosmotic stress for a regulatory purpose. The other option for the future search of interaction can focus on p65 and NFAT5 interaction at nuclear on target gene site such as *IκB α* promoter by implementing CoIP, ChIP and EMSA in U937 cells and in other possible cell lines under hyperosmotic stress. Therefore, the possible interaction can be elaborated on and whether NFAT5 has a tuning role on p65 or a major role on the target gene regulation can be clearer. In addition, SIRT1 and p65 interaction may be examined in future studies. A deacetylating fuction of SIRT1 is estimated to inhibit p65 nuclear translocation and its regulatory function. This possible inhibiting mechanisim can be searched in molecular level through CoIP implementation for acetylated p65 and SIRT1.

All in all, this study indicated that modulation of SIRT1 via a chemical inhibitor or an activator, regulates p65 and its transcriptional target, IκB α under hyperosmotic stress. Under this SIRT1 modulation model of hyperosmotic stress, p65 dependent IκB α transcription may be independent of NFAT5 because physical abundance of NFAT5 is not parallel to p65.

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5. Appendix

6.1 U937 Cell Line Specification Sheet and Cell Culture Protocol

A. U937 Cell Line Specification

U-937 (ATCC® CRL-1593.2™)

Organism: *Homo sapiens*, human / Tissue: Pleura/pleural effusion, lymphocyte, Myeloid / Disease: histiocytic lymphoma

GENERAL INFORMATION | CHARACTERISTICS | CULTURE METHOD | SPECIFICATIONS | HISTORY | DOCUMENTATION

SHARE | EMAIL | PRINT

Permits and Restrictions	View Restrictions
Organism	*Homo sapiens*, human
Tissue	Pleura/pleural effusion, lymphocyte, Myeloid
Product Format	frozen
Morphology	monocyte
Culture Properties	suspension
Biosafety Level	1
Disease	histiocytic lymphoma
Age	37 years
Gender	male
Ethnicity	Caucasian
Applications	This cell line is suitable as a transfection host
Storage Conditions	liquid nitrogen vapor phase

Derivation	The U-937 cell line was derived by Sundstrom and Nilsson in 1974 from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma.
Clinical Data	37 years Caucasian male The U-937 cell line was derived by Sundstrom and Nilsson in 1974 from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma.
Receptor Expression	complement (C3)
Genes Expressed	lysozyme; beta-2-microglobulin (beta 2 microglobulin); tumor necrosis factor (TNF), also known as tumor necrosis factor alpha (TNF-alpha, TNF alpha), after stimulation with phorbol myristic acid (PMA)
Comments	Studies since 1979 have shown that U-937 cells can be induced to terminal monocytic differentiation by supernatants from human mixed lymphocyte cultures. The cells are negative for immunoglobulin production and Epstein-Barr virus expression. The cells express the Fas antigen, and are sensitive to TNF and anti-Fas antibodies. In 1994, PCR and cytogenetic analyses showed that a number of stocks of U-937 were contaminated with the human myeloid leukemia cell line, K-562. In the earliest stocks available, the level of contamination was 0.6%. Distribution was discontinued in March 1994, except if required for patent purposes. Anyone who wishes to receive a sample of this original material should contact the Head of the ATCC Patent Depository. A stock of CRL-1593 found to be free of K-562 was propagated continuously for 8 weeks and tested weekly by PCR. Distribution and seed stocks give DNA profiles characteristic of U-937 only. Such preparations are now offered as authentic U-937 (ATCC CRL-1593.2) and are believed to be free of second subpopulations.

B. U937 Cell Culture Protocol

Note: *Below is the suggested cell culture protocol for U937 cell line. For this research the treatments of U937 cells are done in serum free RPMI not a complete medium.*

Complete Growth Medium	The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Subculturing	Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 to 2 X 10^5 viable cells/mL. **Interval:** Maintain cell density between 1 X 10^5 and 2 X 10^6 viable cells/mL. **Medium Renewal:** Add fresh medium every 3 to 4 days (depending on cell density)
Cryopreservation	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Culture Conditions	**Atmosphere:** air, 95%; carbon dioxide (CO_2), 5% **Temperature:** 37°C

6.2 Specification Sheets of Chemicals

A. EX-527

Specification Sheet

SIGMA-ALDRICH

Product Name	EX-527, ≥98% (HPLC)
Product Number	E7034
Product Brand	SIGMA
CAS Number	49843-98-3
Molecular Weight	248.71

TEST	**SPECIFICATION**
Appearance (Color)	White to Off-White
Appearance (Form)	Powder
Solubility (Color)	Colorless to Very Light Yellow
Solubility (Turbidity)	Clear
	>=30 mg/ml, DMSO
Elemental Composition	Pass
	C13H13ClN2O
Purity (HPLC)	≥98.00 %
Proton NMR spectrum	Conforms to Structure
	Supporting Analytical Data DMSO

B. Resveratrol

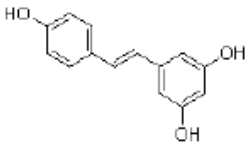
SIGMA-ALDRICH® sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Product Specification

Product Name:
Resveratrol - ≥99% (GC)

Product Number:	**R5010**
CAS Number:	501-36-0
MDL:	MFCD00133799
Formula:	C14H12O3
Formula Weight:	228.24 g/mol
Storage Temperature:	-20 °C



TEST	Specification
Appearance (Color)	White to Off-White
Appearance (Form)	Powder
Solubility (Color)	Faint Yellow
Solubility (Turbidity)	Clear
50 mg/ml, Acetone	
Infrared spectrum	Conforms to Structure
Purity (GC)	≥ 99 %
Purity (TLC)	≥ 99 %
Carbon	73.0 - 74.3 %

6.3 Annexin V FITC-FACS Protocol

- Centrifuge cells (500.000cells/ul / 250.000 cells/ul) with 300 g in FACS tubes.
- Wash pellets with 1ml-500ul cold PBS.
- Vortex slowly
- Centrifuge again.
- Add cold annexin binding buffer cocktail (98ul buffer + 2ul annexin) and resuspend.
- *Note: If there is a no dye group for negative control, do not add annexin.*
- Incubate for 15-20 minutes in dark.
- Add 300 ul annexin binding buffer for dilution and unbounding.
- Adjust FACS voltages to : FAC 132 V, SSC 418V, FITC 260V
- Run and analysis on flow cytometry, follow strictly the FACS-CANTO manual.

6.4 Annexin V Allexis Data Sheet

PRODUCT DATA SHEET

Annexin V (human), (recombinant) (FITC conjugate)

ALX-209-250

Product Number/Sizes

ALX-209-250-T300	300 tests
ALX-209-250-T100	100 tests
ALX-209-250-T020	20 tests

FITC-labelled recombinant human annexin V shows bright green fluorescence (Ex(max): 488nm; Em(max): 530nm).

Product Specifications

MW:	~35.8kDa.
SOURCE:	Produced in *E. coli*.
UNIPROT ID:	P08758
QUANTITY:	20 tests/100µl; 100 tests/500µl; 300 tests/1'500µl.
FORMULATION:	Liquid. In 50mM TRIS, pH 7.4, containing 100mM sodium chloride, 1% BSA and 0.02% sodium azide.
PURITY:	≥98% (SDS-PAGE, HPLC)
SPECIFICITY:	Binds to phosphatidylserine (PS).
BIOLOGICAL ACTIVITY:	Exhibits anti-phospholipase activity.
APPLICATION:	Detection of apoptotic cells by flow cytometry.
LONG TERM STORAGE:	+4°C
HANDLING:	After opening, prepare aliquots and store at -20°C.
PROTOCOL:	**Flow Cytometry Protocol:** - Prepare binding buffer (10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$; filtered through 0.2µm pore filter). - Brief centrifugation of annexin V-FITC before use is recommended - Wash cells in PBS by gentle shaking or pipetting up and down. - Resuspend cells in binding buffer; adjust cell density to 2-5x10^6/ml - Take 195µl cell suspension and add 5µl annexin V-FITC - Mix and incubate 10 min. in the dark at room temperature - Wash cells 1x with PBS and resuspend in 190µl binding buffer - Add 10µl of 20µg/ml propidium iodide stock solution (end concentration 1µg/ml) - FACS analysis

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6.5 *WST-1 ROCHE Data Sheet*

For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Roche

Cell Proliferation Reagent WST-1

A colorimetric assay (WST-1 based) for the nonradioactive quantification of cell proliferation, cell viability, and cytotoxicity
A non-radioactive alternative to the [^{3}H]-thymidine incorporation assay

Cat. No. 11 644 807 001
25 ml for 2500 tests

Version July 2005
Store the kit at −15 to −25°C

1. What this Product Does

Number of Reactions
For 2500 tests

Contents
The Cell Proliferation Reagent WST-1 is a clear, slightly red, ready-to-use solution, containing WST-1 and an electron coupling reagent, diluted in phosphate buffered saline, sterile.

Storage and Stability
The unopened reagent is stable when stored at −15 to −25°C, protected from light, through the control date printed on the label.

⚠ Shipped on dry ice.

If precipitates or turbidity are observed upon thawing, warm the solution to 37°C for 2 – 10 min and agitate to dissolve the precipitates. Centrifugation is not recommended because the working concentration would decrease. After dissolving the WST-1 reagent can be used without any limitations. Please store as follows:

- Once thawed, store at +2 to +8°C, protected from light, for several weeks.
- For longer storage it is recommended to store in aliquots at −15 to −25°C.

Additional Equipment and Reagents Required
Additional reagents and equipment required to perform reactions include:

- Incubator (37°C)
- Centrifuge
- Microplate (ELISA) reader with a filter for a wavelength between 420 – 480 nm (if a reference wavelength is to be subtracted, a filter above 600 nm is recommended)
- Microscope
- Hemacytometer
- Multichannel pipettor (10, 50, 100 µl)
- Sterile pipette tips
- 96-well microplates

For the Cell proliferation assay:

- Culture medium, *e.g.*, RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM Na-pyruvate, 1× non-essential amino acids, and 50 µM 2-mercaptoethanol.
- Optionally, add Penicillin/Streptomycin* or Gentamicin*
- Human IL-2 (10,000 U/ml; 5 µg/ml)*, sterile

For the Cytotoxicity assay (TNF-α):

- Culture medium, *e.g.*, RPMI 1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine, and actinomycin C1 (actinomycin D), 1 µg/ml.
- Optionally, add Penicillin/Streptomycin* or Gentamicin*.
- Human TNF-α (10 µg/ml)*, sterile.

Application
The Cell Proliferation Reagent WST-1 is designed to be used for the non-radioactive, spectrophotometric quantification of cell proliferation, growth, viability, and chemosensitivity in cell populations using the 96-well-plate format. It can be used *e.g.*, for:

- The measurement of cell proliferation in response to growth factors, cytokines, mitogens and nutrients (see figure 1).
- The assessment of growth inhibitory antibodies and physiological mediators (see figure 2).
- Analysis of cytotoxic and cytostatic compounds, such as anti-cancer drugs and other pharmaceutical compounds.

2. How To Use this Product

2.1 Before You Begin

Working concentration
It is recommended to add 10 µl/well Cell Proliferation Reagent WST-1 to the cells already cultured in 100 µl/well (1:10 final dilution).

Using the 100 µl/well cell culture volume, one vial will be sufficient to perform 2500 tests (25 microplates).

⚠ If the cells are cultured in 200 µl/well, add 20 µl/well Cell Proliferation Reagent WST-1.

Determination of optimal incubation periods
The appropriate incubation time after the addition of the Cell Proliferation Reagent WST-1 depends on the individual experimental setup (*e.g.*, cell type and cell concentration used). Therefore, it is recommended to measure the absorption repeatedly at different points in time after the addition of the Cell Proliferation Reagent WST-1 (*e.g.*, 0.5, 1, 2, and 4 h) in a preliminary experiment. This allows you to determine the optimal incubation period for the particular experimental setup used (see figure 5b).

Incubation requirements for high sensitivity
If high sensitivity is required, incubate the cells in the presence of Cell Proliferation Reagent WST-1 for longer periods of time (Figure 5b: half maximum absorbance after incubation with Cell Proliferation Reagent WST-1 for 0.5 h with 2×10^4 cells/well; for 4 h with 0.7×10^4 cells/well).

Initial incubation
If for the initial incubation of the cells an increased volume of culture medium is required, increase the amount of Cell Proliferation Reagent WST-1 correspondingly (*e.g.*, add 20 µl/well Cell Proliferation Reagent WST-1 if cells are cultured in 200 µl/well culture medium).

Control (blank)
Add the same volume of culture medium and Cell Proliferation Reagent WST-1 as used in the experiment into one well (*e.g.*, 100 µl culture medium plus 10 µl Cell Proliferation Reagent WST-1). Use this background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader.

Assay principle

In recent years different tetrazolium salts like MTT (2 – 4), XTT (5 – 7) and MTS (8) have been described which can be used for the measurement of cell proliferation and viability.

① The tetrazolium salts are cleaved to formazan by cellular enzymes (10). An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture.

② Quantification of the formazan dye produced by metabolically active cells by a scanning multiwell spectrophotometer (ELISA reader).
The absorbance of the dye solution is measured at appropriate wavelengths.

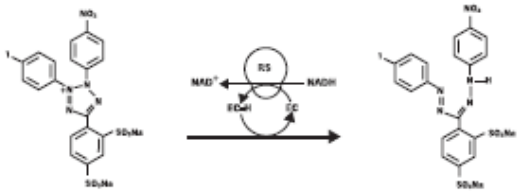

Figure 3: Cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan.
(EC = electron coupling reagent; RS = mitochondrial succinate-tetrazolium-reductase system).

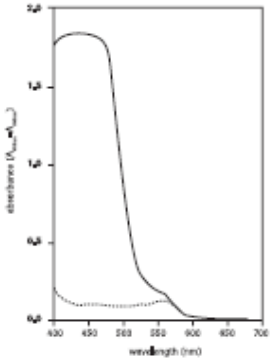

Figure 4: Absorbance spectra of Cell Proliferation Reagent WST-1 (.....) and the reaction product (formazan) (---) after cleavage by mitochondrial dehydrogenase activity.
The Cell Proliferation Reagent WST-1 was diluted 1:10 in a cell suspension in RPMI 1640 containing 10% FCS.

Advantages of WST-1 compared to other cellproliferation agents

The new Cell Proliferation Reagent WST-1 (11–19) (see fig. 5) has several advantages compared to the above mentioned compounds:

- In contrast to MTT which is cleaved to water-insoluble formazan crystal and therefore has to be solubilized after cleavage, WST-1 yields water-soluble cleavage products like XTT and MTS which can be measured without an additional solubilization step.
- In contrast to XTT and MTS, WST-1 is more stable. Therefore, WST-1 can be used as a ready-to-use solution and can be stored at +2 to +8°C for several weeks without significant degradation.
- WST-1 has a wider linear range and shows accelerated color development compared to XTT (see fig. 5a and fig. 5b).

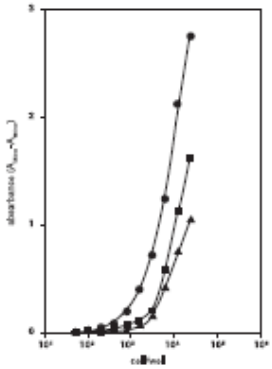

Figure 5a: Comparison of MTT (▲), XTT (■) and Cell Proliferation Reagent WST-1 (●).
P815 cells at cell concentrations indicated in the figure were preincubated for 20 h before the addition of the various tetrazolium salts. After 4 h substrate reaction the absorbance was determined at the respective wavelength with an ELISA reader.

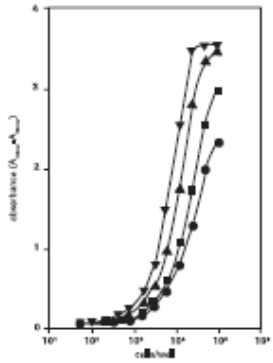

Figure 5b: Kinetics of the metabolism of the Cell Proliferation Reagent WST-1. A549 cells were cultured for 20 h at cell concentrations indicated in the figure, before the addition of Cell Proliferation Reagent WST-1. After 0.5 h (●), 1h (■), 2h (▲) and 4 h (▼) incubation periods the absorbance was determined by an ELISA reader.

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Background absorbance

Slight spontaneous absorbance occurs if Cell Proliferation Reagent WST-1 is added to culture medium in the absence of cells. This background absorbance depends on the culture medium, the incubation time and exposure to light. Typical background absorbance after 2 h is between 0.1 – 0.2 absorbance units.

2.2 General protocol

The incubation period and cell density of the culture depends on the particular experimental conditions and on the cell line used.

⚠ For most experimental setups, a cell concentration between 0.1 and 5×10^4/well and an incubation time of 24 to 96 h is appropriate.

1. Culture cells in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium in a humidifie atmosphere (*e.g.*, 37°C, 5% CO_2).
2. Add 10 µl/well Cell Proliferation Reagent WST-1.
3. Incubate the cells for 0.5 to 4 h in a humidified atmosphere (*e.g.*, 37°C, 5% CO_2).
4. Shake thoroughly for 1 min on a shaker.
5. Measure the absorbance of the samples against a background control as blank using a microplate (ELISA) reader at 420 – 480 nm. The reference wavelength >600 nm.

2.3 Cell proliferation assay

Determination of the activity of human interleukin-2 (IL-2) on the mouse T cell line CTLL-2 (see figure 1).

1. Seed CTLL-2 cells at a concentration of 4×10^3 cells/well in 100 µl culture medium containing various amounts of IL-2 (final concentration *e.g.*, 0.005–25 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
2. Incubate cells for 48 h at 37°C and 5% CO_2.
3. Add 10 µl/well Cell Proliferation Reagent WST-1 and incubate for 4 h at 37°C and 5% CO_2.
4. Shake thoroughly for 1 min on a shaker.
5. Measure the absorbance of the samples against a background control as blank using a microplate (ELISA) reader. The wavelength for measuring the absorbance of the formazan product is between 420 – 480 nm (max. absorption at about 440 nm) according to the filters available for the ELISA reader (see figure 4). The reference wavelength should be more than 600 nm.

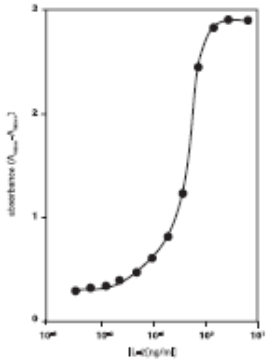

Figure 1: Measurement of proliferation of CTLL-2 cells in response to human IL-2.

2.4 Cytotoxicity assay (TNF-α)

Procedure Determination of the cytotoxic effect of human tumor necrosis factor-α (TNF-α) on the mouse fibrosarcoma cell line WEHI-164 (see figure 2).

1. Preincubate WEHI-164 cells at a concentration of 1×10^6 cells/ml in culture medium with actinomycin C1, 1 µg/ml for 3 h at 37°C and 5% CO_2.
2. Seed cells at a concentration of 5×10^4 cells/well in 100 µl culture medium containing actinomycin C1 (1 µg/ml) and various amounts of TNF-α (final concentration *e.g.*, 0.001 – 0.5 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
3. Incubate cell cultures for 24 h at 37°C and 5% CO_2.
4. Add 10 µl Cell Proliferation Reagent WST-1 and incubate for 4 h at 37°C and 5% CO_2.
5. Shake thoroughly for 1 min on a shaker.
6. Measure the absorbance of the samples against a background control as blank using a microplate (ELISA) reader. The wavelength for measuring the absorbance of the formazan product is between 420 – 480 nm (max. absorption at about 440 nm) according to the filters available for the ELISA reader (see figure 2). The reference wavelength should be more than 600 nm.

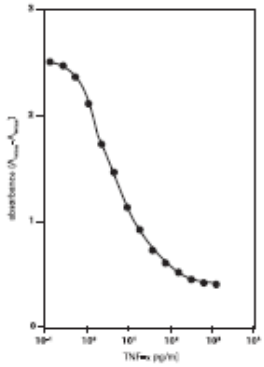

Figure 2: Determination of the cytotoxic activity of human TNF-α on WEHI-164 cells.

3. Additional Information on this Product

Product Description

The measurement of cell proliferation and cell viability has become a key technology in the life sciences. The need for sensitive, reliable, fast and easy methods has led to the development of several standard assays (1).

These include the determination of DNA synthesis by measuring the amount of radioactive labeled nucleosides like [^{3}H]-thymidine incorporated in nucleic acid.

Alternatively, the incorporation of 5-bromo-2'-deoxyuridine (BrdU)* in place of thymidine is used to monitor DNA synthesis and cell proliferation in immunohisto- and immunocytochemistry, in cell ELISA and flow cytometry analysis (kits and reagents for these applications are available from Roche Diagnostic GmbH).

Proliferation assays have become available for analyzing the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. This technique requires neither washing nor harvesting of cells and the complete assay from the onset of the microculture to data analysis by ELISA reader is performed in the same microplate. Together with on-line computer processing consisting of data collection, calculation and report generation, the microtiter tetrazolium assay allows rapid, convenient and automated handling of high number of samples and is thus a viable alternative to the above mentioned methods.

4. Supplementary Information

4.1 Conventions

Text Conventions
To make information consistent and memorable, the following text conventions are used in this package insert:

Text Convention	Use
Numbered stages labeled ①, ②, etc.	Stages in a process that usually occur in the order listed.
Numbered Instructions labeled ❶, ❷, etc.	Steps in a procedure that must be performed in the order listed
Asterisk *	Denotes a product available from Roche Applied Science

Symbols
In this package insert the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

4.2 Changes to previous version

New layout

4.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page www.roche-applied-science.com and our Apoptosis and Cell Proliferation Special Interest Site:
http://www.roche-applied-science.com/sispecial/apoptosis/

	Product	Pack Size	Cat. No.
Kits	BrdU labeling of proliferating cells *in situ* assay		
	BrdU Labeling and Detection Kit I	1 kit (1000 test)	11 296 736 001
	BrdU Labeling and Detection Kit II	1 kit (1000 test)	11 299 964 001
	BrdU Labeling and Detection Kit III	1 kit (1000 test)	11 444 611 001
	In Situ Cell Proliferation Kit, FLUOS	1 kit (100 test)	11 810 740 001
	ELISA		
	Cell Proliferation ELISA, BrdU (colorimetric)	1 kit (1000 test)	11 647 229 001
	Cell Proliferation ELISA, BrdU (chemiluminescent)	1 kit (1000 test)	11 669 915 001
	Measurement of metabolic activity: Quantification in microplates		
	Cell Proliferation Kit I (MTT)	1 kit (2500 test)	11 465 007 001
	Cell Proliferation Kit II (XTT)	1 kit (2500 test)	11 465 015 001
Single Reagents	Human TNF-a recombinant (*E.coli*)	10 µg 1 000 000 U	11 371 843 001
	Human TNF-a recombinant (yeast)	10 µg 1 000 000 U	11 088 939 001
	Interleukin-2, human (h-IL-2) from human lymphocytes	10 000 U (5 µg; 50 ml) 10 000 U (5 µg; 1 ml) 5× 10 000 U (5 × 5 µg; 5 × 50 ml)	10 663 581 001 11 204 700 001 11 680 323 001
	Interleukin-2, human (h-IL-2) recombinant (*E.coli*)	10 000 U (5 µg; 50 ml) 10 000 U (5 µg; 1 ml) 50 000 U (25 µg; 5 ml) 5 × 50 000 U (5 × 25 µg; 5 × 5 ml)	10 799 068 001 11 011 456 001 11 147 528 001 11 722 778 001
	Antibiotics for cell culture		
	Penicillin/Streptomycin	for 20 ml (500x)	11 074 440 001
	Gentamicin	20 ml (500x) sterile	11 059 467 001
	Single reagents for *in situ* assays and ELISA applications		
	Anti-BrdU formalin grade	50 µg (500 µl)	11 170 376 001
	Anti-BrdU-Fluorescein, formalin grade	50 µg (500 µl)	11 202 693 001
	Anti-BrdU-Peroxidase, Fab fragments, formalin grade	15 U	11 585 860 001
	FixDenat	4 × 100 ml (2000 tests)	11 758 764 001

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Use the Product Search function to find Pack Inserts and Material Safety Data Sheets.

Roche Diagnostics

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SANTA CRUZ BIOTECHNOLOGY, INC.

NFAT5 (H-300): sc-13035

BACKGROUND

Members of the NFAT (nuclear factor of activated T cells) family of transcription factors are related to NFκB/Rel proteins and form cooperative complexes with the AP-1 proteins, Fos and Jun, on DNA to regulate cytokine expression in T cells. NFAT proteins are widely expressed and alternatively modified to generate splice variants, and they are localized to both the cytosol (NFATc) and to the nucleus (NFATn). NFAT1, NFAT2, and NFAT4 are predominantly expressed in immune cells, and NFAT2 and NFAT3 are expressed at high levels in cardiac tissues. In addition to activating cytokine gene transcription, NFAT2 is also implicated in cardiac valve development, and NFAT3 is involved in cardiac hypertrophy. NFAT5 is detected in both immune and nonimmune cells and, like other NFAT proteins, contains a highly conserved Rel-like binding domain that mediates NFAT proteins associating with specific consensus sequences on DNA. NFAT proteins are activated by increases in intracellular calcium, which leads to the calmodulin-dependent phosphatase, calcineurin, dephosphorylating NFAT proteins. This activating event induces a conformational change in the protein structure that exposes the nuclear localization signal and facilitates the translocation of NFAT proteins from the cytosol into the nucleus.

REFERENCES

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CHROMOSOMAL LOCATION

Genetic locus: NFAT5 (human) mapping to 16q22.1.

SOURCE

NFAT5 (H-300) is a rabbit polyclonal antibody raised against amino acids 67-300 of NFAT5 of human origin.

PRODUCT

Each vial contains 200 µg IgG in 1.0 ml of PBS with < 0.1% sodium azide and 0.1% gelatin.

Available as TransCruz reagent for Gel Supershift and ChIP applications, sc-13035 X, 200 µg/0.1 ml.

STORAGE

Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment. Non-hazardous. No MSDS required.

RESEARCH USE

For research use only, not for use in diagnostic procedures.

APPLICATIONS

NFAT5 (H-300) is recommended for detection of NFAT5a, NFAT5b, NFAT5c, NFAT5z1 and NFAT5z2 of human origin by Western Blotting (starting dilution 1:200, dilution range 1:100-1:1000), immunoprecipitation [1-2 µg per 100-500 µg of total protein (1 ml of cell lysate)], immunofluorescence (starting dilution 1:50, dilution range 1:50-1:500) and solid phase ELISA (starting dilution 1:30, dilution range 1:30-1:3000).

NFAT5 (H-300) is also recommended for detection of NFAT5a, NFAT5b, NFAT5c, NFAT5z1 and NFAT5z2 in additional species, including equine, canine, bovine and porcine.

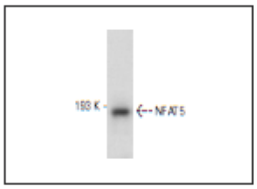
Suitable for use as control antibody for NFAT5 siRNA (h): sc-43968, NFAT5 shRNA Plasmid (h): sc-43968-SH and NFAT5 shRNA (h) Lentiviral Particles: sc-43968-V.

NFAT5 (H-300) X TransCruz antibody is recommended for Gel Supershift and ChIP applications.

Molecular Weight of NFAT5: 170 kDa.

Positive Controls: Jurkat nuclear extract: sc-2132, HEL 92.1.7 cell lysate: sc-2270 or HeLa whole cell lysate: sc-2200.

DATA



NFAT5 (H-300): sc-13035. Western blot analysis of NFAT5 expression in Jurkat nuclear extract.

SELECT PRODUCT CITATIONS

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Santa Cruz Biotechnology, Inc. 1.800.457.3801 831.457.3800 fax 831.457.3801 **Europe** +00800 4573 8000 49 6221 4503 0 **www.scbt.com**

#8469 Store at -20°C

SirT1 (1F3) Mouse mAb

✓100 µl
(10 western blots)

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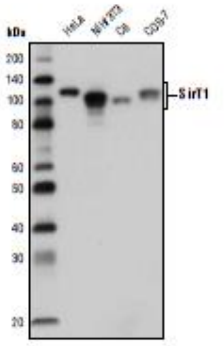
Entrez-Gene ID #23411
Swiss-Prot Acc. #Q96EB6

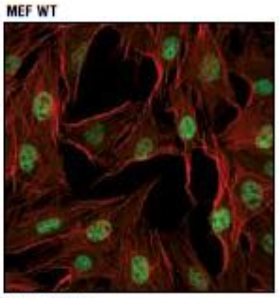
Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, IP, IF-IC	H, M, R, Mk	120 kDa	Mouse IgG1**
Endogenous			

Background: The Silent Information Regulator (SIR2) family of genes is a highly conserved group of genes that encode nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases, also known as class III histone deacetylases. The first discovered and best characterized of these genes is *Saccharomyces cerevisiae* SIR2, which is involved in silencing of mating type loci, telomere maintenance, DNA damage response, and cell aging (1). SirT1, the mammalian ortholog of Sir2, is a nuclear protein implicated in the regulation of many cellular processes, including apoptosis, cellular senescence, endocrine signaling, glucose homeostasis, aging, and longevity. Targets of SirT1 include acetylated p53 (2,3), p300 (4), Ku70 (5), forkhead (FoxO) transcription factors (5,6), PPARγ (7), and the PPARγ coactivator-1α (PGC-1α) protein (8). Deacetylation of p53 and FoxO transcription factors represses apoptosis and increases cell survival (2,3,5,6). Deacetylation of PPARγ and PGC-1α regulates the gluconeogenic/glycolytic pathways in the liver and fat mobilization in white adipocytes in response to fasting (7,8). SirT1 deacetylase activity is inhibited by nicotinamide and activated by resveratrol. In addition, SirT1 activity may be regulated by phosphorylation, since it is phosphorylated on Ser27 and Ser47 *in vivo*, however, the function of these phosphorylation sites has not yet been determined (9).

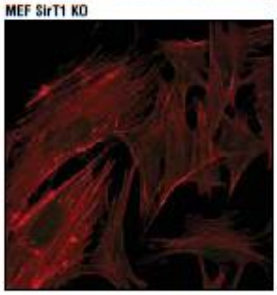


Western blot analysis of extracts from various cell lines using SirT1 (1F3) Mouse mAb

MEF WT



MEF SirT1 KO



Confocal immunofluorescent analysis of MEF WT (upper, positive) and MEF SirT1 KO (lower, negative) cells using SirT1 (1F3) Mouse mAb (green). Actin filaments were labeled with DY-554 phalloidin (red). Wild-type and knockout MEFs were a gift from Wenyi Wei at the Harvard Medical School.

Specificity/Sensitivity: SirT1 (1F3) Mouse mAb recognizes endogenous levels of total SirT1 protein.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a recombinant protein specific to the carboxy terminus of human SirT1 protein.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

***Species cross-reactivity is determined by western blot.**

****Anti-mouse secondary antibodies must be used to detect this antibody.**

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:100
Immunofluorescence (IF-IC)	1:100

For application specific protocols please see the web page for this product at www.cellsignal.com.

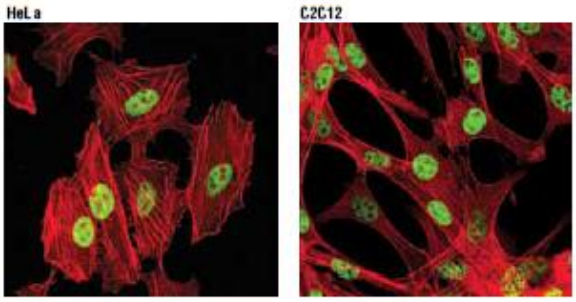
Please visit www.cellsignal.com for a complete listing of recommended companion products.

Background References:

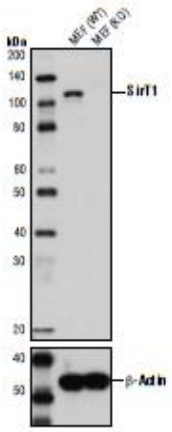
(1) Guarente, L. (1999) *Nat. Genet.* 23, 281-285.
(2) Vaziri, H. et al. (2001) *Cell* 107, 149-159.
(3) Luo, J. et al. (2001) *Cell* 107, 137-148.
(4) Bouras, T. et al. (2005) *J. Biol. Chem.* 280, 10264-10276.
(5) Brunet, A. et al. (2004) *Science* 303, 2011-2015.
(6) Motta, M.C. et al. (2004) *Cell* 116, 551-563.
(7) Picard, F. et al. (2004) *Nature* 429, 771-776.
(8) Rodgers, J.T. et al. (2005) *Nature* 434, 113-118.
(9) Beausoleil, S.A. et al. (2004) *Proc. Natl. Acad. Sci. USA* 101, 12130-12135.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Application Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to reactbased on 100% homology.



Confocal immunofluorescent analysis of HeLa (left) and C2C12 (right) cells using SirT1 (1F3) Mouse mAb (green). Actin filaments were labeled with DY-554 phalloidin (red).



Western blot analysis of extracts from SirT1 wild-type (WT) and knockout (KO) mouse embryo fibroblasts (MEF) using SirT1 (1F3) Mouse mAb (upper) and β-Actin (D6A8) Rabbit mAb #8457 (lower). Wild-type and knockout MEFs were a gift from Wenyi Wei at the Harvard Medical School.

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IκB-α Antibody

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This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

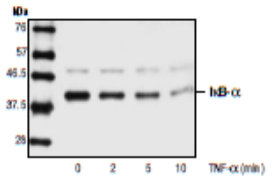
Entrez-Gene ID #4792
Swiss-Prot Acc. #P25963

Applications	Species Cross-Reactivity*	Molecular Wt.	Source
W, IP	H, M, R, Mk, B, Dg, Pg	39 kDa	Rabbit**
Endogenous			

Background: The NF-κB/Rel transcription factors are present in the cytosol in an inactive state complexed with the inhibitory IκB proteins (1-3). Activation occurs via phosphorylation of IκB-α at Ser32 and Ser36 followed by proteasome-mediated degradation, resulting in the release and nuclear translocation of active NF-κB (3-7). IκB-α phosphorylation and resulting Rel-dependent transcription are activated by a highly diverse group of extracellular signals including inflammatory cytokines, growth factors and chemokines. Kinases that phosphorylate IκB at these activating sites have been identified (8). Because phosphorylation of IκB-α at Ser32 is essential for release of active NF-κB, phosphorylation at this site is an excellent marker of NF-κB activation (1-3).

Specificity/Sensitivity: IκBα Antibody detects endogenous levels of total IκBα protein. Under some experimental conditions it binds preferentially to IκBα that is not phosphorylated at Ser32/Ser36.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic peptide surrounding Arg29 of human IκBα. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from HeLa cells treated with TNF-α for the indicated times, using IκB-α Antibody.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:50

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

Background References:

(1) Baeuerle, P.A. and Baltimore, D. (1988) *Science* 242, 540–546.
(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.
(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.
(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.
(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.
(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
(8) Karin, M. and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* 18, 621–663.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow Cytometry E-P—ELISA-Peptide

Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

#4764 Store at –20°C

NF-κB p65 (C22B4) Rabbit mAb

☑100 µl
(10 western blots)

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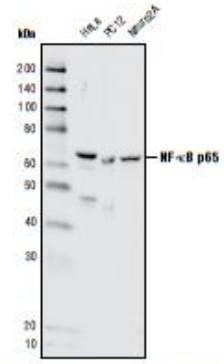
UniProt ID #Q04206

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, IF-IC, F	H, M, R, Mk, B, (Dg)	65 kDa	Rabbit IgG**
Endogenous			

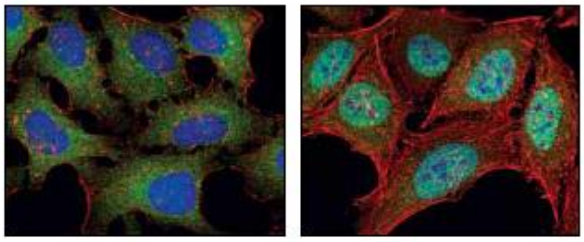
Background: Transcription factors of the nuclear factor κ B (NF-κB)/Rel family play a pivotal role in inflammatory and immune responses (1,2). There are five family members in mammals: RelA, c-Rel, RelB, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). Both p105 and p100 are proteolytically processed by the proteasome to produce p50 and p52, respectively. Rel proteins bind p50 and p52 to form dimeric complexes that bind DNA and regulate transcription. In unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB inhibitory proteins (3-5). NF-κB-activating agents can induce the phosphorylation of IκB proteins, targeting them for rapid degradation through the ubiquitin-proteasome pathway and releasing NF-κB to enter the nucleus where it regulates gene expression (6-8). NIK and IKKα (IKK1) regulate the phosphorylation and processing of NF-κB2 (p100) to produce p52, which translocates to the nucleus (9-11).

Specificity/Sensitivity: NF-κB p65 (C22B4) Rabbit mAb detects endogenous levels of total NF-κB p65 protein. This antibody may also cross-react with the p50 subunit.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues near the amino terminus of human NF-κB p65.



Western blot analysis of extracts from HeLa (human), PC-12 (rat) and Neuro2A (mouse) cell lines using NF-κB p65 (C22B4) Rabbit mAb.



Confocal immunofluorescent analysis of HeLa cells, untreated (left) or TNF-α-treated (#8902, 20ng/ml for 20 min, right), using NF-κB p65 (C22B4) Rabbit mAb (green). Actin filaments have been labeled with Alexa Fluor® 555 phalloidin (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunofluorescence (IF-IC)	1:100
Flow Cytometry	1:400

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween®20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide

Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

#2859 Store at –20°C

Phospho-IκB-α (Ser32) (14D4) Rabbit mAb

☐ Small 100 µl
(10 western blots)

☐ Large 300 µl
(30 western blots)

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Entrez-Gene ID #4792
UniProt Acc. #P25963

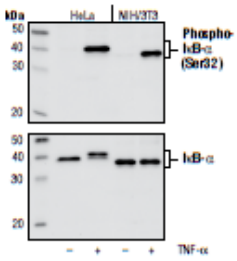
Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, IP Endogenous	H, M, R, Mk, (C, B, Dg, Pg, Guinea pig)	40 kDa	Rabbit IgG**

Background: The NF-κB/Rel transcription factors are present in the cytosol in an inactive state complexed with the inhibitory IκB proteins (1–3). Activation occurs via phosphorylation of IκB-α at Ser32 and Ser36 followed by proteasome-mediated degradation, resulting in the release and nuclear translocation of active NF-κB (3–7). IκB-α phosphorylation and resulting Rel-dependent transcription are activated by a highly diverse group of extracellular signals including inflammatory cytokines, growth factors and chemokines.

Kinases that phosphorylate IκB at these activating sites have been identified (8). Because phosphorylation of IκB-α at Ser32 is essential for release of active NF-κB, phosphorylation at this site is an excellent marker of NF-κB activation (1–3).

Specificity/Sensitivity: Phospho-IκB-α (Ser32) (14D4) Rabbit mAb detects endogenous levels of IκB-α only when phosphorylated at serine 32.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser32 of human IκBα.



Western blot analysis of extracts from HeLa and NIH/3T3 cells, untreated or treated with TNF-α for 5 minutes., using Phospho-IκB-α (Ser32) (14D4) Rabbit mAb (upper), or IκB-α (44D4) Rabbit mAb #4812 (lower).

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:100

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

Background References:

(1) Baeuerle, P.A. and Baltimore, D. (1988) *Science* 242, 540–546.

(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.

(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.

(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.

(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.

(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.

(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.

(8) Karin, M. and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* 18, 621–663.

U.S. Patent No. 5,675,063

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight.

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Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation F—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae All—all species expected Species enclosed in parentheses are predicted to react based on 100% sequence homology.

#2682 Store at –20°C

IKKα Antibody

✓100 µl
(10 western blots)

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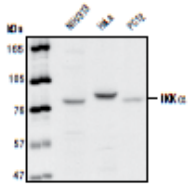
Entrez-Gene ID # 1147
UniProt ID # O15111

Applications	Species Cross-Reactivity*	Molecular Wt.	Source
W, IP Endogenous	H, M, R, Mk, (B)	85 kDa	Rabbit**

Background: The NFκB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IκB proteins (1–3). Most agents that activate NFκB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB (3–7). The key regulatory step in this pathway involves activation of a high molecular weight IκB kinase (IKK) complex, whose catalysis is generally carried out by three tightly associated IKK subunits. IKKα and IKKβ serve as the catalytic subunits of the kinase. IKKγ serves as the regulatory subunit (8–9). Activation of IKK depends on phosphorylation; serines 177 and 181 in the activation loop of IKKβ (176 and 180 in IKKα) are the specific sites whose phosphorylation causes conformational changes resulting in kinase activation (10–13).

Specificity/Sensitivity: IKKα Antibody detects endogenous levels of total IKKα protein. The antibody does not cross-react with IKKβ or IKKγ.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to the 20 amino-terminal residues of human IKKα. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from NIH/3T3, HeLa and PC12 cells, using IKKα Antibody.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C.
Do not aliquot the antibody.

*Species cross-reactivity is determined by western blot.

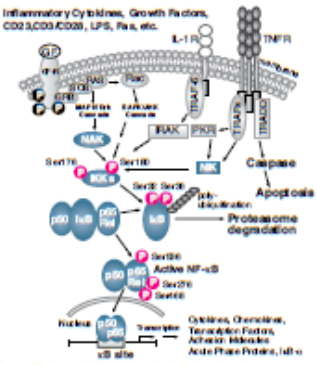
**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:50

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.



Background References:

(1) Baeuerle, P.A. et al. (1988) *Science* 242, 540–546.
(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.
(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.
(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.
(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.
(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
(8) Zandi, E. et al. (1997) *Cell* 91, 243–252.
(9) Karin, M. et al. (1999) *Oncogene* 18, 6867–6874.
(10) DiDonato, J.A. et al. (1997) *Nature* 388, 548–554.
(11) Mercurio, F. et al. (1997) *Science* 278, 860–866.
(12) Johnson, L.N. et al. (1996) *Cell* 85, 149–158.
(13) Delhase, M. et al. (1999) *Science* 284, 309–313.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween®20 at 4°C with gentle shaking, overnight.

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Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Py—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

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IKKβ Antibody

✓100 µl
(10 western blots)

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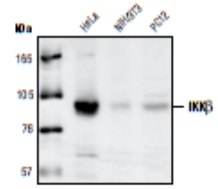
Entrez-Gene ID #3551
UniProt ID #O14920

Applications	Species Cross-Reactivity*	Molecular Wt.	Source
W Endogenous	H, Mk	87 kDa	Rabbit**

Background: The NFκB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IκB proteins (1–3). Most agents that activate NFκB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB (3–7). The key regulatory step in this pathway involves activation of a high molecular weight IκB kinase (IKK) complex, whose catalysis is generally carried out by three tightly associated IKK subunits. IKKα and IKKβ serve as the catalytic subunits of the kinase. IKKγ serves as the regulatory subunit (8–9). Activation of IKK depends on phosphorylation; serines 177 and 181 in the activation loop of IKKβ (176 and 180 in IKKα) are the specific sites whose phosphorylation causes conformational changes resulting in kinase activation (10–13).



Western blot analysis of extracts from HeLa, NIH/3T3 and PC12 cells, using IKKβ Antibody.

Specificity/Sensitivity: IKKβ Antibody detects endogenous levels of total IKKβ protein. The antibody does not cross-react with endogenous levels of IKKα or IKKγ.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues at the carboxy terminus of human IKKβ. Antibodies are purified by protein A and peptide affinity chromatography.

Background References:

(1) Baeuerle, P.A. et al. (1988) *Science* 242, 540–546.
(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.
(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.
(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.
(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.
(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
(8) Zandi, E. et al. (1997) *Cell* 91, 243–252.
(9) Karin, M. et al. (1999) *Oncogene* 18, 6867–6874.
(10) DiDonato, J.A. et al. (1997) *Nature* 388, 548–554.
(11) Mercurio, F. et al. (1997) *Science* 278, 860–866.
(12) Johnson, L.N. et al. (1996) *Cell* 85, 149–158.
(13) Delhase, M. et al. (1999) *Science* 284, 309–313.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C.
Do not aliquot the antibody.

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:
Western blotting 1:1000

For application specific protocols please see the web page for this product at www.cellsignal.com.

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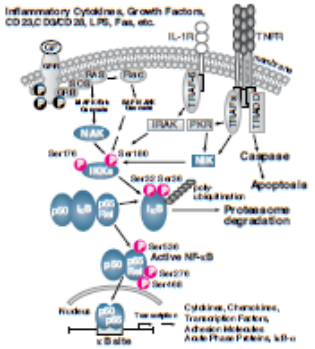

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IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween®20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

#2697 Store at –20°C

Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb

- Small 100 µl (10 western blots)
- Large 300 µl (30 western blots)

rev. 03/21/14

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Web ■ www.cellsignal.com

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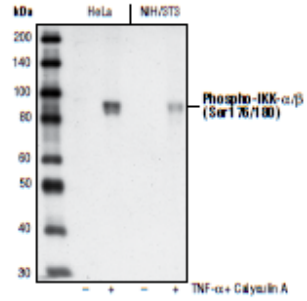
Entrez-Gene ID #1147
UniProt ID #015111

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, IHC-P, IHC-F Endogenous	H, M, R, Mk, (B)	85 kDa IKK-α 87 kDa IKK-β	Rabbit IgG**

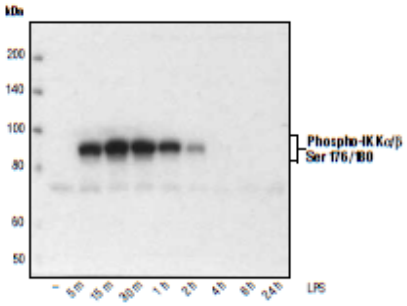
Background: The NFκB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IκB proteins (1–3). Most agents that activate NFκB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB (3–7). The key regulatory step in this pathway involves activation of a high molecular weight $I_{\kappa}B$ kinase (IKK) complex, whose catalysis is generally carried out by three tightly associated IKK subunits. IKKα and IKKβ serve as the catalytic subunits of the kinase. IKKγ serves as the regulatory subunit (8–9). Activation of IKK depends on phosphorylation; serines 177 and 181 in the activation loop of IKKβ (176 and 180 in IKKα) are the specific sites whose phosphorylation causes conformational changes resulting in kinase activation (10–13).

Specificity/Sensitivity: Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb detects IKKα only when phosphorylated at Ser176/180 and IKKβ only when phosphorylated at Ser177/181.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser176/180 of human IKKα.



Western blot analysis of extracts from TNF-α and calyculin A treated HeLa and NIH/3T3 cells, using Phospho-IKK-α/β (Ser176/180) (16A6) Rabbit mAb.



Western blot analysis of extracts from THP-1 cells, differentiated with TPA (#9905, 80 nM for 24h) and treated with 1 µg/ml LPS for the indicated times, using Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween®20 at 4°C with gentle shaking, overnight.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

***Species cross-reactivity is determined by western blot.**

****Anti-rabbit secondary antibodies must be used to detect this antibody.**

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunohistochemistry (Paraffin)	1:150†
Unmasking buffer:	Citrate
Antibody diluent:	TBST-5%NGS
Detection reagent:	SignalStain® Boost (HRP, Rabbit) #8114
Immunohistochemistry (Frozen)	1:150†
Unmasking buffer:	Citrate
Antibody diluent:	TBST-5%NGS
Detection reagent:	SignalStain® Boost (HRP, Rabbit) #8114

†Optimal IHC dilutions determined using SignalStain® Boost IHC Detection Reagent.

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

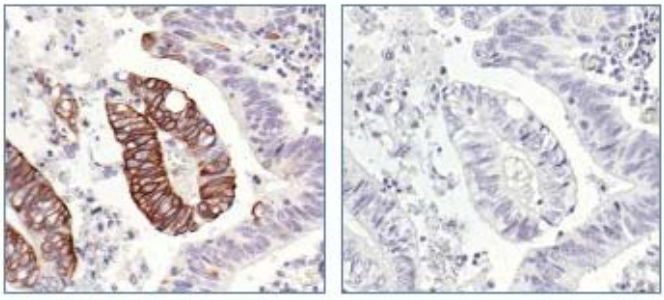
Background References:

(1) Baeuerle, P.A. et al. (1988) *Science* 242, 540–546.
(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.
(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.
(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.
(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.
(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
(8) Zandi, E. et al. (1997) *Cell* 91, 243–252.
(9) Karin, M. et al. (1999) *Oncogene* 18, 6867–6874.
(10) DiDonato, J.A. et al. (1997) *Nature* 388, 548–554.
(11) Mercurio, F. et al. (1997) *Science* 278, 860–866.
(12) Johnson, L.N. et al. (1996) *Cell* 85, 149–158.
(13) Delhase, M. et al. (1999) *Science* 284, 309–313.

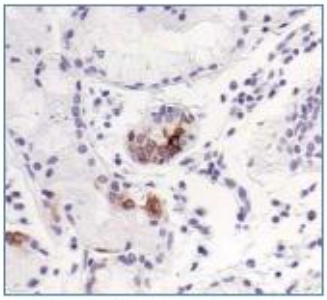
U.S. Patent No. 5,675,063

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide

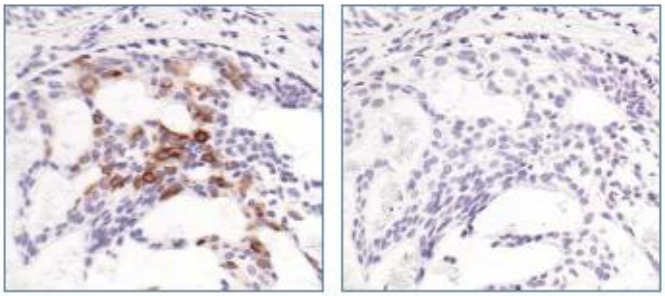
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.



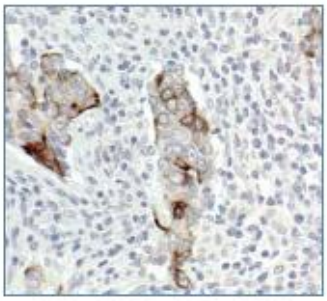
Immunohistochemical analysis of paraffin-embedded human colon carcinoma, untreated (left) or λ-phosphatase-treated (right), using Phospho-IKK-α/β (Ser176/180) (16A6) Rabbit mAb.



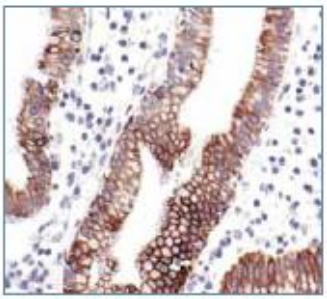
Immunohistochemical analysis of paraffin-embedded human lung (chronic bronchitis), using Phospho-IKK-α/β (Ser176/180) (16A6) Rabbit mAb.



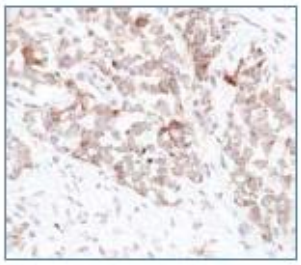
Immunohistochemical analysis of paraffin-embedded human breast carcinoma, using Phospho-IKK-α/β (Ser176/180) (16A6) Rabbit mAb in the presence of control peptide (left) or Phospho-IKK-α/β (Ser176/180) Blocking Peptide #1123 (right).



Immunohistochemical analysis of paraffin-embedded human colon carcinoma, showing cytoplasmic localization, using Phospho-IKK-γ (Ser376/180) (16A6) Rabbit mAb.



Immunohistochemical analysis of paraffin-embedded human gall bladder (chronic cholecystitis), using Phospho-IKK-α/β (Ser176/180) (16A6) Rabbit mAb.



Immunohistochemical analysis of frozen H1650 xenograft, showing cytoplasmic localization using Phospho-IKK-α/β (Ser176/180)(16A6) Rabbit mAb.

#2078 Store at –20°C

Phospho-IKKα (Ser176)/IKKβ (Ser177) (C84E11) Rabbit mAb

☐ Small 100 µl
(10 western blots)

☐ Petite 40 µl
(4 western blots)

rev 03/21/14

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Entrez-Gene ID #1147, 3551
UniProt ID #015111, 014920

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W Endogenous	H, M, (R, Mk, B)	85 (IKKα) 87 (IKKβ)	Rabbit IgG**

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:
Western blotting 1:1000

For product specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

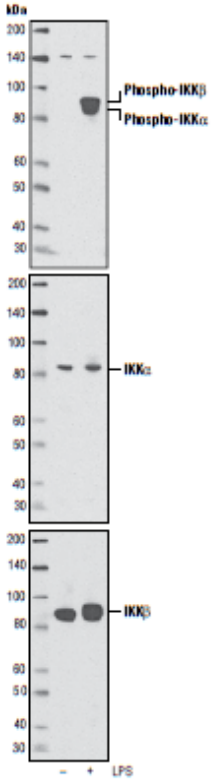
Background: The NF-κB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IκB proteins (1-3). Most agents that activate NF-κB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB (3-7). The key regulatory step in this pathway involves activation of a high molecular weight IκB kinase (IKK) complex, whose catalysis is generally carried out by three tightly associated IKK subunits. IKKα and IKKβ serve as the catalytic subunits of the kinase. IKKγ serves as the regulatory subunit (8,9). Activation of IKK depends on phosphorylation; Ser177 and Ser181 in the activation loop of IKKβ (176 and 180 in IKKα) are the specific sites whose phosphorylation causes conformational changes resulting in kinase activation (10-13).

Specificity/Sensitivity: Phospho-IKKα (Ser176)/IKKβ (Ser177) (C84E11) Rabbit mAb detects endogenous levels of IKKα and IKKβ only when phosphorylated at Ser176 and Ser177, respectively.

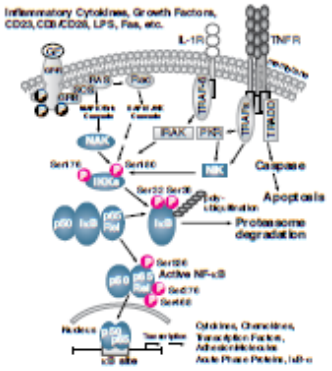
Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser177 of IKKβ.

Background References:

(1) Baeuerle, P.A. et al. (1988) *Science* 242, 540–546.
(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.
(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.
(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.
(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.
(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
(8) Zandi, E. et al. (1997) *Cell* 91, 243–252.
(9) Karin, M. et al. (1999) *Oncogene* 18, 6867–6874.
(10) DiDonato, J.A. et al. (1997) *Nature* 388, 548–554.
(11) Mercurio, F. et al. (1997) *Science* 278, 860–866.
(12) Johnson, L.N. et al. (1996) *Cell* 85, 149–158.
(13) Delhase, M. et al. (1999) *Science* 284, 309–313.



Western blot analysis of extracts from differentiated THP-1 cells, untreated or LPS-treated for 15 minutes, using Phospho-IKKα (Ser176)/IKKβ (Ser177) (C84E11) Rabbit mAb (upper), IKKα Antibody #2682 (middle) and IKKβ (2C8) Rabbit mAb #2370 (lower).



IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight.

U.S. Patent No. 5,675,063
Tween® 20 is a registered trademark of ICI Americas, Inc.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation F—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

#2032 Store at –20°C

Lamin A/C Antibody

✓100 µl
(10 western blots)

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rev 04/25/11

Entrez-Gene ID #4000
Swiss-Prot Acc. #P02545

Applications	Species Cross-Reactivity*	Molecular Wt.	Source
W, IHC-P	H, M, R, (B)	28 kDa, 70 kDa	Rabbit**
Endogenous			

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunohistochemistry (Paraffin)	1:100†
Unmasking buffer:	Citrate
Antibody diluent:	SignalStain® Antibody Diluent #8112
Detection reagent	SignalStain® Boost (HRP, Rabbit) #8114

†*Optimal IHC dilutions determined using SignalStain® Boost IHC Detection Reagent.*

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

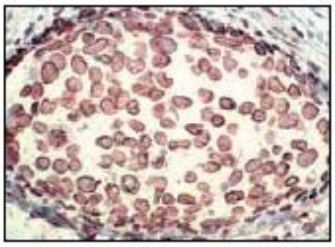
Background: Lamins are nuclear membrane structural components that are important in maintaining normal cell functions such as cell cycle control, DNA replication and chromatin organization (1-3). Lamin A/C is cleaved by caspase-6 and serves as a marker for caspase-6 activation. During apoptosis, Lamin A/C is specifically cleaved to a large (40-45 kDa) and a small (28 kDa) fragment (3,4). The cleavage of lamins results in nuclear disregulation and cell death (5,6).

Specificity/Sensitivity: Lamin A/C Antibody detects endogenous levels of total full length lamin A (and lamin C) (70 kDa), as well as the small fragment of lamin A (and lamin C) resulting from cleavage at aspartic acid 230 (28 kDa).

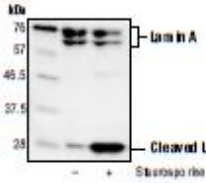
Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Asp230 of human lamin A. Antibodies are purified by protein A and peptide affinity chromatography.

Background References:

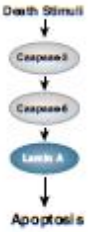
(1) Gruenbaum, Y. et al. (2000) *J. Struct. Biol.* 129, 313–323.
(2) Yabuki, M. et al. (1999) *Physiol. Chem. Phys. Med. NMR* 31, 77–84.
(3) Goldberg, M. et al. (1999) *Crit. Rev. Eukaryot. Gene Expr.* 9, 285–293.
(4) Orth, K. et al. (1996) *J. Biol. Chem.* 271, 16443–16446.
(5) Oberhammer, F.A. et al. (1994) *J. Cell Biol.* 125, 827–837.
(6) Rao, L. et al. (1996) *J. Cell Biol.* 135, 1441–1455.

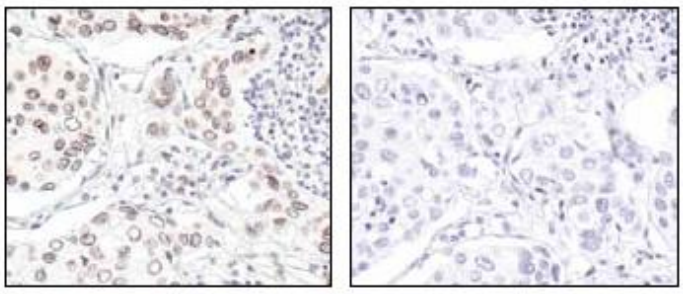


Immunohistochemical staining of paraffin-embedded human breast tumor, showing staining of the nuclear envelope, using Lamin A/C Antibody.



Western blot analysis of extracts from HeLa cells, untreated or staurosporine-treated (1 µM), using Lamin A/C Antibody.





Immunohistochemical analysis of paraffin-embedded human lung carcinoma, using Lamin A/C Antibody in the presence of control peptide (left) or antigen-specific peptide (right).

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

#4967 Store at -20°C

β-Actin Antibody

Small 100 µl
(10 western blots)

Large 300 µl
(30 western blots)

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rev 02/19/13

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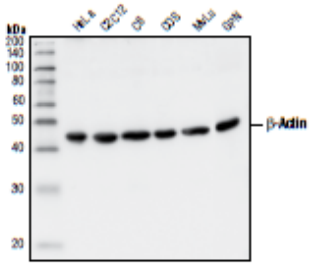
Entrez-Gene ID #60
Swiss-Prot Acc. #P60709

Applications	Species Cross-Reactivity*	Molecular Wt.	Source
W Endogenous	H, M, R, Mk, Mi, Hm, B, Dm, Z, Dg, (Pg, C, X, Hr)	45 kDa	Rabbit**

Background: Actin, a ubiquitous protein in eukaryotes, is the major component of the cytoskeleton. At least six isoforms are known in mammals. Nonmuscle β- and γ-actin, also known as cytoplasmic actin, are predominantly expressed in nonmuscle cells, controlling cell structure and motility (1). α-cardiac and α-skeletal actin are expressed in striated cardiac and skeletal muscles, respectively; two smooth muscle actins, α- and γ-actin, are found primarily in vascular smooth muscle and enteric smooth muscle, respectively. These actin isoforms regulate contractile potentials for muscle cells (1). Actin exists mainly as a fibrous polymer, F-actin. In response to cytoskeletal reorganizing signals during processes such as cytokinesis, endocytosis, or stress, cofilin promotes fragmentation and depolymerization of F-actin, resulting in an increase in the monomeric globular form, G-actin (2). The Arp2/3 complex stabilizes F-actin fragments and promotes formation of new actin filaments (2). It has been reported that actin is hyperphosphorylated in primary breast tumors (3). Cleavage of actin under apoptotic conditions has been observed *in vitro* and in cardiac and skeletal muscle (4-6). Actin cleavage by caspase-3 may accelerate ubiquitin/proteosome dependent muscle proteolysis (6).

Specificity/Sensitivity: β-Actin Antibody detects endogenous levels of β-actin. This antibody may cross-react with the γ-actin (cytoplasmic) isoform. It does not cross-react with α-skeletal, α-cardiac, α-vascular smooth, or γ-enteric smooth muscle isoforms.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to amino-terminal residues of human β-actin. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from HeLa, C2C12, C6, COS, MMu cells and guinea pig neutrophils (GPN) using β-Actin Antibody.

Background References:

(1) Herman, I.M. (1993) *Curr. Opin. Cell Biol.* 5, 48–55.
(2) Condeelis, J. (2001) *Trends Cell Biol.* 11, 288–293.
(3) Lim, Y.P. et al. (2004) *Clin. Cancer Res.* 10, 3980–3987.
(4) Kayalar, C. et al. (1996) *Proc. Natl. Acad. Sci. USA.* 93, 2234–2238.
(5) Communal, C. et al. (2002) *Proc. Natl. Acad. Sci. USA.* 99, 6252–6256.
(6) Du, J. et al. (2004) *J. Clin. Invest.* 113, 115–123.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:
Western Blotting 1:1000

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Hr All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Mini-PROTEAN® 3 Cell

Instruction Manual

Catalog Numbers
165-3301
165-3302

BIO-RAD

Table of Contents

Section 1
General Information

1.1 Introduction

The Mini-PROTEAN 3 cell runs both hand cast gels and Ready Gel precast gels interchangeably. The Mini-PROTEAN 3 system includes a casting stand and glass plates with permanently bonded gel spacers that simplify hand casting and eliminate leaking during casting. The cell can run one or two gels, and the mini tank is compatible with other Bio-Rad electrode modules for tank blotting, 2-D electrophoresis, and electro-elution.

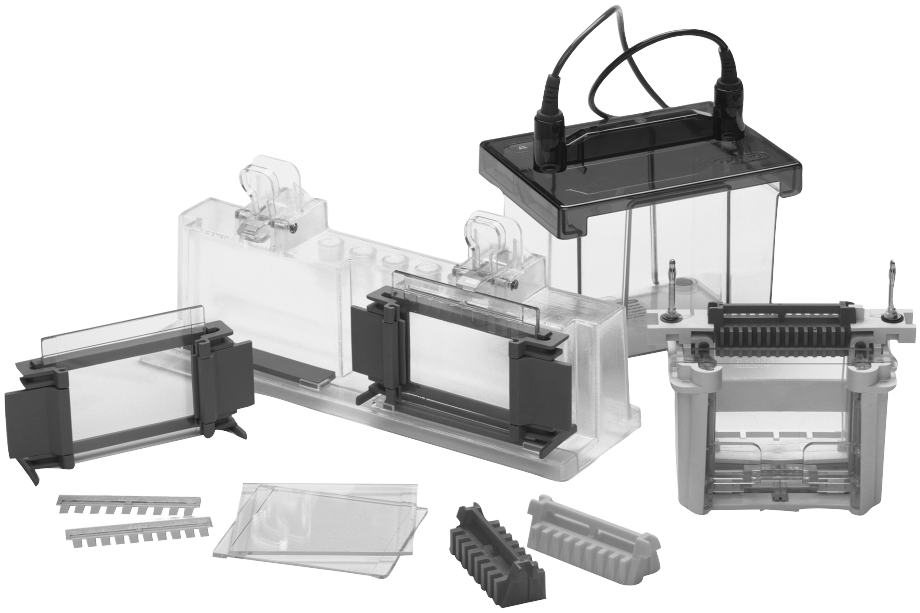


Fig. 1. Mini-PROTEAN 3 system components.

1.2 Components

To get the best performance from your Mini-PROTEAN 3 cell, familiarize yourself with the components by assembling and disassembling the cell before using it (refer to Figures 1 and 2).

Spacer Plate	The Spacer Plate is the taller glass plate with gel spacers permanently bonded. Spacer Plates are available in 0.5 mm, 0.75 mm, 1.0 mm, and 1.5 mm thicknesses, which are marked directly on each Spacer Plate.
Short Plate	The Short Plate is the shorter, flat glass plate that combines with the Spacer Plate to form the gel cassette sandwich.
Casting Frame	The Casting Frame, when placed on the benchtop, evenly aligns and secures the Spacer Plate and the Short Plate together to form the gel cassette sandwich prior to casting.
Gel Cassette Assembly	One Casting Frame, a Spacer Plate, and a Short Plate form one Gel Cassette Assembly.
Casting Stand	The Casting Stand secures the Gel Cassette Assembly during gel casting. It contains pressure levers that seal the Gel Cassette Assembly against the casting gaskets.
Gel Cassette Sandwich	A Spacer Plate and Short Plate with polymerized gel form a Gel Cassette Sandwich after casting.
Combs	A selection of molded combs is available.
Buffer Dam	The molded, one-piece buffer dam is used when running only one gel.
Electrode Assembly	The Electrode Assembly holds the Gel Cassette Sandwich. It houses the sealing gasket, the upper and lower electrodes and the connecting banana plugs. The anode (lower electrode) banana plug is identified with a red marker and the cathode (upper electrode) banana plug with a black marker.
Clamping Frame	The Clamping Frame holds the Electrode Assembly and Gel Cassette Sandwich in place. Its pressure plates and closure cams seal the Gel Cassette Sandwich against U-shaped gaskets on the Electrode Assembly to form the inner buffer chamber.
Inner Chamber	The Electrode Assembly, two Gel Cassette Sandwiches or one gel cassette sandwich and a buffer dam, and the Clamping Frame form the Inner Chamber.
Mini Tank and Lid	The Mini Tank and Lid combine to fully enclose the inner chamber during electrophoresis. The lid cannot be removed without disrupting the electrical circuit. The Mini Tank and Lid are also compatible with other Bio-Rad electrode modules for blotting, first dimension 2-D, and electro-elution.

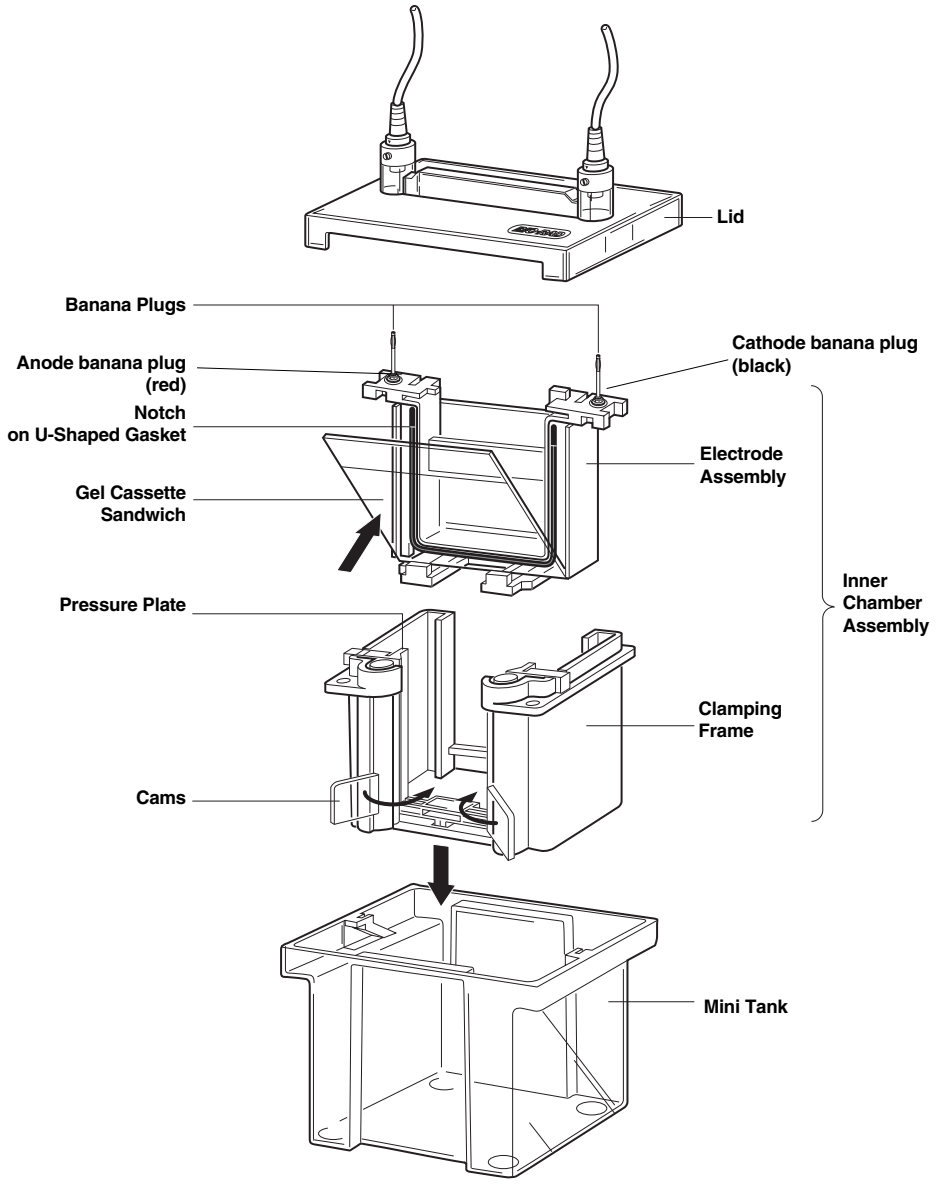


Fig. 2. Assembling the Mini-PROTEAN 3 cell.

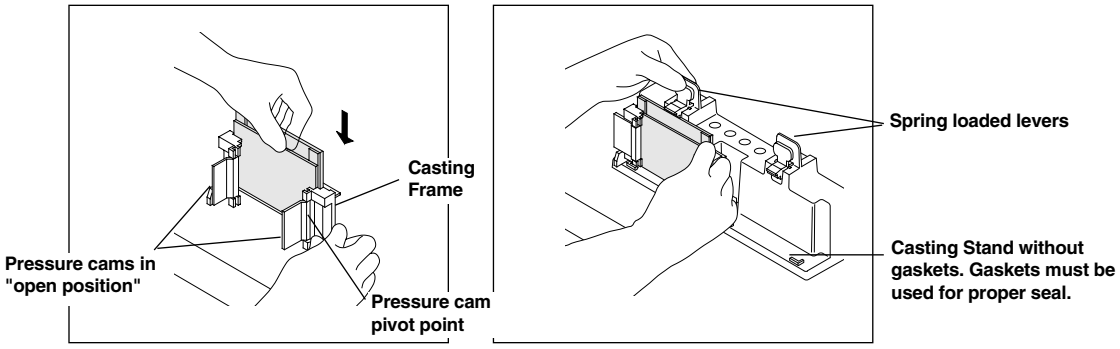


Fig. 3. Assembling the Mini-PROTEAN 3 Casting Frame and Casting Stand.

1.3 Specifications

Casting Stand*	Polycarbonate
Pin, Retaining Ring, and Spring	Stainless Steel
Casting Frames*	Polysulfone
Gray Gaskets	Silicone Rubber (gray)
Clamping Frame**	Glass-filled liquid crystal polymer (Vectra™)
Pressure Plate and Cams	Polycarbonate
Electrode Assembly	Glass-filled liquid crystal polymer
Electrodes	Platinum wire, 0.010 inches diameter
Gasket, electrode inner core	Silicone Rubber (green)
Mini Tank and Lid	Molded Polycarbonate
Sample Loading Guides†	Delrin™
Combs*	Polycarbonate

Maximum Sample Volume Per Well

# wells	Well width	0.5 mm	0.75 mm	1.0 mm	1.5 mm
5	12.7 mm	—	70 µl	105 µl	160 µl
9	5.08 mm	—	33 µl	44 µl	66 µl
10	5.08 mm	22 µl	33 µl	44 µl	66 µl
15	3.35 mm	13 µl	20 µl	26 µl	40 µl
IPG	76.2 mm	—	—	420 µl	730 µl
Prep/2-D					
Reference well	3.1 mm	—	13 µl	17 µl	30 µl
Sample well	71.7 mm	—	310 µl	400 µl	680 µl

Overall Size of cell	16 cm (L) x 12 cm (W) x 18 cm (H)
Gel Size	8 cm (W) x 7.3 cm (H)
Inner Plate	10.1 cm (W) x 7.3 cm (H)
Outer Plater	10.1 cm (W) x 8.3 cm (H)
Precast Gel Compatibility	Ready Gels
Voltage Limit	600 VDC and 15 watts
Shipping Weight	2.0 kg

1.4 Chemical Compatibility

Mini-PROTEAN 3 components are not compatible with acetone, ethanol, or butanol. Use of organic solvents voids all warranties. Call 1-800-4-BIORAD or your local Bio-Rad representative for technical information regarding additional chemical compatibility of the Mini-PROTEAN 3 cell with various laboratory reagents.

The Mini-PROTEAN 3 combs are not compatible with repeated exposure to 100% TEMED. Rubbing the combs with TEMED prior to casting will destroy the structural integrity of the combs over time.

* US patent No. 6,162,342

** US patent No. 5,632,877

† US patent No. 5,656,145

1.5 Safety

Power to the Mini-PROTEAN 3 cell is supplied by an external DC voltage power supply (not included). The output of this power supply must be isolated from external ground to insure that the DC voltage output floats with respect to ground. All Bio-Rad power supplies meet this important safety requirement. Regardless of the power supply used, the maximum specified operating parameters for the Mini-PROTEAN 3 cell are as follows:

- 600 VDC maximum voltage limit
- 15 watts maximum power limit
- 50 °C maximum ambient temperature limit

The current to the cell enters the unit through the lid assembly which provides a safety interlock to the user. The current to the cell is broken when the lid is removed. Always turn off the power supply before removing the lid. **Do not attempt to use the cell without the safety lid.**

Important: This Bio-Rad product is designed and certified to meet *EN61010-1 safety standards. Certified products are safe to use when operated in accordance with the instruction manual. This instrument should not be modified or altered in any way. Alteration of this instrument will

- Void the warranty
- Void the EN61010-1 certification, and
- Create a potential safety hazard.

Bio-Rad is not responsible for any injury or damage caused by use of this instrument for purposes other than those for which it is intended or by modifications of the instrument not performed by Bio-Rad or an authorized agent.

* EN61010-1 is an internationally accepted electrical safety standard for laboratory instruments.

Section 2
Set Up and Basic Operation

2.1 Gel Cassette Sandwich Preparation

Hand Cast Gels

1. Glass Cassette and Casting Stand Assembly

   Note: Ensure the casting stand, casting frames, and glass plates are clean and dry before setting up the casting stand assembly. During regular use, a powder residue may build up behind the pressure cams of the casting frame at the pivot point. This powder should be removed before each use.

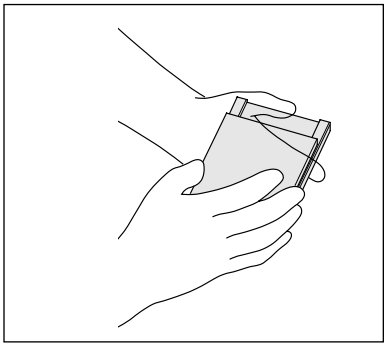
   a. Place the Casting Frame upright with the pressure cams in the open position and facing forward on a flat surface.
   b. Select a Spacer Plate of the desired gel thickness and place a Short Plate on top of it (see Figure 4a).
   c. Orient the Spacer Plate so that the labeling is "up". Slide the two glass plates into the Casting Frame, keeping the Short Plate facing the front of the frame (side with pressure cams) (see Figure 4b).

   Note: Ensure both plates are flush on a level surface and labeling on the Spacer Plate is oriented correctly. Leaking may occur if the plates are misaligned or oriented incorrectly.

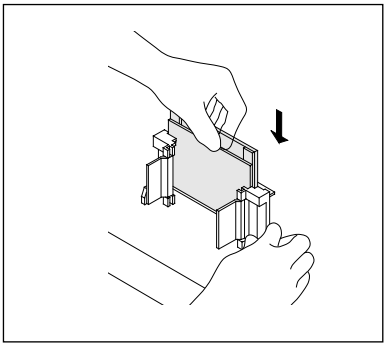
   d. When the glass plates are in place, engage the pressure cams to secure the glass cassette sandwich in the Casting Frame (see Figure 4c). Check that both plates are flush at the bottom.

e. Engage the spring loaded lever and place the gel cassette assembly on the gray casting stand gasket. Insure the horizontal ribs on the back of the Casting Frame are flush against the face of the Casting Stand and the glass plates are perpendicular to the level surface. The lever pushes the Spacer Place down against the gray rubber gasket (see Figure 4d).

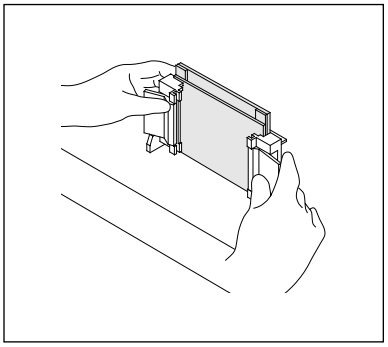
f. Repeat steps a–e for a second gel.



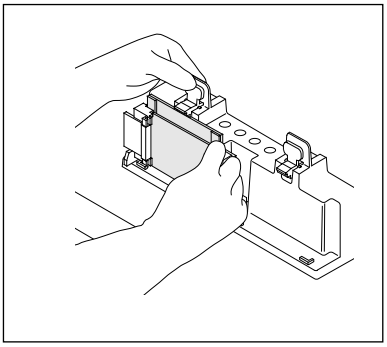
4a. Place a Short Plate on top of the Spacer Plate.



4b. Slide the two plates into the Casting Frame keeping the Short Plate facing front.



4c. Lock the pressure cams to secure the glass plates.



4d. Secure the Casting Frame in the Casting Stand by engaging the spring loaded lever.

Fig. 4. Assembling the Mini-PROTEAN 3 casting stand and frame.

2. Gel Casting

a. **Discontinuous Polyacrylamide Gels**

i. Place a comb completely into the assembled gel cassette. Mark the glass plate 1 cm below the comb teeth. This is the level to which the resolving gel is poured. Remove the comb.

ii. Prepare the resolving gel monomer solution by combining all reagents except APS and TEMED. (Refer to Section 4 for gel formulations.) Degas the solution under vacuum for at least 15 minutes. Do not use a sink water aspirator.

iii. Add APS and TEMED to the degassed monomer solution and pour to the mark using a glass or disposable plastic pipette. Pour the solution smoothly to prevent it from mixing with air.

iv. Immediately overlay the monomer solution with water or t-amyl alcohol.

Note: If water is used, add it slowly and evenly to prevent mixing. **Do not overlay w/butanol or isobutanol.**

v. Allow the gel to polymerize for 45 minutes to 1 hour. Rinse the gel surface completely with distilled water. Do not leave the alcohol overlay on the gel for more than 1 hour because it will dehydrate the top of the gel.

Note: At this point the resolving gel can be stored at room temperature overnight. Add 5 ml of a 1:4 dilution of 1.5 M Tris-HCl, pH 8.8 buffer (for Laemmli System) to the resolving gel to keep it hydrated. If using another buffer system, add 5 ml 1x resolving gel buffer to the resolving gel surface for storage.

vi. Prepare the stacking gel monomer solution. Combine all reagents except APS and TEMED. Degas under vacuum for at least 15 minutes.

vii. Before casting the stacking gel, insert a piece of filter paper to dry the area in between the glass plates above the resolving gel. Take care not to touch the surface of the gel.

viii. Add APS and TEMED to the degassed stacking gel monomer solution and pour the solution between the glass plates. Continue to pour until the top of the short plate is reached.

ix. Insert the desired comb between the spacers starting at the top of the Spacer Plate, making sure that the tabs at the ends of each comb are guided between the spacers. It is easiest to insert the combs starting at an angle and insert well 1 first, then 2, 3, and so on until the combs is completely inserted. Seat the comb in the gel cassette by aligning the comb ridge with the top of the Short Plate.

x. Allow the stacking gel to polymerize for 30–45 minutes.

xi. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.

xii. Rinse the Casting Frame(s) and Stand with distilled, deionized water after use.

b. **Continuous Polyacrylamide Gels**

i. Prepare the monomer solution by combining all reagents except the APS and the TEMED. Degas under vacuum for 15 minutes (Refer to Section 4 for gel formulations).

ii. Add APS and TEMED to the degassed monomer solution and pour the solution between the glass plates. Continue to pour until the top of the Short Plate is reached.

iii. Insert the desired comb between the spacers starting at the top of the Spacer Plate, making sure that the tabs at the ends of each comb are guided between the spacers. It is easiest to insert the combs starting at an angle and insert well 1 first, then 2, 3, and so on until the combs is completely inserted. Seat the comb in the gel cassette by aligning the comb ridge with the top of the Short Plate.

iv. Allow the gel to polymerize for 45 minutes to 1 hour.

v. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.

vi. Rinse the Casting Frame(s) and Stand with distilled, deionized water after use.

Ready Gel Precast Gels

1. Ready Gel Cassette Preparation

Note: The Mini-PROTEAN 3 cell is guaranteed for use only with Bio-Rad's Ready Gel precast gels.

a. Remove the Ready Gel from the storage pouch.

b. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.

c. Cut along the dotted line at the bottom of the Ready Gel Cassette with a razor blade.

d. Pull the clear tape at the bottom of the Ready Gel Cassette to expose the bottom edge of the gel.

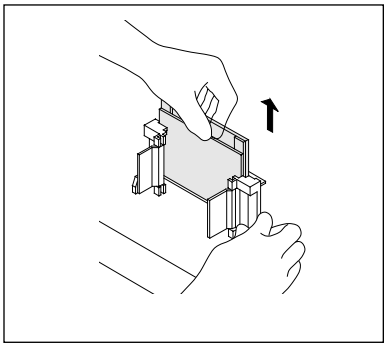
e. Repeat for second Ready Gel.

Note: If only one gel is to be run, use the mini cell buffer dam.

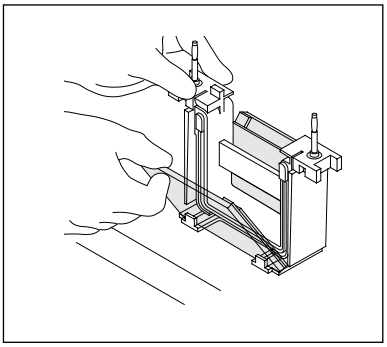
2.2 Mini-PROTEAN 3 Electrophoresis Module Assembly and Sample Loading

Mini-PROTEAN 3 Electrophoresis Module Assembly

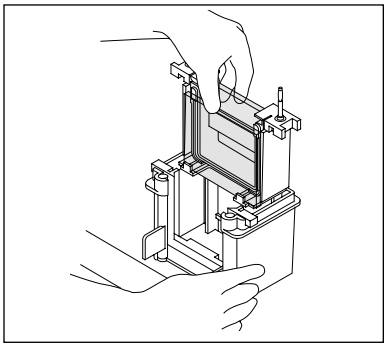
1. Remove the Gel Cassette Assemblies from the Casting Stand. Rotate the cams of the Casting Frames inward to release the Gel Cassette Sandwich (see Figure 5a).
2. Place a Gel Cassette Sandwich into the slots at the bottom of each side of the Electrode Assembly. Be sure the Short Plate of the Gel Cassette Sandwich faces inward toward the notches of the U-shaped gaskets (see Figure 5b).
3. Lift the Gel Cassette Sandwich into place against the green gaskets and slide into the Clamping Frame (see Figure 5c).
4. Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame to form the Inner Chamber and to insure a proper seal of the short plate against the notch on the U-shaped gasket. (see Figure 5d). Short plate must align with notch in gasket.



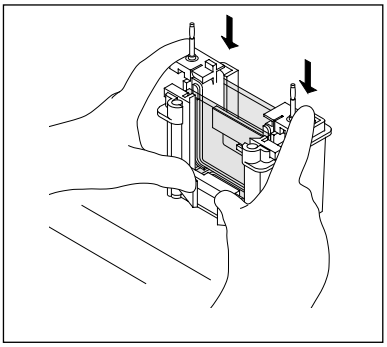
5a. Remove the Gel Cassette Sandwich from the Casting Frame.



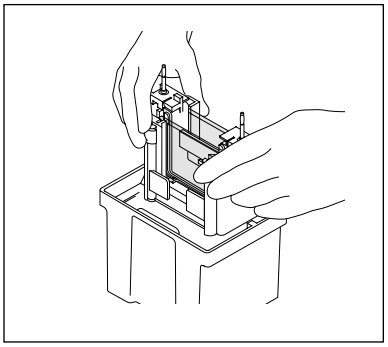
5b. Place Gel Cassette Sandwich into the Electrode Assembly with the Short Plate facing inward.



5c. Slide Gel Cassette Sandwiches and Electrode Assembly into the clamping frame.



5d. Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame.



5e. Lower the Inner Chamber into the Mini Tank.

Fig. 5. Mini-PROTEAN 3 assembly.

Note: Gently pressing the top of the Electrode Assembly while closing the Clamping Frame cams forces the top of the Short Plate on each Gel Cassette Sandwich to seat against the rubber gasket properly and prevents leaking.

5. Lower the Inner Chamber Assembly into the Mini Tank. Fill the inner chamber with ~125 ml of running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the Gel Cassettes.

   Note: Do not overfill the Inner Chamber Assembly. Excess buffer will cause the siphoning of buffer into the lower chamber which can result in buffer loss and interruption of electrophoresis.

6. Add ~200 ml of running buffer to the Mini Tank (lower buffer chamber).

Sample Loading

1. Load the samples into the wells with a Hamilton syringe or a pipette using gel loading tips.
2. If using Bio-Rad's patented sample loading guide, place it between the two gels in the Electrode Assembly. Sample loading guides are available for 9, 10, 12, and 15 well formats.

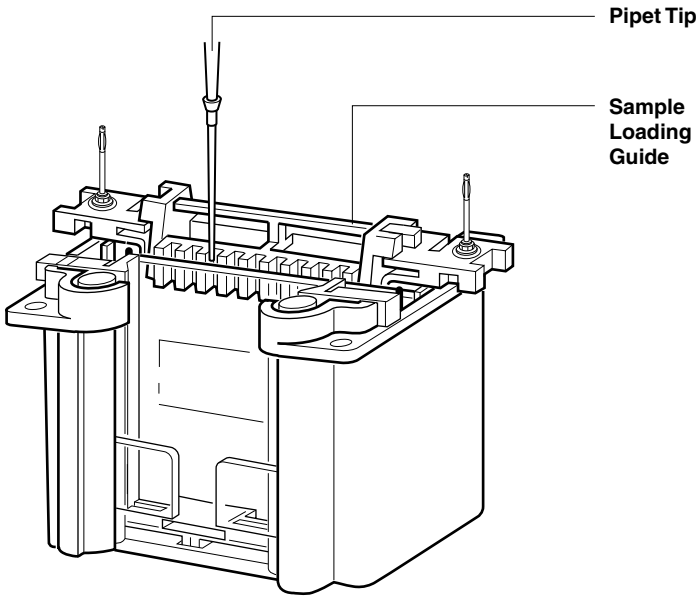


Fig. 6. Using the Sample Loading Guide (patent #5,656,145).

3. Use the Sample Loading Guide to locate the sample wells. Insert the Hamilton syringe or pipette tip into the slots of the guide and fill the corresponding wells.

   Note: Load samples slowly to allow them to settle evenly on the bottom of the well. Be careful not to puncture the bottom of the well with the syringe needle or pipette tip.

2.3 Gel Electrophoresis

Mini Tank Assembly

1. Place the Lid on the Mini Tank. Make sure to align the color coded banana plugs and jacks. The correct orientation is made by matching the jacks on the lid with the banana plugs on the electrode assembly. A stop on the lid prevents incorrect orientation.

Power Conditions

1. Insert the electrical leads into a suitable power supply with the proper polarity.
2. Apply power to the Mini-PROTEAN 3 cell and begin electrophoresis; 200 volts constant is recommended for SDS-PAGE and most native gel applications. Run time is approximately 35 minutes at 200 volts for SDS-PAGE.

Gel Removal

1. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
2. Remove the tank lid and carefully lift out the Inner Chamber Assembly. Pour off and discard the running buffer.

   Note: Always pour off the buffer before opening the cams to avoid spilling the buffer.
3. Open the cams of the Clamping Frame. Pull the Electrode Assembly out of the Clamping Frame and remove the Gel Cassette Sandwiches.
4. Remove the gels from the Gel Cassette Sandwich by gently separating the two plates of the gel cassette. The green, wedgeshaped, plastic Gel Releaser may be used to help pry the glass plates apart.

   Note: To remove the gel from a Ready Gel Cassette, first slice the tape along the sides of the Ready Gel Cassette where the inner glass plate meets the outer plastic plate.
5. Run the sharp edge of the Gel Releaser or a razor blade along each spacer to separate the gel from the spacer. Remove the gel by floating it off the glass plate by inverting the gel and plate under fixative or transfer solution, agitating gently until the gel separates from the plate.
6. Rinse the Mini-PROTEAN 3 cell electrode assembly, Clamping Frame and Mini Tank with distilled, deionized water after use.

Section 3
Separation Theory and Optimization

3.1 Introduction

Polyacrylamide gel electrophoresis separates molecules in complex mixtures according to size and charge. During electrophoresis there is an intricate interaction of samples, gel matrix buffers, and electric current resulting in separate bands of individual molecules. Hence the variables that must be considered in electrophoresis are gel pore size, gel buffer systems, and the properties of the molecule of interest.

Gel Pore Size

Gel pores are created by the crosslinking of polyacrylamide with bis-acrylamide (bis) to create a network of pores. This structure allows the molecular sieving of molecules through the gel matrix. Gel pore size is a function of the acrylamide monomer concentration used (%T). By convention, polyacrylamide gels are characterized by %T which is the weight percentage of the total monomer including the crosslinker. The %T gives an indication of the relative pore size of the gel. In general, pore size decreases with increasing %T.

%T is calculated using the following equation.

$$\%T = \frac{\text{g acrylamide + g crosslinker x 100\%}}{\text{total volume (ml)}}$$

%C is the crosslinker:acrylamide monomer ratio of the monomer solution. %C is calculated using the following equation.

$$\%C = \frac{\text{g crosslinker x 100\%}}{\text{g acrylamide + g crosslinker}}$$

2.67% C is traditionally used for most analytical gels.

Gels can be made as a single continuous percentage throughout the gel, or can be cast as a gradient %T through the gel. Typical compositions are from 7.5% up to 20% for single percentage gels, or gradients ranging from 4–15% to 10–20%.

The total monomer concentration for optimal separation is referred to as optimal %T. Optimal %T will vary depending on the molecular weight of the molecule of interest. Empirically the pore size providing optimum resolution for proteins is that which results in a relative mobility (R_f) value between 0.55–0.6. R_f values for specific proteins are calculated as follows.

$$R_f = \frac{\text{Distance migrated by the protein of interest}}{\text{Distance migrated by the ion front}}$$

Gel Buffer System

The buffer system determines the power requirements and affects separation. The buffer system is composed of the buffer used in the gel and the running buffer. There are continuous and discontinuous buffer systems.

Continuous Buffer Systems

In continuous buffer systems the same buffer ions are present, at constant pH and concentration throughout the system. The gel is typically made of one continuous %T and the sample is loaded directly into the part of the gel where separation will occur. The band width is determined in part by the height of the sample load in the well, so samples should be concentrated and volumes small for best results.

Discontinuous Buffer Systems

In discontinuous buffer systems different buffer ions are present in the gel and electrode reservoirs. By using different buffers in the gel and in the electrode solutions and adding a stacking gel to the resolving gel, samples are compressed into a thin starting band and individual proteins are finely resolved and separated. Discontinuous buffer systems were devised initially for use with undenatured, or native proteins; however the most popular discontinuous system employed is the SDS-PAGE buffer system by Laemmli.[1] Formulations for this system are included in Section 4.1.

3.2 SDS-PAGE (Laemmli) Buffer System

The Laemmli buffer system is a discontinuous buffer system that incorporates SDS in the buffer. In this system, proteins are denatured by heating them in buffer containing sodium dodecyl sulfate (SDS) and a thiol reducing agent such as 2-mercaptoethanol (βME.) The resultant polypeptides take on a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights. Proteins separate according to their molecular weight, making this system extremely useful for calculating molecular weights.

3.3 Native PAGE

Native PAGE is a technique for separating biologically active proteins. In contrast to SDS-PAGE, the mobilities of proteins in a Native PAGE system depend on both size and charge. There is no single electrophoresis buffer system that will optimally separate all proteins in a native gel. Key parameters for separating proteins in a Native PAGE system are pI of the protein of interest and the pH of the electrophoresis buffer

pH and pI

The pH of the electrophoresis buffer must be within the pH range over which the protein of interest is stable and retains biological activity. In addition, the pH of the buffer must impart sufficient charge to the protein for it to move through the gel. Changes in pH will affect both the charge and size (hydrodynamic volume) of the protein of interest and will affect migration rates. For example, a buffer with a pH greater than the pI of the protein will impart a negative charge on the protein and it will migrate toward the positive electrode (anode). Conversely, a buffer with a pH lower than the pI of the protein will impart a positive charge and the protein will migrate to the negative electrode (cathode). A pH equal to the pI will result in no net charge in the protein and it will not migrate in an electric field.

Protein mobilities are best modified by the buffer's pH. Buffers with a pH closer to the pI will provide the best resolution. However run times may be lengthy. Conversely, buffers with a pH further from the pI will allow faster migration but resolution may be compromised. The choice of pH becomes a tradeoff between separation and speed.

How to Choose a Native PAGE system

1. Discontinuous Buffer Systems (Ornstein-Davis[2])

   A discontinuous buffer system should be the first non-denaturing gel system tried. Detailed protocols are provided in Section 4.2. The advantage of a discontinuous system is the use of a stacking gel to concentrate dilute protein samples. However, the stacking phenomena can also cause aggregation of some proteins and interfere with resolution. If protein aggregation occurs, a continuous buffer system should be used.

   Note: The pH attained in the resolving gel of the Ornstein-Davis system approaches pH 9.5, which may be outside the range of stability for some proteins, causing denaturation. Additionally, the pI of the protein of interest may be too close to or above the Ornstein-Davis buffer pH (9.5), which may result in a very low net charge or a positive net charge that may significantly reduce or even prohibit migration to the anode. Alternative discontinuous systems can be found in an article by Chrambach and Jovin.[3]

   Note: It is very desirable to know the pI of the protein of interest before selecting a buffer system.

2. Continuous Buffer Systems

   A continuous buffer system will be required if discontinuous systems cannot be used due to stacking-induced protein aggregation. In a continuous system the same buffer is used in the upper and lower electrode chambers as in the gel. Since stacking does not occur, proteins migrate in bands at least as wide as the height of the applied sample in the well. Consequently, sample volumes should be minimized. The mobility of proteins in a continuous system is dictated by pH rather than by sieving through the polyacrylamide gel. For this reason, 6% polyacrylamide gels are recommended for most applications. For very large proteins, 4% or 5% gels may be used. McLellan describes various continuous buffer systems from pH 3.8–10.2.[4] Detailed protocols are provided in Section 4.3.

Section 4
Reagent Preparation and Stock Solutions

4.1 Volumes Required Per Gel

The volumes listed are required to completely fill a gel cassette. Amounts may be adjusted depending on the application (with or without comb, with or without stacking gel, etc.).

Gel Thickness (mm)	Volume (ml)
0.5	2.8
0.75	4.2
1.0	5.6
1.5	8.4

Note: 10 ml of monomer solution is sufficient for two stacking gels of any thickness.

4.2 SDS-PAGE (Laemmli)[1] Buffer System

Stock Solutions and Buffers

1. Acrylamide/Bis (30% T, 2.67% C)

87.6 g	acrylamide	(29.2 g/100 ml)
2.4 g	N'N'-bis-methylene-acrylamide	(0.8 g/100 ml)

   Make to 300 ml with deionized water. Filter and store at 4 °C in the dark (30 days maximum.)

   or, use:

   Preweighed Acrylamide/Bis, 37.5:1 mixture (30%T, 2.67% C)
   (Bio-Rad catalog number 161-0125, 150 g)

   30% Acrylamide/Bis Solutions, 37.5:1 mixture (30%T, 2.67% C)
   (Bio-Rad catalog number 161-0158, 500 ml)
   (Bio-Rad catalog number 161-0159, 2 x 500 ml)

2. 10% (w/v) SDS

   Dissolve 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water. Alternatively 10% SDS solution (250 ml) can be used (Bio-Rad catalog number 161-0416).

3. 1.5 M Tris-HCl, pH 8.8

27.23 g	Tris base	(18.15 g/100 ml)
80 ml	deionized water	

   Adjust to pH 8.8 with 6 N HCl. Bring total volume to 150 ml with deionized water and store at 4 °C. Alternatively 1.5 M Tris-HCl, pH 8.8 (1 L) premixed buffer can be used (Bio-Rad catalog number 161-0798).

4. 0.5 M Tris-HCl, pH 6.8

6 g	Tris base
60 ml	deionized water

   Adjust to pH 6.8 with 6 N HCl. Bring total volume to 100 ml with deionized water and store at 4 °C. Alternatively 0.5 M Tris-HCl, pH 6.8 (1 L) premixed buffer can be used (Bio-Rad catalog number 161-0799).

5. Sample Buffer (SDS Reducing Buffer)

3.55 ml	deionized water
1.25 ml	0.5 M Tris-HCl, pH 6.8
2.5 ml	glycerol
2.0 ml	10% (w/v) SDS
0.2 ml	0.5%(w/v) bromophenol blue
9.5 ml	Total Volume

Store at room temperature.
Use: Add 50 µl β-Mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95 °C for 4 minutes.

6. 10x Electrode (Running) Buffer, pH 8.3 (makes 1 L)

30.3 g	Tris base
144.0 g	Glycine
10.0 g	SDS

Dissolve and bring total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. Store at 4 °C. If precipitation occurs, warm to room temperature before use. Alternatively, electrophoresis running buffer 10x Tris/Glycine/SDS, 5 L cube (Bio-Rad catalog number 161-0772) can be used.
Use: Dilute 50 ml of 10x stock with 450 ml deionized water for each electrophoresis run. Mix thoroughly before use.

7. 10% APS (fresh daily)

100 mg ammonium persulfate
Dissolved in 1 ml of deionized water.

Gel Formulations (10 ml)

1. Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS. Degas the mixture for 15 minutes.

Percent Gel	DDI H_2O (ml)	30% Degassed Acrylamide/Bis (ml)	*Gel Buffer (ml)	10% w/v SDS (ml)
4%	6.1	1.3	2.5	0.1
5%	5.7	1.7	2.5	0.1
6%	5.4	2.0	2.5	0.1
7%	5.1	2.3	2.5	0.1
8%	4.7	2.7	2.5	0.1
9%	4.4	3.0	2.5	0.1
10%	4.1	3.3	2.5	0.1
11%	3.7	3.7	2.5	0.1
12%	3.4	4.0	2.5	0.1
13%	3.1	4.3	2.5	0.1
14%	2.7	4.7	2.5	0.1
15%	2.4	5.0	2.5	0.1
16%	2.1	5.3	2.5	0.1
17%	1.7	5.7	2.5	0.1

* Resolving Gel Buffer - 1.5 M Tris-HCl, pH 8.8

* Stacking Gel Buffer - 0.5 M Tris-HCl, pH 6.8

2. Immediately prior to pouring the gel, add:

   For 10 ml monomer solution:

   Resolving Gel: 50 µl 10% APS and
   5 µl TEMED

   Stacking Gel: 50 µl 10% APS and
   10 µl TEMED

   Swirl gently to initiate polymerization.

   Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipe. The volumes of APS and TEMED must be adjusted accordingly.

   Warning: The catalyst concentration is very important! Webbing and incomplete well formation can result from inaccurate catalyst concentration.

4.3 Discontinuous Native PAGE (Ornstein-Davis)[2]

Stock Solutions and Buffers

1. Acrylamide/Bis (30% T, 2.67% C)

87.6 g	acrylamide	(29.2 g/100 ml)
2.4 g	N'N'-bis-methylene-acrylamide	(0.8 g/100 ml)

   Make to 300 ml with deionized water. Filter and store at 4 °C in the dark (30 days maximum).

   or, use:

   Preweighed Acrylamide/Bis, 37.5:1 mixture
   (Bio-Rad catalog number 161-0125, 150 g)

   30% Acrylamide/Bis Solutions, 37.5:1 mixture
   (Bio-Rad catalog number 161-0158, 500 ml)
   (Bio-Rad catalog number 161-0159, 2 x 500 ml)

2. 1.5 M Tris-HCl, pH 8.8

27.23 g	Tris base	(18.15 g/100 ml)
80 ml	deionized water	

   Adjust to pH 8.8 with 6 N HCl. Bring total volume up to 150 ml with deionized water and store at 4 °C. Alternatively 1.5 M Tris-HCl, pH 8.8 (1 L) premixed buffer can be used (Bio-Rad catalog number 161-0798).

3. 0.5 M Tris-HCl, pH 6.8

6 g	Tris base
60 ml	deionized water

   Adjust to pH 6.8 with 6 N HCl. Bring total volume up to 100 ml with deionized water and store at 4 °C. Alternatively 0.5 M Tris-HCl, pH 6.8 (1 L) premixed buffer can be used (Bio-Rad catalog number 161-0799).

4. Sample Buffer

5.55 ml	deionized water
1.25 ml	0.5 M Tris-HCl, pH 6.8
3.0 ml	glycerol
0.2 ml	0.5% (w/v) bromophenol blue
10.0 ml	Total Volume

   Store at room temperature.
   Use: Dilute the sample at least 1:2 with sample buffer and heat at 95 °C for 4 minutes.

5. 10x Electrode (Running) Buffer, pH 8.3

30.3 g	Tris base	(15 g/l)
144.0 g	Glycine	(72 g/l)

Bring total volume up to 1,000 ml with deionized water. Do not adjust pH. Alternatively electrophoresis running buffer 10x Tris/Glycine, 1 L (Bio-Rad catalog number 161-0734) can be used.

Usage: Dilute 50 ml of 10x stock with 450 ml deionized water for each electrophoresis run.

Gel Formulations (10 ml)

1. Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS. Degas the mixture for 15 minutes.

Percent Gel	DDI H_2O (ml)	30% Degassed Acrylamide/Bis (ml)	*Gel Buffer (ml)
4%	6.2	1.3	2.5
5%	5.8	1.7	2.5
6%	5.5	2.0	2.5
7%	5.2	2.3	2.5
8%	4.8	2.7	2.5
9%	4.5	3.0	2.5
10%	4.2	3.3	2.5

* Resolving Gel Buffer - 1.5 M Tris-HCl, pH 8.8

* Stacking Gel Buffer - 0.5 M Tris-HCl, pH 6.8

2. Immediately prior to pouring the gel, add:

   50 ml APS and
   TEMED (5 µl for Resolving Gels; 10 µl TEMED for stacking gels)
   Swirl gently to initiate polymerization.

   Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipe. The volumes of APS and TEMED must be adjusted accordingly.

4.4 Continuous Native PAGE

Stock Solutions and Buffers

1. Acrylamide/Bis (30% T, 2.67% C)

87.6 g	acrylamide	(29.2 g/100 ml)
2.4 g	N'N'-bis-methylene-acrylamide	(0.8 g/100 ml)

Make to 300 ml with deionized water. Filter and store at 4 °C in the dark (30 days maximum.)

or, use:

Preweighed Acrylamide/Bis, 37.5:1 mixture
(Bio-Rad catalog number 161-0125, 150 g)

30% Acrylamide/Bis Solutions, 37.5:1 mixture
(Bio-Rad catalog number 161-0158, 500 ml)
(Bio-Rad catalog number 161-0159, 2 x 500 ml)

2. Sample Buffer

1.0 ml	Electrophoresis Buffer
3.0 ml	Glycerol
0.2 ml	0.5% Bromophenol Blue
5.8 ml	Deionized water
10.0 ml	Total Volume

3. Continuous Buffers (McLellan)[4]

McLellan describes various continuous buffer systems from pH 3.8 to pH 10.2. Use the table below to prepare 5x continuous non-denaturing PAGE electrophoresis buffers. Add both the acidic and basic component to 1 liter of water. Do not adjust the pH. If the final pH is outside the listed range discard the buffer and remake.

pH	Basic Component	5x Solution	Acidic Component	5x Solution
3.8	Beta-Alanine (89.09 MW)	13.36 g/L	Lactic Acid 85% Solution	7.45 ml/L
4.4	Beta-Alanine (89.09 MW)	35.64 g/L	Acetic Acid 17.4 M	11.5 ml/L
4.8	GABA (103.1 MW)	41.24 g/L	Acetic Acid 17.4 M	5.75 ml/L
6.1	Histidine (155.2 MW)	23.28 g/L	MES (195.2 MW)	29.5 g/L
6.6	Histidine (155.2 MW)	19.4 g/L	MOPS (209.3 MW)	31.4 g/L
7.4	Imidazole (68.08 MW)	14.64 g/L	HEPES (238.33 MW)	41.7 g/L
8.1	Tris (121.14 MW)	19.38 g/L	EPPS (252.2 MW)	37.85 g/L
8.7	Tris (121.14 MW)	30.29 g/L	Boric Acid (61.83 MW)	7.73 g/L
9.4	Tris (121.14 MW)	36.34 g/L	CAPS (221.3 MW)	44.26 g/L
10.2	Ammonia (14.8 M)	12.5 ml/L	CAPS (221.3 MW)	22.13 g/L

Dilute 200 ml of 5x buffer with 800 ml deionized water to prepare 1x electrophoresis buffer. The final concentrations of buffer components will be.

pH	Basic Component	Acidic Component
3.8	30 mM Beta-Alanine	20 mM Lactic Acid
4.4	80 mM Beta-Alanine	40 mM Acetic Acid
4.8	80 mM GABA	20 mM Acetic Acid
6.1	30 mM Histidine	30 mM MES
6.6	25 mM Histidine	30 mM MOPS
7.4	43 mM Imidazole	35 mM HEPES
8.1	32 mM Tris	30 mM EPPS
8.7	50 mM Tris	25 mM Boric Acid
9.4	60 mM Tris	40 mM CAPS
10.2	37 mM Ammonia	20 mM CAPS

Gel Formulations (10 ml)

1. Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS. Degas the mixture for 15 minutes.

Percent Gel	DDI H_2O (ml)	30% Degassed Acrylamide/Bis (ml)	Continuous Buffer (ml)
4%	6.7	1.3	2.0
5%	6.3	1.7	2.0
6%	6.05	2.0	2.0

Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipe.

2. Immediately prior to pouring the gel, add:

   For 10 ml monomer solution:
   50 µl 10% APS
   10 µl TEMED
   Swirl gently to initiate polymerization.

   Note: Below pH 6, TEMED becomes a less effective catalyst. Increase the concentration of TEMED 5-fold to polymerize gels with a pH range between 4 and 6.

Section 5 References

1. Laemmli, U. K., *Nature*, **227**, 680 (1970).
2. Ornstein, L. and Davis, B. J., *Anal. NY Acad. Sci.*, **121**, 321 (1964).
3. Chrambach, A. and Jovin, T. M., *Electrophoresis*, **4**, 190–204 (1984).
4. McLellan, T., *Analytical Biochemistry*, **126**, 94–99 (1982).

Section 6 Maintenance

Mini-PROTEAN 3 tank and lid, electrode assembly, clamping frame	Rinse thoroughly with distilled water after every use.
Casting stand and frame	Rinse thoroughly with distilled water after every use.
Glass plates and combs	Wash with a laboratory detergent, then rinse thoroughly with distilled water. Limit submersion of Spacer Plates in strongly basic solutions, such as >100 mM NaOH, to less than 24 hours. Limit submersion in chromic-sulfuric acid glass cleaning solution to 2–3 hours. Prolonged submersion compromises the integrity of the adhesive. To preserve the longevity of the adhesive bond, avoid extended submersion (>5 days) in cleaning solutions made from Bio-Rad cleaning concentrate (161-0722) or other strongly basic detergents.

Section 7
Troubleshooting Guide

	Problem		Cause		Solution
1.	"Smile effect" - band pattern curves upward at both sides of the gel.	a.	Center of the gel running hotter than either end.	a.	Buffer not mixed well or buffer in upper chamber too concentrated. Remake buffer, insuring thorough mixing, especially when diluting 5x or 10x stock.
		b.	Power conditions excessive.	b.	Decrease power setting from 200 V to 150 V or fill lower chamber to within 1 cm of top of Short Plate.
2.	Vertical streaking of protein.	a.	Sample overload.	a.	Dilute sample, selectively remove predominant protein in the sample, or reduce voltage by about 25% to minimize streaking.
		b.	Sample precipitation.	b.	Centrifuge sample before addition of SDS sample buffers, or decrease % T of resolving gel.*
				c.	The ratio of SDS to protein should be enough to coat each protein molecule with SDS, generally 1.4:1. It may require more SDS for some membrane protein samples. For example, SDS in sample can be increased to 4% and/or in running buffer increased to 0.4%.
3.	Lateral band spreading.	a.	Diffusion out of the wells prior to turning on the current	a.	Minimize the time between sample application and power start up.
		b.	Ionic strength of sample lower than that of gel.	b.	Use same buffer in sample as in gel or stacking gel.
4.	Skewed or distorted bands.	a.	Poor polymerization around sample wells.	a.	Degas stacking gel solution thoroughly prior to casting; increase ammonium persulfate and TEMED concentrations by 25%; for stacking gel or low%T, leave APS the same and double the TEMED concentration.
		b.	Salts in sample.	b.	Remove salts by dialysis, desalting column, Micro Bio-Spin columns, etc.
		c.	Uneven gel interface.	c.	Decrease the polymerization rate. Overlay gels very carefully.
5.	Lanes constricted at bottom of gel.	a.	Ionic strength of sample higher than that of surrounding gel.	a.	Desalt sample and neighboring samples.
6.	Run taking unusually long time.	a.	Running buffer too concentrated.	a.	Check buffer protocol, dilute if necessary.
		b.	Excessive salt in sample.	b.	Desalt sample.

	Problem		Cause		Solution
7.	Run too fast, poor resolution.	a.	Running or reservoir buffer too dilute.	a.	Check buffer protocol, concentrate if necessary.
		b.	Voltage too high.	b.	Decrease voltage by 25–50%.
8.	Doublets observed where a single protein species is expected (SDS-PAGE)	a.	A portion of the protein may have been reoxidized during the run or may not have been fully reduced prior to run.	a.	Prepare fresh sample buffer solutions if over 30 days old; increase 2-mercaptoethanol concentration in the sample buffer; substitute DTT for BME.
9.	Observe fewer bands than expected and one heavy band at dye front.	a.	Protein(s) migrating at the dye front.	a.	Increase % T of resolving gel.*
		b.	Protein degradation.	b.	Use protease inhibitors, *e.g.* PMSF, etc.
10.	Upper buffer chamber leaks.	a.	Upper buffer chamber over filled.	a	Keep level of buffer below the top of the Spacer Plates.
		b.	Improper assembly.	b.	Be sure u-shaped electrode core gasket is clean, free of cuts, and lubricated with buffer. Be sure Short Plate is *under* the notch on the gasket, not on top of it and press down on electrode assembly when closing cams of the frame.
11.	Leaking during gel casting.	a.	Chipped glass plates.	a.	Insure glass plates are free of flaws.
		b.	Spacer Plate and Short Plate not level.	b.	Insure cassette is aligned correctly.
		c.	Casting Stand gasket is flawed or worn out.	c.	Replace casting stand gaskets.
12.	Poor end well formation.	a.	Incorrect catalyst concentration.	a.	Prepare fresh catalyst solution, or increase catalyst concentration of stacking gel to 0.06% APS and 0.12% TEMED.
		b.	Monomer solution not degassed. Oxygen inhibits polymerization.	b.	Degas monomer solution immediately prior to casting the stacking gel.
13.	Webbing/excess acrylamide behind the comb.	a.	Incorrect catalyst concentration.	a.	Prepare fresh catalyst solution, or increase catalyst concentration of stacking gel to 0.06% APS and 0.12% TEMED.
14.	The pressure cams on the casting frame are difficult to close or make a noise when closed.	a.	A build up of a powder residue at the pivot point of the pressure cams.	a.	Rinse or wipe off the powder residue before each use.

*Polyacrylamide gels are described by reference to two characteristics:

1) The total monomer concentration, (%T) and

2) The crosslinking monomer concentration (%C).

$$\frac{\text{g acrylamide + g bis-acrylamide}}{\text{Total Volume}} \times 100\%$$

$$\frac{\text{g bis-acrylamide}}{\text{g acrylamide + g bis-acrylamide}} \times 100\%$$

Section 8
Product Information and Accessories

Catalog Number	Description
Mini PROTEAN 3 Systems	
165-3301	**Mini-PROTEAN 3 Electrophoresis System,** 10 well, 0.75 thickness, complete system includes 2 combs, 5 sets of glass plates, casting stand, 2 casting frames, sample loading guide, 2 gel releasers, and Electrophoresis Module
165-3302	**Mini-PROTEAN 3 Electrophoresis Module,** for Ready Gel precast gel applications, includes electrode assembly, clamping frame, tank, lid with power cables, mini cell buffer dam, 2 gel releasers
165-3375	**Mini-PROTEAN II Upgrade Kit,** includes Mini-PROTEAN 3 Clamping Frame and Electrode Assembly
165-3314	**Mini-PROTEAN 3 Cell/PowerPac 300 System**, 100/120 V
165-3315	**Mini-PROTEAN 3 Cell/PowerPac 300 System**, 220/240 V
165-3316	**Mini-PROTEAN 3 Cell/PowerPac Junior System,** 100–240 V
165-3317	**Mini-PROTEAN 3 Cell and Mini Trans-Blot® module**

Casting Modules

Each casting module includes 2 combs, 5 sets of glass plates, casting stand, 2 casting frames, and the appropriate Sample Loading Guide.

	0.5 mm spacer	0.75 mm spacer	1.0 mm spacer	1.5 mm spacer
5 well comb	NA	165-3327	165-3332	165-3338
9 well comb	NA	165-3328	165-3333	165-3339
10 well comb	165-3325	165-3329	165-3334	165-3340
15 well comb	165-3326	165-3330	165-3335	165-3341
Prep/2D comb	NA	165-3331	165-3336	165-3342
IPG comb	NA	NA	165-3337	165-3343

Hand Cast Gel Accessories and Replacement Parts

165-3303	**Mini-PROTEAN 3 Casting Stand,** 1
165-3304	**Mini-PROTEAN 3 Casting Frame,** 1
165-3305	**Mini-PROTEAN 3 Casting Stand Gaskets (replacement),** 2
165-3308	**Mini-PROTEAN 3 Short Plates,** 5
165-3309	**Mini-PROTEAN 3 Spacer Plates with 0.5 mm spacers,** 5
165-3310	**Mini-PROTEAN 3 Spacer Plates with 0.75 mm spacers,** 5
165-3311	**Mini-PROTEAN 3 Spacer Plates with 1.0 mm spacers,** 5
165-3312	**Mini-PROTEAN 3 Spacer Plates with 1.5 mm spacers,** 5

Catalog Number	Description
Other Replacement Parts	
165-3306	**Mini-PROTEAN 3 Clamping Frame**, 1
165-3307	**Mini-PROTEAN 3 Electrode Assembly**, 1
165-3201	**Sample Loading Guide, 9 well (red)**, 1
165-3146	**Sample Loading Guide, 10 well (yellow)**, 1
165-3203	**Sample Loading Guide, 12 well (green)**, 1
165-3132	**Sample Loading Guide, 15 well (blue)**, 1
165-3130	**Buffer Dam**, 2
165-3320	**Mini PROTEAN 3 Gel Releaser**, 5
165-3149	**Replacement Electrode Assembly Gaskets**, 2
165-3157	**Gaskets, for precast carbohydrate gels**, 2
161-0990	**Empty Cassettes, 1.0 mm Ready Gel**, 10
165-2975	**Buffer Tank and Lid**, replacement
165-2948	**Replacement Power Cables**
165-2949	**Cell Lid with Power Cables**
900-7680-8	**Replacement Platinum Wire, cathode**, 8 inches
900-7680-13	**Replacement Platinum Wire, anode**, 13 inches

Combs

	0.5 mm spacer	0.75 mm spacer	1.0 mm spacer	1.5 mm spacer
5 well comb	NA	165-3352	165-3357	165-3363
9 well comb	NA	165-3353	165-3358	165-3364
10 well comb	165-3350	165-3354	165-3359	165-3365
15 well comb	165-3351	165-3355	165-3360	165-3366
Prep/2D comb	NA	165-3356	165-3361	165-3367
IPG comb	NA	NA	165-3362	165-3368

Section 9
Warranty Information

The Mini-PROTEAN 3 cell is warranted for 1 year against defects in materials and workmanship. If any defects should occur during this warranty period, Bio-Rad Laboratories will replace the defective parts without charge. However the following defects are specifically excluded.

1. Defects caused by improper operation.
2. Repairs or modifications done by anyone other than Bio-Rad Laboratories or their authorized agent.
3. Damaged caused by accidental misuse.
4. Damage caused by disaster.
5. Common consumable replacement parts including platinum wire, the rubber gaskets, and glass plates.
6. Damage caused by the use of organic solvents.

For inquiry or request for repair service, contact your local Bio-Rad office.

Warranty Information

Model _____

Catalog Number _____

Date of Delivery _____

Serial Number _____

Invoice Number _____

Purchase Order No _____

Bio-Rad Laboratories, Inc.

Life Science Group

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Brazil *Ph. 55 21 507 6191* ***Canada*** *Ph. (905) 712-2771, Fx. (905) 712-2990* ***China*** *Ph. 86-10-8201-1366/68, Fx. 86-10-8201-1367*
Denmark *Ph. 45 44 52-1000, Fx. 45 4452 1001* ***Finland*** *Ph. 358 (0)9 804 2200, Fx. 358 (0)9 804 1100* ***France*** *Ph. 01 47 95 69 65, Fx. 01 47 41 9133*
Germany *Ph. 089 318 84-177, Fx. 089 318 84-123* ***Hong Kong*** *Ph. 852-2789-3300, Fx. 852-2789-1257* ***India*** *Ph. (91-124)-6398112/113/114, Fx. (91-124)-6398115*
Israel *Ph. 03 951 4124, Fx. 03 951 4129* ***Italy*** *Ph. 34 91 590 5200 , Fx. 34 91 590 5211* ***Japan*** *Ph. 03-5811-6270, Fx. 03-5811-6272*
Korea *Ph. 82-2-3473-4460, Fx. 82-2-3472-7003* ***Latin America*** *Ph. 305-894-5950, Fx. 305-894-5960* ***Mexico*** *Ph. 52 5 534 2552 to 54, Fx. 52 5 524 5971*
The Netherlands *Ph. 0318-540666, Fx. 0318-542216* ***New Zealand*** *Ph. 64-9-4152280, Fx. 64-9-443 3097* ***Norway*** *Ph. 47-23-38-41-30, Fx. 47-23-38-41-39*
Russia *Ph. 7 095 979 98 00, Fx. 7 095 979 98 56* ***Singapore*** *Ph. 65-2729877, Fx. 65-2734835* ***Spain*** *Ph. 34-91-590-5200, Fx. 34-91-590-5211*
Sweden *Ph. 46 (0)8-55 51 27 00, Fx. 46 (0)8-55 51 27 80* ***Switzerland*** *Ph. 061-717-9555, Fx. 061-717-9550* ***United Kingdom*** *Ph. 0800-181134, Fx. 01442-259118*

Sig 0101

4006157 Rev B

Mini Trans-Blot® Electrophoretic Transfer Cell

Instruction Manual

Catalog Numbers
170-3930
170-3935
170-3989
170-3836

BIO-RAD

Note

Assembly and Disassembly

To insure best performance from the Mini Trans-Blot® electrophoretic transfer cell, become fully acquainted with these operating instructions before using the cell to transfer samples. Bio-Rad recommends that you first read these instructions carefully. Then assemble and disassemble the cell completely. After these preliminary steps, you should be ready to transfer a sample.

Wash Cell Before Use

Bio-Rad also recommends that all Mini Trans-Blot electrophoretic transfer cell components and accessories be cleaned with a suitable laboratory cleaner (such as Bio-Rad Cleaning Concentrate, catalog number 161-0722) and rinsed thoroughly with distilled water before use.

Warranty

Model _____

Catalog Number _____

Date of Delivery _____

Warranty Period _____

Serial Number _____

Invoice Number _____

Purchase Order Number _____

Bio-Rad Laboratories warrants the Mini Trans-Blot electrophoretic transfer cell against defects in materials and workmanship for 1 year. If any defects occur in the instrument during this warranty period, Bio-Rad Laboratories will repair or replace the defective parts free. The following defects, however, are specifically excluded:

1. Defects caused by improper operation.
2. Repair or modification done by anyone other than Bio-Rad Laboratories or an authorized agent.
3. Use of fittings or other spare parts supplied by anyone other than Bio-Rad Laboratories.
4. Damage caused by accident or misuse.
5. Damage caused by disaster.
6. Corrosion due to use of improper solvent or sample.

For any inquiry or request for repair service, contact Bio-Rad Laboratories after confirming the model and serial number of your instrument.

Table of Contents

Section 1
Introduction

Blotting was first performed by Southern in 1975 with the transfer of DNA from agarose gels to nitrocellulose membranes.[1] Since that time, blotting has been applied to RNA[2-4] and proteins[5, 6] in both agarose and polyacrylamide gels. To circumvent the inefficiencies observed in various capillary transfers, electric current has been adopted for eluting proteins from polyacrylamide gels, as first described by Towbin *et al.* in 1979.[7] The use of electrophoretic transfer has also been applied to DNA and RNA blotting.[8-13, 30] Numerous publications have dealt with the topic of protein electrophoretic transfer techniques.[14-25] There have also been reviews summarizing the expanding literature being generated on electrophoretic blotting methodology.[26, 27, 31]

The Mini Trans-Blot® tank is part of Bio-Rad's modular Mini-PROTEAN® Tetra system. The unique feature of this electrophoresis system is that the electrode modules are interchangeable. After finishing gel electrophoresis, remove the electrode module from the buffer tank, insert a new electrode module, add new buffer, and the next electrophoresis application can be performed.

The Mini Trans-Blot module accommodates two cassettes for electrophoretic transfer. The Mini Trans-Blot module is useful for blotting either protein or nucleic acid from both agarose and acrylamide gels. It is also capable of blotting isoelectric focusing gels from horizontal electrophoresis cells, or DNA and RNA gels from the Mini-Sub® submarine electrophoresis cell. For applications where the gel is larger than 7.5 x 10 cm, or when there are more than two mini gels to be transferred, the larger standard Trans-Blot® cell (catalog #170-3910 or 170-3946), Criterion™ Blotter (catalog #170-4070, 170-4071) or the Trans-Blot® SD semi-dry cell (catalog #170-3940) should be used.

The heart of the Mini Trans-Blot cell is its electrode module. This module has the capacity to hold two gel cassettes between parallel electrodes only 4 cm apart. The driving force for blotting applications is the voltage applied over the distance between the electrodes. This short 4 cm electrode distance allows generation of higher driving forces to produce efficient protein transfers. A second feature of the electrode module is that it is offset to accommodate a blue cooling unit. The cooling unit, which is completely contained within the Mini Trans-Blot cell, absorbs the Joule heat generated during rapid electrophoretic transfers. The advantages of having an internal cooling unit include elimination of an expensive external cooling bath and avoidance of cumbersome cooling tubing. Other features of the Mini Trans-Blot cell include gel holder cassette latches for easy handling, color coordinated cassettes and electrodes to insure proper orientation of the gel during transfer, and an efficient design which simplifies insertion and removal of the cassettes from the electrode assembly. These features result in an electrophoretic transfer system which is easy to use and produces excellent blotting results.

1.1 Specifications

Construction

Electrode module	Molded polysulfone
Gel holder cassettes	Molded polycarbonate
Electrodes	Platinum wire 0.254 mm diameter
Buffer chamber and lid	Molded polycarbonate
Cooling unit	Polyethylene

Overall dimensions

Mini Trans-Blot cell	16 (L) x 12 (W) x 18 (H) cm
Gel holder dimensions	10 x 11 cm

Maximum gel size 7.5 x 10 cm

Buffer capacity

With cooling unit	950 ml
Without cooling unit	1,150 ml

Cleaning Use mild soap and warm water to clean the electrodes, cassettes, and buffer tank. Use special care when cleaning the electrode cards. Avoid stretching or breaking the platinum wires. Do not use abrasives or strong detergents. Rinse the fiber pads under hot water and then in distilled, deionized water.

Chemical compatibility The Mini Trans-Blot cell components are not compatible with chlorinated hydrocarbons (*e.g.*, chloroform), aromatic hydrocarbons (*e.g.*, toluene, benzene), or acetone. **Use of organic solvents voids all warranties.**

1.2 Safety Instructions

Power to the Mini Trans-Blot cell is supplied by an external DC voltage power supply. This power supply must be ground isolated in such a way that the DC voltage output floats with respect to ground. All of Bio-Rad's power supplies meet this important safety requirement. Regardless of which power supply is used, the maximum specified operating parameters for the cell are:

150 VDC	⎓	Maximum voltage limit
40 Watts		Maximum power limit
50°C		Maximum ambient temperature limit

Current to the cell, provided from the external power supply, enters the unit through the lid assembly, providing a safety interlock to the user. Current to the cell is broken when the lid is removed. **Do not attempt to circumvent this safety interlock, and always turn the power supply off before removing the lid, or when working with the cell in any way.**

Important: This Bio-Rad instrument is designed and certified to meet IEC1010-1* safety standards. Certified products are safe to use when operated in accordance with the instruction manual. This instrument should not be modified or altered in any way. Alteration of this instrument will:

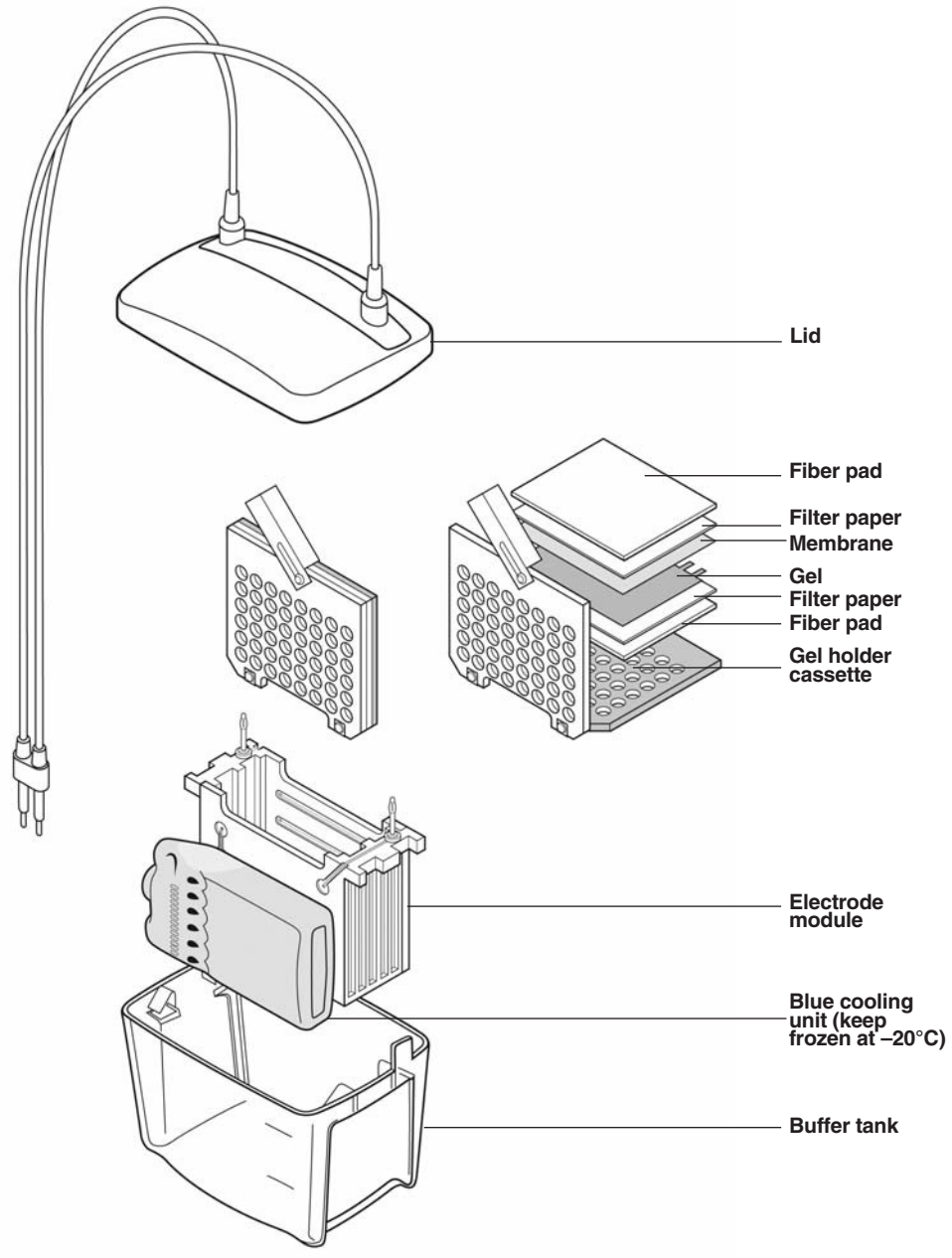
- **Void the manufacturer's warranty**
- **Void the IEC1010-1 safety certification**
- **Create a potential safety hazard**

Bio-Rad is not responsible for any injury or damage caused by the use of this instrument for purposes other than for which it is intended or by modifications of the instrument not performed by Bio-Rad or an authorized agent.

* IEC1010-1 is an internationally accepted electrical safety standard for laboratory instruments.

Section 2
Mini Trans-Blot® Cell Assembly and Preparation for Transfer

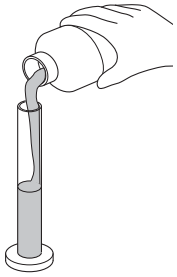
2.1 Mini Trans-Blot Cell Description and Assembly of Parts



2.2 Preparation for Blotting

Store the blue cooling unit in your laboratory freezer at –20°C until ready to use. After use, rinse the outside container with water and return the cooling unit to the freezer for storage.

1. Prepare the transfer buffer. (See Section 3.3 for buffer formulation. Using buffer chilled to 4°C will improve heat dissipation.)



2. Cut the membrane and the filter paper to the dimensions of the gel or use precut membranes and filter paper. Always wear gloves when handling membranes to prevent contamination. Equilibrate the gel and soak the membrane, filter paper, and fiber pads in transfer buffer (15–20 min depending on gel thickness).

3. Prepare the gel sandwich.

   Place the cassette, with the gray side down, on a clean surface.

   Place one pre-wetted fiber pad on the gray side of the cassette.

   Place a sheet of filter paper on the fiber pad.

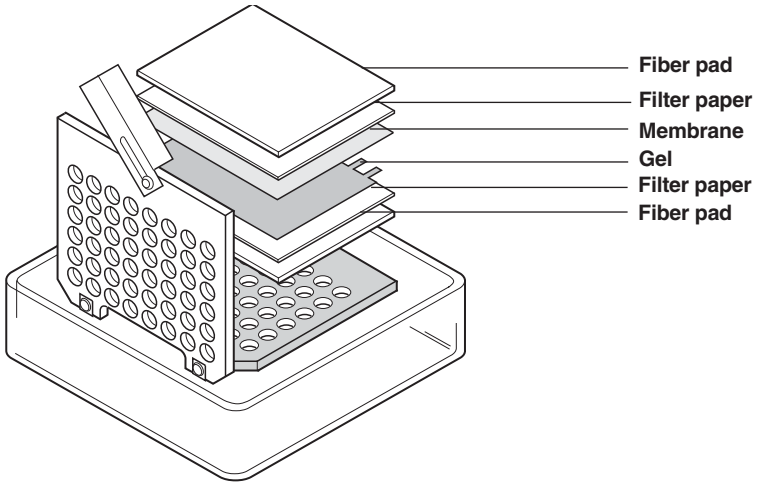
   Place the equilibrated gel on the filter paper.*

   Place the pre-wetted membrane on the gel.*

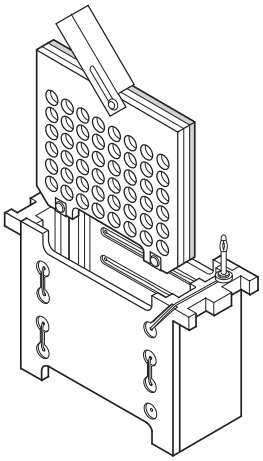
   Complete the sandwich by placing a piece of filter paper on the membrane.*

   Add the last fiber pad.

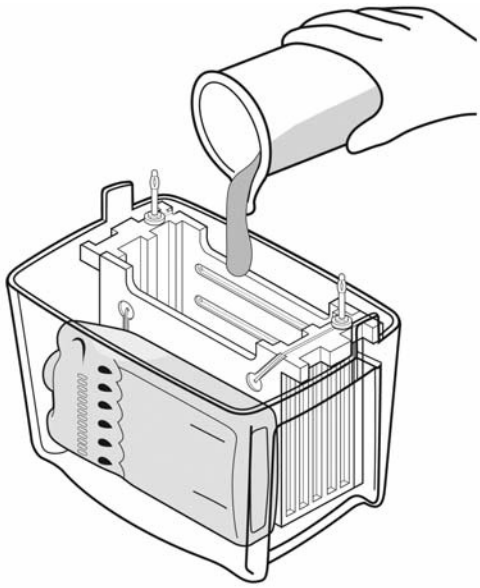
   * Removing any air bubbles which may have formed is very important for good results. Use a glass tube or roller to gently roll out air bubbles.



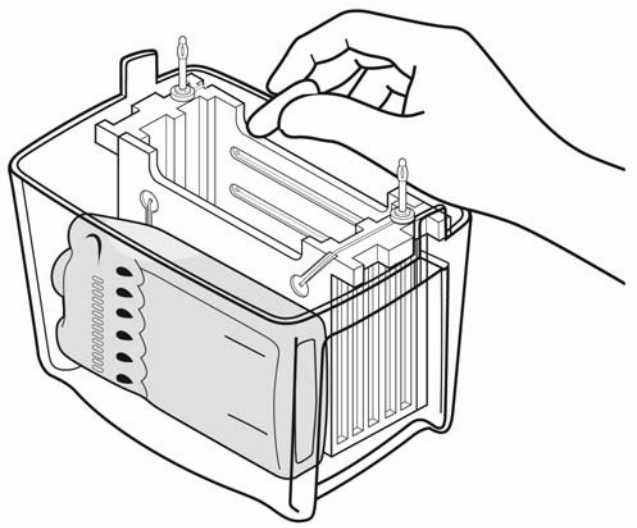
4. Close the cassette firmly, being careful not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.
5. Place the cassette in module. Repeat for the other cassette.



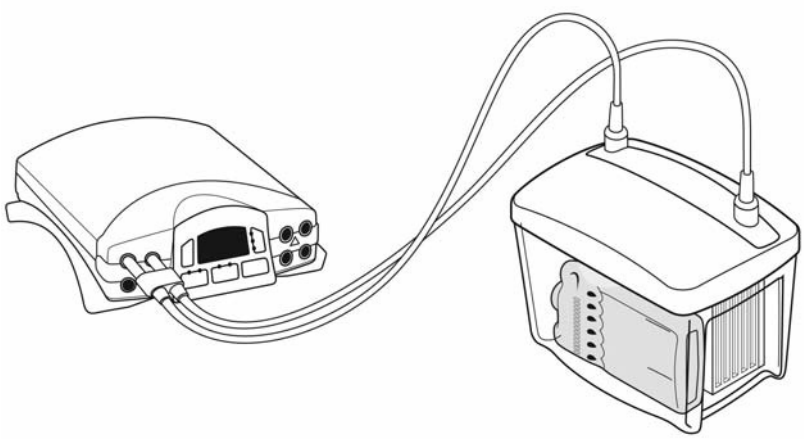
6. Add the frozen blue cooling unit. Place in tank and fill to the "blotting" mark on the tank.



7. Add a standard stir bar to help maintain even buffer temperature and ion distribution in the tank. Set the speed as fast as possible to keep ion distribution even.



8. Put on the lid, plug the cables into the power supply, and run the blot. Refer to Section 3 for run times and voltage settings with various buffers.



9. Upon completion of the run, disassemble the blotting sandwich and remove the membrane for development. Clean the cell, fiber pads, and cassettes with laboratory detergent and rinse well with deionized water.

2.3 Acidic Transfers

If transferring under acidic conditions, switch the gel and membrane in the set up instructions. This will place the membrane on the cathode side of the gel. Under acidic conditions, proteins will transfer in the opposite direction going toward the negative cathode.

Section 3
Transfer Conditions

3.1 General Guide to Transfer Buffers and Running Conditions

Table 3.1 provide guidelines for power conditions using different buffers. Power conditions are provided for various run times. Where multiple conditions are displayed, the higher the voltage, the less time required for the run. Always use the blue cooling unit.

Table 3.1. Guide to Buffers and Running Conditions

Buffer	Standard Field Overnight Transfer	High Intensity Field 1 Hour Transfer
SDS-PAGE Gels	**Buffer A or B or C**	**Buffer A or B or C**
A: 25 mM Tris, pH 8.3, 192 mM glycine, with or without 20% MeOH and .025%–0.1% SDS	30 V, constant 90 mA	100 V, constant 350 mA
B: 48 mM Tris, pH 9.2, 39 mM glycine, with or without 20% MeOH and .025%–0.1% SDS		
C: 10 mM $NaHCO_3$, 3 mM $NaCO_3$, pH 9.9, with or without 20% MeOH and .025%–0.1% SDS		
DNA and RNA		
TAE: 20 mM Tris, pH 7.8, 10 mM sodium acetate, 0.5 mM EDTA.	30 V, constant 100 mA	80 V, constant 500 mA
TBE: 50 mM Tris, pH 8.3, 50 mM sodium borate, 1.0 mM EDTA.		
Native Gels		
25 mM Tris, pH 8.3, 192 mM glycine. No methanol.	30 V, constant 90 mA	100 V, constant 350 mA
Isoelectric Focusing, Native Gels, Basic Proteins, Acid Urea Gels*		
0.7% acetic acid.	30 V, constant 100 mA	100 V, constant 350 mA

*Please refer to Section 2.3 before transfering.

3.2 Notes on Electrophoretic Transfer Conditions

These variables will change total resistance and thus the current readings:

- Alterations in buffer make-up, *i.e.*, addition of SDS, or changes in ion concentration due to addition of acid or base to adjust the pH of the buffers
- Gel pH, ionic strength, and percentage of acrylamide, especially if the gel has not been properly equilibrated
- Number of gels; current increases slightly as the number of gels increases
- Volume of buffer; current increases when volume increases
- Platinum mass; current increases when mass increases
- Transfer temperature; current increases when temperature increases
- Time in transfer at which reading was taken; current normally increases as the buffering capacity diminishes with progress of the run

Pre-equilibration of gels (15–20 min)

All electrophoresis gels should be pre-equilibrated in transfer buffer prior to electrophoretic transfer. Pre-equilibration will facilitate the removal of contaminating electrophoresis buffer salts and neutralization salts (salts resulting from the denaturation of nucleic acids prior to transfer). If the salts are not removed, they will increase the conductivity of the transfer buffer and the amount of heat generated during the transfer. Also, low percentage gels will shrink in methanol buffers. Equilibration allows the gel to adjust to its final size prior to electrophoretic transfer.

Current limits

The PowerPac® Basic power supply is capable of a 75-watt output. Unless a current limit is set, uncontrolled conductivity changes may result in full power being delivered to the Mini Trans-Blot® cell. The gel holders may warp, and the transfer buffer may boil and evaporate (further increasing conductivity). This would result in a potential safety hazard. Refer to the PowerPac Basic power supply instruction manual for setting current limits and run times. The Mini Trans-Blot cell is also compatible with the PowerPac HC power supply.

Use of a stir bar during transfer

For all blotting applications a stir bar must be placed inside the Mini Trans-Blot cell and the entire unit be placed on a stir bar mixer, so that the transfer buffer is stirred during the course of the experiment. This will help to maintain uniform conductivity and temperature during electrophoretic transfer. Failure to properly control transfer buffer temperature results in poor transfer of macromolecules and poses a potential safety hazard.

Transfer buffer pH

Do not adjust the pH of transfer buffers unless specifically indicated. Adjustments of the transfer buffers pH, when not indicated, will result in increased buffer conductivity. This is manifested by a higher than expected initial current output and a decreased resistance. It is recommended that the buffer conductivity and resistance be checked with the PowerPac Basic power supply before starting each transfer.

Transfer buffer recommendations

Use only high quality, reagent grade methanol. Contaminated methanol can result in increased transfer buffer conductivity, as well as poor transfer of macromolecules. Do not reuse transfer buffers or dilute transfer buffers below recommended levels. Reuse of transfer buffers is not advised, since these buffers have most likely lost their ability to maintain a stable solution pH during transfer. Dilution of transfer buffers below their recommended levels is also not advised, since this will decrease buffering capacity.

Voltage limits

Do not increase voltage settings beyond those indicated in Table 3.1. If overnight transfers at low voltages are ineffective for your application, and higher voltages are necessary, transfer times must also be decreased. Failure to do so may result in a potential safety hazard.

3.3 Buffer Formulation

All formulas provided below are for a total volume of 1 L of buffer. Approximately 950 ml of buffer are required for the Mini Trans-Blot cell with cooling unit. Ethanol can be used in place of methanol in all buffer formulations.

Do not add acid or base to adjust pH of the following buffers. Methanol should be analytical reagent grade, as metallic contaminants in low grade methanol will plate on the electrodes.

Note: Some pH electrodes will not perform a proper measurement for the pH of Tris buffers. If the pH of the buffer is off, check to make sure the electrode is designed to work with Tris buffers. If the pH electrode functions properly for Tris buffers and the pH is below 8.0, remake the buffer.

25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3
Mix 3.03 g Tris, 14.4 g glycine, and 200 ml of methanol; add distilled deionized water (dd H_2O) to 1 L.

25 mM Tris, 192 mM glycine, pH 8.3
Mix 3.03 g Tris and 14.4 g glycine; add dd H_2O to 1 L.

48 mM Tris, 39 mM glycine, 20% v/v methanol, pH 9.2
Mix 5.82 g Tris and 2.93 g glycine in ddH_2O, add 200 ml methanol.
Add to 1 L with ddH_2O.

48 mM Tris, 39 mM glycine, pH 9.2
Mix 5.82 g Tris and 2.93 g glycine.
Add ddH_2O to 1 L.

10 mM $NaHCO_3$, 3 mM $NaCO_3$, 20% methanol, pH 9.9
Mix 0.84 g $NaHCO_3$ and 0.318 g $NaCO_3$ in ddH_2O, add 200 ml methanol.
Add to 1 L with ddH_2O.

1.0x TBE (Tris-Borate EDTA), pH 8.3
90 mM Tris-Borate, 1 mM EDTA

5x stock solution
54 g Tris base
27.5 boric acid
20 ml 0.5 M EDTA (pH 8.0)

Add 200 ml 5x stock solution to 800 ml ddH2O to make 1x working solution.

1x TAE (Tris-Acetate EDTA)
40 mM Tris-Acetate, 1 mM EDTA

50x stock solution
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

Add 20 ml 50x stock solution to 980 ml ddH_2O to make 1x working solution.

Section 4
Strategies for Optimizing Electrophoretic Transfer

4.1 Optimizing Protein Transfer

Generally, quantitative elution of denatured high molecular weight proteins is difficult. The following tactics, alone or in combination, will increase transfer efficiency.

Vary gel composition

Gradient gels are often more effective than single gel concentrations for elution of a wide range of molecular weight proteins.

Lower the total monomer to create a more porous gel.

Increase or decrease the percentage of crosslinker. A 5.26% C gel will contain the smallest pore size of all gels no matter what the concentration of acrylamide. Decrease in %C will make gels more porous with little loss in resolution.

$$\%C = \frac{\text{grams bis}}{\text{grams bis + grams acrylamide}} \times 100$$

Increase transfer time

An initial control should be performed to determine the time required for complete transfer.[17, 24] Times may vary from as little as 30 minutes to as long as overnight. Remember all overnight applications should be performed at 30 volts to minimize heating problems.

Increase the power

Initial controls should be performed to evaluate the efficiency of increasing the V/cm as well as its effects on the temperature of transfer. The temperature increase may change buffer resistance and subsequent power delivered, as well as the state of protein denaturation, thus affecting transfer efficiency.

Reduce buffer strength

Dilution of transfer buffer results in lower current at any given voltage. This will allow the use of higher voltages without excessive heating. However, be aware not to dilute the buffer below its buffering capacity.

Vary buffer type and pH

Maximize charge-to-mass ratio. It appears that alcohols present in SDS transfer buffer strip SDS from proteins. Basic proteins in Tris, glycine, methanol buffer at pH 8.3 may assume a state near isoelectric neutrality and thus transfer poorly. For example, lysozyme exhibits this behavior. Buffers with pH of 9.5 to 10.0 have shown much better elution and binding characteristics for basic proteins such as lysozyme and histones.[41]

Different buffer types at similar V/cm may yield different efficiencies. Generally, Tris buffers allow more efficient transfer than acetate or phosphate buffers.

Add detergent

Addition of 0.1% SDS detergent to Tris, glycine, methanol buffer has been reported to increase transfer efficiency.[24] SDS, however, increases relative current, power, and heating. Also, temperatures below 10°C may precipitate the SDS so the starting buffer temperature will be higher. SDS may also affect the antigenicity of some proteins. SDS will aid in eluting the proteins from the gel, but it may reduce the binding efficiency of those proteins to the membrane.[42]

Eliminate alcohol from the transfer buffer

Alcohol in the transfer buffer improves binding of proteins to nitrocellulose only. Elimination of alcohol results in increased transfer efficiency but diminishes binding to nitrocellulose. Transfer efficiency is increased because alcohol causes gel pores to contract resulting in capture of large molecular weight proteins within the gel matrix. Use of PVDF membrane for protein transfers eliminates the alcohol requirement, and constitutes a logical strategy for analysis of high molecular weight or difficult-to-transfer proteins.[26, 27] PVDF must be wetted in 100% methanol but may then be used in buffer without methanol.

Limited protease treatment

A protocol for protease digestion of protein during transfer has been published.[22] Efficient transfer without loss of immunological reactivity was reported.

Alter membrane type

Both nitrocellulose and PVDF can be used for protein transfer.

Alter gel system

If possible, use nondenaturing gradient pore gels for separation of proteins. Isoelectric focusing gels, or native gels, may be considered if separation by molecular weight is not mandatory.

Enhance gel-membrane contact

Failure of molecules to bind efficiently to the membrane, caused by poor gel-membrane contact, is often confused with inefficient elution. Poor contact is usually due to excess moisture in the gel-membrane interface. Proper technique and the use of a test tube or glass pipet as a “rolling pin” should assure good contact. Proper selection of filter paper spacers will help assure good compression. Gel and membrane equilibration in transfer buffer for 15–20 min prior to transfer will help prevent shrinking of either component during transfer, and will eliminate reactants such as urea or SDS from the gel.

4.2 Optimizing DNA and RNA Transfer

Problems with elution of nucleic acids can be solved by altering the gel percentage. It may be somewhat more difficult to quantitatively transfer large amounts of DNA used in genomic blots. Agarose gels over 6 mm thick are not compatible with the Mini Trans-Blot. The following tactics should be considered for optimizing elution in such transfers.

Alter gel composition

Lower % total monomer or % crosslinker for polyacrylamide gels.
Lower % agarose. This allows better elution of high molecular weight DNA.

Alter DNA denaturants

It has been found that glyoxal denaturation allows more efficient elution of DNA than NaOH. Boiling polyacrylamide gels to denature DNA has also been found to give excellent results.[11] Base denaturation often causes polyacrylamide gels to weaken and stick to blotting membranes.

Section 5
Choice of Blotting Membranes

5.1 Protein Blotting Membranes

Nitrocellulose Membrane

Nitrocellulose membranes have been used extensively for protein binding and detection.[7, 20, 23, 24, 27] They can be easily stained for total protein by a dye stain (Amido Black, Coomassie Blue, Ponceau S, Fast Green FCF, etc.),[27] or the more sensitive Colloidal Gold Total Protein Stain, and also allow either RIA, FIA, or EIA.[7] Nitrocellulose has a high binding capacity of 80–100 µg/cm^2. Nonspecific protein binding sites are easily and rapidly blocked, avoiding subsequent background problems. No pre-activation is required. Low molecular weight proteins (especially <15,000 daltons) may be lost during post transfer washes, thus limiting detection sensitivity.[19] Smaller pore size nitrocellulose membrane
(0.2 µm), has been shown to be effective in eliminating this loss.[37] Large proteins (≥100,000 daltons) denatured by SDS may transfer poorly due to the addition of alcohol to the transfer buffer. Alcohol increases binding of SDS-proteins to nitrocellulose, but decreases pore sizes in the gel. Elimination of alcohol from SDS-protein transfers results in considerably diminished binding. Adding SDS (up to 0.1%) to the transfer buffer increases the transfer efficiency of proteins, but reduces the amount of binding to the membrane.[17] Also, SDS increases the conductivity of the buffer and the heat generated during transfer.

PVDF Membrane

Polyvinylidene difluoride (PVDF) membrane is an ideal support for amino-terminal sequencing, amino acid analysis and immunoassays of blotted proteins. PVDF retains proteins under extreme conditions of exposure to acidic or basic conditions, and in the presence of organic solvents. Greater retention during sequencing manipulations enhances the likelihood of obtaining information from rare, low abundance proteins, by increased initial coupling and higher repetitive yields. In addition, PVDF membrane exhibits better binding efficiency of blotted material in the presence of SDS in the transfer buffer. PVDF must first be wetted in 100% MeOH but can then be used in buffer, which does not contain MeOH.

5.2 DNA and RNA Blotting Membranes

Zeta-Probe® Nylon Membrane

Nitrocellulose is not a suitable medium for electrophoretic transfer of nucleic acids, as high concentrations of salt (≥10x SSC) are required for efficient binding.[13] Molecules ≤500 bp are not bound at all, even at high salt. Low resistance results when an electric current is passed through a solution of high salt. This causes potentially damaging high currents (and power) even at very low voltages. Since V/cm is the eluting force, inefficient transfer occurs under conditions required for proper binding. Zeta-Probe membrane allows efficient binding of all sizes of single stranded DNA and RNA in the presence of low ionic strength buffers.[13] Zeta-Probe membrane is an ideal alternative to nitrocellulose for the transfer of nucleic acids. Binding is more stable through post transfer washes, and reprobing may be performed as many as 10 times.

Table 5.1 Guide to Protein Blotting Membranes

A variety of blotting membranes is available for immunoblotting, each with particular advantages depending on the needs of the experiment. The physical properties and performance characteristics of a membrane should be evaluated when selecting the appropriate transfer conditions.

Membrane	Pore Size	Binding Capacity ($\mu g/cm^2$)	Notes
Nitrocellulose	0.45 µm 0.2 µm	80–100	General purpose protein blotting membrane
Supported Nitrocellulose	0.45 µm 0.2 µm	80–100	Pure nitrocellulose cast on an inert synthetic support; increased strength for easier handling and for reprobing
PVDF	0.2 µm	170–200	High mechanical strength and chemical stability, used for protein sequencing and western blotting; enhanced binding in the presence of SDS. Must be wet in alcohol before equilibration in buffer
Nylon	0.2 µm	170	Recommended for nucleic acids

Note: Nucleic acids cannot be transferred to nitrocellulose by electrophoretic blotting. Use Zeta-Probe membrane.

Section 6
Troubleshooting Guide

6.1 Electrophoretic Transfer

Poor electrophoretic transfer (as detected by staining the gel)—proteins

1. Transfer time is too short.
   - Increase the transfer time
2. Power is too low.
   - Always check the current at the beginning of the run. The current may be too low for a particular voltage setting. If the buffer is prepared improperly, the conductivity may be too low, and not enough power will be delivered to the cell. See the power guidelines for specific applications in Section 3
   - Remake the buffer or increase the voltage
   - Try the high intensity blotting option
3. Power supply circuit is inoperative, or an inappropriate power supply was used.
   - Check the fuse. Be sure the voltage and current output of the power supply match the needs of the blotting instrument
4. Transfer apparatus is assembled incorrectly, and the proteins are moving in the wrong direction.
   - The gel/membrane sandwich may be assembled in the wrong order or the cassette is inserted in the tank facing the opposite orientation. Check the polarity of the connections to the power supply
   - Use a pre-stained protein standard to assess transfer efficiency after blotting

5. Charge-to-mass ratio is incorrect.
    - Try a more basic or acidic transfer buffer to increase protein mobility. Proteins near their isoelectric point at the pH of the buffer will transfer poorly. (It has been suggested that buffer pH should be 2 pH units higher or lower than the pI of the protein of interest for optimal transfer efficiency.)
6. Protein is precipitating in the gel.
    - Try using SDS in the transfer buffer. SDS can increase transfer efficiency, but can also reduce binding efficiency to nitrocellulose and affect reactivity of some proteins with antibodies
    - An excess of methanol will lead to protein precipitation. Try decreasing methanol content
7. Methanol in the transfer buffer is restricting elution.
    - Reduction of methanol results in increased transfer efficiency of proteins from the gel, but it also diminishes binding to nitrocellulose
8. Gel percentage too high.
    - Reduce %T (total monomer) or %C (crosslinker). A 5.26% C (with bis as the crosslinker) will produce the smallest pore size gel. Decreasing from this concentration will increase the pore size and increase transfer efficiency

Poor transfer—nucleic acid

1. Gel percentage is too high.
    - Reduce the %T or %C in the acrylamide gel or reduce % agarose in an agarose gel
    - Prior to transfer, cleave DNA in 0.25 M HCl or RNA in dilute NaOH
2. Transfer time is too short or power conditions are too low.
    - Increase the transfer time, or try high intensity transfer
3. DNA or RNA cannot be transferred electrophoretically to nitrocellulose, since high salt concentrations are required for efficient binding.
    - Use Zeta-Probe membrane instead of nitrocellulose

Swirls or missing bands; diffuse transfers

1. Poor contact between the membrane and the gel. Air bubbles or excess buffer remain between the blot and gel.
    - Use a test tube or pipet as a rolling pin, and roll over the membrane carefully in both directions until air bubbles and excess buffer are removed from between gel and membrane, and complete contact is established
    - Use thicker filter paper in the gel/membrane sandwich
    - Replace the fiber pads. Pads will compress with time, and will not hold the membrane to the gel
2. Power conditions are too high.
    - Always check the current at the beginning of the run. The current may be too high for a particular voltage setting. If the buffer is prepared improperly, the conductivity may be too high, resulting in excessive power delivered to the cell. See the power guidelines for specific applications in Section 3

3. The membrane is not properly wet or has dried out.
    - White spots on the nitrocellulose membrane indicate dry areas where protein will not bind. If wetting does not occur immediately by immersion of the sheet in transfer buffer, heat distilled water until just under the boiling point, and soak the membrane until completely wet. Equilibrate in transfer buffer until ready for use
    - Because of the hydrophobic nature of PVDF, the membrane must be prewet in methanol prior to equilibration in aqueous transfer buffer. Do not let membrane dry after wetting. Rewet in methanol if necessary
4. The gel electrophoresis may be at fault.
    - Artifacts of electrophoresis may be produced by poor polymerization, inappropriate running conditions, contaminated buffers, sample overload, etc

Gel cassette pattern transferred to blot

1. Contaminated or thin fiber pads are used.
    - Replace the fiber pads, or thoroughly clean the contaminated pads
2. Excessive amounts of protein were loaded on the gel, or too much SDS was used in the transfer buffer. Proteins can pass through the membrane without binding, and recirculate through the tank blotting system.
    - Reduce the amount of protein on the gel, and SDS in the transfer buffer. Reduce transfer duration or add a second sheet of membrane to bind excess protein
3. The transfer buffer is contaminated.
    - Make fresh solutions. Transfer buffer solution cannot be reused

Poor binding to the membrane—nitrocellulose

1. Nitrocellulose requires 20% methanol in the transfer buffer for optimal protein binding.
    - Make sure the buffer contains the proper amount of methanol
2. Proteins may be transferring through the nitrocellulose.
    - Use PVDF (higher binding capacities) or 0.2 µm nitrocellulose (smaller pore size). Decrease the voltage if using the high intensity option
3. Mixed ester celluloses bind proteins poorly.
    - Use pure nitrocellulose
4. Proteins <15,000 daltons may show diminished binding to 0.45 µm nitrocellulose, or may be washed from the membrane during assays.
    - To increase stability of binding, proteins can be crosslinked to nitrocellulose with glutaraldehyde
    - Use PVDF membrane, which has higher binding capacities
    - Use Tween-20 detergent in the wash and antibody incubation steps. Reduce or eliminate the more stringent washing conditions
5. SDS in the transfer buffer will reduce binding efficiency of proteins.
    - Reduce or eliminate the SDS from the transfer buffer

6. The membrane may not be completely wet.

    - White spots on the membrane indicate dry areas where protein will not bind. If wetting does not occur immediately by immersion of the sheet in transfer buffer, heat distilled water until just under the boiling point, and soak the membrane until completely wet. Equilibrate in transfer buffer until ready for use

Poor binding to the membrane—PVDF

1. The membrane may not be completely wet.

    - Because of the hydrophobic nature of PVDF, the membrane must be prewet in alcohol prior to equilibration in aqueous transfer buffer. Follow the directions in the product insert

2. The membrane may have been allowed to dry during handling.

    - A completely wet membrane has a gray, translucent appearance. White spots will form on the surface of the membrane, indicating that it has been allowed to dry. Since proteins will not bind to the dry spots, rewet the membrane with methanol and re-equilibrate in transfer buffer

Section 7
Product Information

Catalog Number	Product Description
Mini Trans-Blot® Cell	
170-3930	**Mini Trans-Blot Electrophoretic Transfer Cell,** includes 2 gel holder cassettes, 4 fiber pads, modular electrode assembly, blue cooling unit, lower buffer chamber, and lid with cables
170-3935	**Mini Trans-Blot Module,** same as 170-3930 without lower buffer chamber and lid
170-3989	**Mini Trans-Blot Cell and PowerPac Basic Power Supply**
170-3836	**Mini Trans-Blot Cell and PowerPac HC Power Supply**
Mini Trans-Blot Cell Accessories	
170-3931	**Mini Gel Holder Cassette**
170-3932	**Filter Paper,** 7.5 x 10.5 cm, 50
170-3933	**Fiber Pads,** 8 x 11 cm, 4
170-3934	**Blue Cooling Unit**

Section 8
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