User Manual

TransFactor Kits User Manual

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* "Designated" refers to the Individual, Profiling, and Family Kits.

I. Introduction & Protocol Overview

TransFactor Kits provide rapid, high-throughput detection of transcription factor activities in cell extracts. Using an enzyme-linked immunosorbent assay (ELISA)-based format, the TransFactor kits detect DNA binding by specific transcription factors (Shen *et al.*, 2002). This method is faster, easier, and significantly more sensitive than electrophoretic mobility shift assays (EMSA; Benotmane *et al.,* 1997) and does not require the use of radioactivity.

The TransFactor assay is well suited to various applications. For example, it can be used to investigate what transcription factors might be induced by a stimulus; or to investigate the transcription factor response under a variety of conditions, or to a variety of stimuli; or to study DNA binding by a transcription factor once a cell line has been transfected with a specific gene of interest.

Our TransFactor kits are available in a designated assay format for studying a specific predetermined transcription factor or set of factors. The included Data Sheet (PT3594-3) lists the transcription factors that can currently be studied with designated TransFactor Kits. We also offer Universal TransFactor kits, which provide a flexible assay format for studying any transcription factor .

A. Designated TransFactor Kits

Each designated TransFactor Kit is provided in a 96-well format with oligonucleotides containing the consensus DNA binding sequences for each transcription factor coated on the wells. When cell extracts containing the transcription factors are incubated in the wells, the transcription factors bind to their consensus sequence. Bound transcription factors are then detected by a specific Primary Antibody.

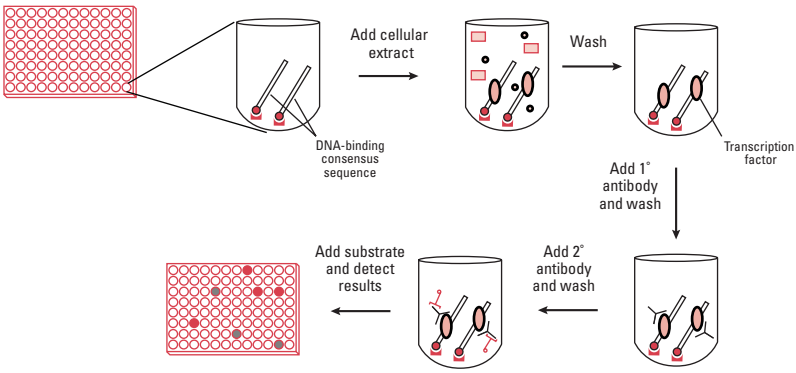

Figure 1. TransFactor method. When samples containing the transcription factors are applied to the wells, the transcription factors recognize their specific consensus DNA binding sequence and bind to form a DNA-transcription factor complex. Those transcription factors are then detected by a specific antibody. Once the Primary Antibody has bound to the transcription factor, horseradish peroxidase (HRP)-conjugated Secondary Antibody detects the complex. HRP reacts with added substrate to produce an enzymatic product that can be measured with an absorbance microtiter plate reader for colorimetric detection or a luminometer for chemiluminescent detection.

I. Introduction & Protocol Overview *continued*

A horseradish peroxidase-conjugated Secondary Antibody is then used to detect the bound Primary Antibody (Figure 1). After substrate is added, the enzymatic product can be measured with an absorbance microtiter plate reader for the colorimetric kits, or a luminometer for the chemiluminescent kits. The **designated** TransFactor kits are available in several formats:

- **Individual Kits:** These kits are most useful for studying a specific transcription factor in detail. In these kits, 80 wells are coated with oligos containing the wild-type consensus binding sequence, and 16 wells are coated with an oligo containing a mutant sequence. The mutant sequence-coated wells serve as a negative control. The results shown in Figure 2 are an example of data that were obtained with the TransFactor NFκB p50 individual kit.
- **Family Kits:** These kits are designed for studying a transcription factor family (Brivanlou & Darnell, 2002). In these kits, all 96 wells of the plate are coated with the same DNA consensus sequence so any well can be used to detect any of the transcription factors that bind to it. The results shown in Figure 3 are an example of data that was obtained with the TransFactor NFκB Family Kit.
- **Profiling Kits:** These kits are more useful for studying multiple transcription factors in parallel, or for helping to identify what transcription factor(s) might be activated by a specific stimulus. Figure 4 shows an example of results that were obtained using the TransFactor Profiling Kit–Inflammation I. These kits are divided into six sets of 16 wells. Each set of wells is coated with oligos containing the wild-type consensus sequence of a different transcription factor.

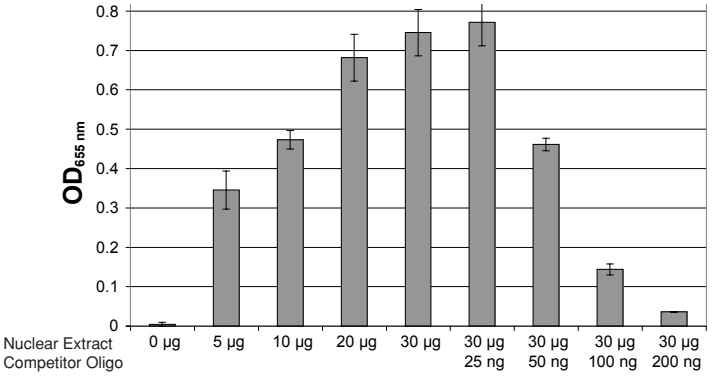

Figure 2. NFκB p50 Kit Positive Control Nuclear Extract binding and Competitor Oligo assay. On the left side of the graph, the quantity of HeLa + TNF-α Positive Control Nuclear Extract correlates to an increase in signal, indicating a corresponding increase in transcription factor DNA-binding activity. On the right side of the graph, increasing the amount of Competitor Oligo corresponds to a decrease in signal because transcription factor binding decreases as it is competed away from the Oligo-coated surface of the TransFactor well.

I. Introduction & Protocol Overview *continued*

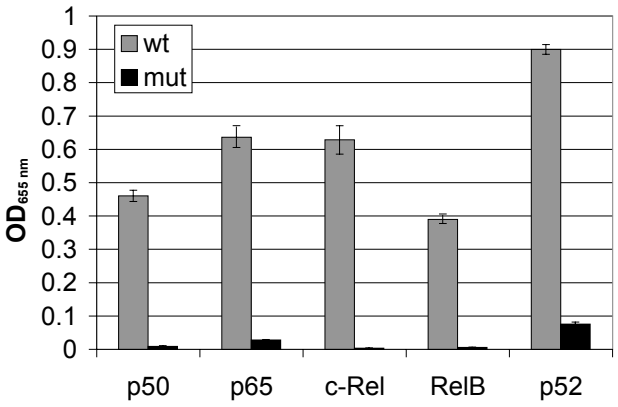

Figure 3. Detection of the NFκB family of transcription factors. The TransFactor Family Kit-NFκB was used to detect DNA binding of NFκB family members. Raji cell nuclear extract (30 µg) was applied to both wild-type DNA oligo-coated wells and mutant DNA oligo-coated wells and analyzed according to the User Manual.

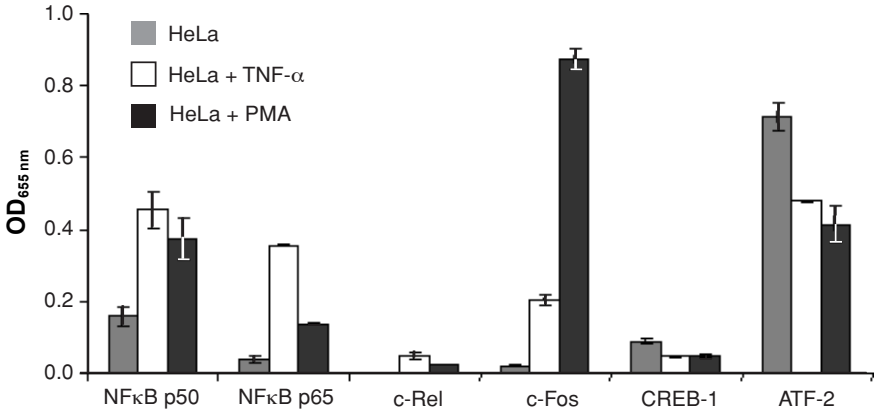

Figure 4. Transcription factor profiling in HeLa nuclear extracts. The TransFactor Profiling Kit–Inflammation I was used to analyze nuclear extracts from HeLa cells, HeLa cells stimulated with TNF-α, and HeLa cells stimulated with PMA. This Profiling Kit detects the indicated transcription factors. The unstimulated HeLa cells exhibit low levels of all transcription factors except ATF-2. HeLa cells stimulated with TNF-α exhibit higher levels of NFκB p50 and NFκB p65, and HeLa cells stimulated with PMA exhibit higher levels of c-Fos.

I. Introduction & Protocol Overview *continued*

B. Universal TransFactor Kits

These kits provide a flexible ELISA-based assay format designed for studying any transcription factor, not just a specific, predetermined factor or set of factors. They are available with either colorimetric or chemiluminescent detection reagents.

The TransFactor Universal assay procedure is shown in Figure 5. First design and synthesize a biotinylated double-stranded DNA oligo containing the consensus binding sequence for the target transcription factor (see Section IV.A). Mix the binding oligo with the cellular extract in a microcentrifuge tube, incubate briefly on ice, and add the mixture to a streptavidin-coated well to capture the transcription factor-oligo complex. Then wash away the unbound proteins and add primary antibody specific for the target transcription factor. Finally, detect the antibody bound to the transcription factor using the horseradish peroxidase-conjugated secondary antibody and a colorimetric or chemiluminescent substrate.

The TransFactor Universal assay makes it possible to detect multiple transcription factors in your sample in a single experiment (with a single plate). It is useful for studying the interactions between different transcription factor pathways. Figure 6 shows an example of results that were obtained using the TransFactor Universal Colorimetric Kit.

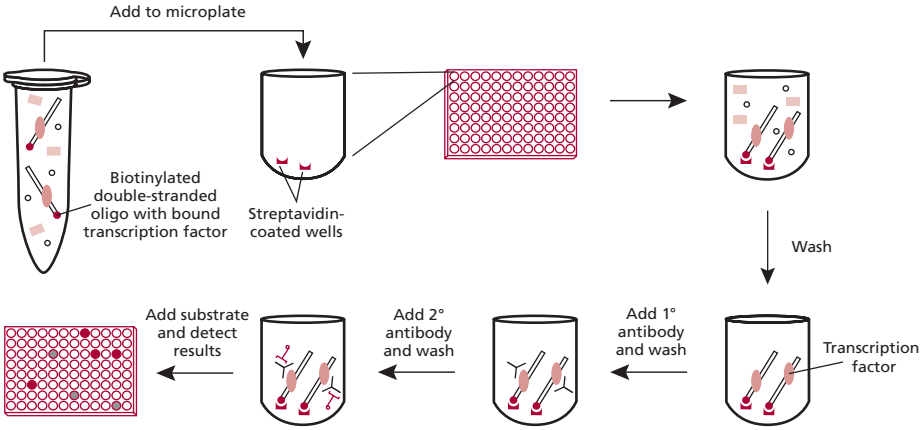

Figure 5. The TransFactor Universal Kits provide a flexible assay format. A sample containing a transcription factor is combined with a biotinylated oligo containing the corresponding consensus DNA binding sequence and incubated to form a DNA-transcription factor complex. The mixture is then applied to a streptavidin-coated well, and a specific antibody is used to detect the transcription factor. Once the primary antibody has bound to the transcription factor, a horseradish peroxidase (HRP)-conjugated secondary antibody detects the complex. After washing, HRP reacts with the added colorimetric or chemiluminescent substrate to produce an enzymatic product that can be detected with a standard absorbance plate reader or a luminometer, respectively.

I. Introduction & Protocol Overview *continued*

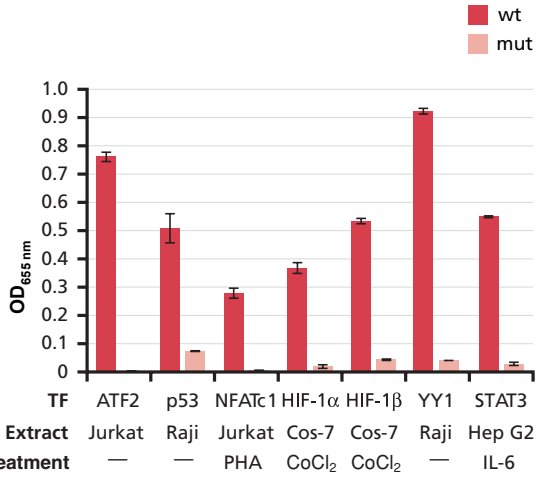

Figure 6. Simultaneous analysis of multiple transcription factors using the TransFactor Universal Colorimetric Kit. Whole cell extracts containing each transcription factor were incubated with 2 pmol of a biotinylated double-stranded oligo containing the corresponding consensus DNA binding sequence. For each assay, oligos containing mutant binding sequences served as negative controls. The following cell extracts were used for assaying the indicated transcription factors: Jurkat cells for ATF2, Raji cells for p53, phytohemagglutinin-treated Jurkat cells for NFATc1, $CoCl_2$-treated Cos-7 cells for HIF-1α and HIF-1β, Raji cells for YY1, and IL-6 treated Hep G2 cells for STAT3. Signal was detected at 655 nm with a Bio-Rad Model 550 microplate reader. The data demonstrates the ability of the TransFactor Universal assay to detect binding of several transcription factors in a single experiment.

The TransFactor Universal kits are available in two formats. Core kits can be used to study any transcription factor. Specific kits are offered as a supplement to the core kits for detecting specific transcription factors.

- **Core Kits**

  Each core kit provides two streptavidin-coated 96-well plates—clear plates for colorimetric assays and white plates for chemiluminescence assays. The kits also include TransFactor binding buffer, blocking reagent, Poly dIdC, colorimetric or chemiluminescent substrate and secondary antibodies. Just add your own biotinylated oligos and primary antibody to detect your transcription factor of interest.

- **Specific Kits**

  Specific kits provide all the additional components you need to study specific transcription factors. These kits contain biotinylated wild-type and mutant binding oligos, a nonbiotinylated wild-type competitor oligo, a primary antibody, and a positive control cellular extract. Our specific kits are optimized for each transcription factor and enable you to design your own plate.

I. Introduction & Protocol Overview *continued*

C. TransFactor Detection Methods

TransFactor kits are available with either colorimetric or chemiluminescent detection reagents.

- **Colorimetric Detection**

  The TransFactor Colorimetric Kits provide sensitive ELISA-based assays that use blue color development for detecting the presence of the transcription factor. Colorimetric detection is ten times more sensitive than traditional gel-shift assays.

  The TransFactor Plates are provided in clear pouches. Each plate permits flexible assay design through the removable, 8-well strips (color-coded, in Pathway Profiling Kits). These strips can be broken apart to provide as many individual assay wells as desired. A TransFactor Rack that fits into any standard microtiter plate reader is provided for holding the separated assay wells. This flexible assay design allows for optimization of sensitivity as well as testing of a dynamic range of parameters.

- **Chemiluminescent Detection**

  Our TransFactor Chemiluminescent Kits are also based on the ELISA technology for detection of transcription factors, but they use chemiluminescence as the detection method. These kits can detect transcription factor binding activity in as little as 0.01 µg of nuclear or whole-cell extract (Figure 7). They are at least ten times more sensitive than their colorimetric counterparts.

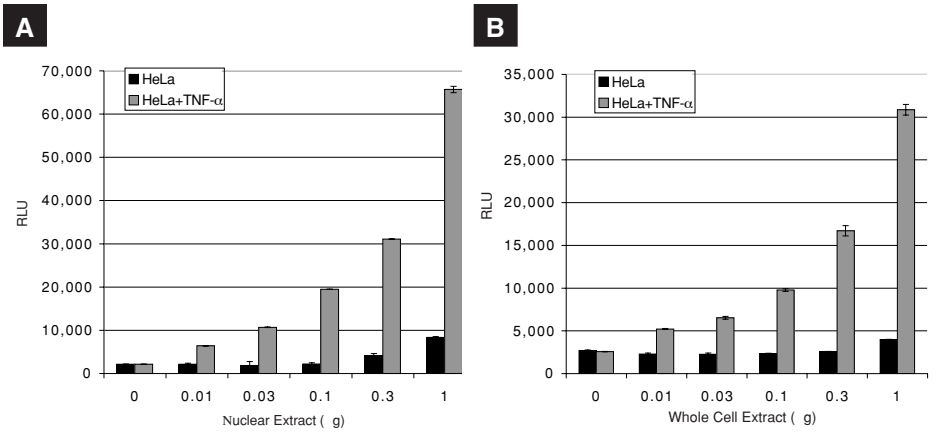

Figure 7. Sensitivity of chemiluminescent detection of NFκB p65. Increasing amounts of uninduced and TNF-α induced HeLa nuclear extracts (**Panel A**) or whole cell extracts (**Panel B**) were tested with the chemiluminescent NFκB Family Kit by following the procedure listed in the User Manual. Chemiluminescent signals were detected by using the Monolight™ 3096 Microplate Luminometer (Cat. No. 551280).

I. Introduction & Protocol Overview *continued*

In these kits, two TransFactor Plates are provided in aluminum pouches. In addition, the Chemiluminescent plates have an 8-well strip configuration instead of the individual snap-off wells of the colorimetric kits. For your convenience, in these kits we provide antibody in excess. This allows you to assay 192 wells of the transcription factor, and also perform a Western blot, if desired.

D. TransFactor Controls

A number of control reagents are provided with each TransFactor Kit. Refer to the Product Analysis Certificate for a complete list of materials provided with your kit.

- **Cell Extract Controls:** Cellular extracts are provided as positive controls along with each TransFactor Kit. These have been tested and shown to have a standard amount of the desired transcription factor DNA-binding activity (Figures 2–4). See the TransFactor Data Sheet (PT3594-3) to determine which extract is recommended for each TransFactor assay. These extracts were generated specifically for the TransFactor assay using our TransFactor Extraction Kits (see Related Products). Extract-to-extract or plate-to-plate comparisons are not recommended unless the lots and methods used for each are identical. When using the Cellular Extract Controls, you may see a loss of DNA-binding activity with repetitive freezing and thawing of those samples.
- **Mutant DNA-Coated Well Control:** The mutant DNA sequence-coated wells are provided in Pathway Profiling Kits as internal negative controls. The transcription factor consensus binding sequence in those wells has been altered so that the factor will bind very weakly to the DNA. Mutant sequence wells provide a background control, eliminating the interference of nonspecific DNA binding from the readings. For Universal kits, Section IV.A contains guidelines for designing biotinylated mutant binding oligos to serve as negative controls.
- **Competitor Oligo Control:** Competitor Oligos are provided in the TransFactor kits for competition assays that demonstrate the binding specificity between DNA and the transcription factor. These Oligos have the same DNA sequence as the oligo-coated wells, and so they act as an effective competitor for transcription factor binding (Figure 2). This assay is similar to the competition assays of EMSA and may be run to demonstrate the specificity of the DNA-protein interactions. However, the Competitor Oligo controls need not be run every time. For Universal kits, Section IV.A contains guidelines for designing biotinylated wild-type binding oligos. Nonbiotinylated forms of these oligos may be synthesized for use in competition assays.

II. List of Components

Store the Positive Control Cellular Extract at –70°C. Avoid repeated freezing and thawing.

Store the Competitor Oligos and TransFactor Buffer at –20°C for long-term storage. They can be stored at 4°C for a month or less.

Store all other components at 4°C.

TransFactor Colorimetric Kits Components

- TransFactor Plate (sealed in a clear pouch)
- TransFactor Rack
- Positive Control Cellular Extract
- Primary Antibody
- Secondary Antibody
- Wild-Type Competitor Oligo
- Poly dIdC (included in some kits)
- TMB Substrate
- 10X TransFactor Buffer
- Blocking Reagent
- Stop Solution (0.009 M Na Azide) included in some kits
- TransFactor Kits Data Sheet (PT3594-3)
- TransFactor Protocol-at-a-Glance (PT3594-2)

TransFactor Chemiluminescent Kit Components

- TransFactor Chemiluminescent Plates (2 plates, sealed in an aluminum pouch)
- TransFactor Rack
- Positive Control Cellular Extract
- Primary Antibody
- Secondary Antibody
- Wild-Type Competitor Oligo
- Poly dIdC (included in some kits)
- 10X TransFactor Buffer
- Blocking Reagent
- Chemiluminescent Substrate A
- Chemiluminescent Substrate B
- TransFactor Kits Data Sheet (PT3594-3)
- Chemiluminescent TransFactor Protocol-at-a-Glance (PT3757-2)

II. List of Components *continued*

TransFactor Universal Colorimetric Kit Components

- Streptavidin-coated 96-well plates (2)
- TransFactor Rack
- Secondary Antibodies
  - — Anti-mouse secondary antibody
  - — Anti-rabbit secondary antibody
- Poly dIdC
- TMB Substrate
- 10X TransFactor Buffer
- Blocking Reagent
- TransFactor Kits Data Sheet (PT3594-3)
- TransFactor Protocol-at-a-Glance (PT3594-2)

TransFactor Universal Chemiluminescent Kit Components

- Streptavidin-coated 96-well plates (2)
- TransFactor Rack
- Secondary Antibodies
  - — Anti-mouse secondary antibody
  - — Anti-rabbit secondary antibody
- Poly dIdC
- Chemiluminescent Substrate A
- Chemiluminescent Substrate B
- 10X TransFactor Buffer
- Blocking Reagent
- TransFactor Kits Data Sheet (PT3594-3)
- TransFactor Protocol-at-a-Glance (PT3757-2)

TransFactor Universal Specific Kit Components

- Positive Control Cellular Extract
- Primary Antibody
- Biotinylated Wild-Type Binding Oligo
- Biotinylated Mutant Binding Oligo
- Wild-Type Competitor Oligo
- TransFactor Kits Data Sheet (PT3594-3)
- TransFactor Protocol-at-a-Glance (PT3594-2)
- TransFactor Protocol-at-a-Glance (PT3757-2)

III. Additional Materials Required

The following materials are required but not supplied:

- **TransFactor Nuclear Extraction Kit** (Cat. No. 631921)
  For preparation of nuclear or cytosolic extracts
- **TransFactor Whole Cell Extraction Kit** (Cat. No. 631946)
  For preparation of whole cell extracts
- **Pipettor**
- **Pipette tips**
- **Multichannel pipet**
- **Distilled water**
- **Whatman filter paper** (Folded grade 113V; Whatman Cat. No. 1213-125)

Additional Materials Required for TransFactor Colorimetric Kits

- **Microtiter plate reader** (We recommend Bio-Rad Model 550)
- **Optional**

  — 0.009 M Na Azide (stop solution, for kits that do not include it)

  — 1 M H_2SO_4 (alternative stop solution, recommended for certain assays as described in the Data Sheet)

Additional Materials Required for TransFactor Chemiluminescent Kits

- **Luminometer** (We recommend the Monolight™ 3096 Microplate Luminometer (Cat. No. 551280). Multifunctional readers such as the Gemini System from Molecular Devices are not recommended for detection.)

IV. General Considerations for Universal Kits

A. Design and Synthesis of Specific DNA-Binding Oligos

1. Wild-Type Binding Oligo Design Guidelines

   Each DNA-binding oligo contains a specific transcription factor-binding consensus sequence and a short flanking sequence on both the 5' and 3' ends of the consensus sequence. The flanking region consists of a short DNA sequence normally ranging from 3 to 10 base pairs, which does not contain binding sites for other transcription factors. To determine the consensus binding sequence, refer to the following sources:

- For the specific transcription factors detected by our designated TransFactor Kits, we have listed the binding consensus sequences on the Data Sheet provided with each TransFactor kit (PT3594-3).
- If you are interested in a factor that is not listed on our Data Sheet, or you are seeking alternative binding sites, the binding sequence information may be obtained from the scientific literature or from transcription factor binding sequence databases.
- Recommended databases include the commercially available TransFac Database from BioBase Biological Databases (Wolfenbüttel, Germany) at http://www.biobase.de/ and the public databases at **http://www.cbrc.jp/research/db/TFSEARCH.htm** and at **http://www.modor.cgb.ki.se/sgi-bin/jaspar2005/jaspar_db.pl** In all of these databases, a field labeled MATRIX lists the highly conserved binding sequence for each transcription factor compiled from mutiple known binding sequences. The consensus sequence is the portion that is very highly conserved. MATRIX also includes some flanking sequences that are not as highly conserved. The public JASPAR database at **http://www.modor.cgb.ki.se/sgi-bin/jaspar2005/ jaspar_db.pl** is an open-access database of annotated, high-quality, matrix-based transcription factor binding factors binding site profiles for eukaryotes developed by the Center for Genomics and Bioinformatics, Karolinska Instituret, Stockholm, Sweden (Sandelin, et at., 2004). This information may be used aas a basis for designing your oligos.
- **An oligo that contains two concatenated copies of the binding sequence produces a stronger binding signal than a single copy** Increasing the sequence copy number may not necessarily raise the binding efficiency any further (data not shown).

IV. General Considerations for Universal Kits *continued*

2. Control Oligo Design Guidelines

- Mutant Binding Oligos: Mutant binding oligos serve as negative controls for the binding assay. To design a mutant binding oligo, replace the most highly conserved nucleotides in the consensus sequence (which are most likely to be the nucleotides that interact directly with the transcription factor) with other nucleotides. To make the mutant oligo a good negative control, it is best to limit the number of nucleotides that are changed to no more than 4 within a given DNA binding site.
- Wild Type Competitor Oligos: A wild-type competitor oligo has the same sequence as a wild-type binding oligo, but it is not biotinylated.

3. Oligo Synthesis and Annealing

Generate a double-stranded DNA-binding oligo as follows:

a. After the DNA-binding consensus sequence is determined, synthesize two complementary oligos. Biotinylate one of the two oligos at its 5' end.

b. Combine the two complementary oligos at an equimolar ratio and an approximate concentration of 100 µM, in a volume of 100–500 µl.

c. Heat the oligo mixture at 95°C for 10 min in a microcentrifuge tube in a heating block, and allow the block containing the mixture to cool down slowly to room temperature.

d. After diluting the double-stranded oligo to its desired concentration (see Section V.C.3 or VI.C.3), it is ready for use.

B. Optimization of Primary Antibody Dilutions

You may use either your own transcription factor-specific antibodies or commercially available transcription factor-specific antibodies in TransFactor Universal assays.

1. To optimize the dilution factor for a primary antibody, begin with the following dilutions:

- For colorimetric assays, use a 1:100 dilution.
- For chemiluminescent assays, use a 1:500 dilution.

2. Based on your results, increase or reduce the dilution of your antibodies as appropriate.

V. Colorimetric TransFactor ELISA Procedure

A. General

- Microtiter plate readers frequently require a background control when reading the absorbances of the wells. A background control consists of a well containing TransFactor/Blocking Buffer (and no cellular extract sample). This control is useful to provide a normalization control against which the plate reader compares the other wells.
- Quantitative results may require more purified samples than the Cellular Extracts provided. The Positive Control Cellular Extract sample provides a positive control. However, for quantitative results, the appropriate purified transcription factor can be used to generate a standard curve.
- Optimize the TransFactor assay for your samples by plotting a dose response curve (see Figure 2 for an example). Competition assays can also be optimized by adding different amounts of Competitor Oligo.
- The color development time may require optimization to meet your particular assay requirements.
- The microtiter plate wells can be read at 655 nm. If desired (optional), add Stop Solution (0.009 M Na Azide), which is included in some kits, to stop the reaction before measuring the absorbance. If no Stop Solution is added, the same wells can be read at various time points after addition of TMB substrate to determine the optimal time for color development.
- As an alternative to Na Azide, 1 M H_2SO_4 can also be used as a stop solution. However, samples stopped with 1 M H_2SO_4 must be read at 450 nm. This stop solution is recommended for certain TransFactor assays because it yields threefold higher readings at 450 nm than the same samples stopped with Na Azide and read at 655 nm.
- Refer to Table II on the TransFactor Data Sheet (PT3594-3) to determine the appropriate stop solution for your assay.
- To prepare nuclear extracts, we recommend using the TransFactor Nuclear Extraction Kit (Cat. No. 631921). To prepare whole cell extracts, we recommend using the TransFactor Whole Cell Extraction Kit (Cat. No. 631946).

B. Prepare 1X TransFactor Buffer and Block Wells

1. Determine the amount of 1X TransFactor Buffer required:

   (No. assay wells) x 3.0 ml = Total Volume TransFactor Buffer

   Dilute the 10X TransFactor Buffer 1:10 with distilled water to obtain the above volume.

V. Colorimetric TransFactor ELISA Procedure *continued*

2. Prepare 1X TransFactor/Blocking Buffer:

1.8	ml/assay	1X TransFactor Buffer
0.06	g/assay	Blocking Reagent
1.8	ml/assay	Total Volume TransFactor/Blocking Buffer

Notes

- Mix the Blocking Reagent with the 1X TransFactor Buffer until the Blocking Reagent completely dissolves, then filter the TransFactor/Blocking Buffer through Whatman filter paper before use.
- Keep the remaining 1.2 ml/assay of 1X TransFactor Buffer to use as wash after the Secondary Antibody incubation.

C. Add Sample to TransFactor Well

1. Thaw the nuclear or whole cell extract slowly on ice.

   Note: After the nuclear or whole cell extract is thawed, we recommend centrifuging the sample at 20,000 g for 5 min at 4°C to remove residual cell debris. Including this step will decrease the variability of your results.

2. Proceed to Step 3 for designated TransFactor kits or Step 4 for TransFactor Universal Kits.

3. **For designated TransFactor Kits only:**

   a. Add 150 µl 1X TransFactor Blocking Buffer per well and incubate at room temperature for 15 min.

   b. Prepare sample by diluting the desired amount of cell extract to a final volume of 50 µl with 1X TransFactor/Blocking Buffer. Add the appropriate amount of Poly dIdC when required (see PT3594-3). Proceed to Step 5.

   Notes

   - Optimal extract concentration may vary depending on the transcription factor, cell type, and extraction method. To optimize the assay, perform a dose response curve with your cellular extract (see Figure 2). We find that 30 µg of the provided Positive Control Cellular Extract usually works well as a starting point; however, some extracts perform better at lower concentrations.
   - For a background control, use 50 µl 1X TransFactor/Blocking Buffer alone.
   - Optimal Competitor Oligo concentration may vary depending on the transcription factor. For competition assays, add 1 µl Competitor Oligo (500 ng) to the sample and reduce the 1X TransFactor/Blocking Buffer volume sufficiently to maintain a total assay volume of 50 µl. If this does not generate an adequate decrease, then add more Competitor Oligo in subsequent competition assays.

V. Colorimetric TransFactor ELISA Procedure *continued*

4. **For TransFactor Universal Kits only:**
   a. Prepare sample by mixing the desired amounts of cell extract and Poly dIdC (see Notes below) with 2 pmol biotinylated oligo, and diluting the mixture to a final volume of 50 µl with 1X TransFactor/Blocking Buffer.

      Notes

      - Optimal extract concentration may vary depending on the transcription factor, cell type, and extraction method. To optimize the assay, perform a dose response curve with your cellular extract (see Figure 2). We find that 30 µg of the provided Positive Control Cellular Extract usually works well as a starting point; however, some extracts perform better at lower concentrations.
      - For a background control, use 50 µl 1X TransFactor/Blocking Buffer alone.
      - Optimal Competitor Oligo concentration may vary depending on the transcription factor. For competition assays, add 20 pmol Competitor Oligo to the sample and reduce the 1X TransFactor/Blocking Buffer volume sufficiently to maintain a total assay volume of 50 µl. If this does not generate an adequate decrease, then add more Competitor Oligo in subsequent competition assays.
      - The biotinylated oligo can be a wild-type or mutant oligo.
      - Optimal Poly dIdC concentration can vary with different transcription factors. We find that 0.5 µg of Poly dIdC per reaction is a good starting point.
      - When multiple repeats of the same binding assay are desired, you can multiplex the same amount and mix in the same tube.

   b. Incubate the samples on ice for 15 min.
   c. Meanwhile, add 150 µl of 1X TransFactor/Blocking Buffer per well and incubate at room temperature for 15 min. Proceed to Step 5.
5. Remove the 1X TransFactor/Blocking Buffer.
6. Add the 50 µl sample to the well, and incubate for 60 min at room temperature.
7. Wash the wells 3 times with 150 µl 1X TransFactor/Blocking Buffer per well. Allow 4 min for each wash. After the final wash, remove 1X TransFactor/Blocking Buffer from the wells.

D. Incubate with Primary Antibody

1. Dilute Primary Antibody in 1X TransFactor/Blocking Buffer (see Data Sheet for required dilutions). Dilute sufficient Primary Antibody to yield 100 µl per assay well.
2. Add 100 µl of diluted Primary Antibody to each well. Incubate at room temperature for 60 min.
3. Wash the wells 4 times with 150 µl 1X TransFactor/Blocking Buffer per well. Allow 4 min for each wash. After the final wash, remove 1X TransFactor/Blocking Buffer from the wells.

V. Colorimetric TransFactor ELISA Procedure *continued*

E. Incubate with Secondary Antibody

1. Dilute Secondary Antibody in 1X TransFactor/Blocking Buffer (1:1,000 dilution for Anti-rabbit IgG-HRP and 1:200 dilution for Anti-mouse IgG-HRP). Refer to the Data Sheet to identify the correct Secondary Antibody for your assay.
2. Add 100 µl of diluted Secondary Antibody to each assay well and incubate at room temperature for 30 min.
3. Wash the wells 4 times with 250 µl of **1X TransFactor Buffer** per well (no Blocking Reagent). Allow 4 min for each wash. After the final wash, remove 1X TransFactor Buffer from the wells.

   Note: The use of 1X TransFactor/Blocking Buffer for this wash increases background. Therefore, only use 1X TransFactor Buffer, without Blocking Reagent, after the Secondary Antibody incubation.

F. Colorimetric Development

1. Add 100 µl of TMB substrate to each well. Incubate at room temperature for 10 min.

   Note: The color development time may have to be adjusted in order to optimize readings (see Section V.A).
2. After seeing the blue color develop, measure the absorbance of the plate at 655 nm with a microtiter plate reader (We recommend the Bio-Rad Model 550 microplate reader).

   Notes

   • If desired (optional), add 100 µl per well of Stop Solution (0.009 M Na Azide; included in some kits) to stop the reaction before measuring the absorbance.

   • 1 M H_2SO_4 (100 µl per well) can also be used as a stop solution and is recommended for some assays. In these cases, the absorbance should be read at 450 nm. (See Section V.A as well as the TransFactor Data Sheet PT3594-3 for details.)

VI. Chemiluminescent TransFactor ELISA Procedure

Before you start:

- To prepare nuclear extracts, we recommend using the TransFactor Nuclear Extraction Kit (Cat. No. 631921). To prepare whole cell extracts, we recommend using the TransFactor Whole Cell Extraction Kit (Cat. No. 631946).

A. Prepare 1X TransFactor Buffer and Block Wells

1. Determine the amount of 1X TransFactor Buffer required: (No. assay strips) x 24.0 ml = Total Volume TransFactor Buffer Dilute the 10X TransFactor Buffer 1:10 with distilled water to obtain the above volume.
2. Prepare 1X TransFactor/Blocking Buffer:

1.8	ml/assay	1X TransFactor Buffer
0.06	g/assay	Blocking Reagent
1.8	ml/assay	Total Volume TransFactor/Blocking Buffer

Notes

- Mix the Blocking Reagent with the 1X TransFactor Buffer until the Blocking Reagent completely dissolves, then filter the TransFactor/Blocking Buffer through Whatman filter paper before use.
- Keep the remaining 1.2 ml/assay of 1X TransFactor Buffer to use as wash after the Secondary Antibody incubation.

B. Add Sample to TransFactor Well

1. Thaw the nuclear or whole cell extract slowly on ice.

   Note: After the nuclear or whole cell extract is thawed, we recommend centrifuging the sample at 20,000 g for 5 min at 4°C to remove residual cell debris. Including this step will decrease the variability of your results.

2. Proceed to Step 3 for designated TransFactor kits or Step 4 for TransFactor Universal Kits.
3. **For designated TransFactor Kits only:**
   a. Add 150 µl 1X TransFactor Blocking Buffer per well and incubate at room temperature for 15 min.
   b. Prepare sample by diluting the desired amount of cell extract to a final volume of 50 µl with 1X TransFactor/Blocking Buffer. Add the appropriate amount of Poly dIdC when required (see PT3594-3). Proceed to Step 5.

   Notes

   - Optimal extract concentration may vary depending on the transcription factor, cell type, and extraction method. To optimize the assay, perform a dose response curve with your cellular extract (see Figure 2). We find that 5 µg of the provided Positive Control Cellular Extract usually works well as a starting point; however, some extracts perform better at lower concentrations.
   - For a background control, use 50 µl 1X TransFactor/Blocking Buffer alone.

VI. Chemiluminescent TransFactor ELISA Procedure *cont.*

- Optimal Competitor Oligo concentration may vary depending on the transcription factor. For competition assays, add 1 µl Competitor Oligo (500 ng) to the sample and reduce the 1X TransFactor/Blocking Buffer volume sufficiently to maintain a total assay volume of 50 µl. If this does not generate an adequate decrease, then add more Competitor Oligo in subsequent competition assays.

4. **For TransFactor Universal Kits only:**

   a. Prepare sample by mixing the desired amounts of cell extract and Poly dIdC (see Notes below) with 2 pmol biotinylated oligo, and diluting the mixture to a final volume of 50 µl with 1X TransFactor/Blocking Buffer.

   Notes

   - Optimal extract concentration may vary depending on the transcription factor, cell type, and extraction method. To optimize the assay, perform a dose response curve with your cellular extract (see Figure 2). We find that 5 µg of the provided Positive Control Cellular Extract usually works well as a starting point; however, some extracts perform better at lower concentrations.
   - For a background control, use 50 µl 1X TransFactor/Blocking Buffer alone.
   - Optimal Competitor Oligo concentration may vary depending on the transcription factor. For competition assays, add 20 pmol Competitor Oligo to the sample and reduce the 1X TransFactor/Blocking Buffer volume sufficiently to maintain a total assay volume of 50 µl. If this does not generate an adequate decrease, then add more Competitor Oligo in subsequent competition assays.
   - The biotinylated oligo can be a wild-type or mutant oligo.
   - Optimal Poly dIdC concentration can vary with different transcription factors. We find that 0.5 µg of Poly dIdC per reaction is a good starting point.
   - When multiple repeats of the same binding assay are desired, you can multiplex the same amount and mix in the same tube.

   b. Incubate the samples on ice for 15 min.

   c. Meanwhile, add 150 µl of 1X TransFactor/Blocking Buffer per well and incubate at room temperature for 15 min. Proceed to Step 5.

5. Remove the 1X TransFactor/Blocking Buffer.
6. Add the 50 µl sample to the well, and incubate for 60 min at room temperature.
7. Wash the wells 3 times with 150 µl 1X TransFactor/Blocking Buffer per well. Allow 4 min for each wash. After the final wash, remove 1X TransFactor/Blocking Buffer from the wells.

VI. Chemiluminescent TransFactor ELISA Procedure *cont.*

C. Incubate with Primary Antibody

1. Dilute Primary Antibody in 1X TransFactor/Blocking Buffer (see Data Sheet for required dilutions). Dilute sufficient Primary Antibody to yield 100 µl per assay well.
2. Add 100 µl of diluted Primary Antibody to each well. Incubate at room temperature for 60 min.
3. Wash the wells 3 times with 150 µl 1X TransFactor/Blocking Buffer per well. Allow 4 min for each wash. After the final wash, remove 1X TransFactor/Blocking Buffer from the wells.

D. Incubate with Secondary Antibody

1. Dilute Secondary Antibody in 1X TransFactor/Blocking Buffer (1:10,000 dilution for Anti-rabbit IgG-HRP and 1:2,000 dilution for Anti-mouse IgG-HRP). Refer to the Data Sheet to identify the correct Secondary Antibody for your assay.
2. Add 100 µl of diluted Secondary Antibody to each assay well and incubate at room temperature for 30 min.

   Note: Remove Chemiluminescent Substrates from the refrigerator and allow them to come to room temperature before use.
3. Wash the wells 4 times with 250 µl of **1X TransFactor Buffer** per well (no Blocking Reagent). Allow 4 min for each wash. After the final wash, remove 1X TransFactor Buffer from the wells.

   Note: The use of 1X TransFactor/Blocking Buffer for this wash increases background. Therefore, only use 1X TransFactor Buffer, without Blocking Reagent, after the Secondary Antibody incubation.

E. Chemiluminescent Signal Development

1. Calculate the total volume of chemiluminescent substrate by multiplying the number of wells in the assay by 100 µl/well.
2. Mix the appropriate volume of chemiluminescent substrate by mixing 1/2 volume Substrate A with 1/2 volume Chemiluminescent Substrate B.

   Note: Allow the Chemiluminescent Substrates to come to room temperature before use.
3. After mixing, allow the substrate mixture to incubate at room temperature for at least 2 min before adding it to the wells.
4. Add 100 µl of mixed substrate to each well.
5. Measure chemiluminescent intensities with a luminometer. For highest sensitivity, read the signals immediately after adding the substrate.

   Notes

   • The signals are stable within10 minutes (See Figure 8 in Section VIII).

   • We recommend the Monolight 3096 Microplate Luminometer (Cat. No. 551280).

VII. Troubleshooting Guide for Colorimetric Kits

A. Lack of signal or weak signal in all wells

Upon development, readings at 655 nm are either absent or lower than desirable.

Possible explanations:

- Omission of a reagent or a step.
- Improper preparation or storage of a reagent (e.g., overdilution of the Primary Antibody).
- Reagent expired (e.g., 1X TransFactor buffer should not be stored for extended periods at 4°C).
- Assay performed before allowing reagents to fully thaw.
- Low volumes of reagents were used.
- Low sample concentration: Sample was overdiluted or nuclear extracts contained less than the optimal total protein concentration.
- Plate reader did not perform well (e.g., Plate reader UV light is not warmed up).

B. High signal and background in all wells

Possible explanations:

- Improper or inadequate washing: e.g., 1X TransFactor/Blocking Buffer should not be used for washing after the Secondary Antibody incubation. 1X TransFactor Buffer should be used instead. Make sure that the recommended volumes and number of repetitions of washes were used.
- Improper dilution of antibody.
- Overdeveloping: Decrease the incubation time before the Stop Solution is added.

C. High background in sample wells

Possible explanations:

- Sample concentration too high: Cellular extracts of approximately 5 µg/µl total protein concentration usually work well (see Data Sheet). It is best to adjust the concentration before the sample is frozen. Optimize by generating a dose response curve with your sample.
- Improper dilution of antibody.

D. Weak signal in sample wells

Possible explanations:

- Sample concentration too low: A cellular extract concentration of approximately 5 µg/µl total protein usually gives an adequate signal if active transcription factors are present. However, low transcription factor levels or poor sample preparation could result in a false negative. Optimize by performing a dose response curve with your sample.
- Improper dilution of antibody.

VIII. Troubleshooting Guide for Chemiluminescent Kits

A. Lack of signal or weak signal in all wells

Upon development, readings are either absent or lower than desirable.

Possible explanations:

- Omission of a reagent or a step.
- Improper preparation or storage of a reagent (e.g., overdilution of the Primary Antibody).
- Reagent expired (e.g., 1X TransFactor buffer should not be stored for extended periods at 4°C).
- Assay performed before allowing Chemiluminescent Substrates to come to room temperature.
- Low volumes of reagents were used.
- Low sample concentration: Sample was overdiluted or cellular extracts contained less than the optimal total protein concentration.
- Luminometer did not perform well.

B. High signal and background in all wells

Possible explanations:

- Luminometer did not perform well.
- Improper or inadequate washing: e.g., 1X TransFactor/Blocking Buffer should not be used for washing after the Secondary Antibody Incubation. 1X TransFactor Buffer should be used instead. Make sure that the recommended volumes and number of repetitions of washes were used.
- Improper dilution of antibody.
- Overdeveloping: Decrease the incubation time.

C. High background in sample wells

Possible explanations:

- Sample concentration too high: Cellular extracts of approximately 5 µg/µl total protein concentration usually work well (see Data Sheet). It is best to adjust the concentration before the sample is frozen. Optimize by generating a dose response curve with your sample.
- Improper dilution of antibody.

D. Weak signal in sample wells

Possible explanations:

- Sample concentration too low: A cellular extract concentration of approximately 5 µg/µl total protein usually gives an adequate signal if active transcription factors are present. However, low transcription factor levels or poor sample preparation could result in a false negative. Optimize by performing a dose response curve with your sample.
- Improper dilution of antibody.

VIII. Troubleshooting Guide for Chemiluminescent Kits *cont.*

E. Sensitivity is low.

Possible explanations:

- Luminometer did not perform well.
- Did not read signal fast enough. In order to obtain the best sensitivity, we recommend reading the signals as soon as the substrate is added. To obtain the best signals, multiple readings may be performed. We find that the signals are stable within 10 min after adding the substrate (Figure 8).

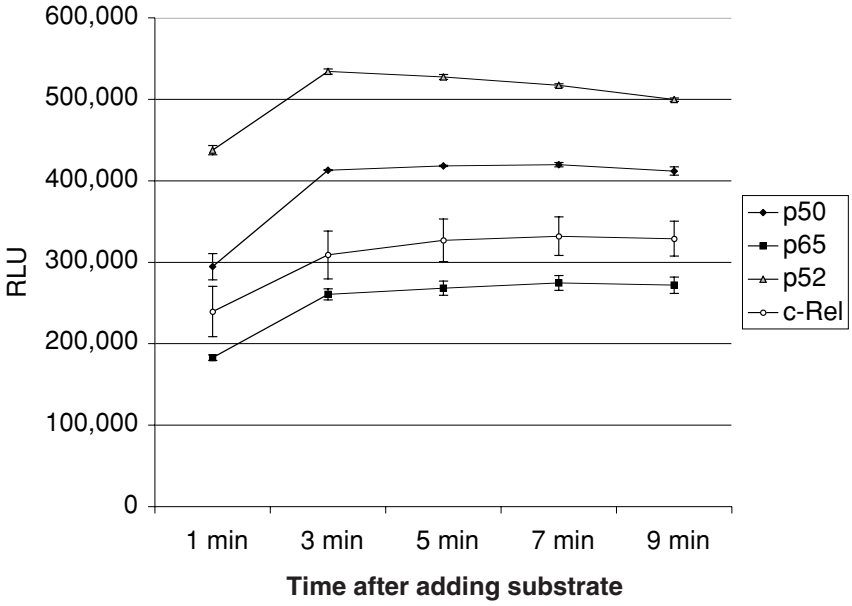

Figure 8. Stability of chemiluminescent signals. Raji nuclear extract (1 µg) was used to test chemiluminescent signal stability with the chemiluminescent NFκB Family Kit. Signals for each factor were detected at the indicated times.

IX. Troubleshooting Guide for Universal Kits

A. Lack of signal or weak signal in all wells

Possible explanations:

- Improper design of binding oligo. Refer to Section IV.A for oligo design instructions.
- Primary antibody is too dilute. Increase the concentration used in the antibody incubation step.
- Primary antibody does not function in the binding reaction. Choose a different primary antibody.
- Poly dIdC concentration used in the binding assay is too high. Omit Poly dIdC or use a lower concentration in the experiment.
- No activity in cellular extract. This may be due to improper or inefficient induction of the cells, or improper isolation or storage of the cellular extract. Check the literature for the appropriate cell induction reagent and kinetics, and use our recommended nuclear or whole cell extraction kits (see Section III).

B. High signal in mutant wells

Possible explanations:

- Improper mutant oligo design. Refer to Section IV.A for oligo design instructions.
- Primary antibody is too concentrated. Increase the dilution factor of the primary antibody.
- Primary antibody does not function in the binding reaction. Choose a different primary antibody.
- Insufficient Poly dIdC. Increase the amount of Poly dIdC in each reaction.
- Improper isolation of the cellular extract.

C. No competition, or low competition

Possible explanations:

- Improper design of oligo. Refer to Section IV.A for oligo design instructions.
- Primary antibody is too concentrated. Increase the dilution factor of the primary antibody.
- Primary antibody does not function in the binding reaction. Choose a different primary antibody.
- Insufficient Poly dIdC. Increase the amount of Poly dIdC in each reaction.
- Improper isolation of the cellular extract.

X. References

Benotmane, A. M., Hoylaerts, M. F., Collen, D. & Belayew, A. (1997) Nonisotopic quantitative analysis of protein-DNA interactions at equilibrium. *Anal. Biochem.* **250**:181–185.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**:248-54.

Brivanlou, A. H. and Darnell, J. E. (2002) Signal Transduction and the control of gene expression. *Science* **295**:813–818.

Dignam, J. D., Lebovitz, R. M. & Roeder R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475–89.

Dyer, R. B. & Herzog, N. K. (1995) Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. *BioTechniques* **19**:192–195.

Feifel, E., Obexer, P., Andratsch, M., Euler, S., Taylor, L., Tang, A., Wei, Y., Schramek, H., Curthoys, N. P. & Gstraunthaler, G. (2002) p38 MAPK mediates acid-induced transcription of PEPCK in LLC-PK1-FBPase$^+$ cells. *Am. J. Physiol. Renal Physiol.* **283:**678–688.

Fiorucci, S., Antonelli, E., Distrutti, E., Del Soldato, P., Flower, R. J., Clark, M. J. P., Morelli, A., Perretti, M. & Ignarro, L. J. (2002) NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice. *Proc. Natl. Acad. Sci.* **99**:15770–15775.

Hehner, S. P., Heinrich, M., Bork, P. M., Vogt, M., Ratter, F., Lehmann, V., Schulze-Osthoff, K., Droge, W., and Schmitz, M. L. (1998) Sesquiterpene lactones specifically inhibit activation of NFκB by preventing the degradation of IκB-α and IκB-β. *J. Biol. Chem.* **273**:1288–1297.

Lee, K. A., Bindereif A. & Green, M. R. (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene. Anal. Tech.* **5**:22–31.

O'Sullivan, B. J. & Thomas, R. (2002) CD40 Ligation Conditions Dendritic Cell Antigen-Presenting Function Through Sustained Activation of NFkB. *J. Immunol.* **168**:5491–5498.

Sandelin, A., Alkema, W. Engstrom, P., Wasserman, W.W., and Lenhard, B. (2004) JASPAR: an open-access database for eukaryotic transcription factor profiles. *Nucleic Acids Res.* **32**: D91-D94.

Shen, Z., Peedikayil, J., Olson, G. K., Siebert, P. D., and Fang , Y. (2002) Multiple transcription factor profiling by enzyme-linked immunoassay. *BioTechniques* **32**(5):1168–1177.

XI. Related Products

For a complete listing of all Clontech products, please visit:
www.clontech.com

Product	Cat. No.
TransFactor Related Products	
• Universal STAT3 Specific Kit	631958
• Universal STAT1 Specific Kit	631959
• Universal c-Jun Specific Kit	631960
• TransFactor Nuclear Extraction Kit	631921
• TransFactor Whole Cell Extraction Kit	631946
• CalPhos™ Mammalian Transfection Kit	631312
• Clonfectin™ Transfection Reagent	631301
• Great EscAPe™ SEAP Chemiluminescence Detection Kit	631701 631702
• Great EscAPe™ SEAP Fluorescence Detection Kit	631704
• Luciferase Reporter Assay Kit	631714
TransFactor Colorimetric Kits	
• NFκb p50 Kit	631916
• NFκb p52 Kit	631949
• NFκb p65 Kit	631930
• NFκb Family Kit	631945
• STAT3 Kit	631953
• NFATc1 Kit	631954
• Universal Kit	631956
TransFactor Chemiluminescent Kits	
• NFκb p50-p65 Kit	631947
• NFκb Family	631948
• STAT3 Kit	631952
• NFATc1 Kit	631955
• Universal Kit	631957

XI. Related Products *continued*

Product	Cat. No.
TransFactor Colorimetric Profiling Kits	
• Inflammation 1	631919
• Inflammation 2	631935
• Oncogenesis 1	631936
• Oncogenesis 2	631937
• Oncogenesis 3	631938
Pathway Profiling Kits	
• SEAP System	631910
• SEAP System 2	631920
• Luciferase System 1	631911
• Luciferase System 2	631912
• Luciferase System 3	631913
• Luciferase System 4	631914
• Luciferase System 5	631915
Reporter and Control Vectors	
• pAP1(PMA)-TA-Luc Vector	631906
• pAP1(PMA)-SEAP Vector	631907
• pAP1-SEAP Vector	631903
• pSRE-SEAP Vector	631901
• pGRE-SEAP Vector	631902
• pNFκB-SEAP Vector	631905
• pNFκB-Luc Vector	631904
• pCMV-β Vector	631719
• pSEAP2-Control	631717
• pAcGFP1-C1 Vector	632470
• pAcGFP1-N1 Vector	632469

XI. Related Products *continued*

Product	Cat. No.
Dominant Negative Vector Sets	
• p53	631922
• CREB	631925
• Ras	631924
• Raf	631926
• IκBα	631923
NucleoBond® Plasmid Kits	
• Midi Kit	635929 635930 635931
• Maxi Kit	635933 635934 635935

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