

# A Method for Simultaneously Measuring the Activation of Multiple Transcription Factors in Nuclear Extracts Prepared from Stimulated Whole Blood

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## Introduction

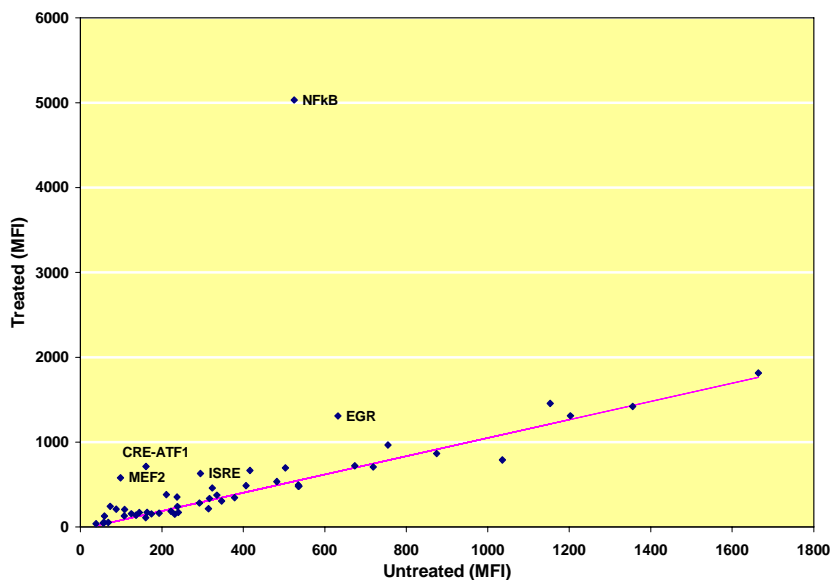
Regulation of gene expression is controlled by the binding of transcription factors to specific DNA sequences in gene promoter regions. Multiple transcription factor binding events are involved in the regulation of cellular processes. Traditional methods such as gel shift (EMSA), gene reporter assays and immunoassays measure the DNA-binding activity of one transcription factor in a single sample and provide limited information regarding the control of the cellular processes controlling gene expression. Described here is a method to simultaneously measure the binding activity of 50 different transcription factors in a single sample of nuclear extracts prepared from treated whole blood. The results obtained using this method gives a more complete picture of the regulatory mechanisms involved in the biological response than can be obtained with traditional methods. Whole blood provides a valuable sample to monitor the control of biological processes since it gives an indication of the “health” of the immune system and is readily available from individuals. Whole blood cells can also be taken from all animal models and is used, among other applications, to monitor disease states and drug therapies. In the following application note whole blood was treated with PMA plus ionomycin, and nuclear extracts were analyzed for changes in the binding activity of transcription factors to their cognate recognition sequences using Marligen’s 50-plex transcription factor testing service assay.

## Method and Experimental Results

Briefly, whole blood was collected in heparinized tubes, diluted in RPMI 1640, and treated for 48 hours with PMA plus ionomycin. The blood was incubated in the presence of 100 nM PMA plus 500 nM ionomycin for 48 hours. Untreated whole blood was used as a control. Nuclear extracts were prepared using Marligen’s Nuclear Extraction Kit (Cat. No. 11906-100) and 10 µg of each nuclear extract was assayed on the 50-plex assay that included the following transcription factor binding sites: AP-1, AP2, AML-1, AP4, AR, CRE-ATF, C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\gamma$ , CREB, E2F1-5, E2F6, EGR, ER, ETS, GATA, HFH2/3, HIC-1, HIF, HNF-1, HNF-3, HNF-4, HSF-1, ISRE, MEF-2, MTF1, c-Myb, Myc-Max, NFAT, NF-1, NF $\kappa$ B, NFY1, Octamer, p53, PAX6, PBX, PLZF, PPAR, SAF-1, SOX9, STAT, SREBP, SMAD1/5, SMAD 2/3, SP1, SRE, TGIF, TAL1, TCF/LEF, and YY1. A detailed protocol for preparing and analyzing the samples is provided in the following section.

The assay data was acquired on a Luminex 100™ Instrument and analyzed using the StarStation™ software. The results were plotted on a scatter-plot with the median fluorescent intensity (MFI) from the untreated blood graphed on the x axis and the MFI generated from the PMA/ionomycin-treated whole blood graphed on the y axis. Each point on the graph represents an individual transcription factor activity assay (as above). All of the points above or below the line represent transcription factor activities that are changed when whole blood is treated with PMA and ionomycin. All of the points that fall on the line are transcription factor activities that were not changed with this treatment. All of the changes observed in transcription factor activity in human whole blood are summarized in the table below.





Transcription Factor Assay	Fold Induction Of TF Activities in Whole Blood treated w/PMA/IM for 48 hours
CRE-ATF	4.9
CREB	1.4
EGR	2.0
GATA	1.2
HSF1	1.5
ISRE	2.2
MEF2	7.3
Myc-Max	1.6
NF1	4.7
NFkB	9.3
Octamer	3.1
PPAR	1.5
SRE	2.3
Sterol	1.1

## Detailed Experimental Protocols

### Required Reagents

ACK Lysis Buffer prepared using the following:

- 0.15 M NH<sub>4</sub>Cl (Sigma Cat. No. 09718) 8.3g
- 10 mM KHCO<sub>3</sub> (Sigma Cat.No. P9144) 1g
- 0.1 mM EDTA (Sigma Cat.No. E7889) 0.2 mL
- q.s. to 1L with nuclease free water

Nuclear Extraction Kit (Marligen Cat. No. 11906-100)

Protease Inhibitor Cocktail (Sigma Cat. No. P8340)

Phosphatase Inhibitor Cocktail (Sigma Cat. No. P5726)

PMSF (Sigma Cat. No. P7626)

Dithiothreitol (Sigma Cat. No. D9779)

Marligen's 50-plex Transcription Factor Service Testing Assay (Marligen Cat. No. 11907-003)

### Recommended Reagents (not required)

Protein Control (NF-1/NFkB) (Marligen Cat. No. 11934-001)

HL-60 Nuclear Extract Controls (Untreated and Treated) (Marligen Cat. No. 11938-001)

## Protocols

### Part 1: Whole Blood Collection

1. Pipette 2 mL RPMI 1640 supplemented as desired, into a 50 mL conical tube. Allow the medium to warm in a 37°C incubator.
2. Collect whole blood in heparinized tubes. Citrate tubes are also suitable for this application, but their use has not been extensively validated.
3. Pipette 2 mL of the collected whole blood into the warmed RPMI 1640. Cover the tube and mix by gentle inversion.

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[Note: To avoid clotting of blood samples, the whole blood should not be allowed to sit for long periods of time prior to the addition of RPMI.]

4. Maintain the whole blood/RPMI 1640 mixture at 37°C until ready to stimulate as desired.

### Part 2: Whole Blood Stimulation

1. Stimulate the whole blood/RPMI 1640 mixture as desired.

[Note: For this protocol, whole blood cells were treated for 24 hours with 100nM PMA (phorbol12-myristate 13-acetate) (Sigma Cat. No P8139) plus 500nM ionomycin (Sigma Cat. No. 3909). Depending on the experiment, there may be cases where it is desirable to treat whole blood samples at different times after the collection. Whole blood samples may be treated any time after they are placed in culture medium. When experiments do not require stimulation prior to nuclear extraction, red blood cell lysis can be done immediately. In this circumstance proceed to Part 3: Red Blood Cell Lysis.]

### Part 3: Red Blood Cell Lysis

1. At the end of the stimulation period, add 36mL of ACK Buffer to 4mL of blood culture in a 50mL conical tube.

[Note: the whole blood in this procedure is diluted in RPMI 1640 during stimulation. For whole blood that is not diluted with RPMI media, use 38mL of ACK Lysis Buffer per 2mL of whole blood.]

2. Mix by inversion five times and incubate the reaction on ice for 5 minutes. The lysis of the red blood cells should be evident during this incubation (e.g. the solution turns a clear deep red color.)

3. Pellet the cells by centrifugation at 300 x g for 7 minutes, and then pour off the supernatant.

4. The resulting cell pellet should be white or off-white. If the pellet is darker than pink, repeat steps 1-3 one more time.

5. Gently resuspend the pellet in 20 mL 1X PBS and centrifuge again for 7 minutes at 300 x g.

6. Pour off the supernatant and resuspend the pellet in 500 µL of 1X PBS.

7. Using a clean transfer pipet, transfer the cell suspension to a clean 1.5 mL microcentrifuge tube.

8. Pellet the cells by centrifugation at 700 x g for 5 minutes. Use a transfer pipet to remove and discard the supernatant.

[Note: be careful not to disturb the cell pellet.]

9. Proceed with the nuclear extraction procedure

### Part 4: Nuclear Extraction of Mononuclear Cells

Marligen's Nuclear Extraction Kit (Cat. No. 11906-100) has been optimized to provide samples containing activated transcription factors with little or no carry over of cytoplasmic proteins. This section describes the procedure for preparing nuclear extracts from the lysed whole blood.

[Note: all of the following steps should be performed on ice or in a refrigerated microcentrifuge.]

1. Immediately prior to use, prepare Complete Hypotonic Lysis Buffer in the prechilled container according to the chart below. Five (5) pellet volumes of Complete Hypotonic Lysis Buffer will be used for each sample. A minimum of 500 µL will be used for cell pellets of less than 100 µL. Volumes can be directly scaled to accommodate larger pellet volumes based on the minimum requirement of 500µL Hypotonic Lysis Buffer for 100µL of cell pellet. Once prepared, place Complete Hypotonic Lysis Buffer on ice.

Reagent	Quantity needed for 500 µL
Hypotonic Lysis Buffer	500 µL
Protease Inhibitor	5 µL
Phosphatase Inhibitor	5 µL
0.1 M DTT	5 µL
0.1 M PMSF	0.5 µL

[Note: the Complete Hypotonic Lysis Buffer should be used within 10 minutes of preparation.]

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2. Add 500  $\mu\text{L}$  or 5 pellet volumes (whichever is greater) of Complete Hypotonic Lysis Buffer to each cell pellet. Resuspend each pellet by pipeting up and down and transfer to clean prechilled 1.5 mL microcentrifuge tubes. Incubate on ice for 10 minutes.
3. Add 5  $\mu\text{L}$  of Detergent Solution for each 100  $\mu\text{L}$  of Complete Hypotonic Lysis Buffer used in step 2. Vortex for 5 seconds and centrifuge for 5 minutes at 800 x g (~3000 rpm) in a refrigerated microcentrifuge. During this centrifugation step prepare Complete Wash Solution as outlined in Step 5.
4. Remove supernatant from the nuclear pellet by using a clean transfer pipet. This supernatant contains the cytoplasmic extracts that can be stored at  $-80^{\circ}\text{C}$ , if desired.
5. Immediately prior to use, prepare Complete Wash Solution in the prechilled container. Each sample will require 1mL of Complete Wash Solution.

Reagent	Quantity needed for each sample
Complete Wash Solution	1000 $\mu\text{L}$
Protease Inhibitor	10 $\mu\text{L}$
Phosphatase Inhibitor	10 $\mu\text{L}$
0.1 M DTT	10 $\mu\text{L}$
0.1 M PMSF	1 $\mu\text{L}$

6. Add 500 $\mu\text{L}$  of cold Complete Wash Solution to each nuclear pellet and mix by gently pipetting up and down. Centrifuge for 5 minutes at 800 x g (~3000 rpm) in a refrigerated microcentrifuge and discard supernatant. Estimate and record pellet volume.

[IMPORTANT: This estimated pellet volume will be used to calculate the amount of complete Extraction Buffer 1 and Extraction Buffer 2 required for Step 8 below.]

7. Repeat the Wash step as described in step 6 until the supernatant is clear.

[Note: Save the excess Complete Wash Solution to be used later for preparing a sample blank for determining protein concentration.]

8. Prepare the Complete Extraction Buffers 1 and 2. Use the pellet volumes estimated in Step 6 to prepare Complete Extraction Buffers 1 and 2 as described in the table. Complete Extraction Buffers 1 and 2 should be prepared in prechilled containers immediately prior to use.

Complete Extraction Buffer 1		Complete Extraction Buffer 2	
Reagent	Quantity for 200 $\mu\text{L}$ nuclear pellet	Reagent	Quantity for 200 $\mu\text{L}$ nuclear pellet
Extraction Buffer 1	100 $\mu\text{L}$	Extraction Buffer 2	100 $\mu\text{L}$
Protease Inhibitor	1 $\mu\text{L}$	Protease Inhibitor	1 $\mu\text{L}$
Phosphatase Inhibitor	1 $\mu\text{L}$	Phosphatase Inhibitor	1 $\mu\text{L}$
0.1 M DTT	1 $\mu\text{L}$	0.1 M DTT	NONE
0.1 M PMSF	0.1 $\mu\text{L}$	0.1 M PMSF	0.1 $\mu\text{L}$

9. Add one-half ( $\frac{1}{2}$ ) pellet volume (~10  $\mu\text{L}$  for  $5 \times 10^6$  cells) of Complete Extraction Buffer 1 followed by  $\frac{1}{2}$  pellet volume (~ 10  $\mu\text{L}$  for  $5 \times 10^6$  cells) of Complete Extraction Buffer 2 to each cell pellet Vortex the sample immediately for 5 seconds.

[Note: Save the excess Complete Extraction Buffers 1 and 2 to be used later for preparing a sample blank for determining protein concentration.]

10. Incubate on ice for 30 minutes. Vortex the sample momentarily at high speed every 10 minutes.
11. Centrifuge at high speed for 30 minutes in a refrigerated microcentrifuge.
12. Remove and save supernatant in a chilled tube. This is the nuclear extract.

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13. Quantitate extracts prior to freezing using The Bradford assay or Absorbance at  $A_{280}$

[To prepare a sample blank for the protein assay mix two volumes of Complete Wash Buffer with one volume Complete Extraction Buffer 1 and one volume of Complete Extraction Buffer 2.]

14. Dilute the extracts to the desired concentration (5-10  $\mu\text{g}/\mu\text{L}$  is recommended) in Extract Dilution Buffer prepared according to the table below.

Buffer	To Prepare 100 $\mu\text{L}$	To Prepare 200 $\mu\text{L}$	To Prepare 500 $\mu\text{L}$
Wash Solution	50 $\mu\text{L}$	100 $\mu\text{L}$	250 $\mu\text{L}$
Extraction Buffer 1	25 $\mu\text{L}$	50 $\mu\text{L}$	125 $\mu\text{L}$
Extraction Buffer 2	25 $\mu\text{L}$	50 $\mu\text{L}$	125 $\mu\text{L}$

### Part 5: Assaying the Nuclear Extracts on Marligen's Multiplex Transcription Factor Assay

In this protocol, the nuclear extracts are assayed using Marligen's 50-plex Transcription Factor Testing Services Assay. This simple and rapid protocol is used for all of Marligen Multiplex Transcription Factor Assays. See specific product package insert for complete instructions on running the assay as protocols may vary slightly.

#### Preparation

1. Prior to starting the assay carry out the following preparation steps:

- a. Initiate the Luminex Instrument warm up cycle.
- b. Prepare reagents.
  - Allow all reagents to warm to room temperature for 30 minutes.
  - Place the Hybridization Buffer in a 37°C water bath to dissolve precipitate.
  - Briefly microfuge the Detection Reagent vial and the Digestion Reagent vial to bring their contents to the bottom of the vials.
- c. Allow the Protein Controls, Nuclear Extract Controls, and nuclear extract samples to thaw on ice.
- d. Determine the number of wells required for the assay. Each assay requires a Bead Control well, Negative Reagent Control wells, Positive Reagent Control wells, plus wells for the samples under investigation. The number of replicates should be determined by the user.

We recommend that with the initial use of this kit, the Negative Reagent Control wells, Positive Reagent Control wells, Protein Control, Unstimulated and Stimulated Nuclear Extract Controls, and samples under investigation be analyzed in triplicate. With subsequent analyses, the number of sample replicates may be decreased, and the Protein Control and the Unstimulated and Stimulated Nuclear Extract Controls may be omitted.

#### Binding Step 1

1. Pipette 15  $\mu\text{L}$  of the 1x Binding Mix 1 into each well of a 96 well PCR plate (provided).
2. Add 3  $\mu\text{L}$  sample volume Protein Control, Unstimulated and Stimulated Nuclear Extract Controls to designated well. Mix by gently pipetting up and down two times.
3. Add 3  $\mu\text{L}$  of sample nuclear extract, diluted in **Extraction Dilution Buffer**, to designated wells. Mix by gently pipetting up and down two times.

[Note: it is critical that 3 $\mu\text{L}$  and no more than 3 $\mu\text{L}$  of sample containing nuclear extract is added to each well.]

4. Seal plate with an adhesive plate cover and incubate at 25°C for 20 minutes in a PCR thermocycler.

#### Binding Step 2

1. Add 15  $\mu\text{L}$  of Binding Mix 2 to each well and mix by pipetting up and down two times.

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2. Seal plate with an adhesive plate cover and incubate at 25°C for 20 minutes in a PCR thermocycler.
3. During the last 5 minutes of this step, prepare the Complete Digestion Mix according to the table presented below. All wells require the Complete Digestion Mix except for the Positive Reagent Control wells. It is suggested sufficient volume be made for at least two extra wells. Space is provided in the table below for performing calculations. Mix the Complete Digestion Mix by gently pipetting up and down ten times.

Complete Digestion Mix	Volume per 1 Reaction	Number of Reactions + 2	Volume x (Number of Reactions +2)
Digestion Buffer	30 µL		
Digestion Reagent (nuclease)	0.25 µL		
Total Volume	30 µL		

[Notes: Do not use protein binding materials such as polystyrene for the preparation of Complete Digestion Buffer. The Complete Digestion Mix should be kept on ice and used within 15 minutes of preparation.]

### Digestion

1. At the end of the 20 minute Binding Step 2 incubation, remove the 96 well plate from the PCR thermocycler. Adjust the thermocycler temperature to 37°C.
2. Add 30 µL of Complete Digestion Mix to all wells except the Positive Reagent Control wells. Mix by gently pipetting up and down two times.
3. Add 30 µL of Digestion Buffer to the Positive Reagent Control wells. Mix by gently pipetting up and down two times.
4. Seal the plate with an adhesive plate cover.
5. Incubate wells for 20 minutes at 37°C in the PCR thermocycler.
6. During this incubation step, prepare for the next phase of the assay.
  - a. Mix Hybridization Buffer by inversion and check for precipitate. If precipitate is present, return the Hybridization Buffer to the 37°C water bath until the solution is clear.
  - b. Mix the Hybridization Buffer again by inversion. Mix the Hybridization Accelerator by brief, gentle vortexing and briefly microfuge the vial to bring the contents to the bottom.
  - c. Resuspend the Bead Mix by vortexing for 10 seconds and then incubating for 2 minutes in a sonicating water bath.
  - d. Prepare the Complete Hybridization Mix according to the table presented below. Space is provided to perform calculations of volumes for each experiment. All wells require the Complete Hybridization Mix. It is suggested that sufficient volume be made for at least two extra wells. Vortex the Complete Hybridization Mix for 10 seconds, then incubate the Complete Hybridization Mix for 2 minutes in a sonicating water bath. Protect this mixture from light by wrapping the tube in aluminum foil, as the beads are light-sensitive.

Complete Hybridization Mix	Volume per 1 Reaction	Number of Reactions	Volume x (Number of Reactions )
Hybridization Buffer	15.0µL		
20x Bead Mix	1.0 µL		
Hybridization Accelerator	1 µL		
Total Volume	17 µL		

### Hybridization

- At the end of the 20 minute digestion incubation step, remove the 96 well plate from the PCR thermocycler. All subsequent procedures will be performed at room temperature.
- Add 15 µL of the Complete Hybridization Mix to each well. Mix by pipetting up and down two times. [Note: this Complete Hybridization Mix must be protected from light.]
- Seal the plate with an adhesive plate cover. An aluminum foil wrapped adhesive plate cover is recommended to shield the beads from light.
- Incubate wells for 45 minutes at room temperature on an orbital shaker (500-600 rpm). Protect the wells from light during this incubation.
- During this incubation step, prepare the 1x Assay Wash Buffer and Complete Detection Mix.
  - Prepare the 1x Assay Wash Buffer according to the table presented below. Space is provided to allow calculations of volumes for each experiment. Mix the 1x Assay Wash Buffer by inverting several times. It is suggested sufficient volume of this reagent be made for at least two extra wells.

1x Assay Wash Buffer	Volume per 1 Reaction	Number of Reactions +2	Volume x (Number of Reactions +2)
10x Assay Wash	60 µL		
Deionized Water	540 µL		
Total Volume	600 µL		

- Prepare the Complete Detection Mix, according to the table presented below. Space is provided to perform calculations of volumes for each experiment. Mix the Complete Detection Mix by inverting several times. It is suggested sufficient volume of this reagent be made for at least two extra wells.

Complete Detection Mix	Volume per 1 Reaction	Number of Reactions + 2	Volume x (Number of Reactions +2)
1x Assay Wash Buffer	49.5 µL		
Detection	0.50 µL		
Total Volume	50 µL		

## Detection

1. Pre-wet the 96 well filter plate by adding 50  $\mu$ L 1x Assay Wash Buffer to each well designated for the assay.
2. Remove the 1x Assay Wash Buffer from each well by gentle aspiration with the vacuum manifold.  
[Notes: for this and the following wash steps use a vacuum manifold. Do not exceed ~5mm Hg. Do not allow the filter plate to dry out. Blot residual liquid from the bottom of the plate with absorbent paper towels.]
3. Carefully transfer the contents of the wells of the PCR plate to the corresponding wells of the pre-wetted filter plate. A multi-channel pipette is desirable for this transfer. Care must be taken not to puncture the membranes of the filter plate or cross-contaminate the samples.
4. Wash each well by adding 100  $\mu$ L 1x Assay Wash Buffer. Remove the 1x Assay Wash Buffer by gentle aspiration with the vacuum manifold. Repeat this washing step two more times for a total of three washes. Blot residual liquid from the bottom of the plate with absorbent paper towels.
5. Add 50  $\mu$ L of Complete Detection Mix to each well.
6. Incubate for 5 minutes at room temperature. Shield the plate from light during this incubation with an aluminum foil-wrapped plate cover.
7. Remove the liquid from the wells of the filter plate by gentle aspiration with the vacuum manifold.
8. Wash each well by adding 100  $\mu$ L 1x Assay Wash Buffer. Remove the 1x Assay Wash Buffer by gentle aspiration with the vacuum manifold. Blot residual liquid from the bottom of the plate with absorbent paper towels.
9. Add 100  $\mu$ L of 1x Assay Wash Buffer to each well. Shake the plate on an orbital shaker (500-600 rpm) for 2-3 minutes to resuspend the beads.
10. Read on the Luminex Instrument.

## Reading the plate

1. The assay was analyzed on the Luminex 100™ instrument.  
[Note: this step may vary slightly for different models of Luminex instrument.]
  - a. Set bead events to 50.
  - b. Enter the number of samples.
  - c. Set Sample Size to 65  $\mu$ L.
  - d. Enter the bead region numbers as indicated on the specific product package insert.
  - e. Check the probe height to accommodate the filter plates.
  - f. Perform one alcohol flush and one sheath fluid wash.
  - g. Insert the plate into the instrument and initiate the analysis.

[Note: we recommend the use of StarStation™ software supplied by Applied Cytometry Systems (England, U.K.) for the analysis of data. However different softwares can be used. To get a full list of vendors for instruments and software visit [www.luminexcorp.com](http://www.luminexcorp.com).]