A Method for Simultaneously Measuring Multiple Transcription Factors Activities in Nuclear Extracts Prepared from Fresh or Frozen Mouse Tissues

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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 full spectrum of cellular processes involved in controlling gene expression. Tissue samples from patients or from animal models of disease can provide powerful insights leading to better understanding of disease mechanisms, approaches to monitor disease progression and for development of new drugs. However, current methods for determining mechanisms that control gene expression are tedious and require large amounts of tissue for each assay, thereby limiting the amount of information that it is practical to collect on any given sample. Described here is a method for simultaneously measuring the binding activity of 50 different transcription factors isolated from nuclear extracts prepared from flash-frozen mouse heart, lung and brain tissues. The resulting data can give an overview of the regulatory mechanisms involved in the biological processes controlling healthy tissues. In animal models this method can be used to identify the underlying mechanism of a disease and can be used to monitor disease progression in these animal models. Moreover this provides a powerful tool to evaluate and monitor the effectiveness of drug therapies.

Method and Experimental Results

Briefly, nuclear extracts were prepared from frozen mouse heart, lung, and liver tissue using Marligen's Nuclear Extraction Kit (Cat. No. 11906-100). 10 µg of each nuclear extract was assayed using Marligen's 50-plex Transcription Factor Testing Services Assay (Marligen Cat. No. 11907-003), which includes the following transcription factor binding assays: AP-1, AP2, AML-1, AP4, AR, CRE-ATF, C/EBPα, C/EBPβ, C/EBPγ, 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, NFκB, 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. The multiplexed assays were read on the Luminex 100™ Instrument and the StarStation™ software used for data acquisition. [Note: A detailed protocol is provided in the following section.]

The background subtracted median fluorescent intensity (MFI) obtained from each of the transcription factor activities measured in the nuclear extracts from the tissues are shown in the table below. Notably each tissue type has a distinct transcription factor activity profile. This is demonstrated further in Figure 1 where nuclear extracts from lung are observed to have the highest levels of AP-1, PPAR, NFAT, NF1 and NFκB binding activity. STAT binding activity is highest in nuclear extracts from heart, and elevated PPAR and NF1 activity are observed in nuclear extracts prepared from liver.

Figure 1

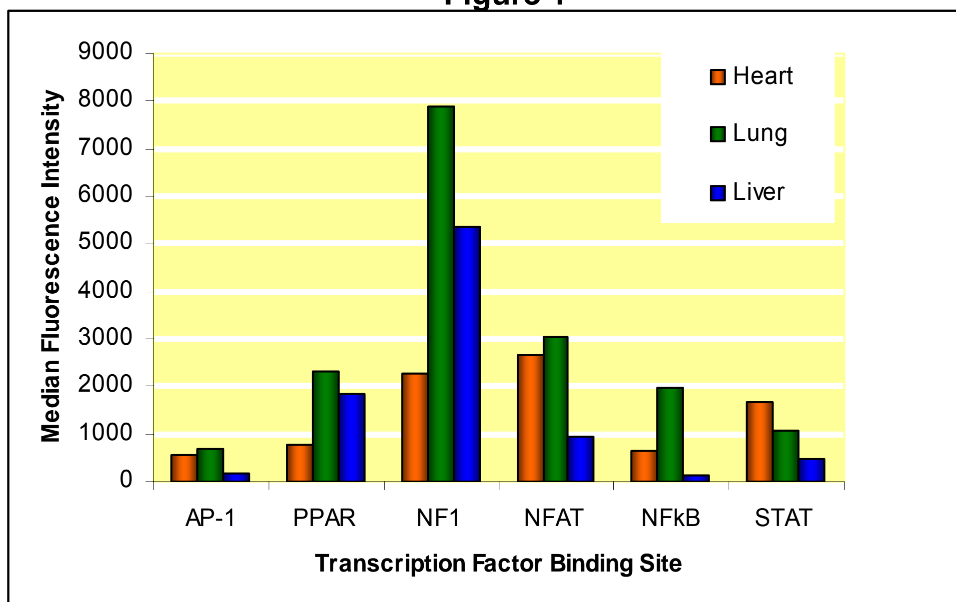

Table1

Transcription Factor Binding Site	Median Fluorescent Intensity of TF binding Site			Transcription Factor Binding Site	Median Fluorescent Intensity of TF binding Site		
	Heart	Lung	Liver		Heart	Lung	Liver
AML1	1292	1076	386	LEF1	66	164	71
AP-1	560	692	158	MEF2	765	2474	301
AP2	2094	3434	489	MTF1	2737	2438	479
AP4	398	497	122	Myc-Max	1290	2100	272
AR	962	968	371	NF1	2282	7866	5360
CEBPα	1037	500	246	NFAT	2670	3061	945
CEBPβ	497	424	250	NFκB	634	1977	132
CEBPδ	168	159	72	NFY1	672	344	146
C-MYB	1826	1253	570	Octamer	764	1588	273
CRE-ATF	762	1929	148	P53	740	771	203
CREB	1215	1577	426	PAX6	872	982	269
E2F1-5	2133	2715	448	PBX	317	194	125
E2F6	1543	1456	224	PLZF	748	588	321
EGR	1246	1992	388	PPAR	788	2317	1855
ER	627	208	198	SAF1	1061	1453	322
ETS	852	1629	395	SMAD 1/5	1880	1438	372
GATA	629	1261	174	SMAD 2/3	847	633	334
HFH3	826	435	451	SOX9	1254	708	358
HIC1	1594	1213	426	SP1	442	1193	124
HIF	666	473	115	SRE	674	854	229
HNF1	1636	2312	734	SREBP	204	754	143
HNF3	1182	676	438	STAT	1652	1068	468
HNF4	1422	1135	355	TAL1	1720	792	426
HSF1	810	268	263	TGIF	1265	710	462
ISRE	1590	1300	376	YY1	243	244	58

Detailed Experimental Protocols

Required Reagents

Liquid Nitrogen
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 Testing Services 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: Tissue Collection.

1. Heart, lung and liver tissue were excised and immediately flash frozen in liquid nitrogen.
2. The frozen tissues were then stored at -80°C prior to preparing the nuclear extracts.

[Notes: Once frozen, the tissue can be stored at -80°C for up to 6 months prior to processing.]

3. Proceed with the nuclear extraction procedure

Part 2: Preparation of Nuclear Extraction from Fresh or Frozen Tissue

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 mouse tissues.

[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. 1 mL of hypotonic lysis buffer is required for each tissue sample (up to 150mg of tissue). 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.]

2. Pipet 1 mL of Complete Hypotonic Lysis Buffer into a chilled 5mL polypropylene snap cap tube or equivalent and place on ice. Place the fresh or frozen tissue sample into the 1mL of hypotonic lysis buffer.

[Note: to minimize protein degradation, it is important that frozen tissues do not thaw before placing in Complete Hypotonic Lysis Buffer.]

3. Disrupt the tissue with a homogenizer keeping samples on ice at all times to avoid heating. It is also important to minimize foaming of the sample during homogenization.

[Note: for most tissues, especially "tough" tissue such as cardiac muscle a hand-held electric homogenizer such as Ultra-Turrax T8 disperser with 5mm dispersing element (VWR Cat. No. 33994-400)
3 X 5 second pulses on a setting of 5.gives best results.]

4. Transfer samples to pre-chilled polypropylene microcentrifuge tubes and incubate on ice for 10 minutes.
5. Add 50 µL of Detergent Solution for each 1 mL of tissue lysate. Vortex for 5 seconds and centrifuge for 5 minutes at 800 x g (3000rpm) in a refrigerated microcentrifuge.
[Note: certain mouse and human tissues may require higher centrifuge speeds to pellet the nuclear material. If a pellet cannot be seen after the first 5 minute spin, re-spin the samples at 1500g (4000pm). During this centrifugation step, prepare Complete Wash Solution as outlined above in Step 7. For tissues, 2mLs of wash solution will be required for each sample.
6. Remove supernatant from the nuclear pellet using a clean transfer pipet. This supernatant contains the cytoplasmic extracts that can be stored at –80ºC, if desired.
7. Immediately prior to use, prepare Complete Wash Solution in the prechilled container. Each sample will require 2mL of Complete Wash Solution.

Reagent	Quantity needed for each sample
Complete Wash Solution	2000 µL
Protease Inhibitor	20 µL
Phosphatase Inhibitor	20 µL
0.1 M DTT	20 µL
0.1 M PMSF	2 µL

8. Add 500µL of cold Complete Wash Solution to each nuclear pellet and mix by gently pipetting up and down. [Note: It is not necessary to disperse the nuclear pellet.]
9. Centrifuge for 5 minutes at 800 x g (~3000 rpm) in a refrigerated microcentrifuge and discard supernatant.
10. Repeat the Wash step as described in step 8 until the supernatant is clear.
[IMPORTANT: It is important that the nuclear extracts are washed sufficiently to avoid nuclease contamination in the transcription factor assay. The pellet can be washed up to five times and ensure that the supernatant is clear before proceeding to Step 11.
IMPORTANT: Estimate and record pellet volume.
This estimated pellet volume will be used to calculate the amount of complete Extraction Buffer 1 and Extraction Buffer 2 required for Step 11 below.
Note: Save the excess Complete Wash Solution to be used later for preparing a sample blank for determining protein concentration.]
11. Prepare the Complete Extraction Buffers 1 and 2. Use the pellet volumes estimated in Step 10 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 µL nuclear pellet	Reagent	Quantity for 200 µL nuclear pellet
Extraction Buffer 1	100 µL	Extraction Buffer 2	100 µL
Protease Inhibitor	1µL	Protease Inhibitor	1µL
Phosphatase Inhibitor	1µL	Phosphatase Inhibitor	1µL
0.1 M DTT	1µL	0.1 M DTT	NONE
0.1 M PMSF	0.1µL	0.1 M PMSF	0.1µL

12. Add one-half (½) pellet volume (~10 µL for 5 x 10^6 cells) of Complete Extraction Buffer 1 followed by ½ pellet volume (~ 10 µL for 5 x 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.]

13. Incubate on ice for 30 minutes. Vortex the sample momentarily at high speed every 10 minutes.
14. Centrifuge at high speed for 30 minutes in a refrigerated microcentrifuge.
15. Remove and save supernatant in a chilled tube. This is the nuclear extract.
16. 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.]

17. Dilute the extracts to the desired concentration (5-10 µg/µL is recommended) in Extract Dilution Buffer prepared according to the table below.

Buffer	To Prepare 100 µL	To Prepare 200 µL	To Prepare 500 µL
Wash Solution	50 µL	100 µL	250 µL
Extraction Buffer 1	25 µL	50 µL	125 µL
Extraction Buffer 2	25 µL	50 µL	125 µL

Part 3: Assaying the Nuclear Extracts on Marligen's Multiplex Transcription Factor Assay

The procedure for assaying the presence of transcription factors in nuclear extracts using Marligen's 50-plex Transcription Factor assay is described. 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 Cell Nuclear Extract Control, Stimulated Cell Nuclear Extract Control, and samples under investigation be analyzed in triplicate. With subsequent analyses, the number of sample replicates may be decreased, and the Protein Control, the Unstimulated Cell Nuclear Extract Control, and the Stimulated Cell Nuclear Extract Control may be omitted.

Binding Step 1

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

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[Note: avoid bubbling when mixing samples by pipeting as this may denature active transcription factor proteins.
Note: it is critical that 3µL and no more than 3µL 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 µL of Binding Mix 2 to each well and mix by pipetting up and down two times.
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, including the Bead Control well. 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

1. 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.
2. 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.
3. Seal the plate with an adhesive plate cover. An aluminum foil wrapped adhesive plate cover is recommended to shield the beads from light.
4. Incubate wells for 45 minutes at room temperature on an orbital shaker (500-600 rpm). Protect the wells from light during this incubation.
5. During this incubation step, prepare the 1x Assay Wash Buffer and Complete Detection Mix.
   a. 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		

   b. 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 µL 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 µL 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 washings. Blot residual liquid from the bottom of the plate with absorbent paper towels.
5. Add 50 µL 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 µL 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 µL 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 µL.
    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 acquisition and analysis of data. However different softwares can be used. To get a full list of vendors for instruments and software visit www.luminexcorp.com