

**Catalog No. 11954-096 96 Reactions**

**Overview and Intended Use**

Marligen's proprietary transcription factor profiling technology allows simultaneous measurement of twenty transcription factors present in nuclear extracts. The assay measures the binding of transcription factor complexes to DNA binding probes carefully mined for affinity and specificity. The assay is configured in the Luminex xMAP® format and is read on the Luminex instrument, allowing the analysis of up to 96 samples in less than three hours. Marligen's Multiplex Transcription Factor Profiling Kits are designed and optimized for use with nuclear extracts prepared with the Marligen Nuclear Extraction Kit (Catalog # 11906-100). For additional information on related assays, sample preparation, nuclear extracts, protein controls or for additional technical and reference information, consult the Marligen website: [www.marligen.com](http://www.marligen.com).

**Table of Binding Sites and Bead Regions**

Bead	Site	Bead	Site	Bead	Site
4	CRE-ATF	34	E2F6	57	AP2
6	AP-1	35	SP1	59	SMAD 2/3
10	NFκB	38	Myc-Max	64	HNF-1
30	AR	41	EGR	65	HNF-4
31	CREB	44	ISRE	73	NF-1
32	YY1	49	p53	99	PPAR
33	E2F1-5	52	GATA		

**IMPORTANT INFORMATION**

**Terms and Conditions**

By opening the package containing this Assay Product (which contains fluorescently labeled microsphere beads authorized by Luminex Corporation) or using this Assay Product in any manner, you are consenting and agreeing to be bound by the following terms and conditions. You are also agreeing that the following terms and conditions constitute a legally valid and binding contract that is enforceable against you. If you do not agree to all of the terms and conditions set forth below, you must promptly return this Assay Product for a full refund prior to using it in any manner.

You, the customer, acquire the right under Luminex Corporation's patent rights, if any, to use this Assay Product or any portion of this Assay Product, including without limitation the microsphere beads contained herein, only with Luminex Corporation's laser based fluorescent analytical test instrumentation marketed under the name Luminex Instrument.

**Software Recommendation**

Marligen recommends StarStation™ software from Applied Cytometry Systems with the Luminex Instrument. However, other Luminex software is also acceptable.

**Components included with this kit**

Reagents	Volume
Sample Diluent	0.75 ml
Binding Mix 1	1.6 ml
Binding Mix 2 (20-plex)	1.6 ml
Digestion Reagent B	30 µl
Digestion Buffer	3.2 ml
Hybridization Accelerator	120 µl
Hybridization Buffer	1.8 ml
Bead Mix (20-plex)	110 µl
10X Wash Buffer	6.5 ml
Detection Reagent	55 µl
Filter Plate	1
Aluminum Plate Sealers	2
PCR Plate	1

**Equipment and materials required but not supplied**

PCR thermal cycler	Luminex Instrument
Vortex Mixer	Sonicated waterbath
96-well filter plate vacuum manifold	Plate Shaker

**ASSAY NOTES**

**READ ENTIRE PROTOCOL BEFORE STARTING THE PROCEDURE.**

**Special Handling Instructions**

- Use only with nuclear extracts prepared with the Marligen Nuclear Extraction Kit.
- Do not mix or interchange different reagent lots from various kits.
- Maintain Digestion Reagent on ice or at -20°C at all times. DO NOT place Digestion Reagent or Detection Reagent at temperatures below -20°C.
- DO NOT vortex Digestion or Detection Reagents or samples.
- **Thaw samples (nuclear extracts or proteins) on ice and maintain on ice throughout the procedure.**

**CONTROLS**- Two reagent controls should be included in every assay. The **Positive Reagent Control** provides the maximum signal for each site and the **Negative Reagent Control** provides the background signal for each site. DO NOT add any sample to the wells for the reagent controls. Instructions for the reagent control wells are outlined below:

- For Binding Step 1, add 3 µl of Sample Diluent to each of the reagent control wells.
- For the Digestion Step, add Digestion Buffer only to the **Positive Reagent Control wells**; add the Complete Digestion Buffer to the **Negative Reagent Control wells**.

For all other steps in the assay, the Positive and Negative Reagent Control wells should be treated like the samples. Samples and controls should be tested in triplicate until reproducible results have been obtained in several assays.

Thereafter, Positive Reagent Control and samples may be tested in duplicate or singlicate. Negative Reagent Control should always be tested in triplicate.

**FILTER PLATE** - The filter plate provided is used after hybridization. The filter membrane should be pre-wet with 50 µl of 1X Wash Buffer immediately prior to the transfer step. Use a 96-well plate vacuum manifold (not to exceed ~5mm Hg) to filter the wash before transferring the assay. **DO NOT ALLOW THE FILTER MEMBRANE TO DRY THROUGHOUT THE TRANSFER, WASH, AND DETECTION STEPS!!!**

**ALUMINUM PLATE SEALERS** - Two aluminum plate sealers are provided to cover the plate during the Digestion and Hybridization steps. Standard plate sealers compatible with the PCR thermal cycler can be used to cover the plate during other incubations. **TO AVOID SPLASHING OF SAMPLES AND CROSSCONTAMINATION OF WELLS** when removing plate sealers, it is **VERY IMPORTANT** to ensure that the PCR plates are supported in a 96 well plate holder.

#### ASSAY SETUP

1. Allow the Sample Diluent, Binding Mixes (1 and 2), Digestion Buffer, Hybridization Accelerator and 10X Wash Buffers to thaw at room temperature, for approximately 15-20 minutes.
2. Place the Hybridization Buffer in a 37°C water bath until ready to use. Make sure the precipitate has dissolved before using.
3. Warm up the Luminex Instrument and set up the assay as described in the Luminex Instrument's User Manual. Setup details specific to this assay are described below:
  - The XY platform heater should be off.
  - Set the bead events to 50.
  - Set the minimum events to 20.
  - Enter the number of samples.
  - Set sample size to 50 µl.
  - Set flow rate to Fast.
  - Enter the bead region numbers as indicated in the table above.
  - Check the probe height and adjust it, if necessary to accommodate the filter plate.
  - Perform 1 alcohol flush, 1 sheath fluid wash, and 1 prime.
4. Vortex the Sample Diluent to mix well and normalize nuclear extract samples by diluting them with Sample Diluent so that all control and experimental samples are the same concentration. Three microliters of sample will be used for each replicate well. For most cell types, a sample concentration ranging from 1-3 µg/µl is recommended. Keep samples on ice at all times.

#### ASSAY PROCEDURE

1. **Binding Step 1** (The volumes listed under a-c in Binding Step 1 assume samples are run in triplicate. If more or fewer replicates are desired, volumes can be scaled proportionally based on the number of replicates.)
  - a. To run samples in triplicate, label one polypropylene microcentrifuge tube for each group of replicates. Add 45 µg of room temperature Binding Mix 1 to each of the labeled microfuge tubes.
  - b. Add 9 µl of each sample to the appropriately labeled tube. Add 9 µl of sample diluent to the tubes designated for the positive and negative reagent controls.
  - c. Mix well by slowly pipetting up & down 3-5 times. (IT IS IMPORTANT TO AVOID SAMPLE BUBBLING as this may denature active transcription factors.)
2. **Addition of Binding Mix 2** (The volumes listed under a and b in Addition of Binding Mix 2 assume samples are run in triplicate. If more or fewer replicates are desired, volumes can be scaled proportionally based on the number of replicates.)

- a. Briefly vortex room temperature Binding Mix 2. Add 45 µl of Binding Mix 2 to each well containing samples or reagent controls.
  - b. Mix by slowly pipetting up & down 2 times. (IT IS IMPORTANT TO AVOID SAMPLE BUBBLING as this can denature active transcription factors.)
3. **Binding Step 2**
    - a. Transfer 30 µl of each reaction to each of replicate well in the 96 well PCR plate.
    - b. Cover and incubate 20 minutes at 25°C in a thermal cycler or other temperature-controlled Microplate block.
  4. **Digestion**

**Warning: Do not use protein binding materials such as polystyrene for the preparation of Complete Digestion Buffer. The use of polypropylene is recommended.**

- a. During the last five minutes of Binding Step 2 incubation, prepare the Complete Digestion Mix. To do this, mix Digestion Buffer well by gently vortexing and prepare Complete Digestion Buffer according to the chart below. (IMPORTANT: To insure assay performance, the Complete Digestion Buffer must be used within 5 minutes of its preparation.

Complete Digestion Buffer (per well)	
0.25 µl	Digestion Reagent B
30 µl	Digestion Buffer

IMPORTANT: DO NOT vortex to mix the Digestion Buffer and Digestion Reagent B. Mix by gently pipetting up and down and avoid introducing bubbles.

- b. Following the 20 minute incubation under Binding Step 2, remove the PCR plate from the thermocycler and carefully remove the plate cover (The use of a 96 well plate holder is highly recommended to minimize the risk of splashing and cross-contamination of samples).
  - c. Add 30 µl to Complete Digestion Buffer to each well **EXCEPT the positive reagent control wells**. (IT IS IMPORTANT TO AVOID SAMPLE BUBBLING as this may reduce the activity of the Complete Digestion Buffer.)
  - d. Add 30 µl of Digestion Buffer (not containing digestion reagent) to each **positive reagent control** well.
  - e. Mix all wells gently by pipetting up & down 2 times. (IT IS IMPORTANT TO AVOID SAMPLE BUBBLING as this may reduce the activity of the Complete Digestion Buffer.)
  - f. Cover the PCR plate with an aluminum plate sealer and incubate 20 minutes at 37°C in a thermal cycler. This timing is **IMPORTANT!** Incubations longer than 22 minutes, shorter than 18 minutes and 4 degree cooling cycles may negatively impact assay performance. **IMMEDIATELY** after the 20 minute incubation, the PCR plate should be removed from the thermocycler and Hybridization Mix must **IMMEDIATELY** be added to each well as described below.
5. **Hybridization**

**The Hybridization Buffer should be prepared during the last 10 minutes of the 20 minute Digestion step.**

- a. Vortex the Bead Mix on high for 10 seconds, & place in sonicating waterbath for 1-3 minutes before use.

- b. Prepare the Hybridization Mix as indicated in the chart below by adding all components and vortexing briefly.

Hybridization Mix (per well)	
15 µl	Hybridization Buffer
1 µl	Hybrid. Accelerator
1 µl	Bead Mix

- c. **IMMEDIATELY** after **Digestion step 3f**, add 15 µl of Hybridization Mix to each well.
- d. Apply a new aluminum plate sealer and shake on a plate shaker for 45 minutes at room temperature protected from light.

## 6. Transfer and Wash

- a. Prepare 1X Wash Buffer as described below.

1X Wash Buffer (per well)	
65 µl	10X Wash Buffer
585 µl	Deionized water

- b. Prepare the pre-wet filter plate as described in ASSAY NOTES.
- c. Transfer the reactions from the PCR plate to the pre-wet filter plate. (IMPORTANT! Use care to not pierce the Filtration membranes with the pipet tip during sample transfer or wash steps.) Use a vacuum manifold to filter the assay (do not exceed -5mm Hg).

**\*\* After each step requiring vacuum, it is VERY IMPORTANT to remove excess liquid from the bottom of the filter plate by momentarily blotting on an absorbent paper towel.**

- d. Wash the wells by adding 100 µl of the 1X Wash Buffer to each of the assay wells and filtering with a vacuum manifold.
- e. Repeat step d. two more times for a total of 3 washes.

## 7. Detect and Read

- a. Prepare the Detection Mix as described in the subsequent table.

Detection Mix (per well)	
0.5 µl	Detection Reagent
50 µl	1X Wash Buffer

- b. Add 50 µl of Detection Mix to each well. Incubate for 5 minutes at room temperature protected from light. (Shorter incubations or longer incubations may decrease significantly assay sensitivity or increase assay background.)
- c. Filter the Detection Mix from the PCR plate using the vacuum manifold, blot dry, add 100 µl of 1X Wash Buffer, and filter the plate again. **\*\*Dry the bottom of the filter plate well by pressing it onto an absorbent paper towel.**
- d. Remove the filter plate from the absorbent towel, add 100 µl of 1X Wash buffer to each well and read using the Luminex Instrument.

## DATA ANALYSIS

1. Use the MFI data output from the Luminex 100 to calculate the percent digestion of the Negative Reagent Control by the following equation:

$$\text{Percent Digestion} = 100 * \left[ 1 - \frac{\bar{x}MFI_{\text{Negative Reagent Control}}}{\bar{x}MFI_{\text{Positive Reagent Control}}} \right]$$

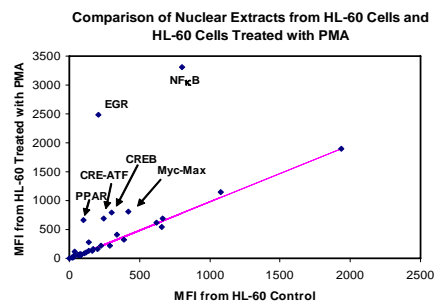
The Percent Digestion should be  $\geq 95\%$ .

2. Subtract the mean Negative Reagent Control from the mean MFI values of the samples.
3. Compare the background-subtracted MFI values of treated and untreated samples.

## EXAMPLE DATA

	Positive Reagent Control (MFI)	Negative Reagent Control (MFI)	Untreated Extracts	Treated Extracts	Percent Digestion (%)
NFkB	13513	41	332	947	99.7
CREB	11279	10	51	91	99.9
ISRE	9851	10	23	64	99.9
EGR	12833	36	500	2941	99.7
NF-1	10927	116	2188	2767	98.9
GATA	10043	12	1124	1727	99.9
PPAR	11393	32	217	661	99.7
AP-1	9309	9	97	213	99.9
SMAD 2/3	12961	80	269	457	99.4
HNF-4	15612	111	291	472	99.3
HNF-1	13091	80	630	993	99.4
CRE-ATF	12510	5	359	608	100.0
E2F1-5	11881	25	343	667	99.8
E2F6	13493	379	515	702	97.2
Myc-Max	11907	18	586	1080	99.8
YY1	9559	5	55	127	99.9
SP1	9153	32	1253	1571	99.7
p53	12720	20	41	91	99.8
AP2	11842	35	210	418	99.7

4. To clearly determine differences between untreated and treated samples the data can be analyzed by a scatter-plot method. Construct a plot of the Treated extracts MFI (y-axis) versus the Untreated extracts MFI (x-axis) in Excel or similar program. Most of the points should lie on a straight line. Draw a line through the points that form the line. The points lying significantly above the line represent activated transcription factors while points below the line represent transcription factors that are inhibited. An example of this method of data analysis is shown below.



5. Marligen offers customers additional data analysis tools to further quantify changes in the transcription factor activity. These are available at no additional cost to customers who purchase Marligen's Multiplex Profiling Kits. Please contact [customer.service@marligen.com](mailto:customer.service@marligen.com) or call us at 877-484-4990 for further information.