Multiplex Transcription Factor Profiling Kit (Custom Panel)

Store at –20°C

Catalog No. 11955-096 96 Reactions

Overview and Intended Use

Marligen's Transcription Factor Profiling Kit allows simultaneous measurement of up to 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.

The specific transcription factors detected in this profiling kit are listed in Table 1.

Table 1 – Binding Sites and Bead Regions

Bead	Site	Cat #
4	CRE/ATF1	11956-096
6	TRE/AP1	11957-096
10	NFkB	11958-096
30	AR	11959-096
31	CREB	11960-096
32	YY1	11961-096
33	E2F1-5	11962-096
34	E2F6	11963-096
35	SP1	11964-096
38	Myc-Max	11965-096

Bead	Site	Cat #
41	EGR	11966-096
44	ISRE	11967-096
49	p53	11968-096
52	GATA	11969-096
57	AP2	11970-096
59	SMAD 2/3	11971-096
64	HNF-1	11972-096
65	HNF-4	11973-096
73	NF-1	11974-096
99	PPAR	11975-096

Principle of Method

Nuclear extracts are prepared with Marligen's Nuclear Extraction Kit (Kit sold separately. Catalog # 11906-100). The nuclear extracts are incubated with Binding Mix 1 that contains specific biotinylated DNA binding probes. Active transcription factor complexes bind to their specific probes, whereas unbound probes are removed utilizing Marligen's patented digestion step. The remaining probes are incubated with a Bead Mix containing a mixture of different fluorescently dyed xMAP® beads. Each different xMAP® bead is coupled with a unique probe that recognizes a specific DNA binding probe and the probes hybridize to their specific bead. Following hybridization, the samples are incubated with streptavidin-phycoerythrin (SAPE), which binds to the biotin on the DNA binding probe. The samples are read on Luminex™ or Luminex-based instruments (e.g. BioPlex®), which detect the specific transcription factors activated in the sample by their unique bead region and quantified by the intensity of the SAPE signal.

Terms and Conditions

By opening the package containing this Assay Product (which contains flourescently 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 under the name Luminex Instrument.

Safety and Use Statement

All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of potentially infectious or hazardous agents.

This product is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic application. Uses other than the labeled intended use may be a violation of applicable law.

If you have any questions concerning the use of this product, please contact Marligen Biosciences, Inc. at (866) 464 4990 or visit www.marligen.com.

Components included with this kit

Sample Diluent	0.75 mL
Binding Mix 1	2 x 1.6 mL
Digestion Reagent B	30 µL
Digestion Buffer	3.2 mL
Hybridization Accelerator	120 µL
Hybridization Buffer	1.8 mL
10X Wash Buffer	6.5 mL
Detection Reagent	55 µL
Filter Plate	1
Aluminum Plate Sealers	2
PCR Plate	1

Storage and Handling Instructions

The Transcription Factor Profiling Kit is shipped on ice pack. Upon receipt, the components should be stored at -20ºC.

Materials and Equipment Required But Not Supplied:

PCR Thermocycler
Plate Shaker
Vortex Mixer
Sonicating waterbath
96-well filter plate vacuum manifold
Luminex Instrument

Important Information

READ ENTIRE PROTOCOL BEFORE USE

1. Special Handling Instructions

a. Use only with nuclear extracts prepared with the Marligen Nuclear Extraction Kit (Catalog # 11906-100).
b. DO NOT mix or interchange different reagent lots from various kits.
c. Maintain Digestion Reagent on ice or at –20°C at all times.
d. DO NOT place Digestion Reagent or Detection Reagent at temperatures below -20°C.
e. DO NOT vortex Digestion Reagent or Detection Reagent.
f. Thaw samples on ice and maintain on ice throughout the procedure. DO NOT vortex samples.

2. Assay controls

a. 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:
   i. For Binding Step 1, add 3 µl of Sample Diluent to each of the reagent control wells.
   ii. 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.
b. Two additional sets of assay controls are also available from Marligen. The HL-60 Nuclear Extract Control (Cat. No. 11938-001) contains two vials; one contains a nuclear extract prepared from untreated HL-60 cells and the other contains nuclear extracts prepared from HL-60 cells incubated with 100nM PMA plus 500nM ionomycin for 24 hours. A Protein Control (Cat. No. 11934-001) contains purified NF-1 and NFκB p50 protein.

Set-up Prior to Starting Assay Procedure

1. To create a custom TF multiplex from individual sites, create a 1X Bead Mix or Binding Mix 2 by combining equal volumes of the 20X stock from each site and add an appropriate volume of the reagent diluent. The total volume per well is 15 µl. Use 0.75 µl/well of the 20X stock for each site in the multiplex. Use the calculations below to determine the appropriate volumes for your custom TF multiplex. Prior to preparing the 1X Bead Mix, place the 20X Bead Mixes in a 37°C water bath to dissolve precipitate. Once the precipitate is dissolved, vortex each individual Bead Mix on high for 10 seconds and place in a sonicating waterbath for 5 minutes.

   The reagent diluents are:
   - 20X Binding Mix 2: *Binding Mix 1*
   - 20X Bead Mix: *Hybridization Buffer*

   Each Individual Binding Mix 2/Bead Mix
      # wells x 0.75 µl = Each individual (a)
   Total Individuals
      # sites x (a) = Total Individuals (b)
   Diluent Required
      (# wells x 15 µl) – (b) = Volume of diluent required (c)

   Combine the required volume of Each Individual Mix (a) then add the required volume of Diluent (c) in an appropriately sized tube.
2. Prepare nuclear extracts using Marligen's Nuclear Extraction Kit (Catalog # 11906-100).
3. 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.
4. Place the Hybridization Buffer in a 37°C water bath until ready to use. Make sure the precipitate has dissolved before using.
5. 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.
6. Warm up the Luminex or Luminex-based instrument.

Luminex Instrument Setup

1. **Set up the instrument as described in the user's manual.** Setup details specific to this kit are described below:
   a. The XY platform heater should be off
   b. Set the bead events to 50
   c. Set the minimum events to 20
   d. Enter the number of samples
   e. Set the sample size to 50 µL
   f. Set the flow rate to Fast

g. Enter the bead region numbers as indicated in the Table 1.
h. Check the probe height and adjust it, if necessary to accommodate the filter plate
i. Perform 1 prime with sheath fluid, 1 alcohol flush, and 2 sheath fluid washes.

2. Adjusting Luminex Instrument to High Gain Setting

A high gain setting for the Luminex instrument is recommended to provide the best results. Each specific software used with the Luminex or Luminex-based instrument may have different instructions for obtaining the high gain setting. Below are instructions using the Luminex 2.3™ software. Please see manufacturer's guidelines for instrument/software specific instructions (e.g. BioPlex®)

a. Create a new lot number for CAL2 and enter lot number with an HG at the end to designate High Gain.
b. Record the CAL2 Calibrator target "RP1" which is usually around 3832
c. Multiply the CAL2 Calibrator target "RP1" by 4.55 to get a new target value of approximately 17,436.
d. Enter the new Calibrator target "RP1" as the value for your New CAL2 lot.
e. Run the CAL2 calibration.

Assay Procedure

1. **Binding Step 1**

   NOTE: 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 µL of Binding Mix 1 warmed to room temperature 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.

   NOTE: It is important to avoid sample bubbling as this may denature active transcription factors.

   d. Cover and incubate for 20 minutes at room temperature.

2. **Addition of Binding Mix 2**

   NOTE: 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 Binding Mix 2 warmed to room temperature. Add 45 µL of Binding Mix 2 to each well containing samples or reagent controls.
   b. Mix by slowly pipetting up & down 2 times.

   NOTE: It is important to avoid sample bubbling as this may 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**

   NOTE: 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 the Binding Step 2 incubation, prepare the Complete Digestion Mix according to the volumes stated in Table 2. Mix Digestion Buffer well by gently vortexing and add Digestion Reagent B.

Table 2 - Complete Digestion Buffer (per well)	
0.25 µL	Digestion Reagent B
30 µL	Digestion Buffer

NOTES ON PREPARING COMPLETE DIGESTION BUFFER:

NOTE: To insure assay performance, the Complete Digestion Buffer must be used *within 5 minutes* of its preparation.

NOTE: Do not vortex to mix the Complete Digestion Buffer. Mix by gently pipetting up and down and avoid introducing bubbles.

b. Following Binding Step 2, remove the PCR plate from the thermocycler and carefully remove the plate cover.

NOTE: 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.
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.

NOTE: 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.

NOTE: 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 be added to each well as described below.

5. **Hybridization**

NOTE: 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 Table 3 by adding all components and vortexing briefly.

Table 3 - Hybridization Mix (per well)	
15 µL	Hybridization Buffer
1 µL	Hybridization Accelerator
1 µL	Bead Mix

c. IMMEDIATELY after Digestion step 4f, 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**

NOTE: 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 ~5 mmHg) to filter the wash before transferring the assay. Do not allow the filter membrane to dry throughout the transfer, wash and detection steps.

NOTE: It is important to apply a slight vacuum of ~5 mmHg during all wash steps. Higher vacuum may result in the loss of xMAP® beads and reduce bead count.

NOTE: During all wash steps, cover unused wells with a plate sealer to ensure a seal necessary to pull a vacuum.

a. Prepare 1X Wash Buffer as described below in Table 4.

Table 4 - 1X Wash Buffer (per well)	
65 µL	10X Wash Buffer
585 µL	Deionized water

b. Prepare the pre-wet filter plate as described in Important Information.

c. Transfer the reactions from the PCR plate to the pre-wet Filter Plate, using care not to 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).

NOTE: 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 Table 5.

Table 5 - 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 30 minutes at room temperature protected from light.

c. Filter the Detection Mix through the filter plate by using the vacuum manifold, blot dry, add 100 µL of 1X Wash Buffer, and filter the plate again.

d. Dry the bottom of the filter plate well by pressing it onto an absorbent paper towel.

e. Remove the filter plate from the absorbent towel, add 100 µL of 1X Wash Buffer to each well.

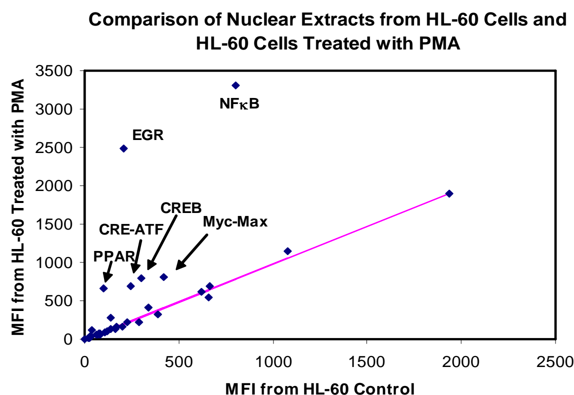
f. Read the filter plate in Luminex instrument at high gain setting (see Luminex Instrument Set-up).

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 ≥ 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.
4. Differences between untreated and treated samples may 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.



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Example Data

TF	Positive Reagent Control (MFI)	Negative Reagent Control (MFI)	Untreated Extracts	Treated Extracts	% Digestion
NFκB	24090	121	304	3334	99.5
CREB	25688	42	544	1614	99.8
ISRE	24695	14	16	37	99.9
EGR	24602	90	287	3705	99.6
NF1	25106	58	2278	2381	99.8
GATA	23844	41	53	134	99.8
PPAR	24644	57	100	324	99.8
AP-1	25638	273	316	363	98.9
SMAD 2/3	25950	136	250	269	99.5
HNF-4	25730	102	118	106	99.6
HNF-1	24903	114	168	154	99.5
CRE-ATF	23972	65	113	380	99.7
E2F1-5	25280	76	133	145	99.7
E2F6	25342	349	304	308	98.6
Myc-Max	23318	61	510	634	99.7
YY1	24006	17	23	37	99.9
SP1	24369	142	836	720	99.4
p53	24850	44	32	51	99.8
AP2	24438	93	249	278	99.6
AR	26144	45	70	140	99.8

Technical Support

For further technical assistance please contact us at (866) 464 4990 ext 102 or by email at technical.support@marligen.com. Technical support and troubleshooting guides for these products can also be found on our website at www.marligen.com

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2502 Urbana Pike Ijamsville, MD 21754 USA
301-874-4990 (phone)
301-874-4993 (fax)
Customer.Service@marligen.com www.marligen.com