CRE-ATF Transcription Factor Microplate Assay

Store at –20°C

Catalog No. 11983-096   96 Reactions

Overview and Intended Use

Marligen's proprietary transcription factor profiling technology allows the measurement of activated transcription factors from nuclear extracts. The assay measures the binding of transcription factors to their cognate DNA binding sequences and is configured in a standard microplate format to facilitate the analysis of up to 96 samples in less than four hours. Marligen Transcription Factor Microplate Assays 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, nuclear extracts and controls and for additional technical and reference information, consult the Marligen website www.marligen.com.

Important Information

READ ENTIRE PROTOCOL BEFORE STARTING THE PROCEDURE.
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 throughout procedure.
The volumes in this kit are sufficient for four uses.
Spin small-volume reagents and samples prior to use.
DO NOT vortex Digestion Reagent, conjugate, or samples.
Thaw samples (nuclear extracts or proteins) on ice and maintain on ice throughout the procedure.

Components included with this kit:

Reagents	Volume
Sample Diluent	0.75 ml
Binding Mix 1	1.6 ml
CRE-ATF Binding Mix 2	1.6 ml
CRE-ATF Digestion Buffer	2.75 ml
Digestion Reagent B	30 µl
Capture Microplate	1 Each
Hybridization Accelerator	120 µl
Hybridization Buffer	1.2 ml
10 X Wash Buffer	28 ml
Conjugate Diluent	12 ml
Streptavidin-Alkaline Phosphatase Conjugate (SA-AP)	120 µl
Chemiluminescent Substrate	9 ml
PCR Plate	1 Each
Plate Sealers	2 Each
Microplate Cover	1 Each

Note: Once diluted, the Wash Buffer may be stored at room temperature, however, to avoid microbial contamination during long time storage, a preservative, such as Sodium Azide, may be added.

Materials and equipment required but not supplied:

Additional Wash Buffer necessary for priming an automated plate washer (Marligen Catalog # 60319)
1M HCl
PCR thermal cycler
Vortex mixer
Rotary shaker for microplate
Manual or automated plate washer
Microplate luminometer

Protocol

1. Setup
   a. Thaw Sample Diluent at room temperature, vortex to mix and then place on ice. Place Hybridization Buffer at 37ºC until use. Warm both Binding Mixes, Digestion Buffer and Hybridization Accelerator at room temperature for 30 minutes.
   b. Dilute samples with Sample Diluent so that control and experimental samples are the same concentration. Three microliters of sample will be used for each replicate well. Keep samples on ice at all times. Note: For most cell types tested, 2µg of nuclear extract is sufficient for this assay. However, optimal extract concentration may vary depending upon the transcription factor, cell or tissue source, and it may be necessary to titrate the extract amount to obtain optimal results. A titration of nuclear extract ranging from 100ng-15µg is suggested.
   c. Determine the number of wells needed for samples and controls. Two reagent controls should also 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 sample wells. Samples and controls should be run in triplicate until reproducible results have been obtained in several runs. Thereafter, Positive Reagent Control and samples may be run in duplicate or singlicate. Negative Reagent Control should always be run in triplicate.
   d. In the Capture Plate, remove or seal unused wells that will not be used in the assay and store at 4°C.
2. Binding
   a. Mix Binding Mix 1 by inversion. Place 15 µl of Binding Mix 1 into the required number of wells of a PCR Plate.
   b. Add 3 µl of Sample Diluent to the Positive and Negative Reagent Control wells.

c. Add 3 µl of nuclear extract or proteins to the sample wells containing Binding Mix 1.
d. Mix each well by gently pipetting up and down 2-3 times and cover the plate with microplate cover.
e. Incubate binding reactions at room temperature for 20 minutes.
f. Mix Binding Mix 2 by inversion. Add 15 µl of Binding Mix 2 into the wells containing Binding Mix 1.
g. Mix each well by gently pipetting up and down 2-3 times and seal wells with plate sealer.
h. Incubate binding reactions at 25°C for 20 minutes in the thermal cycler.

3. Digestion
   a. Mix Digestion Buffer by gently vortexing and spin down if necessary. Add 25 µl of Digestion Buffer to *Positive Reagent Control wells only*.
   b. During the last five minutes of the binding step incubation, prepare Complete Digestion Buffer according to the following table: **IMPORTANT: Do not use protein binding materials such as polystyrene for the preparation of Complete Digestion Buffer. The use of polypropylene is recommended.**

Complete Digestion Buffer	Digestion Buffer	Digestion Reagent	Final Volume
Volume per Reaction	25 µl	0.25 µl	25.25 µl

   c. Pipette the Complete Digestion Buffer up and down until completely mixed. DO NOT VORTEX ! Add 25 µl of Complete Digestion Buffer to all sample wells and Negative Reagent Control wells. Do not add Complete Digestion Buffer to the Positive Control wells.
   d. Mix contents of all wells by gently pipetting up and down two to three times, and cover wells with plate sealer. Avoid making bubbles.
   e. Incubate wells for 20 minutes at 37°C in a thermal cycler or a heat block.

4. Hybridization
   a. Mix the Hybridization Buffer by vortexing briefly and then check for precipitate. If precipitate is present return to 37°C waterbath until the solution is clear.
   b. Mix the Hybridization Buffer and the Hybridization Accelerator prior to use by vortexing briefly. Spin the vials in a microcentrifuge to bring the liquid to the bottom of the tube.
   c. Prepare Complete Hybridization Buffer according to the following table:

Complete Hybridization Buffer	Hybridization Buffer	Hybridization Accelerator	Final Volume
Volume per Reaction	10 µl	1.0 µl	11 µl

   d. Mix the Complete Hybridization Buffer by vortexing briefly. Spin the vial in a microcentrifuge to bring the liquid to the bottom of the tube.
   e. Add 10 µl of Complete Hybridization Buffer to each well of the Capture Microplate that will be used.
   f. Transfer 50 µl of each sample from the PCR plate to the corresponding wells of the Capture Plate. Cover the plate with a microplate cover and incubate for 45 minutes at room temperature with shaking. Shaking speed should be as fast as possible without spilling anything from the wells.

5. Wash
   a. During the 45 minute incubation in step 4.f, prepare 1X Wash Buffer by diluting 10X Wash Buffer 1:10 with de-ionized water (e.g. 1 ml 10X Wash Buffer plus 9 ml water). A total of 3 mls of 1X wash buffer will be needed for each well. If using an automatic plate washer, prepare extra wash buffer for priming. Additional 10X Wash Buffer (Catalog # 60319) can be ordered from Marligen if needed.
   b. After preparation of the 1X Wash Buffer and also during the 45 minute incubation in step 4.f, prepare Detection Reagent by diluting the streptavidin-alkaline phosphatase conjugate 1:100 in Conjugate Diluent. Use the following table as a guide. Mix by inverting 3-5 times and spin to bring liquid to the bottom of the tube.

Detection Reagent	Conjugate Diluent	SA-AP Conjugate	Final Volume
Volume per Reaction	100 µl	1.0 µl	101 µl

   c. Remove plate from shaker and aspirate contents. Wash plate four times with 1X Wash Buffer (300 µl/well).

6. Detection
   a. Add 100 µl of Detection Reagent to each well. Cover plate with Microplate cover and incubate for 30 minutes at room temperature with shaking.
   b. Carefully aspirate Detection Reagent from the wells. Wash the plate four times with 1X Wash Buffer (300 µl/well).
   c. Add 75 µl of Chemiluminescent Substrate to each well. Cover plate with the microplate cover and incubate at 37°C for 30 minutes.
   d. Read light intensity with a microplate luminometer and record the results as relative light units (RLUs).
   e. Remove plate from plate reader.
   f. Add 100 µl of 1M HCl to the wells containing the Positive Reagent Control and mix. This step is necessary to stop the production of light from the Positive Control wells. Otherwise, the high level of light produced by the Positive Reagent Control wells may affect the readings of adjacent wells.
   g. Read plate again and record the results as RLUs.

7. Example Data

	Positive Reagent Control	Negative Reagent Control	Untreated Nuclear Extract	PMA-treated Nuclear Extract
Replicate 1	844250	9166	40102	101548
Replicate 2	853300	9162	40482	95211
Replicate 3	783750	9817	45874	87534
Mean	827100	9382	42153	85383
%CV	4.6%	4.0 %	7.7 %	8.2 %
Background Subtracted	817718	0	32771	76001

% Digestion = 1 – (mean Neg./mean Pos.) = 1 – (9382/827100) = 98.9%

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