Signosis
Innovative Plate Assay Solutions

Promoter-Binding TF Profiling Plate Array I

Catalog Number FA-2001 **(For Research Use Only)**

Introduction

To characterize transcription factors (TFs) that bind to a specific promoter or that regulate the expression of a specific gene via its upstream promoter, two common approaches are applied. First is to employ gel shift assay with DNA binding sites of TFs that are silico-identified within the promoter. Second is to remove or knockout the binding site(s) of a specific TF in order to measure whether the expression of a promoter-linked reporter is increased or decreased. Because many binding sites of one or a few TFs are present within a promoter, it is required to make a series of reporter constructs with the promoter deletions or mutations. **Signosis, Inc.** has developed a fast method to facilitate the characterization through a revised TF activation array. This assay will help to test whether a selected 48 TFs bind to the promoter or not.

Principle of the assay

Promoter-Binding TF Profiling Assay is a competition of **Signosis, Inc.'s** *TF Activation Plate Array I*. In the *TF Activation Plate Array I*, if all of 48 targeted transcription factors exist in the assayed samples, they will form 48 types of complexes, each TF with its corresponding biotin-labeled oligo (similar to the complex in the gel shift assay). After a simple spin separation of the complexes from unbound free biotin-labeled oligos with a membrane-based column, TF-bound probes are eluted from the column and used for plate hybridization. The captured probes are then detected with Streptavidin-HRP Conjugate and a chemiluminescent substrate. If no TF is present, no complex will form leading to no detection of TF in the plate assay. In promoter-binding TF profiling assay, PCR fragment containing the promoter of your interest is mixed with a set of 48 biotin-labeled oligos corresponding to 48 TFs along with an assayed sample. If DNA fragment contains a TF binding sequence, it will compete with the biotin-labeled oligo to bind to the TF in the sample, leading to no or less complex formation and no or lower detection. Through comparison in the presence and absence of the competitor plasmid or DNA fragment, promoter TFs can be identified.

Materials Provided with the Kit

Component	Qty	Store at
96-Well Plate (with aluminum adhesive seal)	2	RT
Isolation Columns	4	RT
Elution Buffer	400µL	RT
TF Plate Hybridization Buffer	20mL	RT
5X Plate Hybridization Wash Buffer	60mL	RT
5X Detection Wash Buffer	60mL	RT
Blocking Buffer	60mL	4°C
Filter Wash Buffer	10mL	4°C
Filter Binding Buffer	1mL	4°C
Substrate A	2mL	4°C
Substrate B	2mL	4°C
Streptavidin-HRP Conjugate	40µL	4°C
Substrate Dilution Buffer	16mL	4°C
TF Binding Buffer Mix	60µL	-20°C
TF Probe Mix I	20µL	-20°C

Material Required but not Provided

- Nuclear Extraction Kit from Signosis (SK-0001)
- DNA PCR product fragment
- PCR machine
- Microcentrifuge working at 4 °C
- Hybridization incubator
- Shaker
- Plate reader for luminescent detection
- ddH2O (RNase free)

Before Starting the Experiment Prepare the Following:

- Place *Filter Binding Buffer* and *Filter Wash Buffer* on **ice** so they are chilled for the assay (for at least **10 minutes**).
- Warm up *TF Plate Hybridization Buffer*, *Blocking Buffer*, and *Hybridization Wash Buffer* to **42˚C** before use.
- Aliquot **500µL** of ddH_2O in a 1.5mL microcentrifuge tube **per sample** on ice so that it is chilled for the assay (for at least **10 minutes**).
- Dilute **60mL** of *5X Plate Hybridization Wash Buffer* with **240mL** of ddH2O before use.
- Dilute **60mL** of *5X Detection Wash Buffer* with **240mL** of ddH2O before use.
- Dilute **40µL** *Streptavidin-HRP* in **20mL** Blocking

Please Read the Assay Procedure Before You Begin

Assay Procedure
Read the procedure carefully before you start

TF DNA Complex Formation

1. Mix the following components for each reaction in a tube or one well of a PCR plate:

   15µL *TF Binding Buffer Mix*
   3µL *TF Probe Mix I*
   2-5µL Promoter PCR fragment (0.1-0.5µM)
   XµL Nuclear Extract (5µg-15µg)
   YµL ddH2O
   30µL *Reaction Mix*

2. Incubation at room temperature (20-23°C) for **30 minutes**.

Separation of TF DNA Complex from Free Probes

3. Equilibrate the Isolation Column by adding **200µL** *cold Filter Binding Buffer*, and centrifuge **at 6,000 rpm** for **1 minute** in microcentrifuge at room temperature.
4. Transfer the **30µL** reaction mix directly onto the center of the Isolation Column.
5. Incubate the column on ice for **30 minutes**.
   DO NOT incubate longer than 30 minutes; this results in high background.
6. Add **500µL** *cold Filter Wash Buffer* to the column, and incubate for **2-3 minutes** on ice.
7. Centrifuge at **6,000 rpm** for **1 minute** in microcentrifuge at **4°C**, and discard the flow through.
8. Wash the column by adding **500µL** *cold Filter Wash Buffer* to the column on ice.
9. Centrifuge for **1 minute** at **6,000 rpm** in microcentrifuge at *cold Filter Wash Buffer*, and discard the flow through.
10. Repeat the steps 8-9 so that the columns are washed an 3 times.

Elution of Bound Probe

11. Move the column to a new collection tube. Add **100µL** of *Elution Buffer* onto the center of column and at room temperature for **5 minutes**.
12. Centrifuge the column and tube at **10,000 rpm** for **2 minutes** at room temperature.
13. If you have yet to do so, chill **500µL** ddH2O (DNase free) in a **1.5mL** microcentrifuge tube on ice for at least **10 minutes**, and **keep on ice**.
14. Transfer the eluted probe to a PCR tube and denature the eluted probes at **98°C** for **5 minutes**.
15. **Immediately** transfer the denatured probes to the chilled ddH2O from step 13. and place **on ice**. The samples are ready for hybridization. You store -20ºC for the future use (the probe must be denatured again before use, but do not dilute in water again).

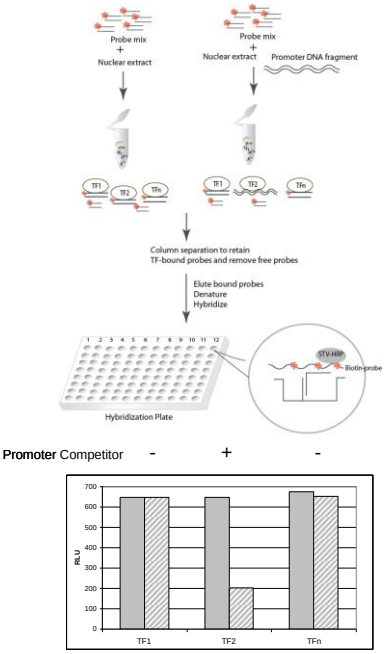


Diagram of Promoter –Binding TF Profiling Assay

Hybridization of Eluted Probe with the Plate

16. Remove the sealing film from the plate.
17. Add **5mL** warmed Hybridization buffer to a dispensing reservoir (DNase free) and then add 600ul denatured probes. Mix them together by gently shaking the reservoir.
18. Dispensing 100ul of the mixture into the corresponding wells with 8 multi-channel pipette **immediately**.
    Note: the 96 well hybridization plate is divided into two sections. Section one (Column 1-6) for one sample and section two (Column 7-12) for another sample.
    If a blank well is desired to perform, select one TF well you may not be interested in from the diagram below as a blank well and add 1X Hybridization buffer only without the eluted probe
19. Seal the wells with foil film securely and hybridize at **42°C** for overnight. Ensure the numbers and letters on the plate are clearly visible from under foil seal by pressing the foil down on every single experimental well.

Detection of Bound Probe

20. Remove the foil film from the experimental wells with a blade. Keep the unused well sealed.

21. Remove the foil film from the experimental wells with a blade. Keep the unused well sealed.
22. Invert the Hybridization Plate over an appropriate container and expel the contents forcibly, and wash the plate 3 times by adding 200ul of pre-warmed 1x Plate hybridization wash buffer to each well. At each wash, incubate the plate for 5 minutes with gently shaking at room temperature.
23. Complete removal of liquid at each wash by firmly tapping the plate against clean paper towels.
24. Add 200µl of Blocking buffer to each well and incubate for 15 minutes at room temperature with gently shaking
25. Invert the plate over an appropriate container to remove block buffer.
26. Add **40µL** of *Streptavidin-HRP Conjugate* in 20mL Blocking Buffer (1:500 dilution), enough for two plates. Add **95µL** of *diluted Streptavidin-HRP Conjugate* to each well and incubate for **45 minutes** at room temperature with gently shaking.
27. Wash the plate **3 times** by adding **200µL** *1X Detection Wash Buffer* to each well. At each wash, incubate the plate for 10 minutes with gently shaking at room temperature.
28. Complete removal of liquid at each wash by firmly tapping the plate against clean paper towels. At the last wash, invert plate on clean paper towels for **1-2 minutes** to remove excessive liquid.
29. Freshly prepare the substrate solution:
    For the whole plate:
    1mL *Substrate A*
    1mL *Substrate B*
    8mL *Substrate Dilution Buffer*
    10mL *Substrate Solution*

30. Add **95µL** substrate solution to each well and incubate for **1 minute**.
31. Place the plate in the luminometer. Allow plate to sit inside machine for **5 minutes** before reading. Set integration time to 1 second with no filter position. For the best results, read the plate within **5-20 minutes**.

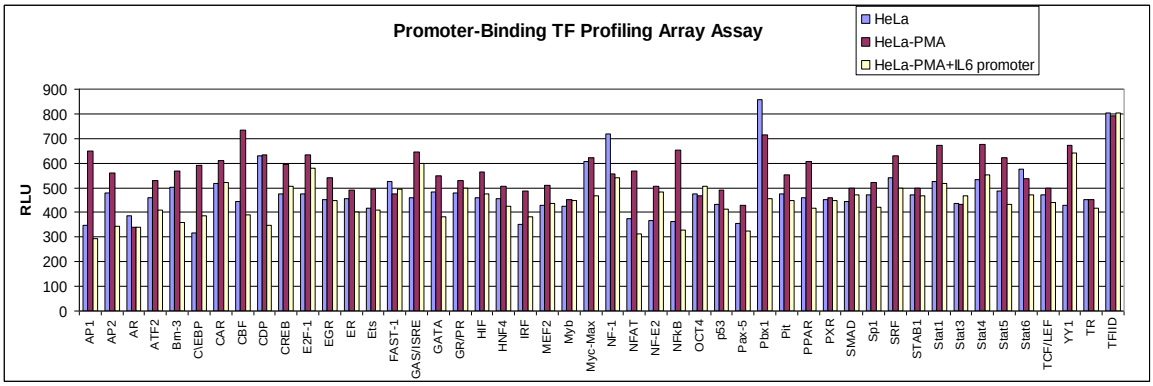
TF Promoter Binding Array I Diagram

	1	2	3	4	5	6	7	8	9	10	11	12
A	AP1	CDP	GATA	NF-1	Pit	Stat3	AP1	CDP	GATA	NF-1	Pit	Stat3
B	AP2	CREB	GR/PR	NFAT	PPAR	Stat4	AP2	CREB	GR/PR	NFAT	PPAR	Stat4
C	AR	E2F-1	HIF	NF-E2	PXR	Stat5	AR	E2F-1	HIF	NF-E2	PXR	Stat5
D	ATF2	EGR	HNF4	NFkB	SMAD	Stat6	ATF2	EGR	HNF4	NFkB	SMAD	Stat6
E	Brn-3	ER	IRF	4-Oct	Sp1	TCF/LEF	Brn-3	ER	IRF	4-Oct	Sp1	TCF/LEF
F	C\EBP	Ets	MEF2	p53	SRF	YY1	C\EBP	Ets	MEF2	p53	SRF	YY1
G	CAR	FAST-1	Myb	Pax-5	SATB1	TR	CAR	FAST-1	Myb	Pax-5	SATB1	TR
H	CBF	GAS/ISRE	Myc-Max	Pbx1	Stat1	TFIID	CBF	GAS/ISRE	Myc-Max	Pbx1	Stat1	TFIID

TF Description Chart:

TF	Gene Description	TF	Gene Description
AP1	Activator protein 1 (JUN/FOS)	NF-1	Nuclear factor 1
AP2	Activator protein 2	NFAT	Nuclear factor of activated T-cells
AR	Androgen receptor	NF-E2	Nuclear factor (erythroid-derived 2)
ATF2	activating transcription factor 2	NFkB	gene
Bm-3	POU domain, class 4, transcription factor 1	OCT4	POU class 5 homeobox 1
C/EBP	CCAAT/enhancer binding protein (C/EBP),alpha	p53	Tumor protein p53
CAR	nuclear receptor subfamily 1, group I, member 3	Pax-5	Paired box 5
CBF	CCAAT/enhancer binding protein (C/EBP), zeta	Pbx1	Pre-B cell leukemia transcription factor-1
CDP	cut-like homeobox 1; CCAAT displacement protein	Pit	Pituitary specific transcription factor 1
CREB	cAMP responsive element binding protein 1	PPAR	Peroxisome proliferator-activated receptor
E2F-1	E2F transcription factor 1	PXR	Pregnane X Receptor
EGR	Early growth response	SMAD	SMAD family
ER	Estrogen receptor	Sp1	SP1 transcription factor
Ets	v-ets erythroblastosis virus E26 oncogene homolog 1	SRF	Serum response factor
FAST-	Forkhead box H1	SATB1	Special AT-rich sequence binding protein 1
GAS/ISRE	IFN-stimulated response element	Stat1	Signal transducer and activator of
GATA	GATA transcription factor	Stat3	Signal transducer and activator of
GR/PR	Glucocorticoid receptor/Progesterone receptor	Stat4	Signal transducer and activator of
HIF	Hypoxia inducible factor	Stat5	Signal transducer and activator of
HNF4	Hepatocyte nuclear factor 4	Stat6	Signal transducer and activator of
IRF	Interferon regulatory factor	TCF/LEF	Runt-related transcription factor 2
MEF2	Myocyte enhancer factor 2	YY1	YY1 transcription factor
Myb	v-myb myeloblastosis viral oncogene homolog	TR	Thyroid hormone receptor
Myc-Max	v-myc myelocytomatosis viral oncogene homolog (avian)	TFIID	TATA box binding protein

Data Example



AP1

TGCTGAGTCACTAATAAAAGAAAAAAAGAAAGTAAAGGAAGAGTGGTTCTGCTTCTTAGCGCTAGCCTCAATGACGAC

C/EBP

CTAAGCTGCACTTTTCCCCCTAGTTGTGTCTTGCCATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAAT

NFkB

CAGCCCCACCCGCTCTGGCCCCACCCTCACCCTCCAACAAAGATTTATCAAATGTGGGATTTTCCCATGAGTCTCAATA

TTAGAGTCTCA

Figure. Promoter-Binding TF Profiling Assay I. Promoter-Binding TF Profiling Assay: HeLa cells were treated with or without PMA. PMA was used to active TFs including AP1 and NFkB. Nuclear extracts were prepared and incubated with TF binding oligo probe mix: control HeLa cells without PMA treatment with the probe mix (blue); PMA-treated HeLa cells with the probe mix alone (red) and the probe mix plus IL6 promoter DNA fragment (yellow).