Signosis
BioSignal Capture

Promoter-Binding TF Profiling Assay I

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

Introduction

To characterize transcription factors (TFs) that binds 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 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' 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 and a chemiluminescent substrate. If any TF is not present, it will not form a complex, 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 complete 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.

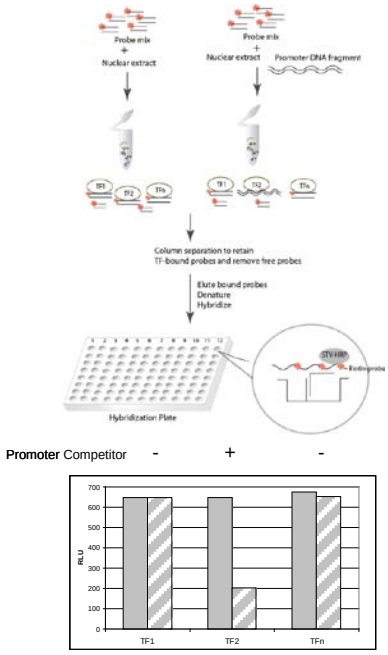


Diagram of Promoter –Binding TF Profiling Assay

Materials provided with the kit

- Two 96-well Hybridization Plate (RT)
- Four isolation columns (RT)
- TF binding buffer mix (-20 °C)
- TF Probe mix I (-20 °C)
- Filter binding buffer (4 °C)
- Filter wash buffer (4 °C)
- Elution buffer (RT)
- Streptavidin-HRP conjugate (4°C)
- Plate hybridization buffer (RT)
- 5x Plate hybridization wash buffer (RT)
- Blocking buffer (RT)
- 5x Detection wash buffer (RT)
- Substrate A (4°C)
- Substrate B (4°C)
- Substrate dilution buffer (RT)
- Foil film

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 (RNAase free)

Reagent preparation before starting experiment

- Keep Filter binding buffer and Filter wash buffer on ice
- Warm up Plate Hybridization Buffer and Hybridization Wash buffer at 42 ^{o}C before use.
- Dilute 30ml of 5x Plate Hybridization wash buffer with 120 ml of dH_2O before use.
- Dilute 40ml of 5x Detection wash buffer with 160 ml of dH_2O before use.
- Dilute 500 times of streptavidin-HRP with blocking buffer before use.

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
   15ul TF binding buffer mix
   3ul TF Probe mix I
   2-5ul Promoter PCR fragment (2-3uM)
   Xul Nuclear extract (5µg-15µg)
   Xul ddH2O
   30ul
2. Incubation at room temperature (20-23^{o}C) for 30 minutes.

Separation of TF DNA Complex from Free Probes

3. Equilibrate the Isolation Column by adding 200ul cold Filter binding buffer, and centrifuge at 6000 rpm for 1 min in microcentrifuge at room temperature.
4. Transfer the 30ul reaction mix directly onto the center of the Isolation Column.
5. Incubate on ice for 30 minutes.
   Don't incubate longer than 30 minutes, which results in high background.
6. Add 500ul cold Filter wash buffer to the column, and incubate for 2-3 minutes on ice.
7. Centrifuge at 6000 rpm for 1 min in microcentrifuge at 4^{o}C, and discard the flow through.
8. Wash the column by adding 500ul cold Filter wash buffer to the column on ice.
9. Centrifuge for 1 min at 6000 rpm in microcentrifuge at 4^{o}C, and discard the flow through.
10. Repeat the step 8-9 for additional 3 time washes.

Elution of Bound Probe

11. Add 100ul of Elution buffer onto the center of column, and incubate at room temperature for 5 minutes.
12. Put the column on a 1.5 ml microcentrifuge tube, and centrifuge at 10,000 rpm for 2 minutes at room temperature.
13. Chill 500ul ddH2O (DNAase 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 ^{o}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 or store -20 ^{o}C for the future use (the probe must be denatured again before use).

Hybridization of Eluted Probe with Hybridization Plate

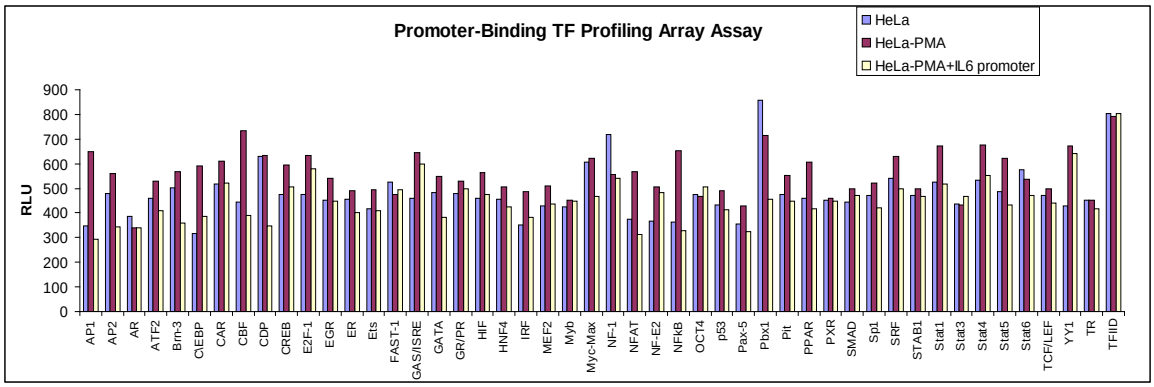
16. Remove the sealing film from the plate.
17. Add 5ml warmed Hybridization buffer to a dispensing reservoir (DNase free) and then add 600ul denatured probes. Mix them together by gently shaking the reservoir.
18. Dispensing 100ul of the mixture into the corresponding wells with 8 multi-channel pipette **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 ^{o}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. *Put an open container with water in the incubator to keep humidity and prevent evaporation from experimental wells.*

Detection of Bound Probe

20. Invert the Hybridization Plate over an appropriate container and expel the contents forcibly, and wash the plate 3 times by adding 200µl of pre-warmed 1x Plate hybridization wash buffer to each well.
21. Complete removal of liquid at each wash by firmly tapping the plate against clean paper towels.
22. Add 200µl of Blocking buffer incubate for 15 minutes at room temperature with gently shaking.
23. Invert the plate over an appropriate container to remove block buffer.

24. Add 20 µl of streptavidin-HRP conjugate in 10ml blocking buffer (1:500) dilution, enough for two plates. Add 95 µl of diluted streptavidin-HRP conjugate to each well and incubate for 45 min at room temperature with gently shaking.
25. Wash the plate with 200ul 1X Detection wash buffer for 5 minutes. Complete removal of liquid at each wash by firmly tapping the plate against clean paper towels.
26. Repeat step 25 for additional 2 time washes.
27. Freshly prepare the substrate solution:
    For the whole plate:
    1ml Substrate A
    1ml Substrate B
    8ml Substrate dilution buffer
28. Add 95µl substrate solution to each well and incubate for 1 min.
    Notes: Substrate solution can be added to one plate first. After the measurement of the first plate is done, the substrate solution can be then added to the second plate.
29. Place the plate in the luminometer. Allow plate to sit inside machine for 5min before reading. Set integration time to 1 second with no filter position. For the best results, read the plate within 5-20 minutes.

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).

TF Activation 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	OCT4	Sp1	TCF/LEF	Brn-3	ER	IRF	OCT4	Sp1	TCF/LEF
F	C\EBP	Ets	MEF2	p53	SRF	TR	C\EBP	Ets	MEF2	p53	SRF	TR
G	CAR	FAST-1	Myb	Pax-5	SATB1	YY1	CAR	FAST-1	Myb	Pax-5	SATB1	YY1
H	CBF	GAS/ISRE	Myc-Max	Pbx1	Stat1	TFIID	CBF	GAS/ISRE	Myc-Max	Pbx1	Stat1	TFIID

Notes: TFIID can be used to normalize the readings for comparison between two samples if the promoter of interest doesn't contain TFIID binding site, TATA box. The TATA box has the core DNA sequence 5'-TATAAA-3' or a variant, which is usually followed by three or more adenine bases. It is usually located 25 base pairs upstream of the transcription site.

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
Brn-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