Signosis
Innovative Plate Assay Solutions

AP1 Filter Plate Assay

Catalog # FA-0004 **(For Research Use Only)**

Introduction

AP1, a dimeric protein complex composed of Jun and Fos proteins, plays a key role in the control of cell death, proliferation and transformation. AP1 protein can be activated by many stimuli including TPA, serum, growth factors, oncoproteins, TNF and IL-1. It can also be activated by a variety of environmental stresses, such as UV radiation. When activated the AP1 protein binds to a AP-DNA recognition element within the promoter regions of target genes and regulates the gene expression. **Signosis, Inc**. has developed AP1 filter assay for monitoring DNA binding activity of AP1, which can be used for analyzing multiple samples simultaneously.

Principle of the Assay

Signosis, Inc.'s *AP1 Filter Plate Assay* is a plate-based analysis for monitoring the activity of AP1. In the assay, biotin labeled AP1 probes are mixed with nuclear extract to allow formation of AP1-DNA complex. The filter plate is used to retain bound AP1 probes and remove free DNA probes. The bound pre-labeled AP1 probe is then eluted from the filter plate and hybridize to the corresponding well of 96 well of Hybridization Plate for quantitative analysis. The captured AP1 probe is further detected with streptavidin-HRP. Luminescence is reported as relative light units (RLUs) on a microplate luminometer.

Material Required but Not Provided

- Nuclear Extraction Kit from **Signosis, Inc**. (SK-0001)
- Wash Collection Plate (Any 96-Well ELISA plate or equivalent 96-well plastic plate)
- 96-Well PCR plate
- Microplate centrifuge working at 4°C
- PCR machine and PCR tubes
- Hybridization incubator at 42°C
- Plate-Shaker
- Plate reader for luminescent detection
- ddH2O (DNAase-free)
- 8 and 12 Multi-channel pipettes

Materials Provided with the Kit

Component	Qty	Store at
96-Well Plate (with aluminum adhesive seal)	1	RT
96-Well Filter Plate	1	RT
Elution Buffer	5mL	RT
TF Plate Hybridization Buffer	10mL	RT
5X Plate Hybridization Wash Buffer	30mL	RT
5X Detection Wash Buffer	40mL	RT
Blocking Buffer	30mL	4°C
Filter Wash Buffer	100mL	4°C
Filter Binding Buffer	25mL	4°C
Substrate A	1mL	4°C
Substrate B	1mL	4°C
Streptavidin-HRP Conjugate	20µL	4°C
Substrate Dilution Buffer	8mL	4°C
TF Binding Buffer Mix	1mL	-20°C
AP1 Probes	200µL	-20°C

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* **45˚C** before use.
- Dilute **30mL** of *5X Plate Hybridization Wash Buffer* with **120mL** of ddH2O before use.
- Dilute **40mL** of *5X Detection Wash Buffer* with **160mL** of ddH2O before use.
- Dilute **20µL** *Streptavidin-HRP* in **10mL** Blocking Buffer (1:500 dilution).

Please Read the Assay Procedure Before You Begin

Assay Procedure

TF DNA Complex Formation

1. Mix the following components for each single reaction in a tube or one well of a PCR plate
   10µL *TF Binding Buffer Mix*
   2µL *AP1 probes*
   XµL Nuclear Extract (2µg-10µg recommended)
   YµL ddH2O (add up to final volume)
   20µL Reaction Mix [final volume]
2. Incubate at **room temperature** for **30 minutes**.

Separation of TF DNA Complex from Free Probes

3. Based on the number of samples for the experiment, allocate the same number of wells on the *96-Well Hybridization Plate*. Remove the clear adhesive seal that covers the allocated selection by using a razor blade. Keep all unused-wells sealed.
4. Acquire the *96-Well Filter Plate* and remove the top hard-plastic plate guard (clear). Do **NOT** remove the semi-translucent filter bottom of the plate.
5. Place the *Wash Collection Plate* on the bottom of *96-Well Filter Plate* during the following steps to assemble Filter Plate/Wash Collection Plate.
6. Pre-wet the *96-Well Filter Plate*
   a. Add **250µL**/well cold *Filter Binding Buffer* to each experimental well of *96-Well Filter Plate*.
   b. Centrifuge *96-Well Filter Plate/Wash Collection Plate* at **600 g** for **1 minute**, and discard the flow-through from Wash Collection Plate.
7. Add **20µL** cold *Filter Binding Buffer* to each **Reaction Mix** tube or well, and transfer **35µL** of the mixture to the center of the filter in the corresponding well of *96-Well Filter Plate*.
8. Incubate the *96-Well Filter Plate* on ice for 30 minutes. **Don't incubate longer than 30 minutes, which results in high background.**
9. Centrifuge *96-Well Filter Plate/Wash Collection Plate* at **600g** for **2 minutes** and discard the flow-through from the Wash Collection Plate.
10. Add **250µl** cold *Filter Wash Buffer* to each experimental well of *96-Well Filter Plate* and incubate for **2-3 minutes** on ice.
11. Centrifuge *96-Well Filter Plate/Wash Collection Plate* again at **600g** for **2 minutes**, and discard the flow-through from Wash Collection Plate.
12. Repeat the steps 10-11 for 3 more times for total of 4 washes.

Elution of Bound Probe

13. Add **60µL** of *Elution Buffer* to the center of each experimental well in *96-Well Filter Plate*.
14. Place a *96-Well PCR Plate* on the top of Wash Collection Plate or a 96-Well PCR plate rack.
15. Place *96-Well Filter Plate* on the top of a *96-well PCR Plate* so that the column tips align to dispense into the *PCR plate*. Ensure each well on the top plate matches the bottom ones. For added stability, fix the assemblies with tape on sides of the plates.

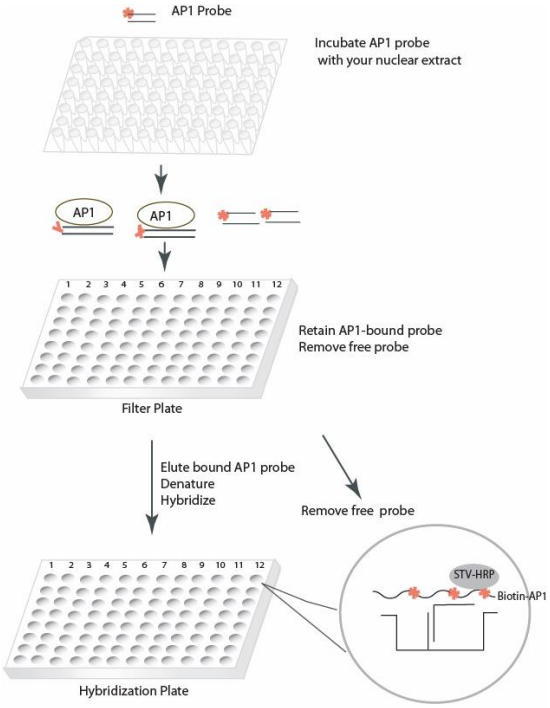

Figure 1: AP1 Filter Plate Assay Procedure

16. Incubate for **5 minutes** at room temperature.
17. Centrifuge the assembly at **600g** for **2 minutes**. The **60µL** of sample should be in each well of the *96-Well PCR plate*.
18. Denature the eluted samples in the *96-Well PCR Plate* at **95ºC** for **3 minutes** in a PCR machine, and transfer on ice **immediately**. The samples are ready for hybridization or store -20°C for the future use.

Hybridization of Eluted Probe with Hybridization Plate

19. Add **60µL** *Hybridization Buffer* to each experimental well of *96-Well Hybridization Plate*. Then transfer **30µL** of denatured sample to the corresponding well and mix gently.
20. Seal the wells with provided foil film. Seal the wells firmly. Hybridize the samples to the plate by placing the plate in a incubator set at **42°C** overnight. Ensure the numbers and letters are clearly visible from under foil seal. This will help orient you around the plate.

Detection of Bound Probe

21. Add **20 µL** of *Streptavidin-HRP Conjugate* in **10 mL** *Blocking Buffer* (1:500 dilution). This is sufficient for the entire plate.
22. Remove the foil film from the experimental wells with a razor blade. Keep the unused wells sealed.
23. Invert the *96-Well Hybridization Plate* over an appropriate container or sink and expel the contents forcibly. Tap the plate over paper towels to remove residual liquid in the wells.

24. Wash the plate by adding **200µL** of pre-warmed *1X Plate Hybridization Wash Buffer*. Decant the wash buffer forcibly after the wash. Completely remove any residual liquid from each well by firmly tapping the plate against clean paper towels. Repeat this step two more times for a total of **three** washes.
25. Wash the plate once by adding **200 µL** of *Blocking Buffer* to each well. Decant the buffer forcibly after the wash. Completely remove any residual liquid by firmly tapping the plate against clean paper towels.
26. Add **95µL** of Streptavidin-HRP Conjugate/Blocking Buffer solution to each well and incubate for **45 minutes** at room temperature with gentle shaking on the Plate-Shaker.
27. After the 45 minutes have elapsed, decant the wells' contents in the sink or in an appropriate container.
28. Tap the plate over paper towels to remove any residual liquid.
29. Wash the plate 3 times by adding **200µL** *1X Detection Wash Buffer* to each well. Completely remove the liquid at the end of each wash by firmly tapping the plate against clean paper towels.
30. At the last wash, invert plate on clean paper towels for 1-2 min to remove residual liquid.
31. Freshly prepare the **Substrate Solution**:
    1 part *Substrate A* + **1 part** *Substrate B* + **8 parts** *Substrate Dilution Buffer*.
    For example, for the whole plate:
    1mL *Substrate A*
    1mL *Substrate B*
    8mL *Substrate Dilution Buffer*
    10mL *Substrate Solution*
32. Add **95µL** *Substrate Solution* to each well and incubate at room temperature for **1 min.**
33. 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**.

Data Example

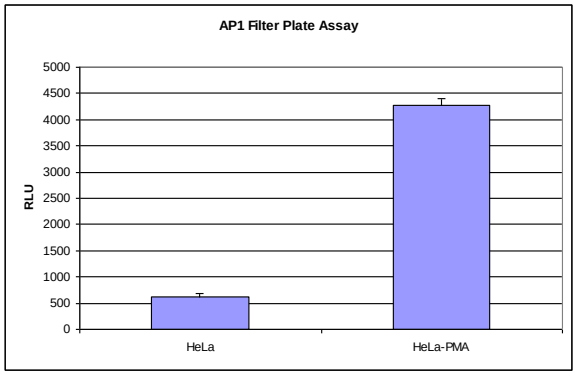

Figure 2. AP1 Filter Plate Assay analysis. HeLa cells were starved with serum-free medium for 6 hours, and treated without and with 20ng/ml PMA for 30 minutes. The nuclear extracts were prepared, and subjected to AP1 Filter Plate Assay.