



HIF Filter Plate Assay

Catalog # FA-0002

(For Research Use Only)

Introduction

Hypoxia-inducible factor (HIF) has been recognized primarily for its role in the maintenance of oxygen and energy homeostasis. HIF-1 and HIF-2 are heterodimeric transcription factors composed of HIF-1 α and HIF-2 α , respectively, which dimerize with a constitutively expressed β subunit and subsequently bind to hypoxia response elements in the promoters of target genes. The activity of HIF is regulated by a variety of stimuli, including changes in cellular oxygen concentration, growth factors and oncogenic activation. **Signosis, Inc.** has developed the *HIF Filter Plate Assay*. Unlike conventional gel shift assays, this filter assay is a high throughput assay and it can analyze DNA binding activities of HIF in multiple samples simultaneously. Additionally, the assay can be set up for a few samples with less tedious than non-radioactive gel shift assay or much safer than ^{32}P -based gel shift assay.

Principle of the Assay

Signosis, Inc.'s *HIF Filter Plate Assay* is a plate-based analysis for monitoring the activity of HIF. In the assay, biotin labeled DNA binding sequence of HIF is mixed with nuclear extract to allow formation of HIF-DNA complex. A filter plate is used to retain bound HIF probe and remove free DNA probe. The bound pre-labeled HIF probe is then eluted from the filter and hybridize to the corresponding well of 96 well of Hybridization Plate for quantitative analysis. The captured HIF 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	20 μ L	4°C
Substrate Dilution Buffer	8mL	4°C
TF Binding Buffer Mix	1mL	-20°C
HIF Probes	200 μ L	-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 **20 μ L** *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 reaction in a tube or one well of a PCR plate
10µL TF Binding Buffer Mix
2µL HIF 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.

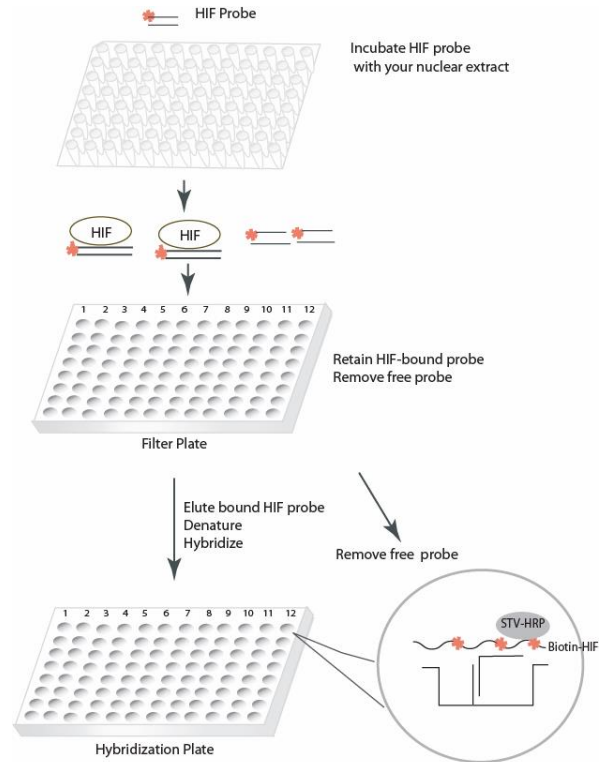


Figure 1: HIF Filter Plate Assay Procedure

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 **95°C** 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 *IX 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. 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
30. Add **95µL Substrate Solution** to each well and incubate at room temperature for **1 min.**
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**.

Data Example

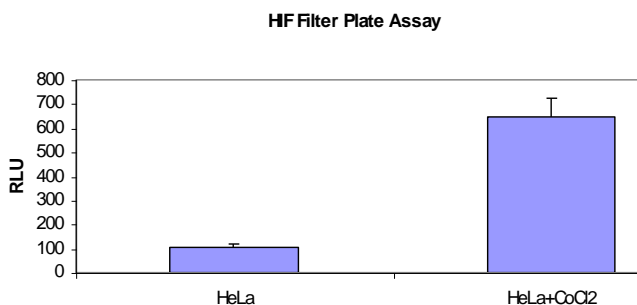


Figure 2. HIF filter plate assay analysis. HeLa cells were treated with or without CoCl₂ for 8 hours. Nuclear extracts were prepared and subjected to HIF Filter Plate assay.