RayBio® Human Nanog Transcription Factor Activity Assay Kit

Catalog #: TFEH-Nanog

User Manual
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RayBio® Human Nanog TF-Activity Assay Kit Protocol

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Please read the entire manual carefully before starting your experiment.

I. INTRODUCTION

Homeobox-containing transcription factor Nanog is essential in maintaining the pluripotent cells of the inner cell mass and in the derivation of embryonic stem cells (ESCs). Overexpression of Nanog is capable of maintaining the pluripotency and self-renewing characteristics of ESCs under what normally would be differentiation-inducing culture conditions. Concomitant with this essential function in pluripotent cell maintenance, expression of Nanog has restricted patterning in the inner cells of the morula, blastocyst, and epiblast during different developmental processes. Additionally, Nanog acts as a molecular gatekeeper to suppress or ensure differentiation in response to events in fluctuating environments and the presence of stimuli. Nanog has a single homeodomain that binds to DNA through the consensus sequence TAATGG. Coordinating with Oct4 and Sox2, Nanog regulates a large cohort of genes involved in pluripotency and development in later stages.

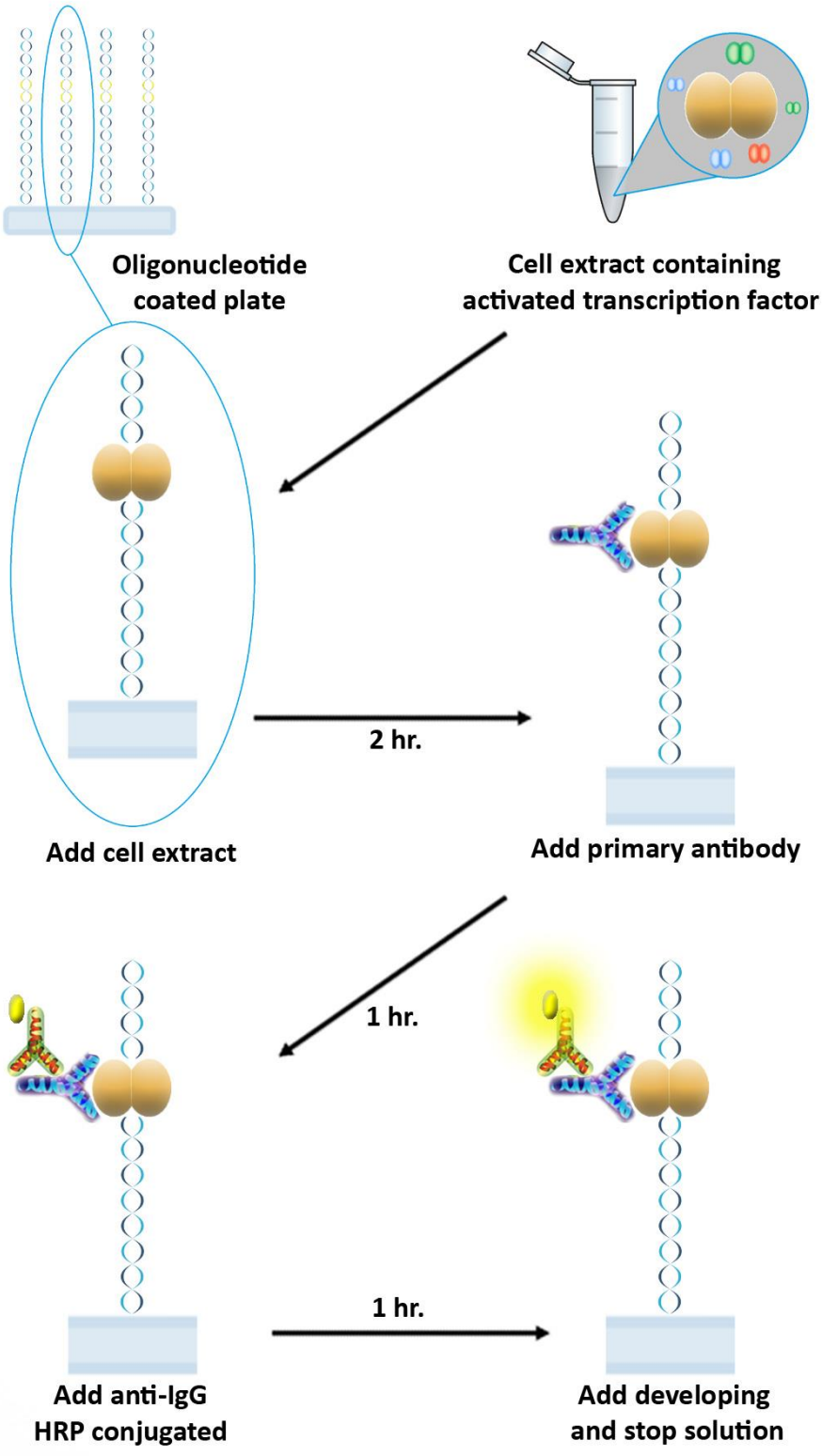
Accurate monitoring of the level of activated Nanog in cells, tissues or animal models is required for both science research investigating signal transduction pathways and applications such as drug development, and simple, speedy and high-throughput methods are needed for this purpose.

Traditionally, western blots to detect the expression of Nanog, electrophoretic mobility shift assays (EMSA) to detect the DNA binding capacity of Nanog, or transfection of reporter genes such as luciferase and β-galactosidase with Nanog binding sites in culture cells are used in evaluation of Nanog reactivity. However, these methods are time consuming, laborious, and sometimes require the use of radioactivity.

The RayBio® Nanog TF-Activity Assay (Transcription Factor-Activity Assay) kit is a non-radioactive transcription factor assay with an ELISA format. It offers an easy, speedy, sensitive and high-throughput method to detect the activation of transcription factors. In 96-well plates, double stranded oligonucleotides containing Nanog

binding sequence have been coated. These oligonucleotides specifically capture the active Nanog contained in whole cell lysate or nuclear extracts after a short incubation. Subsequently, the primary antibody against Nanog recognizes the Nanog-DNA complex in each well, and a HRP-conjugated secondary antibody is then used for detection. After washing away any unbound antibody, signal can be obtained easily through a colorimetric assay with a spectrophotometric plate reader at 450 nm. The specificity of the reaction between active Nanog and the DNA probe is additionally stringent because of the establishment of specific competitive DNA and non-specific competitive DNA probes in this reaction system.

II. HOW IT WORKS



III. STORAGE

Upon receipt, the positive control should be removed and stored at -20° or -80°C. The remainder of the kit can be stored for up to 6 months at 2-8°C from the date of shipment. Opened Microplate Wells or reagents may be stored for up to 1 month at 2-8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: The kit can be used within one year if the whole kit is stored at -20°C upon receipt. Avoid repeated freeze-thaw cycles.

IV. REAGENTS

Component	Description	Size
Nanog DNA Probe Microplate	96 wells (12 strips X 8 wells) coated with Nanog probes	1 plate
DNA Binding Buffer	5X concentrated Buffer	4 ml
Positive Control	Cell nuclear extracts	1 vial (20 µl)
Specific Competitor DNA Probe	Free DNA probes that compete with the coated probes by binding with activated Nanog.	1 vial
Non-specific Competitor DNA Probe	Free DNA probes with mutations of the coated DNA probe. Cannot bind activated Nanog.	1 vial
Assay Reagent	1X solution	1 vial (200 µl)
DTT	300 mM DTT	1 vial (200 µl)
Wash Buffer Concentrate (20X)	20X concentrated solution	25 ml
Nanog Primary Antibody	Anti- Nanog antibody	1 vial
HRP-conjugated Secondary Antibody	Anti-IgG HRP conjugated antibody	1 vial
Antibody Diluent Buffer	Buffer solution for diluting primary and secondary antibodies	25 ml
TMB One-Step Substrate Reagent	3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	12 ml
Stop Solution	0.2 M sulfuric acid	8 ml

V. ADDITIONAL MATERIALS REQUIRED

1 Microplate reader capable of measuring absorbance at 450 nm.
2 Precision pipettes to deliver 1 μl to 1 ml volumes.
3 Adjustable 1-25 ml pipettes for reagent preparation.
4 100 ml and 1 liter graduated cylinders.
5 Absorbent paper.
6 Distilled or deionized water.
7 Tubes to prepare positive or sample mixtures.

VI. REAGENT PREPARATION

1. Preparation of samples:
   Prepare nuclear extraction or whole lysate containing targeted protein Nanog from cell culture or tissue. We recommend using the RayBiotech Nuclear Extraction Kit (Cat#: NE-50) to isolate nuclear proteins for subsequent use in this transcription factor activity assay.
2. Preparation of transcription factor binding reaction system:
   Bring all reagents to room temperature (18 - 25°C) before use. Thaw the positive control and samples and keep them on ice before adding into wells. Prepare 100 μl transcription factor binding reaction system for each well with 5 x TF-Activity Assay DNA Binding Buffer, TF-Activity Assay Reagent, DTT, Specific Competitor DNA Probe, Non-specific Competitor DNA Probe, and Positive Control or samples containing targeted proteins. Typical examples are shown in the table below.

   Note:
   Each reaction may be prepared in a labeled microfuge tube or directly in the coated plate well. If the reaction system is prepared directly in the coated plate wells, please add the reagents sequentially as shown in the table to get the best results.

	REACTION				
COMPONENT	Positive control	Sample	Specific competitor	Non-Specific competitor	Blank
5x TF-Activity Assay DNA Binding Buffer	20 μl	20 μl	20 μl	20 μl	20 μl
TF-Activity Assay Reagent	1.5 μl	1.5 μl	1.5 μl	1.5 μl	1.5 μl
DTT	1 μl	1 μl	1 μl	1 μl	1 μl
Specific Competitor	-	-	10 μl	-	-
Non-specific Competitor	-	-	-	10 μl	-
Control/Sample containing proteins	5 μl	* μl	* μl	* μl	-
Total volume	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water	bring final volume to **100μl** with deionized water

* Please note that the amount of total protein containing the target protein to be used in this test can be optimized and must be determined by the investigator.

3. Preparation of primary antibody:
   Briefly spin down the TF-Activity Assay Nanog Primary Antibody vial. Add 100 μl of Antibody Diluent Buffer into the vial to prepare a primary antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The primary antibody concentrate should then be diluted 100-fold with the Antibody Diluent Buffer and used in step 4 of Part VII Assay Procedure.

4. Preparation of secondary antibody:
   Briefly spin down the TF-Activity Assay HRP-conjugated Secondary Antibody vial before use. Add 100 μl of Antibody Diluent Buffer into the vial to prepare a detection antibody

concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should then be diluted 100-fold with the Antibody Diluent Buffer and used in step 6 of Part VII Assay Procedure.

5. Preparation of 1x Wash Buffer:
   Dilute 25 ml of the 20x Wash Buffer Concentrate into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If the Wash Buffer Concentrate (20x) contains visible crystals, warm to room temperature and mix gently until dissolved.

   Note:
   All reagents containing protein (positive control, samples) should be kept on ice to maintain protein stability.

   If the reaction system is prepared directly in the coated plate wells, please add the reagents sequentially as shown in the table to get the best results.

   To observe the specificity of the DNA binding activity, the amount of protein used in wells of sample, specific competitor and non-specific competitor must be the same.

   A positive control should be included every time to confirm correct operation of experiment, however it is not necessary to run specific competitor and non-specific competitor for each sample and every time.

VII. ASSAY PROCEDURE:

1. Bring the 96-well plate to room temperature (18 - 25°C) before use. If the whole plate will not be used in this assay, place remaining wells back to 2 to 8°C or -20°C. It is recommended that all positive control and samples be run at least in duplicate.

2. Add 100 μl of each prepared transcription factor binding reaction system (see Reagent Preparation step 2) including positive control, specific competitor, non-specific competitor and sample into appropriate wells. Cover wells and incubate for 2 hours at room temperature or overnight at 4°C with gentle shaking.

3. Discard the solution and wash 4 times by filling each well with 300 μl of 1x Wash Buffer (Reagent Preparation step 5) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 μl of prepared TF-Activity Assay Nanog Primary Antibody (Reagent Preparation step 3) to each well. Incubate for 1 hour at room temperature with gentle shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 100 μl of prepared TF-Activity Assay HRP-conjugated Secondary Antibody (see Reagent Preparation step 4) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution. Wash as directed in step 3.

8. Add 100 μl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

9. Add 50 μl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples, and controls as instructed.

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2. Add 100 μl reaction mixture to each well.
   Incubate 2 hours at room temperature or overnight at 4°C.

⇩

3. Add 100 μl prepared primary antibody to each well.
   Incubate 1 hour at room temperature.

⇩

4. Add 100 μl prepared secondary antibody.
   Incubate 1 hour at room temperature.

⇩

5. Add 100 μl TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

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6. Add 50 μl Stop Solution to each well.
   Read at 450 nm immediately.

VIII. TYPICAL DATA

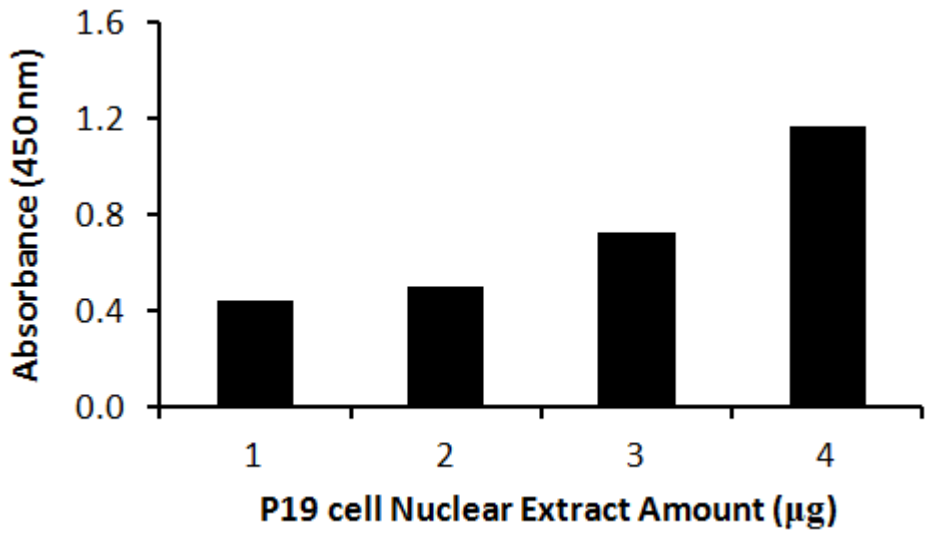

Figure 1: Transcription factor activity assay of Nanog from nuclear extracts of p19 cells with the RayBio® Nanog TF-Activity Assay Kit (cat # TFEH- Nanog).

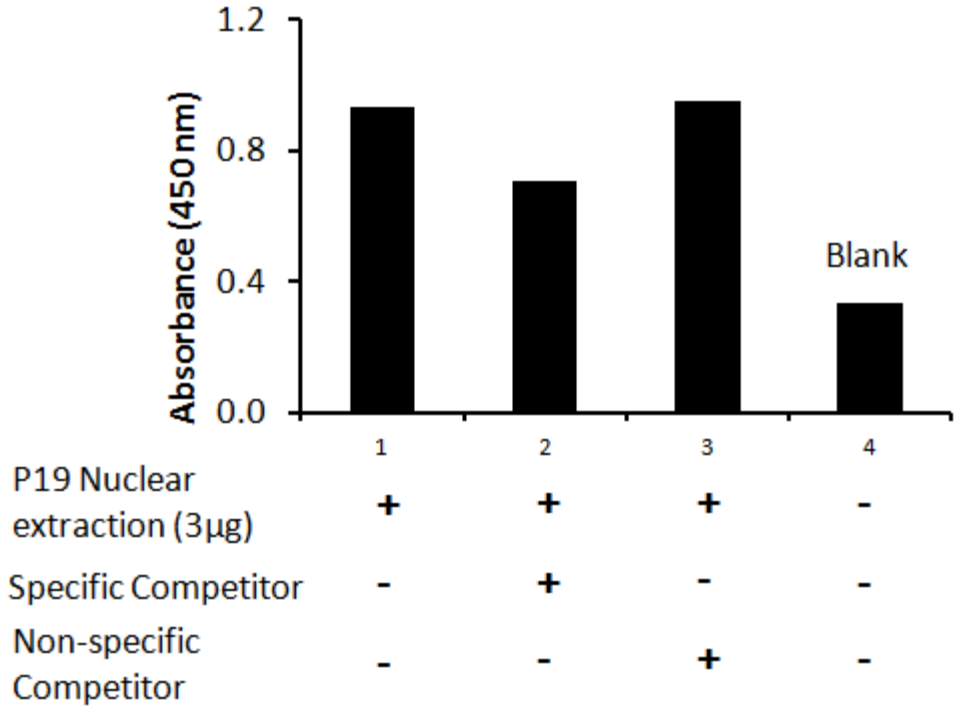

Fig. 2: Transcription factor activity assay of Nanog from nuclear extracts of P19 cells with the specific competitor or non-specific competitor. The result shows specific binding of Nanog to the conserved DNA binding site.

TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; assay procedure step 2 change to overnight
	2. Missed key reagent, inadequate reagent volumes or improper dilution	2. Check all reagents have been added and check pipettes to ensure correct preparation
	3. Not enough targeted protein per well	3. Check positive control wells and increase the amount of sample.
	4. Inadequate development in colorimetric assay	4. Ensure correct developing buffer and enough time used
2. Large CV	1. Inaccurate pipetting	1. Check pipettes
	2. Wells cross contamination	2. Be careful when preparing samples between wells
3. High background	1. Plate is insufficiently washed	1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed
	2. Contaminated wash Buffer	2. Make fresh wash buffer
	3. Incorrect antibody dilution	3. Check antibody dilutions

RayBio® TF-Activity Assay kits:

Choose TF-Activity Assay kits with more targets for human, mouse, rat and a variety of other species. Visit www.raybiotech.com for the complete list.

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