Certificate of Analysis

pNLF1-HIF1a [CMV/neo] Vector:

Part No.	Size
N138A	20µg

Part# 9PIN138

Revised 9/16

AF9PIN138 0916N138

Description: The pNLF1-HIF1a [CMV/neo] Vector[a,b] encodes a HIF1A-NanoLuc® fusion protein under control of a mammalian (CMV) promoter. The NanoLuc® gene encodes an engineered luciferase optimized for extreme brightness, small size and robust thermostability. The vector backbone contains a neomycin phosphotransferase gene to allow selection in *E. coli* with kanamycin and in mammalian cell lines with neomycin. This product also includes Transfection Carrier DNA for dilution of the reporter DNA, allowing titratable intracellular expression of the HIF1A-NanoLuc® fusion protein.

Concentration: 1µg/µl.

GenBank® Accession Number: KF853601.

Storage Buffer: The pNLF1-HIF1a [CMV/neo] Vector is supplied in 10mM Tris-HCl, 1mM EDTA (pH 7.4).

Storage Conditions: See the Product Information Label for storage recommendations and expiration date. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes. These fluctuations can greatly alter product stability. See label for expiration date.

Usage Note: Concentration gradients may form in frozen products and should be dispersed upon thawing. Mix well prior to use.

Quality Control Assays

Contaminant Assays

Contaminating Nucleic Acids: RNA, single-stranded DNA and chromosomal DNA are not evident in specified quantities of the vector as determined by agarose gel electrophoresis.

Endotoxin Concentration: Endotoxin Units (EU) are obtained using Limulus amoebocyte lysate testing. The specification is <100EU/mg of plasmid DNA.

Nuclease Assay: Following incubation of 1µg of the vector in Restriction Enzyme Buffer at 37°C for 16–24 hours, no evidence of nuclease activity is detected by agarose gel electrophoresis.

Physical Purity: $A_{260}/A_{280} \geq 1.80$, $A_{260}/A_{250} \geq 1.05$.

Functional Assays

Identity Assay: The vector has been sequenced completely and has 100% identity with the published sequence available at: www.promega.com/vectors/

Restriction Digestion: The functional purity of the vector DNA is verified by successful digestion with restriction enzymes at the optimal temperature for one hour. Samples are examined by agarose gel electrophoresis, comparing cut and uncut vector DNA with marker DNA.

Signed by:

R. Wheeler, Quality Assurance

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In addition, researchers must:

(1a) use Nano-Glo®-branded luminescent assay reagents (LARs) for all determinations of luminescence activity of this product and its derivatives; or

(1b) contact Promega to obtain a license for use of the product and its derivatives with LARs not manufactured by Promega.

For uses of Nano-Glo®-branded LARs intended for energy transfer (such as bioluminescence resonance energy transfer) to acceptors other than a genetically encoded autofluorescent protein, researchers must:

(2a) use NanoBRET™-branded energy acceptors (e.g., BRET-optimized HaloTag® ligands) for all determinations of energy transfer activity by this product and its derivatives; or

(2b) contact Promega to obtain a license for use of the product and its derivatives for energy transfer assays to energy acceptors not manufactured by Promega.

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[b]U.S. Pat. No. 8,557,970 and other patents pending.

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Promega

Usage Information

pNLF1-HIF1a [CMV/neo] Vector Features

The following features are present in the vector based on nucleotide sequence.

Feature	Position
CMV immediate early enhancer/promoter	1–742
Chimeric Intron	857–989
T7 RNA polymerase promoter (–17 to +3)	1033–1052
HIF1A coding region	1065–3542
Linker	3552–3563
NanoLuc® coding region	3564–4073
SV40 late poly(A) region	4210–4431
SV40 early enhancer/promoter	4530–4942
EM7 bacterial promoter	4956–5022
Neomycin phosphotransferase coding region	5036–5830
Synthetic poly(A) signal	5894–5942
ColE1-derived plasmid replication origin	6178–6214

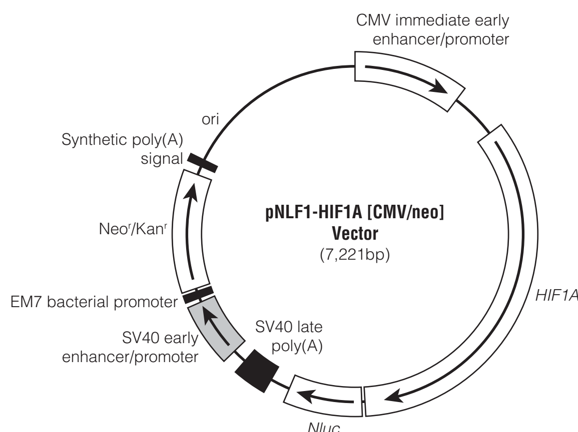

Figure 1. pNLF1-HIF1a [CMV/neo] Vector circle map.

Sample Protocol

In this sample protocol, the pNLF1-HIF1a [CMV/neo] Vector is used to measure activation of hypoxic signaling in HCT-116 cells upon treatment with chemical hypoxia mimetics, such as phenanthroline. The Transfection Carrier DNA is used as a diluent to lower expression of the HIF1A-NanoLuc® fusion protein. This protocol is designed for use with HCT-116 cells. If other cell types are used, protocol optimization may be needed to ensure the best transfection and hypoxia response.

Materials to be Supplied by User

- Complete medium (McCoy's 5A; Life Technologies Cat.# 16600) + 10% FBS (Life Technologies Cat.# 16000)
- Dulbecco's PBS (DPBS; Life Technologies Cat. # 14190)
- 0.05% Tryspin-EDTA (Life Technologies Cat.# 25300)
- Opti-MEM® I (Life Technologies Cat.# 31985)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- 1,10 Phenanthroline (Sigma 131377). Prepare 50mM (1,000X) stock in pure DMSO.
- DMSO (Sigma Cat.# D2650)
- Nano-Glo® Luciferase Assay System (Cat.# N1110)
- white 96-well plates (Corning Cat.# 3917)
- HCT-116 Cells

Day 1: Plate Cells

1. Grow HCT-116 cells in complete medium (McCoy's 5A + 10% FBS + 1X sodium pyruvate). Wash with DPBS and treat with one volume of 0.05% trypsin-EDTA. Resuspend the cells in at least four volumes of complete medium.
2. Quantify the cells and dilute to 5×10^4 cells/ml in complete medium.
3. Plate 90µl of cells per well to a solid, white 96-well plate.
4. Incubate for 18–24 hours in a 37°C, 5% CO_2 incubator.

Day 2: Transfection

1. Prepare a 10µg/ml solution of total DNA in Opti-MEM® I. **Note:** To ensure accuracy of DNA concentrations, it may be necessary to use serial dilution steps in a DNA-compatible buffer.
   a. Add Transfection Carrier DNA to 10µg/ml.
   b. Add pNLF1-HIF1a [CMV/neo] Vector DNA to 0.01ng/ml in Opti-MEM® I (including 10µg/ml Transfection Carrier DNA). **Note:** To achieve optimal assay results, it may be beneficial to adjust the amount of pNLF1-HIF1a [CMV/neo] Vector DNA to optimize HIF1A-NanoLuc® fusion expression.
2. Add FuGENE® HD to a 3:1 lipid to DNA ratio (µl FuGENE®HD to µg DNA). Mix gently by inversion several times. Incubate at room temperature for 20 minutes.
3. Add 10µl of transfection complex per well (100ng of DNA/well) and incubate for 16–20 hours in a 37°C, 5% CO_2 incubator.

Day 3: Treat Cells

1. Prepare a 10X stock solutions of serially-diluted positive control agonist 1,10 phenanthroline beginning at 500µM (**Note:** 1, 10 phenanthroline can be prepared as a 1,000X stock in pure DMSO). Prepare a 10-point, 2-fold serial dilution of agonist, including a vehicle-only control. Do not exceed 2% DMSO in the 10X stock.
2. Prepare 10X stock solutions of serial-diluted test compounds/unknowns. Do not exceed 2% DMSO in the 10X stock so that the final concentration of DMSO on cells does not exceed 0.2%.
3. Add 10µl of the 10X controls or test compounds to each well of the 96-well plate, and incubate for 2–4 hours in a 37°C, 5% CO_2 incubator.

Measure Luminescence

1. Remove plates from the 37°C, 5% CO_2 incubator and allow to cool to room temperature for approximately 15 minutes.
2. Add 100µl per well of the Nano-Glo® Luciferase Assay System detection reagents and measure luminescence following the recommended protocol (see the *Nano-Glo® Luciferase Assay System Technical Manual* #TM369 for details).

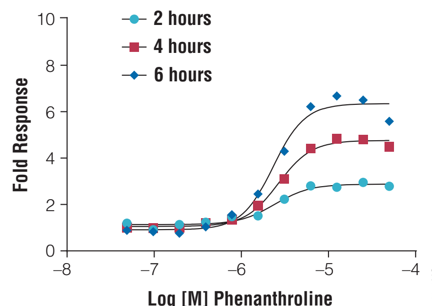

Figure 2. Representative data for the pNLF1-HIF1a [CMV/neo] Vector in HCT-116 cells upon stimulation with phenanthroline. HCT-116 cells were transiently transfected with the pNLF1-HIF1a [CMV/neo] Vector and Transfection Carrier DNA, stimulated with phenanthroline, and assayed in 96-well format. Luminescence was detected after addition of Nano-Glo® reagent, using a GloMax® 96 Instrument with a 0.5 second integration time. Fold response was determined by dividing the average RLU of the stimulated sample by the average RLU of the unstimulated control.

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