PRODUCT INFORMATION & MANUAL

NFkB Secreted Alkaline Phosphatase Assay Kit

NBP2-25286

Plasmids included in the kit is for one 96 well plate transfection. Other reagents included in the kit are for four 96 well plate SEAP Assays.
Research use only. Not for diagnostic or therapeutic procedures

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Novus kits are guaranteed for 6 months from date of receipt

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I. Introduction

Placental alkaline phosphatase (PLAP) is one of the most stable isoenzymes, only existing in the placenta of higher primates. These characteristics make placental alkaline phosphatase suitable to use as a reporter gene for the analysis of promoter activity and gene expression in cell culture and animal serum. The natural form of PLAP is membrane anchored. The recombinant form of placental alkaline phosphatase (secreted alkaline phosphatase, SEAP) is used for reporter gene function. SEAP is designed by inserting a translational terminator after amino acid 489 (Berger, et al., Gene 66 (1): 10 (1988). This mutation converts the membrane-bound PLAP protein into the secreted protein SEAP.

As a major transcription factor NF-κB plays a key role in regulating genes responsible for the innate and adaptive immune responses. In unstimulated cells, the NF-κB dimers are held in cytoplasm by IκBs that masks the nuclear localization signals of NF-κB. Upon cell stimulation, which leads to IκB degradation, NF-κB quickly translocates to the nucleus and activates various genes that have DNA-binding sites for NF-κB.

Our NF-κB SEAPorter™ Assay Kit is designed to measure NF-κB activation using SEAP protein secreted to the culture media as a read-out.

SEAP Reporter Advantages:

- Sampling Ease: SEAP is secreted into the media so no cell lysis is required for the detection of its enzymatic activity.
- A secreted indicator function permits multiple kinetics experiments using only one culture through sequential sampling of the medium, and also allows the cells to be used for other purposes, such as RNA extraction, western blot analysis, and other assays.
- Heating samples at 65ºC for 10-30 mins can destroy endogenous alkaline phosphatase but not SEAP.
- SEAP is stable in serum, allowing it to be used as a reporter assay *in vivo*.
- SEAP catalyzes the hydrolysis of pNitrophenyl phosphate (PNPP) producing a yellow product that can be read in a spectrophotometer or ELISA reader at 405 nm.

II. Kit Description:

The NF-κB SEAPorter™ Assay Kit includes the pNF-κB/SEAP plasmid, which expresses SEAP protein under the control of the NF-κB promoter. The NF-κB promoter can be activated or suppressed by using ligands or inhibitors. For instance, TNFα can activate NF-κB resulting SEAP expression. TLR ligands can also activate NF-κB leading to an increase in SEAP expression. This enables researchers to screen for new TLR ligands.

This Kit provides sufficient reagents for 400 quantitative measurements of SEAP protein in 96-well microtiter format. All 96 wells can be used at one time or you may use only the wells as needed by your experimental design. Use of duplicate wells for the SEAP standard is recommended to obtain accurate results. This kit can be used with supernatant from transfected cells as described or serum as determined by your experimental protocol. The pCMV/SEAP plasmid is included as a positive control for transfection efficiency.

The kit advantages include:

- Multiple samples can be analyzed in a low-volume, high through put experiment
- Full analysis complete in under 1 hr
- Quantitative nature of assay allows direct measurement of SEAP protein in cell supernatant and serum

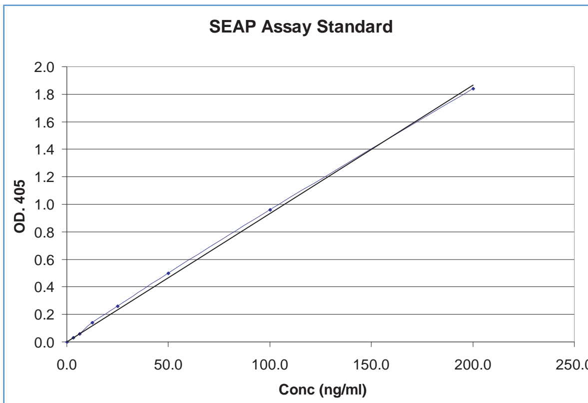

Figure 1: Standard curve for the SEAP protein provided in the NF-κκB SEAPorter™ Assay Kit. (NBP2-25285) A serial dilution of SEAP protein was added to wells of a 96-microtiter plate and the stan-dard curve was generated according to the Kit assay protocol.

III. Kit Components and Storage

The components included in this kit need to be stored at the specified temperatures:

	Kit Components	Quantity	Storage
	Secreted Placental Alkaline Phosphatase standard, SEAP (200 µg/ml)	10 µl	-20ºC
	PNPP Substrate	8 x 5 mg	-20ºC
	pNF-κB/SEAP plasmid (0.5 µg/µl)*	2 x 10 µg	-20ºC
	pCMV/SEAP plasmid (0.5 µg/µl)*	2 x 10 µg	-20ºC
	Sample Dilution Buffer (10X)	10 ml	4ºC
	PNPP Buffer (10X)	4 ml	4ºC
	96 well Assay Plates	4	RT
	Plate Sealer	4	RT

****Note:*** Twenty micrograms of each plasmid DNA is included in this kit which is enough for transfection of 5 wells of a 6 well plate or one 24 well plate or one 96 well plate. For repeat experiments, the plasmid DNA should be transfomed into a suitable bacterial strain, such as DH5a or Top10 cells. The pCMV/SEAP plasmid should be grown in LB or other bacterial medium containing 50 µg/ml of Ampicillin. The pNF-κB/SEAP plasmid should be grown in LB or other bacterial medium containing 50 µg/ml of Ampicillin.*

Additional materials needed but not provided in the kit:

- Centrifuge
- 37°C CO_2 incubator
- 65°C water bath or heating block
- ELISA plate reader
- Multichannel pipette
- Lipid for transfection

IV. Protocol

A. Reagents Preparation

Note: *The protocol has been optimized using the buffers and reagent in the kit. Substitution with other reagents may not give optimal results. Reagents are prepared at room temp. just prior to the Assay.*

Note: *The supernatant can be used immediately or stored at -70°C for later use.*

SEAP Standard Stock Solution (400 ng/ml):
The SEAP protein standard is supplied at 200 µg/ml. Prepare stock solution by diluting 1 µl of SEAP protein to 499 µl of dilution buffer. Diluted Standard can be stored at -20ºC for future use. An excessive amount of SEAP standard is provided to maximize test size flexibility.

PNPP Substrate:

1. PNPP buffer (1X): Dilute 10X PNPP buffer with sterile. H_2O (e.g. 1 ml to 9 ml sterile H_2O). Store at 4ºC.
2. Dissolve one 5 mg PNPP substrate tablet in 5 mls of 1X PNPP Buffer (giving a final concentration of 1 mg/ml). Prepare just before use.

Sample Dilution Buffer:
Dilute 10X Dilution Buffer to 1X with sterile H_2O (e.g. 1 ml of 10X dilution buffer to 9 ml of sterile H_2O).

B. Transfection:

1. In a six-well or 35 mm tissue culture plate, seed ~5 x 10^5 cells per well in 2 ml DMEM containing 10% FBS with nonessential amino acids and without any antibiotics.
2. Incubate the cells at 37ºC in a CO_2 incubator until the cells are 70-80% confluent. This will usually take 18-24 hrs.
3. Prepare the following solutions in 12 x 75 mm sterile tubes:

   Solution A: For each transfection, dilute 4 µg DNA (plasmid) in 250 µl serum-free DMEM (containing nonessential amino acids).

   Solution B: For each transfection, dilute 10 µl Lipofectamine 2000 (Life Technologies, CA) reagent in 250 µl serum-free DMEM.

4. Combine solutions A and B, mix gently, and incubate at room temperature for 15-45 min. The solution may appear cloudy; however, this will not impede the transfection.
5. Aspirate media from plate, add 1.5 ml DMEM with 10% FBS to each well. Do not add any antibiotics to media during transfection. Add 500 ul of DNA/lipid mixture from Step 4 to the plate drop by drop. Mix gently.
6. Incubate at 37ºC in a CO_2 incubator for 48-72 hrs.
7. Add appropriate inhibitor or ligands and incubate for 6-24 hrs or as appropriate for each agent.
8. Collect cell supernatant for SEAP assay. Supernatant can be used immediately or stored at -70ºC.

V. Measurement of SEAP

This kit allows quantitative measurements of SEAP protein in 96-well microtiter formats. All 96 wells can be used at one time or you may use only the wells as required by your experimental design. Use of duplicate wells for each time point or control is recommended to obtain accurate results. This kit can be used for transfected cell supernatant or serum.

1. **SEAP standard:** Label eight eppendorf tubes from A-H. Add 50 µl of Dilution Buffer to tubes A-H. Add 50 µl of 400 ng/ml SEAP standard to tube A. Mix well by pipetting up and down a few times. Take 50 µl from tube A and transfer it to tube B. Continue this serial dilution to tube G. Tube H only contains 50 µl of Dilution Buffer. This will serve as a blank for generating the standard curve. The remaining diluted standard can be stored at -20ºC for future use (aliquot to minimize freeze-thaw cycle).
2. **Samples:** Transfected samples can be diluted 1:2 or 1:10 in Dilution Buffer depending on the efficiency of transfection.
3. **Load samples and standard to microtiter plate:** Add 10 µl of diluted SEAP standard to columns 1 and 2 of the microtiter plate. Add 10 µl of diluted samples to the plate. Add 10 µl of H_2O to each well containing a sample or standard.
4. Seal the microtiter plate with plate sealer and incubate at 65ºC for 30 min. to inactivate any endogenous Alkaline Phosphatase in the super-natant and allow for precise quatitation of SEAP.
5. Spin plate briefly in a centrifuge to return all liquid to the bottom of the well. If this equipment is not available, gently tap the sealed plate on a hard surface until all of the liquid is down.
6. Add 100 µl of 1mg/ml PNPP substrate solution to each well. Incubate at room temp.
7. Take absorbance readings at 405 nm after 30 mins and 1 hr in ELISA plate reader.

		1	2	3	4	5	6	7	8	9	10	11	12
200 ng/ml	A												
100 ng/ml	B												
50 ng/ml	C												
25 ng/ml	D												
12.5 ng/ml	E												
6.25 ng/ml	F												
3.1 ng/ml	G												
0 ng/ml	H												

See Appendix B for use of this kit in TLR mediated NF-κB signaling.

VI. References

1. Cullen B.R. and Malim M.H, *Method Enzymol*. 216: 362-368 (1992).
2. Berger J and Cullen B.R, *Gene* 66: 1-10 (1988).

VII. Trouble Shooting

Problem	Possible Cause	Suggestion
Weak or no SEAP expression in the supernatant	Poor transfection efficiency	Use pCMV/SEAP plasmid to check transfection efficiency
	Expired substrate	Use freshly prepared substrate
	Insufficient incubation time	Incubate longer

If you require additional assistance, please contact Novus Technical Service:

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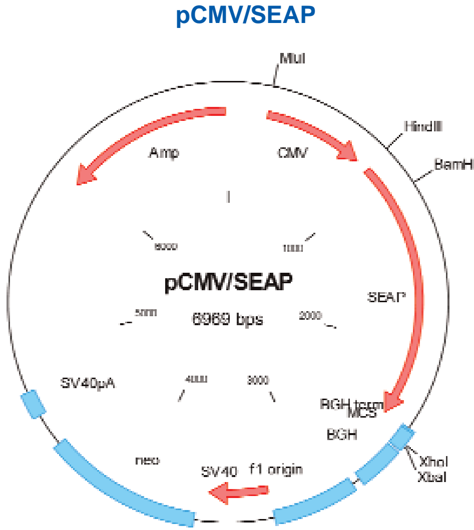
Antibodies:

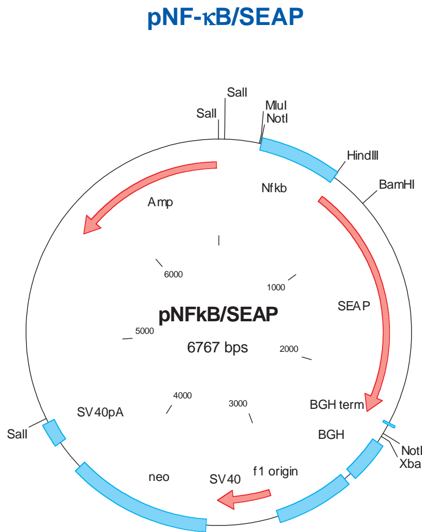
For a complete list of antibodies against TLR and NF-κB signaling pathway, please visit our website: www.novusbio.com

Appendix A. Schematic maps of plasmid vectors:

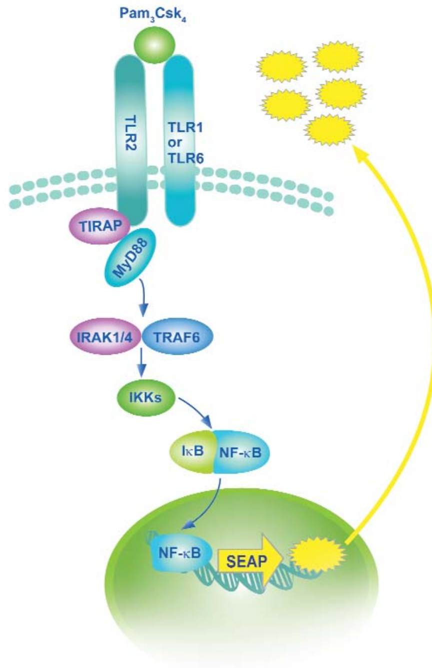
pCMV/SEAP

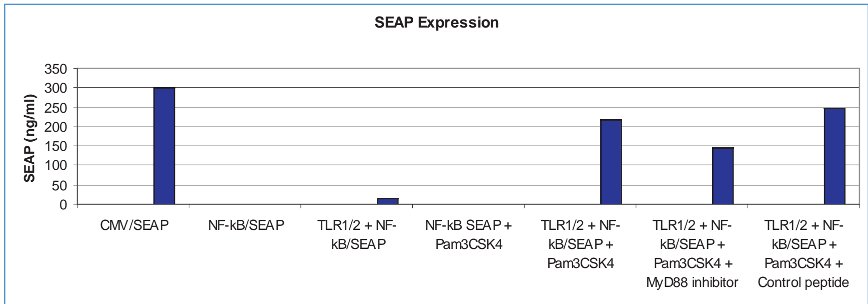


pNF-κB/SEAP



Appendix B. Use of NF-κB SEAPorter™ Assay Kit in detecting ligand-induced NF-κB in TLR expressing cell lines.





293 cells were co-transfected with pCMV/TLR1/2 and pNF-κB/SEAP plasmids using Lipofectamine 2000 (Life Technologies, CA). After 48 hrs of transfection, MyD88 inhibitory peptide (100 nmole/ml) or control peptide (100 nmole/ml) was added and incubated at 37°C. After 24 hrs of incubation Pam_3Csk_4 ligand was added (1 µg/ml). Cells were incubated at 37°C for 24 hrs. Transfected supernatant was collected and analyzed using The NF-κB SEAPorter™ Assay Kit. pCMV/SEAP plasmid was used to check transfection efficiency.