

NF- κ B SEAPorter™ Assay Kit

Secreted Alkaline Phosphatase Reporter Assay
for fast and sensitive detection of NF- κ B activity

(Version 4/09)

Catalog No.: IMK-515

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

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

Our NF- κ B SEAPorter™ Assay Kit is designed to measure NF- κ B 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 65°C 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- κ B SEAPorter™ Assay Kit includes the pNF- κ B/SEAP plasmid, which expresses SEAP protein under the control of the NF- κ B promoter. The NF- κ B promoter can be activated or suppressed by using ligands or inhibitors. For instance, TNF α can activate NF- κ B resulting SEAP expression. TLR ligands can also activate NF- κ B 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

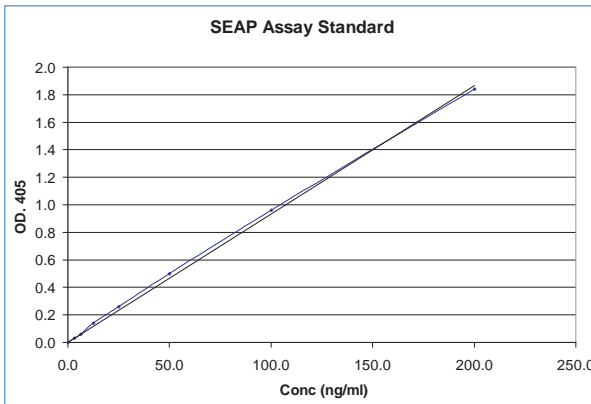


Figure 1: Standard curve for the SEAP protein provided in the NF- κ B SEAPorter™ Assay Kit (IMK-515). A serial dilution of SEAP protein was added to wells of a 96-microtiter plate and the standard curve was generated according to the Kit assay protocol.

III. Kit Components and Storage

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

Cat. No.	Kit Components	Quantity	Storage
IMK-515-1	Secreted Placental Alkaline Phosphatase standard, SEAP (200 µg/ml)	10 µl	-20°C
KC-103	PNPP Substrate	8x5 mg	-20°C
IMK-515-3	pNF-κB/SEAP plasmid (0.5 µg/µl)*	2x10 µg	-20°C
IMK-515-4	pCMV/SEAP plasmid (0.5 µg/µl)*	2x10 µg	-20°C
IMK-515-6	Sample Dilution Buffer (10X)	10 ml	4°C
IMK-515-7	PNPP Buffer (10X)	4 ml	4°C
	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 transformed 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 25 µg/ml of kanamycin. 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 CO₂ 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 at IMGEX 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 $\mu\text{g/ml}$. Prepare stock solution by diluting 1 μl of SEAP protein to 499 μl of dilution buffer. Diluted Standard can be stored at -20°C 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 4°C .
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 $\sim 5 \times 10^5$ cells per well in 2 ml DMEM containing 10% FBS with nonessential amino acids and without any antibiotics.
2. Incubate the cells at 37°C 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 μl of DNA/lipid mixture from Step 4 to the plate drop by drop. Mix gently.
6. Incubate at 37°C 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 -70°C .

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 μ l of Dilution Buffer to tubes A-H. Add 50 μ l of 400 ng/ml SEAP standard to tube A. Mix well by pipetting up and down a few times. Take 50 μ l from tube A and transfer it to tube B. Continue this serial dilution to tube G. Tube H only contains 50 μ l of Dilution Buffer. This will serve as a blank for generating the standard curve. The remaining diluted standard can be stored at -20°C 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 μ l of diluted SEAP standard to columns 1 and 2 of the microtiter plate. Add 10 μ l of diluted samples to the plate. Add 10 μ l of H₂O to each well containing a sample or standard.
4. Seal the microtiter plate with plate sealer and incubate at 65°C for 30 min. to inactivate any endogenous Alkaline Phosphatase in the supernatant and allow for precise quantitation 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 μ l 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- κ B signaling.

VII. 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 IMGEX Technical Service:

Tel: 858-642-0978

Toll free: 888-723-4363

Fax: 858-642-0937

Email: info@imgenex.com

VIII. Related Products

TLR ligands

Cat. No.	Description
IMG-2201	Pam ₃ Csk ₄ - TLR1-2 Ligand
IMG-2203	Poly (I). Poly (C) - TLR3 Ligand
IMG-2204	LPS - TLR4 Ligand
IMG-2205	Flagellin - TLR5 Ligand
IMG-2206	MALP 2 - TLR6 Ligand
IMG-2207	Imiquimod - TLR7 Ligand
IMG-2209H	CpG - TLR9 Ligand

Inhibitory Peptides

Cat. No.	Description
IMG-2000	IKK γ NEMO Binding Domain
IMG-2001	NF- κ B p65 (Ser 276)
IMG-2003	NF- κ B p65 (Ser 529/536)
IMG-2004	NF- κ B p50
IMG-2005	MyD88
IMG-2006	TIRAP

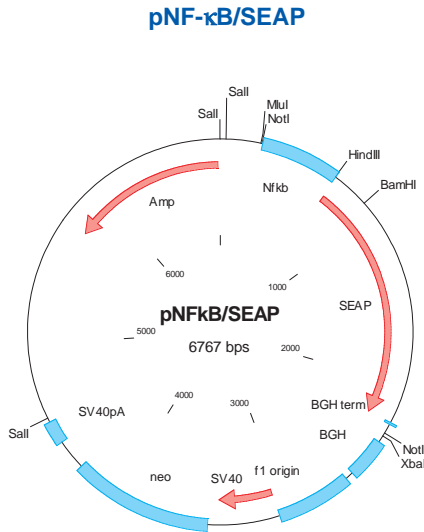
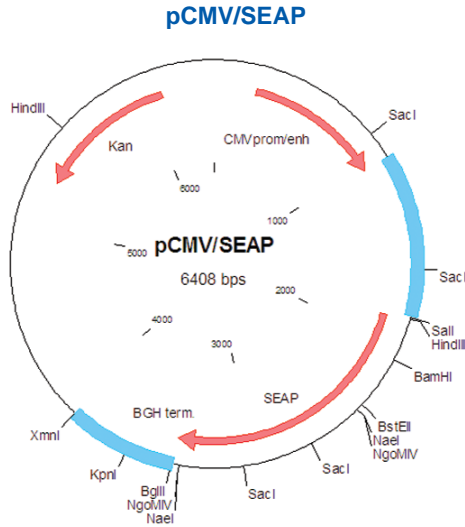
ELISA Kits

Cat. No.	Description
IMK-501	Phospho-I κ B α ActivELISA Kit
IMK-503	NF- κ B/p65 ActivELISA Kit

Antibodies

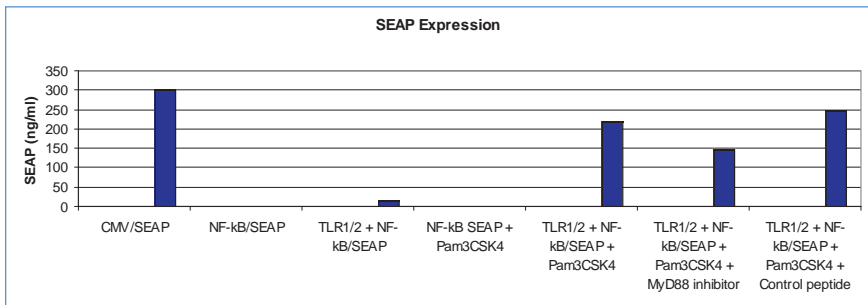
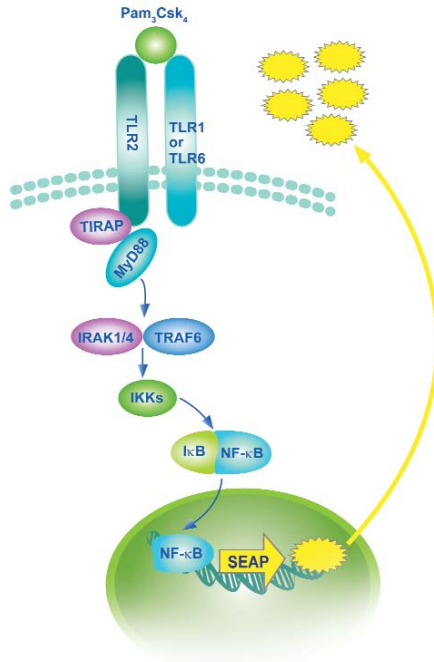
For a complete list of antibodies against TLR and NF- κ B signaling pathway, please visit our website www.imgenex.com

Appendix A. Schematic maps of plasmid vectors:



You can view and download the sequence at http://www.imgenex.com/getfile.php?manual_id=47

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



293 cells were co-transfected with pCMV/TLR1/2 and pNF- κ B/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 (IMG-2005) and incubated at 37°C. After 24 hrs of incubation Pam₃Csk₄ ligand (IMG-2201) was added (1 μ g/ml). Cells were incubated at 37°C for 24 hrs. Transfected supernatant was collected and analyzed using The NF- κ B SEAPorter™ Assay Kit. pCMV/SEAP plasmid was used to check transfection efficiency.

Notes