Next-Generation Chemiluminescent Secreted Placental Alkaline Phosphatase (SEAP) Reporter Gene Assay

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OVERVIEW

NovaBright Secreted Placental Alkaline Phosphatase (SEAP) Enzyme Reporter Gene Chemiluminescent Detection System 2.0 → Next-generation chemiluminescent SEAP reporter assay system providing:

- √ Highest sensitivity and wide assay dynamic range
- √ Long-lived light emission kinetics compatible with HTS batch-mode processing
- √ Simplified assay reagents and protocol
- √Multiple microplate density formats enabled
- √ Versatile assay applications

INTRODUCTION

The NovaBright™ Secreted Placental Alkaline Phosphatase (SEAP) Enzyme Reporter Gene Chemiluminescent Detection System 2.0 is designed for rapid and sensitive detection of SEAP reporter enzyme in cell culture media. SEAP is a truncated form of human placental alkaline phosphatase (PLAP) that is secreted into culture media when expressed in mammalian cells (1). SEAP activity is quantitated by testing media aliquots, leaving cells intact. The assay is ideal for measuring reporter gene expression in cell lines and transfected primary cells. Our next-generation SEAP assay system has improved assay performance, ease-of-use, and time-to-result compared to our widely-used first-generation SEAP assay, the Applied Biosystems® Phospha-Light™ SEAP Reporter Gene Assay System. This next-generation assay incorporates a new substrate/enhancer reagent that provides 5-fold higher signal intensity and signal-to-noise than the original assay. Reagents are provided ready-to-use, eliminating both preparation and assay steps, with a streamlined protocol decreasing overall assay duration. The entire assay is performed within 30 minutes. The glow luminescent signal reaches a maximum within 20 minutes, enabling read-time flexibility. The assay is highly sensitive with a detection limit of ~10 fg/mL of purified PLAP, and has a wide linear assay range over 4-5 decades of enzyme concentration, and can be performed using standard cell culture media, including media containing serum and phenol red.

Secreted reporter enzymes enable time-course expression studies as well as additional testing of cell culture media or cells for other experimental read-outs. Alternatively, the SEAP assay can be performed directly in the culture well without media removal, desirable for high throughput screening assays. The assay is compatible with either 96- or 384-well microplate formats, transiently- or stably-transfected cell lines, and can also used for quantitation of non-secreted PLAP reporter enzyme, which is anchored in the cell membrane. This next-generation chemiluminescent SEAP assay system provides the highest sensitivity of all commercial chemiluminescent SEAP assays, together with a stream-lined assay protocol. This robust assay system enables the use of SEAP reporter for a wide variety of research and screening applications.

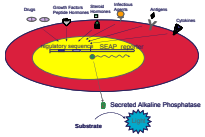
Chemiluminescent SEAP assays have also been used to measure gene expression for viral research, including gene expression, replication, fusogenicity, infectivity, antibody neutralization, and to measure SEAP levels in transgenic animal sera for gene therapy research. Additional applications demonstrated include using SEAP as a functional reporter for protease-mediated secretion, secretion pathway activity, and siRNA-mediated protein knockdown.

MATERIALS AND METHODS

Assay Reagents: Purified PLAP enzyme (Sigma P-3895); Invitrogen Lipofectamine™ 2000 transfection reagent; pCMV-SEAP expression vector; NovaBright™ SEAP 2.0 assay kit (Invitrogen Cat. Nos. N10577, N10578); Applied Biosystems Phospha-Light™ Reporter Gene Assay System (Applied Biosystems Cat. Nos. T1015, T1016, T1017); commercially available chemiluminescent SEAP assay kits.
Cell Lines: CHO-K1; NIH/3T3
NovaBright SEAP 2.0 Assay protocol:
25 µL – 100 µL of sample
+ 50 µL Assay Buffer
→ 5 min incubation @ 65°C
+ 50 µL Reaction Buffer
→ Incubate for 20 min at RT
→ Measure light emission with microplate luminometer

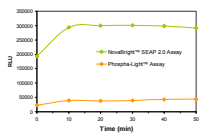
RESULTS

Figure 1. Reporter Gene Assay with a Secreted Reporter Protein



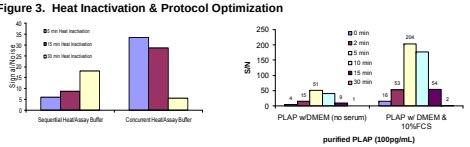
SEAP reporter enzyme is used to characterize regulatory sequences, transcription factor activity, cell signaling and response pathways. Chemiluminescent detection and quantitation provides sensitive and convenient read-out of gene expression from target gene regulatory sequences. Secreted reporter enzymes preserve cells for performing time course studies or measuring additional cellular responses.

Figure 2. Signal Intensity Optimization & Extended Glow Light Emission



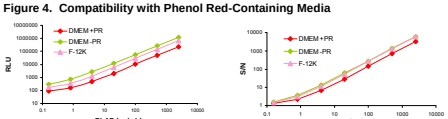
The NovaBright™ SEAP 2.0 assay incorporates a next-generation chemiluminescence enhancer that provides ~5-fold higher light signal intensity with similar light emission kinetics as the Phospha-Light™ assay. Light signal reaches max within 10-20 minutes, with a half-life of several hours.

Figure 3. Heat Inactivation & Protocol Optimization



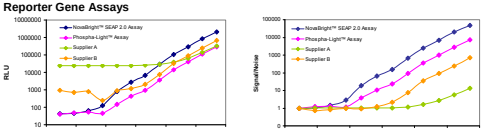
Heat inactivation (@ 65°C) is typically used with SEAP reporter assays to provide preferential inactivation of serum alkaline phosphatase (AP) to minimize assay background and provide optimal assay sensitivity. When the heat inactivation is performed after the addition of Assay Buffer, the optimum incubation time is 5 min, reducing the total assay time and combining two assay steps. The Assay Buffer contains chemical inhibitors of non-placental AP activity, and together with heat inactivation, provide complete inhibition of background AP activity.

Figure 4. Compatibility with Phenol Red-Containing Media



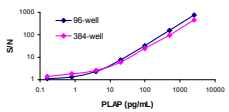
The NovaBright™ SEAP 2.0 assay is compatible with the use of cell culture medium containing phenol red. Purified PLAP (non-secreted human placental alkaline phosphatase) serial dilutions in culture media containing differing concentration of phenol red indicator demonstrate effect of phenol red on the assay. In DMEM containing phenol red, signal intensity is ~5-fold lower, but low-end assay sensitivity is only slightly reduced and assay detection range is similar.

Figure 5. Sensitivity Comparison to Alternative Chemiluminescent SEAP Reporter Gene Assays



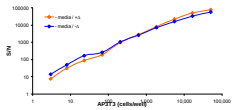
NIH-3T3 cells were transfected with pCMV-SEAP expression vector, which provides constitutive expression of SEAP, using Invitrogen Lipofectamine™ 2000 reagents, and serial dilutions seeded into a 96-well plate. Aliquots of cell culture medium were assayed with each of the SEAP assays using their supplied protocols. The NovaBright SEAP 2.0 assay provides the highest signal intensity and highest assay sensitivity.

Figure 6. Assay Performance in 96- and 384-Well Microplate Formats



In the 384-well microplate formate, NovaBright™ SEAP 2.0 assay sensitivity and dynamic range are identical to the 96-well format. Sample and reagent volumes are scaled down four-fold for the higher-density format, typically used for high throughput screening assays.

Figure 7. Detection of Non-Secreted PLAP Enzyme



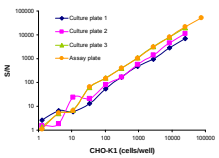
Non-secreted PLAP bears an additional 12 amino acid sequence at its carboxyl-terminus, which provides a membrane anchoring peptide. NovaBright SEAP 2.0 assay is also used to measure activity of non-secreted PLAP enzyme, which remains bound to the surface of cells. Highly sensitive detection of PLAP on cells is achieved, demonstrated with AP3T3 cells constitutively expressing PLAP reporter enzyme.

Table 1. Assay Protocol Streamlining

Phospha-Light™ Assay	NovaBright™ SEAP 2.0 Assay
1. Prepare 1x Dilution Buffer from 5X Dilution Buffer.	
2. Dilute CSPD 1:20 into Reaction Buffer.	
3. Dilute samples with 1x Dilution Buffer.	
4. Heat inactivate samples for 30 min.	1. Add undiluted sample aliquots to assay plate.
5. Add heated, diluted samples to assay plate.	2. Add Assay Buffer to plate.
6. Add Assay Buffer, incubate 5 min.	3. Heat inactivate plate for 5 min.
7. Add Reaction Buffer, incubate 20 min.	4. Add Reaction Buffer, incubate for 20 min.
8. Read	5. Read
~55 minutes = assay time	~25 minutes = assay time

- NovaBright™ SEAP 2.0 assay has no sample prep step, no reagent prep steps, and only two assay reagents.
- NovaBright™ SEAP 2.0 assay has only two reagent addition steps.
- NovaBright SEAP 2.0 assay is completed in < 30 min.

Figure 8. Assay Performance Directly in Cell Culture Plate



Format	Sample	Assay Buffer	Reaction Buffer	Protocol Description
Culture plate 1	100 µL	50 µL	50 µL	entire culture volume/standard reagent volume
Culture plate 2	100 µL	50 µL	100 µL	entire culture volume/higher reagent volume
Culture plate 3	25 µL	50 µL	50 µL	remove 75 µL of culture volume/standard reagent volume
Assay plate	25 µL	50 µL	50 µL	aliquot of culture media assayed in separate plate/standard reagent volume

Secreted reporters enable removal of aliquots of cell culture media for assay in a separate plate, preserving the cell culture for additional time points or other experimental read-outs. However, it may also be desirable for high throughput screening assays to measure SEAP activity directly in the cell culture plate, without media removal to a separate assay plate. CHO-K1 cells transfected with pCMV-SEAP were serially diluted in a 96-well culture plate, and SEAP activity expressed in the cell culture media was assayed either after removal to an assay plate or directly in the cell culture plate without media removal. Using identical reagent volumes, SEAP activity is quantitated over a wide range. Signal/noise is lower when the larger volume of culture medium is used, due to increase in assay background from serum AP.

Table 2. SEAP Reporter Assay: Versatility for Multiple Applications (2)

Reporter Gene (Gene Expression) Applications	Functional Reporter (Non-Gene Expression) Applications	Cell Types/Samples Assayed
Gene expression assays – study gene regulatory sequences and transcription factor activity	Receptor-ligand binding (SEAP-ligand fusion)	Mammalian cell lines – CHO, HEK293, A10, C2C12, C17, HeLa, HepG2, HuH-7, CV-1, A549, Jurkat, Ba/F3, 3T3, et al.
Signal transduction pathway/second messenger activation	Protease-mediated secretion with SEAP fusion protease target	Mammalian primary cells – aortic VSMCs, glial, PBMCs, mast, hepatocytes, neurons, lung epithelia
Gene knockdown/RNAi experimental read-out	Secretion pathway monitoring	Tissues/animals - serum from mouse, rat, marmoset, monkey, pig, allantoic fluid from chicken egg
Virus function assays with reporter read-out – gene expression, replication, fusogenicity, Ab-neutralization, cell-cell fusion, infectivity	Protein aggreagation assay	
"In vivo" – serum samples from live animals – gene therapy, gene delivery, tissue-specific targeting and expression studies	Secreted protein expression quantitation (SEAP fusion)	

CONCLUSIONS

The NovaBright™ Secreted Placental Alkaline Phosphatase (SEAP) Enzyme Reporter Gene Chemiluminescent Detection System 2.0 provides next-generation assay performance with fewer assay reagents and a more streamlined assay protocol than the first-generation Phospha-Light™ Reporter Gene Assay system.

- ✶ Highest signal intensity chemiluminescent SEAP assay
- ✶ Highest sensitivity SEAP assay
- ✶ Wide assay dynamic range – 4-5 orders of magnitude
- ✶ Total assay time < 30 min
- ✶ Long-lived light emission for assay read-time flexibility and high throughput assay compatibility
- ✶ Efficient reduction of background serum AP activity with reduced heat inactivation time
- ✶ Compatibility with 384-well microplate format for HTS
- ✶ Detection of SEAP or non-secreted PLAP
- ✶ Compatible with multiple types of culture media with differing concentrations of phenol red
- ✶ Versatile assay format:
  - Assay performance with culture aliquots enables and preserves precious cell samples for additional cellular read-out
  - Assay performance in cell culture plate eliminates need for separate assay plate
- ✶ More environmentally-friendly buffer incorporated in new assay reagents
- ✶ Wide variety of applications demonstrated with chemiluminescent SEAP assays
- ✶ Ideal for use with transgenic animal "in vivo" studies
- ✶ Ideal for high throughput cell-based gene expression assays

REFERENCES

1. Berger, J, J Hauber, R Hauber, R Geiger and BR Cullen (1988). Secreted placental alkaline phosphatase: A powerful new quantitative indicator of gene expression in eukaryotic cells. Gene 66(1):1-10.
2. > 125 publications. "References_Phospha-Light™ Reporter Gene Assay System v2.05.04.09.doc" available upon request or on www.appliedbiosystems.com.

TRADEMARKS/LICENSING