应用发光检测的方法定量检测分泌型碱性磷酸酶（SEAP）

在 TD-20 /20 发光检测仪上的应用

1. 应用说明

A major area of current research in eukaryotic molecular biology focuses on factors which control the level of gene expression at the transcriptional and post-transcriptional levels. One approach is to use "reporter genes" which encode enzymes or proteins that can be quantitatively assayed. Many genes such as *B*-galactosidase, galactokinase, and CAT have been used for this purpose. Often, these assays are expensive, time-consuming, and/or require the use of radioactive substances. The luminescent detection of a secreted form of human placental alkaline phosphatase[1] (SEAP), however, is an inexpensive, fast, simple, highly-sensitive bioluminescent alkaline phosphatase assay[2].

The Turner BioSystems TD-20/20 Luminometer combined with CLONTECH's Great EscAPe™ SEAP Reporter System 2 provides a simple, sensitive method for monitoring the activity of eukaryotic promoters and enhancers. The SEAP reporter gene encodes a truncated form of the placental enzyme which lacks the membrane anchoring domain, thereby allowing the protein to be efficiently secreted from transfected cells. Chemiluminescent alkaline phosphatase substrate CSPD™ and an enhancer are then added to amplify the signal, resulting in a highly sensitive assay with linear range approximately from 10e-13 units/mL to 10e-9 units/μl alkaline phosphatase.

Materials Required

- TD-20/20 Luminometer
- Adjustable 10-100 μL volume pipetter and tips
- Adjustable 1–10 μL volume pipetter and tips
- Microcentrifuge tubes
- Centrifuge
- Deionized water
- Great EscAPe™ SEAP Reporter System 2 (#k2042-1) which includes:
  - 10 μg of each plasmid (Basic, Enhancer, Promoter, Control)
  - 0.6 mL 25mM CSPD™ Chemiluminescent substrate
  - 12 mL Chemiluminescent enhancer
  - 12 mL Assay buffer
  - 5 mL 5X Dilution buffer
  - 30 μL Positive control placental alkaline phosphatase

Proper Use of Controls

3.1 Negative Controls - A negative control is necessary to determine background signals from the cell culture media. Assay 25 μL of medium from cells transfected with the pSEAP2-Basic Vector and subtract this obtained value from all results.

3.2 Positive Control for transfection and expression of exogenous DNA - A positive control is used to confirm transfection and expression of exogenous DNA and to verify the presence of active SEAP in the culture media.

Assay 25µl of medium from cells transfected with the pSEAP2-Control Vector. Cells transfected with this plasmid vector should yield greater than 100RLU chemiluminescence within 48-72 hours after transfection for optimum assay results. If yield is low, increase number of cells in media. If yield is above 5000 RLU, decrease number of cells in media or dilute media.

3.3 Positive Control for detection method - The positive control placental alkaline phosphatase can be used to confirm that the detection method is working. To do this, add 2 mL of the positive control placental alkaline phosphatase to 23 µL of culture medium from untransfected cells. This should give a strong positive signal. Serial dilution of this control can be used to determine the linear range of the assay.

Note: Before beginning, read through entire method.

Instrument Setup

4.1 Turn on instrument and allow to warm up for at least 5 minutes.

4.2 Set instrument to:

Delay period: 10 seconds

Integrate period: 10 seconds

Number of replicates: 1

Standard Preparation

5.1 Allow a sufficient amount of Chemiluminescent enhancer (95 µL per sample tube) and Assay buffer (100 µL per sample tube) required for the entire experiment to equilibrate to room temperature (20-25° Celsius).

5.2 Prepare the required amount of 1X Dilution buffer (75µl needed per sample tube) by adding 1 part 5X Dilution buffer to 4 parts deionized water.

5.3 Prepare 1.25 mM CSPD™ substrate by adding 1 part 25m M CSPD™ Chemiluminescent substrate to 19 parts Chemiluminescent enhancer. Store in the dark until use.

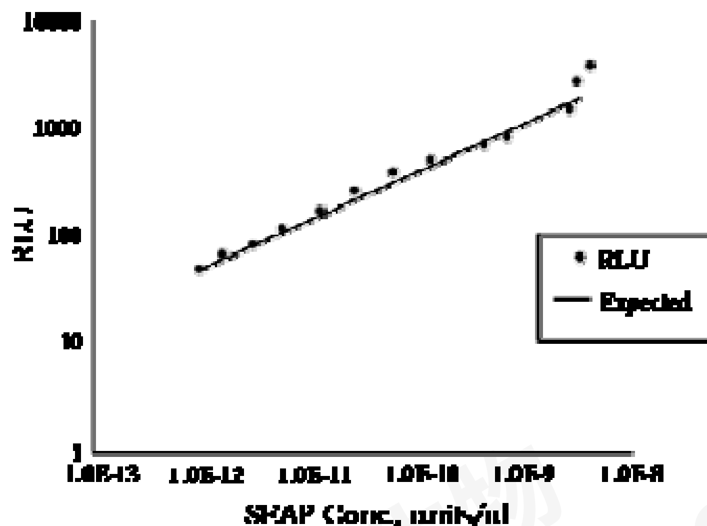
5.4 Add 2 µL of positive control human placental alkaline phosphatase to 23 µL of culture medium from untransfected cells.

5.5 Incubate for 10 minutes at room temperature.

5.6 Read standard in luminometer every 5 minutes to determine point of maximum light emission. This is the incubation time that should be used for the rest of your samples.

Determining Linearity

6.1 Serial dilutions of the standard in step 5.6 can be used to determine the linear range of the luminometer. Plot [SEAP] vs. RLU on a log-log graph. On the TD-20/20, linearity is maintained down to 10e^(-13)units/µL as shown in Graph 1.



Graph 1: 2µl of positive control alkaline phosphatase were added to 100µl of 1.25mM CSPD™ and incubated at room temperature. Serial dilutions of this standard were taken to determine linear range.

Transfection of Mammalian Cells with SEAP Expression Vectors

7.1 The SEAP expression vectors may be transfected into eukaryotic cells by a variety of techniques, including those using calcium phosphate3, DEAE-dextran, various liposome-based transfection reagents4, and electroporation. A method that works well with one cell line may not work well with another. When working with a cell line for the first time, compare the efficiencies of several transfection protocols using the pSEAP2-Basic and pSEAP2-Control Vectors as described in section 3.

7.2 Each different construct should be transfected(and subsequently assayed) in triplicate to minimize variability among treatment groups.

7.3 When monitoring the effect of promoter and enhancer sequences on gene expression, it is critical to include an internal control that will distinguish differences in the level of transcription from variability in the efficiency of transfection5. This is done by cotransfecting a second plasmid which expresses an activity different from SEAP. The level of the second enzymatic activity can then be used to normalize the levels of SEAP among different treatment groups. Reporter proteins frequently used for this purpose include E. coli *b*-galactosidase, which is expressed intracellularly, and human growth hormone(hGH), which is secreted extracellularly[6].

Preparation of Samples

8.1 48-72 hours (time for collecting samples will vary amongst different cell types, cell densities, and experimental conditions) after cell transfection, remove 110 mL of cell culture medium and transfer to a microcentrifuge tube.

8.2 Centrifuge at 12,000 x g for 10 seconds to pellet any detached cells present in the culture medium.

8.3 Transfer 100 mL of supernatant to a fresh microcentrifuge tube.

8.4 Store at -20 degrees Celsius until ready for assay.

Chemiluminescent Detection of SEAP

9.1 Thaw samples of cell culture medium and place 25 µL into a 12x50 mm polypropylene test tube.

9.2 Add 75 µL of 1X dilution buffer to sample tube. Mix gently.

9.3 Incubate samples for 30 minutes at 65 degrees Celsius using a heating block or water bath.

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9.4 Cool samples by placing them in an ice bath for 2-3 minutes. Remove from ice bath and allow to equilibrate to room temperature.

9.5 Add 100 mL of assay buffer to sample tube and incubate 5 minutes at room temperature.

9.6 Add 100 mL of 1.25 mM CSPD to sample tube and incubate until maximum signal(time is determined in step 4.9) is achieved.

9.7 Read on TD-20/20 Luminometer.

References

1,2 Berger, J., Hauber, J., Hauber, R., Gelger, R., & Cullen, B.R. © 1988 Secreted placental alkaline phophatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* 66:1-10.

3 Chen, C. and Okayama. H. © 1988 Calcium Phosphate mediated gene transfer: A highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* 6:632-638.

4 Kain, S.R. and Ganguly, S. © 1996 Use of secreted alkaline phosphatase as a reporter of gene expression in mammalian cells. *Methods in Molecular Biology*, vol. 63, Humana Press, Totowa, NJ.

5,6 Sambrook, J. © 1989 *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.