应用发光检测的方法定量检测 Poly(A) mRNA

在 TD-20 /20 发光检测仪上的应用

1. 应用说明

The concentration and quality of mRNA is an important parameter to consider prior to generating cDNA probes for microarray analysis. Conventional spectrophotometric methods cannot be used to distinguish contaminating rRNA and tRNA species in a total RNA preparation. A sensitive and specific Poly(A) mRNA Detection System has been developed, based on the pyrophosphorylation activity of DNA Polymerase I (Klenow; Exonuclease minus) that acts only on DNA strands perfectly matched at their 3-termini. Anchored oligo(dT) primers hybridize to poly(A) mRNA, followed by a coupled pyrophosphorylation and transphosphorylation reaction, resulting in the production of ATP in a quantity proportional to the amount of poly(A) tails present. ATP concentration is then measured in the highly sensitive TD-20/20 Luminometer using recombinant luciferase. A standard curve is generated using a purified 1.2kb synthetic Kanamycin transcript to estimate the number of mRNA molecules present in unknown samples.

2. MATERIALS REQUIRED

- TD-20/20 Luminometer
- Adjustable 10-100 µL volume pipetter and RNAse-free tips
- Adjustable 1-10 µL volume pipetter and RNAse-free tips
- 0.5mL and 1.5mL Microcentrifuge tubes
- Vinyl or latex gloves
- Microcentrifuge
- 37° C and 65° C water bath or heating block
- RNAse-free, sterile, deionized water
- Poly(A) mRNA Detection System (Promega Cat. # K4040) which includes:
  - · 1 mL DNA Polymerase 10X Buffer
  - · 50 µL Sodium Pyrophosphate (40 mM)
  - · 150 µL ADP (20µM)
  - · 15 µL Nucleoside 5´ Diphosphate Kinase (NDPK) (1 unit/µL)
  - · 1000 units DNA Polymerase I, Klenow Fragment Exonuclease Minus
  - · 300 µL mRNA Detection Oligo Mix (1 µg/µL)
  - · 20 µL 1.2 kb Kanamycin mRNA (0.5µg/µl)
  - · 1 vial rLuciferase/Luciferin Reagent (luciferase Reagent)
  - · 1 vial ENLITEN® Reconstitution Buffer (12 mL)

3. METHODS

N.B. Gloves should be worn during the reconstitution of luciferase reagent and while performing the assay to avoid contamination with ATP and RNAses. Luciferase enzyme activity will decline with multiple freeze-thaw cycles.

A. Instrument Setup

- Turn on instrument and allow to warm up for at least 5 minutes.
- Set instrument to:
- Delay period: 3 seconds
- Integrate period: 15 seconds
- Number of replicates: 1

B. Luciferase Reagent Preparation

- Reconstitute the vial of Luciferase reagent with 12 mL of the ENLITEN Reconstitution Buffer.
- Gently invert the vial several times to dissolve the contents, and allow the reconstituted reagent to equilibrate to room temperature for at least 60 minutes.
- The luciferase reaction is temperature-sensitive; therefore, ensure that the temperature of the reagent does not fluctuate during a set of readings.
- After the reconstitution step is completed, the prepared reagent may be kept at room temperature (up to 8 hours). Unused reconstituted reagent should be dispensed, as single-use aliquots, into sterile microfuge tubes and frozen at –20° C.

C. Sample Preparation: Hybridization

- Dilute 5 µL of the 1.2kb Kanamycin mRNA 1:10 or 1:100 using RNase-free, sterile deionized water. Prepare 5-7 concentrations between 40 pg/µL and 2 ng/µL.
- If no estimate exists for the concentration of mRNA in a sample, perform the assay with multiple dilutions in order to obtain a reading that falls in the linear range of the standard curve.
- For each dilution of standard and sample, prepare a hybridization reaction containing the following:

  - 10 µL l of sample to be tested
  - 3 µL of Oligo Mix
  - 7 µL of RNase-free, sterile, deionized water

- As a negative control also prepare hybridization reactions without mRNA:
  - 3 mL of Oligo Mix
  - 17 mL of RNase-free, sterile, deionized water
- Heat samples and standards at 65° C for 15 minutes.
- Allow the tubes to cool by placing them at room temperature for 15 minutes.
- Microfuge at 12,000 x g for 10 seconds to collect condensation.

D. Reaction Mix Preparation

- Prepare the reaction mix just prior to use.
- Determine the number of reactions needed, and add the following volumes of individual components per reaction. All samples and controls should be assayed in triplicate.

- Thaw the enzyme and buffer solutions and keep on ice. Mix the reagents by gentle vortexing or inversion.

- Prepare a reaction mix as follows. Add the components in the order listed to a fresh microfuge tube. Mix gently and keep the solution on ice.

Note: If the reaction mix is kept at room temperature, a precipitate may form. Do not use.

10.4 µL Sterile, deionized water

2.0 µL 10X DNA polymerase buffer

0.5 µL sodium pyrophosphate

1.0 µL ADP

0.1 µL NDPK enzyme solution

1.0 µL Klenow exonuclease minus for a total volume of 15.0 µL

E. Pyrophosphorylation / Transphosphorylation Reaction

1. Add 15 µL of the above Reaction mix into labeled microfuge tubes.
2. Add 5 µL of each hybridization reaction to each of the tubes. Vortex gently. Assay each reaction in triplicate.
3. Incubate at 37°C (in a heating block or water bath) for 30 minutes. All samples must be incubated for the same period of time.
4. Add 100 µL of the reconstituted luciferase reagent into luminometer tubes, preparing one tube per reaction.
5. After the 30-minute incubation, place reactions on ice and analyze as soon as possible (within 2 hours).
6. Remove 15 µL from one reaction mix and place into 100 µL of reconstituted luciferase reagent. Mix gently.
7. **Immediately** read the sample once it has been added to the luciferase reagent, as the enzyme rapidly begins to utilize the ATP.
8. Repeat steps 6 & 7 above until all samples are read.

F. Calculation of mRNA Concentration

- Average triplicate sample values.

- Subtract the averaged value for the blank reaction, i.e., no RNA, from the values obtained from samples. This yields net light units.

- Generate the standard curve by plotting mRNA concentration versus net light units and drawing a best-fit line.

- Estimate the concentration of the unknown samples by comparing the net light units (LU) seen for these samples against those seen for the standard curve.

Further Reading

- Deuscher, M.P. and Kornberg, A. (1969) Enzymatic synthesis of deoxyribonucleic acid. The pyrophosphate exchange and pyrophosphorolysis reactions of deoxyri-bonucleic acid polymerase. J. Biol. Chem.**244**, 3019.
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- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A
- Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press.
- Lewin, B. (1997) Genes VI, Oxford University Press.

This Poly(A) mRNA method was adapted from a Note (#77) written by Promega Corporation scientists. Data obtained from this Note indicates that the assay is linear over the 20 pg/mL to 2 ng/mL concentration range. Further details about the system can be found on their website.

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