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Fast Chemiluminescent Measurement of RNA Polymerase Activity Based on Photon Counting Technology

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ABSTRACT

A fast and simple assay for T7 RNA polymerase based upon chemiluminescent detection of the synthesized, digoxigenin-labeled RNA on a nylon membrane with anti-digoxigenin coupled alkaline phosphatase and CSPD™ as substrate is described. Activity of RNA polymerase is determined with high sensitivity by quantifying the emitted light of the microplate-formatted dot-blot membrane with a photon counting microplate luminometer and a specially designed filter adapter. The described method is one example for the application of this new adapter to measure luminescent membrane filters.

INTRODUCTION

Bioluminescent and chemiluminescent detection of biological compounds has gained widespread acceptance as an alternative to radioactive detection methods. Mainly within the last decade, domains that seemed to be restricted to isotopic labels, e.g., radio immunoassay, were successfully replaced by luminescence technologies maintaining identical or even increased sensitivity (33). In molecular biology, sensitive chemiluminescent detection of nucleic acids is either performed in solution (e.g., DNA-probe assay for detection of pathogens) or on membranes (for review see Reference 24). In the latter case, the respective DNA or RNA molecules are often detected through the activity of an enzyme, e.g., alkaline phosphatase, which is linked to the probe. In a final step, a chemiluminescent substrate is degraded by the enzyme resulting in light emission (5, 30).

DNA-dependent RNA polymerases play an important role in modern molecular biology. Several bacteriophage enzymes have been cloned [e.g., SP6 (20), T3 (9) and T7 (11) RNA polymerase] and are now used on a large scale for generating specific RNA for a number of purposes (13, 21, 27). To quantify the specific activity of the enzyme for these applications and during its preparation from an overexpressing strain, a fast, sensitive and simple activity assay is needed. Traditional activity tests for polymerases measure the incorporation of a radioactive nucleotide into the produced polymer (8). The need to carry out large numbers of routine radiometric enzyme assays has led to the development of several scanning systems to quantify radioisotopes on membranes (for review see Reference 28). Recently, several non-isotopic assays for DNA polymerase activity, based on detection of the generated double-stranded nucleic acids, have been described (1, 18, 19 and 29). These methods are not as sensitive and as easily quantifiable as radioactive assays. In addition, they are not suitable for detection of RNA polymerase activity, since transcribed RNA is single-stranded.

Here, we describe a fast and sensitive assay for RNA polymerases, which is based upon a dot blot technique using a chemiluminescent label. The development of a special filter adapter for membrane filters allows the use of a commercial photon counting microplate luminometer to quickly obtain quantitative results.

MATERIALS AND METHODS

General Materials

T7 RNA polymerase at a concentration of 50 U/µl and the endonuclease *Pvu*II were purchased from Stratagene (Heidelberg, FRG). The DIG RNA Labeling Kit including plasmid pSPT18, DIG Nucleic Acid Detection Kit and RNase-free

tRNA from *Escherichia coli* MRE 600 were obtained from Boehringer Mannheim (Mannheim, FRG). The 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate (CSPD™) chemiluminescent substrate was purchased from Serva (Heidelberg, FRG). Zeta-Probe® Membranes and the Bio-Dot® Microfiltration Apparatus were obtained from Bio-Rad (München, FRG). Ribonucleotides were purchased from Pharmacia Biotech (Freiburg, FRG) and other reagents used for buffer preparation were from Baker (Deventer, The Netherlands) or Fluka (Neu-Ulm, FRG).

T7 RNA Polymerase Activity Assay and Blotting of the Reaction Products on Membranes

The activity assay for T7 RNA polymerase was performed according to Davanloo et al. (11) in 40 mM Tris-HCl, pH 7.9, 8 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 4 mM spermidine-HCl and 0.4 mM each of ATP, GTP and CTP. Furthermore, it contained 0.26 mM UTP, 0.14 mM digoxigenin (DIG)-UTP and 1 µg pSPT18 (carrying the T7 promoter) linearized with *Pvu*II in a final reaction volume of 50 µl. After addition of 2 µl of an appropriate dilution of T7 RNA polymerase, the reaction mixture was incubated for 15 min at 37°C. Then 450 µl of an ice-cold solution containing 10 mM NaOH, 3.75 mM EDTA and 15 µg/ml tRNA were added to stop the polymerization reaction and to denature the transcribed DIG-labeled RNA for the blotting procedure. Blotting on Zeta-Probe membranes was performed with a Bio-Dot Microfiltration Apparatus according to the manufacturer's instructions. The RNA was cross-linked on the membrane by UV irradiation at 254 nm for 90 s.

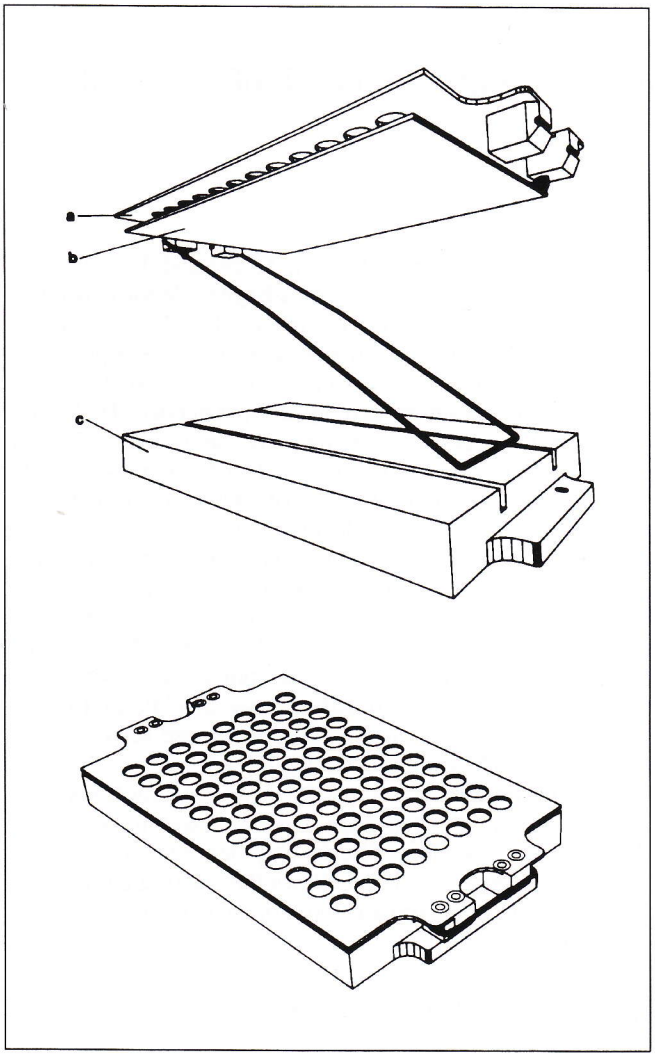
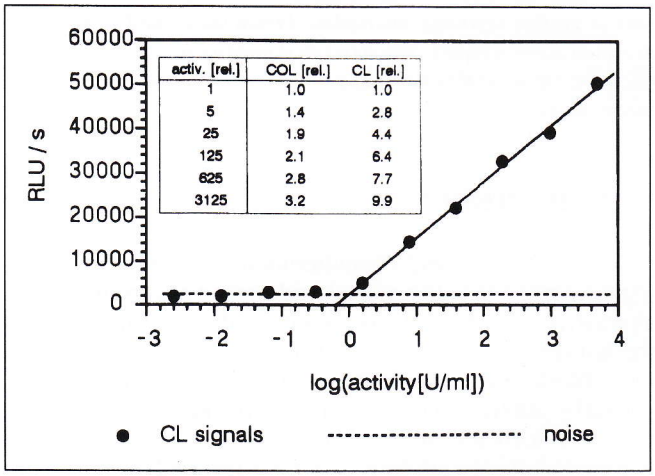


Figure 1. Filter adapter for microplate luminometer MicroLumat LB 96 P. The dot-blot filter (b), sandwiched between two layers of plastic wrap, is matched precisely to the 12 × 8 dot matrix of the diaphragm (a) and fixed by the attached spring-stirrup. Subsequently, the diaphragm is snapped into the support unit (c), thus finally aligning the filter. The complete filter adapter is mounted in the microplate luminometer and measured like standard microplates.

Detection of Blotted, DIG-labeled RNA

The blotted DIG-labeled RNA was detected by the anti-DIG-alkaline-phosphatase immunoassay as described in the instructions for DIG Nucleic Acid Detection (Boehringer Mannheim). For colorimetric detection, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used, which formed a blue precipitate on the membrane during overnight incubation (16 h), the amount of which was measured by a Densitometer CD 60 (DESAGA, Heidelberg, FRG) in remission mode.

For chemiluminescent detection, the last step of the NBT/BCIP system was replaced by an incubation of the membrane for 5 min in a 0.25 mM solution of CSPD in 100 mM diethanolamine, pH 10.0, 1 mM $MgCl_2$ and 0.02% NaN_3 (7). Decomposition of the enzymatically dephosphorylated 1,2-dioxetane substrate CSPD on dot-blot filters results in production of single photons (25). These were quantified using the microplate luminometer MicroLumat LB 96 P (EG&G Berthold, Wildbad, FRG) featuring photomultiplying technology for quantitative photon counting (3, 31). To



activ. [rel.]	COL [rel.]	CL [rel.]
1	1.0	1.0
5	1.4	2.8
25	1.9	4.4
125	2.1	6.4
625	2.8	7.7
3125	3.2	9.9

Figure 2. Chemiluminescent membrane signals of blotted DIG-labeled RNA generated by a serial dilution of T7 RNA polymerase. A serial dilution of commercial T7 RNA polymerase was subjected to a novel non-isotopic activity assay, the reaction products were blotted on positively charged membranes and detected by chemiluminescent measurement of the RLU/s in the microplate luminometer MicroLumat LP 96 P. The correlation coefficient for the obtained regression line is 0.998. The inset shows a table comparing the dynamic range of the chemiluminescent signal (CL) measured within 10 min after incubation of the membrane with CSPD and a colorimetric signal (COL) after a 16-h incubation with BCIP/NBT and subsequent densitometric scanning as described in Materials and Methods.

allow easy and comfortable measurement of microplate-formatted dot-blot filters in this instrument, a special adapter was designed, which is now available from EG&G Berthold (Figure 1). The filter adapter consists of two parts. The upper black-colored dot diaphragm can be separated from the support unit. The dot-blot filter, which is placed between two layers of plastic wrap, is matched precisely to the 12 × 8 dot matrix of the diaphragm and fixed by the attached spring-stirrup. Subsequently, the diaphragm is snapped into the support unit, thus finally aligning the filter. The filter adapter is mounted in the microplate luminometer MicroLumat LB 96 P and measured like standard microplates. A measuring time of 1 s per well is sufficient even for very low signals. Measurement of all 96 dot positions is completed in 2 min. Numeric results are either printed out or transferred to an external host computer for further data evaluation.

RESULTS AND DISCUSSION

To evaluate the capability of the described filter adapter for measuring chemiluminescent dot-blot membranes in a commercial microplate luminometer, a traditional isotopic activity assay based on filter binding of RNA polymerization products (8) was adapted to a nonradioactive labeling procedure. Since T7 RNA polymerase is known to incorporate DIG-labeled UTP into transcribed RNA (15), and DIG-labeled nucleic acids can be detected by chemiluminescence (16), the DIG system was chosen to establish a non-isotopic activity assay for this polymerase. Several assay conditions (like buffer formulation, template concentration, reaction time, etc.) and different blotting procedures for the reaction products were tested to optimize sensitivity of the assay (data not shown). Positively charged nylon membranes (e.g., Zeta-Probe) were chosen for their strong luminescent signal intensity (16). CSPD served as a chemiluminescent substrate for alkaline phosphatase (AP) because of its high signal-to-noise ratio and high sensitivity (6, 7), while the BCIP/NBT system (26) was used for permanent, colorimetric record of membranes.

A serial dilution of T7 RNA polymerase of known, isotopically determined concentration showed a linear relation between applied enzyme activity and the chemiluminescent signal (Figure 2). The dynamic range of the signal is higher than with colorimetric detection by the BCIP/NBT system and subsequent densitometric scanning (inset Figure 2). Moreover, while colorimetric detection requires overnight incubation for a sensitivity comparable with the chemiluminescent assay, chemiluminescent measurement of AP-labeled dot-blot membranes with up to 96 dots takes only minutes.

The time course of the luminescence signal shown in Figure 3 reveals the typical kinetics of a consecutive reaction. The anion produced upon dephosphorylation of CSPD is moderately stable and accumulates on the membrane until a flat, steady state maximum of chemiluminescence is reached. The faster CSPD is decomposed, the stronger the signal and the sooner the maximum of light emission will be reached (25). This phenomenon can be seen both for the AP-labeled dots and for the unspecific reagent background or noise on AP-free membrane dots. (The unspecific signal exceeds 40 times the noise of the photon counter.) In Figure 4, the kinetics of signal-to-noise ratios of the strongest and the weakest

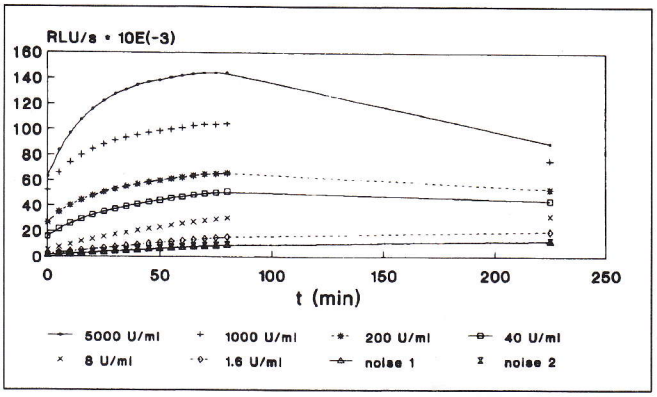


Figure 3. Time course of chemiluminescent signals on a membrane. The signals of a T7 RNA polymerase dilution series with 5000 to 1.6 U/ml subjected to the non-isotopic activity assay with chemiluminescent detection using CSPD were followed with time by automatic repeat measurement. Single photons were counted and luminescence intensity is given in RLU/s, with 1 RLU corresponding to 10 photons. Two of the shown signals are due to unspecific background or noise on the membrane.

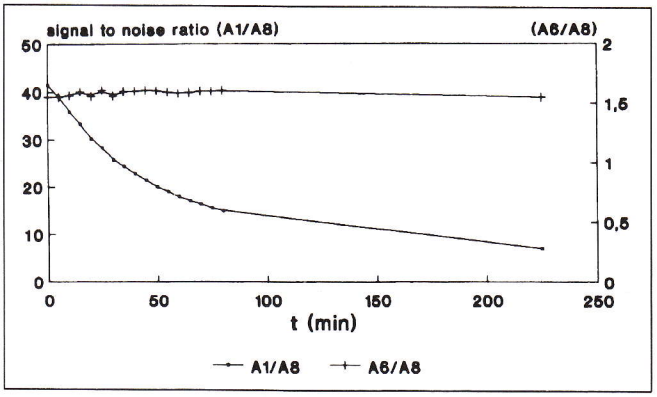


Figure 4. Signal-to-noise ratio for a strong and a weak chemiluminescent signal. A1, A6 and A8 are signals of chemiluminescent dots on a membrane blotted with reaction products of activity assays with different amounts of T7 RNA polymerase. While A1 represents a strong signal (due to 2 µl of 5000 U/ml T7 RNA polymerase in a final reaction volume of 50 µl), A6 is a weak signal (1.6 U/ml) and A8 is caused by unspecific noise.

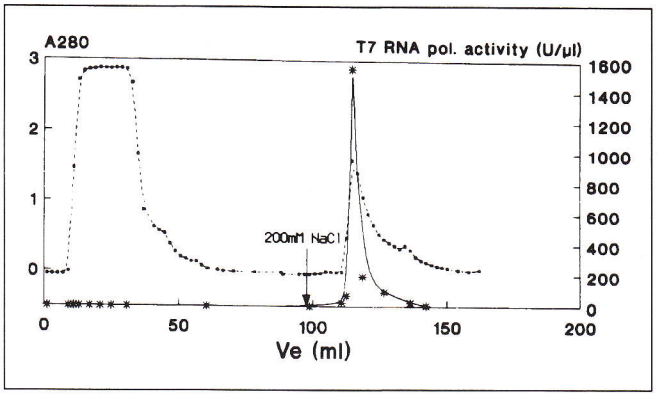


Figure 5. Anion-exchange chromatography of T7 RNA polymerase on IBF SP-Trisacryl M. After ammonium sulfate precipitation and resuspension, the enzyme was applied at a flow rate of 40 ml/h to an anion-exchange chromatography on IBF SP-Trisacryl M (bed volume 25 ml) equilibrated with buffer C (20 mM sodium phosphate, pH 7.7, 1 mM trisodium EDTA, 1 mM DTT, 5% glycerol) plus 50 mM NaCl. The column was washed with equilibration buffer and the enzyme was eluted with buffer C containing 200 mM NaCl (arrow). Fractions of 2 ml were collected and assayed for protein content (A_{280}-profile). T7 RNA polymerase activity was measured by the described chemiluminescent assay.

signal of Figure 3 are compared. While the signal-to-noise ratio for the weak signal remains nearly unchanged and results in a time-stable detection limit for the assay, the ratio for the strong signal decreases exponentially. For linearity of the assay, it is therefore recommended to measure chemiluminescence within the first minutes after incubation with CSPD.

Thus, quantitative detection of AP-labeled membranes, including incubation with CSPD and luminescence measurement, is completed within 10 min for 96 samples.

To evaluate the feasibility of the assay for routine analysis, the specific activity of chromatographic fractions during purification of T7 RNA polymerase according to Grodberg and Dunn (14) was measured. As an internal standard, a dilution series of T7 RNA polymerase of isotopically determined activity was included on each quantified membrane, thus allowing direct comparison with conventional isotopic activity assay units (one unit is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of AMP in 1 h at 37°C) (2, 10). The eluted fractions from the ion-exchange columns were suitably diluted with enzyme storage buffer to fit the calibration curve (1–3500 U/ml).

Figure 5 shows an elution profile of the anion-exchange chromatography on SP-Trisacryl M (IBF-Biotechnics, Villeneuve la Garenne, France) determined by the new chemiluminescent assay. The curve corresponds to that described in literature (14). The detection limit of the chemiluminescent assay for chromatographic fractions is about 1 U/ml T7 RNA polymerase leading to 2500 relative light units per second (RLU/s) on the membrane dot; thus, the detection limit is at least as sensitive as the isotopic assay (5 U/ml) (10). Reproducibility of activity values lies in the range of about ±5%, while the luminometer itself is more accurate (data not shown).

CONCLUSIONS

The described procedure featuring a non-isotopic polymerase activity assay represents an alternative to traditional methods using radioactive nucleotides. While maintaining the same sensitivity, the non-isotopic assay displays several advantages. Besides reduced costs, inconveniences of radioactive labels (e.g., ^{32}P), such as short half-life time of labeled probes, special training of staff, possible hazards to health, and disposal of radioactive waste are eliminated by the use of luminescent labels (22). Since DIG-labeled (d)UTP can serve as substrate for a number of polymerases (4), the described chemiluminescent assay should be easily transferable to enzymes such as SP6 and T3 RNA polymerase, *E. coli* polymerase I (holoenzyme, Klenow fragment), T4 and *Taq* DNA Polymerase, avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (M-MuLV) reverse transcriptase or terminal transferase. In our view, the benefits from a fast non-isotopic activity assay fully compensate the initially needed optimization of reaction conditions for chemiluminescent detection, especially for routine analysis of large numbers of samples.

Furthermore, the photon counting microplate luminometer MicroLumat LB 96 P, together with the described membrane filter adapter, provides an ideal measuring device for all types of chemiluminescent dot-blot assays, such as filter binding or hybridization assays (Southern, Northern and Western blots)

(16, 24). The overall processing time may be reduced to 2–3 h after blotting and nevertheless results in highly sensitive and quantitative signals. Besides these dot-blot applications, completely different assay systems in molecular biology can be transferred from isotopic to luminescent methods. Important examples are the luciferase and the galactosidase reporter gene assays (12, 17, 31) that were demonstrated to be even more sensitive than the traditional radioactive chloramphenicol acetyl transferase (CAT) assay (17, 32). Moreover, bioluminescence and chemiluminescence have become increasingly popular in other areas of biology, biochemistry and medicine. Applications include analysis of many enzymes and metabolites, measurements of cellular luminescence (phagocytosis assay), luminescence and enzyme immunoassays as well as tumor chemo-sensitivity assays (for review see Reference 23). Thus, the design of a universal research luminometer, like the MicroLumat LB 96 P, should allow definition of a variety of completely different measuring protocols, including possibility of reagent injection. Looking into the near future, one can assume that in life science laboratories a luminometer will become a routine analytical instrument as the scintillation counter is today.

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