Bioluminescence and Chemiluminescence

Fundamentals and Applied Aspects

Proceedings of the 8th International Symposium on Bioluminescence and Chemiluminescence, Cambridge, September 1994

Edited by

A K Campbell, L J Kricka and P E Stanley

JOHN WILEY & SONS
Chichester · New York · Brisbane · Toronto · Singapore

SCREENING FOR POLYMERASE ACTIVITIES BY FAST QUANTIFICATION OF CHEMILUMINESCENT DOT BLOT MEMBRANES USING A FILTER ADAPTER IN A PHOTON COUNTING MICROPLATE LUMINOMETER

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Introduction

In recent years, an "irrational design" method for the generation of enhanced or altered protein enzyme activities has successfully been developed (1-3). In contrast to the classical "rational design" approach involving site-directed mutagenesis, the technique works well in the absence of detailed structural information or when the molecular basis for catalytic activity is poorly understood. The underlying principle is to produce a large variety of mutant enzymes by random mutagenesis of the wild type gene, to express this library, and to select or screen for mutants with the desired catalytic properties. These experimental steps can be repeated sequentially to further optimize the final product for a specific application (3), thereby even more closely mimicking molecular evolution (4). Furthermore, the technique allows researchers to establish structure-function relationships for the mutagenized enzyme by characterizing mutants with altered properties.

The availability of the cloned gene for bacteriophage T7 RNA polymerase (5) (EC 2.7.7.6) has stimulated the search for determinants of catalytic activity (6) and promoter specificity (7) of this enzyme by characterization of "irrationally designed" mutants. The crucial step in finding mutants with altered enzymatic activities is a fast and reliable screening assay. Here, we describe a dot-blot format chemiluminescence assay to screen for variable T7 RNA polymerase activities as they occur in crude fractions during purification of the enzyme (5). The development of a special filter adapter for dot-blot membranes allows the use of a commercial photon counting microplate luminometer to quickly obtain quantitative results (8). This progress in instrumentation should make possible the application of similar kinds of chemiluminescence assays in the described molecular evolution experiments.

Materials and Methods

Instrumentation We used the Bio-Dot microfiltration apparatus with Zeta Probe membranes (Bio-Rad, München, Germany), a densitometer CD 60 (Desaga, Heidelberg, Germany) and the photon counting microplate luminometer MicroLumat LB 96 P with a special adapter for dot-blot filters (EG&G Berthold, Wildbad, Germany).

Reagents T7 RNA polymerase (at 50 U/µl) and endonuclease *Pvu*II (Stratagene, Heidelberg, Germany); digoxigenin (DIG) RNA labeling and detection reagents, plasmid pSPT18 and RNase-free tRNA from E. coli MRE 600 (Boehringer, Mannheim, Germany); 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenylphosphate (CSPD) (Serva, Heidelberg, Germany).

Methods The activity assay for T7 RNA polymerase was performed similar to (9) 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 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 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 was added to stop the polymerization reaction. Blotting on Zeta Probe Membranes was performed with a Bio-Dot microfiltration apparatus according to the manufacturer´s instructions. The RNA was crosslinked on the membrane by UV irradiation at 254 nm for 90 s.

The blotted DIG-labeled RNA was detected by anti-DIG-alkaline-phosphatase immunoassay as described by the manufacturer. For colorimetric detection, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were applied in an overnight incubation. The resulting blue precipitate was quantified by densitometrical scanning in remission mode at 530 nm.

For chemiluminescence detection, the membrane was incubated 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 (10). Decomposition of the dephophorylated 1,2-dioxetane substrate on the dot-blot filters resulted in emission of single photons (wavelength 477 nm). These were quantified using the photon counting microplate luminometer MicroLumat LB 96 P with a specially designed filter adapter (Figure 1). 1 s per dot was sufficient to measure even very low signals. Measurement of all 96 dot positions was completed within 2 min.

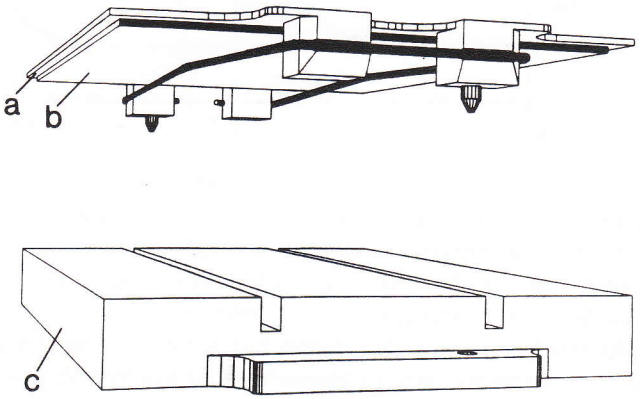

Figure 1. Filter adapter for the microplate luminometer MicroLumat LB 96 P. The dot-blot membrane (b), sandwiched between two layers of plastic wrap, is matched precisely to the 8 x 12 dot matrix of the diaphragm (a) and fixed by the attached spring-stirrup. Subsequently, the diaphragm is snapped into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

Results and Discussion

Screening for T7 RNA polymerase activity in *E. coli* strains overexpressing mutant enzymes following random mutagenesis (6, 7) comprises isolation or compartmentation of bacterial clones and comparison of enzyme activities in crude cell extracts. Traditionally, polymerase activity assays are based on quantification of filter-bound, radioactively labeled polymerization products (11). In order to make use of the advantages of a non-isotopic assay and at the same time compare large numbers of mutant enzymes, we adapted the traditional activity assay to nonradioactive DIG-labeling coupled with dot-blotting. To measure the chemiluminescent dot-blot membranes obtained after an anti-DIG-alkaline-phosphatase immunoassay with CSPD as substrate (10), we developed a special filter adapter for a commercial photon counting microplate luminometer (Figure 1). Together with common techniques of bacterial growth and handling in standard microplates, this allows the parallel processing and comparison of up to 96 isolated clones.

As a model system to test the performance of both the activity assay and the filter adapter, we analysed crude fractions as they occur during purification of wild type T7 RNA polymerase from the overexpressing *E. coli* strain BL21/pAR1219 (5) by the described procedure. Since we did not only intend to compare different enzyme fractions, but also wanted to express the activity in enzyme units (1 U being defined as the amount of enzyme catalyzing the incorporation of 1 nmol of AMP in 1 h at 37°C (12)), we included a dilution series of commercially available T7 RNA polymerase of isotopically determined activity as internal standard on the same dot-blot membrane (8). A typical elution profile obtained by this method is shown in Figure 2.

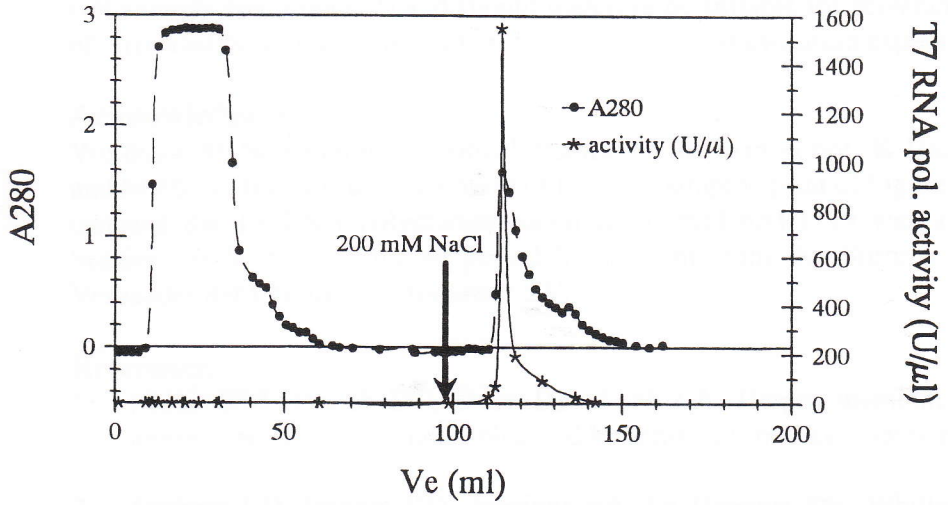

Figure 2. Anion-exchange chromatography of T7 RNA polymerase on IBF SP-Trisacryl M. The *E. coli* BL21/pAR1219 cell extract with the overexpressed enzyme was precipitated with ammonium sulphate, the precipitate resuspended and applied to an anion-exchange chromatography on IBF SP-Trisacryl M as described (5). The enzyme was eluted with 200 mM NaCl (arrow). Fractions of 2 ml were collected and assayed for protein content (A_{280}-profile). T7 RNA polymerase activity was determined by the described chemiluminescence assay.

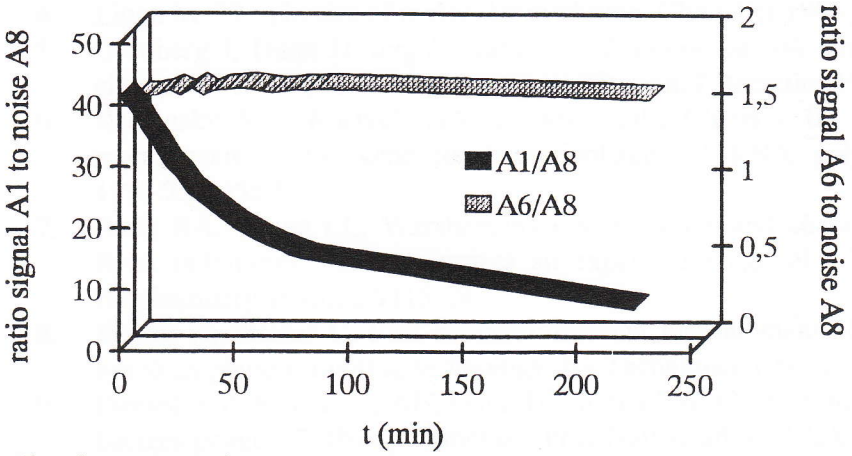

Figure 3. Signal-to-noise ratio for a strong and a weak chemiluminescent signal. A1, A6 and A8 are signals of chemiluminescent dots on a membrane blotted with reaction products of the described activity assay, but with different amounts of T7 RNA polymerase (A1: 10 U; A6: 3.2 mU; A8: 0 U, unspecific background or "noise").

The corresponding regression line for the internal standard dilution series (from 3.2 mU to 10 U T7 RNA polymerase in 50 µl final assay volume) yielded a correlation coefficient of 0.998,

indicating a broad linear range of the assay (data not shown). The chemiluminescent signal had a dynamic range threefold higher than the colorimetric one quantified by densitometric scanning. Moreover, while colorimetric detection required overnight incubation with NBT and BCIP for a sensitivity comparable with the luminescence assay, the chemiluminescent quantification of 96 dots including incubation with CSPD and measurement was completed within 10 min. We found that immediate luminometric measurement after incubation in CSPD yielded the highest signal-to-noise ratio (Figure 3). The detection limit for crude extracts under these conditions was 2 mU T7 RNA polymerase in 50 µl reaction volume and comparable with the isotopic assay (12). The chemiluminescence assay was insensitive to other components of cell extracts (see Figure 2) and should therefore be suitable for screening for altered activities of "irrationally designed" mutant enzymes in molecular evolution experiments (6, 7).

Acknowledgements

We thank M Nöllenburg for critical reading of the manuscript, K Hafner for CAD-drawing and M Meyer for careful preparation of the photographic print of Figure 1. *E. coli* strain BL21 carrying the T7 RNA polymerase encoding plasmid pAR1219 was a generous gift of FW Studier. NGW was partly supported by a grant from the Stiftung Stipendien-Fonds des Verbandes der Chemischen Industrie e.V.

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