

Benchmarks

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Simple Method for High Sensitivity Chemiluminescence ELISA Using Conventional Laboratory Equipment

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Enzyme-linked immunosorbent assays (ELISAs) have a unique importance in numerous different analytical procedures due to their specificity and sensitivity. Moreover, the use of the 96-well format allows the quantification of high numbers of samples in parallel. Most commonly, the binding of antibodies to antigens in an ELISA is detected by the cleavage of a chromogenic substrate by an enzyme conjugate, for instance alkaline phosphatase or horseradish peroxidase (HRP) coupled to either an antibody or to streptavidin. Thus it is usually a col-

ored reaction product that is quantified by measuring the absorbance. Assays with higher sensitivities can be set up based on the principle of chemiluminescence (2-5), but equipment for chemiluminescence measurements in a 96-well format are more expensive than the usual ELISA plate readers and therefore not available in many laboratories.

We developed a chemiluminescence ELISA system with a very simple detection of the immobilized HRP activity that does not require any sophisticated equipment and which is still much more sensitive than a conventional photometric assay with *o*-phenylenediamine as the substrate. Since the chemiluminescence detection that we used is based on the HRP immobilized to the bottom of the plate only, the volume of the coating antibody (or protein) and all subsequent antibody solutions can be as low as 50 μ L (or even 30 μ L, which is about the minimal volume to cover the bottom surface).

For a model system, we used an ELISA for the detection of cyclophilins (1) with a cyclosporine/bovine serum albumin (BSA)-conjugate coated to flat-bottom plates (Immuno Maxi-SorpTM; Nunc, Roskilde, Denmark). In brief, the plates were coated overnight at 4°C with the cyclosporine/BSA conjugate (1 μ g/mL; 50 μ L/well) in coating buffer (50 mmol/L NaHCO₃, pH 9.6). Unspecific binding was blocked by incubation with 2% BSA in phosphate-buffered saline (PBS)/0.5% Tween[®] 20 (PBS/T) for 1 h at room temperature. Then the plates were washed three times with PBS/T (all washing steps were carried out with 200 μ L per well). This was followed by incubation with different concentrations of cyclophilin A (50 μ L per well) for 1 h at 37°C, then by 7 washing steps as above, incubation with rabbit anti-cyclophilin A (polyclonal antibody against human cyclophilin A at 50 μ L per well) at 1:100 overnight at 4°C, washing as above and finally incubation with peroxidase-conjugated anti-rabbit IgG [50 μ L per well, F(ab')₂ fragments; Amersham International, Little Chalfont, Bucks, England, UK] at 1:2000 for 1 h at 37°C. After thorough washing, the plates were then either processed conventionally by incubation with *o*-phenylenediamine sub-

strate solution, or the chemiluminescence-based detection was carried out. The conventional assay was done by adding 50 μ L per well substrate buffer (1 mg/mL *o*-phenylenediamine in buffer containing 7.3 g/L Na₂HPO₄, 5.64 g/L citric acid monohydrate, pH 5.0, and 0.4 μ L/mL 30% H₂O₂). After stopping the reaction with 10 μ L per well with 2 mol/L H₂SO₄, the color was measured at 492 nm with reference reading at 620 nm with an ELISA reader (SLT Labinstruments, Salzburg, Austria).

For the chemiluminescence detection, 50 μ L of substrate solution were added per well (enhanced chemiluminescence solution 1 and 2 from Amersham in the ratio 1:1), and the plate was exposed as soon as possible to a suitable film (HyperfilmTM-ECL; Amersham) in the darkroom. The film was cut somewhat smaller than the size of the plate and put on the plastic cover of a 96-pipet tip box. Thus, the plate could be put onto the film in a way that the flat bottom of the wells was in direct contact with the film. After various exposure times, the film was developed and fixed according to the manufacturer's protocol. A blackening of the film was observed exactly in the size of the

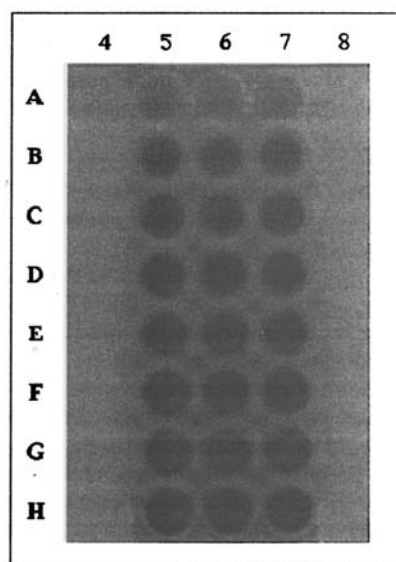


Figure 1. Film after exposure to chemiluminescence ELISA plate for 5 s (triplicates of cyclophilin standard dilutions: 10, 25, 33, 50, 62.5, 83, 125 and 250 ng per well). Note that no signal is observed at adjacent well positions without samples, indicating that there is no cross-talk of signal.

wells (Figure 1). There was no significant cross-talk of the signal to adjacent well positions up to an optical density of about 1.2 (with film background of 0.2 optical density units). For quantification, the film was fixed on top of an empty 96-well plate so that the blackened spots were exactly at their corresponding well positions and the optical density was measured with a 690-nm filter by a usual ELISA plate reader (SLT Labinstruments) in a single wavelength mode (other filters are possible as well, but the highest wavelength available should be used to get the maximum signal). Under these conditions, a dynamic range of 50–100 was achieved.

A direct comparison between the conventional detection with *o*-phenylenediamine and the chemiluminescence detection system described here, showed a 20-fold higher sensitivity of the chemiluminescence system (Figure 2), which also exhibited a better linear range (about 0–60 ng per well compared to 0–30 ng per well for the conventional assay). Another very important advantage of the chemiluminescence detection by exposure to film is

the possibility to accumulate the signal for a longer period of time (under the given conditions that the chemiluminescence lasts for several hours) (Figure 3). This cannot be achieved with the conventional technique because prolonged incubations with *o*-phenylenediamine lead to a high background due to oxidation of the substrate with H₂O₂. Although more sophisticated detection systems for chemiluminescence, like single-photon counting with scintillation counters in a 96-well format, offer a much higher dynamic range (Top-Count™ Scintillation Counter; Packard Instruments, Meriden, CT, USA) of about three orders of magnitude higher than that of the film detection method, they do not provide the opportunity to accumulate the signal as is possible with film detection. This feature lowers the limit of detection significantly, thus allowing the quantification of extremely small amounts of antigen. With regard to this aspect, the film detection of the chemiluminescence signal can compete with scintillation counters.

In conclusion, several features make this assay system attractive for a wide spectrum of users. The sensitivity is

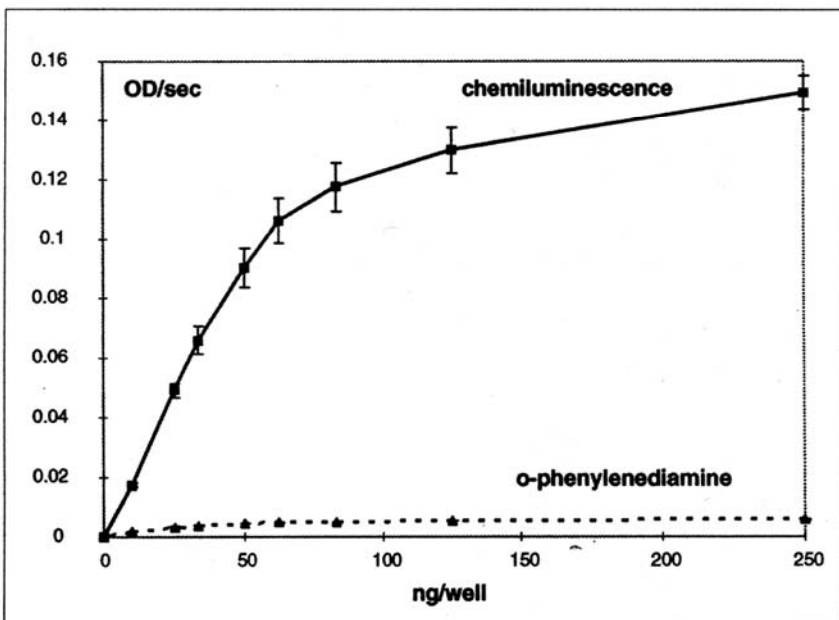
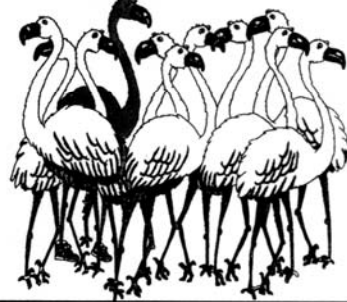


Figure 2. Signal of the chemiluminescence ELISA in comparison to a conventional ELISA detection system. The signal is related to the time of incubation (conventional ELISA), respectively, and to the time of exposure to the film (chemiluminescence ELISA) because the high sensitivity of the latter did not allow a comparison with the same incubation time. (Exposure of the chemiluminescent plate to the film was for 5 s and the incubation with the *o*-phenylenediamine was for 2 min). Error bars represent S.E.M. values.

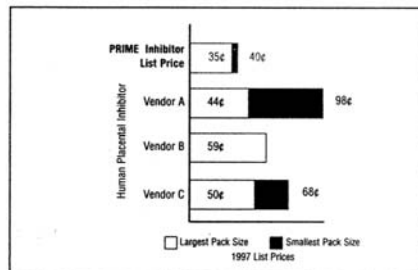
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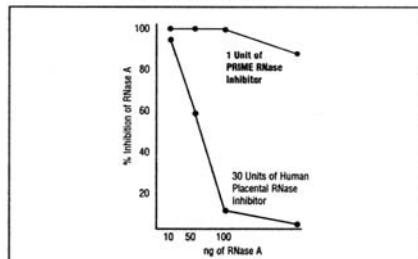
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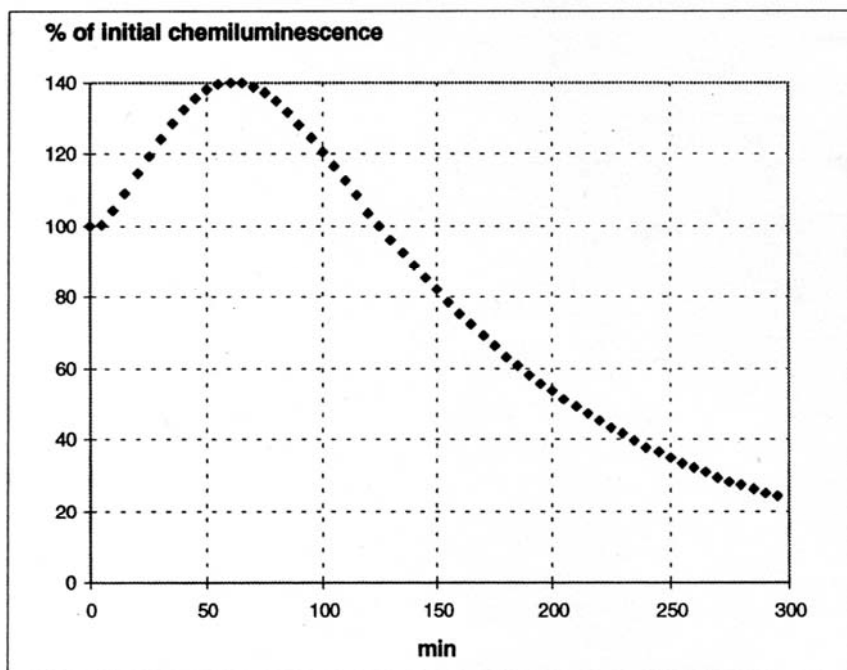


Figure 3. Kinetics of the chemiluminescence decay in 96-well plates. The decay of the chemiluminescence signal was determined by repetitive measurement of a standard sample at 25°C with a 96-well format scintillation counter in a single-photon counting mode (TopCount).

about 20-fold higher compared to a conventional photometric quantification (under the same ELISA conditions with *o*-phenylenediamine as substrate), which reduces the use of expensive antibodies. Moreover, sophisticated detection systems like luminometers or scintillation counters for the 96-well format with capability for single-photon counting are not necessary. Thus, normal, transparent flat-bottom ELISA plates can be used, for which the individual assay was set up (irrespective of the manufacturer), and it is not necessary to switch to white or black plates (as would be for measurements with 96-well luminometers). This aspect can be very important, because significant differences can occur between the plates of different suppliers.

Another drawback of scintillation counters is that the samples are usually not measured at the same time, with the consequence that the decay curve of the chemiluminescence has to be considered. This is not true for our method, since all samples of the plate are exposed simultaneously to the film. Furthermore, it is not necessary to quantitate the signal on the film with a laser scanning densitometer, since the spots

on the film show a homogeneous blackening exactly in the diameter of the wells, thus allowing a simple measurement with an ELISA reader.

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Method for Preparation of Epidermal Imprints Using Agarose

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The epidermal layer of plants carries unique and distinctive features that may serve, in most cases, as morphological markers to distinguish different plant species. These morphological features may include relative size and shape of component cells, quality and thickness of the cuticle and nature and distribution of specialized cells such as the stomata and trichomes. The venation patterns of leaves may also provide important identification points. Studies of epidermal traits gain special importance for the identification and phenotypic characterization of mutations that result in alterations of surface cells or their derivatives, and their characterization generally involves scanning electron microscopy (1).

The preparation and scanning of plant samples not only requires care and expertise but also an electron microscope. Such studies are therefore greatly curbed by the available financial and technological resources of a laboratory. Alternatively, but with a much lower degree of resolution, silicone rubber latex, collodion, cellulose acetate films, dental wax and clear lacquer/acrylic polymer have been used to obtain surface impressions, which are then recorded on photographic film for further analyses (2-4). While these substances provide good imprints, the preparation methods are also time-con-