A non-radioactive assay for the detection and quantitation of a DNA binding protein

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We have developed an alternative method for the assay of DNA binding activity of a known DNA binding protein. In contrast to standard gel retardation assays, this method is non-radioactive, quantitative and yields results within hours of commencement of the assay. Using an enzyme-linked immunosorbent assay (ELISA), the DNA binding activity of protein preparations can be easily compared. While this assay has been developed for the measurement of active human papillomavirus type 16 (HPV 16) E2 protein, it could be easily adapted for application to any known DNA binding protein for which antibodies are available.

The DNA binding sequence of HPV 16 E2 has been previously reported as being 5'-ACCGNNNNCGGT-3' (1) and single nucleotide changes at two positions to 5'-ACCANNNNTGGT-3' reduce the ability of the protein to bind to DNA significantly. Both forward and reverse oligonucleotides of the sequences 5'-ACCAGAAATCGGTTGAACCGAAACCGGT-3' and 5'-ACCACCCGCTGGTTGAACCACCGCTGGT-3' were synthesised with sticky ends using conventional methodology. Oligonucleotides were annealed by cooling from 95°C and ligated by incubation with T4 ligase overnight at 15°C. Following phenol extraction and ethanol precipitation, oligonucleotides were biotinylated using photoprobe (Vector Laboratories) according to the manufacturer’s instructions. For a control in the assay, an unrelated oligonucleotide 5'-ATATAATAATACTAAACTACAATAAT-TC-3' was also biotinylated. Ligated oligonucleotides were resuspended at a concentration of 1mg/ml and stored at −20°C until required.

The E2 protein used in the development of this assay was expressed as a fusion with glutathione-S-transferase (GST). The HPV 16 E2 open reading frame was amplified by polymerase chain reaction from pBR-HPV16 (2) and cloned into pGEX (Pharmacia). Bacteria induced to express the GST-E2 fusion protein were harvested by centrifugation and disrupted by sonication. Soluble protein was affinity purified on glutathione resin. The GST portion of the protein was expressed in bacteria containing the pGEX plasmid with no insert and was purified as described above. This protein was used as a control in the assay.

MaxiSorp Immuno plates (Nunc) were coated overnight at 37°C by allowing 100 μl of a 5 mg/ml solution of streptavidin in dH2O to dry onto the wells. The washing procedure was carried out between each of the incubation steps. Plates were washed by three cycles of filling wells with phosphate buffered saline containing 0.1% Tween 20 (PBS-T20) and incubating at RT for 5 min. The plate was then inverted and knocked vigorously on absorbent paper to ensure thorough removal of the wash buffer. Biotinylated oligonucleotides were attached to the streptavidin coated plates by diluting the 1 mg/ml stocks 1:1000 in PBS-T20 and adding 100 μl to each well. Plates were incubated for 15 min at 37°C on a shaking platform for this and each of the subsequent steps. Protein samples to be tested were diluted serially in buffer then further diluted 1:2 in 40 mM TRIS–HCl, pH 8.0, 140 mM KCl, 4 mM MgCl2, 40 μM zinc acetate, 0.4 mM dithiothreitol, 12% glycerol, 0.2% Tween 20, 4% bovine serum albumin and 40 μg/ml calf thymus DNA. A volume of 100 μl of each sample was transferred into the test plate and incubated. A mouse monoclonal antibody, TVG261, which interacts with the N-terminal region of E2, was used for the detection of E2 protein in the assay. After incubation with 100 μl of a 1:10 dilution of TVG261 supernatant, the monoclonal antibody was detected using a 100 μl per well of an appropriate dilution of a peroxidase labelled rabbit anti-mouse polyclonal.

Figure 1. Specificity and quantitation of protein binding to E2 specific oligonucleotide. Either GST-E2 or GST alone was serially diluted and incubated with the E2 specific oligonucleotide 5'-ACCGNNNNCGGT-3', attached to plastic. Protein was previously quantified by Bradford assay and results are expressed as moles of protein. The assay was performed in triplicate and bars represent standard error of the mean (SEM) values for each data point.

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antibody (Dakopatts). The colour reaction was developed by the addition of 100 μl of HRP substrate to each well (4 mg orthophenylene diamine and 0.03% H2O2 in 10 ml of 0.2 M phosphate-citrate buffer, pH 5.0) and incubation at room temperature for 20 min. At this time the reaction was terminated by the addition of 50 μl 2 M H2SO4 to each well. Absorbance at 490 nm was read using a microplate reader.

The amount of GST-E2 used in the assay was quantified using a Bradford based assay (BioRad). Using the molecular weight of the monomeric protein estimated at 67 000 daltons, the OD(490 nm) was correlated with moles of protein, generating a standard curve (Fig. 1). This standard curve could then be used to determine the quantity of active E2 expressed in the pET system, during various stages of its purification.

Results from this assay indicate that, in line with other published reports (3), E2 protein specifically binds the sequence 5'-ACCGNNNCGGT-3' and that changing the nucleotide at position 4 from G to A and position 9 to from C to T and changing the NNNN from predominantly A and T to G and C reduced the interaction of this protein with DNA significantly (Fig. 2), to a level comparable with the control oligonucleotide. The GST protein showed no specific binding to the oligonucleotide that was bound by GST-E2, indicating that binding was specific to the E2 protein (Fig. 1).

This assay could be applied to any DNA binding protein and it is both oligonucleotide and protein specific. An assay of function such as this is ideal in assessing protein activity, an important factor in the development of any purification strategy. As well as the advantages of speed and quantitation, the microassay format permits large numbers of samples to be easily tested and may, for example, be applied to large scale screening of inhibitors of DNA–protein interactions.

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