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(54) Title: IMMUNOASSAY FOR CERVICAL CANCER

(57) Abstract

A method of stabilising HPVE7 protein in a sample by boiling the sample and/or treating the sample with a denaturing agent which may be sodium dodecyl sulphate which may also be employed in a final concentration of between 0.01-0.1 %. The sample may be also boiled for 10-15 mins. prior to treatment by sodium dodecyl sulphate.

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TITLE

"IMMUNOASSAY FOR CERVICAL CANCER"

FIELD OF THE INVENTION

THIS INVENTION relates to detection of cervical cancer in humans associated with human papilloma virus. In particular, the invention relates to the determination of the presence or absence of specific viral antigens in a fluid sample from a patient suspected of suffering from cervical cancer which may be used as the basis for detection of human cervical cancer.

PRIOR ART

It is well known (e.g. in "Papilloma Viruses and Human Cancer" edited by H. Pfister and published by CRC Press Inc. in 1990) that papilloma viruses can be classified into several distinct groups based on the host in which they infect. Human papilloma viruses (HPV) can be further differentiated into types 1-56 depending DNA sequence homology. Types 16, 18 and 42 are associated with the majority of *in situ* and invasive carcinomas which may occur in the anogenital tract and in particular the cervix. In this regard, a number of cervical intra epithelial neoplasias and carcinomas of the cervix have been associated with HPV 16 and HPV 18. (Lancaster *et al.* 1987: Cancer Metast. Rev. 6 653 and Pfister 1987. Adv. Cancer Res 48 113). These same two references also point out that papilloma viruses are small DNA viruses encoding up to 8 early and 2 late genes.

The protein from the expression of the early gene E7 varies between 93 to 127 amino acid residues. The E7 protein is the most abundant viral protein in HPV 16 containing CaSki and SiHa squamous carcinoma cell lines and in HPV-18 containing HeLa and C4-1 lines. (Seedorf *et al.* 1987. EMBO J. 6, 139). DNA tranfection experiments implicate the E6 and E7 ORF proteins in *in vitro* transformation of mouse

fibroblasts (Yasumoto *et al.* 1986. J. Virol, 57, 572), rat epithelial cells (Matlashewski *et al.* 1987. EMBO J. 6, 1741) and primary human keratinocytes (Schlegel *et al.* 1988, EMBO J. 7, 3181; Pirisi *et al.* 1987. J. Virol. 61, 1061). Co-operation with an active *ras* oncogene leads to full transformation (Matlashewski *et al.* 1987. EMBO J. 6, 1741) and there is a requirement for continued expression of the E7 gene to maintain the transformed phenotype (Crook *et al.* 1989. EMBO J. 8, 513). The E7 protein may be recognised by the immune system, since anti E7 antibodies can be detected in the serum of approximately 20% of patients with HPV 16 associated cervical lesions (Jenison *et al.* 1988. J. Virol. 62, 2115; Jochmus-Kudielka *et al.* 1989. J. Natl. Cancer Institute 81, 1698; Smillie *et al.* 1990. Immunol. Infect. Dis. 1, 13).

At present, HPV infection is confirmed by histological and cytological criteria or by detection of HPV DNA or RNA (McDougall *et al.* 1986. In Papillomaviruses, CIBA Foundation Symposium 120. Evered and Clark eds. John Wiley and Sons. Chichester. p86). Tests for detecting antibody against papillomaviruses are difficult and not well standardised. Many have been developed as experimental techniques but none is in routine laboratory use. The basic problem is the lack of standard antigen. Only small amounts of HPV proteins have been isolated from tissues infected with HPV 16 and 18. (Androphy *et al.* 1987. EMBO J. 6, 1989; BANKS *et al.* 1987. J. Gen. Virol. 68, 1351; Firzlaff *et al.* 1988. Virology 164, 467; Oltersdorf *et al.* 1987. J. Gen. Virol. 68, 2933; Schneider-Gadicke *et al.* 1988. Cancer Res. 48, 2969; Seedorf *et al.* Embo J. 6, 139; and Smotkin and Wettstein 1986. PNAS 83, 4680). Also papillomaviruses cannot be grown in tissue culture. There is no epithelial cell culture permissive for HPV (Taichman *et al.* 1984. J. Invest.

Dermatol. 83, 25). As discussed in an article by Spradbrow in the 1990 reference by Pfister discussed above there is no conventional neutralisation test. Neutralising bodies directed against bovine fibro-papillomaviruses have been assayed *in vivo* using cattle or horses as the indicator systems. Similar tests have been performed with SHOPE cottontail papillomavirus and with canine oral papillomavirus and would be possible in other species. The ability of the viruses causing bovine fibro-papillomavirus to transform cultured cells has been used in serological tests, using mouse cells or bovine cells as substrate. Transformation was inhibited with the addition of antibodies producing in effect a neutralisation test. The transformation inhibition test however was complement dependent and the activity of the serum was absorbed by both virions and transformed cells. Also bovine fibro-papilloma virus together with mouse erythrocytes have been used in haemaglutination reactions. Haemaglutination reactions were inhibited by specific antiserum (Spradbrow 1990).

Tests for the detection of viral antigens in cells are also typical and suffer from similar problems as tests which detect antibodies. The common tests are indirect immuno fluorescents on frozen sections or the peroxidase-antiperoxidase (i.e. PAP) test on sections from formalin-fixed tissues. Antigens for these tests may be derived from tissue from different HPV lesions or tumours or particles from HPV1, cotton tail rabbit papillomavirus (CRPV) or bovine papillomavirus (BPV) disrupted by treatment with a detergent. The PAP test uses rabbit anti-HPV antibodies to complex with an antigen in the section followed by the complexing with a second labelled antibody that is specific for rabbit antibody, that is, for example, goat anti-rabbit antibody linked to an enzyme label such as peroxidase.

To overcome the problem of lack of standard antigen recombinant HPV 16 and 18 E7 proteins have been expressed from procaryote and eucaryote cells (Sato *et al.* 1989. Virology 170, 311; and Smotkin and Wettstein. 1986. PNAS 83, 4680). Recombinant viral proteins produced in procaryotes are not ideal for the study of immune responses to the native viral protein. First, they may show glycosylation and phosphorylation differences from the viral protein produced by infected eucaryote cells and secondly they are commonly produced as bacterial/viral fusion proteins derived from viral genomic DNA rather than from cDNA produced from viral encoded message. It is therefore necessary to demonstrate that the antibodies raised from such proteins recognise the native viral protein. In Selvey *et al.* 1990. J. Immunology 145, 3105 and Tindle *et al.* 1990. J. Gen. Virology 71, 1347 there was raised a panel of murine monoclonal antibodies against a MS2 replicase/HPV 18 E7 and MS2 replicase/HPV 16 E7 respectively. Both groups by ELISA detected reactive monoclonal antibodies which were bound to an antigen comprising the above fusion proteins which antigen was bound to an insert support. The ELISA system used horseradish peroxidase linked sheep anti-mouse Ig as a second antibody wherein the reaction between the peroxidase enzyme and the substrate hydrogen peroxidase was assayed using 0-phenylene diamine as indicator. In the Tindle 1990 reference use was also made of ^{125}I conjugated anti-mouse Ig as a second antibody. Both references demonstrated that the majority of raised monoclonal antibodies recognise linear epitopes of the corresponding viral protein. Selvey *et al.* concluded that the B cell epitopes on the HPV 18 E7 protein are located in the end terminal region of the molecule and that the E7 protein is either present in HeLa cells at a concentration too low to be detected by indirect

immunofluorescence or the end terminal epitopes are masked by protein confirmation or interaction with cellular or other viral components. Tindle *et al.* similarly defined the B cell epitopes of the E7 protein of HPV 16 to be located in the end terminal region of the molecule.

Reference may also be made to Tindle *et al.* PNAS 88 5887-5891 (1991) which describes a major T cell epitope in the E7 ORF of HPV 16.

To add to the frustration of obtaining large amounts of standard antigen, the HPV 16 E7 protein has been reported to have a short half life (about an hour) in CaSki and SiHa cells (Smotkin and Wettstein. 1986. PNAS 83 4680).

In relation to cervical cancer, this disease remains the fifth commonest cause of cancer deaths amongst women in Australia. Pap smear programmes are effective for preventing cervical cancer but have a high cost per prevented cancer because many abnormal pap smears are not due to potentially malignant cervical lesions.

Currently, abnormal pap smears are repeated and a gynaecologic opinion sought if the smear remains abnormal. Much of the expense of the pap smear programme arises from the cost of the gynaecologic opinion and biopsy, which often shows disease of no malignant potential.

Cervical cancer cells, and the epithelial cells of its precursor lesion, cervical intraepithelial neoplasia, generally contain HPV DNA. HPV 16, or closely related genotypes, is found in >95% of squamous cervical cancers if a sufficiently sensitive test is employed, whereas adenocarcinomas contain HPV 18 or HPV 16. Epidemiologic evidence supports the hypothesis that HPV 16 and 18 commonly act as initiators of cervical cancer, but measurement of HPV DNA in the cervix has not proved useful as a

screening test for risk of cervical cancer, because HPV 16 infection is common in the cervix, and the risk of progression of HPV infection to significant premalignant disease is less than 1%.

Recently, the molecular mechanisms which allow HPV DNA to initiate malignant transformation of the cervical epithelium have become clear. Two early proteins of HPV (E6 and E7 which are discussed above) are together able to transform, with great efficiency, normal human epithelial cells to a malignant phenotype (independent growth in soft agar, tumour formation in nude mice). Transformation is probably initiated by binding of these proteins to two anti-oncogenes, p53 and RB. Infection of epithelium with HPV produces short lived expression of E6 and E7 in relatively undifferentiated basal cells. As the cells mature, trans acting regulatory mechanisms involving the HPV E2 protein normally down regulate E6 and E7 expression, such that little or no message for these proteins is seen in superficial keratinising epithelium. However, HPV DNA integrates occasionally into the host genome, and integration of HPV DNA with disruption of the E2 open reading frame has been found in all cervical cancers and cancer cell lines examined. Thus, the necessary step for initiation of malignant transformation by HPV DNA seems to be integration, with disruption of HPV E2 open reading frame (ORF) and in consequence continued high level expression of E6 and E7, and this in turn results in failure of differentiation of epithelium in the cervix. Premalignant lesions associated with HPV 16 or HPV 18 should therefore have significant levels of E7 protein in superficial cells accessible by cervical scrape, but E7 should not be found in benign lesions associated with episomal HPV 16 or 18 infection, nor in non-HPV related cervical abnormalities.

Screening for premalignant cervical lesions

is generally carried out by pap smear. While this screening programme is effective at detecting cervical lesions with malignant potential, the overall prevalence of abnormal pap smears is high and there is a significant 'false positive' rate, whether this is defined as the number of women with an abnormal pap smear for whom a repeat smear is negative, or the number for whom a repeat smear is abnormal, but for whom a cervical biopsy shows a lesion without preneoplastic change and for whom no therapy is therefore indicated. Thus there is a place for a simple non-invasive confirmatory test for the malignant potential of a cervical lesion to be performed on women with an abnormal pap smear, and which like the pap smear could be carried out by the primary physician without referral of the patient for specialist advice and biopsy.

Research to evaluate the utility of three potential screening tests for cervical premalignant lesions: i.,e. (i) detection of HPV DNA using PCR, (ii) detection of antibodies to HPV E7 protein in serum, and (iii) evaluation of an HPV 18 E7 ELISA capture assay has now taken place. These assays were evaluated serially. It was first demonstrated that there is a total lack of utility of the E7 antibody assay as a screening test despite claims of utility by others. It has also been demonstrated that the PCR based screening is impractical because of the high false positive rate amongst 'normal' women. The ELISA screening assay for E7 protein of HPV 18 gave encouraging preliminary results, in that cervical cancer cells tested positive, as did a small proportion of samples from women with abnormal pap smears.

Reference may also be made to Selvey *et al.* Journal of Virological Methods 37 (1992) 119-128 entitled "An ELISA capture assay for the E7

transforming proteins of HPV16 and HPV 18" which referred to the establishment of ELISA capture assays for the E7 transforming proteins of HPV16 and HPV18 based on a range of previously characterised polyclonal and monoclonal antibodies. No cross reactivity was observed in the ELISAs between HPV16 E7 and HPV18 E7. Immunoreactive E7 protein (iE7) was measured in a series of HPV-transformed cell lines, and ranged from 0.6 to 17.7 ng iE7/mg cell protein. iE7 was labile at 22°C ($t_{1/2}$ = 37 min) but relatively more stable at 4°C ($t_{1/2}$ = 210 min). HPV16 E7 protein at concentrations from 0.10 to 0.69 ng iE7/mg cell protein was detected in 5 of 13 smears from women with abnormal cervical cytology. It was therefore considered that assay of E7 protein may play a role in the detection of HPV-induced cervical lesions with malignant potential.

The Selvey *et al.* reference above also established that in regard to assays for both HPV16 E7 and HPV18 it was necessary to utilise a combination of monoclonal antibodies and polyclonal antibodies to give adequate sensitivity and specificity. The majority of the E7-specific monoclonal antibodies which were raised against an MS2/E7 fusion protein and have already been demonstrated to be specific for defined linear or conformational epitopes of E7 as described in the Selvey *et al.* 1990 and Tindle *et al.* 1990 references were nevertheless ineffective as reagents for the capture ELISA assays. The main reason for this result was that presumably many of the linear epitopes for E7 are internal, and antibodies directed against these epitopes do not see E7 in solution in the ELISA. The Selvey *et al.* reference also established that E7-dependent cell lines contain about 10^{-11}M immunoreactive E7. this level is at the threshold of sensitivity of ELISA assays, and this and the relative instability of iE7

at room temperature and 4°C presents problems for the measurement of this protein in clinical samples. Attempts to stabilise the E7 by a number of conventional techniques including addition of a wide range of protease inhibitors and manipulation of divalent cations showed no improvement in E7 stability.

The Selvey *et al.* reference concluded that an assay which allowed detection of the transforming protein of HPV16 in cells exfoliated from the cervix would be of considerable potential clinical utility as less than 1% of abnormal PAP smears classified as CIN are from lesions with potential for progression to malignancy whereas routine detection of HPV16 DNA is non specific because of the high frequency of positive results in clinically and histologically normal cervix.

Therefore up to the present time there has not been discovered a reliable and efficient assay for detection of HPV16 or HPV18 E7 in a fluid sample from a patient suspected of having cervical cancer.

To this end, it must be appreciated in relation to a quantitative measurement of a particular substance in a clinical sample that it is essential to have (i) the appropriate reagents (ii) the relevant detection parameters and (iii) the proper preparation of clinical samples. For example in relation to (i) when assaying for antigen it is necessary to have antibodies that are specific for a certain epitope. In this regard the Selvey *et al.* and Tindle *et al.* references referred to above have described a variety of epitopes for both HPV16 and HPV18 and it is therefore not clear which of these epitopes will react quantitatively with an E7 antibody although this problem has been clarified to a certain extent by the Selvey *et al.* (1992) reference discussed above. In relation to (ii) detection parameters such as buffer,

pH, salt concentration and incubation temperature must be considered in establishing the appropriate assay conditions. In regard to (iii) generally preparation of antigen from serum is more complex than preparation of antibodies from serum. In this regard it has already been established that in the case of E7 protein that this protein has only a short half life (refer to Smotkin *et al.* above).

SUMMARY OF THE INVENTION

It is therefore an object of the invention to provide an assay for detection of E7 protein that is efficient and which may be used for quantitative analysis.

It is a further object of the invention to provide a test system and test kit which may be used for quantitative detection of E7 protein to thereby provide an efficient diagnosis of cervical cancer.

The present invention therefore provides an assay for determination of E7 protein which includes the following steps:

(i) obtaining a sample from a patient suspected of suffering from cervical cancer and boiling the sample and/or treating the sample with a denaturing agent which may include sodium dodecyl sulfate (SDS);

(ii) assaying said sample with antibodies raised against E7 protein;

(iii) determining the presence or absence of E7 in the sample by the determining of binding between the E7 protein and the antibody.

In step (i) it has been found that in relation to the HPV16 E7 antigen boiling may increase the level of this antigen by a mean of about 100% and has little effect on the half life of the E7 protein.

Also in relation to step (i) SDS treatment

above has little effect on the amount of detectable antigen, but stabilises antigen, prolonging the $T\frac{1}{2}$ of E7 at room temperature; the kinetics of the decay of E7 are first order and the $T\frac{1}{2}$ is approximately 10 fold increased at any given E7 concentration and temperature.

However, the combination of boiling and SDS treatment achieves the benefits of each of these steps as set out above.

The concentration of SDS that may be used is 0.01% to 0.1% and the antigen may be boiled sufficiently long for the purpose intended. However an optimal boiling time may be of the order of 5-30 minutes and more suitably 10-15 minutes.

The invention therefore in another aspect refers to a method of stabilizing HPV E7 protein by the use of step (i) above. The HPV E7 samples may be collected into an SDS vehicle of 0.01-0.1% concentration and boiled for an appropriate time at the point of collection in a simple manner, such that the stablized antigen may then be transported to the laboratory in such a way that transport conditions are not critical. Thus, an assay for HPV E7 may be carried out on the same day and at room temperature as opposed to the antigen being snap frozen at -70°C as was the case previously.

Preferably in regard to stabilisation of HPV16E7, the sample is preferably boiled for 10-15 mins and also treated with a final concentration of 0.01-0.1% SDS.

In regard to stabilisation of HPV18E7, the final concentration of SDS is preferably from 0.01 to 0.1%. However, boiling is preferably avoided.

Preferably prior to carrying out of the assay the sample from the patient is subjected to a mild lysis process whereby any infected cells may be lysed so that minimal disruption occurs. To this end

harsh or structurally disruptive lysis techniques are preferably avoided. Suitably the sample is placed in a mild lysis buffer such as a non-ionic detergent.

Suitable non-ionic detergents include BRIJ56, BRIJ58, BRIJ35, Triton X-45, Triton X-114, Nonidet P-40, Triton X-100, Triton N-101, Tween 20, Tween 40, Tween 80, and oxtyl-β-glucoside. Nonidet P40 is the preferred non-ionic detergent. The concentration of the non-ionic detergent may vary between 0.01% to 1% in buffered solution. Preferably a 0.1% solution in phosphate buffered saline or other suitable buffer is used.

After the sample has been placed in a mild lysis buffer, the cells may be subsequently disrupted in a variety of ways but preferably by means of external pressure whereby the sample may be forced under pressure through a small orifice or tubular passage of small cross section such as a hollow bore or cannula of a needle. Alternatively, a French press may be used where large volumes of sample are being processed. The disruption of the cells may occur in the presence of a protease inhibitor such as phenylmethylfluorane sulphonate.

Subsequently the sample may be incubated in boiling water for a suitable time span (e.g. 5 min-30 min) and suitably a denaturing agent such as SDS is added which may have the effect of stabilising any E7 protein located in the sample. Any appropriate amount of SDS may be utilised such as 1-20µl of 10% SDS which may be added to 500 ml samples for example.

Subsequently the lysed sample may be purified by centrifugation of filtration and especially ultrafiltration wherein insoluble matter may be discarded.

With respect to the assay procedure use may be made of a first antibody that is specific for the E7 protein and demonstrates high reactivity to the E7

protein. The first antibody is preferably monoclonally derived and is suitably raised against MS2 replicase/E7 fusion protein described above in the Tindle 1990 *et al.* and Selvey *et al.* 1990 references. The level of reactivity is determined by the ability with which the antibody may bind to the E7 protein under assay conditions. High reactivity is when significant binding occurs. The abovementioned Selvey *et al.* 1992 reference has demonstrated that different antibodies which recognise different epitopes of the E7 protein were more reactive than others, and that antibodies which recognise confirmational epitopes were more reactive than antibodies which recognised linear epitopes. Suitable first antibodies of HPV16E7, that is anti-E7 HPV16MAb include 10F and 6D referred to in the Tindle *et al.* 1990 reference above. Suitable first antibodies for HPV18E7, that is anti-E7 HPV18MAb include 7E10, 15H4, and monoclonal antibodies that recognise epitopes A, B, C, CONF1 and CONF2 referred to in the Selvey *et al.* 1990 reference above. Preferably, anti-E7 HPV18MAb7E10 is used for the capture of HPV18E7 protein and anti-E7 HPV16MAb10F is used for the capture of HPV16E7 protein. The first antibody is preferably bound to a solid support surface. The solid support surface may be of any suitable material including hydrocarbon polymers such as polystyrene, polyethylene and polybutylene; polyesters and polyamides; cellulose and cellulosic derivatives; and vinyl polymers. A preferred form of the solid support surface is a test tube or a microtitre plate well.

After the first antibody is bound to the solid support surface binding sites on the solid support surface may be blocked by any suitable material but preferably by skim milk powder.

The sample from the patient may then be applied to the first antibody in any suitable manner.

After this procedure a second antibody may then be applied in the assay specific for the E7 protein. The second antibody may be monoclonally derived or from polyvalent antiserum. Suitable monoclonal antibodies that can serve as a second antibody are the monoclonal antibodies that recognise the epitopes in epitope C and CONF2. Suitable polyvalent antiserum may be raised in rabbits against purified or partially purified E7 protein. A source of E7 protein may be obtained from the expression of a recombinant vector which encodes the E7 gene. A suitable E7 protein is the MS2-replicase fusion protein expressed from the MS2/E718 or MS2/E716 vectors discussed previously. Preferably rabbit polyvalent antiserum of appropriate specificity is used as the source of the second antibody.

The first antibody and the second antibody may be added together in the one reaction mix in order to achieve a simultaneous immunoassay although preferably the antibodies are added in a step-wise addition in order to achieve a forward immuno assay reaction.

After use is made of the second antibody in the assay procedure of the invention a labelled third antibody may then be utilised which may bind to the second antibody. Alternatively, the second antibody may be labelled but the former procedure is preferred for convenience. A suitable label may be selected from a radioactive isotope, an enzyme, or a fluorometric molecule. Enzyme labels may include catalase, peroxidase, urease, glucose oxidase and alkaline phosphatase. The third antibody is preferably labelled with a peroxidase enzyme label. Standard techniques known to a person expert in the art may be used to determine the concentration of the labelled antibody in the third complex or the unreacted labelled antibody.

The first antibody may be selected from any of those mentioned above but preferably anti-E7 HPV18 MAb 7E10 anti-E7 HPV16 MAb 10F. Preferably the first antibody is provided in the form of a coating on a solid support such as on the surface of the wells of a microtitre tray. A blocking solution may be optionally provided. The preferred block solution is a 5% skim milk powder in 0.15 molar phosphate buffer saline PH 7.4. An ELISA wash buffer may be optionally provided. The preferred ELISA wash buffer consists of 0.1% skim milk powder and 0.1% tween 20 in phosphate buffer saline.

The second antibody may be selected from those mentioned above. For HPV 16E7 antigen antibodies of appropriate specificity from polyvalent rabbit anti-E7 sera are preferably used. For HPV 18E7 antigen antibodies of appropriate specificity from rabbit polyvalent anti-E7 18 sera are preferably used.

The third antibody may consist of any appropriate label but preferably a peroxidase enzyme label. The third antibody is preferably a goat anti-rabbit immunoglobulin coupled to horseradish peroxidase. Alternatively, if the second antibody is derived from a mice or mice cell line, then the third antibody may be rabbit anti-mice immunoglobulin coupled to horseradish peroxidase. A OPD substrate buffer may be optionally provided. The preferred OPD substrate buffer comprises of 0.012% hydrogen peroxide, 0.2M o-phenylenediamine, 25 mM citric acid, and 50 mM disodium hydrogen phosphate PH 5.0. A stop solution may be optionally provided. The preferred stop solution is 2.5 M HC1.

It will be appreciated that the scope of the present invention includes the detection and determination of E7 antigens from other papilloma viruses and that types HPV 16 and HPV 18 are used here by way of example.

It will also be appreciated that the assay procedure of the invention may be utilised in regard to not only a forward type reaction in which the antibody bound to the solid phase is first contacted with the sample so as to form a solid phase antibody:antigen complex and then subsequently detected with a second (or third) labelled antibody; but also a simultaneous type reaction which involves a single incubation step where the antibody bound to the solid support and the labelled antibody are both added to the sample at the same time.

The present invention may be further described in the following preferred embodiment which relates to an appropriate experimental procedure.

Having regard to the foregoing, the assay of the present invention reduces the cost of screening for cervical cancer by allowing simple further evaluation by a medical officer who first performs the test of the malignant potential of an abnormal pap smear.

Also it will be appreciated that the assay of the present invention allows for evaluation of abnormal pap smears for malignant potential by a further cervical scrape at the time of the repeat pap smear, and only those women with a positive screening test for malignant potential would require further specialist evaluation.

This may reduce the cost and increase the acceptability of pap smear programmes and thus may also indirectly increase uptake of the programme.

The assay of the invention may also involve collection of one extra cervical scrape from women with known abnormal pap smears who are undergoing routine evaluation of their lesion by a gynaecologist. It is clear that no extra discomfort is envisaged.

It will also be appreciated that the assay of the invention may be utilised as a confirmatory

test for the diagnosis of cervical disease of significant malignant potential.

The assays of the invention are reproducible, sensitive and HPV genotype specific and give positive results with cervix cancer cells but negative results with non-cervical tissues. The assays of the invention may also be used as confirmatory assays in the field, using patients with abnormal pap smears who are to proceed to biopsy for confirmation of the nature of their disease.

EXPERIMENTAL

EXAMPLE 1

By way of example, one particular method of performing the assay is outlined below. The method presented below is a sandwich ELISA method including the steps of treating the sample with SDS and boiling.

Preparation of Tissue Samples

1. Cells from cervical smears are collected in sodium lactate, spun down, the pellet transferred to 500ul of lysis buffer (0.1% NP40 in PBS (0.15 M phosphate-buffered saline pH 7.4)) and immediately stored at -70°C. Tissue samples collected are immediately snap frozen in a dry ice/ethanol bath or in liquid nitrogen after which they are crushed with a mortar and pestel or tissue homogeniser, resuspended in 500 µl of lysis buffer and snap frozen again.
2. Samples are thawed on ice and if possible passed through a 27 gauge needle five times. Samples are then spun down (top speed microfuge for 5 min) and 30 µl removed from the supernatant into a separate tube for total protein assay.
3. 5 µl of 10% SDS is then added to each 500 µl sample and the sample resuspended followed by incubation in a boiling water bath for 10 mins.
4. Samples are then placed on ice and spun down, after which the supernatant can be added to the

assay plate.

Making Elisa Plates

For the detection of HPV16E7 the monoclonal 6D is preferably used whereas for the detection of HPV18E7 the monoclonal 7E10 is preferably used.

1. Dilute 6D monoclonal antibody 1:1000 in PBS.
2. Add monoclonal antibody, 50 µl/well (except first 4 wells of column 1 which are to be incubated with distilled water or PBS, as blanks to zero the plate reader) and incubate at 4°C ON in an ELISA plate shaker.
3. Wash 2X with cold PBS and block with 5% skim milk powder in PBS (200 µl/well), for 1 h RT in the plate shaker.
4. Wash 2X with cold PBS, tap dry, add 10 µl of cold wash buffer (0.1% tween 20, 0.1% skim milk powder in PBS) to all wells (prevents drying) and use immediately.

Elisa Reaction

1. Have a plan for the assay ready.
2. Use 16 and 18E7MS2 fusion protein (alternatively 16E7GST fusion protein or the cleaved E7 derived therefrom) as positive and negative controls and for the production of a standard curve. Make serial dilutions in PBS of 16E7MS2 fusion protein starting from 150 ng and continue diluting 1:2 for 5 dilutions. Use 18E7MS2 fusion protein as the cross fusion protein and zero value for 16E7, diluted 1:1000 in PBS. Make 4 replicates of each dilution.
3. Add samples to the ELISA plate, 50 µl/well, (4 replicates of each). Add 50 µl of distilled water (or PBS or wash buffer (0.1% skim milk powder, 0.1% Tween 20 in PBS)) to the wells for blanking.
4. Incubate plate for 1-1.5 h at 4°C in the plate shaker. Meanwhile measure the total protein of

the cell lysates (see below).

5. Wash 10X in wash buffer.

6. Add appropriate polyclonal antiserum (rabbit anti-E7 already in 25 µl aliquots) diluted 1:200 in wash buffer or PBS, 50 µl/well. Also add 50 µl of distilled water (or PBS or wash buffer) to the blanking wells and incubate for 1h at 37°C.

7. Wash 10X in wash buffer.

8. Add peroxidase labelled anti-rabbit antiserum - diluted 1:1000 in wash buffer or PBS (50 µl/well). Add 50ul of distilled water (or PBS or wash buffer) to the blanking wells and incubate for 45 min at 37°C.

9. Wash 10X in wash buffer.

10. Add OPD substrate (125 µl) and hydrogen peroxide (5 µl) to 12.5 ml OPD substrate buffer, and add to plate 50 µl/well, cover in foil and incubate at 37°C for 15-30 mins depending on colour reaction.

11. Stop reaction with 25 µl/well of 2.5M HCl, burst any bubbles with sterile needles or toothpicks, wipe bottom of plate and read plate at OD492 in ELISA plate reader (mode 1, filter 4). Blank plate in air and then on single well uncoated with monoclonal antibody.

OPD (O Phenylenediamine)

TOXIC-prepare in fume hood!

Dissolve 400 mg in 10 ml dimethyl formamide (use glass pipette).

Store in glass bottle wrapped in foil at 4°C.

OPD substrate buffer (500 ml)

citric acid 2.5g

disodium hydrogen orthophosphate 3.3 g

dissolve in distilled water, pH 5.0, autoclave.

Determination of Protein Content

Use the BCA (Bicinchoninic Acid - specific reagent for cuprous ion) kit and a 96 well assay plate with lid.

Follow instructions with kit, but use 5 µl of each sample and BSA protein standards instead of 10 µl.

Prepare your own protein standards using BSA (10.0 mg/ml, 5.0 mg/ml, 2.5 mg/ml, 1.0 mg/ml, 0.5 mg/ml) and store in the cold room (replace every month or so).

Include lysis buffer and distilled water as blanks in this assay (Blank the plate on distilled water, the lysis buffer should give a similar reading).

For samples, BSA standards and blanks prepare 3 replicate wells.

After reaction measure the OD at 540 nm (on ELISA plate reader, mode 1, filter 6).

Results

Draw a total protein standard curve with OD vs mg/ml total protein (from BCA assay). Determine the value of the total protein mg/ml of the sample from the standard curve.

Draw a standard curve for the positive control fusion protein with OD vs ng E7/ml. Determine the value of E7 ng/ml of the sample from the standard curve.

The 16E7 fusion protein curve should cross the y axis at an OD point approximately equal to the mean of the negative control values (distilled water treated with OPD etc and cross fusion protein).

Any sample with an OD (492) reading greater than the mean of the negative controls plus 2 standard deviations is considered positive.

The ng E7/mg total protein of these positive samples can then be calculated by dividing the value obtained for 16E7 mg/ml by total protein mg/ml.

EXAMPLE 2

Determination of the amount of 16E7 in a Caski cell sample

A sample of *Caski* cells grown *in vitro* under conventional conditions was taken and subjected to the assay outlined in Example 1. *Caski* cells have been known to contain HPV16E7.

The results of the experiment are given in Tables 1 and 2 and FIGS. 1 and 2. Bovine serum albumin (BSA) was used as the total protein standard. The fusion protein 16E7MS2 was used as the standard for E7 ng/ml determinations. When using 16E7MS2 (or 18E7MS2) as an E7 standard only half the total protein concentration is taken into account as approximately only half of the fusion protein is E7. Thus, for example, if the total protein concentration of 16E7MS2 is 600 µg/ml, only a figure of 300 µg/ml is used with respect to E7 ng/ml. Further, 18E7MS2 is used as a control for the E7 ng/ml determinations and is the zero or base line value as there is no cross reactivity with the anti-16E7 monoclonal antibody. (Alternatively any protein that does not cross react with the anti-16E7 monoclonal antibody, may be used as the control.)

The OD reading at 540 nm of the *Caski* cell sample prepared for total protein determination was 0.429 which from the total protein standard curve (FIG. 1) gave a total protein concentration of 1.95 mg/ml. The OD reading at 492 nm of the *Caski* cell sample prepared for 16E7 ng/ml determination was 0.688 which from the 16E7 standard curve (FIG. 2) gave a 16E7 concentration of 41.75 ng 16E7/ml. The amount of 16E7 per total protein in the *Caski* cell sample was 21.41 mgE7 per mg total protein. The mean OD reading at 492 nm of the negative controls plus two standard deviations was 0.023. This value is lower than the equivalent OD reading for the *Caski* cell sample, thus confirming the presence of 16E7 in the *Caski* cell sample.

EXAMPLE 3

Determination of the amount of 16E7 in a cervical scrape sample from a human subject

A cervical scrape sample was obtained from a human subject and assayed in accordance with the assay

in Example 1. Standard total protein and 16E7 values were determined and appropriate standard curves were prepared. The values of the standards and the standard curves were substantially identical to those given in Example 2 (Tables 1 and 2; FIGS. 1 and 2).

The OD reading at 540 nm of the cervical scrape sample prepared for total protein determination was 0.042 which from the total protein standard curve gave a total protein concentration in the sample of 0.107 mg/ml. The OD reading at 492 nm of the cervical scrape sample prepared for 16E7 concentration determination was 0.034 which from the 16E7 standard curve gave an 16E7 concentration of 0.252 ng/ml. The amount of 16E7 per total protein in the cervical scrape sample was 2.36 16E7 ng/total protein mg. The mean OD at 492 nm of the 18E7 MS2 plus two standard deviations was 0.037. As this value was close to the OD 492 value for the cervical scrape sample (i.e. 0.034), it was considered worthwhile to re-examine the subject for the presence of 16E7.

EXAMPLE 4

Determination of E7 assay conditions

A series of experiments were conducted to demonstrate the advantages of including the SDS and boiling steps in the E7 assay. The procedure employed was the same as used in Example 1 with various modifications outlined in each experiment. In all the experiments in this Example, the amount of E7 per total protein in each sample was determined according to the assay procedure and these values were compared to determine which modification gave the best results.

Experiment 1

USE OF PROTEASE INHIBITORS

Samples of *Caski* cells were used in the assay. The monoclonal antibody 6D specific for 16E7 was used All samples were boiled for approximately 10 minutes and then:

(i) kept at room temp for 3 hrs then stored at -70°C.

(ii) immediately stored at -70°C.

(iii) treated with N ethyl malemide, kept at room temp for 3 hrs, then stored at -70°C.

(iv) treated with antipain, kept at room temp for 3 hrs, then stored at -70°C.

(v) treated with leupeptin, kept at room temp for 3 hrs, then stored at -70°C.

Results:

Modifications	Mean OD at 495 nm	16E7 ng/total protein mg
i	1.002	8.96
ii	1.398	14.8
iii	0.242	2.37
iv	0.922	7.92
v	0.914	8.81

Boiling for approximately 10 minutes and then immediately storing at -70°C (i.e. modification (ii)) gave comparatively better results than did the use of protease inhibitors.

Experiment 2

BOILING

Caski cell samples were used. The monoclonal antibody 6D specific for 16E7 was used. *Caski* cell lysates were syringed and boiled prior to assay.

(i) kept at room temp for 3 hrs then stored at -70°C.

(ii) immediately stored at -70°C.

(iii) no boiling and immediately stored at -70°C

(iv) No boiling, kept at room temp for 3 hrs and then stored at -70°C

(v) N ethyl malemide added, kept at room temp for 3 hrs and stored at -70°C

Results:

Modifications	Mean OD at 495 nm	16E7 ng/total protein mg
i	1.002	8.96
ii	1.398	14.8
iii	1.049	7.10
iv	0.740	5.36
v	0.002	0.00

The step of boiling as per procedure (ii) gave comparatively better results.

Experiment 3

BOILING TIME

Caski cell lysates were boiled for different time periods. The monoclonal antibody 6D specific for 16E7 was used.

Modifications	Mean OD at 495 nm	16E7 ng/total protein mg
(i) no treatment	0.730	33.28
(ii) 2 min boil	0.325	39.93
(iii) 10 min boil	0.636	161.73
(iv) 15 min boil	0.682	135.30

Boiling for 10 mins (modification (ii)) gave better results than boiling for any other length of time tested.

Experiment 4

TREATMENT WITH SDS

Samples of the 16E7 GST fusion protein, were used. The samples were treated with and without the addition of 0.1% SDS (final concentration) followed by 30 min incubation on ice.

Results:

Modifications	Mean OD at 495 nm	16E7 µg/ml
addition of SDS	1.235	155
no SDS	0.846	70

The addition of 0.1% SDS gave comparatively better results for 16E7 determination.

Experiment 5

ADDITION OF SDS AND BOILING

(a) *Caski* and *STO* cell lysates with combination of SDS and boiling (final concentration of SDS is given as a percentage). The monoclonal antibody 6D specific for 16E7 was used. The fusion protein (FP) used was 16E7MS2. Boiling was for approximately 10 mins. *STO* cells do not contain E7 protein.

Modifications	Mean OD at 495 nm	16E7 µg/ml
(i) *Caski*	0.379	8.03
(ii) *Caski* + 0.01% SDS	0.289	15.49
(iii) *Caski* + 0.1% SDS	0.549	12.07
(iv) *Caski* + 1.0% SDS	0.080	1.79
(v) *Caski* + 0.01% SDS + boil	0.244	5.13
(vi) *Caski* + 0.1% SDS + boil	**0.689**	**22.90**
(vii) *Caski* + 1.0% SDS + boil	0.081	2.54
(viii) *Caski* boil	0.485	13.02
(ix) *STO*	0.014	---
(x) *STO* + FP	0.084	3.03
(xi) *STO* + 0.01% SDS	---	---
(xii) *STO* + FP + 0.01% SDS	**0.062**	**1.23**
(xiii) *STO* + 0.1% SDS	---	---
(xiv) *STO* + FP + 0.1% SDS	0.176	7.63
(xv) *STO* + 0.01% SDS + boil	---	---
(xvi) *STO* + FP + 0.01% SDS + boil	**0.577**	**32.16**
(xvii) *STO* + 0.1% SDS + boil	---	---
(xviii) *STO* + FP + 0.1% SDS + boil	0.442	25.08
(xix) *STO* boil	---	---
(xx) *STO* + FP + boil	0.389	25.16

Comparatively better results were obtained for *Caski* cells treated with 0.1% SDS and boiled for 10 mins and for *STO* cells with 16E7MS2 treated with 0.01% SDS and boiled for 10 mins.

(b) *Caski* cells lysates with combination of SDS and boiling (final concentration of SDS is given as a percentage). Monoclonal antibody 6D specific for 16E7 was used. Boiling was for approximately 10 mins.

Modifications	Mean OD at 495 nm	16E7 µg/ml
(i) *Caski*	0.803	21.87
(ii) *Caski* + 0.01% SDS	0.961	25.09
(iii) *Caski* + 0.1% SDS	1.109	31.99
(iv) *Caski* + 0.01% SDS	1.025	30.34
(v) *Caski* + 0.1% SDS + boil	**1.187**	**36.92**
(vi) *Caski* boil	0.869	25.47

(c) Hela and *STO* cell lysates with combination of SDS and boiling (final concentration of SDS is given as a percentage). Hela cells have been shown to contain 18E7 protein. The monoclonal antibody 7E10, specific for 18E7 was used. The fusion protein (FP) used was 18E7MS2. Boiling was for approximately 10 mins. *STO* cells do not contain E7 protein.

Modifications	Mean OD at 495 nm	16E7 µg/ml
(i) Hela	0.970	› 25.56
(ii) Hela + 0.01% SDS	0.695	27.50
(iii) Hela + 0.01% SDS + boil	0.200	3.95
(iv) Hela + 0.1% SDS	0.810	› 23.32
(v) Hela + 0.1% SDS + boil	0.466	12.47
(vi) Hela boil	0.112	0.79
(vii) *STO*	0.043	---
(viii) *STO* + FP	0.820	› 77.67
(ix) *STO* + 0.01% SDS	0.044	---
(x) *STO* + 0.01% SDS + boil	0.041	---
(xi) *STO* + FP + 0.01% SDS	**0.692**	**84.56**
(xii) *STO* + FP + 0.01% SDS + boil	0.082	---
(xiii) *STO* + 0.1% SDS	0.087	---
(xiv) *STO* + 0.1% + boil	0.131	3.67
(xv) *STO* + FP + 0.1% SDS	0.496	31.42
(xvi) *STO* + FP + 0.1% SDS + boil	0.231	12.48
(xvii) *STO* boil	0.034	---
(xviii) *STO* + FP + boil	0.059	---

For Hela cells treatment with 0.01% SDS produced the best results. For *STO* cells with 18E7MS2 treatment with 0.01% SDS produced the best results.

TABLE 1

Concentration of BSA (mg/ml)	OD 540
0.25	0.096
0.5	0.145
1.0	0.269
2.5	0.548
5.0	1.012

TABLE 2

OD 492	16E7 ng/ml
1.230	150
0.887	75
0.699	37.5

TABLE LEGENDS

TABLE 1

Standards for total protein concentration

TABLE 2

Standards for 16E7 concentration

FIGURE LEGENDS

FIG. 1

Total protein concentration standard curve

FIG. 2

16E7 concentration standard curve

CLAIM

1. A method of stabilising HPVE7 protein in a sample by boiling the sample and/or treating the sample with a denaturing agent.

2. A method as claimed in Claim 1 wherein the sample is boiled and treated with a denaturing agent.

3. A method as claimed in Claims 1 or 2 wherein the denaturing agent is sodium dodecyl sulphate.

4. A method as claimed in Claim 3 wherein the final concentration of sodium dodecyl sulphate is used between 0.01% to 0.1%.

5. A method as claimed in any one of the preceding claims wherein boiling is for 10-15 mins.

6. A method as claimed in any one of the preceding claims wherein the HPVE7 protein is HPV16E7 and the sample is boiled for 10-15 mins and treated with a final concentration of 0.01-0.1% sodium dodecyl sulphate.

7. A method as claimed in any one of the preceding claims wherein the HPVE7 protein is HPV18E7 and the sample is treated with a final concentration of 0.01-0.1% sodium dodecyl sulphate.

8. A method as claimed in any one of the preceding claims wherein the sample is a human cervical-derived sample.

9. A method for determination of E7 protein in a sample including the steps of:-

(i) boiling the sample and/or treating the sample with a denaturing agent;

(ii) assaying the sample with antibodies raised against E7 protein; and

(iii) determining the presence or absence of E7 protein in the sample by the determination of binding between the E7 protein and

the antibody.

10. A method as claimed in Claim 9 wherein the denaturing agent is sodium dodecyl sulphate.

11. A method as claimed in Claim 10 wherein the final concentration of sodium dodecyl sulphate is used between 0.01% to 0.1%.

12. A method as claimed in any one of Claims 9-11 wherein boiling is for 10-15 mins.

13. A method as claimed in any one of Claims 9-12 wherein the HPVE7 protein is HPV16E7 and the sample is boiled for 10-15 mins and treated with a final concentration of 0.01-0.1% sodium dodecyl sulphate.

14. A method as claimed in any one of Claims 9-13 wherein the HPVE7 protein is HPV18E7 and the sample is treated with a final concentration of 0.01-0.1% sodium dodecyl sulphate.

15. A method as claimed in any one of Claims 9-14 wherein the sample is a human cervical-derived sample.

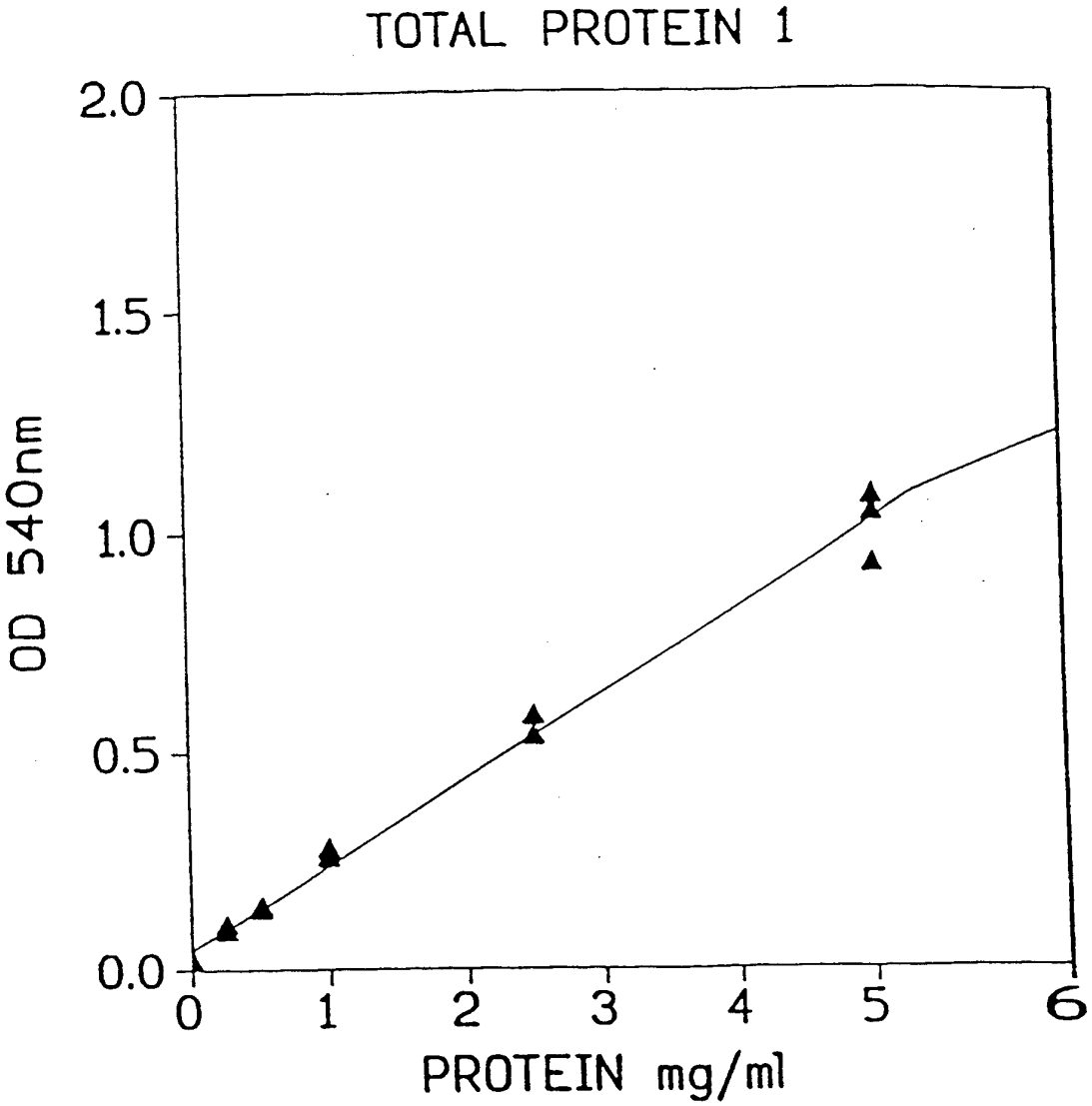


FIG 1

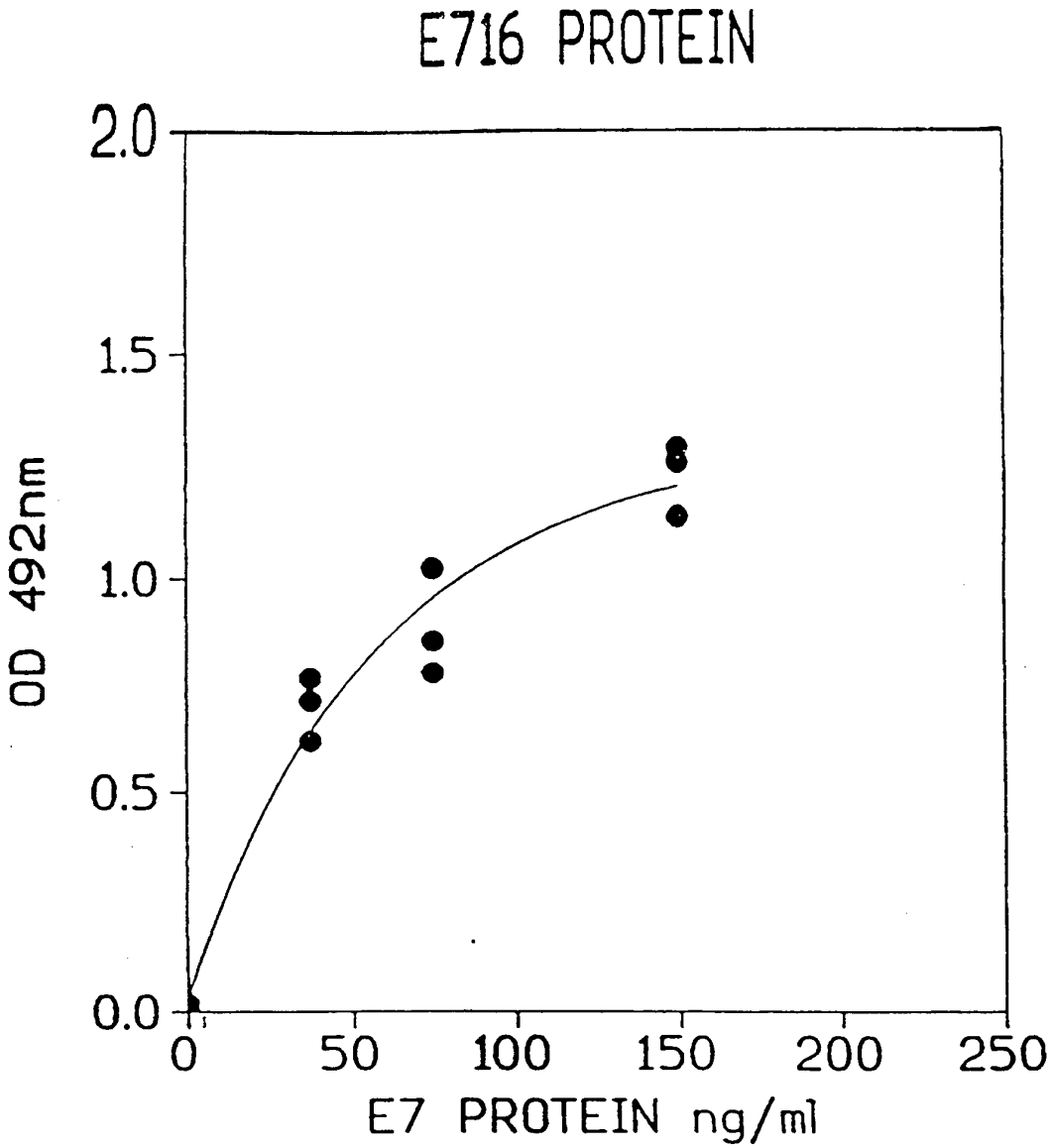

E716 PROTEIN
2.0
1.5
1.0
0.5
0.0
OD 492nm
0
50
100
150
200
250
E7 PROTEIN ng/ml

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00741

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[6] G01N, 33/531, 33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: G01N: Derwent database - World Patent Index (WPAT);
American Chemical Society data base - Chemical Abstracts (CA)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC: AU Database G01N, 33/531, 33/574; Derwent database -
Biotechnology Abstracts (BIOT)

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
CASM:HPVE7, E7, human()papillona()virus; protein#, antigen#, Ag, peptide#; denatur:(W) agent, detergent#, SDS, dodecyl(W) sulfate, doclecyl(W) sulphate, boil;
BIOT: as above plus stabil: WPAT: as above plus shelf(W)life, half(W)life, half(W)lives, stabil:; electrophoresis, western(W)blot; C07K/IC, G01N/IC; heat:

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP 495401 (KAO CORPORATION) 22 July 1992 (22.07.92)	1-14
A	JP 55-136233 (MIDORI JUJI K.K.) 23 October 1980	
A	WO 82/04396 (THE SECRETARY OF STATE FOR SOCIAL SERVICES IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND) 22 December 1982 (22.12.82)	

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 1995 (09.02.95)	22 Feb 1995 (22.2.95)

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INTERNATIONAL SEARCH REPORT
Information on patent family membe

International application No.
PCT/AU 94/00741

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
EP	495401	US	5296367	US	5344770

END OF ANNEX