

Full Length Research Paper

Production of recombinant proteins GST L1, E6 and E7 tag HPV 16 for antibody detection of Tunisian cervical cancer patients

Achour, M.^{1*}, Ben Younes, R.¹, Kochbati, L.², Kahla, S.¹, Zeghal, D.³, Maalej, M.², Zouari, F.³ and Oueslati, R.¹

¹Laboratory of Environmental Immuno-Microbiology and Cancerogenesis (IMEC Unit), Faculty of Sciences Bizerta 7021 Zarzouna, Tunisia.

²Service of Radiotherapy, Institute of Cancer Salah Azaeiz, Tunisia.

³Service of Gynecology- Obstetrics, Center of Maternity and Neonatology, Tunisia.

Accepted 2 June, 2008

In the present work recombinant proteins were produced for used in LUMINEX in order to undergo serological study of Tunisian female population. HPV types 16 L1, E6 and E7 sequences fused to their 3'-end to a sequence encoding the terminal undecapeptide of the SV40 large T-antigen (tag) were isolated from plasmids and inserted into a pGEX vector for expression as GST fusion proteins in *Escherichia coli*. Coding sequences for L1tag, E6tag and E7tag of HPV 16 respectively were mobilized by digestion with enzymes and ligated into digested plasmids downstream of the GST domain. An expression plasmid for GST tag was constructed by inserting a fragment coding for the tag epitope. Data showed that the lysates were stable for detection and they were used in Luminex for detection of antibodies in female Tunisian female patients. This assay showed that the sero-positivity towards the different antigens depends upon the group studied and differences between cases and controls were significant ($P < 0.001$). Elevated percentage of positivity was found for E7 (61%) versus 44 and only 21% for E6 and L1 antigens, respectively, and the intensity of the antibody response towards the late antigen L1 and the early antigens E6 and E7 were different.

Key words: T-antigen, LUMINEX, cervical cancer, HPV type 16, L1, E6 and E7.

INTRODUCTION

On a global level, human papillomavirus (HPV) is estimated to cause almost half a million cases and more than 270,000 deaths from cervical cancer, corresponding to more than 2.5 million years of life lost (YLL) annually (Sue et al., 2007). HPV type 16 (and to a lesser degree HPV type 18) is linked with more rare cancers, namely cancer of the vulva, vagina, penis, anus, oropharynx and larynx. Effective prophylactic vaccines have been developed (Dillner et al., 2007). Molecular epidemiological studies have demonstrated that specific subtypes of HPV are associated with cervical cancer (Castle and Giuliano, 2003; Dillner and Brown, 2004).

The HPV group of viruses today consists of more than 100 completely characterized types. Partial sequences of additional isolates indicate that at least another 100 HPVs exist. Of these, 15 genital HPVs are established as oncogenic in humans. HPV type 16 is by far the most important virus, accounting for more than 50% of all cervical cancers. HPV16 is even more dominating as an etiology of the noncervical HPV-associated cancers (Dillner, 2005). HPV serology is complex for several reasons. Different types of HPV can infect the epithelia of skin or mucosa and induce proliferative diseases. HPV antibodies are type specific. Those targeting the major viral capsid protein L1 are markers of infection, and those targeting the viral oncoproteins E6 and E7 are markers for HPV-associated cancer (Waterboer et al., 2005). Conventional serologic methods such as ELISA allows the analysis of sera for antibodies to only 1 antigen per

*Corresponding author. E-mail: mahaachour2000@yahoo.fr.
Tel: 216 72 591 845 .Fax: 216 72 590 566.

well. Previous studies have determined antibodies against early antigens as E6 protein by ELISA methods that use small, linear epitopes of the proteins but sometimes they show low sensitivities and specificities. To improve the immunologic method, other approaches have been advanced like radioimmunoprecipitation assays (RIPAs) with whole native proteins and sandwich ELISAs with full-length (Meschede et al., 1998). Today, HPV serology is performed mostly in a limited number of laboratories, but it is likely to become widely used in clinical laboratories in the post-HPV vaccination sera. Previous studies showed that the LUMINEX method constitutes an attractive method for HPV serology in high-throughput laboratories (Waterboer et al., 2006).

In the present report, viral antigens were expressed with pGEX vectors in *Escherichia coli* as double fusion proteins with N-terminal GST and a C-terminal peptide (tag) consisting of the 11 C-terminal amino acids from the large T antigen of simian virus 40. The protein concentrations of the cleared lysates have been determined using the Bradford-reagent and the characterization of the full-length recombinant proteins was verified by coomassie-stained SDS-PAGE. Furthermore, the titration of antigen lysates was done using mouse anti-tag antibodies. These antigens were then used in LUMINEX assay for antibody detection in Tunisian female population.

MATERIALS AND METHODS

HPV 16 L1

A modified pGEX vector was constructed for expression of GST fusion protein with an additional C-terminal fusion tag in *E. coli*. HPV 16 L1 coding sequence lacking the 10 N-terminal residues was amplified by polymerase chain reaction PCR with SmaI/SalI ends and inserted into pGEX4T3tag opened by EcoRI digestion. PCR primers:

HPV	16	L1	forward
5'GCAGTCCCCGGGGTCTACTTGCCTCCTGTCC3'			
HPV	16	L1	reverse
5'GCATGAGTCGACCAGCTTACGTTTTTTCGTTTAGC3'			

E. coli BL21 cells transformed with the pGEX plasmids were grown at room temperature in Luria Bertani medium containing 1 mM ampicillin. At an OD600 of 0.3 recombinant protein expression was induced by adding 0.25 mM isopropyl- β -D-thio-galactoside (IPTG) to the medium. The bacteria were harvested by centrifugation 15 h after induction. Pelleted bacteria were re-suspended in 40 mM Tris pH 8, 200 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid) and 2 mM DTT (dithiothreitol) supplemented with complete protease inhibitor cocktail and lysed using a high pressure homogenizer. ATP (adenosine triphosphate) and MgCl₂ were added to final concentration of 2 mM and 5 mM, respectively (Sehr et al., 2001).

HPV 16 E6 and E7

HPV type 16 E6 and 16 E7 coding sequence fused at its 3'-end in frame to a sequence encoding the terminal undecapeptide of the

SV40 large T-antigen (tag) is isolated from bluescript plasmid and inserted into a pGEX vector for expression as GST fusion protein in *E. coli*. These coding sequences for E6tag and E7tag were mobilized by digestion and ligated into digested plasmid downstream of the GST domain. Then, *E. coli* BL 21 cells transformed with the pGEX plasmids were grown and induction protein expression was induced as previously described for L1. The bacteria were harvested 6 h after induction by centrifugation. Pelleted bacteria were resuspended in PBS containing 2 mM DTT, 1% Triton X-100, and complete protease inhibitor cocktail and lysed with the high-pressure homogenizer. Lysates were then cleared by centrifugation and stored in aliquots at -20 °C (Sehr et al., 2002).

Titration of proteins

To determine the concentrations of different lysates the Bradford-reagent method as previously described was used. Optical density has been measured at 595 nm (Bradford, 1976). The separation of recombinant proteins has been done by migration on 12% polyacrylamide gel electrophoresis and bands were revealed by coomassie staining.

Titration of antigens

Lysates corresponding to GST L1tag and GST E6tag and GST E7tag were diluted in 1/3 steps, starting with a concentration of 2 μ g/ μ l and the detection of antigens was allowed by mouse anti-tag antibodies diluted to 1/4000. The optical density was then determined at 450 nm.

Luminex

Hardware and software Measurements were performed on a Luminex 100 Total System comprising the Luminex 100 analyzer, Luminex XYP plate handler, Luminex SD sheath fluid delivery system, a Pentium 4 personal computer (Dell) running Windows 2000 (Microsoft Corp.), and Luminex IS 2.2 SP1 software (Waterboer et al., 2005). Multiplex serology has been described in detail by Waterboer et al. (2005). With the Luminex analyzer, reporter fluorescence of the beads was determined and expressed as median fluorescence intensity (MFI).

Viral antigens were expressed with pGEX vectors in *E. coli* as double fusion proteins with N-terminal GST and a C-terminal peptide (tag) consisting of the 11 C-terminal amino acids from the large T antigen of simian virus 40. The expression constructs for E6, E7, and L1 of HPV types 16 as GST fusion proteins have been described (Sehr et al., 2001, 2002).

Bacterial lysate was diluted to 1 g/L in casein buffer (1 g/L casein in PBS, pH 7.4). For each antigen, GC beads were loaded with GST fusion proteins directly in the lysate and incubated for 1 h at room temperature in the dark on a shaker. The beads were then washed 3 times with 1 mL of casein buffer. Human sera from a case-control study divided into 70 controls and 71 cervical cancer cases. Ethics committee approval and informed consent of study participants for HPV serology were obtained. Sera were preincubated at a 1:50 dilution on a shaker for 1 h at room temperature in a serum preincubation buffer based on casein buffer and additionally containing 2 g/L lysate from bacteria expressing GST alone to block antibodies directed against residual bacterial proteins and GST.

Multiplex assay

Bead sets carrying different antigens were mixed and 50 μ L each of

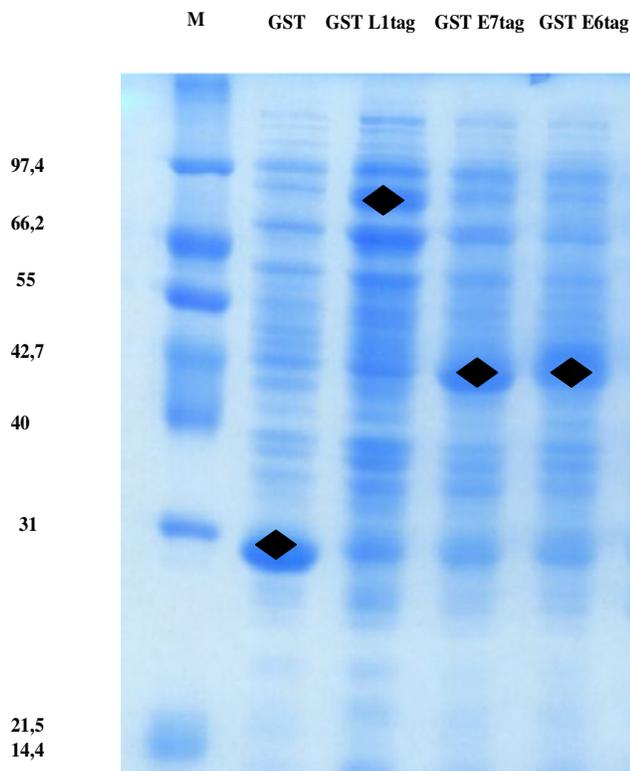


Figure 1. Separation of GST L1tag, GST E6tag and GST E7 tag by gel electrophoresis using the coomassie staining M: Molecular weight marker in Kda.

preincubated diluted serum and mixed beads were combined in 96-well plates with filter bottoms and incubated on a shaker for 1 h at room temperature in the dark. The beads were washed 3 times in 100 μ L of casein buffer on a vacuum manifold. Biotinylated secondary antibody (goat anti-human IgG diluted 1:1000 in casein buffer) was added and incubated as before. After washing, detection conjugate (streptavidin-R-phycoerythrin) diluted 1:1000 in casein buffer was incubated with the beads for 30 min. The beads were washed again, and the wells were filled with casein buffer. Reporter fluorescence of the beads was determined with the LUMINEX analyzer and expressed as median fluorescence intensity (MFI). To calculate antigen-specific reactivity, the MFI of GST tag was subtracted from the antigen MFI. This multiplex HPV serology method used *in situ* affinity-purified viral antigens developed yet for a conventional GST capture ELISA as previously described (Sehr et al., 2001).

RESULTS

Using the Bradford method, we have determined concentrations of the three protein lysates by the measurement of optical density at 595 nm. GST L1tag, GST E6tag and GST E7tag have the following concentrations: 16, 19 and 23.5 μ g/ μ l, respectively. The lysates were from bacteria over expressing GST L1tag (lane 3), GST E6tag (lane 5), GST E7tag (lane 4) and GST tag (lane 2) (Figure 1). Proteins were separated by gel electrophoresis and stained with coomassie. M. molecular weight marker with

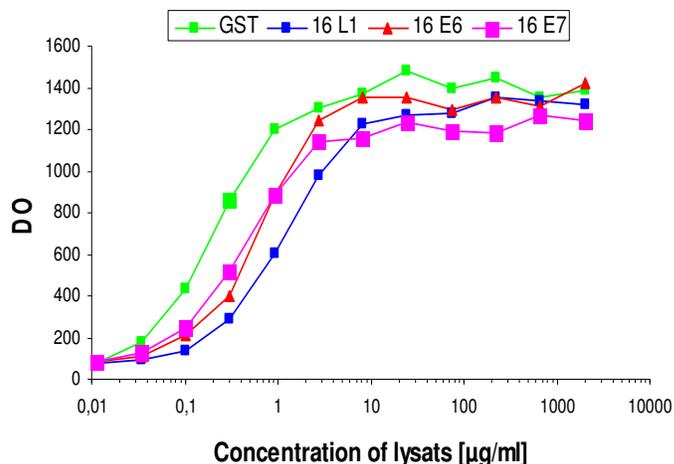


Figure 2. Titration of antigen lysates using mouse anti-tag antibodies.

molecular mass in kDa indicated in the left (lane 1). As shown in Figure 1, the different bands obtained correspond to the different recombinant proteins showing different apparent molecular weights. GST L1tag showed by migration in gel electrophoresis an 82 kDa molecular weight, and approximately 40-45 kDa for HPV 16 E7 and E6 GST fusion proteins respectively. However, the GSTtag showed a band of about 31 kDa.

For the determination of limit of detection of these proteins, different dilutions have been effectuated. As shown in Figure 2, and using lower concentrations, the best detection was obtained for GST alone, followed by E7 then E6 and L1 antigens.

Data from Figure 3 showed elevated percentages of sero-positivities in cervical cancer cases compared to controls toward the three antigens L1, E6 and E7 analysed using the LUMINEX assay: 21, 44 and 61% respectively and differences in results between cases and controls were significant ($p < 0.001$).

In addition, the technology LUMINEX allowed us to determine the intensity of the antibody response by analysing the MFI values determined for the different groups of patients towards the three antigens tested. Data showed that among cervical cancer cases, the distribution of the MFI values was different and it depends on the antigen type and the higher intensity of fluorescence was noted for the early antigens E6 and E7 compared to the late antigen L1. In fact, these values did not exceed 5609 units for L1 while for E6 and E7; higher MFI values reaching 13317 and 13235 were noted for E6 and E7 antigens respectively (Figures 4 and 5).

DISCUSSION

In the present work, we have produced three recombinant proteins for HPV 16 as GST L1tag, GST E6tag and GST E7tag in *E. coli*. After production, the verification of the size of these proteins by separation on gel

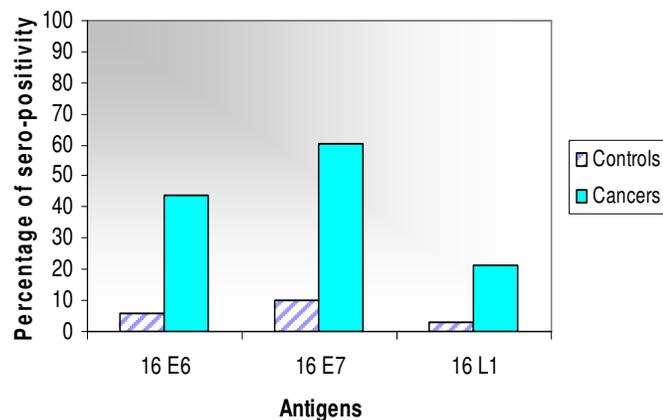


Figure 3. Sero-positivity toward L1, E6 and E7 HPV 16 antigens detected by LUMINEX for the different groups of patients.

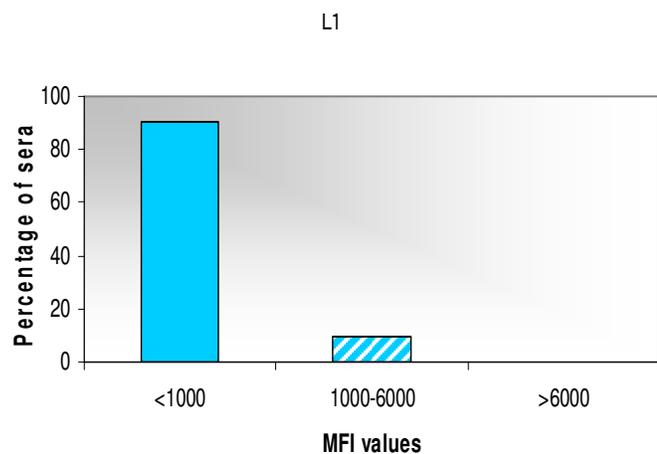


Figure 4. Distribution of cervical cancer cases upon their MFI values for L1 HPV 16 antigen.

electrophoresis and results obtained were in agreement with the literature (Sehr et al., 2001, 2002). Previous studies showed that different systems can be used for the production of viral expression for the HPV 16 (Biemelt et al., 2003). In fact, virus-like particles (VLP) have been expressed in various systems: mammalian cells, yeast, baculovirus, transgenic plants, *Semliki Forest Virus*, *Salmonella* as well as *E. coli* (Kimbauer et al., 1993; Hagensee et al., 1993; Sasagawa et al., 1995; Lyengar et al., 1996; Nepper et al., 1996). In fact, previous studies have expressed HPV 16 E7 proteins in *Schizosaccharomyces pombe* and they have used a protocol of purification (Braspenning et al., 1997).

Yeast such as *Sz. pombe* is a system that has several advantages (Yuko et al., 1999). In fact, it can be handled conveniently, low-cost synthetic medium is used, and several milligrams of recombinant proteins can be produced. Braspenning et al. (1997) have developed

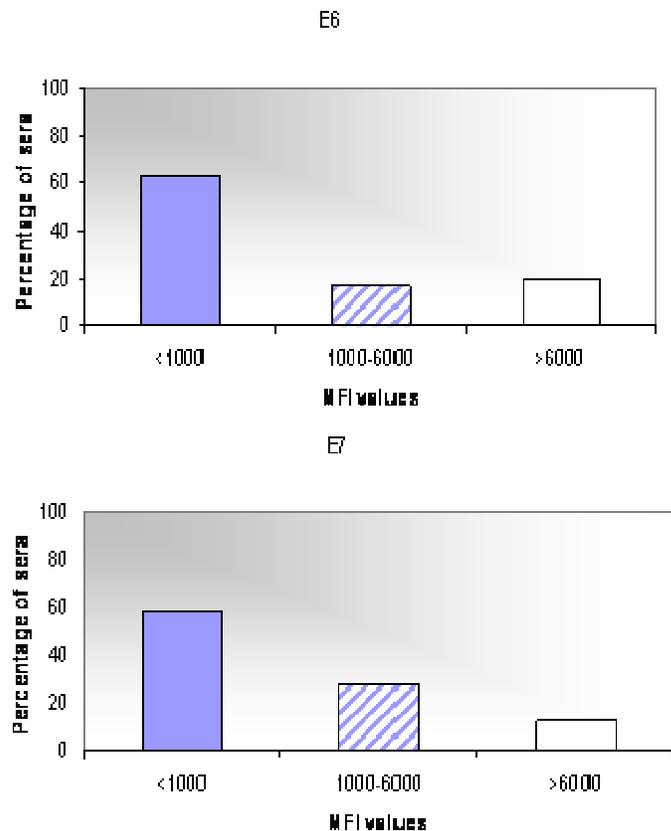


Figure 5. Distribution of cervical cancer cases upon their MFI values for E6 and E7 HPV 16 antigens.

purification protocol to obtain human papillomavirus HPV type 16 E7 proteins expressed in the yeast *Sz. pombe* by chromatography. In previous works, authors have expressed recombinant E6tag and E7tag proteins for HPV 16 and HPV 18 in *Sz. Pombe* (Meschede et al., 1998). The purified recombinant proteins were separated in silver-stained sodium dodecyl sulphate polyacrylamide gels. Production of antigens in plants is known to be safe and potentially very cost-effective alternative to traditional expression systems. HPV 16 L1 major capsid protein has been expressed in *Nicotiana tabacum* cv. Xanthi plants in order to produce prophylactic vaccines. This system is not easy to practise frequently (Varsani et al., 2003).

Compared to others, the *E. coli* system used in our work, has the advantage of the ease of antigen production and purification and provides large amounts of proteins which can be used for a wide range of studies including antigen and vaccine production; molecular immunology and structural biochemical and cell biology studies. Although a wide variety of *E. coli* host strains can be used for cloning and expression with the pGEX vectors, some strains as *E. coli* strain BL 21 maximize expression of full-length fusion proteins. This strain is defective in Omp T and Lon protease production and is the only strain able to express the fusion protein in a solu-

ble intact form. In addition, the glutathione-S-transferase (GST) gene fusion system provides an integrated system for the expression, purification and detection of glutathione-S-transferase fusion proteins using *E. coli* (Saluta and Bell, 1998).

More recently, researchers have reported the transient expression mediated by a potato virus X derived vector of the E7 protein targeted to the secretory system of *Nicotiana benthamiana* (Franconi et al., 2006).

In addition, production of recombinant proteins L1, E6 as well as E7 and their stability have been verified. Higher detection was obtained for GST and at the same concentration, the detection of E6 and E7 is higher than L1. The differences noted concerning the production as well as the capacity of detection with the mouse anti-tag antibodies of the different recombinant proteins GST L1tag, GST E6tag and GST E7tag may be explained by the fact that L1 protein has relatively high molecular weight that is why it needs more steps of purification for the liberation of the bacterial proteins that can grow with the recombinant proteins. This is in agreement with the work of Waterboer et al. (2005).

These protein antigens prepared with the concentrations of 16, 19 and 23.5 µg/µl for L1, E6 and E7, respectively, have been used in LUMINEX assay for antibody detection in Tunisian female human sera. This multiplex system enables antibody analyses of large numbers of sera against the different antigens in parallel and has the potential to replace ELISA technology. Consequently, this method which allows the simultaneous analysis of large numbers of serum samples for antibodies against multiple viral antigens would be useful for sero-epidemiologic studies on prevalence and disease association of human papillomaviruses (HPVs).

The literature has reported that HPV antibodies are type specific. Those targeting the major viral capsid protein L1 are markers of infection and those targeting the viral oncoproteins E6 and E7 are markers for HPV-associated cancer (Dillner, 2005; Waterboer et al., 2005). Data reported in the present work showed that the intensity of the antibody response was important for the early antigens E6 and E7 compared to L1 antigen and this may be explained by differences in characteristics of these proteins. The antibody response to HPV is, in general, type specific, and HPV serology is an important technology for determining the spread of type-specific HPV infections in populations and monitoring of the effect of HPV vaccines in inducing protective antibodies. The literature has shown that the antibodies against the major HPV capsid protein, L1, are induced after infection and usually stay detectable for many years after clearance of the infection, since they belong to the class of antibodies that mark past exposure to an infection. The concentrations of these antibodies correlate well with protection, and it is for L1 antibodies that there is an urgent need for efficient, standardized HPV serologic methods for use in vaccination implementation/evaluation efforts and for epidemiologic monitoring of the type-specific spread of

HPV infections (Dillner, 2005).

In the present work, uses of the Luminex improved the sensitivity of antibody response and can replace conventional serologic methods such as ELISA which allows the analysis of sera for antibodies to only 1 antigen per well. However, this method for multiplex HPV serologic analysis combines the fluorescent bead array with a generic method allowing *in situ* affinity purification of any glutathione S-transferase (GST) fusion protein developed for conventional ELISA.

High-density planar arrays allow the analysis of very large numbers of targets in a reasonable time frame at acceptable costs.

As a conclusion, production of recombinant proteins in *E. coli* enabled us to obtain recombinant proteins of HPV types as well as many other proteins relatively easy and in large quantities. Increased sensitivity and low imprecision of the Luminex-based method and the possibility for easy combination of different antibody assays lead to renewed interest in the use of these antibodies in predictive oncology. Furthermore, using the LUMINEX technology and in countries lacking a cervical screening programme as our country we are able to investigate the feasibility of the serology analysis for sero-epidemiologic study or for vaccination purpose in Tunisian female population by parallel testing for antibodies against three antigens (E6, E7, and L1 proteins of HPV 16 type).

ACKNOWLEDGEMENTS

We would like to thank Dr. Pawlita, M., Dr. Sehr, P. and Dr Waterboer, T. from the DKFZ Heidelberg Germany for providing all the products and materials necessary for the production of viral antigens and for the LUMINEX application.

REFERENCES

- Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Muller M (2003). Production of Human Papillomavirus Type 16 Virus-Like Particles in Transgenic Plants. *J. Virol.* 77: 9211-9220.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Braspenning J, Manetti R, Zumbach K, Meschede W, Gissman L, Tommasino M (1997). A General Purification Protocol for E7 Proteins from "High-and Low-Risk" Human Papillomavirus Types Expressed in the Yeast *Schizosaccharomyces pombe*. *Prot. Expr. Purif.* 10: 192-201.
- Castle PE, Giuliano AR (2003). Genital tract infections, cervical inflammation, and antioxidant nutrients – assessing their roles as human papillomavirus cofactors. *J. Natl. Cancer. Inst. Monogr.* 31: 29-34.
- Dillner J, Brown DR (2004). Can genital-tract human Papillomavirus infection and cervical cancer be prevented with a vaccine? *Exp. Rev. Mol. Med.* 9: 1-21.
- Dillner J (2005). Toward "Serolomics": Papillomavirus Serology Is Taking a Technologic Lead in High- Throughput Multiplexed Antibody Analysis. *Clin. Chem.* 51: 1768-1769.
- Dillner J, Arbyn M, Dillner L (2007). Translational Mini-Review Series on Vaccines: Monitoring of human papillomavirus vaccination. *Clin. Exp.*

- Immunol. 148: 199-207.
- Franconi R, Massa S, Illiano E, Mullar A, Cirilli A, Accardi L, Di Bonito P, Giorgi C, Venuti A (2006). Exploiting the plant secretory pathway to improve the anticancer activity of a plant derived HPV 16 E7 vaccine. *Int. J. Immunopathol. Pharmacol.* 19: 187-197.
- Hagensee ME, Yaegashi N, Galloway Dan (1993). Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by co-expression of the L1 and L2 capsid proteins. *J. Virol.* 67: 315-322.
- Lyengar S, Shah KV, Kotloff KL, Ghim SJ, Viscidi RP (1996). Self-assembly of in vitro-translated human papillomavirus type 16 L1 capsid protein into virus-like particles and antigenic reactivity of the protein. *Clin. Diagn. Lab. Immunol.* 3: 733-739.
- Kirnbauer R, Taub J, Greenstone H, Roden R, Durst M, Gissmann L, Lowy DR, Schiller JT (1993). Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J. Virol.* 67: 6929-6936.
- Meschede W, Zumbach K, Braspenning J, Scheffner M, Bribiesca LB, Luande J, Gissman L, Pawlita M (1998). Antibodies against early proteins of human papillomaviruses as diagnostic markers for invasive cervical cancer. *J. Clin. Microbiol.* 36: 475-480.
- Neeper MP, Hofmann KJ, Jansen KU (1996). Expression of the major capsid protein of human papillomavirus type 11 in *Saccharomyces cerevisiae*. *Gene.* 18: 1-6.
- Saluta M, Bell PA (1998). Troubleshooting GST fusion protein expression in *E. coli*. *Life Sci News.* 1: 1-3.
- Sasagawa T, Pushko P, Steers G, Gschmeissner SE, Hajibagheri MA, Finch J, Crawford L, Tommasino M (1995). Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology.* 206:126-135.
- Sehr P, Zumbach K, Pawlita M (2001). A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: Validation for HPV serology. *J. Immunol. Methods* 253: 153-162.
- Sehr P, Muller M, Hopfl R, Widschwendter A, Pawlita M (2002). HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase. *J. Virol. Methods.* 106: 61-70.
- Sue JG, Jane JK, Katie K, Jeremy DG, Joshua S, Meredith KH O'Shea, F. Xavier B, Silvia de Sanjos'e c, Eduardo LF (2007). Cost-effectiveness of HPV 16, 18 vaccination in Brazil. *Vaccine.* 25: 6257-6270.
- Varsani A, Williamson AL, Rose RC, Jaffer M, Ribicki EP (2003). Expression of Human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. *Arch. Virol.* 148: 1771-1786.
- Waterboer T, Sehr P, Pawlita M (2006). Suppression of non-specific binding in serological Luminex assays. *J. Immunol. Methods* 309: 200-204.
- Waterboer T, Sehr P, Michael KM, Franceschi S, Nieland JD, Joos TO, Templin MF, Pawlita M (2005). Multiplex human papillomavirus serology based on in situ-purified glutathione S-transferase fusion proteins. *Clin. Chem.* 51: 1845-1853.
- Yuko GH, Hiromichi K (1999). Expression system for foreign genes using the fission yeast *Schizosaccharomyces pombe*. *Biotechnol. Appl. Biochem.* 30: 235-244.