New molecular diagnostic approach of human papillomavirus detection: the role of experimental design in the development, analytical and clinical evaluation

Doctoral (Ph.D.) thesis

Csaba Jeney, MD

Semmelweis University, School of Ph.D. Studies

Pathology Interdisciplinary Doctoral School

Program 8/3: Studies on the effects of microorganisms and their products at molecular, cellular and organism levels

Program/project leader: Prof. Ferenc Rozgonyi, MD, PhD, DSc

Opponents: Judit Deák, PhD, István Sziller, PhD

Chairman: Prof. Zoltán Papp, MD, PhD, DSc
Memebers: Prof. Erzsébet Nagy, MD, PhD, DSc
Prof. Ilona Kovalszky, MD, PhD, DSc

Budapest, 2008

Table of contents

Abbreviations

% V(e) - degrees of contribution calculated on the basis of the variance of pooled error

% Ve - degrees of contribution calculated on the basis of variance of error factor factor e

ANOVA – analysis of variance

AP - alkaline phosphatase

ASCUS - atypical squamous cells of undetermined significance

BSA – bovine serum albumin

CF - correction factor

CIN - cervical intraepithelial neoplasia

CIN2+ - cervical intraepithelial neoplasia grade 2 or above

CNI - copy number imbalance

DNA – dezoxynucleic acid

dNTP – dezoxynucleoitide triphosphates

dsDNA – double stranded DNA

ED50 - dose causing a 50% maximal response

EDTA-Na2 – dinatrium ethylenediamine tetraacetic acid

ELISA – enzyme linked immonosorbent assay

F - degrees of freedom

F - F-test value

FDA – Federal

GUSCN – guanidinium thiocyanate

HCII, HC2 - Hybrid Capture II

HG-SIL - high grade SIL

HPLC – high pressure liquid chromatography

HPPA - hydroxiphenyl-propionic acid

HPV – human papillomavirus

HR – high risk

HRPO - horse-radish peroxidase

LBC – liquid based cytology

LG-SIL - low grade SIL

LiPA - line probe assay

LR – low risk

NCBI – National Center for Biotechnology Information

OPD – ortho-phenylenediamine

ORF - open reading frame

Pap – Papanicolaou

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PEG – polyethylene glycol

PID - pelvic inflammatory disease

RFLP - restriction fragment length polymorphism

RNA – ribonucleic acid

S - variation term

Sa, Sb, etc. - variation terms of the factors A, B, etc.

ScFv – single chain .fragment of variable

SigLimit% - significance limit

SIL - squamous intraepithelial lesion

SSC – sodium chloride sodium citrate

St - total variation

STD - sexually transmitted disease

TRIS – trishydroxymethylaminomethane

U.S. Pat. No. – United States Patent Number

URR - upper regulatory region

V - variance

Introduction

Experimental design is widely used in science, however some special implementation would greatly help research in that particular area. The original goal of the Taguchi method was to find the optimum conditions and to estimate its numerical parameters (Taguchi, 1993). The method has been very effective in the determination of the optimum conditions even in the presence of multifactorial interactions. However, the Taguchi system contains much more information because it could calculate the numerical parameters not only for the optimum conditions but for any combinations of the factors. We used this method to determine factors that affect the results to the greatest extent or, on the contrary, to determine which factor's effect is negligible. Following this process we could omit or include protocol steps, or we could use an advantageous condition which is not the best choice but only slightly affects the result. Moreover, on this basis the recalculation of data it possible to tailor the assay to the given circumstances and evaluate the analytical behavior of the assay.

Another advantage of the method is that the optimization and the determination of the analytical parameters of the assay can be carried out in one step. Furthermore, the recalculation of these parameters can be carried out, which is impossible in the traditional methods. The analysis of variance used here makes the predictions more robust because the calculations are based on all the experiments conducted during the optimization. The method accepts qualitative conditions which permit the incorporation of all the circumstances important in an ELISA procedure. Moreover, the calculated weighting of the factors make realistic assumptions about the system so it is possible to control the critical conditions. The developed method was applied generally to determine the multiparametric optimization of different analytical systems, e.g. multiplex PCR methods to detect human papillomaviruses during the later stages of the work, which was exemplified by the optimization of the primer concentrations in a highly multiplex PCR reaction.

Considering the role of HPVs in malignant lesions (zur Hausen et al., 1975), the accurate and high throughput detection and genotyping of HPVs is of great importance. The

diversity of the HPV spectrum and the high incidence of multiple infections make it necessary to establish reliable methods for identification of the various HPV genotypes, not only for epidemiologic studies, but also for patient management.

DNA based methods (PCR and hybridization) are widely used to detect of HPV infections. Besides being the most sensitive diagnostic methods, these supply important genotyping information for counseling, selection of therapy, follow-up and vaccine monitoring.

The hybrid capture technique, the most frequently used diagnostic method, is based on the formation of RNA-DNA hybrids between HPV DNA and the complementary unlabelled HPV RNA probes. Positivity or negativity is based on comparison to a standard positive reference (Peyton et al., 1998). However, as a consequence of crosshybridizations , false positive reactions were described (Vernon et al., 2000).

There are several detection assays using amplification methods, which differ in the selected amplified genome segment, number of primers, and the applied detection technique. The most frequently used primer sets are the GP5+/6+, MY9/MY11, PGMY, SPF, and there are different type-specific PCR reactions as well.

The GP5+/6+ (de Roda Husman et al., 1995) primer set consists of a fixed nucleotide sequence for each primer and detects a wide range of HPV types by using a lowered annealing temperature during PCR, but the system shows difficulties to detect multiple infections (Qu et al., 1997). However the combination of GP5+/6+ PCR with reverse line blot analyses was successful in detecting multiple HPV infections as well as single infections, in case of 37 mucosotropic HPV types (van den Brule et al., 2002).

The MY9/MY11 primer system is considered to be the reference reaction in this field. However its major drawback is the difficulty to control the degenerated synthesis of the primers, e.g. the relative ratio of the primer species is varying from synthesis to synthesis (Coutlee et al., 2002), resulting unpredictable changes in the analytical behavior of the PCR reaction. It is important to note that this reaction can amplify the fewest types, compared to the other reactions (Gravitt et al., 1998). A related method, the PGMY09/PGMY11 system (Gravitt et al., 2000) is improved by using definitive primer mixes. Although an increase in sensitivity by the new PGMY primers is achieved, the high stringency line blot using the

original MY09/11 probes is still unlikely to detect the presence of sequence variants (Stewart et al., 1996) of HPV 45, HPV 52 and HPV 58.

Several authors tried to combine the latter two approaches in order to obtain a better HPV detection system. Recently Fuessel Haws et al. (Fuessel Haws et al., 2004) created a nested PCR system combining the PGMY and GP5+/6+ primer sets claiming a more sensitive system, but at the expense of double amplification.

The SPF system has been described as a very sensitive HPV detection system (Kleter et al., 1999; Kleter et al., 1998) featuring a very short amplicon length (65 bp). However the diversity of the 22 bp interprimer region does not allow adequately specific and robust discrimination of different HPV genotypes. The amplicons present high similarity, there are genotypes which differ in only one bp from the other (HPV 31-33 having 1 bp difference), or even showing no difference at all (HPV types 68-73). SPF was the first method to address genus specific detection, however due to the limited amplicon length, the same interprimer region is used for probe binding.

Another method described by Yoshikawa et al. (Yoshikawa et al., 1991) employs an L1–based PCR system consisting of two consensus primers and a restriction fragment length polymorphism analyses (RFLP). This PCR system is not sensitive enough for many clinically important genotypes (there are even important genotypes which are not amplified or amplified at a very low sensitivity, e.g. HPV 16), it is time-consuming, and not a feasible method for HPV genotyping high number of clinical samples. A new method was devised to address the unmet requirements of the clinical detection of human papillomavirus.

To evaluate the performance of this new HPV detection method clinical studies and comparisons were carried out.

Human papillomavirus and its significance

According to the World Health Organization (WHO), cervical cancer is the second most common cause of cancer death in women. The presence of HPV infection has been implicated in more than 99% of cervical cancers worldwide. As estimated, more than

500,000 women worldwide develop cervical cancer in every year, and more than 273,000 of the cases are fatal. Even with Pap screening programs, a significant number of women die from cervical cancer each year. In Hungary the incidence of low-risk (LR), high-risk (HR) and double infections were 2.6%, 30.8% and 1.7% respectively using HCII test (Sapy et al., 2007), but the overall rate varied with geographical regions (Kornya et al., 2002)

HPV infection is the most frequent sexually transmitted disease (STD) worldwide, and up to 75% of sexually active women will be infected by HPV in the genital tract once in their lifetime (Cox, 2006). Irrespective of HPV infection status, fewer than 1 in 10,000 women will develop invasive cervical cancer. The fact that most HPV-infected women do not develop cytological anomalies or cancer underlines the importance of factors modulating the progression of cervical disease to cancer in HPV-infected women. These factors may include the HPV genotype and molecular variant, the HPV viral load, persistence of HPV infection, co-infection with other STD agents, the immune status of the host and environmental factors such as smoking (Castellsague and Munoz, 2003). In Hungarian population similar factors were verified in the background of having cervical lesions at higher risk (Nyari et al., 2001)

Papillomaviruses are small DNA viruses that infect mammalian epithelial cells, causing epithelial proliferative lesions which may be benign, e.g., fibropapillomas (warts), or which may be malignant. All papillomaviruses are similar in that the genome size, organization, open reading frames, and protein functions are shared. Many, but not all, genome regions are conserved among the various papillomaviruses.

Because of the close association between the papillomavirus life cycle and the differentiation state of the host cell, the details of the papillomavirus life cycle have not been completely elucidated. It is known that papillomaviruses infect host epithelial basal cells, where the viral genomes become established and are maintained as low copy-number episomes that replicate in coordination with host cell replication. As the infected cells

differentiate into keratinocytes, viral DNA is amplified, the late genes are induced, and vegetative replication of the papillomavirus follows.

Papillomaviruses infect a wide variety of animals, including humans. The human papillomaviruses (HPV) (including Papillomaviridae family, Alpha-, Beta-, Gamma-, Delta-, Mupapillomavirus and unclassified Papillomaviridae genera) are common causes of sexually transmitted disease. Several types of HPV have been identified by DNA sequence data, and 96 HPV genotypes have been fully sequenced to date. Genotyping of HPV is based on DNA sequences of the L1, E6, and E7 genes. A 10% difference in sequence with respect to previously established strains is sufficient to define a new type of virus. The heterogeneity of the human papillomavirus group is generally described (deVilliers, 1989, J. Virology 63:4898-4903).

HPVs are DNA tumour viruses whose genome is organized in three regions: the early gene (E1 to E7), the late gene (L1 and L2) regions and the upper regulatory region (URR) or long control region (LCR). The URR possesses binding sites for many repressors and activators of transcription, suggesting that it may play a part in determining the range of hosts for specific HPV types. E1 and E2, meanwhile, encode proteins that are vital for extrachromosomal DNA replication and the completion of the viral life cycle. The E2 encodes two proteins: one, which inhibits transcription of the early region; and the other, which increases the transcription of the early region. A hallmark of HPV-associated cervical carcinoma is loss of the expression of the viral E2 proteins. Recently a new E2 protein, consisting of the product of the small E8 ORF with the part of the E2 protein, was described. This protein able to repress both viral replication and transcription, and is therefore believed to have a major role in viral latency.

The E4 protein is expressed in the later stages of infection when complete virions are being assembled, and is not known to have transforming properties; however it is considered to play an important role for the maturation and replication of the virus. The E4 protein also

induces the collapse of the cytoplasmic cytokeratin network in human keratinocytes, a situation which may assist the release of virions from the infected cell.

The E5 open reading frame (ORF), meanwhile, is often deleted in cervical carcinoma cells, indicating that it might not be essential in maintaining the malignant transformation of the host cell. When present, E5 interacts with various transmembrane proteins like the receptors of the epidermal growth factor, platelet-derived growth factor β, and colony stimulating factor. A study using HPV 16-infected cells found the E5 protein to possess weak transforming activity.

In carcinogenesis, the E6 and E7 ORF are considered to play the most major roles. These two units encode for oncoproteins that allow replication of the virus and the immortalization and transformation of the cell that hosts the HPV DNA.

The late region units, L1 and L2 encode viral capsid proteins during the late stages of virion assembly. The protein encoded by L1 is highly conserved among different papilloma virus species. The minor capsid protein encoded by L2 has more sequence variations than that of the L1 protein.

HPV can infect the basal epithelial cells of the skin or inner tissue linings, and are, accordingly, categorized as either cutaneous or mucosal (anogenital) type.

The HPV DNA is usually extrachromosomal or episomal in benign cervical precursor lesions (Stubenrauch and Laimins, 1999). However, in many cervical cancer cells as well as in cervical cancer cell lines and HPV-transformed human keratinocytes in vitro, the HPV DNA is integrated in the host genome.

Cancer tissues may contain both episomal and integrated HPV DNAs at the same time, although integration appears to occur more frequently in HPV 18-associated cervical cancer than in HPV 16-associated cervical cancer (Hudelist et al., 2004). Integrated HPV 16 is

present in some premalignant lesions but is not always present in carcinomas. During HPV DNA integration, the viral genome usually breaks in the E1/E2 region. The break usually leads to the loss of the E1 and E2 regions. The loss of E2, which encodes proteins including one that inhibits the transcription of the E6 and E7 regions, has been known to result in uncontrolled and increased expression of E6 and E7 oncogenic proteins. Increased expression of E6 and E7, meanwhile, has been observed to lead to the malignant transformation of the host cells and to tumour formation (Stanley, 2001). HPV viral integration into the host genomic DNA is associated with progression from polyclonal to monoclonal status in cervical intraepithelial neoplasia (CIN), and these events play a fundamental role in the progression from low-grade to high-grade cervical neoplasia (Ueda et al., 2003).

Patterns of DNA copy number imbalance (CNI) are characteristic of cervical squamous intraepithelial lesion (SIL) grade, human papillomavirus (HPV) status and postoperative recurrence (Alazawi et al., 2004). While some CNIs were seen at similar frequencies in HG-SIL (high grade SIL) and LG-SIL (low grade SIL), others, including gain on 1q, 3q and 16q, were found frequently in HG-SIL but not in LG-SIL. There were significantly more CNIs per case in HG-SILs showing loss of the HPV16 E2 gene and in HG-SILs that subsequently recurred. The data are consistent with sequential acquisition of CNIs in cervical SIL progression. Higher frequency of CNI in association with E2 gene loss supports in vitro evidence that high-risk HPV integration is associated with genomic instability.

Based on the available molecular, clinical and epidemiologic data, a subset of HPVs is unequivocally the etiologic agents for cervical cancers and their precursors. HPVs have been detected in about 90% of cervical adenocarcinomas and squamous cell carcinomas. The majority of HPV infections clear spontaneously, but persistent infection with HPV DNA has been found in metastases arising from cervical tumours. Nevertheless known high-risk (or oncogenic) HPV types are a significant risk factor for cervical cancer and are increasingly recognized for their role in other cancers. Virtually all cervical cancers (99%)

contain the genes of high-risk HPVs, most commonly types 16, 18, 31, and 45. Other high-risk types include types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73. HPVs 31, 33, 35, 51 and 52 are sometimes regarded as “intermediate risks” because they are more common in mild or severe dysplastic lesions than in carcinomas (Bosch and Munoz, 2002). Among the high-risk strains, HPV 16 and 18 are the most closely associated with cervical carcinoma. The HPV16 DNA has been found in more than 50% of squamous cell carcinomas, while the HPV18 DNA has been found in more than 50% of adenocarcinomas. However, the great majority of anogenital HPVs have oncogenic potential (Wiley and Masongsong, 2006). The risk of HPV16/18 genotypes is signified (Hernadi et al., 2006)

HPV's interaction with host cells has two principal biological consequences:
a) All anogenital HPVs induce low grade squamous lesions, which are the morphologic correlate of a productive infection and the immortalisation phenotypes exerted by normal E6, E7 expression. The immortalisation is an inherent strategy of papillomaviruses to mobilize resources for the DNA replication and produce new progeny.
b) Rarely, HPVs induce a proliferative epithelial phenotype recognized as a high grade lesion and that is the proximate cytohistologic precursor of invasive cervical carcinoma, which might involve uncontrolled E6, E7 expression.

To date the clinical diseases, which are associated with HPV infections and the potential field of applications of HPV detection and typing methods include condyloma acuminatum, lichen sclerosus, squamosus cell hyperplasia, vulvar intraepithelial neoplasia, squamosus cell carcinoma, cervical intraepithelial neoplasia, cervical carcinoma, adenocarcinoma of the cervix, anal intraepithelial neoplasia, penile intraepithelial neoplasia, adenocarcinoma of the larynx, recurrent respiratory papillomatosis, and epidermodysplasias verruciformis. Recent evidence suggests that HPV may play a role in the development of prostate cancer in men as well.

Cervical cancer precursor lesions (intraepithelial lesions) or cytological abnormalities are tested using Papanicolaou Stain, known as the Pap Smear after the inventor George

Papanicolaou. The technique involves smearing cervical scrapes on a glass slide, and staining the cells obtained from the ano-genital tract with hematoxylin, a nuclear stain. The Pap smear, however, has a lack of repeatability and it is not sufficiently predictive of impending HPV-induced neoplasias. It has been shown that 25% of patients with advanced in situ carcinoma may present normal Pap smears a few years before diagnosis or the last negative cytology was uniformly positive in cervical cancer cases on re-examination (Martin-Hirsch et al., 2002). An increasingly prevalent problem is the occurrence of invasive cancer within 3 years of a negative Pap smear.

The current acceptable rate of false negatives (i.e., women who do have dysplasia according to an expert panel of pathologists looking at tissue biopsies rather than smear samples, but are not diagnosed that way during the routine smear screening) is roughly 5-10% but recent studies suggest that the actual rate may be much higher. Furthermore, in approximately 7-8% of cases, the Pap smear demonstrates atypical squamous cells of undetermined significance (ASCUS). In an additional 20-30% of cases, the Pap smear may be insufficient for interpretation due to the presence of inflammatory cells (Schneider et al., 2001). In the case of the cervix, flat warts (visualised by colposcopy) are suspected premalignant lesions. Histopathological progression of flat warts to carcinoma in situ and cervical cancer has been well described.

Intraepithelial lesions are common early events among women with incident HPV infection, and the interval between incident HPV-16 or HPV-18 infection and biopsy-confirmed CIN grade 2-3 appears to be relatively short (Duggan, 2002). However studies have demonstrated that infection with high-risk HPV types is usually transient (Snijders et al., 2006). Persistence of HPV infection substantially increases the risk of progression to high grade intraepithelial lesions and invasive disease.

The progression of the disease is variable and it is associated with the loss or persistence of HPV. Significant numbers of dysplastic lesions regress spontaneously, others fail to progress, while a few progress rapidly.

As a consequence of the preferential role of high-risk genotypes in cervical cancer and because of the different, consequential and characteristic type patterns for the other pathological conditions, both identification and typing of HPV is highly important. Additionally different types of high-risk HPV pose different risks to the affected individuals. For instance, HPV16 and HPV18 have been more consistently identified in higher grades of cervical dysplasia and carcinoma than other HPV types HPV16 is also more prevalent in squamosus carcinoma cases, and HPV18 is more prevalent in adenocarcinoma cases.

HPV diagnostics

From 1980, several viral genomes have been cloned and used as type-specific probes in the diagnosis of HPVs. Filter hybridization techniques have been used to detect HPV DNA in cervical scrapes collected in parallel with samples for routine cytology. HPV DNA probes have been used in different hybridization-based assays such as Southern and hybrid Dot/Southern assays to detect HPV DNA in clinically-derived tissue samples. Additionally, purified biopsy DNA and in situ hybridizations in preserved tissue specimens, that is, direct localization within the intact cell of those sequences complementary to the nucleic acid probes have been demonstrated (Warner and McCance, 1989) (Kiss et al., 1993; Orosz et al., 1994).

A method for detecting HPV DNA types that utilizes a reverse-blotting procedure has been reported. The procedure involved forming a membrane to which genomic DNA from different HPV types was bound and then hybridizing labeled DNA from a biological sample to the DNA bound to the membrane.

Numerous methods have been developed to detect human papillomavirus types using type-specific reaction, detecting one HPV type at a time. The Polymerase Chain reaction (PCR)

has been used to amplify and detect the presence of HPV16 and HPV18 DNA , in particular to detect HPV16 in oral and cervical biopsies. A mixture of primers has been described for the specific amplification by PCR of HPV sequences in types 1a, 5, 6a, 8, 11, 16, 18, and 33. U.S. Pat. Nos. 4,683,195 and 4,683,202 disclose PCR and the use of PCR to detect the presence or absence of nucleic acid sequence in a sample. However numerous publications target the detection of HPV in clinical samples, providing the PCR approach as de facto gold standard of the field (Denny and Wright, 2005). However due to the number and variability of the HPV genomic sequences, these techniques evolved continuously providing better and better approaches, which fulfills the clinical needs (Nijhuis et al., 2006).

U.S. Pat. No. 5,447,839, discloses a method for detection and typing of HPV. In this method, HPV DNA sequences in a sample are amplified by PCR using consensus primers which amplify both oncogenic and non-oncogenic HPV types. Thus, the presence of HPV in the sample is indicated by the formation of amplification products. HPV is then typed using type-specific DNA probes which hybridize with the amplified region of DNA. The type-specific hybridization probes disclosed in this patent are capable of identifying and distinguishing among five known oncogenic types of HPV, namely HPV-6, HPV-11, HPV-16, HPV-18 and HPV-33.

A variety of methods for detecting high-risk types of HPV have been devised (Hubbard, 2003). Many of these rely on the detection of unique sequences in the HPV genome. For example, DNA or RNA probes complementary to a portion of the genes of a particular high risk HPV strain have been reported, as useful in screening for the presence of a particular strain of high-risk HPV in patient samples (U.S. Pat. No. 4,849,332, incorporated herein by reference). U.S. Pat. No. 5,705,627., reports use of PCR to amplify and detect HPV DNA using degenerate or mixed consensus primers, followed by typing using a mixture of genotype-specific DNA probes. Other examples of using consensus primers can be found in U.S. Pat. No. 5,364,758, and (Kleter et al., 1998).

There is a commercial method available, which is based on hybridisation and signal amplification. (Hybrid Capture II, Digene Corp.) However, this method reportedly has specificity problems due to the high sequence homology of some part of the HPV genomes (Carozzi et al., 2007) (Konya et al., 2000) Currently the Hybrid Capture 2 test (HC2, Digene Corporation Inc, Gaitersburg, Md) is the only HPV test to have approval from the FDA for in-vitro diagnostic use. However, at present it is only approved for use as an adjunct to Pap testing in cervical cancer screening rather than as a stand-alone screening test. LBC samples can be used in the HC2 system which provides a positive or negative test result at a threshold of approximately 1pg/ml of viral DNA. This assay employs HPV genotype specific RNA probes, which bind to DNA targets. Hybrids, consisting of target HPV bound to RNA probes, are bound or "captured" by antibodies recognizing RNA-DNA hybrids. Addition of a second antibody tagged with alkaline phosphatase allows detection of the bound hybrid using a chemiluminescence detection system. Within the HC2 system, genotype specific HPV probes are mixed to form low-risk (6, 11, 42, 43, and 44) and high/intermediate-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) HPV cocktails. The HC2 system provides an-easy-to-use method, allowing triage of patients with HPV infection into low and high risk groups for the development of cervical dysplasia (Hubbard, 2003). However, the HC2 test has a number of limitations including the inability to identify specific HPV types or mixed HPV infections. In addition, incorporation of HPV DNA testing into primary screening remains controversial. This is because, while HPV testing shows high sensitivity in the detection of high grade CIN, it has low specificity (Snijders et al., 2003).

In order for high-grade cervical lesions and cervical cancer to develop, persistent activity of HPV E6 and E7 oncogenes is necessary. Therefore, testing for HPV oncogenic activity rather than simply the presence of HPV DNA may be a more relevant clinical indicator of disease. Detection of HPV E6/E7 messenger RNA (mRNA) is an indicator of HPV oncogenic activity and may be used as a clinically predictive marker to identify women at risk of developing high-grade cervical dysplastic lesions and cervical carcinoma. HPV mRNA can be detected using the PreTect HPV-Proofer Kit (NorChip AS, Norway). The

HPV-Proofer Kit is a molecular test kit based on Real-Time NASBA (nucleic acid sequence based hybridisation) technology, which detects the presence of high-risk HPV types 16, 18, 31, 33 and 45. Initial studies comparing HPV DNA and RNA detection (using consensus PCR and PreTect HPV-Proofer), in women with ASCUS and mild dysplasia on cervical cytology, showed that twice as many women were positive for HPV DNA compared to those with HPV RNA positivity. They also demonstrated that while both tests were highly sensitive for detection of CIN2+ (85.7%), HPV RNA detection was far more specific for detection of high grade lesions (84.9% vs 50%). PreTect HPV-Proofer positive women were 69.8 times more likely to be diagnosed with CIN2+ within 2 years of testing than PreTect HPV-Proofer negative women (Molden et al., 2005). Thus, the addition of HPV mRNA triage to cervical screening programmes may decrease the incidence of false negative results while also allowing the time interval between screening events to be increased for those women who are HPV DNA and RNA negative.

The amplification based methods consist of a part responsible for sensitivity (amplification), which is separated from those parts responsible for specificity (detection by hybridisation). These techniques differ in the amplified genome section, the number of primers and the techniques of detection. The most often used amplification methods are GP5+-GP6+ (general primer - GP) (Jacobs et al., 1995), MY9-MY11 (Gregoire et al., 1989), PGMY (Coutlee et al., 2002), SPF (Kleter et al., 1998), L1C (Yoshikawa et al., 1991) and the type specific PCR reactions (Gravitt and Manos, 1992). The most often used detection techniques are sequence specific hybridization, restriction fragment length polymorphism (RFLP) and line probe assay (LiPA). Sometimes, but rarely, sequencing or other methods are applied. The analytical properties of the amplifications vary within a wide range and are characterised by the number genotypes, which can be amplified, the analytical sensitivity, and specificity of the amplification/detection of genotypes and also by the differences of sensitivities between genotypes.

Cervical screening strategies

Cytological screening has greatly reduced the incidence of invasive cervical cancer in many industrialized nations (Davey, 2003). State-of-the-art cervical cancer prevention is costly, however, and includes cytological screening at repeat intervals, confirmation of abnormalities by colposcopic biopsy, and treatment of precancerous lesions. In resource-limited settings, accessibility to prevention programs for cervical cancer is often poor, or such programs are simply unavailable or inadequately supported. This disease, therefore, remains a leading form of cancer among women living in low-resource regions, and over 250,000 women worldwide die from cervical cancer each year. Persistent cervical infection with one of approximately 15 carcinogenic human papillomavirus (HPV) types causes virtually all invasive cervical cancer and its precursor abnormalities, which can be detected by cytological screening. Genital HPV infections are primarily transmitted via sexual intercourse. HPV testing has been widely adopted for the triage of patients after a cervical cytology screening test (Papanicolaou smear or liquid-based cervical cytology such as ThinPrep or SurePath) interpretation of atypical squamous cells of undetermined significance (ASCUS), and HPV testing is increasingly used for screening in conjunction with cervical cytology (Ronco et al., 2007). Although cervical cytology is a highly effective screening test for cancer, it has limited specificity for clinically significant lesions in cases with low-grade cytological abnormalities. Up to a quarter of all patients may have a false-negative result on the basis of cervical cytology testing alone. Recent decades have witnessed a reduction in the incidence of cervical cancer in countries where screening programmes have achieved broad coverage. The recognized importance of high-risk HPV (human papillomavirus) infection in the aetiology of cervical cancer may introduce a role for HPV DNA testing in cervical screening programmes. Positive HPV DNA tests indicate women at risk of cervical cancer with greater sensitivity, but reduced specificity, compared with exfoliative cytology (Nijhuis et al., 2006) Combining HPV testing with cytology may be useful in the triage of minor cytological abnormalities into those requiring referral to colposcopy (HPV positive) compared with those who can be safely managed by cytological surveillance (HPV negative). With its high sensitivity and high-negative-predictive value,

HPV testing may also be useful for predicting treatment failure, since residual disease is very unlikely in the event of a negative HPV test. Ultimately, prevention is better than cure, and the advent of HPV prophylactic vaccines may obviate the need for population-based cervical screening programmes in the future. A multivalent vaccine administered to adolescents prior to the onset of sexual activity and boosted at regular intervals throughout their sexually active life may provide protection against type-specific HPV infection, malignant precursors and invasive cervical disease. Several large randomized placebo-controlled trials have been conducted with promising results (Goldie, 2006). For those generations of women already exposed to high-risk HPV infection, therapeutic vaccines may offer advantages over conventional treatment, although much work still needs to be done (Mahoney, 2006). The vaccine against HPV includes virus-like particles, composed of the major viral capsid protein of HPV without the carcinogenic genetic core. Large-scale studies have shown that the vaccine is tolerated well, leads to high antibody levels in both men and women, and prevents chronic HPV infection and its associated diseases (Monk and Mahdavi, 2007). To achieve effective coverage the vaccine should be given prior to sexual debut. Introduction of the vaccine into specific countries, should take into account the local incidence of cervical cancer as well as the increasing incidence of precancerous cervical lesions and genital warts, which reduce quality of life and are associated with considerable costs.

One promising prophylactic HPV vaccine is available and others continue in development as primary cervical cancer prevention strategies in younger women. As secondary interventions, HPV tests are simultaneously evolving for use in cervical cancer screening programs, including routine screening of older women. HPV testing is more sensitive and reproducible than cytology with colposcopy for the detection of cervical precancer and cancer.

Experimental design

The enzyme linked immunosorbent assay (ELISA) is a widely used antigen and antibody detection technique and recently other biomolecular interactions e.g. DNA hybridisation with a versatility making it easy to tailor the method to a particular application. Nevertheless, significant variation exists at the protocol level and these optional or alternative protocol steps may or may not be important in an experimental situation. During optimization of a new ELISA system the experimenter should pay attention to these details. Also, further refinement is needed to optimize quantitative variables, e.g., concentrations of the reagents used in the assay. But other variables are also important, e.g., incubation time, buffer composition and pH, blocking protocols, etc. In this combinatorial process, the most widely used techniques are the intuitive experimentation following the one-step- at-a-time method and the 'criss-cross' dilution technique (Hornback, 1991). The most difficult problem during the optimization of a given ELISA procedure is the treatment of multifactorial interactions. The equilibrium binding process is heavily influenced by the concentrations of the analytes and by the concentration of available binding sites, which is a common source of the interactions. The standard experimental designs are frequently coupled to tedious experimentation and/or the usage of complex statistical methods (Sittampalam et al., 1996). This might be one reason for the fact that, very few experimental designs have been applied to optimize ELISA procedures. Those found in the literature are fairly complex and unsuitable for wide application. We have attempted to find a good balance between effectiveness and complexity. The method used in our approach is based on the experimental design called Taguchi, which has been successfully applied in PCR optimization (Cobb and Clarkson, 1994) and baculovirus expression (Burch et al., 1995). This method is straightforward, easy to follow, and needs no guesswork to take the initial experimental steps. The proposed layout for assignment of factors can suppress the effects of the interactions occurring between factors. With a special assignment e.g., the antigen concentration assigned to the six-level factor we could extend the number of

parameters which could be calculated and almost all the important biochemical parameters of an ELISA system could be assessed as a lower bound value. The method can also be used to eliminate the multifactorial effects contributing to the background. All the necessary calculations directly provide the widely known parameters of the ELISA systems such as calibration curve, its parameters, sensitivity and intra-assay and inter-assay variability. Taguchi optimization is a powerful method to screen the parameters to find near optimal experimental setups in a multiparametric experimental space.

Aims of this thesis

1. To use new experimental design method in the development of new human diagnostic devices. To demonstrate the application of Taguchi optimization by the examples of an ELISA and a PCR method.
2. To improve the efficacy of the Taguchi method a further improvement is needed to be devised in the field of ELISA optimization: the calibration curve, the sensitivity of the optimum assay, the intra-assay and inter-assay variability can be directly estimated.
3. To achieve optimum primerset concentration and usage in highly multiplex PCR systems detecting human papillomavirus an improved Taguchi optimization is need to be proposed.
4. To create a new PCR based detection system, involving experimental design approaches, with the following features: it detects a broader spectrum of HPV genotypes, is dynamic to recognize newly emerging HPV types, yields a balanced amplification and features an automatizable genotyping method (hybridization).
5. To the aim of point 4. the improvement of the Yoshikawa L1 (Yoshikawa et al., 1991) amplicon based method is proposed.
6. To compare the system to other systems, to access its analytical characterization analytical evaluation is needed to be carried out.
7. To access clinical behavior of the system a clinical evaluation of the system is devised.
8. As a part of the clinical evaluation the pelvic inflammatory disease proposed to be studied as a predictor of cervical cancer.

Materials and methods

HPV controls.

We subcloned the following HPV genotypes: 2a, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 28, 29, 31, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55/44, 57, 58, 59, 61, 66, 67, 68, 72, 73 into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA). Plasmids contained subgenomic fragments of the HPV genomes which embody the L1F/L1R1 amplicons. Clinical samples were used in case of other sequencing verified genotypes (HPV types 30, 43, 56, 70, 74, 82, 84, 89, 90, 91).

Primers.

The oligonucleotide primers were synthetized by IDT (Coralville, IA). We used the set of primers described by Yoshikawa et al. (Yoshikawa et al., 1991) (L1C1, L1C2 and newL1C2) and our L1F/L1R set (Tables 7-8). The L1C1 and L1F (forward set of primers) were biotin-labeled.

Polymerase Chain Reaction protocols

The PCR protocol used for amplification with the L1C primer set was described by Yoshikawa H. et al. (Yoshikawa et al., 1991)

Our improved PCR protocol using the L1F/L1R primers was the following: the amplification reaction contained a mixture of 3.4 μM L1F and 3.6 μM L1R primer set, 2.5 μl 10× PCR Gold buffer (Applied Biosystems, Foster City, CA), 1 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA), 2 mM of MgCl2 solution (Applied Biosystems, Foster City, CA) and 250 μM of each dNTP (Promega, Madison, WI). The amplification profile was 95°C for 10 minutes, 10 cycles of 95°C for 30 seconds, 45°C for 30 seconds,

72°C for 30 seconds, 35 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 30 seconds, and terminal extension 72°C for 4 minutes. The reaction volume was 25 µl containing 5 µl DNA sample. PCR products were subjected to electrophoresis followed by hybridization.

Molecular Cloning of HPV DNA sequences. The HPV DNA samples were cloned into pCR 2.1. Topo vector with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), according to the instructions of the manufacturer. Plasmids were prepared using the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and concentration of plasmids was measured with the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands).

Sequencing

PCR products were treated by the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). This template was then sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). Sequencing reaction products were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The DNA sequences were aligned with the public database on NCBI homepage using BLAST algorithm.

Hybridization probes

The probes (IDT, Coralville, IA) used for solid-phased hybridization were fluorescein-labelled in case of genus (Table 11) and genotype (Table 13) specific oligonucleotides, and digitoxigenine-labelled in case of HPV-Internal Control specific oligonucleotides. All labeled nucleotides were HPLC purified.

HPV detection and typing by solid-phased hybridization

The biotinilated PCR products (products of amplification by one labelled primer) are captured onto the surface of a streptavidin-coated microplate well. The complementary chain is eluted from the immobilized PCR product and the hybridization is carried out in the presence of specific fluorescein- (in case of genus and genotype specific probes) and digitoxigenin- (in case of internal control specific probe) labelled probes. The bound probes are reacted with anti-fluorescein-HRPO (HRPO-horse-radish peroxidase) and anti-DIG-AP (AP-alkaline phosphatase) antibody. Substrate development is done sequentially (separately). The HRPO substrate yields fluorescent signals measured at ex/em: 324/410, while the substrate of AP yields fluorescein signals measured at ex/em: 355/460.

Polystyrene plates (Costar) were coated with streptavidine (Sigma, St. Louis, MO) (0.02 mg/ml streptavidine in PBS solution) overnight. Plates were washed with 250 µl washing solution [25 mM TRIS pH7.5, 125 mM NaCl, 20 mM MgCl2, 3% Tween-20]. 20 µl PCR product was diluted in 140 µl ultrapure water, and 5 µl from this solution was mixed with 45 µl binding puffer [25 mM TRIS pH7.5, 125 mM NaCl, 5 mM EDTA-Na2, 5× Denhardt´s solution, 0.1% Tween-20] and dispensed into the wells of the streptavidine coated plate. The reaction was incubated at room temperature for 30 minutes with constant shaking. Then 50 µl elution buffer [100 mM NaOH, 300 mM NaCl] was added to the mixture, incubated for 3 minutes at room temperature and the plates were washed three times with 250 µl washing solution [25 mM TRIS pH7.5, 125 mM NaCl, 20 mM MgCl2, 3% Tween-20]. Next 50 µl hybridization buffer [(5× SSC (0.3 M Na-citrate pH7, 3 M NaCl), 1× Denhardt´s solution, 0.1% SDS], containing fluorescein labelled probes (5 nM per probe) was added to the wells. The mixture was incubated for 30 minutes at 55°C with constant shaking, and washed six times with 250 µl high stringency washing solution [0.05× SSC, 0.3% Tween-20]. Next 50 µl conjugation buffer [125 mM TRIS pH7.5, 125 mM NaCl, 2 mM MgCl2, 0.3% Tween-20, 1% BSA], containing Anti-Fluorescence-POD (Roche Diagnostics GmbH, Mannheim, Germany) antibody (0.0015 E/reaction), was added to the reaction. Plates were incubated for 30 minutes at room temperature with shaking, and washed six times with 250 µl low stringency washing solution [25 mM TRIS pH7.5, 125

mM NaCl, 20 mM MgCl2, 3% Tween-20]. For development 135 µl substrate solution (5 volume [45 mM hydroxiphenyl-propionic acid (HPPA), dissolved in 0.1 M TRIS-HCl pH9.0 buffer], + 1 volume [0.6 g/l H2O2 in 20 mM citrate-phosphate buffer]) was added. After 20 minutes of incubation 65 µl stop solution [0.75 M glycin pH10.3] was added to the reaction mixture. The fluorescent signal was measured by SpectraMax plate-fluorometer (Molecular Devices, Sunnyvale, CA) at 324/410 nm. Samples were considered positive if their value was higher than three times the mean of 3 parallel negative control sample values. This value is higher than the cutoff value established as mean +3 SD of the controls (mean=858, SD=81, the value of mean + 3 SD=1101, the value of three times the mean: 2574). Our preliminary experiments showed no differences between the two approaches, based on positivity. All substances mentioned above were purchased from Sigma, St. Louis, MO.

Sample acquisition and preparation

Cervical specimens were collected by cervical brush samplers and transported in PBS from STD (sexually transmitted disease) outpatient clinics. The samples were processed by centrifugation (2000 g, 10 minutes), the pellet was washed in PBS, lysed in 250 µl lysis solution containing HPV internal controls (0.5 mg/ml proteinase K, 0.01 M TRIS-HCl pH8, 0.001 M EDTA pH8). The samples were then vortexed and incubated for 30 minutes at 56°C. The following steps were carried out on a TECAN RSP150 (Tecan Group Ltd., Maennendorf, Switzerland) robot. 200 µl binding solution (5.5 M GUSCN, 20 mM EDTA, 10 mM TRIS-HCl pH6.5, 65 mM dithiothreitol, 40 g/l silicate) was added, and the silica was separated by vacuum filtration from the soluble components. Next silica was washed twice with 200 µl binding solution without silica, and twice with 200 µl washing solution (25% isopropyl-alcohol, 25% ethanol, 0.1 M NaCl). Finally 200 µl ethanol is applied. After air-drying the silica, DNA was eluted in 200 µl 10 nM TRIS solution pH8. All substances mentioned above were purchased from Sigma, St. Louis, MO.

Comparison of L1F/L1R system and the Hybrid Capture II test

Cervical samples issued as ASCUS (Atypical Squamous Cells of Undetermined Significance) or higher in prior cytological examinations were analyzed using the Hybrid Capture II test (HC II, Digene Corporation, Silver Spring, MD) and the L1F/L1R system, following the instructions of the manufacturers. The samples included in this study were collected by Digene Cervical Sampler and were split: one part was used for Hybrid Capture II detection, the other part was subjected to DNA extraction by High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The amplicons of discordant results were sequenced in order to establish the true high-risk positivity/negativity.

Testing the system on clinical samples

Clinical samples issued as ASCUS or higher from a cohort of 360 persons were tested by the L1F/L1R system. Numbers of positive and negative cases were recorded, together with the prevalence of the different high-risk HPV types. Percentages from prevalence of 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) were compared to those published by Coutlee et al. (Coutlee et al., 2002).

Statistical methods

Two-sided McNemar's chi-square analysis for matched-pair data and the Yule statistic were performed to analyse contingency tables comparing HCII and L1F/L1R. The two-tailed Fisher Exact Probability Test (http://faculty.vassar.edu/lowry/tab2x2.html) was used to analyse contingency tables comparing L1F/L1R with the sequencing-clarified results. The unweighted kappa statistic was calculated to adjust for chance agreement between the two systems. In general, a kappa value above 0.75 indicates excellent agreement, between 0.40 and 0.75 indicates fair to good agreement, and below 0.40 represents poor agreement beyond chance.

The Wilcoxon signed-ranks test and the Mann-Whitney U-test were used when comparing PGMY and L1F/L1R results. The percentages from prevalence of 14 high-risk HPV types in case of both systems were considered and agreement was calculated.

Antigens and antibodies

Phages were prepared by transforming SURE E. coli cells with a ScFv phagemid pCANTAB5 from .Pharmacia, Uppsala, Sweden containing a rearranged adenovirus hexon monoclonal antibody Mab gene Ruzsics et al., 1994 and rescued with M13KO7 helper phage. The preparation of phages followed the protocols described in the Pharmacia Recombinant Phage Antibody System Expression Module kit. After precipitation of the phages with PEG/NaCl we further purified the phages on a CsCl step gradient in a Beckman ultracentrifuge using a SW60Ti rotor .30 000 rpm, 48C, overnight .Finally, the collected bands were dialysed against TE 10 mM TRIS pH .8.0, 1 mM EDTA buffer. The anti-M13-phage polyclonal serum were raised using purified phages as antigen as previously described Cooper and Paterson (Cooper and Paterson, 1991).The serum was purified by ammonium sulphate precipitation and cation exchange chromatography: particulate material being removed by ultracentrifugation (Andrew, 1991). The biotinylated anti-rabbit antibody donkey was from Amersham Buckinghamshire, UK and Extravidin-HRPO was from Sigma Deisenhofen, .Germany.

ELISA for detection of ScFv phages

All reagents were prepared in PBS and the concentrations and conditions were changed according to the experimental layout forced by the Taguchi array Table 1. All incubations were carried out at 37^{o}C. Every condition was tested in triplicate. For coating of the wells the dilutions of the phages 12 600, 6300, 630, 6.3, 0.063, 0.0063 ng/ml were prepared in PBS. The protein concentrations were measured by the Coomassie binding method.

	A	B	C	D	E	F	e
1	1	1	1	1	1	1	1
2	1	2	2	2	2	2	2
3	1	3	3	3	3	3	3
4	2	1	1	2	2	3	3
5	2	2	2	3	3	1	1
6	2	3	3	1	1	2	2
7	3	1	2	1	3	2	3
8	3	2	3	2	1	3	1
9	3	3	1	3	2	1	2
10	4	1	3	3	2	2	1
11	4	2	1	1	3	3	2
12	4	3	2	2	1	1	3
13	5	1	2	3	1	3	2
14	5	2	3	1	2	1	3
15	5	3	1	2	3	2	1
16	6	1	3	2	3	1	2
17	6	2	1	3	1	2	3
18	6	3	2	1	2	3	1

Table 1 .The layout of the Taguchi experiments L18 array. The factor levels are shown in each experiment. On the left side, the numbers indicate the experiments, and the letters at the top of the columns indicate the factors variables

After overnight incubation, the coated wells were washed four times with the washing buffer milliQ water, .PBS, PBS-0.05% Tween 20 and the wells were blocked with 0.5% BSA-0.05% Tween 20-PBS for 1 h. The rabbit anti-phage polyclonal antibody was used as primary reagent dilutions: 1:5000, 1:10000 or 1:20000 for 1 h. The wells were washed four

times and were either blocked for 30 min or were further processed without blocking according to the experimental layout. Biotinilated anti-rabbit donkey antibody was used as secondary reagent for 1 h dilutions: 1:500, 1:1000, 1:2000 . Again, a four- fold wash stage with or without blocking was used. Extravidin-HRPO conjugate was added 1:500, .1:1000, 1:2000 to detect the biotin label and it was washed four times without blocking. To detect the enzyme label, OPD substrate was used and the reaction was stopped after a period of time 5, 8, 12 .min by the addition of 4 N sulfuric acid. The plates were measured at 492 nm with background subtraction at 620 nm. Parallel control experiments were run omitting the coat but using the Taguchi layout. Also, every single reagent was excluded systematically to detect cross-reactions these wells should result in low values and every reagent was also coated and only the subsequent steps were added to these wells to check out possible reagent deterioration.

Taguchi method

Calculations are performed with Microsoft Excel spreadsheet macro written in our laboratory. All the experiments were carried out in triplicate. The Taguchi experiments followed an experimental layout called the L18 array (Table 1). We chose the L18 array because it has a few advantages over the others in this particular application. For complete optimization the protocol consists of only 18 parallel setups in one experiment and is also characterized by a specific feature suppressing the interaction effects which otherwise can bias the calculation of optimum conditions. The latter is important because the interaction of factors is very common in ELISA. One modification used with the original L18 array is a new six-level factor A combining the first two original factors of the array assigned to the concentration of the coated material. The levels of this factor should be set so as to cover at least four magnitudes in concentration to serve as the basis for calculating the calibration curve. Other factors were assigned to conditions which should be optimized to give better performance in the assay (Table 2). The last factor was not assigned and was used as an error factor factor e. The 18 experiments of the L18 array were also carried out without phage coating for the purpose of background subtraction and for determination of the

interaction causing background effects. The triplicate experiments were averaged and the background average was subtracted for each experiment. The subtracted values were transformed to normalize the distribution according to the so called ‘ Larger-Is-Better’ characteristic transformation Taguchi, 1993 a special kind of log transformation included in the spreadsheet . These transformed values were used to calculate the level totals by adding together the transformed values of those

	Name	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
A	Phage concentration in coat buffer (ng/ml)	6300	630	157	63	6,3	0,063
B	Dilution of anti-phage polyclonal antibody (rabbit)	5000	10000	20000			
C	Dilution of anti-rabbit biotinylated antibody	500	1000	2000			
D	Development time of the OPD substrate	5 min	8 min	12 min			
E	Extra blocking step after each wash	Yes	Yes	No			
F	Dilution of Extravidin-HRPO conjugate	500	1000	2000			

Table 2 The assigned levels of the factors

The assigned values of the factors are given in the table (the letters correspond to the letters in Table 1).

The seventh factor was used as an error factor and was not assigned experiments which have the same level of a given factor. Because of the orthogonality of the layout, the effects of the other factors will cancel out each other’s effects and give a relative value representing the effect of a level compared to the other levels of a given factor. This table of level totals can be used to judge the optimum condition, namely, those levels that have the largest total value of a given factor. To break down the system variation an ANOVA table was constructed so that variation term S and variance V for each factor could be calculated. The F value was used to test the significance of a factor and for factors which were significant we calculated the relative contribution in the whole variation in percentage

Ve and V(e) . The two values differ in calculation of the error variance: the Ve value is based on the error factor variance factor e , however, for the V(e) value calculation the non-significant factors had been pooled with the error and this pooled variance was used. We used a 5% level of significance. The curve fitting was carried out by the four-parameter logistic technique and the Newton–Gauss iteration method using the least-square approach with the built-in Excel add-in called 'Solver'. It has proved to be the best curve fitting method in this area (Plikaytis et al., 1991). The reliability of fit was judged by the Pearson test. The biochemical parameters were calculated as follows:

i. sensitivity: adding standard error twice to the lower infinite value of the logistic curve at the estimation of this value the standard deviation of the 18 experiments .was calculated and the largest was chosen for use;
ii. intra-assay variability: the average of the coefficient of variation of the triplicates of the 18 experiments;
iii. inter-assay variability: the coefficient of variation of the triplicate averages of the 18 experiments plus the intra-assay variability.

The half maximal value is the **c** value of the four logistic curve parameters, **a** is the lower limit, **b** is the slope and **d** is the upper limit.

One-step-at-a-time method

All factors were kept constant except for one and the first observation was taken at the third level of that factor. The second observation was taken at the second level. If the response on the second observation was larger, a third observation was taken at the first level. Finally, keeping the largest response level of that factor constant a new factor was chosen and the procedure was repeated in the same manner.

Taguchi optimization for mapping the optimum concentration of the primersets

The experimental layout is on Table 3.

	A	B	C	D	E	e	F	G
1	1	1	1	1	1	1	1	1
2	1	1	2	2	2	2	2	2
3	1	1	3	3	3	3	3	3
4	1	2	1	1	2	2	3	3
5	1	2	2	2	3	3	1	1
6	1	2	3	3	1	1	2	2
7	1	3	1	2	1	3	2	3
8	1	3	2	3	2	1	3	1
9	1	3	3	1	3	2	1	2
10	2	1	1	3	3	2	2	1
11	2	1	2	1	1	3	3	2
12	2	1	3	2	2	1	1	3
13	2	2	1	2	3	1	3	2
14	2	2	2	3	1	2	1	3
15	2	2	3	1	2	3	2	1
16	2	3	1	3	2	3	1	2
17	2	3	2	1	3	1	2	3
18	2	3	3	2	1	2	3	1

Table 3 .The layout of the Taguchi experiments the modified L18 array. The factor levels are shown in each experiment. On the left side, the numbers indicate the experiments, and the letters at the top of the columns indicate the factors variables. ‘e’ is the error factor.

The factors, which are groups of primers, are according to the phylogenetic tree determined by ClustalW and on Table 4.

No. of experiment	A Group 1 primerset	B Group 2 primerset	C Group 3 primerset	D Group 4 primerset	E Group 5 primerset	F Group 6 primerset	G Group 7 primerset
1	0,15	0,4	0,45	0,2	0,6	0,35	0,2
2	0,15	0,4	0,9	0,4	1,2	0,7	0,4
3	0,15	0,4	0,225	0,1	0,3	0,175	0,1
4	0,15	0,8	0,45	0,2	1,2	0,175	0,1
5	0,15	0,8	0,9	0,4	0,3	0,35	0,2
6	0,15	0,8	0,225	0,1	0,6	0,7	0,4
7	0,15	0,2	0,45	0,4	0,6	0,7	0,1
8	0,15	0,2	0,9	0,1	1,2	0,175	0,2
9	0,15	0,2	0,225	0,2	0,3	0,35	0,4
10	0,3	0,4	0,45	0,1	0,3	0,7	0,2
11	0,3	0,4	0,9	0,2	0,6	0,175	0,4
12	0,3	0,4	0,225	0,4	1,2	0,35	0,1
13	0,3	0,8	0,45	0,4	0,3	0,175	0,4
14	0,3	0,8	0,9	0,1	0,6	0,35	0,1
15	0,3	0,8	0,225	0,2	1,2	0,7	0,2
16	0,3	0,2	0,45	0,1	1,2	0,35	0,4
17	0,3	0,2	0,9	0,2	0,3	0,7	0,1
18	0,3	0,2	0,225	0,4	0,6	0,175	0,2

Table 4. Amount of group of primers used in the experiments. The amounts are microliters and color coded, the benchmark amount is yellow, the green indicates twice the original amount and the blue is for half of the original amount of groups of primers.

The PCR and the hybridization was carried out as described above, samples are dilutions of plasmids of the cloned genotypes (100, 10, 1 copies/ul). The evaluation was a comparison of the sensitivity (detecting lower amount of target or with higher signal) between the benchmark (L1F/L1R) reaction (experiment 1) and the others. The signals are calculated as fold signals over the corresponding genotypes specific NTC signals.

Study to access the pelvic inflammatory disease as risk factor for cervical cancer

The study population consisted of two groups of women. The 2,215 patients with signs and symptoms of PID will be referred to as Group 1, they were enrolled during 1999-2001. Diagnosis of PID was based on physical, clinical and laboratory findings. The clinical minimum criteria used to diagnose pelvic inflammatory disease were: lower abdominal tenderness, bilateral adnexal tenderness, cervical motion tenderness, no evidence of competing diagnosis, and negative pregnancy test. Additional criteria were one of more of the following: fever (at least 38.3^{o}C), C-reactive protein >20mg/l, or erythrocyte sedimentation rate >40mm/h, leucocytosis (white blood cell count > 10^{10}/l), abnormal cervical or vaginal discharge, evidence of N. gonorrhoeae or C. trachomatis in the endocervix, and purulent material form peritoneal cavity by culdocentesis or laparoscopy. Definitive criteria for PID were: abdominal or transvaginal sonography showing thickened fluid-filled tubes with or without free pelvic fluid or tubo-ovarian complex, and laparoscopic abnormalities consistent with PID.

Group 2 consisted of 4,217 women who attended gynecological visit in 2000, patients with signs or symptoms of PID were excluded from this group. HPV was detected according to the protocols above.

Atypical squamous cells of undetermined significance (ASCUS) refers to cytologic changes suggestive of SIL but qualitatively or quantitatively insufficient for a definitive interpretation. We used this term according to the Bethaesda 2001, the subcategory of ASCUS, ASCUS, favor low-grade SIL (LSIL) was used as ASC-US, and the subcategory, ASC, cannot exclude high-grade SIL (HSIL) or ASC-H, was applied. In essence, ASC-US reflects the difficulties in distinguishing between reactive changes and LSIL in equivocal cases, whereas ASC-H reflects the difficulties in distinguishing between immature squamous metaplasia and HSIL.

Results

Taguchi optimization of the parameters of ELISA

To test the Taguchi method in the optimization of ELISA an antigen detection problem was chosen that had proven to be very troublesome, i.e., detecting antigen bound ScFv phages with polyclonal antibodies. Dealing with the ELISA of ScFv phages we found high backgrounds and low sensitivity hampering our efforts to find antigen specific phage clones. To circumvent this problem, a new anti-phage serum was raised in rabbits and we faced a multi-variable optimization of the ELISA assay. Although the phage clone used in this experiment had proven binding ability to HAdV1 hexon protein (Ruzsics et al., 1994) we decided to refine the system to detect coated ScFv phages only and not the antigen bound form, because used in this way the optimized protocol becomes independent from the antigen binding characteristics of the phage. All the possible important factors had been assigned Table 2 and the experiments were carried out according to the Taguchi array layout described in Section 2. One set of Taguchi experiments was carried out using phage coated wells and a parallel set of wells without antigen coat. The corresponding background was subtracted and the ‘ Larger-the-better’ transformation was used. The results of the calculations, the table of .level totals and optimum condition A and the ANOVA table B are shown in Table 5. The optimum conditions are shown in Table 5 these levels are given in bold .

A) Totals and optimum conditions*

	A	B	C	D	E	F	e
1	**-4,984645**	**-25,21168**	**-51,66**	-83,62616	-58,9605	**-55,16216**	-60,17344
2	-14,27968	-63,62286	-57,55495	-55,05401	-61,61203	-57,78318	-54,85343
3	-20,54273	-87,39957	-67,01916	**-37,55395**	**-55,66158**	-63,28878	-61,20724
4	-28,09862						
5	-42,0939						
6	-66,23455						
	-176,2341	-176,2341	-176,2341	-176,2341	-176,2341	-176,2341	-176,2341

B) ANOVA**

	f	*S*	*V*	*F*	% Ve	% V(e)	CF	St	SigLimit%
Sa	5,00	807,60	161,52	416,87	59,16	58,37			
Sb	2,00	328,23	164,11	169,43	24,05	23,73			
Sc	2,00	20,01	10,01	10,33					
Sd	2,00	180,29	90,15	93,06	13,08	12,76			
Se	2,00	2,96	1,48	1,53					
Sf	2,00	5,74	2,87	2,96					
Serror	2,00	3,88	1,94						
S(e)	8,00	32,58	4,07			5,13			
Sum	17,00	1348,70				100,00			
							1725,47	1348,701	5

Table 5

Taguchi calculations on the signals of ScFv ELISA background subtracted.

*The column letters indicate the factors, and the numbers on the left are the factor levels. The totals of levels were calculated according to Section 2. The sums of totals for every given factor were calculated and are given at the bottom of the columns. The optimum levels of the factors are given in bold the largest value in the column.

**Abbreviations: f—degrees of freedom; S—variation term; Sa, Sb, etc.—variation terms of the factors A, B, etc.; V—variance; F—F-test value; % Ve—degrees of contribution calculated on the basis of variance of error factor factor e.; % V(e).—degrees of contribution calculated on the basis of the variance of pooled error, see Section 2.; CF—correction factor; St—total variation; SigLimit%—significance limit.

(see further details in the text)

Based on column %V e values the significant factors have been determined at the p s 0.05 level the contribution of these factors to .the final result is not contingent . In the case of the .non-contributory factors C, E the optimum levels were not used for reasons discussed later. To test the predictions, a control experiment was conducted using more data points to obtain the calibration curve .of the system under the optimum conditions Fig. 1 .

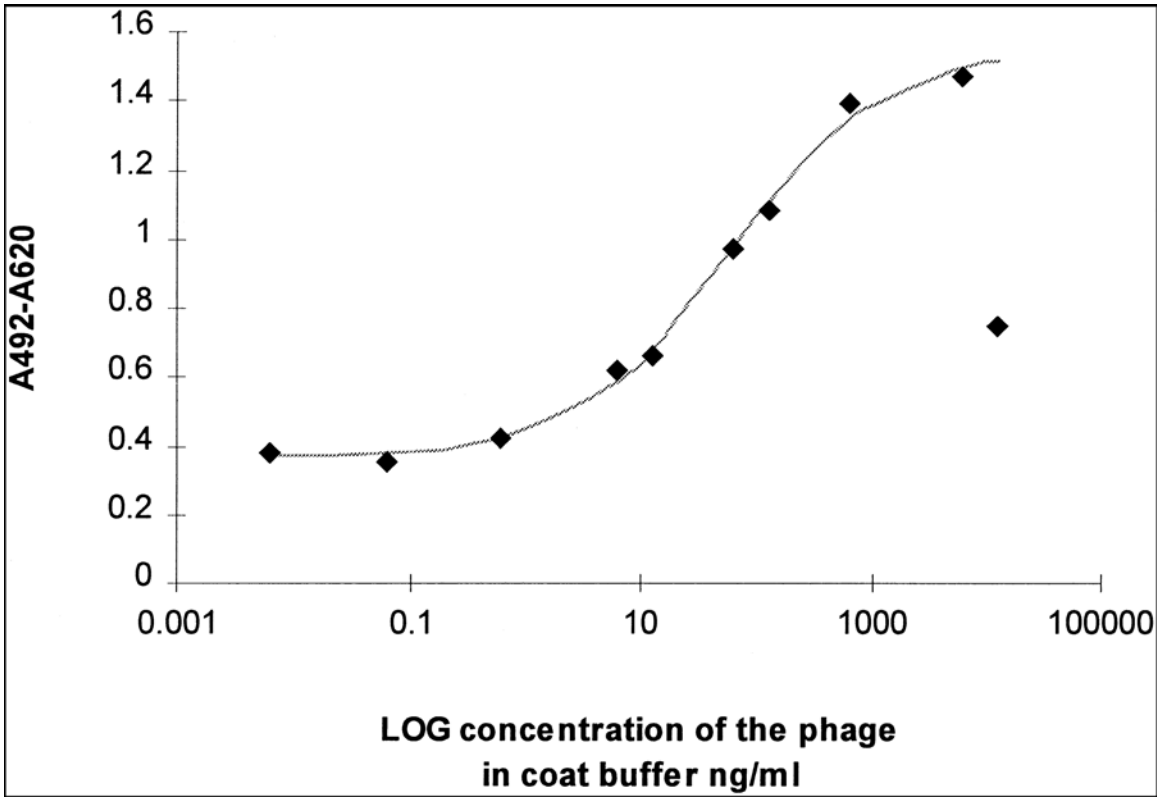


Figure 1. Calibration curve of ScFv ELISA after optimization by the Taguchi method.

The calculated parameters display an ED50 of 55.80 ng/ml logistic curve parameters: a = 0.37, b = .0.68, c = 55.80, d = 1.54 and 1.47 ng/ml sensitivity (Chard, 1997), an inter-assay coefficient of variation of 6.35% and an intra-assay coefficient of variation of 4.06%. To check out the prediction we performed the one-factor-at-a-time method to find .the optimum condition (Anderson and McLean, 1974) which resulted in a very similar set of optimum conditions. The only difference between the two method results was the concentration of Extravidin peroxidase conjugate and the extra blocking step .factor E , the effects of these factors are non-contributory as judged by the ANOVA table Table 5 and for

this reason too close to apply the method of one-factor-at-a-time. The two curves' ED50 and slope were not statistically different $p > 0.05$ $F[1,18] = 0.001618$ for ED50 and $F[1,18] = 0.000742$ for slope . For the curve shown in Fig. 2.

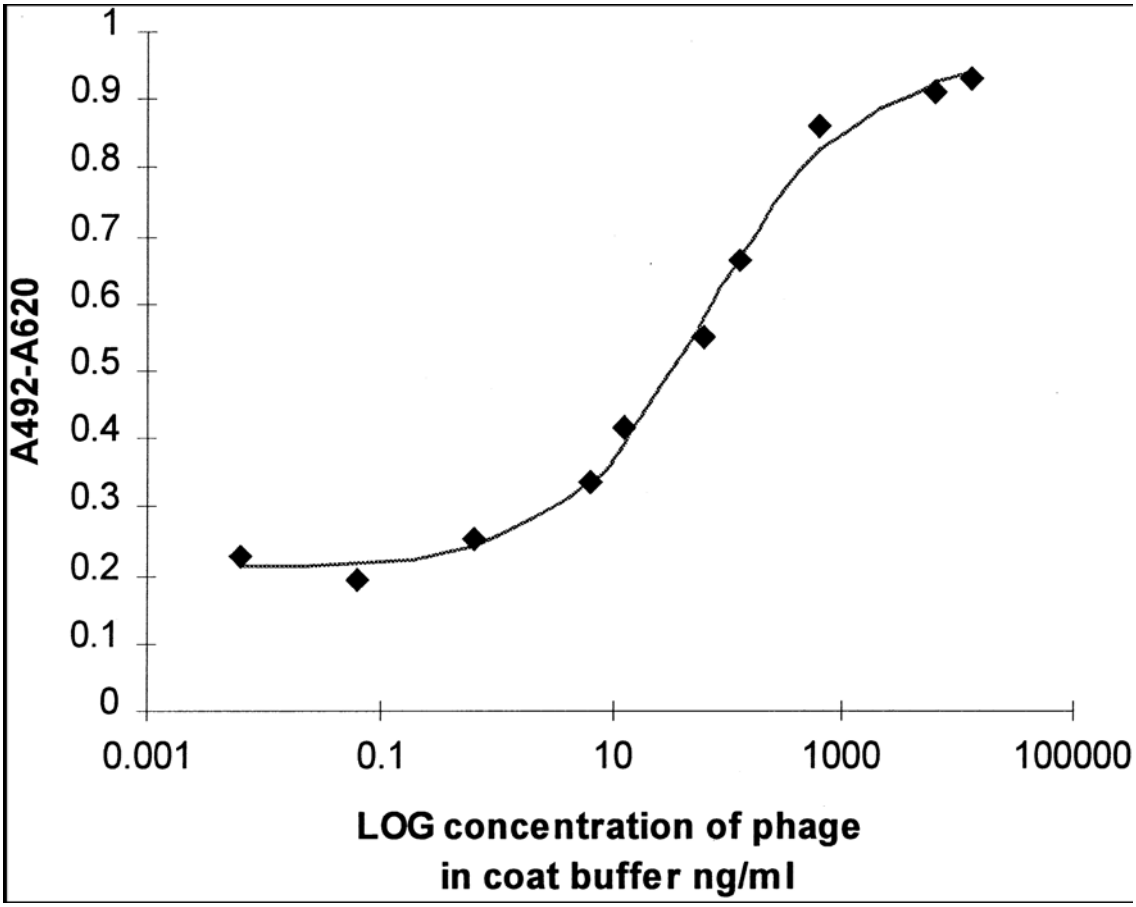


Figure 2. Calibration curve of ScFv ELISA after optimization by the 'one factor at a time' method.

parameters display an ED50 of 66.17 ng/ml a = 0.212, b = 0.69, c = 66.17, d = 0.958 and a sensitivity of 2.60 ng/ml.

Estimation of the ELISA assay parameters at the optimum levels of the factors

To access not only the optimum conditions but also more useful analytical parameters of

the assay; we have taken advantage of the proposed special assignment with six levels of coated antigen concentration. Using the result of the Taguchi optimizations, based on this six-level factor, the standard curve of the optimum conditions can be calculated. Taking the optimum levels of the contributory factors, and using each level of factor A, the corresponding estimated results can be calculated and, using them, the calibration curve can be constructed. Nevertheless, the factor interactions can bias the calculations because the interactions can partially be confounded with the factors even in the L18 array used. This leads to overestimation of absorbance values mostly in the upper part of the standard curve causing a predicted higher level of saturation. To get a better estimation, we suggest that the highest absorbance level during the Taguchi optimization experiments should be used as the saturation absorbance value and for the iteration process, approaching the logistic curve parameters, this saturation value was forced. This will result in a fairly close but generally lower saturation value because, due to the orthogonality of the system, one of the experiments frequently approaches the real optimum. The other important consideration is the determination of the factors which can reduce the background but not contribute significantly to the performance of the assay. To pinpoint these factors we again used the original Taguchi experimental results of non- .coated background wells and their factor contributions were compared to the original factor contributions. The result of these calculations is shown in Table 6.

A)Level totals and optimum conditions*

	A	B	C	D	E	F	e
1	-73,39971	-103,7578	-136,2375	**-174,9395**	**-159,5855**	-154,1624	-152,3689
2	-76,19112	-169,8722	-156,801	-151,3519	**-159,6109**	-151,4609	-153,3077
3	-80,29342	**-184,8282**	**-165,4199**	-132,1669	-139,2619	-152,8349	-152,7818
4	-76,62429						
5	-74,74861						
6	-77,20116						
	-458,4583	-458,4583	-458,4583	-458,4583	-458,4583	-458,4583	-458,4583

B) ANOVA**

	f	*S*	*V*	*F*	% Ve	% V(e)	CF	St	*A x B*	SigLimit%
Sa	5	9,208	1,841500	249,52290						
Sb	2	620,400	310,200000	16812,66000	68,61	68,61				
Sc	2	74,931	37,465000	2030,60800	8,28	8,04				
Sd	2	152,996	76,498200	4146,15600	16,91	16,68				
Se	2	45,951	22,975700	1245,27100	5,07	4,84				
Sf	2	0,608	0,304100	16,48278						
Serror	2	0,074	0,369000							
S(e)	9	9,890	1,098846			2,07				
Sum	17	904,170				100,00				
							11676,89	904,1686	0	5

Table 6

Taguchi calculations of the background of ScFv ELISA.

* The optimum levels of the factors are given in bold the smallest value in the column.. *The column letters indicate the factors, and the numbers on the left are the factor levels. The totals of levels were calculated according to Section 2. The sums of totals for every given factor were calculated and are given at the bottom of the columns. The optimum levels of the factors are given in bold the largest value in the column.

**Abbreviations: f—degrees of freedom; S—variation term; Sa, Sb, etc.—variation terms of the factors A, B, etc.; V—variance; F—F-test value; % Ve—degrees of contribution calculated on the basis of variance of error factor factor e.; % V(e).—degrees of contribution calculated on the basis of the variance of pooled error, see Section 2.; CF—correction factor; St—total variation; SigLimit%—significance limit

(see further details in the text)

Factors with a significant %V e value contribute to the background and if we use the .lowest levels in bold we reduce the background and remove some of the confounding interactions making the estimations better. The comparison also reveals the interactions showing different patterns of percentage values and so the different levels of contribution in the presence of antigen and without it. Factor C and factor E have unique features. They show significant contributions in the absence of antigen indicating that usage of the lowest levels of these factors influences only the background and not the overall performance of the assay. The background optimum levels are shown in Table 6A. These considerations and the treatment of the data cannot remove all the effects of the interactions but the calculation after the data treatment gives a very close estimation of the calibration curve. In Fig. 3 the comparison of the standard curve calculated after the proposed data treatment and the experimentally determined standard curve are shown.

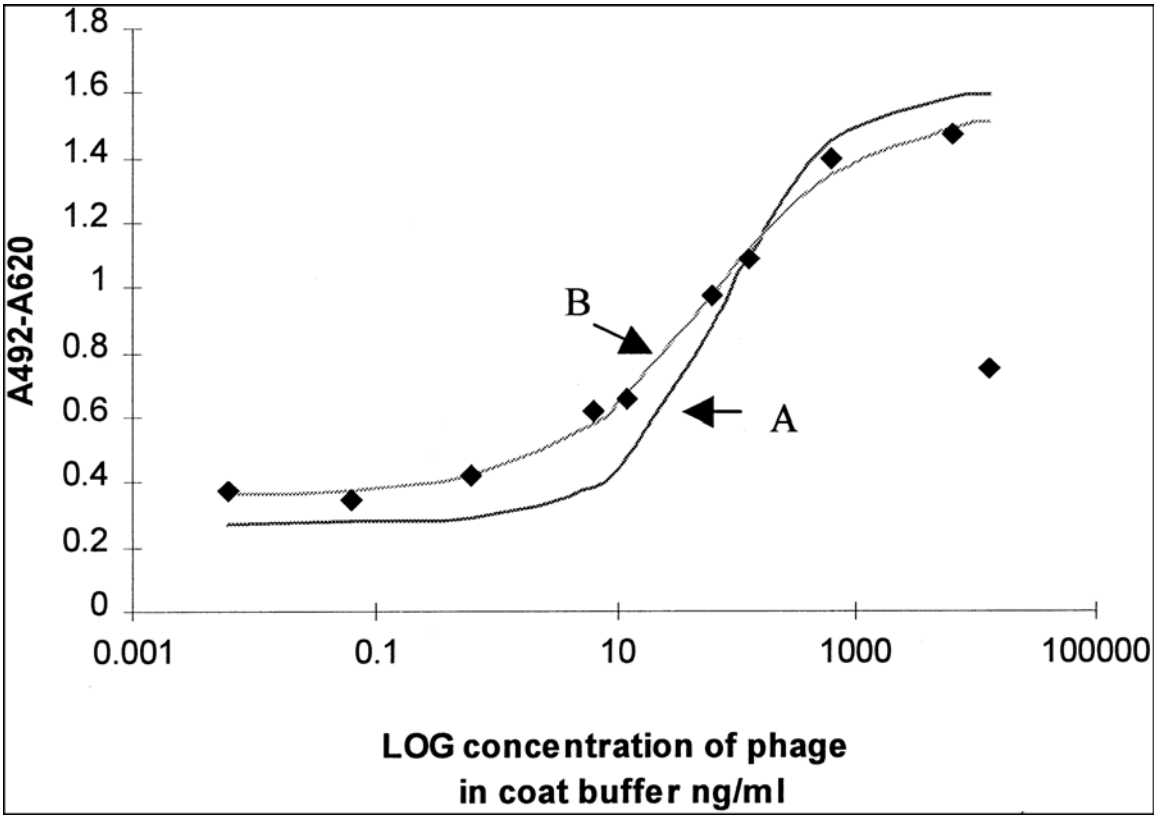


Figure 3. Comparison of the experimentally determined calibration curve of ScFv ELISA to the calculated calibration curve. _A. The calculated curve, _B. the experimentally determined curve.

The analytical parameters of the fitted curve agreed very closely with the estimated values parameters for A: a = 0.15, b = 0.68, c = 145.91, d = 1.27 for B: a = 0.37, b = 0.68, c = 55.80, d = 1.54 and according to the chi-squared test the two sets of points were similarly distributed with a probability of 0.99. Nevertheless, the calculated and the determined sensitivities were fairly close 7.22 ng/ml and 5.57 ng/ml, respectively . Finally, the inter-assay and the intra- assay variability estimates were 7.02% and 4.66%, respectively, compared to 6.35% and 4.06%. The results confirmed the predicted values suggest that the method is effective in the estimation of these parameters. We have described a new experimental method in the field of optimization of ELISA methods. The original goal of

the Taguchi method was to find the optimum conditions and to estimate its numerical parameters (Taguchi, 1993). The method has been very effective in the determination of the optimum conditions even in the presence of multifactorial interactions. However, the Taguchi system contains much more information because it could calculate the numerical parameters not only for the optimum conditions but for any combinations of the factors. We used this method to determine factors that affect the results to the greatest extent or, on the contrary, to determine which factor's effect is negligible. Following this process we could omit or include protocol steps, or we could use an advantageous condition which is not the best choice but only slightly affects the result. Moreover, on this basis the recalculation of data it possible to tailor the assay to the given circumstances and evaluate the analytical behavior of the assay. By introducing the Taguchi method to optimize ELISA procedures we demonstrate the advantages of this approach over the more traditional labor-intensive methods. Another advantage of the method is that the optimization and the determination of the analytical parameters of the assay can be carried out in one step. Furthermore, the recalculation of these parameters can be carried out, which is impossible in the traditional methods. The analysis of variance used here makes the predictions more robust because the calculations are based on all the experiments conducted during the optimization. The method accepts qualitative conditions which permit the incorporation of all the circumstances important in an ELISA procedure. Moreover, the calculated weighting of the factors make realistic assumptions about the system so it is possible to control the critical conditions.

The Taguchi optimization of this pilot experiment established its further use, during the development of the L1F/L1R primer system and optimization of the application of DNA ELISA for the detection of the L1F/L1R amplicons this experimental design method proved to be very successful.

Development of the L1F/L1R primers.

During the development we first determined the L1C-PCR sensitivity spectrum over 46 HPV genotypes. On the basis of these results and the sequence data of the primer binding regions of the theoretically amplifiable genotypes, we designed 15 forward and 16 reverse primers (Table 7-8). The priming efficiency of the primer set was optimized by the relative concentration of the primers and also by the design of the primer sequences. Introducing mismatches could help to reduce internal competition of the primers, but mismatches are kept at a minimum especially at the 3' end of the primers. This approach resulted in a smooth priming efficiency distribution. The optimized reaction has very high sensitivities and uniform amplification power. Notably the reaction also has very broad spectrum over the genotypes.

L1C1	CGTAAACGTTTTCCCTATTTTTTT
L1F2	CGTAAAGCTATACCATATTTTTTT
L1F3	CGTAAACACCTTCCTTATTTTTTT
L1F4	CGTAAACGTGTTTCCTATTTTTTT
L1F5	CGTAAACGTATTCCCTTATTTTTT
L1F6	CGTAAACGTTTACCATATTTTTTT
L1F7	CGTAAACCTGTACCATATTTTTTT
L1F8	CGTAAACGTCTGTCATATTTTTTT
L1F9	CGTAAACATGTTCCCTTTTTTTTT
L1F10	CGTAAACGTATGCCTTATTTTTTT
L1F11	CGAAAGCGCATATTAG
L1F12	CGTAAACGTGTGCCCTATTCTTTT
L1F13	CGTAAACGTGTCCACTATTTCTTT
L1F14	CGTAAACGTGTCTCATATTTTCTT
L1F16	CATAAACGTATTCACTATTCTTTT

Table 7. The L1F primers.

L1C2	CAATACAGAGTATTTAGGGTA
L1C2new	CAATATAGGGTATTTAGGGTA
L1R3	CAGTACAGAGTTTTTAGAATT
L1R4	CAATACAGAGTATTTAGAGTA
L1R5	CAATATAGAGTGTTTAAGGTA
L1R6	CAATACAGGGTATTTAGAATA
L1R7	CAGTACAGAGTATTTAGAGTT
L1R8	CAATATAGGGTTTTTAAGATG
L1R9	CAATACAGGGTGTTTAGGGTT
L1R10	CAATATAGGGTTTTTCGCGTT
L1R11	CAGTATAGGGTCTTTAGGGTA
L1R12	CAGTATAGGGTATTTAGAGTT
L1R13	CAATATCGTGTATTTCACGTT
L1R14	CAGTACAGGGTGTTCCGTGTG
L1R15	CAATATAGGGTGTTTAGGGTG
L1R16	CAGTATCGAGTGTTTCGGGTT

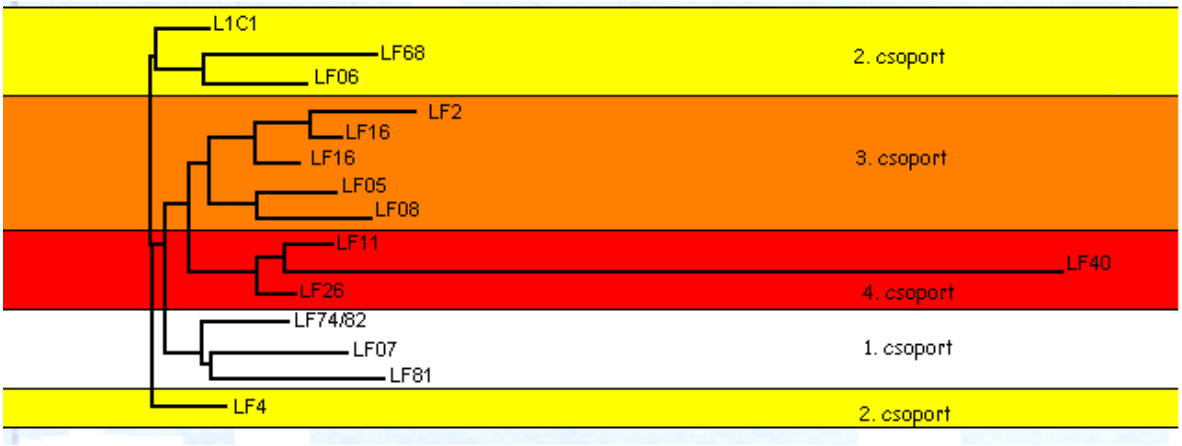
Table 8. The L1R primers.

Primer sequences were aligned with the public database on NCBI homepage using BLAST algorithm to test primer specificity. Primers were specific to HPV.

While the SPF system is able to detect 39 genotypes (Kleter et al., 1999), and the MY09/11 system detects 27, with 12 additional genotypes (Gravitt et al., 1998; Qu et al., 1997), the L1F/L1R system detects theoretically 52 genotypes, from which the amplification and detection capabilities of 46 genotypes were tested in this study. Further studies are needed to provide data about HPV 69, 81, 83(MM7), 85 and the novel genotypes HPV 86, 87 (Menzo et al., 2001).

After the initial design, we evaluated the possible experimental space to find better solution considering the relative concentration of the phylogenetic groups of primers. Using the clustalw algorithm we constructed the corresponding phylogenetic trees for both the forward and the reverse primer sets (Figure 4.).

A.



B.

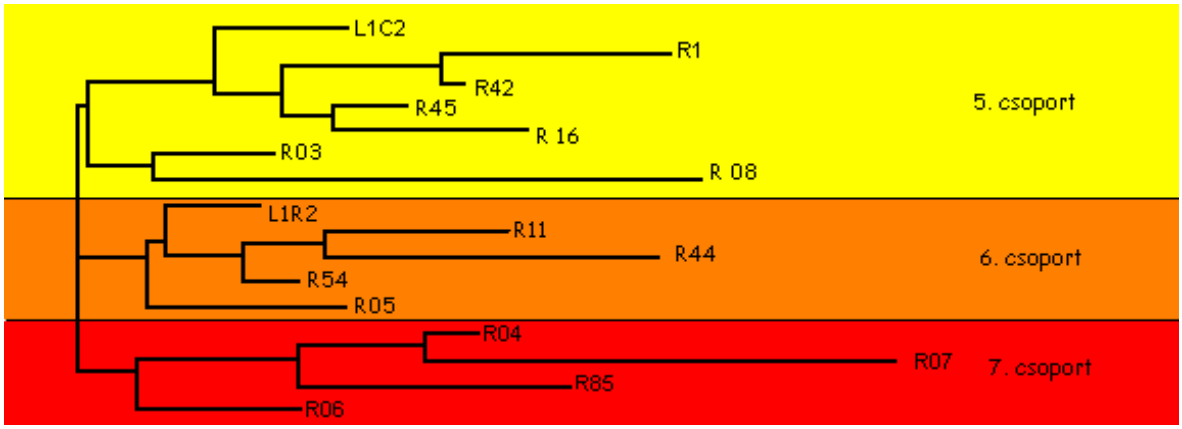


Figure 4. Panel A. ClustalW phylogenetic tree of the primers and the groups formed on the basis of their phylogenetic relatedness. Panel A. Forward primerset. Panel B. Reverse primerset.

In the next step the optimization experiments were carried out to access the optimum conditions to achieve the highest sensitivity over several genotypes. We used the Taguchi design to set up the experiment, however because the Taguchi optimization calculations are based on the assumption of the low factor interactions, and this requirements is no realistic in our system (see above) we used a slightly modified the evaluation of the method. We carried out the experiments devised by the method, however we evaluated the results

directly from these experiments, choosing the best experiment as an optimum. The Taguchi design uses orthogonal matrixes to separate the experiments evenly in the experimental space, by this way effectively sampling the possible experimental space. In several other cases we have found (data no shown) that this method is sensitive to find both local and global optimums of the systems in question. Both local and global optimum solution could be further mapped by setting up the factors around of the found optimum. However, usually only one step of optimization is needed to find a satisfactory solution. The experiments were carried out according as described in the Material and Methods section.

The results are summarized on Table 9.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
6	61	35	46	33	31	23	33	13	27	40	33	3	23	47	16	4	52	11
	9	5	7	neg	neg	neg	8	neg	neg	7	5	neg	4	9	neg	neg	5	neg
	neg	neg	3	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
11	32	49	34	29	53	6	77	3	45	75	35	6	62	34	14	4	92	30
	4	5	8	neg	6	neg	15	neg	3	7	3	neg	6	8	neg	2	18	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
16	19	11	25	15	15	4	16	5	16	23	19	4	15	23	3	2	26	5
	neg	neg	5	3	neg	neg	4	neg	3	3	3	neg	neg	4	neg	neg	neg	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
18	31	12	30	16	46	13	36	9	55	66	53	18	53	66	10	7	71	21
	5	neg	12	neg	7	neg	7	neg	11	32	6	neg	11	10	neg	neg	24	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	4	neg	neg	neg	neg	neg	neg	4	neg
31	48	31	42	11	22	6	43	6	27	46	29	3	27	19	2	2	23	3
	5	3	5	neg	4	neg	5	neg	neg	15	4	neg	7	10	neg	neg	7	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
33	120	84	122	83	108	74	101	39	92	107	77	27	83	107	16	11	107	19
	47	18	80	16	43	32	29	10	22	38	22	neg	22	33	4	3	26	3
	neg	neg	3	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
35	53	35	44	11	34	13	78	12	35	144	30	9	47	59	18	5	60	6
	4	4	4	neg	neg	neg	5	neg	4	13	4	neg	5	14	neg	neg	8	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
39	93	75	87	31	73	58	84	41	82	122	97	17	112	99	19	13	85	50
	10	6	27	3	21	6	16	4	11	47	11	2	32	16	3	2	15	3
	3	neg	7	neg	12	3	neg	neg	neg	neg	neg	neg	neg	5	neg	neg	4	neg
42	167	168	127	161	151	135	169	162	131	158	164	96	156	158	134	70	137	117
	66	58	103	60	48	22	78	52	32	74	70	17	56	100	12	9	73	9
	8	4	6	3	5	neg	5	neg	4	7	5	neg	4	7	2	neg	10	neg
43	68	47	45	20	35	18	53	50	24	60	55	10	66	87	15	8	56	7
	4	4	6	neg	3	3	4	3	neg	6	7	neg	9	7	neg	neg	neg	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
44	83	61	83	62	85	99	79	43	43	74	60	12	84	38	1	1	1	1
	44	11	27	9	17	15	22	7	10	34	16	3	58	32	11	3	10	4
	4	neg	4	neg	neg	neg	3	neg	neg	3	neg	neg	neg	3	neg	neg	neg	neg
45	133	87	129	100	148	123	78	74	95	133	111	36	148	140	80	27	151	40
	6	8	38	6	26	5	10	3	5	25	13	neg	29	36	8	neg	38	4
	2	2	2	neg	7	neg	neg	neg	4	3	neg	neg	3	3	neg	neg	4	neg
51	102	83	100	87	119	96	97	60	74	126	97	35	81	120	45	24	115	49
	7	9	30	6	42	15	16	5	9	40	11	4	23	29	6	4	24	3
	neg	neg	6	neg	neg	neg	neg	neg	neg	4	neg	neg	6	4	neg	neg	neg	neg
52	107	62	73	60	38	36	64	77	68	149	120	70	115	120	66	62	122	38
	12	8	28	8	7	9	16	9	17	49	22	7	12	40	8	5	19	4
	neg	neg	neg	neg	neg	neg	neg	neg	neg	5	neg	neg	neg	3	neg	neg	neg	neg
56	62	28	83	29	65	67	63	31	50	114	36	18	58	81	19	23	72	11
	8	7	17	neg	13	6	8	3	6	20	10	3	12	10	4	4	13	neg
	neg	neg	neg	neg	4	neg	neg	neg	neg	neg	neg	neg	neg	5	neg	neg	neg	neg
58	47	25	56	17	42	25	58	33	35	79	36	18	58	37	18	8	65	11
	7	4	22	4	32	13	6	5	neg	19	neg	neg	5	6	neg	neg	11	neg
	neg	neg	5	neg	neg	neg	neg	neg	neg	4	neg	neg	neg	3	neg	neg	4	neg
59	55	26	67	21	43	30	64	40	55	71	61	11	46	89	13	11	84	10
	4	neg	23	neg	4	6	6	neg	5	12	neg	neg	neg	4	3	neg	10	neg
	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
66	102	87	108	67	115	123	92	69	93	112	100	16	71	98	29	22	105	37
	23	6	41	4	66	40	17	4	16	36	20	neg	19	22	3	4	17	3
	neg	neg	neg	neg	4	5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
68	51	58	79	40	90	64	55	31	31	85	36	9	37	33	20	7	58	21
	10	8	20	3	5	5	19	5	8	17	3	3	13	12	5	neg	7	neg
	neg	neg	3	neg	4	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

Table 9. The results of the Taguchi optimization. The rows are the dilutions of the detected genotypes (three dilutions for each). The fold signals were calculated over the

corresponding genotype specific non-template controls signal. The sensitivity is color coded, better sensitivity than the benchmark is red, lower sensitivity is blue and the approximately the same sensitivity is white. The first column is the benchmark experiment. According to the results the optimum solution (experiment 3) is slightly better than the benchmark.

According to the results there is a slightly better solution than the benchmark setup, as on Table 4, this is an experiment with reduced amount of primers in several primer groups. It was discovered in other systems (at low multiplicity) that the primer concentration is usually have a concentration optimum (Linz et al., 1990), however in such highly multiplex primers sets this is the first proof.

Evaluation of amplification

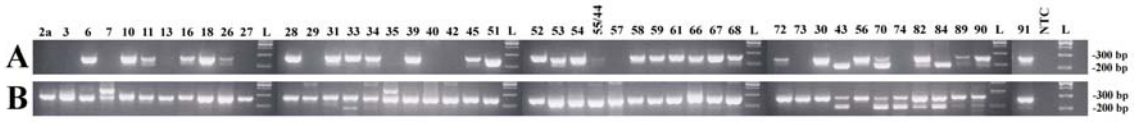
We compared the Yoshikawa system, using the original reaction conditions as described (Yoshikawa et al., 1991), to our L1F/L1R system. We tested the clinically important HPV genotypes in dilution series (HPV low-risk: 6, 11, 42, 55/44, HPV high-risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 66, 68) in order to compare the differences in sensitivity (Table 10). When we compared the lowest detectable copy numbers/reaction, the L1F/L1R system proved to be more sensitive with orders of magnitude.

HPV type	Yoshikawa-PCR	L1F/L1R
6	$1.6x10^5$	16
11	$7.4x10^7$	7.4
42	$7x10^7$	70.5
55/44	$1.2x10^9$	116
16	$9.5x10^5$	97
18	$7x10^2$	7
31	$6.5x10^5$	6.5
33	$1.6x10^3$	16
35	$1.3x10^7$	13.5
39	$2.2x10^4$	22.5
45	$3.0x10^6$	30.5
51	$7.25x10^3$	7
52	$2.7x10^6$	26.5
58	$9.5x10^2$	9.5
59	$2.9x10^3$	2.95
66	$7.5x10^5$	7.5
68	$2.6x10^5$	26.3

Clinically important genotypes were analyzed for sensitivity by both L1C-PCR and L1F/L1R systems. The numbers represent the lowest detectable copy numbers/reaction. The L1F/L1R system proved to be more sensitive with orders of magnitude.

Table 10. The lowest detectable copy numbers by L1C-PCR and L1F/L1R systems.

Both systems were evaluated to assess the genotype spectrum of the reactions. This latter was carried out detecting 46 genotypes at high copy number. The Yoshikawa system could not amplify or poorly amplified a number of HPV genotypes (e.g. 2a, 3, 7, 13, 27, 29, 35, 40, 42, 43, 55/44, 57, 73, 74, 84), while the L1F/L1R had a well balanced and much more sensitive amplification compared to the L1C-PCR (Figure 5).



Panel A shows the results obtained by the Yoshikawa system, panel B shows results obtained by the L1F/L1R amplification system. L is a 100 bp DNA ladder and NTC is for no template control.

The following HPV DNAs were from cloned genomes: 2a, 3, 6, 7, 10 , 11, 13, 16, 18, 26, 27, 28, 29, 31, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55/44, 57, 58, 59, 61, 66, 67, 68, 72, 73(MM9), and from clinical samples (containing internal control DNA, lower band): 30, 43, 56, 70, 74, 82(MM4), 84(MM8), 89, 90, 91.

The Yoshikawa PCR system could not amplify or poorly amplified a number of HPV types (e.g. 2a, 3, 7, 13, 27, 29, 35, 40, 42, 43, 55/44, 57, 73, 74, 84) while the L1F/L1R had a well balanced and much more sensitive amplification compared to the Yoshikawa PCR.

Figure 5. Comparison of amplification with the Yoshikawa PCR and L1F/L1R (L1F/L1R) systems.

The Yoshikawa PCR system showed a significantly lower sensitivity for the low-risk genotypes generally e.g. HPV 11, HPV 42, HPV 44/55 and for some high-risk genotypes (HPV 16, HPV 35), these could be detected only at very high copy numbers which is clinically unacceptable. Considering that clinical samples could contain only a small

number of HPV infected cells, the general level of sensitivity of the L1C-PCR was also too low.

Specificity of the amplification was tested on several genital pathogenic agents, such as: Herpes simplex virus, Chlamydia trachomatis, Ureaplasma urealyticum, Neisseria gonorrhoeae, and Mycoplasma genitalium. The L1F/L1R system showed amplification only for HPV (data not shown).

Genus-specific detection

For clinical applications it is advantageous if general detection of HPV amplicon is also possible. The amplification systems frequently cover a much broader genotype range than the detection systems, so the uncovered genotypes could be detected only by general detection of the amplicon. This helps to avoid the technically false negative results, moreover the detection of these genotypes might be clinically important. The general detection and genotyping of the same amplicon must produce concordant results, which virtually eliminates detection errors.

The L1F/L1R amplicon contains a conservative region that allows designing genus-specific probes. Using sequence alignments we devised a probe mixture containing 16 oligonucleotide probes (Table 11).

KP1	CGCACCAACATATTTTATTATGG
KP2	CGCACAAGCATCTATTATTATGC
KP3	CGCACAAGCATATTTTATCATGC
KP4	CGCACCAGTATATTTTATCATGC
KP5	CGCACAAGCATTTACTATCATGC
KP6	CGCACCAACTACTTTTACCATGC
KP7	CGTACCAGTATTTTCTACCACGC
KP8	CGCACAGGCATATATTACTATGC
KP9	CGCACCAACATATATTATCATGC
KP10	CGTACCAACCTGTACTATTATGG
KP11	CGCACCAACTTATTTTACCATGC
KP12	CGCACCAACCTCTTTTATTATGG
KP13	AGCACAAATATATATTATTATGG
KP14	CGCACCGGATATATTACTATGCA
KP15	CGCACAAATATTTATTATTATGC
KP16	CGGACGAATGTTTATTACCATGG

Table 11. Genus specific hybridization probe set.

Based on Chan et al. (Chan et al., 1995) we chose one HPV type from each Group (A1-A11) of Supergroup A to test the performance of the genus-specific hybridization. The results (Table 12) indicate that the genus-specific hybridization could detect all HPV genotypes representing all phylogenetic groups with very similar sensitivities.

Group	HPV type	Hybridization signal with HPV(1-16) genus-specific probe mix
A1	HPV42	42310
A2	HPV3	26097
A3	HPV72	32053
A4	HPV27	25114
A5	HPV26	32739
A6	HPV53	40403
A7	HPV45	47049
A8	HPV40	31035
A9	HPV67	30267
A10	HPV13	26182
A11	HPV34	12666
	NTC	892

For the genotypes shown in the table the hybridization signals are given in RFU. NTC is for no template control representing the background signal of hybridization.

Table 12. Hybridization signal with HPV(1-16) genus-specific probe mix.

Genotype- specific detection

The differences in DNA genomic sequences and the probes are shown in Table 13. The probes were designed for a unique highly diverse region, which is nested by conserved regions. As shown, several micro- insertions and/or deletions improved the diversity of the regions. This allows highly specific probe design for each genotype.

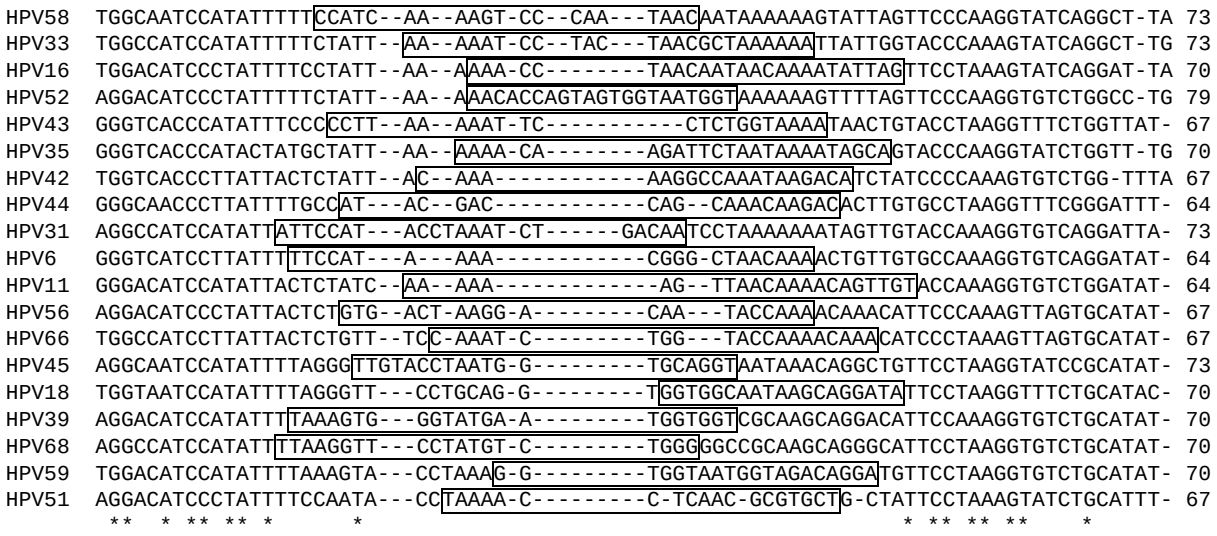
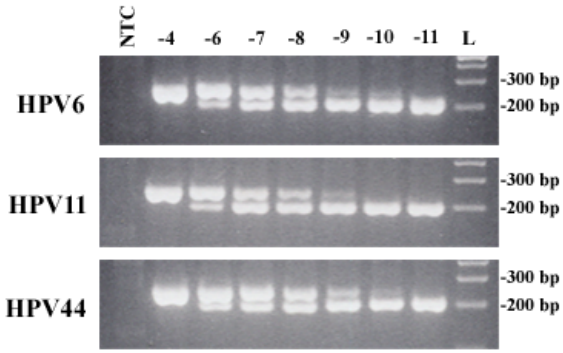


Table 13. The alignment of the genotype specific probes of HPV L1F/L1R amplicons of clinically important genotypes. The boxes emphasize the specific probes, "-" stands for gaps in the alignment, "*" shows the consensus. The numbers at the end of each row represent the length of the sequences.

Chan et al. (Chan et al., 1995) compared a 291 bp conserved region from a 460 bp region of the L1 gene amplified by the MY09-MY11 primer pair in order to show phylogenetic relationship among 95 HPV types. Based on this data we chose the HPV types 6, 11, 44 from the Group A10 in order to test the performances of our hybridization system. We used these genotypes because they are highly similar to each other (Chan et al., 1995). To test the discrimination between these closely related genotypes we amplified a serial dilution of these genotypes with a sample containing internal control DNA and the tested DNA. Each PCR product was hybridized with the HPV 6, 11, 44 type specific oligos. Crosshybridization levels were assessed by comparing the hybridization signals with matching and not matching oligos. All genotypes discriminated from the others, e.g. the 5 mismatches that occur between the HPV 6 DNA and the HPV 11 probe were discriminated perfectly even in case of high copy number samples (Figure 6). Based on these experiments we generally applied this minimum 5 base mismatch rule during the design of the probes as criteria to design specific probes.



B

Serial dilution of **HPV 6 DNA**		NTC	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
Probe	**HPV6P**	**694**	**39935**	**30939**	**22365**	**12071**	**2593**	**1945**	**728**
	HPV11P	698	1051	1044	893	718	644	662	668
	HPV44P	713	706	669	674	702	686	713	740

Serial dilution of **HPV 11DNA**		NTC	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
Probe	HPV6P	791	747	771	720	735	765	721	718
	HPV11P	**813**	**78465**	**77453**	**52804**	**42786**	**17583**	**854**	**748**
	HPV44P	814	823	813	831	827	849	763	879

Serial dilution of **HPV 44 DNA**		NTC	10^{-4}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
Probe	HPV6P	849	912	811	800	756	832	828	866
	HPV11P	804	1046	1003	1056	803	788	769	786
	HPV44P	**862**	**77830**	**75509**	**76036**	**62107**	**35545**	**17457**	**872**

C

```
HPV6     CATCCTTATTTTTCCATAAAACGGGCTAACAAAACTGTTGTGCCAAA
HPV11P   ------------------AAAAAAGTTAACAAAACAGTTGT------
                           ***   * ********* *****
HPV6     CATCCTTATTTTTCCATAAAACGGGCTAACAAAACTGTTGTGCCAAA
HPV44P   ----------------ATACGACCAGCAAACAAGAC-----------
                         ***  **  ** ***** **
HPV11    ACATCCATATTACTCTATCAAAAAAGTTAACAAAACAGTTGTACCAAA
HPV44P   ----------------ATACGACCAGCAAACAAGAC------------
                         **   *  **  ***** **
HPV11    ACATCCATATTACTCTATCAAAAAAGTTAACAAAACAGTTGTACCAAA
HPV6P    ------------TTCCATAAAACGGGCTAACAAA--------------
                     ** ** ***   * *******
HPV44    CAACCCTTATTTTGCCATACGACCAGCAAACAAGACACTTGTGCCTAA
HPV6P    ------------TTCCATAAAACGGGCTAACAAA--------------
                    * *****  **  ** *****
HPV44    CAACCCTTATTTTGCCATACGACCAGCAAACAAGACACTTGTGCCTAA
HPV11P   ------------------AAAAAAGTTAACAAAACAGTTGT------
                           *  **  ***** *** ****
```

Panel A presents a serial dilution of HPV types 6, 11, 44 amplified in the presence of an internal control. The upper band represents the amplicon of the HPV DNA, while the lower band is the amplified internal control. L is a 100 bp DNA ladder and NTC is for no template control. Dilution factor is indicated only by the negative power of dilution (e.g. –4 stands for 10^{-4}, -5 for 10^{-5}, etc.).

Panel B shows the correlation between the hybridization signals and the corresponding serial dilution value. Even for high copy numbers, the probes show positive signals only for their matching genotypes. Samples were considered positive if their value was higher than three times the average of 3 parallel negative control sample values.

Panel C presents the genotype specific probes and the corresponding probe binding regions of the other genotypes. At least five mismatches exist for each genotype. Probe sequences are presented in "bold face" below the type specific sequence, while "*" shows the matching bases.

Figure 6. Hybridization specificity for HPV 6, 11, 44.

Sequences containing the L1F/L1R amplicons of the theoretically amplified 52 HPV genotypes were aligned to determine the specificity of type-specific oligonucleotide probes. Except the very closely related (Menzo et al., 2001) low-risk genotypes HPV 44 (Lorincz et al., 1992)and HPV 55 (Favre et al., 1990), all genotype-specific oligonucleotide probes contained five or more mismatches with the probe-binding region.

Clinical evaluation

Clinical performances of our system and the Hybrid Capture II test were compared on a set of 81 samples that have been issued as ASCUS or higher. The study design was to compare the detection capabilities of several clinically relevant, high-risk genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in both systems. In the cases of conflicting classification sequencing was carried out to clarify discordant results. Additionally we genotyped the low-risk and the high-risk samples.

After the first round of analyses we found concordant positive samples for both systems in 28 cases, while 40 samples were concordant negative. There were 6 samples which were L1F/L1R positive- HCII negative, and 7 samples L1F/L1R negative- HCII positive (Table 14.).

	L1F/L1R positive	**L1F/L1R negative**	**Total**
HCII positive	28	7	35
HCII negative	6	40	46
Total	34	47	81

Table 14. Results of the comparison between L1F/L1R and HCII systems. There was a good agreement of 83.9% (68 of 81 samples) between the two systems (kappa value=0,67, McNemar's chi square, P<0,0001; Q=0.65, Yule test).

There was a good agreement of 83.9% (68 of 81 samples) between the two systems (kappa value=0,67, McNemar's chi square P<0,0001; Q=0.65, Yule test).
After this first step samples showing discordant results were sequenced in order to establish true high-risk positivity/negativity. Analysis showed that in 10 of 13 discordant cases L1F/L1R had indicated the correct results, 3 of 13 discordant samples were considered as L1F/L1R false positive cases. There were no L1F/L1R false negative samples (Table 15) and there was an excellent agreement of 96.2% (78 of 81 samples) between the two systems (p=2,58*10-19, two-tailed Fisher Exact Probability Test).

	L1F/L1R positive	**L1F/L1R negative**	**Total**
Consensus positive	31	0	31
Consensus negative	3	47	50
Total	34	47	81

Table 15. Results of the comparison between L1F/L1R and HCII systems considering sequencing data of the previously discordant results. There was an excellent agreement of 96.2% (78 of 81 samples) between the two systems (p=2,58*10-19, two-tailed Fisher Exact Probability Test). L1F/L1R's estimated sensitivity was 91,1% (31/34) and estimated specificity was 100% (47/47).

L1F/L1R's estimated sensitivity was 91,1% (31/34) and estimated specificity was 100% (47/47). The discordant results are based on the higher sensitivity attributable to the PCR technique used by the L1F/L1R test and also on the already known cross-reactions of HCII (HPV 6, 42, 53) . We have findings showing HCII cross-reactions with other genotypes (HPV 30, 82) as well, but this was not further investigated.

After additional tests (semiquantitative determination of the amount of HPV DNA, individual genotyping, and also additional analytical data) the 3 false positive samples may be classified differently. We tried to detect a cross-reaction between HPV 90 and HPV 45 (HPV 90 was proved to be present by sequencing, where HPV 45 was detected by the L1F/L1R system), but we were not able to detect any cross-reaction using pure DNA. As a conclusion both HPV 90 and HPV 45 DNA were probably present in the sample and sequencing could not provide information about the minor components of the mixed infections.

In order to further test the L1F/L1R system, samples issued as ASCUS from a cohort of 360 persons were tested. There were 198 negative samples, while 162 were positive, of which 120 samples showed to contain only one HPV type, 33 cases showed infection with 2 HPV types, and in 9 cases multiple infections with 3 HPV types was found. The most frequent type was HPV 16, which was encountered in 36 cases, followed by HPV type 31 in 23 cases. 19 cases presented infections with low-risk HPV types (Figure 7). We compared our results with data obtained by Coutlee et al. (Coutlee et al., 2002)by superimposing the percentages from prevalence of 14 high-risk HPV types, and we found similar distributions, slightly influenced probably by the populational differences (Figure 8). Statistical analysis shows strong correlation between the results of the two systems. Both the Wilcoxon signed-ranks test ($t= 1.005398$, $P< 0.05$) and the Mann-Whitney U-test ($z= -0.781111$, $P< 0.05$) show high concordance between the two systems.

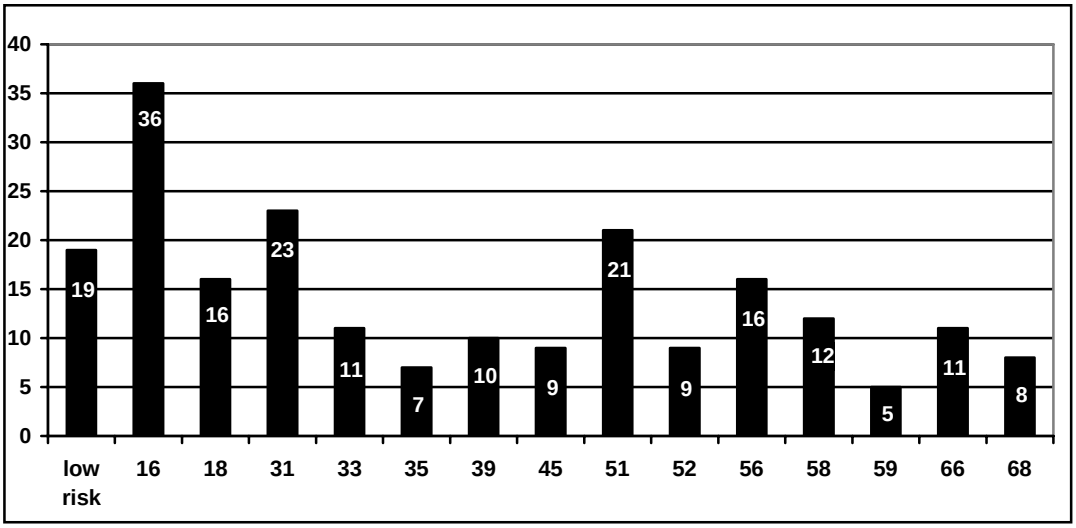


Figure 7. Prevalence of the different HPV genotypes among the 162 positive samples, where genotyping was performed. Numbers within the columns count the appearances of the different genotypes in the examined population.

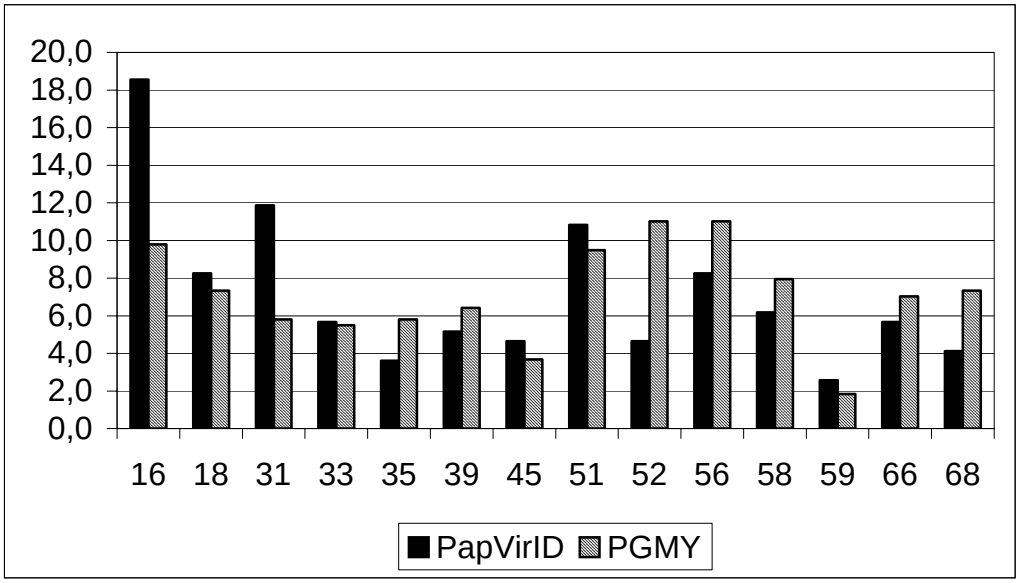


Figure 8. Comparison of L1F/L1R and PGMY, data superimposed by the percentage of frequencies of the indicated high-risk HPV types. The two systems show similar distributions of high-risk HPV types. Statistical analysis shows strong correlation, both the Wilcoxon signed-ranks test ($t= 1.005398$, $P< 0.05$) and the Mann-Whitney U-test ($z= -0.781111$, $P< 0.05$) indicate high concordance between the two systems.

In the cervical samples of our 2,215 patients with PID (Group 1) the most prevalent agent was HPV as it was detected in 33.74%. In patients without signs or symptoms of PID (Group 2), similarly to group HPV was the most frequently detected agent, its frequency (26.40%) was significantly lower than in Group 1 ($p<0.001$).

The age-distribution of women with cervical HPV infection was similar in both groups. The age distribution of patients with cervical HPV infection from both groups showed a peak of 32.2% at 20-24 years of age and almost 60% of the patients were between 20 and

29 years old. High risk HPV types were identified in 75.21% of HPV-positive patients if they suffered from PID, and 66.86% if they did not suffer from this disease ($p<0.001$).

Discussion

Diagnosis of HPV infection requires the specific and sensitive detection of viral DNA. The L1 region of the HPV genome has been used for the development of widely applied and general PCR primer sets, such as MY09/11, PGMY, SPF and GP5+/6+ (Kleter et al., 1999). In this article we described a novel human papillomavirus detection method, which shows significant improvements over the previously published methods. Sequence database searching has revealed a highly variable and a highly conserved region in the L1 gene of HPV genomes in close proximity. These regions were flanked by a known consensus amplification primer set (Yoshikawa et al., 1991), which amplicon was used to develop the final system. To achieve sensitive, not biased amplification of the genotypes, a multiprimer consensus primer system was devised. The highly specific genotyping and the simultaneous general detection of HPV DNA are mandatory to design clinically relevant HPV detection tests.

Balanced amplification is not thoroughly addressed in the literature, but the consensus amplification is widely studied. Single consensus primer pair system and multiprimer system are the two basic approaches. One primer pair approach is exemplified by the GP5+/6+ system, which shows highly sensitive amplification for several genotypes due to its short amplicon and the high priming efficiency of the primers, but its sensitivity is biased for some genotypes (Qu et al., 1997). This also influences the detection of the multiple HPV infections and overall the two effects significantly alter the prevalence of detected HPV genotypes (Kleter et al., 1999; Qu et al., 1997). This unbalanced behavior cannot be changed when only two primers are used.

The other approaches e.g. MY09/11, PGMY and SPF use several primers to cover the sequence variations occurring at the primer binding regions. To produce large sets of different primers degenerate synthesis was applied in case of the MY09/11 system. This approach has been proved to be inferior compared to a mixture of individual primers, based on Qu (Qu et al., 1997) and Gravitt (Gravitt et al., 2000). This is underlined by the introduction of PGMY system, a redesigned version of the MY09/11 primer set, where a

mixture of individual primers was used and this change resulted in significant improvements (Coutlee et al., 2002).

Recent advances in HPV vaccination (Munoz et al., 2004) (Berencsi et al., 2006) require the exact genotyping favorably based on those regions of the viral nucleic acid, which are evolutionary responsible for genotype-specific segregations. Given that the crystallography model predicts the hypervariable regions of the L1 protein to be displayed as surface-exposed loops (Chen et al., 2000)and that the loops are highly immunogenic, it is quite likely that the loop epitopes are the determinants of genotype-specificity. The genotype specific probes hybridize to one of these hypervariable regions. The goal is to harness the close relatedness of the genotype specific sequence regions and the biological niche of the genotype to provide more relevant clinical information. This is highly stressed for vaccination, where a prior natural infection may exclude the administration of the vaccine. One of the hypervariable regions (Olcese et al., 2004)was also used to design genotype-specific detection in case of the GP5+/6+ system, but neither the MY09/11 nor the SPF system use those.

Nevertheless the clinical application of a HPV detection system requires sensitive and specific differentiation of the genotypes. Using highly stringent conditions the normally occurring sequence variants of the HPV genotypes could cause detection problems. Stewart et al. (Stewart et al., 1996) discussed the effect of these variants on the performance of the detection in case of both MY09/11 and GP5+/6+ systems. They concluded that at the applied stringency both systems would miss the detection of some sequence variants. As a solution more than one probe (Stewart et al., 1996)could be used to cover the variations of a given genotype, but the yet unknown sequence variants could make it worse by introducing further diversity. It is also published (Stewart et al., 1996)that these variations in sequence could have diverse geographical distributions, which could influence the applicability of a given test in a country where it was not previously evaluated.

The parallel use of genus- and genotype specific detections improves overall reliability of the detection. Moreover the genus specific approach itself allows the detection of new, unknown HPV types, and provides semi-quantitative information on the HPV DNA

amount. In our practice we have found concordant genus and genotype specific signals in the cases of single infections, which is possibly a tool to rule out multiple infections.

The specific detection of the genotypes is also important in case of multiple infections, which are highly prevalent in the infected population. Tests that could not discriminate all the genotypes correctly should not be advised for clinical application. Using them one could not follow up the history of the infections correctly, which could be an essential prognostic information (Kleter et al., 1999).

The hybridization of the genotype specific probes to the highly diverse region of L1F/L1R amplicon allows the detection at medium stringency with high specificity (Figure 6). Variations in sequence would be tolerated up to 2 mismatches. Given that genotype diversity is attributed to small deletions and insertions (Table 13) and much of the natural variants have base changes, this still makes possible the design of genotype specific probes. The general detection probes have the same 2-3 mismatches and by this approach 16 probes detect 52 genotypes with balanced signals, as exemplified on Table 12. Even this approach could fail in cases where the sequence variation differs significantly from the original sequence, but for known changes new probes could be introduced, these probes would further extend the detection range at medium stringency. In summary, the devised approach would combine the specificity and the tolerance for sequence variations.

The achievable performance was evaluated using a modified Taguchi optimization method. According to the results there is a slightly better solution than the benchmark (L1F/L1R) setup. As it is proved in other systems (at low multiplicity) that the primer concentration is usually has a concentration optimum (Linz et al., 1990), however in such highly multiplex primers sets our results indicating the same, which indicates the affect of the concentration of primers on amplification efficacy, regardless of primers sequences specificity.

The benchmark system was developed in a stepwise manner and its performance is excellent providing sensitivity below 100 copies per reaction for more than twenty genotypes. The development of these systems took a considerably long developmental time, however the Taguchi optimization achieved a slightly better solution in only one experiment proving its extraordinary efficacy. We used this experiment to evaluate our previous benchmark reaction, if we would have found a more advantageous setup.

Although there is a better solution, but this is only slightly better in sensitivity and other factors, namely the expertise what we already have with the benchmark reaction (over several thousand reaction were done), its robustness, stability balance this result, and makes the original reaction more preferable.

We compared the L1F/L1R system to Hybrid Capture II (HCII) to assess clinical performances. HCII has a high reputation in this field, and the wide clinical application of this test makes it a standard in many aspects. The two systems gave concordant (either positive or negative) results in 68 of 81 cases. For the establishment of high-risk true positivity/negativity among the discordant results we proceed the direct sequencing of the PCR products. The inherent limitation of this approach is the inability to detect multiple infections correctly, the sequencing would reveal the sequence of the high abundance genotype only. Nevertheless we have not used PCR approaches for the validation, because they could have biased amplification sensitivities and this could add more uncertainty. Taking together the original HCII and the sequencing results the analysis revealed excellent performance of the L1F/L1R (Tables 7-8), 78 of 81 samples being concordant.

We determined the prevalence of the genotypes on a cohort of 360 persons, as shown on Figure 7-8. A very similar distribution of genotypes was published by Coutlee at al. (Coutlee et al., 2002)using the PGMY system, statistical analysis indicating close concordance between the two systems. In the future we plan to carry out a more comprehensive comparison of the two systems.

We developed a HPV detection and genotyping method that in several aspects performs better than the previously published systems. The clinical application of these diagnostic procedures is constantly expanding, national authorities consider the introduction or already introduced the HPV testing in screening programs to improve the effectiveness of such preventive measures. Epidemiologic studies report that countries where HPV screening has been implemented, the incidence of cervical cancer has shown decreasing tendencies (Berkhof et al., 2005). Vaccination soon would further increase the demand for this testing. With this background the improvement of the current diagnostic procedures is mandatory to eliminate the still existing problems.

HPV is mostly sexually transmitted, and its spread may be associated with the transmission of other agents that can cause pelvic inflammatory disease (PID) with complaints serious enough to force the patients to seek medical help. It has been demonstrated that CIN occurs more frequently in women treated for PID, our study was aimed to detect a possible association of PID with cervical HPV infection.

In patients with PID the prevalence of cervical HPV infection was found to be significantly higher than a patient without PID. This finding was expected, since sexual behavior is an epidemiological factor for both PID and cervical HPV infection. The prevalence of high-risk HPV types in women with PID was found to be significantly higher than in those who had no signs or symptoms of PID ($p<0.001$).

Conclusions

1. A new experimental design method was devised in the development of new human diagnostic devices. To demonstrate the application of Taguchi optimization, the examples of an ELISA and a PCR method were used. In the field of ELISA the optimization using an experimental design method a modified Taguchi method is proposed. This can be used to compare the net effects of different conditions which can be both qualitative and quantitative in nature. The method reduces the effects of the interactions of the optimized variables making it possible to access the optimum conditions even in cases where there are large interactions between the variables of the assay. The method is fast, accessing the results in one step, compared to the traditional, time-consuming 'one-step-at-a-time' method. We exemplify the procedure with methods to optimize the detection of ScFv single chain .fragment of variable phages by ELISA and a PCR system.
2. The efficacy of the Taguchi method is further improved by a special assignment of factors, which makes possible to calculate the biochemical parameters of the ELISA procedure carried out under optimum conditions. Thus, the calibration curve, the sensitivity of the optimum assay, the intra-assay and inter-assay variability can be estimated.
3. The optimum primerset concentration in the case L1F/L1R PCR system, which is a highly multiplex PCR method detecting human papillomavirus is proved, with an improved Taguchi optimization.
4. A new PCR based detection system was developed which detects a broader spectrum of HPV genotypes, is dynamic to recognize newly emerging HPV types, yields a balanced amplification and features an automatizable genotyping method (hybridization). The Yoshikawa L1 (Yoshikawa et al., 1991) amplicon sequence itself is suitable for consensus amplification of HPV genotypes and it is also possible to design both separate genus and genotype specific probes to the same amplicon. So we created a new PCR detection system, using an

optimized primer set for amplification and an automatizable genotyping approach.

5. We compared it to other systems and also tested its analytical and clinical performances. The method is based on a new L1F/L1R primerset, where the primers are independently synthesized molecules, therefore their relative ratio can be easily controlled and optimized. During the development of the method key target was the clinically desirable balanced sensitivity of amplification. The novel approach using two separate regions for the genus- and genotype specific detection of the amplicons allows highly optimized probe design for both applications.
6. We compared the L1F/L1R system to Hybrid Capture II (HCII) to assess clinical performances. HCII has a high reputation in this field, and the wide clinical application of this test makes it a standard in many aspects. This clinical evaluation study revealed excellent performance of the L1F/L1R for the examined parameters. L1F/L1R's estimated sensitivity was 91,1% and estimated specificity was 100%. Taking together the original HCII and the verifying sequencing results the analysis revealed excellent performance of the L1F/L1R (Tables 7-8), 78 of 81 samples being concordant.
7. As a part of the clinical evaluation the pelvic inflammatory disease studied as a predictor of cervical cancer. Although HPV spreads together with agents causing pelvic inflammatory disease (PID) with complaints forcing the patient to seek medical advice, PID has not yet been evaluated as a predictor of cervical cancer. The study aimed to determine the relationship between PID and HPV in order to evaluate the possible risk factor role of PID for cervical cancer. As a part of the clinical evaluation the pelvic inflammatory disease as a risk factor were studied: (i) 2,215 women with PID; (ii) 4,217 women participating in a cervical cancer screening program who were found to have cytological atypia, mucopurulent cervicitis or other colposcopically detected disorders but were free of symptoms of PID were examined. The presence of HPV and other STD agents in cervical smears was detected with L1F/L1R reaction and other PCR

methods. HPV prevalence was 33.74% in patients with PID and 26.40% in the group of women without PID ($p < 0.001$). This suggests that patients suffering from PID apparently have a higher risk for aquiration of HPV infections.

Summary

By introducing the Taguchi method to optimize ELISA procedures we demonstrate the advantages of this approach over the more traditional labor-intensive methods. We have described a new experimental design method, which better handles the basic parameters of the underlying system: the calibration curve, the sensitivity of the optimum assay, the intra-assay and inter-assay variability can be directly estimated. The modified strategy was further exemplified by the demonstration of the existence of a general primer optimum in a highly multiplex PCR reaction. On the basis of these work we developed a new detection method (L1F/L1R) that is suitable for sensitive and balanced amplification and specific genotyping of HPV DNA from clinical samples. It amplifies 46 HPV genotypes: 2a, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 28, 29, 30, 31, 33, 34, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 55/44, 56, 57, 58, 59, 61, 66, 67, 68, 70, 72, 73(MM9), 74, 82(MM4), 84(MM8), 89, 90, 91. Key elements of the L1F/L1R system: the special selection of the amplified region, a novel and optimized amplification primer set, circumspectly designed genus and genotype specific oligonucleotide probes. The detection is based on solid phase hybridization in microtiter plate format using geno- and type specific probes at medium stringency, which makes the detection robust in case of small sequence variants. The assay is highly reproducible and suitable for automatization. The method was compared to Hybrid Capture II test. This study revealed excellent performance of the L1F/L1R for the examined parameters. L1F/L1R's estimated sensitivity was 91,1% and estimated specificity was 100%. As a part of the clinical evaluation the pelvic inflammatory disease as a risk factor were studied: (i) 2,215 women with PID; (ii) 4,217 women participating in a cervical cancer screening program who were found to have cytological atypia, mucopurulent cervicitis or other colposcopically detected disorders but were free of symptoms of PID were examined. The presence of HPV and other STD agents in cervical smears was detected with L1F/L1R reaction and other PCR methods. HPV prevalence was 33.74% in patients with PID and 26.40% in the group of women without PID ($p < 0.001$). This

suggests that patients suffering from PID apparently have a higher risk for aquiration of HPV infections.

Összefoglaló

A Taguchi kísérlet tercezési módszer bevezetése az ELISA eljárások optimalizálására bizonyította, hogy az új módszer hatékonyabb, mint a régebbi bonyolultabb eljárások. Egy olyan új eljárást vezettünk be, ami jobban képes megragadni az optimalizálandó rendszer alapvető analitikai paramétereit, így a kalibrációs görbe, az optimált eljárás szenzitivitása, a vizsgálaton belüli és vizsgálatok közötti variancia közvetlenül megbecsülhető. A módosított stratégia hatékonysága további példákkal is bizonyítható, így, erősen multiplex PCR reakció esetén, a konszenzus primerek optimumának léte is bizonyítható volt. Ezen eredmények alapján egy új humán papillómavírus (HPV) detektáló és genotipizáló rendszer került kifejlesztésre (L1F/L1R), amely teljesíti az érzékeny és kiegyensúlyozott amplifikáció és specifikus genotipizálás követelményeit, klinikai mintákon. A módszer 46 HPV genotípust amplifikál: 2a, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 28, 29, 30, 31, 33, 34, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 55/44, 56, 57, 58, 59, 61, 66, 67, 68, 70, 72, 73(MM9), 74, 82(MM4), 84(MM8), 89, 90, 91. A L1F/L1R rendszer kulcselemei, az amplifikációs terület gondos kiválasztása, az új és optimalizált primer rendszer, a körültekintően tervezet genusz és genotípus specifikus oligonukleotid próbák. A detektálás szilárdfázisú, mikrotiter lemez formátumú, közepes specificitású hibridizálás, amely az igen specifikus genusz és genotípus próbákkal együtt lehetővé teszi, hogy a rendszer toleráns legyen a kisebb szekvencia variánsokkal szemben. A módszer megbízhatósága igen jó és automatizálható. Az eljárást összehasonlítottuk a Hybrid Capture II eljárással. Az L1F/L1R eljárás jól teljesített az összes vizsgált paraméter tekintetében. A L1F/L1R eljárás érzékenysége 91,1%-ak, míg specificitása 100%-nak adódott. A klinikai vizsgálatok része, hogy a kismedencei gyulladást (PID), mint a cervix carcinóma kockázati tényezőjét vizsgáltuk: (i) 2215 beteget pozitív PID diagnózissal, és (ii) 4217 nőgyógyászati vizsgálaton átesett citológiai diagnózis, mucopurulens cervicitis vagy kolposzkóposan pozitív, de PID negatív nőbeteget vontunk be a vizsgálatba. A HPV és más STD kórokozók kimutatását az L1F/L1R eljárással és más PCR módszerekkel végeztük. A HPV prevalanciája 33,7% volt az első, PID pozitív csoportban, míg 26,4% azoknál a nőbetegeknél, ahol a PID diagnózisa

negatív volt ($p < 0{,}001$). Így feltételezhető, hogy PID esetén magasabb a HPV infekció kockázata.

References

Summary of Safety and Effectivness Data, Digene High-Risk HPV DNA Test, Hybrid Capture 2,.P890064/S009.

Alazawi, W., M. Pett, S. Strauss, R. Moseley, J. Gray, M. Stanley, and N. Coleman. 2004. Genomic imbalances in 70 snap-frozen cervical squamous intraepithelial lesions: associations with lesion grade, state of the HPV16 E2 gene and clinical outcome. *Br J Cancer*. 91:2063-70.

Anderson, L.A., and R.A. McLean. 1974. Univariate Method or One-Factor-at-A-Time in Statistics: Textbooks and Monographs. *In* Design of Experiments, A Realistic Approach. Vol. Vol. 5. M. Dekker, editor, New York. 361 – 362.

Andrew, S.M. 1991. Purification of immunoglobulin G. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. Greene Publishing and Wiley-Interscience, New York. 2.7.1 – 2.7.12.

Berencsi, G., I. Sziller, and M. Melles. 2006. [Prevention of malignant tumors caused by human papilloma virus (HPV) by vaccination and with the methods of classical gynecologic diagnostics]. *Orv Hetil*. 147:1449-53.

Berkhof, J., M.C. de Bruijne, G.D. Zielinski, and C.J. Meijer. 2005. Natural history and screening model for high-risk human papillomavirus infection, neoplasia and cervical cancer in the Netherlands. *Int J Cancer*. 115:268-75.

Bosch, F.X., and N. Munoz. 2002. The viral etiology of cervical cancer. *Virus Res*. 89:183-90.

Burch, G.J., C.H. Ferguson, G. Cartwright, and F.Y. Kwong. 1995. Application of Taguchi experimental design to the optimisation of a baculovirus expression system. *Biochem Soc Trans*. 23:107S.

Carozzi, F., S. Bisanzi, C. Sani, M. Zappa, S. Cecchini, S. Ciatto, and M. Confortini. 2007. Agreement between the AMPLICOR Human Papillomavirus Test and the Hybrid Capture 2 assay in detection of high-risk human papillomavirus and diagnosis of biopsy-confirmed high-grade cervical disease. *J Clin Microbiol*. 45:364-9.

Castellsague, X., and N. Munoz. 2003. Chapter 3: Cofactors in human papillomavirus carcinogenesis--role of parity, oral contraceptives, and tobacco smoking. *J Natl Cancer Inst Monogr*:20-8.

Chan, S.Y., H. Delius, A.L. Halpern, and H.U. Bernard. 1995. Analysis of genomic sequences of 95 papillomavirus types: uniting typing, phylogeny, and taxonomy. *J Virol*. 69:3074-83.

Chard, T. 1997. Characteristics of binding assays sensitivity. *In* Laboratory Techniques in Biochemistry and Molecular Biology. An Introduction to Radioimmunoassay and Related Techniques. R.H. Burdon and P.H. van Knippenberg, editors. Elsevier, Amsterdam. 161 – 174.

Chen, X.S., R.L. Garcea, I. Goldberg, G. Casini, and S.C. Harrison. 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol Cell*. 5:557-67.

Cobb, B.D., and J.M. Clarkson. 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Res*. 22:3801-5.

Cooper, H.M., and Y. Paterson. 1991. Production of antibodies. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. Greene Publishing and Wiley-Interscience, New York. 2.4.1 – 2.4.7.

Coutlee, F., P. Gravitt, J. Kornegay, C. Hankins, H. Richardson, N. Lapointe, H. Voyer, and E. Franco. 2002. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. *J Clin Microbiol*. 40:902-7.

Cox, J.T. 2006. Epidemiology and natural history of HPV. *J Fam Pract*. Suppl:3-9.

Davey, D.D. 2003. Cervical cytology classification and the Bethesda System. *Cancer J*. 9:327-34.

de Roda Husman, A.M., J.M. Walboomers, A.J. van den Brule, C.J. Meijer, and P.J. Snijders. 1995. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol*. 76 (Pt 4):1057-62.

Denny, L.A., and T.C. Wright, Jr. 2005. Human papillomavirus testing and screening. *Best Pract Res Clin Obstet Gynaecol*. 19:501-15.

Duggan, M.A. 2002. A review of the natural history of cervical intraepithelial neoplasia. Vol. Feb. G.T.K. Ryoho, editor. Suppl 1:176-93.

Favre, M., D. Kremsdorf, S. Jablonska, S. Obalek, G. Pehau-Arnaudet, O. Croissant, and G. Orth. 1990. Two new human papillomavirus types (HPV54 and 55) characterized from genital tumours illustrate the plurality of genital HPVs. *Int J Cancer*. 45:40-6.

Fuessel Haws, A.L., Q. He, P.L. Rady, L. Zhang, J. Grady, T.K. Hughes, K. Stisser, R. Konig, and S.K. Tyring. 2004. Nested PCR with the PGMY09/11 and GP5(+)/6(+) primer sets improves detection of HPV DNA in cervical samples. *J Virol Methods*. 122:87-93.

Goldie, S. 2006. A public health approach to cervical cancer control: considerations of screening and vaccination strategies. *Int J Gynaecol Obstet*. 94 Suppl 1:S95-105.

Gravitt, P.E., and M.M. Manos. 1992. Polymerase chain reaction-based methods for the detection of human papillomavirus DNA. *IARC Sci Publ*:121-33.

Gravitt, P.E., C.L. Peyton, T.Q. Alessi, C.M. Wheeler, F. Coutlee, A. Hildesheim, M.H. Schiffman, D.R. Scott, and R.J. Apple. 2000. Improved amplification of genital human papillomaviruses. *J Clin Microbiol*. 38:357-61.

Gravitt, P.E., C.L. Peyton, R.J. Apple, and C.M. Wheeler. 1998. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. *J Clin Microbiol*. 36:3020-7.

Gregoire, L., M. Arella, J. Campione-Piccardo, and W.D. Lancaster. 1989. Amplification of human papillomavirus DNA sequences by using conserved primers. *J Clin Microbiol*. 27:2660-5.

Hernadi, Z., L. Gazdag, K. Szoke, T. Sapy, Z.T. Krasznai, and J. Konya. 2006. Duration of HPV-associated risk for high-grade cervical intraepithelial neoplasia. *Eur J Obstet Gynecol Reprod Biol*. 125:114-9.

Hornback, P. 1991. Enzyme-linked immunosorbent assays. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. Greene Publishing and Wiley-Interscience, New York. 2.1.17 – 2.1.18.

Hubbard, R.A. 2003. Human papillomavirus testing methods. *Arch Pathol Lab Med*. 127:940-5.

Hudelist, G., M. Manavi, K.I. Pischinger, T. Watkins-Riedel, C.F. Singer, E. Kubista, and K.F. Czerwenka. 2004. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. *Gynecol Oncol*. 92:873-80.

Jacobs, M.V., A.M. de Roda Husman, A.J. van den Brule, P.J. Snijders, C.J. Meijer, and J.M. Walboomers. 1995. Group-specific differentiation between high- and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. *J Clin Microbiol*. 33:901-5.

Kiss, M., S. Husz, J. Soos, and A. Dobozy. 1993. [A new method, based on in situ DNA hybridization, for the detection of papillomavirus in condyloma patients and their partners]. *Orv Hetil*. 134:1739-42.

Kleter, B., L.J. van Doorn, L. Schrauwen, A. Molijn, S. Sastrowijoto, J. ter Schegget, J. Lindeman, B. ter Harmsel, M. Burger, and W. Quint. 1999. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol*. 37:2508-17.

Kleter, B., L.J. van Doorn, J. ter Schegget, L. Schrauwen, K. van Krimpen, M. Burger, B. ter Harmsel, and W. Quint. 1998. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol*. 153:1731-9.

Konya, J., G. Veress, A. Juhasz, K. Szarka, T. Sapy, Z. Hernadi, and L. Gergely. 2000. Additional human papillomavirus types detected by the hybrid capture tube test

among samples from women with cytological and colposcopical atypia. *J Clin Microbiol*. 38:408-11.

Kornya, L., I. Cseh, J. Deak, M. Bak, and V. Fulop. 2002. The diagnostics and prevalence of genital human papillomavirus (HPV) infection in Hungary. *Eur J Obstet Gynecol Reprod Biol*. 100:231-6.

Linz, U., U. Delling, and H. Rubsamen-Waigmann. 1990. Systematic studies on parameters influencing the performance of the polymerase chain reaction. *J Clin Chem Clin Biochem*. 28:5-13.

Lorincz, A.T., R. Reid, A.B. Jenson, M.D. Greenberg, W. Lancaster, and R.J. Kurman. 1992. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. *Obstet Gynecol*. 79:328-37.

Mahoney, M.C. 2006. Protecting our patients from HPV and HPV-related diseases: the role of vaccines. *J Fam Pract*. Suppl:10-7.

Martin-Hirsch, P.L., G. Koliopoulos, and E. Paraskevaidis. 2002. Is it now time to evaluate the true accuracy of cervical cytology screening? A review of the literature. *Eur J Gynaecol Oncol*. 23:363-5.

Menzo, S., A. Monachetti, C. Trozzi, A. Ciavattini, G. Carloni, P.E. Varaldo, and M. Clementi. 2001. Identification of six putative novel human papillomaviruses (HPV) and characterization of candidate HPV type 87. *J Virol*. 75:11913-9.

Molden, T., J.F. Nygard, I. Kraus, F. Karlsen, M. Nygard, G.B. Skare, H. Skomedal, S.O. Thoresen, and B. Hagmar. 2005. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-proofer and consensus PCR: A 2-year follow-up of women with ASCUS or LSIL Pap smear. *Int J Cancer*. 114:973-6.

Monk, B.J., and A. Mahdavi. 2007. Human papillomavirus vaccine: a new chance to prevent cervical cancer. *Recent Results Cancer Res*. 174:81-90.

Munoz, N., F.X. Bosch, X. Castellsague, M. Diaz, S. de Sanjose, D. Hammouda, K.V. Shah, and C.J. Meijer. 2004. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer*. 111:278-85.

Nijhuis, E.R., N. Reesink-Peters, G.B. Wisman, H.W. Nijman, J. van Zanden, H. Volders, H. Hollema, A.J. Suurmeijer, E. Schuuring, and A.G. van der Zee. 2006. An overview of innovative techniques to improve cervical cancer screening. *Cell Oncol*. 28:233-46.

Nyari, T., I. Cseh, M. Woodward, J. Szollosi, M. Bak, and J. Deak. 2001. Screening for human papillomavirus infection in asymptomatic women in Hungary. *Hum Reprod*. 16:2235-7.

Olcese, V.A., Y. Chen, R. Schlegel, and H. Yuan. 2004. Characterization of HPV16 L1 loop domains in the formation of a type-specific, conformational epitope. *BMC Microbiol*. 4:29.

Orosz, Z., N. Udvarhelyi, and Z. Szentirmay. 1994. Non-isotopic in situ hybridization of human papilloma virus on histologic sections: an amended protocol. *Anticancer Res*. 14:1991-4.

Peyton, C.L., M. Schiffman, A.T. Lorincz, W.C. Hunt, I. Mielzynska, C. Bratti, S. Eaton, A. Hildesheim, L.A. Morera, A.C. Rodriguez, R. Herrero, M.E. Sherman, and C.M. Wheeler. 1998. Comparison of PCR- and hybrid capture-based human papillomavirus detection systems using multiple cervical specimen collection strategies. *J Clin Microbiol*. 36:3248-54.

Plikaytis, B.D., S.H. Turner, L.L. Gheesling, and G.M. Carlone. 1991. Comparisons of standard curve-fitting methods to quantitate Neisseria meningitidis group A polysaccharide antibody levels by enzyme-linked immunosorbent assay. *J Clin Microbiol*. 29:1439-46.

Qu, W., G. Jiang, Y. Cruz, C.J. Chang, G.Y. Ho, R.S. Klein, and R.D. Burk. 1997. PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. *J Clin Microbiol*. 35:1304-10.

Ronco, G., J. Cuzick, N. Segnan, S. Brezzi, F. Carozzi, S. Folicaldi, P. Dalla Palma, A. Del Mistro, A. Gillio-Tos, P. Giubilato, C. Naldoni, E. Polla, A. Iossa, M. Zorzi, M. Confortini, and P. Giorgi-Rossi. 2007. HPV triage for low grade (L-SIL) cytology is appropriate for women over 35 in mass cervical cancer screening using liquid based cytology. *Eur J Cancer*. 43:476-80.

Ruzsics, Z., T. Takacs, E. Adam, and I. Nasz. 1994. Production of antibodies against adenovirus hexons in Escherichia coli by recombinant phages (a note). *Acta Microbiol Immunol Hung*. 41:473-4.

Sapy, T., R. Poka, K. Szarka, J. Konya, S. Huga, and Z. Hernadi. 2007. Age-specific prevalence of high-risk human papillomavirus infection in a Hungarian female population with positive cytology. *Eur J Obstet Gynecol Reprod Biol*.

Schneider, V., M.R. Henry, M. Jimenez-Ayala, L.S. Turnbull, and T.C. Wright. 2001. Cervical cancer screening, screening errors and reporting. *Acta Cytol*. 45:493-8.

Sittampalam, G.S., W.C. Smith, T.W. Miyakawa, D.R. Smith, and C. McMorris. 1996. Application of experimental design techniques to optimize a competitive ELISA. *J Immunol Methods*. 190:151-61.

Snijders, P.J., R.D. Steenbergen, D.A. Heideman, and C.J. Meijer. 2006. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol*. 208:152-64.

Snijders, P.J., A.J. van den Brule, and C.J. Meijer. 2003. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. *J Pathol*. 201:1-6.

Stanley, M.A. 2001. Human papillomavirus and cervical carcinogenesis. *Best Pract Res Clin Obstet Gynaecol*. 15:663-76.

Stewart, A.C., A.M. Eriksson, M.M. Manos, N. Munoz, F.X. Bosch, J. Peto, and C.M. Wheeler. 1996. Intratype variation in 12 human papillomavirus types: a worldwide perspective. *J Virol*. 70:3127-36.

Stubenrauch, F., and L.A. Laimins. 1999. Human papillomavirus life cycle: active and latent phases. *Semin Cancer Biol*. 9:379-86.

Taguchi, G. 1993. Taguchi Methods. ASI Press, Dearborn, Michigan, .

Ueda, Y., T. Enomoto, T. Miyatake, K. Ozaki, T. Yoshizaki, H. Kanao, Y. Ueno, R. Nakashima, K.R. Shroyer, and Y. Murata. 2003. Monoclonal expansion with integration of high-risk type human papillomaviruses is an initial step for cervical carcinogenesis: association of clonal status and human papillomavirus infection with clinical outcome in cervical intraepithelial neoplasia. *Lab Invest*. 83:1517-27.

van den Brule, A.J., R. Pol, N. Fransen-Daalmeijer, L.M. Schouls, C.J. Meijer, and P.J. Snijders. 2002. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol*. 40:779-87.

Vernon, S.D., E.R. Unger, and D. Williams. 2000. Comparison of human papillomavirus detection and typing by cycle sequencing, line blotting, and hybrid capture. *J Clin Microbiol*. 38:651-5.

Warner, C.A., and D.J. McCance. 1989. Screening for cervical carcinoma. *Med Lab Sci*. 46:215-22.

Wiley, D., and E. Masongsong. 2006. Human papillomavirus: the burden of infection. *Obstet Gynecol Surv*. 61:S3-14.

Yoshikawa, H., T. Kawana, K. Kitagawa, M. Mizuno, H. Yoshikura, and A. Iwamoto. 1991. Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. *Jpn J Cancer Res*. 82:524-31.

zur Hausen, H., L. Gissmann, W. Steiner, W. Dippold, and I. Dreger. 1975. Human papilloma viruses and cancer. *Bibl Haematol*:569-71.

Publications

Publications directly related to the thesis

Jeney C, Takacs T, Sebe A, Schaff Z.

Detection and typing of 46 genital human papillomaviruses by the L1F/L1R primer

system based multiplex PCR and hybridization.

J Virol Methods. 2007 Mar;140(1-2):32-42.

Skapinyecz J, Smid I, Horvath A, Jeney C, Kardos L, Kovacs P.
Pelvic inflammatory disease is a risk factor for cervical cancer.
Eur J Gynaecol Oncol. 2003;24(5):401-4.

Jeney C, Dobay O, Lengyel A, Adam E, Nasz I.
Taguchi optimisation of ELISA procedures.
J Immunol Methods. 1999 Mar 4;223(2):137-46.

Other publications

Antus B, Sebe A, Fillinger J, Jeney C, Horvath I.
Effects of blockade of the renin-angiotensin and endothelin systems on experimental bronchiolitis obliterans.
J Heart Lung Transplant. 2006 Nov;25(11):1324-9.
PMID: 17097496 [PubMed - indexed for MEDLINE]

Antus B, Fillinger J, Sebe A, Jeney C, Soltesz I, Horvath I.
No gender difference in development of obliterative airway disease in rat tracheal allografts.
Exp Mol Pathol. 2006 Dec;81(3):235-8. Epub 2006 Jul 21.
PMID: 16859678 [PubMed - in process]

Skapinyecz J, Smid I, Juhasz A, Jeney C, Rozgonyi F, Kovacs P.
Planning of empirical antibiotic therapy for women with pelvic inflammatory diseases: a geographical area-specific study.
Eur J Obstet Gynecol Reprod Biol. 2003 Dec 10;111(2):183-8.
PMID: 14597249 [PubMed - indexed for MEDLINE]

Jeney C, Banizs B, Dobay O, Glatz K, Huszar T, Adam E, Nasz I.
The endosomal epsilon-coatomer protein is involved in human adenovirus type 5 internalisation.
Acta Vet Hung. 2002;50(4):481-9.
PMID: 12498152 [PubMed - indexed for MEDLINE]

Varvolgyi C, Buban T, Szakall S, Hargitai Z, Galuska L, Jeney C, Kakuk G, Gaal J.
Fever of unknown origin with seronegative spondyloarthropathy: an atypical

manifestation of Whipple's disease.
Ann Rheum Dis. 2002 Apr;61(4):377-8. No abstract available.
PMID: 11874851 [PubMed - indexed for MEDLINE]

Huszar T, Mucsi I, Terebessy T, Masszi A, Adamko S, Jeney C, Rosivall L.
The use of a second reporter plasmid as an internal standard to normalize luciferase activity in transient transfection experiments may lead to a systematic error.
J Biotechnol. 2001 Jul 12;88(3):251-8.
PMID: 11434970 [PubMed - indexed for MEDLINE]

Huszar T, Mucsi I, Antus B, Terebessy T, Jeney C, Masszi A, Hunyady L, Mihalik B, Goldberg HJ, Thekkumkara TJ, Rosivall L.
Extracellular signal-regulated kinase and the small GTP-binding protein p21Rac1 are involved in the regulation of gene transcription by angiotensin II.
Exp Nephrol. 2001 Mar-Apr;9(2):142-9.
PMID: 11150863 [PubMed - indexed for MEDLINE]

Ghidan A, Jeney C, Marodi CL, Csiszar K, Rozgonyi F.
PCR detection of the vanA gene in a vancomycin-resistant Enterococcus faecalis clinical isolate from Hungary.
J Antimicrob Chemother. 2000 Aug;46(2):325-6. No abstract available.
PMID: 10933665 [PubMed - indexed for MEDLINE]

Lakatos B, Farkas J, Adam E, Dobay O, Jeney C, Nasz I, Ongradi J.
Serological evidence of adenovirus infection in cats.
Arch Virol. 2000;145(5):1029-33.
PMID: 10881688 [PubMed - indexed for MEDLINE]

Szabo D, Filetoth Z, Szentandrassy J, Nemedi M, Toth E, Jeney C, Kispal G,

Rozgonyi F.
Molecular epidemiology of a cluster of cases due to Klebsiella pneumoniae producing SHV-5 extended-spectrum beta-lactamase in the premature intensive care unit of a Hungarian hospital.
J Clin Microbiol. 1999 Dec;37(12):4167-9.
PMID: 10565955 [PubMed - indexed for MEDLINE]

Endresz V, Kari L, Berencsi K, Kari C, Gyulai Z, Jeney C, Pincus S, Rodeck U, Meric C, Plotkin SA, Gonczol E.
Induction of human cytomegalovirus (HCMV)-glycoprotein B (gB)-specific neutralizing antibody and phosphoprotein 65 (pp65)-specific cytotoxic T lymphocyte responses by naked DNA immunization.
Vaccine. 1999 Jan;17(1):50-8.
PMID: 10078607 [PubMed - indexed for MEDLINE]

Jeney C, Dobay O, Adam E, Nasz I.
Fast method to remove UV absorbing agarose gel contamination from DNA samples.
Acta Microbiol Immunol Hung. 1998;45(2):253-6.
PMID: 9768293 [PubMed - indexed for MEDLINE]

Acknowledgements

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I want to thank the Department of Medical Microbiology enabling me to do the necessary research work and supporting me during the years. I have furthermore to thank the former Heads of Department, Dr. Nász István, Dr. Rozgonyi Ferenc and the present director of the department, Dr. Nagy Károly, who gave the permission and encouraged me to go ahead with my thesis.

I am deeply indebted to my supervisors Dr. Ádám Éva, Dr. Gönczöl Éva whose help, stimulating suggestions and encouragement helped me in all the time of research for and writing of this thesis.

My former and present colleagues from the Department of Medical Microbiology supported me in my research work. I want to thank them for all their help, support, interest and valuable hints. Especially I am obliged to Dr. Dobay Orsolya, Takács Tibor, Dr. Kiss Alexi, Dr. Ruzsics Zsolt, Dr. Berencsi Klára, Dr. Skapinyecz József, Dr. Schaff Zuszsa, Dr. Kiss András and Dr. Fejér György.

I would like to deeply thank the various people who, during the several months in which this endeavor lasted, provided us with useful and helpful assistance.

I am bound to the owners and the staff of Genoid Ltd., without their belief and their care and consideration, this would likely not have matured. Especially to Takács Tibor, whose friendship and help is greatly appreciated and to Dr. Sebe Attila, his help was indispensable during the preparation of the manuscript and in research.

Especially, I would like to give my special thanks to my wife Erika whose patient love enabled me to complete this work.