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Detection of bacteria and virus-associated Pancreatic Ductal Adenocarcinoma by cell-free protein microarray

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Abbreviations

6xHis	Hexa-histidine
BLAST	Basic local alignment search tool
bp	Base pair
°C	Degrees Celsius
$CaCl_2$	Calcium chloride
CagA	Cytotoxin associated protein A
CI	Confidence interval
CP	Chronic pancreatitis
CPI	Cag pathogenicity island
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DKFZ	Deutsches Krebsforschungszentrum
E. coli	*Escherichia coli*
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
F532	Foreground intensity measured at excitation wavelength 532nm
F635	Foreground intensity measured at excitation wavelength 635nm
g	Gram
GI	Gastrointestinal
H. pylori	*Helicobacter pylori*
HUSAR	Heidelberg Unix Sequence Analysis Resources
HVT	Herpes Virus of Turkey
KH_2PO_4	Potassium phosphate monobasic
MFI	Median Fluorescence Intensity

$MgCl_2$	Magnesium chloride
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
M	Molar
mM	Millimolar
Na_2HPO_4	Sodium phosphate dibasic
NCBI	National Center for Biotechnology Information
Ni	Nickel
$NiSO_4$	Nickel (II) sulfate
NTA	Nitrilotriacetic acid
OR	Odds ratio
ORF	Opening reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween-20
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
RBS	Ribosome binding site
SB	SuperBlock Buffer
SD	Standard deviation
SNR	Signal-to-noise ratio
T4SS	Type IV secretion system
UTR	Untranslated region
VacA	Vacuolating cytotoxin A
V5	V5 epitope

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1. Introduction

1.1 *Helicobacter pylori*

Helicobacter pylori (H. pylori) characteristically is a gram-negative bacterium of human gastric. Special shape and characteristics of this bacterium lead to colonization in the human gastric mucosa. *Helicobacter pylori* results in acute inflammation, chronic inflammation and gastric cancer. This bacterium was first isolated in 1983. After a short time, it was reported that this bacterium is associated with peptic ulcer. This finding in 2005 led to Nobel prize (Fock et al. 2013). *H. pylori* infection currently influences almost 50% of the world's population and it is considered as a cause of gastric and duodenal ulcer, chronic gastritis, gastric mucosa associated lymphoid-tissue (MALT) lymphoma, and gastric adenocarcinoma. People usually get the infection in childhood and they carry the infection for whole life. *H. pylori* is known for several genes and proteins which help the bacterium to be a pathogen to cause different diseases and epidemiological conditions. Frequency and severity of *H. pylori* could be different between different populations (Correa and Piazuelo 2008). *H. pylori* uses different ways of transmission such as oral and fecal ways. *H. pylori* has abilities to persist in the gastric and acidic situation due to special spiral form and flagella which lead to propel into lumen, penetration and colonization. Moreover *H. pylori* produces urease, an enzyme which produces ammonium to neutralize gastric acid. Most of the *H. pylori* Infections do not cause severe diseases and obvious symptoms, so it is left without treatment and special care (Thaker et al. 2016).

1.1.1 Epidemiology of *H. pylori*

H. pylori is estimated to spread from East Africa around 58,000 years ago. Currently childhood is the age of infection onset. *H. pylori* infection is more common in developing countries than developed countries (Linz et al. 2007). *H. pylori* transmission is accompanied by poor household sanitation and healthy conditions. The bacterium is disappearing in good hygienic societies with clean water and high standards of living, but remains common where these conditions are still missing (Aitila et al. 2019). The incidence of *H. pylori* shows large epidemiological differences. The infection positivity could vary from 80% of some populations to 40% of the populations with high level of sanitation and economic situation. The incidence of *H. pylori* is significantly lower in children than in adults (Genta 2002).

H. pylori a permanent-colonizing bacterium, usually occurs in early childhood and lasts for life. *H. pylori* infection happens in childhood and remains persistently for the rest of life. "The active elimination of *H. pylori* from the population with enhanced hygiene and housing conditions resulted in a lower infection rate in children, which reflects the infection age of *H. pylori* in the childhood and persistence for life" (Kusters et al. 2006).

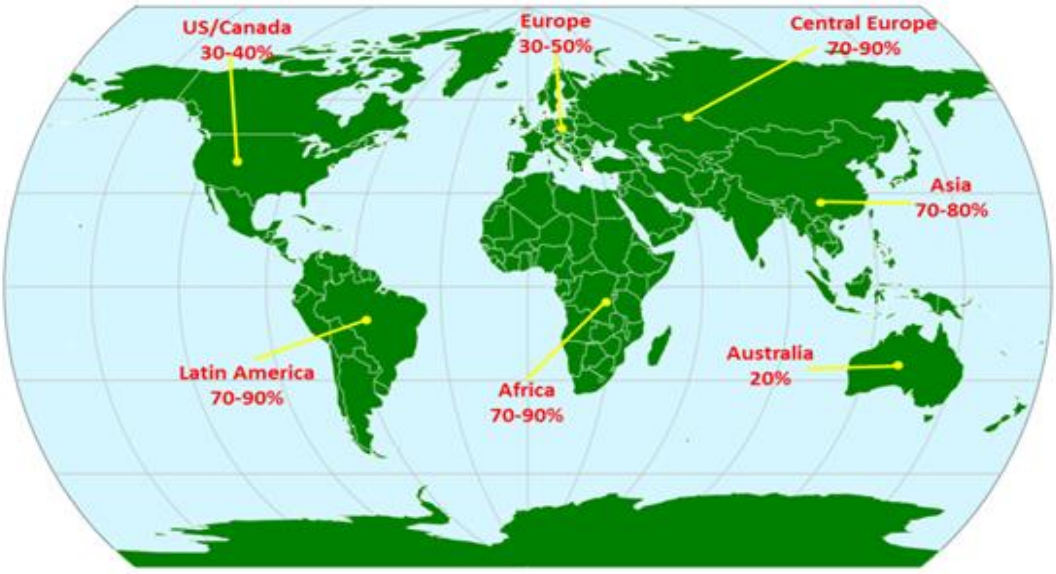


Figure 1. Global prevalence of *H. pylori*. Frequency of *H. pylori* across the world shows high frequency in developing countries and low frequency in industrialized countries (Zamani et al. 2018; K. Y. Hooi et al. 2017).

1.1.2 Infection mechanisms of *H. pylori*

H. pylori is adapted to maintain and live for a life in the acidic gastric condition. These bacteria have a strong adaptation to its natural habitation and they can modify their environment to reduce the acidity and scape of harsh situation. Whereas infection typically does not show symptoms, it can lead to other diseases such as gastric adenocarcinoma, ulcer diseases (gastric and duodenal ulcer) and MALT lymphoma. *H. pylori* causes a mixed acute and chronic inflammatory reaction, stimulating neutrophils, eosinophils, mast cells and dendritic cells. However, *H. pylori* has been considered a non-invasive pathogen, it is an intracellular bacterium of innate immune cells with ability of interfering with the phagosome maturation which could explain the difficulty in eliminating the bacteria (Diaconu et al. 2017; Kusters et al. 2006).

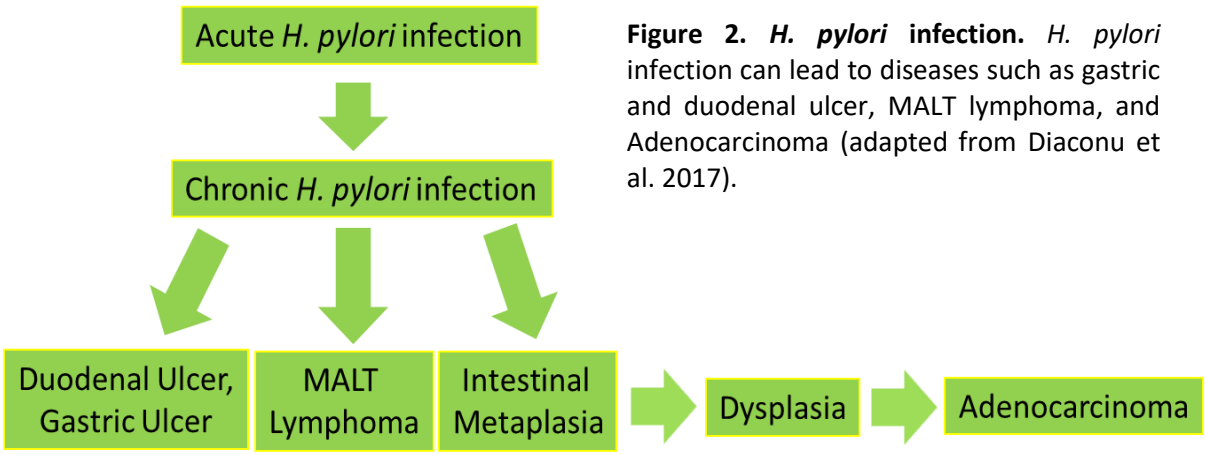


Figure 2. *H. pylori* infection. *H. pylori* infection can lead to diseases such as gastric and duodenal ulcer, MALT lymphoma, and Adenocarcinoma (adapted from Diaconu et al. 2017).

1.1.2.1 Survival of *H. pylori*

Usually human gastric lumen is able to block all of the ingested bacteria by using natural defense mechanisms. Any microorganism which can have successful gastric colonization in gastric lumen should have various abilities to suppress host acidic conditions in the mucosal lumen. Acidic pH is not the optimum condition for *H. pylori*, however it can survive in the acidic condition. The ability to defeat acidic condition is largely due to production of urease enzyme in the bacterial cytosol and bacterial surface. Stomach with acidic condition (pH 1-2) is the first barrier for *H. pylori* survival. *H. pylori* can neutralize acidic condition to have a less acidic environment (pH 5). Many bacteria cannot live and survive in PH 5 (Robinson et al. 2017; Scott et al. 2016). Urease sounds to be the most important *H. pylori* factor to survive in acidic condition. Urease is able to hydrolyze urea to produce ammonia, which acts as a receptor for H+ ions and therefore it leads to ammonium production which then creates a neutral microenvironment. Urease enzyme can shift gastric pH from 1-2 to 2-6 and as a result *H. pylori* can survive in the gastric condition (Figure 3) (Robinson et al. 2017; Talebi Bezmin Abadi 2017).

1.1.2.2 Penetration of *H. pylori to* mucus layer

H. pylori has to move into the mucus layer for colonization and helical shape of *H. pylori* has an important role in this penetration. Acidic environment can cause harm to *H. pylori* and the bacterium has to escape from gastric lumen to gastric mucus (Atuma et al. 2001). *H. pylori* can penetrate into the gastric mucosal layer with its 2 to 6 polar flagella and also through the production of urease, which allows it to meet friendlier environment (pH 5-6) (Figure 3) (Uemura et al. 2001). *H. pylori* mutants which have defects in helical shape are unable to penetrate inside the gastric mucus (Dunne et al. 2014).

1.1.2.3 Adherence of *H. pylori* to epithelial cells

Studies have shown that only a small fraction of *H. pylori* bacteria in gastric lumen can penetrate into gastric mucus and contact with the epithelial cells which is important in *H. pylori* pathogenesis (Dunne et al. 2014). *H. pylori* outer membrane proteins (OMP) family aligns with other *H. pylori* adhesion proteins assist in the successful binding of the bacterium to the epithelium. A variety of virulence factors including BabA, HorB, HomB, IceA2, AlpA, AlpB, DupA, OipA, SabA, HopZ, CagA, DupA and Lectin ensure the *H. pylori* attachment to the gastric epithelium. BabA is a major member of OMPs and it is one of the significant protein involving in the attachment for the persistent colonization. Secreted lectin by *H. pylori* is able to induce small cytoplasmic changes for the bacterial attachment to the epithelial cell (Kusters et al. 2006; Talebi Bezmin Abadi et al. 2015). Interestingly, although *H. pylori* adherence is mediated by surface proteins, different studies could not suggest a specific molecule to have the main role in the bacterial adherence (Oleastro and Ménard 2013).

1.1.2.4 Injection of virulence factors to epithelial cells

Clinical outcomes of *H. pylori* infection are associated with expression of certain *H. pylori* virulence factors such as CagA and VacA. The most known virulence factor is the cytotoxin-associated gene A (CagA). "Following binding of *H. pylori* to epithelial cells the CagA protein is injected into the epithelial cells via a Type IV secretion system (T4SS) encoded by genes present on a pathogenicity island (cagPAI)" (Dunne et al. 2014). When CagA is translocated into the cell, phosphorylation independent pathways as well as phosphorylation dependent pathways are performed to undertake the infected cell signaling mechanisms. Serious forms of diseases are associated with CagA expressing. (Figure 3) (Dunne et al. 2014). Studies have shown that CagA expression can result in oncogenesis features (Bonsor et al. 2018).

The *H. pylori* gene VacA encodes a secreted cytotoxin (VacA) which induces vacuolation in the host cell. VacA secretion is done by type V secretion system. The physiological importance of vacuolation during *H. pylori* infection is that vacuolation affects cell membranes and secretion pathways. Another function of VacA is related to mitochondria. VacA can enter to mitochondria to initiate apoptotic cascades (Palframan et al. 2012). VacA is able to induce vacuolation and also VacA is responsible to host immune response modulation that permits *H. pylori* long-term colonization (Figure 4) (Polk and Peek 2010).

1.1.3 *H. pylori* in pancreatic cancer

"Pancreatic cancer is the 14th most common cancer worldwide and it is the 7th highest cause of cancer mortality in the world in males and females. Pancreatic cancer has one of the highest mortality rates among all cancers with median survival of 3-6 months" (McGuigan et al. 2018). High mortality rate of pancreatic cancer indicates importance of early detection especially for pancreatic ductal adenocarcinoma (PDAC) which is the most common type of pancreatic cancer. "Europe and North America has the highest pancreatic cancer frequency while Africa and South Central Asia indicate the lowest frequency" (Figure 5) (McGuigan et al. 2018). Due to lack of effective screening, most PDAC patients show advanced disease or metastatic. Finding new methods and strategies for PDAC detection and management is related to finding new risk factors by case-control and cohort studies (Trikudanathan et al. 2010).

In the past it was confidently established that *H. pylori* has an important role in gastric diseases and gastric cancer (Marshall 1995, Correa and Houghton 2007). Some studies including meta-analyses suggest that *H. pylori* might be associated with PDAC and the bacterium has a role in the progression of pancreatic cancer (De Martel et al. 2008; Risch et al. 2010).

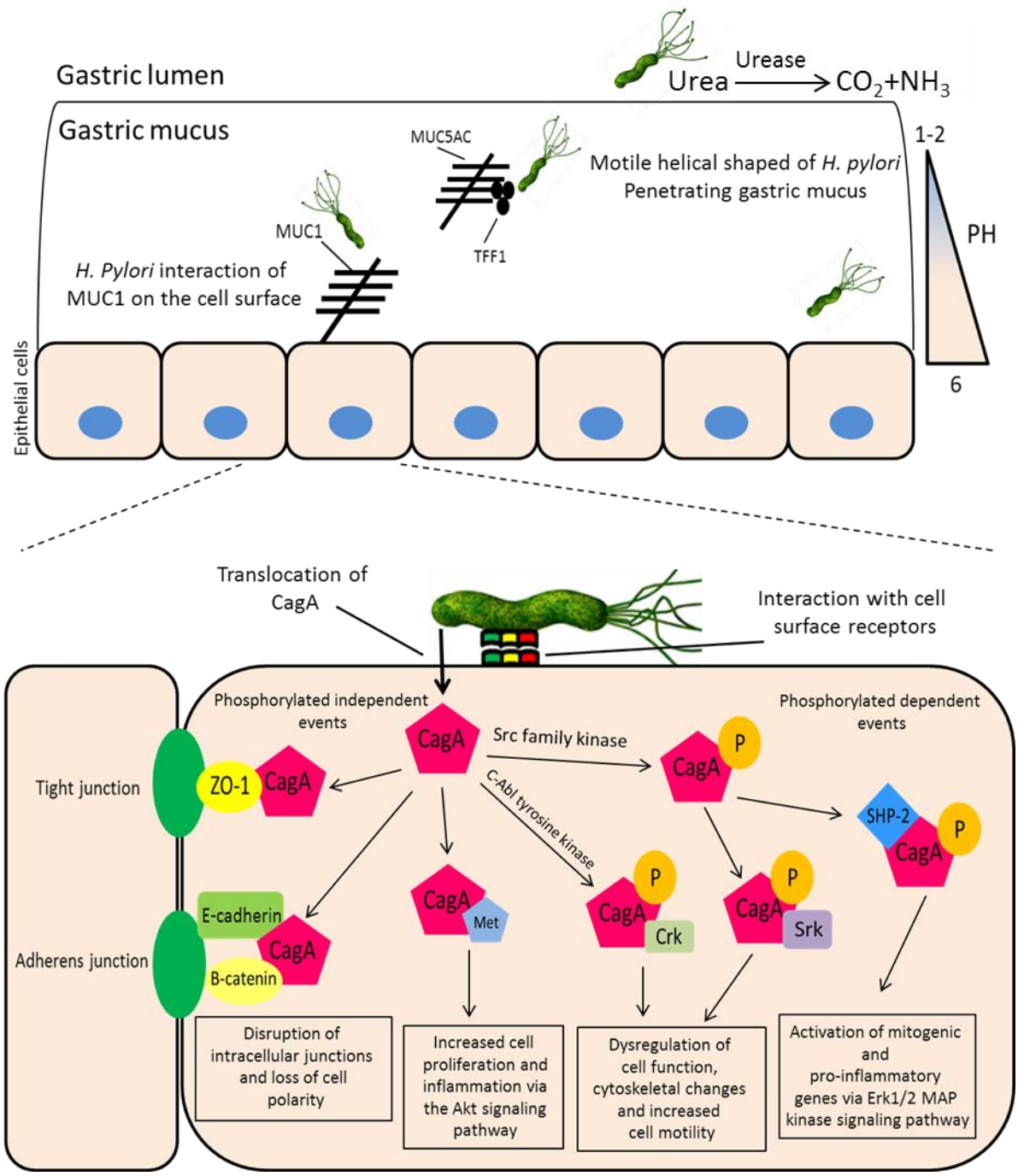


Figure 3. Colonization and infection model by *H. pylori*. Urease generates ammonia from urea to increase the pH to protect the bacteria from gastric acid. Movement of helical shaped bacteria helps it to enter gastric mucus and also helps to survive in the acidic situations of the lumen. Only a small fraction of the bacteria can attach to the epithelial cell. Outer membrane proteins (OMPs) and other adhesive mediators help bacteria attachment. *H. pylori* pathogenic factors such as CagA is injected into the epithelial cell by Type IV secretion system and starting CagA phosphorylation dependent and CagA phosphorylation independent pathways to transform epithelial cell function (adapted from Dunne et al. 2014).

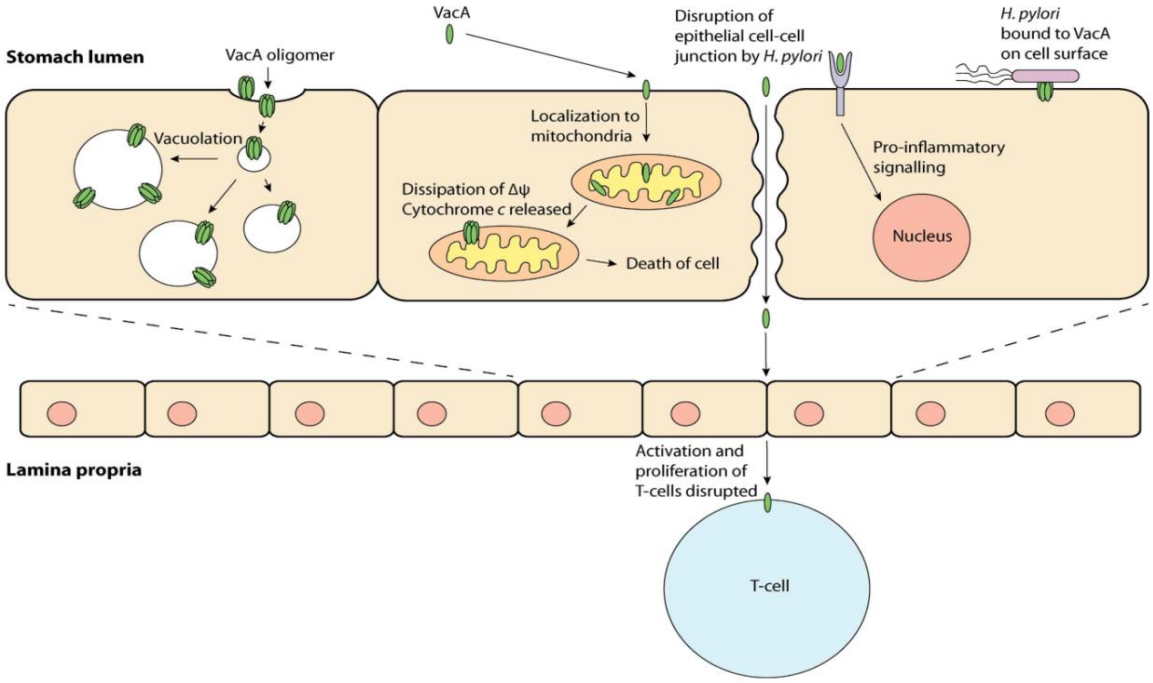


Figure 4. VacA, a multi-functional toxin. VacA induces vacuolation in the host cell. VacA can release cytochrome c, which initiates apoptosis. VacA can cause inflammation and finally VacA blocks T-cell proliferation (Palframan et al. 2012).

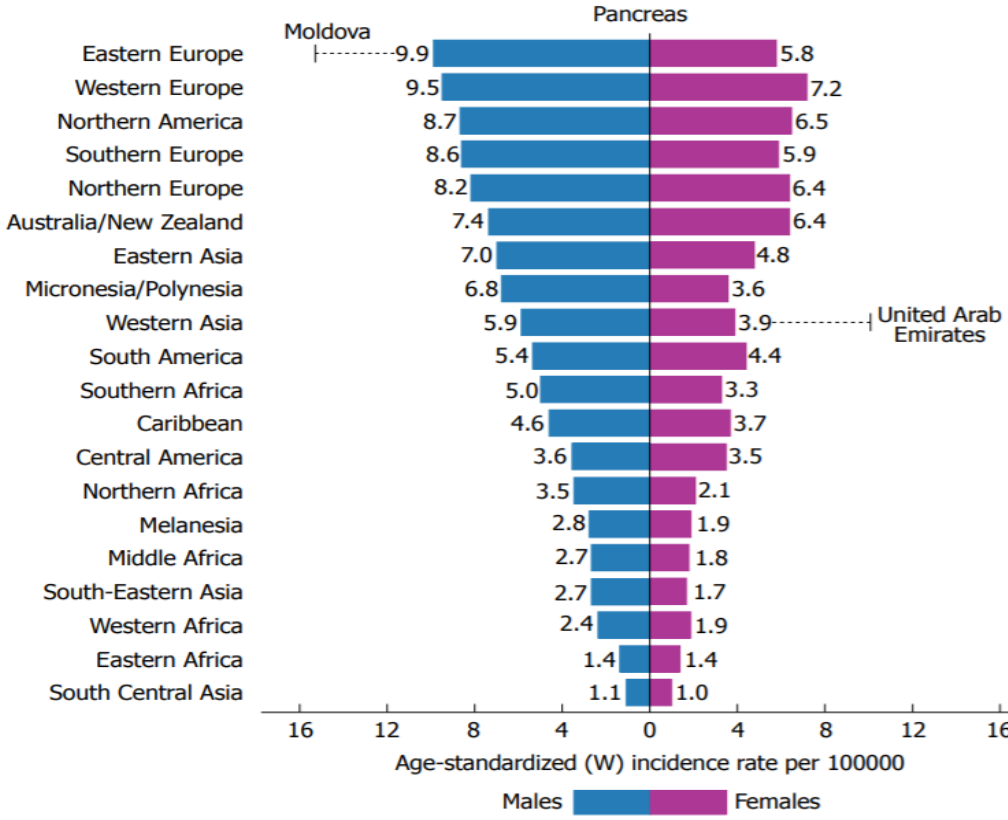


Figure 5. Incidence of pancreatic cancer. The age-standardized incidence shows pancreatic cancer frequency across the world (McGuigan et al. 2018).

Studies have shown antibody level against *H. pylori* is increased in pancreatic cancer (Bulajic et al. 2014). Some studies show that the risk of pancreatic cancer and autoimmune pancreatitis is increased by *H. pylori* infection (Culver et al. 2017). Moreover, numerous epidemiological publications also showed that patients with *H. pylori* infection are likely to have pancreatic cancer (Lindkvist et al. 2008). "Because of the small number of cases and different study methods, the meta-analysis results were limited and there was lack of power to confirm the high prevalence of *H. pylori* in pancreatic cancer rather than control group" (Wu et al. 2016). Finally people with *H. pylori* infection have a higher risk of developing PDAC (Becker 2014; Benzel et al. 2018). Different mechanisms and theories have been suggested to clarify how *H. pylori* infection causes carcinogenesis (Trikudanathan et al. 2010).

First possible mechanism emphasizes in inflammation, inflammatory mediators such as CagA and inactivation of tumor-suppressor genes in *H. pylori* infections which cause malignant transformation in pancreas. Second possible mechanism emphasizes in IL-8, VEGF and nuclear factor kB. Generally, the *H. pylori* infection increases in inflammatory cytokine and angiogenic factor secretion, ending in malignant transformation of the pancreatic cell line (Takayama et al. 2007). Third possible mechanism emphasizes in *H. pylori* colonization after decreases in gastrin and gastric acid. Gastrin is important in gastric acid production. This low acidity assists bacterial growth and increases production of N-nitroso (Annibale et al. 2002).

1.2 Herpesviridae family

"Herpesviridae family has evolved 180–220 million years ago and the present study highlights that it is still evolving and more genes can be added to the repertoire of this family" (Sehrawat et al. 2018). Herpesviridae family members have double strand DNA in nucleocapsid which is covered by tegument. The tegument is then covered by virus envelope which consists of a lipid membrane and glycoproteins (Figure 6). Herpesviruses are able to attach and penetrate to host cells. After releasing of viral DNA inside the host cell, transcription and translation of viral genes are performed. DNA replication is done by rolling circle mechanism in the nucleus of the host cell and after viral assembly and packaging, new virions release to outside by exocytosis mechanism (Figure 7). Herpesviridae family is considered as DNA viruses which consist of major pathogens with an extensive range of hosts. "Herpesviridae family has zoonotic potential thus they are responsible for a broad range of diseases in humans and animals" (Wozniakowsk and Salamonowicz 2015). Herpesviridae family includes nine types of viruses which can cause human infection: herpes simplex viruses (HSV), Varicella zoster virus (VZV), Epstein Barr virus (EBV), human cytomegalovirus (CMV), human herpesvirus 6, 7 and Kaposi's sarcoma-associated herpesvirus (KSHV). So far more than 130 herpesviruses from mollusks, birds, fish, reptiles, amphibians and mammals have been known (Brown and Newcomb 2011).

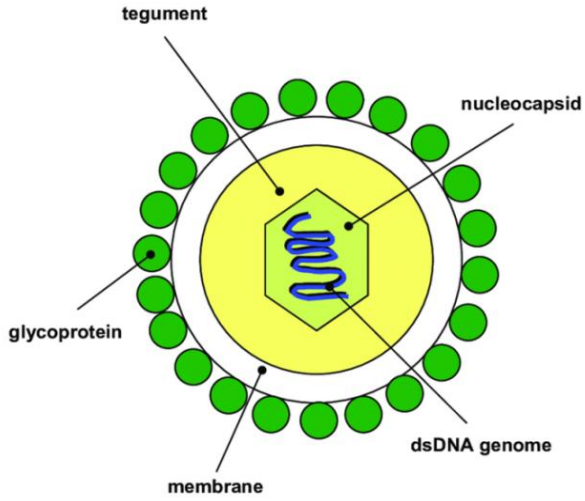


Figure 6. Structure of herpesviruses. The tegument surrounds the nucleocapsid containing the double stranded DNA. A lipid bilayer membrane covered by glycoproteins forms the virus envelope (Zimmerli 2005, P. 43).

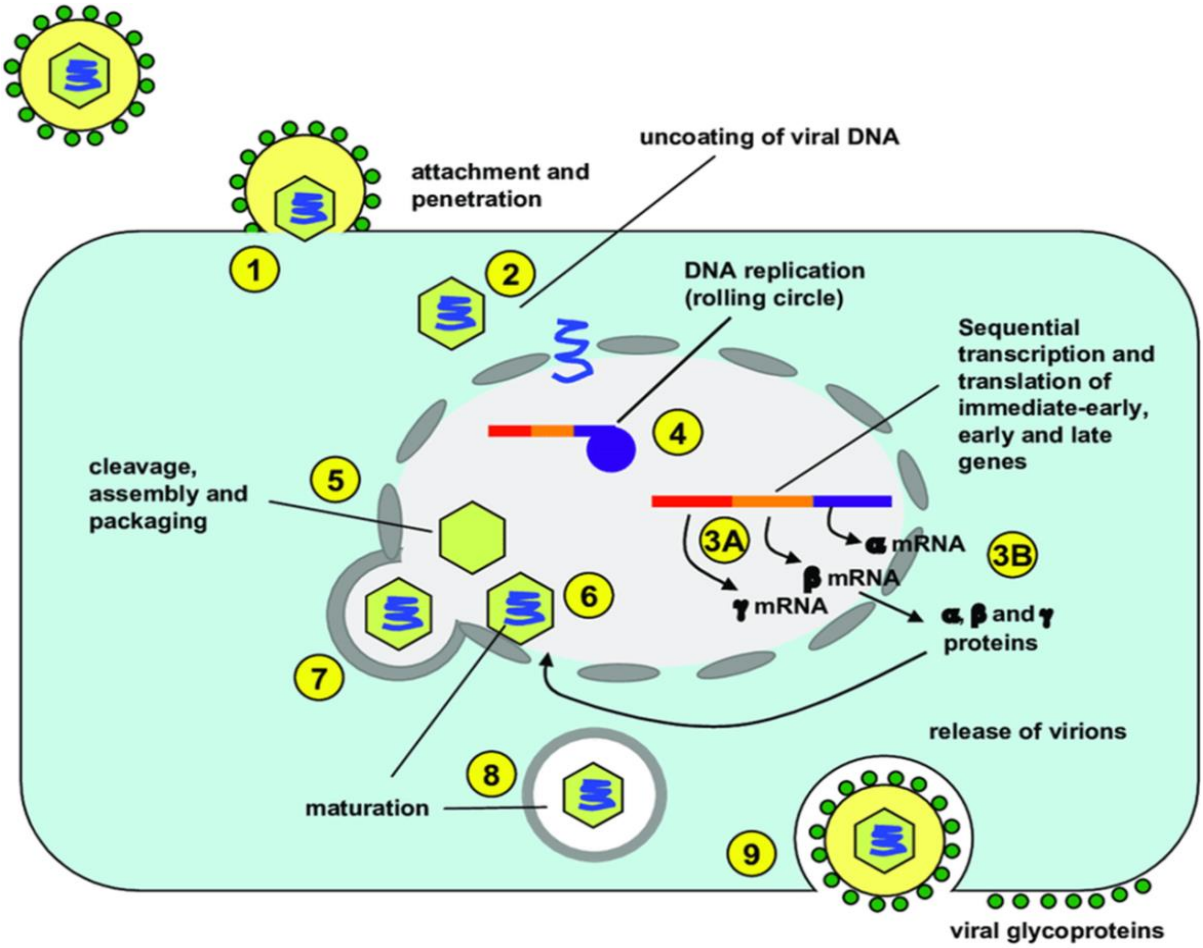


Figure 7. Life cycle of herpesviruses. 1. Herpesviruses move into host cells following attachment and penetration. 2. Releasing of viral DNA inside the host cell. 3. Transcription and translation (A, B). 4. DNA replication machinery in the nucleus of the host cell. 5. Virus association. 6. Assembling and maturation. 7. Budding. 8. Final maturation inside the cell. 9. Exocytosis of the virion (Zimmerli 2005, P. 44).

1.2.1 Herpesviridae family classification

"The subfamilies of Herpesviridae family are classified based on their cytopathic effect, replication strategies, genetic organization and hosts into alpha (α), beta (β), and gamma (γ)

subfamilies" (Table 1) (Whitley 1996). Among 130 herpesviruses, Cytomegalovirus, HSV 1, 2, Epstein Barr and Varicella zoster are example of herpesviruses can cause diseases in humans (Boshoff and Weiss 2001; Virgin 2014).

Subfamily	Characteristics	Examples
α herpesviruses	short replicative cycle, induce cytopathology in monolayer cell cultures, and have a broad host range	HSV1, HSV2 Varicella-zoster, HVT
β herpesviruses	long replicative cycle and restricted host range	CMV and human herpesviruses 6, 7
γ herpesviruses	very restricted host range	Epstein-Barr virus and human herpesvirus 8

Table 1. Classification of Herpesviridae family. Herpesviridae family is categorized into three subfamilies in the basis of cytopathic effect, host range, genetic material and replicative cycle (Adapted from Whitley 1996).

1.2.2 Infection mechanisms of herpesviruses

Following a primary exposure, herpesviruses can live and maintain in appropriate hosts for a long time mainly because of having the latency and the lytic cycles and also having the ability for immune system evasion which make herpesviruses successful pathogens (Uppal et al. 2014; Virgin 2014). As they have zoonotic potential to cross infection between species; infection in non-native hosts could result in more severity. HSV infection in mice (non-native host) is highly virulent and cause lethal encephalitis, a very rare outcome in humans. "Another example is herpes B virus, α-herpesvirus of monkeys causes lethal encephalitis in humans" (Wozniakowski and Samorek-Salamonowicz 2015).

1.2.3 Immune system evasion of herpesviruses

Herpesviruses have the ability of immune system evasion and immune system decrease by using latency phase and using immunomodulatory mechanisms which allows lowest immune recognition (Rezaee et al. 2006; Hengel et al. 1997). Herpesviruses are host immune modifiers (Litjens et al. 2018).

1.2.4 Turkey herpesvirus (HVT)

Turkey herpesvirus (HVT) is also called as the third serotype of the Marek's disease virus (MDV), is one of the alpha herpesviruses. HVT could be defined as an abundant virus in domestic turkeys and almost a nonpathogenic virus. MDV group is avian herpesviruses with an affinity to lymphocytes. MDV serotype 1 (MDV1) is the cause of the Marek's disease in chickens which brings economic problems. "HVT is nonpathogenic in chickens, but it does

induce a viremia which is associated with induction of protective immune responses against MDV1" (Witter 1997). So far there is no report regarding detection of HVT in human beings.

1.2.5 HVT Pathogenicity

HVT is non-pathogenic for turkeys. In chickens, HVT does not lead to tumorigenesis, but other problems have been observed (Witter et al. 1970). Some evidences such as induction of wounds and lesions in the chickens emphasize in HVT transformation ability (Purchase and Sharma 1974; Prasad 1979). Unpublished data in our division including the Next-generation sequencing result, digital PCR and real-time PCR confirms that HVT associates with PDAC (Mohan 2015, P. 49).

1.3 Cell-free protein microarray as a powerful technology for antigen screening

Cell-free protein microarray is a strong and pioneering technology for the detection of positive candidates, novel biomarkers and protein-antibody reactivities. Protein purification is expensive and time-consuming and could destroy the protein structure and function. Cell-free protein microarray technology avoids protein purifications by replacing expression *in situ* with an *in vitro* transcription-translation. This technology allows doing high-throughput approaches for the analysis of the proteome at large scales (Merbl and Kirschner 2010). In comparison to other proteomics methods, cell-free protein microarray avoids the necessity of purification a sample into small parts and therefore complex samples can be immediately applied for research (Hanash 2003).

In the cell-free protein microarray, proteins are produced from a template which has the regulatory sequences for *in vitro* transcription and translation. Then the new synthesized protein is captured by affinity reagents (Díez et al. 2015). In our method the DNA template is an expression construct with His-Tag and V5-Tag which is flanked by regulatory sequences, made by two successive PCRs and after *in vitro* transcription-translation, nascent protein binds to Ni surface by His-Tag. In this thesis *H. pylori* whole-proteome microarray and HVT whole-proteome microarray were produced from *H. pylori* ORFeome and HVT ORFeome.

1.4 Aims of the project

After generation of *H. pylori* whole-proteome microarray and HVT whole-proteome microarray the aim of this study was to identification of potential biomarkers for *H. pylori*-associated PDAC and HVT-associated PDAC. The long-term goal of this project is finding diagnostic biomarkers for PDAC detection purposes.

Overall aims of this project are:

- Detection of *H. pylori*-associated antigens to PDAC.
- Detection of HVT-associated antigens to PDAC.
- Identification of *H. pylori*-associated antigens for diagnostic purposes.
- Identification of HVT-associated antigens for diagnostic purposes.

2. Materials and Methods

2.1 Chemicals and labwares

Table 2. Chemicals and labwares used during this thesis

Product	Company	Catalog number
6x DNA Loading dye	Fermentas	R0611
Adhesive PCR plate foil	Thermo Scientific	AB0626
Microcentrifuge tube 1.5 ml	Eppendorf	0030120086
Microcentrifuge tube 2 ml	Eppendorf	0030120094
GeneRuler 1 kb DNA ladder	Thermo Scientific	SM 0311
XCEED Nitrile Exam Glove	Microflex	108-60299
Nickel sulfate	Sigma-Aldrich	227676
Nickel nitrilotriacetic acid	Sigma-Aldrich	72560
Nuclease-free water	Life Technologies	AM9939
NAP-Blocker	G-Biosciences	786-190S
PCR tubes,8 strip 0,2 ml	Life Technologies	AM12230
Sodium chloride	Sigma-Aldrich	S9888
Sodium phosphate dibasic	Sigma-Aldrich	S9763
SuperBlock blocking Buffer	Thermo Scientific	37515
PCR plate, 96-well, non-skirted	Thermo Scientific	AB0600L
Blocking solution	Candor bioscience	110 050
Betaine	Sigma-Aldrich	61962
Pipettes tips	Biozym	720031, 720230, 721014
Nexterion Block E	SCHOTT	1066069
Glacial Acetic Acid	Sigma-Aldrich	A6283-1L
Potassium phosphate monobasic	Sigma-Aldrich	P9791-500G
dNTPs	Genaxxon	M3015.4100
Microplate, 384 Well, Pp, V-Bottom	Greiner	781280
Epoxysilane slide	Schott	1066643
Milk powder	Bio RAD	1706404
ProPlate® Multi-Well Chambers Well Slide Module delrin clip	Graco Bio-Labs	246861
Agarose BioReagent, for molecular biology, low EEO	Sigma-Aldrich	A9539-500G

2.2 PCR reagents and expression kits

Table 3. Kits and enzymes used in this thesis

Product	Company	Catalog number
QIAamp DNA Mini Kit/for isolation of bacterial and viral genome	Qiagen	51304
Qiagen Long Range PCR kit	Qiagen	206403
S30 T7 High-Yield Protein Expression Kit	Promega	L1110
Taq DNA Polymerase	Qiagen	201207

2.3 Antibodies

Table 4. Source of antibodies used

Product	Company	Catalog number
Alexa Fluor-647 AffiniPure Goat Anti-Human IgA+IgG+IgM (H+L)	Jackson ImmunoResearch	109-605-064
Monoclonal Anti-V5, Cy3 conjugate	Sigma-Aldrich	V4014-100UG
Penta-His Alexa Fluor-647 Conjugate	Qiagen	35370

2.4 Buffers and media

50xTAE buffer:

Tris Base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA	100 ml
ddH2O	Up to 1000 ml

10xPBS:

Tris Base	242 g
NaCl	80 g
KH2PO4	2 g
Na2HPO4	11.1 g
ddH2O	Up to 1000 ml

1xPBST:

10xPBS	100 ml
10% Tween-20	5 ml
ddH2O	Up to 1000 ml

1% Agarose gel:

1xTAE buffer	200 ml
Agarose	2 g

2.5 Equipments

Table 5. Source of equipments

Name	Company
Gel Documentation System	Azure Biosystems
Heating Block	Grant Instrument
Ice maker	Scotsman
Laminar Hood	Hereaus instruments
Microcomputer electrophoresis power supply	Renner GmbH
Microwave oven	Bosch
Mixmate Microplate Mixer	Eppendorf
Nanoplotter 2.1	GeSIM
Orbital Shaker	Edmund Bühler
PowerScanner	Tecan
Thermocycler	LifeEco
TKA MilliQwatter supply	Millipore
Ventillated oven	Kendro Instruments
Vortex Mixer	Neolab

2.6 DNA extraction

H. pylori genomic DNA was supplied by ATCC (ATCC™, 700392D-5) as pure genomic DNA but for HVT (ATCC™, VR-584B) there was not any source of DNA. After ordering the virus I received cell pellet and supernatant. Since it was not known if this virus would be present in the cell pellet or in the supernatant, DNA extraction was performed using both supernatant and cell pellet and results were confirmed by PCR. For this purpose, very specific forward and reverse primers were designed which just amplified HVT sequence (HVT075) and the primers didn't amplify any other viruses or organisms.

Table 6. Forward and reverse primers used for HVT confirmation

Forward primer	5´-AAGTGGGCGTGCAATGATAC-3´
Reverse primer	5´-TCTTCACACACGCCCTGTAT-3´

2.7 Primer design and strategy

For primer design all of the open reading frame (ORF) sequences were extracted from databases. Each ORF starts with ATG codon and ends at stop codons. To have an expression construct each ORF should be in frame and stop codons should be removed from the sequence. Forward primer starts at the ATG codon and reverse primer starts at the end of the ORFs. To have the best PCR result every primer has the length of 16-23 nucleotides, 50% GC content and 50°C Tm. Then binding specificity of each primer was checked by blast tools. Because of different GC content at the beginning and at the end of a gene, primer designing with the similar criteria was not always possible and also in cell-free protein expression technique there are some difficulties in expression of ORFs more than 5 kb, therefore with splitting a gene into two or three smaller sections it was possible to have a better primer design for PCR and also to be sure about full protein expression. Splitting a gene can result in missing natural protein structures which by sequence overlapping it is possible to overcome this problem to some extent. *H. pylori* primers were designed already by HUSAR (Heidelberg Unix Sequence Analysis Resources) and I designed HVT primers in our laboratory. For these purpose I used different databases and tools (Table 7). Moreover, these specific primers were flanked by 2 overhangs which then used for binding sites of primer pairs in the second PCR. In the second PCR regulatory sequences for transcription and translation were added by using second primers.

Table 7. Name of databases and tools for primer design

Name	Address
NCBI	https://www.ncbi.nlm.nih.gov
NCBI blast	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Primer3	http://primer3.ut.ee/
In Silico PCR	http://rohsdb.cmb.usc.edu/GBshape/cgi-bin/hgPcr
OligoCalc	http://biotools.nubic.northwestern.edu/OligoCalc.html
Integrated DNA Technology	https://eu.idtdna.com/site/account/

Table 8. Overhangs primers

Forward primer overhang	5’-ATGCACCAAACCCAA-3’
Reverse primer overhang	5’-CGCACTGGCATCATC-3’

Table 9. Second primer sequences used to generate expression construct

Forward primer	5’-gaaattaatacgactcactatagggagaccacaacggtttccctctagaaataattttgtttaaga aggagatatacatatgcatcatcatcatcatcatatgcaccaaacccaa-3’
Reverse primer	5’-ctggaattcgccctttttattacgtagaatcgagaccgaggagagggttagggataggcttacccgc actggcatcatc-3’

2.8 PCR protocols

For generating of whole-proteome microarray first step is to produce expression constructs. For this purpose, two successive PCRs by using specific primers for the first PCR and common primers for the second PCR were performed. For PCR products more than 1 kb length, Long Range Enzyme (Qiagen) was used instead of Taq DNA polymerase (Qiagen). First PCR products were used as the templates for the second PCRs to generate the final library which were used to produce whole-proteome microarray. Second primers bind to the overhang sequences in upstream and downstream of the first PCR product. Second primers include regulatory sequences: T7 promoter (Pro), 5’ untranslated region (UTR), ribosome binding site (RBS), start codon, 6xHis-Tag, V5-Tag and T7 terminator (Ter) to each ORF.

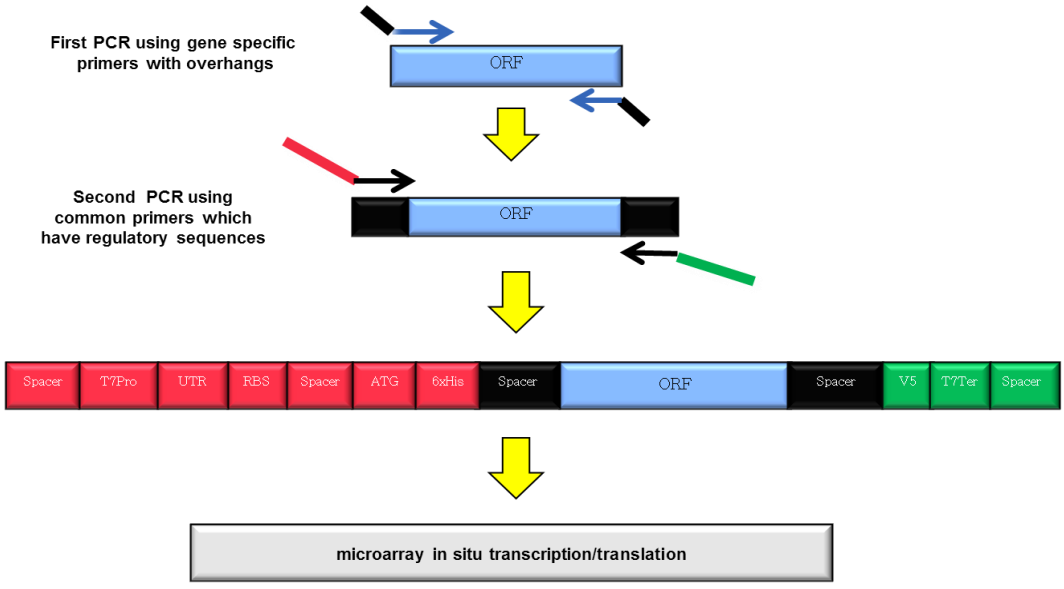


Figure 8. Generation of expression construct by two successive PCRs. PCR plan for generating of *H. pylori* ORFeome and HVT ORFeome with using gene specific primers (dark blue) which binds specifically to the beginning and to the end of the ORF (light blue). First primers also have overhangs (black) which used as the binding sites for the second primers. The product of the first PCR is used as the template for the second PCR. In the second PCR the regulatory sequences for transcription and translation (green and red), which are in the second primers, are added to the ORF.

First PCR reaction and program were prepared according to the following protocols:

First PCR reaction mixture:

PCR buffer 10x	2 µl
DNase-free water	8.25 µl
dNTPs 10 mM	1 µl
$MgCl_2$ 25 mM	2.5 µl
Betaine 5 M	2 µl
Forward primer 10 µM	1 µl
Reverse primer 10 µM	1 µl
*H. pylori/*HVT DNA 1 ng	2 µl
Taq DNA polymerase	0.25 µl
Total	20 µl

First PCR Program:

Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	30 cycles
Annealing	45-60°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	10 min	
Hold	10°C	∞	

Second PCR reaction and program were prepared according to the following protocols:

Second PCR reaction mixture:

PCR buffer 10x	5 µl
DNase-free water	23.5 µl
dNTPs 10 mM	1 µl
Betaine 5 M	5 µl
Forward primer 10 µM	1 µl
Reverse primer 10 µM	1 µl
First PCR product	2 µl
Taq DNA polymerase	0.5 µl
Total	50 µl

Second PCR program:

Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	30 cycles
Annealing	50°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	5 min	
Hold	10°C	∞	

2.9 Positive controls

To generate whole-proteome microarray and evaluation the expression assay and immunoassay, positive PCRs were used which they have known expression constructs and after spotting and incubation, the expressed protein was used as endogenous positive control. P18 capsid antigen of Epstein-Barr-Virus (EBV) and F11 capsid antigen of Poliovirus were used as templates for Positive PCRs. Epstein-Barr-Virus DNA was in B070 laboratory library and Poliovirus F11 sequence was ordered as the template to be synthesized. Gene specific primers were designed for the First PCR. Second primers which include regulatory sequences for all of the expression constructs were used for the second PCRs.

2.9.1 EBV p18 positive control

EBV p18 control PCRs produced by the following protocols:

Table 10. Forward and reverse primers used for EBV p18

EBV p18_F	5´- ATGGCACGCCGGCT-3´
EBV p18_R	5´- CTGTTTCTTACGTGCCCCG-3´

First PCR reaction mixture for EBV p18:

Buffer 10x	2.5 µl
Betaine	5 µl
dNTP 10 mM	1 µl
$MgCl_2$ 25 mM	2 µl
Forward primer 10 µM	1 µl
Reverse primer 10 µM	1 µl
EBV DNA 1 ng/µl	2 µl
Taq DNA polymerase	0.25 µl
DNase-free water	10.25 µl
Total	25 µl

Second PCR reaction mixture:

PCR buffer 10x	5 µl
DNase-free water	23.5 µl
dNTPs 10 mM	1 µl
Betaine 5 M	5 µl
Forward primer 10 µM	1 µl
Reverse primer 10 µM	1 µl
First PCR product	2 µl
Taq DNA polymerase	0.5 µl
Total	50 µl

First PCR program for EBV P18:

Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	30 cycles
Annealing	51°C	30 s	
Extension	72°C	30s	
Final extension	72°C	5 min	
Hold	10°C	∞	

Second PCR program:

Initial denaturation	94°C	2 min	
Denaturation	94°C	30 s	30 cycles
Annealing	50°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	5 min	
Hold	10°C	∞	

2.9.2 Poliovirus F11 positive control

First PCR primers and protocols for Poliovirus F11 positive control were prepared according to the following protocol. Second PCR reaction mixtures and programs for Poliovirus F11 are the same as EBV p18.

Table 11. Forward and reverse primers used for Poliovirus F11

Polio F11_F	5´- AAGGAAATTCCAGCACTC -3´
Polio F11_R	5´- TGTGGCCCCAGTTTCCA -3´

Table 12. Poliovirus F11 sequence to be synthesized as the template for PCR

5´-AAGGAAATTCCAGCACTCACCGCAGTGGAAACTGGGGCCACA-3´

First PCR reaction mixture for Poliovirus F11:

PCR buffer 10x	2 µl
DNase-free water	9.8 µl
dNTPs 10 mM	1 µl
$MgCl_2$ 25 mM	2 µl
Betaine 5 M	2 µl
Forward primer 10 µM	1 µl
Reverse primer 10 µM	1 µl
Taq DNA polymera	0.2 µl
Polio DNA 1 ng/µl	1 µl
Total	20 µl

2.10 Agarose gel electrophoresis

All of the PCRs were performed using 96 well plates and the Thermocycler (MJ Research LifeEco) and they were stored in -20°C for quality control and spotting. Quality and length of the final PCR products were always verified with gel electrophoresis using 1% agarose gel in TAE buffer. After each PCR the success of PCRs was checked by Agarose Gel Electrophoresis. For this purpose, 1 gram of Agarose powder (Biozyme) was dissolved in 1X TAE buffer by heating then it was poured in a special cast to cool down to form a gel. 5 µl of PCR products and 1 µl of 6X DNA Loading dye (Fermentase) were mixed and loaded on the gel. Beside that to check the size of the PCR products, 5 µl of 1 kb DNA ladder (Thermo Scientific) was loaded on the gel. The electrophoresis was performed by using the Microcomputer electrophoresis power supply system (Renner GmbH) at 100 Voltage for 30 minutes. Gel documentation was performed by Gel Documentation System (Azure biosystems) at UV 302 nm, 5 seconds exposure.

2.11 Ni-NTA slides preparation

To generate Ni-NTA slides, the epoxysilane slides were treated with a mixed solution of 0.63 M NTA and 2.38 M sodium bicarbonate for overnight (12 hours). Then, the slides were washed twice with Millipore water and air-dried. Subsequently, the slides were immersed in $NiSO_4$ solution for 6 hours, and then washed again. The slides were finally treated with 0.2 M acetic acid, 0.2 M $CaCl_2$ and 0.1% Tween-20 for 30 minutes, washed again, air-dried and stored at 4°C. All steps were done under sterile hood.

2.12 Spotting of protein microarrays

After two successive PCRs, *H. pylori* ORFemoe and HVT ORFeome were generated. These products are expression constructs and they have sequence of gene of interest flanked by regulatory sequences which are necessary for transcription and translation. 45 µl of each PCR product was combined with 5 µl of 5 M betaine then tranfered into 384-well plates (Whatman). All of the samples were spotted using the Nanoplotter 2.1 (GeSIM). The spotter system and pins were washed once with 1% Hellmanex and twice with miliQ water for at least 20 minutes before and after of each spotting to prevent pin blockage and to keep the spotter clean. Then Z-Values were measured for all of the slides to estimate the position and distance of each slide. A transfer file which has all the commands and information of spotting were prepared in Notepad. 1.2 nl of the PCR products as two droplets were first spotted on the slide. Before each spotting the sample uptake was checked and confirmed by stroboscope automatically. In the meantime of spotting, S30 T7 High-Yield Protein Expression mix (Promega) was prepared as follows for 5 slides:

S30 Premix Plus:	20 µl
T7 S30 Extract:	18 µl
Nuclease free water:	12 µl

The mixture should be homogenized while avoiding any bubble formation. 4.8 nl of the expression mix was then spotted on the exact same locations where the PCR products were spotted. Afterwards in order to allow on-chip protein expression, the slides were placed in a humidified incubation chamber containing 30 µl nuclease free water at each chamber reservoir. Then all of the slides were placed in incubation boxes containing wet tissue and incubated at 37°C, 50% humidity for 1 hour, and subsequently at 30°C, 50% humidity for 12 hours in ventilated oven. After 12 hours incubation the slides were transferred into a slide rack and then kept at -20°C for 24 hours.

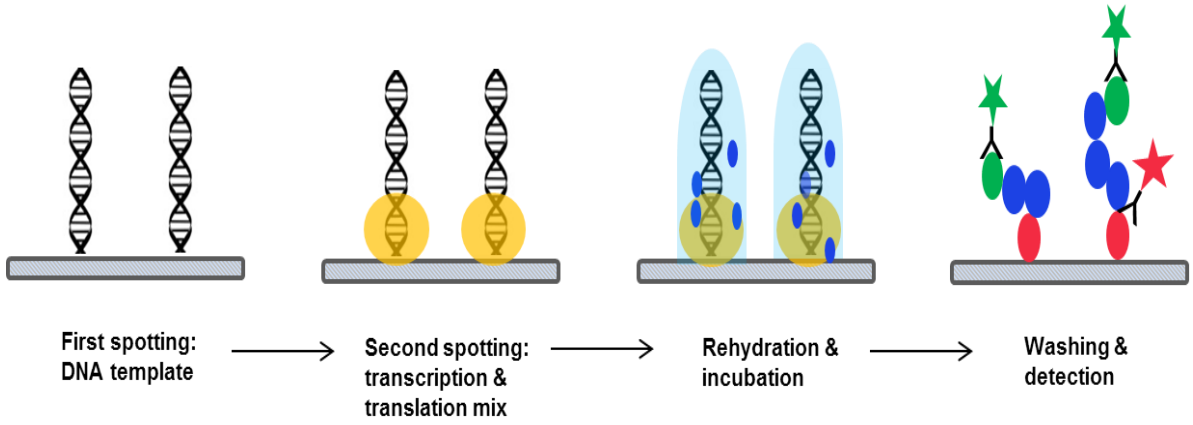


Figure 9. Overview of spotting and expression of *H. pylori* and HVT proteins. All of the ORFs of HVT and *H. pylori* which produced by two successive PCRs are ready for spotting and expression. In the first step of spotting, Nanoplotter spots all of the second PCR products on slide. In the second step of spotting transcription and translation mixes are added on the spots. After rehydration and incubation, the ORFs are transcribed and translated into proteins to have a whole-proteome array. Finally, the expressed proteins are used for detection.

2.13 Expression assay and immunoassay

After producing protein microarrays, the existence of the expressed protein was checked by performing expression assay and after that, the reactivity of these expressed proteins on the array was checked with serum antibodies of cancer donors as well as cancer free donors by immunoassay.

2.13.1 Expression assay

After spotting and incubation the protein microarrays were produced and the expressed proteins on the array should be checked in terms of quality of protein expression. The presence of the N-terminal 6xHis-Tag and a C-terminal V5-Tag as fusion tags allows testing the expression of *H. pylori* and HVT antigens. For this purpose, Anti-V5 antibody (Cy3 conjugated, Sigma) and anti-His antibody (Alexa Fluor-647 conjugated, Qiagen) with green and red signals respectively were applied during expression assay (Table 13 and Figure 10).

Table 13. Procedures of expression assay for protein microarray

Expression assay	
1.	Preparing fresh blocking buffer: 2% BSA in 1X PBST. Adding 0.2 g BSA in 10 ml 1X PBST, vortexing it to dissolve. Then adding 2 ml of the blocking buffer on each slide on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
2.	Decanting the supernatant and washing twice with 2 ml 1X PBST, each wash is 5 minutes on an orbital shaker at 50 rpm speed at room temperature.
3.	Preparing and adding 1 ml of antibody dilution (1:1000) for each slide. 1 µl of anti V5 sigma (cy3, Green, 532nm, V4014/100UG) and 1 µl of anti penta His, Qiagen (Alexa Flour 647, 635 Red) in 1 ml of the blocking buffer. Putting it on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
4.	Removing the slide from the chamber and washing with 6 ml 1X PBST 3 times and each wash for 10 minutes on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
5.	Rinsing the slides in Millipore water until the spots on the slide become visible.
6.	Drying the slides in a ventilated oven at 30°C for 15 minutes.
7.	Acquiring microarray image using Tecan PowerScanner at the wavelength of 532 nm (cy3) and 635 nm (Alexa Flour).

2.13.2 Immunoassay

After detection of the expressed protein on the array, immunoassay was performed using serum samples from healthy, chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) patients. The serum samples should consist 3% of the serum dilution and 45 µl serum was used for each slide. Pooled sera were prepared by mixing 9 µl of each 5 individual serum samples to a total 45 µl serum for one slide. *E. coli* antibodies which are common in human sera were neutralized by using *E. coli* wildtype lysate (BL21) in the serum dilution to have a minimum background. In principle, the specific antibody from the serum sample binds to the spotted pathogen candidates on the array. If there is specific antibody against proteins of *H. pylori*, the antibody binds to the protein on the microarray and this binding is detected by using a secondary antibody which is conjugated to Alexa Flour fluorescence dye with red signal and secondary antibody binds to Fragment crystallizable (Fc) region of IgA, IgG and IgM (Table 14 and Figure 10).

2.14 Image acquisition

After immunostaining, the slides were transferred to Tecan PowerScanner for intensity measurement. For expression assay signals from Cy3 and Alexa Fluor-647 fluorescent dyes were captured using 532 nm and 635 nm excitation wavelengths respectively. For immunoassay signal from Alexa Fluor-647 fluorescent dye were captured using 635 nm excitation wavelength. The laser intensity was kept at 75% and autogain was generally used

for the gain setting. The scanner was in general configured with a 40 µm resolution and a high scanning speed setting over high sensitivity. All slide images were collected as 2200 pixels by 7500 pixels TIFF file for further analysis.

Table 14. Procedures of immunoassay for protein microarray

Immunoassay	
1.	Preparing 1.5 ml serum dilution with *E. coli* lysate, and blocking buffer: 45 µl of serum + 1383 µl blocking buffer + 72 µl *E. coli* lysate (20 mg/ml) and incubate it for 2 hours in 4°C. The main blocking buffers used in this thesis are SuperBlock buffer (Thermo Scientist) and Candor Blocking Solution (Candor bioscience).
2.	Gently adding 2 ml of blocking buffer to the slide and putting the slide on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
3.	Decanting the supernatant and wash twice with 2 ml 1X PBST, each wash 5 minutes on an orbital shaker at 50 rpm speed at room temperature.
4.	Adding the 1.5 ml diluted serum dilution to the slides and putting them on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
5.	Decanting the supernatant and washing twice with 2 ml 1X PBST, each washing 5 minutes on an orbital shaker at 50 rpm speed at room temperature.
6.	Preparing and adding 1.5 ml of secondary antibody which is 4.5 µl of goat anti-human IgG, IgA, IgM Antibody (Jackson ImmunoResearch) in 1.5 ml of the blocking buffer. Putting them on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
7.	Removing the slide from the chamber and washing 3 times with 6 ml 1X PBST, each wash for 10 minutes on an orbital shaker at 50 rpm speed at room temperature for 1 hour incubation.
8.	Rinsing the slides in Millipore water until the spots on the slide become visible.
9.	Drying the slides in a ventilated oven at 30°C for 15 minutes.
10.	Acquiring microarray image using Tecan PowerScanner at the wavelength of 635 nm.
•	The liquid amount was used for one slide. In case of dividing one slide into eight blocks, all the amounts should be divided respectively.

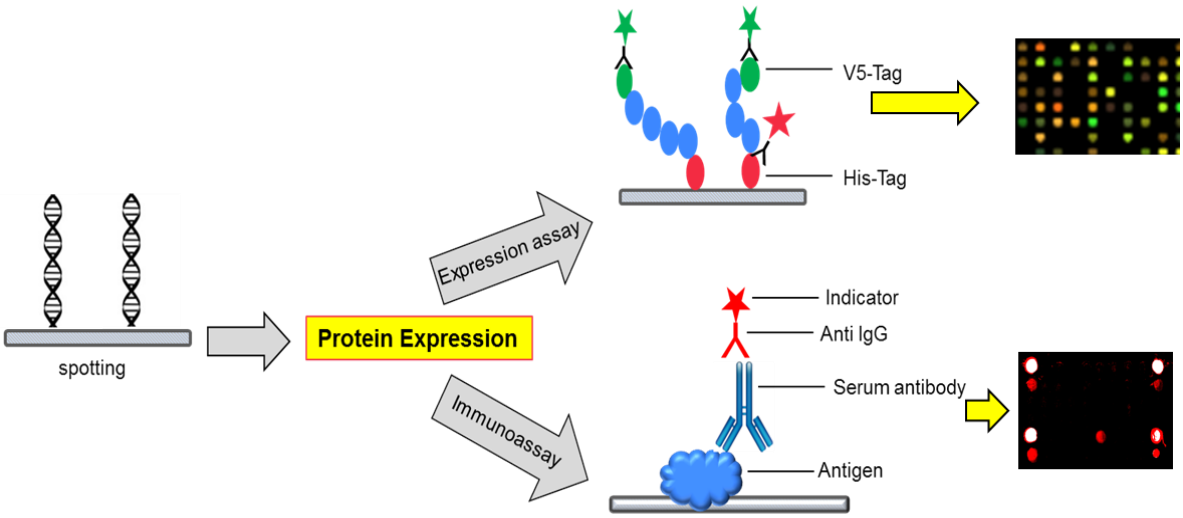


Figure 10. Overview of cell-free protein microarray for antigen detection. After spotting and incubation, whole-proteome on the array is used for checking the expression, which is called expression assay by using conjugated antibody with Alexa Fluor-647 and Cy3 against His-Tag and V5-Tag respectively. Whole-proteome also used for serum antibody reactivity, which is called immunoassay. In immunoassay if there is any reactivity between serum antibody and expressed protein on the array, this reactivity can be detected by using secondary antibody conjugated with Alexa Fluor-647 with red signal but in saturated condition it shows white signal.

2.15 Data analysis and statistics

All TIFF images were analyzed using GenePix Pro 6.0 and gal.file, which represents the order and position of each protein on the array and after analyzing, the quantitative data were extracted. After processing the Median Fluorescence Intensity (MFI) was collected. For comparison of Ni and Epoxy surface, Signal to Noise Ratio (SNR) was used, which considered Foreground signal (F), Background signal (B) and Standard deviation (SD) of the Background. While a protein has a MFI larger than the cut off (Average of negative MFIs + 2.5 SD of negatives) it is considered as a positive candidate. Statistical analysis was performed using GraphPad Prism 6 and Microsoft Excel 2010. A provisional Odds Ratio (OR) to compute the corresponding 95% Confidence Interval (CI), was calculated to estimate the importance of each antigen. Online databases and analytical tools for further analysis were mentioned in Table 15.

Table 15. Databases and analytical tools for data analysis

Name	Address
NCBI	https://www.ncbi.nlm.nih.gov/
The Human Protein Atlas	https:// www.proteinatlas.org/
The GeneCards human gene databases	https://www.genecards.org/
Uniprot	https://www.uniprot.org/
Kyoto Encyclopedia of Genes and Genomes	https://www.genome.jp/kegg/
Stand protein BLAST	https://blast.ncbi.nlm.nih.gov/
MEDCALC	https://www.medcalc.org/index.php/

3. Results

3.1 HVT DNA extraction and PCR confirmation

H. pylori genomic DNA was provided as a pure genomic DNA, but for HVT there was not any source of pure DNA. Since it was not known if this virus would be present in the cell pellet or in the supernatant, DNA extraction was performed using both supernatant and cell pellet and results were checked by PCR. For this purpose, specific primers were designed to amplify a short sequence of HVT (HVT075) while these primers did not bind to any DNA of other viruses or organisms. After DNA extraction there were two sources of extracted DNA, DNA from the supernatant and DNA from the cell pellet. PCRs were performed at the same condition by using the same amount of DNA from the supernatant and the cell pellet for templates. PCR results confirmed existence of HVT DNA in both supernatant and cell pellet but DNA from the cell pellet produced better PCR results (Figure 11).

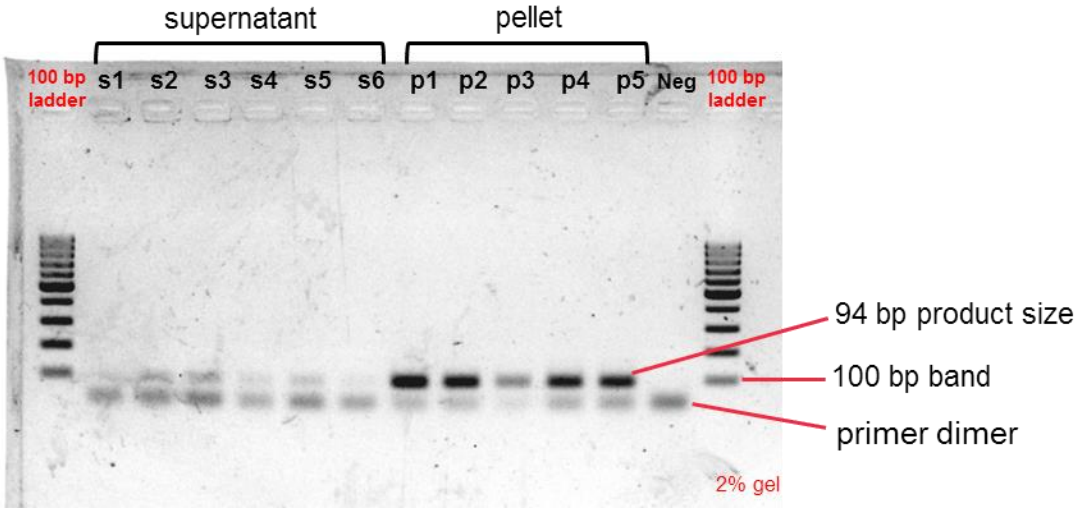


Figure 11. PCR confirmation of HVT DNA. HVT DNA extraction was performed using the virus supernatant and the virus cell pellet separately. After DNA extraction, PCR was performed to confirm HVT DNA in the supernatant and in the cell pellet. The specific 94 bp PCR band, confirms HVT DNA in the both supernatant and cell pellet whereas HVT DNA in the cell pellet was much more than supernatant. 94 bp is the specific product size, s: PCR with the supernatant DNA as the template, p: PCR with the pellet DNA as the template, numbers 1-6 show different column washes to yield HVT DNA. Neg: negative control.

3.2 Whole-ORFeome PCRs for *H. pylori* and HVT

H. pylori genome has 1437 open reading frames (ORFs) and HVT has 100 open reading frames (ORFs). PCR primers were designed for all of the ORFs as mentioned in section 2.7. After extracting of sequences from database and finding ORFs, primer design was performed for start and end of the ORFs. To have an expression construct ORF should be in frame, without stop codons and it should start with ATG. Forward primer starts at the ATG codon and reverse primer starts at the end of the ORFs. Each primer was considered to have 16-23

nucleotides, 50% GC content and 50°C Tm. Then binding specificity of each primer was checked by online blast tools. Because of different GC content at the beginning and at the end of a gene, primer designing with the similar criteria was not always possible and also in cell-free protein expression there were some difficulties in expression of ORFs more than 5 kb, therefore with splitting a gene into two or three smaller sections it was possible to have better primer designs for PCR and also to have full protein expressions. Specific primers were flanked by two overhangs which then used for binding sites of second primers. In the second PCR regulatory sequences for transcription and translation and also His-Tag and V5-Tag sequences were added to ORFs. In the first PCR, specific primers amplified ORFs and in the second PCR, regulatory sequences were added to the ORFs (Figure 8). All of the PCRs were done on 96-well plates. The expression construct was generated by two successive PCRs and then checked for quality control by Gel Agarose Electrophoresis (Figure 12 and Figure 13). The PCRs were repeated to obtain the optimum result if they didn't work or if they were evaporated especially at the corners of 96-well plates. After two successive PCRs, *H. pylori* ORFemoe and HVT ORFeome were generated. All of the PCR products were spotted using Nanoplotter 2.1 (GeSIM). Then the expression mix was added on the exact positions of the spotted PCR products. Afterwards in order to on-chip protein expression, the slides were placed in a humidified incubation chamber at 37°C, 50% humidity for 1 hour, and subsequently at 30°C, 50% humidity for 12 hours in a ventilated oven. Spotting was performed using the Nanoplotter 2.1 (GeSIM) and with a transfer file which has all the spot positions and commands. After producing protein microarrays, the existence of the expressed protein was checked by doing expression assay and after that the reactivity of these expressed proteins on the array with serum antibodies was checked by immunoassay.

In the expression assay two antibodies with different fluorescent dyes were used to bind and detect the N-terminal His-Tag and C-terminal V5-Tag of the expressed proteins with red and green signals respectively. For this purpose, anti-His antibody (Alexa Fluor-647 conjugated, Qiagen) and anti-V5 antibody (Cy3 conjugated, Sigma) were applied during expression assay. In the immunoassay if there was specific antibody against proteins of *H. pylori*, the antibody would bind to the protein on the microarray and this binding was detected by using a secondary antibody (goat anti-human IgG, IgA, IgM Antibody, Jackson ImmunoResearch) which is conjugated to Alexa Flour-647 fluorescence dye.

For expression assay signals from Alexa Fluor-647 and Cy3 fluorescent dyes were captured at the ratio of 635/532 nm in image acquisition. For immunoassay signal from Alexa Fluor-647 fluorescent dye were captured at 635 nm wavelength in image acquisition.

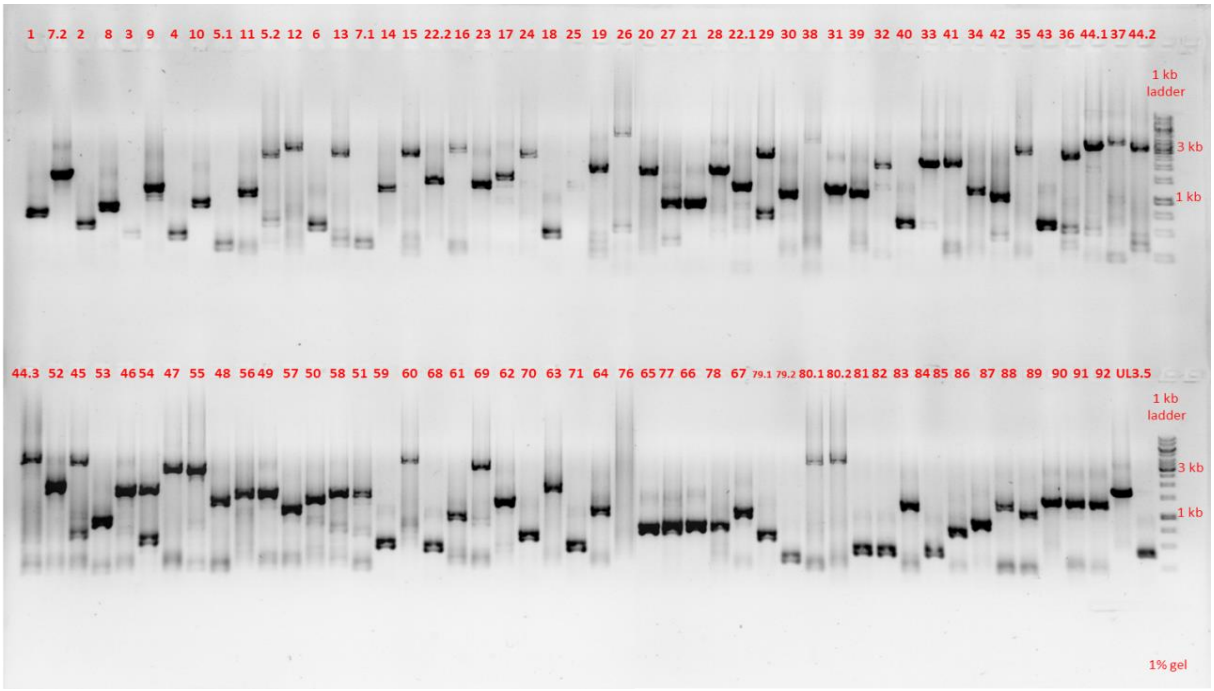


Figure 12. Whole-ORFeome PCR for HVT genes. All of the HVT ORFs were amplified by two PCRs to generate expression constructs. All of the products were loaded on 1% agarose gel. 1 kb ladder was used to verify the correct length of PCR products. The number on each product represents the HVT gene symbol.

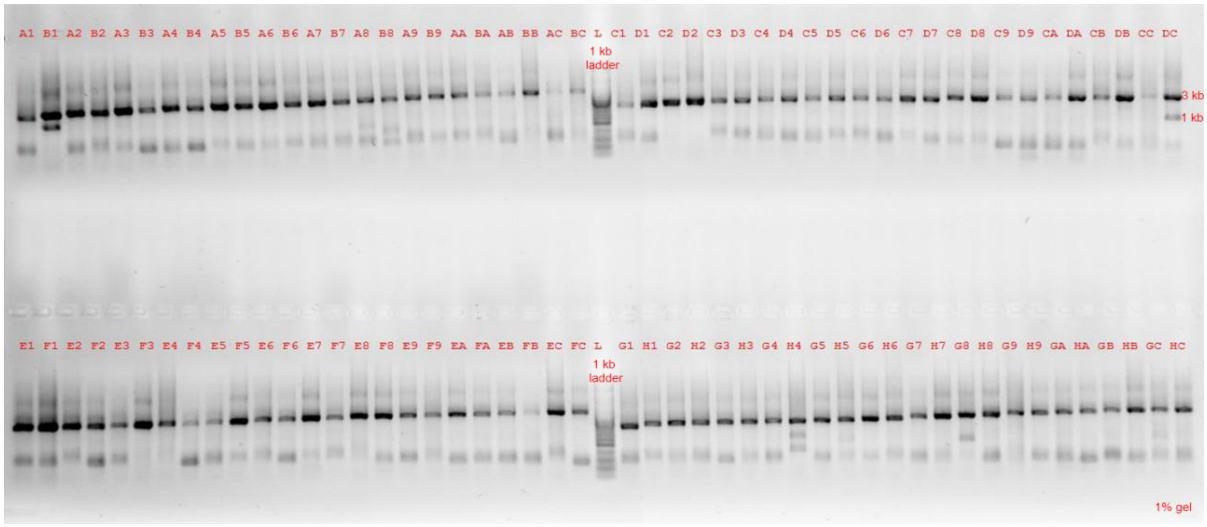


Figure 13. Whole-ORFeome PCR for *H. pylori* genes. Example of *H. pylori* ORFs after two PCRs to generate expression constructs. All of the products were loaded on 1% agarose gel. 1 kb ladder was used to verify the correct length of PCR products. The sign on each product represents the *H. pylori* gene position.

3.3 Positive controls for whole-proteome microarray

To generate whole-proteome microarray and evaluation the expression assay and the immunoassay, negative and positive controls were produced by the same PCR plan that was used for all of the ORFs in this thesis. Positive PCRs contain known expression constructs which after incubation, the expressed protein was used as endogenous positive control. P18

antigen from Epstein-Barr-Virus (EBV) and F11 antigen from Poliovirus were used as positive controls. Negative PCRs which don't have any expression construct were used as negative controls which also called Non Template Controls (NTC).

3.3.1 EBV p18 PCR

Epstein-Barr virus (EBV) is ubiquitous, and about 90% of adults throughout the world have IgG, IgM and IGA antibodies against p18 antigen. EBV p18 is also called Virus-Capsid-Antigen (VCA). ORF of EBV p18 was amplified by two successive PCRs to produce expression constructs.

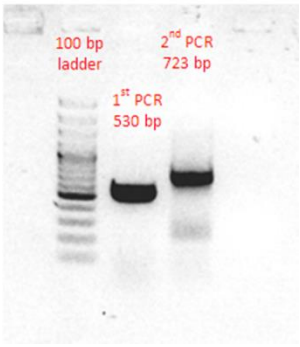


Figure 14. EBV p18, first and second PCRs. For generation expression constructs, ORF of EBV p18 was produced by two successive PCRs. The first PCR product is 530 bp and the second PCR product is 723 bp. The difference between two PCR product sizes is related to the length of the second PCR primers.

3.3.2. Poliovirus F11 PCR

Poliovirus F11 is a capsid antigen and ORF of Poliovirus F11 was amplified by two successive PCRs to produce expression constructs.

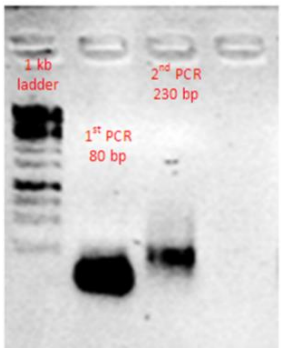


Figure 15. Poliovirus F11, first and second PCRs. For generation expression construct, ORF of Poliovirus F11 was ordered and synthesized. Then two successive PCRs were performed to generate expression construct. The difference between two PCR product sizes is related to the length of the second PCR primers.

3.4 Comparison of whole-proteome microarray on Epoxysilane (Epoxy) and Ni-NTA (Ni) surfaces

For whole-proteome microarray optimization, two different surfaces were used as the solid supports for expressed proteins. Spotting of antigens was performed on Ni and Epoxy slides and after incubation the obtained proteins were subjected to expression assay and immunoassay. The result was important to determine the solid surface which has the better result in case of protein expression and immunoassay. All of the procedures for Epoxy surface and Ni surface were performed at the same condition. The result was analyzed and calculated in GenePix pro 6.

For comparison of two different surfaces, it was necessary to consider the background signal and standard deviation of the background. Thereby Signal to Noise Ratio (SNR) was used to compare the two different surfaces. The result was analyzed in terms of expression assay and immunoassay.

3.4.1 Expression assay comparison of Epoxy and Ni surfaces

After spotting and incubation, expression assay was performed for Epoxy slide and Ni slide and these two surfaces were subjected at wavelengths 635 nm (red signal) and 532 nm (green signal) which represent the His-Tag and V5-Tag of the expressed proteins respectively. In terms of quality and morphology for both the Ni and Epoxy surfaces, there was not detected smearing problem, rush on the slide and merged spots (Figure 16 a). Ni surface was observed to have a bigger spot size. Then the result was calculated to have a quantitative comparison. SNRs of 635 nm and 532 nm for each spot were calculated and compared. Higher SNR represents how much a signal of one spot, is higher than its background. Epoxy surface showed higher SNRs at both wavelengths 532 nm and 635 nm (Figure 16 b). In summary, Epoxy surface showed higher SNRs in compare of Ni surface however it is not significant. The higher SNR signal in Epoxy surface could be due to the smaller spot size.

3.4.2 Immunoassay comparison of Epoxy and Ni surfaces

Immunoassay was performed at the same situation for both Epoxy and Ni slides and the result was captured at wavelength 635 nm. Since in immunoassay not all of the spots were reactive to serum samples, positive controls which were reactive to serum samples were used to have a robust calculation. In terms of quality in Epoxy surface there was strong background which could mask the specific signal but in Ni slide, background signal was very low which led to significant positive signal.

In terms of quantity, Signal to Noise Ratio (SNR) of Ni surface is much higher than Epoxy surface and also in Ni surface the difference between SNRs of Positives (Pos) and Negatives (Neg) is so much higher than Epoxy surface.

In summary in Epoxy surface the specific signal could be masked by background. In Ni surface the specific signal was much more detectable because of low background. Based on this result, Ni surface was selected to be used for further experiments (Figure 17).

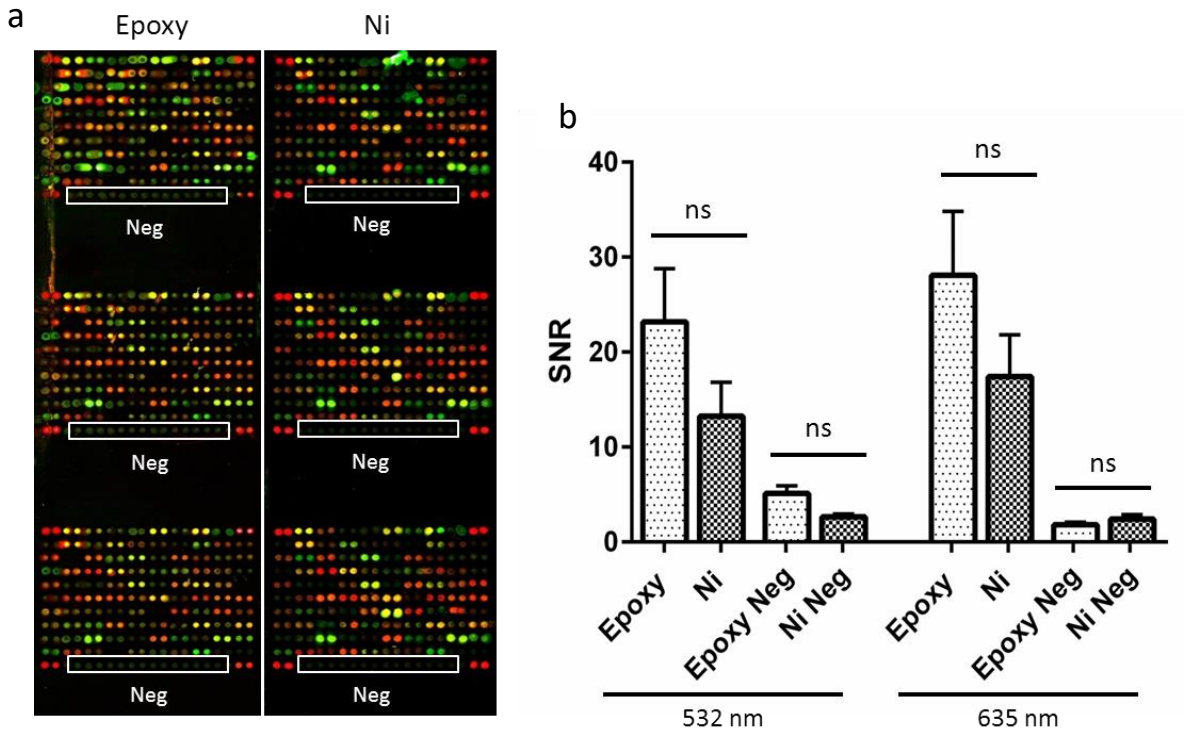


Figure 16. Qualitative and Quantitative comparisons of Epoxy and Ni slides in expression assay. After protein expression and immunostaining, Epoxy and Ni slides were scanned and the image was captured at the ratio of 635/532 nm. Regarding quality and morphology the spots on both Ni and Epoxy surfaces were resembled however spot size on Ni was larger (a). In quantitative comparison Epoxy and Ni slides were analyzed in terms of SNR. At both wavelengths 532 nm and 635 nm, Epoxy surface showed higher SNRs in compare of Ni surface (b). Red signal represented anti-His-Tag binding antibody at N-terminal of proteins. Green signal represented anti-V5-Tag binding antibody at C-terminal of proteins and also green signal was a sign of protein full expression. Yellow signal represented combination of red and green signals. This image was taken by GenePix pro 6. $*$: $P \leq 0.05$, ns: $P > 0.05$.

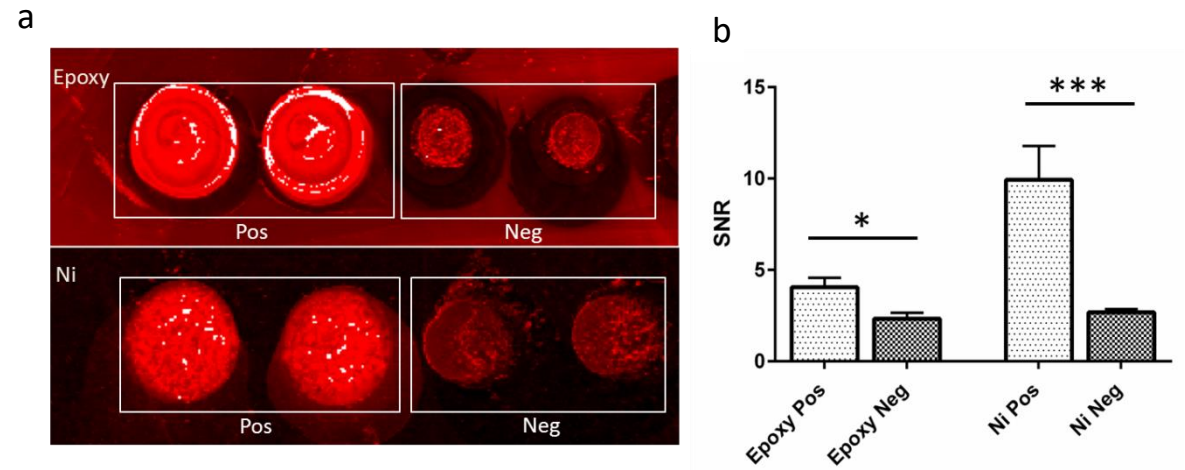


Figure 17. Qualitative and quantitative comparisons of Epoxy and Ni slides in immunoassay. In Epoxy surface, background signal was too high and it masked the positive signal. In Ni slide the background signal was low and the positive signal was completely significant. This result was captured at wavelength of 635 nm and 4x magnification by GenePix pro 6 (a). After calculation of both Ni and Epoxy surfaces, Ni slide showed a higher positive signal than Epoxy slide (b). *: $P \leq 0.05$, ***: $P \leq 0.001$.

3.5 Optimization of *E. coli* lysate in Immunoassay

One of the important things in immunoassay is reducing background to obtain optimum result. High background interferes with specific signal. Without reducing background, it is very hard to discriminate between positive and negative signals. Result showed that using *E. coli* lysate was very important in reducing background signals by collecting *E. coli* antibodies in serum. Serum sample should be incubated with *E. coli* lysate before using it on the slide. In another experiment different timepoints of incubation were tested and the result showed that two hours serum incubation with *E. coli* lysate provided the best result.

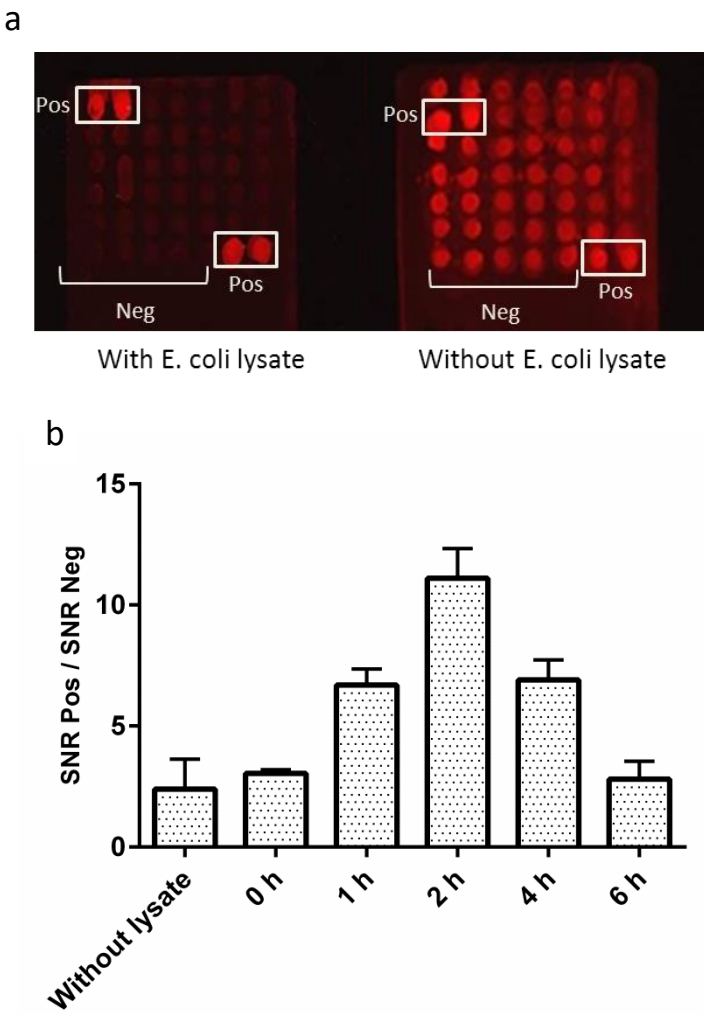


Figure 18. Effect of *E. coli* lysate in immunoassay. The result of *E. coli* lysate incubation with serum samples is shown**.** Without *E. coli* lysate the difference between positive and negative signals was very low and positive signal was hardly detectable. This data showed using the *E. coli* lysate reduced background and nonspecific signal. P represented positive controls which are in duplicate and Neg represented negatives (a). To check the effect of incubation time of *E. coli* lysate, 5 different timepoints were tested and the results were compared. The result was analyzed based on SNR (b). This data showed two hours *E. coli* lysate incubation with serum sample before applying it on the slide, presented the highest signal.

3.6 Optimization of Betaine effect in whole-proteome microarray

To prepare PCRs for spotting, Betaine 5 M was added to prevent evaporation and also it helps to increase transcription and translation by preventing secondary structure. 10% and 20% of Betaine 5 M were used in total of spotting volume and the results were compared. When the Betaine amount was increased from 10% to 20%, morphology problems such as merged spots and rush on the slide were observed.

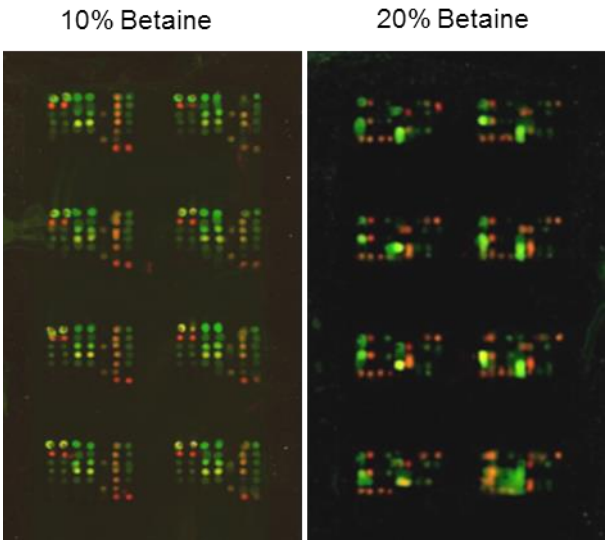


Figure 19. Effect of Betaine amount. 10% Betaine 5 M is the optimum amount in PCRs for spotting. 20% Betaine 5 M in PCRs would result in merged spots and rush on the slide. Image was captured at the ratio of 635/532 nm.

3.7 Inconsistent spotting

During optimization, inconsistent spotting was noticed. After expression assay the spots were only visible on some parts of the slide. This problem could be mechanical (spotter) or could be technical (during expression assay). To solve it, after spotting, exogenous protein fused to His-Tag was added on 4 places on the slide. Expression assay was performed and after staining the exogenous His-Tag protein was detected by antibody against His-Tag. This result showed that staining procedure was homogenous across the slide.

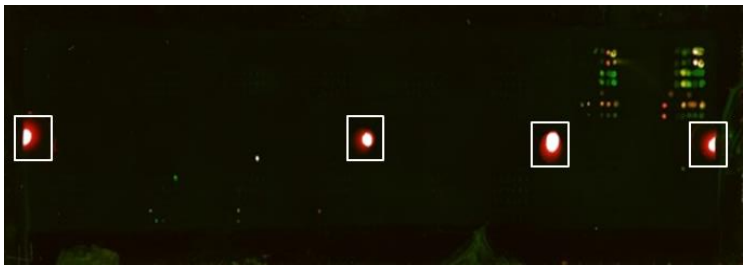


Figure 20. Inconsistent spotting. Using His-Tag fused protein exogenously and staining it during expression assay represented homogenous staining across the slide. His-Tag proteins are shown in white squares. Image was captured at the ratio of 635/532 nm.

3.8 Whole-proteome microarray of *H. pylori*

All of the *H. pylori* ORFs were spotted on Ni slide to produce *H. pylori* protein microarray. After incubation the expressed proteins on the array were used for antibody staining to determine the protein expression. Two different conjugated antibodies with fluorescence labels were applied and the image was captured using ratio of 635/532 nm. Alexa Flour fluorescence dye reflects red and Cy3 fluorescence dye reflects green. One antibody is anti-His-Tag which is labeled with Alexa Flour fluorescence dye. This antibody detects and binds to His-Tag of the expressed proteins. Another antibody is anti V5-Tag which is labeled with Cy3 fluorescence dye. This antibody detects and binds to V5-Tag of the expressed proteins on the array. The C-terminal V5-Tag of the expressed proteins was reflected as the green signal and green signal was the sign of protein full expression however the N-terminal His-Tag of the expressed proteins was reflected as the red signal. The N-terminal His-Tag serves immobilization of the expressed proteins to Ni slide.

H. pylori proteins were expressed on the array. *H. pylori* proteins with similar protein lengths spotted close to each other and protein lengths were increased from top to down of the array. EBV p18 and Poliovirus F11 were used for positive controls which they have known antigens to react to human serum samples whereas PCR without template was used for negative controls (Figure 21).

3.9 Immunoassay of *H. pylori*

Immunoassay was performed using healthy, chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) serum samples. If there was specific antibody against proteins of *H. pylori*, the antibody would bind to the protein on the microarray and this binding was detected by using a secondary antibody which was conjugated to Alexa Flour fluorescence dye. Secondary antibody binds to Fragment crystallizable (Fc) region of IgA, IgG and IgM. Immunoassay was performed initially by using *H. pylori* positive and negative serum samples. The presence of antibodies and also their binding to the antigens on the microarray were proved by obtaining specific signal. Moreover, spotting of positive and negative controls on the array provided a valuable estimation of detecting positive candidates. Positive candidate should have a higher signal than negative controls. When a protein has a Median Fluorescence Intensity (MFI) larger than the cut off (Average of negative MFIs + 2.5 SD of negatives) it is considered as a positive candidate (Figure 22).

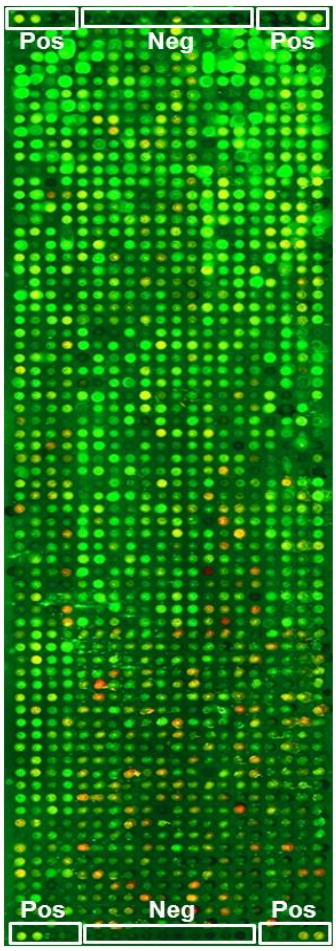


Figure 21. Expression assay result. *H. pylori* ORFeome was spotted on Ni surface and after incubation whole-proteome microarray was generated. Immunostaining was performed using two antibodies against His-Tag (red signal) and V5-Tag (green signal). His-Tag is in the N-terminal of the newly expressed protein and serves to bind to Ni surface. V5-Tag is in the C-terminal of the newly expressed protein and it is a sign of full expression. Pos represents positive controls at four corners and Neg represents negative control on top and bottom of the slide. This image was captured at the ratio of 635/532 nm.

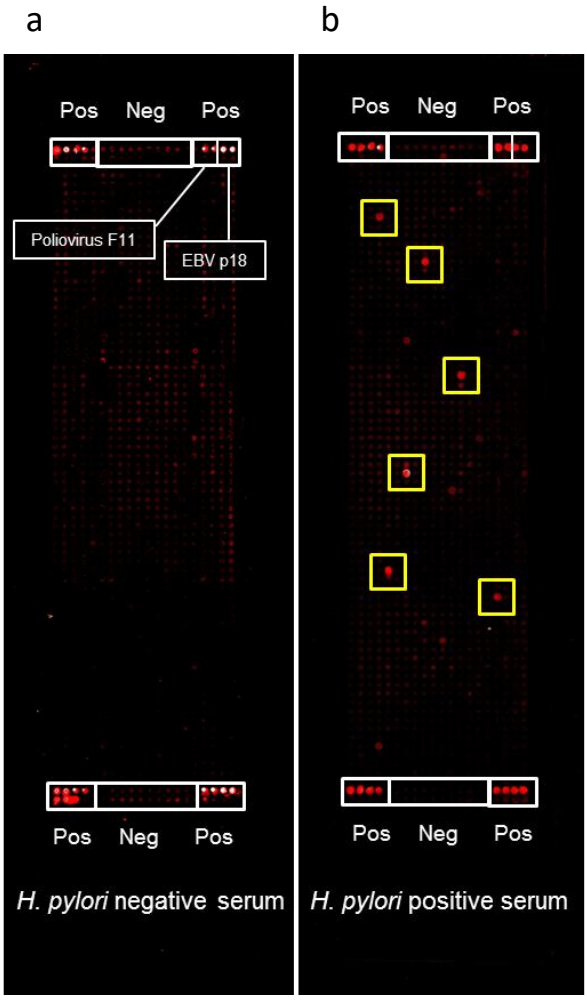


Figure 22. Immunoassay of *H. pylori* negative and positive serum samples. All of 1437 *H. pylori* proteins were spotted on Ni surface and positive controls which were EBV p18 and Poliovirus F11 also spotted on the corners. Negative controls were spotted in the middle of positive controls. *H. pylori* negative and positive serum samples were used for immunoassay. By using *H. pylori* negative serum sample there were not any positive candidates but positive controls were detected as a sign of technical performance (a). Specific positive signals identified by using *H. pylori* positive serum sample (b). Pos represents positive controls. Neg represents negative controls. This image was captured at wavelength 635 nm.

3.9.1 Screening of *H. pylori* antigens in PDAC and non-PDAC serum pools

After validation of immunoassay by using *H. pylori* negative and positive serum samples, serum samples of healthy, CP and PDAC were applied to the array. Whole-proteome microarrays comprised of 1437 *H. pylori* proteins were prepared and used to test with pooled serum samples to find the most promising candidates that might be relevant to PDAC condition. Some proteins were detected just in PDAC serum samples and could be relevant to PDAC pathogenesis and tumorigenesis. As *H. pylori* is a common infection in human being, it is possible to detect this bacterium in non-PDAC donors (healthy and CP) as well as PDAC patients, so I have also detected shared proteins between healthy, CP and PDAC (Figure 23).

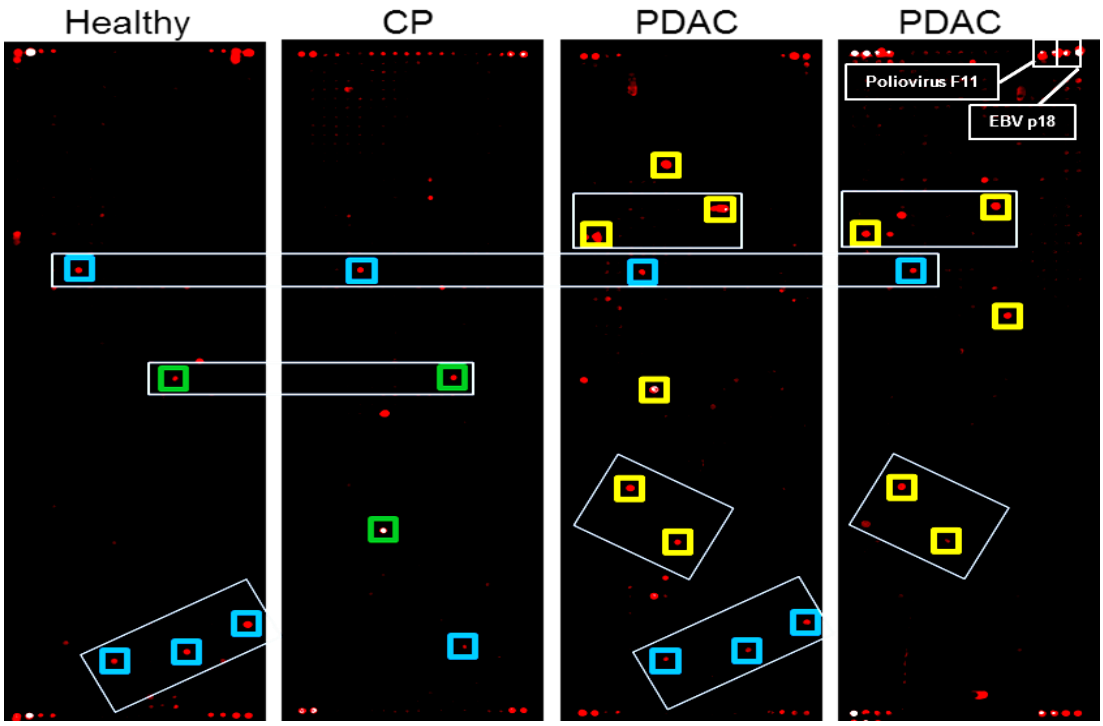


Figure 23. Example screening of positive candidates. 1437 *H. pylori* proteins were spotted on the array. Healthy, CP and PDAC serum samples were applied during immunoassay. *H. pylori* antigens were recognized based on the position on the array. Differential screening of *H. pylori* positive candidates showed some antigens were in common between healthy, CP and PDAC (blue squares), some antigens were detected just in healthy and CP (green squares) and some were specific for PDAC (yellow squares). To keep the clarity, just some of the antigens were labeled.

Immunoassay was performed using pooled serum samples of CP and PDAC from Heidelberg University Clinic and healthy from Mannheim Blood Center. In this thesis this dataset is briefly called Heidelberg serum samples. 50 serum samples from healthy donors, 45 serum samples from CP donors and 50 from PDAC patients were included. 5 sera of each group were combined as one pool, resulting in a total of 10 healthy pools, 10 PDAC pools, and 9 CP pools. Figure 23 shows a descriptive image of whole-proteome microarrays incubated with PDAC serum samples which represents some of the detected antigens and Figure 24 shows the total detected antigens. In this screening, the prevalence of detected antigens in PDAC group and non-PDAC groups (healthy and CP) were estimated by the frequency of

seropositivity. A total of 46 proteins were detected in this experiment. The shortlisted candidates were ranked according to the frequency of seropositivity and the association with PDAC and non-PDAC (Figure 24). Antigen associations were measured and reported by Odds Ratio (OR) and 95% Confidence Interval (CI):

OR=1 Exposure does not affect odds of outcome

OR>1 Exposure associated with higher odds of outcome

OR<1 Exposure associated with lower odds of outcome

3.9.2 Screening of *H. pylori* antigens in individual PDAC and non-PDAC serum samples

Using serum pools can be a fast way to find the reactive antigens but when pooling serum samples to have one serum pool with the same volume there are some considerations. Firstly, it is possible to dilute antibody level in a positive serum sample in other non-positive serum samples which can eliminate the positive signal (false negative). Secondly, combining different sera in one pool might have accumulative effect since different sera can recognize multiple epitopes of one antigen on the array. So some antigens can be detectable in pooled serum samples while they are not detectable in individual serum samples (false positive). Thirdly, combining different serum samples in one pool is reducing the size of dataset. For these reasons serum pools might not reflect all of the positive candidates and frequencies. For a better estimation it was important to apply a bigger dataset with individual serum samples.

After screening of positive candidates in serum pools, to have a better estimation, individual serum samples of PDAC and non-PDAC (healthy and CP) were applied to the array. Whole-proteome microarrays comprised of 1437 *H. pylori* proteins were prepared and used to test with individual serum samples to find the most promising candidates that might be relevant to PDAC condition. Immunoassay was performed using individual serum samples of healthy, CP and PDAC. 47 serum samples from PDAC patients and 38 serum samples from non-PDAC donors (19 serum samples from healthy and 19 serum samples from CP) were included. In this screening, the prevalence of the antigens in PDAC patients and non-PDAC donors (healthy and CP) were estimated by the frequency of seropositivity. A total of 62 proteins were detected in this experiment. The shortlisted candidates were ranked according to the frequency of seropositivity and the association with PDAC and non-PDAC serum samples. Antigen associations were measured and reported by Odds Ratio (OR) and 95% Confidence Interval (CI) (Figure 25).

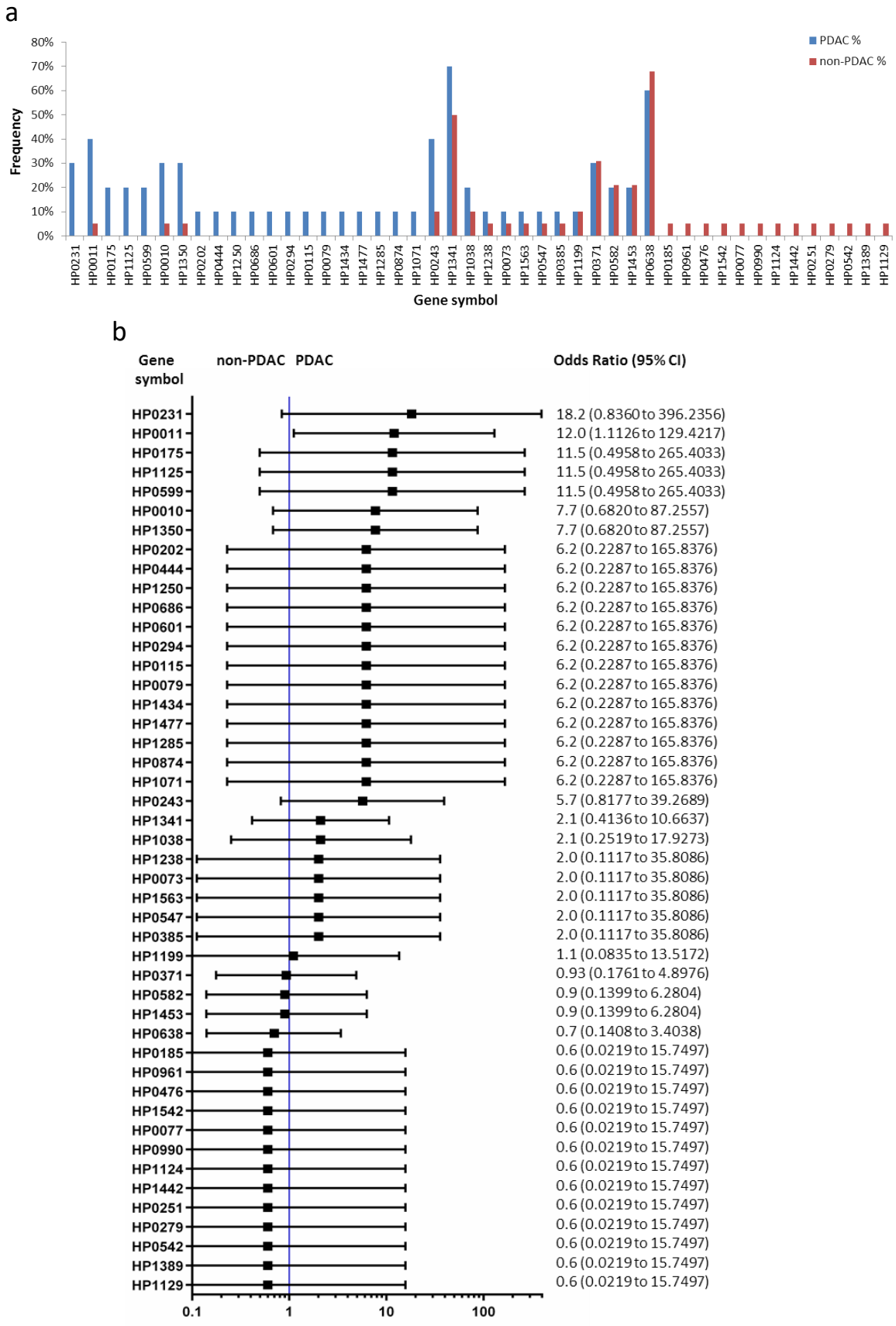
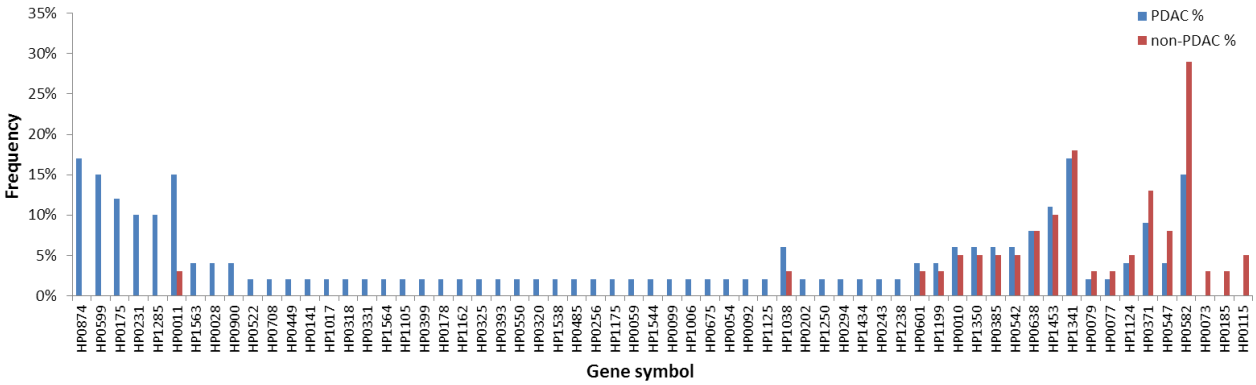


Figure 24. Association of *H. pylori* positive candidates with PDAC serum pools. The frequency of positive candidates in PDAC and non-PDAC serum pools (a) and the positive candidates were ranked based on Odds Ratio and 95% CI (b).

a



b

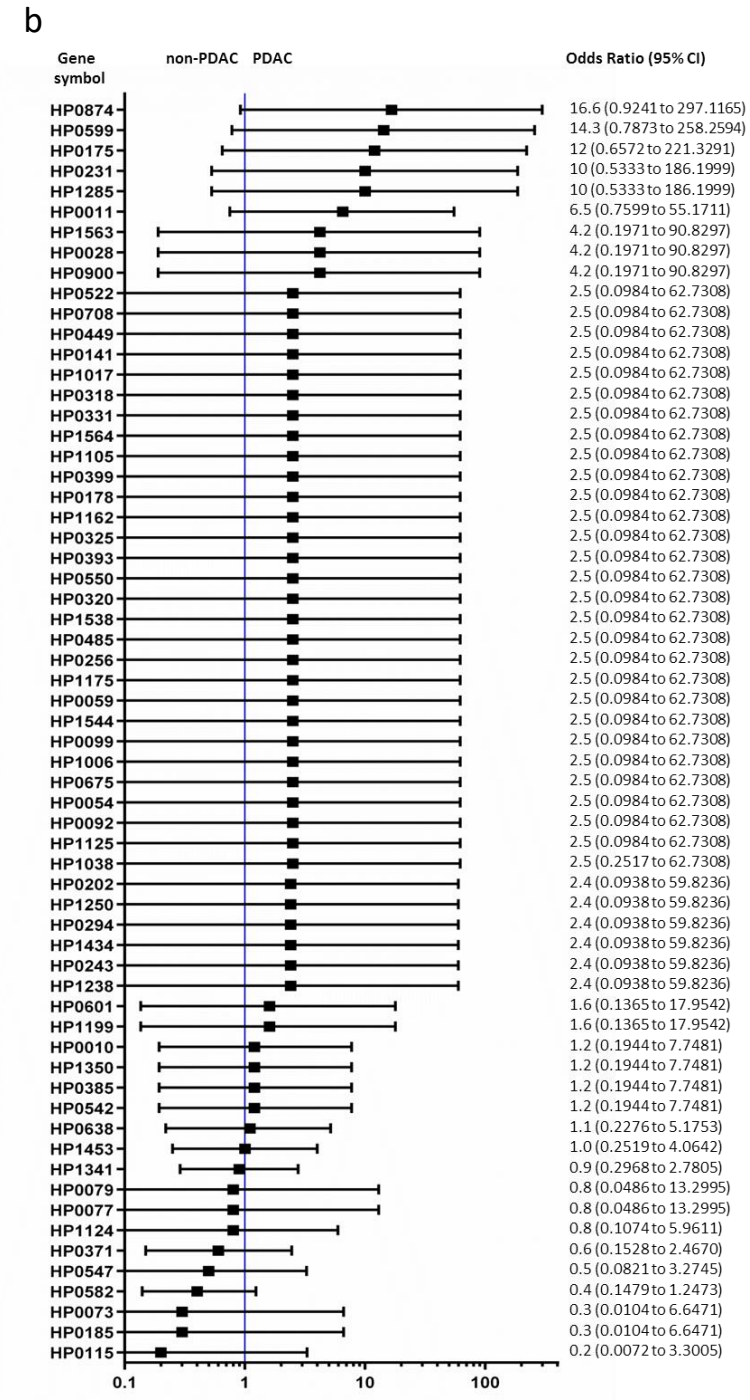


Figure 25. See next page.

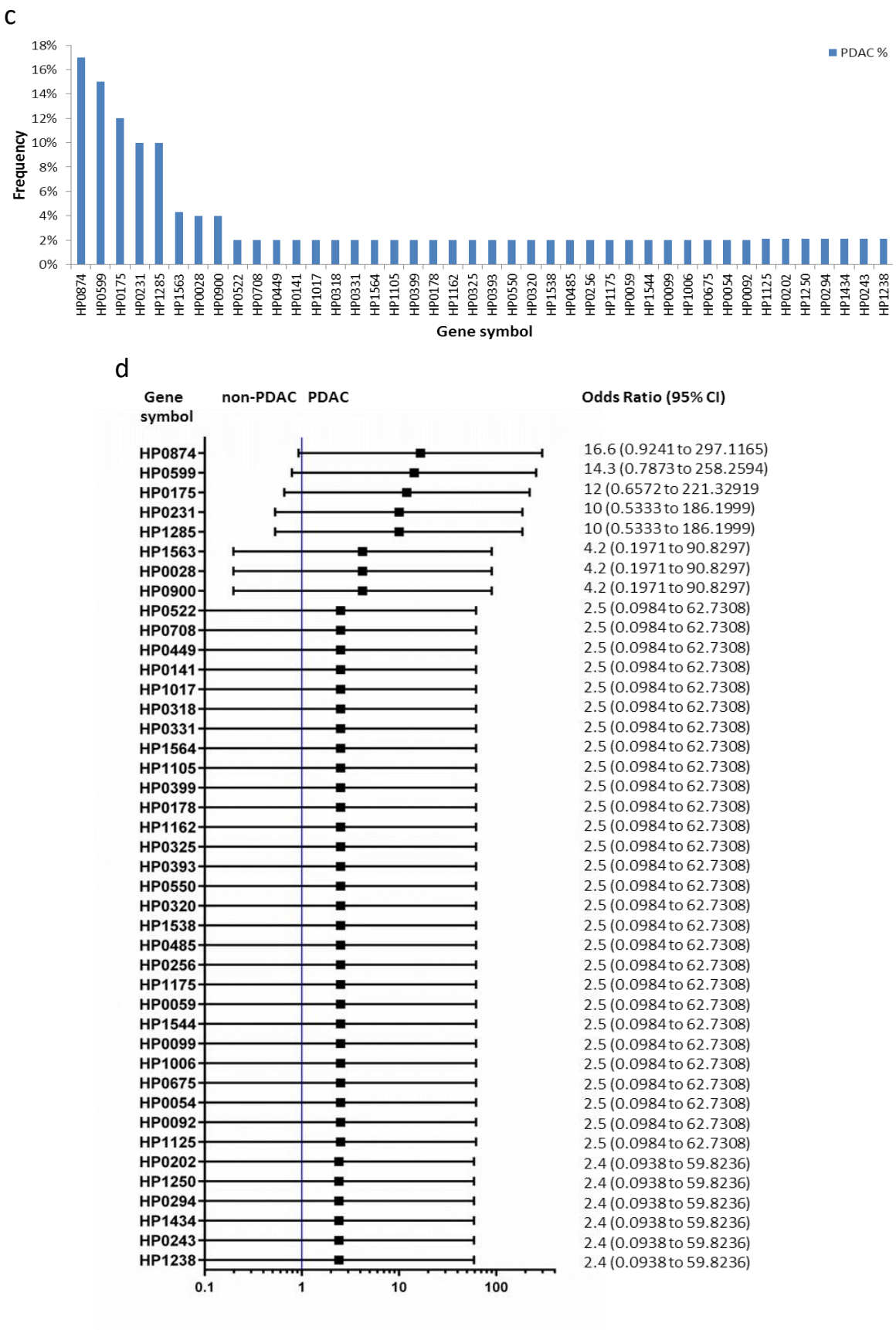


Figure 25. Association of *H. pylori* positive candidates with PDAC serum samples. The frequency of positive candidates in individual PDAC and non-PDAC serum samples (a) and the ranking based on Odds Ratio and 95% CI (b), the frequency of positive candidates which were detected just in PDAC serum samples (c) and the ranking based on Odds Ratio and 95% CI (d).

3.9.3 Comparison of *H. pylori* antigen screenings in pools and individual serum samples

In the first screening of *H. pylori* antigens, 10 PDAC pools, 10 healthy pools and 9 CP pools were applied while in the second screening, 47 PDAC serum samples and 38 non-PDAC serum samples (19 healthy and 19 CP) were applied. Serum pools and individuals were from Heidelberg University Clinic (healthy samples were from Mannheim Blood Center). Antigen associations were measured and reported by Odds Ratio (OR) and 95% Confidence Interval (CI). In applying individual serum samples, the number of detected antigens is higher than using pools (62 antigens vs 46 antigens) (Figures 24 and 25). 33 antigens were in common between individuals and serum pools. Figure 26 a, b shows the frequency of the detected antigens between individuals and pools is not the same and Figure 26 c, d shows Odds Ratio (OR) of the antigens is not the same. Association patterns of most of the antigens were similar between individuals and pools while minority of the antigens such as HP0115 showed different patterns. Antigens were classified based on OR and +/+ means OR>1 and +/- means OR≤1 (Table 16). Figure 26 e, f shows 29 detected antigens in individual serum samples and they are not in pools, moreover these antigens only belong to PDAC serum samples but in low frequency and it might be due to higher number of PDAC serum samples. Figure 26 g, h shows 13 detected antigens just in serum pools and they belong to PDAC or non-PDAC serum pools. This data emphasized the role of individual serum samples for obtaining larger antigen list and better evaluation with exact frequency and Odds Ratio.

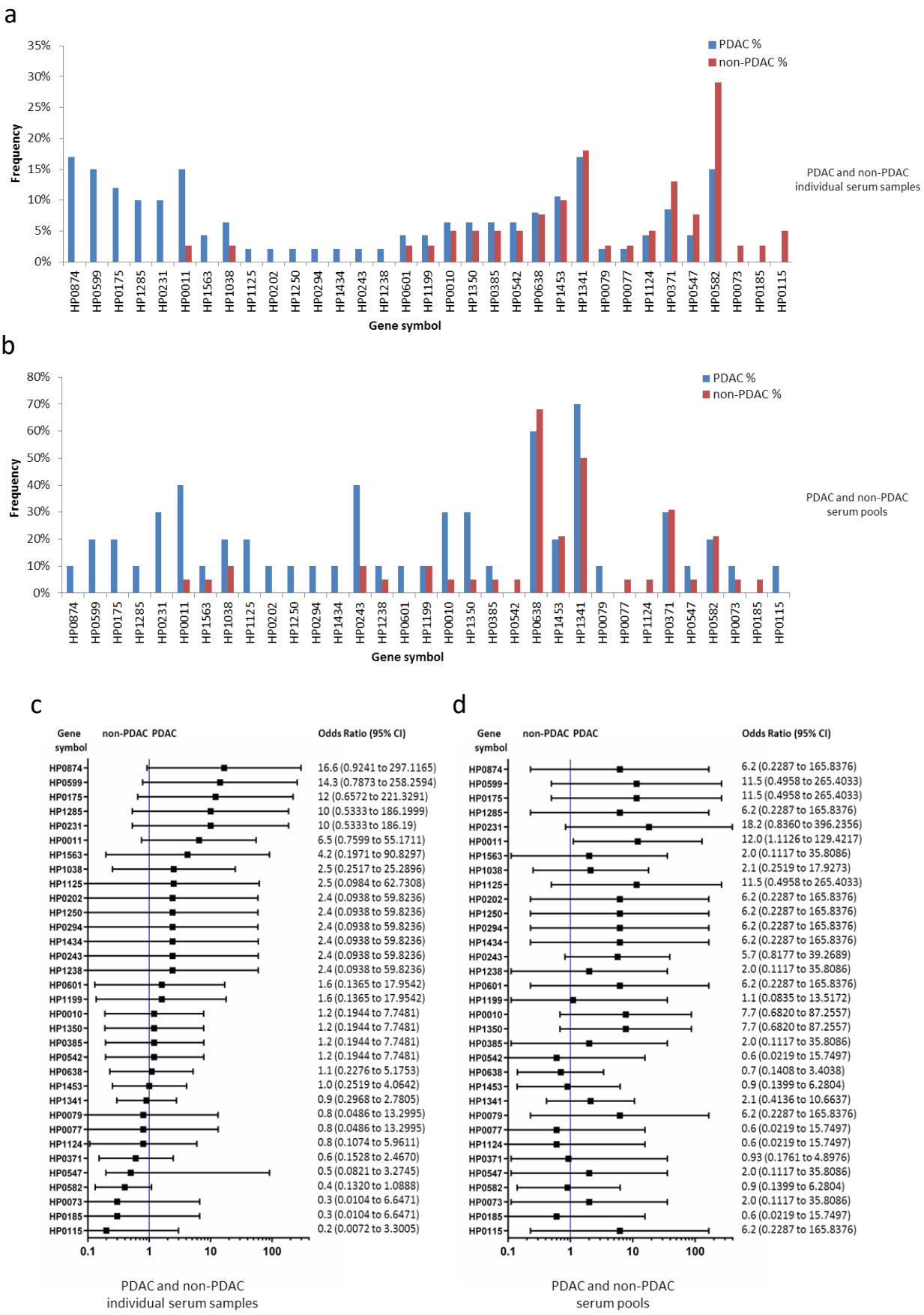


Figure 26. See next page.

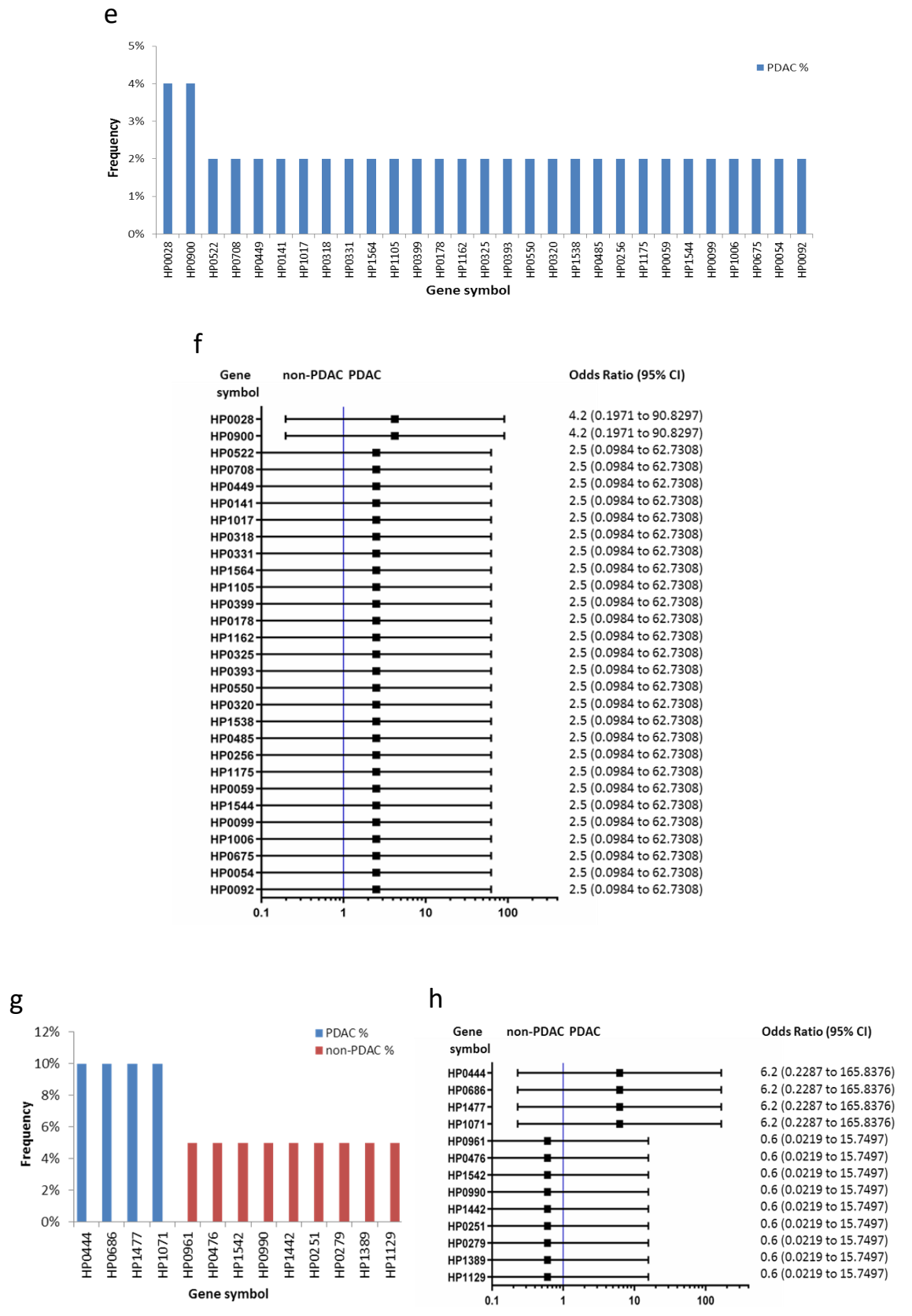


Figure 26. Comparison of *H. pylori* antigen screenings in pools and individual serum samples. The frequency of positive candidates between PDAC and non-PDAC individual samples (a) and in PDAC and non-PDAC serum pools (b). The positive candidates were ranked based on Odds Ratio and 95% CI in individual serum samples (c) and in serum pools (d). The frequency of positive candidates which were detected just in individual PDAC serum samples (e), and the ranking based on Odds Ratio and 95% CI (f). The frequency of positive candidates which were detected just in PDAC or non-PDAC serum pools (g) and the ranking based on Odds Ratio and 95% CI (h).

Gene symbol	Heidelberg individual serum samples	Heidelberg serum pools	Odds Ratio average
HP0231	+/+	+/+	14.1
HP0599	+/+	+/+	12.9
HP0175	+/+	+/+	11.7
HP0874	+/+	+/+	11.4
HP0011	+/+	+/+	9.2
HP1285	+/+	+/+	8.1
HP1125	+/+	+/+	7.0
HP0010	+/+	+/+	4.5
HP1350	+/+	+/+	4.5
HP0202	+/+	+/+	4.3
HP1250	+/+	+/+	4.3
HP0294	+/+	+/+	4.3
HP1434	+/+	+/+	4.3
HP0243	+/+	+/+	4.0
HP0601	+/+	+/+	3.9
HP1563	+/+	+/+	3.1
HP1038	+/+	+/+	2.3
HP1238	+/+	+/+	2.2
HP0385	+/+	+/+	1.6
HP1199	+/+	+/+	1.3
HP0542	+/+	+/-	0.9
HP0638	+/+	+/-	0.9
HP1453	+/-	+/-	1.0
HP0371	+/-	+/-	0.8
HP0077	+/-	+/-	0.7
HP1124	+/-	+/-	0.7
HP0582	+/-	+/-	0.7
HP0185	+/-	+/-	0.4
HP0079	+/-	+/+	3.5
HP0115	+/-	+/+	3.2
HP1341	+/-	+/+	1.5
HP0547	+/-	+/+	1.3
HP0073	+/-	+/+	1.1

Table 16. *H. pylori* antigens in Heidelberg individual serum samples and serum pools. 33 antigens were detected in both individual serum samples and serum pools and they were ranked based on association pattern and Odds Ratio average. +/+: OR>1 and +/-: OR≤1.

Gene symbol	Heidelberg individual serum samples	Heidelberg serum pools	Odds Ratio average	Definition	Pathway
HP0231	+/+	+/+	14.1	protein-disulfide isomerase	Chaperones and folding catalysts
HP0599	+/+	+/+	12.9	hemolysin secretion protein	Chemotaxis
HP0175	+/+	+/+	11.7	peptidylprolyl isomerase	Pro apoptosis and angiogenesis
HP0874	+/+	+/+	11.4	hypothetical protein	Resistance to hydrogen peroxide
HP0011	+/+	+/+	9.2	co-chaperonin GroES	Protein folding
HP1285	+/+	+/+	8.1	hypothetical protein	Cell binding
HP1125	+/+	+/+	7.0	peptidoglycan associated lipoprotein	Interferon-γ modulation
HP0010	+/+	+/+	4.5	molecular chaperone GroEL	Chaperone and heat shock protein
HP1350	+/+	+/+	4.5	protease	Carboxyl-terminal processing protease

Table 17. Top *H. pylori* positive candidates in Heidelberg individual serum samples and serum pools. Definition and function of top *H. pylori* positive candidates with the same association pattern.

3.9.4 Screening of *H. pylori* antigens in PDAC and non-PDAC serum pools with gastrointestinal conditions

In another screening, 19 serum pools with gastrointestinal disorders from Spanish National Cancer Research Centre (Madrid) which 11 serum pools were PDAC and 8 of them were non-PDAC were applied to find *H. pylori* positive antigens (Table 18). Immunoassay was performed to find the antigens in PDAC and non-PDAC serum pools (Figure 27). A total of 59 antigens were detected in this experiment and they were ranked based on frequency and Odds Ratio with 95% CI (Figure 28 a, b).

Serum pool	Description
1-PDAC & HP group	1-pool.HP-(cases)<2
2-PDAC & HP group	2-pool.HP-(cases)>2
3-PDAC & HP group	3-pool.HP-(cases)>2
4-Cancer free, HP group	4-pool.HP-(cntr)>2
5-Cancer free, HP group	5-pool.HP-(cntr)>2
6-PDAC & UL group	6-pool.UL-(cases)>2
7-PDAC & UL group	7-pool.UL-(cases)>2
8-Cancer free & UL group	8-pool.UL-(cntr)>2
9-Cancer free & UL group	9-pool.UL-(cntr)>2
10-PDAC & Heartburn group	10-pool.H-(cases)<2
11-PDAC & Heartburn group	11-pool.H-(cases)<2
12-PDAC & Heartburn group	12-pool.H-(cases)>2
13-Cancer free & Heartburn group	13-pool.H-(cntr)>2
14-Cancer free & Heartburn group	14-pool.H-(cntr)>2
15-PDAC & Acid regurgition group	15-pool.AR-(cases)<2
16-PDAC & Acid regurgition group	16-pool.AR-(cases)<2
17-PDAC & Acid regurgition group	17-pool.AR-(cases)>2
18-Cancer free & Acid regurgition group	18-pool.AR-(cntr)>2
19-Cancer free & Acid regurgition group	19-pool.AR-(cntr)>2

Table 18. List of PDAC and non-PDAC serum pools from Madrid. PDAC and non-PDAC serum pools with gastrointestinal conditions. PDAC groups are in gray and non-PDAC groups are in white. UL: Ulcer disorder, H: Heartburn disorder, AR: Acid regurgitation disorder. Case: PDAC, cntr: non-PDAC. >2: gastrointestinal disorder was detected more than 2 years, <2: gastrointestinal disorder was detected less than 2 years.

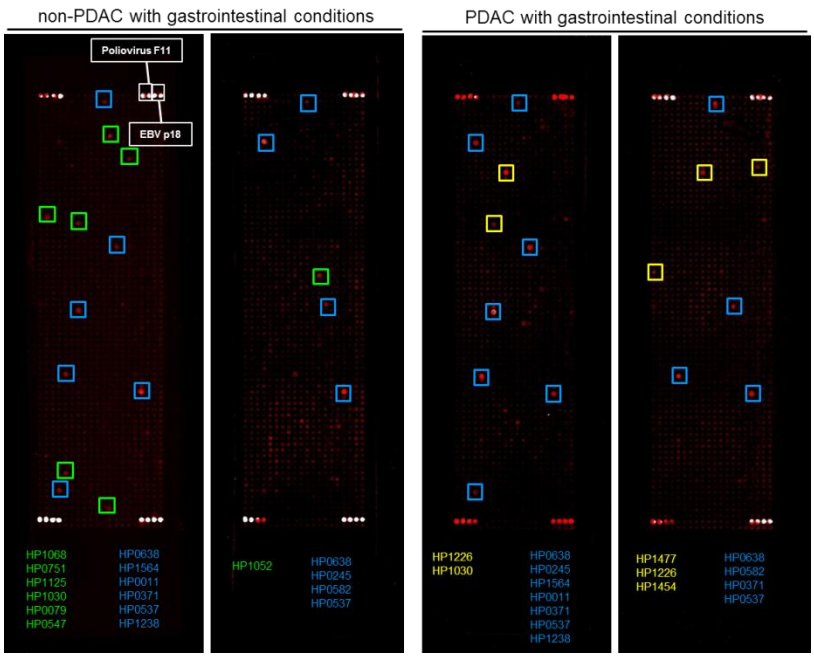
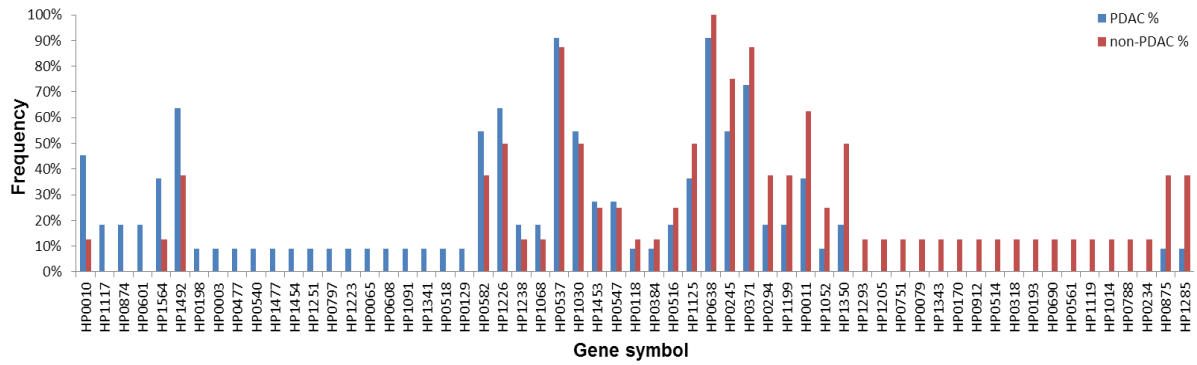


Figure 27. Example of *H. pylori* immunoassay result and antigen detection in PDAC and non-PDAC serum pools with gastrointestinal conditions. Whole-ORFeome of *H. pylori* were spotted in microarray and after transcription and translation the expressed proteins were applied to Immunoassay by using PDAC and non-PDAC serum pools with gastrointestinal conditions. Green shows the specific antigen in non-PDAC pools, yellow shows the specific antigens in PDAC pools and blue shows the antigen in PDAC and non-PDAC pools.

a



b

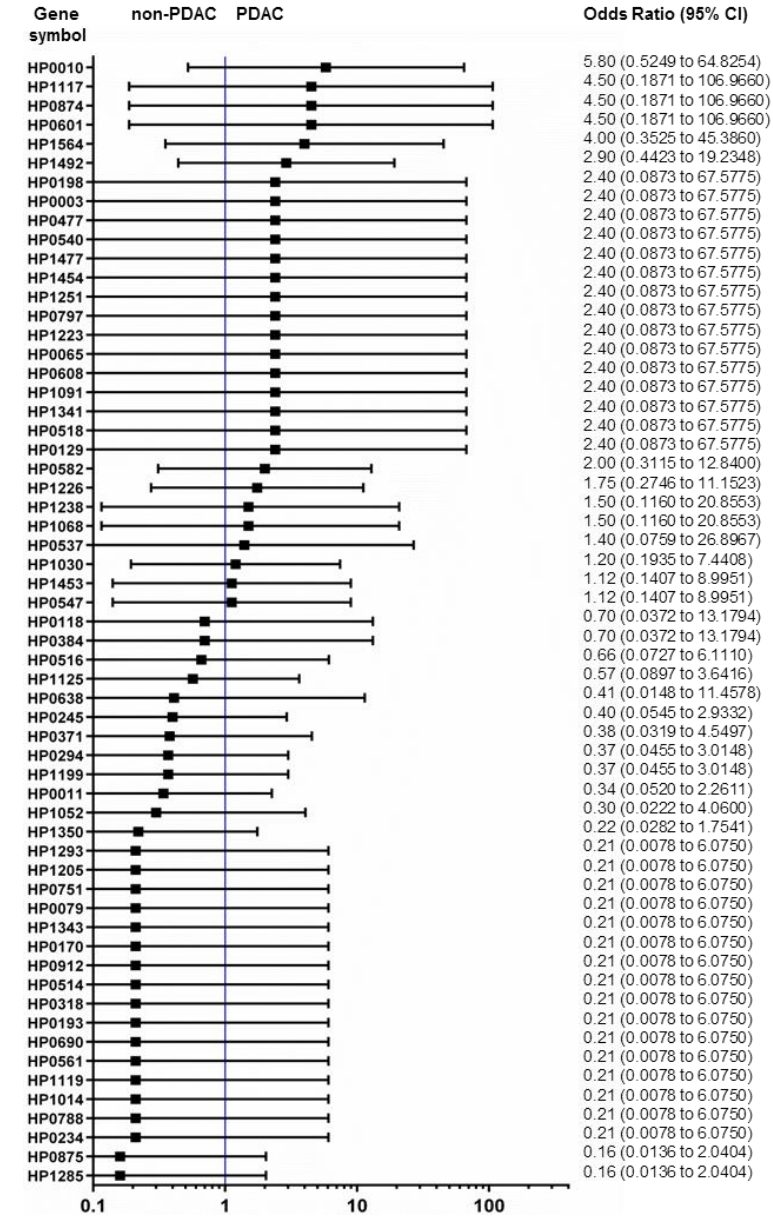


Figure 28. Association of *H. pylori* positive candidates with PDAC serum pools in gastrointestinal conditions. The frequency of positive candidates in PDAC and non-PDAC serum pools (a) and the positive candidates were ranked based on Odds Ratio and 95% CI (b).

3.9.5 Comparison of *H. pylori* screenings in three serum datasets

First screening was performed using PDAC and non-PDAC (CP and healthy) serum pools from Heidelberg University Clinic and Mannheim Blood Center and the second screening was performed using PDAC and non-PDAC (CP and healthy) individual serum samples from the same center. Third screening was performed using PDAC and non-PDAC serum pools with gastrointestinal conditions. Each screening has its advantage: in the first and second screening using CP and healthy groups as controls, could help to have a better screening of PDAC associated antigens. In the first screening, serum pools have presented a fast way of antigen screening (Figure 29 b, e) however pooling serum samples could have some considerations such as false negative antigens due to dilution effect of specific antibodies in pools and false positive due to accumulation effect of different antibodies for different epitopes of one antigen. To have a better estimation of antigen value, in the second screening, individual serum samples from the same center were applied instead of serum pools (Figure 29 a, d). In the third screening, PDAC and non-PDAC serum pools with gastrointestinal conditions from Madrid were applied to microarray. In this screening using serum pools with gastrointestinal disorders could help to subtract *H. pylori* gastrointestinal antigens from PDAC antigens (Figure 29 c, f). In the third screening by using PDAC and non-PDAC serum pools with gastrointestinal conditions the Odds Ratios are lower in compare of the first and second screening.

In the next step three serum datasets were compared to find the *H. pylori* antigens. A total of 167 *H. pylori* antigens were detected while 36 *H. pylori* antigens were detected in three serum datasets. Figure 30 shows the frequency of the antigens in three serum datasets with the ranking based on Odds Ratio (OR) and the result was summarized in Table 19. Because of different serum sources, serum numbers and using pooled or individual serum samples in each experiment there was a small variation in antigen frequency and Odds Ratio in the three serum datasets. Some antigens such as HP0318 were detected in only two datasets but with different association patterns (OR>1 and OR≤1). Some antigens such as HP0599 and HP1477 were detected in two serum datasets with the same association pattern (OR>1). Some antigens such as HP1285 and HP0011 were detected in three datasets but only in two datasets they showed the same association pattern (OR>1). Finally, some antigens were detected in three datasets with the same association pattern: HP0874, HP01238, HP0601 and HP0010 showed the same association pattern in three serum datasets (OR>1). Table 20 shows description of top candidates in Heidelberg and Madrid serum datasets.

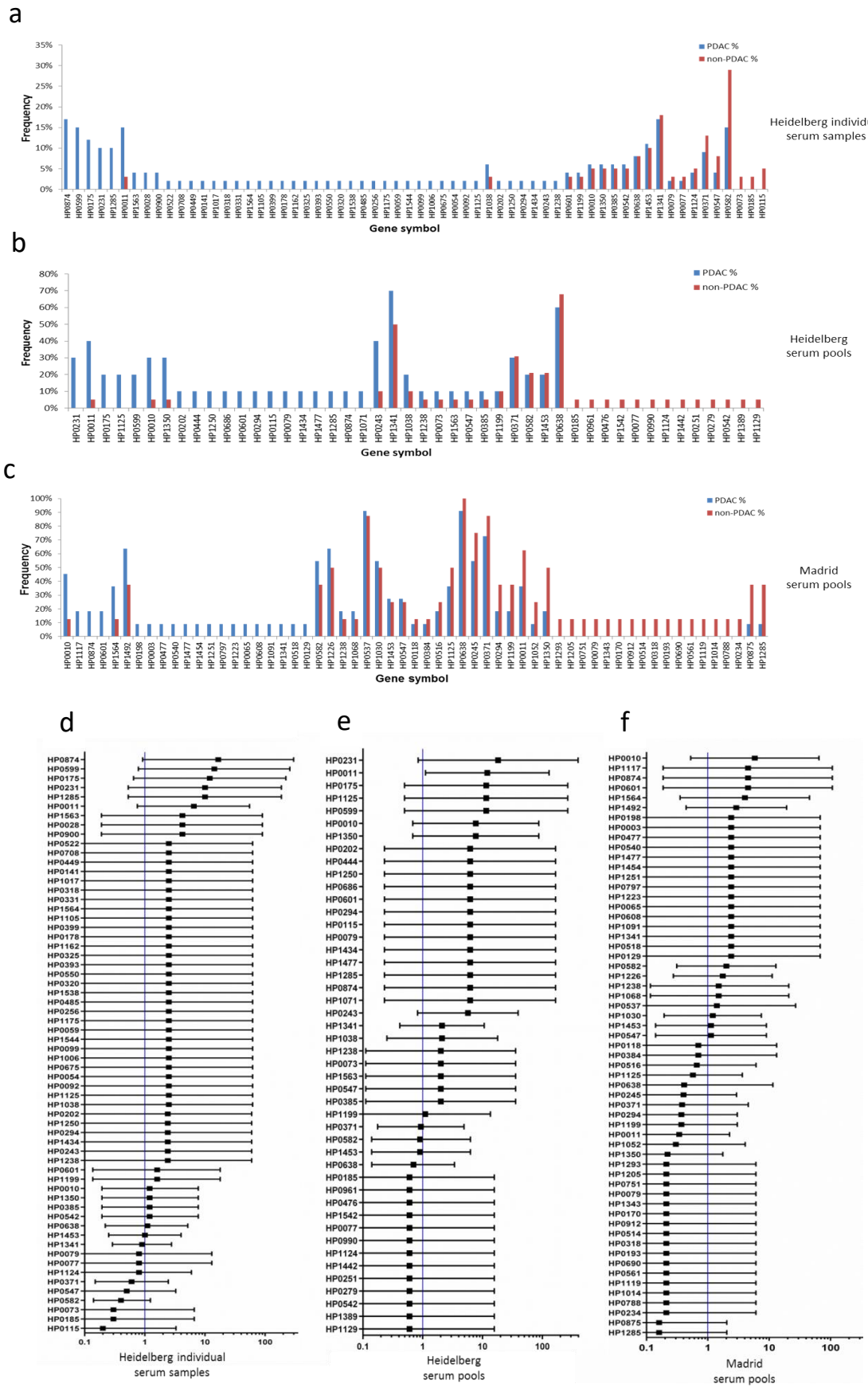


Figure 29. *H. pylori* antigen screenings by using three serum datasets. 1437 *H. pylori* antigens were spotted in microarray. The expressed proteins were applied to Immunoassay by using PDAC and non-PDAC serum samples. The frequency of detected antigens in Heidelberg individual serum samples (a) in Heidelberg serum pools (b) and in Madrid serum pools (c). Detected antigens were ranked based on Odds Ratio and 95% CI in Heidelberg individual serum samples (d), in Heidelberg serum pools (e) and in Madrid serum pools (f).

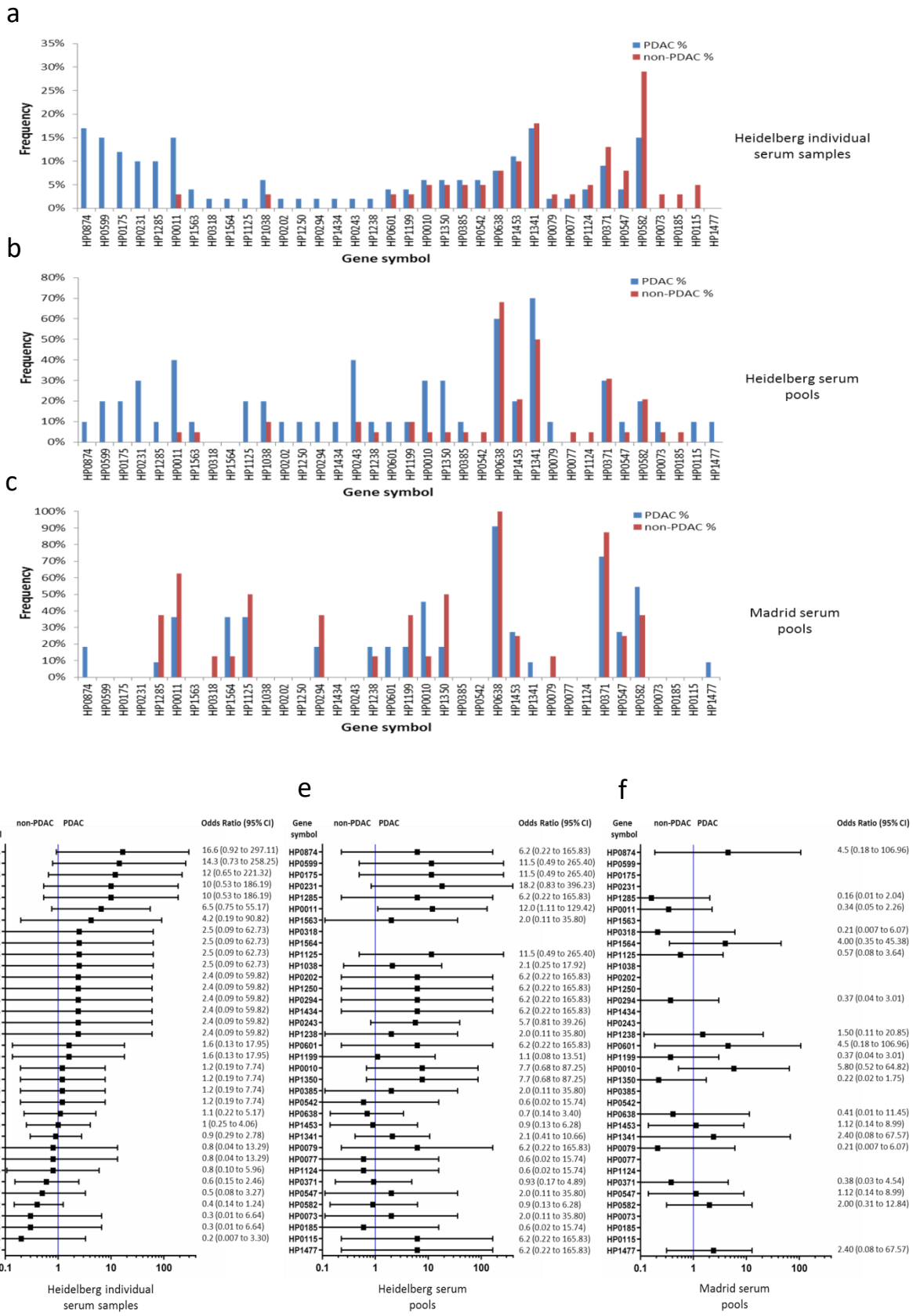


Figure 30. Comparison of *H. pylori* antigens in three serum datasets. After screening of *H. pylori* antigens by using serum datasets from Heidelberg and Madrid, 36 shared antigens were detected. The frequency of 36 antigens in PDAC and non-PDAC (CP and healthy) individual serum samples from Heidelberg (a), in PDAC and non-PDAC (CP and healthy) serum pools from Heidelberg (b) and in PDAC and non-PDAC serum pools with gastrointestinal conditions from Madrid (c). The antigens were ranked based on Odds Ratio and 95% CI in Heidelberg individual serum samples (d), in Heidelberg serum pools (e) and in Madrid serum pools (f).

Gene symbol	Heidelberg individual serum samples	Heidelberg serum pools	Madrid serum pools	Odds Ratio average
HP0874	+/+	+/+	+/+	9.1
HP0010	+/+	+/+	+/+	4.9
HP0601	+/+	+/+	+/+	4.1
HP1238	+/+	+/+	+/+	2.0
HP0231	+/+	+/+	-/-	9.4
HP0599	+/+	+/+	-/-	8.6
HP0175	+/+	+/+	-/-	7.8
HP0202	+/+	+/+	-/-	2.9
HP1250	+/+	+/+	-/-	2.9
HP1434	+/+	+/+	-/-	2.9
HP0243	+/+	+/+	-/-	2.7
HP1563	+/+	+/+	-/-	2.1
HP1038	+/+	+/+	-/-	1.5
HP0385	+/+	+/+	-/-	1.1
HP1564	+/+	-/-	+/+	2.2
HP1477	-/-	+/+	+/+	2.9
HP0011	+/+	+/+	+/-	6.3
HP1285	+/+	+/+	+/-	5.4
HP1125	+/+	+/+	+/-	4.8
HP0294	+/+	+/+	+/-	3.0
HP1350	+/+	+/+	+/-	3.0
HP1199	+/+	+/+	+/-	1.0
HP0318	+/+	-/-	+/-	0.9
HP0542	+/+	+/-	-/-	0.6
HP0638	+/+	+/-	+/-	0.7
HP1341	+/-	+/+	+/+	1.8
HP0547	+/-	+/+	+/+	1.2
HP0115	+/-	+/+	-/-	2.1
HP0073	+/-	+/+	-/-	0.8
HP0079	+/-	+/+	+/-	2.4
HP0371	+/-	+/+	+/-	0.6
HP0582	+/-	+/-	+/+	1.1
HP1453	+/-	+/-	+/+	1.0
HP0077	+/-	+/-	-/-	0.5
HP1124	+/-	+/-	-/-	0.5
HP0185	+/-	+/-	-/-	0.3

Table 19. *H. pylori* antigens in three serum datasets. 36 antigens were detected in Heidelberg individual serum samples, Heidelberg serum pools and in Madrid serum pools. The result was ranked based on OR. Each group was separated based on association patterns in the three serum datasets. +/+: OR>1, +/-: OR≤1 and -/-: antigen was not detected.

Gene symbol	Heidelberg individual serum samples	Heidelberg serum pools	Madrid serum pools	Odds Ratio average	Definition	Pathway
HP0874	+/+	+/+	+/+	9.1	hypothetical protein	Resistance to hydrogen peroxide
HP0010	+/+	+/+	+/+	4.9	molecular chaperone GroEL	Chaperone and heat shock protein
HP0601	+/+	+/+	+/+	4.1	flagellin	Flagellar assembly
HP1238	+/+	+/+	+/+	2.0	formamidase	Nitrogen and carbon metabolism
HP0231	+/+	+/+	-/-	9.4	protein-disulfide isomerase	Chaperones and folding catalysts
HP0599	+/+	+/+	-/-	8.6	hemolysin secretion protein	Chemotaxis
HP0175	+/+	+/+	-/-	7.8	peptidylprolyl isomerase	Pro apoptosis and angiogenesis
HP0202	+/+	+/+	-/-	2.9	3-oxoacyl-ACP synthase III	Fatty acid biosynthesis
HP1250	+/+	+/+	-/-	2.9	hypothetical protein	-----
HP1434	+/+	+/+	-/-	2.9	formyltetrahydrofolate deformylase	Glyoxylate and dicarboxylate metabolism
HP0243	+/+	+/+	-/-	2.7	DNA protection during starvation protein	Oxidative stress
HP1563	+/+	+/+	-/-	2.1	peroxiredoxin	Resistance to peroxidase activity
HP1038	+/+	+/+	-/-	1.5	3-dehydroquinate dehydratase	Biosynthesis of amino acids
HP0385	+/+	+/+	-/-	1.1	hypothetical protein	Threonyl and Alanyl tRNA synthetase
HP1564	+/+	-/-	+/+	2.2	ABC transporter	Drug resistance
HP1477	-/-	+/+	+/+	2.9	flagellar P-ring biosynthesis	Flagellar assembly

Table 20. Description of top *H. pylori* positive candidates. Definition and function of *H. pylori* positive candidates with triple +/+, with double +/+ and without +/-. +/+: OR>1, +/-: OR≤1 and -/-: antigen was not detected.

3.10 Whole-proteome microarray of turkey herpesvirus (HVT)

HVT ORFeome were spotted on Ni slide to produce HVT protein microarray. After incubation, the expressed proteins on the array were used for immunostaining against His-Tag and V5-Tag to determine the protein expression as mentioned in Table 13. EBV p18 antigen was used for positive control which it has known antigen to react to human serum samples whereas, Non Template Controls (NTC) were used for negative controls.

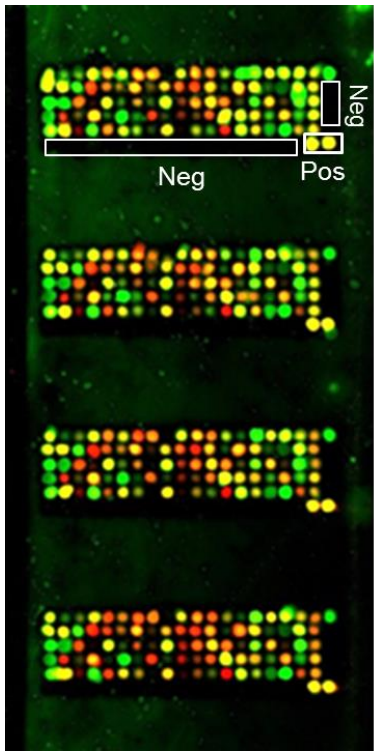


Figure 31. Expression assay result of HVT microarray. HVT ORFeome were spotted on Ni slide and after incubation, immunostaining was performed using two antibodies against His-Tag (red signal) and V5-Tag (green signal). P represents positive controls and Neg represents negative controls.

3.11 Immunoassay of HVT

3.11.1 Analysis of different blocking agents for immunoassay

HVT immunoassay was performed with the same protocol as mentioned in Table 14, but the result was not sharp enough and there was not a big difference between positive candidates and other signals. So I decided to test different blocking agents to check which one ensured the highest sensitivity to find the optimum situation for HVT immunoassay. 11 different blocking buffers in total of 14 situations were tested (Table 21). Five blocking agents which have the highest positive signals and the lowest negative signals (Candor, SuperBlock, Nexterion Block E, NAP blocker and Milk powder) were selected. After obtaining these top five candidates, I tested again just these five blocking agents to find the best one. The result presented that Candor had the highest SNR Pos / SNR Neg in compare of other blocking agents (Figure 32). I used Candor as the blocking agent in HVT immunoassay. Candor (Candor Bioscience) and Milk powder (Bio-Rad) showed similar SNRs because they both contain segmented casein as the main agent in blocking buffer.

#	Blocking agents
1	SuperBlock
2	10% SuperBlock in 1X PBST
3	Rockland MB 70
4	NAP blocker
5	Candor
6	Rabbit gamma globulin
7	PVP polyvinyl pyrrolidone
8	ECL prime blocking agent
9	HSA Human Serum Albumin
10	Pierce protein free
11	Pluronic F-127
12	Nexterion Block E
13	2% Milk powder in 1X PBST
14	SuperBlock without *E. coli* lysate

Table 21. List of blocking agents were analyzed in HVT immunoassay. 11 blocking agents in total of 14 situations were tested and evaluated to find the most sensitive in immunoassay.

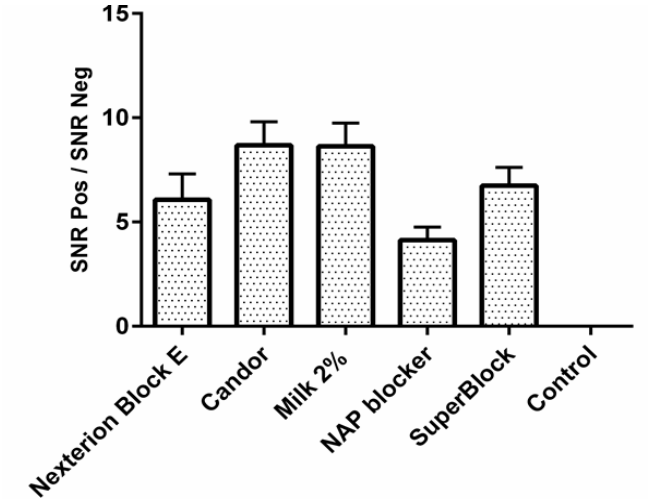


Figure 32. Optimization result of HVT immunoassay. Top five blocking agents which were obtained from the primary experiment were confirmed to find the most optimum situation based on Signal to Noise Ratio (SNR). Candor and Milk powder showed the highest SNR. Candor was used in HVT immunoassay because of lower negative signal (data not shown). Water was used as controls instead of blocking buffers.

3.11.2 Screening of HVT candidates in PDAC and non-PDAC serum pools

Immunoassay was performed initially by using 50 serum samples of PDAC, 100 serum samples of non-PDAC serum samples (CP and healthy). Serum pools and individuals were from Heidelberg University Clinic (healthy samples are from Mannheim Blood Center). 5 sera of each respective group were combined as one pool, resulting in a total of 10 healthy pools, 10 PDAC pools, and 10 CP pools. Candor was used as the blocking buffer and all of the settings and protocols were the same as explained in Table 14. When a protein showed Median Fluorescence Intensity (MFI) larger than the cut off (Average of negative MFIs + 2.5 SD of negatives) it was considered as a positive candidate. Two PDAC pools showed positive signals whereas healthy and CP pools showed no positive signal. Then individual serum

samples of each positive pool were tested, and 3 individual serum samples were positive in each pooled serum (Figure 33). By using serum pools there were detected three positive candidates (HVT059, HVT062 and HVT079) which were ranked based on frequency and Odds Ratio with 95% CI (Figure 34).

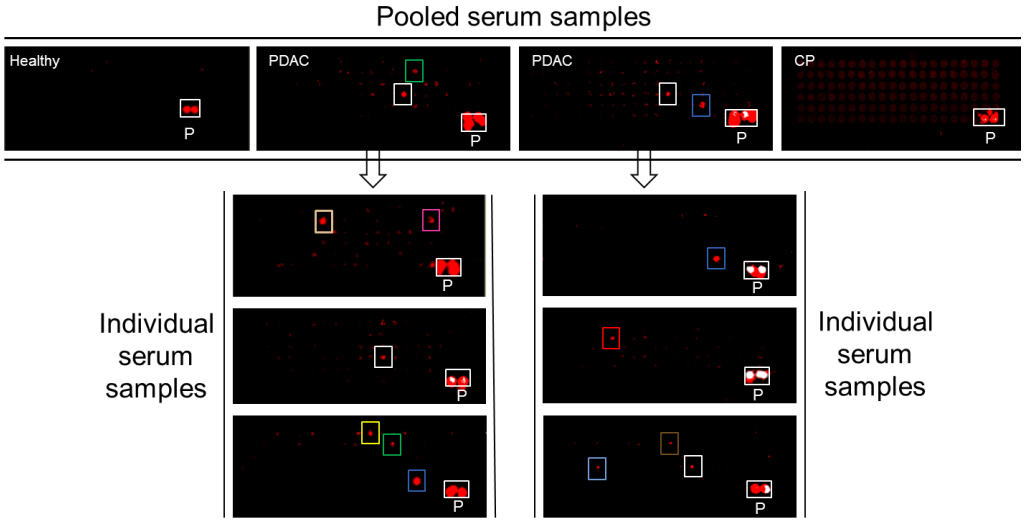


Figure 33. Immunoassay result of HVT microarray. HVT ORFeome were spotted on Ni slide and after protein expression healthy, CP and PDAC serum pools were applied during Immunoassay. HVT antigens were recognized based on the position on the array. HVT proteins were detected in two PDAC serum pools, and then these two pools were analyzed for individual serum samples. Three individual serum samples were positive in each serum pool.

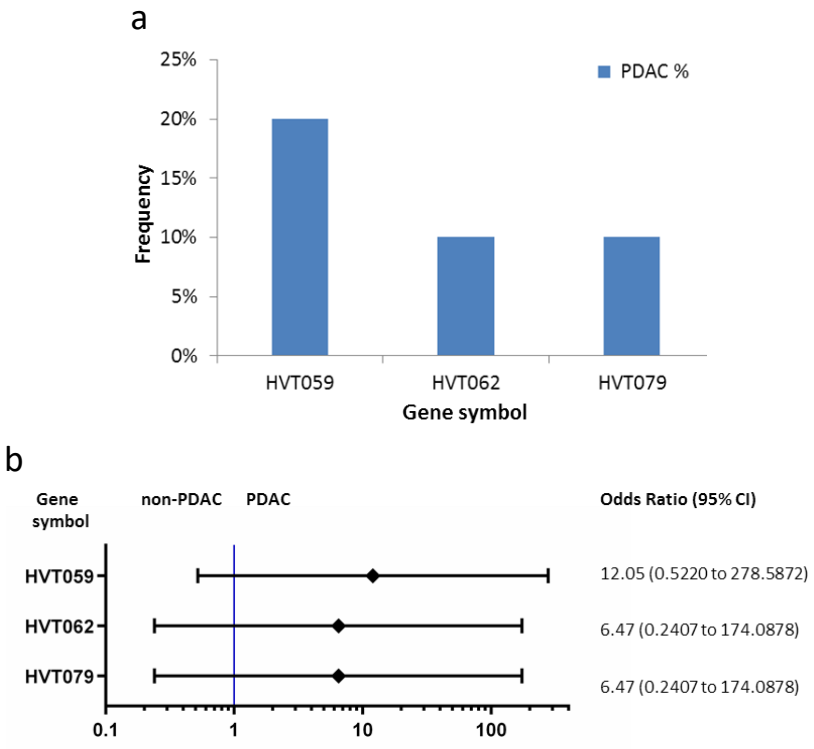


Figure 34. Association of HVT positive candidates with PDAC serum pools. The frequency of positive candidates: HVT059, HVT062 and HVT079 in PDAC serum pools (a) and the positive candidates were ranked based on Odds Ratio with 95% CI (b).

3.11.3 Screening of HVT antigens in PDAC and non-PDAC serum samples

After immunoassay of serum pools, Individual serum samples were analyzed. 74 PDAC serum samples and 46 non-PDAC serum samples (24 CP serum samples and 22 healthy serum samples) were applied to HVT arrays. HVT positive candidates were detected in 21 (28%) PDAC serum samples whereas HVT positive candidates were not detected in non-PDAC (CP and healthy) serum samples. By using individual serum samples there were detected 25 HVT positive candidates which then ranked based on frequency and Odds Ratio with 95% CI. The detected candidates in pooled serum samples were also detected in individual serum samples (Figure 35). Top candidates were listed with gene description in Table 22.

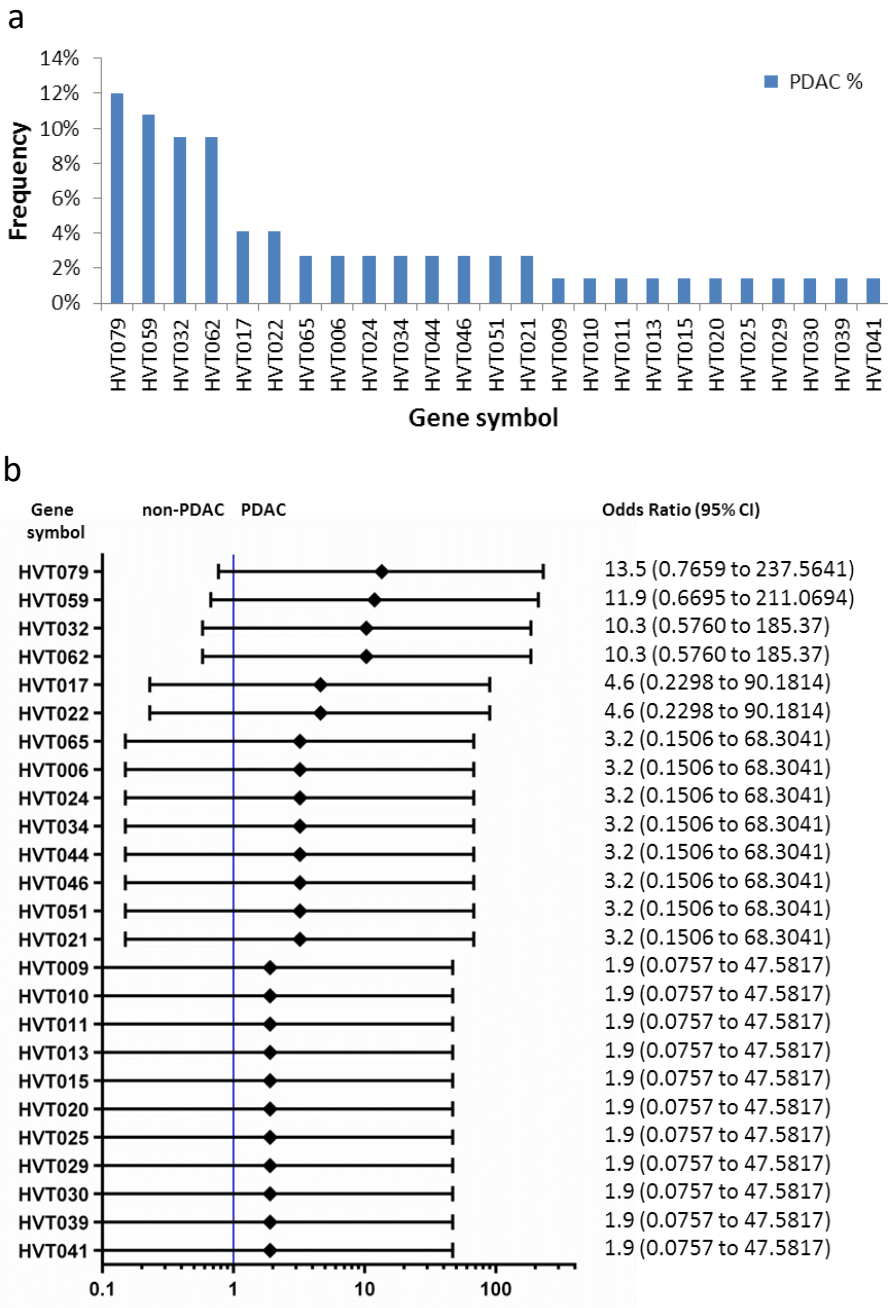


Figure 35. Frequency and association of HVT positive candidates in PDAC serum samples. The frequency of positive candidates in PDAC serum samples (a) and the positive candidates were ranked based on Odds Ratio with 95% CI (b).

Gene symbol	Heidelberg individual serum samples	Heidelberg serum pools	Odds Ratio average	Cellular component	Name
HVT059	+/+	+/+	12	membrane	envelope/tegument protein N
HVT079	+/+	+/+	10	capsid	apoptosis inhibitor Bcl-2
HVT062	+/+	+/+	8.4	membrane	envelope glycoprotein K
HVT032	+/+	-/-	5.2	capsid	viral DNA packaging tegument UL25
HVT017	+/+	-/-	2.3	membrane	envelope glycoprotein M
HVT022	+/+	-/-	2.3	capsid	viral DNA packaging terminase UL15

Table 22. Description of HVT positive candidates. HVT top candidates were classified based on detection in individual serum samples and pools. HVT059, HVT079 and HVT062 were detected in both Individuals and pools while HVT032, HVT017 and HVT022 were detected in individual serum samples. +/+: OR>1 and -/-: no detection.

4. Discussion

4.1 Cancer and infection

Cancer is a complex disease with multiple causes. Among these causes, infection and its association to cancers have been studied for many years. Infection is the cause of nearly 20% of all cancers. Infectious agents such as the bacterium *Helicobacter pylori* (*H. pylori*) as the first known bacterial carcinogen and viruses from Herpesviridae family are involved in the pathogenesis of different cancers. Infection carries risks for disease development to drive carcinogenesis. Screenings of bacteria and virus antigens in cancer may uncover areas in the identification and optimizing cancer control strategies.

Infection with *H. pylori*, which is a known carcinogen, may result in the development of adenocarcinoma. *H. pylori* has a strong adaptation to its natural habitat and they can modify their environment. *H. pylori* causes a mixed acute and chronic inflammatory reaction. Whereas infection typically does not show symptoms, it can lead to other diseases such as ulcer (gastric and duodenal) and gastric adenocarcinoma.

Herpesviruses have applied number of immune evasion mechanisms. There are several carcinogenic mechanisms exploited by herpesviruses, including inhibiting apoptosis and tumor suppressors. Moreover, they are responsible for changing the microenvironment, promoting cellular migration, metastasis, angiogenesis, and also mutagenesis. Some of the members of Herpesviridae, such as Epstein-Barr virus (EBV) cause of lymphoma, cytomegalovirus (CMV) cause of brain tumors and Kaposi's Sarcoma-associated herpesvirus (KSHV) are officially recognized as carcinogens.

4.2 Whole-ORFeome for *H. pylori* and HVT

Pure *H. pylori* genomic DNA was provided by ATCC. After checking the quality, it was used for PCR template. For HVT DNA there was not any pure DNA, so the virus was ordered with the infected cell. HVT DNA was extracted by using the supernatant and the cell pellet. Result showed that although the infected cells were harvested after cytopathic effect, HVT DNA was much more in the cell pellet rather than supernatant and this data could emphasize that HVT in viral culture is a cell associated virus and for preparing cell-free HVT, special treatment is needed (Zanella and Granelli 1974).

H. pylori genome has 1437 open reading frames (ORFs) and HVT has 100 open reading frames (ORFs). PCR primers were designed for all of the ORFs to have whole-ORFeome. Each ORF sequence was obtained from database. To have an expression construct ORF should be in frame and without stop codons. Forward primer starts at ATG codon and reverse primer starts at end of the ORFs. Each primer was considered to have 16-23 nucleotides, 50% GC content and 50°C Tm. Then binding specificity of each primer was checked by online blast

tools. Because of different GC content at the beginning and end of a gene, primer designing with the similar criteria was not always possible and also in cell-free protein expression there were some difficulties in expression of long ORFs, therefore with splitting a gene into two or three sections it was possible to have better primer designs and to have full protein expressions. In this thesis as an example, HVT079 was divided into two sections and the first part of HVT079 showed positive signal. Specific primers were flanked by two overhangs which then used for binding sites of second primers. HVT specific primers were designed in our laboratory and *H. pylori* specific primers were designed by Heidelberg Unix Sequence Analysis Resources (HUSAR). Specific primers were designed and ordered to synthesize. The specific primers were used in first PCR were purified by chromatography. Second primer pair which was quite long was purified by high performance liquid chromatography (HPLC).

First PCR was performed by using specific primers to amplify the ORFs. Primer Tm is very important in PCR optimization. There are different types of Tm(s) such as basic Tm, salt adjusted Tm and nearest neighbor Tm. Nearest neighbor Tm is very close to annealing temperature. Choosing nearest neighbor Tm prevents try and error to find the optimum annealing temperature. Using Betaine 5 M was helpful to enhance Taq polymerase activity and also it helps to open secondary structures. Since the specific primers were designed with the similar criteria the first PCR was performed with Taq DNA Polymerase (Qiagen). Second PCR was performed by using second primer pair for all of the PCRs and it seemed to be straightforward but in the second PCR, minority of reactions didn't work and it was due to long product size and long primer size. By changing the polymerase from Qiagen Taq DNA Polymerase to Qiagen Long Range PCR kit, the optimum result was obtained. After two successive PCRs regulatory sequences for transcription, translation and protein tags were added to ORFs to generate expression constructs which then were used for whole-proteome microarray.

4.3 Whole-proteome microarray for *H. pylori* and HVT

After production of whole-ORFeome for *H. pylori* and HVT, they were spotted on slide. 45 µl of PCR product with 5 µl Betaine 5 M prepared for spotting. Then expression mix was added on top of each spot. For protein expression the whole array was incubated in a ventilated oven to produce whole-proteome microarray. This whole-proteome microarray was subjected to expression assay to check the protein expression and to immunoassay to check the seropositivity. Before doing expression assay and immunoassay there were some issues such as using Epoxy surface or Ni surface, importance of *E. coli* lysate and its optimum incubation time in immunoassay, using Betaine 5 M in spotting and inconsistence spotting.

First of all, spotting was done on both Epoxy and Ni surfaces and each slide was subjected to expression assay and immunoassay to see the effect of the surface in protein expression and immunoassay. In the expression assay Epoxy and Ni surfaces showed acceptable similar quality without merged spots and rush on the slide and any other artifacts. Regarding spot size, Epoxy surface showed smaller and condensed spot in compare to Ni surface and it

might be the reason of higher SNR in Epoxy surface. In the immunoassay the Epoxy surface showed significantly lower SNR as a result of higher background. In the Ni surface, background was very low and foreground signal was much more visible.

Testing immunoassay without using *E. coli* lysate showed huge background so even the positive controls could not be detected. Generally human sera contain antibody against *E. coli* and using *E. coli* lysate reduced background by absorbing these antibodies. Actually two hours serum incubation with *E. coli* lysate showed the best result.

Each spotting solution was prepared by mixing of secondary PCR product with 10% Betaine 5 M. Increasing Betaine 5 M from 10% to 20% resulted in rush on the slide and merged spots. Betaine 5 M helps to open secondary structures and also prevents evaporation. It seems increasing Betaine 5 M to 20% would decrease viscosity.

Before doing the main experiments and during testing a few PCR products, inconsistent spotting was noticed. Same protein expressions should be visible across the slide but it was visible only on upper part of the slide. Adding exogenous protein fused to His-Tag across the slide showed this problem arose from spotting and not from expression assay. Spotter optimization solved this problem.

H. pylori and HVT whole-ORFeome were spotted on Ni surface and after incubation whole-proteome microarray were produced. This microarray was checked for protein expression and immunoassay respectively. EBV p18 and Poliovirus F11 antigens were used as endogenous positive controls and their expression and detection showed technical performance.

4.4 Identification and screening of PDAC associated antigens

Each person shows specific antibodies for infection agents and disease conditions. Antibodies detect and bind to pathogens and toxins to facilitate phagocytosis of foreign substances by phagocytic cells. After phagocytosis the antigens are digested to present part of the antigens as epitopes to T cells. Epitope presentation is done by MHC class I to cytotoxic T cells for viruses and MHC class II to helper T cells for bacteria. Because of different genetic background, type of infection and immune responses over time, antibody patterns could be different between people. As a result, it is possible that special antibody patterns which are associated with disease conditions could be detectable for diagnosis. Using *H. pylori* positive and negative serum samples showed specific signals in *H. pylori* positive serum sample whereas there were not specific signals in *H. pylori* negative serum sample. While a protein showed MFI larger than the cut off (Average of negative MFIs + 2.5 SD of negatives) it was considered as a positive candidate. This data showed that serum antibodies of patients can detect and bind to specific *H. pylori* antigens among many other antigens on the microarray. During HVT experiment also it was noticed that only some serum samples showed antibody against HVT proteins on the microarray. In conclusion due

to specific antibody patterns and seropositivity I was able to detect and screen *H. pylori* and HVT antigens based on frequency, Odds Ratio and association patterns to PDAC. In this work three screenings were performed for *H. pylori* and two screenings were performed for HVT.

4.5 *H. pylori* positive candidate screening

Firstly, immunoassay was performed using pooled serum samples of CP and PDAC from Heidelberg University Clinic and healthy from Mannheim Blood Center. In this thesis this dataset is briefly called Heidelberg. 50 serum samples from healthy donors, 45 serum samples from CP donors and 50 from PDAC patients were included. 5 sera of each group were combined as one pool, resulting in a total of 10 healthy pools, 10 PDAC pools, and 9 CP pools. In this screening, the prevalence of detected antigens in PDAC groups and non-PDAC groups (healthy and CP) were estimated by the frequency of seropositivity. The shortlisted candidates were ranked according to the frequency of seropositivity and the association with PDAC and non-PDAC. A total of 46 proteins were detected in this experiment and five of them showed higher association to PDAC: HP0231, HP011, HP0175, HP1125 and HP0599. "HP0231 is a dimeric oxidoreductase that functions in an oxidizing disulfide bonds pathway of *H. pylori*. Many *H. pylori* virulence factors are stabilized by the formation of disulfide bonds and the lack of *H. pylori* HP0231 damaged CagA translocation into gastric epithelial cells and reduced VacA vacuolation" (Zhong et al. 2016). HP011 is a co-chaperonin in protein folding. "HP0175 induces apoptosis of gastric epithelial cells" (Basak et al. 2005). HP1125 is an outer membrane protein (omp18) which altered IFN-gamma to avoid immune response (Shan et al. 2015). HP0599 is a hemolysin secretion protein and it is important in *H. pylori* chemotaxis (Scott et al. 2007).

Secondly, using serum pools can be a fast way to find the reactive antigens but when pooling serum samples to have one serum pool with the same volume there are some considerations. It is possible to dilute antibody level of a positive serum sample in other non-positive serum samples which can eliminate the positive signal (false negative). Combining different sera in one pool might have accumulative effect since different sera might recognize and bind to multiple epitopes of an antigen on the array. So some antigens can be detectable in pools while they are not detectable in individual serum samples (false positive). Moreover, in combining different serum samples in one pool we are reducing the size of dataset. For these reasons serum pools might not reflect all of the positive candidates and the frequencies. For a better estimation I needed to apply a bigger dataset with individual serum samples. So this time individual serum samples from Heidelberg were applied to microarray. In this section 47 PDAC serum samples and 38 non-PDAC serum samples (19 healthy and 19 CP) were applied. A total of 62 proteins were detected in this experiment and six antigens showed higher association to PDAC: HP0874, HP0599, HP0175, HP0231, HP01285 and HP0011. Four antigens of six antigens were already mentioned in Heidelberg pools and here another two antigens are mentioned: HP0874 and HP01285; HP0874 (KapA) is responsible for *H. pylori* hydrogen peroxide resistance (Harris et al. 2002). HP1285 is a hypothetical protein.

Thirdly, in another experiment 19 serum pools with gastrointestinal disorders from Spanish National Cancer Research Centre (Madrid) including 11 PDAC and 8 non-PDAC serum pools were applied to find *H. pylori* positive antigens. Immunoassay was performed to find the antigens in PDAC and non-PDAC serum pools. In total of 59 antigens were detected in this experiment and top six of detected antigens are: HP0010, HP1117, HP0874, HP0601, HP1564 and HP1492. HP0010 is a chaperone. HP1117 is a hypothetical protein and assumed to be a Sel1 repeat-containing protein. HP0601 is flagellin and important in *H. pylori* colonization. HP1564 is ABC transporter and HP1492 is a hypothetical protein which is suggested for *H. pylori* oxidative stress resistance (Benoit et al. 2018). In this screening by using PDAC and non-PDAC serum pools with gastrointestinal conditions the Odds Ratios are lower in compare of the first and second screenings and it could be due to existence of the same gastrointestinal conditions in PDAC and non-PDAC groups.

4.6 Comparison of *H. pylori* screening results in three serum datasets

First screening was performed by using PDAC and non-PDAC (CP and healthy) serum pools from Heidelberg and the second screening was performed by using PDAC and non-PDAC (CP and healthy) individual serum samples from the same center. Third screening was performed by using PDAC and non-PDAC serum pools with gastrointestinal conditions from Madrid. Each screening has its advantage: in the first and second screenings using CP and healthy groups as controls could help us to have a better screening of PDAC associated antigens. In the first screening, serum pools have presented a fast way of antigen screening however pooling serum samples could have some considerations such as false negative antigens due to dilution effect of specific antibodies and false positive due to accumulative effect of different sera for multiple epitopes of one antigen. So some antigens could be detectable only in pooled serum samples or in individual serum samples. To have a better estimation of antigen value, in the second screening individual serum samples from the same center were applied instead of serum pools. In the third screening PDAC and non-PDAC serum pools with gastrointestinal conditions from Madrid were applied to microarray. In this screening using serum pools with gastrointestinal disorders can help us to subtract *H. pylori* gastrointestinal antigens from PDAC antigens.

Comparison result of two serum datasets from Heidelberg (pools and individual serum samples) showed that in applying individual serum samples the number of detected antigens is higher than using pools (62 antigens vs 46 antigens). 33 antigens were in common between individuals and serum pools. Odds Ratio (OR) of the antigens was not exactly the same but association patterns of most of the antigens were similar between individuals and pools while minority of the antigens such as HP0115 showed different patterns (OR>1 and OR≤1). 26 antigens showed the same association pattern and 20 of them showed positive associations to PDAC in pools and individuals (OR>1). This data emphasized the importance of individual serum samples for obtaining a larger antigen list and a better evaluation with exact frequency and Odds Ratio however using serum pools was a fast way to obtain top candidates. In the next step three serum datasets were compared to find the shared *H. pylori*

antigens. In total of 167 *H. pylori* detected antigens, 36 *H. pylori* antigens were detected in common in three serum datasets. Because of different serum sources, serum numbers and using pooled and individual serum samples there was expected some variations in antigen frequency and Odds Ratio. Some antigens such as HP0318 were detected in only two datasets but with different association patterns to PDAC (OR>1 and OR≤1). Some antigens such as HP0599 and HP1477 were detected in two serum datasets with the same association pattern (OR>1). Some antigens such as HP1285 and HP0011 were detected in three datasets but only in two datasets they showed the same association pattern to PDAC (OR>1). Finally, four antigens were detected in three datasets with the same association pattern to PDAC (OR>1): HP0874, HP01238, HP0601 and HP0010. Table 20 shows description of top candidate in Heidelberg and Madrid serum datasets. HP0874 (KapA) is responsible for *H. pylori* hydrogen peroxide resistance (Harris et al. 2002). HP1238 is a formamidase which involves in nitrogen and carbon metabolism. HP0601 is flagellin and important in *H. pylori* colonization. HP0010 is a GroEL chaperone.

4.7 HVT positive candidate screening

Immunoassay was performed initially by using CP and PDAC serum samples from Heidelberg University Clinic and healthy serum samples from Mannheim Blood Center. In this thesis this dataset was briefly called Heidelberg serum samples. 50 serum samples of PDAC, 100 serum samples of non-PDAC serum samples (CP and healthy) were applied. 5 sera of each respective group were combined as one pool, resulting in a total of 10 healthy pools, 10 PDAC pools, and 10 CP pools. The first immunoassay by using Superblock as blocking buffer didn't show very good signal. Then 11 blocking buffers were tested to find the most sensitive one and Candor was used as the blocking buffer. Two PDAC pools showed positive signals whereas healthy and CP pools showed no positive signal. By using serum pools there were detected three positive candidates (HVT059, HVT062 and HVT079) in PDAC serum pools. After immunoassay of serum pools, Individual serum samples were analyzed. 74 PDAC serum samples and 46 non-PDAC serum samples (24 CP serum samples and 22 healthy serum samples) were applied to HVT array. HVT positive candidates were detected in 21 (28%) PDAC serum samples whereas HVT positive candidates were not detected in non-PDAC (CP and healthy) serum samples. By using individual serum samples there were detected 25 HVT positive candidates. The detected candidates in pooled serum samples (HVT059, HVT062 and HVT079) were also detected in individual serum samples. HVT059 is defined as envelope/tegument protein, HVT079 is defined as apoptosis inhibitor Bcl-2 which regulates cell death and HVT062 is envelope glycoprotein K. HVT032 and HVT022 are involved in viral DNA packaging.

5. Conclusion

Cell-free protein microarray is a strong and pioneering technology for the detection of positive candidates, novel biomarkers and protein-antibody reactivities. Protein purification is expensive and time-consuming and could destroy the protein structure and function. Cell-free protein microarray technology avoids protein purifications by replacing expression *in situ* with an *in vitro* transcription-translation. This technology allows doing high-throughput approaches for the analysis of the proteome at large scales (Merbl and Kirschner 2010). In comparison to other proteomics methods, cell-free protein microarray avoids the necessity of purification a sample into small parts and therefore complex samples can be immediately applied for research (Hanash 2003).

In cell-free protein microarray, the expressed proteins are produced from a special template with transcription and translation elements on the surface and the expressed protein is captured by affinity reagents. In our method the DNA template was an expression construct with His-Tag and V5-Tag which was flanked by regulatory sequences, made by two successive PCRs and after *in vitro* transcription-translation, nascent protein bound to the Ni surface by His-Tag. In this study after technique establishment, *H. pylori* whole-proteome microarray and HVT whole-proteome microarray were produced from *H. pylori* ORFeome and HVT ORFeome. Cell-free protein microarray allows performing high-throughput approaches for the analysis of the proteome at large scales for diagnostic purposes (Díez et al. 2015).

In this study firstly, whole-ORFeome and whole-proteome were produced and after checking the protein expression on the array, immunoassay was performed by using PDAC and non-PDAC serum samples. After screening of positive candidates which should have signal larger than the cut off (Average of negative MFIs + 2.5 SD of negatives), the result was screened based on frequency in each PDAC and non-PDAC groups and also the positive candidates were ranked based on Odds Ratio to see the association patterns to PDAC and non-PDAC groups. Moreover, some serum samples were tested randomly to check the reproducibility. In this study for *H. pylori* screening three serum datasets were used. Result for each dataset could be valuable for diagnostic purposes but a strict comparison of results was performed to detect positive candidates in three datasets. In *H. pylori* screenings, among 167 positive candidates four of them (HP0874, HP0010, HP0601 and HP1238) were detected to be associated with PDAC condition in three datasets (triple +/+) which might have diagnostic value. In comparison of three serum datasets the difference between detected antigens was expected. Using individual serum samples and pooled serum samples could bring different data. In individual serum samples there were detected more antigens with different frequencies but association patterns of most of the antigens were similar to pooled serum samples. Comparison of Heidelberg and Madrid serum samples showed less similarity however finding 36 *H. pylori* antigens between Heidelberg and Madrid was promising. This difference between Heidelberg and Madrid is firstly because of different ethnicity and

different genetic background and secondly is due to the special gastrointestinal conditions in Madrid serum samples.

In this study for HVT screening two serum datasets were used. Result for each dataset could be valuable for diagnostic purposes but comparison of results was performed to detect shared positive candidates in two datasets. In HVT screenings three antigens were detected (HVT059, HVT062 and HVT079) to be associated with PDAC condition in pooled and individual serum samples.

Cell-free protein microarray has shown numerous benefits but it is possible that cell-free protein microarray is not able to detect all of the reactive antigens. There are several reasons for missing of some reactive antigens. Firstly, it can be related to the protein expression on the array. Antibody binding to antigen on the array is prevented if the protein is not fully expressed. As a result, antibody could not bind to antigen. Secondly, using strict cut off to screen positive candidates might result in missing of some antigens which are reactive but the signal is under cut off. Thirdly protein folding can mask the epitope, so antibody could not access to epitope and also adding protein tags could also mask the epitope. Lastly, using pooled serum samples leads to some considerations such as antibody dilution in a pool which results in negative signal.

In this study for *H. pylori* and HVT three and two list of promising antigens were produced which can be valuable for further PDAC diagnostic purposes. Moreover, strict antigen comparison was performed to find the antigens which are associated to PDAC in all of the used serum datasets.

Summary

Background

Infection is the cause of nearly 20% of all cancers. Infectious agents such as the bacterium *Helicobacter pylori* (*H. pylori*) as the first known bacterial carcinogen and viruses from Herpesviridae family are involved in the pathogenesis of different cancers. Screening for antibodies against bacterial and viral antigens in cancer patients may therefore uncover potential markers and targets for optimized cancer control strategies. To these ends, I studied the antibody composition in the peripheral blood of patients with pancreatic ductal adenocarcinoma (PDAC) and appropriate non-cancer donors as controls.

Methods

All 1437 open reading frames (ORFs) of *H. pylori* and the 100 ORFs of Turkey herpesvirus (HVT) were produced by two successive PCRs and spotted on nickel-coated glass surfaces. By *in vitro* transcription-translation, whole-proteome microarrays were produced for *H. pylori* and HVT. Immunoassays were performed with PDAC and non-PDAC serum samples to detect antibody binding patterns and to differentiate between PDAC and non-PDAC groups. For presenting the association pattern of each detected antigen with PDAC, the result was calculated as Odds Ratio (OR) with 95% Confidence Interval (CI). Also, functional annotations were performed.

Results

At first, the *H. pylori* proteome was screened using serum pools of 10 PDAC, 10 healthy and 9 CP (chronic pancreatitis). A total of 46 proteins were detected, of which 29 showed association to PDAC (OR>1). After screening for candidates in the serum pools, individual serum samples of PDAC and non-PDAC (healthy and CP) were applied to the microarray. In total, 47 PDAC and 38 non-PDAC samples (19 healthy and 19 CP) were analyzed. A total of 62 proteins were detected. Most of them showed association to PDAC (OR>1); 33 antigens had also been found in the pool experiments, such as HP0874, HP0599 and HP0175. In further experiments, 11 PDAC and 8 non-PDAC serum pools with gastrointestinal conditions were applied to find *H. pylori* positive antigens. In total, 59 antigens were detected in this experiment of which 29 showed association to PDAC (OR>1).

The HVT proteome was firstly screened using serum pools (10 PDAC, 10 healthy and 10 CP). In this step, three antigens were detected in PDAC pools. Subsequently, screening was performed using individual samples. Altogether, 74 PDAC and 46 non-PDAC serum samples (24 CP patients and 22 healthy donors) were applied to the HVT microarray. In total, 25 HVT candidates were identified including the antigens which were detected in the pools: HVT079, HVT059 and HVT062.

Conclusions

H. pylori and HVT screenings were performed to find antigens which are associated with the occurrence of PDAC using serum pools and individual serum samples. Each screening produced a candidate list, which is a valuable resource for diagnostic purposes. Among the

167 *H. pylori* detected antigens, four antigens were detected in all three datasets with strong association to PDAC: HP0874, HP0010, HP0601 and HP01238. Among the 25 HVT proteins that were detected, three were detected commonly: HVT079, HVT059 and HVT062. These results could help PDAC diagnostics and provides targets for interfering with PDAC development and progression.

Zusammenfassung

Hintergrund

Infektion ist die Ursache von fast 20% aller Krebsarten. Infektionserreger wie das Bakterium *Helicobacter pylori* (*H. pylori*) als erstes bekanntes bakterielles Karzinogen und Viren aus der Familie der Herpesviridae sind an der Pathogenese verschiedener Krebsarten beteiligt. Das Screening auf Antikörper gegen bakterielle und virale Antigene in Krebspatienten kann daher potenzielle Marker und Ziele für optimierte Strategien zur Krebsbekämpfung aufdecken. Zu diesem Zweck untersuchte ich die Antikörper-Zusammensetzung im peripheren Blut von Patienten mit duktalem Adenokarzinom des Pankreas (PDAC) und geeigneten Nicht-Krebs-Spendern als Kontrollen.

Methoden

Alle 1437 offenen Leserahmen (ORFs) von *H. pylori* und die 100 ORFs des Truthahn Herpesvirus (HVT) wurden durch zwei aufeinanderfolgende PCRs hergestellt und auf nickelbeschichteten Glasoberflächen aufgebracht. Durch *in vitro* Transkription/Translation wurden Mikroarrays des gesamten Proteoms von sowohl *H. pylori* als auch HVT hergestellt. Immunoassays wurden mit PDAC- und Nicht-PDAC-Serumproben durchgeführt, um Antikörperbindungsmuster nachzuweisen und zwischen PDAC- und Nicht-PDAC-Gruppen zu unterscheiden. Zur Darstellung der Assoziation jedes nachgewiesenen Antigens mit PDAC wurde das Ergebnis als „Odds-Ratio“ (OR) mit 95% Konfidenzintervall (CI) berechnet. Auch funktionale Annotationen wurden durchgeführt.

Ergebnisse

Zunächst wurde das *H. pylori*-Proteom mit Serumgemischen aus Blutproben von 10 PDAC-Patienten, 10 gesunden Personen und 9 Patienten mit chronischer Pankreatitis (CP) untersucht. Insgesamt wurden 46 Proteine nachgewiesen, von denen 29 eine Assoziation mit PDAC zeigten (OR> 1). Nach der Analyse der Serumgemische wurden einzelne Serumproben von PDAC und Nicht-PDAC (gesund und CP) auf den Microarrays untersucht. Insgesamt wurden 47 PDAC- und 38 Nicht-PDAC-Proben (19 gesunde und 19 CP) analysiert. Insgesamt wurden 62 Proteine nachgewiesen. Die meisten von ihnen zeigten eine Assoziation mit PDAC (OR> 1); davon waren 33 Antigene auch in den Analysen mit Probengemischen gefunden worden, wie beispielsweise HP0874, HP0599 und HP0175. In weiteren Experimenten wurden 11 PDAC- und 8 Nicht-PDAC-Serumgemische untersucht, für die eine genaue Annotation des gastrointestinalen Gesundheitszustands vorlag, um wiederum *H. pylori*-Antigene zu finden. Insgesamt wurden in diesem Experiment 59 Antigene nachgewiesen, von denen 29 eine Assoziation mit PDAC zeigten (OR> 1).

Die HVT-Proteine wurden zunächst unter Verwendung von Serumgemischen (10 PDAC, 10 gesunde und 10 CP) analysiert. In diesem Schritt wurden in den PDAC Serumgemischen Antikörper gegen drei spezifische Antigene nachgewiesen. Anschließend wurde ein „Screening“ mit Einzelproben durchgeführt. Insgesamt wurden 74 PDAC- und 46 Nicht-PDAC-Serumproben (24 CP-Patienten und 22 gesunde Spender) auf den HVT-Microarrays

untersucht. Dabei wurden 25 HVT-Proteine identifiziert, einschließlich der Antigene, die bereits in den Gemischen nachgewiesen wurden: HVT079, HVT059 und HVT062.

Schlussfolgerungen

Analysen von Serumproben auf Darstellungen des Proteoms von *H. pylori* und HVT wurden durchgeführt, um Antigene zu finden, die mit dem Auftreten von PDAC assoziiert sind, wobei Serumpools und einzelne Serumproben verwendet wurden. Aus jedem „Screening“ wurde eine Kandidatenliste erstellt, die eine wertvolle Ressource für diagnostische Zwecke darstellt. Unter den 167 *H. pylori*-Proteinen wurden vier Antigene in allen drei Datensätzen mit starker Assoziation zu PDAC nachgewiesen: HP0874, HP0010, HP0601 und HP01238. Unter den 25 nachgewiesenen HVT-Proteinen wurden folgende drei häufig nachgewiesen: HVT079, HVT059 und HVT062. Diese Ergebnisse könnten die PDAC-Diagnose unterstützen und Zielmoleküle für eine mögliche Behandlung der PDAC Entwicklung und Progression liefern.

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Appendix 1: Result of *H. pylori* screening with Heidelberg serum pools

Gene symbol	10 PDAC	9 CP	10 healthy	19 non-PDAC	PDAC %	non-PDAC %	Odds Ratio	95% CI
HP0231	3	0	0	0	30%	0%	18.2	0.8360 to 396.2356
HP0011	4	0	1	1	40%	5%	12.0	1.1126 to 129.4217
HP0175	2	0	0	0	20%	0%	11.5	0.4958 to 265.4033
HP1125	2	0	0	0	20%	0%	11.5	0.4958 to 265.4033
HP0599	2	0	0	0	20%	0%	11.5	0.4958 to 265.4033
HP0010	3	0	1	1	30%	5%	7.7	0.6820 to 87.2557
HP1350	3	0	1	1	30%	5%	7.7	0.6820 to 87.2557
HP0202	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0444	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP1250	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0686	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0601	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0294	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0115	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0079	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP1434	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP1477	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP1285	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0874	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP1071	1	0	0	0	10%	0%	6.2	0.2287 to 165.8376
HP0243	4	1	1	2	40%	10%	5.7	0.8177 to 39.2689
HP1341	7	5	5	10	70%	50%	2.1	0.4136 to 10.6637
HP1038	2	0	2	2	20%	10%	2.1	0.2519 to 17.9273
HP1238	1	0	1	1	10%	5%	2.0	0.1117 to 35.8086
HP0073	1	0	1	1	10%	5%	2.0	0.1117 to 35.8086
HP1563	1	0	1	1	10%	5%	2.0	0.1117 to 35.8086
HP0547	1	0	1	1	10%	5%	2.0	0.1117 to 35.8086
HP0385	1	0	1	1	10%	5%	2.0	0.1117 to 35.8086
HP1199	1	1	1	2	10%	10%	1.1	0.0835 to 13.5172
HP0371	3	2	4	6	30%	31%	0.93	0.1761 to 4.8976
HP0582	2	2	2	4	20%	21%	0.9	0.1399 to 6.2804
HP1453	2	2	2	4	20%	21%	0.9	0.1399 to 6.2804
HP0638	6	6	7	13	60%	68%	0.7	0.1408 to 3.4038
HP0185	0	1	0	1	0%	5%	0.6	0.0219 to 15.7497
HP0961	0	1	0	1	0%	5%	0.6	0.0219 to 15.7497
HP0476	0	1	0	1	0%	5%	0.6	0.0219 to 15.7497
HP1542	0	1	0	1	0%	5%	0.6	0.0219 to 15.7497
HP0077	0	1	0	1	0%	5%	0.6	0.0219 to 15.7497
HP0990	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP1124	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP1442	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP0251	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP0279	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP0542	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP1389	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497
HP1129	0	0	1	1	0%	5%	0.6	0.0219 to 15.7497

Appendix 2: Result of *H. pylori* screening with Heidelberg serum individuals

Gene symbol	47 PDAC	19 CP	19 healthy	38 non-PDAC	PDAC %	non-PDAC %	Odds Ratio	95% CI
HP0874	8	0	0	0	17%	0%	16.6	0.9241 to 297.1165
HP0599	7	0	0	0	15%	0%	14.3	0.7873 to 258.2594
HP0175	6	0	0	0	12%	0%	12	0.6572 to 221.3291
HP0231	5	0	0	0	10%	0%	10	0.5333 to 186.1999
HP1285	5	0	0	0	10%	0%	10	0.5333 to 186.1999
HP0011	7	1	0	1	15%	3%	6.5	0.7599 to 55.1711
HP1563	2	0	0	0	4%	0%	4.2	0.1971 to 90.8297
HP0028	2	0	0	0	4%	0%	4.2	0.1971 to 90.8297
HP0900	2	0	0	0	4%	0%	4.2	0.1971 to 90.8297
HP0522	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0708	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0449	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0141	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1017	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0318	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0331	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1564	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1105	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0399	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0178	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1162	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0325	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0393	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0550	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0320	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1538	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0485	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0256	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1175	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0059	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1544	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0099	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1006	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0675	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0054	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP0092	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1125	1	0	0	0	2%	0%	2.5	0.0984 to 62.7308
HP1038	3	0	1	1	6%	3%	2.5	0.0984 to 62.7308
HP0202	1	0	0	0	2%	0%	2.4	0.0938 to 59.8236
HP1250	1	0	0	0	2%	0%	2.4	0.0938 to 59.8236
HP0294	1	0	0	0	2%	0%	2.4	0.0938 to 59.8236
HP1434	1	0	0	0	2%	0%	2.4	0.0938 to 59.8236
HP0243	1	0	0	0	2%	0%	2.4	0.0938 to 59.8236
HP1238	1	0	0	0	2%	0%	2.4	0.0938 to 59.8236
HP0601	2	0	1	1	4%	3%	1.6	0.1365 to 17.9542
HP1199	2	0	1	1	4%	3%	1.6	0.1365 to 17.9542
HP0010	3	0	2	2	6%	5%	1.2	0.1944 to 7.7481

HP1350	3	0	2	2	6%	5%	1.2	0.1944 to 7.7481
HP0385	3	0	2	2	6%	5%	1.2	0.1944 to 7.7481
HP0542	3	1	1	2	6%	5%	1.2	0.1944 to 7.7481
HP0638	4	1	2	3	8%	8%	1.1	0.2276 to 5.1753
HP1453	5	1	3	4	11%	10%	1	0.2519 to 4.0642
HP1341	8	4	3	7	17%	18%	0.9	0.2968 to 2.7805
HP0079	1	0	1	1	2%	3%	0.8	0.0486 to 13.2995
HP0077	1	1	0	1	2%	3%	0.8	0.0486 to 13.2995
HP1124	2	0	2	2	4%	5%	0.8	0.1074 to 5.9611
HP0371	4	2	3	5	9%	13%	0.6	0.1528 to 2.4670
HP0547	2	0	3	3	4%	8%	0.5	0.0821 to 3.2745
HP0582	7	4	7	11	15%	29%	0.4	0.1479 to 1.2473
HP0073	0	0	1	1	0%	3%	0.3	0.0104 to 6.6471
HP0185	0	0	1	1	0%	3%	0.3	0.0104 to 6.6471
HP0115	0	1	1	2	0%	5%	0.2	0.0072 to 3.3005

Appendix 3: Result of *H. pylori* screening with Madrid serum pools

Gene symbol	11 PDAC groups	PDAC %	8 non-PDAC groups	non-PDAC %	Odds Ratio	95% CI
HP0010	5	45%	1	13%	5.8	0.5249 to 64.8254
HP1117	2	18%	0	0%	4.5	0.1871 to 106.9660
HP0874	2	18%	0	0%	4.5	0.1871 to 106.9660
HP0601	2	18%	0	0%	4.5	0.1871 to 106.9660
HP1564	4	36%	1	13%	4.00	0.3525 to 45.3860
HP1492	7	64%	3	38%	2.90	0.4423 to 19.2348
HP0198	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0003	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0477	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0540	1	9%	0	0%	2.40	0.0873 to 67.5775
HP1477	1	9%	0	0%	2.40	0.0873 to 67.5775
HP1454	1	9%	0	0%	2.40	0.0873 to 67.5775
HP1251	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0797	1	9%	0	0%	2.40	0.0873 to 67.5775
HP1223	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0065	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0608	1	9%	0	0%	2.40	0.0873 to 67.5775
HP1091	1	9%	0	0%	2.40	0.0873 to 67.5775
HP1341	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0518	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0129	1	9%	0	0%	2.40	0.0873 to 67.5775
HP0582	6	55%	3	38%	2.00	0.3115 to 12.8400
HP1226	7	64%	4	50%	1.75	0.2746 to 11.1523
HP1238	2	18%	1	13%	1.50	0.1160 to 20.8553
HP1068	2	18%	1	13%	1.50	0.1160 to 20.8553
HP0537	10	91%	7	88%	1.40	0.0759 to 26.8967
HP1030	6	55%	4	50%	1.20	0.1935 to 7.4408

HP1453	3	27%	2	25%	1.12	0.1407 to 8.9951
HP0547	3	27%	2	25%	1.12	0.1407 to 8.9951
HP0118	1	9%	1	13%	0.70	0.0372 to 13.1794
HP0384	1	9%	1	13%	0.70	0.0372 to 13.1794
HP0516	2	18%	2	25%	0.66	0.0727 to 6.1110
HP1125	4	36%	4	50%	0.57	0.0897 to 3.6416
HP0638	10	91%	8	100%	0.41	0.0148 to 11.4578
HP0245	6	55%	6	75%	0.40	0.0545 to 2.9332
HP0371	8	73%	7	88%	0.38	0.0319 to 4.5497
HP0294	2	18%	3	38%	0.37	0.0455 to 3.0148
HP1199	2	18%	3	38%	0.37	0.0455 to 3.0148
HP0011	4	36%	5	63%	0.34	0.0520 to 2.2611
HP1052	1	9%	2	25%	0.30	0.0222 to 4.0600
HP1350	2	18%	4	50%	0.22	0.0282 to 1.7541
HP1293	0	0%	1	13%	0.21	0.0078 to 6.0750
HP1205	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0751	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0079	0	0%	1	13%	0.21	0.0078 to 6.0750
HP1343	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0170	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0912	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0514	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0318	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0193	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0690	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0561	0	0%	1	13%	0.21	0.0078 to 6.0750
HP1119	0	0%	1	13%	0.21	0.0078 to 6.0750
HP1014	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0788	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0234	0	0%	1	13%	0.21	0.0078 to 6.0750
HP0875	1	9%	3	38%	0.16	0.0136 to 2.0404
HP1285	1	9%	3	38%	0.16	0.0136 to 2.0404

Appendix 4: Result of HVT screening with Heidelberg serum pools

Gene symbol	10 PDAC	10 CP	10 healthy	PDAC %	non-PDAC %	Odds Ratio	95% CI
HVT059	2	0	0	20%	0	12.05	0.5220 to 278.5872
HVT062	1	0	0	10%	0	6.47	0.2407 to 174.0878
HVT079	1	0	0	10%	0	6.47	0.2407 to 174.0878

Appendix 5: Result of HVT screening with Heidelberg serum individuals

Gene symbol	74 PDAC	PDAC %	non-PDAC%	Odds Ratio	95% CI
HVT079	9	12%	0%	13.5	0.7659 to 237.5641
HVT059	8	10.8%	0%	11.9	0.6695 to 211.0694
HVT032	7	9.5%	0%	10.3	0.5760 to 185.37
HVT062	7	9.5%	0%	10.3	0.5760 to 185.38
HVT017	3	4.1%	0%	4.6	0.2298 to 90.1814
HVT022	3	4.1%	0%	4.6	0.2298 to 90.1815
HVT065	2	2.7%	0%	3.2	0.1506 to 68.3041
HVT006	2	2.7%	0%	3.2	0.1506 to 68.3042
HVT024	2	2.7%	0%	3.2	0.1506 to 68.3043
HVT034	2	2.7%	0%	3.2	0.1506 to 68.3044
HVT044	2	2.7%	0%	3.2	0.1506 to 68.3045
HVT046	2	2.7%	0%	3.2	0.1506 to 68.3046
HVT051	2	2.7%	0%	3.2	0.1506 to 68.3047
HVT021	2	2.7%	0%	3.2	0.1506 to 68.3048
HVT009	1	1.4%	0%	1.9	0.0757 to 47.5817
HVT010	1	1.4%	0%	1.9	0.0757 to 47.5818
HVT011	1	1.4%	0%	1.9	0.0757 to 47.5819
HVT013	1	1.4%	0%	1.9	0.0757 to 47.5820
HVT015	1	1.4%	0%	1.9	0.0757 to 47.5821
HVT020	1	1.4%	0%	1.9	0.0757 to 47.5822
HVT025	1	1.4%	0%	1.9	0.0757 to 47.5823
HVT029	1	1.4%	0%	1.9	0.0757 to 47.5824
HVT030	1	1.4%	0%	1.9	0.0757 to 47.5825
HVT039	1	1.4%	0%	1.9	0.0757 to 47.5826
HVT041	1	1.4%	0%	1.9	0.0757 to 47.5827

Appendix 6: Serum samples for *H. pylori* screening

ID	Kennung	Alter	Geschlecht	Klassifikation	Herkunft	Histologie_Klinik	Tumorhistologie (IPMN old classif)
1	Se0001	51	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
2	Se0002	52	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
3	Se0003	48	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
4	Se0004	43	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
5	Se0005	22	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
6	Se0006	19	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
7	Se0007	42	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
8	Se0008	65	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
9	Se0009	39	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
10	Se0010	40	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
11	Se0011	23	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
12	Se0012	33	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
13	Se0013	54	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
14	Se0014	48	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
15	Se0015	59	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
16	Se0016	37	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
17	Se0017	60	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
18	Se0018	34	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
19	Se0019	59	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
20	Se0020	49	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
21	Se0021	47	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
22	Se0022	27	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
23	Se0023	40	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
24	Se0024	43	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
25	Se0025	47	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
26	Se0026	54	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
27	Se0027	22	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
28	Se0028	50	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
29	Se0029	53	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
30	Se0030	52	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
231	Se0219	66	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
232	Se0220	76	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor

233	Se0221	72	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
234	Se0222	72	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
235	Se0223	67	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
236	Se0224	79	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
237	Se0225	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
238	Se0226	82	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
239	Se0227	72	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
240	Se0228	51	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
241	Se0229	74	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
242	Se0230	70	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
243	Se0231	62	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
244	Se0232	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
245	Se0233	53	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
246	Se0234	77	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
247	Se0235	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
248	Se0236	67	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
249	Se0237	82	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
250	Se0238	76	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
251	Se0239	62	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
252	Se0240	69	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
253	Se0241	52	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
254	Se0242	78	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
255	Se0243	70	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
256	Se0244	67	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
257	Se0245	54	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
258	Se0246	63	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
259	Se0247	48	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
260	Se0248	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
73	Se0073	22	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
74	Se0074	58	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
75	Se0075	74	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
76	Se0076	47	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
77	Se0077	50	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
78	Se0078	40	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
79	Se0079	49	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor

80	Se0080	59	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
81	Se0081	53	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
82	Se0082	67	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
83	Se0083	58	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
84	Se0084	51	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
85	Se0085	50	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
86	Se0086	56	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
87	Se0087	41	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
88	Se0088	41	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
89	Se0089	49	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
90	Se0090	52	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
91	Se0091	39	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
92	Se0092	52	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
93	Se0093	43	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
94	Se0094	54	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
95	Se0095	37	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
96	Se0096	39	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
97	Se0097	49	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
98	Se0098	42	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
99	Se0099	45	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
100	Se0100	50	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
101	Se0101	53	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
103	Se0103	44	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
104	Se0104	55	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
105	Se0105	74	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
106	Se0106	78	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
107	Se0107	43	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
108	Se0108	24	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
109	Se0109	62	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
110	Se0110	53	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
111	Se0111	59	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
112	Se0112	76	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
508	Se0508	65	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
509	Se0509	42	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
510	Se0510	45	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
511	Se0511	32	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
512	Se0512	69	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
513	Se0513	54	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
514	Se0514	37	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
515	Se0515	47	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor

516	Se0516	55	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
517	Se0517	49	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
518	Se0518	54	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
519	Se0519	61	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
520	Se0520	49	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
521	Se0521	59	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
522	Se0522	29	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
523	Se0523	64	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
524	Se0524	44	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
525	Se0525	55	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
526	Se0526	42	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
527	Se0527	52	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
457	Se0457	56	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
458	Se0458	72	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
459	Se0459	51	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
460	Se0460	77	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
461	Se0461	74	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
462	Se0462	73	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
463	Se0463	42	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
464	Se0464	63	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
465	Se0465	52	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
466	Se0466	63	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
467	Se0467	67	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
468	Se0468	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
469	Se0469	69	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
470	Se0470	56	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
471	Se0471	63	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
472	Se0472	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
473	Se0473	81	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
474	Se0474	61	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
475	Se0475	76	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
476	Se0476	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
477	Se0477	70	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
479	Se0479	79	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)

480	Se0480	82	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
481	Se0481	66	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
482	Se0482	60	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
483	Se0483	58	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
484	Se0484	72	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
485	Se0485	70	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
486	Se0486	63	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
487	Se0487	78	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
488	Se0488	57	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
489	Se0489	72	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
490	Se0490	47	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
491	Se0491	85	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
492	Se0492	78	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
493	Se0493	80	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
494	Se0494	70	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
495	Se0495	53	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
496	Se0496	72	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
497	Se0497	75	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
498	Se0498	50	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
499	Se0499	62	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
500	Se0500	76	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
501	Se0501	76	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
502	Se0502	62	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
503	Se0503	76	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
504	Se0504	76	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
505	Se0505	50	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
506	Se0506	50	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
507	Se0507	58	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
560	Se0560	79	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
561	Se0561	58	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
562	Se0562	56	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
563	Se0563	72	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)

564	Se0564	69	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
565	Se0565	78	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
566	Se0566	58	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
567	Se0567	65	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
568	Se0568	76	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
569	Se0569	63	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
570	Se0570	70	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
571	Se0571	61	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
572	Se0572	77	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
573	Se0573	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
574	Se0574	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
575	Se0575	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)

Appendix 7: Serum samples for HVT screening

ID	Kennung	Alter	Geschlecht	Klassifikation	Herkunft	Histologie_Klinik	Tumorhistologie (IPMN old classif)
1	Se0001	51	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
2	Se0002	52	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
3	Se0003	48	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
4	Se0004	43	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
5	Se0005	22	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
6	Se0006	19	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
7	Se0007	42	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
8	Se0008	65	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
9	Se0009	39	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
10	Se0010	40	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
11	Se0011	23	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
12	Se0012	33	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
13	Se0013	54	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
14	Se0014	48	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
15	Se0015	59	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
16	Se0016	37	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
17	Se0017	60	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
18	Se0018	34	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
19	Se0019	59	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
20	Se0020	49	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
21	Se0021	47	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
22	Se0022	27	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
23	Se0023	40	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
24	Se0024	43	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
25	Se0025	47	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
26	Se0026	54	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
27	Se0027	22	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
28	Se0028	50	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
29	Se0029	53	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
30	Se0030	52	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
231	Se0219	66	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
232	Se0220	76	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor

233	Se0221	72	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
234	Se0222	72	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
235	Se0223	67	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
236	Se0224	79	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
237	Se0225	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
238	Se0226	82	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
239	Se0227	72	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
240	Se0228	51	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
241	Se0229	74	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
242	Se0230	70	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
243	Se0231	62	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
244	Se0232	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
245	Se0233	53	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
246	Se0234	77	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
247	Se0235	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
248	Se0236	67	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
249	Se0237	82	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
250	Se0238	76	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
251	Se0239	62	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
252	Se0240	69	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
253	Se0241	52	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
254	Se0242	78	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
255	Se0243	70	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
256	Se0244	67	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
257	Se0245	54	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
258	Se0246	63	w	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
259	Se0247	48	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
260	Se0248	69	m	healthy	Blutspendedienst Mannheim	healthy	kein Tumor
528	Se0528	49	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
529	Se0529	64	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
530	Se0530	44	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
531	Se0531	45	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
532	Se0532	56	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
533	Se0533	77	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
534	Se0534	66	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor

535	Se0535	42	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
537	Se0537	41	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
538	Se0538	43	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
539	Se0539	69	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
540	Se0540	49	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
541	Se0541	47	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
542	Se0542	50	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
543	Se0543	64	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
544	Se0544	45	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
545	Se0545	39	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
546	Se0546	41	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
547	Se0547	42	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
548	Se0548	56	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
549	Se0549	50	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
550	Se0550	73	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
551	Se0551	63	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
552	Se0552	61	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
553	Se0553	40	w	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
554	Se0554	46	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
555	Se0555	63	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
556	Se0556	70	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
557	Se0557	48	m	CP	Uni Heidelberg	Chronische Pankreatitis	kein Tumor
558	Se0558	42	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
576	Se0576	60	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
577	Se0577	60	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
578	Se0578	60	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
579	Se0579	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
580	Se0580	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
581	Se0581	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
582	Se0582	79	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
583	Se0583	79	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
584	Se0584	79	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
585	Se0585	66	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
586	Se0586	66	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
587	Se0587	67	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
588	Se0588	69	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
589	Se0589	56	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)

590	Se0590	71	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
591	Se0591	44	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
592	Se0592	70	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
593	Se0593	59	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
594	Se0594	75	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
595	Se0595	70	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
596	Se0596	80	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
597	Se0597	59	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
598	Se0598	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
599	Se0599	44	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
600	Se0600	63	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
601	Se0601	60	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
602	Se0602	75	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
603	Se0603	79	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
604	Se0604	54	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
605	Se0605	83	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
607	Se0607	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
608	Se0608	64	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
609	Se0609	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
610	Se0610	80	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
611	Se0611	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
612	Se0612	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
613	Se0613	58	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
614	Se0614	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
615	Se0615	50	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
616	Se0616	58	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
618	Se0618	83	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
619	Se0619	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
620	Se0620	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
622	Se0622	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
623	Se0623	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
624	Se0624	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)

625	Se0625	50	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
626	Se0626	66	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
627	Se0627	60	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
628	Se0628	58	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
629	Se0629	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
630	Se0630	63	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
631	Se0631	57	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
632	Se0632	83	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
633	Se0633	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
634	Se0634	83	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
635	Se0635	50	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
636	Se0636	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
637	Se0637	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
638	Se0638	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
639	Se0639	60	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
640	Se0640	50	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
641	Se0641	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
642	Se0642	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
643	Se0643	52	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
644	Se0644	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
645	Se0645	64	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
646	Se0646	64	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
647	Se0647	60	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
648	Se0648	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
649	Se0649	60	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
650	Se0650	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
651	Se0651	51	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
652	Se0652	58	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
653	Se0653	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
654	Se0654	62	w	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)
655	Se0655	57	m	PDAC	Uni Heidelberg	Pankreastumor	Adenocarcinom ductal (auch szirrhöses)

Appendix 8: Serum pools for *H. pylori* screening from Madrid

Serum pool	Description
1-PDAC & HP group	1-pool.HP-(cases)<2
2-PDAC & HP group	2-pool.HP-(cases)>2
3-PDAC & HP group	3-pool.HP-(cases)>2
4-Cancer free, HP group	4-pool.HP-(cntr)>2
5-Cancer free, HP group	5-pool.HP-(cntr)>2
6-PDAC & UL group	6-pool.UL-(cases)>2
7-PDAC & UL group	7-pool.UL-(cases)>2
8-Cancer free & UL group	8-pool.UL-(cntr)>2
9-Cancer free & UL group	9-pool.UL-(cntr)>2
10-PDAC & Heartburn group	10-pool.H-(cases)<2
11-PDAC & Heartburn group	11-pool.H-(cases)<2
12-PDAC & Heartburn group	12-pool.H-(cases)>2
13-Cancer free & Heartburn group	13-pool.H-(cntr)>2
14-Cancer free & Heartburn group	14-pool.H-(cntr)>2
15-PDAC & Acid regurgition group	15-pool.AR-(cases)<2
16-PDAC & Acid regurgition group	16-pool.AR-(cases)<2
17-PDAC & Acid regurgition group	17-pool.AR-(cases)>2
18-Cancer free & Acid regurgition group	18-pool.AR-(cntr)>2
19-Cancer free & Acid regurgition group	19-pool.AR-(cntr)>2

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