Dissertation

Pre-analytic procedures for biomarker analysis.

Submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Parts of this thesis are based on the following original publications that are referred to in the text wherever required:

Loibner M, Buzina W, Viertler C, Groelz D, Hausleitner A, Siaulyte G, Kufferath I, Kölli B, Zatloukal K. Pathogen Inactivating Properties and Increased Sensitivity in Molecular Diagnostics by PAXgene, a Novel Non-Crosslinking Tissue Fixative. PLoS One. 2016;11(3):e0151383.

Loibner M, Oberauner-Wappis L, Viertler C, Groelz D, Zatloukal K. Protocol for HER2 FISH using a non-cross-linking tissue fixative to combine advantages of Cryo-preservation and formalin fixation. J Vis Exp. 2017 Dec 25;(130). doi: 10.3791/55885.

Loibner M, Hagauer S, Schwantzer G, Berghold A, Zatloukal K. Limiting Factors for Wearing Personal Protective Equipment (PPE) in a Health Care Environment Evaluated in a Randomised Study. In revision.

Oberauner-Wappis L, Loibner M, Viertler C, Groelz D, Wyrich R, Zatloukal K. Protocol for HER2 FISH determination on PAXgene-fixed and paraffin-embedded tissue in breast cancer. International Journal of Experimental Pathology. 2016;97(2):202-206. doi:10.1111/iep.12185.

Kap M, Aron G, Loibner M, Hausleitner A, Siaulyte G, Zatloukal K, Murk JL, Riegman P. Inactivation of Influenza A virus, Adenovirus, and Cytomegalovirus with PAXgene tissue fixative and formalin. Biopreserv Biobank. 2013 Aug;11(4):229-34.

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Abbreviations (alphabetical order)

A: adenine

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

ATCC: American Type Culture Collection

bp: basepair(s)

Bs: Bacillus subtilis

BSL: Biosafety level (synonymous to RG)

CBRN-PPE: Chemical, Biological, Radiation and Nuclear Personal Protective Equipment

CCHFV: Crimean-Congo hemorrhagic fever virus

cDNA: complementary DNA (DNA transcribed from RNA)

CE IVD: European Conformity *In Vitro* Diagnostics

CEN: Comité Européen de Normalisation, Center of European Norms

cfu/mL: colony forming units per milliliter

CISH: chromogenic *in situ* hybridization

CLSM: confocal laser scanning microscope

CMV: human cytomegalovirus

CPE: cytopathic effects

Cs: Clostridium sporogenes

Ct: Cycle threshold (this value is generated by real time-PCR)

C: cytosine

DAPI: 4',6-diamidino-2-phenylindole (fluorescent stain)

DGR: Dangerous Goods regulations (http://www.iata.org/publications/dgr/Pages/index.aspx)

DGHM: Deutsche Gesellschaft für Hygiene und Mikrobiologie (German Society of Hygiene and Microbiology)

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)

ECDC: European Centre for Disease Prevention and Control

ERK: extracellular-signal-regulated kinase

ERINHA: European Research Infrastructure for Highly Pathogenic Agents, www.erinha.eu

FDA: U.S. Food and Drug Administration

FF: formalin-fixed

FFP3: filtering face piece class 3

FFPE: formalin-fixed paraffin-embedded

FISH: fluorescence *in situ* hybridization

G: guanine

GAPDH: Glyceraldehyde-3-phosphate-dehydrogenase gene

GCP: Good Clinical Practice

HCWs: health care workers

HEPA: High Efficiency Particulate Air

HER2: human epidermal growth factor receptor 2

HR: heart rate

Hsp70: heat shock protein 70

IARC: International Agency for Research on Cancer

IATA: International Air Transport Association
(http://www.iata.org/publications/dgr/Pages/index.aspx)

IFD: invasive fungal disease

IHC: immunohistochemistry

ISH: *in situ* hybridization

kbp: kilo basepairs

MEM: Minimum Essential Medium

MERS-CoV: *Middle East respiratory syndrome corona virus*

miRNA: micro ribonucleic acid

MRC-5: Medical Research Council cell strain 5

Ms: Mycobacterium smegmatis

Mt: Mycobacterium terrae

NIH: National Institutes of Health (U.S. Department of Health and Human Services)

ONC: over-night culture

Pa: Pseudomonas aeruginosa

PAPR: Powered Air Purifying Respirator

PAXgene: PAXgene Tissue® (containing PAXgene Fixative and PAXgene Stabilizer)

PAXgene Fix: PAXgene Fixative®

PAXgene Stab: PAXgene Stabilizer®

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PF: PAXgene-fixed

PFPE: PAXgene-fixed paraffin-embedded

PPE: personal protective equipment

RG: risk group (synonymous to BSL: biosafety level)

RIN: RNA integrity number

RNA: ribonucleic acid

rpm: rounds per minute

18S/28S rRNA: ribosomal ribonucleic acid (size S is represented in Svedberg units)

RT: room temperature

RT-PCR: reverse transcription polymerase chain reaction

rt-PCR: real-time polymerase chain reaction = qPCR: quantitative PCR

RT-qPCR: reverse transcription quantitative polymerase chain reaction

Sa: Staphylococcus aureus

SARS-CoV: severe acute respiratory syndrome corona virus

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNV: single nucleotide variant

T: thymine

TRS1: terminal right short 1 (early immediate *CMV* gene)

TSE: Transmissible Spongiform Encephalopathy

U: uracil

UN numbers: United Nations numbers

WHO: World Health Organization

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Zusammenfassung

Präanalytische Prozesse, die biologische Proben in Forschung und Diagnostik durchlaufen, haben Einfluss auf nachfolgende Ergebnisse aller Analysemethoden. Probenahme, Transport- und Lagerbedingungen, physiologische Faktoren der Probenspender (Alter, Geschlecht, Lebensstil, etc.) und Probenvorbereitung beinhalten viele Variable, die nicht nur reproduzierbare Ergebnisse für einzelne Biomarker verhindern, sondern auch hohe Kosten verursachen. Standards für molekularanalytische *in vitro* diagnostische Verfahren für Vollblut, formalinfixiertes (FF) und schockgefrorenes Gewebe und der daraus gewonnen Nukleinsäuren (NS) und Proteine sind erst seit kurzem verfügbar. Formalin verbindet NS und Proteine mit- und untereinander, was wiederum harsche chemische Bedingungen zur Isolierung von NS erfordert, die dadurch in ihrer Qualität beeinträchtigt und für nachfolgende Analysemethoden eingeschränkt geeignet sind. Hochsensitive molekularbiologische Methoden erfordern höchste Qualität des eingesetzten und limitierten Probenmaterials. Diese Arbeit beleuchtet präanalytische Prozesse von Probenahmen mit besonderem Infektionsschutz über Pathogeninaktivierung- und detektion bis zu einem ausgewählten Beispiel in der Routinediagnostik in vier Kapiteln.

Kapitel 1: Die Probenahme an Patienten oder Verstorbenen mit noch unklarem Infektionsstatus (auch für Pathogene mit hoher Risikogruppe) und zur Klärung von Komorbiditäten ist seit 2016 im Steirischen Seuchenplan vorgeschrieben. Die persönliche Schutzausrüstung (PSA) schützt das Laborpersonal bei der Probenverarbeitung vor Infektionen muss aber trotzdem genaues Arbeiten ermöglichen. In einer Probandenstudie wurden PSA Systeme bei simulierter Laborarbeit getestet. Entgegen den Erwartungen sind mit fortschreitender Versuchsdauer die Fehlerquoten nicht angestiegen, obwohl körperliche Anstrengungen deutlich messbar waren. Diese Ergebnisse wurden im neuen Hochsicherheitsbereich des Institutes für Pathologie bei der Auswahl der PSA herangezogen.

Kapitel 2: Zur Verbesserung der Probenqualität wurde vom Industriepartner des Christian Doppler Labors für biologische Proben und Biobanktechnologien (Institut für Pathologie) ein formalinfreies Fixativ, PAXgene entwickelt und in einem EU-Projekt getestet. Die Gewebemorphologie ist gleich gut wie bei FF Proben, die Qualität der NS signifikant höher. Ob die Pathogeninaktivierung durch PAXgene gleich effizient ist, wie bei Formalin

wurde durch Austestung an Bakterien, Pilzen und einem Virusstamm getestet. Bis auf das sporenbildende *Clostridium sporogenes* inaktiviert PAXgene alle Pathogene gleich gut wie Formalin, das auch keine vollständige Inaktivierung erzielte. Diese Ergebnisse zeigen, dass Humanproben nach Fixierung potentiell infektiös sein können und dieselben Sicherheitsmaßnahmen für Formalin auch für PAXgene anzuwenden sind.

Kapitel 3: Durch die gute Qualität von NS aus PAXgene fixierten (PF) Proben stellte sich die Frage, ob auch die Sensitivität eines Virusnachweises im Vergleich zu FF Proben einen Vorteil bringt. Dazu gab es bisher, wie für die Pathogeninaktivierung, noch keine publizierten Daten. Bei verschiedenen PCR-Methoden war der Virusnachweis bei PF signifikant besser als bei FF Proben.

Kapitel 4: Die Statusbestimmung von HER2 (human epidermal growth factor receptor) bei Brustkrebspatientinnen mittels FISH (fluorescence *in situ* hybridization) und einem *in vitro* Diagnostik Kit indiziert die Behandlung mit Herceptin (Trastuzumab®), falls eine Amplifikation von HER2 vorliegt. Der für FF Proben entwickelte Diagnostik Kit brachte bei FP Proben vorerst keine Ergebnisse, erst bei einer präanalytischen Formalinbehandlung von mindestens16 Stunden waren die Signale auswertbar. Die gleichzeitig bessere RNA Qualität der PF Proben zeigte, dass multiple Analysen von derselben Probe möglich und PAXgene als Alternative für Formalin besonders bei limitierter Probengröße verwendet werden kann.

Abstract

Pre-analytic processes to which biological samples are subjected influence the outcome of all analytic methods. Sample taking, transport, storage conditions, physiological factors of the donors (age, sex, life style, etc.), and sample preparation comprise variables that not only impede reproducible results for biomarkers but also provoke tremendous costs in the health care system. Standards for molecular *in vitro* diagnostic examinations for snap frozen and formalin-fixed (FF) tissue, and DNA, RNA and protein isolation are available recently. Formalin crosslinks nucleic acids and proteins which requires harsh chemical conditions to isolate nucleic acids which hence become degraded. This negatively impacts the outcome of subsequent analyses. Highly sensitive molecular methods require high sample quality especially when sample size is limited. In addition during sample taking health care workers (HCWs) have to be protected from possible infections by adequate personal protective equipment (PPE). This work covers pre-analytic aspects from sample taking, biosafety issues and pathogen detection and ends at a selected example in routine diagnostic analysis over four chapters.

Chapter 1: Sample taking from potentially high risk group infected or deceased patients became mandatory since an updated version of the Styrian Contagion Plan has been published in 2016. PPE protects HCWs but shall allow the same laboratory procedures without massive restrictions. A study with volunteers has been designed to test PPE systems by performing different tasks of simulated laboratory work. Contrary to expectations error rates did not increase and performances improved with ongoing study duration, although physical strain was measured. The results obtained were used to select the adequate PPE for the staff working in the newly built high security laboratory at the Institute of Pathology.

Chapter 2: To improve sample quality, PAXgene Tissue, a non-crosslinking fixative was developed by the industrial partner of the Christian Doppler Laboratory for Biospecimen Research and Biobanking Technologies (Institute of Pathology). PAXgene, already extensively tested in an EU-project, preserves tissue morphology similar to FF tissue but the quality of isolated nucleic acids is superior. Hence, the question arose whether inactivation of bacterial, fungal and virus strains by PAXgene is as efficient as it is supposed to be with formalin. Except for one spore forming strain PAXgene inactivated all pathogens as well as formalin, which did also not achieve a sufficient inactivation. These

results indicate that human samples may remain infectious after fixation. The same biosafety measures used for formalin can be applied for PAXgene.

Chapter 3: The proven good quality of nucleic acids isolated from PAXgene-fixed (PF) samples raised the question of whether the sensitivity of virus detection may be improved when compared to FF material. No publications on this question as well as on the pathogen inactivation properties of PAXgene are available so far. The comparison of virus detection with two PCR methods showed a significantly higher sensitivity for PF compared to FF samples.

Chapter 4: The determination of the HER2 (human epidermal growth factor receptor) status of breast cancer patients by FISH (fluorescence *in situ* hybridization) with an IVD-approved kit was used to investigate whether it can be applied on PF samples. The treatment of patients with herceptin is indicated when the HER2 gene locus is amplified. PF samples did not yield interpretable results before a pre-analytic treatment with formalin was performed for at least 16 hours. RNA quality tested from the same sample confirmed the feasibility of performing multiple analyses from a single PF sample with at least similar results for FISH and superior RNA quality when compared to FF material. Small samples indivisible for different analysis methods indicate the use for PAXgene as an alternative to formalin.

General introduction

Relevance of pre-analytic processes in research and diagnostics

"Pre-analytic" is a term used for any kind of procedure before any kind of analysis takes place. Dependent on the discipline countless variables with their individual values are able to affect the outcome of a subsequent analysis method. Pre-analytic steps comprise initial procedures performed by healthcare personnel outside of the laboratory and also without the control of a clinical laboratory. These steps are patient preparation, sample collection, transport, preparation and storage (Plebani, 2012). In 2000 S. Narayanan stated that *"the pre-analytic phase is an important component of total laboratory quality"* and defined three comprehensive groups for pre-analytic variables for laboratory results: physiology, specimen collection and influence or interference factors. Physiologic variables are age, sex, time of day, season, altitude, pregnancy, and lifestyle. Specimen collection variables, e.g. blood collection, fasting, tourniquet application, different anticoagulants and stabilizing additives, as well as duration and temperature of specimen storage. Interference factors are of endogenous type such as circulating antibodies, hemolysis and enzymes (Narayanan, 2000). All variables of these groups can be continued and subdivided again and allocated to different types of specimen such as tissues, blood and other body fluids like urine, saliva, liquor, synovial, pleural fluid, ascites, and more, beside blood and its solid and liquid components. Molecular components of these specimens, so-called biomarkers, are used in medicine for the analysis of the health status, aiming at health prevention, diagnostics, aetiopathology and helping to define treatment plans and therapy tracking. Sample taking of different kinds of specimen (blood, tissue and other body fluids) and subsequent pre-analytic preparation to make the samples suitable for the following analysis have a major impact on the analysis outcome.

The term "biomarker" has been defined by the NIH (National Institutes of Health) Biomarkers Definitions Working Group as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" (NIH-Biomarkers-Definitions-Working-Group, 2001). A second definition *"any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease"* has been published by world leader organizations in coordination of the International Programme on Chemical Safety, led by the WHO (World Health Organization) with the United Nations and the International Labor Organization (WHO-

International-Programme-on-Chemical-Safety-Biomarkers-in-Risk-Assessment-Validity-and-Validation, 2001).

Storage and transport conditions, temperature shifts, chemical additives for sample stabilization, mechanic forces like centrifugation, and filtration have a significant impact on changes of macromolecules (e.g. nucleic acids, proteins, etc.) and metabolites (e.g. amino acids, peptides and other small molecules) analyzed and interpreted for diagnostic statements. Surveys on errors in laboratory medicine revealed that up to 68% of total errors occur before a test has been performed (Plebani, 2006) mostly due to mishandling procedures during collection, preparing or storing of samples (Lippi et al., 2011).

"Analysis of past studies indicates that the cumulative (total) prevalence of irreproducible preclinical research exceeds 50%, resulting in approximately US$28,000,000,000 (US $28B)/year spent on preclinical research that is not reproducible - in the United States alone." (Freedman et al., 2015).

Selective overview on analytic methods and problems

The so-called "garbage in - garbage out" problem, originally shaped by computer sciences, describes that the quality of data input mirrors the quality of the results which can also be applied to natural and medical sciences. Reproducible and reliable results require precedingly defined pre-analytical protocols and procedures (Pazzagli et al., 2013, Kamlage et al., 2014). Studies examining the results of the same analysis methods by using the same sample category (blood plasma with citrate as stabilizing additive) but collected according to the institutions internal procedures revealed a clustering of institutions within their own sample results (unpublished data from P.M. Abuja, Institute of Pathology, Medical University Graz). The plasma metabolome (= composition of all metabolites in a biological sample) is reported to be significantly affected by common pre-analytical variations like non-cooling (Kamlage et al., 2014).

Highly specified molecular analysis methods using miniscule amounts of sample material, for example nano- or picograms of nucleic acids or even single molecules or cells can already be declared as "state of the art" in research applications. Some of those like reverse transcription polymerase chain reaction (RT-PCR), different types of DNA and RNA sequencing on various platforms, fluorescence or chromogenic *in situ* hybridization

(FISH, CISH), immunohistochemistry (IHC), and others are already standardized and certified for diagnostics of human diseases since years or even decades. In contrast a standardization of pre-analytic sample treatment was only released from the Centre of European Norms – CEN in 2015 describing a selection of technical specifications for molecular *in vitro* diagnostic examinations for tissue and blood specimen and subsequent DNA and RNA extraction.

Some examples below underline the need to standardize pre-analytic procedures. A study investigating alterations of the human liver metabolome at different cryostorage temperatures and repeated transfer of samples between storage and retrieval environments revealed a distinct change of the metabolite signature (Abuja et al., 2015). Preparation, storage and handling of blood samples for prognostic, diagnostic and predictive biomarker analysis, for example, miRNA (Glinge et al., 2017), influence the analysis in clinical practice. The quality of RNA extracted from tissue samples is additionally highly dependent on cold and warm ischemia times, method and length of tissue fixation, processing and storage conditions (Groelz et al., 2013, Masuda et al., 1999). A standardized score for RNA quality measurement is the so-called RIN (RNA integrity number, ranging from 1 for low to 10 for best quality) obtained by an automated microcapillary electrophoretic analyzer using an algorithm calculated ratio of 18S and 28S rRNA. Studies have shown that RNA quality determination solely by RIN is not reliable for fixed tissue specimen and may require additional evaluation such as a quantitative amplicon length qRT-PCR. Differences in RNA quality were detected when tissue samples were pre-analytically processed by different methods (Viertler et al., 2012, Mathieson et al., 2016).

Pre-analytic procedures performed on biological samples

The method of choice is to stabilize samples as early as possible in the pre-analytic process. Stabilization or fixation methods for tissues and other biological specimens have to provide several properties. They have to block all chemical processes, preserve the morphology, allow the retrieval of antigens, snap-shot the current bio-molecular status and inactivate pathogens.

Stabilization by freezing

A method to obtain high quality samples is cryopreservation by snap-freezing of tissue specimen. Slow freezing and the temperature gradient within a sample that is directly submerged into liquid nitrogen (-196 °C) leads to conversion of liquid to gaseous nitrogen which acts as an insulator slowing down the freezing process and leading to ice crystal formation and disruption of cell membranes. Biological material is a poor thermal conductor, therefore, ice crystal formation may occur in inner layers of the specimen. During very fast cooling of water vitreous ice is formed without expansion as it occurs from ice crystals (Jongebloed, 1999). To speed up the freezing process without nitrogen gas formation ultra-cooled isopentane is used to quickly freeze the tissue sample without penetrating it and without the formation of ice-crystals. Vitreous frozen tissue has to be stored as cool as possible because vitreous ice is not stable and will slowly form crystalline ice, the warmer, the faster (Peters, 2010). This method is best suited for subsequent molecular and also microscopic analyses but cannot be broadly applied in routine health care for logistical, financial and ethical reasons, e.g. interference with established routine procedures that may negatively affect histopathological diagnosis. Splitting of samples for both methods, formalin fixation (described below) and cryopreservation, is controversial because of tumor heterogeneity and potential difficulties to distinguish tumor regions from normal tissue by gross examination. Acquisition and long-term storage of cryopreserved samples are complicated, cost intensive and need specific biosafety measures due to not inactivated pathogens as they are present in fresh samples.

Furthermore, it has been reported that liquid nitrogen tanks can become contaminated with viruses (Tedder et al., 1995), and disease transmissions due to cross-contamination between liquid nitrogen and embryos may occur (Bielanski, 2014).

Stabilization with formaldehyde, chemical aspects and consequences

Up to now fixation of bio-specimen using formaldehyde solution (formalin) is the gold standard method for tissue preservation. The first publication by Blum in 1893 described the use of formalin as a hardener to provide cutting of thin sections for microscopy as well as by Dell´Isola in 1895 (Blum, 1893, Dell'Isola, 1895). Furthermore, pathogen inactivation by formalin for example in vaccine production (Ulmer et al., 2006) and its efficacy is still indisputable.

Formaldehyde (CH_2O) is a colorless, pungently smelling and highly reactive gas with a molecular weight of 30.03 g/mol. In aqueous solution formaldehyde is hydrated to formaldehyde monohydrate ($CH_2(OH)_2$, synonymous to methylene glycol) and is also present as low molecular weight polymeric hydrates, $HO(CH_2O)_n$ H. Oxidation of formaldehyde leads to formic acid (CH_2O_2), all those reactions described by J. Frederic Walker in 1944 (Walker, 1944) (Figure 1).

By complete dissolution in water the resulting solution contains 37 – 40% formaldehyde which is known as "formalin" (Kim S Suvarna, 2013). Published in CEN/TS (Center of European Norms / Technical Specifications) 16827-1-3 in 2015, formalin is literally defined as a "*saturated formaldehyde solution containing mass fraction of 37% (corresponding to a volume fraction of 40%) formaldehyde, termed 100% formalin*". For tissue fixation with formaldehyde a standard buffered formalin solution shall be used determined as follows: "*10% formalin solution containing a mass fraction of 3.7% (corresponding to a volume fraction of 4%) formaldehyde buffered to pH 6.8 to pH 7.2. Standard buffered formalin solution often contains methanol to inhibit oxidation and polymerization of formaldehyde." (CEN, 2015).*

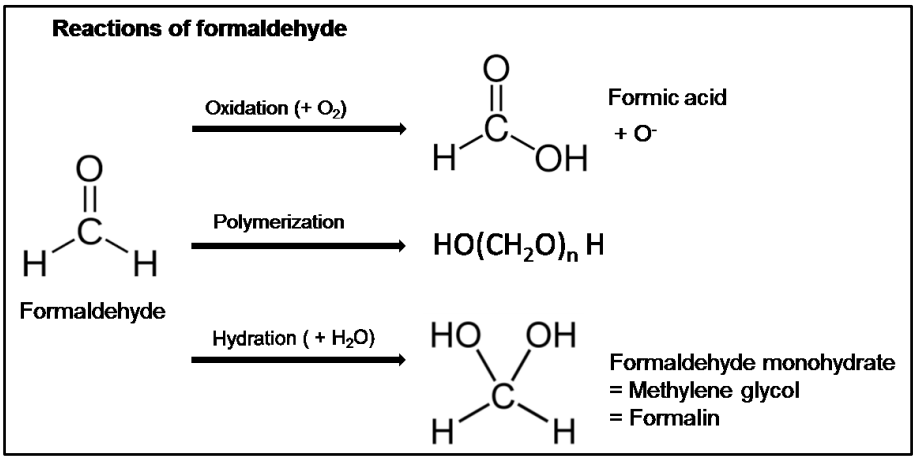


Figure 1: Reactions of formaldehyde. Formaldehyde can be oxidized to formic acid. Polymerization and hydration occur in aqueous solution resulting in formalin, which has several different types of chemical nomenclature.

Various and complex reactions of formaldehyde and macromolecules are described in literature (Kim S Suvarna, 2013). The fixation process initially starts with an alcohol fixation phase followed by the aldehyde-mediated crosslinking phase (Hewitt et al., 2008)

between proteins and nucleic acids and chemical adducts (McDonnell and Russell, 1999) (Figure 2).

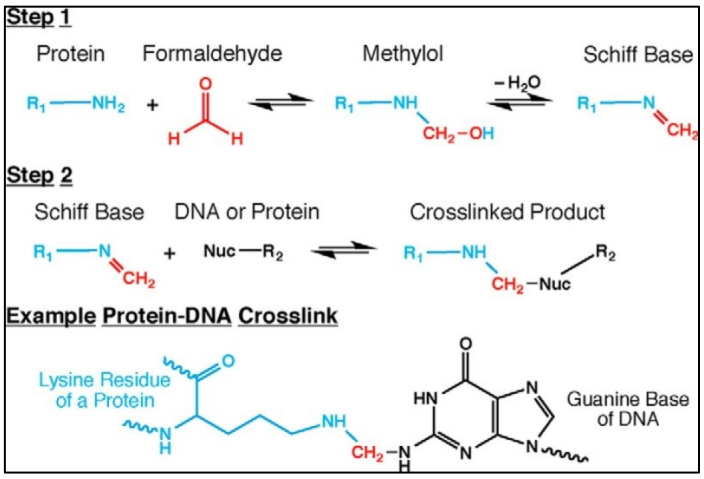


Figure 2: Formaldehyde crosslinks in two steps. At step one formaldehyde (red) most commonly reacts with a lysine residue of a protein (blue). This strong nucleophile forms methylol that splits water off resulting in an imine, the Schiff base (double bond between N and CH_2). At step two the Schiff base again reacts with a nucleophile, e.g. an amino group of a DNA base (black) forming a crosslink. Here, as an example, a protein-DNA crosslink is pictured. The second nucleophile could also be from another or even the same protein as in step one, so many variations of this crosslink may occur. Adapted from (Hoffman et al., 2015) and reprinted by permission from the American Society for Biochemistry and Molecular Biology, Copyright 2015.

Another serious modification of DNA due to formalin is the conversion of cytosine (C) to uracil (U) by hydrolytic deamination (Figure 3) that leads to sequence artifacts in different DNA sequencing methods. The DNA polymerase used in PCR reactions prior to sequencing incorporates an adenine (A) opposite to the uracil (U) (Do and Dobrovic, 2015). But on this position there should be a guanine (G). Opposite of an adenine should be a thymine (T), an artificial C:G>T:A single nucleotide variant (SNV) is generated. This process also occurs in normal living cells and is reversible by the cells own uracil-DNA-glycosylase. In formalin-fixed specimen the uracil remains. These SNVs cannot be distinguished from true sequence changes in cancer detection (Do and Dobrovic, 2015).

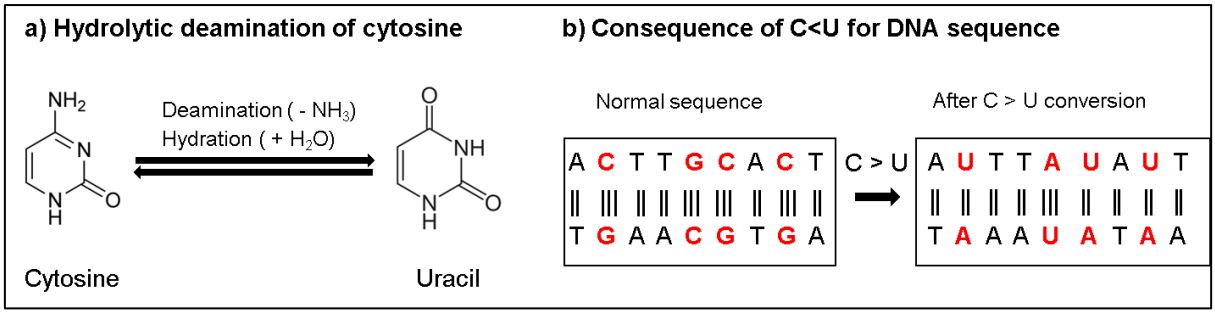


Figure 3: Hydrolytic deamination of cytosine to uracil. a) Hydrolytic deamination of cytosine to uracil takes place in normal cells and is reversible by the cells own repair mechanisms but remains in formalin-fixed samples. b) When C is converted to U the DNA polymerase inserts an A instead of a G in the opposite DNA strand (red letters). This failure is continued by PCR reactions prior to sequence analysis.

Various chemical reactions within macromolecules triggered by formalin are summarized in Figure 4.

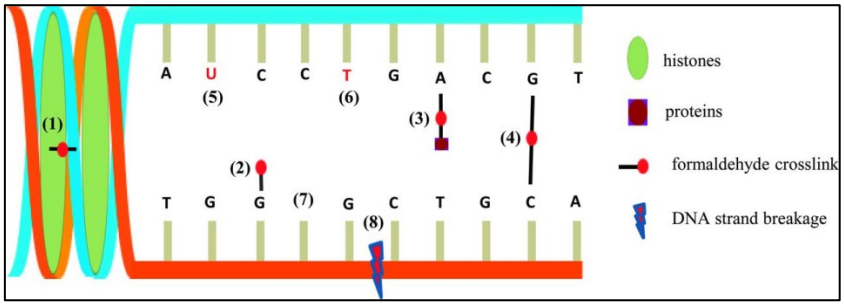


Figure 4: Formalin fixation in tissues causes various types of DNA damage. Formaldehyde reacts with DNA bases and proteins. (1) Histone–DNA crosslinks, (2) formaldehyde–DNA adducts, (3) DNA–protein crosslinks and (4) DNA–DNA crosslinks. (5) Uracil and (6) thymine are generated by deamination of cytosine and 5-mC, respectively. (7) DNA bases may also be lost, resulting in abasic sites. (8) Broken DNA strands lead to fragmentation of DNA. Adapted from (Do and Dobrovic, 2015) and reprinted by permission from Clinical Chemistry. Copyright 2015.

The formation of crosslinks and the subsequently necessary harsh conditions to break them again for nucleic acid extraction lead to DNA and RNA degradation and limit the usability of formalin-fixed bio-samples for molecular diagnostics (Groelz et al., 2013) especially for PCR applications (Bridge, 2017, Viertler et al., 2012, Kashofer et al., 2013).

The RNA quality of FFPE (formalin-fixed and paraffin-embedded) samples also decreases with prolonged fixation times (Mathieson et al., 2016, Kashofer et al., 2013, Viertler et al., 2012) (Figure 5 E). This has also been shown by comparison of Ct (cycle threshold) values generated by qRT-PCR for 92 genes using RNA extracted at different time points from cryopreserved and FFPE liver samples (Kashofer et al., 2013, Viertler et al., 2012) (Figure 5 G). Diminished RNA quality extracted from formalin-fixed samples was detected by several research groups by evaluation of RIN (Bioanalyzer, Agilent Technologies) (Figure 5 F) and qRT-PCR of templates with different lengths (between 65 and 942 base pairs) (Kashofer et al., 2013, Viertler et al., 2012, Mathieson et al., 2016).

DNA fragmentation due to formalin fixation is a major form of damage that negatively influences PCR amplification rates as a consequence of the reduced amount of DNA templates which is additionally more pronounced in long term stored fixed samples (Do and Dobrovic, 2015, Viertler et al., 2012).

In addition to the adverse effects caused by formaldehyde regarding molecular analysis formaldehyde (1 ppm = 1.23 mg/m^3 at 1 atm and 25 °C) in general is a biohazard and causes respiratory irritation. It is genotoxic, has a clastogenic effect (= leads to disruption of chromosomes) and it is also classified as a common indoor air pollutant. *"A recent update by IARC* (International Agency for Research on Cancer) *(2012) classified FA* (formaldehyde) *as "carcinogenic to humans (Group 1)" on the basis that FA may cause cancer of the nasopharynx and leukaemia, whereas there was limited evidence for association with sinonasal cancer. However, a consistent finding is the observed occurrence of nasal cancer in rats and mice at high FA exposures"* (Nielsen et al., 2017).

Although the use of the low-cost formalin is state of the art and analysis methods are tailored to counterbalance the adverse effects of formalin fixation, efforts are made to stepwise substitute formalin due to the altered demands on sample quality, and not to forget, the health hazardous impact for personnel.

Alternative stabilization and comparison with formaldehyde and freezing

A variety of alternative tissue preservation methods, such as alcohol-based fixatives UMFix (Sakura Finetek, Torrance, CA), 70% ethanol, modified Carnoy's, (Cancer Diagnostics, Durham, NC, USA; Carnoy´s solution contains ethanol : chloroform : acetic acid glacial, 6:3:1), modified methacarn (Leica Biosystems, Vienna, Austria; Methacarn contains methanol : chloroform : acetic acid glacial, 6:3:1), modified Davidson's II (Ricca

Chemical Company, USA), picrate fixative Bouin´s solution (Newcomer Supply, Middleton, WI), 30% sucrose, HOPE (Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect) (DCS, Hamburg, Germany), zink-based Z7, RCL2 (Alphelys, Plaisir, France), PAXgene Tissue (PreAnalytix, Hombrechtikon, Switzerland), Allprotect (Qiagen, Hilden, Germany), RNAlater (Invitrogen, Life technologies, UK), Cell-Block (Bio-Optica, Milano, Italy), Neo-Fix (Merck, Italy) and FineFixx (Milestone, Sorisole, Italy) were developed and tested as to whether they fulfil the required features of optimal preservation of tissue morphology, antigen retrieval, quality of extracted nucleic acids and their applicability for molecular analysis.

Wiedorn et al. 2002 designed HOPE (DCS, Germany) and evaluated HOPE-fixed and paraffin-embedded tissues that are up to 5 years old for downstream applications of high molecular weight DNA and RNA of > 20 kbp for PCR and RT-PCR, IHC and *in situ* hybridization (ISH) (Wiedorn et al., 2002).

Cox et al. 2006 compared morphology and RNA quality of tissues fixed with 70% neutral-buffered formalin, 70% ethanol, modified Carnoy's, modified Davidson's II, modified methacarn, picrate fixative Bouin´s solution, UMFIX, phosphate-buffered saline, or 30% sucrose. For tissue morphology they assessed modified methacarn, 70% ethanol, and modified Carnoy's solution as reasonable alternatives to formalin and modified methacarn and UMFIX for best RNA quality (Cox et al., 2006).

Dotti et al. 2010 tested methacarn, FineFixx and formalin and found high rRNA degradation at tissue RNA but conversely not at RNA extracted from a cell line model. Here, methacarn-fixed mRNA levels were well preserved and when compared to fresh frozen samples mRNA levels from FineFix and formalin samples they were notably lower (Dotti et al., 2010).

Staff et al. 2013 analysed the preservation of nucleic acids, tissue morphology and immunohistochemical (IHC) staining properties with zink-based Z7, RCL2, PAXgene, Allprotect and RNAlater fixed and paraffin-embedded samples with FFPE and cryopreserved samples as references. They received good IHC results (primary antibodies vimentin, smooth muscle actin, oestrogen and progesterone receptor, pancytokeratin, Ki-67 and cytokeratin 19) with all fixatives except Allprotect and RNAlater fixed samples. Only PAXgene, RCL2 and Z7 fixed samples showed a comparable morphology with FFPE sections. RNA quality assessed with RIN and qRT-PCR analyses

were only comparable between cryopreserved and PAXgene tissues. DNA analyses were equal in all samples (Staff et al., 2013).

Zanini et al. 2012 compared the commercially available fixatives Cell-Block, RCL2 and Neo-Fix with laboratory made Zinc fixatives (ZBF and Z7) and PAGA (polyethylenglycol, ethyl alcohol, glycerol, acetic acid), with FineFixx and formalin only from post-mortem examination as reference. They observed a higher staining affinity and tissue shrinkage in alcohol based fixed specimen compared to formalin-fixed samples. RNA concentrations extracted from Z7, PAGA and RCL2 were higher but equal in quality when compared to the other fixatives used in this study.

PAXgene tissue (PAXgene) was developed as an alternative tissue preservation system by PreAnalytix (Hombrechtikon, Switzerland), a joint venture company of Qiagen GmbH (Hilden, Germany) which is the industrial partner of the Christian Doppler Laboratory for Biospecimen Research and Biobanking Technologies at the Medical University Graz, Austria. PAXgene was designed to maintain tissue quality for morphological assessment and to allow molecular analysis from the same sample. It contains a non-crosslinking and non-carcinogenic formalin-free fixation and a subsequently used separated stabilization solution based on a mixture of different alcohols, acetic acid and a soluble organic compound.

The morphology of PAXgene-fixed paraffin-embedded (PFPE) tissue was assessed to be as good as the corresponding formalin-fixed paraffin-embedded (FFPE) tissue (Kap et al., 2011, Gundisch et al., 2014, Viertler et al., 2012, Mathieson et al., 2016) (Figure 5 A –D).

The quality of RNA extracted from PFPE samples determined by RIN was less than of cryopreserved samples (Viertler et al., 2012) but superior to FFPE samples (Viertler et al., 2012, Mathieson et al., 2016) (Figure 5 F), whereas the quality of PFPE-RNA evaluated by qRT-PCR was almost as high as from cryopreserved samples and both far better than from FFPE-RNA (Viertler et al., 2012, Mathieson et al., 2016, Groelz et al., 2013, Loibner et al., 2017) (Figure 5 E, G).

Proteins isolated from corresponding FFPE, PFPE and cryo-preserved spleen, breast, duodenum and stomach specimen were investigated by Western blot analysis using E-cadherin, ß-actin, ERK (extracellular-signal-regulated kinase), p-ERK and Hsp70 (heat shock protein) antibodies for detection. E-cadherin (responsible for cellular adhesion and tumor marker) and Hsp 70 were mostly less or not detected in FFPE in every tissue when

compared to cryo or PFPE. Minor differences were obvious in other tissue types but PFPE samples always resulted in visible detection (Figure 5 H) (Ergin et al., 2010).

DNA quality of samples isolated from five corresponding FFPE, PFPE and cryo-preserved colorectal cancer cases was evaluated by agarose gel (1%) electrophoresis. High molecular weight DNA bands were visualized by the intercalating dye ethidium bromide. DNA bands from PFPE and cryo cases are distinctly visible in contrast to FFPE samples which did not result in a band in any sample (Figure 5 I) (Viertler et al., 2012).

Figure 5 shows a summary of comparison studies on morphology (Figure 5 A - D), RNA (Figure 5 E - G), protein (Figure 5 H), and DNA (Figure 5 I) quality of different tissue types fixed with PAXgene and formalin and also with some corresponding cryopreserved samples.

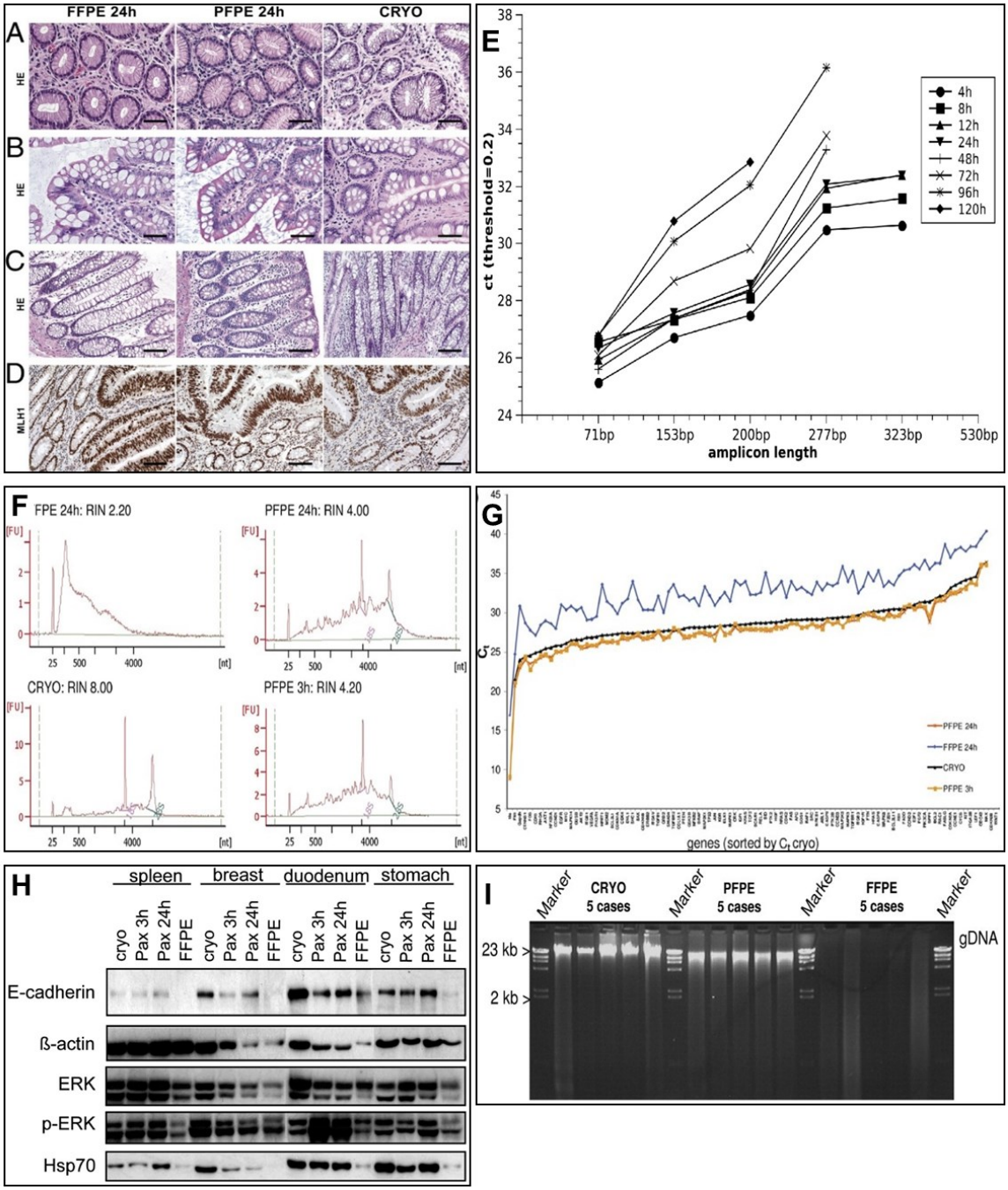


Figure 5. Comparison of morphology, antigenicity, RNA, DNA and protein quality of FFPE and PFPE human tissue. A–D: Comparison of morphology and antigenicity of FFPE, PFPE and cryopreserved human stomach tissue. Human tissue samples were fixed in formalin (FFPE 24h), PAXgene fixative and stabilizer each for 24 hours (PFPE 24h), or cryopreserved (CRYO). H&E (hematoxilin and eosin) staining of A: stomach (antrum), B: ileum and C: rectum. Morphology of PFPE is similar to FFPE samples. Less preservation (e.g. chromatin structure) is observed in cryopreserved samples. D: MLH1, an immune-histochemical screening marker routinely used for analysis for hereditary

colon cancer. The staining pattern of cancer and non-neoplastic colon tissue is similar in FFPE, PFPE and cryopreserved samples. A and B: magnification x 400, scale bar: 50 µm. C and D: magnification x 200, scale bar: 100 µm.

E: RNA quality of aliquots of an FFPE human liver sample was measured by real-time reverse transcription PCR with different lengths of *Glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH)* amplicons. y-Axis: Ct (cycle threshold) values are shown for different amplicon lengths (71 bp, 153 bp, 200 bp, 275 bp, 323 bp, 530 bp) on the x-axis. Samples were formalin-fixed for 4 up to 120 hours before paraffin embedding. Ct values rise correspondingly with increasing fixation time and amplicon length. Amplification of fragments longer than 277 bp was impossible with prolonged formalin fixation periods indicating RNA fragmentation.

F: RNA quality measured by RIN analysis: Electropherograms of RNA from FFPE tissue show the most degraded RNA (= lowest RIN values). PFPE tissues (fixed for 3 or 24 hours and stabilized for 24 hours) achieve higher RIN values and comparable peaks with frozen samples. RNA extracted from cryopreserved (CRYO) samples shows clear peaks corresponding to 18S and 28S rRNA.

G: A qRT-PCR (gene expression analysis) with 92 genes (x-axis) was performed with corresponding FFPE (blue), cryo (black) and PFPE (yellow, 3 or 24 hours fixed) samples on the TaqMan “Human Molecular Mechanisms of Cancer” panel. The y-axis shows the Ct values. Genes are sorted by increasing Ct value, the cryopreserved sample is used as reference.

H: Western blot analysis of proteins extracted from corresponding FFPE, PFPE (fixation time 3 and 24 hours) and cryo-preserved human nonmalignant spleen, breast, duodenum and stomach samples. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) with protein extracts and immunoblotting onto nitrocellulose membrane with the indicated antibodies, E-cadherin, ß-actin, ERK (extracellular-signal-regulated kinase), p-ERK and Hsp70 (heat shock protein) was performed.

I: DNA integrity. Genomic DNA was extracted from five corresponding FFPE, PFPE and cryo-preserved colorectal cancer cases. Agarose gel (1%) electrophoresis was performed and DNA visualized with ethidium bromide. No recovery of DNA isolated from FFPE samples is obvious. DNA bands from PFPE cases are distinctly visible.

A - D, F, G, I: adapted from (Viertler et al., 2012) and reprinted by permission from The Journal of Molecular Diagnostics. Copyright 2012.

E: adapted and reprinted with permission from (Kashofer et al., 2013).

H: Reprinted with permission from (Ergin et al., 2010), Copyright 2010. American Chemical Society.

Goal of the thesis

The goal of this work is to investigate key pre-analytic aspects of sample workflows in a clinical setting starting with acquisition of highly infectious specimen and the specific requirements for personal protective equipment. It continues with evaluation of sample stabilization and pathogen inactivation by comparing the current gold standard formalin and a formalin-free method. As pathogen detection and various applications of analysis methods require high quality samples it was investigated whether the formalin-free method may fulfil the requirements for a robust and specific diagnostic use. These aspects are addressed and described in four separate chapters and the corresponding hypotheses expressed below.

Hypotheses

Stabilization and preparation of potentially infectious human specimen are essential factors in pre-analytics. These factors must provide optimal quality for diagnostic analysis and highest safety and protection levels for the performing persons (physicians and laboratory workers) at the same time. The preparation of fresh specimen requires specific precautions depending on the risk group of the potential pathogen. Since PAXgene resulted in excellent preservation of morphology and biomolecules as described above the question arose whether pathogens present in human tissue samples are properly inactivated as it is supposed to be in formalin-fixed material. Studies in the past investigating the inactivation capabilities of formalin were performed on specific pathogens but not on a variety of different pathogens over the taxonomic kingdoms of bacteria, fungi and non-cellular organisms like viruses in parallel. Furthermore, an alternative fixative needs to be evaluated for its applicability in a research or routine laboratory. Associated pre-analytic steps may require adaptations which have to be identified by research work to achieve comparable results as obtained by gold standard procedures like formalin fixation.

Chapter 1: Evaluation of personal protective equipment (PPE) systems for highly infectious sample taking.

Two different personal protective equipment (PPE) systems were tested by nineteen volunteers on ergonomics and physically limiting factors due to PPE-induced restrictions. Potential error rates due to extended working times and increased ambient temperature

and the applicability in a health care setting were evaluated and quantifiable data generated.

Hypothesis 1: PPE-induced restrictions lead to increased error rates when simulated laboratory work, concentration and reaction performance are evaluated in a study with volunteers.

Chapter 2: Relevance of pathogen inactivation within a pre-clinical process.

To investigate whether specific safety precautions are needed for handling potentially infectious human bio-specimens that are either fixed with formalin or PAXgene, various *in vitro* inactivation assays for different types of bacteria, fungi and the highly sero-prevalent virus *CMV* (*human cytomegalovirus*) had to be developed and performed.

Hypothesis 2: The capability of PAXgene to well preserve biomolecules diminishes its pathogen inactivation efficiency when compared to formalin.

Chapter 3: Effect of PAXgene on pathogen detection by *CMV* analysis.

Due to the reported high quality of extracted nucleic acids from PAXgene-fixed samples pathogen detection is supposed to be different from formalin-fixed samples.

Hypothesis 3: Detection of *CMV* is expected to be more sensitive for PAXgene than for formalin-fixed samples.

Chapter 4: Clinical applicability of PAXgene for fluorescence *in situ* hybridization (FISH).

It was shown that PAXgene excellently preserves biomolecules but the applicability of PAXgene-fixed samples for *in vitro* diagnostic test kits compared to formalin-fixed samples has not yet been evaluated. A test for a breast cancer biomarker on FFPE tissues was selected and evaluated for its applicability for PFPE tissue. Different fixatives may require

different hybridization conditions that consequently might not be compliant with *in vitro* diagnostic regulations. In case of negative results a pre-analytic post-fixation procedure was evaluated as to whether this would allow the utilization of PAXgene-fixed samples for this application.

Hypothesis 4: The determination of the human epidermal growth factor receptor 2 (HER2) status by fluorescence *in situ* hybridization (FISH) can be performed with PAXgene-fixed paraffin-embedded (PFPE) breast cancer specimen after a pre-analytic post-fixation treatment with formalin.

Chapter 1 - Evaluation of personal protective equipment (PPE) systems for highly infectious sample taking.

Introduction

Prior to stabilization the sample has to be obtained from a person or a patient not only by meeting sample quality criteria but also biosafety criteria regarding protection of the health care workers (HCWs) like physicians, nurses and laboratory personnel. Tissues or other samples collected for diagnostic or research purposes from patients suffering from blood borne pathogens or who are at risk for being infected require that all personnel is educated in biohazards and the appropriate PPE (personal protective equipment) has to be provided from the employer and deployed by the HCWs. Diseases with endemic potential e.g. severe acute respiratory syndrome *coronavirus* (*SARS-CoV*), *Crimean-Congo hemorrhagic fever virus* (*CCHFV*), *Middle East respiratory syndrome corona virus* (*MERS-CoV*), *West Nile fever virus* and especially the increase of emerging antibiotic resistant mycobacterial strains (Jeanes and O'Grady, 2016) require enhanced preparedness to protect HCWs and their environment, and safe sample workflows. Unpublished data of the Institute of Pathology of the Medical University Graz have shown that between 2000 and 2015 the following risk group-3 (RG-3) pathogens (please see explanation of risk group categories below) were identified in clinical specimen by medical diagnosis: *Escherichia coli* (enterohemorrhagic), *Mycobacterium tuberculosis, Fransisella tularensis, Rickettsia, Shigella dystenteriae, Hantavirus, Hepatitis B, C, E, Cryptococcus, Echinococcus, Leishmania, Trypanosoma cruzi, HIV (Human Immunodeficiency Virus)*, TSE (transmissible spongiform encephalopathy), and *influenza virus* H1N1. Between January and June 2017 the Austrian Ministry of Health has registered among other certifiable pathogens 2 cases of infections with *Chicungunyavirus*, 44 x *Denguevirus*, 19 x *Echinococcus*, 56 x *Hantavirus* (50 cases only in Styria), 283 x *Mycobacterium tuberculosis*, 36 cases of Malaria and 3 cases of TSE, all of which are RG-3 pathogens as well.

Biosafety measures and relevance for acquisition of biohazardous samples

Biological samples may contain biohazardous microorganisms which are classified by the WHO into risk groups 1 to 4 (WHO, 2004) as follows:

"Risk Group 1: No or low individual and community risk. A microorganism that is unlikely to cause human or animal disease.

Risk Group 2: Moderate individual risk, low community risk. A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.

Risk Group 3: High individual risk, low community risk. A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

Risk Group 4: High individual and community risk. A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available."

Most research and diagnostic laboratories are equipped, registered, and the personnel educated and trained to handle specimen containing RG-2 microorganisms as well as the microorganisms solely as cultures. Manipulation of classified RG-3 samples requires additional large-scale biosafety measures. *"Enhanced environmental and personal protection may be required by the agent summary statement, risk assessment, or applicable local, state, or federal regulations"* (Chosewood, 2007). According to Austrian legislation (Verordnung der Bundesministerin für Arbeit, Gesundheit und Soziales über den Schutz der Arbeitnehmer/innen gegen Gefährdung durch biologische Arbeitsstoffe, BGBl. II Nr. 237/1998) a BSL-3 laboratory shall provide the following structural enhancements additional to a BSL-2 laboratory standard listed in Table 1.

Anteroom for clean storage of equipment
Anteroom for supplies with dress-in, shower-out capabilities
Separate rooms for storage of private clothes, PPE-undergarments, and PPE
Autoclave (pass-through)
Communication system
HEPA-filtration (High Efficiency Particulate Air) of the laboratory-exhaust air
Laboratory effluent decontamination system

Gas tight dampers to facilitate laboratory isolation
Negative pressure (+ checking devices inside + outside + acoustical alarm) to prevent exhaust of contaminated air
Emergency power supply
Advanced access control devices
Decontamination systems for laboratory equipment, PPE, sample devices to be locked out, waste material
Room decontamination system

Table 1: Mandatory enhancements for BSL-3 facilities additional to a standard BSL-2 standard facility.

For the intended manipulation of RG-3 manipulated samples it is recommended to use additional PPE (personal protective equipment) such as water-repellent suits and sleeves, a double layer of gloves, goggles, a respiration mask class 3 (FFP3 = filtering face piece class 3, which at least protects from 99% of droplets, particles and microorganisms), and a decontamination or waste system for the PPE.

Until recently the Styrian “Epidemic Plan Regulations” defined the post-mortem handling of persons deceased from RG-3 or 4 pathogens to that effect that any manipulation was prohibited unless adequate equipment is available. In 2016 a new edition of the Styrian “Epidemic Plan Regulations” came into force claiming the following in Table 2.

An autopsy of a deceased patient infected with agents of Risk Group-3 or 4 poses an increased risk for clinical staff and shall be performed only if BSL-3 or 4 containment, equipment and trained personnel are available.
Clarification of cases suspected of involving BSL-3 or 4 agents should nonetheless be pursued, also post mortem.
The presence or suspicion of a BSL-3 or 4 infectious disease must be noted on the instruction for autopsy.
The classification of the biosafety level is based on international criteria (CDC, WHO, ECDC).
If no risk classification has yet been assigned for a particular agent or death was elicited by an unknown pathogen, at least BSL-3 conditions shall be applied.

Table 2: Styrian “Epidemic Plan Regulations”.

Most laboratories and hospitals may not be equipped with rooms and PPE fulfilling the requirements to handle high risk (RG-3 and RG-4) specimen and patients suffering from those. Re-emerging diseases, unexplained deaths due to unidentified pathogens or risk evaluation at the start of epidemics, the evaluation of the role of comorbidities and the increase in multi-resistant bacteria may legitimate the preparedness for HCWs to use proper PPE to protect themselves and their environment. The UK National Health Service stated a poor preparedness regarding chemical incidents in a study published in 2000. After that they equipped all emergency departments with chemical, biological, radiation and nuclear personal protective equipment (CBRN-PPE) due to the escalation in world terrorism (Horby et al., 2000, Castle et al., 2009).

Prior to sampling and sample preparation of high risk specimen an appropriate PPE has to be selected from a variety of PPE systems that are commercially available. A full PPE consists of a protective suit or coverall, gloves (double layer is mostly requested), a respiratory protection and boots that are tightly sealed to the suit with adhesive tapes. Firstly, every component has to be selected according to the appropriate safety classification to provide the optimal protection. Secondly, the best ergonomic features and the tolerance should be based on evidence by the wearers. Various constrictions arise when wearing a full PPE and performing hazardous laboratory or clinical work for several hours. These constrictions are limited dexterity due to double layer of gloves, heat-stress and eye fatigue caused by stressful observation through light reflecting and refracting foils or face shields.

A study performed in the UK revealed that HCWs wearing CBRN-PPE and performing intubation and intravenous cannulation are significantly slower or even unsuccessful compared to personnel with the same skills doing the same work under standard conditions mimicking time critical emergency casualties caused by a CBRN incident (Castle et al., 2009). Tests mimicking the event of a biological threat have been performed in different contexts, for example, the assessment of whole hospitals by the French “Biotox-Pirotox” Network (Merens et al., 2012), performance of resuscitation skills while wearing PPE (Garner et al., 2004, MacDonald et al., 2006), and evaluation of PPE protection factors (Steward and Lever, 2012, Zamora et al., 2006). Furthermore, in China valuable experience was obtained from performing autopsies in BSL-3 facilities of patients deceased from severe acute respiratory syndrome coronavirus (SARS-CoV) and the quick spread of SARS-CoV originated in the Guangdong Province of China to 33 countries worldwide within 6 months (Li et al., 2005). Another example for an emerging virus is the

Crimean-Congo hemorrhagic fever virus (CCHFV, RG-4) having a zoonotic potential due to the transmission via arthropods that themselves are directly affected by climate changes and expansion of their natural habitat. CCHFV infections were noticed in 30 Asian countries, southeastern Europe, Africa and the Middle East (Mertens et al., 2013). The outbreak of Ebola (RG-4) in West Africa in 2013 demonstrated the absolute need for a fast response to unusual emerging infections using rapidly deployable field laboratory equipment and that wearing of PPE has several limitations under hot climatic conditions (Honda and Iwata, 2016, Wolfel et al., 2015, CDC, 2015). The need for measures to protect HCWs and the population and the increased awareness of the impact from biological threats has been triggered by different infections and their modes of transmission. Additional research and comparative studies on various types of PPE are needed to determine optimal PPE for HCWs as demonstrated by recent studies (Hersi et al., 2015, Sprecher et al., 2015). Testing of actual PPE configurations used under simulated environmental conditions is recommended to determine the length of time they could be safely worn (Sprecher et al., 2015).

How to test PPE for highly infectious sample preparation

A study was designed to identify parameters that affect the performance and tolerability of wearing PPE and how PPE influences physical performance, concentration, individual wellbeing, and error rates (e.g. wrongly processed items in different tasks). Different tasks simulating typical working steps of handling infectious materials in a health care environment under normal (22 °C) and increased (28 °C) working temperatures had to be performed in dedicated series. In particular, advantages and disadvantages of two different PPE systems were evaluated and it was investigated which parameters of the above mentioned were the most limiting for working under the tested conditions.

Material and Methods – Chapter 1

General information

Re-emerging diseases and pandemics challenge the health care system to be prepared for patient care and sample logistics employing easily available but best possible personal protective equipment (PPE) for health care workers. Quantifiable data on ergonomics of PPE applicable in a health care setting were generated in a study by measuring error rates and physically limiting factors due to PPE-induced restrictions.

This study corresponds to the study described in (Loibner et al., 2018) which is currently in revision. Approval was obtained from the Ethical Committee of the Medical University Graz, Austria (No. 23-321 ex 10/11) and all persons gave written informed consent according to the Helsinki Declaration. Furthermore, the study was registered at ClinicalTrials.gov (NCT03004690, “Testing of Personal Protective Equipment (PPE)) after its completion since the aim was not to provide data for a certification or approval process for medical products or devices. Regarding data protection and privacy the individual-related information connected with data generated was exclusively stored in a coded way in a database with restricted access and password protection. For communication and contacting the subjects a separate contact database with restricted access and password protection was established. For analysis and publication the data generated were exclusively used in a coded way.

Study participants

Nineteen healthy study volunteers were recruited for this study following good clinical practice guidelines by a voluntary registration in response to a public announcement. For every subject key lifestyle and medical parameters were documented. Exclusion criteria were latex and polyvinyl chloride allergy, pregnancy, claustrophobia, hypotension, cardiovascular and pulmonary diseases, history of vein thrombosis, chronic obstructive pulmonary disease, epilepsy, and infectious diseases. Ten male and nine female volunteers were recruited, aged between 21 and 38 years with body-mass-indices from 17.3 to 32.5 (Table 3).

	Subjects	Male (m)	Female (f)	Mean Age (SD)	Mean Body Mass Index (SD)
Suit A	10	6	4	26.5 (5.2)	22.5 (4.3)
Suit B	9	4	5	25.2 (3.8)	21.4 (2.5)

Table 3: Participants baseline data. Allocation of suits to the participants, and their mean age and body-mass-indices (Loibner et al., 2018).

The participants were allocated to wear one of two different types of randomly selected PPE suits. All subjects had to take off their personal clothes except their underwear and received uniform green cotton shirts and trousers to be worn under the PPE. They had to perform four different tasks six times at 22 °C on one day and four times at 28 °C on another day in an adapted room at the local core facility clinical research center of the Medical University Graz. The increased working temperature of 28 °C was selected as an additional parameter to mimic moderate heat stress in a field laboratory or a not air-conditioned environment as it may occur in a situation for sample taking outside of a BSL-2 or 3 facility. The web-based randomizer software (www.randomizer.at) released by the Institute for Medical Informatics, Statistics and Documentation of the Medical University Graz (IMI) was used for randomization (allocation of suits and the first task). The software's GCP-compliance (Good Clinical Practice) has been confirmed by the Austrian Agency for Health and Food Safety.

Personal protective equipment

Suit A: TychemR F overall whole-body (DuPont de Nemours and Company, 3M) suit including socks, a reusable light hood Versaflo™ S-655 (3M) with a stable face shield and an external 3M Jupiter™ Powered Air Turbo Unit (3M Austria) providing head-only positive pressure. Ten participants were randomly allocated to wear suit A (Figure 6 a).

Suit B: 3M™ JS-series Typ 3 Chemical and Respiratory Protective Suit (CRPS, 3M) with an integrated foil cap and an integrated respirator 3M Jupiter JP-ER-03 Powered Air Purifying Turbo (3M) fixed as a rucksack generating whole-suit positive pressure. Nine participants were randomly allocated to wear suit B (Figure 6 b).

With both suits, Sempercare surgical gloves (Sempermed, powder-free 150; Semperit) as the first layer, Ansell Sol-Vex gloves 37-900 (Ansell, Medical GBU, VWR, Austria) as a second glove layer and white rubber boots were worn. Gloves and boots were sealed to

the suit with an adhesive tape. Tasks I and II (Table 4) were performed in a mock-up glove box providing a third layer of latex gloves.

Figure 6: Two different PPE types used for the study. Suits tested vary in their respiration system and face shields. a: Suit A, head ventilation with external fixation of the air filters on a belt. b: suit B, total suit ventilation with internal respiration system worn on a rucksack inside the suit (Loibner et al., 2018).

Recording of physical parameters and tasks for simulation of laboratory work

Physical parameters like heart rate (HR) were measured with a wireless heart rate monitor placed below the sternum directly on the skin (Garmin Forerunner 305) during the task series described below. HR data before and after the task series were used for statistical analysis. Other parameters documented were body temperature assessed by tympanic infrared temperature measurement and body weight measured without PPE and undergarments at the beginning and after the last test (measurement accuracy 0.1 kg) for calculating dehydration. Four tasks had to be repeatedly performed as well as an assessment on comfort and general condition before the tasks started and after every four-task series (Table 4).

Task I comprised the correct assembly and position of coloured and numbered 2 mL cryo-tubes and their corresponding screwcaps (colour + number) in a storage box according to a given pattern to test fine motor skills, concentration and error rate (Table 4, Figure 7a).

Task II was employed to evaluate the same skills in a different approach by pipetting different volumes of coloured water into a 96-well microliter plate according to a given pattern. Task I and II were performed in a simulated glovebox made of plexiglass and two glove ports equipped with an additional third layer of a latex gloves (Table 4, Figure 7b).

Task III was a timed test of selective attention and a standardized refinement of a visual cancellation, the commercially available “d2 Test of Attention” (Brickenkamp R, 1998). In response to the discrimination of similar visual stimuli, the test measures processing speed, rule compliance, and quality of performance, allowing estimation of individual attention and concentration performance (Bates and Lemay, 2004), (Zillmer and Kennedy, 1999) (Table 4, Figure 7c).

Task IV investigated mobility, reaction time, speed and physical exercise by tapping touch sensors directed by signs on a screen (tapping test by talent-systems sportconsulting GmbH, www.werthner.at) (Table 4, Figure 7d).

All subjects documented their general condition, individual comfort and limiting factors in a structured assessment sheet before the tasks started to receive a baseline reference and after every series of tasks (Table 4) by rating every requested status from 1 to 10 on the assessment sheet. The highest ranking of 10 was used as a decisive factor for the immediate termination of the study.

After randomisation (www.randomizer.at) of the starting exercise all subjects performed the tasks in the given order (Table 5). Subject one started with test I while subject two started with test II at the same time. After ten minutes working time and a five minute break, subject one carried on with task II and subject two with task I. After a five minute break, subject one started with task III while subject two performed task IV over a 10 minute period. Again, after five minutes break, subject one continued with task IV and subject two with task III. All four tasks had to be repeated 6 times at 22 °C resulting in 6 hours absolute working time on the first study day and four times at 28 °C resulting in 4 hours absolute working time on the second study day. The waiting times between the first and the second study day were between 4 and 21 days (mean waiting time 14 days).

Task	Task Description	Readout	Mode	Position
I	**Tubes and caps** (Figure 7a) - Closing coloured and numbered tubes with corresponding coloured and numbered screwtops together. - Arranging closed tubes in a box following a certain pattern.	Comparison of total amount of handled tubes and wrong combinations.	Simulated glovebox	sitting
II	**Pipetting** (Figure 7b) - Pipetting a defined volume from three coloured water reservoirs into a 96-well plate following a certain amount and pattern.	Comparison of total amount of filled wells and wrong or omitted wells.	Simulated glovebox	sitting
III	**"d2 Test of attention"** (Figure 7c) - Ticking off every "d"-item with 2 bars in a pattern of "d" and "p" with different numbers and adjustments of bars. 14 rows with 47 items each have to be checked within 20 seconds per row (658 items in total).	A standardised matrix reveals correctly, wrongly or omitted "d" items.	Handwritten	sitting
IV	**Power of reaction test "TDS (test your talent)"** (Figure 7d) Digital readouts on a screen indicating to beat four touch sensors with the hands located on the left and right side in front of and behind the subject and two additional touch sensors on the floor for the legs.	Recording of reaction time.	Computer	standing
	Assessment of individual perception: - subjective temperature - sweating - dizziness - sickness - headache - hunger - thirst - subjective concentration - view - respiration - strangury - fine motor skills - mobility - back pain - other problems - general condition	Ranging from 1 (low interference) to 10 (high interference, resulting in termination of the study).	Handwritten	sitting

Table 4: Detailed description of all four tasks and an assessment sheet to evaluate the individual perception. (Loibner et al., 2018)

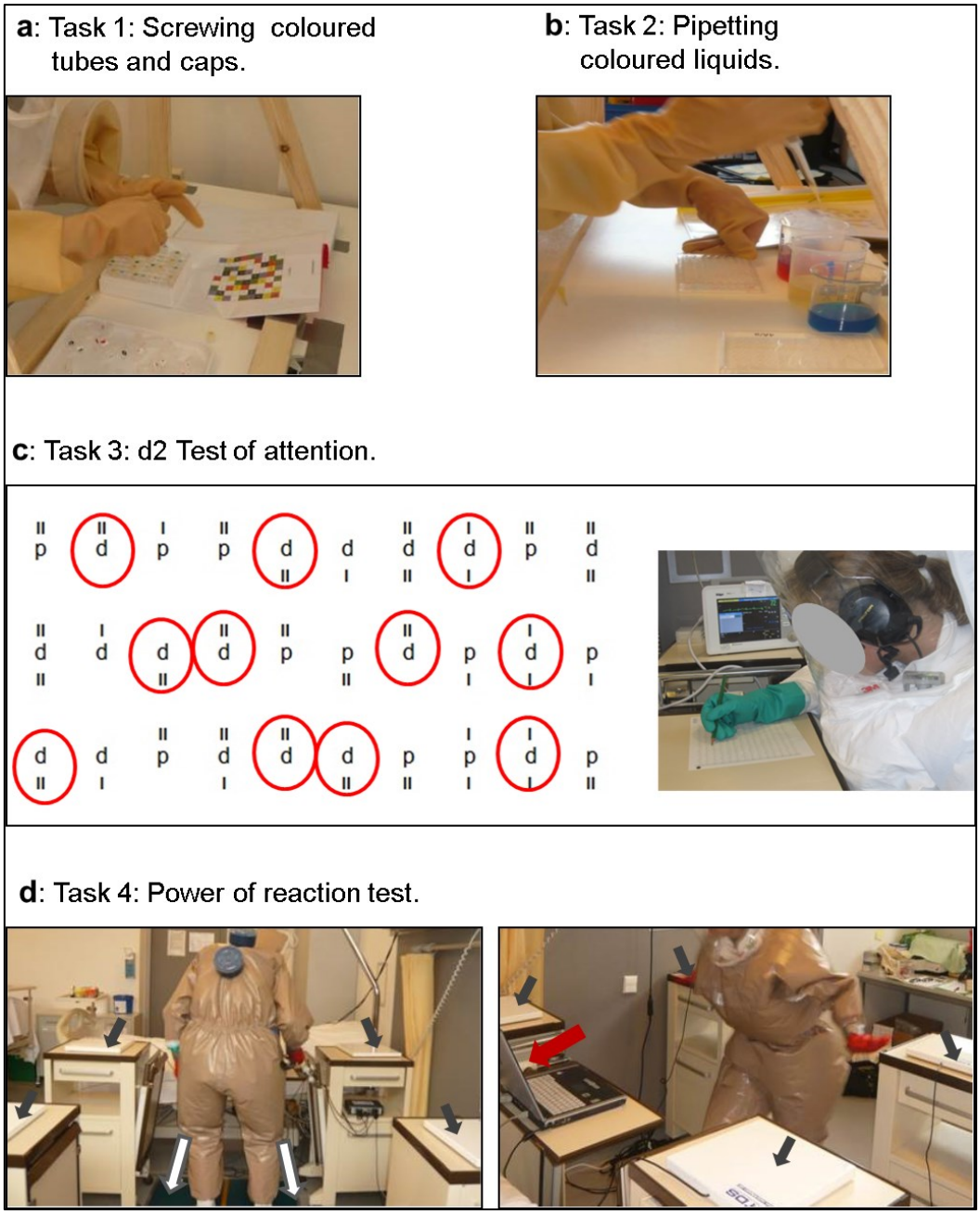


Figure 7: Overview on tasks 1 – 4 according to the descriptions in table 4. All tasks are described in detail in Table 4.

a: Task 1: Correspondingly coloured and numbered tubes and caps had to be closed and placed into the given position in a box. b: Task II: Coloured water had to be pipetted into the given position in a 96-well plate. c: Task III: d2 Test of attention, where only “d”s with two bars had to be identified. Red circles indicate the correct items. d: Task IV: Power of reaction test. Subjects had to tap onto four sensors with their hands (grey arrows) and feet (white arrows) according to the instructions indicated on the screen (red arrow) as fast as possible.

Task Schedule		
Time	**Suit A**	**Suit B**
10 min	Task I	Task II
5 min	Break	Break
10 min	Task II	Task I
5 min	Break	Break
10 min	Task III	Task IV
5 min	Break	Break
10 min	Task IV	Task III
1 min	Break	Break
3 min	Assessment	Assessment
1 min	Break	Break

Table 5: Schedule of task series 1 – 4, breaks and assessment of individual perception. The allocation of the starting task and the suit as well as the allocation of the suit to the subjects has been randomized by an online randomizer tool (Loibner et al., 2018).

Statistical analysis

Descriptive statistics for all task performances, physical measurements and assessment data are given as mean and standard deviation (SD).

The physical measurements like heart rate, body temperature and fluid loss (determined on the basis of body weight reduction) were documented before and after the task series. Heart rate was recorded during the whole study phase to identify the most challenging task. This should allow comparison of different tasks, error rates and reaction times. For each of the two working temperatures the differences between the two suits regarding heart rate, body temperature and fluid loss were assessed separately with an analysis of covariance (ANCOVA). Whether the assumptions for performing an ANCOVA were fulfilled was checked prior to analysis.

The task performance data were recorded 6 times at 22 °C, and 4 times at 28 °C. Assessment data of individual perception were recorded 7 times at 22 °C and 5 times at 28 °C. The first assessment data form was completed before the series started to provide a baseline result, then after every completed task series.

For each of the two study temperatures a repeated measurements analysis of variances (rmANOVA) was calculated to assess the effects of the working time as a within subject

factor. For the suits (A, B) also rmANOVA was calculated as a between subject factor on the amount of processed tubes and the amount of wrongly screwed or arranged tubes in task I. The same analysis was performed for the assessment data and task IV for exploratory purposes. Descriptive statistics were calculated for the data of task III.

For data management, descriptive analysis and figures Microsoft Excel 2003 (Version 11 for Windows 2003. Redmond, Washington, USA: Microsoft Corporation) was used. IBM SPSS Statistics (Release 20.0.0.2 2011. Armonk, New York, USA: International Business Machines Corporation) was employed for analyses of variance. Greenhouse-Geisser correction of within subject effects was used whenever indicated. P-values less than 0.05 were considered as statistically significant.

Results – Chapter 1

The results obtained correspond to the study described in (Loibner et al., 2018) which is currently in revision. In this study nineteen volunteers (10 male, 9 female), aged between 21 and 38 years, were recruited to perform four different tasks and assessments of individual perception wearing two types of PPE at two ambient temperatures on two different study days. Three subjects (2 x suit B, 1 x suit A) dropped out of the study before all task series were completed. Two indicated score 10 on their assessment forms on the first test day at 22 °C after 4 series due to paranasal sinus obstruction and after 5 series due to hunger, respectively. Both of them performed the complete series on the second study day at 28 °C. The third subject allocated to suit B completely resigned from the study after the first test day. The performance data of those tasks that were completed by the resigned subjects were included in the analysis.

Task II (pipetting a defined volume from three differently coloured water reservoirs into a 96-well plate following a certain amount and pattern) could not be evaluated due to a technical failure in the plate reading system and had to be excluded from statistical analysis.

Results, physical parameters

The ascertained fluid loss difference between subjects wearing the head ventilated suit A (-0.53 kg, SD 0.67) and the total ventilated suit B (-0.32 kg, SD 0.34) at 22 °C (ANCOVA, $p = 0.985$) was not statistically significant. However, a higher dehydration at 28 °C when subjects wore suit A (-1.27 kg, SD 0.62) compared to suit B (-0.59 kg, SD 0.34) (ANCOVA, $p = 0.069$) was obvious but without statistical significance (Figure 8 a, Figure 9).

The mean body temperature of subjects who wore suit A increased by 0.43 °C (SD 0.84) at 22 °C and 0.42 °C (SD 0.65) at 28 °C. Wearing suit B at 28 °C led to a minor increase of 0.39 °C (SD 0.42). A mean decrease of the body temperature at suit B at 22 °C was observed (-0.51 °C, SD 0.85). In general no significant body temperature effects between suit A and B were calculated by ANOVA at both working temperatures (Figure 8 b).

The heart rates slightly decreased in both suits during all six series at 22 °C. An increase of the heart rate throughout all four series was measured in subjects wearing suit A for 15.3 beats per minute (SD 19.81) and for 10.4 beats per minute (SD 14.15) at suit B

(ANCOVA, p = 0.724) at 28 °C. All differences were of no statistical significance (Figure 8 c).

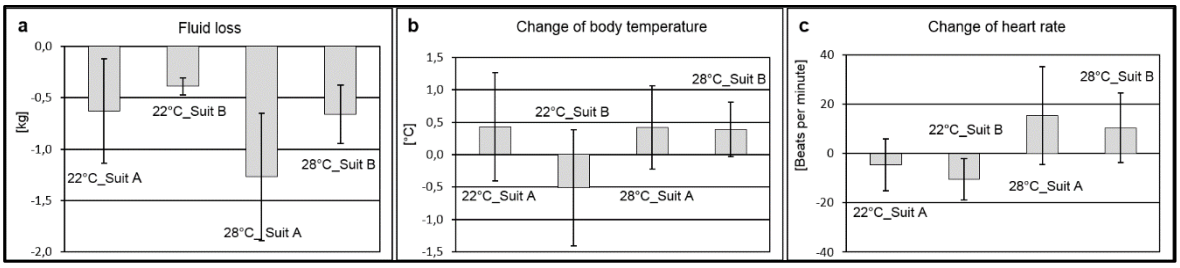


Figure 8: Change of physical parameters of subjects wearing PPE (suit A or suit B). All parameters were measured right before the start and immediately after termination of the task series at 22 °C (6 working hours) and 28 °C (4 working hours) and differences calculated. a: Mean fluid loss was determined by body weighing. b: Body temperature was measured with a tympanic infrared temperature device. c: Heart rate was recorded by a wireless heart rate monitor placed below the sternum. Mean values and plus/minus standard deviation values are graphically indicated.

Figure 9: Fluid loss illustrated by sweat soaked undergarment. After termination of all four series at 28 °C the fluid loss was visible by the differently sweat soaked undergarment. The left subject was allocated to suit B, the right subject to suit A.

Results, task performances

Task I, simulated laboratory work

Coloured and numbered tubes had to be closed with corresponding coloured and numbered screwtops and arranged in a box following a certain colour pattern.

The amount of processed tubes increased significantly from series to series at each working temperature independent from the suits (rmANOVA, $p < 0.001$ for 22 °C and $p = 0.013$ for 28 °C) (Figure 10). At 22 °C subjects with suit A processed significantly more tubes (rmANOVA, $p = 0.030$) than subjects who wore suit B. This tendency, without significance, was observed at 28 °C as well (rmANOVA, $p = 0.094$). A constantly low amount of wrong tubes (wrong position or wrong cap) was observed at both temperatures independent from the suits. In subjects wearing suit B a stable heart rate at both temperatures was recorded. Those wearing suit A had a slight decrease of their heart rates between series 2 and 4 at 22 °C and a not significant continuous increase during all four series at 28 °C.

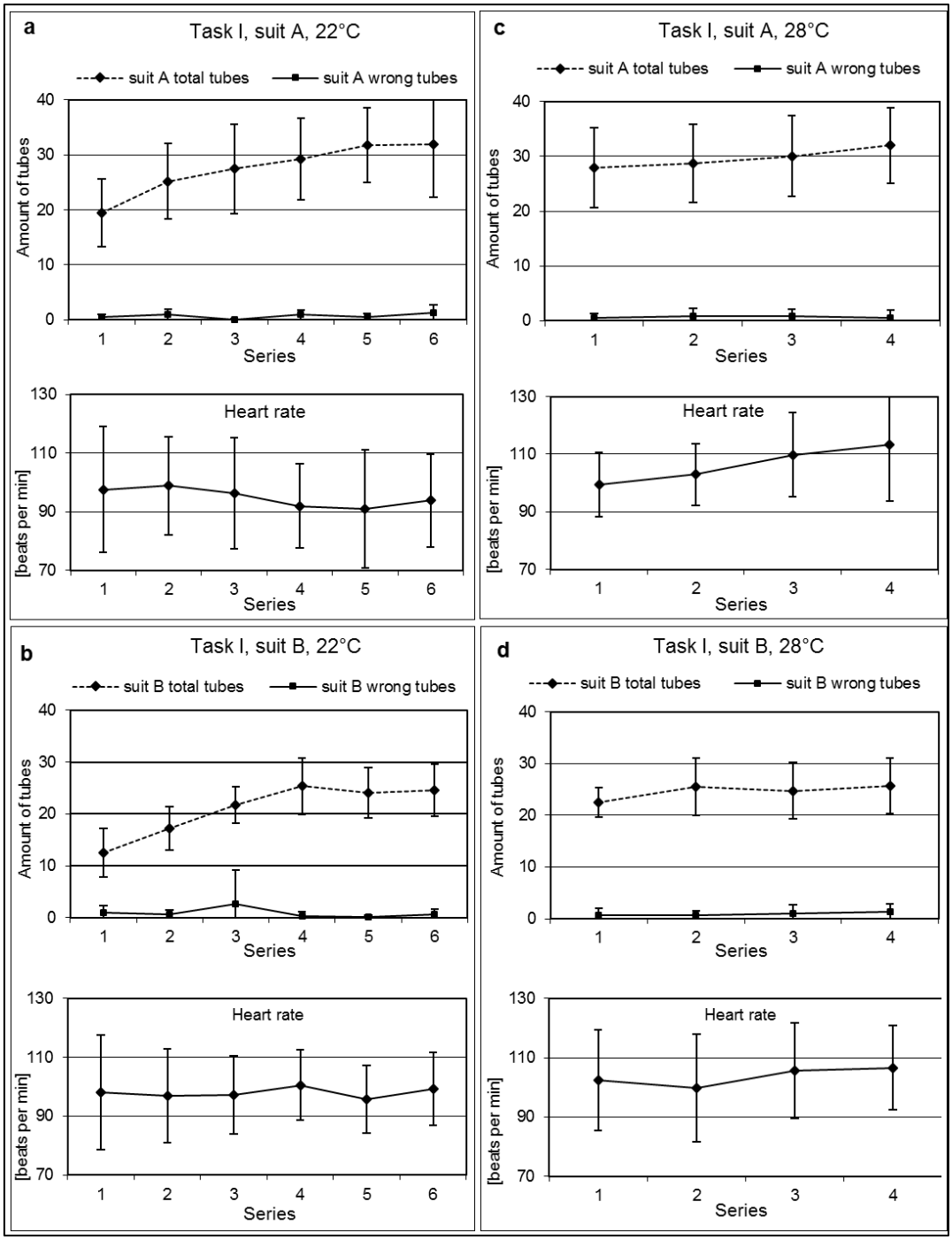


Figure 10: Results of task I (laboratory exercise): Average amount of correctly and wrongly screwed tubes and corresponding heart rates. The graphs show mean values and standard deviations on the y-axis, calculated from the amount of all processed (dashed line) and wrongly screwed tubes (continuous line). The x-axis indicates the amount of series comprising all tasks. a: Suit A at 22 °C, 6 series. b: Suit B at 22 °C, 6 series. c: Suit A at 28 °C, 4 series. d: Suit B, 28 °C, 4 series. The corresponding heart rates whilst the task series are mapped in the adjunctive panels below.

Task III, "d2 Test of attention"

The "d2 Test of attention" was used to evaluate correctness, working rate and accuracy of discrimination by ticking off every "d"-item with 2 bars in a pattern of "d"s and "p"s with different numbers and adjustments of bars. Fourteen rows with 47 items each have to be checked within 20 seconds per row. The maximum number of items is 658. The characteristic numbers obtained from this test are determined as follows:

TN is the total number of items processed.
E is the error score comprising the sum of all mistakes. These include:

E1, the errors of omission and

E2, the errors of commission, i.e. wrongly identified character, wrong dashes and wrong letters).

E% is calculated as the proportion of E (error score) and the number of all items processed.
TN-E is calculated as the total number of items processed minus E (error score).
CP is the index of concentration performance which is calculated as the difference of correct items and E2 (errors of commission) (Brickenkamp R, 1998, Zillmer and Kennedy, 1999).

CP was at least constant or even increased with progression of the working time (Figure 11, green bars). TN-E (black bars) was also almost constant for every participant in all series. From the first to the second or third series for almost all participants an increase of TN-E was observed at 22 °C which was not observed at 28 °C and was of no statistical significance. During the first four series E% (red bars) decreased but remained constant until the last series for five subjects at 22 °C working temperature. Four subjects had a worse performance indicated by an increase of E% at the last two series. The trend of decreasing E% could not be observed when subjects wore suit B at 28 °C. The heart rates of subjects at 22 °C were below 100 beats per minute independent of the suit. An average increase of the heart rate from 100 to 110 beats per minutes was observed in subjects wearing suit A at 28 °C. This increase was not obvious in subjects wearing suit B. The mean values of heart rates were calculated at each time point for every subject (Figure 11 c, d, g and h). The mean values of TN-E, E% and CP were calculated from all subjects at every time point and connected to receive a trend line (Figure 11 i, j, k and l). The CP trend line shows a slight increase and the E% trend line a clear decrease from the first to the last series but without statistical significance.

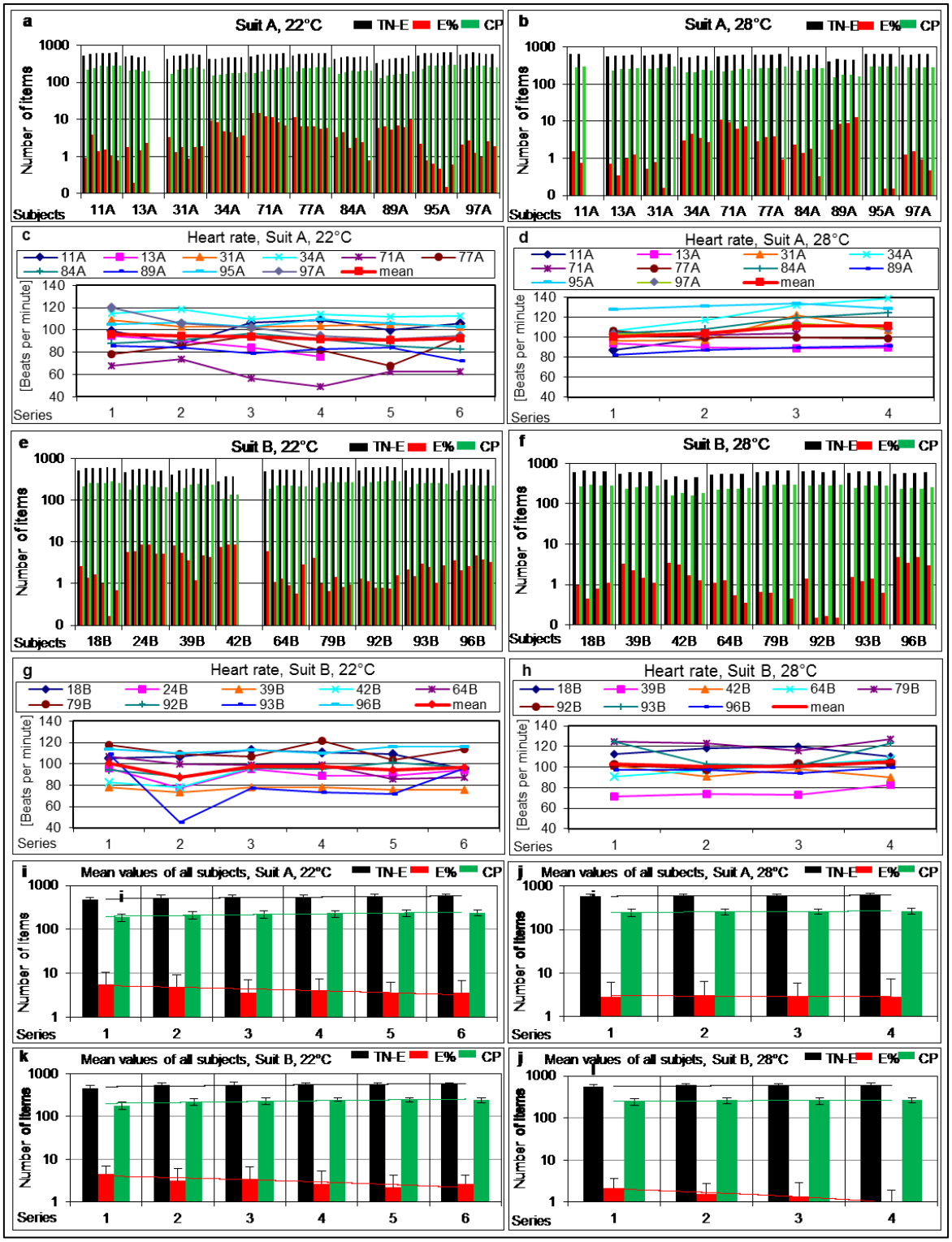


Figure 11: Results of task III "d2 Test of attention" per subject, corresponding heart rates (a – h) and mean values of all subjects (i – l). a, b, e, f: y-axis: The graphs show TN-E (black bars), indicating the amount of symbols recognized correctly; E% (red bars), the proportion of errors and number of all items processed, and CP (green bars), the index of concentration performance. x-axis: Individual performance values of each subject

who performed six series at 22 °C with a: suit A, e: suit B. Four tests were performed at 28 °C shown at b: suit A and f: suit B. Individual performances per subject are divided by vertical black lines. c, d, g, h: Courses of individual heart rates corresponding to the d2 test performances are indicated during the six or four tests, respectively. i, j, k and l: Mean values of all subjects were calculated per test series resulting in a trend line which shows the average development of TN-E and E% in 6 series at 22 °C and 4 series at 28 °C, respectively.

Task IV, power of reaction test "TDS (test your talent)"

Digital readouts on a screen indicated to beat four touch sensors with the hands located on the left and right side in front of and behind the subject and two additional touch sensors on the floor for the feet.

A reduction of reaction time from the first to the following test series was measured in subjects at 22 °C independent from the suit (rmANOVA, $p < 0.001$) (Figure 12 a). A performance improvement equated with the decrease of reaction time was observed at 28 °C when subjects wore suit A which was significantly more pronounced to those who wore suit B (rmANOVA interaction, $p = 0.016$). The heart rates of subjects with suit B at 28 °C was significantly higher than those who wore suit A (Figure 12 b). The mean values show a reduced reaction time (Figure 12 c) and a corresponding higher heart rate (Figure 12 d) at 28 °C when compared to 22 °C.

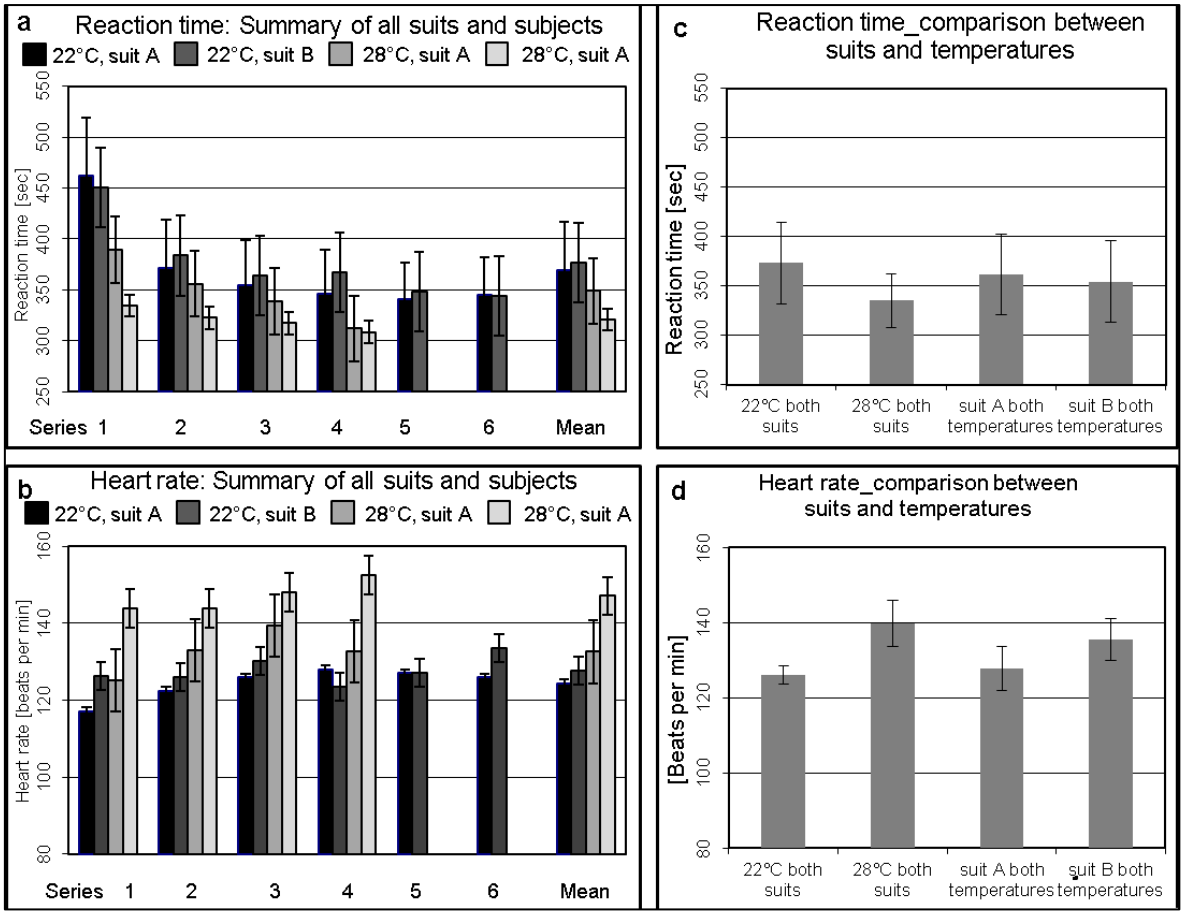


Figure 12: Results of task IV, reaction test and corresponding heart rates. The reaction time was evaluated by digital readouts on a screen in front of the subjects indicating to tap four touch sensors with the hands which were located on the left and right side in front of and behind the subject and two touch sensors for the feet. a: The bars indicate the mean values and standard deviations of the reaction times measured and calculated from all subjects within the same suits and temperatures (y-axis). This resulted in four groups: 22 °C, suit A; 22 °C, suit B; 28 °C, suit A; 28 °C, suit B. The x-axis indicates the series 1 - 6 and 1 - 4, respectively, as well as the mean values from all four groups. b: The bars indicate the heart rates (beats per minute) on the y-axis from the respective groups (x-axis) as described for a. c: The bars show the differences of the reaction times of the combined groups. Both suits at 22 °C are compared to 28 °C and suit A at both temperatures is compared to suit B at both temperatures. d: Comparison of the heart rates according to the combined groups as described for c.

Results, assessment of individual perception under PPE conditions during task performances

The assessment sheets comprised sixteen statements of individual perception including the general condition. The rating ranged from 1, indicating a low interference to 10, indicating the highest interference. This was stated three times and led immediately to termination of the study as described above. The assessment sheets were filled in before the task series started and after each task series at 22 °C and 28 °C, respectively (Figure 13). No major limitations by the parameters thirst, dizziness, restricted respiration, concentration, strangury, fine motor skills, mobility or other problems were stated at 22 °C (Figure 13 a and b). Considerable but not significant differences due to the different PPE types were detected for temperature perception and sweating as well as for headache and hunger, which were more pronounced for suit A. A significant restriction of the view was ascertained at suit B (Figure 13 b) due to the flexible face shield in contrast to the fixed shield fitted with suit A (rmANOVA, $p < 0.001$). At 22 °C this difference became more pronounced with proceeding working time (rmANOVA interaction, $p < 0.021$). Back pain was in general noted to be more pronounced when subjects wore suit B (Figure 13 b) compared to suit A (Figure 13 a), however, these differences were not of statistical significance ($p = 0.096$).

At 28 °C on the second study day the view was verified to be more restrictive with suit B (Figure 13 d) compared to suit A (Figure 13 c) (rmANOVA, $p = 0.027$). The discomfort due to sweating increased for most subjects wearing both suits ($p < 0.001$), but significantly more with suit A (Figure 13 c) than with suit B (Figure 13 d) (rmANOVA interaction, $p = 0.003$). The mean rating nearly stepped up to 7 (Figure 13 c) but this situation was still tolerable for all subjects.

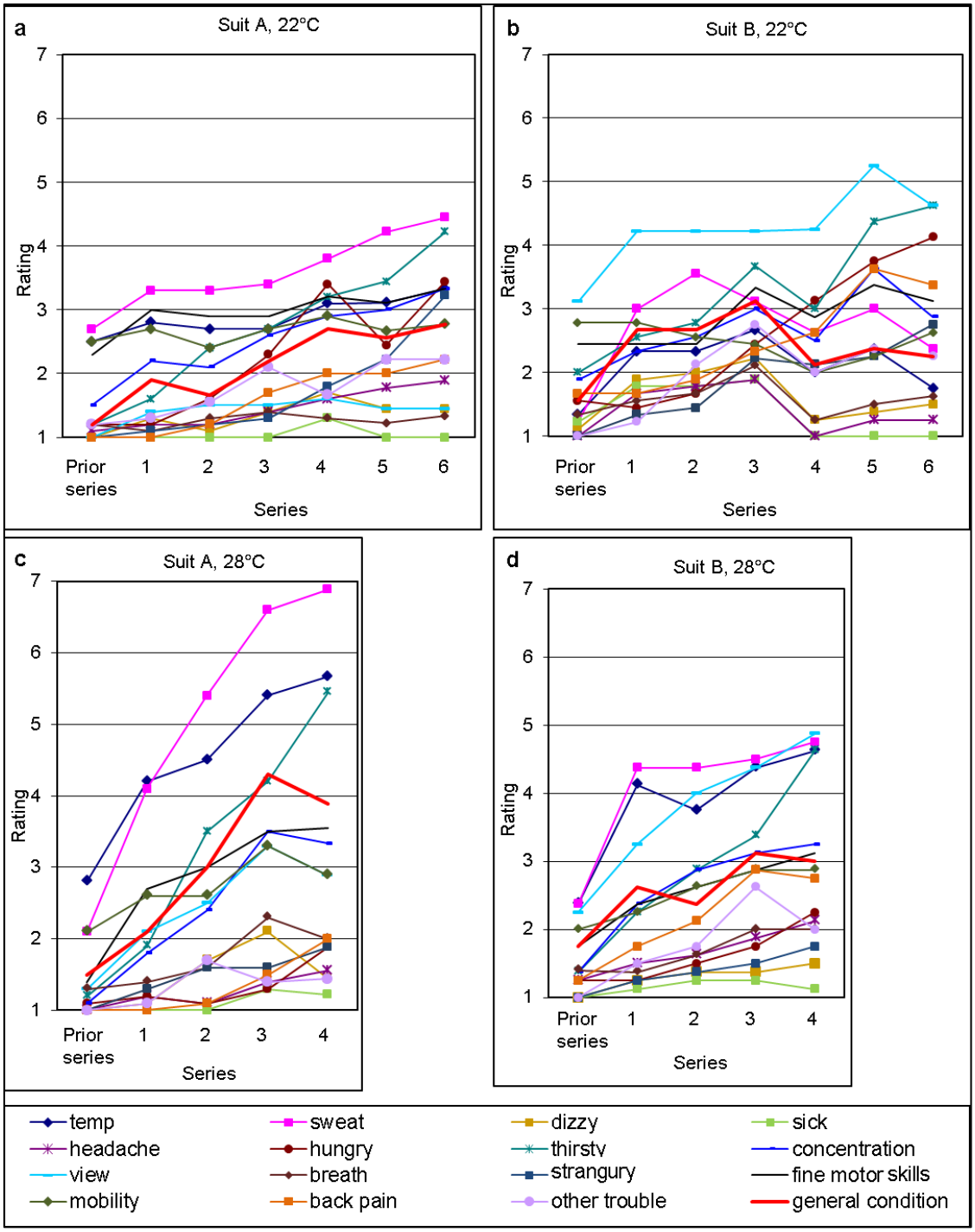


Figure 13: Results of the assessment of individual perception. All participants rated their individual perception, general condition and comfort in an assessment sheet comprising sixteen basic and PPE related concerns. y-axis: The rating was scaled from 1 (= low interference) to 10 (= high interference, which would lead to termination of the study). Mean values from all subjects per concern are indicated. x-axis: The assessment sheets were filled in before and after every tasks series, hence, 7 times at 22 °C and 5 times at 28 °C.

Discussion - Chapter 1

Hypothesis 1: PPE-induced restrictions lead to increased error rates when simulated laboratory work, concentration and reaction performance are evaluated in a study with volunteers.

Due to the specific requirements of an autopsy workflow that includes the direct contact of pathologists with high risk pathogenic samples e.g. excision and macroscopic inspection of organs and tissues, which cannot only be performed in class II lamina air flow, as well as the sample preparation performed by assistant personnel, the use of additional PPE is required. Working procedures may induce the spill of contaminated body fluids resulting in a need for mandatory whole body protection for all personnel working in the laboratory. These PPE types have to comprise an air-tight and chemically-resistant (for subsequent chemical decontamination) suit and boots, and two pairs of gloves (virus-tight and chemically-resistant) worn one above the other, and fixed to the suit with adhesive tape, PAPR (powered air purifying respirator), and optionally cut resistant gloves which depends on the planned lab work. The complete set of PPE protects from exposure to pathogens but constricts mobility, dexterity, view and causes heat stress. It can be assumed that these restraints could impact concentration and may increase error rates due to fatigue or heat stress. Combined evaluation of biophysical conditions and working performance of test persons has been published by a UK hospital group mimicking vascular access skills (Castle et al., 2009), a Swiss BSL-4 laboratory group with a physical workout (Kümin et al., 2011) and a US Army Research Institute (O'Brien et al., 2011) with partly different but also similar approaches.

Briefly, the vascular access skills evaluated in the UK study were endotracheal intubation, intra-osseous and intra-venous cannulation and laryngeal mask airway placement on special training devices. All skills were performed twice whilst PPE-suited and once unsuited. Participants were anaesthetists, paramedics, pre-hospital care doctors, resuscitation officers and emergency physicians (n = 64). The time to complete attempt 2 was shorter than in the first attempt in general. Unsuited conditions led to 100% completion of all attempts whereas 12% of intra-venous cannulation and 8% of intubation remained uncompleted when suited (Castle et al., 2009).

The Swiss study tested 3 different fully ventilated suits on their material robustness with 2.5% per-acetic acid which is used for the decontamination and locking out process. A program of physical activity was performed by volunteers to evaluate how the characteristics of the suit change during deflating and quick re-inflating during strenuous exercises and when the inhaling frequency is markedly increased. The participants feedback after a 3-hour test program revealed clear preferences for two suits and major disadvantages of one suit due to the high noise level inside the suit and diminished view due to the position of the suit zipper. This study underlines the importance of suit testing by future users not only by the user´s feedback but also on the basis of scientific data collected (Kümin et al., 2011).

The US Army Research Institute of Environmental Medicine recommends to setup PPE testing by starting with a biophysical assessment of the respective suit material by using hot plates followed by thermal manikins to evaluate thermal resistance and water vapour permeability. Then PPE was evaluated by test persons under different laboratory conditions covering heat and cold stress during rest and exercise periods and recording of body temperature, heart rate and sweating rate as well as the individually rated perception of the increase of skin wetness, skin temperature and garment comfort (O'Brien et al., 2011).

Here, it was accomplished to match different parameters and criteria as described in the different studies above such as mimicking working skills, physical activity, heat stress and the impact on physical parameters at once. The goal in the underlying study was to find out how a PPE combination with the lowest restrictions on the wearer´s performance can be selected and easily and widely implemented in health care. Not surprisingly, the measured physical parameters, for example the amount of fluid loss was dependent on the working temperature and on the suit type. The average fluid loss was higher in subjects wearing suit A (head ventilated) at 22 °C and 28 °C, but not statistically significant compared to suit B (total body ventilated) and when compared between both temperatures (Figure 8 a, Figure 9). An unexpected observation but in hindsight logical consequence was the measured but statistically not significant decrease of body temperature immediately after subjects finalised the 6 hour series at 22 °C who were wearing the total body ventilated suit B but not for those wearing the head ventilated suit A. Although measuring the tympanic temperature may include a factor of variability this decrease might nonetheless indicate a cooling effect due to the continuous ventilation within the whole suit for 6 hours at 22 °C. This cooling effect was not observed at 28 °C.

Here, the mean body temperature increased by the same value for both suits (Figure 8 b). In general, dangerous heat stress is defined as an increase over 39 °C (CDC, 2015), which was not measured in any of the subjects.

Another unexpected observation was the decrease of heart rates at 22 °C measured at the time points before the series started and after completion of all series, which, however, did not show statistical significance. It can be assumed that on the first test day the process of donning the equipment for the first time and facing the test setup may have led to excitement and increased heart rates, both of which decreased again after adaptation to the conditions and repetition of the test series. The increased heart rates at 28 °C measured for both suits also indicated increased heat stress which met the expectations (Figure 8 c). However, the individual perception of increase of body temperature as well as sweating and the measured fluid loss were perceived as moderate to high stress. But unexpectedly, these were not limiting factors even after the 4 hour working series at the high temperature of 28 °C.

Another surprising observation was made in the error analysis of task I (screwing coloured tubes and caps together and placing them into a colour patterned box). At both suits and working temperatures an increased number of correct and a constant number of wrong tubes was registered which consequently led to a better ratio of total to wrong tubes at all conditions. There were, however, differences between suit A and B with respect to the total number of correctly processed tubes which was higher at suit A (more than 30 at the last series compared to markedly less than 30 at suit B). This difference is most likely explained by the improved dexterity and better fitting gloves fixed at the mock-up glove box allocated to suit A than those of suit B as it was also registered in the assessment sheets by rating fine motor skills (Figure 13). The obvious training effect exceeds the effects of fatigue under these experimental conditions. Since the training effect was also registered at the second task series performed on another day at the higher temperature of 28 °C, however, in lower markedness, it can be concluded that training effects did not interfere with general results of the tasks performed but may rather serve as a relative indicator for stress and fatigue intensity.

Similar observations regarding general performance and training effects were made for the d2 Test of attention (task III). Here, the analysis of individual performances (Figure 11 a, b, e, f) demonstrated that some subjects could decrease their errors (E%, red bars) and raise their index of concentration performance (CP, green bars) with proceeding numbers

of task series. This can also be generalised when a trend line was created with the mean values of the respective characteristic numbers (Figure 11 i, j, k, l). Significant differences between performances of the d2 Test were observed neither for the two suit types nor for the two working temperatures as, for example, no fine motor skills as for task I were required. The heart rates also remained nearly unchanged, except outliers at 22 °C, suit B, series 2 and suit A at series 3 and 4.

Results of the reaction test (task IV) also showed that training effects were more pronounced than fatigue effects. This is indicated by the continuous decrease of reaction times with increasing number of task series (Figure 12 a). As this test required physical activity, the low reaction time is a sign of ambitious activity. This also corresponds with increased heart rates. These two parameters were further verified by higher temperature perception. The heart rates of subjects wearing suit B at 28 °C were, in general for this task, higher than for suit A (Figure 12 d). This could be interpreted as a result of increased physical activity indicated by the shorter reaction time as well as of increased stress during performing in a fully ventilated suit (suit B). Unexpectedly, mean values of reaction times (Figure 12 c) for both suits showed lower reaction times at 28 °C than 22 °C which also corresponds with the higher heart rates. Another interpretation for the decreasing reaction times and also for the enhancement of other tasks might be the individual challenge the subjects underwent, meaning to boost their own performance from series to series.

The assessment of discomfort and restrictions caused by the different suit types demonstrated that for subjects wearing suit B, restricted view caused by the flexible face shield was the most distracting parameter at 22 °C and at 28 °C together with increased sweat and temperature perception. Additionally, the associated respiration system that was worn as a rucksack led to striking back pain with long-lasting series. In contrast, subjects wearing suit A (head ventilation) reported that temperature and sweating were the most irksome factors which was expected for this suit type. Surprisingly, none of these parameters caused a decrease of performance or led to earlier termination of the study (Figure 13). It can be supposed that the cooling of the head only via the respiration system in suit A may induce a better heat tolerance. A study investigated the impact of face cooling during passive heat stress. Eleven subjects that wore a temperature controlled suit underwent measurements of cardiac parasympathetic activation, vascular resistance and blood pressure. Their intestinal temperature was increased by up to 1 °C and face cooling performed for 3 minutes with water bags with 0 °C. The generated data

indicated that passive heat stress diminished face-cooling-induced increases in cardiac parasympathetic activation, vascular resistance and blood pressure. The increase in mean arterial pressure during heat stress was reduced with face cooling compared to normothermia. It is supposed that face cooling may be used as a countermeasure for increased blood pressure during orthostasis and heat stress (Schlader et al., 2018). Although the cooling in our suit study was performed only by air ventilation around the head and not by a cold shock of 0 °C like in the described study the air ventilation may be regarded as a cooling factor when compared to the increased temperature in a gastight full body suit and the subjective feeling of a cool surrounding of the head may induce a better heat tolerance.

Although the subjects knew about the long-lasting working hours, repeated series and tasks and the possible boredom that might occur they were highly motivated, carried out the program, and motivated and pushed themselves to increase their performance, documented as increased reaction times and no increase of errors, by means of sportsmanship. These results can be underlined by a study with 10 participants wearing PPE that revealed that no increase of risk propensity by light exertion for 60 minutes under heat stress (32 °C) could be detected (Schlader et al., 2016).

The maximum possible working time was not evaluated in this study since typically four hours are the usual working time in BSL-4 suit laboratories as stated by participants of the ERINHA projects (European Research Infrastructure for Highly Pathogenic Agents, www.erinha.eu). These projects (FP7 - ERINHA and H2020 - ERINHA2) aimed at building a European research infrastructure to support the European coordination and capacities of high containment facilities and expert institutes for the study and the surveillance of highly pathogenic micro-organisms. The Institute of Pathology has participated in both projects. Here, this maximum working time was knowingly exceeded to challenge the subjects and provoke them to make mistakes and errors.

The use of PAPRs that provided constant ventilation and cooling of the head in suit A leads to the assumption that this effect might have increased the tolerability of heat stress which probably would not have been the case with simple FFP3 face masks or unpowered respirators. The comparison of types and effects of various PAPRs with other devices was not the focus of this study. PAPRs reduce the risk of unintentional contamination of the face which markedly increases safety when compared to the combination of goggles and face masks. Consequently, PAPRs are recommended by WHO, CDC US (Centre of

Disease Control and Prevention), and the Public Health Agency of Canada to protect from airborne infections, e.g. tuberculosis, and as also reported from the West African outbreak of Ebola virus disease and toxic aerosol generating procedures in general (MacIntyre et al., 2014). Interestingly, several reports are published with partly contradictory conclusions regarding restrictions caused by PAPRs. Studies published in the 1990s (Caretti, 1999, Caretti, 1997, Zimmerman et al., 1991) stated that no changes in cognitive performance could be determined when subject wore respirators. This contradicts a study from 2013 (AlGhamri et al., 2013) that asserted significant effects and errors when cognitive tasks were performed when full-face respirators were used.

Conclusions

The hypothesis that restrictions due to the use of PPE would induce increased error rates has to be rejected contrary to expectations because this study revealed that both suit types were well tolerated in general. Specific restrictions for every PPE type perceived by the majority of the subjects did not lead to an increase of errors during any of the tasks performed. To identify limiting parameters it was decided to evaluate the subjective perception of restrictions and discomfort and physical parameters in parallel. The tasks were chosen to simulate parts of sample processing, analysis and documentation combined with standardized concentration and reaction tests and maximum protection of HCWs. The generated data and error rates obtained from wearing PPE under prolonged and stressful working conditions can be used to define working procedures and biosafety measures. This study may be used to help HCWs to evolve trust and confidence in the PPE and raise awareness that working under special biosafety conditions can be well tolerated and also trained to develop safe working procedures. The learning curve observed here was also observed by the UK group testing PPE and this fact underlines the importance of training skills whilst wearing PPE (Castle et al., 2009). Furthermore, this study should also support the decision process and facilitate the selection of appropriate PPE in a hospital environment especially by involving the staff.

Outlook and connection to chapter 2

After selection of the ergonomically best fitting PPE, donning and doffing has to be trained by the respective persons that work together in health care facilities. Contamination of HCWs has been reported to frequently occur during removal of gloves or gowns due to inappropriate use (Tomas et al., 2015, Reidy et al., 2017). The procedures of

decontamination of PPE and locking out the staff from the BSL-3 area would be the next steps in the biosafety sample workflow process.

Procedures for preparation of samples obtained from an autopsy or shipped samples with this new facility as recipient as would be the case here, require further logistic and biosafety relevant techniques. In case an immediate evaluation of the specimen is required, frozen sections are prepared from not fixed native and highly infectious sample material and stained for microscopic inspection. Trained pathologists evaluate the samples and decide about the next steps (e.g. further analysis, activation of a contagion plan, shipment to reference laboratories).

Vessels with fixed and consequently inactivated specimens, with a minimum risk left, have to be decontaminated and locked out for transport to the respective departments for further evaluation. Vessels containing active pathogens that are required, for example, for further cultivation or evaluation in other specific BSL-3 or BSL-4 laboratories need to be threefold packed according to IATA (International Air Transport Association) and DGR (Dangerous Goods Regulations). The certified shipper must declare UN numbers (United Nations numbers) identifying the hazardous materials, the name of the dangerous product, weight, hazard class, packing list and personal contact information of the shipper and the recipient. Violations of the IATA Dangerous Goods Regulations have criminal consequences.

The expected rarely emerging cases in Europe, for example, imported VHF (Virus Haemorrhagic Fever) nonetheless demand safe sample handling procedures for HCWs. The ECDC (European Centre for Disease Prevention and Control) has reported 203 cases of West Nile Fever (RG-4) in Europe (4 cases in Austria) in 2015 (ECDC-Communicable-Disease-Threats-Report, 2017) and 2889 bank vole-transmitted Hantavirus infections (RG-3) (22 cases in Austria) (ECDC-Hantavirus-infection_Annual-epidemiological-report-for-2015, 2017).

As also reported by the ECDC, estimated 323,000 new TB (tuberculosis) cases and relapses in the WHO European Region and 60,195 cases of reported TB in 2015 in 30 European Union/European Economic Area (EU/EEA) countries show a declining trend on one hand but a remaining unchanged treatment success rate over the last 10 years on the other hand. Particular concerns exist about MDR TB (multidrug-resistant tuberculosis) and its control which will be a major challenge in the European region (ECDC/WHO-Europe/Tuberculosis-surveillance-and-monitoring-in-Europe, 2017). As reported by the

ECDC recently arrived migrants with MDR TB from Africa were identified in Switzerland in December 2016. An Early Warning and Response System (EWRS) of the European Commission to the other member states led to the realization that also cases in Germany, Austria, Finland, France and Sweden could be identified to be linked to the pathogen cluster identified in Switzerland by whole genome sequencing (total cases 28). Therefore, it is important to quickly investigate itineraries of patients, their contacts, risk factors, and to share this information within the EU (ECDC-Multidrug-resistant-tuberculosis-in-migrants, 2017).

To avoid unnecessary cutting and splitting of highly infective specimen for different analysis methods it is recommended to fix the samples with multi-purpose fixatives that provide good morphology, stability of nucleic acids as well as a good preservation of the pathogen. All those requirements need to be provided under the aspects of safe biohazard conditions. This leads to the aims of chapter 2.

Chapter 2 – Relevance of pathogen inactivation within a pre-clinical process.

Introduction

The ascertained good quality features of nucleic acids and proteins raised the question whether PAXgene fixation would also result in adequate inactivation of pathogens compared to formalin, or whether additional biosafety requirements would need to be established for HCWs handling infectious human samples. Only one study so far investigated virus inactivation properties of PAXgene compared to formalin, using solely immunocytochemistry assays. It was shown that PAXgene inactivates *influenza A virus*, a*denovirus* and *human cytomegalovirus (CMV)* at least as well as formalin (Kap et al., 2013). However, information on further tests on other microbiological species is lacking. Hence there is a major demand for additional analysis of the pathogen inactivating properties of PAXgene compared to formalin to ensure safe specimen handling.

Pathogen inactivation by tissue fixatives - bacteria, fungi and viruses

Biosafety guidelines for tissue preservatives regarding their pathogen inactivation properties are not available. One study published in 2005 comparing the pathogen inactivating effects on bacteria, fungi and viruses of UMFix, an alcohol based tissue fixative, and formalin revealed that spores of *Bacillus subtilis* could not be inactivated by either fixative within 20 minutes, and with UMFix not even after 24 hours (Cleary et al., 2005). Practical experience with formalin on biosafety risks exists for decades but documentation in literature is rare (Sagripanti et al., 1997). Therefore, guidelines used for accreditation of disinfectants released by the DGHM (German Society of Hygiene and Microbiology) had to be adapted to test PAXgene (Loibner et al., 2016). The selection of appropriate microorganisms was also conducted by using the DGHM guidelines as well as sterilization procedures for bone transplants (Pruss et al., 2001, Pruss et al., 1999) and CEN/CT 216 EN 14485 (Chemical disinfectants and antiseptics in human medicine).

Bacteria

Specific structures on the bacterial cell surface (capsules, O antigen, pili, lipoteichoic acids) act as adhesion molecules at the beginning of a bacterial infection. Different lytic and cytotoxic enzymes like proteinases, fibrolysine, nucleases, phosphatases and hyaluronidase secreted by bacteria are responsible for invasion and bacterial

dissemination in the infected individual. Most bacteria cause acute as well as chronic purulent inflammation with varying general symptoms (fever, tachycardia) up to sepsis (Boecker W., 2012).

Six bacterial strains were selected for inactivation experiments by considering the guidelines mentioned above but also covering all possible life conditions like spore forming or not, anaerobic or aerobic and gram negative and positive strains. All strains were obtained from ATCC (American Type Culture Collection) or DSMZ (German Collection of Microorganisms and Cell Cultures) (Table 6) (Loibner et al., 2016).

Bacterial strain	Abbreviation	Shape	Gram stain	Spore forming	Growth condition	ATCC / DSMZ
Clostridium sporogenes	*Cs*	rod	positive	yes	anaerobic	DSM 1446
Staphylococcus aureus	*Sa*	coccus	positive	no	aerobic	ATCC 29213
Bacillus subtilis	*Bs*	rod	positive	yes	aerobic	DSM 347
Pseudomonas aeruginosa	*Pa*	rod	negative	no	aerobic	ATCC 27853
Mycobacterium terrae	*Ms*	rod	positive	no	aerobic	DSM 43227
Mycobacterium smegmatis	*Mt*	rod	positive	no	aerobic	ATCC 356

Table 6: Bacteria selected for inactivation experiments. Overview of all bacterial strains used for inactivation experiments, their habitus and life conditions.

Clostridium sporogenes (Cs) was selected for experimental series as a surrogate representative for gram positive rods and its abilities for anaerobic growth and spore forming. The genus of *clostridium* comprises various species. The pathogenicity of *clostridium sp.* is due to their exotoxins which are highly toxic and immunogenic proteins secreted from living bacteria. Infections of wounds under anaerobic conditions by *C. perfringens* and *C. tetani* cause gas gangrene with quickly proceeding necrosis of the affected tissue and tetanus with endoneural spread leading to tonic spasms, respectively (Boecker W., 2012).

Staphylococcus aureus (Sa) is the most relevant agent causing purulent infections of the skin. From infected wounds dissemination is enabled to other organs and visceral cavities (pleura cavity, joints). Secondary infections of kidney, lung or central nervous system can occur by spreading via the vascular system up to development of sepsis. *Sa* is also capable of producing enterotoxins causing gastroenteritis due to food poisoning as well as the toxic shock syndrome (Boecker W., 2012).

Bacillus subtilis (Bs) was selected as a surrogate representative of the bacillus species to be one of the test organisms because of its ability to develop resistant spores and its low risk group affiliation, RG 1. Another representative of this genus is *bacillus anthracis*, a highly infectious RG-3 agent causing anthrax. The infection is determined by the entry into the body via skin, lung or intestine leading to hemorrhagic reactions and toxic shock (Boecker W., 2012). As our laboratory was not equipped to handle RG-3 pathogens it was decided to employ *Bs* as a representative of spore forming aerobically growing rods.

Pseudomonas aeruginosa (Ps) the only gram negative strain tested is the most common agent causing hospital-acquired infections such as pneumonia and wound infections in immune deficient patients (Boecker W., 2012).

Mycobacterium smegmatis (Ms) and M*ycobacterium terrae (Mt)* are the representatives of the genus *mycobacteria* used as surrogate strains for the experimental series. Both of them are agents of RG-1 but comprising the same properties regarding growth conditions and resistance against disinfectants as the RG-3 species *M. tuberculosis*. The cell wall of *mycobacteria* contains an extraordinarily high (60%) amount of lipids and waxes leading to a high resistance in the environment which allows transmission via dust and infects individuals via inhalation. Mycobacteria are obligatory intracellular pathogens inducing typical macrophage activity because of their ability to survive within the phagosomes of macrophages. Components of the cell wall determine the immunoreactivity, mostly a delayed hypersensitivity, of the infected individual. Tuberculosis is reported to be the most eminent infectious disease with an estimated 60 million cases, 3 million deaths and 20 million cases with open lung tuberculosis as highly infectious source (Boecker W., 2012).

Fungi

Fungi may become pathogen for humans when they undermine the immune system e.g. via mucoid capsules or affect immunocompromised persons (after organ transplantations,

AIDS, traumata). Medical relevant fungi can be divided into three major groups which are dermatophytes, yeasts and mould fungi. Dermatophytes are equipped with keratolytic enzymes that enable digestion of keratin containing cells and material (skin, nails, hair).

Twenty-two human pathogenic fungi strains containing 8 yeasts and 14 moulds were selected for inactivation experiments (Table 7). Strains were either obtained from ATCC, DSMZ or the Global Catalogue of Microorganisms (CBS, formerly: Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection; new name: Westerdijk Fungal Biodiversity Institute, Netherlands). Strains with WB numbers are patient´s isolates and archived in the Biobank at the Biobank of the Medical University Graz.

	Fungus / Group	Strain numbers
1	*Candida albicans* / Yeast	ATCC 90028, WB 005.09, WB 036.00
2	*Candida glabrata* /Yeast	DSMZ 11226, WB 015.09, B 011.02
3	*Candida parapsilosis* /Yeast	ATCC 22019, WB 005.01, WB 030.01
4	*Candida krusei /Yeast*	ATCC 6258, WB 022.03, WB 012.02
5	*Candida tropicalis* /Yeast	ATCC 90874, WB 004.04, 002.02
6	*Cryptococcus neoformans* /Yeast	ATCC 90112, WB 015.07, 011.05
7	*Geotrichum candidum* /Yeast	DSMZ 6401, WB 020.03, WB053.02
8	*Exophiala dermatitidis* /Black Yeast	CBS 207.35, WB 012.05, WB 028.11
9	*Aspergillus fumigatus* /Mould	ATCC 204305, WB 002.10, WB042.11
10	*Aspergillus flavus* /Mould	ATCC 204304, WB 038.11, WB011.03
11	*Aspergillus niger* /Mould	ATCC 16404, DSMZ 1988, WB032.08
12	*Aspergillus terreus* /Mould	DSMZ 826, WB 016.02
13	*Scedosporium apiospermum* /Mould	WB 002.12, WB 008.05, WB 017.08
14	*Fusarium solani* /Mould	WB 045.00, WB 030.11, WB 023.08
15	*Scopulariopsis brevicaulis* /Mould	WB 060.11, WB 007.05, WB 052.02
16	*Alternaria alternata* /Mould	CBS 109803, WB 004.06, WB 015.02
17	*Paecilomyces lilacinus* /Mould	WB 021.03, WB 034.00

18	*Penicillium chrysogenum* /Mould	WB 021.03, WB 034.00
19	*Rhizopus oryzae* /Mould	WB 012.06, WB 055.02, WB 027.08
20	*Rhizomucor pusillus* /Mould	WB 051.04
21	*Lichtheimia corymbifera* /Mould	WB 019.03, WB 037.11
22	*Cunninghamella bertholletiae* /Mould	WB 030.09, WB 014.03, WB 056.02

Table 7: Yeast and mould strains selected for inactivation experiments. Different fungi strains were tested for their viability after PAXgene and formalin treatment. Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Yeasts: The most common agent of (often endogenous) mycosis is *Candida albicans,* followed by *C. glabrata*, *parapsilosis, krusei* and *tropicalis* affecting mouth, skin, gastrointestinal organs, lung and other organs which may lead to systemic diseases like sepsis, endocarditis and meningitis. The entry of *Cryptococcus neoformans* is mostly via inhalation of pigeon excrement contaminated dust but stays without symptoms for immunocompetent persons. It may cause severe diseases of lung or central nervous system of immunocompromised patients. *Geotrichum candidum* is less frequently reported to cause invasive fungal disease (IFD) but may cause disease with high mortality (Duran Graeff et al., 2017).

Moulds: *Aspergillus fumigatus, flavus, niger* and *terreus* are the major agents causing IFD (Duran Graeff et al., 2017). *Scedosporium apiospermum,* a saprophytic mould, compromises patients with underlying diseases e.g. chronic lung disease, cystic fibrosis. It causes invasive fungal diseases in immunocompromised hosts like soft tissue infections, septic arthritis, osteomyelitis, ophthalmic infections, sinusitis, pneumonia, meningitis and brain abscesses, endocarditis, and disseminated infection (Goldman et al., 2016, Cooley et al., 2007). *Fusarium solani* is the second most filamentous fungus causing opportunistic infections (Chiewchanvit et al., 2017). *Scopulariopsis brevicaulis* is a commonly found dermatophyt usually not associated with invasive diseases. However, fatal diseases were reported from a high-dose chemotherapeutically treated large B cell lymphoma patient and other cases (Iwen et al., 2012). *Alternaria alternata* is known as an aeroallergen as it is one of the most common airborne moulds (Hedayati et al., 2009) but is also of invasive potential as reported from bone marrow transplanted patients (Ferreira Ide et al., 2013). *Paecilomyces lilacinus* rarely afflicts humans, causing ocular, sinonasal and cutaneous

infections (Ciecko and Scher, 2010). *Penicillium chrysogenum* is a ubiquitous organism and rare human pathogen but involved in infections of immunocompetent and immunosuppressed persons (Geltner et al., 2013). *Rhizopus, Rhizomucor* and *Lichtheimia* are the most common isolated genera causing mucormycosis, which is an uncommon opportunistic but rapidly progressive lethal infection (e.g. rhinocerebral, pulmonary, gastrointestinal and skin type) (Rodriguez-Lobato et al., 2017). *Cunninghamella bertholletiae,* together with *Rhizopus,* and *Lichtheimia,* were reported to be the most frequently isolated mucormycosis-causing strains in a Spanish hospital with increasing incidence (Guinea et al., 2017).

Viruses

Immunocytochemistry assays with *Influenza A virus*, *Adenovirus* and *CMV* showed that PAXgene inactivates these viruses as effectively as formalin (Kap et al., 2013, Loibner et al., 2016). *Influenza A* and *Adenovirus* are non-enveloped viruses. The virus capsid that contains the nucleic acids is a protein sheath that is more resistant to chemical and thermal attacks than enveloped viruses like *CMV* which is coated with a lipid envelope that is more sensitive to degradation especially by detergents. The species *human cytomegalovirus (HCMV)* or *human herpesvirus 5 (HHV-5)* is a member of the *herpesviridae* family and the subfamily of *β-herpesvirinae*. Its genome, like for all other *herpesviridae*, is linear double-stranded DNA (~ 235kbp) packed into an icosahedral capsid, surrounded by the tegument, a protein layer which again is enclosed by an envelope, a lipid bilayer containing glycoprotein (Shenk T., 2008). *CMV* was chosen to be investigated in more detail for several reasons. First, it is highly sero-prevalent, gradually increasing with age from 36.3% in 6 - 11 year olds to 90.8% in over 80 year olds which was evaluated in the Third National Health and Nutrition Examination Survey from 1988 to 1994 in the United States (Staras et al., 2006). Second, *CMV* causes a widespread and persistent infection not leading to a clinical disease in immunocompetent persons. In immunosuppressed patients like transplant recipients and HIV-positive persons *CMV* can cause severe illnesses such as colitis, encephalitis and retinitis (Poncet et al., 2006). Third, infections during pregnancy can lead to neurological damage of premature newborns (deafness, learning disabilities). During an active infection *CMV* can be detected in various tissues (Sinclair and Sissons, 2006).

Material and Methods - Chapter 2

General information

All inactivation experiments with PAXgene were also performed with formalin and mock-treated samples with PBS (phosphate buffered saline). The latter served as reference to calculate the growth reduction and as a pathogen negative control. Measuring the amount of cfu/mL (colony forming units per milliliter) was used to assess the inactivation properties of PAXgene and formalin for bacteria and fungi. The goal was to reduce the amount of bacteria for at least 10^5 or of fungi for at least 10^4 compared to not inactivated samples as recommended by the DGHM guidelines. The inactivation of *CMV* was evaluated by RTq-PCR and immunocytochemistry.

Inactivation of bacteria

The cultivation of aerobic *Sa, Bs* and *Pa* bacterial strains was performed as recently described (Loibner et al., 2016). Starting with an ONC (overnight culture) a single colony was inoculated into the appropriate liquid medium (Table 8) and incubated under shaking at 200 rpm (rounds per minute) overnight at 37 °C. The ONCs for *Cs* were hermetically sealed for anaerobic conditions and incubated at 37 °C without shaking. On the next day 1 mL of each bacterial suspension was filled into 4 tubes per strain, centrifuged for 20 minutes at 1,200 x g and the supernatant was discarded. To start the cultivation of mycobacteria the cells were washed with PBS from a confluent cell layer of an agar plate. Because of extensive clotting of *Mt* these cells were dissociated with the GentleMACS Dissociator (Miltenyi, Bergisch-Gladbach, Germany). Mycobacteria suspensions were also filled into 4 tubes (1 mL each) and centrifuged at 10,000 x g for 5 minutes. Each resulting bacterial cell pellet was re-suspended in 1 mL of the respective inactivation solution, either PAXgene Fix, formalin or PBS (Figure 14).

Bacterial strain	Liquid media (bioMerieux, France)	Solid media
Clostridium sporogenes (Cs)	Bouillon Schaedler + vitamin K3	Schaedler Agar + 5% sheep blood (bioMerieux, France)
Staphylococcus aureus (Sa)	Trypcase Soy broth	Columbia ANC Agar + 5% sheep blood (bioMerieux, France)
Bacillus subtilis (Bs)	Trypcase Soy broth	Chocolate Agar + PolyViteX (bioMerieux, France)
Pseudomonas aeruginosa (Pa)	Trypcase Soy broth	Mc Conkey Agar (bioMerieux, France)
Mycobacterium smegmatis (Ms)		CSA_Casein Soya bean digest agar; Blood agar + 5 - 10% human blood (Oxoid, England)
Mycobacterium terrae (Mt)		Middlebrook 7H10 Agar (Becton Dickenson, Germany)

Table 8: Overview of bacterial strains and specific media used for inactivation experiments. Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Because the PAXgene treatment comprises two steps (i.e. PAXgene Fix followed by PAXgene Stab), both steps were tested separately and in combination. After 30 minutes incubation with either 1 mL PAXgene Fix, formalin or PBS at room temperature (RT) cells were centrifuged to pellets. One of the two PAXgene-fixed samples was re-suspended in PBS. The second PAXgene-fixed sample was incubated with 1 mL PAXgene Stab for another 30 min, centrifuged again and re-suspended in PBS. Dilution series of 1:10 were prepared and 100 µL were plated on the adequate agar medium (Table 8). *Bs, Sa* and *Pa* were cultivated under aerobic conditions at 37 °C for 24 - 48 hours. *Cs* was cultivated anaerobically using the GENbag anaer system (bioMérieux) at 37 °C for 24 - 48 hours. *Ms* and *Mt* had to be incubated at 37 °C for four and fifteen days, respectively, which is typical for mycobacteria. Six independent series of assays were performed with *Ba*, *Sa, Ms* and *Mt*, seven with *Cs* and four with *Pa*.

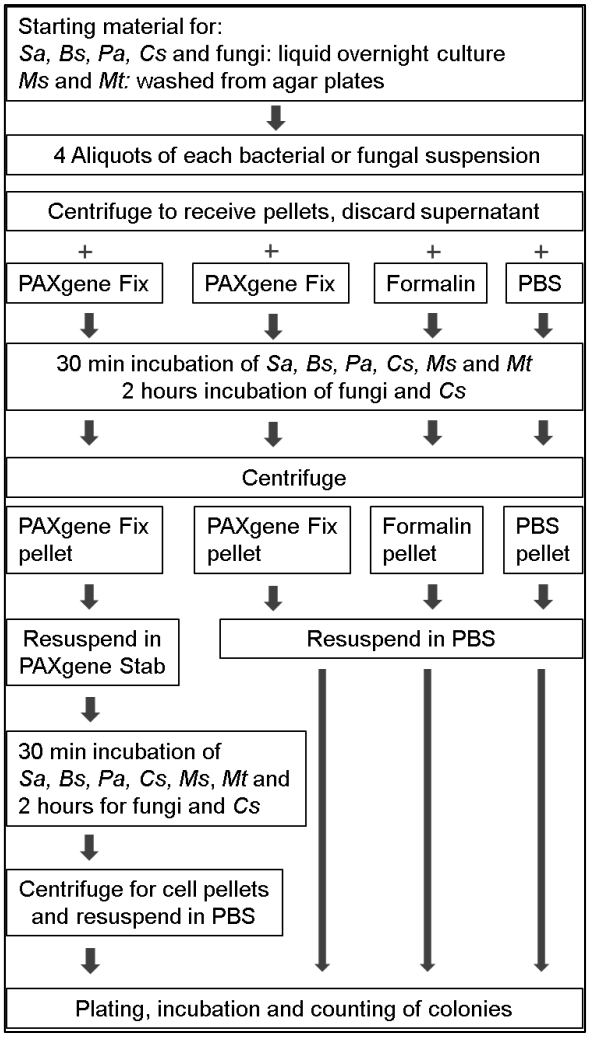


Figure 14: Workflow of bacterial and fungal inactivation experiments. Experimental series with bacteria were performed in four independent experiments with *Pa* because no colony was detected after any inactivation series. Six experiments were performed with both mycobacteria strains and with *Bs* and *Sa* because some colonies were detected. Seven series with an incubation time of 30 minutes were done with *Cs* because this strain has been identified as the most variable. For this reason three additional two hour treatment series were performed as well. Two hour treatments in general were undertaken with fungi for two experiments with all strains listed in Table 7. Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Inactivation of fungi

Twenty-two human pathogenic fungi strains containing 8 yeasts and 14 moulds were used for inactivation experiments (Table 7).

Cultivation and inactivation experiments were performed as recently described (Loibner et al., 2016) according to the DGHM guidelines for disinfectants with modifications as follows. Four samples of yeast cells or mould spore suspensions were prepared by scraping off the cells from a solid agar plate with sterile cell scraper. The suspension was adjusted to a McFarland turbidity equivalent of 4 and centrifuged to receive cell pellets as described for bacteria (Figure 14). The cell pellets were incubated for 2 hours with either 1 mL of PAXgene, formalin or PBS as reference. Two hour incubations were performed due to the known higher resistance of spores. One hundred microliters of each of the fixed samples and 1:10 dilution series of PBS control samples up to 10^{-12} were plated onto Sabouraud agar plates (Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 hours. For each fungus strain two independent series of experiments were performed.

Inactivation of *cytomegalovirus* (*CMV*)

MRC-5 cells (Medical Research Council cell strain 5, which is a human lung fibroblast cell line, LGC Promochem, ATCC #CCL-171) were selected to be the host cells for *CMV* propagation. They grow as a monolayer and were cultivated as recently described (Loibner et al., 2016) in 182.5 cm^2 cell culture flasks (VWR) with MEM (Minimum Essential Medium) supplemented with GlutaMax (Gibco), 10% fetal calf serum (Gibco) and 1% Pen-strep (Gibco) at 37 °C and 5% CO_2 in a cell culture incubator (Binder) until they were confluent to 60 - 70%. Cells were inspected with a light optical microscope (Nikon Eclipse E 600 with 10 x, 20 x, 40 x, 60 x objectives). Infection was induced with 2 mL of *human cytomegalovirus* AD 169 (HPA #622, former Health Protection Agency, actually Public Health England, UK) suspension containing 900 plaque forming units/mL. A virus negative control (equivalent to MRC-5 growth control) was incubated with culture medium without virus inoculum. Cells were continuously cultured and passaged as required for 12 and 19 days respectively after infection until massive cytopathic effects (CPEs) were observed (Figure 15). The second sample set harvested on day 19 was to help to verify the virus propagation in the PBS mock-treated samples. MRC-5 cells were harvested on day 12 and 19, respectively, by aspiration of the growth medium and washing 3 x with PBS. Application of 0.05% trypsin-EDTA (Gibco) solution for 2 minutes at 37 °C dissociated the cells from the culture dish. The EDTA activity was stopped by adding culture medium. The cells were aspirated with a pipette and transferred into a centrifugation tube. See Figure 16 for the graphical description of the sample workflow. Cell pellets were obtained by gentle centrifugation (500 x g for 5 min), the supernatant was discarded and resulting cell

pellets were washed with PBS. Pellets were gently re-suspended and distributed in equal amounts to 6 reaction tubes (1.5 mL). Two tubes were incubated either with PBS (*CMV* positive control), PAXgene Fix or formalin for one hour. After removing PAXgene Fix by centrifugation, cells were stabilized for one hour with PAXgene Stabilizer (PAXgene Stab). The reaction tubes were centrifuged and cell pellets were washed with PBS. One set of differently fixed cells was injected into 500 µL liquid 5% low melt agarose (Carl Roth) prefilled in a 1.5 mL reaction tube and immediately cooled on ice. Resulting agarose plugs were placed in tissue cassettes (VWR) and embedded into paraffin in an automated tissue processor (Tissue Tek VIP, Miles Scientific, Sanova). The paraffin embedding process for tissue and subsequent staining for histopathological diagnosis as performed in a routine pathology laboratory was experimentally done for the agarose plugs comprising the following conditions: 4 increasing ethanol steps (70% - 99%) within four hours, 2 hours isopropanol, 2 hours xylene and 3 hours molten paraffin at 55 °C.

The second set of samples was not processed and paraffin-embedded. All cell samples (paraffin-embedded and not paraffin-embedded) were dissociated in 5 mL MEM using a GentleMacs Dissociator (Miltenyi) to release virus particles from the cytoplasm. Floating paraffin was removed, cell lysates from each fixed and control sample were applied to separate new MRC-5 monolayers pre-cultured in 75 cm^2 cell culture flasks and cultivation was performed until CPEs appeared in PBS-mock-treated cells (virus positive control). Cells from all samples were harvested on day 12 and 19 after infection.

For evaluation of virus inactivation in fixed samples and simultaneous verification of virus viability in the PBS-mock-treated samples two different assays, RT-qPCR and immunocytochemistry were performed.

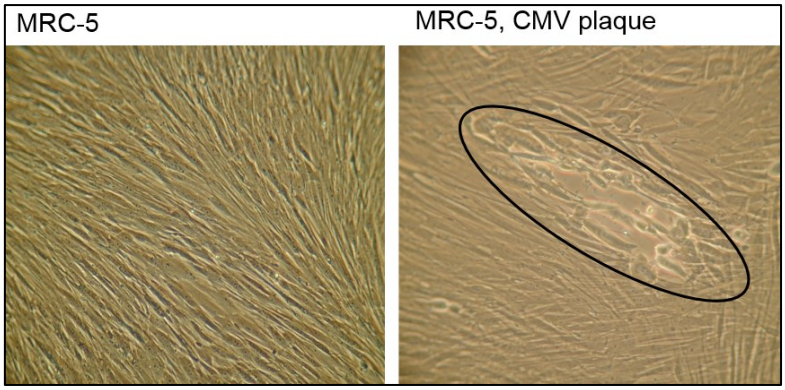


Figure 15: MRC-5 cell layers. Pictures of a dense MRC-5 cell layer (left) ready for passage and a *CMV* plaque in a MRC-5 cell layer (right).

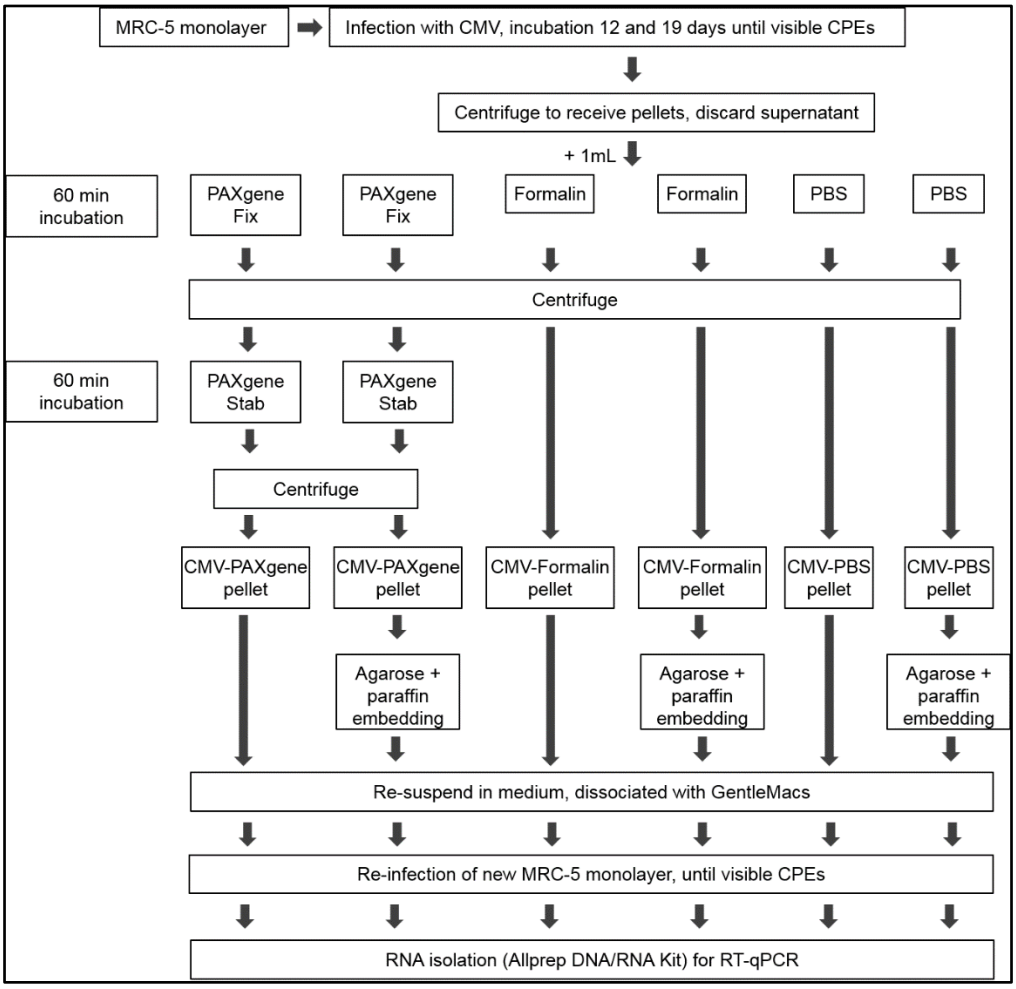


Figure 16: Workflow of *CMV* inactivation for detection after re-infection with RT-qPCR. MRC-5 cells were infected with *CMV,* cell pellets prepared and fixed with PAXgene or formalin, PBS was used as control. After embedding in agarose, fixed and control samples were again used to infect new MCR-5 cells. RNA was isolated from every sample for RT-qPCR.

CMV detection by RTq-PCR after re-infection

To investigate the viability of *CMV* on the basis of viral transcripts, RNA was isolated from all inactivated and the PBS control samples (see Figure 16 for the workflow) using AllPrep DNA/RNA/Protein Mini Kit (Qiagen) as recently described (Loibner et al., 2016). RNA quantification was performed on a NanoDrop 100 Spectrophotometer (PeqLab). Reverse transcription to obtain cDNA and DNAse-I-digestion to remove any DNAse from the

sample were performed using QuantiTect Reverse Transcription Kit (Qiagen). Primers for immediate early *CMV* gene *TRS1* (terminal right short 1) (NCBI Reference Sequence: NC_006273.2) were designed and blasted using the NCBI primer design tool (www.ncbi.nlm.nih.gov/tools/primer-blast).

Forward primer: ACACAGATGGAACAAAAGCAGA.
Reverse primer: ACGCTGTGGTTTGGAGATTGA.
Amplicon lenght 170 bp (Eurofins MWG Operon).

RT-qPCR was performed on the Applied Biosystems 7900HT Fast Real Time PCR machine (Applied Biosystems) using a TaqMan-specific set of PCR reagents following the manufacturer's instructions. Human *Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)* was used as a reference gene. The PCR assay was performed in triplicates.

Forward primer: CCACATCGCTCAGACACCAT.
Reverse primer: GTAAACCATGTAGTTGAGGTC.
Amplicon length 153 bp (Eurofins).

CMV detection using immunocytochemistry after re-infection

MRC-5 cells were cultivated in 9 cell culture flasks as recently described (Kap et al., 2013). Cells in 6 flasks were infected with *CMV* until CPEs appeared as described above and 3 flasks were used as controls. Cells were harvested, and infected and not infected cells were pooled. Each sample was washed in PBS and counted with a haemocytometer counting chamber (0.2 mm depth, 0.0625 mm^2, Fuchs-Rosenthal). Series of aliquots of 10^5 cells/mL were prepared and centrifuged at 200 x g for 5 minutes to remove excess medium. Cell pellets were incubated with 10 mL either of PAXgene Fix, formalin or PBS for 15 min, 1, 3, 6 and 24 hours and centrifuged again (200 x g for 5 min) to remove fixatives and PBS. After re-suspension of the cell pellets with 3 mL medium the cells were dissociated with the GentleMacs Dissociator (Miltenyi) and centrifuged at 500 x g for 5 minutes to remove cell debris. Each of the resulting supernatants from the fixed and the control samples (1.5 mL) was used to infect new MRC-5 monolayers cultivated on square cover slips (22 mm x 22 mm) in 6-well cell culture plates until 50% CPEs were observed in the PBS control cells. See Figure 17 for description of the workflow.

For immunocytochemistry the medium was removed and the cover slips placed into a 35 mm petri dish. Cells on the cover slips were fixed with methanol/acetic acid (3:1) for 10 minutes at - 20 °C. After fixation the cells were washed 3 x with PBS, blocked with 8 ml methanol + 300 mL H_2O_2 (30%) for 10 minutes, washed again 3 x with PBS and incubated with 1:50 diluted monoclonal mouse anti-CMV antibody (M085401, former Dako, now Agilent) for 60 min. The first antibody was removed, cells were washed 3 times with PBS and incubated with the second anti-mouse antibody (EnVision Ready-to-Use, Dako) for 30 minutes and washed again. Staining was performed with the AEC Substrate System (Ready-To-Use, Dako) containing 3-amino-9-ethylcarbazole that forms a red coloured product with the *CMV* target antigen. This reaction was stopped with water and counterstaining was performed with hematoxilin for 1 minute. Aquatex (Merck) was used to cover the slides for microscopy (Kap et al., 2013).

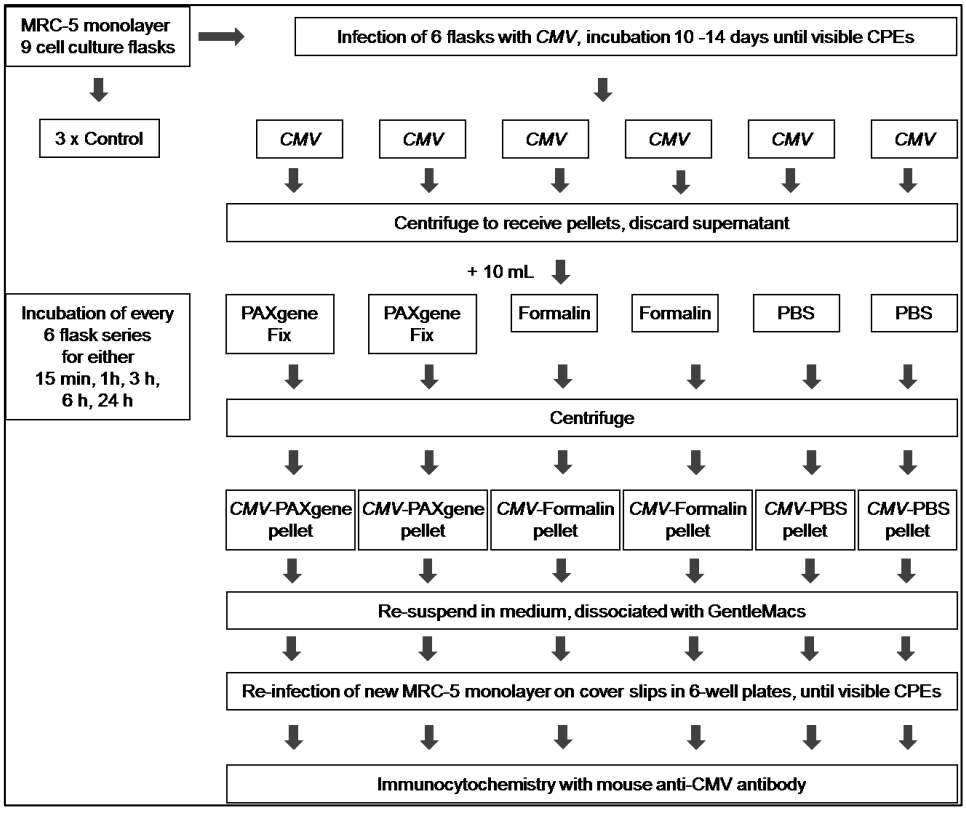


Figure 17: Workflow of *CMV* inactivation for detection with immunocytochemistry. Preparation of the cell culture workflow and *CMV* inactivation experiments with PAXgene and formalin for the subsequent detection of *CMV* with immunocytochemical staining.

Results - Chapter 2

Results, bacteria inactivation

Six bacterial strains (Table 6) were cultivated and their cells pellets were incubated either with PAXgene (PAXgene Fix as well as Fix and Stab as separate experimental series), formalin or PBS that served as a growth control. The cell suspensions were diluted and 100 µL of each of the dilution series were plated on agar plates. After incubation the colonies were counted and the cfu/mL calculated. According to the DGHM guidelines for disinfectants a reduction of more than 10^5 cfu/mL was considered as sufficiently inactivated. To obtain comparable results within the experimental series of each bacterial strain the counted and calculated cfu/mL were normalized to 10^6 (Loibner et al., 2016).

Sa was treated in six series (Figure 18 a) resulting in a reduction of more than 10^5 in all six series with PAXgene Fix and Stab as well as with formalin. PAXgene Fix alone led to a reduction of 10^3 in two and more than 10^5 in the other four series.

Bs was inactivated by formalin in all six series and by PAXgene Fix and PAXgene Fix and Stab in four series (Figure 18 b).

Cs was inactivated by formalin in six out of seven series. In one series cell growth was detected close to the threshold and in another one reduced cell growth was detected but with a sufficient reduction of 10^6. PAXgene Fix inactivated *Cs* in two out of seven series. Cell growth above the threshold was detected in the other five series. PAXgene Fix and Stab inactivated *Cs* in three series and cell growth was not sufficiently reduced in the other four series (Figure 18 c). Due to the various results within the seven *Cs* experiments with incubation periods of 30 minutes additional series with three experiments and 2 hour incubation times were performed (Figure 18 d). These also resulted in variable and inefficient reduction for all PAXgene experiments but also in one formalin experiment.

Pa (Figure 18 e) and *Ms* (Figure 18 f) were sufficiently inactivated by PAXgene and formalin in all experiments performed.

Mt was sufficiently inactivated by PAXgene Fix and Stab as well as by formalin. Cell growth below the reduction threshold was detected in two out of six experiments at both fixatives. PAXgene Fix alone did not sufficiently inactivated *Mt* in one experiment and in another one cell growth was detected closely below the threshold (Figure 18 g).

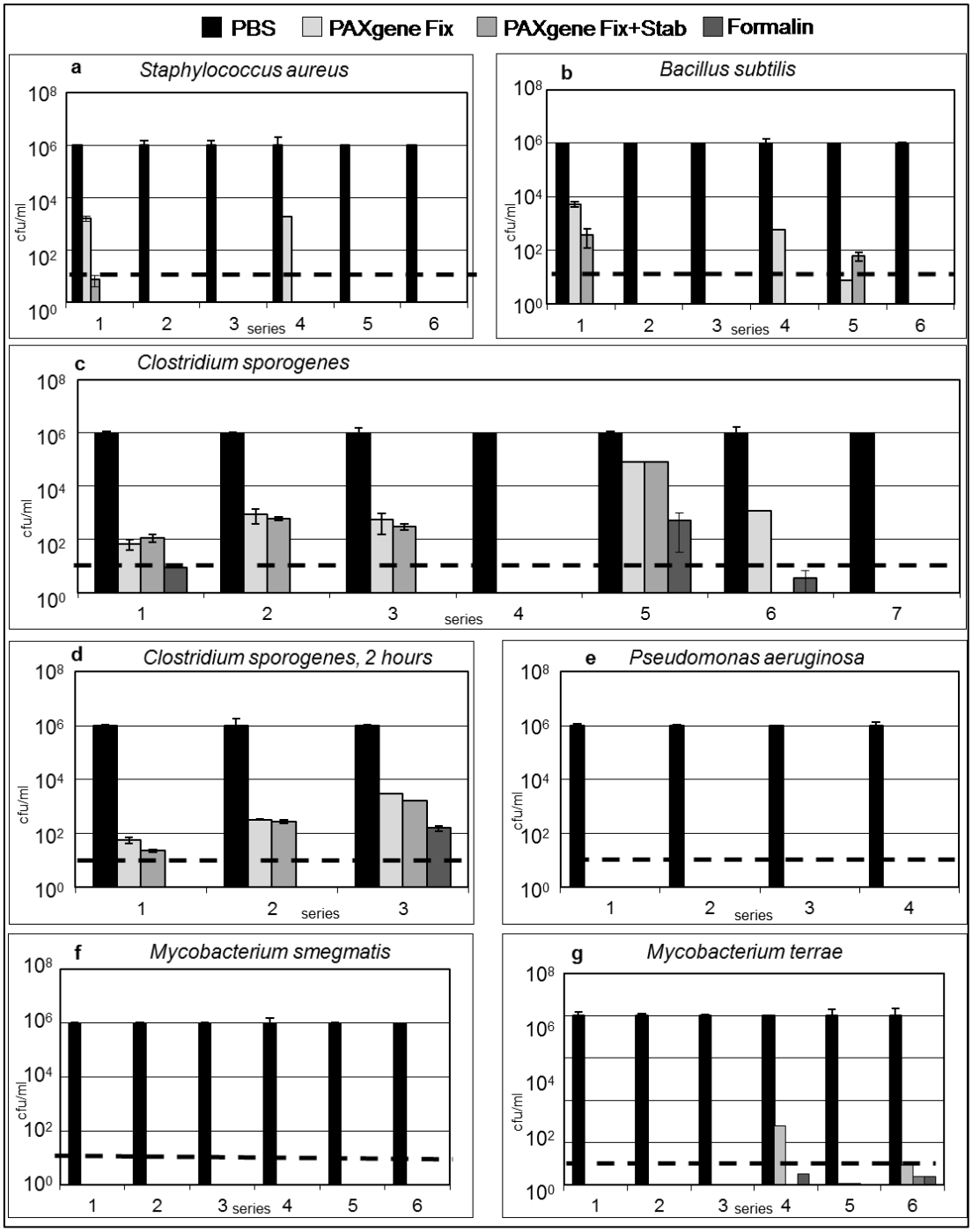


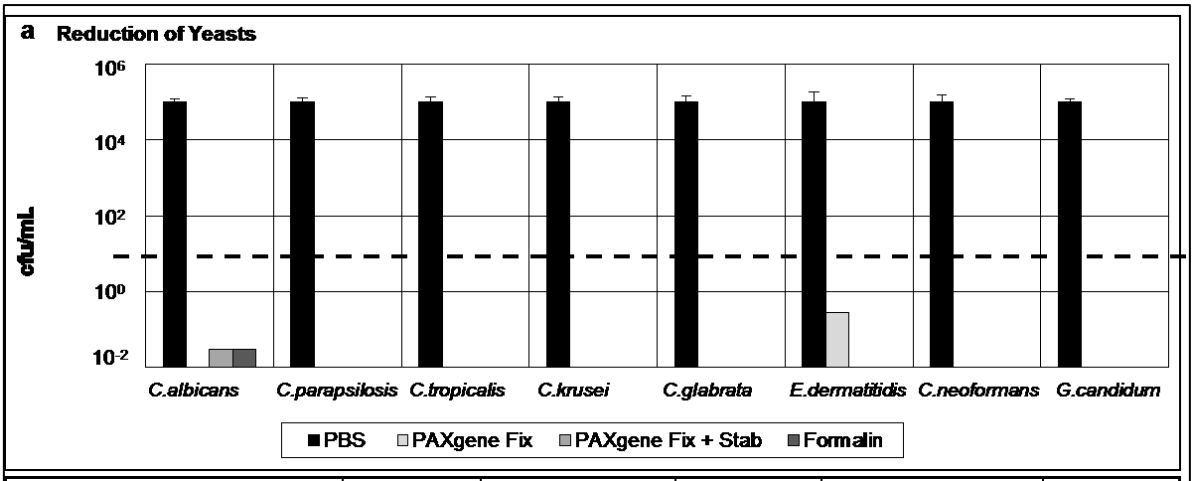
Figure 18: Results of experiments to inactivate bacteria with PAXgene and formalin, with PBS as growth reference. Different bacterial strains show a variable reduction of viability after separate treatment with PAXgene Fix, PAXgene Stab, formalin, and PBS as calculation reference and viability control. The x-axis indicates the inactivation series, the y-axis the cfu/ml. Bacteria were incubated for 30 minutes in series a, b, c, e, f and g. An additional treatment was performed with *Sa* for 2 hours in series d. To obtain comparable results within the experimental series of each bacterial strain the counted and calculated cfu/mL were normalized to 10^6. The columns represent the mean values and error bars

which were calculated from the counted cfu of a series containing different dilution series for each experiment. The dashed lines indicate the threshold of the reduction limit of 10^5. Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Results, fungi inactivation

The spectrum of human pathogenic microorganisms was expanded from bacteria to various species of fungi (Table 7). All fungi inactivation series started with the determination of cell concentration by adjusting the turbidity equivalent of a McFarland 4 standard. The PBS mock-treated assays were diluted by 1:10 series until single colonies were countable to calculate the reduction. The PAXgene and formalin treated assays were not diluted. Hundred µl were plated on each agar plate. The requested reduction for fungi according to DGHM guidelines and European standards (EN 1275) is more than 10^4 cfu/mL which was reached by PAXgene Fix, PAXgene Fix and Stab as well as for formalin in all experimental series performed. Some single *Candida albicans* colonies were detected after treatment with PAXgene Fix and Stab and formalin but the required reduction was fulfilled in all series. *Exophiala dermatitidis* developed some colonies only after PAXgene Fix inactivation but the reduction was sufficient as well. The two other yeast species *Cryptococcus neoformans* and *Geotrichum candidum* were inactivated as required. Each column represents a repeated series of all three strains per species as stated in Table 7. The calculated cfu/mL of the growth controls were normalized to 10^5 to receive comparable results (Figure 19 a).

The PBS mock-treated samples of moulds for growth control had to be diluted up to 10^{-12} to receive a countable amount of colonies. Single colonies, far below the reduction threshold were detected in some strains as follows: After PAXgene Fix treatment all four strains of *Aspergillus* and *Penicillium chrysogenum* yielded 0.8 to 8 cfu/mL. PAXgene Fix and Stab treatment yielded 0.1 to 0.3 cfu/mL for *Aspergillus niger*, *Penicillium chrysogenum* and *Rhizopus oryzae.* In two *Aspergillus* strains, *Penicillium chrysogenum* and *Cunninghamella bertholletiae,* 0.1 cfu/mL to 6.8 cfu/mL were detected after formalin treatment (Loibner et al., 2016).



b Reduction of Mould Fungi	Strains + experiments n =	Mean dilution of PBS growth controls	cfu/ml after PAXgene Fix	cfu/ml after PAXgene Fix + Stabilizer	cfu/ml after formalin fixation
Aspergillus flavus	6	10^{-12}	**0.8**	0.0	**0.1**
Aspergillus fumigatus	6	10^{-12}	**8.0**	0.0	**4.0**
Aspergillus niger	3	10^{-12}	**2.0**	**0.3**	0.0
Aspergillus terreus	6	10^{-12}	**8.0**	0.0	0.0
Penicillium chrysogenum	6	10^{-12}	**3.1**	**0.3**	**6.8**
Absidia corymbifera	6	10^{-12}	0.0	0.0	0.0
Rhizopus oryzae	6	10^{-12}	0.0	**0.1**	0.0
Cunninghamella bertholletiae	6	10^{-12}	0.0	0.0	**3.0**
Rhizomucor pusillus	2	10^{-12}	0.0	0.0	0.0
Alternaria alternata	2	10^{-12}	0.0	0.0	0.0
Scedosporium apiospermum	6	10^{-12}	0.0	0.0	0.0
Fusarium solani	6	10^{-12}	0.0	0.0	0.0
Paecilomyces lilacinus	4	10^{-12}	0.0	0.0	0.0
Scopulariopsis brevicaulis	6	10^{-12}	0.0	0.0	0.0

Figure 19: Results of inactivation experiments with human relevant fungi. Inactivation was performed by 2 hours incubation with PAXgene Fix, PAXgene Fix and Stab and formalin. PBS was used as a mock treatment to obtain growth standard cfu/ml for calculation of the reduction rate. At least two assays per species comprising 1 - 3 strains (see Table 7) were performed. a: For the calculation of the reduction rate of yeasts the cfu/mL were normalized to 10^5. The dashed line indicates the threshold for the minimum reduction of 10^4 for fungi. b: The reduction of moulds is shown in a table. Bold printed numbers indicate the detected cfu/mL which is in any case far below the threshold of a 10^4 reduction due to the high dilution of the PBS growth controls. Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Results, *CMV* detection with RTq-PCR

As described in Figure 16 *CMV* was cultivated for 12 and 19 days, respectively, before the inactivation with PAXgene and formalin was performed. It was decided to extend the *CMV*

cultivation to 19 days to obtain the evidence that the detected virus is active. The relative expression of *TRS1* as the *CMV* specific gene was calculated against *GAPDH* as a reference gene. The increase in the PBS-mock-treated sample as positive control was nearly 100-fold. This indicates that *CMV* was actively producing RNA transcripts. No gene expression was detected in all paraffin-embedded and inactivated samples (Figure 20).

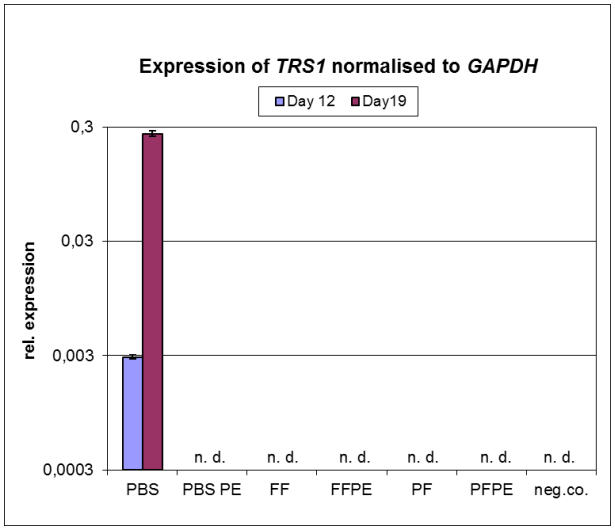


Figure 20: Results of *CMV* inactivation and detection with RTq-PCR. The PBS-mock-treated samples revealed active *CMV* virus content as shown by the increase of relative expression from day 12 to day 19 after infection. No *CMV* transcripts were detected either after paraffin embedding (PE) and inactivation with formalin and PAXgene (n.d.: not detectable).

Results, *CMV* detection with immunocytochemistry

CMV-infected MRC-5 cells were incubated with formalin and PAXgene, respectively (see Figure 17 for the workflow) for different time periods, i.e. 15 minutes, 1, 3, 6 and 24 hours. Not treated *CMV*-infected and not infected MRC-5 cultures served as *CMV*-positive and *CMV*-negative controls. After re-infection of new MCR-5 cell cultures with the respectively treated samples the immunocytochemical reaction revealed a complete inactivation of *CMV* already after 15 minutes incubation with formalin and PAXgene. This is apparent by the lack of *CMV*-positive cells on the new MCR-5 cell culture. The *CMV*-positive control (Figure 21 upper lane left) shows clear and distinct red stained *CMV*-positive MRC-5 cells as expected. No red stained cells are visible in the *CMV*-negative controls (Figure 21

upper lane right) (Kap et al., 2013). A reduced amount of re-infected MCR-5 cells was observed at samples from 1 hour formalin treatment onwards. This was not obvious at the PAXgene-treated assays.

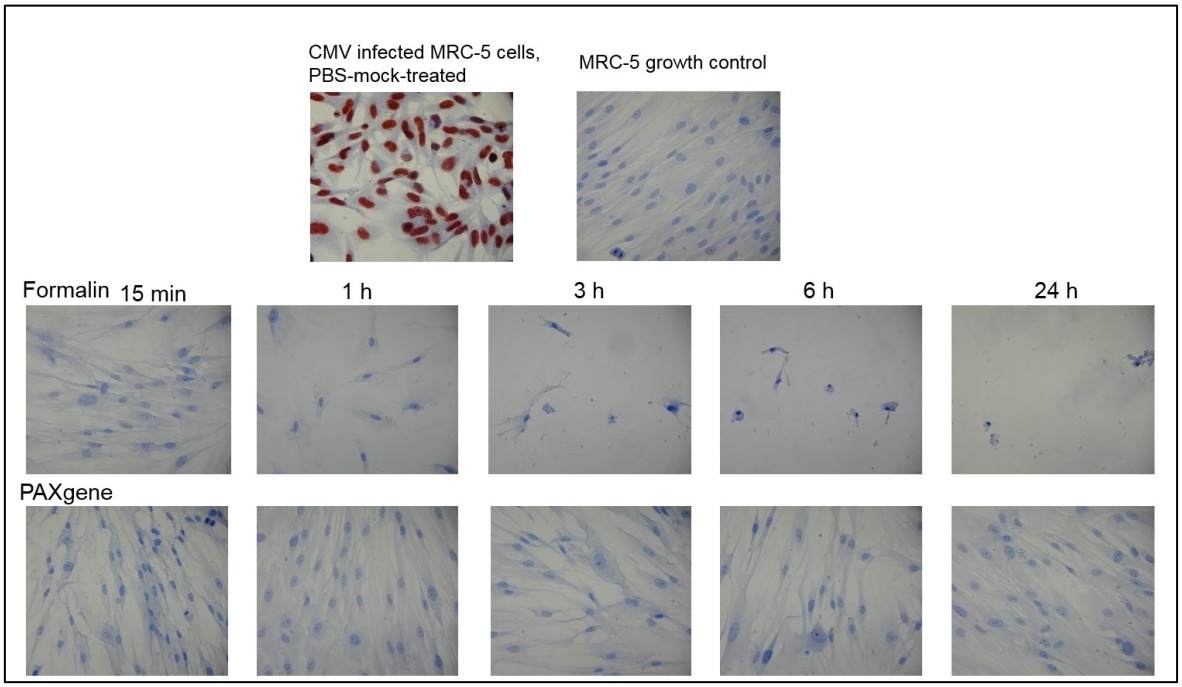


Figure 21: Immunocytochemical assay to detect *CMV* after inactivation.

Upper lane, left: Red staining of *CMV* positive PBS-mock-treated MRC-5 cells (*CMV* positive control). Right: MRC-5 growth control, not treated cells (*CMV* negative control).

Middle lane: Formalin treated *CMV* infected MRC-5 cells. Inactivation was performed with increasing incubation times starting with 15 minutes (left), 1, 3, 6 to 24 hours (right).

Lower lane: PAXgene treated *CMV* infected MRC-5 cells. Incubation periods were the same as indicated for formalin.

Discussion – Chapter 2

Hypothesis 2: The capability of PAXgene to well preserve biomolecules diminishes its pathogen inactivation efficiency when compared to formalin.

The combined analysis of classical histo-pathological features and molecular biomarkers are the intended applications of fixatives increasingly in demand for personalized medicine and this may also develop their eligibility for highly hazardous samples. Increased sensitivity for diagnosis of infectious diseases is an advantage that may be obtained by using PAXgene for tissue fixation and this would allow the molecular detection of pathogens and the evaluation of morphological alterations in tissues from the same specimen. PAXgene, which has been intensively evaluated in the context of the European Framework Programme 7-funded project SPIDIA (www.SPIDIA.eu), met both requirements. However, the exceptionally good preservation of biomolecules in PAXgene-fixed tissues (Staff et al., 2013, Viertler et al., 2012, Groelz et al., 2013, Ergin et al., 2010, Gundisch et al., 2013, Mathieson et al., 2016), superior to formalin and mostly equal to cryo-preserved tissue (Viertler et al., 2012, Groelz et al., 2013, Mathieson et al., 2016) raised concerns as to whether it adequately inactivates pathogens. Hence, information on the pathogen inactivation capabilities of PAXgene were required to determine whether PAXgene can be used following the same biosafety rules as established for HCWs who process formalin-fixed biological samples.

The results obtained by *in vitro* inactivation series of all classes of microorganisms and a selected virus showed a nearly similar inactivation activity of PAXgene and formalin with a small but not significant advantage for formalin only in series with *Cs* (Loibner et al., 2016). It is difficult to interpret and compare the results of the less efficient inactivation of *Cs* by PAXgene especially because very few systematic studies on the inactivation capabilities of formalin have been published. Specific guidelines for fixatives are lacking completely. Interestingly, these studies revealed that even formalin could not kill *Cs* at the desired reduction of more than 10^5 cfu/mL in three out of ten assays independent of the inactivation period. Published reports on formalin regarding incomplete inactivation of picornaviruses causing poliomyelitis and foot-and-mouth disease (Brown, 1993) and a study evaluating the inactivation of different bacterial strains by several chemical germicides (Sagripanti et al., 1997) are in line with the observations made here. Apart from that tissue fixation in a routine laboratory process is followed by tissue processing comprising several inactivation conditions i.e. the exposure of potentially infectious

samples to dehydrating conditions by increasing alcohol concentrations (70% to 100%) and paraffin embedding at more than 55 °C. Indeed, the absolute inactivation of the most resistant strain tested (*Cs*) was reached, as expected, by the alcohol processing steps in a separate study (data not shown).

However, the only not tested infectious agent here, prions, require extremely harsh inactivation conditions going beyond the properties formalin and alcohol can provide, for example, hypochlorus acid (Hughson et al., 2016), and as mostly used, 2 M sodium hypochlorite (Taylor, 1999, Rutala et al., 2010).

Anyhow, with respect to the results obtained from the inactivation series here, fixed tissues should not be considered as sufficiently inactivated in general. Therefore, it is recommended to handle fixed tissues with care following biosafety regulations (Grizzle and Fredenburgh, 2001) and use adequate PPE independent of the fixation method.

The inactivation experiments with formalin and PAXgene performed with *CMV* here confirmed the not quantitative immunocytochemistry results as previously reported by Kap et al. (Kap et al., 2013). To detect early transcripts from active *CMV,* RT-qPCR was chosen here as a more sensitive method to additionally show that *CMV* is sufficiently inactivated by PAXgene as well as by formalin.

Conclusions

The *in vitro* experiments performed could refute the hypothesis for all bacterial strains except *Cs*. Only the inactivation of *Cs* by PAXgene was less efficient than that by formalin. The additional outcome that even formalin could not completely inactivate *Cs* may attenuate the lower inactivation capability of PAXgene.

Outlook and connection to chapter 3

The attributes of PAXgene for good preservation of DNA, RNA and proteins, as well as for tissue morphology can be rated as of promising relevance for molecular analysis and stepwise substitution of formalin where appropriate. In the context of transplantation medicine and in addition to infectious samples assessing morphological features and highly sensitive pathogen detection both could be performed from the same specimen, which leads to chapter 3.

Chapter 3 - Effect of PAXgene on pathogen detection by *CMV* analysis

Introduction

The better quality of nucleic acids isolated from PAXgene-fixed specimen compared to formalin was repeatedly proven by independent studies (see general introduction). This fact led us to consider whether PAXgene fixation may result in better sensitivity or earlier pathogen detection whenever PCR-based assays are employed (Loibner et al., 2016). Series of experiments investigating higher sensitivity for pathogen detection due to better preserved biomolecules as obtained by PAXgene were not performed and published yet.

Material and Methods – Chapter 3

CMV detection with reverse transcription real-time PCR

The *CMV* inactivation and detection assays was performed as recently described (Loibner et al., 2016). MRC-5 cells were infected with *CMV*, harvested seven days after infection, centrifuged to obtain cell pellets and fixed either with PAXgene, formalin or PBS as *CMV* positive control (Figure 22 a). Three independent series with triplicate samples were performed. RNA isolation of formalin-fixed cells was done with the RNeasy FFPE kit (Qiagen) without applying the deparaffinization step at the beginning. RNA of PAXgene-fixed cells was extracted with the PAXgene tissue RNA Kit (PreAnalytiX). For RNA isolation of PBS mock-treated (*CMV* positive control) and not infected *CMV* negative cells the RNeasy Mini Kit (Qiagen) was used and all kits were used following manufacturer´s instructions if not otherwise stated.

To exclude potential differences of PCR sensitivity due to different RNA isolation methods, RNA from an additional set of samples was isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) for all fixation types. Three biological samples and duplicates from every sample (PAXgene, formalin and PBS) were used.

Reverse transcription was performed with 1 µg RNA (measured by NanoDrop 100 Spectrophotometer, PeqLab) per assay as described above and 100 ng cDNA per tube were used for RT-qPCR performed on a Rotor Gene Q 6000 Cycler (Qiagen) using the Rotor Gene SYBR Green PCR Kit (Qiagen). The PCR assay was performed in triplicate. The *TRS1* gene was used as a *CMV* specific primer and *GAPDH* as reference for MRC-5 cells (see page 82 for description).

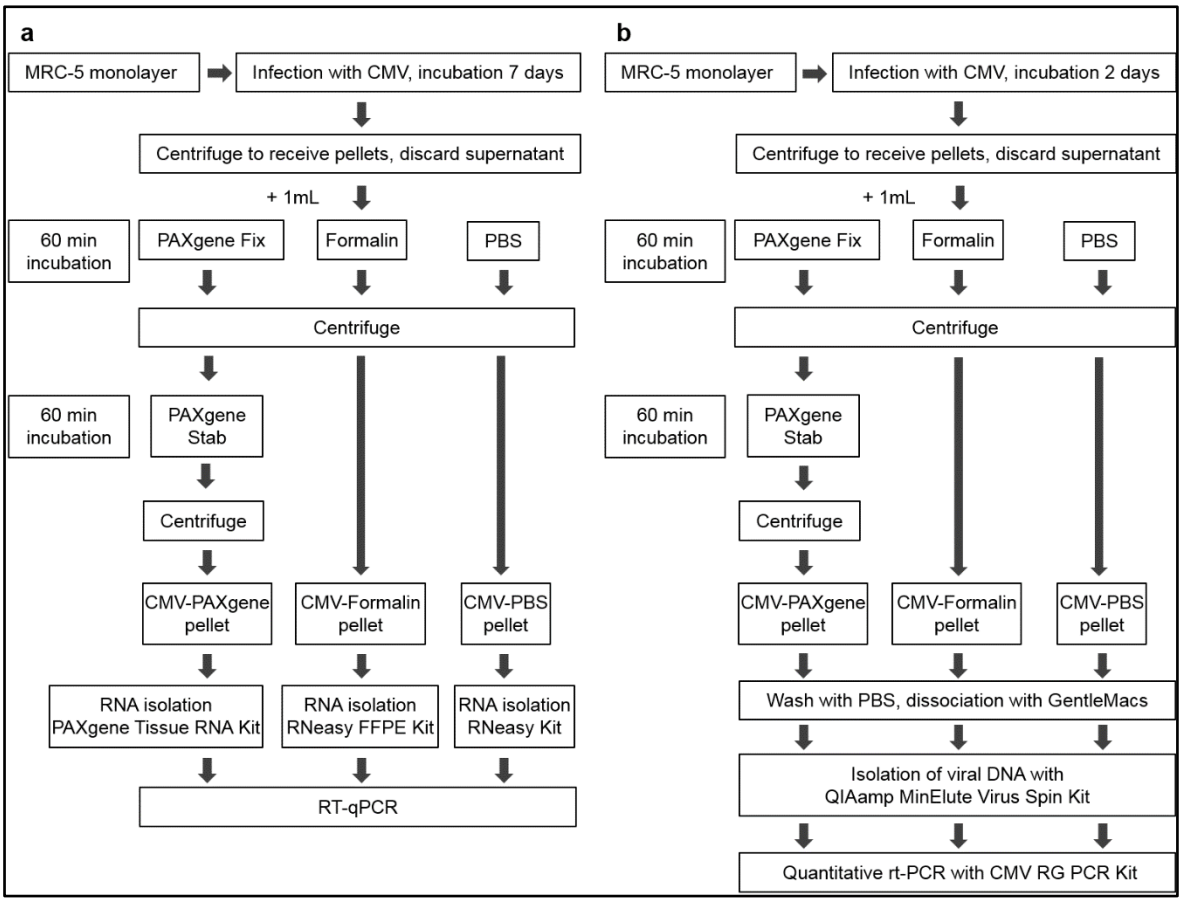


Figure 22: Workflows of fixation experiments to detect *CMV*. For detection of *CMV* two workflows with different *CMV* infection periods and sample preparation methods for analysis assays were employed:

a) *CMV* incubation for 7 days, pellet preparation and RNA isolation according to the fixation method and reverse transcription qPCR (RT-qPCR).

b) *CMV* incubation for 2 days, pellet preparation, cell disruption, isolation of viral DNA and quantitative real-time PCR (q-rt-PCR).

CMV detection with quantitative real-time PCR

MRC-5 cells were cultivated, infected with *CMV*, harvested two days after infection, pelleted and fixed either with PAXgene, formalin or mock-treated with PBS (for control) as described above (Figure 22 b). To challenge the sensitivity of the assay cells were already harvested two days after infection. Cell pellets were washed with PBS and homogenized with the GentleMacs Dissociator (Miltenyi) to release virus particles from the cells. Viral DNA was isolated using the QIAamp MinElute Virus Spin Kit (Qiagen) and 20 µL of template-DNA were used for detection of *CMV* performed with the IVD-approved "artus *CMV* RG PCR" kit (Qiagen) in duplicates according to manufacturer´s instructions.

Statistical analysis of PCR data

RT-qPCR and qPCR data on sensitivity (delivered from Rotor Gene Q Series Software 2.0.2, dynamic tube normalization) were analysed with IBM SPSS Statistics 22, Kolmogorov-Smirnov-Tests of Normality with Lillefors Significance Correction and T-test for paired samples ($\alpha = 0.05$).

Results – Chapter 3

Results, *CMV* detection with reverse transcription real-time PCR

CMV-infected MRC-5 cells were treated either with formalin, PAXgene or PBS for control as described above. RNA was isolated with the fixative-respective kit, reverse transcription to receive cDNA and the qPCR performed (Figure 22 a). *TRS1* was detected at the same cycle number (~ 13) for the untreated PBS control and the PAXgene treated samples. The formalin treated samples were not detected before cycle 17.5 which indicates a significantly higher sensitivity for the PAXgene treated samples ($p < 0.0001$). This effect was more pronounced in the *GAPDH* detection. The formalin fixation led to a *GAPDH* detection of 10 cycles later than obtained after PAXgene treatment (Figure 23).

To exclude that the measured differences in RT-qPCR sensitivity were due to the different fixation-specific RNA isolation methods, RNA from an additional sample set (formalin, PAXgene and PBS) was isolated with the same kit (Allprep RNA, Qiagen). However, no measurable RNA for further reverse transcription assays could be isolated from PAXgene or from formalin treated samples. Fixed samples were not suited for Allprep RNA isolation (Qiagen) which works well for PBS treated samples. A direct comparison of the isolation methods was not feasible (data not shown) (Loibner et al., 2016).

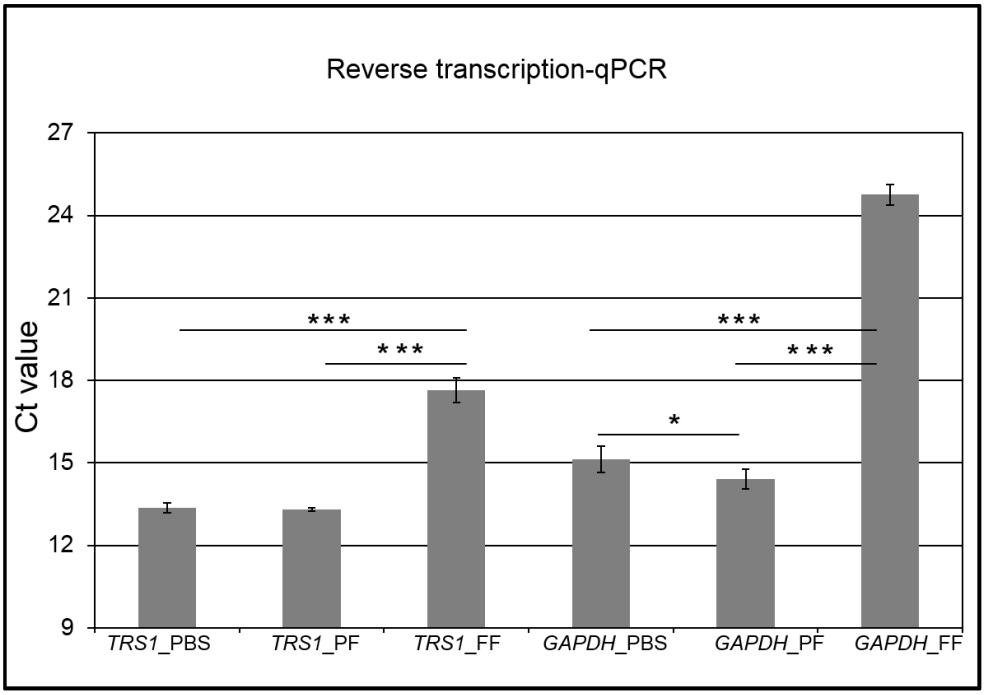


Figure 23: Comparison of Ct values of formalin and PAXgene-fixed CMV-positive samples. Differences in sensitivity between formalin and PAXgene-fixed samples was assessed by detection of the *CMV* early-immediate gene *TRS1* and the reference gene *GAPDH* by RT-qPCR. y-axis indicates the Ct value. The bars show the measured Ct value for the respective treated specimen: PAXgene-fixed (*TRS1*_PF, *GAPDH*_PF), formalin-fixed (*TRS1*_FF, *GAPDH*_FF) and PBS control samples (*TRS1*_PBS; *GAPDH*_PBS) calculated for triplicate biological samples. Low Ct values indicate early detection. Statistical significance was $p < 0.0001$ (***) or $p < 0.03$ (*). Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Results, *CMV* detection with quantitative real-time PCR

To verify the results obtained from the self-designed sensitivity assay with a commercially available and IVD-approved *CMV* detection kit, the "artus *CMV* RG PCR" Kit (Qiagen) was employed. Two days after infection with *CMV* the cells were harvested and viral DNA isolated (Figure 22 b). The assays were performed in triplicate biological samples. In PBS mock-treated control samples (*CMV*_PBS) 8,000 copies/µL were detected. In PAXgene (*CMV*_PF) and formalin-fixed samples (*CMV*_FF) 1,000 copies/µL and 165 copies/µL were detected respectively (Figure 24). The copy number difference between PBS and both fixed samples (formalin and PAXgene) was highly significant ($p < 0.0001$). However, a signifcant difference was also obvious between PAXgene and formalin-fixed samples ($p < 0.05$). An earlier detection of *CMV* in PAXgene-fixed samples compared to formalin is evident.

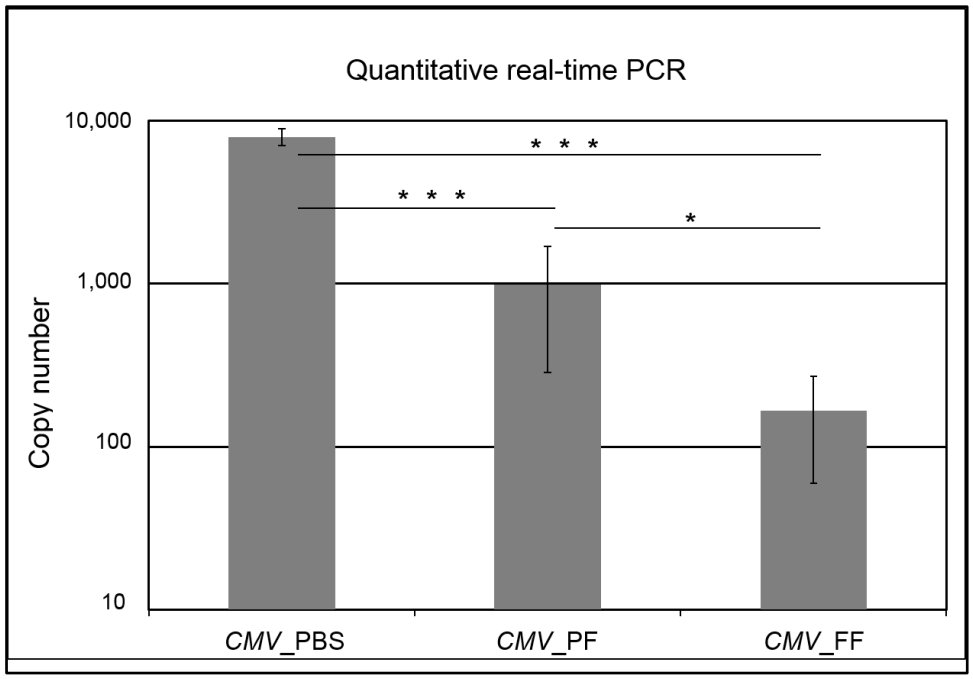


Figure 24: Quantitative real-time PCR to compare detected *CMV* copy numbers of PAXgene and formalin-fixed samples. *CMV* DNA copy numbers were detected by the IVD-approved "artus *CMV* RG PCR" Kit (Qiagen). A significantly higher copy number was detected when samples were fixed with PAXgene (*CMV*_PF) compared to formalin-fixed (*CMV*_FF) *CMV* infected samples. Unfixed *CMV* samples (*CMV*_PBS) show the highest copy number. *CMV* infected MRC-5 cells were cultured in triple biological samples. Statistical significance was $p < 0.0001$ (***) or $p < 0.05$ (*). Adapted from (Loibner et al., 2016) and reprinted by permission from PLOS ONE. Copyright 2016.

Discussion – Chapter 3

Hypothesis 3: Detection of *CMV* is expected to be more sensitive for PAXgene than for formalin-fixed samples.

The observation of the superior attributes of PAXgene for molecular diagnosis of pathogens has not yet been published. This may become a useful application as there is a need for more accurate and sensitive detection of pathogens, especially in the field of transplantation medicine (Hayden et al., 2013) and pathogen detection in general regarding biosafety issues. With different and independent PCR assays using multiple samples with the same amount of starting material for each assay a 16-fold significantly increased sensitivity to detect *CMV* transcripts with a self-designed RT-qPCR kit and a 6-fold increased sensitivity for *CMV* DNA detection by employing an IVD-approved kit, PAXgene-fixed samples were proven to provide superior pathogen detection than samples fixed with formalin (Loibner et al., 2016).

Dilution series to evaluate the limit of detection of PAXgene compared to formalin treated samples could be considered to be performed in future experiments. Larger test series on different tissue types obtained from autopsies of *CMV* positive deceased patients could be an interesting future approach to detect traces of either early transcripts of a possibly active *CMV* or DNA from latent *CMV* in various specimen. These advanced studies on different infected human tissue specimen could be contemplated to complement and confirm the *in vitro* results.

Due to the fact that the chemically different fixation methods require different nucleic acid extraction methods (Thatcher, 2015, Kashofer et al., 2013) the direct comparison of both fixatives is not possible. The RNA isolation from the sample set prepared for both fixatives and the PBS control with the same kit for all samples did not result in any measurable RNA content for both fixatives as described on page 90. Therefore, the optimal isolation protocols were used for each fixative to compare the best sensitivity achieved by both fixation methods and the unfixed native PBS mock-treated samples.

Conclusions

PAXgene-fixed specimens were found to rather resemble unfixed samples when used for PCR assays. These results match the observations that PAXgene interferes less than formalin regarding sample pre-analytics as previously published (Viertler et al., 2012). The hypothesis that PAXgene-fixed samples enable earlier pathogen detection was confirmed.

Outlook and connection to chapter 4

The pre-analytic features of PAXgene raise the question of whether further analytic applications would be appropriate for the use of PAXgene-fixed samples and whether similar results could be obtained as those from FFPE fixed specimen which leads to chapter 4.

Chapter 4 – Clinical applicability of PAXgene for fluorescence *in situ* hybridization (FISH)

Introduction

FISH (fluorescence *in situ* hybridization) is a method to detect specific regions of nucleic acids by applying complementary nucleic acids labelled with a fluorescent dye directly onto a section of fixed tissue (= *in situ*). Detection of the examined region is performed with a confocal laser scanning microscope (CLSM). Chromosomal alterations like translocations, deletions and amplifications can be detected by FISH (Oberauner-Wappis et al., 2016). This method is routinely used in personalized medicine for breast cancer specimen to investigate whether the human epidermal growth factor receptor 2 (HER2) gene is amplified or not. HER2 is located on the chromosome 17q12 and becomes an oncogene in case of amplification which stimulates cell growth (Boecker W., 2012). This amplification occurs in twenty percent of invasive breast cancer tumors (Pauletti et al., 1996, Owens et al., 2004, Wolff et al., 2007, Boecker W., 2012) and is associated with a poor prognosis (Press et al., 1997, Yamauchi et al., 2001). Determination of the HER2 amplification is used to evaluate whether breast cancer patients are susceptible for anti-HER2 directed therapy, e.g. trastuzumab (Herceptin®) which is a companion diagnostic listed by the U.S. Food and Drug Association (FDA) (http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm) (Wolff et al., 2007, Olsen and Jorgensen, 2014). FISH-based assays for companion diagnostics in health care were developed for FFPE tissues to allow a broad application.

The enhancement of personalized medicine requires that different types of analysis ranging from classical histology to molecular analysis and various *in situ* techniques are performed on a tumor to select the best therapy and avoid ineffective treatments and heavy side effects caused by cytostatic drugs. Therefore, it is necessary to investigate techniques for processed tissues to enable various analytical technologies from a single specimen. The challenge of using PFPE tissue for FISH by applying protocols developed for FFPE tissue is that typical hybridization conditions and chemical modifications determined for formalin are not appropriate for PFPE samples. Tissues fixed with PAXgene did not allow the generation of satisfying FISH results so far as stated by Kap et

al. 2011 (Kap et al., 2011). The aim was therefore to modify PFPE samples to serve as FISH substrates using the example of an assay for HER2 amplification.

Material and Methods – Chapter 4

General information

The sample preparation and hybridization assay was performed as recently described (Loibner et al., 2017, Oberauner-Wappis et al., 2016). Eight samples from breast cancer patients were obtained in Graz, approved by the Ethics Committee of the Medical University Graz (20-066). Five samples were obtained from Qiagen with the agreement from Cureline (South San Francisco, CA). All women (aged between 39 and 88 years, mean age 64) underwent an excision of the primary invasive breast cancer tumor. The HER2/CEN17 dual color probe provided in the commercially available and CE/IVD approved HER2/CEN17 Dual Color Probe Kit (ZytoVision GmbH, Bremerhaven, Germany) is a mixture of a red fluorochrome-labelled CEN17 probe specific for the alpha satellite centromeric region of chromosome 17 (D17Z1) as reference and a green fluorochrome-labelled HER2 probe specific for the HER2 gene (probe location 17q12) (Wolff et al., 2014, Wolff et al., 2007).

Tissue fixation, processing and embedding

From each of the excised specimen two 4 x 15 x 15 mm samples were cut, put into a tissue cassette and fixed either in formalin or PAXgene Fix for 24 hours at RT. The PF (PAXgene-fixed) sample was removed from PAXgene Fix and submerged into PAXgene Stab for another 24 hours (Figure 25, step 1). Prior to further tissue processing and paraffin embedding the FF (formalin-fixed) tissue was submerged into 70% ethanol for 30 minutes to avoid formalin contamination of the tissue processing device and the PAXgene-fixed samples. All samples were processed according to routine laboratory protocols in a Spin Tissue Processor (ThermoFisher Scientific) by stepwise dehydration in 70% (2 x 15 min), 80% (30 min), 90% (60 min), and 99% (2 x 60 min) ethanol, followed by xylene (2 x 60 min) and infiltration with paraffin (4 x 45 min). The tissue cassettes were put into metal moulds which were then filled up with 5 – 10 mL molten paraffin (solidification point 56 - 58 °C) by using a paraffin embedding instrument (Sakura Tissue-Tek TEC) and cooled on a cooling plate. The resulting paraffin blocks, PFPE (PAXgene-

fixed paraffin-embedded) and FFPE (formalin-fixed paraffin-embedded), were recovered from the metal moulds and stored at 4 °C in the dark until further use (Figure 25, step 2).

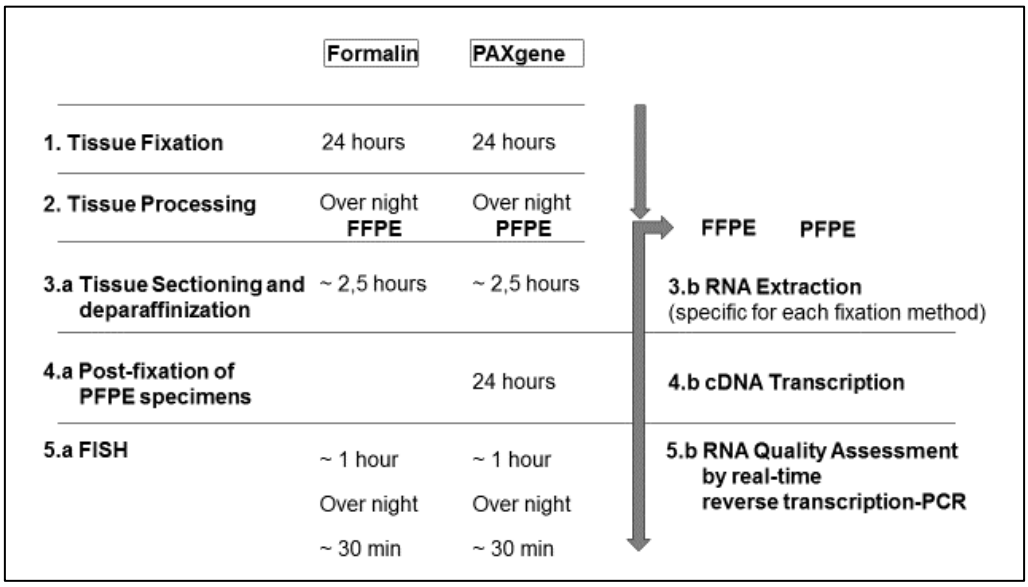


Figure 25: Workflow for preparation of FFPE and PFPE samples for FISH and RNA quality assessment from the same sample. The diagram shows the workflow for FFPE and PFPE tissue preparation with the crucial formalin post-fixation step before FISH (Fluorescence in-situ hybridization) and RNA analysis. Adapted from (Loibner et al., 2017) and reprinted by permission from JoVE. Copyright 2017.

Slide preparation for FISH (fluorescence *in situ* hybridization)

According to the routinely performed process, 2 - 3 µm thick sections of the tissue in the paraffin block were cut with a microtome and put into a cold water bath. The floating sections were picked up with a brush and a coated microscope slide, and transferred into a warm water bath (40 °C). By slowly pulling the slide out of the water bath the section adhered stretched without wrinkles. The sections on the slides needed to dry for about one hour at RT. To remove the paraffin the slides were heated at 70 °C in an incubator in a first step. Thenceforth, several slides can be horizontally arranged on a bracket and processed in parallel. The sections were rehydrated stepwise at RT in glass jars containing 100% xylene (2 x 15 min), 96% ethanol (2 x 15 min), 90% ethanol (2 min), 80% ethanol (2 min), 70% ethanol (2 min), 50% ethanol (2 min), and finally distilled water (2 x 10 min) (Figure 25, step 3.a). PFPE sections were post-fixed with formalin at RT in a glass

jar in several experimental time series starting with 1 minute up to 24 hours (Figure 25, step 4.a). Excess formalin was removed by washing with PBS (3 x 10 min) and distilled water (2 x 10 min).

FISH

FISH was performed using the commercially available and European Conformity *In Vitro* Diagnostics (CE IVD) approved HER2/CEN17 Dual Color Probe Kit (ZytoVision) according to the manufacturer's instructions (Figure 25, step 5.a). Briefly, the slides were incubated at 98 °C for 15 min in a pre-warmed (water bath) Heat Pre-treatment Solution Citric. After washing (3 min) with distilled water the slides were put into a temperature controlled hybridization system at 37 °C. For proteolysis pepsin solution was immediately and dropwise applied onto the tissue section and incubated for 9 min. Slides were put back into the brackets and washed in Wash Buffer SSC (5 min) in a glass jar and then rinsed in distilled water (1 min). Sections were gradually dehydrated in 50%, 70%, 90% and 100% ethanol (each for 1 min) and air dried. Protected from light exposure, 10 µL of HER2/CEN17 Dual Color Probe for hybridization were applied onto the dried tissues, covered with a coverslip and sealed with rubber cement. Probe and specimen DNA were co-denatured at 75 °C in the hybridization system for 10 min where the hybridization is performed at 37 °C overnight. The rubber cement and the cover slides were removed and the sections washed at 37 °C with pre-warmed Wash Buffer A (2 x 5 minutes). The sections were dehydrated in gradually increasing ethanol (70%, 90% and 100%, each for 1 min), and air dried before staining with 30 µL DAPI solution (4',6-diamidino-2-phenylindole). The hybridized area is covered with a cover slip and slides were incubated for 15 minutes (protected from light). The slides were stored at 2 – 8 °C for up to two weeks until evaluation by CLSM. The images were acquired with filters for green (excitation: 503 nm, emission: 528 nm) and orange (excitation: 547 nm, emission: 572 nm) channels.

For interpretation at least 20 cells which are located in two different regions of the invasive component of the carcinoma as defined by a pathologist have to be evaluated. Clearly distinguishable well distributed DAPI-blue stained nuclei in the counting area must be included. Regions at the boundary as well as retracted or squeezed areas must be excluded from the counting tissue. Tumor specimens with two green HER2 signals and two red CEN17 signals denoted as a HER2 : CEN17 ratio ≤ 2.0 per nucleus are scored as

normal. Those with a HER2 : CEN17 ratio ≥ 2.0 are scored as amplified (Wolff et al., 2014, Wolff et al., 2007).

Assessment of RNA Quality

Differences in RNA quality after formalin and PAXgene fixation employing real-time reverse transcription PCR has already been stated by several research groups (Viertler et al., 2012, Kashofer et al., 2013, Mathieson et al., 2016). However, here, corresponding FFPE and PFPE specimens from 2 different cases were used to demonstrate that different molecular analyses (FISH and RT-qPCR) can be performed from a single sample with similar and even superior quality when fixation has been performed with a non-cross-linking fixative such as PAXgene as recently described (Loibner et al., 2017).

Sections of 5 µm from FFPE and PFPE sample blocks were cut with a microtome and RNA was extracted employing the RNeasy FFPE kit (Qiagen) and the PAXgene Tissue RNA kit (PreAnalytix), respectively, according to the manufacturer´s instructions. For reverse transcription 500 ng RNA per sample to cDNA the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used. cDNA was stored at -20 °C until further use. The Power SYBR Green PCR Master Mix (ThermoFisher Scientific) was prepared according to the manufacturer´s instructions. Four microliters of 1:16 diluted cDNA was used as PCR template. The PCR assays were performed in triplicates on a 384-well-plate on a QuantStudio 7 Flex Real-Time PCR System (ThermoFisher Scientific). The primers for GAPDH detection were designed to obtain five different amplicons (Viertler et al., 2012):

GAPDH forward	5´-CCACATCGCTCAGACACCAT-3´;	
GAPDH reverse	71 bp:	5´-ACCAGGCGCCCAATACG-3´;
	153 bp:	5´-GTAAACCATGTAGTTGAGGTC-3´;
	200 bp:	5´-TTGACGGTGCCATGGAATTT-3´;
	277 bp:	5´-ACTTGATTTTGGAGGGATCT-3´;
	323 bp:	5´-AAGACGCCAGTGGACTCCA-3´;
	530 bp:	5´-ACGATACCAAAGTTGTCATG-3´.

To exclude nonspecific PCR products from further data analysis of the Ct values, the melting curves received by the SYBR Green technology were evaluated by using the QuantStudio™ Software V1.3—for QuantStudio™ 6 and 7 Flex and ViiA™ 7 Real-Time PCR Systems.

Results – Chapter 4

Results, FISH

Repeated inspections by CLSM of the slides prepared in different time series, starting with post-fixation times of 1 minute and subsequently stepwise increasing revealed that interpretable data for PFPE slides could not be obtained before 16 to 18 hours of post-fixation with formalin (data not shown). Red and green hybridization signals inside of the blue DAPI-stained nuclei were not as clear as those obtained from FFPE slides at shorter post-fixation times. In Figure 26, both pictures A (FFPE) and B (PFPE after 24 hours of formalin post-fixation) show the green signal of the same quality. The red reference signals (CEN 17 gene locus) are of the same signal quality as well and both signals provide a clear and distinct intensity that allows an interpretation of the HER2 status according to the requested amount of cells. An amplified case (HER2 : CEN17 ratio ≥ 2.0) means that this patient would be susceptible for an anti-HER2 targeted therapy (e.g. trastuzumab, Herceptin ®). Figure 26 C shows two green signals, a not amplified HER2 gene locus and two red signals as reference in the green circle. The red circles flag n amplified loci with more than two green signals and two red signals as reference (Loibner et al., 2017, Oberauner-Wappis et al., 2016).

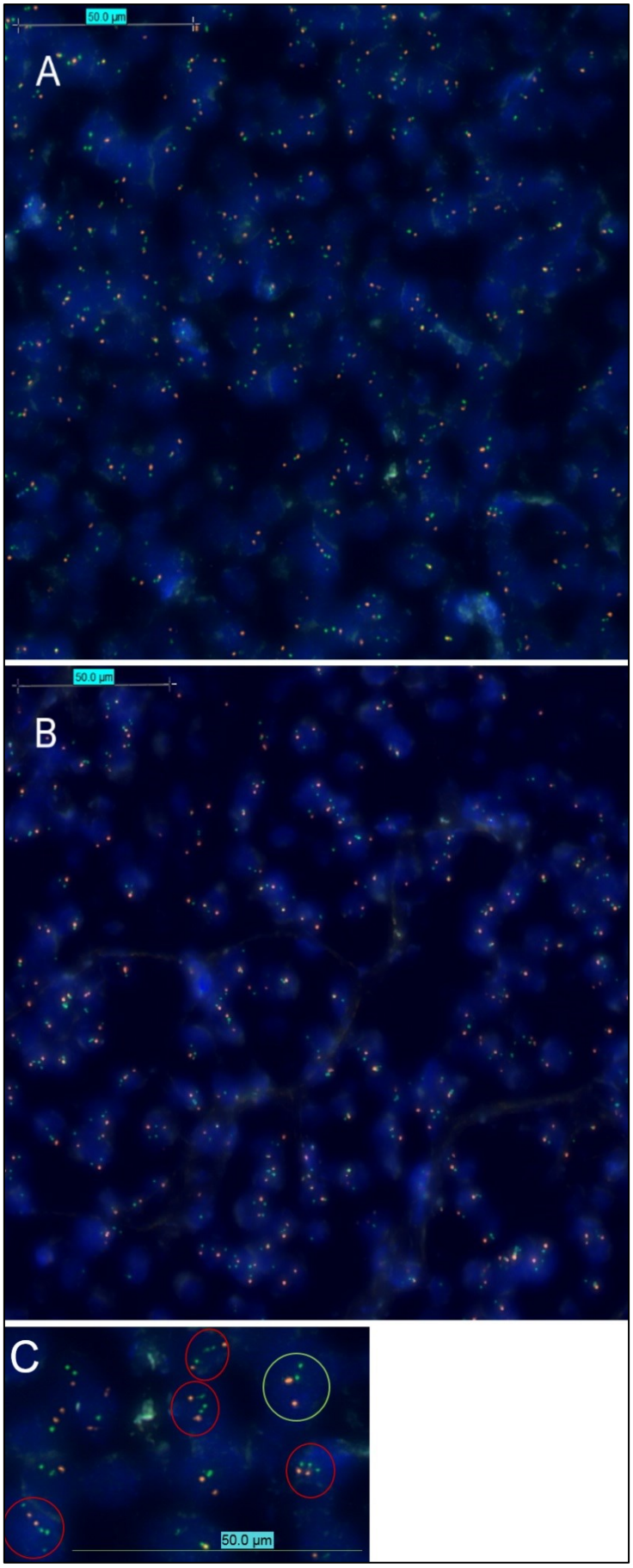


Figure 26: Results of HER2-FISH for formalin and PAXgene-fixed breast cancer tissue sections. CEN17 (red) and HER2 (green) hybridization signals are detected inside of the blue DAPI-stained nuclei.
A: FFPE section of mamma tissue.
B: PFPE section from the same sample and 24-hour post-fixed with formalin. The HER2 amplification status is identical in both FFPE and the post-fixed PFPE sections. The signals are of the same quality and intensity (A, B).
C: Normal interphase cells (green circle) show two green (HER2) and two red (CEN17) signals. In cells with an amplified HER2 gene locus multiple copies of green signals (red circles) are detected. Adapted from (Loibner et al., 2017) and reprinted by permission from JoVE. Copyright 2017.

Results, assessment of RNA quality

RNA was isolated from corresponding FFPE and PFPE specimens that were already used for FISH. Reverse transcription and a qPCR to detect *GAPDH* were performed. The obtained results show different amplification efficiencies for different amplicon lengths (71 bp, 153 bp, 200 bp, 275 bp, 323 bp) of the *GAPDH* mRNA (Figure 27). All Ct values for all amplicons obtained from FFPE samples were higher than those obtained from PFPE. The Ct values increased for the 277 bp and 323 bp amplicons in both fixation methods. This increase was more pronounced in the FFPE samples. The Ct values obtained by the 530 bp amplicon were excluded from analysis due to their unspecific melting curves evaluated after termination of all PCR cycles.

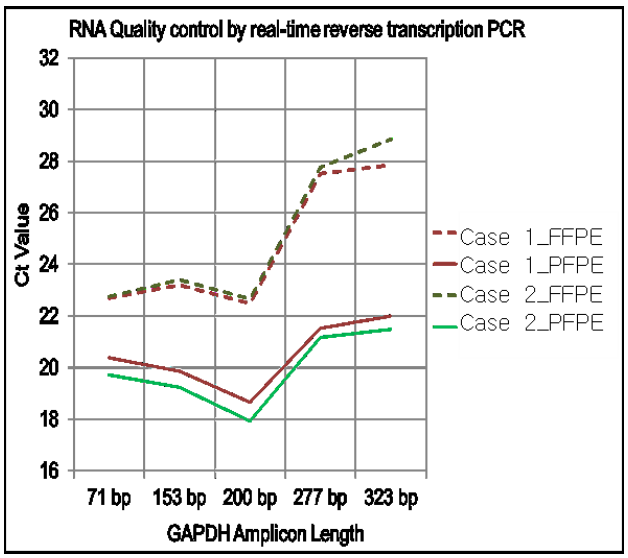


Figure 27: RNA quality of corresponding FFPE and PFPE samples from human breast cancer samples. Real-time reverse transcription PCR was performed with different lengths of *Glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) amplicons. mRNA was isolated from FFPE and PFPE of two different breast cancer tissue samples processed in parallel and transcribed to cDNA for PCR.
y-Axis: Ct (cycle threshold) values are shown for different amplicon lengths (71 bp, 153 bp, 200 bp, 275 bp, 323 bp) indicated on the x-axis. Adapted from (Loibner et al., 2017) and reprinted with permission from the Journal of Visualized Experiments. Copyright 2017.

Discussion – Chapter 4

Hypothesis 4: The determination of the human epidermal growth factor receptor 2 (HER2) status by fluorescence *in situ* hybridization (FISH) can be performed with PAXgene-fixed paraffin-embedded (PFPE) breast cancer specimen after a pre-analytic post-fixation treatment with formalin.

As a selected example*, in situ* hybridization with PAXgene-fixed tissue could not yet be successfully applied yet as stated by Kap et al. 2011. Due to the assumption of the respective study group that DNA in PFPE samples is more natively preserved and therefore more easily accessible for probes, they performed FISH without protein digestion which led to increased auto fluorescence. Heating the slides improved the signal to noise ratio but the results were not satisfying. PAXgene-fixed samples for FISH as a widely used method in cancer diagnostics and personalized medicine would require further investigations to develop adaptations in order to obtain similar results as from formalin-fixed specimen.(Kap et al., 2011).

For example, a diagnostic kit used for FISH to evaluate the amplification status of the HER2 gene of breast cancer patients is listed by the FDA for diagnostic use. The protocols listed there must not be changed (e.g. omitting the protein digestion or additional cooking steps of the samples) in order to avoid losing the FDA approved status. FISH-based assays were primarily developed and validated for FFPE samples according to their chemical modification of proteins and nucleic acids and building of cross-links by formalin fixation.

Therefore, it was proposed here to modify PFPE samples before the FISH analysis and to keep the FISH protocol itself unchanged. The idea was to convey PFPE samples through a process that ends with FISH compatible samples comprising all necessary chemical modifications. A simple post-fixation step of deparaffinized and re-hydrated PFPE sections with formalin was performed. The experimental incubation times tested for post-fixation varied from one and a few minutes, and hours up to 24 hours. Although the PFPE sections were of only 2-3 µm thickness, short post-fixation times of minutes up to approximately eight hours revealed no positive effect on the FISH result. The signals were unclear and background fluorescence made an evaluation for pathologists who are trained to assess the quality of FFPE-FISH slides impossible. Not until longer formalin post-

fixation times of 18 to 24 hours did the signal quality become similar to that obtained from FFPE samples.

As dehydration by PAXgene has a minimal impact on nucleic acids the detection of chromosome segments by FISH can be performed after formalin post-fixation. By considering the reported chemical reactions of formalin and dehydrating fixatives, and the observation made here by extending the formalin post-fixation times to achieve similar FISH signals for PFPE than for FFPE, it was determined to add a 24-hour formalin post-fixation step to the pre-analytic protocol before FISH analysis can be performed. This also keeps the laboratory process as easy as possible (Oberauner-Wappis et al., 2016, Loibner et al., 2017).

The different Ct values obtained from RT-qPCR (Figure 27) demonstrate a lower amplificability of mRNA isolated from FFPE breast cancer specimen compared to PFPE material. This confirms again a more pronounced chemical modification. Additionally, the higher Ct values of amplicons larger than 200 bp in FFPE samples, compared to shorter amplicons indicate a higher fragmentation of mRNA. Here, corresponding FFPE and PFPE samples from only two cases were used to demonstrate the feasibility to perform different analysis methods from the same sample with PFPE material. This low number may be justified as comparative RNA quality studies for FFPE and PFPE have already been published with reasonable certainty by several research groups (Viertler et al., 2012, Groelz et al., 2013, Mathieson et al., 2016). Thirteen breast cancer cases were used to demonstrate the effect of formalin post-fixation for PFPE samples for FISH analysis (Oberauner-Wappis et al., 2016).

Conclusions

Whenever a small amount of cancer tissue limits the number of investigations (e.g. gene expression studies, protein phosphorylation status, DNA methylation, *in situ* hybridization etc…) that have to be performed from a single specimen the use of PAXgene may substitute formalin with the constraint that every method has to be tested explicitly for the use of PFPE material. As demonstrated here, the use of the same FISH protocol that has been originally approved for FFPE can be applied on PFPE after a 24 hour post-fixation of the tissue sections with formation to obtain identical results.

General discussion

The goal of this work is to investigate key pre-analytic aspects of sample workflows in a clinical setting starting with acquisition of highly infectious specimen and the specific requirements for personal protective equipment. It continues with evaluation of sample stabilization and pathogen inactivation by comparing the current gold standard formalin and a formalin-free method. As pathogen detection and various applications of analysis methods require high quality samples it was investigated whether the formalin-free method may fulfil the requirements for a robust and specific diagnostic use. These aspects are addressed and described in four separate chapters and the corresponding hypotheses expressed below.

Requirements for safe sample acquisition and preparation

The route a clinical specimen takes until it reaches its destination and fulfils its purpose, namely a reliable diagnostic result crucial for a therapy concept, needs to follow predetermined and robust pathways and is always conducted by qualified human resources. Ideally one sample allows a variety of analyses which leads the sample to crossroads and branches along its processing route. Starting with the sample taking from a patient under different conditions in a clinical setting a two-part assurance needs to be provided. On the one hand the human resources need to be protected from the presently unknown biological hazard and on the other hand the sample quality needs to be preserved with all its biological markers to ensure its utilization for subsequent multi-purpose analysis including sensitive pathogen detection. However, a limitation for multiple analysis methods may occur due to a small sample size which does not allow a splitting for different analysis pathways. Therefore, a secure method of pathogen inactivation and preservation of biological markers needs to be applied at the very first step of pre-analytic sample handling. At the end the sample should have been processed in a way that allows established analysis methods to be employed although the pre-analytic process may have been modified to fulfil all requested demands.

Recently, the Institute of Pathology of the Medical University Graz has received a newly built BSL-3 autopsy facility that is unique in the civil hospital landscape in Europe as stated by the members of ERINHA (European Research Infrastructure for Highly

Pathogenic Agents, www.erinha.eu). Furthermore, the local Styrian government has adapted the “Epidemic Plan Regulations” regarding the modus operandi for persons deceased due to RG-3 or RG-4 pathogens as described in Table 2 (page 41).

To set up the sample acquisition workflows and processes under BSL-3 conditions a detailed planning was done already several years earlier. One part of this detailed planning included the selection of adequate PPE involving the workforce and independent volunteers as reported in chapter 1. BSL-3 laboratories have to comprise additional constructional enhancements compared to BSL-2 facilities as regulated by the Austrian legislation (Verordnung der Bundesministerin für Arbeit, Gesundheit und Soziales über den Schutz der Arbeitnehmer/innen gegen Gefährdung durch biologische Arbeitsstoffe, BGBl. II Nr. 237/1998) (Table 1, page 40) and the European Commission Directive “Protection of workers from risks related to exposure to biological agents at work”.

This thesis describes the implementation of revised legal foundations regarding biosafety for HCWs, the approach to fulfil increased demands regarding sample quality for molecular analyses and biosafety, and the applicability of an alternative tissue fixative for routine diagnostics.

Approach to understanding

Formalin rapidly penetrates tissues and cells as reported by Baker et al. already in 1958, reaching 5 mm in 2 hours (Baker et al., 1958), and Hewitt et al. 2008 proclaimed an average rate of 1 mm per hour depending on the tissue type (Hewitt et al., 2008). However, the penetration only cannot be the critical factor leading to the results obtained after 18 hours of formalin post-fixation on 2 - 3 µm PFPE breast cancer tissue sections as the experiments showed for the adaptation of PFPE samples for FISH analysis. The first most common chemical reaction within the formalin fixation process is the formation of methylol out of formaldehyde and a lysine residue or other various side chains of proteins or peptides containing lysine (Hoffman et al., 2015) (Figure 2), cysteine, histidine, arginine, tyrosine and the hydroxyl groups of serine and threonine (Means and Feeney, 1995) (Figure 28) to form hydroxyl-methyl side groups (-CH_2-OH) which are again reactive. Then methylol is dehydrated to a Schiff base which again reacts e.g. with an amino group of a nucleic acid resulting in a crosslink (Hoffman et al., 2015) (Figure 2). Gustavson et al. reported in 1956 that the longer the fixation time, the greater the degree of crosslinking (Gustavson, 1956). In the case of short fixation times like hours to days with formalin (10% neutral buffered, as most commonly used in diagnostic pathology) the

formation of hydroxyl-methyl side chains may be the primary reaction. Actual crosslink formation is reported to be rare at short fixation times (Kim S Suvarna, 2013).

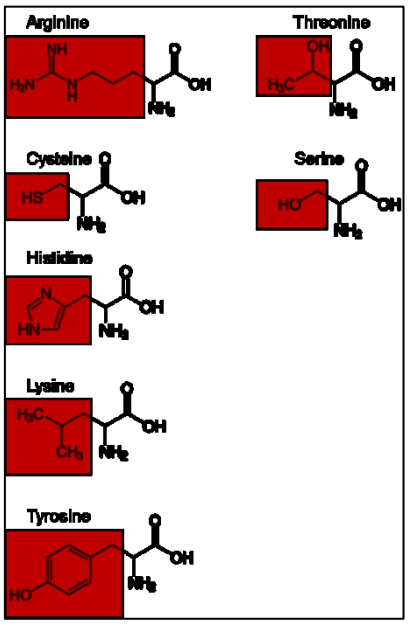


Figure 28: Most reactive side chains of proteins with methylene glycol. Different amino acids contain reactive components. The uncoloured part shows the basic structure of amino acids with the amino and the carboxyl groups (COOH and NH_2). In the red box the amino acid residues with different reactive groups (SH, OH, NH_2, COOH) are shown. Left: Arginine, cysteine, histidine, lysine and tyrosine have the highest affinity for formaldehyde. Right: Threonine and serine possess highly reactive hydroxyl groups (OH).

Formaldehyde permeates between proteins and nucleic acids, thus stabilizing their connection. As with proteins formaldehyde also reacts with free amino groups (-NH_2) of nucleic acids (McGhee and von Hippel, 1975a, McGhee and von Hippel, 1975b, McGhee and von Hippel, 1977a, McGhee and von Hippel, 1977b). Typical fixation times in a routine laboratory are approximately 24 hours with a minimum formalin-to-tissue ratio of 1:10 and up to 36 hours for larger samples or even during a weekend. Overfixation leads to RNA degradation and altered histomorphology and immunoreactivity (Hewitt et al., 2008). Fox et al. reported that tissue sections fixed in formaldehyde would reach an equilibrium status in 24 hours at RT (Fox et al., 1985).

PAXgene is composed of a mixture of different alcohols, acetic acid and a soluble organic compound (Belloni et al., 2012, Ergin et al., 2010, Groelz et al., 2013, Gundisch et al., 2013, Gundisch et al., 2014, Viertler et al., 2012, Kap et al., 2011, Loibner et al., 2016,

Mathieson et al., 2016) and therefore belongs to formalin-free, non-cross-linking dehydrating fixatives. Ethanol, methanol and acetone dehydrate proteins by removing bound and free water which changes the protein tertiary structure leading to precipitation. Nucleic acids remain mostly unchanged. The extraction of lipids implicates shrinking of tissue components as well as destruction of the ultrastructure of organelles including mitochondria (Kim S Suvarna, 2013), both of which has been observed in own experiments with PAXgene-fixed samples at the Institute of Pathology, Medical University Graz. Shrunk tissue occurred mostly in mamma specimen due to the high amount of fat tissue.

Electron microscopy revealed the destruction of the organelle ultrastructure (Figure 29). Fixation with formalin and glutaraldehyde the latter is the standard fixation for electronmicroscopy, allows to clearly identify membrane formations, the nucleus and the rough endoplasmic reticulum (rer) (Figure 29 A, B, C). At samples fixed with PAXgene (Figure 29 D, E, F) the nucleus is distinctly marked off and other membrane formations appear to have lost their structures.

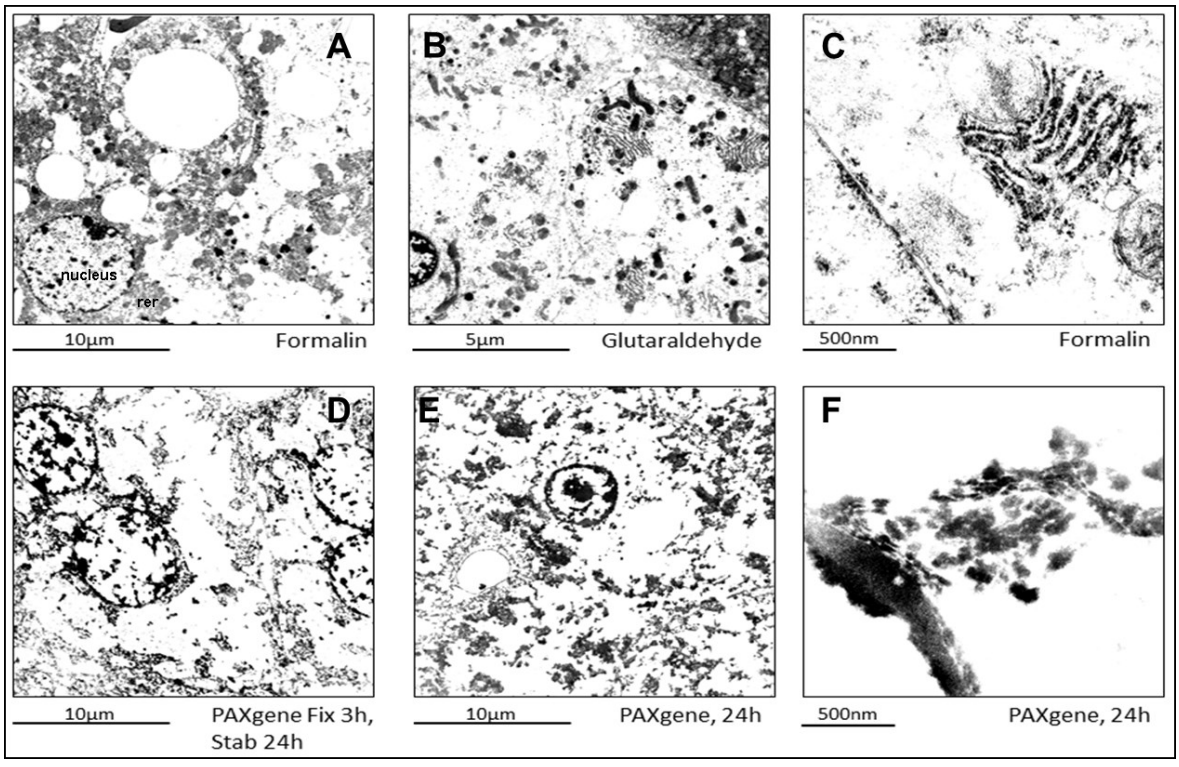


Figure 29: Electron microscopy pictures of mouse liver. Mouse liver samples were fixed with A, C: formalin; B: glutaraldehyde. Nuclei and rough endoplasmic reticulum (rer) are clearly visible. D: PAXgene (3 hours fixation, 24 hours stabilization); E and F: PAXgene (24 hours each for fixation and stabilization). Membranes appear destroyed.

Conclusions

***Hypothesis 1**: PPE-induced restrictions lead to increased error rates when simulated laboratory work, concentration and reaction performance are evaluated in a study with volunteers.*

***Hypothesis 2**: The capability of PAXgene to well preserve biomolecules diminishes its pathogen inactivation efficiency when compared to formalin.*

***Hypothesis 3**: Detection of CMV is expected to be more sensitive for PAXgene than for formalin-fixed samples.*

***Hypothesis 4**: The determination of the human epidermal growth factor receptor 2 (HER2) status by fluorescence in situ hybridization (FISH) can be performed with PAXgene-fixed paraffin-embedded (PFPE) breast cancer specimen after a pre-analytic post-fixation treatment with formalin.*

All hypotheses have been worked through with different scientific approaches like a proband study following the setup and guidelines of a clinical trial. Different pre-analytic scenarios were compared with respect to quality and biosafety aspects and different analysis methods and evaluation of their chemical backgrounds were investigated. Hypothesis 1 was rejected contrary to expectations, Hypothesis 2 could be refuted as well with the minor restriction regarding the extraordinary resistance of *Clostridium sporogenes* against inactivation by PAXgene and also formalin. Hypothesis 3 and 4 were confirmed and may help to further establish formalin-free sample workflows.

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Appendix

Equipment and Reagents

Cell culture

Anti-CMV antibody, M085401, Dako, Agilent, USA

Clean Air Biohazard safety cabinet, type CA / REV 6 (Class II), Heraeus, Hanau, Germany

Coverslips, 22 mm x 22 mm, Assistant, Germany

GentleMACS Dissociator, Miltenyi, Bergisch-Gladbach, Germany

Haemocytometer counting chamber; 0.2 mm depth; 0.0625 mm^2; Fuchs Rosenthal, Assistant, Germany

Holten LaminAir HB 2448 Fume Cabinet (Class II), Thermo Elektron LED GmbH, Wien, Austria

Human cytomegalovirus AD 169, HPA #622, former Health Protection Agency, actually Public Health England, UK

MRC-5 cells, human lung fibroblast cells, LGC Promochem, Germany, ATCC #CCL-171

Chemicals, reagents, kits

Acetic Acid, Merck Millipore, Munich, Germany

AEC Substrate System, Dako, Agilent, USA

AllPrep DNA/RNA/Protein Mini Kit, Qiagen, Hilden, Germany

Aquatex, Merck, Germany

Artus CMV RG PCR kit CE, Qiagen, Hilden, Germany

EnVision Detection System, Peroxidase/DAB, Dako, Agilent, USA

Ethanol abs., Merck Millipore, Munich, Germany

Formaldehyde 4% buffered, SAV LP GmbH, Flintsbach a. I., Germany

Hematoxilin solution modified acc. to Gill II, Merck, Darmstadt, Germany

High-Capacity cDNA Reverse Transcription Kit, ThermoFisher Scientific, Darmstadt, Germany

Low melt agarose, Carl Roth GmbH, Karlsruhe, Germany

Methanol, Merck Millipore, Munich, Germany

Paraffinum solidum F43/46 Grad C, ACM Herba Chemosan Apotheker AG, Vienna, Austria

Paraplast Plus (56°), Leica Mikrosysteme Handels GmbH, Vienna, Austria

PAXgene Tissue Container, PreAnalytix, Hombrechtikon, Switzerland

PAXgene Tissue RNA Kit, PreAnalytix, Hombrechtikon, Switzerland

Power SYBR Green PCR Master Mix, ThermoFisher Scientific, Darmstadt, Germany

QIAamp MinElute Virus Spin Kit, Qiagen, Hilden, Germany

QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany

RNeasy FFPE RNA Kit, Qiagen, Hilden Germany

RNeasy Mini Kit, Qiagen, Hilden Germany

Rotor Gene SYBR Green PCR Kit, Qiagen, Hilden, Germany

Xylene, VWR Chemicals, Darmstadt, Germany

General technical equipment

Centrifuges

Megafuge 1.0, Heraeus Sepatech, Thermo Fisher Scientific, Darmstandt, Germany

MiniSpin, Eppendorf, Hamburg, Germany

Incubators and Shakers

Incubator Shaker Infors HT Ecotron, Infors, Bottmingen, Switzerland

CO_2 Incubator C150, Binder, Tuttlingen, Germany

Incubator Heraeus, Hanau, Germany

Microscopes

Digital Camera, Canon S3,

Light optical microscope, Nikon Eclipse E 600 (10x, 20x, 40x, 60x objectives), Japan

Pannoramic Confocal Digital Scanner, 3DHISTEC, Budapest, Hungary

PCR machines

Gene Amp PCR System 9700 PE, Applied Biosystems, Foster City, USA

QuantStudio 7 Flex Real-Time PCR System, ThermoFisher Scientific

Rotor Gene 5Plex HRM, Qiagen, Hilden, Germany

Spectrophotometer

NanoDrop 100 Spectrophotometer, PeqLab, Erlangen, Germany

Thermo block

Thermomixer comfort, Eppendorf, Hamburg, Germany

Water bath

Julabo SW 20, Seelbach, Germany

In situ hybridization

HYBrite Hybridization System, Vysis/Abbott Laboratories. Abbott Park, Illinois, U.S.A

ZytoLightSPEC ERBB2/CEN 17 Dual Color Probe, ZytoVision GmbH, Bremerhaven, Germany

Media, cell culture additives and chemicals

Blood agar + 5-10% human blood, Oxoid, Basingstoke, UK

Bouillon Schaedler + vitamin K3, bioMerieux, France

Chocolate Agar + PolyViteX, bioMerieux, France

Columbia ANC Agar + 5% sheep blood, bioMerieux, France

CSA_Casein Soya bean digest agar, Oxoid, Basingstoke, UK

Fetal calf serum, Gibco, Life Technologies, UK

GlutaMax, Gibco, Life Technologies, UK

McConkey Agar, bioMerieux, France

Middlebrook 7H10 Agar, Becton Dickenson, Germany

Minimum Essential Medium, Gibco, Life Technologies, UK

PBS (phosphate buffered saline), cell culture grade, Gibco, Life Technologies, UK

Pen-strep, Gibco, Life Technologies, UK

Sabouraud agar plates, Oxoid, Basingstoke, UK

Schaedler Agar + 5% sheep blood, bioMerieux, France

Trypcase Soy broth, bioMerieux, France

Trypsin-EDTA, Gibco, Life Technologies, UK

Plasticware

Cell culture plates, 6-well, 182 cm^2 and 75 cm^2 vented cell culture flasks, VWR, Vienna, Austria

GENbag anaer system, bioMérieux, Marcy L'Etoile, France

Petri dish, 35 mm, 90 mm, VWR, Vienna, Austria

PCR strips (0.2 mL and 0.1 mL), Biozym, Qiagen

Reaction vials and tubes, 1.5 mL to 50 mL, Corning, Eppendorf and Sarstedt

Cryo tubes (1,8m L), VWR, Vienna, Austria

PPE

JS-series Typ 3 Chemical and Respiratory Protective Suit, CRPS, 3M, Austria

Jupiter JP-ER-03 Powered Air Purifying Turbo, 3M, Austria

Sempercare surgical gloves, Sempermed, powder-free 150; Semperit, Austria

Sol-Vex gloves 37-900, Ansell, Medical GBU, VWR, Austria

TychemR F overall whole-body, DuPont de Nemours and Company, 3M, Austria

VersafloTM S-655 (3M, Austria)

VersafloTM S-655 Hood, 3M, Austria

Heart rate monitor Garmin Forerunner 305, Garmin Austria GmbH, Premstätten, Austria

Primers

Cytomegalovirus TRS1: Forward: 5´-ACACAGATGGAACAAAAGCAGA-3´
Reverse: 170 bp: 5´-ACGCTGTGGTTTGGAGATTGA-3´
(NCBI Reference Sequence: NC_006273.2)
(www.ncbi.nlm.nih.gov/tools/primer-blast)

Human Glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*)
Forward: 5´-CCACATCGCTCAGACACCAT-3´
Reverse: 71 bp: 5´-ACCAGGCGCCCAATACG-3´
153 bp: 5´-GTAAACCATGTAGTTGAGGTC-3´
200 bp: 5´-TTGACGGTGCCATGGAATTT-3´
277 bp: 5´-ACTTGATTTTGGAGGGATCT-3´
323 bp: 5´-AAGACGCCAGTGGACTCCA-3´

530 bp: 5´-ACGATACCAAAGTTGTCATG-3´

(Eurofins MWG Operon, Ebersberg, Germany)

Software

Randomizer (www.randomizer.at) Medical University Graz, Institute for Medical Informatics, Statistics and Documentation, Austria

Microsoft Excel 2003, Version 11 for Windows 2003. Redmond, Washington, USA: Microsoft Corporation

IBM SPSS Statistics, Release 20.0.0.2 2011. Armonk, New York, USA

Rotor Gene Q Series Software 2.0.2, Qiagen, Hilden, Germany

QuantStudio™ Software V1.3—for QuantStudio™ 6 and 7 Flex and ViiA™ 7 Real-Time PCR Systems, Thermo Fisher Scientific

Tissue processing

FLEX IHC Microscope Slides, Dako Denmark A/S, Glostrup, Denmark

Microtome HM430 Histocom, Medizintechnik Vertriebs GmbH, Wiener Neudorf, Austria

Sakura Tissue-Tek TEC Instrument, Sanova Diagnostik, Vienna, Austria

Spin Tissue Processor Microm STP 120-2, ThermoFisher Scientific