The Pre-analytical Phase of Pathology Research and Diagnostics

De Pre-analytische Fase van Pathologie Onderzoek en Diagnostiek

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Cover: Time is a major pre-analytical factor and in time, histology will be replaced by sequencing techniques.

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CONTENTS

General Introduction

Scope and context of the thesis

For the development of personalized medicine [1-3] it is necessary to know if patient biomolecular profiles are not qualitatively, or quantitatively altered by variation in the pre-analytical work flow [4-6]. Quality and quantity of biomolecules are known to vary during the time between taking a sample from the patient to the sample is processed [7-13], but also during processing before further analysis [14-18]. These pre-analytical changes can obscure disease specific changes in molecular profiles by causing high levels of background signals, especially when the actual disease biomarkers are also responsive to the pre-analytical phase. So in order to avoid losing crucial biomolecular information due to background signals during translational research, it is necessary to eliminate pre-analytical variation as much as possible. Therefore, the effects of pre-analytical variables on the biomolecular profile must be investigated to find which pre-analytical variables cause unwanted changes in the biomolecular profile. It is crucial to fully understand the pre-analytical work flow and which actions and/or circumstances can influence biomolecular profiles.

Before tissue can be used for diagnostics or research it must first be collected and then processed to enable subsequent histotechnical or molecular analysis. For tissues, the pre-analytical phase can be divided in three sub-phases; the pre-acquisition, acquisition and post-acquisition phase. During these three phases many parameters can influence the quality of tissue samples as well as their molecular profiles and can therefore influence outcome and interpretation of the subsequent analysis (see figure 1).

Practically, the tissue collection starts at the operating theatre (in case of surgical resections) or at the outpatient clinic (in case of biopsies). During surgery the surgical specimen is exposed to anesthetics and warm ischemia (WI). Biopsies are usually taken without anesthesia, and not exposed to WI. In general the surgical resection specimens are transported to the pathology laboratory unfixed. Most biopsies, however, are transported fixed in formalin, but sometimes, fresh unfixed biopsies are required for diagnosis. In case of unfixed tissue, the enzyme driven processes in the tissue cells go on and can cause unwanted biomolecule variation. As soon as the surgical specimens arrive at the pathology department, the pathologist can determine the tissue that is not needed for diagnosis and will be left over. From the left over part of the specimen, samples can be taken for research while the rest of the organ is macroscopically examined and subsequently fixed in formalin until the next day. The samples are preserved by snap freezing at ultra-low temperatures. Formalin fixed biopsies undergo macroscopic examination and are enclosed in histo cassettes for rapid processing. Unfixed biopsies are snap frozen for later analysis. Formalin fixation is always done in 4% neutral buffered

formalin, where the only variable is the duration of formalin fixation. The next day the surgical specimens are taken out of the formalin solution and samples for diagnostic purposes are taken. Depending on the fixation grade (did the fixative reach the core of the specimen, is it fatty tissue?) the tissue is fixed for another day or processed later that day. If unfixed specimens are received on Wednesday afternoon, chances are that the samples are processed during the weekend, causing a considerable variation in formalin fixation time, which is hard to standardize. After processing, the samples are embedded in paraffin and sections can be cut for diagnostic use. If the pathologist has completed his diagnostic report, the paraffin embedded tissue samples are stored in the archive.

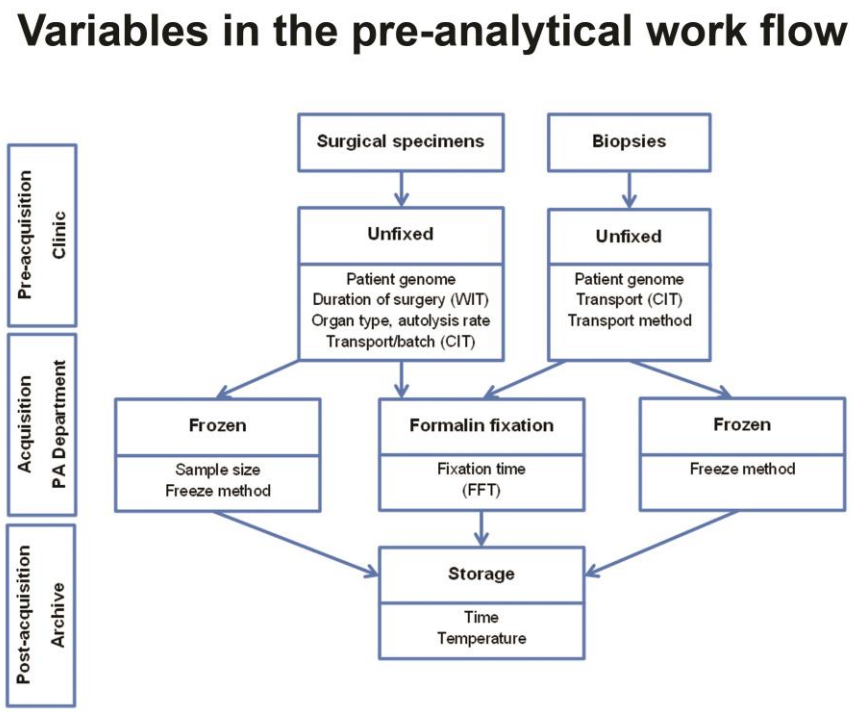

Figure 1. Variables in the pre-analytical work flow of tissue based diagnostics and research. During the pre-acquisition phase most factors (except cold ischemia) cannot be standardized. The patient genome determines how a patient sample will react to warm- and cold ischemia. The acquisition phase can only partly be standardized. Especially formalin fixation time is difficult to standardize due to fixation of different tissue types and weekend processing. During the post-acquisition phase only storage temperature can be standardized, since time is an ongoing phenomenon.

By designing specific bio specimen research studies, it is possible to simulate the entire pre-analytical workflow to study the adverse effects of varying pre-analytics on biomolecular profiles. After these pre-analytical variables are determined, it is possible to assess whether it is possible to eliminate the variability by 1) standardization of the pre-analytical workflow, 2) adaptation of analytical protocols or 3) taking note of the pre-analytical variation during data analysis. The research described in this thesis was done within the European Commission FP7 SPIDIA project. SPIDIA is an acronym for “Standardization and improvement of generic pre-analytical tools and procedures for in-vitro diagnostics”. During this project many different aspects of the pre-analytical phase of tissue based diagnostics and research were studied. One of the main goals of the project, was to use the forthcoming results to describe new (International Standardization Organization) ISO standard operating procedures (SOPs) for accreditation and/or certification [19] of both diagnostic laboratories and therewith hospital integrated biobanks.

To provide high quality care at low cost it is necessary to maintain an elaborate quality control and quality assurance system. This is achieved partly by automation and hiring low paid and relatively unskilled employees to man the machines, but mainly by implementation of a quality management system, based on the Lean/SixSigma/4P (Philosophy, Process, People&Partners and Problem solving) principle as developed by Toyota [20]. If a lab can keep track of and measure the quality of the output, the effectiveness of responses to incidents can also be measured. This will eventually lead to improvement of the workflow and in the end, it saves a lot man hours, reagents loss by unguided trouble shooting and therefore money. The entire laboratory work flow is described in a much needed fool proof network of SOPs and Work Instructions (WIs). Also, work spaces are designed to be efficient. Utensils, reagent storage, waste disposal and machinery are situated in central, strategic places in the department. Within this well-oiled machine there is hardly room for errors and if errors do occur, the incidents can be addressed and handled within the digital quality management system. Regular checks of the incident list are performed in order to continually improve the work flow, as described in the Deming “Plan, Do, Check, Act” cycle (figure 2) [21] and other ISO, Lean and Six-Sigma protocols [22].

The results and conclusions derived from experiments described in this thesis are of crucial importance to provide evidence based ISO norms, which in return will further optimize and simplify the routine pathology workflow. The seven chapters of this thesis give a chronological overview of the pre-analytical work flow of tissue based diagnostics and research; from surgery, cold ischemia, transport and freezing methods, storage of frozen samples, to immunohistochemistry on PAXgene or formalin fixed paraffin embedded tissue samples.

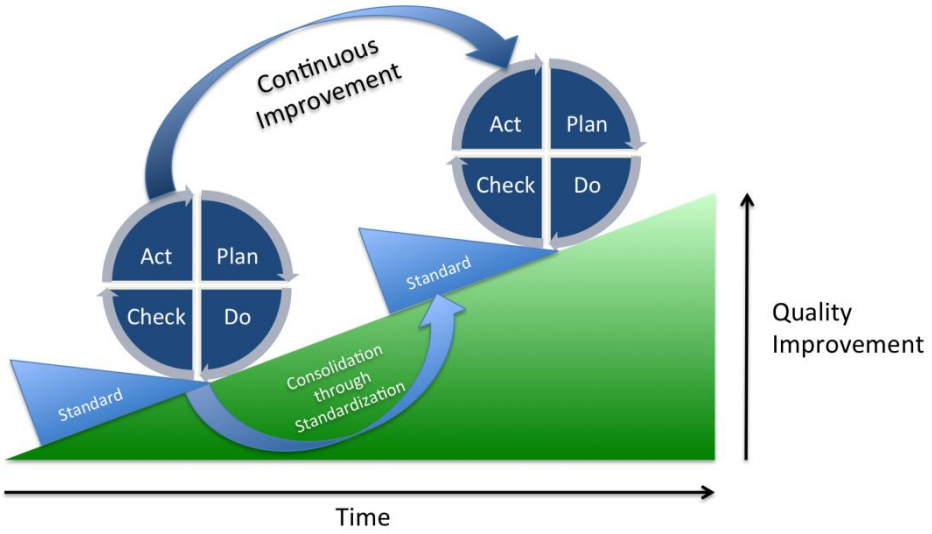

Figure 2. The Deming PDCA cycle. Continuous improvement of quality and efficiency can only be achieved by smart **p**lanning of work, **d**oing things exactly as prescribed in SOPs, **c**hecking results and **a**cting upon unexpected failure or aberration of expected results. Evidence based standardization efforts, as described in this thesis, should be made to reach increasing levels of quality and (cost) efficiency.

Variables in the pre-analytical work flow

Patient variability

During the pre-acquisition phase two main factors can influence quality and quantity of biomolecules in patient tissue specimens; the patient and the surgical technique used to obtain the tissue specimen. The patient has of course a personal geno- and phenotype upon which the concepts of “multi-omic” biomarker discovery programs and personalized medicine research are based [22-27]. The patient’s genome determines if and how a patient develops and reacts to a disease [28]. Vineis [28] described five models of carcinogenesis in which the patient’s genome plays a role. When DNA repair mechanisms fail, mutations due to exposure to carcinogens can occur (chemicals or microorganisms), genomic instability (hereditary/familiar diseases, susceptibility genes), epigenetic changes due to diet or hormones, cell selection by chemotherapy (eradicating only a subset of tumour cells) and general tissue organization (micro environment) are seen as major factors in carcinogenesis. Specific diets and habits like smoking, recreational drugs or alcohol use are notorious health risks [29-33]. Exposure to micro-organisms can

cause a wide variety of diseases, including cancer in case of human papilloma virus and Helicobacter pilori [34-37]. Recently it was discovered that even a patients' intestinal microbiome can influence disease progress and drug response [38].

It is important to realize that the patient's genome, including all the genetic predispositions a patient may have, can also influence how an individual tissue sample (taken from that individual patient) reacts to pre-analytical variables. Furthermore, these genetic and demographic factors cannot be controlled by any form of procedural standardization. For proper analysis and subsequent interpretation of multi omic profiling data it is of crucial importance to consider all patient related meta data and record the most influential parameters.

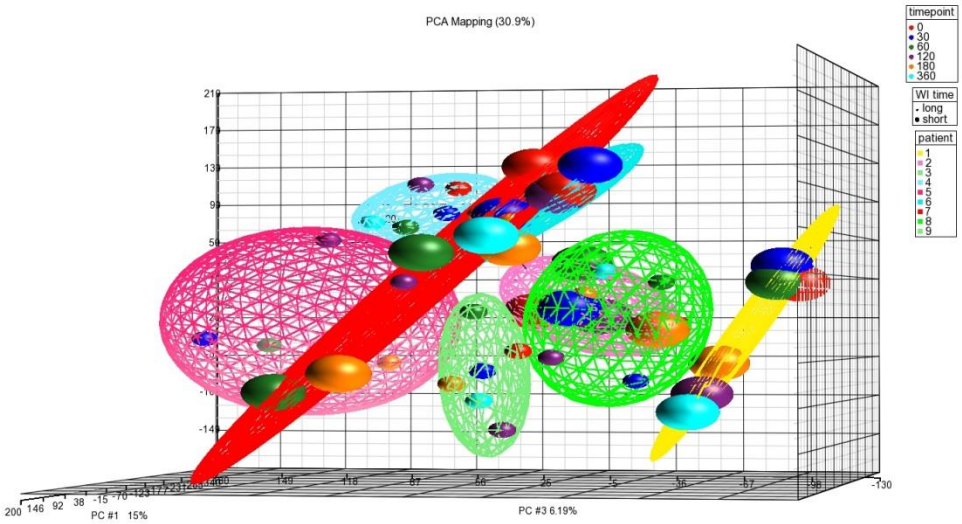

Figure 3. Patient variation as observed in the SPIDIA Ischemia RNA study. The large spheroids represent the individual patients. The solid globes within these large spheroids represent samples taken from the patients, divided by cold ischemic time (color code) and warm ischemic time (size of the globes). We can see that the samples are clustered by patient and not by ischemia related parameters. This indicates that patient variation is a stronger factor for gene expression variability than cold ischemia.

Warm and cold ischemia

In general there are two ways to obtain tissue specimens; invasive surgery and taking minimally invasive biopsies. Curative- or palliative invasive surgical procedures are the most common source of tissue specimens in hospital based tissuebanks. During surgery, the individual patient [28], is exposed to a number of procedure related and coincidental variables. As a consequence, as mentioned

above, biomolecular profiles in tissue specimens from individual patients can react differently to surgery. The adverse effects of warm ischemia, induced by clamping of blood vessels to prevent excessive blood loss, on gene and protein expression were described before, albeit it mostly in animal models or in small human cohorts [9-13, 39-41]. During warm ischemia, enzyme driven cellular processes in the organ continue under low oxygen conditions, and can cause up- or down regulation of genes and proteins which are sensitive to hypoxia [9-13, 39-41]. Disturbingly, well known tumor biomarkers like JUN, FOS, MYC and VEGF are also influenced by (predominantly) warm and (in less extent) cold ischemia [12,40].

The effects of different anesthetic methods or coincidental events (excessive blood loss, blood transfusion or intra-surgical heart failure) on biomolecular profiles in tissue were never assessed. It is, however, imaginable that these parameters can cause a general state of shock in the entire body and as a consequence, cause the biomolecular profile to change dramatically.

Despite the fact that surgical procedures are precisely performed according to well defined and standardized protocols, the individual patient's needs may cause aberrations. The duration of surgery can depend on the severity of the disease, complications and surgical skills. Therefore, also the surgical parameters described above need to be considered while analyzing profiling data.

When an organ is entirely or partly removed from the patient it has to be transported to the pathology department for further processing. Since in most cases the pathologist is responsible for further diagnostics, the decision on what is left over tissue not needed for diagnosis and further sampling needs to be carried out under pathology supervision. To save time and resources it is quite common that tissue is transported to the pathology department in batches of specimens collected at several operating theatres during the morning or afternoon. During this period of batch collecting and transport, the same enzyme driven cellular processes continue, but now the enzymes driving these processes are less active due to the lower temperature. This cold ischemic phase is a well described part of the pre-analytical process. Some articles mention major variability in gene and protein expression [9-13, 39-41], whereas other reports show only minor, or even no changes at all [42]. Especially when surgically removed tissue specimens remain intact, RNA can remain quite stable for up to 16 hours after surgery [42]. Vacuum packaging of surgical specimens [43-45] may even prolong that period. Bussolati [43], who first described the vacuum preservation concept as a replacement for formalin at operating theatres in 2008, showed that RNA was still readily detectable after the organ was vacuum sealed and stored at 4 degrees for 48 hours. This was confirmed by Kristensen [45] in 2011, furthermore he found that tissue quality was acceptable for up to 92 hours when vacuum sealed and stored at 4 degrees. Since gene array analysis has not yet been performed on vacuum stored tissue, it is yet unknown whether this vacuum preservation really preserves all macro molecules.

This period of cold ischemia, contrary to the surgery related warm ischemia period, can be standardized by managing logistics. Studying the cold ischemic phase has

led to tissue procurement guidelines, which prescribe that surgical specimens should be transported on ice (to stop enzymatic cellular processes) and that the time between surgical removal of the specimen and sample fixation (either by snap freezing or by formalin) should be less than half an hour [46,47]. This can be accomplished in a few time and money consuming steps. The first step in this standardization process is maintaining a maximum distance of 500 meters between the operating theatre and the pathology department. The second step is to avoid the batch effect. It is better for diagnostics and research alike if each specimen is transported individually, instead of collecting a batch and save some time and money on transport. For smaller samples, for which rapid transport is most crucial, this can be arranged by implementation of transport tubes between the clinic and the pathology department. Also at the pathology department, direct macroscopical examination and sampling of individual unfixed tissue specimens is preferable over batching. This implies that a pathologist must be willing to be standby during the day (and come to the grossing room for each individual specimen) to take fresh samples for the frozen tumor tissuebank and to pre-treat the specimen for further processing (ink margins, cut open to allow fixation, macroscopical examination) [46].

Freezing method

Tissue can be frozen in various ways [48], but the goal is always to reach the critical temperature of -40 degrees centigrade as rapid as possible. The faster the temperature of a tissue sample drops from room temperature to -40 degrees, the fewer (and smaller) disruptive ice crystals are formed. Tissue can be frozen directly in liquid nitrogen, which is a relatively slow process due to the Leidenfrost effect, which causes a gas layer of boiling nitrogen around the tissue sample, insulating the heat flux from the tissue . This effect is correlated to the size of the tissue sample; large samples (with a large exposed surface) will take longer to freeze entirely than small samples [48]. This Leidenfrost effect can be overcome by freezing tissue samples in pre cooled iso pentane, the cold liquid (-160°C) encloses the sample completely allowing a faster temperature exchange. Therefore theoretically, freezing small tissue samples in pre cooled isopentane would result in optimally preserved frozen tissue samples. However, cutting tissue samples into smaller aliquots may cause a release of endogenous RNase [49] and thereby, relatively stronger RNA degradation than when a tissue sample is left intact.

Figure 4. Freezing tissue in pre cooled iso-pentane. Since the establishment of the Erasmus MC tissuebank, tissue was always frozen in pre cooled iso-pentane. A glass cylinder is filled for 1/3 with iso-pentane and then lowered into a dewar vessel filled with liquid nitrogen. As soon as the iso-pentane looks misty, or feels more viscous when the sample basket is moved around in it, the accurate freezing temperature is reached to freeze tissue samples almost instantaneously.

Formalin fixation

Of course, the cold ischemia effects on biomolecules, as described above, can also be observed in tissue that will be chemically fixed. Moreover, fixation of large specimens is a slow process, formalin penetrates the tissue at 2 mm per hour [50]. If an organ is kept intact during formalin fixation, the core will remain unfixed for hours and the cold ischemia will eventually lead to cell death and autolysis. This implies that large tissue specimens must always be cut open (intestine, stomach), sliced (liver, kidney) or injected with formalin (prostate, lungs) to allow rapid fixation.

Although formalin is the most widely used fixative for over a century [50,51] and many “omic” techniques have been adapted for use with formalin fixed and paraffin embedded (FFPE) tissue [52-57], it has certain disadvantages for use in molecular research.

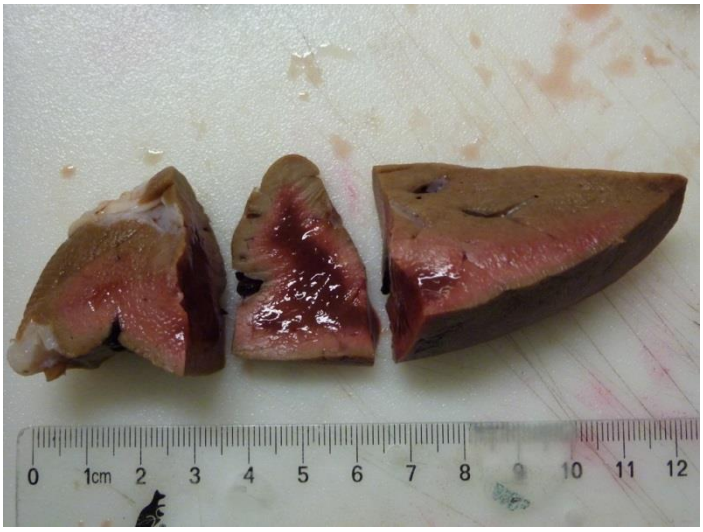

Figure 5. Formalin fixation is a slow process. Therefore, it is necessary to cut larger specimens open to allow thorough fixation. Here we see a liver segment of 2 cm thick which is not yet thoroughly fixed after overnight formalin fixation. The core of the specimen is therefore still unfixed and has been exposed to hours of cold ischemia.

Routine immunohistochemistry (IHC) relies on FFPE tissue for diagnostic use. Formalin fixation causes formation of chemical cross links between proteins, sometimes changing their three dimensional conformation [14-18]. Such conformational changes can obscure epitopes which are targets for monoclonal antibodies used in immunohistochemistry (IHC) assays. The longer a tissue sample is fixed in formalin, the more crosslinks are formed, the stronger epitopes can become masked [14,58-60]. Therefore, the most practical solution to this problem would be standardization of the formalin fixation time [14,61,62]. According to literature, fixation times between 24 and 48 hours should be considered optimal [14,63]. It is, however, not always feasible to standardize fixation time [63].

Therefore, it is more feasible to adapt the analytical phase in such a way that the IHC assays are robust enough to overcome fixation time dependent loss of antigenicity [58-60]. Boenisch [58,59] described that in theory it would be possible to obtain optimal antigen retrieval, if protein expression patterns in frozen section were used as reference. Shi [60] described that he succeeded to optimize IHC protocols to overcome formalin fixation time related antigen masking.

The last decades, alcohol based/non-cross linking fixatives were developed to replace the allegedly carcinogenic formalin fixative [64-65]. Alcohol tissue fixation is based on dehydration and subsequent coagulation of proteins and nucleic acids, rather than the formation of crosslinks [65-67]. Therefore alcohol based fixatives are believed to enhance the use of tissue for molecular diagnostics and research. With variable success, these alternative fixatives were assessed for use in

molecular research [65-67]. Morphological aspects of these alternative fixatives are also not completely the same as those of formalin fixed tissues, which pathologists have come to appreciate for over a century [50,51]. This is one of the reasons why, despite the fact that formalin is considered to have a mild carcinogenic effect, alternative fixatives never got a foothold in routine pathology laboratories. Another reason is the vast archive of FFPE blocks available for research use. If a new fixative (X) were to be implemented, a mixed archive would be generated comprising both FFPE and "X"FPE blocks, leading to problems in the analytical phase (i.e. FFPE needs different antigen retrieval than "X"FPE) of large retrospective studies. Besides that, it is yet unknown how long "X"FPE blocks can be stably stored.

Biopsies

In the case of (needle) biopsies, the entire pre analytical phase is quite different compared to surgically obtained tissue samples. Every step can in principle be standardized. There is no warm ischemia which could influence the biomolecular profile. Without these pre-analytical disturbances, it is possible to observe disease specific changes more precise. This is why the use of biopsies is preferred in prospective disease biomarker discovery studies [68].

Depending on the diagnostic question biopsies are directly put in formalin or transported unfixed to be snap frozen at the pathology department. During transport, unfixed biopsies are exposed to cold ischemia. To avoid adverse cold ischemia effects on tissue RNA, RNAlater is often used for biopsies collected for clinical trials or medical studies in general. The beneficial effects of RNAlater on RNA preservation in small tissue samples are well described [69]. However, tissue morphology is not optimally preserved [69,70]. For diagnostic purposes, or the selection of tumor cells from the biopsy reasonable frozen section morphology is obligatory [48].

Storage of tissue samples

Biobanks have been using either mechanical -80ºC freezers or liquid nitrogen storage (-196ºC) to store tissue samples for decades. During this period the follow up data on treatment effects and outcome can be enriched. In literature there is, however, no evidence that one storage method is better than the other, so it is a matter of making rational decisions based on weighing disadvantages and advantages of both methods. Storing frozen tissue in liquid nitrogen is advantageous because of the lower temperature and the fact that a liquid nitrogen tank doesn't run on electricity, which makes it less sensitive to power failure. There must, however, be a secure supply of liquid nitrogen and also the liquid nitrogen tanks are not easily accessible for automated storage and subsequent track and trace possibilities. For relatively small scale biobanks with limited financial

resources it is, however, a very good option. Large population based biobanks, which are mostly government and commercially funded, make use of sophisticated -80ºC warehouses where samples are inserted and retrieved by robots [71]. Ingenious precautions like power back up plans and well-chosen warehouse locations guarantee the required level of security [71].

In The Netherlands the FFPE archive is maintained for decades for juridical reasons. In theory, the FFPE archive is a good and abundant source of bio-information [72,73]. Many “omic” techniques have been adapted to enable the use of FFPE blocks from the archive. When miRNA, mRNA, DNA and proteins retrieved from FFPE blocks are qualitatively and quantitatively compared to those retrieved from fast frozen tissue samples, in general about 80% of all markers can still be detected [74-77]. Further refinement of extraction protocols and/or development of novel sequencing methods are expected to further enhance the use of FFPE tissue samples for “multi-omic” research.

Niland [78] showed that DNA of reasonable quality could still be retrieved from 70 year old formalin fixed celloidin-embedded blocks . Paraffin was not yet widely used in the 1930’s. Although DNA from the celloidin blocks seemed to be of inferior integrity, it still performed equally well in PCR procedures when compared to FFPE derived DNA. The proteome is quite stable in FFPE tissue, no significant differences in the amount of detectable proteins were observed when 18 months old blocks were compared to 10 years old blocks [79]. However, Von Ahlfen [80] showed that RNA degradation in FFPE blocks is an ongoing process, for unknown reasons. When FFPE blocks are stored at room temperature for longer than one year, RT-qPCR performance is adversely influenced. It is therefore advisable to store FFPE blocks that are to be used for research at 4°C or lower to preserve RNA and use the blocks for RNA isolation as soon as possible after embedding the tissue in paraffin.

Figure 6. The FFPE block archive. Blocks are normally stored at room temperature. Considering that the average academic hospital annually collects around 100.000 blocks, it is infeasible to store all blocks at the suggested 4ºC. However, if blocks are prospectively collected for research purposes, humidity controlled 4ºC storage is advisable.

The FFPE block archive is not only a rich source of tissue for biomarker discovery. It can also be used for biomarker validation. By making tissue micro arrays (TMAs) it is possible to take small cores of individual tissue blocks (donor blocks) and place those cores in a new block (recipient block) (figure 7) [81]. By doing so, one recipient block can contain up to 600 small tissue cores from 200 donor blocks, which corresponds with 200 patients. Subsequently, tissue sections can be cut from the recipient block and these can be used to uniformly detect proteins (immunohistochemistry) or nucleic acids (RNA or DNA *in situ* hybridization).

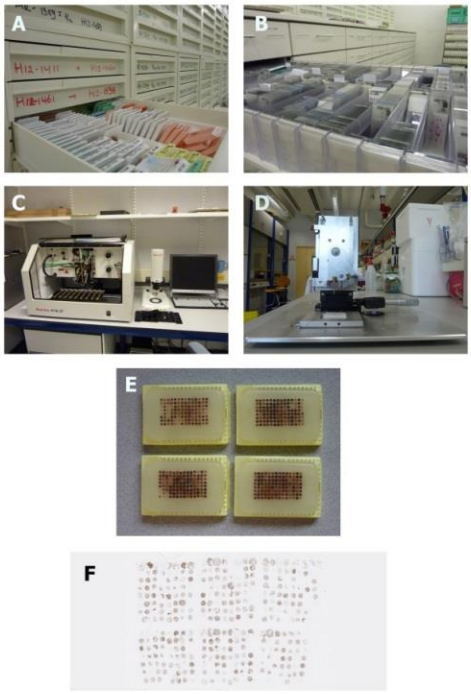

Figure 7. How to make tissue micro arrays. Archived FFPE blocks (A) can be used to take small cores of the right tissue areas (which can be selected in the corresponding slides (B)) with a fully automatic (C) or manual (D) tissue arrayer. The resulting blocks € contain hundreds of patient samples and can be stained on one slide (F).

Researchers collect tissue for genomic- and proteomic research on the frozen samples and TMA research on the adjacent formalin fixed, paraffin embedded samples. Parsons et al [82], described 5 types of TMAs that can answer specific scientific questions:

1. “Predictive TMAs”; In this type of TMA the tissue samples are taken from and arranged by responders and non-responders.

2. “Control tissue TMAs”; For routine IHC, positive control TMA can be useful for quality control and assurance of the IHC protocols.

3. “Biomarker validation TMAs”; These TMAs are often comprised of normal tissues and diseased tissues of the same patient group used for the preceding genomic research.

4. “Prognostic TMAs”; In this type of TMA usually normal tissue, pre-malignant tissue, malignant tumour tissue, lymph node metastases and distant metastases are included.

5. “Progression TMAs” are very similar to “Prognostic TMAs” when it comes to tissue content, only the scientific reasoning is quite different. With this TMA, comprised of also normal tissue, pre-malignant tissue, different stages of cancer tissue, LNM and DM, it is possible to follow the expression of one certain protein (IHC) or gene (RNA ISH) during the biological progression of the carcinogenesis. This type of TMA can also be helpful in pathway analysis.

When the TMAs are used for IHC or ISH techniques, the staining patterns have to be scored and correlated to the clinical data of the tissue samples. Scoring of staining patterns by pathologists leads to highly variable results [83] and has a negative impact on the validation of biomarkers. Auxiliary techniques like virtual microscopy and subsequent image recognition can help to make morphology and/or staining intensity based analysis more objective.

When biomarkers are validated, they can become useful in diagnostics, or for further discrimination between patient groups within a disease, or treatment cohort. When the disease cohort can be split up in, for instance, receptor positive or negative, the initial biomarker discovery data can be analyzed again to see if patients form clusters when sorted by the new discriminating receptors. This means that every tissue specimen from every patient can be used for discovery, validation and refinement of tumor geno- and/or phenotype. This continuous process will eventually lead to personalized medicine. The reliability of this process and therefore, the development of personalized medicine, can be greatly enhanced by knowing exactly what has happened with the samples during the pre-analytical phase.

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Chapter 1

Development of a RT-qPCR Fingerprint to Evaluate Pre-analytical Surgical Effects in Human Liver

M. Kap*, A.M. Sieuwerts*, A. Heine, M. Smid, C. Verhoef, C. Viertler, D Svec, M Kruhoffer, K Zatlaoukal, K-F. Becker, P. Riegman

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Abstract

Variation in pre-analytical procedures can have effects on RNA expression in tissue. Standardization of the pre-analytical workflow is not always an option, therefore it is necessary to know which factors might be of influence and should be noted as meta data in tissuebank databases. Here we describe the discovery and potential use of a RT-qPCR fingerprint of genes sensitive to the ischemic period.

The biomarker discovery cohort existed of ten patients undergoing curative operations for metastases in the liver. Sample series taken just after the start and directly after surgery followed by a time series ranging from 30 to 360 minutes enabled RNA expression monitoring during the Warm (37^0C) and Cold (RT) ischemic time.

After gene array analysis 1,146 probe sets were found to react significantly to the surgical phase only. A selection of the identified genes were confirmed by RT-qPCR and enabled the design of a pre-analytical gene expression fingerprint, comprised of six genes. This fingerprint was validated using twenty-one liver needle biopsies (no ischemia) and excision samples (warm and cold ischemia), as well as *in silico* analysis of seven publicly available liver gene array data sets. The samples from these data sets were divided in three sample type groups, which reflect the approximate length of the surgical procedure.

Gene expression *in vivo* can deviate from what is measured in *ex vivo* tissue samples. The pre-analytical RT-qPCR fingerprint showed low levels of expression before surgery and higher levels after surgery. Needle biopsies showed lower expression levels than samples exposed to surgery. *In silico* analysis showed that fingerprint expression levels are correlated with surgical technique/gross duration of surgery. Further research is necessary to find a possible linear proportional function between duration of surgery and fingerprint gene expression, to establish an evidence based normalization of gene array data.

Introduction

Finding a diagnostic biomarker or drug target with RNA based genomic platforms in multi-centre, tissue based translational studies has proven to be difficult due to limited signal to noise ratios caused by both sample handling [1] and sub optimal use of data in databases [2]. Pre-analytical parameters are believed to influence RNA expression levels as well as RNA quality [3]. Implementation of International guidelines, best practices and common minimal standards for biobanking in Standard Operating Procedures (SOPs) for tissue procurement contribute to minimize the effects of pre-analytical variation. However the biobank can only implement such SOPs from the moment the tissues are received for transport, which can be arranged as soon as the surgical specimen is taken from the body. From transport until freezing, also referred to as the *acquisition phase*, includes controllable variables like: transport time, transport temperature, freezing methodology and storage temperature [4]. However, during the *pre-acquisition phase* variables like genetic background, gender, general condition, concomitant disease, drugs, response to treatment, intervention / operation differences in terms of tools, equipment, procedure, time are parameters that cannot be standardized, simply because the priority of individual patient needs during treatment.

Of all parameters, (warm and cold) ischemic time is the most obvious and also easy to replicate in a controlled lab environment and the effects of ischemia on RNA quality and gene expression were described before [5-7]. However, in most publications the terms “warm ischemia” and “cold ischemia” were not always used in the same context. More specifically, the term warm ischemia can be used for body temperature (37^0C) or room temperature (RT), whereas cold ischemia could mean RT or cooled by refrigerator or on ice (4 - 0^0C) [5-7]. Therefore, the “Warm ischemic time (WIT)” is here defined as the period the tissue is in the body at body temperature during vessel ligation or clamping, whereas “cold ischemic time (CIT)” is the period that starts as soon as the organ or biopsy is taken from the body and is left without fixation at RT or at 4^oC. Enzyme driven cellular processes are still active under ischemic conditions [8]; therefore the hypothesis was that this would especially be the case at enzyme friendly body temperature, i.e. warm ischemia during the surgical procedure. Within the SPIDIA project, Pazzagli, et al.[9] have performed a large scale, pan-European blood RNA quality ring trial. This research resulted in a set of genes which can be used as quality biomarkers for blood samples. The goal of the research described here, was to find similar RNA quality biomarkers for tissue samples. For the EU FP7 project, SPIDIA (www.spidia.eu) samples were collected at the start of surgery and after surgery in the Erasmus Medical Center Rotterdam.

Here the separation between pre-acquisition (warm ischemia, surgery) and acquisition (cold ischemia) effects on tissue derived RNA gene expression is presented. Furthermore, with RT-qPCR assays designed to detect genes that were found to be influenced by effects during the surgical procedure, we show in this study that it is possible to formulate a pre-analytical RT-qPCR fingerprint which is able to distinguish the effects on gene expression levels during surgery in an external sample cohort. *In silico* fingerprint analysis on seven publicly available

gene array data sets, of normal/unaffected liver samples which were acquired by five different surgical procedures, resulted in further proof of concept.

Materials and Methods

Tissue collection for marker discovery

To bring pre-analytical variation caused by sampling errors due to tissue heterogeneity to a minimum, unaffected liver was considered the tissue of choice.
Macroscopically normal (non-tumorous/unaffected) liver samples from ten patients (n = 9 diagnosed with colorectal carcinoma metastases in the liver, n = 1 diagnosed with melanoma metastases in the liver) were taken during partial hepatectomy directly after first incision in the liver (T0). Directly after removal of the surgical specimen from the body, a second sample (T1) was taken, adjacent to the first, guaranteeing maximal exposure to warm ischemia and maximal tissue homogeneity. Both samples cover the period where effects like warm ischemic time, anaesthetics and pressure by manipulation of the organ for the duration of surgery (which ranged from 40-240 minutes) are induced. These samples were frozen in liquid nitrogen in the operating theatre, with a cold ischemia delay between 10 and 90 seconds.
After the resected specimen was transported (5 minutes) to the pathology laboratory, additional samples (also from the same region of the first two samples) were taken for a cold ischemia time series. Samples T2 were frozen after 30 minutes of cold ischemia at room temperature in a closed container (to prevent air-drying artefacts). Samples T3, T4, T5 and T6 were frozen after respectively 60, 120, 180 and 360 minutes. Due to logistical reasons, T6 samples from only six patients could be collected (see supplementary **Table S1** for sample data). In general the sample size was around 8 x 4 x 4 mm. The approval to use the tissue samples within the SPIDIA project was obtained from Erasmus MC Medical Ethical Commission under number MEC-2008-397.

RNA isolation, microarrays, QC, and normalization procedures

For RNA extraction from liver tissue the miRNeasy Mini Kit (Qiagen) was used following the manufacturers' instructions. Briefly, frozen liver samples (max 50 mg) were quickly transferred to 2 mL Safe-lock tubes on ice each containing 700 µL QIAzol (Qiagen, Germany) and 2 steel balls. The tissue was disrupted on a Tissuelyser II in two bursts 2 min at 20 Hz. The RNeasy kit (Qiagen, Germany) was used according to the manufacturers' description to isolate the RNA. As quality control before micro array analysis, concentration and purity was measured with the NanoDrop 2000 to obtain measurable concentration as input for the bioanalyzer (Bioanalyzer 2100, Agilent, USA) where RIN values, concentration and purity were measured .
For microarray analysis 300 ng of total RNA was labelled using the 3 IVT Express Kit according to the manufacturers' manual. The resulting copy RNA was

hybridised to the Affymetrix U219 array plate on the GeneTitan system. Wash, staining and scanning of the microarray plates was automatically performed after hybridisation on the GeneTitan system.
The Human Genome U219 microarray detects 3'-labeled RNAs and thus the polyadenylated transcriptome is interrogated. Affymetrix Gene Expression Console software was used to perform QC of data, and subsequently normalize the raw microarray data. First, the microarrays were quality checked by analysing microarray pictures to look for dots and scratches. Next, microarray performance was assessed by analysing the distribution of raw microarray signal intensity data.
The (robust multi array) RMA normalized and 2-log converted gene array data, delivered in an Excel file by AROS, were analyzed using the Time Course Analysis and the Class Comparison univariate two-sample T-test analysis modules of the BRB ArrayTools Excell plugin. After the gene array data analysis (more details in Results section), 1,146 probe sets were found to significantly react to the effects during the surgical procedure (T0 compared to T1). None of the probe sets reacted significantly to cold ischemia (T1-T6).

Primary marker validation with RT-qPCR and Fingerprint design

Remaining RNA of above mentioned n=67 samples were sent back from AROS to Erasmus MC for RT-qPCR experiments using these assays to detect above mentioned genes. Samples from the patient diagnosed with melanoma metastases were not used for RT-qPCR to avoid possible disease induced heterogeneity of the normal liver samples, leaving n=60 samples for qPCR validation.
cDNA from 2 µg input RNA was prepared using the Fermentas cDNA synthesis kit (Fermentas GmbH, Germany) as described before [11]. To prevent amplification of gDNA, potentially still present in these RNA preparations, gDNA was removed by an heat-labile double strand specific DNase (HL-dsDNase) step as described by the manufacturer (ArcticZymes, Norway) prior to preparing the cDNA. Complete removal of gDNA after this step was confirmed with ValidPrime (TATAA Biocenter, Sweden).
Before the actual PCR, the samples were diluted 20 times to result in a final cDNA concentration of 2.3 ng/ µL. PCR was performed on the Agilent Stratagene Mx3000P platform (Agilent, USA) using 96-well plates. Eight target genes were validated (small (83 – 105 bp) and large (250-389 bp) amplicons, so 16 assays), furthermore, 3 reference genes (*TBP, HPRT1* and *HMBS*; 94-192 bp amplicons) were included in the analysis (see **Table 1**). The reference genes were readily available as SYBR green primer assays (Invitrogen, The Netherlands). The run protocol for the SYBR Green primer assays consisted of: Activation; 95°C 15min (1 cycle), Amplification; 95°C 15s, 62°C 30s, 72^{o}C 30s, 79^{o}C 30s (40 cycles), followed by a melting curve analysis. A reaction volume of 25 µL was used for all RT-qPCR runs (5 µL cDNA and 20 µL Master Mix; 330 nM primers and 50% v/v master mix). The target gene assays (see **Table 1**) were FAM-BHQ1 labelled primer/probe assays, provided by SPIDIA partner TATAA Biotechnology. The run protocol for the FAM primer/probes assays consisted of: Activation; 95°C 15min (1 cycle), Amplification; 95°C 30s, 60°C 60s, (40 cycles). A reaction volume of 20 µL was

used for all RT-qPCR runs (5 µL cDNA and 15 µL Master Mix; 200 nM primers, 50 nM probe and 25% v/v master mix).
Raw Cq values were transformed into relative expression by subtracting the target gene Cq values from the average Cq values of the three reference genes. Mann-Whitney U tests confirmed the significant up and down regulation of 5 of the 8 target genes. The down regulated (in the array analysis) gene *GLG1* and stable genes *NONO* and *ALDH2* did not show any significant changes (see Results section for further details).
Before further validation, an algorithm was calculated which consisted of the relative expression of the 6 significantly changed genes, multiplied by the Z-value derived from the Mann-Whitney U test for determination of the weight of the factor. Since the secondary validation would be comprised of comparing needle biopsies to excision specimens, we analyzed gene expression differences between T0 samples and T1 to T6 samples (covering the entire cold ischemia period, in which no significant gene expression changes occurred according the array data) in order to create the pre-analytical fingerprint values (see **Table 2**). For this, all samples from T1 to T6 combined were included to represent all excision specimens with unknown post-surgical ischemia times. Down regulated genes were subtracted from - and up regulated genes were added to- the equation. With this formula, a new variable was generated and subsequently fingerprint values for all samples were calculated.

Fingerprint Value = (*relative expression up regulated gene* * Z-value) **-** (relative expression down regulated gene * Z-value) + (relative expression up regulated gene * Z-value)

Validation of the fingerprint

To validate the RT-qPCR markers on an independent cohort, RNA was isolated from n = 21 liver needle biopsies and n = 21 liver excision samples. Needle biopsies were taken without the influences of warm ischemia, anaesthetics and organ handling, whereas excision samples had been exposed to these factors. This approach would resemble comparing, respectively, T0 and T1-T6 samples from the discovery cohort. The samples were randomly taken from the tissue bank quality control cohort wherein liver samples stored for 15+ years at -80^{o}C were tested for RNA integrity (see supplementary **Table S2**). RNA was isolated by cutting 20 µm frozen sections and using the same RNA isolation kit as described above, except in this case, tissue was homogenized by shaking the frozen sections in the QIAzol solution. Contrary to the above described experiment, the underlying disease was not exclusively colorectal carcinoma metastasis (see supplementary **Table S2**). The RNA concentration (median 65 ng/µL, range 35-188 ng/ µL) in the biopsy group was too low to perform our standard cDNA synthesis (starting with 100 ng/µL), therefore the samples were vacuum dried (SpeedVac AES1010, Savant, USA) for 15 min at 45°C and reconstituted in the required volume of RNase/DNase-free water to reach a final RNA concentration of 100 ng/µL. To assure equal sample treatment, equal amounts of RNA were taken from the

Table1. Primer and probe sequences.

Small amplicon target gene qPCR assays

Gene name	Ref seq	Forward primer, sequence 5'--> 3'	Reverse primer, sequence 5'--> 3'	Probe (FAM-BHQ1 labeled)	PCR product size (bp)
HSPA1A	*NM_005345*	GGGCTTCGGGGCTCA	AACAGCAATCTTGGAAAGGC	CCTGACCCAGACCCTCCCTTGGGACC	89
NONO	*NM_001145410.1*	CGGATGGAACTTTGGGATTG	GTTGAATGCAGGAGGAGTTC	TCCATTGTAGCAGCCTGACCAAAGCGT	104
MYCL1	*NM_001033081.2*	GGTCCGAGAGCCCAAG	GCTTCCGAATACCCAGAGA	CTTCTCTACTGTCACAACATCAATTTCTTCATTCTCCG	84
ALDH2	*NM_000690.3*	CATGGACGCATCACACAG	TTGTCCAGGGTCTCCAAGG	TGCTGAACCGCCTGGCCGATCTGAT	96
JUN	*NM_002228.3*	CAGCCCAAACTAACCTCAC	TTGAGGGCATCGTCATAGAA	TGCAGTCATAGAACAGTCCGTCACTTC	83
ADAMTS4	*NM_005099.4*	CTCCTCTTGGAAGCCCC	CATCTTGTCATCTGCCACC	CCCCAGACCCCGAAGAGCCAAGC	98
GLG1	*NM_001145666**	AAGGTCAACCTGCTCAAGAT	GTACCGGGTCAACAAAGATG	ATTGTGTAAAAAGGAAGTGCTAAACATGCTGAAGGAA	97
PLAUR	*NM_001005377.2**	GAGGTTGTGTGTGGGTTAGA	GTCTGATGAGCCACAGGAAA	AACTCTGGCCGGGCTGTCACCTATTCC	105

Large amplicon target gene qPCR assays

Gene name	Ref seq	Forward primer, sequence 5'--> 3'	Reverse primer, sequence 5'--> 3'	Probe (FAM-BHQ1 labeled)	PCR product size (bp)
HSPA1A	*NM_005345*	GGAGGCGGAGAAGTACAAA	AACAGCAATCTTGGAAAGGC	CCTGACCCAGACCCTCCCTTGGGACC	389
NONO	*NM_001145410.1*	CACAACCAAGAGGTGCAAAA	GTTGAATGCAGGAGGAGTTC	TCCATTGTAGCAGCCTGACCAAAGCGT	378
ALDH2	*NM_000690.3*	CATGGACGCATCACACAG	GCTACCTTCATCACAACCAC	TGCTGAACCGCCTGGCCGATCTGAT	348
JUN	*NM_002228.3*	GACTTTTCAAAGCCGGGTAG	TTGAGGGCATCGTCATAGAA	TGCAGTCATAGAACAGTCCGTCACTTC	307
ADAMTS4	*NM_005099.4*	AGCCCTGTTAGGCGTG	CATCTTGTCATCTGCCACC	CCCCAGACCCCGAAGAGCCAAGC	250
GLG1	*NM_001145666**	GGTATCCTGACTAAGGCCAA	GTACCGGGTCAACAAAGATG	ATTGTGTAAAAAGGAAGTGCTAAACATGCTGAAGGAA	346
PLAUR	*NM_001005377.2**	TGCATGCAGTGTAAGACCAA	GTCTGATGAGCCACAGGAAA	AACTCTGGCCGGGCTGTCACCTATTCC	300

* variant used for design

Cybr Green assays	**Forward primer, sequence 5'--> 3'**	**Reverse primer, sequence 5'--> 3'**	**product size (bp)**
HPRT1	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG	192
HMBS	CATGTCTGGTAACGGCAATG	GTACGAGGCTTTCAATGTTG	139
TBP	TTCGGAGAGTTCTGGGATTG	ACGAAGTGCAATGGTCTTTAG	94

excision group and also diluted to 100 ng/µL. Next, these samples were similarly vacuum dried and reconstituted in water, to assure equal treatment of all samples. After assessing the quantity and purity by Nanodrop and the integrity of the RNA with the Agilent BioAnalyzer, cDNA was prepared from 1 µg input RNA using the same procedure as described above. The same analyses were performed on these cDNA samples, using the same amount of input cDNA and PCR assays as described before.

In silico validation of the RT-qPCR Fingerprint

After the biopsy vs excision sample experiment was performed and analysed, it was clear that the fingerprint could differentiate samples that had, or had not been exposed to surgery. The next question that arose was; would the fingerprint be sensitive enough to distinguish different sample types, obtained during different surgical procedures (variable duration of surgery/exposure to warm ischemia)? To investigate this question, publicly available gene array data from seven different studies, human liver (normal/unaffected), all analysed with Affymetrix HG-U133-Plus-2 chips (no sufficient data obtained with Affymetrix U219 chips was available), was downloaded (CEL files) from Geo Datasets [12-18]. The related articles [19-25] were downloaded and relevant pre-analytical data; sample type/surgical procedure and RNA isolation method was used during further analysis (see supplementary **Table S3**).
The samples were divided into three groups: excision (n = 62), transplantation (n = 17) and biopsies (n = 69). Excision samples consisted of samples taken during curative surgery, these procedures typically take 2 to 5 hours during which the tissue is exposed to warm ischemia, handling and wound trauma. Transplantation liver samples were taken from donors and receivers (explant livers). In the case of explant liver specimens (n = 10), the organ is removed in its entirety, on average this procedure takes 1.5 hours. Donor liver samples (n = 7) were taken from organ transplant donors who had been brain dead for less than 3 hours. Although not mentioned in the original article, typically organ donors are perfused with cooled perfusion fluid to avoid warm ischemia during harvesting of the donor organs. So even when the liver is not the first organ harvested, the warm ischemia effects will be minimal. The surgical procedure to remove the donor organ also takes 1.5 hours on average. Since the donor and explant procedure are quite comparable when the procedures are considered, it was deemed acceptable to put the donor and explant data in one group to enhance statistical power. General surgical procedure duration numbers and details were kindly provided by dr. M.C. Warlé, transplant/vessel surgeon at Nijmegen University Medical Center. Wedge biopsies (n = 4) were taken during gall bladder surgery and were least likely to be subjected to ischemia or handling, since the liver was not the main subject of surgery and therefore

probably no major blood vessels had been clamped. The surgical biopsies (n = 65) were taken while patients were anesthetized, no further details were described, but this description implies that the samples were taken during surgery and are of comparable quality as the wedge biopsies. To enhance statistical power, it was deemed correct to combine the two types of biopsies in one group.
To summarize the assumed ischemia effects: excision samples would be subjected to the highest level of ischemia, transplantation samples would have intermediate levels of ischemia, whereas the biopsies would not have been subjected to ischemia at all. Therefore, it was our hypothesis that fingerprint expression in biopsies would be low, intermediate expression was expected in transplant samples and high expression was expected in excision samples.
The CEL files were put in R array analysis software [26], gene array data was RMA normalized and log2 transformed. Principal component analysis (PCA) with the top 2,000 variable probe sets was performed. The 6 fingerprint genes, selected during the marker discovery, were isolated from the array data and the PCA was repeated using only the 6 fingerprint genes.
The gene chip expression values of the 6 fingerprint genes were used as virtual PCR data to determine the fingerprint values as described above. The virtual fingerprint data was then analysed to find fingerprint expression differences between sample type (i.e. approximate duration of surgery/exposure to warm ischemia). Effects of the RNA isolation methods on gene expression were analyzed by considering *GAPDH* expression as well as expression of the two stable genes *NONO* and *ALDH2* in the three sample type groups.

Statistical analysis

BRB Arraytools was used for Time Course analysis to find significantly deregulated genes (2-fold change, false discovery rate of 0.05) during the entire ischemic time series (T0-T6) and the cold ischemic time series (T1-T6) in the discovery cohort. Gene expression analysis between T0 samples and T1 samples was performed using the Class Comparison univariate two-sample T-test (2- fold up or down regulation, permutation p-values for significant genes were computed based on 10,000 random permutations, nominal significance level of each univariate test: 0.001).
RT-qPCR results were analysed with the Mann-Whitney U test in SPSS, all tests were 2-sided, and unless indicated otherwise, $P<0.05$ was considered statistically significant.
The downloaded CEL files, used for the *in silico* finger print validation, were put in R array analysis software [26], gene array data was RMA normalized and log2 transformed. PCA with the top 2000 variable probe sets was performed. The 6 fingerprint genes, selected during the marker discovery, were isolated from the array data and the PCA was repeated using only the 6 fingerprint genes.

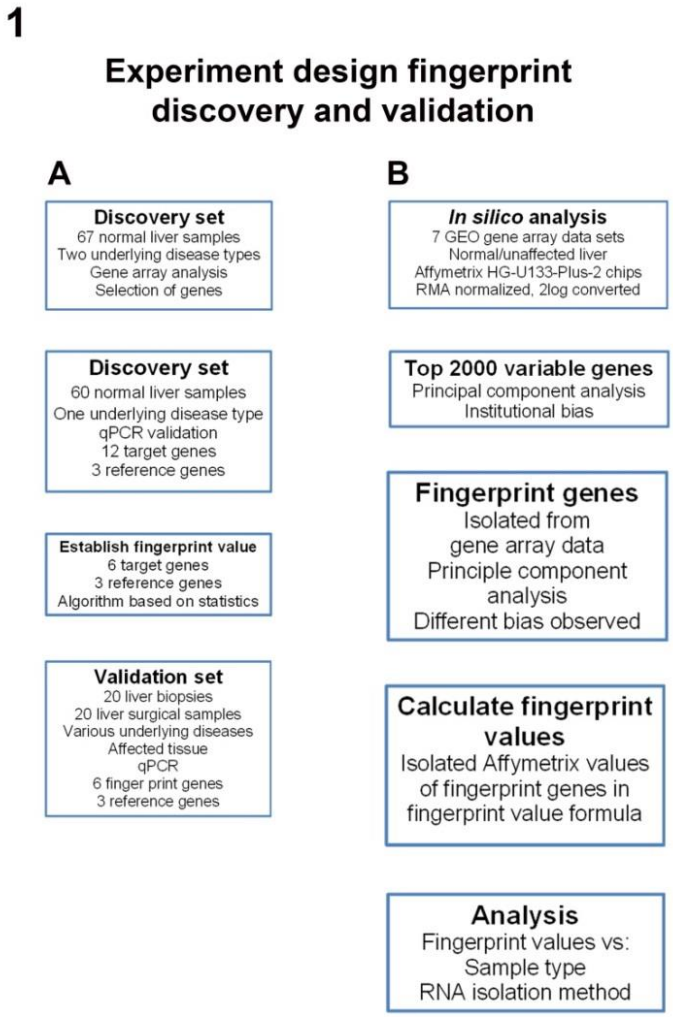

Figure 1. Experiment design fingerprint discovery and validation. Panel A shows the actual performed experiments from gene array analysis, validation of gene array results with qPCR, the establishment of the fingerprint values to the validation of the fingerprint with qPCR on an external sample cohort. Panel B shows how the fingerprint was validated on virtual normal liver samples.

Results

Marker discovery

BRB ArrayTool Time Course analysis of all time points (WIT and CIT combined) showed that 2,713 of all probe sets show significant changes in expression level (supplementary **Table S4**).
When T0 was excluded from the Time Course analysis, thus leaving CIT as only variable, no significantly up or down regulated genes were found, indicating no adverse effects on gene expression in human liver during six hours of cold ischemia. These findings showed that up- or down regulation is only observed during surgery and gave no significant increase or decrease expression during the entire cold ischemia time series.
Since significant gene expression alterations were found only to occur between time points T0 and T1, T0 was compared with T1 using Class Comparison Two-T-test analysis. 1,146 of 26,813 probe sets that passed the 2-fold change and False Discovery Rate (FDR) 0.05 filtering criteria showed significant 2-fold lower or higher expression changes (704 up regulated and 444 down regulated genes; supplementary **Table S5**). Remarkably, down regulated genes did not show fold changes higher than 3, whereas up regulated genes showed up to 12 fold increase of expression.
A selection from these genes based on unanimous high up- or down regulation and on expression in most human tissue types (according to The Human Protein Atlas) [27], resulted in 4 significantly up regulated genes (*HSPA1A*, *ADAMTS4*, *JUN* and *PLAUR*), 2 significantly down regulated genes (*GLG1* and *MYCL1*) and 2 stable genes (*NONO* and *ALDH2*) were selected for primer/probe design and subsequent RT-qPCR validation (**Figure 2**).

Primary marker validation with RT-qPCR and Fingerprint design

RT-qPCR was performed on the remaining cDNA of the n=60 (only samples from patients with underlying colorectal metastases were used) samples from the discovery set. RT-qPCR with primers and probes designed to prove these genes exhibit the same patterns of expression as found in the gene arrays when relative expression (target gene relative to three reference genes) was calculated. *HSPA1A*, *ADAMTS4*, *JUN* and *PLAUR* showed a significant up regulation between time points T0 and T1-T6, whereas *MYCL1* and *GLG1* showed significant down regulation (*P* values are shown in **Table 2**). The stable genes *NONO* and *ALDH2* show no significant reaction to WIT or CIT.

Table 2. Statistical significance of fingerprint genes T0 vs T1-6

Statistical analysis	*HSPA1A*	*MYCL1*	*JUN*	*ADAMTS4*	*GLG1*	*PLAUR*
Mann-Whitney U	12.000	58.000	26.000	17.000	110.500	13.000
Wilcoxon W	57.000	1384.000	71.000	62.000	1436.500	58.000
Z	**-4.503**	**-3.551**	**-4.213**	**-4.399**	**-2.464**	**-4.482**
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.014	0.000

Fingerprint Value = rel. expr. *HSPA1A* * 4.503 - rel. expr. *MYCL1**3.551 + rel. expr. *JUN**4.213 + rel. expr. *ADAMTS4**4.399 - rel. expr. *GLG1**2.464 + rel. expr. *PLAUR**4.482

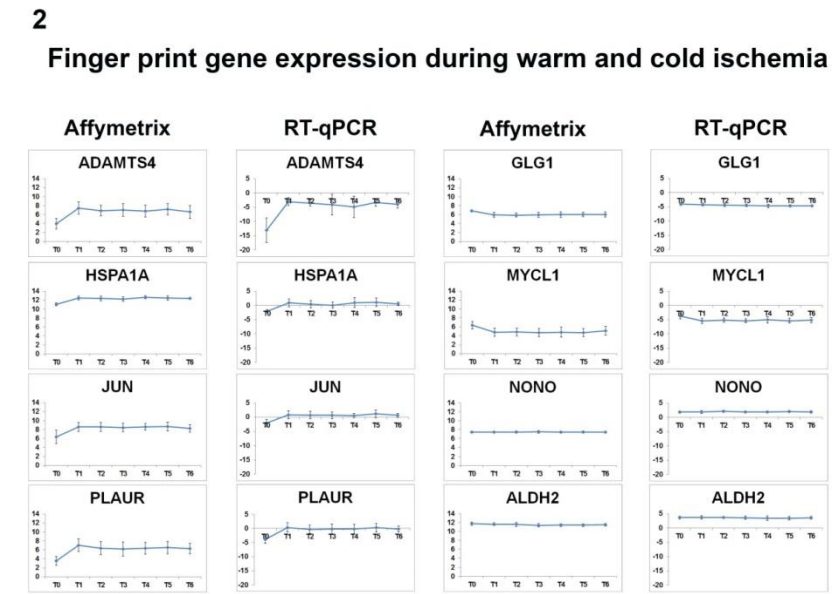

Figure 2: Comparison of affymetrix and RT-qPCR gene expression variation during WIT (T0-T1) and CIT (T1-T6) of the selected gene set. Affymetrix: On the Y-axis, the 2-log expression after RMA normalization is given, on the X-axis the seven ischemia time points are shown. **RT-qPCR:** On the Y-axis relative expression (Cq average 3 ref - Cq target gene) is shown, on the X-axis the ischemia time points are given.

Secundary marker validation and primary fingerprint validation

The fingerprint RT-qPCR assays that were found to be able to distinguish the T0 samples (no WIT) from the T1-6 (WIT + CIT) samples: *HSPA1A*, *ADAMTS4*, *JUN*, *PLAUR, MYCL1* and *GLG1* and the reference genes were used to detect fingerprint expression levels in liver biopsies (no WIT) and liver excision samples (variable WIT + CIT). Not all individual gene expression levels, as found in the discovery cohort, were significantly different between these two sample groups. However, the fingerprint values of the biopsy group were significantly lower than those of the excision group (**Figure 3**).

In silico external validation of the fingerprint

To further explore the fingerprint, which is able to distinguish between samples exposed to WIT and biopsies which had not been exposed to WIT, seven gene array data sets were downloaded. The samples used to generate these data sets had all been procured during different surgical procedures, which could be divided into three main WIT categories. When principal component analysis (PCA) with the top 2,000 variable genes was performed on the RMA normalized, 2log transformed downloaded gene array data, distinct clustering of the individual institutes could be observed (**Figure 4A**). When PCA was performed with the six fingerprint probe sets, a moderate degree of clustering was found (**Figure 4B**). The pattern was, however, less distinct and also the clustering seemed to be based on other factors than institutional bias.

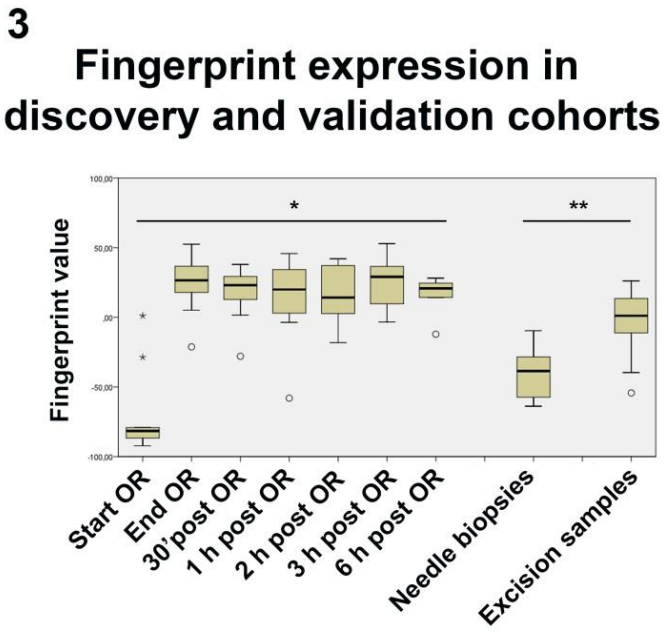

Figure 3. Fingerprint expression profiles of the discovery and biopsy/excision cohorts. At the start of surgery (start OR), the fingerprint values (Y-axis) are low. Whereas after surgery (end OR) the fingerprint levels are significantly elevated (* $P < 0.001$), but not further altered by cold ischemia (30' to 6 h post OR). Also, the samples obtained from the Erasmus MC tissue bank originating from biopsies have a lower expression than the excision samples (** $P < 0.001$), which are both procedures that have an avarage shorter warm ischemic time (excision) to almost no warm ischemic time (needle biopsies) then the discovery cohort in comparison to the OR cohort. The observations obtained from the biobanked samples are in agreement with the discovery cohort results.

Next, the external gene array data of the six fingerprint gene probes were used to calculate the fingerprint values with the same algorithm as designed during the marker discovery, after which the data was analysed against pre-analytical variables derived from the respective articles. Five different sample types; excision, explant, donor, wedge biopsies and biopsies taken under anaesthesia were described in the original articles. To enhance statistical power, the samples were combined into three groups; biopsies, transplant samples and excision samples. When the fingerprint values were analysed, significantly different expression levels were found between the three different sample types. The biopsies showed relatively low fingerprint

expression levels, the transplantation samples showed intermediate expression and the excision samples showed the highest expression levels (**Figure 5A**). Expression levels of reference gene *GAPDH* and stable genes *NONO* and *ALDH2* are relatively stable between the different sample types and therefore between the different RNA isolation methods (**Figure 5B**).

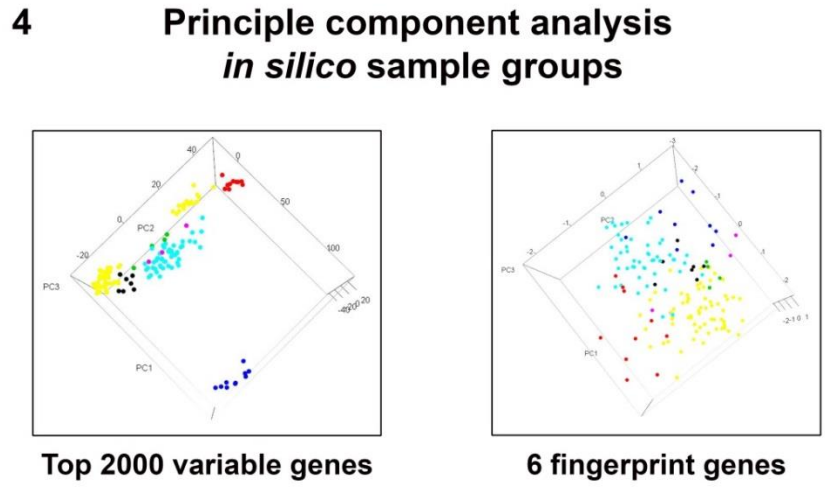

Figure 4. Principal component analysis (PCA) of seven virtual sample cohorts. Figure 4A shows a PCA plot based on the top 2,000 variable genes as found in the *in silico* analysis. Here, the seven virtual sample cohorts, representing seven institutes (represented by the different colors), form clusters. Figure 4B shows a PCA plot of the same virtual sample cohorts based on the six selected fingerprint genes. Here, the clusters are less distinct and not based on the individual institutes.

Discussion

Variation in the pre-analytical phase of tissue sample procurement is known to cause unwanted bias in gene array measurements, which may lead to poor reproducibility of results, or to poor results during identification and validation of biomarkers [1]. In literature, mostly the effects of cold ischemia have been described [5-7]. Here, the first distinct difference between warm ischemia and cold ischemia in human tissue was studied in samples taken at the start and the end of surgery (warm ischemia) and in a cold ischemia time series (0 to 360 minutes post-surgery).

Gene expression patterns in this discovery cohort showed that significant changes in gene expression only occurred during surgery. The cold ischemia time series showed no significant changes. It is remarkable that the gene expression level in the post-surgical samples (e.g. the cold ischemia time series) was caused by changes during surgery, therefore it seems that gene expression variation is mainly based on surgery related gene expression changes. During surgery, many parameters may influence gene expression. Warm ischemia due to clamping of blood vessels, but also the type of anaesthetics (metabolized in the liver), complications leading to blood loss and tissue handling by the surgeon will probably play a role. It is feasible to assume that the duration of surgery is therefore proportional to gene expression variation in post-surgical tissue samples.
Putative pre-analytical RNA quality biomarkers were found with gene expression array analysis. For a selection of 8 genes, these findings were confirmed and validated with RT-qPCR on the discovery samples. To robustly measure the effects of warm ischemia on gene expression variation, a fingerprint of 6 up and down regulating genes was designed. It was found that pre-surgical samples (no warm ischemia) showed low fingerprint levels, whereas post-surgical samples (warm ischemia and cold ischemia) showed high fingerprint levels (see **Figure 3**). This finding was validated by comparing fingerprint expression levels of needle biopsies (no warm ischemia, thus comparable to the pre-surgical samples) to post-surgical (excision) samples (variable cold ischemia, thus comparable to the cold ischemia samples). In agreement with the findings in the discovery cohort, the needle biopsies showed low finger print levels and the excision samples showed high fingerprint levels (see **Figure 3**).

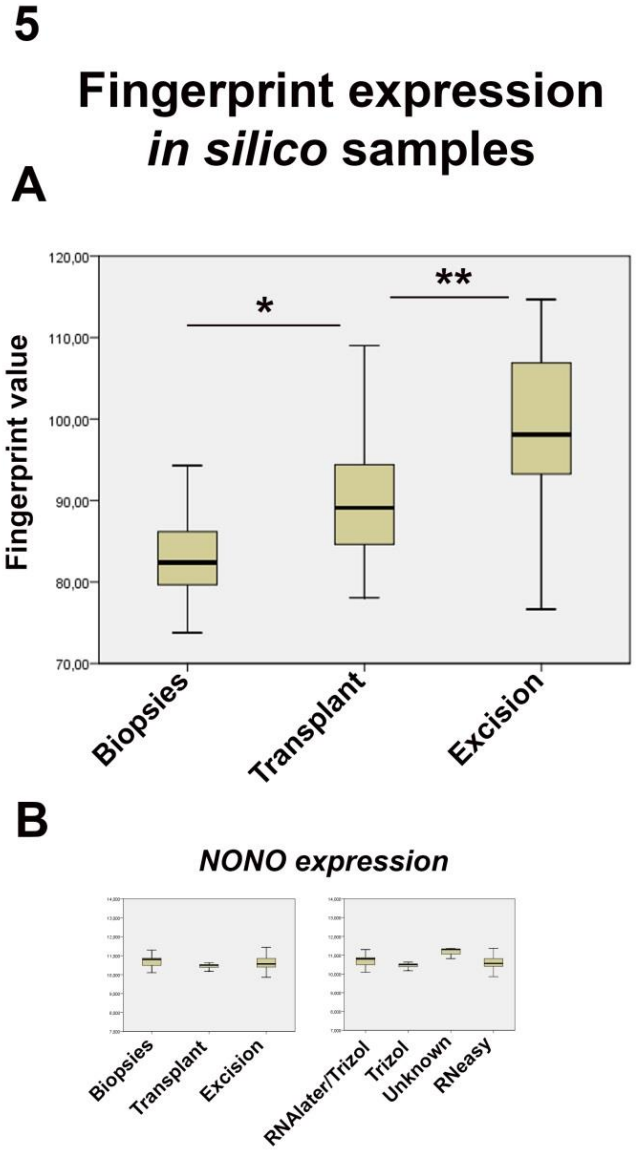

Figure 5A. Fingerprint data analysis of the *in silico* sample cohorts. The fingerprint expression levels of the different sample types are shown. Biopsies show significantly lower expression levels than transplant samples (* $P = 0.001$), while transplant samples show significantly lower expression levels than the excision samples (** $P = 0.003$). **Figure 5B** shows the expression levels of stable gene *NONO*, in relation to sample type and RNA isolation method in approximately the same scale as the finger print expression in **Figure 5A**.

An *in silico* analysis on gene array data downloaded from the GEO database revealed a correlation of fingerprint expression level with the surgical procedure during which the samples were taken. As expected (with regard to the results found in the discovery and biopsy versus excision experiments); excision samples (long surgery; relatively long warm- and cold ischemia) showed high fingerprint levels, transplantation samples (relatively short surgery; relatively short warm and cold ischemia) showed intermediate levels and the biopsy samples (only cold ischemia) showed the lowest fingerprint expression levels. The influence of the different RNA isolation methods (which partly overlapped with the sample types; see supplementary **Table S3**) on gene expression in the *in silico* cohorts are minor, considering the expression levels of stable gene NONO (**Figure 5B**). Therefore, the observed significant fingerprint expression level differences in the *in silico* cohorts (**Figure 5A**) can be attributed to the sample type, hence the gross duration of surgery, rather than the coincidentally correlated RNA isolation methods.

All experiments were done with normal/unaffected human liver samples; however, the biomarkers were selected because of the wide expression in most tissue types in the human body. *JUN, PLAUR, ADAMTS4, HSPA1A*, *MYCL-1* and *NONO* code for functional proteins and are known or suspected oncogenes. *PLAUR* and *ADAMTS4* are involved in extra cellular matrix degradation, which would, as a prelude to breaking off dead tissue, explain up regulation during hypoxic stress (warm ischemia). *HSPA1A* is also known as heat shock protein *HSP70-1*, which has been previously described (together with JUN) to react to ischemia in colon samples [28]. During the RT-qPCR validation, these biomarkers were also measured in randomly picked colon, lymph node and kidney tissue (data not shown). The expression levels of the genes in these tissue types seemed to be sufficient for accurate measurement. Of course, the separate RT-qPCR assays must be validated on a wide variety of tissue types (obtained by different surgical procedures) to see if the fingerprint can be used in this configuration, or if there is a need to develop tissue type specific pre-analytical fingerprint algorithms.

The discovery cohort was too small to find a significant correlation between the duration of surgery and gene expression levels. However, the *in silico* analysis showed that fingerprint expression is correlated to the surgical procedure and therefore, in a somewhat crude way, the duration of surgery. Based on statistical methods described by SPIDIA partner Pazzagli, et al. [9], it would be possible to use the fingerprint as a RNA quality biomarker for tissue derived RNA. If future research would result in a proportional function between fingerprint expression levels and duration of surgery, the duration of surgery might become a crucial parameter to help normalize tissue RNA based genomic data in an evidence based way, rather than a statistical way.

In conclusion, when surgical tissue specimens are used for RNA based transcriptomic research, it is advisable to record parameters of patient,

disease/treatment status and surgical data that might be of influence on gene expression, when analysing gene array/RNA expression data.

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Chapter 2

Post-mortem tissue biopsies obtained at Minimally Invasive Autopsy: an RNA-quality analysis

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(submitted for publication)

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Anita van der Linden*, Britt M. Blokker*, Marcel Kap, Annick C. Weustink, Peter H.J. Riegman, J. Wolter Oosterhuis

Abstract

Introduction: Bereaved relatives often refuse to give consent for post-mortem investigation of deceased cancer patients, mainly because of the mutilation due to conventional autopsy (CA). Minimally invasive autopsy (MIA) may be a more acceptable alternative and, if implemented in clinical practice, creates an opportunity to more often obtain post-mortem tissue samples of (recurred) primary tumors and metastases for molecular research. As a measure for overall tissue quality for molecular studies, we hereby present a feasibility study, comparing the RNA quality of MIA and CA samples, and fresh frozen samples as reference.
Materials and methods: Tissue samples of heart, liver and kidney were prospectively collected from 24 MIAs followed by CA, and compared to corresponding archival fresh frozen tissue. After RNA isolation and RT-qPCR, RNA integrity numbers (RIN) and *GAPDH* expression (six amplicon sizes ranging from 71 to 530 base pairs) were measured. RIN values and *GAPDH* Cq values were analyzed and compared between all sample groups and post-mortem intervals (PMI).
Results: RIN values in MIA samples were significantly higher than those in CA samples. *GAPDH* was significantly higher expressed in MIA samples than in CA samples and 530bp PCR products could be measured in all cases. *GAPDH* expression was significantly lower in samples with PMI >15 hours. As expected, the samples of the fresh frozen reference standard performed best in all analyses.
Conclusion: MIA samples showed better RNA quality than CA samples, likely due to shorter PMI. Both had lower RNA quality and expression levels than fresh frozen tissue, however, remaining *GAPDH* RNA was still sufficiently intact. Therefore, other highly expressed genes are probably detectable. Gene array analysis should be performed to gain insight into the quality of entire post-mortem genomes. Reducing PMI will further improve the feasibility of demanding molecular research on post-mortem tissues, this is most likely better feasible with MIA than CA.

Introduction

In most cancer patients, only tissues from the primary tumors are biopsied or resected for diagnostic and therapeutic purposes. Outside the context of studies clinicians do not biopsy metastatic disease, unless it has therapeutic significance. This hampers the molecular comparison of primary and metastatic disease. Even though it is now known, that there is not just intra-tumor heterogeneity (1) but also considerable molecular differences between primary tumors and metastases (2, 3). Chemotherapeutic and other systemic treatments based on genetic characteristics of the primary tumor, may not work effectively on metastases, due to changes of molecular targets, such as receptor conversion (4, 5). Knowing the molecular characteristics of metastases may help to target them specifically.

It is, therefore, necessary to pursue molecular research, comparing primary tumors and metastases. Post-mortem investigation is an opportunity to obtain tissue samples from (recurred) primary tumors and metastases for comparative molecular studies (6, 7). The “rapid autopsy”, by which tumor samples are collected within 1 to 3 hours after death minimizes the post-mortem RNA degradation, and allows for procurement of high quality tumor tissue (8, 9).

Unfortunately, autopsies are rarely performed on patients died of cancer. Bereaved relatives are often not willing to give their consent for conventional autopsy (CA), mainly because they feel that their loved one has suffered enough from the disease and consider (further) mutilation of the deceased’s body undesirable (10-12). Minimally invasive autopsy (MIA), however, may be an acceptable alternative to CA (13), because with MIA, the body is imaged by CT and MRI and tissue samples from a (recurred) primary tumor and metastases are obtained through CT-guided biopsies, leaving the body intact.

Here we have investigated RNA in such biopsies as a measure of overall quality of the tissue for molecular studies. We studied whether the biopsies yielded: a) a sufficient amount of RNA and b) RNA of sufficient quality for downstream RNA analysis. By using RNA isolated from MIA, CA and fresh frozen ex vivo tissue in a RT-qPCR amplicon size assay, we were able to determine the levels of post-mortem RNA degradation and quality.

Materials and Methods

In this prospective study RNA quality of post-mortem tissues was examined and compared to fresh frozen samples. The post-mortem tissues were obtained from two types of post-mortem examination: MIA and CA. Heart, kidney and liver tissue samples were collected at both MIA and subsequent CA in the same case, thereby excluding inter-patient variation between the two types of post-mortem samples.

Fresh frozen samples of the same three organ types, which had been obtained from living subjects, were culled from our frozen tissue bank. The three tissue types were selected based on accessibility during MIA, different rates of postmortem autolysis, availability in the frozen tissue bank and previous studies, showing acceptable results for basic molecular research with these tissue types (14, 15). The collected tissue samples were not always free from pathological changes.

Subject inclusion and clinical states

This study was approved by the Erasmus MC Medical Ethical Committee (file MEC-2011-055-amendment 002). All cases of in-hospital deceased adult patients whose bereaved relatives have given signed informed consent for both MIA and CA could potentially be included in this study protocol. Samples of fresh frozen residual tissue of heart, kidney and liver derived from surgical specimens or biopsies were provided by the Erasmus MC Tissue Bank and used according to the Dutch Code of Conduct 2011.
The autopsy samples were collected in the period between 11-28-2012 and 11-27-2013 whenever the responsible researcher (AvdL) was available for tissue sampling.
The time of death, entered by the subject's physician, and the time of tissue sampling at MIA and CA were registered. The time elapsed between death and the freezing of the sampled tissue was defined as the post-mortem interval (PMI). The MIA was always performed at the evening before the CA, therefore the PMI was longer in tissues collected at CA. Medical data from the last phase of life (fever and hypoxia) and patients body mass index (BMI) were obtained from the subject's medical records and the autopsy forms filled in by the subject's physician.

Tissue sampling

At MIA tissue samples were obtained with CT guided biopsies, using a 12-gauge needle. Immediately after each biopsy the sampled tissue was snap frozen in a 50 ml tube filled with pre-cooled isopentane in dry ice (16). The samples were then placed in a pre-cooled aluminum vial, stored temporarily in a -80°C freezer and finally transferred into liquid nitrogen storage.
Immediately after MIA the body was returned to the mortuary with an ambient temperature of 4 ^{o}C. The following day tissue samples of approximately 0.5 cm^3 were collected from the same organs in the same subject during CA. Due to logistics at CA, snap freezing immediately after harvesting was not possible in all cases. In two cases the tissue samples were temporarily stored at 4°C before snap freezing. All collected samples were eventually stored in liquid nitrogen until RNA could be isolated.
Fresh frozen tissues were either derived from surgically resected tissues or from biopsies (heart). These tissue samples were stored in liquid nitrogen in

the Erasmus MC Tissue Bank after being snap frozen using pre-cooled isopentane and liquid nitrogen.
Two extra heart samples (1 from MIA, 1 fresh frozen from the Erasmus MC Tissue Bank) were collected for training purposes. To prevent selection bias, these extra samples were both included in the analyses.

RNA extraction, RIN measurement and frozen H&E sections

Depending on the size of the sample, 10 to 20 10 µm thick frozen sections were cut on a cryostat microtome (Microm HM560, Adamas, The Netherlands). The sections were transferred to 700 µL Qiazol (Qiagen, Hilden, Germany) and RNA was extracted from these sections using the miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was eluted in 40 µL RNase-free water and 1 µL of RNase inhibitor (20 U/µL; AB, California, USA) was added to avoid RNA degradation during further handling.
The RNA integrity number (RIN value) (17) of the extracted RNA and the RNA concentration (ng/µL) was assessed by on-chip-electrophoresis, using the BioAnalyzer (Agilent RNA 6000 Nano kit and BioAnalyzer 2100 Expert, Agilent Technologies, USA).

5 µm sections were cut and stained with Haematoxylin and Eosin (H&E) for morphological investigation of the samples used for RNA isolation. The morphologic tissue quality assessment comprised of 1) the representativeness of the sample (yes or no); 2) the presence of necrosis (yes or no); 3) scoring the amount of autolysis: no (morphology unaffected), moderate (mild loss of staining of nuclei; some detachment of endothelium in vessels), or severe (severe/complete loss of staining of nuclei; complete detachment of endothelium in vessels).
5 fresh frozen samples and 9 MIA samples contained so little tissue, that it was all needed for the RNA isolation and no histologic slide could be made. For 3 fresh frozen samples, 2 MIA samples and 6 CA samples the necrosis could not be scored, due to freezing artifacts rendering the interpretation of the morphology uncertain. These cases were registered as 'no scoring possible' (n/s), due to severe autolysis.

cDNA synthesis and RT-qPCR

Some smaller tissue samples did not yield the required concentration of 100 ng/µL RNA for cDNA synthesis. In order to achieve a concentration of 100 ng/µL total RNA per sample, a volume containing 1 µg of RNA from all samples (to assure equal treatment amongst all samples) was transferred to another test tube and air dried by Vacuspin (SpeedVac AES1010, Savant, USA) for 30 minutes at 45°C. 10 µL of RNase free water was then added to reconstitute the RNA samples to the required 100 ng/µL total RNA.

cDNA synthesis of all samples, including a positive control (MCF7 cell line RNA), as well as a no template control (NTC) was performed as previously described (3).
Three pools of randomly picked cDNA samples were made by taking 1 µL of cDNA of 10 fresh frozen (pool 1), 10 MIA (pool 2) and 10 CA (pool 3) samples and diluting them twenty times in water. A fourfold dilution series was made of the initial pool samples to create a sensitivity curve. This additional step, consisting of fifteen samples in total, was added to each PCR assay to assess the efficiency of the assays.
To determine the quantity (degradation in post-mortem samples compared to the fresh frozen samples) and quality (acceptable length of RNA fragments for demanding downstream RNA based genomic techniques), quantitative real time polymerase chain reaction (RT-qPCR) with the *GAPDH* amplicon size assay (table 1), as previously described by Viertler et al (18), was performed on all patient and control samples.

Table 1. *GAPDH* primer sequences and base pair lengths used for RT-qPCR analyses

Primer	**Primer sequence (5' --> 3')**	**Amplicon size** base pairs	**PCR efficiency** POWER (10(-1/slope))
GAPDH common fwd	CCA CAT CGC TCA GAC ACC AT		
GAPDH rev	ACC AGG CGC CCA ATA CG	71	1,94
GAPDH rev	GTA AAC CAT GTA GTT GAG GTC	153	1,93
GAPDH rev	TTG ACG GTG CCA TGG AAT TT	200	1,86
GAPDH rev	ACT TGA TTT TGG AGG GAT CT	277	1,81
GAPDH rev	AAG ACG CCA GTG GAC TCC A	323	1,82
GAPDH rev	ACG ATA CCA AAG TTG TCA TG	530	1,71

Data analysis

Differences in RIN values and Cq values, with respect to tissue types (heart, kidney and liver); sample types (fresh frozen, MIA and CA); PMI; clinical/patient related data and morphology scores, were analyzed in SPSS (IBM SPSS Statistics, version 21.0), using the Mann-Whitney U test and the Paired Kruskal-Wallis test.
For the analyses of RT-qPCR outcomes multiple testing was applied, because the Cq values were measured for 6 *GAPDH* base pairs per tissue sample. Therefore the significance level of P=0.05 had to be divided by 6, and a significance level of P=0.00833 was used.

Results

Sample collection

A total of 218 tissue samples from three different tissue types were collected. In 23 out of 24 autopsy cases the MIA and CA samples were collected from the same corpse. In 1 autopsy case, the samples had only been collected during MIA and not during the CA; therefore CA samples from another autopsy case were collected from the Erasmus MC Tissue Bank. For training purposes two additional heart samples were collected, one fresh frozen sample and one MIA sample. These were both included in the analyses.
All fresh frozen tissue samples were culled from the Erasmus MC Tissue Bank. Per sample type three tissue types were collected (see table 2).

Table 2. Number of tissue samples per sample type per tissue type

		Sample type			
		Fresh Frozen	**MIA**	**CA**	
Tissue type	**Heart**	25*	25*	24	74
	Kidney	24	24	24	72
	Liver	24	24	24	72
	Total	73	73	72	218

** extra samples collected*

The collected tissues were categorized based on post-mortem interval (PMI). The post-mortem intervals of our cases ranged from 10 to 59 hours. Six PMI categories were created, starting with the first category of up to 12 hours, each following category consisting of a post-mortem interval of 10 hours, and a last category consisting of 14 hours. An overview of the distribution of PMI categories among the included cases is given in table 3.

An overview of the available (and potentially relevant) clinical variables is shown in table 4 and an overview of the morphologic tissue quality assessment of the samples is shown in table 5.

Table 3. Distribution of tissue samples per sample type over post-mortem intervals

	Sample type			
PMI	**Fresh frozen**	**MIA**	**CA**	**Total**
0h	73	-	-	73
≤12h	-	12	-	12
13h-24h	-	21	6	27
25h-34h	-	31	18	49
35h-44h	-	6	36	42
≥45h	-	3	12	15
Total	73	73	72	218

Table 4. Available patient conditions in (agonal phase)

		MIA and CA
Condition	**Fever**	40%
	Hypoxia	57%
	BMI >25	60%

Table 5. Morphologic tissue quality assessment per sample type

		Sample type		
		Fresh frozen n = 73	**MIA n = 73**	**CA n = 72**
Representative	**yes**	88%	88%	100%
	no	5%	0%	0%
	No H&E-slide*	7%	12%	0%
		n = 68	**n = 64**	**n = 72**
Autolysis	**severe**	3%	11%	21%
	moderate	16%	50%	44%
	no	81%	39%	35%
Necrosis	**yes**	3%	3%	6%
	no	93%	94%	86%
	n/s**	4%	3%	8%

** No H&E-slide, all tissue used for RNA isolation*

*** n/s = no scoring possible*

RNA integrity

RIN values were established for 199 samples. In 6 fresh frozen samples, 3 MIA samples and 2 CA samples the RNA integrity could not be established, because the RNA yield of the sample was too low. These samples were excluded from the analyses, for not being able to establish RIN values was due to the size of the tissue samples instead of the tissue quality.
In 4 MIA samples and 4 CA samples the RNA integrity could not be established, due to extensive RNA degradation (RIN <1.0). These samples were included in the analyses with an RIN-value of 0 (zero), resulting in a total of 207 samples for the analysis.
The median RIN value and interquartile range for all three sample types are shown in figure 1A. The RIN values in fresh frozen samples were significantly higher than those in post-mortem samples ($P < 0.001$, Unpaired Mann-Whitney test, significance level 0.05), and the RIN values in MIA samples were higher than those in CA samples ($P = 0.032$, Unpaired Mann-Whitney test, significance level 0.05).
Figure 1B shows the median RIN values and interquartile range per sample type and tissue type. For each tissue type the RIN values of fresh frozen samples were significantly higher than those in post-mortem samples ($P < 0.001$, unpaired Mann-Whitney test, significance level 0.05). No significant differences in RIN value per tissue type were found between MIA and CA. The morphological factor autolysis adversely influenced RNA integrity ($P <$

0.001). Of the patient related agonal factors, hypoxia and BMI did not influence RNA integrity, and fever did have an adverse effect on the RIN value in post-mortem samples (P = 0.006, unpaired Mann-Whitney test, significance level 0.05).

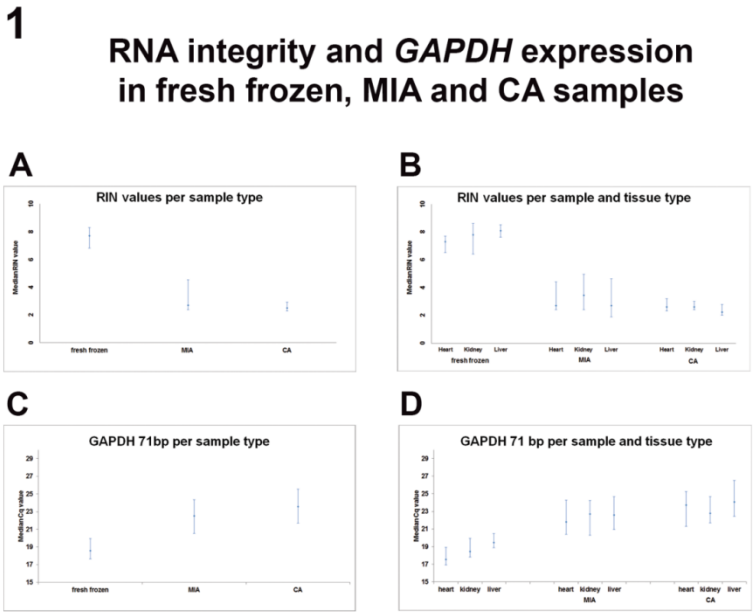

Figure 1: RNA integrity and GAPDH expression in fresh frozen, MIA and CA samples. Figure 1A shows median RIN values (X-axis) of fresh frozen, MIA and CA samples (Y-axis). The fresh frozen samples, taken from surgical specimens, yielded high quality RNA. Whereas both types of post mortem samples MIA and CA yielded RNA of lower quality. In figure 1B the samples are divided into 3 tissue types; heart, kidney and liver respectively. In figures 1C and 1D RT-qPCR results of the same samples are shown, in the same order. Since all GAPDH RT-qPCR assays in the 6-amplicon size assay showed similar results, only the results of the 71 bp assay are depicted here. The Cq value (Y-axis) obtained in fresh frozen samples is low, i.e. the GAPDH expression level is high. In the post mortem samples MIA and CA, GAPDH RNA is partly degenerated, resulting in higher Cq values corresponding with lower GAPDH expression levels.

Table 6. P-values comparing sample types per GAPDH size assay

Sample type	*P*-value					
	71 bp	**153 bp**	**200 bp**	**277 bp**	**323 bp**	**530 bp**
Fresh frozen vs. MIA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Fresh frozen vs. CA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
MIA vs. CA	0.009	0.003	0.005	0.009	0.003	0.016

Kruskal Wallis, significance level 0.00833

RT-qPCR

RT-qPCR was perfomed on all 218 samples and Cq values could be established for 216 samples. In one case the RNA yield was too low, in the other case the Cq value could not be measured due to technical failure. These samples were excluded from the analyses concerning Cq values. Figure 1C shows that median Cq values are lowest in fresh frozen samples for the *GAPDH* 71 bp assay. The Cq values in CA samples are highest. Figure 1D show the Cq values per tissue type. Liver tissues have the highest Cq values for all sample types.

Figure 2 shows the Cq values of all *GAPDH* amplicon sizes per sample type. The difference in Cq values between fresh frozen and post-mortem samples (MIA and CA) is significant for all *GAPDH* amplicon sizes (P< 0.001, Paired Kruskal-Wallis test, significance level 0.00833). The difference in Cq values between MIA and CA samples is not significant for all *GAPDH* amplicon sizes, as shown in table 6. When the Cq values were stratified per tissue type, there were no significant differences between MIA and CA samples.

2 *GAPDH* size assay in fresh frozen, MIA and CA samples

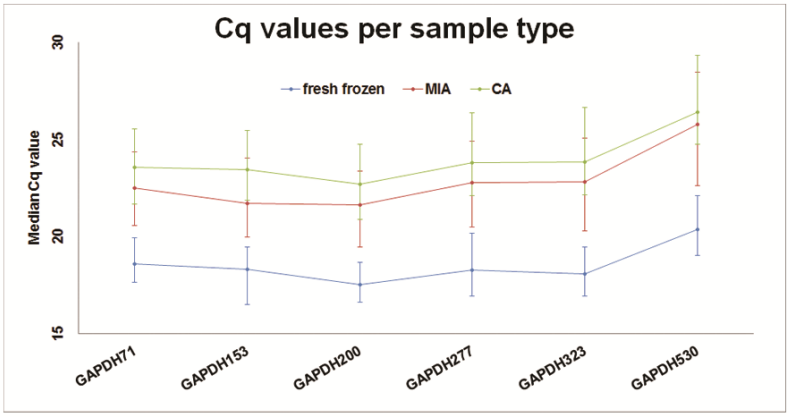

Figure 2: GAPDH size assay in fresh frozen, MIA and CA samples. The median Cq values (X-axis) of RNA derived from fresh frozen, MIA and CA samples obtained by RT-qPCR with various PCR product lengths (Y-axis) are shown. The fresh frozen samples always show lower Cq values, whereas the MIA samples show intermediate and the CA samples show high Cq values. This indicates that RNA in MIA samples is less degraded than RNA in CA samples. The parallel increasing Cq values between GAPDH323 and GAPDH530 indicate sub optimal RT-qPCR performance, rather than decreased GAPDH expression levels.

The RNA integrity of all samples decreases with increasing post-mortem interval (see figure 3A). Figure 3B shows the RIN values of the three different tissue types per PMI. The fresh frozen samples are represented by PMI category “0”. The RIN value in this category was significantly higher than in all other categories, but there were no significant differences in RIN values between PMI categories 1 to 5. According to figure 3C, the *GAPDH* 71 bp assay Cq values increase with PMI (i.e. decrease of *GAPDH* expression level). Figure 3D shows the GAPDH 71 bp assay Cq values per tissue type per PMI. Cq values in PMI category 2 are significantly higher than those in PMI category 1.
The morphological factors necrosis and autolysis had an adverse effect on *GAPDH* expression, with *P*-values ranging from <0.001 to 0.008 depending on amplicon sizes (results not shown). The *GAPDH* expression differed between patients with a BMI up to 25 and those with a BMI higher than 25 (P range <0.001 to 0.015 depending on amplicon size), whereas fever and hypoxia did not influence *GAPDH* expression.

3

RNA integrity and *GAPDH* expression versus post-mortem intervals in MIA and CA samples

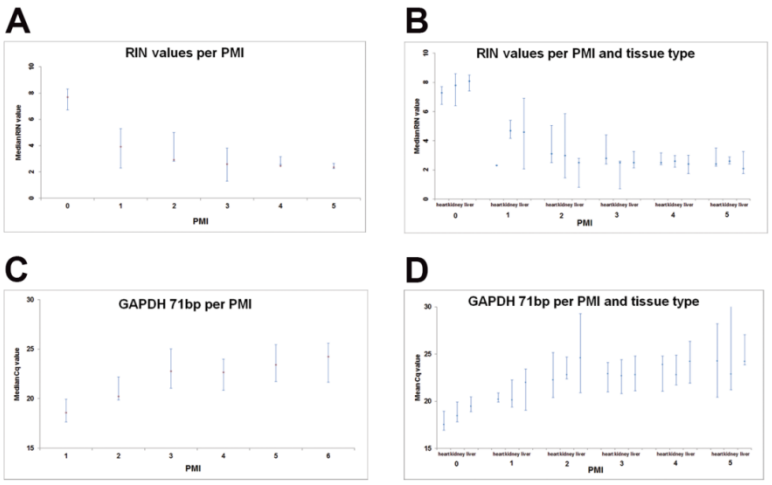

Figure 3: RNA integrity and GAPDH expression versus post-mortem intervals in MIA and CA samples. In figure 3A and B the median RIN value (X-axis) decreases with the post mortem interval (PMI; Y-axis). The fresh frozen specimens (PMI = 0) show the highest RNA integrity. PMI 1 is exclusively comprised of MIA samples. PMI 2 to 5 are comprised of both MIA and CA samples. The actual PMI intervals are given in table 3. In figures 3C and D the results of RT-qPCR with GAPDH 71 bp are shown in the same context. Here we can appreciate that RT-qPCR is a more sensitive method of RNA integrity measurement, since it was possible to find significant differences not only between PMI 0 and PMI 1, as was also measured with RIN values, but also between PMI 1 and PMI 2.

Conclusion and Discussion

RIN values were significantly higher in fresh frozen samples than in post-mortem samples. MIA samples showed higher RIN values than CA samples. Also, *GAPDH* expression was significantly higher in fresh frozen samples than in post-mortem samples. There were differences in *GAPDH* expression between MIA and CA samples, but only for 3 out of 6 *GAPDH* amplicon sizes they were significant. Since *GAPDH* amplicon sizes of up to 530 base pairs could be detected well within the limits of the assay's sensitivity, we

conclude that the post-mortem samples still contained RNA of reasonable quality.
It is generally believed that post-mortem tissues with poor RNA integrity values (RIN value <5) are not suitable for molecular techniques (19). In this study we showed that samples with low RIN values could still be used for determining gene expression with RT-qPCR.

The Affymetrix and Illumina gene array platforms respectively use 25-mer or 50-mer probes to detect gene expression. Since it is possible to amplify 530 bp *GAPDH* RNA fragments, this implies that detection of *GAPDH* with gene array technology must be possible. *GAPDH* expression in post-mortem tissue is 4 Cq, equivalent to approximately 16-fold lower than in fresh frozen tissue (see figure 2). Since *GAPDH* is an abundantly expressed housekeeping gene (i.e. high copy number per cell), the transcript could still be measured in post-mortem tissue derived RNA with qPCR. Assuming post-mortem RNA degradation is a random process, all transcripts (i.e. the entire transcriptome) will be subjected to it to the same degree (20). Therefore, less abundantly expressed genes (i.e. low copy number per cell) may become undetectable after this rate of degradation. Nonetheless, successful gene array analysis of post-mortem heart tissue was previously described (6).

Handling and processing RNA may have major impact on RNA quality and therefore on the outcomes of the study. Inconsistency in executor and work performance, and contamination of RNA (with RNase or other genetic material) must be avoided when working with RNA (14, 21). The experiments were therefore performed by one properly trained and experienced researcher (AvL). To all RT-qPCR assays a dilution series was added to assess the performance of both the assay and the researcher's skills. The efficiency numbers (table 1) show that the RT-qPCR assays were all well performed, although the efficiency decreases with amplicon size. The observed (parallel) increase of Cq values in all samples (i.e. post-mortem as well as fresh frozen; figure 2) in relation to the increasing amplicon size may thus be due to decreasing PCR efficiency, rather than increased RNA degradation.
Our results suggest that RNA in MIA tissue is less degraded than RNA derived from CA tissue. However, in this study CA was always performed after MIA, so the longer PMI is probably the explanation for this finding. Yet it is possible that differences intrinsic to MIA and CA play a role as well (5, 7, 22) . More extensive exposure of tissues to air, which occurs during conventional autopsies, could also have a negative influence on tissue quality. It is known that vacuum sealing of tissue specimens has a beneficial effect on RNA preservation (23).
The integrity of post-mortem tissues is subject to various factors (24). Agonal factors, such as hypoxia and fever are known to have a negative influence on RNA quality (25). Our analyses show inconsistent results: whereas

hypoxia and Body Mass Index (BMI) did not cause significant differences between RIN values, and hypoxia and fever did not cause significant differences between Cq values, the RNA quality according to the RIN values was significantly lower in cases with reported fever, and the RNA quality according to the Cq values was significantly lower in cases with a BMI of >25. This latter phenomenon is probably due to the temperature insulation by fatty tissue, whereby high BMI bodies cool down more slowly, exposing RNA to a longer period of warm ischemia. The somewhat inconsistent results on the influence of agonal factors and BMI in our study are probably due to the low number of samples per condition..

In conclusion, although RNA integrity is lower in MIA and CA samples than in fresh frozen tissues, MIA and CA samples can be used to detect *GAPDH* PCR products up to 530 base pairs. This implies that tissue obtained by MIA yields a sufficient amount of RNA with a sufficient quality for gene array based research. Therefore, the MIA procedure is a new method for researchers to obtain metastatic tumor tissue for molecular translational research.
Potential advantages of MIA over CA for obtaining metastatic tumor tissue are the higher chance of getting consent from bereaved relatives, and the better feasibility to reduce PMI, which is the most crucial factor for high quality tissue for molecular analyses.

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Chapter 3

The Influence of Tissue Procurement Procedures on RNA Integrity, Gene Expression and Morphology in Porcine and Human Liver Tissue

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Abstract

The advent of molecular characterization of tissues has brought an increasing emphasis on the quality of biospecimens, starting with the tissue procurement process. RNA levels in particular are affected by factors in the collection process, but the influence of the different pre-analytical factors is not well understood. Here, we present a comprehensive study of the influence of tissue sample size as well as transport and freezing protocols on RNA quality.

Porcine liver samples of predetermined sizes were stored either dry, on moist gauze, or in salt solution for various times and then either frozen in liquid nitrogen or in pre cooled isopentane. Large (8 x 4 x 4 mm) and small (2 x 2 x 4 mm) human liver samples were frozen in pre-cooled isopentane either immediately or after one hour at room temperature. The small samples were stored dry, on moist gauze, or in salt solution. RNA was isolated and RIN values were measured. Human liver RNA was analyzed by RT-qPCR for six standard reference genes, and tissue morphology was assessed for artifacts vis à vis pre-analytical conditions.

Human liver samples showed significant RNA degradation after one hour of cold ischemia, which was more pronounced in small samples. RNA integrity was not significantly influenced by the transport or freezing method, but changes in gene expression were observed in samples either transported on gauze or in salt solution. Salt solution also had a minor adverse effect on tissue morphology.

In conclusion, based on observations in liver samples, small samples are more subject to gene expression variability introduced by post-excision sample handling than larger samples. When working with small biopsies, dry transport in air-tight containers placed on ice is recommended. It is also important to freeze biopsies within 30 minutes after they are procured from the patient.

Introduction

Residual tissues procured from surgical specimens is an important source of samples for clinical research studies. Solid tissue samples are most often excised from tissue collected during routine surgical procedures and stored as archival FFPE or frozen residual tissue in hospital tissue banks that can later be used in translational research studies. Surgical specimens are, however, subject to warm ischemia during surgery when the blood supply is clamped. In addition, after the surgical specimen is removed from the patient it undergoes a period of cold ischemia during transport to the pathology laboratory. The ischemic periods may affect RNA [1, 2] and metabolite [3] expression and, to a lesser extent, protein expression [4,5]. Since biopsies are not subject to long periods of warm ischemia, this sample type is often preferred in clinical trials [6]. Ideally, when biopsies are snap frozen or formalin fixed immediately after collection, much of the pre-analytical variation is avoided minimizing post sampling distortions of biomolecule levels [6]. However, in-hospital tissue banks, it is normal procedure to transport such solid tissue biopsies to the pathology laboratory where they are registered, snap-frozen, and stored as part of the diagnostic process. Under these conditions, cold ischemia during transport, the transport method and temperature, as well as the freezing method may contribute to RNA degradation and affect gene expression.

While cold ischemia time (CIT) is known to compromise RNA integrity and gene expression [7-9], it is not known if the sample size and the transport method can influence RNA quality. When tissue is procured during routine macroscopic examination of a surgical specimen, it can be desirable to divide large tissue samples into smaller pieces to avoid repeated handling of the frozen tissue sample like cutting or breaking. In cases when material is limited it is not even possible to procure large samples. Tissues contain nucleases that vary in amount and composition with tissue type [10]. Cutting tissue into small pieces might release these damaging enzymes, degrading the RNA. Small samples are alos exposed to air and may sustain damage through oxidation. Evaporation of water during storage and transport increases salt concentration which may affect expression as well as degradation.

Slow freezing may lead to variation in RNA quality between the core of a larger sample that freezes later compared to its outer surfaces. Freezing tissue samples directly in liquid nitrogen or in pre-cooled isopentane are considered the best methods to avoid the formation of ice crystals that damage cells and local accumulation of salt that may lyse cells [11] exposing RNA to extracellular nucleases. Snap freezing is also preferred for conserving tissue morphology. When tissue samples are frozen directly in liquid nitrogen an insulating layer of nitrogen vapour forms around the

sample that slows the freezing process (the Leidenfrost effect) [12]. The Leidenfrost effect can be avoided by freezing tissue samples in an empty metal container that floats on the liquid nitrogen. This procedure also avoids the formation of large ice crystals in the tissue [11]. Pre-cooled isopentane can also be used to aoid the Leidenforst effect and is widely accepted as the optimal way to preserve morphology of snap frozen tissue samples [11]. Acknowledging the preserving effects on morphology, isopentane was not found to be advantageous to nitrogen concerning RNA quality in a previous study [13].

Small samples such as needle biopsies can suffer air drying artifacts that damage tissue morphology and possibly also compromise RNA. To minimize evaporation clinicians often send small samples to the laboratory either on moist gauze or submerged in isotonic salt solution. When biopsies are submerged in salt solution, the salt diffuses into blood vessels and other hollow structures in the tissue and may precipitate upon freezing the biopsy. Cutting frozen sections for RNA isolation, excess salt may also be carried over to the isolated RNA compromising the yield and interfering with downstream biochemical processing. Excess salt may also affect tissue morphology.

In summary, many possible complications have been described, but yet there is little data showing if/how of these various factors really are affecting RNA quality. Here, the effects of sample size in combination with transport and freezing conditions on tissue morphology, RNA integrity, and gene expression are reported.

Materials and Methods

Exploratory experiments were performed on porcine liver to determine main aspects of the relation between sample size, transport- and freezing methods on RNA integrity. Based on these results, parameters were selected for study on human liver, Quality parameters to assess the conditions were the RNA Integrity Number (RIN) and RT-qPCR Cq values of the reporter genes: HPRT1, HMBS, GUSB, PPIB, GAPDH and TBP. The reporter genes were selected for expression stability and belong to the group commonly used as reference genes and occasionally referred to as ‘housekeeping’ genes. These genes are highly expressed in all cell types at rather constant levels [14,15]. Variation in their expression therefore reflect changes introduced by the pre-analytical process.

Tissue samples

Normal (porcine) and (human) liver tissue was chosen for its homogeneity to minimize variation due to sampling in this the study.
Porcine liver tissue, was procured under approval number EMC 1878 (130-09-07) after study review by the Erasmus University DEC (animal experiment committee). The animals, a mix breed of Landrace x Great Yorkshire, were acquired from a commercial pig breeder (meat production) and had been housed on bedded concrete floors with floor heating and had unlimited access to water and food (as specified in the ETS No 123 and Dutch regulations). Animals, weighing around 65 kilograms, were anesthetized (with a cocktail of propofol, isoflurane, suffentanyl and succinylcholine) and used for endoscopic operation technique training. Within 15 minutes after sacrifice (intra venous overdose of sodium-pentobarbital) by properly trained and certified (as described in article 12 of the Dutch animal experiment law; WOD) veterinarian technicians at the Erasmus MC Skills Lab whole livers were removed and transported in less than 15 minutes to the pathology lab at room temperature.

To investigate the influence of sample size on RNA integrity, 12 large (8 x 4 x 4 mm), 12 medium (4 x 4 x 4 mm) and 12 small (2 x 2 x 2 mm) porcine liver samples were excised from a single liver. The samples were snap frozen on the bottom of an aluminium vial (PA6015, Sanbio, Uden, The Netherlands) floating on liquid nitrogen (“without isopentane”) either immediately (T0), or after 5, 15, 30 or 60 minutes storage at room temperature (**Figure 1A**).

To test how different freezing methods affect tissue morphology, 6 large (8 x 4 x 4 mm), 6 medium (4 x 4 x 4 mm), and 6 small (2 x 2 x 2 mm) porcine liver samples were prepared as described above and immediately either snap frozen without isopentane or in pre-cooled isopentane. Tissue sections (4 µm) were cut and stained with haematoxylin and eosin (H&E), to assess the morphology (**Figure 1B**).

To study the effect of transport or freezing method on RNA integrity and tissue morphology, needle biopsies (28 gauge, 2 cm) of porcine liver were taken and frozen immediately. Replicate needle biopsies were also stored for one hour either dry, on moist gauze, or submerged in physiological salt solution (0.9% NaCl) at room temperature or on ice and then snap frozen. Three biopsies were taken for each condition studied (**Figure 1C**).

Human residual liver samples were procured according to the Dutch Code of Conduct legislation concerning the use of residual tissue for research. The Code of Conduct maintains an opt-out consent system and therefore, no written informed consent was required. The study and the consent regulation, as described in the Code of Conduct [16], was approved by the

Erasmus MC Medical Ethical Commission, under number MEC-2008-397. The tissue specimen was obtained after 3.5 hours of surgery (warm ischemia) and 30 minutes of transport at room temperature (cold ischemia). All human liver tissue samples were collected during routine macroscopic examination of the tissue specimen. Small (2 x 2 x 4 mm) and large (8 x 4 x 4 mm) samples (n = 6 each) were obtained and frozen on the bottom of a metal container cooled in LN2. The large samples were frozen either immediately, or after being stored dry for 60 minutes at room temperature. The small samples were frozen either immediately or after 60 minutes at room temperature either dry, stored on moist gauze, or submerged isotonic salt solution (0.9% NaCl). From all tissue samples, sections (4 µm) were cut and stained with H&E and submitted for morphological assessment (**Figure 1D**).

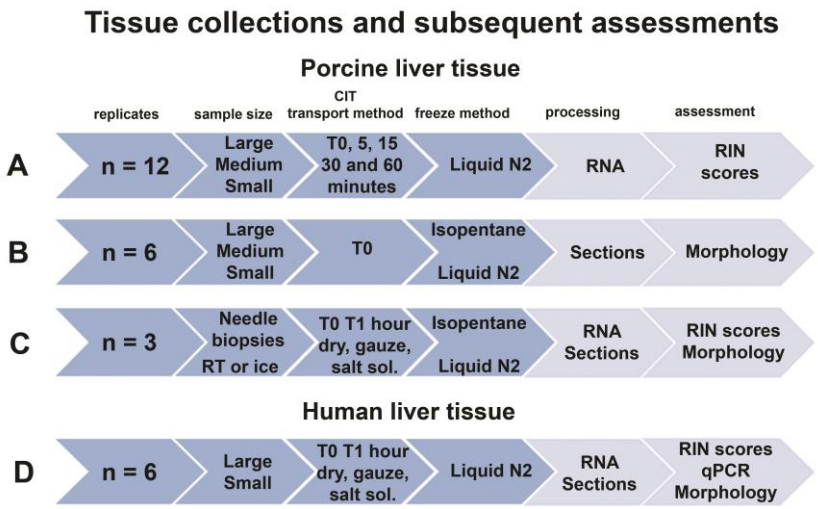

Figure 1. Tissue collections and subsequent assessments. Here the different tissue collections and subsequent assessments are shown. For each condition tested, n samples were used. E.g. for the top experiment RIN scores were assessed of 12 x 3 (large, medium, small) x 5 (T0, 5, 15, 30 and 60 minutes) = 180 samples. Samples were frozen either in liquid nitrogen or in pre-cooled isopentane after which RNA was isolated (for RIN score and RT-qPCR) and/or frozen sections were cut (for morphological assessment).

RNA isolation and RIN measurement

Frozen 10 µm sections were cut and manipulated with pre-cooled tools and immediately placed in 700 µL Qiazol (Qiagen, Hilden, Germany). The amount of tissue used for RNA isolation was the same for samples, using a smaller number of sections for the larger samples (10 sections from large samples, 20 from medium samples and 40 from small samples). The time in the Qiazol solution was limited by analysing no more than six samples at each time The tissue sections were disrupted in the Qiazol by shaking the tubes vigorously for about five seconds. Total RNA was then isolated according to the manufacturer's protocol using the (mi)RNeasy mini kit (Qiagen, Hilden, Germany). The obtained RNA was placed on ice prior to RIN assessment on an Agilent bioanalyzer (Bioanalyzer 2100, Agilent, The Netherlands).

RT-qPCR on human liver tissue derived RNA samples

Complementary DNA (cDNA) was prepared from 2 µg (measured with nanodrop, Isogen Life Science, The Netherlands) RNA using the Thermo Scientific. RevertAid H Minus M-MuLV First Strand cDNA Synthesis Kit, followed by a ribonuclease H step to degrade any remaining RNA as described previously [17]. As the *GAPDH* assay used here also amplifies genomic DNA (gDNA) and to prevent amplification of gDNA potentially still present in these RNA preparations, the samples were treated with a heat-labile double strand specific DNase (HL-dsDNase) as described by the manufacturer (ArcticZymes, Norway) to remove any gDNA. Complete removal of the gDNA was confirmed using ValidPrime (TATAA Biocenter, Sweden).

The samples were diluted 20 times to a final cDNA concentration of 2.38 ng/µL prior to qPCR analysis, which was performed on the Stratagene Mx3000P platform (Agilent, The Netherlands) using 96-well plates. Six reporter transcripts were included in the analysis. Assays for *TBP*, *HPRT1* and *HMBS* (designed by A. Sieuwerts and ordered from Invitrogen (Invitrogen, The Netherlands), were readily available as SYBR green primer assays (see **Table 1**). The run protocol for the SYBR Green primer assays consisted of: Activation; 95°C 15min (1 cycle), Amplification; 95°C 15s, 62°C 30s, 72°C 30s, 79°C 30s (40 cycles), followed by a melting curve analysis. A final reaction volume of 25 µL containing 11.9 ng cDNA, 330 nM of each primer and 50% (v/v) SYBR qPCR Master Mix with ROX (2-fold concentrated stock) from Abgene/Thermo Scientific was used for SYBR qPCR runs. The other three reference genes (*GUSB*, *PPIB* and *GAPDH*; designed by TATAA, ordered at Eurofins MWG, Germany) were FAM-BHQ1 labelled primer/probe assays (see **Table 1**). The run protocol for the

primer/probes assays consisted of: Activation; 95°C 15min (1 cycle), Amplification; 95°C 30s, 60°C 60s, (40 cycles). A reaction volume of 20 µL containing 11.9 ng cDNA, 200 nM primers, 50 nM probes and 25% (v/v) Absolute qPCR Master Mix with ROX (2-fold concentrated stock) from Abgene/Thermo Scientific was used for all FAM-BHQ1 qPCR runs.

A standard curve consisting of a serial diluted pool of cDNA samples, as well as a negative control consisting of gDNA (5 ng) and samples without reverse transcriptase enzyme, were included in each PCR plate to assess the efficiency and specificity of the reactions (**Table 1**).

For gene expression analysis, the difference between the Cq value of the stored sample and the average Cq values of large samples frozen at T0 were calculated by using the formula: ΔCq = Cq size Tx – Cq large T0. A ΔCq value of 1.0 corresponds to a two-fold decrease of template RNA.

Morphological assessment

The morphological quality of all samples of both porcine and human origin were assessed, blinded to the transport or freeze method. For each of the described experiments (**Figure 1**), the assessing pathologist made two groups of slides; no considerable morphological artifacts versus morphological artifacts that could compromise diagnosis. If no considerable morphological differences were observed within one experiment, the pre-analytical parameters studied did not influence morphology adversely and were not presented. The Fisher exact test was used to analyse whether there was a significant correlation between aberrant morphology and the transport methods.

Statistical analysis

All RIN scores and RT-qPCR Cq values were collected in IBM SPSS version 21 for statistical analysis. Owing to the small test numbers used for each sample handling condition, the non-parametrical Mann-Witney U test was performed to assess differences between the experimental groups and the Fisher's exact test to analyse the associations between categorical variables. All tests were 2-sided, and unless indicated otherwise, $P<0.05$ was considered statistically significant. For RT-qPCR data and in consideration of the small number of samples and the relatively high number of measured genes, a Bonferroni-Holm multiple testing corrected cut-off *P*-value of 0.0083 was considered significant.

Table 1. RT-qPCR primers and probes and qPCR efficiency

FAM-BHQ1-labeled assays	Forward primer, sequence 5'--> 3'	Reverse primer, sequence 5'--> 3'	PROBE	product size (bp)	PCR efficiency
GAPDH	CCTCCACCTTTGACGCT	TTGCTGTAGCCAAATTCGTT	AGCTTGACAAAGTGGTCGTTGAGGGCAATG	91	1.97
PPIB	GGCAAAGTTCTAGAGGGCA	GCGATGATCACATCCTTCAG	CTCTCCACCTTCCGCACCACCTCC	92	2.07
GUSB	AGAAACGATTGCAGGGTTTC	CCCAGATGGTACTGCTCTAG	ACTTTTCTGGTACTCTTCAGTGAACATCAGAG	87	2.08

SYBR Green assays	Forward primer, sequence 5'--> 3'	Reverse primer, sequence 5'--> 3'	product size (bp)	PCR efficiency
HPRT1	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG	192	1.90
HMBS	CATGTCTGGTAACGGCAATG	GTACGAGGCTTTCAATGTTG	139	1.95
TBP	TTCGGAGAGTTCTGGGATTG	ACGAAGTGCAATGGTCTTTAG	94	2.01

Results

RIN scores and morphology of porcine liver tissue

Sample size

The results of the RNA integrity analysis expressed as RIN scores in relation to sample size (experiment described in **Figure 1A**) are shown in **Figure 2**. Large samples showed no significant decrease in RIN score during the time course (0 – 60 min). Medium sized samples showed significant RNA degradation after 60 minutes (T0 vs T60, $P = 0.001$; T5 vs T60, $P = 0.041$; T15 vs T60, $P = 0.006$; T30 vs T60, $P = 0.001$; Mann-Witney U test). Although small samples seem to show earlier RNA degradation, this decrease is not significant ($P = 0.05$; Mann-Witney U test) when measured between T0 – T60.

Transport and freeze method

The transport method, simulated by storing porcine liver needle biopsies for 60 minutes either dry, on a moist gauze, or in salt solution (experiment described in **Figure 1C**) did not significantly influence RNA integrity. Keeping the biopsies in a container placed on wet ice for 60 minutes gave significantly better results ($P = 0.026$; Mann-Witney U test) in contrast to keeping the biopsies at room temperature for the same amount of time.

Frozen section artifacts such as vacuoles in nuclei and ice damage in the cytoplasm (**Figure 3**) were observed in 3 of the 17 sections when needle biopsies were stored dry, in 6 of the 18 sections when stored on moist gauze, and in 9 of the 17 sections when stored in salt solution ($P = 0.07$, Fisher's exact test) for dry versus salt solution.

When morphology between liver samples snap frozen without isopentane was compared to samples frozen in pre-cooled isopentane (experiment described in **Figure 1B**), no considerable differences were observed.

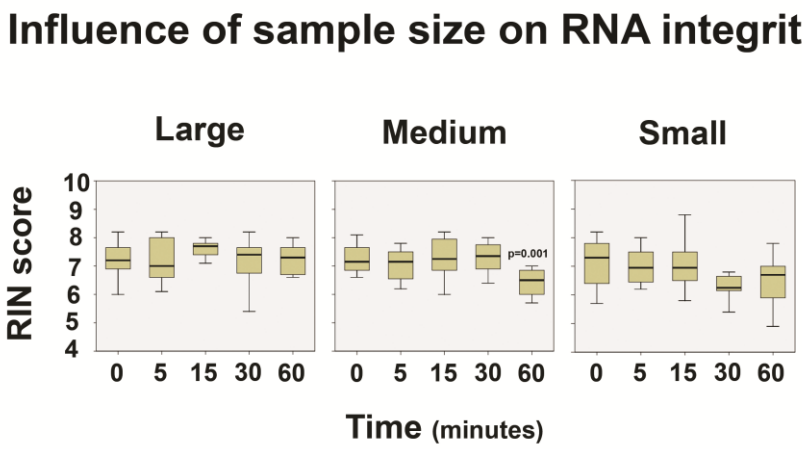

Figure 2. Influence of sample size on RNA integrity in porcine liver samples. On the x-axis the time points of the porcine liver sample collection until freezing in liquid N2 are shown. The y-axis shows the RIN score. The only significant change was (p=0,001) measured between T15 and 60 of the medium samples all other differences were not significant.

RNA integrity (RIN scores) and gene expression of human liver tissue

Sample size

There was a significant decrease in average RIN score between large samples frozen T0 and samples held for 60 minutes at room temperature (ΔRIN = 0.3; *P*= 0.015; Mann-Witney U test) (**Figure 4**). This decrease in RNA integrity was more pronounced in small samples (ΔRIN = 0.85; *P*= 0.015; Mann-Witney U test) (T0 versus T60 dry).

To measure the effect of cold ischemia and sample size on gene expression, six common house hold genes were assessed with RT-qPCR. Using the formula: ΔCq = Cq large T1 – Cq large T0, our results indicated that one hour of cold ischemia time did not appear to significantly alter gene expression in large samples (**Figure 5A**), while in small samples, levels of three reference genes were significantly lower (ΔCq = Cq small T1 dry – Cq small T0; **Figure 5B**). *P*-values of the genes with significantly altered expression are presented in **Table 2**.

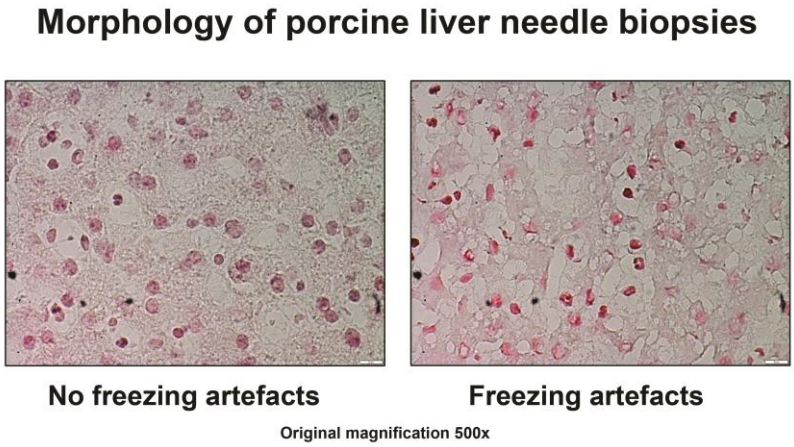

Figure 3. Morphology of porcine liver needle biopsies. The left image shows relatively unaffected liver morphology, which was mostly observed in liver needle biopsies that were transported in a dry container. The right image shows damaged liver morphology most observed in liver needle biopsies which were transported on moist gauze or submerged in salt solution.

Transport method

There was no significant difference in RIN scores among the three transport methods (dry, moist gauze, and salt solution), but data suggested that RNA degrades to a greater extent when small tissues are transported in salt solution (**Figure 4,** S(mall) T1 D(ry) vs S(mall) T1 S(alt solution), P = 0.08; Mann-Witney U test).

When small samples were transported on moist gauze (ΔCq = Cq small T1 moist gauze – Cq small T0; **Figure 5C**) or in salt solution (ΔCq = Cq small T1 salt solution – Cq small T0; **Figure 5D**), levels of all six reference genes were significantly lower compared to the dry transported samples. *P*-values of the genes with significantly altered expression are presented in **Table 2**.

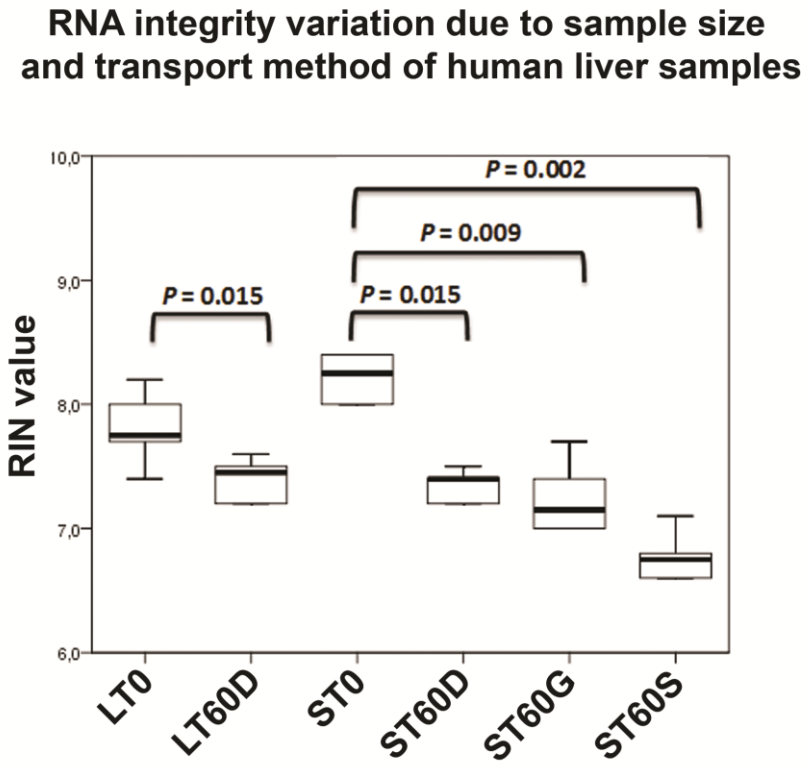

Figure 4. RNA integrity variation due to sample size and transport method of human liver samples. On the y-axis the RIN scores are shown. The x-axis shows respectively the large (L) samples at T0 and T60 (60 minutes), transported dry (D). The small (S) samples at T0, T60 transported dry (D), on moist gauze (G) and in salt solution (S). The level of RNA degradation in large samples is relatively low ($\Delta RIN_{LT0-LT60D} = 0.3$) compared to that in small samples ($\Delta RIN_{ST0-ST60D} = 0.85$).

Table 2. *P*-values of all sample size and transport method statistical analyses

Tested conditions	RIN score*	Gene expression**					
		GAPDH	***GUSB***	***PPIB***	***HMBS***	***HPRT1***	***TBP***
Large T0 vs Large T1	**0.015**	0.485	0.589	0.699	0.015	0.015	0.132
Large T0 vs Small T0	0.132	0.818	0.589	0.937	0.180	0.093	0.589
Large T1 vs Small T1	0.394	**0.004**	0.093	0.093	**0.004**	**0.002**	0.009
Small T0 vs Small T1 Dry	**0.015**	0.009	0.093	0.132	**0.002**	**0.002**	**0.002**
Small T0 vs Small T1 Moist gauze	**0.009**	**0.002**	**0.002**	0.009	**0.002**	**0.002**	**0.002**
Small T0 vs Small T1 Salt solution	**0.002**	**0.002**	**0.002**	0.009	**0.002**	**0.002**	**0.002**
Small T1 Dry vs Small T1 Moist gauze	0.537	0.093	0.485	0.937	0.818	0.180	0.699
Small T1 Dry vs Small T1 Salt solution	0.082	0.240	0.589	0.699	0.485	0.310	0.310
Small T1 Moist gauze vs Small T1 Salt solution	0.093	0.394	0.589	0.394	0.699	1.000	0.699

P-values Mann-Whitney U test

* Significance level RIN score $P < 0.05$ (**bold**)

Bonferoni corrected for multiple testing P-value ($P < 0.0083$) (bold**)

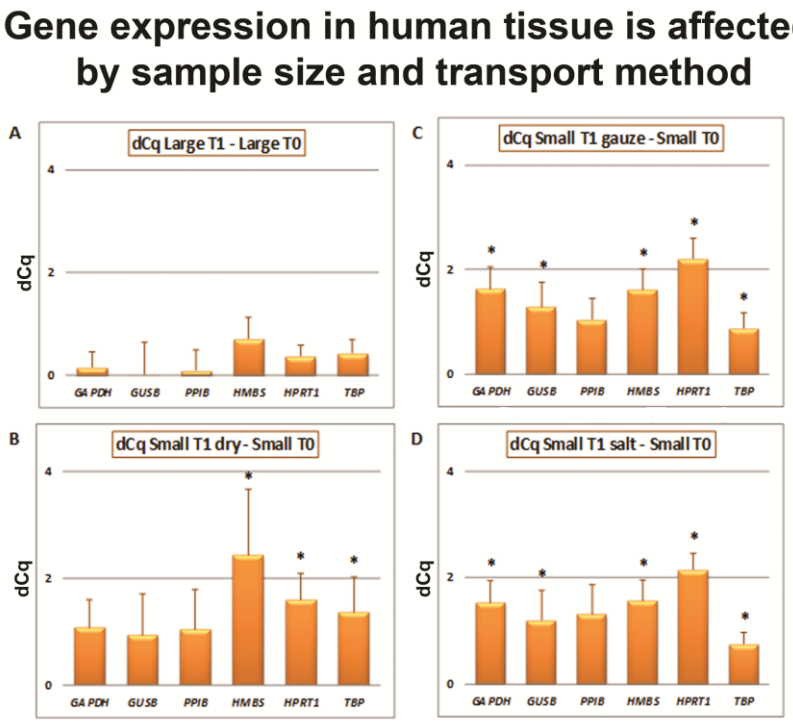

Figure 5. Gene expression in human tissue is affected by sample size and transport method. Figures 5A, 5B, 5C and 5D depict ΔCq's at T60, relative to T0 (y-axis) for the six reference genes (x-axis). A positive ΔCq indicates a decrease in gene expression of the test time point or condition as compared to the control. Significant ΔCq's after multiple correction are marked with an asterisk (*). The error bars represent standard deviations for n = 6 measurements. One ΔCq corresponds to a two-fold decrease in gene transcript copy number (original number of mRNA copies per cell). Figure A shows that no significant gene expression alterations take place during one hour of cold ischemia in large samples. Figure B shows that expression of 3 transcripts is significantly influenced by 1 h of cold ischemia in small samples. Figures C and D show that transport on a moist gauze and in salt solution, respectively, amplifies the adverse effects on gene expression.

Conclusion and Discussion

We have evaluated RNA integrity, gene expression and tissue morphology in human and porcine liver samples of various sizes subjected to different transport and freezing conditions. Our results indicate that gene expression profiles are more distorted by cold ischemia in smaller samples. Furthermore, small samples are more prone to morphological freezing artifacts.

Liver was chosen for these experiments because of tissue homogeneity. Therefore, the effects of sample size and transport methods in other tissue types may be different or non-existent and need further investigation.

When different transport methods of human liver samples were compared, no significant differences in RNA integrity (RIN scores) were observed. At the gene transcript level, however, it was found that transport on moist gauze and in salt solution resulted in a lower gene transcript levels compared to dry transport. This result in addition shows that measuring gene expression is a more sensitive way to monitor RNA quality than a single determination of RIN score.

In porcine liver needle biopsies, no significant differences in RIN scores were found between transport (dry, moist gauze or salt solution) and snap freezing methods (without isopentane or pre-cooled isopentane). In addition, one hour of cold ischemia time did not significantly influence RNA integrity. This experiment did show, however, that sample transport (regardless of the three conditions described above) on ice resulted in a significantly higher RIN score.

Transport of porcine liver needle biopsies on moist gauze, or in salt solution showed a minor, yet significant, increase in damaged tissue morphology compared to samples that were transported dry. This effect was not observed in the human liver samples. This discrepancy can be explained by the slightly different sample sizes. The 28 gauge needle biopsies were only 1 mm thick, while the “small” human samples were 2 mm thick.

The morphologic artifacts induced by the transport method could have overshadowed the artifacts caused by the freezing method, but the two experiments combined led to the conclusion that freezing small liver samples in isopentane caused minor morphological artifacts, but did not adversely affect RNA integrity. It seems that freezing average sized biobank samples, as represented by the three chosen sample sizes (8 mm^3 - 128 mm^3), can be performed using the same freezing method without isopentane thus avoiding the use of isopentane. Since this was only done in liver, we have also snap frozen kidney, lung and colon tissue without isopentane. The morphology of these tissues appeared to have no damage acquired from the freezing process (data not shown). Since RNA integrity of several tissue types is not influenced by the freezing method [13] and morphology is not harmed, tissue banks may consider to terminate the use of isopentane after

further internal validation. However, for diagnostic purposes like enzyme histochemistry on muscle biopsies, where optimal morphology is of crucial importance, it is still necessary to use isopentane for optimal results [15].
The discrepancy between RNA integrity differences in human (small samples) and porcine tissue (needle biopsies) in more or less comparable experimental settings might be explained by the fact that the liver was taken from the animals only minutes after the animals were euthanized. The organs suffered only a short period (<15 minutes) of post mortem warm ischemia, and because samples were taken shortly after organs were transported to the pathology department, whole organ cold ischemia time (15 minutes) and damage to RNA was probably minimized. The human tissues, taken from a surgically removed liver segment, had been exposed to relatively extensive surgical warm ischemia (report is inconclusive, but at least 3.5 hours) and cold ischemia times (30 minutes consisting of transport and waiting for macroscopical examination), which may have been responsible for more extensive RNA degradation than that seen in porcine tissues.
Initial experiments were conducted on porcine liver to determine the relationship of sample size to RNA integrity. While RIN scores did not change for a long time post-excision in large samples, the RIN scores decreased disproportionately in smaller samples. Based on the results of these initial porcine studies, it was decided to only study the extreme handling and storage parameters for the human liver experiments with RIN values and RT-qPCR data of a set of reference genes as end points. Reference genes, commonly referred to as 'housekeeping' genes, are abundantly expressed in all cell types and are considered to be expressed in relatively constant quantities. Therefore, six standard reference genes were used to determine the influence of tissue handling on gene expression levels. For the studies involving human liver samples, the ΔCq as defined in the Materials and Methods section was between 1.5 and 2 Cq (**Figure 3**) which reflects a three- to four-fold variation in gene transcript copy number. It is difficult to identify which cellular stress mechanisms, in addition to the decrease of available intact RNA molecules, might have driven the variable transcript levels of the standard reference genes as observed between tissue samples exposed to different handling conditions. But it was surprising to us that sample size, was a greater driver of differential gene transcript level than cold ischemia time. Moreover, as soon as biopsies had been placed on moist gauze or in salt solution, this effect seemed to be more pronounced for some genes, suggesting some additional regulation besides poor RNA quality due to one hour of cold ischemia played a role.
In conclusion, based on observations in liver tissue (other tissue types may behave differently), however it seems best not to procure small samples or divide large samples into smaller pieces when the possibility to procure larger samples exists. By RT-qPCR, rather than relatively crude RIN assessment, we were able to conclude that when small biopsies are transported to the pathology department, it is best to keep them in a dry

environment with the container cooled on ice to avoid loss of morphological integrity and to minimize gene expression variability. To prevent air drying artifacts, it is advisable to use small containers which are tightly closed. To avoid RNA degradation and post-surgical gene expression variation, it is of high importance to freeze small biopsies within 30 minutes (preferably immediately) after they have been procured from the patient.

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Chapter 4

Histological Assessment of PAXgene Tissue Fixation and Stabilization Reagents

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(PLoS ONE 2011 6(11): e27704. doi:10.1371/journal.pone.0027704)

Inactivation of Influenza A virus, Adenovirus, and Cytomegalovirus with PAXgene Tissue Fixative and Formalin

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Histological Assessment of PAXgene Tissue Fixation and Stabilization Reagents

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Abstract

Within SPIDIA, an EC FP7 project aimed to improve pre analytic procedures, the PAXgene Tissue System (PAXgene), was designed to improve tissue quality for parallel molecular and morphological analysis. Within the SPIDIA project promising results were found in both genomic and proteomic experiments with PAXgene-fixed and paraffin embedded tissue derived biomolecules. But, for this technology to be accepted for use in both clinical and basic research, it is essential that its adequacy for preserving morphology and antigenicity is validated relative to formalin fixation. It is our aim to assess the suitability of PAXgene tissue fixation for (immuno)histological methods. Normal human tissue specimens (n = 70) were collected and divided into equal parts for fixation either with formalin or PAXgene. Sections of the obtained paraffin embedded tissue were cut and stained. Morphological aspects of PAXgene-fixed tissue were described and also scored relative to formalin-fixed tissue. Performance of PAXgene-fixed tissue in immunohistochemical and *in situ* hybridization assays was also assessed relative to the corresponding formalin-fixed tissues. Morphology of PAXgene-fixed paraffin embedded tissue was well preserved and deemed adequate for diagnostics in most cases. Some antigens in PAXgene-fixed and paraffin embedded sections were detectable without the need for antigen retrieval, while others were detected using standard, formalin fixation based, immunohistochemistry protocols. Comparable results were obtained with *in situ* hybridization and histochemical stains. Basically all assessed histological techniques were found to be applicable to PAXgene-fixed and paraffin embedded tissue. In general results obtained with PAXgene-fixed tissue are comparable to those of formalin-fixed tissue. Compromises made in morphology can be called minor compared to the advantages in the molecular pathology possibilities.

Introduction

Formalin has been the fixative of choice for many decades, and as a result, pathology departments have collected vast archives of formalin-fixed and paraffin embedded (FFPE) samples. These archives of well-defined and documented tissue samples are frequently used in medical research [1]. Histomorphology together with immunohistochemistry (IHC) are the foundations on which all diagnostic and pathological research is based. IHC, the most commonly used tool for tumor phenotyping can, with or without application of antigen retrieval, be performed on FFPE sections providing pathologists with both excellent morphology and reproducible results. Multi-center ring trials, however, show that reproducibility of IHC in FFPE tissue is often compromised by the degree of tissue fixation and the various antigen retrieval protocols [2] used in different laboratories (for more information see www.nordiqc.org). The last 20 years molecular diagnostics were added to the histological assays to enable further discrimination of patient groups and subsequent treatment. In our department for molecular diagnosis we noted that fixation level dependent variation limits our ability to apply techniques used in routine diagnostics to molecular research. While (mi)RNA, DNA and proteins can be isolated from FFPE samples [3-8], assay reproducibility; hence diagnostic value may be limited. This is due to the poor quality of the derivates caused by varying fixation times which results in high variation of the number of cross-links in any individual sample [9].

Limited possibilities for reproducible application of molecular diagnostics and research on paraffin embedded tissue is one of two major reasons why formalin fixation should be replaced by a non-cross-linking (alcohol based) fixative. The second reason is the alleged carcinogenicity of formalin [10,11]. Despite the fact that pathology laboratories have invested in advanced air conditioning units to avoid formalin toxicity, formalin substitutes continue to be developed [12-14]. An impediment to the wide acceptance of formalin substitutes, however, is the fact that pathologists prefer formalin fixation because they have been trained in the assessment of common artifacts in FFPE tissues. Formalin is also a cheap reagent compared to commercially available alcohol based fixatives. Furthermore, morphology of tissue fixed with non-formalin fixatives is, with few exceptions [15], not the same as the morphology of FFPE tissue.

The European FP7 project SPIDIA (Standardisation and improvement of generic Pre-analytical tools and procedures for In-vitro DIAgnostics), a unique consortium of European universities and biotechnology companies, aims at standardization and improvement of generic pre-analytical tools and procedures for *in vitro* molecular diagnostics. The goal of the consortium is to develop pre-analytical tools for molecular diagnostics which improve the stabilization, handling and study of biomolecules in blood, plasma, serum and tissues. By standardizing pre-analytic and analytic procedures, it is foreseen that the ultimate result of this effort will lead to significant

improvement in patient care. We therefore seek to standardize a tissue fixation process that, along with preserving histomorphology, guarantees the extraction of the maximum yield of high-quality nucleic acids from diseased and normal tissue. Along with an improvement in the sensitivity and specificity of routine molecular diagnostic tests, we anticipate that new preanalytical tools for tissue fixation will result in a tissue archive comprised of samples in which high quality biomolecules are preserved. The development of new non-cross-linking fixation reagents, such as the PAXgene tissue fixation and stabilization reagents, therefore, is expected to be pivotal in the standardization of diagnostic and research procedures and may also find applications in biobanking. According to the manufacturer's product description, the PAXgene fixation reagent is a non-carcinogenic, non-cross-linking mixture of different alcohols, acid and a soluble organic compound that rapidly preserves morphology and all bio-molecules. The PAXgene stabilization reagent contains a mixture of alcohols and is applied after fixation. It acts as a storage and transport medium in which morphology and all bio-molecules in the tissue are stabilized before processing. During the development of PAXgene other alcohol based fixatives and transport media like RNA later were tested for both histological and molecular features. PAXgene was the only fixation method which resulted in optimal conditions for both histology and molecular aspects (unpublished observation).
In this paper we compare morphology, histochemistry and antigenicity of PAXgene-fixed and paraffin embedded (PFPE) with formalin-fixed and paraffin embedded (FFPE) tissues. Three independent (no affiliation with any company) pathology labs within the SPIDIA consortium participated in this study, using their own established protocols and automated procedures for tissue processing, H&E staining and IHC procedures. Simultaneously, two other pathology labs within the SPIDIA consortium worked on the proteomic and genomic assessments of the PAXgene fixative. The proteomic data are published elsewhere [16] and the manuscript reporting stabilization of nucleic acids in PAXgene-fixed and stabilized tissue is submitted for publication.

Materials and Methods

Human tissue collection

Tissue samples used for this study were unfixed specimens, collected during routine grossing at the respective pathology departments of the Erasmus MC (Rotterdam, The Netherlands)(EMC), the Medical University Graz (Graz, Austria)(MUG) and the Technical University Munich (Munich, Germany)(TUM). Tissue was collected with consent according to each

institute's local legislation and policies. EMC declares the following; The use of residual tissue accompanied by data on tissue type and disease state is approved by the Erasmus MC Medical Ethical Commission under number MEC-2008-397. Since the Dutch Code of Conduct legislation concerning the use of residual tissue for research is adhered to, no informed consent was necessary for this work. TUM declares the following; All patients gave written informed consent, and the study was approved by the Ethics Committee of the Klinikum rechts der Isar of the Technische Universität München, Germany (reference number 2336/09). MUG declares the following; All sample donors provided written informed consent and the study was approved by the Ethics Committee of the Medical University of Graz, Austria (reference number 20-066).

For each tissue studied, a 4 mm thick tissue specimen was removed from an organ or tumor. This specimen was then subdivided into three approximately equal samples for treatment by either formalin or PAXgene or to be snap-frozen in liquid nitrogen (LN2). Frozen tissue samples were archived for reference in proteomic and genomic research. The following tissues were collected: adrenal gland (1), bladder (2), bonemarrow (5), colon (4), colon cancer (1), esophagus (2), fat (5), fibroma (1), kidney (3), renal cancer (1), liver (9), lung (4), lymphoma (3), breast cancer (2), striated muscle (2), myoma (2), ovarian carcinoma (3), pancreas (1), placenta (1), prostate (2), rectum (5), skin (4), small intestine (4), spleen (2), soft tissue (1), stomach (6), testis (1), thyroid (2) and uterus (1).These were fixed either in formalin for 24 hours (4% neutral buffered formaldehyde) or in PAXgene tissue fixation reagent (Paxgene Tissue System, PreAnalytix GmbH, Hombrechtikon, CH) for either 3 or 24 hours. After fixation in PAXgene tissue fixation reagent, tissues were transferred to PAXgene tissue stabilizer reagent and stored for a minimum of 24 hours or up to one week at room temperature. PAXgene treated samples were processed in a formalin-free tissue processor (ASP3000, Leica). Six changes, 1 hour each, of 100% ethanol and 3 changes of xylene, 1 hour each, were applied in a vacuum at room temperature before tissue was impregnated with 3 changes of low melting point paraffin at 60^{o}C for no longer than 3 hours. Bonemarrow samples were decalcified by submerging the tissue in 10% formic acid (Merck, Germany) for 48-72 hours. Other participating laboratories used essentially the same processing protocol but with different processing instruments.

Tissue samples from human breast cancer were acquired with informed consent from Cureline Inc. (San Francisco, USA). After resection, tumor specimens were divided, one part fixed in neutral buffered formalin (NBF) for 24 hours at room temperature, and the other part fixed and stabilized in the PAXgene Tissue Container (Cat.# 765112, PreAnalytiX, Hombrechtikon, Switzerland). Fixation was performed at room temperature in chamber 1 of the container. Fixation was stopped after 2-4 hours by transfer into chamber 2. Samples were stored for up to 5 days at 4°C until they were processed manually with 80%, 90%, 95%, 99% ethanol(2x), followed by isopropanol

(2x), xylene (2x), and infiltration and embedding in low-temperature melting paraffin. Formalin-fixed samples were processed according to Cureline SOP with 70%, 80%, 90%, 100% (3x) alcohol, xylene (3x), and infiltration and embedding with high-grade melting paraffin. All blocks of formalin-fixed, paraffin-embedded (FFPE) and PAXgene-fixed, paraffin-embedded (PFPE) tissues were stored at 4°C in the dark until use.

H&E staining

Sections of FFPE and PFPE tissue, 4 μm thick were cut on a microtome (HM335E, Microm GmbH, Germany). Slides were dried for 15 minutes at 42^{o}C on a slide warmer (Slide warmer SW85, Adamas Instruments B.V., The Netherlands). In our department H&E staining was performed according to a routine standard operating procedure using a Leica Multistainer (ST5020, Leica). Slides were dewaxed and rehydrated with successive applications of xylene, alcohol 100%, alcohol 70% and tap water. Haematoxylin (Mayer's, Klinipath, Benelux) was applied for 4 minutes followed by a 20 second differentiation in ammonia after which eosin (Eosin Y A+B, Klinipath, Benelux) was applied for 20 seconds. At the other laboratories H&E staining was performed using similar protocols, but with different reagents and machines. Only one H&E stained slide was prepared from each FFPE or PFPE tissue sample.

Virtual microscopy

To ensure that all pathologists examined the exact same tissue section, slides were completely digitized using the Hamamatsu Nanozoomer Digital Pathology (NDP) slide scanner (Hamamatsu, Japan) at a resolution of 40x which is comparable to 400x magnification on a microscope. The areas to be scanned and focus points were manually set. The obtained files were uploaded into a secured internet environment to which the pathologists had access. NDP Slideviewer software; NDP server and NDP View, provided by Hamamatsu, were used to score morphologic features. Images shown in this paper are sections of the files exported from the digital slides.

Morphology scoring

This part of the study was not performed blind to the fixative, because the primary goal was to evaluate potential morphological artifacts of PAXgene-fixed tissues. Four pathologists were asked to assess and compare overall morphology, contrast and nuclear, cytoplasmic, membrane detail and the most obvious fixation artifacts on paired PFPE and FFPE slides. Morphology

of tissue fixed for 24 hours in formalin was defined as the baseline (score = 0). Five grades were used to compare PFPE morphology to FFPE morphology. PFPE morphology could be equal to (0), better (1), considerably better (2), worse (-1) or considerably worse (-2) than FFPE morphology. “Considerably worse” was considered unacceptable for diagnostic purposes, “Worse” is considered to represent poor quality, but nonetheless acceptable for diagnostic purposes. Scores from all pathologists were recorded for tissue type, fixation method and duration. The average score and standard deviation for each tissue type were calculated (see table 1) in order to depict the relative performance of PAXgene tissue fixation reagent and inter-observer variance in one graph.

Histochemical stains

Frequently used histochemical and histological staining procedures were selected for assessment of PFPE tissue. Periodic acid schiff (PAS) and resorcin fuchsin (RF; elastin stain) are commonly used histochemical stains, sirius red (SR; collagen stain) is a typical histological stain, and Gomori (GOM; reticulin stain) represents a silver-gold enhanced histochemical staining procedure.
RF and SR stains were performed according to standard FFPE based protocols [17]. PAS and GOM stains were performed using an Artisan stainer (Artisan, DAKO, Denmark) and DAKO Artisan staining reagents (Artisan PAS kit, AR165, DAKO, Denmark and Artisan reticulin-Nuclear Fast Red kit, A179, DAKO, Denmark). Sections, 4 μm thick, of FFPE and PFPE colon tissue were dewaxed and rehydrated (except sections for RF staining, which were dewaxed and stored in 70% ethanol) before staining.

IHC

Sections, 4 μm thick were cut from PFPE and FFPE tissue blocks right after embedding and after two years of room temperature storage (IHC with CD3, CD20, CD68, KERPAN, KER8.18 and S100 was repeated on blocks that were stored for two years). PFPE and FFPE sections were mounted on the same glass slide to ensure equal treatment of all sections. The slides were dried for 15 minutes at 42^{o}C and baked at 65^{o}C for 15 minutes to enhance adherence. For the first PFPE IHC assessment, we subjected the slides to standard (as used for FFPE) heat induced epitope retrieval (HIER). To demonstrate any changes in the level of antigen masking due to absence of cross-links, we also omitted HIER. At Erasmus MC, HIER was performed using a DAKO PT module (PT Link, Dakocytomation, Denmark) with DAKO PT High pH Buffer (Envision FLEX target retrieval solution high pH 50x, K8004, Dakocytomation, Denmark) at 99 degrees centigrade for 15 minutes. For DAKO PharmaDX EGFr IHC the slides were treated with proteinase K (5

minutes at room temperature) provided with the ready to use kit (EGFR PharmDX For Autostainer, K1494, Dakocytomation, Denmark). The IHC procedure was performed using a DAKO Stainer (DAKO Autostainer plus, Dakocytomation, Denmark). At Erasmus MC the sections were incubated with PBS/3%H_2O_2 for ten minutes to block any endogenous peroxidase activity. Slides were incubated with primary antibodies for 30 minutes followed by DAKO envision conjugate for 30 minutes. The conjugate's peroxidase label was visualized by DAB+ (DAKO Real, K5007, Dakocytomation, Denmark). Slides were counterstained with Mayer's Heamatoxylin (Mayer's, Klinipath, Benelux) for 1 minute, dehydrated and a cover slip applied (Leica multistainer/coverslipper station, resp. ST5020 and CV5030, Leica). The different antigen retrieval methods of partner labs and IHC protocols are listed in table 2. Specificity of IHC stains from both untreated and HIER treated PFPE sections were compared to the FFPE sections. If immunohistochemical reactions in PFPE sections were different to those in FFPE sections, only the antibody dilution was altered except for the progesterone receptor antibody for which another clone was used to replace the non-reacting clone.

HER2 IHC

Sections of PFPE and FFPE human breast cancer tissue with a thickness of 4 μm were mounted on SuperFrost Plus slides (Cat.# 631-0108, VWR, Darmstadt, Germany). Immunohistochemistry for determination of HER2 protein overexpression was performed with the HercepTest kit (Code K5204, Dako, Glostrup, Denmark). All samples were treated according to manufacturer's instructions with the exception of the epitope retrieval step with PFPE sections. In the case of FFPE tissue, the sections were placed in a staining dish filled with epitope retrieval solution (vial no. 7, HercepTest kit) and incubated for 40 minutes at 98°C in a water bath. PFPE tissue sections were incubated for 5 minutes at 98°C in a pH9 target retrieval solution (Code S2367, Dako, Glostrup, Denmark). Counterstaining was done with all samples for 2 minutes with hematoxylin.

HER2 CISH

For determination of HER2 gene amplification sections of FFPE and PFPE human breast cancer samples with a thickness of 6 μm mounted on SuperFrost Plus slides (Cat.# 631-0108, VWR, Darmstadt, Germany) were de-waxed, pretreated, and hybridized with reagents from the SPOT-Light HER2 CISH kit (Cat.#84-0150, Invitrogen Corporation, Camarillo, USA). Sections from FFPE tissue were processed according to manufacturer's instructions including heat treatment and a 5 minutes protease digestion

step. For PFPE tissue sections, the heat pretreatment and enzyme digestion steps were omitted. After deparaffinization, sections were air-dried and covered with the HER2 probe for denaturation and hybridization. Counterstaining was performed with all samples for 5 seconds with hematoxylin (Reagent J, SPOT-Light HER2 CISH kit).

Results

Morphology

In the evaluation of morphology of human tissue, the morphology of PFPE tissue appeared clearly comparable to FFPE tissue (see figure 1a). Morphology of PFPE liver was crisper and showed slightly more contrast as compared to FFPE tissue. In striated muscle tissue, contrast is also slightly enhanced after PAXgene fixation. The same evaluation was noted for thyroid and adrenal gland tissue in which the contrast between FFPE and PFPE is somewhat stronger in the PAXgene-fixed tissues. Overall, the differences between FFPE and PFPE tissues were deemed to be minimal. Elevated levels of eosinophilia, while noted in PFPE tissues, did not seem to hinder observations. PFPE lung tissue appears swollen in relation to FFPE lung tissue. This may be due to the fact that red blood cells (RBC), normally found in and around alveoli are damaged and appear empty, or are, in some cases, completely lysed in PFPE tissue. In the lung, only minor variations are observed with slightly less contrast in the lung tissues after PAXgene fixation compared to formalin fixation. In tissues from stomach, prostate and small intestine PAXgene tissue fixation reagent seems to cause small deviations in morphology (see figure 1b). In stomach tissue, the PAXgene fixation shows less contrast between glands and infiltrate, and discrimination between parietal and chief cells is difficult. In the colon, morphology of PFPE and FFPE tissues is nearly identical, but Paneth cells are less readily identified after PAXgene fixation. In prostate, PAXgene fixative causes slight pyknosis of nuclei and minor cell shrinkage compared to formalin fixation.

The overall scores of the H&E staining evaluation by four pathologists are presented in a comprehensive overview in figure 2. For gastric tissues, the detailed scores are all better for PAXgene fixation and only the overall morphology scores are negative. In small intestine, there is a decrease in the cytoplasmic and membrane details in PFPE tissue that results in this overall negative score, whereas for prostate the only feature better for PAXgene fixation is contrast. Figure 2 shows that, considering the standard deviation, the differences between 3 hours and 24 hours fixation in PAXgene are minor. In general, the variation in estimating various tissue features indicates that observation of morphology is quite subjective. No serious

statistical analysis was possible with this number of data points. The average and standard deviation were calculated to indicate the subjectivity of these observations. Except for pathologist TUM2, pathologists do not prefer their trusted in-house H&E stain above H&E stains from other sites. One pathologist (MUG) seems to be very critical, giving mostly negative scores for PFPE tissue, however the overall morphology scores, both positive and negative, are mostly close to the neutral baseline, indicating only minor differences between PFPE and FFPE (table 1).

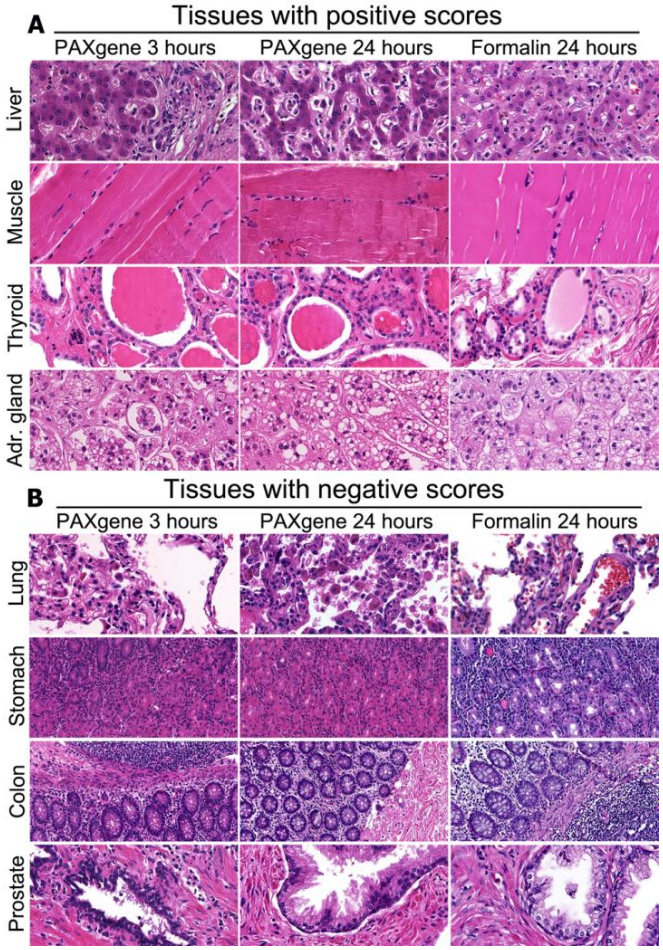

Figure 1
Morphology of various normal tissues.
Figure 1a shows H&E stains in tissues fixed for 3 hours in PAXgene (left column), 24 hours in PAXgene (centre column) and for comparative purposes routine fixation in formalin for 24 hours (right column). In liver PAXgene fixation scores better on

contrast. Striated muscle shows more detail after PAXgene fixation. Thyroid tissue scores better in all scored details. And adrenal gland tissue predominantly scores better in membrane detail. (400x original magnification)
Figure 1b shows H&E stains in tissues fixed for 3 hours in PAXgene (left column), 24 hours in PAXgene (center column) and for comparative purposes routine fixation in formalin for 24 hours (right column). Lung tissue appears swollen. In gastric tissue the cell differentiation is harder to distinguish. In colon the distinctive granules in Paneth cells are less easily detectable after PAXgene fixation and in prostate the contrast of the epithelium is lower in PFPE tissue compared to FFPE tissue. (Lung and prostate 400x; gastric and colon tissue 200x original magnification)

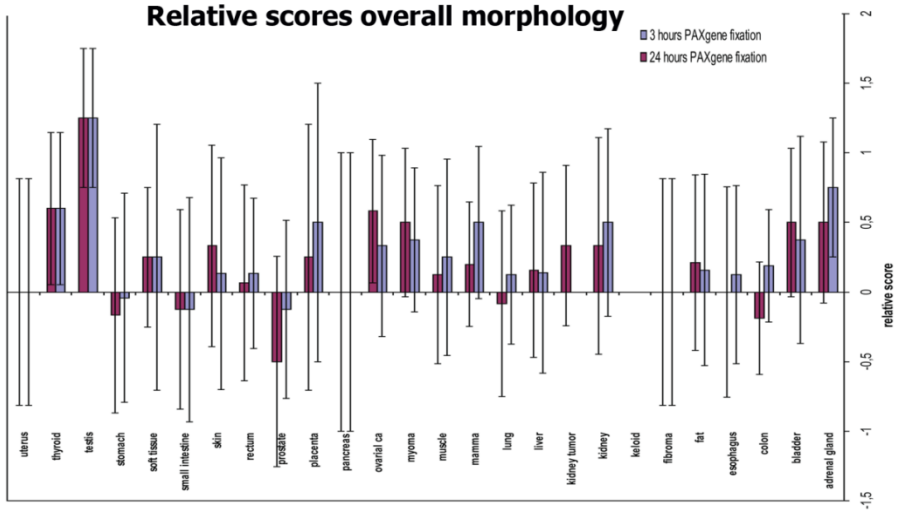

Figure 2
Relative morphology scores of human tissues.
In this graph relative scores of overall morphology of 3 hours fixation and 24 hours fixation are compared. All PAXgene-fixed tissue is scored relatively to 24 hours formalin-fixed tissue (zero base line). Error bars, representing inter-observer variance, are shown.

Table 1.

	Pathologist TUM1		Pathologist TUM2		Pathologist MUG		Pathologist EMC	
	average score	*st. dev*	*average score*	*st. dev.*	*average score*	*st. dev.*	*average score*	*st. dev.*
all scores scores	0.16	0.44	0.16	0.56	-0.05	0.60	0.13	0.61
EMC slides scores	0.14	0.47	0.10	0.50	0.07	0.66	0.04	0.66
MUG slides scores	0.22	0.42	0.21	0.67	-0.15	0.40	0.17	0.52
TUM slides	0.17	0.38	0.31	0.54	-0.28	0.59	0.41	0.50

Since red blood cells (RBC) are damaged during PAXgene fixation, we examined seminoma tissue (seminoma tumor cells are fragile cells which barely express any intermediate filaments [18]), in order to investigate possible limitations of the PAXgene tissue fixative. PAXgene fixation did not damage or lyse the seminoma cells. PFPE seminoma tissue did, however, show some dissociation and slightly decreased membrane detail. Immunohistochemistry assessment, however, showed that membranes, cytoplasmic as well as nuclear, in PFPE seminoma tissue express all the antigens also found in regular, FFPE based IHC. The staining patterns show that the cell membranes are intact and that the dissociation did not lead to structural or molecular integrity loss. Furthermore, Glycophorin C was stained to show that, although RBC appear to be empty, the membrane antigen Glycophorin C is still present on RBC (figure 3).

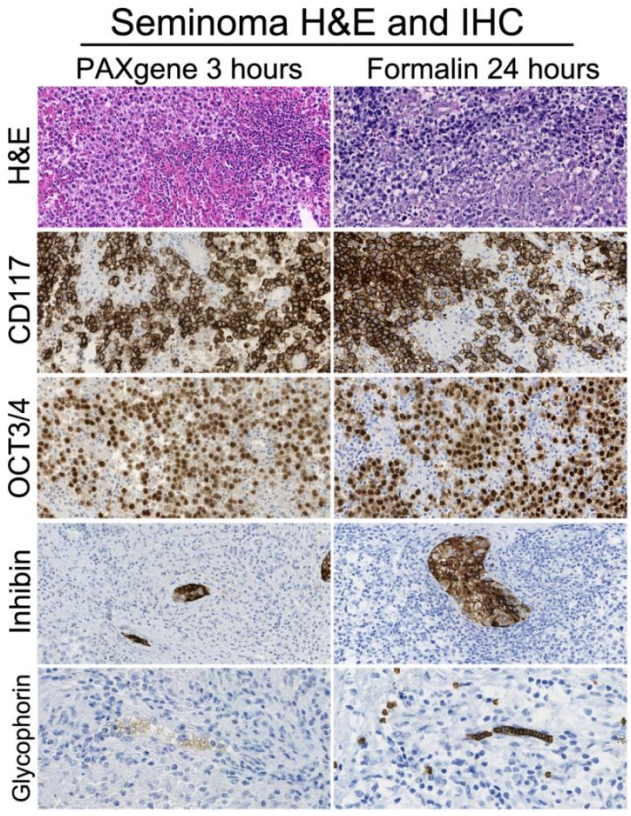

Figure 3
Despite of morphological differences, diagnosis can be confirmed by immunohistochemistry. The left column shows PAXgene-fixed sections, the column on the right shows formalin-fixed sections. Although PAXgene-fixed seminoma tissue seems more compact and cells tend to be rounder and less interconnected the membranes, stained with CD117, are intact. The nuclear antigens OCT3/4 are well preserved. IHC was not optimized for this antibody, therefore the staining is somewhat lighter. The Inhibin stain shows that this particular cytolplasmic antigen is well preserved and did not leak out of the cells. The red blood cells in PAXgene-fixed tissue are damaged, but still express some Glycophorin C. (200x original magnification)

The above described artefacts raised the question whether hematopathological diagnostic would be hindered by PAXgene fixation of lymphoid tissue. Red blood cells are often used as internal cell size reference, differentiation of the lymphoid and myeloid lineages needs proper nuclear details and normal hematoxylin/eosin contrast. In spleen tissue the adverse effects of PAXgene fixation became apparent. We observed loss of red blood cells, alteration of nuclear detail and disproportionate cell shrinkage. These artefacts resulted in such morphological changes that the pathologist needed to re-evaluate the cell types by comparing the PAXgene-

fixed tissue directly with the formalin-fixed section. Morphology of PFPE bonemarrow and lymphoma samples was comparable to that of FFPE tissue. However, the higher eosinophilia made recognition of the different lineages difficult (figure 4)

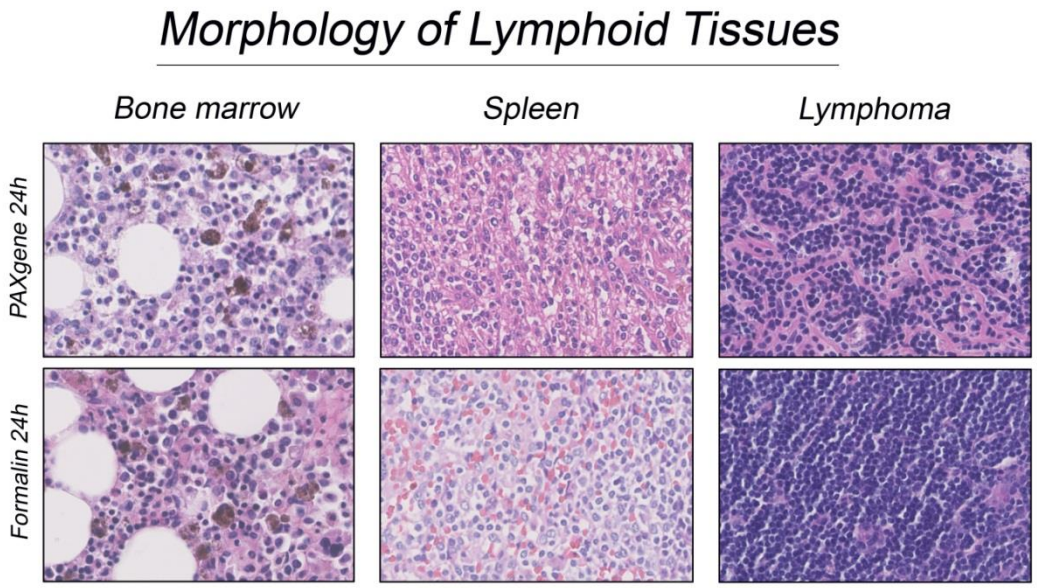

Figure 4
Morphology of lymphoid tissues.
The left panel shows bonemarrow after PAXgene or formalin fixation, followed by decalcification in 10% formic acid. The middle panel shows that the PAXgene specific artefacts like red blood cell lysis, aberrant nuclear details and higher eosiniphilia result in a very different picture compared to FFPE tissue. The PFPE lymphoma tissue in the right panel is slightly over stained with eosin which negatively influences the differentiation of hematopoetic lineages. (H&E, 400x original magnification)

Immunohistochemistry

Of all routinely used immunohistochemical antibodies tested, only a few exhibited lower levels of immune reactivity on PFPE sections compared to mirrored FFPE sections. This was overcome by omitting antigen retrieval, adjustment of antibody concentration, or using another clone (see table 2 for details). Antigen retrieval could be omitted for the following antibodies: CD3, CD20, CD68, KER8.18 and S100. For S100 there was no clear difference in immuno staining with or without antigen retrieval. Neuroendocrine cells in colon mucosa were intensely and specifically stained regardless of whether

or not antigen retrieval was applied. The IHC procedure on PFPE tissue with antibodies: BCL-2, BCL-6, CD2, CD4, CD5, CD7, CD8, CD10, CD21, CD23, CD31, CD117, D2-40, ER, EGFR, Her2Neu, PR, glycophorin-C, Inhibin, Ker5/6, KERPAN, KI-67, OCT3/4, P53, PLAP and vimentin still required an antigen retrieval procedure. Typical examples of IHC stains used for determination of prognosis or pharmacodiagnostics are shown in figure 5a. Both nuclear antigens ER and PR were stained equally well in breast carcinoma after both modes of fixation. PR clone PgR636 (DAKO, M3569), however, needed to be replaced by clone 1A6 (Bioprime, PR500) to obtain these results. HER2NEU was stained in 5 different breast tumor samples. The HER2NEU staining pattern in PFPE sections did not always resemble the pattern found in mirrored FFPE sections. In general the PFPE HER2NEU signal is stronger and crisper compared to the signal found in FFPE sections. EGFR in skin looks somewhat more granular in PFPE sections compared to FFPE sections, nevertheless the signal is specifically located in the epidermis and in hair follicles. Commonly used antigens for tumor typing are shown in figure 5b. CD3 is stained in a lymph node and although the staining intensity is somewhat lower in PFPE tissue than in FFPE tissue, the signal is specifically found in all T-cells. S100 stains neuroendocrine cells in colon mucosa. Epithelial cells in colon tissue are stained positive for pankeratin. In colon, almost all cell types are vimentin positive. Epithelium however, is vimentin negative. To investigate epitope stability in PFPE blocks, IHC of CD3, CD20, CD68, KERPAN, KER8.18 and S100 was repeated on blocks that were stored for two years. No deterioration of antigenicity was observed.

Table 2

Antibody	Clone	Company and code number	Dilution	Antigen retrieval	
				PAXgene fixation 3 hours	*Formalin fixation 24 hours*
BCL-2	124	DAKO M0887	1:100	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
BCL-6	GI191E/A8	ITK CMC796	1:100	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD2	AB75	MONOSAN MONX10830	1:50	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD3	polyclonal	DAKO A0452	1:150	none	DAKO PT Hi 15 minutes
CD4	4B12	MENARINI 35066	1:10	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD5	4C7	MONOSAN MONX10335	1:100	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD7	CBC.37	DAKO M7255	1:10	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD8	C8/144B	DAKO M7103	1:200	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD10	56C6	MONOSAN MONX10354	1:20	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD20	L26	DAKO M0755	1:400	none	DAKO PT Hi 15 minutes
CD21	1F8	DAKO M0784	1:30	DAKO PT Lo 15 minutes	DAKO PT Lo 15 minutes
CD23	SP23	Neomarkers RM-9123-S	1:25	DAKO PT Lo 15 minutes	DAKO PT Lo 15 minutes
CD31	JC70A	DAKO M0823	1:50	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD68	KP-1	DAKO M0814	1:1600	none	DAKO PT Hi 15 minutes
CD79a	JCB117	DAKO M7050	1:100	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
CD117	YR145	Cellmarque 117R-16	1:50	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
D2-40	D2-40	DAKO M3619	1:50	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
EGFR	2-18C9	DAKO K1494	R.T.U.*	protK 5 minutes R.T.	protK 5 minutes R.T.
ER	1D5	DAKO M7047	1:35	TE pH9 98°C 20 minutes	TE pH9 98°C 20 minutes
glycophorin C	RET40f	DAKO M0820	1:600	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
Her2Neu	Herceptest	DAKO K5204	R.T.U.*	Kit buffer 98°C 40 minutes	Kit buffer 98°C 40 minutes
Her2Neu	4B5	Roche, 790-2991	R.T.U.*	HIER cell conditioner (Benchmark)	HIER cell conditioner (Benchmark)
Inhibin	R1	DAKO M3609	1:50	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
KER5/6	D5/16B4	DAKO M7237	1:50	HIER cell conditioner (Benchmark)	HIER cell conditioner (Benchmark)
KER8&18	5D3	Neomarkers MS 743-S	1:100	none	DAKO PT Hi 15 minutes
KER-PAN	AE1/AE3	Neomarkers MS 343-P	1:200	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
KI-67	MIB-1	DAKO M7240	1:100	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
OCT3/4	C-10	Santa Cruz sc-5279	1:80	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
P53	DO-7	DAKO M7001	1:400	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
PLAP	8A9	DAKO M7191	1:100	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes
PR	1A6	Bioprime PR500	R.T.U.*	TE pH9 98°C 20 minutes	TE pH9 98°C 20 minutes
S100	polyclonal	DAKO Z0311	1:3200	none	DAKO PT Hi 15 minutes
Vimentin	V9	DAKO M0725	1:1000	DAKO PT Hi 15 minutes	DAKO PT Hi 15 minutes

*R.T.U. = ready to use

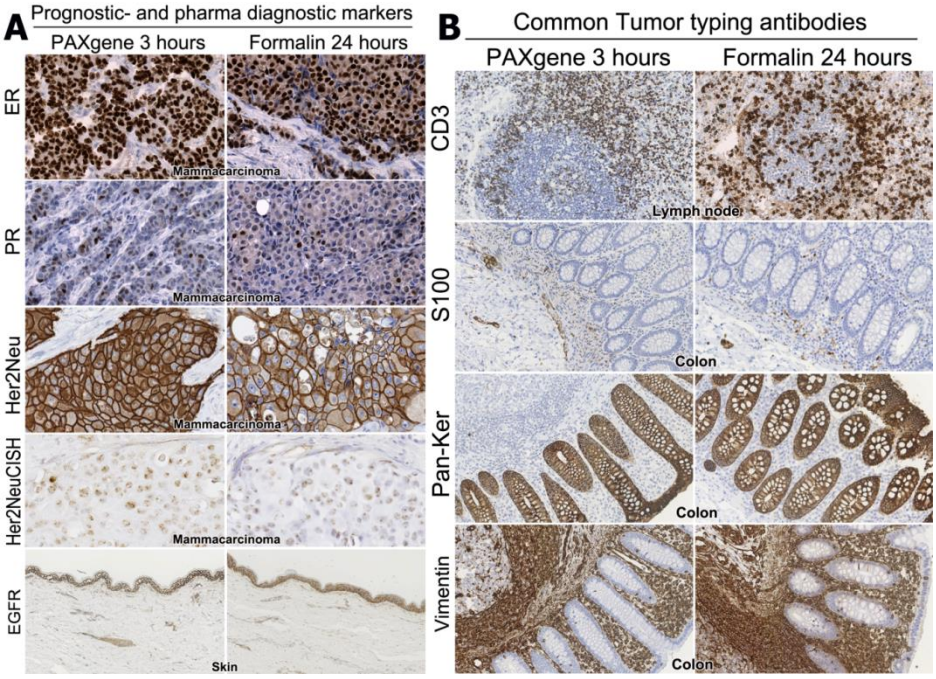

Figure 5
Immunohistochemistry .
In **figure 5a** the most common prognostic (ER and PR) and pharmaco diagnostic markers (Her2Neu and EGFR) are shown. ER, PR are stained in serial sections of the same breast tumor sample. Her2Neu IHC as well as CISH was performed on a breast cancer sample with Her2Neu over expression and gene amplification. EGFR is stained in skin samples. The left column shows PAXgene-fixed sections, the column on the right shows formalin-fixed sections. (ER, PR, Her2Neu 400x; EGFR 100x original magnification)
In **figure 5b** the most common tumor differentiation markers are shown. CD3 is stained in lymph node. S100, Pan-Keratin and vimentine are stained in colon tissue. The left column shows PAXgene-fixed sections, the column on the right show formalin-fixed sections. (200x original magnification)

Histochemical stains

The results of histochemical stains are depicted in figure 6. Different levels of staining intensity were observed between the two fixation methods. After PAS staining, the goblet cells in colon are more intensely stained in PFPE sections as compared to FFPE sections. The staining intensity of basal membranes in blood vessels, however, is the same in both fixation methods.

This indicates that specifically mucin in Goblet cells is more condensely present after PAXgene fixation. The RF stain, which in FFPE results in black elastin fibers, red collagen fibers and yellow muscle fibers, shows the same results in PFPE tissue. However, after 3 hours of PAXgene fixation Goblet cells are stained light purple. This stain is less distinct when tissue is fixed in PAXgene for 24 hours. Collagen fibers were stained with the SR staining method. Collagen fibers are stained red, whereas muscle fibers are stained yellow. In PFPE sections the collagen fibers between the crypts appear crisper than in FFPE tissue. In FFPE sections, this pattern is also present, but the intensity is much lower. The reticulin stain (Gomori silver impregnation) shows that reticulin fibers are stained less intensely in PFPE sections as compared to FFPE sections. The staining in PAXgene tissue fixed for 3 hours is crisper than that of tissue fixed for 24 hours PAXgene.

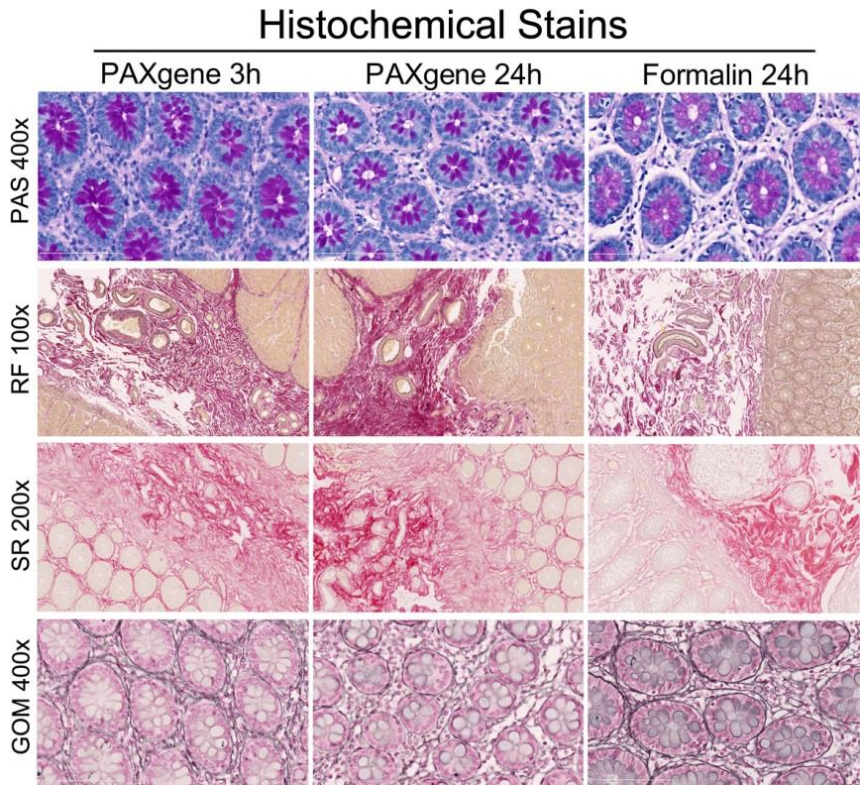

Figure 6

Histochemical stains show some qualitative differences between PAXgene and formalin fixation.

In PAXgene-fixed tissue PAS staining results in more condense staining of Goblet cells in colon. The RF, elastin stain of PFPE tissue is similar to the staining pattern in FFPE tissue. The SR, collagen stain in PFPE tissue is stronger compared to the stain in FFPE tissue. The GOM, reticulin stain is crisper in FFPE tissue. In PFPE tissue

short fixation results in a stronger, crisper stain compared to longer PAXgene fixation. (PAS and GOM 400x; RF 100x and SR 200x original magnification)

HER2 CISH

Her2Neu CISH is possible on PFPE breast cancer tissue. Figure 5a shows that the breast cancer tissue (ductal *in situ* carcinoma) which also over-expresses the Her2Neu protein shows Her2 gene amplification. Although the signal in the PFPE section is stronger as compared to the signal in the FFPE section, the results show that in both sections HER2NEU gene amplification is present.

Discussion

The results of our exploratory investigation clearly show that despite slight differences seen between FFPE and PFPE tissues, PAXgene tissue fixation and stabilization reagents indeed preserve morphology, histochemical features, and antigenicity such that the reagents can eventually be used in routine pathology laboratories. Molecular diagnostics and medical research can benefit from this fixative, because (described in a separate publication) it appears to preserve the macromolecules in the tissue in a more native state than in tissue fixed with formalin [16]. All diagnostic techniques described in this paper can be performed on PFPE material without major protocol adaptations. Furthermore, a future PFPE archive may provide researchers with high quality and easily accessible bio-molecules which can be harvested from the same morphologically well described and diagnosed tissue blocks [16]. For contemporary basic pathology research, this means that it would no longer be necessary to divide small specimens into two even smaller samples: one for morphology and IHC (FFPE) and another for molecular research (snap freezing or immediate RNA isolation). PAXgene seems to provide the answers to many questions, but in the meantime, some critical questions are raised.

According to participating pathologists, the most salient feature of PFPE tissue was the quality of the H&E staining. They noted that in general, the staining for both haematoxylin and eosin were more intense in the PFPE tissues than in corresponding FFPE tissues. In many cases this effect resulted in increased contrast between the components of the various tissues. Details such as nuclear and cytoplasmic detail were also affected by the choice of fixative. Surprisingly however, these details were variously scored as either positive or negative factors in the evaluation of tissue morphology. This indicates that the individual pathologist can sense that there is a shift in morphological features, but that it is difficult to evaluate

whether this has a definitive positive or negative effect on how they evaluate tissue. It is known that morphologic evaluation of tissue is subject to considerable inter- and intra-observer variability, therefore, it could be that the effect of the PAXgene fixation on tissue morphology, despite the fact that it is unequivocally observed, falls within the range of inter- and intra-observer variability in general. Of course this is all speculative, since we chose not to perform a blind, but a comparative study. This approach uncovered a number of morphologic features characteristic of PAXgene fixation that are not seen in FFPE tissue, the most noticeable of which are the loss of granules in Paneth cells and fragmentation of red blood cells. This effect is probably due to the acetic acid component of the fixative which seems to dissolve some constituents of cytoplasm or cell membrane. Whether these artifacts influence the establishment of a proper diagnosis in cancer tissue will be further investigated during a world wide SPIDIA ring trial, planned in the near future. One problem mentioned by the hematopathologist is that red blood cells are often used as a cell size reference in lymphoma diagnostics. Since the cell shrinkage factor in PFPE tissue seems slightly different opposed to FFPE tissue, a new reference is needed. Endothelial cells could be of use, since the capillaries in PFPE tissue are more open and easily detected. The nuclear detail in lymphoid cells in spleen tissue is slightly different in PFPE tissue compared to FFPE tissue. This effect underlines the need for a new learning curve for pathologists to get acquainted with the PAXgene fixation induced artefacts.

PAS, reticulin, elastin and sirius red stains were performed to investigate whether the appearance of histochemical stains on PFPE tissue and FFPE tissue were similar. The protocols used for these stainings were not optimized for use with PAXgene-fixed tissue. However, follow up experiments showed certain tissue substances stained during these procedures react differently to PAXgene fixation compared to formalin fixation. The findings do, however, indicate that with some fine-tuning of protocols the PFPE results can become the same as the standard FFPE result. Considering that different pathology labs use different protocols they will need to adopt their own workflow.

The results also show that immunohistochemistry in PFPE sections is very comparable to IHC in FFPE sections. We observed some limitations which needed further investigation. The progesterone receptor antibody had to be replaced by an antibody from a different clone to obtain results seen in FFPE sections. Antigens remain stable in the tissue blocks for at least two years. When IHC was repeated on two year old blocks, the specificity and sensitivity where comparable to those of fresh blocks. Chromagen in situ hybridization (CISH) was performed without any problems, whereas fluorescence in situ hybridization (FISH) needs further investigation. Because DNA is more natively preserved it is easier accessible for the used probes, even without protease digestion or cooking the slides. Without protein digestion (PFPE tissue is more prone to over-digestion) auto fluorescence is a major obstacle in some tissue types. Cooking the slides did

improve the signal to noise ratio, but results are not yet optimal. Specific tumor types are now being collected to study the effectiveness of improved FISH protocols.

Like Kryofix or Boonfix, PAXgene tissue fixation is an alcohol based fixative and could therefore lead to further standardization of IHC [13] and histological procedures in general. Furthermore, prolonged fixation in PAXgene tissue fixative will not result in increased numbers of cross-links, an effect known to occur after prolonged formalin fixation, and one which often leads to false negative or doubtful IHC results and the resulting clinical impact [19]. Using not recommended alternative longer stabilization times showed that tissues fixed for 24 hours and than stored in stabilizer for 3 months, both morphology and antigenicity (CD31, KER-07, synaptophysin and chromogen A) remain intact. It was, however, necessary to mount the sections of long term stabilized fatty tissue on poly L-lysin coated slides to avoid loosing sections after antigen retrieval. It is, however, not common that tissue stored for that amount of time in stabilizer fluid (or presently formalin) is used for diagnostic purposes.

Although this work focuses on the morphological and immunohistochemical aspects of PAXgene tissue fixation, other members of the SPIDIA consortium have performed extensive investigations of the nature of proteins and nucleic acids in FFPE and PFPE tissues. Proteins derived from PFPE tissue show reactivity patterns analogous to proteins derived from frozen tissue, as opposed to proteins isolated from FFPE tissue which show decreased activity in proteomic testing [16]. Furthermore, when the properties of the new fixative with regard to stabilization of nucleic acids were investigated, preliminary data suggested that RNA is preserved far better in PAXgene-fixed as compared to formalin-fixed tissue. A detailed study will be reported in the near future by Viertler *et al.* (submitted for publication).

Although biomolecules appear to be preserved in a more native state, it is not yet known how long these molecules remain stable in PFPE tissue blocks. It is known that RNA deteriorates even after tissue is embedded in paraffin [20]. In FFPE tissue, the RNA is cross-linked and therefore of inferior quality to start with, but conversely, these cross-links provide stability. PAXgene fixation leaves RNA intact from the beginning, but since no cross-links are formed by PAXgene fixation, stability may be an issue [21]. Ongoing experiments on storage conditions and their effect on diverse tissue derivates will provide more insight into this matter.

Since the time when formalin replaced alcohol fixation over a century ago [22], analytical technologies were developed for use on formalin-fixed tissue. Nowadays, technology and medical care have reached a point at which a non-cross-linking fixative like PAXgene is once again preferable. Medical care demands standardized diagnoses and the use of a broader spectrum of diagnostic tools. Gene chips, which require high quality tissue RNA, have been developed and will one day be widely used for diagnostics [23]. Although genomic and proteomic techniques can be applied to biomolecules

derived from FFPE tissue [3-8], the fixation time dependent variability of the number of cross-links in each tissue sample makes standardization, and therefore reproducibility, of downstream procedures virtually impossible. If standardization of formalin fixation were possible, it would have easily been established during the last century. The standardization and nucleic acid and protein friendly tissue fixation that the PAXgene fixative can offer may provide answers to these demands. Implementation of PAXgene fixation in routine pathology has one added advantage in that, as opposed to formalin fixation, PAXgene fixation reagents have no known carcinogenic effects (according to manufacturer's material safety data sheet). Proper air regulation as installed to avoid formalin toxicity, will keep alcohol toxicity levels to the required minimum. Therefore, introduction of this fixative could lead to the much desired formalin-free environment in pathology laboratories. Preliminary results obtained by fixation of whole pig organs show that, when tissue is pretreated as usual (e.g. cutting open to allow fixation) PAXgene is able to fix organs overnight. A beneficial side effect of the alcohol based fixation is that small lymph nodes embedded in fatty tissue are easily detectable by visual inspection. When specimens were stored in the stabilizer reagent for up to 3 months morphology remained optimally preserved. Furthermore, histology of tissues fixed in PAXgene for only 3 hours were comparable to that of tissues fixed in formalin or PAXgene for 24 hours indicating that eventual use of PAXgene in routine pathology could shorten the time from tissue acquisition to pathology result thus improving laboratory work flow. When tissue is placed in PAXgene tissue stabilizer, the fixation process stops while all tissue components are stable until processing. Potentially, the stabilization step could be performed in any processing machine so that the laboratory workflow does not need to be adapted to a two reagent system. PAXgene fixation of post mortem bone marrow samples showed that decalcification is still necessary, eventhough the fixative contains acetic acid. This decalcification step (10% formic acid 48 to 72 hours) did not influence morphology. Of course, before PAXgene can be fully implemented in routine pathology, more specific questions must be answered. While preparing this manuscript, tissue collection was ongoing to build a shadow archive of PFPE tissue. This archive will be subjected to all imaginable diagnostic testing and basic research techniques. A tissue atlas will be produced to serve as a reference manual for pathologists. When all tissue types are explored and described it will be more acceptable for pathologists to learn a new set of artefacts. Issues like biomolecule stability in this archive will be addressed, as well as the true versatility of the fixative in a clinical pathology setting. This stepwise introduction will build confidence in the fixative while exploring the molecular diagnostic and research possibilities. Only when trustworthy results are obtained in all fields of expertise can this fixative be implemented without obstacles.

In conclusion, PAXgene tissue fixation and stabilization reagents provide contemporary tissue related molecular medical research the best of both worlds. Although the technique is not without compromise, all currently

assessed conventional histological techniques are fully applicable on PFPE tissue. Data produced within the SPIDIA project show that proteins and nucleic acids are preserved in a more native state in PFPE tissue compared to FFPE tissue [16]. We believe that after more extensive assessment in routine (molecular) pathology, the PAXgene fixation method can be used for primary diagnosis by routine H&E and IHC while enabling more accurate proteomic and molecular profiling for determination of prognosis and drug response.

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Inactivation of *Influenza A virus*, *Adenovirus* and *Cytomegalovirus* with PAXgene Tissue Fixative and Formalin

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Abstract

Formalin fixation is known to inactivate most viruses in a vaccine production context, but nothing is published about virus activity in tissues treated with alternative, non-crosslinking fixatives. We used a model assay based on cell culture to test formalin and PAXgene Tissue fixative for virus inactivating abilities.
MDCK, A549 and MRC-5 cells were respectively infected with *Influenza A* virus, *Adenovirus* and *Cytomegalovirus*. When 75% of the cells showed a cytopathic effect (CPE), the cells were harvested and incubated for 15 minutes, one, three, six or twenty-four hours with PBS (positive control), 4% formalin or PAXgene Tissue Fix. The cells were disrupted and the released virus was used to infect fresh MDCK, A549 and MRC-5 cells, cultured in 24 wells cover slip plates. The viral cultures were monitored for CPE and by immunocytochemistry to record viral replication and infectivity.
Inactivation of *Adenovirus* by formalin occurred after 3 hours, while *Influenza A* virus as well as *Cytomegalovirus* were inactivated by formalin after 15 minutes. All three virus strains were inactivated by PAXgene Tissue fixative after 15 minutes. We conclude that PAXgene Tissue fixative is at least as effective as formalin in inactivating infectivity of *Influenza A virus*, *Adenovirus and Cytomegalovirus.*

Introduction

Manipulating potentially virus-infected human tissue is an occupational hazard pathologists and technicians face on a daily basis. Despite many personal safety precautions like protective clothing, masks, glasses and directional air, the risk of viral infection is ever present. Although most casualties due to seasonal and non-pandemic pathogens are reported amongst nurses and physicians [1,2], the rise of pandemic viruses like Severe Acute Respiratory Syndrome (SARS) and avian influenza bring potential biohazards inside the pathology departments and morgues.

Not much is known about the stability of virus in tissue or cytological specimens, but *Influenza A* virus has been found to be infective on smooth surfaces and bank notes for several hours. The duration of infectiveness is prolonged up to 24 hours when the virus is embedded in protein, like dried mucus or culture medium [3]. This indicates that virus infectivity is highest as long as the virus is stored in the host environment. As long as tissue is not chemically fixed, virus infection can occur by exposure to aerosols (opening a tissue container) or by percutaneous exposure (scalpel stick accidents during grossing). In the field of vaccine preparation, formalin is a widely used agent to inactivate viruses although accidents caused by incomplete inactivation were reported in the past [4]. As soon as tissue is thoroughly fixed in formalin, most viruses are fully inactivated [5]. However, tissue fixation takes several hours, depending on the size of the specimen. For formalin the average penetration rate of tissue is 2 mm/hour. Large organs or specimens can take 24 hours or longer to be completely fixed. Some viruses are very resilient and are only inactivated after long exposure to glutaraldehyde [6], indicating that tissue fixation in formalin may not be sufficient to assure a safe working environment for pathologists.

PAXgene tissue fixation reagent (PAXgene) is a novel commercially available (provided as ready to use solution) fixative that is based on alcohol/acid mix which results in tissue morphology and antigenicity comparable to that of formalin fixed tissue. The major advance is that macro molecules are preserved in a more native state compared to macromolecules derived from formalin fixed tissue. In proteomic and genomic assays, proteins and nucleic acids derived from PAXgene fixed and paraffin embedded (PFPE) tissues react as those derived from fast frozen tissues [7,8, 9, 10]. Recently, Cadoret, et al. [11] showed that DNA, isolated from PAXgene fixed tissue of Atlantic salmon gills, could be used to detect *Neoparamoeba perurans* DNA by PCR. Also, conventional histological stainings and IHC could be performed on PAXgene fixed tissue to visualize the amoeboid parasite in salmon gills.

Although alcohol can be used to decontaminate virus contaminated surfaces [12], it is not known if inactivation by alcohol-based tissue fixatives occurs. Since PAXgene fixation leaves macromolecules in their native form [7,8, 9, 10], it could be that upon rehydration a virus could become re-activated. Formalin can be used to inactivate viruses for vaccine production [4] and recently, Kading, et al. [13] described inactivation of Rift Valley fever virus during paraformaldehyde fixation of mosquito specimens. To provide a standardised method of virus inactivation monitoring, we chose to use this virus cell culture approach to find out whether the novel fixative would inactivate viruses and that viruses would remain inactive upon rehydration.

In the cell culture virus inactivation assay, we used *Influenza A* virus (RNA virus, *Orthomyxovirus, Influenzavirus A*) and *Adenovirus* (DNA virus, *Adenoviridae, Human adenovirus type 2*) as model viruses, because these viruses can be transmitted in aerosols [14,15] that may form during tissue handling, and are known to remain active for hours to days outside the host

environment [3]. Human cytomegalovirus (DNA virus, *Herpesvirales*, *Herpesviridae*, *Cytomegalovirus*) was chosen because of its overall prevalence in nearly 60% of the population in the U.S. [16]. It has the ability to infect a variety of human cell types such as fibroblasts, smooth muscle cells, macrophages, and cells of the bone marrow [17]. Also its ability to establish a lifelong latency after primary exposure in specific types of the myeloid lineage [16,18] makes it relevant for investigating safety matters in tissue processing.

Materials and Methods

Primary infection

A monolayer of MDCK cells (Madin-Darby Canine Kidney Cells, ATCC, CCL-34) in a 75 cm^2 culture flask was incubated with 0.01 multiplicity of infection (MOI) *Influenza A* virus (H3N2, reference strain A/Perth/16/09) containing medium EMEM (Eagle's Minimal Essential Medium, Lonza, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS), 100IU/ml penicillin, 100 μg/ml streptomycin, 2mM glutamine, 1.5 mg/ml sodium bicarbonate (Cambrex), 10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid, Lonza) and non-essential amino acids (MP Biomedicals Europe, Illkirch, France) for 1 hour at 37^oC. The medium was removed, the flask was washed with PBS (phosphate buffered salt solution, generic) and new medium was added. After 2 days of incubation 75% CPE (cytopathic effect) was reached.
A monolayer of A549 cells (human lung carcinoma cells, LGC Promochem, United Kingdom, ATCC-CCL-185) in a filter top 162 cm^2 flask was incubated with 0.01 MOI *Adenovirus* type 2 (human clinical isolate from Erasmus MC diagnostic virology -80^oC archive) containing medium (Ham's F12 Medium, Lonza, The Netherlands) for one hour at 37^oC. After 1 hour of incubation 40 ml of medium was added. After 4 days of culture 75% CPE was reached.
MRC-5 cells (human lung fibroblast cells, LGC Promochem, Germany, ATCC #CCL-171) were cultivated in 9x 175cm^2 cell culture flasks with MinimumEssential Medium + GlutaMax (Gibco, Life Technologies, UK) supplemented with 10% FCS (Gibco, Invitrogen, UK) and 1 % Penstrep (Gibco, Invitrogen, UK) at 37°C and 5% CO_2 until 60-70% confluency. Infection was performed in 6 flasks with 2ml suspension of human cytomegalovirus AD 169 (HPA #622) (900 pfu/ml) per flask (0.03 MOI). Multiple negative controls without virus infection were performed for every cell line. Cells were continuously cultured undergoing one passage because of high density of cells until 75% CPE was observed (10-14 days). (Figure 1, I)

Cell harvest, fixation and disruption

After CPE was observed, cells were harvested using 0,05% Trypsin-EDTA (Gibco, Life Technologies, UK). Cells were collected, centrifuged for 5 minutes at 200 x g, washed in PBS and counted. Duplicate aliquots of $1 \cdot 10^5$ cells/ml were made, centrifuged at 200 x g for 5 minutes, in order to discard excess medium (Figure 1, II) and fixed at room temperature with 10 ml of formalin (4% buffered formalin, Klinipath, The Netherlands), PAXgene (Paxgene Tissue System, PreAnalytix GmbH, Hombrechtikon, CH) or incubated with PBS (positive control) for 15 minutes, 1, 3, 6 and 24 hours (Figure 1, III). After fixation or incubation with PBS cells were centrifuged (200 x g for 5 minutes) to remove the fixative / PBS (Figure 1, IV) Influenza A and Adenovirus: The pelleted cells were resuspended in 3 ml medium and subsequently disrupted by vortexing in glass bead tubes (UTM kit 3 ml, Copan, ITK Diagnostics BV, The Netherlands) to release intracellular virus (Figure 1, Va). The CMV procedure was slightly different; cells were disrupted in a Gentle Macs Dissociator (Miltenyi, Germany) (Figure 1, Vb). The obtained suspension was centrifuged (500 x g for 5 minutes) to remove cell debris (Figure 1, VI).

Virus quantification and infection of recipient cells

After fixation and disruption of the virus positive cells, polymerase chain reaction (PCR) was performed to prove presence of virus in the supernatant. Since in all cases the same amount of supernatant (derived from the same number of cells) was used for PCR, cycle threshold (Ct) values could be used to show that in all cases the same amount of virus was introduced into the recipient cell culture (Figure 1, VI).
Influenza A specific PCR [19], performed on 200 µL of the supernatant, confirmed the presence of virus in the supernatant, with average Ct values of 15.46. Adenovirus specific PCR [20], performed on 200 µL of the supernatant, confirmed the presence of virus in the supernatant, with average Ct values of 12.96. CMV DNA specific PCR performed on 200 µL supernatant using QIAamp MinElute Virus Spin Kit for isolation of viral nucleic acids and artus CMV RG PCR Kit (both from Qiagen, Hilden, Germany), according to the protocol provided with the kit, confirmed the presence of CMV in all samples (average Ct 22.88) except negative controls.
The Influenza A virus supernatant was transferred to fresh MDCK cells (1 ml/well) on cover slips in 24 wells plates (in duplicate). Negative control wells were cultured in the presence of culture medium only. The Adenovirus supernatant was added to recipient A549 cells in coverslips in 24 wells plates (1 ml/well, duplicate wells). Medium without virus was added to the negative control wells (Figure 1, VIIa).
1.5ml of each CMV supernatant was used for reinfection of a MRC-5 monolayer with 60-70% confluency in a $25cm^2$ flask (Figure 1, VIIb). Cells

were incubated until 50% CPE was observed in the positive control wells (Figure 1, VIII).

Detection of virus activity with immunocytochemistry

Medium was removed from the wells, the wells were washed with PBS and finally the cover slips were fixed in 80% acetone (Influenza A and Adenovirus, Figure 1, IXa) or methanol/acetic acid (CMV, Figure 1, IXb)) for immunocytochemistry (ICC). The Influenza A ICC procedure was adapted from the one step fluorescence kit, without further need of optimization. After air drying overnight the coverslips were incubated for 30 minutes at room temperature with monoclonal mouse anti-Influenza A virus-FITC antibody (Imagen™ Influenza Virus A, REF K610511-2,Oxoid), diluted 1:3 in normal antibody diluent (NAD, ScyTek Laboratories, Logan, Utah).
The Adenovirus ICC (peroxidase-DAB) procedure was also adapted from the one step fluorescence kit. Background staining, predominantly found in dividing cells was eliminated by diluting the primary antibody until negative control cells were free of any background. Adenovirus ICC was performed with a 1:160 diluted monoclonal mouse anti-Adenovirus-FITC antibody (Imagen™ Adenovirus, REF K610011-2. Oxoid). Slides were repeatedly washed with PBS/0.05%Tween and the primary antibody was detected by peroxidase labeled envision (Chemvision, DAKO, Denmark). The peroxidase label was visualized with DAB (Chemvision, DAKO, Denmark) and the cells were counterstained with haematoxylin (Figure 1, Xa).
For CMV, contrary to the other assays, the coverslips were not air dried overnight, but washed in PBS prior to the ICC procedure. 1:50 diluted monoclonal mouse anti-CMV (Dako M085401, Denmark) was incubated for 60 minutes. After repeated washing the second antibody EnVision ready to use (Dako, Denmark) was incubated for 30 min, washed again and staining was performed with AEC ready to use (Dako, Denmark, 3-amino-9-ethylcarbazole that forms a red end-product at the site of the target antigen). The reaction was stopped with water and slides were counterstained with haematoxylin for 1 min. Slides were covered with Aquatex (Merck, Germany) (Figure 1, Xb).

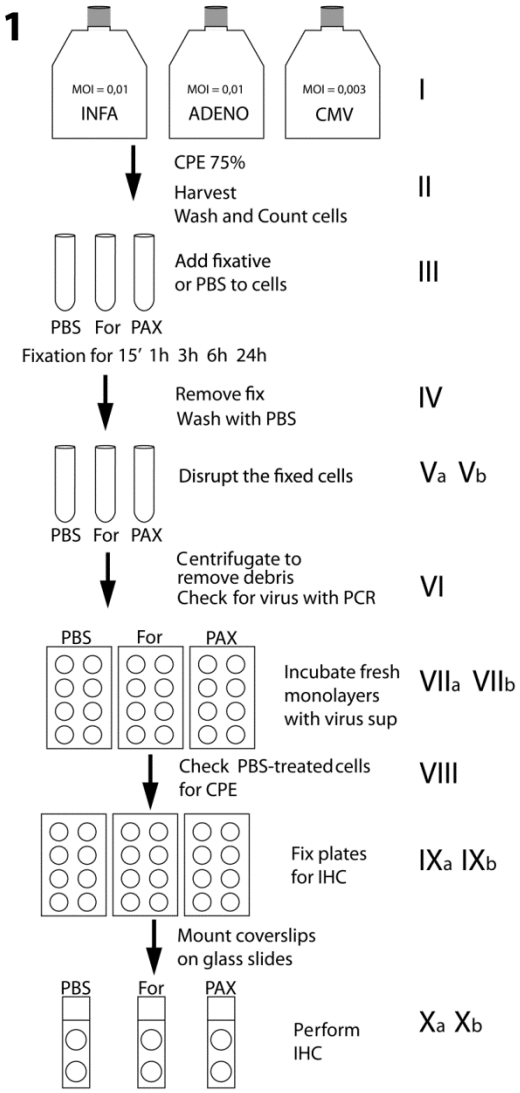

Fig 1 Experimental workflow

I: Primary culture of virus infected cells. II: When 75% CPE was reached, the cells were harvested and counted. III: Fixatives or PBS (positive control) were added to the virus infected cells IV: Fixative was removed and cells were washed with PBS. Va and b: Two different protocols were used to disrupt the cells. VI: Cell debris was removed by centrifugation and presence and quantity of virus in the remaining supernatant was detected with PCR. VIIa and b: Recipient cells were incubated (either in 24 well plates or in small flasks) with the virus containing supernatant. VIII: The PBS treated recipient cells were monitored for CPE. IXa and b: When 50-75% CPE was observed, the coverslips were fixed (in 80% acetone or in methanol/acetic acid) for ICC. Xa and b: ICC was performed (different visualization steps) to detect virus infected cells.

Results

Formalin and PAXgene inactivated Influenza A virus within 15 minutes of fixation (see figure 2). No viral activity was found after 1, 3, 6 and 24 hours of fixation (not shown). The positive control showed a strong signal, while the negative control showed no virus specific signal, nor any background staining. The monolayer of MDCK cells to which formalin fixed virus was applied was no longer intact and most cells looked damaged and deformed. The deformed cells were virus negative. Viable cells were, however, also detected and a specific ICC signal was not seen in these cells as well, indicating absence of active virus.

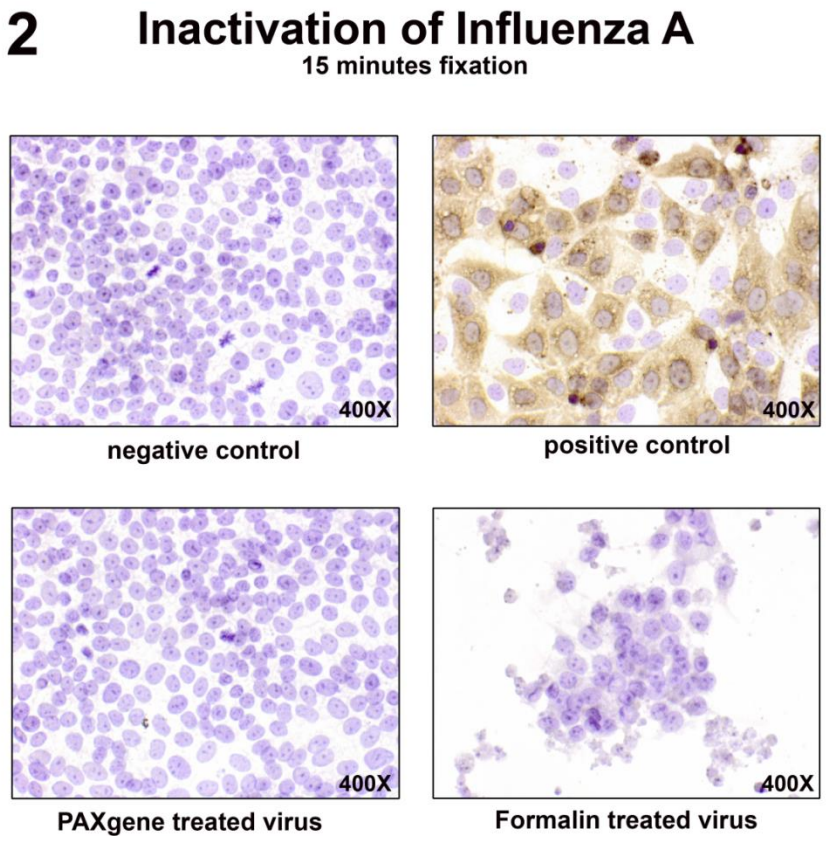

Fig 2 Inactivation of Influenza A
The upper panel shows the negative and positive control MDCK cells stained for Influenza A virus. The lower panel shows staining of monolayers inoculated with virus that was fixed for 15 minutes with PAXgene and formalin. Original magnification 400x

When A549 cells infected with *Adenovirus* were fixed with formalin, we observed total inactivation only at 6 and 24 hours of fixation. PAXgene inactivated *Adenovirus* within 15 minutes (see figure 3).

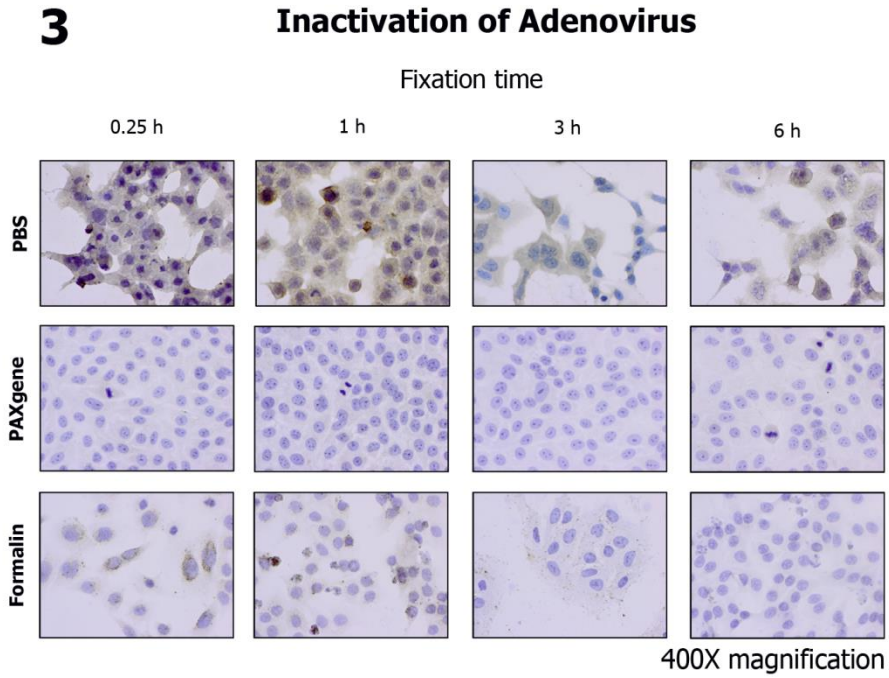

Fig 3 Inactivation of Adenovirus
The upper row shows the positive control A549 cells stained for Adenovirus. The middle row shows staining of cells inoculated with PAXgene fixed virus and the bottom row shows staining of cells inoculated with formalin fixed virus. Cells inoculated with virus that was fixed with formalin for 3 h show weak staining. After 6 hours of formalin fixation, no staining is observed. Original magnification 400x

Human Cytomegalovirus was inactivated after 15 minutes incubation by formalin and by PAXgene. The MRC-5 monolayer showed a mild degree of cell damage after formalin treated cell supernatant was applied. The amount of damaged cells seems to be dependent on formalin incubation duration. Strong signals at PBS control samples were detected at immune histological staining. No signals of active CMV at immune histological staining was seen after PAXgene and formalin fixation from 15 minutes onwards (see figure 4).

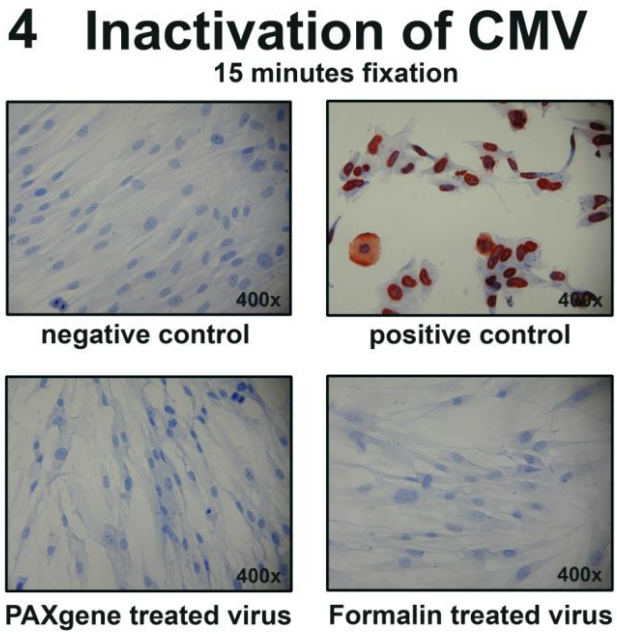

Fig 4 Inactivation of Cytomegalovirus
The upper panel shows the negative and positive control MRC-5 cells stained for Cytomegalovirus. The lower panel shows staining of monolayers inoculated with virus that was fixed for 15 minutes with PAXgene and formalin. Original magnification 400x

Discussion and Conclusion

The aim of this study was to test the virus inactivating properties of formalin and PAXgene in a virus culture assay. Since we found no literature with precedent evidence or insights regarding inactivation of virus in tissue samples when we planned our experiments, we designed a cell culture model to investigate whether formalin and PAXgene would inactivate the viruses. This cell culture model does not resemble solid tissue fixation exactly, but it does allow us to gain insight into how different virus strains, while actively infecting a cell layer, react to tissue fixatives. Any extrapolation of this cell culture data towards solid tissue fixation is based on known dynamics of solid tissue fixation and commonalities between cell fixation and solid tissue fixation. All model viruses remained infective for at least 24 hours in a PBS solution at room temperature. Therefore, we may conclude that virus also remains infective for at least 24 hours in unfixed tissue specimens. The penetration rate of formalin is around 2 mm per hour. Large clinical tissue specimens are almost always cut open to enhance fixation, but

some parts of the specimen could remain too thick to allow thorough fixation overnight. Our observation that the time needed to inactivate viruses differs between type of virus and type of fixative, argues for vigilance when working with large solid organs, even after overnight gross fixation.
We have chosen immunocytochemistry (ICC) instead of PCR as the virus replication read out system. With PCR, differentiation between infective and inactivated virus is not possible. PCR was only used to show that virus was present in the medium used for re-infection of the recipient cells. The PCR kits are not validated for use with cell culture material, calculating copy numbers could, therefore, be misleading. Since we have taken equal amounts of material for PCR, the Ct values do serve as a measure for the amount of virus put into the recipient culture and confirm that equal amount of virus were actually present in the supernatants.
To avoid false positive ICC results (i.e. detection of fixed virus-positive donor cells) the fixed cells and control cells were disrupted before the inoculation of fresh recipient cells. The culture was ended when the positive control wells showed 50-75% CPE. Prolonged culture would have resulted in complete lysis of the positive control cells, which would eliminate positive ICC by lack of cells. The damaged cell layers observed after treatment with formalin fixed virus, could be explained by a cyto-toxic effect of residual formalin cross links in the virus homogenate. The number of affected cells seems to increase in correlation with the formalin fixation/inactivation time, indicating that an increase of cross links or any chemical alteration in the cells may lead to loss of cells in the culture. Nevertheless, we did find viable, healthy looking cells in these wells. Those were virus negative, as were the affected cells. It is safe to conclude that, although a cyto toxic effect may have taken place, the virus was unable to infect the remaining cells and therefore inactivated by formalin fixation.
Inactivation of *Adenovirus* by formalin seems to be a slower process than by PAXgene. This may be due to the fundamental difference between the two methods of fixation and the different virus structures. The virus envelope in the case of *Influenza A* and *CMV* provides more molecules for formalin cross links. The non-enveloped *Adenovirus* is more resilient to formalin inactivation, since fewer surface molecules are available for cross linking [21]. Formalin forms cross links between proteins and nucleic acids, whereas alcohol-acetic acid based fixatives, like PAXgene, precipitate macromolecules. Since the standard formalin solution contains only 4% formaldehyde, the amount of molecules in the aqueous solution may be insufficient to form cross links in small virus particles in a short period of time. The high (not disclosed, but at least 50% to 70%) alcohol concentration in PAXgene may deliver an abundance of molecules to precipitate the virus proteins. More research in the field of virus induced pathologies is needed to explore the benefits of PAXgene for this specific subject. However, previously published articles [7, 8, 9, 10] show that histological techniques as well as proteomic and genomic techniques are applicable to PAXgene fixed tissue. The possibility to detect parasite-DNA in DNA extracted from

PAXgene fixed, paraffin embedded salmon gills [11], suggests that in future experiments virus RNA, DNA and proteins maybe easily detected in PAXgene fixed and paraffin embedded tissue.
In conclusion: our virus inactivation experiments show that the investigated viruses, *Influenza A* virus, *Adenovirus* and *Cytomegalovirus* can be inactivated by PAXgene tissue fixative and formalin. In other words; when virus was rehydrated after PAXgene fixation (i.e. resuspension in medium), re-activation did not occur. Therefore, PAXgene is, at least regarding inactivation of these three viruses, a safe replacement of formalin in clinical pathology labs.

Acknowledgments

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Chapter 5

A New Reference Image Based Method for Optimization of Clinical Immunohistochemistry

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(submitted for publication)

A New Reference Image Based Method for Optimization of Clinical Immunohistochemistry

M. Kap, K.H. Lam, P. Ewing-Graham, P. Riegman

Abstract

Introduction:

Cold ischemic- and formalin fixation time (CIT and FFT) are considered to be crucial parameters for intra-laboratory variation in immunohistochemistry. Here we describe a new method to optimize IHC, by using control tissue blocks with known pre-analytic history and comparing the IHC outcome with digitized reference slides.

Methods:

Tissue specimens (n = 2 per tissue type) were divided in eight samples, which were subjected to different CIT and FFT. Immunohistochemistry was performed with 34 routinely used antibodies, following standard operating procedures. Relative staining intensity of 4 sections per slide was scored. Of the antibodies studied; 7 were influenced by CIT, 13 by FFT and 5 by both parameters. IHC protocols were adapted until most sections on the slide showed the same intensity.

Results:

Changing the antibody dilution for 10 and the antigen retrieval method for 6 protocols, improved the consistency of the IHC staining. 9 protocols couldn't be optimized. The optimized staining results were compared to reference slides and were found to be of adequate quality.

Conclusions:

It was possible to optimize most IHC protocols by adapting the analytical phase, rather than the pre-analytical phase. If global references could be established, this method could decrease inter-laboratory variation, without standardization of the pre-analytical workflow.

Introduction

In diagnostic pathology laboratories, inter- and intra-laboratory variability of immunohistochemistry (IHC) due to pre-analytical and analytical factors remains a major problem [1-5]. Research studies on pre-analytical factors [5-7] resulted in guidelines and recommendations [8,9] aimed at improving reproducibility within and among laboratories. Most of these guidelines have been developed for (quantitative) diagnostic tests with pharmaco-therapeutic implications, such as ER/PgR [8] and HER2NEU. However, common qualitative IHC assays also need to be reliable in terms of specificity and sensitivity [10], but despite all effort,s adequate standardization of the pre-analytical and analytical workflows is often not feasible [4,11].

The next best approach is avoidance of the need to standardize the pre-analytical phase by adaptation of the analytical test. In routine pathology laboratories, positive control tissue sections are used to optimize IHC protocols. The end result in IHC procedures is a specific staining pattern with a constant level of staining intensity. To maintain a constant staining intensity comparison methods have been designed, such as the CQPath database (www.cqpath.nl) [11]. It contains digitized reference images, which were selected after years of experience using the antibodies in a diagnostic setting and by participation in ring trials such as NordiQC [10]. By comparing daily IHC output and antibody titrations (performed on control tissues mounted on the same slide as the patient tissue), to the reference images the quality level of the output, as well as of the IHC protocol can be maintained.

Although such outcome based methods reduce variations, there is also a potential pitfall: Variation of pre-analytical factors such as CIT and FFT cause variation in the intensity of the IHC staining in random positive control blocks . This may lead to a decision to change the primary antibody concentration simply because a new positive control block has been used (Figure 1). This in turn can influence the outcome of the staining of patient material with unknown pre-analytical status, in a negative way. Hence, a solution in the form of standardization of the pre-analytical phase would be advantageous. Standardization of FFT, however much desirable is not a practical solution [12]. Moreover, it is known that different antigen retrieval (AR) methods may eliminate some of the FFT effects [7, 13]. Boenisch,[13] took a pragmatic approach towards the problems of IHC inconsistency caused by under- or over-fixation in formalin. By adapting the AR procedure where necessary, the intensity of 30 IHC assays could be normalized amongst differently fixed tissue samples. Using snap frozen tissue as a standard, Shi, et al.[7] stated that with adaptation of the AR procedure, it could be possible to reach 100% antigen recovery.

In this paper a method for routine IHC laboratories to optimize IHC protocols based upon the two principles described above is presented. This method is designed to avoid the adverse influence of pre-analytical variables that may interfere with the desired final IHC result. However, instead of snap frozen

tissue as used by Shi, et al. [7], we have used digital reference images of optimally stained sections as standard [10,11,14]. Comparison of the results of the optimization experiments to the digitized reference slides enabled monitoring of the accuracy of the IHC stains (i.e. specificity and sensitivity after adapting the protocols). The low cost and easy-to-implement approach proposed here provides IHC laboratories with a means to optimize their IHC protocols and reduce the effects of CIT and FFT. If a set of publically available digitized references images could be established, this easy and low cost approach could lead to global equalization of IHC staining results without cumbersome standardization of the pre-analytical workflow.

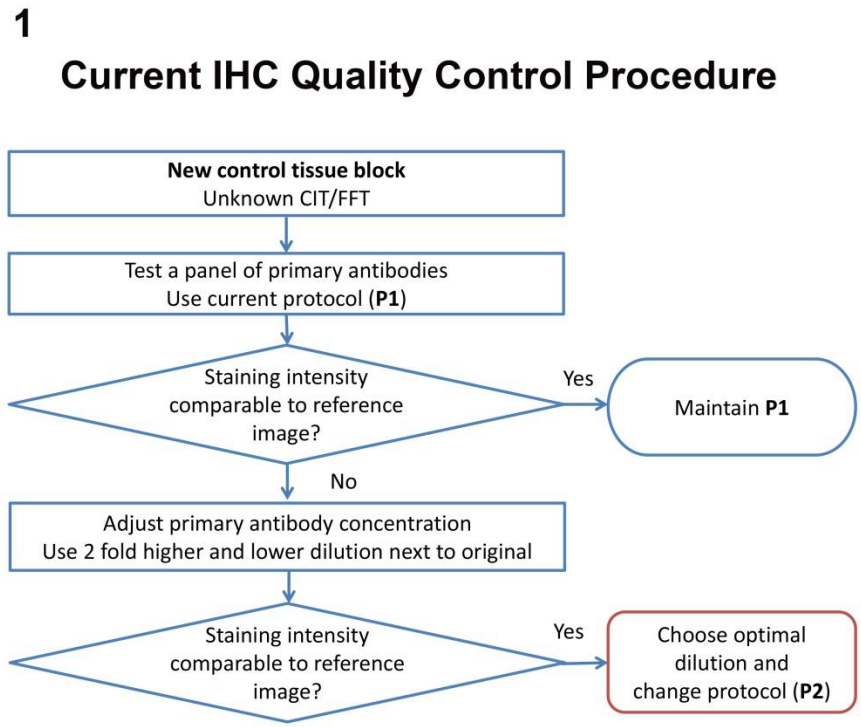

Figure 1. Current IHC Control Tissue Quality Control Procedure. In this figure the standard procedure for testing antigenicity of a new control tissue block is depicted. If for whatever reason (e.g. cold ischemia time (CIT), formalin fixation time (FFT)) the IHC staining intensity deviates from the reference image, the primary antibody concentration is adapted to fit the reference. The new primary antibody dilution is then recorded in the new protocol (P2). Since this has to be done every time a new control tissue block is taken, the overall IHC efficiency may become unnecessarily variable, depending on the control tissue alone.

Materials and Methods

New IHC optimization method

A new optimization method was designed for efficiently adjusting IHC protocols to avoid variations in the control tissues which occurred with the method currently in use (Figure 1). Tissue sample cohorts with controlled CIT and FFT were collected and tested according the new method (Figure 2)

Tissue collection

The use of residual tissue accompanied by data on tissue type and disease state is approved by the Erasmus MC Medical Ethical Commission under number MEC-2008-397. According to the Dutch Code of Conduct concerning the use of residual tissue for research, no (written) informed consent was necessary for this work.

Two (n=2) samples of thyroid tumor and normal/non tumorous skin, kidney, colon, liver, tonsil and pancreas tissue were collected during routine grossing. These tissue types are used as positive controls for the majority of the IHC antibodies studied [12] (Table 1). Eight aliquots of approximately 4 x 4 x 4 mm were prepared. Four aliquots were subjected to a cold ischemia time series of 0, 1, 3 and 6 hours (to cover also long cold ischemia times during logistical incidents) by leaving the samples at room temperature in a closed container in order to prevent them from drying out. These aliquots were subsequently fixed in 4% neutral buffered formalin (Klinipath, Benelux) for 24 hours. The FFT series aliquots were fixed in formalin for 3, 24, 72 and 168 hours to mimic the fixation times related to: rapid processing, standard and over-weekend/prolonged fixation of fatty tissues, respectively. To assure a well-defined FFT until processing, the tissue samples were transferred to 70% ethanol after three hours of fixation. Samples fixed longer than three hours were processed after the designated fixation time without additional formalin fixation in the tissue processor (Excelsior, Thermo Scientific, The Netherlands). The samples were embedded in paraffin and 4 µm sections were cut for H&E staining to confirm presence of relevant cell types.

Immunohistochemistry

4 µm tissue sections were cut and mounted on adhesive slides. For each case, all four sections in the time series were mounted on one slide to ensure the same treatment during antigen retrieval and further processing for IHC. Immunohistochemistry with routinely used monoclonal antibodies was performed using Ventana Benchmark Ultra stainers (Roche, Netherlands). The AR protocols and buffers as performed within the Ventana stainers are described in Table 1.

When differences in staining intensity were observed while using the antibody dilution regarded until then as optimal (screening phase; see Figure 2-1), the antibody was titrated in a twofold dilution series around the original titer (titration phase; see Figure 2-2). This allowed adjustment of the dilution to overcome eventual adverse pre-analytical effects (Table 2).

Exceptions to this approach were the ready-to-use (RTU) antibodies against Calretinin, CD10, CD31, CD34, CDX2, Cyclin-D1, EGFR and Synaptophysin. These were applied without further dilution. For some antibodies it was necessary to change the antigen retrieval protocol to yield optimal results (antigen retrieval phase; see Figure 2-3). This was done by repeating the staining with either shorter heat induced epitope retrieval (HIER), prolonged HIER or (in two cases) protease digestion (Table 2).

Evaluation of staining intensity

The staining intensities of individual slides were scored (blind to the pre-analytical values) by two pathologists. The hemato-pathologist (KHL) mainly scored the lymphoma markers, whereas the other markers were scored by an all-round pathologist (PE). The scoring method is an adaptation of the scoring method used by Boenisch et al [13]. When sections showed specific staining, the intensity of the stain was scored relatively to the lightest stained section of the four sections of the slide (score 1). Since 4 sections were mounted on one slide, the score ranged from 1 (the weakest stained section of the slide) to 4 (the strongest stained section of the slide). When there was staining of scattered cells (for example CD56 in tonsil), rather than massive tissue structures (for example CD20 in tonsil), the “weakest staining” was defined as having fewer cells stained positively. Slides were not scored relative to each other, but remarkable intensity differences between slides were noted.

In Figure 3 the three most frequently observed scenarios are depicted to explain the scoring method. The slides with optimized staining were visually compared to the references in the CQPath data base [11] to determine whether the results after adapting the IHC protocols (specificity and sensitivity) were still acceptable for routine IHC application.

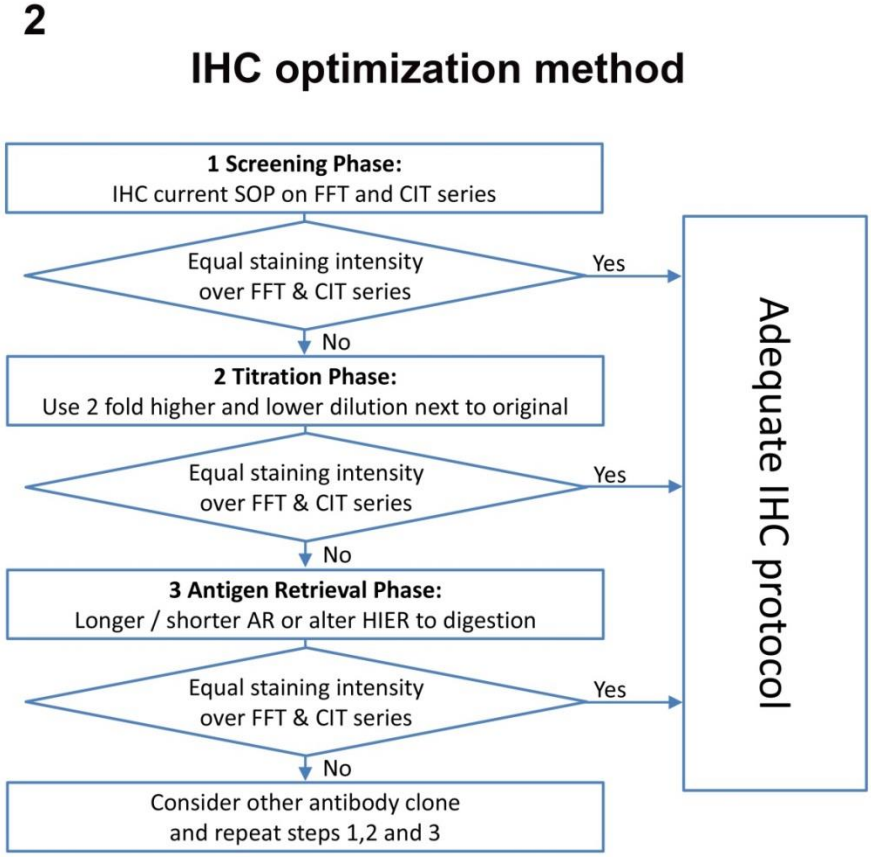

Figure 2. IHC optimization method. When this workflow is applied to control tissue sections which have been subjected to a series of predetermined fixation or cold ischemia times, it will be possible to optimize (most, but not all) IHC protocols in such a way that the assays are no longer influenced by these most common pre-analytical factors. When equal staining over FFT & CIT series is observed, always compare the outcome with the reference image to assure adequate sensitivity. By this approach the continuous adaptation of the protocol as depicted in the red box in **Figure 1** can be avoided.

Results

Screening phase

During the screening phase (see Figure 2-1), no obvious intensity differences were observed for the following antigens; Calretinin, CD30, CD31, CD34, CD45, CD79a, ChromograninA, e-cadherin, EGFr, Factor8, Cyto-keratin18, MelanA, KI-67, Renal carcinoma, S100, Synaptophysin, Thyroglobulin and TTF1. See Table 1 for more details on the antibodies used. In Figure 3, a typical example of a stable antigen (CD79a) is shown. The staining intensity for these antibodies was comparable with the intensity as recorded in the reference image data base [11].
The following antigens showed obvious intensity differences due to FFT: BEREP4, CD3, CD10, CD20, CD23, CD31 (only in cirrhotic liver), CD56, CD99, CD117, CD138, CyclD1, SMA and Vimentin. BCL6 showed only slightly lower intensity at 3 hours formalin fixation. Whereas BCL6, CD3, CD10, CD20, CDX2, Desmin and Vimentin were influenced by CIT (Table 1)

Titration phase

When antibody dilutions were adapted during the titration phase (see Figure 2-2) the following antigens, influenced by FFT, showed improvement of results; CD3, CD23, CD56, CD117 and SMA. Adjustment of antibody dilution resulted in improved results for the CIT sensitive antibodies BCL6, CD3, CD20 and Vimentin (Table 2).

Antigen retrieval phase

After adjusting the antigen retrieval protocol during the antigen retrieval phase (see Figure 2-3); BEREP4, CD10 (RTU), CDX2 and CYCL-D1 showed more stable results (Table 2). In Figure 4a the graphs with scores during the screening, titration and AR adjustment phases are shown. The ready to use antibodies for CD10 and CDX2 (Table 2) showed better consistency when the antigen retrieval protocols were adjusted during the antigen retrieval phase (see Figure 1). Figure 4b shows a typical example of improvement of the IHC consistency by changing the antigen retrieval (AR) method. Figure 5 shows graphs in which the evolution of protocol improvement is depicted. The specificity and sensitivity (staining intensity dependent) after adapting the IHC protocol, were comparable with the intensity as recorded in the reference image data base.
Sample variability
The observed IHC intensity differences due to FFT or CIT were not always consistent between various tissue types or even between different samples of the same tissue type. The CIT-dependent IHC intensity differences observed for CDX2 were most prominent in pancreas, whereas in colon

tissue hardly any difference was found. The detection of CD31 in liver with different FFTs strongly depended on the individual liver samples. In one sample of healthy liver tissue, no intensity differences were observed, whereas in the diseased, cirrhotic sample a time-dependent intensity gradient was observed (Figure 6)

Table 1. Antibody data and IHC staining intensity scores during the screening phase

ntibody data					Screening phase		FFT				CIT			
Antigen	Clone	Company	Catalog number	Control tissue	Dilution*	AR**	3h	24h	72h	168 h	0h	1h	3h	6h
BCL6	LN-22	Novocastra	NCL-L-DCL-6-564	tonsil	1:100	cc1 64' 97 C	1	2	2	2	3	2	2	1
BEREP4	Ber-EP4	Dakocytomation	M0804	colon	1:200	cc1 64' 97 C	1	2	2	3	1	1	1	1
CALRET †	SP65	Ventana	790-4464	colon	RTU	cc1 16' 100 C	1	1	1	1	1	1	1	1
CD1A †	010	Immunotech	1590	skin	1:10	none	1	1	1	1	1	1	1	1
CD3	polyclonal	Dakocytomation	A0452	tonsil	1:300	cc1 64' 97 C	1	2	3	3	1	1	2	3
CD10	SP67	Ventana	790-4506	tonsil	RTU	cc1 64' 97 C	1	2	3	2	2	1	3	3
CD20	L26	Dakocytomation	M0755	tonsil	1:400	cc1 64' 97 C	1	2	2	2	1	1	2	2
CD23	SP23	Thermo Scientific	RM-9123-S	tonsil	1:25	cc1 64' 97 C	1	3	3	2	1	1	1	1
CD30 †	BER-H2	Dakocytomation	M0751	tonsil	1:20	cc1 64' 97 C	1	1	1	1	1	1	1	1
CD31	JC70	Cell Marque (ITK)	760-4378	liver	RTU	cc1 64' 97 C	1	1	1	1	1	1	1	1
CD34 †	QBEND/10	Ventana	n.a.***	liver	RTU	cc1 64' 97 C	1	1	1	1	1	1	1	1
CD45 †	2B11+PD7/26	Dakocytomation	M0701	tonsil	1:200	cc1 64' 97 C	1	1	1	1	1	1	1	1
CD56	MRQ-42	ITK	156R-95	tonsil, pancreas	1:200	cc1 64' 97 C	1	1	3	2	1	1	1	1
CD79 †	SP18	Ventana	790-4432	tonsil	1:50	cc1 64' 97 C	1	1	1	1	1	1	1	1
CD99	12E7	Dakocytomation	M3601	tonsil	1:40	cc1 64' 97 C	1	2	2	2	1	1	1	1
CD117	YR145	Cell Marque (ITK)	117R-16	colon, tonsil	1:50	cc1 64' 97 C	1	2	3	3	1	1	1	1
CD138	B-A38	IQ Products	IQP-153P	tonsil	1:100	cc1 64' 97 C	1	2	3	3	1	1	1	1
CDX2	EPR2764Y	Cell Marque	760-4380	pancreas	RTU	cc1 64' 97 C	1	1	1	1	3	3	2	1
CHROMOA †	polyclonal	Dakocytomation	A0430	colon	1:600	cc1 64' 97 C	1	1	1	1	1	1	1	1
CYCLD1	SP4-R	Ventana	790-4508	tonsil	RTU	cc1 64' 97 C	1	2	2	3	1	1	1	1
DESMIN	D33	Monosan	MON3001	colon	1:100	cc1 64' 97 C	1	1	1	1	4	3	2	1
ECADH †	NCH-38	Dakocytomation	M3612	liver	1:15	cc1 64' 97 C	1	1	1	1	1	1	1	1
EGFR †	3C6	Ventana	790-2988	skin	RTU	P1 8'	1	1	1	1	1	1	1	1
F8 †	polyclonal	Dakocytomation	A0082	liver	1:8000	cc1 64' 97 C	1	1	1	1	1	1	1	1
KER18 †	5D3	Neomarkers	MS 743-S	liver	1:100	cc1 64' 97 C	1	1	1	1	1	1	1	1

MELANA †	A103	Ventana	790-2990	skin	1:200	cc1 64' 97 C	1	1	1	1	1	1	1	1
MIB1 †	KI-67	Dakocytomation	M7240	tonsil	1:200	cc1 64' 97 C	1	1	1	1	1	1	1	1
RENALCA †	PN-15(AB-1)	Neomarkers	MS-409-P	kidney	1:200	cc1 64' 97 C	1	1	1	1	1	1	1	1
S100 †	polyclonal	Dakocytomation	Z0311	colon	1:3200	cc1 64' 97 C	1	1	1	1	1	1	1	1
SMA	HHF35	Dakocytomation	M0635	liver	1:100	none	2	2	2	1	1	1	1	1
SYNAPTO †	SP11	Ventana	790-4407	colon	RTU	cc1 32' 100 C	1	1	1	1	1	1	1	1
TG †	polyclonal	Dakocytomation	A0251	thyroid tumor	1:8000	none	1	1	1	1	1	1	1	1
TTF1 †	SPT24	Monosan	MONX10584	thyroid tumor	1:100	cc1 64' 97 C	1	1	1	1	1	1	1	1
VIM	V9	Dakocytomation	M0725	colon	1:1200	cc1 64' 97 C	1	2	2	2	3	3	2	1

* proposed optimal dilution used in routine at that time
** proposed optimal antigen retrieval used in routine at that time
*** test kit for evaluation of RTU clone
CIT = cold ischemia time
FFT = formalin fixation time

cc1 = buffer pH8.4, Ventana Ultra Cell ref# 950224
x' = time in minutes

score 1 = least intensely stained section on slide
score 2 = intermediate or most intensely stained section
score 3 = intermediate or most intensely stained section
score 4 = most intensely stained section
If no differences in staining intensity were observed, all sections are scored equally

P1 = 0.38 mg/mL protease Ventana ref# 760-2018
y C = degrees Centigrade

Table 2. IHC intensity scores after adjustment of antigen retrieval protocols.

Antigen retrieval phase			Formalin fixation time (FFT)					Cold ischemia time (CIT)				
			3h	24h	72h	168h		0h	1h	3h	6h	
Antigen	**Dilution**	**Antigen retrieval**					**Remarks**					**Remarks**
BCL6	1:100	Shorter (32')*	1	2	2	2	minor effect	ND	ND	ND	ND	Was optimal after titration
BCL6	1:100	Longer (92')	1	2	2	2		ND	ND	ND	ND	Was optimal after titration
BEREP4	1:200	Protease digestion (4')	1	2	2	2		1	1	2	2	minor effect
CALRET	RTU	Shorter (8')	1	1	1	1		1	1	1	1	
CALRET	RTU	Longer (32')	1	1	1	1		1	2	1	1	minor effect
CD3	1:150	Shorter (32')*	1	2	2	2						Was optimal after titration
CD3	1:150	Longer (92')	1	2	2	2		ND	ND	ND	ND	Was optimal after titration
CD10	RTU	Longer (92')	1	2	2	2	minor effect	1	1	2	2	minor effect
CD10	RTU	Shorter (32')	1	2	2	1		1	1	1	2	
CD23	1:15	Shorter (32')*	1	2	2	2		ND	ND	ND	ND	Was not influenced by CIT
CD23	1:15	Longer (92')	1	2	2	2		ND	ND	ND	ND	Was not influenced by CIT
CD31	RTU	Longer (92')	1	1	1	1		ND	ND	ND	ND	Was not influenced by CIT
CD31	RTU	Shorter (32')	1	1	1	1		ND	ND	ND	ND	Was not influenced by CIT
CD56	1:400	Longer (92')	1	1	1	1		ND	ND	ND	ND	Was not influenced by CIT
CD56	1:400	Shorter (32')	1	1	1	1		ND	ND	ND	ND	Was not influenced by CIT
CD117	1:200	Longer (92')	1	2	2	2		ND	ND	ND	ND	Was not influenced by CIT
CD117	1:200	Shorter (32')*	1	2	2	2		ND	ND	ND	ND	Was not influenced by CIT
CDX2	RTU	Shorter (32')	1	2	2	2		1	1	2	1	
CDX2	RTU	Longer (92')	1	2	2	2		2	2	2	1	
CYCLD1	RTU	Shorter (32')	1	2	2	2		ND	ND	ND	ND	Was not influenced by CIT
CYCLD1	RTU	Longer (92')	1	2	2	2		ND	ND	ND	ND	Was not influenced by CIT
DESMIN	1:50	Shorter (32')*	1	2	2	2	minor effect	3	2	2	1	No improvement
DESMIN	1:50	Longer (92')	1	2	2	2	minor effect	3	2	2	1	No improvement

ND = not done
Optimal or most time efficient protocol marked blue.
Staining consistency was not always improved by changing the protocol (marked with *), in that case the shortest antigen retrieval procedure was chosen for sake of time efficiency.

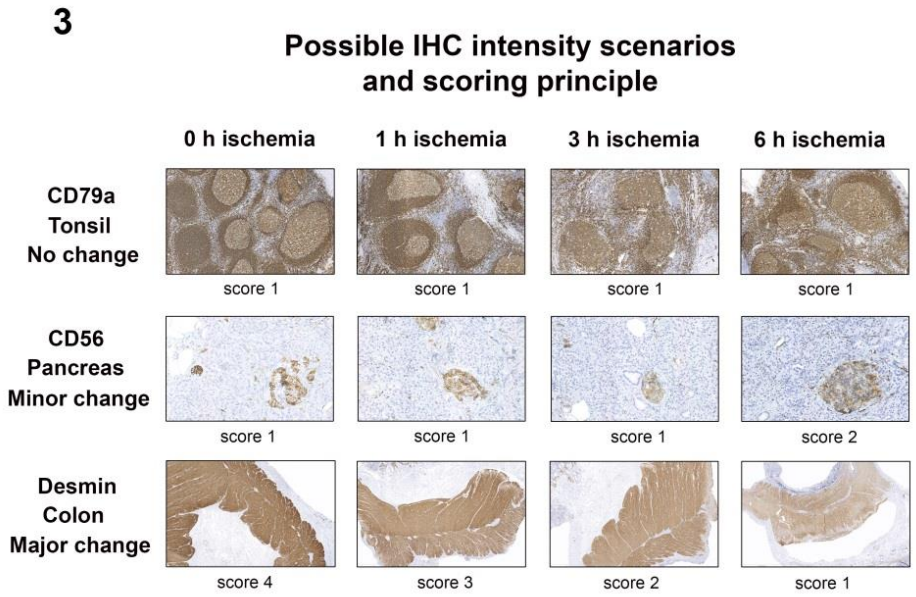

Figure 3. Typical examples of intensity variability and subsequent scores. If no differences in staining intensity were observed, as shown for CD79a in tonsil, all sections were given score 1. In most cases, the intensity variability was minor, as shown for CD56 in pancreas, islets of Langerhans. In this case the most intensely stained section was given score 2, whereas the other sections all were given score 1. When an intensity gradient is observed, as was the case for desmin in colon tissue, the most intensely stained section was given score 4 and the least intensely stained section score 1. The sections in between were given scores 2 and 3 in this case.

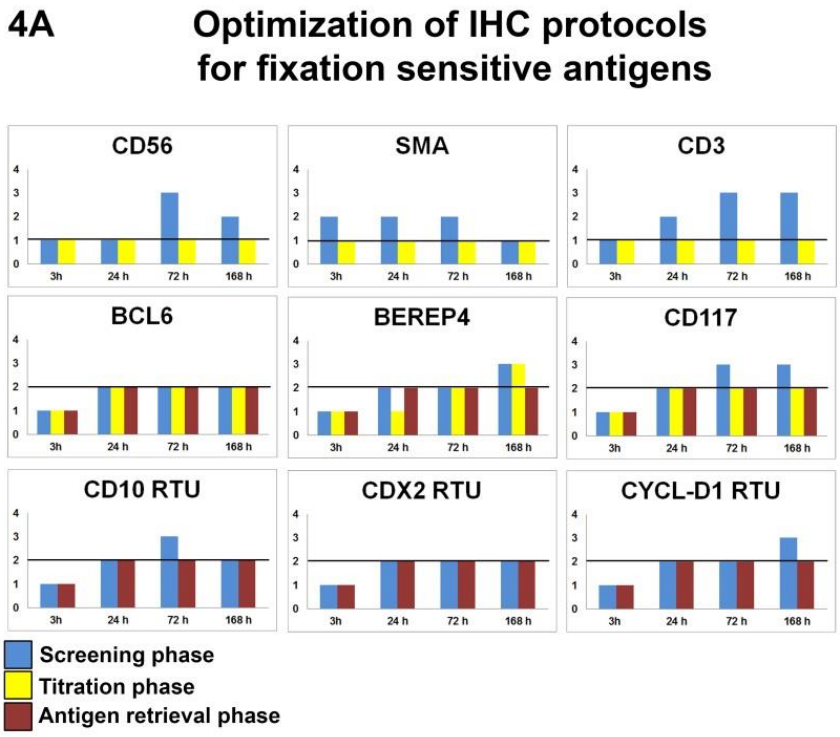

Figure 4A. Improvement of IHC protocols for FFT sensitive antigens. On the X-axis the FFT is shown (3, 24, 72 and 168 hours). The IHC intensity scores are given on the Y-axis, where score 1 is the least intensely stained section on the slide. RTU, or Ready To Use antibodies were used as provided, no further dilution was made. The lines indicate the cut off where all sections show equal (or in some cases almost equal) staining intensities. The BCL6 IHC protocol, which showed only a slightly lower intensity at 3 hours of formalin fixation, could not be improved. In some cases, adjusting the antibody titer was sufficient to equalize the intensity scores (CD56, CD117, SMA and CD3), whereas for BEREP4, CD10 RTU and CYCL-D1 RTU it was necessary to adjust the antigen retrieval protocol to obtain optimal results.

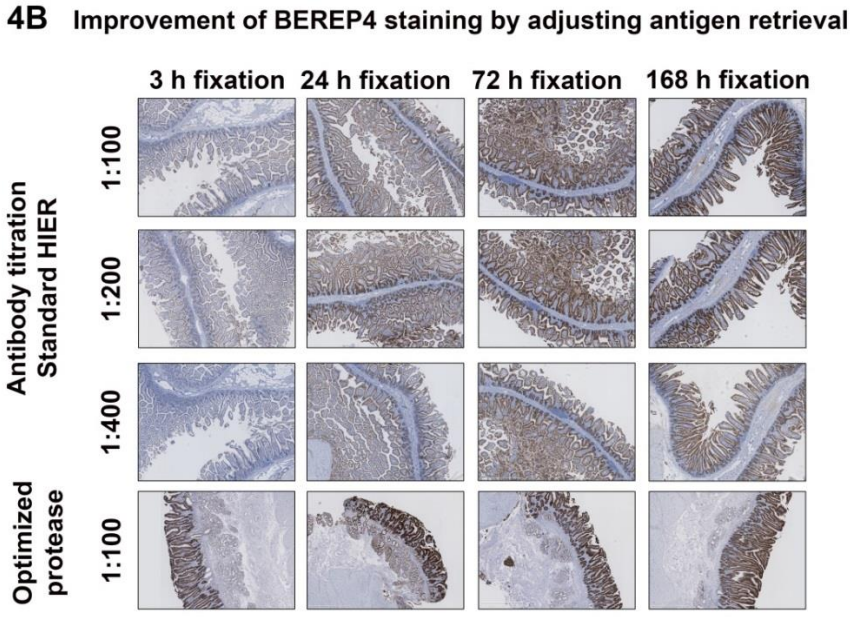

Figure 4B. Improvement of BEREP4 staining by adjusting antigen retrieval. The antibody dilution during the screening phase was 1:200 and standard HIER was applied. During the titration phase, one dilution lower and higher than the original dilution was applied with the same AR protocol. The intensity gradient remained, so the AR protocol was adjusted by applying protease digestion (as suggested by Ventana). This resulted in a more stable IHC performance. **Tables 1** and **2** show the AR protocols and intensity scores in more detail.

5 Optimization of IHC protocols for ischemia sensitive antigens

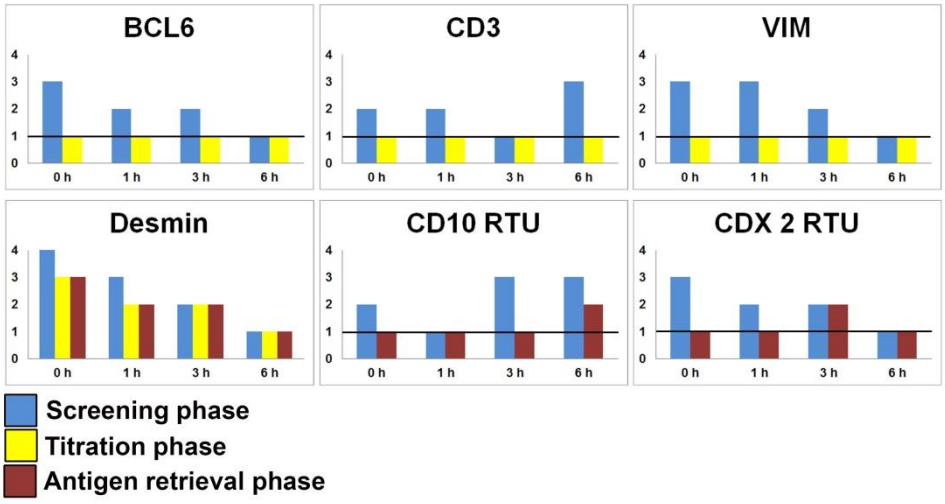

Figure 5. Improvement of IHC protocols for CIT sensitive antigens. On the X-axis the CIT is shown (0, 1, 3 and 6 hours). The IHC intensity scores are given on the Y-axis, where score 1 is the least intensely stained section on the slide. The lines indicate the cut off where all sections show equal (or in some cases almost equal) staining intensities. The desmin stain remains sensitive to CIT, despite several protocol modifications.

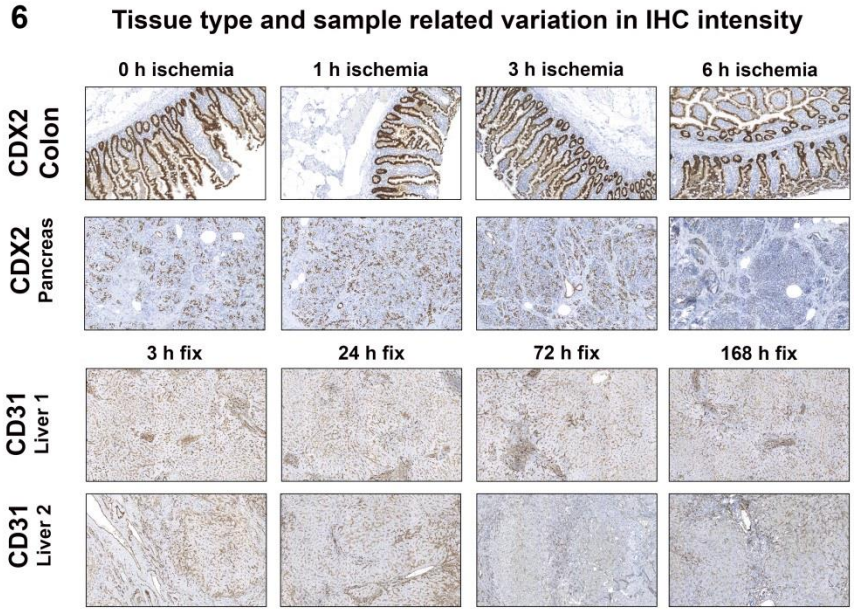

Figure 6. Tissue type and sample type related variation in IHC intensity. The expression level of CDX2 in pancreas is lower than in colon. This may explain why no intensity changes due to CIT can be observed in colon, but these changes are observed in pancreas, where the intensity distribution is quite irregular. Regardless of FFT or CIT, patient variability, as shown for the CD31 staining in two different liver samples, also influences staining intensity distribution. Liver 1 shows no considerable intensity differences, whereas (cirrhotic) liver 2 shows irregular CD31 staining.

Sample variability

The observed IHC intensity differences due to FFT or CIT were not always consistent between various tissue types or even between different samples of the same tissue type. The CIT dependent IHC intensity differences observed for CDX2 were most prominent in pancreas, whereas in colon tissue hardly any differences were found. The detection of CD31 in liver with different FFTs strongly depended on the individual liver samples. In one unaffected sample, no intensity differences were observed, whereas in the other, cirrhotic sample a time dependent intensity gradient was observed (**Figure 6**).

Discussion

The primary goal of this study, was to design a method (Figure 2) to avoid unnecessary adaptation of IHC protocols due to pre-analytical variation of control samples during routinely QC/QA interventions (Figure 1) as much as possible. The results show that the new IHC optimization method can efficiently correct for this variation for many antibodies used in diagnostic pathology labs. By adjusting the antibody dilution and/or the AR method, IHC intensity fluctuations due to FFT and CIT can be reduced, although not entirely eliminated for all antibodies studied (Table 2).

The optimization process of BEREP4 as illustrated in Figure 4B demonstrates how using control tissue with unknown pre-analytical history can lead to variable, or even false negative results. The optimal staining intensity, as indicated by the digital reference image for BEREP4 IHC on a control tissue sample fixed for 72 hours (over weekend fixation/processing), would be achieved by using the antibody diluted 1:400 after standard HIER. If this particular protocol were then to be used for a patient tissue specimen fixed for 3 hours (rapid processing), or for 24 hours (week day fixation/processing), the result could be a false negative. However, when the antigen retrieval protocol and thereby the consistency of IHC intensity is adjusted using different FFTs as described in the new optimization method (Figure 2), the chance of obtaining a false negative result is greatly reduced. If this pre-analytical optimization approach were then to be combined with standardization of formalin fixation, the overall reliability of the IHC procedure would be expected to increase even more.

However, FFT standardization remains very difficult to achieve. Optimal FFT also depends on the tissue type (e.g. fatty tissue needs longer fixation) and on the day of the week that the specimen was brought to the pathology lab (i.e. weekend processing) [12]. These problems may be solved by fixation of large specimens in cold (4ºC) formalin for exactly 24 hours, followed by cold 95% ethanol, before further processing and paraffin embedding, but automation of this method is still in development and it is therefore not yet widely used [15].

Most CIT artifacts that were observed in this study occur after 1 hour of CIT. Therefore, in practice CIT is not the major issue, since most pathology laboratories already strive to fix tissue specimens within an hour after removal from the patient even in hospitals that have formalin free operating

theatres. Furthermore, when tissue is transferred to the pathology lab in vacuum sealed plastic bags, autolysis and decay of nucleic acids and proteins is inhibited for at least 48 hours [16-18], making adverse influence of CIT on proteins unlikely.

In some cases, it was observed that patient variation may be the cause of differences in staining intensity. The variation in staining intensity of CD31 in Figure 6 may be due to the underlying condition of the tissue (unaffected vs cirrhotic liver). It is therefore important to use only homogeneous, healthy control tissue samples.

CDX2 is known to be expressed in lower quantities in pancreas, as compared to intestinal tissue [10]. It is, however, very important for the overall sensitivity of an IHC assay to use control tissue with low expression of the antigen in question [8]. Obviously, when low expression levels in a certain tissue type can be detected, the assay will certainly be sensitive enough to detect high(er) expression levels in clinical (tumorous) specimens. However, it must be taken into account that the lower expression of an antigen in the control tissue probably makes the protocol more prone to pre-analytical artifacts.

This study was performed on an IHC platform using Ventana machines and reagents (e.g. antigen retrieval buffers, detection antibody conjugates and chromogen substrate) within specific protocols. The specific findings described here may of course be different in other IHC laboratories using different platforms and/or detection methods (e.g. different antibodies may react to FFT or CIT due to less sensitive detection methods). Nevertheless, we think that the essence of the method described here should be widely applicable. Because, the optimal staining compared to a common reference as the end result of any IHC procedure/protocol will be the same in all laboratories, regardless of the platform used.

For this study the CQPath image database that was developed and implemented at Erasmus MC pathology for Quality Assurance of routine IHC was used as a source of reference images [10,11]. The reference images provide criteria against which any individual laboratory can validate [19] their IHC protocols.

In conclusion, it is possible to optimize IHC protocols in order to minimize staining intensity variations by finding the optimal protocol for all the positive control tissue with pre-determined CIT and FFT. Hence, the optimized protocols allow antigens to be detected with the same sensitivity, regardless

of FFT and CIT. If laboratories achieve consensus based on an agreed upon reference image, a major step in global equalization of IHC results (i.e. elimination of inter-laboratory variation) can be achieved without cumbersome standardization of every detail in the pre-analytical phase.

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Chapter 6

Assessment of “Fit-for-Purpose” Frozen Tissue Collections by RNA Integrity Number-Based Quality Control at the Erasmus Medical Center Tissue Bank

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Abstract

Every year around 5,000 frozen tissue samples are collected by the Erasmus Medical Center tissue bank. Two percent of these samples are randomly selected annually for RNA isolation and RNA Integrity Number (RIN) measurement. A similar quality assessment was conducted during centralization of a 20 year-old tissue collection from the cancer institute, a 15 year-old liver sample archive (-80^{o}C), and a 13 year-old clinical pathology frozen biopsy archive (Liquid Nitrogen). Samples were divided into either high quality (RIN≥6.5) or low quality overall categories or into four “fit-for-purpose” quality groups: RIN <5 - not reliable for demanding downstream analysis; 5≤ RIN <6 - suitable for RT-qPCR); 6≤ RIN <8 - suitable for gene array analysis; and RIN ≥8 - suitable for all downstream techniques. In general, low RIN values were correlated with fatty, fibrous, pancreatic or necrotic tissue. When the percentage of samples with RIN≥6.5 is higher than 90%, the tissue bank performance is adequate. The annual 2011 quality control showed that 90.3% (n = 93) of all samples had acceptable RIN values; 97.4% (n = 39) of the cancer institute collection had RIN values above 6.5; and 88.6% (n=123) of samples from the liver sample archive collection had RIN values higher than 6.5. As the clinical pathology biopsy collection contained only 58.8% (n = 24) acceptable samples, the procurement protocols of these samples needed immediate evaluation. When the distribution of RIN values of the different collections were compared, no significant differences were found, despite differences in average storage time and temperature. According to the principle of “fit-for-purpose” distribution, the vast majority of samples are considered good enough for most downstream techniques. In conclusion, an annual tissue bank quality control procedure provides useful information on tissue sample quality and sheds light on where and if improvements need to be made.

Introduction

Ever since the human genome project [1] was completed and high-throughput genomics became available, the demand for high-quality human samples for medical research increased. In the case of rare diseases, research teams joined their efforts in consortia sharing samples [2] to form large enough cohorts for solid statistical analysis [3]. In general, professional biobanking of various samples such as blood, serum, urine and tissue increased. Soon after formation of the first international cooperative groups, inter-center variation (institutional bias) due to lack of standardization of sample procurement protocols was observed, and standardization of sample procurement procedures became desirable to enhance exchangeability and comparability of samples. This need for harmonization and standardization led to the establishment of the Best Practices for Repositories by the International Society for Biological and Environmental Repositories (ISBER) [4]. While biorepositories evolved from activities within clinics or pathology laboratories into professional institutes, the need to maintain appropriate levels of sample quality also grew. Quality control (QC) and quality assurance (QA) schemes were designed [5] and are now embedded in the routine procedures of many biobanks.

The main benefit of these QC (measurement) and QA (improvement) exercises is to establish an efficient and constant tissue bank workflow in which all protocols are designed to deliver high-quality samples. This automatically implies that tissue banks need dedicated personnel who are not only skilled in procuring samples the right way, but who can also, on a multi-disciplinary level, improve the sample quality. Tissue biobank personnel should give clinicians directions on how to send in their specimens under the specific conditions needed for optimal tissue sample procurement. In addition, biobank personnel must be able to facilitate clinical trials, which may require specialized tissue procurement protocols. Furthermore, biobank personnel must also be able to perform the QC and subsequent QA to determine systematic errors and noncompliance with Standard Operating Procedures (SOPs). There are several steps involved in the process of QC, ranging from traceability of samples to RNA integrity measurement. The kind of QC performed by a biobank depends on the kinds of samples preserved and in what way. For instance, a biobank with blood and serum samples will most probably focus on the quality of DNA and serum proteins [6], while a tissue biobank will also have to assess tissue morphology (percentage of tumor or necrosis in a sample) [5], and molecular integrity. The latter will be described in this article.

At our facility, the accuracy of sample storage (i.e., the position of a sample in the freezer as recorded in the tissue bank database) is assessed by randomly selecting 2% of samples [7], and confirming in the database whether the numbers of the tubes match the contents of the tube. For this

reason, frozen hematoxylin and eosin (H&E) sections are prepared from the randomly selected samples and compared to a formalin-fixed and paraffin-embedded H&E section of an adjacent piece of tissue that was taken and processed during sample procurement. In this way the tissue and disease type can be determined and compared to the description in the biobank database to confirm proper annotation and storage position. In addition, the quality of frozen tissue samples is assessed by RNA isolation and RNA Integrity Number (RIN) measurement. In order to achieve reliable QC results, biobank personnel must know how to isolate RNA in the most reproducible and accurate manner. If the RNA isolation is done with sub-standard materials or following protocols which lead to RNA degradation, the RIN values may be lower than expected for technical reasons. This would result in misleading performance indicators because they would be based on technical short comings rather than true tissue quality. The ISBER RNA Proficiency Testing Program confirms that the method used for assessment of RNA quality is accurate [8]. The QC and QA programs allow tissue bank personnel to trust their product and their skills and then provide advice to researchers who want to do high-quality research on tissues. Because of the high-quality standards pursued by the central tissue bank, many researchers have transferred their private collections into the biobank in order to obtain and maintain a higher level of sample quality, and to take advantage of the tissue bank's expertise and expanding facilities.

In this article we describe how and what we have learned about our frozen tissue sample collections during our standard QC and QA process. Tissue bank performance was assessed by measuring RIN values of a random selection of samples which were acquired over the past six years; this was primarily to determine systematic errors or non-compliance with SOPs. In addition, the quality of several centralized frozen tissue collections which had been received from researchers from several sites within our institute, was assessed. Samples in these collections had been procured using different protocols and/or were stored under different conditions. These assessments gave insight into the quality of the collections considering the different procurement and storage conditions, and the influence of these conditions on RNA, and therefore sample integrity.

Materials and Methods

Quality assurance procedures and quality criteria

The Erasmus MC tissue bank QC procedure is based on the fact that RNA is unstable and therefore sensitive to pre-analytical factors. RNA integrity can be reliably measured using micro capillary gel electrophoresis from which

the RIN value can be calculated. In the literature RNA with RIN values <5 have been deemed to be of a quality that is too low for demanding downstream RNA analysis; RIN values higher than 8 have been considered to be perfect [9]. The cut-off RIN values for samples used in gene expression arrays appear to be between 6 and 7 [10, 11]. Considering these literature references, we used a RIN value of 6.5 as a cut-off for RNA quality and therefore, tissue quality. In other words, if RNA with RIN values >6.5 can be derived from a tissue sample, the tissue sample is assumed to also provide DNA and proteins of sufficient quality for demanding techniques.

The assessment of tissue bank performance can be determined by RIN values but RIN values can be influenced by tissue type. A low RIN value is not always due to procurement errors; certain tissue types notoriously yield too little RNA for exact RIN measurement, or contain naturally degraded RNA. This is why frozen sections are also made during RNA isolation for morphological assessment. “Badly-procured” samples are those with low RIN values even though the cells are morphologically viable and there is enough RNA for measurement. Fatty tissue has a low cell density and contains lipids which interfere in the RNA isolation protocol (low RNA yield). Pancreatic tissue contains high levels of endogenous RNase in combination with proteolytic enzymes, which can cause RNA to be degraded when the isolation is not performed fast enough. Fibrous and muscle tissues are difficult to disrupt and do not contain that much RNA. And necrotic tissue does not contain viable cells [12].

Tissue bank performance is more accurately assessed by omitting from the calculations the results from notoriously low-yielding tissues. By taking into account only those tissue types where it is possible to routinely collect good quality samples (and true “badly-procured”,samples can therefore be recognized) can tissue bank performance be calculated as the percentage of “badly-procured” samples. It has been suggested that if this percentage is below 10% [13], the tissue bank can be deemed to function well. As soon as this percentage rises above 10%, it suggests that systematic errors may have occurred or that there has been non-compliance with tissue bank protocols. In that case, the tissue bank protocols and procedures, as well as the practice of individual tissue bank technicians must be evaluated to improve the overall quality.

To gain additional insight in the scientific value of the samples, they were divided into four different “fit-for-purpose” quality groups based on information from the literature [9-11]: RIN <5 - not reliable for demanding downstream analysis; 5≤ RIN <6 - suitable for RT-qPCR only; 6 ≤ RIN <8 - suitable for RT-qPCR and gene expression arrays; and RIN ≥8 - suitable for all downstream techniques. This approach provides a more comprehensive “fit-for-purpose” quality assessment of the biobank, since a single RIN cut-off may lead to underestimation of tissue sample quality and thus the scientific

value of the biobank. The notoriously low-yielding tissues were also omitted from these calculations. This way, only the true “badly-procured” samples are taken into account.

The average number of frozen tissue samples collected annually in the Erasmus MC tissue bank has grown to about 5000 and each year approximately 2% are randomly chosen for QC [7]. In the case of rare tumors, other samples from the same acquisition date were selected. In the earlier years lower percentages were used. Recently, the tissue bank has assumed and centralized three peripheral tissue collections and the same QC scheme was used to assess their value.

Tissue collections

Annual QC of centralized tissue bank: Tissues of various organs and disease types (mostly tumors) were collected during routine macroscopic examination of the specimens. The transport time from the operating theater to the pathology laboratory varied between thirty minutes and two hours; the longer time was due to batching of specimens for more cost efficient transport. Samples were placed on a piece of filter paper covered cork sheet for orientation reasons. The tissues were snap frozen in pre-cooled isopentane and placed in pre-cooled vials with screw lids (3 mL cryo tubes, Sanbio B.V., The Netherlands). The samples were stored in liquid nitrogen for a year before the QA procedure took place. For the 2011 QA RNA isolation procedure, 103 samples (2.1% of the 4394 collected samples) were randomly picked from the biobank database (Table 1). Samples collected during autopsy were not selected for RNA isolation. QA assessments for the preceding years (2006-2010), were performed on 1.3% (33 of 2631), 0.7% (17 of 2316), 0.5% (18 of 3484), 1.7% (67 of 3905), and 2.2% (98 of 4458) of the samples, respectively, using comparable tissue types (no further details shown).

Tissue type	Average RIN	Standard Deviation	N	N with RIN<6.5
Abdomen	7.80		1	
Adnex	6.90		1	
Adrenal gland	8.03	0.76	3	
Bladder	8.07	0.60	3	
Breast	8.45	0.07	2	
Cervix	9.10		1	
Colon	7.44	0.80	5	
Esophagus	7.83	1.01	3	
Ethmoid	8.80		1	
Gall bladder	7.10		1	
Kidney	8.17	0.25	3	
Larynx	8.77	0.21	3	
Liver	8.09	0.76	7	
Lung	6.99	0.60	7	1
Lymph node	7.75	1.00	6	
Nose	8.30		1	
Ovarium	5.33	2.78	3	2
Pancreas	7.97	0.29	3	
Parotis	8.90		1	

Tissue type	Average RIN	Standard Deviation	N	N with RIN<6.5
Penis	7.60		1	
Peritoneum	7.80	0.42	2	
Prostate	7.95	0.21	2	
Rectum	7.77	1.81	3	1
Skin	4.95	3.46	2	1
Soft Tissue	8.00	0.42	5	
Spleen	5.70		1	1
Stomach	7.60	1.13	3	1
Testis	7.80	0.71	2	
Thorax	7.90		1	
Thymus	8.05	0.78	2	
Thyroid	8.20	0.26	3	
Tongue	7.40		1	
Tonsil	7.03	1.52	3	1
Tuba	7.75	0.35	2	
Ureter	7.90		1	
Uterus	5.50	4.38	2	1
Vulva	8.05	0.35	2	

Low yielding tissue samples excluded from analysis

Tissue type	RIN	Reason for exclusion
Adrenal gland	5,6	partially necrotic
Adrenal gland	N/A	totally necrotic

Bladder	N/A	TUR heat damage**
Muscle	N/A	fibrosis
Nerve	N/A	low cellular content
Omentum	5,2	fatty
Ovarium	N/A	partially necrotic
Soft Tissue	1,1	fatty
Bladder	4,8	TUR heat damage*
Omentum	5,8	fatty

* From 2011 annual QC evaluation
** TUR = Trans Urethral Resection (with electric scalpel)
N/A = Not Acquired

Cancer institute archive collection: Tissues of various organs and disease types (mostly tumors) were collected during routine macroscopic examination of the specimen. The transport time was between five and thirty minutes, due primarily to the short distance between the operating theater and the pathology laboratory. Samples were directly frozen in liquid nitrogen and stored in the same type of containers as described above. The samples had been collected and stored in mechanical -80^{o}C freezers for over 20 years before they were transferred to the central tissue bank in 2005. Forty-eight (48) samples (2% of the approximately 2500 collected) were randomly selected for the QA RNA isolation procedure (Table 2).

Liver sample archive collection: Liver samples were collected at the clinic (needle biopsies); the operating theater (wedge biopsies of liver transplantation patients), or during routine macroscopic examination of the specimen. The transport time of these samples was not recorded but was known to vary between thirty minutes and several hours. The longer waiting time was one reason to take over this collection and to expand it under control of the biobank. There is no record of how the samples were frozen during the first 5 to 7 years, but from 2003 till the present, samples were snap frozen in pre-cooled isopentane. Samples were stored in a -20^{o}C freezer for several weeks to months before being transferred to a -80^{o}C freezer where they were stored for at least 15 years before transfer of the collection to the central liquid nitrogen facility in 2007. One hundred and twenty-three (123) samples (2% of the approximately 5000 collected) were randomly selected for QA RNA isolation (Table 3). Since most of the samples were very small, morphology was not assessed.

Clinical pathology frozen biopsy archive collection: Biopsy samples of various tissue types were sent to the pathology laboratory from the outpatient clinics. Sample transport could have taken place under a variety of conditions (e.g., on ice, at room temperature, dry, on moist gauze, in salt solution). Unfortunately, there is no record of this. Since unfixed biopsies are treated with high priority by clinicians, the transport from the outpatient clinic to the pathology laboratory never took longer than thirty minutes. The samples were frozen in pre-cooled isopentane and stored in liquid nitrogen. Interestingly, these biopsies were collected for histotechnical procedures that required fresh frozen (i.e., not formalin-fixed) tissue. Nineteen of these biopsies were used for cutting frozen sections and were put back after use. The archive dates back 13 years and samples were centralized in 2011. Of this relatively small collection (approximately 1000 samples), 24 samples (2%) were randomly taken for RNA isolation for QA (Table 4). Since samples were very small, morphology was not assessed.

Table 2: Average RIN Values of Selected Tissue from the Cancer Institute Archive Collection

Tissue type	Average RIN	Standard Deviation	N
Breast	8.20		1
Colon	8.20	1.27	2
Larynx	8.15	1.48	2
Liver	8.23	0.71	4
Lung	8.50		1
Lymph node	7.63	0.92	15
Mouth	7.70		1
Omentum	6.20		1
Ovarium	7.25	0.35	2
Pancreas	8.30		1
Penis	7.40		1
Rectum	7.70	1.13	2
Salivary gland	8.67	0.38	3
Thymus	6.90		1
Thyroid	8.80		1
Tonsil	6.90		1

Low yielding tissue samples excluded from analysis

Tissue type	Average RIN	Standard Deviation	N	Reason for exclusion
Soft tissue	2.40		1	necrosis
Colon	2.40		1	necrosis
Skin	2.60		1	necrosis
Lymph node	3.83	1.08	3	fatty
Liver	5.50		1	partially necrotic
Breast	3.00		1	fatty
Pancreas	3.70		1	fatty

Table 3: Average RIN Values of Selected Tissues from the Liver Sample Archive Collection

Diagnosis	Average RIN	Standard Deviation	N	N RIN<6.5
Abcess	9.10		1	
Adenoma	7.44	1.06	6	1
Alcohol hepatitis	6.30	1.41	2	1
Carcinoma	8.80		1	
Cellular decay	5.90		1	1
Cholestasis	8.00		1	
Chronic hepatitis	7.85	0.35	2	
Circulation disorder	8.28	0.59	5	
Cirrhosis	7.51	0.95	9	2
Fibrosis	8.90		1	
FNH	7.00		1	
HBV	7.35	1.66	17	2
HCC	7.70	0.69	10	
HCV	7.46	0.91	19	2
Hemangioma	7.40		1	
Inflammation	7.77	1.74	18	1
Ischemia	7.63	0.87	3	
Metastasis	7.84	0.65	8	
NEC	8.60		1	
Necrosis	3.00		1	1
Normal	8.20	0.68	4	
PSC	7.20	0.66	3	
Pseudo tumor	7.80		1	
Rejection	7.60	0.46	3	
Fatty liver	8.20	0.71	2	
Storage disorder	6.90		1	
Toxic reaction	5.30		1	1

FNH = focal nodular hyperplasia
HBV = hepatitis B virus
HCC = hepatocellular carcinoma
NEC = neuroendocrine carcinoma
PSC = primary sclerosing cholangitis
HCV = hepatitis C virus

Table 4: Average RIN Values of Selected Tissue From the Clinical Pathology Biopsy Archive Collection

Tissue type	Average RIN	Standard Deviation	N	N with RIN<6.5	Used for histology
Cervix	8.10		1		no
Colon	4.00		1	1	yes
Conjunctiva	9.50		1		yes
Esophagus	7.60		1		no
Nasopharynx	7.90		1		no
Palatum	2.20		1	1	yes
Rectum	8.43	0.68	3		yes
Skin	7.63	1.68	11*	4**	10 yes/1 no
Small intestin	7.90	1.41	2		yes
Stomach	8.00		1		no
Stomach/esophagus	5.80		1	1	no

* for two samples, no RIN value was available. These were excluded from the calculation of the average RIN value

** two samples with RIN values lower than 6.5 and two samples where no RIN value could be acquired

RNA isolation, RIN measurement and frozen tissue morphology

Performed during all QC assessments

A small drop of OCT (Sakura TissueTec, KliniPath, The Netherlands) was used to mount the selected tissue samples on the cryostat microtome object holder. The samples were not submerged in OCT to avoid interference with the RNA isolation procedure.

2006-2008 QC assessments

Frozen sections of 20 µm thickness were cut and placed frozen in pre-cooled tubes with 1 mL RNA-Bee (Amsbio, Oxon, UK). The sections were disrupted by shaking the tube vigorously for about five seconds. Chloroform (200 µL) was added, the mixture was shaken for 15 seconds and placed on ice for 5 minutes. After centrifugation at 12,000 xg at 4^{o}C, the upper aqueous phase was transferred to a clean tube. An equal volume of cold (-20^{o}C) isopropanol was added and the RNA was allowed to precipitate for 30 minutes in the -20^{o}C freezer. After centrifugation for 30 minutes at 12,000 xg, the supernatant was decanted and the RNA pellet washed with 80% ethanol. The RNA solution was centrifuged for 8 minutes at 12,000 xg and the supernatant decanted. The RNA pellet was air dried and subsequently dissolved in 100 µL nuclease-free water.

2009-2011 QC assessments

Frozen sections of 10 μm thickness were cut and placed in 700 μL Qiazol (Qiagen, Germany). The tissue sections were disrupted by shaking the tubes vigorously for about five seconds. To avoid chemical degradation of RNA by the Qiazol cell lysis solution, no more than six samples were isolated at once. Total RNA was isolated according to the protocol supplied with the miRNeasy kit (Qiagen, Germany). The RNA samples obtained were placed on ice for approximately 30 minutes prior to RIN value measurement with RNA Nano Chips (Bio Analyzer 2100, Agilent, California/USA). After the 10 μm sections for RNA isolation were cut, a 4 μm section was cut and mounted on a slide for H&E staining (LiniStainer, Leica, Germany).

Results

Annual QC: Of the 103 samples tested, 19 (18.4%) had a RIN value lower than 6.5 or the RIN value could not be determined (Table 1). Of these 19 samples, 9 (9.68%) had RIN <6.5 and 10 were comprised of notoriously low-yielding tissue types - fibrous, fatty, or necrotic tissues, or tissues with otherwise low cellular content (see Figure 1 for photographs of some typical examples).

According to the principal of “fit-for-purpose” distribution, the annual QC of the Erasmus tissue bank in 2011 (n = 93 after correction for notoriously low yielding tissue types) showed that 4.3% (n = 4) of samples were unreliable for demanding downstream procedures (RIN <5); 3.2% (n = 3) of the samples should only be used for RT-qPCR (5≤ RIN <6); 49.5% (n = 46) of the samples can be used for gene array work (6 ≤RIN <8); and 43% (n = 40) of samples are considered fit for all demanding downstream techniques (RIN ≥8) (Figure 2). The numbers in Table 1 reflect average RIN values of N samples while the numbers cited here reflect sample counts based on individual RIN values.

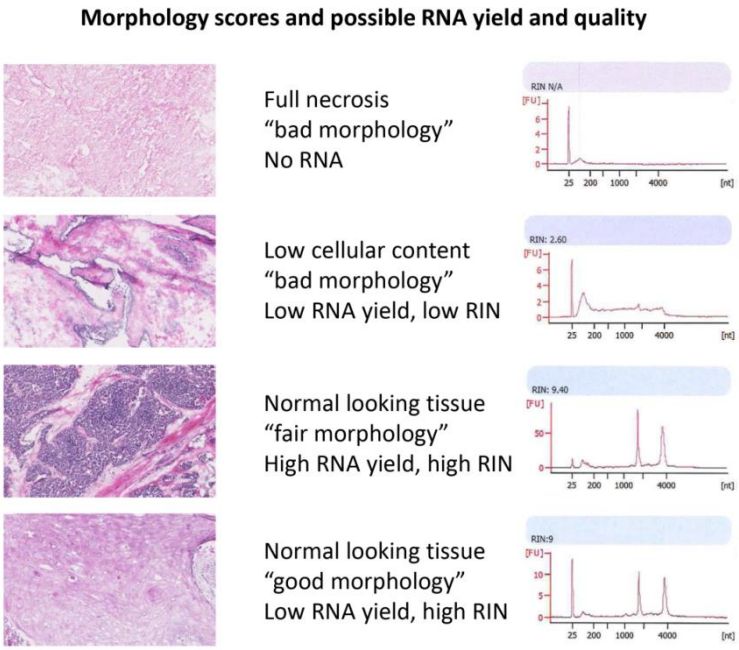

FIG. 1. Morphology scores and possible RNA yield and quality. Low RIN values are mostly correlated with morphology. Samples comprised of completely necrotic tissue (top panel) mostly do not yield RNA at all. When tissue is comprised of structures with low cell density (second from top) and the morphology shows either freeze artifacts or an abundance of noncellular matrix, RNA yield and integrity is mostly low. Normal looking tissue with fair morphology (third from top) is defined as tissue with high cellular content, absence of freeze artifacts, and relatively low amounts of fatty, necrotic, or fibrous tissue components. In some cases, excellent morphology and high cellular content does not correlate with high RNA yield. In the squamous cell carcinoma shown in the bottom panel, the RNA yield is low because the cells are hard to disrupt during RNA isolation; the RNA integrity, however, is high.

Figure 2 Fit for purpose distribution

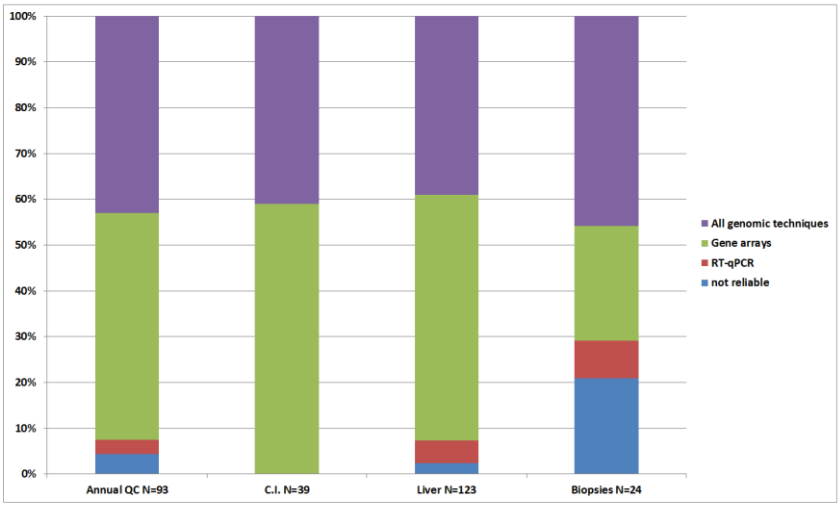

FIG. 2. Fit-for-purpose distribution of all assessed tissue collections. Samples were divided
by RIN values. Samples with RIN < 5 are not reliable for demanding downstream genomic techniques; samples with 5 ≤ RIN < 6 are only reliable for RT-qPCR; samples with 6 ≤ RIN <8 are reliable for RT-qPCR and gene array analysis; and samples with RIN ≥ 8 are reliable for all imaginable genomic
techniques. The latter two categories combined generally represent the tissue quality level that is likely reliable for most other ‘‘omics’’ research such as proteomics and metabolomics. C.I. = Cancer Institute.

Results of the preceding QC assessments (2006-2010) were analyzed in the same fashion (data not shown). The changes in “fit-for-purpose” distribution (Figure 3A), as well as the tissue bank performance (Figure 3B) from 2006-2010 are shown in Figure 3. The number of samples unreliable for demanding downstream analysis decreases, while the number of samples suitable for RT-qPCR increases. In the high RIN value side of the spectrum, the number of samples suitable for gene array analysis increase, while there is a small decrease of samples suitable for all genomic analyses (Figure 3A). To give a clearer presentation of deterioration or improvement of the tissue bank performance, the percentages of “badly-procured” samples (RIN <6.5) from all annual QC assessments were converted to negative figures (Figure 3B). This shows a three year period of substandard performance (2007-2009), which ultimately resolved during the last two years (2010-2011).

Cancer institute archive collection: Out of 48 tested samples, only 28 showed a RIN value above 6.5 after the first RNA isolation (Table 2). RNA

isolation was repeated for 20 samples which showed low RIN values. After this re-assessment, RIN values improved and only 10 samples (20.8%) scored RIN values below 6.5. For 9 of these 10 samples, the low RIN values could be explained by tissue type and these were excluded from the analysis: five samples showed necrosis or a low number of cells, and the other four had good to fair morphology but it is notoriously difficult to obtain reasonable amounts of RNA from these tissue types (1 pancreatic tumor, 2 fatty lymph nodes and 1 fatty breast) [5, 12]. One sample had a RIN value lower than 6.5 (2.6%, n = 39) that could not be explained by morphology. The numbers in Table 2 reflect average RIN values of N samples while the numbers cited here reflect sample counts based on individual RIN values.

The “fit-for-purpose” distribution of the cancer institute collection (n = 39 after correction for notoriously low-yielding tissue types) shows that none of the samples are unreliable for demanding downstream procedures (RIN <5); no samples should only be used for RT-qPCR (5 ≤ RIN <6); 59% (n = 23) of the samples can be used for gene array work (6 ≤RIN <8); and the remaining 41% (n = 16) of samples are considered fit for all demanding downstream techniques (RIN ≥8) (Figure 2).

Liver sample archive collection: Out of 123 tested samples, 12 samples (9.8%) had RIN values below 6.5 (Table 3). The relatively high percentage of “badly- procured’ samples cannot be correlated with morphology since morphology was not assessed for this collection. The “fit-for-purpose” distribution of the liver sample collection shows that 2.4% (n = 3) of samples are unreliable for demanding downstream procedures (RIN <5); 4.9% (n = 6) of the samples should only be used for RT-qPCR (5≤ RIN <6); 53.7% (n = 66) of the samples can be used for gene array work (6≤ RIN <8); and the remaining 39% (n = 48) of samples are considered fit for all demanding downstream techniques (RIN ≥8) (Figure 2). The numbers in Table 3 reflect average RIN values of N samples while the numbers cited here reflect sample counts based on individual RIN values. Clinical pathology frozen biopsy archive collection: Out of 24 tested samples, 7 samples (41.2%) had RIN values lower than 6.5 (Table 4). Nineteen biopsies were previously used to cut frozen sections for diagnostic reasons. Pearson Chi-Square analysis showed the use for histology was not correlated to low RIN value.

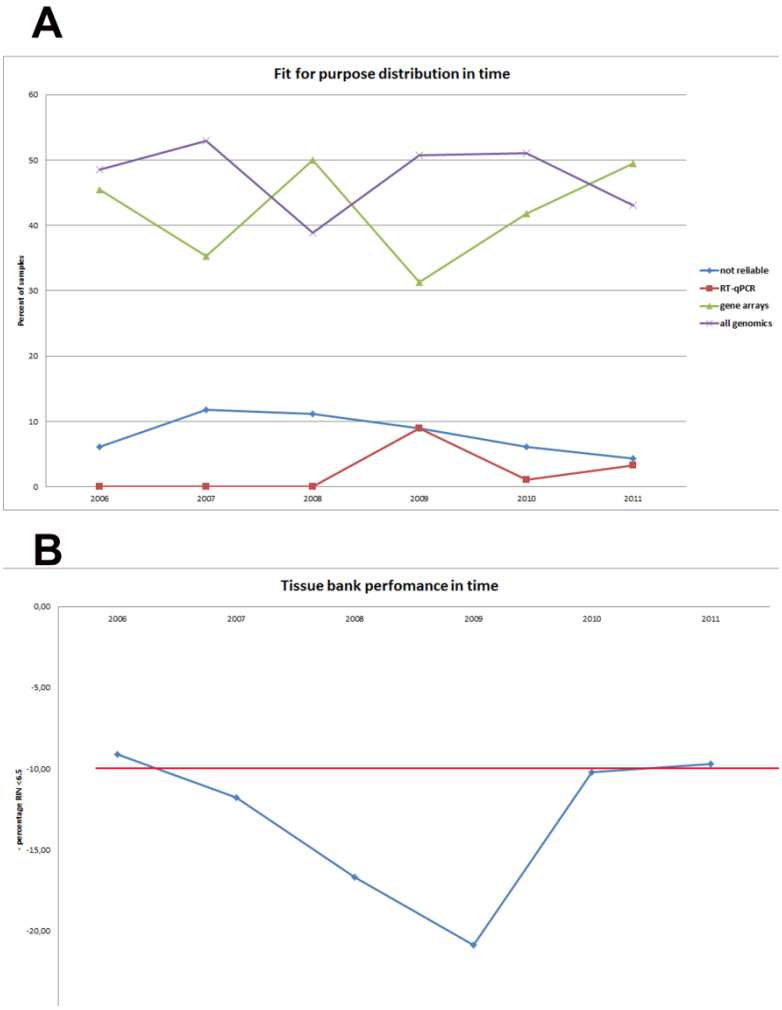

FIG. 3. Fit-for-purpose distribution and tissue bank performance over the past 6 years. (**A**) shows how the fit-for-purpose distribution of quality assessed tissue bank samples has changed over the past 6 years. The percentage of samples that are deemed unreliable for downstream techniques has decreased steadily since 2008. The percentage of samples that are deemed perfect for all genomic techniques also decreased, while the percentage of samples suitable for gene array analysis increased. (**B**) shows how the tissue bank performance level (displayed as a negative percentage of samples with RIN < 6.5) has improved after being substandard for 3 years. Tissue bank performance is considered adequate when the percentage of samples with RIN ≤ 6.5 exceeds 90% and thus - 10% is considered the cut-off.

In the diagnostic pathology reports we did not find morphological characteristics which could explain low RIN values. The “fit-for-purpose”

distribution of the clinical pathology biopsy sample collection shows that 20.83% (n = 5) of samples are unreliable for demanding downstream procedures (RIN <5); 8.33% (n = 2) of the samples should only be used for RT-qPCR (5≤ RIN <6); 25% (n = 6) of the samples can be used for gene array work (6 ≤RIN <8); and the remaining 45.83% (n = 11) of samples are considered fit for all demanding downstream techniques (RIN ≥8) (Figure 2). The numbers in Table 4 reflect average RIN values of N samples while the numbers cited here reflect sample counts based on individual RIN values.

We performed statistical analysis of RIN vs sample type (biopsy vs excision); RIN vs storage type (-80º C vs liquid nitrogen); and RIN vs storage time (annual QC samples stored for one year vs long-term stored collections). None of the analyses showed significant differences in RNA integrity.

Conclusions and Discussion

RNA isolation and RIN measurement was used as a tool for tissue quality assessment. We designated a cut-off RIN value of 6.5 as an indicator of adequate tissue quality. It is not standard practice to assess the applicability of demanding proteomic or metabolomic techniques using RIN values, but it is safe to assume that as RNA is an unstable tissue derivative that can be reliably tested, RNA RIN values can be used as a surrogate indicator of good tissue quality. When RIN values lower than 6.5 were found in most cases this could be correlated with overall low cellular content or with notoriously low-yielding tissue types [5]. For some specific tissue types, like pancreas and fatty (adipose) tissue, special RNA isolation procedures [14] and kits (Qiagen, RNeasy Lipid Tissue Mini Kit) are available. It may be useful to use these specialized kits in future tissue bank QC exercises, especially when a research group works with these tissue types. When these tissue types are left out of the QC exercise, the results are a better indicator of the tissue bank's performance.

Tissue bank performance was above the threshold standard of 10% of samples with RIN <6.5 (10% RIN <6.5) for two years (see Figure 3B). In the first three years, during the developmental phase of our QC procedure, we aspired to gain all necessary information by testing as few samples as possible (n = 20) rather than a percentage of all samples. The old RNA isolation protocol was very time consuming and RIN values were still a novelty. In 2009, when it was repeatedly observed that the amount and quality of RNA isolated from tissue depended heavily on tissue type, we decided to take more samples for QC purposes. From 2010 onward, we have been taking 2% of all annually collected samples for QC. This also became more feasible due to the use of the RNeasy kits, which made it possible to isolate RNA in under one hour.

In the tissue bank QC reports before 2009 there was also no mention of the 10% RIN <6.5 standard performance cut-off. That makes sense because the cut-off was first described in the ISBER Best Practices document of 2008. Nevertheless, in retrospect, the tissue bank performance in 2009 was substandard compared to earlier years and may have been due to major reconstruction of the pathology cutting room. This reconstruction forced the pathology laboratory, as well as the tissue bank facility, to move the entire cutting room to the morgue which is situated far away from the rest of the pathology department. Although most of the logistics, like transport of tissue specimens from the clinic to the morgue facility, remained the same as before, pathologists had to walk a fair distance to examine fresh specimens, from which samples were then taken for the tissue bank. This caused a batching effect and thus longer cold ischemia periods occurred during which RNA could possibly have degraded more.

The "fit-for-purpose" distribution over the course of six years (Figure 3A) shows that the number of unreliable samples decreased while the number of samples useful for RT-qPCR increased. This implies that the low performing end of the sample spectrum improved. On the other hand, the opposite is observed in the high performing end of the spectrum, where fewer samples became fit for all genomic techniques and more samples became fit for use in gene array studies. The decrease in the number of top-quality specimens may also be due to financial reorganizations within the pathology department, especially after the temporary move to the morgue in 2010/2011. The tissue bank strives to collect and freeze samples within 30 minutes after specimens are taken from the patient at the operating theater. But due to budget cuts, the specimen transport frequency was decreased and specimens arrived at the pathology laboratory often after two hours of waiting in refrigerators in the clinic.

In the case of the clinical pathology frozen biopsy collection, a correlation between tissue type and low RIN values could not be established. Since these samples were collected for histological purposes and would have been disposed of after 10 years, the procurement protocols did not anticipate use of these samples for future molecular research. Another reason for the high number of samples with low RIN values in this collection could be that after sectioning for histological procedures, the tissue was thawed while taking it off the cryostat microtome object holders. The clinical pathology biopsies are now handled by the central tissue bank in a standardized manner. However, the biopsies are still used for diagnostic purposes before storage in the tissue bank, so education of technicians on how to handle this tissue properly (e.g., don't let the samples thaw while putting them back in the vials) is necessary to preserve these useful samples for future research. Again, this emphasizes the need for dedicated biobank personnel who can

educate others on how to collect high-quality samples which are also suitable for medical research purposes.

RIN measurements have always been performed using Agilent Bio Analyzer RNA Nano Chips. In some cases a proper RIN value could not be established because the RNA yield was too low. Although the amount of tissue used for RNA isolation was always the same (the area and number of sections were observed and accounted for - e.g., 10 sections of 1 cm^2 area or 20 sections of 0.5 cm^2 area were cut), the use of RNA Pico Chips could give a better impression of RNA quality when low RNA yields are obtained. However, the occurrence of low RNA yield is small and the majority of the Pico Chips would expire before being put to use. Therefore, the use of these chips is financially unfeasible in the QC procedure.

We have also noticed that when tissue morphology would predict a higher-than-measured RIN value, it is best to repeat the RNA isolation. It is possible that something can go wrong at any time during the RNA isolation procedure. Although the pre-PCR laboratory where RNA isolation is performed is clean, an airborne speck of dust may contaminate the sample, causing RNA degradation by nucleases.
For the 2011 QC report, only the cases where no valid explanation for low RIN values could be determined (i.e., high enough RNA yield and tissue types not suspected to yield low RNA quality, but RIN value lower than 6.5) were accounted as “badly-procured” samples. Therefore, the biobank does not need evaluation of the methods used since the performance is good, with only 9.68% insufficiently procured samples. Of course, only 2% of all samples were actually tested. Before using the remaining 98% untested samples for further genomic analysis, it is advisable to measure RNA integrity in order to be able to take corrective measures during data analysis.

By doing QC we showed that long term storage of various tissue types in either liquid nitrogen or in -80^{o}C freezers preserves tissue well enough to achieve a high proportion with RIN values that we have characterized as good for most downstream “omic” techniques. Furthermore, our results suggest that the mechanical freezers used to store these collections have not failed and the samples did not thaw accidentally, since this would likely have lead to significantly lower average RNA RIN values. However, more than 10% of the liver samples had RIN values that suggested that they had been badly procured, since these numbers cannot be explained by morphological features, because morphology was not assessed. Temporary storage (weeks to months) of liver samples at -20^{o}C may have harmed RNA integrity in some of these cases. However, when statistical analysis was performed and different aspects of the collections (sample type, storage temperature and procurement method) compared, no significant correlation between RIN values and these factors were found. Therefore it seems that

sample storage (time or temperature) does not significantly influence RNA integrity.

When samples were divided into four “fit-for-purpose” groups, it was observed that the majority of samples can be designated as useful for downstream techniques varying from RT-qPCR and gene expression array analysis to next generation sequencing, based on cut-off RIN values found in the literature [9-11]. This “fit-for-purpose” distribution can be helpful for researchers who need samples for certain downstream techniques. It would facilitate making statistical calculations on the number of samples needed for a study, and the expected availability of those samples in a tissue bank. There is no known correlation between RIN value and proteomic, DNA or metabolomics research performance. Some reports describe the simultaneous isolation of RNA, DNA and proteins [13, 15-17]. There seems to be a correlation between RNA, DNA and protein quality when different sample types like paraffin embedded tissue and serum are considered. However, this does not say anything about an eventual correlation between RIN value and DNA/protein quality within one specific sample type group, namely frozen tissue. The amount of information found in these articles on RIN vs DNA/protein quality is too low (either all RIN values are high or RIN isn’t measured at all) to draw final conclusions. So, although the RIN value should provide an indication for overall tissue quality, these “fit-for-purpose” groups only apply for RNA-based genetic research. It is recommended that for all downstream tests an appropriate “fit-for-purpose” test should be considered, especially where signals of very unstable components need to be determined.

The minority of samples labeled as “not reliable for downstream techniques” can still be valuable, albeit for less demanding techniques. Ongoing research at our laboratory shows that RNA with RIN values as low as 1.5 can be used for RT-qPCR with 530 bp amplicons (data not shown). This implies that these samples could also be suitable for gene array analysis, a technique which uses smaller probes on chips or beads. Viljoen et al. [18] recently showed that statistical correction of gene array data can be used to correct for low RIN or degraded RNA samples, making the worst samples seem useful, even for (allegedly) demanding downstream analyses. The same goes for high quality samples to some extent. For now, we maintain a cut-off of RIN ≥8 as fit for all downstream techniques, but future research (with new techniques) may very well show that all downstream techniques can be performed on tissues with lower RIN values. Thus, the cut-off values used here are not necessarily permanent. Since RNA quality is not yet correlated with performance of “omic” techniques outside the RNA spectrum, it is impossible to even imply that low RIN tissue would also be unsuitable for proteomic or metabolomics techniques. Therefore, it is necessary to develop easy to assess tissue quality markers which are tailored to these techniques.

In conclusion, the annual QC as well as incidental quality assessments of peripheral frozen tissue collections can provide useful information on how tissue procurement and storage can influence tissue quality. It will eventually lead to improvement of procurement protocols and guarantee a high level of sample quality, which will instill confidence for both the tissue banker and the researchers who use the samples.

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General conclusions and discussion

From surgery to tissue fixation, many pre-analytical parameters can be identified which can cause unwanted variations in the final test results and alter for instance gene expression, RNA integrity, morphology and antigenicity. Some of these pre-analytical parameters can be standardized, others can be avoided or eliminated by adapting the analytical phase, but some can only be dealt with by adding relevant variables (recorded as sample meta data) to the statistical analysis of research data. The results presented in this thesis have a direct impact on translational research, where the potential of combining huge numbers of multi-disciplinary data points might be necessary before being able to design the best personalized medicine for the millions of individual patients. Here a future perspective is presented where insights obtained during the SPIDIA project are put into a context of a possible future diagnostic/research scenario.

In case therapy theoretically becomes really personalized to the individual level and dependent on the individual patient's biomolecular profile, it will become necessary to implement a new diagnostic workflow, based on molecular profiling of patient derived samples. During treatment of the individual patient, the therapy response must be measured and related to clinical parameters and/or clinical end stage of the disease [1]. This "n = 1" genome wide therapy response diagnostics can only be performed in the same multi-disciplinary set up as used in the initial therapy target discovery study; where the attending physician gathers general patient data, for instance in the case of cancer, the radiology department measures the decrease of tumor size, and the clinical diagnostic laboratories (clinical chemistry, hematology, immunology and pathology) will perform profiling techniques to find a drug response profile [1].

To identify functional disease biomarkers it would be useful to implement a systems biology approach performing DNA analysis (sequencing, SNP arrays, methylation assays) in parallel to RNA analysis (gene arrays or sequencing) to be able to correlate individual gene expression to individual DNA aberrations. Additional proteomic profiling can be performed in order to investigate whether the genetic biomarkers are functional in terms of protein expression.
In case what was determined in this thesis for liver is also valid for other tissue types, DNA is quite stable under ischemic conditions [2-4], however, since RNA **(chapter 1**), proteins [5] and metabolites [6] are influenced by warm and to a lesser extend cold ischemia. The correlation between DNA and the other tissue derivatives could be skewed and give an unrealistic representation of the comprehensive, interconnected functional bio profile. It is therefore crucial to be aware of variability of the pre-analytical workflow and the impact it has on biomolecular profiles. When surgically removed liver specimens are used for RNA based biomarker discovery studies, it is

important to know that surgery causes significant up- or down regulation of 4.2% of all genes during liver surgery (**chapter 1**). Although results described in chapter 2 only suggest that the length of surgery is proportional to gene up or down regulation during liver surgery, it is advisable to take note of such surgical parameters when analyzing RNA based genomic data. This way it will become possible to either discard genes influenced by surgery from the data analysis, or to use the length of surgery as a correction factor for gene expression. The latter option requires more research, but seems feasible. Also cold ischemia has an impact on certain genes, in some individual patients. This seems to be highly dependent on the patient's genomic background, since most patients show no reaction to cold ischemia at all. The effects of cold ischemia are, however, amplified in small liver samples (**chapter 3**), so it is preferable to use larger specimens if available. In the same liver samples as used for this RNA study, the same conclusions can be drawn for proteomic [5] and metabolomic [6] variability. When the SPIDIA project was written and submitted (2007/2008), techniques like RNA sequencing were not yet widely established. Therefore, it has not been possible to investigate effects of all described pre-analytical variables on next generation "omic" techniques. With regard to the article by Marioni, et al. [7] in which RNA-sequencing performance was compared to that of gene array analysis, it is expected that "post-SPIDIA" techniques are also adversely influenced by pre-analytical variability.

One way to avoid pre-analytical bias related to working with surgically removed tissue specimens, is the use of (CT guided) biopsies, provided they are fixed right after they are taken in vivo from the patient [8]. The principle of the MIA (Minimal Invasive Autopsy) group (**chapter 2**) may prove to be of great value for future translational research. When the MIA procedure can be used as a diagnostic tool to determine the cause of death equally accurate as conventional autopsies (ongoing research), then it is not unimaginable that CT guided biopsies taken from living patients, can be used for translational research and future diagnostics. One drawback could be that biopsies possibly will not always purely consist of tumor tissue, which can influence the accuracy and effectiveness of the biomolecular profile Most research described in this thesis was performed on liver tissue, where it was deliberately chosen for its homogeneity and enables a more controlled investigation of pre-analytical variation. This way sampling errors caused by different ratios of structures, which typically can be caused by tissue heterogeneity. Tissue heterogeneity remains a difficult issue, since even copy number variations in DNA can be variable in different cell types [9]. Especially in tumorous tissues, several different tumor cell types can play various roles in tumor development and persistence during treatment. It is widely accepted that tumors are not monoclonal and that quiescent tumor stem cells, rapidly dividing cells and metastasizing cells can have different profiles and subsequent physical properties [10]. When developing personalized medicine, which will most probably be aimed to target quiescent tumor stem cells and metastasizing cells which are not or hardly

affected by conventional treatments, it may be necessary to study the primary tumor and local/distant metastases on a cellular level [11]. Laser guided micro dissection [12] on PAXgene fixed and paraffin embedded sections can be performed to isolate specific cell types for specific molecular profiling. Therefore, it is necessary to first or in parallel make a histology based diagnosis (H&E, IHC and/or ISH), before multi omic research can be performed. The PAXgene fixative (**chapter 4**) was designed for this purpose. PAXgene fixation results in acceptable morphology and SPIDIA partners in Graz and Munich have shown that respectively nucleic acids [13] and proteins [14] are very well preserved by PAXgene fixation. Current omic assays can be applied to as little as 50.000 cells [15]. And when isolation kits like Allprep are used; DNA, RNA and proteins can be isolated from tissue simultaneously [16,17]. Strict standardization of protocols which enable working with small amounts of cells (i.e. random amplification of RNA [18,19]) is mandatory to guarantee reproducible results. Another benefit of immediate PAXgene fixation is the elimination of cold ischemia and bias caused by various transport methods, to which needle biopsies are quite sensitive (**chapter 3**).

Before the newly discovered biomarkers can be used for diagnostic purposes, they have to be validated. Validation of newly discovered tissue based biomarkers can be performed, using different techniques than were used during the discovery. Sequencing of selected DNA regions and RNA based RT-qPCR can confirm respectively mutations/LOH/CNV/SNPs and gene expression differences between patient groups. But also RNA in situ hybridization can be performed to detect the mRNA markers in situ, giving an indication which cell types are responsible for up or down regulation of genes. RNA will eventually be translated to proteins, so also IHC can be used to confirm up or down regulation of genes. In case of IHC it is advisable to design TMAs with FFPE tissue from all patients of the discovery cohort to verify the assumption the alternative approach works. But here different formalin fixation times can cause technical bias. Therefore, it is necessary to optimize the IHC assays as described in chapter six. By using control tissue that has known formalin fixation times and cold ischemia times it is possible to adapt the IHC protocol in such a way it is robust enough to stain sufficiently, regardless of these pre-analytical variables. In the same way also RNA and DNA fluorescence (or chromogen) in situ hybridization protocols may be optimized. By using fully optimized procedures, the validation will be more successful. Through validation, a diagnostic test can be developed in such a way that a fool-proof diagnosis and therapy inclusion criteria (for curative treatment, clinical trials, or division of patients in groups for further translational research) can be established.

Considering this future perspective, it is very important to know that the way a tissue sample is procured and handled until analysis can cause pre-analytical artefacts. At this moment, reproducibility of whole-genome sequencing is still an issue, regardless of the pre-analytical phase [20]. Therefore, the development of effective diagnostic profiles as personalized

therapy inclusion criteria, therapy response monitoring and subsequent feedback/refinement of diagnostics and therapy will be a challenge as long as pre-analytical variation remains an issue. It is therefore crucial to standardize, eliminate or record the pre-analytical variables.

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Summary

The chapters in this thesis describe in chronological order which pre-analytical variables can occur during the tissue based diagnostics and research workflow and their effect on various tissue based analyses, as outlined in the general introduction.
The effects of warm and cold ischemia on gene expression in liver tissue and the development of a RT-qPCR fingerprint assay to measure the effect of surgery on gene expression are described in **chapter 1**. Here we found that in liver tissue (selected for homogeneity of morphology) only 4.6% of all genes measured show significant up or down regulation due to intra-surgical warm ischemia. The extent of up or down regulation varies from patient to patient. Remarkably, the average expression during 6 hours of post-surgical cold ischemia did not lead to significant gene expression variation.
Here we assume that gene expression levels we measure with a finger print (a combination of above described genes) in ex vivo samples, are caused by gene expression up/down regulation due to surgery. This assumption is based on the fact that finger print expression levels are low at the start of surgery (no ischemia at all) and high after surgery (warm and cold ischemia). By comparing needle biopsies (only inert cold ischemia) with excision samples (warm and cold ischemia) this was confirmed, since the needle biopsies showed low finger print levels (in concordance with pre-surgical specimens) while high finger print levels were measured in the excision samples (concordant with the post-surgical samples in the discovery cohort).
An in silico analysis of downloaded gene array data of normal liver samples, obtained by 5 different surgical procedures provided further indication that the finger print expression level is proportional to the (approximate) duration of surgery. The assumption that finger print expression is proportional to duration of surgery is supported by the fact that also in this in silico analysis biopsies (not exposed to warm ischemia) show low finger print levels and excision samples (relatively long warm ischemia and inert cold ischemia) show high finger print levels. Interestingly, donor and recipient liver samples, taken during relatively shorter surgical procedures (i.e. shorter warm ischemia), showed intermediate finger print expression levels.
Further research with a larger well defined liver sample cohort may lead to identification and quantification of a evidence based correction factor for gene data analysis. If a linear, proportional correlation between finger print expression level and duration of surgery could be established, then taking note of duration of surgery would suffice for applying a correction factor for surgically induced warm ischemia during gene array analysis.

Chapter 2 in which RNA integrity and gene expression in post-mortem tissue is described, can be considered as a prolongation of the warm and cold ischemia time line described in **chapter 1**.

The Minimally Invasive Autopsy (MIA) study was not intended to be a pre-analytical study per se, however, the outcome is very useful to learn more about how RNA degrades while it is still in intact tissue and how that degradation can be accurately measured. Here we measured RNA integrity (RIN values) and gene expression with RT-qPCR in *ex vivo* and post mortem tissue. Ex vivo liver and kidney samples were surgically removed, whereas *ex vivo* heart samples consisted only of small biopsies. The post mortem tissue samples of the same types were taken either during MIA procedures (CT guided needle biopsies), or during routine autopsies (organs were taken out of the body) of the same individuals after the MIA procedure. The *ex vivo* samples contained RNA of good integrity (in average RIN7). The post mortem samples however, showed RNA had poor integrity (in average RIN3). When RT-qPCR was performed to detect a housekeeper gene (GAPDH) with primers and probes designed to replicate different length amplicons, it was found that also in post mortem tissue long strands of RNA could still be detected. The amount of RNA however was 16 to 20 fold lower in post mortem tissue as opposed to ex vivo tissue. This implies that while the body cools down after death (first at room temperature, then at 4°C), a warm ischemia process is induced which seems to end after approximately 24 hours, since no further decrease in RIN value was observed after 24 hours of death.
It seems RNA in post mortem tissue is still of good enough quality to perform gene array analysis, however the quantity of low expressed genes may become an issue when detection limits of gene array techniques are considered.

In **chapter 3** the first part of the pre-analytical phase that actually takes place at the pathology laboratory (acquisition phase) is described. When a pathologists selects a piece of tissue from a large specimen, depending on the size of the available tumor, a small or larger sample can be taken for the tissue bank frozen tissue collection. Tissue samples waiting to be registered and frozen are exposed to cold ischemia, as well as to the air which might cause air drying artefacts like oxidation, or increased intracellular salt concentrations caused by evaporation of water from cells, which could damage the RNA. It could, therefore, be possible that RNA would degrade faster in smaller samples than in larger samples. To test this hypothesis, tissue samples of different sizes were collected and subjected to one hour of cold ischemia. RNA was isolated and RIN values as well as gene expression levels (RT-qPCR) were measured. It was found that RNA degradation is indeed more pronounced in small samples and also gene expression levels of genes known to react to ischemia (see chapter 1) are decreased more in small samples.
In this chapter is also described how the transport method (dry, on moist gauze, in salt solution, cooled or at room temperature) and freeze method (liquid nitrogen or pre cooled iso-pentane) of small biopsies can have an additional (to one hour of cold ischemia) effect on RNA degradation and

gene expression. When RNA integrity is considered (RIN value) these factors don't contribute to further RNA degradation. The expression of certain genes, however, is significantly decreased when small biopsies are transported on moist gauze or in salt solution. When all conditions were generalized and the transport temperature was analyzed, it became clear that cooled transport preserves RNA significantly better. The freezing method does not influence RNA integrity in any way, tissue morphology of small samples, however, is damaged by freezing in pre cooled iso-pentane.
It is advisable to transport small biopsies dry and cooled on ice. The cold ischemia time or air exposure must be kept to a minimum, freezing small samples must be done a priori.

Chapter 4 describes an alternative for formalin tissue fixation. Chemical tissue fixation has a major advantage over frozen tissue, it enables working with tissue specimens at room temperature. This property has enabled the entire pathology diagnostic workflow as we know it today. Formalin forms cross links between proteins and nucleic acids which hinder molecular techniques. Furthermore, the longer a sample is fixed in formalin, the more of these cross links are formed. Also, it is known that the watery substance (formalin is a 4% solution of formaldehyde in water) causes random degradation of nucleic acids by hydrolysis.
To avoid these formalin fixation related disadvantages a new, alcohol and acid based fixative, PAXgene, was designed. This fixation method is based on precipitation of biomolecules instead of the formation of cross links, which in theory would enhance tissue based protein and nucleic acid research. In this chapter morphology and antigenicity of PAXgene fixed tissue are described.
Results of this study show that morphology of PAXgene fixed tissue is found acceptable and is by some pathologists preferred above formalin fixation. There are some critical artifacts, of which damage to red blood cells is the most prominent. Red blood cells are often used as an internal reference for cell size. This disadvantage can be eliminated by using endothelial cells as a new cell size reference, but this will take some training. In general, immunohistochemistry is as applicable in PAXgene fixed tissue, as it is in FFPE tissue. For most antibodies tested the original FFPE based protocols could be applied, for some antibodies the antigen retrieval step had to be adapted. Only in case of progesterone receptor a different clone had to be used to obtain results comparable to FFPE.
Thorough validation of techniques is mandatory when implementing a new fixative, the diagnostic value must be guaranteed. Considering the variety of machines and reagents used in individual laboratories, it is advisable to let individual laboratories perform their own assessment.
Safety is another aspect of implementation of a new tissue fixative. Formalin is known to inactivate viruses and bacteria. Because PAXgene fixates biomolecules in a more native form than formalin, the question arose whether PAXgene would inactivate viruses as well as formalin does. In

cooperation with the Department of Virology a virus culture experiment was developed to investigate the virus inactivation properties of formalin and PAXgene. The results show that viruses are inactivated after fifteen minutes of exposure to PAXgene. A coincidental find was that in case of Adenovirus, formalin needs at least six hours to completely inactivate the virus. Combined with the fact that unfixed virus remained active for at least 24 hours, this implies that as long as large tissue specimens are not thoroughly fixed they should be considered contagious.
PAXgene inactivates viruses equally well, or in case of Adenovirus better than formalin. It is therefore safe to work with PAXgene. It is important to be aware that large specimens, which are not thoroughly fixed after overnight fixation, may still contain active viruses and should be handled with caution.

In **chapter 5** a new method to overcome cold ischemia and formalin fixation time related antigenicity problems by adapting the analytical phase of immunohistochemistry (IHC) is described

The problem that led to the experiments was as follows: If at day 1 a positive control tissue fixed for 72 hours is used and at day 2 one that has been fixed for 24 hours, the protocol will be adjusted in such a way that the possible fixation time discrepancy between control tissue and patient tissue becomes problematic. For example: If positive control tissue is fixed for 24 hours and the patient tissue for 72 hours, the protocol may be suitable for the positive control, but not for the patient tissue. This could lead to false negative results and wrong diagnosis.
Standardization of the formalin fixation time would be the ultimate solution. But some tissue types require prolonged fixation and also weekend fixation is unavoidable. Therefore, the only remaining possibility to optimize IHC is to adjust the protocols in such a way that sensitivity is optimal, regardless of formalin fixation time. This was achieved by using positive control tissues which were fixed for 3, 24, 72 and 168 hours. Of all 4 tissue samples, sections were cut and mounted on one slide to guarantee equal treatment during the entire IHC procedure. When IHC was done, the intensity of all sections was scored. If all sections had equal intensity, the protocol was optimal. If different intensities were observed, the concentration of the primary antibody, or the antigen retrieval method was adjusted. If the adjustment resulted in equally stained sections, the protocol was optimized and robust enough to overcome formalin fixation artifacts. This approach worked for the majority of antibodies, only some still showed minor variation. When the staining intensity, achieved by the optimal protocol was compared to the digital reference, the intensity was found sufficient for diagnostic use.
If standardization of the pre-analytical phase is not feasible, it is necessary to optimize the analytical phase in such a way that effects of pre-analytical variation are eliminated. For some antibodies, the protocols could not be fully optimized. It may be advisable to find antibody clones which are not sensitive to formalin fixation time. As concluded in earlier chapters, it is

always smart to take note of the variables in the pre-analytical phase, the laboratory information system should contain formalin fixation time data for use in data analysis and trouble shooting.

Chapter 6 describes the final stage of the pre-analytical phase, where tissue has been collected and only the storage time and temperature remain as major variables. Annually, 2% of all collected samples are randomly picked from the frozen tissue archive for quality assessment. Since RNA is one of the least stable tissue components, RNA integrity can be considered as a yardstick for tissue quality. If RNA is of good quality, more stable tissue components like DNA and proteins are most likely of good quality as well. RNA integrity can be easily an reproducibly assessed by measuring the RIN value. If the RIN value is high, the RNA is not degraded and if the RIN value is low, RNA is degraded which is an indication of low tissue quality.
For this research a new division of RNA quality was designed. RNA quality of tissue samples stored in the frozen tissuebank is now divided in four “fit for purpose” groups. This means that, depending of the RIN value, a tissue sample can be reliably used for certain downstream techniques. RNA with RIN<5 can, according to literature, only be reliably used for RT-qPCR.
Because recently several external frozen tissue collections were added to the central tissue bank, it became possible to analyze any storage time and temperature related effects on RNA quality. The collection of the central tissuebank is relatively young and all samples were always stored in liquid nitrogen. Another collection is over 15 years old and was stored at -80oC, whereas a third 20 years old collection had always been stored in liquid nitrogen. From all external collections RNA quality was assessed as described above, before it was decided to add the collection to the central biobank. This provided RNA quality data related to storage time and temperature for the analysis.
None of the parameters has adversely influenced RNA quality. Needless to say, also the way the tissues were primarily frozen, either in liquid nitrogen or in pre cooled iso-pentane had no effect on RNA quality.
Storage temperature and duration has no influence on RNA quality and also the freezing method, as confirmed with the experiment described in **chapter 3**, does not adversely influence RNA quality.

Samenvatting

De hoofstukken in dit proefschrift gaan in chronologische volgorde in op welke pre-analytische variabelen binnen en buiten het pathologie laboratorium kunnen optreden en de effecten die zij kunnen hebben op diverse weefsel gebonden analyses, zoals beschreven in de algemene inleiding.
Hoofdstuk 1 behandelt het effect dat warme en koude ischemie hebben op genexpressie in lever weefsel en de ontwikkeling van een RT-qPCR fingerprint waarmee het effect van de operatie op genexpressie niveaus te meten is. Hier zien we dat voor slechts 4.6% van alle gemeten genen de grootste effecten te vinden zijn in de intra-operatieve, warme ischemie, fase. Genen kunnen hierdoor overmatig tot expressie komen, of hun expressie kan geremd worden. De mate van expressieverschil verschilt per patiënt.
Hier gaan we er dus vanuit dat elk genexpressie niveau dat we met een combinatie van die genen, samengevoegd in een finger print, meten in *ex vivo* weefsel wordt veroorzaakt door de operatie gerelateerde op regulatie van deze fingerprint. Dit werd tijdens de validatie van het ischemie onderzoek gevonden en bevestigd toen lever naaldbiopten vergeleken werden met leverweefsel afkomstig van *ex vivo* operatieweefsel. Biopten hebben een lage expressie en operatieweefsel een hoge expressie. Een analyse op gedownloade genexpressie data van lever weefsel dat met verschillende operatie procedures verkregen was leerde ons dat de mate van fingerprintexpressie samen lijkt te hangen met grofweg de duur van de operatie. Hier werd gevonden dat biopten een lage expressie hadden, donor en ontvanger weefsel van lever transplantaties een middelmatige expressie en de *ex vivo* operatieweefsels een hoge expressie. Dit lijkt te kloppen met de verwachting, want een biopt nemen gebeurt snel en zonder warme ischemie. Een stukje weefsel van een donor of ontvanger orgaan wordt genomen na een redelijk snelle operatie en ondergaat dus minder warme ischemie. En ten slotte duurt een operatie waarbij slechts een deel van de lever wordt verwijderd het langst en is het meest blootgesteld aan warme ischemie.
Verder onderzoek zou kunnen leiden tot een technische correctiefactor voor de analyse van genexpressie data. Als er inderdaad een lineair en evenredig verband is tussen fingerprintexpressie en de duur van de operatie, dan is het noteren van de duur van de operatie voldoende om te kunnen corrigeren voor door warme ischemie ontstane genexpressie variatie.

Hoofdstuk 2 behandelt RNA integriteit en genexpressie in post mortem weefsel en sluit mooi aan op de warme en koude ischemietijdlijn. In **hoofdstuk 1** hebben we gezien dat vooral warme ischemie genexpressie beïnvloedt en dat zes uur koude ischemie geen invloed heeft op genexpressie, maar ook niet op RNA kwaliteit.

Hier werd weefsel van levende patiënten, verkregen na standaard operaties (nier en lever) of biopteren (hart) (*ex vivo*), vergeleken met obductieweefsel en MIA weefsel van overleden patiënten (post-mortem). In *ex vivo* weefsel is het RNA goed intact, zoals gemeten in de vorm van RIN waarden. In post-mortem weefsel was het RNA echter behoorlijk afgebroken. Zodra er met kwantitatieve PCR werd gekeken naar de expressie van een huishoudgen, dat door de keuze van specifieke primers en probes in verschillende lengtes gerepliceerd werd, werd duidelijk dat zelfs het RNA uit post mortem weefsel nog lange RNA strengen bevatte. De hoeveelheid intact RNA echter was in post mortem ongeveer 16 tot 20 maal lager dan in *ex vivo* weefsel. Dit betekent dat terwijl het ontzielde lichaam afkoelt (eerst bij kamer temperatuur en later in de koeling), er een langdurig warm ischemie proces op gang komt, dat pas na een uur of 24 uitdooft. Daarna is er geen verdere RNA afbraak waarneembaar, wat de stabiliteit van koude ischemie lijkt te benadrukken.
Het lijkt erop dat er in post mortem weefsel nog RNA van voldoende kwaliteit te vinden is voor genetisch onderzoek. De gevonden mate van afbraak kan problematisch zijn voor genen die slechts laag tot expressie komen.

Hoofdstuk 3 behandelt voor het eerst een deel van de pre-analytische fase beschreven dat daadwerkelijk plaats vindt op de pathologie afdeling. Als een stukje weefsel wordt uitgesneden voor de weefselbank kan dat, afhankelijk van de grootte van het beschikbare weefsel, verschillende groottes hebben. Als het vervolgens even duurt eer de weefselbankers worden opgepiept om het weefsel te komen in te vriezen dan kan het zomaar zijn dat RNA in een klein weefselstukje sneller degradeert dan in een groot stukje. Om dit te testen zijn van stukjes weefsel van verschillende groottes de RIN waarden en genexpressie gemeten. Inderdaad is de mate van RNA degradatie afhankelijk van de grootte van weefselstukjes. In kleine stukjes degradeert RNA sneller en worden ook genen die reageren op ischemie effecten sneller afgebroken, zoals aangetoond met kwantitatieve PCR. In dit hoofdstuk staat ook beschreven hoe de transport methode van biopten; gekoeld, bij kamer temperatuur, droog, op een vochtig gaasje en in een zoutoplossing, RNA afbraak gedurende een uur koude ischemie beïnvloedt. Hoewel er op RIN waarde niveau geen noemenswaardige verschillen te vinden zijn, zijn er op genexpressie niveau enkele genen die sterker afbreken wanneer het biopt wordt getransporteerd in een vochtige omgeving. Over de hele lijn is het beter om kleine biopten gekoeld te transporteren. Tevens is er bestudeerd welke invriesmethode, direct in vloeibare stikstof of in voorgekoelde iso-pentaan, de beste resultaten oplevert. Het is dus aan te raden om kleine stukjes weefsel snel in te vriezen en biopten droog en gekoeld te transporteren.

Hoofdstuk 4 gaat over weefsel fixatie. Naast het invriezen van weefsel voor onderzoek is het ook mogelijk om onderzoek te doen op formaline gefixeerd

en paraffine ingebed weefsel. Een groot voordeel van chemische fixatie is dat het weefsel na inbedden in paraffine bij kamer temperatuur bewaard en gemanipuleerd kan worden. Op deze eigenschap is de gehele pathologie work flow gebaseerd. Formaline is echter een stof die storende verbindingen maakt tussen eiwitten en nucleïnezuren (DNA en RNA). Doordat de verbindingen overal aan- en tussen zitten wordt de vorm van biomoleculen verstoord en dus moeilijker te meten met gen- of eiwittechnieken. Hoe langer weefsel in formaline wordt gefixeerd, hoe meer van deze storende verbindingen ontstaan. Tevens is het zo dat de waterige formaline oplossing op willekeurige plaatsen de nucleïnezuren kan afbreken door hydrolyse.
Om dit soort formaline fixatie artefacten te voorkomen is het PAXgene fixatief ontwikkeld. PAXgene is een op alcohol en zuur gebaseerd fixatief dat geen storende verbindingen maakt en dus beter zou zijn voor gen- en eiwitonderzoek. In dit hoofdstuk is gekeken naar hoe vergelijkbaar weefselmorfologie en antigeniciteit is van weefsel dat is gefixeerd in PAXgene fixatief.
Het blijkt dat de morfologie acceptabel wordt bevonden en dat sommige pathologen de morfologie van PAXgene gefixeerd weefsel zelfs prefereren ten opzichte van formaline gefixeerd weefsel. Toch zijn er enkele PAXgene specifieke artefacten waarneembaar, waaronder aantasting van rode bloedcellen die vaak gebruikt worden als visuele maatstaf voor celgrootte. Het is echter wel mogelijk om bijvoorbeeld endotheelcellen te gebruiken als referentiepunt, maar dit zal enige training en gewenning vergen. In principe is immunohistochemie relatief probleemloos toepasbaar op PAXgene gefixeerd weefsel. Voor de meeste antilichamen was het standaardprotocol zoals gebruikt voor formaline gefixeerd weefsel te handhaven. Voor sommige antilichamen moest met name de antigen retrieval worden aangepast en in het geval van de progesteron receptor moest het antilichaam worden vervangen door een andere clone.
Het testen van dergelijke nieuwe fixatieven zal altijd grondig moeten gebeuren. Daarnaast is uitvoerige validatie onontbeerlijk, de diagnostische waarde moet gewaarborgd zijn. Dit zal elk laboratorium zelf moeten doen, want niet elk laboratorium gebruikt dezelfde apparatuur en reagentia.
Een ander aspect van implementatie van een nieuw fixatief is de veiligheid. Het is bekend dat virussen geïnactiveerd worden door formaline. Omdat PAXgene fixatie biomoleculen in relatief naieve vorm fixeert rees de vraag of PAXgene evenals formaline virussen zou inactiveren. Middels een *in vitro* virus celkweekexperiment, ontwikkeld in samenwerking met afdeling Virologie, bleek dit inderdaad het geval te zijn. Zijdelings bleek zelfs dat formaline er in het geval van Adenovirus aanzienlijk langer over doet om dit virus te inactiveren. Ook is het zo dat ongefixeerd virus minstens 24 uur actief blijft in kweekmedium. Dit impliceert dat zolang weefsel dat besmet is met Adenovirus besmettelijk blijft zolang het niet door en door gefixeerd is.
PAXgene inactiveert virussen minstens even goed en in geval van Adenovirus zelfs sneller dan formaline. Het is belangrijk om te weten dat

grote preparaten die na overnacht fixatie niet volledig gefixeerd zijn alsnog als potentieel besmettelijk te beschouwen.

Hoofdstuk 5 beschrijft een methode om antigeniciteit problemen veroorzaakt door koude ischemie en formaline onder- of over fixatie te niet te doen door het aanpassen van de analytische fase van immunohistochemie.

Het probleem dat leidde tot dit experiment was het volgende: Als op dag 1 een stukje controleweefsel wordt gebruikt dat 72 uur gefixeerd is en op dag 2 een nieuw stukje dat slechts 24 uur gefixeerd is moet door dit verschil het protocol worden aangepast. Deze aanpassing kan ervoor zorgen dat de fixatie tijd discrepantie tussen controle-en patiëntweefsel hinderlijk wordt. Bijvoorbeeld: Als positief controleweefsel 24 uur gefixeerd is en patiëntweefsel 72 uur, dan is het protocol geoptimaliseerd voor 24 uur fixatie en kan het patiëntweefsel als vals negatief worden uitgegeven. Dit kan gevolgen hebben voor de diagnose en dus de behandeling van de patiënt.
Standaardisatie van de formaline fixatie tijd zou de beste oplossing zijn. Dit is echter niet altijd mogelijk, daar sommige weefseltypes langere fixatie nodig hebben en ook omdat weekend fixatie onoverkoombaar is. Om toch betrouwbare IHC te kunnen uitvoeren rest uiteindelijk alleen het zodanig optimaliseren van het protocol, dat de invloed van de pre-analytische fase te niet wordt gedaan. Dit is ten dele bereikt door controleweefsels die 3, 24, 72 en 168 uur gefixeerd werden te gebruiken. Van deze vier stukjes weefsel in de fixatie tijdreeks werden coupes gesneden en op één enkel glaasje geplakt om er zeker van te zijn dat alle vier de coupes dezelfde behandeling kregen gedurende het gehele IHC protocol. Na de IHC procedure werd de intensiteit van elke coupe gescoord. Als alle coupes dezelfde intensiteit hadden, was de procedure optimaal. Als er intensiteitsverschillen werden geobserveerd, dan werd de verdunning van het primaire antilichaam of de antigen retrieval methode aangepast om deze verschillen te elimineren. Als de aanpassingen leidden tot gelijke intensiteitsniveaus in alle coupes op het glaasje, was de procedure geoptimaliseerd en robuust genoeg om, ongeacht variabele fixatietijd altijd het eiwit met afdoende gevoeligheid aan te tonen. Deze tactiek werkte voor het merendeel van alle antilichamen, hoewel enkele antilichamen nog steeds lichte variatie vertoonden.
Als standaardisatie van de pre-analytische fase praktisch niet mogelijk is, dan kan het aanpassen van de analytische fase in de meeste gevallen soelaas bieden. Voor de gevallen waar ook dit niet mogelijk is zou gezocht kunnen worden naar alternatieven (andere antilichaam clone, welke niet gevoelig is voor ischemie of fixatie artefacten). Ook hier geldt dat het noteren van de factoren in de pre-analytische fase kan worden aanbevolen om de kwaliteit van diagnostiek te verbeteren.

Hoofdstuk 6 beschrijft kwaliteit issues tijdens de opslag van de gevroren weefsels welke aan het einde van de pre-analytische fase te vinden is.

Hierin is weefsel dat op verschillende manieren opgeslagen in het vriesweefsel archief werd onderzocht. Tijdens de standaard weefselbankkwaliteitcontrole wordt in een steekproef van 2% van alle dat jaar verzamelde weefselstukjes gekeken naar de RNA kwaliteit. RNA wordt beschouwd als een van de minst stabiele weefselcomponenten. Dus als RNA intact is, dan is DNA en eiwit ook intact. De RNA integriteit is heel makkelijk en reproduceerbaar te meten en wordt uitgedrukt in een simpel getal, de RIN waarde. Als de RIN waarde hoog is, dan is RNA intact, als de RIN waarde laag is dan is RNA afgebroken. Voor dit onderzoek is een nieuwe indeling van RNA kwaliteit ontworpen. De RNA integriteit in weefselstukjes die zijn opgeslagen in de weefselbank is ingedeeld in “fit for purpose” groepen. Dit betekent dat afhankelijk van de RIN waarde het RNA betrouwbaar te gebruiken is voor bepaalde technieken. RNA met een RIN waarde lager dan 5 kan bijvoorbeeld, volgens de literatuur, niet gebruikt worden voor betrouwbaar gen array onderzoek, maar wel voor de minder veel eisende RT-qPCR techniek. Zodoende zijn er vier RNA kwaliteitsgroepen ontworpen die elk gerelateerd zijn aan een gebruiksadvies. Doordat er in de loop der jaren enkele externe weefselverzamelingen aan de centrale weefselbank werden toegevoegd werd het mogelijk om deze verzamelingen met elkaar te vergelijken. De verzameling van de centrale weefselbank is relatief jong en altijd heeft de opslag plaatsgevonden in vloeibare stikstof. Een andere verzameling is ouder en ook opgeslagen in stikstof, terwijl een derde verzameling ouder is en altijd opgeslagen was in een -80˚ C vriezer. Van elke verzameling is voordat deze definitief werd overgenomen steekproefsgewijs de RNA kwaliteit bepaald volgens bovenstaand principe. Hierdoor was het dus mogelijk om de invloed van opslagduur en temperatuur te analyseren. Het blijkt dat geen van deze parameters een significant verschil in RNA kwaliteit teweeg brengt. Hetzelfde geldt voor de manier waarop weefsel in de verschillende verzamelingen is ingevroren; direct in vloeibare stikstof of in voorgekoelde iso-pentaan.
Het maakt niet uit bij welke temperatuur weefsel wordt opgeslagen. En ook de methode van invriezen heeft geen invloed op RNA kwaliteit, zoals ook werd gevonden in **hoofdstuk 3**.

Appendices

List of publications

Bilge Ergin, Stephan Meding, Rupert Langer, **Marcel Kap**, Christian Viertler, Christina Schott, Uta Ferch , Peter Riegman, Kurt Zatloukal, Axel Walch, and Karl-Friedrich Becker Proteomic Analysis of PAXgene-Fixed Tissues. J. Proteome Res., **2010**, 9 (10), pp 5188–5196 doi: 10.1021/pr100664e

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The entire SPIDIA consortium was of great value during the numerous discussions we had during the SPIDIA meetings all over Europe. Some results were pretty straight forward, others were absolutely surprising and hard to process. General conclusions and optimal tissue, blood and serum/plasma procurement protocols are being molded into new ISO guidelines and regulations. I think we can all be proud of our joint contribution to a more regulated/standardized pre-analytical phase of many research and diagnostic fields! Of course, I want to mention Uwe Ölmüller, the leader of the SPIDIA consortium specifically. Thanks to this fabulous project, I have been able to do loads of interesting experiments in many fields of pathology research and diagnostics and write this thesis as a result thereof. The meetings, in all their aspects, were as inspiring as unforgettable. Many, many thanks to you!

Anieta Sieuwerts: Hello daaaarling… Van jou, een van de besten in de wereld, heb ik RT-qPCR en alles wat erbij komt kijken geleerd. Netjes werken, vlot doorwerken, ijklijnen, positieve- en negatieve controles en goed plannen zijn dingen die onmisbaar zijn als je van RNA bruikbaar cDNA wilt maken. Het lijkt wel of RNA van gewapend beton gemaakt is als je jouw tips en tricks in acht neemt, al vacuüm spin je het compleet droog, RIN waarden en de daar op volgende PCR resultaten blijven prima! Onder ’t genot van een biertje/droge witte wijn en een portie bitterballen hebben we vaak genoeg gepraat over het reilen en zeilen op de diverse afdelingen van het JNI, onderzoek en van alles en nog wat. En toch ook weer gelachen, onderzoek doen is op die manier best uit te houden.

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maar dragen' doorslaggevend voor een geslaagde dag. Natasja is met haar positieve (feest) geest en goede muzieksmaak de aangewezen persoon om alles in goede banen te leiden. Op het moment dat ik dit dankwoord schrijf realiseer ik me dat we nog veel moeten plannen. De boekjes uitdelen, de uitnodigingen verzorgen en natuurlijk het feestje achteraf! Maar ik weet zeker dat het dankzij jullie een mooie dag gaat worden.

Natuurlijk, ook zij die niet persoonlijk genoemd zijn; enorm bedankt voor alle hulp en steun!!

Tot mijn eeuwige verdriet, is mijn vader op veel te jonge leeftijd komen te overlijden aan de gevolgen van darmkanker. Ik mis mijn vader, hoewel het gat in m'n ziel inmiddels goeddeels is gevuld met dankbaarheid voor alles wat hij voor mij betekend heeft. Ik weet zeker dat hij het prachtig had gevonden, het hele promotie gebeuren.

Gelukkig heb ik altijd, maar voornamelijk ook nadat pa er niet meer was, veel liefde en steun gehad van m'n moeder en m'n broer. Om de andere zaterdag naar de visboer in Rockanje en samen gezellig eten is uitgegroeid tot een heerlijke traditie. Heel veel dank voor jullie gezelligheid, liefde en steun de afgelopen jaren.

Curriculum Vitae

Personal information

Name:	Marcel
Surname:	Kap
Date of Birth:	9 March 1974
Place of Birth:	Rockanje

Education

Hoger Laboratorium Onderwijs

HLO Delft, Biomedical/Zoological Techniques, 1992-1997

- Internship at the department of Immunology, Erasmus MC, 1996-1997

PhD research

Tissuebank/Tissue Research Support Unit, Department of Pathology, Erasmus MC, Rotterdam, The Netherlands, 2009-2014

Experience

TNO Immunological and Infectious Diseases, Leiden, The Netherlands
Research Technician, September 1997-December 1997

Lever Transplantatie Unit, Erasmus MC, Rotterdam, The Netherlands
Research Technician, 1998-2002

Immunohistochemistry Unit, Genmab B.V., Utrecht, The Netherlands
Research Associate, 2002-2003

Department of Pathology, Daniel den Hoed Cancer Clinic/Josephine Nefkens Instituut, Erasmus MC, Rotterdam, The Netherlands
Core Technician, 2003-2009

Department of Pathology, Josephine Nefkens Building, Erasmus MC Cancer Institute, Rotterdam, The Netherlands
PhD candidate, 2009-2014

Technical Manager CQPath Ltd. 2008-present

PhD Portfolio

Name PhD Student:	Marcel Kap	PhD period:	2009-2014
Department:	Pathology	Promotor:	prof. dr. J.W. Oosterhuis (em)
Research School:	Molecular Medicine	Copromotor:	dr. P.H.J. Riegman

1. Courses

Course	Date	ECTS
Molecular Diagnostics IV	May 28-29 2009	1.00
Biomedical Research Techniques VIII	Oct 12-16 2009	1.60
Basic and Translational Oncology	Nov 09-13 2009	1.60
BAGE V	Apr 28-29 2010	1.00
Course in Virology	May 31- Jun 4 2010	1.60
Partek Training Course	May 24-26 2011	1.20
Partek Training Course	Jun 12-14 2012	1.20

2. Presentations

Oral presentations

JNI lectures 2009, 2010, 2011, 2012	2.00
ISBER Annual meeting, Rotterdam, The Netherlands, 2010	0.50
ISBER Annual meeting, Bethesda, USA, 2011	0.50
Lecture at TATAA Biocenter, Prague, Czech Republic, 2011	1.00
Invited speaker at Dakocytomation User Meeting, Copenhagen, Denmark, 2012	1.00
ESBB Annual meeting, Granada, Spain, 2012	0.50

Poster presentations

PhD-day, 2010	0.25
ESP Annual Congress, Helsinki, Finland, 2011 (2 posters)	0.50
BRN Meeting, Bethesda, USA, 2012	0.25
ESBB Annual meeting, Granada, Spain, 2012	0.25

3. Teaching activities

2011 (sep/oct)

VO 2A1 introductie epitheliale tumoren	14 uur	0.50
VO 2A9 kleincellig en niet-kleincellig longcarcinoom	14 uur	0.50

2012 (sep/oct)

VO 2A1 introductie epitheliale tumoren	14 uur	0.50
VO 2A5 diagnose stadiumbepaling kanker	14 uur	0.50

2013 (sep/oct)

VO 2A1 introductie epitheliale tumoren	14 uur	0.50
VO 2A5 diagnose stadiumbepaling kanker	14 uur	0.50

2013 (jan/aug)

Practical supervisor fourth year elective research program (A. van der Linden)	4.00

Total ECTS **22,95**

Supplemental tables Chapter 1

Table S1. Discovery cohort sample data.

Sample number	AROS RIN	EMC after transport RIN	Patient number	Gender	Age	Time point	Duration of resection (T0-T1)	Excised segment	Steatosis	Dilation	Fibrosis
1	7,3	7	1	F	48	T0	1:45 hours	8	0	2	0
2	6,5	6,4	1	F	48	T1	1:45 hours	8	0	2	0
3	6,7	6,5	1	F	48	T2	1:45 hours	8	0	2	0
4	7,0	6,9	1	F	48	T3	1:45 hours	8	0	2	0
5	6,2	6,1	1	F	48	T4	1:45 hours	8	0	2	0
6	6,7	6,5	1	F	48	T5	1:45 hours	8	0	2	0
7	6,4	6,3	1	F	48	T6	1:45 hours	8	0	2	0
8	6,9	6,6	2	F	74	T0	20 minutes	7+8	1	1	1
9	6,6	6,4	2	F	74	T1	20 minutes	7+8	1	1	1
10	5,7	5,6	2	F	74	T2	20 minutes	7+8	1	1	1
11	7,2	7	2	F	74	T3	20 minutes	7+8	1	1	1
12	6,1	6,3	2	F	74	T4	20 minutes	7+8	1	1	1
13	6,0	5,8	2	F	74	T5	20 minutes	7+8	1	1	1
14	7,7	7,3	3	F	58	T0	1:48 hours	right half	3	1	0
15	7,8	7,6	3	F	58	T1	1:48 hours	right half	3	1	0
16	8,0	7,5	3	F	58	T2	1:48 hours	right half	3	1	0
17	7,7	7,4	3	F	58	T3	1:48 hours	right half	3	1	0
18	7,9	7,4	3	F	58	T4	1:48 hours	right half	3	1	0
19	8,2	7,8	3	F	58	T5	1:48 hours	right half	3	1	0
20	7,7	7,6	3	F	58	T6	1:48 hours	right half	3	1	0
21	6,5	6,2	4	M	60	T0	2:44 hours	right half	2	2	0
22	7,3	7	4	M	60	T1	2:44 hours	right half	2	2	0
23	6,4	6,2	4	M	60	T2	2:44 hours	right half	2	2	0
24	6,3	6	4	M	60	T3	2:44 hours	right half	2	2	0
25	5,8	5,6	4	M	60	T4	2:44 hours	right half	2	2	0

26	6,0	5,9	4	M	60	T5	2:44 hours	right half	2	2	0
27	3,5	2,9	4	M	60	T6	2:44 hours	right half	2	2	0
28	7,1	6,4	5	F	70	T0	2:05 hours	8	1	1	0
29	4,2	n.a	5	F	70	T1	2:05 hours	8	1	1	0
30	5,1	4,8	5	F	70	T2	2:05 hours	8	1	1	0
31	6,6	6,2	5	F	70	T3	2:05 hours	8	1	1	0
32	6,0	5,7	5	F	70	T4	2:05 hours	8	1	1	0
33	5,5	n.a	5	F	70	T5	2:05 hours	8	1	1	0
34	6,2	5,9	6	M	51	T0	1:33 hours	2	1	2	0
35	7,1	6,6	6	M	51	T1	1:33 hours	2	1	2	0
36	5,4	5,1	6	M	51	T2	1:33 hours	2	1	2	0
37	3,3	n.a	6	M	51	T3	1:33 hours	2	1	2	0
38	5,0	5,1	6	M	51	T4	1:33 hours	2	1	2	0
39	5,6	5,9	6	M	51	T5	1:33 hours	2	1	2	0
40	5,7	5,3	6	M	51	T6	1:33 hours	2	1	2	0
41	7,2	6,9	7	M	53	T0	2:03 hours	6	3	1	1
42	7,9	7,7	7	M	53	T1	2:03 hours	6	3	1	1
43	7,6	7,4	7	M	53	T2	2:03 hours	6	3	1	1
44	6,9	6,7	7	M	53	T3	2:03 hours	6	3	1	1
45	7,9	7,6	7	M	53	T4	2:03 hours	6	3	1	1
46	7,3	7,3	7	M	53	T5	2:03 hours	6	3	1	1
47	6,2	6	8	F	58	T0	2:42 hours	4	0	3	2
48	5,9	5,9	8	F	58	T1	2:42 hours	4	0	3	2
49	5,2	5	8	F	58	T2	2:42 hours	4	0	3	2
50	6,5	6,3	8	F	58	T3	2:42 hours	4	0	3	2
51	3,9	3,9	8	F	58	T4	2:42 hours	4	0	3	2
52	4,6	4,4	8	F	58	T5	2:42 hours	4	0	3	2
53	7,0	6,6	8	F	58	T6	2:42 hours	4	0	3	2
54	6,6	6,2	9	M	63	T0	2:45 hours	5+6	1	1	0
55	6,5	6,2	9	M	63	T1	2:45 hours	5+6	1	1	0
56	5,3	5,3	9	M	63	T2	2:45 hours	5+6	1	1	0
57	7,1	6,7	9	M	63	T3	2:45 hours	5+6	1	1	0
58	6,3	5,8	9	M	63	T4	2:45 hours	5+6	1	1	0
59	7,0	6,5	9	M	63	T5	2:45 hours	5+6	1	1	0
60	6,2	5,9	9	M	63	T6	2:45 hours	5+6	1	1	0

Steatosis: 0 = none, 1 = less than 1%, 2 = less than 5% and 3 = >20%
Dilation: arbitrary observation 0 = none, 1 = hardly , 2 = mild, 3 = severe
Fibrosis: 0 = none, 1 = starting lobular structures and 2 = strong lobular structures, possible cirrhosis
n.a. = not assessed

Table S2. Sample data liver biopsies and excision samples

Needle biopsies			**Excision samples**		
N	RIN value	Diagnosis	N	RIN value	Diagnosis
1	7,7	HBV	1	8,7	HBV
2	6,9	HBV	2	8,5	Normal/unaffected
3	7,8	pseudo tumor	3	7,5	Normal/unaffected
4	7,0	HCV	4	7,8	Normal/unaffected
5	7,4	HBV	5	7,8	HBV/HCC
6	7,2	HBV/HCV	6	8,9	HBV
7	5,8	HCV	7	8,6	Colorectal cancer metastasis
8	7,2	HCV	8	7,7	HCC
9	6,6	AIH/PSC	9	9,2	HCC/Adenoma
10	6,9	Stacking disorder	10	6,9	HCC/Dysplasia
11	7,9	HBV	11	7,1	PSC
12	8,0	Cholestasis	12	6,9	Ischemia
13	7,2	HBV	13	8,6	Ischemia
14	8,3	HBV/HCV	14	7,6	Circulation disorder
15	8,1	HCV	15	7,8	Circulation disorder
16	8,1	Chronic hepatitis	16	7,0	Adenocarcinoma metastasis
17	8,3	HCV	17	8,0	HCV
18	7,5	HCV	18	8,2	HBV/HCC
19	7,3	AIH	19	9,0	Normal/unaffected
20	8,6	HBV	20	7,5	Circulation disorder
21	7,0	HCV	21	7,1	HCC

HBV; hepatitis B virus, **HCV**; hepatitis C virus, **AIH**; auto immune hepatitis,

PSC; primary sclerotizing cholangitis; **HCC**; hepatocellular carcinoma,

Table S3. Meta data of the *in silico* cohort

Institute	Sample type	N samples	RNA isolation method	Original CEL files
1	Donor liver	7	Trizol	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28619
2	Explant liver	10	Trizol	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38941
3	Wedge biopsies	4	Rneasy	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7117
4	Excision	10	Rneasy	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41804 (selection: minor non-tumor)
5	Excision	49	Rneasy	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40873
6	Excsion	3	unknown	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33006 (selection: adjacent normal liver)
7	Surgical biopsies	65	RNAlater/Trizol	http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40231 (selection: liver)

Sample group	Combined institutes	N samples
Biopsies	3 + 7	69
Transplant	1 + 2	17
Excision	4 + 5 +6	62

Table S4. Top 100 probe sets influenced by surgery

Rank	Probe set	Gene symbol	p-value	FDR	T0	T1
1	11763310_at	MIR21	0.0000	0.0000	10.365	-0.1356
2	11759135_x_at	NR4A1	0.0000	0.0000	11.215	-0.1562
3	11757908_x_at	RALGDS	0.0000	0.0000	0.9241	-0.1144
4	11757865_a_at	GADD45B	0.0000	0.0000	10.520	-0.1452
5	11757721_s_at	CSRNP1	0.0000	0.0000	11.708	-0.1541
6	11757367_s_at	HSPA6 /// HSPA7	0.0000	0.0000	0.9131	-0.1146
7	11757312_x_at	SERPINE1	0.0000	0.0000	13.737	-0.1876
8	11757115_s_at	SNORD14E	0.0000	0.0000	11.684	-0.1778
9	11756465_s_at	RND3	0.0000	0.0000	10.438	-0.1464
10	11756406_x_at	ADM	0.0000	0.0000	12.111	-0.1634
11	11756358_a_at	PLK3	0.0000	0.0000	12.879	-0.1731
12	11756316_a_at	CHI3L1	0.0000	0.0000	0.9841	-0.1144
13	11755653_a_at	RFX2	0.0000	0.0000	0.9430	-0.1145
14	11754662_s_at	ADAMTS1	0.0000	0.0000	13.072	-0.1804
15	11754026_a_at	IL8	0.0000	0.0000	0.9334	-0.1165
16	11753823_a_at	S100A8	0.0000	0.0000	0.9637	-0.1123
17	11753427_a_at	RND3	0.0000	0.0000	11.034	-0.1560
18	11753131_x_at	TM4SF1	0.0000	0.0000	10.211	-0.1372
19	11753130_at	TM4SF1	0.0000	0.0000	0.9838	-0.1291
20	11753129_a_at	TM4SF1	0.0000	0.0000	10.301	-0.1371
21	11751914_s_at	ELF3	0.0000	0.0000	11.358	-0.1478
22	11750411_x_at	HSPA1A /// HSPA1B	0.0000	0.0000	12.971	-0.1666
23	11749905_a_at	CYR61	0.0000	0.0000	12.153	-0.1711
24	11748180_a_at	SERPINB8	0.0000	0.0000	11.220	-0.1508
25	11746954_s_at	CCL4 /// CCL4L1 /// CCL4L2	0.0000	0.0000	10.267	-0.1421
26	11746856_a_at	SERPINE1	0.0000	0.0000	10.270	-0.1385
27	11745724_at	MALAT1	0.0000	0.0000	0.8486	-0.1000
28	11745021_a_at	MYC	0.0000	0.0000	15.826	-0.2143
29	11745020_a_at	TNFRSF12A	0.0000	0.0000	11.597	-0.1577
30	11744751_a_at	SERPINB9	0.0000	0.0000	0.9751	-0.1266
31	11744413_x_at	HSPA1A	0.0000	0.0000	0.9723	-0.1262
32	11742752_a_at	ETS2	0.0000	0.0000	0.9657	-0.1273
33	11739797_a_at	RFX2	0.0000	0.0000	0.8273	-0.0958
34	11738053_x_at	PLAUR	0.0000	0.0000	11.779	-0.1597
35	11737952_a_at	RALGDS	0.0000	0.0000	0.8925	-0.1094
36	11732999_a_at	ICAM1	0.0000	0.0000	10.240	-0.1408
37	11730923_x_at	ADAMTS4	0.0000	0.0000	13.938	-0.1933
38	11729120_x_at	HSPA1B	0.0000	0.0000	14.108	-0.1831

39	11729119_a_at	HSPA1A /// HSPA1B	0.0000	0.0000	13.953	-0.1814
40	11729058_s_at	NR4A3	0.0000	0.0000	13.539	-0.1940
41	11728190_s_at	CXCR4	0.0000	0.0000	10.508	-0.1492
42	11726611_x_at	MAFF	0.0000	0.0000	11.411	-0.1638
43	11725056_a_at	NEDD9	0.0000	0.0000	0.9936	-0.1340
44	11724828_at	CCL20	0.0000	0.0000	10.522	-0.1387
45	11724037_at	PTGS2	0.0000	0.0000	11.365	-0.1581
46	11721308_at	PHLDA1	0.0000	0.0000	14.008	-0.1960
47	11721307_at	PHLDA1	0.0000	0.0000	16.111	-0.2203
48	11721306_at	PHLDA1	0.0000	0.0000	10.466	-0.1484
49	11721305_a_at	PHLDA1	0.0000	0.0000	17.884	-0.2455
50	11721092_a_at	THBS1	0.0000	0.0000	11.080	-0.1643
51	11721090_at	THBS1	0.0000	0.0000	11.361	-0.1630
52	11720029_a_at	LDLR	0.0000	0.0000	0.9786	-0.1297
53	11719899_at	LOC387763	0.0000	0.0000	11.532	-0.1622
54	11719366_s_at	CXCL1	0.0000	0.0000	11.371	-0.1585
55	11719344_a_at	ATF3	0.0000	0.0000	15.497	-0.2194
56	11719218_at	SOCS3	0.0000	0.0000	17.499	-0.2454
57	11718983_x_at	CCL4 /// CCL4L1 /// CCL4L2	0.0000	0.0000	11.016	-0.1568
58	11718982_s_at	CCL4 /// CCL4L1 /// CCL4L2	0.0000	0.0000	10.965	-0.1533
59	11718904_s_at	FILIP1L	0.0000	0.0000	11.708	-0.1698
60	11718841_s_at	IL8	0.0000	0.0000	17.857	-0.2228
61	11718397_s_at	JUN	0.0000	0.0000	11.124	-0.1508
62	11718396_x_at	JUN	0.0000	0.0000	12.076	-0.1615
63	11718395_s_at	JUN	0.0000	0.0000	13.179	-0.1740
64	11718394_at	JUN	0.0000	0.0000	13.121	-0.1735
65	11717994_a_at	NR4A1	0.0000	0.0000	15.305	-0.2109
66	11717594_s_at	ELF3	0.0000	0.0000	10.527	-0.1379
67	11717582_a_at	C13orf15	0.0000	0.0000	13.314	-0.1847
68	11717565_s_at	EMP1	0.0000	0.0000	13.073	-0.1777
69	11717345_a_at	FOSB	0.0000	0.0000	16.245	-0.2255
70	11717256_at	PIM1	0.0000	0.0000	10.146	-0.1363
71	11716710_a_at	ADM	0.0000	0.0000	11.647	-0.1541
72	11716384_at	CCL2	0.0000	0.0000	13.157	-0.1880
73	11715636_a_at	SERPINE1	0.0000	0.0000	12.284	-0.1668
74	11715493_a_at	CYR61	0.0000	0.0000	13.452	-0.1892
75	11732158_a_at	ADAMTS1	0.0000	0.0000	10.260	-0.1432
76	11722852_s_at	BIRC3	0.0000	0.0000	10.110	-0.1415
77	11722851_at	BIRC3	0.0000	0.0000	0.9974	-0.1377
78	11718905_x_at	FILIP1L	0.0000	0.0000	10.554	-0.1538
79	11760535_at	VNN3	0.0000	0.0000	0.9928	-0.1381

80	11727215_a_at	NRG1	0.0000	0.0000	0.8826	-0.1112
81	11725496_a_at	AGPAT9	0.0000	0.0000	0.9892	-0.1372
82	11719898_s_at	HBEGF	0.0000	0.0000	0.9847	-0.1352
83	11719754_s_at	IL1RN	0.0000	0.0000	0.9642	-0.1305
84	11715931_s_at	SGK1	0.0000	0.0000	0.9122	-0.1179
85	11753632_x_at	ATF3	0.0000	0.0000	10.466	-0.1575
86	11737750_s_at	SGK1	0.0000	0.0000	0.9030	-0.1165
87	11720558_a_at	GEM	0.0000	0.0000	10.324	-0.1525
88	11720028_x_at	LDLR	0.0000	0.0000	10.139	-0.1451
89	11717619_at	EPHA2	0.0000	0.0000	10.070	-0.1435
90	11750185_a_at	FLJ36031	0.0000	0.0000	0.9890	-0.1396
91	11717863_a_at	DUSP5	0.0000	0.0000	0.9345	-0.1253
92	11753631_a_at	ATF3	0.0000	0.0000	10.358	-0.1583
93	11746681_a_at	VNN3	0.0000	0.0000	0.9470	-0.1292
94	11733698_s_at	SGK1	0.0000	0.0000	0.9047	-0.1186
95	11718026_a_at	NCOA7	0.0000	0.0000	0.9805	-0.1379
96	11716049_a_at	TRIB1	0.0000	0.0000	10.214	-0.1515
97	11737278_at	CYP7A1	0.0000	0.0000	-0.7135	0.0772
98	11736555_s_at	SLC2A14 /// SLC2A3	0.0000	0.0000	0.9893	-0.1412
99	11717995_x_at	NR4A1	0.0000	0.0000	0.9489	-0.1299
100	11715487_a_at	MCL1	0.0000	0.0000	0.9994	-0.1446

Table S5. Top 100 probe sets with at least two-fold up/down regulation due to surgery

Rank	Parametric p-value	FDR	Permutation p-value	Geom mean of intensities T0	Geom mean of intensities T1	Fold-change	Probe set	Gene symbol
1	< 1e-07	< 1e-07	< 1e-07	123	1012.19	0.12	11729120_x_at	HSPA1B
2	1,00E-07	0.00067	< 1e-07	145.93	1106.35	0.13	11750411_x_at	HSPA1A /// HSPA1B
3	1,00E-07	0.00067	< 1e-07	11.87	24.05	0.49	11729607_at	PGM2
4	1,00E-07	0.00067	< 1e-07	205.31	1593.33	0.13	11729119_a_at	HSPA1A /// HSPA1B
5	2,00E-07	0.000766	< 1e-07	2184.07	5590.32	0.39	11757907_x_at	HSPA1A
6	2,00E-07	0.000766	< 1e-07	59.5	22.88	2.6	11736765_at	C14orf28
7	2,00E-07	0.000766	< 1e-07	721.16	3401.83	0.21	11744413_x_at	HSPA1A
8	7,00E-07	0.00235	< 1e-07	18.56	102.51	0.18	11753427_a_at	RND3
9	8,00E-07	0.00238	< 1e-07	34.96	13.39	2.61	11735717_a_at	ZNF226
10	9,00E-07	0.00241	< 1e-07	56.94	250.39	0.23	11725496_a_at	AGPAT9
11	1.2e-06	0.0029	< 1e-07	302.59	451.36	0.67	11719046_a_at	SNRK
12	1.3e-06	0.0029	< 1e-07	50.94	28.55	1.78	11758163_s_at	ZFP62
13	1.5e-06	0.00306	< 1e-07	33.29	14.28	2.33	11741856_s_at	LOC653501 /// ZNF658 /// ZNF658B
14	1.6e-06	0.00306	< 1e-07	24.69	73.34	0.34	11718503_a_at	RP5-1022P6.2
15	1.9e-06	0.00322	< 1e-07	462.49	1363.46	0.34	11737760_s_at	RND3
16	2.1e-06	0.00322	1,00E-04	185.09	402.06	0.46	11731574_at	FRMD4B
17	2.3e-06	0.00322	< 1e-07	174.99	117.61	1.49	11717366_at	ZC3HAV1
18	2.6e-06	0.00322	< 1e-07	326.69	452.73	0.72	11717866_s_at	PSME4
19	2.8e-06	0.00322	< 1e-07	55.3	24.11	2.29	11758424_s_at	UNC119B
20	2.9e-06	0.00322	< 1e-07	36.63	118.09	0.31	11745724_at	MALAT1
21	3,00E-06	0.00322	1,00E-04	105.83	350.9	0.3	11758889_a_at	FAM46A
22	3.1e-06	0.00322	< 1e-07	420.92	690.69	0.61	11756210_a_at	RCL1
23	3.1e-06	0.00322	< 1e-07	33.59	161.99	0.21	11756465_s_at	RND3
24	3.3e-06	0.00322	< 1e-07	13.81	44.23	0.31	11759765_at	PLSCR1
25	3.5e-06	0.00322	< 1e-07	122.57	86.9	1.41	11758638_s_at	MKL2
26	3.7e-06	0.00322	< 1e-07	26.55	112.34	0.24	11727215_a_at	NRG1
27	3.7e-06	0.00322	< 1e-07	79.48	133.05	0.6	11744445_a_at	DUSP14
28	3.8e-06	0.00322	< 1e-07	492.39	1762.61	0.28	11743136_x_at	CEBPD
29	3.8e-06	0.00322	< 1e-07	95.82	290.19	0.33	11715673_x_at	JUNB
30	4.1e-06	0.00322	< 1e-07	78.54	480.55	0.16	11756406_x_at	ADM
31	4.1e-06	0.00322	< 1e-07	47.77	25.06	1.91	11727040_at	MAVS
32	4.3e-06	0.00322	< 1e-07	16.89	54.18	0.31	11740347_x_at	NRG1
33	4.4e-06	0.00322	< 1e-07	138.61	209.72	0.66	11756233_a_at	AKIRIN2
34	4.5e-06	0.00322	< 1e-07	19.02	63.22	0.3	11740346_a_at	NRG1
35	4.6e-06	0.00322	< 1e-07	38.16	59.07	0.65	11740031_a_at	DUSP14
36	4.6e-06	0.00322	1,00E-04	37.97	89.75	0.42	11736478_a_at	ELL2

37	4.6e-06	0.00322	1,00E-04	69.16	257.44	0.27	11730235_a_at	MYADM
38	4.8e-06	0.00322	< 1e-07	334.27	976.71	0.34	11715482_a_at	MCL1
39	4.8e-06	0.00322	< 1e-07	56.35	98.22	0.57	11722796_a_at	MAPK6
40	4.8e-06	0.00322	1,00E-04	205.69	698.56	0.29	11723686_s_at	FAM46A
41	5.3e-06	0.00338	< 1e-07	113.09	61.54	1.84	11718565_at	KIAA1147
42	5.3e-06	0.00338	< 1e-07	901.38	2935.33	0.31	11743135_s_at	CEBPD
43	5.6e-06	0.00349	2,00E-04	1148.79	2992.36	0.38	11715483_s_at	MCL1
44	5.8e-06	0.00353	< 1e-07	102.38	65.26	1.57	11757695_s_at	ZNF512
45	6.3e-06	0.0037	< 1e-07	21.16	76.52	0.28	11715119_s_at	FAM148B
46	6.4e-06	0.0037	2,00E-04	58.09	214.65	0.27	11735128_a_at	TSC22D2
47	6.9e-06	0.0037	1,00E-04	68.47	533.62	0.13	11754662_s_at	ADAMTS1
48	6.9e-06	0.0037	< 1e-07	43.29	92.48	0.47	11717926_a_at	FOXK2
49	6.9e-06	0.0037	< 1e-07	59.95	589.87	0.1	11753823_a_at	S100A8
50	6.9e-06	0.0037	1,00E-04	13.45	74.55	0.18	11719898_s_at	HBEGF
51	7.5e-06	0.00385	1,00E-04	16.78	175.77	0.095	11730923_x_at	ADAMTS4
52	7.7e-06	0.00385	1,00E-04	41.29	136.09	0.3	11721543_a_at	CSRNP1
53	7.8e-06	0.00385	< 1e-07	88.1	161.08	0.55	11720532_s_at	C9orf72
54	7.9e-06	0.00385	< 1e-07	45.52	459.51	0.099	11717565_s_at	EMP1
55	7.9e-06	0.00385	< 1e-07	105.25	146.77	0.72	11757874_x_at	PFDN1
56	8.4e-06	0.00391	< 1e-07	13.35	132.72	0.1	11738053_x_at	PLAUR
57	8.4e-06	0.00391	< 1e-07	71.7	208.17	0.34	11757945_s_at	TP53BP2
58	8.7e-06	0.00391	< 1e-07	63.22	336.1	0.19	11716710_a_at	ADM
59	8.8e-06	0.00391	3,00E-04	51.92	267.04	0.19	11757721_s_at	CSRNP1
60	8.9e-06	0.00391	2,00E-04	20.22	299.09	0.068	11719218_at	SOCS3
61	8.9e-06	0.00391	2,00E-04	51.73	187.78	0.28	11723687_a_at	FAM46A
62	9.1e-06	0.00394	2,00E-04	23.33	134.32	0.17	11732158_a_at	ADAMTS1
63	9.5e-06	0.00395	< 1e-07	112.61	70.56	1.6	11723115_s_at	MTX3
64	9.6e-06	0.00395	1,00E-04	27.57	86.65	0.32	11758556_s_at	PGM2
65	9.7e-06	0.00395	2,00E-04	528.17	222.1	2.38	11740331_at	RTP3
66	9.8e-06	0.00395	< 1e-07	10.83	32.6	0.33	11746350_a_at	PIM1
67	1.01e-05	0.00395	1,00E-04	16.07	186.96	0.086	11729058_s_at	NR4A3
68	1.02e-05	0.00395	< 1e-07	29.72	18.01	1.65	11741074_s_at	C1orf156
69	1.06e-05	0.00395	< 1e-07	34.88	97.13	0.36	11720211_a_at	TP53BP2
70	1.07e-05	0.00395	3,00E-04	780.9	2552.72	0.31	11739000_a_at	SLC38A2
71	1.07e-05	0.00395	4,00E-04	184.63	594.94	0.31	11754933_s_at	TSC22D2
72	1.08e-05	0.00395	2,00E-04	100.74	201.45	0.5	11736477_a_at	ELL2
73	1.08e-05	0.00395	< 1e-07	131	284.75	0.46	11717892_a_at	PTP4A1
74	1.09e-05	0.00395	< 1e-07	16.77	11.3	1.48	11718825_a_at	FAM125B
75	1.12e-05	0.00397	2,00E-04	65.89	244.73	0.27	11748213_a_at	TSC22D2
76	1.13e-05	0.00397	2,00E-04	815.17	2517.39	0.32	11752869_s_at	CEBPD
77	1.14e-05	0.00397	1,00E-04	1009.51	3725.37	0.27	11759525_at	GADD45B
78	1.18e-05	0.00406	1,00E-04	33.2	74.99	0.44	11744289_a_at	JMJD6
79	1.21e-05	0.00406	< 1e-07	490.5	284.28	1.73	11718566_s_at	KIAA1147
80	1.21e-05	0.00406	1,00E-04	46.48	162.2	0.29	11746689_a_at	FAM46A
81	1.26e-05	0.00414	< 1e-07	52.06	35.34	1.47	11756637_a_at	RNF219

82	1.28e-05	0.00414	< 1e-07	25.64	216.52	0.12	11716523_at	S100A9
83	1.28e-05	0.00414	< 1e-07	42.05	198.98	0.21	11732234_a_at	RND1
84	1.32e-05	0.00416	< 1e-07	115.96	79.93	1.45	11719710_a_at	PDPK1
85	1.33e-05	0.00416	< 1e-07	238.43	142.67	1.67	11718789_x_at	ZNF302
86	1.34e-05	0.00416	< 1e-07	61.12	139.23	0.44	11719261_s_at	LMNA
87	1.37e-05	0.00416	< 1e-07	206.23	119.78	1.72	11755756_a_at	NHSL1
88	1.37e-05	0.00416	5,00E-04	277.25	515.98	0.54	11718164_at	PPP1R15B
89	1.43e-05	0.00416	1,00E-04	71.63	187.4	0.38	11723685_a_at	FAM46A
90	1.44e-05	0.00416	< 1e-07	136.17	102.46	1.33	11756199_a_at	ATMIN
91	1.44e-05	0.00416	1,00E-04	747.69	2602.98	0.29	11715691_s_at	ZFP36
92	1.45e-05	0.00416	< 1e-07	210.7	367	0.57	11739303_a_at	DLC1
93	1.45e-05	0.00416	< 1e-07	77.99	37.76	2.07	11727039_at	MAVS
94	1.46e-05	0.00416	3,00E-04	44.69	34.39	1.3	11724333_a_at	USP8
95	1.5e-05	0.00419	3,00E-04	90.52	369.72	0.24	11742752_a_at	ETS2
96	1.51e-05	0.00419	< 1e-07	89.86	50.9	1.77	11757795_s_at	RCOR3
97	1.52e-05	0.00419	< 1e-07	61.39	40.77	1.51	11737955_a_at	C15orf40
98	1.53e-05	0.00419	< 1e-07	27.57	137.79	0.2	11717256_at	PIM1
99	1.55e-05	0.0042	1,00E-04	207.72	620.31	0.33	11730236_s_at	MYADM
100	1.58e-05	0.00424	1,00E-04	41.52	77.82	0.53	11749208_a_at	ELL2

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Cover: Time is a major pre-analytical factor and in time, histology will be replaced by sequencing techniques.

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