Master Thesis

Quality Control from Day 1: determining a long-term quality control panel for a neurodegenerative diseases biorepository

Submitted by

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Sworn Declaration

I declare on my honor that I have written this dissertation independently and without assistance, that no sources other than those cited were used and that the sources used verbatim or in substance have been marked as such.

Bonn, 2023 Cristina Hagmann

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And last but not least, my family: Now you can see what all my computer video calls were about and why I got to visit Graz not just once, but twice. Your support, your humor ("*another* thesis"?) and your cheerleading ("my mom works with robots but they don't really do what she tells them to") brightened every day.

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Abbreviations and Explanations

BDNF – Brain-derived neurotrophic factor

BIMS – Biorepository Information Management Software

CD8 – cluster of differentiation 8

cDNA – copy/complementary DNA

cfDNA – cell-free DNA

CRP – C reactive protein

CSF – cerebrospinal fluid

DELFIA – dissociation-enhanced lanthanide fluorescence immunoassay

DZNE – German Center for Neurodegenerative Diseases

ELISA – enzyme-linked immunosorbent assay

EMSA – electrophoretic mobility shift assay

FACS – fluorescence activated cell sorting

GC – gas chromatography

gDNA – genomic DNA

HDL – High density lipoprotein

HEK 293 – human embryonic kidney cells

HISCL – high sensitivity chemiluminescent enzyme immunoassay

HPLC – High-performance liquid chromatography

HspA1A – Heat shock protein A1A

IARC – International Agency for Research on Cancer

IL-6, 18 – Interleukin 6, 18

IMR – immunomagnetic reduction

ISBER - International Society for Biological and Environmental Repositories

LC-MS – liquid chromatography – mass spectrometry

LDL – Low density lipoprotein

LN_2/LIN – Liquid Nitrogen

miRNA – micro RNA

Nano-CLIA – nano chemiluminescence immunoassay

OECD – Organisation for Economic Co-operation and Development

PBMC – Peripheral Blood Mononuclear Cells

PCR – polymerase chain reaction

PKC – Protein Kinase C

PSA – prostate specific antigen

QMS – Quality Management System

qtPCR – quantitative PCR

ROM – reactive oxygen metabolites

RT-PCR – reverse transcription PCR

scRNA – single cell RNA sequencing

SPIDIA – Standardisation and improvement of generic pre-analytical tools and procedures for in vitro diagnostics

TGF β – Transforming Growth Factor beta

TNFα – Tumor Necrosis Factor alpha

Treg – regulatory T cells

WBC – white blood cells

WHO – World Health Organisation

Glossary

ELISA – enzyme – linked immunoassay. Used to detect presence of molecules via specific antibodies, performed on coated plates. Readout performed in a plate reader

Fluorometric assay – laboratory technique to monitor the kinetics of enzyme reactions via fluorescence emission

Preanalytical variables – variables that can influence biosample quality prior to biosample testing

qPCR or RT-PCR – real-time polymerase chain reaction. This method monitors the amplification of a particular DNA molecule during the PCR procedure

Quality Control – collection of procedures or workflows within an company or department with the aim to maintain or attain adherence to a defined set of quality criteria

Vitrification – method to cryopreserve cells avoiding harmful ice formation

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Zusammenfassung

Neue und belastbare Erkenntnisse in der Medizin und Forschung beruhen auf intrinsische Art und Weise auf der Verfügbarkeit von hochqualitativen Proben, deren Ergebnisse reproduzierbar sind.

Die Lagerung von Bioproben in dafür vorgesehenen Lagerorten (Gefrierschränken oder Stickstofftanks) ist ein Bestandteil einer solchen Qualitätssicherung der Proben.

Das DZNE Biorepository ist in seiner Mission, als Dienstleistungseinrichtung für die Wissenschaftler:innen des Forschungsinstitutes eine reine Probenlagerung anzubieten, deshalb in der einzigartigen Lage, zusätzlich zum Service der Probenlagerung in Eigenarbeit Biorepository-interne Proben einzulagern, diese an genau definierten Zeitpunkten zu entnehmen und mit vorher festgelegten Assays auf Degradation oder Abbau bestimmter Merkmale und Biomarker zu testen.

Das Ziel dieser Arbeit ist es, mit Hilfe von Feedback der Forscher:innen des Institutes und aktuellen wissenschaftlichen Erkenntnissen ein Qualitätssicherungspanel zu erstellen. Dieses Panel soll aus einem oder mehreren Biomaterialien bestehen, welche auf die vorherrschende Lagertemperatur abgestimmt sind. Die Entnahmefrequenzen zum Testen werden im Abgleich mit dem Ergebnis der Forscher:innenbefragung und der Literaturrecherche festgelegt. Die Assays sollen einen eventuellen Abbau oder Anstieg von Biomarkern spezifisch widergeben können; so sollen auch die Biomarker so ausgewählt werden, dass sie sensibel auf Temperaturunterschiede und Freeze-Thaw Zyklen reagieren. Als letztes Kriterium zur Panelerstellung müssen natürlich die Ressourcen des Biorepository einfließen – so sind personelle, finanzielle und zeitliche Ressourcen nicht unendlich, umso mehr als dass dieses Panel der reinen internen Qualitätskontrolle dienen soll.

Nach Forscher:innenbefragung und Literaturrecherche kommt diese Arbeit zum Schluss, dass ein Panel in monatlichen Abständen Proben der humanen Zelllinie HEK293 entnehmen und auf Veränderungen des Proteins HspA1A testen wird. Als paralleler Readout werden Proben humanen Serums auf Harnsäure getestet. Diese Assays werden über 5 Jahre absolviert, mit dem erwarteten Ergebnis, dass Ausfälle der Lagerorte sich in der Probenqualität niederschlagen. Die genauen Testprotokolle sollen der Biobanking-Community zur Verfügung gestellt werden.

Abstract

New and reliable findings in medicine and research are intrinsically dependent on the availability of high-quality samples yielding reproducible results.

The storage of biosamples in designated storage locations (ultra-low temperature freezers or nitrogen tanks) is part of the quality assurance process of the samples.

In its mission to offer the scientists of the research institute high-quality sample storage, the DZNE biorepository is in the unique position to store biorepository samples for test purposes (i.e. not stored for clients or customers) in the biorepository, in addition to the service of sample storage. This allows to retrieve them at precisely defined times and to test them with predetermined assays for decay or degradation of certain traits and biomarkers.

The aim of this work is to create a biorepository sample-based quality assurance panel with the help of feedback from the institute's researchers and current scientific knowledge. This panel should consist of one or more biomaterials that are tailored to the prevailing majority sample storage temperature. The sampling frequencies for testing are determined in comparison with the results of the researcher survey and the literature research. The assays should be able to specifically reflect any reduction or increase in biomarkers; the biomarkers should also be selected in such a way that they react sensitively to temperature differences and freeze-thaw cycles. The last criterion for panel creation is of course to consider the resources of the biorepository as well – staff, financial and time resources are not infinite, all the more so since this panel is intended for pure internal quality control only.

After interviewing researchers and researching the literature, this work comes to the conclusion that the best suited panel will consist of samples of the human cell line HEK293, retrieve them at monthly intervals and test them for changes of the protein HspA1A. As a parallel readout, samples of human serum are to be tested for uric acid. These assays will be completed over 5 years, with the expected result that storage location failures will be reflected in sample quality. The exact test protocols are to be made available to the biobanking community.

Introduction

Introduction to the DZNE Biorepository and justification of research question

The German Center for Neurodegenerative Diseases (DZNE) is planning to offer a central biosample storage service to its researchers and clinicians. I was tasked to establish such a Biorepository.

Before expanding on this, a quick comparison between biorepositories and biobanks is in order:

A biobank is a *"structured collection of biological samples and associated data"* (1).

Biobanks handle and store tissues from humans, animals, cell and bacterial cultures. Importantly, they also handle the information pertaining to their biosamples, including (in the case of human samples) lifestyle, demography, illness and clinical history, or (generally speaking) date of collection, genetic makeup, any assays run with the sample or preanalytical variables prior to storage. Biobanks are then able to communicate this data appended to any samples requested by researchers or clinicians.

In the case of the DZNE Biorepository, it was clear from its conception that the goal was not to establish a biobank; rather, the Biorepository is planned to supply a service of storing the samples researchers and clinicians had collected and generated. The only data the DZNE Biorepository was to obtain from the researchers sending in the samples was the code on the vial and the temperature at which the vial was to be stored. This is particularly useful when considering data protection and patient confidentiality laws. Thus, the DZNE Biorepository was planned as a pure storage facility for samples of DZNE researchers and clinicians – and not as a biobank storing samples and data to be delivered to those scientists asking for such samples/data.

The status of the Biorepository when I started working there was purely hypothetical with many different stakeholders delaying the project to an extent that not even the storage temperature could be agreed upon. The requirements regarding the Biorepository were finally summarized as follows in Table 1 (see also Figure 1):

Table 1: Requirements for a central DZNE Biorepository
- Available to all DZNE researchers, with 10% of storage capacity reserved for external clients
- Automated at LN_2 temperatures, with temperatures never going above -130°C (vitrification point of water)
- Housed as securely and safely as possible (considering environmental, technological and human risk factors)
- Sustainable – service must come with a price that reflects the cost of the Biorepository to the institute
- Available in a timely manner: sample requests and sample storage needed to be done with a turnaround time of 4000 samples per work day
- An interim storage needed to be made available to researchers and clinicians for the duration of Biorepository setup

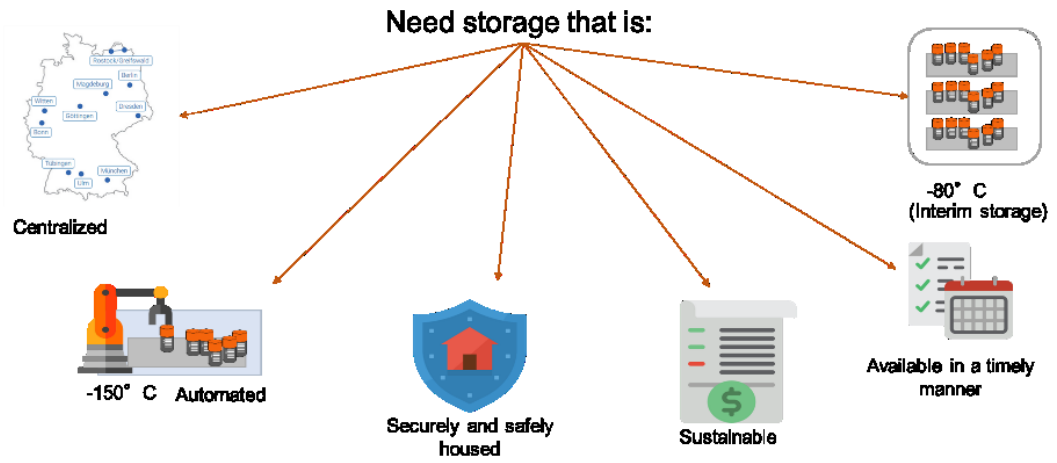

Fig. 1 and Table 1: Main requirements for Biorepository storage parameters. Clinicians and scientists requested storage that was safe, secure, dependable, prompt and easily available. The Biorepository also needed to be sustainable and provide interim storage until operability could be provided (Icons made by Freepik from www.flaticon.com).

It was my job to bring everyone to the table to establish a budget, timeline, scientific and managerial requirements, organizational positioning, and administrative directives.

The main objective of the Biorepository was therefore the development of a state-of-the-art automated cryogenic Biorepository Facility at the DZNE in Bonn, making biospecimens collected from DZNE sites/projects available to support translational fundamental, clinical and population research throughout the DZNE and with external partners.

We now have a new building nearing completion, awarded tenders for the Biorepository Information Management Software (BIMS) and the automated LN_2 storage system and are currently busy nailing down everything else. As the DZNE Biorepository is not a typical biobank, the samples entrusted to us come with very little information and the directive to only retrieve samples for the researcher who submitted them in the first place. This, however, does not preclude rigorous quality control of our facility, including quality control of our sample storage setup.

Briefly, the plan is to store a large amount of identical samples on the first day the Biorepository can actively store samples, then to remove three samples per storage location (LN_2 tank or freezer) per month and run as yet to be determined assays with them. It is my goal to assemble a panel of predetermined material (serum vs plasma, urine vs PBMCs, murine vs human cell lines, tumor vs primary cells, and so on) and settle on two or three highly standardized assays (IL-6, PKC, TNFα) that are cost-efficient and user-friendly to run. Performing the same standardized tests and obtaining the corresponding readouts of such a tightly controlled process on a monthly basis over the next two decades will enable the Biorepository to offer unprecedented quality control going back to the first day of operability of the Biorepository.

In order to choose which assays and which material best reflects the samples we are storing, I aim to interview our main clients as to what kind of material they will be storing with us and, importantly, which assays they will run with their material (2). Following that, I will make a table of common materials and biomarkers used in freeze-thaw experiments in published literature and evaluate them as to their suitability for the DZNE Biorepository. Combining finding and reports from peer-reviewed scientific literature, client feedback, user experience questionnaires and biobank network best practice recommendations, this thesis will present viable sample-assay combinations for such a large-scale sample quality control approach.

Introducing the concepts of quality, cold chain, and biomarker degradation

As enticing it is to simply store as many samples as possible, only samples obtained in a standardized fashion and which yield reproducible results in assays are high quality samples worth storing and using for research, as irreproducibility results in research that cannot be translated into clinical applications (3,4). However, sample quality is a characteristic that is, unfortunately, beholden to many parameters (5). An

obvious countermeasure, is, of course, to standardize and monitor sample generation in its pre-analytical phase, as most quality issues have been traced to the time between sample generation and sample storage (6). Various organizations, from ISBER (International Society for Biological and Environmental Repositories) (7) to the OECD (Organisation for Economic Co-operation and Development) (8) and WHO/IARC (World Health Organisation and International Agency for Research on Cancer, respectively) (9), have put in great efforts to improve reliability and reproducibility of analytical data via (international) standardization of pre-analytical procedures. Fig. 2 below gives a brief overview of the numerous parameters (10) to be considered in the pre-analytical phase of sample management if consistently high sample quality is to be achieved.

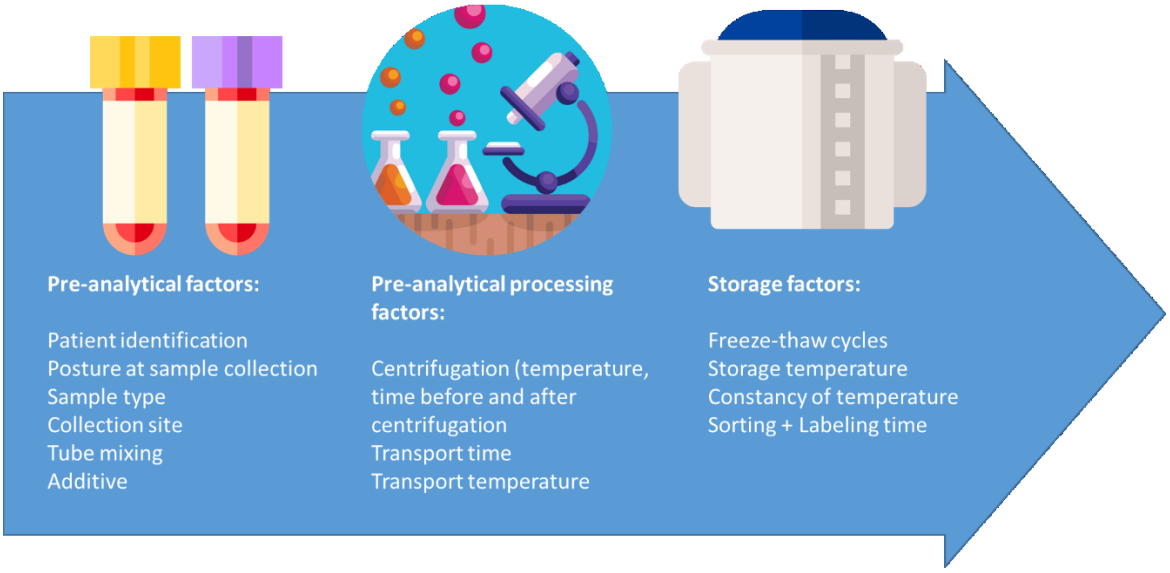

Fig 2: Selection of factors that influence sample quality in the pre-analytical phase (adapted from (10)).This thesis will concentrate on determining a test panel involving storage-associated factors influencing sample quality. (Icons made by Freepik from Flaticon.com)

Regarding quality-assured biobanking, specific workflows and processes are deemed to be essential, irrespective of biomaterial type, applying equally to both liquid and tissue biobanking (11). Documentation of sample-associated parameters is of utmost importance, as well as the communication of this data to the biobank. Examples of measures that contribute to high-quality samples are listed in Table 2:

Table 2: Procedures to ensure sample quality
- Clear identification of the sample and correct assignment to the donor
- Data on the sample material
- Collection time, location and type of collection
- Recording of clinical information as well as diagnostic and therapeutic measures
- Type and scope of the donor's declaration of consent
- Transport time and temperature
- Transport medium
- Frequency of transport
- Clear sample designation
- Integrity of the sample container
- Documentation of incoming temperature of the samples when cooled samples are received
- Appropriate encryption of the sample and donor data
- Adequate processing of the samples for proper storage (e.g. number of aliquots, type of sample containers) up to permanent storage (storage temperature, storage format, allocation of storage space, entry of associated patient and sample data)

Table 2: Procedures and documentation points to ensure sample quality for biobanks (12–15)

Broadly speaking, there should always be complete documentation of the sample history from sample collection, processing and storage to sample distribution. One option is to implement time stamps and the use of bar-coded sample tubes. The continuous temperature monitoring of the storage, registration of possible temperature fluctuations up to possible complete functional failures of the sample storage is also essential for ensuring a consistently high sample quality, so that - within the framework of an emergency management - an immediate reaction can be taken (12).

The abovementioned measures of bar-coded samples tubes, a software-aided timestamp system and continuous temperature monitoring, have all been documented in the quality mission statement of the DZNE Biorepository.

Conventionally, samples are stored at temperatures below -80°C because of sample degradation at higher temperatures (11,16).

Biomarkers are defined as

“A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (17)”.

These molecules found in biological samples can be independently measured and therefore be used to monitor for diseases and other pathologies, which makes them highly valuable research subjects. Temperature, however, is key. It has been shown that cytokine readout changes within even a two hour window following blood draw (18). Assays performed at multiple storage and temperature combinations (room temperature, 4°C) demonstrate the importance of temperature fidelity (16) at sample collection. Long-term stability, however, can only be attained at lower temperatures; even storage at -80°C results in degradation of some cytokines over the course of one year (19), two years and five years (20). RNA and DNA, which have come into their own regarding assay suitability and precision, were demonstrated to have high levels of stability even when stored for 10 years at liquid nitrogen temperatures (21).

These findings, combined make an urgent case for maintaining the temperatures samples are stored at in order to avoid degeneration and reduced quality. Once a sample has been collected and prepared, avoiding freeze-thaw cycles and suboptimal storage temperatures is mandatory (22,23). Nevertheless, sample quality even prior to storage, albeit a factor out of the DZNE Biorepository’s realm of responsibility, remains paramount ensuring overall sample quality.

Overview of conventional sample quality assurance methods

Documenting sample collection parameters and sample storage parameters as extensively as possible are the main contributors to sample quality when data is concerned (24). Of course, preserving and optimizing biosample integrity itself in order to enable scientifically sound research results is the guiding star of sample management (25).

While aiming for the greatest level of standardization and documentation is highly beneficial to sample quality, in the case of the DZNE Biorepository, the samples of the customers themselves are not to be handled beyond storage, retrieval and possible disposal, as the Biorepository is most emphatically not considered a biobank. This

signifies that any and all pre-storage parameters and conditions are not within the realm of the DZNE Biorepository. The stipulations are merely to communicate to the DZNE Biorepository how the samples are to be stored and to deliver the samples in standardized vials to enable automated handling.

This means that conventional quality assurance measures regarding sample collection and pre-analytical phases are not part of the DZNE Biorepository's quality assurance protocol and cannot be altered. This leaves a smaller window of processes for the DZNE Biorepository to ensure sample quality (see Fig. 3):

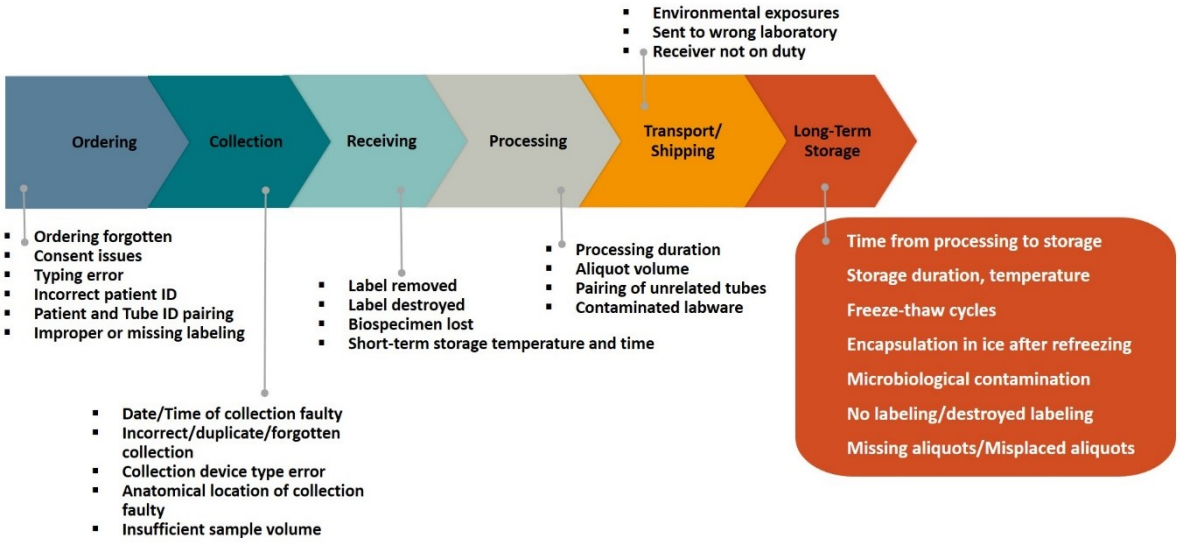

Fig. 3: DZNE Biorepository's scope of control to assure sample quality with regards to preanalytical variables. The DZNE Biorepository will be able to control for the variables in the dark orange box.

Demonstrating the urgent need for a sample quality control panel as explored in this thesis

Quality control is being brought to the forefront in biobanking circles. With biobanking leaving the realm of niche academia, both for providers and requesters of samples, quality control, audit trails and certifications are gaining more and more traction when considering sample provenance (reviewed in (14,26)). One of the resulting developments is the focus on pre-analytical variables regarding sample quality. The field of biospecimen research aims to solidify and cement the knowledge surrounding this highly complex area influencing experimental reproducibility. Closely associated and intrinsically relevant to sample quality and reproducibility, the factors of process optimization and validation or equipment qualification are much less publicized, even

though their contribution to consistency is no less vital. Among others, UK Biobank (27) and Biobank Graz (28) have been implementing manufacturing and SOP standards to monitor and optimize sample processing. Regarding biobank hardware validation and performance qualification (ISO 11139:2018), only a single conference abstract was found ((29), abstract QAC18).

Understandably, with the wide range of storage options, types of material to store, and intended readouts upon sample retrieval, this thesis will aim to present a quality control panel tailored to the specific DZNE Biorepository needs.

Low-temperature storage research is not a novel research field. Its origins date back to the 1800s ((30), reviewed in (31)). Early setbacks in sample thawing could be explained in the 1950s with the discovery of osmotic stress during the process (32). Current knowledge has established incremental temperature change during the freezing or thawing process, together with selection of appropriate cryoprotectant agents, as most favorable for high survival rates of cryopreservation (31). Halting the cellular metabolism at storage temperatures of -196°C is one of the main reasons the DZNE Biorepository has been chosen to concentrate their services at cryostorage temperatures below -130°C and not at -80°C.

As a DZNE Biorepository, we will not be analyzing biosamples entrusted to us as to their viability upon retrieval, which is why we will commence DZNE Biorepository-internal quality control panels with assays to be presented in this thesis.

This comes with the abovementioned advantage that the quality-control panels investigated in this thesis aim to be easily repeatable by all sample storage institutions, not just biobanks. This includes service providers with less immediate access to relevant biosamples, be it because they are not affiliated with a hospital or funding issues with regards to ethics committees.

Of course, norms and whitepapers have already been published with regards to minimizing preanalytical variability, ISO 15189 and ISO 20387 among them (33),(34). One of the most crucial factors regarding sample quality, time, is however understandably only measurable with significant investment in all the relevant resource parameters of a sample storage service facility: storage time, sample storage space, sample quality readout method, relevant data collection and storage, as well as

personnel. Which is why attempting a long-term, large-scale storage quality readout approach such as outlined in this thesis, aims to serve as a kind of template, with benchmark values at selected time points for other institutions.

Hypothesis and Aims

This master thesis hypothesizes that biosamples and biomarkers within those samples can serve a purpose beyond a research or clinical assay a biosample is used for. It is posited that biosamples and their markers, when paired with the appropriate assays, can be used to document and chronicle the temperature of storage locations over a long period of time.

This master thesis aims to test this hypothesis by searching biomedical literature databases for publications documenting biomaterial and biomarkers of interest and pairing these finding with responses from an exploratory questionnaire submitted to research group leaders from the institute. The goal then is to find a panel combining a documented assay with a relevant biomarker and planning a setup that will suit the DZNE Biorepository's needs.

Materials and Methods

Client questionnaire and evaluation

The DZNE Biorepository will not handle client samples beyond storing and retrieving them. Nevertheless, it is wise to implement a quality control panel that reflects the sample types and readouts generated by the clients themselves. To this end, I initiated a two-pronged approach to generate a wide dragnet of possible sample/readout combinations that would suit the DZNE Biorepository needs and resources: an exploratory client questionnaire and a systematic literature review of relevant cell types and assays.

Survey Respondents

In order to obtain an overview of client needs regarding DZNE Biorepository storage services, an exploratory questionnaire (see Annex Item 1) was generated and distributed by email to all 36 research group leaders of the institute (15 junior group leaders, 21 senior group leaders). Responses were graphed and evaluated.

Data analysis

As this was an exploratory questionnaire, no correction method was applied to the data set. Only completed surveys were included. One round of reminder emails was sent out to group leaders who had not replied, which resulted in additional completed questionnaires. These were not analyzed separately.

Systematic literature review

Pubmed and Embase were searched for studies (both observational and interventional) with the keywords biomarkers, long-term storage, low temperatures, and stability (for search strategy see figure 4); no publication date restrictions were entered. After removal of duplicates and screening, 63 full-text articles were sought. An additional 16 articles were removed from consideration due to assay use, storage duration or cell type. This left 47 articles to analyze with regards to assay and cell type. Of these 47, the assay costs and run time were compared and contrasted to determine the best fit for the DZNE Biorepository.

Data items

Articles were sorted according to which biomarkers were analyzed, which assay types were used, which sample types were selected for analysis, the storage duration available and the storage temperature of the samples used.

- Author, year
- Biomarker
- Assay type
- Sample type
- Storage duration
- Storage temperature

Search strategy

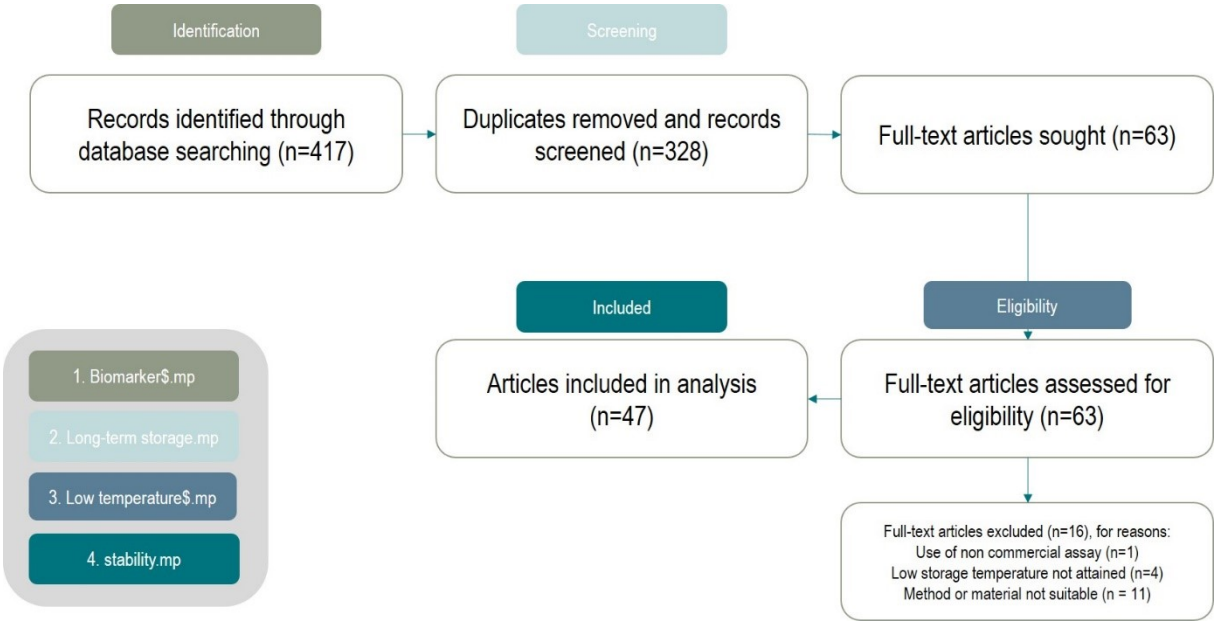

Fig. 4: Search strategy flowchart.

Results

Client questionnaire results

Response rate

The questionnaire (see item 1 in annex) was assembled following informal inquiries with interested members of the institute (Fig. 5).

Briefly, scientists were asked to consider their storage needs and their assay types with the biosamples stored in a DZNE Biorepository facility. The aim was to use this feedback and current published assay options (see Literature research results section below) to find a quality control panel that would best suit the DZNE Biorepository resources.

36 questionnaires were sent out via email, with a reminder email sent out after 14 days.

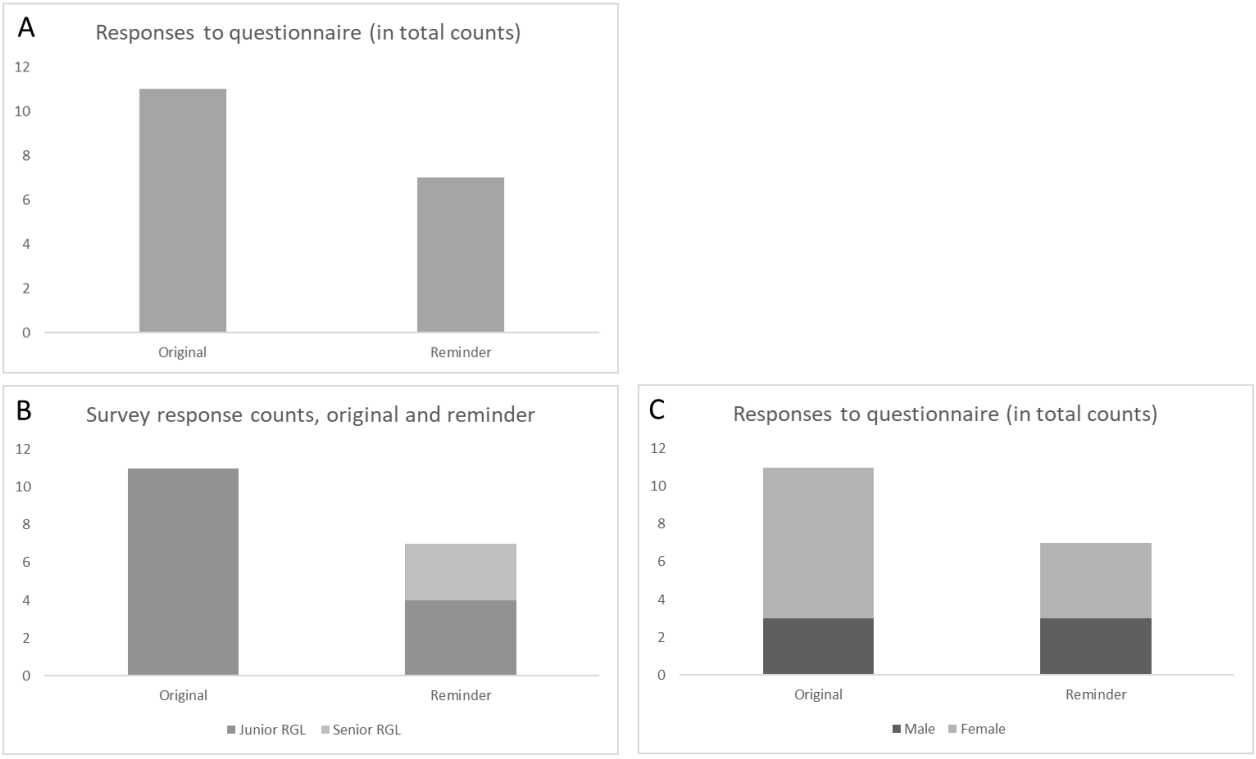

Fig. 5a: Questionnaire response rate (in total counts). 36 questionnaires were sent out via email, with a reminder email sent out after 14 days. In total, a 50% response rate could be obtained, with n=18 for total responses. b. Questionnaire response rate (in total counts), split between junior and senior research group leaders, with n=11 for the response to the original email and n = 4 for the junior research group leader response and n = 3 for the senior group leader to the reminder email. c. Questionnaire response count split between female and male responders and original and reminder emails.

Questionnaire response analysis: sample storage needs

The first question was to gauge the number of samples the group leaders planned to store for their research groups (Fig. 6). It was important to differentiate between samples stored (i.e. kept for later purposes, such as compliance with biosafety regulations or experiments planned for a later time point) versus samples kept for active research, as these were unlikely to be entrusted to the DZNE Biorepository.

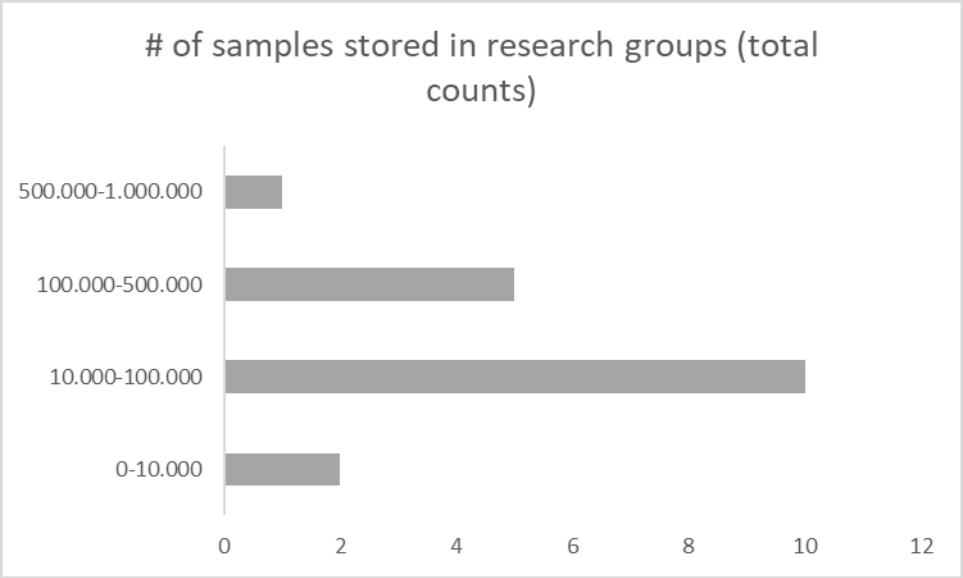

Fig. 6: Stored biosample number ranges (in total count numbers). 15 out of 18 respondents were storing between 10 000 and 500 000 samples outside of active experiments.

Of the completed questionnaires, more than 50% of the respondents were storing between 100 000 and 500 000 samples in addition to those actively being analyzed by their group members. Quality control panel considerations aside, this was encouraging, as storing even a fraction of these samples in the DZNE Biorepository would free up a large number of resources for the research groups, both space- and personnel-wise. This feedback signified that any quality control panel by the DZNE Biorepository could justifiably encompass a minimum of 10 000 samples as, based on questionnaire responses, this was a sample size reflective of almost 90% of research group sample storage cohorts.

Respondents were then asked about sample vial volumes (see Fig. 7). Vial size was dependent on the material, as not all material can provide sufficient substrate for certain assays at low sample volume. Readout for urine proteins, for example, requires a larger volume of urine than analyzing the gene copy number in certain cell types via qPCR.

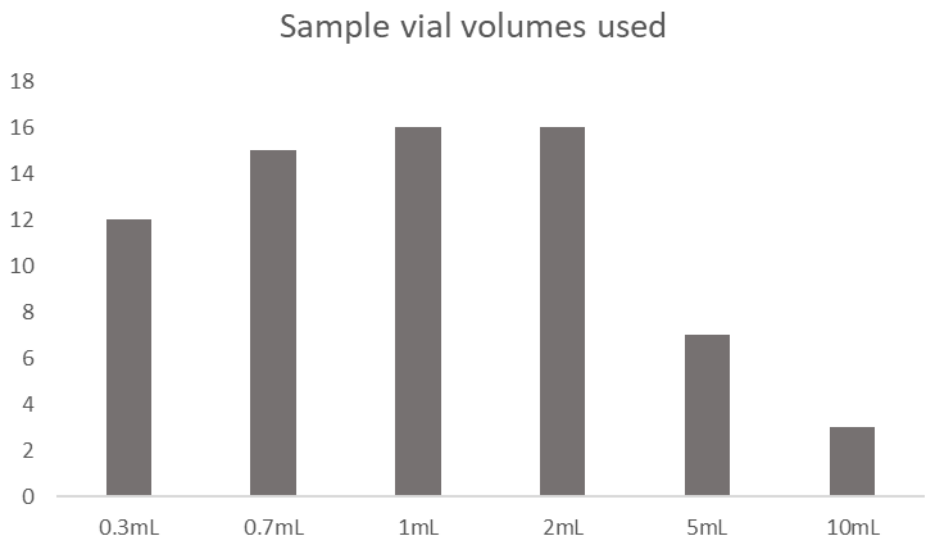

Fig. 7: Sample vial volumes in use by respondents of questionnaire (multiple choices available, total number of responses per option).

Based on the replies to the questionnaire, the quality control panel of the DZNE Biorepository would safely be able to consist of vials in the 0.7mL-2mL range, as more than 50% of respondents used vials of these sizes. Considering budget cuts and energy surcharges currently plaguing most, if not all, large-scale academic infrastructures in Germany, planning a quality control panel as tightly as possible to refrain from unnecessary purchases and storage space occupation is made significantly easier if the vials are 0.7mL vs 2mL or higher (see Table 3). A baseline amount of about 30 000 vials was assumed in order to reflect all the storage locations of the DZNE Biorepository and n=3 for each time point per storage location. For further information on these numbers, please see section ”Required assay parameters regarding DZNE Biorepository equipment”.

Vial Size	Freezer space needed	Operational costs/month
0.7mL	2 shelves	12€
2mL	3 shelves	18€
5mL	5 shelves	30€

Table 3: Operational costs difference (electricity, 2023 values) for storing 30 000 vials for a quality control panel in -80°C freezers. The DZNE Biorepository uses Stirling freezers with a capacity of 75 000 0.7mL vials and 6.67kWh/day energy consumption. Each freezer is divided into 4 horizontal shelving units. Assay costs are not considered here, as assay choice will be discussed at a later time point in this thesis.

Researchers were also asked about their sample storage needs once the samples were no longer in active experimental use (Fig. 8). This question served a dual purpose – one, simply, to ascertain the storage requirements of potential clients, while the secondary reasoning was to determine the time frame of the DZNE Biorepository quality control panel setup. If a large percentage of customers were aiming to work with and retrieve samples after 5+ years, then it would stand to reason that the quality control panel of the DZNE Biorepository should reflect this fact. Results from such a panel would enable assay readouts to be minutely traceable back to the very first month of the panel setup.

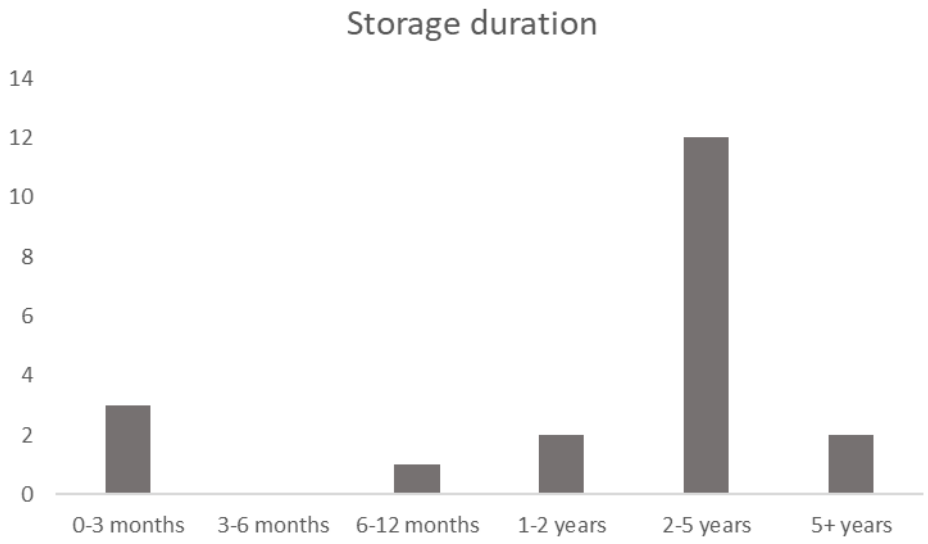

Fig. 8 Storage duration of samples not actively used in experiments. Respondents were asked how long they intended to store samples no longer (or not yet) required for active experimentation. Y axis is number of replies with that answer selected. Multiple replies were not allowed. Most samples are estimated to remain in storage between 2-5 years, with a few very short-term samples only remaining in storage for very few months, and some researchers requiring their samples to be stored for 5 years or more.

Researchers were given 6 options to choose the length of storage duration; from 0-3 months, 3-6 months, 6-12 months, 1-2 years, 2-5 years and over 5 years. These intervals were chosen to represent storage times for research samples as widely as possible. As biomarkers, and specifically neurodegenerative disease biomarkers, have been shown to be highly stable over years and even decades when conserved properly, adding a time interval of 5+ years (35,36) was, unsurprisingly, also chosen by 2 researchers. Very short-term storage (under 6 months) is not done to test

biomarker stability but more along the lines of generating the samples and then testing them in batches when enough samples of the desired characteristics can be tested together. As this option was also selected, however, starting to test the DZNE Biorepository samples from day 1 will not only give an excellent baseline value but also be able to pick up deviations (preanalytic or source-internal) otherwise not explainable by long-term storage artefacts.

The DZNE Biorepository will offer storage at vapor phase LN_2 (between -196°C and -135°C) and -80°C temperatures, with lesser space dedicated to -20°C, 4°C and room temperature storage. The decision was made to invest in automated sample storage at LN_2 temperatures. Regarding the quality control panel setup, however, the aim is to find a biological material that works for both main temperatures communicated by the questionnaire responses (see Fig. 9). The options given were 20°C, 4°C, -20°C, -80°C, LN_2 (vapor phase) and LN_2 (liquid phase), with expected replies to heavily concentrate on -80°C and vapor LN_2.

Precisely pinpointing the ranges at which the quality control panel should be stored is intrinsically important. Storage conditions are a large factor in protecting biological materials and pharmaceuticals, as well as ensuring that quality (and therefore reproducibility) remains consistent. A short overview of the most popular sample storage temperatures that were included in the questionnaire is listed below.

- *Ambient temperature*: While room temperature is not recommended for materials with molecular information that needs to be analyzed, some types of preserved samples (FFPE, DNA blots) remain stable at room temperature for a long time.
- *Refrigerated storage:* Refrigerated storage (controlled and monitored to remain at or around 4°C) is not suited for long-term storage, but can be used for short term storage of materials such as certain cell culture media, some enzymes and selected antibodies. These substances risk to have irrevocable changes to their structural integrity if subjected to freezing and thawing processes.
- Frozen storage at -20°C: If they are suspended in the correct media, -20°C is well suited for storage of RNA and DNA (37).
- Frozen storage at -80°C (ultra-low freezer): At the DZNE Biorepository, samples stored in ultra-low temperature freezers are controlled and monitored to remain

between -80° and around -75°C, with some other facilities aiming for a temperature interval of -70°C to -90°C. Storage at these temperatures was the first kind of long-term storage widely available, as degradation for many types of biological materials (e.g. antibodies, enzymes, robust cell lines, nucleic acids) can be prevented. A crucial factor to consider before storing is, of course, the method of reaching the storage temperature and, upon retrieval, thawing the substance as gently as possible.

- Vapor phase LN_2: Storage in LN_2 tanks usually brings with it the option to store in the LN_2 vapor phase (between -196° and -135°C) versus storing the materials completely submerged in liquid LN_2. This makes for easier access to the materials and is safer for the person retrieving the vials, but does result in higher temperature fluctuations (albeit never above -130°C, if LN_2 supply is not compromised). All biological activity is stopped at these temperatures, which enables cells frozen correctly to be retrieved and used in cell culture months or even years after their storage date.
- Liquid phase LN_2: with the introduction of vapor phase LN_2 storage tanks, liquid phase LN2 storage has seen a steep decline in popularity. Nevertheless, some researchers prefer storing their materials in liquid phase LN_2 due to constraints in lab space (38). One crucial point to consider before submerging samples in liquid phase LN_2 is contamination – certain viruses (39) have been shown to infect individuals simply because they were stored in the same tanks as materials the individuals later came in contact with. The DZNE Biorepository does not offer storage in liquid phase LN_2, but nevertheless included the option in the exploratory questionnaire.

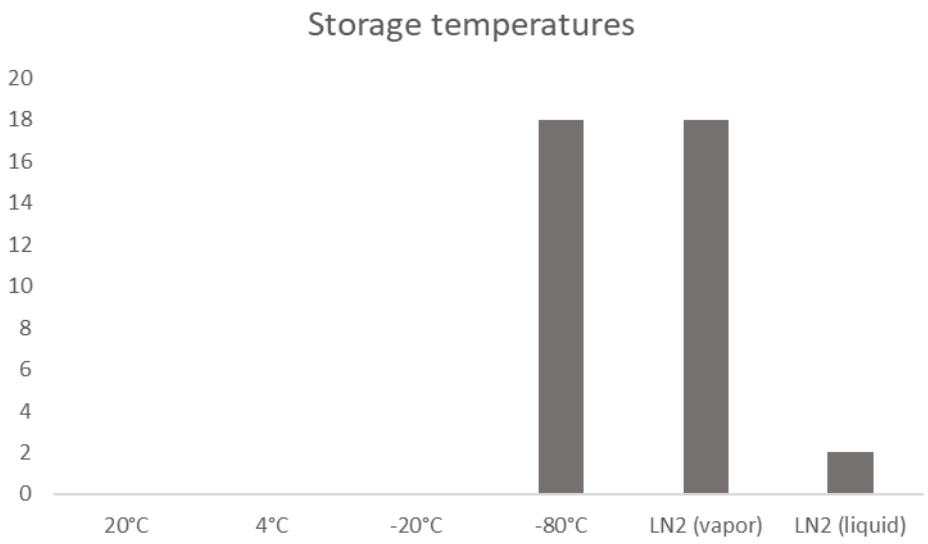

Fig. 9: Storage temperatures. Respondents were asked at which temperatures they planned to store or stored their samples. The Y axis represents the number of times an answer was selected, with multiple answers possible. As all respondents selected both -80°C and LN_2 (vapor) storage, with two respondents also opting for the LN_2 (liquid) answer, a biological material adapted to storage at these temperatures was required.

As seen in figure 9, the storage needs were mostly concentrated on the -80°C and vapor phase LN_2 storage temperature ranges, with two respondents also noting storage in liquid phase LN_2. This was within the realm of the storage options offered by the DZNE Biorepository.

Questionnaire response analysis: sample readout needs

Aside from the storage needs, the questionnaire also contained various questions regarding the readouts performed by the research groups on samples stored. This part of the questionnaire yielded very scattered responses, not only due to the diversity of research fields at the institute, but also because one of the questions had no answers filled out. In figure 10, the biomaterial sources were asked, as the groups at the institute work with anything from *drosophila* to nematodes to clinical studies to experiments in mice.

Neurodegenerative diseases, the focus of the host institute of the DZNE Biorepository, have become more and more prevalent around the world, with the success in treating diseases that would otherwise have prevented the afflicted individuals from reaching an age at which the neurodegenerative disease becomes pathological.

Unfortunately, most of the diseased investigated (e.g. Huntington's, Alzheimer's and Parkinson's) do not have any therapy in place that goes beyond treating and ameliorating the symptoms of the disease in question. This is why the broad approach to biomaterials and other biological substances mimicking the disease in different organisms or experimental in vitro setups is so vital to the understanding of disease progression and treatment (reviewed in (40)). Storing the biomaterials in the best way possible to ensure that any new discoveries are reproducible and applicable to the current clinical state of treatment is a key principle for the DZNE Biorepository.

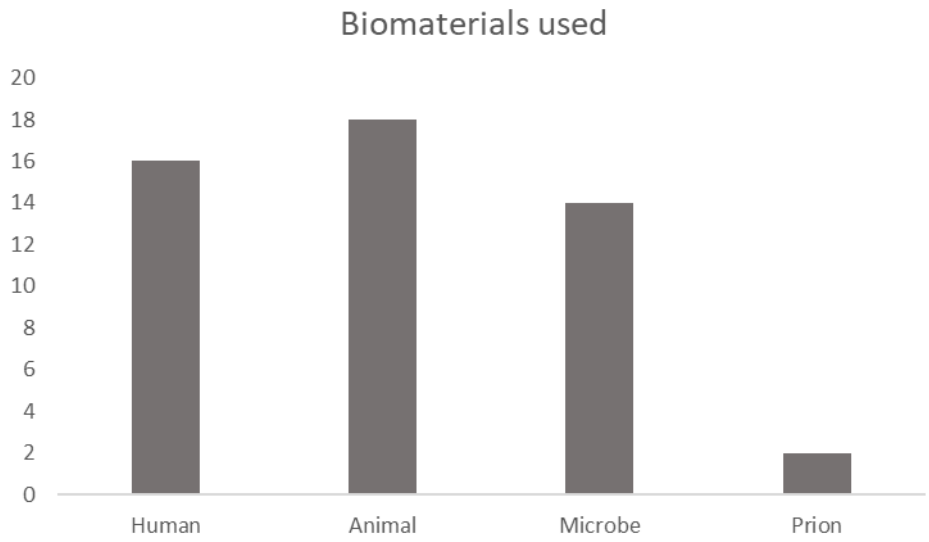

Fig. 10: Biomaterial source: Researchers were given the option to select multiple responses regarding their source material for their samples. The Y axis represents the number of responses selecting each option. All respondents (18/18) used some type of animal material, although the questionnaire did not ask them to specify which species.

After asked to supply the source species for their biomaterials, the answers to the questionnaire indicated that while all respondents used animal source material in their biosamples to some capacity, a large number of researchers also used human material in their experiments. As the availability of tests and assays based on human biomaterial is overwhelmingly extensive, the choice to concentrate on readouts based on human biomaterial was made. This is also advantageous in light of two large research focal points at the institute, which are population-based studies and clinical research groups.

As an added bonus, with the feedback of microbial and prion biosamples also being used in some capacity, the decision was made to upgrade the DZNE Biorepository to biosafety level 2 status in order to be adequately equipped to handle storage of infectious (41,42) and genetically modified samples. Dedicated tanks and freezers will be implemented in order to avoid any cross-contamination at this level.

The researchers were next asked to supply their 5 main readouts used with human biosamples in their group; as expected for open-form questions, the responses were extensively varying (Fig. 11a). No distinct trend could be discernible, apart from the focus on RNA and Viability as readout types. Viability is understandably a highly used readout when storing cell lines and primary cells cryogenically, as this is an indicator for a successful storage process. The multiple responses for RNA were also understandable, as RT-PCR is a widely used method for determining up- or downregulation of gene expression and discovering new transcription products (43,44).

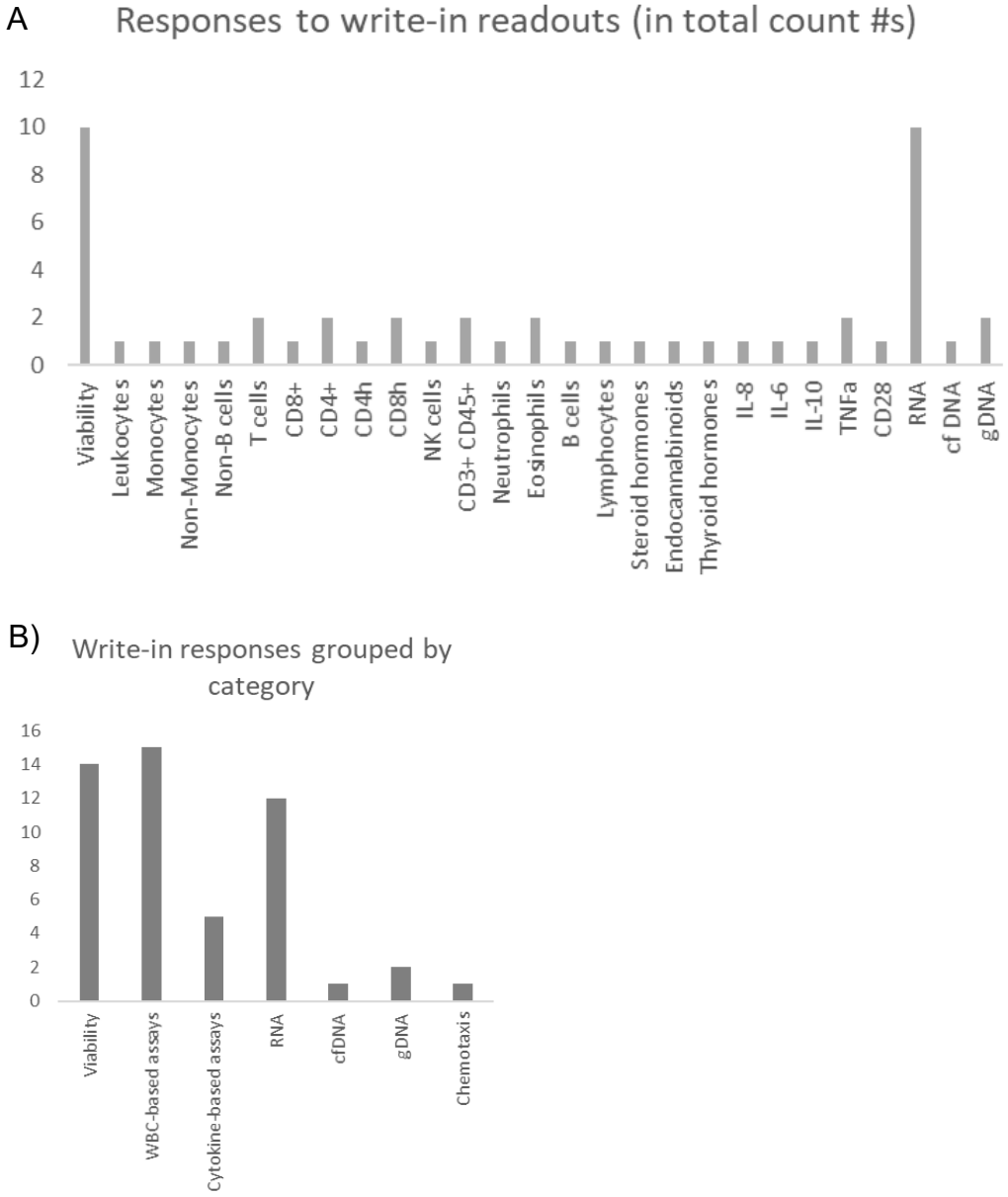

Fig 11: Write-in responses to readout requests. A) Unfiltered responses to the write-in request for readout choices, in total number of replies. Each response got out of the total amounts of questionnaires received. IL-6, Interleukin-6; TNFa, Tumor Necrosis Factor alpha; CD 8+, Cluster of differentiation 8. B) The responses were grouped into 7 main categories, with viability and RNA remaining on their own. Y axis represents the total number of replies mentioning readouts in a specific category; WBC, white blood cell; cfDNA, cell-free DNA; gDNA, genomic DNA.

In a next step, the widely varying replies were grouped into categories and compared to the main responses of RNA and cell viability assays (reviewed in (45)). This could nicely show that while in absolute numbers, white blood cell-based assays were selected more often than cell viability readouts, the sheer breadth of white blood cell populations and the high price did not make this option a promising candidate to take

into further consideration when assembling a cost-efficient panel. Nevertheless, using a subset or population of white blood cells in a readout was not entirely disregarded and would be considered further on when evaluating the results from the literature review.

When asking the researchers about their current readout technology used, irrespective of source material, the results were not quite as striking (Fig. 12). The replies indicated that most readouts were used by at least half of the respondents with no standouts uniquely discernible.

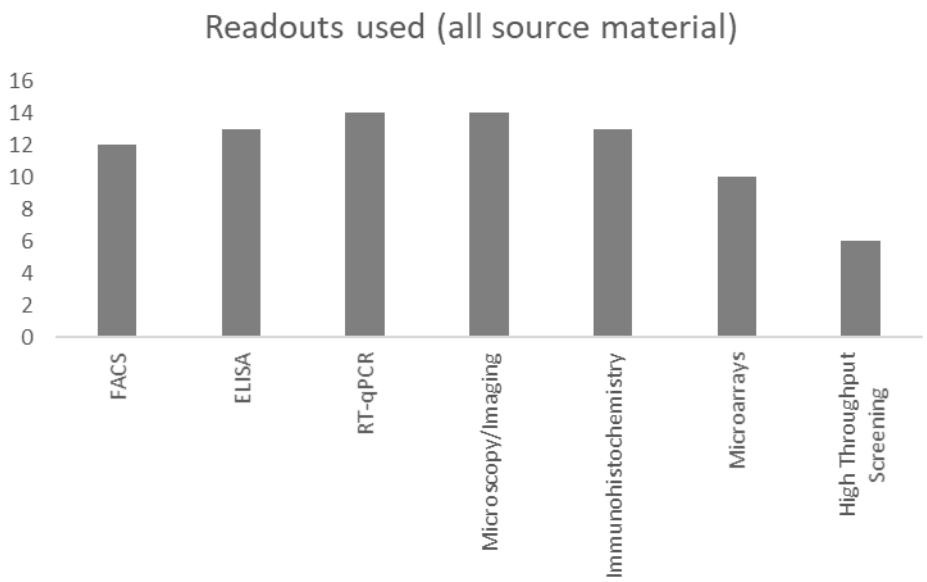

Fig. 12: Readouts used for all source biomaterial, species-independent. The Y axis represents the total number of replies to the question. As expected for a neurodegenerative diseases institute, microscopy and other imaging techniques figure prominently in the response landscape.

In summary, the questionnaire was supremely helpful. It supported the plan to start measuring and evaluating quality control samples from the beginning of the storage period. The sample numbers stored by the respondents to the questionnaire also aided in the planning of the internal quality control panel because the DZNE Biorepository would by far not be storing an unnaturally high number of biosamples when compared to the numbers stored by institute researchers. This would allow the DZNE Biorepository to possess a pool of identically treated samples from which to retrieve samples periodically and measure them via a readout that must be chosen after evaluating current literature and protocols. The readouts selected by the respondents for human biosamples skewed in direction of cell viability and RT-PCR assays, which

will be taken into consideration upon further evaluation of the available readouts on the market. The second factor to use for assembly of a quality control panel is the result of a literature search focused on finding the best assay for the DZNE Biorepository needs, as will be presented in the following section.

Literature research results

In this section, the results of the literature search and analysis are presented. The goal of the search strategy was to identify candidate biomarker assays that would translate well into the everyday DZNE Biorepository processes and protocols. To this end, the biomarkers would have to be shown to be sensitive to temperature changes when freezing and thawing, the assays needed to be time and cost efficient, as well as not subject to the risks of niche availability in 2-5 years' time.

There is an abundance of review articles describing long-term biomarker stability results (16,46–50). The reason for the separate approach performed in this thesis is precisely to find an assay and a substrate that would fit as well as possible to be performed to this specific DZNE Biorepository's needs. With no particularly significant research budget to speak of and no access to native blood, as the DZNE Biorepository is not associated with any clinical trials or hospital entities, budget and feasibility for this approach were not to be dismissed.

Explanation of Literature search strategy and terms

The DZNE Biorepository was conceived primarily as a helpmeet for two of the institute's most prolific sample generating departments, with one of them, a population sciences study, established as a 30-year enterprise. This of course allowed for the unique opportunity to implement a quality control experiment with similarly long run times. While the primary objective of the literature search was not to find a biomarker assay that demonstrated storage times of the exact same duration, nevertheless "long-term storage" was one of the chosen keywords.

As the DZNE Biorepository offers storage of biosamples at LN_2 or -80°C temperatures, it also followed to search available literature for publications concentrating on biomarker assays of samples stored at these parameters, which is why "low temperature" was added to the query strategy.

The quality control test procedure needs to occur with biomarkers that do not degrade easily – the point of the test is to be able to follow biomarker stability over time, with a

change in expression or readout occurring upon thawing. Of course, no biomarker will remain completely unfazed by years' worth of storage; however, aiming for biomarker assays with stable readouts is why the keyword "stability" was added to the query.

And finally, the quality control protocol by manner of regular readouts of DZNE Biorepository-internal samples could also be attempted with non-biomarker setups such as an assortment of liquid suspensions whose rheological properties change over time/when subjected to fluctuating temperatures, but as the DZNE Biorepository is part of a biological sciences research institute, searching for biomarkers was the stated goal, with "biomarkers" the fourth keyword in the search strategy.

Electronic searches with the abovementioned keywords were performed. After screening the abstracts, 63 full-text articles were sought (see Annex Item 2).

Analysis of search results: materials used for assays

As a preliminary overview of the search results, the materials used in the publications were listed. These are presented in Figure 13. A short explanation for how the results were used to further narrow the selection of publications is given below.

- An expected heavy emphasis on the traditional materials usually available and amenable to sample storage was confirmed, with 56 out of 84 total different material mentions being grouped in the blood byproducts list: native blood, whole blood, dried blood, an assortment of sera and plasma configurations, as well as PBMC (peripheral blood mononuclear cells) and Treg (regulatory T cells) subsets. With commercial suppliers available for materials such as pooled sera, this material remains one of the front-runners in the QC panel search.
- Even though highly niche materials such as nanobodies and nanozymes (51–56) are highly intriguing for possible future assays, they are unfortunately not a viable option for the current projected endeavor and the publications using these were struck from the list (Table 4).
- The niche and/or difficult to procure materials such as CSF (cerebrospinal fluid), eye fluid and lung effluent were in themselves unlikely candidates for use, but the assays remained of interest, so this category of material was retained in the assay search list.

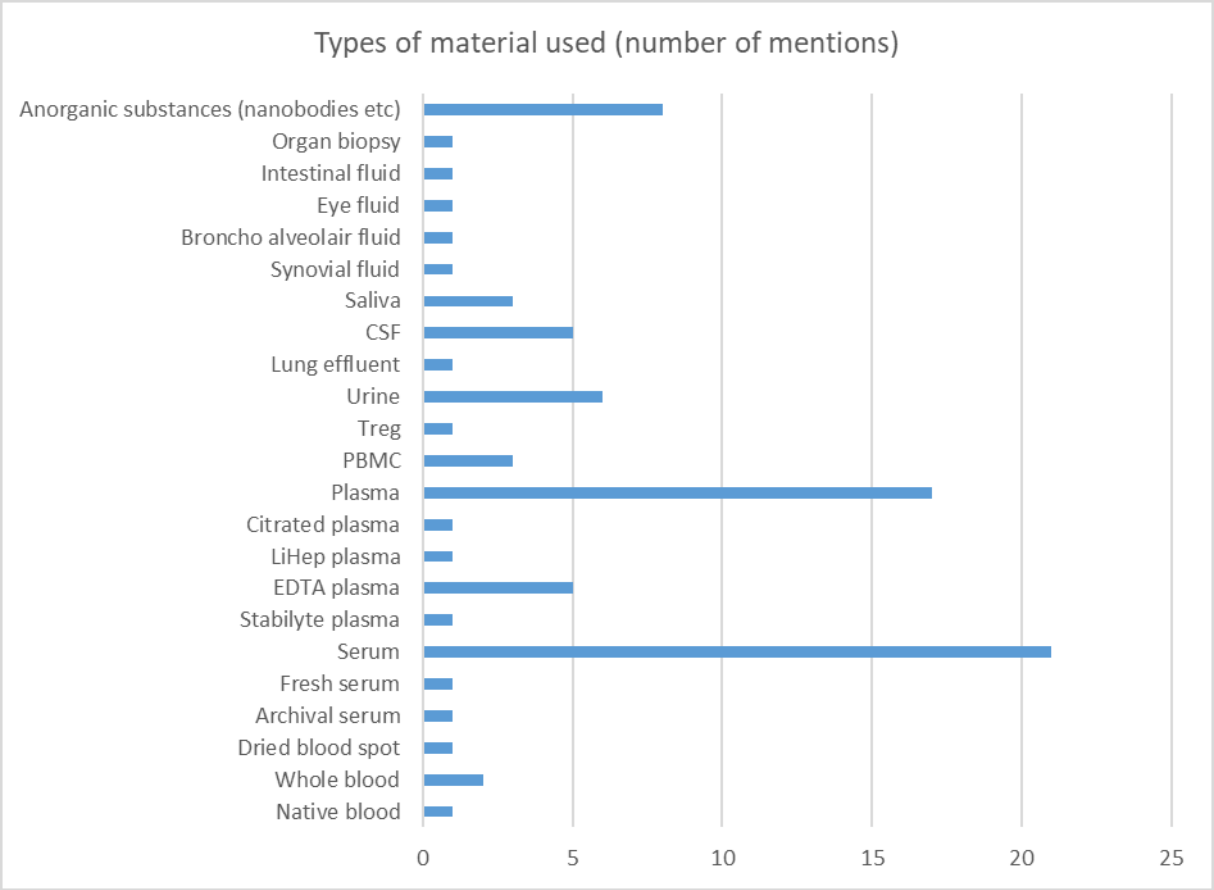

Figure 13: Materials used in publications for preliminary full-text screening. A total of 84 different material mentions were documented spread out over 63 publications. Certain materials are not feasible for the large-scale assay setup considered for the DZNE Biorepository, nevertheless of interest with regards to assay used (to be discussed further on).

Analysis of search results: methods used for assays

The next parameter to be analyzed was the type of method used for the assay in the publication (Figure 14). While certain methods are undeniably attractive because of the wealth of information they yield (microarrays (57) come to mind), this is unfortunately not one of the criteria for an assay panel for the DZNE Biorepository.

The DZNE Biorepository panel needs to only work with one or two easily measurable parameters that can be extracted with as little hands-on time as possible. Selecting a method that would require each specimen to be measured individually or necessitates a long preparatory period for setting up the experiment would not be feasible with the DZNE Biorepository day-to-day activities.

The methods in Figure 14 range from the clinical chemist's workhorse ELISA (enzyme-linked immunosorbent assay) (35,36,55,58–71) to a proprietary use of a high sensitivity chemiluminescent enzyme immunoassay (HISCL).

Publication Nr.	Reason for removal
2.	Temperatures of -70°C or colder not attained
3.	Temperatures of -70°C or colder not attained
9.	PSA antigen assay
12.	Method not storage-associated
17.	Source material (acute kidney injury urine) difficult to obtain
21.	Review article
26.	Method paper on extracting miRNA, not measuring
30.	Review article
34.	Method/technology not feasible yet (nanozymes/nanobodies)
39.	Antibody generation
47.	Review article
49.	Use of proprietary assay documented for the first time
50.	Temperatures of -70°C or colder not attained
55.	Description of a p53 screening method
60.	Method/technology not feasible yet (nanozymes/nanobodies)
62.	Temperatures of -70°C or colder not attained

Table 4: List of publications removed from literature research with the reason appended as to why their methods and results were no longer considered. The numbers in the first column correspond to the numeration of the articles in Annex item 2.

Upon analysis of the pulled publications regarding their method of measuring biomarker presence, prevalence, or stability, a few intriguing options presented themselves that were not considered prior to this thesis.

The breadth of biomarkers measured via LC-MS (liquid chromatography – mass spectrometry) ranged not only from oxysterol, for measuring reactive oxygen species (72) to creatinine and thiocyanate for tobacco use indication (73), but an entire host of serum biomarkers (74). Particularly the biomarkers for cellular stress offer potential as panel candidates. LC-MS as a method would have the advantage of not being as time-sensitive as methods run with live cells and very sensitive compared to routine clinical UV measurement.

Methods such as FACS (fluorescence activated cell sorting) bring with them the unique possibility of using an entire cell population as a DZNE Biorepository panel component.

Freezing, then thawing cells and analyzing them with regards to their surface marker expression might work for setups in which only a few vials are to be measured at a time, but this is not the case with the proposed DZNE Biorepository panel.

Methods encompassing the possibility of running tens or even hundreds of tests at the same time are of course of special interest, so setups such as RT-PCR and qPCR with 96-well or larger plates and the added benefit of only needing a minute amount of sample were a welcome option to be considered as part of the DZNE Biorepository panel.

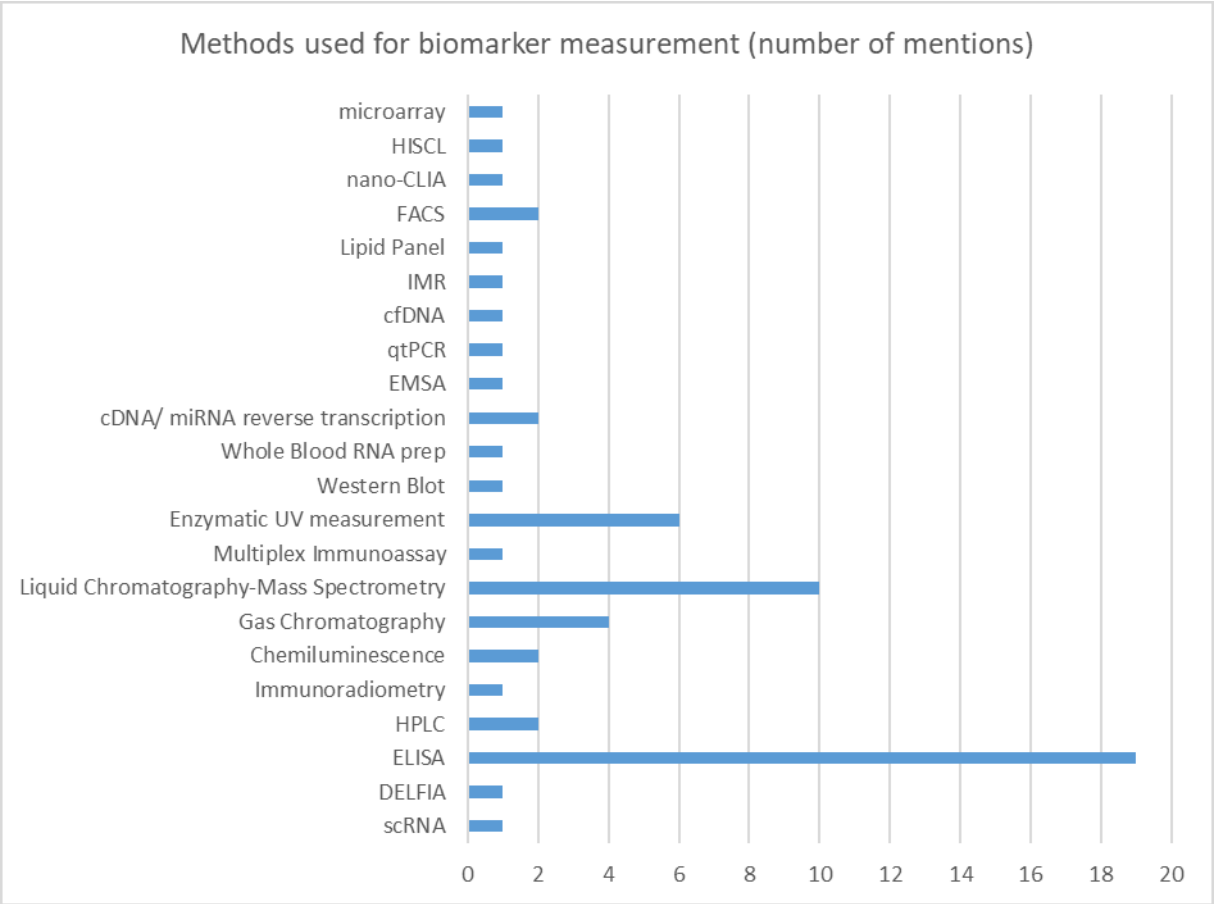

Fig. 14: Methods used for biomarker measurement as found in the publications pulled for full-text screening. ELISA assays for a variety of biomarkers were the method of choice for biomarker measurement, with enzymatic analysis via clinical systems such as Roche Cobas or Siemens Centaur as well as LC-MS (Liquid Chromatography – Mass Spectrometry) likewise broadly represented.

After screening the full-text publications, the methods of HISCL, nano-CLIA, FACS, enzymatic UV measurement, EMSA, Western Blot, LC-MS, HPLC, GC, Immunoradiometry, and scRNA sequencing were eliminated from panel consideration, for various reasons. As certain methods (enzymatic UV analysis, LC-MS, FACS, GC)

required instrumentation not present in the DZNE Biorepository and as there was no feasible purchase in the near future, the emphasis for these publications was no longer the method but the biomarker used therein.

The final two criteria pulled from the screened publications were the biomarkers measured and the testing frequencies performed.

Analysis of search results: biomarkers measured

Biomarkers can be a tricky subject. Ideally, the biomarker found would be sensitive to thawing, with a discernible difference between samples thawed once for analysis and samples thawed repeatedly, be it on purpose or because of defective storage locations.

Additionally, the biomarkers should be stable over a long period of time – some cytokines, such as BDNF, TGF-β (75) change significantly with storage duration, which must be considered prior to choosing material and assay for the panel.

Fortunately, stability of biomarkers has been an object of research interest for a long time, giving a putative DZNE Biorepository panel an abundance of choices (21,36,37,61,66,74,76–90).

In Figure 15, the biomarkers and other substances (55,91) measured in the screened publications were listed with the count number of their mentions forming the column height. It was encouraging to read about long-term stable and still financially viable compounds such as triglycerides, ROM, and IL-18 (66,78,92).

Of course, the decision which biomarker or biomarkers to choose to employ in the DZNE Biorepository QC panel also depends on which biomaterial will be used. Biomarkers only found in material such as cerebrospinal fluid (CSF) or biomarkers for environmental pollutants would be difficult to scale up to the extent required for the amount of samples the DZNE Biorepository panel was aiming to store.

In short, analyzing the screened publications for viable biomarker candidates led to the consideration of not merely aiming for soluble molecules able to be directly analyzed but possibly extending the search criteria to biomaterials such as entire cells. The biomarker to be evaluated would then be the level of gene expression of a target protein.

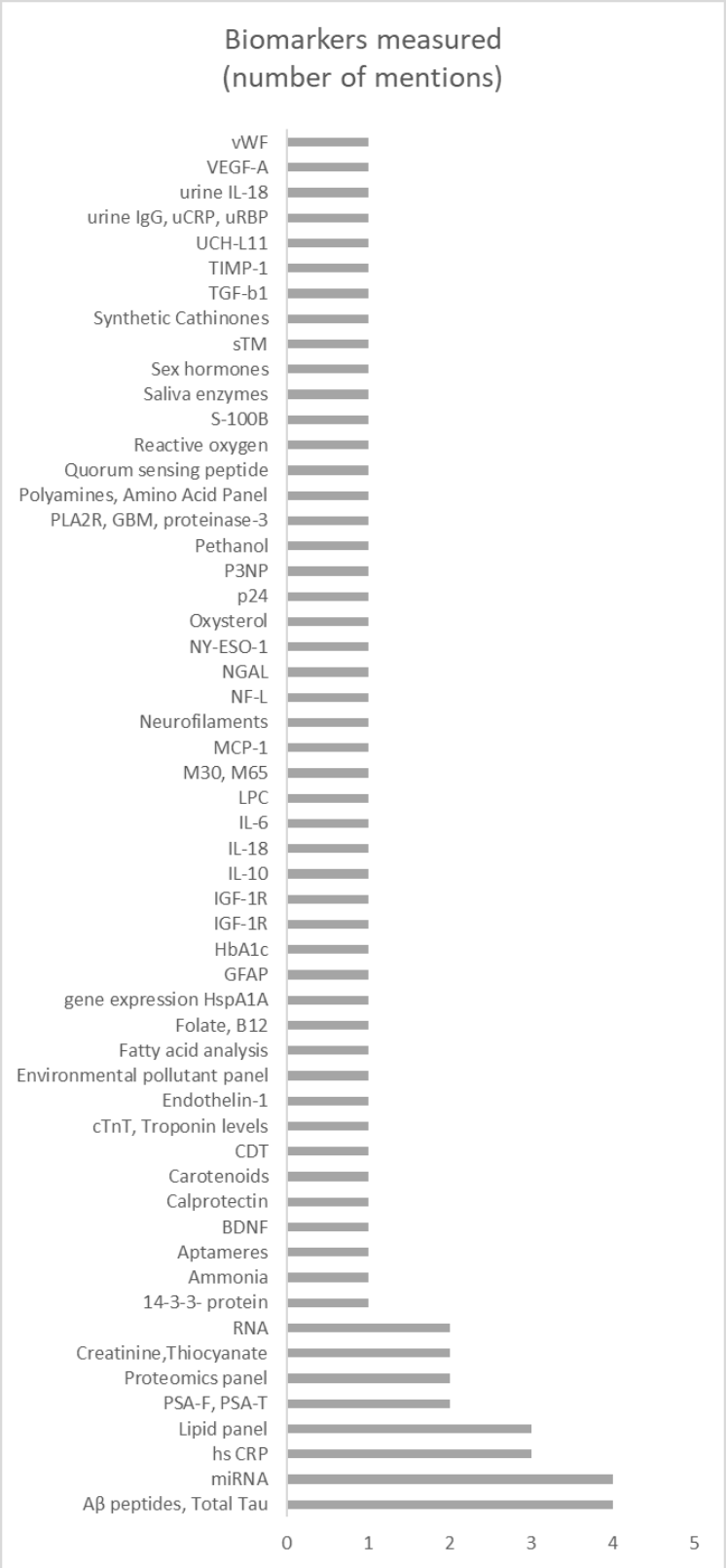

Fig. 15: Biomarkers measured as found in the publications pulled for full-text screening. The breadth of biomarkers used is understandable due to the wide array of subject matter investigated, with a slight emphasis on miRNA, Tau and CRP (C reactive protein).

The current preferred materials and assays, based on the preceding literature search analysis, are easily sourced materials such as pooled sera, and materials where the biomarker in question is not measurable within the sample vial itself, but must be generated first, such as with RT-PCR from cryopreserved cells. Going forward, viewing factors such as costs, time intervals of assays, and biomarkers used, will be with the notion that pooled sera and cryopreserved cells will be the biomaterials most suited to the DZNE Biorepository needs. The exact panel composition will be discussed at a later point.

With these factors (biomaterial, biomarker, and assay type) examined, it must of course remain to be mentioned that the panels will be run at fixed intervals. It would not do to space the tests too closely, which would not result in measurable differences, require

resources the DZNE Biorepository cannot offer, and possibly leads to false expectations extrapolating the current values over time.

Analysis of search results: testing time points

With the screened search results boasting years and even decades of stored samples' analysis it was clear that long testing intervals had been documented (21,35,65,66,74,80,93–95).

The point for this data compilation was, therefore, to find plausible and feasible intervals for the preferred biomaterials of pooled sera and cryopreserved cells, with literature on assays based on cryopreservation as reference.

Concentrating on these biomaterials, in Fig. 16 it can be shown that the literature (23,31,89,90,94,96–98) can group assay intervals in four groups.

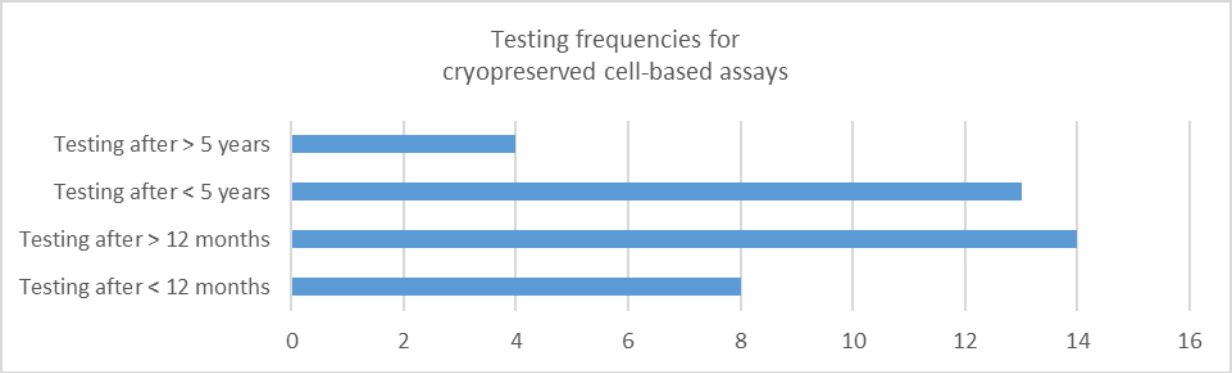

Fig. 16: Testing frequencies for cryopreserved cell-based assays as selected from literature. Published assays were grouped according to time between storage of sample and assay run on the sample. Non cryopreserved cell-based assays were not considered. Multiple mentions were possible.

Together with the consideration of DZNE Biorepository resources, especially regarding purchasing funds for assays and allocation of personnel to run the assays, the testing intervals will be as follows:

Months 0-12: every month
Months 13-60: every 6 months
Months 61+: every 12 months

This is a literature-based compromise between following storage-associated changes in gene expression as closely as possible and being able to run the assay as a routine process in the DZNE Biorepository that will not disrupt its main purpose, the storage and retrieval of samples.

With the general direction of the panel parameters in place, the next step now is to pull up the available resources and equipment at the DZNE Biorepository, which will establish the requirements any candidate assay will have to fulfill.

Required assay parameters regarding DZNE Biorepository equipment

The central DZNE Biorepository has three main storage options: manual LN_2 storage, primarily as a backup option or for samples that will not be accessed frequently, manual -80°C freezers, and an automated LN_2 storage system (see Fig. 17).

The aim of the DZNE Biorepository panel is to have biomaterials stored in each location, with retrievals and assays run at regular intervals. This would mean that any panel will have to work with the sample formats already configured for the storage locations (Table 5).

Storage location	No of units	Formats available	No of Panel vials/time point
Manual -80°C freezer	43	2mL vial boxes SBS racks (0.7mL, 2mL), PAXGene racks 10mL vials	3
Manual LN_2 Tank	10	2mL vial boxes SBS racks (0.7mL, 2mL)	3
Automated LN_2 system	7	0.7mL FluidX vials 0.3mL FluidX vials 2mL FluidX vials 0.7mL Matrix vials 2mL Matrix vials	3

Table 5: List of formats compatible with the storage locations offered by the DZNE Biorepository. Due to the emphasis on automation, the manual storage locations have also been configured with shelves and drawers compatible with automation-friendly sample tube formats.

In order to allow for reproducibility, three vials per time point will be retrieved. Multiplying this with the number of storage locations, this would signify that each time point and assay would require

43 x -80°C freezers + 10 x manual LN_2 tanks + 7 x automated LN_2 tanks) x 3 = 180 vials

This fortuitously fits in quite well as a multiple of the common 96-well format for assays. Two different assays would then require 360 vials per time point.

10 Back-Up manual LN_2 Tanks 43 -80 manual Freezers 7 automated LN_2 tanks

Fig. 17: Three storage options of the DZNE Biorepository. Manual LN_2 storage, manual -80°C storage, and automated LN_2 system are all located at the main DZNE Biorepository location.

Taking these requirements and returning to the preferred biomaterials and biomarkers drawn from the screened literature in the previous section, it becomes easier to discern a way forward for the DZNE Biorepository panel.

Evaluation of candidate assays and samples in view of financial and personnel resources

With large volumes of pooled sera from commercial suppliers and cryopreserved cells, the biomaterials of choice for the panel, candidate assays were assembled and investigated as to their compatibility with available resources at the DZNE Biorepository (Table 6).

Time	Funding	Instrumentation
Max 1 day for 180 samples	Cannot exceed 10k€/ year	Either present or, if purchased, cannot exceed 30k€

Table 6: Resource requirements of the DZNE Biorepository for the panel

Time: The DZNE Biorepository is run by a core team of 5 full-time employees. This means that every single moment that cannot be dedicated to supplying clients with their samples or storing the samples entrusted to the DZNE Biorepository needs to be wisely apportioned, as delays in sample storage and retrieval will result in lower customer satisfaction. The current sample handling processes are such that no samples are received on Mondays, which would indicate this to be a possible option to dedicate one Monday a month for panel assays.

Funding: The DZNE Biorepository aims to be as transparent as possible in its charging policy and, as such, factors in its entire operating costs for appropriate reflection of its pricing structure. As such, in the general operational budget there is an allowance of

10k€ per year for chemicals and other consumables that are not explicitly charged to DZNE Biorepository services. A provision for higher setup costs for the first year of the panel has been inserted in the budget; nevertheless, the 10k€ will remain as a benchmark.

Instrumentation: The DZNE Biorepository already possesses a luminescence plate reader, which is why 96-well assays compatible with existing instrumentation will be favorably viewed. Additionally, the investment budget allows for a one-time purchase of an instrument up to 30k€. This would permit for an assay panel to consist of two different methods.

The candidate assays would ideally be run with biomarkers particularly well suited to LN_2 temperature as well as -80°C. To this end, the following candidates for the panel have been assembled (Table 7):

Name	Biomarker	Biomaterial	Instrument	Time/96 tests	€/96 tests
Single cell sequencing (97)	HspA1A gene	Human T cells	Illumina	Multiple days (external service)	12K€
RT qPCR (99)	H2AXγ	PBMCs	Applied Biosystems Quantstudio	8-10 hours (including prep work)	56€
RT qPCR(100)	IFNG gene	PBMCs	Life Technologies	8-10 hours (including prep work)	56€
Colorimetric assay (22)	Uric Acid (UA)	Serum	Roche Cobas	4 hours (including prep work)	ca 200€
Colorimetric assay (101)	Uric Acid	Serum	Abbott Aeroset	4 hours (including prep work)	ca 200€
Fluorometric assay (102)	Uric Acid	Plasma	Information requested		

Table 7: Description of 6 candidate assays deemed to work best with DZNE Biorepository resources. Focus was on assays compatible with cryopreserved cells and pooled sera.

With these candidates, the biomaterial itself must not be disregarded. Particularly cells such as Human T cells would not be a financially viable solution to use at a rate of 180 vials per month for the first year.

Description and justification of recommended QC assay panel(s) and sample material

With the information from Table 7 and the DZNE Biorepository resources available to dedicate to such a process, be it monthly, bi-annually, or yearly, it might seem as though PBMCs and pooled sera would be the biomaterial of choice, with RT-qPCR and colorimetric/fluorometric assays the analysis methods for the respective biomaterials, with single cell sequencing and the HspA1A gene an unlikely candidate.

However, the reason the HspA1A was so intriguing was not because of the biomaterial or the method – it was the gene that was upregulated as a response to thawing and the possibility of quantifying its expression in a different, financially more interesting cell line.

And indeed; HspA1A is highly expressed in the HEK293 cell line (103). This is fortuitous, as commercial suppliers of these immortalized cell lines are of course easier to use than being dependent on primary cells or cells that are more difficult to cultivate. Measuring HspA1A via RT-qPCR brings with it the additional justification that RT-qPCR is a widely performed method entailing many suppliers with even more analysis kits available.

Regarding the second candidate assay, the decision to go with human sera was quite straightforward, again mostly based on the easy supply of pooled sera from commercial suppliers. Uric acid (UA) has been published as being sensitive to changes in thawing, strengthening its position as a suitable biomarker. UA testing as an assay is also widely distributed, both on clinical and fundamental research levels, so that assays can be purchased at different price points depending on if a result needs to be clinically dependable (i.e. for the Roche Cobas instrument, in clinical labs) or reproducible in a fundamental science setting. This is why the final decision was made for RT-qPCR analysis of HspA1A in HEK293 cells and uric acid measured via fluorometer from pooled sera.

In summary, being able to freeze single vials at 0.7mL volume or lower will save the DZNE Biorepository precious storage space. The timing of running 360 samples, albeit likely staggered over the course of two days, is feasible at current DZNE Biorepository

staffing plans. Using existing plate readers and applying the investment funding for a 96-well RT-PCR machine will allow additional investments to remain at a minimum. The biomarkers, biomaterials and assays selected are widely known, broadly applied and easily available. The final layout and outlook of the panels to be run at the DZNE Biorepository will follow in the next section.

Final layout and plan for freezing and testing samples, with aliquot and vial numbers, retrieval dates, assays run, cost estimates

Taking all the preceding information into consideration, the criteria for the DZNE Biorepository were as follows:

Small volume test vials: this was possible by selecting assays that were sensitive and did not require large amounts of test material.

Financially responsible assay selection: by choosing two assays with various applications and multiple suppliers in laboratories across the world, costs were able to be kept low.

Scientifically sound assay detection: the most financially responsible assay would be of no use in a DZNE Biorepository QC panel if it did not respond to thawing with altered concentrations or values. Both HspA1A and uric acid have documented temperature sensitivity in the readouts selected for the panel.

Speedy assay performance: Including prep work and documentation, assays of one panel were able to be run in one work day, preferably on a day that did not see much incoming sample traffic.

Time points: Literature of cryopreserved cells and their analysis intervals pointed to retrieving test vials on a monthly basis for the first 12 months of the setup, with bi-annually frequencies thereafter and then yearly testing after year 5.

The findings are summarized below in Table 8. An overview of the experimental setup and process of the panel implementation can be seen in Figure.18.

Biomaterial	Volume	Cost (biomaterial)	Time points	Assay	Time per assay	Cost (assay)
Human pooled serum	0.3mL	5000€/5L	Monthly, bi-annually, then annually	Uric Acid (Fluorometric)	40 minutes (96 well plate)	Ca. 1000€/time point
HEK293 cells	2x10^6 cells/vial to be frozen	4500€ for 10 vials	Monthly, bi-annually, then annually	RT PCR of Heat Shock Protein A1A	60 minutes (light cycler)	Ca. 1500€/time point

Table 8: Summary of panel components, time points, and costs. Of note, the implementation costs and monthly assay runs in the first year will result in higher expenses the first year of panel testing.

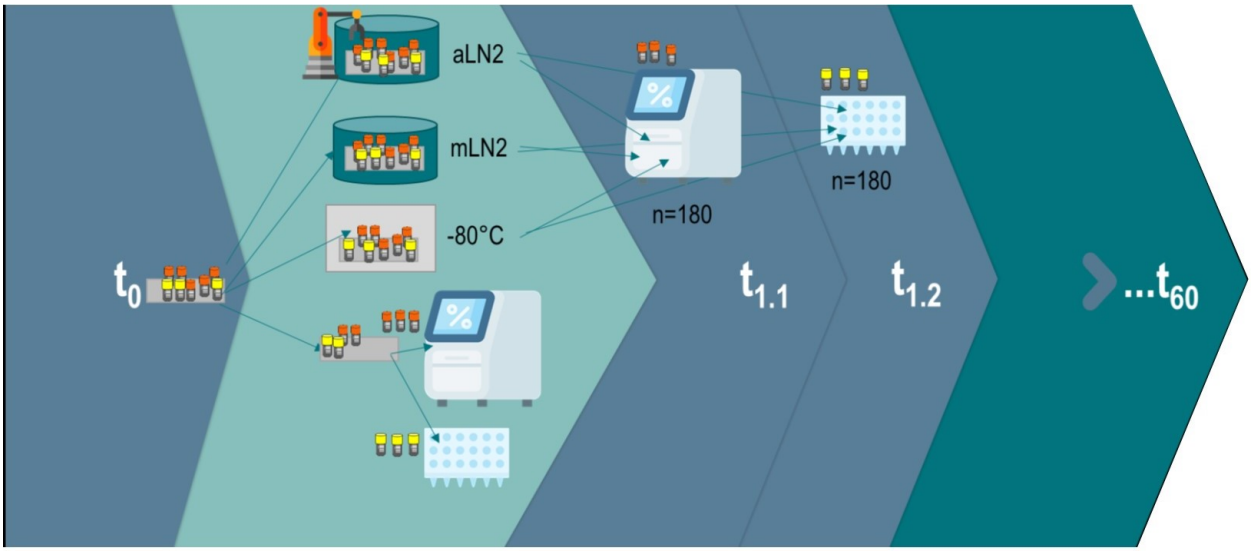

Fig. 18: Timeline of the DZNE Biorepository QC panel as described in this thesis. Select icons courtesy of Flaticon.com

Briefly, all the biomaterials will be frozen at the same time point (t_0). This signifies that for a run time of at minimum 5 years, the panels would require

$$\underline{360 \text{ vials per time point for two assays } x \ (12_{(\text{year 1})}+8_{(\text{years 2-5})}) = 7200 \text{ vials}}$$

for the first 5 years, with another 360 vials for every additional year of panel assay that is to be run.

Pooled human sera and Hek293T cells will be cryopreserved at -80°C and LN_2 temperatures. Both biomaterials will be stored in all three storage locations (automated LN_2, manual LN_2, and -80°C). For the first year, on sequential days, the vials for the RT-qPCR assay and then for the fluorometric assay will be retrieved and the assays performed. The first year will see monthly assay intervals, with the years 2-5 only testing biannually. From year 5 onwards, the samples will then be tested annually.

Of course, there are still various factors to be considered before starting the panel at the DZNE Biorepository, which will be discussed below.

Discussion

The DZNE Biorepository was established primarily in order to offer a centralized sample storage service for the scientists and clinicians of the institute. To this end, as mentioned in the introduction, the primary mission of the DZNE Biorepository is to keep the samples safe and easily retrievable but not to perform any kind of quality assurance or quality control on them for the depositing research groups.

This signifies, however, that the DZNE Biorepository has the task of keeping the parameters outside of the sample stable and secure, with close monitoring of temperature and location of storage units, but has no influence or knowledge of any preanalytical variables that might have impacted the sample before it was stored in the DZNE Biorepository.

As the DZNE Biorepository is a new service unit for the research groups of the institute, this setup prior to begin of operations opened a fascinating new option for the DZNE Biorepository and its processes. The DZNE Biorepository had the unique possibility to store long-term samples of its own choosing, analyze them according to assays that would measure biomarkers best suited to its readout needs, and timing this so the procedures would work around the core services offered by the DZNE Biorepository to the research groups. This assay panel would be able to begin its run time together with the storage of the first institute samples and be set to deliver results over 5 years or more.

Of course, any panel selected would also need to fulfill various requirements aside from the practical ones mentioned above. Research group leaders of the institute were sent a questionnaire in order to develop a general sense of what sample storage and assay parameters were prevalent at the institute. This was helpful in the sense that aiming for human material in the DZNE Biorepository control panel would then be an accurate reflection of the sample types stored by the research groups. Likewise, the volumes of the sample vials were of interest, mostly for compatibility purposes – this way, no new storage formats would have to be organized simply to accommodate the

DZNE Biorepository panel samples. The sample storage times were interesting to review, as most of the storage requirements were short-term; long-term storage would mostly be for the population sciences cohort as well as to keep in compliance with German genetic safety legislation.

As a second step, biomedical literature databases were searched for publications using the key words long-term, stability, biomarker and low temperature. The number of publications regarding biomarkers and their storage stability is daunting; nevertheless, choosing to analyze screened publications brought with it the hope to possibly unearth an assay that would fit the DZNE Biorepository needs.

The database search results were pared down to 63 full-text articles, and those were again reduced to 47. Among those, the prevalence of ELISA assays and colorimetric assays dependent on clinical laboratory instruments such as the Roche cobas was present as expected. Plotting the different parameters of the assays in the publications, the option of using RT-qPCR as a method specifically for cryopreserved cell cultures was more and more a method of interest, as it united many of the criteria a DZNE Biorepository panel had to fulfill – namely, available to analyze many samples at the same time, speedy completion with a day, widespread use of a method, and a viable option for biomaterial stored at LN_2 temperatures. With the main storage option of the DZNE Biorepository being the automated LN_2 storage system, this made RT-qPCR highly interesting for further investigation. Cryopreserved cells are some of the main biomaterials where storage is recommended at LN_2 temperatures; combining this biomaterial with such an exquisitely highly sensitive assay such as RT-qPCR was an intriguing option for the DZNE Biorepository panel. The choice to perform this assay with a gene from the heat shock protein family in a cell line that has a demonstrated high expression of this gene was the result of choosing results from this wide net cast into the sea of biomedical literature databases. As a parallel assay, pooled sera as a biomaterial suited to storage at -80°C with the widely used biomarker uric acid would form a fine counterpoint to the cell culture analysis. Together, the aim is that both biomaterial and biomarker readouts show temperature changes, possibly long-term storage changes and be available for the foreseeable future as assay substrate due to their widespread use in the world of research and clinical science today. To start with, enough vials for tests to run the next five years will be frozen and stored at the different storage locations. Budget allowing, it would of course be very interesting to extend the

panel run time for as long as possible, perhaps spacing the timing of the retrievals of the first year, while they still occur on an monthly basis out to every two months.

Challenge analysis of selected assays for the DZNE Biorepository panel

Of course, all precautions were taken to establish a DZNE Biorepository QC panel that fulfilled all the requirements of the DZNE Biorepository. Using feedback from the research group leaders and the database searches resulted in a preliminary panel setup that will hopefully run smoothly and give reproducible results for years to come without impacting the DZNE Biorepository core processes or budget in an exaggerated manner. Nevertheless, risks remain (they always do). Some of the possible pitfalls and future challenges have been assembled in Figure 19 below.

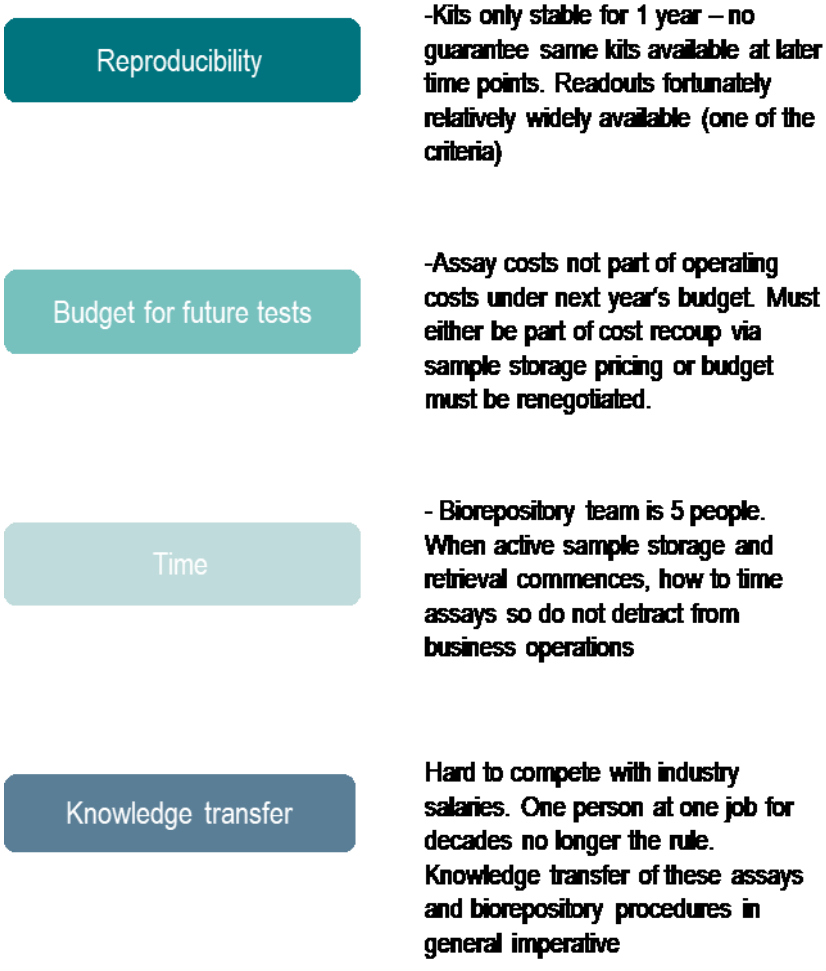

Fig. 19: List of possible future challenges for the DZNE Biorepository panel

The risk of kits or reagents not becoming available in the future has been attempted to be mitigated by using biomarker readout and methods that are offered by more than one supplier, as well biomarkers whose sequence is known and therefore (in the case of RT-qPCR) in which the kits necessary for the method can, at a pinch, be assembled elsewhere.

Another challenge is one afflicting all publically-funded institutions – budget. Simply because a budget has been granted one year does not mean that the same budget

will be available the next year. Although the assay costs, once the original setup expenses of biomaterial acquisition, kit purchase and instrument purchase are completed, will only be a small amount of the yearly operating costs it is still pertinent to have a backup plan. In this case, it is relatively easy – should the costs for the assays no longer be in the budget, the assays will simply need to be spaced out more, which might even have the unexpected bonus of therefore being able to observe the biomaterial characteristics for longer than planned.

The staffing issues and budget issues go hand in hand, unfortunately. The core team of 5 staff members will not change for the next years, which means that if one staff member is sick and the other on vacation, it might not be feasible to run the DZNE Biorepository panel assay for one time point. As this is an internal QC panel and not an external service for customers of the DZNE Biorepository, running the assay has lower priority than ensuring that the core processes of the DZNE Biorepository are still available to the research groups and the assay will have to be delayed until the next time point or (if the intervals are yearly) the next moment when staffing allows for retrieval and analysis of the samples.

Knowledge transfer is an issue if staff is not simply just not there for a short amount of time but leaves the DZNE Biorepository entirely. Having to document as much as possible regarding SOPs, processes, monitoring technologies, samples storage, and client interfacing is a slightly annoying endeavor now, but has already proven very helpful for troubleshooting and onboarding purposes. This will expand to include the DZNE Biorepository QC panel in a way that all assay results and protocols are uploaded and stored in a common server file, with standard onboarding protocol also including training on the assay panel test run for any new hires.

Discussion on possible alternative processes

The concept of the DZNE Biorepository QC panel is not a core process to the DZNE Biorepository service mission. There are parameters set in place to monitor temperature, sample location, storage volume, cooling agent supply, and more. Emergency cooling systems and generators are present in case of systems failure. The servers with the database on all the sample storage locations are regularly copied to the backups in case of data loss. All this to say that the DZNE Biorepository panel is not implemented as a measure to enable an immediate response to instrument or

technical failure; its aim is to follow the storage history of all the DZNE Biorepository storage locations, compare the sample readouts to each other over time, and collect enough data points and information to allow for extrapolations on temperature and other fluctuations and their effect on the biomaterial stored.

Should the DZNE Biorepository panel in its entirety not be feasible for some reason, a more technical way to compile a storage location history would be to simply assemble all of the temperature monitoring data, service visits, error reports, and so on of each location and form recommendations based on these findings. They would, of course not be comparable to the purpose the DZNE Biorepository panel was attempting to fulfill, but allow for extensive and comprehensive data regarding freezer or other storage location maintenance requirements.

Outlook and applicability for other biobanks or biosample repositories

Finally, with all its restrictions as to its purpose and mission, the DZNE Biorepository prides itself on aiming to keep biosamples safe and easily retrievable. Expanding its function to also investigating the long-term effects on cryopreservation of cells and pooled sera, specifically concerning the two biomarkers of HspA1A and uric acid, will hopefully serve to aid other repositories or biobanks looking for an independent sample assay that can serve to accompany their storage locations for the duration of their use while in the facility. Using biomarkers that will respond to temperature changes will give readouts that can trace differences in results to differences in temperature storage. Implementation of this panel is set for 2023, with hopes to extend it beyond the primary 5 years' worth included in preliminary calculations. Beyond that, perhaps a new record can be achieved; after all, the population cohort is set to run for 30 years.

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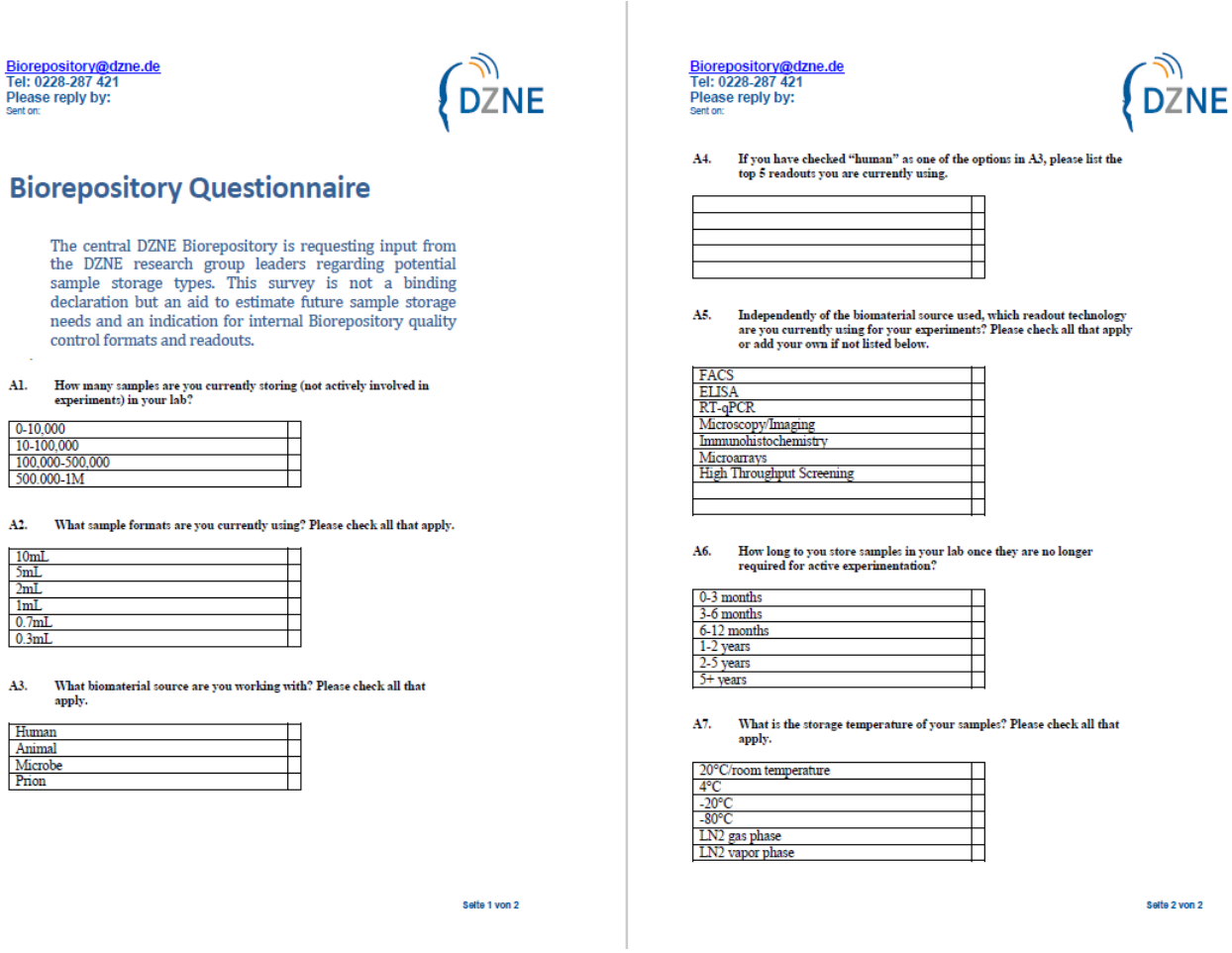
Annex

Item 1: Client Questionnaire



Biorepository@dzne.de
Tel: 0228-287 421
Please reply by:
Sent on:

DZNE

Biorepository Questionnaire

The central DZNE Biorepository is requesting input from the DZNE research group leaders regarding potential sample storage types. This survey is not a binding declaration but an aid to estimate future sample storage needs and an indication for internal Biorepository quality control formats and readouts.

A1. How many samples are you currently storing (not actively involved in experiments) in your lab?

0-10,000	
10-100,000	
100,000-500,000	
500,000-1M	

A2. What sample formats are you currently using? Please check all that apply.

10mL	
5mL	
2mL	
1mL	
0.7mL	
0.3mL	

A3. What biomaterial source are you working with? Please check all that apply.

Human	
Animal	
Microbe	
Prion	

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Biorepository@dzne.de
Tel: 0228-287 421
Please reply by:
Sent on:

DZNE

A4. If you have checked "human" as one of the options in A3, please list the top 5 readouts you are currently using.

A5. Independently of the biomaterial source used, which readout technology are you currently using for your experiments? Please check all that apply or add your own if not listed below.

FACS	
ELISA	
RT-qPCR	
Microscopy/Imaging	
Immunohistochemistry	
Microarrays	
High Throughput Screening	

A6. How long to you store samples in your lab once they are no longer required for active experimentation?

0-3 months	
3-6 months	
6-12 months	
1-2 years	
2-5 years	
5+ years	

A7. What is the storage temperature of your samples? Please check all that apply.

20°C/room temperature	
4°C	
-20°C	
-80°C	
LN2 gas phase	
LN2 vapor phase	

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Item 1: Client Questionnaire. Questionnaire sent to group leaders requesting information about sample storage needs and sample readouts performed.

Item 2: Table of publications reviewed for sample QC readouts

Publication Nr.	Title,Authors,Journal/Book,Publication Year,DOI
1.	Effects of Cryopreservation and Thawing on Single-Cell Transcriptomes of Human T Cells. Lee JS, Yi K, Ju YS, Shin EC Immune Netw. 2020 Jul 15;20(4):e34. doi: 10.4110/in.2020.20.e34.
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Item 2: Table of publications selected from literature search with focus on long-term, stability, biomarkers and low temperatures.