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Review Article

How standardization of the pre-analytical phase of both research and diagnostic biomaterials can increase reproducibility of biomedical research and diagnostics

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A B S T R A C T

Comparison of published biomedical studies shows that a large proportion are irreproducible, causing severe damage to society and creating an image of wasted investments. These observations are of course damaging to the biomedical research field, which is currently full of future promise. Precision medicine and disease prevention are successful, but are progressing slowly due to irreproducible study results. Although standardization is mentioned as a possible solution, it is not always clear how this could decrease or prevent irreproducible results in biomedical studies. In this article more insight is given into what quality, norms, standardization, certification, accreditation and optimized infrastructure can accomplish to reveal causes of irreproducibility and increase reproducibility when collecting biomaterials. CEN and ISO standards for the sample pre-analytical phase are currently being developed with the support of the SPIDIA4P project, and their role in increasing reproducibility in both biomedical research and diagnostics is demonstrated. In particular, it is described how standardized methods and quality assurance documentation can be exploited as tools for: 1) recognition and rejection of 'not fit for purpose' samples on the basis of detailed sample metadata, and 2) identification of methods that contribute to irreproducibility which can be adapted or replaced.

Introduction

Irreproducible study results slow the progress of biomedical research and as a consequence inhibit innovation of healthcare [1–12]. The economic damage of irreproducible preclinical research is estimated to exceed 50% of the total biomedical research budget, which amounts in the US alone to about US $28 billion of pharma industry spending on preclinical research [1]. In the same study the biological reagents and reference materials could account for 36.1% and laboratory protocols for another 11% of nonreproducible data. A large part of medical research is based on tests performed on human biomaterials. There are estimates that human biomaterials are used in about 40% of the research findings in biomedical science publications [1]. Such numbers could be seen as endangering the reputation of the field of biomedical research where even such harsh terms as "translational research crisis", "scientific waste" and "irreproducibility crisis" are increasingly being used [2–6,8,9,11]. In addition, such estimates are also prompting action and indeed publications as well as research organizations have come forward with possible solutions [1–12]. Placing focus on greater control of study design and publication of results looks very promising.

It has been shown in studies performed within the SPIDIA consortium that biomaterial sample variations can markedly disturb test results [13–26]. Variations can be introduced into biomaterials before they are finally used in research. The chain of events before the sample is used in a test or measurement is called the pre-analytical phase. This starts with the patient or research subject from whom the samples are taken and ends when the analytes (such as DNA, RNA or proteins) are

Abbreviations: BBMRI-ERIC, Biobanking and BioMolecular resources Research Infrastructure - European Research Infrastructure Consortium; BBRB, Biorepositories and Biospecimen Research Branch; ESBB, European, Middle Eastern and African Society for Biopreservation and Biobanking; CEN, European Committee for Standardization; FFPE, Formalin-Fixed Paraffin-Embedded; ISBER, International Society for Biological and Environmental Repositories; ISO, International Organization for Standardization; NCI, National Cancer Institute; NIH, National Institute of Health; SPIDIA4P, Standardisation of generic Pre-analytical procedures for In vitro DIAgnostics for Personalized Medicine

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enriched or isolated for the research to be measured or tested in an assay. The influence of possible pre-analytical variations is often ignored, but small as they might seem their influence might be more severe than anticipated.

Signals measured and noise

Where a large number of aberrant signals caused by variations seem random, it is referred to as noise. Noise caused by sample variations results in a signal-to-noise ratio that can be confined to certain areas within the measurement as a noise pattern. Where different methods are used, different noise patterns can be formed that can completely hide, partly hide or not hide signals. Next to these false negative signals, different methods can also trigger false positive signals. The noise patterns can depend on the variations introduced by the methods used and the sensitivity of the measured analytes for the variations. This means that noise patterns can vary between collections, institutes and studies, which are observed as batch effects [27–30]. Batch effects are of influence when building upon a study or comparing one study to another. Currently sample variations are not controlled due to the lack of sufficient tools to determine the impact and the exact source of such variations. Reports on optimization of methods in the pre-analytical phase are numerous but fragmented and usually focus on or solve specific methods that only deal with a specific part of the pre-analytical phase in a particular situation. In fact, these reports again confirm that different pre-analytical methods can cause variations in the test result. In this overview the focus is on the influence of the sample pre-analytical phase on sample variation and what can be done to control this.

Sample variation damage

Sample variation can have an influence on:

1 A single sample, such as an individual diagnostic test result, where as a consequence this can result in a wrong diagnosis, which might lead to non-optimal medical treatment.
2 Multiple samples, where the introduced variations can cause noise that completely, partly or not at all hides and/or upregulates the actual test results being measured.
3 Multiple samples in multi-center studies, where different sources of variation (or methods used) can cause different batch effects that can hide and/or upregulate, or not, different parts of the signals to be measured.
4 Published results, where also different sources of variation (or methods used) can again cause different batch effects that can hide and/or upregulate, or not, different parts of the signals to be measured, disturbing reproducibility that can inhibit building upon a study for further research or commercial test development.

Sources of sample variations

The influence of sample variations increases where different methods are used to:

- Treat patients (patient condition and genetic background can play a role).
- Handle samples: collect, transport, process, stabilize and store the samples.
- Isolate or enrich the analyte from the samples.

The influence of variations also increases if the analyte is unstable in terms of quality (e.g. general stability of DNA, RNA and protein) as well as quantity (such as the difference between the expression of housekeeping genes or rapidly processed regulatory genes or proteins). When measuring rapid expression processes where the measured analyte is rapidly processed, the influence of variations introduced during patient treatment or transport increases. Here, the expression in the cell, degradation rate or molecular form can be affected. This currently makes genomic DNA isolated from blood and analyzed by sequencing one of the most accessible and reliable sources of information [31]. However, considering the image of a butterfly and its caterpillar which originate from the same DNA, there is so much more depth in the knowledge involved in disease development and progression that can be obtained from epigenetics, RNA and protein expression and/or metabolomics in the different cells or circulating in the body [32].

In the diagnostic laboratory, standardization and the use of proper controls, together with certification or accreditation-related areas like audits, standard operating procedures and quality improvement programs, have helped to bring down the number of failing individual tests based on stable analytes of that laboratory. The (commercially) available laboratory tests used nowadays are very robust, because they have survived the obligatory validation for intended use with diagnostic samples that needs to be performed in all the different certified or accredited diagnostic laboratories using the test. This does not however mean that the workflow in the different laboratories is the same. As a consequence, laboratory specific variations are introduced to the sample. Validation for intended use is key for the certification or accreditation of the laboratories. It is defined as: confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled. This means the tested or measured analyte is not found to be sensitive to the sample variations in that specific laboratory when tested in a representative series for intended use. For instance, the formalin fixed and paraffin embedded sample is seen by many as a standard way of fixing tissues. However, a closer look reveals differences in fixation time. Fixation can cause variations in the form of chemical changes to the analytes, which increase over time, and this variation can be found in almost all pathology laboratories. Pathology laboratories leave all tissues over the weekend in formalin fixative if they cannot be embedded in paraffin before the weekend. Dehydration starts on a Sunday night just before embedding the tissues on the Monday morning. This may lead to over-fixation which can cause impaired test results [14,33–36]. Between laboratories, different formalin formulas or durations in the dehydration process or temperatures, as well as durations when embedding the tissues in paraffin, can be found. For blood samples, variation in a simple step such as inverting the tube after blood collection can affect results. For example, when a tube is inverted vigorously rather than gently, blood cells can lyse which can impair test results. When blood collection tubes are not inverted, blood coagulation may occur in some parts of the tube and cells might be lysed because of a high concentration of anticoagulant elsewhere in the tube. A laboratory quality management system decreases this variation with audits and quality assurance programs. Without the instruction of the test manufacturer, the storage temperature and the time interval allowed between specimen receipt and sample processing can vary between laboratories, which can introduce variations in sample quality. This however can differ between accredited and certified laboratories. Such differences are even allowed in ISO 15189 (hospital laboratories) accredited laboratories. As long as all the tests performed on the samples are validated for intended use all these internal variations in methods used are allowed.

Some variations originate from sources that cannot be standardized to be performed in the same way, as for instance during patient care. This may include the treatment or intervention in the disease, concomitant disease, infections or pain relief, where drugs might be given just before the sample is collected and is not yet cleared from the cells. In addition, the patient condition, environment and genetic background can play a role in treatment responses and/or hospital inclusion criteria for allowing a certain treatment. As a consequence, both the treatment response and the hospital inclusion factors for treatment can later influence the population of a research cohort selection. For tissues sampled from a surgical specimen, the warm and cold ischemic times can be

of influence or the anesthetics or type of surgical treatment chosen. Sample transport cannot always be performed in the same way and can also introduce variations.

Another source of sample variation can be identified where the outcome of diagnostic evaluations is used to define the cohorts in a study. For instance, an intra- and inter-observer variation among pathologists can cause a variation in the inclusion of samples or patients in the different study legs. Therefore, a complete review by one pathologist or a consensus group of pathologists reviewing all the study cases is often included in the study design. Such an effort cannot, however, be undertaken when comparing studies for reproducibility.

The role of biobanks

Biobanks play a pivotal role in the collection, stabilization, storage and transport of human biomaterials and cover most of the pre-analytical phase. It has already been suggested that for biomedical research studies, human biomaterials can best be collected in specialized, centralized facilities within academic hospitals [37]. Such biobank facilities have the overall aim of supporting medical translational research and increasing the study impact. They aim to provide exchangeable high quality samples and can often support study design as well as offer support for sample specific techniques. International biobanking societies like ISBER or ESBB and organizations like BBMRI-ERIC, NIH, NCI and BBRB focus on evidence-based biobanking and sample quality as a topic [38–45]. Integration within the hospital care systems, making use of the already implemented protocols to collect samples, can be beneficial to all parties involved [43]. Departments such as clinical chemistry, pathology, hematology and microbiology can form a solid basis for collecting the vast majority of biomaterials needed for most biomedical studies. Here, the knowledge exists on how to procure samples in a standardized way and what can or cannot be measured in a given sample. Often these departments can also provide specialized techniques dedicated to handling the sample to make the final test results more reliable. Biobank networks can support upon request the involvement of disciplines in the study design or contact their external network of standardized biobanks to speed up the collection process.

Within one institute, a well-managed biobank can already contribute to more reliable results if the collection, stabilization, storage and transport procedures are performed by dedicated personnel according to biobank standard operating procedures. This quality level can be improved if the biobank workflow is performed according to ISO standards with QA/QC programs [46]. In such situations, all procedures are covered from the moment the sample is received up to its release for research purposes. This is, however, not the complete pre-analytical phase, which starts already with the patient. In the workflow starting with the patient until receipt, most parameters cannot be standardized to always be performed in the same way, simply because the first priority is patient care. This part can bring in unexpected variations during treatment and transport as already mentioned. Despite the current guidelines, best practices, certification and accreditation for biobanks, through which many processes have already been normalized, there is still room for batch associated pre-analytical sample variation, even if all the procedures listed are followed [37–45].

Fit for purpose

An argument often used is that a study needs so called “fit for purpose” samples. This argument is a logical one, but can currently leave extra room for variations when wide margins are chosen and only few upfront criteria are defined and met. The pre-analytical events not included do not seem to matter. Such a situation does not allow checking, other than observing during analysis, whether something might have been wrong with some samples, which are subsequently interpreted as outliers. The outliers are usually taken out of the analysis without presenting a solid reason for why the sample was not fit for purpose. This could be interpreted as p-harking, but more importantly, leaves out the chance to learn more about the underlying parameters causing the sample to behave aberrantly.

Where samples defined as fit for purpose are chosen with wide margins and the analysis results in conclusions important for understanding a disease process, one should realize that sample variation could lead to wrong conclusions in a batch effect. The findings should be validated in a high quality sample set before conclusions can be drawn with certainty. For instance, if by using NGS on FFPE tissue samples certain mutations are concluded as being important for disease development, one should realize that chemical changes by formalin fixation can influence the sequence results. Verification should be done on fresh frozen tissue samples for confirmation.

Samples collected as fit for purpose might also mean that they are collected under strictly standardized circumstances. These high quality samples are not directly comparable to those used in diagnostics, due to batch effects. In these cases, it is recommended to verify the findings on diagnostic samples and not only on samples collected under the same conditions. This way it is clear at an early stage whether findings are, or are not, robust enough to be reproducible in the diagnostic setting.

Documentation

Not all methods can be fully standardized to a point where the method is performed in the same way for all samples without introducing variations. To obviate sample pre-analytical variations that cannot be controlled, the next step is to try an alternative method that can be fully standardized and avoid the method that cannot be standardized. Where full standardization or avoidance of non-standardized methods is not possible, the introduced variation can be documented. This method can be easily remembered by the abbreviation SAD, which needs to be followed when optimizing and developing standard operating procedures:

1 **S**tandardization (method performed in the same way for all samples); if not possible,
2 **A**void (find an alternative method that can be standardized in the same way for all samples or has the least impact on variation); if not possible,
3 **D**ocument or record the variation

The documentation can be used and kept with the sample data as sample metadata for both diagnostics and research. Documentation of pre-analytical factors before the sample is received in the biobank are in most cases already stored in the hospital database for documentation of the treatment of the disease of the patient, but are not yet accessible as sample metadata. This means that the diagnostic department and the biobank should have access to this data, to collect it as sample metadata.

Controlling pre-analytical sample variations

Standardization on a high level such as that of ISO with stricter and well-determined standards can increase sample quality and exchangeability and thereby increase reproducible results. However, gaining complete control over pre-analytical sample variation cannot be achieved in a short time. The proper documentation of the steps and methods that have an influence on sample variation in the pre-analytical phase (sample metadata) needs to be collected and analyzed.

SPIDIA4P, a European project, is supporting the development of ISO standards setting these norms and is focused on the pre-analytical phase for sample collection. The aim is to develop 21 selected, high priority, sample pre-analytical CEN and ISO standard documents as well as corresponding External Quality (EQA) schemes and implementation tools. The CEN and ISO documents set requirements and recommendations for critical steps and methods in the workflow for

sample collection, transport, processing, stabilization, storage and analyte isolation or enrichment as well as the documentation requirements, making the samples more comparable and exchangeable. The pre-analytical history of the samples can be traced in detail in the sample metadata.

The development of the first documents started at the end of the SPIDIA project (2008–2012 and predecessor of SPIDIA4P) where pre-analytical sample variations were studied. The first five ISO documents were published in January 2019 [47–51]. This was done under the Vienna agreement, a technical cooperation between ISO and CEN, harmonizing European and International standard work wherever possible. These ISO documents describe the international standards for the sample pre-analytical phase for diagnostic laboratories and the industry supplying laboratory equipment and tests, as well as biobanks. Those recently published describe: FFPE tissue in combination with tests on isolated RNA (part 1) [47], isolated proteins (part 2) [48] or isolated DNA (part 3) [49] and frozen tissue samples in combination with tests on isolated RNA (part 1) [50] or isolated proteins (part 2) [51]. ISO documents on whole blood with isolated cellular RNA (part 1), isolated genomic DNA (part 2) and isolated circulating cell free DNA (part 3) as targets in the tests, are due to be published at the beginning of 2019.

Use of sample metadata

New standards and recommendations reduce variations in the samples by preventing divergent methods or circumstances affecting the final results. This will bring the quality of collected samples closer to the level where they can be compared and enable more sensitive tests on less stable molecules to be performed. The sample metadata will enable researchers to gain more insight into where critical steps are in the pre-analytical phase of a sample as they identify the outliers and allow their correlation with the critical pre-analytical steps. This information can be used in the inclusion criteria for selecting samples or a cohort for a study. It can also form an incentive to examine that critical method to see if the variation really cannot be avoided with an alternative fully standardized method. Finally, better arguments can be put forward to explain outliers in publications. Bridging the gap between differing sample quality and recognizing if samples are fit for purpose and comparable is not achievable in a short time. Perhaps a more productive environment for that can be found in the diagnostic setting.

Accredited diagnostic departments continuously need to improve quality and learn from every failure. Within quality management systems the PDCA (Plan, Do, Check, Act) cycle is often applied for continuous quality improvement. The collected sample metadata can be used in a PDCA cycle to improve the output of diagnostics laboratories. The following PDCA cycle can be developed:

- PLAN: collect and compare correlations over the years for detection of trends.
- DO: determine if correlations are significant and if so, adjust the standard operating procedures using the SAD method to act and list when samples cannot be used for a particular test.
- CHECK: for false positive or negative results that can be correlated to pre-analytical events.
- ACT: use the new methods and reject samples that are not fit for purpose and order new samples instead.

The difficulty in this schema is to recognize all the false positive and negative results, because the real positive or negative result is not always known at the time the diagnostic test is performed. Including patient follow up data and checking if the test result is in line with the disease development and patient response to treatment over the years would increase that chance. The cycle can identify the most critical events in the pre-analytical phase. It could be that methods in the pre-analytical phase are revealed as having a large negative influence on sample variation; they could be reconsidered using the SAD method. The significant findings should be presented or published in a suitable way for the diagnostic and research community, so it can help others to improve as well. Within the diagnostic community, it can be used to exclude samples from a test and order new ones and improve methods used, whereas in the scientific community the sample metadata can be used as inclusion factors for sample and/or cohort selection.

Big data

The new ‘big data’ approaches of this era can be exploited to speed up the process. A diagnostic laboratory can set up an artificial intelligence system to discover false positives and negatives on the bases of patient follow up data, which can subsequently be correlated with sample variations based on sample metadata to identify the possible causes of the false positives and negatives. The duration could be shortened if diagnostic laboratories formed networks in which this data could be shared and stored for trend analysis. This centralized database could publish its findings online for the diagnostic laboratories that share the data. These laboratories could publish their findings to the scientific community. A very important prerequisite for such a plan is that the diagnostic laboratories and research laboratories that have organized biobanks have access to the data already stored in the hospital information systems in such a way that the data is accessible and can be added as sample metadata to the sample data. This is a recommendation in the ISO documents, where this is needed.

Future fit for purpose samples

The sample or cohort selection to be used in a study can become much more detailed on the basis of sample metadata. During the study selection process, samples can be rejected early on, according to inclusion criteria based on detailed arguments which should be published when reporting the study. Outliers can be correlated to pre-analytical events and reported. Within publications a reference can be made to the reason for rejection of certain data sets based on publications on known (correlated) variations in the pre-analytical phase or on sample metadata. Samples are best documented with their metadata and can be kept as raw data within the study. When comparing studies for their reproducibility, the sample metadata can be taken into account in the comparison. Companies that propose to use study results as a basis for developing a new product for the diagnostic market can use the sample metadata to estimate if the samples used in the study are comparable to the diagnostic situation or whether they might first require a validation before product development is considered.

Conclusions

If both the research and the diagnostic communities implement the ISO documents on the sample pre-analytical phase, sample quality would be brought to a higher and more comparable level thereby reducing irreproducible results. As a consequence, tests can be less robust than they need to be nowadays to find their way into the diagnostic laboratory, because validation for intended use has become more reproducible. In addition, the acquired sample documentation could be used as sample metadata to further increase reproducibility of biomedical research and diagnostics. It will take time and organization of infrastructure in order to share the benefits of sample metadata in the future, but when employed, sample metadata can provide two important tools:

- Tool 1 - Identification of the source of variation in the sample pre-analytical phase.
- Tool 2 - Provide guidance for sample exclusion before a diagnostic test or during study cohort sample selection.

Academic hospitals would need to provide infrastructure for diagnostics laboratories and biobanks to make the first steps towards collections with availability to sample metadata as a tool for identification and control of sample variation:

- Access to the required data in the hospital information system available to all diagnostic and research collections as part of the sample metadata.
- Organize biobanking in a central biobank facility, preferably using collection points in the different key departments in the academic hospital.
- Implementation of the ISO documents on sample collection in the diagnostic laboratories and biobanks.
- Organize national and international standardization via existing collaborating biomedical diagnostic laboratory and research biobank platforms.

Such an approach could result in well documented samples with exchangeable or comparable quality in diagnostic laboratories and biobanks worldwide.

Currently we do not have sufficient tools which properly identify if or what sample variation could be the cause in relation to divergent test results. This means that if studies are currently declared as irreproducible, the individual studies might actually still be solidly based on their study results. The conclusion that the studies are not solidly based on the results cannot be drawn directly because the real cause of the deviation might be that different pre-analytical methods were used. It is not known if there might have been influences from variations in the pre-analytical phase, causing different batch effects hiding or showing different or upregulated parts of the test signals. In such cases irreproducibility might confirm that the pre-analytical variations are not yet under control.

In the end, the robust tests are the best ones to use for diagnostic purposes. The comparison of studies on reproducibility [1–12] and the studies to correct batch effects [27–30] is very useful to reveal common and robust results. Where comparisons do not reveal a common signal, it may pinpoint areas where the biomedical field is not yet in control of the entire chain of events leading up to the end result. It could be the study design, analysis methods used and or reporting of the results that are not up to standard, but it might also confirm that the pre-analytical sample variations are not yet under control.

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