Official International Association for Therapeutic Drug Monitoring and Toxicology guideline

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Published in:
Therapeutic Drug Monitoring

DOI:
10.1097/FTD.0000000000000643

Publication date:
2019

Therapeutic Drug Monitoring Publish Ahead of Print
DOI: 10.1097/FTD.0000000000000643

Official International Association for Therapeutic Drug Monitoring and Toxicology guideline: Development and Validation of Dried Blood Spot-based Methods for Therapeutic Drug Monitoring

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Funding

the artwork was financially supported by IATDMCT, UGent and UMCG. Furthermore, S. Capiau wishes to acknowledge the FWO Research Foundation Flanders for granting her a PhD fellowship (application number 11R7316N).

Acknowledgement

The authors thank M. Volmer of the UMCG for his assistance in writing the statistical paragraph and Anoek Houben for preparing the art work.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Keywords

Dried Blood Spots, Guideline, validation, microsampling, VAMS

Abbreviations Page

CLSI, Clinical and laboratory standards institute

CV, coefficient of variation

DBS, Dried blood spot(s)

DPS, Dried plasma spot

EBF, European bioanalysis forum

EMA, European medicines agency

EVF, Erythrocyte volume fraction

FDA, Food and drug administration

HT, Hematocrit

IS, Internal standard

ISR, Incurred sample reanalysis

LLOQ, Lower limit of quantification

LoA, Limits of agreement

LSS, Limited sampling strategy

ME, matrix effect

QC(s), Quality control(s)

RE, Recovery

RSD, Relative standard deviation

TDM, Therapeutic drug monitoring

ULOQ, Upper limit of quantification

VAMS, Volumetric absorptive microsampling

Content

1. INTRODUCTION

Dried Blood Spot (DBS) analysis has been introduced more and more into daily practice.[1] To assure the quality of bioanalytical methods and to assure that the results obtained with those methods are valid, it is of utmost importance that newly developed methods are fit for purpose. Those methods must have undergone adequate method validation and are monitored *via* a suitable quality control (QC) program. Absence of DBS-specific method validation guidelines results in DBS-based methods lacking essential validation aspects with reduced credibility.[1-4] Validation requirements described in guidelines for the quantitative analysis of traditional matrices (i.e. liquid blood, plasma or serum) are not always easily translated to analysis of dried blood spots.[5,6] Moreover, several additional parameters, like volume- and hematocrit (HT) effects, which are not part of traditional guidelines, are often overlooked or not adequately assessed.[7]

Therefore, this guideline aims at defining the parameters necessary for the validation of quantitative DBS-based methods and to provide advice on how these can be assessed. In addition, guidance is given on the application of validated methods in a routine context. The recommendations in this guideline are based on existing guidelines for traditional matrix analysis -in particular the bioanalytical method validation guidelines issued by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA),[5,6] the guideline for measurement procedure comparison provided by the Clinical and Laboratory Standards Institute (CLSI)[8], several white papers on dried matrix analysis,[9-11] as well as other published work and the personal experience of the authors.

The focus of this guideline is the analysis of DBS for the quantitative determination of small molecule drugs and drug metabolites using chromatographic techniques for therapeutic drug monitoring (TDM) purposes. However, many elements of this guideline are also relevant for the analysis of samples obtained *via* volumetric absorptive microsampling (VAMS) and for

dried plasma spot (DPS) analysis, as well as for the analysis of DBS for purposes other than TDM.

As the successful validation of a DBS-based analytical method starts with method development, this guideline commences by outlining the potential pitfalls encountered during that stage (part 2A, 2B, 2C). Furthermore, the importance of pre-validation stress testing is highlighted (2D). In a next section, the actual method validation is extensively discussed (part 3 and 4). This validation section encompasses both the analytical validation (comprising both the classical and the DBS-specific validation parameters) and the clinical validation (i.e. demonstration of equivalence between DBS-based results and results obtained in the classical matrix). Finally, quality control is briefly discussed (part 5). A summary of this guideline can be found in Supplement S-1, http://links.lww.com/TDM/A342.

2. METHOD DEVELOPMENT: CONSIDERATIONS FOR SUCCESSFUL VALIDATION

Before embarking on the set-up of a DBS-based procedure, it is essential to carefully think about the purpose of the method. Certain considerations need to be made to ensure the suitability of the method for a given application (i.e. to ensure the method is fit for purpose) already in this early stage. These considerations are discussed below, and the different options are schematically summarized in Figure 1. Furthermore, stress testing of the method during method development will allow potential issues to be detected at an early stage, which will eventually increase the chances of a successful method validation and application.

A. CONSIDERATIONS REGARDING SAMPLE COLLECTION

Collection procedure

Nowadays, the most frequently used dried blood sample collection method is the collection of a non-volumetric drop of blood (DBS), free falling or by touching onto a filter paper (i.e. directly from a finger prick or heel stick). Alternatively, the blood sample may be deposited volumetrically using a capillary or a pipette. Furthermore, several blood collection strategies exist in which a volumetric dried blood sample can be directly generated from a non-volumetric drop of blood, without the use of pipettes or handheld capillaries. These strategies include i.e. HemaXIS[12], HemaPEN®[13], Capitainer[14-16] and VAMS.[17-19] In addition, dried plasma spot (DPS) may be collected rather than DBS. These DPS may be generated either by centrifugation of a liquid blood sample and subsequent application of an amount of plasma onto a filter paper or by using a device which allows *in situ* DPS generation.[20-23]

While some of the above-mentioned collection strategies may allow patient self-sampling (e.g. non-volumetric DBS collection[24], VAMS™[25], *in situ* generated DPS), other collection methods (e.g. volumetric DBS collection using exact volume capillaries, DPS generation following centrifugation) require trained professionals and/or laboratory equipment. Although the latter strategies are not suitable for home sampling, they may still be valuable in another context. DPS generation *via* whole blood centrifugation and pipetting may, for example, be a suitable approach if DPS are prepared in a laboratory in a remote or resource-limited setting to allow more convenient transport to a centralized or reference laboratory.[26] Additionally, other parameters such as required sample volume, automation capabilities, commercial availability, the cost of a given microsampling device, as well as overall costs, may also play an important role in the selection of the sample collection method.

Selection of the type of filter paper

If samples are to be collected on filter paper, the type of filter paper (card) that will be used needs to be carefully chosen. The type of filter paper may affect the occurrence of interferences, the blood's spreading behavior, sample homogeneity, as well as analyte stability and recovery.[27-29] Commercially available filter paper can either be untreated (e.g. Whatman® 903, Ahlström 226, DMPK-C), or pretreated with e.g. denaturing agents or enzyme inhibitors (e.g. DMPK-A or DMPK-B).[30] Furthermore, in certain DBS-based methods, in-house pretreated filter paper has been used to increase analyte stability or recovery.[31-34] Moreover, some types of collection devices have been reported to be less affected by the HT effect and may help to overcome this issue.[35,36] Additionally, chitosan and alginate foams have been proposed as collection substrates to help increase analyte recovery, as they dissolve during sample extraction.[37] Although most DBS-based bioanalytical methods use regular, cellulose-based, untreated filter paper (cards), for certain applications it may be valuable to evaluate the use of pretreated or non-cellulose-based alternatives. However, it needs to be kept in mind that the use of non-commercially available substrates may hinder a generalized application of the method and requires in-house assessment of batch to batch quality.[38]

Interferences originating from the collection substrate

It is advised to analyze some blank collection cards during early method development to assess whether the collection material itself is blank and whether there are any interferences present that need to be separated chromatographically from the target compound(s).[28] If one of these issues occurs, it might also be valuable to evaluate different collection substrates.

Sample volume

The amount of sample that is required for a certain analysis will mainly depend on the envisaged lower limit of quantitation (LLOQ) and is inherently linked to the available instrumentation. However, the minimally required volume should always relate to how the samples are collected. For the set-up and validation of the method a sample volume representative of the sample volume of the patient samples needs to be employed. Most people will typically generate DBS of 20 to 70 µL if free falling drops of blood are collected, whereas somewhat smaller DBS-typically 15 to 50 µL-will be obtained if a hanging blood drop is collected by bringing it into contact with the filter paper. With the latter approach, it is essential that only the blood drop and not the fingertip touches the filter paper. If a DBS is smaller than what is typically expected, this may be an indication that the fingertip came into contact with the filter paper. On the other hand, if a DBS is larger than expected, multiple drops were likely collected. Obviously, whenever samples are collected volumetrically, the sample volume will be determined by the employed device. If a larger volume of blood is required to reach the LLOQ, sometimes punch stacking is used.[39] Nonetheless, the number of punches required for a single analysis should remain as small as possible, to limit the amount of good quality samples that needs to be collected and to allow incurred sample reanalysis.

Drying and storage process

A parameter that is often neglected in DBS-based methods is the impact of drying time. If the sample is not completely dry before putting it in a zip-locked bag for storage, microbiological growth may occur and compromise sample quality.[40] Furthermore, improper drying might also affect analyte stability and recovery.[41,42] Therefore, it is advised to dry samples at least 3 hours under ambient conditions (preferably without direct sunlight) and to store them with a

desiccant, which will remove an additional 5% of water from the dried samples.[40,43] In certain settings, however, the required drying time may be longer, as this depends on the ambient temperature and humidity, the sample volume and the type of filter paper.[42] In other settings, shorter drying times may suffice. Therefore, it is relevant to evaluate during early method development whether the drying time is adequate under the conditions likely to be encountered during the collection of the patient samples. This evaluation is preferably performed using DBS with a HT in the upper range of the HT of the target population and, if applicable, a large sample volume, as these will dry the slowest.[27] Furthermore, the ambient temperature and humidity during drying have been suggested to affect DBS homogeneity (although this effect also depends on the type of filter paper that is used).[44] Similarly, also the storage conditions should mimic the ambient conditions encountered during patient sample transport/storage.[45]

B. CONSIDERATIONS REGARDING SAMPLE PREPARATION

Punch size

For volumetric DBS applications, the punch size needs to be large enough to punch out the entire DBS, independent of the HT of the sample. Hence, it is advised to select the required punch size based on samples with a HT of approximately 0.15, since this HT level will be lower than the lowest HT level of the patient population and will therefore yield DBS that are (slightly) larger than the largest expected patient DBS. The punches can either be made after application of the blood spot to the substrate or in advance.[46-48] For non-volumetric DBS applications, partial DBS punches are made that exclude the outer edge of the sample. If relatively small punches are made (≤ 4 mm or approximately 5.7 µL), most patients should be able to generate multiple DBS that are large enough to analyze. However, larger punch sizes

may be required to obtain the desired LLOQ to increase method accuracy and imprecision or to exclude DBS homogeneity issues. Although generating larger DBS will be somewhat more difficult for a patient, when properly educated and trained, the vast majority of patients will be able to provide at least 1 or 2 samples that are large enough to make punches up to 8 mm (± 20 µL). The latter will also be easier if falling-drop-collection is used rather than hanging-drop-collection.

Internal standard incorporation

Ideally, an internal standard (IS) is mixed homogenously with the biological sample before sample preparation to compensate for any variability throughout the entire analytical process. Unfortunately, this is difficult to achieve with a DBS. For DBS analysis, the closest alternative is to spray the IS evenly onto the sample prior to extraction.[49] However, this requires the availability of a validated dedicated spraying system, which is not available in the majority of laboratories. Another option is to pre-coat the filter paper with the IS.[50] However, in that case the IS needs to be applied to a larger surface, as it is not known where exactly the sample will be deposited. Furthermore, the IS should be stable for a sufficiently long period of time (i.e. during sample collection, transport, storage and analysis). In addition, the same batch of IS solution should be used for calibrators, QCs and patient sample collection cards, which is not feasible on a large scale. Another potential side-effect of pre-coating filter paper with IS (in the absence of matrix) is that the IS may show different recovery than the target analyte. To the best of the authors' knowledge, such strategies have not yet been evaluated for other dried blood samples nor has a successful application of IS-pre-coated micro capillaries been described. Again, such an approach would require the availability of tailor-made devices, which will be at the expense of additional costs. In the majority of DBS-based methods, the IS is added to the extraction solution or directly to the

DBS punch before extraction and will hence not compensate for variability in analyte recovery.[9,51] Therefore, analyte recovery must be investigated extensively under different conditions (see below) during method development and validation.

C. OTHER IMPORTANT CONSIDERATIONS

Type of blood used

For the set-up of calibration curves and internal QCs, it is from a practical point of view impossible to use capillary blood samples derived from a finger prick. Instead, spiked samples generated from venous whole blood containing an anticoagulant are used. Which type of blood is best suited for this purpose largely depends on how patient samples will be collected. If the DBS collection device that is used to generate the patient DBS contains a certain anticoagulant, the venous whole blood also needs to contain that same anticoagulant. On the other hand, if no anticoagulant is used during the collection of the patient samples, theoretically, the blood used to set up the calibration curves and QCs also has to be non-anticoagulated. Unfortunately, it is very impractical to prepare spiked samples from non-anticoagulated blood, as blood will start coagulating almost immediately after collection. Therefore, in most cases, a suitable anticoagulant will have to be selected. It is essential that the use of this anticoagulant does not impact the obtained results and that the stability of calibrators and QCs reflects that of real samples. Hence, we strongly advise to compare in an early stage results obtained from a non-anticoagulated sample with results from patient samples anticoagulated with different anticoagulants.[52] These blood samples should all be obtained venously from the same volunteer or patient at (approximately) the same time and should be analyzed in quintuplicate. Based upon the knowledge about the (lack of) impact of certain anticoagulants in liquid blood, some anticoagulants may readily be excluded. For

example, if analytes are e.g. stabilized by oxalate/NaF, this type of blood should preferentially not be used to assess the analyte's stability in DBS (which in practice would not contain that stabilizing anticoagulant). On the other hand, if the anticoagulant stabilizes the analyte, and anticoagulant-containing DBS are commutable in any other way with DBS without anticoagulant, the former could be used for the set-up of calibrators and QCs as the prolonged analyte stability could help ensure consistent calibration.

Preparation of spiked samples

A first step in the preparation of spiked samples is to adjust the HT or erythrocyte volume fraction (EVF) of the whole blood to the desired HT value. For most experiments the latter will correspond to the mean or median HT value of the target population.[53] Although there are several ways of preparing samples with a certain HT, the preferred procedure is to measure the HT of the original blood sample with a hematology analyzer and to calculate how much plasma needs to be added or removed to obtain the desired HT value.[54] After the addition or removal of the plasma, it is important to measure the HT again, to assure the sample was prepared correctly.

In a next step, the analyte needs to be spiked into the blood. It is important to only spike a limited volume of analyte solution to the blood (i.e. < 5% of the sample and preferably even less) to not change the nature of the sample.[5] Moreover, the addition of a larger volume of solvent would also change the sample's viscosity and/or cause cell lysis, thereby affecting its spreading behavior through the DBS filter paper. Furthermore, organic solvents may denature proteins. To further minimize the effect of the spiking volume on the sample's spreading behavior, stock solutions can be diluted with plasma, rather than with water or another solvent, if solubility allows for it. After spiking the blood with the target analyte, the samples

should equilibrate for a sufficient amount of time at a suitable temperature to mimic the analytes' *in vivo* RBC/plasma distribution.[55]

D. PREVALIDATION – STRESS TESTING

Exploratory tests

As with a traditional bioanalytical method, several exploratory tests need to be performed to assess whether a developed method is good enough to proceed towards validation. As with any chromatographic method, several technical aspects should be checked early on during method development, e.g. the absence of carryover and the influence of the sample matrix on the chromatographic method. Furthermore, the stability of the stock solutions used for the spiking of the calibrators and QCs should be guaranteed. Particular points of attention during prevalidation for DBS-based methods are short-term stability and extraction efficiency.

Although DBS generally tend to improve analyte stability, this is not always the case. Enzymatic analyte degradation may readily occur during the drying process.[56] Furthermore, oxidation sensitive analytes are likely to suffer from stability issues, since DBS are exposed to air during drying and/or storage.[30] If low signals are obtained from fresh samples (e.g. compared to a standard solution with the same concentration), this might be due to stability issues during the drying process. In addition, these low signals may also be caused by matrix effects, poor extraction efficiency or a combination of the above.

When using LC-MS/MS, the presence of matrix effects can be evaluated using post column infusion. If present, these matrix effects may be eliminated by further optimization of the sample preparation and/or the chromatography. Poor extraction efficiency may be due to the analyte's interaction with the carrier or with endogenous matrix compounds.[29,57,58] However, the differentiation between extraction efficiency issues and actual analyte instability may not

be so straightforward.[34] To get an idea about potential stability issues, existing literature about the stability of the analyte in whole blood or about the chemical and physical properties of the analyte may be a good starting point. If degradation during sample drying is anticipated (e.g. for compounds with a very short *in vitro* half-life), flash heating may improve the analyte's stability (at least if the analyte is thermostable), as this inactivates the enzymes.[56] Unfortunately, this strategy is not suitable for home sampling. Nonetheless, it may help to figure out the cause of the poor method outcome. Other strategies to help improve the analyte stability may include pre-impregnating the collection substrate with anti-oxidants or buffers.[34,59] However, these strategies may hamper generalized application of the method. For some analytes, instability issues remain unsolved, when taking into account a restrictive time frame for transportation of DBS. In those cases, it should be decided that dried blood sampling for that analyte is not feasible. In specific situations, a volumetrically obtained sample could be brought into a stabilizing sampling buffer shortly after.[60] When poor extraction efficiency is suspected, further optimization of the extraction procedure may be required (i.e. the evaluation of different extraction solvents, additives and extraction temperatures, as well as more rigorous extraction techniques (like sonication). Furthermore, the use of different (pre-treated) collection cards/devices may also help to improve the extraction efficiency.

At this stage, it should also be evaluated whether the obtained results are affected by the time between sample collection and analysis. More particularly, the results from samples analyzed at T_0 (typically between 30 minutes-3 hour after sample generation, depending on the required drying time) should be compared with results obtained at later time points, preferably up to 48 or 72 hours. This experiment is important since time-dependent extraction issues have been described.[61] More specifically, if the recovery decreases for the first (couple of) time points, but remains stable afterwards, it may still be possible to obtain good

analytical results. This implies to only analyze samples older than a specified time point. Obviously, this strategy should not only be implemented for the patient samples, but also for the calibrators and QCs.

Evaluation of the robustness of the extraction procedure and short-term stability

In a next step, the robustness of the extraction procedure should be thoroughly investigated. This is a crucial experiment, since in most DBS applications, the IS is not capable of correcting for variability in extraction efficiency. The extraction efficiency may be concentration, HT and time-dependent and, importantly, these parameters may also affect each other.[41,62-64] HT-dependent extraction efficiency may be present or more pronounced at one concentration level compared to another.[64] Similarly, time-dependent extraction efficiency issues may occur earlier at a more extreme HT level.

For non-thermolabile compounds, the occurrence of HT- and time-dependent extraction issues can be evaluated by comparing the results from fresh DBS at low, medium and high HT levels (with these HT levels encompassing the HT range of the target population; e.g. 0.20, 0.40, 0.60) with a second set of samples stored at 50-60°C for at least two days. This second set mimics thoroughly dried (aged) samples. This experiment should be performed at both the low and high QC level (see Figure 2). Furthermore, to simultaneously determine the actual extraction efficiency at both QC levels, and to evaluate the presence of matrix effects, also samples spiked after extraction and standard solutions should be included in this experiment. Moreover, each of these samples should be analyzed in quintuplicate. Additionally, along with these samples, a calibration curve and QCs have to be analyzed. Importantly, in case of partial DBS analysis, these samples should be prepared by the

accurate pipetting of a fixed amount of blood onto pre-punched filter paper disks to rule out any influence of the HT spreading effect on the amount of sample being analyzed.

When no relevant differences (i.e. ≤15%) can be observed between the results obtained from fresh DBS and those stored at 50-60°C, it is unlikely storage will have an impact on extraction efficiency. A good outcome in this set-up may also readily indicate good stability under ambient conditions, although this needs to be formally evaluated during method validation. However, it needs to be mentioned that the latter can also be affected by other parameters such as humidity and exposure to sunlight. Furthermore, by comparing the results of the samples at the three different HT levels (both for the fresh and the stored samples), the occurrence of HT-dependent extraction efficiency issues can be evaluated. Moreover, using the Matuszewski approach, recovery and matrix effect can be evaluated at both concentration levels and at three HT levels.[65] While performing this experiment may seem fairly elaborate at first, it may prevent serious problems at a later stage, which may require a complete revalidation (e.g. if the extraction needs to be adapted). Moreover, if successful, the evaluation of matrix effect and recovery may not have to be repeated at different HT levels during the actual method validation, as long as the method remains unchanged. Also, the evaluation of short-term stability at fairly extreme storage conditions (i.e. 50-60°C) is already incorporated in this experiment (cfr. section 3A).

For more thermo-labile compounds, a similar experiment can be performed with samples stored at room temperature for two weeks instead of at 60°C for two days. Although this is a less harsh experiment than the previously described one, it does cover a time span in which most clinical samples in a laboratory will have been analyzed. Alternatively, even lower storage temperatures may be used. However, if the analyte is not stable at room temperature

for at least a couple of days, the method will not be suitable. Obviously, if satisfactory, these data can also be used as part of the stability data required for method validation.

To minimize the number of samples that has to be analyzed at this stage, a simplified experimental set-up is suggested in Figure 3. In particular, this set-up does not include 'spiked after extraction' samples or standard solutions, and all samples are only analyzed in triplicate. This simplified set-up offers the advantage that if the extraction procedure has to be adjusted (and consequently, this evaluation has to be repeated), the number of samples that needs to be analyzed will not increase drastically. However, with this experiment, recovery and matrix effect will still need to be evaluated at different HT levels in a separate experiment during method validation.

If the results of the above-mentioned experiments are non-satisfactory, this may be due to instability of the target analyte or to extraction efficiency issues. If the results for the different HT levels differ significantly and/or substantially (i.e. >15%), this is due to a HT-dependent extraction efficiency issue and the extraction procedure needs further optimization. In this context, heated extraction, as well as the use of a mixture of organic solvents rather than a single organic solvent may be helpful.[62,63,66,67] Furthermore, the use of a different collection card may also help to resolve this problem. Possibly, depending on the target population, the procedure can be repeated with less extreme low and high HT values, to evaluate whether acceptable results are obtained for a more limited HT span.

A difference between the fresh and the stored samples, on the other hand, might be due both to a time-dependent extraction efficiency issue and to actual instability of the target analyte.[68] However, if this difference is not observed at all HT levels, it is unlikely that analyte instability is the culprit. If the difference is observed at all HT levels, it may be worthwhile to repeat the experiment at a lower storage temperature, as this may indicate analyte instability.

DBS homogeneity

In case of partial DBS analysis, it is essential to evaluate DBS homogeneity, i.e. to assess whether results from central punches are equivalent to peripheral (or decentral) ones.[69] By already evaluating this parameter during pre-validation, one knows whether during the next experiments it is required to make a central punch or whether a peripheral punch or multiple punches can be made from a single DBS.

This evaluation must be performed at two concentration levels (low QC and high QC), at different HT levels (low, medium and high) and at sample volumes representative of the anticipated patient sample volumes. Each of the evaluated conditions should be analyzed in quintuplicate. All samples should be compared to a calibration curve prepared with samples of medium HT level and average volume, of which a central punch was extracted. When both central and peripheral punches yield results within the standard bioanalytical acceptance criteria (typically, within 15% of their target value), the use of both types of DBS punches is considered acceptable.[69]

Obviously, this experiment only needs to be conducted if a central and a more peripheral punch can be made from a sample, which in turn will depend on the used punch size. When making peripheral punches, the very outer edge of the DBS should be excluded, as this has a different composition than the rest of the DBS (e.g. a higher amount of red blood cells, when using conventional Whatman® 903 filter paper). In addition, the back of the filter paper should always be checked to ensure that the peripheral punch is made in a part of the DBS in which the filter paper is saturated. Importantly, the samples should be prepared under similar conditions as the patient samples, as the drying process is known to influence DBS homogeneity.[27,70] Other parameters that may influence the equivalence between central and peripheral punches include the filter paper type, the position of the DBS card during drying, and the punch size (with larger punches being less affected by inhomogeneities within the

DBS sample). The presence of an anticoagulant on the other hand, does not seem to influence DBS homogeneity.[27]

3. ANALYTICAL VALIDATION

None of the currently existing bioanalytical validation guidelines have been set up for dried blood sample-based methods. Certain experiments described in these guidelines may not be applicable (e.g. freeze-thaw stability, depending on the storage and transport conditions), whereas others may require some refinement (see section A). Moreover, some additional parameters will have to be evaluated (see section B).[9,71] An overview of the required additional investigations can be found in Table 1. These will result in a slightly larger number of samples that will have to be analyzed during method validation (see Table 2). Before starting any analytical validation, it is essential to contemplate what the desired quality of the method should be. Although the analytical performance requirements described in e.g. the FDA or EMA guidelines are widely applied and accepted, they may not always be suitable for DBS methodology. Depending on the analyte and the purpose of the method, these requirements can be set either more or less strict based on scientific evidence. In this context, some have suggested to use acceptance criteria based on biological variation, as is common practice in other areas of clinical chemistry.[72]

A. CLASSICAL VALIDATION PARAMETERS TO BE EVALUATED

Most of the validation parameters described in traditional bioanalytical method validation guidelines will have to be assessed for DBS-based methods as well.[5,6] Therefore, those documents will need to be consulted too when performing a DBS method validation. However, the particular points of attention when evaluating those classical validation

parameters in the context of a DBS method, are given below. Furthermore, to assist the reader, a brief overview of these classical validation parameters is given in Table 3.

Selectivity

To assess the selectivity of the method, blank matrices of at least six different individuals should be analyzed without IS, as well as two zero samples (blank DBS extracted with extraction solvent containing IS). These blank samples should be obtained using the same sampling approach as the one that will be used to collect the patient samples. In addition, DBS prepared from blank blood spiked with common co-medications, metabolites, and other potential interferences could be tested. At this stage, it may also be worthwhile to run a few authentic patient samples to ascertain there is no non-anticipated co-elution of a metabolite that may not be available as a standard.

Calibration model, accuracy and precision, measurement range

For the evaluation of the calibration model, the lower and upper limits of quantitation (LLOQ and ULOQ), accuracy and precision, all experiments should be performed in accordance with existing guidelines.[5,6] The only difference is that all calibrators, blank, zero and QC samples should be prepared in blood with the median HT of the target population and should have a volume representative of the patient samples.[53] As with any bioanalytical method, the measurement range should be representative of the concentration range in patient samples. For the purpose of TDM, a calibration range minimally spanning from half of the lower end of the therapeutic interval to twice the upper end of the therapeutic interval should suffice. Furthermore, intra- and inter-card variability do not need to be evaluated separately, as these variables will be inherently included throughout the method validation.[9] For a method to be

applied in a routine context, inter-batch variability should be assessed. The latter can be done by including cards from multiple batches in the validation experiments. However, if non-certified filter paper is used, a more elaborate evaluation of the filter paper may be warranted.

Dilution integrity

Contrary to traditional liquid blood samples, DBS cannot be diluted directly. Hence, to analyze samples with a concentration above the measurement range, DBS extracts are typically diluted with blank DBS extracts or extraction solvent. Furthermore, IS-tracked dilution can be performed.[6,73] With this approach a higher concentration of IS is added to the extraction solvent, with the exact amount of IS depending on the envisaged dilution factor. This approach renders the dilution a volume-non-critical step. In addition, for DBS, the donut punch approach can be used.[74] With this approach, a small central punch (i.e. smaller than the regular punch size for a given DBS method) is made from a DBS sample and is extracted simultaneously with a donut punch prepared from a blank DBS sample. This donut punch is a regular sized DBS punch from which a small central punch (with the same punch size as used for the actual DBS sample) has been removed. However, to use the latter approach successfully, DBS homogeneity should be adequate for the small punch size and the extraction efficiency should not depend on the punch size.

Carryover

Aside from classical carryover, in a DBS workflow, the punching step could be considered a potential source of contamination. Hence, we propose to include in the method validation the processing of one or more blanks following the processing of the highest calibrator.[9] To the authors' knowledge, however, no punch-mediated carryover has been described for

(therapeutic) drugs, although it has been observed for PCR-based methods.[75] In addition, physical carryover between cards should be avoided by storing the cards separately. However, if multiple cards will be stored together, potential carryover between cards requires evaluation.[9] The same acceptance criteria as for classical carryover should be applied.[5,6]

Matrix effect, recovery and process efficiency

Matrix effect, recovery and process efficiency should be evaluated in line with the set-up proposed by Matuszewski et *al*. (also see section 2).[65] For this experiment, blood from at least six different donors should be used and two concentration levels should be evaluated (i.e. low and high QC level). In addition, since it is known that the HT may strongly impact the recovery -and possibly also the matrix effect- it is essential to evaluate recovery and matrix effect at different HT levels, prepared from the blood of at least one donor. These HT levels should encompass the anticipated HT range of the target population. Alternatively, this experiment could also be performed using five HT levels (0.20, 0.30, 0.40, 0.50, 0.60). The latter set-up has the advantage that whenever the most extreme HT values do not yield acceptable results, a narrower, acceptable HT range (regarding recovery and matrix effect) may still be determined, without having to repeat the experiment. This set-up is schematically depicted in Figure 4. As mentioned before, to accurately perform this experiment, a fixed volume of blank or spiked blood needs to be applied on pre-punched filter paper discs.

Although matrix effects are preferably as small as possible, recovery and process efficiency as high as possible, the exact values are not that relevant. It is essential, though, that they are reproducible (i.e. relative standard deviation or % RSD within 15% after IS-normalization). It is relevant to note that observations by Abu-Rabie et *al*. suggest that extraction procedures with lower recoveries may be more subject to an impact of HT (see 3B).[49]

Stability

The stability assessments performed during method validation should be representative of the ambient conditions encountered during sample transport, storage and processing. Therefore, stability should be evaluated at room temperature (the exact temperature depending on where the method will be applied) and the investigated time frame should cover the maximum expected time frame between sample collection, analysis and potential re-analysis. Furthermore, since temperatures may be significantly higher during transport (e.g. in a mail box in the sun during summer time), short-term stability at elevated temperatures (i.e. 2 or 3 days at 50°C – 60°C, or higher temperatures depending on the country) should also be tested.[45,76] If stability under ambient conditions is only sufficient for a couple of days (but long enough to allow transport to the laboratory), it may be evaluated if storage at lower temperatures in the lab may help stabilize the DBS until (re)analysis.

Importantly, stability may also be affected by other parameters such as humidity and exposure to (sun)light, conditions which are harder to replicate in the laboratory. To evaluate the effect of actual sample transport, samples which are generated in the laboratory can be analyzed immediately after drying, after storage for a certain time under controlled conditions and after sending them to the laboratory via mail service. Preferably the samples are deposited in a mail box which is relatively far from the laboratory. Furthermore, it may be relevant to repeat this experiment under different weather conditions, to rule out any seasonal effects on the stability of the samples. Although stability is typically evaluated using spiked samples, it may be worthwhile to also evaluate the stability of incurred samples, as spiked samples may not always display the same stability profile as actual samples.[77] Additionally, post-preparative stability should be assessed.

B. DRIED BLOOD SPOT-SPECIFIC VALIDATION PARAMETERS

The analytical validation of DBS methods requires the evaluation of several additional parameters (see Table 2): i.e. the volume effect, the volcano effect (i.e. DBS homogeneity) and the HT effect.[1,9,71] It is essential that these parameters are assessed simultaneously, as they may affect one another. These parameters can be evaluated in a single day experiment in which the obtained results are compared to those obtained from the reference condition (i.e. central DBS punches generated from DBS of average or median volume and HT). Alternatively, this evaluation can be combined with the accuracy and precision experiments (i.e. by measuring two series of DBS samples with different volumes, different HT levels etc. on each of three days). The latter approach has the advantage that accuracy profiles can be established.[78,79] Importantly, if a certain effect is observed (i.e. a relevant volume, HT or volcano effect) appropriate measures need to be taken to ensure patient samples are within the validated limits and patient results are reliable. Obviously, it should also be demonstrated that these measures are indeed adequate.

Volume effect

The volume range in which DBS-based results are still acceptable should be defined during method validation. Typical volume ranges to be evaluated are 10-50 µl for hanging-drop-collection and 20-70 µl for falling-drop-collection. The volume effect should also be evaluated at low (0.30), medium (0.40) and high (0.50) HT and at both low and high QC as shown in Figure 5. Whether a sufficient volume is collected from a patient should always be evaluated in the laboratory before DBS analysis. This evaluation should be performed based on the diameter of the DBS. More particularly, the diameter of the patient DBS should be between the diameter of the DBS prepared from the smallest validated volume at low HT and

the diameter of the DBS prepared from the largest validated volume at high HT. To help patients to collect DBS of adequate volume, filter paper with two concentric circles may be used (see Figure 5).[80] These circles should correspond to the minimally required volume and the maximally allowed volume (also taking into account different HT levels, as described above).[80] It should be noted, however, that this type of filter paper is not commercially available. Furthermore, although these circles may be printed onto commercially available filter paper, it should be considered that the printing itself may affect the analysis (interferences from ink or toner, potential effect on blood flow e.g. caused by paper compression or wax-like materials present in toner). Therefore, the printed filter paper should be used during the entire method validation. Alternatively, equivalence between the in-house printed filter paper and the filter paper used during validation should be demonstrated at both low and high QC levels, and at low, medium and high volume and HT. In addition, the volcano effect might have to be re-evaluated, depending on the DBS punch size. Another option is to use a phone app to assess whether the generated DBS are within the validated volume ranges.[81] Again, correct performance of the app should be verified during method validation using samples of known volume, covering the entire validated volume and HT range.

Volcano effect

Spot homogeneity should be evaluated when embarking upon partial-spot analysis (also see part 2, pre-validation). If a relevant volcano effect is observed (e.g. punches from the central part of the spot yield different analytical results then punches from edges of the spot), only central punches should be analyzed.

Hematocrit effect

As mentioned before, it is important to actually determine the HT of the calibrators and the samples used during method validation. This will assure the exact HT value and, consequently, the validated HT range. At least three HT levels should be evaluated, more particularly, a QC generated with blood that has the same HT as the blood that was used to generate the calibrators, bracketed by HT values that encompass the expected patient HT range. At each HT level, two concentrations should be tested. The HT range that needs to be evaluated depends on the target population (see Figure 6). For a quasi-universal method, the range should span from 0.20 to 0.65, although a narrower range will suffice for most applications.[80] The exact range will depend on the target population and should encompass at least 95% of the target population.[53]

Unless no relevant HT effect is observed over the entire HT range (both during analytical and clinical validation, cfr. part 4) or unless it is reasonable to assume that all patient HT values will be within the validated HT range, a method should be used to assess the HT of the patient samples. Besides confirming that the HT of the patient sample effectively lies within the validated HT range, this may also allow to perform a HT correction, to alleviate the HT bias.[82,83] Other options are to use volumetric dried blood samples (if there is no HT effect on recovery or matrix effect) or DPS (if there is no HT effect on DPS generation).[36]

C. VALIDATION OF ONLINE DBS ANALYSIS

Whether the sample preparation and analysis are performed online or not does not affect the validation parameters that need to be evaluated. The way in which certain parameters (more particularly, recovery, matrix effect and process efficiency) are evaluated, however, will need to be adapted.[84-87]

Recovery is typically evaluated by comparing the peak areas from blank matrix samples spiked before extraction with the peak areas from blank matrix samples spiked after extraction. However, with an online sample preparation procedure, there is no option to spike the samples after extraction. Instead, the analytes are introduced to the system during the extraction step. Depending on the type of system used, this can be done via the IS loop or by spiking the extraction solvent. The results of the samples spiked during extraction are then compared to those of DBS samples containing the same absolute amount of analyte. This requires the entire DBS to be analyzed. When adding the analyte during extraction, the analyte passes through the filter paper and dried blank blood matrix, during which, theoretically, some analyte adsorption may occur. If such adsorption occurs, this will yield a falsely lowered '100% extracted' reference value, which in turn will result in an overestimation of the analyte's recovery. Alternatively, recovery may be evaluated by comparing the peak area resulting from a single extraction with the sum of peak areas resulting from, for example, 10 consecutive extractions. It needs to be considered that even after 10 extractions, not all the analyte may be extracted, again leading to an overestimation of the recovery. Moreover, these multiple extractions may technically not be possible because of filter paper deterioration (depending on the type of filter paper used).

For the evaluation of the matrix effect, the peak areas resulting from the analysis of blank DBS samples and blank DBS cards can be compared. In both cases the analyte will be introduced during extraction.

4. CLINICAL VALIDATION

It is generally accepted that a DBS sampling method can only be implemented in the routine care for the purpose of TDM − and thereby (partly) replacing the standard venous whole blood sampling with blood, serum or plasma analysis − after it has been successfully validated in a clinical validation study.[1,88-91] In a clinical validation study, paired DBS and venous blood, plasma and/or serum samples are obtained and analyzed. The analytical results are compared and statistically evaluated. The purpose of a clinical validation is to demonstrate that results from DBS are interchangeable with those obtained with the standard method used for TDM, i.e. a blood, serum or plasma analysis. The aim of this part of the guideline is to provide recommendations on how to clinically validate a DBS assay for TDM in daily practice. Current recommendations regarding clinical validation are largely based on published clinical validation studies that used genuine finger prick blood-derived DBS, paired DBS and traditional matrix samples from at least 20 patients, and appropriate statistical analysis to compare both methods.[90-102]

Concentration range, number of clinical samples and patients

The concentration range that needs to be covered during clinical validation depends on the sampling time points of interest (i.e. trough, peak) and the shape of the pharmacokinetic time curve of a particular drug and the intra- and/or interindividual variability.[2] The CLSI guideline states that at least 40 patient samples should be analyzed for a clinical validation, ideally covering the entire measuring interval of the measurement procedures.[8] This sample size is based on linear regression described by Linnet *et al.*[103] The sample size that is necessary mostly depends on the coefficient of variation (CV%) of the method and the range ratio (maximum value divided by minimum value). Because most DBS methods have a CV%

> 5% and a range ratio > 25, the number of samples needed following Linnet's calculation will always be 36 or 45. Therefore, using fewer than 40 samples is only possible if the CV% of the method is <5% and/or the range ratio <25. Depending on the situation, these 40 samples could either be paired capilarry DBS-venous blood samples from at least 40 different patients collected at a single time point (i.e. trough or peak), or paired samples taken at 2-3 time points and from a smaller cohort, covering the whole concentration range of interest.[8,103] Ideally, a total of 80 samples obtained from at least 40 different patients should be acquired for validation. This allows using one set of 40 randomly selected samples for fitting a line between DBS and blood (or serum or plasma) concentrations using appropiate statistical tests (see next paragraphs). If required, this will derive a conversion formula or factor to convert e.g. capillary DBS concentrations into venous plasma concentrations. The other set of 40 samples can be used to validate this conversion.[104] Despite the limitation of collecting multiple samples from the same patient this approach does not require a new cohort of 40 subjects. If the amount of patients is limited and multiple samples from the same patient (e.g. trough and peak) are acquired, it is our recommendation to have a minimum of 40 samples from at least 25 different patients to account for variation in matrix effects. In those cases where there is only a limited number of paired samples available, the conversion of a concentration in one matrix to that of another can also be checked for by a jackknife method. In this approach, the original set of n samples is resampled n times by systematically creating all possible subsets of n-1 samples. Each of these subsets is then used to set up a conversion equation, which is subsequently applied to the n^{th} sample (i.e. that sample which was not included in the subset that was used to set up the conversion equation).[105] To assess the predictive performance of the conversion equation, the Median Percentage Predictive Error (MPPE) = median (corrected $[Analyte]_{Test\ matrix}$ - $[Analyte]_{Reference\ matrix}$)/ $[Analyte]_{Reference\ matrix}) * 100\%$ and Median Absolute Percentage Predictive Error

(MAPE) = median ($\left|\text{corrected } [Analyte]_{Test\ matrix} - [Analyte]_{Reference\ matrix}\right| / [Analyte]_{Reference\ matrix}) * 100\%$ can be calculated. These provide a measure of bias and imprecision, respectively. [106,107]

Comparing DBS concentrations to plasma or whole blood concentrations and effects of hematocrit

Peripherally collected blood consists of a mixture of venous and arterial blood and interstitial fluids. Therefore, the drug concentration in peripherally collected blood may differ from venously collected blood. This effect is mostly present during the distribution phase of the drug. Although drugs are usually rapidly distributed throughout the body, this process sometimes can take up to several hours, leading to unreliable results when samples are collected during the distribution phase.[2,108-110] To detect a potential capillary-venous difference (Figure 7), the results obtained from a DBS collected from a finger prick (sample A) can be compared with those from a DBS prepared from venously collected blood (sample B). This venous blood (sample C) can be used to generate plasma (sample D). Both sample C and D can be compared to blood collected by finger prick (sample A). Alternatively, another blood sample needs to be collected at the same time point if serum (sample E) is to be prepared. Serum or plasma is typically used for routine TDM. It is essential that samples B and C should give the same result. If they do not, this points to an effect of the DBS approach *in se*.

In vivo, drugs can bind to components of plasma or accumulate in red blood cells, leading to differences between observed concentrations in whole blood (and, hence DBS) and in plasma (or serum, depending on the matrix that is routinely used for an analyte).[98,108] The difference in drug concentration between blood (DBS) and plasma can be explained by the fraction of

drug in plasma relative to whole blood, the HT and the drug's affinity for red blood cells. The study design may allow the generation of this blood-plasma relationship. If a blood concentration has to be expressed as a plasma or serum concentration for easy interpretation by the clinician, HT values should ideally be measured, known or calculated for each blood (DBS) sample. Furthermore, when acceptance limits for the HT have been set based upon the analytical validation, one should actually know whether the HT of a given sample effectively lies within these limits. When comparing capillary DBS values to reference whole blood values, correction factors (sometimes based on HT) can be necessary and should be derived from clinical validation studies comparing whole blood values to fingerprick (capillary) DBS values.[89,91,92,95,97,111-115]

If, for a specified HT range, the analytical validation has demonstrated that a DBS analytical method is independent of HT (or dependency is within acceptable analytical limits, see above), confirmation is required in a clinical validation study by plotting the differences between DBS results and reference method results *vs* the HT. The slope of the resulting curve should not be significantly different from zero.[80] When this has been confirmed, plasma or serum concentrations can be calculated based on the equation derived from the Passing-Bablok or weighted Deming regression line.[91,101,116-120] If an analytical method has proven to be dependent on HT values during analytical and clinical validation using appropriate statistical tests, a conversion formula should include a correction for HT.[121,122] An example is the estimation of plasma values from DBS concentrations using the formula $1 - (\frac{HT}{100})$.[122] This will only be possible if there is a systematic effect from HT on estimated venous blood concentrations which is fixed within the relevant clinical range.[123] If this is not the case, the method might not be suitable for clinical application. If an HT-dependent method is to be used in routine care, the HT of the DBS should ideally be known. Procedures to derive HT from a DBS card include potassium measurements[80], noncontact diffuse reflectance

spectroscopy[52,83], near-infrared spectroscopy[124] or the use of sulfolyser reagent.[125] If for a HT-dependent method it is- because of technical or other reasons- not possible to know the HT of a DBS, clinical validation can be performed for a specific patient population, provided the HT range in that specific population is narrow and lies within the method's acceptance limits (Figure 6).[94,98] In many instances, the mean or median HT and range for a given patient population can be calculated from historical patient data.[53] For a different patient population, it should be determined whether a new clinical validation should be performed.[10,98,122] Another approach to cope with the HT effect is whole blood spot analysis using a fixed spot volume. A volumetric capillary or pipet can be used to apply a fixed volume of finger prick blood to the filter paper.[14,126,127] In this situation, no conversion formula to correct for HT is needed. However, it should be clear from the analytical validation that the HT has no impact on recovery or matrix effects.[89,91,95,97,115] Moreover, this can be at the expense of the simplicity of sampling and/or bring along additional costs.

Statistical methods and interpretation

Technically, a DBS clinical validation is a cross validation study because a candidate method (DBS-based) is compared to a reference method (blood-, serum- or plasma-based). Although guidelines from the EMA, FDA and CLSI include cross validation and subsequent statistical analysis of results, this paragraph provides additional recommendations and guidance for the interpretation of results.[1,5,6,8]

As part of a clinical validation, the results obtained from DBS and the reference method should be compared using appropiate statistical tests. To compare two methods, regression analysis should be performed to measure the correlation, followed by an agreement and bias estimation test.[8] As both the reference and the DBS method have some inherent variability so

that either Passing-Bablok or weighted Deming regression should be used instead of standard linear regression.[8,128-130] Both approaches have been used in various clinical validation studies.[91-102,131] Deming regression takes variability of both x and y into account, Passing-Bablok regression makes no assumptions about the distribution of data points and is more resistant towards outliers.[8,129,132] Various clinical validation studies have shown that the absolute difference between results from a reference and a DBS method is propoportional to the concentration, at least at higher concentrations. However, in these studies, sometimes only a few high concentration samples were available.[91,96,120] Theoretically, an outlier in this region would impose an inflated or deflated estimate of proportional difference. In this case, a Passing-Bablok regression analysis is the preferred statistical method.[8,133] Following regression analysis, a Bland-Altman difference plot should be made to assess the agreement between both methods and estimate the bias.[8] When using a (HT-dependent) conversion formula obtained from Passing-Bablok or weighted Deming regression, the Bland-Altman difference plot should be made using the (blood, plasma or serum) concentrations that were calculated from the DBS concentrations.[1,91]

Most clinical validation studies show some level of bias when performing a Bland-Altman test. While it may seem obvious that Bland-Altman graphs should be generated and interpreted in a correct manner, this is not always the case.[133] Several things can be deduced from a Bland-Altman difference plot. First, it can be observed whether there is an average bias between both methods and whether the 95% CI of this bias contains zero. Importantly, if the latter is not the case, it should have been formally decided beforehand what a clinically relevant or acceptable bias and corresponding limits of agreement (LoA) should maximally be. For instance, for tacrolimus, where trough concentrations in blood are usually between 5-20 µg/L, a bias of 0.28 µg/L (LoA -0.45 µg/L – -0.12 µg/L), which is at most a bias of 5.6% (LoA 9.0%-2.4%) would not impact clinical decision making, whereas a higher bias or LoA

might.[134] Second, the LoA's can be derived from the Bland-Altman plot. Here, the same holds true: pre-set criteria are needed to define what concentration or % difference span between the LoA's is still considered acceptable. This is a critical point that in many instances is lacking: e.g. whereas on average there may be no bias between a DBS- and blood-based procedure, the span of the LoA's may be too wide (implying there is too much variation) to be acceptable. What is considered acceptable in terms of bias or LoA will largely depend on the clinical setting, the lab's internal policy, the availability of guidelines (e.g. RCPA criteria)[135] and the drug of interest. Acceptance criteria should be decided by a multidisciplinary team of experts based on both clinical and analytical acceptance criteria. In addition, during a clinical validation, it can be investigated for each measured pair of samples whether the clinical decision by the healthcare provider would differ, based on the DBS concentration *versus* the concentration in the reference sample.[92,93,99,136] Again, acceptance criteria should be stated beforehand in the study protocol. The EMA guideline states for cross-validation study samples, 'the difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats'.[5] It has been suggested that this guideline could also be aplied to assess agreement between DBS-based analytical results and reference results.[1] For example, a study, in which for 30% of the samples a difference of more than 20% of the mean is observed, would theoretically fulfill the criteria put forward by the EMA guideline. However, this would likely be clinically unacceptable and in this case stricter limits of agreement would be preferred. It is also possible that, at lower concentrations, a maximum absolute deviation may be tolerated, while at higher concentrations a maximum alowable percentage deviation may be set.

Type of card/paper used

In a clinical validation study, it should be stated which type of paper or DBS card is used. This type of paper should be the same as the one that was used during analytical validation.[29]

Sampling method and spot quality

A major problem during clinical validation is that the provided DBS may be of insufficient quality for analysis due to incorrect sampling.[42,137] Therefore, during clinical validation, the method of sampling and spot quality assessment by either an analyst or an automated quality assessment method should be mentioned in the study protocol.[138,139] As drug concentrations are dynamic, it is important to collect all paired samples within 5-10 minutes of each other.[91,116] Time-dependent changes in drug concentration are determined by pharmacokinetics and should be taken into account for the preparation of a sampling scheme. This is particularly relevant for drugs with a very short half-life or during the absorption and distribution phase of the drug.

The sampling method that is used during clinical validation should be the same as the sampling method that will be used in daily practice. For example, if the method is intended for home sampling by patient finger prick, the DBS samples obtained for clinical validation should also be obtained by finger prick. Spotting of venous blood on a DBS card is only appropriate if in clinical practice venous blood will be spotted on DBS cards. For instance, this may be the case when transport of tubes of whole blood is not possible due to instability of the compound or because of logistic difficulties (e.g. in remote areas or in resource-limited settings).[58] This is highly relevant as for some analytes venous-capillary differences may, or are known to, be present.

If a method is designed for home sampling, patients should ideally perform a finger prick to collect a DBS sample themselves during clinical validation. However, in most clinical validation studies, a trained phlebotomist collects or helps to collect samples, to rule out variability due to inexperienced sampling by the patient.[91,95,97,99,116,123] Alternatively, both approaches can be used successively during clinical validation.

Proper finger prick DBS sampling technique has been described earlier by the WHO, CLSI and in several studies[11,42,131,138,140,141] and is also shown in supplemantary Figure S-2, http://links.lww.com/TDM/A342. In short, sampling should be done after disinfecting the finger without excessive 'milking' or squeezing of the puncture site to avoid hemolysis or dilution by tissue fluid. When possible, finger prick blood should fall on the sampling paper instead of applying the droplet of blood to the sampling paper with the finger (without touching the sampling paper with the finger). Both patient and phlebotomist should be trained before samples can be obtained. This training should include practicing the whole sampling procedure under supervision of someone experienced in DBS sampling using either a test kit or a real finger prick aided by educational material such as a movie or a written instruction.[25,131,137,138,140]

All spots provided in a clinical validation study should be checked for quality by an experienced analyst or *via* a validated automated quality assessment method. Some requirements for a good quality spot depend on the analytical method and should be stated on beforehand, such as minimum spot size imposed by punching size. Other requirements are independent of the analytical method. Criteria are stated in supplementary Figure S-3, http://links.lww.com/TDM/A342. In short, all spots should be round, dried, consisting of one droplet of blood, and not touching other droplets.

Incurred sample reanalysis, duplicates and outliers

In their guideline, the FDA mentions incurred sample reanalysis (ISR) as a validation parameter for DBS methods.[6] In a clinical validation, ideally at least two replicate spots are available for analysis, to allow ISR and/or duplo analysis. However, reanalysis of the same spot (via a second punch) will not be possible when the protocol involves the use of larger punching sizes (e.g. 6 or 8 mm).[64] During clinical validation, it is recommended to analyze 2 different spots per sample, when possible, to evaluate within-card precision which can be calculated as the percentage difference $\%difference = \frac{repeat\ value - initial\ value}{mean\ value} * 100$.[5,24] The %difference between duplicates should not be greater than 20% of their mean for at least 67% of the samples.[5,6] In addition, ISR of the same spot is recommended when decentral punches may be used, provided spot homogeneity is supported by the analytical validation and small puch sizes (e.g. 3 mm) are used.[27]

The presence of an outlier may be explained by several reasons such as contamination of the sample, errors in sampling, extreme drying or storage conditions during transport or analytical errors.[42] In a clinical validation study most of the possible errors can be accounted for by, for instance, checking of spot quality of the sample upon arrival in the lab or checking and logging the drying time. When an outlier cannot be explained by such errors, the extreme studentized deviate technique[8] or a standardized score test can be used to exclude outliers.[121] However, outliers should be discussed in the context of clinical application of the DBS method. Therefore, outliers require an argumented discussion considering clinical setting and the aforementioned statistics tests.[8]

Clinical validation of automated analysis methods

Automation of a DBS assay could improve DBS sample- and workflow efficiency and reproducibility. Several examples exist of automated (on- or off-line) DBS assays using techniques like online extraction and solid phase extraction.[87,142,143] If an automated method is designed without a prior manual DBS method, the same recomendations for clinical validation apply. If a manual DBS assay used in clinical practice is replaced by an automated DBS method which is fully analytically validated, it is recommended to perform a cross validation including sample size of 40 samples from at least 25 different patients.[5,6,8] Due to the nature of DBS, it will most likely be challenging in real practice to measure the same spot using both an on- and offline method. Therefore, if during the clinical validation the within-card precision is found to be acceptable and two spots per finger prick DBS sample are provided, it is recomended to analyze one spot using the automated method and one spot using the manual method. Evaluation of agreement can again be performed by Passing-Bablok or Deming analysis and via a Bland-Altman plot, as described earlier.

Quality control

Laboratories should participate in external QC programs if a DBS assay is implented in routine care or provide objective evidence for determining the reliability of their results.[2,38] Apart from a prociency test pilot for the immunosuppresant tacrolimus, no external QC programs are currently available for DBS assays for drugs.[144] There is an urgent need for DBS proficiency testing programs to facilitate the uptake of DBS in routine care. Although external QC materials developed for the evaluation of liquid blood-based methods may be used to evaluate the quality of a DBS-based method, it should be taken into account that these materials typically have a different viscosity than true blood samples and will therefore yield

DBS of deviating sizes. Therefore, when using these materials, they should always be analyzed using a full spot approach.[145] Furthermore, the extraction efficiency of an artifical matrix may always differ from the extraction efficiency of an actual sample. Since most external QC materials are only available for plasma analysis and not for whole blood analysis, anopther option might be to remove part of the plasma of a blank whole blood sample and to replace it with the external QC material. The resulting blood can then be used to generate DBS, as was successfully applied for e.g. conventional antiepileptics.[67]

5. CROSS-VALIDATION

Once a DBS assay has been successfully applied in clinical practice, it is possible that changes have to be made to the sampling method, filter paper or analytical method. For some of these changes the standard guidelines for cross-validation are applicable.[5,6] This part will focus on additional recommendations when DBS assays or sampling methods are altered.

Different punch size

As stated before (see section 2), a punch size is preferably less than 4 mm because punching the sample in the lab will be easier and patients do not need to produce large blood spots. When the desired LLOQ, accuracy and precision can be met with a different punch (e.g. smaller or 'donut' punch)[74] than currently used in practice, a cross-validation study should be performed. If during the clinical validation the within-card precision is within analytical limits and two spots per sample are provided, it is reccomended to analyze 1 spot with the new punch size and 1 with the old punch size. In total, 40 samples of at least 25 different patients should be analyzed. In addition, extraction efficiency and DBS homogeneity should be re-evaluated. The extraction volume used with smaller punches can be downscaled

accordingly. Although theoretically possible, we do not recommend to use a surface-based formula to convert a result from a small (e.g. 3-mm) DBS punch to a theoretical bigger (e.g. 6-mm) DBS equivalent.

Different type of filter paper

In routine practice, several types of DBS filter paper are used such as the Whatman® 903, Whatman® FTA DMPK cards (type, A, B and C) (GE Healthcare, Chicago, IL, USA), and Perkin Elmer 226 cards (Ahlstrom, Helsinki, Finland).[29] Although performance of the FDA-approved Whatman® 903 (GE Healthcare, Chicago, IL, USA), and Perkin Elmer 226 paper is consistent and comparable in newborn screening,[146] the influence of drug concentration and HT can lead to a difference in recovery of up to 20% between cards.[29,147] This may be caused by the drugs' ability to form hydrogen bonds with the cellulose paper, leading to decreased recoveries[57], differences in spot homogeneity or differences in background signal.[27] Not only the recovery of the analyte may be altered, also matrix, volcano, volume and HT effects may have changed, as well as the analyte's stability. These parameters should all be re-evaluated as discussed before. Furthermore, QC samples for the new filter paper should be made using the same method as was done for the old filter paper.[54] Both old and new QC samples should be analyzed and the obtained mean accuracy should be within 15%.[5] The equivalence between both filter papers should be confirmed using a minimum of 40 samples obtained from at least 25 different patients. If not all parameters prove to be similar for both types of filter paper, a full analytical and clinical validation are required.

Different sampling method

Switching the sampling method will, most likely, be accompanied by some change in the method. For instance, it is likely that whole spot analysis rather than partial-punch analysis will be performed when a fixed volume of finger prick blood is deposited on a card instead of direct application of blood from the fingertip to the card. Moreover, it is possible that DBS-based assays are replaced by newer alternatives such as the earlier discussed VAMS technique because of the convenience of sampling and/or automation possibilities.[25] Importantly, as stated earlier, volumetric sampling does not necessarily eliminate the effect of HT or ageing on recovery, so this remains an important parameter to be studied.[7,29,57,62,148] In addition, a new sampling technique might influence spot homogeneity, thereby introducing a possible unknown error in analytical results.[27] Therefore, when changing sampling technique, sample vehicle or changing to whole spot analysis, it is recommended to perform a full clinical validation study, comparing the new method to the reference method, provided this change has been appropriately analytically validated.[25]

6. CONCLUSION

To successfully incorporate DBS-based methods in routine practice, good quality methods are a prerequisite. Since the quality of a method starts with its design, a sound method set-up not only ensures the method is suitable for a given application, it also increases the chances of a successful method validation. The quality of a method needs to be assessed both during analytical and clinical validation and should be compared with pre-set acceptance criteria. This is the first guidance document discussing how to evaluate the quality of a DBS-based method. This guideline outlines which traditional and non-traditional validation parameters should be assessed for this type of method and provides suggestions on how to do this. Most

importantly, each parameter should be evaluated in a way that reflects the real-life situation in which the method will eventually be applied. Furthermore, to ensure the method's quality on a day-to-day basis the first QC programs for quantitative DBS-based methods have been established recently. It is important to keep in mind that DBS for TDM applications only has a future if the quality of the result can be guaranteed. A proper analytical and clinical validation are essential to achieve this.

References

1. Enderle Y, Foerster K, Burhenne J. Clinical feasibility of dried blood spots: Analytics, validation, and applications. *J Pharm Biomed Anal*. 2016;130:231-243.

2. Wilhelm AJ, den Burger JCG, Swart EL. Therapeutic drug monitoring by dried blood spot: Progress to date and future directions. *Clin Pharmacokinet*. 2014;53:961-973.

3. Freeman JD, Rosman LM, Ratcliff JD, et al. State of the science in dried blood spots. *Clin Chem*. 2018;64:656-679.

4. Antunes MV, Charão MF, Linden R. Dried blood spots analysis with mass spectrometry: Potentials and pitfalls in therapeutic drug monitoring. *Clin Biochem*. 2016;49:1035-1046.

5. European Medicines Agency, London, UK. Guideline on bioanalytical method validation. 2011.

6. Food and Drug Administration, US Department of Health and Human Services, Rockville, MD, USA. Guidance for Industry, Bioanalytical Method Validation. 2018.

7. Velghe S, Delahaye L, Stove CP. Is the hematocrit still an issue in quantitative dried blood spot analysis? *J Pharm Biomed Anal*. 2018;163:188-196.

8. CLSI. Measurement Procedure Comparison and Bias Estimation Using Patient Samples; Approved Guideline - Third Edition. *CLSI document EP09-A3 Wayne, PA: Clinical and Laboratory Standards Institute*. 2013.

9. White S, Timmerman P, Globig S, et al. EBF recommendation on the validation of bioanalytical methods for dried blood spots. *Bioanalysis*. 2011;3:1567-1575.

10. Evans C, Arnold M, Bryan P, et al. Implementing dried blood spot sampling for clinical pharmacokinetic determinations: Considerations from the IQ consortium microsampling working group. *AAPS J*. 2015;17:292-300.

11. Timmerman P, White S, Cobb Z, et al. Update of the EBF recommendation for the use of DBS in regulated bioanalysis integrating the conclusions from the EBF DBS-microsampling consortium. *Bioanalysis*. 2013;5:2129-136.

12. Leuthold LA, Heudi O, Déglon J, et al. New microfluidic-based sampling procedure for overcoming the hematocrit problem associated with dried blood spot analysis. *Anal Chem*. 2015;87:2068-2071.

13. Neto R, Gooley A, Breadmore MC, et al. Precise, accurate and user-independent blood collection system for dried blood spot sample preparation. *Anal Bioanal Chem*. 2018;410:3315-3323.

14. Beck O, Modén NK, Seferaj S, et al. Study of measurement of the alcohol biomarker phosphatidylethanol (PEth) in dried blood spot (DBS) samples and application of a volumetric DBS device. *Clinica Chimica Acta*. 2018;479:38-42.

15. Lenk G, Sandkvist S, Pohanka A, et al. A disposable sampling device to collect volume-measured DBS directly from a fingerprick onto DBS paper. *Bioanalysis*. 2015;7:2085-2094.

16. Velghe S, Stove CP. Evaluation of the Capitainer-B microfluidic device as a new hematocrit-independent alternative for dried blood spot collection. *Anal Chem*. 2018;90:12893-12899.

17. Spooner N, Denniff P, Michielsen L, et al. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis*. 2015;7:653-659.

18. Denniff P, Spooner N. Volumetric absorptive microsampling: a dried sample collection technique for quantitative bioanalysis. *Anal Chem*. 2014;86:8489-8495.

19. Hauser J, Lenk G, Hansson J, et al. High yield passive plasma filtration from human finger prick blood. *Anal Chem*. 2018;90:13393-13399.

20. Li Y, Henion J, Abbott R, et al. The use of a membrane filtration device to form dried plasma spots for the quantitative determination of guanfacine in whole blood. *Rapid Commun Mass Spectrom*. 2012;26:1208-1212.

21. Kim J, Woenker T, Adamec J, et al. Simple, miniaturized blood plasma extraction method. *Anal Chem*. 2013;85:11501-11508.

22. Sturm R, Henion J, Abbott R, et al. Novel membrane devices and their potential utility in blood sample collection prior to analysis of dried plasma spots. *Bioanalysis*. 2015;7:1987-2002.

23. Ryona I, Henion J. A book-type dried plasma spot card for automated flow-through elution coupled with online SPE-LC-MS/MS bioanalysis of opioids and stimulants in blood. *Anal Chem*. 2016;88:11229-11237.

24. Ingels AME, Hertegonne KB, Lambert WE, et al. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients

using dried blood spot sampling at home: An exploratory study. *CNS Drugs*. 2013;27:233-237.

25. Verougstraete N, Lapauw B, Van Aken S, et al. Volumetric absorptive microsampling at home as an alternative tool for the monitoring of HbA1c in diabetes patients. *Clin Chem Lab Med*. 2017;55:462-469.

26. Barfield M, Wheller R. Use of dried plasma spots in the determination of pharmacokinetics in clinical studies: validation of a quantitative bioanalytical method. *Anal Chem*. 2010;83:118-124.

27. Ren X, Paehler T, Zimmer M, et al. Impact of various factors on radioactivity distribution in different DBS papers. *Bioanalysis*. 2010;2:1469-1475.

28. Chen X, Zhao H, Hatsis P, et al. Investigation of dried blood spot card-induced interferences in liquid chromatography/mass spectrometry. *J Pharm Biomed Anal*. 2012;61:30-37.

29. Koster RA, Botma R, Greijdanus B, et al. The performance of five different dried blood spot cards for the analysis of six immunosuppressants. *Bioanalysis*. 2015;7:1225-1235.

30. Majumdar TK, Howard DR. The use of dried blood spots for concentration assessment in pharmacokinetic in pharmacokinetic evaluations. Pharmacokinetics in Drug Development. Springer, Boston, MA

31. Mess J, Taillon M, Côté C, et al. Dried blood spot on-card derivatization: an alternative form of sample handling to overcome the instability of thiorphan in biological matrix. *Biomed Chromatogr*. 2012;26:1617-1624.

32. Van der Heijden J, De Beer Y, Hoogtanders K, et al. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. *J Pharm Biomed Anal*. 2009;50:664-670.

33. Blessborn D, Römsing S, Annerberg A, et al. Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of lumefantrine in capillary blood on sampling paper. *J Pharm Biomed Anal*. 2007;45:282-287.

34. Liu G, Ji QC, Jemal M, et al. Approach to evaluating dried blood spot sample stability during drying process and discovery of a treated card to maintain analyte stability by rapid on-card pH modification. *Anal Chem*. 2011;83:9033-9038.

35. Mengerink Y, Mommers J, Qiu J, et al. A new DBS card with spot sizes independent of the hematocrit value of blood. *Bioanalysis*. 2015;7:2095-2104.

36. De Kesel PM, Capiau S, Lambert WE, et al. Current strategies for coping with the hematocrit problem in dried blood spot analysis. *Bioanalysis*. 2014;6:1871-1874.

37. Eibak LEE, Hegge AB, Rasmussen KE, et al. Alginate and chitosan foam combined with electromembrane extraction for dried blood spot analysis. *Anal Chem*. 2012;84:8783-8789.

38. International Organization for Standardization. *ISO 15189. Medical laboratories - Requirements for quality and competence. Paragraph 5.6.3.2*: Geneva, Switzerland; 2012.

39. Shi Y, Jiang H. Assay dynamic range for DBS: battles on two fronts. *Bioanalysis*. 2011;3:2259-2262.

40. Denniff P, Spooner N. Effect of storage conditions on the weight and appearance of dried blood spot samples on various cellulose-based substrates. *Bioanalysis*. 2010;2:1817-1822.

41. Parker SL, Guerra Valero YC, Lipman J, et al. Effect of time on recovery of plasma microsamples for the quantitative determination of vancomycin. *Bioanalysis*. 2016;8:2235-2242.

42. Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit*. 2009;31:327-336.

43. Hannon W, Boyle J, Davin B, et al. Blood Collection on Filter Paper for Neonatal Screening Programs, 4th Approved Standard, NCCLS Document A4-A5. Vol 23. Wayne, PA: National Committee for Clinical Laboratory Standards; 2003:21.

44. Li L, Chen T, Ren Y, et al. Mini 12, Miniature Mass Spectrometer for Clinical and Other Applications- Introduction and Characterization. *Anal Chem*. 2014;86:2909-2916.

45. Bowen CL, Dopson W, Kemp DC, et al. Investigations into the environmental conditions experienced during ambient sample transport: impact to dried blood spot sample shipments. *Bioanalysis*. 2011;3:1625-1633.

46. Meesters RJ, Zhang J, van Huizen NA, et al. Dried matrix on paper disks: the next generation DBS microsampling technique for managing the hematocrit effect in DBS analysis. *Bioanalysis*. 2012;4:2027-2035.

47. Li F, Zulkoski J, Fast D, et al. Perforated dried blood spots: a novel format for accurate microsampling. *Bioanalysis*. 2011;3:2321-2333.

48. Youhnovski N, Bergeron A, Furtado M, et al. Pre-cut dried blood spot (PCDBS): an alternative to dried blood spot (DBS) technique to overcome hematocrit impact. *Rapid Commun Mass Spectrom*. 2011;25:2951-2958.

49. Abu-Rabie P, Denniff P, Spooner N, et al. Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias. *Anal Chem*. 2015;87:4996-5003.

50. Mommers J, Mengerink Y, Ritzen E, et al. Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. *Anal Chim Acta*. 2013;774:26-32.

51. van Baar BL, Verhaeghe T, Heudi O, et al. IS addition in bioanalysis of DBS: results from the EBF DBS-microsampling consortium. *Bioanalysis*. 2013;5:2137-2145.

52. Capiau S, Wilk LS, Aalders MCG, et al. A novel, nondestructive, dried blood spot-based hematocrit prediction method using noncontact diffuse reflectance spectroscopy. *Anal Chem*. 2016;88:6538-6546.

53. De Kesel PM, Sadones N, Capiau S, et al. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis*. 2013;5:2023-2041.

54. Koster RA, Alffenaar JW, Botma R, et al. What is the right blood hematocrit preparation procedure for standards and quality control samples for dried blood spot analysis? *Bioanalysis*. 2015;7:345-351.

55. Saha A, Kumar A, Gurule S, et al. Role of RBC partitioning and whole blood to plasma ratio in bioanalysis: A case study with valacyclovir and acyclovir. *Mass Spectrom Purif Tech.* 2017;3:119.

56. Blessborn D, Sköld K, Zeeberg D, et al. Heat stabilization of blood spot samples for determination of metabolically unstable drug compounds. *Bioanalysis*. 2013;5:31-39.

57. Koster RA, Alffenaar JC, Botma R, et al. The relation of the number of hydrogen-bond acceptors with recoveries of immunosuppressants in DBS analysis. *Bioanalysis*. 2015;7:1717-1722.

58. Vu D, Koster RA, Bolhuis M, et al. Simultaneous determination of rifampicin, clarithromycin and their metabolites in dried blood spots using LC-MS/MS. *Talanta.* 2014;121:9-17.

59. Gelzo M, Dello Russo A, Corso G. Stability study of dehydrocholesterols in dried spot of blood from patients with Smith-Lemli-Opitz syndrome, using filter-paper treated with butylated hydroxytoluene. *Clin Chim Acta*. 2012;413:525-526.

60. Verougstraete N, Stove V, Stove C. Wet absorptive microsampling at home for HbA1c monitoring in diabetic children. *Clin Chem Lab Med (CCLM)*. 2018;56.12:e291-e294.

61. Koster RA. The influence of the dried blood spot drying time on the recoveries of six immunosuppressants. *J Appl Bioanal*. 2015;1:395.

62. De Kesel PMM, Lambert WE, Stove CP. Does volumetric absorptive microsampling eliminate the hematocrit bias for caffeine and paraxanthine in dried blood samples? A comparative study. *Anal Chim Acta*. 2015;881:65-73.

63. Hempen CM, Maarten Koster EH, Ooms JA. Hematocrit-independent recovery of immunosuppressants from DBS using heated flow-through desorption. *Bioanalysis*. 2015;7:2019-2029.

64. Koster RA, Alffenaar JWC, Greijdanus B, et al. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. *Talanta*. 2013;115:47-54.

65. Matuszewski B, Constanzer M, Chavez-Eng C. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC– MS/MS. *Anal Chem*. 2003;75:3019-3030.

66. Ye Z, Gao H. Evaluation of sample extraction methods for minimizing hematocrit effect on whole blood analysis with volumetric absorptive microsampling. *Bioanalysis*. 2017;9:349-357.

67. Velghe S, Stove CP. Volumetric absorptive microsampling as an alternative tool for therapeutic drug monitoring of first-generation anti-epileptic drugs. *Anal Bioanal Chem*. 2018;410:2331-2341.

68. Delahaye L, Dhont E, De Cock P, et al. Volumetric absorptive microsampling as an alternative sampling strategy for the determination of paracetamol in blood and cerebrospinal fluid. *Anal Bioanal Chem*. 2018:1-11.

69. Cobb Z, de Vries R, Spooner N, et al. In-depth study of homogeneity in DBS using two different techniques: results from the EBF DBS-microsampling consortium. *Bioanalysis*. 2013;5:2161-2169.

70. Lenk G, Hansson J, Beck O, et al. The effect of drying on the homogeneity of DBS. *Bioanalysis*. 2015;7:1977-1985.

71. Jager NG, Rosing H, Schellens JH, et al. Procedures and practices for the validation of bioanalytical methods using dried blood spots: a review. *Bioanalysis*. 2014;6:2481-2514.

72. Fraser CG. Desirable standards of performance for therapeutic drug monitoring. *Clin Chem*. 1987;33:387-389.

73. Liu G, Snapp HM, Ji QC. Internal standard tracked dilution to overcome challenges in dried blood spots and robotic sample preparation for liquid chromatography/tandem mass spectrometry assays. *Rapid Commun Mass Spectrom*. 2011;25:1250-1256.

74. Ewles MF, Turpin PE, Goodwin L, et al. Validation of a bioanalytical method for the quantification of a therapeutic peptide, ramoplanin, in human dried blood spots using LCMS/MS. *Biomed Chromatogr*. 2011;25:995-1002.

75. Mitchell C, Kraft K, Peterson D, et al. Cross-contamination during processing of dried blood spots used for rapid diagnosis of HIV-1 infection of infants is rare and avoidable. *J Virol Methods*. 2010;163:489-491.

76. Black J, Layloff T. Summer of 1995-Mailbox temperature excursions in St. Louis. 1996;22:3305-3305.

77. Castillo-Mancilla JR, Zheng J, Rower JE, et al. Tenofovir, emtricitabine, and tenofovir diphosphate in dried blood spots for determining recent and cumulative drug exposure. *AIDS Res Hum Retroviruses*. 2013;29:384-390.

78. Feinberg M. Validation of analytical methods based on accuracy profiles. *J Chromatogr A*. 2007;1158:174-183.

79. González AG, Herrador MÁ. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends Anal Chem*. 2007;26:227-238.

80. Capiau S, Stove VV, Lambert WE, et al. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. *Anal Chem*. 2013;85:404-410.

81. Veenhof H. Performance of an app measuring spot quality in dried blood spot sampling. 2016. Poster session presented at 9th International Workshop on Clinical Pharmacology of Tuberculosis Drugs 2016, Liverpool, United Kingdom.

82. De Kesel PM, Capiau S, Stove VV, et al. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots. *Anal Bioanal Chem*. 2014;406:6749-6755.

83. Capiau S, Wilk LS, De Kesel PM, et al. Correction for the hematocrit bias in dried blood spot analysis using a nondestructive, single-wavelength reflectance-based hematocrit prediction method. *Anal Chem*. 2018;90:1795-1804.

84. Oliveira RV, Henion J, Wickremsinhe ER. Automated direct extraction and analysis of dried blood spots employing on-line SPE high-resolution accurate mass bioanalysis. *Bioanalysis*. 2014;6:2027-2041.

85. Oliveira RV, Henion J, Wickremsinhe ER. Automated high-capacity on-line extraction and bioanalysis of dried blood spot samples using liquid chromatography/high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom*. 2014;28:2415-2426.

86. Duthaler U, Berger B, Erb S, et al. Automated high throughput analysis of antiretroviral drugs in dried blood spots. *J Mass Spectrom*. 2017;52:534-542.

87. Verplaetse R, Henion J. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. *Drug Test Anal*. 2016;8:30-38.

88. Zakaria R, Allen KJ, Koplin JJ, et al. Advantages and challenges of dried blood spot analysis by mass spectrometry across the total testing process. *EJIFCC*. 2016;27:288-317.

89. Hoogtanders K, van der Heijden J, Christiaans M, et al. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. *J Pharm Biomed Anal*. 2007;44:658-664.

90. Kloosterboer SM, de Winter BCM, Bahmany S, et al. Dried blood spot analysis for therapeutic drug monitoring of antipsychotics: Drawbacks of its clinical application. *Ther Drug Monit*. 2018;40:344-350.

91. Veenhof H, Koster RA, Alffenaar JWC, et al. Clinical validation of simultaneous analysis of tacrolimus, cyclosporine a and creatinine in dried blood spots in kidney transplant patients. *Transplantation*. 2017;101:1727.

92. Zwart TC, Gokoel SR, van der Boog, Paul JM, et al. Therapeutic drug monitoring of tacrolimus and mycophenolic acid in outpatient renal transplant recipients using a volumetric dried blood spot sampling device. *Br J Clin Pharmacol*. 2018.

93. Berm EJ, Odigie B, Bijlsma MJ, et al. A clinical validation study for application of DBS in therapeutic drug monitoring of antidepressants. *Bioanalysis*. 2016;8:413-424.

94. Boons CCLM, Chahbouni A, Schimmel AM, et al. Dried blood spot sampling of nilotinib in patients with chronic myeloid leukaemia: a comparison with venous blood sampling. *J Pharm Pharmacol*. 2017;69:1265-1274.

95. Dickerson JA, Sinkey M, Jacot K, et al. Tacrolimus and sirolimus in capillary dried blood spots allows for remote monitoring. *Pediatr Transplant*. 2015;19:101-106.

96. Enderle Y, Meid AD, Haefeli WE, et al. Dried blood spot technique for the monitoring of ambrisentan, bosentan, sildenafil, and tadalafil in patients with pulmonary arterial hypertension. *Anal Chem*. 2015;87:12112-12120.

97. Hinchliffe E, Adaway J, Fildes J, et al. Therapeutic drug monitoring of ciclosporin A and tacrolimus in heart lung transplant patients using dried blood spots. *Ann Clin Biochem*. 2014;51:106-109.

98. Jager NGL, Rosing H, Schellens JHM, et al. Use of dried blood spots for the determination of serum concentrations of tamoxifen and endoxifen. *Breast Cancer Res Treat*. 2014;146:137-144.

99. Kromdijk W, Mulder JW, Rosing H, et al. Use of dried blood spots for the determination of plasma concentrations of nevirapine and efavirenz. *J Antimicrob Chemother*. 2012;67:1211-1216.

100. Taylor RR, Hoffman KL, Schniedewind B, et al. Comparison of the quantification of acetaminophen in plasma, cerebrospinal fluid and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal*. 2013;83:1-9.

101. Nijenhuis CM, Huitema ADR, Marchetti S, et al. The use of dried blood spots for pharmacokinetic monitoring of vemurafenib treatment in melanoma patients. *J Clin Pharmacol*. 2016;56:1307-1312.

102. Foerster KI, Huppertz A, Meid AD, et al. Dried-blood-spot technique to monitor direct oral anticoagulants: Clinical validation of a UPLC–MS/MS-Based Assay. *Anal Chem*. 2018;90:9395-9402.

103. Linnet K. Necessary sample size for method comparison studies based on regression analysis. *Clin Chem*. 1999;45:882-894.

104. Das S, Fleming D, Mathew B, et al. Determination of serum carbamazepine concentration using dried blood spot specimens for resource limited settings. *Hosp Pract*. 2017;34:46-50.

105. Ting LS, Villeneuve E, Ensom MH. Beyond cyclosporine: a systematic review of limited sampling strategies for other immunosuppressants. *Ther Drug Monit*. 2006;28:419-430.

106. Barraclough KA, Isbel NM, Kirkpatrick CM, et al. Evaluation of limited sampling methods for estimation of tacrolimus exposure in adult kidney transplant recipients. *Br J Clin Pharmacol*. 2011;71:207-223.

107. Sheiner LB, Beal SL. Some suggestions for measuring predictive performance. *J Pharmacokinet Biopharm*. 1981;9:503-512.

108. Rowland M, Emmons GT. Use of dried blood spots in drug development: pharmacokinetic considerations. *The AAPS J*. 2010;12:290-293.

109. Hahn RZ, Antunes MV, Costa Arnhold P, et al. Determination of topiramate in dried blood spots using single-quadrupole gas chromatography-mass spectrometry after flash methylation with trimethylanilinium hydroxide. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2017;1046:131-137.

110. Mohammed BS, Cameron GA, Cameron L, et al. Can finger-prick sampling replace venous sampling to determine the pharmacokinetic profile of oral paracetamol? *Br J Clin Pharmacol*. 2010;70:52-56.

111. Webb NJA, Roberts D, Preziosi R, et al. Fingerprick blood samples can be used to accurately measure tacrolimus levels by tandem mass spectrometry. *Pediatr Transplant*. 2005;9:729-733.

112. van Boekel GA, Donders AR, Hoogtanders KE, et al. Limited sampling strategy for prolonged-release tacrolimus in renal transplant patients by use of the dried blood spot technique. *Eur J Clin Pharmacol*. 2015;71:811-816.

113. Sadilkova K, Busby B, Dickerson JA, et al. Clinical validation and implementation of a multiplexed immunosuppressant assay in dried blood spots by LC-MS/MS. *Clinica Chimica Acta*. 2013;421:152-156.

114. Leino AD, King EC, Jiang W, et al. Assessment of tacrolimus intrapatient variability in stable adherent transplant recipients: Establishing baseline values. *Am J Transplant*. Available at https://doi.org/10.1111/ajt.15199. Accessed November 14th, 2018.

115. Koop DR, Bleyle LA, Munar M, et al. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013;926:54-61.

116. Jager NG, Rosing H, Schellens JH, et al. Determination of tamoxifen and endoxifen in dried blood spots using LC-MS/MS and the effect of coated DBS cards on recovery and matrix effects. *Bioanalysis*. 2014;6:2999-3009.

117. Koster RA, Greijdanus B, Alffenaar JC, et al. Dried blood spot analysis of creatinine with LC-MS/MS in addition to immunosuppressants analysis. *Anal Bioanal Chem*. 2015;407:1585-1594.

118. Nijenhuis CM, Rosing H, Schellens JH, et al. Quantifying vemurafenib in dried blood spots using high-performance LC-MS/MS. *Bioanalysis*. 2014;6:3215-3224.

119. Linder C, Andersson M, Wide K, et al. A LC-MS/MS method for therapeutic drug monitoring of carbamazepine, lamotrigine and valproic acid in DBS. *Bioanalysis*. 2015;7:2031-2039.

120. Linder C, Beck O, Gustafsson LL, et al. Comparison between dried blood spot and plasma sampling for therapeutic drug monitoring of antiepileptic drugs in children with epilepsy: A step towards home sampling. *Clin Biochem*. 2016;50:418-424.

121. Kong ST, Lim S, Lee WB, et al. Clinical validation and implications of dried blood spot sampling of carbamazepine, valproic acid and phenytoin in patients with epilepsy. *PLoS ONE*. 2014;9:e108190.

122. Arpini J, Antunes MV, Pacheco LS, et al. Clinical evaluation of a dried blood spot method for determination of mycophenolic acid in renal transplant patients. *Clin Biochem*. 2013;46:1905-1908.

123. Koster RA, Veenhof H, Botma R, et al. Dried blood spot validation of five immunosuppressants, without hematocrit correction, on two LC-MS/MS systems. *Bioanalysis*. 2017;9:553-563.

124. Oostendorp M, El Amrani M, Diemel EC, et al. Measurement of hematocrit in dried blood spots using near-infrared spectroscopy: Robust, fast, and nondestructive. *Clin Chem*. 2016;62:1534-1536.

125. Richardson G, Marshall D, Keevil B. Prediction of haematocrit in dried blood spots from the measurement of haemoglobin using commercially available sodium lauryl sulphate. *Ann Clin Biochem*. 2018;55:363-367.

126. Weber J, Oberfeld S, Bonse A, et al. Validation of a dried blood spot method for therapeutic drug monitoring of citalopram, mirtazapine and risperidone and its active metabolite 9-hydroxyrisperidone using HPLC-MS. *J Pharm Biomed anal*. 2017;140:347-354.

127. Spooner N, Olatunji A, Webbley K. Investigation of the effect of blood hematocrit and lipid content on the blood volume deposited by a disposable dried blood spot collection device. *J Pharm Biomed Anal*. 2018;149:419-424.

128. Cornbleet PJ, Gochman N. Incorrect least-squares regression coefficients in method-comparison analysis. *Clin Chem*. 1979;25:432-438.

129. Linnet K. Evaluation of regression procedures for methods comparison studies. *Clin Chem*. 1993;39:424-432.

130. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. *Clin Chem Lab Med*. 1983;21:709-720.

131. Al-Uzri AA, Freeman KA, Wade J, et al. Longitudinal study on the use of dried blood spots for home monitoring in children after kidney transplantation. *Pediatr Transplant*. 2017; 21:e12983. https://doi.org/10.1111/petr.12983 Accessed November 14th, 2018.

132. Johnson R. Assessment of bias with emphasis on method comparison. *Clin Biochem Rev*. 2008;29:S37-S42.

133. Dewitte K, Fierens C, Stöckl D, et al. Application of the Bland-Altman plot for interpretation of method-comparison studies: a critical investigation of its practice. *Clin Chem*. 2002;48:799-780.

134. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Transplant*. 2009;9:S1-155.

135. Jones GR, Sikaris K, Gill J. Allowable limits of performance' for external quality assurance programs - An approach to application of the stockholm criteria by the RCPA quality assurance programs. *Clin Biochem Rev*. 2012;33:133-139.

136. Patteet L, Maudens KE, Neels H, et al. Are capillary DBS applicable for therapeutic drug monitoring of common antipsychotics? A proof of concept. *Bioanalysis*. 2015;7:2119-2130.

137. Panchal T, Spooner N, Barfield M. Ensuring the collection of high-quality dried blood spot samples across multisite clinical studies. *Bioanalysis*. 2017;9:209-213.

138. World Health Organization. Participant Manual Module 14 Blood Collection and Handling - Dried Blood Spot (DBS). 2005;Module14:EQA.

139. Dantonio PD, Stevens G, Hagar A, et al. Comparative evaluation of newborn bloodspot specimen cards by experienced laboratory personnel and by an optical scanning instrument. *Mol Genet Metab*. 2014;113:62-66.

140. Abdi A, Emming G, Koster R. University Medical Centre Groningen - DBS Instruction, 2017. Available at: www.driedbloodspot.umcg.nl. Accessed December 19th, 2017.

141. CLSI. Blood collection on Filter Paper for Newborn Screening Programs; Approved Standard - Sixth Edition. *CLSI Document NBS01-A6. Wayne, PA: Clinical and Laboratory Standards Institute*. 2013;NBS01-A6.

142. Ooms JA, Knegt L, Koster EH. Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE-MS/MS. *Bioanalysis*. 2011;3:2311-2320.

143. Meesters RJ, Hooff GP. State-of-the-art dried blood spot analysis: an overview of recent advances and future trends. *Bioanalysis*. 2013;5:2187-208.

144. Robijns K, Touw DJ, Koster RA. Therapeutic drug monitoring by dried blood spot: Progress to date and future directions. *Clin Pharmacokinet*. 2014.

145. Keevil BG. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. *Clin Biochem*. 2011;44:110-118.

146. Mei JV, Zobel SD, Hall EM, et al. Performance properties of filter paper devices for whole blood collection. *Bioanalysis*. 2010;2:1397-1403.

147. Vu DH, Koster RA, Alffenaar JW, et al. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2011;879:1063-1070.

148. Mano Y, Kita K, Kusano K. Hematocrit-independent recovery is a key for bioanalysis using volumetric absorptive microsampling devices, MitraTM. *Bioanalysis*. 2015;7:1821-1829.

Figures Legends

Figure 1: Flowchart depicting different options for the set-up of a dried-blood-spot-based method which can be used before setting up a dried blood spot-based procedure. The highlighted 'flow path' shows the procedure for TDM of immunosuppressants following home sampling by adult patients and partial spot analysis of DBS cards sent to the laboratory. Reprinted with permission from Anoek Houben. Copyright 2018

Figure 2: Schematic set-up of the experiments needed to assess the robustness of the extraction procedure and short-term stability. The total amount of samples to be analyzed for this experiment is 100 (plus calibrators and QC samples). Reprinted with permission from Anoek Houben. Copyright 2018

Figure 3: A simplified schematic set-up of an experiment to assess the robustness of the extraction procedure and short-term stability, requiring a minimum number of samples. The total number of samples to be analyzed for this experiment is 36 (plus calibrators and QC samples). Reprinted with permission from Anoek Houben. Copyright 2018

Figure 4: A schematic set-up for the evaluation of matrix effect (ME) and recovery (RE). The experiment can either be performed at five hematocrit (HT) levels or at three (i.e. without the grey samples). This experiment allows to evaluate whether ME & RE are constant for different matrices and for different HT levels. Each condition is analyzed in quintuplicate. Reprinted with permission from Anoek Houben. Copyright 2018

Figure 5: Example of filter paper with two concentric samples corresponding to the minimally required volume (e.g. 20 µL) and the maximally allowed volume (e.g. 50 µL), also taking into account different hematocrit (HT) levels. Figure adapted from Capiau *et al.*[80] Reprinted with permission from Anoek Houben. Copyright 2018

Figure 6: Overview of the expected hematocrit (HT) range in different patient populations. The boxplots depict the distribution of hematocrit values per patient population. The boxes show the HT values between the 25th and 75th percentile, as well as the median HT value. The flags show the 2.5% and 97.5% percentiles. Adapted from De Kesel *et al.*[53] Reprinted with permission from Anoek Houben. Copyright 2018

Figure 7: A schematic overview of the samples that could be collected during a clinical validation study. The bold blue lines depict which samples could be compared to one another. The grey lines show which samples can be generated from which sampling method. Reprinted with permission from Anoek Houben. Copyright 2018

Table 1: Overview of the analytical validation parameters that require additional evaluation in dried blood spot-based methods, and how to assess them.

Validation parameter	Evaluation	Statistical test/ Acceptance criterion
Recovery, matrix effect, process efficiency	Evaluate at both high and low QC levels using 6 different donors, (with one donor evaluated at minimally 3 HT levels), with each condition determined in quintuplicate*.	Should be reproducible, both between matrices and HT values (%RSD ≤ 15%).
Volume effect	Evaluate at both high and low QC levels and at least at 3 HT levels and 3 volumes*.	One-way ANOVA with bonferroni post-hoc analysis ($p \leq 0.05$). Back calculated values deviate ≤15 % of medium volume.
Hematocrit effect	Evaluate at both high and low QC levels and at least at 3 HT levels*.	One-way ANOVA with bonferroni post-hoc analysis ($p \leq 0.05$). Back calculated values deviate ≤15 % of medium HT values.
Volcano effect	Compare central and peripheral measurements. Evaluate at both high and low QC levels and at least at 3 HT levels and one volume (typically, the highest) *.	Paired t-test ($p \leq 0.05$) Back calculated 'peripheral' values deviate ≤15% of 'central' values

*HT levels should cover the entire HT range of the target population and the volumes should be representative of the sample volumes that will be generated by the patient.

Table 2: An overview of the minimally required amount of analyses for the analytical validation of dried blood spots vs. whole blood.

Validation parameter	**Amount of samples (dried blood spot-based)**	**Amount of samples (liquid whole blood)**
Selectivity	n = (6 + 6) x 1 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo	n = (6 + 6) x 1 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo
Calibration model	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo
Accuracy & precision	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate
Dilution integrity	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate
Carry-over	n = (1 + 1) x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo	n = (1 + 1) x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo
Recovery, matrix effect, process efficiency	n = 2x (2 x 5 **x 1** x 1 x 5) + 2x (2 x 1 **x 3** x 1 x 5) + (2 x 1 x 5) = 170 2 QC levels, 6 donors, of which 1 donor at **3 HT levels**, 1 day, in quintuplicate (spiked before/after)	n = 2x (2 x 6 **x 1** x 1 x 5) + (2 x 1 x 5) = 130 2 QC levels, 6 donors, **1 HT level**, 1 day, in quintuplicate (spiked before/after) 2 QC levels, 1 day, quintuplicate (standard

	2 QC levels, 1 day, quintuplicate (standard solutions)	solutions)
Stability	n = 2 x 1 x 4 x 5 = 40 2 QC levels, 1 HT level, **4** points: T_0, T_{1w}, T_2w @ RT, T_{2d} @ 60°C, in quintuplicate	n = 2 x 1 **x 7** x 5 = **70** 2 QC levels, 1 HT level, 7 points, in quintuplicate: Bench-top stability: T_0 & T_{24h} @ RT Storage stability: T_{1w}, T_{2w} @ 4°C/-20°C **Freeze thaw stability: min. 3 cycles**
Volume effect, hematocrit effect, volcano effect	**n = 2 x 3 x 4 x 5 = 120** **2 QC levels, 3 HT levels, low, medium and high-volume central punch + high volume peripheral punch, all in quintuplicate**	**N.A.**
TOTAL	**412**	**282**

RT = room temperature, T = time point, T_0 = starting point = at the minimum drying time (e.g. 2 hours) = at the minimum drying time (e.g. 2 hours), d = day, w = week.

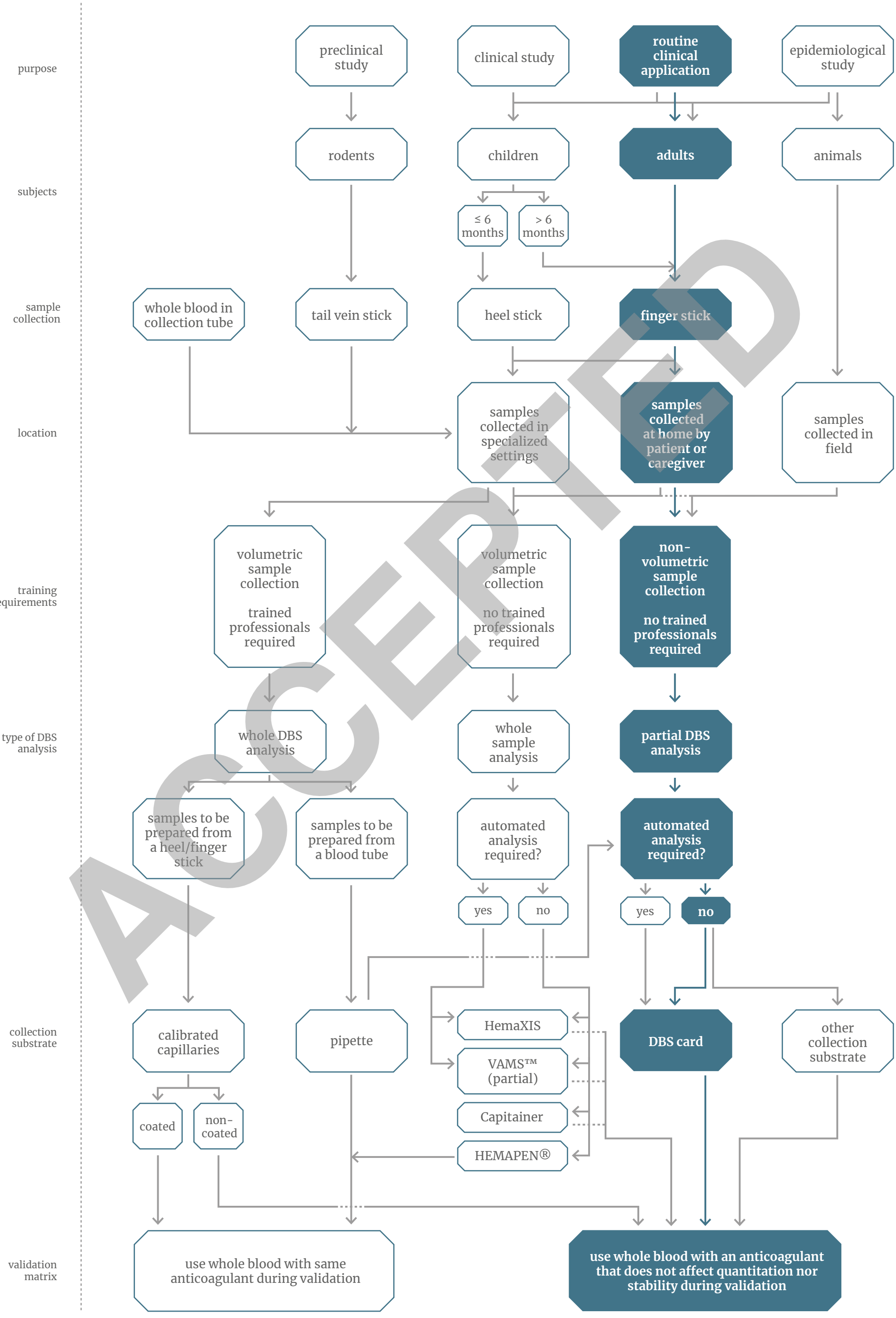
*samples are prepared in blood of median HT, unless mentioned otherwise.

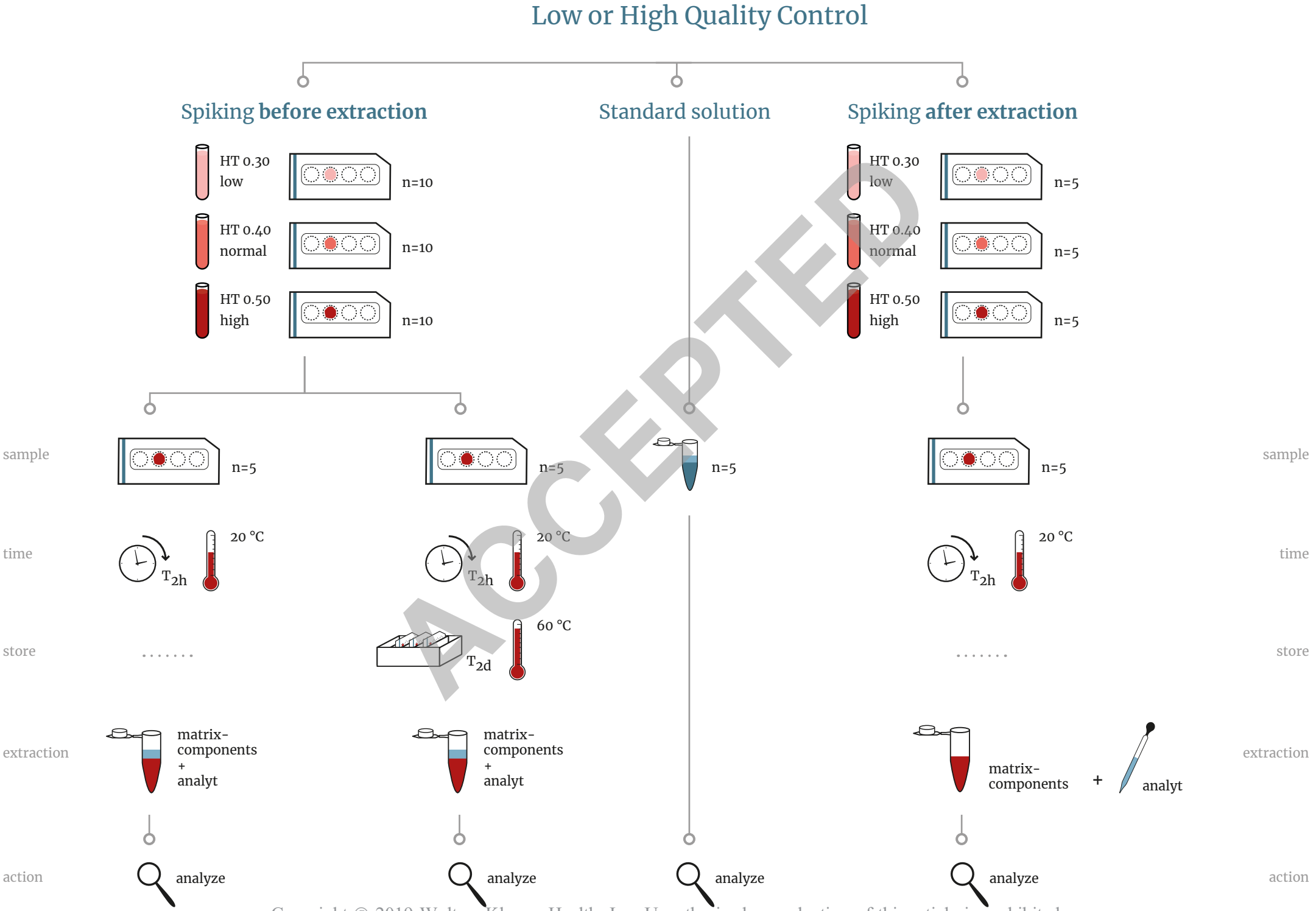
Table 3: An overview of the classical validation parameters and how to assess them.

Validation parameter	**Evaluation**	**Statistical test/ Acceptance criterion**
Selectivity	6 individual blank matrices	≤ 20% of LLOQ (analyte) ≤ 5% (IS)
Calibration model	Use min. 6 calibrators + zero + blank. Zero and blank samples should not be included in the calibration curve.	Back calculated concentrations ≤ 15% of nominal value (≤ 20% at LLOQ). ≥ 75% of all calibrators and ≥ 50% per calibration level should comply.
Accuracy & precision	Evaluate at 4 QC levels: - LLOQ - Low = ≤ 3 x LLOQ - Medium = 30 - 50% of range - High = ≥ 75% of highest calibrator	≤ 20% for LLOQ ≤ 15% for other QC levels
Dilution integrity	Evaluate a dilution factor (e.g. 1:9) applicable to the patient samples.	Accuracy and precision ≤ 15%
Carry-over	The analysis of (zero and) blank samples after the highest calibrator	≤ 20% of LLOQ (analyte) ≤ 5% (IS)
Recovery, matrix effect, process efficiency	Evaluate at both low and high QC, using 6 different blank matrices. - Recovery: spiked before/spiked after. - Matrix effect: spiked after/ standard solutions - Process efficiency: spiked before/	CV ≤ 15%

	standard solutions	
Stability	Evaluate at both low and high QC levels. Store stability QCs under representative conditions for a representative time frame and measure against fresh calibrators.	≤ 15% of nominal value (or ≤ 15% of value at T_0)

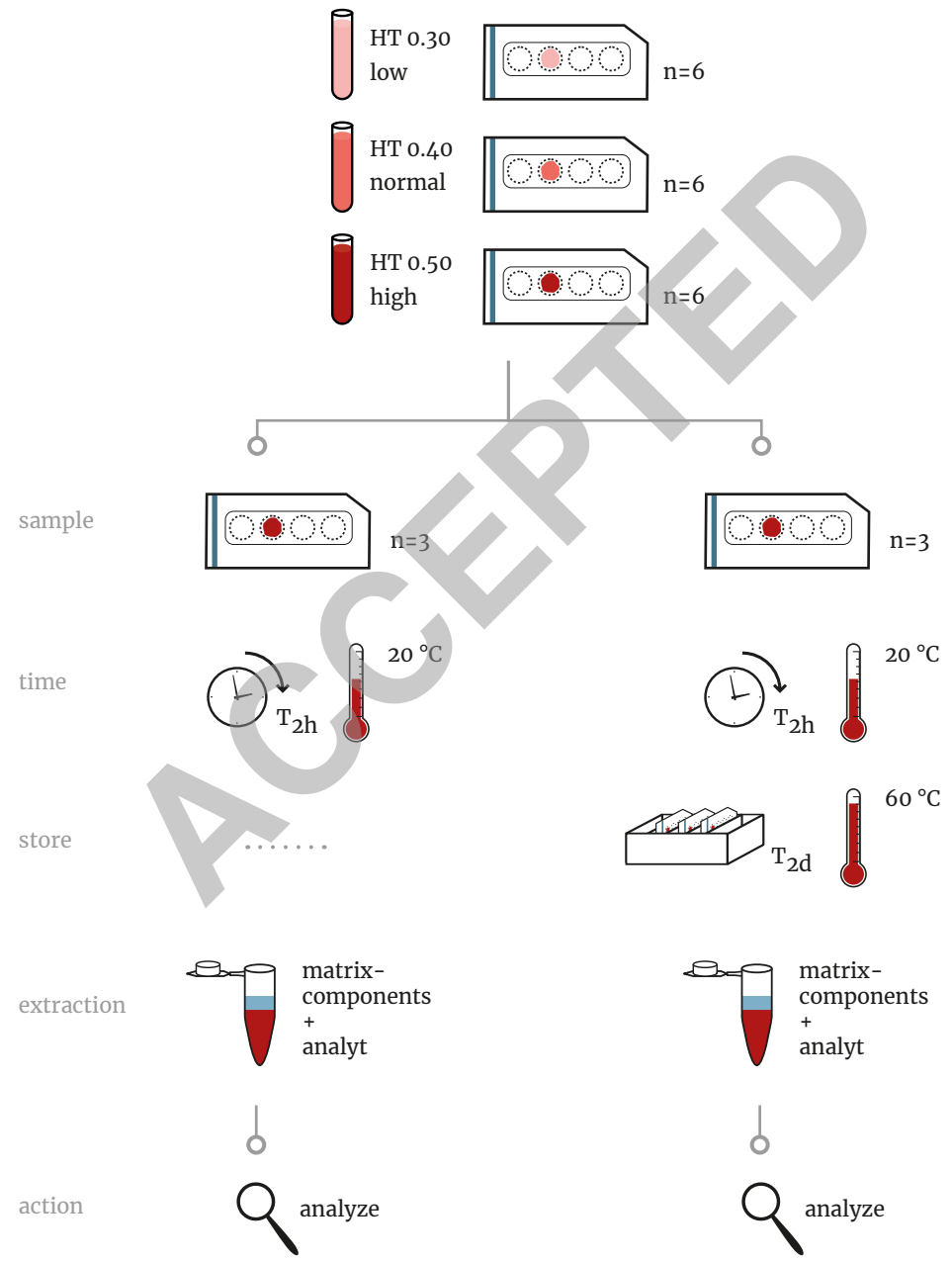
T_0 = starting point = when samples were fresh.

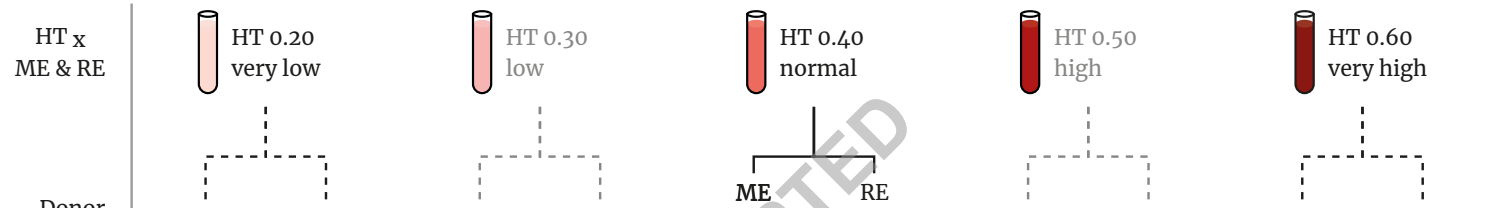




Low or High Quality Control

Spiking **before extraction**





Donor	HT 0.20 very low	HT 0.30 low	HT 0.40 normal	HT 0.50 high	HT 0.60 very high
A	$ME_{0.20A}$ & $RE_{0.20A}$	$ME_{0.30A}$ & $RE_{0.30A}$	$ME_{0.40A}$ & $RE_{0.40A}$	$ME_{0.50A}$ & $RE_{0.50A}$	$ME_{0.60A}$ & $RE_{0.60A}$
B	...	...	$ME_{0.40B}$ & $RE_{0.40B}$	...	...
C	...	...	$ME_{0.40C}$ & $RE_{0.40C}$	...	...
D	...	...	$ME_{0.40D}$ & $RE_{0.40D}$	...	...
E	...	...	$ME_{0.40E}$ & $RE_{0.40E}$	...	...
F	...	...	$ME_{0.40F}$ & $RE_{0.40F}$	...	...

HT 0.30
low

HT 0.50
high

volume
spotted
(µl)