Supplement S1 – Guideline summary

SCOPE

This guideline aims to define the elements necessary for the validation of quantitative dried blood spot-based methods. The main focus of this guideline is the analysis of dried blood spots (DBS) for the quantitative determination of small molecule drugs and drug metabolites using chromatographic techniques for therapeutic drug monitoring (TDM) purposes.

METHOD DEVELOPMENT

Before embarking on the set-up of a dried blood spot-based procedure, it is essential to carefully think about the purpose of the method.

Sampling method: which sample collection method is best suited for a certain application largely depends on the context in which the method will be applied (e.g. home sampling). Also, the required sample volume, automation capabilities, commercial availability and cost may play an important role in the selection of the sample collection method.

Filter paper: 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. Analyze some blank collection substrates to assess whether the collection material itself is blank and whether there are any interferences present.

Sample volume: the required sample volume will mainly depend on the envisaged lower limit of quantitation (LLOQ). 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. The number of punches required for a single analysis should remain as small as possible (preferably one).

Drying and storage process: dry samples at least 3h under ambient conditions outside of direct sunlight and store them with desiccant. Evaluate whether the drying time is adequate under the conditions likely to be encountered during the collection of the patient samples using dried blood samples with a high HT (0.50 – 0.65, depending on the target population) and, if applicable, a large sample volume, as these will dry the slowest. The ambient conditions during drying may also affect DBS homogeneity.

Punch size: for volumetric DBS applications, the punch size needs to be large enough to punch out the entire DBS, independently of the HT of the sample. Hence, select the required punch size based on samples with a HT of approximately 0.15.
For non-volumetric DBS applications, partial DBS punches are made that exclude the outer edge of the sample. The punch size largely depends on the desired LLOQ and DBS homogeneity. Typical punch sizes are between 2 and 8 mm.

Internal standard incorporation: the IS is typically added to the extraction solution or directly to the DBS punch before extraction and will not compensate for variability in analyte recovery. Therefore, analyte recovery has to be investigated extensively.

Type of blood used: for the set-up of calibration curves and QCs it is impossible to use finger prick samples. Instead, spiked samples generated from venous whole blood containing an anticoagulant, are used. 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. If no anticoagulant is used during the collection of the patient samples, the anticoagulant should not impact the obtained results nor the analyte's stability. Compare results obtained from a non-anticoagulated sample with results from samples anticoagulated with different anticoagulants. These samples should be obtained venously from the same person at the same time.

Preparation of spiked samples: first adjust the hematocrit (HT) of the whole blood to the desired HT value. For most experiments the latter will correspond to the median HT value of the target population (e.g. for the calibration curve). Measure the HT, to assure the sample was prepared correctly. 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. Equilibrate for a sufficient amount of time at a suitable temperature, to mimic the analytes' *in vivo* RBC/plasma distribution.

PREVALIDATION

Proper 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.

Extraction efficiency and short-term stability

Since time-dependent extraction issues shortly after sample generation have been described, results from samples analyzed at T_0 (typically 30min – 3h after sample generation) should be compared with results obtained at later time points, preferably up to 48h or 72h. If the measured analyte concentration only decreases for the first time points, but remains stable for the later ones, it is possible to obtain good analytical results if only samples older than a certain age (depending on the time point from which stable results were obtained) are measured.

The extraction efficiency may be concentration, HT and time-dependent and, importantly, these parameters may also affect one another. For non-thermolabile compounds, this can be evaluated by comparing the results from fresh dried blood samples 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. Importantly, in case of partial DBS analysis, these samples should be prepared by pipetting a fixed amount of blood onto prepunched filter paper disks to rule out any influence of the HT effect on the amount of sample being analyzed. For more thermo-labile compounds a similar experiment can be performed with samples stored at e.g. room temperature for two weeks instead of at 60°C for two days.

ANALYTICAL VALIDATION

Selectivity: blank samples should be obtained using the same sampling approach as the one that will be used to collect the patient samples

Calibration model, accuracy and precision, measurement range: all experiments should be performed in accordance with existing guidelines. 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. For the purpose of TDM, a calibration range spanning from half of the lower end of the therapeutic interval to twice the upper end of the therapeutic interval suffices.

Dilution integrity: to analyze samples above the measurement range, dried blood sample extracts are typically diluted with blank dried blood spot extracts or extraction solvent. Furthermore, IS-tracked dilution or the donut punch approach can be used.

Carry over: aside from classical carryover, in a DBS workflow, the punching step could be considered a potential source of contamination. Hence, we propose to include the processing of one or more blanks following the processing of the highest calibrator.

Matrix effect, recovery and process efficiency**:** these should be evaluated in line with the set-up proposed by Matuszewski et *al*. Blood from at least six different donors should be used and two concentration levels should be evaluated. 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. To accurately perform this experiment, a fixed volume of blank or spiked blood needs to be applied on pre-punched filter paper discs or VAMS tips.

Stability: the stability assessments should be representative of the ambient conditions encountered during sample transport, storage and processing 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) should also be tested. Additionally, post-preparative stability should be assessed.

DBS-specific validation parameters

The analytical validation of DBS methods requires the evaluation of the volume effect, the volcano effect and the HT effect. It is essential that these parameters are assessed simultaneously, as they may affect one another. If a relevant volume, HT or volcano effect is observed, appropriate measures need to be taken to ensure patient samples are within the validated limits and are reliable. Obviously, it should also be demonstrated that these measures are indeed adequate.

a) 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, medium and high HT and at both low and high QC.

b) Volcano effect (DBS homogeneity): in case of partial DBS analysis, it is essential to assess whether results from central punches are equivalent to peripheral ones. This evaluation must be performed at low and high QC, at different HT levels (low, medium and high) and at sample volumes representative of the patient sample volumes (n = 5). All samples should be compared to a calibration curve prepared with samples of medium HT and volume, of which a central punch was extracted. If a relevant volcano effect is observed, only central punches should be analyzed.

c) Hematocrit effect: 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. At each HT level, two concentrations should be tested.

An overview of the minimally required analyses for the analytical validation of DBS.

Validation parameter	**Number of samples**
Selectivity	n = 6 x 1 x 2 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
Accuracy & precision	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
Carry-over	n = 2 x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo
Recovery, matrix	n = 2x (2 x 5 **x 1** x 1 x 3) + 2x (2 x 1 **x 3** x 1 x 3) + (2 x 1 x 3) =

effect, process efficiency	102 2 QC levels, 6 donors, of which 1 donor at **3 HT levels**, 1 day, in triplicate (spiked before/after) 2 QC levels, 1 day, triplicate (standard 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
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, in quintuplicate**
TOTAL	**344**

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

CLINICAL VALIDATION

Comparison between DBS and traditional matrix

- Samples should be collected after the distribution phase and within 5 to 10 minutes from each other. They should be collected as will be done during routine use and should be processed and analyzed as was done during analytical validation.
- Samples should be collected after disinfecting the finger without excessive milking or squeezing of the puncture site. The drop of blood should either freely fall onto the DBS card or should be deposited onto it by gently bringing the drop of blood into contact with the filter paper without the finger touching the filter paper.
- Ideally, collect at least 80 samples from at least 40 patients (if samples are difficult to obtain, collect at least 40 paired samples from at least 25 patients).
- Collect finger prick DBS (at least in duplicate) and venous whole blood
- Analyze capillary DBS, venous DBS, venous liquid whole blood and, depending on the traditional matrix used, also venous plasma or serum.

- Compare venous whole blood and venous DBS: a difference between both methods indicates a problem with the DBS approach *per se*.

  By plotting the differences between the DBS results and whole blood results *vs*. HT, the presence of a HT effect can be evaluated. If there is no HT effect, the slope of the resulting curve should not be significantly different from zero.
- To find out a potential capillary-venous difference, the results obtained from fingerprick DBS can be compared with the corresponding venous DBS.
- The differences in analyte concentration between liquid whole blood and plasma depend on the distribution of an analyte between red blood cells and plasma and may be HT-dependent (the latter can be evaluated as described earlier).
- Eventually capillary DBS results should be compared with reference method results. Conversion between both may be HT-dependent or not (which can be evaluated as described earlier) and will be the result of the of the differences observed during the above-mentioned comparisons.
- Whenever two matrices are compared, a conversion factor, if required, can be derived from the Passing Bablok or weighted Deming regression analysis (if the conversion is not HT-dependent). If the conversion is HT-dependent the conversion should also include the patient's HT value. A conversion factor should be determined on the first 40 randomly selected samples and should be validated by applying it to the other 40 samples.
- If fewer samples are obtained, conversion factors may be validated using a jack-knife approach.

<u>Required statistics</u>

- Passing Bablok or weighted Deming regression analysis to assess the correlation between two matrices and the presence of proportional and/or systemic errors.
- Bland and Altman plot to assess both bias and agreement between both matrices.

- Acceptance criteria for bias and limits of agreement should be agreed upon beforehand by a multidisciplinary team of experts based on clinically and analytically relevant parameters.
- These statistics should be ran before and after conversion calculations have been performed.
- Incurred sample reanalysis (to assess method reproducibility, as described in FDA guideline draft). The % difference between duplicates should not be greater than 20% of their mean for at least 67% of the samples. These duplicates may be two punches from the same DBS or two punches from two different DBS on the same card (also depends on punch size).

How to ensure good spot quality?

- Patient education
- Cards with two concentric circles corresponding to the minimally and maximally required sample volume that should be collected (taking into account the Ht-range of the population).
- Checking the spot quality in the laboratory, either by lab technicians or by an automated system.
- The same method to ensure/check spot quality should be used during clinical validation and routine use.

How to deal with volume, volcano and hematocrit effect?

a) Volume effect

    - Train patient to collect samples of adequate volume

- To ensure the spot volume is within the validation volume range a card with two concentric circles (corresponding to the minimally and maximally required volume may be used).

b) Volcano effect

- If a relevant volcano effect is present, only use central punches (both during validation and routine use).

c) Hematocrit effect

- Unless no relevant HT effect is observed over the entire HT range (both during analytical and clinical validation), 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.
- If the target population has a narrow HT-range, the median HT value of the target population may be used instead of the actual patient HT (e.g. for DBS – plasma concentration conversion).

Quality control

- Join a dried blood sample-based EQC program if available
- EQC materials for liquid blood-based methods can be used if whole sample analysis is performed (although differences in extraction efficiency between the EQC and a genuine dried blood sample cannot be ruled out).
- EQC materials for plasma-based methods can be used to replace (part of) the plasma of a blank donor sample. The spiked sample can then be used to generate a dried blood sample

(again whole sample analysis is required and differences in extraction efficiency cannot be ruled out).

CROSS VALIDATION

Different punch size

- Compare 40 samples of at least 25 different patients with both punch sizes.
- Thoroughly re-evaluate extraction efficiency for potential concentration, time and HT-dependent issues.
- Re-evaluate DBS-homogeneity (although the DBS homogeneity will not change *per se*, smaller punch sizes may be more sensitive to this phenomenon).

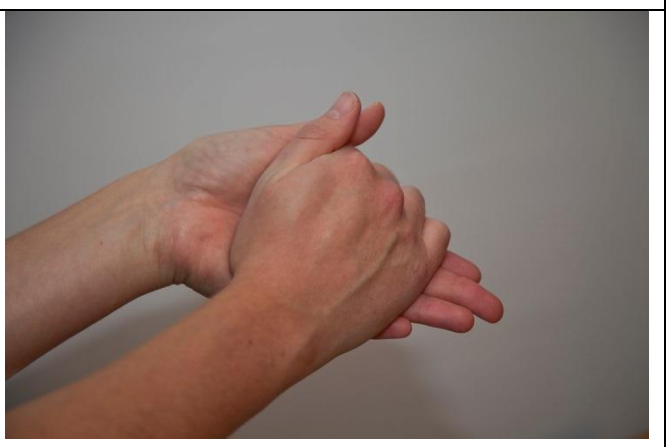
Different type of filter paper

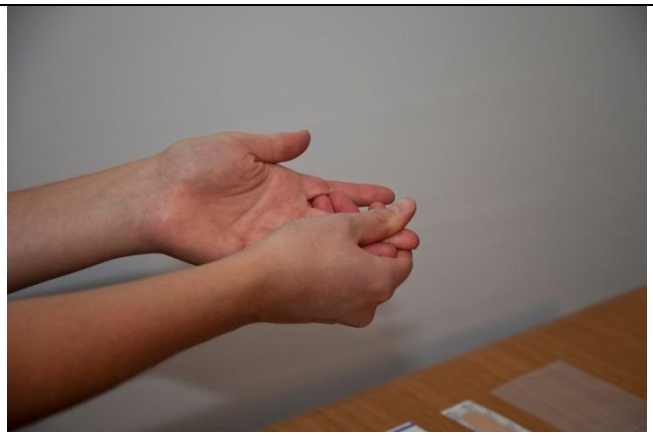
- Compare 40 samples of at least 25 different patients with both types of filter paper.
- Thoroughly re-evaluate extraction efficiency and matrix effect for potential concentration, time and HT-dependent issues.
- Re-evaluate DBS-homogeneity, stability as well as volume and HT effect.

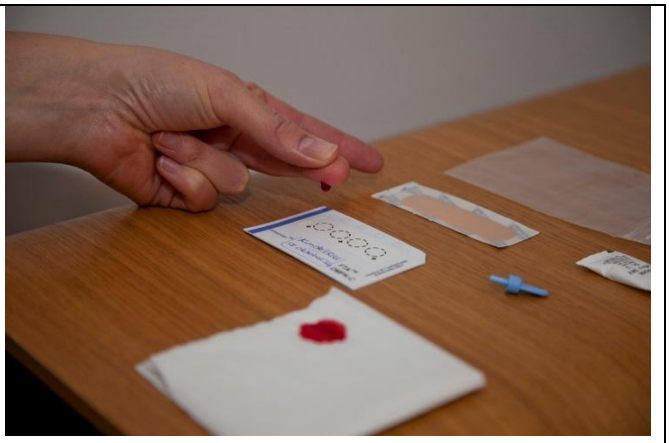
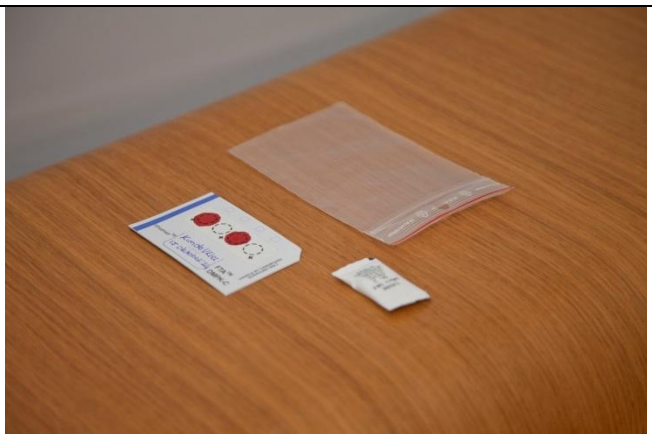
Different sampling method

- Requires a completely new analytical and clinical validation

Supplement S-2 DBS sampling procedure for sampling at home

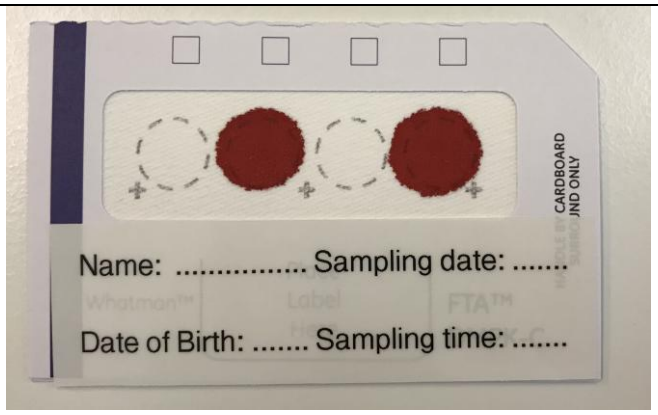
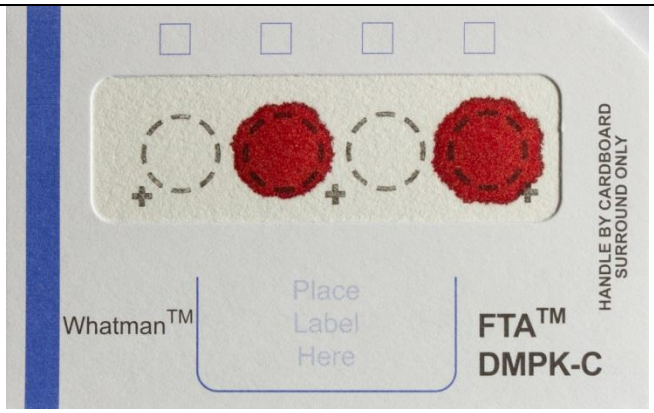
1. Place all sample accessories (blood collection card, lancets, sealable plastic bag, desiccant pack, band-aid, alcohol prep) on a smooth and clean surface. Do not touch the filter paper.	
2. Wash hands with soap and warm water for at least 30 seconds. Thoroughly dry hands.	
3. Warm hands by rubbing the hands together to increase blood flow. Afterwards, disinfect the top of the middle- or ring finger with the alcohol prep and let dry for at least 40 seconds.	

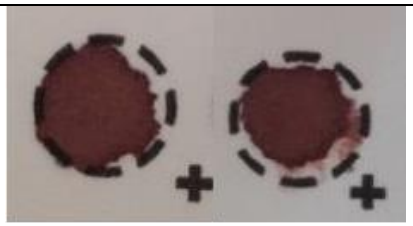
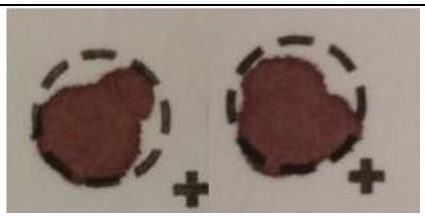
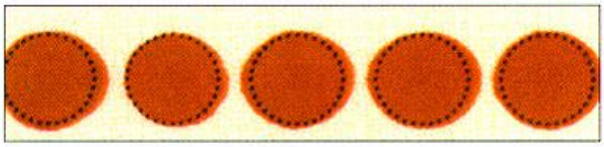
4. Prepare the lancet by removing the plastic cover.	
5. Gently massage the fingertip in a downward motion to increase blood flow.	
6. Place lancet on the finger and push to prick the finger.	
7. Allow a blood drop to form and wipe away the first drop with a tissue or cotton ball.	

8. Allow a large drop to form. If a drop doesn't form, gently massage the finger downwards without excessive 'milking' of the finger. Ideally, a single drop falls on the sampling card filling the circle completely. If the drop doesn't fall within 30-60 seconds, lightly touch the circle with the blood drop without touching the filter paper with the finger and without layering successive blood drops.	
9. Fill at least 2 circles completely, without the droplets touching each other. Puncture another finger (repeat steps 4-8) if the blood flow from the first puncture is insufficient.	
10. Clean the finger(s) and, if needed, place a band-aid on the finger(s). Dispose of all lancets appropriately.	
11. Record time and date of sampling and patient details or study number on the sampling card. Dry for a least 3 hours in a non-humid environment at ambient temperature on a clean and flat surface. Keep away from direct sunlight.	

12. After drying, place each individual sampling card inside a plastic (biohazard) seal bag with a desiccant pack and transport as soon as possible to the laboratory using regular mail or other transportation methods.	

Supplement S-3: spot quality assessment

General - Patient details / study number provided. - Date and time of sampling provided.	
Package and shipping - DBS is sealed in a plastic bag with desiccant sachet. - DBS and plastic bag are intact and blood is only applied to filter paper. - Time between sampling and analysis is within validated stability range.	
Valid samples - Circles should be filled with enough blood to meet criteria set by the lab. - Both sides of the filter paper should be equally soaked with blood. - Blood drops should not touch each other. - Blood drops should be round and consisting of one droplet.	
Invalid samples	
Lighter circles around the darker blood spots due to applying heat, contamination or excessive squeezing of the puncture site.	

The blood spots are crusted on the card, causing the DBS card to be contaminated. Also, finger smearing is visible on the right spot.	
The premarked circle contains multiple overlapping blood drops.	
Sample not dried before mailing.	
Clotted or layered spots.	
Serum rings, possible due to contact with alcohol or excessive squeezing of the puncture site.