

МЕТОД ФАРМАКОГЕНЕТИЧЕСКОГО ТЕСТИРОВАНИЯ ПОСЛЕ ПРОСТОГО ВЫДЕЛЕНИЯ ДНК

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Реферат. Существует несколько коммерческих наборов для выделения ДНК, пригодных для экстракции геномной ДНК из образцов цельной крови, но эта процедура требует определенных затрат времени, ручной работы и довольно дорога. Альтернативной техникой мог бы быть метод исследования сухого пятна крови (СПК), который позволяет быстрее выделять ДНК, более дешевый и простой. Мы разработали некоммерческий метод сухого пятна крови и оценили достаточны ли качество и количество ДНК, выделенной из СПК и неинвазивно получаемого мазка со щеки. Изоляты ДНК, полученные из образцов крови с этилендиаминтетрауксусной кислотой и пятен крови на фильтровальной бумаге, были сопоставлены после выделения ДНК посредством колонки и двумя альтернативными методами. Образцы крови были получены посредством трения стерильным сухим хлопковым тампоном по внутренней поверхности щеки испытуемого. Кроме того, было измерено качество выделенной ДНК, полученной разными методами, проведен анализ кривых плавления 5 цитохромов P450 и 3 АТФ-связанных кассетных полиморфизмов посредством полимеразной цепной реакции (ПЦР) в реальном времени. Во всех случаях результаты генотипирования были полными. Более того, производные кривых плавления образцов ДНК из капиллярной крови и альтернативных образцов были сопоставимы и высоковоспроизводимы. Средние измеренные концентрации ДНК составили 16,0 нг/мкл (12,6—19,4 нг/мкл) и 70,2 нг/мкл (57,3—83,1 нг/мкл) соответственно для сухого пятна и образца со щеки ($p < 0,001$). Выделение ДНК методом СПК является альтернативным существующим коммерческим набором для выделения ДНК и более простым методом для установления различий по генотипам. Метод расширяет возможности быстрого и простого выделения ДНК. В частности, неинвазивный метод получения материала со щеки может стать хорошей альтернативой инвазивному пути получения образцов.

Ключевые слова: метод исследования сухого пятна крови и образца со щеки, ДНК, генотипирование.

PHARMACOGENETIC TESTING AFTER A SIMPLE DNA ISOLATION METHOD

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Abstract. Several commercial DNA isolation kits are available for extracting genomic DNA from whole blood samples, but these procedures require certain consumption of hand and work time and are rather expensive. An alternative technique could be dried blood spot (DBS) sampling, with which DNA isolation is faster, cheaper and logistics are easier. We have developed a non-commercial DBS method and examined whether the quality and quantity of DNA, isolated from DBS and noninvasively obtained buccal swab (BS) samples, was satisfactory. DNA isolation from EDTA blood samples and blood spots on filter paper were compared after DNA isolation with a column method and two different DBS methods. BS samples were obtained by rubbing a sterile, dry cotton swab against the inside of the subject's cheek. In addition, the quantity of the obtained DNA was measured and melting curve analyses of 5 cytochrome P450 and 3 ATP-binding cassette polymorphisms were performed with real-time PCR FRET assays to establish the quality of the obtained DNA from both the DBS and BS samples. In all cases the genotype results corresponded completely. Moreover, the derivative melting curves of the DNA samples obtained from the capillary blood and BS were comparable and highly reproducible. The mean DNA concentrations measured were 16,0 ng/μl (12,6—19,4 ng/μl) and 70,2 ng/μl (57,3—83,1 ng/μl), respectively, for DBS and BS samples ($p < 0,001$). The DBS DNA isolation appears to be a useful alternative for the commercially available DNA isolation kits and an extremely useful method to discriminate between genotypes. This expands the possibilities of this quick and easy DNA isolation procedure. In particular, the noninvasive BS sampling method appeared to be a good alternative to invasive sampling methods.

Key words: dried blood spot and buccal swab, sampling method DNA, genotype.

Introduction. Pharmacogenetics, the study of the role of inheritance in the individual variation in drug response, is still a growing field and it is gaining more importance in the treatment and investigation of the cause of certain diseases, symptoms and adverse drug reactions (ADR). The promise of pharmacogenetics lies in its potential to identify the right drug and dose for each patient. Even though individual differences in drug response can result from the effects of age, sex, disease or drug interactions,

genetic factors also influence both the efficacy of a drug and the likelihood of an adverse reaction occurring [1—3]. There is an increasing number of examples in which pharmacogenetic studies have indicated that a genetic test prior to treatment may be useful either for setting the individual dose or making a decision to use a particular drug [4—6]. Therefore, genetic testing of individuals, specific patient groups and possibly their family members or genotyping prior to prescribing certain drugs is becoming

more important. The materials used for genotyping are mostly ethylene diamine tetra-acetic acid (EDTA) whole blood obtained by venous sampling and tissue samples. However, both require an invasive sampling method and for a number of subjects, this can be a reason to refrain from signing up to an investigation or trial.

Several commercial DNA isolation kits are available for extracting genomic DNA from EDTA whole blood samples. To obtain DNA from whole blood, commercially available DNA isolation procedures, require approximately 1 h hands on time of a technician and are rather expensive (approximately €4 per isolation). Simplification of the standard DNA isolation procedures by the dried blood spot method (DBS) might be advantageous. DBS sampling has already become common practice for newborns. Over the past decade many applications have been reported for both qualitative and quantitative screening of metabolic disorders [7]. Possible advantages of genotyping with DBS are.

1. The patient does not have to leave home and no phlebotomist is necessary.
2. Genotyping results are known when the patient visits the clinician and the clinician can take these results into account when he prescribes (other) drugs.
3. Transport is easy and there is a decrease in transport costs because only an envelope with the DBS of the patient is required.
4. Lower isolation costs and faster handling: DNA isolation with DBS is rapid, simple and no expensive DNA isolation kits are necessary.

Previously, the usefulness of DBS genotyping has been described [8, 9]. However, a non-commercial method for DNA isolation with DBS in association with pharmacogenetics has not yet been reported in literature. In the present study a new non-commercial DBS method was developed for isolating DNA from capillary blood and this method is validated *versus* a standard commercial available DNA isolation kit and also compared to an already existing extraction method for DBS described by Fischer et al. [10].

The aim of this study was also to test if the same DNA isolation protocol as applied to DBS could be used for isolating DNA from noninvasive buccal swab (BS) samples. Subsequently, the DNA quality and quantity of the BS samples were compared with the obtained DBS samples. Real-time PCR melting curve analyses were performed and DNA yields were measured using UV spectrometry. Additional storage tests were performed in order to ascertain whether delays in sending the sample to the laboratory or rather time from acquiring the BS sample to isolation, had an influence on the final result. Moreover, establishing the turnaround time and storage conditions required to avoid problems allows storage recommendations to be provided for the patient to follow.

Materials and methods. Samples and materials. EDTA whole blood samples and blood spots on filter paper made from the same samples were obtained from 106 Chinese renal transplant recipients and isolated by using 200 µl EDTA whole blood with a column method QIAamp blood mini kit, (Qiagen, Leusden, The Netherlands) according to manufacturer's instructions and the DBS method developed by our lab. Additionally, DNA was isolated from 10 samples obtained from healthy volunteers using the column method for EDTA blood and simultaneously obtained finger prick blood was isolated using the DBS method described in this study and the one described by Fischer et al.

Furthermore, another 100 DBS samples were collected by finger prick from healthy volunteers to examine our DBS method in clinical practice. When using the DBS method capillary blood can be obtained by a finger prick by the patients themselves. The drop of blood is spotted on filter paper (Whatman® Schleicher and Schuell, code 903 (2992), Whatman®, Dassel, Germany).

Buccal swabs were obtained by thoroughly rubbing a sterile, dry cotton stick (Copan plain swab sterile plastic applicator rayon tipped, ref 155C, Copan, Brescia, Italy) twice up and down against the inside of the individual's cheek on the one side of the mouth. One DBS and one BS sample were collected at the same time from 25 healthy volunteers, resulting in a total of 25 DBS and 25 BS samples to examine the two sampling methods that could be used in clinical practice. In addition, the samples were isolated according to our DBS protocol.

After obtaining 12 BS samples per individual from a total of five healthy volunteers for a storage test, four swabs from each subject were kept at room temperature, four were put in a refrigerator, and four in a freezer. The BS samples kept at room temperature were isolated at day 1, 3, 5 and 7 after sampling. The samples kept at 4–8°C were isolated at 1, 2, 3 and 4 weeks and the BS samples stored at -20°C were isolated at 1, 2, 3 and 4 months after sampling.

The DNA concentrations of the samples were measured on the NanoDrop® ND-1000 UV Spectrophotometer (Witec ag, Littau, Switzerland).

Written informed consent for participation in this study was obtained from all subjects.

DNA isolation. EDTA whole blood samples, capillary DBS and BS samples were collected. In the laboratory, after drying for 1/2 h a 3 mm paper disk was cut out from the blood spot sample with a puncher (Harris Uni-core□) or the tip was cut off the cotton stick (*fig. 1*). In addition, the 3 mm bloodstained paper disk or the cotton tip was placed into a cup, 500 µl sterile water was added and vortexed three-times for 5 s each. The water was pipetted off. After adding 200 µl 10% Chelex® 100 solution (Bio-Rad Laboratories, CA, USA), the cup was placed in a water bath at 95°C for 30 min. Finally, this DNA solution was pipetted into a new cup and the DNA was ready for use.

With the method described by Fischer et al. [10] the 3 mm disk is placed in an Eppendorf□ cup and washed twice with 1 ml of phosphated-buffered saline — 0,1% Tween during 10 min with shaking. After transferring the disk to a 2 mL screw-cap cup and adding 200 µl 5% Chelex-100 solution (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), the cup is placed in a water bath at 60°C for 30 min, followed by 30 min in a water bath boiling at 100°C.

Genotyping. A total of five previously described cytochrome P450 (CYP) and three ATP-binding cassette (ABCB1) polymorphisms were performed in this study, using real-time PCR fluorescence resonance energy transfer (FRET) assays (TIB MOLBIOL, Berlin, Germany), namely: *CYP3A4-V A-392G*, *CYP3A5*3 A6986G*, *CYP2C9*2 C430T/*3 A1075C*, *CYP2C19*2 G681A/*3 G636A*, *CYP2D6*3 2549delA/*4 G1846A*, *ABCB1 C1236T*, *ABCB1 G2677T/A*, and *ABCB1 C3435T* [11–15]. The single nucleotide polymorphisms (SNPs) were genotyped by examining the melting curves from the aforementioned FRET assays using the LightCycler® (Roche Diagnostics, Basel, Switzerland).

For comparison an EDTA sample, isolated on the MagNA Pure Compact (Roche Diagnostics), was genotyped within



a



b

Fig. 1. Sampling of dried blood spots and buccal swabs: (a) dried bloodspot disk sampling; (b) buccal swab sampling

the same run as the DBS and BS samples. Furthermore, a heterozygote plasmid sample supplied with the primer/probes sets of each assay and a negative water control were analysed within each run.

Statistical analysis. The paired t-test was used to compare the two sampling methods.

Melting curves were generated by the LightCycler® and its software/integration program calculates melting temperature(s) for each detected peak.

Statistical analyses were performed with SPSS 15,0 (SPSS, Inc., IL, USA) for Windows. A p-value of less than 0,05 (two sided) was considered to indicate statistical significance.

Results. After analysing the melting curves obtained by real-time PCR FRET assays for the different DNA isolation methods, the results obtained with the DNA extracted with the column method were in complete concordance with the results of the DNA isolated with our DBS method. Additionally, the DNA isolation with the QIAamp blood mini kit and the two DBS methods performed for 10 healthy volunteers were also completely in concordance. Genotyping with the DNA isolated with our DBS method for the 100 healthy volunteers gave also satisfactory melting curves.

Although analysis of the column and previously described DBS DNA isolation methods were performed for five different real-time PCR FRET assays, an overview of the results is only given for the *CYP3A5* A6986G polymorphism. Melting of a sample homozygous for the 6986G allele produced a melting peak at approximately $57,1^{\circ}\text{C}\pm 0,1$ (mean ± 2 SD) and $57,3^{\circ}\text{C}\pm 0,2$, and the 6986A allele gave a melting peak at approximately $62,3^{\circ}\text{C}\pm 0,2$ and $62,5^{\circ}\text{C}\pm 0,5$, for respectively the QIAamp blood mini kit and our DBS DNA isolation method. Heterozygous samples contained both type of method and thus generated both peaks. In addition, for heterozygous samples ($n=40$), the difference between the two melting temperatures ($5,2^{\circ}\text{C}\pm 0,1$) had a coefficient of variation (CV) of respectively 0,64% and 1,24%.

Hence, the derivative melting curves of the commercial DNA isolation method and our DBS method were highly reproducible and can therefore be used perfectly for discrimination between the *CYP3A5**1 and *CYP3A5**3 allele, which is illustrated in *fig. 2*. The results of the other real-time PCR FRET assays were similar to the results presented for the *CYP3A5* A6986G polymorphism.

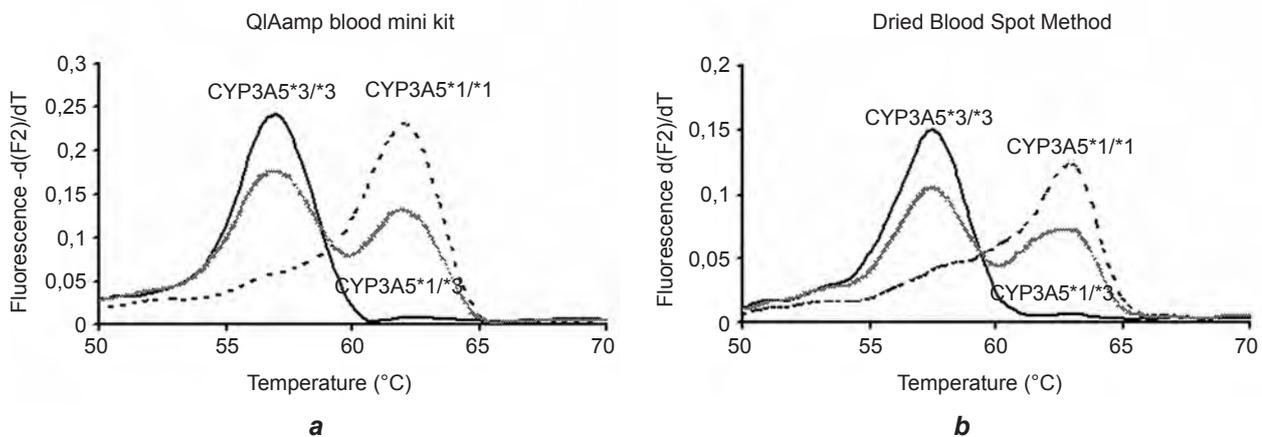


Fig. 2. Examples of melting curves from DNA isolated with the QIAamp blood mini kit from EDTA whole blood, and the dried blood spot method from capillary blood.

Genotyping of the A6986G polymorphism of the *CYP3A5* gene with allele specific fluorescent probes by derivative melting curve plots using DNA samples isolated with the QIAamp blood mini kit (a) and with our dried blood spot DNA isolation method (b).

The $-dF2/dT$ derivative melting curves are read in channel 2 for the detection of the *CYP3A5* fragment. The derivative melting curves is plotted for a similar sample homozygous for the 6986G (*CYP3A5**3/*3) allele (Tm 57°C); a similar heterozygous sample (Tm 57°C (*CYP3A5**3) and 62°C (*CYP3A5**1)) and a similar sample homozygous for the 6986A (*CYP3A5**1/*1) allele (Tm 62°C)

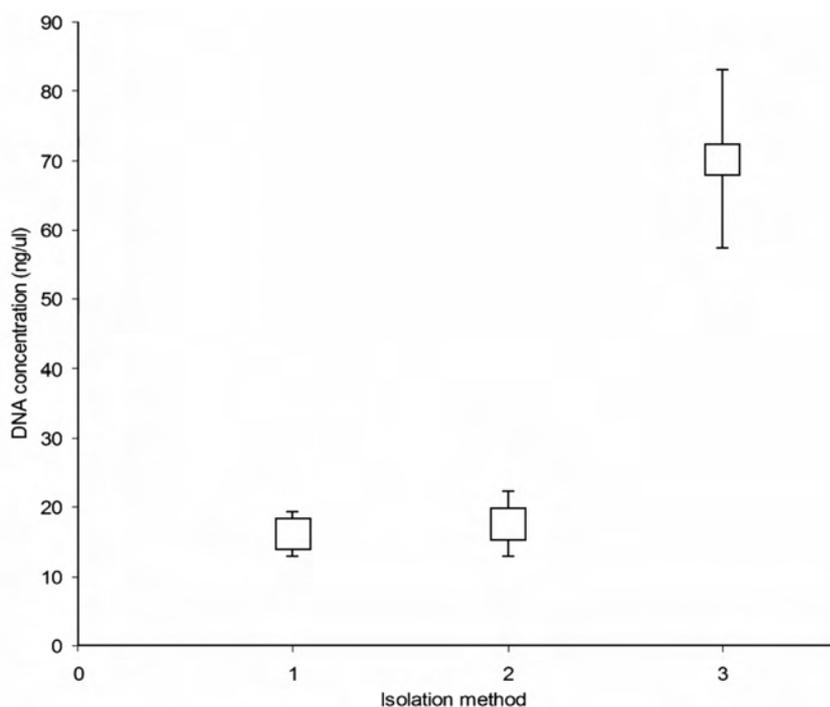


Fig. 3. Mean DNA quantities, isolated with two different DBS DNA isolation protocols, using capillary blood DBS and BS samples:

1. DBS sample isolated using our method.
2. DBS sample isolated using the method described by Fischer et al. [10].
3. BS sample isolated using our method

BS: Buccal swab; DBS: Dried blood spot.

An issue that may be of importance when the DBS method is used for DNA isolation is a possible contamination risk. Because it is likely that the filter paper used will be contaminated before and/or after the blood spot is actually on the filter paper. Therefore, we examined whether the DBS DNA isolation procedure is robust enough to discriminate the blood spot of the patient from the contamination caused by rubbing vigorously over respectively the dry filter paper, the dry filter paper before adding a blood spot and the dried blood spot on the filter paper. In the cases with the blood sample only the genotypes of the patients were seen and in the case of the no blood blank disk, no signal was found, which implies that there is no disturbing influence when the filter paper is contaminated by hands before or after adding the blood spot on the filter paper.

The DNA concentrations of the DBS and BS samples resulted in mean concentrations of 16,0 ng/μl (12,6—19,4 ng/μl) and 70,2 ng/μl (57,3—83,1 ng/μl), respectively ($p < 0,001$) obtained using our isolation method. As a comparison, the samples isolated using the dried blood spot method previously described by Fischer et al. [10] resulted in a mean concentration of 17,5 ng/μl (12,8—22,2 ng/μl) for these DBS samples (fig. 3).

The derivative melting curves of the DNA samples obtained from capillary blood and BS samples were comparable and highly reproducible for all SNPs studied. As a representative example, the melting results of the CYP2C9*2 C430T SNP are hereby presented below. Melting of a sample homozygous for the 430T allele produced a melting peak at approximately $50,6^{\circ}\text{C} \pm 0,4$ and $50,7^{\circ}\text{C} \pm 0,4$ for a BS and a DBS sample, respectively. In a sample homozygous for the 430C allele a melting peak was obtained at approximately $59,5^{\circ}\text{C} \pm 1,2$ and $59,6^{\circ}\text{C} \pm 1,3$, respectively, for a BS and a DBS sample. Heterozygous samples contained both type of targets and therefore generated both melting peaks, with a mean \pm SD difference of $9,6^{\circ}\text{C} \pm 0,4$ and $9,5^{\circ}\text{C} \pm 0,7$ for the BS and DBS samples, respectively. An overview of all the melting temperature ranges for the SNPs

investigated, stratified per sample type, are listed in table.

Genotyping confirmed the results demonstrating the same amount of peaks at the same melting temperatures and concurrent results were obtained for the DBS and BS sample types per subject for the CYP3A4-V, CYP3A5, CYP2C9, CYP2C19 and CYP2D6 SNPs that were investigated (table). An EDTA sample, isolated with the QIAamp blood mini kit, was also analyzed in the same run to illustrate and compare the height and position of the melting curves of the different sample types. A representative example of a FRET melting curve analyses is shown for the CYP2C9*2 assay in fig. 4.

Furthermore, we investigated the stability of the BS samples obtained using a plain cotton swab under different storage conditions. When kept at room temperature, the performed FRET assays gave only a weak signal that was difficult to interpret, or no signal at all, after isolating the swabs after a 7 day period. In contrast, when isolating the BS samples at day 5 all the samples still performed well. Storage at $4-8^{\circ}\text{C}$ extended that period to 1 month, whereas storing the BS samples at -20°C gave good results up until at least 4 months.

Discussion. A large difference was found for labour time and costs between the QIAamp blood mini kit and our DBS method: it took approximately 1 h and €4.00 per blood sample to isolate DNA from EDTA whole blood with the QIAamp mini blood kit while DNA isolation with the DBS method takes about 45 min hands on time and less than €2.00 per capillary blood sample. Moreover, there was also a significant difference when comparing the two DBS methods, not so much in costs, but in hands on and total time. The method described by Fischer et al. [10] takes about 1 h more before DNA is obtained. Furthermore a Tween/phosphate-buffered saline solution is necessary for washing the DBS. For DBS samples, there was virtually no difference in DNA yield between the isolation method previously described by Fischer et al. [10] and the one described in this study. Moreover, an advantage of our method is that the use of chemicals is minimal, except for

Overview of melting temperatures ± 2 standard deviations per sample type for the CYP450 SNPs examined and isolated with our dried blood spot method

CYP450	SNP	Tm	BSS ($^{\circ}$ C)	DBS ($^{\circ}$ C)	Expected Tm ($^{\circ}$ C)
CYP2D6	*3 2549delA	Tm1	NA	NA	52,1 \pm 0,4
		Tm2	58,8 \pm 0,6	58,8 \pm 0,6	59,4 \pm 0,2
	*4 G1846A	Tm1	57,7 \pm 1,2	58,0 \pm 1,9	58,2 \pm 0,2
		Tm2	65,2 \pm 1,5	66,9 \pm 1,8	66,4 \pm 0,8
CYP2C9	*2 C430T	Tm1	50,6 \pm 0,4	50,7 \pm 0,4	50,5 \pm 0,2
		Tm2	59,5 \pm 1,2	59,6 \pm 1,3	60,4 \pm 1,0
	*3 A1075C	Tm1	50,1 \pm 0,2	50,2 \pm 0,2	50,2 \pm 0,3
		Tm2	NA	NA	59,4 \pm 0,1
CYP2C19	*2 G681A	Tm1	49,3 \pm 1,0	49,0 \pm 1,0	49,3 \pm 0,1
		Tm2	55,4 \pm 1,2	55,2 \pm 0,8	56,1 \pm 0,1
	*3 G636A	Tm1	54,0 \pm 0,6	54,2 \pm 0,2	54,1 \pm 0,2
		Tm2	NA	NA	61,4 \pm 0,2
CYP3A4-V	A-392G	Tm1	NA	NA	50,1 \pm 0,4
		Tm2	58,5 \pm 0,8	58,6 \pm 0,3	58,8 \pm 0,4
CYP3A5	*3 A6986G	Tm1	57,6 \pm 0,2	57,5 \pm 0,2	57,4 \pm 0,2
		Tm2	NA	NA	62,6 \pm 0,2

BSS: Buccal swab sample; CYP450: Cytochrome P450; DBS: Dried blood spot sample; Expected Tm: Positive control, a plasmid sample; NA: Not available; Tm: Melting temperature.

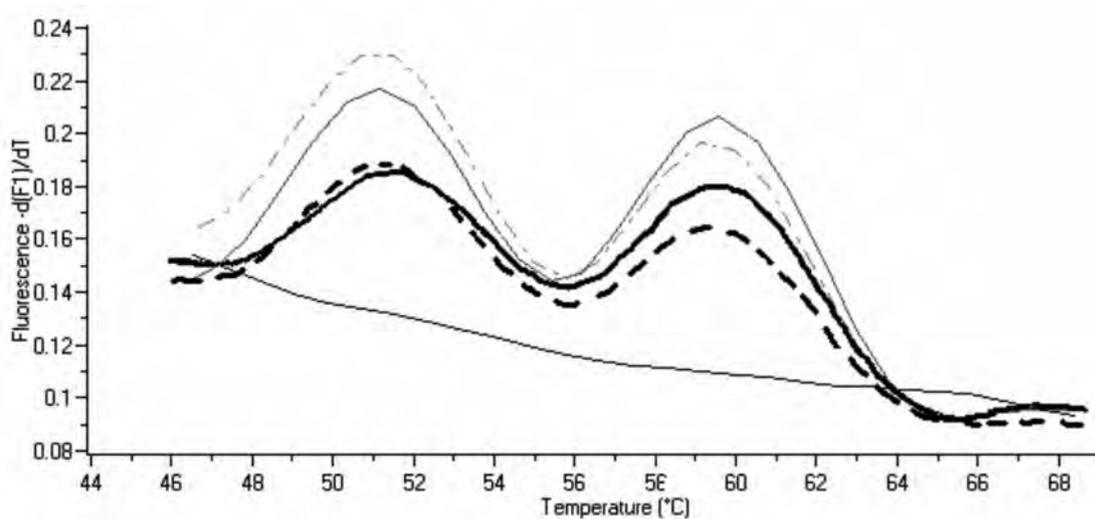


Fig. 4. An example of completely concurrent CYP2C9*2 melting curves of an EDTA blood sample, a DBS sample and a BS sample

Positive control □ □ □ □ • □ □ □ □ □
 EDTA sample □ □ □ □ □ □ □ □ □ □
 DBS sample □ □ □ □ □ □ □ □ □ □
 BS sample —————
 Negative control □ □ □ □ □ □ □ □ □ □

The positive control is a heterozygote plasmid sample, supplied with the primer/probes of each assay. The negative control is sterile water (no peaks).

BS: Buccal swab; DBS: Dried blood spot; EDTA: Ethylenediaminetetraacetic acid.

a 10% Chelex solution, and it has an incubation time of only 30 min.

Although the amount of blood required for this DBS DNA isolation is less, an invasive finger prick is still required to obtain capillary blood. Another restriction of this DBS method can be the sometimes relatively low amount of DNA obtained from the blood spots.

Combined with the noninvasive BS as a sampling method, this provides a patient friendly and easy way of obtaining DNA samples that can be used for genotyping. Especially when conducting a large trial including family members, the BSs can be sent by post. Furthermore, attending the hospital in order to have a blood sample drawn would no longer be necessary. The sending by post and not having to travel to a hospital are advantages that also apply to DBS samples. However, when using that sampling method there is still the need to draw blood. The noninvasive nature of a BS sample combined with the other benefits implicate that there are even fewer drawbacks and people will be more motivated to participate.

We used plain sterile swabs that are readily available and cheap (approximately €0.10 each) to investigate if buccal-cell collection and subsequent isolation would work. Isolation with our method only takes 30 min in a water bath, and adding preparation and pipetting time, the procedure is completed well within an hour and with minimal chemical use and cost. On the other hand, commercially available saliva collection methods (up to approximately €18.00 per collection vial) currently being used in most community-based studies require an additional kit to isolate DNA, and all at considerably higher costs. The obtained BS samples, when using plain sterile cotton swabs, should be isolated within 5 days from the time of sampling to prevent loss of signal when performing melting curve analyses. The fact that we did not get a signal in the cases that were a week old was a strong indication that amplification of the desired fragment was no longer possible and DNA degradation was present. Whenever isolation within 5 days time is not possible, the BS samples can be stored for up to a month at 4–8°C or for up to at least 4 months at -20°C prior to isolation. Therefore, amending the instructions to the patient to include: «Store in the refrigerator when you are not able to send it off by mail the same day», is an important addition that will improve the final result.

After isolation, the ready to use DNA solution (approximately 150 µl per isolation), when pipetted off the Chelex and transferred into a new vial, can be stored at 4–8°C or at -20°C for more than 1 year.

In conclusion, the DNA isolation method described appeared to be extremely useful for the different sampling procedures (capillary blood and BS). Furthermore, it is able to isolate DNA with less hands-on time, is less invasive for the patient, and saves transport expenses and DNA isolation costs. Comparison of our DBS method to another non-commercially available DBS method resulted in similar DNA yields, less use of chemicals and it was less time consuming. All these advantages make our DSB method very useful in clinical practice.

Moreover, it appeared to be a useful alternative for commercially available DNA isolation kits. The DNA yields of BS samples were considerably higher compared with the yields of DBS samples. For DNA isolated from BS

samples could be used perfectly to distinguish genotypes or polymorphisms. This expands the possibilities of this quick and easy DNA isolation procedure. Especially, the noninvasive BS sampling method appeared to be a good and patient friendly-alternative to invasive sampling methods.

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