**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**INSTITUTO DE BIOCIÊNCIAS**

**CURSO DE CIÊNCIAS BIOLÓGICAS - BACHARELADO**

**TRABALHO DE CONCLUSÃO DE CURSO**

**Padronização de um protocolo de extração de DNA a partir de Sangue Impregnado em Papel Filtro para uso na triagem neonatal de doenças lisossômicas selecionadas**

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**Novembro de 2020**

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## 1.   RESUMO

A Triagem Neonatal consiste no rastreamento neonatal de crianças portadoras de doenças que devem ser diagnosticadas e tratadas o mais precocemente possível a fim de evitar sequelas para o paciente. No Brasil, o Programa Nacional de Triagem Neonatal (PNTN) inclui o exame Teste do Pezinho (TP), onde gotas de sangue são impregnadas em papel filtro (SIPF). As doenças lisossômicas de depósito (DLD) são um grupo de doenças onde há acúmulo progressivo de substâncias não metabolizadas, no interior do lisossomo, principalmente devido a uma deficiência enzimática. Os pacientes geralmente são assintomáticos ao nascimento, mas apresentam progressão rápida da doença e manifestações irreversíveis. Atualmente, nenhuma DLD faz parte do TP disponibilizado pelo SUS, de maneira que a inclusão dessas doenças no TP terá um ganho significativo na qualidade de vida futura dos recém-nascidos. A triagem é composta por um conjunto de exames realizados na amostra de SIPF, dentre eles diferentes testes bioquímicos. Além disso, o SIPF pode ser utilizado para a extração de DNA e então para a aplicação em diversas técnicas moleculares. O presente trabalho foi realizado no Laboratório de Genética Molecular do Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre. O objetivo do trabalho foi padronizar um protocolo de extração orgânica de DNA de SIPF utilizando uma quantidade mínima de amostra, 3 discos de 3-mm, para ser utilizado, principalmente, no Sequenciamento de Nova Geração (NGS). O estabelecimento de um protocolo de extração padronizado possibilita que o SIPF enviado ao Serviço de Genética Médica para testes enzimáticos seja reaproveitado na análise molecular, reduzindo significativamente o tempo de confirmação diagnóstica. A padronização do protocolo foi realizada utilizando vinte amostras controle, quantificadas após a extração de DNA, e analisadas através das técnicas de PCR, PCR-RFLP e Sequenciamento de Sanger. Uma vez estabelecida a padronização do método, foram

analisadas através de NGS, três amostras de SIPF de pacientes com diagnóstico bioquímico prévio pertencentes a um projeto piloto de triagem neonatal para DLDs selecionadas, para testar a aplicabilidade clínica do protocolo padronizado e assim confirmar o diagnóstico bioquímico inicial através do diagnóstico molecular. Os resultados demonstram eficiência na padronização do protocolo de extração orgânica, possibilitando o desenvolvimento das diversas técnicas moleculares propostas: PCR, PCR-RFLP, Sequenciamento de Sanger e Sequenciamento de Nova Geração (NGS). Foi possível determinar o genótipo das três amostras encaminhadas pelo projeto piloto de triagem neonatal, confirmando a aplicabilidade clínica da padronização do protocolo de extração orgânica de DNA de SIPF, utilizando apenas 3 discos de 3-mm.

**Palavras-chave:** Extração de DNA, sangue impregnado em papel filtro, diagnóstico genético-molecular, triagem neonatal, sequenciamento de nova geração.

## 2.    INTRODUÇÃO

A Triagem Neonatal é uma ação preventiva para a identificação de doenças em fase pré-sintomática em recém-nascidos, possibilitando assim o tratamento precoce e, consequentemente, a redução da morbi/mortalidade gerada pelas doenças triadas.[1,2] No Brasil, o Programa Nacional de Triagem Neonatal (PNTN) do Sistema Único de Saúde (SUS) inclui o exame denominado Teste do Pezinho (TP),[1,3] onde são colhidas algumas gotas de sangue do calcanhar do recém-nascido (entre o terceiro e quinto dia de vida) que são impregnadas em papel filtro (SIPF). Após a coleta, o SIPF é utilizado para a execução dos diferentes testes bioquímicos de triagem. Na hipótese de um resultado alterado, a família é contatada e uma segunda amostra (soro, sangue total, urina ou SIPF) é coletada para a realização de testes confirmatórios, diferenciando os resultados positivos dos falsos-positivos.[4-6] Nos dias de hoje, as doenças que são englobadas no TP disponibilizado pelo SUS são: Fenilcetonúria, Hipotireoidismo congênito, Doença falciforme e outras hemoglobinopatias, Fibrose cística, Hiperplasia adrenal congênita e Deficiência de biotinidase.

No Brasil, o período entre o nascimento do bebê afetado, a realização do TP e a obtenção do resultado do exame confirmatório é muito grande. No RS, a média do tempo desde a chegada das amostras do papel filtro ao laboratório é de 4 dias, a da emissão de resultados laboratoriais de triagem é de 2 dias e a de retorno dos pacientes reconvocados é de 18 dias.[7] Uma vez que ensaios bioquímicos e moleculares podem ser realizados a partir do mesmo cartão de coleta, reaproveitando o material coletado no TP,[8] a possibilidade de realizar a análise molecular a partir de DNA extraído do SIPF coletado no TP reduziria significativamente o tempo de confirmação diagnóstica.

## 2.1 EXTRAÇÃO DE DNA A PARTIR DE SIPF

A extração de DNA de SIPF é uma tarefa mais exaustiva e desafiadora quando comparada àquela feita a partir de sangue periférico, pois a amostra de sangue é coletada no papel filtro na ordem de 50 até 100µl de sangue por círculo (*spot*). São fatores limitantes da técnica a quantidade de material disponível, a dificuldade de extração sem contaminação e a obtenção de uma quantidade suficiente para futuras análises. Além disso, a qualidade do papel filtro utilizado também desempenha um papel importante na coleta de amostras.[9] Por estes motivos é importante estabelecer um método eficiente de extração.

Há vários protocolos disponíveis, tanto *in-house* como comerciais, para a extração e purificação de DNA a partir de SIPF.[10-13] O método de extração orgânica (fenol/clorofórmio) de DNA é o mais usual na genética forense,[14] sendo o método recomendado quando é necessária a extração de DNA de alta massa molecular; é um método mais complexo e demorado, mas com um custo baixo. É considerado um dos métodos mais eficientes, abrangendo a capacidade de remover a maioria de inibidores da Reação em Cadeia da Polimerase (PCR).[15] Portanto, neste trabalho, a extração de DNA genômico (gDNA) será realizada seguindo um protocolo *in-house* de extração orgânica, que utiliza fenol/clorofórmio.[16] Este protocolo baseia-se na incubação *overnight* com um tampão de lise celular e proteinase K para romper as membranas plasmáticas. Em seguida é realizada uma purificação para limpeza das proteínas, utilizando fenol e recuperando a fase aquosa. Após, a fase aquosa é submetida a uma segunda purificação com clorofórmio. A fase aquosa obtida neste último passo é precipitada com sal e etanol absoluto, lavada com etanol 70% e o *pellet* obtido, que contém o DNA, é ressuspendido em água.

No período pós-natal, amostras de SIPF como as coletadas no TP, são utilizadas para identificar o defeito enzimático das doenças por testes bioquímicos. Atualmente, este mesmo

material biológico tem sido introduzido nas práticas de pesquisa e diagnóstico como fonte de DNA podendo ser utilizado em diferentes metodologias, como a Reação em Cadeia da Polimerase (PCR), PCR quantitativo em tempo real (qPCR), *Multiplex Ligation- Dependent Probe Amplification* (MLPA), *Restriction Fragment Length Polymorphism* (RFLP), sequenciamento convencional (Sanger), Sequenciamento de Nova Geração (NGS), entre outros.[17,18] O Sequenciamento de Nova Geração tem a capacidade de analisar, de maneira massiva e simultânea, diferentes regiões do genoma ou todo genoma (painéis de genes ou *Whole Exome Sequencing* - WES).[19-21] Diferentes estudos mostraram que é possível obter DNA com quantidade e qualidade suficiente a partir do SIPF para a sua utilização em diversas técnicas moleculares, incluindo NGS.[13,18,21-24] Os painéis de genes (*Targeted Next-Generation Sequencing* - TNGS) permitem o sequenciamento de vários genes conjuntamente com custo relativamente baixo, sendo uma alternativa atrativa para a execução dos testes moleculares necessários para a confirmação diagnóstica na triagem neonatal. Esta abordagem é capaz de detectar, de maneira altamente específica, variantes *missense*, *nonsense*, de sítio de splicing, pequenas deleções, pequenas inserções, pequenas *indels*, e algumas grandes deleções.[25]

## 2.2    DOENÇAS LISOSSÔMICAS DE DEPÓSITO

As doenças lisossômicas de depósito (DLDs) são um grupo de doenças raras que fazem parte dos erros inatos do metabolismo (EIM). Elas são causadas pelo progressivo acúmulo de metabólitos não degradados devido a deficiência de enzimas lisossômicas solúveis, proteínas de membrana ou proteínas lisossômicas acessórias que levam a um *turnover* defeituoso de macromoléculas complexas, incluindo glicosaminoglicanos (GAGs), proteínas e lipídios.[26-28] A triagem neonatal dessas patologias é muito relevante, pois são doenças graves e de difícil manejo. Ainda não existe concordância sobre qual estratégia de tratamento é a mais adequada

para cada DLD, porém não há dúvida de que o diagnóstico precoce seguido de um acompanhamento adequado oferece um melhor prognóstico para a doença,[29,30] melhorando a qualidade de vida do paciente e da família. Atualmente, as DLDs são consideradas candidatas para a triagem neonatal, pois os pacientes geralmente são assintomáticos ao nascimento, mas apresentam progressão rápida da doença e manifestações irreversíveis depois de estabelecidas. Existem inúmeras opções de tratamento disponíveis para várias dessas condições.[31] Visto que a triagem neonatal viabiliza o diagnóstico precoce, possibilitando que doenças genéticas possam ser tratadas antes do aparecimento de manifestações irreversíveis, a inclusão das DLDs no PNTN ofereceria benefícios para o paciente e familiares.

O Serviço de Genética Médica (SGM) do Hospital de Clínicas de Porto Alegre (HCPA) é reconhecido como centro de referência nacional, latino-americano e até mesmo internacional para Doenças Lisossômicas de Depósito,[32] compreendendo a sede da Rede DLD Brasil, uma colaboração que envolve diversos serviços que atendem pacientes com doenças lisossômicas e que tem como objetivos principais facilitar o acesso ao diagnóstico, dispor uma caracterização completa (incluindo definição genotípica), permitir o manejo adequado e apoiar o desenvolvimento de pesquisas na área.

O projeto "Avaliação do potencial de um programa de triagem neonatal para doenças lisossômicas", nº 17-0445, desenvolvido no SGM do HCPA, é um projeto piloto para a triagem de DLDs selecionadas. As doenças selecionadas para o projeto incluem: doença de Gaucher, doença de Fabry, doença de Pompe, doença de Krabbe, doença de Niemann-Pick A/B e Mucopolissacaridose tipo I. Este projeto trabalha com diagnóstico bioquímico das DLDs, através da espectrometria de massas em tandem, e também prevê a análise molecular dos respectivos genes para as amostras cujas enzimas forem deficientes e os biomarcadores forem alterados. Como o diagnóstico bioquímico está passível ao diagnóstico de

pseudodeficiências, as quais estão relacionadas a uma redução ou não detecção da atividade enzimática *in vitro*, porém *in vivo* ocorre o funcionamento enzimático normal, somente o diagnóstico molecular pode diferenciar um paciente afetado de um paciente com pseudodeficiência.

## 3.    JUSTIFICATIVA

Em nosso serviço, o diagnóstico consiste de duas etapas realizadas subsequentemente. A primeira etapa do processo diagnóstico inclui ensaios enzimáticos específicos realizados em diferentes matrizes celulares, tendo o SIPF se mostrado uma boa opção para várias doenças lisossômicas. Na etapa seguinte, que inclui o desenvolvimento das técnicas moleculares, o SIPF também tem se adequado como matriz celular para extração de DNA, demonstrando desempenho semelhante ou apenas minimamente inferior quando comparado ao padrão ouro, como sangue periférico.[33] Desde 2017 o Laboratório de Genética Molecular (LGM) passou a usar o DNA genômico (gDNA) extraído de SIPF através de um método de extração orgânica,[15] que foi validado internamente com seis discos de 3-mm. Esta metodologia tem se mostrado confiável para o desenvolvimento de diversos testes moleculares, consistindo em um protocolo de baixo custo. A expectativa de incluir as doenças lisossômicas de depósito no Programa Nacional de Triagem Neonatal do Brasil e a circunstância das técnicas moleculares serem realizados após os ensaios bioquímicos, limitando a quantidade de amostra, torna importante determinar o volume mínimo de amostra necessário para obtenção de um bom rendimento de gDNA para a realização de diferentes ensaios moleculares; uma vez que a disponibilidade da amostra é limitada e a re-coleta de amostras de SIPF. Logo, é importante estabelecer um método de coleta eficaz, como o SIPF, que permite um transporte conveniente, requer apenas uma quantidade mínima de sangue e é compatível com as análises bioquímicas e moleculares a serem realizadas.

## 4. OBJETIVOS

### 4.1 OBJETIVO GERAL

O objetivo deste estudo foi padronizar o protocolo de extração orgânica empregado para isolar gDNA de SIPF, anteriormente validado com seis discos de 3-mm, utilizando um mínimo de amostra, determinado como três discos de 3-mm, para ser usado principalmente no Sequenciamento de Nova Geração. O desempenho das amostras foi testado em diferentes métodos de biologia molecular além do TNGS para verificar a qualidade e eficiência da extração.

### 4.2 OBJETIVOS ESPECÍFICOS

- Padronizar um protocolo de extração orgânica de DNA de SIPF, utilizando um volume mínimo de amostra;

- Confirmar a efetividade da padronização do protocolo de extração orgânica de SIPF através das técnicas de biologia molecular como PCR, RFLP e Sequenciamento de Sanger;

- Validar a aplicabilidade clínica do protocolo padronizado usando amostras obtidas de um projeto de triagem neonatal para DLDs através do Sequenciamento de Nova Geração;

- Confirmar os resultados possivelmente alterados da triagem neonatal para DLDs, através do Sequenciamento de Nova Geração, proporcionando um diagnóstico mais robusto.

JOURNAL OF BIOMOLECULAR TECHNIQUES

# Standardization of an organic DNA extraction method from dried blood spots and its downstream molecular applications for neonatal screening and diagnostic confirmation of lysosomal disorders

**Running title: Organic DNA extraction from DBS**

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**Requested article type:** Article

## 5.1 ABSTRACT

Dried blood spot (DBS) samples have been used for diagnostic purposes since its introduction for the neonatal screening of phenylketonuria almost 50 years ago. From that time onwards, the range of its applications has been extended until today, including next-generation sequencing (NGS) for molecular genetics diagnosis. This study aimed to evaluate a standardized organic method to extract DNA from DBS samples, for its use in the diagnostic setting. The clinical applicability of the method was tested using three samples collected in a newborn screening project for lysosomal storage diseases, allowing the determination of the genotype of the individuals. DNA was extracted from three 3-mm diameter DBS punches. Quality, purity, and concentration were determined, and its performance was assessed through standard PCR, Restriction Length Polymorphism, Sanger sequencing, and Targeted Next-generation sequencing (TNGS). Results were compared with the ones obtained with DNA samples extracted following the internally validated in-house extraction protocol that used six 3-mm punches of DBS and samples extracted from whole blood. This organic method proved to be effective in obtaining high-quality DNA from DBS, being compatible with several downstream molecular applications, in addition to presenting a lower cost per sample.

**Keywords:** DNA extraction, dried blood spots, molecular genetics diagnosis, newborn screening, target next-generation sequencing.

## 5.2 INTRODUCTION

The collection of dried blood spots (DBS) on filter paper is a useful approach for large scale analysis, such as screening programs. The DBS was first introduced in 1963 as an alternative to blood sampling and screening of phenylketonuria (PKU) in a large population of newborn infants.[1] Since then, DBS sampling has emerged as an appropriate method for collection, transport, analysis, and storage of biological fluids, due to its simplicity, less invasiveness, and low cost.[2-4] Therefore, it is also the best option when venipuncture, transportation, and storage conditions are not favorable,[5] in the way that until the present day DBS is used as sample for newborn screening (NBS).[6]

DBS use in the diagnostic setting has increased throughout the years[7] and besides some limitations (small amount of material available, risk of DNA degradation, etc.), its performance in downstream molecular processes is similar, or just slightly inferior, when compared to gold standard sample types.[2] To date, DNA obtained from DBS has been used for diagnostic purposes using different molecular biology techniques such as standard polymerase chain reaction (PCR),[5] real-time PCR (q-PCR),[8] multiplex ligation-dependent probe amplification (MLPA),[9] high-resolution melting (HRM) analysis[10], and different next-generation sequencing (NGS) approaches: Targeted NGS (TNGS), whole-exome sequencing (WES) and whole-genome sequencing (WGS).[11-15]

The performance and reliability of the different molecular downstream processes are strongly influenced by both the quantity and quality of DNA.[16] Therefore, it is highly required to establish an efficient methodology for DNA extraction that maximizes both yield and quality of DNA with minimal co-extraction of inhibitors.[17] To achieve these parameters there is a wide range of commercial and non-commercial methods for purifying DNA from DBS.[5,18-20]

The organic extraction, a non-commercial method, is well-known for having high extraction

efficiency, as well as for removing a majority of PCR inhibitors in comparison with other methods.[21]

The Medical Genetics Service of Hospital de Clínicas de Porto Alegre is a reference center for lysosomal storage diseases (LSD) diagnosis in Brazil and Latin America[22] that receives a large number of samples from different services of this region. Hence, it is important to establish an effective collection method, such as DBS, that allows for convenient worldwide shipment and requires only a minimal amount of blood. Few years ago we have started to use genomic DNA (gDNA) extracted from DBS by an organic extraction method[23] that was internally validated using six punches of 3-mm. The possibility of including the lysosomal storage diseases, a group of rare diseases,[24-25] in Brazil's neonatal screening program, makes it necessary to determine the minimum amount of sampling needed to obtain a good gDNA yield to perform different molecular assays, since sample availability is limited and it is not always possible to obtain a new DBS sample from babies.

The aim of the present study was to standardize the organic extraction method previously used for isolating gDNA from DBS, using three punches of 3-mm, instead of six punches, to make it suitable to be used in newborn screening. Its performance was tested in different molecular biology methods including NGS, to check for the quality and efficiency of sampling.

## 5.3 MATERIAL AND METHODS

### Sample Collection

Twenty control samples provided by the manufacturer of the newborn screening kit (Perkin Elmer®, USA) that were also used as biochemical controls, were used for method standardization. Additionally, to prove the validation of the method on clinical applicability, three newborns with previous biochemical diagnosis for Fabry disease, Pompe disease and

Mucopolysaccharidosis type I, participating in a project that evaluates a potential NBS program for lysosomal storage diseases, whose DBS samples have been stored at the Medical Genetics Service of Hospital de Clínicas de Porto Alegre, were analyzed. Control samples were collected in a sterile environment, through venipuncture. The blood was spotted on PerkinElmer® 226 Paper Grade and dried for 3 hours at room temperature before storage. The samples were stored at 4ºC until analysis.

**DNA Extraction**

The organic extraction (GE Healthcare, Reliable extraction of DNA from Whatman$^{TM}$ FTA$^{TM}$ cards) was performed using three punches (3-mm). Three discs were punched out from a full dried blood spot into a 1.5mL microcentrifuge tube using a metal hole puncher and six blank discs were punched between each sample to avoid cross-contamination. DBS punches were incubated at 56°C overnight in constant agitation of 650 RPM with 500µL of Extraction Buffer (10mM Tris-HCl pH 8.0, 10mM EDTA disodium salt pH 8.0, 100mM NaCl$_2$ and 2%w/v SDS) and 6µL of proteinase K (10mg/ml) (Promega, USA). The lysate was treated with the same volume of buffered phenol (pH 8.0) (Sigma®, Germany), vortexing and centrifugation at 14.000 RPM for 10 minutes at room temperature. The aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube with 500µL of chloroform (Sigma®) for subsequent vortexing and centrifugation at 14.000 RPM for 10 minutes at room temperature. The upper aqueous phase was transferred to a new 1.5mL microcentrifuge tube and precipitated with 45µL of 3M sodium acetate pH 5.2 and 100% absolute ethanol (Merck, Germany) and incubated at -20°C overnight. The next day the solution was centrifuged at 14.000 RPM for 30 minutes at 4ºC to recover the DNA. The supernatant was removed, the pellet was washed with 70% ethanol and centrifuged again at 14.000 RPM for 30 minutes at

4ºC. The supernatant was again removed and the pellet was dried out at 60ºC for 30 minutes. DNA was resuspended in 13µL of water.

**DNA yield and quality**

DNA integrity (5µl) was checked on 0.8% agarose gel electrophoresis. Concentration was measured using Qubit® double stranded DNA (dsDNA) High Sensitivity (HS) Assay kit (Invitrogen - Thermo Fisher Scientific, USA), which calculates concentration based on the fluorescence of a dye that binds to double stranded DNA. Concentration values provided by Qubit® dsDNA HS were used as a reference for the TNGS analysis. Additionally, the concentration was measured using NanoDrop[TM] 2000 Spectrophotometer (Thermo Fisher Scientific, USA) that also provides DNA purity (A260/A280nm ratio).

As calculated by Saavedra-Matiz and colleagues,[20] a 100% efficient DNA extraction is expected to yield 3,47ng/µL of DNA per 3-mm DBS. Accordingly, a yield of approximately 10,41 ng/µL is expected for three punches of 3-mm; this will be our reference to calculate the extraction efficiency.

**Molecular biology techniques**

To evaluate the quality of the DNA extracted by this standardized method and to determine whether any inhibitory material was interfering with the reaction, PCR was performed using Veriti 96 Well Thermal Cycler (Applied Biosystems®, USA) of different size fragments using primers flanking exonic and intronic regions of the following genes: 297bp (*TPP1*, exon 8), 367bp (*GAPDH*, exon 4) and 1315bp (*IDS*, intron 7). The products were checked on 1.5% agarose gel to demonstrate amplification. PCR of the *TPP1* gene was followed by BigDye sequencing using AB 3500xl 96 capillary DNA analyzer (Thermo Fisher Scientific) and the sequence was analyzed on BioEdit Sequence Alignment Editor. Restriction Fragment Length Polymorphism (RFLP) was performed following the amplification of intron 7 of the

*IDS* gene, using *Hinf*1. The reaction was incubated at 37ºC overnight and the product was checked on 3.0% agarose gel to demonstrate the digestion.

**Targeted next-generation sequencing (TNGS)**

Briefly, 10ng of DNA samples were used to prepare the enrichment of targets with the specifically customized panels, consisting of 2 different PCR primer pools (Thermo Fisher Scientific). The amplification was done by multiplex PCR using the Ion AmpliSeq™ Library kit (Thermo Fisher Scientific), followed by the connection to IonXpress™ barcodes (Thermo Fisher Scientific). These unamplified libraries were purified with magnetic beads, Agencourt™ AMPure™ XP Reagent (Beckman Coulter, USA), and quantified (Qubit® dsDNA HS kit, Thermo Fisher Scientific). The quantified libraries were prepared and pooled in equimolar concentrations of 100pM each. Barcoded libraries were submitted to template preparation on the Ion Chef™ Instrument (Thermo Fisher Scientific) where the Ion 510™ chip (Thermo Fisher Scientific) is loaded. The chip was transferred to the Ion S5™ Sequencer (Thermo Fisher Scientific) where the libraries are sequenced. Raw sequencing data were processed and analyzed using Torrent Suite Software (Thermo Fisher Scientific). After that, a list of detected sequence variants, including SNPs and small insertions/deletions were imported into Ion Reporter™ Software (Thermo Fisher Scientific) for interpretation. Alignments were visually verified with the Integrative Genomics Viewer (IGV, USA) v2.3. Run metrics and coverage analyses were performed to identify technical deficiencies.

We used two different customized gene panels[26]: Panel I (26.75 Kb) - with 8 targets and 138 amplicons to analyze sample 1 (Mucopolysaccharidosis type 1), and Panel II (13.1 Kb) - with 4 targets and 72 amplicons to analyze sample 2 (Pompe disease) and 3 (Fabry disease). On account of internal planning and budgeting, sample 1 was analyzed in two separate runs. First, it was sequenced with DNA extracted from six (3-mm) punches and then with DNA

extracted from three (3-mm) punches. Samples 2 and 3 were sequenced in the same run, using both six punches and three punches.

**Ethical approval**

The study received ethical approval in 2019 from the HCPA Ethics Committee (Project: 19-0754).

**5.4    RESULTS**

**EVALUATION OF EXTRACTED DNA**

**DNA yield and quality**

An extraction efficiency of 63.6 % was achieved - mean 6.62 ng/µL with the DNA extracted from three 3-mm punches, provided by Qubit® dsDNA HS (Invitrogen, Thermo Fisher Scientific). An average concentration of 16.6 ng/µL was provided by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific). Purity was about 1.8 (A260/A280nm ratio), indicating good quality of the extracted DNA (Fig. 1). The quality of the DNA was also checked by its ability to be amplified through different molecular assays as reported below.

**Molecular biology techniques performed**

All samples had amplified PCR products, with recognizable bands of the different targeted genomic regions. Figure 2 shows the amplification of different PCR products from 1315bp to 297bp on the *IDS* and *TPP1* genes, respectively, comparing the amplification between a PCR product obtained through the amplification of DNA extracted from DBS using six punches and DNA extracted from whole blood (WB) - gold standard. Figure 3 show the digestion performed using *Hinf1* of the intron 7 of the *IDS* gene through RFLP. Figure 4 shows BigDye sequence analysis for the beginning of exon 8 of the *TPP1* gene, which is a homopolymeric region of difficult amplification.

**Target Next Generation Sequencing**

Results from TNGS have shown good sequencing data, adequate depth coverage for variant calling and acceptable concordance between the extractions from the same DBS sample using six (3-mm) punches and three (3-mm) punches (Table 1). Furthermore, TNGS using DNA obtained from DBS has shown high similarity with results obtained from TNGS using DNA extracted from WB. The mean coverage of the DBS samples was 195,88; likewise, the mean coverage of the WB samples was 200,71. These values were obtained using the coverage 100x of the amplicons.

## 5.5 DISCUSSION

Extraction of pure, intact, and double stranded DNA is a requirement for successful and reliable downstream molecular applications.[27] Peripheral blood is the preferred material for DNA extraction when regarding type of collection matrix; however, when its collection is not feasible, blood can be collected as dried spot in filter paper,[28] as in newborn screening, which blood collection by heel prick in dried blood spots is the gold standard.

Several methods for DNA isolation from DBS have been described,[5,19-20,28-30] overcoming the limitations presented by this type of sampling. Some of the limitations consist of the low amount of DNA available on the sample, which can be a problem for the quantification of the DNA extracted, making it difficult to standardize the amount of DNA per reaction.[5] Also, the chemicals included in some protocols, such as phenol and salt, can be a source of contamination causing problems if not removed properly, because they are considered inhibitors for PCR assays.[19,28] When taken into account the amount or viscosity of the blood, cold or dehydrated patients can lead to unequal saturation of the filter paper, what can entail to an imprecise estimation of starting volume from a DBS punch.[31] The open-air drying before storage can be a source for contamination too, along with the possibility of being

affected by the surrounding temperature and humidity conditions. The open-air drying also precludes the retention of volatile organic compounds, which are often lost during drying; besides, even though most of the pathogens are inactivated by drying, some pathogens remain active for a few days, like dengue, hepatitis B, and group A streptococci.[31] Secondly, the general recommendation to sample storage is at -20ºC to prevent DNA degradation,[32] but when budget is considered, which is the case of our laboratory, DBS stored at room temperature provides economic benefits like reduced cost and space requirements.[30] Despite the limitations mentioned above, DBS sampling allows for convenient transportation, without the necessity of refrigeration conditions, which facilitates its transportation in relation to whole blood, that requires refrigeration and biological material safety conditions.

To this extent, the implementation of an efficient and cost-effective standardized DNA extraction method is fundamental for the molecular diagnosis of genetic diseases in routine laboratories like ours. Considering that in the dynamics of our center the enzymatic assays are performed first and we receive samples from several places, sometimes from long distances; which makes it complex to recollect DBS samples, it is necessary to develop a DNA extraction method that is adaptable with the minimum volume of sample available and also provides suitable DNA to perform the molecular assays. Several commercial kits are available[28] and even some of them have been tested in comparison to the organic extraction method, showing that the Chelex-100 would be the optimal choice for DNA extraction.[33] However, for the reality of our laboratory it ends up being expensive to purchase commercial kits since our demand does not compensate for the large-scale purchase of these kits.

Some studies have shown that different modified phenol-chloroform methods yielded suitable amounts of DNA from several sample types.[19,34-37] Here, standardization of a phenol-chloroform extraction method was examined for effectiveness along with efficiency in

extracting and purifying DNA from DBS using a minimum amount of sample, equivalent to three (3-mm) punches.

The DNA concentration obtained from most DBS samples was 6.62 ng/µL. We observed a good quality of DNA, which was not degraded following vortex, repeated pipetting and other forms of mechanical stress that it was exposed (Fig. 1). The housekeeping gene *GAPDH* was used as an internal control for normalization of the gDNA obtained from the standardized organic extraction method, which obtained amplified PCR products, indicating that the DNA is amplifiable for specific short regions (data not shown). Neduvat and colleagues[38] used coagulation factor XIII (*F13*) gene as an internal control for normalization of gDNA and cited some of the housekeeping genes that are frequently used as internal control in mammalian cells - *GAPDH, β-actin, β2-microglobulin, cyclooxygenase 1, hypoxanthine phosphoribosyl transferase 1, glucose-6-phosphate dehydrogenase, cyclophilin A, tubulin, transferrin receptor,* and *18S ribosomal RNA*.

Different studies have demonstrated that it is possible to obtain DNA with both sufficient quality and quantity from DBS for its use in different molecular techniques.[11,12,14,20,39] The molecular processes assessed in this study (conventional PCR followed by RFLP, conventional PCR followed by Sanger sequencing and targeted next-generation sequencing) were performed, and similar results compared with those obtained from DNA isolated from whole blood, were observed (Fig. 2 - 4).

Regarding the TNGS used to prove the clinical applicability of the standardization of the extraction method, despite the data obtained from the sequencing performed using three punches of DBS appeared to be inferior to the data obtained from the sequencing performed using six punches of DBS (Table 1), it was possible to identify the same variants in all samples. This indicates that it is feasible to use the DNA extracted through the standardized

method for TNGS and still provide the molecular diagnosis. It is clear that sample 1 demonstrated to have low run metrics values; this is because it is a poor quality sample that is fragmented. Even though, it was possible to amplify the sample, sequence through TNGS, and identify the molecular variants related to the disease according to the biochemical pattern obtained previously.

Our laboratory used this method for more than three years to extract DNA using six punches of 3-mm as an internal validation, mainly for TNGS for lysosomal storage disorders, enabling molecular diagnosis. The opportunity to standardize the method using the least possible amount of samples and still enable molecular diagnosis is an achievement, since these molecular assays are presently used as second-tier tests.

The NBS programs based on biochemical methods are progressively adding molecular tests as second-tier tests, generating the necessity for a reliable, inexpensive and practical DNA extraction method from DBS.[20] In addition, the possibility to include lysosomal diseases in Brazil's NBS program emphasizes the demand for an optimal DNA extraction method that requires small amounts of sample. As already stated, the application of DBS-based TNGS assay for the precise and rapid diagnosis of inborn error of metabolism, which includes lysosomal storage diseases, is viable, efficient and beneficial.[12,40-41]

In summary, we have shown in this report that the organic method of DNA extraction using only three punches of 3-mm from DBS offers an acceptable yield of DNA concentration and purity, enabling its use for target next-generation sequencing analysis, combining simplicity and cost effectiveness, being a suitable alternative for reference laboratories in developing countries.

## 5.6   ACKNOWLEDGEMENTS

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**5.8    FIGURE LEGENDS**

**Figure 1:** Quality of genomic DNA was checked on 0.8% agarose gel showing DNA concentration. Lane 1-5: Extraction of DNA using the standardized organic method (three punches of 3-mm). Lane 7: Extraction of DNA using the previous internally validated organic method (six punches of 3-mm). Lane 8: Extraction of DNA from whole blood (WB). Lane 1: 34.4ng, Lane 2: 22.6ng, Lane 3: 18.05ng, Lane 4: 34.4ng, Lane 5: 34.2ng, Lane 6: 100bp mass ladder (Invitrogen$^{TM}$, USA), Lane 7: 24.5ng, Lane 8: 35.0ng.

**Figure 2:** Quality of DNA extracted from DBS using three punches was checked based on the amplification of PCR products of different genes on 1.5% agarose gel, in comparison to the previous internally validated organic method (six punches of 3-mm) and to whole blood (WB). Lane 1-7: *IDS*, intron 7 (1315bp) with DNA from three punches, Lane 8: *IDS*, intron 7 (1315bp) with DNA from six DBS punches, Lane 9-10: *IDS*, intron 7 (1315bp) with DNA from WB, Lane 12: 100bp ladder (Invitrogen$^{TM}$), Lane 13: *TPP1*, exon 8 (297 bp) with DNA from three punches, Lane 14: *TPP1*, exon 8 (297 bp) with DNA from six punches, Lane 15: *TPP1*, exon 8 (297 bp) with DNA from WB, Lane 11 and 16 correspond to no DNA template control.

**Figure 3:** Restriction Fragment Length Polymorphism products of the *IDS* gene were checked on 3.0% agarose gel. Lane 1-7: DNA from DBS using three punches of 3-mm, Lane 8: DNA from DBS using six punches of 3-mm, Lane 9: DNA from whole blood (WB), Lane 10: DNA from WB showing a control, Lane 11: No DNA template control.

**Figure 4:** Sanger Sequencing of exon 8 of the *TPP1* gene using gDNA extracted from 3 DBS punches. (A) Bigdye sequence analysis showing the chromatogram peaks of the homopolymeric region in exon 8 of the *TPP1* gene (amplicon size = 297bp). (B) Bigdye sequence analysis showing the alignment with the reference sequence. PCR products were

purified with ExoSAP-IT$^{TM}$ (ThermoFisher Scientific) and cycle sequenced according to standard manufacturer recommendations.

## 5.9    TABLES

**Table 1:** Results of TNGS from the Ion Torrent Reporter softaware™.

|  | Sample 1 | | Sample 2 | | Sample 3 | |
|---|---|---|---|---|---|---|
|  | **6 spots** | **3 spots** | **6 spots** | **3 spots** | **6 spots** | **3 spots** |
| **Number of mapped reads** | 56,312 | 118,058 | 29,904 | 21,481 | 66,679 | 35,520 |
| **Percent reads on target** | 36.41% | 27.73% | 98.57% | 98.96% | 97.00% | 95.51% |
| **Average base cover depth** | 150.7 | 233.3 | 401.3 | 290.2 | 895.6 | 451.1 |
| **Uniformity of base coverage** | 94.24% | 95.42% | 91.41% | 91.92% | 98.99% | 98.88% |

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