

CPT1A Diagnosis Five Years after a Presumptive Positive Newborn Screen
J. Bartley, Loma Linda University Children’s Hospital, San Bernardino, CA

Abstract

A five year old boy was hospitalized for his first known episode of hypoglycemia and cardiomegaly the day following hiking and horseback riding. His serum glucose was 15 mg/dl. The plasma acylcarnitines had a high acetylcarnitine (33.08 umol/l, normal <27.57 umol/l). The free carnitine (FC) was 31 umol/l and the total carnitine was 44 umol/l, both in the normal range. His mother recalled he had an abnormal newborn screen but no disorder was diagnosed. The review of the newborn screening result showed he was presumptive positive for CPT1 with a FC/(C16 + C18) ratio of 775 (normal < 100), but confirmatory testing was interpreted as normal. However, retrieval of the 2007 confirmatory testing showed a FC/(C16 + C18) ratio of 525 using the FC value from the butanol-HCL extracted free and total carnitine specimen and 527 using the FC value from the acylcarnitines. These ratios are usually not reported because of the two different extraction methods used for determining free and total carnitine versus acylcarnitines. Application of the R4S post-analytical tool (2004-2013 Region 4 Genetics Collaborative) to the newborn screening ms/ms values gave a case score of 389; where a case score of greater than 90 indicates the condition is very likely CPT1. The FC/(C16 + C18) ratio using the 2013 clinical lab values (even with the caveat that the free carnitine may be falsely high because of the extraction method) was 344. CPT1A sequencing show two heterozygous mutations in trans configuration: c.946C>G (p.R316G) pathogenic (reported in 2004 Mol Genet Metab 82:59 in a patient with CPT1A) and c.1966C>G (p.H656D) novel (predicted to be deleterious by SIFT and PolyPhen-2 because histidine has been at amino acid position 656 from yeast to human). He has had no further episodes of hypoglycemia or cardiomegaly while consuming a low fat-high carbohydrate diet and treating catabolic conditions with intravenous glucose at a glucose infusion rate of 10.

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Summary

Reviewing the newborn screening results regardless of the age of the patient is an important aspect of the past medical history. In this case mother remembered that he had a positive newborn screen but on further testing she was told that her son was normal. The newborn screen was presumptive positive for CPT1 with a high Free Carnitine/ (C16 +C18) ratio—the ratio was 775 with normal <100. The confirmatory testing using the free and total carnitine results and the acylcarnitine profile results was interpreted as normal by the metabolic center reviewing the confirmatory testing results. However retrieval of the confirmatory lab values allowed an estimate of the Free Carnitine/ (C16 + C18) ratio which was 525, even though this ratio is usually not reported because one extraction method is used to
Free and Total Carnitine and another extraction method is used to determine the Acylcarnitine Profile.

Application of the CPT1 R4S post-analytical tool developed by Dr. Piero Rinaldo at Mayo Clinic (2004-2013 Region 4 Genetics Collaborative) to the ms/ms newborn screening results gave a case score of 389. A case score of greater than 90 indicates the condition is very likely CPT1.

CPT1A gene sequencing was done and two heterozygous mutations in trans were found. One mutation c.946C>G (p. R316G) has been reported in a patient with CPT1A (2004 Mol Genet Metab 82:59) and is considered pathogenic. The second mutation c.1966C>G (p.H656D) has not been reported but is predicted by SIFT and PolyPhen-2 to be deleterious as histidine has been at amino acid position 656 from yeast to human.

During the acute hospitalization he was treated initially with intravenous glucose at a glucose infusion rate of 10 to prevent catabolism and to treat his hypoglycemia and cardiomegaly. As he recovered he was given a low-fat heart healthy diet (20% or less calories from fat). He has grown well and had no further episodes of hypoglycemia or cardiomegaly during the 18 months since his hospitalization.

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Newborn Screening for Duchenne Muscular Dystrophy: Rationale, Options, and Laboratory Challenges
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Abstract

Problem Studied: Duchenne muscular dystrophy (DMD) is a candidate for universal newborn screening (NBS) because of the benefit of early treatment with corticosteroids, the feasibility of more efficient methods of population-based laboratory screening, and the prospect of approval of genetic treatments. The success of the cystic fibrosis newborn screening (CF-NBS) is of particular relevance in developing a DMD-NBS program.

Objective: To identify the key elements required for an effective DMD-NBS program using CF-NBS as a model.

Methods: We reviewed the literature on DMD-NBS and CF-NBS, noting technical, medical, programmatic, and ethical concerns.
**Results:** Highly specific DMD-NBS is possible on dried blood spots, by screening to detect CK elevations followed by DNA analysis; however, there has been little evidence that previous DMD-NBS programs have lead to meaningful improvement in DMD outcomes. CF-NBS has resulted in documented improvement in CF outcomes into adulthood, through practices that include:

- attention to improving laboratory guidelines to promote efficient screening,
- consistent confirmatory testing and immediate referral of all newly diagnosed infants to designated CF care centers that follow established practice guidelines, and,
- ongoing evaluation of CF care centers via a centralized clinical database.

Improved methods for DMD-NBS screening algorithms are apparent from carefully examining the algorithms that have made CF-NBS successful. The experiences of CF-NBS suggest that improved DMD outcomes can result from DMD-NBS if there is close cooperation between public health laboratory and the DMD clinical centers where follow-up care can be standardized and optimized while a centralized registry oversees outcome and practice data.

**Conclusions:** DMD-NBS would allow all patients to receive early treatment with corticosteroids and standardized care. Newborn screening would also identify those who could benefit from new genetic therapies. The lessons learned from the past 20 years of CF-NBS are applicable to DMD-NBS: screening algorithms to identify infants at risk for DMD; standardized protocols for all patients identified by DMD-NBS; longitudinal follow-up in multidisciplinary clinics; coordinated oversight of these clinics.

**Presenter:** Jennifer Kwon, MD, MPH, University of Rochester Medical Center, Neurology & Pediatrics, Rochester, NY, Phone: 585.273.5264, Email: jennifer_kwon@urmc.rochester.edu

**Summary**

Duchenne muscular dystrophy (DMD) is an X-linked disorder affecting 1:5000 male births. DMD a candidate for universal newborn screening (NBS) because of data about the benefit of early treatment with corticosteroids, the feasibility of more efficient methods of population-based laboratory screening, and the prospect of approval of genetic treatments which could help even younger boys with DMD. The success of the cystic fibrosis newborn screening (CF-NBS) is of particular relevance in developing a DMD-NBS program. Like DMD, CF is an inexorably debilitating and ultimately fatal disorder. Over the decades, however, CF clinical outcomes, including median age of survival, have steadily and consistently improved, due in large part to the CF Foundation’s reliance on collecting best clinical practice data via their CF Registry and then using their findings to improve care across all “certified” CF Centers (certification again coming from the CF Foundation.)

Our objective was to identify the key elements required for an effective Duchenne muscular dystrophy newborn screening program (DMD-NBS) by looking at CF-NBS and how patients are diagnosed and then managed in CF centers. We reviewed the literature on DMD-NBS and CF-NBS, noting technical, medical, programmatic, and ethical concerns.

Newer methodologies have been developed to provide highly specific DMD-NBS on dried blood spots (DBS), using a two-tier (or possibly a three-tier) system. First, screening on DBS to detect CK elevations (in the three-tier model, CK screening would be done twice) followed by dystrophin DNA analysis. This screening workflow is anticipated to efficiently diagnose DMD. What remains then, is to outline a
pathway where this early diagnosis is linked to a system of clinical care that can meaningfully improve DMD outcomes.

Using the example of CF-NBS, several practices have resulted in documented improvement in CF outcomes into adulthood, including:

• attention to improving laboratory guidelines to promote efficient screening,
• consistent confirmatory testing and immediate referral of all newly diagnosed infants to designated CF care centers that follow established practice guidelines, and,
• ongoing evaluation of CF care centers via a centralized clinical database.

The experiences of CF-NBS suggest that improved DMD outcomes can result from DMD-NBS if there is close cooperation with DMD clinical centers where follow-up care can be standardized and optimized while a centralized registry oversees outcome and practice data. The key elements of a successful DMD-NBS would include:

1. A CK assay with high specificity and sensitivity that can be used at the state newborn screening laboratory, with criteria in place for state-lab-based dystrophin mutational testing.
2. Follow-up at a DMD specialty care center within 4 weeks of confirmation of abnormal DMD screen.
3. Identified and “certified” care centers with neuromuscular specialist, nurse practitioners, physical therapist, social worker, and genetic counselor.
4. Documented consensus and evidence based guidelines for patients from infancy to adulthood, including evaluation for early intervention components.
5. A patient registry with documented outcome measures.
6. Cardiology and Pulmonology follow up within local hospital systems.
7. An overseeing organization with financial support for the care centers and for the patient registry.
8. Care center adherence to guidelines with overseeing by a central organization.

In conclusion, DMD newborn screening would allow all patients to receive early treatment with corticosteroids and standardized care. Newborn screening would also identify those who could benefit from new genetic therapies. The lessons learned from the past 20 years of CF-NBS are applicable to DMD-NBS: standardized protocols for all patients identified by DMD-NBS; longitudinal follow-up in multidisciplinary clinics; coordinated oversight.

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Arkansas Newborn Screening Long-Term Follow-up Cohort Study - Year 2
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Abstract

Problem/Objectives: Every year approximately 38,000 Arkansas newborns receive a newborn screen for the 28 primary core panel metabolic conditions and hearing; about 70 infants are diagnosed with a metabolic condition and 70 are diagnosed with hearing loss. Prior to January 2012, data systems were
not in place to capture the long-term health outcomes of the hundreds of infants diagnosed with a newborn screening (NBS) condition in Arkansas. The Arkansas NBS Long-Term Follow-up (LTFU) Cohort Study was established for the purpose of tracking and monitoring the clinical care and public health outcomes for children diagnosed with a NBS condition and to follow them until 21 years of age.

**Methodology:** The Arkansas NBS LTFU Cohort Study is a longitudinal, observational study conducted by the University of Arkansas for Medical Sciences (UAMS), Pediatric Genetics Section in partnership with the Arkansas Children’s Hospital (ACH) and its Research Institute (ACHRI). The primary aim of the Study is to record demographics, characteristics of disease and treatment, utilization patterns, quality improvement measures, and clinical outcomes in Arkansas children with NBS conditions. The study database was developed using REDCap (Research Electronic Data Capture) hosted by the UAMS Translational Research Institute (NCRR/NIH 1 UL1 RR02988).

**Significant Results:** After receiving Institution Review Board approval September 2011, the database was implemented January 2012. Based on projections, the enrollment goal for the Study is a total of 3,000. At the end of Year 1, the database contained information on 307 subjects representing 311 newborn screening cases. Second year data (2013) and comparison data with Year 1 (2012) will be presented at the Symposium.

**Conclusions/Implications:** The Arkansas NBS LTFU Cohort Study will provide the opportunity to monitor and track health outcomes over time, and this could lead to improvements in health care for this population and ultimately the lives of children diagnosed with these conditions in the future.

**Presenter:** Jo Ann Bolick, BSN, MA, University of Arkansas for Medical Sciences/Arkansas Children’s Hospital, Department of Pediatrics, Division of Genetics, Little Rock, AR, Phone: 501.364.1906, Email: jabolick@uams.edu

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**Summary**

**Problem/Objectives:** Every year approximately 38,000 Arkansas newborns receive a newborn screen for the 28 primary core panel metabolic conditions and hearing; about 70 infants are diagnosed with a metabolic condition and 70 are diagnosed with hearing loss. Prior to January 2012, data systems were not in place to capture the long-term health outcomes of the hundreds of infants diagnosed with a newborn screening (NBS) condition in Arkansas. The Arkansas Newborn Screening (NBS) Long-Term Follow-up (LTFU) Cohort Study was established for the purpose of tracking and monitoring the clinical care and public health outcomes for children diagnosed with a NBS condition and to follow them until 21 years of age.

**Methodology:** The Arkansas NBS LTFU Cohort Study is a longitudinal, observational study conducted by the University of Arkansas for Medical Sciences (UAMS), Pediatric Genetics Section in partnership with the Arkansas Children’s Hospital (ACH) and its Research Institute (ACHRI). The funding for the Study is provided by the Antenatal and Neonatal Guidelines, Education, Learning System (ANGELS), a program at UAMS. The primary aim of the Study is to record demographics, characteristics of disease and treatment, utilization patterns, quality improvement measures, and clinical outcomes in Arkansas children with NBS conditions. The study database was developed using REDCap (Research Electronic Data Capture) hosted by the UAMS Translational Research Institute (NCRR/NIH 1 UL1 RR02988). REDCap is a secure, web-based application designed to support data capture for research studies.
The Arkansas NBS LTFU Database Study consists of 4 cohorts:

1) ACH patients with newborn screening conditions meeting enrollment criteria. The Study has received a waiver of HIPAA and consent forms for these subjects.

2) Non-ACH patients with newborn screening conditions meeting enrollment criteria. Study staff will obtain informed consent from these subjects.

3) Abstracting data from the Cystic Fibrosis Registry on enrolled subjects. Study staff will obtain informed consent from subjects meeting enrollment criteria. (Note: To date, this component has not been implemented.)

4) Obtaining health information from the Arkansas Department of Health (ADH).

The Database was created by abstracting and compiling information from the medical record, and the data is entered into a Database that includes a set of common data elements gathered on every subject and eight disease-specific data sets. The Study utilized the common data elements developed by the Newborn Screening Translational Research Network (NBSTRN) as its starting point. The Study collaborated with the clinical experts at ACH to develop the data elements for the eight disease-specific data sets (Cystic Fibrosis, Galactosemia, Congenital Hypothyroidism, Congenital Adrenal Hyperplasia, Biotinidase Deficiency, 3 Sickle Cell Hemoglobinopathies (Sickle Cell Disease, Sickle C Disease, and Sickle/Beta Thalassemia), MS-MS Disorders (20 Disorders screened via Tandem Mass Spectrometry), and Hearing Loss.

The Study has been working in close collaboration with the ADH and has established a Memorandum of Agreement (MOA) with ADH for the exchange of information between ADH and the Study. Parental consent is required before health information housed at ADH can be shared with the Study. The Institution Review Board (IRB) approved the ADH component of the Protocol prior to implementation. The current MOA is effective through December 31, 2015.

**Significant Results:** After receiving Institution Review Board approval September 2011, the database was implemented January 2012. There will be no defined timeframe that the Study will end. Based on projections, the enrollment goal for the Study is a total of 3,000. At the end of Year 1 (2012), the database contained information on 307 subjects representing 311 newborn screening cases.

At the end of Calendar Year 2013, there were a total of 452 patients in the database representing 457 NBS cases enrolled in Years 2012 and 2013 (Figure 1). Note: Five children have 2 NBS conditions.
During Year 2, the Study staff reviewed and refined the data entry forms, and this process will be conducted annually. Data analysis will be conducted on a yearly basis.

Conclusions/Implications: The Arkansas NBS LTFU Cohort Study will provide the opportunity to monitor and track health outcomes over time and this could lead to improvements in health care for this population and ultimately the lives of children diagnosed with these conditions in the future.

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Arkansas Newborn Screening Short- and Long-term
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Abstract

Problem/Objectives: Between September 2000 and up to 07-01-2008, Arkansas screened for 7 of the 29 newborn screening (NBS) conditions recommended on the Recommended Uniform Screening Panel (RUSP): PKU, Galactosemia, Congenital Hypothyroidism, 3 Hemoglobinopathies (Sickle Cell Disease, Sickle C Disease, and Sickle/ Beta Thalassemia), and Hearing. On 07-01-2008, Arkansas implemented expanded NBS which included all of the 29 conditions on the RUSP. With NBS expansion, processes and linkages were put in place with the University of Arkansas for Medical Sciences (UAMS)/Arkansas Children’s Hospital (ACH) to strengthen the tracking and monitoring, follow-up components for NBS.

Methodology: In July 2008, a contract was established between UAMS/ACH and the Arkansas Department of Health (ADH) to provide physician consultation, coordination of referrals, and tracking.
and monitoring of follow-up services for the NBS Program. As part of this contract, a UAMS/ACH NBS Coordinator position was established on the ACH campus to serve as a liaison and channel of communication between ADH, ACH pediatric specialists, and the ACH Laboratory.

The Arkansas NBS Long-Term Follow-up Database Study received IRB approval September 2011. The Database was implemented January 2012. The Database consists of a set of common data elements gathered on every subject and eight disease-specific datasets. A NBS Nurse Data Coordinator leads the Study Team on the data-abstraction process.

**Significant Results:** The NBS Coordinator coordinates daily communications between ADH and UAMS/ACH; coordinates consultations/referrals to ACH specialty clinics from providers across the State; and serves as a resource for all pediatric providers throughout AR. The NBS Nurse Data Coordinator’s main tasks are the identification of subject eligibility and abstraction of data from medical records. The short and long-term follow-up processes will be described in the Poster presentation at the Symposium.

**Conclusions/Implications:** Through the collaboration between ADH and UAMS/ACH, newborns are receiving appropriate and timely follow-up on positive newborn screenings. The ultimate goal is to identify newborns with these NBS conditions as early as possible and get them into appropriate treatment and medical/case management.

**Presenter:** Tiffany Moore, BSN, RN, University of Arkansas for Medical Sciences, Little Rock, AR, Phone: 501.364.1608, Email: tpmoore@uams.edu

**Summary**

**Problem/Objectives:** Between September 2000 and June 2008, Arkansas screened for 7 of the 29 newborn screening (NBS) conditions currently on the Recommended Uniform Screening Panel (RUSP): PKU, Galactosemia, Congenital Hypothyroidism, 3 Hemoglobinopathies (Sickle Cell Disease, Sickle C Disease, and Sickle/ Beta Thalassemia), and Hearing. On July 1, 2008, Arkansas implemented expanded NBS which included all of the 29 conditions on the RUSP. NBS expansion called for processes and relationships to be put in place between the University of Arkansas for Medical Sciences (UAMS), Arkansas Children’s Hospital (ACH) and Arkansas Department of Health (ADH) to strengthen the tracking, monitoring and follow-up components for NBS.

**Methodology:** In July 2008, a contract was established between UAMS/ACH and the Arkansas Department of Health (ADH) to provide physician consultation, coordination of referrals, and tracking and follow-up services for the NBS Program. As part of this contract, a UAMS/ACH NBS Short Term Follow-up (STFU) Coordinator position was established on the ACH campus to serve as a liaison between ADH, UAMS/ACH pediatric specialists and the ACH Laboratory.

The Arkansas NBS Long-Term Follow-up (LTFU) Database Study received IRB approval September 2011 and was implemented January 2012. The Database consists of a set of common data elements gathered on every subject and eight disease-specific datasets. A NBS LTFU Coordinator leads the Study Team on the data-abstraction process.

**Significant Results:** The NBS STFU Coordinator manages daily communications between ADH and UAMS/ACH; coordinates physician consultations/referrals to ACH specialty clinics; and serves as a resource for all pediatric providers throughout AR. The NBS LTFU Coordinator’s main tasks are the
identification of subject eligibility and abstraction of data from medical records. For more details on responsibilities, see Figure 1.

Figure 1

<table>
<thead>
<tr>
<th>NBS Short-Term Follow-up Coordinator Essential Functions</th>
<th>NBS Long-Term Follow-up Coordinator (for Study) Essential Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Serves as the channel of communication between ADH, UAMS/ACH for abnormal screenings consults, coordination, and referrals with the sub-specialists.</td>
<td>1. Identifies subject eligibility for Study.</td>
</tr>
<tr>
<td>2. Informs ADH and provides follow-up on 2nd tier screenings performed in the ACH Laboratory (CAH &amp; CF).</td>
<td>2. Oversees data abstraction process for the Study.</td>
</tr>
<tr>
<td>3. Arranges sweat tests for those with NBS CF positive 2nd tier screens, provides education for parents/families, and arranges genetic counseling.</td>
<td>3. Performs data abstraction for new cases &amp; file updates.</td>
</tr>
<tr>
<td>4. Maintains files and NBS database for tracking &amp; monitoring positive screenings.</td>
<td>4. Maintains data integrity via review &amp; Reports.</td>
</tr>
<tr>
<td>5. Provides NBS education to providers daily</td>
<td>5. Assists with NBS educational efforts</td>
</tr>
</tbody>
</table>

The timeframe for Short Term Follow-up is defined as that which begins with a positive screening and extends until a diagnosis is confirmed or ruled out. Long-term Follow-up (per the Study) is defined for those subjects meeting enrollment criteria, which includes the monitoring and tracking provided until 21 years of age.

A NBS Flow chart for the NBS Short-Term Follow-up Coordinator processes will be described in the poster.

A description of the data abstraction flow for the NBS Long-Term Follow-up Coordinator is described below in Figures 3 & 4:

Figure 3

Figure 4

**Conclusions/Implications:** Through the collaboration between ADH and UAMS/ACH, newborns are receiving appropriate and timely follow-up on positive newborn screenings. The ultimate goal is to identify newborns with these NBS conditions as early as possible and get them into appropriate treatment and medical/case management. Then afterwards to provide long-term monitoring and tracking on diagnosed conditions over time.

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**Novel Techniques for Preparing Dried Blood Spot Proficiency Testing Specimens for Sickle Cell Disease Newborn Screening Tests**

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**Abstract**

**Introduction:** All US newborn screening (NBS) laboratories screen dried blood spot (DBS) samples for sickle cell disease (SCD). Producing proficiency testing (PT) specimens for NBS SCD tests is challenging because the predominant hemoglobin (Hb) species in newborns—Hb F—is not normally present in adult blood. We devised novel techniques for producing simulated newborn Hb variant carrier specimens and simulated newborn SCD specimens.

**Materials and Methods:** Umbilical cord blood (UCB) units from Hb S or Hb C carriers were used to prepare DBS Hb carrier specimens for those Hb variants. UCB was combined with venous blood from homozygous Hb DLA and Hb E donors to produce simulated DBS Hb carrier specimens. Residual venous blood samples, collected for confirmation of presumptive SCDs identified by NBS, were combined to produce DBS SCD specimens.

All blood samples were subjected to: 1) high performance liquid chromatography (HPLC) to determine presumptive Hb phenotype; 2) tandem mass spectrometry (MS/MS) analysis to identify amino acid substitutions characteristic of specific Hb variants; and 3) mutation analysis to confirm Hb species present in each blood sample.

**Results:** HPLC results, expressed as percent of total peak area attributable to each Hb species, show that the levels of Hb F, Hb A, and all Hb variants in the simulated NBS DBS specimens are consistent with levels found in natural NBS specimens. MS/MS results are consistent with the presumptive Hb variants identified by HPLC. DNA analyses confirm the presence of the β-globin gene point mutations that produced the Hb variants.

**Discussion:** All PT specimens prepared by methods described here are suitable for all SCD NBS tests used by US laboratories. Because of the limited residual blood volumes available after SCD confirmatory tests, the number of DBS in each SCD PT specimen is only sufficient to supply domestic laboratories. Additional blood sources for production of larger PT pools are being sought.

**Presenter:** Victor De Jesus, PhD, Centers for Disease Control and Prevention, Atlanta, GA, Phone: 770.488.7963, Email: foa5@cdc.gov
The Stability of Hexacosanoyl Lysophosphatidylcholine in Dried-Blood Spot Quality Control Materials for X-linked Adrenoleukodystrophy Newborn Screening

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Abstract

Background: X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder caused by mutations in the peroxisomal transmembrane ALD protein (ALDP, ABCD1). The biochemical defect associated with X-ALD is an accumulation of very long-chain fatty acids, which has been shown to result in the accumulation of C26:0-lysophosphatidylcholine (C26:0-LPC). Some states are either already performing newborn screening for X-ALD (NY) or may do so in the near future (CT). Dried-blood spots (DBS) were prepared with C26:0-LPC-enriched whole blood, and stored under different conditions for up to six months. C26:0-LPC was measured to determine its stability under different storage conditions to evaluate candidate QC materials for X-ALD newborn screening.

Methods: DBS were prepared by enriching whole blood with C26:0-LPC to target concentrations of 0 µM (no enrichment), 1 µM, and 10 µM. C26:0-LPC was measured in the DBS to assess homogeneity. The means and standard deviations were determined by analysis on 20 different days. The throughput of a HPLC-ESI-MS/MS method to detect C26:0-LPC in DBS (Haynes et al. 2013) was improved to achieve an HPLC analysis time of 1 minute per sample.

Results: Linear regression of the C26:0-LPC levels in DBS stored with desiccant for at least 6 months resulted in low slopes at 4 temperatures [-20 C (-0.0003), 4 C (-0.0005), ambient (-0.001), and 37 C (-0.0029)]. Linear regression of the measured C26:0-LPC resulted in a slope of -0.0005 for DBS stored at 4 C for 30 days with desiccant but removed daily from refrigeration with opening of their storage bag simulating daily use of QC materials. Storage at ambient temperature with relative humidity > 90% for 50 days resulted in a linear regression of C26:0-LPC with a slope of -0.0115.

Conclusions: Results indicate that C26:0-LPC is stable in DBS under all of the conditions tested, as evidenced by low linear regression values. X-ALD QC materials are available currently on a pilot basis.

Presenter: Christopher Haynes, PhD, Centers for Disease Control and Prevention, NSMBB, Atlanta, GA, Phone: 770.488.7459, Email: cph7@cdc.gov

Important Factors in Designing Accurate and Reliable Next-generation Sequencing (NGS) Assays

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Abstract

Problem: NGS applications are increasing in newborn screening (NBS), including reducing false-positives, confirming positive screens, identifying carriers, and providing timely information for appropriate initiation of treatment. NBS laboratories, commercial providers, and researchers are developing NGS assays for genes, gene panels, and the whole human exome and genome, however, there are challenges in designing accurate and reliable assays.

**Design and Methodological Challenges:** New data from the GENCODE Project, an international consortium, is providing information on a large number of pseudogenes in the human genome. These pseudogenes are distributed throughout the genome and most have high homology to their parent functional genes. These data indicate that approximately 17% of the roughly 20,000 human genes have pseudogenes. The very mutations that are sought in sequencing the parent genes can be present in the normal sequence of the pseudogenes. Therefore, it is likely that false reports of mutations in the parent genes will result from these homologous regions unless appropriate methods are used. This has implications for all standard approaches to targeted, whole exome, and whole genome NGS. Additionally, special care is needed in designing 1) primers and hybrid capture arrays, 2) depth of coverage, 3) and data analysis to maximize the accuracy and reliability of NGS.

**Current Approaches and Tools:** Available approaches and tools include specific long-range amplicons, software for determining primer and oligonucleotide specificity, designing appropriate coverage for the quality score used, and appropriate data trimming.

**Implications:** New approaches are needed to improve the accuracy and reliability of NGS especially when screening newborns, children and adults, because the use of this technology in a non-symptomatic population increases the risk of a false result compared to sequencing a clinical population symptomatic or diagnosed with genetic disease.

**Presenter:** Patricia Mueller, PhD, Centers for Disease Control and Prevention, NSMBB, Atlanta, GA, Phone: 770.488.4015, Email: pwm2@cdc.gov

**Summary**

There is a widespread problem of pseudogenes in the development of next-generation sequencing (NGS) applications.

Problem exists in the design of assays for:
- genes and gene panels
- whole exome sequencing (WES)
- whole genome sequencing (WGS)

Problem exists for all standard approaches:
- standard primer design software for PCR enrichment
- enrichment of DNA fragments using standard hybrid capture arrays and bead sets
- non-enriched fragmentation (WGS).

An example of this problem is the difficulty of sequencing CYP21A2, the gene that causes congenital adrenal hyperplasia (CAH).
- CYP21A2 has a highly homologous pseudogene (>98%) in the high-repeat HLA region of chromosome 6
- CYP21A2 is an important Newborn Screening gene and sequencing could potentially be used to help reduce false positives for CAH from first tier biochemical screens
- However, standard enrichment and WGS sequencing approaches will result in false mutation reports for the functional gene (1,2) because of
  - lack of specificity in primers
  - lack of specificity in hybridization oligonucleotides

• misalignment of nonspecific fragmented DNA
• Other methods such as using a long-range amplicon that is specific to the functional gene as DNA template for sequencing are needed for genes such as CYP21A2
• Consider that:
  • The very mutations in functional genes that cause disease are frequently present as normal sequence in highly homologous pseudogenes because
  • pseudogenes accumulate mutations in their normal sequence over time
  • many functional gene mutations result from rearrangements and recombinations with a highly homologous pseudogene

**Human genome pseudogene content**

In the human genome, approximately 20,000 genes including exons, introns, and regulatory sequences make up about 25% of the DNA sequence. Large portions of the remaining genome include repetitive DNA (approximately 59%). Approximately 12,683 to 19,724 regions distributed throughout the human genome have been identified that are likely to encode pseudogenes, and 3,391 parents (known functional genes) have been identified for more than 9,368 pseudogenes (3,4). Approximately 2/3 of these parent genes are associated with one pseudogene, and the remaining parent genes are associated with large numbers of pseudogenes. Pseudogenes have mean identities with their parents’ coding sequences of 76% to 80%.

**Expect that ~17% of known genes will have pseudogenes.**

(3,391 functional parents among approximately 20,000 functional human genes)

**Tools to identify pseudogenes**

- NCBI Blast searches: BLAST parameters determine the stringency of the BLAST search, and the number of homologous sequences will vary widely depending on the search parameters.
- Pseudogene.org (Mark Gerstein’s Laboratory at Yale): a pilot pseudogene data set as part of the GENECODE project that currently contains data on 11,763 pseudogenes (NCBI GRCH37) linked to parents.
  • The pseudogenes range from 22 bp to 220,910 bp with a median length of 675 bp
  • Their percent identity with the parent genes ranges from 40% to 99.97% with a median percent identity of 84.4%
- Ensembl: Ensembl lists pseudogenes on the “location tab” in the genomic region of the gene of interest, however they may or may not be related to the gene of interest.
- Genome search engines to search primer specificity:
  • University of California, Santa Cruz (UCSC) In-Silico PCR: http://genome.csdb.cn/cgi-bin/hgPcr

**Enrichment considerations**

- Check primer specificity for PCR-based amplification of genomic DNA
- Overlap amplicons so that the primer regions of the target genes are sequenced
- In primer design, map and avoid regions with known mutations and known variants
- PCR-based amplification of specific long-range amplicon DNA templates may be needed for genes with highly homologous pseudogenes and can also be used as confirmation of standard NGS results
• Check for nonspecific oligonucleotides in genes of interest when using hybrid capture enrichment

Coverage and depth of coverage
• Coverage will depend on the total output of a run for the specific NGS sequencer used, the total number of samples, and the total number of base pairs in the target region(s) or genome
• Coverage is the average number of reads for a given nucleotide in a given target area, and depth of coverage refers to the number of times a given nucleotide is read

NGS data trimming
• When using a microfluidic plate for multiplexing samples and library preparation by PCR amplification, trim the consensus sequences
• Trim NGS sequencer adaptors
• Trim sample barcodes
• Trim primer sequences at the ends of reads so that the sequence obtained from primer regions is from sample DNA as opposed to synthesized primer DNA

Phred Quality score
A quality score of 30, a commonly used cut off, means that the probability of an incorrect base call is 1 in 1,000. At this quality score cut-off expect

**Expect ~1 million incorrect base calls / Gb of data**
• Examine samples with known mutations and known normals to determine what percentage of reads is needed to call a heterozygote to increase reliability
• Confirm low percentage heterozygous mutations

Conclusions
• Expect ~17% of human genes to have pseudogenes
• Determine if the gene or genes of interest have pseudogenes and use appropriate sequencing methods
• Check specificity of primers and hybridization oligonucleotides
• Map and avoid known mutation and variant sites in primer design
• Design sufficient coverage for the entire target region and verify the coverage
• Be aware of the expected number of incorrect reads depending on the quality score used and determine what percentage of reads is needed to reliably call a heterozygote
• Overlap and trim primers so that the final sequence for the primer regions is from the target DNA not synthesized primer DNA

References


The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

P-56 – Withdrawn

P-57

Long Term Stability of Long-Chain Acylcarnitines in Enriched Dried Blood Spots Stored at Various Temperatures
P. Pickens, T. Lim and V. De Jesus, Centers for Disease Control and Prevention, Atlanta, GA

Abstract

Background: The Newborn Screening Quality Assurance Program (NSQAP) added hydroxystearoylcarnitine (C18OH) to its proficiency testing (PT) and quality control (QC) dried blood spot (DBS) materials in mid-2013. C18OH is an important secondary biomarker associated with Long-Chain 3-Hydroxyacyl CoA-Dehydrogenase (LCHAD) Deficiency. To assure stability and improve service to newborn screening laboratories, the long term stability of C18OH and other long-chain acylcarnitines (C14, C16, C16OH, C18, and C18:1) enriched in DBSs stored under various temperatures was examined.

Methods: DBSs enriched with C14, C16, C16OH, C18, C18:1, and C18OH were placed in Mylar foil bags containing desiccant packets to maintain humidity at <30%. Identical sets of these sample bags were grouped and stored in four different conditions: 37°C, ambient temperature, 4°C, and -20°C, for a 12 month storage period. Samples were collected on designated pull dates and re-stored in a freezer set to -70°C until the last sample bags were collected. DBS samples were then analyzed using tandem mass spectrometry. Recoveries were calculated as percent of initial concentrations remaining after each storage interval using a simple Arrhenius model.

Results & Discussion: DBSs stored for up to one year with humidity controlled at <30% retained the following percentages of their initial concentrations after one year: 100% at -20°C, 100% at 4°C, 70% at ambient temperatures, and 27% at 37°C for C18OH. Other long-chain acylcarnitines showed comparable results.

Conclusions: Long-chain acylcarnitines—including C18OH—enriched in DBSs maintained with humidity <30% are stable at least up to 6 months if stored at 4°C and for at least 100 days if stored at ambient temperatures. The recommended storage condition for our semi-annual acylcarnitine QC DBS at -20°C with routine working bag stored at 4°C is validated for C18OH and other long-chain acylcarnitines based on this stability study.

Presenter: Patrick Pickens, Centers for Disease Control and Prevention, Atlanta, GA, Phone: 678.230.2976, Email: xglO@cdc.gov
Summary

The Newborn Screening Quality Assurance Program (NSQAP) added hydroxystearoylcarnitine (C18OH) to its proficiency testing (PT) and quality control (QC) dried blood spot (DBS) materials in mid-2013. C18OH is an important biomarker associated with long-chain 3-hydroxyacyl CoA-dehydrogenase (LCHAD) deficiency.

To assure stability and improve service to newborn screening laboratories, the long term stability of C18OH and other long-chain acylcarnitines (C14, C16, C16OH, C18, and C18:1) enriched in DBSs stored under various temperatures was examined. The objective of this experiment was to examine and provide long term stability data on C18OH and other long-chain acylcarnitine-enriched DBSs stored at various temperatures.

DBSs enriched with hydroxystearoylcarnitine (C18OH) and other long chain acylcarnitines (C14, C16, C16OH, C18, and C18:1) were made from red blood cells purchased from Tennessee Blood Bank (Memphis, Tennessee, USA). The red blood cells were washed with saline and adjusted to 50% +/- 1% hematocrit using charcoal-stripped serum from SeraCare (Milford, Massachusetts, USA). After adjusting the hematocrit, the red blood cells were poured in Nalgene storage bottles and stored at -20°C. After two weeks, the cheesecloth-filtered blood was enriched with hydroxystearoylcarnitine (C18OH) and other long chain acylcarnitines (C14, C16, C16OH, C18, and C18:1). Using a QuadFlex liquid handling system, the blood was spotted onto filter paper cards; each spot was 75 µL of blood. The DBSs dried overnight. Subsequently, the DBSs cards were packaged in sets of twenty in Bitran bags with desiccant packets.

These DBSs enriched with hydroxystearoylcarnitine (C18OH) and other long chain acylcarnitines (C14, C16, C16OH, C18, and C18:1) were brought to room temperature and placed in Mylar foil bags containing desiccant packets to maintain humidity at <30%. Identical sets of sample bags were grouped and stored at four different conditions: 37ºC, ambient temperature, 4ºC, and -20ºC. One sample bag from each stored condition was collected on designated pull dates (0, 28, 56, 84, 112, 140, 168, 196, 224, 252, 280, 308, 336, and 364 days) and re-stored into a freezer set to -80ºC. DBS samples from different pull dates were then analyzed with tandem mass spectrometry in three runs of triplicates1.

A Waters TQD quadrupole tandem mass spectrometer with MassLynx 4.1 and NeoLynx data processing system was used and operated with electrospray ionization (ESI) of 10 µL samples supplied by a Waters Acquity autosampler (Waters, Milford, Massachusetts, USA). Flow injection analysis (FIA) was used in analyzing these samples.

The recoveries of C18OH and other long-chain acylcarnitines were calculated as percentages of initial concentrations remaining after each storage interval. In addition, slopes were calculated based on the concentration (µmol/ L whole blood) versus time in days over the duration of 364 days. DBSs stored for up to one year with humidity controlled at <30% retained the following percentages of their initial concentrations after one year: 100% at -20ºC, 100% at 4ºC, 70% at ambient temperatures, and 27% at 37ºC for C18OH. Other long-chain acylcarnitines showed comparable results. A slope representing the degradation of concentration over a duration of 364 day period was calculated for C18OH for each condition: 1.476E-05 at -20ºC, -5.492E-04 at 4ºC,-5.492E-04 at ambient temperatures, and 1.674E-03 at 37ºC. Other enriched long-chain acylcarnitines showed comparable slope values.

Long-chain acylcarnitines—including C18OH—enriched in DBS maintained with humidity <30% are stable at least up to 6 months if stored at 4ºC and for at least 100 days if stored at ambient temperatures and -20ºC. The recommended storage condition for our semi-annual acylcarnitine QC DBS at -20ºC with routine working bag stored at 4ºC is validated for C18OH and other long-chain acylcarnitines based on this stability study.

Reference
1. Dhillon, KS et al. Improved tandem mass spectrometry (MS/MS) derivatized method for the detection of tyrosinemia type 1, amino acids, and acylcarnitine disorders using a single extraction process. Clinica Chimica Acta, 2011; 412(11-12): 873-879

P-58

Proficiency Testing for Lysosomal Storage Disorders in Dried Blood Spots to Detect Krabbe and Pompe Diseases
J. Mei, H. Zhou, F. Lee, I. Williams, S. Zobel, V. De Jesus and R. Vogt, Centers for Disease Control and Prevention, Atlanta, GA

Abstract

Background: In 2013 The Newborn Screening Quality Assurance Program (NSQAP) initiated a pilot proficiency (PT) program to detect enzyme deficiencies in dried blood spots (DBS) associated with the lysosomal storage disorders (LSD) Krabbe and Pompe diseases.

Methods: DBS specimens were produced from disease-specific Epstein-Barr virus transformed cell lines. PT panels consisted of 5 blind-coded specimens, with one specimen in each panel deficient for either galactocerebrosidase (GALC), the biomarker for Krabbe disease; or acid alpha-glucosidase (GAA), the biomarker for Pompe disease. Participating laboratories tested the PT specimens and reported the quantitative values for each enzyme (µmol/L/h), clinical interpretation, method, and analytical cutoff.

Results: We summarized quantitative and qualitative results for five quarters from 2013-2014. For GALC (Krabbe biomarker) no false negative results and no false positive results were reported for the PT specimens. For GAA (Pompe biomarker) no false negative and one false positive result (0.7% false positive rate) were reported for the PT specimens. Three methods were reported: flow injection tandem mass spectrometry; liquid chromatography tandem mass spectrometry; and digital microfluidics (GAA only). All methods identified the screen negative specimens and the specimens with low enzyme activities that needed follow-up.

Conclusions: Reference materials produced at CDC were suitable for use in a pilot PT program to detect LSD enzyme deficiencies in DBS. Low false positive rates and no false negative results were observed for the PT specimens over 5 quarters. PT is needed for continuous quality improvement and can identify sources of errors to help reduce risk.

Presenter: Joanne Mei, PhD, Centers for Disease Control and Prevention, Newborn Screening Quality Assurance Program, Atlanta, GA, Phone: 770.488.7945, Email: jmei@cdc.gov
Summary

Background: In 2013, the Newborn Screening Quality Assurance Program (NSQAP) initiated a pilot proficiency testing (PT) program to detect enzyme deficiencies in dried blood spots (DBS) associated with the lysosomal storage disorders (LSD) Pompe and Krabbe diseases. The program was offered to US newborn screening laboratories. PT panels were sent six times between January 2013 and April 2014. Methods: DBS specimens were produced from disease-specific Epstein-Barr virus (EBS) transformed cell lines. DBS with “normal” enzyme activity were prepared from freshly-collected umbilical cord blood. Normal and condition-specific DBS were randomized into PT panels of five blind-coded specimens, with one specimen in each panel deficient for either acid alpha-glucosidase (GAA), the biomarker for Pompe disease; or galactocerebrosidase (GALC), the biomarker for Krabbe disease. Participating laboratories tested the PT specimens and reported the quantitative values for each enzyme (µmol/L/h), clinical interpretation, method, and analytical cutoff.

Results: We summarized quantitative and qualitative results for six quarters from 2013-2014 (Figures 1 and 2). For GAA (Pompe biomarker) no false negative and one false positive result (0.74% false positive rate) were reported for the PT specimens (Table 1). For GALC (Krabbe biomarker) no false negative results and no false positive results were reported for the PT specimens. Three methods were reported: flow injection tandem mass spectrometry, liquid chromatography tandem mass spectrometry, and digital microfluidics (GAA only). All methods identified the screen negative specimens and the specimens with low enzyme activities that needed follow-up.

Conclusions: Reference materials produced at CDC were suitable for use in a pilot PT program to detect LSD enzyme deficiencies in DBS. EBV-transformed lymphoblast cells derived from LSD patients provided a sustainable resource for condition-specific reference materials. Low false positive rates and no false negative results were observed for PT specimens distributed over six quarters. PT is needed for continuous quality improvement and can identify sources of errors to help reduce risk.

![Figure 1. Performance of Methods that Detect GAA Enzyme Deficiency in DBS PT Specimens (2013-2014)](image-url)

Table 1. Proficiency Testing for Pompe and Krabbe in DBS. Data compiled from six quarters, five specimens per proficiency testing panel.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. US Labs Reporting</th>
<th>Negative Specimens Assayed (N)</th>
<th>False Positive Errors (%)</th>
<th>Positive Specimens Assayed (N)</th>
<th>False Negative Errors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pompe (GAA)</td>
<td>7</td>
<td>136</td>
<td>0.74</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Krabbe (GALC)</td>
<td>6</td>
<td>124</td>
<td>0</td>
<td>31</td>
<td>0</td>
</tr>
</tbody>
</table>

P-59

Development of Dried Blood Spot Reference Materials for Fragile X
J. Lyons, P. Mueller and G. Kerr, Centers for Disease Control and Prevention, Atlanta, GA

Abstract

Objective: Fragile X syndrome is the most common known single-gene cause of autism and the most common inherited cause of intellectual disability. It is associated with the expansion of the CGG repeat in the promoter region of the Fragile X Mental Retardation 1 (FMR1) gene on the X chromosome. A definitive diagnosis of fragile X syndrome is made through genetic testing to identify expanded CGG repeats. Early intervention provides important services for identified newborns and their families. The goal of this study was to develop dried blood spots using characterized cell lines to serve as quality assurance (QA) materials.

Methodology: We created DBS using twice filtered red blood cells. After testing negative for any donor FMR1 genes, blood was reconstituted using serum. This blood was inoculated with either 10x106
transformed lymphocyte cells/ml (samples) or phosphate buffered saline (base pool). Samples included both genders and gray zone, premutation, and full mutation expansions. Ten spots per card were created using 75 µl of blood per spot; 3-mm punches were collected from one spot from every 5th card for each sample for each DNA extraction method. Four methods for isolating DNA were compared, and the commercially available Amplidex® kit was used to test for FMR1.

**Results:** All samples except the base pool produced FMR1 PCR products with the Amplidex® kit. Appropriate CGG expansion results were obtained using the Qiagen spin column method, and results varied with other DNA extraction methods.

**Conclusions:** In order to assure proper reference materials are available for FMR1 genetic testing, we demonstrated that lab-created DBS can serve as QA material for certain molecular methods. Further work is needed to optimize for screening protocols.

**Presenter:** Justine Lyons, PhD, Centers for Disease Control and Prevention, NSMBB, Atlanta, GA, Phone: 770.448.7704, Email: vro9@cdc.gov

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**Summary**

**Introduction:** Fragile X syndrome (FXS) is a genetic condition that is the most common known single-gene cause of autism and the most common inherited cause of intellectual disability. It is associated with the expansion of the CGG trinucleotide repeat in the promoter region of the Fragile X Mental Retardation 1 (FMR1) gene on the X chromosome. Depending on the length of uninterrupted CGG repeats, an allele may be classified as normal (5 – 44 repeats; unaffected by the syndrome), a premutation (55–200 repeats; at risk of adult-onset fragile X associated disorders), or full mutation (>200 repeats; usually affected by the syndrome).

Several studies have investigated the feasibility of large-scale screening for Fragile X in newborns (Chow 2003, Fernandez-Carvajal 2009, Saul 2008). The recent development of a rapid, inexpensive molecular screening test that is capable of using DBS to register expanded FMR1 alleles (Tassone 2008) may help facilitate the introduction of newborn screening for FMR1 mutations in the future. The Newborn Screening and Molecular Biology Branch offers technical support to laboratories using DBS. As such, we provide subject matter expertise for screening platforms and reliable quality assurance materials. DBS are collected universally from newborns and may be valuable for the detection of expanded CGG repeats. In this study we developed dried blood spots using Fragile X characterized cell lines to serve as quality assurance (QA) materials for method development and proficiency testing.

**Methods:** Normal newborn blood contains approximately 14x10^6 white blood cells/ml. We previously created and tested three titrations of Coriell cell line concentrations to test higher and lower ranges of detection in dried blood spots (10x10^6 cells/ml; 15x10^6 cells/ml; 20x10^6 cells/ml) and found no differences in obtaining expected PCR results across the range. Therefore, we created DBS using the lowest concentration of cells which is near the lower range of normal for newborns.

In order to deplete donor leukocytes, the red blood cells (RBCs) received from TN Blood Bank had been filtered twice. We then used serum to adjust the hematocrit of the blood to 55%. Aliquots of RBCs, serum, and plasma all tested negative for presence of donor FMR1 using a PCR method developed by Tassone et al. (2008).
We created dried blood spots (DBS) from four Fragile X cell lines representing both a heterozygous and a homozygous full mutation, a heterozygous grey zone mutation, and a heterozygous premutation, plus a base pool (2x filtered, leukocyte-depleted (LD) red blood cells hematocrit-adjusted with serum to 55% with no Fragile X cells added). Cards were dried overnight at room temperature, then stored at -20°C until DNA isolation.

To characterize the homogeneity across samples, three-mm punches were collected from one spot from every 5th card for each sample. To test within-spot homogeneity, punches were collected from the center, north, south, east and west regions of each spot. DNA was extracted from all samples (n=175) using a saline-sodium phosphate EDTA (SSPE)/Chelex protocol which is a modified version of that used by LaFauci et al., (2013; DNA extraction methods comparison, #1; modified protocol provided courtesy of W. Ted Brown, Institute for Basic Research, Staten Island, NY). One sample from each cell line, plus the base pool (n=5), was also tested using the Asuragen reagents and FragileX primers according to manufacturer’s instructions. DNA concentration was measured using RNase P (Life Technologies™). In addition to the SSPE/Chelex protocol, four other methods for isolating DNA from DBS were compared using one sample from each cell line (n=5 for each method). All protocols are for isolation of genomic DNA from blood card punches with diameters of approximately 3 mm (1/8 inch) and are suitable for blood treated with the anticoagulant CPDA-1. The SSPE/Chelex method performed the best and was therefore used for the rest of the extractions.

DNA was tested for presence of expanded CGG repeats using a PCR protocol developed by Tassone et al (2008). This method uses forward and reverse primers to amplify the CGG repeat region and then uses a chimeric PCR primer that targets randomly within the expanded CGG region, such that the presence of a broad distribution of PCR products represents a positive result for an expanded allele. The method is applicable for screening both males and females and can detect expansions, flagging the sample for second-tier testing and specific sizing using clinical reagents. Furthermore, the method is capable of rapid detection of expanded alleles using as little as 1% of the DNA from a single dried blood spot.

**Results:** DNA concentrations for the 3-mm punches ranged from 1.05 – 11.19 ng/ul (Table 2) with a mean of 2.67 +/- 1.31 ng/ul as determined using RNase P. Concentration of DNA extracted from the center of the spot was not significantly different from that of DNA extracted from the periphery (north, south, east, or west regions) of the spot (mean center: 2.79 +/- 1.99 ng/ul; mean periphery: 2.65 +/- 1.09 ng/ul). Samples from the Base pool (no cells added) yielded no DNA (0.00 ng/ul). All samples showed the appropriate and expected results when tested for CGG repeat length using a PCR developed by Tassone et al (2008, data not shown).

**Conclusions:** We developed and characterized dried blood spots for four Fragile X cell lines to be used as reference material for newborn screening. Cell lines were cultured in the lab and then mixed with leukocyte-depleted (twice filtered) red blood cells hematocrit adjusted with serum, then spotted. We tested several DNA isolation methods and determined that a rapid, inexpensive method using an in-house prepared SSPE solution in combination with Chelex provided the highest concentrations of DNA. Mean DNA concentrations did not vary greatly across the cell lines, nor between the center and the periphery. All samples produced PCR products of the expected size when characterized using a molecular method developed by Tassone et al (2008), confirming that even minute concentrations of DNA (>1 ng/ul) isolated from DBS can successfully determine the presence of an expanded CGG repeat.

**Improved Method for the Detection of Hemoglobinopathies via an Enhanced Trypsin Digestion/Extraction and Sample Analysis via HPLC-ESI-MS/MS**

D. Foreman, C. Haynes, V. De Jesus, Centers for Disease Control and Prevention, Atlanta, GA

**Abstract**

**Background:** Sickle Cell disease (SCD) is the most prevalent hereditary disorder in humans, resulting from a point mutation at the first glutamate residue of β-Globin. The two most common clinically significant hemoglobin variants are Hb S and Hb C. Recently, newborn screening, better medical care, and parent education have successfully reduced the morbidity and mortality due to Hb S; however, improving the detection of hemoglobinopathies in newborns could be accomplished with mass spectrometry (MS). MS could serve as a confirmatory tool for IEF and HPLC, as well as, providing a more sophisticated analysis for complex hemoglobinopathy cases.

**Methods:** A previously published HPLC-ESI-MS/MS method to detect hemoglobinopathies was modified to resolve hemoglobin peptides over a shorter time (Haynes et al. 2013). Data acquisition was performed via single-reaction monitoring (SRM).

**Results:** A comparison of the TIC (summed peptide content) for digestion conditions A (4hr at 37°C) and B (10 min at 50°C) showed similar signal intensity. Optimal digestion conditions for the peptides of interest were determined to be 30 minutes at 50°C. The use of a shorter analytical LC column reduced the sample analysis time from 27 minutes to 9 minutes per sample. Overall, the changes applied to the published method (Haynes et al., 2013) have resulted in an assay that is approximately 75% faster than its original counterpart. The use of SRM instead of single ion monitoring (SIM) with complementing product ions scans improve the semi-quantitative data analysis of this assay as SRM acquisition produced well defined peaks thus eliminating the need for manual integration.

**Summary:** Performing the trypsin digestion/extraction step at a higher temperature produced higher peptide intensities to a published method; thus significantly enhancing the efficiency of the assay.

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Summary

Hemoglobinopathies is the term used to describe a class of disorders that are caused by mutations to the globin chains (α-, β-, γ-) of the protein hemoglobin. The protein hemoglobin is found within red blood cells and is responsible for the transport of oxygen from the lungs to the tissues where it is used to produce energy. The more prevalent mutations occur on the β-Globin chains of the tetramer resulting in structural change to the red blood cell shape leading to what is commonly known as sickle cell and thus sickle cell disease. When this occurs, it becomes challenging for blood to flow freely throughout the body’s vasculature leading to anemia, fatigue and possible damage to blood vessels and vital organs.

The implementation of newborn screening coupled with comprehensive care has decreased hemoglobinopathy morbidity and mortality rates significantly to approximately 1.8%\(^2\). The most commonly used methodologies for the detection of hemoglobinopathies are isoelectric focusing (IEF) and high performance liquid chromatography coupled with UV (HPLC-UV) each of which rely on the migration and elution of hemoglobin tetramers relative to a given standard. The use of the aforementioned methods work well for a wide variety of hemoglobinopathies but can prove challenging with variants that share migration and elution with disease-specific tetramers\(^3\).

A developing approach for the rapid detection of Hb variants is the use of HPLC-ESI-MS/MS. HPLC-ESI-MS/MS coupled with a peptide database provides one with the ability to determine amino acid sequences of proteins. This sequence deduction is made possible through the use of trypsin to “digest” proteins resulting in a peptide mixture that can be subsequently analyzed. This method is particularly useful as it aids in the resolution of Hb variants that are challenging for HPLC and IEF.

The method detailed in Haynes et al., 2013 employs HPLC-ESI-MS/MS for the detection of hemoglobinopathies by identifying disease specific peptides. The sample prep included the tryptic digestion/extraction of a dried-blood spot (DBS) at 37°C for 4 hours. The HPLC method incorporated a Phenomenex Jupiter C12 2.1 x 150 mm column with a binary solvent system of isopropanol and water with 0.1% formic acid (27 minutes)\(^4\). Though this assay performed well in detecting hemoglobinopathies, the sample prep (4 hr) and analysis times (27 min) were longer than ideal. Our initial efforts were focused on the reduction of the sample prep time, where we were interested in exploring the idea of performing the tryptic digestion/extraction at a higher temperature for a shorter period of time. Figures 1-3 summarize the results of this experiment where the digestion/extraction time was held constant over a range of temperatures (Fig. 1), where the temperature was held constant over a range of times (Fig. 2), an a inter-prep comparison of three different sample prep conditions (Fig. 3). From these results, it was determined that performing the extraction/digestion step at 50°C for 30 minutes proved the best compromise for all the conditions considered. This modification represented a significant reduction in sample prep time.

The analysis time in Haynes et al., 2013 was 27 minutes per sample. The improved HPLC analysis incorporated the use of a Phenomenex Jupiter C12 2.1 x 50 mm column with a binary solvent system of acetonitrile and water with 0.1% formic acid. In addition to the modification to the HPLC method, we also modified the acquisition of data, changing from data dependent product-ion scans to single-ion reaction monitoring (SRM) which collectively served to improve both the throughput and sensitivity of this assay. The change in organic solvent, length of HPLC column, and type of acquisition resulted in an analysis time of 18 min per sample. A representative data file of both the 27 and 18 minute analysis are
shown in Figures 4 and 5, respectively. Note that in Figure 4 the data acquisition window ends at 15 minutes, however there is an additional 12 minutes of column flush and re-equilibrating that is included in the final 27 minutes but not shown in the chromatogram. In figure 5, the column flush and re-equilibrating time is included in the acquisition window for a total of 18 minutes. A review of both figures 4 and 5 indicates that the HPLC profile used in the improved method resulted in well-resolved peaks. Improved peak shapes simplified the data integration necessary for data analysis. The reduction in both the sample prep and sample analysis times resulted in an assay that is significantly shorter than its original counterpart, with no significant loss in overall peptide abundance and data quality.
Figure 4: Total-Ion Chromatogram and Extracted-Ion Chromatograms obtained from 27 minutes analysis

![Figure 4](image)

Figure 5: Total-Ion Chromatogram and Extracted-Ion Chromatograms obtained from 18 minutes

![Figure 5](image)

Figure 5: Sample chromatogram acquired from a digested DBS using the improved method

References