PETITION FOR ADOPTION, AMENDMENT, OR REPEAL OF A STATE ADMINISTRATIVE RULE

Print Form

In accordance with RCW 34.05.330, the Office of Financial Management (OFM) created this form for individuals or groups who wish to petition a state agency or institution of higher education to adopt, amend, or repeal an administrative rule. You may use this form to submit your request. You also may contact agencies using other formats, such as a letter or email.

The agency or institution will give full consideration to your petition and will respond to you within 60 days of receiving your petition. For more information on the rule petition process, see Chapter 82-05 of the Washington Administrative Code (WAC) at http://apps.leg.wa.gov/wac/default.aspx?cite=82-05.

CONTACT INFORMATION *(please type or print)*

Petitioner's Name: Dr. Sihoun Hahn, M.D, Ph.D.

Name of Organization: Key Proteo, Inc.

Mailing Address: 720 Broadway

City: Seattle State: WA Zip Code: 98122

Telephone: 206-399-7515 Email: steve.hahn@keyproteo.com

COMPLETING AND SENDING PETITION FORM

- Check all of the boxes that apply.
- Provide relevant examples.
- Include suggested language for a rule, if possible.
- Attach additional pages, if needed.
- Send your petition to the agency with authority to adopt or administer the rule. Here is a list of agencies and their rules coordinators: http://www.leg.wa.gov/CodeReviser/Documents/RClist.htm.

INFORMATION ON RULE PETITION

Agency responsible for adopting or administering the rule: Washington State Board of Health

☒ **1. NEW RULE - I am requesting the agency to adopt a new rule.**

☒ The subject (or purpose) of this rule is: Institute newborn screening of Wilson Disease for Washington State.

☒ The rule is needed because: NBS and early diagnosis for Wilson Disease is currently an unmet need.

☒ The new rule would affect the following people or groups: Newborns with Wilson Disease; Parents and families negatively impacted by association.

☐ **2. AMEND RULE - I am requesting the agency to change an existing rule.**

List rule number (WAC), if known: _____

☐ I am requesting the following change: _____

☐ This change is needed because: _____

☐ The effect of this rule change will be: _____

☐ The rule is not clearly or simply stated: _____

☐ **3. REPEAL RULE - I am requesting the agency to eliminate an existing rule.**

List rule number (WAC), if known: _____

(Check one or more boxes)

☐ It does not do what it was intended to do.

☐ It is no longer needed because: _____

☐ It imposes unreasonable costs: _____

☐ The agency has no authority to make this rule: _____

☐ It is applied differently to public and private parties: _____

☐ It conflicts with another federal, state, or local law or rule. List conflicting law or rule, if known: _____

☐ It duplicates another federal, state or local law or rule. List duplicate law or rule, if known: _____

☐ Other (please explain): _____

[Supplemental Documents]

1. Criteria Justification
2. Petition Letter
3. WA State Pilot Study Overview Slide deck
4. NBS Kit validation for FDA submission Slide deck
    a. IFU
    b. Analytical & Clinical Validation data for FDA submission
5. References
    a. APHL abstract 2024
    b. Ann. N.Y. Acad. Sci: Population screening for Wilson disease
    c. Clinical and Translational Perspectives on Wilson disease. Edited by Nanda Kerkar and Eve A. Roberts
        i. Chapter 26. Population screening for Wilson disease
        ii. Chapter 17. Wilson disease in Infancy through Adolescence
    d. Handbook of Clinical Neurology. Wilson Disease Edited by Anna Czlonkowska and Michael L. SChilsky
        i. Chapter 3.The genetics of Wilson Disease
    e. J. Proteome Res. 2017: Quantification of ATP7B Protein in Dried Blood Spots by Peptide Immuno-SRM as a Potential Screen for Wilson's Disease
    f. Gastroenterology 2021: Direct Measurement of ATP7B Peptides Is Highly Effective in the Diagnosis of Wilson Disease
        i. Editorial comments: Expanding the Diagnostic Toolkit of Wilson Disease with ATP7B Peptides
    g. JPGN 2020: Management of Wilson Disease Diagnosed in Infancy: An Appraisal of Available Experience to Generate Discussion
    h. GeneReviews: Wilson disease: Wilson Disease - GeneReviews® - NCBI Bookshelf (nih.gov)
6. Letter of Support
    a. Wilson Disease Association, U.S.A.
    b. Wilson Disease Association, Spain
    c. Experts
        i. Dr. Karl Weiss in Germany
        ii. Dr. Michael Schilsky at Yale University
    d. Family members:
        i. Letters from US

- Alice William Family
- Christopher and Rachel Johnson
- Nora Closser
- Erin Brooks
- Dr. Kirk Vestal
- Thomas Sandall
- Marilee and Gary Wolter
- Maxine and Michael Bonn
- Janet Laubgross

ii. Letter from Poland

- Anna Aniol

THREE GUIDING PRINCIPLES: Three guiding principles govern all aspects of the evaluation of a candidate condition for possible inclusion in the NBS panel.
• Decision to add a screening test should be driven by evidence. For example, test reliability and available treatment have been scientifically evaluated, and those treatments can improve health outcomes for affected children.
• All children who screen positive should have reasonable access to diagnostic and treatment services.
• Benefits of screening for the disease/condition should outweigh harm to families, children and society

[Criteria for Testing]

Available Screening Technology: Sensitive, specific and timely tests are available that can be adapted to mass screening.

The base technology is tandem mass spectrometry (LC-MS/MS), which is already being used for NBS conditions such as LSD (Lysosomal Storage Disorders) and XLD (X-linked Adrenoleukodystrophy) in Washington State. Quantification of ATP7B protein using LC-MS/MS utilizes trypsin to digest protein and quantify the resulting signature surrogate peptides of the target protein, ATP7B. It has been well demonstrated that the assay can provide the diagnosis and newborn screening of Wilson disease with sensitivity and specificity both above 92%. To date, over 25,000 newborns have been successfully tested in Washington State. A total of 4 presumptive positive cases have been detected. Two of them were false positive (no variants or carriers) and two of them were likely true positive. The sample to sample run time is less than 3 minutes which is appropriate for high throughput analysis.

Diagnostic Testing and Treatment available: Accurate diagnostic tests, medical expertise, and effective treatment are available for evaluation and care of all infants identified with the condition.

Biochemical and genetic testing are widely available to confirm the diagnosis. Treatment includes a copper restricted diet, zinc or chelating agents such as penicillamin or syprine. Gene therapy is currently under clinical trial. Liver transplantation is required in advanced patients with decompensated liver cirrhosis and acute liver failure. The cost for the maintenance treatment with zinc is a penny a day ($.01/day).

Prevention Potential and Medical Rationale: The newborn identification of the condition allows for early diagnosis and intervention. Important considerations:

• *There is sufficient time between birth and onset of irreversible harm to allow for diagnosis and intervention.*
The newborn identification of the condition allows for early diagnosis and preemptive intervention. There has been a strong need for NBS of Wilson disease (WD), which progresses to an irreversible multi-systemic disease. The patient mortality rate is 100% if left untreated. The copper accumulation starts right after birth but as the disease progress is slow, WD is usually diagnosed in adolescents after significant complications occur. Nonetheless, it is now well established that Wilson disease can present with clinical disease, mainly hepatic, in very young children <5 years old. Since the first detailed and well-documented report of a 3-year-old with cirrhosis, numerous reports have appeared providing additional evidence to institute NBS for Wilson disease.

Unlike other metabolic disorders, infants with Wilson disease do not present with any acute deterioration in the first few years of life. It is generally recommended to start the zinc maintenance

treatment during 12-24 months of life and monitor the labs every 6 months or a year. There is ample time to allow for diagnosis, counseling and initiation of treatment.

• *The benefits of detecting and treating early onset forms of the condition (within one year of life) balance the impact of detecting late onset forms of the condition.*
The symptoms of Wilson disease are frequently nonspecific. Approximately one-third of children with clinically evident WD are present with cirrhosis. Acute liver failure poses significant diagnostic problems. Although neurologic Wilson disease is less common in pediatric patients with Wilson disease, it has been reported in children as young as 6 years old. Initial neurological symptoms could be extremely nonspecific further delaying the diagnosis and appropriate management. Treatment for neurological Wilson disease is challenging as they could develop further deterioration with chelating agents.

The cost for zinc maintenance treatment is only a penny a day ($.01/day) when started before any complications occurred and the patients live a completely normal life. The benefits of detecting and treating the condition before symptoms occurred have been well described in many literatures.

• Newborn screening is not appropriate for conditions that only present in adulthood.
As many genetic disorders have a spectrum of clinical severity, some patients with Wilson disease may not have the symptoms until later in life. However, all those biochemically and genetically confirmed Wilson disease patients should start the treatment regardless of the symptoms or lab results at the time of the diagnosis.

Public Health Rationale: Nature of the condition justifies population-based screening rather than risk-based screening of other approaches.

Effective treatment for Wilson disease is widely available and affordable, highlighting the importance of early detection and intervention. These treatments are highly effective in preventing the downstream of morbidity and early mortality associated with Wilson disease. While there are effective treatments available, it is unfortunate that most patients come to the clinic after developing serious complications. Furthermore, many patients suffer from delayed diagnosis due to the nature of nonspecific and considerably variable clinical presentations. Half of the affected patients with neurological symptoms may develop permanent neurological damage. Patients with chronic liver disease may progress to cirrhosis and hepatocellular carcinoma.

Importantly, patients with Wilson disease can live a completely normal life when the treatment is started early in life. Early initiation of therapy before the onset of symptoms makes a critical difference in their outcome. The cost for zinc treatment is nominal compared to other cell based or gene therapies. The best clinical outcome could be achieved only through NBS.

Cost-benefit/Cost-effectiveness: The outcome outweighs the costs of screening. All outcomes, both positive and negative, need to be considered in the analysis. Important considerations to be included in economic analyses include:

Newborn screening of Wilson disease will change the diagnostic course and clinical outcome of WD patients. As many WD patients undergo liver transplantation, this opportunity for early initiation of therapy before the onset of symptoms will significantly make a critical and huge difference in their long-term clinical outcome. Ultimately, we anticipate there will be savings that come from converting patients who would otherwise be consumers of high-intensity medical care, long-term convalescent care, and disability resources, to functional individuals capable of holding jobs and contributing to the tax base.

- *The prevalence of the condition among newborns.*

The prevalence of WD is approximately 1 in 30,000 newborns, with a carrier frequency of 1 in 90 (higher in certain populations). However, regional variations exist. Costa Rica, Sardinia, the Canary Island and Crete have all reported to have increased incidence.

• *The positive and negative predictive values of the screening and diagnostic tests.*

ATP7B 887 analysis was found to have a sensitivity of 91.2%, specificity of 98.1%, positive predictive value of 98.0%, and a negative predictive value of 91.5%. ATP7B 1056 showed a positive predictive value of 96.1% and negative predictive value of 91.3%.

• *Variability of clinical presentation by those who have the condition.*

Wilson disease presents a variety of symptoms depending on which organs are more affected. Wilson disease can lead to hepatic failure and/or severe neurological disability and ultimately death if untreated.

• *The impact of ambiguous results. For example, the emotional and economic impact on the family and medical system.*

The current pilot study in Washington State indicates the false positive rate is extremely low compared to the other conventional newborn screening methods.

• *Adverse effects or unintended consequences of screening.*

As with other screening programs, there could be false negative cases from this screening.

720 Broadway
Seattle, WA 98122—4302
206-339-7515
KEY PROTEO

To: Washington State Department of Health
Board of Directors

From: Sihoun Hahn, MD, PhD
Professor of Pediatrics, University of Washington
Seattle Children's Hospital
Founder and CMO, Key Proteo, Inc

Re: Letter from Petitioner

Date: July 26, 2024

Dear Washington State Department of Health Board of Directors,

On behalf of Key Proteo, I am writing to request your review of our Petition for Rulemaking to adopt a new administrative rule to screen for Wilson Disease (WD). Within our petition package you will find evidence to support Washington States Qualifying Assumption, evidence to support the Three Guiding Principles, as well as evidence to support the 5 Criteria used to evaluate conditions for possible inclusion in the newborn screening panel.

Wilson Disease is an autosomal recessive disease of copper metabolism caused by pathogenic mutations in the *ATP7B* gene, a copper-transporting ATPase. About 1 out of 30,000 individuals are affected by WD. Early diagnosis and pre-emptive therapy before the manifestation of symptoms are crucial for successful clinical outcomes that can lead to a completely normal life with a cost of a penny a day ($.01/day) with zinc treatment. Without early intervention, individuals can develop significant life-threatening complications, including liver cirrhosis, acute kidney failure, and brain and nerve damage. Key Proteo's Immuno-Selected Reaction Monitoring (SRM) platform is a novel proteomic screening solution that uniquely delivers rapid, scalable, and cost-effective detection of extremely low abundance peptide biomarkers in newborn dried blood spot specimens with high accuracy, effectiveness, and efficiency via familiar mass spectrometry workflows currently being used in the Washington State Newborn Screening Laboratory. By quantifying the ATP7B peptide, Key Proteo's technology provides accurate results to identify affected patients allowing them to be treated clinically leading to healthier outcomes. We have been successfully running a pilot study in collaboration with WA State DOH Newborn Screening Laboratory (Director Dr. John Thompson) for over two years screening ~25,000 newborn babies for Wilson disease. The progress report is included in the supplemental package.

Our petition package includes letters of support from the Wilson Disease Association (US), Wilson Disease Association (Spain), experts in the field such as Drs. Karl Heinz Weiss and Michael Schilsky, and families from the U.S., and Poland providing additional support as well as sharing their stories. We've also included evidence to support the need to adopt this test including validation data, Instructions for Use, criteria justification, as well as literature references.

It is our immense hope that Washington State will adopt and require this test for all newborns screened in the newborn screening program which will be the first model to all other states and the world. Together we can provide the community with early diagnosis, healthier outcomes, and relieve the heavy

720 Broadway
Seattle, WA 98122—4302
206-339-7515
KEY PROTEO

burden these families carry when confronted with unexpected but life-saving decisions due to late diagnosis.

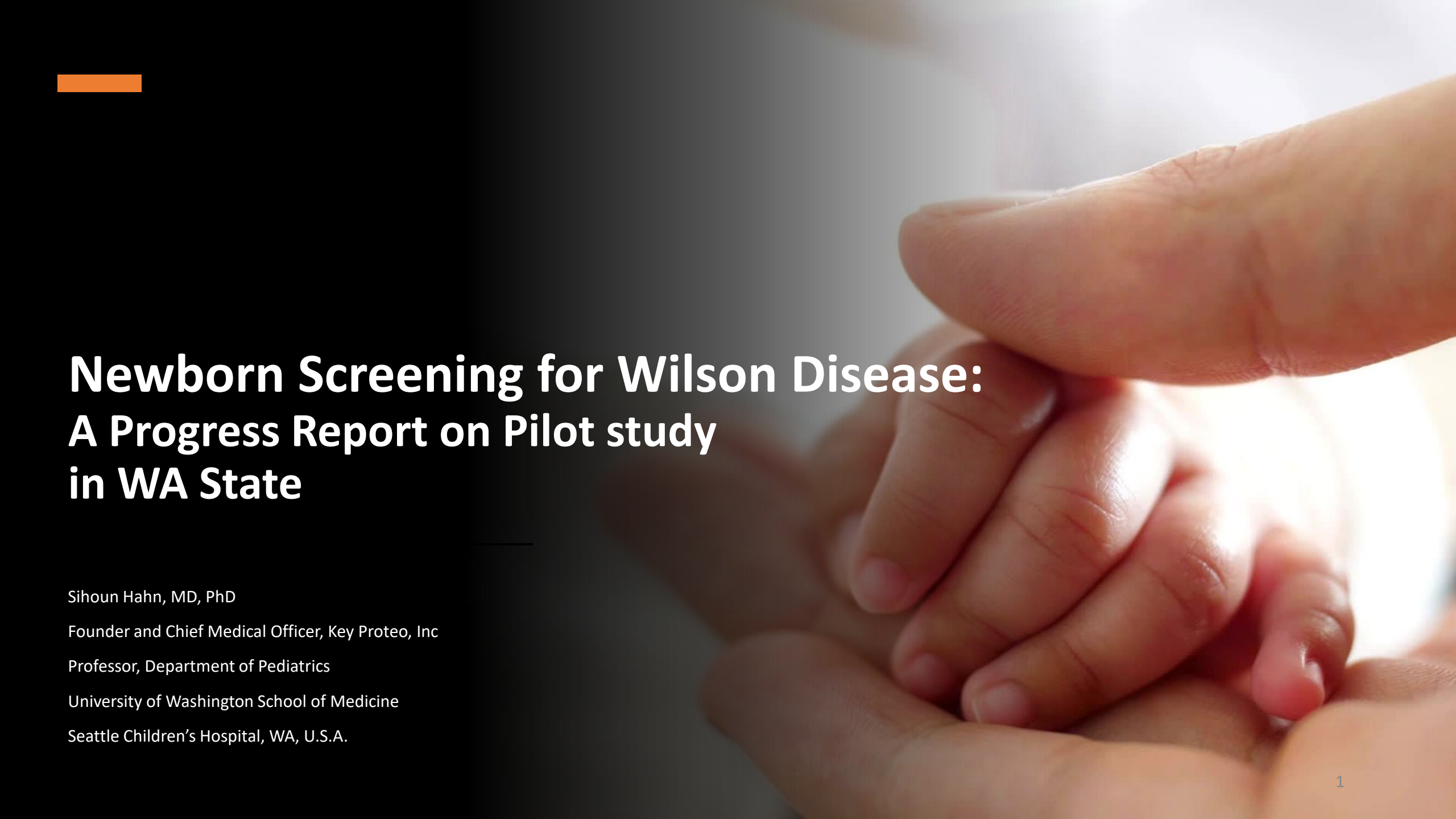
We appreciate your consideration and look forward to your review. Please do not hesitate to reach out with any questions whatsoever.

Sincerely,

Sihoun Hahn Verified by pdfFiller
07/26/2024

Sihoun Hahn, MD, PhD
Director, Center of Excellence for Wilson disease
Seattle Children's Hospital
Founder and CMO, Key Proteo, Inc

Newborn Screening for Wilson Disease:
A Progress Report on Pilot study in WA State



Sihoun Hahn, MD, PhD

Founder and Chief Medical Officer, Key Proteo, Inc

Professor, Department of Pediatrics

University of Washington School of Medicine

Seattle Children's Hospital, WA, U.S.A.

FINANCIAL DISCLOSURES

Sihoun Hahn, MD, PhD, is a member of the Seattle Children's Hospital workforce and is serving as Chief Medical Officer of Key Proteo, Inc. He is an inventor of intellectual property that has been licensed to Key Proteo, Inc. Dr. Hahn is the founder of Key Proteo, Inc. and has ownership equity interests in the company.

Topics to discuss

- Background of Wilson disease
- Proof of evidence study on a large-scale patient cohort
- Manufactured kit validation for public health transition
- Pilot study in WA state

Background of Wilson Disease

Wilson Disease

- Autosomal Recessive Copper Transport Disorder
- Incidence: ~1/30,000
- Early diagnosis and pre-emptive treatment can lead to a normal quality of life

Dr. Samuel A. K. Wilson (1878-1937)

- Progressive, fatal disease if untreated
- ***ATP7B* gene**: ATPase, copper transporter
  - >1700 variants (Varsome)
  - p.H1069Q : ~35-45% in European origin
  - p.R778L: ~30% in Far East Asian origin

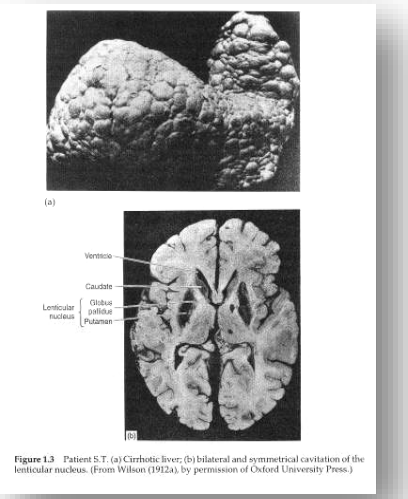


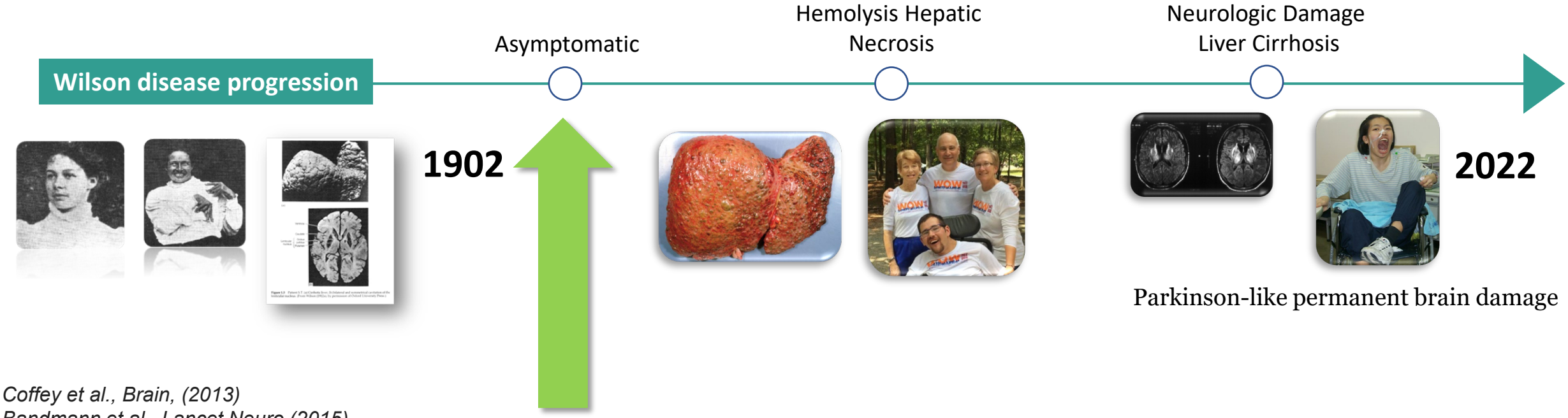
Figure 1.3 Patient S.T. (a) Cirrhotic liver; (b) bilateral and symmetrical cavitation of the lenticular nucleus. (From Wilson (1912a), by permission of Oxford University Press.)

Hoogenraad TU, Wilson's disease, Intermed Medical Publishers

- Diagnosis: high copper in the liver/urine, low serum ceruloplasmin, KF ring in the cornea
- Treatment: Chelators (Trientine), Zinc salts, Gene therapy under trial
- ***Immuno-SRM analysis is promising for NBS***
  - ***Majority of mutations results in markedly decreased level of ATP7B protein due to enhanced degradation, absence or decay of mRNA*** *(Hepatology, 2009; Proc Natl Acad Sci U S A, 1998; Gastroenterology, 2007; Curr Issues Mol Biol, 2001; Proteins, 2008; Genetics, 1998; Blood, 2005; Mol Genet Metab, 2005; BMC Gastroenterol, 2010; Nat Genet, 2004; Annu Rev Biochem, 2007)*

With early intervention, we can change the clinical course

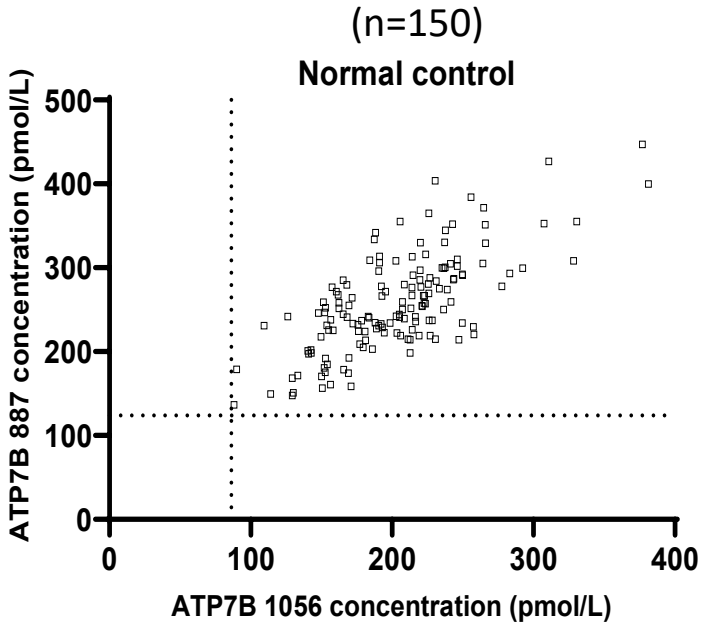
- Unrecognized in a substantial portion of affected individuals [1-2]
  (At least half of patient with WD are **never diagnosed** and die of untreated disease)
- Late diagnosis is the most common cause of death [3]
- Early diagnosis improves clinical outcome [4]
- Currently, there is no screening method while effective treatments are available

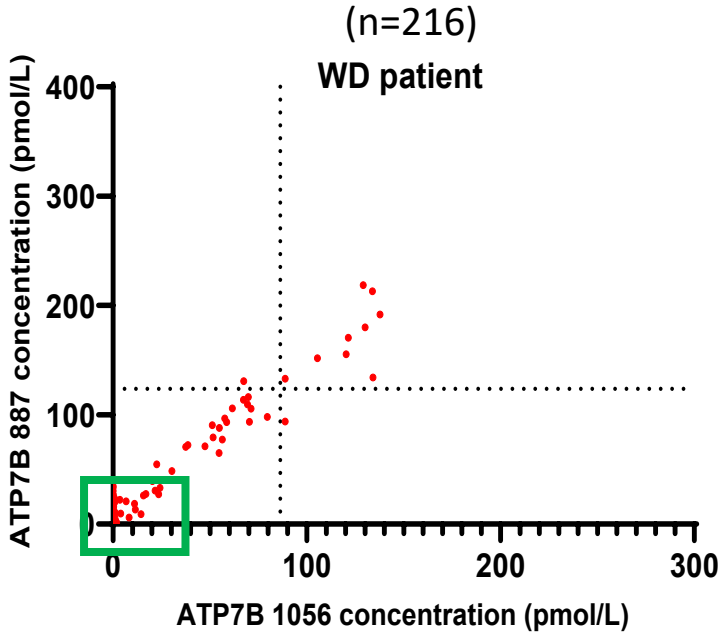


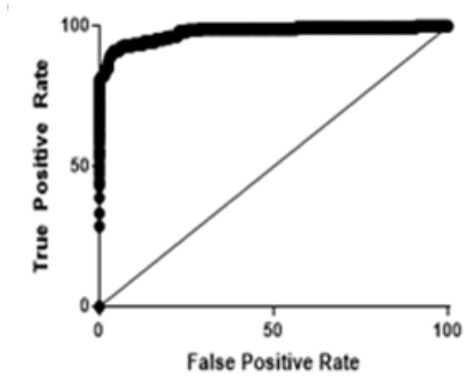
1. *Coffey et al., Brain, (2013)*
2. *Bandmann et al., Lancet Neuro (2015)*
3. *Walshe . Mov Disord. (2007)*
4. *Beinhardt et al, Clini Gastroenterol Hepatol (2014)*

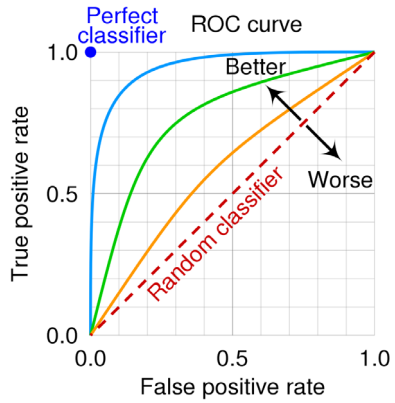
Proof-of-Evidence Study on a Large-Scale Patient Cohort

Wilson Disease: Most patients are deficient in ATP7B peptides







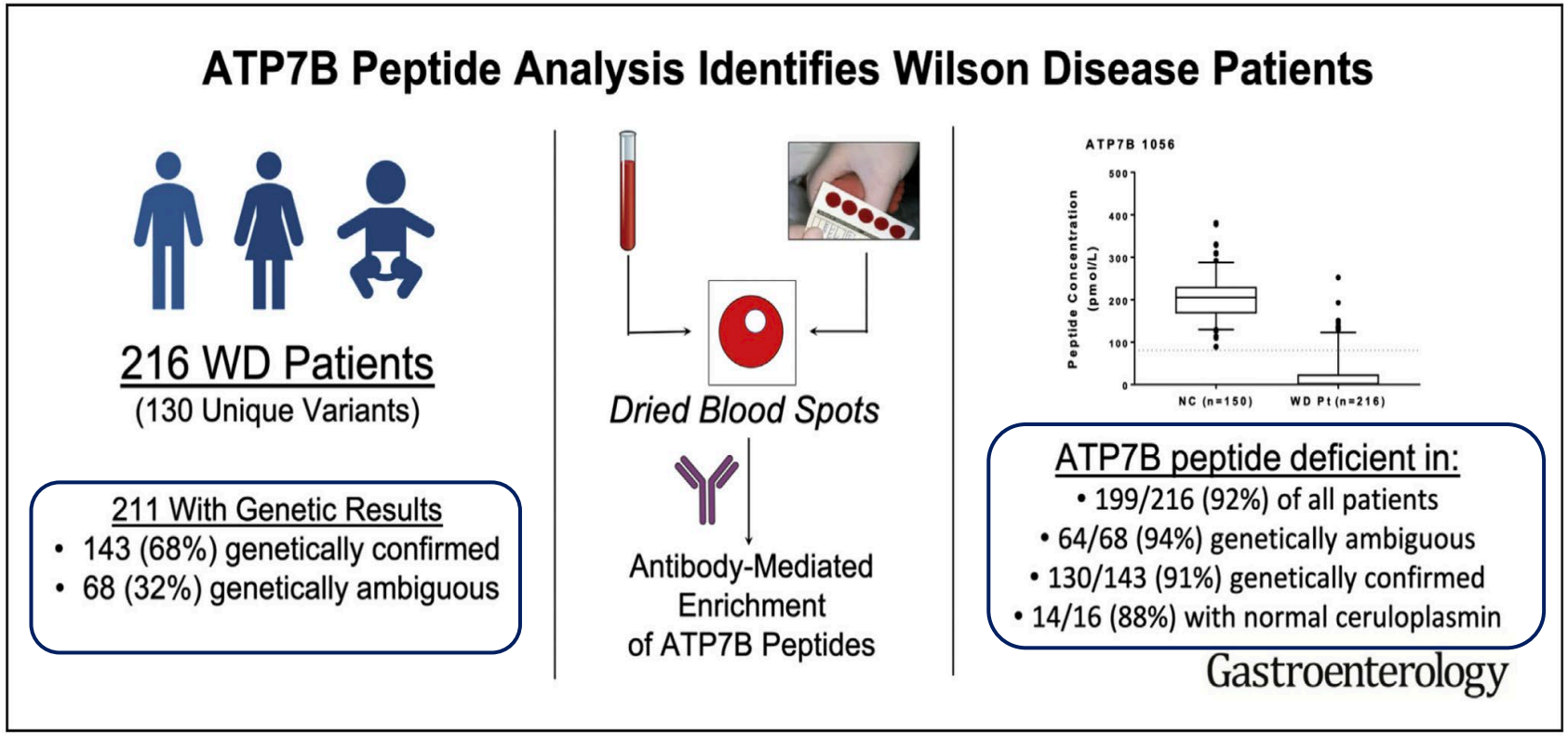


- **Large Retrospective Study**
  - 216 WD patients, 48 carriers,150 healthy controls
  - ATP7B peptides enriched from DBS
- 199 / 216 patients (92.1%) had at least 1 ATP7B peptide below diagnostic cutoff.
  - ~80% of patients had ATP7B < 32 pmol/L and <56 pmol/L for ATP7B 1056 and ATP7B 887 respectively
- ROC curve shows AUC 0.98, sensitivity 91.2%, specificity 98.1%, PPV 98.0% and NPV 91.5%

Collins C.J. et al. Gastroenterology. (2021)

Wilson Disease: Direct measurement of ATP7B peptides reduces clinical ambiguity

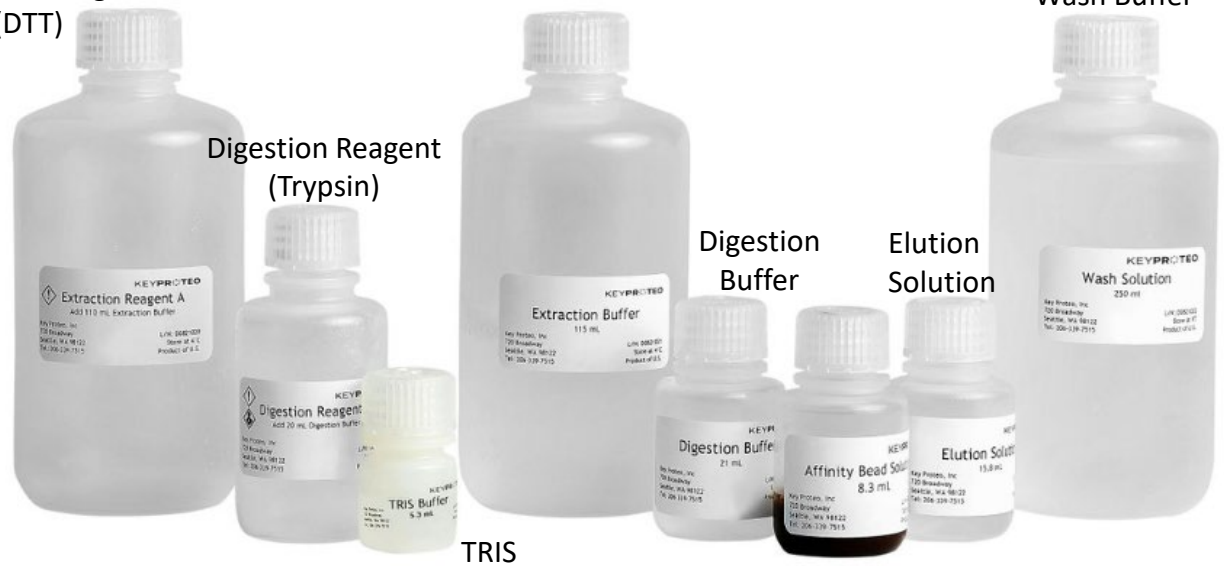
- *Collins C.J. et al. Gastroenterology. 2021, 160(7):2367-2382*
- *Editorial comment. Expanding the Diagnostic Toolkit of Wilson Disease with ATP7B Peptides*



Manufactured Kit Validation for Public Health Transition

Public Health Transition: Prototype 4-plex Screening Reagents

- Prototype reagents for assay performance have been manufactured at GMO facility
- Affinity beads mixed into a single solution
- Internal Standard Peptides lyophilized into 96-well plates
- DBS Control Cards
  - 3 levels of DBS spots
  - Negative, Low, High

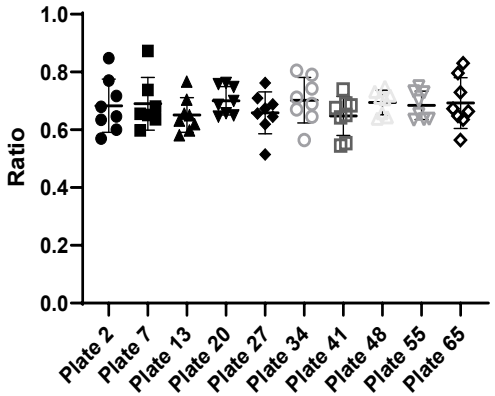


Internal Standard Plates

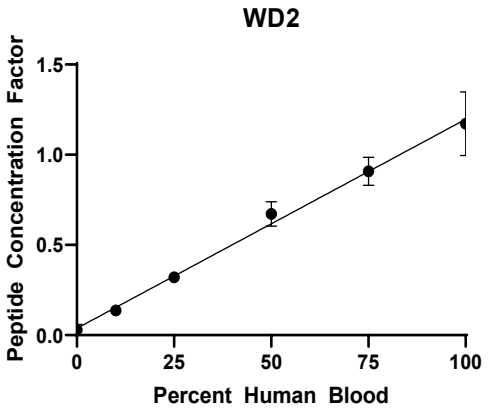
Control Cards

Prototype reagents are not FDA approved and currently intended for investigational use only

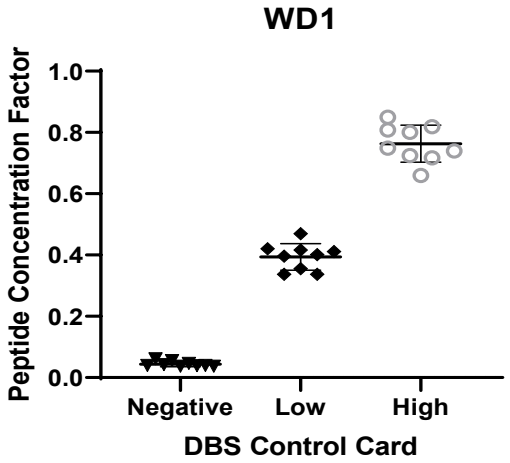
Performance: *Full assay performance and DBS control cards*



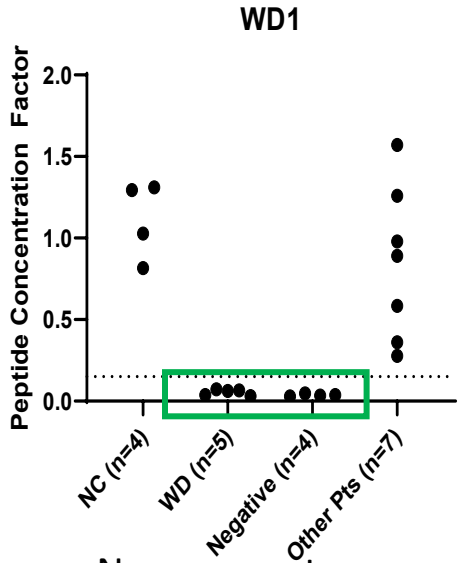
- *IS Plates ensured consistent peptide measurements and maintain consistent cutoff*
  - No dissolution/dilution of IS stock



- *Linear response from decreasing percentage of human blood.*
  - 0, 10, 25, 50, 75 and 100% human



- *DBS control cards perform as expected.*
  - Linear change in measured concentration



- New reagents, workflows, and analysis positively identify blinded patient samples.

Pilot Study in WA State

WA State Pilot Study

In Q1 2022, a large-scale pilot study began in WA State

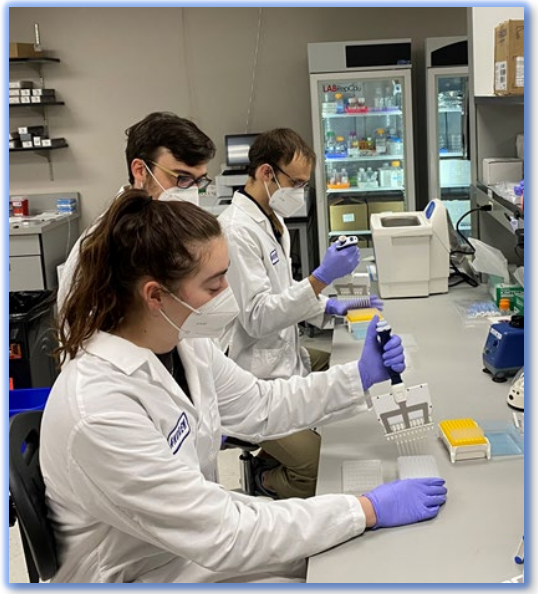
- In conjunction with the ***WA State Public Health Newborn Screening Laboratory***
- De-identified newborn DBS collected through routine course

Multiplexed screening utilizing the Neo-WA kit reagents

- Screening for WD, WAS, XLA, and ADA Deficiency
- 50,000-75,000 newborn samples targeted

Samples below initial cut-offs are sequenced for confirmation

WA PHL beginning KP Pilot



Analyzing KP Pilot Samples

WA Pilot Study of Newborn Screening:
Cut-off as of 7/2024

- Initially, the ionKey Microfluidic Separation Chromatography was used, which was later changed to more affordable low-flow ESI probe assembly fitted with a narrow bore capillary suitable for use with flow rates from 5 µL/min to 100 µL/min

- *Ionkey (plates 1-166)*

	ATP7B 887	ATP7B 1056
MEDIAN	359.8	292.4
SD	149.7	99.2
CV	41.6	33.9
Number	14,607	14,607
CUTOFF	68.4	55.5

- *ESI Low Flow (plates 179-current)*

	ATP7B 887	ATP7B 1056
MEDIAN	272.0	270.2
SD	98.1	90.3
CV	36.1	33.4
Number	10,139	10,141
CUTOFF	76.2	75.7

*cutoff is tentative as for now

WA Pilot Study of Newborn Screening: *Demographics*

- A total of ~25,000 newborns have been analyzed to date (7/2024).

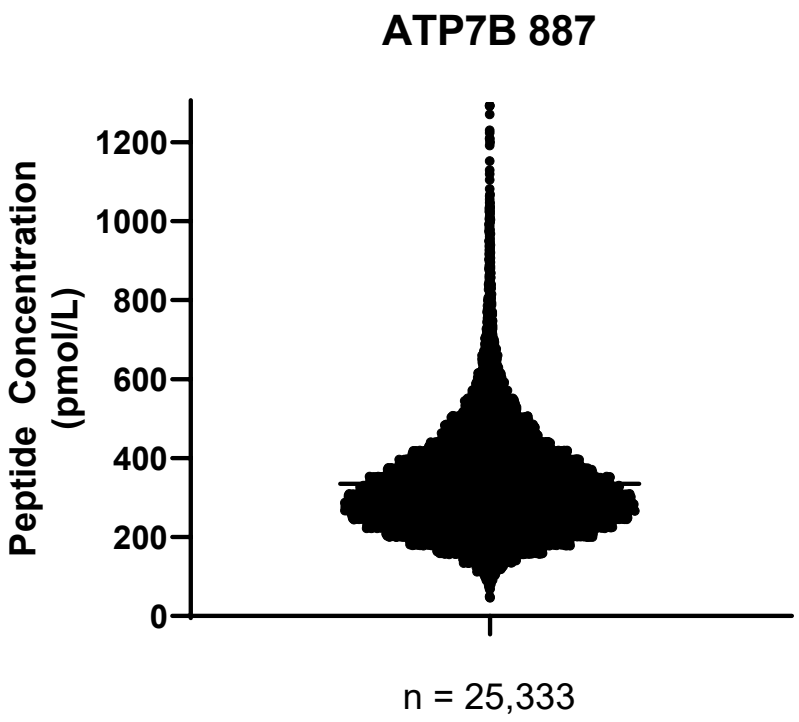
Category	Number
Male	13,035
Female	12,298
< 1500 g BW	258
1500 - 2500 g BW	1,533
2501 – 3500 g BW	13,995
> 3500 g BW	9,547

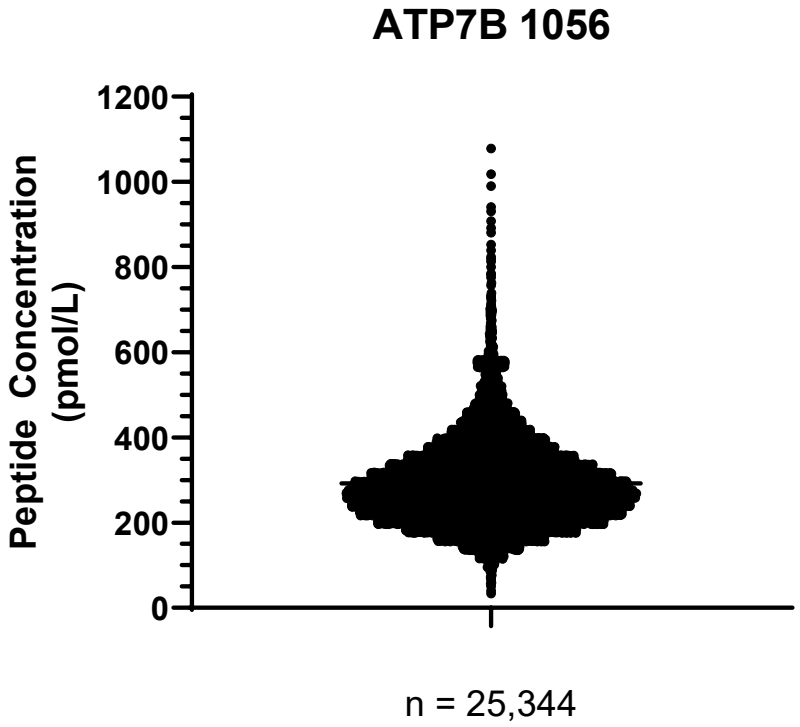
DOC After Birth	Number
0 day (< 24 hours)	4,044
1 day	10,345
2 day	5,608
3 day	3,285
4 day	1,165
5 day	479
6 - 14 days	407

Ethnicity	Number
White	15,603
Hispanic	4,967
Asian	2,813
Black	1,803
Native American	518
Other	910

WA Pilot Study of Newborn Screening: *Peptide Conc.*

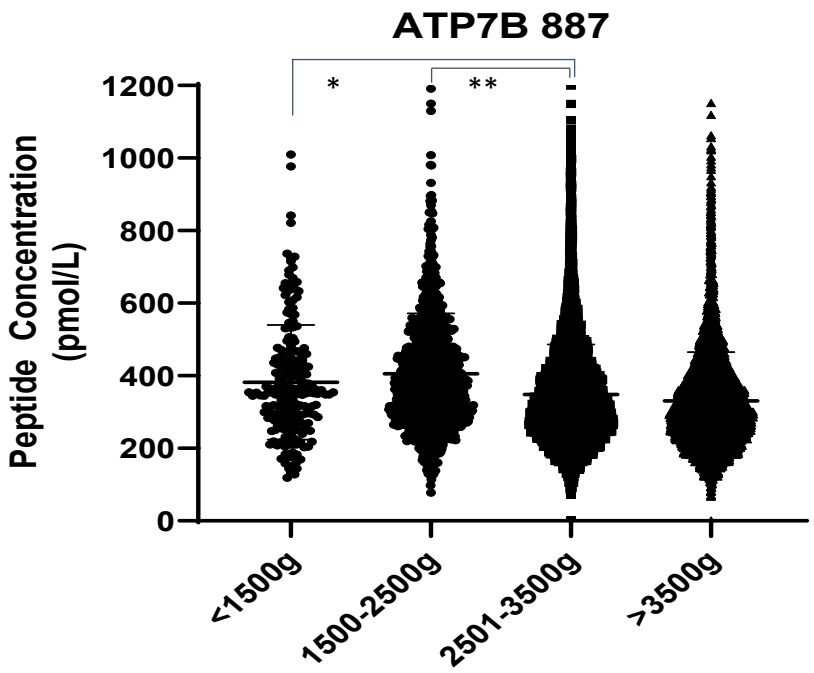
- *Two peptide concentration distributions were identical*

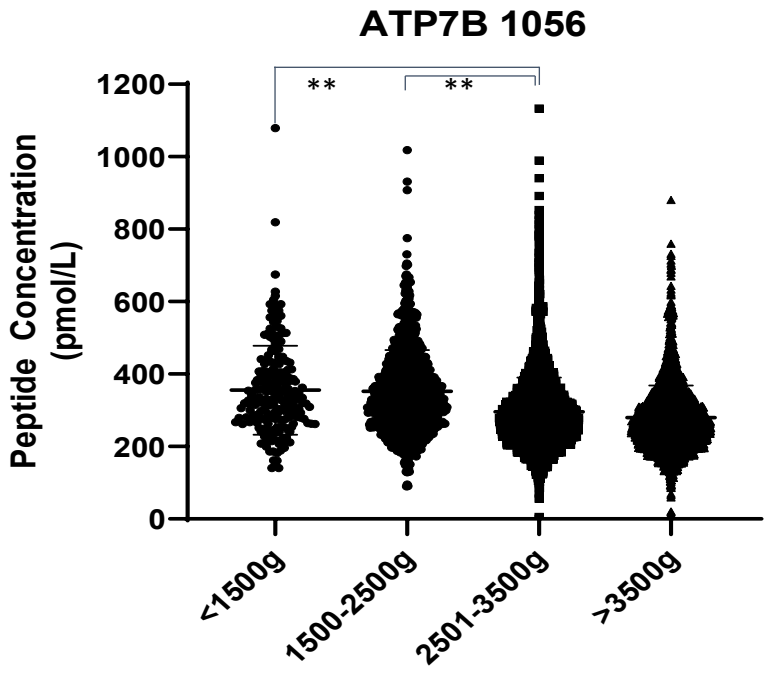




WA Pilot Study of Newborn Screening : *Birth Weight*

- *Peptide concentration was slightly higher in low birth weight but did not impact the overall cutoff range*





*P<0.01
**p<0.001

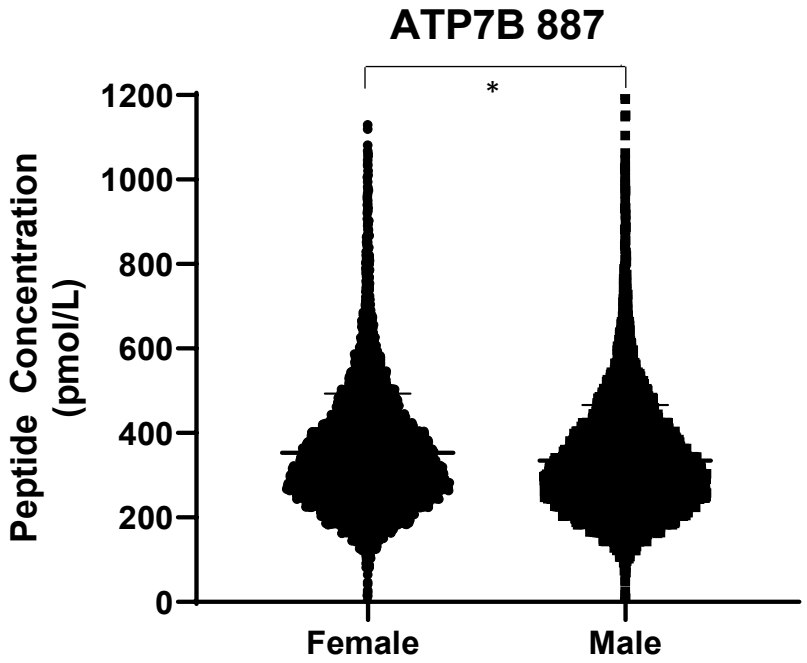
WA Pilot Study of Newborn Screening : *Birth Weight*

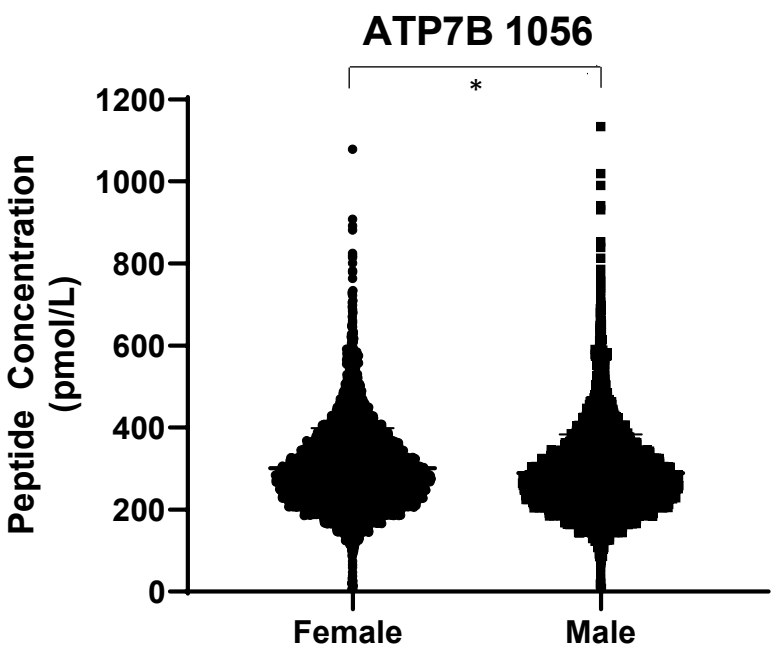
Statistical Analysis

Peptide	<1500g mean (pmol/L)	1500-2500g mean (pmol/L)	>2500g Mean (pmol/L)	p-val <1500g vs >2500g	p-val 1500-2500g vs. >2500g
887	382.0	405.6	341.6	0.0008	<0.0001
1056	355.3	352.4	289.6	<0.0001	<0.0001

WA Pilot Study of Newborn Screening : *Gender*

- *Difference between gender is minimal though statistically significant due to high number of samples tested*





*P<0.001

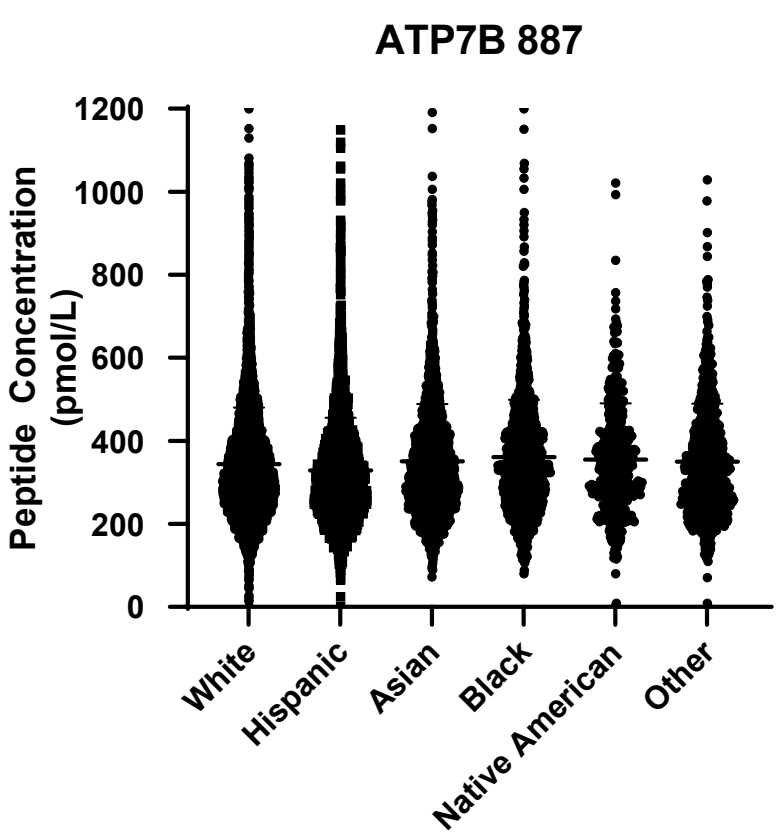
WA Pilot Study of Newborn Screening : *Gender*

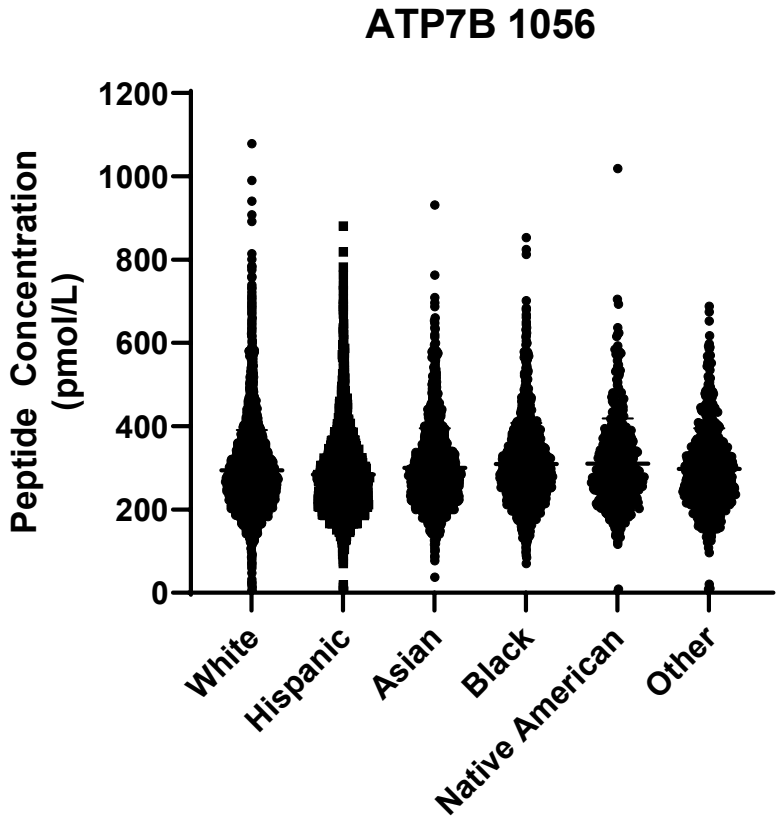
Statistical Analysis

	Female mean (pmol/L)	Male mean (pmol/L)	Difference between means	p-value
887	352.2	334.4	18.4	<0.0001
1056	288.7	301.1	12.4	<0.0001

WA Pilot Study of Newborn Screening : *Ethnicity*

- *No difference in various ethnicities*





Four presumptive positive cases detected

- Please note that current cut-off was tentatively set which can be readjusted in the future
- Two False positive (2/25,000 = 0.008%)
- One likely true positive with two VUS
- One likely true positive DNA pending

	BW (g)	Sex	ATP7B 887 (pmol/L)	ATP7B 1056 (pmol/L)	Genotype	Note
1	3690	M	67.6	134.5	c.3402del/no second variant	carrier
2	4105	M	71.0	96.5	p.Pro610Leu/p.Arg1224Leu	Presumptive Positive
3	3870	M	66.4	70.0	NO VARIANTS	Not affected
4	3840	F	64.3	59.2	PENDING	DNA pending

1: IonKey column (cutoff 68.4 and 55.5 respectively)

2-4: ESI low flow column (cutoff 76.2 and 75.7 respectively)

Summary

- These studies highlight the use of a novel IVD assay demonstrating the feasibility of LC-MS/MS proteomics for NBS of Wilson disease
- The false positive rate is extremely low
- FDA study shows the performance and precision of the manufactured kit were reliable, highly sensitive, and specific for targeted peptides as surrogate markers for ATP7B protein (please see separate PowerPoint slide)
- LC-MS/MS has been adopted globally in clinical laboratories and newborn screening. We anticipate the assay kit can be successfully utilized for clinical/public practice
- Other global feasibility studies are on going:
    - Sardinia (Dr. Georgios Loudianos): 41/44 cases below cutoff (manuscript in preparation)
    - Grand Canary Island, Spain (Drs. Luis García Villarreal/Antonio Tugores)
    - Costa Rica (Dr. Monica Portman Penon)

Acknowledgements

- **Key Proteo, Inc**

  Science Team:

  Claire Klippel, BS
  Jiwoon Park, PhD
  Moumita Dutta, PhD

  Business/Admin Team:

  Sean Sandin, COO
  Mark Willig, CEO

- **Seattle Children's Research Institute**

  Phi Duong, BS
  Tim Grotzer, BS

- **University of Washington**

  Mike Gelb, PhD

- **Washington State NBS Laboratory**

  John Thompson, PhD
  Aranjeet Singh
  Jonathan Hill
  Brandon Officer
  Tareq Shahbal
  Emily Hamacher

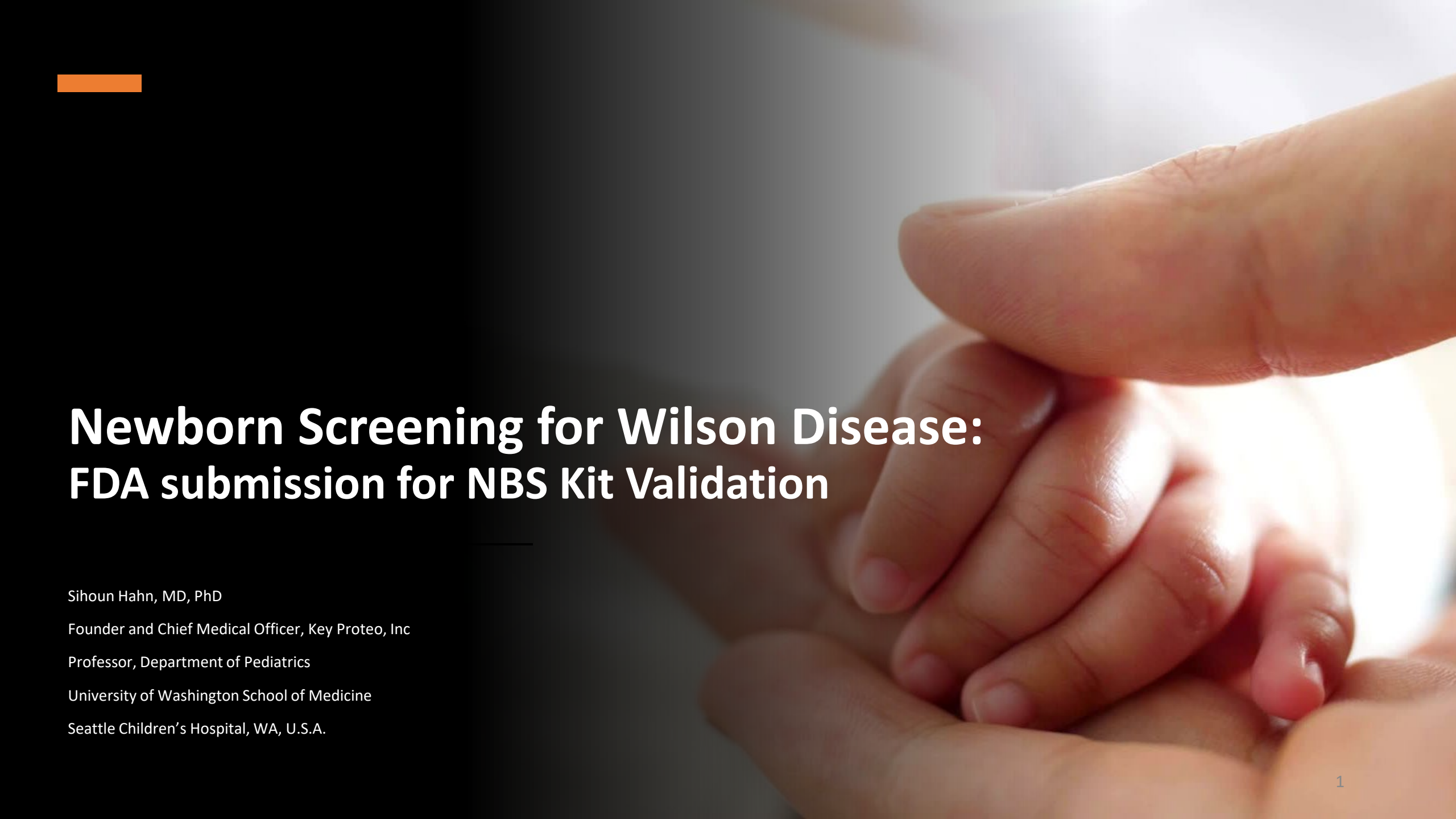
- **Alberta Precision Laboratories**

  Dennis Orton, PhD
  Tara Winstone, PhD
  Xue Chen, PhD

- **Seattle Children's Hospital**

  Hans Ochs, MD

Newborn Screening for Wilson Disease:
FDA submission for NBS Kit Validation



Sihoun Hahn, MD, PhD

Founder and Chief Medical Officer, Key Proteo, Inc

Professor, Department of Pediatrics

University of Washington School of Medicine

Seattle Children's Hospital, WA, U.S.A.

FINANCIAL DISCLOSURES

Sihoun Hahn, MD, PhD, is a member of the Seattle Children's Hospital workforce and is serving as Chief Medical Officer of Key Proteo, Inc. He is an inventor of intellectual property that has been licensed to Key Proteo, Inc. Dr. Hahn is the founder of Key Proteo, Inc. and has ownership equity interests in the company.

Key Proteo Newborn Screening Kit for Wilson Disease

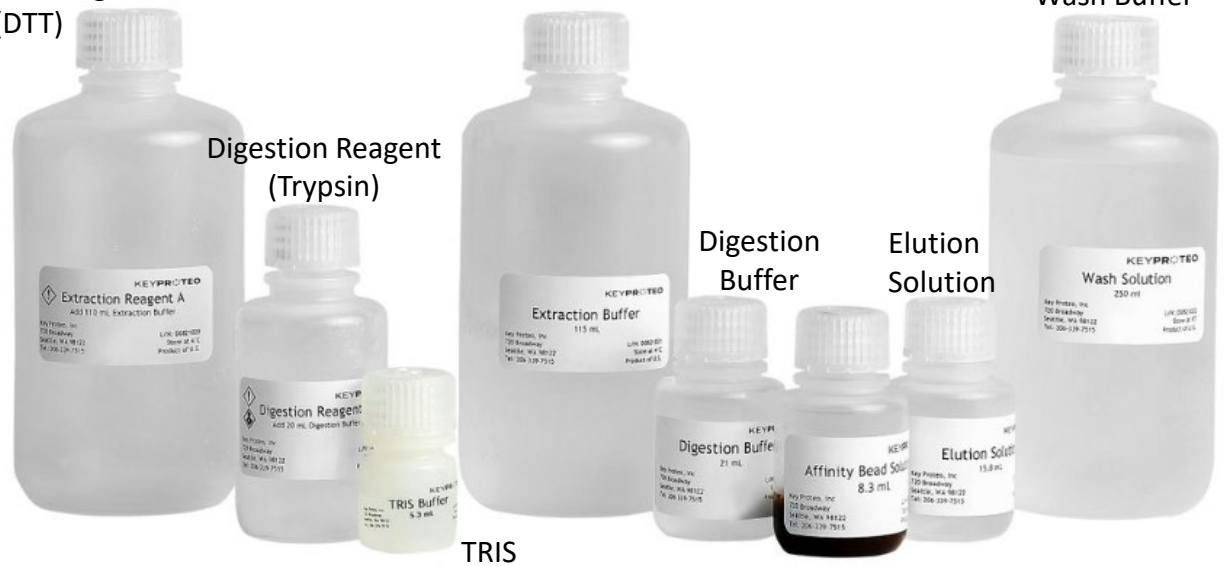
Intended Use:

- The Key Proteo Newborn Screening test is a quantitative LC-MS/MS assay, for **combined and simultaneous detection** of biomarker peptides representing (1) ATP7B, (2) BTK, (3) WASP, and (4) ADA proteins from dried blood spots. The deficiency of these proteins are diagnostic for *Wilson disease*, *X-linked agammaglobulinemia, Wiskott-Aldrich syndrome, and ADA deficiency*, respectively.

- Key Proteo Newborn Screening assay is indicated for use in a population screening of newborns in public health labs as an initial screen for the listed conditions. Those indicated as potential patients would be guided to follow up with specialists in the indicated diseases. This will allow for additional follow up testing as deemed appropriate by healthcare providers to provide or rule out a definitive diagnosis and initiate appropriate pre-emptive treatment.

Public Health Transition: Prototype 4-plex Screening Reagents

- Prototype reagents for assay performance have been manufactured at GMO facility
- Affinity beads mixed into a single solution
- Internal Standard Peptides lyophilized into 96-well plates
- DBS Control Cards
  - 3 levels of DBS spots
  - Negative, Low, High



Internal Standard Plates

Control Cards

Prototype reagents are not FDA approved and currently intended for investigational use only

Analytical Validation Study for FDA

- *Kit was classified as De novo, Class II by FDA: First-of-its kind proteomic-based NBS IVD kit*

Reproducibility/Precision:

The precision studies were performed following the recommendations in the CLSI EP05-A3 guideline at three different laboratories. Repeatability (within-run precision), between-day precision, and reproducibility (between operators) were conducted utilizing a six level multi-analyte reproducibility panel (0,20,40,60,80, and100% human blood).

Study sites 1 and 2 were conducted over 5 days, with 2 plates per day and 3 replicate measurements of DBS samples with identical peptide concentrations by two operators for a total of 360 measurements. Each study site used 1 lot of reagents and 1 instrument.

Study site 3 was conducted over 20 days, with 2 plates per day and 3 replicates per plate by two operators for a total of 720 measurements.

Site to Site Reproducibility Study

- The repeatability and within-laboratory precision of the assay for each multiplexed peptide were determined by replicate measurement of DBS samples with 6 different peptide concentrations created by serial dilution of NHC with fish blood.
- Assessment of within-laboratory precision was done using studies based on the CLSI EP5-A3 document and the modified 20 x 2 x 2 study for internal site and 5 x 2 x 2 study for two external sites.
- The study was conducted using a total of 720 measurements for each multiplexed peptide at the internal site and 180 measurements at each external site. Each kit on the three sites was from three different lots. A two-way nested ANOVA model was used to calculate the site-specific precision estimates.
- Overall, the repeatability, between-day and reproducibility on 100% human sample showed CVs of less than 30% for ATP7B peptides in all three sites.

Peptide	Panel	Mean (pmol/L)	Intra-day (%)	Between Day (%)	Between operator (%)
ATP7B 1056	Panel A	NA	NA	NA	NA
	Panel B	56.0	6.7	6.3	6.2
	Panel C	99.7	11.3	12.1	12.0
	Panel D	168.9	9.9	13.8	13.4
	Panel E	230.9	9.7	12.1	11.8
	Panel F	312.2	12.3	13.6	12.9
ATP7B 887	Panel A	NA	NA	NA	NA
	Panel B	56.8	9.7	11.2	10.6
	Panel C	101.0	13.9	13.8	14.1
	Panel D	154.6	8.3	10.9	10.6
	Panel E	200.5	8.8	11.1	10.6
	Panel F	246.7	11.1	11.0	10.9

Analytical Validation Study for FDA

Analytical Sensitivity:

The limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) for the test kit were determined following the recommendations in the CLSI EP17-A2 guideline with consultation of CLSI document C64 “Quantitative Measurement of Proteins and Peptides by Mass Spectrometry”, involving replicate analysis of a set of blank and low-level samples using multiple lots and a single instrument

Detection Capability (pmol/L)	N	ATP7B 887	ATP7B 1056
Limit of Blank	120	15.5	13.7
Limit of Detection	120	24.5	22.7
Limit of Quantification	180	40.2	49.6

Analytical Validation Study for FDA

Carry-Over:

Carryover was determined by replicate measurements of blank samples with and without carryover for each multiplexed peptide. The blank samples were run followed by a DBS sample with high analyte concentrations for each multiplexed peptide and repeated to evaluate change in concentration due to carryover. The carryover effects of all peptides except ADA 93 were negligible. Based on the results, a specimen with ADA concentration within or slightly higher than the borderline cutoff should be retested if a specimen with ADA concentration within the normal range is present in the same column

Target Peptide	Carryover (%)
ATP7B 887	0.2-4.1
ATP7B 1056	0.9-4.2

Analytical Validation Study for FDA

Interference:

Analytical specificity was determined in accordance with the CLSI EP7-A2 guideline. The interferents tested for the kit are as follows: Hematocrit, Unconjugated Bilirubin, Conjugated Bilirubin, Galactose, Glucose, EDTA, Heparin, Total Protein, Hemoglobin, and Triglyceride. Blood pools were made in both 100% fish blood and a 30% mixture of NHC and fish blood. Test pools were spiked with interfering substances. None of the 11 interferents tested in this experiment had a significant effect on the concentrations of all six peptides at the recommended initial interferent concentration levels (below 20%)

Interference	Recommended Concentration	Unit
Unconjugated Bilirubin	0.4	mg/mL
Conjugated Bilirubin	0.4	mg/mL
Galactose	0.6	mg/mL
Glucose	10	mg/mL
EDTA	3.4	µM
Heparin	3.3	µg/mL
Total Protein	50	mg/mL
Hemoglobin	10	mg/mL
Cholesterol	400	mg/dL
Triglycerides	1500	mg/dL
Gamma-globulin	2.5	g/dL

Analytical Validation Study for FDA

Stability:

Stressed stability test was conducted in collaboration with Nelson Laboratories (Salt Lake City, UT). The study was conducted to determine the stability of screening kit using a classical and real-time approach as described in the CLSI EP17-A2 guideline. On-board stability testing was conducted by replicate panel of eluted samples placed into the instrument sample manager and measuring across time (0, 4, 8, 24, 32 and 48 hours). The kit was stable up to 6 month or even longer at -20'C. DBS stability was determined up to 5 days at three different temperatures. ATP7B peptides were stable at all conditions with no statistically significant differences.

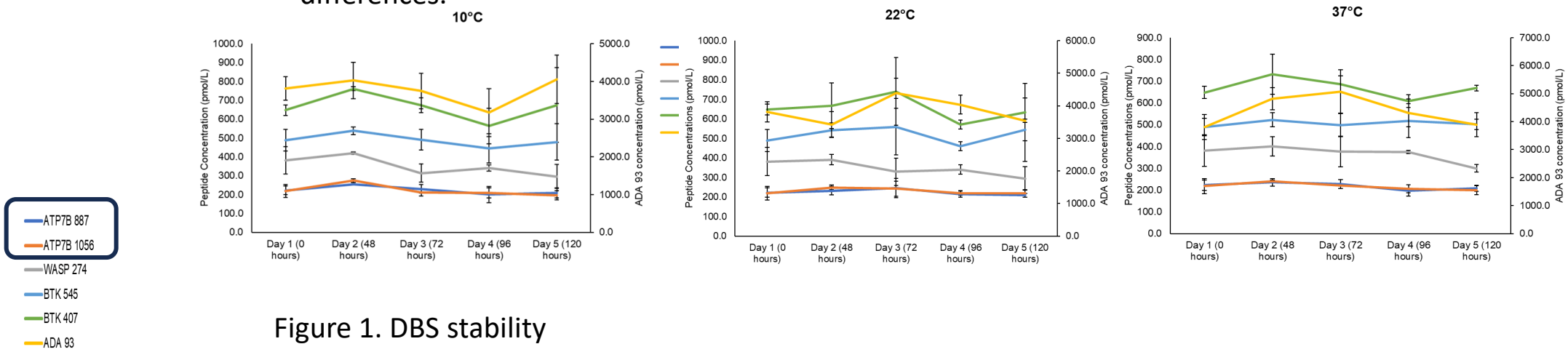


Figure 1. DBS stability

Clinical Validation Study for FDA

- A total of 3,294 newborns and 32 WD patient samples (including retrieved original NBS samples) were blindly tested at three sites (SCH, APL, KP).
  - No presumptive positive cases were detected in presumably normal newborn samples
  - All confirmed 32 positive Wilson disease cases were screened positive, and repeats were concordant with initial results
- To estimate the potential for false negative results of the test, the samples presumed to be normal (total 33 cases above the cutoff out of 3,294 newborns) were sequenced for *ATP7B* gene in a clinical molecular laboratory
  - 10 Carriers: Six (6) VUS and four (4) pathogenic variants. No second variants.
  - One uncertain case: Two variants of VUS were detected in which the peptide result was normal. The clinical significance is yet uncertain.
  - 22 cases with no variants

Additional FLEX studies

- **Upon requests, following FLEX study results can be provided:**
  - DBS punch test
  - Freeze-Thaw cycle test
  - Kit inversion test
  - Temperature/Time/Volume test
  - Hematocrit test
  - Inter-injection variation test

Summary

- FDA study demonstrated that the performance and precision of the manufactured kit was reliable, highly sensitive, and specific for targeted peptides as surrogate markers for ATP7B protein

- These studies highlighted the use of a novel IVD assay demonstrating the feasibility of LC-MS/MS proteomics for NBS of Wilson disease

- LC-MS/MS has been adopted globally in clinical laboratories and newborn screening. We anticipate the assay kit can be successfully utilized for clinical/public practice

Acknowledgements

- **Key Proteo, Inc**

  Science Team:

  Claire Klippel, BS
  Jiwoon Park, PhD
  Moumita Dutta, PhD

  Business/Admin Team:

  Sean Sandin, COO
  Mark Willig, CEO

- **Seattle Children's Research Institute**

  Phi Duong, BS
  Tim Grotzer, BS

- **University of Washington**

  Mike Gelb, PhD

- **Washington State NBS Laboratory**

  John Thompson, PhD
  Aranjeet Singh
  Jonathan Hill
  Brandon Officer
  Tareq Shahbal
  Emily Hamacher

- **Alberta Precision Laboratories**

  Dennis Orton, PhD
  Tara Winstone, PhD
  Xue Chen, PhD

- **Seattle Children's Hospital**

  Hans Ochs, MD

Key Proteo Newborn Screening Kit 1 Product Insert

Newborn Screening Kit 1

Wilson Disease | X-Linked Agammaglobulinemia | Wiskott-Aldrich Syndrome | ADA Deficiency

Reagents and Consumables for 440 specimens

Instructions For Use

Important Notice
Please read these Instructions For Use fully. If you have any questions or comments, please contact Key Proteo Customer Support at support@keyproteo.com or call (+1) 206-339-7515.

IVD
For *In Vitro Diagnostic Use*

Key Proteo, Inc.
720 Broadway
Seattle, WA 98122, USA
www.keyproteo.com

SYMBOLS GUIDE

The following symbols may be found on Newborn Screening product packaging and labeling:

	Manufacturer		*In vitro* diagnostics
	Date of Manufacture		Consult Instructions For Use
	Expiration Date		Customer Support
	Reference Item Number		Temperature Limits
	Lot Number		Do Not Reuse
	Serial Number		Control
	Caution		Keep dry
	Biohazard		Number of Tests
	Keep Away From Sunlight		

Contents

1. Intended Use

The Key Proteo Newborn Screening Kit 1 – WD|XLA|WAS|ADA is intended for quantitative measurement of levels of specific peptides that identify high-risk populations for WD, XLA, WAS, and ADA deficiency, respectively, from newborn dried blood spot (DBS) specimens, using the XEVO-TQXS mass spectrometer as an aid in screening newborns for WD, XLA, WAS, and ADA deficiency. Reduced levels of the measured peptides may be indicative of Wilson's Disease and the inborn errors of immunity (IEI) such as XLA, WAS or ADAD. The peptides measured by the Key Proteo NBS Screening Kit – WA|XLA|WAS|ADA and their associated genetic disorders and IEI are listed below.

Protein (Abbreviation)	Disease (Abbreviation)
Copper-transporting ATPase2 (ATP7B)	Wilson Disease (WD)
Bruton's Tyrosine Kinase (BTK)	X-linked Agammaglobulinemia (XLA)
Wiskott-Aldrich Syndrome Protein (WASP)	Wiskott-Aldrich Syndrome (WAS)
Adenosine Deaminase (ADA)	Adenosine Deaminase Deficiency (ADAD)

2. Indications for Use

The Key Proteo Newborn Screening Kit 1is a quantitative LC-MS/MS assay, for combined detection of ATP7B, BTK, WASP, and ADA proteins from dried blood spots. The deficiency of each of these proteins indicates high risk for Wilson's Disease, X-linked Agammaglobulinemia, Wiskott-Aldrich Syndrome, and ADA deficiency, respectively. The Key Proteo Newborn Screening assay is indicated for use in a population screening of newborns as an initial screen for the listed conditions. Those indicated as potential patients would be guided to follow up with specialists in the indicated diseases. This will allow for additional follow up testing as deemed appropriate by healthcare providers to provide or rule out a definitive diagnosis and initiate pre-emptive appropriate treatment.

Warnings: For *In Vitro Diagnostic Use*

3. Summary and Explanation of the Test

Wilson Disease (WD) is an autosomal recessive copper transport disorder with an estimated incidence of 1 in 30,000 individuals and with a carrier frequency of 1 in 90 [1]. WD is caused by mutations in the *ATP7B* gene, which encodes a copper-transporting ATPase. Copper accumulation in WD is present at birth and continues until toxic levels in the brain and liver produce noticeable symptoms which eventually lead to disease diagnosis. Symptoms usually present in the first decade of life, with most cases occurring between the ages of 5 and 35 years old [2]. Hepatic symptoms predominate in younger patients in their first decade of life, and neurological symptoms occur in older patients in the second or third decade of life [3]. Patients left undiagnosed and untreated during this accumulation phase develop irreversible brain damage, often in the form of Parkinsonism, and require liver transplants due to liver cirrhosis. Early treatment, with either a chelating agent such as Trientine or with zinc salts, has been proven to be highly effective in preventing these negative sequelae and allows patients to live essentially normal lives [4]. Nevertheless, since WD is rare, there is a significant challenge in rapid screening, thus treatment is often started only after the development of severe and permanent complications. Previous pilot studies using ceruloplasmin as a potential biomarker were unsuccessful due to high false positive and negative rates [5].

There are >1300 variants in *ATP7B* reported worldwide [6, 7] (www.varsome.com). Most mutations are rare, and the five most prevalent mutant alleles are estimated to be responsible for ~70% of the disease spectrum [8]. The two most common mutations, p.H1069Q (~35% in the European population) and p.R778L (~30% in the East Asian population) [8-10], result in markedly decreased levels of ATP7B protein, presumably due to enhanced degradation [10-12]. This is in line with observations that disease-causing missense mutations [13-16], protein-truncating nonsense mutations (~13% of known point mutations) [17], and frameshift mutations [8] result in the absence or decay of mRNA [18, 19] and therefore absent or diminished levels of the protein. ***Taken together, it is expected that most patients with WD have an absence or decreased level of ATP7B protein***. Our previous studies have confirmed this protein reduction in patient samples studied thus far [20-23].

Inborn Errors of Immunity (IEI) are a group of over 416 genetic disorders that compromise the health of affected individuals due to an improperly functioning or wholly absent immune system. These conditions often arise from genetic variants that lead to deficiencies in the abundance and/or activity of various proteins critical for immune function in the crucial time between birth and diagnosis. During this time period, serious complications related to IEI can emerge, including profound and frequent infections, autoimmunity, malignancy, and death. Although individually quite rare with varying frequency, the combined prevalence of all IEI is estimated to be about 1 in 1,200 [24-26]. Fortunately, if diagnosed early and accurately, appropriate treatments can dramatically improve, and in some cases, save the lives of the patients [27, 28]. Depending on the disorder, effectively curative methods exist such as hematopoietic stem cell transplantation (HSCT) and gene therapy [28-34]. Undoubtedly, early detection of IEI is essential to manage and prevent potentially life-threatening infections and chronic negative sequelae [35, 36].

(a) X-Linked Agammaglobulinemia (XLA) is *the most common primary immunodeficiency in men*. It is caused by a single genetic defect encoding **Bruton's tyrosine kinase (BTK) protein** and occurs with an incidence of 1:200,000 [37]. Loss of function of this kinase in males leads to a block in B cell development and B cell lymphopenia. Patients are therefore unable to make productive antibody responses and are prone to severe, life-threatening infections. Most patients with XLA who receive immunoglobulin on a regular basis will be able to lead relatively normal lives [27]. They do not need to be isolated or limited in

their activities. For **XLA**, ~60% of patients lack expression of BTK, and another 20-25% demonstrate markedly decreased levels [38, 39]. We have confirmed this reduction in 97% of patients from previous studies [21, 22].

(b) Wiskott-Aldrich Syndrome (WAS) is an X-linked congenital immunodeficiency in which affected males have very small, dysfunctional platelets and consequently have moderate to severe thrombocytopenia and a tendency to have bleeding problems with a combined immunodeficiency caused by defects in **WAS Protein (WASP)** expression [40]. It has an estimated incidence between 1-10 in 1 million [41]. The only permanent cure for WAS is transplantation of stem cells from bone marrow, peripheral blood, or cord blood. Thirty years ago, WAS was a fatal disorder with a life expectancy of only two to three years. Follow-up of the earliest WAS bone marrow transplant recipients for more than 30 years has demonstrated that this therapy is curative [31, 40]. The recent success of gene therapy for WAS holds promise for being the treatment of choice for this disease in the future [42]. In **WAS** patients, ~60% have a complete absence of WASP protein expression and another ~35% demonstrate decreased levels [43, 44]. We have confirmed this reduction in 100% of patients from previous studies [21, 22].

(c) Adenosine Deaminase (ADA) is expressed in erythrocytes, lymphoid cells, and plasma, and is mutated in ADA-deficient $T^-B^-NK^-$ SCID [45]. Inherited ADA deficiency causes a severe form of SCID that may have a somewhat variable phenotypic spectrum, with 10%-15% of patients having a 'delayed' clinical onset presenting by 6 to 24 months, and a smaller percentage of patients having a 'later' onset, diagnosed from age 4 years to adulthood. This form of SCID can be missed by the current SCID NBS method [46]. We have confirmed this reduction in previous studies [21].

4. Principles of Procedure

In the reagent Kit, protein concentrations are analyzed by measuring the amounts of representative peptides that are specific to the targeted protein. An internal standard peptide nearly identical to the target sequence is included at a known concentration and analyzed along with the peptide biomarker. The peptide concentration in blood is then calculated using the ratio of the peptide in the dried blood spots (DBS) to the internal standard.

From each DBS sample, proteins in the specimen are extracted using 50 mM ammonium bicarbonate solution. Disulfide bond reduction is performed using 2 M DTT. Trypsin enzyme is added to initiate digestion of proteins to peptides. Heavy isotope-labeled synthetic peptide internal standards (IS) are incorporated at optimized concentrations for quantification by MS/MS. Target peptides are then captured and enriched using anti-peptide monoclonal antibodies immobilized on magnetic protein G beads. Samples are incubated overnight at 4˚C with shaking. After incubation, anti-peptide MAb beads containing captured peptides are collected using a magnet and washed to remove non-target peptides. Finally, peptides are eluted with 30 µL of 5% acetic acid/3% acetonitrile. Since the internal standard and target peptide are chemically identical, any loss of target peptide (incomplete MAb capture, absorption to vessel walls, etc.) is accounted for. Target peptide-containing supernatant is transferred to a new well for subsequent MS/MS analysis.

LC-MS/MS is performed using a Waters Xevo TQ-XS. LC runs are performed with an elution gradient using H_2O + 0.1 % formic acid and acetonitrile + 0.1% formic acid before column washing and re-equilibration for a total run-time of 2.4 minutes for multiplexed analysis of 12 peptides (six endogenous peptides extracted from DBS and six isotopically labeled IS) covering 4 target conditions. The mass spectrometer is operated in MS/MS mode that allows the first mass-selective filter (quadrupole) to pass the target peptide-derived parent ion. This is followed by collision-induced dissociation of the peptide in the second quadrupole followed by a third quadrupole to allow a specific peptide-derived fragment to pass to the detector (triple-quadrupole MS/MS). This is referred to as single reaction monitoring mode (SRM). A collection of SRMs, one for each target peptide, is cycled rapidly so that all target peptides are detected in the same LC-MS/MS run (multiple reaction monitoring, MRM). The Reagent Kit contains the necessary reagents and consumables for completion of the assay before LC-MS/MS analysis.

5. Kit Contents

Each reagent kit contains sufficient reagents for 440 newborn specimens and quality controls, totaling 1760 tests as each specimen is tested for up to 4 different protein deficiencies. The expiration date for the unopened kit is on the outer label. An expiration date is noted on the labels of the individual kit components. The components are provided in two boxes- one cold (2-8 °C) box containing reagents and one room temperature (15-30 °C) box containing plastics and consumables. The kit components along with their quantity and storage conditions are listed in **Table 1** and **2.**

5.1. Materials Provided:

Table 1. Refrigerated (2-8 °C)/Frozen box contents.

Item	Composition	Delivery State	Number in Kit	Initial Storage	Storage After Initial Use
DBS Calibrator Cards	QC Card: Negative	Dried Blood Spot	1 Card x 5 spots	-20°C	-20°C
	QC Card: Low	Dried Blood Spot	1 Card x 5 spots	-20°C	-20°C
	QC Card: High	Dried Blood Spot	1 Card x 5 spots	-20°C	-20°C
Internal Standard	IS Mix in Deep Well Plate	Lyophilized	5 plates	-20°C	-20°C
Extraction Reagent A	DTT	Solid	1 Bottle x 101.8 mg	2-8°C	Aliquot and store at -20°C
Extraction Buffer	0.1% Triton X-100 in Ammonium Bicarbonate	Liquid	1 Bottle x 115 mL	2-8°C	
Digestion Reagent A	Trypsin	Solid	1 Bottle x 20 mg	2-8°C	Aliquot and store at -20°C
Digestion Buffer	50 mM Acetic Acid	Liquid	1 Bottle x 21 mL	2-8°C	
Tris Solution	1M Tris pH 8.0	Liquid	1 Bottle x 5.3 mL	2-8°C	2-8°C
Bead Mix	In 1X PBS + 0.03% CHAPS	Liquid	1 Bottle x 9.2 mL	2-8°C	2-8°C
Wash Solution	0.1X PBS + 0.01% CHAPS	Liquid	1 Bottle x 250 mL	2-8°C	2-8°C
Elution Solution	H_2O + 5% acetic acid + 3% ACN	Liquid	1 Bottle x 15.8 mL	2-8°C	2-8°C

Table 2. Room temperature (15-30 °C) box contents.

Item	Number In Kit
50 mL Reservior	2
10 mL Reservior	3
10 mL Reservior PPE	1
DWP for Extraction	5
MS Plate	5
Plate Seal	20
MS Plate Seal	5

5.2 Internal Standard Concentrations

Internal standard peptides are utilized to indicate the concentrations of the target peptides in the blood specimen. These internal standards are mixed in the correct ratios and lyophilized into 96-well *Internal Standard Plates*. Each well of the *Internal Standard Plate* contains enough internal standard peptide for 1 experiment. The amounts of each peptide lyophilized into each well is listed below in **Table 3**.

Table 3. Internal Standard amounts in each well.

Peptide	Femtomole Peptide
BTK 407	25
WAS 274	25
ATP7B 1056	5
ATP7B 887	5
BTK 545	50
ADA 93	100

5.3 Calibration

Included in each concentration calculation is a constant factor that calibrates the measured peptide concentration values between lots. This ensures consistency of the measured concentrations between lots and is designed to help maintain consistent cutoffs. The constant value by which the blood peptide/internal standard ratio should be multiplied is provided in a lot-specific quality control certificate.

5.4 Quality Control DBS

The quality control dried blood spots include 3 levels of control material: QC negative, QC low, and QC high. The composition of the quality control DBSs is summarized in **Table 4** below. The peptide concentrations in the QC dried blood spots are provided on a lot-specific quality control certificate included in each assay kit. Each laboratory should establish its own mean and acceptable range.

Table 4. Quality Control Materials.

Quality Control Level	Composition
QC Negative	Fish Whole Blood drawn with Sodium Heparin anticoagulant before spotting
QC Low	A mixture of Normal Human and Fish Whole Blood drawn with Sodium Heparin anticoagulant before spotting
QC High	Human Whole Blood drawn with Sodium Heparin anticoagulant before spotting

5.5 Materials required but not provided:

- DBS puncher
- Personal protective equipment: gown and gloves.
- Adjustable single-channel pipettes: as needed.
- Single-channel pipette tips: as needed.
- Multichannel pipettes: 10 µL, 100 µL, 300 µL, 1200 µL
- Multichannel pipette tips: 10 µL, 100 µL, 200 µL, 1200 µL
- Alpaqua Magnum EX universal magnet plate, A000380 (Beverly, MA) or equivalent.
- KimWipes, Kimberly-Clark, Fisher Scientific. (Chicago, IL) or equivalent.
- Vortex Mixer, Four E's (Amazon, M/N: MI0101002) or equivalent.
- Orbi-Shaker MP, BenchMark Scientific (Genesee Scientific) or equivalent.
    - For use at 15-30 ℃ and 2-8 ℃
- Incu-Mixer MP Plate Shaker, BenchMark Scientific (Genesee Scientific) or equivalent.
    - For use at 37+/-1 ℃
- Centrifuge 5810, Eppendorf (AG, Germany) or equivalent.
    - Capable of centrifuging deep well plates/microtiter plates
- Waters Xevo TQ-XS MS with ESI source and Low-Flow probe connected to Waters M-Class Gradient and Loading pumps, Waters (Milford, MA).
- nanoEase M/Z Peptide Colum; P/N 186009257, Waters (Milford, MA).
- 2x nanoEase loading column; P/N: 186009251; Waters (Milford, MA).
- Acetonitrile (no. A955, LCMS optima grade); Thermo Fisher Scientific (Waltham, MA) or equivalent; Stored at 15-30 °C.
- Water (no. W6, LCMS optima grade); Thermo Fisher Scientific (Waltham, MA) or equivalent; Stored at 15-30 °C.
- Formic Acid (no. A117-50, LCMS optima Grade); Fisher Scientific (Waltham, MA) or equivalent; Stored at 2-8 °C.

6. Warnings and Precautions

- For in vitro diagnostic use.
- For professional use only.
- The Key Proteo Newborn Screening Kit 1 should be used by adequately trained personnel.
- Gloves should be worn whenever working with blood samples. All blood samples, containers, and materials that contact blood should be handled as if capable of transmitting infectious disease and discarded into a biohazard waste container after use.
- This kit contains quality control DBS that were produced using human blood specimens. All quality control DBS were tested for HIV, Hepatitis B, Hepatitis C, and other bloodborne pathogens by FDA-approved or equivalent methods and found to be negative for all infectious agents. However, all recommended precautions for handling blood specimens as specified in the document provided by U.S. Health and Human Services titled "Biosafety in Microbiological and Biomedical Laboratories" should be followed.
- *Internal standard plates, QC dried blood spots, and lot-specific QC reports are all lot specific and should not be mixed between lots.*
- Disposal of all waste should be in accordance with local regulations.
- Ensure that materials from the Key Proteo Newborn Screening Kit 1 are treated as biohazard waste.
- Do not use any reagent kit components after their expiration dates.
- Do not use any damaged reagent kit components.
- Store all reagent kit materials as described in section 5.1, Materials Provided.
- Keep work areas clean per standard practices.
- Wash and dry hands before and after testing. Wear gloves and appropriate PPE.

7. Limitations

- Test results are intended to be used in conjunction with clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, and clinical evaluation as appropriate.
- Reduced or absent protein concentrations must be confirmed by standard diagnostic methods, such as gene sequencing.
- This test is a screening test and is not intended to diagnose the genetic conditions listed herein.
- As with other screening tests, negative results do not rule out the diseases being screened.
- Conditions that are known to cause anomalous results are[47]:
    - Specimen spot not uniformly saturated with blood.
    - Specimen spot punched too close to the edge of the blood spot.
    - Poorly collected and improperly dried specimens.
    - Non-eluting blood spot due to deterioration of specimen caused by exposure to heat and humidity.
    - NBS samples collected from premature infants or sick/transfused infants may require mandated repeat specimen.
- The Key Proteo Newborn Screening Kit 1 may result in:
    - False positives by detecting carriers for the targeted diseases.
    - False negatives if disease-causing mutations do not affect protein concentration.

    - Please also refer to the Section titled "Procedural Notes".
- The test is specific for the targeted peptides. Other peptides that may be present in the blood do not react in/are not identified by the test.
- Certain regions or demographics may have differences in average protein concentrations or the incidences of false positives and negatives.
- The performance of the Key Proteo Newborn Screening Kit 1will be determined using the procedures provided in these Instructions For Use. Failure to follow these procedures and precautions as directed may alter test performance.
- Improper sample handling (through collection, storage, or transport) may impact results or lead to invalid results.
- This reagent kit cannot identify peptide levels associated with diseases other than those stated in this Instructions For Use.

8. Specimen Collection, Storage, and Handling

Blood specimens should be taken using the standard procedures utilized by public health workflows. Neonatal screening programs differ from one another in the type of specimen required. In the United States, the recommendation is that a blood spot, approximately 12.7 mm (0.5 inch) in diameter, be collected by heel prick and spotted onto filter paper. Blood from a newborn heel prick is usually collected 24-48 hours after birth. However, in some screening programs, the specimen from the neonatal heel prick may be collected 2-6 days after birth. Consult local regulations for appropriate timing and screening specimen collection. The manufacturer recommends following the procedures listed in the CLSI document number NBS01 - A6- "Blood collection on filter paper for newborn screening programs; Approved standard"- Sixth edition (2013)[47] and some important points are mentioned below:

- Ensure that the expiration date of the blood collection card has not passed.
- Wipe the newborn skin with 70% isopropanol and allow the skin to dry.
- Puncture the infant's heel with a heel incision device or a sterile lancet by making a standardized incision of 1.0 mm deep. Ensure that the puncture does not exceed 2.0 mm in depth since a deep incision may cause bone damage in small infants.
- Wipe away the first drop of blood with a sterile gauze pad and allow a large drop of blood to form. Touch the filter paper against the large drop of blood and allow a sufficient quantity of blood to soak through and fill the entire pre-printed circle. Examine both sides of the paper to ensure that the blood uniformly penetrated and saturated the filter paper.
    - o Do not excessively squeeze the puncture since it may cause hemolysis of the specimen or result in a mixture of tissue fluids with the specimen and might adversely affect the assay result.
    - o Do not apply successive blood drops to the same printed circle or already partially dried spots can result in "caking".
- Allow the blood specimen to air dry on a horizontally level, non-absorbent, open surface for at least 3 hours at an ambient temperature, away from direct sunlight. Ensure that the specimens are not stacked to avoid cross-contamination.
- Ensure that the required information on the dried blood spot card is completed which includes:
    - o Last name, First name, Sex, Birth date, Birth weight and Patient identification number
    - o First and last name of mother
    - o Date of specimen collection
    - o Name and address of the submitter
    - o Name and phone number of the physician
    - o Name of the newborn screening program and address
    - o Each card should have a unique serial number
- Follow the basic triple packaging system i.e., blood absorbed into paper, a fold-over flap or inner envelope, and an outer envelope of high-quality paper. Ensure that the local regulation and institutional policies are followed when shipping dried blood spot specimens.
- Transport the dried blood spot specimens within 24 hours of collection unless otherwise directed by the newborn screening laboratory.
- Humidity and moisture are detrimental to the quality of the dried blood spot. Special attention must be paid to the storage and transportation conditions of the dried blood spot specimens. Storage of specimens in an environment with elevated temperatures and humidity may increase the risk of false positive results.

8.1. Target Peptide Stability in Dried Blood Spots

This plan governs the determination of DBS stability for the Key Proteo Newborn Screening Kit 1 before analysis. As this is a multiplexed test, stability was determined for each of the six peptide targets. Assay design involves replicate analysis of a panel of eluted samples and measured across time up to 5 days at 10°C, 25°C, and 37°C. Through this protocol, it was determined how long the DBS samples can be expected to measure consistent concentrations over time at different temperature conditions. Quality control and 100% normal human control blood(NHC) , samples were processed per the IFU. Three (3) replicates of each sample were injected at each subsequent day and change in concentrations over time were monitored.

All six target peptides were stable for up to 5 days at all three different temperatures (10°C, 22°C and 37°C) with no statistically significant differences.

9. Assay Procedure

The Key Proteo Newborn Screening Kit 1 assay procedure is detailed below.
**NOTE*: All frozen and refrigerated reagents, components, and samples must be brought to room temperature (15-30°C) before use.*

9.1. Plate and Specimen Preparation

- Wear nitrile gloves and clean work bench with 70% Ethanol.
- Initialize automated DBS puncher or equivalent for use.
- If using a standard hole puncher, clean standard hole puncher and tweezer by spraying and wiping with 70% EtOH.
- Pre-heat Incu-Mixer MP Plate Shaker or equivalent to 37+/-1°C.
- Punch out controls and samples into wells (1/8-inch punches from filter paper disks).
    - To minimize variation in controls it is advisable to avoid punching solely from the perimeter of the dried blood spot.
    - A recommended punching layout is represented below where the three punches needed for each well can be taken from each of three DBS sections below (**Figure 1**).

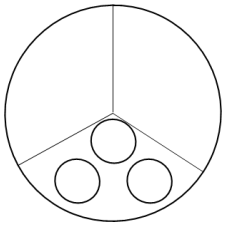


Figure 1. Diagram of recommended DBS punching locations. Three punches can be taken from each of three DBS sections.

Table 5. Recommended Quality Control DBS punch location

A12	IS
B12	IS
C12	QC Negative
D12	QC Low
E12	QC High
F12	QC Negative
G12	QC Low
H12	QC High

- A sample plate layout is shown below in **Figure 2**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	IS
B	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	IS
C	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	QC Neg
D	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	QC Low
E	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	QC High
F	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	QC Neg
G	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	QC Low
H	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	NBS	QC High

Figure 2: Recommended plate layout

- Thaw Extraction Solution aliquots or make Extraction Solution per DBS Protein Extraction protocol below.
- For each DBS sample, punch 3 x 3.2-mm punches (equivalent to ~10.5 µl blood per punch). DBS spots are punched (using Panthera or equivalent) or placed (using tweezers) into 96-well Masterblock Extraction Plate wells.
- Generate Plate Layout document (each sample has a designated well location, i.e. A1, A2…).

8.2. DBS Protein Extraction

- Add 110 mL of Extraction Buffer into bottle containing Extraction Reagent A.
- Invert 10 times to dissolve and mix.
- Transfer resulting Extraction Solution to 50 mL Reagent Reservoir as needed.
- Add 200 µL of this Extraction Solution into each well.
- Seal plate with a new foil plate seal.
- Place the plate into Incu-Mixer MP Plate Shaker or equivalent and incubate at 37+/-1°C for 25-35 min at 1000 RPM.
- NOTE: Store remaining Extraction Solution in 21-ml aliquots in -20°C freezer.
    - To thaw:
        - remove aliquot from -20°C freezer.
        - Thaw to 15-30°C using one of the following options: let sit at 15-30°C, or incubate at 37+/-1°C.

8.3.Trypsin Digestion

- Transfer 20 mL of Digestion Buffer into bottle containing Digestion Reagent A. ***Do Not Vortex!***
- Pipette to dissolve and mix.
- Transfer resulting Digestion Solution to 10 mL Reagent Reservoir.
- Remove plastic plate seal, then add 37.5 µL of Digestion Solution to each well.
- Seal plate with new foil plate seal.
- Incubate mixture on Incu-Mixer MP Plate Shaker or equivalent at 37+/-1°C for 2 hours +/- 10 min to digest.
    - NOTE: Store remaining Digestion Solution in 4-ml aliquots in -20°C freezer.
    - To thaw:
        - remove aliquot from -20°C freezer.
        - Thaw by letting sit at 15-30°C.

8.4. Peptide Enrichment

- Remove plate seal from the Internal Standard Plate.
- Remove foil plate seal from the Extraction Plate, then transfer 200 µL of the resulting DBS digest to its corresponding well on the Internal Standard Plate.
- Remove TRIS Buffer from 2-8°C refrigerator and mix gently by swirling or pipetting up/down.
    - Do Not Vortex.
- Transfer TRIS Buffer into 10 mL reservoir for ease of addition to plate.
- To each sample, add 10 µL of TRIS Buffer.
- Mix for 1-3 minutes on Orbi-Shaker MP at 1000 RPM at 15-30°C.

- Remove Affinity Bead Solution from 2-8°C refrigerator and mix gently by pipetting.
- Transfer Affinity Bead Solution into 10 mL Affinity Bead Reservoir for ease of addition to plate.
- To each sample, add 18 µL of Affinity Bead Solution.

- Seal plate with a new foil plate seal (foil if leftover lyo spots).
- Incubate samples overnight on Orbi-Shaker MP at 1000 RPM at 2-8°C to allow for peptide capture.

8.5. Washing Beads

- The next day, remove foil plate seal from Internal Standard Plate, then place plate on Alpaqua Magnum EX magnetic plate rack and leave for 1-3 min to collect beads.
- Remove Wash Solution from 2-8°C refrigerator and invert 10-15 times to mix.
- Transfer Wash Solution to 50 mL reagent reservoir to aid in transfer to plate.
- Transfer supernatant from beads to corresponding wells in new DWP. Store plate containing transferred digest in -20°C freezer.
- Remove plate from Alpaqua Magnum EX magnetic plate rack.
- Add 220 µL of Wash Solution to each well.
- Pipette up/down 5-10 times to mix.
- Place plate on Alpaqua Magnum EX magnetic plate rack and leave for 1-3 min to collect beads.
- Remove supernatant from beads and discard.
- Remove plate from Alpaqua Magnum EX magnetic plate rack.
- Repeat wash by adding 220 µL of Wash Solution to each well.
- Pipette up/down 5-10 times to mix.
- Place plate on Alpaqua Magnum EX magnetic plate rack and leave for 1-3 min to collect beads.
- Remove supernatant from beads and discard.
- Remove plate from Alpaqua Magnum EX magnetic plate rack.

8.6. Peptide Elution

- Add Elution Solution to 10ml reservoir.
- After discarding the final supernatant, add 30 µL of Elution Solution to each well.
  - NOTE: Pipet up/down 10-20 times (until fully homogenized).
- Seal plate with a new foil plate seal.
- Shake for 4-6 minutes on Orbi-Shaker MP or equivalent at 1000 RPM at 15-30°C to elute peptides.
- Centrifuge in Eppendorf 5810 or equivalent at 600 RPM for 15-25 seconds.
- Remove plate seal, then place plate on Alpaqua Magnum EX magnetic plate rack. Leave for 1-3 min to collect beads.
- Transfer 14 µl of eluted peptide solution into corresponding 96-well MS Plate.
  - Take care not to disturb or pipette the beads collected on the bottom of the well.
- NOTE: If beads are disturbed or aspirated, return beads to well, wait 20-30 seconds to allow to settle, ensure pipet is placed in center of well, then re-attempt aspiration.
- Seal plate with new MW Waters plate seal.
- Centrifuge in Eppendorf 5430 centrifuge or equivalent at 3000 rpm for 25-35 seconds.
- Transfer plate to MS for analysis.

8.7. Liquid Chromatography Conditions and Peptide Transition List

[Liquid Chromatography (LC) Inlet Method]

- The provided Key Proteo Newborn Screening Kit 1 standard LC conditions are below.
- Run time, load time, and LC mode are listed in **Table 6**.

Table 6. Inlet method run time, loading time, and mode.

Run Time (Min)	2.4
Loading Time (Min)	1
Mode	Trapping

- Trapping Conditions for both the gradient and loading pumps are listed in **Table 7**.

Table 7. LC Inlet method trapping conditions.

Trapping - Gradient Pump			
Time (min)	Flow (µL/min)	% A	% B
Initial	8	95	5

Trapping - Loading Pump			
Time (min)	Flow (µL/min)	% A	% B
Initial	40	98	2

- Analytical conditions for both the gradient and loading pumps are listed in **Table 8**.

Table 8. LC Inlet method gradient conditions

Analytical - Gradient Pump			
Time (min)	Flow (µL/min)	% A	% B
Initial	8	95	5
0.1	8	95	5
0.3	8	80	20
0.55	8	80	20
0.95	8	70	30
1.3	8	5	95
1.75	8	5	95
1.9	8	95	5
2.4	8	95	5

Analytical - Loading Pump			
Time (min)	Flow (µL/min)	% A	% B
Initial	40	98	2

- Trap valve manager settings should be set to “Toggle” as shown in **Figure 3**.

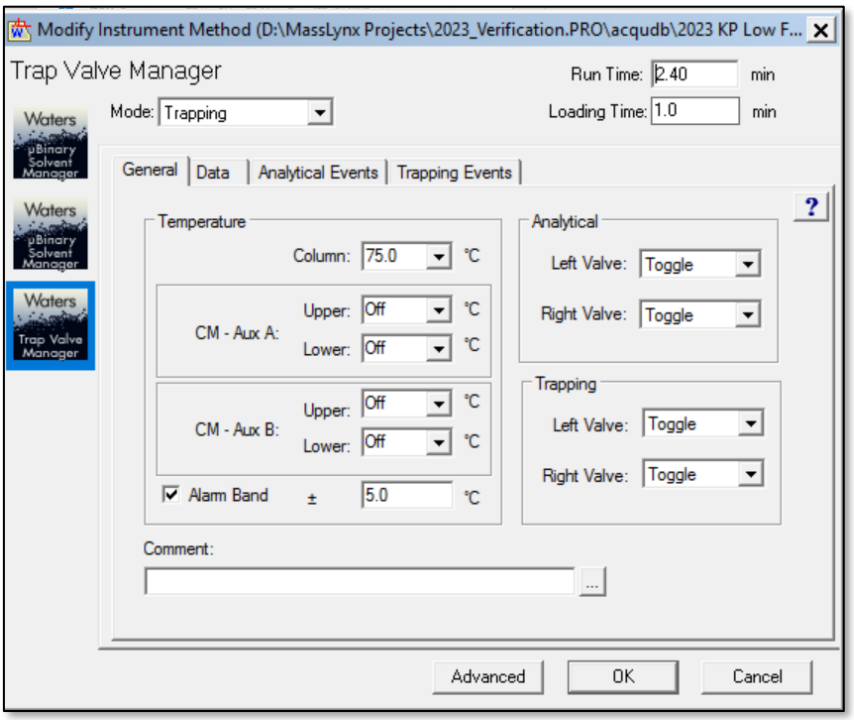


Figure 3: Trap Valve Manager Settings

- µSample Manager settings should be set as in **Figure 4**.

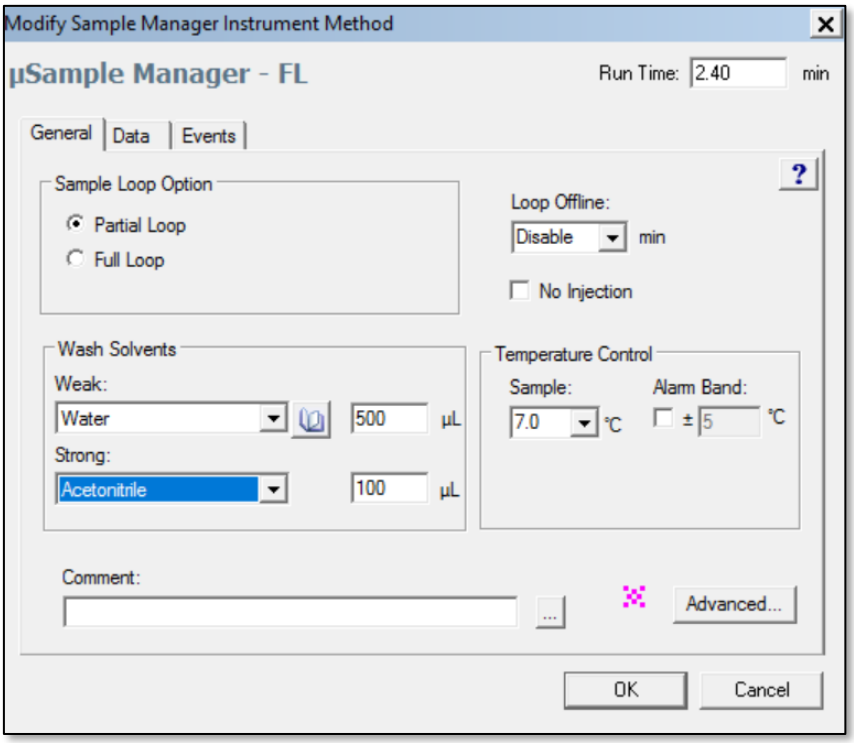


Figure 4: µSample Manager Settings

- Trapping mode advanced settings should be selected as shown in **Figure 5**.

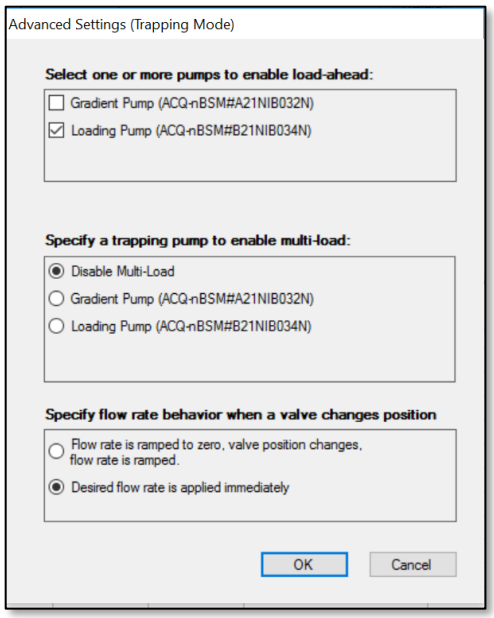


Figure 5. Advanced Trapping Mode Settings

[Mass Spectrometry (MS) Method]

- The provided Key Proteo Newborn Screening Kit 1 standard MS method conditions are below.
- **Table 9** lists the parent ions, fragment ions, cone voltages, and collision energies for each target peptide.

Table 9. Transition information for the Key Proteo Newborn Screening Kit 1 Standard MS Method.

		Parent Mass (m/z)	Fragment Mass (m/z)	Cone Voltage (kV)	Collision Energy (kV)	Fragment
Endogenous (Light)	ATP7B 887 L	523.6	558.2	0	14	y5+
		523.6	671.4	6	14	y6+
		523.6	695.3	0	14	b7+
		523.6	772.6	2	12	y7+
Internal Standard (Heavy)	ATP7B 887 IS	526.3	566.2	0	14	y5+
		526.3	679.4	6	14	y6+
		526.3	695.3	0	14	b7+
		526.3	780.6	2	12	y7+
Endogenous (Light)	ATP7B 1056 L	712.6	827.4	0	18	y17++
		712.6	877.0	0	18	y18++
		712.6	926.5	0	18	y19++
Internal Standard (Heavy)	ATP7B 1056 IS	715.2	831.4	0	18	y17++
		715.2	881.0	0	18	y18++
		715.2	930.5	0	18	y19++
Endogenous (Light)	WASP 274 L	761.0	650.7	8	23	y6
		761.0	751.5	0	22	y7
		761.0	864.6	8	24	y8
		761.0	1063.5	0	24	y10+
Internal Standard (Heavy)	WASP 274 IS	764.9	658.7	8	23	y6
		764.9	759.4	0	22	y7
		764.9	872.4	8	24	y8
		764.9	1071.5	0	24	y10+
Endogenous (Light)	ADA 93- L	525.4	403.6	5	12	y3+
		525.4	502.3	5	14	y4
		525.4	548.3	10	14	b5
Internal Standard (Heavy)	ADA 93 IS	530.5	413.6	5	12	y3+
		530.5	512.3	5	14	y4
		530.5	548.3	10	14	b5
Endogenous (Light)	BTK 545 L	781.8	828.4	0	28	y8+
		781.8	1187.5	0	20	y11+
		781.8	1300.6	0	28	y12+
Internal Standard (Heavy)	BTK 545 IS	785.8	836.4	0	28	y8+
		785.8	1195.5	0	20	y11+
		785.8	1308.6	0	28	y12+
Endogenous (Light)	BTK 407 L	568.1	402.3	2	16	y4+
		568.1	446.8	8	13	y9++
		568.1	892.5	2	16	y9+
Internal Standard (Heavy)	BTK 407 IS	571.9	410.3	2	16	y4+
		571.9	450.8	8	13	y9++
		571.9	900.5	2	16	y9+

[MS Analysis Procedure]

1. Open the MassLynx environment.

2. Open or generate the necessary sample list.
    a. Navigate to File -> Open -> *Select the relevant sample list.*
    b. Alternatively, generate the necessary sample list by entering the information for all analyte and QC samples.

3. Ensure the correct columns are present in the MassLynx Window.
    a. File Name
    b. Bottle/Vial
    c. Injection Volume
    d. Inlet Method
    e. MS File
    f. MS Tune File
    g. Sample Type
    h. ATP7B 887 (CONC A)
        i. If this column is headed with "CONC A", right click the column and use the "Properties" selection to rename the column to the peptide name.
    i. ATP7B 1056 (CONC B)
        i. If this column is headed with "CONC B", right click the column and use the "Properties" selection to rename the column to the peptide name.
    j. WASP 274 (CONC C)
        i. If this column is headed with "CONC C", right click the column and use the "Properties" selection to rename the column to the peptide name.
    k. ADA 93 (CONC D)
        i. If this column is headed with "CONC D", right click the column and use the "Properties" selection to rename the column to the peptide name.
    l. BTK 545 (CONC E)
        i. If this column is headed with "CONC E", right click the column and use the "Properties" selection to rename the column to the peptide name.
    m. BTK 407 (CONC F)
        i. If this column is headed with "CONC F", right click the column and use the "Properties" selection to rename the column to the peptide name.
    n. Quan Meth
    o. File Text
    p. If these fields are not present Right Click on the column heading bar and select "Customize Display". Then select the fields above.

4. Enter Standard Methods for control of MS, LC, and Tune page (**Figure 6**).
    a. Inlet File -> *Standard LC Method*
    b. MS File -> *Standard MS Method*
    c. MS Tune File -> *Standard Tune File*
        i. This file should be generated by Waters upon installation or calibration.

5. Enter Sample Specific Information.
    a. Under Sample Type
        i. Designate QC samples as "QC" using the dropdown menu.
        ii. Designate all other samples as "Analyte"
            1. Use Right Click -> Fill down to rapidly fill menus.

6. Enter Lot Specific QC Information.

a. Concentration values for each peptide will be provided on a lot-specific QC certificate.
b. Values will be provided for all QC DBS cards including Negative, Low, and High controls.
c. Enter these values into rows containing the QC DBS Cards
    i. Be sure to enter the values in the columns corresponding to the correct peptides.

7. Highlight all samples to be run and hit the play button in the toolbar to begin the analysis.
8. In the pop-up window, under operations select the following:
    a. Integrate Samples.
    b. Calibrate Standards
    c. Quantify Samples
9. Under the method dropdown menu select the correct TargetLynx Method.
10. Press OK.

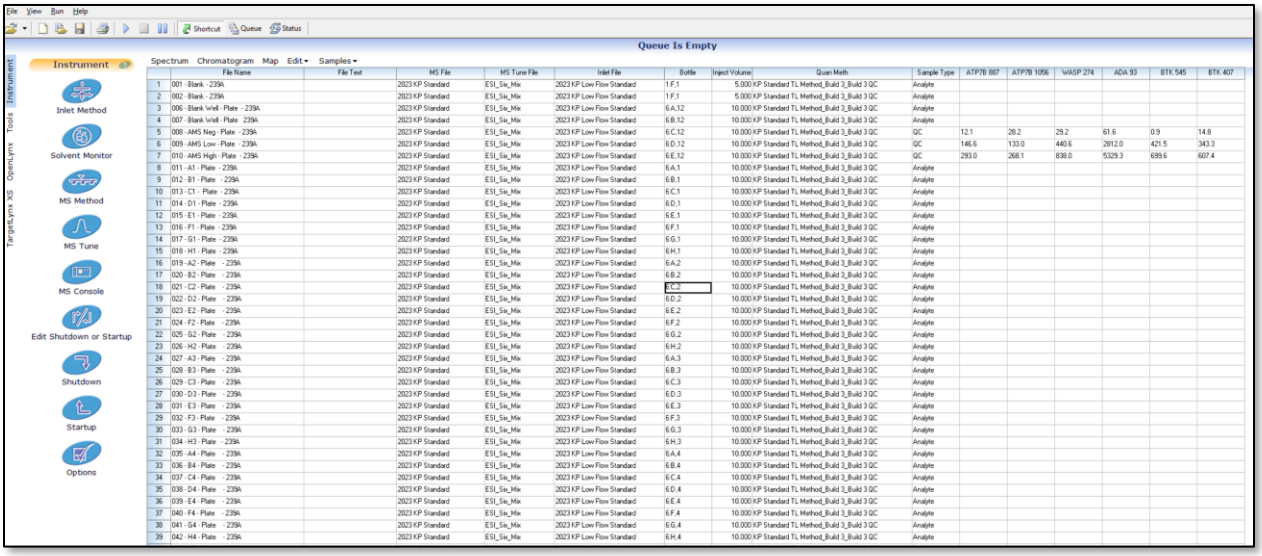


Figure 6. Example MassLynx Sample Layout

8.8. TargetLynx Results Procedure

1. On the left side of the MassLynx window, select “TargetLynx XS”.
    a. Select “Edit Method”.
    b. The Key Proteo Standard TargetLynx Method Editor should open.
    c. If it does not, navigate to File -> Open -> MethDB folder
        i. Select the Key Proteo Standard TargetLynx Method.

2. Set the lot specific Concentration Units for each peptide internal standard (**Figure 7**).
    a. There will be 12 compounds in the compound list.
        i. There will be an internal standard (IS) and an endogenous (light, L) compound for each of the six target peptides.
    b. Above the window on the right, select the “Calibration Properties” tab.
        i. For each IS compound in the compound list, there will be a lot-specific constant to enter under “Concentration of Standard”.
        ii. For each IS peptide, enter the constant value from the QC certificate next to “concentration of standard”.

1. This value incorporates blood volume, IS concentration, dilution, and lot specific calibration to generate a peptide concentration as an output.

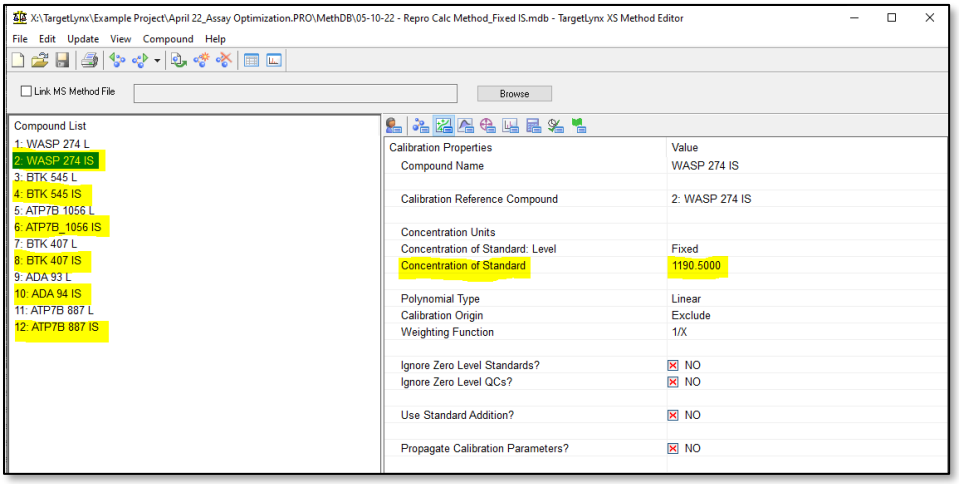


Figure 7. TargetLynx Concentration of Standard

3. Set the lot specific QC settings for each endogenous (Light) peptide (**Figure 8**).
    a. Above the window on the right of the TargetLynx XS method editor, navigate to the "QCMonitor" tab.
        i. For each L compound in the compound list, there will be a lot-specific QC and percent deviation values to enter under "QC Settings".
        ii. For each L peptide, enter the values from the QC certificate.
            1. These values set the allowable deviations from the pre-determined QC card concentrations and enable QC flagging.

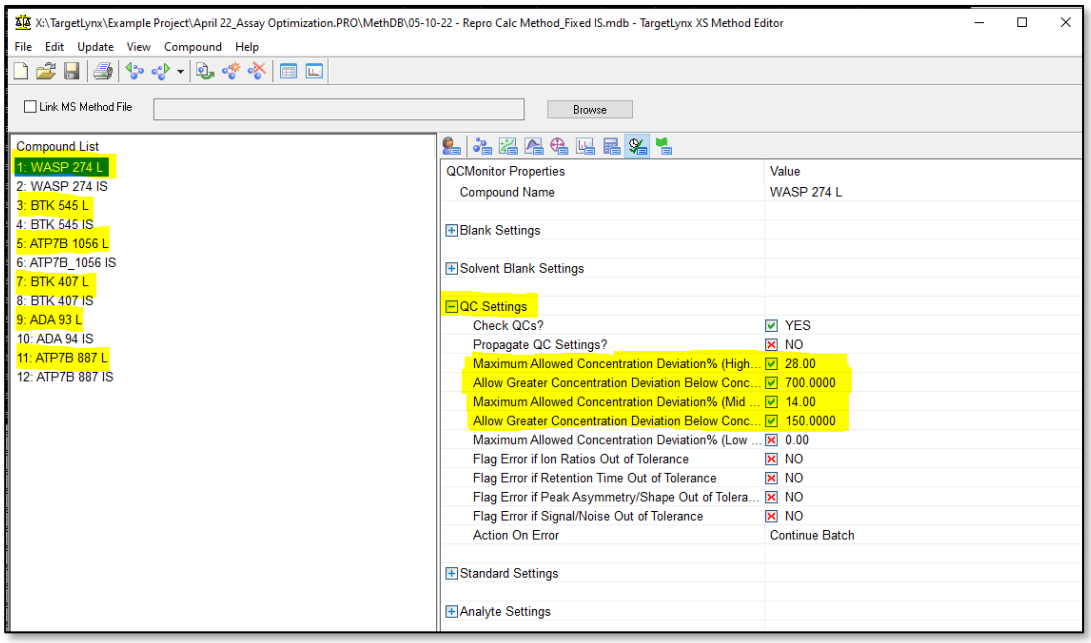


Figure 8. TargetLynx QC Monitor

4. TargetLynx method should now be prepared for sample processing.

8.9. Process Samples After Data Collection

1. Sample processing can be done after data collection.
    a. Open MassLynx software.
    b. Open the sample list that requires sample processing.
    c. Select all sample rows that require processing by clicking and dragging along the leftmost column.
    d. Highlight the “TargetLynx XS” tab along the leftmost side of the MassLynx window.
        i. Select “Process Samples” under the TargetLynx XS menu.
    e. A “Create TargetLynx XS Dataset” window will appear.
        i. Under operations select the following
            1. Integrate Samples
            2. Calibrate Standards
            3. Quantify Samples
        ii. Under the method dropdown menu select the “Key Proteo Processing Method”.
    f. Select OK.

8.10. TargetLynx Reports, Layouts, and Cutoffs

1. After processing, a TargetLynx Quantify MFC application window will open.

2. Two layouts will be provided that will format this TargetLynx window and allow for application of peptide cutoffs.
    a. Each layout will contain cutoffs for 3 peptides.
        i. KP_Layout 1_ATP7B_WASP contains cutoffs for ATP7B 887, ATP7B 1056, and WASP 274.
        ii. KP_Layout 2_ADA_BTK contains cutoffs for BTK 545, BTK 407, ADA 93.

3. Apply a Key Proteo layout.
    a. Select File -> Apply Layout
        i. A MethDB folder window will open.
        ii. Select one of the KP Peptide Layouts.

4. The Key Proteo Newborn Screening Reagent Kit Peptide Layouts will report Areas, Concentrations, Standard Concentrations, Deviations, Concentration Flags, and Cutoffs in columns.
    a. Chromatograms can be reviewed sample by sample.
    b. Deviations from QC concentrations can be monitored.

5. Peptide concentration cutoffs can be changed as needed (Figure 9).
    a. Load the TargetLynx layout that contains the peptide cutoff that needs to be changed.
        i. Right click the column containing the peptide of interest.
        ii. Select “Edit Column Properties”
        iii. Select “Formula”
        iv. A formula will be displayed of the format:
            1. Peak Response<=*[CUTOFF]*?1:0
        v. A formula will be preloaded with a recommended cutoff value e.g.

1. Peak Response<=100?1:0

vi. Changing the number between "=" and "?" will apply a new cutoff.
   1. Change this number
   2. Select "OK".

vii. The column will now flag any peptide with a concentration below the new cutoff with a "1".

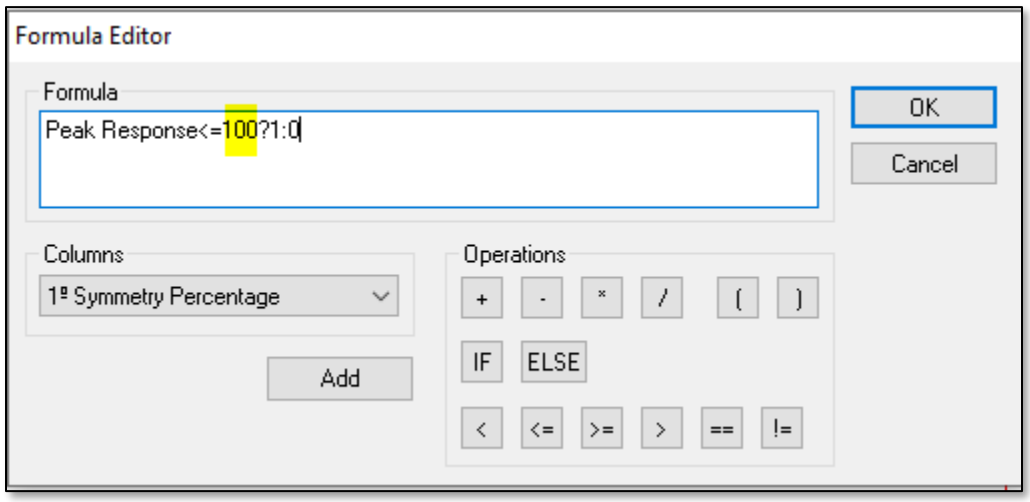


Figure 9: Setting TargetLynx Cutoffs

6. Each Layout can flag 3 cutoffs. Both layouts will be needed to monitor the six target peptides of interest.
7. To Add Concentrations that are generated.

8. Reports will need to be generated from both layouts.

8.11 . Procedural Notes:

- ***Internal standard plates, QC dried blood spots, and lot-specific QC reports are all lot specific and should not be mixed between lots.***

- Store and handle IS plates right-side up as lyophilized internal standard peptide pellets can become dislodged and lyophilized pellet dust can get stuck to adhesive of plate seal.
- The pellet itself can move around and potentially stick to adhesive.
  - If a pellet is loose in the well or stuck to the adhesive, clean forceps can be used to gently move the pellet to base of well to ensure accurate reading of sample.
- Ensure that all Wash Solution is removed from wells before Elution Solution is added.

10. Results and Reporting

10.1 Result Calculation: Target peptide concentrations are calculated using a ratio. The specimen peptide is determined as a specific concentration in the blood. Internal standard peptide is incorporated into the assay at a specific and known concentration. The ratio of the specimen peptide concentration to the internal standard peptide concentration gives the concentration of each peptide in the injected samples. This ratio is then multiplied by a constant value incorporating the assumed blood volume and between-lot calibration factors. Results are reported out in pmol/L of each peptide.

10.1. Run Failure:

10.1.1. Internal standard signals are monitored in wells A1 and A2 where no DBS samples are located. Lack of signal for any internal standard analyte in these wells constitutes a failure of the assay for that specific analyte. The sample run is recommended to be stopped and/or the samples are recommended to be repeated. The results are considered INVALID.

10.1.2. It is possible that an individual sample may experience a loss of chromatographic performance during a run. Any peptide signal presenting as a smear of signal across the gradient time or at a retention time different from the retention time of all other samples in the plate should be considered a failure and reported as INVALID and that specific sample should be re-tested. It is recommended that a third test is run to clarify the result.

10.1.3. Human blood is positive for all target peptides in the multiplex. The fish blood mixture is negative for all target peptides. DBS samples containing different levels of peptide targets will be created by serial dilution of NHC with negative fish blood. QC DBS cards will be processed twice during each run. These values for each individual peptide analyte at each of the low and high concentration levels should fall within the established ± 3 SD limits for the run to be accepted outright. Individual peptide values for the negative QC DBS cards should fall below pre-defined clinical cutoff concentrations to be accepted. Values outside of the established range across all QC samples on a plate will result in failure of a run and need to be retested.

10.1.4. Quality control DBS cards are supplied and must be processed alongside the assay on every plate. These standard deviation ranges are reported on lot specific quality control reports. QC DBS cards will be processed twice during each run. It is recommended that each lab establishes mean and SD values for each peptide and determine their own acceptable range of peptide values for QC samples. Control DBS results should be interpreted based on the laboratory's established criteria for acceptability and any state or federal requirements.

10.1.5. Presumptive positive results should only be reported from plates with passing DBS QC values as established by laboratory's internal guidance.

10.2. Values below LOQ: Values reported below the LOQ of the assay are positive for the targeted disease(s). Samples should be repeated to confirm peptide deficiency and reported as positive for the specific condition.

10.3. Values above Linear Range: Values reported above the linear range of the assay are likely negative for the targeted diseases and should be reported as negative (normal)

11. Quality Control

11.1. Materials: The quality control dried blood spots include 3 levels of control material: QC negative, QC low, and QC high. These spots contain mixtures of human and fish blood with varying concentrations of target peptides.

11.2. Use: It is recommended that QC samples are utilized to monitor the day-to-day validity of testing results. Control DBS at three levels are included in the kit. These controls should be run on each plate in accordance with the assay protocol. If more than one plate is run, these controls should be included on each plate. Peptide concentrations for all 6 target peptides are determined along with the newborn specimens. Lot-specific mean peptide concentration values and standard deviations (SD) will be provided with each Newborn Screening reagent kit. It is recommended that each lab establishes mean and SD values for each peptide and determines their own acceptable range of peptide values for QC samples. Patient results should be reported only if the control results meet the laboratory's established criteria for acceptability.

11.3. External Quality Control: QC testing should be performed in accordance to local, state and federal regulations. It is recommended to participate in external quality assurance programs such as CDC's NSQAP (Centers for Disease Control Newborn Screening Quality Assurance Program). It is also recommended that laboratories monitor trends in peptide concentrations for the QC samples on a weekly basis and compare them with the established limits.

12. Analytical Performance Characteristics:

12.1. Reproducibility/Precision: The precision studies were performed following the recommendations in the CLSI EP05-A3 guideline at three different laboratories. Repeatability (within-run precision), between-day precision, and reproducibility (between operators) were conducted utilizing a six level multi-analyte reproducibility panel (0,20,40,60,80, and100% human blood).

Study sites 1 and 2 were conducted over 5 days, with 2 plates per day and 3 replicate measurements of DBS samples with identical peptide concentrations by two operators for a total of 360 measurements. Each study site used 1 lot of reagents and 1 instrument.

Study site 3 was conducted over 20 days, with 2 plates per day and 3 replicates per plate by two operators for a total of 720 measurements.

Table 10. Site 1 Precision study (N=180)

Peptide	Panel	Mean (pmol/L)	Intra-day (%)	Between Day (%)	Between operator (%)
ADA 93	Panel A	379.6	26.8	24.2	35.2
	Panel B	900.8	6.1	11.2	10.4
	Panel C	1390.1	7.6	11.8	11.2
	Panel D	2263.5	20.8	24.4	24.4
	Panel E	2587.5	10.4	16.1	15.0
	Panel F	3324.0	8.2	14.8	14.0
ATP7B 1056	Panel A	NA	NA	NA	NA
	Panel B	59.1	5.1	8.5	7.9
	Panel C	79.2	11.8	12.9	12.3
	Panel D	117.5	9.9	11.3	10.9
	Panel E	156.0	6.0	9.7	9.5
	Panel F	223.0	9.3	14.6	13.5
ATP7B 887	Panel A	94.8	48.0	63.5	68.8
	Panel B	66.7	14.9	23.6	22.1
	Panel C	109.9	17.0	20.4	20.8
	Panel D	160.8	10.5	11.2	12.8
	Panel E	184.8	7.7	9.5	10.2
	Panel F	248.0	13.5	12.5	12.8
BTK 407	Panel A	53.9	6.8	10.3	10.1
	Panel B	107.3	21.7	23.5	25.5
	Panel C	192.3	34.5	35.2	35.9
	Panel D	268.2	12.5	17.5	18.9
	Panel E	335.3	21.6	21.8	21.8
	Panel F	405.0	11.6	10.9	11.0
BTK 545	Panel A	NA	NA	NA	NA
	Panel B	152.9	3.3	31.7	32.9
	Panel C	238.4	46.8	41.9	42.3
	Panel D	384.8	13.2	16.3	15.4
	Panel E	533.0	24.3	24.8	25.0
	Panel F	707.4	14.5	13.1	13.4
WASP 274	Panel A	190.1	19.3	21.8	20.8
	Panel B	188.7	7.4	34.9	31.4
	Panel C	226.1	9.8	35.3	31.8
	Panel D	279.9	11.0	26.0	24.0
	Panel E	324.5	10.8	26.5	24.1
	Panel F	380.9	10.7	17.1	15.9

Table 11. Site 2 precision study (N=180)

Peptide	Panel	Mean (pmol/L)	Intra-day (%)	Between Day (%)	Between operator (%)
ADA 93	Panel A	337.9	13.6	57.6	55.4
	Panel B	828.6	7.4	11.5	10.8
	Panel C	1387.8	8.2	9.7	9.2
	Panel D	2180.1	5.7	9.0	10.0
	Panel E	2854.8	6.5	9.3	9.5
	Panel F	3780.2	9.9	11.0	10.5
ATP7B 1056	Panel A	NA	NA	NA	NA
	Panel B	56.0	6.7	6.3	6.2
	Panel C	99.7	11.3	12.1	12.0
	Panel D	168.9	9.9	13.8	13.4
	Panel E	230.9	9.7	12.1	11.8
	Panel F	312.2	12.3	13.6	12.9
ATP7B 887	Panel A	NA	NA	NA	NA
	Panel B	56.8	9.7	11.2	10.6
	Panel C	101.0	13.9	13.8	14.1
	Panel D	154.6	8.3	10.9	10.6
	Panel E	200.5	8.8	11.1	10.6
	Panel F	246.7	11.1	11.0	10.9
BTK 407	Panel A	NA	NA	NA	NA
	Panel B	141.8	26.2	27.1	26.5
	Panel C	282.3	15.8	16.2	15.7
	Panel D	441.9	11.2	12.4	12.7
	Panel E	616.0	27.3	24.7	30.0
	Panel F	716.3	24.3	22.6	22.2
BTK 545	Panel A	NA	NA	NA	NA
	Panel B	145.5	21.1	21.0	21.6
	Panel C	299.8	20.0	20.3	19.7
	Panel D	497.1	15.7	16.9	16.7
	Panel E	728.9	28.7	26.9	30.5
	Panel F	865.2	22.7	21.4	20.9
WASP 274	Panel A	NA	NA	NA	NA
	Panel B	126.1	23.9	21.9	21.6
	Panel C	217.3	20.3	21.0	20.5
	Panel D	297.7	14.0	15.8	15.0
	Panel E	397.2	15.6	15.2	14.8
	Panel F	559.7	17.2	16.6	16.7

Table 10. Site 3 Precision study (N=720)

Peptide	Panel	Mean (pmol/L)	Intra-day (%)	Between Day (%)	Between operator (%)
ADA 93	Panel A	391.3	31.7	37.5	47.6
	Panel B	1066.2	22.0	36.0	33.7
	Panel C	1501.3	21.7	25.2	24.0
	Panel D	2224.6	18.7	24.0	25.1
	Panel E	2920.6	15.9	20.9	23.8
	Panel F	4178.2	20.2	31.0	34.6
ATP7B 1056	Panel A	68.0	NA	NA	NA
	Panel B	63.1	18.9	19.2	18.5
	Panel C	80.9	28.0	28.4	27.7
	Panel D	128.4	23.1	25.2	25.9
	Panel E	178.6	21.2	24.4	23.4
	Panel F	259.3	24.7	26.7	25.5
ATP7B 887	Panel A	71.3	36.8	37.8	38.1
	Panel B	92.4	33.0	35.6	37.3
	Panel C	123.7	24.9	26.9	28.1
	Panel D	156.9	20.8	25.5	25.9
	Panel E	197.0	21.5	22.5	21.8
	Panel F	260.6	20.6	23.3	22.3
BTK 407	Panel A	85.9	38.1	49.2	55.0
	Panel B	101.7	38.1	51.7	49.4
	Panel C	191.5	39.9	41.2	40.6
	Panel D	276.8	21.6	23.8	23.6
	Panel E	371.4	21.9	24.7	23.6
	Panel F	482.2	21.3	22.9	23.0
BTK 545	Panel A	166.0	NA	NA	NA
	Panel B	153.3	19.5	22.6	21.5
	Panel C	230.5	38.5	42.4	47.1
	Panel D	360.0	30.6	32.2	31.4
	Panel E	528.1	23.0	24.9	25.8
	Panel F	670.5	24.7	24.3	23.5
WASP 274	Panel A	83.5	50.4	48.3	50.3
	Panel B	114.8	30.1	40.7	38.0
	Panel C	181.8	32.0	33.6	32.2
	Panel D	244.4	21.8	22.6	23.5
	Panel E	321.2	22.6	26.9	26.1
	Panel F	402.7	24.8	26.2	25.7

12.2 Analytical Sensitivity: The limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) for the test kit were determined following the recommendations in the CLSI EP17-A2 guideline with consultation of CLSI document C64 "Quantitative Measurement of Proteins and Peptides by Mass Spectrometry", involving replicate analysis of a set of blank and low-level samples using multiple lots and a single instrument.

The LOB, LOD and LOQ are summarized in **Table 13**.

Table 13. Analytical sensitivity

Detection Capability (pmol/L)	N	ATP7B 887	ATP7B 1056	WASP 274	ADA 93	BTK 407	BTK 545
Limit of Blank	120	15.5	13.7	6.2	77.5	7.5	2.6
Limit of Detection	120	24.5	22.7	23.9	172.7	21.4	13.3
Limit of Quantification	180	40.2	49.6	51.4	197.8	41.2	108.8

5 outliers were identified and removed them before determining the LOD and LOQ, as detailed below:

ATP7B 887: outlier A (z-score 2.4), C (z-score 2.5) and D (z-score 2.2)
ATP7B 1056: outlier A (z-score 2.0) and B (z-score -1.9)
WASP 274: outlier C (z-score 3.0)
BTK545: outlier E (z-score 3.5)
BTK407: outlier D (z-score 3.0) and E (z-score 3.2)

12.3 Linearity: The linearity study was performed following the recommendations in the CLSI EP06 guideline using two lots of reagents. The linearity of the assay was determined by replicate measurements of DBS samples with a range of endogenous peptide concentrations. These specimens consisted of fish blood and normal human control (NHC) blood in percentages from 0-100% NHC. For each peptide, the assay shows linearity for the intervals reported below, with deviations from linearity within 30%.

Table 14. Linearity range

Target Peptide	Linearity Range (pmol/L)
ATP7B 887	269.8-430.8
ATP7B 1056	70.3-451.9
WASP 274	51.4-547.0
ADA 93	197.8-3753.5
BTK 545	108.8-311.4
BTK 407	55.3-1290.7

12.4 Carry-Over: Carryover was determined by replicate measurements of blank samples with and without carryover for each multiplexed peptide**.** The blank samples were run followed by a DBS sample with high analyte concentrations for each multiplexed peptide and repeated to evaluate change in concentration due to carryover. The carryover effects of all peptides except ADA 93 were negligible. Based on the results, a specimen with ADA concentration within or slightly higher than the borderline cutoff should be retested if a specimen with ADA concentration within the normal range is present in the same column.

Table 15. Carry-over

Target Peptide	Carryover (%)
ATP7B 887	0.2-4.1
ATP7B 1056	0.9-4.2
WASP 274	0.1-0.6
ADA 93	5.0-21.2
BTK 545	0.4-2.0
BTK 407	0.0-0.8

12.5 Interference: Analytical specificity was determined in accordance with the CLSI EP7-A2 guideline. The interferents tested for the kit are as follows: Hematocrit, Unconjugated Bilirubin, Conjugated Bilirubin, Galactose, Glucose, EDTA, Heparin, Total Protein, Hemoglobin, and Triglyceride. Blood pools were made in both 100% fish blood and a 30% mixture of NHC and fish blood. Test pools were spiked with interfering substances. None of the 11 interferents tested in this experiment had a significant effect on the concentrations of all six peptides at the recommended initial interferent concentration levels (below 20%).

Table 16. Interference

Interference	Recommended Concentration	Unit
Unconjugated Bilirubin	0.4	mg/mL
Conjugated Bilirubin	0.4	mg/mL
Galactose	0.6	mg/mL
Glucose	10	mg/mL
EDTA	3.4	µM
Heparin	3.3	µg/mL
Total Protein	50	mg/mL
Hemoglobin	10	mg/mL
Cholesterol	400	mg/dL
Triglycerides	1500	mg/dL
Gamma-globulin	2.5	g/dL

The effect of hematocrit was tested by adjusting the amount of red blood cells to 40%, 50%, and 60% following the CLSI EP7-A2 guideline. The samples were constructed from mixtures of human and fish blood product. Human plasma and packed RBCs were mixed as described by CLSI guidelines NBS-04, Appendix F. 0%, 30%, and 100% human blood at 40/50/60% hematocrit was tested. The assay was not subject to interference for samples with low (40%) or high (60%) hematocrit with 30% human blood concentrations. However, the assay was subject to interference for five peptides (ATP7B 1056, ATP7B 887, ADA 93, BTK 407, and BTK 545) in samples in low hematocrit (40%) with 100% human blood concentration.

12.6 Outlier Rate: As detailed in section 11.4, a total of 5 outliers were observed in all the analytical studies from three sites (LOB/LOD/LOQ). Specifically, outliers were observed at a rate of 0.71% for ATP7B 887, 0.48% for ATP7B 1056 and BTK 407, and 0.24% for WASP 274 and BTK 545.

12.7 Stability: Stressed stability test was conducted in collaboration with Nelson Laboratories (Salt Lake City, UT). 3 kits were randomly selected from the manufactured lot and placed into controlled chambers. These kits were taken through a specified stress sequence (40 ˚C, -20˚C, 30˚C, and 2-8˚C) while the remaining the kits in the lot were stored at the recommended conditions of 2-8˚C. Temperature extremes affected ATP7B 887, ATP7B 1056, and WAS 274 concentrations.

The study was conducted to determine the stability of screening kit using a classical and real-time approach as described in the CLSI EP17-A2 guideline. In-use 30 days after open kit testing showed stable for up to 1 month, however the difference bias has increased at 6 months. Real time stability at 4˚C at 3 months and 6 months showed high bias likely due to degradation of IS plate. Frozen stability at -20˚C showed stable peptide concentration through 6 months with low CV and bias. We recommend storing the kit at -20˚C to prevent the internal standard from degrading over time and the properly stored kits are stable up to 6 months.

On-board stability testing was conducted by replicate panel of eluted samples placed into the instrument sample manager and measuring across time (0, 4, 8, 24, 32 and 48 hours). Differences in the six peptide concentrations across all time points were below 25%.

13 Key Proteo Newborn Screening Clinical Validation Study and Cutoff Determinations

12.1. Clinical Sensitivity
Not applicable

12.2. Clinical Specificity
Not applicable

12.3. Other Clinical Supportive Data

12.3.1. To establish the initial cutoff values, Key Proteo performed a preliminary study prior to the clinical study by analyzing 1,000 presumed normal de-identified samples and 12 positive samples. The cutoffs were selected to ensure that all known affected specimens would be detected and to minimize the false positive rate. The cut-off for ATP7B 887, 1056 was set for 25% of median values in pmol/L, 20% of median values in pmol/L for ADA 93 and the rest, BTK 407, 545 and WASP274 were set for 10% of median values in pmol/L. If the target peptide concentration was lower than cutoff, retest on remaining sample was performed. Only male newborns were reported for WAS and XLA test results. All positive samples were sequenced.

The initial cutoff used at the start of the study is shown in **Table 17**. The average peptide concentrations were not affected by the age of sample collection up to 7 days or gender for WASP274, BTK545 and BTK407.

Table 17. Initial proposed cutoff

	ATP7B 887	ATP7B 1056	WASP 274	ADA 93	BTK 545	BTK 407
Initial	66.6	67.5	226.4	1145.6	156.4	112.2

12.3.2. The screening performance of the kit was determined in a prospective clinical study of routine newborn screening samples and previously confirmed positive patient samples which have been saved in the biorepository at -20C for WD, XLA, WAS and ADAD. Six of them were original newborn blood spots from affected patients. A total of 3,294 newborns and 49 genetically confirmed positive samples were tested at three sites. No presumptive positive cases were detected at the three sites.

Table 18. Clinical study cohort

Site	NBS samples	Screen Negative	False Positive rate
1	601	601	0
2	601	601	0
3	2092	2092	0

Table 19. Blinded patient study cohort

Site	Patient samples	Screen Positive	False Negative rate
1	15	15	0
2	14	14	0
3	20	20	0

All confirmed 49 positive cases were screen positive, and repeats were concordant with initial results. Four confirmed Wilson disease cases had ATP7B 887 level above the cutoff while ATP7B 1056 level were below the cutoff. Two Wilson disease patients had ATP7B 1056 level above the cutoff but ATP7B 887 level were below the cutoff. In one confirmed case of Wilson disease, WAS 274 level was below cutoff. Sequencing showed the variant of uncertain significance.

Table 20. Confirmed patient cohort

Site	WD	XLA	WAS	ADAD	Total
1	10	2	2	1	15
2	9	3	1	1	14
3	14	3	2	1	20

12.3.3. To estimate the potential for false negative results of the test, the samples presumed to be normal were evaluated using next generation sequencing in a clinical molecular laboratory. Specimens included 20% or higher of the median values in pmol/L and were pulled from three different sites. (A total of 100 newborn samples).

ATP7B gene: Heterozygous genetic variants of uncertain significance (VUS) were detected in six routine samples and heterozygous pathogenic variants were detected in two routine samples that were screened negative. The second variants were not detected in those samples. In one routine case, two variants of uncertain significance were detected in which the screen was negative. The clinical significance is yet uncertain.

ADA gene: Three heterozygous variants of uncertain significance and one heterozygous pathogenic variant was detected that were screen negative. The second variants were not detected in all these cases.

BTK gene: One variant of uncertain significance was detected in a case that was screened negative. The clinical significance is yet uncertain.

12.4. Interpretation of Results

12.4.1. Applying cutoff and retesting

- Specimens with initial test results above the cutoff for all six peptides were presumed negative (or normal). No additional test was taken.
- Specimens below initial cutoff was retested and reported presumed positive when the target concentration was still below the cutoff.
- For XLA and WAS, the specimens from females were not reported regardless of the test results.

- The specimen in which two or more target peptides were below cutoff was considered potentially poor-quality sample.

13. **Expected Values and Interpretation of Results**

Please note that the values described in this insert should be used as a guideline only and each laboratory should establish their own cutoffs for their own populations.

- The measurement of target peptide concentrations for WD, XLA, WAS, and ADAD from the dried blood spots specimens is performed by using a cutoff concentration that distinguishes between presumed affected and presumed normal newborns. Each laboratory should follow its own procedures for establishing cutoffs. For the diseases targeted by the Newborn Screening kit, specimens with peptide concentrations below the cutoff are highly likely to have the specific diseases and should be referred for diagnostic follow-up.

- It is recommended that each laboratory establish cutoffs per laboratory guidelines and any applicable local, state, and/or federal requirements. It is recommended to include as many true positive samples as possible to estimate the cutoff values with higher confidence levels.

- The cutoffs should be selected to ensure that all known affected samples will be detected and to minimize the false positive rates, keeping in mind the expected incidence of the diseases.

- As larger numbers of samples are screened and presumed positive results are obtained, cutoffs should be reviewed in consultation with specialists who can provide additional guidance based on incidence rates, disease severity, and typical profiles of known positive patients. It is also recommended that the performance of the test be monitored for seasonal variability (for example by monitoring daily or weekly medians). If a laboratory chooses to apply a two-tiered cutoff, it is recommended that a borderline cutoff value for each analyte be set above the cutoff.

- Samples with results above the cutoff for all target peptides are considered low risk and should be presumed negative (normal). Local regulations and guidelines should be followed for the handling and reporting of presumed normal results. Samples that fall below the cutoff should be retested for confirmation. If the repeated sample results are different from the first result, repeat test for a third time for clarification.

14. **Appendices**

15. References

1. Ala, A., et al., *Wilson's disease.* Lancet, 2007. **369**(9559): p. 397-408.
2. Lin, L.J., et al., *Comprehensive analysis on clinical features of Wilson's disease: an experience over 28 years with 133 cases.* Neurol Res, 2014. **36**(2): p. 157-63.
3. Wiernicka, A., et al., *Early Onset of Wilson Disease: Diagnostic Challenges.* J Pediatr Gastroenterol Nutr, 2017. **65**(5): p. 555-560.
4. Beinhardt, S., et al., *Long-term outcomes of patients with Wilson disease in a large Austrian cohort.* Clin Gastroenterol Hepatol, 2014. **12**(4): p. 683-9.
5. Hahn, S.H., *Population screening for Wilson's disease.* Ann N Y Acad Sci, 2014. **1315**: p. 64-9.
6. Schilsky, M.L., *Wilson disease: current status and the future.* Biochimie, 2009. **91**(10): p. 1278-81.
7. Kenney, S.M. and D.W. Cox, *Sequence variation database for the Wilson disease copper transporter, ATP7B.* Hum Mutat, 2007. **28**(12): p. 1171-7.
8. Vrabelova, S., et al., *Mutation analysis of the ATP7B gene and genotype/phenotype correlation in 227 patients with Wilson disease.* Mol Genet Metab, 2005. **86**(1-2): p. 277-85.
9. Shah, A.B., et al., *Identification and analysis of mutations in the Wilson disease gene (ATP7B): population frequencies, genotype-phenotype correlation, and functional analyses.* Am J Hum Genet, 1997. **61**(2): p. 317-28.
10. van den Berghe, P.V., et al., *Reduced expression of ATP7B affected by Wilson disease-causing mutations is rescued by pharmacological folding chaperones 4-phenylbutyrate and curcumin.* Hepatology, 2009. **50**(6): p. 1783-95.
11. de Bie, P., et al., *Distinct Wilson's disease mutations in ATP7B are associated with enhanced binding to COMMD1 and reduced stability of ATP7B.* Gastroenterology, 2007. **133**(4): p. 1316-26.
12. Payne, A.S., E.J. Kelly, and J.D. Gitlin, *Functional expression of the Wilson disease protein reveals mislocalization and impaired copper-dependent trafficking of the common H1069Q mutation.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10854-9.
13. Waters, P.J., *Degradation of mutant proteins, underlying "loss of function" phenotypes, plays a major role in genetic disease.* Curr Issues Mol Biol, 2001. **3**(3): p. 57-65.
14. Arredondo-Vega, F.X., et al., *Adenosine deaminase deficiency: genotype-phenotype correlations based on expressed activity of 29 mutant alleles.* Am J Hum Genet, 1998. **63**(4): p. 1049-59.
15. Voskoboinik, I., M.C. Thia, and J.A. Trapani, *A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis.* Blood, 2005. **105**(12): p. 4700-6.
16. Lappalainen, I., et al., *Genome wide analysis of pathogenic SH2 domain mutations.* Proteins, 2008. **72**(2): p. 779-92.
17. Merle, U., et al., *Truncating mutations in the Wilson disease gene ATP7B are associated with very low serum ceruloplasmin oxidase activity and an early onset of Wilson disease.* BMC Gastroenterol, 2010. **10**: p. 8.
18. Mendell, J.T., et al., *Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise.* Nat Genet, 2004. **36**(10): p. 1073-8.
19. Chang, Y.F., J.S. Imam, and M.F. Wilkinson, *The nonsense-mediated decay RNA surveillance pathway.* Annu Rev Biochem, 2007. **76**: p. 51-74.
20. Collins, C.J., et al., *Direct Measurement of ATP7B Peptides Is Highly Effective in the Diagnosis of Wilson Disease.* Gastroenterology, 2021. **160**(7): p. 2367-2382.e1.
21. Collins, C.J., et al., *Multiplexed Proteomic Analysis for Diagnosis and Screening of Five Primary Immunodeficiency Disorders From Dried Blood Spots.* Front Immunol, 2020. **11**: p. 464.
22. Collins, C.J., et al., *Rapid Multiplexed Proteomic Screening for Primary Immunodeficiency Disorders From Dried Blood Spots.* Front Immunol, 2018. **9**: p. 2756.

23. Jung, S., et al., *Quantification of ATP7B Protein in Dried Blood Spots by Peptide Immuno-SRM as a Potential Screen for Wilson's Disease.* J Proteome Res, 2017. **16**(2): p. 862-871.
24. Tangye, S.G., et al., *Human Inborn Errors of Immunity: 2019 Update on the Classification From the International Union of Immunological Societies Expert Committee.* Journal of Clinical Immunology, 2020.
25. McCusker, C., J. Upton, and R. Warrington, *Primary immunodeficiency.* Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology, 2018. **14**(Suppl 2): p. 61-61.
26. Kobrynski, L., R.W. Powell, and S. Bowen, *Prevalence and morbidity of primary immunodeficiency diseases, United States 2001-2007.* J Clin Immunol, 2014. **34**(8): p. 954-61.
27. Kaveri, S.V., et al., *Intravenous immunoglobulins in immunodeficiencies: more than mere replacement therapy.* Clin Exp Immunol, 2011. **164 Suppl 2**: p. 2-5.
28. Raje, N. and C. Dinakar, *Overview of Immunodeficiency Disorders.* Immunology and allergy clinics of North America, 2015. **35**(4): p. 599-623.
29. Aydin, S.E., et al., *DOCK8 deficiency: clinical and immunological phenotype and treatment options - a review of 136 patients.* J Clin Immunol, 2015. **35**(2): p. 189-98.
30. Gaspar, H.B., et al., *How I treat ADA deficiency.* Blood, 2009. **114**(17): p. 3524-32.
31. Moratto, D., et al., *Long-term outcome and lineage-specific chimerism in 194 patients with Wiskott-Aldrich syndrome treated by hematopoietic cell transplantation in the period 1980-2009: an international collaborative study.* Blood, 2011. **118**(6): p. 1675-84.
32. Parta, M., et al., *Allogeneic Reduced-Intensity Hematopoietic Stem Cell Transplantation for Chronic Granulomatous Disease: a Single-Center Prospective Trial.* Journal of Clinical Immunology, 2017. **37**(6): p. 548-558.
33. Staal, F.J.T., A. Aiuti, and M. Cavazzana, *Autologous Stem-Cell-Based Gene Therapy for Inherited Disorders: State of the Art and Perspectives.* Frontiers in Pediatrics, 2019. **7**(443).
34. Ferrua, F., et al., *Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study.* Lancet Haematol, 2019. **6**(5): p. e239-e253.
35. Grunebaum, E., et al., *Bone marrow transplantation for severe combined immune deficiency.* JAMA, 2006. **295**(5): p. 508-18.
36. Kanariou, M., K. Spanou, and S. Tantou, *Long-term observational studies of chronic granulomatous disease.* Curr Opin Hematol, 2018. **25**(1): p. 7-12.
37. Chun, J.-K., et al., *Analysis of Clinical Presentations of Bruton Disease: A Review of 20 Years of Accumulated Data from Pediatric Patients at Severance Hospital.* Yonsei Med J, 2008. **49**(1): p. 28-36.
38. Futatani, T., et al., *Bruton's tyrosine kinase is present in normal platelets and its absence identifies patients with X-linked agammaglobulinaemia and carrier females.* Br J Haematol, 2001. **114**(1): p. 141-9.
39. Lopez-Granados, E., et al., *A genotype-phenotype correlation study in a group of 54 patients with X-linked agammaglobulinemia.* J Allergy Clin Immunol, 2005. **116**(3): p. 690-7.
40. Shin, C.R., et al., *Outcomes following hematopoietic cell transplantation for Wiskott-Aldrich syndrome.* Bone Marrow Transplant, 2012. **47**(11): p. 1428-35.
41. Ochs, H.D. and A.J. Thrasher, *The Wiskott-Aldrich syndrome.* Journal of Allergy and Clinical Immunology, 2006. **117**(4): p. 725-738.
42. Bosticardo, M., et al., *Gene therapy for Wiskott-Aldrich Syndrome.* Curr Gene Ther, 2014. **14**(6): p. 413-21.
43. Lutskiy, M.I., F.S. Rosen, and E. Remold-O'Donnell, *Genotype-proteotype linkage in the Wiskott-Aldrich syndrome.* J Immunol, 2005. **175**(2): p. 1329-36.

44. Jin, Y., et al., *Mutations of the Wiskott-Aldrich Syndrome Protein (WASP): hotspots, effect on transcription, and translation and phenotype/genotype correlation.* Blood, 2004. **104**(13): p. 4010-9.
45. Al-Herz, W., et al., *Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency.* Front Immunol, 2011. **2**: p. 54.
46. la Marca, G., et al., *Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase deficiency.* J Allergy Clin Immunol, 2013. **131**(6): p. 1604-10.
47. CLSI. *Blood Collection on Filter Paper for Newborn Screening Programs: Approved Standard*. 6 ed. Clinical and Laboratory Standards Institute (CLSI). 2013.