A Targeted Next Generation Sequencing (tNGS) Screening Assay for Menkes Disease and its Implications for Primary DNA-based Newborn Screening

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Background

• Genomic sequencing technology can now allow for pathogenic variant identification on a small DNA sample in a clinically relevant timeframe.

• Menkes disease is a X-linked recessive neurodegenerative disorder with onset in early infancy.

• The impact of existing and emerging treatments for Menkes would be enhanced through early detection by newborn screening (NBS), given the irreparable brain injury apparent by 6 weeks after birth of affected infants.
Menkes Disease

- X-linked recessive disorder that affects Cu\(^{2+}\) levels in the body leading to Cu\(^{2+}\) deficiency.
- Incidence 1 /100,000
- Due to variants in the \(ATP7A\) gene, which produces a protein that regulates Cu\(^{2+}\) transport.
- Presents in early infancy with lethargy, hypotonia, poor feeding, failure to thrive, and seizures, with CNS deterioration. Brain MRI eventually reveals white matter loss. Other manifestations may include skeletal, cardiac and bladder abnormalities. “Kinky” hair.
- Plasma and CSF catecholamine levels are abnormal. Elevations in the levels of dihydroxyphenylalanine and dihydroxyphenylacetic acid, with low levels of norepinephrine metabolites, are characteristic. Beyond 6 weeks of life, serum Cu\(^{2+}\) and ceruloplasmin levels are low.
- Untreated, survival is generally < 3 years
- Cu\(^{2+}\) histidine therapy is efficacious in some patients; promising gene therapy in mice
- No analytes measurable by current NBS methodologies.
ATP7A Gene and Protein

Over 300 pathogenic variants have been reported. 70% are SNVs and 30% duplications/deletions. Rate of de novo variants ~30%.

These characteristics mean low sensitivity with the use of 2nd tier DNA testing via a fixed variant panel, if there were to be a primary analyte to measure.


https://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pk=143
ATP7A traffics Cu\(^{2+}\) down axons and mediates copper release from axonal membranes of motor and at neuromuscular junctions.

Lack of Cu\(^{2+}\) in the CNS leads to demyelination, gliosis, and neuron loss.

Transmembrane Cu\(^{2+}\) transporting ATPase that cycles between the trans-Golgi (delivery of Cu\(^{2+}\) to the secreted Cu\(^{2+}\) enzymes) and the plasma (export of surplus intracellular Cu\(^{2+}\) ) membranes.

Defects lead to trapping of Cu\(^{2+}\) at the intestinal mucosa and kidney and failure of delivery to the CNS and connective tissues.

Lutsenko S et al Physiological Reviews (2007) 87:1011-1046
Biomarkers

• Presymptomatic detection is hindered by the inadequate sensitivity and specificity of diagnostic tests (serum [Cu$^{2+}$]).

• The deficiency of the Cu$^{2+}$ based enzyme, dopamine-β-hydroxylase, has been shown to be of diagnostic usefulness in the newborn through measurement of plasma neurochemical levels. These measurements have not been amenable to MS/MS.

Treatment

- Treatment with subcutaneous Cu$^{2+}$ injections must be initiated early.
- With early treatment, significant improvement in neurologic function can occur, and normal neurologic development is possible.
- 10% protein function is adequate
- 12 patients diagnosed as newborns received daily subcutaneous Cu$^{2+}$ injections until age 3 years.
  - 11/12 survived.
  - 2/12 had normal neurologic development at a median follow-up of 4.6 years.
  - It is postulated that the children with normal development likely had mild variants and some residual Cu$^{2+}$-transporting ATPase activity.

Treatment

• In patients with mutations that are unresponsive to Cu$^{2+}$ therapy, gene therapy has shown promise in mouse models of brain directed (choroid plexus) ATP7A gene therapy via AAV5.
• We developed and validated a DBS-based targeted next generation sequencing (tNGS) method with rapid turnaround time for prompt, accurate identification of affected newborns that would allow for early treatment.

• We applied this method to characterize 24 blinded specimens from unaffected control or Menkes disease subjects enrolled in a current clinical trial (ClinicalTrials.gov number, NCT0081785).
Assay
Panel of Panels

NBS - hearing
CMV
Hearing Loss subpanel

Metabolic subpanel
CF
SCID subpanel
Endocrine (thyroid, adrenal) subpanels

Common Nursery/NICU Phenotype Subpanels
- Seizures
- Macrocephaly
- Microcephaly
- Hypotonia
- Respiratory distress
- Pancreatic function
- Liver dysfunction
- Hyperbilirubinemia
- Hypoglycemia
- Hypo/hypercalcemia
- Anemia/Clotting/Thrombocytopenia/Neutropenia
- Renal abnormalities
- Skeletal/Connective tissue disorders
- Skin disorders
- Eye disorders
- Cancer predisposition
- PGx

NBS - DBS

544 genes
Critical Assay Characteristics

• Optimized for DBS as DNA source
• Rapid TAT
• Relatively lower cost
# Neurologic Disease Panels NBDx v.1.0

## SEIZURES

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<td>KCNQ3</td>
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<td>CDKL5</td>
<td>SPTAN1</td>
<td>STXBP1</td>
<td>SLC2A1</td>
<td>ATP7A</td>
<td>MEF2C</td>
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<td>TCF4</td>
<td>CSTB</td>
<td>EPM2A</td>
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<td>ALDH7A1</td>
<td>SCN1A</td>
<td>SUOX</td>
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<td>TSC1</td>
<td>TSC2</td>
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## MACROCEPHALY

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## MICROCEPHALY

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<td>SLC25A19</td>
<td>UBE3A</td>
<td>SLC9A6</td>
<td>FOXG1</td>
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<td>IER3P1</td>
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<td>ASPM</td>
<td>CDK5RAP2</td>
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<td>CEP152</td>
<td>MCPH1</td>
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<td>DHCR7</td>
<td>RAB3GAP2</td>
<td>RAB18</td>
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## HYPOTONIA/MYOPATHY

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<td>MPZ</td>
<td>PMP22</td>
<td>MPI</td>
<td>PMM2</td>
<td>LAMA2</td>
<td>POMT1</td>
<td>POMT2</td>
<td>FKTN</td>
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<td>ABAT</td>
<td>WNK1</td>
<td>CEP290</td>
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<td>ACTA1</td>
<td>NDN</td>
<td>SNRPN</td>
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## NEONATAL ABSTINANCE SYNDROME

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<td>COMT</td>
<td>OPRM1</td>
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ATP7A Coverage Across Exons

Exon 9
- G666R

Exon 10
- G727R

>20x coverage

Ctrl 1574

NA12878

User Supplied Track
**Workflow**

**Sample Isolation**
- DBS
- No repeat collection
- Automated

**DNA Capture & Sequencing**
- Panel specific to
  - DNA based screening genes
  - Panel covering exons+
  - Multiple technologies available-leveraging latest advancements

**Raw Data Management**
- Optimized Data Processing and Analysis Using Miseq onboard tools

**Analysis, Database & Interpretation**
- Interpretation using internal Variant Curated Database
  - (Fabric Genomics-Opal)
Results

• 23 samples were from Menkes patients, 21 of which had previously characterized mutations

• 100% of the known mutations were correctly identified
  – 14 SNVs (missense, splice site, indel, stop)
  – 8 CNVs
    • 6 deletions
    • 2 duplications
  – 1 not detected
  – Of 2 Menkes samples for which clinical sequencing had not previously discovered mutations, a causative mutation was newly identified in 1 patient

• 1 control correctly identified.

• 23/24 samples were correct identification = 95.8% detection rate
### SNV (mutations, splice site, INDELs)

<table>
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<tr>
<th>New Sample ID</th>
<th>Transcript Variant</th>
<th>Protein Variant</th>
<th>Position</th>
<th>dbSNP</th>
<th>MAF</th>
<th># Reads</th>
<th>Evidence</th>
<th>Clinvar ID</th>
<th>Turner 2013 Ref</th>
<th>ExAC/GnomAD</th>
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<tbody>
<tr>
<td>NBS-1</td>
<td>c.601C&gt;T</td>
<td>p.Arg201Ter</td>
<td>chrX:77244218 s1151340633</td>
<td>0</td>
<td>85</td>
<td>Likely Pathogenic</td>
<td>KO</td>
<td>NO *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBS-2</td>
<td>c.1139_1140delTG</td>
<td>p.Val380AspTer4</td>
<td>chrX:77245255-77245256</td>
<td>n.a.</td>
<td>87</td>
<td>Likely Pathogenic</td>
<td>KO</td>
<td>NO *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBS-3</td>
<td>c.2129_2132delCTGT</td>
<td>p.Ser710LeufsTer2</td>
<td>chrX:77267128-77267131</td>
<td>n.a.</td>
<td>55</td>
<td>Pathogenic</td>
<td></td>
<td>Kaler 2008, Skjorringe 2011</td>
<td>NO</td>
<td></td>
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<tr>
<td>NBS-4</td>
<td>c.2172+6T&gt;G (c.IVS9+6T&gt;G)</td>
<td>n.a.</td>
<td>chrX:77267177 n.a.</td>
<td>0</td>
<td>113</td>
<td>Pathogenic</td>
<td>210424</td>
<td>NO</td>
<td></td>
<td></td>
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<tr>
<td>NBS-5</td>
<td>c.2357T&gt;G</td>
<td>p.Met786Arg</td>
<td>chrX:77268560 s797045354</td>
<td>0</td>
<td>62</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>similar splice site affected</td>
<td>NO</td>
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<tr>
<td>NBS-6</td>
<td>c.2627-10G&gt;A (c.IVS12-10G&gt;A)</td>
<td>n.a.</td>
<td>chrX:77275740 n.a.</td>
<td>0</td>
<td>66</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>NO *</td>
<td></td>
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<tr>
<td>NBS-7</td>
<td>c.30140T</td>
<td>p.Glu1005Val</td>
<td>chrX:77248484</td>
<td>n.a.</td>
<td>51</td>
<td>Pathogenic</td>
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<td>194455/RCV000193101.1</td>
<td>KO</td>
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<td>NBS-8</td>
<td>c.3041C&gt;T</td>
<td>p.Thr1014Ile</td>
<td>chrX:77248401 n.797044648</td>
<td>0</td>
<td>80</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>NO *</td>
<td></td>
<td></td>
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<tr>
<td>NBS-9</td>
<td>c.3445C&gt;T</td>
<td>p.Gln1149Val</td>
<td>chrX:77289253</td>
<td>n.a.</td>
<td>75</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>NO *</td>
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<td>NBS-10</td>
<td>c.3526C&gt;T</td>
<td>p.Ile1176Ter</td>
<td>chrX:77294348</td>
<td>n.a.</td>
<td>62</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>NO *</td>
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<tr>
<td>NBS-11</td>
<td>c.3753delT</td>
<td>p.Leu1252Ter</td>
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<td>n.a.</td>
<td>80</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>NO *</td>
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<tr>
<td>NBS-12</td>
<td>c.3946dupA</td>
<td>p.Ile1316AsnTer2</td>
<td>chrX:77298226 n.a.</td>
<td>0</td>
<td>40</td>
<td>Likely Pathogenic</td>
<td>NO</td>
<td>similar splice site affected</td>
<td>NO</td>
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<td>NBS-13</td>
<td>c.4006-1G&gt;A (c.IVS20-1G&gt;A)</td>
<td>n.a.</td>
<td>chrX:77299894 n.a.</td>
<td>0</td>
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<td>Gourdon 2011</td>
<td>KO</td>
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<tr>
<td>NBS-15</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>NBS-16 blinded negative control</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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### CNV (deletion, duplication)

<table>
<thead>
<tr>
<th>CNV Type</th>
<th>Description</th>
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<tbody>
<tr>
<td>NBS-17</td>
<td>Deletion</td>
</tr>
<tr>
<td>NBS-18</td>
<td>Deletion</td>
</tr>
<tr>
<td>NBS-19</td>
<td>Duplication</td>
</tr>
<tr>
<td>NBS-20</td>
<td>Duplication</td>
</tr>
<tr>
<td>NBS-21</td>
<td>Deletion</td>
</tr>
<tr>
<td>NBS-22</td>
<td>Deletion</td>
</tr>
<tr>
<td>NBS-23</td>
<td>Deleted</td>
</tr>
<tr>
<td>NBS-24</td>
<td>Deleted</td>
</tr>
</tbody>
</table>
Samples 1084452-2, 1084481-1, 1084477-5 displaying copy number variants identified by the CNV caller as intervals beneath the coverage track.

Duplications are green and deletions are red.

Sample 1084452-2 shows a duplication of exons 7-12.

Sample 1084481-1 shows a homozygous deletion of exons 13 and 14.

Sample 1084477-5 shows a homozygous deletion after exon 6 (the deletion continues for the remaining exons).
Would Criteria be Met for NBS?

• Incidence appropriate
• Early onset of disease
• Early initiation of treatment critical
• Therapy available
• Screening assay with high detection rate
• TAT- under 7 days
• Confirmatory test available
• Potential for low cost
Big Picture

- Disorder with an available therapy
- Can’t measure analytes
- Variant panel would not detect
- Primary DNA testing could identify these affected babies
Limiting Factors

- Introduction of new technology to PHLs
- Cost
- False Negative: estimate ~5%
- False Positive: estimate <0.05% (ExAc <1/213 (258/60,706))
- Consent for genetic testing?
- Carrier detection
Would cost be a limiting factor?
Cost estimate for 2\textsuperscript{nd} tier single gene vs. tNGS NBS panel

- Consider a tNGS NBS panel of 200 genes that cover all genetic disorders in the RUSP + other actionable disorders without a current screen (including ATP7A/Menkes).
- Consider disorders for which there are currently 2\textsuperscript{nd} tier DNA based tests (many of which would be optimized by tNGS sequencing, with or without an \textit{in silico} filter for specific variants as a first pass and reflex to analysis of unmasked remaining sequence:
  - CF, Galactosemia, SCID, BTD, Hemoglobinopathies, CAH, MCAD, other MS/MS detected IEMs
- Disorders that are actionable but for which there is no current screen:
  - e.g. Menkes, Wilson, Cystinosis, DMD, Fragile X, MPS/IDU, Pompe, XALD, Krabbe, G6PD, GBJ2
## Theoretical Break Even/Savings Point

<table>
<thead>
<tr>
<th>Disease</th>
<th>Total Screen/yr</th>
<th>Presumptive Positive (PP)</th>
<th># PP cases</th>
<th>Presumed 200 gene tNGS panel costs $200/sample</th>
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</thead>
<tbody>
<tr>
<td>CF</td>
<td>250000</td>
<td>5%</td>
<td>12500</td>
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<tr>
<td>Pompe</td>
<td>250000</td>
<td>0%</td>
<td>120</td>
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<tr>
<td>DMD</td>
<td>250000</td>
<td>1%</td>
<td>2500</td>
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<tr>
<td></td>
<td></td>
<td>6%</td>
<td>15120</td>
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If every baby gets a $200 test, the cost is $50 million/yr.

<table>
<thead>
<tr>
<th>PP</th>
<th>NGS 2nd tier Volume</th>
<th>Offset</th>
<th>Primary assay</th>
<th>Break even point</th>
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<td>2% PP</td>
<td>5000</td>
<td>$2,000,000</td>
<td>$192</td>
<td>$50,000,000</td>
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<td>10 diseases</td>
<td>50000</td>
<td>$20,000,000</td>
<td>$120</td>
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<td>$100,000,000</td>
<td>$-200</td>
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</table>
Conclusions

• Our results suggest that a primary tNGS algorithm can be applied with high sensitivity and specificity to detect ATP7A variants predictive for Menkes disease, a medically actionable disorder of copper metabolism.

• The assay generated 95.8% correct calls across both SNV/CNV.

• Early detection by genomic screening, in concert with advances in ATP7A gene therapy, suggest the potential to transform the natural history of Menkes disease.