Two Tier CK & CKMB Assay for Duchenne Muscular Dystrophy in Dried Blood Spots by Fluorometry.

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This is part of a 3-phase collaborative project on Duchenne Muscular Dystrophy funded by the Centers of Disease Control (CDC).
BACKGROUND

- Duchenne Muscular Dystrophy (DMD) is a debilitating sex-linked disorder produced when the dystrophin gene undergoes mutation or damage.
- Creatine kinase (CK) is markedly elevated in DMD patients
- Creatine Kinase: a dimeric molecule with distinct subunits M & B
- Isozymes: CK-MM found mostly in skeletal muscle
  CK-MB found mostly in heart muscle
  CK-BB found mostly in brain

ASSAY PRINCIPLE

- CK Assay
- CKMB Assay

PROTOCOL: Endpoint Assay by Fluorometry

DATA

ASSAY ALGORITHM

CONCLUSION / RECOMMENDATION
Reaction Sequence

1. Enzyme Activation: N-acetyl-L-cysteine (NAC)

2. ATP Production:
   - CK
     \[ \text{ADP} + \text{Creatine Phosphate} \rightarrow \text{ATP} + \text{Creatine} \]
   - AK*
     \[ \text{ATP} + \text{AMP} \rightarrow 2 \text{ATP} \]
   * Inhibited by Diadenosine pentaphosphate

3. ATP Monitoring: Kinetic vs. Endpoint
   - Biological luminescence
     \[ \text{luciferase} \]
     \[ \text{ATP} + \text{luciferin} + \text{O}_2 \rightarrow \text{AMP} + \text{PPI} + \text{CO}_2 + \text{oxyluciferin} + \text{light} \]
     Measured by a luminometer, the light intensity is proportional to the ATP concentration or CK Activity.

FLUOROMETRY/SPECTROPHOTOMETRY
   through a Series of Coupled Reaction

- HK
  \[ \text{ATP} + \text{Glucose} \rightarrow \text{G-6-P} + \text{ADP} \]
  \[ \text{G-6-PDH} \]
  \[ \text{G-6-P} + \text{NADP}^+ \rightarrow 6\text{-Phosphogluconate} + \text{NADPH}* + \text{H}^+ \]
*Can be monitored by Spectrophotometry: @ 340 nm
or by Fluorometry: @ 355 nm Excitation and 460 nm Emission

Signal is proportional to NADPH/ATP/CK Activity
CKMB Assay

- CK activity is measured in the presence of an antibody to the CK-M monomer.
- Creatine Kinase
dimeric molecule
  immunologically distinct subunits M and B.
- Isozymes:
  CK-MM (skeletal muscle) - completely inhibited
  CK-MB (heart) - half the activity
  CK-BB (brain) - not affected
- Due to the negligible concentration of CK-BB in the circulation, the remaining activity multiplied by 2, represents the activity of the CK-MB isozyme.
DMD CK Assay Protocol (End Point)
1. Prepare all needed reagents per reagent preparation protocol.
2. Punch patient samples, standards and controls into black microtiterplates.
3. Add 50 uL of the Diadenosine Pentaphosphate Reagent
4. Incubate for 30 minutes at 25 degrees C with mild shaking.
5. Add 50 uL of the CK Reagent Mixture.
6. Incubate for 10 minutes with mild shaking at room temperature.
7. Add 100 uL 95% Ethanol and allow precipitate to completely settle at the bottom of the wells. (10-15 minutes).
8. Handling gently so as not to disturb the precipitate, read at 355 nm (excitation) and 460 nm (emission).

DMD CKMB Assay Protocol (End Point)
1. Prepare all needed reagents per reagent preparation protocol.
2. Punch patient samples, standards and controls into black microtiterplates.
3. Add 50 uL of the Diadenosine Pentaphosphate Reagent
4. Incubate for 30 minutes at 37 degrees C with mild shaking.
5. Add 50 uL of the CKMB Reagent Mixture.
6. Incubate for 10 minutes with mild shaking at 37 degrees C.
7. Add 100 uL 95% Ethanol and allow precipitate to completely settle at the bottom of the wells. (10-15 minutes)
8. Handling gently so as not to disturb the precipitate, read at 355 nm (excitation) and 460 nm (emission).
### TOTAL CK POPULATION STATISTICS

<table>
<thead>
<tr>
<th>Counts</th>
<th>Mean (U/L)</th>
<th>SD</th>
<th>Mean + 1SD</th>
<th>Mean + 2SD</th>
<th>Mean + 3SD</th>
<th>Mean + 4SD</th>
<th>Mean + 5SD</th>
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<tbody>
<tr>
<td>All Infants Screened</td>
<td>30547</td>
<td>247.915</td>
<td>109.4</td>
<td>357.36</td>
<td>471.21</td>
<td>585.06</td>
<td>698.91</td>
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<td><strong>By Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Males</td>
<td>15446</td>
<td>251.52</td>
<td>113.85</td>
<td>365.37</td>
<td>479.22</td>
<td>593.07</td>
<td>706.92</td>
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<tr>
<td>Females</td>
<td>14983</td>
<td>246.39</td>
<td>113.56</td>
<td>359.95</td>
<td>473.51</td>
<td>587.07</td>
<td>700.63</td>
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<td><strong>Total</strong></td>
<td>30429</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>By Weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&gt;2500 grams</td>
<td>27506</td>
<td>250.61</td>
<td>115.99</td>
<td>366.60</td>
<td>480.16</td>
<td>593.72</td>
<td>707.28</td>
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<td>&gt;2000 - 2500 grams</td>
<td>1555</td>
<td>231.68</td>
<td>87.78</td>
<td>319.46</td>
<td>433.02</td>
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<td>1500 - 2000 grams</td>
<td>538</td>
<td>210.41</td>
<td>71.01</td>
<td>281.41</td>
<td>394.97</td>
<td>508.53</td>
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<tr>
<td>&lt; 1500 grams</td>
<td>573</td>
<td>226.36</td>
<td>75.88</td>
<td>302.24</td>
<td>415.80</td>
<td>529.36</td>
<td>642.92</td>
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<td><strong>Total</strong></td>
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<td><strong>By Age at Sample Collection</strong></td>
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<td>48 Hrs. &amp; less</td>
<td>27065</td>
<td>253.37</td>
<td>116.99</td>
<td>370.36</td>
<td>483.92</td>
<td>597.48</td>
<td>711.04</td>
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<tr>
<td>&gt; 48 Hrs. but &lt; 120 Hrs.</td>
<td>2572</td>
<td>207.56</td>
<td>68.51</td>
<td>276.07</td>
<td>389.63</td>
<td>503.19</td>
<td>616.75</td>
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<td>&gt;120 Hrs.</td>
<td>596</td>
<td>201.64</td>
<td>63.54</td>
<td>265.18</td>
<td>378.74</td>
<td>492.30</td>
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<tr>
<td><strong>Total</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Cut-off (U/L)</strong></td>
<td>500</td>
<td>600</td>
<td>700</td>
<td>800</td>
<td>1000</td>
<td></td>
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<tr>
<td><strong>Presumptive positives</strong></td>
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<tr>
<td>Based on CK Screens</td>
<td>650</td>
<td>228</td>
<td>106</td>
<td>63</td>
<td>29</td>
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<tr>
<td>Percentage</td>
<td>2.13</td>
<td>0.75</td>
<td>0.35</td>
<td>0.21</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Based on CK &amp; CKMB</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
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</table>
CK vs. CKMB in Male Newborns

Activity (U/L)

Male Newborn Samples

CK
CKMB
CK vs. CKMB in DMD Patients
(Childrens Hospital)

DMD Patient Samples

Activity (U/L)
Comparison Between ODH & Emory CK Assay

ODH vs. Emory CK Activity in DMD Patients
(Children's Hospital)

ODH vs. Emory CK Activity in DMD Patients
(Emory University Hospital)
The scatter plot shows the relationship between CK Activity (U/L) and CKMB Activity (U/L) for n=222 cases. The equation of the line of best fit is:

\[ y = 0.4904x + 212.56 \]

The coefficient of determination, \( R^2 \), is 0.051, indicating a weak linear relationship between the two variables.
CK & CKMB in Female & Male Newborns

**CK vs. CKMB Activity in Female Newborns**

- **y = -3.5221x + 3191**
- **R² = 0.0754**

**CK vs. CKMB Activity in Male Newborns**

- **y = 1.2162x + 127.77**
- **R² = 0.5699**
CK & CKMB ASSAY ALGORITHM

CK SCREEN

NOTE:
RPT: MEAN @ 30% CV
(SUM OF INTER- AND INTRA-ASSAY CV)

≥500 U/L
Run CK & CKMB in duplicates

<500 U/L
LOW RISK

DECISION TREE

CK RPT
<500 U/L

CK RPT
≥500 & <600 U/L

CK RPT
≥600 U/L
HIGH RISK

CKMB
?< U/L
LOW RISK

CKMB
≥? U/L
MODERATE RISK

CKMB
?< U/L
MODERATE RISK

CKMB
≥? U/L
HIGH RISK

ODH/MSI: 8/10/06
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutation</th>
<th>Time – tCK</th>
<th>Age / Hx</th>
<th>Diagnosis</th>
<th>ODH tCK</th>
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<tr>
<td>DOBS001</td>
<td>Exons 18-39 are deleted plus promoter DMDp260</td>
<td>10:06 – 31,825</td>
<td>7yo Ambulatory No steroids</td>
<td>DMD OF deletion</td>
<td>3,982</td>
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<tr>
<td>DOBS002</td>
<td>Exons 18-39 are deleted plus promoter DMDp260</td>
<td>10:05 – 26,576</td>
<td>7yo Ambulatory No steroids</td>
<td>DMD OF deletion</td>
<td>3,882</td>
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<td>DOBS003</td>
<td>Exons 46 and 47 are deleted</td>
<td>Not done</td>
<td></td>
<td>DMD OF deletion</td>
<td>865</td>
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<td>DOBS004</td>
<td>Exons 46-53 are deleted</td>
<td>10:30 – 4,338</td>
<td>10yo Ambulatory On steroids</td>
<td>DMD OF deletion</td>
<td>~1,000</td>
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<td>DOBS005</td>
<td>Exons 4-30 are deleted plus promoter DMDp260</td>
<td>10:05 – 9,483</td>
<td>9yo Ambulatory On steroids</td>
<td>BMD? IF deletion</td>
<td>1,150</td>
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<td>DOBS006</td>
<td>Exons 45-57 are deleted plus promoter DMDp116</td>
<td>09:25 – 871</td>
<td>22yo Not Ambul On steroids</td>
<td>BMD IF deletion</td>
<td>252</td>
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<tr>
<td>DOBS007</td>
<td>Exons 46-50 are deleted</td>
<td>09:08 – 2,362</td>
<td>15yo Not Ambul No steroids</td>
<td>DMD OF deletion</td>
<td>236</td>
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<td>DOBS010</td>
<td>Exons 6-7 duplicated</td>
<td>10:35 – 14,191</td>
<td>10yo Ambulatory On steroids</td>
<td>DMD OF duplication</td>
<td>1,425</td>
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</table>
Compelling Reasons to Screen for Duchenne Muscular Dystrophy

• Morbid disorder resulting in death at an early age.
• Incidence is 1 in 3500 – 6000 in male births which is higher than many of the disorders being screened by most States.
• Availability of early intervention & management.
• Simple robust laboratory screening protocol.
• Low capital outlay. Instrument already in most screening laboratories.
• Reagents and reagent kits are readily available.
• Assay can be automated for high throughput.
• Availability of Confirmation Protocol: DNA Assay.
Two Tier CK & CKMB Assay for Duchenne Muscular Dystrophy in Dried Blood Spots by Fluorometry.

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