

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:** $\text{ADP} + \text{Creatine Phosphate} \xrightarrow{\text{CK}} \text{ATP} + \text{Creatine}$

$\text{ATP} + \text{AMP} \xrightarrow{\text{AK}^*} 2 \text{ATP}$

** Inhibited by Diadenosine pentaphosphate*

3. **ATP Monitoring:** Kinetic vs. Endpoint
BIOLUMINESCENCE

$\text{ATP} + \text{Luciferin} + \text{O}_2 \xrightarrow{\text{Luciferase}} \text{AMP} + \text{PPi} + \text{CO}_2 + \text{oxyluciferin} + \text{light}$
Measured by a luminometer, the light intensity is proportional to the ATP concentration or CK Activity.

FLUOROMETRY/SPECTROPHOTOMETRY
through a Series of Coupled Reaction

- $\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$
- $\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} 6\text{-Phosphogluconate} + \text{NADPH}^* + \text{H}^+$

*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).**

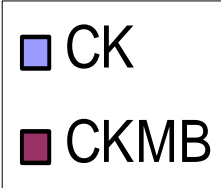
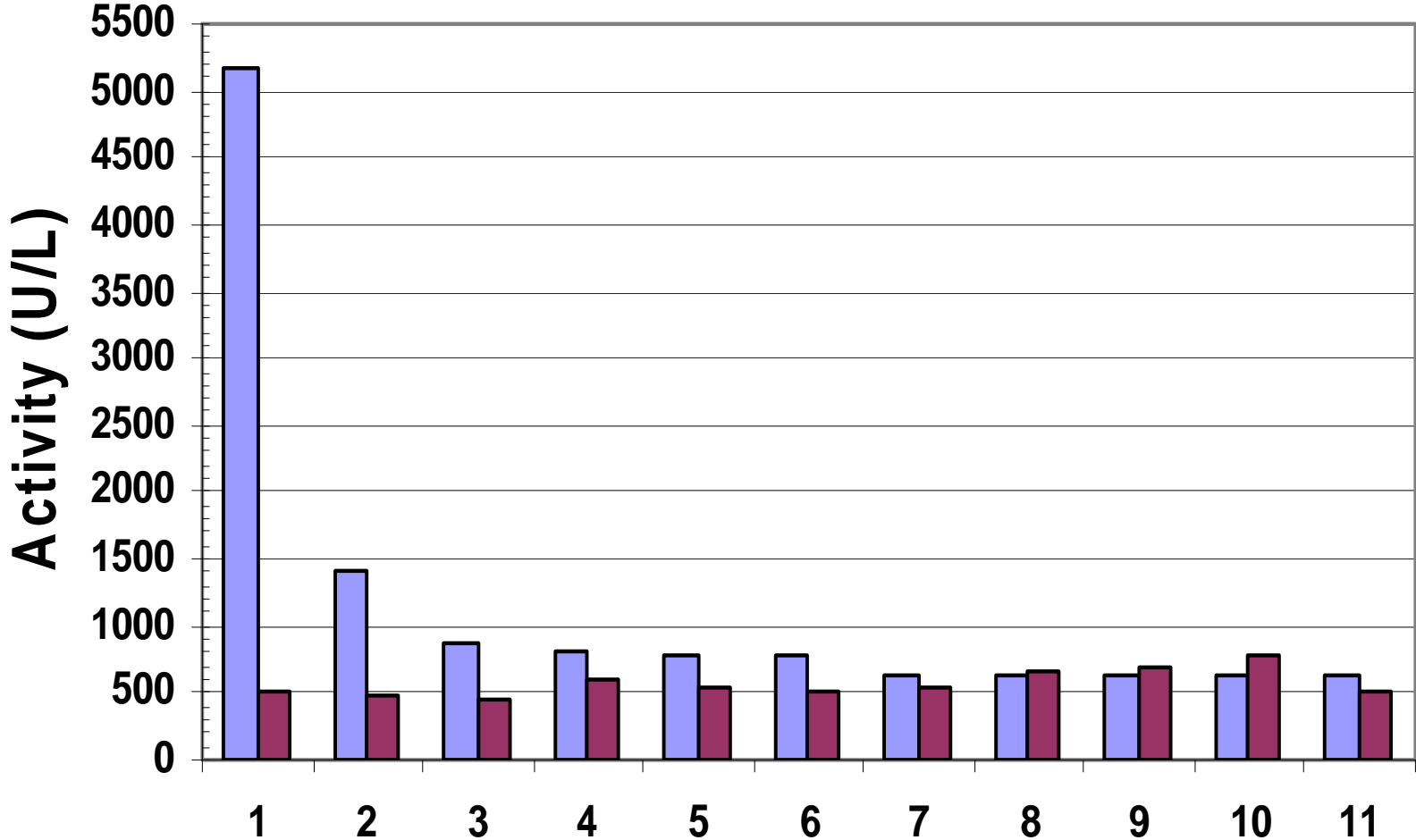
DMD CKMB Assay Protocol (End Point)

- 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

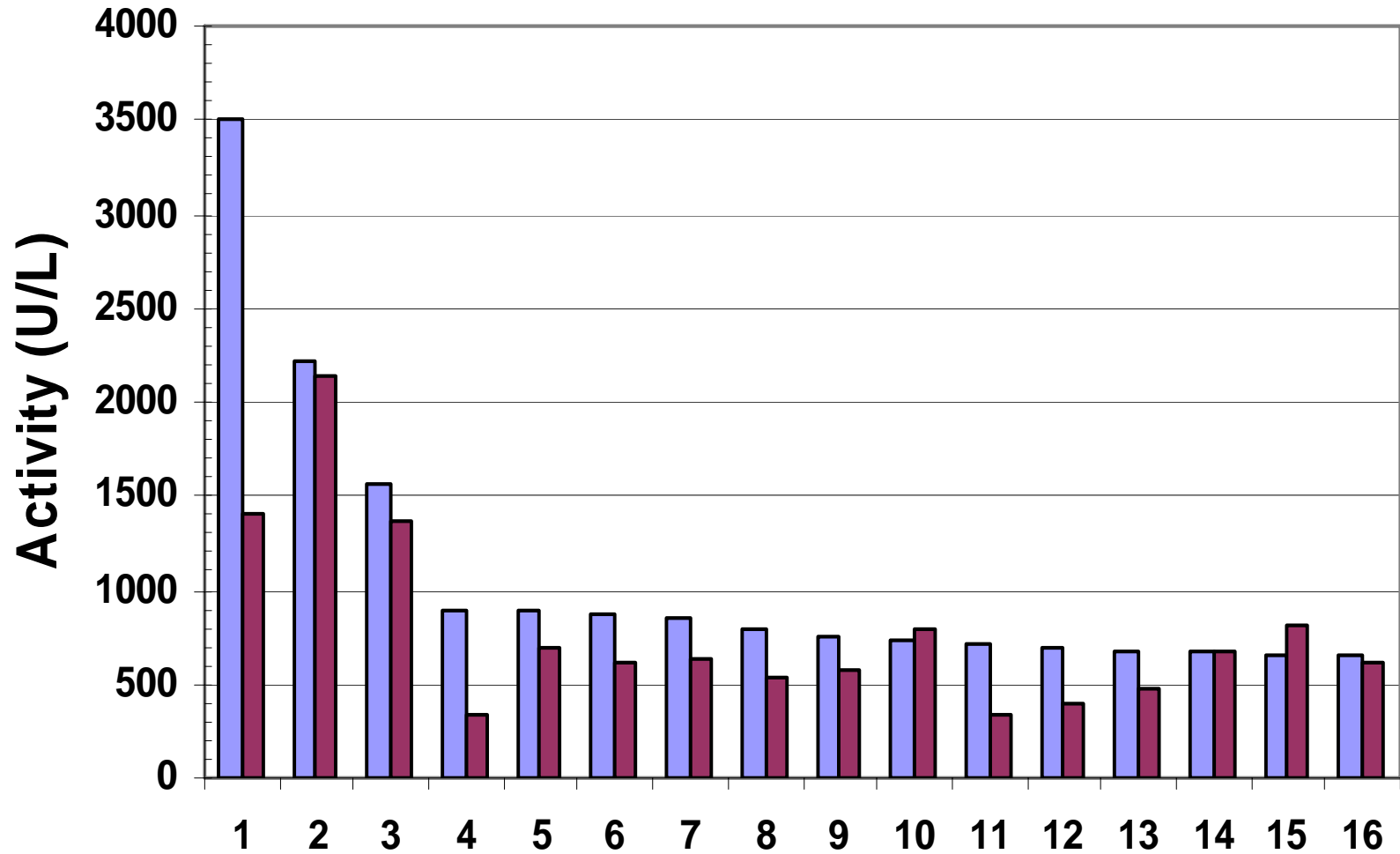
	Counts	Mean (U/L)	SD	Mean + 1SD	Mean + 2SD	Mean + 3SD	Mean + 4SD	Mean + 5SD
All Infants Screened	30547	247.915	109.4	357.36	471.21	585.06	698.91	812.76
By Sex								
Males	15446	251.52	113.85	365.37	479.22	593.07	706.92	820.77
Females	14983	246.39	113.56	359.95	473.51	587.07	700.63	814.19
Total	30429							
By Weight								
>2500 grams	27506	250.61	115.99	366.60	480.16	593.72	707.28	820.84
>2000 - 2500 grams	1555	231.68	87.78	319.46	433.02	546.58	660.14	773.70
1500 - 2000 grams	538	210.41	71.01	281.41	394.97	508.53	622.09	735.65
< 1500 grams	573	226.36	75.88	302.24	415.80	529.36	642.92	756.48
Total	30172							
By Age at Sample Collection								
48 Hrs. & less	27065	253.37	116.99	370.36	483.92	597.48	711.04	824.60
> 48 Hrs. but < 120 Hrs.	2572	207.56	68.51	276.07	389.63	503.19	616.75	730.31
>120 Hrs.	596	201.64	63.54	265.18	378.74	492.30	605.86	719.42
Total	30233							
Cut-off (U/L)	500	600	700	800	1000			
Presumptive positives								
Based on CK Screens	650	228	106	63	29			
Percentage	2.13	0.75	0.35	0.21	0.09			
Based on CK & CKMB	?	?	?	?	?			
Percentage	?	?	?	?	?			

CK vs. CKMB Activity in Female Newborns



Female Newborn Samples

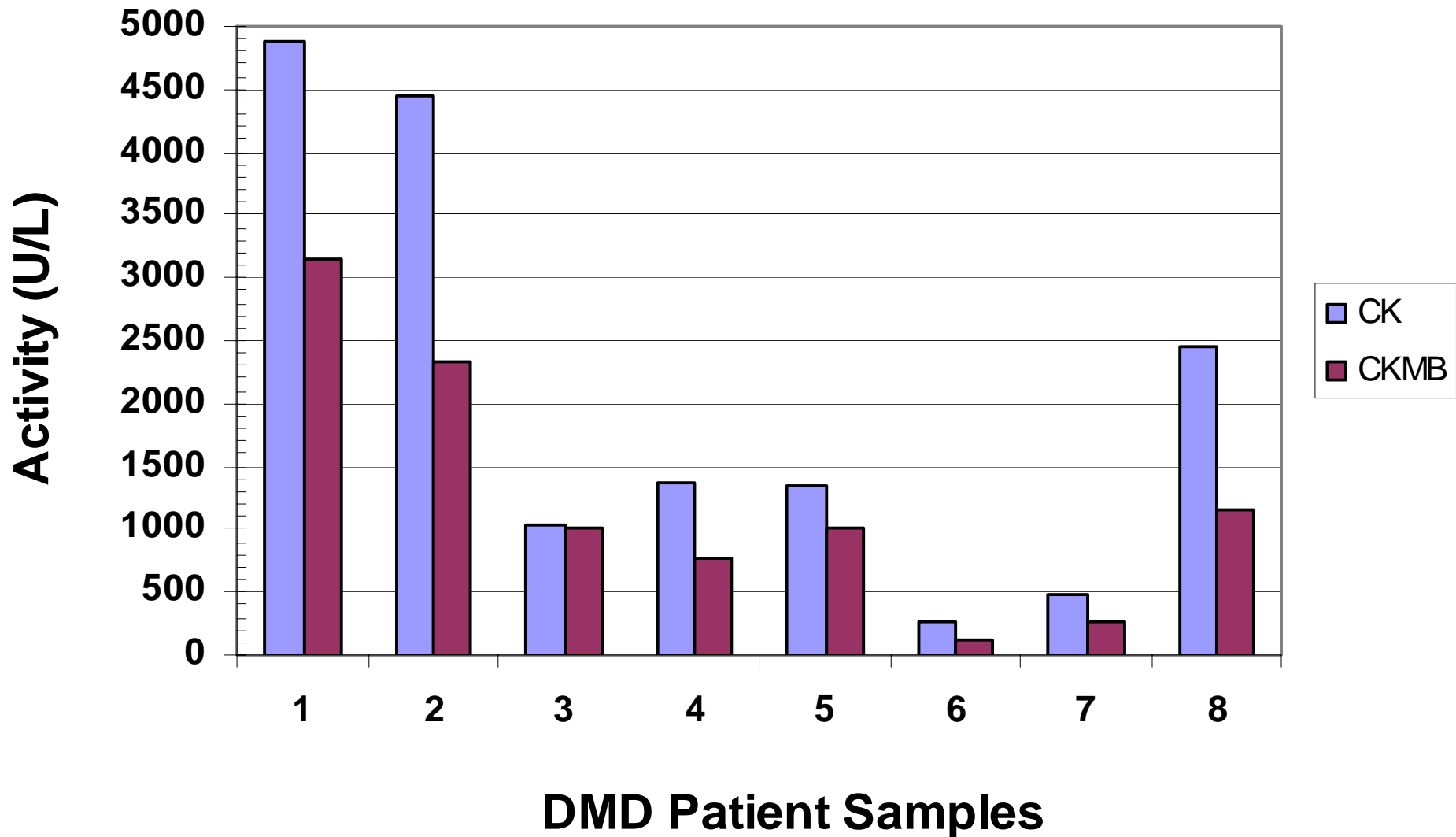
CK vs. CKMB in Male Newborns



■ CK
■ CKMB

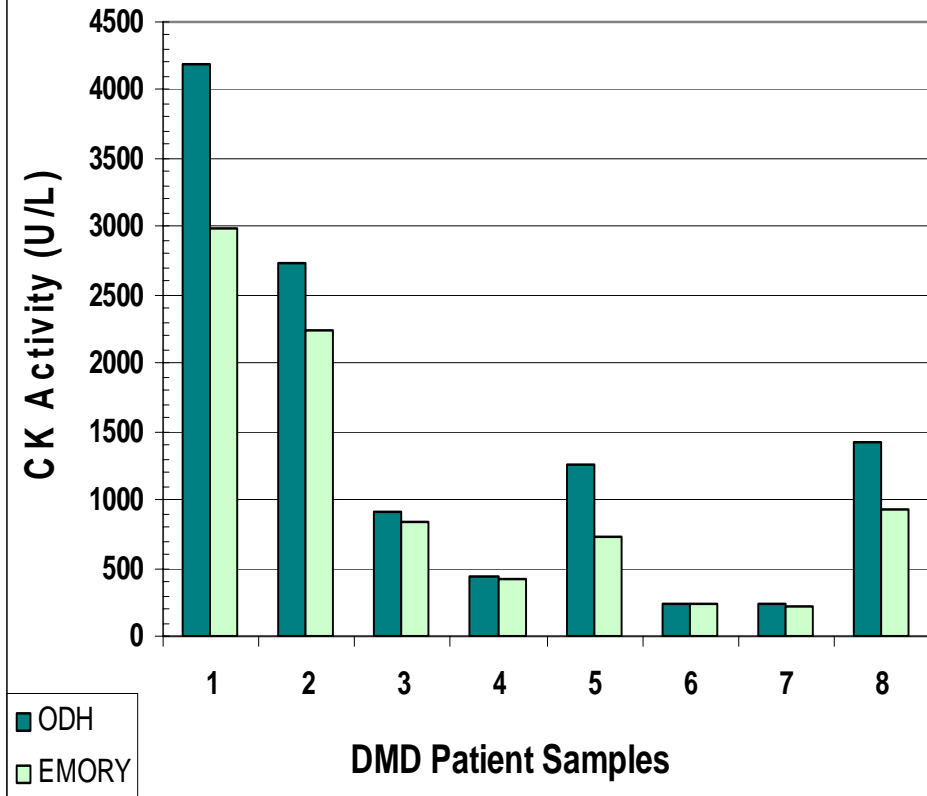
Male Newborn Samples

CK vs. CKMB in DMD Patients (Childrens Hospital)

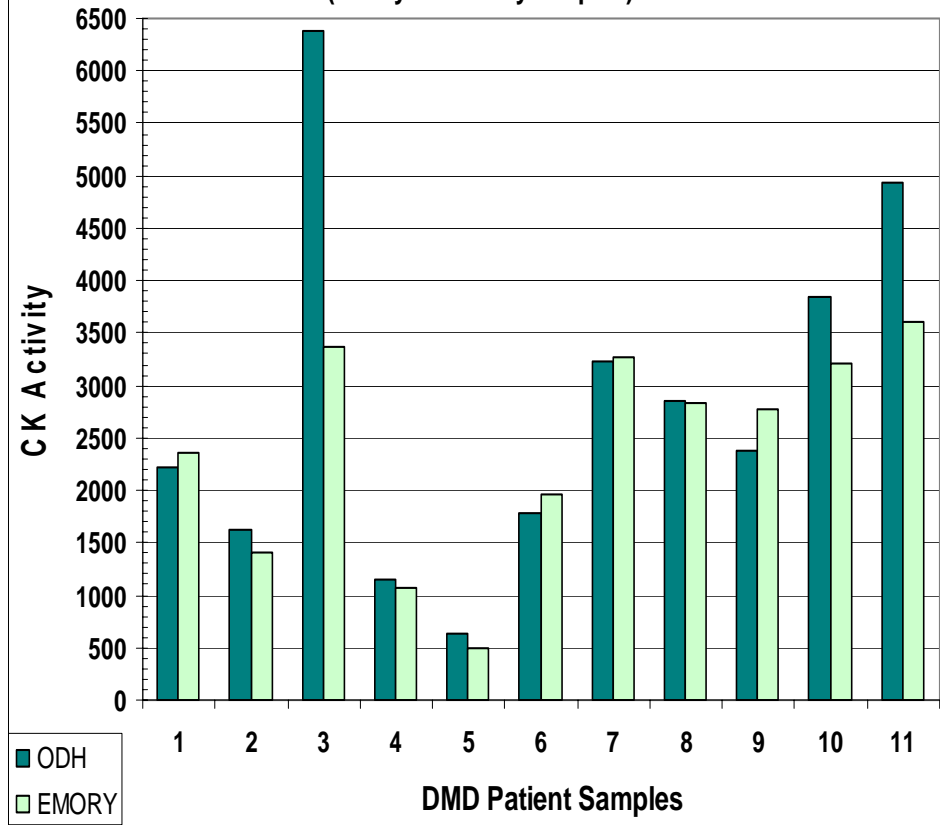


Comparison Between ODH & Emory CK Assay

ODH vs. Emory CK Activity in DMD Patients (Childrens Hospital)

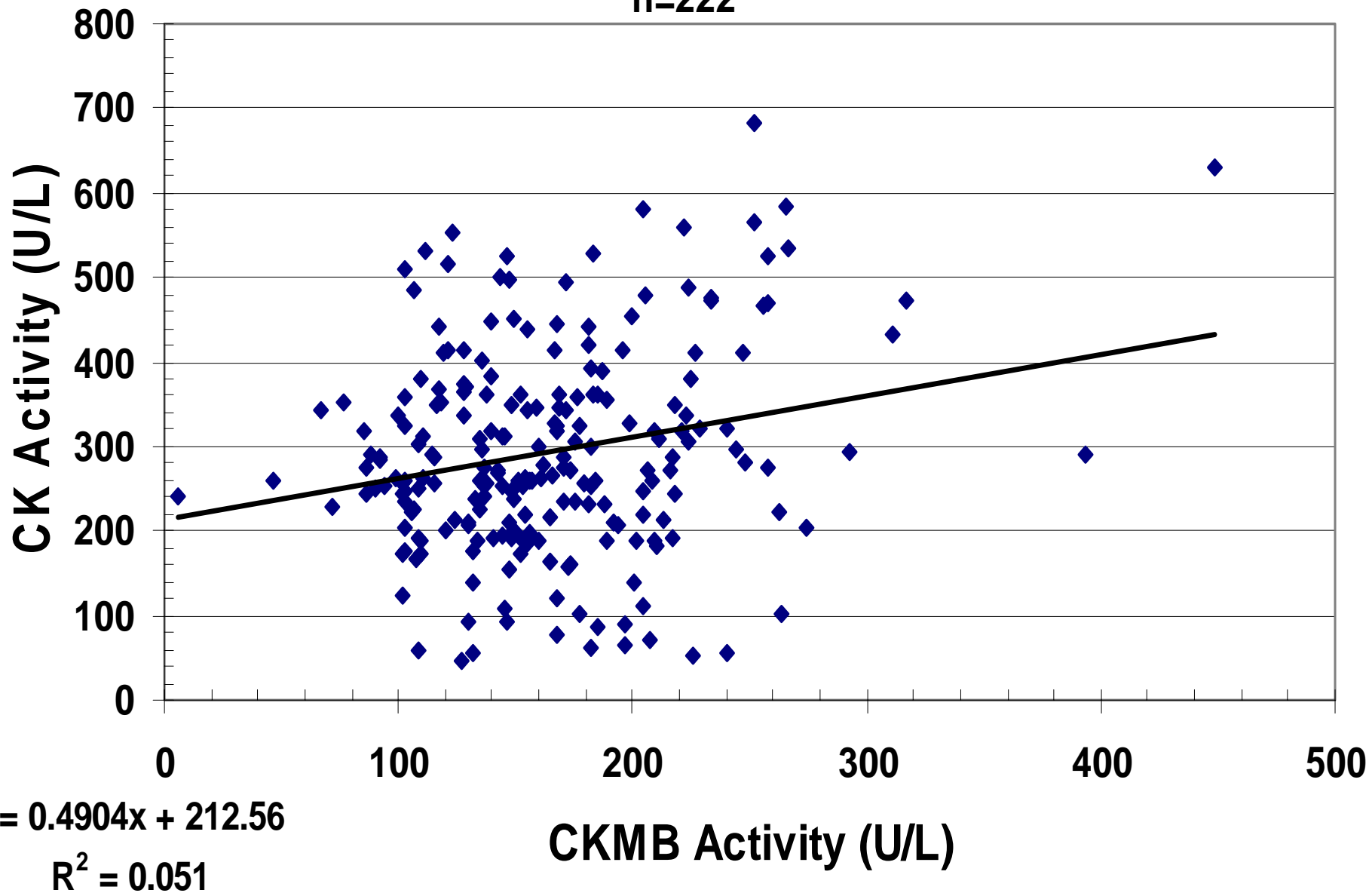


ODH vs. Emory CK Activity in DMD Patients (Emory University Hospital)



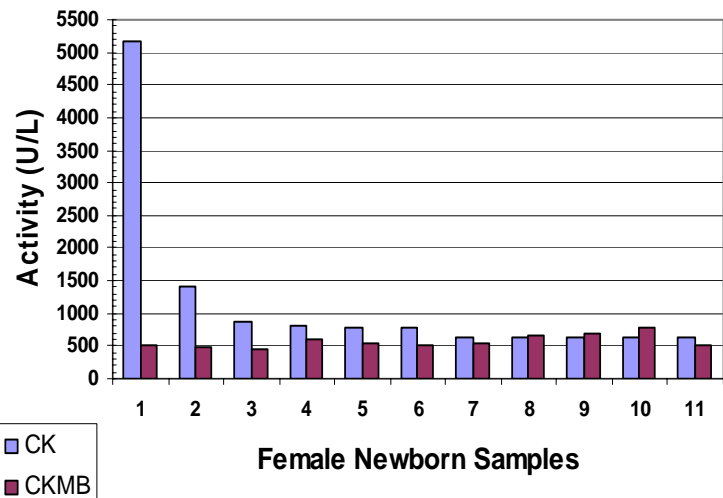
CK vs. CKMB

n=222

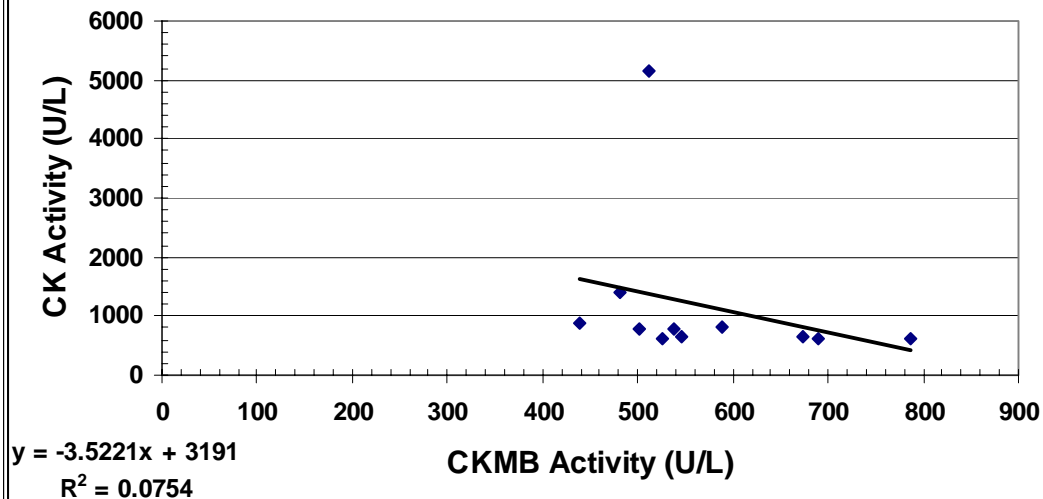


CK & CKMB in Female & Male Newborns

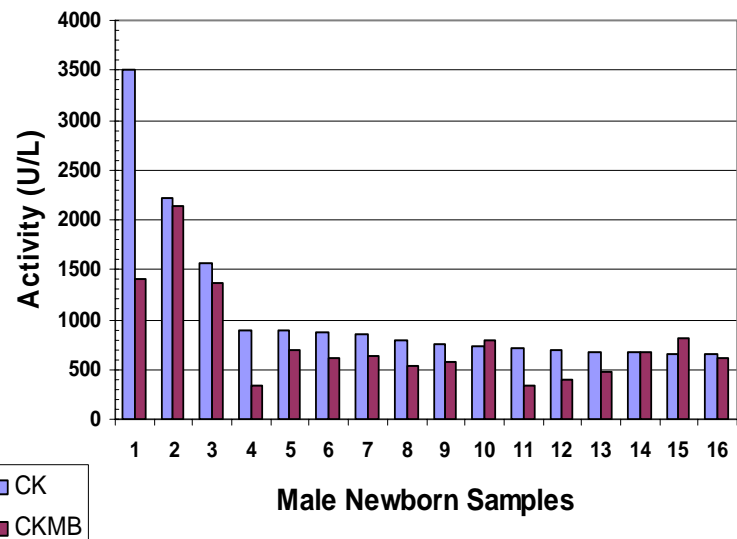
CK vs. CKMB Activity in Female Newborns



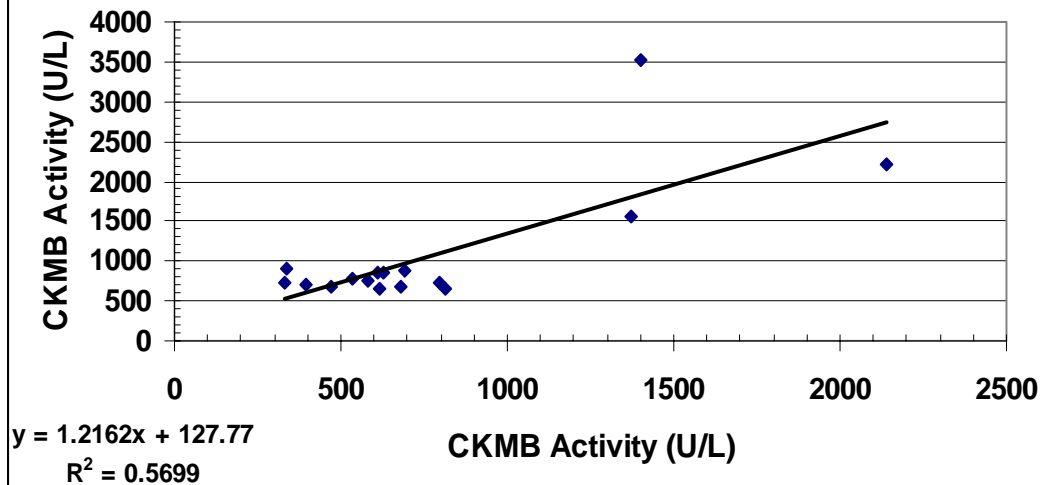
CK vs. CKMB in Female Newborns



CK vs. CKMB in Male Newborns



CK vs. CKMB in Male Newborns



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

Columbus Childrens Hospital Muscular Dystrophy Patients

Sample	Mutation	Time – tCK	Age / Hx	Diagnosis	ODH tCK
DOBS001	Exons 18-39 are deleted plus promoter DMDp260	10:06 – 31,825 16:30 – 38,228	7yo Ambulatory No steroids	DMD OF deletion	3,982 4,381
DOBS002	Exons 18-39 are deleted plus promoter DMDp260	10:05 – 26,576 16:22 – 33,077	7yo Ambulatory No steroids	DMD OF deletion	3,882 2,739
DOBS003	Exons 46 and 47 are deleted	Not done		DMD OF deletion	865 942
DOBS004	Exons 46-53 are deleted	10:30 – 4,338 15:15 – 4,787	10yo Ambulatory On steroids	DMD OF deletion	~1,000 1,379
DOBS005	Exons 4-30 are deleted plus promoter DMDp260	10:05 – 9,483 15:30 – 19,133	9yo Ambulatory On steroids	BMD? IF deletion	1,150 1,358
DOBS006	Exons 45-57 are deleted plus promoter DMDp116	09:25 – 871 15:15 – 985	22yo Not Ambul On steroids	BMD IF deletion	252 224
DOBS007	Exons 46-50 are deleted	09:08 – 2,362 15:25 – 2,291	15yo Not Ambul No steroids	DMD OF deletion	236 197
DOBS010	Exons 6-7 duplicated	10:35 – 14,191 14:20 – 13,415	10yo Ambulatory On steroids	DMD OF duplication	1,425 1,409

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.

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