CYP21A2 Variant Panel as a 2nd Tier for Congenital Adrenal Hyperplasia Newborn Screening: Metrics from the Minnesota Experience

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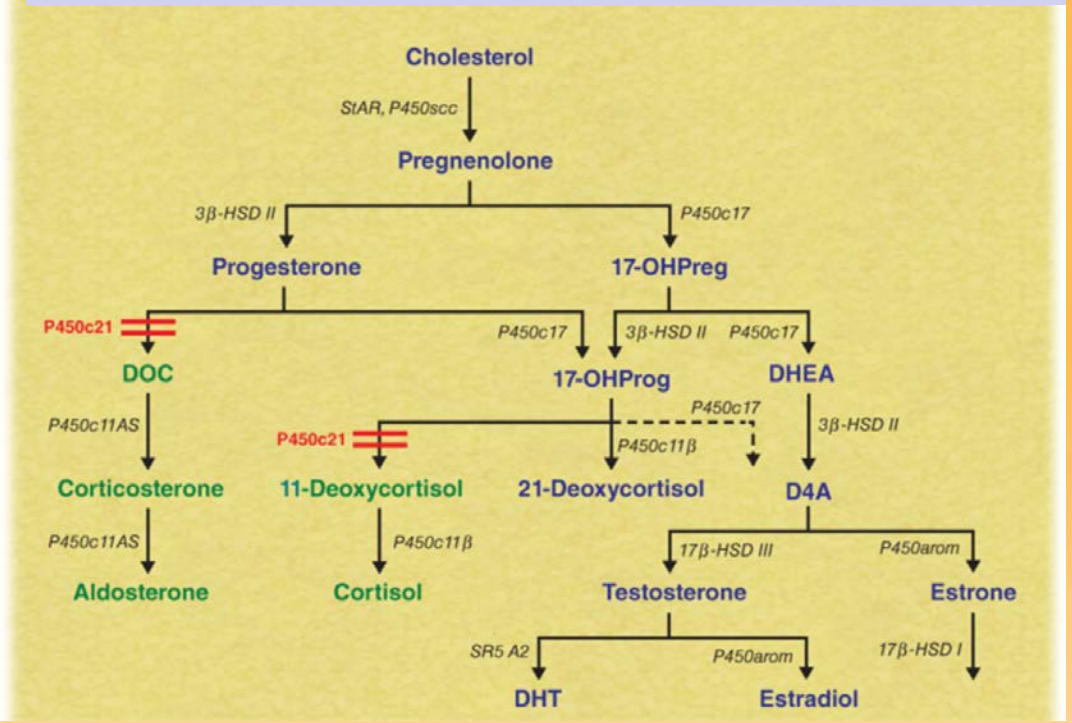
CAH due to 21-hydroxylase deficiency



Both salt wasting and simple virilizing forms of CAH can cause life threatening adrenal crises.

17-hydroxyprogesterone production is increased in CAH and is the metabolite measured in NBS for CAH.

Adrenal androgen production is increased as its synthesis does not require 21 hydroxylase enzyme

			CLINICAL PRESENTATION	
FORM	**FINDINGS**		**BIRTH**	**CHILDHOOD & ADOLESCENCE**
21-OHD (classic)	↑ K	↑ 17-OHP	Females: Always present at birth with ambiguous genitalia; virilization of external genitalia from clitoromegaly to phallic urethra	Males with salt-wasting CAH present at 2–3 weeks of life in salt-wasting crisis
	↓ Na	↑ Androgens		
Salt wasting	↑ PRA	↓ F		
	↓ Aldo			
Simple virilizing	nl-↑ PRA	↑ 17-OHP	Males: Normal external genitalia with hyperpigmentation of scrotum and postnatal virilization	Males with simple virilizing typically present in infancy or early childhood with growth acceleration and virilization
	nl-Lytes	↑ Androgens		
	nl-Aldo	↓ F		
21-OHD (non-classic)	nl-PRA	↑ 17-OHP	Normal genitalia in both sexes	May present with premature adrenarche, growth acceleration, bone advancement, PCO, irregular menses, acne, hirsutism, infertility
	nl-Lytes	↑ Androgens		
	nl-Aldo	nl-F		

Newborn Screening for CAH

- 4 million infants screened each year in U.S.
- Target is to identify classic forms (SW and SV)
- Blood spot on filter paper taken 24-48 hours of life in the U.S.
  - Europe >36 hours; U.K on day 5
- 17OHP measured by fluroimmunoassay (FIA)
  - Cutoff levels vary by state and assay kit

The Basics of NBS for CAH

- Screening protocols
  - One tier screening (FIA)
    - If equivocal, repeat screen
    - If positive, serum confirmatory testing by Peds Endo
  - Routine 2nd screening (FIA)
    - screen repeated 1-2 weeks later regardless of result of 1st screen
  - Two tier screening (FIA then either organic extraction or LC-MS/MS)
    - Initial screen (1st tier)
      - If positive or equivocal then,
    - 2nd tier screen automatically performed
      - **On original filter paper card**

Key Problems in Steroid Assays in NBS for CAH

- High false negative rate (FNR)
  - SV and SW are missed (22% missed by both FIA and LC-MS/MS methods in MN)
    - Sarafoglou K, Banks K, Kyllo J, Pittock S, Thomas W. Cases of congenital adrenal hyperplasia missed by newborn screening in Minnesota. *JAMA* 2012 Jun 13;307(22):2371-4.
- High false positive rate (FPR)
  - Especially in premature infants
    - Sarafoglou K, Banks K, Gaviglio A, Hietala A, McCann M, Thomas W. Comparison of one-tier and two-tier newborn screening metrics for congenital adrenal hyperplasia. *Pediatrics* 2012 Nov;130(5):e1261-8.
    - Sarafoglou K, Gaviglio A, Hietala A, Frogner G, Banks K, McCann M, Thomas W. Comparison of newborn screening protocols for congenital adrenal hyperplasia in preterm infants. *Journal of Pediatrics*. 2014 May;164(5):1136-40

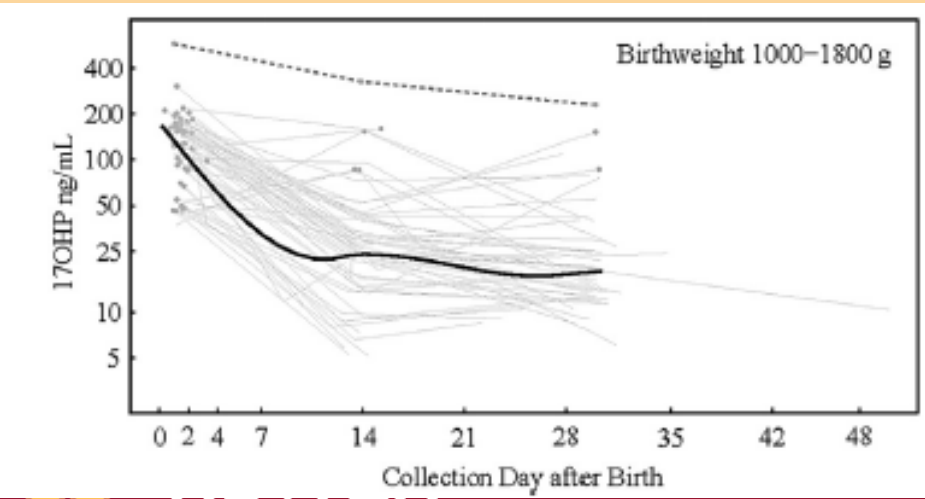
Why are FNR and FPR high with steroid assays in NBS for CAH?

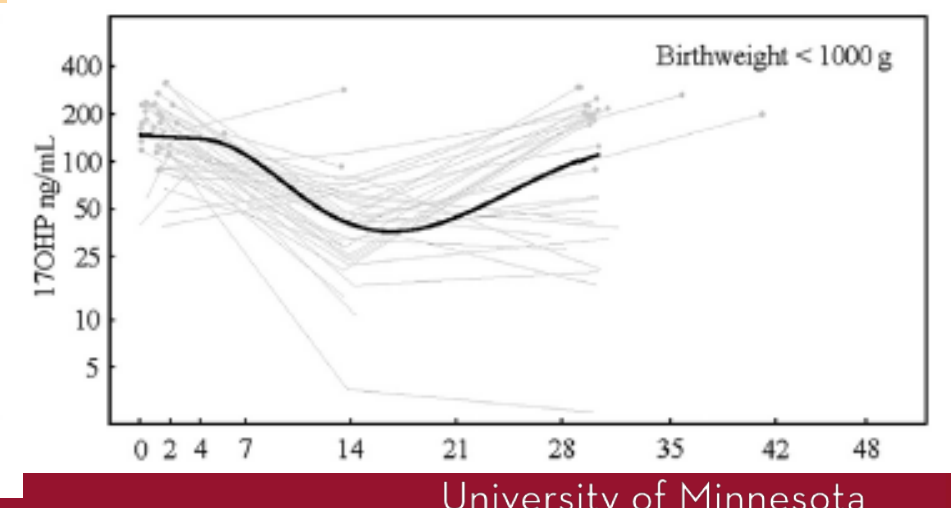
- 17OHP levels reflect the activity of the hypothalamic-pituitary-adrenal (HPA) axis at the time the DBS sample is collected
- HPA axis is highly dynamic and can be affected at birth by:
  - stress during delivery (↑ 17OHP)
  - immaturity of the adrenal glands (↑ 17OHP)
  - Increased fetal glucocorticoid sensitivity (↓ 17OHP)
  - Increased maternal cortisol during fetal life (↓ 17OHP)

- These factors may cause 17OHP levels to be ***transiently higher or lower*** at 24-48 hours when the DBS sample is collected.
- Therefore 17OHP levels measured by FIA or LC-MS/MS may not accurately represent the ***presence or absence*** of newborn's disease state at time of sample.

HPA axis activity at time of DBS collection is the problem and **NOT** the steroid assays

- FIA and LC-MS/MS report 17OHP levels at timing of sample. But if the HPA axis is causing transiently high or low 17OHP levels:
  - CAH-affected newborns may have 17OHP levels below the cutoff
    - Increased fetal glucocorticoid sensitivity (↓ 17OHP)
    - Increased maternal cortisol during fetal life (↓ 17OHP)
  - Unaffected newborns may have increased 17OHP levels
    - stress during delivery (↑ 17OHP)
    - immaturity of the adrenal glands (↑ 17OHP)





Sarafoglou K, Gaviglio A, Hietala A, Frogner G, Banks K, McCann M, Thomas W. Comparison of newborn screening protocols for congenital adrenal hyperplasia in preterm infants. Journal of Pediatrics. 2014 May;164(5):1136-40

What is needed for improvement of NBS for CAH?

- A newborn screening test that is not influenced by timing of sample collection, prematurity, birth stress, or cross reactivity with other steroids

Research Question

- Would molecular testing as a 2nd tier NBS test identify cases of CAH currently missed by NBS using steroid assays (FIA, LC-MS/MS, organic extraction)?

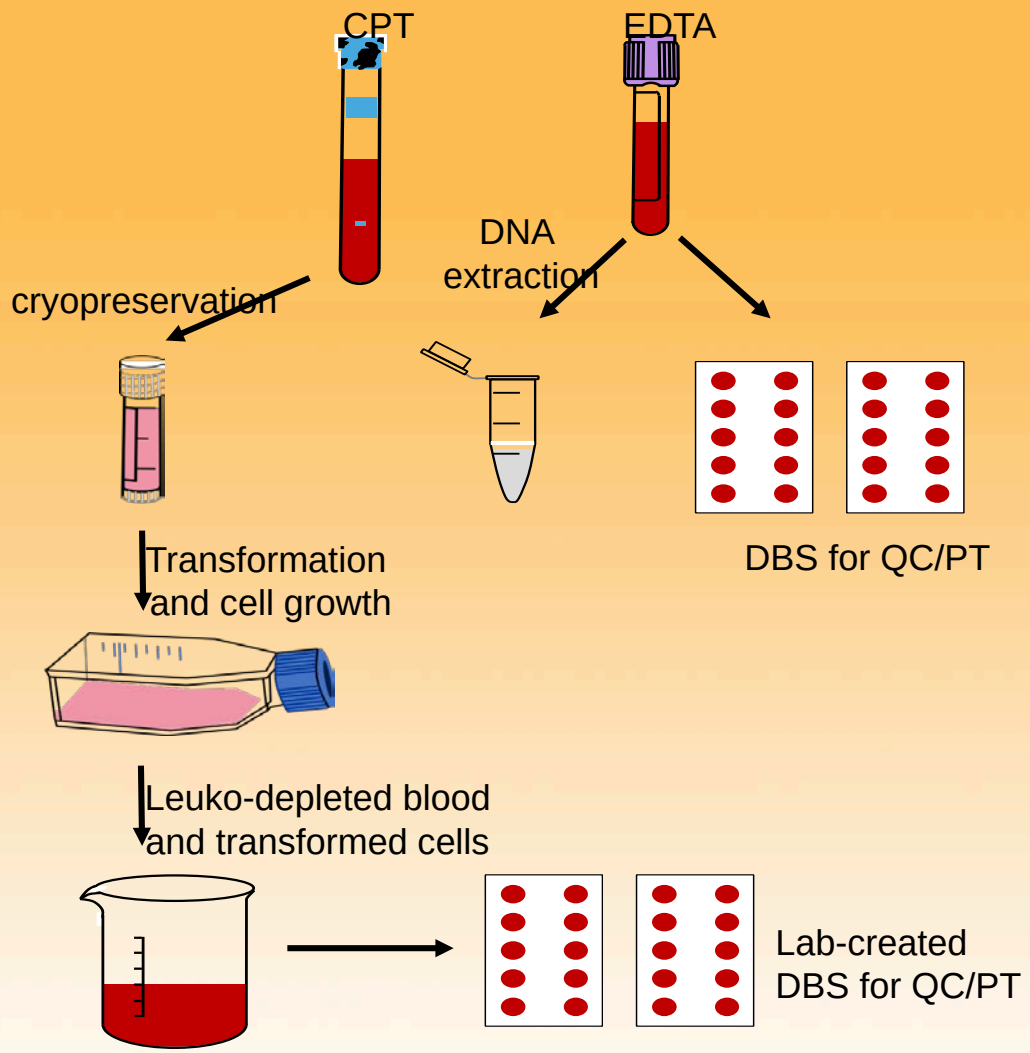
Federal, State and University Collaboration

- This question led to a unique collaboration between the Centers for Disease Control (CDC), Minnesota Department of Health, University of Minnesota, Mayo Clinic College of Medicine, and Children's Hospitals of Minnesota to examine molecular testing in the NBS for CAH

Objectives

1. Characterize the *CYP21A2* mutation spectrum in a large cohort of Minnesota CAH patients to create a Minnesota specific *CYP21A2* mutation panel
2. Have the CDC develop a mass screening assay that was accurate and reproducible in identifying mutations causing CAH disease from dried blood spots
3. Perform a 1 year retrospective analysis of molecular testing as a 2nd tier NBS test

Characterization of *CYP21A2* in MN



103 Minnesota families and 114 affected individuals including their parents were recruited for characterization of *CYP21A2* using a CDC developed assay long-range amplification-based method (LRAM) and complete gene Sanger sequencing of *CYP21A2* gene

Storage of specimens for future transformation to immortalized lines

Creation of dried blood spots for DNA mutation analysis by the CDC molecular assay for validation

Characterization of *CYP21A2* in MN

CYP21A2 Pathogenic Variant - Single	Predicted Phenotype	Count	MN Frequency	Finkielstain et al. Frequency[a]	New et al. Frequency[b]
p.Pro30Leu (c.92C>T)	NC	2	1.00	0.8	2.6
IVS2-13G (c.293-13A/C>G)	SW/SV	51	25.37	23.4	22.9
p.Gly110del8 (c.332_398del8)	SW	2	1.00	0.5	2.1
p.Ile172Asn (c.518T>A)	SV	29	14.43	12.6	8.2
p.Val281Leu (c.844G>T)	NC	17	8.46	12.6	23.9
p.Leu307fs (c.921ins1)	SW	1	0.50	0.3	---
p.Leu307Val* (c.922T>G)	SW	1	0.50	---	0.03
p.Gln318X (c.955C>T)	SW	2	1.00	3.3	3.5
p.Arg356Trp (c.1069C>T)	SW	10	4.98	3.6	3.6
p.His365Tyr* (c.1096C>T)	SW	1	0.50	0.8	0.067
IVS9+1T* (c.1219+1G>T)	SW/SV	1	0.50	---	---
p.Gly424Ser* (c.1269G>A)	SV	1	0.50	---	0.03
p.Arg426Cys* (c.1276C>T)	SW/SV	1	0.50	0.3	0.03
p.Arg426Pro* (c.1277G>C)	SW/SV	1	0.50	0.3	---
p.Arg444X* (c.1330C>T)	SW	1	0.50	0.3	0.03
p.Arg453Ser (c.1360C>T)	NC	2	1.00	0.5	0.57
p.Arg483fs* (c.1448_1449delGGinsC)	SW	1	0.50	---	
CYP21A2 Variants in cis	**Predicted Phenotype**	**Count**	**MN Frequency**	**Finkielstain et al. Frequency[a]**	**New et al. Frequency[b]**
p.His62Leu* - p.Pro453Ser (c.185A>T - C1360C>T)	NC	2	1.00	---	---
IVS2-13G - p.Val281Leu (c.293-13A/C>G - C.844G>T)	SW/SV	1	0.50	---	---
IVS2-13G - p.Pro453Ser (c.293-13A/C>G - C.1360C>T)	SW/SV	3	1.49	---	---
p.Arg149Cys* - p.Val281Leu (c.445C>T - c.844G>T)	NC	1	0.50	---	---
p.Ile236Asn - p.Val237Glu - p.Met239Lys - p.Val281Leu - p.Leu306fs (c.710T>A - C.713T>A - C.719T>A - c.844G>T - c.921ins1)	SW	1	0.50	---	---
p.Ile172Asn - p.Val281Leu (c.518T>A - c.844G>T)	SV	1	0.50	---	---
p.Ile172Asn - p.Arg453Ser (c.518T>A - c.1360C>T)	SV	2	1.00	---	---
p.Val281Leu - p.Leu306fs - p.Gln318X - p.Arg356Trp (c.844G>T - c.921ins1 - c.955C>T - c.1069C>T)	SW	1	0.50	---	---
p.Leu306fs - p.Gln318X - p.Arg356Trp (c.921ins1 - c.955C>T - c.1069C>T)	SW	1	0.50	---	---
CYP21A2 Gene Recombinants	**Predicted Phenotype**	**Count**	**MN Frequency**	**Finkielstain et al. Frequency[a]**	**New et al. Frequency[b]**
30kb Deletion	SW	57	28.36		
Possible A2 Deletion	SW	7	3.48	30.5	20
Large Gene Conversion (I172N-I236N-V337E-M239K-V281L-F306fs)	SW	1	0.50		

NC, Non-Classic; SW, Salt-Wasting; SV, Simple Virilizing

*Variants detected in Minnesota Family samples that are not present on core 12 *CYP21A2* variant panel

[a]Finkielstain et al. (2011) Comprehensive genetic analysis of 182 unrelated families with CAH due to 21-hydroxylase deficiency. J Clin Endocrin Metab 96, E161–172.

[b]New et al. (2013). Genotype–phenotype correlation in 1,507 families with CAH owing to 21-hydroxylase deficiency. PNAS 110.7, 2611-16.

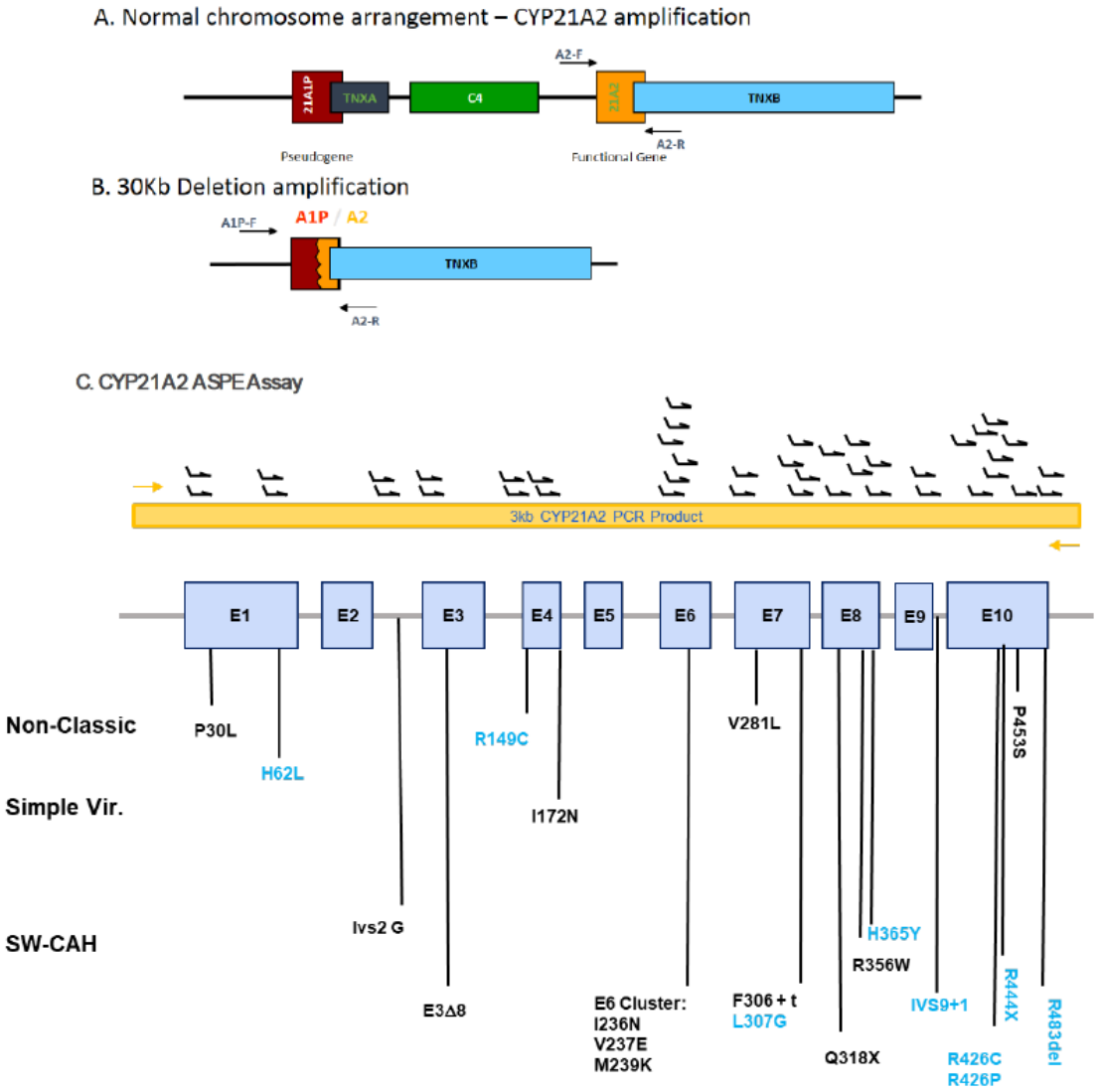
Minnesota specific *CYP21A2* panel

***CYP21A2* Allele-Specific Primer Extension Assay Genotyping Panel**			
Common Diagnostic panel	**Gene Position**	**Allele based on Ref Sequence NM_00500.6**	**Predicted Clinical manifestation**
p.Pro30Leu	Exon 1	c.92C>T	NC
IVS2-13 A/C>G	Intron 2	c.293-13A/C>G	SV-SW
p.Gly110del8	Exon 3	c.332_339del8	SW
p.Ile172Asn	Exon 4	c.518T>A	SV
p.Ile236Asn	Exon 6	c.710T>A	SW
p.Val237Glu	Exon 6	c.713T>A	SW
p.Met239Lys	Exon 6	c.719T>A	Benign
p.Val281Leu	Exon 7	c.844G>T	NC
p.Leu307fs	Exon 7	c.921Ins1	SW
p.Gln318X	Exon 8	c.955C>T	SW
p.Arg356Trp	Exon 10	c.1069C>T	SW
p.Pro453Ser	Exon 10	c.1360C>T	NC
Additional Minnesota Patient Pathogenic Variants:	**Gene Position**	**Allele Bases on Ref Sequence NM_00500.6**	**Clinical Manifestation**
p.His62Leu	Exon 1	c.185A>T	NC-SV
p.Arg149Cys	Exon 4	c.445C>T	NC
p.Leu307Val	Exon 7	c.922T>G	SW
p.His365Tyr	Exon 8	c.1096C>T	SW
IVS9+1 G>T	Intron 9	c.1219+1G>T	Predicted SW
p.Arg426Cys	Exon 10	c.1276C>T	SW
p.Arg426Pro	Exon 10	c.1277G>C	SV
p.Arg444X	Exon 10	c.1330C>T	SW
p.Arg483fs	Exon 10	c.1448_1449delGGinsC c.1448_1449delGG	SW

Development of CAH 2nd tier molecular assay by the CDC

- The next step was the development of a molecular CAH assay that combines a PCR detection assay for common *CYP21A2* gene deletions and a multiplex 21-variant Allele Specific Primer Extension (ASPE) assay to detect pathogenic variants identified in Minnesota families

CAH Molecular 2nd-Tier Assay



The CAH molecular assay consists of a Luminex-based method that detects 21 *CYP21A2* mutations and gene conversions, as well as a QIAxel capillary electrophoresis method to detect the 30 kb mutations.

***CYP21A2* Amplification**: Most common chromosomal arrangement of the *CYP21A2* functional gene and *CYP21A1P* pseudogene (98% identical). *CYP21A2* and *CYP21A1P* are present as direct repeats on chromosome 6. The functional gene is amplified using a *CYP21A2* specific forward and reverse primers (A2-F and A2-R).

30kb Deletion Amplification: The 30Kb deletion alleles are a fusion of *CYP21A1P* and *CYP21A2* resulting from homologous recombination. The 30 kb deletion alleles are PCR amplified using a *CYP21A1P* specific forward primer and *CYP21A2* gene reverse primer (A1P-F and A2-R).

***CYP21A2* ASPE Assay:** ASPE detection of 21 *CYP21A2* variants found in the Minnesota population. The *CYP21A2* gene is PCR amplified as a ~3kb fragment and 43 allele specific primers are used in a primer extension genotyping assay with detection on Luminex instruments. Primer extension probes are shown above the PCR fragment, and the location of each variant is indicated by gene position, and CAH phenotype in the vertical dimension. Common CYP21A2 alleles commonly included in clinical testing panels are in indicated in black and alleles detected from Minnesota family characterization are indicated in blue.

Validation of CDC 2nd tier molecular assay

- The *CYP21A2* pathogenic variants and resulting genotypes identified by the ASPE assay demonstrated 100% accuracy and reproducibility on 900 DBS tested compared to the genotypes identified by complete *CYP21A2* gene sequencing of the respective blood ***(Please see CDC poster #P-027 for more details on the creation and validation of the assay)***
- The molecular assay was transferred to Minnesota Department of Health

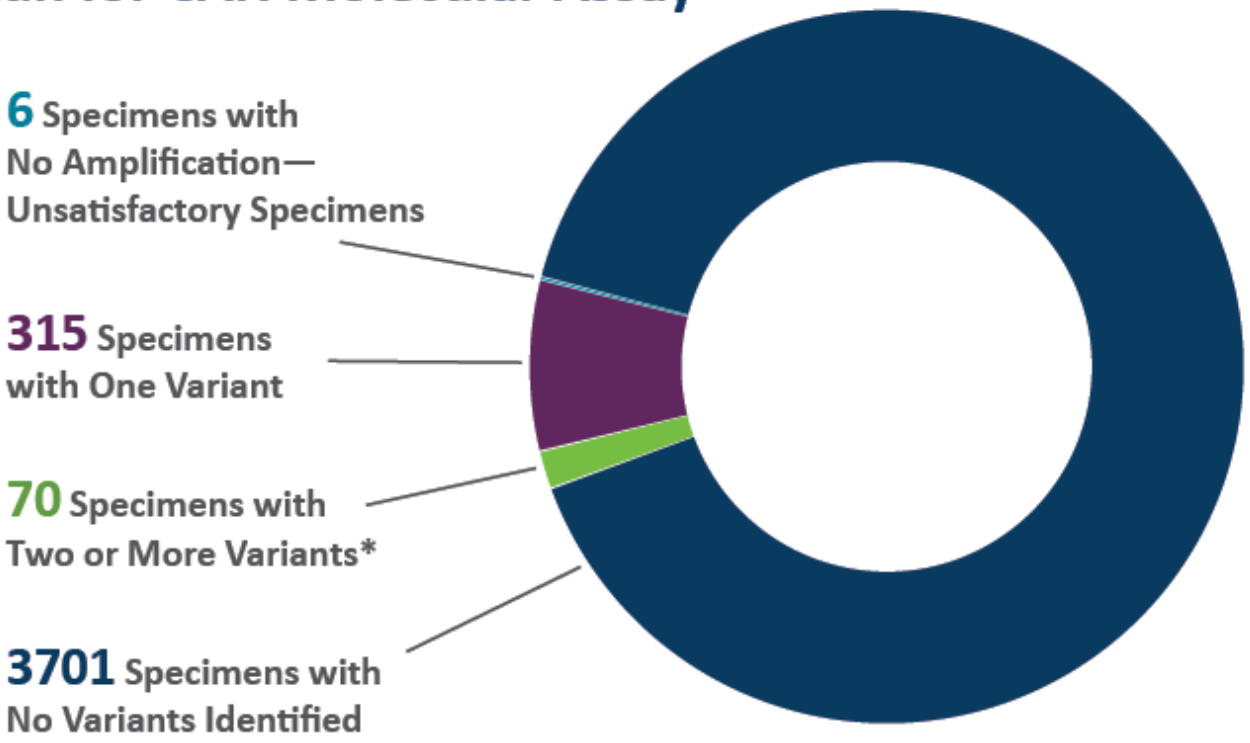
Method for Retrospective Study

- MDH performed a one-year retrospective analysis of 2nd tier molecular testing of de-identified specimens with elevated 17OHP levels.
- Used a 17-OHP cutoff level (measured by FIA) that was decreased by at least 33% for each birth weight category bringing the cutoff levels below values previously associated with false-negative CAH cases in Minnesota.

Table 3: Proposed and Current 1st-Tier 17OHP Cut-offs Stratified by Birthweight

Birthweight	Proposed 17OHP Cut-off	Current 17OHP Cut-off
<1500 g	≥53 ng/mL	≥100 ng/mL
1500-2499 g	≥30 ng/mL	≥45 ng/mL
≥2500 g	≥16 ng/mL	≥30 ng/mL

4092 Retrospective Specimens Run for CAH Molecular Assay



*1 Confirmed Positive and 2 False Negative Specimens Identified

CDC performed full Sanger sequencing that had one or more variants in order to confirm MDH findings

- Of the 2 false negative cases missed by MN's current steroid assay, one case was one missed by the 1st-tier assay (17OHP value 28.3 ng/mL) and one missed by 2nd tier assay (17OHP value 12.50 ng/ml). Both were identified by the 2nd tier molecular assay.

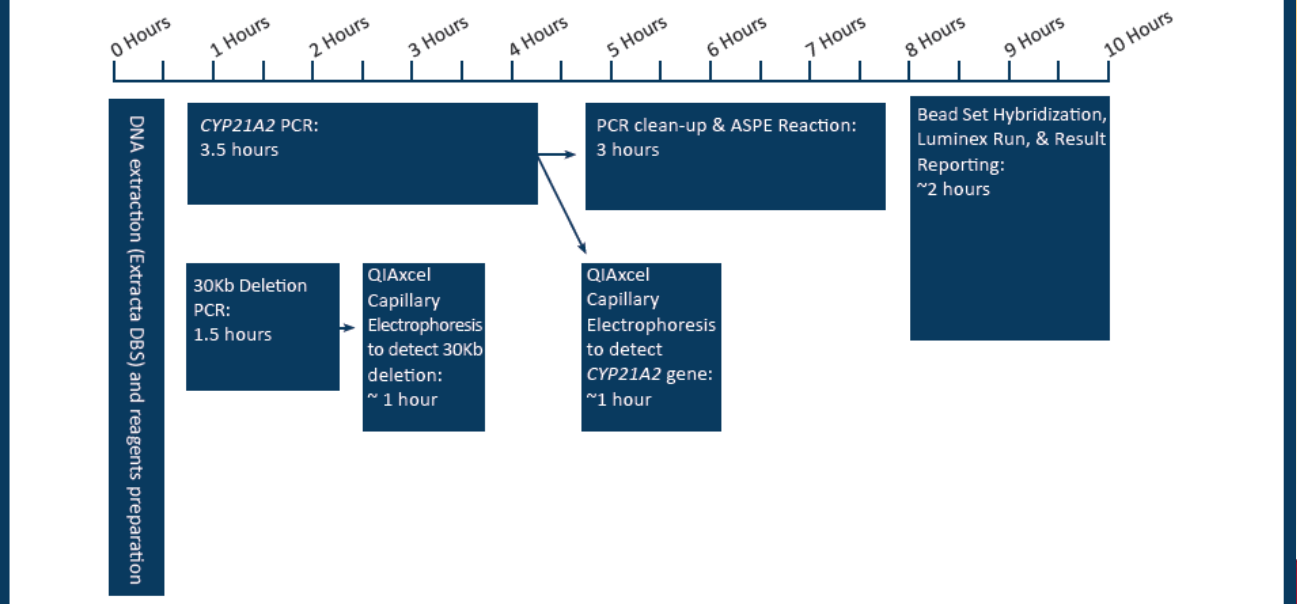
Results of 1 year retrospective analysis

- 2nd -tier molecular testing would have decreased the FPR by 32% (67 FPs molecular assay vs 99 steroid assay) if samples with two or more pathogenic variants were deemed positive and referred for diagnostic evaluation.
- The FPR for premature babies in the <2500 gram category (13 vs. 80 false positives) would also have been reduced under the two or more pathogenic variants scenario.
- Found much higher number of specimens with single variant (315 specimens) than projected (72 specimens expected based on MN's carrier rate of 1:57)
- FPR would be higher in infants with a single variant and present challenges for implementation of 2nd tier molecular testing into state NBS programs ***(please see MDH's poster #P-077 for a detailed discussion of these issues)***

Results

- The assay workflow was approximately 10 hours from start to finish with 1-2 laboratory staff. Staggered work hours could potentially allow the assay to be completed in 1 day.

Assay Workflow and Timeline



- The assay cost was estimated to be around $25 per specimen. MDH would reflex around 4% of the population for the CAH molecular assay.

Conclusions

- Evaluation of CAH molecular 2nd-tier assay shows both high specificity and sensitivity
- Correctly identified >0.999 of all ASPE *CYP21A2* genotypes
- 100% agreement with deletion detection between MDH and CDC
- Low incidence of individual chromosomes with severe CAH alleles not on variant panel of 0.00131 (1/762 chromosomes)

Conclusions

- The number of infants with single variants was higher than expected. Possible reporting mechanisms:
  - Request a Repeat Dried Blood Spot—Approximately 0.4%
  - Complete Sequencing of the CYP21A2 Gene as a 3rd Tier Assay
  - Report Single Variants as Potential Carriers with an Assumption of Risk Based on Coverage of Panel
- An in-depth reporting infrastructure needs to be created to handle the additional molecular testing results.
- A review of the utility of the CAH molecular assay in a two-screen state and further research in establishing a comprehensive panel for diverse state populations is warranted.

Future Directions

- Novel State/Federal/Academic collaboration is a model for future NBS molecular test development
- Development of chromosomal phasing assay from DBS specimens to eliminate need for family testing
  - 12 specimens with greater than 2 variants and phasing test can determine if all variants on the same or separate chromosomes
- Define common *CYP21A2* haplotypes for assay interpretation
- Development of Version 2 molecular CAH assay to include modified ASPE probes and new *CYP21A2* alleles

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