Development of a Multiplex CYP21A2 Genotyping Assay for Congenital Adrenal Hyperplasia Screening

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Forms of Congenital Adrenal Hyperplasia (CAH)

- **Classic CAH**
  - Salt Wasting: Severe to complete loss of 21OH activity
    - Elevated Stress response
    - Loss of electrolyte homeostasis
    - Adrenal crisis can lead to hypotension and cardiac arrest
  - Simple Virilizing: Partial 21OH activity
    - Normal sodium balance
    - Elevated androgen production, partial to complete masculinization in females
- **Non-Classic CAH**
    - Late-onset: slight decrease of 21OH activity
    - Not life-threatening but results in significant quality of life issues

Primary CAH Newborn Screen

- Primary newborn screening assay by time-resolved fluoroimmunoassay (FIA) for 17-α OHP
- FIA high false-positive rate
  - 17-α OHP levels are high in premature and/or stressed babies
    - Birth weight or gestational age stratification for 17OHP cut-offs
    - Second specimen screening programs
    - Second-tier LC-MS/MS steroid profiling
  - Lack of specificity with FIA
    - Second-tier organic extraction of DBS

Rationale for CAH Molecular Second Tier

- Minnesota Department of Health identified classic CAH children missed by newborn screening
- Many of false negative results had 17OHP levels below FIA assay cutoff
- Steroid profiling and organic extraction 2nd-tier methods reduce false positive rate but do not improve false negative rate

Pilot to test if lowered 17OHP cutoffs and 2nd-tier mutation detection increases overall sensitivity for detecting CAH while retaining assay specificity

CAH Molecular Second Tier Screening Study

- "Can molecular testing improve newborn screening performance and outcomes for CAH?"
  - University of Minnesota Masonic Children's Hospital
  - Minnesota Department of Health
  - CDC's Newborn Screening & Molecular Biology Branch
  - Project funded by 

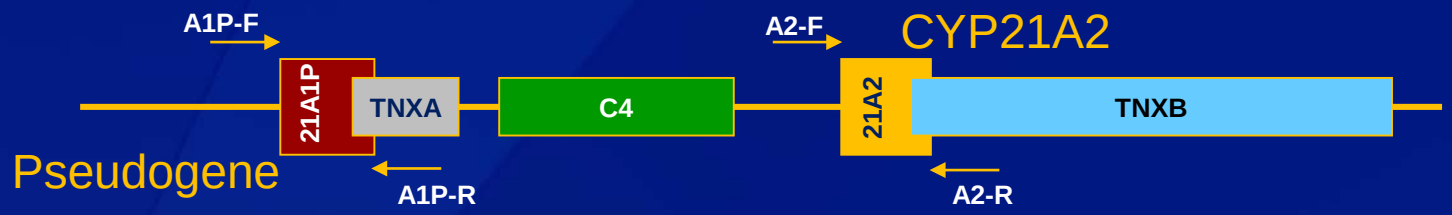
- Three major goals:
  - Determine Minnesota population CYP21A2 mutation panel
  - Develop genotyping assay appropriate for NBS laboratory
  - Pilot test molecular second-tier method and evaluate assay performance and cost effectiveness

Goal 1: CYP21A2 Mutations in Minnesota

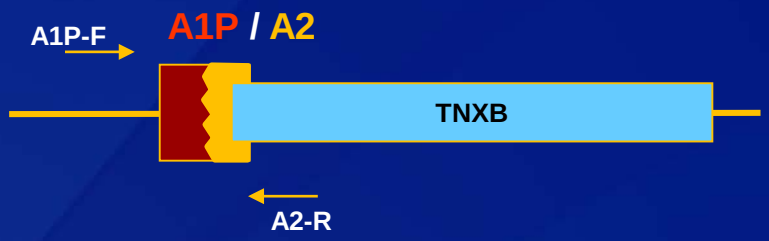
- Enrolled 83 families affected by CAH
  - 200 total specimens
  - ~70% with prior genotype information
- Long-range PCR and DNA sequencing
  - Confirm CYP21A2 genotypes and genotype unknown samples
  - Characterized 30kb Deletion and Gene Conversion samples
- Identified 22 CYP21A2 mutations
  - 12 common diagnostic panel mutations
  - 10 additional non-panel mutations
  - Novel IVS9+1 G >T splice site mutation

PCR-Based Detection of Chromosome Deletion and Gene Conversion Alleles

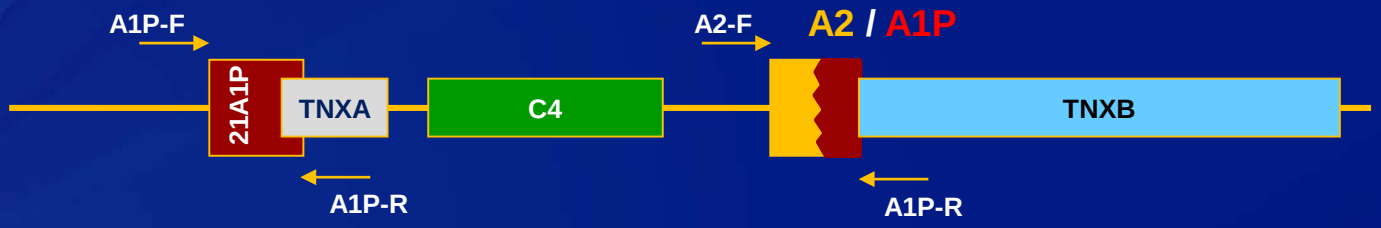
Most-common chromosome arrangement in normal population



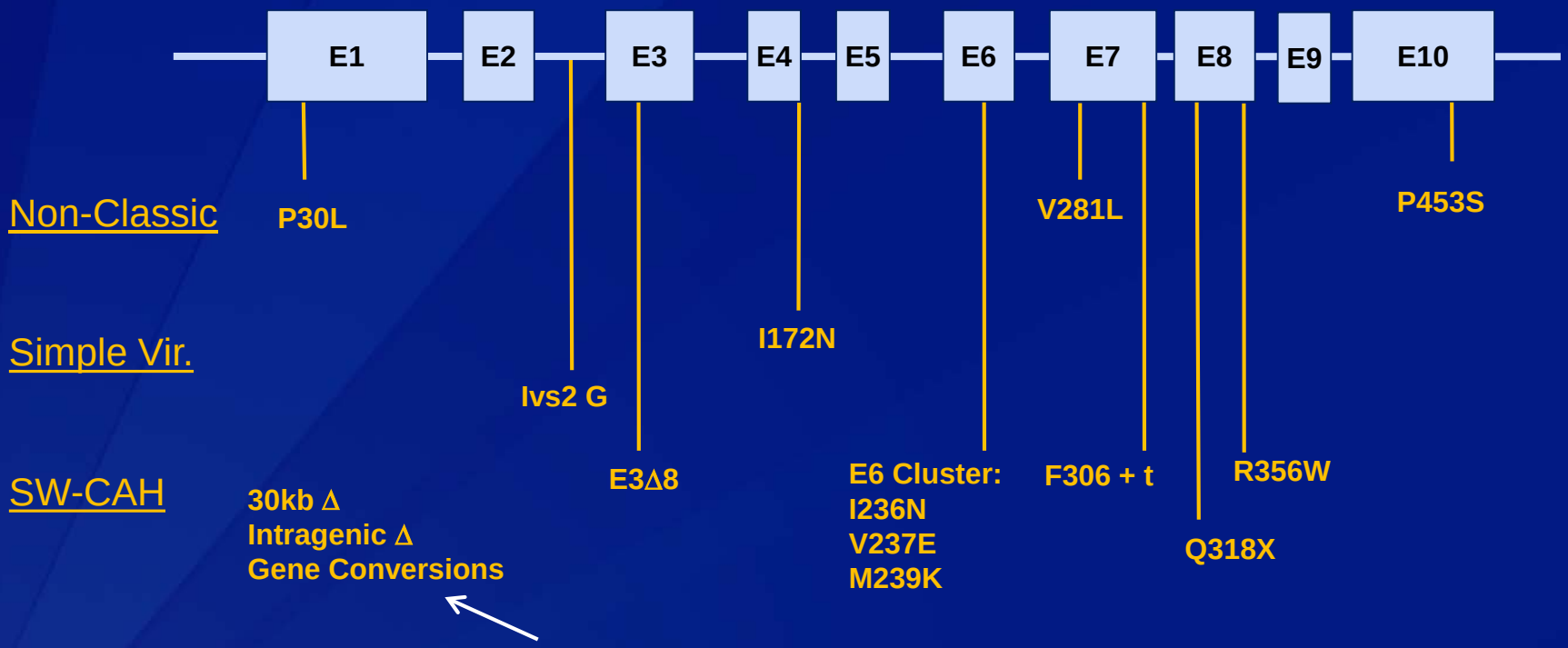
30Kb Deletion



Gene Conversion

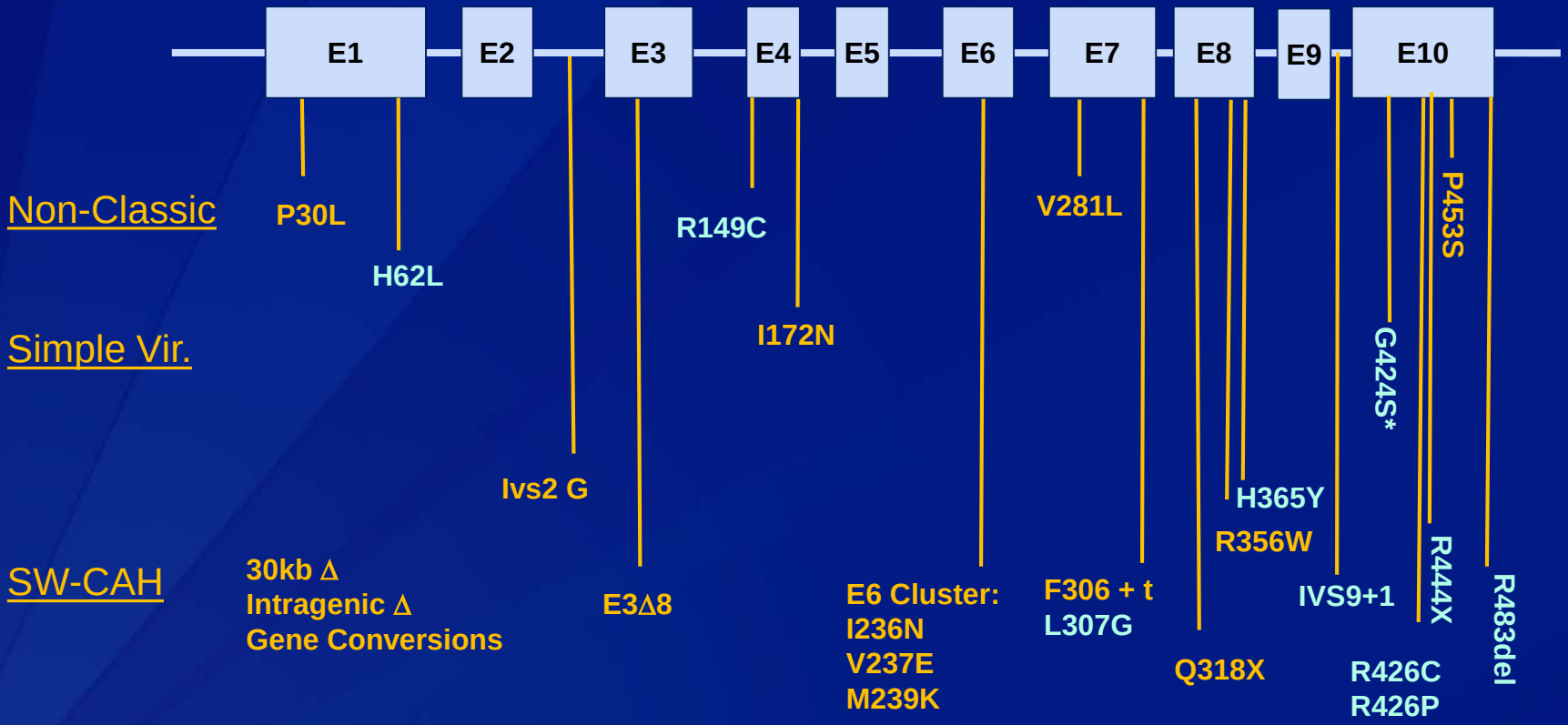


Common Diagnostic CYP21A2 Mutation Panel



Recombination events account for ~30% of CAH-causing mutations

Expanded Minnesota CYP21A2 Mutation Panel



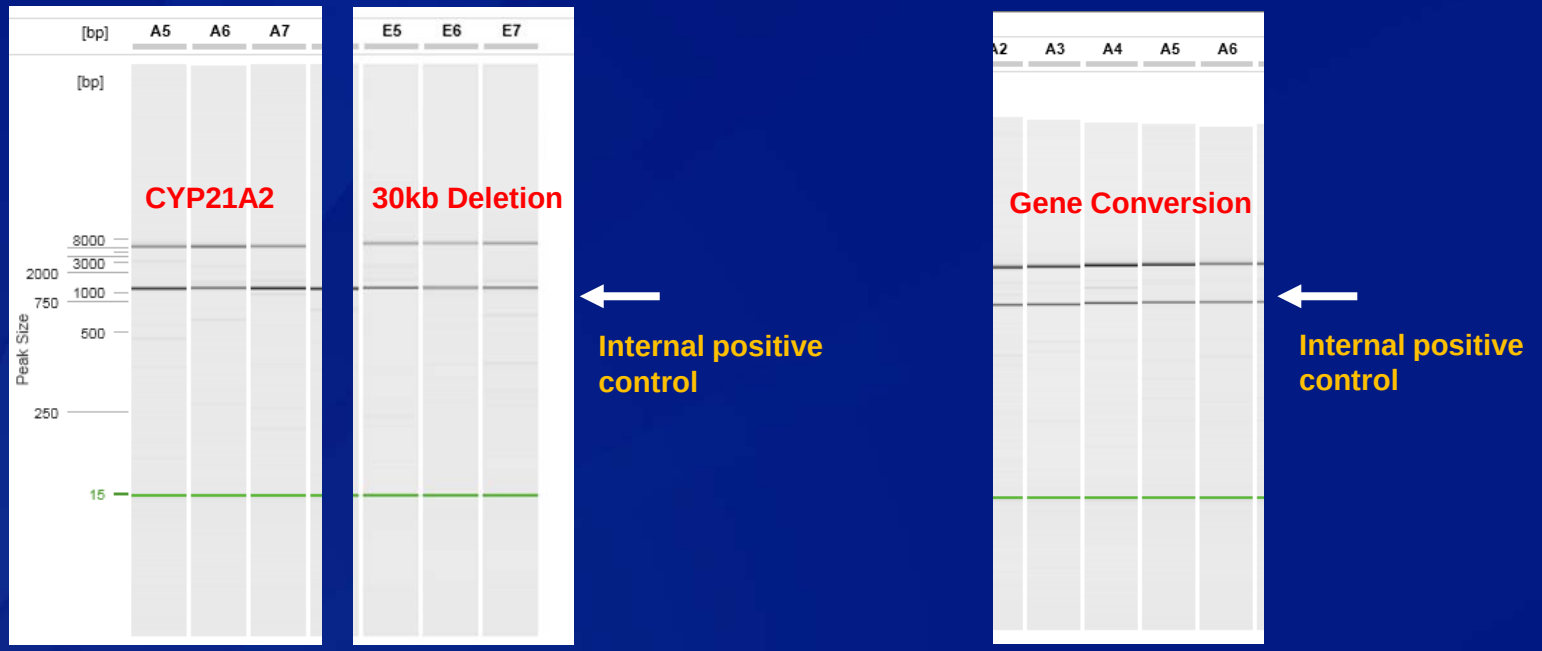
Poster 60: Minnesota Population Spectrum of Congenital Adrenal Hyperplasia Causing Mutations in the CYP21A2 Gene; Detwiler

Goal 2: NBS Genotyping Method Development

- Detection of 30 kb deletions and gene conversion alleles
  - Benchtop capillary electrophoresis and automated data capture
- Multiplex mutation detection method
  - Allele-Specific Primer Extension for 21 mutations
  - Luminex instrument also used for CFTR at MDH laboratory
- Validation and accuracy of lab-developed method compared to provided genotypes
  - Sensitivity
  - Specificity
  - Positive Predictive Value and Negative Predictive Value

PCR-Based Detection of 30kb Deletions and Gene Conversions

Automated capillary electrophoresis and data capture of CYP21A2 functional gene, 30kb Deletion, and Gene Conversion alleles



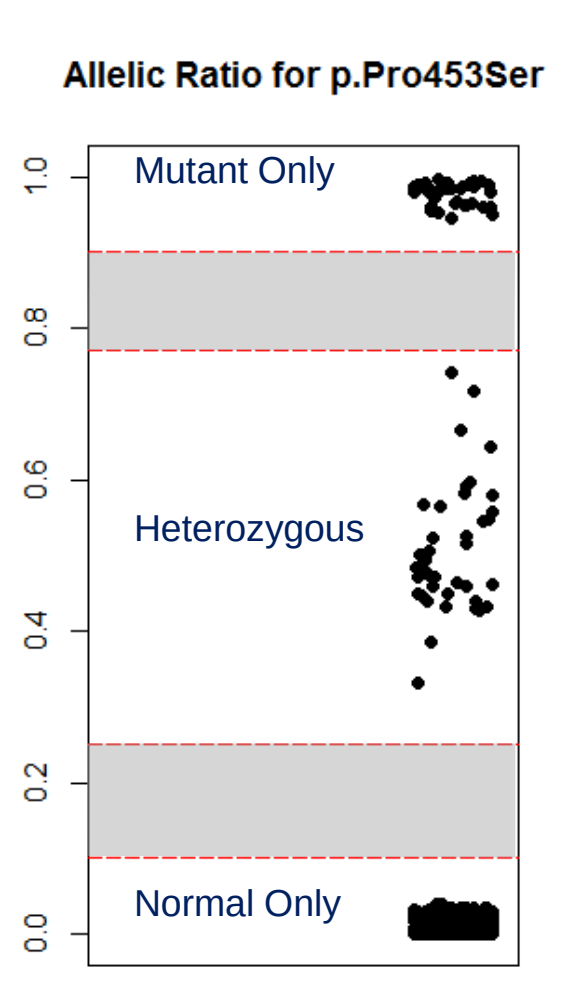
100% reproducibility for same day and between day repeats

Performance of PCR-Based Assay to Detect 30kb Deletions and Gene Conversions

	CYP21A2 Detection	30kb Deletion Detection
True Positives	131	59
True Negatives	9	122
False Positives	0	0
False Negatives	1*	2**
Sensitivity	0.992 (0.958 – 0.999; 95% CI)	0.967 (0.881 – 0.991; 95% CI)
Positive Predictive Value	100%	100%
Negative Predictive Value	90%	93.8%

*CYP21A2 false negative paired with 30kb deletion
**Both 30kb false negatives with potential hemizygous CYP21A2 allele

Allelic Ratios for Robust ASPE Genotyping



- Normalized plot representing the signal for each allele

$$\frac{\text{Mutant signal}}{(\text{Mutant signal} + \text{Normal signal})}$$

- Luminex default ratio values
  - $0.75 \leq 1.00$ = Mutant only
  - 0.25 to 0.75 = Heterozygous
  - $0.00 \leq 0.25$ = Normal only
- Final ratios determined empirically for each probe using inter and intra day repeats

Performance of Genotyping Assay to Detect CYP21A2 Mutations

- **Highly specific and accurate on initial test of 190 patient and family samples**
  - No False Positive Calls
  - No False Negative Calls
- **186 samples passed for all probe sets (97.9%)**
  - 4 out of 190 samples gave an equivocal result
    - 3 normal specimens with EQ-Low for p.Arg426Cys
    - 1 specimen EQ-Low IVS2-13 A/C > G and EQ-High p.Ile172Asn
  - 99.9% robustness with 100% accuracy per genotype

Conclusions and Next Steps

- **CYP21A2 genotyping method is sensitive and accurate**
- **Method transferred in February 2016 to MDH**
- **Validation for use with newborn DBS ongoing**
- **MDH pilot of molecular second tier to start in 2016**
  - Describe assay efficacy and utility in newborn screening
  - Analyze cost effectiveness

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CYP21A2 Genotyping Assay Workflow