Molecular Methods Used in NBS Genotyping

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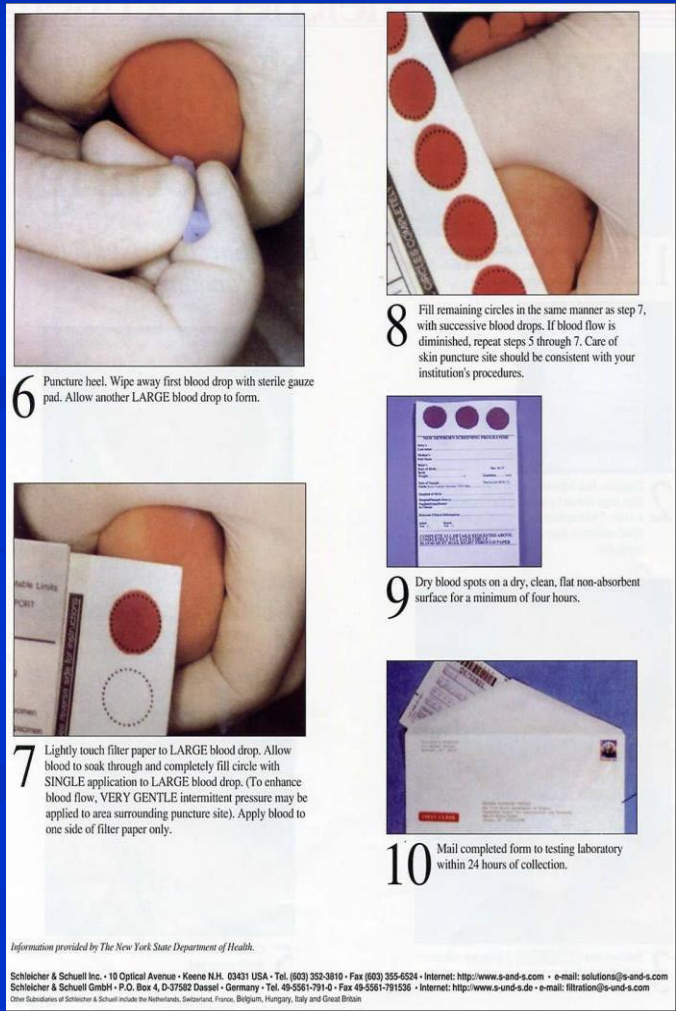
APHL-CDC

Dried Blood Spot (DBS) "Guthrie" Card

- S&S® 903™ Cotton Paper
- 3.1 mm DBS ~ 3.1 uL whole blood.
- Benefits:
  - Rapid absorption (~ 10 seconds)
  - Easy transportation
  - Blood constituents "easily" eluted
  - Typeable DNA from 3-15 years.

- > 1600 articles in PubMed
- > 1 Million Google "hits"



Clin Chem 59:7; 1045-1051 (2013)

Cost-Effective and Scalable DNA Extraction Method from Dried Blood Spots.

Saavedra-Matiz CA, Isabelle JT, Biski CK, Duva SJ, Sweeney ML, Parker AL, Young AJ, Diantonio LL, Krein LM, Nichols MJ, Caggana M.

Source

Newborn Screening Program, Division of Genetics, Wadsworth Center, New York State Department of Health, Albany, NY.

Abstract

BACKGROUND: Dried blood spot (DBS) samples have been widely used in newborn screening (NBS) for the early identification of disease to facilitate the presymptomatic treatment of congenital diseases in newborns. As molecular genetics knowledge and technology progresses, there is an increased demand on NBS programs for molecular testing and a need to establish reliable, low-cost methods to perform those analyses. Here we report a flexible, cost-efficient, high-throughput DNA extraction method from DBS adaptable to small- and large-scale screening settings.METHODS: Genomic DNA (g.DNA) was extracted from single 3-mm diameter DBS by the sequential use of red cell lysis, detergent-alkaline, and acid-neutralizing buffers routinely used in whole blood and plant tissue DNA extractions. We performed PCR amplification of several genomic regions using standard PCR conditions and detection methods (agarose gel, melting-curve analysis, TaqMan-based assays). Amplicons were confirmed by BigDye® Terminator cycle sequencing and compared with reference sequences.RESULTS: High-quality g.DNA was extracted from hundreds of DBS, as proven by mutation detection of several human genes on multiple platforms. Manual and automated extraction protocols were validated. Quantification of g.DNA by Oligreen® fluorescent nucleic acid stain demonstrated a normal population distribution closely corresponding with white blood cell counts detected in newborn populations.CONCLUSIONS: High-quality, amplifiable g.DNA is extractable from DBSs. Our method is adaptable, reliable, and scalable to low- and high-throughput NBS at low cost ($0.10/sample). This method is routinely used for molecular testing in the New York State NBS program.

Clin Chem 59:7; 1011-1013 (2013) **Editorials**

Newborn Screening by Sequence and the Road Ahead.

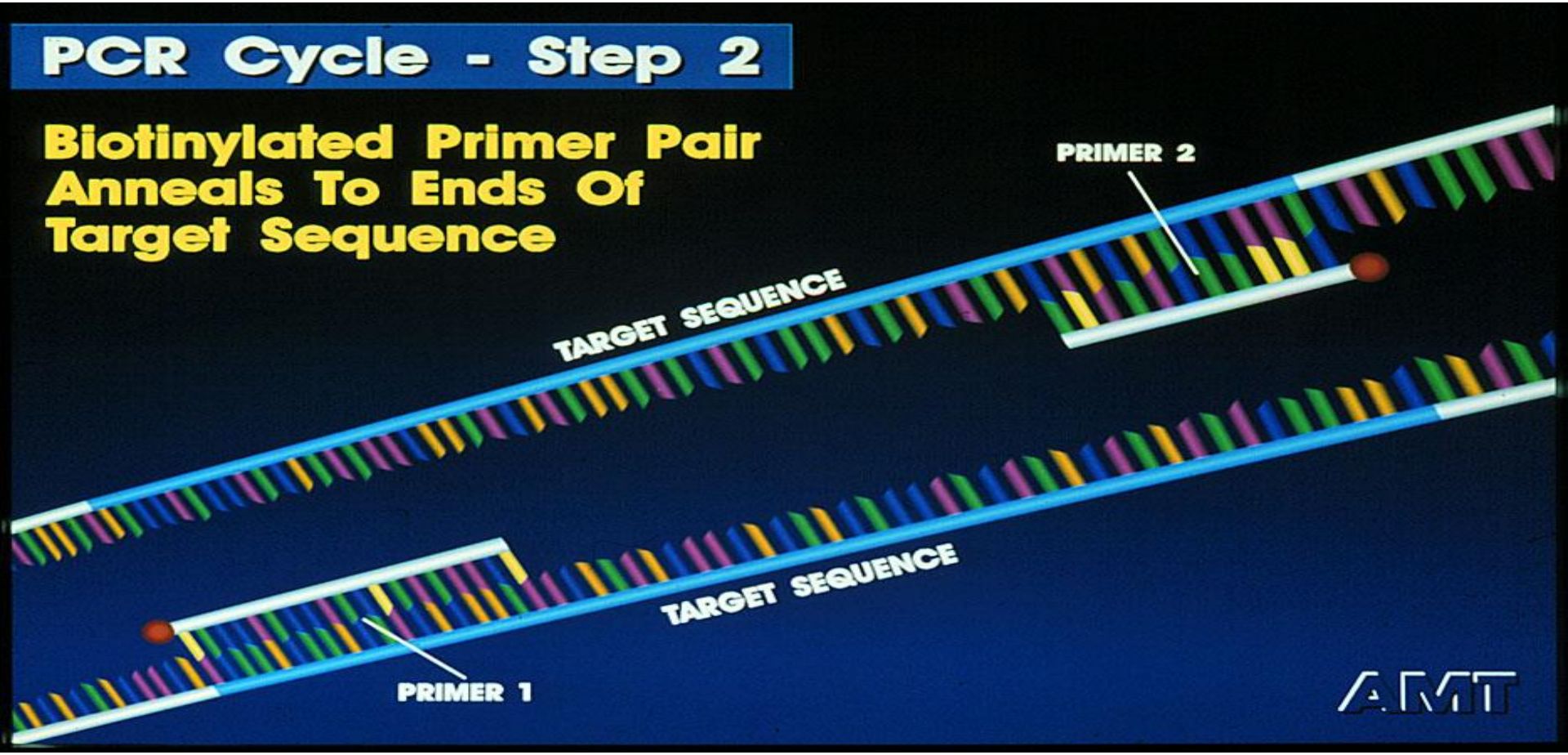
Sondheimer N.

Source

Department of Pediatrics, University of Pennsylvania, and Section of Biochemical Genetics, Children's Hospital Philadelphia, Philadelphia, PA.

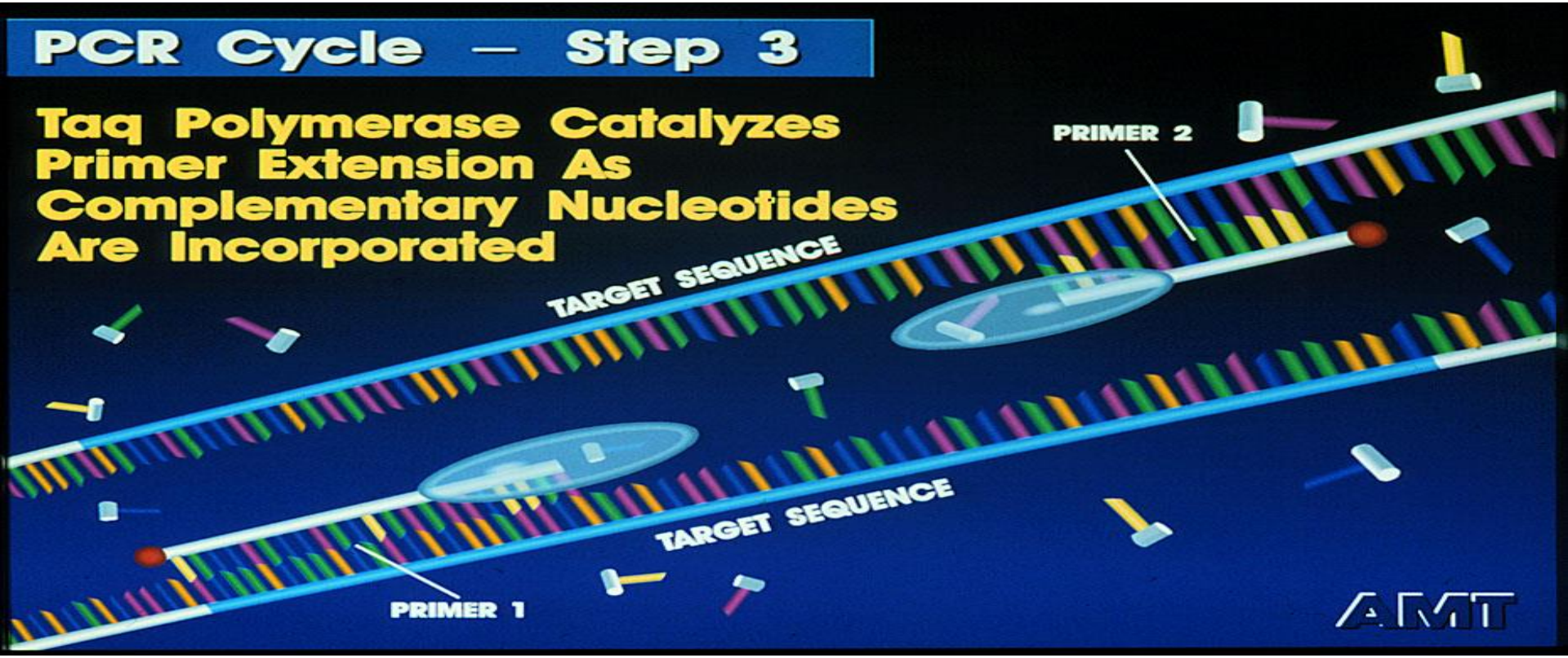
- **Heat denaturation typically 95 degrees C.**
- **Provides sufficient energy to break hydrogen bonds.**

POLYMERASE CHAIN REACTION



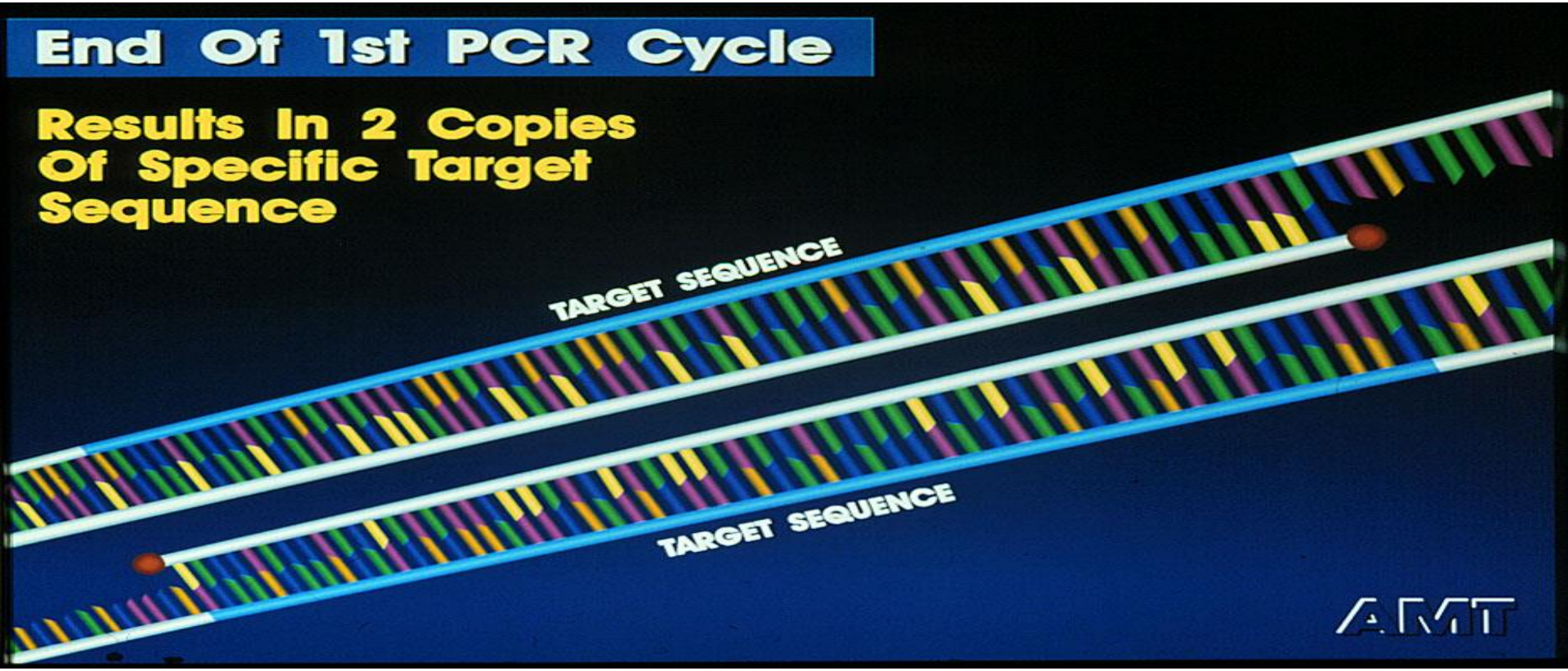
- Primer annealing typically 55-65 degrees C.
- Takes advantage of base complementarity.

POLYMERASE CHAIN REACTION



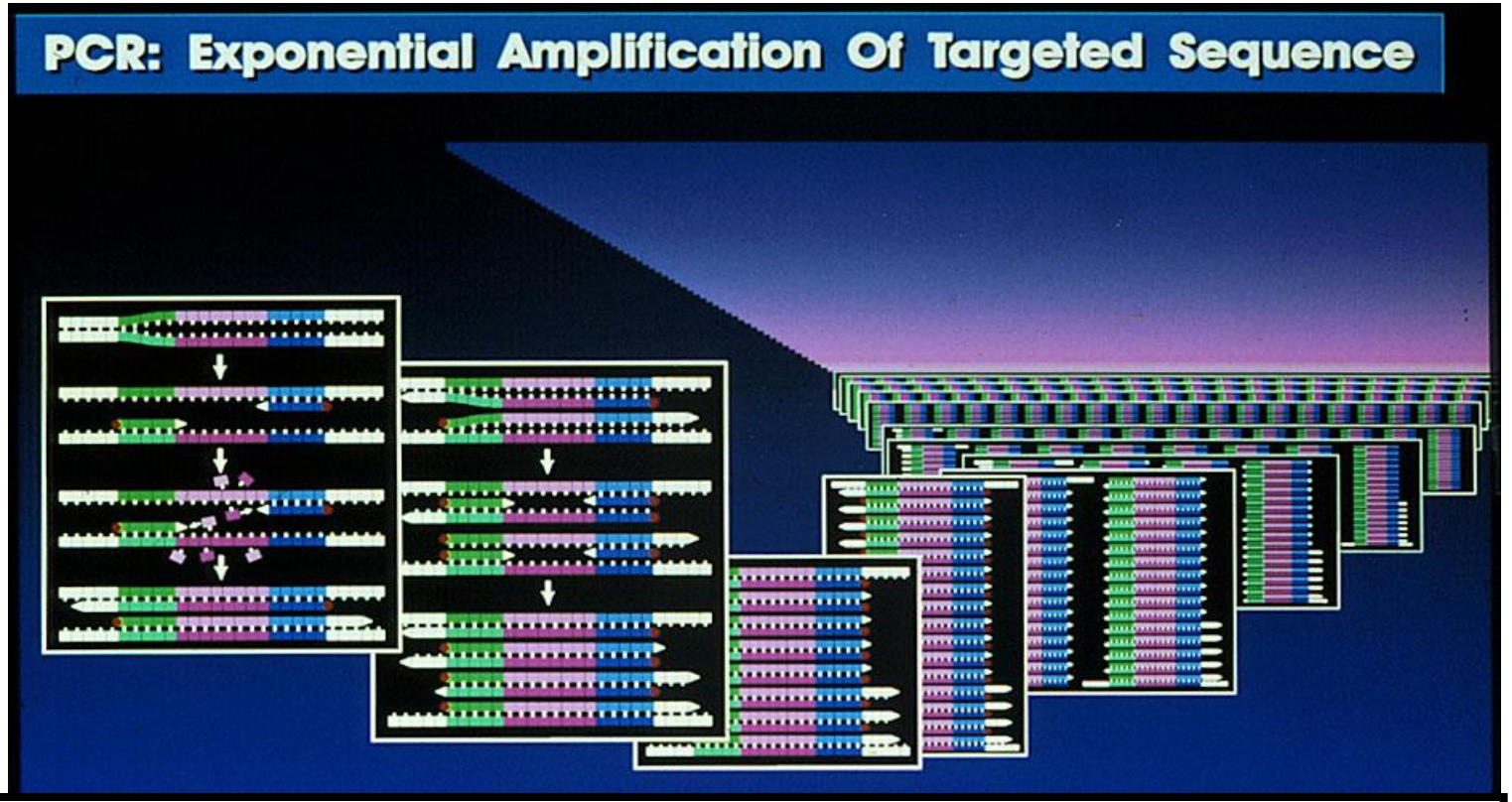
- **Polymerization at 72 degrees C.**
- **Requires availability of Thermus aquaticus DNA polymerase (heat stable).**

POLYMERASE CHAIN REACTION



- After the first cycle, there are 2 copies of the original double helix.
- Continue cycling….

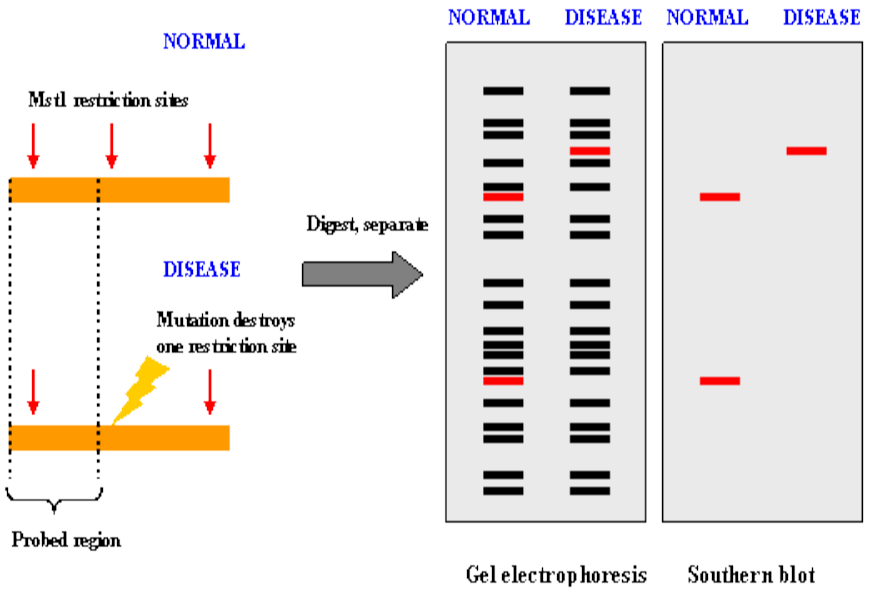
POLYMERASE CHAIN REACTION

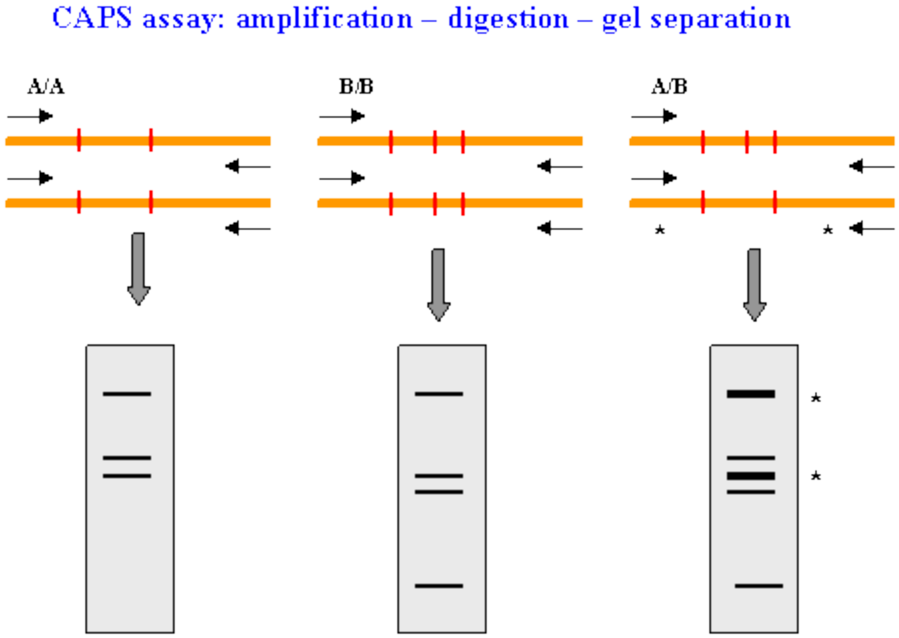


- **And so on…for 30-35 cycles**
- **Result is billion-fold amplification of target.**

POLYMERASE CHAIN REACTION

Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination.

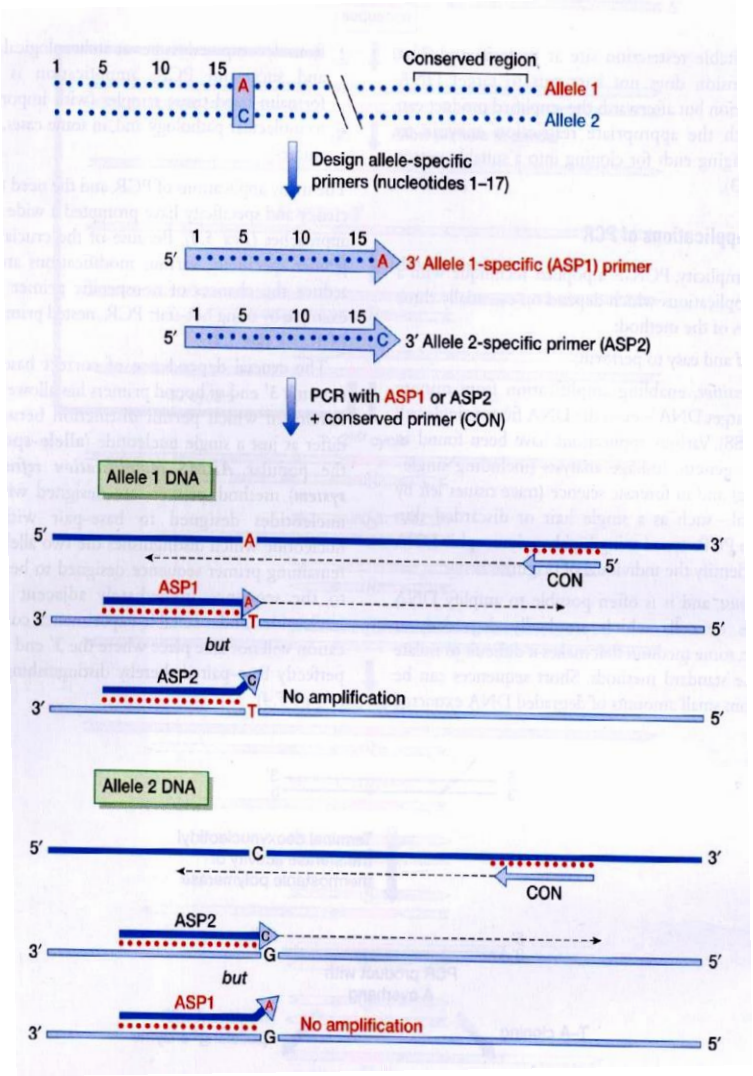




Cleaved Amplified Polymorphic Sequences (**CAPS**) polymorphisms are differences in restriction bit lengths caused by SNPs or INDELs that create or abolish restraint endonuclease recognition sites in PCR amplicons produced by locus-specific oligonucleotide primers.

http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechRFLP.shtml

ARMS:
Amplication Refractory Mutation System



Human Molecular Genetics 3. Strachan & Read. Garland Science

Tetra-primer ARMS-PCR

Context sequence for Q188R (GALT)

AGTCACAGAGGAGCTGGGTGCCCAGTACCCTTGGGTGCAGGTTTGTGAGGTCGCCCCTTCCCCTGGATG
GGCAGGGAGGGGGTGATGAAGCTTTGGTTCTGGGGAGTAACATTTCTGTTTCCACAGGGTGTGGTCAG
GAGGGAGTTGACTTGGTGTCTTTTGGCTAACAGAGCTCCGTATCCCTATCTGATAGATCTTTGAAAACAA
AGGTGCCATGATGGGCTGTTCTAACCCCCACCCCCACTGCC(G/A)GGTAAGGGTGTCAGGGGCTCCA
GTGGGTTTCTTGGCTGAGTCTGAGCCAGCACTGTGGACATGGGAACAGGATTAATGGATGGGACAGAG
GAAATATGCCAATGATGTGGAGGCTTGGAGGTAAAGGACCTGCCTGTTCTTCTCTGCTTTGCCCC

Q188R-Inner-F: CTGTTCTAACCCCCACCCCCACTGACG (mutant)
Q188R-Inner-R: CCCACTGGAGCCCCTGACACCCTTAACT (wildtype)
Q188R-Outer-F: AGTCACAGAGGAGCTGGGTGCCCAGTACC
Q188R-Outer-R: GGGGCAAAAGCAGAGAAGAACAGGCAGG

c.563A>G=p.Q188R

*The bases in **blue** represent intentional mismatches in the primer design to increase annealing temperature stringency

Product of Outer-F / Outer-R: 405 bp

- This product should always be present. It does not involve the mutation

Product of Outer-F / Inner-R: 276 bp

- Amplification of this product signifies a normal sequence

Product of Inner-F / Outer-R: 184 bp

- Amplification of this product signifies a mutant sequence

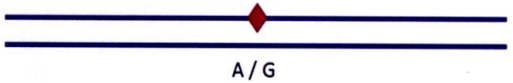
Expected PCR Products and Size:

Homozygous Normal: **405** bp and **276** bp
Heterozygote: **405** bp, **276** bp, and **184** bp
Homozygous Mutant: **405** bp and **184** bp

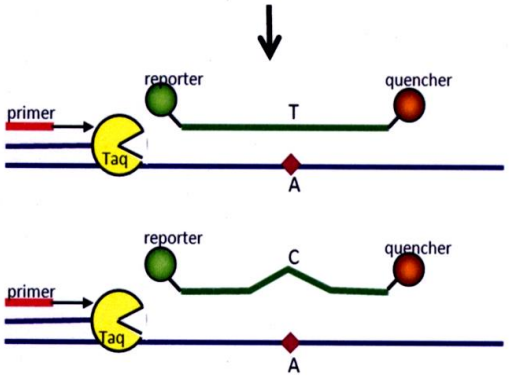
Dr. Mei Baker

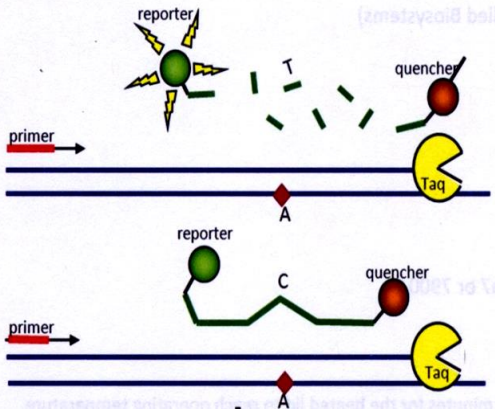
TaqMan Allelic Discrimination

GALT gene region containing Q188R mutation - Normal sequence contains an A and Q188R mutation is a G

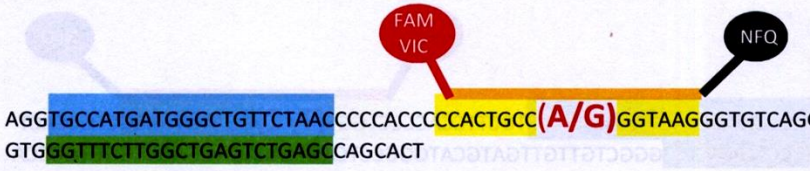


Probes anneal to their targeted sequence. A normal individual will have an A in the position of interest, so the probe with the complimentary T probe will bind. The probe with a C will not. Each probe has a unique reporter molecule in order to differentiate between the two.



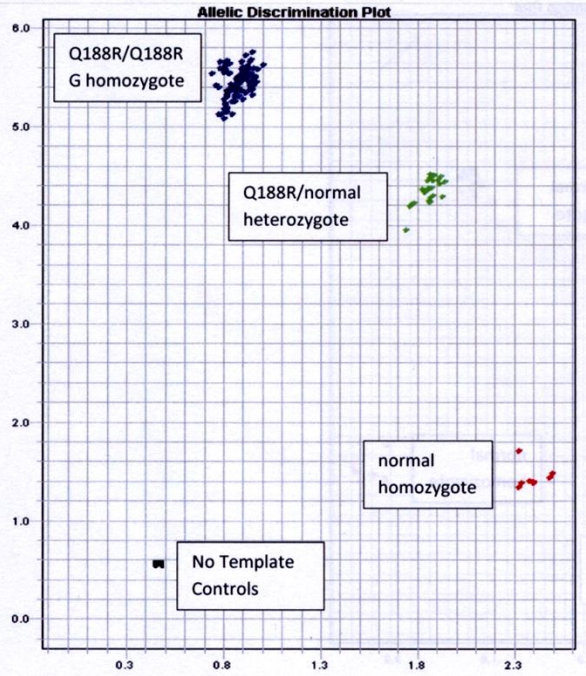


Context sequence for Q188R (GALT)



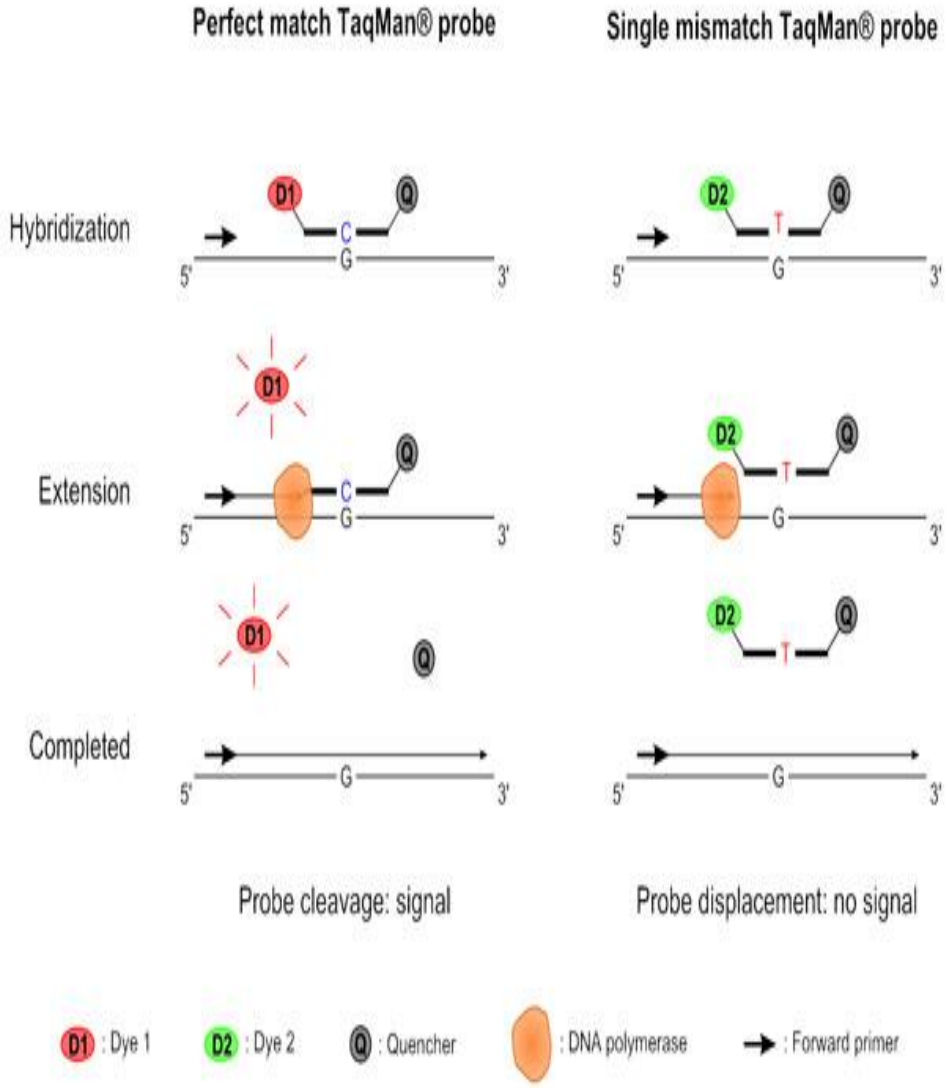
Forward Primer Sequence: TGCCATGATGGGCTGTTCTAAC
Reverse Primer Sequence: GCTCAGACTCAGCCAAGAAACC
Reporter 1 Sequence: [VIC]-CCACTGCCAGGTAAG-[NFQ] (wild type)
Reporter 2 Sequence: [FAM]-CACTGCCGGGTAAG-[NFQ] (mutant)

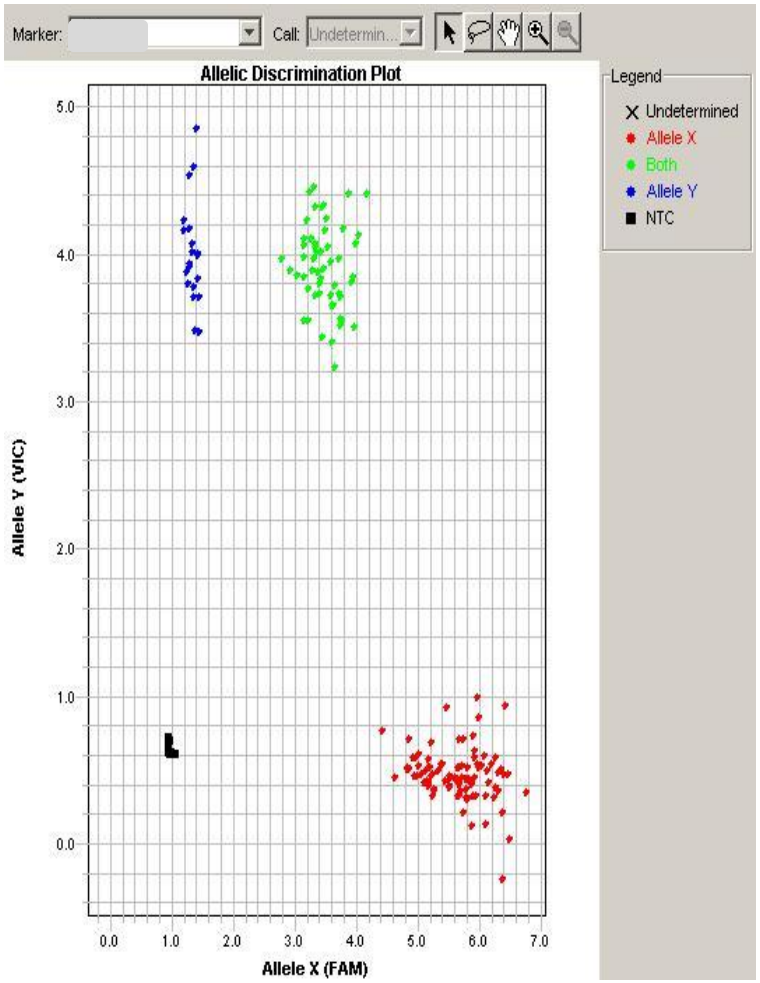
Expected Results:



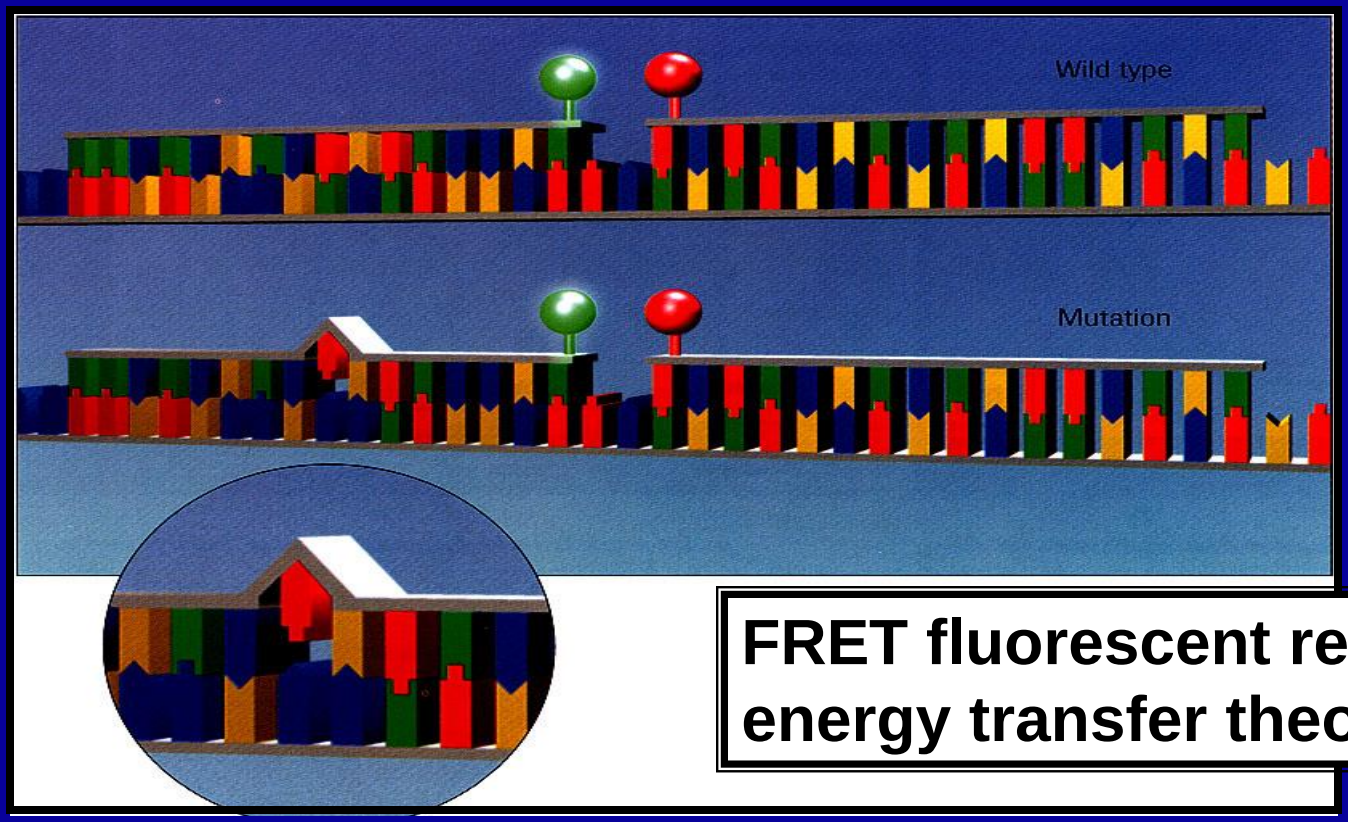
Tim Davis, MS

TaqMan Allelic Discrimination





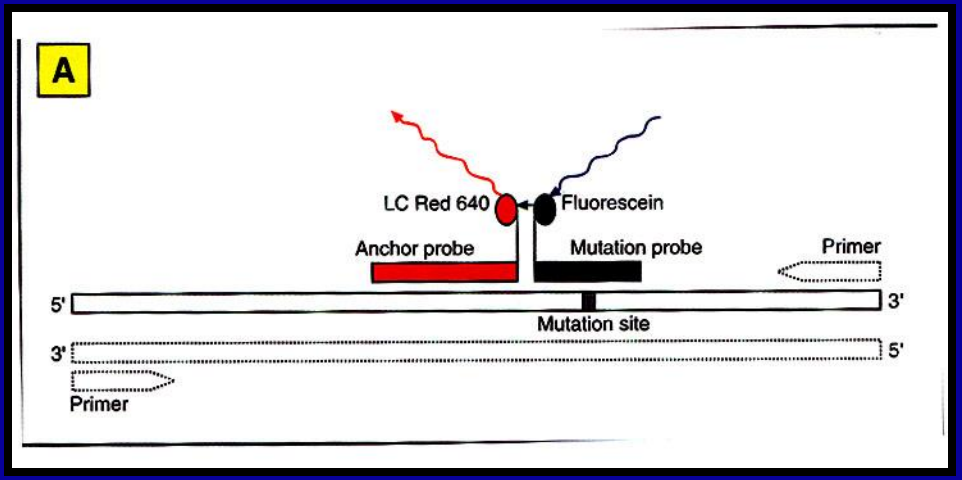
http://www.applied-maths.com/applications/taqman-based-snp-genotyping

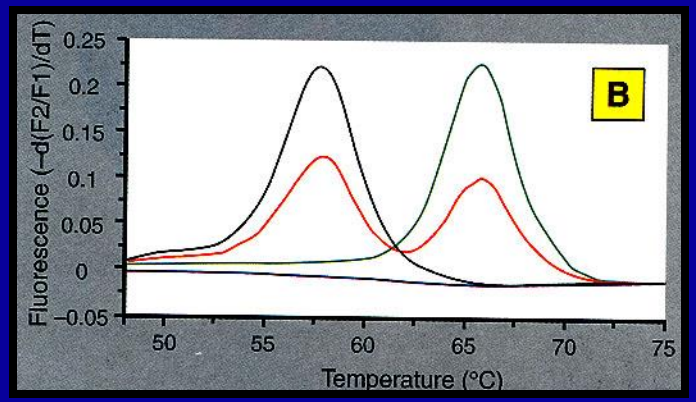


Normal

Mutation Present

FRET fluorescent resonance energy transfer theory





Roche

Wadsworth Center

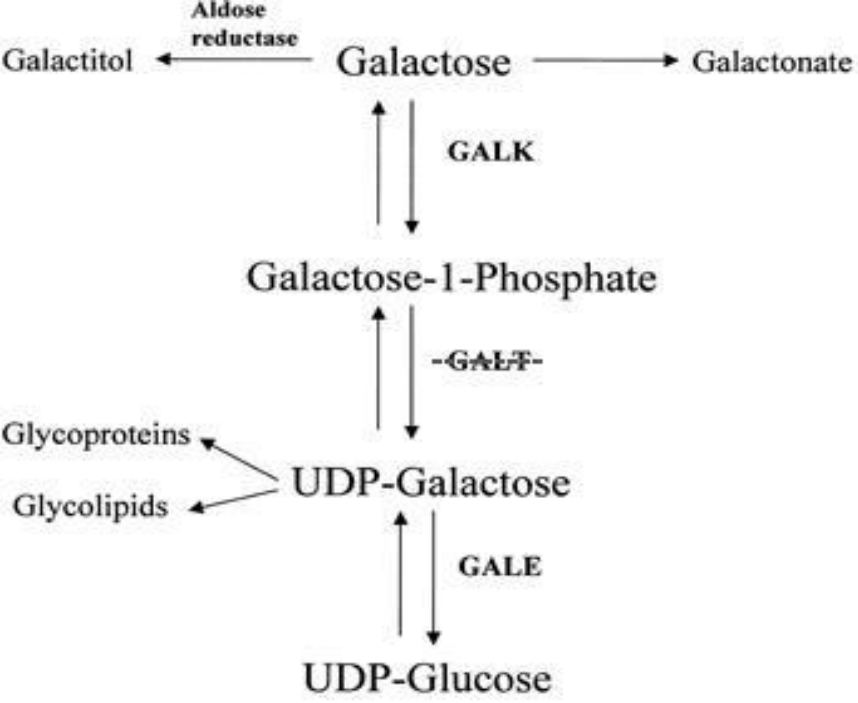
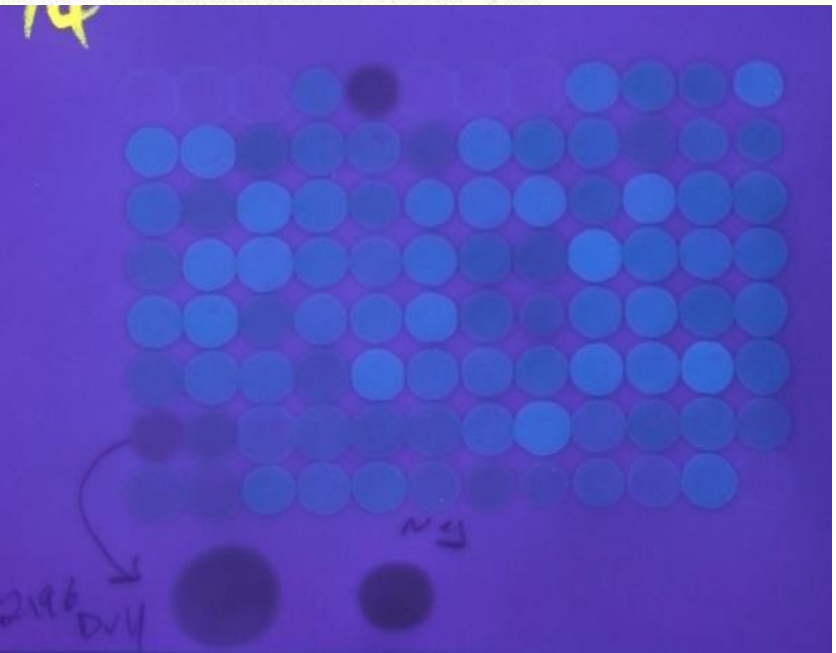


Fig 1. Galactose Metabolism



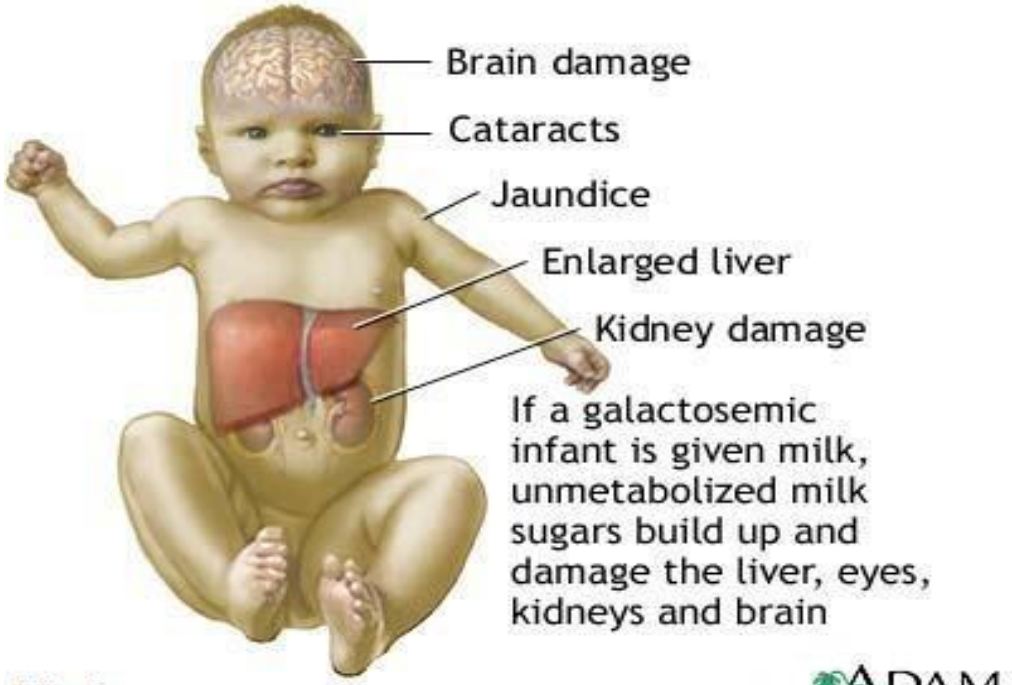


Fig 2.

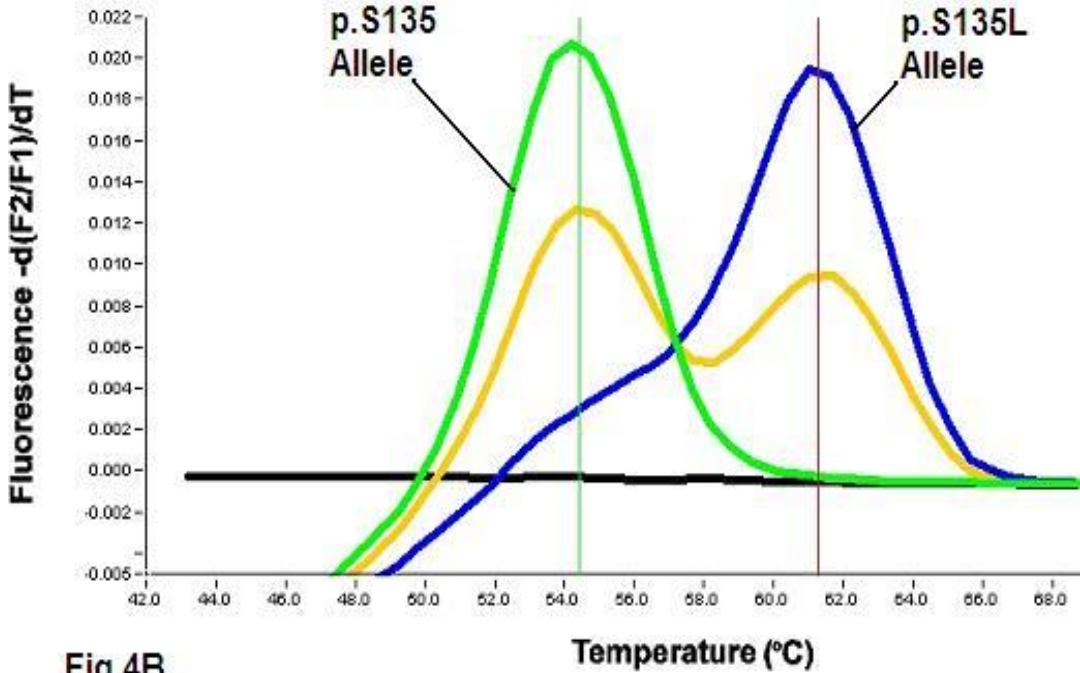
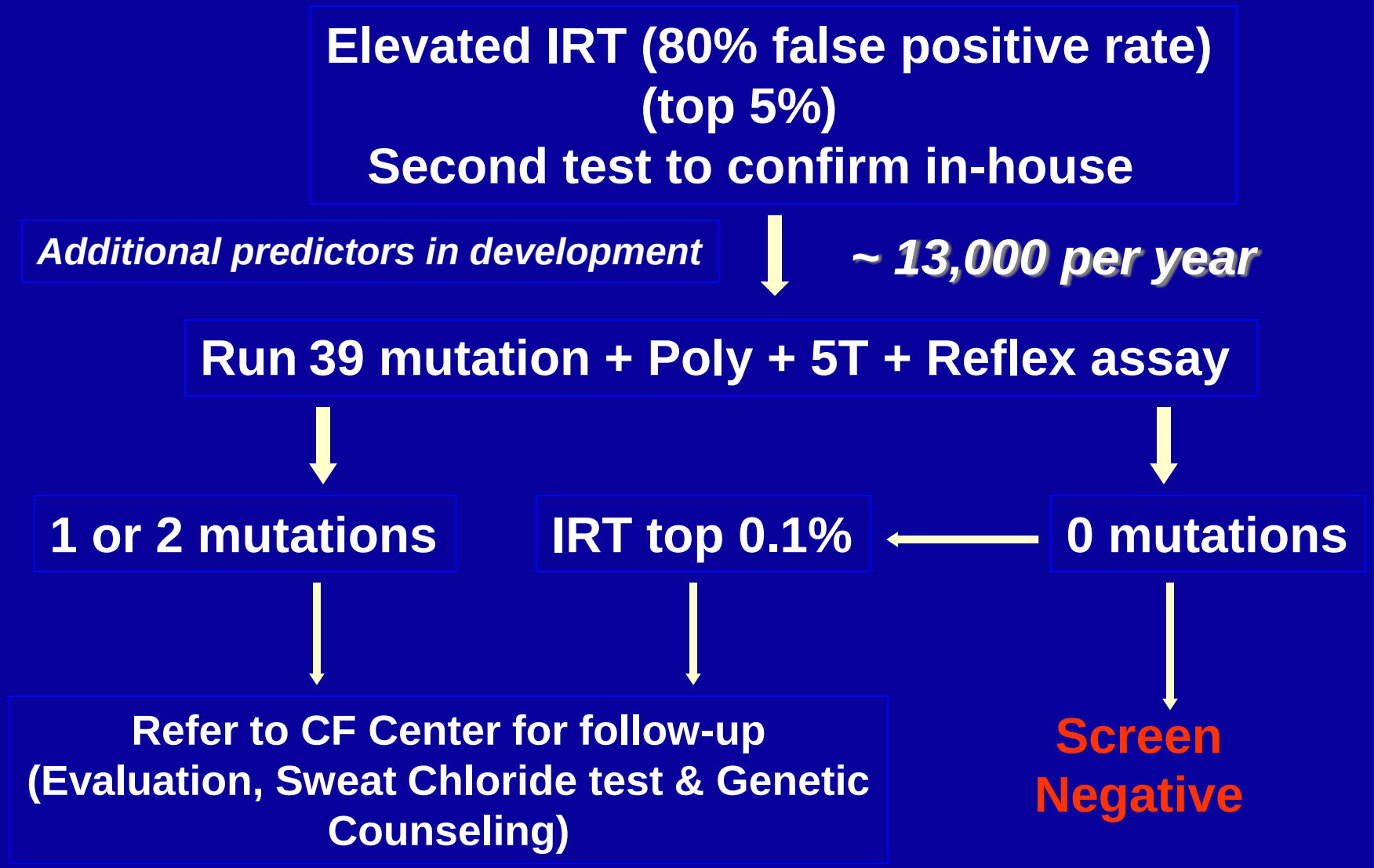


Fig 4B.

Cystic Fibrosis Algorithm

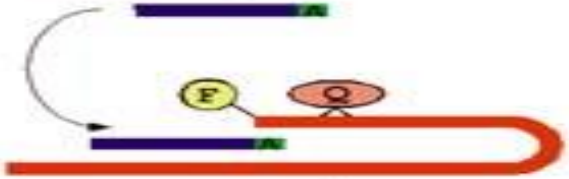


INVADER ASSAY

A.

Structure: Invasive complex forms (one base invasion)

Result: Cleavage



Result: Cleavage and detection

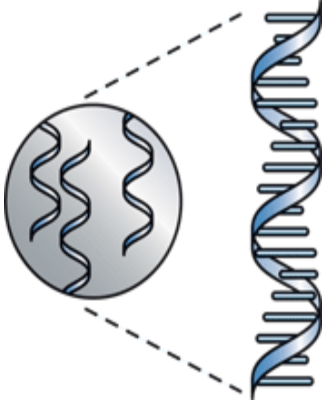
Fluorescence for WT

B.

Structure: No invasive complex forms; MT probe and WT target are not complementary at base of interest.

Result: No Cleavage

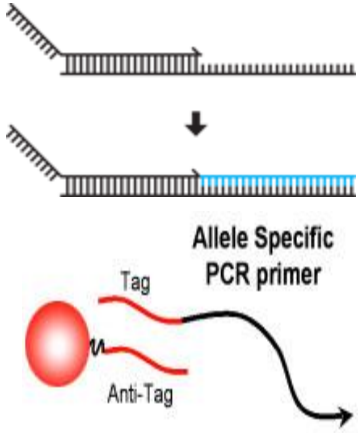
The Invader™ assay from Third Wave Technologies, Inc. relies upon the specificity of the Cleavase® enzymes, which recognize only the invasive complex, permitting discrimination of single base changes.



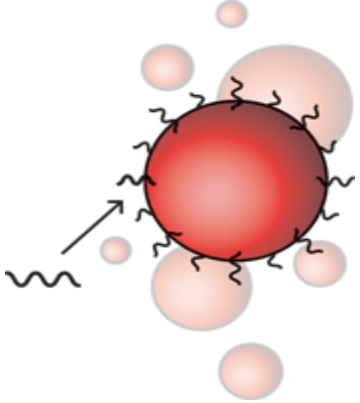
Nucleic Acid Extraction and Purification
A optimal input quantity of 50ng (range of 10 ng to 1.5 ug) per sample is required to perform the assay.

Step 1 - Multiplex PCR Reaction will make multiple copies of multiple DNA targets within the CFTR gene.

Step 2 - Amplicon Treatment
Enzymatic treatment of amplified PCR products cleaves unused reagents (primers and dNTPs) left over after PCR.

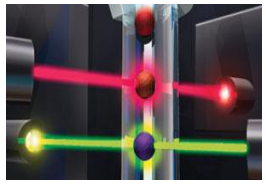


Step 3 - Allele-specific primer extension (for CF)
The amplified DNA is mixed with short sequences (TAG primers) of DNA specific to each target. If the target is present, the primer will bind and will be lengthened through a process called Allele specific extension. During this extension, a reporter label is incorporated.

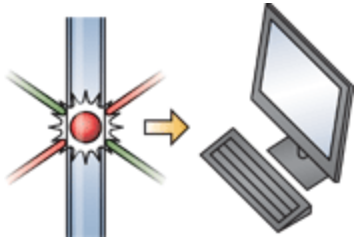


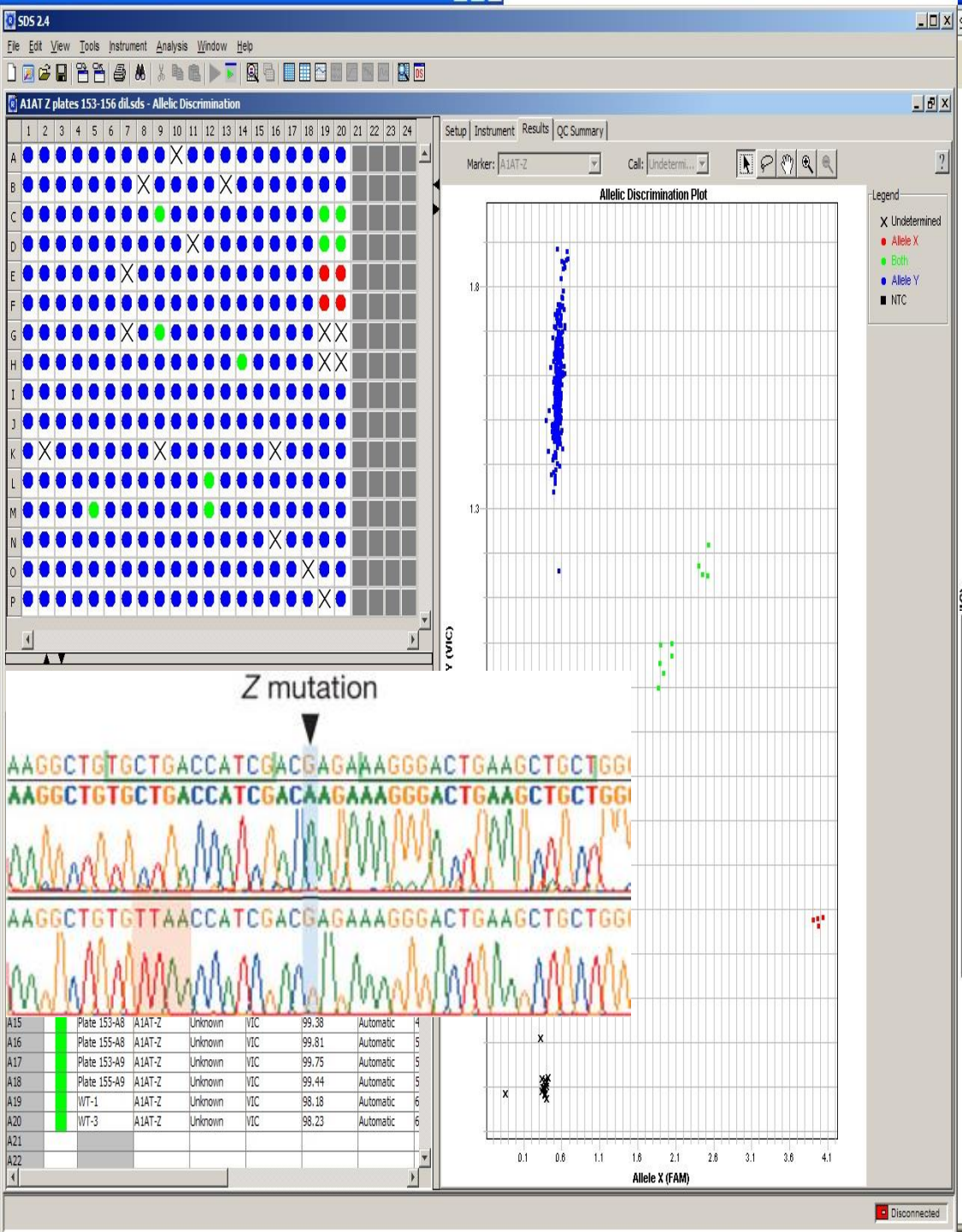
Step 4 - Bead Hybridization
Color-coded beads are added to identify the tagged primers. Attached to each differently colored bead is an anti-TAG sequence specific to *one of the extended TAG primers. Each anti-TAG* only binds to the complementary TAG sequence on the primer.

Step 5 - Addition of Reporter Molecule
The reporter solution is the Streptavidin, R-Phycoerythrin conjugate and will be used to detect the target.

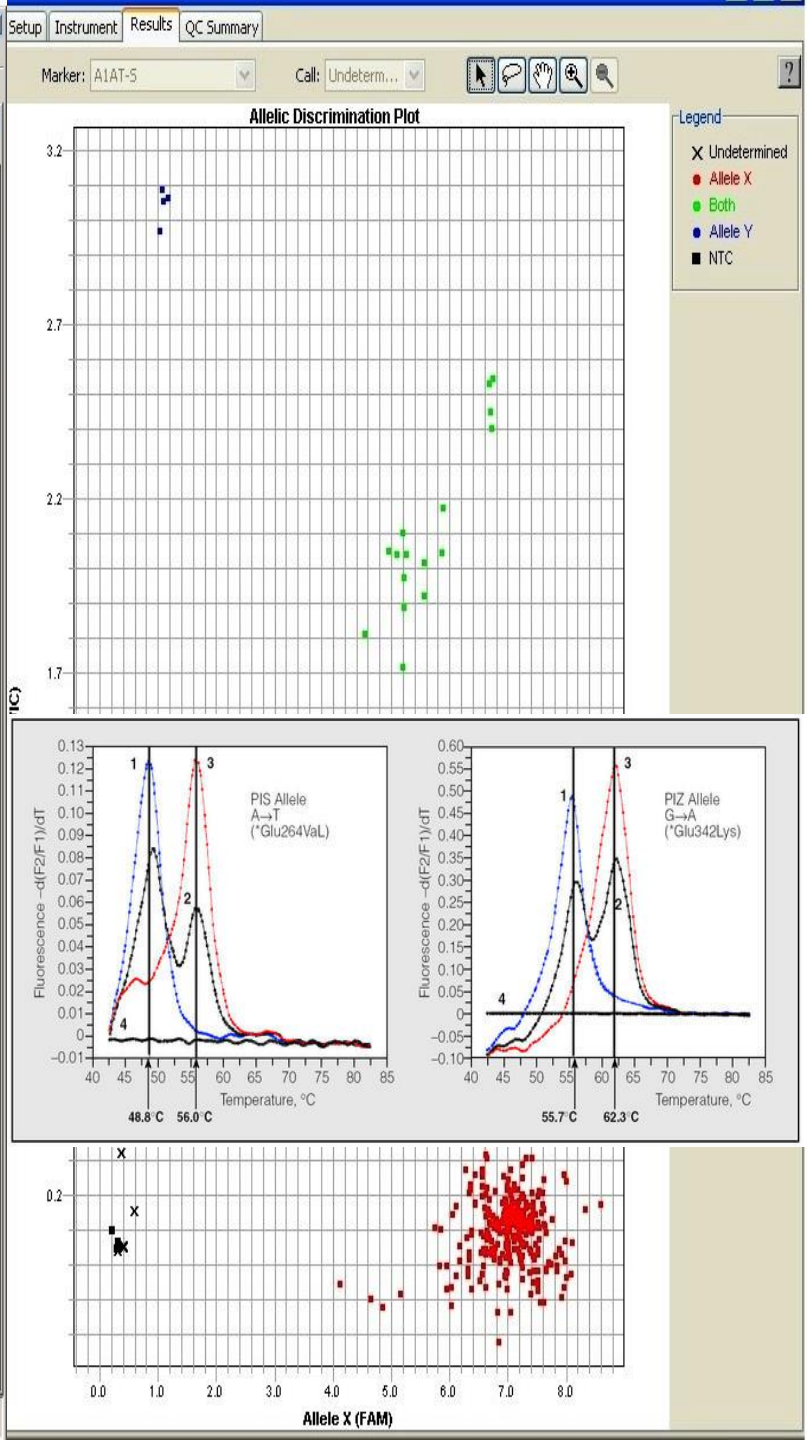


Step 6 - Data Acquisition on Luminex Analyser
Samples are then placed in a Luminex xMAP® instrument where beads are read and analyzed by lasers. The lasers identify the color of the bead and the presence or absence of the labeled target. For each sample, these signals are interpreted by the xTAG Data Analysis Software to determine whether the wild-type and/or mutant alleles for each of the variations have been detected



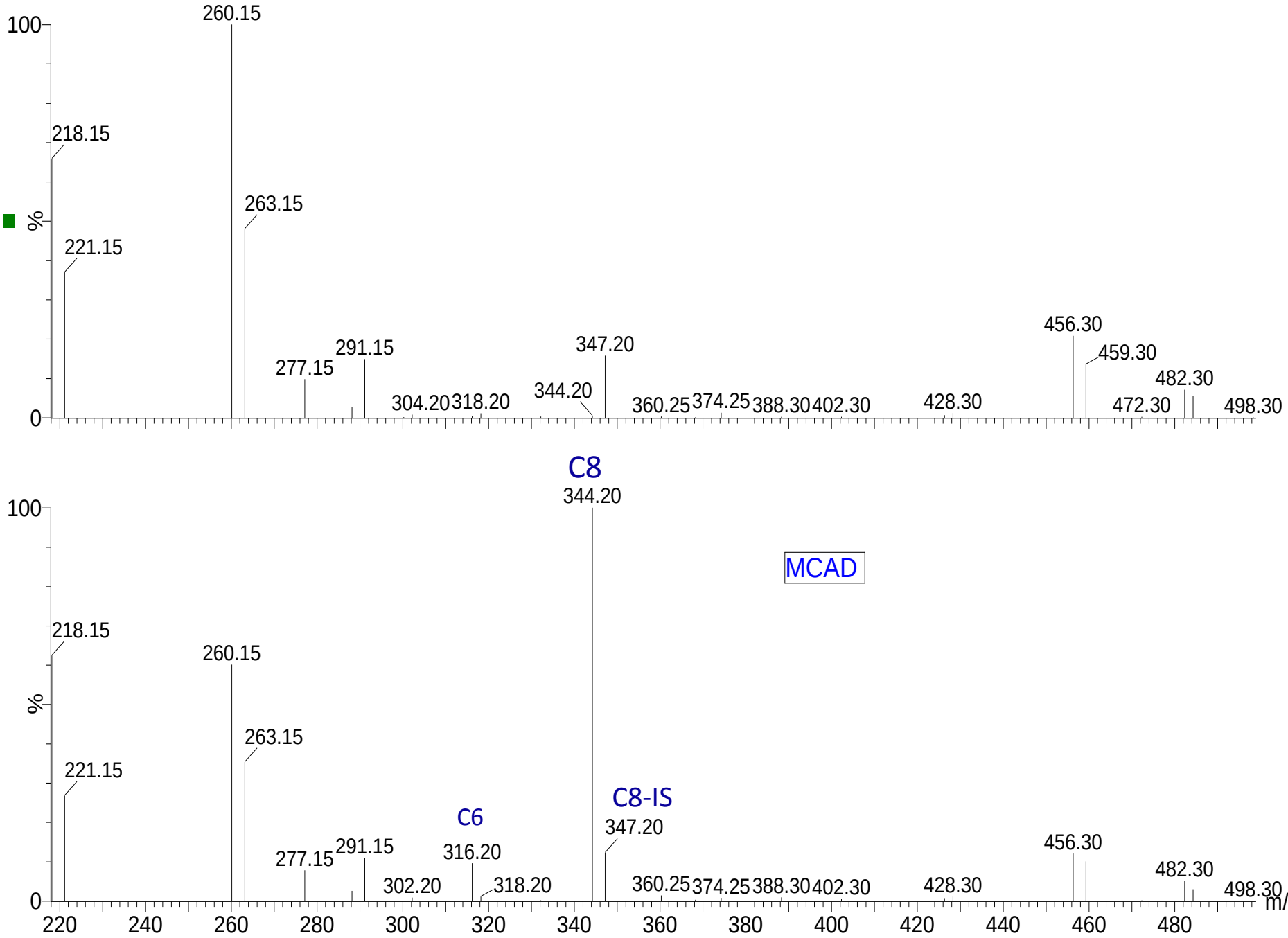


A15	Plate 153-A8	A1AT-Z	Unknown	VIC	99.38	Automatic	4
A16	Plate 155-A8	A1AT-Z	Unknown	VIC	99.81	Automatic	5
A17	Plate 153-A9	A1AT-Z	Unknown	VIC	99.75	Automatic	5
A18	Plate 155-A9	A1AT-Z	Unknown	VIC	99.44	Automatic	5
A19	WT-1	A1AT-Z	Unknown	VIC	98.18	Automatic	6
A20	WT-3	A1AT-Z	Unknown	VIC	98.23	Automatic	6
A21							
A22							



NYSDOH-NBSP MCADD MS/MS Detection

Courtesy Dr. M. Morrissey NBSP-NYSDOH

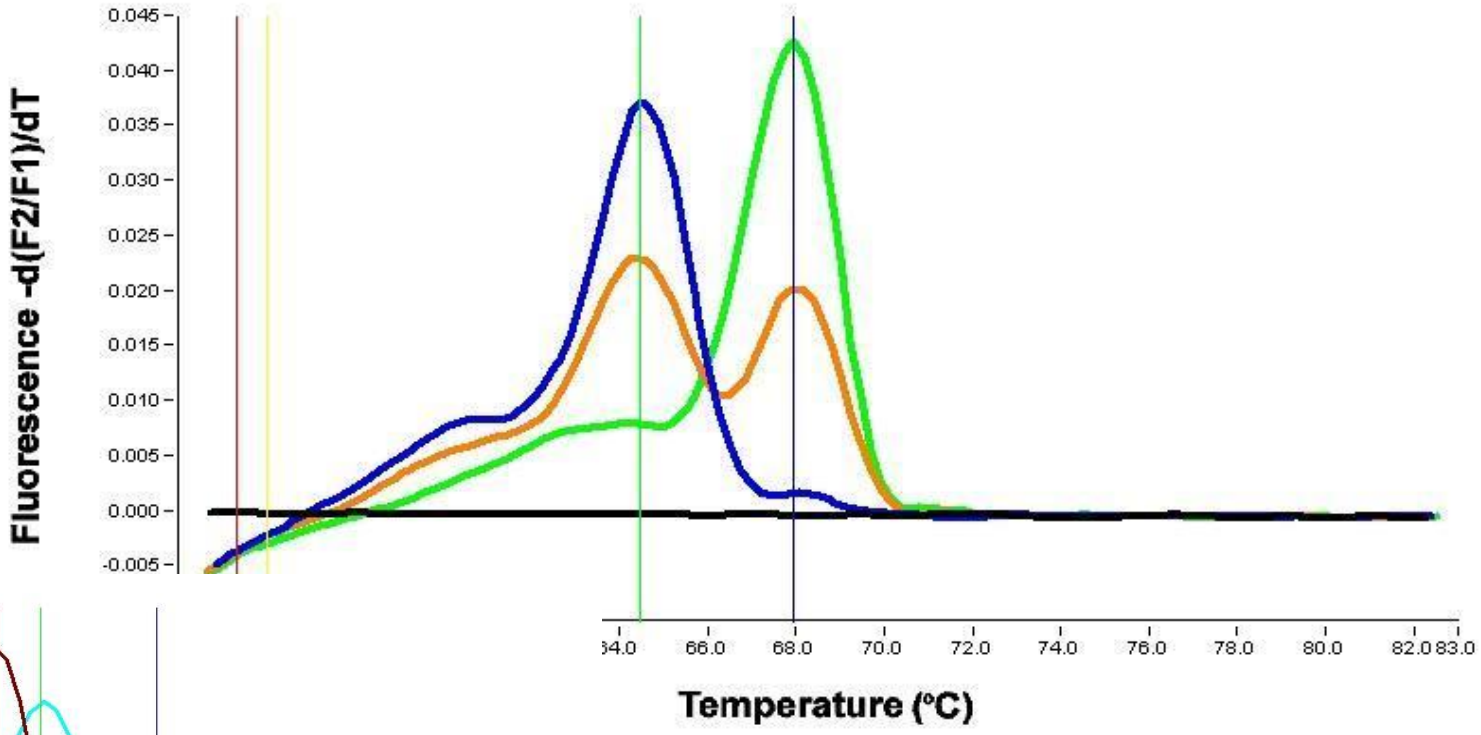


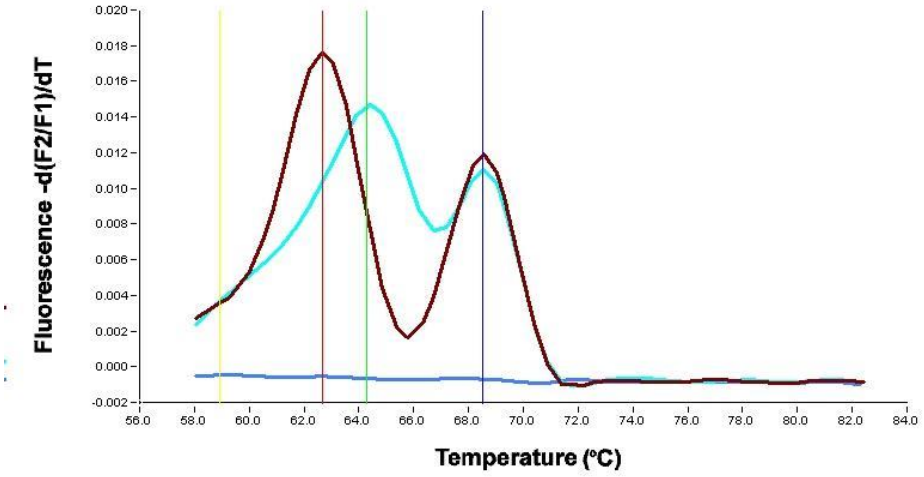
NYSDOH-NBSP

FRET analysis most common *ACADM* Mutation

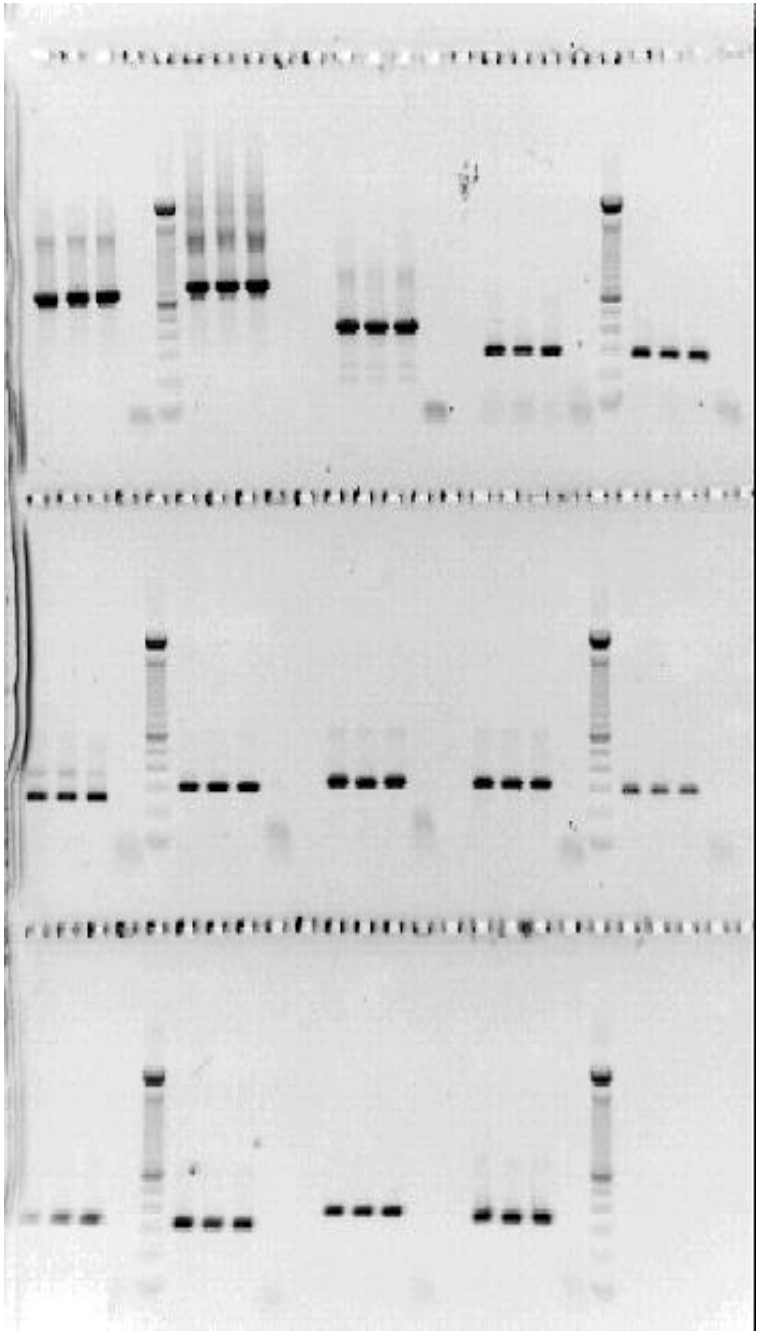
MCADD

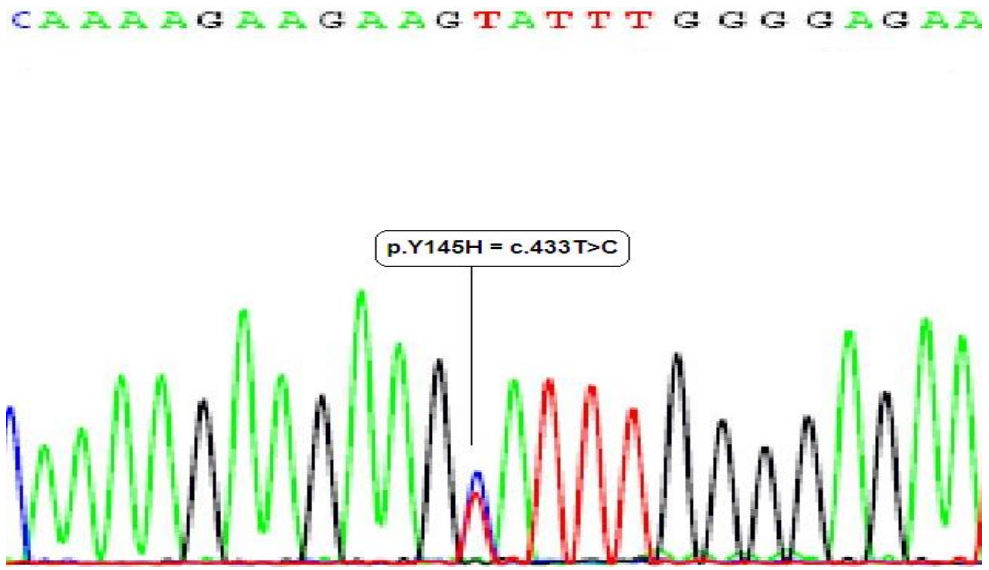
c.985A>G Wt

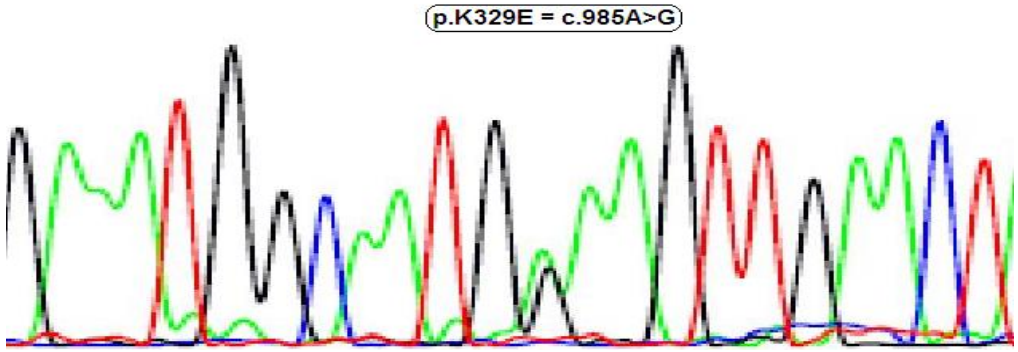




c.985A>G = p.K329E (K304E)




p.Y145H = c.433T>C

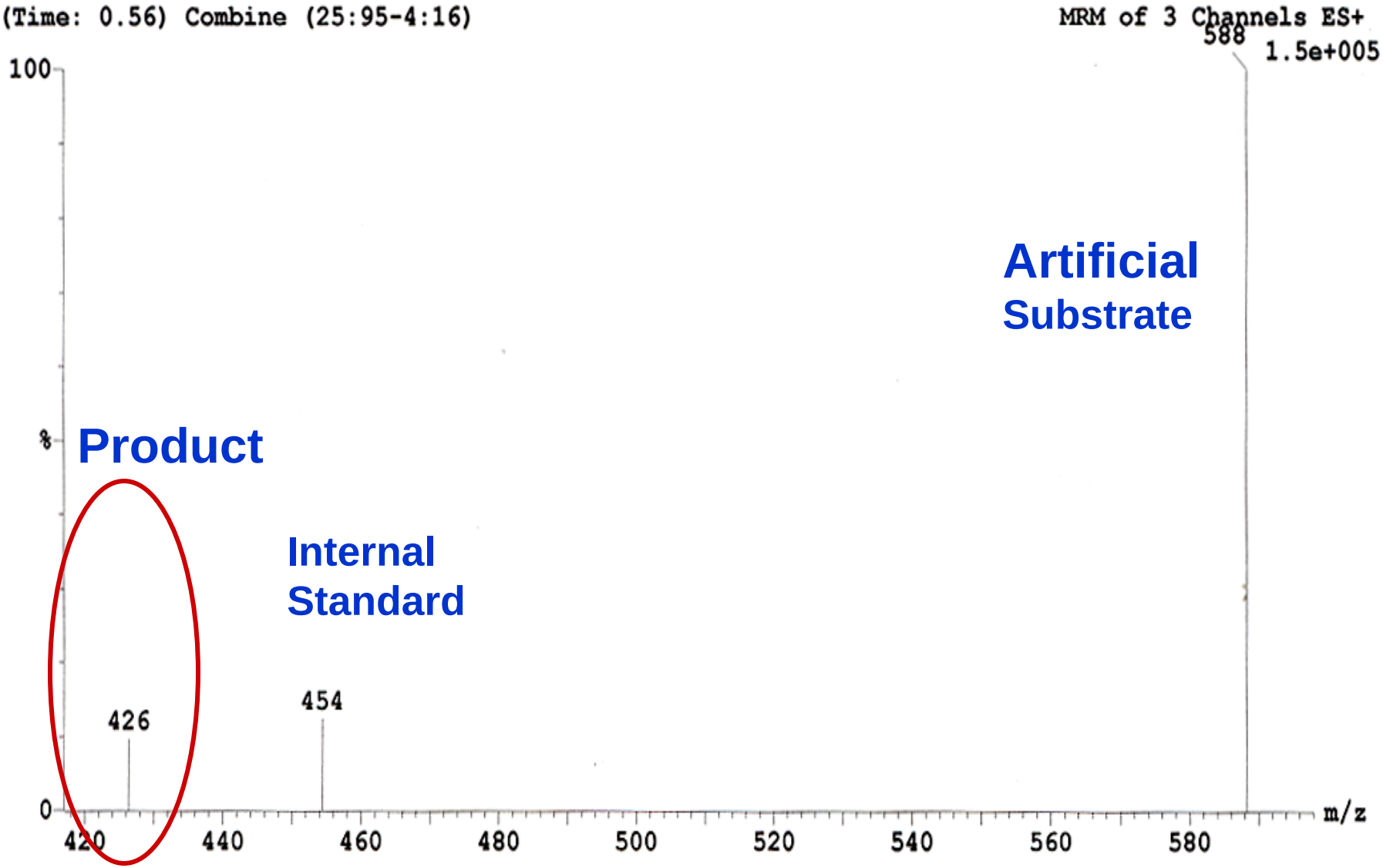

p.K329E = c.985A>G

Sample Report:

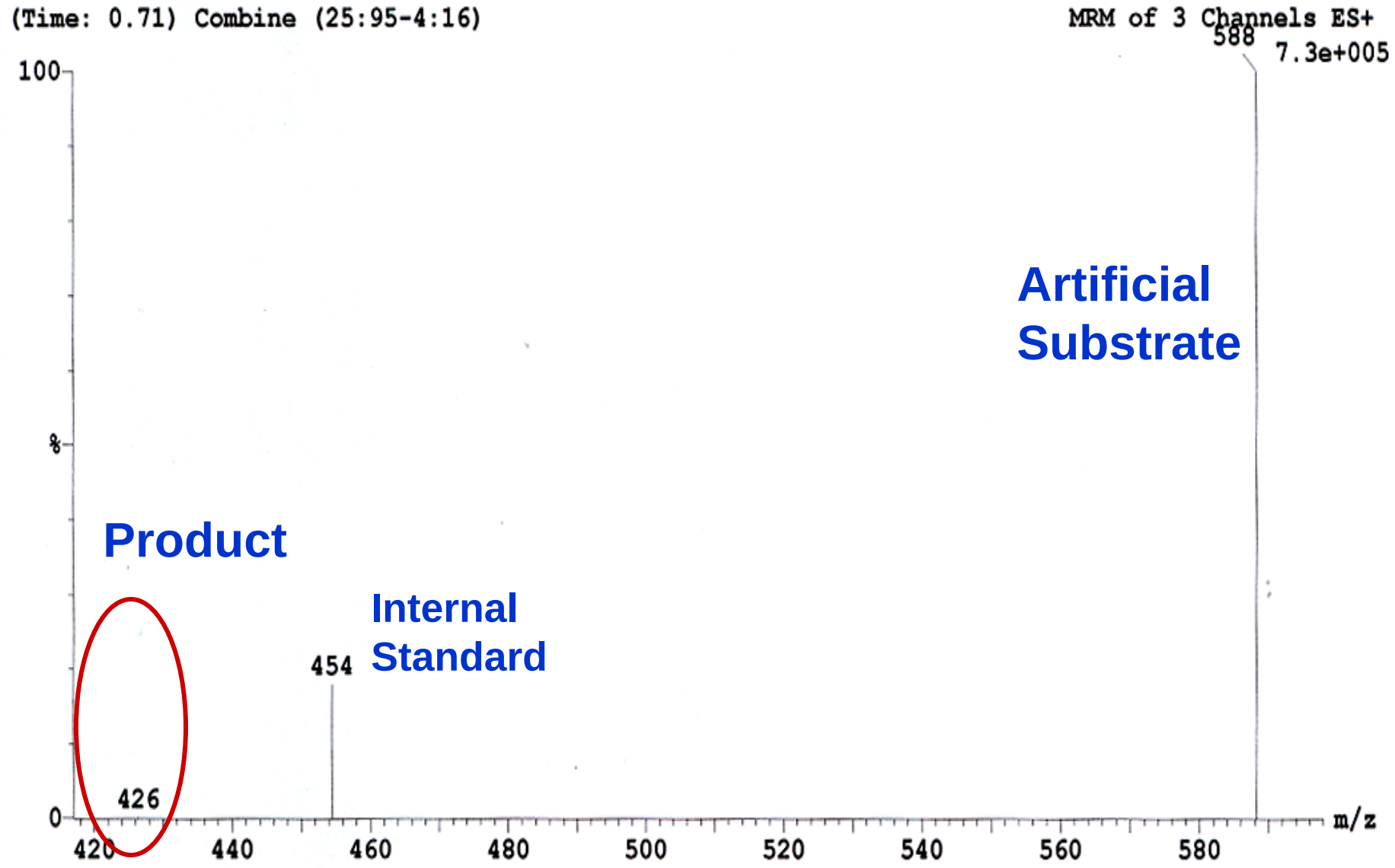
MRM of 3 Channels ES+

(Time: 0.56) Combine (25:95-4:16)

NORMAL



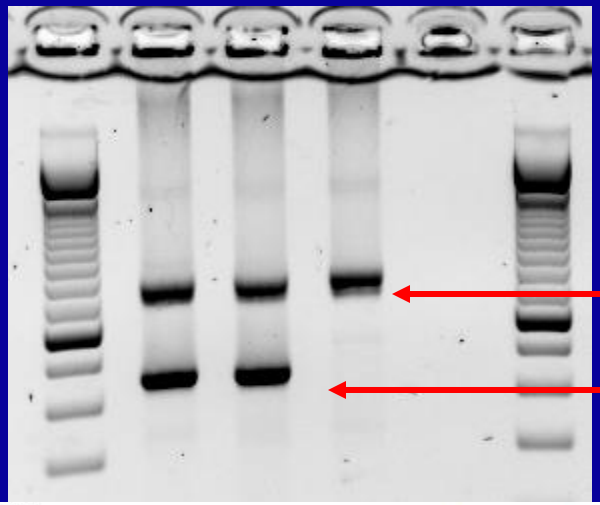
KRABBE DISEASE

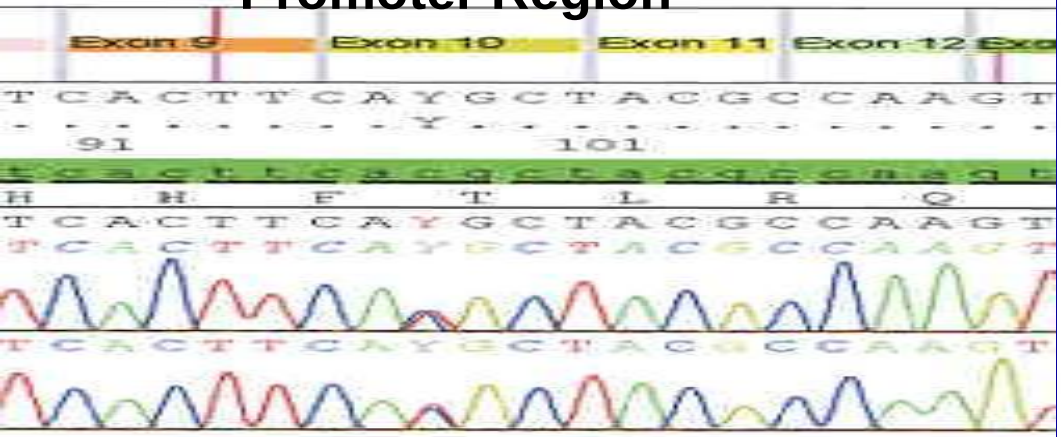


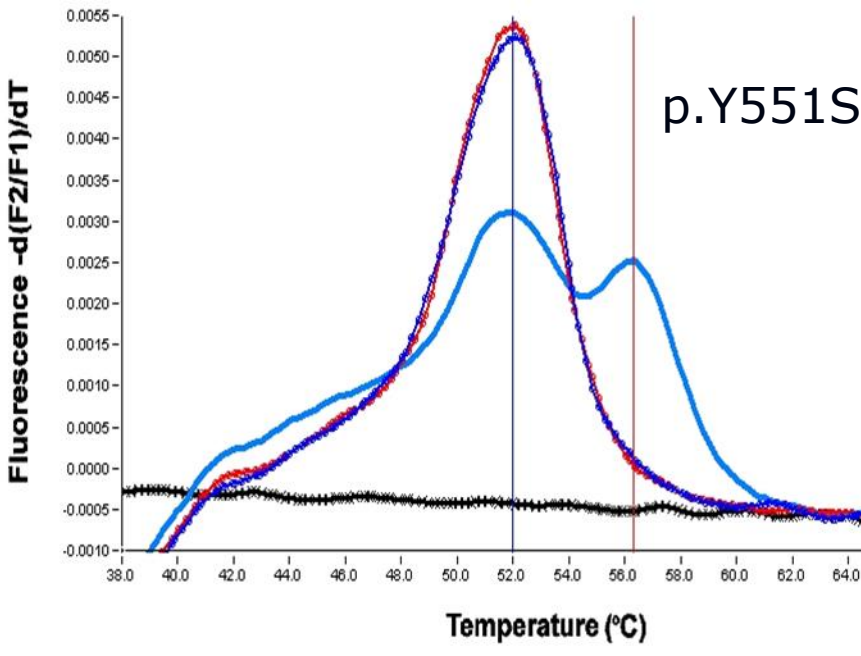
Molecular Analysis of GALC

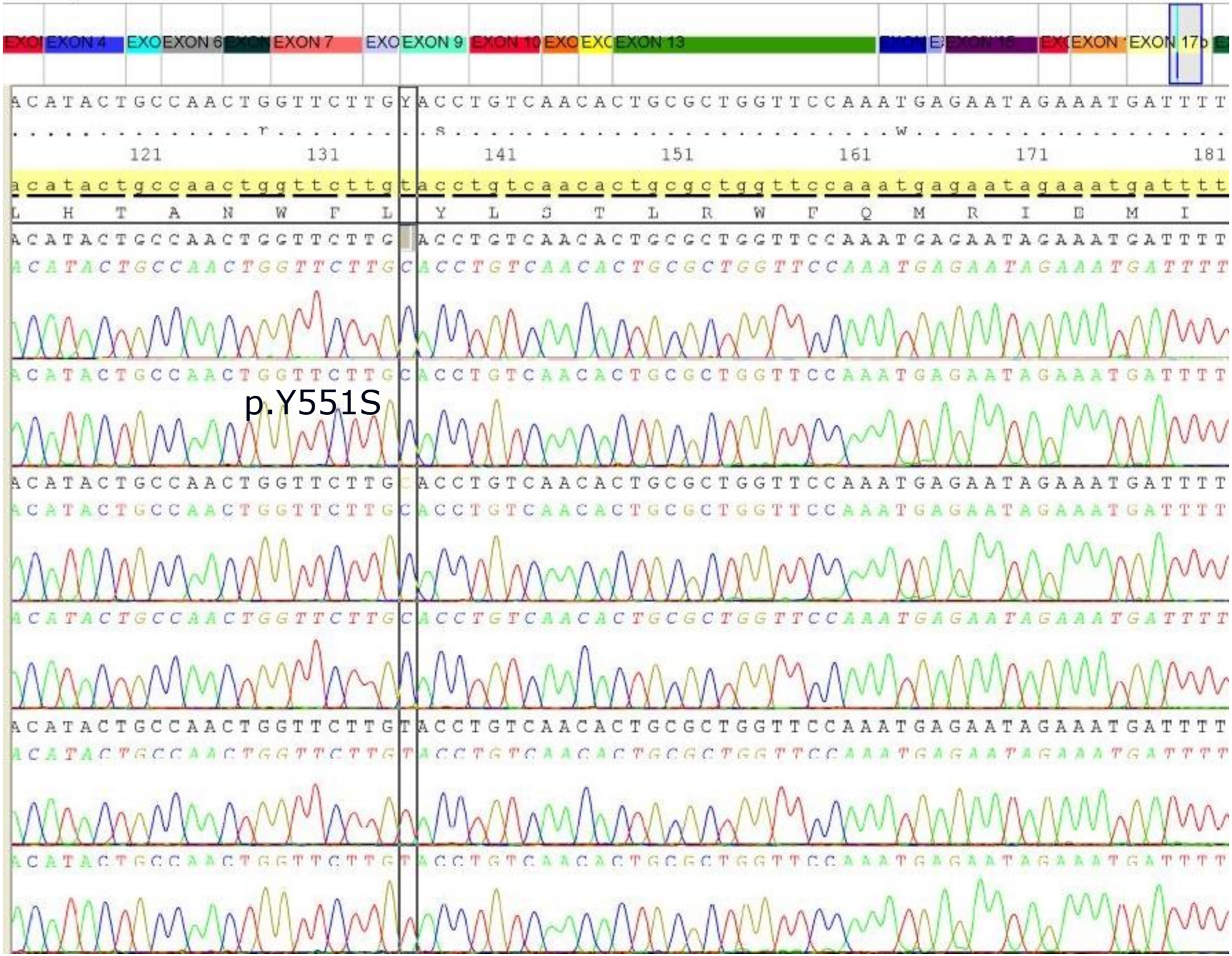
- Reduce number of false positive screens
- Predict phenotype (?)
- Method:
  - Agarose gel for 2 common deletions
    - 30 kb
    - 7 kb
  - Probes for 7 common mutations/polymorphisms
  - **Sequence all 17 exons and Promoter Region**

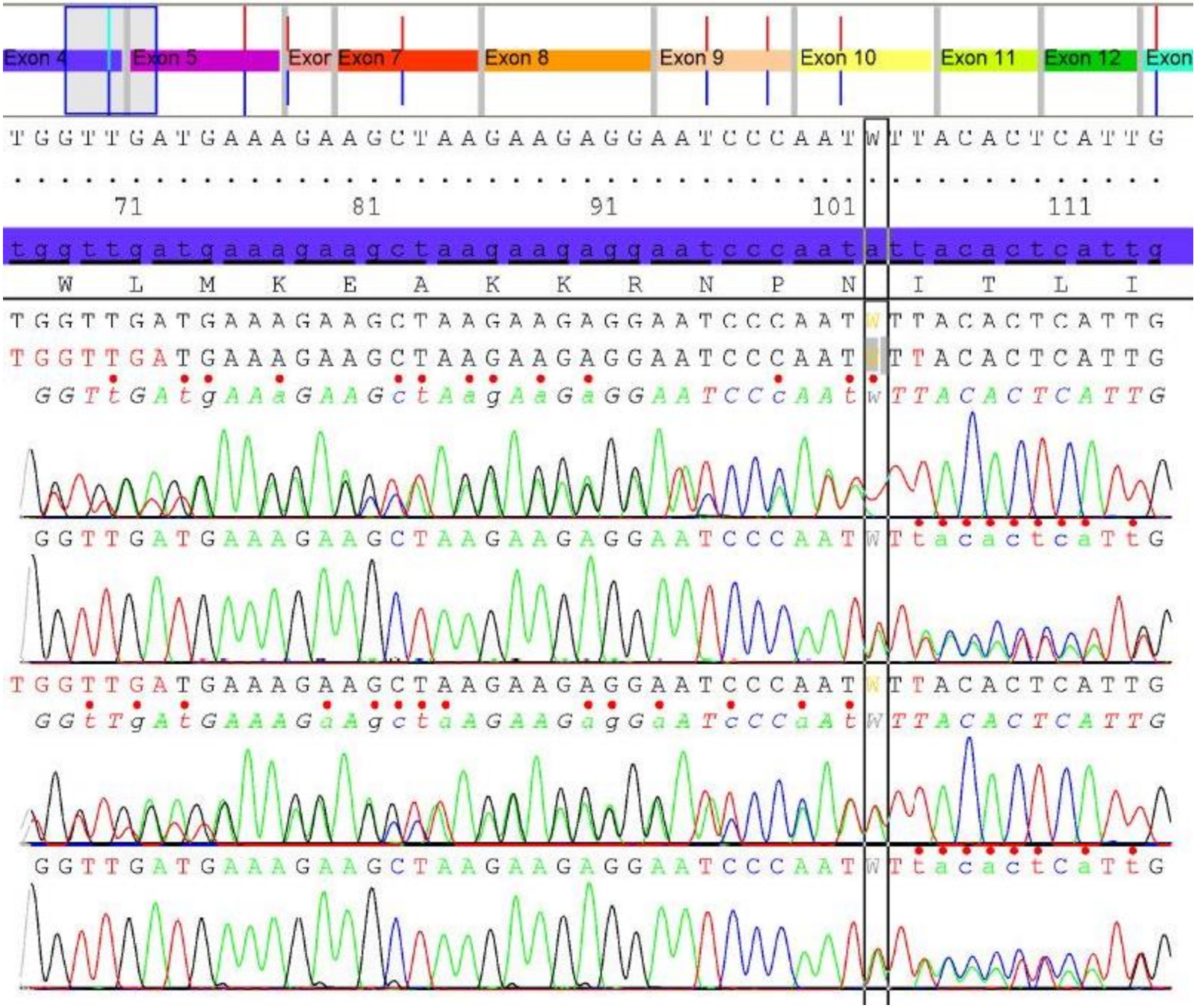
30 kb deletion








EXON 4
EXON 6
EXON 7
EXON 9
EXON 13
EXON 17b
ACATACTGCCAACTGGTTCTTGTACCTGTCAACACTGCGCTGGTTCCAAATGAGAATAGAAATGATTTT
121
131
141
151
161
171
181
acatactgccaactggttcttgtacctgtcaacactgcgctggttccaaatgagaatagaaatgatttt
L H T A N W F L Y L S T L R W F Q M R I E M I
p.Y551S


Exon 4
Exon 5
Exor
Exon 7
Exon 8
Exon 9
Exon 10
Exon 11
Exon 12
Exon
71
81
91
101
111
W L M K E A K K R N P N I T L I

Wadsworth Center *(Newborn Screening DNA Lab)* Adrenoleukodystrophy DNA Sequence Analysis Protocol	**DNA_9.34** **Effective Date:** signature and date of author 1 of 46

Purpose (or Intended Use)

To provide instructions for PCR amplification and bidirectional sequence analysis of the X-linked Adrenoleukodystrophy (*ABCD1)* gene.

Summary and Explanation:

In January 2014, the NYCRR10, Public Health Law 2500-a will be amended to include, "Aidan's Law". Aidan's law requires the New York State Department of Health's Newborn Screening Program to screen for Adrenoleukodystrophy (ALD). X-linked ALD is caused by mutations on the *ABCD1* gene, locus Xq28 (OMIM, X-linked Adrenoleukodystrophy Database). *ABCD1* is a member of the ATP-binding cassette (ABC) transporter superfamily and encodes for the ABC subfamily D, member 1 protein (ABCD1),….

Principle of Procedure

Newborn blood samples are tested by MS/MS. Specimens with C26:0 values above 0.40µmole/L are tested using a HPLC assay to determine if the elevated C26:0 is real or artificial due to an interferent. If the true C26:0 (C26:0 LPC) level is above …

Equipment

DNA Engine Thermal Cycler (BioRad #PCT-0200G)
BioRad T100 Thermal Cycler (BioRad #186-1096)
96 Well PCR Microplate (Axygen #PCR=96-C)
3730 Genetic Analyzer (Life Technologies #3730-1)
48x36cm Capillary Array (Life Technologies #4331247)
Perkin Elmer JANUS Automated Workstation

Reagents

PCR Master Mix (Roche #2158825)
Taq-start Antibody (Clontech #S1476)
EXOSAP-IT® (USB #78205)
5x Sequencing Buffer (Life Technologies # 4336699)
Big Dye Terminator 3.1 Kit (Life Technologies #4337456)
3730-10x Buffer/EDTA (Life Technologies #4335613)
POP-7 Polymer (Life Technologies #4363929

List of PCR Primers (M13 tags in bold)**:**

PCR #1 (promoter region) – 1,084 bp
ABCD1-P-F 37 MER 5'- **GTAAAACGACGGCCAGT**GCCTCTCACACGCAGGTAGG-3'
ABCD1-P-R 34 MER 5'- **ACCAGCTATGACCATG**GAGCACCGGCATGTCACC-3'

PCR #2 (exon 1) – 1,122bp
ABCD1-E1-F 35 MER 5'- **GTAAAACGACGGCCAGT**ACAACAGGCCCAGGGTCA-3'
ABCD1-E1-R3 33 MER 5'- **ACCAGCTATGACCATG**ACACGCTTCCCTCCGTG-3'

PCR #3 (exon 2) – 332bp…

8.2.8 Dispense 28 μL of each reaction mix into the corresponding wells of the labeled 32-well PCR plate (see Figure 1)

Figure 1. PCR reaction and sample order for the PCR plate.

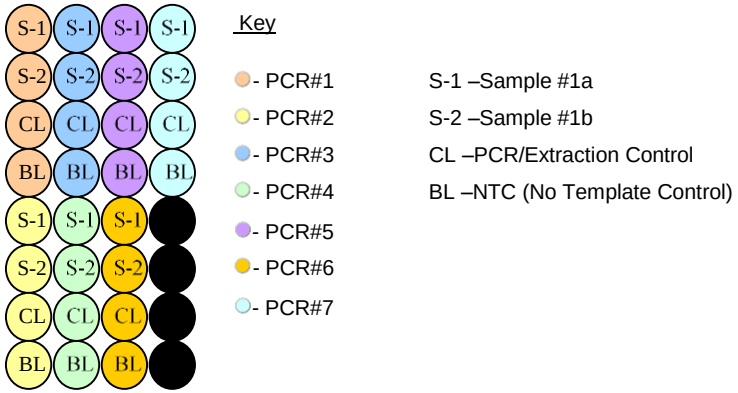
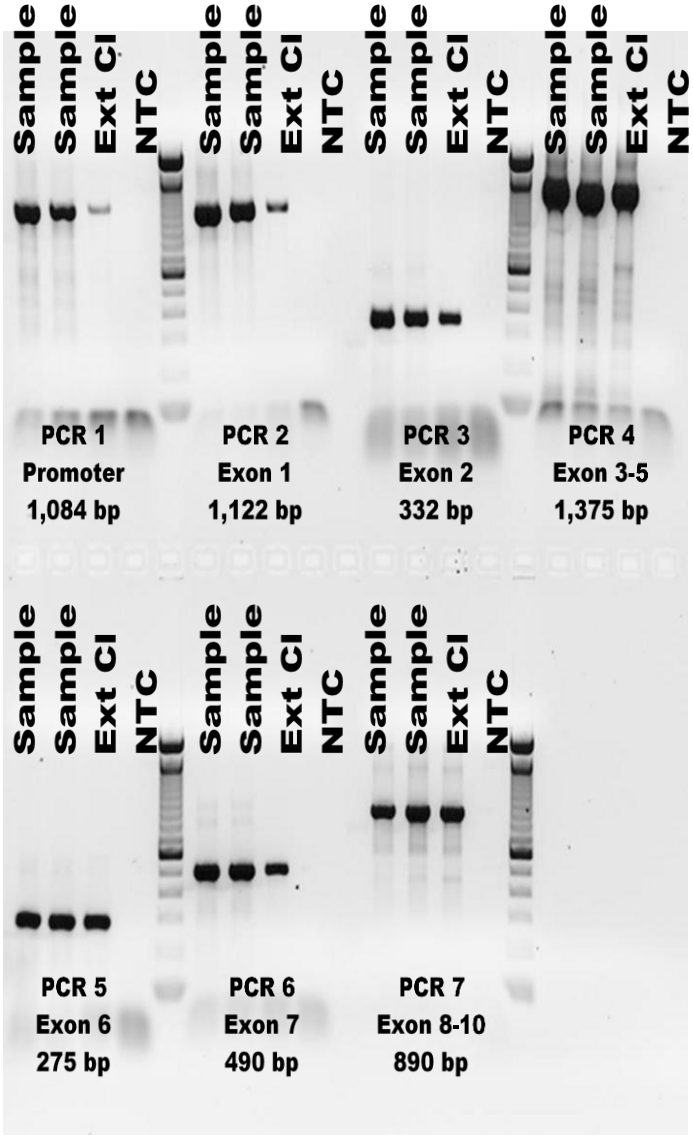


Figure 2: Typical gel electrophoresis of ALD PCR amplification of the *ABCD1* gene. The DNA ladder is a 100bp ladder.



ALD PCR Program Parameters

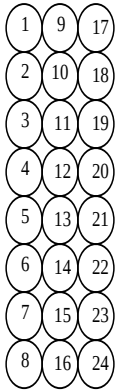
Denature -	95°C for 10 minute
Amplification 1 -	7 cycles
	95°C for 30 seconds
	65°C for 30 seconds

8.3 Procedure for PCR clean up

8.3.1 Pipette 2µL of EXOSAP-IT in each well of a 24-well Axygen PCR plate.

8.3.2 Add 5 µL of each PCR reaction to the wells as shown in Figure 3. Cap with 8 well strips caps and place in a thermal cycler.

Figure 3. PCR addition order for the EXOSAP-IT plate.



1- PCR #1 sample-1a
2- PCR #1 sample-1b
3- PCR #1 sample-1a
4- PCR #1 sample-1b
5- PCR #2 sample-1a
6- PCR #2 sample-1b
7- PCR #2 sample-1a
8- PCR #2 sample-1b
9- PCR #3 sample-1a
10- PCR #3 sample-1b
11- PCR#4 sample-1a
12- PCR#4 sample-1b
13- PCR #4 sample-1a
14- PCR #4 sample-1b
15- PCR #5 sample-1a
16- PCR #5 sample-1b
17- PCR #6 sample-1a
18- PCR #6 sample-1b
19- PCR #7 sample-1a
20- PCR #7 sample-1b
21- PCR #7 sample-1a
22- PCR #7 sample-1b
23- PCR#7 sample-1a
24- PCR#7 sample-1b

17.1 *ABCD1* gene, genomic sequence

Homo sapiens chromosome X genomic contig, GRCh37.p9 Primary Assembly

NCBI Reference Sequence: NG_009022.1

```
CCGCTCCTCCAGGTGGGGCTTCACCGCCCCCCGCCCCGCCCCCGAGACCAGCTTCTAGAGGCGCCGCCCGGT
                      ABCD1-P-F→
TTCCCCTCGCCCCTGCCTCTCACACGCAGGTAGGCTGCGGGCCCCGAGATTCCCCGGCCCCCGGGCCTCCCC

GCGCCGCTCGCCTCTCTCCCTCGTCGATGGGCCGGGGAGCCTCCGCGGTCCCGGAGCCCAGCCCGGCGCGCG
```

```
 C   H   F   K   R   P   R   E   L   E   D   A   Q   A   G   S   G   T
TGT CAC TTC AAG AGG CCC AGG GAG CTA GAG GAC GCT CAG GCG GGG TCT GGG ACC
                           |                   |
                           c.1316 G>T (p.E439X)  c.1330 C>T (p.Q444X)
```

17.4 ALD cycle sequence plate map

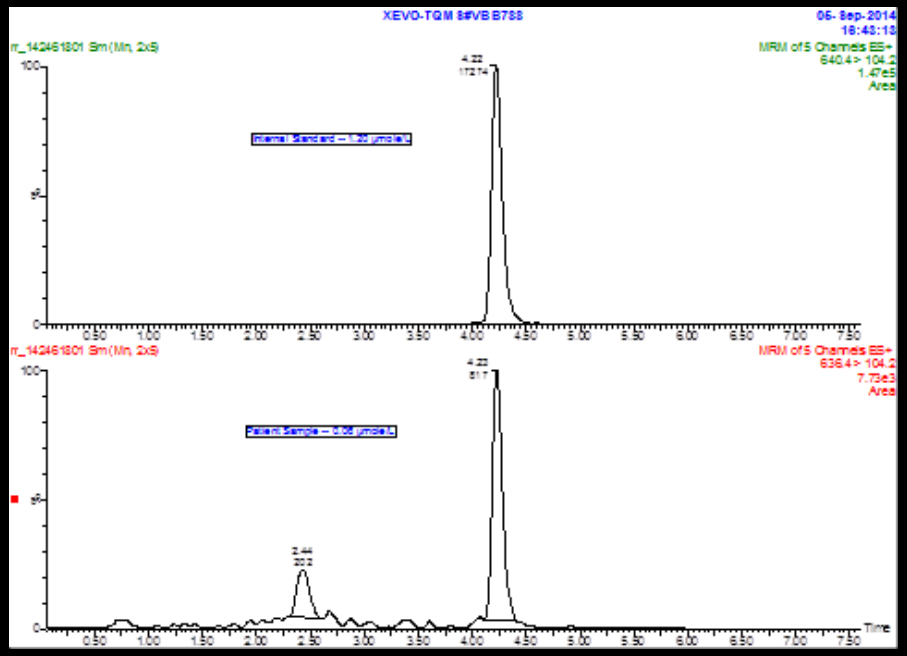
	1	2	3	4	5	6
A	M13-F	M13-R	M13-F	M13-R	M13-F	M13-R
B	M13-F	M13-R	M13-F	M13-R	M13-F	M13-R
C	P-Fseq	P-Rseq	M13-F	M13-R	M13-F	M13-R
D	P-Fseq	P-Rseq	M13-F	M13-R	M13-F	M13-R
E	M13-F	M13-R	E5-Fseq	E4-Rseq	E9-Fseq	E9-Rseq
F	M13-F	M13-R	E5-Fseq	E4-Rseq	E9-Fseq	E9-Rseq
G	E1-Fseq	E1-Rseq	M13-F	M13-R	E10-Fseq	
H	E1-Fseq	E1-Rseq	M13-F	M13-R	E10-Fseq	

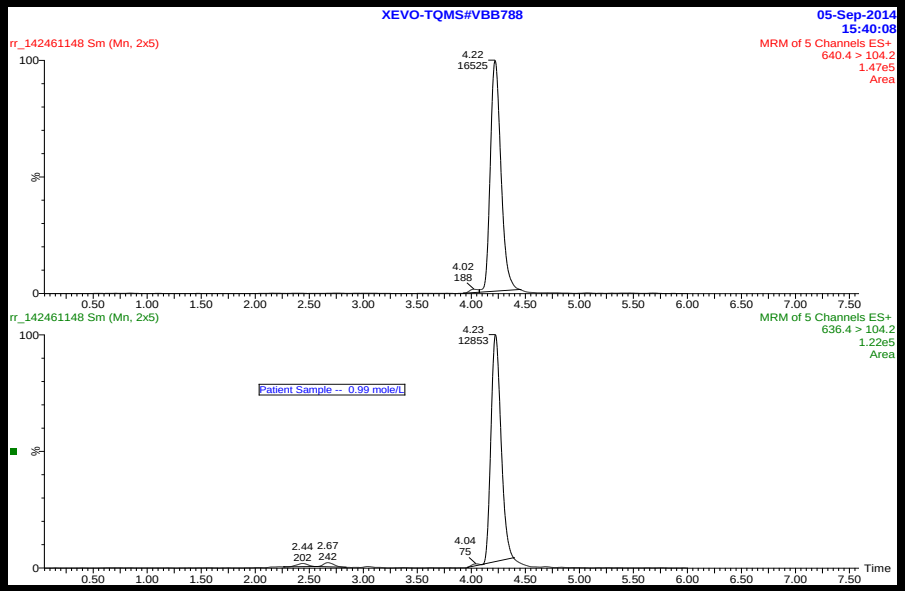
Second Tier: HPLC-MS/MS

Methanol Extract

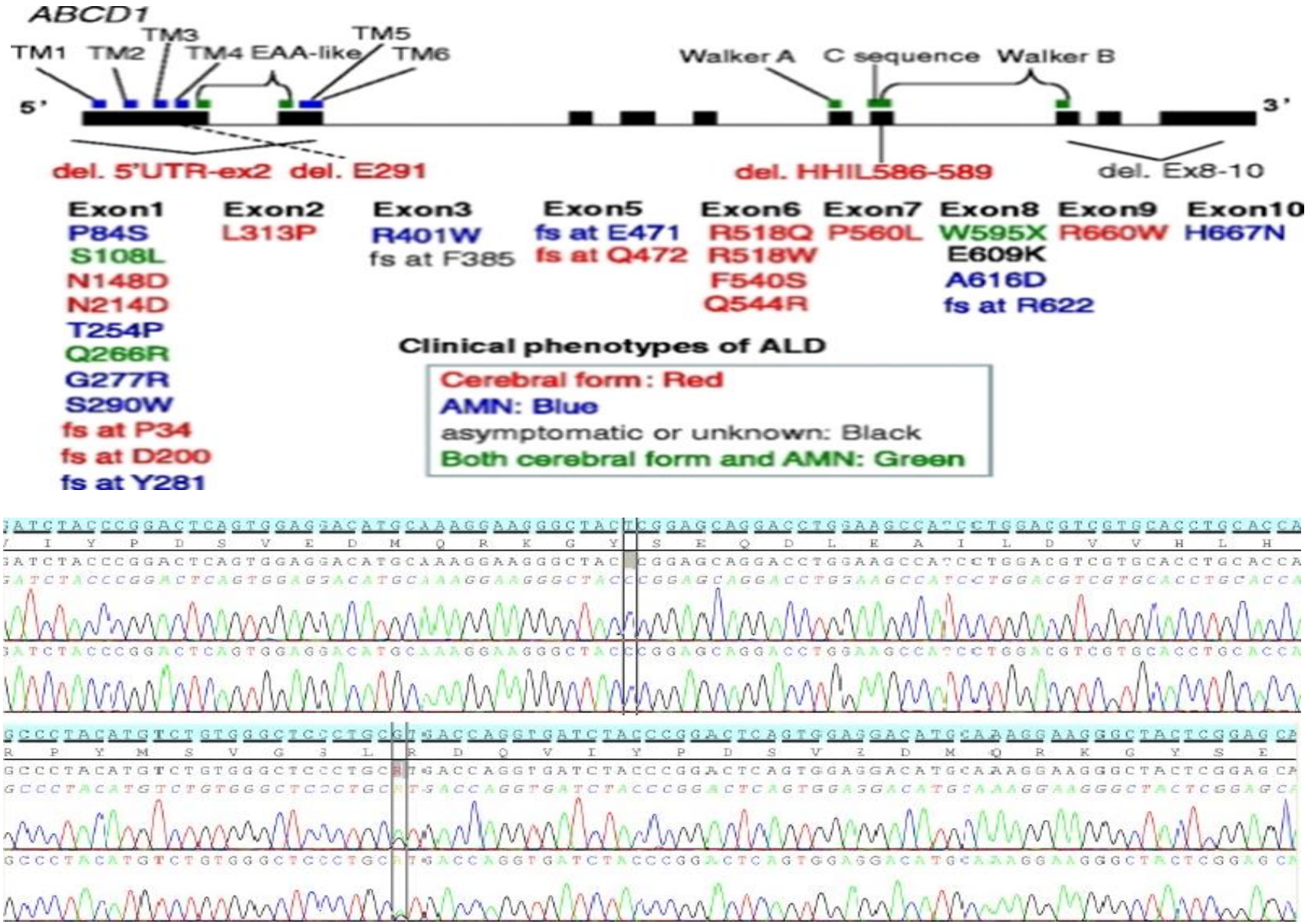
Column:
Waters Xterra C8
2.5 uM particle size

Linear Gradient
Mobile A: 20:80
(MeOH/H20)
Mobile B: 100 MeOH





Sample ID	Sample Result (µMole/L) 1st Tier	2nd Tier
NB_3223	0.40	0.12
NB_3231	0.41	0.08
NB_3251	0.48	0.11
NB_3286	0.40	0.09
NB_3288	0.42	0.08
NB_3337	0.40	0.07
NB_3364	0.42	0.06
NB_3387	0.40	0.08
NB_3542	0.41	0.08
NB_3544	0.92	0.08
NB_3556	1.09	0.10
NB_3669	0.43	0.07
NB_3948	0.45	0.11
NB_5395	0.41	0.09
NB_5997	0.55	0.10
NB_6009	0.42	0.08
NB_6251	0.42	0.09
NB_6253	0.40	0.07
NB_6771	0.43	0.09
NB_6772	0.53	0.08
NB_6919	0.41	0.05
NB_6981	0.53	0.36
NB_8321	2.44	0.14
NB_8322	1.62	0.07
NB_8326	0.42	0.08
NB_8327	0.64	0.11
NB_8424	1.81	0.08
NB_8439	0.58	0.09
NB_8451	0.51	0.08
NB_8511	0.40	0.09
NB_8599	0.97	0.08
NB_8600	0.50	0.09
NB_8601	0.43	0.09
NB_8846	0.61	0.36
NB_8872	0.41	0.08
NB_8895	0.40	0.14
NB_9001	1.02	0.87
NB_9148	1.06	0.08
NB_9160	0.95	0.10
NB_9172	0.41	0.07
NB_9220	0.43	0.08
NB_9480	0.41	0.08
NB_9589	0.42	0.10
NB_9663	0.40	0.14
NB_9669	0.45	0.09



Top: VOUS TCG>CCG (Ser>Pro) Hemizygous (BB)

Bottom: c.1661 G>A (p.Arg554His) Heterozygous (BG). Childhood cerebral & adult onset X-ALD

NEW YORK STATE DEPARTMENT OF HEALTH
Wadsworth Center, Empire State Plaza, Biggs Laboratory, Albany, NY 12201-0509, Phone: (518) 473-7552, Fax: (518) 474-0405

Infant:	Lab ID.:	Submitter Code:
Gender:	Accession No.: 20134521772	Hospital:
Birth Date:	Prior Accession No:	City:
Mother: ,	Specimen Date:	Med. Rec. No.:
Address:	Date Received: 12/04/2013	Physician:
,	Date Reported: 12/06/2013	
Telephone:	Multiple Birth:	

SCREEN POSITIVE NEWBORN SCREENING RESULT
PROMPT CONSULTATION WITH SPECIALTY TREATMENT CENTER REQUIRED

Condition	*Analyte*	*Reference Range*	*Result*
ALD	C26:0	< 0.40	High result C26:0, 0.41 µmole/L.
ALD	HC26:0	< 0.24	Second tier test for C26:0, 0.43 µmole/L.
ALD	ALD_DNA	No Mutations Detected	One Mutation Detected

ALD summary: This sample had a high level of the C26:0 marker when tested by a selective, second-tier method. This may be indicative of ALD or other peroxisomal disorder. Prompt consultation with a specialty care center is requried.

ALL OTHER TESTS SCREEN - NEGATIVE - SEE NEXT PAGE
See reverse side for Screened Disorders and Reference Ranges

DNA Results - See Last Page

Infant: ,
Accession No.: 20134521772
Lab ID:
Submitter Code:
Hospital: Unknown Submitter:

Result: **DNA Results**
One p.Gly266Arg ABCD1 Gene Mutation Detected

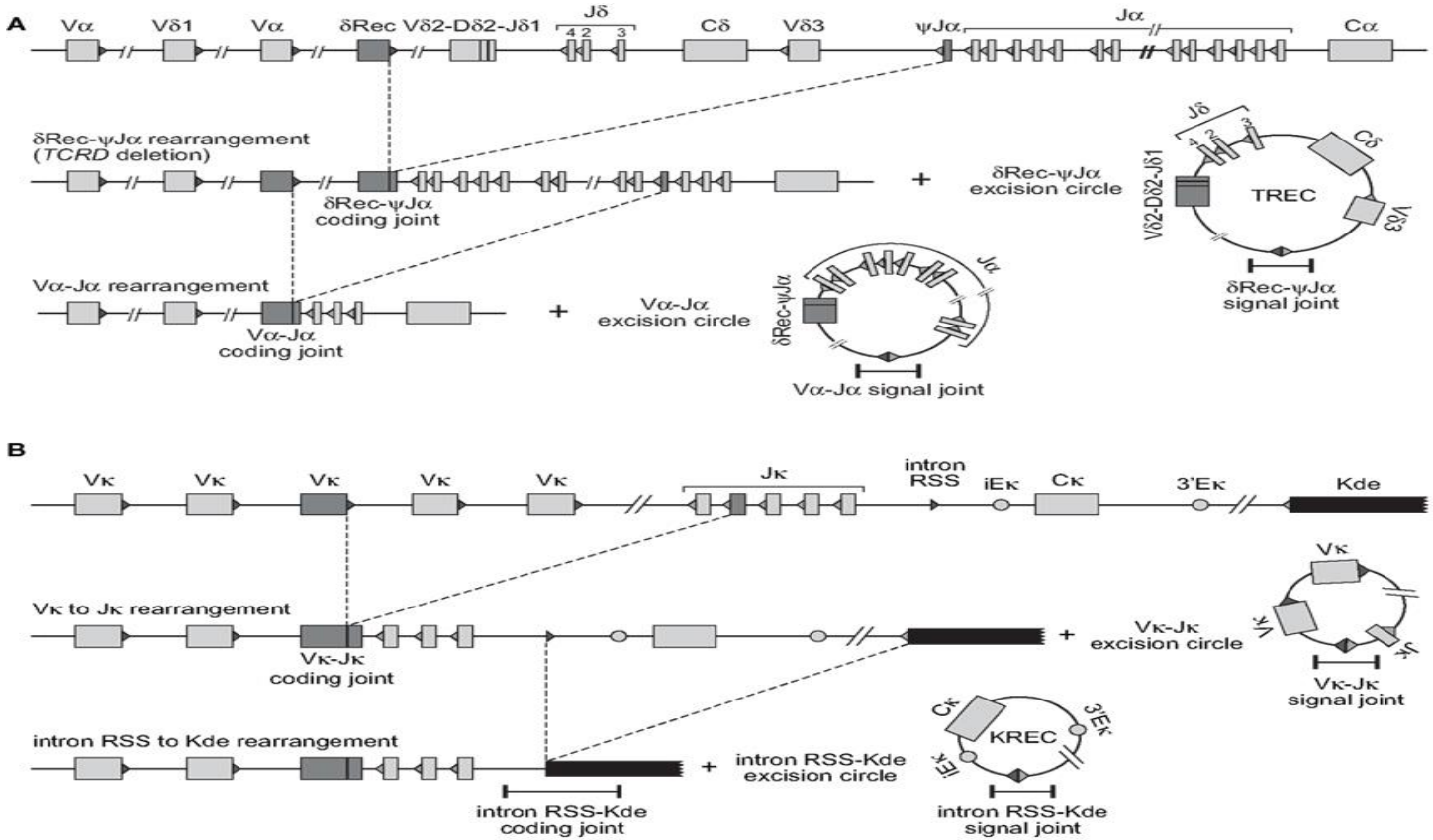
Mutations in the ATP-binding cassette, subfamily D, member 1 (ABCD1) gene cause adrenoleukodystrophy (ALD), a cerebral demyelinating and adrenocortical insufficient (~ 65% cases) peroxisomal disorder. Babies with peroxisomal disorders have elevated very long-chain fatty acids (VLCFA) in plasma. ALD is an X-linked (X-ALD) disease thus X-ALD is more common in males. Approximately 20% of X-ALD female carriers develop clinical symptoms. This baby boy has elevated VLCFA (C26) and one mutation in the ABCD1 gene. The p.Gly266Arg (c.1182 G>A) was detected; this mutation has been associated with the childhood-onset form of X-ALD. Due to this molecular result and the elevated C26:0, referral to a metabolic specialist for evaluation, confirmatory testing, follow-up and genetic counseling is recommended. The molecular test was performed using the polymerase chain reaction (PCR) followed by fluorescence-based sequence analysis of the promoter region, all 10 exons and the intron/exon boundaries of the ABCD1 gene.

ALD Mutations Screened:

C26:0

This type of analysis generally provides highly accurate genotype information. Despite this high level of accuracy, it should be kept in mind that there are potential sources of diagnostic error, including misidentification of samples, polymorphisms or other rare genetic variants that interfere with the analysis. Families should understand that rare diagnostic errors may occur for these reasons.

This test was developed and its performance characteristics determined by the Wadsworth Center Molecular Newborn Screening Laboratory. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. Pursuant to the requirements of CLIA'88, this laboratory has established the test's accuracy and precision.



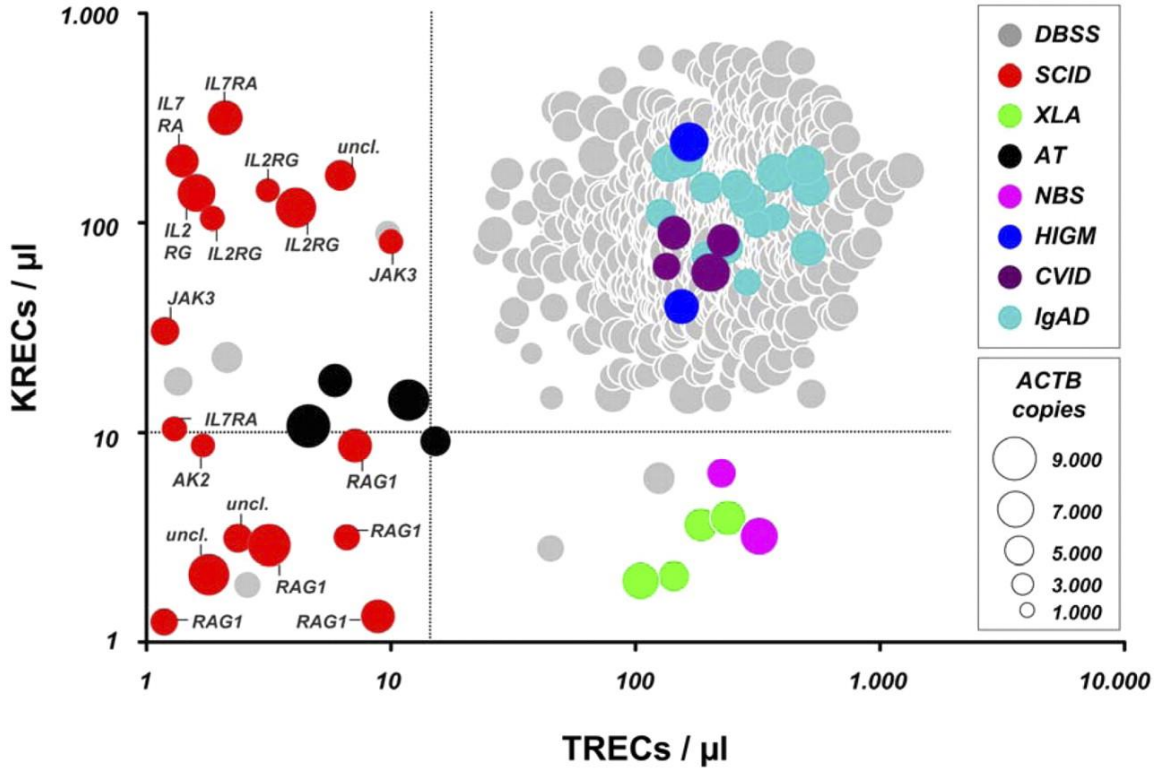
C

Target	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman probe (5'-3')	Amplicon size (bp)
cj ψJα-δREC[a]	AAAAAGCAACATCACTCTGTGTCT	GGCACATTAGAATCTCTCACTGA	CCAGAGGTGCGGGCCCCA	197
sj TREC ψJα-δREC[b]	CCATGCTGACACCTCTGGTT	TCGTGAGAACGGTGAATGAAG	CACGGTGATGCATAGGCACCTGC	131
cj int-Kde[c]	CCCGATTAATGCTGCCGTAG	CCTAGGGAGCAGGGAGGCTT	AGCTGCATTTTTGCCATATCCACTATTTGGAGT	144
sj KREC int-Kde[c]	TCAGCGCCCATTACGTTTCT	GTGAGGGACACGCAGCC	CCAGCTCTTACCCTAGAGTTTCTGCACGG	148
human albumin	TGAACAGGCGACCATGCTT	CTCTCCTTCTCAGAAAGTGTGCATAT[d]	TGCTGAAACATTCACCTTCCATGCAGA[d]	118
human cα[a]	CCTGATCCTCTTGTCCCACAG	GGATTTAGAGTCTCTCAGCTGGTACA	ATCCAGAACCCTGACCCTGCCG	71

a. (Dik et al.,2005)
b. (Hazenberg et al., 2000)
c. (van Zelm et al., 2007a)
d. (Pongers-Willemse et al., 1998)

cj, coding joint; sj, signal joint

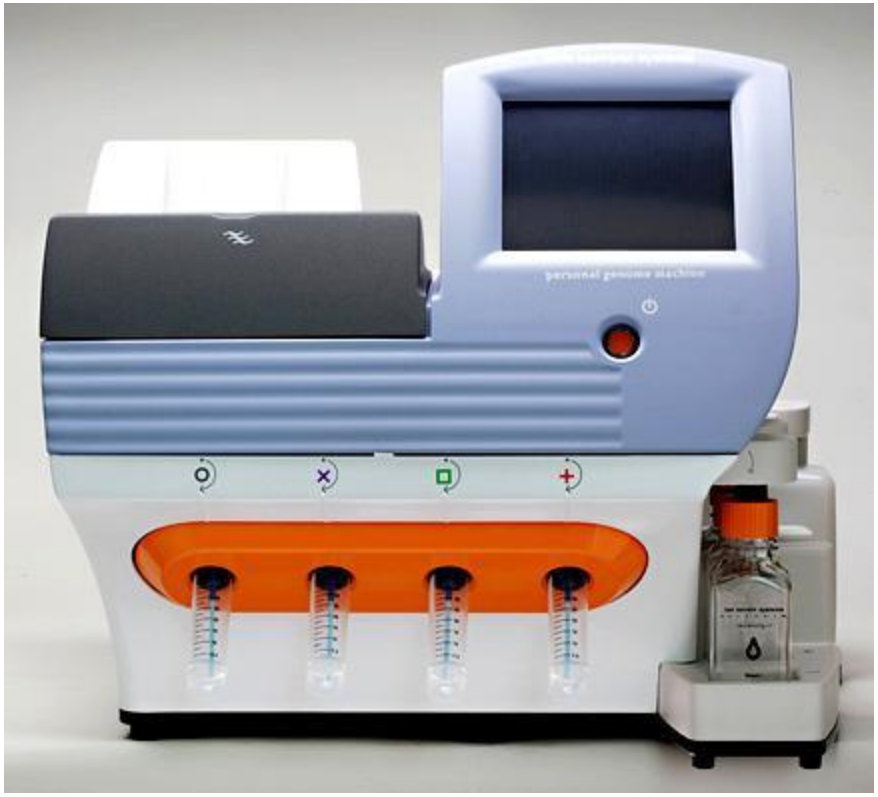
TREC and KREC copy numbers in dried blood spot samples (DBSS) from anonymized Guthrie cards and retested samples and in patients diagnosed with SCID, XLA, AT, NBS, X-HIGM, CVID, or IgAD.



Stephan Borte et al. Blood 2012;119:2552-2555

blood

The Ion Torrent Personal Genome Machine (PGM™) sequencer enables researchers to obtain highly accurate sequence in record time.



Ion Semiconductor Sequencing Chip	Output	Read Length		Total Sequencing Time
314	> 10Mb	2011	2012	< 2 hours
316	> 100Mb	> 200bp	> 400bp	
318	> 1Gb			
Accuracy:	>99.99% consensus accuracy and >99.5% raw accuracy.			

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MiSeq removes the barriers of questionable data, inefficient workflows and slow results. By bringing the most accurate, top of the line next-gen sequencing technology right to your desktop, MiSeq quickly and affordably delivers the highest yield of error-free reads. So you can count on us for answers. And we know we can count on you for the questions. The only limits are your curiosity and your drive. And we know we can count on that too.

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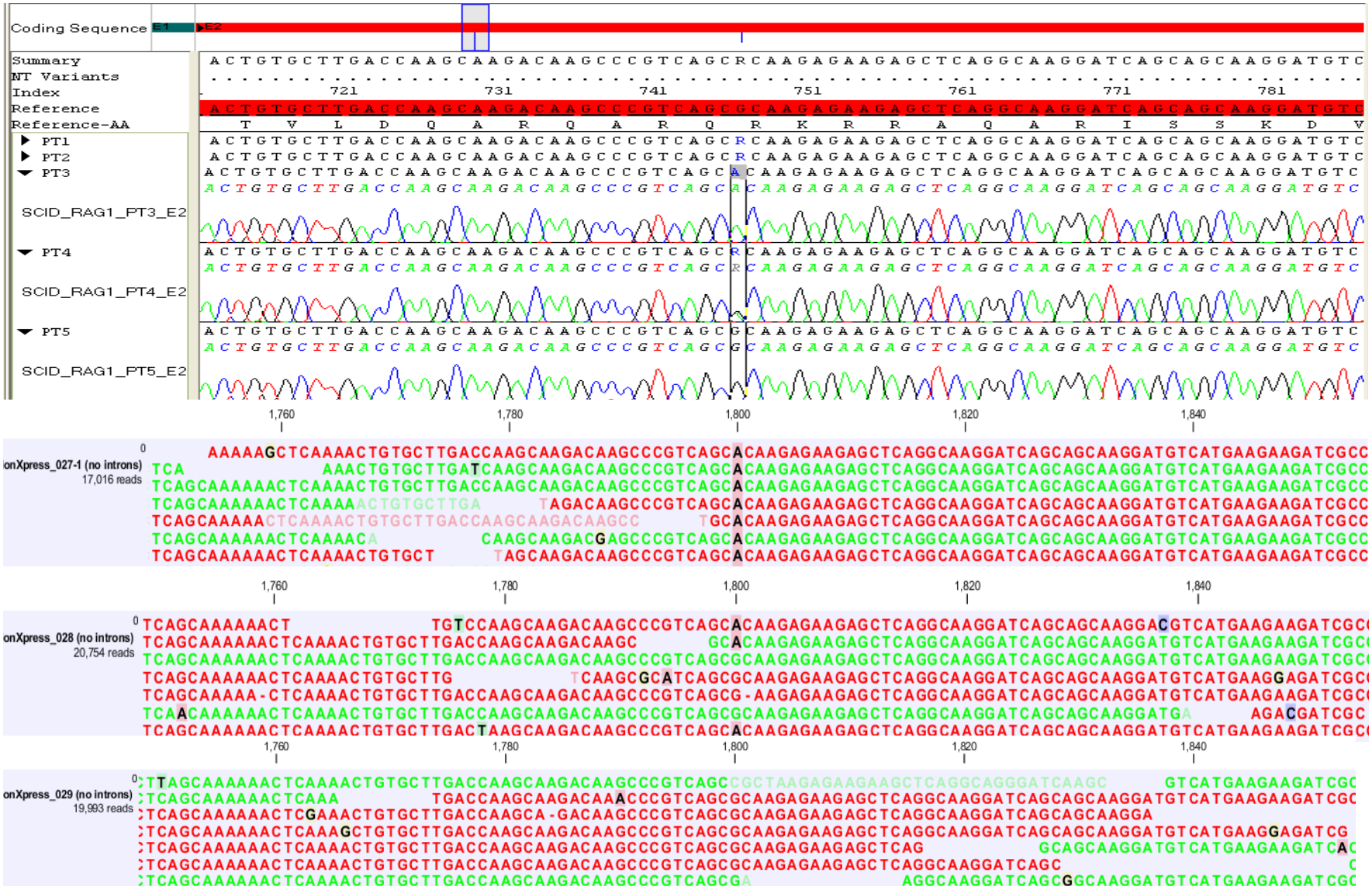

***RAG1* E2: c.746 G>A = p.Arg249His**

Take Home Points for NBS Genotyping

Multiple methods can be used with the following considerations:

- Lab resources
- Natural history and genetics of a disease - is there a common mutation responsible for many cases?
- Number of samples to be tested – depends on population size and whether the assay is first, second or third tier
  - Eg: First (SCID), second (CF, Krabbe, Pompe, MCAD, GALT) or third (X-ALD, GALT) tier

Take Home Points for NBS Genotyping Cont.

- Best technology for program throughput
  - Lower throughput assays: RFLP, ARMS, or allele size detection using agarose gels, FRET analysis, invader, allele-specific primer extension (luminex)
  - Higher throughput assays: Real-time PCR using 96 or 384 well plates *Robotics often necessary!
  - Gene sequencing assays: Needed when natural history of a disease is incomplete - 16, 24, 32, 48 and 96 sample throughput
- NGS sequencing is slowly going to be stablished – will be useful for genotyping multigene diseases
  - Eg: underlying genetic cause of SCID or other lymphopenias


Atlantic Ocean
VENEZUELA
GUYANA
SURINAME
COLOMBIA
ECUADOR
PERU
BRAZIL
BOLIVIA
PARAGUAY
URUGUAY
ARGENTINA
South
Pacific
Ocean
Atlantic Ocean

Newborn screening is an extraordinarily successful example of the application of advances in medical genetics to personal and public health benefit."

Editorial , Nature Genetics, October 2010

Luis Eduardo Saavedra Rodriguez
1926-2013

Soledad Matiz de Saavedra
1924-2010