Capillary Electrophoresis: Genetic Disease Testing for Challenging Targets in the Modern Clinical Lab

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Overview

- Rare Genetic Diseases: What are they, why do they matter, and how rare is rare?
- Nucleic Acid Testing in the Clinic: Diagnosis and Screening
  - Newborn Screening and Carrier Screening
- A Brief History of DNA Sequencing
- Capillary Electrophoresis (CE): Overview and applications
  - Dark DNA: Repetitive DNA elements and their role in disease
  - Other Challenging Applications with CE: Quantifying gene copy numbers
  - Comparing CE to Next-Gen Sequencing and qPCR
- Bringing an Assay Into the Clinic

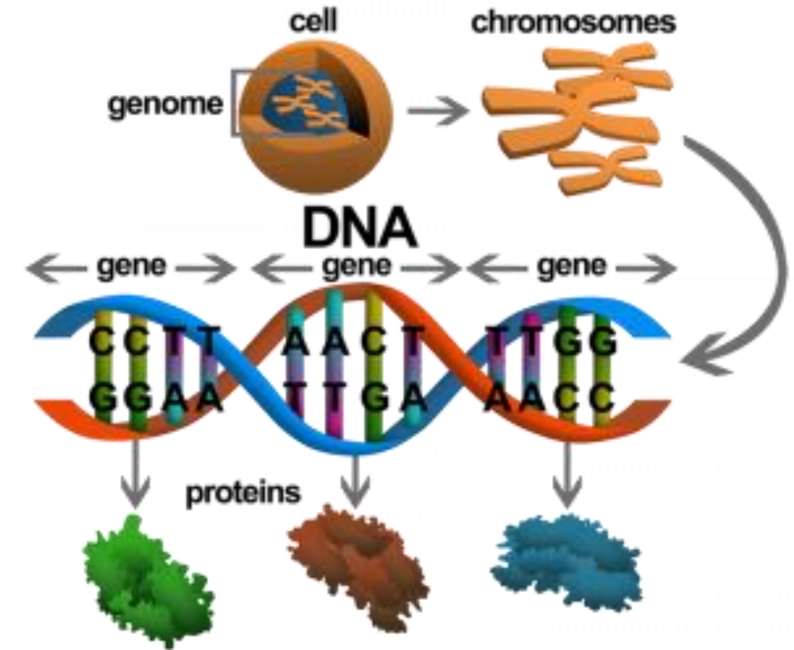
Learning Objectives

- Evaluate the strengths and weaknesses of capillary electrophoresis for nucleic acid diagnostics compared to other technologies
- Describe the importance of genetic mutation testing for carrier screening and newborn screening applications
- Discuss how the roles of capillary electrophoresis and genetic mutation testing have changed over time—and how they might change in the future

Rare Genetic Diseases

What are they?

- Genetic diseases are caused by one or more abnormalities (mutations) in the genome
  - Can occur in a single gene or location (most common)
  - Can also be due to chromosomal abnormalities (gain or loss; e.g. Downs Syndrome)
- Can be Recessive (mutation in both gene copies causes disease) or Dominant (mutation in either gene copy causes disease)



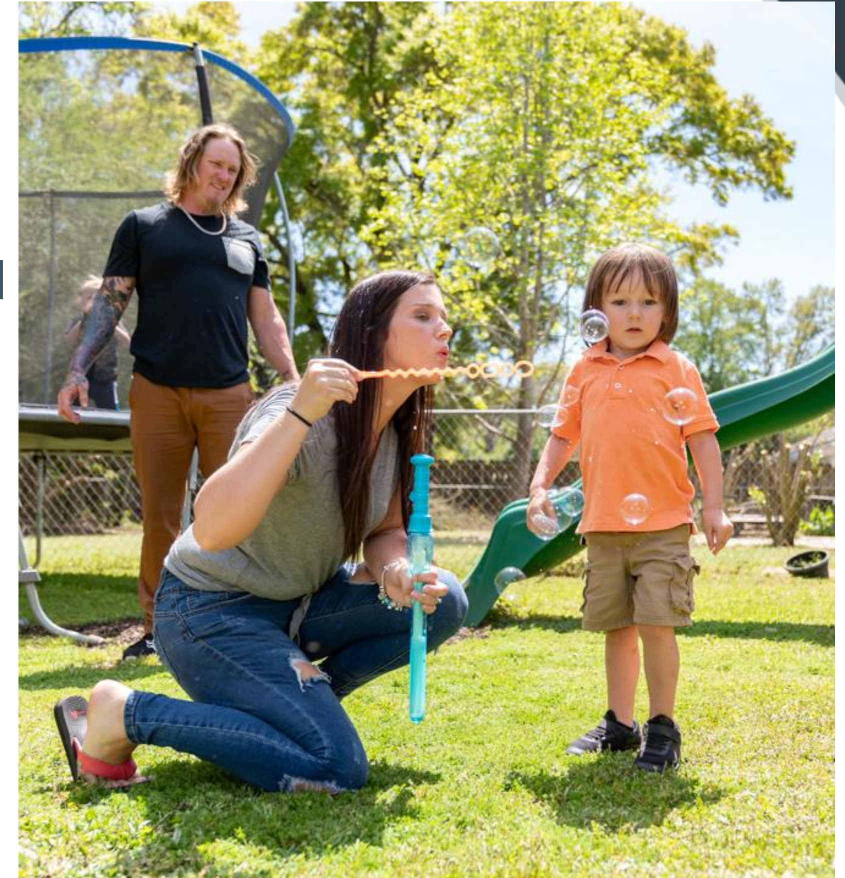
A typical human cell has 23 pairs of chromosomes, or 46 total, with one copy of each chromosome coming from each parent

http://www.thinnergene.com/about-thinnergene/genetics-101/

Rare Genetic Diseases

Why do they matter?

- The global prevalence of all single gene diseases at birth is approximately 1/100 [1], and include:
  - Fragile X syndrome, the leading known cause of inherited intellectual disability/autism, **1 in 3,500-6,000** [2]
  - Spinal Muscular Atrophy (SMA), once the leading genetic cause of infant death, **1 in 6,000-10,000** [3]
  - Over 10,000 other debilitating diseases, including Cystic Fibrosis, Sickle Cell Anemia, Tay Sachs, Thalassaemia [1]
- Breakthroughs in modern medicine and diagnostics improve prognosis and reduce healthcare burden
  - With SMA, new treatments save lives, and children walk who would have struggled to even sit [4]



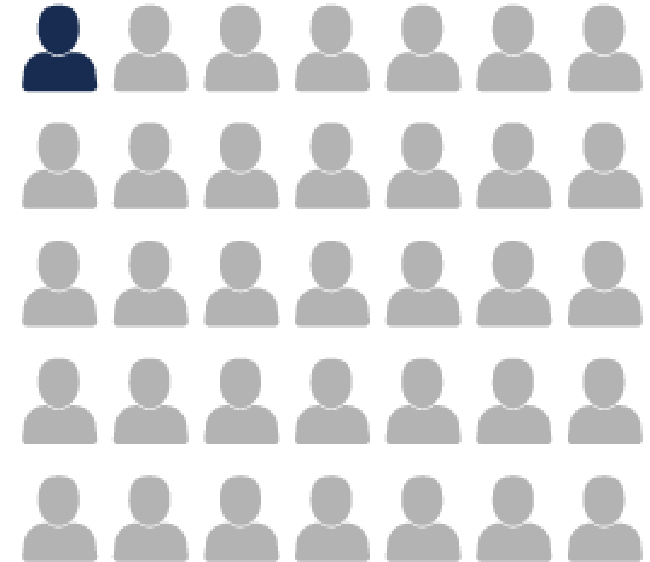
Chance, diagnosed with SMA before birth, plays with his parents in the park after successful treatment. Previously, SMA Type 1 and Type 2 patients were never able to walk or sit without assistance. [4]

[1] World Health organization, https://www.who.int/genomics/public/geneticdiseases/en/index2.html
[2] National Fragile X Foundation, https://fragilex.org/understanding-fragile-x/fragile-x-101/31-shareable-fragile-x-facts/
[3] SMA Foundation, http://www.smafoundation.org/about-sma/
[4] MultiVu, https://www.multivu.com/players/English/8560251-biogen-nurture-study-spinraza-spinal-muscular-atrophy-treatment-data/

Rare Genetic Diseases

How rare is “rare”?

- In the US: a rare disease is defined as a disease that affects “populations smaller than 200,000 people in the United States”[1] or ~ 1 in 1,500 people (varies elsewhere)
- Consider this:
  - In recessive disorders, individuals with a mutation in only one gene copy are **carriers**, and have a **25% chance** of having affected offspring **if the other parent is also a carrier**
  - An autosomal recessive disorder with a prevalence of only **1 in 5,000** has an estimated carrier rate of **1 in 35** people



A “rare” recessive disorder with a prevalence of just **1 in 5,000** will have a carrier rate of ~**1 in 35**

[1] Rare Diseases Act of 2002, https://www.govinfo.gov/content/pkg/PLAW-107publ280/html/PLAW-107publ280.htm

Nucleic Acid Testing in the Clinic

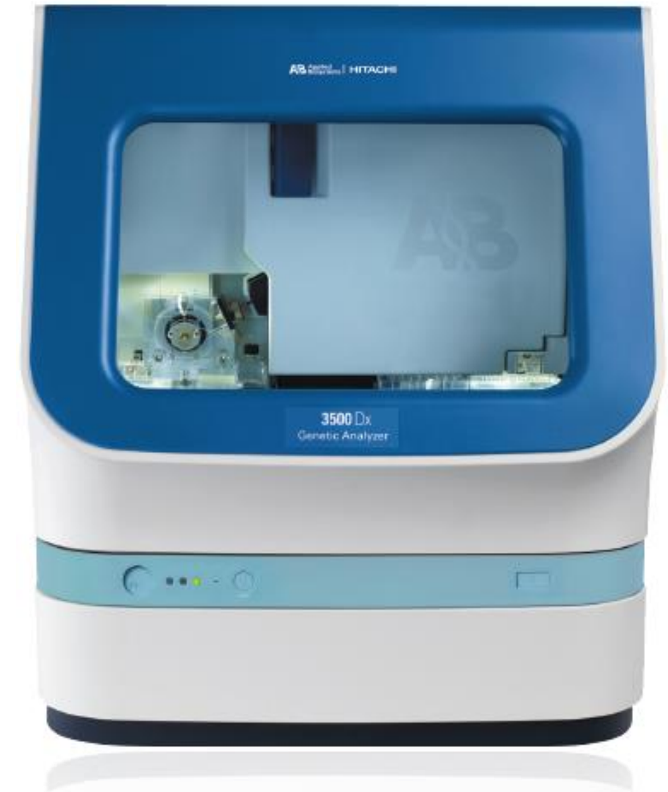
Diagnosis and Screening

- Diagnostic testing is used to confirm/deny a suspected disease state in an individual that is symptomatic
  - May provide additional information beneficial for treatment and characterization of the disease
- Screening is used to detect mutations linked to disease or carrier status in a general population that is not symptomatic
  - Carrier Screening is used to identify carriers of recessive disorders in individuals before or during pregnancy to **assess risk of children having the disorder**
    - Commonly includes testing for fragile X, spinal muscular atrophy, cystic fibrosis
  - Newborn Screening is used to identify genetic diseases in infants before symptoms occur to **enable effective and timely treatment/management**
    - For genetic diseases, may include spinal muscular atrophy, cystic fibrosis, severe combined immunodeficiency (SCID), sickle cell disease, thalassemia, many others

Nucleic Acid Testing in the Clinic

Diagnosis and Screening

- For both screening and diagnostic testing of genetic disorders, clinicians test DNA to characterize relevant genes and mutations
- DNA testing methods include:
  - Polymerase Chain Reaction (PCR), which amplifies the gene or mutation in question to determine **mutation status** and quantify **copy number**
  - Sequencing, which determines the nucleotide sequence of the gene(s) in question to determine **mutation status** and quantify **copy number** (in some applications)
  - Southern Blot, which uses probes to determine **mutation status** and quantify **copy number**
    - Less common now due to amount of time, labor, and interpretation required

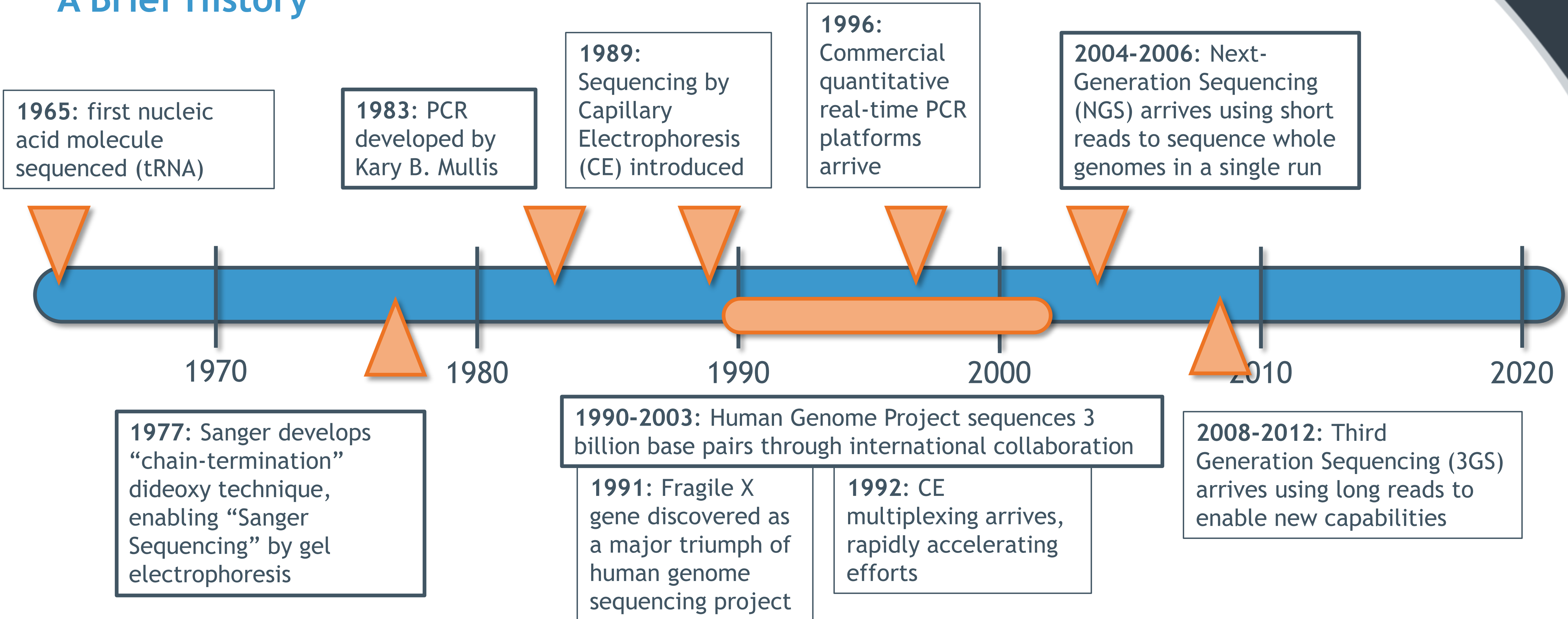


Modern Capillary Electrophoresis (CE) Sequencers* can sequence multiple samples in parallel

*3500 Dx instrument (pictured) intended for the sequencing (detection and identification) of fluorescently-labeled deoxyribonucleic acid (DNA) by capillary electrophoresis

Nucleic Acid Testing

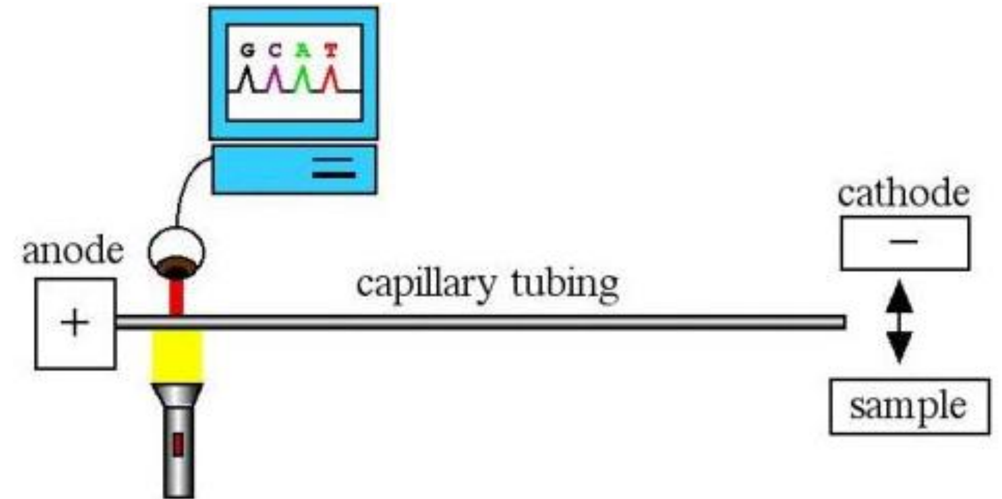
A Brief History



Capillary Electrophoresis for DNA Analysis

How it works

- CE developed as a platform for rapid, multiplexed Sanger sequencing
  - Modern systems are benchtop-sized, inexpensive, easy to maintain, and automated
- DNA migrates through capillary toward anode when voltage is applied, separating by size and charge
- CCD camera detects fluorescently-labeled fragments as they pass
  - Fragments can be accurately sized/resolved to single base pairs
  - Sanger method translates fragment size to sequence position, with a unique fluorescent label for each nucleotide



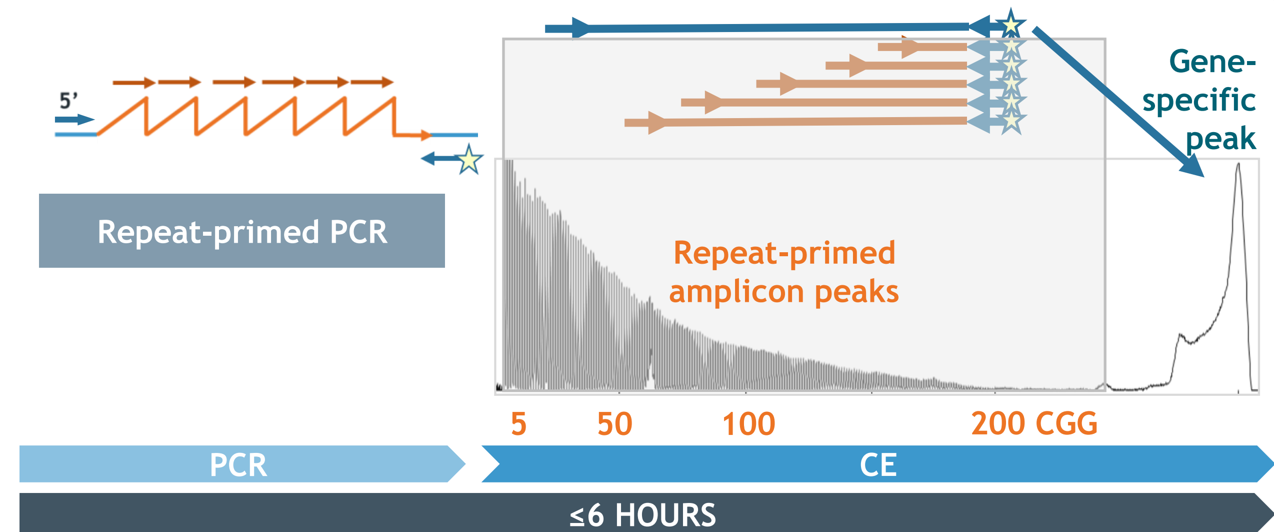
Modern instruments simplify the CE workflow and reduce cost barriers, making CE more accessible to labs*

Workflow Image Copyright 2002 Department of Biology, Davidson College: http://legacy.jyi.org/volumes/volume5/issue9/features/lovejoy.html
*SeqStudio pictured. For Research Use Only. Not for use in diagnostic procedures.

Capillary Electrophoresis for DNA Analysis

Other applications: Repeat DNA Analysis

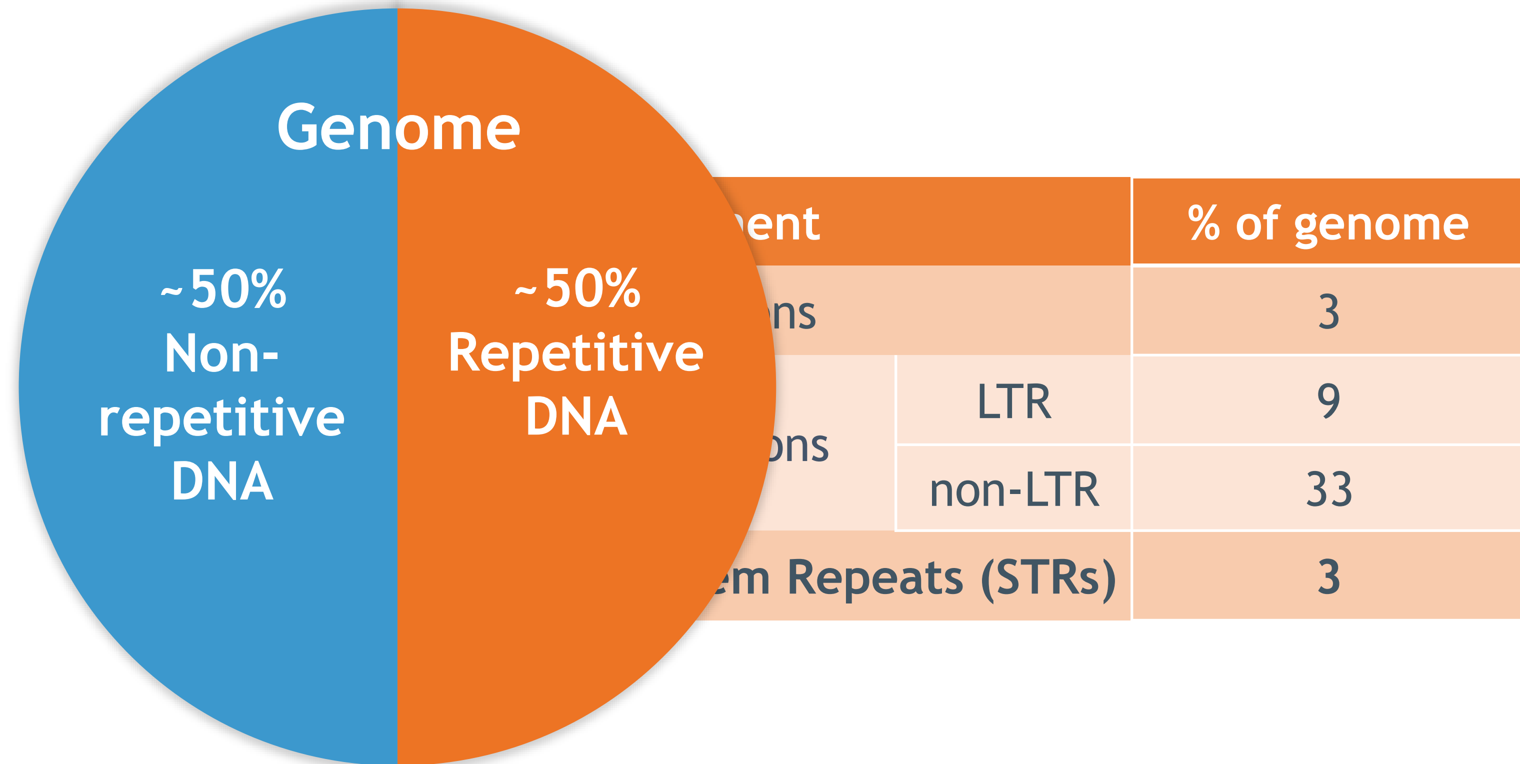
- **Problem:** Short tandem repeats (STRs) are difficult to amplify/ characterize but clinically important
  - Most sequencing methods (including NGS) cannot resolve STRs
- With specially-designed long-read PCR, STRs can be amplified and accurately sized using CE
- STRs are crucial in understanding and diagnosing >30 debilitating genetic diseases
  - Also important for forensic analysis, as STRs known as microsatellites have unique “fingerprint” patterns



Filipovic-Sadic, S. et al (2010)
Chen, L. et al (2010)

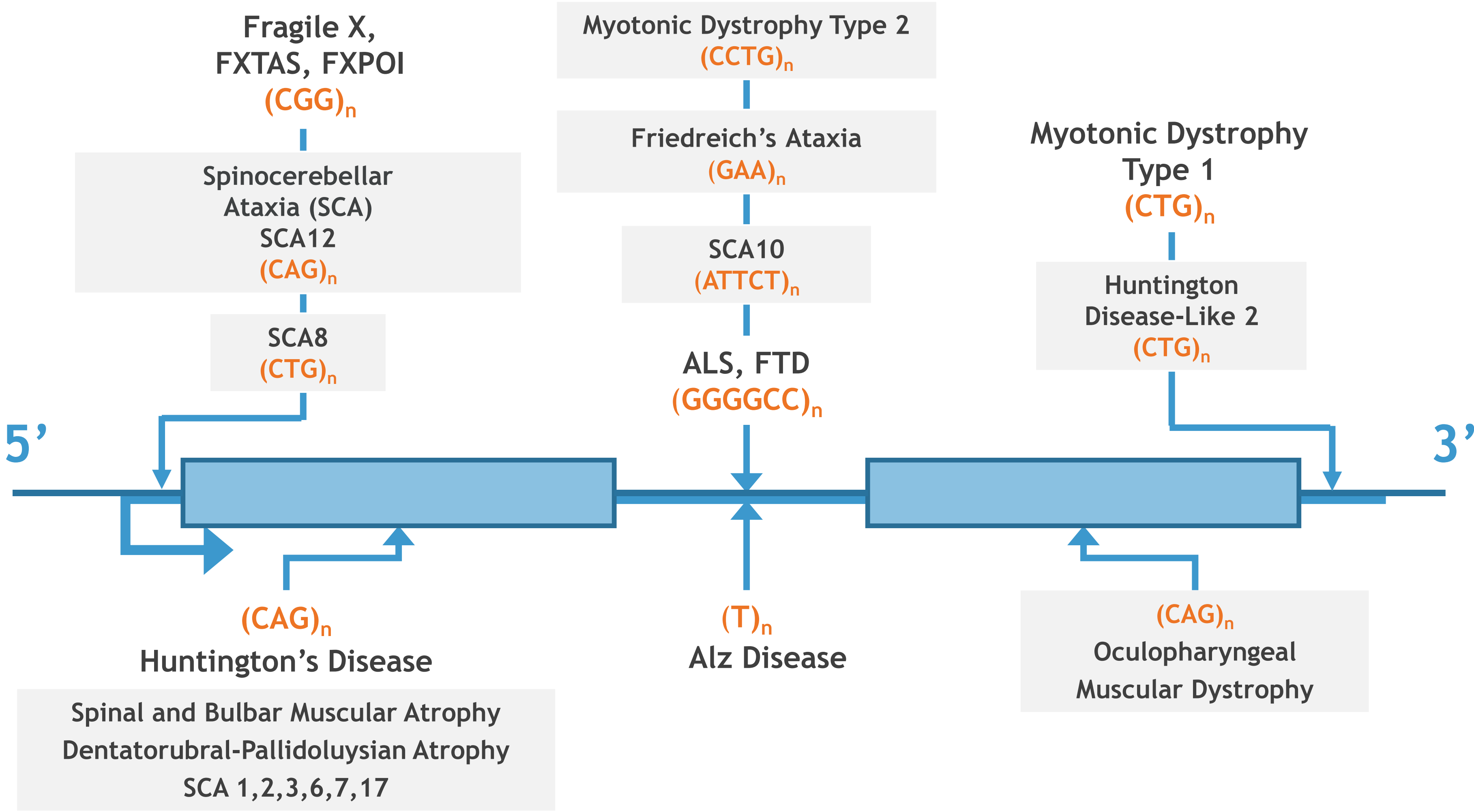
Uncovering “Dark” DNA

Repeat DNA comprises half the genome yet is mostly “dark” in function



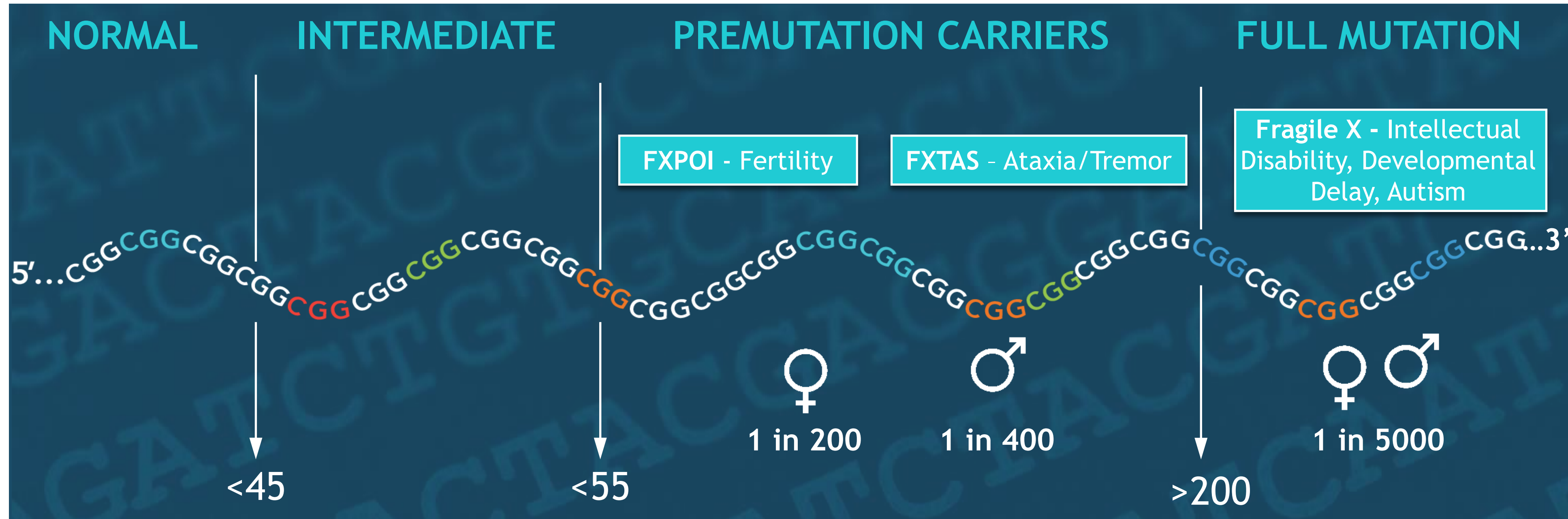
More than 30 Neurological Disorders Associated with STRs

Illuminating repeat DNA and structural variants has greatly advanced molecular basis of genetic disease

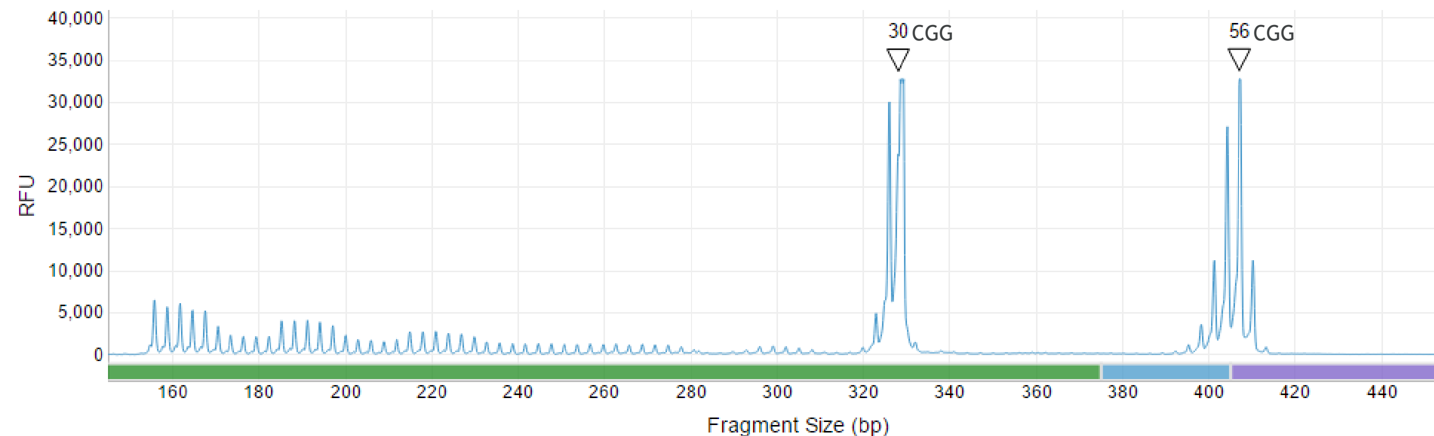


Clinical Disorders associated with *FMR1* Expansions

A case study in the complexity of STR-associated disorders



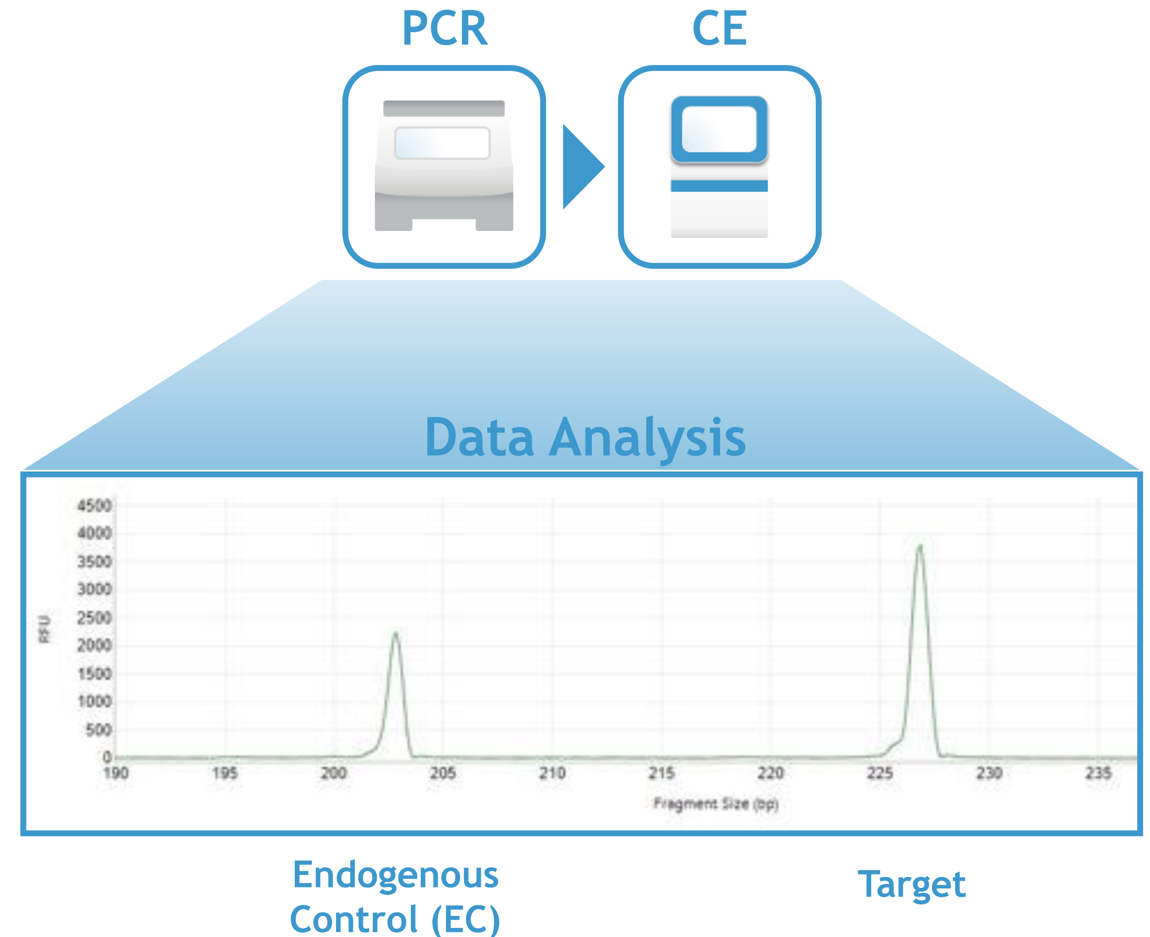
Accurate STR sizing with PCR/CE technology is critical for characterizing repeat-associated disorders



Capillary Electrophoresis for DNA Analysis

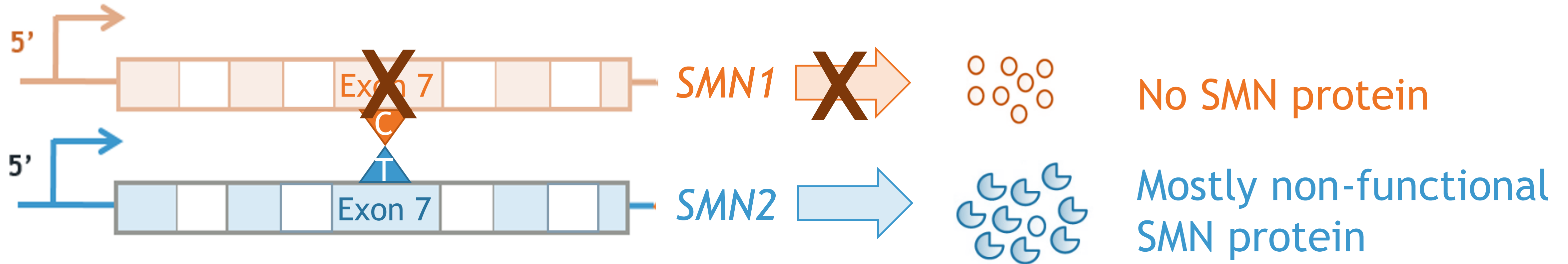
Other applications: Copy Number Variation (CNV)

- **Problem**: CNVs are clinically relevant, but difficult to accurately characterize, especially for paralogs and CNV heterogeneity
  - Some CNVs can be detected by NGS, e.g. Downs, where whole chromosome is gained
- Peak height/area used to determine copy number
- CNVs are a critical factor in many genetic diseases, most notably Spinal Muscular Atrophy (SMA)
  - Can also be associated with Alzheimer's, Autism, Parkinson's, HIV risk…



SMN1, *SMN2*, and Spinal Muscular Atrophy

SMA carriers and patients are informed by *SMN1* or *SMN2* copy numbers



1 copy

Unaffected

Carrier

1 in ~50

Up to 95%
heterozygous deletion detection rates

0 copy
0-8 copy

No *SMN1*
Variable *SMN2*

SMA

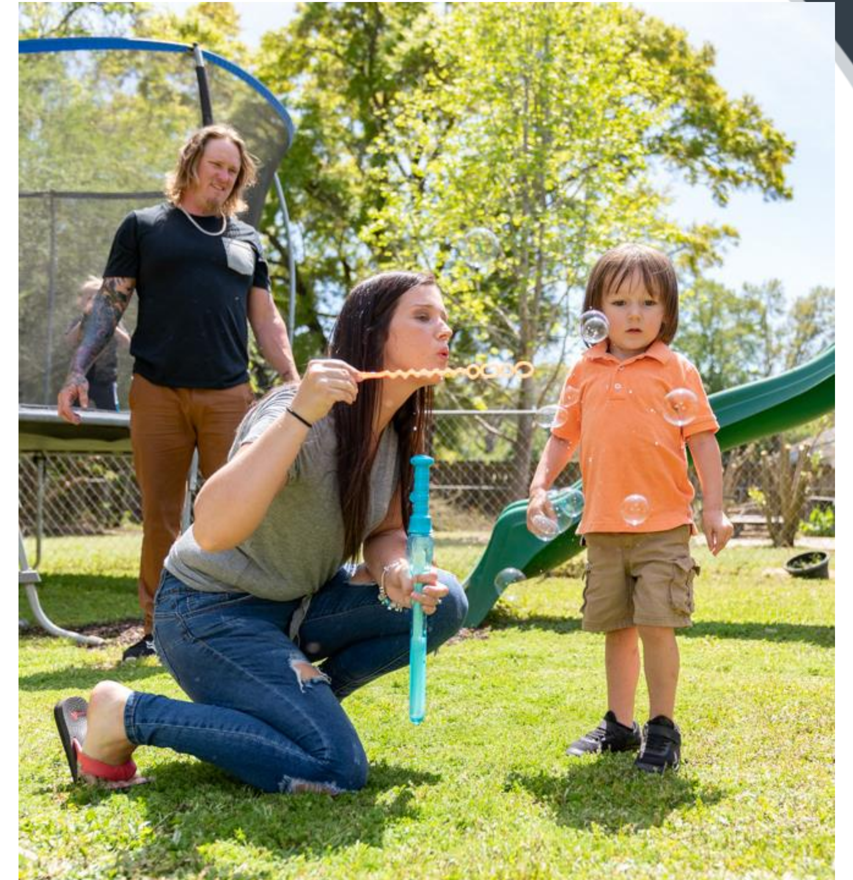
1 in ~10,000

Type	Highest Function	*SMN2* Copy #
1	Never Sit	2
2	Never Stand	3
3	Stand alone	3-4
4	Stand alone	>4

Emerging Therapies Drive Testing Needs

SMA is a key example of emerging trends

- Anti-sense and gene therapies are providing unprecedented treatment of genetic diseases
  - In SMA, drugs correct splicing of *SMN2*, providing fully functional SMN protein
- The timing and accuracy of DNA diagnostics are crucial for successful treatment
  - Novel gene therapies are revolutionizing treatment for SMA if detected early[1]
  - Treatment guidelines differ depending on SMN2 copy number in patients (and by country/region)
- Testing guidelines now recommend screening every woman considering pregnancy for SMA [2]



Chance, diagnosed with SMA before birth, plays with his parents in the park after successful treatment. Previously, SMA Type 1 and Type 2 patients were never able to or sit without assistance. [1]

[1] MultiVu, https://www.multivu.com/players/English/8560251-biogen-nurture-study-spinraza-spinal-muscular-atrophy-treatment-data/
[2] ACOG, https://www.acog.org/About-ACOG/News-Room/News-Releases/2017/ACOG-Recommends-Offering-Additional-Carrier-Screening-to-All-Women-Regardless-of-Ethnicity?IsMobileSet=false

How Does CE Compare to Other Nucleic Acid Testing Methods?

	CE, PCR/CE	NGS	Quantitative PCR
Concept	PCR products/DNA are injected into capillaries, separated by size/charge	DNA is fragmented, attached to a chip, and sequenced in massively parallel process	PCR is monitored during amplification with fluorescent probes/dyes
Detection	Fluorescently labeled DNA	Fluorescently labeled DNA (common); Chemiluminescence; pH	Fluorescently labeled DNA
Workflow	**PCR:** 1 - 4 hr **Instrument:** 1 hr/inj **Analysis:** 0.25 – 1 hr	**Library Prep:** 24 - 48 hr **Instrument:** 4 - 55 hr **Analysis:** 0.5 – 8 hr	**Prep:** 0.5 – 1 hr **Instrument:** 0.5 – 2 hr **Analysis:** 0.25 – 1 hr

*Changing with modern instruments

How Does CE Compare to Other Nucleic Acid Testing Methods?

	CE, PCR/CE	NGS	Quantitative PCR
Useful For	Difficult targets; Fragment sizing; Sanger sequencing	Whole Genome & Targeted Sequencing; Rare SNP detection; Transcriptome sequencing	Absolute/relative quantitation; SNP detection; Pathogen detection
Strengths	Most accurate sizing method (STRs, etc); Cheap for sequencing; Short turnaround time	Genome-wide sequencing in one run; High resolution for rare/localized variants	Cheapest for gene quantification; Short turnaround time; Low analysis burden
Weaknesses	High instrument cost/maintenance*; Medium analysis burden; Targeted sequencing only	Highest instrument/run cost; Highest analysis burden; Highest turnaround time; Short reads can limit utility	Medium instrument cost; Limited to one or a few target genes;

*Changing with modern instruments

Establishing a New Clinical Assay

Considerations for the clinical lab

COST

- Reagents?
- Instruments?
- Time?

OPERATOR SKILL

- What expertise is required to run the assay?

PERFORMANCE

- Does the assay meet the required specifications?

REAGENTS

- Commercial kit?
- If not, is reagent supply sustainable/ reliable?

WORKFLOW

- Does the assay fit with the lab workflows?
- Is automation needed?
- Computational needs?

Establishing a New Clinical Assay

Commercial Kit Vs. Laboratory-Developed Tests

	Commercial	Lab-Developed (LDT)
Strengths	• Automated data analysis • Plug and Play; sample to answer • Limited verification burden • Reliable reagent source & QC • Customer support • No R&D cost	• Customized to lab workflow/needs • May reduce operating costs • Develop internal expertise through assay R&D
Weaknesses	• Can be more expensive (per test cost) • May not fit with existing workflow	• Assay development is labor/time-intensive • Method devt expertise <u>required</u> • High validation burden • Analysis may be more subjective/require more work • QC of materials required • Reagents less sustainable

Conclusions

- Initially developed to accelerate Sanger sequencing, Capillary Electrophoresis can be a powerful platform for testing difficult targets that are relevant to many serious genetic disorders
- Our understanding of the human genome and the importance of certain genetic elements is changing rapidly
  - Many unique features like repeat elements, copy number variants, and others are only beginning to be fully understood in a clinical context
  - As discoveries advance our understanding of phenotypic impact, so too must our ability to characterize them in order to make these discoveries “actionable”
- As new therapies and are developed, carrier screening for reproductive planning and newborn screening to detect disease prior to symptoms will become increasingly important for effective treatment strategies

Further reading

- Filipovic-Sadic, S. et al (2010). A Novel FMR1 PCR Method that Reproducibly Amplifies Fragile X Full Mutations in Concordance with Southern Blotting and Reliably Detects Low Abundance Expanded Alleles. Clin Chem. 56(3): p.399–408
- Chen, L. et al (2010). An information-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for southern blot analysis. J Mol Diagn. 12(5): p. 589-600
- Genetic Support Foundation: Expanded Carrier Screening Practice Guideline Summaries, 2018
- SMA ACOG Guideline Updates, 2017
- For more information on these and many other relevant genetic disorders:
  - https://asuragen.com/
- For additional information about CE instruments:
  - https://thermofisher.com/geneticanalyzer/