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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

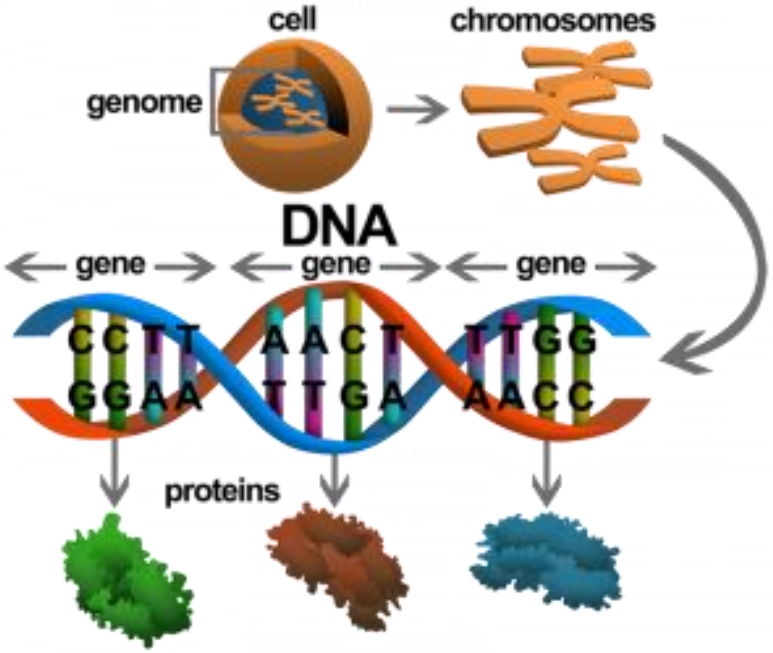
Learning Objectives

- 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)



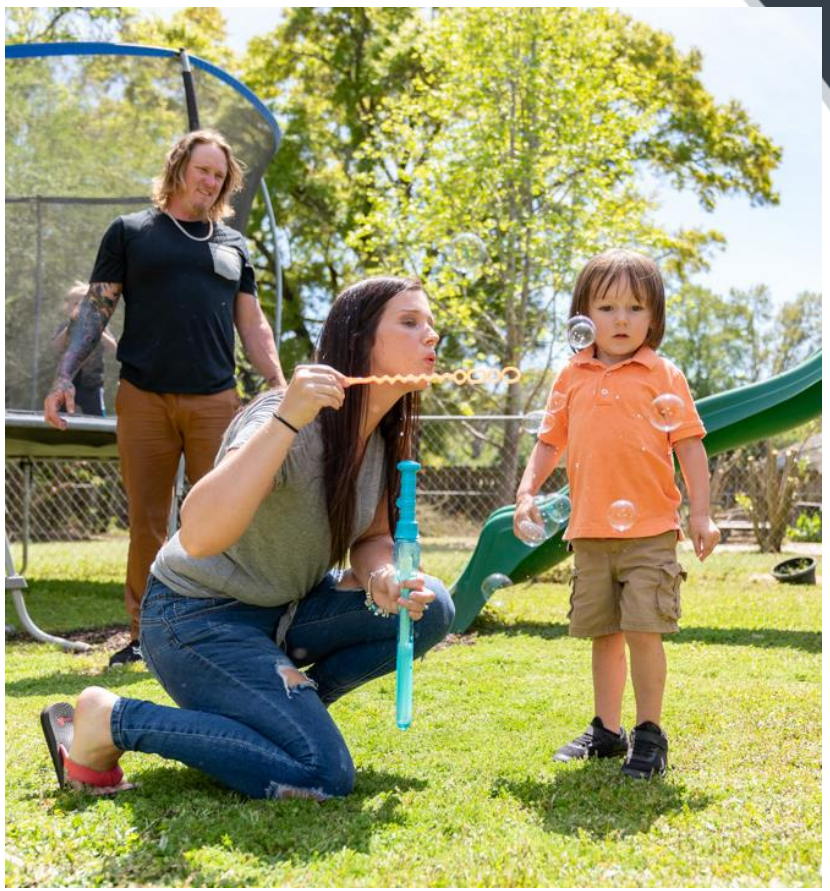
A typical human cell has 23 pairs of chromosomes, or 46 total, with one copy of each chromosome coming from each parent

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]



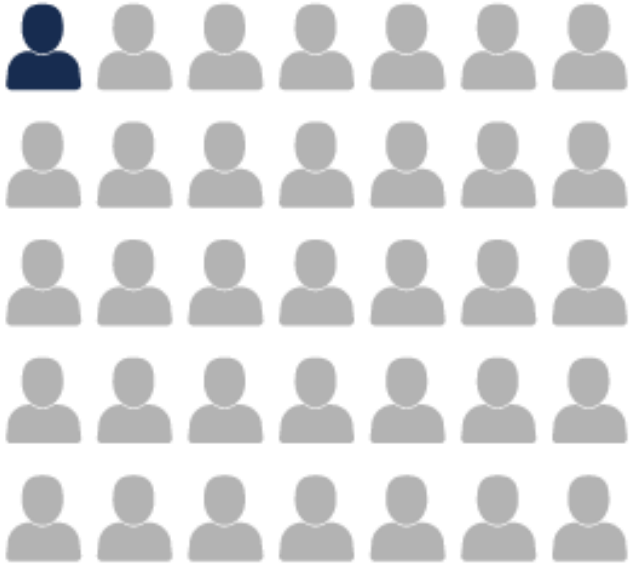
Chance, diagnosed with SMA before birth, plays with his parents in the park after successful treatment. Previously, SMA Type 1 and Type 2 patients were never able to walk or sit without assistance. [4]

[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

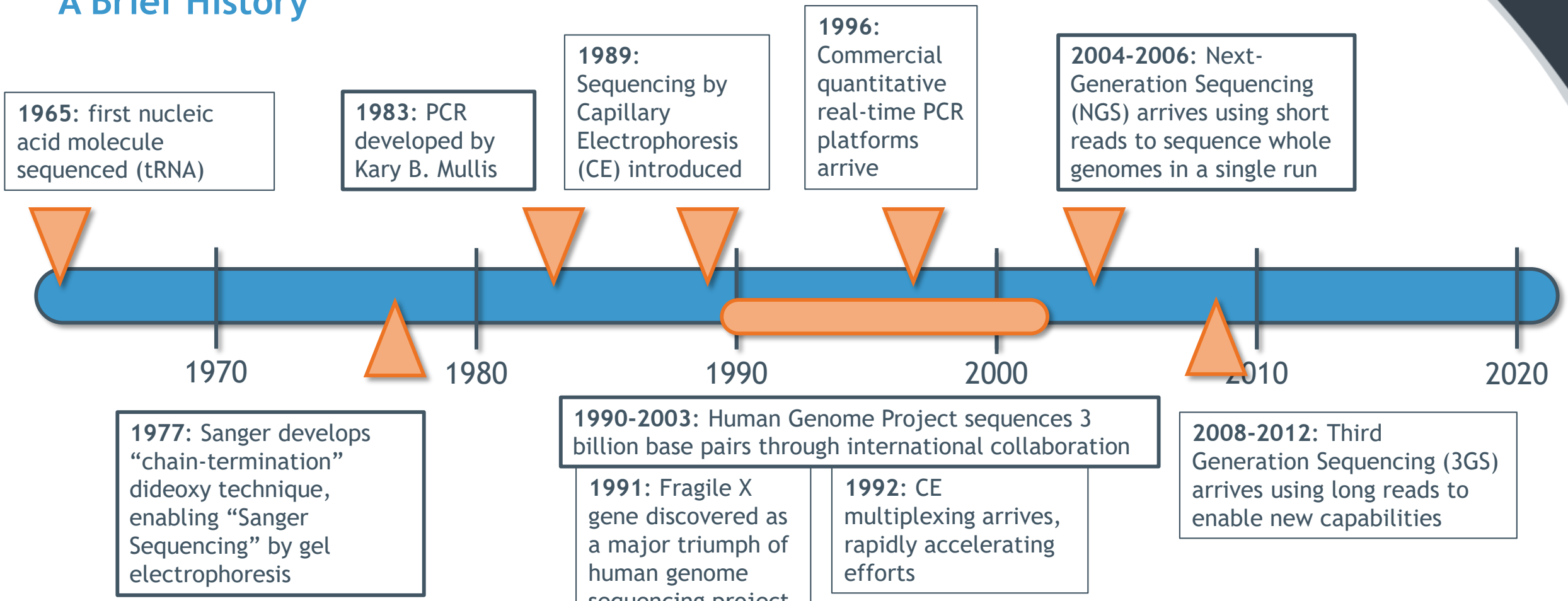
- For both screening and diagnostic testing of genetic disorders, clinicians test DNA to characterize relevant genes and mutations
- DNA testing methods include:
  - Polymerase Chain Reaction (PCR), which amplifies the gene or mutation in question to determine **mutation status** and quantify **copy 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

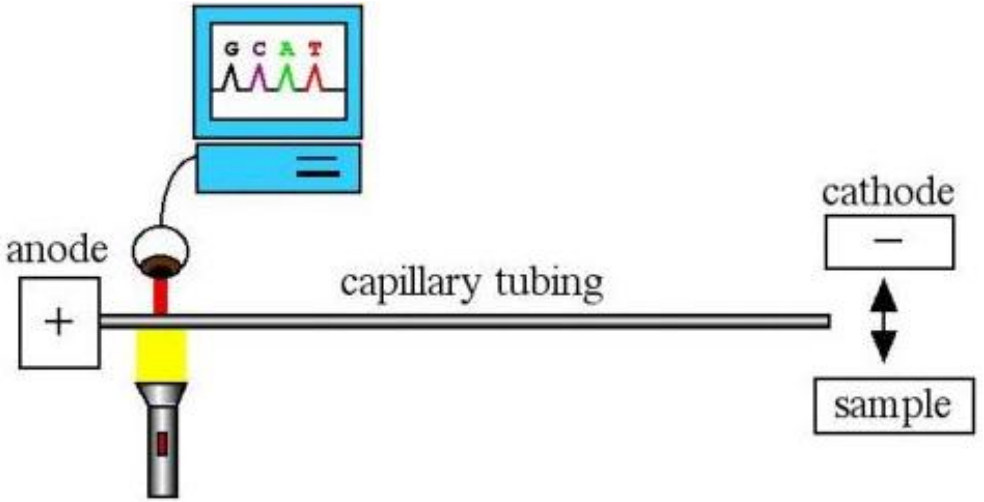
A Brief History



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*

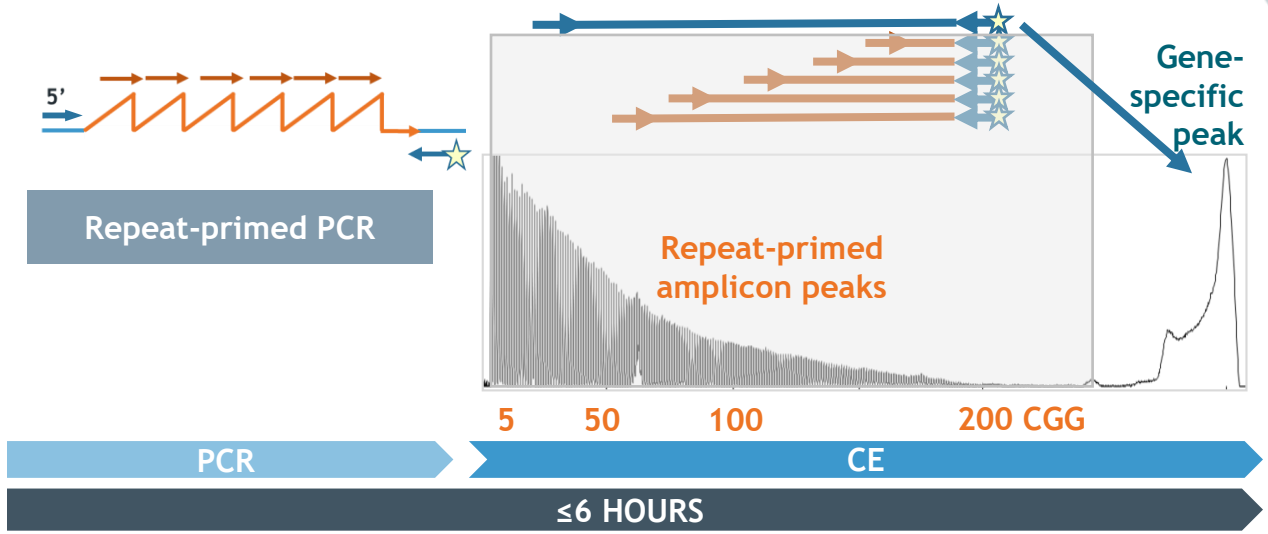
Workflow Image Copyright 2002 Department of Biology, Davidson College: http://legacy.jyi.org/volumes/volume5/issue9/features/lovejoy.html

*SeqStudio pictured. For Research Use Only. Not for use in diagnostic procedures.

Capillary Electrophoresis for DNA Analysis

Other applications: Repeat DNA Analysis

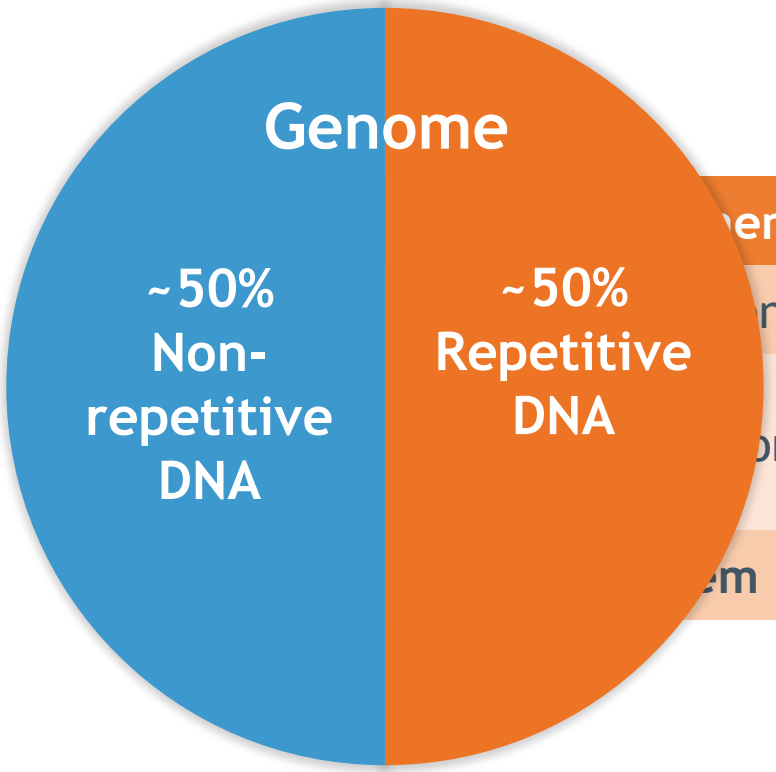
- **Problem:** Short tandem repeats (STRs) are difficult to amplify/ characterize but clinically important
  - Most sequencing methods (including NGS) cannot resolve STRs
- With specially-designed long-read PCR, STRs can be amplified and accurately sized using CE
- STRs are crucial in understanding and 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

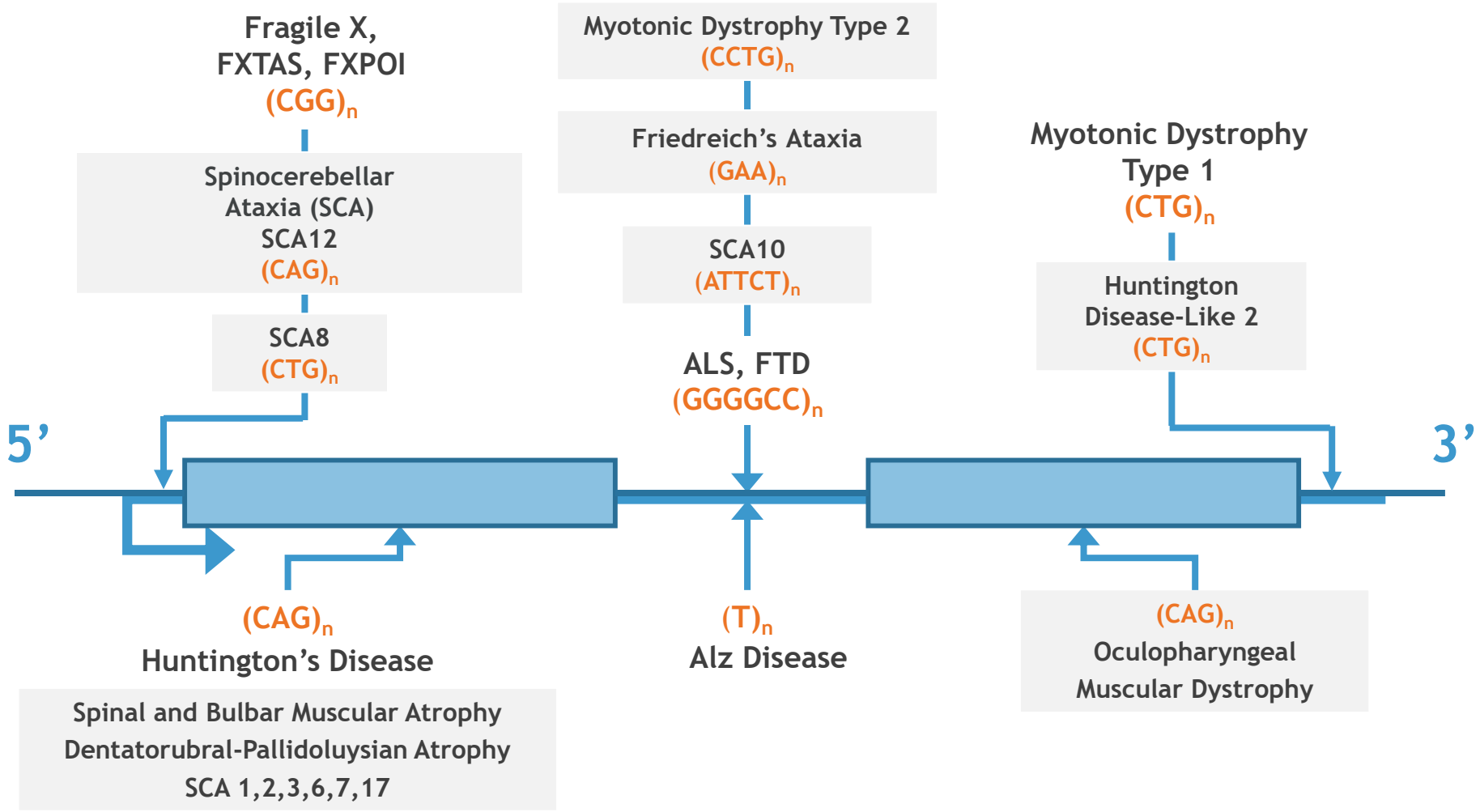
Repeat DNA comprises half the genome yet is mostly “dark” in function



ent		% of genome
ns		3
ons	LTR	9
	non-LTR	33
em Repeats (STRs)		3

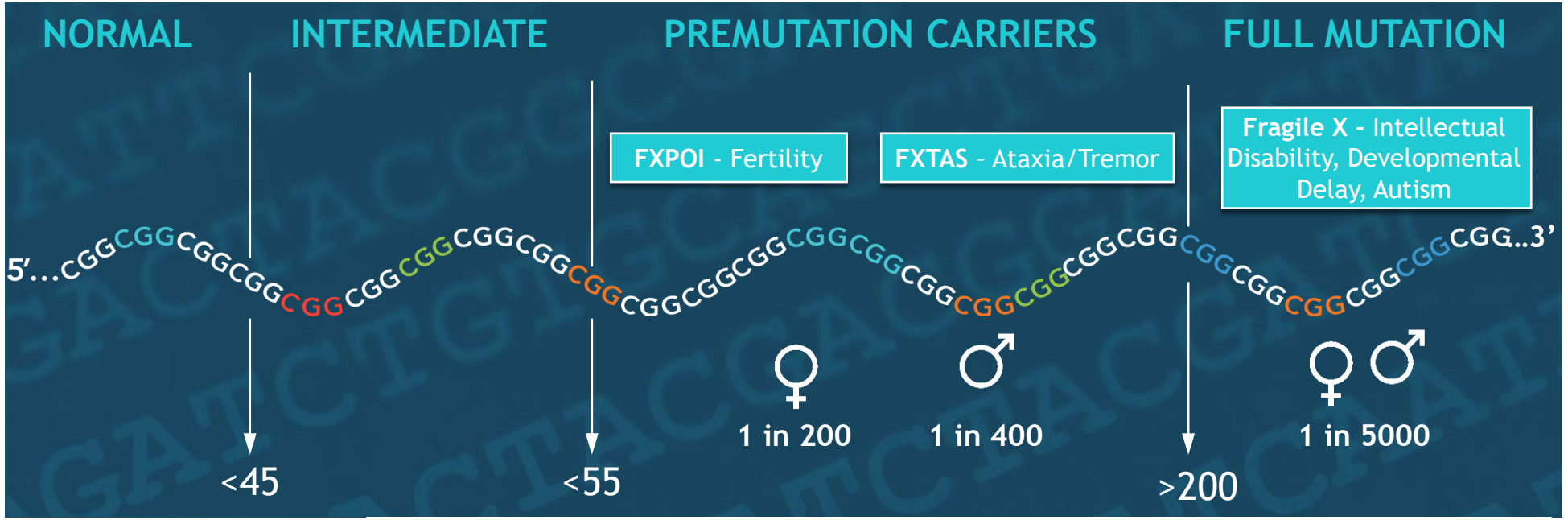
More than 30 Neurological Disorders Associated with STRs

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

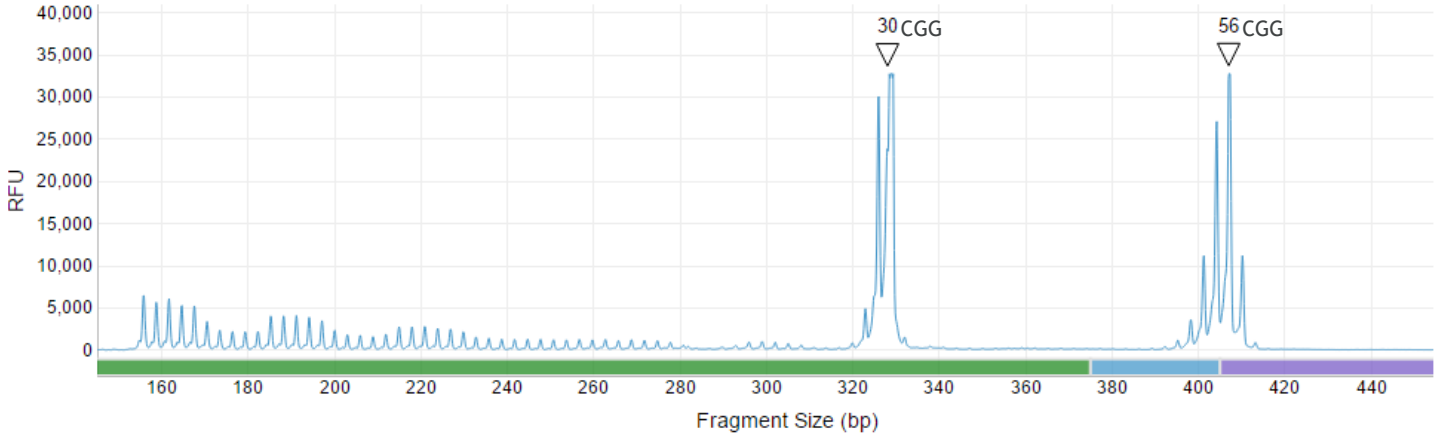


Clinical Disorders associated with *FMR1* Expansions

A case study in the complexity of STR-associated disorders



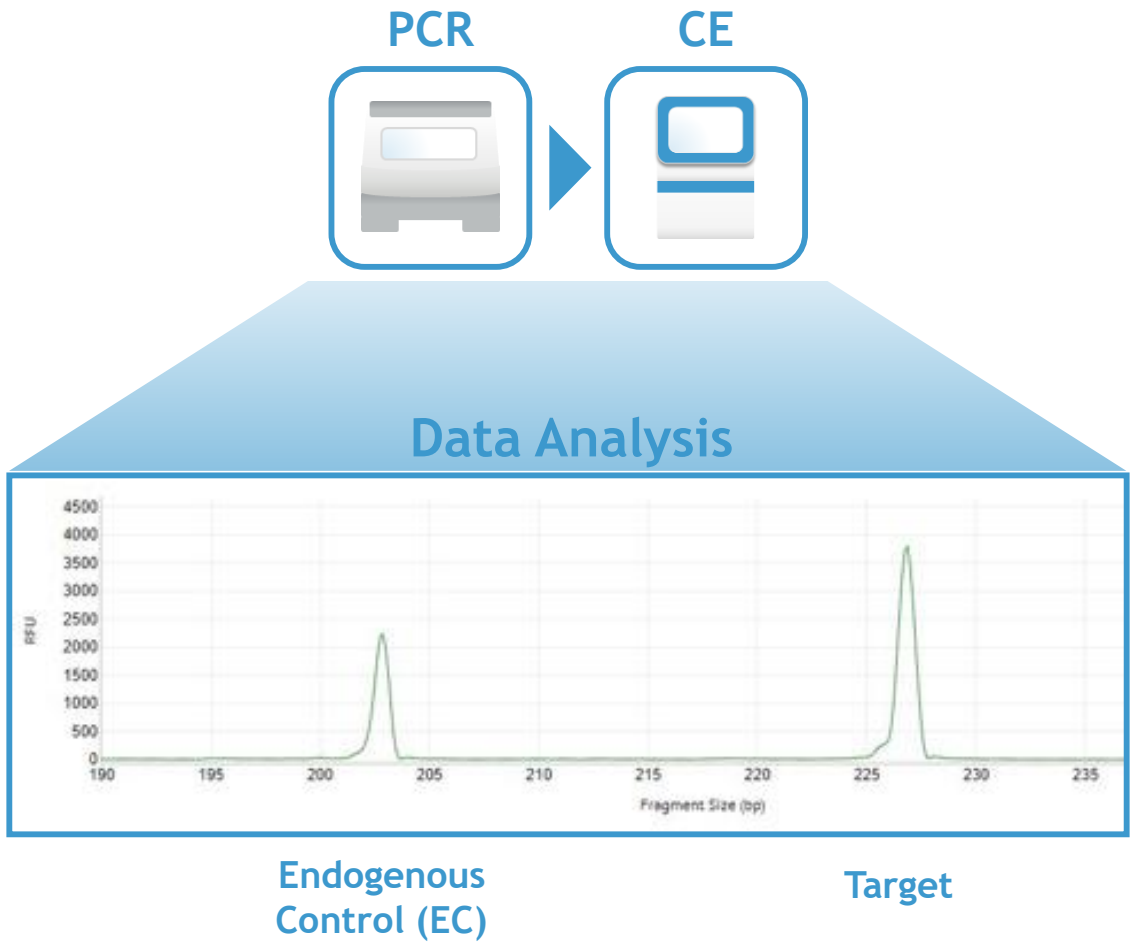
Accurate STR sizing with PCR/CE technology is critical for characterizing repeat-associated disorders



Capillary Electrophoresis for DNA Analysis

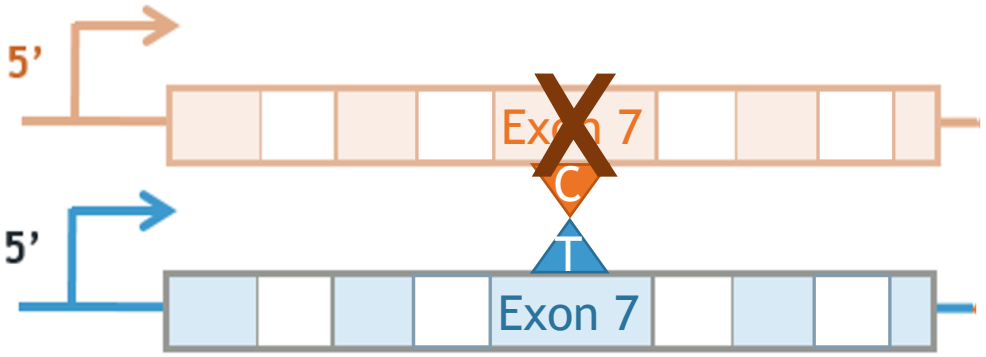
Other applications: Copy Number Variation (CNV)

- **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



SMN1

No SMN protein

SMN2

Mostly non-functional SMN protein

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 required • 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/