DNA Separation

This presentation is made possible through the following financial assistance award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST)

Slides prepared by Kelly M. Elkins, Ph.D., Towson University, 2025

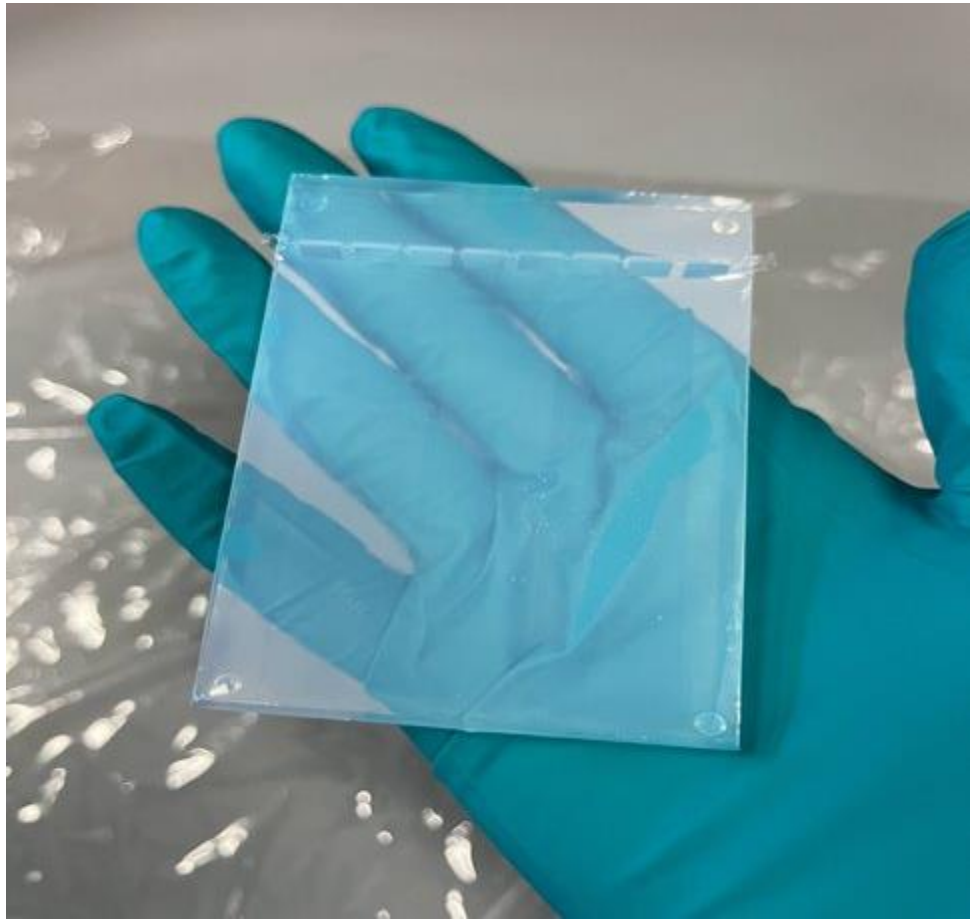
Background

In October 2023, Towson University was awarded a cooperative agreement from NIST to develop a standardized DNA training curriculum for the United States that addresses the components in ANSI/ASB Standard 115, *Standards for Training in Forensic Short Tandem Repeat Typing Methods Using Amplification, DNA Separation, and Allele Detection*. 2020. 1st Ed.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3c in ANSI/ASB Standard 115.

Learning Objectives

1. theory of electrophoresis;
2. capillary electrophoresis
   i. advantages and
   ii. disadvantages;
3. function of reagents;
4. electrokinetic injection;
5. DNA sieving;
6. sample preparation;
7. electrophoresis artifacts;
8. contamination and quality control;
9. limitations of the technology.

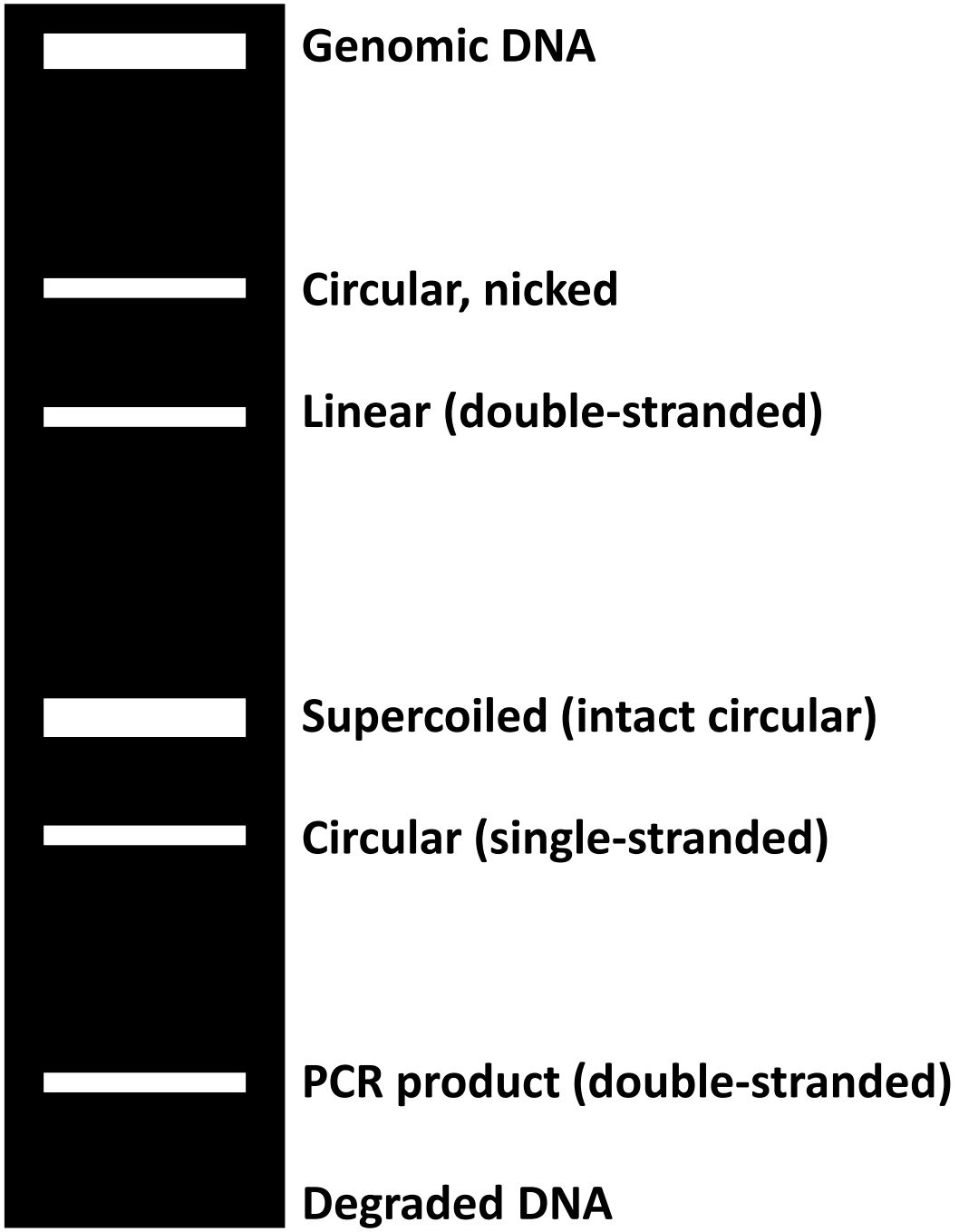


Terms & Definitions (ANSI/ASB 115)

- **Allele.** One of two or more versions of a genetic sequence at a particular location in the genome.
- **Amplification.** An increase in the number of copies of a specific DNA fragment. In forensic DNA testing laboratories, this refers to the use of the PCR technique to produce many more copies of fragments at specific genetic loci from samples of known and unknown origin for the purpose of generating DNA profiles for comparison.
- **Electrophoresis.** A technique used in laboratories to separate macromolecules based on size and charge. Negatively charged molecules (e.g. DNA and RNA) migrate towards a positively charged pole through a sieving matrix, which permits a size-dependent separation.

Theory of Electrophoresis

- An electric field is applied to separate charged molecules, such as DNA or proteins in a liquid or gel medium
  - Electro (elektro) refers to electron flow or electricity
  - Phoresis means travel or movement
- Separation is influenced by several factors
  - molecule length
  - charge and relative charge
  - fold or shape
  - complexation



Theory of Electrophoresis

- DNA fragment size can be determined using a gel
  - Smaller fragments travel further in a given time
  - Larger fragments interact with the gel and are decelerated or retarded
  - Size can be determined using a ladder of known sizes

Theory of Electrophoresis

Separation of DNA molecules is dependent on the following factors:

1. Polymer
2. Capillary
3. Buffer
4. Voltage applied/electric field strength

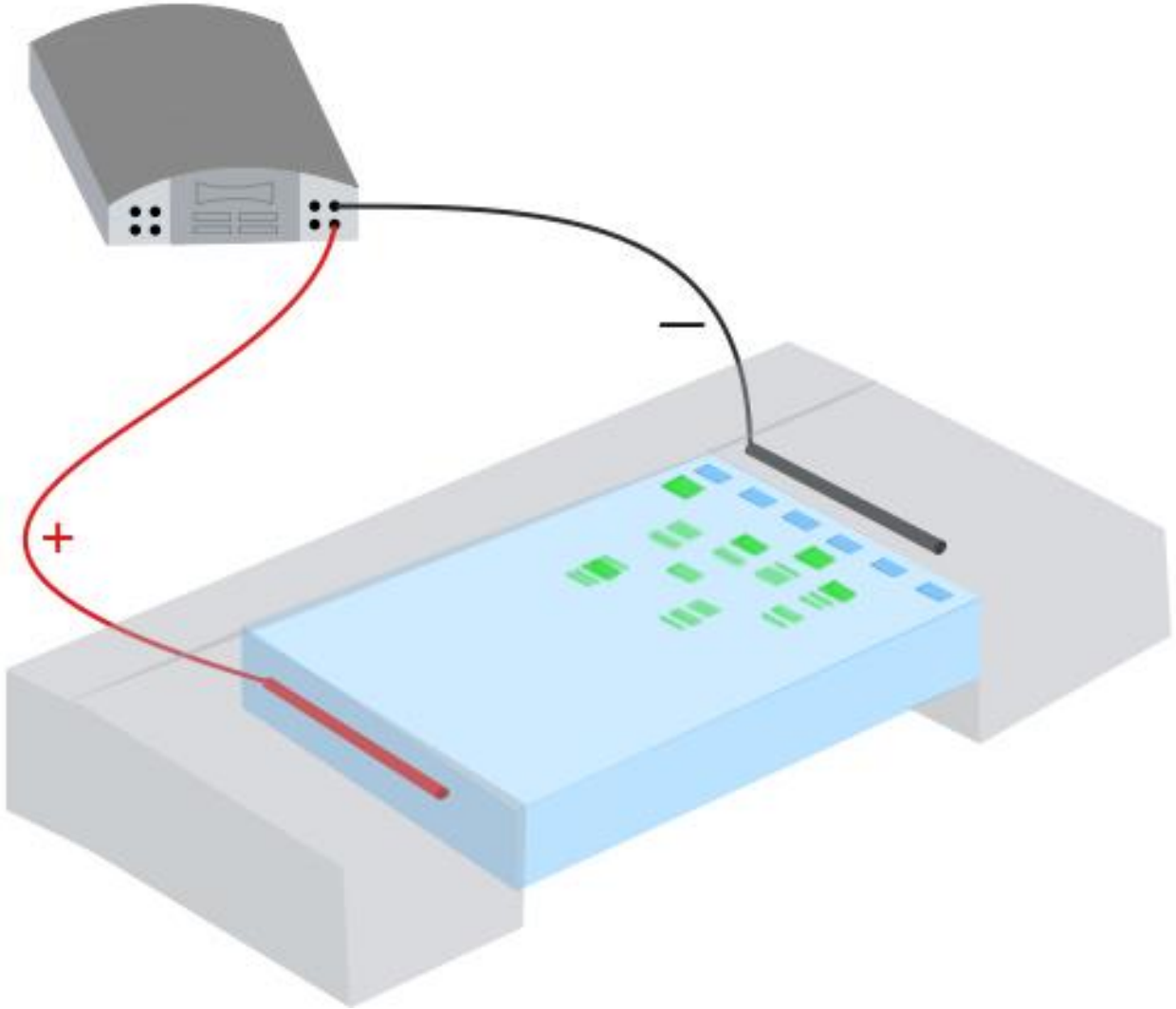
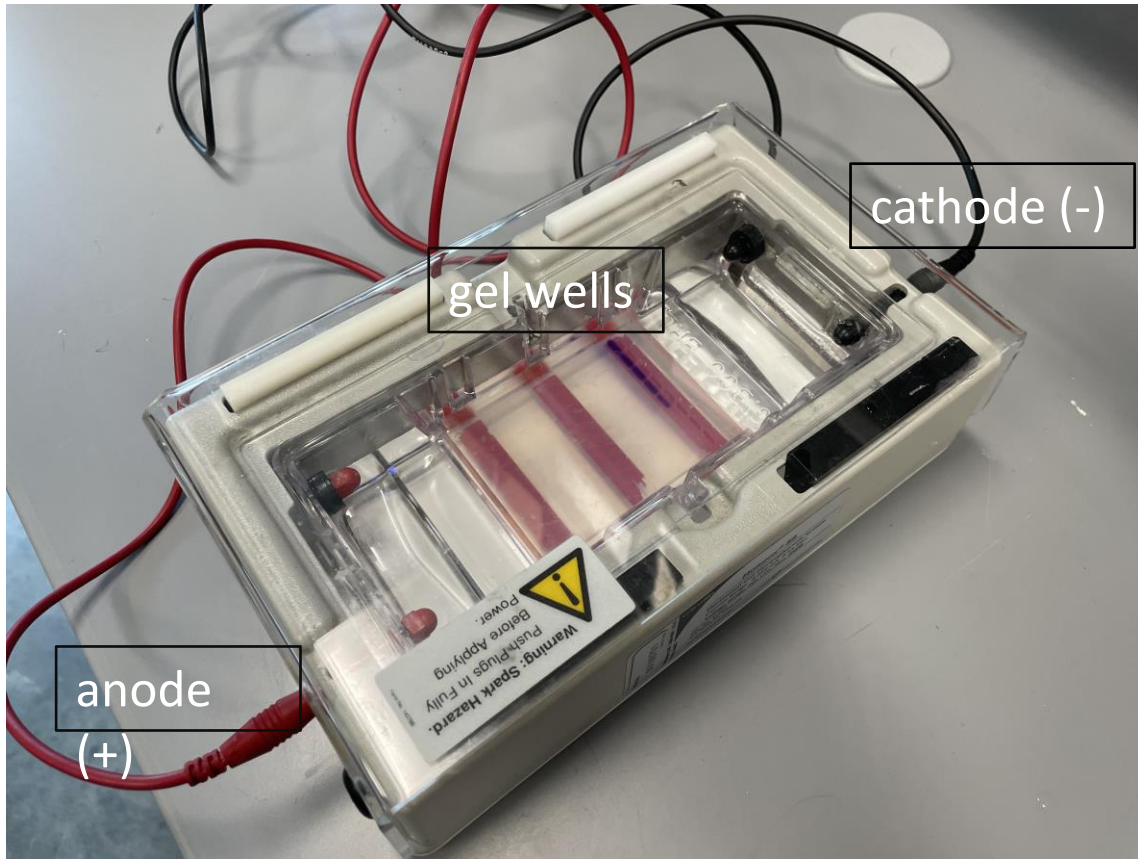


Figure credit: Nihal Chana

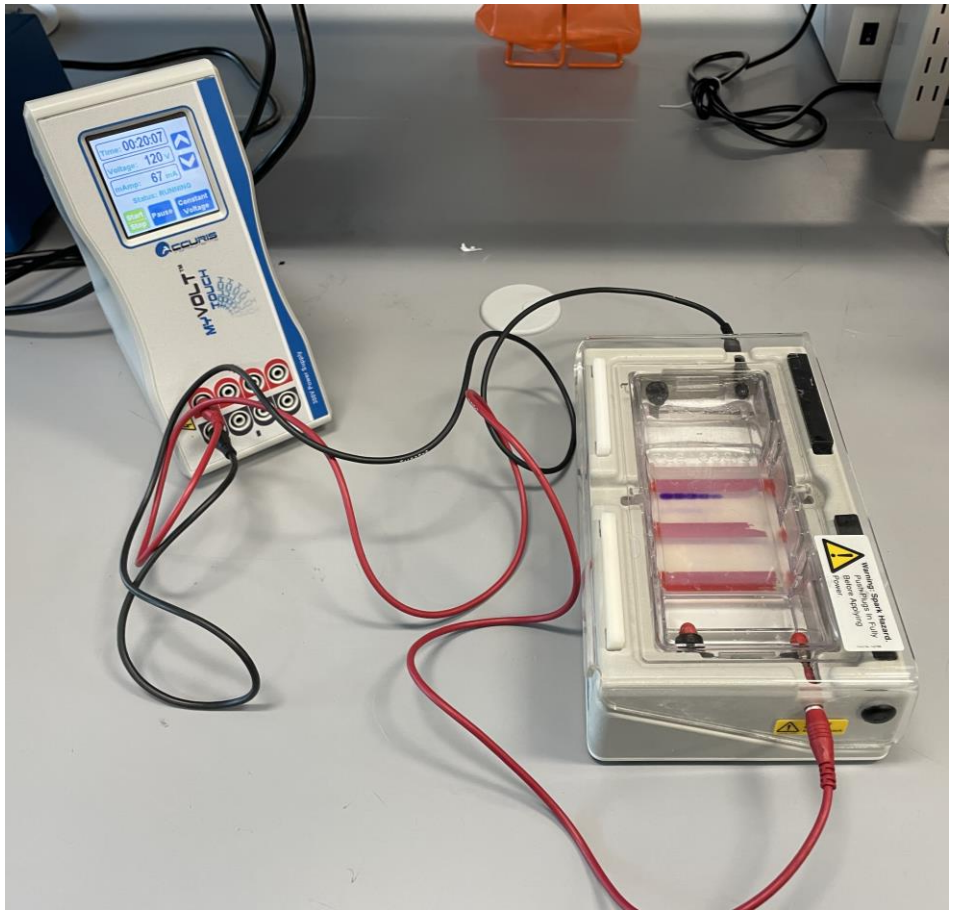
Theory of Electrophoresis

- DNA is negatively charged (-)
- The sample is introduced, or injected, in a well or capillary at the negative electrode or cathode (-)
- Buffer (e.g., 1X TAE, 1X TBE, 1X CE buffer) is introduced to the anode and cathode wells and to fully cover the gel and complete the circuit
- Voltage set to 120 V
- Run 30 min –2 h depending on polymer % and gel or capillary length
- DNA migrates to the anode (+)



Theory of Electrophoresis

- An electric field is generated by plugging into or turning on a power source
- The relative position(s) of the molecule(s) can be determined when the field is terminated on a slab gel using detection methods such as autoradiography, silver staining, or fluorescence by comparison with a ladder
- Fluorescence can be viewed using a UV transilluminator



Theory of Electrophoresis: DNA Sieving

- Size and shape of sieving pores are not uniform
- Two major sieving models
  - Ogston sieving
    - Larger spherical particles are sterically hindered from entering the nanopores, so coiled molecules move more freely through the gel matrix
  - Reptation
    - Complex DNA strands snake or crawl through the gel's network of polymers

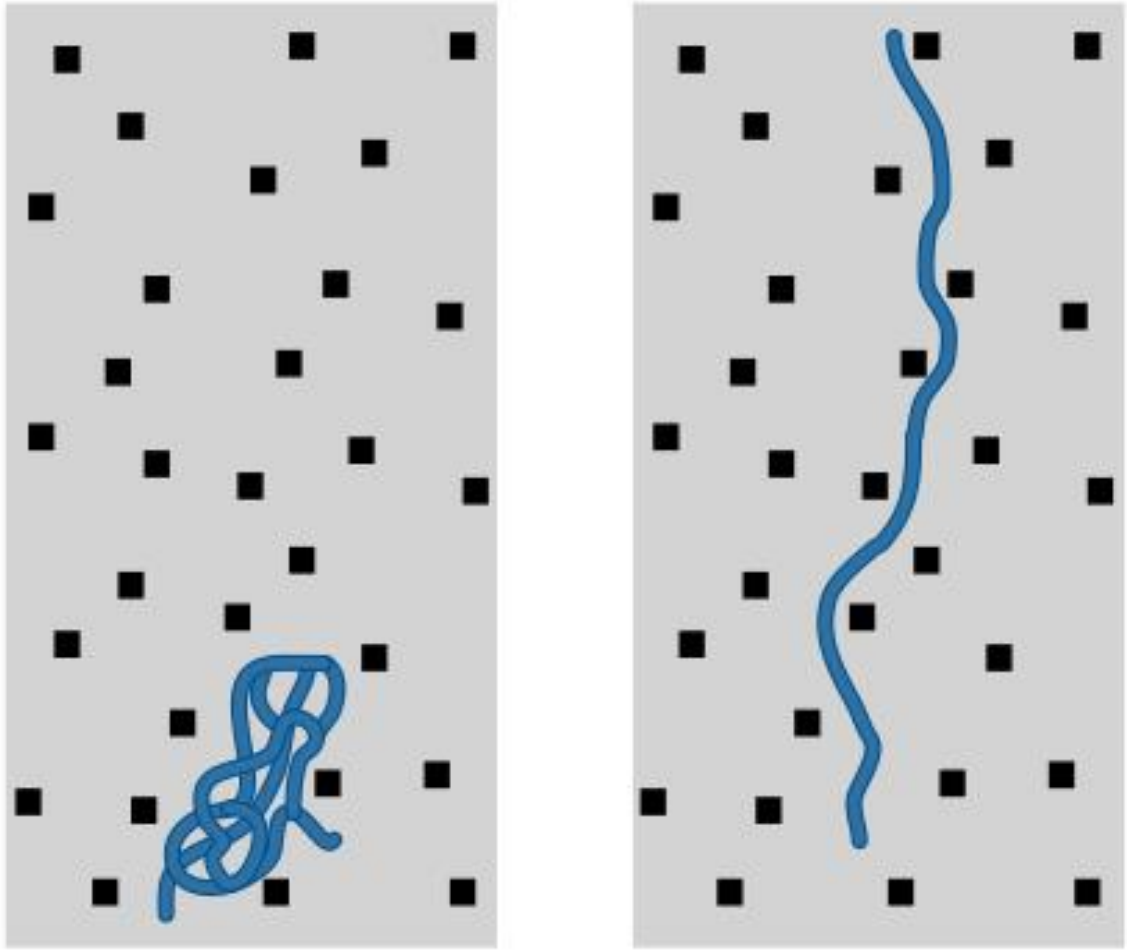
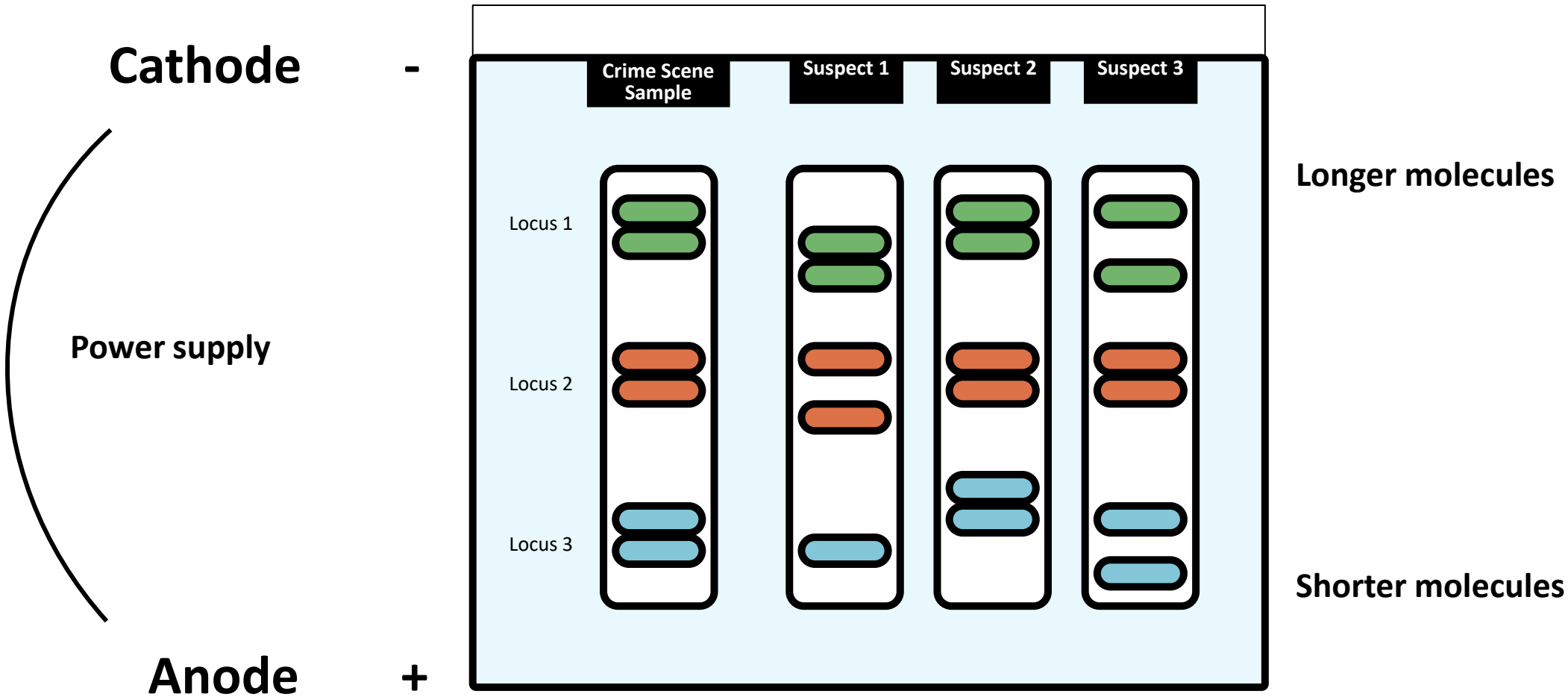


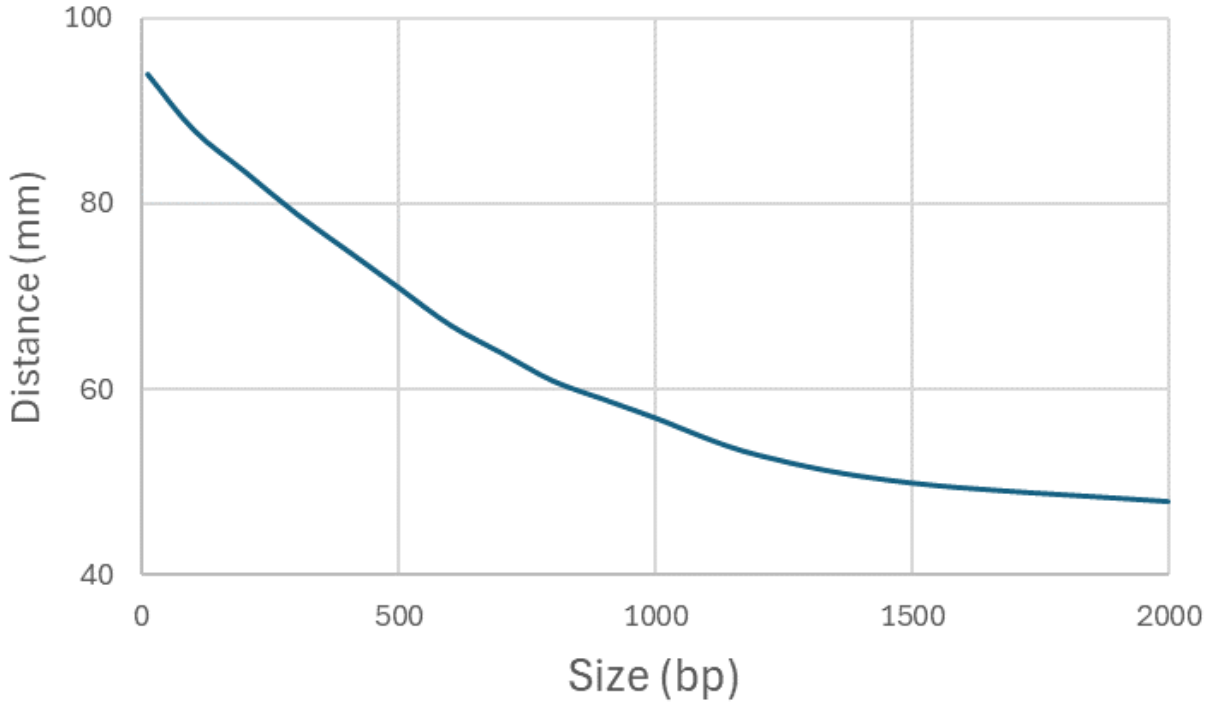
Figure credit: Nihal Chana

Reference: A.G. Ogston, Trans. Faraday Soc. 54, 1754 (1958)

Theory of DNA Gel Electrophoresis: Schematic



Theory of Electrophoresis: DNA Sieving



- Migration of molecules decreases exponentially as the pore size decreases
- Migration distance increases as the gel concentration is decreased

TrackIt 100 bp ladder
(Invitrogen)
1.5% agarose
1x TAE
SYBR Green detection

Capillary Electrophoresis

- The sample is drawn into the capillary by electrokinetic injection at the cathode from the plate
- The capillary must remain immersed in the buffers to complete the circuit
- DNA migrates to the anode due to its negative charge when the electric field is applied
- The DNA molecules are detected using fluorescence when they pass the detection window in the capillary, small fragment reach the detector first
- The eluted DNA molecules are discarded at the anode outlet

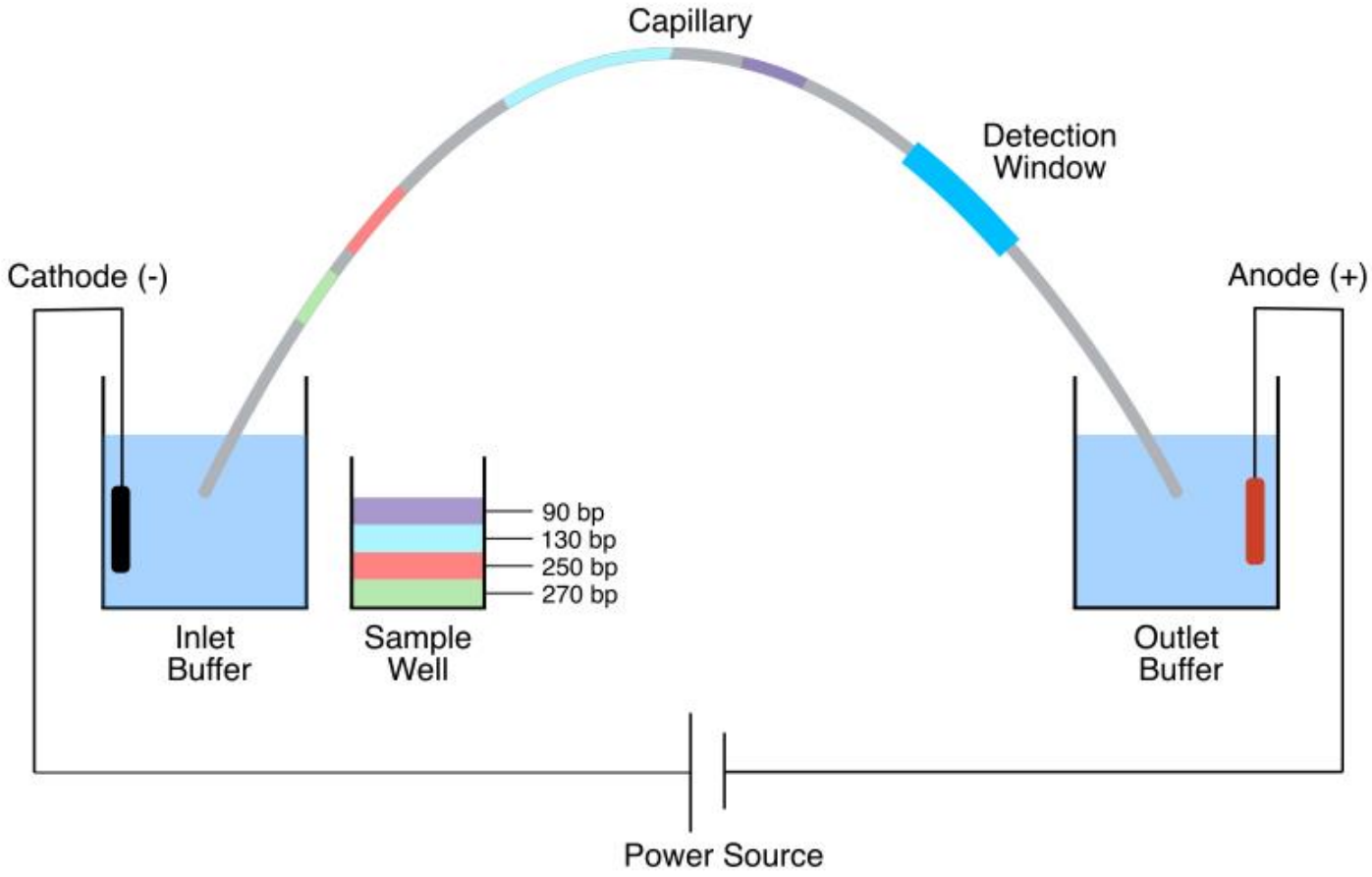


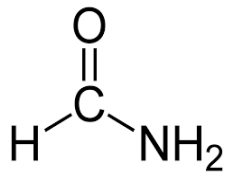
Figure credit: Nihal K. Chana

Anode and Cathode Buffer

- Common buffers can maintain a constant pH and conduct electricity (proton donor acids)
  - 1x Tris-acetate-EDTA (TAE) (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA)
    - Best when working with large DNA fragments or genes
  - Tris-borate-EDTA (TBE) (89 mM tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3)
    - Higher buffering capacity but inhibits many enzymes
    - Higher resolution with small DNA fragments (<2 kb) and longer run times as it is less prone to overheating
- EDTA: chelates metal ions
- pH: 8.0 (range 7.6-8.2)
- Resistance: 1500 - 2000 µS/cm

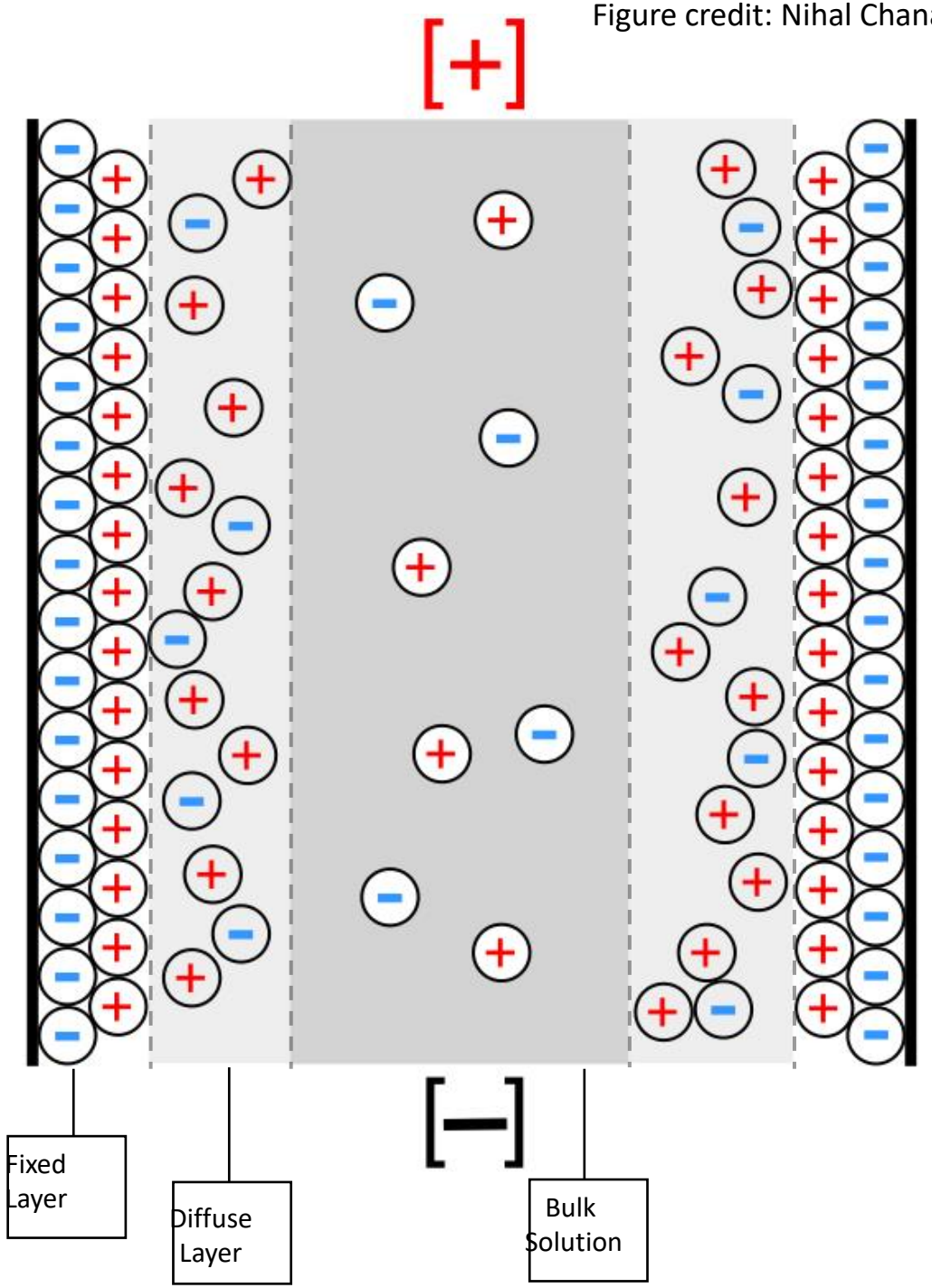
Electrokinetic Injection

- Method of introducing charged samples and controls to the capillary
  - Samples are dissolved in Hi Di formamide which is highly polar



  - Samples are heat denatured to generate single strands and flash cooled
  - Voltage of 5-20 kV (frequently 15 kV) is used
  - Maximum recommended time is 30 seconds, but shorter times are frequently used
  - Negatively charged molecules will be drawn into the capillary by attraction to the opposite charge

Figure credit: Nihal Chana

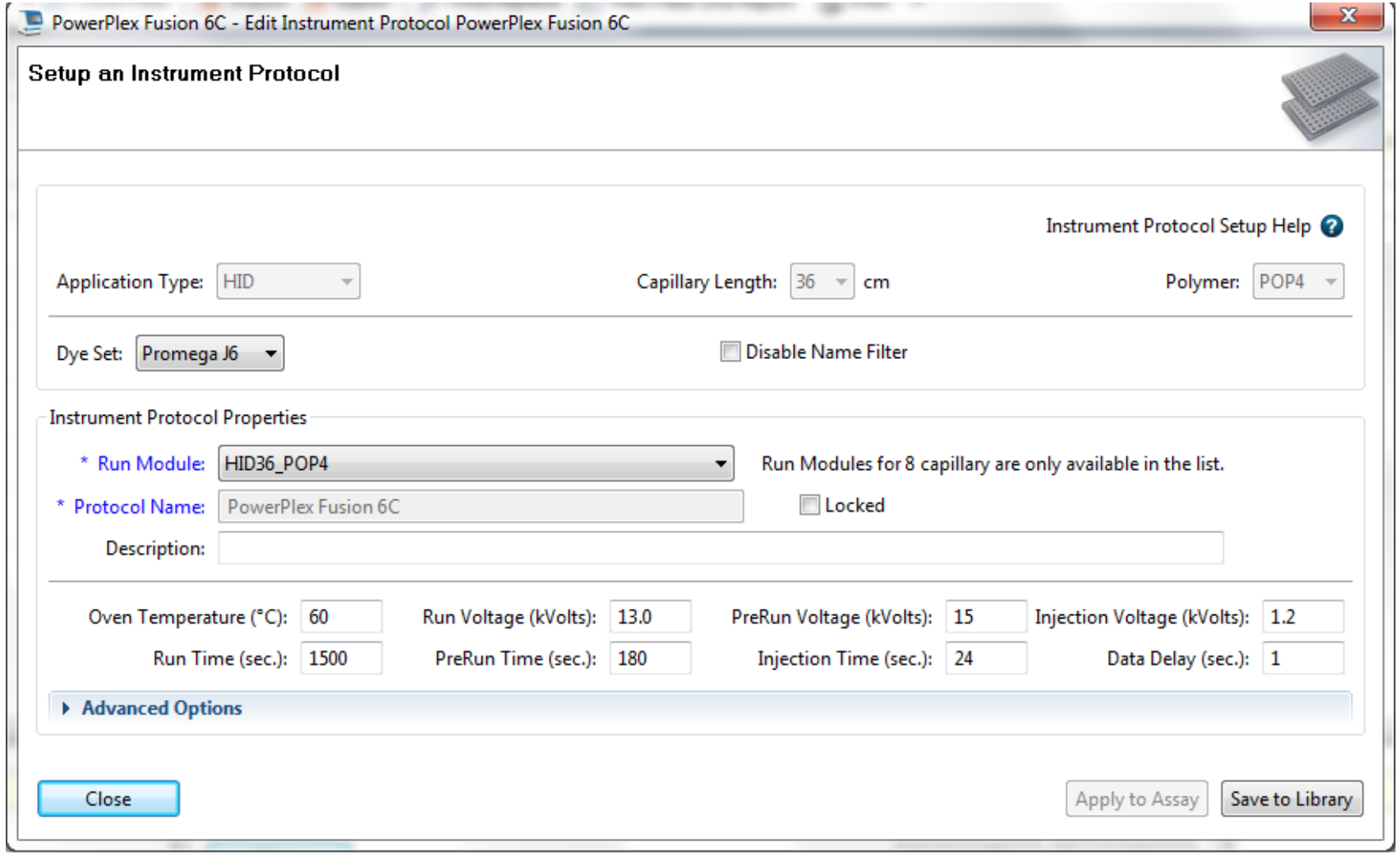


Sample Preparation

- 12 µL Hi-Di (or Seq-Di) formamide
  - Hi-Di is highly deionized
  - Helps in denaturation of double-stranded DNA and for optimal salt levels for electrokinetic injection
- 0.5 µL internal size standard (e.g., BTO, 500 LIZ, 600 LIZ, 500 WEN)
- 1 µL amplified DNA sample
- Heat to denature and snap cool
  - 3-5 minutes at 95 °C
  - 2-3 minutes on ice or 4 °C
- Load to 96-well plate or tubes for CE

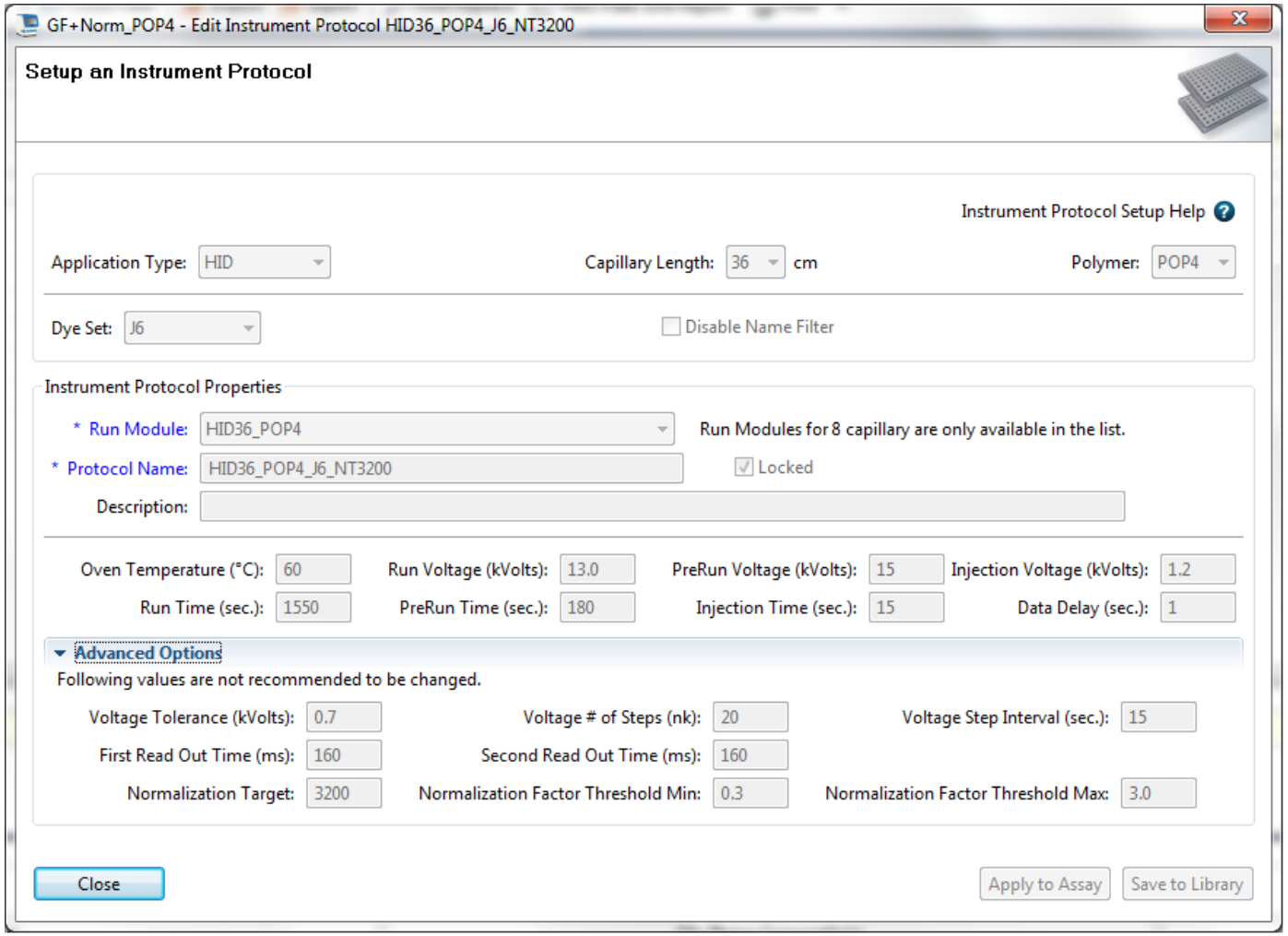
Run Parameters with the Promega PowerPlex Fusion 6C Kit on an ABI 3500 Genetic Analyzer

- Capillary Length: 36cm
- Polymer: POP-4®
- Dye Set: Promega J6
- Run Module: HID36_POP4(xl)
- Injection Time: 15 seconds
- Injection Voltage: 1.2kV
- Run Voltage: 13kV
- Run Time: 25 minutes
- Internal Size Standard: WEN ILS 500



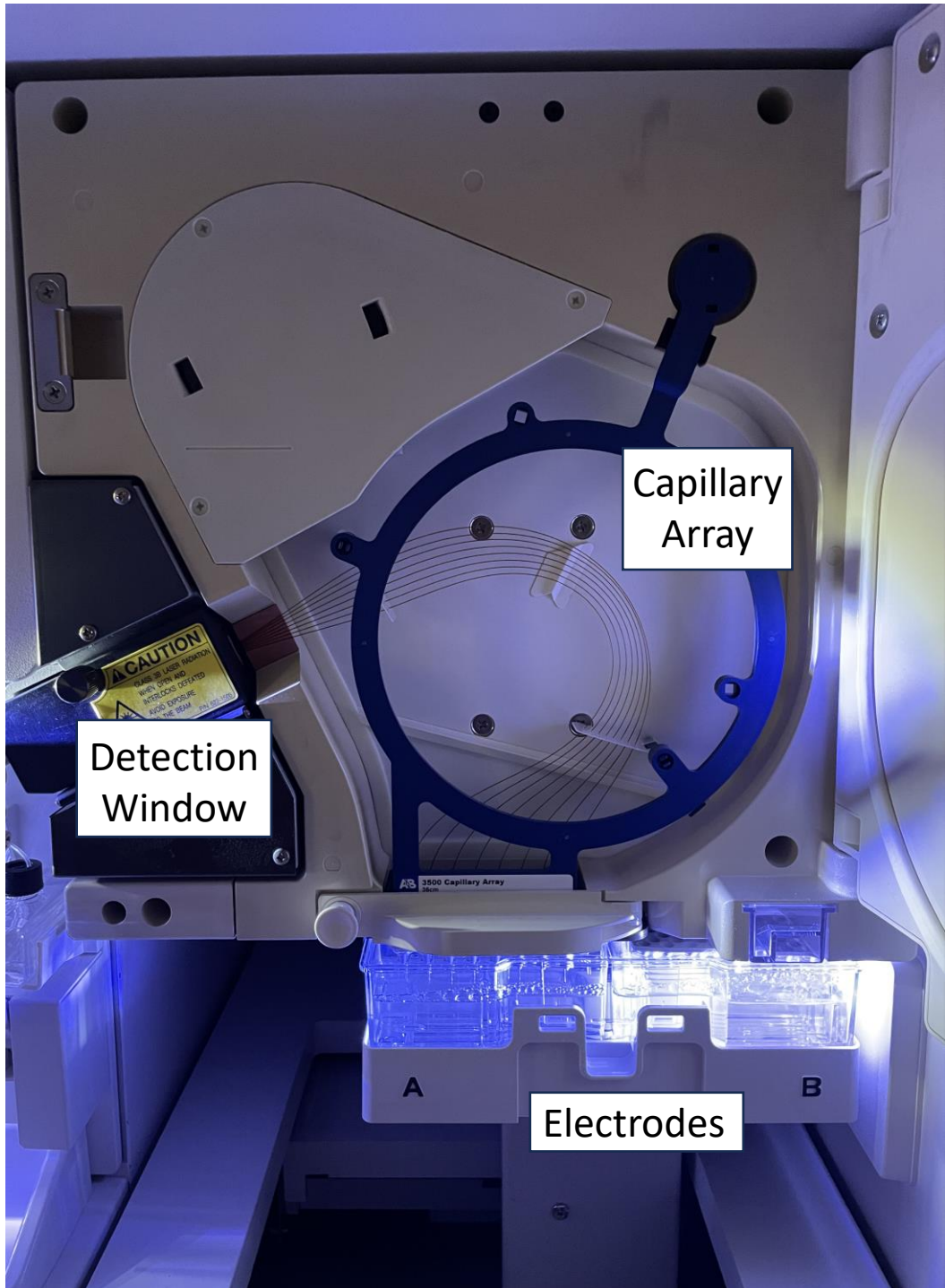
Run Parameters with the Applied Biosystems GlobalFiler Kit on an ABI 3500 Genetic Analyzer

- Capillary Length: 36cm
- Polymer: POP-4®
- Dye Set: J6
- Run Module: HID36_POP4
- Injection Time: 15 seconds
- Injection Voltage: 1.2kV
- Run Voltage: 13kV
- Run Time: 25.8 minutes
- Internal Size Standard: 600 LIZ



Capillary Electrophoresis: Advantages

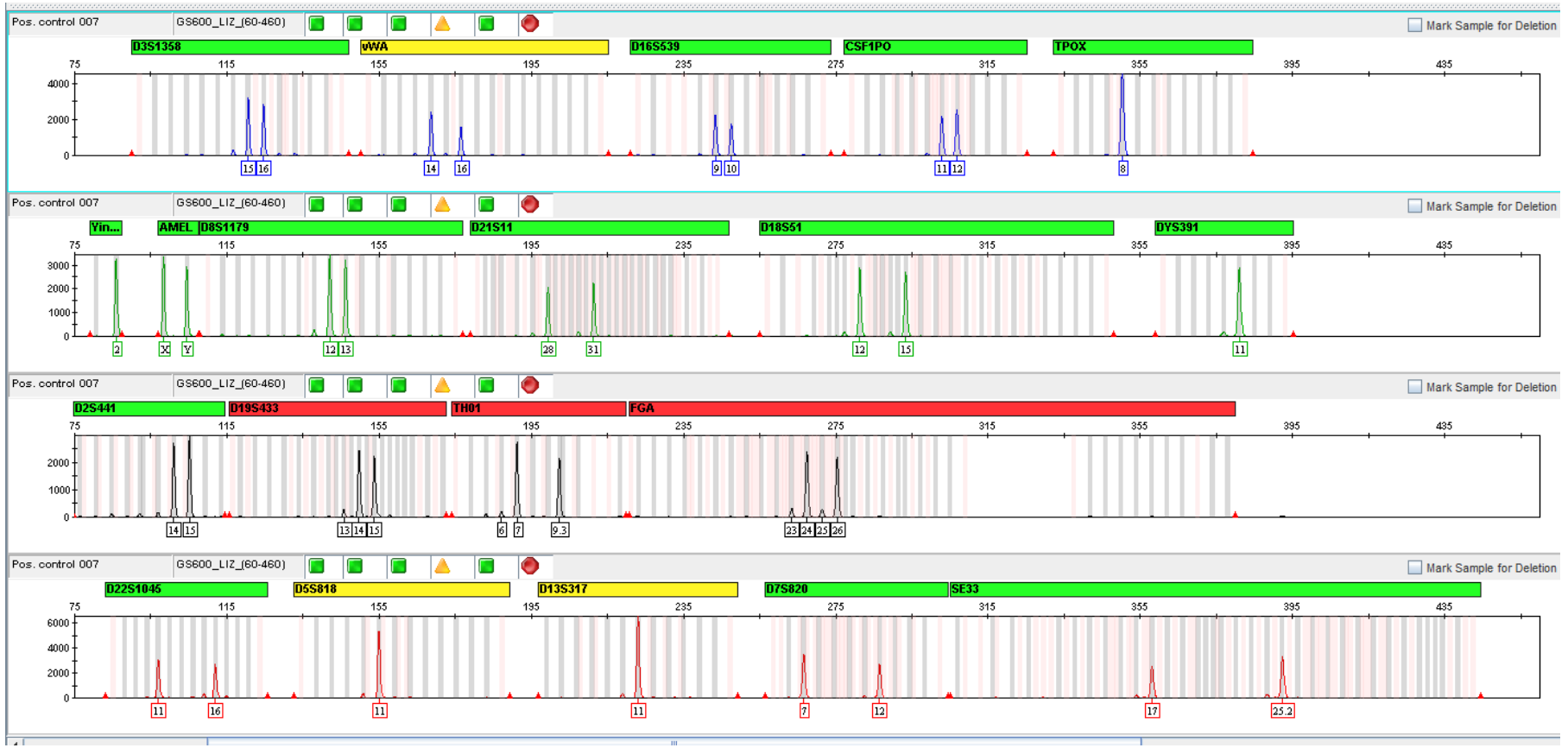
- Requires only a small amount of sample
- DNA cleanup not required
- Samples can be multiplexed
- Automated process
- Fast run times
- Discrete sizing resolution
- High sensitivity
- Cost-effective for large numbers of samples
- Does not require prior knowledge of the fragment size or sequence
- High throughput with multi-capillary arrays
- Data analysis is standardized and (relatively) easy



Capillary Electrophoresis: Disadvantages

- Difficult to discretely separate large molecules
- Sensitive to fluctuations in temperature and buffer composition
- Conductivity failures can occur when bubbles or dust are introduced to the system
- Only cost-effective when all capillaries in the array are utilized
- Poor concentration sensitivity due to nanoliter injection volume small loading capacity
- Salts (e.g., buffer ions, Cl^-) from PCR in samples will lead to poor sample loading
- Prone to artifacts
- Poor matrix can lead to a raised baseline and extra peaks called
- Loci are not sequenced in fragment analysis

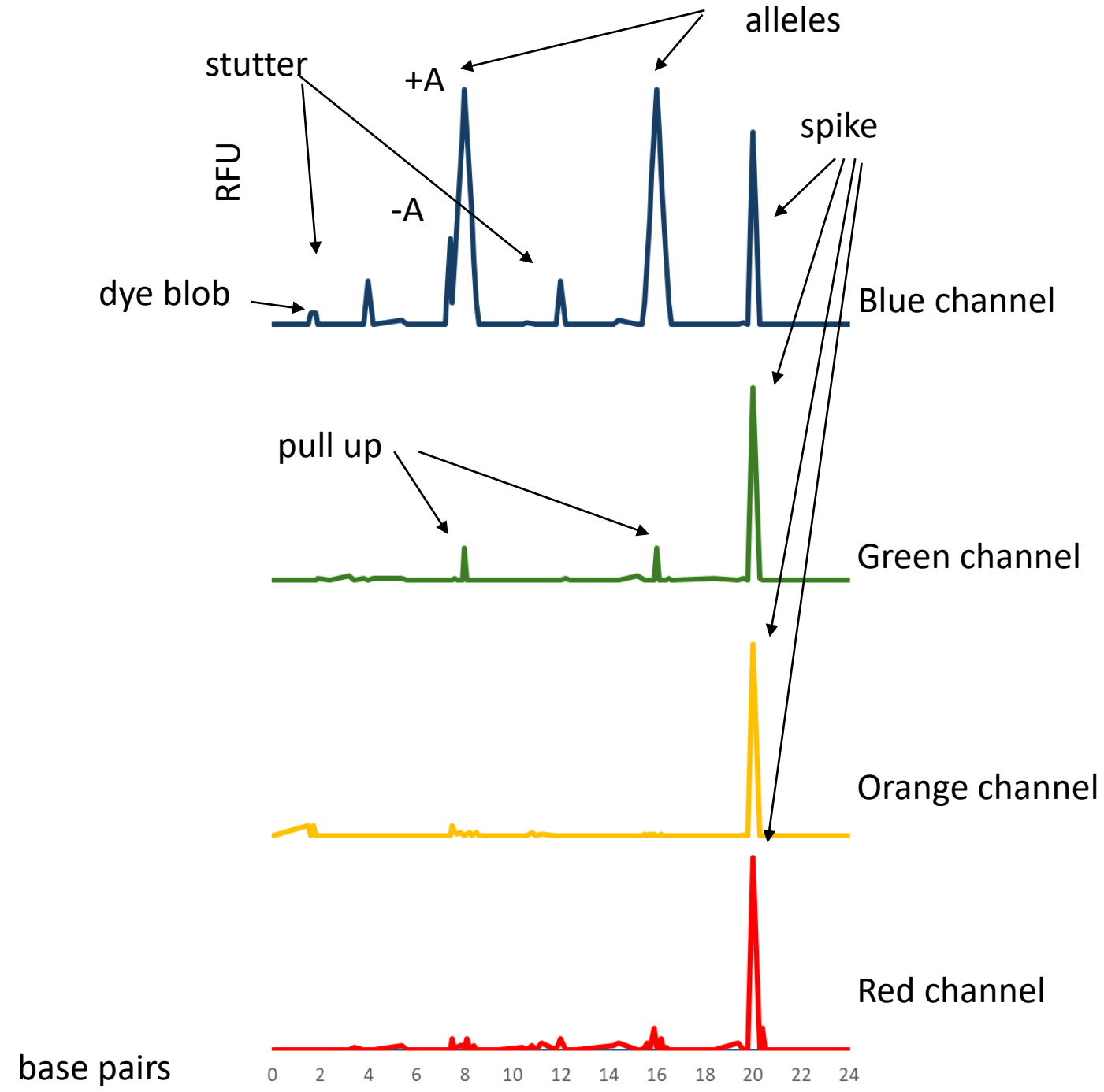
Capillary Electrophoresis Output: Electropherogram



Relative fluorescence units (RFU) vs. Retention time

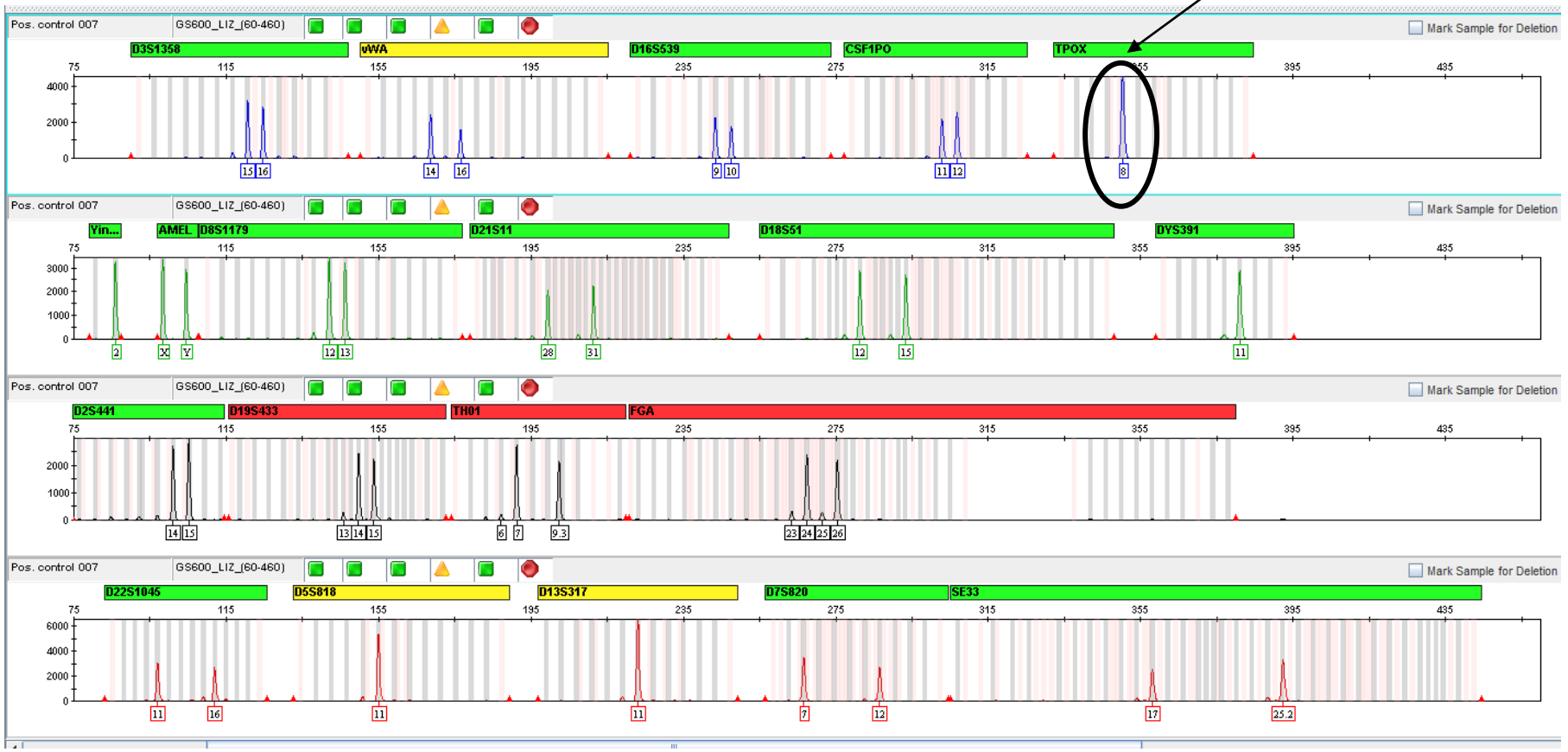
(converted to size using ISS and allele (number of STR repeats) by correlation with a ladder)

Electrophoresis Artifacts



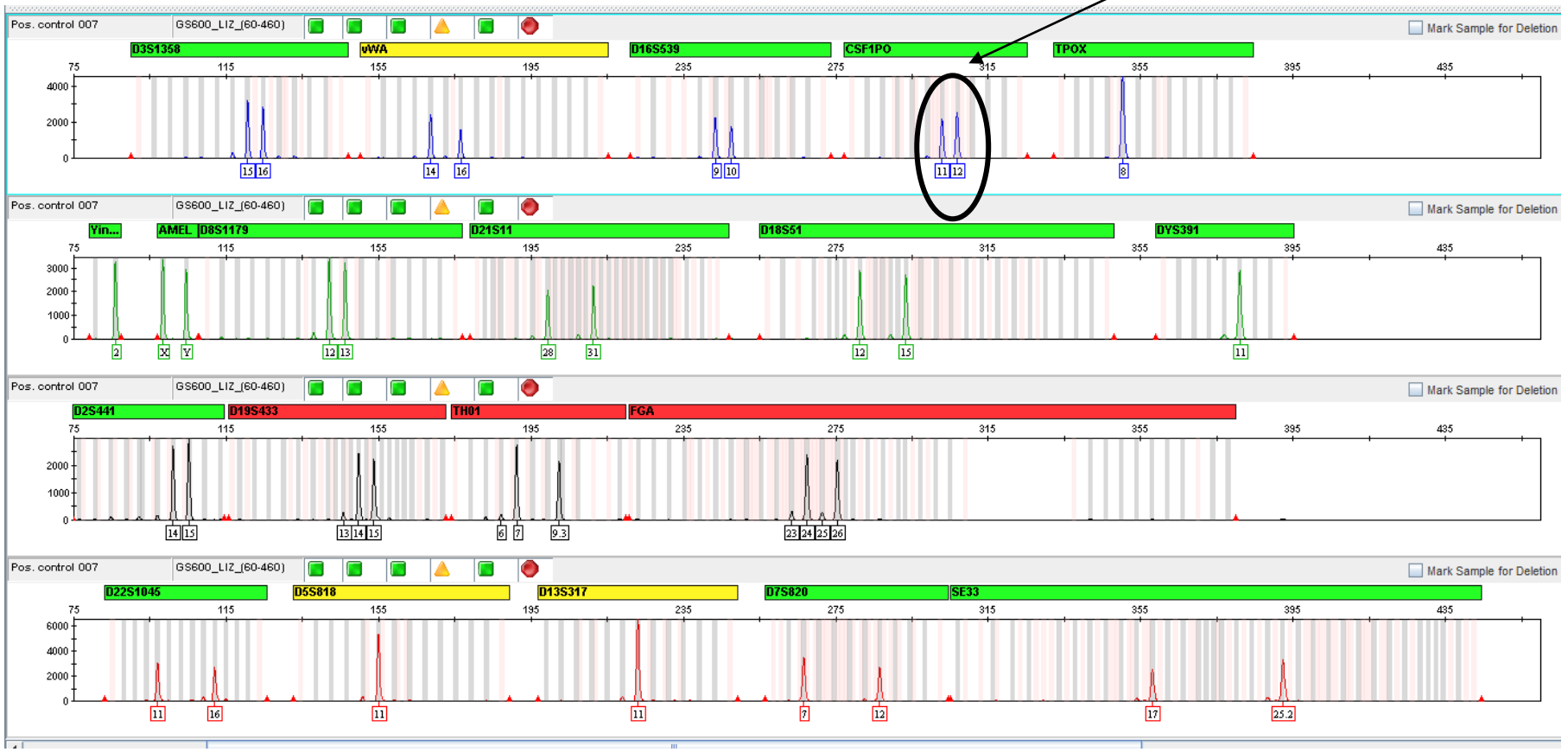
Capillary Electrophoresis: Homozygote

one peak for one allele in a single source sample at a locus



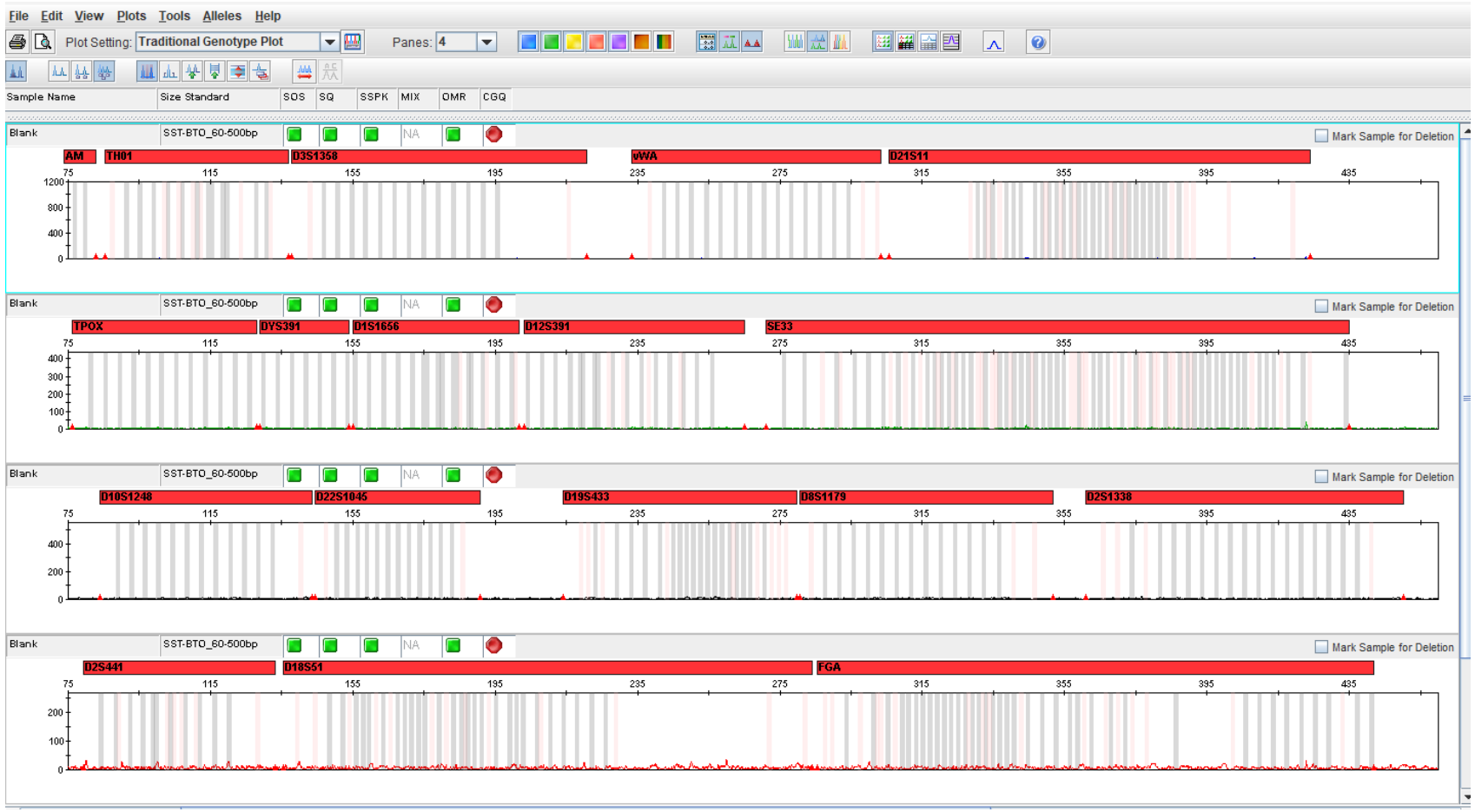
Capillary Electrophoresis: Heterozygote

two peaks for two alleles in a single source sample at a locus



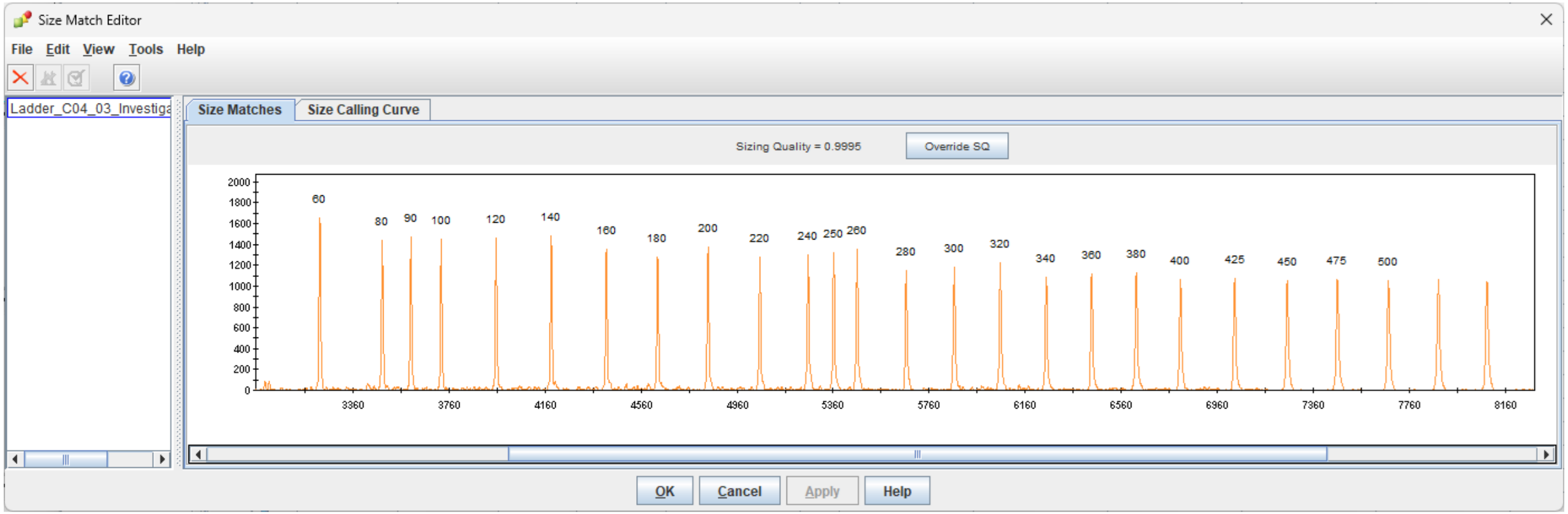
Capillary Electrophoresis: Reagent Blank

No peaks above the thresholds



Capillary Electrophoresis: Sizing with ISS

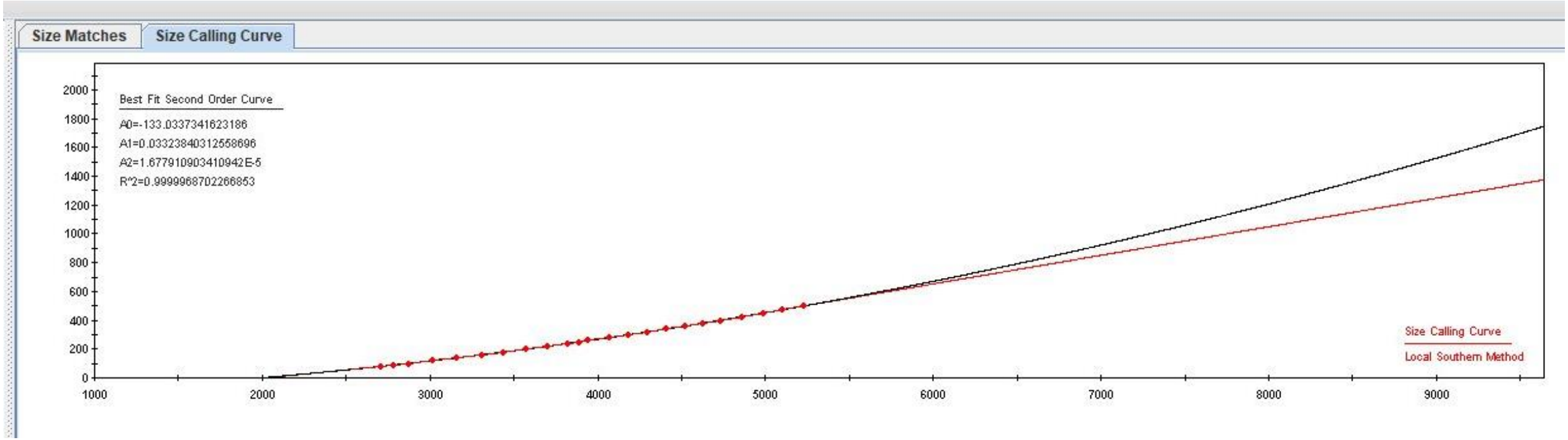
Relative fluorescence units (RFU) vs. Retention time of internal size standard (ISS)



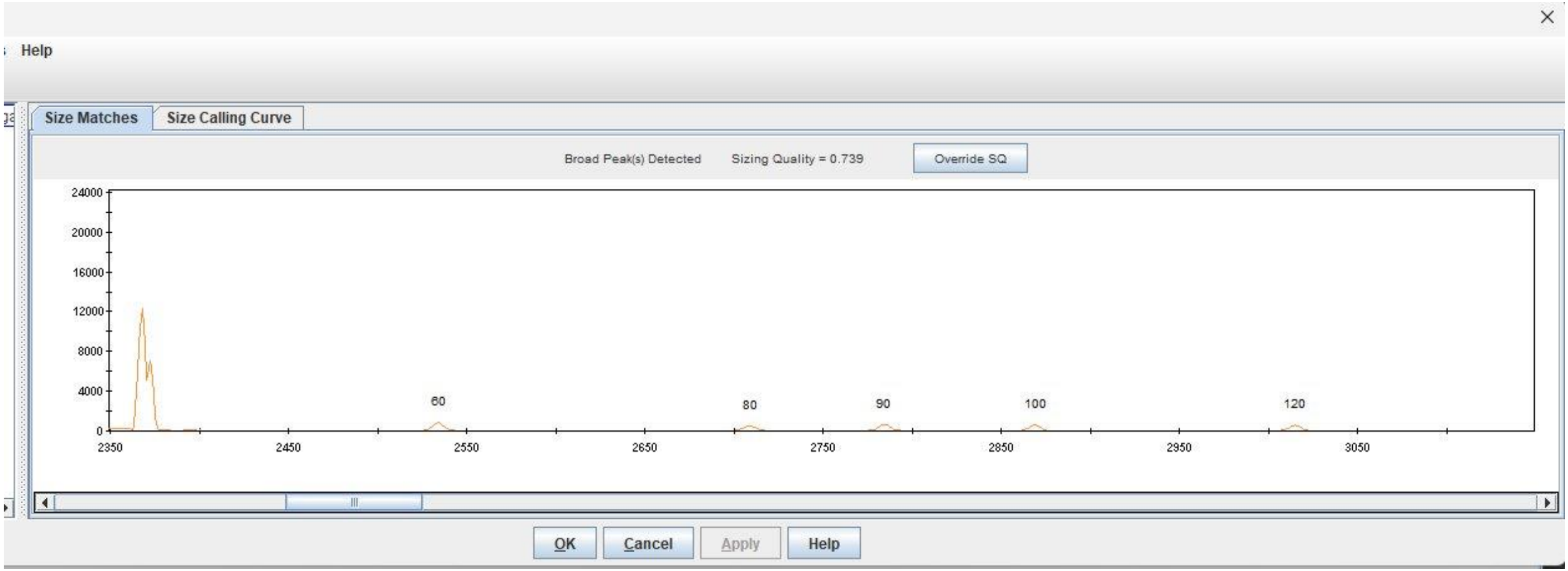
Capillary Electrophoresis: Size Calling Curve

Relative fluorescence units (RFU) vs. Retention time of dye-labelled known size fragments internal size standard (ISS)

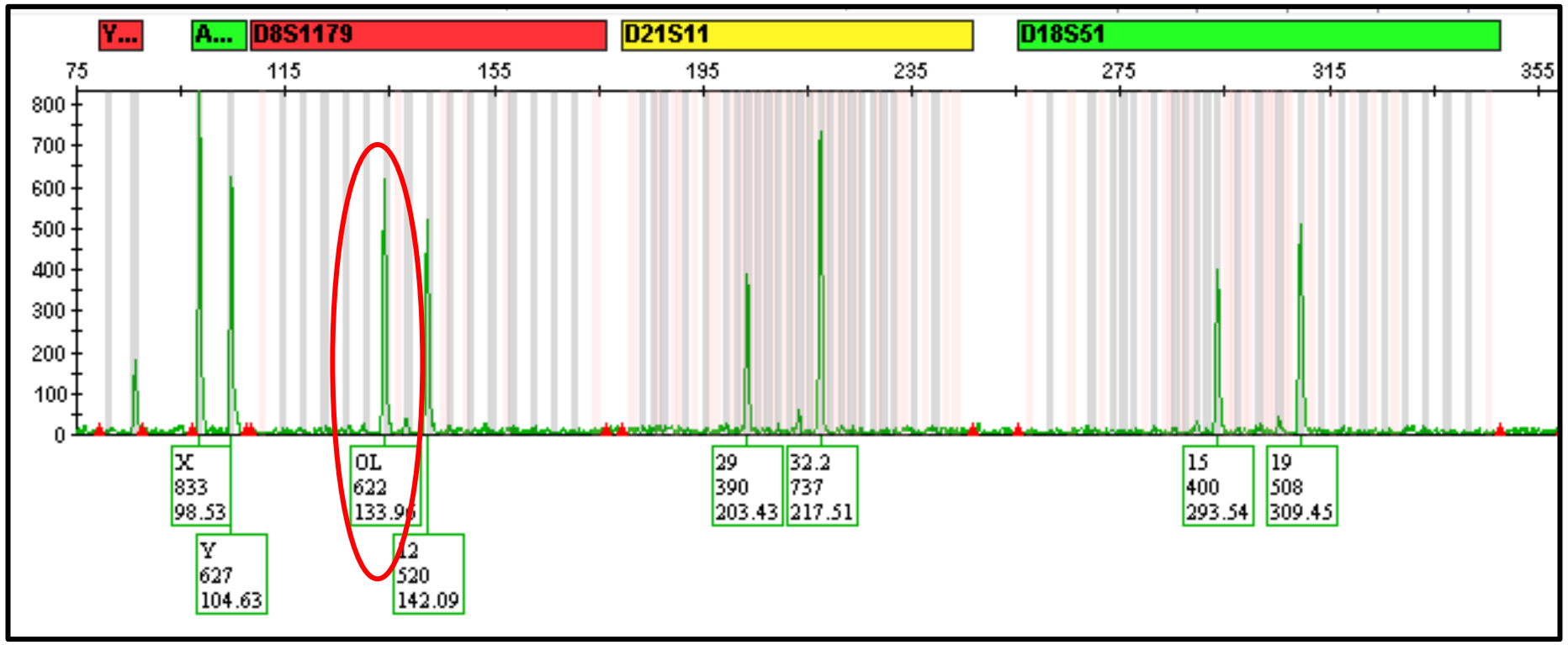
Sizing using Local Southern Method



Capillary Electrophoresis: Low ISS Intensity



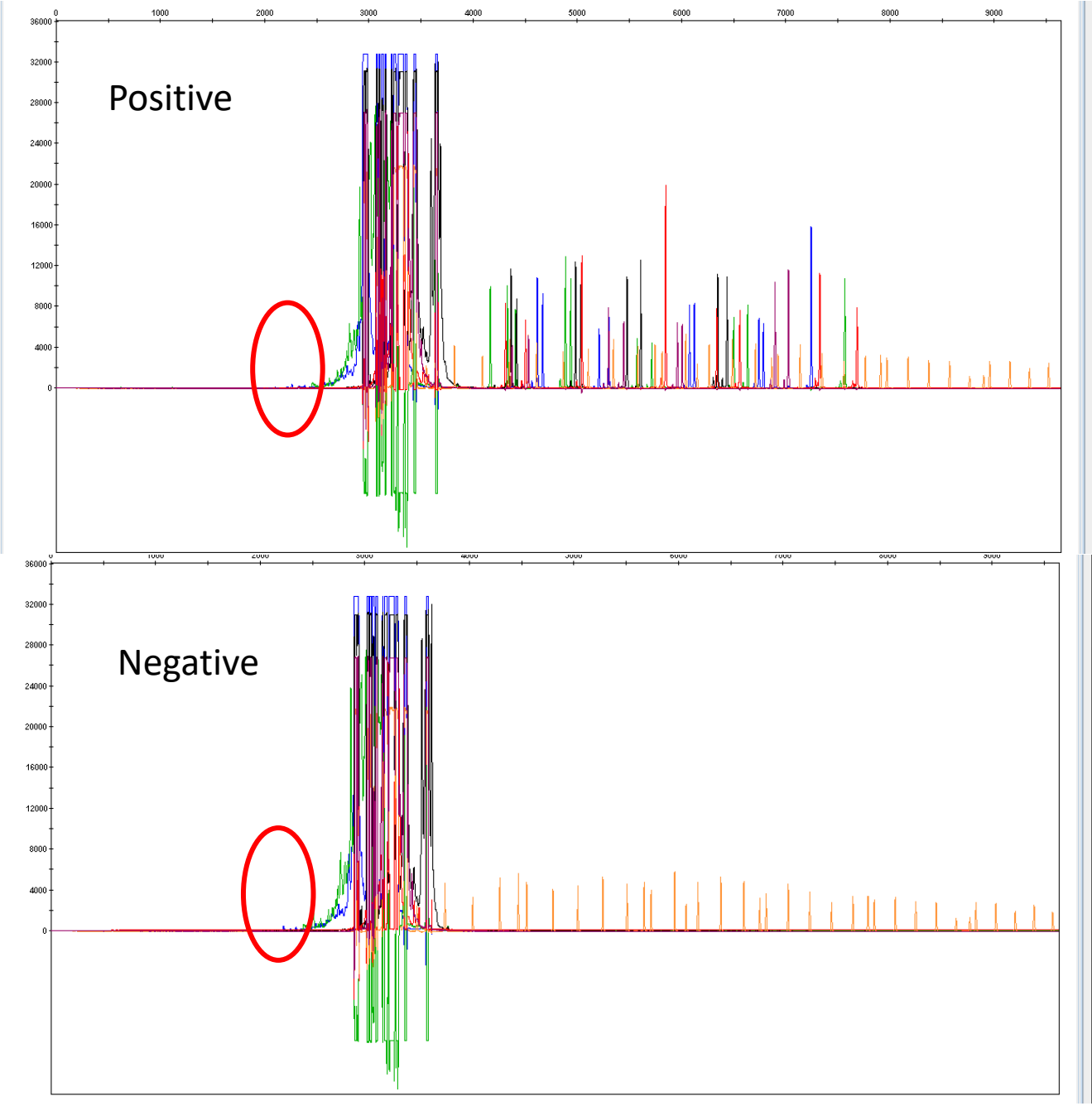
Electropherogram Sizing Issues: Off ladder (OL)



Off-ladder allele peaks are peaks do not correspond to allele sizes in the allele ladder

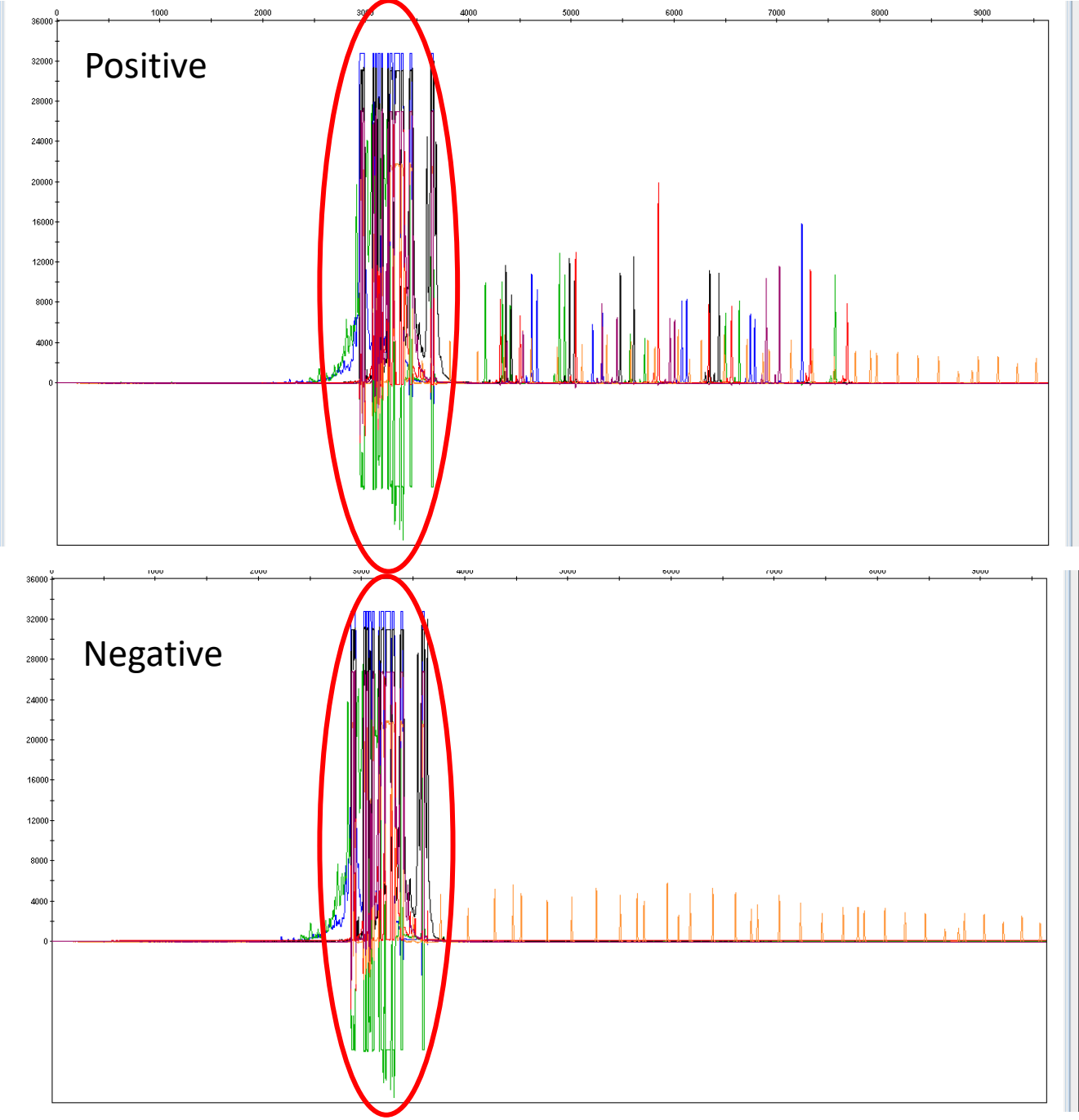
Electrophoresis Artifacts: Dye blobs

Dye blobs are dye labels disassociated from PCR primer oligos that elute early and are detected by the detector.



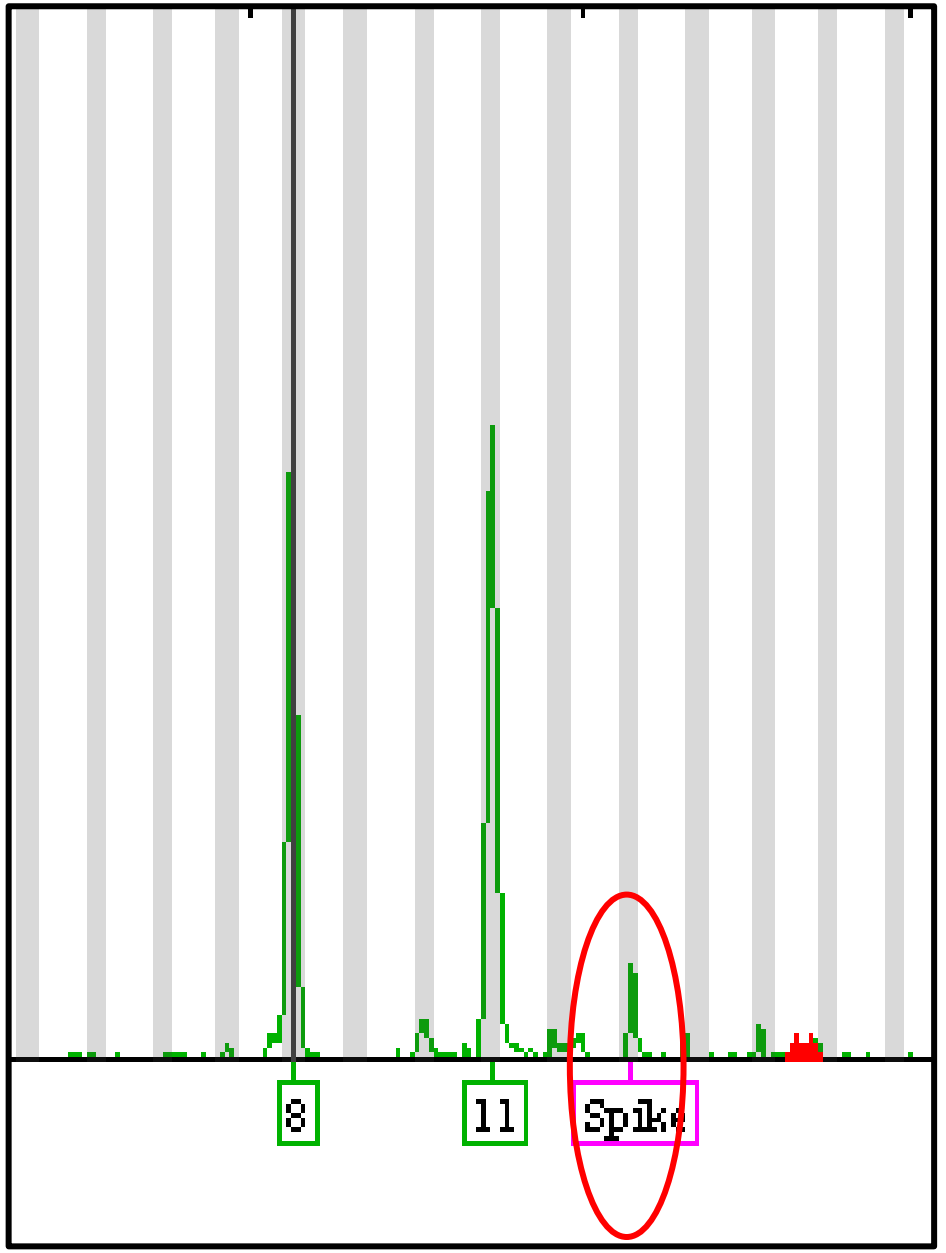
Electrophoresis Artifacts: Unextended Primers / Primer Front

Unextended primers are oligos with dyes attached that elute after dye blobs and are detected by the detector.



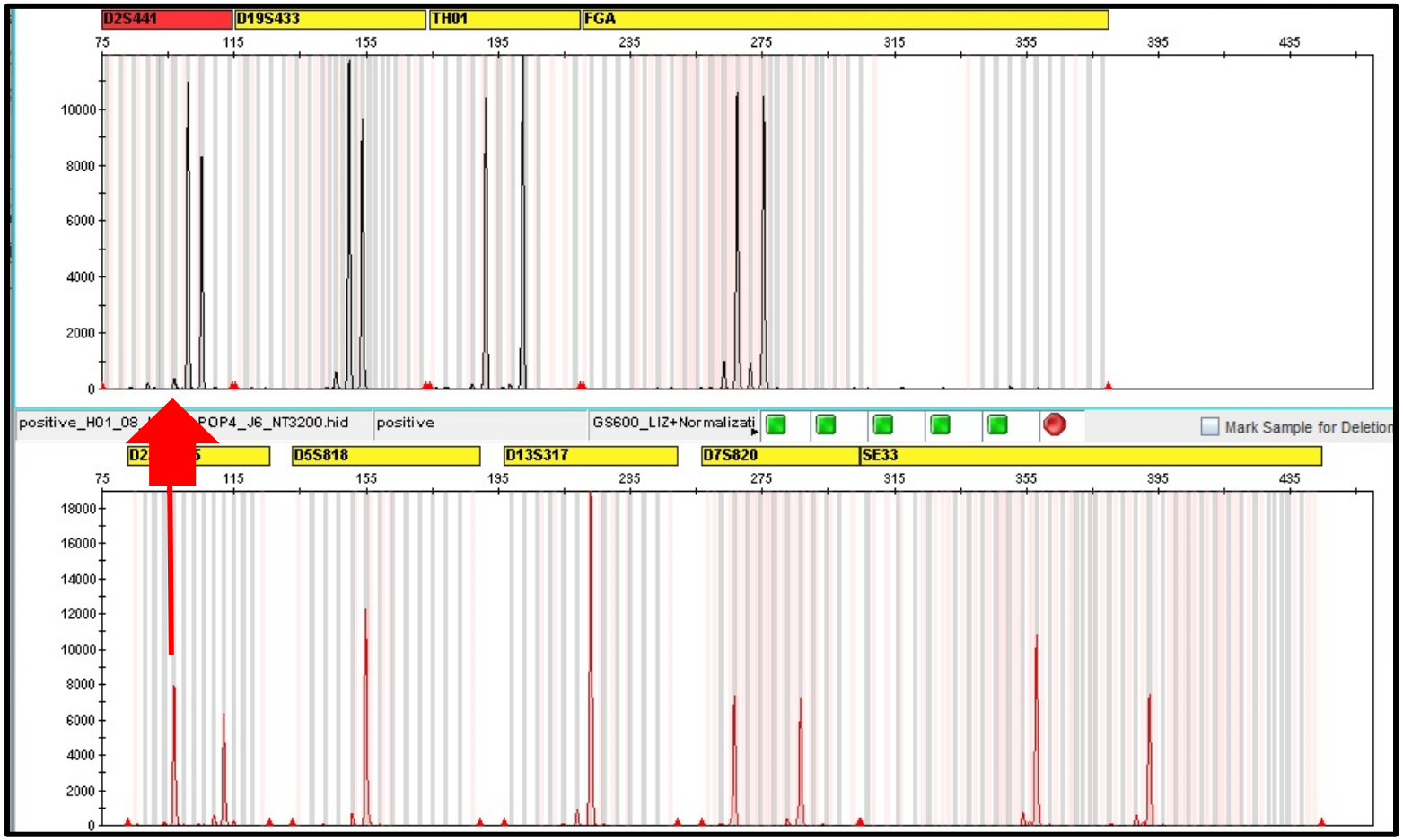
Electrophoresis Artifacts: Spikes

Spikes are intense peaks that appear in all color channels due to voltage fluctuations or air bubbles in the capillary.



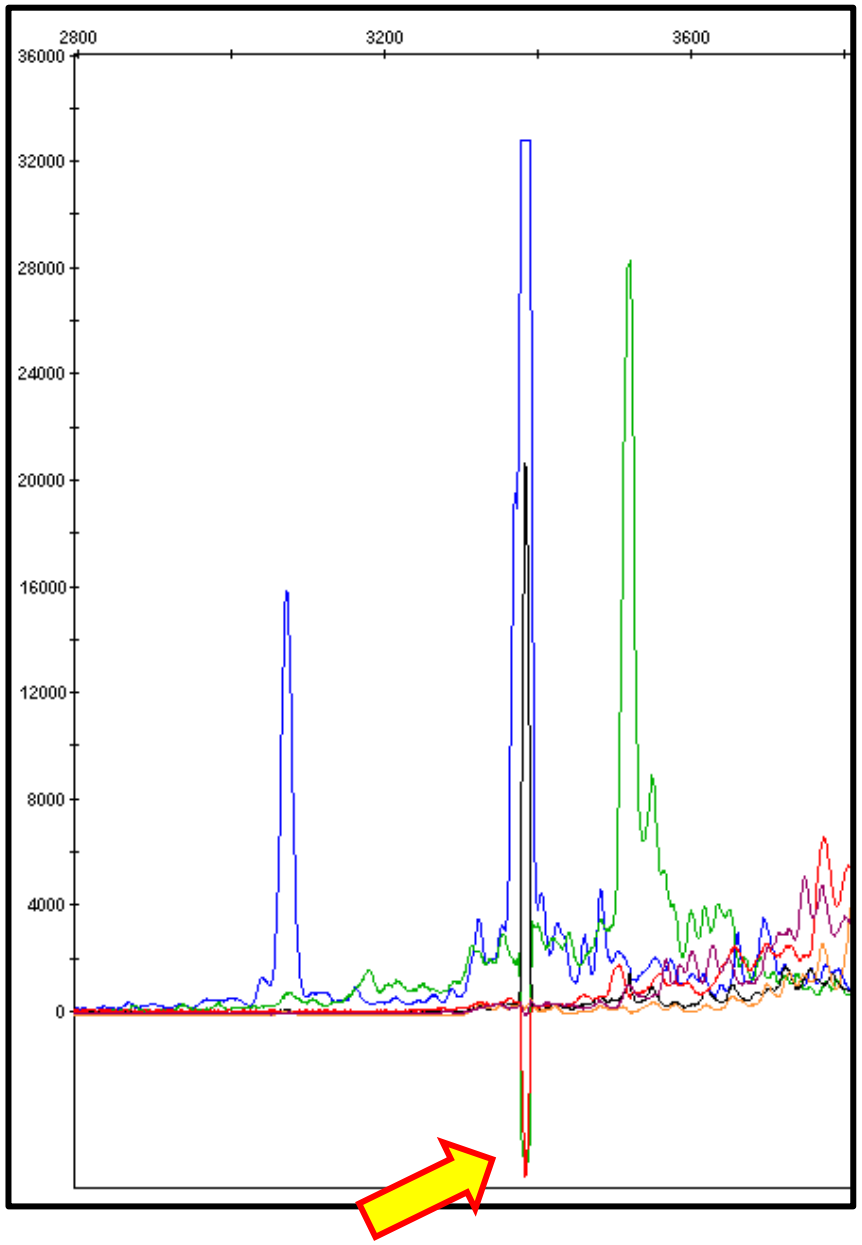
Electrophoresis Artifacts: Pull up / Bleed-through

Pull-up or bleed-through occurs when dyes of similar emission wavelengths are co-detected under the sample peaks due to a failure in the software matrix file to discriminate the colors; they distort the peak height.



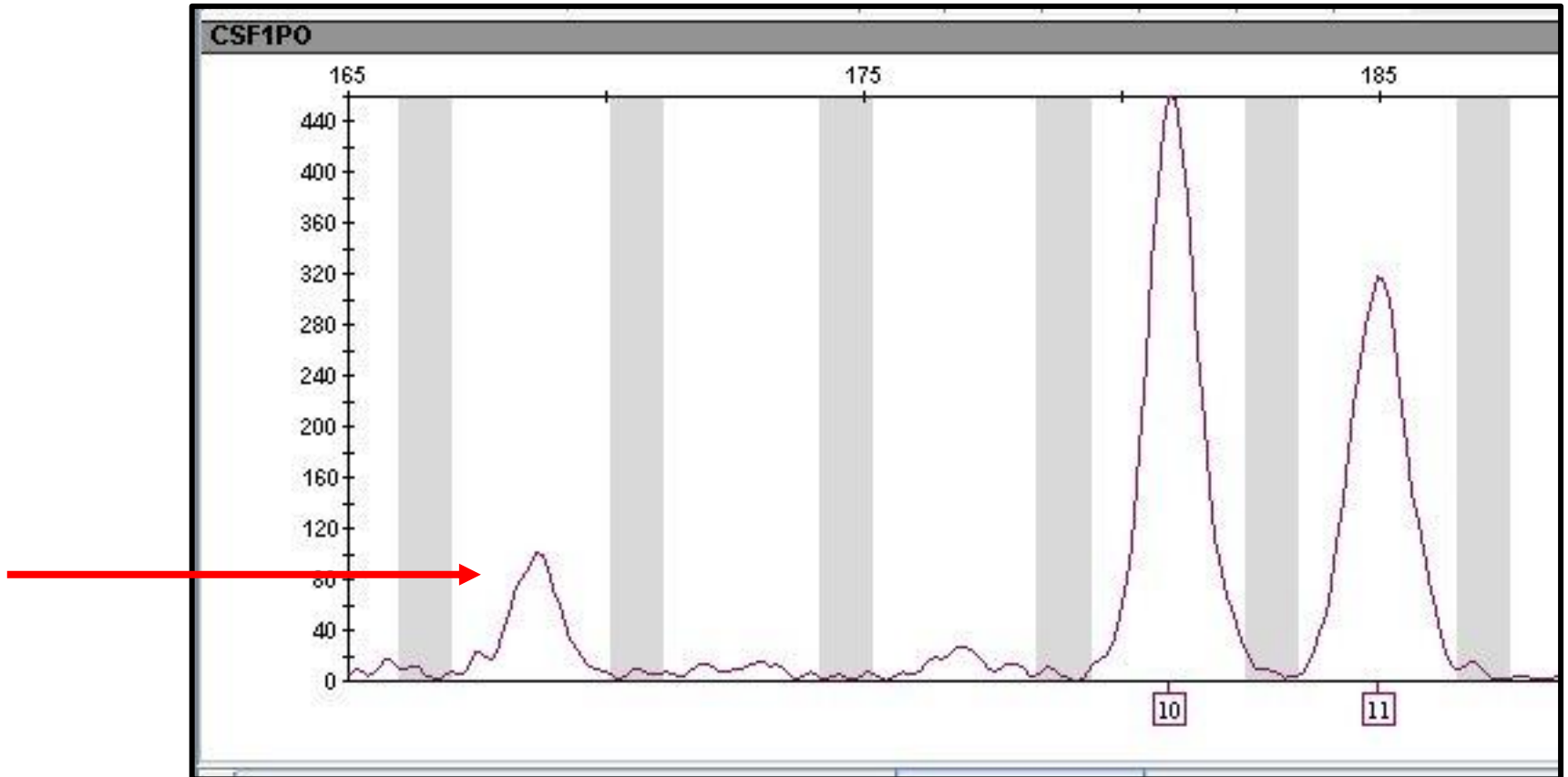
Electrophoresis Artifacts: Pull down

Pull down (or negative pull-up) artifacts occur when dyes of similar emission wavelengths are over subtracted; they distort the peak height.



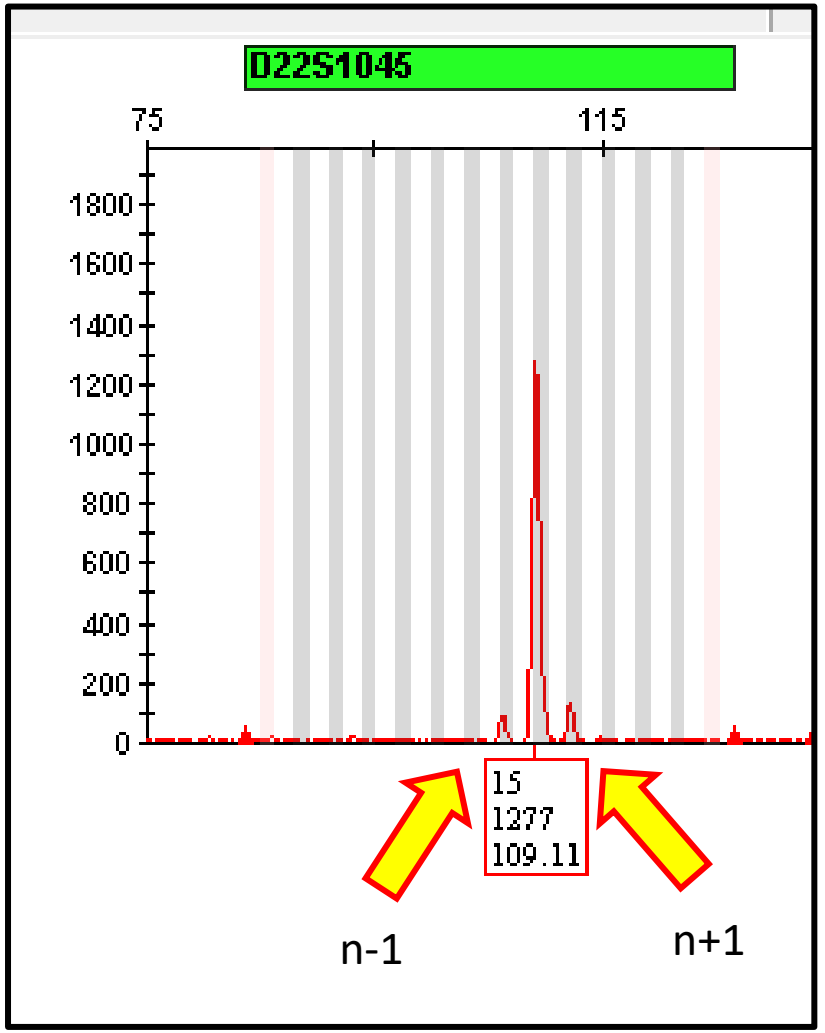
Electrophoresis Artifacts: Shadow Peaks

Shadow peak artifacts are unexpected peaks that arise before the allele peak due to contaminated formamide, rehybridized ssDNA fragments, incompletely denatured dsDNA samples, or interactions with the capillary.



Electrophoresis Artifacts: Stutter

- Stutter is a PCR artifact that is generated with a length of one of more bases more or less than the parent allele targeted in the assay when the DNA template ribbon folds up and two or more bases are skipped or four bases are added when the target folds
  - n+1
  - n-1
  - n-2
  - Half back stuffer
  - Off ladder stutter

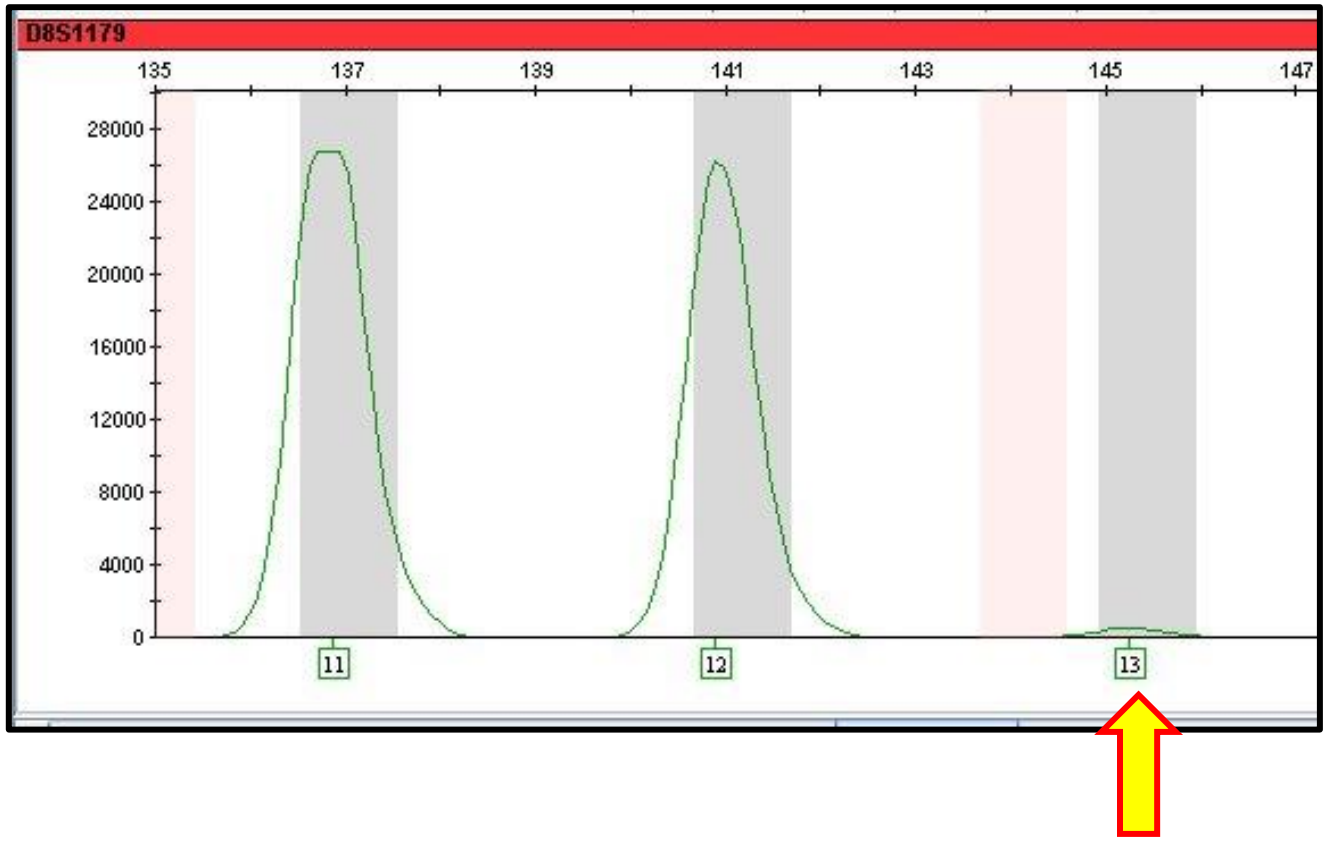


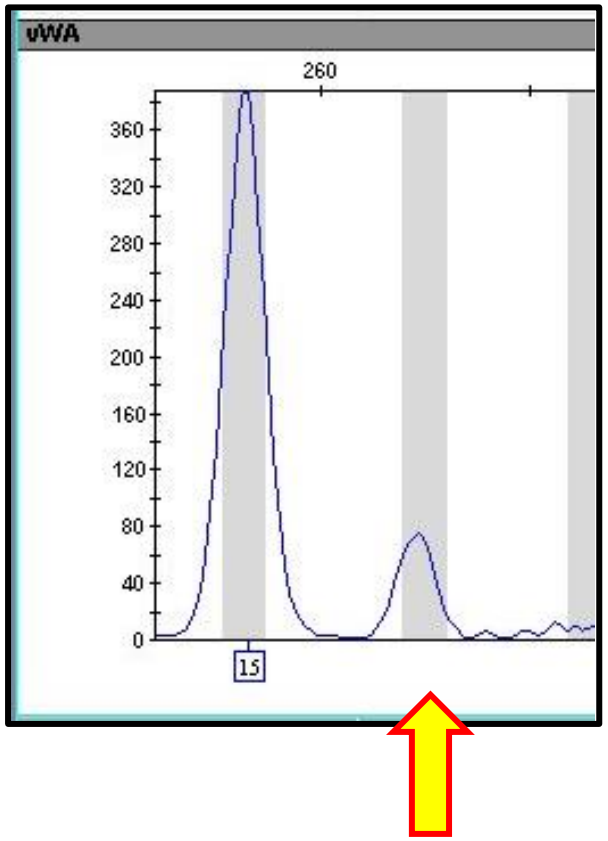
Electrophoresis Artifacts: Stutter

Figure credit: Nihal Chana

Electrophoresis Artifacts: Forward or n+1 stutter

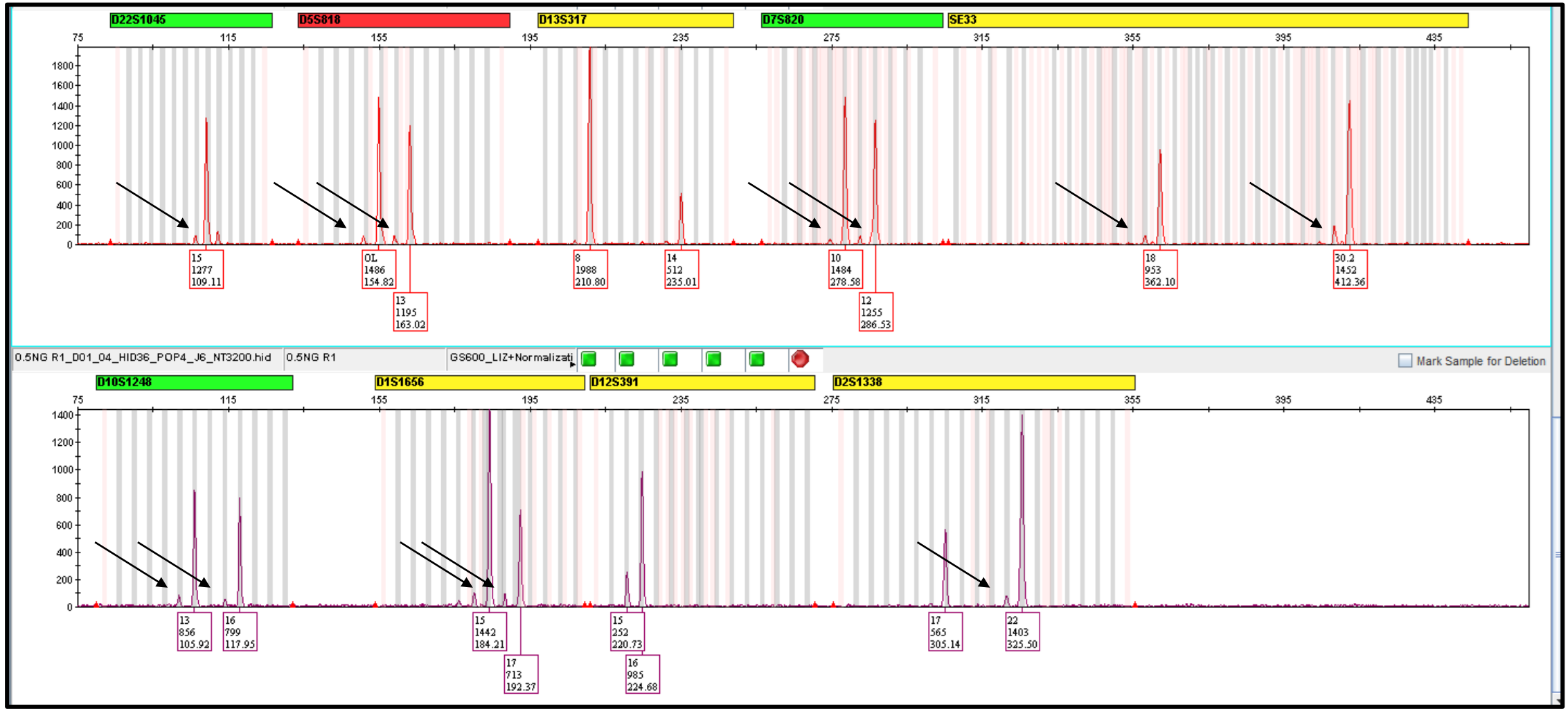
The amplicon has an extra repeat unit than the actual STR allele.





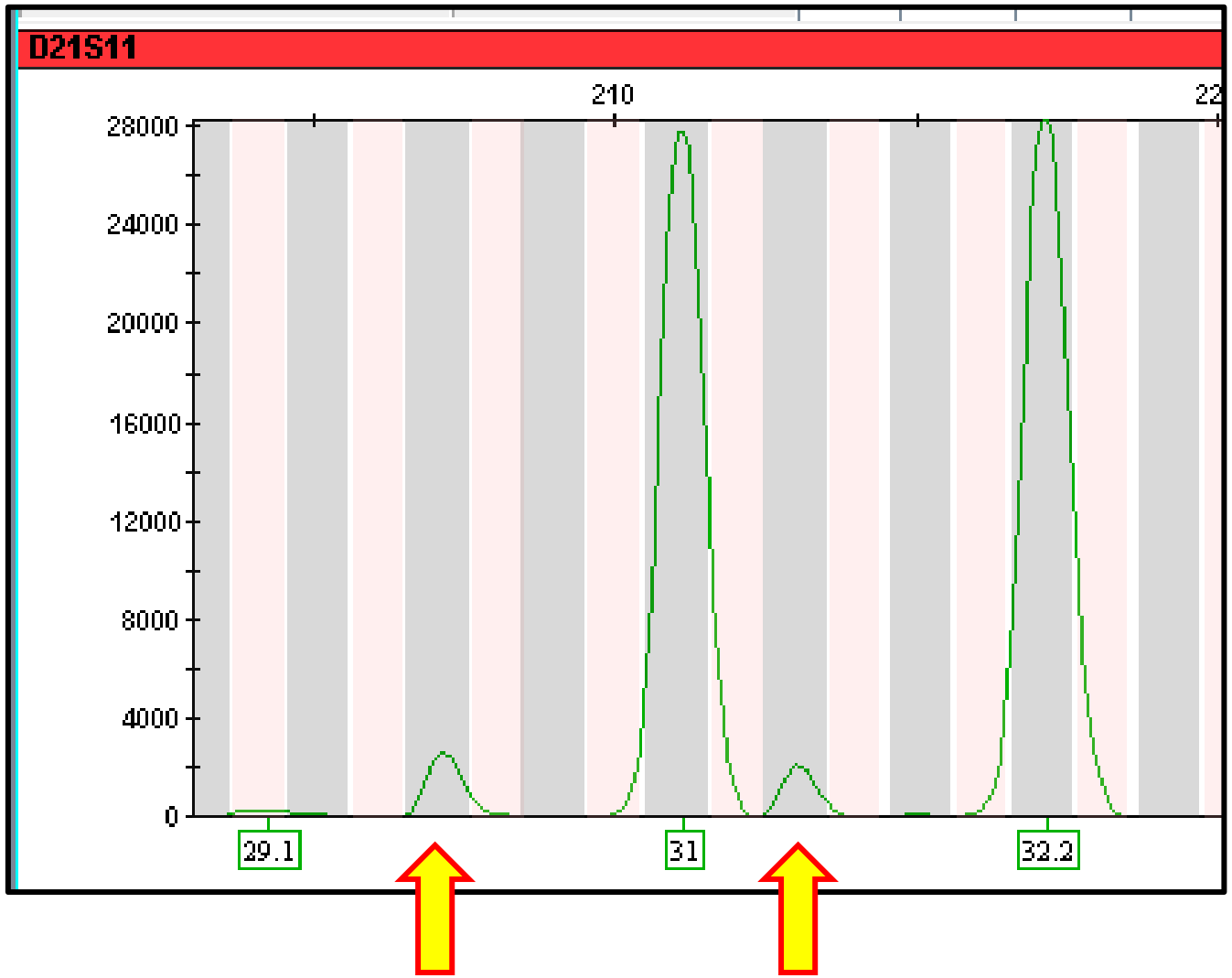
Electrophoresis Artifacts: n-1 stutter

The amplicon has one less repeat unit than the actual STR allele.



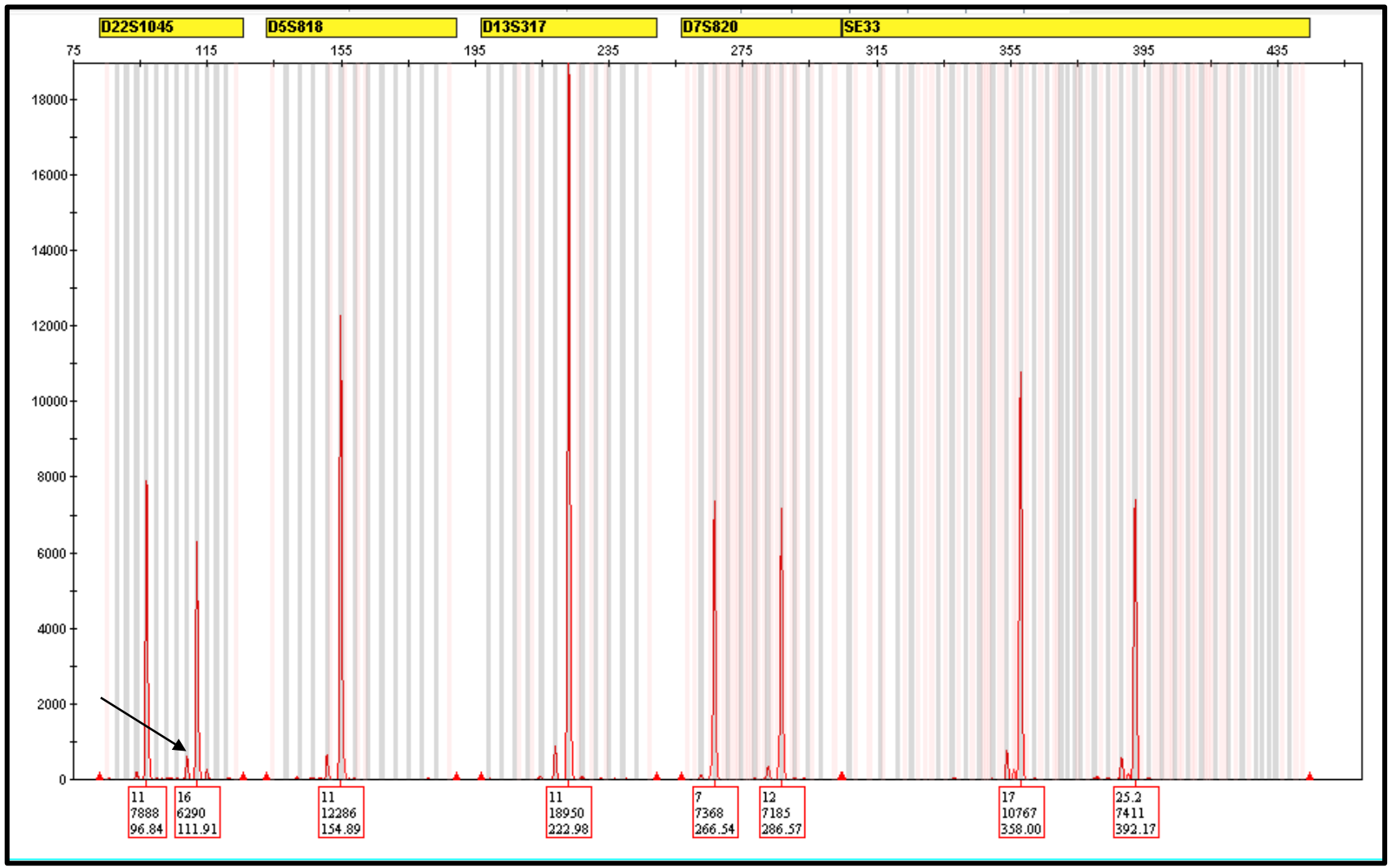
Electrophoresis Artifacts: n-2 stutter

The amplicon has two less repeat units than the actual STR allele.



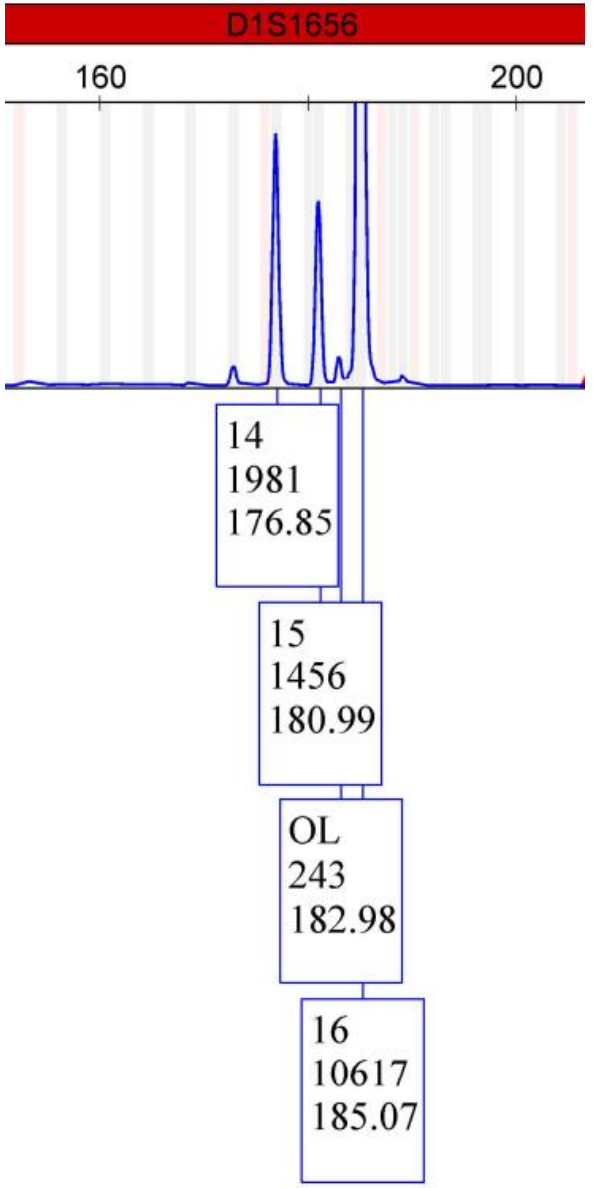
Electrophoresis Artifacts: Half back stutter

The amplicon has two less base pairs than a tetranucleotide STR allele.



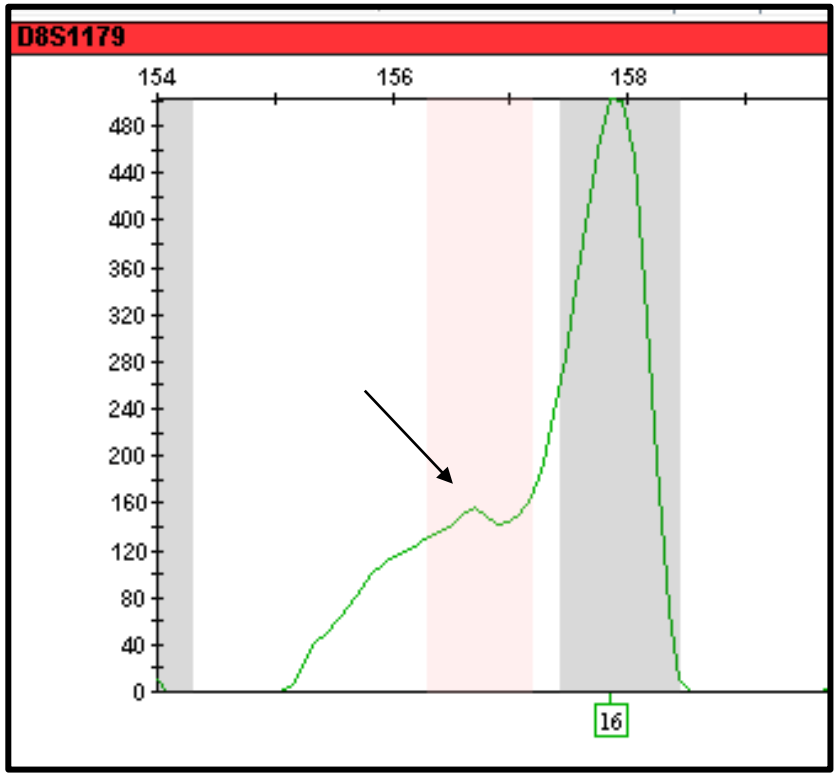
Electrophoresis Artifacts: Off ladder (OL) stutter

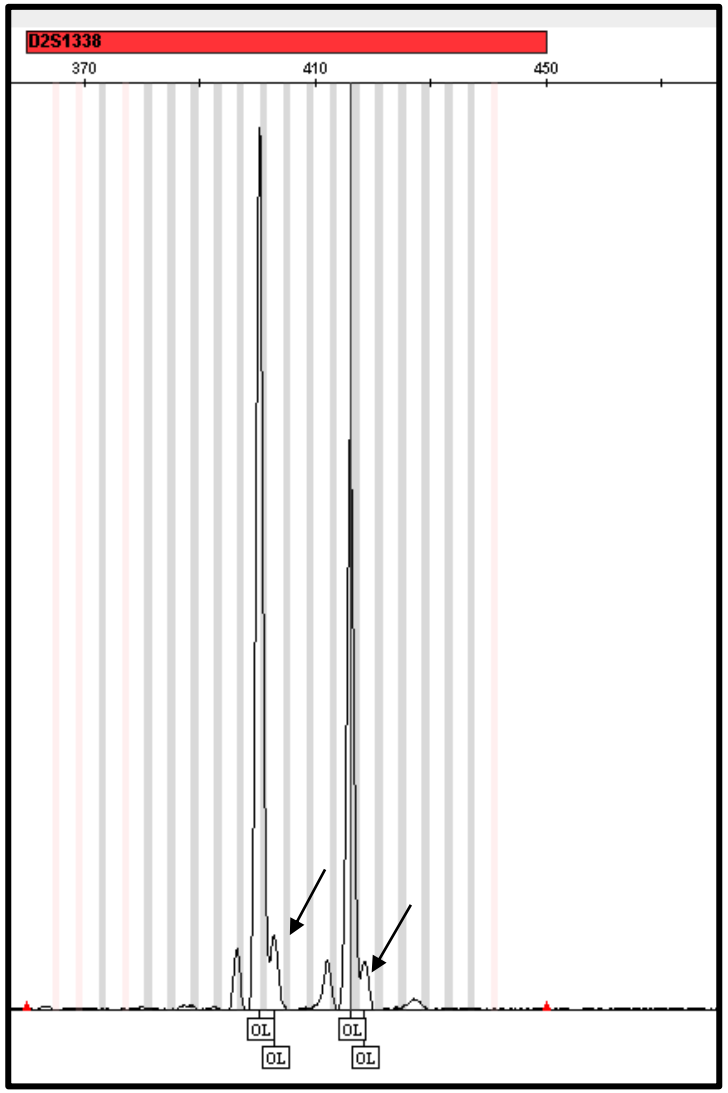
The stutter is in an off ladder position outside of a bin.



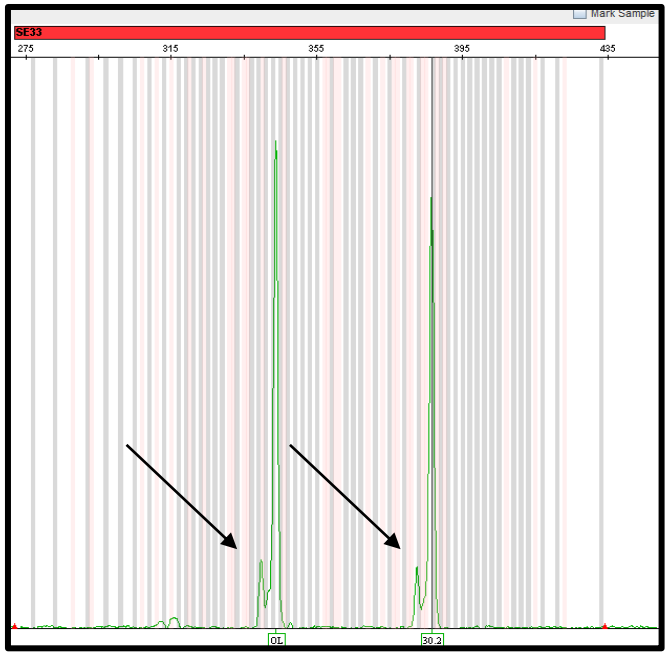
Electrophoresis Artifacts: Shoulder peak

Shoulder peaks are one base pair shorter or longer than the true allele due to issues such as non-template extension or incomplete adenylation.

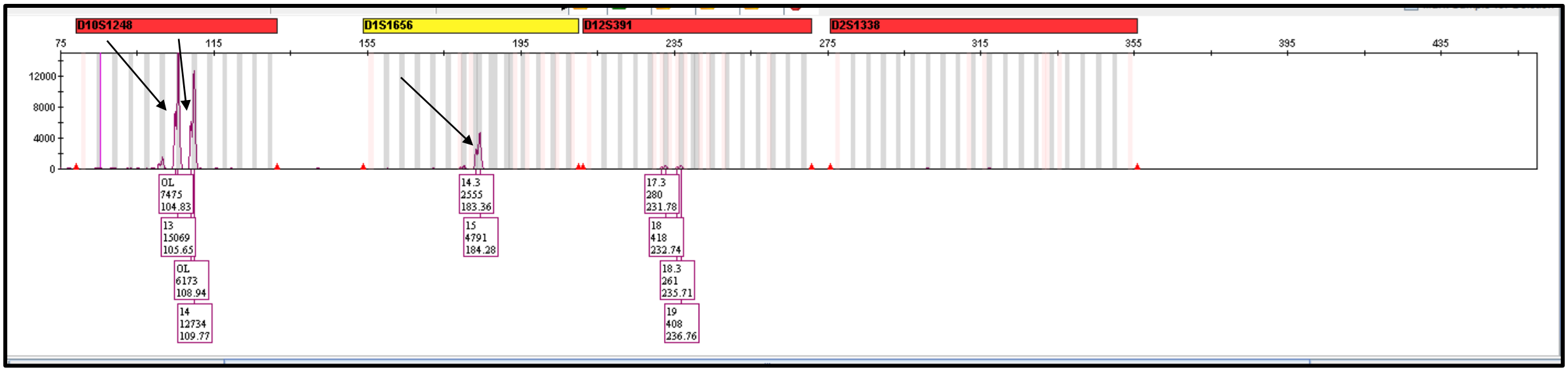




Electrophoresis Artifacts: Split peak due to "N" bands such as +A/-A adenylation

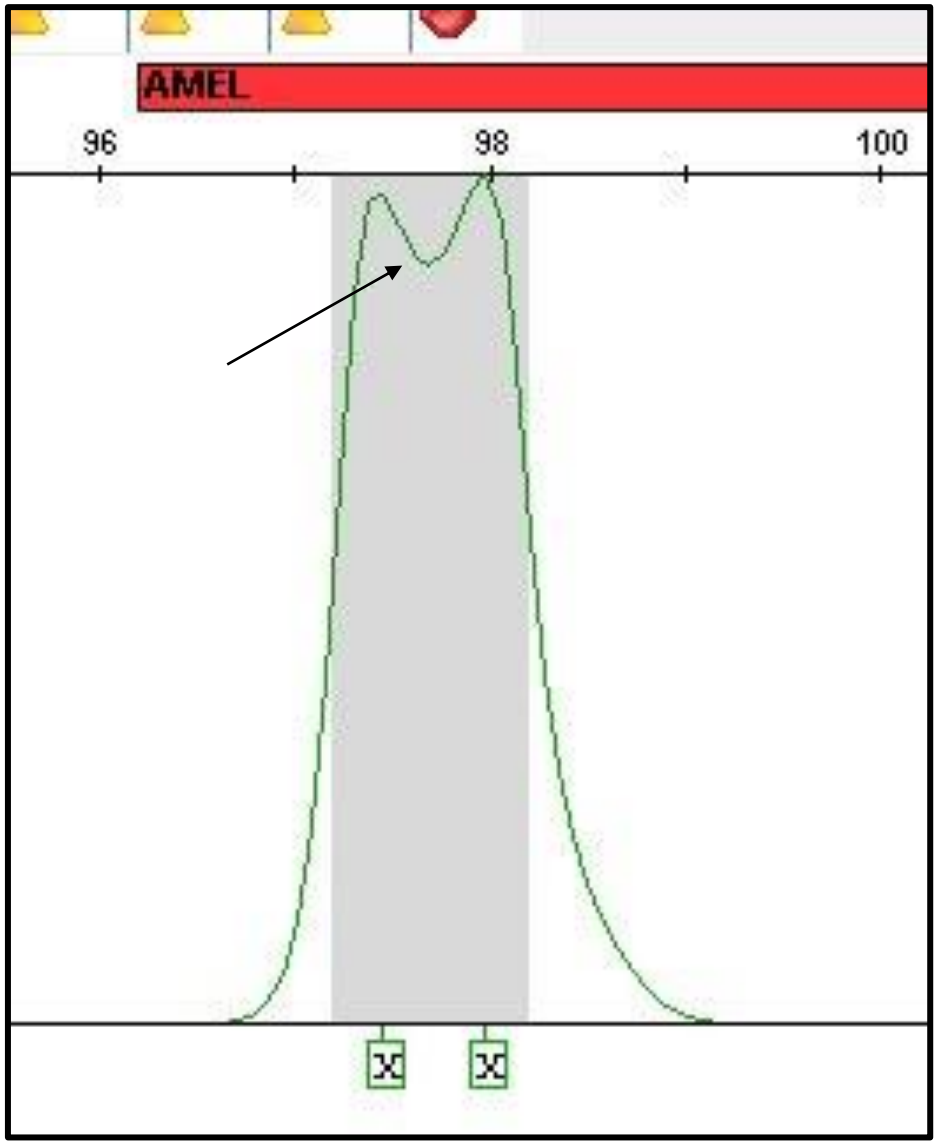


Split peaks are allele peaks that are separated into two due to incomplete adenylation at the terminus of an amplicon.

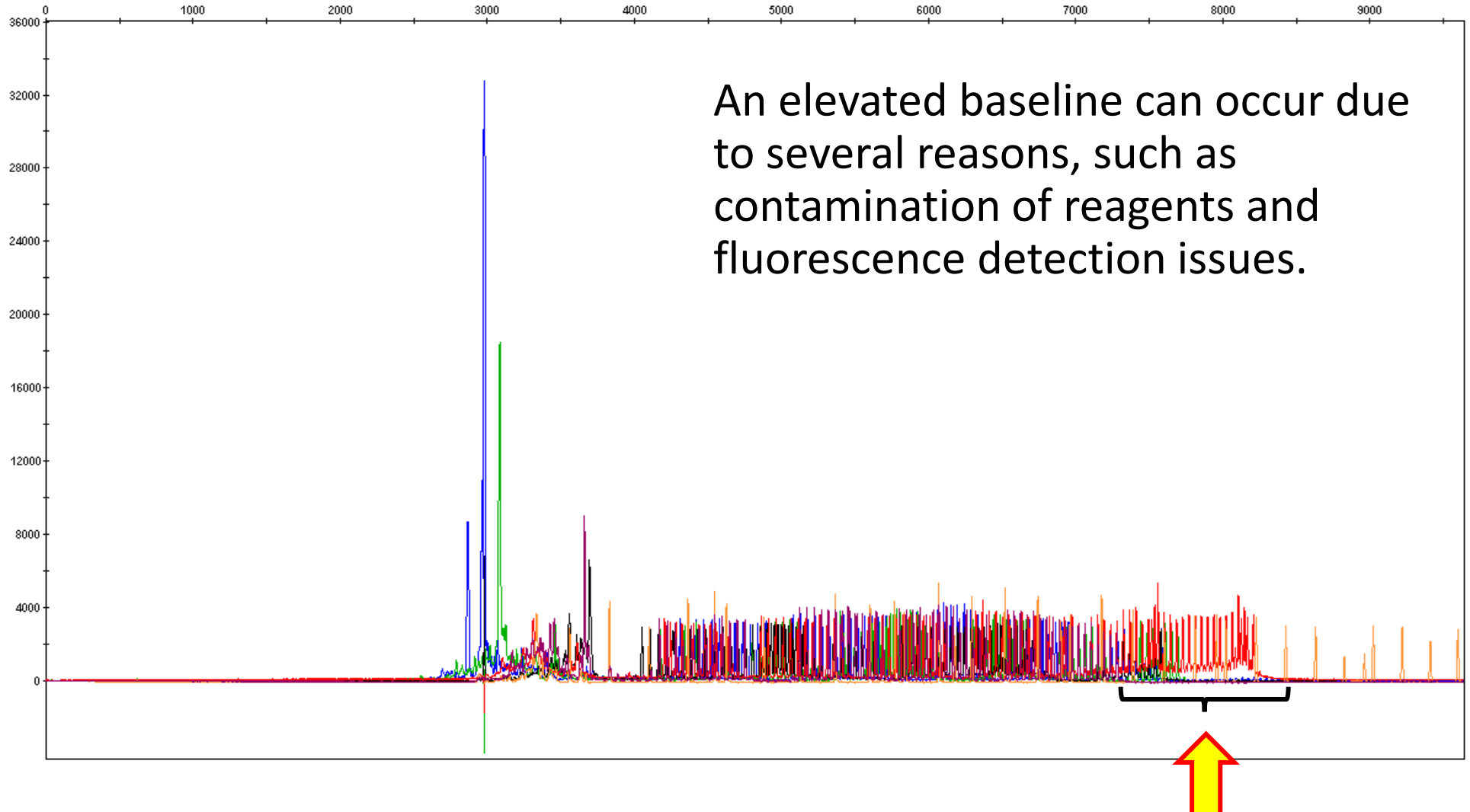


Electrophoresis Artifacts: Split peak due matrix oversubtraction

Matrix oversubtraction from deconvolution software in which overlapping signals are reduced too much can lead to a split peak.



Electrophoresis Artifacts: Elevated Baseline



An elevated baseline can occur due to several reasons, such as contamination of reagents and fluorescence detection issues.

Avoiding Contamination

- Low risk of contamination post-PCR with low level exogenous DNA as it is not fluorescently-tagged
- Post-amp samples should be carefully handled to avoid contaminating any sample
- Waste DNA should be carefully handled to avoid contamination and transfer

Quality Control

- Control samples should be run to verify instrument performance
- Sample locations must be carefully tracked
- Check to ensure sufficient polymer remains for number of samples
- Verify that polymer, buffer, capillary and other products are not expired and are performing as expected
- Internal standard must be included in controls and samples to correlate fragment size with retention time
- Spectral file is needed to quantify dye overlap
- Allele ladder must be run to call the alleles based on size
- Constant room temperature (e.g., 25 °C) must be maintained and heated plate (e.g., 60 °C) is used for to maintain the temperature of the capillary and reduce secondary structures from forming
- Buffers can become depleted and need refreshed
- Capillaries can become clogged and fail (new or cleaned capillaries are required ca. every 100 runs)
- Fresh polymer should be used for best results
    - Polymer polymerizes due to reactions with air over time

Limitations of the Technology

- Variations arise with temperature fluctuations and polymer crosslinking
  - Lower concentration polymers cause fragments to elute faster and may lead to co-elution of fragments of different lengths
  - Higher temperatures will decrease elution times and may lead to co-elution of fragments of different lengths
- Poor performance with large DNA fragments
- Co-eluting fragments of different sequences will not be differentiated
  - STRs of the same length but different sequences will not be differentiated

Study Questions

- Explain how electrophoresis works.
- How does DNA move through a gel?
- What is the purpose of an allelic ladder?
- What is the purpose of the internal size standard?
- Explain how electrokinetic injection works. What can be varied or changed to inject more or less sample?
- Why do the capillaries need to stay immersed in liquid before, during, and after electrophoresis?
- List each of the reagents used to set up CE and describe their function.
- Explain what each of the following artifacts are? Sketch each.
  - Spike
  - Primer peak
  - Pull-up
  - Split peak
  - Reverse stutter
  - Forward stutter
  - Dye blob

Study Questions

- Describe how DNA is separated in capillary electrophoresis.
- Describe how the GeneMapper software analyzes the STR raw data and assigns allele calls to a sample.

Suggested Readings

- ANSI/ASB Standard 115, Standard for Training in Forensic Short Tandem Repeat Typing Methods using Amplification, DNA Separation, and Allele Detection. 2020. 1st Ed. https://www.aafs.org/sites/default/files/media/documents/115_Std_e1.pdf
- Butler, J.M. Advanced Topics in Forensic DNA Typing: Methodology Ch. 6: Capillary Electrophoresis, 2011.