An Exploration of a Sequence-Specific Stutter Model for STR Analysis in Next Generation Sequencing

Damani Johnson, BS; Tim Kalafut, PhD

Department of Forensic Science
Sam Houston State University
Huntsville, TX 77340

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Don’t Let My Relaxed Demeanor Fool You – This Is Exciting

- Being able to identify and filter stutter effectively is important
- Next Generation Sequencing is incredibly powerful and informative, and addressing obstacles can help ease its implementation in crime labs
- Development of software tools can make stutter validation easier and more efficient
- Before and after photos of graph lines getting slightly more linear are very satisfying

Paul

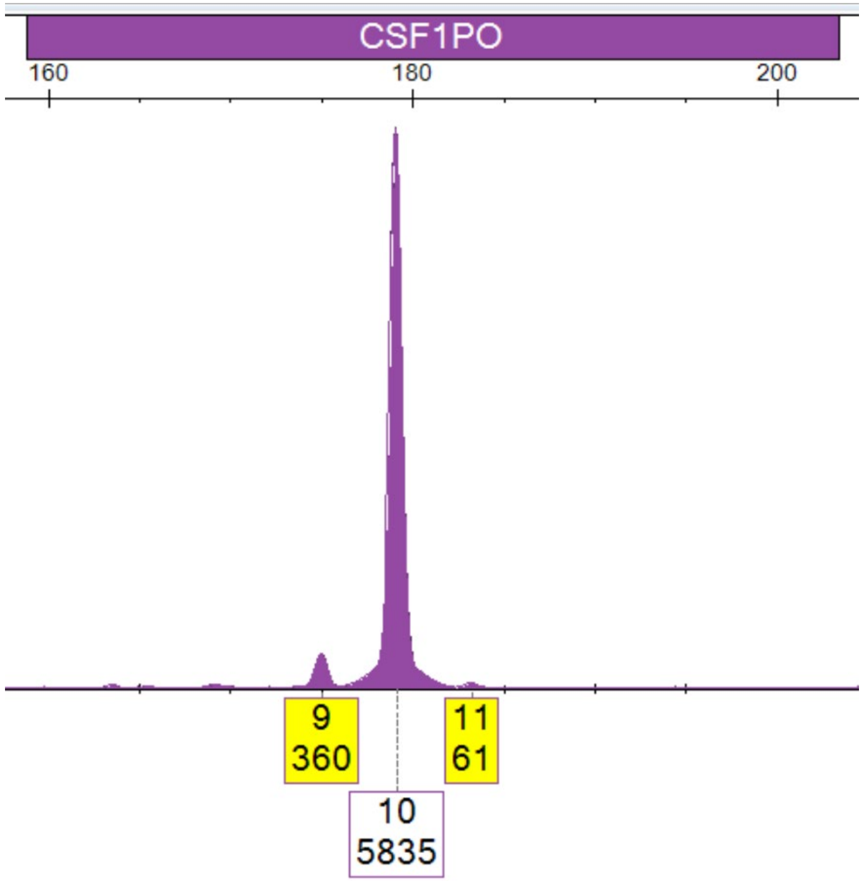
Methods

	CE	NGS
Samples	96 single-source buccal swabs	
Chemistry	Investigator 24plex QS	ForenSeq MainstAY SE
Instrument	3500	Miseq FGx
Data Analysis	OSIRIS/ArmedXpert	MixtureAce

- Additional software tools currently in development were used for calculating and plotting stutter ratios

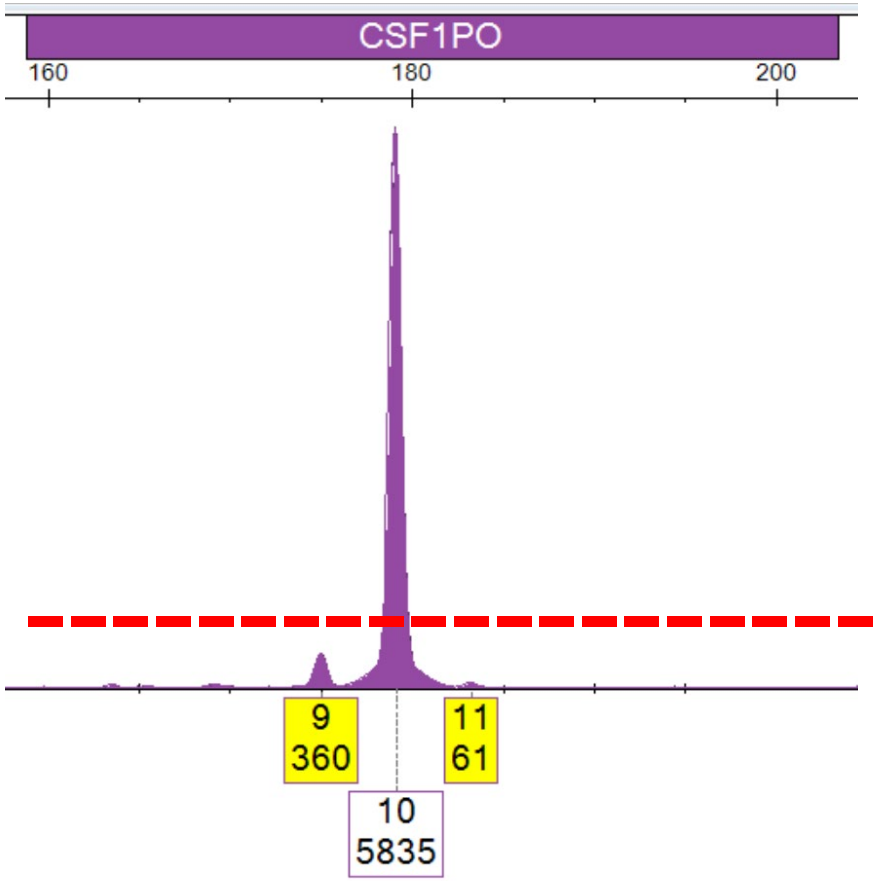
The Issue with Stutter

- Short tandem repeat (STR) markers are susceptible to strand slippage during PCR
- On CE this most commonly results in a stutter peak appearing one repeat unit short of a true allele peak
    - Also seen in a forward +1 or double back -2 position



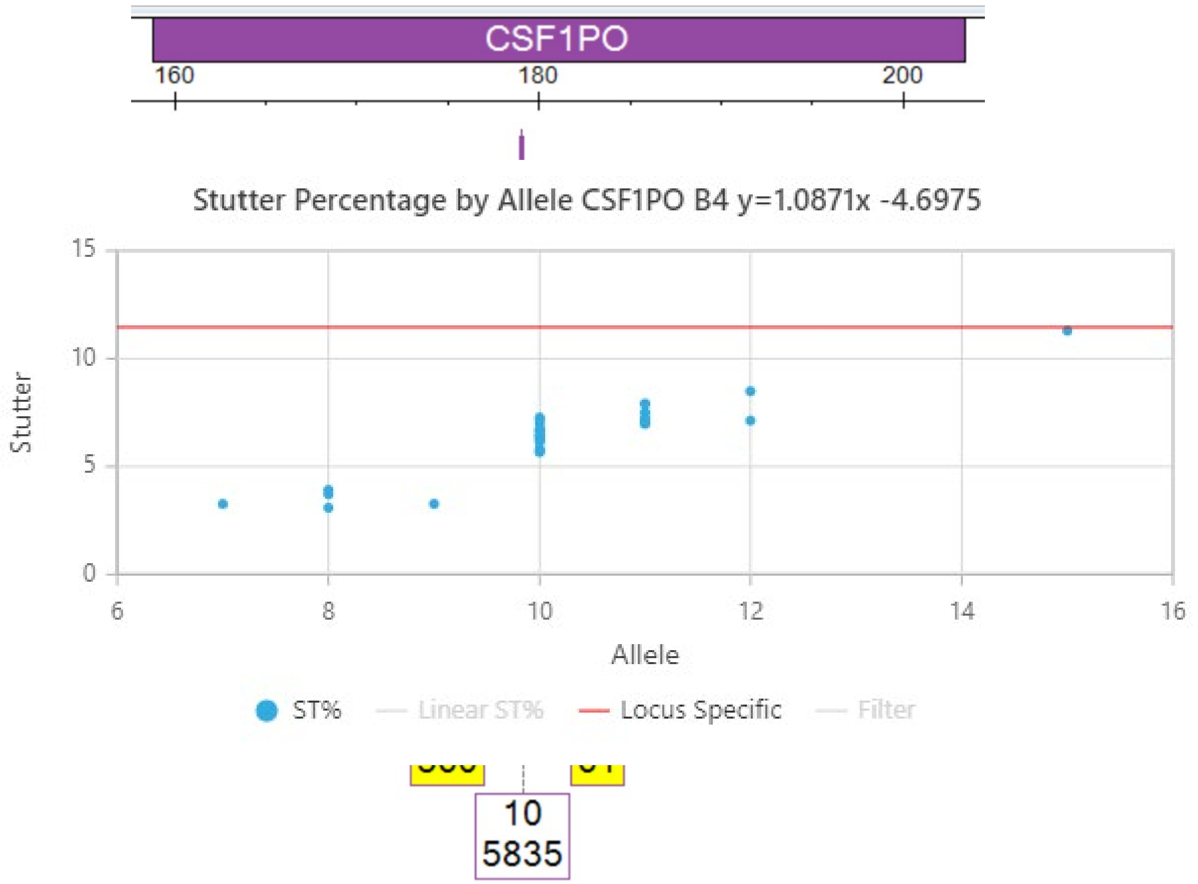
How We Address Stutter

- We use stutter filters in analysis software to help identify stutter peaks automatically
  - Ideally, the better the filter, the less manual editing is needed
- Filters are set through validation of a large single-source sample set
  - Need to validate each kit
  - Need enough samples to have as many alleles and stutter represented as possible



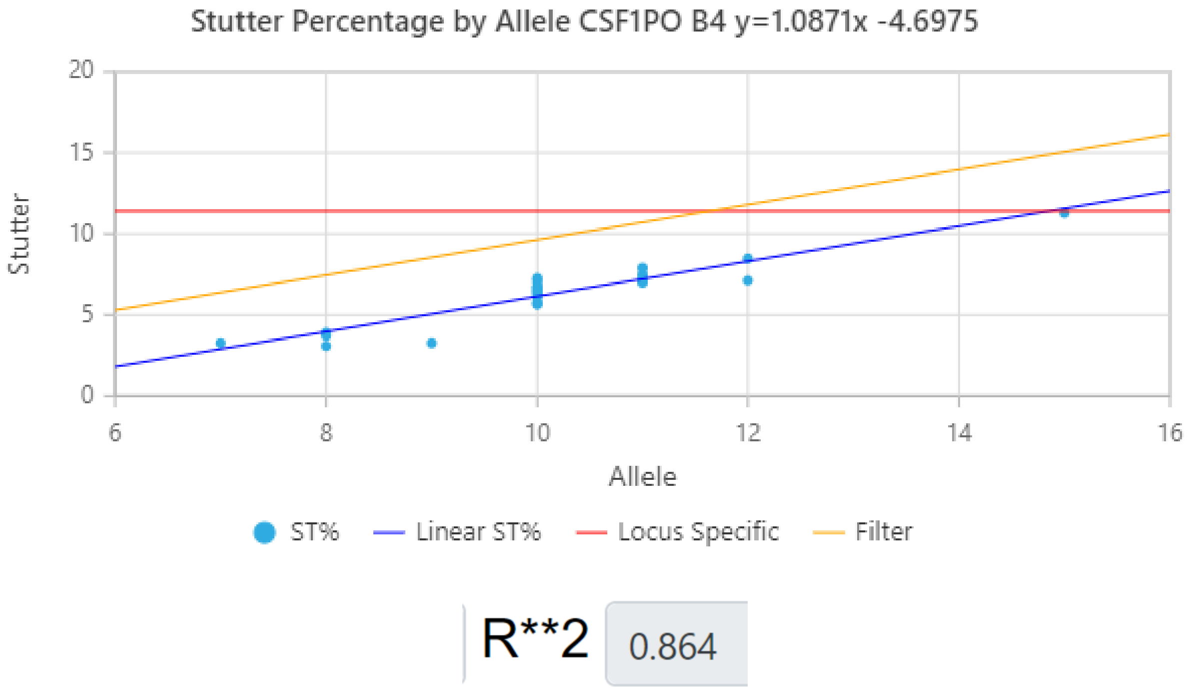
Stutter Filters

- Locus-Specific
  - Static threshold per locus
  - Based on stutter ratio mean and standard deviation
  - Risks over-filtering shorter alleles or under-filtering longer alleles



Stutter Filters

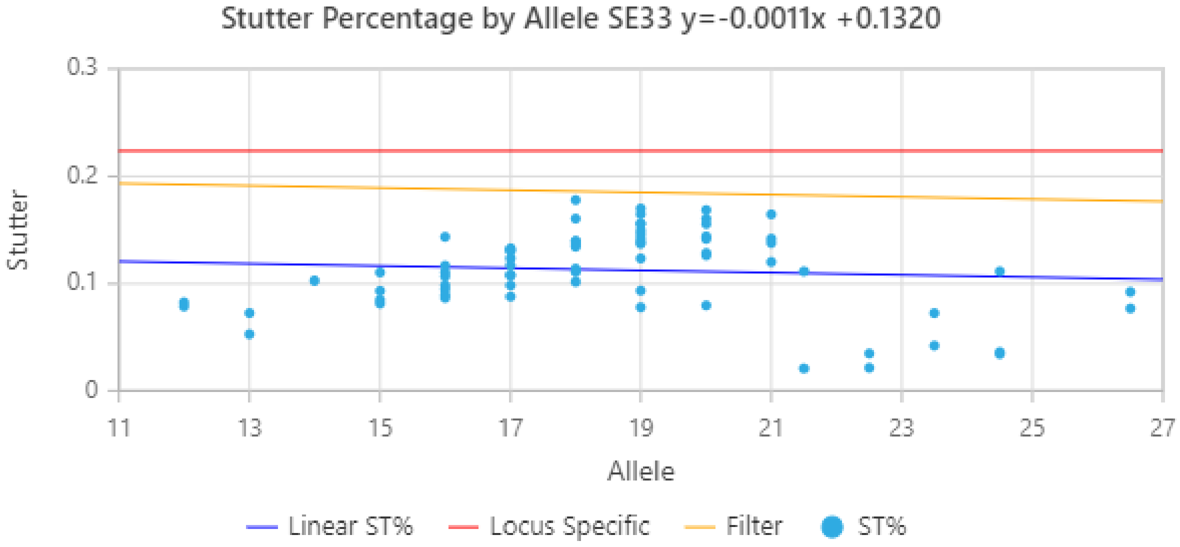
- Locus-Specific
  - Static threshold per locus
  - Based on stutter ratio mean and standard deviation
  - Risks over-filtering shorter alleles or under-filtering longer alleles
- Allele-Specific
  - Based on slope of linear trend line of stutter ratio vs. allele
  - Works very well for loci with simple repeat motifs



R**2 0.864

Allele-Specific Stutter Filter

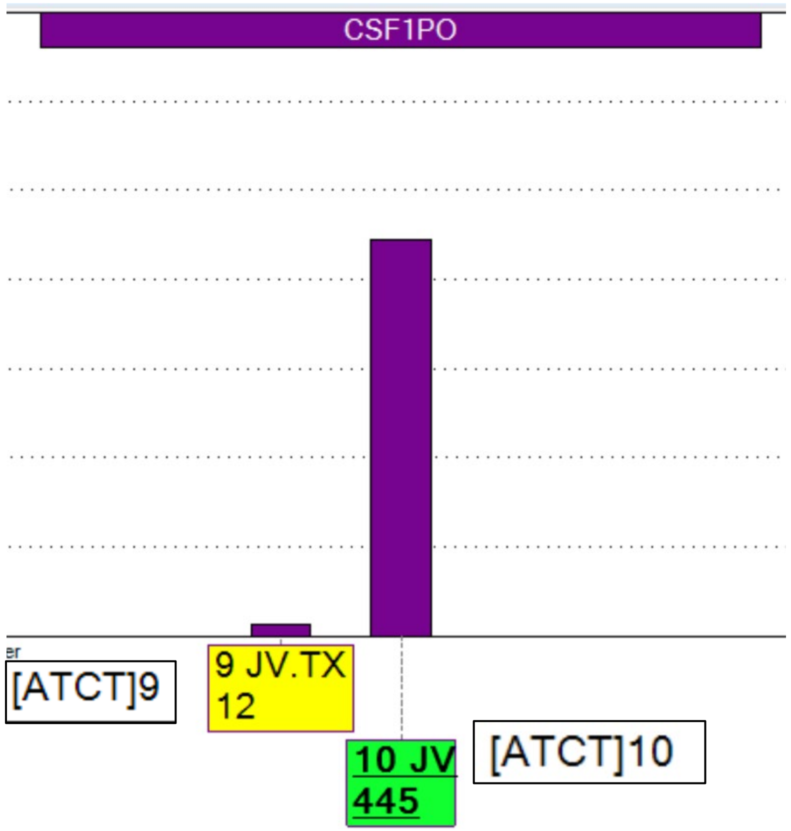
- Current standard for CE based stutter filtering
- Has trouble modeling more complex loci
    - Often appear to have multiple slopes
    - Some loci seem to stutter considerably less at “partial” alleles (.2, .3)
- CE does not give us the information needed to model these more accurately



R**2 0.008

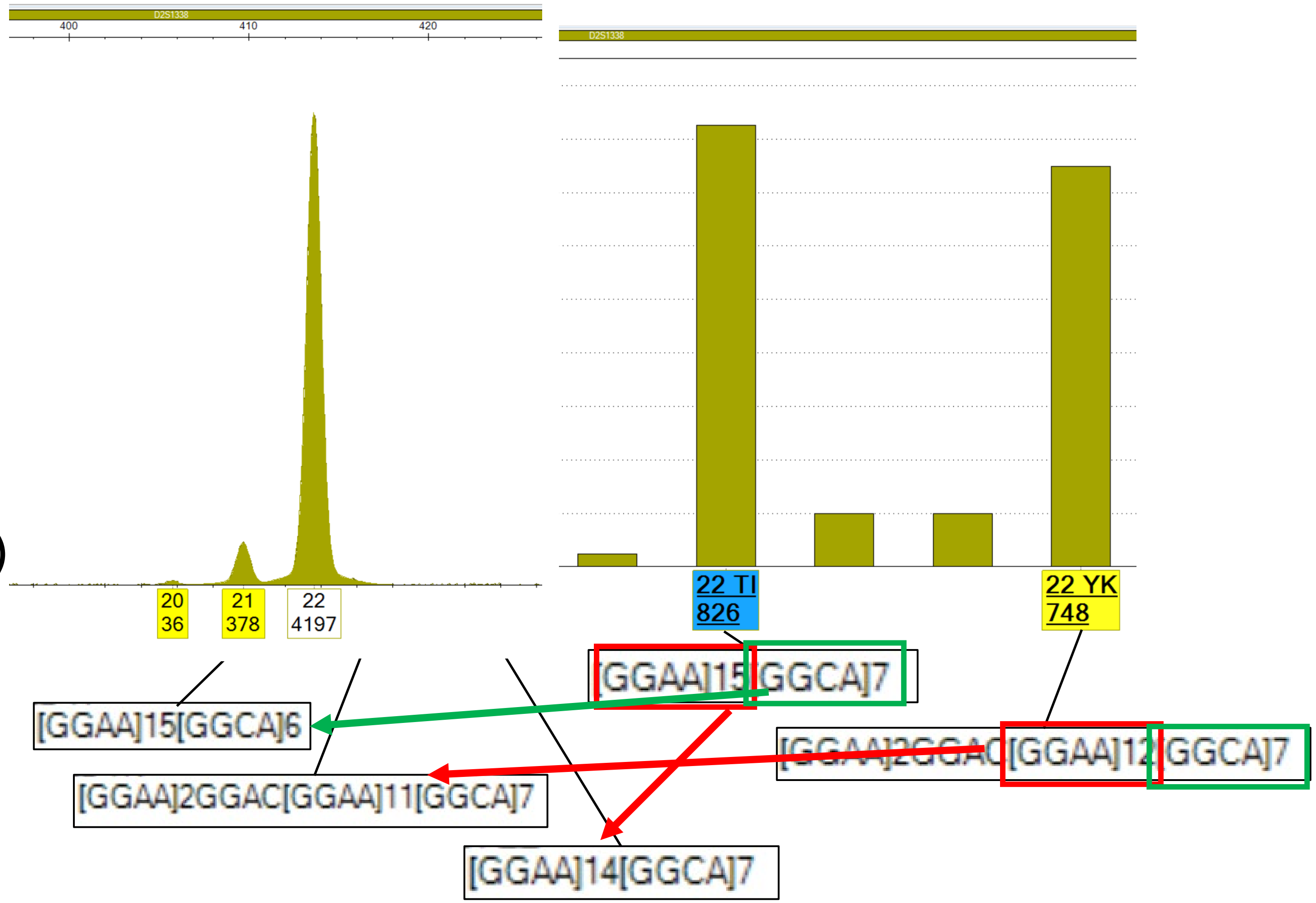
Next Generation Sequencing

- Allows us to view sequence data rather than just length
- When viewed in MixtureAce, alleles are assigned a sequence ID (SID) label that corresponds to a full or bracketed sequence
  - Stutter sequences are associated with their parent allele



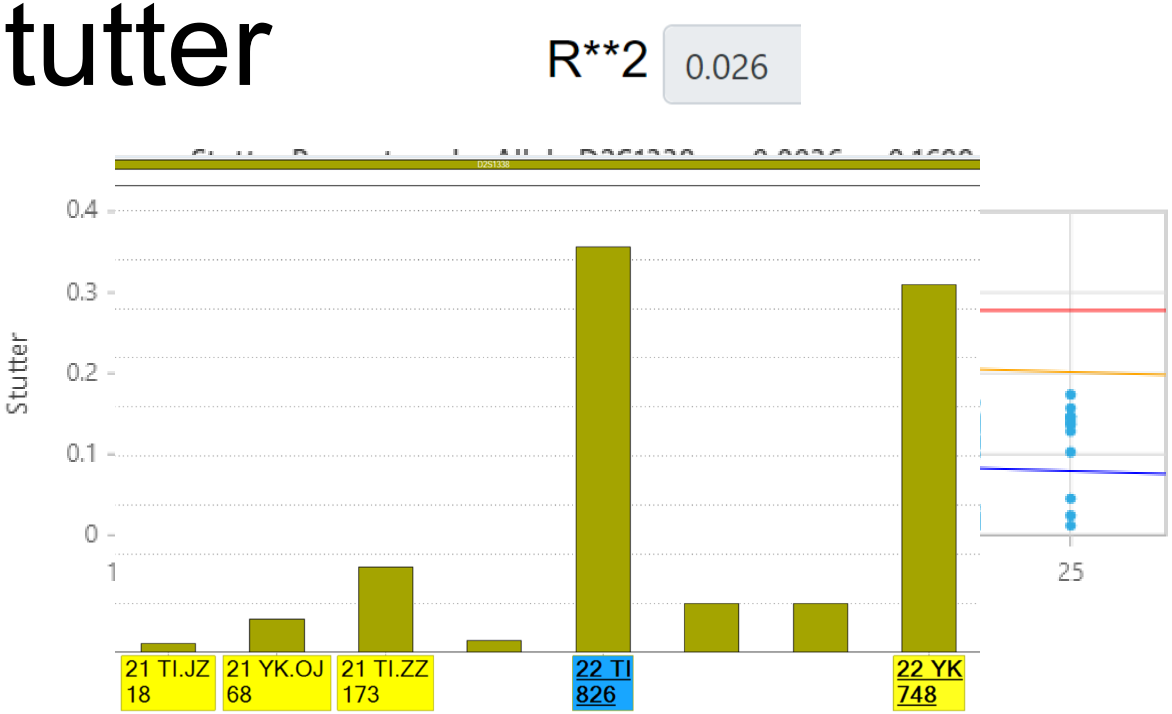
Next Generation Sequencing

- Isoalleles allow us to distinguish alleles of the same length with different sequences
  - Longest Uninterrupted Stretch (LUS)
  - Second Longest Uninterrupted Stretch (SLUS)
- Sequence can be used to associate stutter peaks with correct parent isoallele



Sequence Based Stutter

- These isoalleles from the same sample stutter at very different rates
- Using an allele-specific stutter model treats these all the same

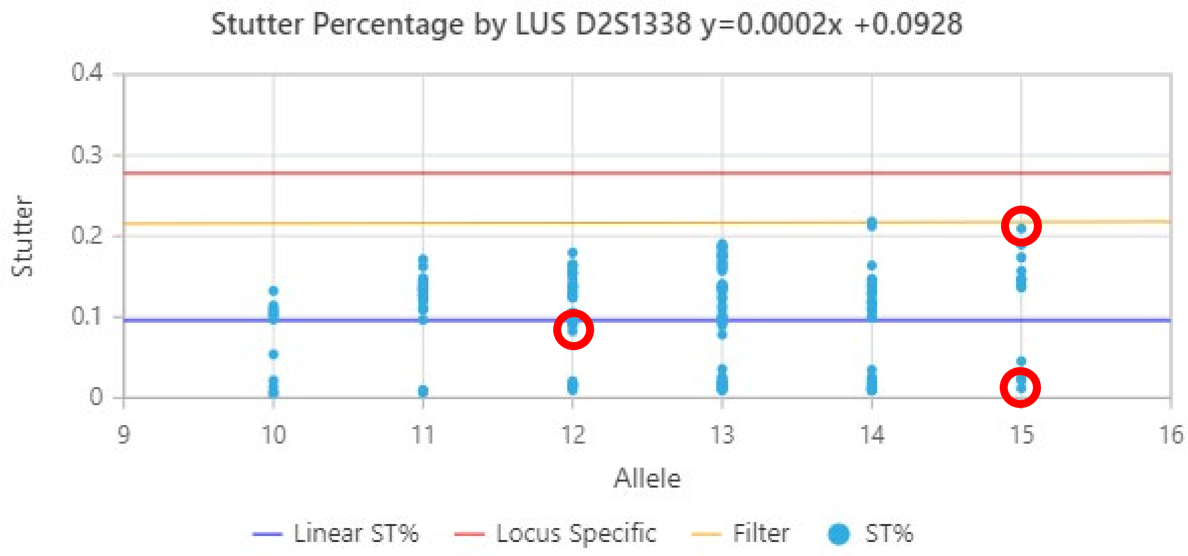


Parent Allele	Stutter Allele	Stutter Ratio	Sequence
22 YK	21 YK.OJ	0.090909	[GGAA]2GGAC[GGAA]12[GGCA]7
22 TI	21 TI.ZZ	0.209443	[GGAA]15[GGCA]7
22 TI	21 TI.JZ	0.021792	[GGAA]15[GGCA]7

Sequence Based Stutter

R**2 0.000

- Plotting by LUS rather than allele number improves slope slightly
- Still not ideal
    - SLUS stutter appears consistently lower
    - R^2 went down



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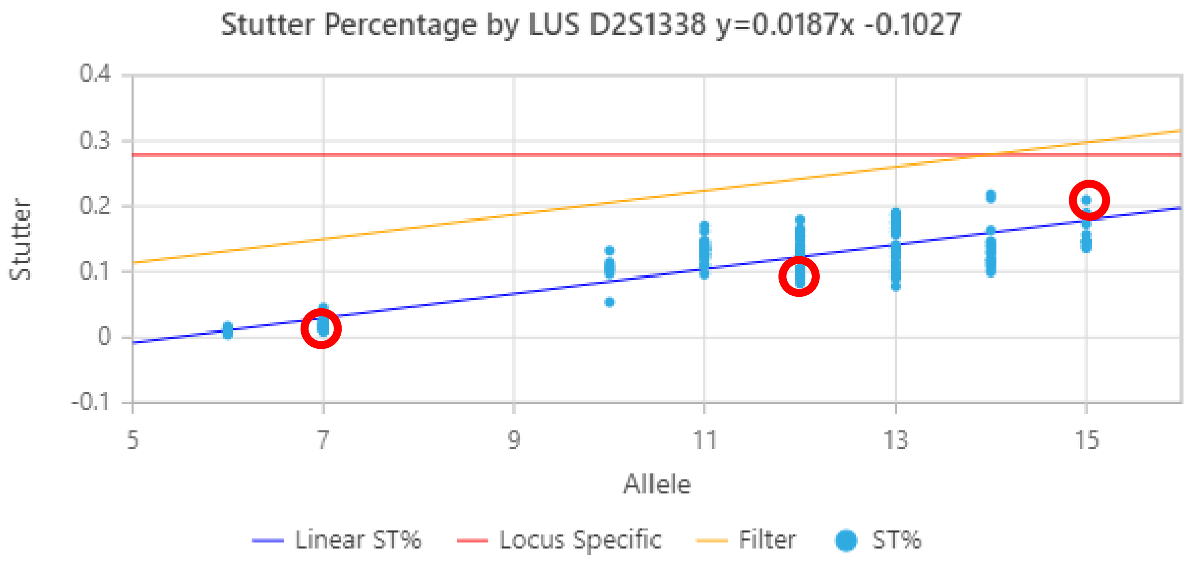
Sequence Based Stutter

R**2 0.795

- Plotting each stutter ratio by the number of the stuttering repeat unit produced the best linear fit



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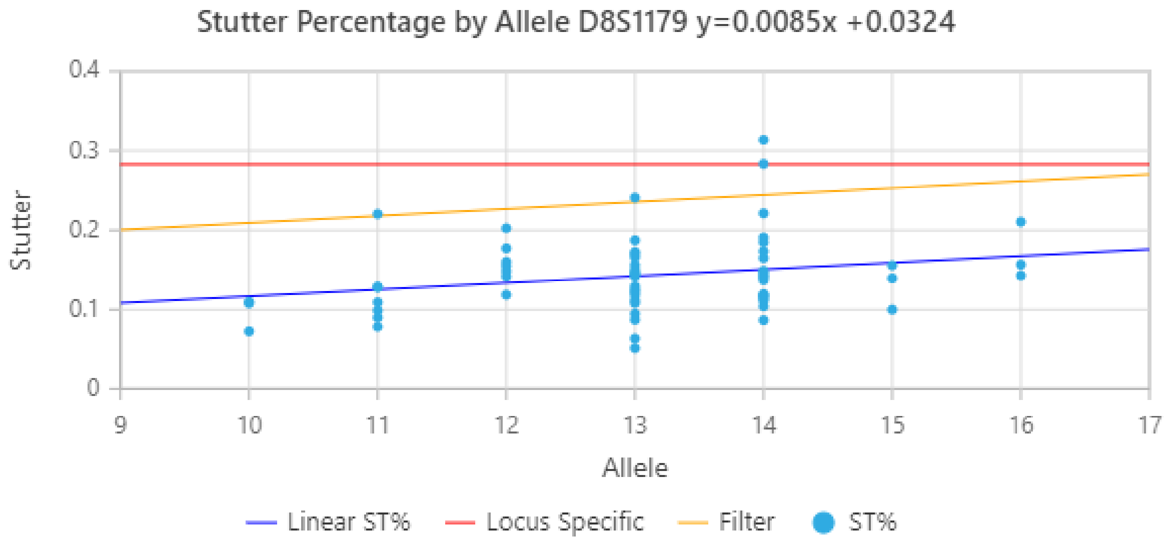
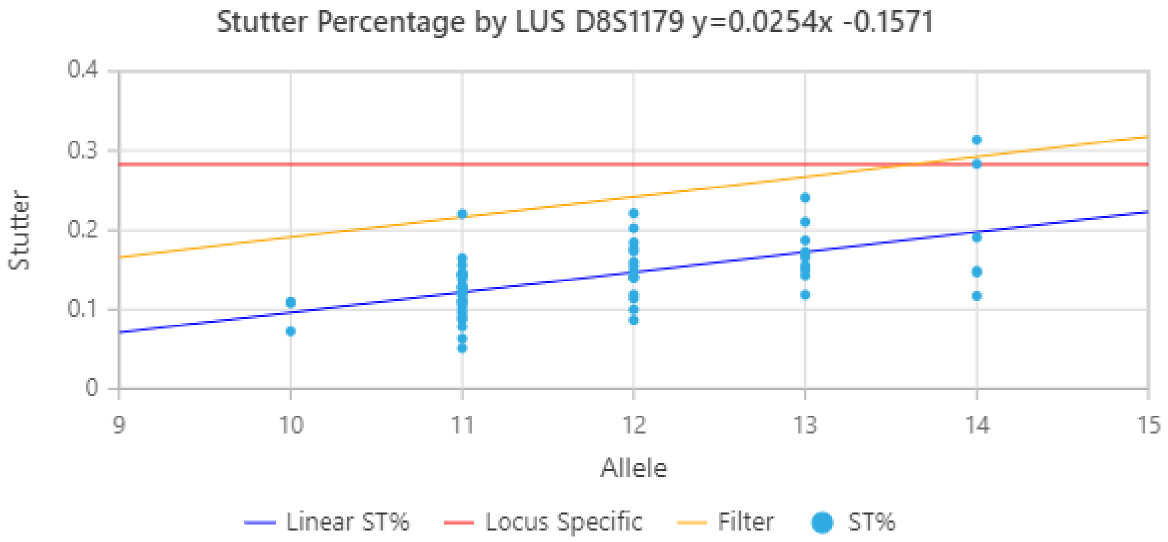
Allele vs Sequence

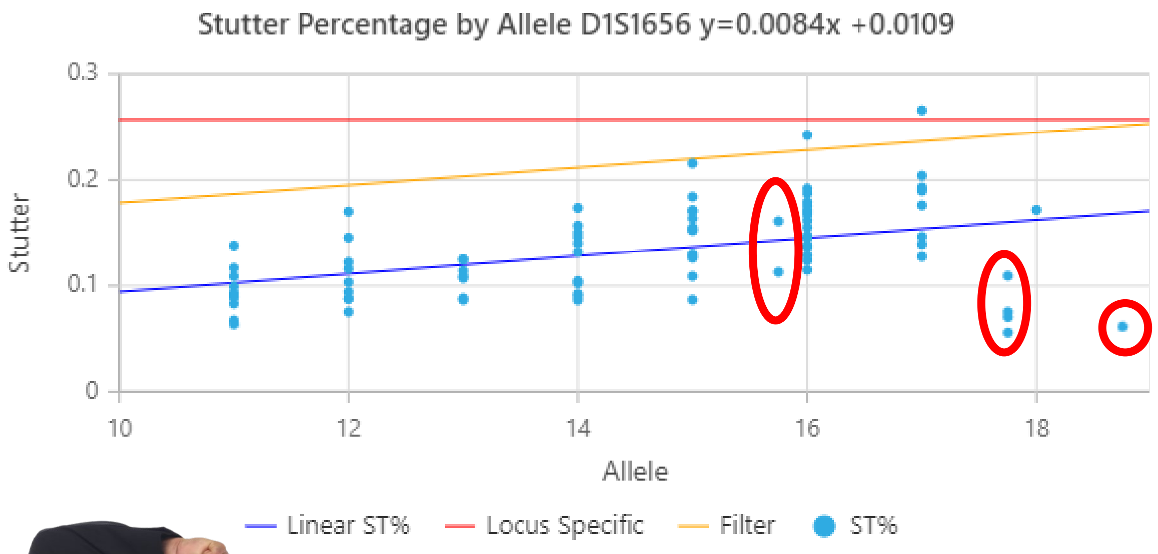

Stutter Percentage by Allele D8S1179 y=0.0085x +0.0324

R**2 0.065

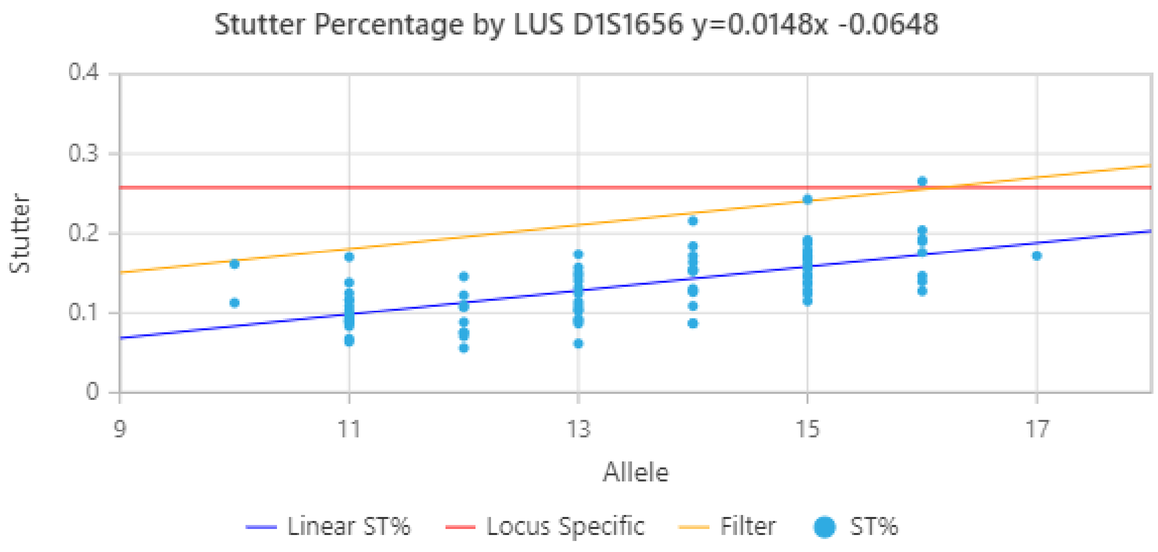

Stutter Percentage by LUS D8S1179 y=0.0254x -0.1571

R**2 0.337

Allele vs Sequence

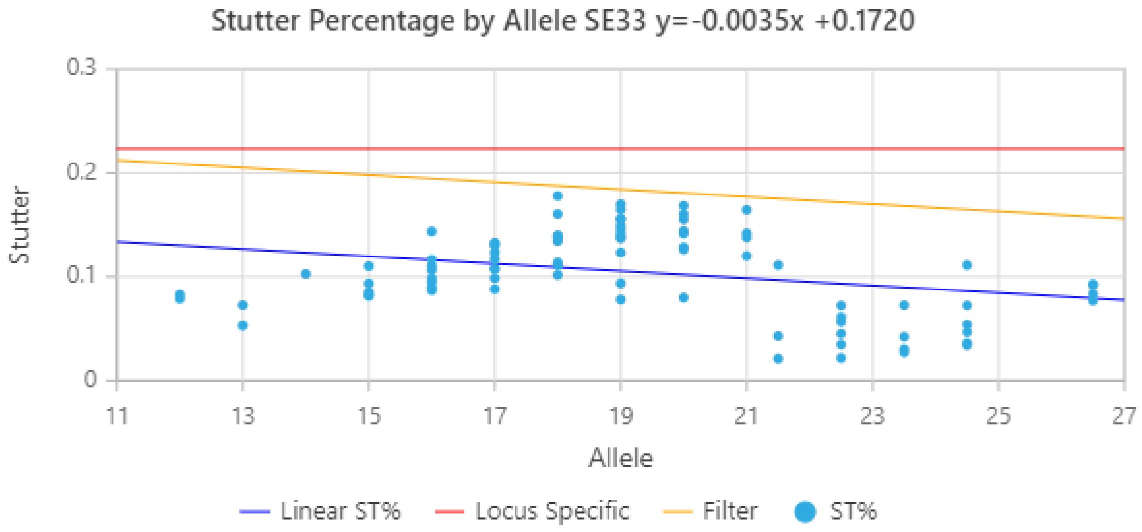


R**2 0.182



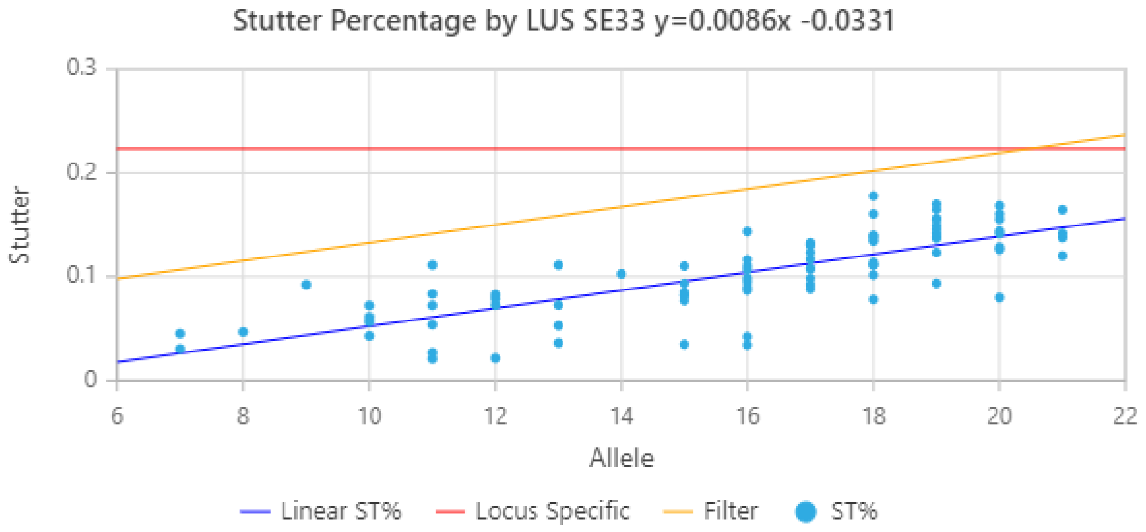
R**2 0.397

Allele vs Sequence



Stutter Percentage by Allele SE33 y=-0.0035x +0.1720

R**2 0.091

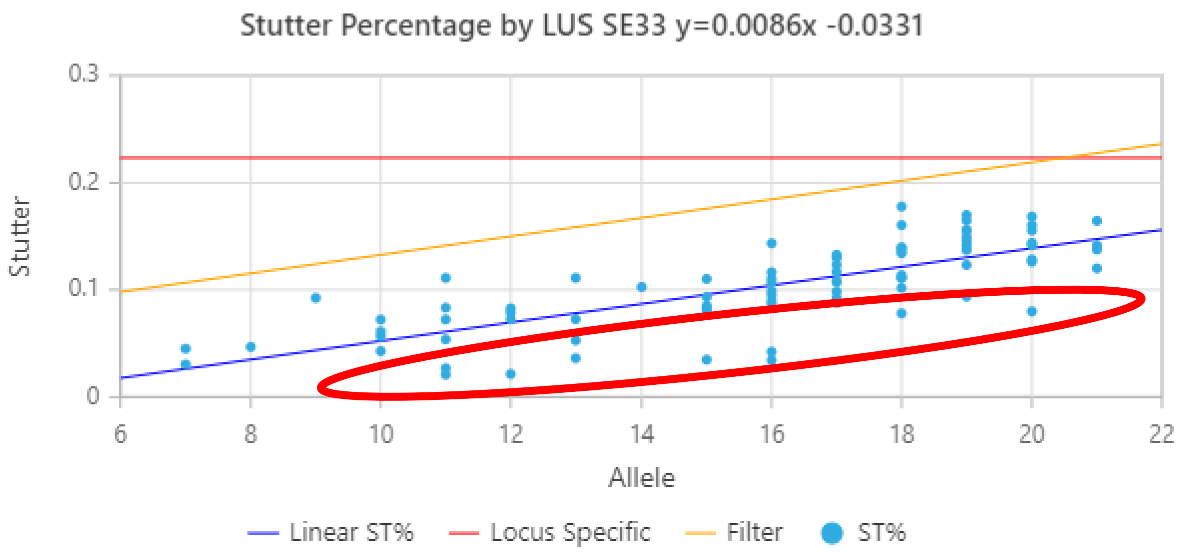


Stutter Percentage by LUS SE33 y=0.0086x -0.0331

R**2 0.588

Additional Considerations

- Small insertion sequences within the STR seem to have further impact on stutter ratio
- SE33 has many ".2" alleles which appear to reduce stutter rates



Allele	Stutter Ratio	Sequence	LUS
16 WD	0.143411	[CTTT]16	16
23.2 PK	0.042157	[CTTT]16TT[CTTT]7	16

R**2 0.506

Final Thoughts

- Plotting stutter by repeat unit rather than allele length improves linearity at many complex loci
- There do appear to be more factors on stutter ratio that will require further study
    - Small insertions within the STR
- Effective stutter validation requires as many observed alleles and stutter as possible
- There is still work to do to make this model and validation tools implementable

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Questions?

Damani Johnson

dtj012@shsu.edu