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A novel and comprehensive testing strategy to identify the genetic etiology of neonatal hypotonia phenotypes

Jasmine A. McQuerry
Children's Mercy Hospital

Margaret Gibson
Children's Mercy Hospital

Scott T. Younger
Children's Mercy Hospital

T Pastinen
Children's Mercy Hospital

Emily G. Farrow
Children's Mercy Hospital

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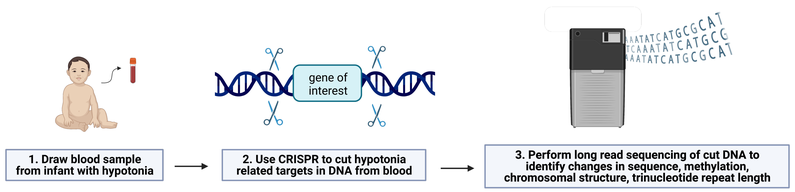
McQuerry, Jasmine A.[1,2]; Gibson, Margaret I.[1,2]; Younger, Scott T.[1,2,3,4]; Pastinen, Tomi [1,2,3,5]; and Farrow, Emily G. [1,2,3]

[1]Genomic Medicine Center, Children's Mercy Kansas City [2]Children's Mercy Research Institute [3]Dept. of Pediatrics, UMKC School of Medicine [4]Dept. of Pediatrics, KU Medical Center [5]Dept. of Cancer Biology, KU Medical Center

Background

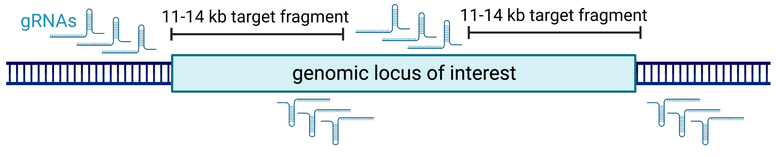
- Neonatal hypotonia (NH) is a non-specific symptom common to a wide variety of congenital disorders.
- The NH phenotype is associated with disorders diverse in genetic etiology including chromosomal aberrations, copy number variation (CNVs), methylation changes, trinucleotide repeat expansions, and single nucleotide variation. Evaluation of these changes currently requires multiple laboratory testing modalities.
- We developed an amplification-free testing approach that uses CRISPR/Cas9 to isolate multiplexed genomic loci associated with NH for native HiFi sequencing using the PacBio Sequel IIe.



Novel comprehensive testing strategy for neonatal hypotonia disorders leverages CRISPR/Cas9 to generate targeted sequencing libraries of native genomic DNA from patient blood and identifies changes using long read/HiFi sequencing.

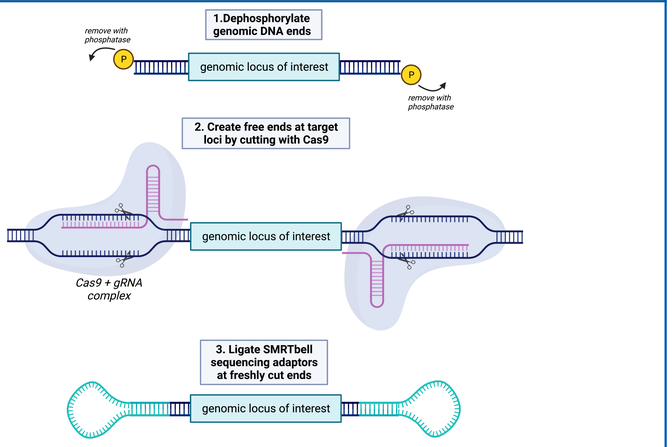
Methods: guide RNA selection

- Target list of 116 NH related loci curated from clinical cases: genes (*GSS*, *TNNT3*, et al.), chromosome regions (Prader-Willi region chr15q11-q13), and trinucleotide repeat expansions (in *ATXN1*, et al.).
- Large loci (genes, chromosome regions) were divided into smaller sections of 11-14 kb in length.
- 3 guide RNAs (gRNAs) were selected up and downstream of each target fragment to maximize cutting efficiency.
- 116 targets divided into 1050 fragments X 6 gRNAs per fragment = 6300 total gRNAs.
- The panel of 6300 gRNAs was transcribed *in vitro* from a DNA oligo pool.



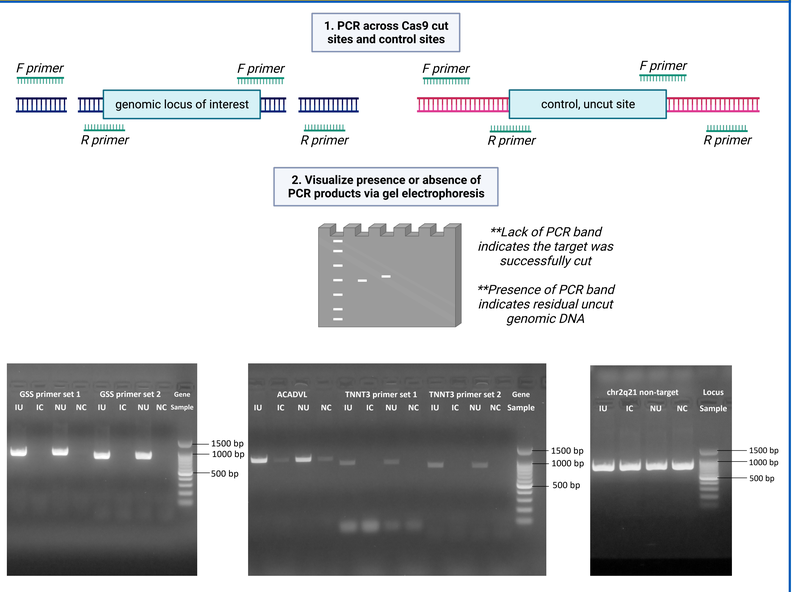
Three guide RNAs were designed up and downstream of each genomic locus with larger loci broken into multiple 11-14 kb fragments. *Note: figure not drawn to scale.*

Methods: Cas9 digestion and library preparation



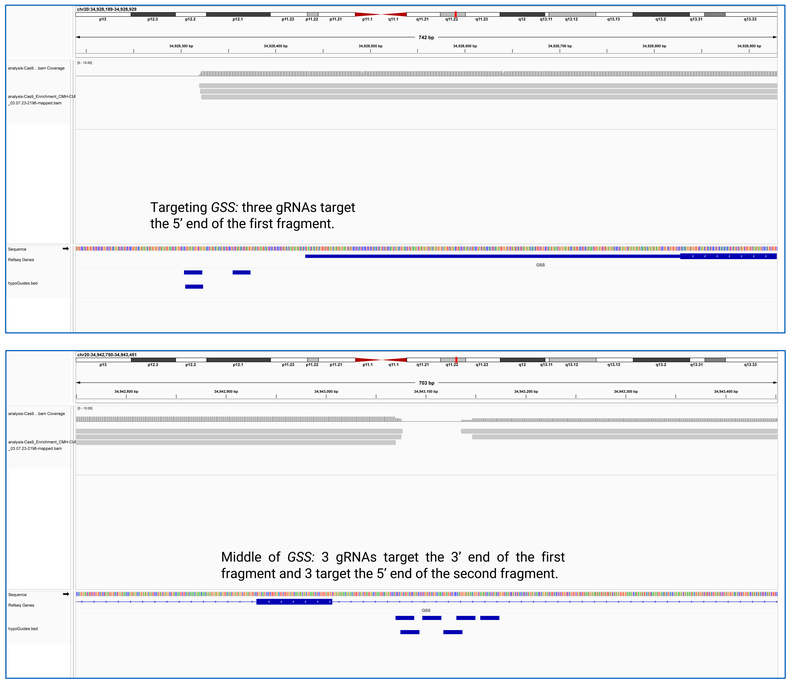
Preparation of sequencing libraries. Following dephosphorylation, patient genomic DNA is subjected to *in vitro* digestion with Cas9 and the panel of 6300 gRNAs. After cleanup, SMRTbell adapters are ligated to freshly cut ends for amplification-free HiFi sequencing on PacBio Sequel IIe.

Results: PCR validation of Cas9 cuts



Cas9 cuts patient genomic DNA at target loci. PCRs across cut sites in *GSS*, *ACADVL*, and *TNNT3* reveal a lack of amplicon in cut samples vs. uncut samples. PCR across a control non-target locus on chr2q21 reveals amplification in all samples, both cut and uncut. *IC*: sample digested with Cas9 from Intact Genomics, *NC*: sample digested with Cas9 from NEB. *IU*: uncut control digest (no gRNAs added) in Intact Genomics buffer, *NU*: uncut control digest in NEB buffer.

Results: HiFi sequencing



HiFi sequencing reads mapped to the GSS gene begin at the Cas9 cut site located 3 bases upstream of the Cas9 protospacer adjacent motif (PAM), the "NGG" sequence directly adjacent to gRNAs which is required for Cas9 cutting. The lower track ("hypoGuides.bed") in each panel depicts locations of gRNAs. The Refseq gene track shows the GSS gene location.

Conclusions

- When complexed with Cas9, a highly multiplexed panel of 6300 gRNAs cuts at on-target NH loci and not the off-target loci tested, as evidenced by PCR.
- Reads from PacBio HiFi sequencing begin, as expected, at the Cas9 cut site 3 bases upstream of the Cas9 protospacer adjacent motif (PAM).
- Future work will address optimization of library recovery to improve coverage across target loci and reduction of the gRNA library to the most efficient guides at each locus.
- This method can be adapted to make targeted sequencing libraries for any desired set of target loci.

Acknowledgements

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- Figures were drawn using BioRender.

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