Project Title: DNA Typing Strategies via Real-Time Nanopore Sequencing for Forensic Analyses

Recipient Organization: UNT Health Fort Worth
3500 Camp Bowie Blvd., Fort Worth, TX 76107

PI: Roxanne R. Zascavage, Ph.D.
Associate Professor
Roxanne.zascavage@unthsc.edu
817-735-5016

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PROJECT SUMMARY

Abstract

Human remains are frequently encountered in forensic laboratories, coming from crime scenes, mass graves, historical samples, mass disasters, and military conflicts. The problem faced by forensic laboratories when analyzing challenged samples of human remains is they must choose between depleting sample volumes repeating individualizing STR analysis or performing costly, time-consuming, and less discriminatory mtDNA analysis. New DNA sequencing methodologies may provide a more effective platform for identification of human remains that overcome the most common challenges associated with processing of bone fragments, aged tissue, and hair samples. While the gold standard for forensic DNA identification is analysis of autosomal STR loci, skeletal remains have often been exposed to environments that trigger degradation of the nuclear DNA and introduce PCR inhibitors. mtDNA can often be extracted and amplified in samples where nuclear DNA analyses fail. However, mtDNA is not individualizing because each person shares their mtDNA with all their maternal relatives. Another potential alternative method is single nucleotide polymorphism (SNP) profiling; SNPs are present throughout the genome and account for 85% of genetic variability observed between individuals. Since SNP genotyping involves testing for variation of at individual nucleotides rather than longer strings of DNA (as with STRs), they are ideal for low template and/or degraded DNA samples; SNP loci panels can provide discriminatory power necessary for human identification. This project aimed to explore alternative sequencing approaches via nanopore sequencing for processes that could potentially interrogate STRs, mtDNA, and SNPs simultaneously. In order to do this, we first updated our software pipeline, STRspy 2.0 to be compatible with the current naming standards and evaluated its applicability on mock casework samples. We then

Goals and Objectives

The primary objective of this project was to develop a comprehensive DNA-based approach to forensic human identification that interrogates standard STR and extensive SNP panels as well as the entire mitochondrial genome using ONT sequencing.

To address the primary objective of this project, we focused on the following specific aims:

Specific Aim 1: Evaluate the effectiveness of rapid, whole genome sequencing for human identification in forensic investigations using robust and challenged samples.

Goal 1.1: Optimize our library preparation methods for PCR-free sequencing of forensic samples.
Sequencing protocols for both Oxford Nanopore (ONT) and Illumina require multiple SPRI-bead clean up steps. Each clean up step results in a loss of DNA, which is significant when working with forensic samples that are limited in quantity. Optimization of the bead clean up process to retain the maximum amount of DNA is essential for success of downstream sequencing of forensic samples.

Goal 1.2: Update STRspy, our custom DNA analysis software to be compatible with all targeted loci and up to date with current nomenclature standards.
STRspy was designed to extract autosomal STR-based data from ONT reads. It was originally tested on control DNA and has yet to be proven reliable for Y-STRs or on casework-type samples, both of which are

essential for utilization of ONT sequencing in forensic settings. Furthermore, the NCBI Bioproject updated all allele calls to match the new recommendations put out by the DNA Commission of the International Society of Forensic Genetics. Errors in allele calls can arise due to inconsistencies in nomenclature, and this needs to be addressed.

Goal 1.3: Test our library preparation method and software on DNA sequences generated using ONT sequencing platforms without any PCR amplification.

Bypassing PCR in forensic analyses will allow for the simultaneous analysis of multiple targets, including mitochondrial DNA, STRs, and SNPs. This process will allow for the most efficient use of forensic samples.

Research question: Can Oxford Nanopore sequencing be utilized to effectively interrogate DNA samples for human identification without the use of PCR?

Specific Aims 2 and 3: Develop a comprehensive probe-capture approach to enrich DNA samples for forensically relevant loci and evaluate its effectiveness on robust and challenged forensic samples.

Research question: Can RNA baits effectively enrich challenged DNA samples for human identification?

Summary of Project Design and Methods

DNA Samples

Control DNAs. Original testing was conducted using 3 NIST traceable standards and the Promega 2800M control (female n = 1; male n = 3) with manufacturer-validated CE and NGS STR profiles.

Fresh Robust Forensic Samples. Blood, soft tissue, and bone samples from seven individuals (female: n=2; male: n=5) were utilized in this project. Samples were obtained from the Donated Body Program at the University of North Texas Health Science Center. Bone was provided as a cross section cutting of approximately 6 inches from an intact femur. Soft tissue was provided as a cutting of approximately 6 square inches from thigh muscle.

Environmental Degradation of Forensic Samples. Tissue samples were cut into four pieces with each sample having a piece in a different exposure: UV, water, soil, and none. Cuttings of each piece in water, UV, or soil were collected on days 3, 10, and 20. Bone samples were degraded through exposure to environmental elements (UV, fresh water, and soil) for a period of up to 20 days. Samples were collected on days 3, 10, and 20, as outlined in Table 1. Degradation was assessed via the QuantTrio kit (Promega) on a subset of six samples and confirmed with a DI above 1.0.

Table 1. Bone exposures to create challenged samples. Bone samples were cut into two or three pieces, depending on the initial size of the bone and exposed to different elements: UV, water, soil, or none. Bone was collected on days 3, 10, or 20, as denoted in the table.

	BONE EXPOSURE (collection day)			
Sample ID	NONE	UV	WATER	SOIL
91845	Day 0	Day 20		
91846				Day 3, Day 20
91847		Day 10		Day 10
91855	Day 0		Day 20	
91895		Day 3	Day 10	
92300	Day 0		Day 3, Day 20	
92301		Day 3		Day 10

Mock-Casework Samples. DNA extracts from six human bone samples (female n = 1; male n = 5) and 16 buccal swab samples (female: n = 6; male: n = 10) were provided from the Center for Human Identification at the University of North Texas Health Science Center and utilized as mock-casework samples in the improvement of STRspy2.0. 19 whole blood samples (female: n = 10; male: n = 9) were selected at random from the PRECISION Pain Research Biobank for utilization in the studies as well.

DNA Extraction. Blood and tissue samples were extracted using the DNeasy Blood & Tissue Kit (Qiagen) spin protocol according to manufacturer's suggestions. DNA was extracted from buccal swab samples according to the QIAamp DNA Mini Blood Kit (Qiagen) spin protocol with the optional centrifugation step at full speed before elution in 50 µL of buffer AE (Qiagen). Bone samples from CHI were extracted using a Demineralization Extraction of Skeletal Remains protocol. Fresh bone samples were pulverized into a powder and DNA extracted using the PrepFiler BTA Forensic DNA extraction Kit (Applied Biosystems).

Specific Aim 1

Goal 1.1. As outlined in Figure 1: DNA was extracted using the Qiagen DNA Investigator Kit following a modified protocol from four whole blood samples. In brief, absolute ethanol was added to the lysate flowthrough which was processed as an additional sample following the manufacturer's guidelines. Extractions were split into six groups. The first four groups (blue boxes) were diluted to 1µg or 500ng. For the last two groups (purple boxes), each sample was pooled with its associated flowthrough and split into fourths. Each group was subjected to a 1.0X AMPure XP bead-clean up following one of six protocols: Beckman-Coulter, ONT Modified, DeepSeq, HMW Ohio, ONT Mod 70, and ONT Mod Shaking. Concentration (ng/µL) was used to calculate the absolute mass remaining (ng) and percent of DNA retained (%).

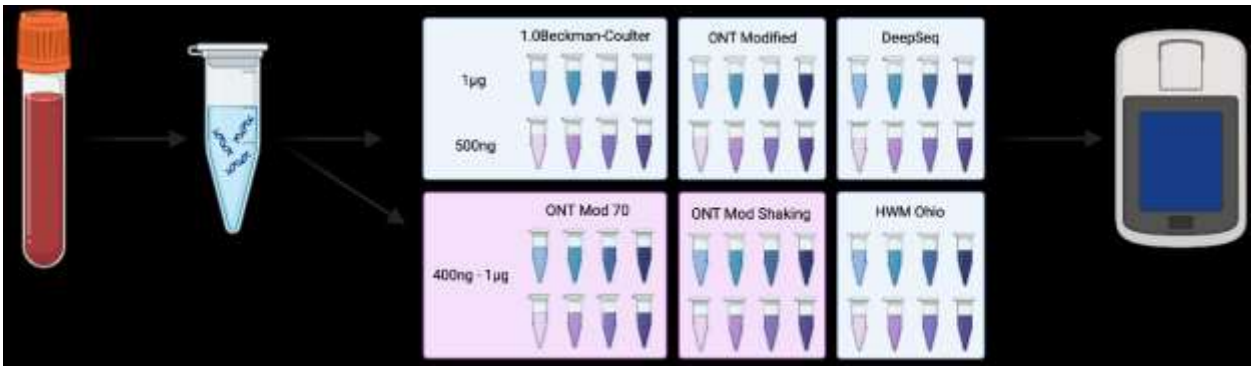

Figure 1. Figure created in BioRender.

Goal 1.2. The 22 autosomal and 23 Y-STRs in the PowerSeq 46GY System were amplified for ONT sequencing using 0.5ng of DNA. STR libraries were prepared using the ONT Ligation Sequencing Kit (SQK-LSK109) with Native Barcoding Expansions 1-12 (EXP-NBD104) and 13-24 (EXP-NBD114) as per the modifications described in Hall et al. [17]. Prepared sequencing libraries were loaded onto primed Min-ION vR9.4D flowcells (FLO-MIN106D, ONT) and sequenced on the MinION device for 72hrs with the MinKNOW control software. Sequence results were processed with the STRspy2.0 command line interface. We assessed concordance between STRspy2.0 predictions and known allele designations, or ground truth profiles, generated using a combination of CODIS-validated CE and NGS methods. These counts were used to calculate the precision, recall, and F1-score of our updated method. The overall performance of STRspy2.0 for autosomal and Y-STRs was evaluated based on F1-score (harmonic mean of precision and recall).

Goal 1.3. Single source DNA samples were prepared for sequencing on the PromethION2 Integrated by ONT using a modified protocol for the Ligation Sequencing Kit gDNA – 24 Native Barcodes (SQK-NBD114.24). Targeted sequencing was performed for 72 hours using adaptive sampling. Our regions of interest included 71 STRs, over 5400 SNPs, mtDNA, and age-related CpG sites. All genomic coordinates for our targeted regions of interest were provided in a BED File, except for mtDNA. We assessed coverage across our targets of interest. Results were evaluated via STRspy2.0 for precision and recall as well.

Specific Aims 2 and 3. RNA-probe assays that target forensically relevant DNA loci were selected and developed in conjunction with Arbor Biosciences. The manufacturer's probe-capture protocol was then combined with either Illumina sequencing or ONT sequencing protocols. Starting sample input was 500pg of DNA. Results were assessed for coverage at the target regions.

Summary of Results and Limitations

Specific Aim 1

Goal 1.1. Among four modified protocols, the Beckman-Coulter manufacturer's protocol retained the highest yields consistently (Figure 2). The DeepSeq protocol also performed well. When comparing the four protocols there were two major differences observed in the DeepSeq and Beckman Coulter protocols that were not observed in the two with poor performance; 70% ethanol as opposed to 80% and consistent shaking of the samples during incubation and elution.

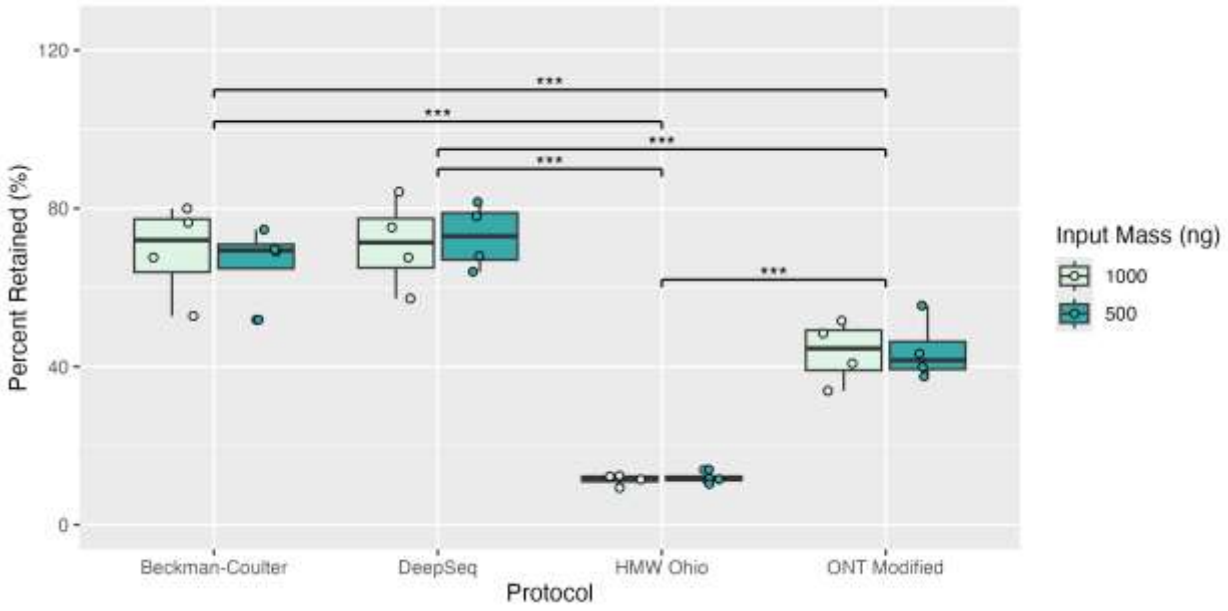

Figure 2. Manufacturer's protocol retains higher yields more consistently than modified protocols. Statistical differences in the percent retained were determined via a two-way, fixed-effects ANOVA using protocol and input mass (1µg or 500ng) as factors followed by a Tukey's Honest Significant Difference test. Statistics were completed in R 4.5.2 and R Studio (v2025.09.2+418). Figures were generated in ggplot2 (v4.5.2). *** p-value < 0.001.

Both adjustments were then made to the protocol suggested by ONT to see if either significantly improved yields. As seen in Figure 3, shaking the samples significantly improved yields, but not above the level seen in the original Beckman-Coulter protocol. Swapping to 70% ethanol increased yields in some samples, but not others.

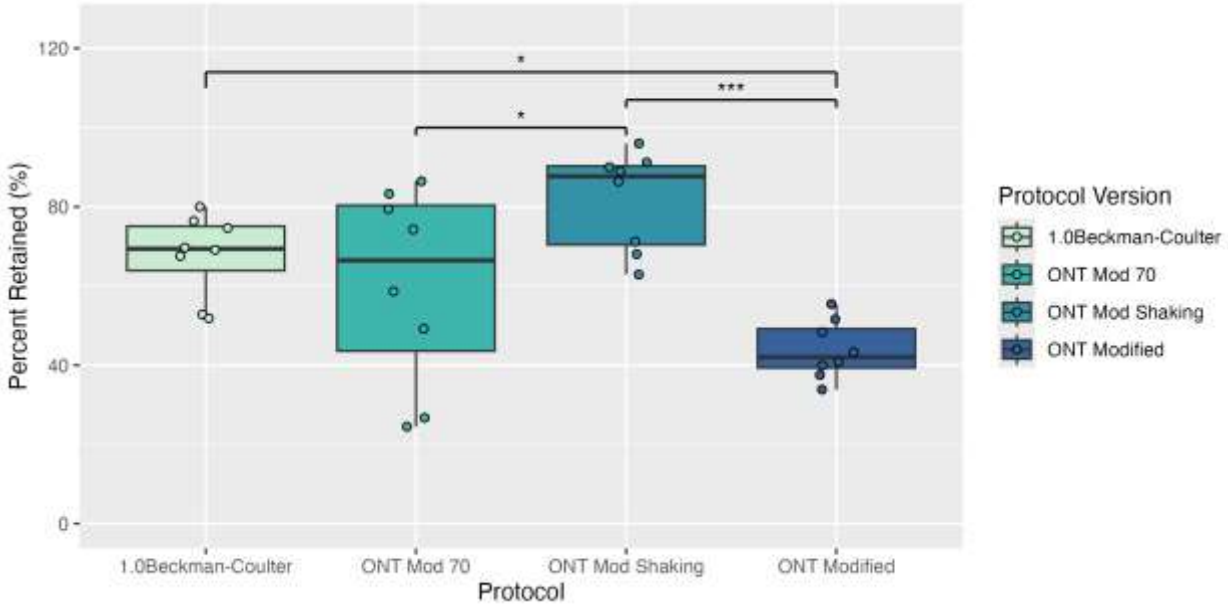

Figure 3. Shaking improves yield of ONT Modified protocol but does not provide higher yields than

manufacturer's protocol. Statistical differences in the percent retained were determined via a one-way ANOVA followed by a Tukey's Honest Significant Difference test. Statistics were completed in R 4.5.2 and R Studio (v2025.09.2+418). Figures were generated in ggplot2 (v4.5.2). * p-value < 0.05. *** p-value < 0.001.

Based on these results, and the fact that the protocol set out by Beckman Coulter is the least time consuming, it was concluded that the manufacturer's protocol should be implemented in all future library preparations.

Goal 1.1 Limitations and Future Directions. These results only compared four protocols using a single consistent input type and quality of DNA. Future analyses should be tested on various sample types and qualities, especially as degraded DNA is likely to lose more in the clean-up process. Future plans also include exploring how common modifications alter DNA binding to SPRI beads and SPRI bead integrity, ascertaining which step in standard SPRI protocols leads to the most significant loss of DNA, and comparing yields of other SPRI technologies like spin columns with bead-based clean-ups. These studies will assist with establishing the ideal protocol for DNA sequencing library preparations when using precious forensic samples.

Goal 1.2. STRspy 2.0 successfully called all alleles and isoalleles in the control DNA samples. Out of 1972 allele calls in the mock casework samples, 1962 were correctly called and 10 were incorrectly identified. Of those 10, two were false negatives – drop out of alleles – and the other 7 were false positives or miscalled alleles (Table 2).

Table 2. STRspy2.0 benchmarking results for control and casework samples. Numbers of correct (true positives, TP), incorrect (false positives, FP), and missed (false negatives, FN) predictions for the control multiplexes (NIST A, NIST B, NIST C, 2800M) and 41 casework-relevant samples (blood, buccal swab, bone) when compared to length-based reference profiles. Recall, precision, and F1-scores are reported as percentages for autosomal and Y-STRs. Y-STRs in the bone samples were not assessed due to a lack of length-based reference profiles.

	Autosomal STRs						**Y-STRs**					
Dataset	TP	FP	FN	Recall	Precision	F1-score	TP	FP	FN	Recall	Precision	F1-score
Control multiplexes												
12-sample	528	0	0	100%	100%	100%	207	0	0	100%	100%	100%
18-sample	792	0	0	100%	100%	100%	299	0	0	100%	100%	100%
24-sample	1056	0	0	100%	100%	100%	404	0	0	100%	100%	100%
Overall	**2376**	**0**	**0**	**100%**	**100%**	**100%**	910	**0**	**0**	**100%**	**100%**	**100%**
Mock casework												
Blood	798	0	1	99.9%	100%	99.9%	166	1	0	100%	99.4%	99.7%
Swab	636	3	1	99.8%	99.53%	99.7%	154	4	0	100%	97.5%	98.7%
Bone	208	0	0	100%	100%	100%	-	-	-	-	-	-
Overall	**1642**	**3**	**2**	**99.9%**	**99.82%**	**99.9%**	**320**	**5**	**0**	**100%**	**98.5%**	**99.2%**

Evaluation of the 10 errors is outlined in Table 3. No two errors were at the same locus. However, 7 of the 10 errors were in swab samples and the other two were in blood samples. No errors were identified in bone extracts.

Table 3. STRspy2.0 genotyping errors in casework samples. Raw and normalized read counts (raw/norm) for the 10 incorrect allele calls observed in blood and swab profiles. Dashes indicate no allele/homozyote identified and bolded alleles denote discrepancies from the ground truth. Read counts displayed represent STRspy calls for the given allele.

Error type	Sample	Locus	Ground truth (raw/norm)	STRspy2.0 calls (raw/norm)
False negative	blood14	Penta D	5 **2.2 (0/0)**	5 **–**
	swab15	vWA	17 **16 (2360/0.2)**	17 –
False positive	swab10	D18S51	16 –	16 **15 (2,858/0.5)**
	swab12	D8S1179	15 **12 (26/0.002)**	15 **17 (10,791/0.9)**
	swab15	FGA	25 –	25 **24 (5,161/0.4)**
	swab02	DYS385ab	**11 (90/0.1)** –	**12 (937/1.0)** **13 (550/0.6)**
	swab14	DYS458	**15.1 (0/0)**	**15 (10,175/1.0)**
	blood07	DYS390	**23 (2,112/0.7)**	**22 (2,865/1.0)**
	swab16	DYS448	**19 (323/0.06)**	**20 (5,274/1.0)**

Goal 1.2 Limitations and Future Directions. While mock casework samples were utilized in this experiment, they were still high quality. STRspy2.0 still needs to be tested on truly challenged samples.

Goal 1.3. Adaptive sampling of our samples showed significant enrichment of on-target regions compared to off-target regions. Autosomal and Y-chromosome targets showed an enrichment of ~285x compared to the off-target regions of the genome. The mitochondrial DNA was not directly targeted, but still yielded over 60x coverage (Figure 4).

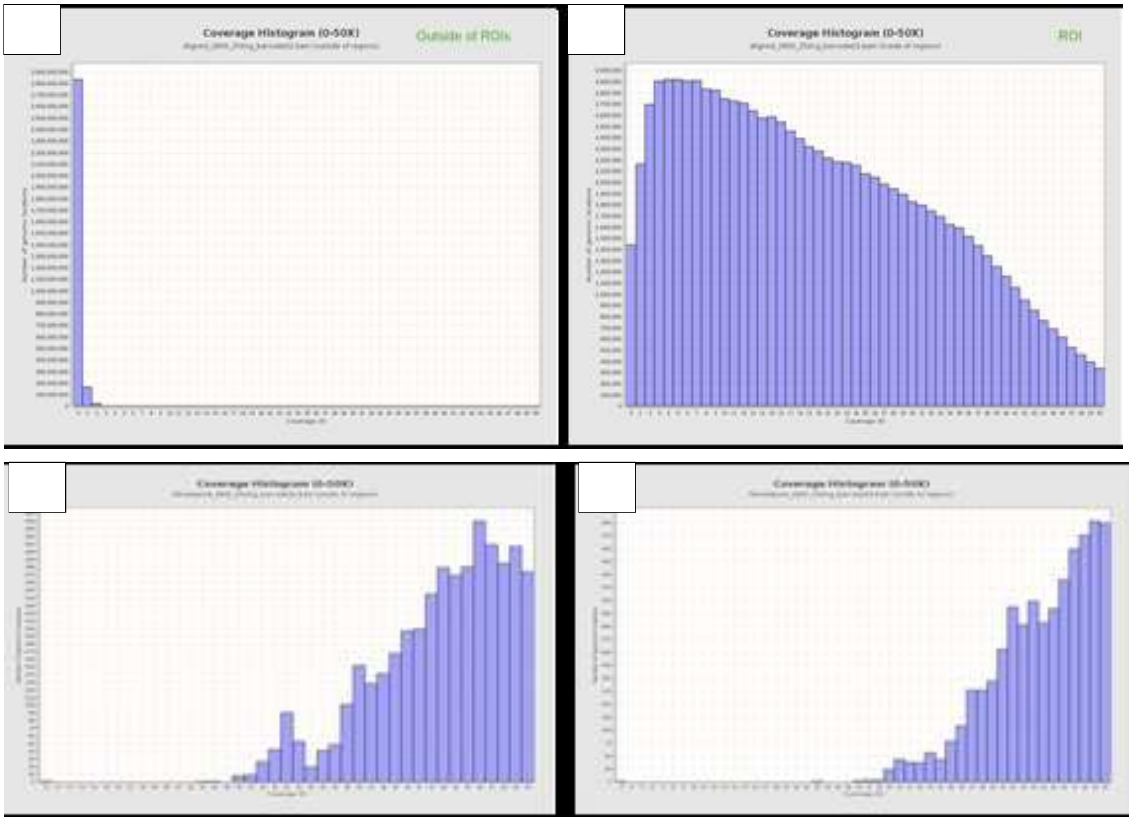

Figure 4. Coverage across regions following adaptive sampling. Genomic coordinates of 71 STRs and over 5400 SNPs were provided in a BED file for the purpose of targeted sequencing via adaptive sampling on the ONT PromethION2 Integrated. Raw reads were basecalled using Dorado and aligned to hg38 using minimap2. Coverage over the regions of interest (ROIs) and off-target regions was calculated using Qualimap (v2.3). We obtained approximately 20x coverage in our ROIs (panel B) compared to approximately 0.07x coverage in off-target regions (panel A). Over 60x coverage across the mitochondrial genome was consistently recorded as well (Panels C and D).

Control sample 2800M with a starting input of 250ng obtained a mean coverage of 20x. At this coverage, STRspy was able to accurately call autosomal and Y-STRs although it was not perfect; precision for the duplicates was 97.7%/93.5%, recall was 89.4%/95.6%, and F1 was 93.3%/94.5%. The results for 2800M with a starting input of only 90ng were very similar – 97.6% precision, 87.2% recall, and 92.1% F1., despite only having a mean coverage of 7x. These results suggest that additional input DNA is not the limiting factor for this method (Figure 5).

Unfortunately, the same methods did not work well for challenged tissues when input DNA was limited. Library preparation began with only 35ng of DNA per samples. In this case, precision was only 45.5%, recall was 27.8%, and the combined F score was 34.5% (Figure 5). Clearly, the quality and quantity of DNA input is important, and perhaps we have found our current minimum is going to be around 90ng of starting material.

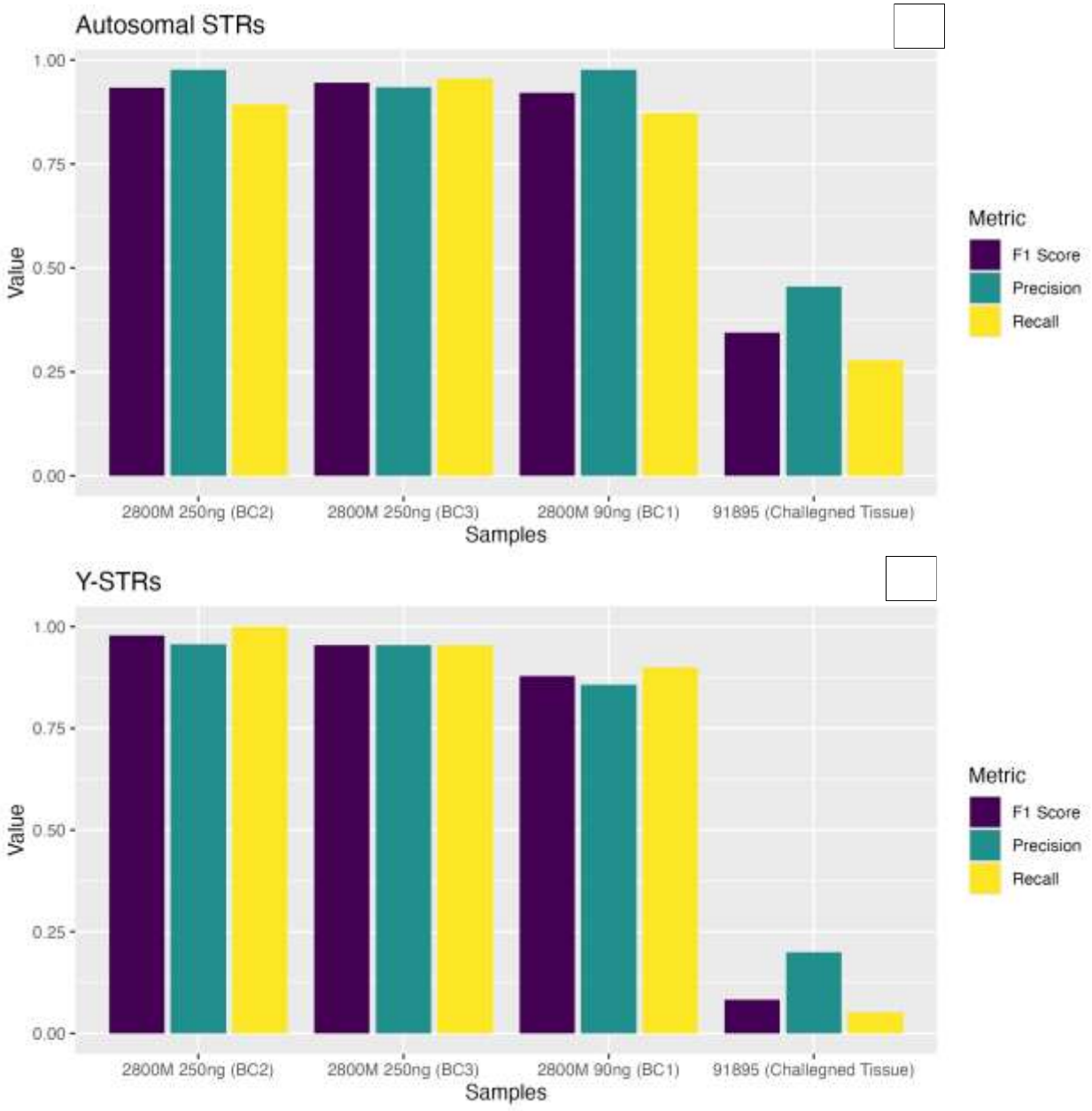

Figure 5. **Precision, recall, and F1 scores following adaptive sampling.** Sequencing was performed for 72 hours on the ONT PromethION2 Integrated following preparation using our modified protocol. Raw reads were basecalled using Dorado, aligned to the human reference genome, and then called using STRspy. The input for the challenged tissue was 35ng. These results indicate that our methods are not appropriate for challenged samples, and therefore needs to be adjusted.

Goal 1.3 Limitations and Future Directions. We understand that in this instance, our dropout rate is too high, which is skewing our results, and we need to work on improving this metric. Interestingly, when recall increased, precision went down by 5%, and in forensics, we would prefer a dropout of an allele over an incorrect call. Furthermore, we can see that when we were only able to start with 35ng of DNA, the quality of results declined. This is much more in line with what would be available in a crime lab in the

best of cases. Ideally, 500pg of DNA or less would be needed for analysis. This further validates the need to improve the library preparation process to retain as much DNA as possible throughout the protocol. Low pass data analysis methods are likely going to need to be implemented for adaptive sampling to work successfully.

Specific Aims 2 and 3.
Results. The average coverage across the autosomal regions per sample was 410.3x with a range from 230.1x – 838.9x). The average coverage across the Y-target regions per sample was 172.6x with a range from 170.3x – 174.8x). These results show that our assay and protocol design are working and that the input amount of DNA can be reduced below 500pg.

Limitations and Future Directions. Sample types were limited and the baiting protocol is labor intensive. Future evaluations will work towards identifying the lower threshold of DNA input, reducing the protocol steps, and evaluating on additional sample types.

Applicability to Criminal Justice

As the first and only third generation sequencing platform-specific method to successfully profile the entire panel of autosomal STRs amplified by a commercially available multiplex, STRspy2.0 significantly increases the feasibility of nanopore sequencing in forensic applications. However, not only is a commercially available STR-kit limited in the type and quantity of data it collects, but not all samples are ideal candidates for PCR amplification. The ability to multiplex types of common forensic targets – STRs, SNPs, mtDNA – simultaneously using varied approaches allows for capture of the most amount of valuable data possible in a sample, allowing forensic laboratories to more efficiently utilize and more successfully identify their limited and challenged samples.

PRODUCTS

Peer Reviewed Publications

1. Hall CL, Kesharwani RK, McBroom Henson KE, Kapema B, Phillips NR, Sedlazeck FJ, Zascavage RR. STRspy2.0: Unlocking the Potential of Long Reads for Forensic DNA Profiling. Int J Mol Sci. 2026 Feb 16;27(4):1889. doi: 10.3390/ijms27041889. PMID: 41752025; PMCID: PMC12940929.

Book Chapters

1. Kapema, B., McBroom, K., Mello, I., and **Zascavage, R.R**. *Lineage markers and their application in Forensic DNA Analysis*. in Advancements in Forensic Biology and Genetics (Publish date: July 2024, Hirak R. Dash, Kelly M. Elkins, and Nora Rashid Al-Snan, eds.). Springer Nature – Humana, New York, NY doi: 10.1007/978-981-96-4585-5_17.

Software

1. STRspy: https://github.com/unique379r/strspy
2. STRspy 2.0: https://github.com/unique379r/strspy/tree/STRspy2.0

Archived Research Data

1. BioProject: PRJNA757759
2. BioProject: PRJNA1248048 SRA: SUB14608125

Conference Presentations

1. **Kesharwani, R.,** Hall, C. L., Phillips, N. R., Planz, J. V., Sedlazeck, F., & Zascavage, R. R. *STRspy-ing Hidden Variation in Forensic DNA Profiles with the MinION.* June 2023. Sequencing, Finishing, & Analysis in the Future Meeting. Santa Fe, New Mexico. (oral presentation)
2. Hall, C. L., Kesharwani, R., Phillips, N. R., Planz, J. V., Sedlazeck, F., & **Zascavage, R. R**. *The Latest on Nanopore Sequencing in Human Identification*. July 2023. Green Mountain DNA Conference, Burlington, Vermont. (Invited oral presentation)
3. **Rupesh K. Kesharwan**, Courtney L. Hall, Bupe Kapema, Nicole R. Phillips, Fritz J. Sedlazeck, Roxanne R. Zascavage. *STRspy 2.0: Unlocking the Potential of Long Reads for Forensic DNA Profiles*. December 2023. Nanopore Community Meeting, Houston, TX. (oral presentation)
4. **Katherine McBroom**, Rupesh Kesharwani, Courtney L. Hall, Bupe Kapema, Nicole R. Phillips, Fritz Sedlazeck, Roxanne R. Zascavage. *Taking the Bait: Utilization of Probe-Capture Enrichment in Human Remains Identification.* February 2024. National Institute of Justice Forensic Science Research and Development Symposium at the American Academy of Forensic Science Conference. Denver, CO. (Oral presentation)
5. Courtney L. Hall, Rupesh Kesharwani, Bupe Kapema, Katherine McBroom, Nicole R. Phillips, Fritz Sedlazeck, **Roxanne R. Zascavage**. *DNA Typing Strategies for Identification of Human Remains via Real-Time Nanopore Sequencing*. February 2024. 7th Annual NIJ Forensic Science Symposium at Pittcon 2024 / NIJ - Advancements in the Analysis of Forensic Trace, San Diego, CA. (Invited oral presentation).
6. **K McBroom**, CL Hall, RK Kesharwani, KB Kapema, NR Phillips, FJ Sedlazeck, RR Zascavage. Taking the Bait: Utilization of Probe-Capture Enrichment in Human Remains Identification. April 2024. Research Appreciation Day. Fort Worth, TX. (award winning poster presentation).
7. McBroom, K., Hall, C. L., Kesharwani, R., Phillips, N. R., Sedlazeck, F., & **Zascavage, R. R**. Evaluation of Improved Typing Strategies for Human Remains Identification using Nanopore Sequencing. July 2024. Green Mountain DNA Conference. Burlington, Vermont. (Invited oral presentation).
8. **Tre Baldwin**, Katherine McBroom, Roxanne R. Zascavage. Determining the Correlation Between the DNA Integrity Number and Degradation Index. July 2024. Summer Research Internship Program Poster Session. Fort Worth, Texas. (poster presentation).
9. **McBroom, K.,** Kesharwani, R., Hall, C. L., Phillips, N. R., Sedlazeck, F., & Zascavage, R. R. Adaptive Sampling for the Simultaneous Analysis of STRs, SNPs, and mtDNA in Human Remains Identification. February 2025. National Institute of Justice Forensic Science Research and Development Symposium at the American Academy of Forensic Science Conference. Baltimore, MD. (Invited oral presentation)
10. **McBroom, K.,** Kesharwani, R., Hall, C. L., Phillips, N. R., Sedlazeck, F., & Zascavage, R. R. Adaptive Sampling for the Simultaneous Analysis of STRs, SNPs, and mtDNA in Human Remains Identification.

March 2025. Research Appreciation Day. University of North Texas Health Science Center. Fort Worth, TX. (oral presentation)

11. **McBroom, K.,** Kesharwani, R., Reynolds, A.W., Phillips, N. R., Sedlazeck, F., & Zascavage, R. R. Adaptive Sampling for the Simultaneous Analysis of Genetic and Epigenetic Loci in Human Remains Identification. June 2025. Gordon Research Symposium. Newry, ME. (Invited oral presentation)
12. **McBroom, K.,** Kesharwani, R., Reynolds, A.W., Phillips, N. R., Sedlazeck, F., & Zascavage, R. R. Adaptive Sampling for the Simultaneous Analysis of Genetic and Epigenetic Loci in Human Remains Identification. June 2025. Gordon Research Conference. Newry, ME. (poster presentation)
13. **K McBroom** and RR Zascavage. Modifications to SPRI Bead Cleanups DO Not Improve Sample Retention. April 2026. Research Appreciation Day. Fort Worth, TX. (poster presentation).

General Press, Podcases, and other Media

1. Zascavage, Roxanne. The Future of Forensics. August 2024. The Analytical Scientist. https://theanalyticalscientist.com/issues/2024/articles/aug/the-future-of-forensics