Same-day, multiplexed, high-accuracy targeted liquid biopsy approach for low-tumor content treatment response monitoring

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Introduction

Motivation

- ***Pediatric brain tumor diagnostics can be difficult:*** Brain tumors make up a significant proportion of pediatric cancers. However, due to their sensitive location, many difficulties exist in providing effective diagnostics, treatments, and treatment response monitoring for these patients. ***MRIs can be difficult to interpret*** due to diffuse disease and radiation induced swelling can masquerade as tumor progression (pseudoprogression), further complicating interpretation.
- ***Quantitative liquid-biopsy can be used to monitor treatment response:*** Prior work in our lab showed that same-day nanopore amplicon sequencing of cerebro-spinal fluid could aid in treatment response monitoring[1]. However, ***CSF is difficult to gather*** in pediatric patients — usually requiring a site visit and general anesthesia—***increasing patient and family burden***.

Problems with state-of-the-art

- ***Nanopore error rates prevent meaningful analysis of plasma cf-tDNA levels:*** Plasma collection is much less invasive and a much more desirable biofluid for analysis. However, the blood-brain-barrier filters cf-tDNA such that meaningful treatment-response patterns in allele fractions ***occur between 0.5% and 0.01%.***
- ***ddPCR-based monitoring has flaws that Nanopore can address:*** Droplet-digital PCR (ddPCR) is a highly sensitive and precise method to quantify rare mutations in patient samples and is the current gold-standard approach for treatment response monitoring; however, ***assay design and validation is time consuming and expensive.***
- ***Concatemeric error correction approaches are promising but slow:*** Prior work leveraging circular consensus sequencing to improve nanopore error rates has promise[2], but *requires long protocol times (~3-days)* preventing same-visit diagnostics and rapid adjustments to patient care.

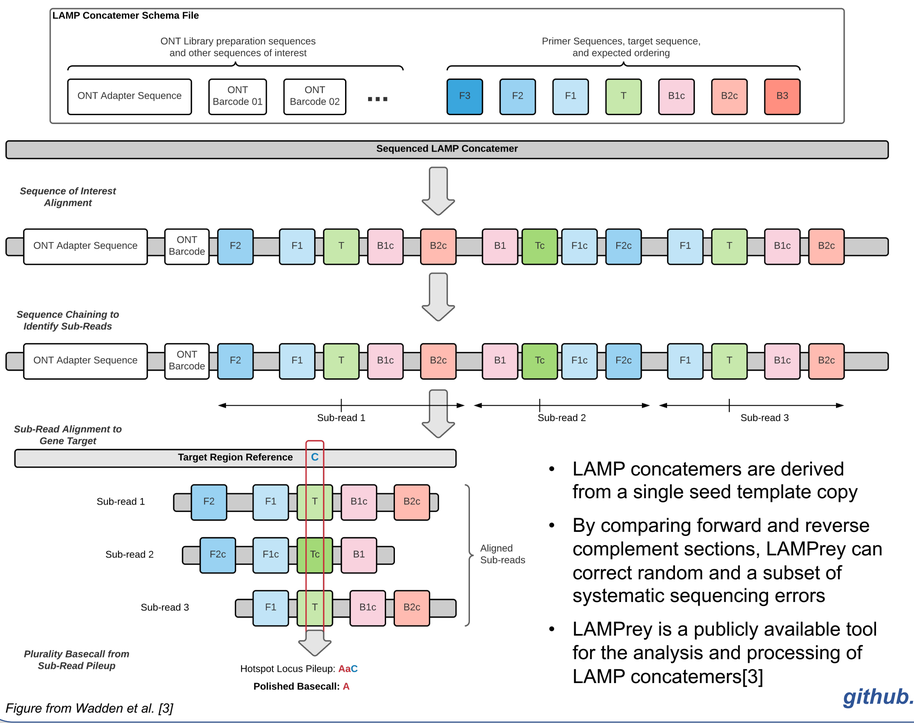
Potential Solutions

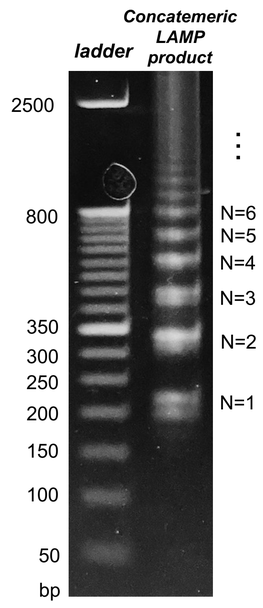
- ***LAMP allows for both rapid and concatemeric amplification:*** Prior work leveraged nanopore sequencing of loop-mediated isothermal amplification (LAMP) for rapid diagnostics ***obtained within 30mins*** of tissue sample acquisition[3], however this approach did not work well at capturing highly fragmented cell-free fragments.
- ***Multiplexed concatemerization of narrow targets can be accomplished using a 2-step amplification approach:*** While LAMP assays are traditionally difficult to design and multiplex, especially for narrow cf-tDNA fragments, ***we solve this problem by co-designing multiplex PCR primers with a narrow, 2-primer "LAMPLite" assays***. We then use a 2-step amplification approach to (1) use multiplex PCR to capture and amplify small cf-tDNA fragments, and (2) parallel singleplex LAMP reactors to concatemerize PCR product.

Research Questions

- **Proof-of-concept:** *Can a two-step amplification approach **capture, amplify, and concatemerize multiple narrow cell-free DNA targets**?*
- ***Speed:*** *Can we perform **same-day** molecular diagnostics on cell-free DNA?*
- **Accuracy:** How does our diagnostic compare to gold-standard methods (e.g. ddPCR genotyping)?

Concatemeric Error Correction with LAMPrey



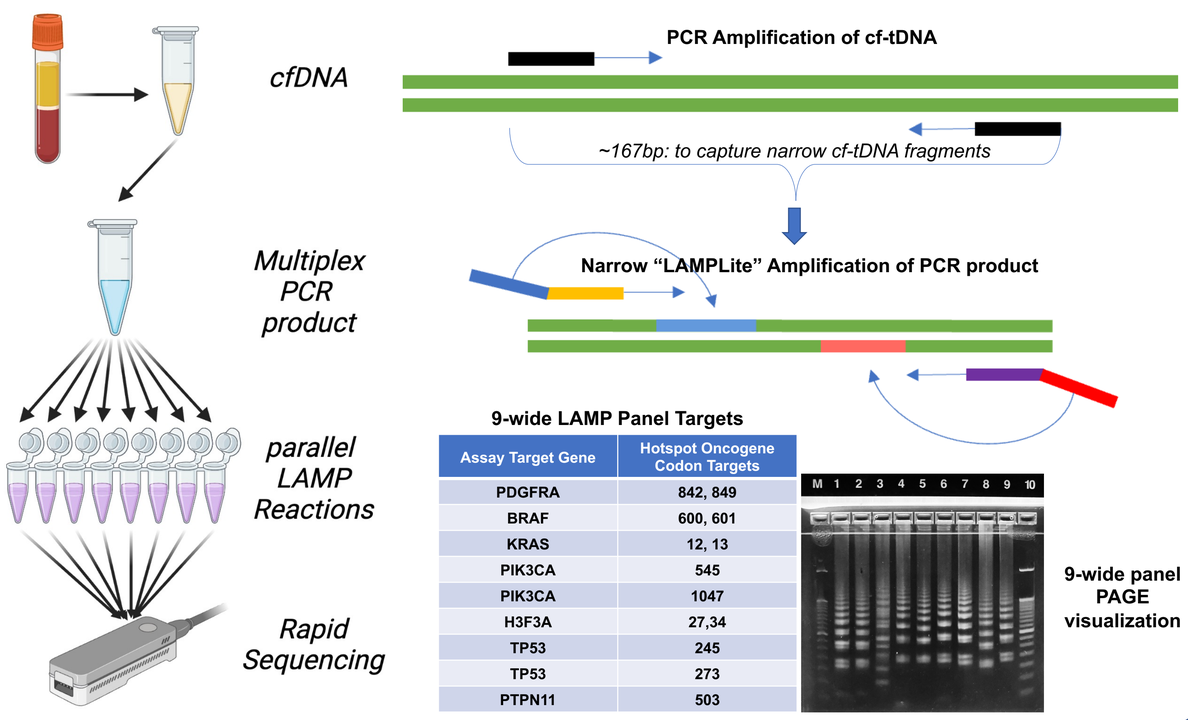


- LAMP concatemers are derived from a single seed template copy
- By comparing forward and reverse complement sections, LAMPrey can correct random and a subset of systematic sequencing errors
- LAMPrey is a publicly available tool for the analysis and processing of LAMP concatemers[3]

github.com/jackwadden/lamprey

Figure from Wadden et al. [3]

2-stage Multiplex Amplification Schema

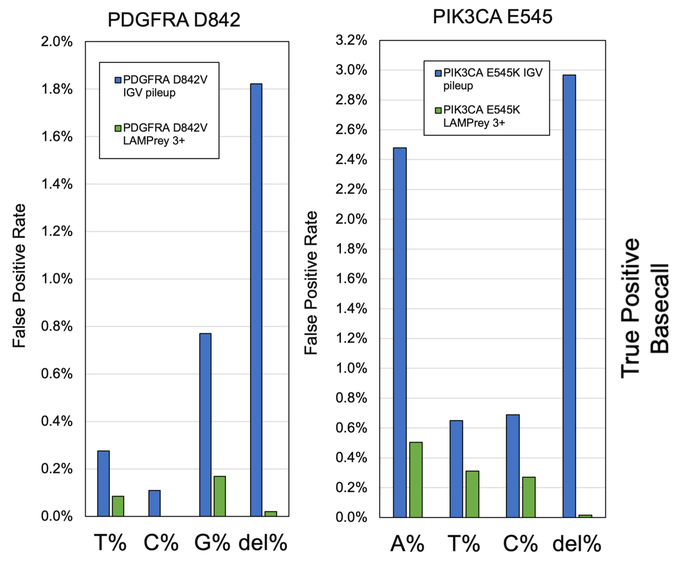


9-wide LAMP Panel Targets

Assay Target Gene	Hotspot Oncogene Codon Targets
PDGFRA	842, 849
BRAF	600, 601
KRAS	12, 13
PIK3CA	545
PIK3CA	1047
H3F3A	27,34
TP53	245
TP53	273
PTPN11	503

9-wide panel PAGE visualization

Error Rate Reduction in 9-wide Multiplex Panel



Improvement in False Positive Error Rate

	A	T	G	C	del
A		1.8x	4.6x	4.8x (3/4 inf)	59.7x (2/4 inf)
T	-		-	-	-
G	5.5x	2.3x		3.0x	124.5x
C	30.4x	3.4x	5.4x		140.7x
del	-	-	-	-	

R10.4.1; SQK-RBK114; 260bp/s; Guppy v6.4.2 "sup"

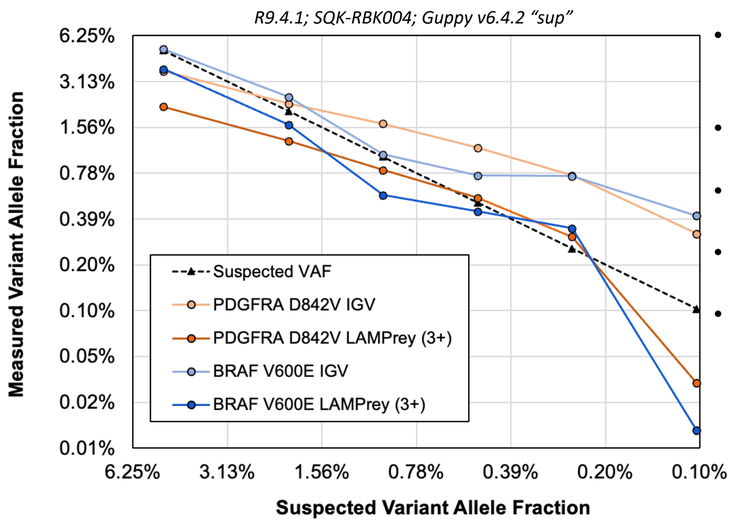
Proof-of-concept: 9-wide multiplex amplification + narrow LAMPLite amplification possible

Accuracy: False positive error rates reduced by 1.8x -> 140.7x, with some errors (inf) eliminated

Limit-of-detection Analysis in Duplex

Cell-free DNA pan-cancer variant reference standards from Twist biosciences (SKU 104549; 5%, 2%, 1%, 0.5%, 0.25%, 0.1%) were amplified and sequenced using our 2-stage approach

R9.4.1; SQK-RBK004; Guppy v6.4.2 "sup"



- ***Stage 1:*** *30ng of Twist bioscience pan-cancer reference standard amplified via PCR (**60mins**)*
- **Stage 2:** 1ul of PCR product taken into parallel LAMP reactors (***30-90mins***)
- **Library prep:** Product combined and prepped via RBK004 (***30mins***)
- **Sequencing:** Sequenced via MinION R9.4.1 flow-cell (***1-4hrs***)
- **Informatics** (***Realtime***)**:**
  - *Basecalled using Guppy 6.4.2 super accuracy model*
  - *Lamplicons aligned using minimap2 (standard ONT options)*
  - *Processed using LAMPrey, requiring 3+ concatemers*

Proof-of-concept: Duplex assay was able to capture fragmented mutant DNA down to ~0.25-0.1% VAF

Speed: Results can be available within ~6-8hrs of sample acquisition. Similar to ddPCR-based analysis.

Accuracy: LAMPrey error correction improves resolution of small cf-tDNA VAFs below 1%

Real-patient Plasma Sample Analysis Agrees with ddPCR

	H3.3K27M Allele Fraction
ddPCR	***0.13%***
UltraPCR[4]	***0.13%***
LAMPLite	***0.13%***
LAMPLite Negative Control	***0.05%***

- *Pediatric diffuse-intrinsic pontine glioma (DIPG) patient diagnosed and scheduled for brainstem biopsy.*
- *Biopsy confirmed H3.3K27M mutation.*
- *Post-radiation blood-draw was assayed for H3.3K27M Mutant cf-tDNA content using 1ml of plasma.*
- *Identical target capture PCR-product enabled direct comparison to ddPCR assay*
- *UltraPCR was performed by Enumerix Inc.*

Accuracy: *H3.3 K27M allele fractions measured via LAMPLite Nanopore sequencing closely match those measured using a gold-standard ddPCR assay and novel UltraPCR[4]*

Conclusions and Ongoing Work

Conclusions

- **Proof-of-concept:** Can a two-step amplification approach capture, amplify, and concatemerized multiple narrow cell-free DNA targets? ***YES. This technique was used to accurately quantify multiple synthetic targets in a reference standard and a 0.13% H3K27M mutation in real patient plasma***
- ***Speed:*** Can we perform ***same-day*** molecular diagnostics on cell-free DNA ***YES. A confident, multi-target diagnostic can be performed within 6-8 hours;*** sequencing time to reach statistically significant depth of coverage increases time-to-result for wide panels.
- **Accuracy:** How does our diagnostic compare to expected reference standard allele fractions and competitive gold-standard methods like ddPCR? ***IDENTICAL TO GOLD STANDARD. Results from all three methods on patient sample produced identical allele fractions indicating high accuracy.***

Ongoing Work

- Full evaluation of 9-wide multiplex panel on Twist pan-cancer reference standards is complete.
- Serial timepoint evaluation of patients with 2+ mutations covered by the validated 9-wide panel.
- Improvement of length distribution of concatemers to eliminate high-fraction of short reads that cannot be error corrected.
- Improvements to LAMPrey algorithm to reduce false positive target calls and improve variant calling accuracy.
- Comparison with multiplex amplicon sequencing via ligation (SQK-LSK114) to identify LAMP assay-induced errors versus naïve approach.
- Multiplex consensus basecalling using forward/reverse lamplicon sub-reads to improve accuracy of concatemers with a small number of sub-reads (e.g. N=2).
- Speed and assay design complexity improvements to allow for rapid assay validation and implementation.

References

[1] Bruzek et al. "Electronic DNA Analysis of CSF Cell-free Tumor DNA to Quantify Multi-gene Molecular Response in Pediatric High-grade Glioma." *Clinical cancer research. 2020:* https://doi.org/10.1158/1078-0432.CCR-20-2066

[2] Marcozzi et al. "Accurate detection of circulating tumor DNA using nanopore consensus sequencing." *NPJ Genomic Medicine. 2021:* https://doi.org/10.1038/s41525-021-00272-y

[3] Wadden et al. "Ultra-Rapid Somatic Variant Detection via Real-Time Targeted Amplicon Sequencing." *Communications Biology. 2022:* https://www.nature.com/articles/s42003-022-03657-6

[4] Shum et al. "Next-Generation Digital Polymerase Chain Reaction: High-Dynamic-Range Single-Molecule DNA Counting via Ultrapartitioning." *Analytical Chemistry. 2022:* https://pubs.acs.org/doi/10.1021/acs.analchem.2c03649

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