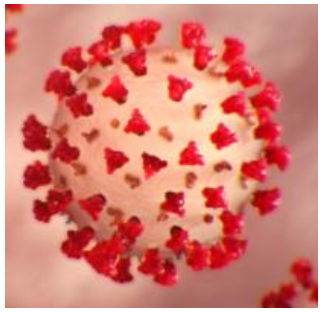
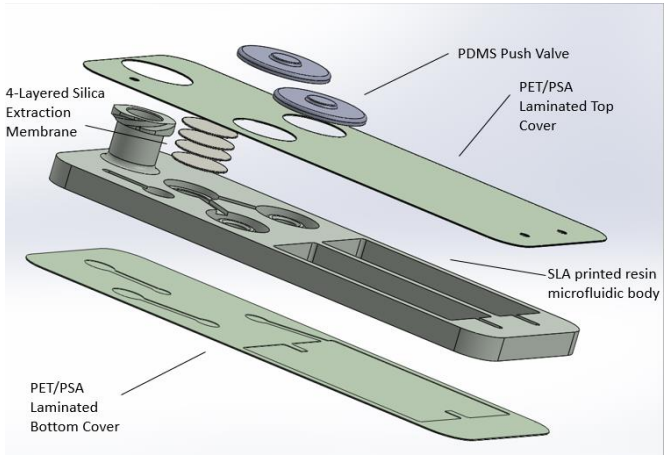
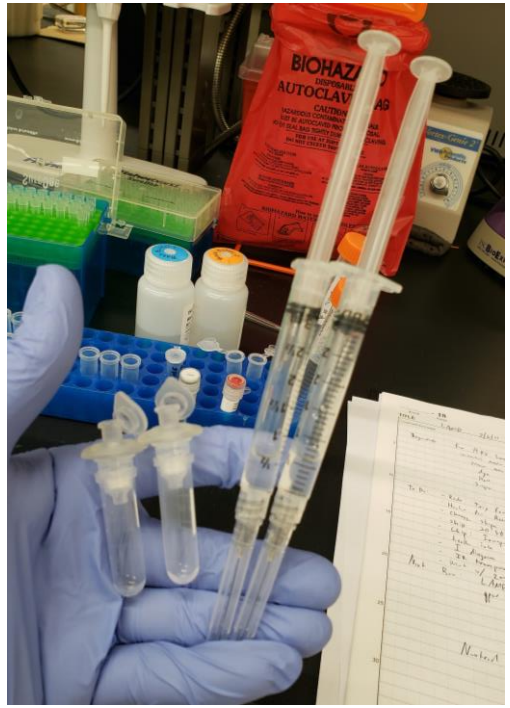
RNA Extraction from Saliva for Covid-19 Point-of-Care Diagnostics

Michael Hansen[1], Vi Nguyen[1], Clifford Anderson[1], Jennifer Blain Christen[1]
[1] Arizona State University



Coronavirus (causative agent of Covid-19), a ssRNA virus, with characteristic spike proteins on the surface





Sensor Signal and Information Processing Center
https://sensip.asu.edu

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

Motivation

- Many areas of lower resources are underserved regarding access to healthcare and timely or affordable laboratory testing.
- Point of Care devices continue to offer the promise of improvements to access and cost in these types of areas.
- Extraction from challenging matrices such as saliva or blood could vastly expand both the range of accuracy of any potential tests and assays based upon nucleic acids.

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

Project Aim

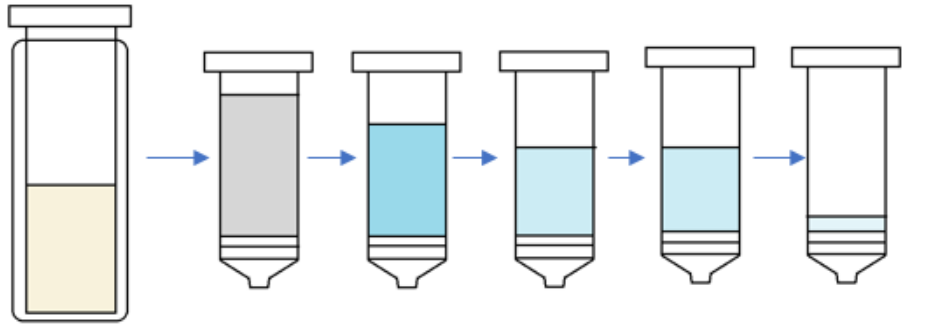
High purity and reliability of extraction are a requirement to using nucleic acid based pathogen/disease detection

- Successfully extract SARS-CoV-2 genomic RNA without requiring a laboratory centrifuge or vacuum manifold using solid-phase extraction (silica membranes)
- Analyze the samples for purity using UV Spectroscopy (NanoDrop™)
- Achieve a purity sufficient to successfully amplify in a RT-LAMP reaction within a microfluidic

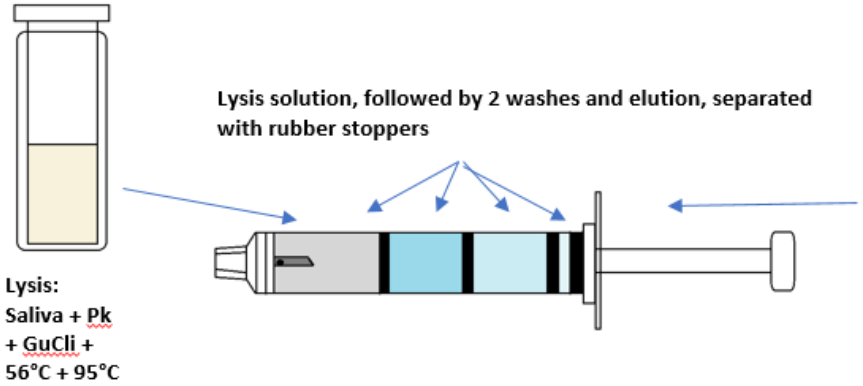
REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

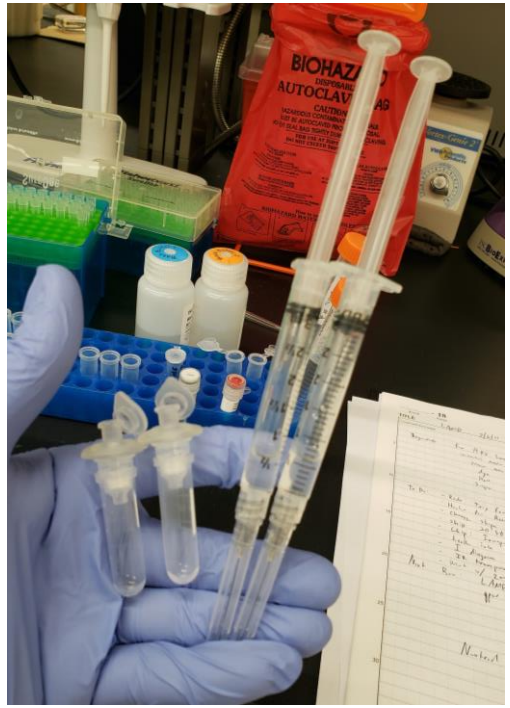
Silica (solid-phase extraction) workflow: Column Centrifuged vs Plunger Driven Syringe

Traditional Spin Column method (requires a centrifuge, pipets)



Multi-chamber syringe completes 5 steps with a single syringe plunger push





REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

UV Spectroscopy using a NanoDrop One™

- **Nucleic Acids Absorb at 260nm [7]**
- **Common organic contaminants absorb strongly under 240nm**
- **Chaotropic salts absorb strongly at 270-290nm, generally can be seen as a bump following the 260nm peak [8]**
- **Desirable 260/230 and 260/280nm ratios are ~2.0 for both RNA and DNA [7]**

The Beer-Lambert Law

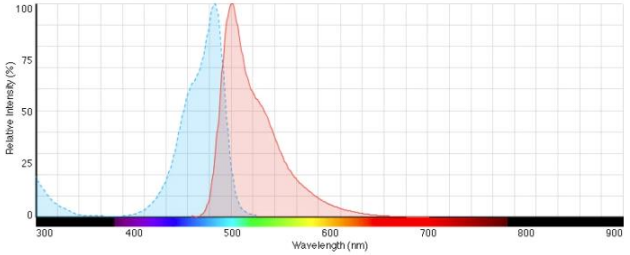
$A = \varepsilon c l$

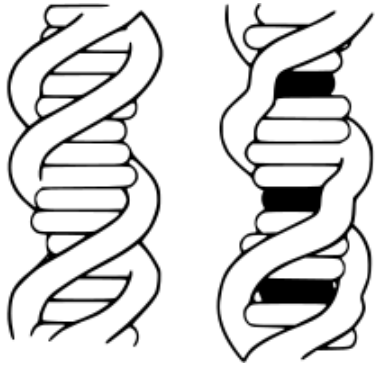
Where A=absorbance, ε=extinction coefficient, c=concentration and l=path length. [8]

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

RT-LAMP w/ and Intercalating Fluorescent Dye

- RT-LAMP: Reverse Transcription Loop-Mediated Isothermal Amplification
- Uses a DNA-pol with high strand displacement
- Separates and amplifies cDNA at 65°C
- Requires 3 sets of primers, leading to the formation of loops that amplify exponentially
- An intercalating dye, Syto 9, increases substantially in fluorescence after integrating in between nucleotides in dsDNA
- Using filters (470nm Excitation, 535nm emission), a handheld reader can be used to look for a substantial increase in fluorescence





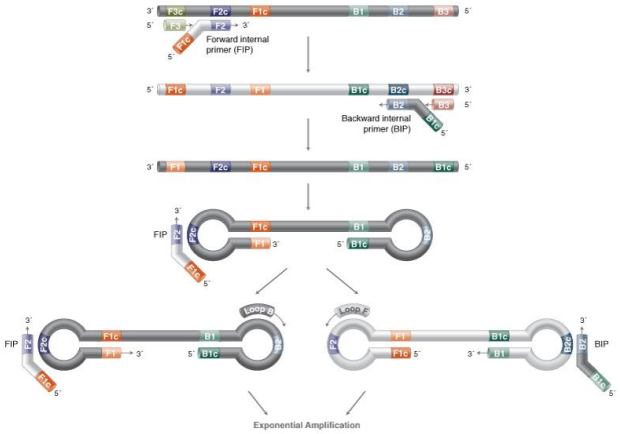


Image credit: NEB

Syto™ 9 Intercalating Dye

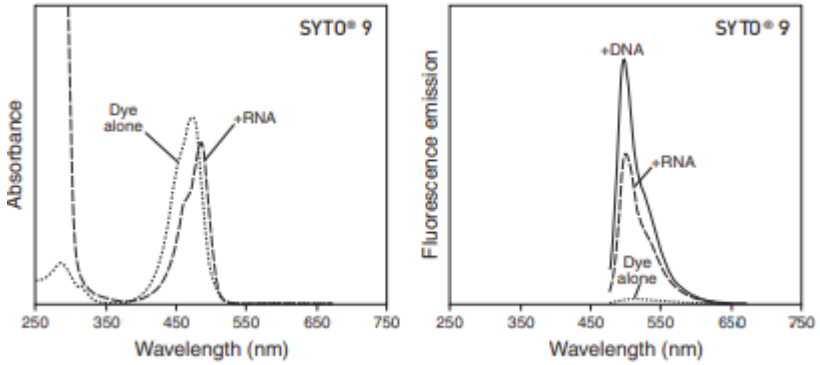


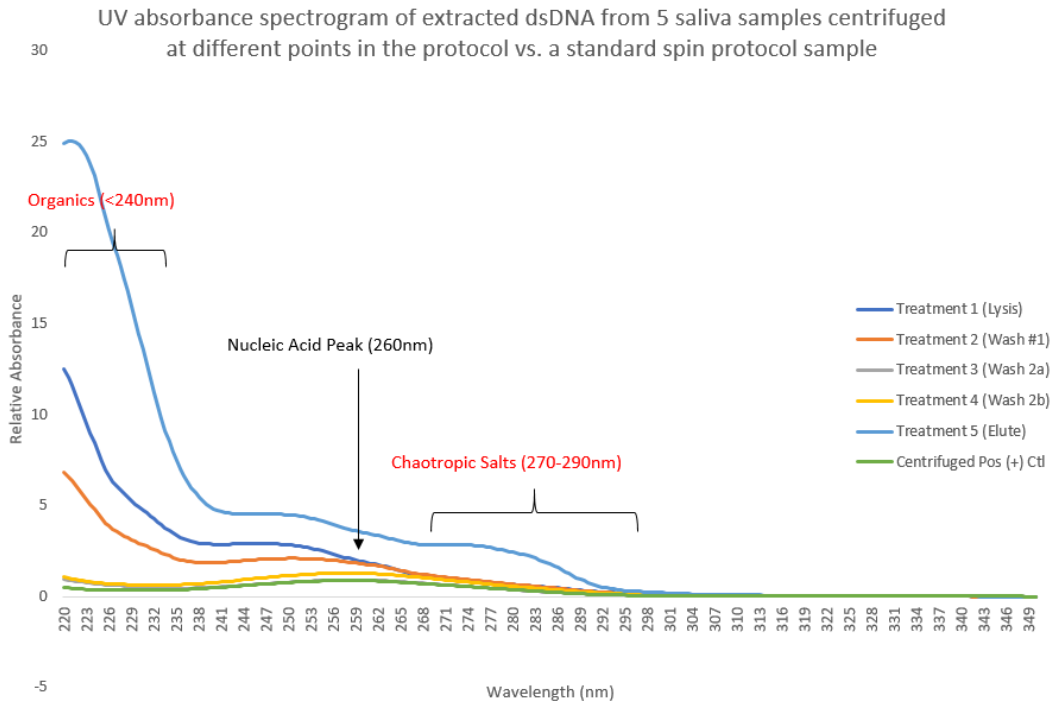
Image Credit: Thermo Fisher, and Wikipedia

Sensor Signal and Information Processing Center
https://sensip.asu.edu

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

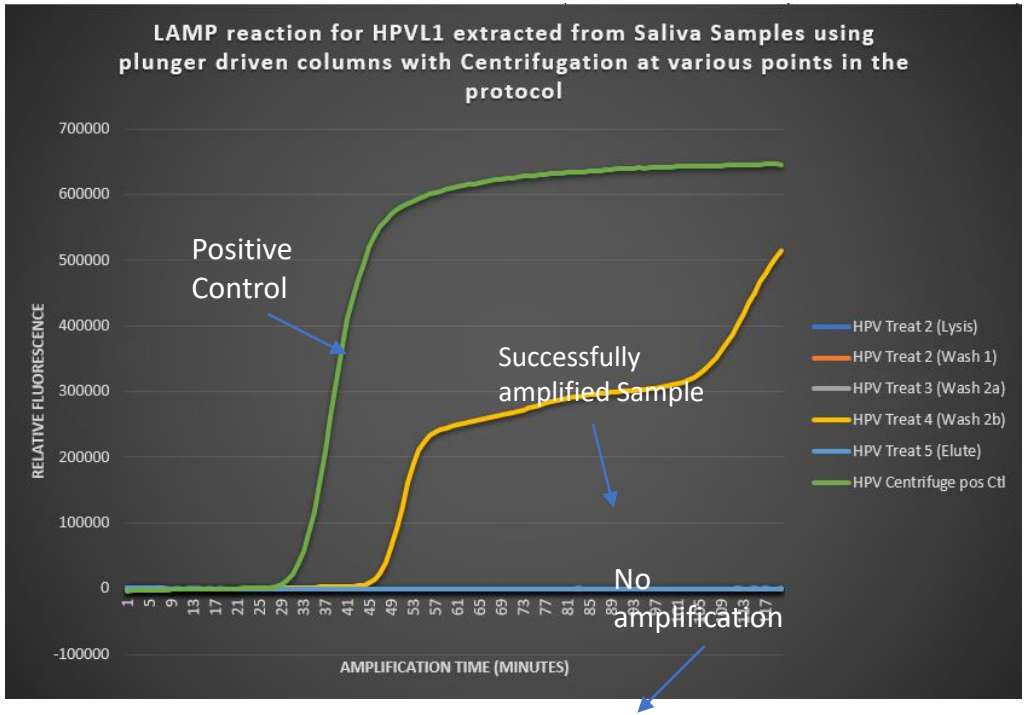
Preliminary Results

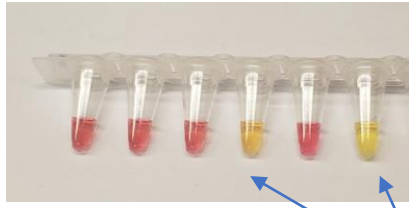
UV Spectroscopy Results



Date	Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
7/13/2021 17:56	HPV Treatment 1 (Lysis)	95.054	2.904	0.388	1.901	0.655
7/13/2021 17:57	HPV Treatment 1 (Wash 1)	88.789	2.659	0.607	1.776	0.668
7/13/2021 17:57	HPV Treatment 1 (Wash 2a)	43.988	2.432	1.606	0.88	0.362
7/13/2021 17:58	HPV Treatment 1 (Wash 2b)	63.221	2.315	1.978	1.264	0.546
7/13/2021 17:58	HPV Treatment 1 (Elution)	175.171	1.442	0.244	3.503	2.43
7/13/2021 17:59	HPV Centrifuged Pos Ctl	43.192	2.377	2.559	0.864	0.363

LAMP Results (HPV dsDNA Template)





Colorimetric version (phenol red) version of above

Amplified Wells

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

Conclusion

- Preliminary results are encouraging but indicate major hurdles to overcome
- UV Spectroscopy, specifically the 260/280nm and the 260/230nm, are strong indicators of the likelihood that a LAMP reaction is possible
- The salts and ethanol required for silica column binding and protein precipitation are potent inhibitors of RT-LAMP
- Amplification of DNA extracted from saliva using plunger driven silica columns is possible as long as sufficient wash solution (primarily ethanol) is eliminated

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

Ongoing Work and Future Plans

- Complete a functional prototype of a handheld saliva sample prep device, both as a separate microfluidic, as well as a syringe integrated version of the hand
- Develop a method to ensure complete elimination of all trapped salts and solvents within the silica membranes (primary source of contaminate retention)
- Continue LAMP and qPCR testing to determine acceptable levels of downstream contamination lysis/wash reagents
- Improve correlation of UV spectroscopy results to likelihood of successful amplification of template
- Find alternative direct sample amplification methods not requiring extraction

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

References

[1] Mauk MG, Liu C, Sadik M, Bau HH. Microfluidic devices for nucleic acid (NA) isolation, isothermal NA amplification, and real-time detection. Methods Mol Biol. 2015;1256:15-40. doi:10.1007/978-1-4939-2172-0_2

[2] Oberkanins C, Berndt A, Hauser B, Puehringer H. AB079. Use of saliva and salivary DNA for comprehensive genotyping based on RealFast and StripAssays. *Ann Transl Med*. 2017;5(Suppl 2):AB079. doi:10.21037/atm.2017.s079

[3] Ali N, Rampazzo RCP, Costa ADT, Krieger MA. Current Nucleic Acid Extraction Methods and Their Implications to Point-of-Care Diagnostics. Biomed Res Int. 2017;2017:9306564. doi:10.1155/2017/9306564

[4] Thompson RE, Duncan G, McCord BR. An investigation of PCR inhibition using Plexor(®) -based quantitative PCR and short tandem repeat amplification. J Forensic Sci. 2014 Nov;59(6):1517-29. doi: 10.1111/1556-4029.12556. Epub 2014 Sep 3. PMID: 25182468.

[5] McCord B, Opel K, Funes M, Zoppis S, Jantz LM. An Investigation of the Effect of DNA Degradation and Inhibition on PCR Amplification of Single Source and Mixed Forensic Samples. NCJRS. https://www.ojp.gov/pdffiles1/nij/grants/236692.pdf

[6] Katevatis C, Fan A, Klapperich CM (2017) Low concentration DNA extraction and recovery using a silica solid phase. PLOS ONE 12(5): e0176848. https://doi.org/10.1371/journal.pone.0176848

[7] Matlock B. Assessment of Nucleic Acid Purity. Thermo Fisher Scientific Technical Bulletin. https://assets.thermofisher.com/TFS-Assets/CAD/Product-Bulletins/TN52646-E-0215M-NucleicAcid.pdf

[8] Interpreting Nanodrop (Spectrophotometric) Results. University of Arizona. http://www.u.arizona.edu/~gwatts/azcc/InterpretingSpec.pdf

[9] Hagan KA, Bienvenue JM, Moskaluk CA, Landers JP. Microchip-based solid-phase purification of RNA from biological samples. Anal Chem. 2008 Nov 15;80(22):8453-60. doi: 10.1021/ac8011945. Epub 2008 Oct 15. PMID: 18855414.

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763

Challenges

1. Patience waiting for materials to arrive for construction of prototypes, reagents for LAMP
2. LAMP is a very sensitive assay and contamination with Covid-19 control RNA has been a persistent problem that resulted in us switching temporarily to a dsDNA virus as the source template for extraction trials, HPV
3. LAMP uses 6 different primers (2 is typical for a non-multiplex PCR) and acquisition and creation of said primers can be a lengthy process

REU project sponsored by NSF Award 1659871 and Arizona Department of Health Services award CTR051763