Seek Amplification™: Introducing a Sensitive, Specific, and Equipment-Free DNA/RNA Amplification Technology to Revolutionize Molecular Diagnostic Testing for the Point of Care

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1 BACKGROUND

- Rapid and accurate molecular diagnostics are critical for timely detection and treatment of infectious human, veterinary, or agricultural pathogens.
- Current molecular diagnostic (Dx) technologies typically require specialized, expensive, lab-based nucleic acid amplification and detection equipment (e.g., qPCR instruments, thermocyclers, centrifuges, and hazardous chemicals). As such, these lab-based technologies cannot be easily deployed for point-of-care (PoC) and low-resource scenarios or field testing thus leading to delays in diagnosis and treatment.
- Healthcare consumers are increasingly favoring affordable and accessible technologies. Indeed, the Covid-19 Pandemic highlighted the necessity for instrument-free, cost-effective molecular Dx technologies that are user friendly, deliverable, and can be rapidly configured for various disease targets.
- A key component in molecular Dx technology is DNA/RNA amplification, the process for copying target DNA/RNA sequences exponentially.
- The gold standard amplification technology, PCR, requires specialized thermocycling equipment, trained (skilled) technical operators, and stringent experiment design, among other constraints.
- Unlike PCR, isothermal amplification technologies–such as LAMP, HDA, and RPA–do not require thermocyclers but still require equipment to maintain a constant elevated temperature. Additionally, these isothermal technologies are prone to non-specific amplification and cannot easily differentiate single nucleotide mismatches in target sequences. These techniques also require an additional enzymatic method for specific and sensitive detection in PoC settings (e.g., CRISPR/Cas-based SHERLOCK and DETECTR technologies). Consequently, these methods increase total detection time, manufacturing costs, and assay design complexity.

Here, we introduce our innovative amplification platform, Seek Amplification™, which overcomes the limitations of existing molecular Dx: equipment requirements, reliance on trained technicians, temperature controls, complex experiment design, incompatibility with developing technology, etc.

- Seek Amplification combines our novel Annexing Isothermal Nucleotide Amplification (ANINA) primer and probe approach with a proprietary enzymatic formulation optimized for instrument-free amplification at ambient or room temperatures.
- This technology has promise for reliable and rapid amplification outside of the laboratory, opening development for next-generation diagnostic technologies.

2 TECHNOLOGY OVERVIEW

Seek Amplification utilizes a single-reaction isothermal nucleotide amplification and detection approach, which contains an Annexing Probe (AP) alongside two or three target-specific primers that exponentially amplify a DNA or RNA target sequence at room temperature (20°-25°C) and up to 40°C. Seek Amplification can be lyophilized for long-term ambient temperature storage and is capable of integration with a variety of detection platforms including lateral flow readouts for attomolar sensitive detection.

- **Enzymes:** Seek Amplification comprises a recombinase enzyme, a single-strand binding enzyme, and a strand displacing DNA polymerase. RNA amplification additionally utilizes a reverse transcriptase (RT) enzyme in the same reaction mix to convert RNA target sequences to cDNA.
- **Annexing Oligos:** Seek Amplification uses a target-specific AP with a 5' Fluorescein (FAM) label and a 3'amplification-blocking terminus as well as two target-specific primers: Forward Primer (FP) and Reverse Primer (RP). For further sensitivity and increased amplification, an additional Reverse Primer (RP2) is required.
- **Target Template:** Genomic DNA (gDNA) or RNA extracted from pathogen samples can be used as template.
- **Reaction Process:** Seek Amplification consists of a single-reaction mixture. The amplification reaction begins immediately upon addition of target-specific primers and template. The reaction occurs at room temperature (20°-25°C) and is functional up to 40°C. The specific details of the reaction process as shown in Figure 1 are as follows:
  - *Binding*: The recombinase enzyme and single-strand binding enzyme enable AP to form a complex with its binding region in the target template.
  - *Annexation*: AP annexes FP to its complementary region.
  - *Extension*: Polymerase extends the 3' ends of FP and RP while displacing the AP.
  - *Annexing Amplification*: Target dsDNA undergoes multiple cycles of binding, annexation, and extension resulting in exponential amplification of biotinylated dsDNA.
  - *Detection*: The Fluorescein (FAM)-labelled AP can re-bind its target region in the biotinylated DNA amplicon. This amplicon-probe complex can be detected with fluorescence assays or on the test line of a molecular Lateral Flow Assay (mLFA) without any additional steps.

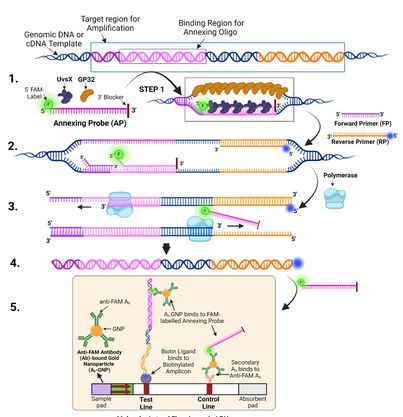

Figure 1. General schematic of Seek Amplification utilizing ANINA primer and probe approach

3 CAPABILITIES

Seek Amplification specifically amplifies DNA/RNA from pathogens in presence of host gDNA. Without specialized equipment or stringent temperature requirements, Seek Amplification amplified Epstein Barr Virus (EBV), a DNA virus that causes mononucleosis and certain types of cancer (Fig. 2); *Neisseria gonorrhoeae* (*N.g*), a bacterium that causes gonorrhea (Fig. 3); and Respiratory Syncytial Virus (RSV), an RNA virus (Fig. 4).

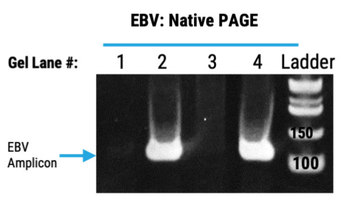

Figure 2. Native PAGE results from EBV amplification: *Lane 1*. No template control (NTC); *Lane 2*. EBV gDNA (Positive Control); *Lane 3*. gDNA from non-infected human saliva (Host gDNA only); *Lane 4*. gDNA from EBV-spiked human saliva.

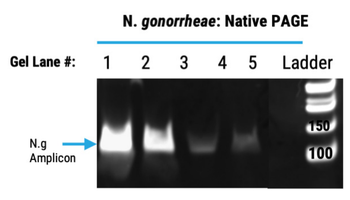

Figure 3. Native PAGE results from *N.g*: *Lane 1*. *N.g* gDNA (Positive Control); *Lane 2*. gDNA from *N.g*-spiked human swab samples 1:1 dilution; *Lane 3*. 1/10 dilution; *Lane 4*. 1/100 dilution; *Lane 5*. NTC.

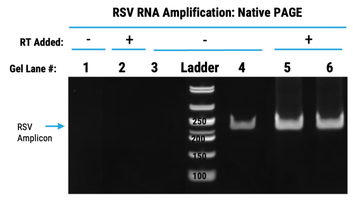

Figure 4. Native PAGE results from RSV: *Lane 1*. RSV RNA (no RT control) *Lane 2*. Human gDNA (+RT); *Lane 3*. Human gDNA (no RT); *Lane 4*. RSV cDNA (Positive Control); *Lane 5*. RNA from RSV-spiked human saliva; *Lane 6*. RSV RNA (+RT).

To demonstrate the sensitivity of Seek Amplification, a single copy of viral genome can be amplified at room temperature (20°-25°C) in <30 minutes to a detectable range (10⁷ copies/uL) without compromising specificity, as illustrated in Fig. 5-6, which show amplification sensitivity and detection on native PAGE and mLFA with White Spot Syndrome Virus (WSSV) (Fig. 5) and EBV (Fig. 6). Seek Amplification RP is 3'- biotinylated for mLFA detection compatibility.

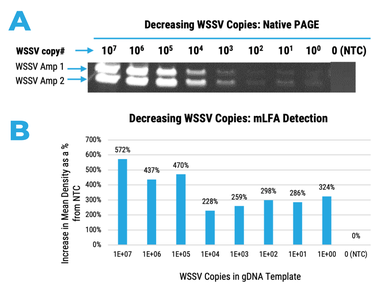

Figure 5. (A) Native PAGE results from amplification of WSSV at concentrations ranging 10^0 to 10^7 copies/uL. **(B)** Mean density of detected amplicons using mLFA strips.

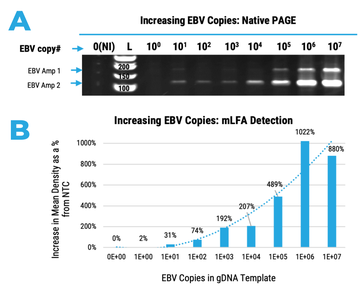

Figure 6. (A) Native PAGE results from amplification of EBV at concentrations ranging 10^0 to 10^7 copies/uL. **(B)** Mean density of detected amplicons using mLFA strips.

Seek Amplification is a highly versatile technology that can be utilized more broadly when combined with existing fluorescence detection systems to measure amplification quantitatively in real time. Detection using Seek Amplification occurs in less than 10 minutes at 39 °C in contrast to traditional qPCR, which can take up to 4 times longer for similar results (Fig. 7).

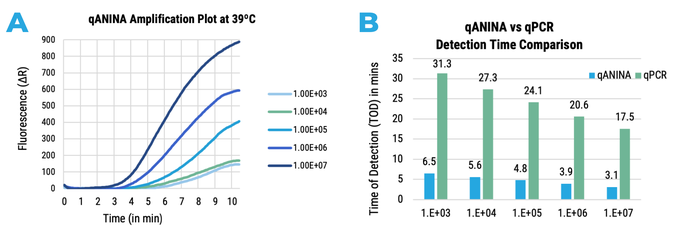

Figure 7. Real-time Seek Amplification (qANINA) for WSSV viral loads at 39°C. **(A)** Amplification plot showing increase in fluorescence of target amplicons in real time. **(B)** Detection times for Seek Amplification (qANINA) as compared to qPCR. **(C)** Standardization curve for real-time Seek Amplification.

Seek Amplification allows for greater flexibility in primer design compared to PCR or existing isothermal amplification methods such as LAMP, HDA, or traditional RPA. The ANINA primer and probe approach efficiently employs primers between 10-35 bases in length (Fig. 8).

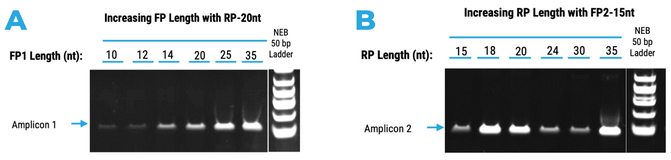

Figure 8. Native PAGE showing amplification from various lengths of **(A)** FP and **(B)** RP lengths.

Seek Amplification permits a larger range of sequence parameters, including primer melting temperature (Tm) and GC% (Fig. 9. A). E.g., Seek Amplification can efficiently amplify a FP-10 nt (30% GC, 18° C Tm) with RP-20nt (55% GC, 57° C Tm) with WSSV-infected shrimp gDNA. Notably, PCR and traditional RPA (TwistDx) are unable to amplify using these primers (Fig 9. B-C).

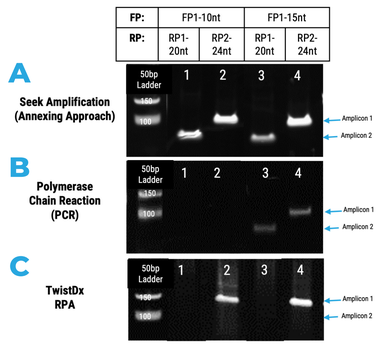

Figure 9. Native PAGE results showing amplification results from different lengths of FP and RP on **(A)** Seek Amplification, **(B)** PCR, and **(C)** TwistDx (RPA). Seek Amplification and TwistDx were run at 25°C for 30 min. PCR was thermo-cycled between 95°C and 60°C for 37 min.

Seek Amplification specifically amplifies and detects two different gene regions by changing the RP Label (Fig. 10).

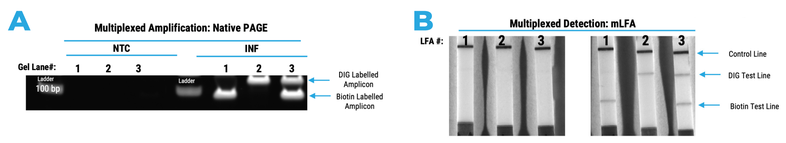

Figure 10. (A) Gel and **(B)** mLFA results showing detected amplicons using 5'-biotin-labelled RP1 (*Lane 1*), 5'- digoxigenin (DIG) labelled RP2 (*Lane 2*), and both 5'-biotin-RP1 and 5'-DIG-RP2 (*Lane 3*).

In addition to working as a detection probe, Seek Amplification's Annexing Probe significantly decreases nonspecific amplification or primer noise common in other isothermal amplification methods (Fig. 11).

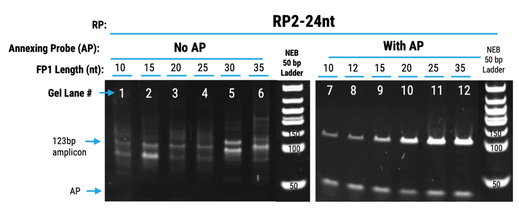

Figure 11. Native PAGE contrasting amplification between Seek Amplification without AP (*Lanes 1-6*) and with AP (*Lanes 7-12*) from shrimp gDNA infected with WSSV at different FP lengths.

4 FUTURE DIRECTIONS

Ongoing studies are focused on Seek Amplification's ability to differentiate single nucleotide polymorphisms (SNP) in gene sequences. This method, combined with the flexibility in primer design and the capability to amplify multiple gene regions simultaneously, could enable the simultaneous detection of multiple pathogen variants and disease mutations.

- FP and RP primers designed with single base substitutions to imitate wild type and SNP mutation.
- Initial experiments show Seek Amplification can differentiate single nucleotide sequences in FP, RP, or FP and RP together (Fig. 12).
- Future experiments will test the functionality of disease-specific SNP.

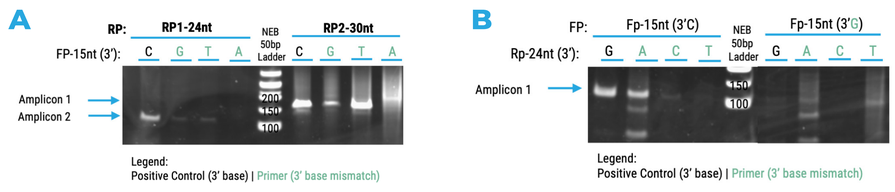

Figure 12. Native PAGE showing amplification with two different FPs, each with a single nucleotide mutation on the 3' end. **(A)** FP-15nt with and without 3' mismatches (C-> G, C-> T, C-> A) with RPs of 2 different lengths: RP1-24nt and RP2-30nt. **(B)** FP-15nt with and without a 3' mismatch (C-> G) tested against RP1-24nt with and without 3' mismatches (G->A, G->C, G->T).

5 CONCLUSION

Seek Amplification is a versatile, efficient, and cost-effective DNA/RNA amplification platform that sensitively and specifically amplifies and detects DNA or RNA targets in the presence of host gDNA at temperatures between 20°C and 40°C in under 30 minutes. Seek Amplification is an optimal technology for rapid and reliable deployment in both laboratory and PoC settings without the constraints of laboratory technologies (e.g., specialized equipment, strict primer design, long incubation or thermocycling time, trained/skilled technical operators, etc.).

- By eliminating equipment, temperature, and time requirements, Seek Amplification is poised as a cost-effective molecular Dx technology for applications in PoC or low-resource scenarios as well as field assays.
- Seek Amplification's versatility enables integration with a variety of detection techniques common to laboratories, such as fluorescent detectors or molecular lateral flow.
- Additionally, Seek Amplification has a longer shelf-life as reactions can be lyophilized with or without primers for storage at ambient temperatures for extended periods. The lyophilized reaction can be deployed in PoC scenarios, low-resource settings, or field assays where cold storage is not available.
- Finally, Seek Amplification's in-reaction probe achieves attomolar sensitivity easily without any post-amplification steps greatly reducing time required by post-amplification detection systems such as SHERLOCK/DETECTR. This sensitivity enables earlier detection for time-sensitive scenarios like disease outbreaks or pandemics.

6 ACKNOWLEDGEMENT

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- We thank Dr. Sharon Hietela for her expertise and insights in laboratory diagnostics and response capabilities.

VISIT SEEK LABS' WEBSITE

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