Rational Design of Duplex Specific Nuclease for One-Step Isothermal Viral RNA Detection

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ABSTRACT

RNA viruses are a potent human adversary, evidenced by several global pandemics including the Ebolavirus in West Africa, the emerging Zika virus, and outbreaks of new Influenza strains and Norwalk virus in the food supply and cruise ships. Despite the virulence of these pathogens, there remains a significant limitation for detecting these viruses in a fast, accurate and cost effective manner. To meet this need we present a modified form of the duplex specific nuclease enzyme from the Paralithodes camtschaticus crab capable of generating an RNA-based signal amplification in a fraction of the time required for standard RT-qPCR. The applicability of this enzyme is demonstrated in an assay for Norwalk virus detection with a lower limit of ~100 viral copies per liter of environmental water.

1. INTRODUCTION

RNA detection can provide a wealth of information used to diagnose diseases [1-3], track cellular growth and development, infections, pollution, as well as many other “omics” applications [4-6]. RT-qPCR remains the gold standard for RNA quantification; however, this technique is limited by time and equipment constraints and can be prone to contamination. To minimize these limitations, isothermal RNA amplification techniques have been developed [7-14], but these still remain dependent on nucleic acid replication and are therefore hindered by polymerase speed and fidelity. Recent RNA detection methods that have employed duplex specific nuclease (DSN) isolated from the Paralithodes camtschaticus crab [15, 16], remain limited to microRNA [17] and are thus unsuitable for longer RNA templates like virus genomic or mRNA targets. DSN is an attractive candidate for the latter, since it preferentially cleaves DNA in a DNA-RNA duplex, leaving ssRNA and dsRNA virtually unchanged [15]. DSN acts as an endonuclease on DNA, cleaving within the phosphate backbone [16], although the direction of digestion is unknown. The enzyme’s preference for DNA has also been applied for many techniques using short RNA sequences [16, 18-29], but applications for long RNA targets have remained elusive.

Norovirus (species Norwalk virus, genus Norovirus, family Caliciviridae, order unassigned) was chosen to develop our DSN assay due to its ubiquity, persistent outbreaks, and reduced virulence compared to other recent pandemic viruses. Worldwide, Norwalk virus is the most common cause of gastroenteritis [40-46], accounting for almost 20% of these cases [47], resulting in 71,000 hospitalizations [48] and approximately 800 deaths per year in the United States [41, 49]. The infectious dose of norovirus is extremely low, around 18 viral particles [50], making it highly contagious and readily transmitted in environments with close interpersonal contact [51-53]. Further, norovirus particles can retain infectivity in ground water for at least two months with viral RNA detectable for up to three years [54]. Given the infectivity and persistence of norovirus, methods to detect viruses in water are of paramount importance to prevent future infections. However, given the limited laboratory options for propagation of norovirus [55, 56] and commercial kits for rapid diagnosis remain underdeveloped [57, 58]. As a result, clinical laboratories commonly use reverse transcription-polymerase chain reaction (RT-PCR), immunoassays or hybridization assays [59-62], which can lead to false positives, provide lower sensitivities and require significant time and materials costs [62-70].
Clearly, there is a need for a sensitive, rapid, more cost effective nuclease detection method. Applying the unique nuclease activity of DSN to broad range RNA detection is limited by the relatively short RNA template range (<20nt), similar to the RISC complex [71], and the reported production/purification difficulty. Here, we report a screening investigation of nuclease inhibitors to challenge DSN to recognize longer RNA templates, which led us to recombinant production of structurally modified DSN that functions without inhibitors on the same larger RNA templates. Targeted point mutations were made based on the predicted secondary structure of DSN, sequence alignment with other nucleases, and the theoretical structural interactions of inhibitors with specific amino acid motifs. Successful detection of norovirus RNA targets was achieved using fluorescently labeled Taqman probes (Figure 1).

Fig. 1: Schematic representation of Norovirus RNA detection based on Duplex-Specific Nuclease. Nuclease activity of DSN digesting the DNA Taqman probe, which provides the fluorescent signal for analyte detection (left). The Taqman-based DNA probes hybridize with the viral RNA template to form a DNA:RNA hybrid and allow DSN to specifically digest the DNA probe within the duplex, generating a fluorescent signal upon the separation of quencher and reporter dye (right).

2. MATERIAL AND METHODS

All chemicals and reagents were purchased from Sigma unless otherwise noted.

4.1 RNA OLIGONUCLEOTIDES AND DNA PROBES

Short RNA oligonucleotides and probes (Table 1) corresponding to the norovirus genogroups GI and GII were synthesized (Operon, USA). The probe sequences were obtained from Hoehne et al. with required modifications to ensure the absence of secondary structure formation using RNA fold [72]. The probes were designed to have an annealing temperature of 60°C, the optimum working temperature for the DSN enzyme. The GI probe was labeled at 5'-end with Cyanine3 (Cy3 fluorophore) and at the 3'-end with BHQ (Black Hole Quencher). The GI probe contained 6-FAM (6-carboxyfluorescein) and BHQ at the 5' and 3' ends, respectively.

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<th>Name</th>
<th>Sequence (5'-3')</th>
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<tr>
<td>NVGI RNA</td>
<td>AAUGAUGAUGGCGUCUAAGGAAGCG</td>
<td>5355-5379</td>
</tr>
<tr>
<td>NVGI probe</td>
<td>Cy3-CCTAGAGCCCATCATCA-BHQ</td>
<td>5376-5359</td>
</tr>
<tr>
<td>NVGII RNA</td>
<td>UUGAAUGAAGAUGGGCGUAUG</td>
<td>5080-5103</td>
</tr>
<tr>
<td>NVGII probe</td>
<td>6-FAM-TGACGCCATCTTCATTC-BHQ</td>
<td>5100-5083</td>
</tr>
<tr>
<td>Cy3:Cyanine3</td>
<td>BHQ:Black Hole Quencher 6-FAM:6-Fluorescein amide</td>
<td></td>
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</tbody>
</table>

4.2 DSN BASED DETECTION FOR RNA OLIGONUCLEOTIDES

For the DSN assay, 25μL reaction was prepared containing 1x DSN buffer A (50mMTris-HCl, pH 8.0; 7mM MgCl2, 1mM DTT), 0.1U DSN enzyme (dissolved in 25 mMTris-HCl, pH 8.0; 50% glycerol), RNA mixture and complimentary probe (50nM). The final reaction mixture was incubated at 60°C for 25 min. Subsequently, 25μl 10mMEDTA was added to the reaction mixture and incubated at 60°C for 5min to inactivate the DSN enzyme and end point fluorescence was recorded. 10-10,000 copies per 25μL reaction with additional no template controls were examined in triplicate. Dilution series for each of the synthetic RNAs were made in either RNase and DNase free H2O or environmental RNA solution to provide a constant background of nonspecific RNA. Enzyme activity ratios were generated by normalizing the signal relative to the minimal fluorescence generated by the template free controls.

4.3 OPTIMIZED DSN REACTION

A volume of 25μL reaction mixture containing 1x DSN buffer B (50 mMTris-HCl, pH 8.0; 10mM MgCl2, 1 mM DTT), 0.1U DSN(dissolved in 25 mMTris-HCl, pH 8.0; 50% glycerol), 0.47 mg/ml ATA, sheared dsDNA (10 copies) to improve accuracy through introduction of potential competitive binders, 10nM probe and RNA template was incubated in a thermal cycler at 60°Cfor25min.Subsequently, 25μL10nMEDTA was added to the reaction mixture and incubated at 60°C for 5 min to inactive DSN enzyme. The fluorescence intensity was recorded at the end of the reaction and normalized RFU were plotted against genomic equivalents of Norovirus RNA. Controls containing no template RNA were used to check for cross contamination and used to normalize the minimal background fluorescent signal.

4.4 LARGE SCALE RNA PRODUCTION

Additional RNA viral target was generated from a DNA template using the RiboMAXLarge Scale RNA Production System (Promega, USA). To obtain the DNA template, extracted viral
RNA was amplified with primers flanking a 950nt genomic target (Table 2) using one step RT-PCR kit (Qiagen, USA). The total reaction mix was 25µl containing 50ng RNA template and 0.6µM each forward and reverse primers. Thermal cycling was performed as follows: 30 min at 50°C for reverse transcription, 15 min at 95°C for initial PCR inactivation of the reverse transcriptase and the initial activation of the polymerase, 40 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C and extension for 1 min at 72°C. The 950bp DNA product obtained was used in a second PCR reaction to attach a T7 promoter (Table 2) on the 5’ end of the amplicon to create a linear template suitable for in-vitro transcription.

The T7 DNA template was used to create the 950nt norovirus RNA replica including homologous regions for DNA probes and the primer and probe binding sites used by Hoehne et al. [73] for real time RT-PCR used for secondary detection and comparison by using the RibofMAX Large Scale RNA Production System according to manufacturer's protocol. The obtained RNA product represents approximately 14% of the total viral genome, containing some of the secondary structures that can be expected from the native Norovirus genome.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Location*</th>
</tr>
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<tbody>
<tr>
<td>NVGI950-Fwd</td>
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<td>4866-4885</td>
</tr>
<tr>
<td>NVGI950-Rev</td>
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<td>5815-5796</td>
</tr>
<tr>
<td>NVGI950-Fwd, T7</td>
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Genome location of primers are based on the sequence of Norwalk/68/US [Gen Bank: M87661].

### 4.5 VIRAL RNA EXTRACTION AND CONCENTRATION

Norovirus GI.1 RNA was kindly donated by Dr. Shay Fout (USEPA, Cincinnati, Ohio). The strain has been isolated from a human stool sample and contained 10⁶ virions/ml. Purification of viral RNA was carried out using QIAamp Viral RNA kit (Qiagen, USA) according to manufacturer’s protocol. The concentration and purity of RNA was determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA extracts were stored at -140°C in the presence of 1U/µL RNase inhibitor (Promega, USA).

Water samples (Little Miami River) were spiked with live norovirus particles ranging from 10⁴ to 10⁶ copies per liter of water. Skim milk solution (1% (w/v)) was prepared by dissolving 1g of milk powder (Difco) in 100ml of water and the pH was set to 3.5 with 1N HCl. The spiked water samples were acidified (pH 3.5) and contained 100ml of 0.2M phosphate buffer at pH 7.5 (1:2, v/v of 0.2M Na₂HPO₄ and 0.2M NaH₂PO₄). The concentrate was used for viral RNA extraction using QIAampViral RNA kit (Qiagen, USA) according to manufacturer's protocol.

### 4.6 RECOMBINANT DSN PRODUCTION IN E. COLI

The DSN gene was synthesized by Genscript (NJ, USA) with an N-terminal 6X His tag followed by a TRX tag to facilitate disulfide bond formation. Three mutant DSN enzymes were created [1] D361N;2) D362N; and 3) D361N, D362N] via site directed mutagenesis and confirmed by Sanger sequencing. Mutant DSN enzymes were expressed in SHuffle T7 Express Competent E. coli cells (New England Biolabs, MA, USA) to enhance disulfide bond formation and proper protein folding. Shuffele cells were grown in Overnight Express at 28°C for 18 hours and harvested at 12,000g for 20 minutes at 4°C. All protein purification steps were done on ice. Cell pellets were lysed in 50mMTris-HCl and 300mMNaCl pH 8.0 using a French press and cleared by centrifugation at 30,000g for 30 minutes at 4°C. Cleared lysate was applied to His60 Superflow Resin washed with 100 column volumes of a 50mMTris-HCl, 300mMNaCl and 20mM imidazole buffer, pH 8.0 and eluted with a 50mMTris-HCl, 300mMNaCl, and 500mM imidazole buffer, pH 8.0. Prior to use, purified enzyme fractions were incubated at 60°C for 15 min and centrifuged at 16,000g to remove any non-thermostable enzymes. DSN enzymes, concentration, molecular weight and purity was verified using SDS PAGE and Bradford assays, while folding was tested via functionality for desired duplex nuclease activity.

### 5. RESULTS AND DISCUSSION

For initial experiments, synthetic oligoribonucleotides (Table 1) representing norovirus genogroup I (GI) and II (GII) in 10-fold serial dilutions (10⁴ to 10⁵ oligo copies) were used for assay optimization and detection of norovirus RNA was carried out by modifying a method described earlier for microRNA [17].

The oligonucleotides were designed to have an annealing temperature of 60°C and were conducted at this temperature, enabling hybridization while the probe is intact and dissociation after probe cleavage, resulting in a temperature dependent fluorescent amplification of target RNA. Using the designed complimentary oligonucleotides, the fluorescent DSN assay showed variable sensitivities to the GI and GII norovirus targets (Figure 2). The optimized assay was unable to distinguish GI RNA at levels lower than 10⁵ genomic equivalents (Figure 2A) while GII RNA was detectable to 10⁴ genomic equivalents (Figure 2B) [46].

Due to the poor resolution observed for the lowest norovirus RNA levels and given that native DSN has limited efficacy on RNA templates longer than ~21nt, increased assay times and temperature cycling were explored to improve detection at lower levels; however, these remedies were ineffective (Figures 3 and 4).
Fig. 2: Plot of Norovirus DSN assay with RNA oligos for (A) Norovirus Genogroup I and (B) Genogroup II. 10-fold serial dilutions of RNA (10⁴ to 10⁰ genomic equivalents per assay) were plotted versus normalized enzyme activity ratios. Enzyme activity ratios for all assays were generated by normalizing the signal relative to the minimal fluorescence generated by the zero template controls. Error bars indicate standard deviations (n=3).

Fig. 3: DSN cycling data using G2 probe and 950bp template. DSN enzyme and the G2 probe were added to the 950nt genomic norovirus template in amounts of 10⁰-10⁴ copies (Figure S1). The temperature was cycled from 50°C for 1 minute to 60°C for 1 minute for a total of 12 cycles.

Fig. 4: Effect of longer incubation time on DSN activity using the G2 probe and the 950nt template. DSN enzyme and the G2 probe were added to the 950nt genomic norovirus template in amounts of 10⁰-10⁴ copies (Figure S2). The activity of the DSN enzyme at 60°C was monitored for 50 minutes.
Assay specificity was further explored by varying concentrations of the single stranded DNA probe to reduce fluorescent signal generated by nonspecific probe cleavage. All probe concentrations tested, 10nM, 30 nM and 50 nM, produced consistent results at varying copy numbers of the Norovirus RNA oligos (Figure 5A). A probe concentration of 10nM was chosen for future experimentation due to its superior sensitivity to varying RNA oligo copy numbers.

Further optimization efforts were undertaken using aurintricarboxylic acid (ATA) and Mg$^{2+}$. ATA was chosen due to its favorable binding outcomes to DD residues, a motif which forms the catalytic center for RNA polymerases and integrases [74, 75] and occurs three times within the DSN amino acid sequence [76]. It was found that 0.47mg/mL ATA provided optimum sensitivity for the fluorescent DSN assay (Figure 5B).

The effect of additional Mg$^{2+}$ in the assay was also examined, given that DSN as well as many other enzymes that act on phosphate containing substrates require magnesium ion for their activity. The impact of varying Mg$^{2+}$ concentration on DSN activity has been well documented [15]. Maximum DSN digestion activity is reached at ~20mM Mg$^{2+}$ and begins to reduce beyond this concentration [15]. Notably, increasing the amount of Mg$^{2+}$ in the reaction mix aided in improving the efficiency of detection in the presence of ATA, and a total Mg$^{2+}$ concentration of 10mM provided superior sensitivity for our norovirus assay (Figure 5C).

The final assay optimization investigated the effects of adding sheared dsDNA to our DSN assay. It has been reported that DSN can demonstrate minor activity against single stranded DNA when both DSN enzyme and substrate (DNA-RNA duplex) are present in high concentrations [27]. This activity, however, is not evident in the presence of competitive dsDNA [27]. Therefore, sheared dsDNA was added to the fluorescent DSN assay to minimize cleavage of unbound ssDNA probe, resulting in non-specific fluorescent signal. It was found that 10 sheared copies of a 1kb DNA sequence resulted in the most consistent results for discrimination between samples (Figure 5D).

After this optimization, we sought to test our improved method on longer RNA targets. We chose a 950nt portion of the
norovirus genome to test the effects of secondary structure on the DSN assay, since this provides a more realistic target size while minimizing exposure to the full virus. The sequence corresponding to norovirus GI was chosen due to its prevalence in waters around Cincinnati. The RNA product contains approximately 14% of the total viral genome, and includes homologous regions for DNA Taqman probes used for our initial DSN assay, as well as the primer and probe RT-qPCR binding sites used by Hoehne et al. [73]. Through the addition of ATA, Mg$^{2+}$ and sheared dsDNA, increased separation for lower norovirus copy numbers was achieved (Figure 6).

In order to verify the efficacy of the optimized assay we performed an experiment spiking environmental water with norovirus particles ($10^0$-$10^4$ particles per liter of water) and extracted the total RNA to mimic a real world testing scenario. In this way the performance of DSN could be evaluated in RNA-water extracts replete with non-target RNA and natural inhibitors which co-elute with the extraction (Figure 7). Performed as a blind test, the concentration of norovirus particles was correctly identified in 89% of the trials with no false positives. RT-qPCR, using the primers and probe developed previously [73], was used as a comparison to test the accuracy of our DSN based quantification method (Figure 8). Larger variation in the $C_t$ values of the RT-qPCR assay were observed (Figures 7 and 8), indicating the higher sensitivity of the DSN assay for the same RNA samples under our operating conditions. Recently, advanced PCR techniques such as digital PCR have been described for absolute RNA quantification [77], but the high equipment cost and time limits the applicability of such techniques. The total reaction time for the presented DSN strategy is less than 30 minutes, compared to the several hours required for RT-qPCR [78], and can be achieved on a simple heat block, rather than a more costly thermocycler.

The assay was independently verified in the lab of Dr. Shay Fout, USEPA, Cincinnati, Ohio (Figure 9). The lower limit of detection was found to be 10 copies per assay, below the minimum concentration required for infection (18 viral copies) [46, 79, 80]. While variation was seen in independent testing for samples containing $10^3$ copies, a reliable separation between 10
and 100 virions was observed, with the most significant signal separation between $10^2$ and $10^3$ viral particles.

Fig. 9: Plot of results obtained through independent verification of the DSN assay at the USEPA. 10-fold serial dilutions of RNA (100 to 104 genomic equivalents per assay) of Norovirus GL. Enzyme activity ratios were generated by normalizing the signal relative to the minimal fluorescence generated by the zero template controls. Error bars represent the standard deviation (n=3).

5.1 Rational Design of DSN Mutants

To engineer the desired enzyme function without the necessity of ATA or other additives, the 3rd DD site (green/red in Figure 10), corresponding to amino acids 361-362, was chosen as the target for mutagenesis. This cluster was chosen because of the classic helix-turn-helix motif (right, Figure 10) in the predicted structure, which is a common DNA interacting domain [81-85]. While both helices contain DD sites, the shorter of the two was chosen for the D to N mutations, since the longer “recognition” helix is often the DNA interacting domain [86, 87]. The Asp amino acids were specifically targeted based on the previous theoretical work with ATA, as well as evidence that these amino acids complex with Ca$^{2+}$, to mediate phosphate interactions of the nucleic acid backbone [86, 87]. Three separate mutants, D362N, D361N, and D361N D362N, along with an unmutated version (DD), were produced in E. coli and screened for nuclease activity using the optimized conditions developed for the commercial enzyme. Enzyme activity ratios for all mutants were determined by normalizing the minimal fluorescent background signal generated by the zero template controls and obtaining the ratio of signal generated in the absence of ATA to the signal generated in the presence of ATA. All enzymes produced in E. coli retained some nuclease activity; however, D361N was found to be the most effective variant, demonstrating selective DNA nuclease activity in the absence of ATA for both the 950nt RNA target (Figure 11) and the shorter 25nt RNA oligo at $10^4$ copies (Figure 11). The high enzyme activity ratios (all $> 4$) indicate improved activity in the absence of ATA, in some cases over 180x greater for the D361N mutant. Surprisingly, the unmutated DD enzyme produced in E. coli also showed improved activity in the absence of ATA, unlike the commercial DSN enzyme. Since norovirus cannot be synthesized via cell culture, and must be purified from the feces of an infected mammalian host, obtaining sufficient quantities of norovirus source material is a challenge, limiting our mutant DSN experiments to synthetic RNA targets.

Fig. 10: Predicted crystal structure of DSN, generated using PyMOL and I-TASSER [88-90]. D amino acid mutation sites are highlighted in green.

Fig. 11: Fluorescent signal ratios for equimolar DSN concentrations in the presence and absence of the ATA inhibitor for A) the 950nt Norovirus genomic fragment and B) the GII RNA oligo. The fluorescent signal ratio was calculated using the ratio of the signal of the non-ATA containing reaction to the reaction containing ATA after normalizing to the zero template controls. Both RNA templates were used at copies. Error bars represent the standard deviation of at least 3 samples.

6. ACKNOWLEDGEMENTS

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7. CONCLUSIONS

The advantage of targeting viral RNA directly provides the opportunity to extend our system to other RNA pathogens such as Influenza, Ebolavirus, Zika, Hepatitis, and Chandipura viruses. Furthermore, DSN has been largely limited to short RNA targets, thus the optimization of our reaction with the use of ATA was important for enabling longer targets. The optimization with ATA ensured specific hybridization of the probes to the long RNA targets and enabled the rational design of DSN mutants that allow for duplex-specific nuclease activity on longer RNA targets. Future work will explore improved DSN mutants beyond the limited number of preliminary mutations to further increase enzyme functionality.

The described method has the opportunity to surpass RT-qPCR as a faster, more cost effective RNA detection scheme and, when multiplexed with different colored Taqman probes, a more versatile approach. The isothermal RNA assay we have developed will find utility where thermocyclers are either too costly or unavailable, enabling future low cost RNA and viral detection sensors.

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Conflict of Interests: There are no conflicts of interest.

8. REFERENCES


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