**Table S1** List of primers, gDNAs and probes used in this study.

| Target | Prime | Sequence(5'-3') | Usage |
|---|---|---|---|
| **RRSV** | RRSV-t7-F | TaatacgactcactatagggATCCATCGACTTGGTTTAGCCAA[a] | PCR amplification of templates for in vitro transcription |
| | RRSV-t7-R | ATAGGATTAGTGATGCTTCCACAG | |
| | RRSV-RPA-F | CGAATCATCACTGAACAAGTATTTGGAGCT | RPA amplification of target sequence |
| | RRSV- RPA-R | ATTGACGAGTCCTCTGGCGGAATGGATGGT | |
| | RRSV-g1 | P-CATTGATAAGACCGATC[b] | Guide pfAgo to a specific target sequence that subsequently cleaved by pfAgo |
| | RRSV-g2 | P-AACTGAATACAACCGAT[b] | |
| | RRSV-g3 | P-GGTATCGGTTGTATTCA[b] | |
| | RRSV-secondary-g4 | P- GTATTCAGTTGATCGGT[b] | gDNA produced from first round cleavage |
| | RRSV-Probe | FAM-cgcaccACCGATCAACTGAATACggtgcg-BHQ1[c] | Molecular beacon |
| **RGSV** | RGSV-t7-F | taatacgactcactatagggAGCATCCTTCAGATCAGCTGTAT[a] | PCR amplification of templates for in vitro transcription |
| | RGSV-t7-R | CTAAAGCTCAGAAGGATAAGCACA | |
| | RGSV- RPA-F | TTTTATGCACTCAATAGCTAATCTGAACAG | RPA amplification of target sequence |
| | RGSV- RPA-R | ACACTAAGTCAGCAATGATAACACACTGCA | |
| | RGSV-g1 | P-GAGATCATCCTTCTACC[b] | Guide pfAgo to a specific target sequence that subsequently cleaved by pfAgo |
| | RGSV-g2 | P-AGCTATCTTATCATCA[b] | |
| | RGSV-g3 | P-TGTCTGATGATAAGATA[b] | |
| | RGSV-secondary-g4 | P-TAAGATAGCTGGTAGAA [b] | gDNA produced from first round cleavage |
| | RGSV-Probe | VIC-cgcaccTTCTACCAGCTATCTTA ggtgcg- BHQ1 [c] | Molecular beacon |
| **RBSDV** | RBSDV-t7-F | taatacgactcactatagggCATGGCAGGTTAAATCTAAAGTT[a] | PCR amplification of templates for in vitro transcription |
| | RBSDV-t7-R | CGCTCAACACTTCGCCAATTTTAC | |
| | RBSDV- RPA-F | TGATGATCCAGATGAATATGAATTGACCA | RPA amplification of target sequence |
| | RBSDV- RPA-R | CATTTCGCCTTTATGAGTTTGAACTACAAC | |
| | RBSDV-g1 | P-CACGTTGCGCACTAATT[b] | Guide pfAgo to a specific target sequence that subsequently cleaved by pfAgo |
| | RBSDV-g2 | P-ACGATGGCACCTCTGCT[b] | |
| | RBSDV-g3 | P-AGAAGCAGAGGTGCCAT[b] | |
| | RBSDV-secondary-g4 | P-GTGCCATCGTAATTAGT[b] | gDNA produced from first round cleavage |
| | RBSDV-Probe | ROX-cgcaccACTAATTACGATGGCAC ggtgcg- BHQ2 [c] | Molecular beacon |

[a] The sequence shown in lower case letters represent the T7 promoter sequence
[b] 5'-phosphorylated ssDNA
[c] Lowercase letters represent hairpin sequences in probe

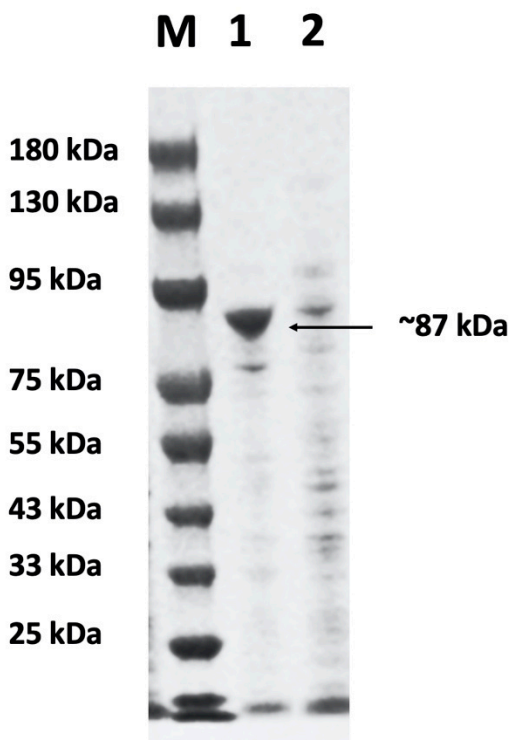


**Figure S1**: Analyzing PfAgo with SDS-PAGE after purification. M: pre-stained protein marker Lane1: purified PfAgo; Lane2: flow-through; The PfAgo protein band was indicated with a black arrow.

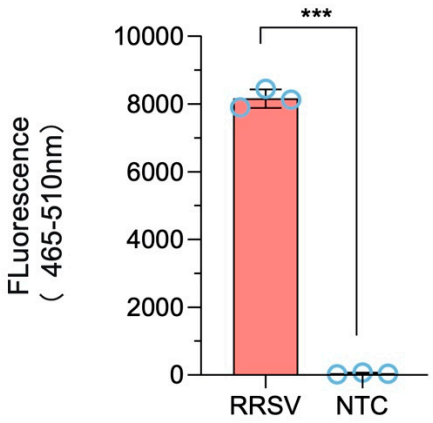


**Figure S2**: Endpoint fluorescence signal for RRSV detection. NTC: non-template control. ***P < 0.001.

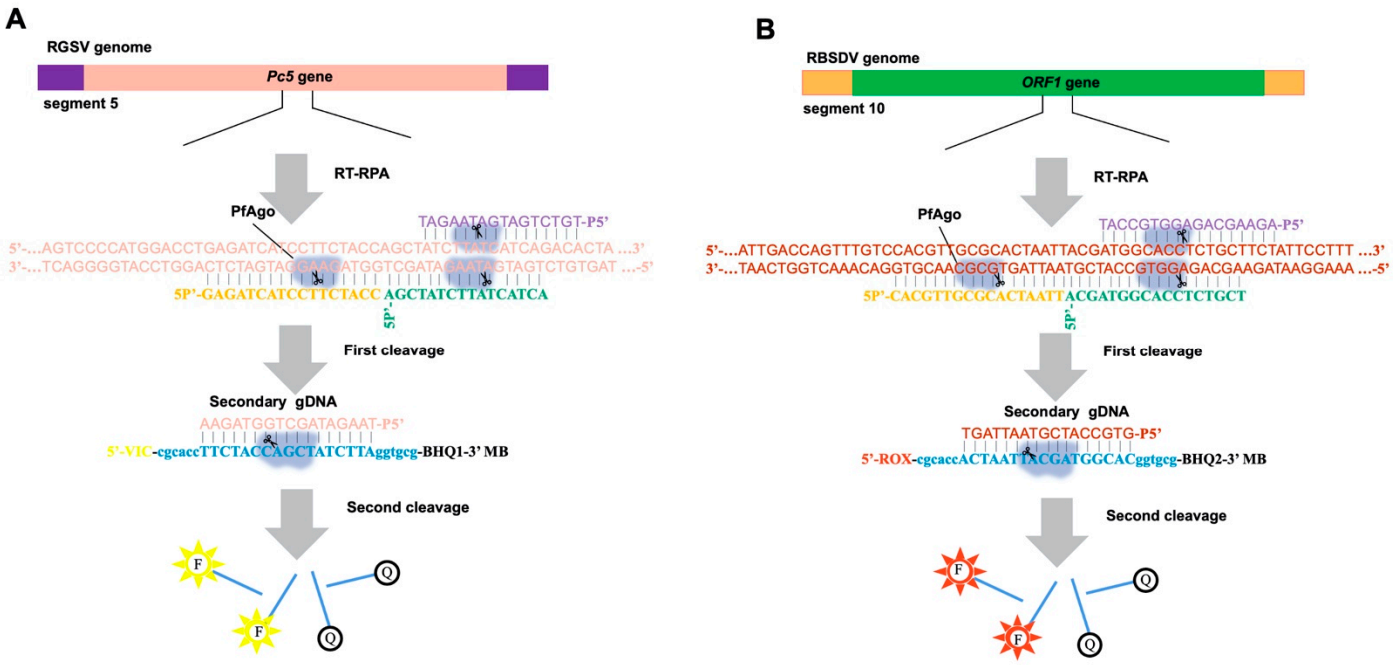


**Figure S3**: Diagram illustrating the gDNAs and specific probes designed for detecting RGSV (A) and RBSDV (B). Three 5-phosphorylated single-stranded DNA guides are depicted in shades of purple, green, and yellow. Molecular beacons are represented and emphasized in blue.

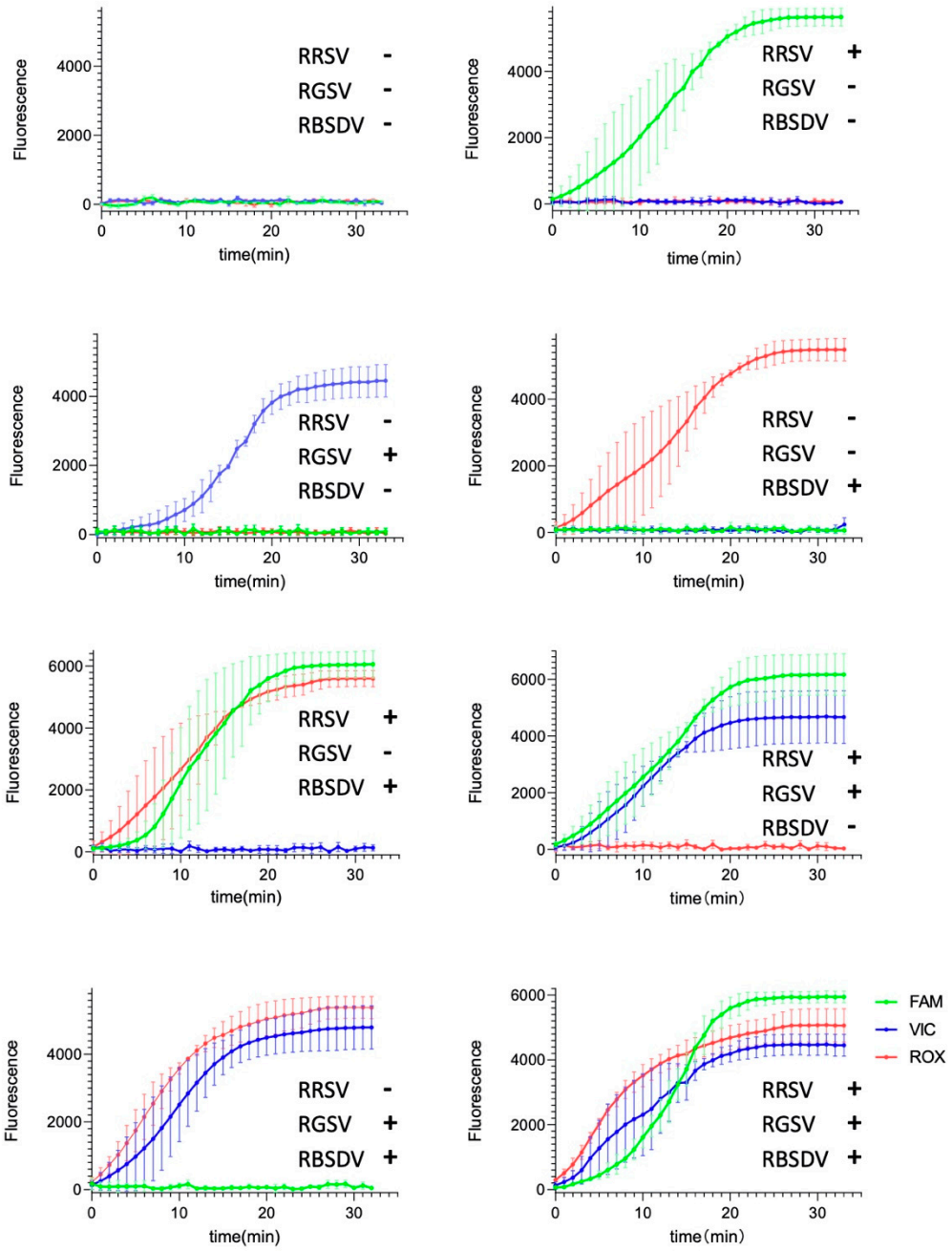


**Figure S4**: Evaluation of the multiplex detection capability of the RT-RPA-PfAgo assay. The assay's capacity to detect RRSV, RGSV, and RBSDV simultaneously was evaluated by utilizing single, double, and triple mixtures comprising 100 copies of reference RNA from each virus. The fluorescence curve obtained by applying the RT-RPA-PfAgo method is evidence for successfully identifying RNA targets.