

# Development of a Rapid, Cost-effective, RT-PCR Based Method to Detect Intergenogroup Reassortant Human Rotavirus

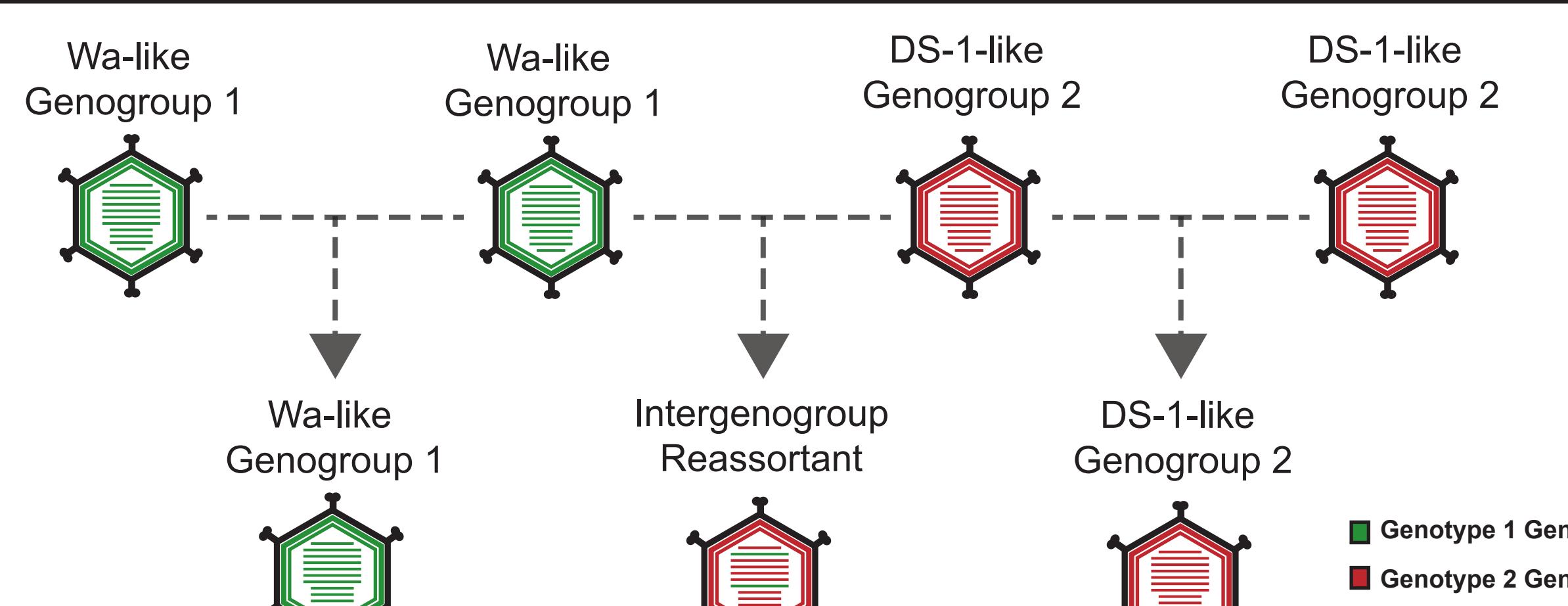
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## Abstract

Complete genome nucleotide sequencing studies have provided evidence for the existence of two dominant human rotavirus (RV) genogroups. Wa-like genogroup 1 RVs exhibit genotype 1 internal protein gene constellations (ie. I1, R1, C1, M1, A1, N1, T1, E1, H1). In contrast, DS-1 like genogroup 2 RVs show genotype 2 internal protein gene constellations (ie. I2, R2, C2, M2, A2, N2, T2, E2, H2). Gene reassortment can occur between genogroup 1 and 2 strains during co-infection; however, the prevalence of intergenogroup reassortants in nature has yet to be systematically analyzed. In this study, we developed an RT-PCR method to distinguish between genotype 1 vs genotype 2 genes of human RVs for the VP1-, VP2-, VP3-, NSP1-, NSP2-, NSP3-, and NSP5/6-coding segments. Oligonucleotide primers designed against conserved gene regions were first used in RT-PCR reactions to convert viral RNAs to cDNAs in a genotype independent manner. Thereafter, genotype 1- or 2-specific primers designed against variable regions were used in traditional PCR reactions with the cDNA templates. Genotype 1 and 2 PCR amplicons differ in size by >50 bps and were easily differentiated following agarose gel electrophoresis. We tested our method on several cell culture adapted human and animal strains with known genotype constellations. The results showed that PCR amplicons of the expected sizes and sequences were generated, indicating that genotype-specific primers recognized their cognate cDNA templates. We anticipate that this RT-PCR method will allow for rapid and cost effective screening of clinical fecal specimens, thereby revealing the extent to which intergenogroup reassortant RVs infect and cause disease in humans.

**Can RT-PCR be used to quickly and accurately genotype all genes of human RV strains?**

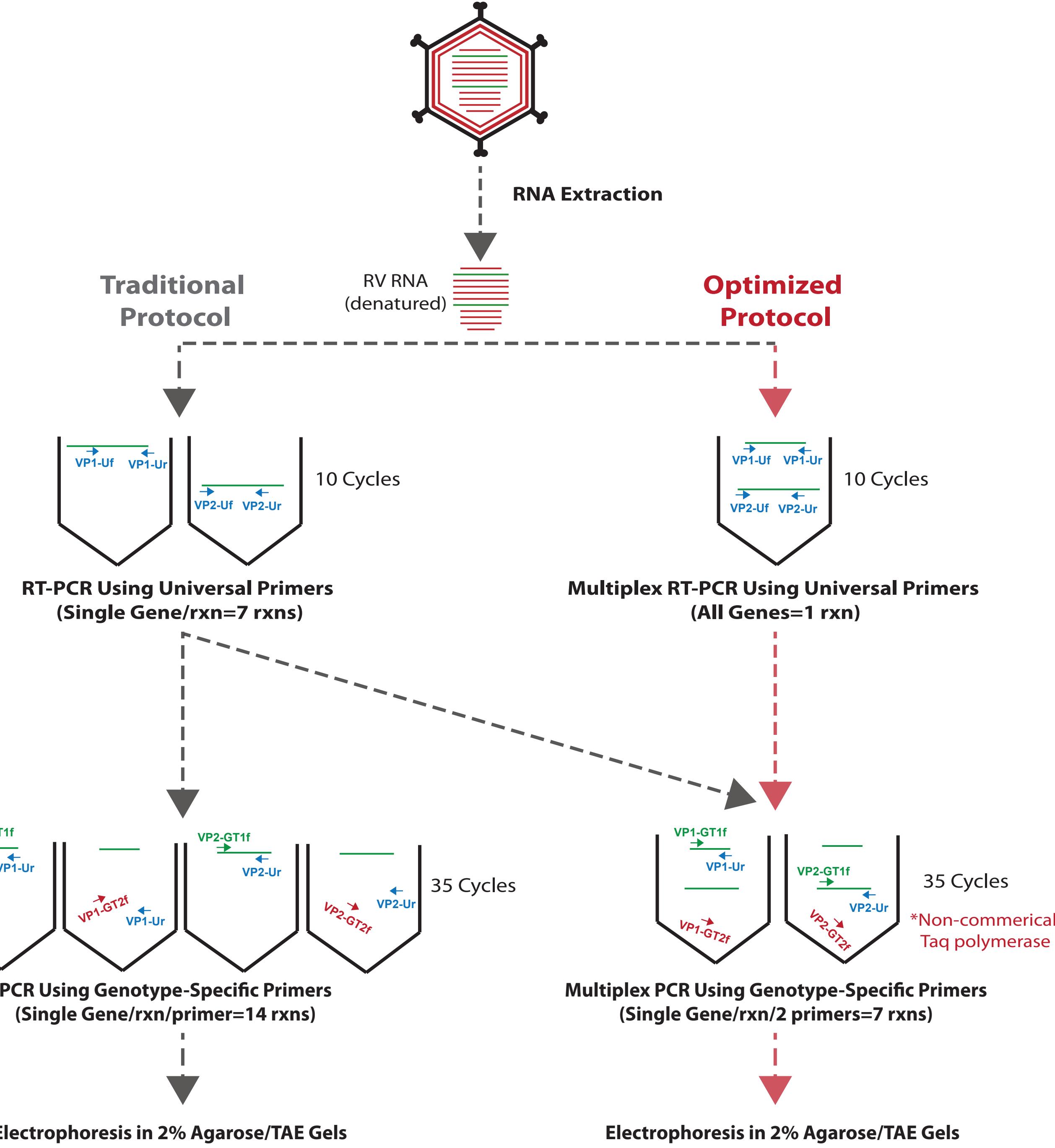


**How common are intergenogroup reassortant human RVs?**

## Aims of the Study

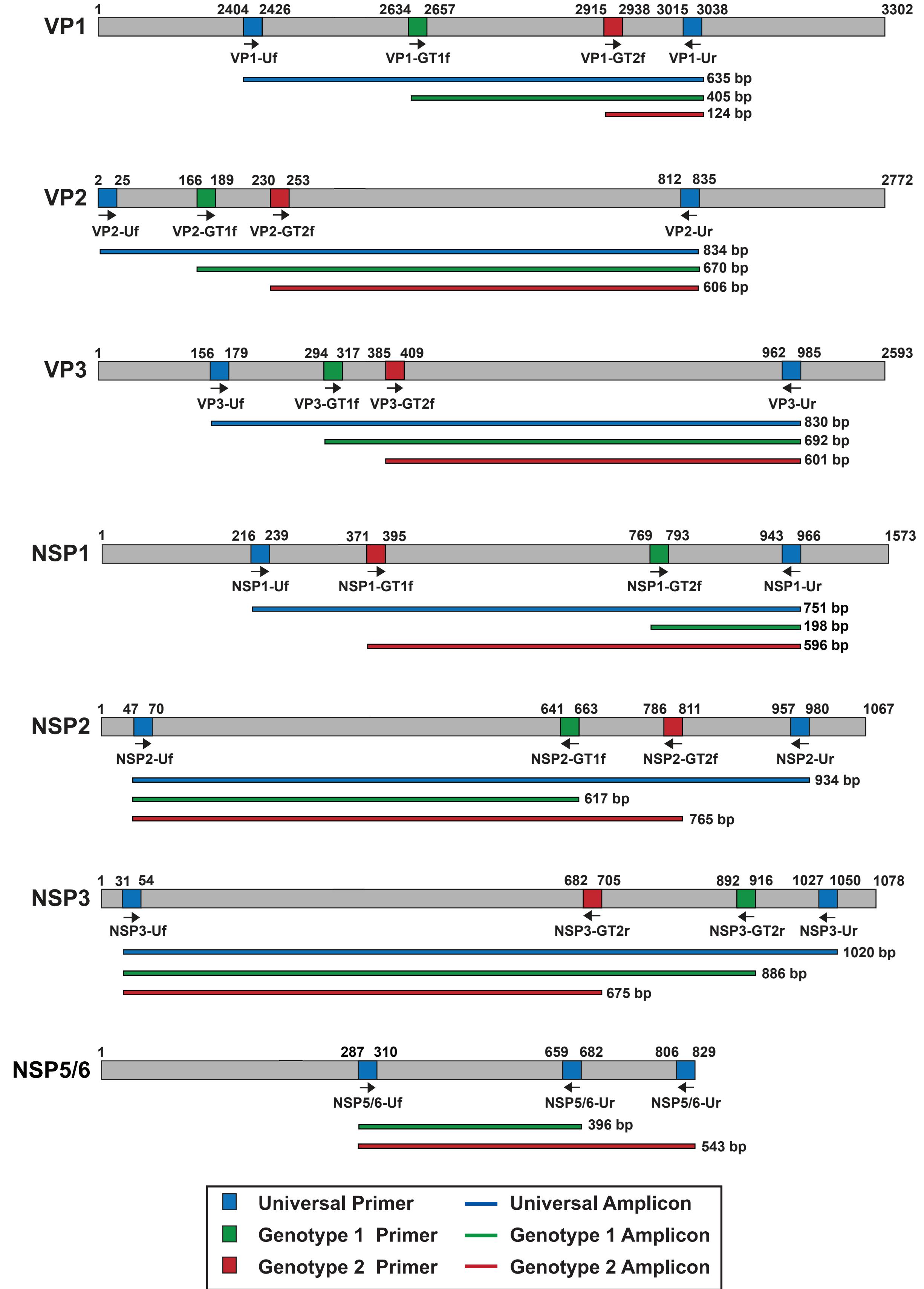
- 1) Develop universal and genotype-specific oligonucleotide primers for the VP1-, VP2-, VP3-, NSP1-, NSP2-, NSP3-, and NSP5/6-coding gene segments of human RV strains.
- 2) Test the specificity of the primers in uniplex and multiplex RT-PCR/PCR reactions using the RNA of genogroup 1 and 2 prototype strains (Wa and DS-1, respectively).
- 3) Validate this RT-PCR/multiplex PCR approach by genotyping common cell culture adapted human and animal strains.
- 4) Determine the feasibility of combining genes in the reactions and using non-commercial Taq in place of commercial polymerase for multiplex PCR to improve cost-effectiveness.

## Overview of Experimental Design



Schematic showing the overall experimental design and protocol optimization. Briefly, RV was grown in MA104 cells and RNA was extracted using Trizol/isopropanol precipitation. Purified RNA was then combined with DMSO and denatured by incubation at 95 degrees C for 10 minutes prior to being used for RT-PCR. 10 cycles of RT-PCR was conducted using Superscript™ One-Step RT-PCR with Platinum® Taq kit (Life Technologies) and universal primers. cDNA products from RT-PCR reactions were then used as a template for 35 cycles of PCR using Accuprime Pfx. Both universal (control-not shown) and genotype-specific primers were used to amplify cDNA in the PCR reactions. Approximately 10 µl of cDNA products from PCR were electrophoresed in 2% agarose/TAE gels and bands were visualized using an ethidium bromide stain. During protocol development, PCR amplicons were gel extracted and sequenced. The protocol was optimized by combining genes at the RT-PCR stage and by using less expensive versions of Taq.

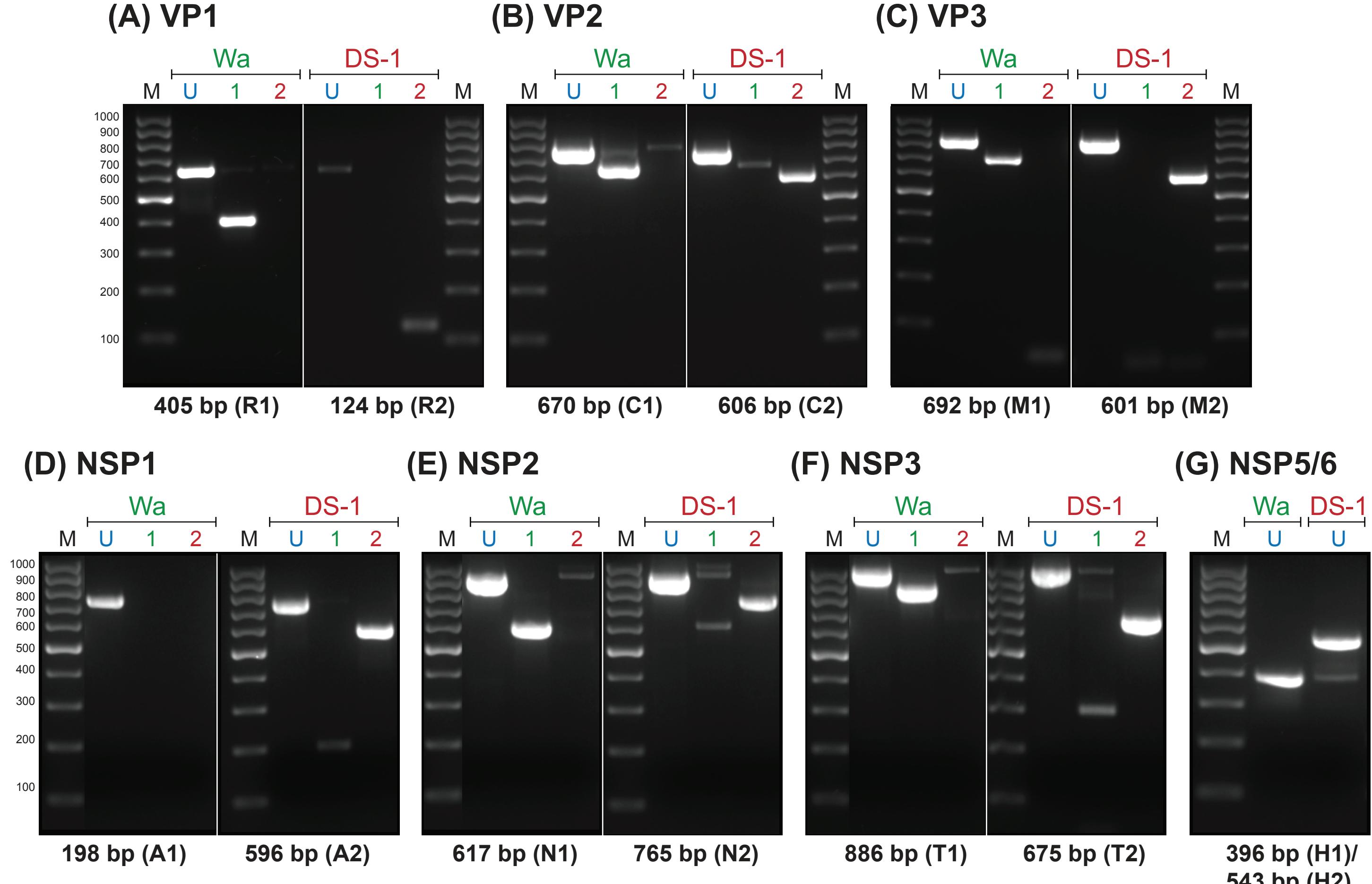
## Schematic of Universal and Genotype-Specific Primer Locations and RT-PCR/PCR Amplicons



Schematic showing the locations of the universal and genotype-specific primers and the expected sizes of RT-PCR/PCR amplicons. The approximate nucleotide numbers are indicated for each gene, as is the direction of the primer. To design the primers, comprehensive nucleotide alignments were performed using the nucleotide sequences of published human RV genotype 1 and 2 genes. Universal primers were designed against conserved regions, while regions of each gene that showed considerable intergenotypic variation (7 to 17 mismatched base pairs when compared to non-cognate genotype), but that remained conserved within a genotype, were targeted for genotype-specific oligonucleotide primer design.

**Can the specific primers distinguish between genotype 1 and 2 RV genes?**

## Initial Testing of Primer Specificity

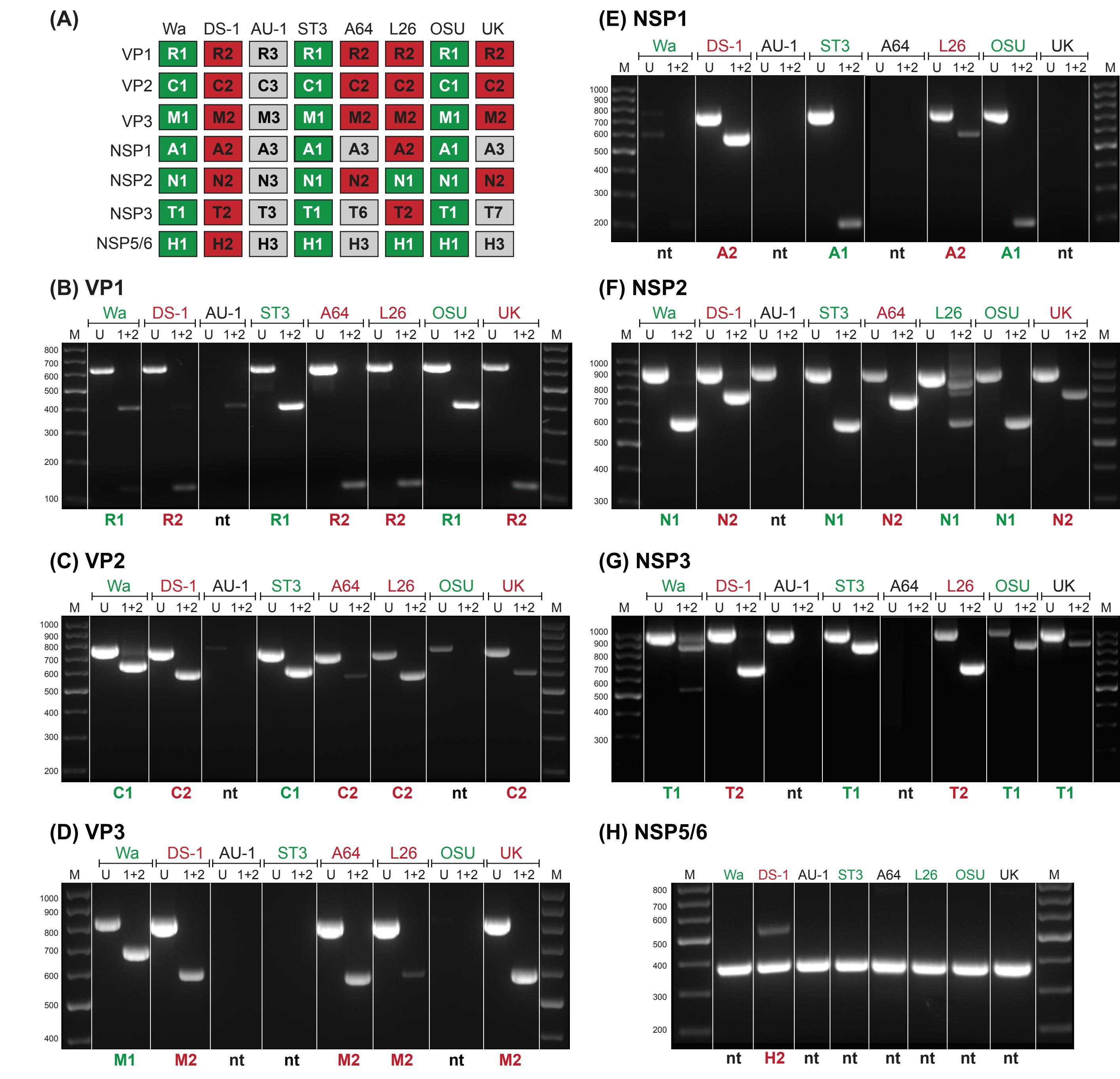


RT-PCR reactions were performed using universal forward and reverse primers with Wa or DS-1 RNA to create cDNA, followed by 10 cycles of amplification. Next, a portion of the cDNA product was used as template for uniplex PCR amplification with either universal (U), genotype 1 (1), or genotype 2 (2) specific primers. The results show that each genotype-specific primer amplifies cDNA of the expected size, suggesting they are able to specifically and efficiently recognize their cognate gene. \*Note that a specific primer is not necessary to differentiate genotype 1 vs 2 NSP5/6 genes. That gene segment is naturally longer for DS-1-like genotype 2 genes viruses and can be distinguished through use of universal primers only.

**Primers amplified cDNAs of the appropriate sizes using the correct genotype templates (i.e. Wa or DS-1).**

## RT-PCR/Multiplex PCR:

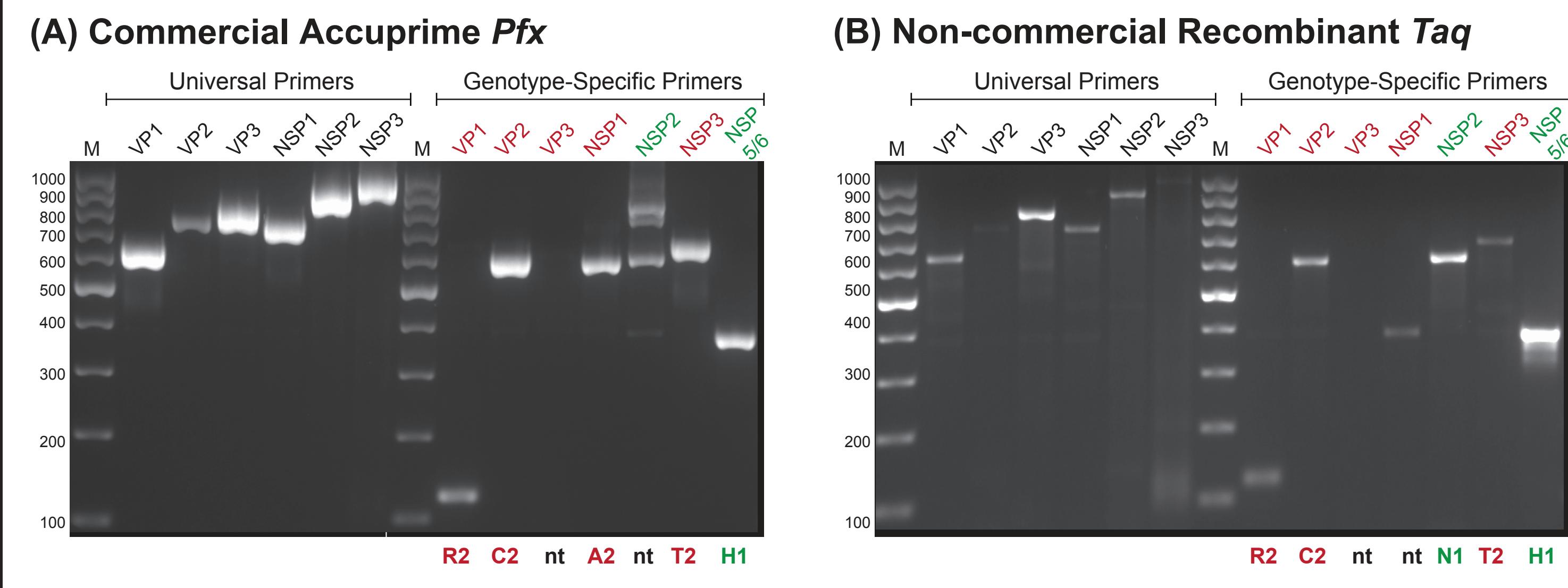
### Cell Culture Adapted Human and Animal RVs



To further validate the specificity of our primers, we tested them on RNA extracted from human and animal RV strains with known genotype constellations (A). In these experiments, RT-PCR and universal primers were used to create cDNA from viral RNA. Next, multiplex PCR using genotype 1- and 2-specific primers were used to amplify that cDNA. (B-H) Results show that the majority of the genotype 1 and 2 genes as well as some genotype 3 genes were amplified by RT-PCR in the presence of the universal primers. Genotype 1-specific primers produced PCR amplicons of expected sizes for most genotype 1 genes as well as genotype 7 genes. Genotype 2-specific primers produced PCR amplicons of the expected sizes for most genotype 2 genes.

**Primers differentiate between genotype 1 and 2 genes for various RV strains in multiplex PCR reactions.**

## Multiplex RT-PCR/Multiplex PCR: Commercial vs. Non-commercial Polymerase



Multiplex RT-PCR reactions were performed by combining all universal primers with strain L26 RNA in a single reaction to create cDNA, using 10 cycles of amplification. Next, a portion of the cDNA product was used as template for multiplex PCR amplification with either commercial (A) Accuprime Pfx or (B) non-commercial recombinant Tag. Results show that both commercial and non-commercial enzyme amplified cDNA of the expected size using universal primers. VP1, VP2, NSP3, NSP5/6 genotype-specific primers produced the expected amplicons using commercial and non-commercial enzyme. The genotype 2 VP3-coding gene was not amplified with either enzyme. The genotype 2 NSP1-coding gene was not amplified using recombinant Tag. NSP2 amplification using genotype specific primers showed nonspecific amplicons using Accuprime Pfx.

**Optimization of this genotyping method at the RT-PCR and PCR steps will allow for substantial cost savings.**

## Conclusions

- 1) We have designed and validated primers that distinguish between the two most common RV genotypes (1 vs. 2) for seven gene segments (encoding VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP5/6).
- 2) In conjunction with previously published primer sets for the VP7-, VP6-, VP4-, and NSP4-coding genes, an RT-PCR based approach for complete genome genotyping now exists. (See also Gouvea et al., 1990; Gentsch et al., 1992; Lin et al., 2008; Rodriguez-Diaz et al., 2008)
- 3) This approach is expected to be a time- and resource-efficient way to screen fecal specimens for the presence of intergenogroup reassortants.

## Acknowledgements

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