Identifying and characterizing Hepatitis C virus mixed infections using a novel deep sequencing-based approach

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Background

Hepatitis C virus (HCV) infection does not elicit a complete protective immune response[1] thus, individuals can be **infected with multiple genotypes or subtypes**. Mixed HCV infections have been observed in **0% to 39% of high-risk** populations[2] and pose a challenge to genotype-specific therapies and vaccine design. More reliable and **standardized methods are needed** to increase our understanding of the prevalence and impact of mixed HCV infections.

Research Goals

- Optimize deep sequencing strategy to detect mixed infections and accurately determine relative genotype proportions
- Develop strict cutoff criteria to eliminate false positive results
- Apply methodology to high-risk HCV infected cohorts

Samples

- ***Artificially mixed Samples:*** Mixed, de-identified clinical samples of known HCV genotypes
- ***ACTIVATE*** [3]: International trial of response-guided PegIFN/RBV therapy in people with HCV G2/3 (96% history of IDU, 73% within 6 months and 59% within one month of enrollment; no HIV)
- ***Dare-C II*** [4]: Australian trial of 6 week sofosbuvir and ribavirin treatment in acute/recent HCV infection (84% history of IDU, 58% within 6 months of screening; 74% HIV)
- ***Canadian Co-infection Cohort (CCC)*** [5]: HIV/HCV co-infected individuals in Canada (mixed infection suspected)

Table 1 - Artificially mixed genotype samples used for testing and validation of mixed genotype methodology

Genotype Mixes	Ratios	Sample Number
1a:1b, 1a:2b, 1a:3, 1a:6, 2b:4, 3a:4, 3a:6, 5:6, 2:3	50:50, 90:10, 10:90, 98:2, 95:5, 5:95, 2:98, 100	72

Table 2 - Clinical samples used for application of mixed-genotype methodology

Cohort	Sample Number	Number of Individuals	Genotypes (Sample Number)
ACTIVATE	114	90	G3 (100); G2 (13); G1a(1)
DARE-C II	26	18	G3 (5); G2 (1); G1a (20)
CCC	3	2	G1a/1b rec (1); G1a/1b mix (1)

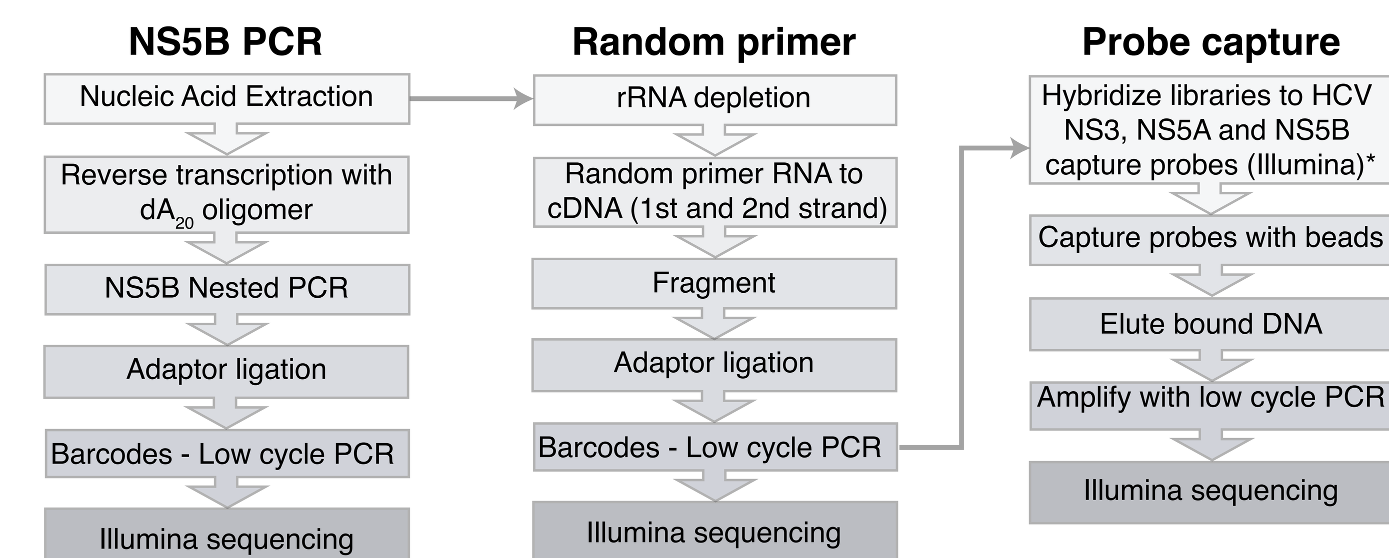

Figure 1. Deep sequencing library preparation methods. Random primer takes ~ 2 days and costs $285 / sample (8 samples / run). Probe capture takes ~3.5 days and costs as little as $165 / sample (24 samples / run). Sequencing is on Illumina MiSeq (2x250 cycle v2). *Probes were custom designed by Illumina to target HCV NS3, NS5A and NS5B and to capture HCV diversity based on over 2400 HCV reference sequences from 7 HCV genotypes and 62 subtypes.*

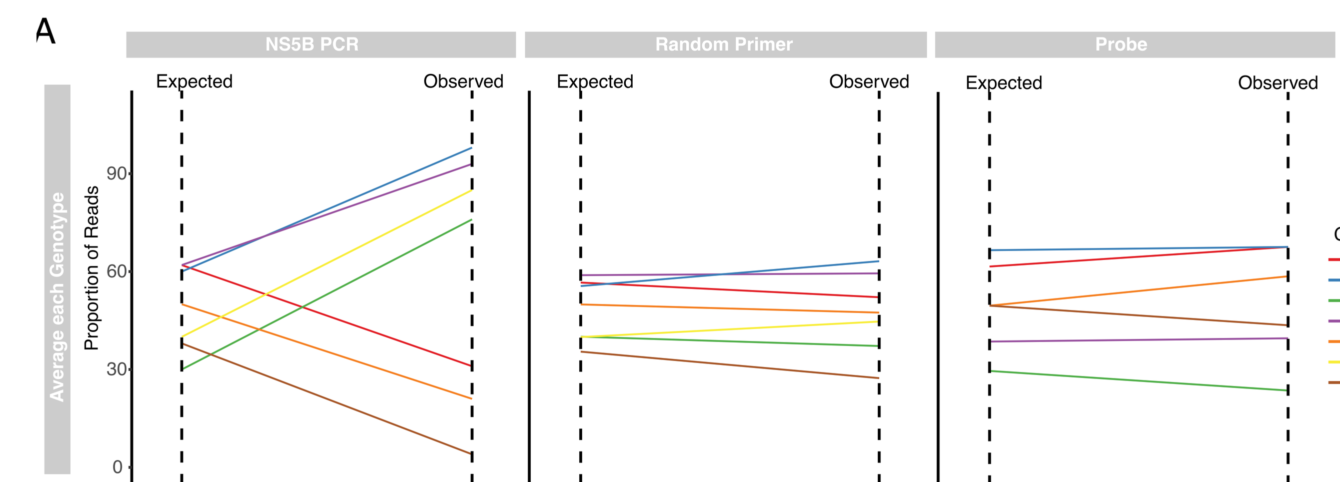

Figure 2. Mean proportion of expected and observed HCV reads for various genotypes in artificially mixed samples sequenced following NS5B PCR, randomly primed cDNA synthesis and probe capture.

NS5B Amplicon sequencing preferentially amplified G1b, G2, G3 and G5 in artificially mixed samples. No genotype bias was observed with Random Primer and Probe Capture sequencing (Figure 2).

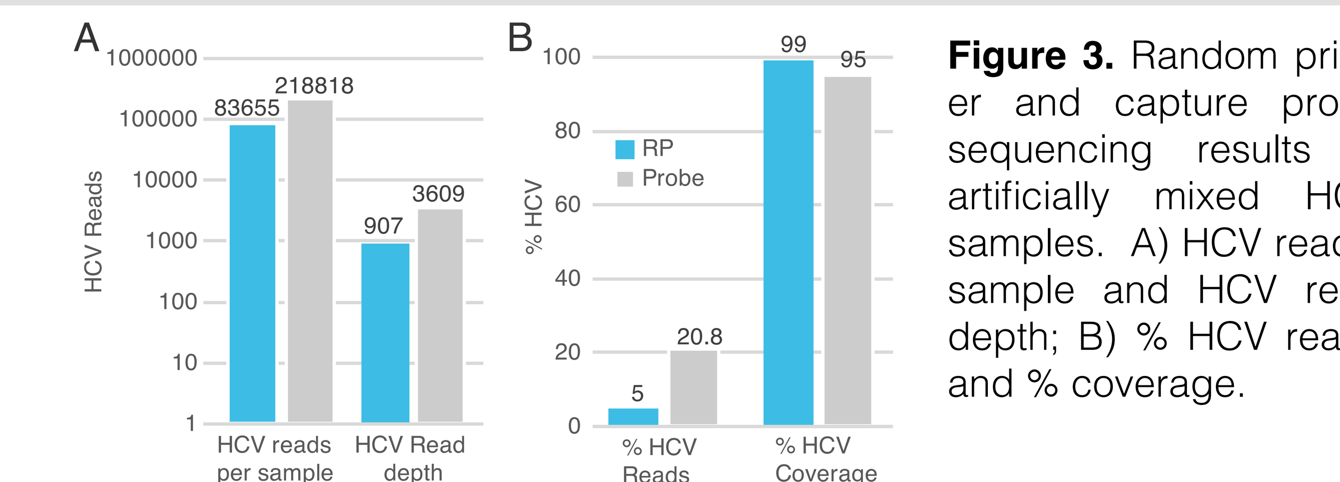

Figure 3. Random primer and capture probe sequencing results in artificially mixed HCV samples. A) HCV reads/ sample and HCV read depth; B) % HCV reads and % coverage.

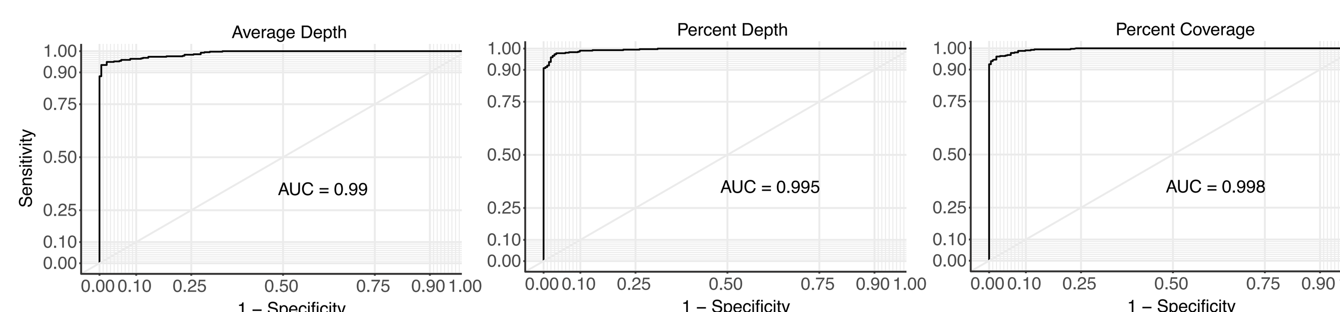

Figure 4. Receiver Operating Characteristic (ROC) curve analysis of 64 artificially mixed genotype HCV samples. AUC = Area Under the Curve.

Based on ROC Curve Analysis (Figure 4), mixed infection HCV genotypes should have a minimum percent depth of 1.8% (out of total HCV depth), minimum percent coverage of 98.2% and an average depth of 39.2 reads per position.

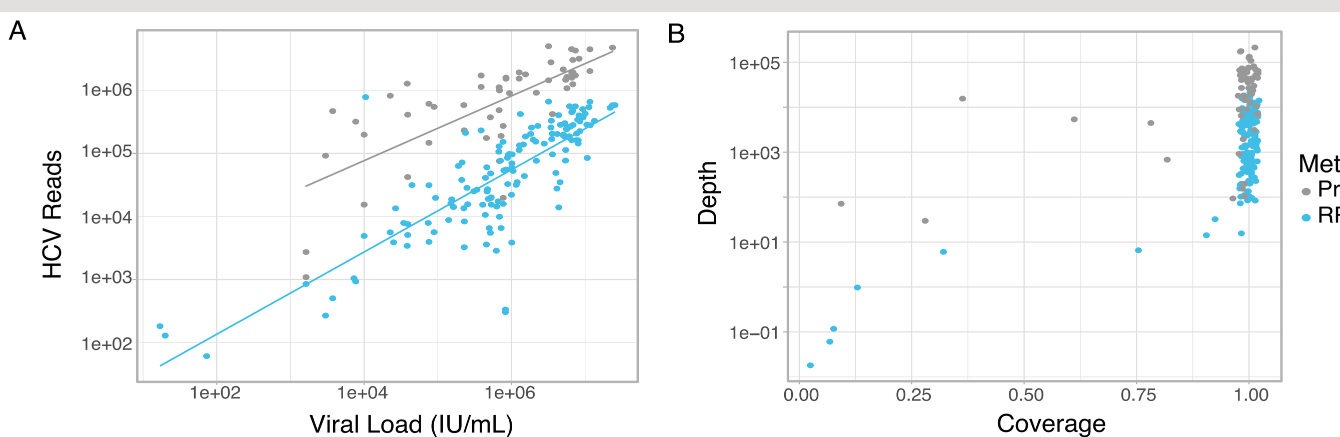

Figure 5. Random primer and probe capture sequencing of ACTIVATE and DARE-C II samples. A) HCV reads vs viral load; B) coverage and depth.

On average, 2% and 1% of HCV reads mapped to a secondary genotype for Random Primer and Probe Capture sequencing, respectively.

None of the secondary genotypes in ACTIVATE or DARE-C II samples passed the mixed infection cutoff criteria.

Phylogenetic Analysis of ACTIVATE and DARE-C II samples confirmed no mixed infections were present (Figure 6). One CCC sample (1_CCC) was confirmed to have a mixed infection (based on cutoffs and phylogenetics) while the other (2_CCC Time Point 1 and 2) contained a recombinant genotype.

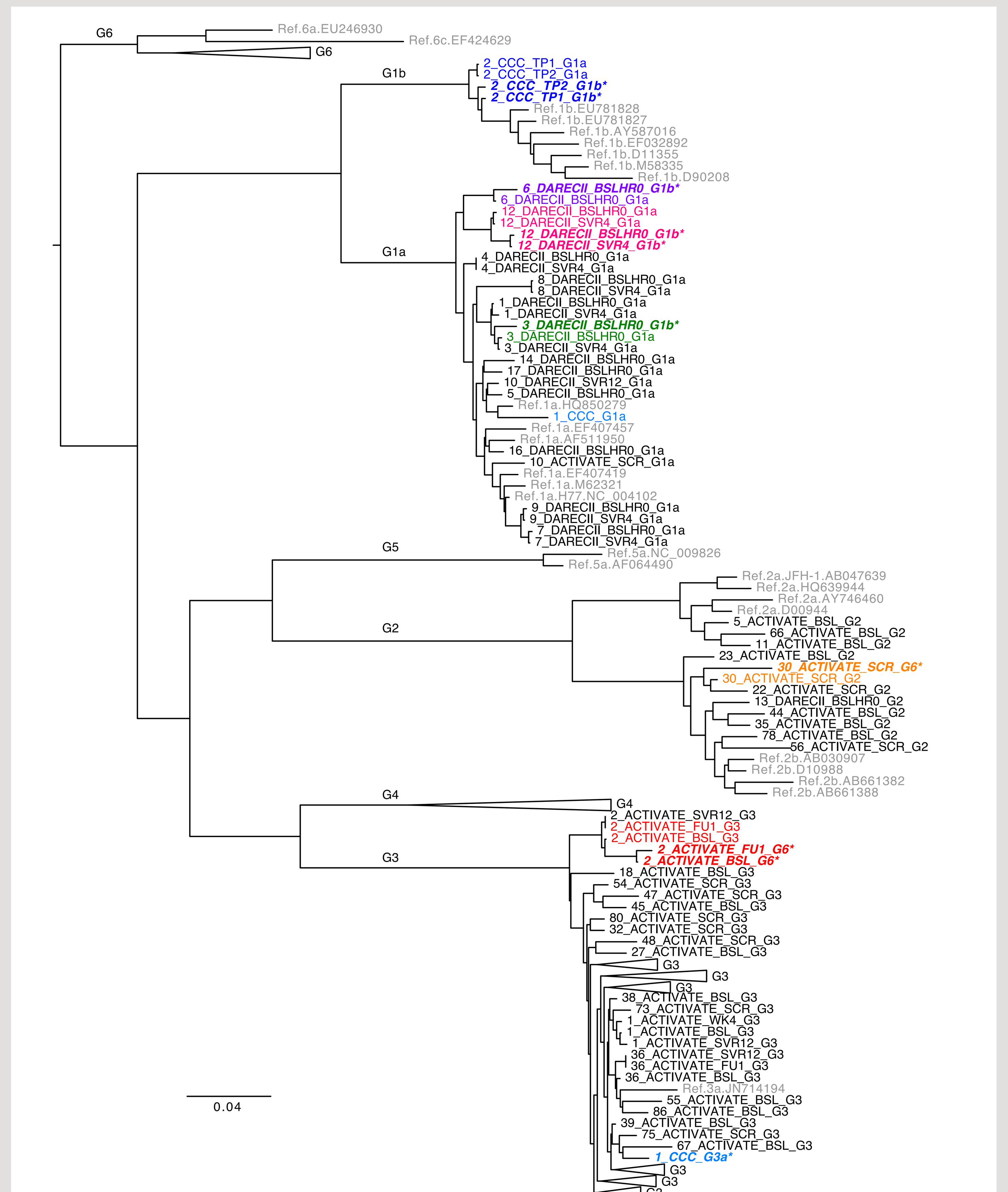

Figure 6. Phylogenetic analysis of primary and secondary NS5B consensus sequences from random primer sequencing of clinical samples. Putative mixed infections are highlighted with different colors. Potential secondary genotypes are bold-italicized with an asterisk. Genotype = G; screening = SCR; baseline = BSL; treatment week 4 = WK4; end of treatment = ETR; 4, 12, 24 weeks post-treatment = SVR4, SVR12, SVR24; follow-up = FU1; time point 1 = TP1; time point 2 = TP2.

Conclusions

- Template independent sequencing methods more accurately capture mixed infection proportions compared to PCR
- Establishing cutoff criteria can rule out false assumptions of mixed genotype infections
- Mixed infections may not be as prevalent in high-risk populations as previously assumed, thus future studies warrant use of methods with standardized cutoff criteria

References

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