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# Novel dPCR Assay Confirms 5'Leader-Defective RNA as the Main Source of Nonsuppressible Viremia

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## Background

Despite effective therapy, residual viremia persists in a large fraction of people with HIV (PWH). In some PHW, HIV RNA remains above the limit of detection (LOD) of clinical assays, a condition referred to as nonsuppressible viremia (NSV), which raises concerns about treatment failure, pathogenesis, and transmission. We and others have shown that NSV may contain 5'Leader (5'L)-defective RNA. The frequency of 5'L defects is unknown, and identifying 5'L integrity by sequencing is labor-intensive. ***To enable feasible profiling of 5'L intact and defective NSV, we developed a new quantitative approach: Capture 5'L Anomalies Without Sequencing (CLAWS).***

## Methods

We recovered 532 5'L RNA sequences from a training set of plasma from PWH with NSV (N=31, **Fig. 1**); 91 unique variants were used to design a dPCR assay based on a single amplicon and two probes annealing to highly conserved regions: a reference probe targeting the *gag* ORF (required for virus production), and a "drop off" probe binding to 10 nucleotides affected by 96% of 5'L defects (**Fig. 2 A-B**). Among the 91 unique 5'L RNA variants recovered, 26% harbored deletions and 30% contained single-nucleotide mutations within the MSD site (**Fig. 1A-B**). Only double-positive HIV RNA molecules are scored as intact (**Fig. 2 C**). Assay performance was validated using intact and defective dsDNA and virus standards, and its agreement was tested against a set of 27 plasma samples from NSV. We also applied the assay to 10 PWH before and post ART introduction and NSV in a PWH from the TITAN clinical trial.

## Results

**CLAWS** correctly scored as defective 18/18 dsDNA standards with single mutations or deletions in the 5'L, and accurately quantified levels of intact RNA in mixtures of wildtype and defective NL4-3 virus (**Fig. 2D-F**). CLAWS is linear (Spearman r=1, p=0.0028) with 95% LOD of 9 copies/ml plasma (**Fig 3G**).

In samples from PWH with NSV, estimates were comparable between single genome sequencing (SGS) and CLAWS (n=27, 4.5% vs. 8.0%, p=0.28; **Fig. 3A**), confirming that most NSV virus is defective. SGS and CLAWS were highly correlated (Spearman r = 0.84, p<0.0001; **Fig. 3B**) with a mean bias of 2.4% (95% CI, ±19.3%; **Fig. 3C**), confirming strong agreement and supporting CLAWS as an alternative to SGS.

Studying pairs of longitudinal plasma samples from 10 participants who were highly monitored from ART initiation to sustained suppression (**Fig. 3D**) with CLAWS denoted low-level defective RNA during the second phase decay of viremia (35 days on ART) in 6/10 individuals and revealed a progressive enrichment of 5'L defects in plasma during long-term ART (98%, 22/23, **Fig. 3F**). The percentage of 5'L defective RNA measured by CLAWS is shown in **Fig. 3E**.

In a TITAN trial participant with NSV, CLAWS revealed that HIV RNA was defective at baseline, but new intact variants were present in rebound viremia and fueled persistent NSV for years after ART reintroduction, in agreement with sequencing data (r=0.96, p<0.0001, **Fig. 6 A-F**).

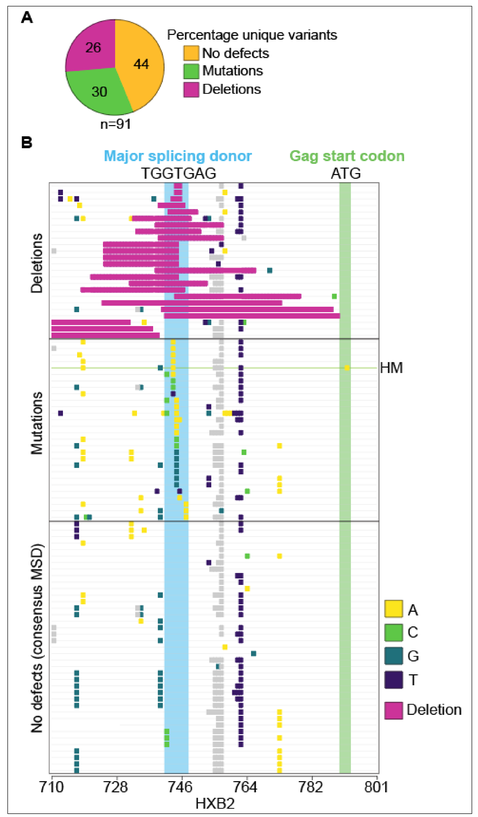



**Figure 1 – Deletions and single nucleotide mutations are narrowly focused on the MSD, affecting HIV-1 RNA splicing.**

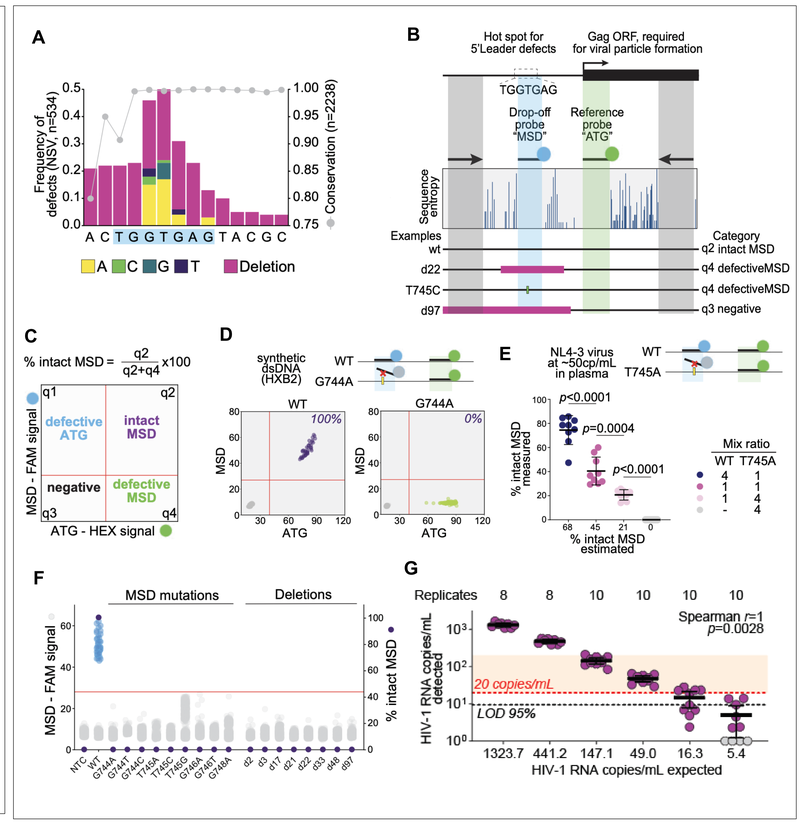



**Figure 2 – A single digital PCR assay allows to distinguish intact and defective 5'L RNA.**

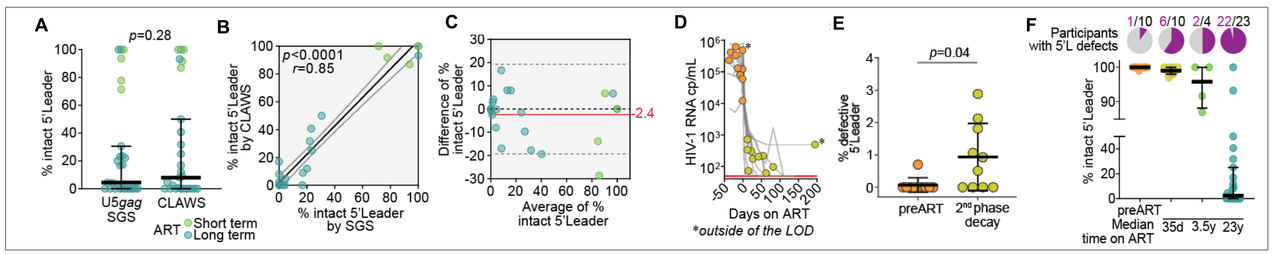



**Figure 3 – CLAWS strongly correlates with 5'L sequencing data, detects rare 5'L defective RNA during 2nd phace decay of viremia, and reveals a progressive increase of 5'L-defective RNA in plasma over time on ART.**

## Conclusion

5'L-defective proviruses are a major source of NSV. CLAWS is a feasible, high-throughput assay that accurately quantifies intact 5'L RNA, which will be useful for investigating persistent viremia and HIV cure clinical trials.

## Additional Key Information

**Contact Information:** Julia Box; jbox2@jh.edu

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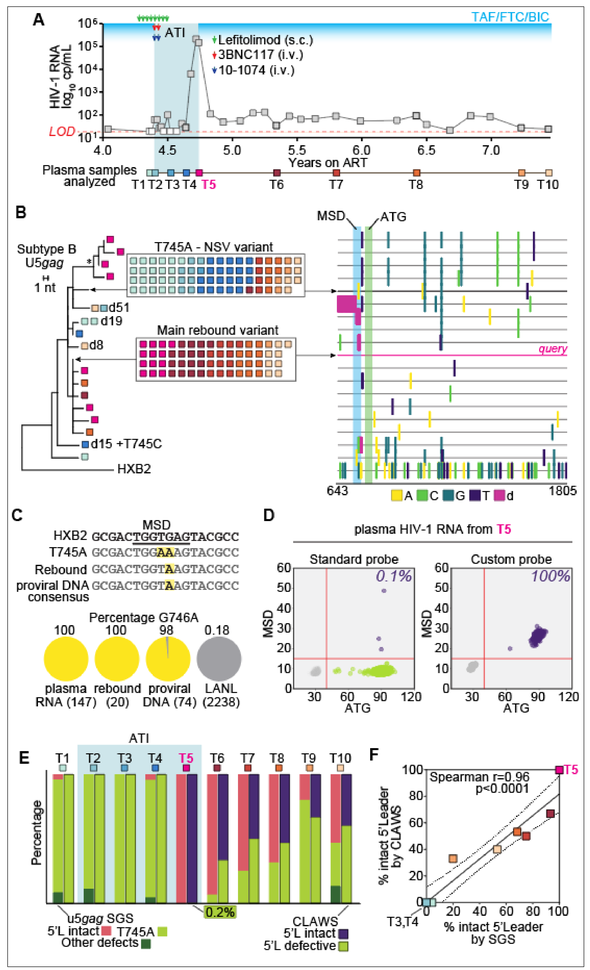



**Figure 4 – 5'L defective RNA contributes to NSV in a participant of the TITAN trial. Viral rebound affected viral populations cvausing NSV after ART re-introduction.**

## Plain Language Summary

We developed a simpler, cheaper method to quantify HIV-1 RNA in plasma and distinguish intact from defective viral RNA, called CLAWS. This novel approach can be used to better understand persistent viremia in both clinical practice and HIV-1 cure-focused trials.