

Design of a SHERLOCK-based low resource screening assay for HIV-1 drug resistance

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ABSTRACT

Recent developments in antiretroviral therapy (ART) have reduced human immunodeficiency virus type 1 (HIV-1) infection from a potent killer to a chronic illness. However, major HIV-1 drug resistance mutations (DRMs) hamper ART's efficacy. Low-resource regions are less capable of screening for DRMs due to poor infrastructure and high testing costs. To overcome this, we propose that Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) can be used for rapid and inexpensive HIV-1 DRM diagnosis. SHERLOCK employs Loop-mediated isothermal Amplification (LAMP) and a gRNA complex to isothermally amplify *protease* sequences and detect any amplified DRM targets, respectively. To account for HIV-1 genetic diversity, LAMP primers and gRNA packages were generated to ensure robust amplification and cleavage. A LAMP package with relatively high predicted sensitivity (66.9%) was generated by scoring each primer by their target diversity and thermodynamic characteristics and then combining the top performing primer sets. Next, DRM-specific gRNAs were ranked by F_3 -score and the top predicted sensitive and specific gRNAs for each DRM were considered for packaging. All gRNA packages demonstrated high predicted specificity (92.2%±4.3%), and 21/24 demonstrated high predicted sensitivity (≥90%). DRM-specific packages were then combined into drug-specific meta-packages with high predicted sensitivity (95.2%±2.1%). Upon *in vitro* validation, one of our generated LAMP primer sets amplified 13/14 patient-derived HIV-1 isolates and outperformed a published *protease*-specific LAMP set, and our gRNA showed some ability to cleave targets. Future directions entail improving LAMP primer and gRNA robustness and performing further *in vitro* validation.

INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) infects an estimated 38 million people globally (UNAIDS 2020), progressively leading to acquired immune deficiency syndrome (AIDS) if left unchecked. Advancements in antiretroviral therapy (ART) prevent infection from progressing to AIDS, relegating HIV-1 to a chronic illness. However, major drug resistance mutations (DRMs) can hinder the efficacy of ART by reducing HIV-1's susceptibility to ART (Wensing et al. 2019; WHO 2017). DRMs can either be acquired or transmitted (Figure 1). While testing for major DRMs is routine in most areas, it is generally unavailable in low-income regions due to high testing costs and poor infrastructure (Clutter et al. 2016). As such, an accessible and affordable point-of-care (POC) DRM detection assay is required to overcome these barriers.

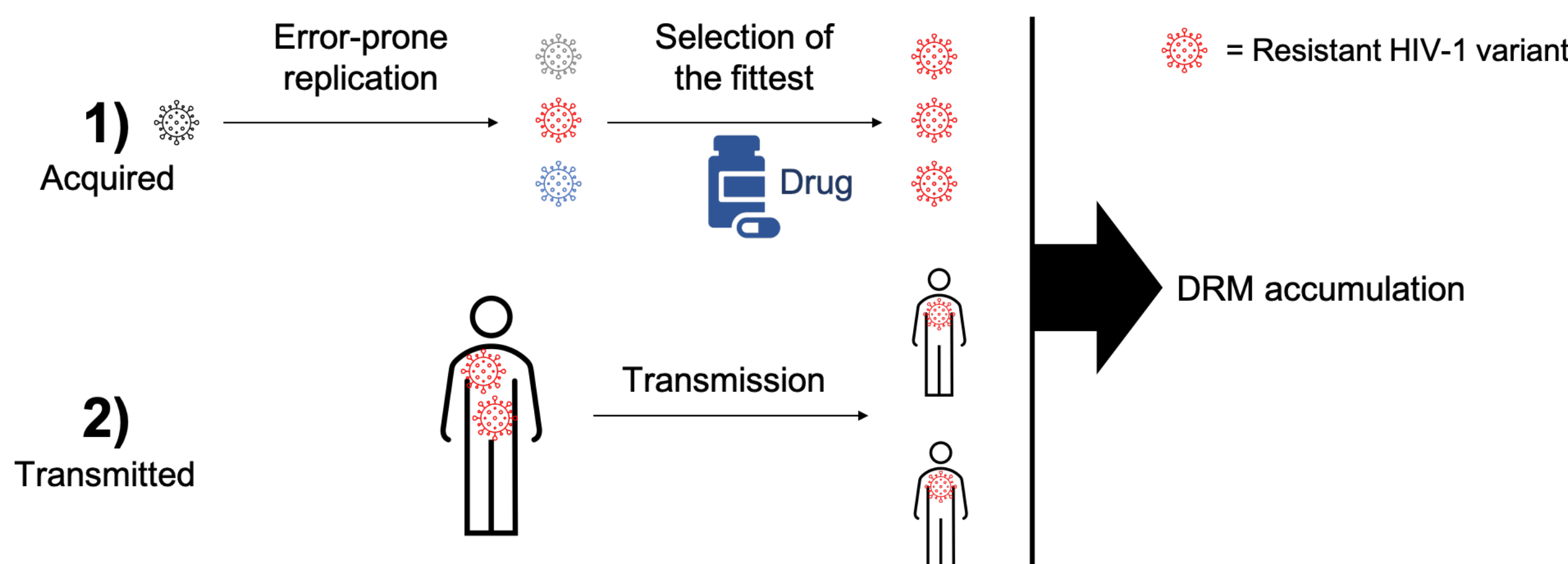


Figure 1: HIV-1 drug resistance is acquired and transmitted. (1) HIV-1's error-prone replication cycle can introduce drug resistance HIV-1 variants. Treatment interruptions and poor drug quality further exacerbate this. (2) Once acquired, drug resistance HIV-1 variants can be transmitted to other people.

Specific High Sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) is a newly developed CRISPR-based assay that has been repurposed as a quick and inexpensive SARS-CoV-2 POC diagnostic with high sensitivity (96.9%) and specificity (100.0%) (Joung et al. 2020). This diagnostic only requires a heat block, reagent mixture, and lateral flow strips, allowing for inexpensive and widespread testing. SHERLOCK uses a target-specific Loop-mediated isothermal Amplification (LAMP), which utilizes four primers to increase target concentration along with a Cas12b enzyme complexed with a predesigned guide RNA (gRNA). After initial activation at a pre-specified target, the enzyme non-specifically cleaves surrounding reporters, whose abundance, and thus target presence, is visualized using an inserted lateral flow strip, making this assay specific and sensitive (Joung et al. 2020; Kellner et al. 2019). Cas12b has been previously used for single nucleotide discrimination (Teng et al. 2019; Li et al. 2019), making it suitable for HIV-1 DRM screening. Given this, our goal is to design an accessible SHERLOCK-based POC major DRM diagnostic assay that overcomes low-income testing barriers. As a proof-of-concept, we present *in silico* LAMP primer and gRNA design pipelines to detect major DRMs within HIV-1 *protease*. A packaging methodology was used to provide sensitivity against the vast HIV-1 diversity (Clutter et al. 2016).

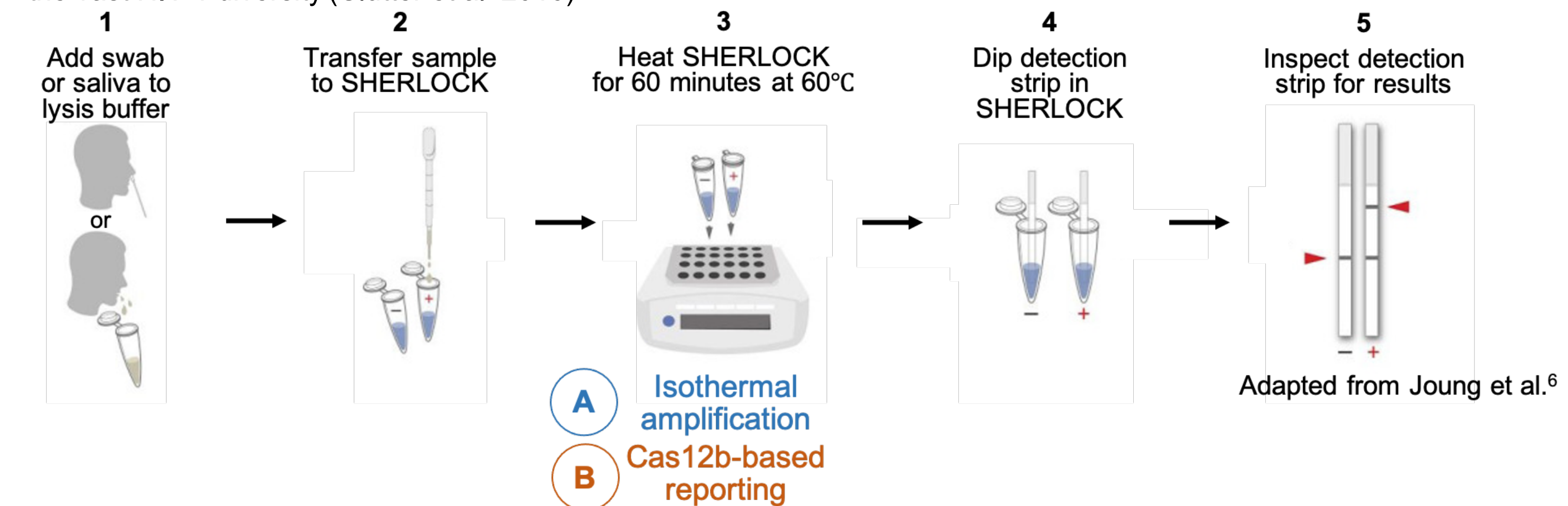


Figure 2: SHERLOCK assay workflow. (1) Samples are extracted and added to a lysis buffer to purify the nucleic acids at 22°C or 60°C for 10 minutes. (2) The samples are mixed with the SHERLOCK master mix, containing LAMP amplification and Cas12b reagents. (3) Then, the SHERLOCK reaction is catalyzed at 60°C for 60 minutes. If present, isothermally amplified target regions are specifically detected by the Cas12b-gRNA complex, cutting surrounding reporters and creating a detectable signal. (4) Lateral flow strips are then dipped into the samples for two minutes, (5) and a positive detection event is called when the intensity of the positive line is greater than the control line.

HIV-1 *protease* variability is an obligatory consideration

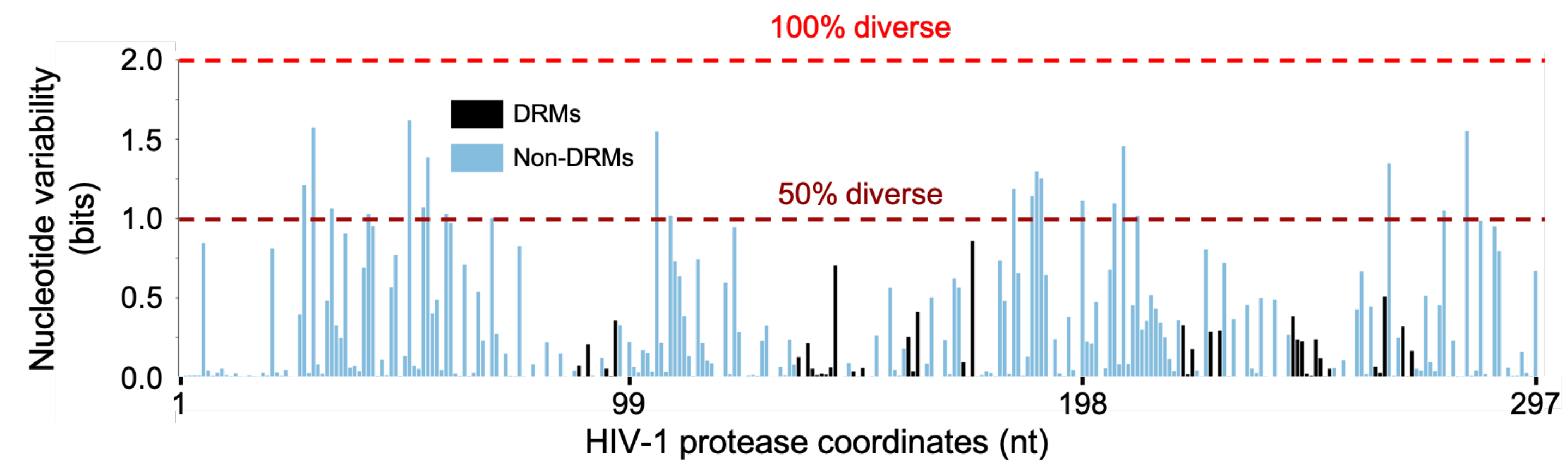
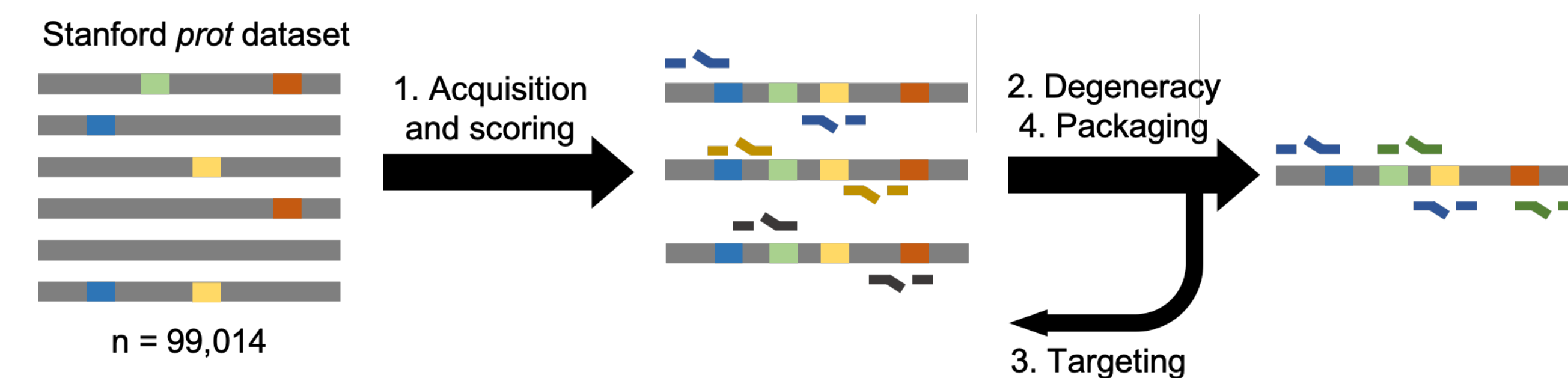


Figure 3: LAMP primer and gRNA design require careful consideration of *prot* variability. 28.6% (85/297) of nucleotides within the HIV-1 *protease* sequence are non-conserved, (variability >0.3 bits). These diverse regions are proximal to all DRM regions. Primer sets and gRNAs were packaged to overcome this diversity barrier.

Probe design pipelines for SHERLOCK

LAMP design pipeline for sensitive amplification



gRNA design pipeline for drug resistance detection



Figure 4: Custom design pipelines for robust SHERLOCK-based detection. (top) LAMP primer design pipeline. All possible primer targets within HIV-1 *prot* were gathered. Primer sets that target conserved regions and have favorable thermodynamic characteristics had ambiguous nucleotides added to bolster sensitivity. Sequences that could not be targeted were reintroduced into the pipeline. After three iterations, the best primer set from each iteration were combined into a single package. (bottom) gRNA design pipeline. All possible gRNAs were derived from sequences within the HIV-1 *prot* dataset without insertions/deletions nor a highly ambiguous nucleotide composition (≥ 2.5%). Each gRNA was ranked by their F_3 -score. Top-performing candidates were grouped to generate a DRM-specific gRNA package. DRM-specific packages were aggregated together to generate drug-specific gRNA meta-packages.

Novel probes are predicted to achieve high coverage while preserving specificity

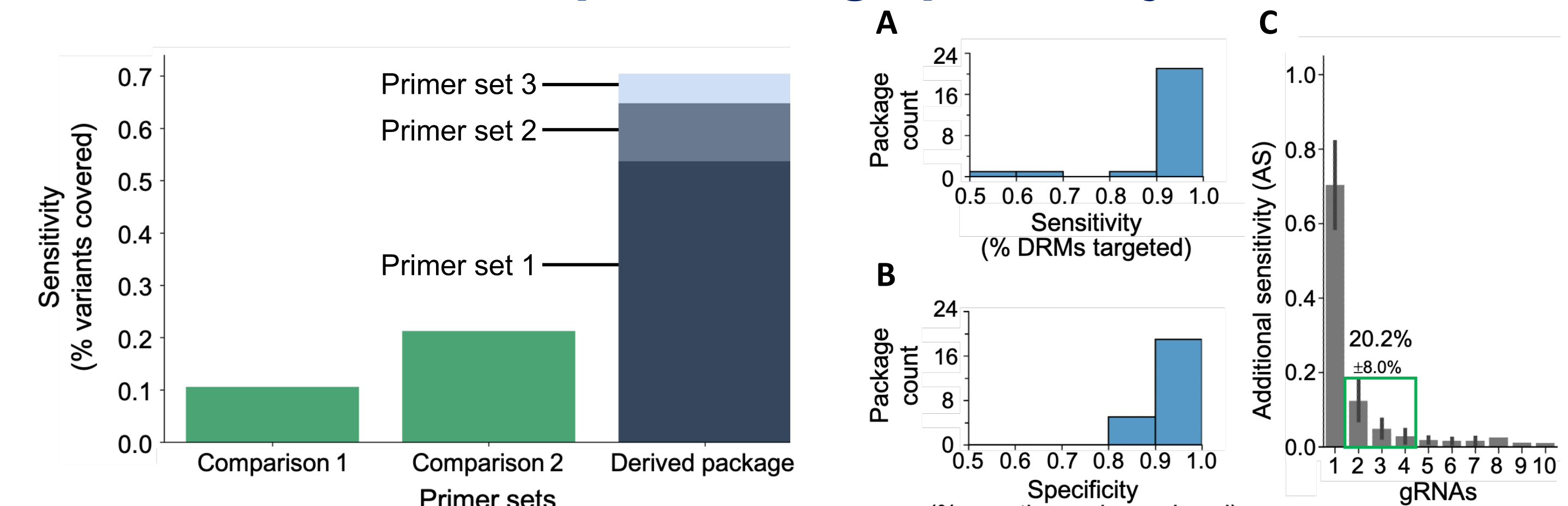


Figure 5: Novel LAMP sets and gRNA packages demonstrate broad-spectrum coverage. (right) Generation of robust *protease* LAMP primer sets. Previously published *protease* LAMP primer sets (Ocwieja et al. 2015; Curtis et al. 2008) were only developed against consensus HIV-1 sequences and have not sufficiently considered HIV-1 diversity. As such, they only conferred 10.5% and 21.2% sensitivity whereas our packaged primers provided 66.9% sensitivity. (left) The targetability of major *protease* DRMs using DRM-specific packages. (A) 21/24 gRNA packages have high predicted sensitivity (>90%) with (B) a high predicted average specificity (92.2%±4.3%) (C) The additional sensitivity (AS) provided when adding multiple gRNAs. Packaging three additional gRNAs confers an AS of 20.2%±8.0% (mean ± STD), considerably improving sensitivity.

Validation of novel LAMP primers

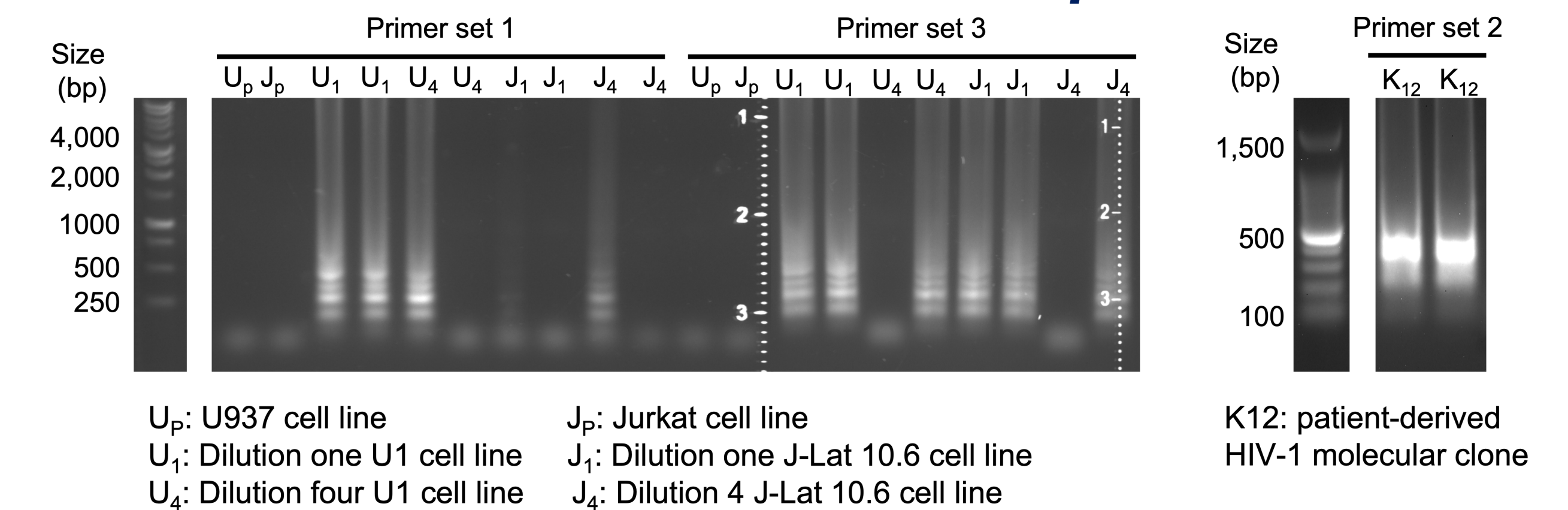


Figure 7: LAMP primers can amplify HIV-1 lab strains. Representative LAMP primer results are shown. Rather than distinct bands, smears appear because LAMP primers generate a variety of amplicon sizes. Primer sets 1 and 3 amplify dilution one targets and maintain some activity against lower dilution targets. Primer set 2 effectively amplifies a clinically-relevant target, containing four major DRMs.

Novel LAMP primers show broad-spectrum amplification

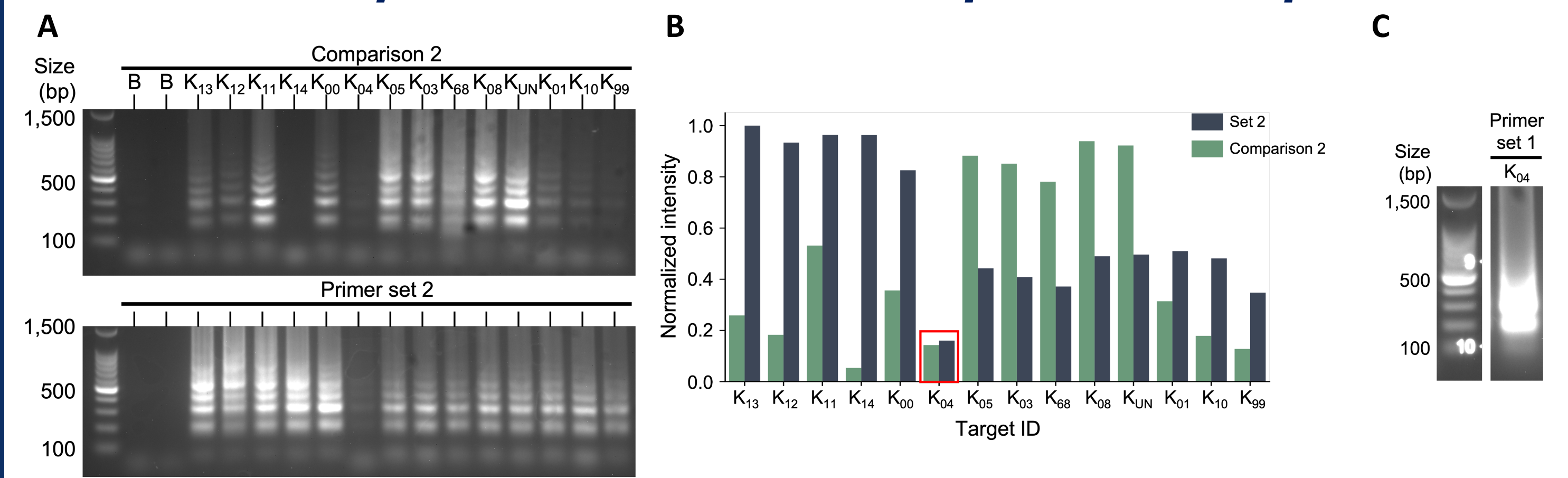


Figure 8: Primer set 2 provides high coverage against patient-derived HIV-1 *protease* targets. (A) Gel readout for LAMP amplification using comparison set 2, the best published set, and our primer set 2 against 14 clinically-relevant HIV-1 isolates. Comparison set 2 fails to achieve consistent amplification efficiency whereas our primer set 2 maintains visibly efficient amplification against 13/14 targets. (B) Quantitative analysis of amplification efficiency. Comparison set 2 only amplifies 5/14 targets more efficiently than primer set 2. Using all possible cutoffs for amplification, our primer set 2 confers on average 60.0% sensitivity while comparison set 2 confers 46.6% sensitivity. Target K_{04} was poorly amplified by both sets, but (C) pooling our primer set 1 achieves amplification of all targets.

Novel gRNA cleaves different dsDNA forms

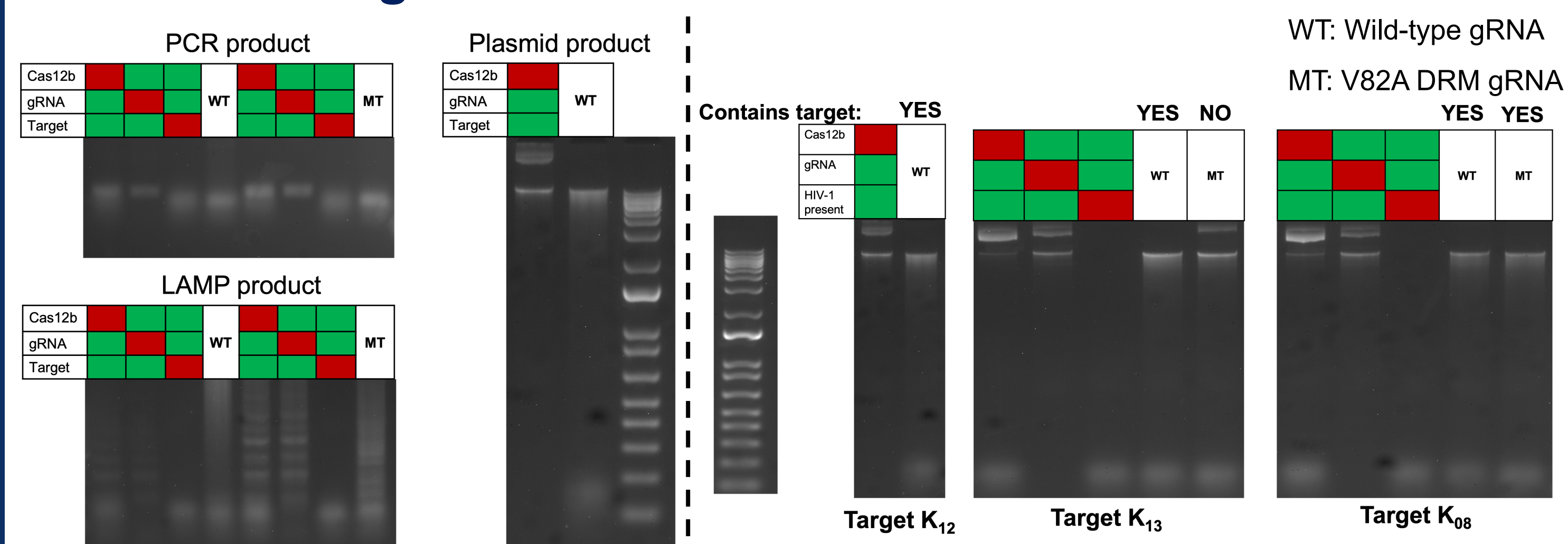


Figure 9: AapCas12b and gRNA complex cuts a variety of dsDNA targets. (left) AapCas12b-gRNA against PCR, LAMP, and plasmid targets. The V82A gRNA was chosen as it displayed high predicted sensitivity while targeting a highly prevalent DRM. AapCas12b-gRNA generated PCR fragments that pooled to the bottom of the gel. The LAMP products' ladder-like pattern was removed as AapCas12b-gRNA cut the concatamer ladder bands containing target sequences. AapCas12b-gRNA cleaved one form of the plasmid target. (right) AapCas12b-gRNA against different positive and negative plasmid targets. Negative target K_{13} maintained an uncut top band as the sequence had four target-MT gRNA mismatches. MT gRNA successfully cut positive targets K_{08} and K_{12} .

SUMMARIZED RESULTS / CONCLUSION

- LAMP package exhibits 66.9% sensitivity
- 21/24 gRNA packages have >90% sensitivity
- Packages have 92.2%±4.3% specificity
- Primer set 2 maintains wide coverage and consistent amplification efficiency
- Our gRNA targets a variety of target types and seems to demonstrate discriminative ability

Our probes show efficacy *in silico* and *in vitro*. Our results suggest that a SHERLOCK-based POC assay to detect HIV-1 drug resistance is feasible. Future directions include validating the combined SHERLOCK assay.

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