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Short communication

Optimization of allele-specific PCR using patient-specific HIV consensus sequences for primer design

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ABSTRACT

Allele-specific PCR based on subtype consensus sequences is a powerful technique for detecting low frequency drug resistant mutants in HIV-1 infected patients. However, this approach can be limited by genetic variation in the region complementary to the primers, leading to variability in allele detection. The goals of this study were to quantify this effect and then to improve assay performance.

Infection with human immunodeficiency virus type 1 (HIV-1) remains a major global health challenge. Antiretroviral therapy (ART) has improved and lengthened the lives of those infected with HIV. Nevertheless, antiviral drug resistance continues to reduce the effectiveness of ART (Coffin, 1995; Martinez-Picado and Martinez, 2008). Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are routinely used for the treatment and prevention of HIV-1 infection. NNRTIs target reverse transcriptase (RT), an enzyme essential for HIV-1 replication (De Clercq, 1994; Deeks, 2001). The single point mutations AAA to AAC or AAT in HIV-1 RT at codon 103 result in a lysine to asparagine substitution (K103N) which causes resistance to efavirenz and nevirapine (Harrigan et al., 2005; Wainberg, 2003). Detection of HIV-1 variants carrying these mutations is important for understanding the emergence of drug resistance and for designing optimal ART strategies. To this end, a sensitive real time allele-specific PCR (ASP) assay for quantifying low frequency HIV-1 variants containing NNRTI-resistant variants in patients infected with HIV-1 has been reported (Palmer et al., 2006a,b). Using this assay, detection of drug resistant variants over a broad range of frequencies (0.1–99.5%) is achievable providing insight into the emergence and persistence of drug resistance after the discontinuation of therapy (Palmer et al., 2006a) and after single-dose nevirapine treatment to prevent mother to child transmission (Palmer et al., 2006b). Other researchers have reported the detection of minor resistant HIV-1 variants using similar techniques (Halvas et al., 2006; Johnson et al., 2005; Loubser et al., 2006; Metzner et al., 2003). As for all PCR-based assays, ASP can be limited by genetic variation in the region complementary to the primers, leading to variability in detection efficiency. The accuracy of ASP may also be influenced by the technique employed and the method of data analysis. A recent report described improved ASP with the use of polymorphism-specific primers (Rowley et al., 2008). It is

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Table 1
Relationship between sequence polymorphism around codon 103 and amplification efficiency.

A. Subtype C Consensus and Primer Sequences

Consensus Subtype C Nonspecific Standard/Primer Sequence

```
RT codon       103 104 105 106 107 108 109 110 111 112 113
             5′ AAA AAA TCA GTG ACA GTA CTA GAT GTG GGG GAT 3′
Primer       3′         AGT CAC TGT CAT GAT CTA CAC CCC CTA 5′
```

Consensus Subtype C 103N (aac) Specific Standard/Primer Sequence

```
RT codon       103 104 105 106 107 108 109 110 111 112
             5′ AAC AAA TCA GTG ACA GTA CTA GAT GTG GGG 3′
Primer[a]     3′   G GTT AGT CAC TGT CAT GAT CTA CAC CC   5′
```

Consensus Subtype C 103N (aat) Specific Standard/Primer Sequence

```
RT codon       103 104 105 106 107 108 109 110 111 112
             5′ AAT AAA TCA GTG ACA GTA CTA GAT GTG GGG 3′
Primer[a]     3′   A CTT AGT CAC TGT CAT GAT CTA CAC CC   5′
```

Consensus Subtype C WT (aaa) Specific Standard/Primer Sequence

```
RT codon       103 104 105 106 107 108 109 110 111 112
             5′ AAA AAA TCA GTG ACA GTA CTA GAT GTG GGG 3′
Primer[a]     3′   T CTT AGT CAC TGT CAT GAT CTA CAC CC   5′
```

B. Sequences Observed in Patient Samples

Sequence	Number of patients	Minus strand sequence 5′ to 3′[b] (codon 112 111 110 109 108 107 106 105 104 103)	PCR efficiencies prior to primer correction (sum of species detected at codon 103)
Consensus[c]	6	CCCACATCTAGTACTGTCACTGATTTT	50 – 100%
I	20	CCCACATCCAGTACTATCACTGATTTT	25 – 100%
II	4	CCCACATCCAGCACTATCACTGATTTT	25 – 72%
III	2	CCCACATCCAATACTGTCACTGATTTT	25 – 66%
IV	2	CCCACATCCAGTACTGTTACTGATTTT	26 – 43%
V	1	CCTACATCCAGTACTGTCACTGATTTT	51%
VI	1	CCCACATCTAGCACTGTCACTGATTTT	70%
VII	1	CCCACATCTAATACTGTCACTGATTTT	36 %
1	1	CCCACATCCAGCACTGTCACTGACTTT	2%
2	1	CCCACATCTAGTACTGTCACTGACCTT	0.5%
3	1	CCCACATCCAACACTGTCACTGATTTT	4%
4	1	CCCACATCCAGCACTGTTACTGATTTT	3%

[a]Penultimate 3′ mismatch shown in blue is built in for discrimination between alleles.
[b]Only the wildtype allele is shown. Patient polymorphisms are denoted in red.
[c]This sequence was common in the LANL database, sequence I was most common in the patient group.

concluded that ASP is a powerful tool only when allele-specific primers perfectly match the patient HIV-1 sequence, requiring a bulk genotypic analysis of the region of interest for each patient plasma sample. However, there is a method to correct for the effects of genetic variation on the quantitation of viral variants at a specific allele and to achieve acceptable assay performance without requiring genotypic analysis of every patient sample. Details of the technique and data analysis that enhance the detection and improve the accuracy of allele quantitation are described.

Viral RNA is extracted from patient samples infected with HIV-1 and a first round amplification is performed using quantitative real time PCR as published (Palmer et al., 2006a). Quantitation of the number of cDNA molecules synthesized and amplified in the first round RT-PCR is important to determine the level of sensitivity of subsequent ASP, without which the assay sensitivity cannot be determined since variation in virus concentration, plasma components, and primer matches to target sequences can lead to variability in efficiency of RNA recovery, cDNA synthesis, and amplification.

Allele-specific primers that amplify selectively subtype-specific HIV-1 mutant or wildtype (WT) alleles at codon 103 are then used in ASP to quantify the proportion of each allele in cDNA derived from patient plasma RNA (Table 1A). Parallel reactions are performed using two primer sets: selective primers to quantify mutant or WT frequencies, and a nonselective primer to quantify total DNA.

The selective primers match a mutant (C or T) or wildtype base (A or G) at the 3′ end of codon 103, but mismatch all templates at the penultimate position, a modification that has little impact on amplification of sequences complimentary to the 3′ end of the primer but reduces markedly amplification (≥1000-fold) when the 3′ end of the primer is also mismatched (Cha et al., 1992; Hance et al., 2001; Palmer et al., 2006a). The specific nucleotide used for the intentional mismatch was given a great deal of attention and the best mismatched pair was determined empirically. For the allele-