Longitudinal Analysis of RAVs by Primer ID 454-Sequencing in the Boceprevir P-05063 Study

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Background

- Although resistance-associated amino acid variants (RAVs) declined to below detectable levels by Sanger sequencing methodology in the majority of patients enrolled in the boceprevir long-term follow-up study P05063, ~30% of these patients had viruses with RAVs detected by population sequencing >2 years after cessation of boceprevir dosing.
- To further investigate the persistence of RAVs and to assess the limits of detection of the population sequencing technique employed in boceprevir clinical trials, RAVs were quantified using a sensitive clonal quantitative nucleotide sequencing method (Primer ID/454 Sequencing).[1]
- Paired samples from 20 patients at baseline (prior to boceprevir treatment) and >2 years after virologic failure with RAVs in a boceprevir clinical study were evaluated for RAVs using Primer ID/454-sequence analysis.
- Virologic failure samples were also evaluated by Primer ID/454 sequencing, where available. However, 8/20 patients did not have virologic failure samples available, so the next available sample was sequenced (Table 1).

1. Jabara CB, Jones CD, Roach J, Anderson JA, Swanstrom R. Accurate sampling and deep sequencing of the HIV-1 protease gene using a Primer ID. *Proc Natl Acad Sci USA*. 2011;108(50):20166-20171.

Table 1. Sample Dates Available for Primer ID/454 Sequence Analysis for 8/20 Patients

Patient	Virologic Failure Date	454-Sequencing Sample Date	Number of Days Since VF
1	09-Dec-2008	30-Sep-2010	659
4	18-May-2009	12-Oct-2010	512
5	10-Mar-2009	20-Jul-2010	497
8	05-May-2009	23-Sep-2010	506
12	27-Mar-2009	13-Dec-2010	626
16	06-Jan-2009	24-Jun-2010	534
18	17-Mar-2009	10-Aug-2010	511
19	08-Dec-2009	25-May-2010	168

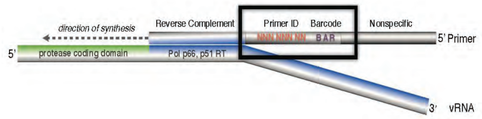
Virologic failure time points were not available for Primer ID/454-Sequencing for 8/20 patients. The next available time point was chosen for each of these patients for Primer ID/454 sequencing. The number of days after virologic failure is indicated.

Methods

- Viral RNA was isolated from blood plasma using the automated MagMAX-96 Viral RNA Isolation Kit (Life technologies). cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen) using the primer (with Primer ID). Approximately 20,000 copies of viral RNA from each sample were present in the cDNA synthesis reaction. The tagging primer used was: 5'ACCTTGCAAGCACGCTCTGGCCTTGAANNNNNNNN CTATA(barcode)GAACACCGGGGACCTCATGGTTGTCT C- 3' (Figure 1).
- The same primer was synthesized with 9 different barcodes, such that multiple samples could be easily pooled into a single lane of the sequencing run.
- The 3' end of the tagging primer targeted downstream of the NS3 coding domain (H77r3971_3945). The oligonucleotides were purchased from IDT and were purified by standard desalting. Following the reaction, RNA in hybrid was removed by RNaseH treatment (Invitrogen).
- Unincorporated cDNA primer was removed using double purification with Ampure^{XP}, and the samples were amplified by nested PCR (25 cycles each) using upstream primers 5'YTGCTYGGRCCRGCYGA- 3' (H77 3373-3390) and 5'AGTGGAGGGTGAGGTCCAGAT- 3' (H77 3503-3523). The downstream primers targeted the 5' portion of the cDNA tagging primer 5'ACCTTGCAAGCACGCTCTGGC -3'and 5'CAAGCACGCTCTGGCCTTGAA- 3'. The PCRs were done using PrimeSTAR GXL DNA polymerase (Takara, cat. #R050Q).
- Once amplified, 3 to 4 tagged samples were combined and sequenced on the 454 GS FLX+ Platform with XLR70 Titanium Chemistry per manufacturer's instructions with ~300,000 beads loaded per region.
- The data generated from the sequencing run is processed using the pipeline outlined in Figure 2.

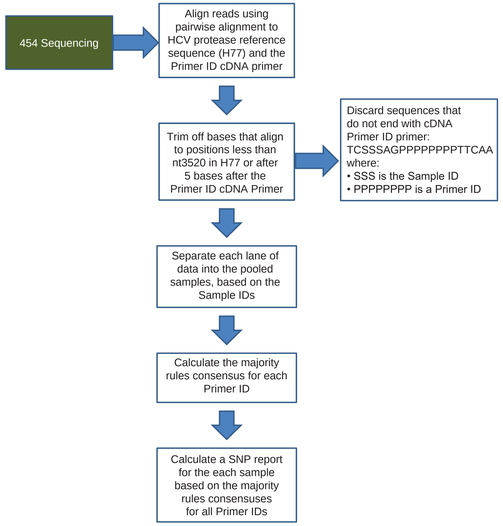
Methods (cont.)

Figure 1. Schematic of Primer ID/454 Sequencing Technology



A random 8-nucleotide tag (NNN NNN NN) is inserted after the reverse-compliment gene-target sequence. A nonspecific primer is added to the 5' end to allow amplification by PCR.

Figure 2. Primer ID Analysis Pipeline



Results

- The Primer ID technique allows the identification and sequencing of individual viral cDNAs from a swarm. The number of individual viral cDNAs sequenced for each time point is listed in Table 1.
- For all 20 patients in the study, the number of independent Primer IDs ranged from 40 to 1398, with an average of 525 at baseline or the 1-2 year follow-up time point. The number of independent Primer IDs proved to be variable in some patients, likely due to the sequences of viruses isolated from these patients not completely matching the cDNA primer and resulting in poor cDNA/PCR amplification.

Baseline Primer ID/454 Sequencing

- 2/20 patients had viruses with RAVs detected at baseline by population sequencing (Patients 2 and 7; Table 2). The same RAVs were detected by Primer ID/454 sequencing and their frequencies could be accurately quantified. In patient 2, this was V36L (93.6%) and V55A (82.2%), while in patient 7, the RAV was V55A (97.9%). An additional RAV (V55P) was identified in Patient 7 by Primer ID/454 sequencing at a low frequency (1.9%)
- Baseline RAVs were identified in a further 4 patients by Primer ID/454 sequencing that were not detected by population sequencing. These RAVs were present at <2.6% of the circulating virus population (Table 1).
- The RAVs detected at baseline by Primer ID/454 sequencing were V36L, T54A, T54S, V55A, V55P and R155K.

Virologic Failure Primer ID/454 Sequencing

- After virologic failure, RAVs were detected in viruses in 12/12 patients by 454/Primer ID sequencing.
- Additional RAVs were identified by 454/Primer ID in viruses from 10/12 patients sequenced at virologic failure. All of these RAVs were detected at less than 15%, below the sensitivity level of population sequencing.

1-2 Year Follow-up Primer ID/454 Sequencing

- 2/20 had viruses with RAVs detected 1-2 years after stopping therapy by population sequencing (Table 2).
- RAVs were detected in viruses from 11/20 patients by 454/Primer ID sequencing (Table 2). The frequencies in the 9/11 patients (that were not detected by population sequencing) with RAVs detected by Primer ID/454 sequencing ranged from 0.18 to 21%. The majority were <4.32%.
- The quantity of RAVs detected by Primer ID/454 sequencing varied, with the highest frequency being in patient 11 (99% T54S and R155K). Interestingly these two variants were detected at high levels in this patient at virologic failure (97.6% and 100%, respectively) and both were detected by population sequencing.
- Patient 20 had low levels of R155K detected at baseline (2.6%) and also experienced virologic failure with R155K. These patients experienced a poor interferon response (<1 $\log_{10}$ decline in HCV RNA by TW4) during the 4-week lead in, indicating they were poorly responsive to interferon/ribavirin (see conclusion).

Table 2. Primer ID Data for 20 Patients Enrolled in P05063

			Baseline					Virologic Failure						Follow-up					
			Population Seq	454-Primer ID Sequencing				Population Seq	454-Primer ID Sequencing					Population Seq	454-Primer ID Sequencing				
Patient Number	RAV Detected by Pop Sequencing (Any Time Point)	RAVs Detected by 454/Primer ID (Any Time Point)	RAVs Detected by Population Sequencing	RAVs Detected at BL	# PID at RAV Position (n)	Clones With RAV (m)	%RAV (m/n)	RAVs Detected by Population Sequence	RAVs Detected at VF	# PID at RAV Position (n)	Clones With RAV (m)	%RAV (m/n)	VF Samples Available for 454-Primer ID?	Days Since Virologic Failure	RAVs Detected by Population Sequence	RAVs Detected in Follow-up	# PID at RAV Position (n)	Clones With RAV (m)	%RAV (m/n)
1	**V36M**	**V36M**	None*	No	361	0	N/A	None	**Yes**	**302**	**5**	**1.7**	No	919	None	**Yes**	**615**	**12**	**2.0**
	R155K	**R155K**		No	362	0	N/A		No	306	0	N/A				**Yes**	**634**	**4**	**0.6**
2	**V36L**	**V36L**	**V36L***	**Yes**	**251**	**235**	**93.6**	**V36L**	**Yes**	**33**	**31**	**93.9**	YES	894	**V36L***	**Yes**	**253**	**249**	**98.4**
	V55A	**V55A**	**V55A***	**Yes**	**253**	**236**	**93.3**	**V55A**	**Yes**	**34**	**31**	**91.2**			**V55A***	**Yes**	**269**	**208**	**77.3**
		V55G		No	253	0	N/A	None	No	34	0	N/A			None	**Yes**	**269**	**57**	**21.2**
		R155K		No	253	0	N/A	None	**Yes**	**34**	**3**	**8.8**			None	No	272	0	N/A
3	**V36M**	**V36M**	None	No	463	0	N/A	**V36M**	**Yes**	**359**	**154**	**42.9**	YES	982	None	No	743	0	N/A
		V36L		No	463	0	N/A	None	**Yes**	**359**	**4**	**1.1**				No	743	0	N/A
		V36A		No	463	0	N/A	None	**Yes**	**359**	**42**	**11.7**				No	743	0	N/A
		T54A		No	463	0	N/A	None	**Yes**	**388**	**10**	**2.6**				No	746	0	N/A
		T54S		No	463	0	N/A	None	**Yes**	**388**	**41**	**10.6**				No	746	0	N/A
		V55A		No	464	0	N/A	**V55A**	**Yes**	**388**	**6**	**1.5**				No	746	0	N/A
	R155K	**R155K**		No	464	0	N/A	**R155K**	**Yes**	**391**	**209**	**53.5**				No	746	0	N/A
4	**V36M**	**V36M**	None	No	250	0	N/A	None	No	124	0	0.0	NO	883	None	No	298	0	0
	A156S	**A156S**		No	254	0	N/A		No	126	0	0.0				No	311	0	0
		D168N		No	254	0	N/A		No	126	0	0.0				**Yes**	**311**	**2**	**0.6**
5	**V36M**	**V36M**	None	No	823	0	N/A	None	**Yes**	**391**	**1**	**0.3**	NO	685	None	No	544	0	N/A
	R155K	**R155K**		No	824	0	N/A		**Yes**	**403**	**1**	**0.2**				No	547	0	N/A
6	**V36M**	**V36M**	None	No	695	0	N/A	**V36M**	**Yes**	**763**	**763**	**100.0**	YES	674	None	No	696	0	N/A
	R155K	**R155K**		No	705	0	N/A	**R155K**	**Yes**	**772**	**768**	**99.5**				No	1052	0	N/A
7		**V36I**		No	528	0	N/A	None	**Yes**	**471**	**1**	**0.2**	YES	528	None	No	809	0	N/A
		V36M		No	528	0	N/A	None	**Yes**	**471**	**93**	**19.7**			None	No	809	0	N/A
	V55A	**V55A**	**V55A**	**Yes**	**526**	**515**	**97.9**	**V55A**	**Yes**	**484**	**477**	**98.6**			**V55A**	**Yes**	**809**	**808**	**99.9**
		V55P		**Yes**	**526**	**10**	**1.9**	None	No	484	0	N/A			None	No	809	0	N/A
	R155K	**R155K**		No	528	0	N/A	**R155K**	**Yes**	**484**	**470**	**97.1**			**R155K**	**Yes**	**809**	**646**	**79.9**
8	**R155K**	**R155K**	None	No	295	0	N/A	None	**Yes**	**598**	**20**	**3.3**	NO	867	None	No	327	0	N/A
		R155G		No	295	0	N/A		**Yes**	**598**	**1**	**0.2**				No	327	0	N/A
9	**V36M**	**V36M**	None	No	123	0	N/A	**V36M**	**Yes**	**40**	**40**	**100.0**	YES	890	None	No	392	0	N/A
		T54I		No	149	0	N/A	None	No	49	0	0.0				**Yes**	**392**	**1**	**0.26**
	R155K	**R155K**		No	156	0	N/A	**R155K**	**Yes**	**51**	**50**	**98.0**				**Yes**	**392**	**1**	**0.26**
		R155T		No	156	0	N/A	None	**Yes**	**51**	**1**	**2.0**				No	392	0	N/A
		D168N		No	156	0	N/A	None	**Yes**	**52**	**1**	**1.9**				No	392	0	N/A
10		**V36M**	None	No	1392	0	N/A	None	**Yes**	**377**	**22**	**5.8**	YES	555	None	**Yes**	**555**	**6**	**1.08**
		T54S		No	1369	0	N/A		**Yes**	**389**	**21**	**5.4**				No	556	0	N/A
		R155K		No	1398	0	N/A		**Yes**	**389**	**57**	**14.7**				No	556	0	N/A
		R155M		No	1398	0	N/A		**Yes**	**389**	**1**	**0.3**				No	556	0	N/A
	R155T	**R155T**		No	1398	0	N/A	**R155T**	**Yes**	**389**	**315**	**81.0**				**Yes**	**556**	**1**	**0.18**
	D168N	**D168N**		No	1397	0	N/A	**D168N**	**Yes**	**389**	**316**	**81.2**				**Yes**	**556**	**1**	**0.18**
11		**V36L**	None	No	533	0	N/A	None	**Yes**	**1092**	**1**	**0.1**	YES	895	None	No	299	0	N/A
		V36M		No	533	0	N/A		**Yes**	**1092**	**27**	**2.5**				No	299	0	N/A
	T54S	**T54S**		**Yes**	**547**	**6**	**1.1**	**T54S**	**Yes**	**1101**	**1075**	**97.6**			**T54S**	**Yes**	**305**	**304**	**99.67**
	R155K	**R155K**		**Yes**	**550**	**7**	**1.3**	**R155K**	**Yes**	**1105**	**1105**	**100.0**			**R155K**	**Yes**	**309**	**307**	**99.35**
		D168N		No	550	0	N/A	None	**Yes**	**1105**	**1**	**0.1**			None	No	309	0	N/A
12	**R155K**	**R155K**	None	No	528	0	N/A	None	**Yes**	**208**	**46**	**22.1**	NO	853	None	**Yes**	**488**	**1**	**0.20**
		R155Q		No	528	0	N/A		No	208	0	N/A				**Yes**	**488**	**1**	**0.20**
		V158I		No	528	0	N/A		**Yes**	**209**	**32**	**15.3**				**Yes**	**488**	**1**	**0.20**
13		**V36M**	None	No	204	0	N/A	None	**Yes**	**394**	**10**	**2.5**	YES	847	None	No	889	0	N/A
	R155K	**R155K**		No	213	0	N/A	**R155K**	**Yes**	**395**	**395**	**100.0**				No	908	0	N/A
	V158I	**V158I**		No	213	0	N/A	**V158I**	**Yes**	**395**	**385**	**97.5**				No	908	0	N/A
14	**V36M**	**V36M**	None	No	675	0	N/A	**V36M/V**	**Yes**	**492**	**462**	**93.9**	YES	961	None	No	223	0	N/A
		V36L		No	675	0	N/A	None*	**Yes**	**492**	**1**	**0.2**				No	223	0	N/A
		T54A		No	679	0	N/A		**Yes**	**493**	**2**	**0.4**				No	231	0	N/A
		T54S		No	679	0	N/A		**Yes**	**493**	**15**	**3.0**				No	231	0	N/A
	R155K	**R155K**		No	683	0	N/A	**R155K**	**Yes**	**493**	**489**	**99.2**				No	232	0	N/A
		R155T		No	683	0	N/A	**R155T**	**Yes**	**493**	**1**	**0.2**							
15	**V36M**	**V36M**	None	No	581	0	N/A	**V36M**	**Yes**	**264**	**215**	**81.4**	YES	688	None	No	349	0	N/A
		T54A		**Yes**	**584**	**1**	**0.2**	None	No	271	0	N/A				No	351	0	N/A
		T54S		No	584	0	N/A	None	**Yes**	**271**	**40**	**14.8**				No	351	0	N/A
		R155I		No	586	0	N/A	None	**Yes**	**274**	**1**	**0.4**				No	352	0	N/A
		R155G		No	586	0	N/A	None	**Yes**	**274**	**1**	**0.4**				No	352	0	N/A
	R155K	**R155K**		**Yes**	**586**	**2**	**0.3**	**R155K**	**Yes**	**274**	**263**	**96.0**				No	352	0	N/A
		A156V		No	586	0	N/A	None	**Yes**	**274**	**1**	**0.4**				No	352	0	N/A
16		**V36A**	None	No	582	0	N/A	None	No	510	0	N/A	NO	902	None	**Yes**	**652**	**1**	**0.15**
	V36M	**V36M**		No	582	0	N/A		**Yes**	**510**	**3**	**0.6**				No	652	0	N/A
		T54S		No	583	0	N/A		**Yes**	**517**	**1**	**0.2**				No	659	0	N/A
	R155K	**R155K**		No	584	0	N/A		**Yes**	**518**	**2**	**0.4**				No	660	0	N/A
		R155T		No	584	0	N/A		**Yes**	**518**	**11**	**2.1**				No	660	0	N/A
		D168N		No	584	0	N/A		**Yes**	**518**	**11**	**2.1**				No	660	0	N/A
17	**V36M**	**V36M**	None	No	359	0	N/A	**V36M**	**Yes**	**259**	**197**	**76.1**	YES	651	N/A	No	63	0	N/A
	R155K	**R155K**		No	374	0	N/A	**R155K**	**Yes**	**397**	**394**	**99.2**				**Yes**	**78**	**1**	**1.28**
18	**V36M**	**V36M**	None	No	745	0	N/A	None	No	139	0	N/A	NO	869	None	No	650	0	N/A
		V36A		No	745	0	N/A		**Yes**	**139**	**1**	**0.7**				No	650	0	N/A
	R155K	**R155K**		No	747	0	N/A		**Yes**	**195**	**7**	**3.6**				No	660	0	N/A
19	**V36A**	**V36A**	None	No	863	0	N/A	None	**Yes**	**351**	**1**	**0.3**	NO	625	None	No	563	0	N/A
		T54A		**Yes**	**872**	**1**	**0.1**		No	356	0	0.0				No	568	0	N/A
20		**V36M**	None	No	193	0	N/A	None	**Yes**	**463**	**5**	**1.1**	YES	814	None	No	387	0	N/A
		T54A		No	196	0	N/A	None	**Yes**	**492**	**4**	**0.8**				No	417	0	N/A
	R155K	**R155K**		**Yes**	**196**	**5**	**2.6**	**R155K**	**Yes**	**495**	**401**	**81.0**				**Yes**	**417**	**18**	**4.32**

*Denotes timepoints where population sequence data was not available at the same time point as 454-Primer ID sequence data. In these cases, population sequence data from the nearest time-point was included.

Summary/Conclusions

- This study used Primer ID to mitigate RT-PCR errors and issues surrounding resampling. Although obtaining a large number of individual Primer IDs for baseline and follow-up time points sometimes requires multiple runs, the technique yielded data where the exact number of unique amplicons being studied can be extremely accurately quantified.
- RAVs circulating at frequencies <21.5% were not detected by standard population sequencing.
- Primer ID/454-sequence analysis detected RAVs at frequencies as low as 0.18%.
- Results obtained by population sequencing revealed that 7/7 poorly interferon responsive patients with baseline RAVs (associated with failure) did not to achieved SVR. The present data extend those findings by identifying two additional patients whose viruses had low-level (~1%) baseline RAVs associated with virologic failure also failed to achieve SVR.
- There did not appear to be a correlation between the frequency of viruses circulating at virologic failure and the longevity of RAVs. In the majority of patients, RAVs were detected at high levels at virologic failure but this did not predict which RAVs would be present after 1-2 years of follow-up. For example, V36M and R155K were present in all of the viruses identified at virologic failure in patient 8. However, after 674 days neither variant was detected.
- Given the success of this technique in studying viral quasi-species before, during and after virologic failure in these 20 patients, the Primer ID/454 sequencing should become the standard approach by which to perform temporal 454-sequence studies.

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