LB-23

Validation of an HCV NS3/4A Sequencing Assay for Evaluating Resistance to Boceprevir, Telaprevir and Protease Inhibitor Candidates in a Clinical Reference Laboratory Setting

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The 62nd Annual Meeting of the American Association for the Study of Liver Diseases
San Francisco, CA
November 4-8, 2011

BACKGROUND and AIMS

• The HCV protease inhibitors boceprevir and telaprevir were recently approved for the treatment of genotype 1 viruses, in combination with pegylated interferon and ribavirin. Several other protease inhibitors are in various stages of clinical development. Protease inhibitor drug resistance-associated variants are frequently observed following treatment failure and may persist for a number of months to years, which could have implications for future treatment options. A sequence-based resistance assay was developed to detect the presence of such variants. Assay performance was characterized in validation experiments designed to assess assay accuracy, precision, reproducibility, linearity, specificity and sensitivity for amplification and detection of minor variants.

METHODS

• The NS3/4A region of HCV is amplified from patient plasma samples by RT-PCR using genotype 1a or 1b specific primers. The nucleotide (nt) and derived amino acid (aa) sequences are determined and aa differences relative to a subtype-specific reference sequence are reported (Fig 1).
• The analytical performance of this assay was characterized in a CLIA/CAP accredited laboratory utilizing reference vectors, vectors containing site-directed mutations (SDMs) that confer resistance to protease inhibitors and patient samples.
• Accuracy was assessed by sequencing well-characterized reference vectors and clones containing SDMs that confer resistance to protease inhibitors.
• Precision was evaluated by determining intra-assay variation from replicate tests of patient samples.
• Reproducibility was evaluated by determining inter-assay variation from replicate tests of patient samples tested on different days. Variations included the use of different lots of critical reagents, different instrumentation and assay operators.
• Amplification sensitivity was assessed by progressive partition analysis and by determining the percent of patient samples successfully amplified at defined viral loads.
• Minor species sensitivity was evaluated using mixtures of wildtype vectors and vectors containing mutations that confer resistance to protease inhibitors.
• Linearity was determined by comparing sequences generated from broad dilutions of plasma samples.
• Specificity was determined by evaluating non-specific amplification and interference from plasma containing HIV-1 or HBV.

Figure 1: HCV NS3/4A Sequencing Assay Workflow

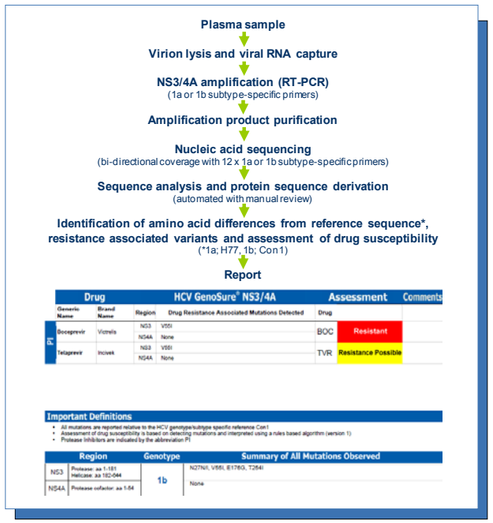
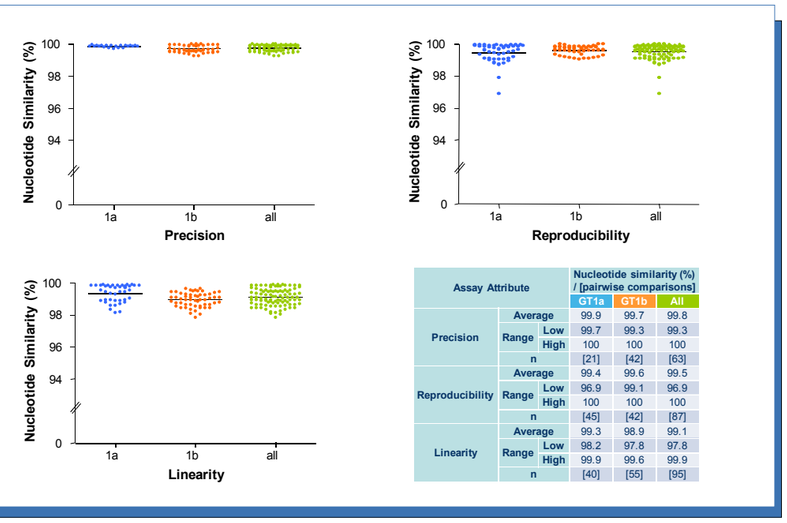
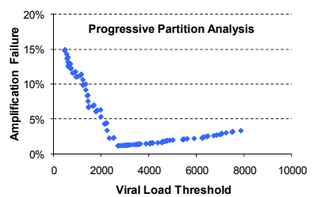


Figure 2: Assay Precision, Reproducibility and Linearity



Assay Attribute			Nucleotide similarity (%) / [pairwise comparisons]		
			GT1a	GT1b	All
Precision	Average		99.9	99.7	99.8
	Range	Low	99.7	99.3	99.3
		High	100	100	100
	n		[21]	[42]	[63]
Reproducibility	Average		99.4	99.6	99.5
	Range	Low	96.9	99.1	96.9
		High	100	100	100
	n		[45]	[42]	[87]
Linearity	Average		99.3	98.9	99.1
	Range	Low	98.2	97.8	97.8
		High	99.9	99.6	99.9
	n		[40]	[55]	[95]

Figure 3: Amplification Sensitivity



• Viral load threshold corresponding to ≥ 95% amplification =1978 IU/mL

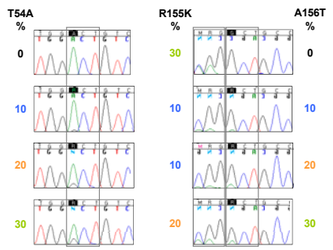
Viral Load (IU/mL)	Number Tested			% Amplified		
	GT1a	GT1b	Total	GT1a	GT1b	Total
>10000	12	7	19	100	100	100
5000-9999	17	14	31	94	100	97
2000-4999	21	22	43	90	95	93
1000-1999	11	13	24	73	54	63
400-999	8	10	18	75	50	61
≥ 2000	50	43	93	94	98	96

• Samples with viral loads of ≥ 2000 IU/mL were amplified in 96% of assays

Figure 4: Sensitivity for Detection of Resistance Mutations

Genotype	Mutation	Mutant detection (n/N assays)			Sensitivity
		10%	20%	30%	
1A	V36M	4/4	4/4	4/4	10%
1A	R155K	3/4	4/4	4/4	10%
1A	A156T	4/4	4/4	4/4	10%
1B	V36M	8/8	4/4	4/4	10%
1B	T54A	1/4	4/4	4/4	20%
1B	R155K	8/8	4/4	4/4	10%
1B	A156T	4/4	4/4	4/4	10%
1B	D168A	6/8	4/4	4/4	10%
1B	V170A	5/8	4/4	4/4	10%

• Subpopulations of ≥ 20% detected in 100% of assays
• Subpopulations of 10% detected in 83% of assays



Example sequence chromatograms

Figure 5: Assay Validation Performance Summary

Assay Attribute	Result
Accuracy	100% average nt concordance with 11 established reference sequences and reference sequences containing resistance association mutations
Precision Intra-assay reproducibility	99.8% average nt similarity from 63 pairwise comparisons
Reproducibility Inter-assay	99.5% average nt similarity from 87 pairwise comparisons
Sensitivity: Amplification	96% of samples with VL ≥ 2000 IU/mL derived from testing samples diluted to a targeted VL of 500-20,000 IU/mL (n=135)
Sensitivity: Minor Species	100% detection of ≥ 20% mixture 83% detection of 10% mixture
Linearity	99.1% average nt similarity from 95 pairwise comparisons of samples serially diluted from a VL of >500,000 IU/ml to a targeted ~2000 IU/mL
Specificity	No false positive amplification or interference observed

Key: nt; nucleotide. VL; viral load

RESULTS

• Accuracy. Assay accuracy was established from concordant sequence data of well-characterized reference vectors (Con1 and H77) and vectors containing SDMs that confer resistance to protease inhibitors (Fig 5).

• Precision. Intra-assay precision was demonstrated by an average nt similarity of 99.8% from 63 pairwise comparisons of replicates tested within any given batch (Fig 2 & 5).

• Reproducibility. Inter-assay reproducibility was established at 99.5% average nt similarity from 87 pairwise comparisons of replicate patient samples tested in different assay batches (Fig 2 & 5).

• Amplification sensitivity. 96% of 93 samples with a viral load of ≥2000 IU/mL were amplified from a plasma panel serially diluted to a target 500-20,000 IU/mL (n=135; Fig 3 & 5).

• Minor species sensitivity. Subpopulations of SDMs representing ≥20% and 10% of a mixture were detected in 100% and 83% of assays, respectively (Fig 4 & 5).

• Linearity. Assay linearity was established at 99.1% average nt similarity from 95 pairwise comparisons of samples serially diluted from >500,000 to approximately 2000 IU/mL (Fig 2 & 5).

• Specificity. Assay specificity was verified by lack of false-positive amplification or interference from plasma containing HIV-1 or HBV (Fig 5).

SUMMARY & CONCLUSIONS

• Validation experiments demonstrated the accuracy, precision, reproducibility, amplification sensitivity, minor species detection sensitivity, linearity and specificity of this HCV NS3/4A sequencing assay.

• This assay can facilitate drug resistance analysis of genotype 1a/b HCV populations derived from patient plasma samples and clones derived from patient virus populations.

• This newly validated assay can be utilized to support the clinical management of patients treated with HCV protease inhibitors, as well as clinical studies (retrospective and prospective) of investigational protease inhibitors.

ACKNOWLEDGEMENTS

We thank all Monogram Biosciences personnel involved in the development and validation of the HCV NS3/4A sequencing assay.