Analytical Validation of an HCV Replicon-based Phenotypic Assay for Evaluating Replication Capacity and Susceptibility of Genotype 1a/1b Patient viruses to Polymerase Inhibitors

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BACKGROUND and AIMS

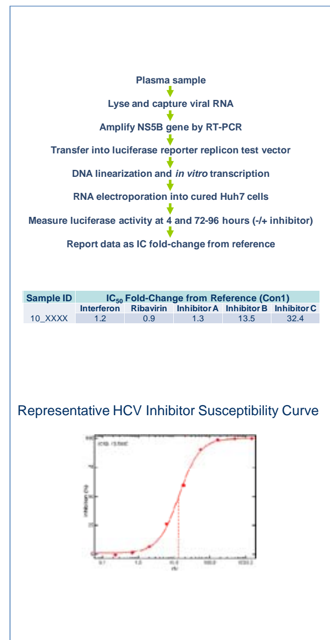
• Several direct-acting antiviral (DAA) agents that target HCV polymerase are in various stages of clinical development. To evaluate DAA resistance, a cell-based phenotypic susceptibility assay for HCV polymerase inhibitors was developed that utilizes reporter replicons containing NS5B regions derived from genotype 1a and 1b patient viruses. The analytical performance of the assay was characterized in validation experiments that assessed accuracy, precision, reproducibility, linearity and sensitivity to detect minor variants.

METHODS

• The entire NS5B region of genotype 1a or 1b HCV is amplified from patient plasma by RT-PCR using subtype-specific primers. Amplification products are cloned into a HCV replicon containing a luciferase-reporter gene. Replicon RNA is introduced into Huh7 cells by electroporation. Replication is determined in the presence and absence of polymerase inhibitors by measuring luminescence (relative light units (RLU)). IC_{50} and IC_{95} fold-changes ($IC_{50}FC$, $IC_{95}FC$) from a reference replicon (Con1) are reported (*Figure 1*). Replication capacity (RC) is determined by evaluating RLUs at 72-96 hours post-electroporation in the absence of inhibitor, relative to RNA input (4hr RLUs) and the reference replicon.

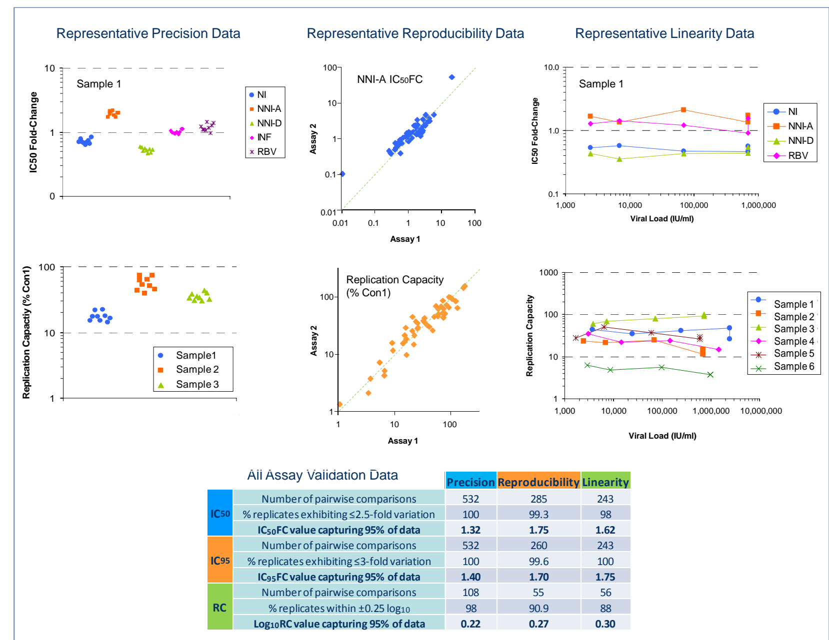
• Assay accuracy was assessed by evaluating well-characterized reference replicons and replicons containing site-directed mutations (SDMs) that confer resistance to polymerase inhibitors. Precision and reproducibility were evaluated by determining intra- and inter-assay variation, respectively, based on repeated testing of patient samples. Linearity was demonstrated by comparing susceptibility measurements generated from serial dilutions of patient plasma samples. Minor species detection was defined using mixtures of drug susceptible and drug resistant replicons.

Figure 1: Assay Workflow



Sample ID	IC_{50} Fold-Change from Reference (Con1)				
	Interferon	Ribavirin	Inhibitor A	Inhibitor B	Inhibitor C
10_XXXX	1.2	0.9	1.3	13.5	32.4

Figure 3: Assay Precision, Reproducibility and Linearity with Patient Samples



All Assay Validation Data

		Precision	Reproducibility	Linearity
IC_{50}	Number of pairwise comparisons	532	285	243
	% replicates exhibiting ≤2.5-fold variation	100	99.3	98
	$IC_{50}FC$ value capturing 95% of data	**1.32**	**1.75**	**1.62**
IC_{95}	Number of pairwise comparisons	532	260	243
	% replicates exhibiting ≤3-fold variation	100	99.6	100
	$IC_{95}FC$ value capturing 95% of data	**1.40**	**1.70**	**1.75**
RC	Number of pairwise comparisons	108	55	56
	% replicates within ±0.25 log_{10}	98	90.9	88
	$Log_{10}RC$ value capturing 95% of data	**0.22**	**0.27**	**0.30**

Figure 2: Evaluation of Assay Accuracy Using Replicons with Mutations in NS5B

Construct	IC_{50} Fold-Change from Con1						
	NI	NNI-A	NNI-B	NNI-C	NNI-D	IFN	RBV
Con1	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Con1 S282T	13.48	0.41	0.77	0.40	0.64	0.68	0.33
Con1 C316Y	0.52	0.39	1.75	6.33	109.66	0.36	0.81
Con1 L392I	0.66	7.34	1.35	1.30	0.51	0.88	0.87
Con1 M423T	ND	ND	>410	ND	ND	1.16	ND
Con1 Y448H	0.59	0.94	0.46	9.77	1.02	0.87	0.81
Con1 P495A	0.97	25.12	0.45	0.30	0.35	0.75	0.90
H77	0.72	0.48	1.38	4.69	0.99	0.92	0.82
H77 S282T	17.68	0.39	0.86	2.57	0.50	1.07	0.35
H77 P495L	0.71	>130.00	2.25	3.63	0.36	0.83	0.80

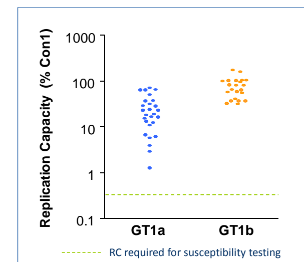
$IC_{50}FC$ ≥2.5 compared to appropriate H77 or Con1 reference
ND = not determined

Figure 4: Evaluation of Minor Species Sensitivity

Mutant	Inhibitor	Phenotypic Data			% mutant detected*	
		$IC_{50}FC$	$IC_{95}FC$	% RC	$IC_{50}FC$	$IC_{95}FC$
Con1 S282T	NI	15.64	19.36	12	100	60
Con1 C316Y	NNI-C	8.15	6.61	87	40	20
	NNI-D	116.91	>53		20	20
Con1 L392I	NNI-A	7.60	5.82	71	40	20
Con1 M423T	NNI-B	>410	>51	71	40	20
Con1 Y448H	NNI-C	16.39	>8	25	100	40
Con1 P495A	NNI-A	21.67	>19	33	100	40
H77 S282T	NI	18.27	>22	2	100	60
H77 P495L	NNI-A	128.95	>27	15	80	20

*ICFC value ≥2. From analysis of samples containing 20, 40, 60, 80 and 100% mutant.

Figure 5: Patient Sample Replication Capacity



RESULTS

• Accuracy. Replicons containing NS5B mutations exhibited expected reductions in susceptibility to nucleoside (NI; S282T mutants) and non-nucleoside polymerase inhibitors targeting site A (NNI-A; L392I and P495A/L mutants), site B (NNI-B; M423T), site C (NNI-C; C316Y and Y448H) and site D (NNI-D; C316Y), demonstrating assay accuracy (*Figure 2*).

• Precision. From analysis of intra-assay variation in inhibitor susceptibility measurements, 95% of replicate $IC_{50}FC$ and $IC_{95}FC$ values were within 1.32 and 1.4-fold, respectively, from 532 pairwise comparisons. 95% of replicate RC values varied by ≤0.22 log_{10} based on 108 pairwise comparisons (*Figure 3*).

• Reproducibility. From analysis of inter-assay variation in inhibitor susceptibility measurements, 95% of replicate $IC_{50}FC$ and $IC_{95}FC$ values were within 1.75 and 1.7-fold, from 285 and 260 pairwise comparisons, respectively. 95% of replicate RC values varied by ≤0.27 log_{10} based on 55 pairwise comparisons (*Figure 3*).

• Linearity. The evaluation of assay linearity over a 3 log_{10} range in viral load demonstrated that 95% of $IC_{50}FC$ and $IC_{95}FC$ values exhibited ≤1.62 and 1.75-fold variation, respectively from 243 pairwise comparisons. 95% of RC values varied by ≤0.3 log_{10} based on 56 pairwise comparisons of serially diluted plasma samples (*Figure 3*).

• Minor species sensitivity. The detection of subpopulations of resistant variants varied from 20-80% depending on the mutation and inhibitor evaluated, likely reflecting the degree of reduced susceptibility and RC conferred by the particular mutation evaluated (*Figure 4*).

• Replication capacity. Replicons derived from a panel of 49 genotype 1a and 1b patient samples all replicated sufficiently to enable phenotypic assessment of polymerase inhibitor susceptibility (*Figure 5*).

SUMMARY & CONCLUSIONS

• We have validated the analytical performance of a phenotypic assay for HCV NS5B that was developed to support clinical studies of investigational inhibitors that target HCV polymerase.

• Validation experiments demonstrated the accuracy, precision, reproducibility, linearity and minor species detection sensitivity of the assay.

• This assay can facilitate analyses of drug resistance and replication capacity of genotype 1a and 1b patient virus populations, as well as molecular clones derived from patient viruses and NS5B sequences containing SDMs.

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