NS5B Sequencing and Phenotypic Resistance Assays for HCV Subtypes 1a and 1b

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5th Intl. Workshop on Hepatitis C Resistance & New Compounds

Presented at the 5th International Workshop on Hepatitis C, Resistance and New Compounds
24-25 June 2010, Boston USA
Several direct acting antiviral agents that target HCV polymerase are in clinical development

Monogram Biosciences, Inc. has developed sequenced-based (GeneSeq® HCV NS5B assay) and phenotypic (PhenoSense® HCV NS5B assay) assays to evaluate polymerase inhibitor resistance for HCV subtypes 1a and 1b
GeneSeq HCV NS5B Assay

Plasma sample
  ↓
Lyse and capture viral RNA
  ↓
Amplify NS5B gene by RT-PCR
    (1a or 1b subtype-specific primers)
  ↓
Gel electrophoresis and DNA purification
  ↓
DNA sequencing
    (bi-directional coverage with 12 x 1a or 1b subtype-specific primers)
  ↓
Sequence analysis
    (automated with manual review)
  ↓
Report amino acid difference from reference sequence
    (1a; H77, 1b; Con1)

<table>
<thead>
<tr>
<th>ID</th>
<th>NS5B Amino Acid Differences from Reference</th>
</tr>
</thead>
</table>

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GeneSeq HCV NS5B Assay Validation

The GeneSeq HCV NS5B assay was validated following an evaluation of the technical performance of the assay in experiments designed to assess:

- Accuracy
- Precision (Intra-assay variation)
- Reproducibility (Inter-assay reproducibility)
- Sensitivity
  - Amplification
  - Minor species
- Linearity
- Specificity
Replicate plasma samples from 3 patients were processed through the assay in parallel (1 batch / patient) and sequence data was evaluated by performing pairwise comparisons of all replicates from the same sample.

From 135 pairwise comparisons:
- Average nucleotide and amino acid similarity >99%
Reproducibility (Inter-assay Variation)

- Replicate aliquots of 50 plasma samples were processed through the assay in different batches by different operators, using different lots of critical reagents on different days. Sequence data was evaluated by performing pairwise comparisons of replicate samples.

From 50 pairwise comparisons:
- Average nucleotide and amino acid similarity >99%
Amplification Sensitivity

The % of diluted plasma samples within viral load groups that produced a sufficient quantity of PCR product for processing was calculated.

<table>
<thead>
<tr>
<th>Viral Load IU/mL</th>
<th>n</th>
<th>% Amplified</th>
</tr>
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<tbody>
<tr>
<td>1000-2000</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>500-999</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>250-499</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>100-249</td>
<td>17</td>
<td>88.2</td>
</tr>
<tr>
<td>50-99</td>
<td>9</td>
<td>88.9</td>
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<tr>
<td>10-49</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>65</td>
<td><strong>92.3</strong></td>
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</table>

- Amplified 100% of 34 samples with viral loads of 250-2000 IU/ml.
### Minor Species Sensitivity

<table>
<thead>
<tr>
<th>Mix</th>
<th>Mixture composition (%)</th>
<th>Mutant detection</th>
<th>% assays</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>L392I</td>
<td>P495L</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>10</td>
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<td>3</td>
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<tr>
<td>6</td>
<td>0</td>
<td>50</td>
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</table>

- Minor species comprising ≥20% of a mixture were detected in 100% of assays.
- Minor species comprising 10% of a mixture were detected in 75% of assays.
Subset of Nucleotide Mixture from Population Sequence Analysis

Sample clonally analyzed to evaluate % minor variant detected
Clonal Sequence Analysis

Sequence analysis of 19 clones

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<tr>
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<th>255</th>
<th>264</th>
<th>337</th>
<th>384</th>
<th>705</th>
<th>801</th>
<th>810</th>
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Clone number

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<th>16</th>
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<td>A</td>
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<tr>
<td>G T A G C T</td>
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<td>C</td>
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</tr>
<tr>
<td>A C G A A</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>C</td>
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<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

% of clones

| nt | A   | C   | A   | A   | T   | C   | G   | G   | A   | G   | T   | C   | T   | T   | T   | T   | T   | T   |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | 15.8| 21  | 31.6| 21  | 10.5| 10.5| 21  | 31.6| 15.8| 26.3| 15.8| 26.3| 21  |
| nt | G   | T   | G   | G   | C   | T   | A   | A   | G   | A   | C   | T   | C   | C   | C   | C   | C   | C   |
| % of clones | 84.2 | 79  | 68.4| 79  | 89.5| 89.5| 79  | 68.4| 84.2| 73.7| 84.2| 73.7| 79  |

Minor variants identified by population sequencing were present in ~11-32% clones

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Sequence data from 13 diluted samples with viral loads of 745-1804 IU/ml were compared pairwise to replicate samples with viral loads of 10,985-1,374,970 IU/ml.

From 26 pairwise comparisons:
- Average nucleotide similarity >98%
- Average amino acid similarity >99%
- No average net loss (~2) or gain (~2) of amino acid mixtures in samples with low viral load.
PhenoSense HCV NS5B Assay

Plasma sample

Lyse and capture viral RNA

Amplify NS5B gene by RT-PCR

Transfer into luciferase reporter replicon test vector

DNA linearization and *in vitro* transcription

RNA electroporation into cured Huh7 cells

Measure luciferase activity at 4 and 72-96 hours (-/+ inhibitor)

Report data as IC50 fold-change from reference

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>IC50 Fold-Change from Reference (Con1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10_XXXXX</td>
<td>Interferon 1.2, Ribavirin 0.9, Inhibitor A 1.3, Inhibitor B 9.5, Inhibitor C 32.4</td>
</tr>
</tbody>
</table>
Representative Susceptibility Curves (SDMs)
PhenoSense HCV NS5B Assay Characterization

The technical performance of the PhenoSense HCV NS5B assay has been characterized in development experiments utilizing reference vectors, site-directed mutants (SDMs) conferring resistance to polymerase inhibitors and patient samples.

Assay characterization:
- Accuracy
- Precision (Intra-assay variation)
- Reproducibility (Inter-assay reproducibility)
- Susceptibility of patient samples to polymerase inhibitors
- Assay modifications to increase the number of phenotypable patient samples
NS5B Site-Directed Mutants Associated with Reduced Drug Susceptibility

Assay accuracy was assessed by evaluating IC50 fold-changes with vectors containing SDMs that reduce susceptibility to specific polymerase inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>Site 1 NNI</th>
<th>Site 2 NNI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBV</td>
<td>NI</td>
</tr>
<tr>
<td>S282T</td>
<td>0.39</td>
<td>11</td>
</tr>
<tr>
<td>N411S</td>
<td>0.74</td>
<td>0.63</td>
</tr>
<tr>
<td>M414T</td>
<td>1.0</td>
<td>0.86</td>
</tr>
<tr>
<td>Y415F</td>
<td>0.82</td>
<td>0.85</td>
</tr>
<tr>
<td>P495A*</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>P495L</td>
<td>1.2</td>
<td>1.7</td>
</tr>
<tr>
<td>G558R</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>V581A</td>
<td>0.94</td>
<td>0.55</td>
</tr>
</tbody>
</table>

*low RLU

SDMs exhibit expected drug susceptibilities.
Precision (Intra-assay Variation)

- Polymerase inhibitor (NNI) susceptibility of 21 patient samples were determined in duplicate within an assay batch.

Pairwise comparison of IC50 fold-changes between replicates:
- 1.2-fold average
- 1.7-fold maximal

IC50’s fold-changes from reference reproducible within ~2-fold.
Reproducibility (Inter-assay Variation)

- Inhibitor susceptibility of 17 patient samples were determined in different assay batches on different days

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Interferon

- Average: 1.6
- Max: 2.3

### Ribavirin

- Average: 1.4
- Max: 2.9

### Polymerase inhibitor (NNI)

- Average: 1.2
- Max: 1.7

Difference in IC50 fold-changes from 17 pairwise comparisons

- IC50’s fold-change from reference reproducible within ~3-fold
Drug Susceptibility of Resistance Test Vectors Containing Patient Derived NS5B

Up to 3 log variability in polymerase inhibitor susceptibility observed
Drug Susceptibility of Resistance Test Vectors Containing Patient Derived NS5B

- Reduced NNI1 and NNI2 susceptibility of 1a relative to 1b samples
Patient Sample Resistance Test Vector Activity

<table>
<thead>
<tr>
<th></th>
<th>ALL</th>
<th>1a</th>
<th>1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>N tested</td>
<td>94</td>
<td>53</td>
<td>41</td>
</tr>
<tr>
<td>N &gt; 2500</td>
<td>55</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td>% &gt; 2500</td>
<td>59%</td>
<td>64%</td>
<td>51%</td>
</tr>
</tbody>
</table>
Optimizing PhenoSense HCV NS5B Assay

- Evaluated approaches to increase the number of samples that can be phenotyped
  - Modified HCV NS5B resistance test vector
    - Alternative approaches to linearize replicon prior to \textit{in vitro} transcription
    - Alternate restriction sites for cloning patient derived NS5B genes into replicon
  - Increased assay signal by optimizing a number of assay steps
    - Replicon test vector reporter
    - Cured Huh7 cells and culture conditions
    - Electroporation conditions
Matrix Optimization of Replicon Activity

Luciferase activity (RLU)

- 4 hr
- 3 days
- 4 days

Original conditions

Presented at the 5th International Workshop on Hepatitis C, Resistance and New Compounds
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Replicon Activity with Original and Enhanced Conditions

- Comparable IC50s, assay precision and reproducibility with original and enhanced conditions
- Evaluate % of phenotypable patient samples with enhanced conditions

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Conclusions

The GeneSeq and PhenoSense HCV NS5B Assays have been developed to support clinical studies of investigational inhibitors that target HCV polymerase.

These assays can facilitate genotypic and phenotypic drug resistance analysis of HCV genotype 1a and 1b patient virus NS5B populations, clones and site-directed mutants.

It will be interesting to determine whether differences in phenotypic baseline susceptibility to polymerase inhibitors influences virologic response.
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