Multi-Laboratory Comparison of Next-Generation to Sanger-Based Sequencing for HIV-1 Drug Resistance Genotyping

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Background

• WHO HIVDR Laboratory Network performs genotyping in support of WHO surveys of HIVDR

• Regional and global analyses depend on standardization of methods to allow comparison of results from different labs
  ◦ Assay validation standards and annual EQA

• Sanger-based sequencing is the norm (kits or in-house)

• NGS methods are being adopted in some labs

• Need to establish parameters to maximize comparability between NGS and Sanger-based methods
Next-generation Sequencing (NGS)

- Full sequence of multiple individual variants
- Objective quantitation of low-abundance variants
- Potential for increased sensitivity (5% or lower) (clinically relevant threshold unknown)
- Potential for lower cost
Study Rationale and Objectives

- In an exploratory manner, compare NGS “consensus” sequences from multiple labs, generated using different thresholds for low-abundance variants, to gold standard Sanger-based reference.
- Evaluate agreement between NGS and Sanger, and between labs.
- Determine the threshold that maximizes agreement and minimizes inter-laboratory variability.
Methods (1)

• Virology Quality Assurance (VQA) HIV genotyping proficiency panels (Rush University Medical Center) 24 and 26 (n=10 specimens) distributed to 10 labs

• VQA (Sanger) consensus sequences
  ◦ The VQA consensus covers PR amino acids 4-99 and RT 38-247
  ◦ Based on over 30 results generated by independent laboratories using a commercial genotyping kit (ViroSeq or TruGene)
  ◦ Where 80% absolute agreement was not reached, an N is inserted at that position, and differences at these positions amongst individual submitted sequences were ignored
Methods (2)

- Assay methodologies
  - Front end (RT-PCR then nested PCR); variable input volume
  - Illumina MiSeq
  - Analysis pipeline (HyDRA, PASEq, MiCall, HMMER, other in-house)
  - Amino acids covered variable

- Labs submitted “Sanger-like” sequences in fasta format based on a minimum nucleotide variant frequency of 5%, 10%, 15% or 20%

- Sequence evaluation
  - vs. VQA Sanger consensus (all positions or just DRM)
  - Pairwise comparisons
  - vs. group consensus (identity vs. majority base)
| Specimen ID | Viral load | Subtype | PR DRM s | RT DRM s | % mixed bases in VQA consensus |
|------------|------------|---------|----------|----------|-------------------------------|
| 24.1       | 7,815      | B       | none     | T215T/C/S | 2.3%                          |
| 24.2       | 18,023     | F       | K20R, M36I | none     | 0.0%                          |
| 24.3       | 26,372     | C       | M36I     | M41L, V75T, V90I, V106M, V179D | 0.0%                          |
| 24.4       | 29,139     | C       | M36I     | M41L, K103N, M184V, T215Y     | 0.1%                          |
| 24.5*      | 6,424      | B       | L10I, L33F, M46L, I54V, A71I/T, V82A, L90M (also N88G) | M41L, E44D, A62V, D67N, L74V, L100I, K103N, H208Y, L210W, T215Y, H221Y, K238K/N (also M184L) | 0.8%                          |
| 26.1       | 16,685     | C       | M36I, T74S | D67N, K70R, V90I, M184V    | 0.9%                          |
| 26.2*      | 4,513      | B       | L10I, L33F, M46L, I54V, A71I/T, V82A, L90M (also N88G) | M41L, E44D, A62V, D67N, L74V, L100I, K103N, H208Y, L210W, T215Y, H221Y (also M184L) | 1.1%                          |
| 26.3       | 18,213     | C       | K20R, M36I | A62A/V, K65K/R, D67D/N, V75V/A/I/T, K101Q, K103N, V106M, E138A, M184V K219K/N | 2.1%                          |
| 26.4       | 6,506      | D       | M36I     | none     | 1.1%                          |
| 26.5*      | 3,656      | B       | none     | V90I, K103K/N | 3.8%                          |

* same donor plasma
### Sequence Identity vs. VQA Sanger Consensus

| LAB THRESHOLD | VQA | Sanger |
|---------------|-----|--------|
|               | 218 | GCAAA |
|               | 219 | GCAAA |
|               | 220 | GCAAA |
|               | 221 | GCAAA |
|               | 222 | GCAAA |

#### Table

| VQA | Sanger |
|-----|--------|
| 1   | 5      | G A C A A R A A A C A Y C A G |
| 1   | 10     | G A C A A R A A A C A Y C A G |
| 1   | 15     | G A C A A R A A A C A Y C A G |
| 1   | 20     | G A C A A R A A A C A Y C A G |
| 2   | 5      | G A C A A R A A A C A Y C A G |
| 2   | 10     | G A C A A R A A A C A Y C A G |
| 2   | 15     | G A C A A R A A A C A Y C A G |
| 2   | 20     | G A C A A R A A A C A Y C A G |
| 6   | 5      | G A C A A R A A A C A Y C A G |
| 6   | 10     | G A C A A R A A A C A Y C A G |
| 6   | 15     | G A C A A R A A A C A Y C A G |
| 6   | 20     | G A C A A R A A A C A Y C A G |
| 9   | 5      | G A C A A R A A A C A Y C A G |
| 9   | 10     | G A C A A R A A A C A Y C A G |
| 9   | 15     | G A C A A R A A A C A Y C A G |
| 9   | 20     | G A C A A R A A A C A Y C A G |
| 10  | 5      | G A C A A R A A A C A Y C A G |
| 10  | 10     | G A C A A R A A A C A Y C A G |
| 10  | 15     | G A C A A R A A A C A Y C A G |
| 10  | 20     | G A C A A R A A A C A Y C A G |
| 11  | 5      | G A C A A R A A A C A Y C A G |
| 11  | 10     | G A C A A R A A A C A Y C A G |
| 11  | 15     | G A C A A R A A A C A Y C A G |
| 11  | 20     | G A C A A R A A A C A Y C A G |
Sequence Identity vs. Sanger Consensus (group 1)
Sequence Identity vs. Sanger Consensus (group 2)

Summary statistics across all labs

|                | 5%  | 10% | 15%  | 20% |
|----------------|-----|-----|------|-----|
| Number         | 94  | 94  | 94   | 85  |
| Minimum        | 95.0| 95.7| 98.2 | 98.3|
| Median         | 98.9| 99.6| 99.7 | 99.9|
| Mean           | 98.7| 99.4| 99.6 | 99.7|
| Std. Deviation | 0.95| 0.63| 0.41 | 0.40|
| Lower 95% CI of mean | 98.5 | 99.2 | 99.5 | 99.6 |
| Upper 95% CI of mean | 98.9 | 99.5 | 99.7 | 99.8 |

All comparisons of % identity between thresholds p<0.0001 by Wilcoxon test or paired t-test
Sequence Identity vs. VQA Sanger Consensus

| LAB THRESHOLD | 218 | 219 | 220 | 221 | 222 |
|---------------|-----|-----|-----|-----|-----|
| VQA           |     | G   | C   | A   | A   |
| Sanger        |     | R   | A   | A   | C   |
| 1             | 5   | A   | A   | Y   | C   |
| 1             | 10  | A   | A   | Y   | C   |
| 1             | 15  | A   | A   | Y   | C   |
| 1             | 20  | A   | A   | Y   | C   |
| 2             | 5   | A   | A   | C   | A   |
| 2             | 10  | A   | A   | C   | A   |
| 2             | 15  | A   | A   | C   | A   |
| 2             | 20  | A   | A   | C   | A   |
| 3             | 5   | A   | A   | C   | A   |
| 3             | 10  | A   | A   | C   | A   |
| 3             | 15  | A   | A   | C   | A   |
| 3             | 20  | A   | A   | C   | A   |
| 4             | 5   | A   | A   | C   | A   |
| 4             | 10  | A   | A   | C   | A   |
| 4             | 15  | A   | A   | C   | A   |
| 4             | 20  | A   | A   | C   | A   |
| 5             | 5   | A   | A   | C   | A   |
| 5             | 10  | A   | A   | C   | A   |
| 5             | 15  | A   | A   | C   | A   |
| 5             | 20  | A   | A   | C   | A   |
| 6             | 5   | A   | A   | C   | A   |
| 6             | 10  | A   | A   | C   | A   |
| 6             | 15  | A   | A   | C   | A   |
| 6             | 20  | A   | A   | C   | A   |
| 7             | 5   | A   | A   | C   | A   |
| 7             | 10  | A   | A   | C   | A   |
| 7             | 15  | A   | A   | C   | A   |
| 7             | 20  | A   | A   | C   | A   |
| 8             | 5   | A   | A   | C   | A   |
| 8             | 10  | A   | A   | C   | A   |
| 8             | 15  | A   | A   | C   | A   |
| 8             | 20  | A   | A   | C   | A   |
| 9             | 5   | A   | A   | C   | A   |
| 9             | 10  | A   | A   | C   | A   |
| 9             | 15  | A   | A   | C   | A   |
| 9             | 20  | A   | A   | C   | A   |
| 10            | 5   | A   | A   | C   | A   |
| 10            | 10  | A   | A   | C   | A   |
| 10            | 15  | A   | A   | C   | A   |
| 10            | 20  | A   | A   | C   | A   |
| 11            | 5   | A   | A   | C   | A   |
| 11            | 10  | A   | A   | C   | A   |
| 11            | 15  | A   | A   | C   | A   |
| 11            | 20  | A   | A   | C   | A   |
Pairwise Sequence Identity

Average % nt identity

VL (SUBTYPE):       7,815 (B)       18,023 (F)      26,372 (C)       29,139 (C)      6,424 (B)*       16,685 (C)       4,513 (B)*      18,213 (C)        6,506 (D)       3,656 (B)

% mixed (VQA):           2.3%                0%                   0%                  0.1%               0.8%               0.9% 1.1%               2.1%                1.1%                3.8%

Legend:
- 5%
- 10%
- 15%
- 20%
Limitations

• Sequence analysis performed over PR-RT uniformly; different optimal thresholds may exist for specific DRM positions

• Many positions with low % identity do not affect the amino acid

• Influence of additional variables not tested
  ◦ Input copy number
  ◦ Sampling bias related to procedural bottlenecks
  ◦ Analysis pipeline methodology
Conclusions

• Highest identity to Sanger consensus and inter-lab agreement was seen at a threshold of 20%

• Lower agreement at <20% was not simply a result of better sensitivity of NGS, as inter-laboratory agreement also decreased

• If clinically relevant thresholds <20% are demonstrated, sources of inter-laboratory variability in sequence determination must be addressed
| Name of laboratory                                                                 | Location of laboratory (City, Country) | Additional contributors                                      |
|-----------------------------------------------------------------------------------|---------------------------------------|-------------------------------------------------------------|
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