Next Gen (deep) Sequencing Discussion

1) **Intro – A. Kwong (2 min)**
2) **Philosophical Considerations (7 min)**

Section lead	On site	Teleconference
Mike Otto	**David Standring** **Rich Colonno**	**Stuart Ray**

3) Technical Considerations (11 min)

Section lead **Lee-**	On site	Teleconference
Jan van Doorn	**Richard Barnard** **Ina vandenbroucke**	**Neil Parkin** **Patrick Harrington**

4) **Discussion – all (4 min)**

Philosophical Considerations in Next Generation (Deep) Sequencing

Mike Otto

HCV DrAG Meeting #6

March 30, 2011

Berlin, Germany

Introduction

- Resistant variants almost certainly exist in each DAA-naïve patient
- They may be enriched during non-SVR DAA-containing treatment
- They may decrease in proportion of quasispecies after non-SVR DAA treatment
- They might reduce efficacy of future DAA treatment

1. Utility of Studying Minor Species

- Clinical impact on response to anticipated/ongoing treatment?
- Clinical impact on regimen selection?
- Implications for viral evolution in vivo (hepatocyte turnover, replication space, fitness)

2. Relevance in Combinations Treatment Regimens

- Relevance likely to depend on therapeutic context
  - Interferon+ribavirin-containing regimens
  - Ribavirin-containing regimens
  - DAA-only regimens (drug concentration comparable to IC_{50} of “mutant” virus)
  - DAA-only regimens (drug concentration greatly exceeding IC_{50} of “mutant” virus)

3. Utility of Studying variants in Retreatment Patients

- Impact on clinical practice?
- Can predictive models be developed?

Technical Considerations in Next Generation (Deep) Sequencing

Lee-Jan van Doorn

HCV DrAG Meeting #6

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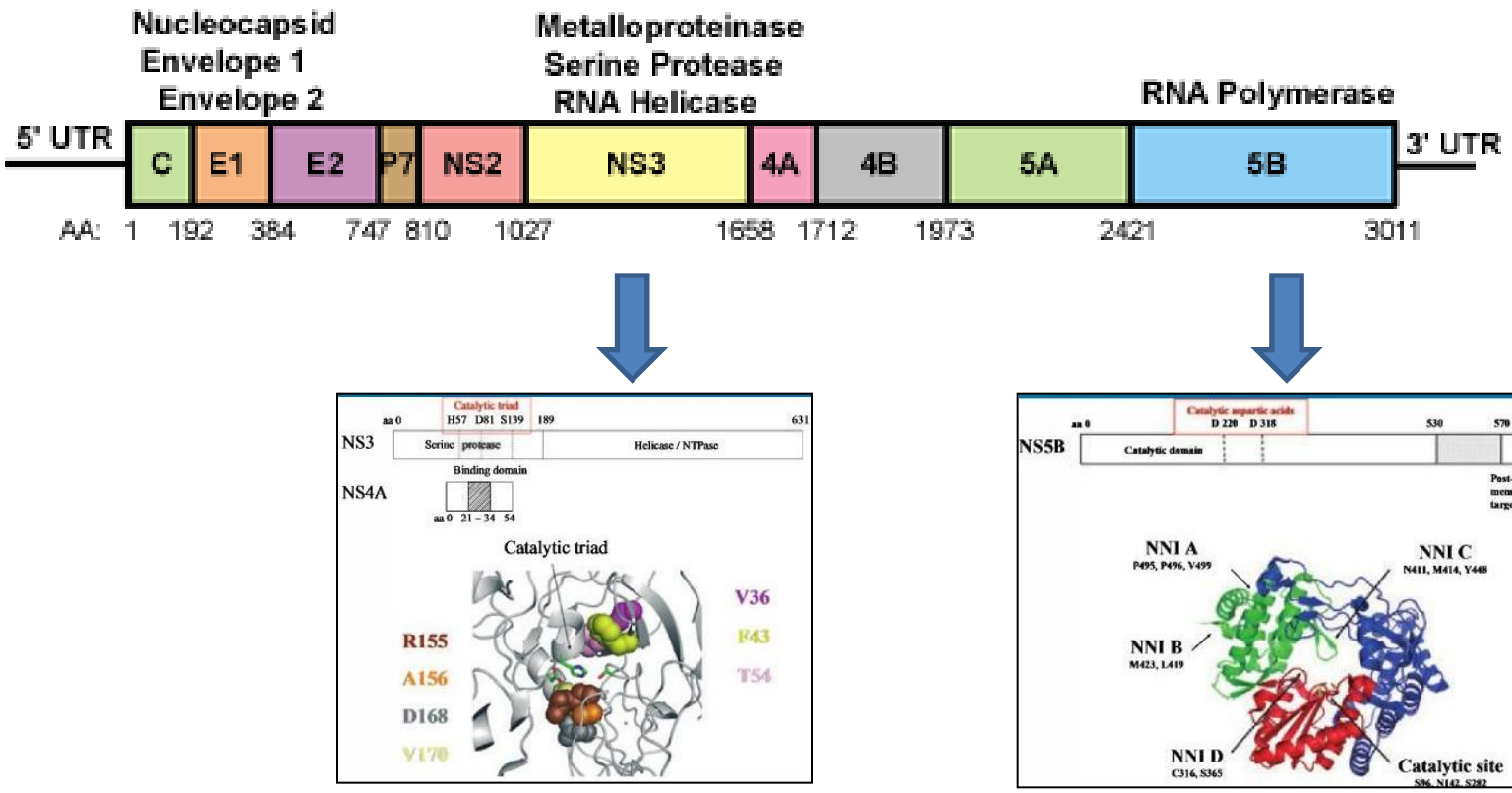
HCV ultradeep sequencing (UDS): technical issues

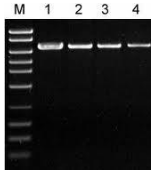


Workflow

Plasma

RNA isolation

RT-PCR

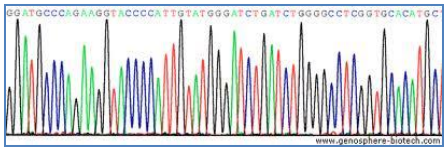


Amplimers

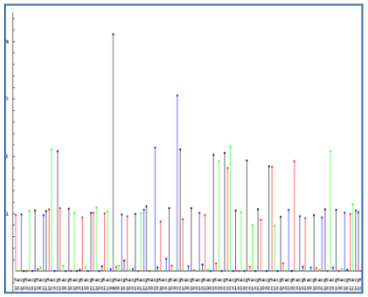
Cloning

Phenotyping

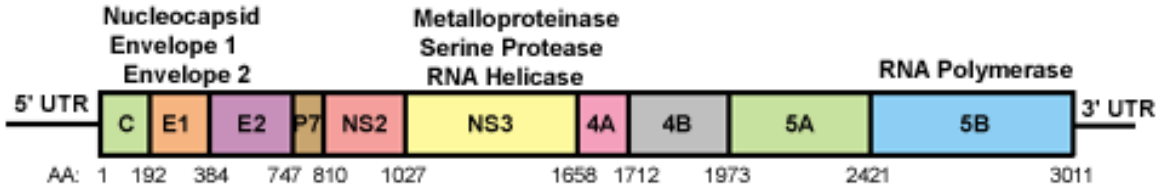
Population sequencing



Ultradeep/next gen seq

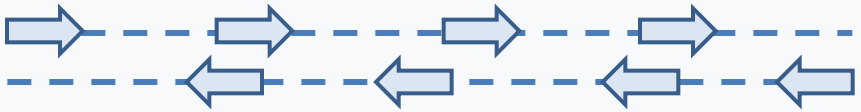


HCV NS5B



~2000 nt

Population sequencing



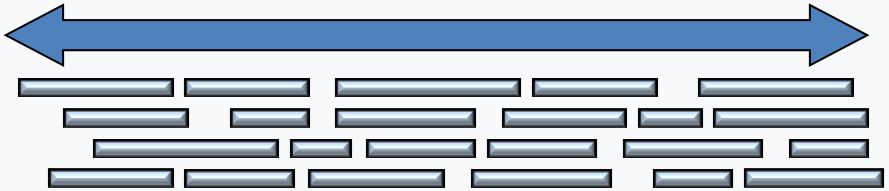
1 consensus sequence

Clonal sequencing

40-90 clonal sequences

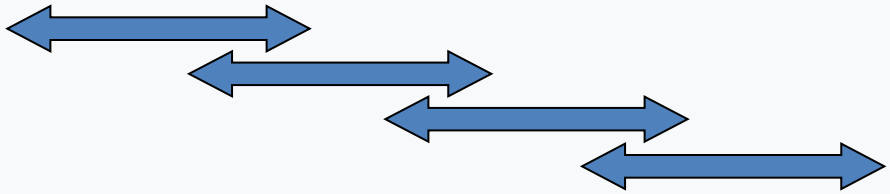
Ultradeep sequencing

A. DNA shearing

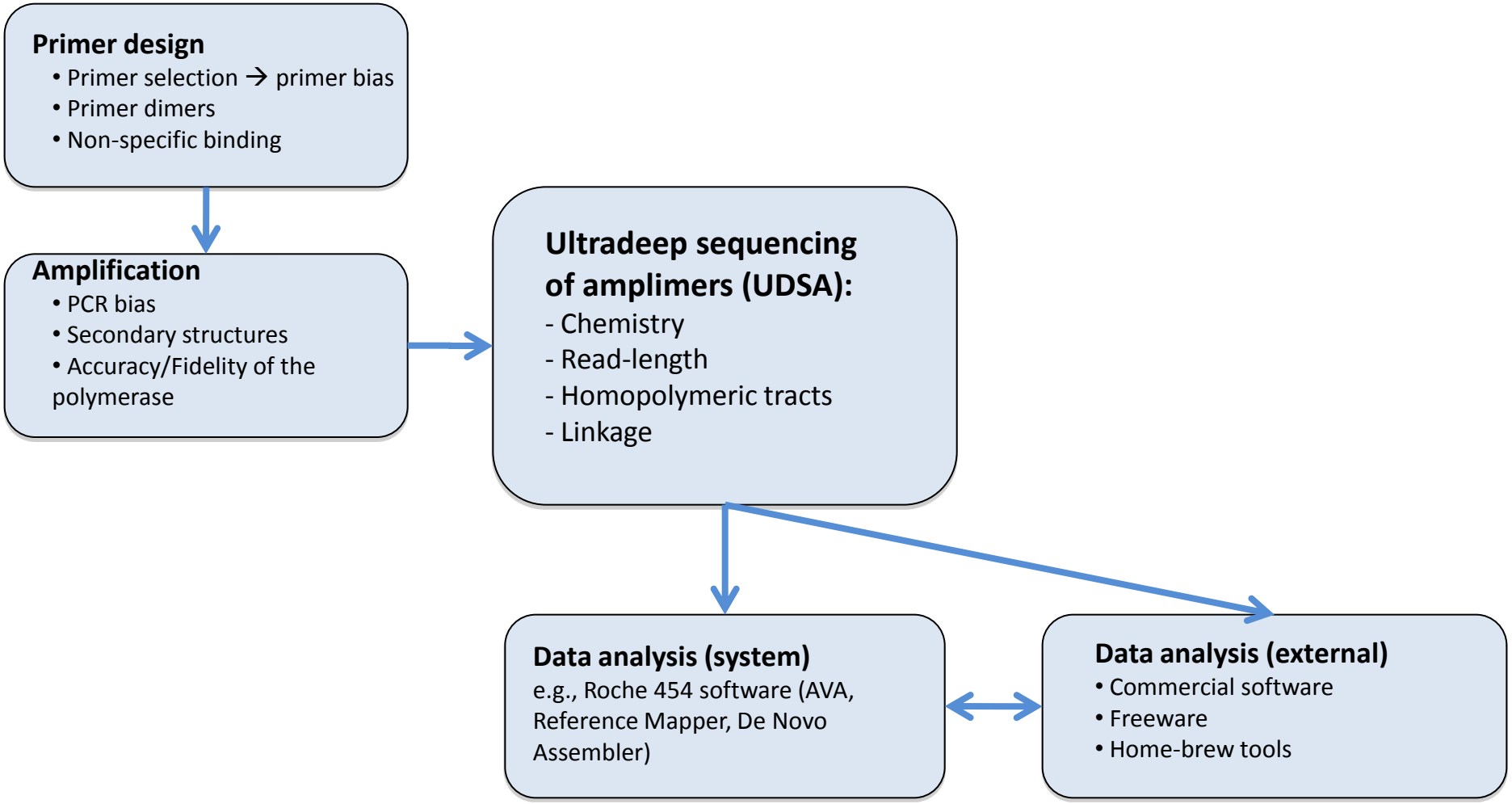


Statistical distribution

B. Subfragment amplification



Potential sources for errors in ultradeep sequencing of amplimers (UDSA)



Provided by Ina Vandenbroucke

Sample preparation

- RNA isolation:
  - no specific technical requirements (high sensitivity/quality; same as for consensus sequencing)
  - Preferably: larger volume, elution in small volume
- Adequate sample ‘representation’:
  - IU/mL in sample → copies per PCR → sensitivity
  - Sampling bias: Representation of minority variants?

Dependence of Sensitivity of Detection of Minor Variants and Input Viral Load

- Assume that 200 µl plasma used for RNA extraction, 25% used for RT-PCR; RT successful for 20% of RNA molecules; minority variant present at 10% of total.

Viral load (copies/ml)	RNA copies in RT rxn	Amplifiable genomes in PCR	Copy no. (minor variant)
100,000	5,000	1,000	100
10,000	500	100	10
1,000	50	10	1

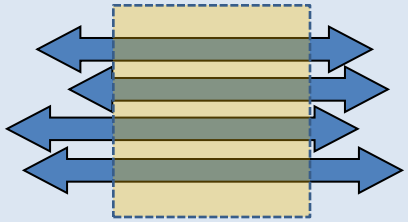
Provided by Neil Parkin

- Low viral load → high sampling bias, high PCR bias
- Threshold ~10,000 IU/ml to perform UDS?

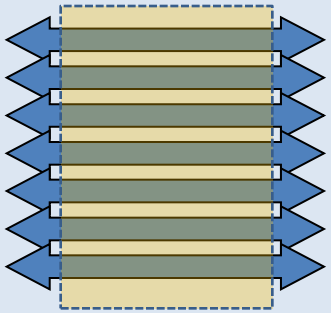
Amplification (Rev. transcription + PCR):

- High-fidelity enzymes RT and PCR step
  - (< 0.1% substitution error rate)
- Amplification bias
  - Robustness of PCR primer sets (genotypes/subtypes)
  - Optimized protocols (Mg^{2+}, temperature,…)

Primer selection:
Overlapping fragments, generated by different PCR primers



PCR bias:
Replicate RT-PCRs



Sampling bias:
- Increase sample input volume
- Replicate entire procedure (RNA isolation, RT, PCR)

Sequencing and data analysis

- Homopolymeric tracts
- Technical sensitivity (# of reads; forward and reverse; coverage per position)
- Analytical sensitivity (sample & viral load)
- Data analysis software (platform specific (454); Bowtie, CLCbio, home-brew).

Reporting issues

- frequency table listing all amino acid positions (also at nt level?), including technical sensitivity
- Assess impact on the protein level → key positions related to resistance
- % wildtype vs. mutant
- Include background sequencing error rate

Reporting to FDA

- No specific reporting format requirements.

Issues to cover (FDA-Division of Antiviral Products; DAVP):

- % of 'resistant' variant relative to wild-type
- absolute concentration of 'resistant' variant (i.e., extrapolating from viral load)
- specifically for long-term persistence analyses:
  - trends over time and modeling approaches to predict return of 'resistant' variant to pre-treatment background level
  - relationship, if any, between 'sensitive' sequence analysis results and population-based results (e.g., is there a relationship between duration while undetectable by population sequencing with time to undetectable by ultradeep sequencing?)
- some descriptive information on technical performance and limitations of the assay (e.g., sensitivity, sampling reproducibility, effect of HCV genotype/subtype, etc.)

Provided by Patrick Harrington

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