Public Comment Speaker #3

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Strategies to establish performance characteristics for NGS-based rare variant oncology panels

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Determining Confidence for Each Rare Variant Fraction Measurement

H23:H520 Cell Line DNA Mixture

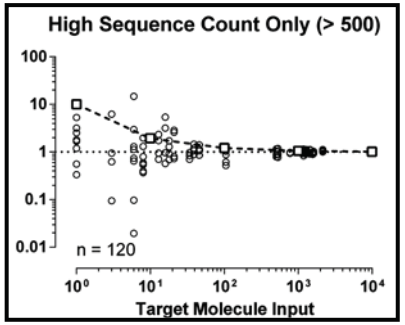
Synthetic Competitive Internal Standards (IS)

Library preparation (target enrichment)

Illumina Hiseq 2500 Sequencing Platform

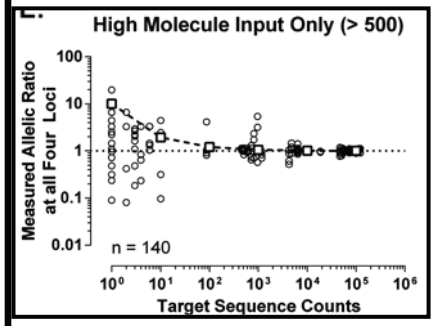
Analysis Pipeline

IS Control for sampling error:
Library prep: Low amplifiable target copies loaded (e.g., FFPE, cytologic)



Symbols:
H23/H520 rs735482 allelic ratio

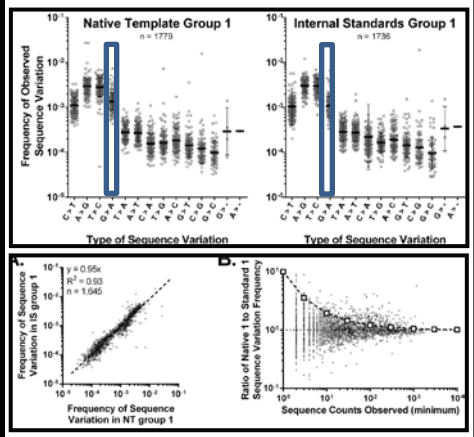
IS Control for sampling error:
Sequencer: Inadequate sequencing space for samples/targets (e.g., excessive/unequal loading)



H23/H520 1:1 library serially diluted
Symbols: rs735482 allelic ratio

Inadequate loading at each step independently increases measurement imprecision

IS Control for polymerase/sequencing error:
Error in IS and target statistically the same



Inter-nucleotide and inter-regional variation in sequence error rate

Determine confidence for each value:

- **Estimated sampling error based on:**
  - known amplifiable copies loaded into library
  - Amplicons loaded into sequencer
- **Estimated sequencing error:**
  - Infer from known error rate in IS

Biomol Detect Quantif. 2015 Sep 1;5:30-37

Determining Confidence for Each Rare Variant Fraction Measurement

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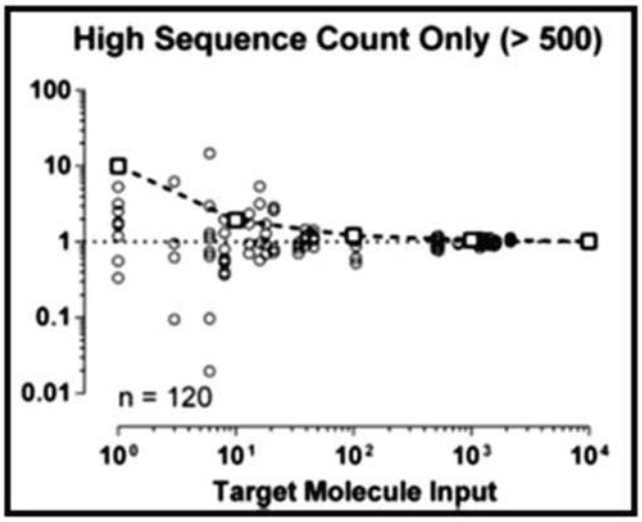

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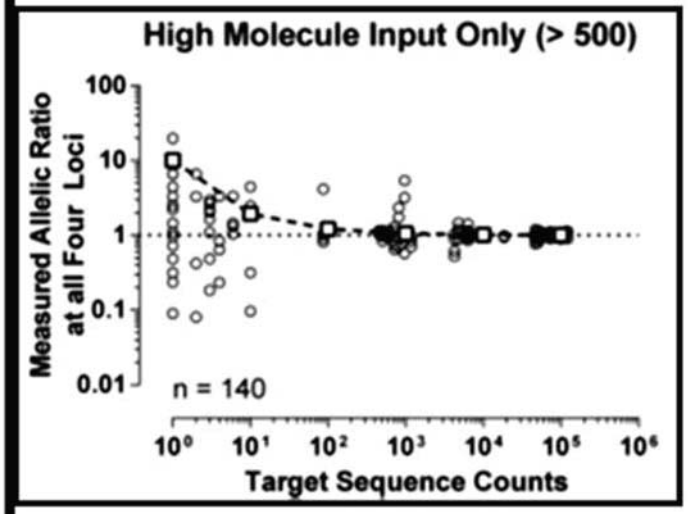

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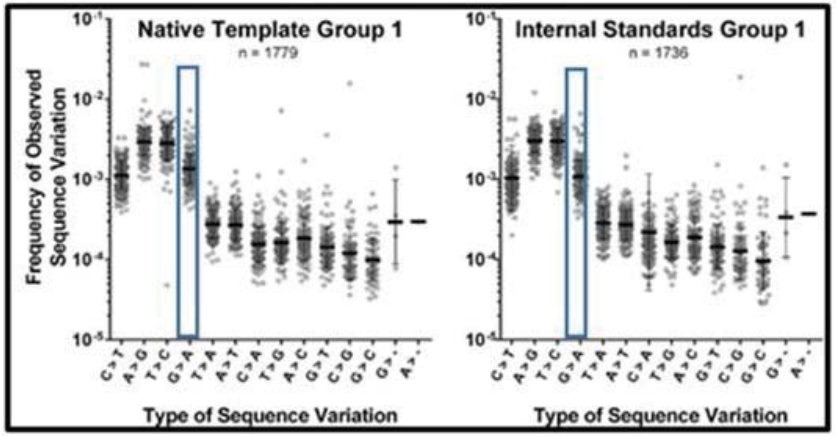

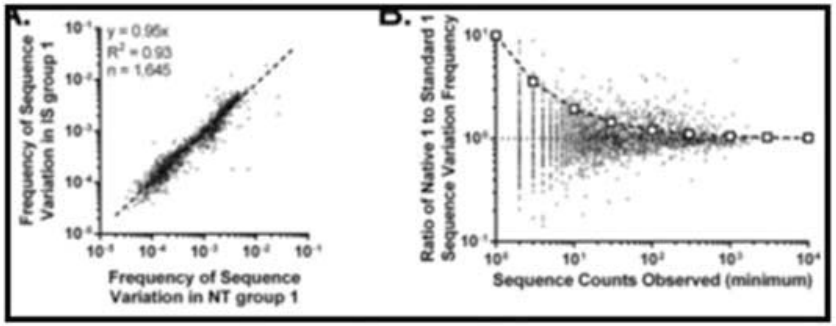

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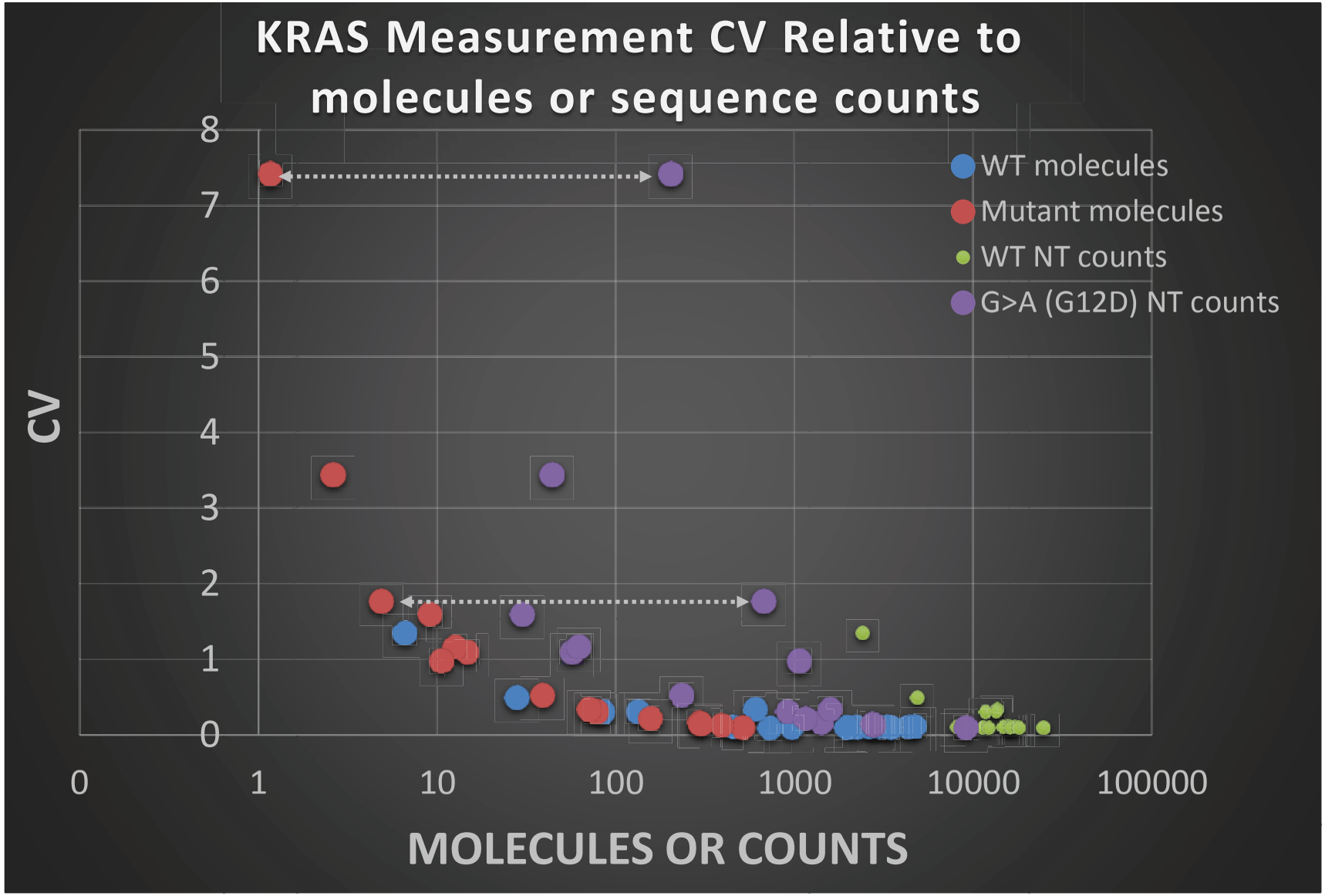

Key Point: Sequencing Depth alone is not sufficient quality control criterion

Conclusions Regarding Analytical Performance:

- CV should be estimated for each variant fraction measurement value based on
  - Molecules loaded into library
  - Library amplicons measured in sequencer
- Synthetic IS in each measurement as process controls is an efficient way to estimate CV for each value and sequencing error at each nucleotide.
- Any departure from optimal conditions will be associated with higher LOD.
- Sub-optimal conditions are frequent, unpredictable, and can render 5% measurement unreliable
  - For example, quality and size of sample, reagents, library preparation.

Conclusions:

- **Under optimal conditions (i.e., 50,000 amplifiable copies loaded into library, 1,000 library amplicons sequenced)**
  - **Limit of quantification (LOD) for KRAS G12D mutation fraction on Illumina Hiseq 2500 will be > 0.004 (>0.4%) assuming:**
    - **200 mutated copies, 50,000 WT copies, 1,000 sequences measured for each value.**
      - **This will be associated with CV = 20%**
    - **0.2% sequencing error on Illumina Hiseq 2500 at KRAS G12D site.**
    - **LOD defined as 3σ above background (sequencing error)***