Standardized Next Generation Sequencing Abundance Measurements (StarSeq) using Competitive Template Mixtures

AccuGenomics Inc.

Need for NGS Standardization

Assuring the quality of next-generation sequencing in clinical laboratory practice

To the Editor:
We direct your readers' attention to the principles and guidelines (Supplementary Guidelines) developed by the Next-generation Sequencing: Standardization of Clinical Testing (Nex-StoCT) workgroup.

The workgroup recommendations are summarized in **Table 1**. Although the workgroup focused on detection of DNA sequence variations associated with heritable human disorders, many of the principles and recommendations described

treatment of cancer and infectious-disease testing.

Validation is the process of establishing analytical performance specifications for a clinical test system developed in house to confirm that the system is suitable for its

		sequencing) to derive high-quality sequence data for problematic genomic regions.
Quality control	Document reliability of the sequence analysis during patient testing	•Utilize a combination of QC materials, both intrinsic and/or spiked in, that mimic genomic complexity and the types of mutations the test is designed to detect. •During patient testing, quality metrics (for example, quality scores, depth and uniformity of coverage, mapping quality, GC bias and transition/transversion ratio) should be assessed and compared to those established during validation. •Clinically actionable findings should be confirmed by independent analysis using an alternate method.
Proficiency	The independent	•PT challenges should target the analysis of both disease-

* Nature Biotechnology November 2012

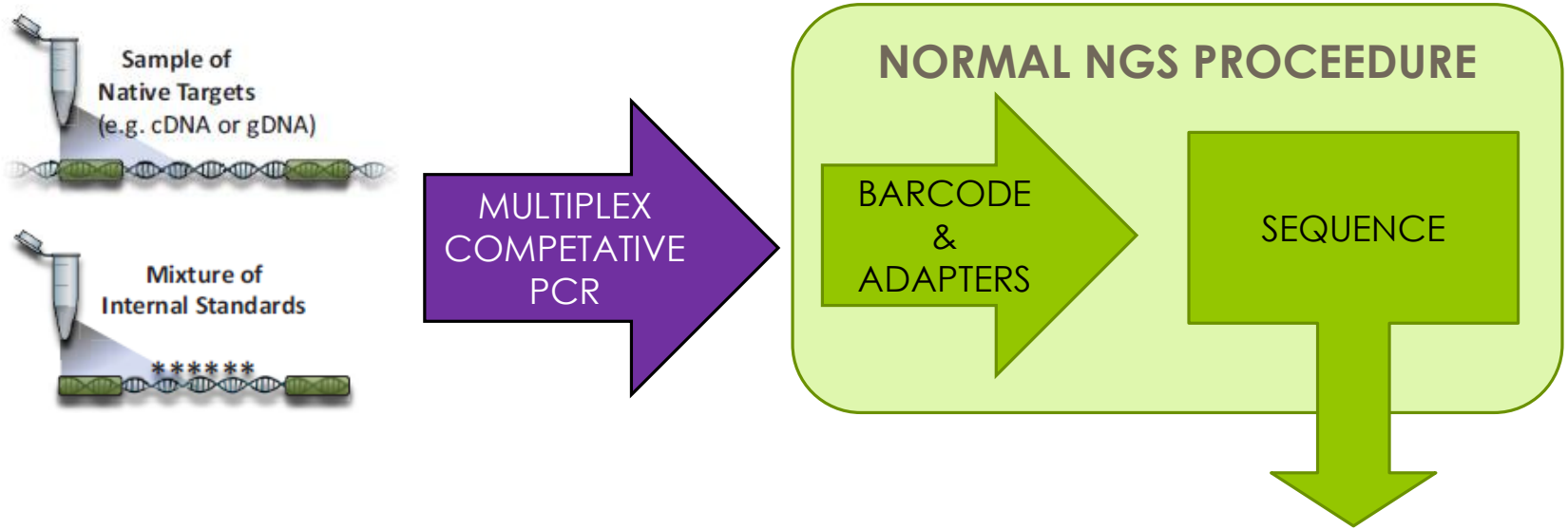
Targeted RNA Sequencing

- More Accurate – Controls for library bias
- Improve Throughput – Amplicon convergence
- QA – every target in every sample

Targeted DNA Sequencing

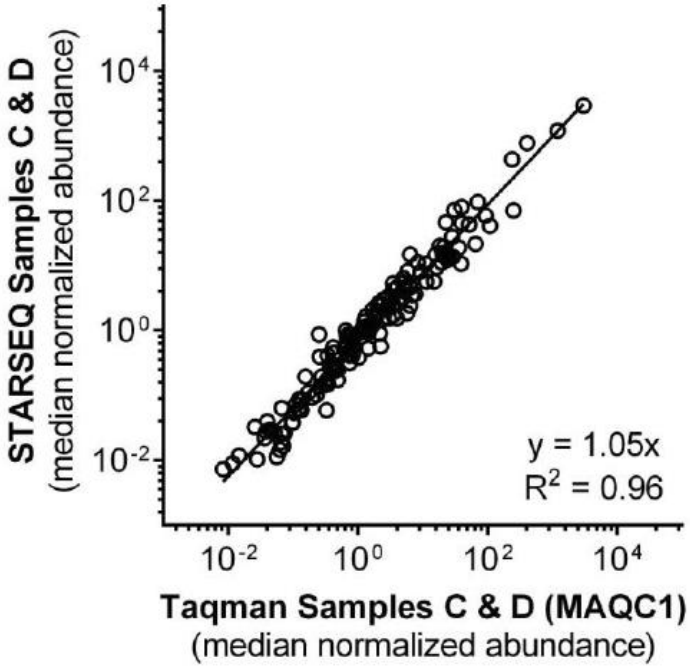
- QA metrics – sensitivity and precision for every mutation

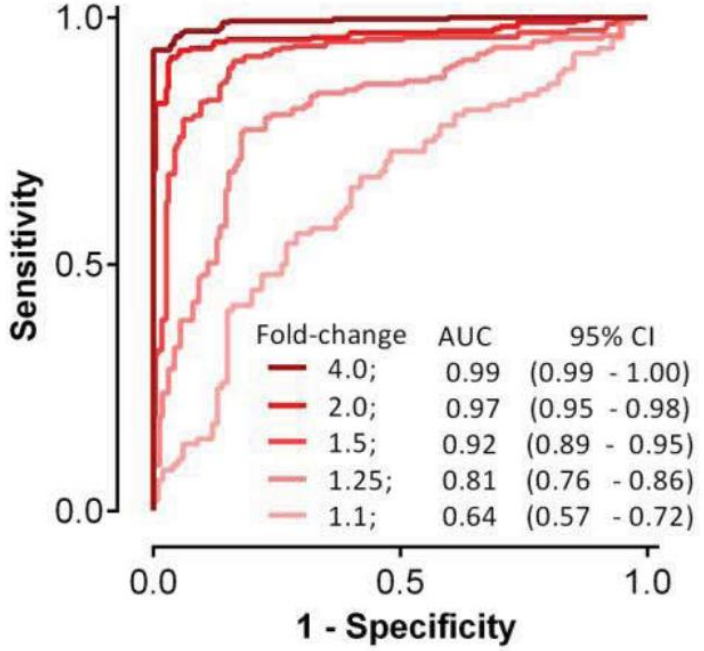
StarSeq Workflow



- **Multiplex PCR to saturation converges high & low abundance targets**
- **NT:CT ratio maintained throughout library construction**
- **Calculate sample abundance from NGS CT and NT counts**

StarSeq ERCC Reference Standards

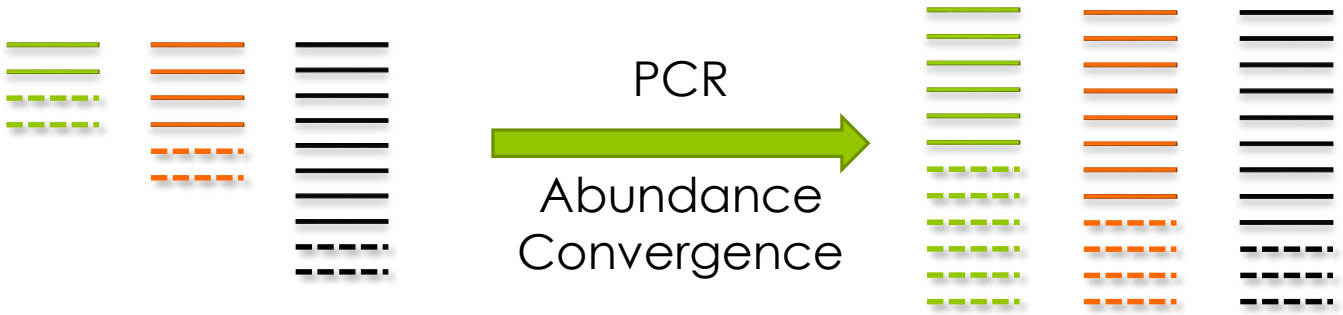




- **Very good correlation between StarSeq vs. real-time qPCR.**
- **ROC curves indicate 95% confidence at detecting two-fold or greater changes.**

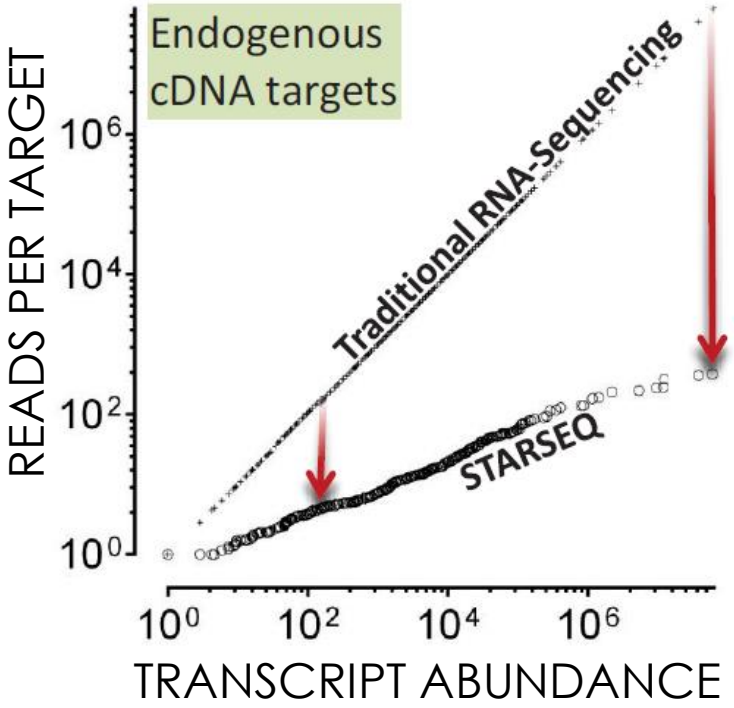
PLoS ONE (2013) 8(11)

Multiplex Competitive PCR Converges High & Low Templates



- **PCR conditions converge amplicons toward equimolar concentrations.**
- **IS:NT ratio maintained while low and high abundance counts converge.**

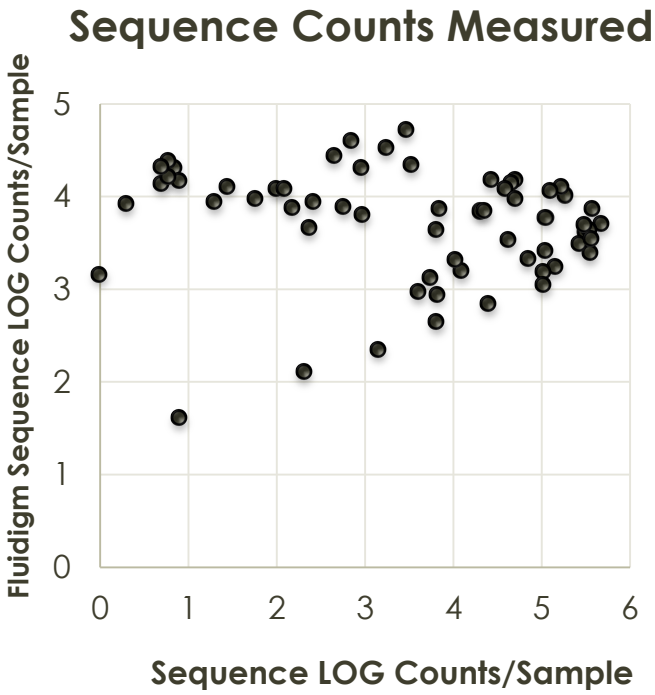
StarSeq Increases Sample Throughput

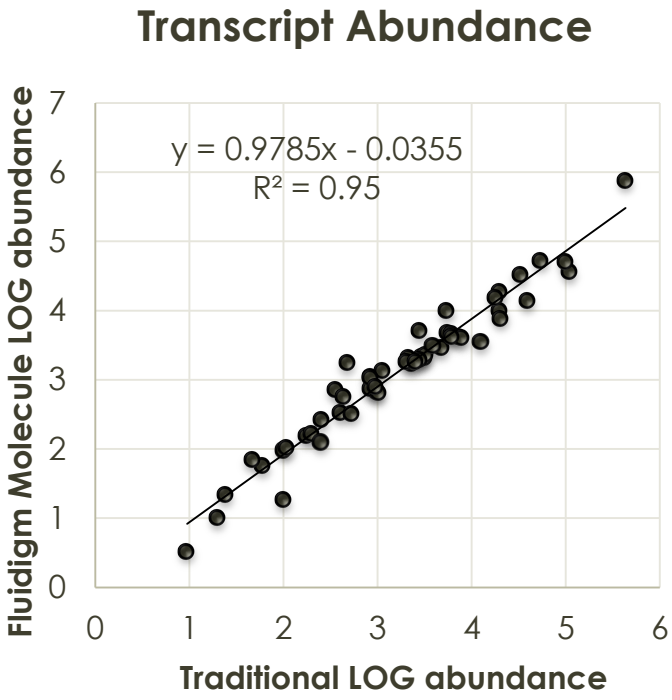


- **StarSeq ratio measurements eliminate the existing requirement for abundance based on total count.**
- **Convergence allows measurement of targets separated over 8 log concentration with <1000 counts**

PLoS ONE (2013) 8(11)

Additional Enrichment for Targeted RNAseq





- **Addition of Fluidigm Access Array™**
  - **Reduces complications of incompatible primer pairs**
  - **Improves convergence**

FFPE Samples

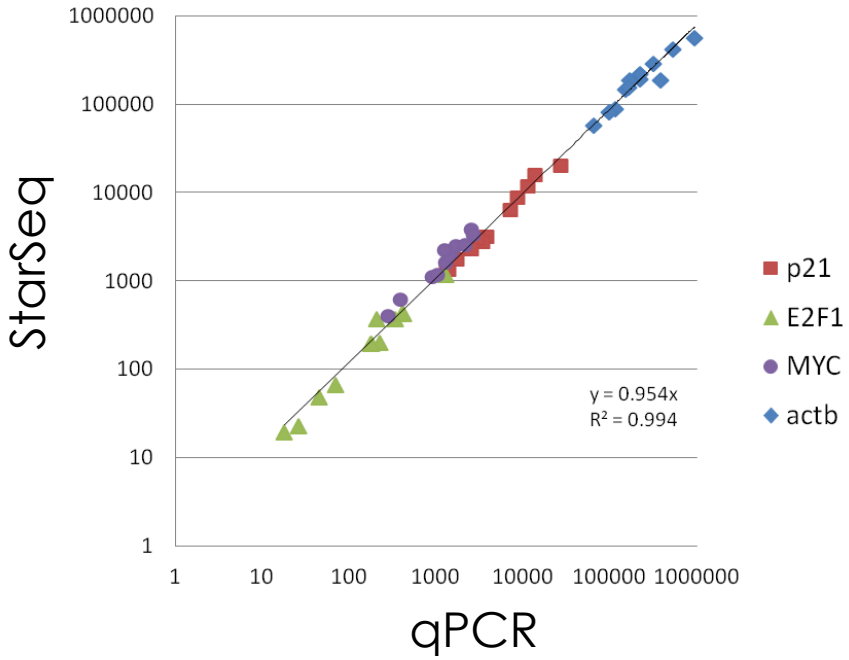
RNA from FFPE samples – 1 cm² area x 10 µm (depth) =
1 ug RNA into a 60 uL Reverse Transcription

↓

1 uL of RT into Pre-amp below
(~17 ng of FFPE RNA derived cDNA)

↓

Multiplex Pre-amplification with Target Specific Primers that have Universal Adapter Tails in the Presence of Competitive Internal Standards



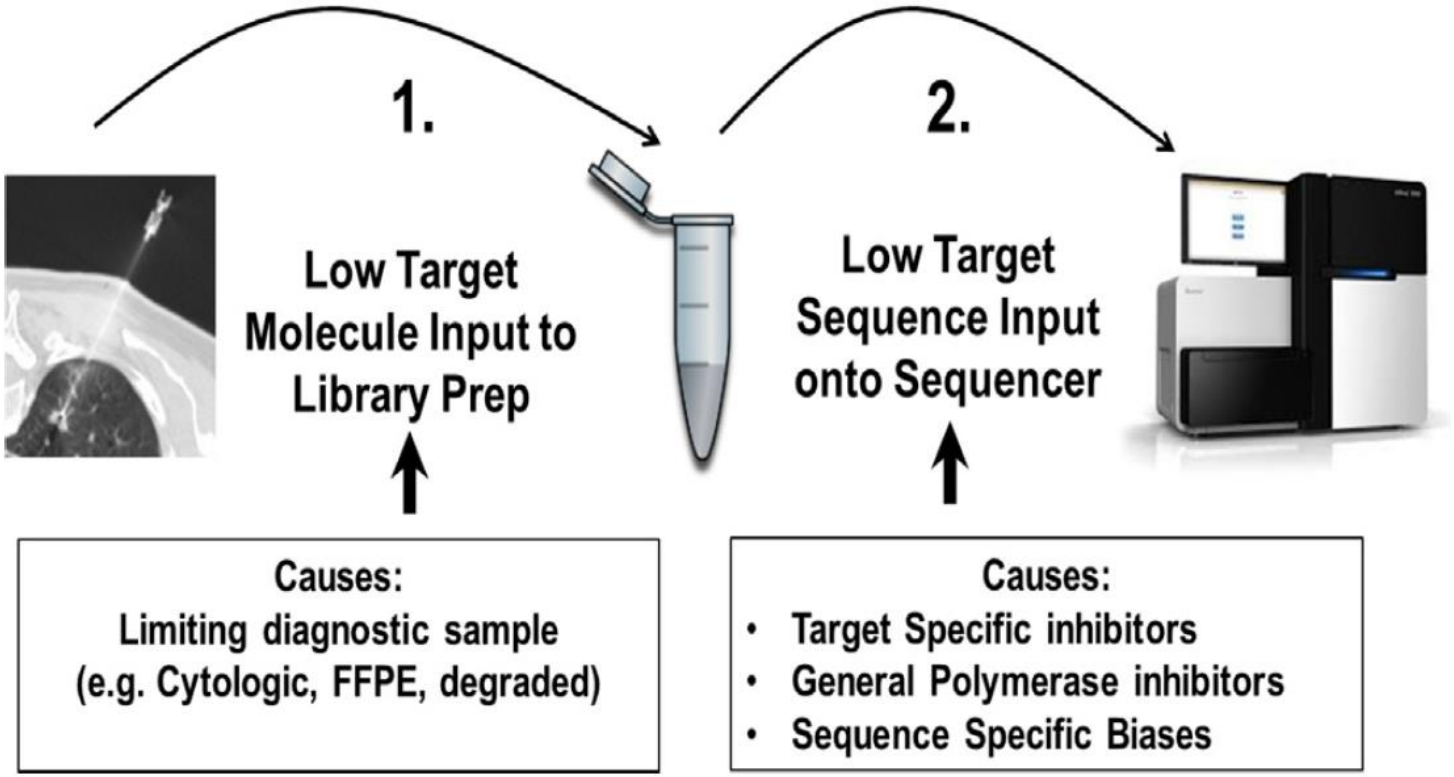
RNAseq Summary

Targeted RNA Sequencing

- **More Accurate – Controls for library bias.**
- **Improve Throughput – Amplicon convergence**
- **QA – every target in every sample**

DNAseq

Allelic Frequency Testing Effected by Sampling Issues

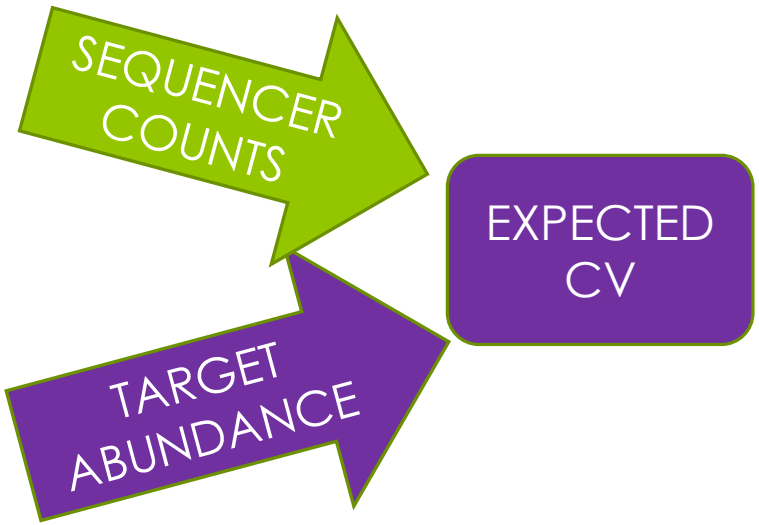


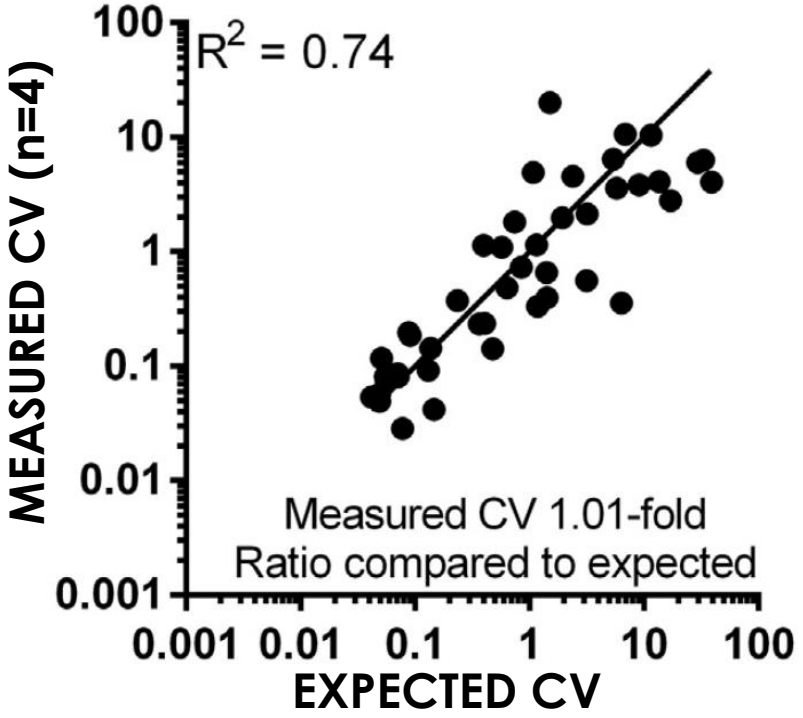
Addition of competitive templates ensures accurate target detection

Biomolecular Detection and Quantification (2015) 5:30–37

Stochastic Error Estimate

H520 x H23 cell lines mixed at different ratios and dilutions for 4 SNP measurements (n=4)

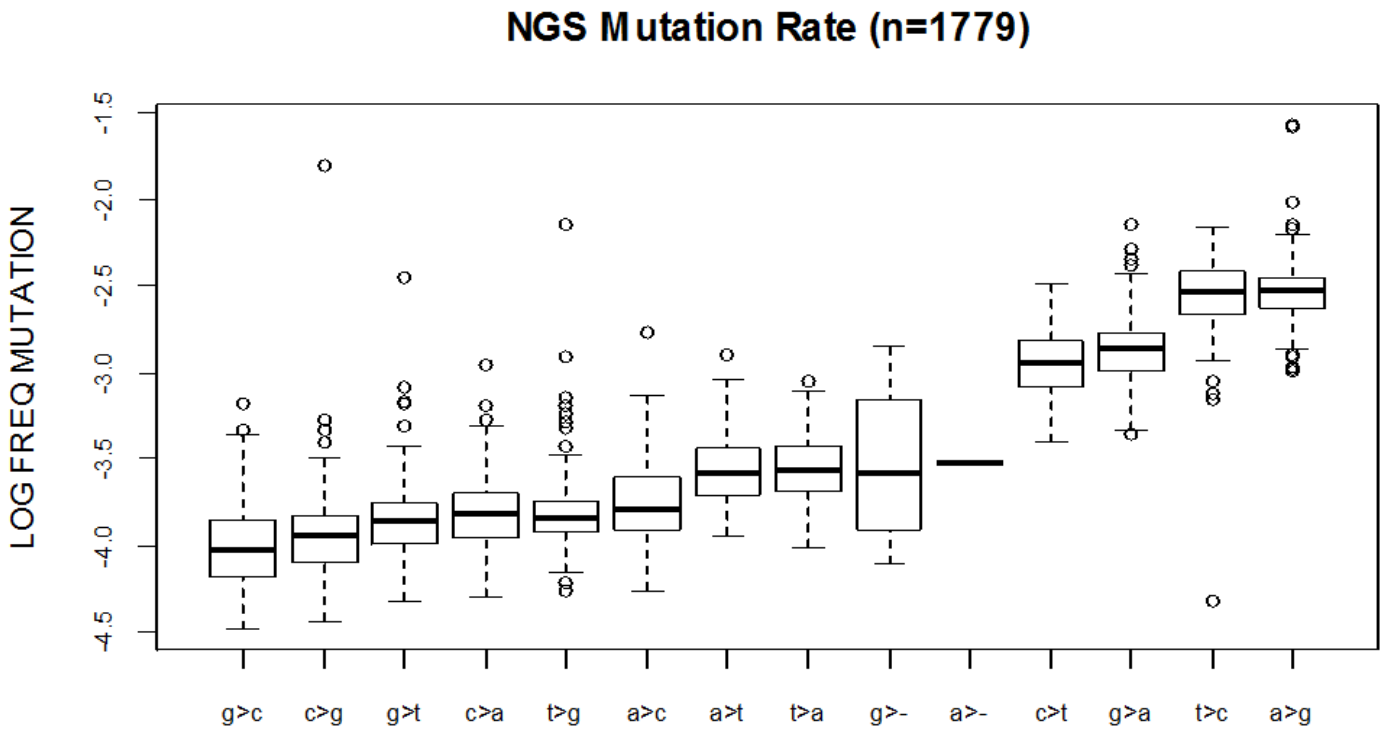




$$Expected\ CV = -1 + 10^{(Molecules\ \ Input\ ^{-0.54} + Sequence\ \ Reads\ ^{-0.54} - [Molecules\ \ Input \times Sequence\ \ Reads\ ]^{-0.54})}$$

Biomolecular Detection and Quantification (2015) 5:30–37

Background Mutations Introduced by NGS Method



Sensitive detection of low frequency mutation is confounded by a variable NGS introduced mutation rate

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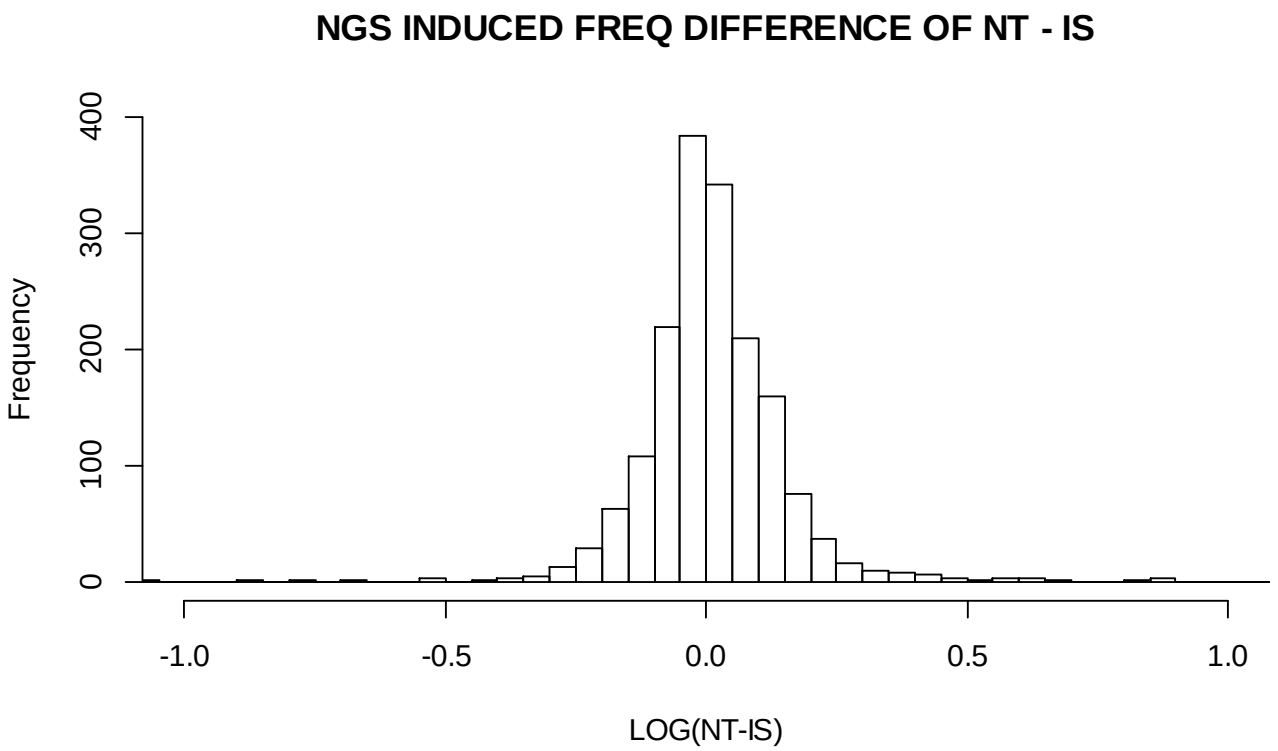
Competitive Templates



Competitive template mutation rate estimates NGS background mutation rate.

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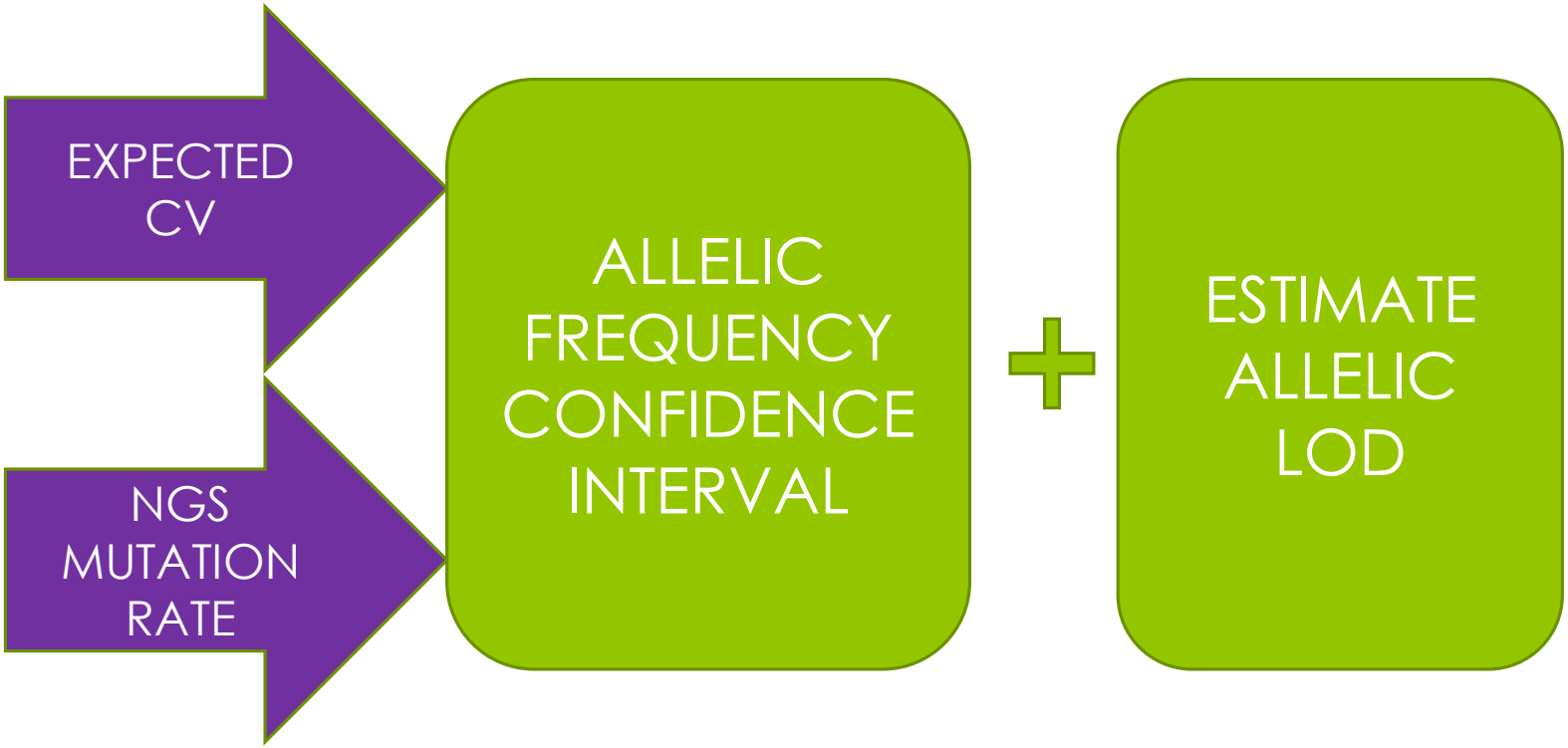
Targeted DNAseq QA



Quality score for every mutation in every sample.

StarSeq Benefits

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- Improve Throughput – Amplicon convergence
- QA – every target in every sample

Targeted DNA Sequencing

- QA metrics – sensitivity and precision for every mutation

Acknowledgements

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