Next Generation Sequencing in the Clinical Laboratory

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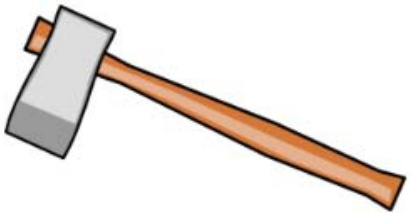
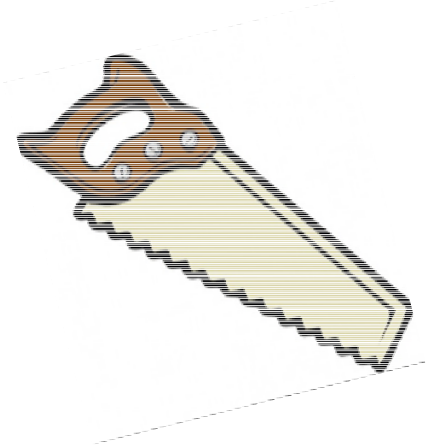
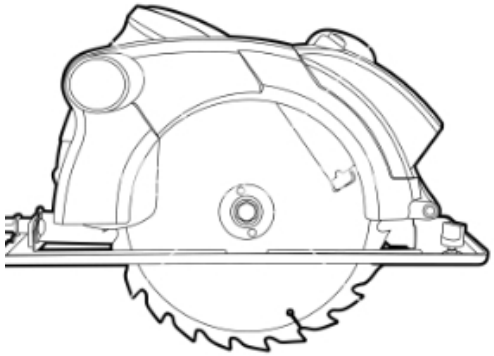
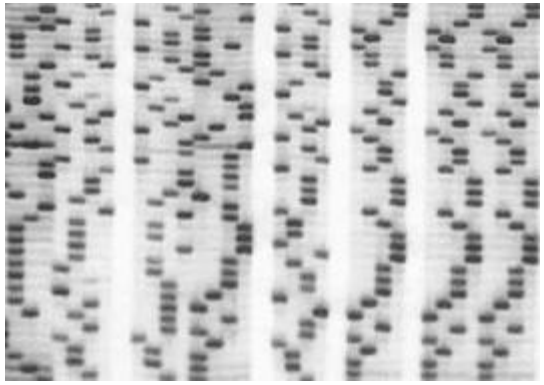
Quest Diagnostics

NGS - An Advancing Technology

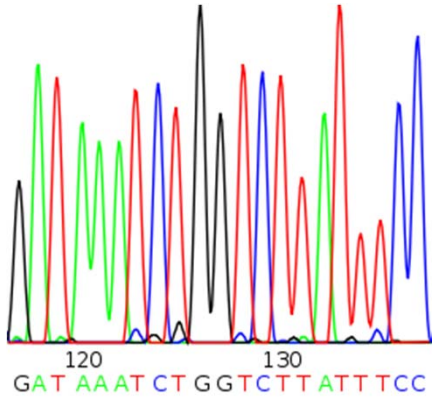




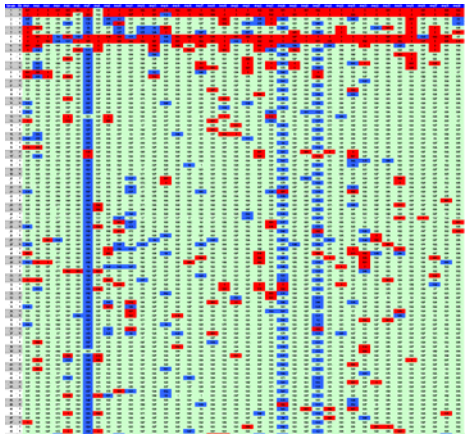




Gel-based Sequencing

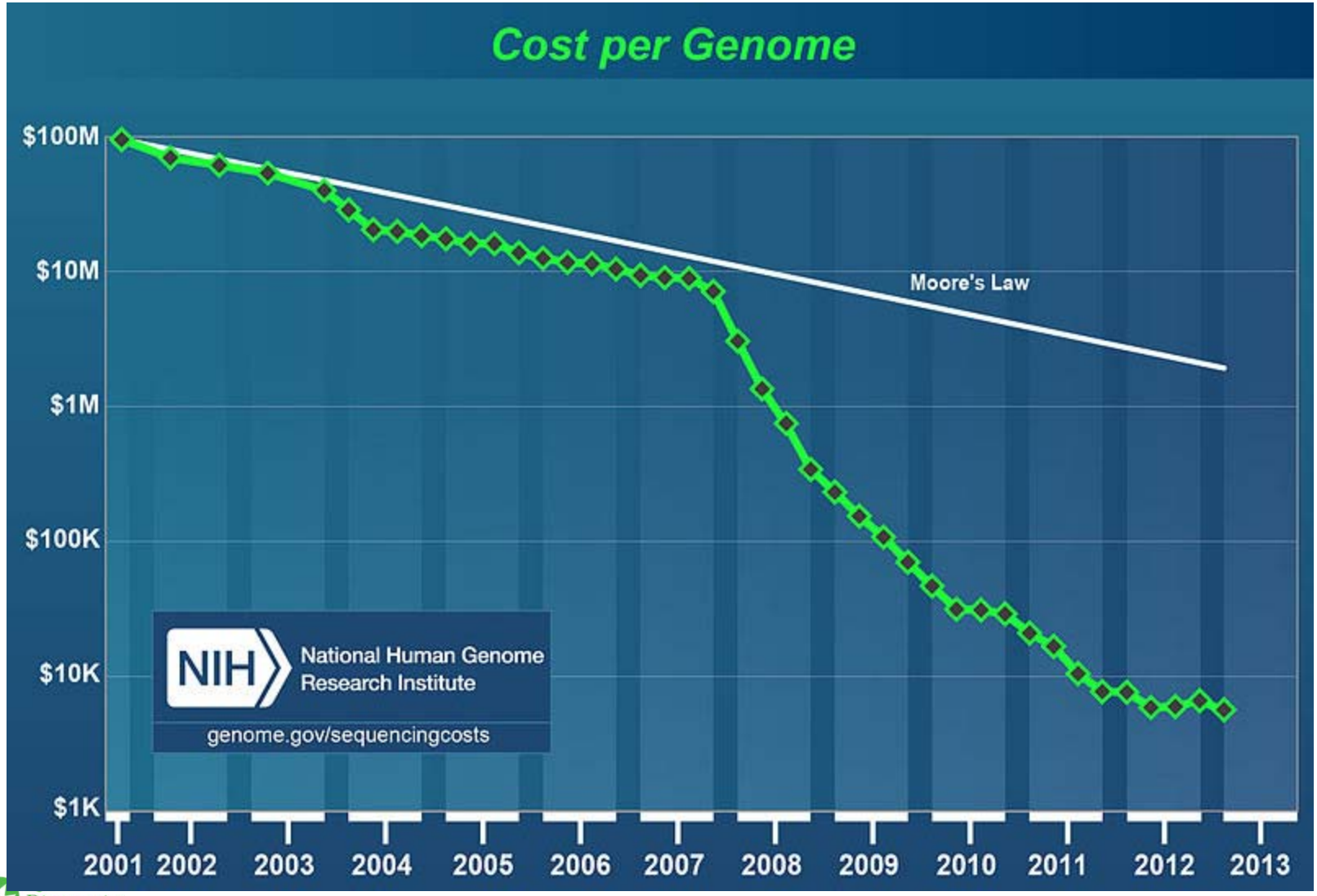


Capillary Electrophoresis



Advanced Sequencing

Cost per Genome Decreasing Dramatically



Diagnostics

Legacy and Next Generation Compared

Sanger Sequencing (Legacy)

- DNA template must be pure
- Sensitivity ≥25%
- High reagent to data ratio
- Low throughput
- Cost per base = $0.50/kb

Advanced Sequencing (Next Gen)

- DNA template can be mixed
- Sensitivity ≥0.1%
- Low reagent to data ratio
- High throughput
- Cost per base ≤ $0.05/kb

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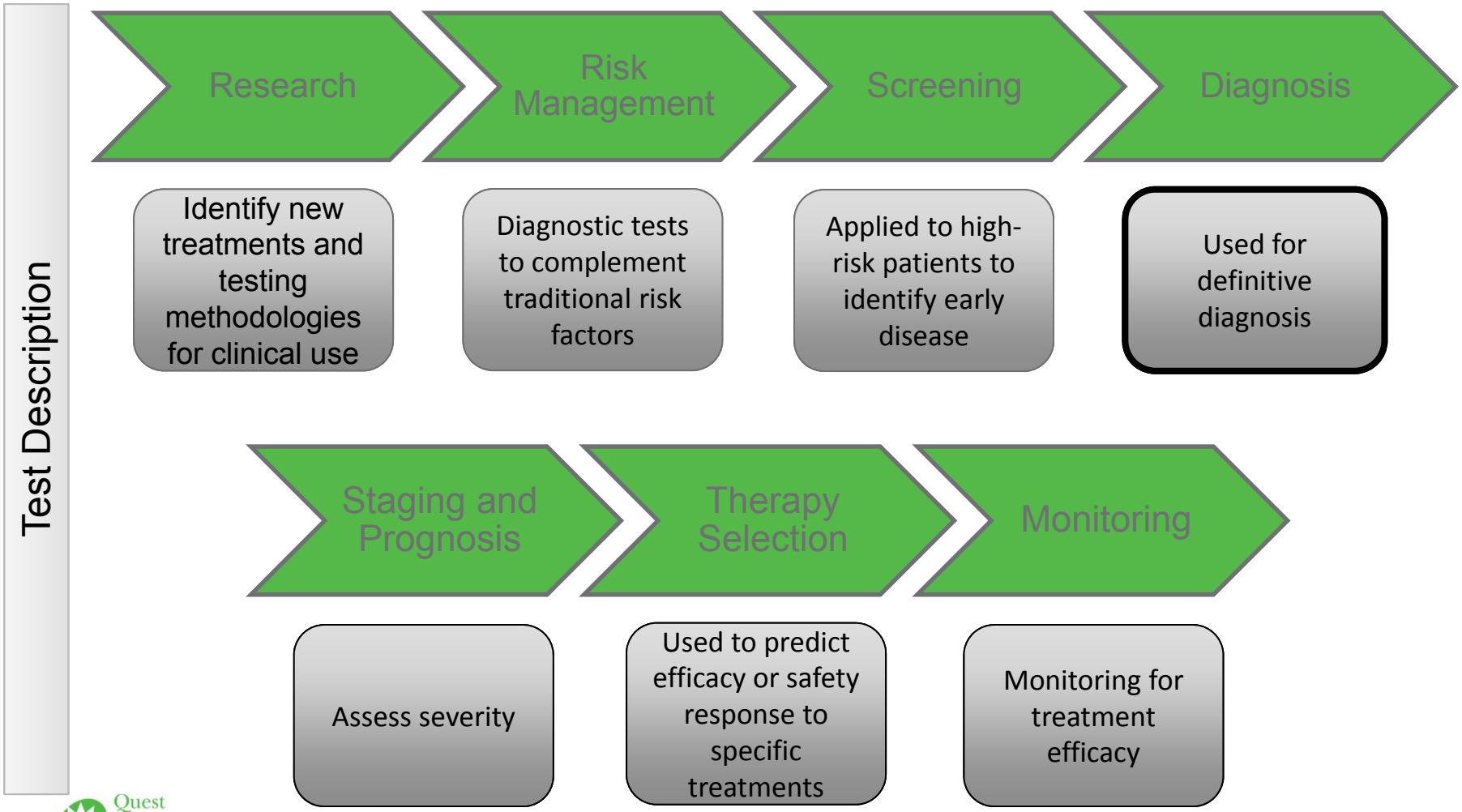
Advantages of NGS Mutation Detection

- High throughput
  - Sequence many samples and many genes in parallel
- Detect most mutation types
  - Single nucleotide polymorphisms (SNPs), copy number variants (CNVs), insertions, deletions, and translocations
- Digital readout of mutation frequency
  - High sensitivity to detect mutations
  - Quantify their frequency in a heterogeneous sample
  - Haplotypes and diplotypes
  - Higher level mutation calling algorithms (vs. Sanger)

Clinical Applications of Sequencing



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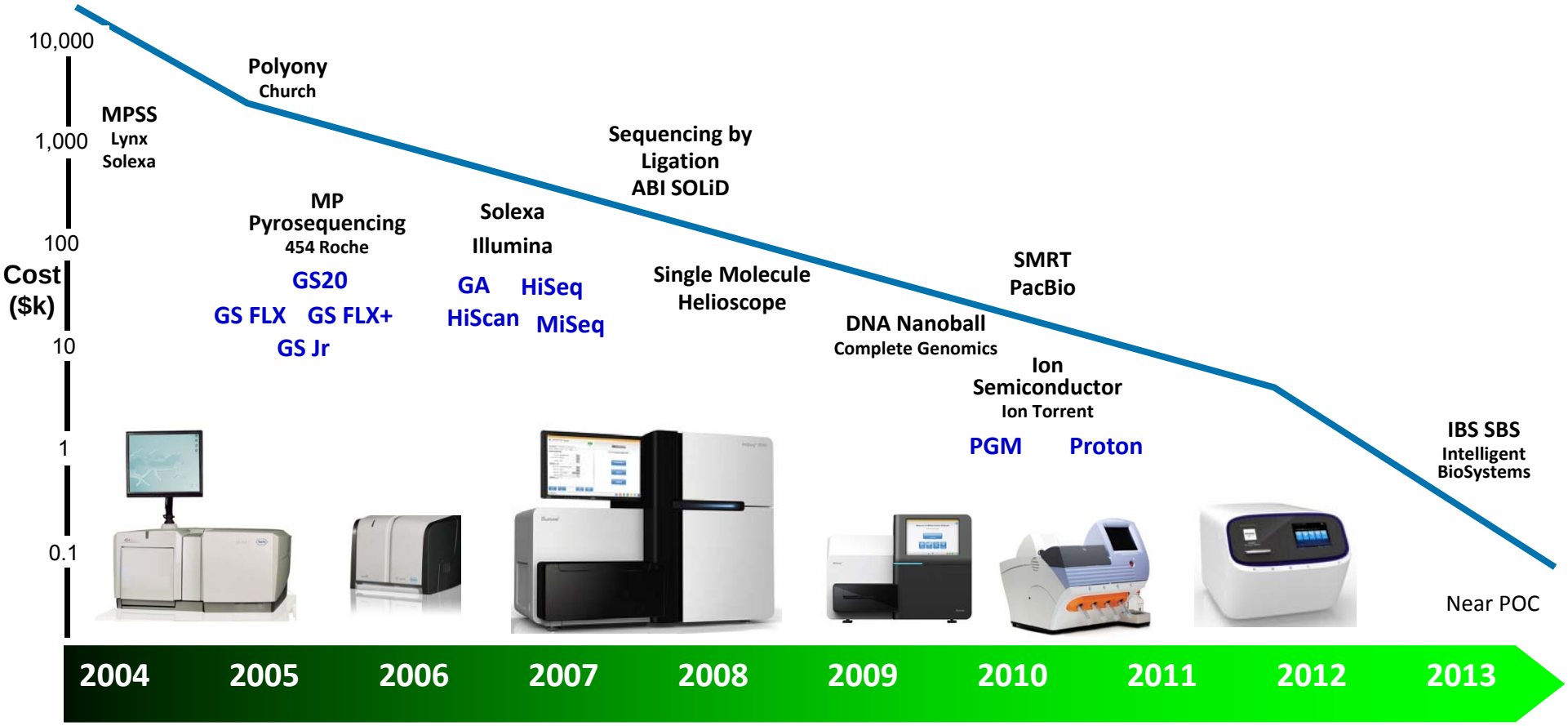
Technology Landscape



- Decreasing cost
- Rapidly developing technology
  - multiple suppliers, multiple platforms in series, changing kits and chemistry

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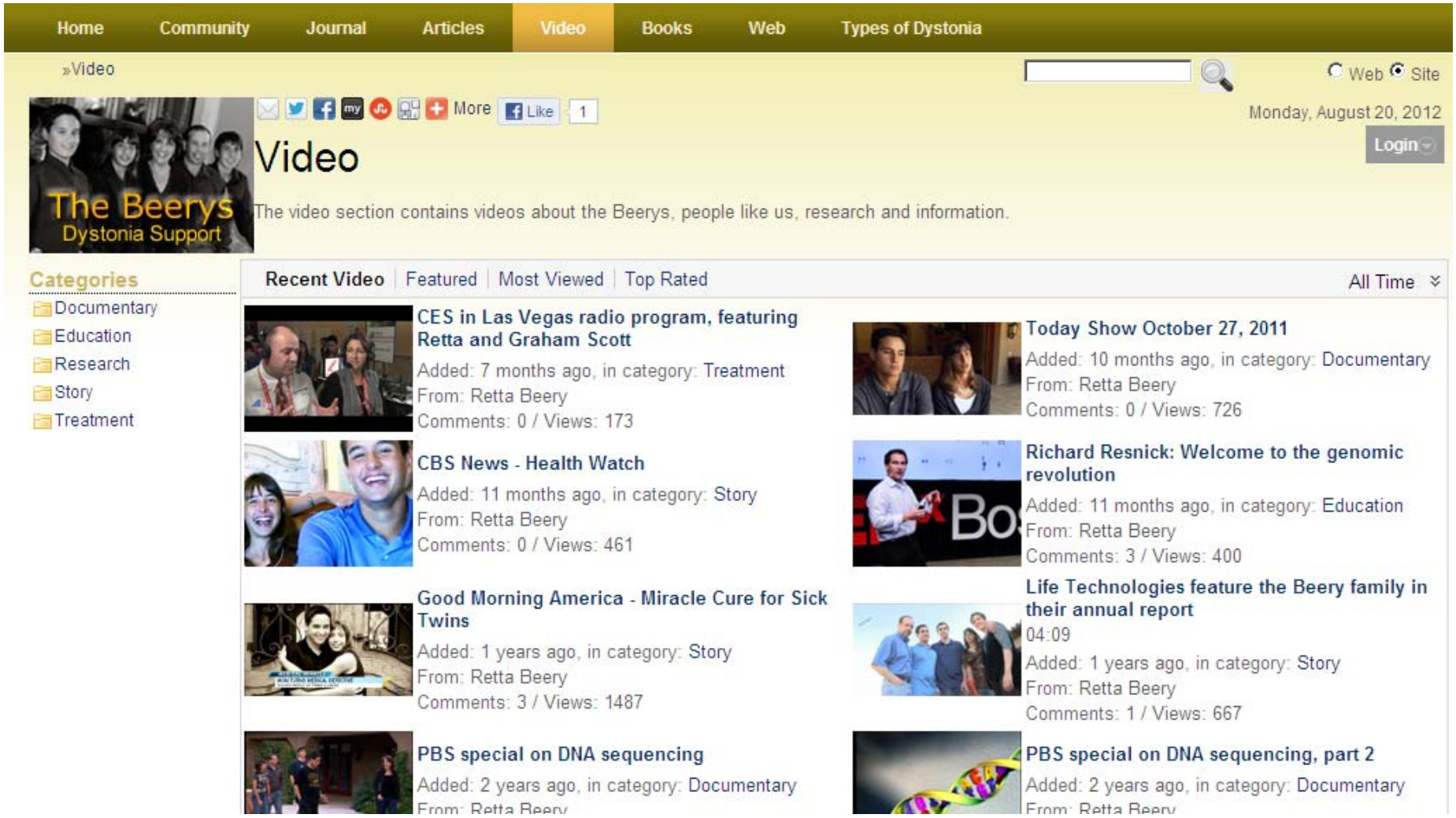
WGS in the Public Eye



Next Generation Sequencing and Guidelines

CLINICAL AND LABORATORY STANDARDS INSTITUTE ™

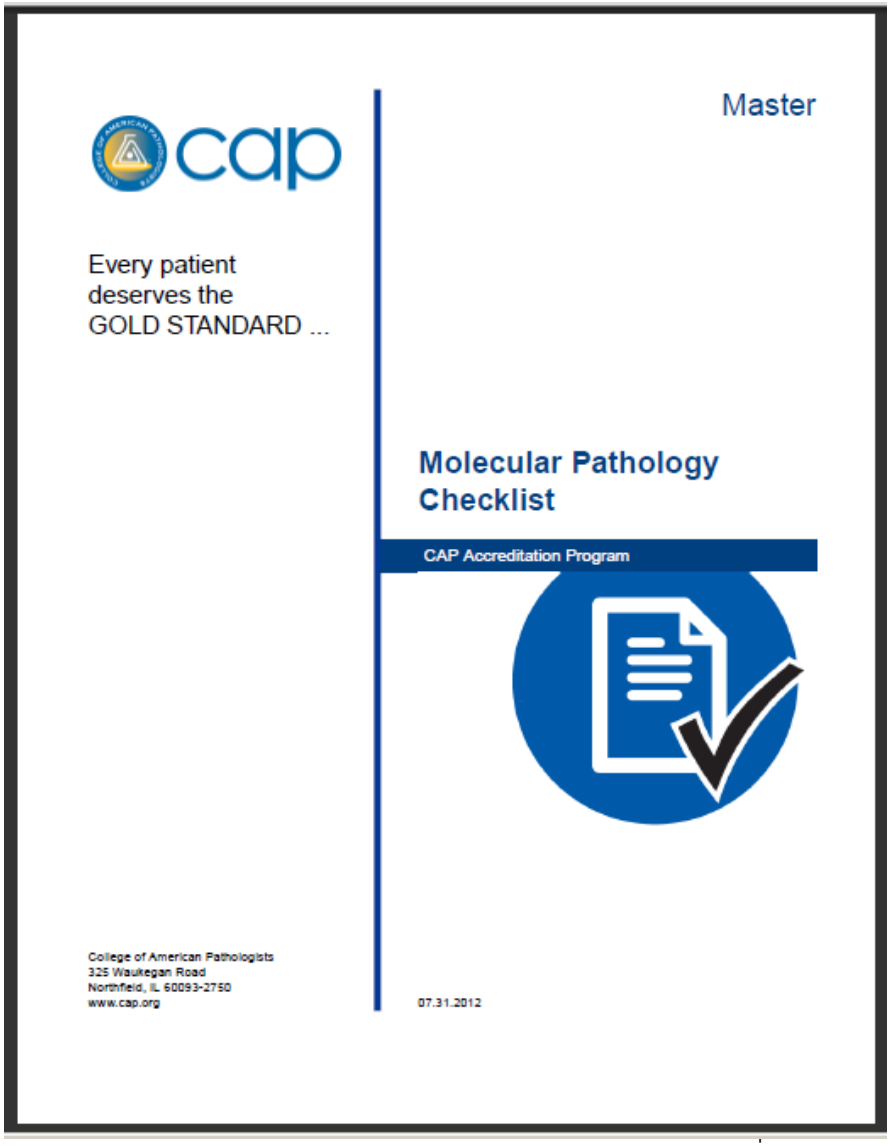
MM09 (Draft)
Vol. 0 No. 0
Replaces MM09-A
Vol. 11 No. 2

Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Draft Guideline—Second Edition

NIST
National Institute of Standards and Technology
U.S. Department of Commerce

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

DOH
STATE OF NEW YORK
DEPARTMENT OF HEALTH



Challenges of NGS for the Clinical Lab

- Goals of Clinical NGS
  - Determine the presence of clinically actionable genetic variation
  - Parallel high throughput analysis replaces historically sequential Sanger sequencing
- How do we move from Research & Discovery into the Clinic?
- Framework to Provide Quality Sequence Information
  - Panel Design
  - Target Enrichment
  - Coverage Depth
  - Bioinformatic Analysis
  - Report

Research vs. Clinical NGS

	Research	Clinical
Sequencing Panel	Targets frequently changed	Fixed & validated panels
Panel Optimization	Some	Robust performance
Sample Prep	Flexible, frequent testing of new methods	Validated and robust
Reagents	Use latest reagents	Reagent change requires validation
Controls	Variable	For each step, unlimited availability
Bioinformatic Analysis	Apply latest tools and algorithms	Locked validated process
Throughput per Project	Many samples over short time	Consistent inflow of samples for years
Report	Not standardized, usually contains all results	Standardized, limit to medically informative findings

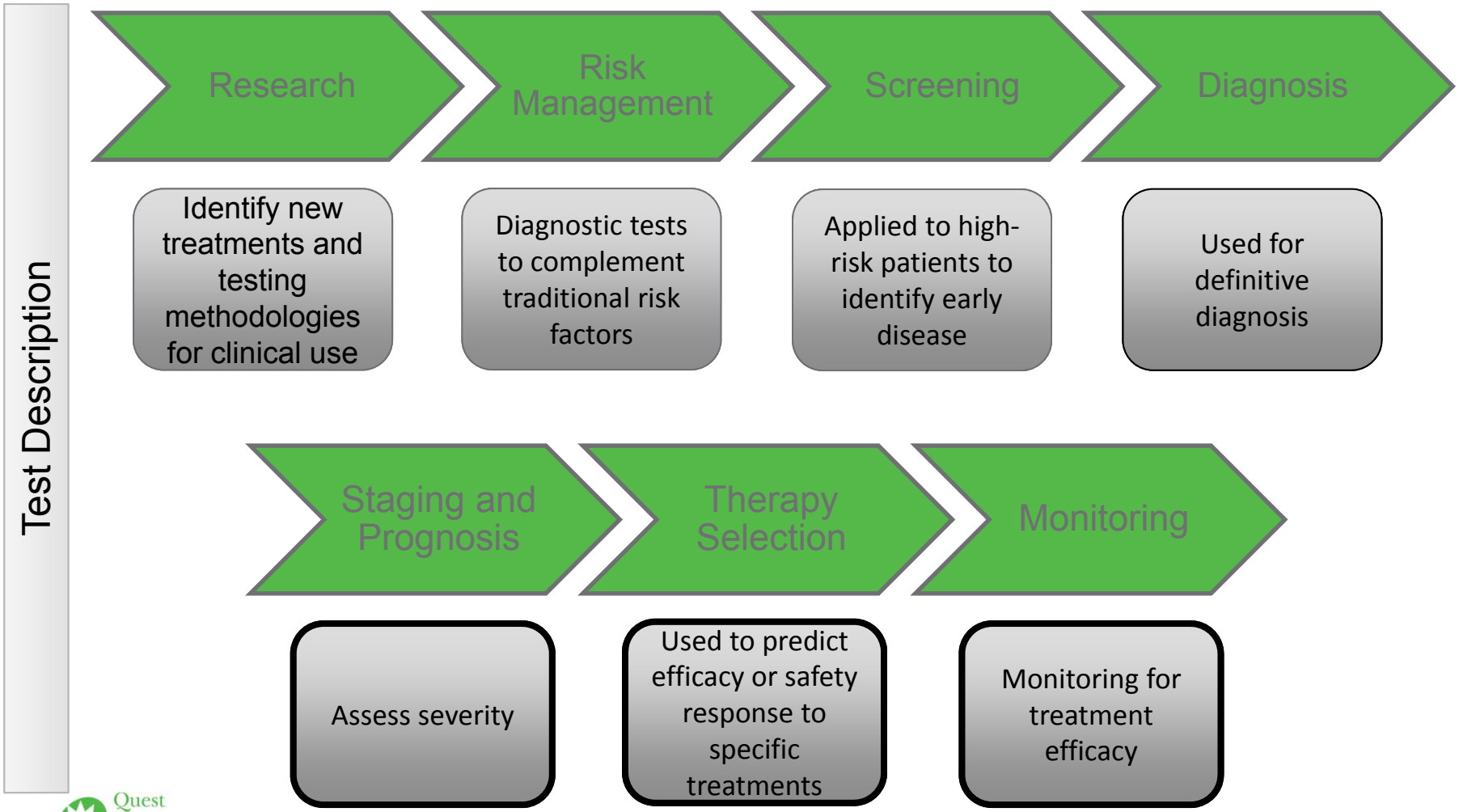
Challenges of NGS

- Many platforms, each with different pros and cons
- Platform specific sequence library preparation
  - Limited or no automation
- Platforms and methods evolve quickly
  - Frequent updates to instruments and reagents
- Huge IT infrastructure costs
- Bioinformatics intensive
  - Rapidly evolving algorithms
  - Accurate and comprehensive reference databases

Other Challenges of NGS in the CLIA Lab

- Staffing and level of training (licensed technologist)
- Data review (needs to be automated)
- Sample prep and workflow
- Reagents and consumables (chips) need to be locked down from vendor
- QC of reagents may require new way of thinking
  - can you phase in every new nanopore chip?
  - Expensive to phase in everything (all reagents)
  - Standards and controls
- Batch size – currently need large batches (few setups) to make assays cost-effective (cost dependent upon assay volume)

Clinical Applications of Sequencing



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Prognosis & Theranosis of Infectious Diseases

Prognosis

- Viral genotyping (Hepatitis C Virus and Hepatitis B Virus)
  - Advantage over Sanger sequencing?

Theranostics and Monitoring

- HIV, HCV, HBV, and bacterial Drug Resistance
- HIV CCR5 entry inhibitor
  - HIV-1 Tropism by Ultra-Deep Sequencing
- May involve amplification
- Clinical Sensitivity

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HIV-1 Coreceptor Tropism Testing

Acknowledgments
Martin Siaw, Ph.D., Staff Scientist
Erik P. Johnson, Ph.D., Senior Scientist
Ron Kagan, Ph.D., Director of Bioinformatics

OPEN ACCESS Freely available online

PLOS ONE

A Genotypic Test for HIV-1 Tropism Combining Sanger Sequencing with Ultradeep Sequencing Predicts Virologic Response in Treatment-Experienced Patients

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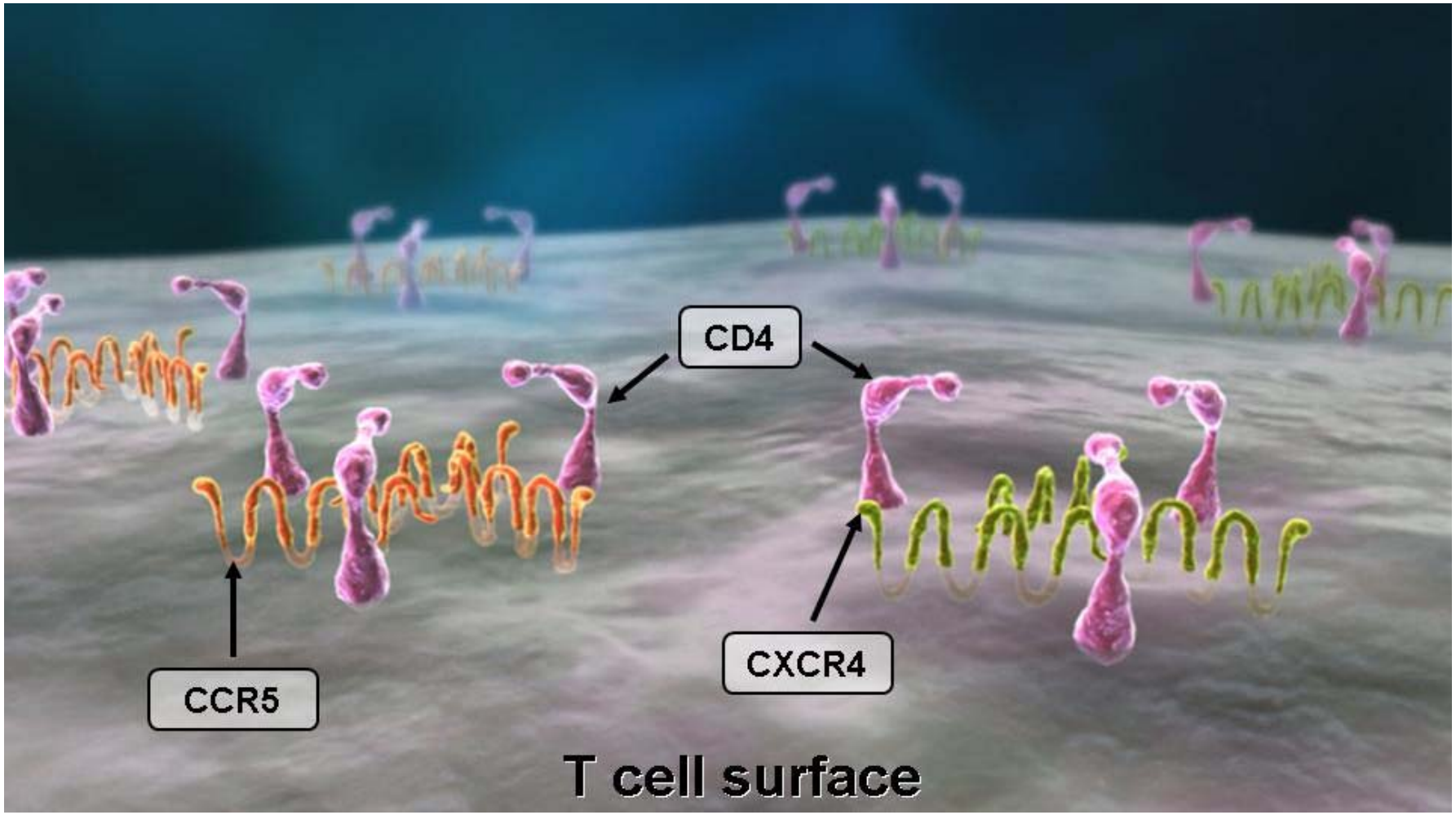
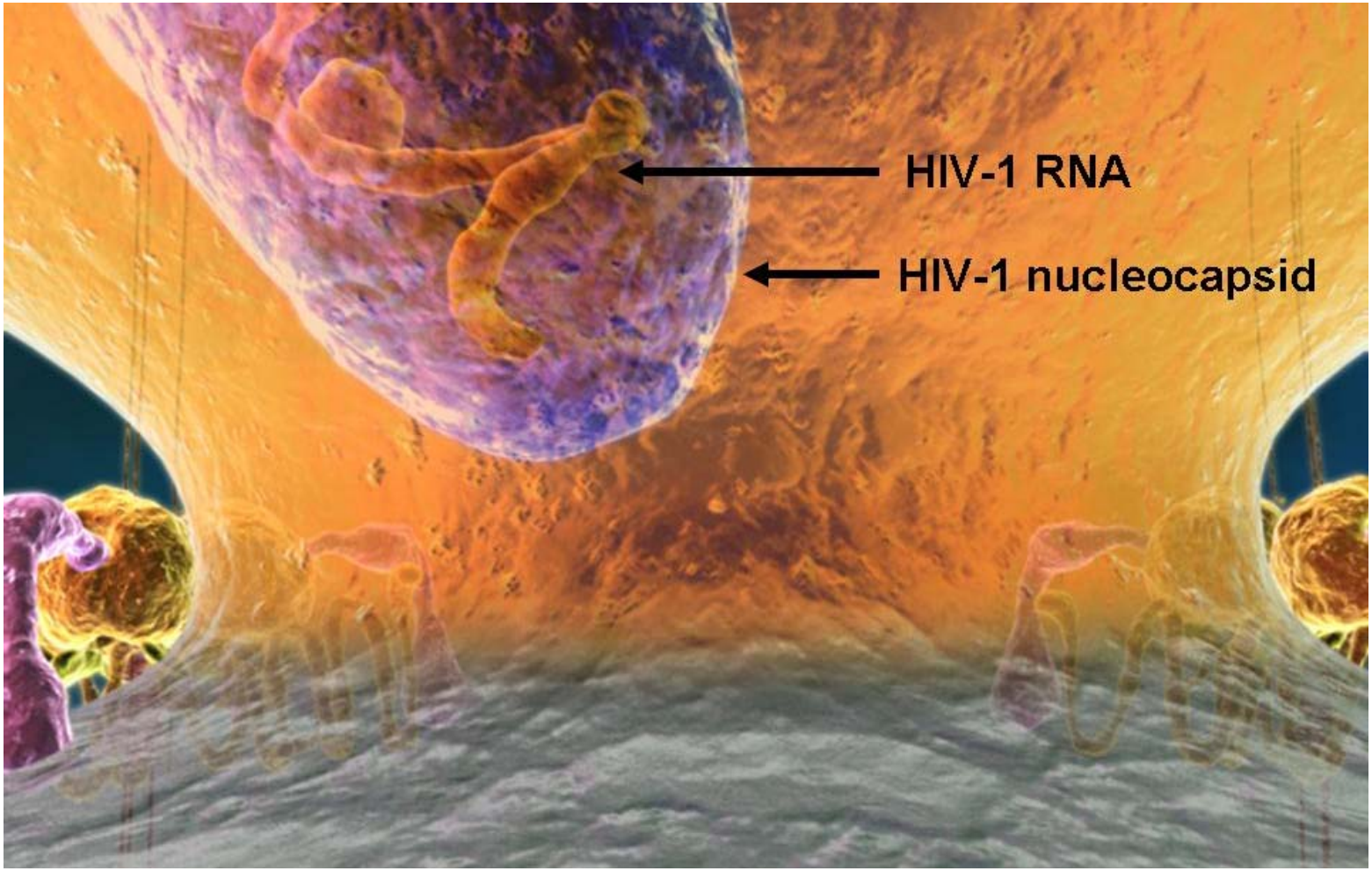
Abstract

A tropism test is required prior to initiation of CCR5 antagonist therapy in HIV-1 infected individuals, as these agents are not effective in patients harboring CXCR4 (X4) coreceptor-using viral variants. We developed a clinical laboratory-based genotypic tropism test for detection of CCR5-using (R5) or X4 variants that utilizes triplicate population sequencing (TPS) followed by ultradeep sequencing (UDS) for samples classified as R5. Tropism was inferred using the bioinformatic algorithms geno2pheno$_{[coreceptor]}$ and PSSM$_{x4r5}$. Virologic response as a function of tropism readout was retrospectively assessed using blinded samples from treatment-experienced subjects who received maraviroc (N = 327) in the MOTIVATE and A4001029 clinical trials. MOTIVATE patients were classified as R5 and A4001029 patients were classified as non-R5 by the original Trofile test. Virologic response was compared between the R5 and non-R5 groups determined by TPS, UDS alone, the reflex strategy and the Trofile Enhanced Sensitivity (TF-ES) test. UDS had greater sensitivity than TPS to detect minority non-R5 variants. The median $\log_{10}$ viral load change at week 8 was −2.4 for R5 subjects, regardless of the method used for classification; for subjects with non-R5 virus, median changes were −1.2 for TF-ES or the Reflex Test and −1.0 for UDS. The differences between R5 and non-R5 groups were highly significant in all 3 cases ($p<0.0001$). At week 8, the

Targets Involved in HIV Entry



Fusion of the Viral and Cell Membranes



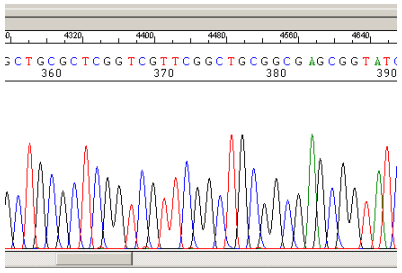
Genotypic Coreceptor Tropism Tests

500 µL plasma → Total RNA

3x sequencing
+ reflex of R5 to ultradeep sequencing (UDS)
- VL ≥1,000 copies/mL
- 2 mL (pref) to 0.6 mL (min) plasma
- CPT code: 87906

DNA RT PCR product x 3
(Env V3 loop)

DNA sequencing x 3



Apply algorithm

Reports in 5-7 days

X4 → **Maraviroc activity: NO**

R5 → **Reflex to UDS**

Why Triplicate Testing?

Replicate Population Sequencing vs Trofile (N=278)

Replicates	Sensitivity	Specificity
triplicate	69%	91%
duplicate	59%	91%
singlicate	48%	91%

Swenson et al. *J Acquir Immune Defic Syndr.* 2010;54:506-10.

Tropism Assay Sensitivity for X4 Virus in Dual/Mixed Samples I

- Technical Sensitivity: the lower limit for an assay's **detection platform**—luciferase assay (Trofile), ABI sequencer (Triplicate Population Sequencing), or Roche/454 instrument (Ultradeep sequencing)—to reproducibly detect a minor species when presented to the detection platform in a **suitable matrix**
  - Original Trofile: 10% (Whitcomb et al 2007)
  - Enhanced Sensitivity Trofile Assay (ESTA): 0.3% (Reeves et al 2009)
  - Ultradeep Sequencing on 454: 0.5%

Tropism Assay Sensitivity for X4 Virus in Dual/Mixed Samples II

- Biological Sensitivity: the lower limit of an **assay system**—Trofile, Triplicate Population Sequencing, Ultradeep Sequencing—to reproducibly detect minority variants in a **mimicked clinical sample**
  - Original Trofile: not published
  - Enhanced Sensitivity Trofile Assay: not published
  - Triplicate Population Sequencing: LOD95 = 20%
  - Ultradeep Sequencing on 454:
    - LOD_{95} = 12% @ 25,000 copies/mL
    - LOD_{95} = 5% @ 100,000 copies/mL

Tropism Assay Sensitivity for X4 Virus in Dual/Mixed Samples: Conclusions

- The technical sensitivity of the Enhanced Sensitivity Trofile and Ultradeep sequencing are essentially the same: **0.5%**
- In biological samples, the ability to detect minority species is **inversely proportional** to viral load for both genotypic and phenotypic technologies
- Ultradeep sequencing is more sensitive than population sequencing for detecting minor X4 variants (**5-12% vs 20%**)
- The **clinical** sensitivity and specificity of Enhanced Sensitivity Trofile Assay and the genotypic tropism test with reflex to UDS are equivalent

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