Genetic Analysis of Pyrazinamide Resistance in MDR/XDR South African *Mycobacterium tuberculosis* Strains Using Next-Generation Ion Torrent Sequencing

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Demystifying Pyrazinamide • September 5-6, 2012

Study Objectives

1. **Develop a standardized sequencing methodology/protocol using the Ion Torrent.**
   - » Streamlined library prep
   - » No ancillary equipment
   - » Reduced laboratory footprint
   - » Safe for standard laboratories

2. **Develop a protocol using 5 full-length MTB genes for detecting and characterizing MDR/XDR strains.**
   - » Proof or principle using 5 genes, expandable to 16+ genes
   - » Standardized amplification and thermocycling

3. **Evaluate the developed protocol using a limited set of retrospectively collected susceptible, MDR and XDR strains.**
   - » Collected from Gauteng and Kwa-Zulu Natal Provinces in South Africa
   - » Clinical isolates collected between July-November, 2011

PrimeStore®

SPECIMEN COLLECTION & PRESERVATION SOLUTION FOR MOLECULAR DIAGNOSTIC APPLICATIONS

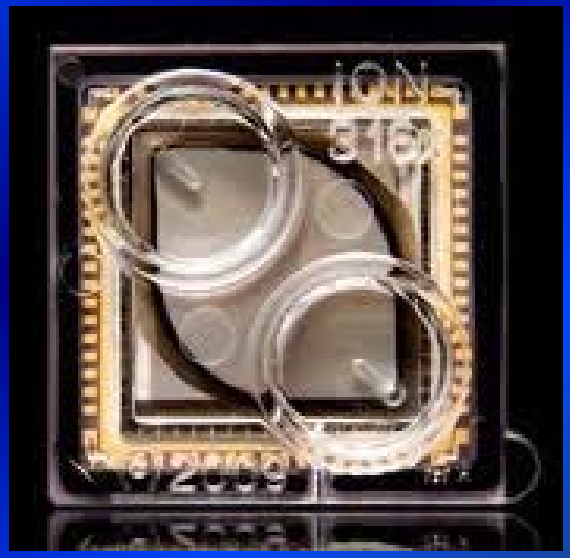
PrimeStore® MTM is a sample collection solution that safely inactivates microbes, and stabilizes/preserves RNA and DNA for downstream Nucleic Acid Testing (NAT)……

…and Next Generation Sequencing

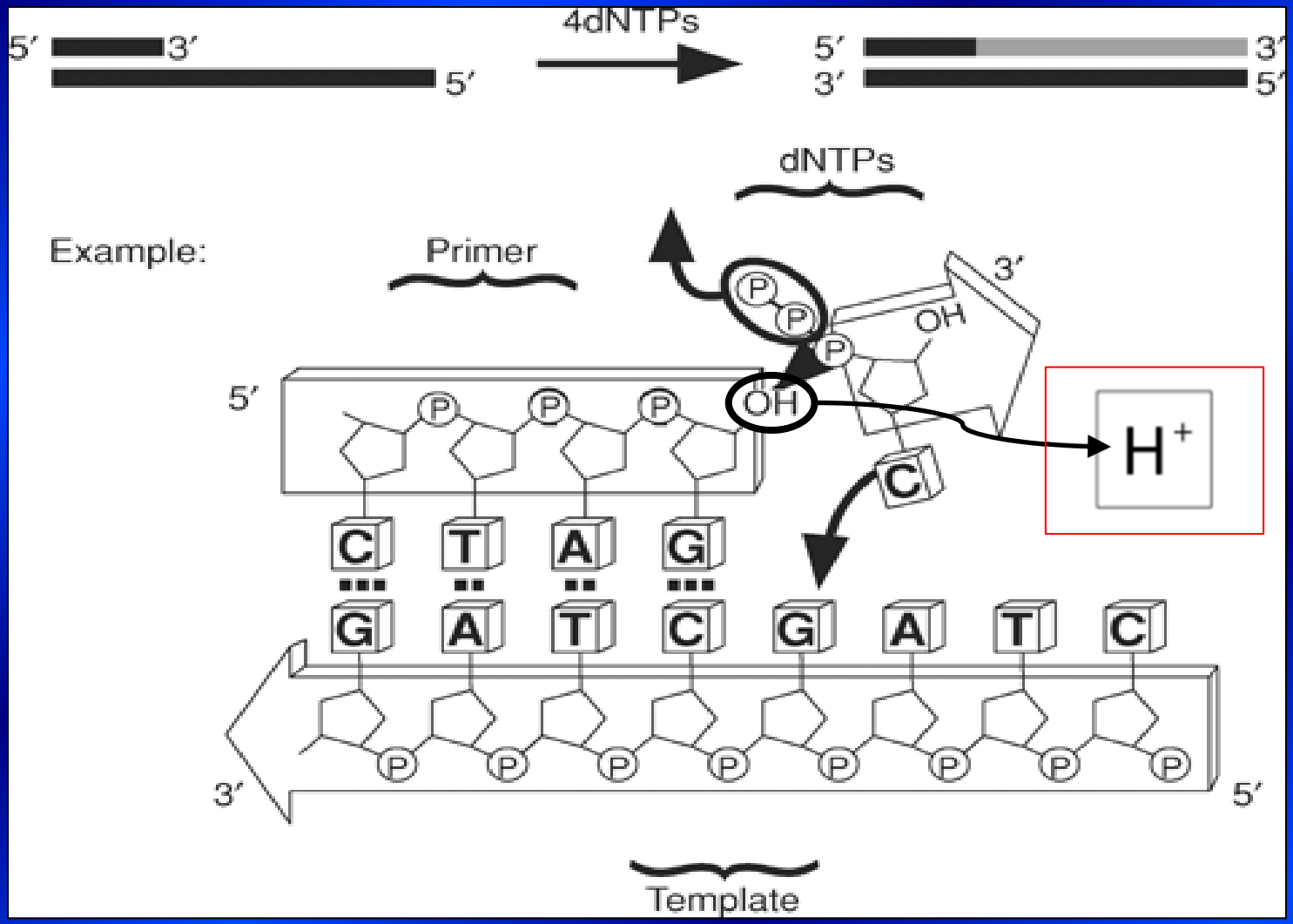
Next Generation Ion Torrent Sequencing

~TB drug resistance chip~

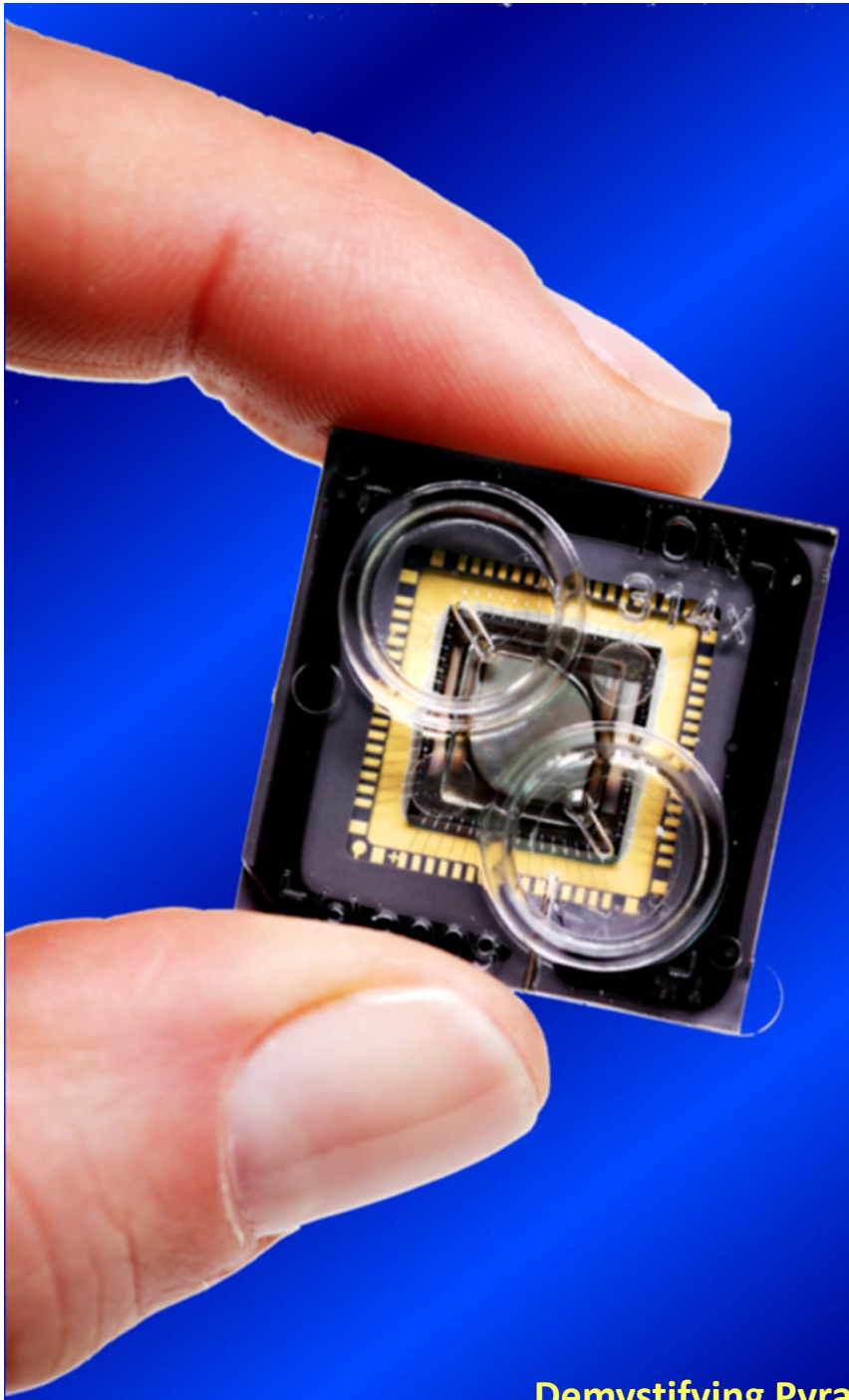
1. *rpoB*
2. *katG*
3. *gyrA*
4. *rrs* (16s rRNA)
5. *pncA*



Ion Torrent-How does it work?



Simple, Natural Chemistry



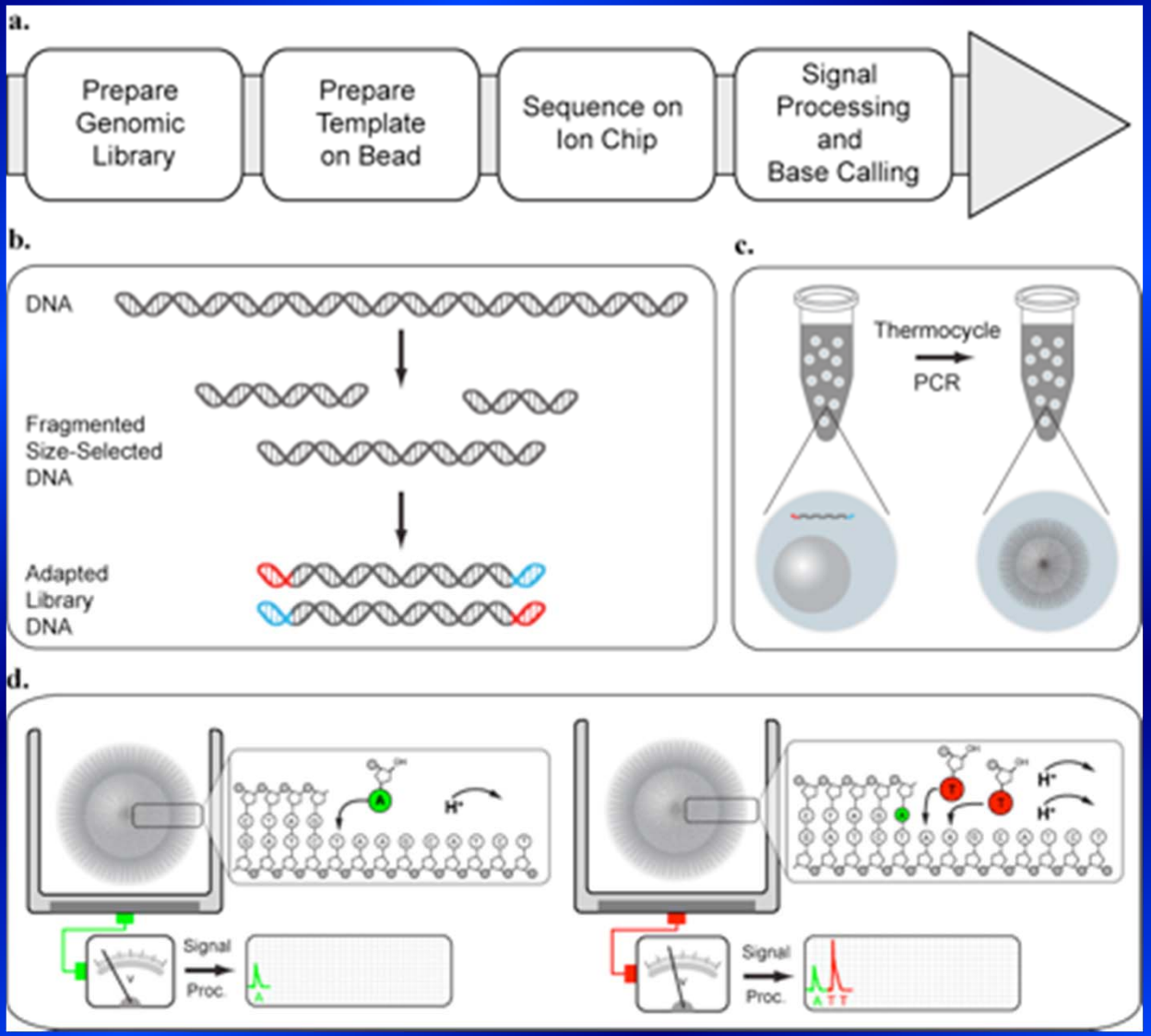
The Chip is the Machine™

Scalability

Simplicity

Speed

Technology Summary



Ion DNA Barcode Adaptor 1-16

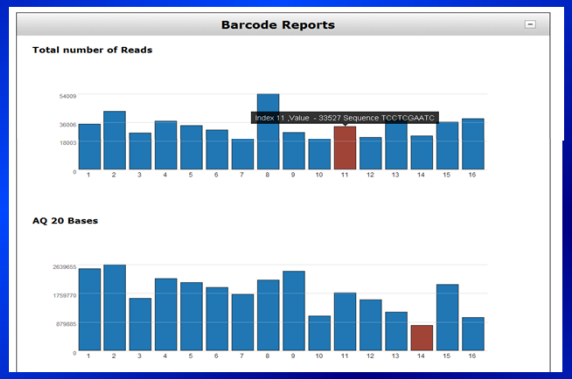
01

Construct Library

1. Enzymatic Fragmentation
2. End Repair and Ligate Adaptors
3. Optional Amplification
4. Normalize and Pool Libraries

1 day

- Efficient multiplexing up to 16 libraries
- Reduces cost/sample
- Minimal adaptor sequence for added sample identification confidence
- Automation compatible



Molecular Basis of Drug Resistance in *Mycobacterium tuberculosis*

Drug	Gene	Function	Prevalence (%)
Isoniazid	*katG*	Catalase peroxidase	~40–60
	inhA	Enoyl-acyl carrier protein reductase	~25
	ahpC	Alkyl-hydroperoxide reductase	~10
	kasA	Ketoacyl acyl carrier protein synthetase	
Rifampicin	*rpoB*	β-subunit of the RNA polymerase	~95
Pyrazinamide	*pncA*	Pyrazinamidase	~95
Streptomycin	*rpsL*	Ribosomal S12 protein	~60
	rrs	16S rRNA	~20
Amikacin/kanamycin	*rrs*	16S rRNA	~70–90
Capreomycin	*rrs*	16S rRNA	~90
	tlyA	rRNA methyltransferase	
Fluorochinolone	*gyrA*, *gyrB*	DNA gyrase	~80–90
Ethambutol	*embCAB*	Arabinosyl transferase	~60

Drug-susceptibility Testing in TB: Current Status and Future Prospects. Elvira Richter; Sabine Rüsch-Gerdes; Doris Hillemann. *Expert Rev Resp. Med*. 2009;3(5):497-510.

Next Generation Ion Torrent Sequencing

COLLECTION

SHIPMENT
Ambient temperature
Standard delivery

DETECTION SEQUENCING

PrimeStore MTM™ collection & shipment from Pretoria, South Africa

4 day transit to San Antonio, Texas, USA

TB drug susceptibility chip

1. *rpoB*
2. *katG*
3. *gyrA*
4. *rrs* (16s rRNA)
5. *pncA**

11,472 bp total
X 16 barcodes
=183,552 bps

ion torrent

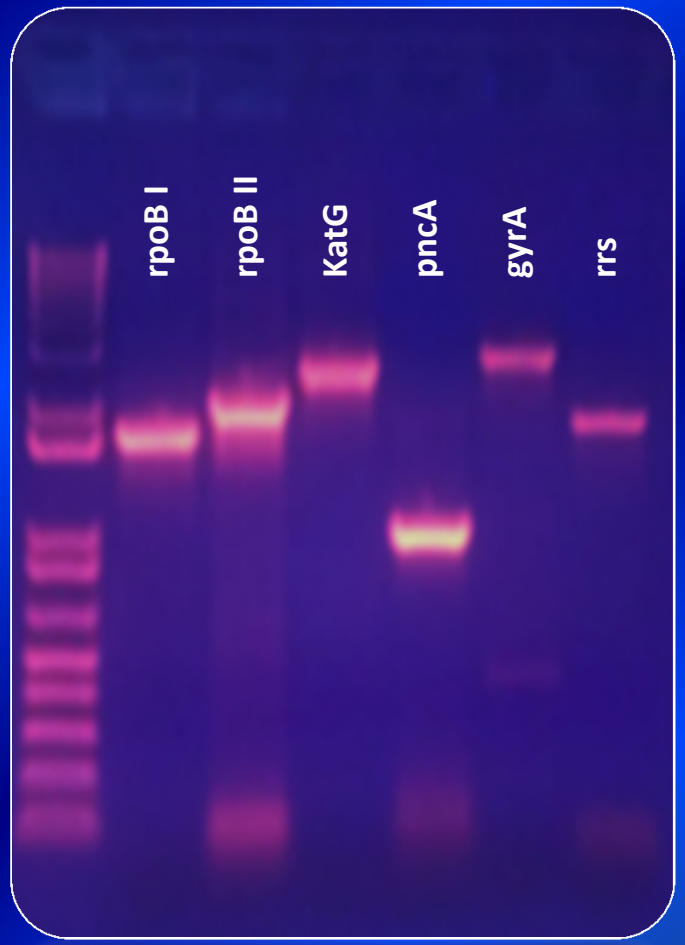
29 GB
(Raw Data)

60 MB
(FASTQ)

Demystifying Pyrazinamide • September 5-6, 2012

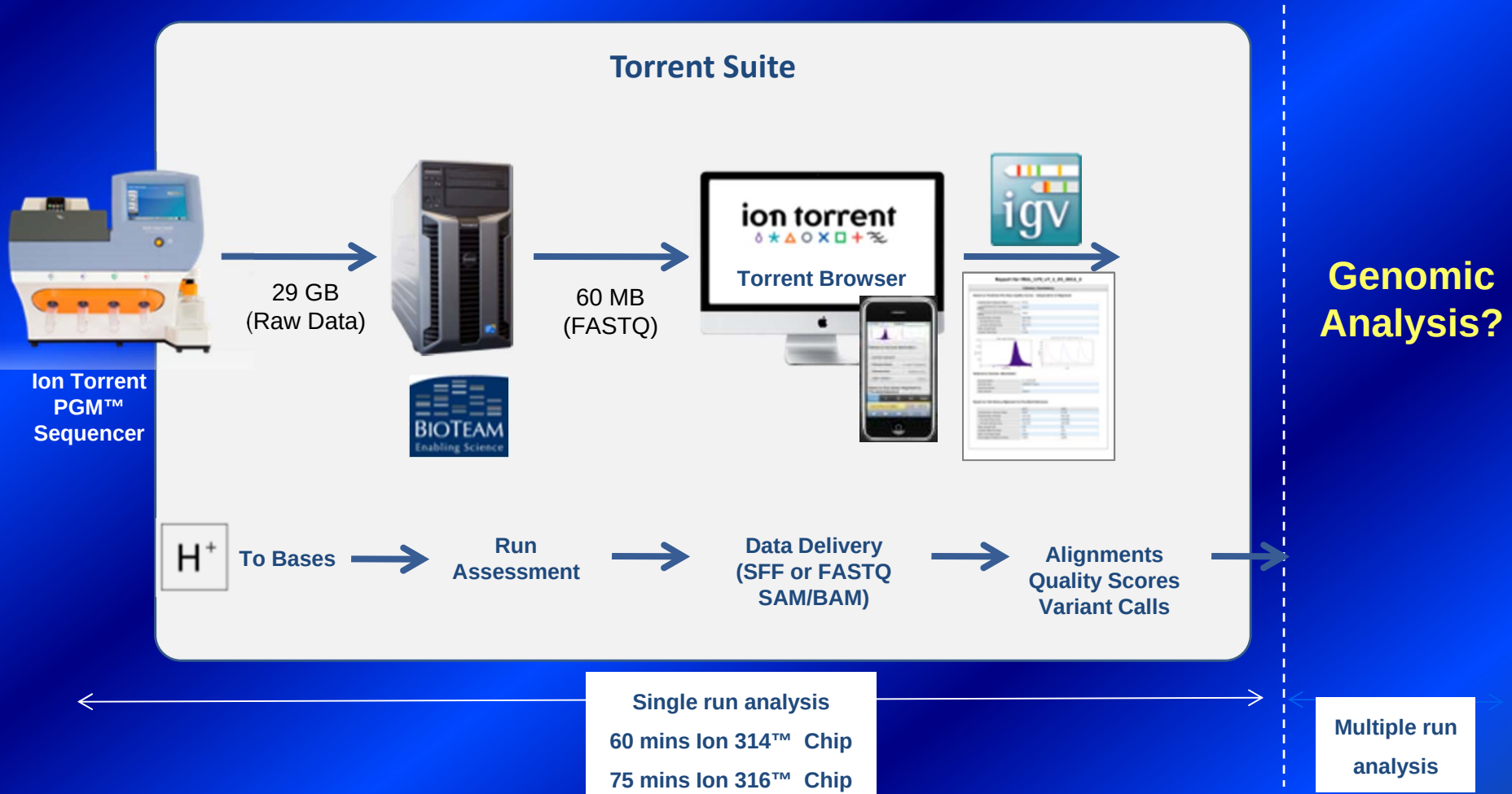
Full-Length Gene Amplification for TB Drug Resistance

- Representative amplicons for 5 MTB genes (*rpoB, katG, pncA, gyrA,* and *rrs*).
- A total of 11,432 bps for five genes were sequenced for each clinical isolate.
- A 1.0% agarose gel with 1 KB ladder is shown.

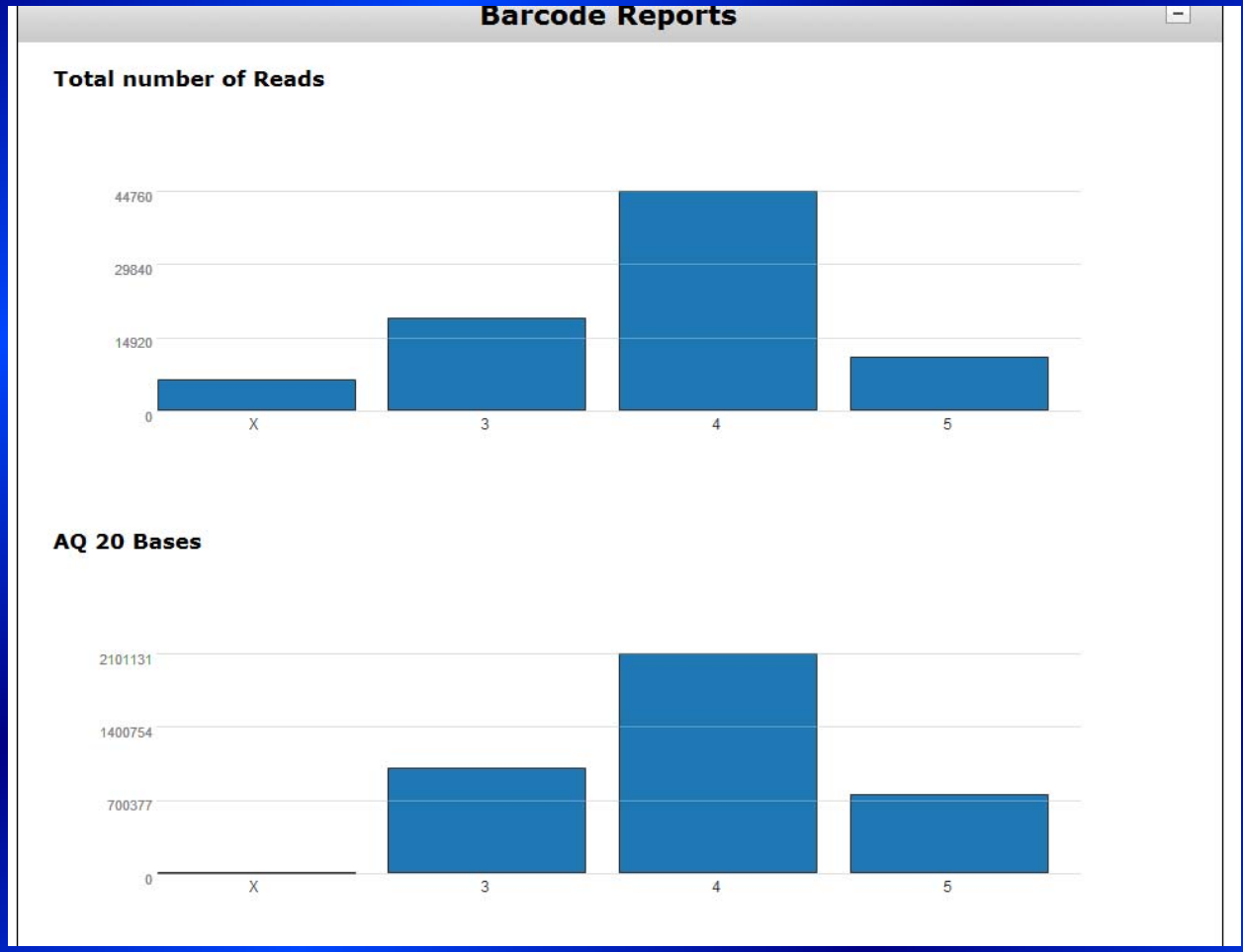


Ion Torrent Server and Suite

Web based data delivery with integrated alignment and variant calling



Barcoded Samples

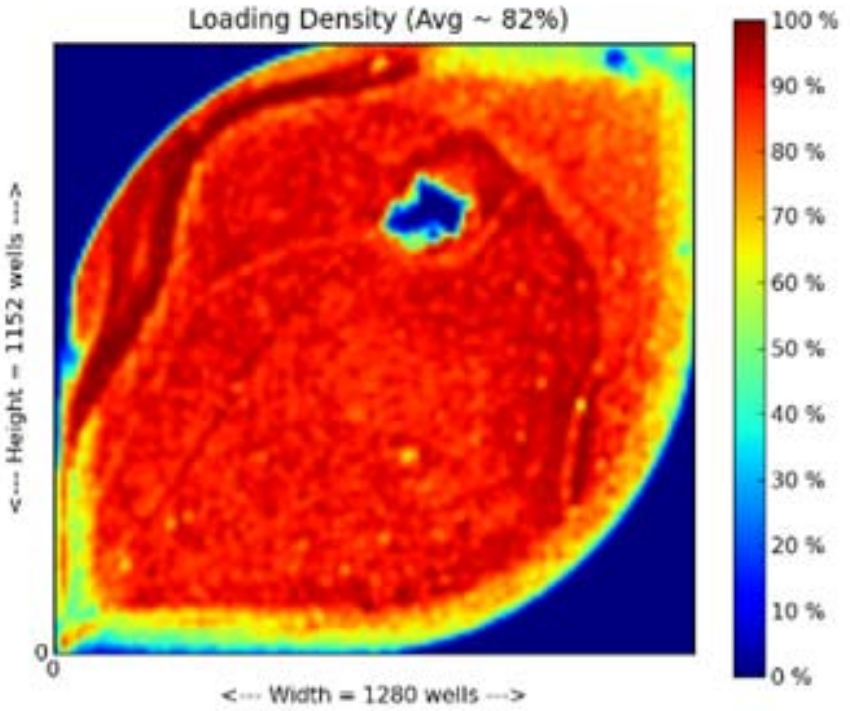


Semiconductor Sequencing for Life™

314 Chip Ion Sphere Particle (ISP) Loading Density Map

	Count	Percentage
Total Addressable Wells	1,262,519	
Wells with ISPs	1,050,930	83%
Live ISPs	1,003,694	96%
Test Fragment ISPs	4,407	<1%
Library ISPs	999,287	100%

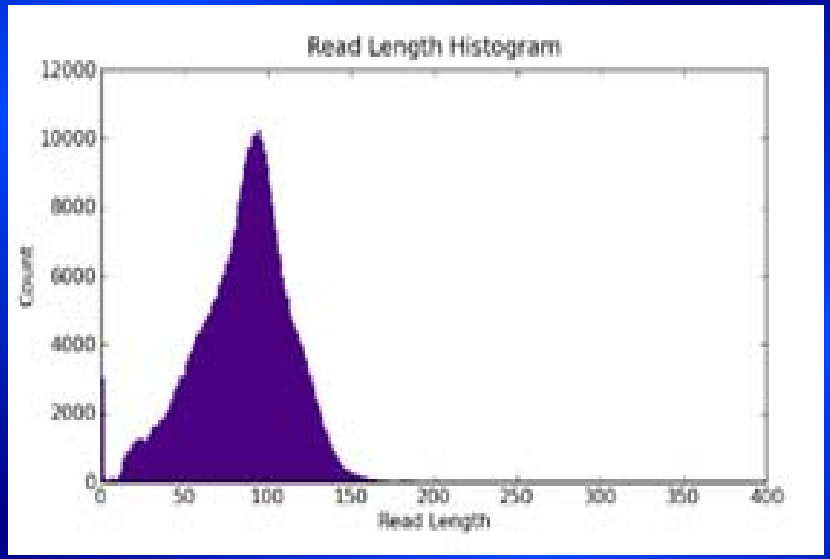
	Count	Percentage
Library ISPs / Percent Enrichment	999,287	95%
Filtered: Polyclonal	260,393	26%
Filtered: Primer dimer	577	<1%
Filtered: Low quality	163,448	16%
Final Library Reads	**574,869**	**58%**



314 Chip Density Map

Semiconductor Sequencing for Life™

MTB Library Run Summary



Semiconductor Sequencing for Life™

life technologies™ | ion torrent

Based on Full Library Alignment to Provided Reference

	AQ17	AQ20	Perfect
Total Number of Bases [Mbp]	10.63	8.31	7.50
Mean Length [bp]	68	60	56
Longest Alignment [bp]	133	133	130
Mean Coverage Depth	1,232.90×	963.10×	868.90×
Percentage of Library Covered	96%	96%	96%

DNASTAR Lasergene®

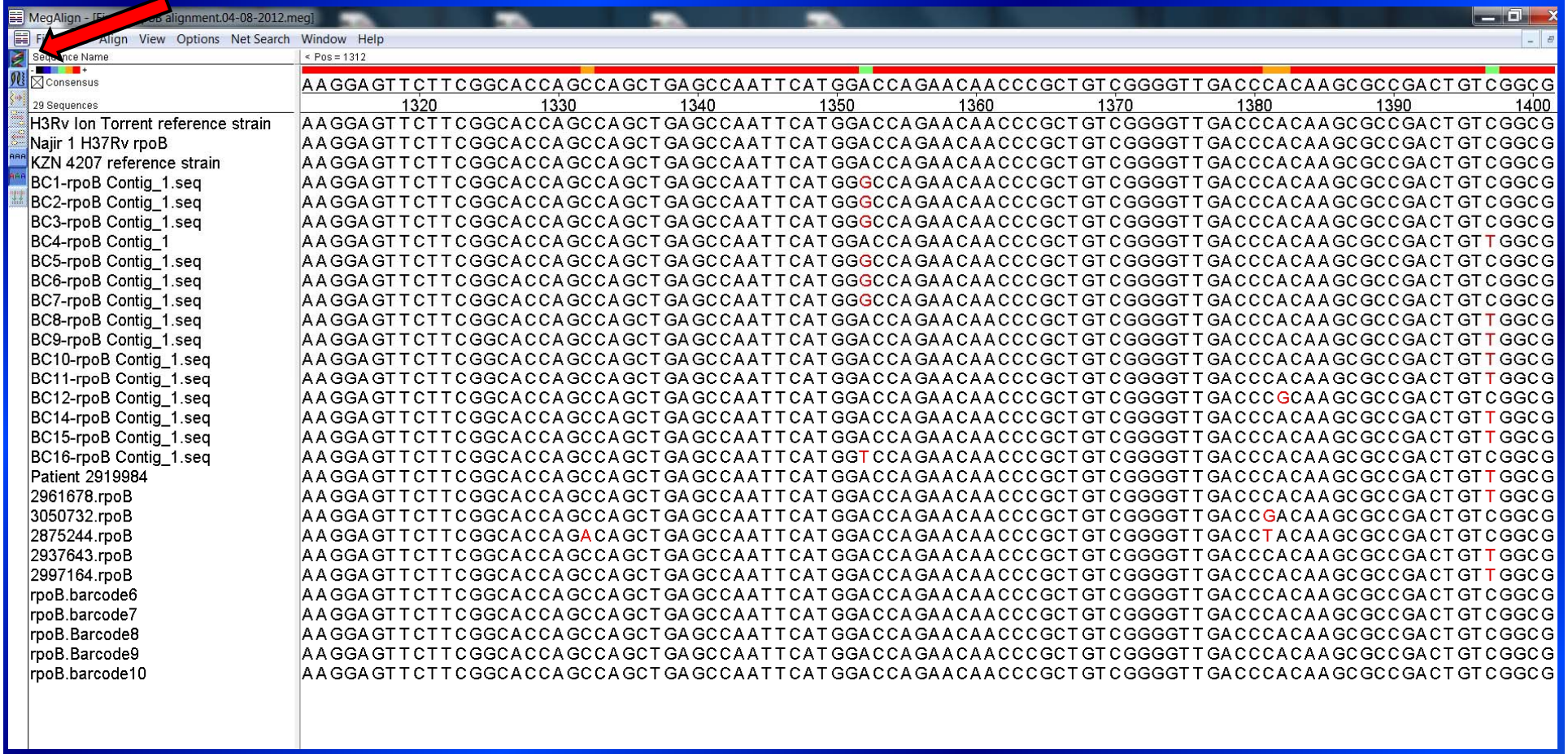
DNASTAR SeqMan NGen

LaserGene 10 Core Suite

- GeneQuest
- GenVision Utility
- GenVision
- MegAlign
- PrimerSelect
- Protean 3D
- Protean
- SeqBuilder
- SeqMan Pro
- SeqNinja
- EditSeq

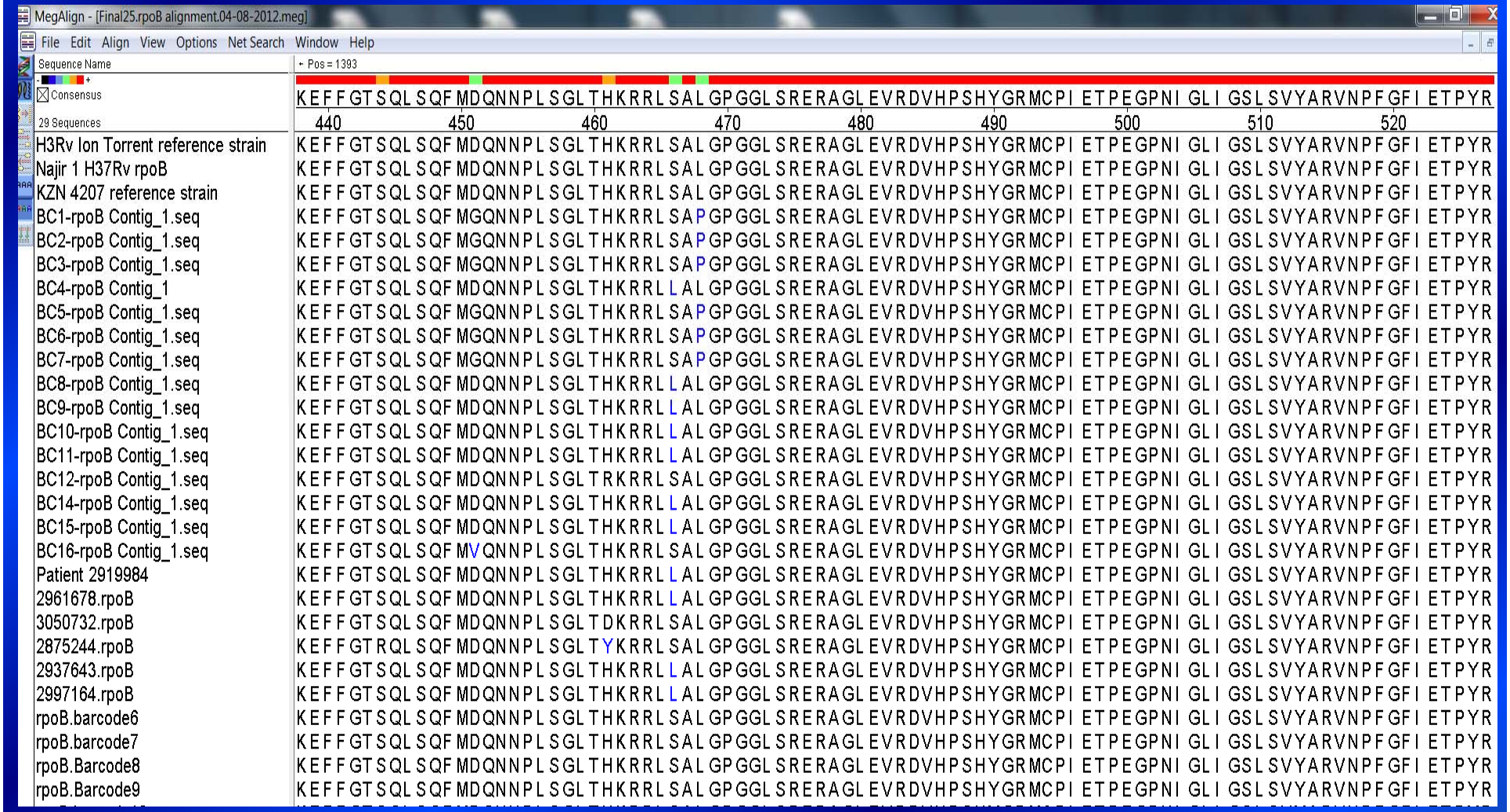
DNASTAR Lasergene®

Compare Assembled Barcoded Samples



Multiple sequence alignments

Protein Translation



amino acid substitution mutations

Ion Torrent Sequencing
Sample 3100029
>100X Coverage

pncA Gene

Raw nucleotides (960 bps)

ACGGATTTGTCGCTCACTACATCACCGGCGTGATCTATCCCGCCGGTTGGGTGGCCGCCGCTCAGCTGGTCATGTTCGCGATCGTCGCGG
CGTCATGGACCCTATATCTGTGGCTGCCGCGTCGGTAGGCAAACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTT
GATCATCGTCGACGTGCAGAACGACTTCCGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCCGCGCTGGCCCGCGCCATCAGCGAC
TACCTGGCCGAAGCGGCGGACTACCATCACGTCGTGGCAACCAAGGACTTCCACATCGACCCGGGTGACCACTTCTCCGGCACACCGGA
CTATTCCTCGTCGTGGCCACCGCATTGCGTCAGCGGTACTCCCGGCGCGGACTTCCATCCCAGTCTGGACACGTCGGCAATCGAGGCGGT
GTTCTACAAGGGTGCCTACACCGGAGCGTACAGCGGCTTCGAAGGAGTCG[illegible]TGAATTGGCTGCGGCA
ACGCGGCGTCGATGAGGTCGATGTGGTCGGTATTGCCACCGATCATTGTGT[illegible]GGTACGCAATGGCTTG
GCCACCAGGGTGCTGGTGGACCTGACAGCGGGTGTGTCGGCCGATACCAC[illegible]GCGCACCGCCAGCGTC
GAGTTGGTTTGCAGCTCCTGATGGCACCGCCGAACCGGGATGAACTGTTG[illegible]CAAGCGGCCGCCGCGC
ACGACCGCGCCGGCTGGGTCGGGTTGTTCACCGGTGACGCGCGGGTCGA[illegible]CAGGTGGGGCATGAG
GCCATCGGCCGCTTCTACGACACCTTCATCGGGCCGCGGGATATCACGTTC[illegible]CGGC

T→C

Full coding region (561 bps)

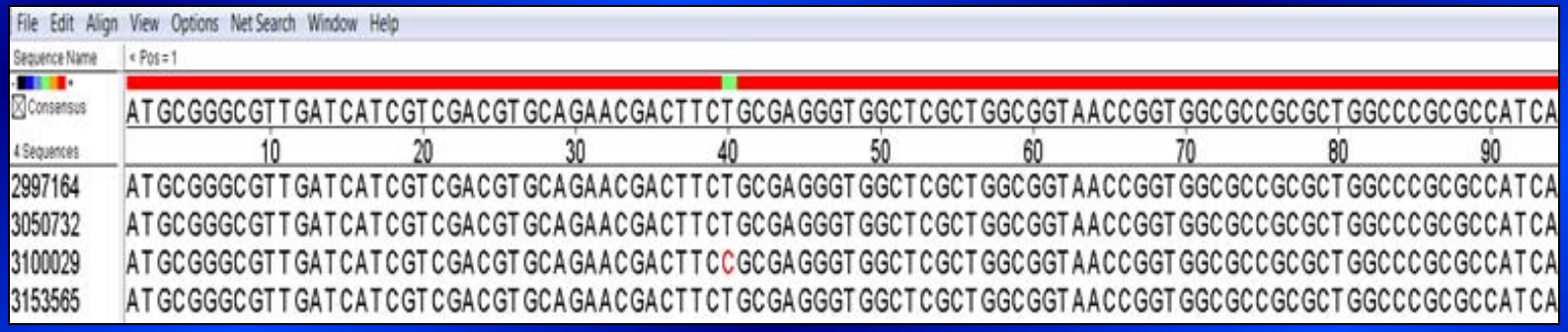
ATGCGGGCGTTGATCATCGTCGACGTGCAGAACGACTTCCGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCCGCGCTGGCCCGCG
CCATCAGCGACTACCTGGCCGAAGCGGCGGACTACCATCACGTCGTGGCAACCAAGGACTTCCACATCGACCCGGGTGACCACTTCTCC
GGCACACCGGACTATTCCTCGTCGTGGCCA[illegible]CCGGCGCGGACTTCCATCCCAGTCTGGACACGTCGGC
AATCGAGGCGGTGTTCTACAAGGGTGCC[illegible]TCGAAGGAGTCGACGAGAACGGCACGCCACTGCTGAA
TTGGCTGCGGCAACGCGGCGTCGATGAG[illegible]CGATCATTGTGTGCGCCAGACGGCCGAGGACGCGGTA
CGCAATGGCTTGGCCACCAGGGTGCTGG[illegible]GGCCGATACCACCGTCGCCGCGCTGGAGGAGATGCGC
ACCGCCAGCGTCGAGTTGGTTTGCAGCTC[illegible]

C→R

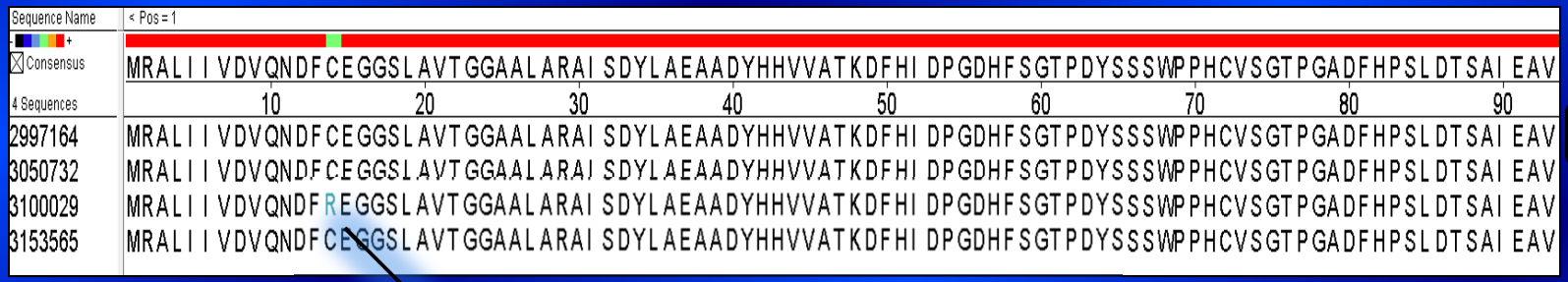
Protein (186 amino acids)

MRALIIVDVQNDFREGGSLAVTGGAALARAISDYLAEAADYHHVVATKDFHIDPGDHFSGTPDYSSS
WPPHCVSGTPGADFHPSLDTSAIEAVFYKGAYTGAYSGFEGVDENGTPLLNWLRQRGVDEVDVVGIA
TDHCVRQTAEDAVRNGLATRVLVDLTAGVSADTTVAALEEMRTASVELVCSS.

Genetic Analysis of *pncA* gene from a Clinical Sample



Nucleotides



Amino Acid

Cys→Arg

$C^{14}R$ substitution
PZA Resistance

PCR amplification primers used for full-length analysis of MTB Genes

Amplification Target	Forward	Reverse	Amplicon (bp)
rrs (16s)	5′ -TTCTAAATACCTTTGGCTCCCT -3′ *22nt*	5′ -TGGCCAACTTTGTTGTCATGCA -3′ *22nt*	1680
rpoB	5′ - TCCTCTAAGGGCTCTCGTT -3′ *19nt*	5′ - GTCAGGTACACGATCTCGT -3′ *19nt*	1625
rpoBII (2nd half)	5' - ATCGAAACGCCGTACCGCAA -3' *20nt*	5' -TGACGTCGAGCACGTAACTCCCT -3' *23nt*	2056
katG	5′ - ACACCAACTCCTGGGAAGGAAT - 3' *21nt*	5′ - TGATCGCACATCCAGCACATTT -3′ *22nt*	2447
gyrA	5' - AAGGATGTTCGGTTCCTGGAT -3′ *21nt*	5′ - TAACACTCGTACCCGGCT -3′ *18nt*	2664
pncA	**5'- GACGGATTTGTCGCTCACTAC-3' *21nt***	**5'- GCCGGAGACGATATCCAGAT-3' *20nt***	**960**

- ✓ **11,432 bases per isolates**
- ✓ **Universal amplification parameters**
- ✓ **Universal thermocycling parameters**

pncA Gene Results

Summary of 6 amino acid mutations in the *pncA* gene of 26 (14 MDR, 7 XDR and 5 fully susceptible) *M. tuberculosis* isolates from South Africa deduced by Ion Torrent sequencing and culture.

No. of isolates *(n=26)*	GenBank Accession No(s).	Amino Acid Substitution(s)‡ in the *pncA* gene (3619 bps)	Pyrazinamide Result by: Ion Torrent†	Pyrazinamide Result by: Bactec MGIT 960
3		**C14R**	**Resistant**	**Resistant**
1		**A102V**	**Resistant**	**Resistant**
1		**Q122(Stop)**	**Resistant**	**Resistant**
16		Wild Type‡	Sensitive	Sensitive
1		**V139G**	**Resistant**	**Resistant**
1		**R154G**	**Resistant**	**Resistant**
2		**L172P**	**Resistant**	**Resistant**
1		**Silent (C195T)¥**	Sensitive	Sensitive

†Pyrazinamide resistance is known to occur in several mutations described by *Mphahele et al.*

‡Compared to H37Rv reference strain.

¥One strain contained a silent (synonymous) nucleotide mutation at position 195 (C→T).

Daum *et al*., under review, *Journal of Clinical Microbiology*, 2012

Conclusions

- Collection and ambient temperature shipment of MTB samples in PrimeStore® MTM provides a safe and cost effective approach for global MTB drug resistance surveillance using Ion Torrent/Next Gen sequencing.
- PZA resistance was observed in 9 of 26 isolates characterized, with 6 different mutations detected in at least one strain.
- Ion Torrent sequencing characterized 5 substitution mutations and an uncommon Q122(Stop) mutation in the *pncA* gene.
- Combined with other full-length resistance genes the developed Ion Torrent method characterizes MDR and XDR strains with overall performance comparable to Hain LPA, and offers potential discovery of novel resistance mutations.
- Future studies are in progress to include ALL (known) genes implemented in TB drug resistance and to compare newly identified and known resistance mutations to quantitative MIC value.

Semiconductor Sequencing Review FROM ION TORRENT

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ISSUE NO. 02
WINTER 2012

Dr. Luke Daum, co-founder of Longhorn Vaccines & Diagnostics, discusses the development of an antibiotic-resistance test for tuberculosis on the Ion Personal Genome Machine™ (PGM™) Sequencer.

Dr. Luke T. Daum, Chief Scientific Officer and a co-founder of Longhorn Vaccines & Diagnostics has developed an assay on the Ion PGM™ Sequencer for detecting antibiotic resistance and sensitivity in tuberculosis strains collected from developing countries, starting with those in Africa. The assay is for epidemiological and surveillance monitoring of geographically significant strains of the disease, which infects one third of the world's population, with upwards of 10 percent of those people developing active tuberculosis. Using current culture methods, it can take up to four months or longer to get results from drug susceptibility tests in developing countries throughout Africa. Dr. Daum's multi-drug resistant (MDR) test would provide results in two days. Ion Torrent products, including the Ion PGM Sequencer, are for Research Use Only. They are not intended for any animal or human therapeutic or diagnostic use. This interview was conducted with Dr. Luke T. Daum who is currently conducting research in Africa. The comments given in the interview relate to the use of the Ion technology in Africa only. No use of the Ion technology in the U.S. is implied. Dr. Daum is not employed by Life Technologies, nor was he paid to take part in this interview.

We want to talk about your test, but before we get into that, can you give us some background on TB in the developing world? How big of a problem is it?

Luke Daum: Tuberculosis or TB is an infectious bacterial disease that is caused by *Mycobacterium tuberculosis*, which infects the lungs. It's transmitted from person to person through droplets from the throat or lungs of people who have active respiratory disease. Tuberculosis is a major problem worldwide, and a lot of people don't understand this. Overall, one third of the world's population is currently infected with TB, and upwards of 10% will develop active tuberculosis. In any given year, on average 1.7 million people die of complications due to active tuberculosis, which equates to an average of 4,700 deaths per day, and the TB problem in Africa specifically is staggering. Among the 15 countries with the highest estimated TB incidence rates, 13 are in Africa. Africa also has a high incidence of HIV infection – about 10% of the population in South Africa has HIV. People that are infected with HIV and TB are about 20 to 40 times more likely to develop active Tuberculosis than people not infected with HIV living in the same country.

DR. LUKE T. DAUM
Co-founder, Longhorn Diagnostics, Inc.

Luke T. Daum earned a Ph.D. in Cell and Molecular Biology at the University of Texas at San Antonio and spent eight years establishing and managing the Air Force's Influenza Strain Surveillance Laboratory. In 2007 he left the Air Force to co-found Longhorn Vaccines & Diagnostics. The company's first product, PrimeStore® MTM, inactivates microbes and then stabilizes and preserves the released RNA and DNA at ambient temperature until the samples can be processed in a molecular diagnostics laboratory by PCR or other nucleic acid tests.

Acknowledgements

Luke T. Daum, Ph.D.

QUESTIONS?

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