Considerations Regarding Forensic DNA Typing and Future Directions

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Technology Works

• Generally speaking this statement is true

• Mitigating Factors
  – Pushing the envelope
  – Uncertainty/Risk
  – Bias
  – Reliability
  – Validation

• Humans are involved!!!
Methodology
Forensic Concerns

• Bad method done poorly
• Bad method done well
• Good method done poorly
• Good method done well, but not accepted in legal system
Historical Example of Method Issues

- Methods can be thought to be reliable
- But sufficient validation studies must be carried out
- Results of validation studies should not be ignored
- HLA-DQA1 and Polymarker
HLA-DQA1 and PM Loci Dot Blots

Control
Victim K
Suspect K
Evidence Q

Control
Victim K
Suspect K
Evidence Q
Errors in Typing Results

• Thermocycler temperature performance affected reliability
  – Four more GC residues in allele 1 than alleles 4, 3, and 2 of DQA1
  – If the denaturing temp is not high enough allele 1 may not denature
  – Causing allele dropout

• Note: manufacturer laboratory scientists were not using outer wells of thermocycler

• Real cases with discrepancies inconsistent with data

Errors in Typing Results

- Amplicon denatured for hybridization to immobilized probes
  - Selective loss of GC B and HLA DQA1 4.1 probe signals
- Primers can re-anneal and extend if the samples is not immediately hybridized
- Blocks the allele variant for hybridization
- Causing allele dropout
- Real cases with discordant DQA1/PM and STR results

Validation

• Requisite!
• Without proper validation the limits are not defined
• Performing validation and ignoring results is unacceptable
Human Failings

• Mistakes will be made by humans with any system
• But some human failings are inexcusable
• FBI misidentification of latent print in Madrid bombing case
• SE33 variants
• One report describes electrophoretic SE33 anomalies
• Another report does not observe it
  – Sampling
  – Not aware and thus looking for it
  – Poorly calibrated instrument
Concordance and population studies along with stutter and peak height ratio analysis for the PowerPlex® ESX 17 and ESI 17 Systems

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Table 4

Discordant results for samples in this study. Null or different alleles due to an insertion or deletion outside of the primer binding site are in bold and underlined.

<table>
<thead>
<tr>
<th>Locus</th>
<th>PP-ESX17</th>
<th>PP-ESI17</th>
<th>Identifier</th>
<th>PPI6</th>
<th>MiniFiler</th>
<th>NIST-NC01</th>
<th>NIST-23plex</th>
<th>PP-SE33</th>
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<td>14, 17</td>
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</table>

\textsuperscript{a}—The compared kit does not provide results for this locus.
\textsuperscript{b}—After inclusion of an additional D22S1045 forward primer to correct the null allele, these samples are not discordant in the commercial PP-ESX17 kit.
Identification and secondary structure analysis of a region affecting electrophoretic mobility of the STR locus SE33

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Life Technologies, 850 Lincoln Centre Drive, Foster City, CA 94404, United States

Table 1
Discordant SE33 samples from the population study. The discordant alleles due to a mobility shift or allele dropout when compared to the SEfiler Plus™ kit are shown in bold and underlined. The kits used in the study were SEfiler Plus™, NGM Select™, SE33 experimental primers, and the Promega ESX-17 and ESI-17 kits. Electropherograms for samples IBB297 and IBB298 are shown in Figs. 2 and 3 respectively. AA, African American; C, Caucasian.

<table>
<thead>
<tr>
<th>Ethnicity (sex)</th>
<th>Sample ID</th>
<th>SEFP</th>
<th>NGM Select</th>
<th>Experimental</th>
<th>ESX-17</th>
<th>ESI-17</th>
<th>Genetic Variation</th>
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<tbody>
<tr>
<td>(1) AA (Male)</td>
<td>IBB039</td>
<td>17.20</td>
<td>17.20</td>
<td>17.20</td>
<td>17.20</td>
<td>17.20</td>
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<td>IBB052</td>
<td>18.20</td>
<td>18.20</td>
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<td>(3) AA (Fem)</td>
<td>IBB114</td>
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<td>18.24</td>
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<td>IBB121</td>
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<td>20.21</td>
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<tr>
<td>(6) AA (Male)</td>
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<td>(14) AA (Male)</td>
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<td>(19) AA (Male)</td>
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<td>20.22</td>
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1) G/A13 SNP in experimental amplicon sequence (Fig. 4B).
2) C/T10 SNP in experimental amplicon sequence (Fig. 4B).
3) G/A11 SNP in experimental amplicon sequence (Fig. 4B).
The ESI-17 kit results yielded a discordant SE33-14.3 allele.
Variants observed for STR locus SE33: A concordance study

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b Universität Bern, Forensische Molekularbiologie, Institut für Rechtsmedizin, Bern, Switzerland
c Landeskrlandesamt Mecklenburg-Vorpommern, Ramae, Germany

Table 1
Discordant SE33 alleles. The discordant alleles are in bold and underlined. The kits used in this study were NGM SElect™, ESX 17, and ESI 17. African ancestry (A); Caucasian ancestry (C) and Unknown ancestry (U).

<table>
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<tr>
<th>Sample</th>
<th>Ethnicity</th>
<th>Sex</th>
<th>ESI-17</th>
<th>ESX-17</th>
<th>NGM SElect</th>
<th>SNP</th>
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<td>18, 19.2</td>
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<td>2</td>
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<td><strong>14.3</strong> 18</td>
<td>14.2, 18</td>
<td>14.2, 18</td>
<td>G/A16</td>
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<tr>
<td>3</td>
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<td>Male</td>
<td>27.3, 30.2</td>
<td>27.2, 30.2</td>
<td>27.2, 30.2</td>
<td>G/A16</td>
</tr>
<tr>
<td>4</td>
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<td><strong>18.1</strong>, 23.2</td>
<td>18, 23.2</td>
<td>18, 23.2</td>
<td>G/A16</td>
</tr>
<tr>
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<tr>
<td>6</td>
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<td>17, 23.2</td>
<td>G/A16</td>
</tr>
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<td>7</td>
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<td><strong>22.2, 20.2</strong></td>
<td>22.2, 20.2</td>
<td>22.2, 20.2</td>
<td>G/A16</td>
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<td>16, 19</td>
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</table>
Sequencing Results Show Shift Due to SNP (not indel)

Fig. 2. The new SNP C/T_{19}. The most stable secondary structure for the sequence encompassing the polymorphic region (using MFOLD computer model). The sequence is annotated with the variant SNPs found in the polymorphic region. The free energy value for the new variant is $\Delta G = -2.44 \text{ kcal/mol}$ as compared to the wild type $\Delta G = -5.79 \text{ kcal/mol}$. 
Initial Low-Copy Number (LCN) Work

- Early work on “touch samples”:

- Application to routine limited quantity casework:

- Note that Touch Samples do not necessarily equate to LCN samples
Comparison of STR Results with Different Amounts of DNA

1ng Standard Result

33pg LT-DNA: 2 reps

- Allele Drop In
- Increased Stutter (43%)
- Allele Drop Out
- Heterozygote peak imbalance (57%)
- Locus Drop Out
- Allele Drop Out
Risk
Risk

• A scientist might say
  – “I am willing to take the risk…”

• But who is really at risk?
  – The scientist?
  – Suspects, Victims, Families, Society??
Bias in Law

DNA is from suspect

$H_p$

DNA is from an unknown person

$H_d$

• Asymmetry of the law - a thousand guilty go free vs one wrongly accused innocent person!

• What about the victim?
Forensic Science & Bias

• Database searches can tolerate false positives more so than false negatives
  – Can resolve with follow up
  – Investigative leads
  – Incumbent on scientist to convey uncertainty

• Casework tolerates false exclusions more so than false inclusions
  – Bias in law
Forensic Science & Bias

• A DNA threshold is a biased tool!
  – Because we are concerned about false inclusions/associations
• Set thresholds sufficiently high to greatly reduce the chance of false inclusions
  – Data below threshold become inconclusive
  – and importantly still can be used for exculpatory purposes
Forensic Science & Bias

• Driven by the degree of risk that should be taken

• What if the scientists do not convey the risk or uncertainty?

• Is that a serious concern or should we turn a blind eye?
New Evolving DNA Reporting Approach

ADDITIONAL REPORT

For previous results, evidence received, and disposition, see report dated October 18, 2007.

SUMMARY OF RESULTS:

The suspect, [redacted], is included as a contributor to the mixtures detected on stain 1A from seat belt “E11” and swab “ES15” from “steering wheel–right”, and he cannot be excluded as a contributor to the mixtures detected on swabs “ES3” from “gear shift” and “ES6” from “brake pedal”, from the report dated October 19, 2007 from case:

<table>
<thead>
<tr>
<th>FB number</th>
<th>victim’s name</th>
<th>report dates</th>
</tr>
</thead>
</table>
Cannot Exclude Interpretation

• Most of the DNA alleles seen in the DNA profile of XXX are seen in the mixture of DNA found on the sample listed below. Since the absence of the alleles can be explained, he cannot be excluded as a possible contributor of the mixture.
Cannot Exclude Interpretation

• No statistics are provided with this statement
• So no risk or uncertainty is conveyed
• We will visit later the bias and allele drop out statistical issues with such interpretations
Relevance

• Some scientists have said
  – “Relevance is for the court to decide”
• Is it up to the court to decide?
• Or are there situations where the scientist should not absolve himself/herself from considering relevance?
• Examples such as the Knox case demonstrate that this simple statement is insufficient for addressing the role of the scientist
• Perhaps it is not so black and white
Amanda Knox Case
The Knife

Selected because it looked cleaner than other knives
Does Evidence Support the Hypothesis?

• Or better posed
  – Is there an alternate hypothesis/interpretation of the findings?

• Should alternate hypotheses be considered?

• We need to develop training in this regard!
Other Tests Were Performed!

- Sample Screening
  - Identification of tissue source
    - Blood
    - Semen
    - Saliva
  - Time, labor, cost
  - DNA decision tree
    - Quality/Quantity of Body Fluid
Presumptive Test

- Sample B from the handle of the knife yielded a negative result for the presumptive tetramethyl benzidine (TMB) test.
  - Extremely sensitive
  - Blood can be diluted 100,000 - 1,000,000 times.
- Knife was collected only 12 days after the crime
- Hemoglobin is fairly stable molecule
- Peculiar and difficult to reconcile that the TMB was negative
Alternate Hypothesis

• Extremely unlikely to have been able to wash away all traces of hemoglobin and preferentially leave behind solely DNA

• General plausible explanations for the presence of DNA on items
  – Contamination
  – Primary and secondary transfer
  – A person’s DNA will be found on his/her items in his/her home, place of work, and other places
  – DNA also can be picked up by others and passed on to other items

• Evidence does not support that DNA on knife was from blood
What should have been done?

• Consider relevance!
  – Background DNA
    • Collect other knives and utensils in drawer
    • Test for presence of DNA

• Incumbent on scientists to consider alternate hypotheses, especially if they are probable

• Understand consequences of low level DNA typing

• Education
Not Unique to This Case
Deceased woman –
- Prosecution hypothesis: offender is male and punched her in the face in committing the offence
- Swabs taken from her face, both cheeks
- Y STR (male) analysis of left and right cheek swabs
- There were two men of interest, A and B - at different times

Yesterday in the High Court in Napier, Justice Denis Clifford granted a defence application led by co-counsel Peter Williams, QC, to dismiss the charge after Crown prosecutor Russell Collins conceded the case against King was not strong enough.

Mr Collins said it was "unsafe" for the Crown to offer its evidence as a reliable basis for a jury to reach a verdict.

From Hawkes Bay Today, 9 February 2010
Left Cheek Swab

A. 3 reactions → no DNA result

B. Peak 54RFU

1 Peak (out of 12 loci)
Right Cheek Swab

A. 3 reactions → no DNA result

B. Peak 70RFU
1 Peak (out of 12 loci)
Case Results

• Two total peaks, observed at two different loci, were seen in only one of four replicates
  – Consensus profile approach requires alleles to be replicated
  – These peaks should not have been reported as alleles
  – These peaks should not have been used for inclusionary or exclusionary purpose
• No peaks at the other loci were detected
• Peaks had very low heights of 54rfu and 70rfu (threshold values range from 50 – 250rfu)
• Violates “Published” Rule and yet was reported!
Bias

• A scientist might say
  – “I am not biased, I am objective and trained to be so”

• However, we are all biased

• Then the scientist says
  – “There is no bias because I detected all the alleles before looking at the reference samples”

• Is that a correct assessment?
Recall Cannot Exclude…

• Most of the DNA alleles seen in the DNA profile of XXX are seen in the mixture of DNA found on the sample listed below. Since the absence of the alleles can be explained, he cannot be excluded as a possible contributor of the mixture.
## Bias Example

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<td>20,25</td>
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</table>

- These 3 suspects are all included but the loci with potential drop-out change
- “Sliding window drop-out”
Bias - LCN and Y STRs

DYS385

DYS635

DYS385

DYS635
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NR = no result  ( ) = weaker allele  * = possible additional allele(s) present below threshold

NR = no result  NS = not searched in database
Interpretation

• Already addressed, but

• A scientist may say
  • “I have a set of defined guidelines and therefore my interpretation of results is reliable”

• What are the protocols?
LCN Transfer Studies

- Secondary transfer studies have thus far concentrated mainly on DNA originating from the epithelial cells of hands
  - Wash hands, shake, evaluate transfer
  - Not realistic
- Saliva is a rich source of DNA that is commonly transferred during normal day-to-day activities:
  - Placing a pen in mouth while studying
  - Licking a thumb before turning a page
Saliva Study

• Study conducted under the hypothesis that saliva, which is rich in DNA, can be a more prevalent source of genetic material during transfer events than hand epithelial cells
  – Saliva-based DNA transfer can result in higher levels of deposited DNA than previously observed by transfer studies
  – The profile of the initial depositor can be more prevalent in secondary transfer samples than previously observed by transfer studies
Bottom Line

• Hand washing studies conclude that last person in transfer line tends to be the dominant profile
• Good shedders and Bad shedders
• Saliva studies show that primary donor can be dominant profile
• No value to shedder status!
• Saliva traces make everyone a good shedder
• Impacts relevance!
FORENSIC DNA TIMELINE

21st Century

- Real-time PCR
- Automation
- Kits
- DNA Repair
- Familial Searching
- Low Copy
- Database Expansion
- Mass Spectrometry
- SNPs
- No extraction
- Interpretation
- Microdevices
- Intelligence Investigations
- Rapid DNA Typing
- Mega multiplexes
- Next Generation Sequencing
- 2000
- 2005
- 2010
- 2015
Kits

- Standard loci
- Quality tested products
- Took burden off analyst
- Greater shared experiences
Bone Sample Amplified with Identifiler
Bone Sample Amplified with Next-Generation Multiplex STR System
Enhancing Databases

More Markers
More Kits
3500 Laser Design

- Smaller, Single Excitation Line Solid State Laser
- Minimal Heat Output
- Standard Voltage Plug
Improved Temperature Control System

- Optional Pre-Heat Control in Data Collection
- Flat Oven Door Seal and New Locking Mechanism
- Smaller Oven
- More Consistent Migration for Better Sizing Precision
McLaren et al, FSI Genetics 2008

- Split peak artifact due to post-PCR reannealing of the unlabeled, unincorporated vWA primer to the 3′-end of the tetramethylrhodamine (TMR)-labeled strand of the vWA amplicon
- Occurs in the capillary post-electrokinetic injection
- Split peak is eliminated by incorporation into the loading cocktail of a sacrificial hybridization sequence (SHS) oligonucleotide that is complementary to the vWA primer
Heating Issues

“Split Peak” at vWA Locus - 3130
Same Sample - 310
### Same Sample – 3100/3500

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Rapid DNA Typing

• Rapidly identify individuals by DNA typing
• Military, forensic, homeland security, and intelligence community
• Self-contained turnkey system
• Swab in --- Result out
• 90 minutes to 2 hours
• Informed identification decisions regarding arrest, detention, or release of suspects, and eventually as it matures analyze crime scene evidence.
• NetBio, IntegenX, MicroLab Diagnostics
• Bottom line – makes DNA typing an actionable tool for investigative leads
Analysis of Difficult Samples

mtDNA is the most successful marker
Mass Spectrometry Advantages

- No labeling
- Mass accuracy
- Multiplexing
- Quantitation - Mixture interpretation
- Automation
- Cost – e.g., mtDNA
PLEX-ID ANALYSIS STRATEGY

PCR amplification of multiple targets

Concordance?
Database search
Assess expected match frequency

Multiplexed PCR

PCR Product(s)

Automated desalting on the PLEX-ID

ESI-MS analysis of complex mixture

Deconvolution

Mapping of products to a coordinate map and development of base composition profile

Data Processing

ESI-MS

m/z

490.43
724.63
1004.38
1329.71
1700.6
2117.06

2906, 154..290: A48 G18 C30 T41
2901, 15893..16012: A47 G18 C25 T30
2892, 16231..16338: A40 G9 C39 T20

Mass (Da)

0.00
31348.48
34045.68
36742.88
39440.08
42137.28
44834.48

0.00
1916.5
3833

Human Mitochondrial Genome 16569bp

12S rRNA
16S rRNA
ND1
ND2
ND5
ND6
ATP6
ATP8
ND3
COX3
COX1
COX2
CYTB
ATP8
ATP6
COX3
COX1
COX2
CYTB

Deconvolution
490.43
724.63
1004.38
1329.71
1700.6
2117.06

Data
Processing

Compare sample and reference base composition profiles

Assess expected match frequency

Concordance?
Database search
Mass Spectrometry
Base composition

Sample 1  ---  A-24, G-30, C-18, T-28

Sample 2  ---  A-23, G-31, C-18, T-28

A to G transition
PLEX-ID: Advances and Applications

Base composition analysis of human mitochondrial DNA using electrospray ionization mass spectrometry: A novel tool for the identification and differentiation of humans

Thomas A. Hall, Bruce Budowle, Yun Jiang, Lawrence Blym, Mark Eshoo, Kristin A. Sannes-Lowery, Ranganjan Sampath, Jared J. Drader, James C. Hannis, Patina Harrell, Vivek Samant, Neil White, David J. Ecker, Steven A. Hofstadler

Automated analysis of sequence polymorphism in STR alleles by PCR and direct electrospray ionization mass spectrometry

John V. Planz, Kristen A. Sannes-Lowery, David D. Duncan, Sheri Manallili, Bruce Budowle, Ranajit Chakraborty, Steven A. Hofstadler, Thomas A. Hall

Research article
Validation of mass spectrometry analysis of mitochondrial DNA

Bruce Budowle, Arthur J. Eisenberg, Suzanne Gonzalez, John V. Planz, Kristin A. Sannes-Lowery, Thomas A. Hall, Jessica E. Paulsen, Steven A. Hofstadler

Base Composition Profiling of Human Mitochondrial DNA Using Polymerase Chain Reaction and Direct Automated Electrospray Ionization Mass Spectrometry

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### Assay Format

- **AMEL**
  - **D8S1179**: 3895, 3886
  - **D13S317** (D5S818): 4755, 4862
  - **D3S1358** (D7S820): 3883, 5559
  - **TPOX** (vWA): 3893, 1185
  - **CSF1PO** (D16S539): 4863, 5690, 3892
  - **THO1**: 1205
  - **D18S51**: 5679
  - **D21S11**: 4976
  - **FGA**: 4976

The table above represents the assay format for the PLEX-ID STR CODIS Core Loci V2.0.
# Biodefense Kit

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The table above lists various SNPs and their associated primer pairs. The image represents a SNP assay with sample information. Each sample (1-12) is tested for the presence of specific SNPs. The color of the dot indicates the presence (positive) or absence (negative) of the SNP in the sample.
Next Generation Sequencing Platforms

Roche 454

Applied Biosystems SOLiD

Illumina Genome Analyzer

Ion Torrent
SOLiD™ Workflow

Application specific sample preparation

Emulsion PCR & substrate preparation

Sequencing chemistry

Imaging and analysis

Application specific Data analysis
**B. anthracis** SNPs
(ambiguities with allelic asymmetry filtered)

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* Four shared ambiguities in imperfect repeat region
Use of strain identification in sexual assault and child molestation

Molecular Evidence of HIV-1 Transmission in Criminal Cases

Structure of Human Immunodeficiency Virus (HIV)
100% of bootstrap replicates place victim sequences within patient sequences

Victim sequences embedded in patient sequences
Dr. Schmidt was found guilty of second degree attempted murder and is serving a 50 year sentence

- The admissibility of the conclusion that the HIV samples were closely related was challenged on appeal

- Use of DNA evidence is well-established in Louisiana, but its use to establish similarities between viral infections was without precedent (Note: no statistical strength)

- The appeal was rejected by the Louisiana State Supreme Court in 2000

- The case was then appealed to the United States Supreme Court, and the appeal was rejected March, 2002
Ion Torrent

Single Day Workflow

• ~2 hour sequencing runs – enabled by PostLight™ Sequencing

• Innovative automated template preparation for PGM sequencer matches the speed of semiconductor sequencing

• Complete end-to-end workflow within 1 day or multiple samples per day
Schematic cross-section of a single well of an Ion Torrent sequencing chip

Semi-conductor technology
**Chemistry**

Eliminate source of sequencing errors:
- Modified bases
- Fluorescent bases
- Laser detection
- Enzymatic amplification cascades

Eliminate source of read length limitations:
- Unnatural bases
- Faulty synthesis
- Slow cycle time

Delivers highly uniform genome coverage

- In principle similar to pyrosequencing
- But simpler
Human Mitochondrial Sequencing

• Deep sequence for heteroplasmy detection
  (> 1000x coverage on Ion 314)

• Ability to do 16 samples per run with barcoding

• Accurate variant calling, especially in hypervariable regions of mitochondria

Amplify mtDNA via two overlapping long range PCR
Fragment via mechanical or enzymatic shearing

Mutation detected on position 15450
Prof. Stefan Schuster Penn. State University
Microbial Sequencing

• Highly uniform coverage (equivalent to predicted) allows more efficient sequencing

• Up to 99.999% consensus accuracy

• 100 bp runs today (200 bp late 2011)
European *E. coli* Outbreak Strain Identified using Ion PGM™ in 3 days

<table>
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<tr>
<th>Day</th>
<th>Activity</th>
<th>Notes</th>
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<td>Library preparation</td>
<td>O104:H4 and HUSC41 samples (reference) strain libraries prepared</td>
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<td>Tuesday</td>
<td>Sequencing runs</td>
<td>0104:H4 amplified and sequenced 2 x 2 runs (Ion 314)</td>
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<td>Wednesday</td>
<td>Sequencing runs</td>
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<td>Thursday</td>
<td>Assembly</td>
<td>Draft Genome identified, Assembled, Submitted and Released from NCBI</td>
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<td>Friday</td>
<td>Assay Design</td>
<td>TaqMan Assays Designed</td>
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*May 22 CEDC reports significant increase in patients with hemolytic uremic syndrome

"The biggest advantage [of the PGM] from my point of view as a public health official is that it's speedy, and speed is what is needed at the moment,“

Prof. Dr. Med Dag Harmen, University Hospital Muenster

"[The PGM] takes the shortest time to generate genomic data."

Junjie Qin, BGI
Acknowledgments

• Angela van Daal
• Life Technologies
• Promega Corporation
• Abbott/Ibis
• Illumina