MultiMaTCH Genotyping


Agilent Technologies, Inc.
Santa Clara, California

Lenore Kelly, Ph.D.
Americas Food Biochemist
Trace-Back: a Situational Analysis

• Serovar typing is a lengthy procedure with turnaround times exceeding the half-life of a typical outbreak
• Sampling during an outbreak is increased an order of magnitude over steady state monitoring
• Outbreaks are increasing in frequency due to globalization of the food supply
• Rapid detection methods desired:
  – Faster identification of problems
  – Better protection of public health
  – Quicker release of non-infected products
Why is Salmonella Serotyping Important?

• Serves as the basis for the National Salmonella Surveillance system
  • “International language” of Salmonella
  • 60+ Years of surveillance data based on serotype
• Approximately 40,000 isolates serotyped each year by state health departments and federal agencies.
• Critical for epidemiologic classification of strains and for outbreak investigations
Disadvantages of Traditional Salmonella Serotyping

- Requires >250 antisera to identify all serotypes
- Production and QC with the antisera is problematic.
- Requires >350 strains and antigens to maintain sera.
- Takes a minimum of 3 days to identify all antigens of an isolate
Salmonella Characterization

- challenging, > 2,500 serovars
- paucity of genome sequence information = difficult molecular subtyping

Project Aims:
Combine 2 of 3 steps
Faster response: ≤ 30 h
More informative results

Detection
Genus, species
qPCR, etc 8-30 h
Traditional: 3-5 days

Serotyping
Serology: 4-5 days

Strain typing
PFGE, MLST, etc.: 5-10 days

Discrimination and Information
Sensitivity
Surveillance
Outbreak response

Specificity
Outbreak investigation
Aim of Our Applied Research

Reliable detection and molecular typing of foodborne pathogens using a single PCR-based assay

- novel labeling technology allowing multiplexed detection (10-40plex)
- probe format
- compatible with real-world sample matrices
- software package allowing simple instrument control and automated results analysis

Current solution with a working prototype

MultiMaTCH methodology with MassCode technology
MultiMaTCH System Workflow

10-40plex

96 samples

6 h post enrichment

primary enrichment → extract gDNA → MultiMaTCH w/MassCode PCR → detect → StrataPrep cleanup

Reagents, consumables, instrumentation and software provided by Agilent
MassCode Tags
A Novel Reporter for Biomolecules

- MassCode labels give biomolecules a **digital code**
- 93 unique tags = high density **multiplexing**
- A **true** liquid array: Solution based; **No beads**; **No solid supports**
Oligos are synthesized by Operon with a 6-amino-1-hexanol linker on the 5’-terminal phosphate. The 6-amino- group is covalently coupled to a photocleavable MassCode tag.
A Stable Modular Design

Mass post-cleavage = 729
Discrete Resolution of 93 tags

Selective Ion Monitoring of tags
No spectral overlap

Benchtop instrument
Simultaneous Monitoring of 44 tags
Detection of 8 tags
MassCode PCR
A Multiplex PCR

Flexible assay design
Dual reporting/target (QC)
High throughput

1 well/sample
20 targets/well

Foodborne Pathogen Panel

<table>
<thead>
<tr>
<th>Target organism</th>
<th>MassCode tag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F primer</td>
</tr>
<tr>
<td>Campylobacter species</td>
<td>352</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>422</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>488</td>
</tr>
<tr>
<td>E. coli species</td>
<td>557</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>621</td>
</tr>
<tr>
<td>non-O157 STEC</td>
<td>688</td>
</tr>
<tr>
<td>ETEC</td>
<td>378</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>442</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>510</td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>577</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>641</td>
</tr>
<tr>
<td>Enterobacter sakazakii</td>
<td>709</td>
</tr>
<tr>
<td>Salmonella species</td>
<td>370</td>
</tr>
<tr>
<td>Salmonella enterica Typhimurium</td>
<td>434</td>
</tr>
<tr>
<td>Salmonella enterica Enteritidis</td>
<td>502</td>
</tr>
<tr>
<td>Listeria species</td>
<td>569</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>633</td>
</tr>
<tr>
<td>Shigella species</td>
<td>701</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>629</td>
</tr>
<tr>
<td>Internal Inhibition Control</td>
<td>697</td>
</tr>
</tbody>
</table>
MultiMaTCH
Method for the Multiplexed Synthesis of Mass Tag Coded Hybrids

Probe detects correctly amplified targets from MassCode PCR
Probe uniquely labeled with MassCode tag
Greater assay specificity
A one-step temperature dependent reaction
 Addition of admixture directly to MassCode PCR reaction tube
Retains dual reporting system
MultiMaTCH

MassCode PCR

Lambda exonuclease digestion

MassCode probe annealing and extension

Mass Tag Coded Hybrid
Clean, Inject and Detect
Salmonella MultiMaTCH Assay Design
A 14plex Hierarchical Subtyping Assay

Allele-specific signatures

Proof-of-concept prototype

Exclusion panel (so far)
- *E. coli* O157:H7
- *E. coli* species
- *Bacillus subtilis*
- Human
- *Campylobacter jejuni*
- *Listeria monocytogenes*

![Diagram of Salmonella MultiMaTCH Assay Design](image-url)
Simultaneous *Salmonella* Detection and Subtyping

**Typhimurium LT2**

error bars = STDEV calculated from 3 biological replicates
individually run in 3 different experiments over a 13 day period

![Graph showing response threshold for various *Salmonella* serovars and serogroups with error bars indicating standard deviation calculated from 3 biological replicates over a 13 day period.](image-url)
Sensitivity of the *Salmonella* MultiMaTCH Assay
Preliminary Results from 3 Isolates

Decrease in sensitivity as # of detected targets increases
This tradeoff provides greater subtyping capabilities in a short amount of time

<table>
<thead>
<tr>
<th>Serovar [SGSC]</th>
<th># Targets Amplified</th>
<th>DNA Copies Detected per Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubislaw [2511]</td>
<td>1</td>
<td>219</td>
</tr>
<tr>
<td>Newport [4910]</td>
<td>2</td>
<td>1,450</td>
</tr>
<tr>
<td>Enteritidis [4901]</td>
<td>3</td>
<td>1,830</td>
</tr>
</tbody>
</table>

Determined by using gDNA extracted from cells cultured in BHI

Right amount of discrimination for rapid response to outbreaks and surveillance?
Detecting Enteritidis Contamination of a Tomato

- Tomato only
- Enteritidis (D1) only
- Tomato + Enteritidis

Serovar:
- Typhimurium
- Arizona
- Paratyphi
- Enteritidis
- Salmonella
- IAC

Sero-group:
- B
- C1
- C2
- D1-A
- E1
- G

Response - Threshold

-2500 -1500 -500 500 1500 2500

- June 7, 2011
- Emerging Technologies in Times of Change
- Agilent Technologies
Robust System Design

Safeguard mechanisms built into assay

- Multiple targets per subtype promotes very low type 1 error rate
- Two tags per target

Systems and Assay Controls

- IAC reduces type 2 errors
- NTC
  - External positive control performed in the same well
  - External negative control
- Instrument integrity control
- Automated instrument calibration
Advantages of the MultiMaTCH System

Multiplexing allows subtyping – 93 tags (not limited to 4 or 5 dyes)
All hybridizations are solution-based
Cost-effective
Rapid method - 6h
Less PCR competition, all amplicons similar in size
Automated data acquisition and analysis
High throughput – a 25plex = 2,400 individual tests for 96-well
Dual reporting per target – built in QC
Greater specificity than PCR alone
Flexible assay design
Not sensitive to ambient light
Agilent Technologies collaboration with California Animal Health and Food Safety

Purpose of collaboration

- Redesign several of the primers, testing amplicons in singleplex
- Validate the 14-plex assay with real samples
- Test specificity against library of pathogen strains
- Develop vertical and horizontal multiplex panels for multiple food pathogens and for Salmonella serovars
### 14-PLEX PRIMERS: WHOLE GENOME BLASTN RESULTS

#### Legend
- **Green** = Present
- **Red** = Absent
- **Purple** = Unexpected Match
- **Yellow** = Target Gene Absent
- **Pink** = Draft Genome
- **Black** = No Genome

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Serogroup</th>
<th>Pan S.e.</th>
<th>O-group B</th>
<th>O-group C1</th>
<th>O-group C2</th>
<th>O-group D1-A</th>
<th>O-group E1</th>
<th>O-group G</th>
<th>sv Typhimurium</th>
<th>sv Agona</th>
<th>sv Enteritidis</th>
<th>sv Dublin</th>
<th>sv Typhi</th>
<th>sv Paratyphi A</th>
<th>NON-SPECIFIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhimurium str. LT2</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhimurium str. D23580</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhimurium str. 14028S</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhimurium str. SL1344</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhimurium str. 4/74</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhimurium str. T000240</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhi str. CT18</td>
<td>D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhi str. Ty2</td>
<td>D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paratyphi A str. ATCC 9150</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paratyphi A str. AKU_12601</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choleraesuis str. SC-B67</td>
<td>C1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paratyphi C str. RKS4594</td>
<td>C1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paratyphi B str. SPB7</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallinarum str. 287/91</td>
<td>D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enteritidis str. P125109</td>
<td>D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schwarzengrund str. CVM19633</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newport str. SL254</td>
<td>C2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heidelberg str. SL476</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dublin str. CT. 02021853</td>
<td>D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agona str. SL483</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weltevreden str. 2007-60-3289</td>
<td>E1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poona strain CDC 99-0277</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
14-PLEX PRIMERS: WHOLE GENOME BLASTN RESULTS

SUMMARY

- Set Eight: Designed to detect ALL serovar Typhimurium strains, but matches only one, sv Typhimurium str. LT2
- Set Nine: Designed to be specific for sv Agona, but has a cross-match with sv Typhimurium str. T000240
- Set Twelve: Designed to detect ALL sv Typhi strains, but matches only one, sv Typhi str. CT18 (no homolog in sv Typhi str. Ty2)
- Set Thirteen: Designed to be specific for sv Paratyphi A, but has a cross-match with sv Weltevreden str. 2007-60-3289-1
- Other primer sets appear OK

<table>
<thead>
<tr>
<th>PRIMER SET</th>
<th>SPECIFICITY</th>
<th>COMMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONE</td>
<td>Pan S.e.e.</td>
<td>OK</td>
</tr>
<tr>
<td>TWO</td>
<td>S.e.e. O-group B</td>
<td>OK</td>
</tr>
<tr>
<td>THREE</td>
<td>S.e.e. O-group C1</td>
<td>OK</td>
</tr>
<tr>
<td>FOUR</td>
<td>S.e.e. O-group C2</td>
<td>OK</td>
</tr>
<tr>
<td>FIVE</td>
<td>S.e.e. O-group D1-A</td>
<td>OK</td>
</tr>
<tr>
<td>SIX</td>
<td>S.e.e. O-group E1</td>
<td>OK</td>
</tr>
<tr>
<td>SEVEN</td>
<td>S.e.e. O-group G</td>
<td>OK</td>
</tr>
<tr>
<td>EIGHT</td>
<td>S.e.e. sv Typhimurium</td>
<td>REDESIGN?</td>
</tr>
<tr>
<td>NINE</td>
<td>S.e.e. sv Agona</td>
<td>REDESIGN?</td>
</tr>
<tr>
<td>TEN</td>
<td>S.e.e. sv Enteritidis</td>
<td>OK</td>
</tr>
<tr>
<td>ELEVEN</td>
<td>S.e.e. sv Dublin</td>
<td>OK</td>
</tr>
<tr>
<td>TWELVE</td>
<td>S.e.e. sv Typhi</td>
<td>REDESIGN?</td>
</tr>
<tr>
<td>THIRTEEN</td>
<td>S.e.e. sv Paratyphi A</td>
<td>REDESIGN?</td>
</tr>
<tr>
<td>FOURTEEN</td>
<td>NON-SPECIFIC</td>
<td>OK</td>
</tr>
</tbody>
</table>
MULTIPLEX PCR: WHAT’S NEXT?

Identification of new targets for serovars Typhirumium, Agona, Typhi, and Paratyphi A is in progress

What’s available?
seven complete genomes for Typhirumium,
two complete genomes each for Typhi and Paratyphi,
and a single complete genome for Agona

Access to more than one complete genome for each serotype will be very helpful for the identification of new targets
How does having more than one complete genome help?

This is a whole genome alignment of six Typhimurium genomes using MAUVE.
It clearly shows that the genomic architecture is highly conserved, and a small genomic island is present in three strains (green block).
How does having more than one complete genome help?

This is a whole genome alignment of seven different strains/serovars of S.e.e. using MAUVE. It shows where the genomic architecture is conserved, and where it varies: there are definitely some genomic hotspots that are prone for variation between strains/serotypes.
The first half of the table is a whole genome comparison of Typhimurium (LT2) with Newport, Heidelberg, Enteritidis, Dublin, Choleraesuis, and Agona using RAST:

`Orthologs` are genes that are in common, and `specific CDS` are genes that are found only strain LT2.