

# Development of Group B *Streptococcus* Multiplex PCR Panel Amendable to Bead-Based and Microarray Detection Systems

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## Introduction/Background:

*Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS), is a common bacterium carried in the vaginal and/or GI tract of between 15-40% of healthy women. Typically, this organism is only problematic when carried by pregnant women who can then pass the bacterium to their infant during the birth process. GBS has been implicated as a leading cause of life-threatening neonatal infections including pneumonia, sepsis and meningitis. If the infected infant can recover from these acute conditions, long-term damage such as developmental disabilities, vision loss and impaired hearing can still occur. Mothers can also be affected by untreated GBS disease, resulting in amniotic infection and possible subsequent stillbirths.

In 2002, the CDC<sup>1</sup> recommended that all pregnant women be screened for Group B *Streptococcus* at 35-37 weeks of pregnancy. If results are positive for the bacterium, the mother will be treated with penicillin or ampicillin through IV during labor. The 'gold standard' method for GBS screening is enrichment culture, followed by a gram stain and catalase test on  $\beta$ -hemolytic colonies, followed by latex agglutination testing for specific identification. Currently, several molecular methods are available for screening; however, these methods vary in sensitivity and specificity and may require specialized equipment and/or expensive reagents to perform.

The goals of this study were 1) to develop and to validate a TEM-PCR™ (Target Enriched Multiplex Polymerase Chain Reaction) panel for detection of *S. agalactiae* in clinical samples and 2) to evaluate different bead- and microarray-based detection platforms for this panel.

## Materials & Methods:

### Development and Validation of TEM-PCR™ GBS Panel



Figure 1. Scheme describing main steps for TEM-PCR™ panel development and validation.

#### DNA Extraction

DNA was extracted from control strains and vaginal/rectal swab specimens on the KingFisher Flex system (Thermo Scientific) using a modification of the Bacterial DNA isolation kit from Omega Bio-tek (Marietta, GA).

#### TEM-PCR™

In order to improve sensitivity and specificity, we designed primers for two gene targets (*pcsB* and *sip*) for specific detection of *S. agalactiae* using TEM-PCR. Each pathogen target is detected by a nested set of primers which, upon specific annealing to and amplification of extracted nucleic acids, will incorporate a biotin label into the resultant PCR product (Figure 2).



Figure 2. TEM-PCR™ Scheme.

Low concentration nested gene-specific primers are (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) designed to enrich the targets during the initial PCR cycles. Later in the procedure, a pair of universal Superprimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection. PCR products are detected by a complementary detection probe covalently coupled to a microsphere or glass microarray substrate.

#### Hybridization and Detection

For initial development of the GBS Panel, a bead-based platform was used for detection (Luminex, Austin, TX). Target-specific detection oligonucleotides were coupled to carboxylated microspheres and PCR products were hybridized to coupled microspheres mixes and were analyzed on a Luminex 100 instrument. Data were reported as median fluorescent intensity (MFI) values, and sample values above cutoff (established at 3x Limit of Blank) were reported as 'Detected'.

#### Validation and Verification

Analytical validation of the GBS TEM-PCR™ panel was performed using titrated *S. agalactiae* 2023 (ZeptoMetrix Corporation, Buffalo, NY) in sample matrix. Limit of detection ( $LoD_{95}$ ) was established by performing replicate analysis ( $n=20$ ) on serial dilutions of organism, followed by data analysis with the Reed-Muench method. Panel inclusivity was tested against 11 reference strains (Table 1) at approximately 1660 GEq/rxn and specificity was tested by examining related strains ( $n=55$  at  $10^6$  organisms/mL) commonly present in the vaginal/rectal area.

To increase sensitivity of the GBS Panel, an enrichment step was performed on clinical samples initially 'Not Detected' on the direct test. The enriched sample was then rerun on the panel (Figure 3).

## Results:

Table 1. Reference strains for inclusivity testing. All strains were evaluated at approximately 1660 GEq/rxn.

| Strain Name and Reference Number               | Organism Notes                                         |
|------------------------------------------------|--------------------------------------------------------|
| <i>Streptococcus agalactiae</i> ATCC® 12386    | Strain O90R                                            |
| <i>Streptococcus</i> sp. ATCC® 12401           | Lancefield's Group B; type Ib                          |
| <i>Streptococcus agalactiae</i> ATCC® 12403    | Lancefield's Group B; type III; Typing strain D136C(3) |
| <i>Streptococcus agalactiae</i> ATCC® 13813    | Lancefield's Group B                                   |
| <i>Streptococcus agalactiae</i> ATCC® 27591    | Lancefield's Group B; serotype Ic                      |
| <i>Streptococcus</i> sp. ATCC® 31475           | Lancefield's Group B; type III                         |
| <i>Streptococcus agalactiae</i> ATCC® 49446    | Strain 3139; Serotype IV                               |
| <i>Streptococcus agalactiae</i> ATCC® 49447    | Serotype V                                             |
| <i>Streptococcus agalactiae</i> ATCC® BAA-1138 | Strain A909                                            |
| <i>Streptococcus agalactiae</i> ATCC® BAA-1177 | Strain 515; Type Ia                                    |
| <i>Streptococcus agalactiae</i> ATCC® BAA-611  | 2603 V/R; Serotype V                                   |

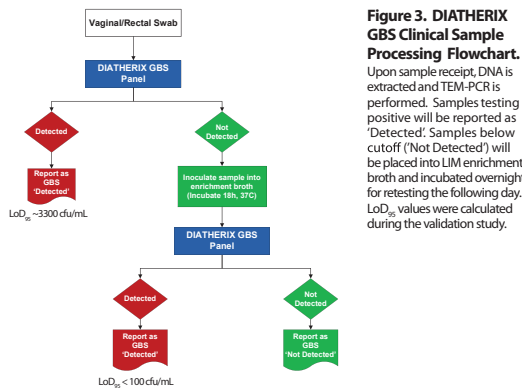


Figure 3. DIATHERIX GBS Clinical Sample Processing Flowchart.

Upon sample receipt, DNA is extracted and TEM-PCR is performed. Samples testing positive will be reported as 'Detected'. Samples below cutoff ('Not Detected') will be placed into LIM enrichment broth and incubated overnight for retesting the following day.  $LoD_{95}$  values were calculated during the validation study.

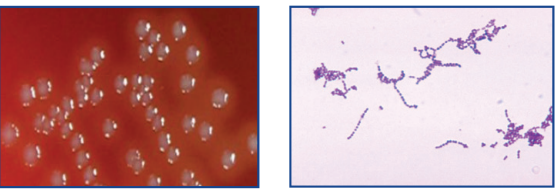


Figure 4. Culture methods for GBS identification.

Representative images of *S. agalactiae* growth on TSA+5% sheep's blood agar (A) and in a Gram stain reaction (B).

Table 2. Culture vs TEM-PCR™.

Clinical samples submitted to Diatherix were inoculated on tryptic soy agar with 5% defibrinated sheep's blood and plates were incubated at 37°C overnight. Plates were inspected and suggestive GBS colonies were selected based on hemolytic zone around growth. Catalase tests and Gram stains were performed on the selected colonies (data not shown). Positive samples were verified with a latex agglutination test (PathoDxtra Strep Grouping Reagent Set, Thermo Scientific).

|                  | TEM-PCR™ |              |       |
|------------------|----------|--------------|-------|
|                  | Detected | Not Detected | Total |
| Culture Positive | 12       | 0            | 12    |
| Culture Negative | 34       | 77           | 111   |
| Total            | 36       | 77           | 123   |

## Results (continued):

### Evaluation of Microarray Detection Platform

- Low-density microarrays on glass substrate were produced by Microarrays Inc. (Huntsville, AL) using proprietary contact printing.
- Detection oligonucleotides used in initial panel development (see part 1) were spotted in triplicate per subarray.
- TEM-PCR™ products from GBS panel were hybridized to detection-specific oligonucleotides on microarrays.
- Streptavidin-phycoerythrin conjugate (SA-PE) was added to detect positive signal.
- Microarray plates were analyzed on a Fluorescent Array Imaging Reader (FLAIR; Sensovation, Germany) (Figure 5).

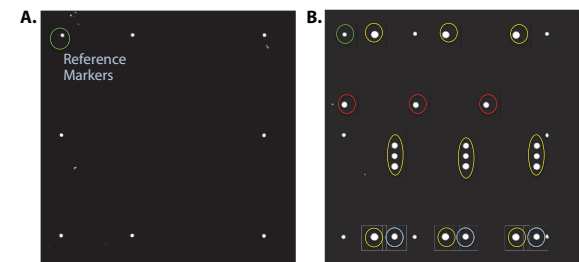


Figure 5. GBS detection on microarray platform.

Representative subarrays of GBS negative control (A) and GBS 'Detected' (B) samples. Reference markers (green); Internal control (yellow); GBS *pcsB* target (red); GBS *sip* target (blue).

Table 3. Clinical comparison of Luminex vs. Microarray platforms for GBS detection.

Clinical samples submitted to Diatherix underwent TEM-PCR™ and were simultaneously evaluated on the Luminex® and microarray detection platforms.

|                  | Microarray |              |       |
|------------------|------------|--------------|-------|
|                  | Detected   | Not Detected | Total |
| Luminex Positive | 36         | 0            | 36    |
| Luminex Negative | 0          | 101          | 101   |
| Total            | 36         | 101          | 137   |

### Evaluation of Bead-Based Detection Platform

- Detection oligonucleotides were coupled to carboxylated, fluorescent microspheres from selected manufacturers (Table 4).
- Positive coupling controls from the GBS panel were hybridized to coupled particles (beads).
- SA-PE was added to detect positive signal.
- Beads were analyzed on an IncellDx Goldfish flow cytometer (Menlo Park, CA) (Figure 6).
- Beads were selected for further evaluation based on bead purity and coupling efficiency (Figure 6A-B).

Table 4. List of fluorescent particles used in evaluation.

| Manufacturer            | Microparticle Name                  | Fluorescence | Bead Size |
|-------------------------|-------------------------------------|--------------|-----------|
| Thermo Scientific       | Cyto-Plex™ Firefly™                 | Red          | 5.4µm     |
| Spherotech Inc.         | SPHERO™ Carboxyl Particle Array Kit | Blue         | 6.0-6.9µm |
| Spherotech Inc.         | SPHERO™ Carboxyl Particle Array Kit | Blue         | 5.0-5.9µm |
| Spherotech Inc.         | SPHERO™ Carboxyl Particle Array Kit | Red          | 6.0-6.9µm |
| Phosphorex Inc.         | Fluorophorex™                       | Green        | 4.2µm     |
| Phosphorex Inc.         | Fluorophorex™                       | Green        | 5µm       |
| Bangs Laboratories, Inc | QuantumPlex                         | Red          | 5.5µm     |

## Results (continued):

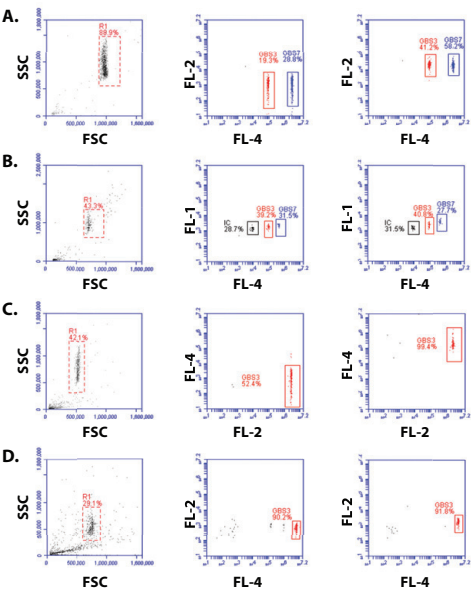


Figure 6. GBS detection on bead-based platform.

Representative scatter plots of microparticles from Thermo Scientific (A); Spherotech Inc. (B); Phosphorex Inc. (C); and Bangs Laboratories (D). The left column illustrates particles in suspension with the R1 population representing the bead subset of interest. Representative negative controls (middle column) and GBS 'Detected' (right column) samples for each microparticle set were examined with appropriate filters to detect phycoerythrin, AlexaFluor 647 and bead fluorescence. FL-1 (533±15 nm); FL-2 (585±20 nm); FL-4 (675±12.5 nm)

## Conclusions:

- Usage of two independent gene targets allows for increased sensitivity and confirmation that negative results are not due to a strain genetic variation.
- The limit of detection for the GBS panel is < 100 cfu/mL, post-enrichment.
- The panel yielded 100% analytical specificity and sensitivity with all reference strains tested.
- TEM-PCR™ GBS panel produced no false-negative results compared with culture.
- TEM-PCR™ GBS panel confirmed 12 positive culture results and identified 34 additional positive results. The identity of additional GBS positive results by PCR will be resolved by sequencing.
- The microarray platform has a significantly increased signal:noise ratio compared to the Luminex platform (data not shown).
- 100% concordance of clinical results observed between the Luminex® and microarray platforms.
- Microparticles from Phosphorex Inc. and Bangs Laboratories showed increased artifacts within the bead suspensions and were not used for further testing.
- Thermo Scientific and Spherotech Inc. microparticle suspensions provided more uniform bead populations with low levels of artifacts.
- The IncellDx platform provided a shorter sample read-time in comparison to the Luminex or microarray platforms (data not shown).
- The TEM-PCR™ GBS panel provides a rapid, sensitive and specific method for GBS detection directly from clinical samples.
- The GBS panel provides higher sensitivity than the current 'gold standard' culture methods.
- This TEM-PCR™ GBS panel can be adapted on different detection systems without compromising panel performance specifications.

#### Reference:

<sup>1</sup> Centers for Disease Control and Prevention. Group B Strep (GBS). <http://www.cdc.gov/groupbstrep/index.html>

#### Acknowledgments:

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