Rapid Detection of *Streptococcus pyogenes* Using the Simplexa® Group A Strep Direct Assay

Heather Gregson, Cindy Cheng, Brian Yu, Ishfaq Maksud, Louis Geller and Michelle Tabb

Focus Diagnostics, Inc., Cypress, CA

***Corresponding author: heatherg@focusdx.com**

Revised Abstract

Introduction

Group A streptococcus (*S pyogenes*) infection can result in serious sequelae if not treated; thus a quick and accurate diagnosis is important. Traditional antigen detection tests have limited sensitivity and require additional confirmatory culture testing to reduce the risk of false negative results. Culture testing typically takes 18 to 24 hours, which may cause treatment delay. In an effort to provide an alternative solution for *S pyogenes* detection, we are developing a real-time PCR assay that detects Group A streptococci (GAS) directly from throat swabs in approximately 1 hour. In this study, we compared the performance of the Simplexa assay to culture testing, evaluated the analytical properties of the assay, and tested the specimen stability.

Methods

The Simplexa Group A Strep Direct Assay (Simplexa assay) targets the conserved exotoxin B gene of *S. pyogenes*. For each assay, 50 µL of patient throat swab and 50 µL of GAS Direct Reaction Mix were added to their respective wells on the Direct Amplification Disc; amplification in the 3M Integrated Cycler followed. Assay results were compared with culture results. Discrepant results were resolved by sequencing a region of the exotoxin B gene that is different from the one targeted by the Simplexa assay. Analytical studies included limit of detection (LoD) for M1, M3, and 21 GAS strains and inter- and intra-assay reproducibility. Viability and detection of *S pyogenes* in the ESwab™ transport system was also evaluated.

Results

Compared to culture, the Simplexa assay agreed in 97.4% of positive results and 95.2% of negative results. The LoD of the Simplexa assay was 6.8 CFU/reaction for the M1 strain and 23.5 CFU/reaction for the M3 strain. Sixty GAS strains were assessed for analytical reactivity. Twenty-one GAS strains were tested at levels near the LoD and were detected by the assay. Additionally, using in silico methods, 39 GAS strains were shown to be detected by the Simplexa assay. The Simplexa assay did not cross react with any of the 62 common pathogens tested, nor did the same pathogens cause inhibition of the assay. No interference was observed with any of the 29 potential interfering substances tested. Inter-site, inter-day, and inter/ intra-assay reproducibility studies yielded ≤5.6% coefficient of variation.

Conclusions

Compared to culture, the Simplexa Group A Strep Direct assay provides quick turn-around time without sacrificing performance and without the need for nucleic acid extraction. The Simplexa Group A Strep Direct Assay is FDA cleared.

Method

Patient Specimen Panel: Prospective: 1352 throat swabs were collected using the ESwab Transport System (Becton Dickinson).. The samples were de-identified, cultured for the presence of group A streptococcus (GAS) and evaluated with the Simplexa assay. Of the positive samples, 91 were used to test stability after 2 cycles of freezing and thawing. Retrospective: 655 throat swabs were collected using the ESwab Transport System. The samples were de-identified, cultured for the presence of group A streptococcus (GAS) and stored in -70C for 3.5 to 4 years before being evaluated with the Simplexa assay.

Simplexa Direct Assay: Sample setup and testing were performed as outlined in Figure 1.

Discrepant Analysis: A SYBR Green-based PCR assay was developed to analyze samples giving discrepant results in the Simplexa Direct assay and culture. Specimens were initially heated at 97ºC for 8 minutes, and 5 µL of sample was then amplified using iTaq™ SYBR Green SuperMix With ROX (Bio-Rad). The amplicons were then sequenced bi-directionally, and the results were compared to available sequences using the Basic Local Alignment Search Tool (BLAST).

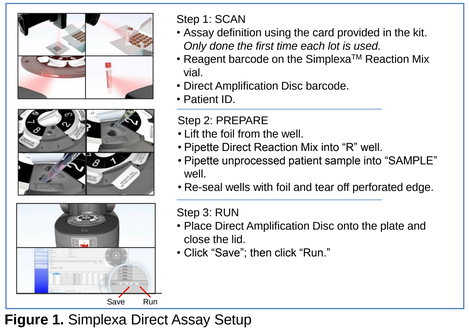

Figure 1. Simplexa Direct Assay Setup

Limit of Detection (LoD): GAS M1 (ATCC# 700294) and M3 (ATCC # BAA-595) strains (American Type Culture Collection [ATCC], Manassas, VA) were used. For each serotype, 8 different concentrations were spiked in simulated matrix from the verified bacterial stock material and confirmed using 32 replicates. The LoD was defined as the lowest concentration at which at least 95% of replicates were detected.

Analytical Reactivity: Twenty-one GAS strains from Zeptometrix (Buffalo, NY) and ATCC were diluted to 2 times the LoD and tested with the Simplexa Direct assay. An additional 39 strains were tested using in silico NCBI BLAST sequence, analysis separate from the wet test strains.

Interference: PCR amplification was performed on a panel of low positive (1X LoD) and moderately positive (3X LoD) concentration samples in the presence of potential interfering substances, including blood, mucin, antibiotics, throat lozenges, nasal sprays, cold and flu medication and pain medications.

Cross Reactivity: A panel of 64 organisms were obtained from ZeptoMetrix and ATCC for cross-reactivity testing. Bacteria were diluted to a concentration of 10^6 CFU/mL and viruses were diluted to 10^5 $TCID_{50}$/mL before being tested on the Simplexa Direct assay.

Method (cont.)

Reproducibility: Low-positive (1X LoD) and moderately-positive (3X LoD) samples were prepared using the M1 and M3 stocks in negative matrix. The study was conducted in triplicate with 6 operators running triplicates (3) for 5 days.

Viability study: M1 strain organisms were diluted into Eswab Transport System at approximately 10^6 cells/mL. Aliquots were run directly on the Simplexa assay and dilutions were plated on to blood agar plates after 0, 1, 2, 3, and 6 days after storage at 2-8˚ C. Colony counts were performed after 24 to 48 hours of growth.

Results

Comparison of Simplexa Direct Assay with Culture Prospective Samples: The Simplexa assay showed 97.4% positive agreement and 95.2% negative agreement with culture (Table 1). Of 1352 specimens, 61 samples were discrepant. Of the 61 discrepant samples, 57 were culture negative and 4 were culture positive. Bidirectional sequencing confirmed that 46 of 57 were GAS positive, 9 of 57 were GAS negative, and 2 were indeterminate. Among the 4 culture positive specimens, 2 were confirmed GAS positive and 2 were GAS negative via sequencing.

Table 1. Simplexa Direct Assay Comparison to Culture

Prospective		Culture: Group A Strep			Percent Agreement
		Detected	Not Detected	Total	
Simplexa Group A Strep Direct	Detected	152	57	209	Sensitivity 97.4% (152/156)
	Not Detected	4	1139	1143	Specificity 95.2% (1139/1196)
	Total	156	1196	1352	

Comparison of Simplexa Direct Assay with Culture Retrospective Samples: The Simplexa assay showed 97.1% positive agreement and 91.5% negative agreement with culture (Table 2). Of 655 specimens, 50 samples were discrepant. Of the 50 discrepant samples, 47 were culture negative and 3 were culture positive. Bidirectional sequencing confirmed that 33 of 47 were GAS positive, 13 of 57 were GAS negative, and 1 was indeterminate. Among the 3 culture positive specimens, 3 were GAS negative via sequencing.

Table 2. Simplexa Direct Assay Comparison to Culture

Retrospective		Culture: Group A Strep			Percent Agreement
		Detected	Not Detected	Total	
Simplexa Group A Strep Direct	Detected	99	47	146	Sensitivity 97.1% (99/102)
	Not Detected	3	506	509	Specificity 91.5% (506/553)
	Total	102	553	655	

Results (cont.)

Limit of Detection: The LoD was determined to be 682 CFU/mL for M1 and 2350 CFU/mL for M3 strains (Table 3).

Table 3. Simplexa Direct Assay Limit of Detection

GAS Strain	Concentration (CFU/mL)
M1	682
M3	2350

Analytical Reactivity: The Simplexa Direct assay detected all 21 of the tested GAS strains at or near LoD concentrations (Table 4). In silico testing of 39 distinct GAS strains also confirmed detection by the Simplexa assay.

Table 4. Simplexa Direct Assay Analytical Reactivity

21 GAS Strains evaluated at concentrations between 1.50 X 10^3 And 5.00 X 10^3 CFU/mL. Detection was observed in 100% of replicates			
M2	M12	M27	M75
M4	M13	M28	M77
M5	M14	M29	M78
M6	M18	M49	M82
M9	M22	M73	M87
			M89

Cross Reactivity: The Simplexa Direct assay did not cross-react with any of the 64 tested organisms.

Interference: Detection was not inhibited by the tested substances, which were present at relevant concentrations.

Reproducibility: The coefficient of variation (CV) value of total variability, including between-instrument, between-operator, between-run, and within-run reproducibility samples, was ≤ 5.6%.

Viability study: The Simplexa Direct assay returned consistent Ct values over the course of study. As shown by the colony counts the M1 organism remained viable for the 7 day study in Eswab Transport System (Table 6).

Table 6. Simplexa Direct Assay Analytical Reactivity

Days of storage at 2-8°C	M1 diluted to 10^6 CFU/ml	colony counts	
		diluted to 100 colonies/mL	diluted to 1000 colonies/mL
	Average GAS Ct	average CFU/mL	average CFU/mL
0	28.7	85	565
1	27.9	100	1060
2	28.0	175	1675
3	28.4	165	1470
6			
7		data to be added by thursday	

Results (cont.)

Fresh and Frozen stability: A total of 91 prospectively collected Group A *Streptococcus* positive samples were included in the study. 92.3% of the samples were detected in both the fresh and frozen samples. (Table 7). Of the 7 discrepant samples, 5 samples had Ct values above 40.2 on the fresh result indicating these are low positive samples. The 2 remaining discrepant samples (2.2%) had Ct values of 33.0 and 32.2 on the fresh result. After freeze/thaw cycles the same samples returned elevated IC (internal control) Ct suggesting that there was some inhibition with these samples after the freeze and thaw cycles. There is minimal effect of freezing samples however this result is within the performance characteristics of the assay.

Table 7. Simplexa Direct Assay Agreement Fresh and Frozen

Agreement Between Fresh and Frozen Results	
Frozen Simplexa™ Group A Strep Direct Results	Fresh Simplexa™ Group A Strep Direct Results
	Detected
Detected	92.3% (84/91) 95% CI: 85.0% to 96.2%
Not Detected	7.7% (7/91) 95% CI: 3.8% to 15.0%
Total	91

Conclusions

- The Simplexa Direct Assay had 95.2% negative agreement and 97.4% positive agreement on prospective samples when compared to culture.
- The Simplexa Direct Assay had 91.5% negative agreement and 97.1% positive agreement on retrospective samples when compared to culture.
- The LoDs of the Simplexa Direct assay were 6.82 CFU per reaction for M1 strain and 23.5 CFU per reaction for M3 strain.
- All analytical reactivity strains were detected by the Simplexa Direct assay.
- No cross-reactivity with common pathogens or interference by tested substances was observed.
- The CV of the Simplexa Direct assay was ≤5.6% in reproducibility tests.
- M1 strain was viable and returned consistent Ct values on the Simplexa Group A Strep direct Assay when stored up to 6 days at 2-8˚ C in Eswab Transport System.
- There is minimal effect of Group A Strep detection after freezing and thawing cycles.
- The Simplexa Group A Strep Direct assay is FDA cleared with a moderate complexity rating.

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