

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

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Revised Abstract

Introduction: Group A streptococcus (*Streptococcus 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 developed 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 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 Simplexa Reaction Mix were added to their respective wells on the Direct Amplification Disc; amplification in the 3M Integrated Cycler followed. Assay results were compared to 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 were evaluated. Performance of the Simplexa assay for fresh versus frozen specimens was also evaluated.

Results: For prospective specimens, the Simplexa assay agreed with culture in 97.4% of positive results and 95.2% of negative results. For frozen, retrospective specimens, the Simplexa assay agreed in 97.1% of positive results and 91.5% 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 and were detected. 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. Samples in Eswab were stable for at least 7 days at 2°C to 8°C and after 2 cycles of freezing and thawing.

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.

Methods

Patient Specimen Panel: Prospective: 1,352 throat swabs were collected using the ESwab Transport System (Becton Dickinson). The specimens were de-identified, cultured for the presence of group A streptococcus (GAS) and evaluated with the Simplexa assay. Of the positive specimens, 91 were used to test stability after 2 cycles of freezing and thawing. Retrospective: 655 throat swabs were collected

Methods (cont.)

using the ESwab Transport System. The specimens were de-identified, cultured for the presence of GAS and stored in -70°C 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.

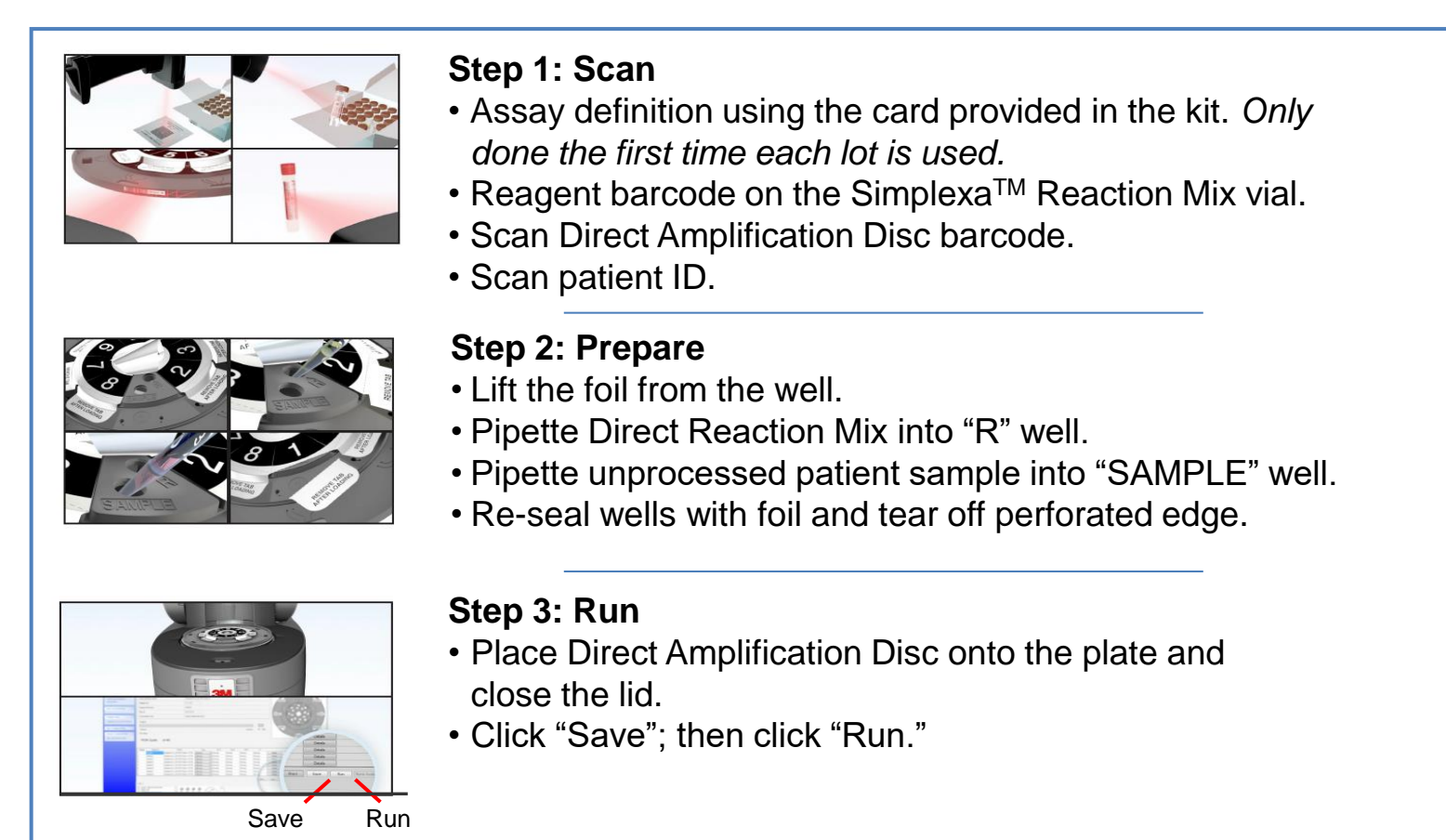


Figure 1. Simplexa Direct Assay Setup

Discrepant Analysis: A SYBR Green-based PCR assay was developed for sequence analysis of discrepant results from the Simplexa Direct assay and culture. Specimens were initially heated at 97°C for 8 minutes, and 5 µL of specimen was then amplified using iTaq[™] SYBR Green SuperMix With ROX (Bio-Rad, Hercules CA). The amplicons were then sequenced bi-directionally, and the results were compared to available sequences using the Basic Local Alignment Search Tool (BLAST).

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 Zeptomatrix (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 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 62 organisms was obtained from ZeptoMatrix and ATCC for cross-reactivity testing. Bacteria were diluted to a concentration of 10⁶ CFU/mL and viruses were diluted to 10⁵ TCID₅₀/mL before being tested on the Simplexa Direct assay.

Methods (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 for 5 days.

Viability study: M1 strain organisms were diluted into Eswab Transport System to approximately 10⁶ cells/mL. Aliquots were run directly on the Simplexa assay and dilutions were plated onto blood agar plates after 0, 1, 2, 3, 6, and 7 days after storage at 2°C to 8°C. Colony counts were performed after 18 to 48 hours of growth.

Results

Comparison of Simplexa Direct Assay with Culture, Prospective Specimens: The Simplexa assay showed 97.4% positive agreement and 95.2% negative agreement with culture (Table 1).

Table 1. Simplexa Direct Assay Comparison to Culture

Prospective Specimens	Culture: Group A Strep			Percent Agreement	
	Detected	Not Detected	Total		
Simplexa Group A Strep Direct	Detected	152	57 ^a	209	Detected 97.4% (152/156)
	Not Detected	4 ^b	1,139	1,143	Not Detected 95.2% (1,139/1,196)
	Total	156	1,196	1,352	

^a Of 57 specimens that were culture-negative, bidirectional sequencing confirmed 46 as GAS-positive and 9 as GAS-negative; 2 were indeterminate.

^b Of 4 specimens that were culture-positive, bidirectional sequencing confirmed 2 as GAS-positive and 2 as GAS-negative.

Comparison of Simplexa Direct Assay with Culture, Retrospective Specimens: The Simplexa assay showed 97.1% positive agreement and 91.5% negative agreement with culture (Table 2).

Table 2. Simplexa Direct Assay Comparison to Culture

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

^a Of 47 specimens that were culture-negative, bidirectional sequencing confirmed 33 as GAS-positive and 13 as GAS-negative; 1 was indeterminate.

^b All 3 specimens that were culture-positive were GAS-negative via sequencing.

Results (cont.)

Limit of Detection: The LoD was determined to be 682 CFU/mL for M1 and 2,350 CFU/mL for M3 strains.

Analytical Reactivity: The Simplexa Direct assay detected all 21 of the tested GAS strains at or near LoD concentrations (Table 3). In silico testing of 39 distinct GAS strains also confirmed detection by the Simplexa assay.

Table 3. 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	M13	M28	M77
M4	M14	M29	M78
M5	M18	M49	M82
M6	M22	M73	M87
M9	M27	M75	M89
M12			

Cross Reactivity: The Simplexa Direct assay did not cross-react with any of the 62 tested organisms.

Interference: Detection was not inhibited by the 29 tested substances.

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 the study as shown by the colony counts. The M1 organism remained viable for the 7 day study in Eswab Transport System (Table 4).

Table 4. Viability in Eswab and detection by Simplexa Direct Assay

Days of Storage at 2-8°C	M1 Diluted to 10 ⁶ CFU/ml Average GAS Ct	Colony Counts	
		Diluted to 100 colonies/mL Average CFU/mL	Diluted to 1000 colonies/mL Average CFU/mL
0	28.7	85	565
1	27.9	100	1,060
2	28.0	175	1,675
3	28.4	165	1,470
6	27.7	105	730
7	28.2	80	1,220

Results (cont.)

Fresh and Frozen Stability: Ninety-one prospectively collected GAS-positive specimens were included in the study. Eighty-four (92.3%) were detected in both the fresh and frozen specimens (Table 5).

Table 5. 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) ^a
	95% CI: 3.8% to 15.0%
Total	91

^a Of the 7 discrepant specimens, 5 had Ct values above 40.2 on the fresh result indicating these are low-positive specimens; 2 (2.2%) had Ct values of 33.0 and 32.2 on the fresh result. After freeze/thaw cycles, the same specimens returned elevated internal control Ct, suggesting that there was some inhibition with these specimens after the freeze/thaw cycles.

Conclusions

- For prospective specimens, the Simplexa Direct Assay had 97.4% positive agreement and 95.2% negative agreement compared to culture.
- For retrospective specimens, the Simplexa Direct assay had 97.1% positive agreement and 91.5% negative agreement compared to culture.
- The LoD of the Simplexa Direct assay was 6.82 CFU/reaction for M1 strain and 23.5 CFU/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 Direct assay when stored up to 6 days at 2°C to 8°C in Eswab Transport System.
- There was minimal effect on GAS detection after freezing and thawing cycles.
- The Simplexa Group A Strep Direct assay is FDA cleared with a moderate complexity CLIA rating.