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

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Revised Abstract

Introduction: Group A streptococcus (GAS) is human pathogen that can cause serious sequelae if not treated, so a quick and accurate diagnosis is important. GAS in throat swabs is commonly detected by rapid antigen tests and/or conventional culture. Rapid antigen tests have good specificity but limited sensitivity, which requires follow-up testing with culture. Culture provides good sensitivity, but turnaround is about a day. To provide an alternative to both methods, we developed a qualitative real-time PCR assay that detects GAS directly from throat swabs in about an hour.

Methods: The Simplexa® Group A Strep Direct Assay (Simplexa assay) targets the conserved exotoxin B gene of *S. Pyogenes* and does not require extraction. 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. The target was amplified and signal was detected in the 3M Integrated Cycler. Performance of the Simplexa assay on patient samples was compared with culture and a rapid antigen test. Discrepant results were resolved by sequencing a region of the exotoxin B gene different from the one targeted by the Simplexa assay. Limit of detection (LoD), analytical reactivity, interference, cross reactivity and reproducibility studies were performed.

Results: Compared to culture, the Simplexa assay returned a 97.0% and 96.3% agreement between positive and negative samples, respectively. When compared to the rapid antigen test, 5 discrepant samples that were Simplexa-negative but rapid antigen test-positive were identified. After performing sequencing, the discrepant samples were confirmed as GAS negative (rapid test false positive). The LoD of the Simplexa assay was 10 CFU/reaction for M1 strain and 50 CFU/reaction for M3 strain. The 21 GAS strains tested were detected at levels near LoD. The Simplexa assay did not cross react with any of the 62 common pathogens tested, and no interference was observed with any of the 29 potential interfering substances tested. Inter- and intra-assay reproducibility assays yielded ≤2.9% coefficient of variation.

Conclusions: Compared to culture and rapid antigen testing, 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 in development; it is not currently for sale and is not FDA approved.

Method

Patient Specimen Preparation: Two unique patient sample panels were collected for method comparison studies. All samples were collected from subjects with presumptive GAS using the ESwab Transport System (Becton Dickinson); samples were de-identified prior to Simplexa testing. The first panel, containing 87 samples, was cultured for the presence of GAS. A different panel of 50 GAS samples that were identified as positive by Sure-Vue Rapid Test was tested 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 were 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).

Method (cont.)

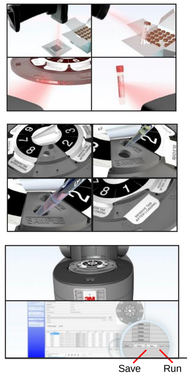

Figure 1. Simplexa Direct Assay Setup

Limit of Detection (LoD): GAS M1 (ATCC# 700294) and M3 (ATCC # BAA-595) strains (American Type Culture Collection [ATCC], Manassas, VA) were serially diluted in negative swab matrix. Twenty replicates of dilutions with concentrations at or near the presumptive LoD were amplified. The LoD was defined as the lowest concentration at which at least 95% of replicates were detected.

Analytical Reactivity (AR): Twenty-one *S. pyogenes* strains obtained from Zeptometrix (Buffalo, New York) and ATCC were diluted to a concentration of 2 times the LoD and tested on the Simplexa Direct assay.

Interference: PCR amplification was performed on a panel of GAS low-positive (3X M1 LoD) samples in the presence of potential interfering substances. These substances included blood, mucin, antibiotics, throat lozenges, nasal sprays, cold and flu medications, and pain medications.

Cross Reactivity (CR): Various organisms were obtained from ZeptoMetrix (Buffalo, New York) and ATCC for testing cross-reactivity. 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.

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

Results

Comparison of Simplexa Direct Assay with Culture: The Simplexa assay showed 97.0% positive agreement and 96.3% negative agreement with culture (Table 1). Of 87 specimens, 52 were negative in both the Simplexa assay and culture, 32 samples were positive in both the Simplexa assay and culture, and 3 samples were discrepant. Of the 3 discrepant samples, 2 historically culture-negative samples were Simplexa-positive. Sequencing confirmed that the 2 samples were GAS positive. The 1 remaining discrepant specimen was historically culture-positive but Simplexa-negative. Initial sequencing showed that this particular sample was negative but repeat sequencing showed that it was positive; this inconsistent detection suggests that the sample was borderline positive.

Results (cont.)

Table 1. Simplexa Direct Assay to Culture

		Culture			Percent Agreement
		GAS-Positive	GAS-Negative	Total	
Simplexa Direct	GAS-Positive	32	2*	34	Positive Agreement 97.0% (32/33)
	GAS-Negative	1*	52	53	Negative Agreement 96.3% (52/54)
	Total	33	54	87	

*Sequencing confirmed all discrepant samples as GAS-positive

Comparison of Simplexa Direct Assay with Rapid Antigen Test: Of 50 samples that were positive using the rapid antigen test, 45 samples were positive when using the Simplexa assay (Table 2). The 5 discrepant samples tested negative when using the Simplexa Direct assay. The samples were evaluated by sequencing, which confirmed that all 5 were negative for GAS. All other GAS specimens matched the expected positive result in the rapid antigen test. This result suggests that the 5 discrepant samples may be false positive on the Sure-Vue Rapid Test. No negative rapid antigen specimens were available for comparison to the Simplexa assay.

Table 2. Simplexa Direct Assay to Rapid Tests

		Rapid Antigen Test	Percent Agreement
		GAS-Positive	
Simplexa Direct	GAS-Positive	45	Positive Agreement 90% (45/50)
	GAS-Negative	5*	
	Total	50	

*Sequencing confirmed all discrepant samples as GAS-negative

LoD: The Simplexa Direct assay LoD was 10 CFU per reaction for M1 strain (average Ct = 37.8) and 50 CFU per reaction for M3 strain (average Ct = 37.4). For each strain, GAS was detected in 20 out of 20 replicates.

Analytical Reactivity: The Simplexa Direct assay detected all of the tested *S. pyogenes* strains at 2X M1 LoD (2,000 CFU/mL) concentrations. The following 21 *S. pyogenes* strains were tested: M2, M4, M5, M6, M9, M12, M13, M14, M18, M22, M27, M28, M29, M49, M73, M75, M77, M78, M82, M87, and M89.

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

Results (cont.)

Cross Reactivity: The Simplexa Direct assay did not cross-react with any of the tested organisms (Table 3).

Table 3. Organisms Tested in the Simplexa Direct Assay

Organisms		
Adenovirus 1	*Lactobacillus acidophilus*	*Streptococcus dysgalactiae*
Adenovirus 7A	*Legionella pneumophila*	*Streptococcus equinus*
Arcanobacterium haemolyticum	Metapneumovirus-9	*Streptococcus gallolyticus*
Bacillus cereus	*Moraxella catarrhalis*	*Streptococcus gordonii*
Bacteroides ovatus	*Mycoplasma pneumoniae*	*Streptococcus intermedius*
Bordetella pertussis	*Neisseria gonorrhoeae*	*Streptococcus mitis*
Burkholderia cepacia	*Neisseria meningitidis*	*Streptococcus mutans*
Campylobacter rectus	Parainfluenza 1	*Streptococcus oralis*
Candida albicans	Parainfluenza 2	*Streptococcus parasanguinis*
Coronavirus	*Peptostreptococcus micros*	*Streptococcus pneumoniae*
Corynebacterium diptheriae	Parainfluenza 3	*Streptococcus sanguinis*
Cytomegalovirus	*Pseudomonas aeruginosa*	*Streptococcus sobrinus*
Enterococcus faecalis vanB	Rhinovirus 1A	*Streptococcus uberis*
Enterovirus 71	RSV A2	*Streptococcus vestibularis*
Epstein Barr Virus	*Staphylococcus aureus* (MRSA)	*Streptococcus zooepidemicus*
Escherichia coli		*Treponema denticola*
Fusobacterium necrophorum	*Staphylococcus epidermidi*	*Veillonella parvula*
Haemophilus influenzae	*Streptococcus agalactiae*	*Tremella fuciformis*
Influenza A	*Streptococcus anginosus*	*Strentophomonas maltophilia*
Influenza B	*Streptococcus canis*	*Streptococcus constellatus*
Klebsiella pneumoniae	*Streptococcus cristatus*	HSV-1

Reproducibility: The coefficient of variation (CV) value of total variability, including between-instrument, between-operator, between-run, and within-run reproducibility samples, was ≤2.9%.

Conclusions

- The Simplexa Direct assay had >95% positive and negative agreement with conventional culture assay.
- Five samples were GAS-positive when using the rapid antigen test but GAS-negative when using the Simplexa Direct assay. These discrepant samples were confirmed as GAS-negative by sequencing, which suggests that false positivity may be observed when using the Sure-Vue Rapid test.
- LoDs of the Simplexa Direct assay were 10 CFU per reaction for M1 strain and 50 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 ≤ 2.9%.
- Simplexa Group A Strep Direct assay is in development; it is not currently available for sale and is not FDA cleared.

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