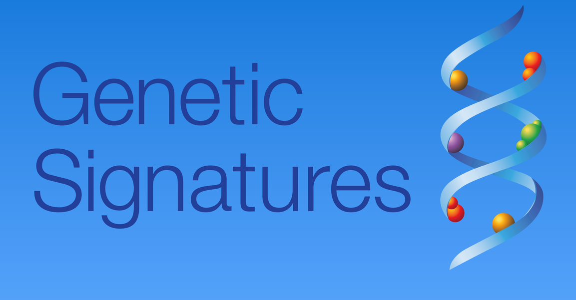
A novel rapid multiplex PCR assay for the detection of 13 bacterial and viral causes of sexually transmitted infection (STI).

John R Melki, Shoo Peng Siah, Kiran Kaur, Jiny Nair, William Rawlinson* and Douglas S Millar

Genetic Signatures, Sydney, Australia, *Virology Division, SEALS Microbiology, Prince of Wales Hospital, Randwick, NSW, Australia.



Abstract

It has been estimated that around 78-300 million new cases of genitourinary tract infections occur annually worldwide. *Neisseria gonorrhoea* and *Chlamydia trachomatis* are considered the most common causes of genital tract infections however other species such as *Mycoplasma* spp., *Trichomonas vaginalis* and *Ureaplasma* spp. are also agents of disease. Furthermore, novel markers have been identified that can improve the specificity for the detection of bacterial vaginosis (1, 2). We have developed a novel technology that has been clinically validated for the detection of HPV (3) and more recently multiplexed PCR panels for the detection of a wide range of Gastrointestinal pathogens (4). Our aims were to produce a rapid multiplex real time assay that could detect the presence of 13 causative agents of STI (see Table 1) in less than 3 hours from primary patient material. The multiplex PCR panels were designed to correspond to clinical symptom groups thus the requesting physician could select the most appropriate panel for the presenting symptoms.

Preclinical Studies

Table 1 Components of the *EasyScreen*™ STI Panels

Panel A	Panel B	Panel C	Panel D
N. gonorrhoeae	*T. vaginalis*	*M. genitalium*	*T. pallidum*
ß-Actin (Control)	ß-Actin (Control)	*C.albicans/galbrata*	ß-Actin (Control)
C. trachomatis	*M. hominis*	BVAB2*	HSV-1
LGV	*Ureaplasma* spp.	Megasphera*	HSV-2

* For the detection of bacterial vaginosis

To assess the sensitivity and specificity of the panels 110bp oligonucleotides were designed containing the identical target region as each of the organisms listed. The oligonucleotides were quantified by Nanodrop™, then diluted to a final concentration of 10^6 copies/µl. The samples were added to the *EasyScreen*™ lysis buffer, heated for 15 minutes, then purified by isopropanol precipitation. Serial dilutions were performed down to 12.5 copies and amplified in the multiplex reaction (see figure 2a). In addition Table 2 shows a list of DNA and whole bacteria that were used for sensitivity and specificity evaluation.

Table 2 Strains and DNA samples used in the study

	Strains/DNA's Sourced	Commercial Source
Panel A	*C. trachomatis*	Zeptometrix CATALOG# 0801482
	C. trachomatis	Serovar E ATCC® VR-348BD
	C. trachomatis	Strain UW-3/Cx ATCC® VR-885D
	N. gonorrhoeae	Zeptometrix CATALOG# 0801850
	N. gonorrhoeae	ATCC® 53424D
	CT/NG verification panel	Zeptometrix CATALOG# NATCT/NGP-C
Panel B	*T. vaginalis*	ATCC® 30001D
	M. hominis	ATCC® 23114D
	M. hyopneumoniae	ATCC® 25934D
	M. hyorhinis	ATCC® 17981D
	M. salivarium	ATCC® 23064D
	M. fermentans	ATCC® 19989D
	Ureaplasma parvum	ATCC® 27815D
Panel C	*Candida albicans*	ATCC® MYA-2876D-5
	Candida glabrata	ATCC® 15545D-5
	Candida kefyr	ATCC® 66028D-5
	Candida parapsilosis	ATCC® 22019D-5™
	Candida tropicalis	ATCC® 66029D-5
	Mycoplasma genitalium	ATCC® 33530D™
Panel D	HSV-1 viral particles	Zeptometrix CATALOG# NATHSV2-0004
	HSV-1 strain McIntyre	ATCC® VR-539D
	HSV-2 viral paticles	Zeptometrix CATALOG# NATHSV1-0005
	HSV-2 strain G	ATCC® VR-734D

Sensitivity & Specificity

The Genetic Signatures sample processing method converts the native nucleic acids from a 4 base code to a 3base™ form. The conversion process simplifies the design of multiplex PCR reactions by eliminating the large Tm differences that can be present when targeting multiple pathogens (see Figure 1). All assay components demonstrated a sensitivity of <10 copies when used in the final multiplex reaction. No inter-panel cross reactivity or cross reactivity with a wide range of non-target organisms at an input of 10^5/copies per PCR reaction was observed.

Conventional Sequence		Tm	3base™ Sequence	Tm
Primer1	GTACACACCGCCCGTCGCTCCTACC	77°C	GTATATATTGTTTGTTGTTTTATT	52 °C
Primer2	GAAGGAGAAGTCGTAACAAG	56 °C	GAAGGAGAAGTTGTAATAAG	50°C
Probe1	TGAATAAAGAGGTGAAATTCTAGG	59 °C	TGAATAAAGAGGTGAAATTTTAGG	59 °C
Probe2	GAAGGGCCGCGAGCCCCCGCGC	87 °C	GAAGGGTTGTGAGTTTTTGTGT	62 °C

Figure 1 The DNA sequence for 2 primers and probes before and after the modification approach

Materials & Methods

Previously characterised CT/NG urine samples (1ml aliquots) and swab samples collected in COBAS sample preparation buffer (1ml) were spun at 13,000rpm for 5 minutes to pellet intact organisms and the pellets resuspended in 250µl of *EasyScreen*™ lysis buffer. The samples were heated at 95°C for 15 minutes and purified as below. Fifty microliters of purified DNA extracts from samples previously tested for HSV were also included in the study. The samples were heated for 15 minutes at 95°C and purified using the EZ1 (Qiagen, Hilden, Germany) or Kingfisher Flex (Thermofisher, Waltham) according to the protocols supplied (Genetic Signatures, Sydney, Australia). Real time PCR analysis can be performed on most common hardware such as the LC480 (Roche, Pleasanton, USA), 7500 fast (Applied Biosystems, Foster City, USA), Rotorgene-Q (Qiagen, Hilden, Germany), the Smartcycler II (Cepheid, Sunnyvale, USA) and the CFX96 (Bio-Rad, Hercules, USA).

Results

The *Easyscreen*™ assays successfully detected all CT/NG samples from a commercially available validation panel (Zeptometrix). Using clinical samples 21/22 *C. trachomatis* positive samples were detected along with 22/24 *N. gonorrhoea*. It is expected that increasing the volume of urine tested would improve sensitivity to 100% while swab samples should be placed directly into GS sample buffer rather than into the COBAS reagent. All 20 HSV samples were detected using the assay. Interestingly HSV-1/2 co-infections were observed in 5/20 patients (25%). Most swab samples and a number of urine samples tested positive for the two markers of bacterial vaginitis thus further studies will be conducted to determine a suitable cut-off for reliable diagnosis of vaginitis. One CT positive sample was determined to be a LGV strain and one negative sample tested positive for the presence of HSV-1.

Results

C. trachomatis *C. trachomatis* *N. gonorrhoea*

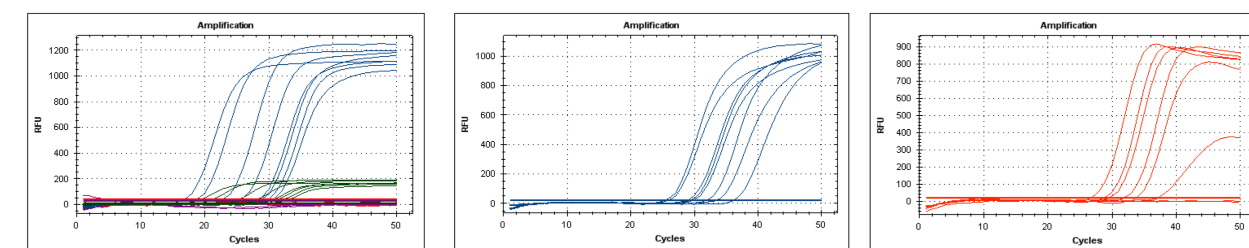


Figure 2a Figure 2b Figure 2c

Panel Member Strain Expected Result	GS *EasyScreen*™ Result
C.trachomatis-High LGVII-434	CT Detected/ NG Not Detected
C.trachomatis-Medium LGVII-434	CT Detected/ NG Not Detected
C.trachomatis-Low LGVII-434	CT Detected/ NG Not Detected
C.trachomatis-High D-UW3	CT Detected/ NG Not Detected
C.trachomatis-Medium D-UW3	CT Detected/ NG Not Detected
C.trachomatis-Low D-UW3	CT Detected/ NG Not Detected
C.trachomatis-High HAR13	CT Detected/ NG Not Detected
C.trachomatis-Medium HAR13	CT Detected/ NG Not Detected
C.trachomatis-Low HAR13	CT Detected/ NG Not Detected
N.gonorrhoeae-High Z017	CT Not Detected/ NG Detected
N.gonorrhoeae-Medium Z017	CT Not Detected/ NG Detected
N.gonorrhoeae-Low Z017	CT Not Detected/ NG Detected
N.gonorrhoeae-High Z001	CT Not Detected/ NG Detected
N.gonorrhoeae-Medium Z001	CT Not Detected/ NG Detected
N.gonorrhoeae-Low Z001	CT Not Detected/ NG Detected
N.cinerea Z104	CT Not Detected/ NG Not Detected
N.flavescens Z061	CT Not Detected/ NG Not Detected

Figure 2a Sensitivity of *C. trachomatis* detection. Figure 2b and 2c results obtained using the GS STI Panel A with the Zeptometrix CT/NG validation Panel.

Clinical Studies

CT/NG *HSV-1/2* *Megasphera/BVAB2*

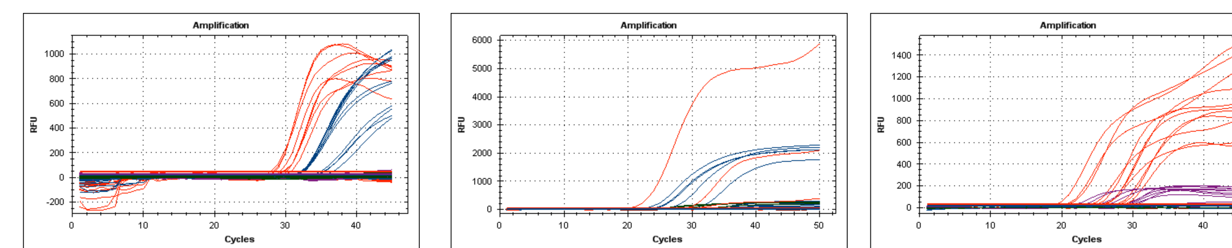


Figure 3a Figure 3b Figure 3c

	GS Positive	Additional Pathogens detected
CT Urine (n=10)	10	
NG Urine (n=12)	11	
CT Swab (n=12)	11	LGV, *Ureaplasma* (3), *M.genetalium*
NG Swab (n=12)	11	*Candida*
HSV Swab (n=20)	20	*Candida*
Negative Urine (n=12)	All CT/NG negative	
Negative Swab (n=12)	All CT/NG negative	HSV-1

Figure 3a. Typical results generated using the STI *EasyScreen*™ Panel A on urine and swab samples containing *C. trachomatis* or *N. gonorrhoea*. Figure 3b. Typical results generated using the STI *EasyScreen*™ Panel D on swab samples containing HSV and 3c. Results obtained using GS STI Panel C on urine and swab samples.

Discussion

The *EasyScreen*™ assays described provide a simple and rapid method that delivers a near complete screen for the presence of STI in less than 3 hours. Sample preparation can be completed on a wide range of automated extraction platforms including the KingFisher Flex (Thermo, Waltham, USA), MagNA™ Pure (Roche, Pleasanton, USA), bioMerieux EasyMag (Marcy l'Etoile, France), Qiagen M48, Qiasymphony and EZ1 workstations (Hilden, Germany). A larger clinical study is now planned to determine the advantages of using a complete STI screening panel to improve patient diagnosis and management.

References

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