A novel multiplex PCR detection method for *Mycobacteria*, *Mycobacterial avium intracellulare* complex (MAIC) and *M. tuberculosis* from clinical samples

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Abstract

According to the World Health Organisation (WHO) there were 8.7 million new cases of tuberculosis (TB) and 1.4 million deaths directly attributable to TB infection in 2011. We have developed a novel technology that has been clinically validated for the detection of HPV (1) and more recently multiplexed PCR panels for the detection of a wide range of Gastrointestinal pathogens (2). We aimed to produce a rapid real time PCR assay that could detect the presence of TB in sputum samples in less than 3 hours. The assay was designed to detect the presence of Mycobacterial species in general, as well as stratifying patients infected with members of the MAIC, which is particularly useful in an immunocompromised setting. Two markers for TB (detected in the same fluorescent channel) were included in the assay to safeguard against false negative reactions as a result of IS*6110* negative *M. tuberculosis* strains.

Background

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).

Conventional Sequence	Tm	3base™ Sequence	Tm
Primer1 GTACACACCGCCCGTCGCTCCTACC	77°C	GTATATATTGTTTGTTGTTTTTATT	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 3base™ modification approach

Sensitivity & Specificity

Table 1 shows the components detected by the GS *EasyScreen*™ *M. tuberculosis* assay. In order to determine the specificity of the multiplex components approximately 10^4 copies of *M. abscessus (*ATCC 19977D-5), *M. avium paratuberculosis (*ATCC BAA-968D-5), *M. gordonae (*ATCC 35760D-5), *M. marinum (*ATCC BAA-535D-5), *M. smegmatis* (ATCC 23037D-5) and *M. tuberculosis* DNA (ATCC 25177D-5) were analysed. All assay components demonstrated a sensitivity of <10 copies when used in the final multiplex reaction with the Mtb component capable of the detection of 1 copy of *M. tuberculosis* DNA (see Figures 2&3). No cross reactivity was observed with a wide range of non-target organisms including organisms found in the respiratory tract at an input of 10^5/copies per PCR reaction.

Preclinical Studies

Table 1. Components detected by the *M. tuberculosis EasyScreen*™ assay.

Component	Channel
MAIC	Fam (Blue)
Extraction Control 16S rDNA	Hex (Green)
M. tuberculosis IS*6110*/Proprietary Marker	TxR (Red)
Mycobacterial 65kDa heat shock protein	Cy5 (Purple)

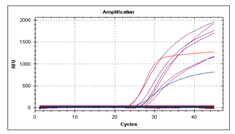

Figure 2. Specificity of the various components of the multiplex reaction. The purple traces correspond to detection of the 65kDa antigen, red trace IS*6110*/proprietary marker and the blue trace indicated the specific detection of MAIC.

M. tuberculoisis *M. gordonae* *M. marinium*

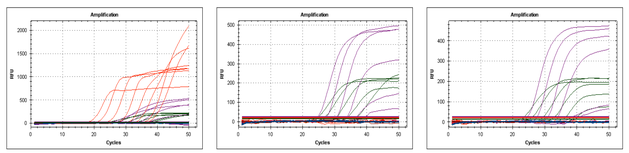

Figure 3a Figure 3b Figure 3c

Figure 3. The sensitivity and specificity of detection of *M. tuberculsosis.*using *M. tuberculosis* (3a), *M. gordonae* (3b) and *M. marinium* (3c).

Clinical Studies

Having assessed the sensitivity and specificity of the assay on a number of purified DNA samples we wished to determine the sensitivity of the assay on clinical isolates and primary patient material. Three sample types were obtained, (i) DNA isolated from patient samples and cultures, (ii) pre-processed sputum samples and (iii) untreated sputum samples.

Materials & Methods

DNA extracts: A total of 20µl of purified DNA was added to 250µl of *EasyScreen*™ lysis buffer. The samples were then heated for 15 minutes at 95°C. Samples were then purified using the EZ1 (Qiagen, Hilden, Germany) or Kingfisher Flex (Thermofisher, Waltham, USA) according to the manufacturer's instructions (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) and the Smartcycler II (Cepheid, Sunnyvale, USA) and the CFX96 (Bio-Rad, Hercules, USA).

***Processed Sputum Samples*:** 150µl of extract was was added to 250µl of *EasyScreen*™ lysis buffer and the samples heated for 15 minutes at 95°C then processed as above.

***Sputum samples*:** Samples were decontaminated using standard procedures (if required) and 1.5ml of sample then spun at 13,000rpm for 5 minutes to pellet intact organisms and the pellets resuspended in 250µl of *EasyScreen*™ lysis buffer. The samples were then heated at 95°C for 20 minutes to simultaneously lyse and convert the target nucleic acids. Sample processing was completed as above.

DNA extracts *Processed sputum* *Unprocessed Sputum*

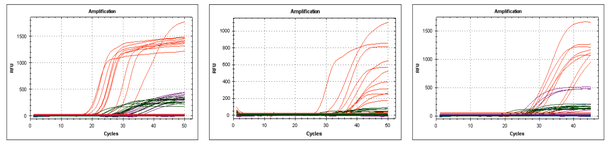

Figure 4a Figure 4b Figure 4c

Sample type	GS EasyScreen™ Positive	Additional Information
DNA extracts (n=10 + 4 TB negative)	10	All sample 65kDa positive
Processed Sputum (n=15)	14	All sample 65kDa positive
Sputum (n=8)	8	All sample 65kDa positive

Figure 4a shows the results obtained using previously prepared DNA extracts obtained from patient samples. Figure 4b shows results obtained using pre-processed sputum samples and finally figure 4c shows the results obtained using sputum obtained directly from the patient.

Discussion

The *EasyScreen*™ assay was able to sensitively detect *M. tuberculosis* down to 1 copy of starting material. The assay performed well using a range of clinical sample including primary patient material. To increase the sensitivity of the method amplification reactions should be performed in duplicate using 10µl of purified material to maximise the chances of detecting low abundance paucibacillary infections. Further optimisation of the sample processing technique will further improve sensitivity using primary patient specimen. A larger scale study is underway to assess the utility of the *EasyScreen*™ assay as a routine screening tool. Furthermore, a reflex assay for the detection of rifampicin resistance is in development so that positive sample can be further screened for the presence of multiple drug resistant (MDR) organisms to prevent the spread of these strains within the community.

Summary

The *EasyScreen*™ assay provides a simple and rapid method for the detection of Mycobacterial infections including *M. tuberculosis* and members of the MAIC. 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). In addition real-time PCR can be carried out on a wide range of instruments providing a solution to existing laboratories without the need for further capital outlay.

References

1. Comparison of a novel HPV test with the Hybrid Capture II (hcII) and a reference PCR method shows high specificity and positive predictive value for 13 high-risk human papillomavirus infections. Baleriola C, Millar D, Melki J, Coulston N, Altman P, Rismanto N, Rawlinson W. *J Clin Virol* 2008;42:22-26.
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