EasyScreen™ multiplexed real-time PCR assays providing rapid and cost effective routine detection of faecal pathogens in a clinical microbiology laboratory

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INTRODUCTION

Gastrointestinal (GI) infections are a major cause of morbidity and mortality world-wide, particularly in developing countries. Diagnosis of the agents of GI infections is challenging as different technologies are typically required for the detection of bacteria, parasites and viruses.

EasyScreen™ assays (see Table 1) utilize a novel chemistry called 3base™ which universally modifies the nucleic acid sequence of pathogens and improves the efficiency of real-time PCR by reducing the temperature variability in multiplexed reactions. All reagents apart from those required for extraction are provided in the kit and results are obtained in approximately 4 hours, greatly reducing the time of diagnosis compared to conventional microbiological techniques.

Table 1: Multiplexed *Easyscreen*™ panels

Panel	Panel Member
EasyScreen™ Enteric Bacterial Detection Kit (Cat#EB001)	*Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia entercolitica*, *Listeria monocytogenes*, *C. difficile*, Extraction control and Internal Process Control
EasyScreen™ Enteric Protozoan Detection Kit (Cat#EP001)	*Giardia intestinalis*, *Cryptosporidium* spp, *Entamoeba* complex, *Dientamoeba fragilis*, *Blastocystis hominis*, Extraction control and Internal Process Control
EasyScreen™ Enteric Viral Detection Kit (Cat#EV001)	Norovirus group I, Norovirus group II, Adenovirus hexon, Adenovirus 40/41, Rotavirus A and B, Astrovirus (group 1-7), Sapovirus , Extraction control and Internal Process Control

METHODS

Evaluation of the *EasyScreen*™ panels

EasyScreen™ Viral, Bacterial and Protozoan kits (Figure 1) were used to evaluate a total of 390 samples (372 fresh and 18 frozen stool samples stored at -20°C) using all 3 *EasyScreen*™ panels (Table 1) simultaneously on each sample. Stools were also tested by conventional stool tests (below) and where possible, confirmatory genetic testing of discrepant or unexpected results by an independent laboratory. Where assay results disagreed with conventional tests or independent confirmatory genetic tests, they were repeated from the same and a new extract.

Stool processing for real-time PCR

Nucleic acids were extracted directly by dipping a flocked swab into unformed stool, transferring the swab to a tube containing 250 µl of extraction buffer (provided with kit) mixing briefly then heating at 95ºC for 15 min. Samples were extracted on an automated extraction system EZ1 BioRobot workstation using the Virus mini kit v2 (Qiagen, Hilden, Germany). Real-time amplification was performed on a CFX96 instrument (Bio-Rad, Hercules, USA) following the manufacturer's instructions (Genetic Signatures, Sydney, Australia) using multiplexed panels (Table 1).

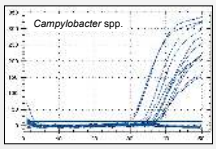
Conventional Stool Tests

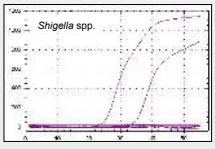
Stools were cultured for *Salmonella*, *Shigella* and *Campylobacter* using commercially available culture media. Samples to be screened for toxigenic *C. difficile* were tested with the TECHLAB C. diff QuikChek GDH/ToxinA/B EIA. GDH positive but ToxinA/B negative samples were tested with the Xpert® *C. difficile* assay (Cepheid, Sunnyvale, CA) to resolve results. All toxin-positive stools were confirmed by culture.

Samples with viruses requested were tested with the RIDA® QUICK Norovirus, or RIDA®QUICK Rotavirus/Adenovirus Combi (R-Biopharm Darmstadt, Germany).

Stools received for culture were screened for parasites. If requested, RIDA® Quick Cryptosporidium/Giardia and concentration of parasites (OCP) tests were performed (Mini PARASEP® SF (Diasys Berkshire, England).

Figure 1: *EasyScreen* ™ kit





RESULTS

Sensitivity

Overall, 100% sensitivity was achieved for most faecal pathogens (Table 2) with 6 samples incorrectly called as negative.

One liquid stool sample grew *S.* Typhimurium yet was repeatedly negative by *EasyScreen*™. An independent genetic test detected *Salmonella*, albeit with a late, weak positive signal. Further testing of positive samples is needed to confirm whether this is a one-off problem.

One sample confirmed as Giardia by microscopy and independent genetic test was *EasyScreen*™ negative.

Initially, 10 samples were incorrectly *EasyScreen*™ *C. difficile* negative. Culture confirmed the Xpert® *C. difficile* assay positive result as correct. Lack of sensitivity for *C. difficile* was attributed to varying concentrations of manufactured primers and repeat testing with standardised primers achieved correct results for 6/10 samples. Quality control implemented now standardises all assay primer concentrations before master mix manufacture.

Specificity

There were no instances of cross-contamination between samples. 100% specificity was achieved for all pathogens except *C. difficile* and *Shigella* spp. (Table 2) and the six apparent false positive results were negative by conventional tests and by independent genetic testing.

Two *C. difficile* positive samples that were positive by *EasyScreen*™ were negative by the Xpert® *C. difficile* test.

Review of two discrepant Astrovirus *EasyScreen*™ positive assay results suggest that the sample cut off for the assay should be cycle 35 instead of 40 as originally indicated.

The two discrepant *EasyScreen*™ *Shigella*-positive/culture-negative samples and seven *EasyScreen*™ *Shigella*-positive/culture-positive samples were analysed using real-time quantitation to determine the absolute copy number. The seven *EasyScreen*™ *Shigella*-positive/culture-positive samples contained between 90 - >3x10^6 genome equivalents. The two *EasyScreen*™ *Shigella*-positive/culture-negative samples contained 2.25 and 0 copies respectively. These samples may well have been below the threshold detection of conventional culture techniques, although were negative when tested at an independent laboratory.

Table 2: Results of clinical evaluation of the *EasyScreen*™ assay

Pathogen Detected	EasyScreen™	Sensitivity %	Specificity %	Additional Pathogens detected
Norovirus	32	100	100	16
Rotavirus	25	100	100	4*
Astrovirus	4	NA	NA	2
Adenovirus	8#	100	100	3
C. difficile	64	93.9	99.7	9
Campylobacter spp.	48	100	100	0
Salmonella spp.	42	97.7	100	1**
Y. enterocolitica	3	NA	NA	2
Shigella spp.	11	100	81.8	0
L. monocytogenes	1*	NA	NA	1
D. fragilis	10	100	100	10*
G. intestinalis	12	92.3	100	7
B. hominis	17	100	100	16*
Cryptosporidium	3	100	100	3
Entamoeba complex	5	NA	NA	5
Totals	285			79

NA = unable to assess sensitivity/specificity as test only performed on *EasyScreen*™-positive samples

*Samples were either insufficient for confirmation of *EasyScreen*™ positive results or independent confirmatory test not available: *Entamoeba* complex (5), *B. hominis* (1), *L. monocytogenes* (1), Rotavirus (1) Adenovirus (1) and *Cryptosporidium* (1)

**One *EasyScreen*™ *Salmonella*-positive sample was culture-negative but was confirmed as positive by an independent PCR test.

#2/8 Adenovirus samples were positive in the Universal Adenovirus test and negative for types 40/41. This would indicate that the adenovirus was unlikely to be causing the gastroenteritis and likely represents ingested respiratory adenovirus.

Sample Inhibition

During the earlier stages of the study, 41 (10.5%) samples were both extraction control and internal control negative. After 1:5 dilution of eluate, 37 of the samples achieved correct results, with re-extraction needed for the remaining 4 samples. A review of technique to minimise sample overload prior to the heat-extraction step and to ensure adequate centrifugation after purification of template via the EZ1 platform prior to testing substantially reduced control failures to <1%.

Cost

Consumable costs for performing an equivalent full faecal screen using conventional testing similar to that provided by all three *EasyScreen*™ panels is estimated at $70/sample. Additional labour costs bring the total cost to $120. All 3 *EasyScreen*™ assays (19 targets) can be performed for under $60 excluding labour, depending on volume and the extraction platform of choice.

CONCLUSIONS

- These assays are suited for any clinical microbiology laboratory equipped with the basic hardware to perform real-time PCR substantially improving time to patient diagnosis and treatment, increasing sensitivity, and importantly, detecting additional pathogens not requested by the consulting clinician.
- The assays are compatible with common nucleic acid extraction platforms and real-time PCR instruments found in hospital and pathology laboratories therefore importantly do not require any further capital outlay by the institution.
- The common 15-minute universal sample processing step allows a near complete gastrointestinal screen from sample to result in around 3 hours. Implementation of robotics would considerably reduce hands-on-time and potential for sample inoculation error as setting up multiplexed panels requires repetitive pipetting and also places excessive demand on machinery for extraction and amplification.
- Further work assessing acceptable Ct values and simplifying the analysis software will only improve this assay.

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