FINAL REPORT
For Research Fellow Position

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TABLE OF CONTENTS

1.0 EXECUTIVE SUMMARY

Dr Ravensdale was employed to supervise and train the PhD students in aspects of Microbiology and Molecular Genetics associated with this project. As part of his administrative duties he has also been responsible for ensuring safety SOP are followed, maintaining laboratory equipment and overseeing the ordering of reagents and equipment at the Curtin University laboratories. His own research project aimed to develop a rapid genetic screening test that can be used by the Meat Industry to identify biological adulterants in their products.

The test relied on DNA-based molecular probes which would been in an unreactive state until a target genetic sequence was introduced. A binding region of the probes would interact with the target sequence and the probes would become reactive with secondary probes. Carbon nanoparticles or a large molecular weight protein (streptavidin) would be used to remove unbound probes before addition of secondary probes. Once added these secondary probes would hybridise to the DNA-probe compounds and form large poly-probe complexes. This would generate either a chromatic or fluorescent signal that indicates a positive reaction.

Using computer generated molecular models and prediction algorithms a range of molecular probe sets were generated. These systems were tested and optimized in the laboratory to detect specific genetic sequences unique to bacterial species of interest. An inexpensive, rapid and robust genetic screening tool was developed which could detect the presence of *Salmonella enterica* in crude DNA extracts. However, the test lacks sensitivity compared to other screening tools and further work is required to increase the sensitivity of the test. Additionally, it was found the probes could cross react with genetic sequences sharing strong homology with the target sequence.

Despite the limitations identified from these preliminary studies, he was awarded a commercialization award and participated in an intensive 6 week CSIRO-funded commercialization workshop. To improve the sensitivity and specificity of the test, new probes have been designed to investigate the physical and chemical properties of the probes which can affect their binding to target sequences. Additionally, reaction conditions such as buffer constituents, reaction temperatures and nanoparticle/streptavidin concentrations. It is anticipated that with further study this test could become a rapid, inexpensive and easy to use microbial screening tool.

All three PhD students have now submitted their thesis for examination. Of these students two have already heard back from their respective examiners. One student has made all the changes and have already submitted it to the University. Once the relevant University committees approve the thesis he would have completed his PhD students. The second student is currently working on the examiners recommended updates.

2.0 INTRODUCTION

Molecular screening techniques have allowed research and diagnostic scientists greater insights into the fundamental processes affecting almost every phenomenon observed in nature. In particular, genetic screening tools allow us to understand the hereditary and microbial basis of disease, determine the lineage of plants and animals, choose appropriate therapeutic agents for multi-drug resistant pathogens, attribute guilt in crimes, and detect microorganisms in different samples. Before molecular hybridisation and polymerase chain

reaction techniques became common in the laboratory, microorganism identification relied solely on culture medium-based growth, staining and biochemical reactions which was time-consuming and biased against microbes that could not be cultivated in a laboratory (1). The ease and sensitivity of these new screening techniques have not only allowed the high throughput screening of larger groups of well-characterised and un-culturable microbes, but also provided greater clarity in assigning taxonomic classification to different organisms (2, 3).

The hybridisation chain reaction (HCR) was first described in 2004 by Dirks and Pierce (4) and its potential applications in *in vitro* fluorescent staining, molecular identification analysis and drug delivery has been extensively studied (5, 6). The basic principle behind the HCR relies on two or more groups of nucleic acid-based probes with complementary regions to each-other flanking a loop sequence that gives the probes a hair pin secondary structure. The reaction is activated by a target initiator sequence which catalyses a hybridisation cascade of the hair pin probes (HPP) resulting in the generation of large hybrid concatemer-like complexes (Fig. 1). The rapid, isothermal, and relatively simple nature of the tests could allow them to be used as signalling components of biosensors or molecular computers (7-9). However, as with most nucleic acid hybridisation techniques large quantities of DNA are required for a signal to be detectable (10, 11).

Choi et al have previously demonstrated that the fluorescent signal generated in fluorescence *in situ* hybridisation could be greatly improved by using linkage probes and fluorescent HCR-HPP compared to direct hybridisation probes (6). The nucleic acid sequence of the RNA-based linkage probes was designed with initiator regions flanking a “detection” region that was complementary for mRNA targets in fixed zebrafish embryos. After the unbound linkage probes were washed off the zebrafish mounts, HPPs linked to quenched fluorophores were added and the 5’ and 3’ initiator regions of the bound linkage sequences would catalyse the HCR; generating a fluorescent signal for each mRNA target (6). Using a similar technique, Yamaguchi et al. (12) demonstrated that bacterial and archael species could be differentiated with similar or greater sensitivity than previously established fluorescence *in situ* hybridisation methods. These studies show the practical advantages of using the HCR in fluorescent staining. However, for routine identity screening fluorescent staining is more time-consuming, expensive and difficult to perform than genetic-screening assays.

Numerous studies have shown the potential of using the HCR as biosensors using synthetically derived nucleic acid initiator targets (5). However, to date no study has shown that the HCR can be used to identify microorganisms or cell types based on genomic extracts. This present study aimed to evaluate the potential of using a modified method described in previous studies (6, 12) to detect target bacteria from a genomic DNA extract with HPP and linkage sequences. Briefly, linkage probes complementary to multiple regions of the *INVA* and *SpiC* genes of *Salmonella* Typhimurium were hybridised to genomic DNA extracts. Un-hybridised linkage probes were removed using graphene oxide nanoparticles, or streptavidin when biotionylated probes were used, and fluorescent HPP were added to the reactions (Fig. 2). Signals were detected using fluorescent spectrometry or agarose gel electrophoresis. Using this technique, *S.* Typhimurium was able to be identified and differentiated from four Gram-negative and Gram-positive bacteria within 20 minutes of commencing the experiment. This assay had substantially less detection sensitivity to PCR screening for the *SpiC* and *INVA* genes. However, the rapid and simple nature of these assays could allow for the development of rapid biosensors for point of care or point of intervention diagnostics if sensitivity can be improved.

3.0 PROJECT OBJECTIVES

a) Develop a rapid genetic screening tool using the HCR

b) Optimise reaction conditions to increase the sensitivity of the test

c) Compare the test with other molecular screening assays

4.0 METHODOLOGY

Probe Design.

Hairpin, linkage and initiator probe sequences are provided in supplementary material. The initial iteration of hairpin probes were designed by following the criteria outlined in the study by Ang and Yung (21). The toehold region was no longer than 12 bp with Cytosine and guanine content kept to 30-40% in the toehold region and 50-60% in the stem regions. These HPP sets had an "A" probe with a fluorescein-dT nucleotide on the 2nd bp position on the 5' stem region and a black hole quencher-1 attached to the cytosine base at the end of the 3' stem region. Linkage probes were designed with a 15-19 bp long target detection region with a minimum of 56% GC content that was complementary to regions of the *Salmonella enterica* serovar Typhimurium (ATCC 13311, Genbank Accession number CP009102.1) *INVA, SpiC, TRRB*, *STM4200*, *STM4467*, *FIMY*, and *FIMZ* genes. The hybridisation overhang of the linkage probes was the reverse compliment of the toehold and 5' stem region of HPPA. Initial HPP and linkage probes were synthesised by Merk Australia (NSW, Australia). Subsequent HPP and linkage probes were synthesised by Integrated DNA Technologies (Australia) and designed using the same parameters with shorter toehold, stem, loop, detection and hybridisation sequences. HPPA probes with biotin linked 5' ends, and HPPA with biotin linked 5' and 3' ends were also synthesized. All probes were solubilised in HpH_2O and kept at -80ºC prior to use. Hybridisation dynamics were simulated using NUPACK software (Caltech, USA; 22). The potential for linkage probes to mis-hybridise with non-Salmonella DNA was evaluated using Basic Local Alignment Search Tool (National Centre for Biotechnology Information, USA) against whole genome sequences of 8 representative strains of bacteria (See supplementary materials).

Bacterial Strains and Culture Conditions

Bacterial strains are listed in supplementary material. *Salmonella* Typhimurium, *Escherichia coli, Klebsiella pneumonia,* and *Staphylococcus aureus* were cultured in LB broth (Oxoid, Basingstoke, UK) while *Enterococcus faecalis* was cultured in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) at 37ºC with 150 rpm shaking. Cell counts were performed by diluting cell culture to McFarland std 2 and plating serial dilutions on LB or BHI plates with 1% agar (Astral Scientific, NSW, Australia). Colonies were counted after 24h incubation at 37ºC.

DNA Extractions

Two methods were used for DNA extraction and purification of bacterial cultures.

1). Bacterial cultures (1 ml) grown overnight were centrifuged at 10,000 x g for 10 minutes and the supernatant was removed. Bacterial pellets were resuspended in 700 µl of HpH_2O and transferred to a 2ml screw cap lysing matrix tubes containing acid washed beads (diameter 0.5 mm and <106 µm; Sigmaaldrich. NSW, Australia). Cells were homogenised by 2 x 45 sec shaking at 6.5 m/s in a Fastprep-24 5G ribolyser (MP Biomedical, NSW,

Australia) followed by centrifugation at 24, 000 x g for 10 minutes. The supernatant was collected and used for downstream applications.

2). Genomic DNA was extracted from 1ml of bacteria cultures grown overnight using the Wizard genomic DNA purification kit (Promega, Wisconsin, USA) following manufactures instructions.

DNA concentration from extracts was estimated using Qubit dsDNA HS assay kit and the Qubit 2.0 fluorometer (Thermofisher, NSW, Australia).

Primer sequences for the *INVA* and *SpiC* amplicons along with reaction conditions are presented in supplementary materials and methods. PCR amplicons were excised from 0.7% agarose TBE gels under UV light and purified using the illustra GFX DNA and Gel Band purification kit (GE Healthcare Life Sciences, NSW, Australia) following manufactures instructions.

Hybridisation Chain Reaction

Hybridisation chain reactions with initiator probes-

0.2μM of initiator was added with 0.1μM of HPPA and 0.1μM HPPB to 80 μl of hybridisation buffer (50mM NaCl, 10mM Tri-Hcl, 10mM $MgCl_2$, 10mM dithiothreitol, 0.1% v/v DMSO) in a Nunc 96-well Polystyrene plate (Thermofisher, NSW, Australia). Negative controls substituted initiator for equal volumes HpH_2O. Fluorescence intensity was measured spectrophotometrically using an EnSight Multimode Plate Reader (PerkinElmer, Buckinghamshire, UK) with excitation of 490nm and emission of 524nm with fluorescence readings taken every three minutes for 30 minutes. Following spectrophotometric analysis 15 μl of sample was mixed with DNA loading dye and separated on a 1.5% SB gel in SB buffer using a 100 V electrophoresis voltage.

Hybridisation Chain Reaction with genomic DNA and linkage probes-

0.2 μM of linkage probes was added with DNA extracts to hybridisation buffer in 500 μl centrifuge tubes. Linkage probe sets are provided in supplementary material. Regardless of the number of different linkage probes in each test set, the total concentration of linkage probes per reaction was kept at 0.2 μM. Tubes were incubated at 95ºC for 5 minutes then left to cool at room temperature for 2-5 minutes. 62 μg/ml of graphene oxide (Graphenea, San Sebastian, Spain) was added to the samples which were immediately centrifuged at 24,000 x g for 2 minutes. For biotinylated probes, 20μg/ml streptavidin was added to the samples, which were incubated at room temperature for 20 minutes followed by centrifugation at 24,000 x g for 2 minutes. The supernatant of all samples was collected and incubated at 95ºC for 2 minutes. When the tubes had cooled to room temperature (~1 minute), 0.1 μM of HPPA and 0.1 μM of HPPB was added to the tubes and signal was detected spectrophotometrically or via agarose gel electrophoresis as described previously.

Hybridisation chain reaction in hybridisation buffer supplemented with PCR inhibitors

50 μl Hybridisation buffer in a 96 well plate was supplemented with 5, 10, 20 and 50% v/v ethanol or isopropanol, 0.1, 0.2 or 0.5% v/v SDS, or 5, 10 or 20% v/v phenol:chloroform:isoamyl alcohol (25:24:1, 10mM tris pH 8, 1mM EDTA; Sigmaaldrich. NSW, Australia). 0.2 μM of initiator and 0.2 μM of HPP probes were added to the wells and the fluorescence intensity was measured over 30 minutes as before.

5.0 PROJECT OUTCOMES AND DISCUSSION

Initial experiments investigated the potential of a HCR being initiated by the toehold and 5' stem region directly binding to a complementary region of DNA of the *INVA* gene of the *S.* Typhimurium genome. Hairpin probe A was designed with a fluorescein-dT that remained quenched when the probes assembled in a hairpin structure. In the presence of initiator sequences the probes would linearise and a fluorescent signal would be detected, with a stronger signal being detected as the concentration of initiator in the reaction increased (Fig. 3A). Agarose gel electrophoresis showed multiple bands of increasing size in initiator positive samples with bands becoming brighter as the concentration of initiator increased. Bands of higher molecular weight tended to assemble in the samples with lower initiator concentrations (Fig. 3B) which has been demonstrated in a previous study (14). This could suggest that in the presence of excess initiator there is more initiator-HPPA hybrids which restricts the assembly cascade of HPPA-HPPB. Purified ATCC 13311 DNA and 1696 bp amplicons of the *INVA* gene which contained the initiator sequence did not initiate the HCR (Fig. 3B).

Hybridisation stability of HPPA to its DNA target may be reduced due to steric hindrance from regions flanking the hybridisation sequence. However, multiple reports have described unpaired overhang sequences, or dangling ends, increasing the stability of DNA duplexes (15, 16). Initiator sequences with 10-20 bp poly-A overhangs were used with the HPP to investigate how the size of the target DNA molecule affected hybridisation to HPPA (Fig 4). The length of the overhang and its position on either the 5' or 3' end of the initiator sequence did not seem to effect hybridisation. A 20 bp overhang on either side of the initiator sequence increased fluorescence intensity generated compared to the other initiator sequences. As previous studies have shown an increase in hybridisation stability as the proportion of purine bases in dangling ends increase (15, 16), these results suggest that targeting regions on genomic DNA flanked by adenine or guanine bases with HPPA could result in greater hybridisation efficiency.

Initial experiments showed that the HCR could not be initiated with genomic DNA extracts (Fig 3B). This may be due to activation of the HCR being dependant on the concentration of target hybridisation sequence present. The total number of initiator molecules required to initiate assembly of HPP chains was calculated to be $3.11*10^{11}$ (See supplementary materials). Assuming the *INVA* gene is only present in the Salmonella genome as a single-copy gene, this could indicate that a minimum of ~$3*10^{11}$ cells/reaction could be required to initiate the HCR. To increase the sensitivity of the tests for detecting Salmonella, linkage probes were designed to hybridise to multiple regions of the *INVA* and *SpiC* genes in a similar method to a previously published report (6). These linkage probes had free 3' "dangling ends" which were complementary to the toehold and 5' stem region of HPPA. It was hypothesised that this would create more activation sites per DNA molecule for the HCR to be initiated and potentially increase the sensitivity of the reaction.

Each linkage set was made up of 5 probes that would bind to different regions of the *INVA* or *SpiC* genes. These sets were used separately and mixed together at equal concentrations with Salmonella DNA to initiate the HCR. Figure 5 shows that an observable increase in fluorescence can be detected using the INVA or SpiC linkage probes for 20 ng of ATCC 13311 DNA. If an equal mix of INVA and SpiC linkage probes are used (SpIN) this seems to further increase the sensitivity of the reaction, with a substantial increase in fluorescence being detected with 10 ng of DNA compared to the DNA negative control after 3 minutes incubation. As the SpIN linkage probe mixture was used at the same concentration as the INVA and SpiC linkage probes, this increase in sensitivity may be explained by a reduced proximity of the HPP to their binding sites rather than a linear increase in concentration of hybridisation initiators. Using 10 linkage probes instead of 5 creates 10 possible sites that

the HPPA can bind to per DNA molecule which increases the proximal area of the DNA that can initiate a HCR.

However, using multiple linkage probes with different detection regions can increase the chance of mis-annealing of these probes to non-Salmonella DNA. The first iteration of the linkage probes showed that 8 of the 10 probes had a moderate chance of annealing to *S. aureus* DNA due to complementary regions between the detector and hybridisation regions of the probes and *S. aureus* DNA. This was confirmed by using the linkage probes with three strains of *S. aureus* (data not shown). Six new linkage probes (3 for *INVA* gene and 3 for *SpiC* gene) were designed with modified and truncated hybridisation regions along with shorter HPPs. Although there appeared to be some faint bands in the *S. aureus* DNA indicating potential mis-annealing of the linkage probes, using this new system substantially reduced the chance of false positives arising with non-Salmonella DNA (Fig. 6).

The sensitivity of this test was compared against PCR using primers covering the 27-1723 region of the *INVA* gene and 42-342 of the *SpiC* gene. Figure 7A shows that by using 6 linkage probes (SIMX), ATCC 13311 DNA was able to be detected at a minimal concentration of 10 ng/ 25 µl. Amplicons were able to be generated using PCR with the INVA and SpiC primers at 5 ng/ 25ul (See supplementary materials). Similarly, DNA extracts from a minimum of 2 *10^8 CFU/ml of ST13311 bacteria seemed to be required to initiate the HCR whereas PCR amplicons of the *INVA* gene were observed with DNA extracted from 2 * 10^3 CFU/ml (Fig 8). To improve the sensitivity of the tests 15 more linkage probes covering regions of the *TRRB*, *STM4200*, *STM4467*, *FIMY*, and *FIMZ* genes were designed and tested with the 6 SIMX linkage probes. Using these 21 linkage probes the HCR was able to be initiated with 2.5 ng of ATCC 13311 DNA (Figure 7B).

Although increasing the amount of linkage probe sets in the reaction appears to increase the sensitivity of the tests by a modest degree, this detection limit is still far below that of PCR. However, the efficiency of the polymerase enzyme can be affected by organic molecules and inorganic salts. For example, detergents, phenol and urea can degrade the polymerase enzyme, EDTA, polyphenols and some proteins can sequester divalent cofactors required to catalyse polymerase reactions, and alcohols can destabilise organised structures of the enzyme protein (17, 18). The HCR relies on hydrogen bonding interactions, base stacking and hairpin structure kinetics. Therefore, PCR-inhibitors which can be co-extracted when preparing the DNA samples should not affect the efficiency of the HCR. To test this, ethanol, isopropanol, sodium dodecyl sulphate (SDS) and phenol:chloroform mix were added to the hybridisation buffer and the hybridisation efficiency of fluorescently labelled HPP in the presence of initiator sequences was compared to reactions in mocked supplemented hybridisation buffer. Figure 9 shows no substantial change in the hybridisation kinetics between mock supplemented and inhibitor supplemented buffer. In contrast, the PCR for *INVA* and *SpiC* genes was inhibited in buffers with concentrations of these compounds ≤10 times lower than what was used in the hybridisation buffers (See supplementary material). The efficiency and reliability of genetic screening assays prone to co-extraction of PCR inhibitors such as those dealing with soil (polyphenols, humic acids), water (metal and salt ions), plants (polysaccharides, phenols) and clinical samples (haemoglobin, bile acids, urea) could be improved by using HCR-based assays instead of polymerase-based assays.

Compared to PCR-based techniques, this HCR assay appears far less sensitive and therefore less practical as a molecular detection technique. However, our results have shown that the HCR can be initiated and produce maximal signal within a matter of minutes (Fig 3,4 and 5) and can function with no loss of efficiency in buffers supplemented with high concentrations of common PCR inhibitors (Fig. 9). The protocol for this assay has the potential to be further optimised, as we observed that a glass fibre filter spin column could be substituted for the removal of graphene oxide and unbound linkage probes which saved time and reduced handling errors (data not shown). Additionally, sensitivity seems to be improved by using more linkage probes designed to hybridise across multiple regions of multiple

genes. Judicious design and screening of these probes could greatly improve the sensitivity of this rapid screening test while maintaining specificity for the target organism.

The three PhD scholarship students have now submitted their thesis for examination. Under Curtin PhD policies and procedures, a PhD research must contribute new knowledge or must be a significant reinterpretation of current knowledge. All three students have contribute new knowledge that is relevant to the red meat industry. The thesis then has to be examined by two independent experts in the respective fields of research. Of the three students, student one and two have already received feedback from their examiners. Student two has already completed the changes recommended by the examiners and have submitted the revised thesis to the University. Once approved by the University's relevant committees, he would graduate. Student one, is in the process of making the examiners' recommended changes and will submit the revised thesis by late February or early March. Student three is still awaiting examiners' feedback.

6.0 CONCLUSIONS/RECOMMENDATIONS

There have been numerous studies published which have shown that the HCR can be used to detect genetic sequences in sub-pM concentrations (5). Although these studies demonstrate rapid, sensitive, and specific detection, the target DNA sequences are synthetically synthesised oligonucleotides ranging in length of 18-46 bp (7, 19, 20) and are not necessarily indicative of the sensitivity of this test if whole genome extracts were used. This proof of concept study has demonstrated that the HCR can be used to identify *S.* Typhimurium from genomic DNA extracts albeit with a lack of sensitivity. The rapid and simplistic nature of the assay could be beneficial for genetic screening applications such as those applicable to the Meat Industry. Additionally, the test appears resistant to many compounds that can inhibit polymerase-based tests and that are commonly co-extracted in DNA preparations from environmental and clinical samples. As such, DNA purification steps for the HCR are minimal which not only reduces time and monetary costs associated with the reaction, but also increases the recovery yield during DNA extraction and may limit the amount of false negatives returned during screening. These preliminary results indicate that the test could be developed into a mobile easy to use microbial screening tool that could be performed with minimal laboratory equipment and by staff with a basic understanding of laboratory techniques. However, the results show that the test is not sensitive or specific enough to be used in its current form. More work will be required to optimise the reaction conditions and pherhaps develop probes with stronger affinity to their target genetic sequence before it could be considered viable for use in the Meat Industry.

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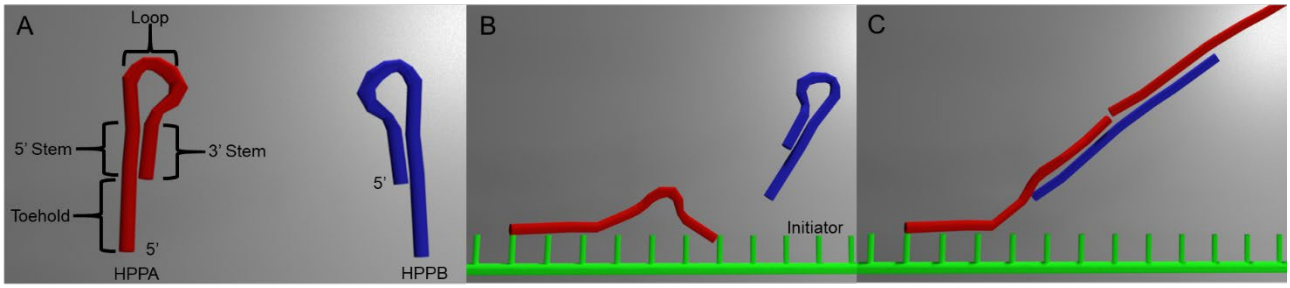


Fig 1

Two or more sets of hairpin pin probes stabilised by 5' and 3' stem regions separated by a loop region. At the 5' end of the leading hairpin probes (HPPA) is a single stranded sequence, known as the toehold sequence, which is complementary to a target nucleic acid "initiator" sequence as well as the loop and 5' stem region of HHPB (A). When the toehold sequence of the 5' stem region of HPPA hybridises with its complementary initiator sequence the kinetic energy stored in the loop region disrupts the basepairing of 5'-3' stem regions allowing the 5' stem region to hybridise with the initiator sequence (B). The 3' stem region is now free to hybridise with the toehold sequence of HPPB which linearises the probe creating another hybridisation target for HPPA (C). In this way, a ssDNA target sequence is able to initiate a hybridisation cascade of hairpin probes, generating large concatemer-like nucleic acid structures.

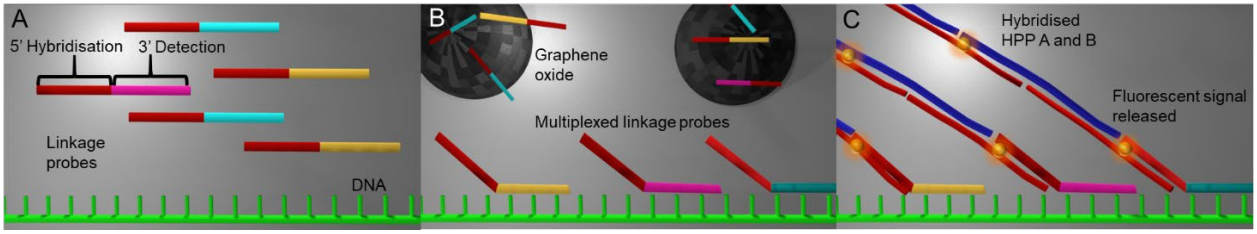
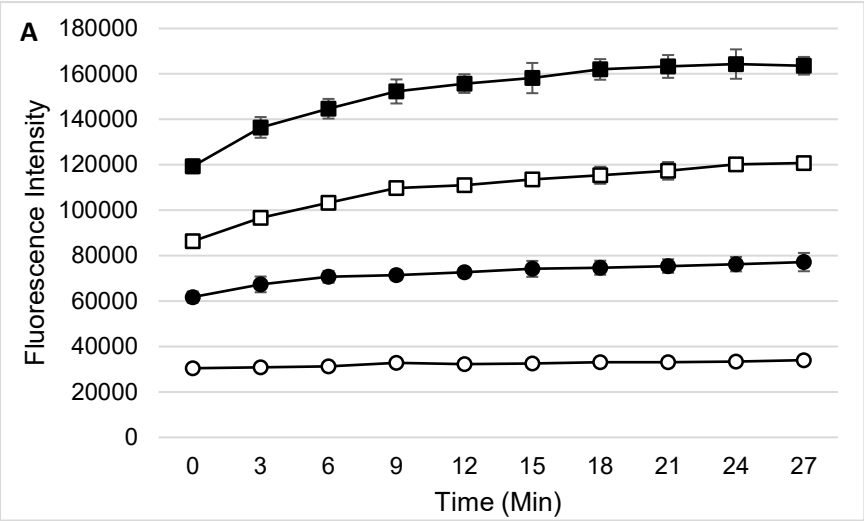


Fig 2

Linkage probes have a 3' detection sequence complimentary for a region on the target gene and a 5' hybridisation region that is complementary to the toehold and 5' stem region of HPPA. Multiple linkage probe sets with different detection sequences that cover multiple regions across multiple genes are used (A). Linkage probes hybridise to their target genes in the absence of HPP. Unbound linkage probes are selectively removed with graphene oxide which preferentially binds smaller ssDNA over dsDNA (B). HPP sets A and B are added and the hybridisation cascade is initiated by the 5' hybridisation regions of the linkage probes that hybridised with the DNA. As HPPAs linearise, a previously quenched fluorophore now emits a detectable fluorescent signal indicating a positive reaction (C).



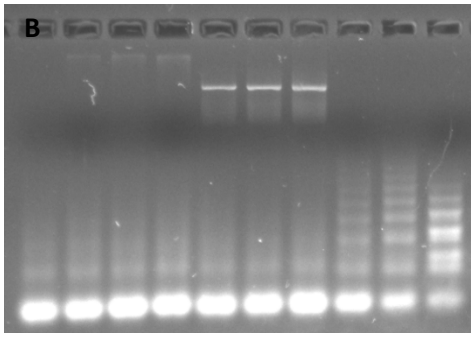


Fig 3.

3A. Fluorescent intensity of HPP over time in the presence of 0 µM initiator sequence (open circles), 0.05 µM initiator sequence (closed circles), 0.1 µM initiator sequence (open squares) and 0.2 µM initiator sequence (closed squares). *Salmonella* DNA extracts and amplicon sequences followed a similar trend line as the 0 µM negative control and are not included in the graph. Each time point is a representative of the average of triplicate samples with error bars representing the standard deviation between the replicates.

3B. Lane 1: 0µM initiator sequence, Lane 2: 10 ng ATCC 13311 DNA, Lane 3: 15 ng ATCC 13311 DNA, Lane 4: 20 ng ATCC 13311 DNA, Lane 5: 10 ng INVA Amplicon, Lane 6: 15 ng INVA Amplicon, Lane 7: 20 ng INVA Amplicon, Lane 8: 0.05 µM initiator sequence, Lane 9: 0.1 µM initiator sequence, Lane 10: 0.2 µM initiator sequence.

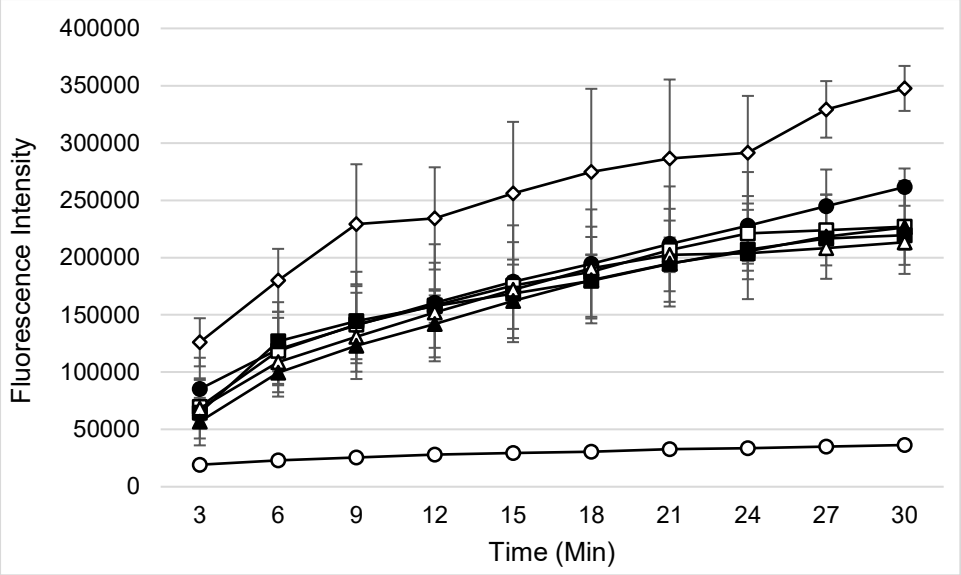


Fig 4.

Fluorescence intensity over time of HPPs in the presence of no initiator sequences (open circles), initiator sequences with 5' 10 bp overhangs (closed circles), 5' 20 bp overhangs (open squares), 3' 10 bp overhangs (closed squares), 3' 20 bp overhangs (open triangles), 5' and 3' 10 bp overhangs (closed triangles) and 5' and 3' 20 bp overhangs (open diamond). Each time point is a representative of the average of triplicate samples with error bars representing the standard deviation between the replicates.

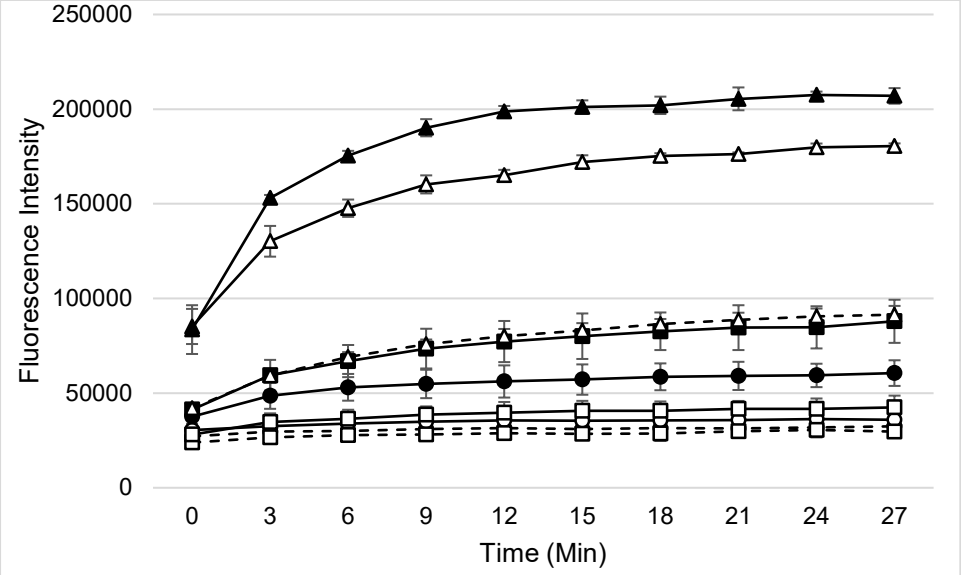


Fig 5.

Fluorescence intensity over time of HPPs in the presence of INVA linkage probes with 0 ng (open circles with dashed lines), 10 ng (open circles with unbroken lines), and 20 ng (closed circles with unbroken lines) ATCC 13311 DNA, SpiC linkage probes with 0 ng (open squares with dashed lines), 10 ng (open squares with unbroken lines), and 20 ng (closed squares with unbroken lines) ATCC 13311 DNA, and an equal mix of INVA and SpiC linkage probes with 0 ng (open triangles with dashed lines), 10 ng (open triangles with unbroken lines), and 20 ng (closed triangles with unbroken lines) ATCC 13311 DNA. Each time point is a

representative of the average of triplicate samples with error bars representing the standard deviation between the replicates.

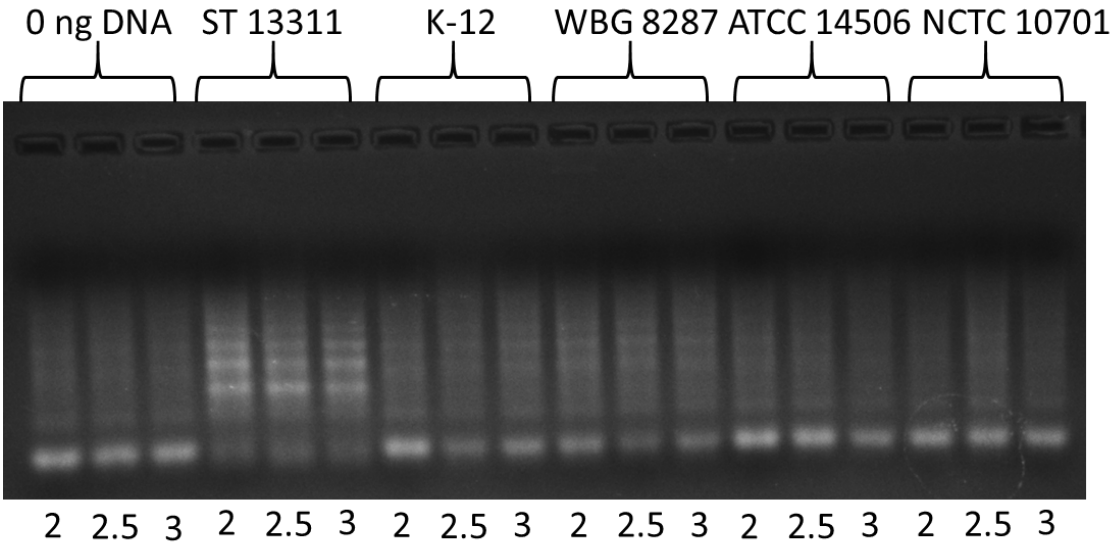


Fig 6.

Hybridisation of SIMX linkage sequences at 2, 2.5 and 3 µM concentrations with 20 ng of *Salmonella* Typhimurium (ATCC 13311), *E. coli* (K-12), *S. aureus* (WBG 8287), *E. faecalis* (ATCC 14506), and *P. aeruginosa* (NCTC 10701). Some light middle bands can be observed in the WBG 8287 samples which could suggest potential mis-annealing of the linkage probes to the DNA.

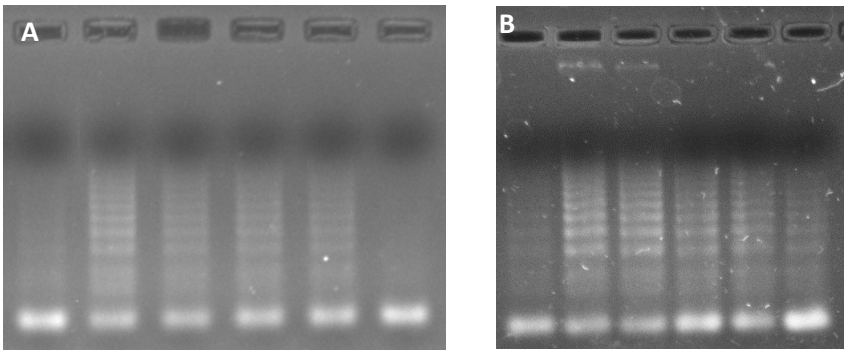


Fig 7.

7A. Hybridisation of SIMX linkage sequences with 0 ng (Lane 1), 40 ng (Lane 2), 30ng (Lane 3), 20 ng (Lane 4), 10 ng (Lane 5) and 5 ng (Lane 6) of ATCC 13311 DNA**.**

7B. Hybridisation of 21 linkage sequences with 0 ng (Lane 1), 25 ng (Lane 2), 20ng (Lane 3), 10 ng (Lane 4), 5 ng (Lane 5) and 2.5 ng (Lane 6) of ATCC 13311 DNA.

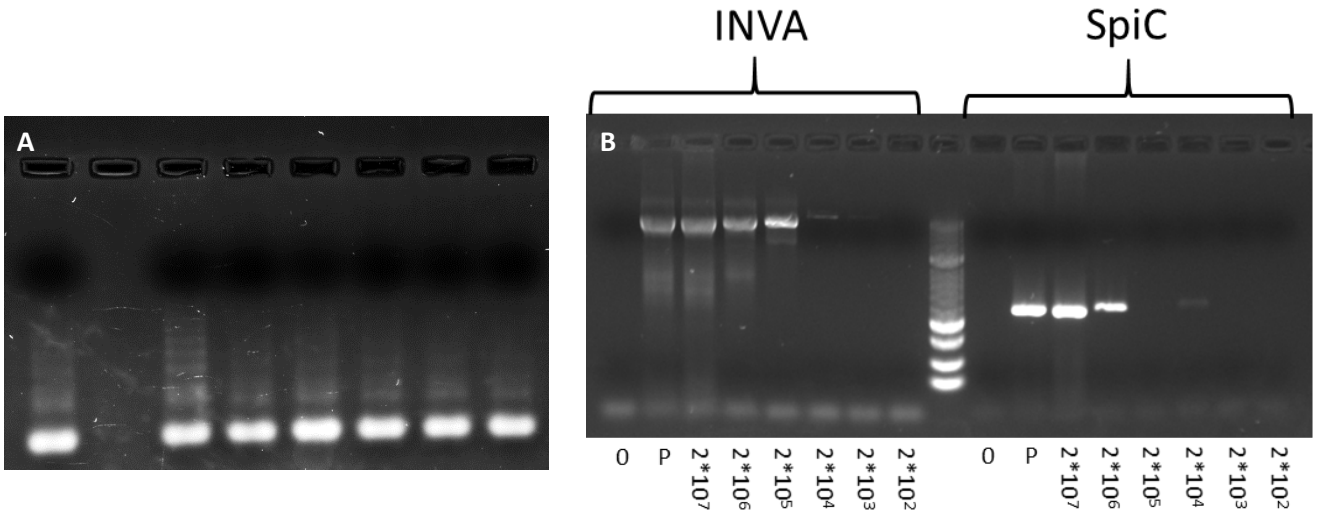


Fig 8.

8A. Hybridisation of SIMX linkage sequences with ATCC 13311 DNA extracted from mock inoculated controls (Lane 1), $2*10^7$ CFU/ml (Lane 3), $2*10^6$ CFU/ml (Lane 4), $2*10^5$ CFU/ml (Lane 5), $2*10^4$ CFU/ml (Lane 6), $2*10^3$ CFU/ml (Lane 7) and $2*10^2$ CFU/ml (Lane 8).

8B. PCR amplicons of primers covering the 27-1723 bp region for the *INVA* gene and the 42-342 bp region for the *SpiC* gene of *Salmonella* Typhimurium. Lanes 1 and 10: 0 ng DNA control, Lanes 2 and 11: 40 ng of ST13311 DNA. Lanes 3 and 12: DNA extracted from $2*10^7$ CFU/ml, Lanes 4 and 13: DNA extracted from $2*10^6$ CFU/ml, Lanes 5 and 14: DNA extracted from $2*10^5$ CFU/ml, Lanes 6 and 15: DNA extracted from $2*10^4$ CFU/ml, Lanes 7-16: DNA extracted from $2*10^3$ CFU/ml, Lanes 8 and 17: DNA extracted from $2*10^2$ CFU/ml, Lane 9: 50 bp ladder. DNA extractions for the HCR and PCR experiments was extracted using Promega Wizard DNA extraction kits.

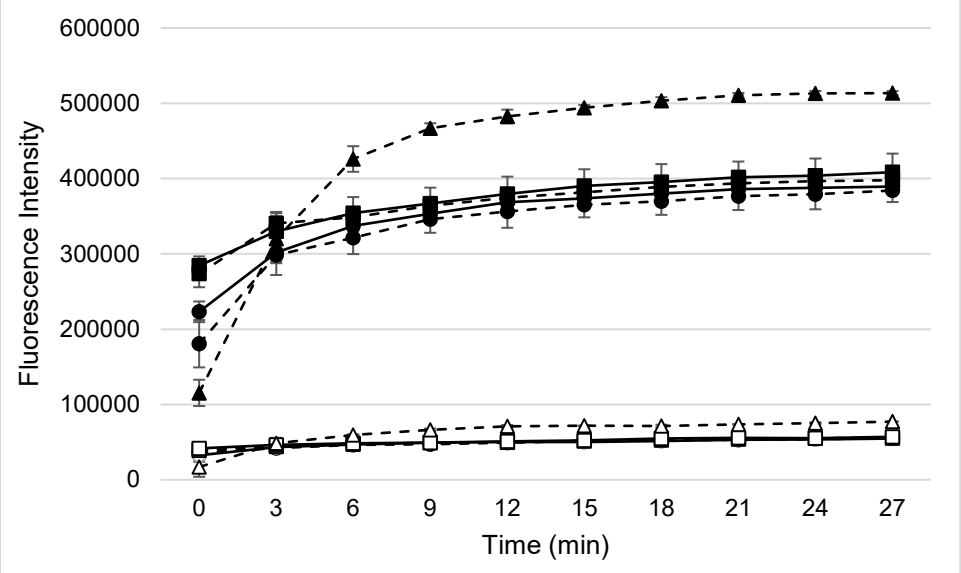


Fig. 9

Fluorescence intensity over time of fluorescently labelled HPP in the presence of initiator sequences and buffer supplemented with different PCR inhibitors. Open circles with dashed lines; mock supplemented hybridisation buffer without initiator sequences. Closed circles with dashed lines; mock supplemented hybridisation buffer with initiator sequences. Open circles with unbroken lines; hybridisation buffer with 50% (v/v) ethanol without initiator sequences. Closed circles with unbroken lines; hybridisation buffer with 50% (v/v) ethanol with initiator sequences. Open squares with dashed lines; hybridisation buffer with 50% (v/v) isopropanol without initiator sequences. Closed squares with dashed lines; hybridisation buffer with 50% (v/v) isopropanol with initiator sequences. Open squares with unbroken lines; hybridisation buffer with 0.5% (v/v) SDS without initiator sequences. Closed squares with unbroken lines; hybridisation buffer with 0.5% (v/v) SDS with initiator sequences. Open triangles with dashed lines; hybridisation buffer with 20% (v/v) phenol: chloroform mix without initiator sequences. Open triangles with dashed lines; hybridisation buffer with 20% (v/v) phenol: chloroform mix without initiator sequences. Each time point is a representative of the average of triplicate samples with error bars representing the standard deviation between the replicates.