

Use of the 3M™ Molecular Detection System for *Salmonella* and *Listeria* spp.

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Current detection of food borne pathogens entails a stepwise approach of pre-enrichment, enrichment, inoculation of selective agar, presumptive identification and confirmation. Each step may require an overnight incubation period which results in delayed release of products, shortening their shelf-life and quality for the consumer. The 3M™ Molecular Detection System was tested for the detection of *Salmonella* serovars Enterica, Typhimurium & Ealing, and *Listeria monocytogenes* after the pre-enrichment step as a potential screening method for the food industry. Testing included detecting the named target organisms which had been used to spike non-sterile milk and minced meat samples in order to include the presence of intrinsic organisms, and complications due to the food matrix (i.e. proteins and fats).

The 3M Molecular Detection System positively detected the laboratory cultures of *Salmonella* serovars and *L. monocytogenes*; *Salm. enterica* serovars Typhimurium NCTC 74, *Salm. Enteritidis* NCTC 3046, *Salm. Ealing*, *Listeria monocytogenes* NCTC 10527, and *L. monocytogenes* NCTC 7973. This was achieved both in pure cultures within 12–30 minutes after the start of the analysis and was well within the pre-set maximum test run of 75 minutes. *Salmonella* serovars and *L. monocytogenes* were also detected in the presence of mixed intrinsic flora of pasteurised milk and minced meat. The intrinsic flora had grown during pre-enrichment to levels in the order of 10^9 – 10^{10} cfu/mL and greatly outnumbered the target organisms by several orders of magnitude. The food matrixes of milk and minced meat did not interfere with the end-detection of the target organisms. The 3M Molecular Detection System, therefore, has the potential to screen samples after the pre-enrichment stage such that only positive samples are further analysed through the further stages to colony isolation. Given the majority of samples are negative for *Salmonella* and *Listeria* spp., screening for positive samples after pre-enrichment would reduce the need for further analysis and costs associated with the preparation and inoculation of enrichment broths and selective agars.

A preliminary detection limit was determined using decimal dilutions of the target organisms. This was in the order of 10^3 colony forming units, in the Lysis Solution Tube. It was deemed highly significant that this small number of organisms were detectable within 45 minutes after starting the test. Although such enumeration after pre-enrichment in itself is not a requirement, it does reflect that the method can detect target organisms at low numbers which may not have grown extensively during the pre-enrichment stage, and therefore considerably out-numbered by non-target organisms. Such a result is clearly highly desirable, and reflects the benefits of the change to DNA sequence-based detection methodology in food microbiology.

► Introduction

The 3M Molecular Detection System is a combination of two techniques to produce a specific and rapid detection method. This is achieved by linking the isothermal amplification of DNA target region(s) with bioluminescence as the end-detection method. The use of DNA sequence-based probes enables the design of a highly specific test procedure and reduces the false positive detection of other organisms which may have similar phenotypic and growth characteristics. Subsequent DNA amplification, following DNA probe binding, is obtained at one temperature (isothermal) which simplifies the procedure and engineering of the equipment. In order to rapidly detect a positive result, bioluminescence has been coupled to the DNA amplification process.

This study was to trial the 3M Molecular Detection System for *Salmonella* and *Listeria* spp. as organisms of serious concern in the food industry for which prolonged detection methods are normally required. Previous studies had focussed on the specificity (inclusion-exclusion strain sets) of the technique and were therefore not included here. The biochemistry and molecular biology of the detection procedure was also not investigated as the standardised protocols provided by 3M were followed. In order to consider the robustness of the method, these experiments were designed to determine if the target organisms could be detected in the presence of mixed bacteria flora — intrinsic to milk and minced meat — and if the food matrix interfered with the result.

► General Methods

Strains

A total of six bacterial cultures were used. These were from the Nottingham Trent University culture collection; *Salmonella enterica* serovars Typhimurium NCTC 74, *Salm. Enteritidis* NCTC 3046, *Salm. Ealing*, *Listeria monocytogenes* NCTC 10527, *L. monocytogenes* NCTC 7973 and *E. coli* K12. *Salmonella* strains were stored on TSA. *L. monocytogenes* strains were stored on Oxford agar, and *E. coli* on LB agar at 4°C.

Growth Conditions

Strains were checked for purity following growth on TSA. Additionally, the *L. monocytogenes* were grown on Oxford agar (37°C) to record colony morphology.

Food

Locally purchased pasteurised whole milk and minced meat were used.

Media

– *Salmonella* spp. testing: 3M™ Buffered Peptone Water (BPW) ISO-Beta, 3M number: 3MBPW500, Batch number: 34-8706-6166-6

– *Listeria* spp. testing: 3M™ Modified *Listeria* Recovery Broth-Beta, 3M number: MLRB500, Batch number: 34-8706-6169-0

3M™ Molecular Detection System Kits

3M™ Molecular Detection Assay *Listeria* and 3M™ Molecular Detection Assay *Salmonella* were evaluated.

General Protocol

The manufacturer's standardised protocols were followed for *Salmonella* serovars and *Listeria* spp. detection; MDAS96 and MDALS96. These should be consulted for experimental details. A brief description of the microbiological procedure was as follows:

1. *Salmonella* spp. were grown in BPW overnight before testing. *Listeria* spp. were grown in the *Listeria* selective broth before testing. Cultures were grown at 37°C with 20 rpm agitation.
2. 20µl of test sample was transferred to a lysis solution tube. The tubes were inverted 3–5 times to mix and then placed in the 3M™ Molecular Detection Heat Block (at 100°C for 15 minutes) for cell lysis. This was followed by being placed on the 3M™ Molecular Detection Chill Block (at -20°C for 10 minutes) and inversion (3–5 times) for clarification. Then left to stand for five minutes.
3. 20µl of the clarified supernatant was transferred to a Reagent Tube. Then mixed with five repeated cycles of the pipette plunger.
4. The Reagent Tube was then placed in a Speed Loader Tray, designed to take up to 96 samples, which was then fitted into the 3M™ Molecular Detection Instrument.
5. An increase in light from the reagent tube indicated DNA amplification had occurred. This was automatically detected by the instrument which was controlled by the 3M™ Molecular Detection Software.
6. Sample details were entered into the spreadsheet prior to starting the test run. The length of the test run was pre-set at 75 minutes.
7. At the end of the test, a report is generated which included automated positive and negative assignment (colour-coded) of the sample results and validation of controls. Individual light curves could be displayed and inspected.

Each test run included the supplied reagent (positive) and negative controls. Standard good laboratory and microbiological practices for containment level 2 bacterial pathogens were followed at all stages of laboratory procedures.

► General Trial Approach

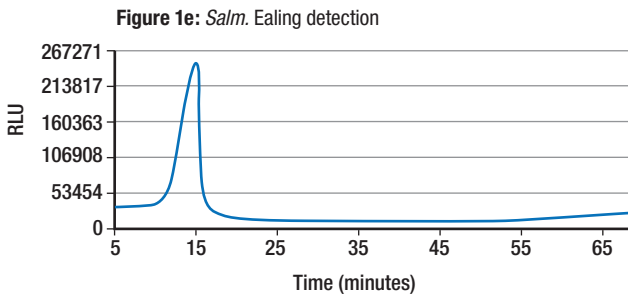
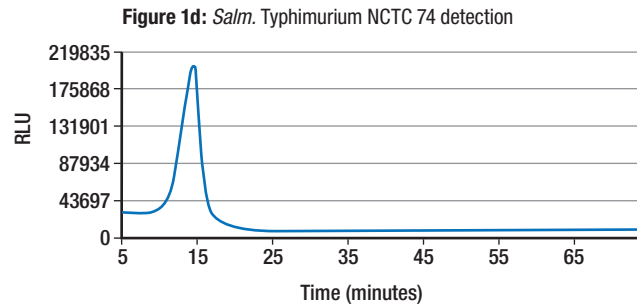
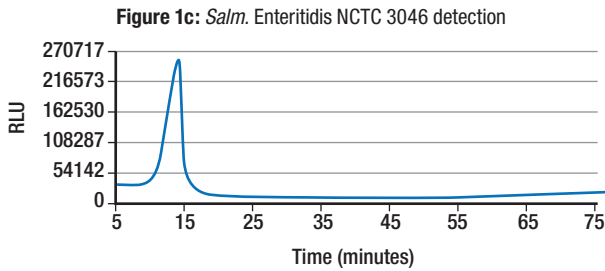
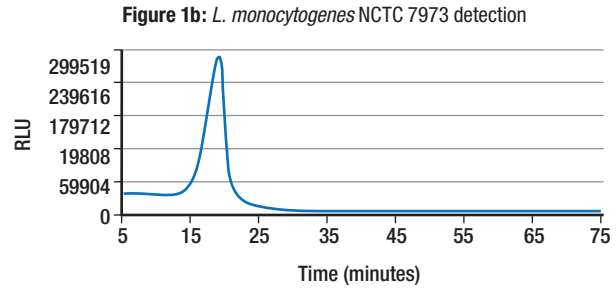
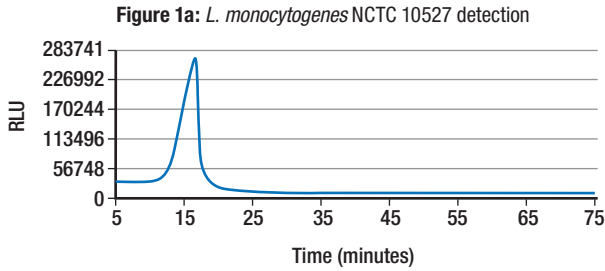
For the evaluation of the 3M Molecular Detection System, a series of experiments were undertaken which used the General Protocol given above but modified the target organism, presence of competing flora and food matrix. These included:

1. Pure cultures of *Salmonella* and *Listeria* spp. to ensure positive detection of target organism.
2. Serial dilutions of *Salmonella* and *Listeria* spp. to determine limit of sensitivity.
3. Mixed pure cultures of target and non-target organisms for initial testing of competing flora.
4. Mixed cultures of target organisms, and intrinsic non-target organisms in milk and minced meat, to test the specificity in the presence of competing flora which had not been chosen by the experimenter.

Results

1. Detection of pure cultures of *Salmonella* serovars and *Listeria* spp.

Using 20µl aliquots of overnight cultures (10^9 – 10^{10} cfu/mL), the system gave a positive result for *Salmonella* Enterica serovars Enteritidis, Typhimurium & Ealing and *Listeria monocytogenes*. This preliminary experiment was for familiarity and basic confirmation of the system. Figures 1a–1e shows the characteristic light curve for a positive detection for the five target organisms. The amount of light increases due to bioluminescence and then decreases.

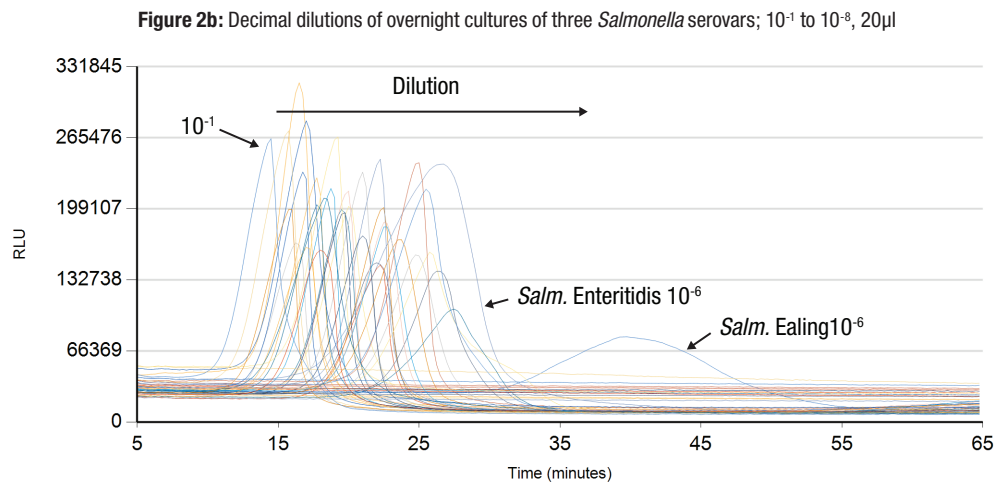
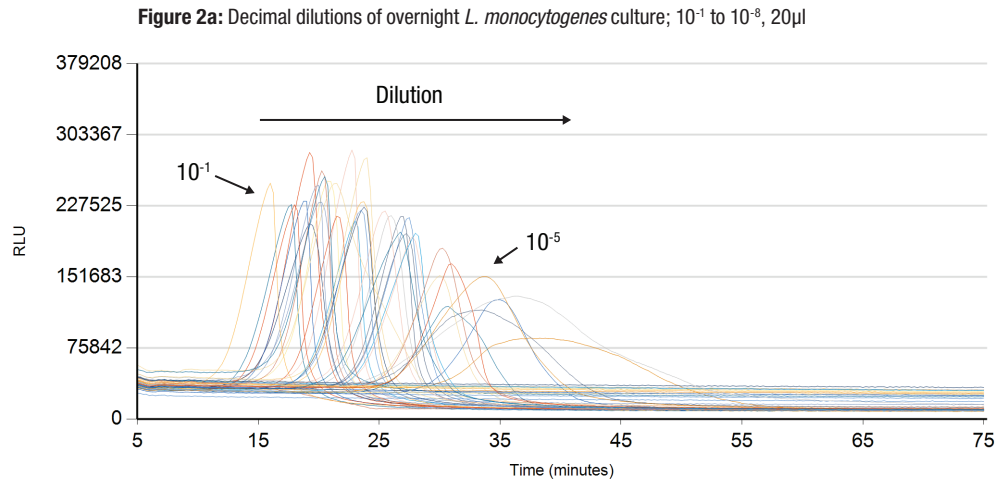


The above curves were obtained using 20µl aliquots of an overnight growth of each organism in pure culture. The peak height of light produced (expressed as relative light units, RLU) was regarded as independent of the amount of bacterial cells in the assay, as shown below.

2. Limit of sensitivity detection for *Salmonella* and *Listeria* spp.

Overnight cultures were decimally diluted in sterile saline; 10^{-1} to 10^{-8} . Standard aliquots (20 μ l) were then tested from step 2 in the General Protocol (page 3). Positive results were obtained for all serial dilutions up to at least 10^{-5} (often 10^{-6}) which was approximately 10^4 – 10^5 cfu/mL (10^3 cfu in the Lysis Solution Tube). Therefore, the detection limit of the 3M Molecular Detection System was in the range of 10^3 bacterial cells and possibly lower.

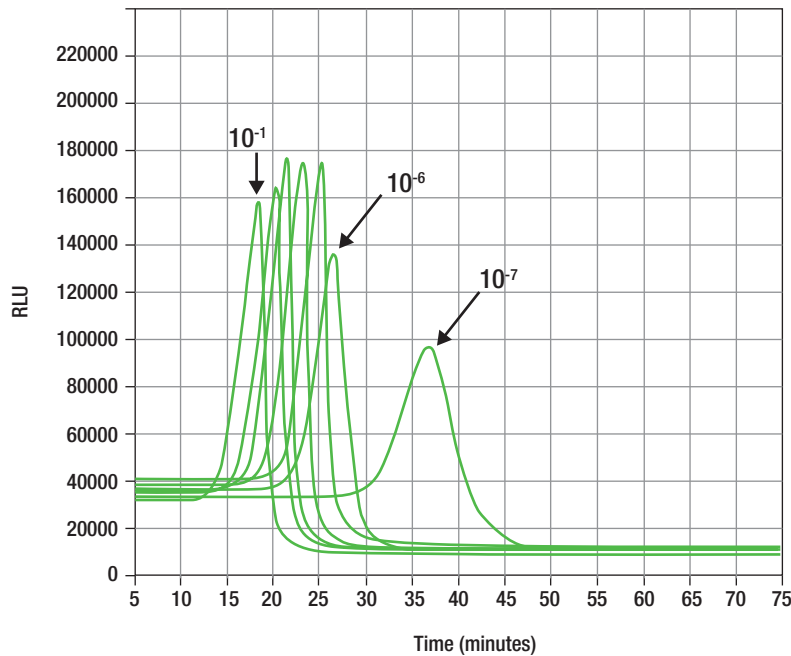
Figure 2a shows the results for *L. monocytogenes* and Figure 2b for three *Salmonella* serovars. It was notable that decreasing numbers of cells resulted in a wider, but lower, light curve. Nevertheless, the shape of the light curve did not result in any false negative results.



3. *Salm. Enteritidis* detection limit and time

For clarity, the experiment on page 5 is reproduced below with just *Salm. Enteritidis* NCTC 3046.

Figure 3: Decimal dilutions of overnight cultures of *Salm. Enteritidis* NCTC 3046, 20 μ l sample size



Given the overnight culture of *Salm. Enteritidis* was $\sim 10^{10}$ cfu/mL, gives a detection limit in 20 μ l (10^{-7}) aliquot of ~ 50 cfu for this illustrated experiment. This is a remarkably low detection value. However, in the light of all the experiments, we have given a more conservative value of 10^3 cfu to be readily obtainable.

4. Mixed pure cultures of target and non-target organisms for initial testing of competing flora

Salmonella and *Listeria* spp. were cultured overnight at 37°C 20 rpm in their pre-enrichment broths. *E. coli* K12 was grown in LB broth. The pure cultures of *E. coli* and *Salmonella* or *Listeria* spp. were mixed in equal volumes and processed as according to the General Protocol.

The presence of *E. coli* did not interfere with the detection of either the *Salmonella* or *Listeria* cultures.

5. Mixed cultures of target organisms, and intrinsic non-target organisms in milk and minced meat, to test the specificity in the presence of competing flora which had not been chosen by the experimenter

Pasteurised milk and minced meat were incubated overnight in BPW (1g : 9mL BPW) and had grown to $\sim 10^{11}$ cfu/mL before spiking with 50 μ l volumes of overnight cultures of *Salmonella* or *Listeria* cultures (inoculum size 10^{10} cfu into 9mL of BPW-food mixture giving 10^9 *Salmonella* or *Listeria* cfu/mL). Following the General Protocol, the *Salmonella* and *Listeria* spp. were still detected in ~ 20 – 30 minutes in the presence of 100-fold greater intrinsic flora, and also in the presence of the food matrixes (proteins and fats).

An example of the above experimentation is given in more detail below. The reason for this emphasis is that we believe that testing for the target organism in the presence of intrinsic, competing flora is very useful for determining the reliability and applicability of the method as opposed to the use of pre-selected laboratory strains.

Salmonella testing is normally presence/absence testing of 25g of food and hence requires the use of a pre-enrichment step for the organism to resuscitate and grow. There is a presumption in this standard approach that any *Salmonella* present will grow to detectable numbers, but this is rarely validated. Therefore, we decided to see how sensitive the 3M Molecular Detection System method was for *Salm. Ealing* in the presence of the intrinsic flora of minced meat. This food was chosen as it would contain a large variety of other bacteria, including potential competing Enterobacteriaceae, as well as being a complex food matrix which could cause other inhibitory conditions for the assay.

Experimental Outline

- Raw minced meat had been incubated in BPW (1:10 ratio) overnight at 37°C.
- *Salmonella Ealing* had been grown in BPW overnight at 37°C.
- Decimal dilutions of *Salmonella* were prepared in saline and used to spike the minced meat-BPW at known levels of *Salmonella*.
- The standard protocol was followed, with 20µl sample removed to the lysis tube, etc.
- Reagent (positive) and negative controls gave the appropriate results.

Results

Total Flora (cfu/mL)	Enterobacteriaceae (cfu/mL)	<i>Salmonella Ealing</i> (cfu/mL)	<i>Salmonella</i> as % of Intrinsic	<i>Salmonella</i> Detected
8 x 10 ⁸	4 x 10 ⁸	4.6 x 10 ⁶	0.5	+
8 x 10 ⁸	4 x 10 ⁸	4.6 x 10 ⁵	0.05	+
8 x 10 ⁸	4 x 10 ⁸	4.6 x 10 ⁴	0.005	+
8 x 10 ⁸	4 x 10 ⁸	4600	0.0005	-/+ ^a
8 x 10 ⁸	4 x 10 ⁸	460	0.00005	-
8 x 10 ⁸	4 x 10 ⁸	46	0.000005	-
8 x 10 ⁸	4 x 10 ⁸	5	0.0000005	-
8 x 10 ⁸	4 x 10 ⁸	-	-	-
8 x 10 ⁸	4 x 10 ⁸	-	-	-
8 x 10 ⁸	4 x 10 ⁸	-	-	-
8 x 10 ⁸	4 x 10 ⁸	-	-	-

^a In duplicate samples, one was positive and the other was negative. The shape of the positive curve was slightly atypical with a shoulder.

This experiment shows the 3M Molecular Detection System can detect *Salm. Ealing* at an initial (enriched BPW) level of 4.6 x 10⁴ cfu/mL when only present at 0.005% of the total flora. This is a considerably small fraction (1/1000) of the expected *Salmonella* spp. level in BPW following normal overnight sample pre-enrichment and reflects the robustness of the detection procedure.

Additional Comments

Use of the 3M Molecular Detection System only required laboratory bench space for the detector and connected laptop computer, plus heating block. These all have small footprints. The chill block could be stored in the -20°C freezer until required.

Care must be executed when handling the Chill Block from the -20°C freezer. Use of the 3M Molecular Detection System was undertaken by undergraduate and graduate-level laboratory personnel after initial training. It is implied that such personnel need to have appropriate microbiological training for the safe use of bacterial pathogens such as *Salmonella* and *Listeria* spp.

► Concluding Statement

The term “rapid detection” has become a cliché in food microbiology and has often equated to “quicker than traditional” which is undefined — or “quicker than before” which is unhelpful. We have considerable experience in the isolation and identification of foodborne bacterial pathogens and are well able to evaluate the potential of the 3M Molecular Detection System. It was not directly relevant to our report here to discuss the background development of combining DNA-sequence amplification and bioluminescence, nor the design and engineering of the equipment. But suffice to say that the use of a single temperature appears to give both a more rapid detection of the target organism compared to PCR while using equipment with a small footprint.

Our preliminary studies with the 3M Molecular Detection System have confirmed its application, after initial overnight pre-enrichment, to detection of the target organisms — *Salmonella* spp. and *Listeria* spp. — within 75 minutes. In fact, to us, the necessary time to detection period could be even shorter and could be reduced to 45 minutes.

Given previous studies had focussed on specificity of the nucleotide amplification using large numbers of relevant bacterial strains, we undertook our studies to primarily consider interference by the food matrix and other intrinsic bacteria. In our opinion the use of non-target organisms chosen from culture collections is informative, but limited due to the users’ pre-selection approach. Our approach was to determine if the target organism was detectable in the presence of intrinsic flora of the food, which had been allowed to grow during the pre-enrichment stage of the detection procedures. We used pasteurised milk and minced meat as the food matrixes in which to detect *Salmonella* serovars and *Listeria* spp. The background flora grew to levels of $\sim 10^{11}$ cfu/mL overnight in the pre-enrichment broths. We were able to detect *Salmonella* serovars and *Listeria* spp. in the presence of such background flora, which were in milk and minced meat.

Although the standard requirement is the presence/absence of *Salmonella* serovars in 25g food material, it was of interest to determine the level of sensitivity. We found the 3M Molecular Detection System detected *Salmonella* serovars at the level of 10^4 and even below in a background of $\sim 10^{10}$ cfu/mL intrinsic flora. This was reassuring as one does not know in the standard pre-enrichment phase how well the *Salmonella* have grown and may be missed in the following culturing stages. **We found it remarkable that the detection of such low numbers of *Salmonella* cells was achieved within 45 minutes.** Consequently, in a commercial situation given that the majority of samples normally being analysed will be *Salmonella*-free, the decrease in samples needing to be processed through to selective agar inoculation could be considerably reduced. This would result in reduced labour-time, consumables and associated costs. This is achievable using a procedure which a reasonably competent laboratory worker could operate. Additionally, the equipment has the benefit of being able to detect different target organisms at the same time.

To us, the 3M Molecular Detection System combined ease of use, reliability and remarkable rapidity into a unit with a small footprint. The ability to use the same unit for different target organisms, with the opportunity to expand to further target organisms, is a significant addition towards food safety.