Development of a rapid on-farm Test for the Detection of *Campylobacter* in Poultry

Final Report

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CONTENTS

Abbreviations

Ab	Antibody
Abs	Antibodies
cfu	Colony forming unit(s)
FSA	Food Standards Agency
LF	Lateral flow
LFD	Lateral flow device
nsb	Non-specific background signal
N/A	Not applicable
SD	Standard deviation
LOD	Limit of detection
~	Approximately
SVS-UoB	School of Veterinary Science, University of Bristol
AFBI	Agri-Food and Biosciences Institute, Belfast
Ag	Antigen
ELISA	Enzyme-linked immunosorbant assay
WB	Western blotting
SDS	Sodium dodecyl sulphate
NP40	Nonidet P-40
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Mabs	Monoclonal antibodies
Pabs	Polyclonal antibodies
mtp	microtitre plate
ncm	Nitrocellulose membrane
N/A	Not applicable
P-01	Prototype 01 (test strip)
IR	Interim Report

1) INTRODUCTION

This Report describes the development of a lateral flow test strip for rapid on-farm detection of *Campylobacter* in poultry. The work was funded by the Food Standards Agency (FSA) and was divided into two Work Packages. In Work Package One, prototype-01 lateral flow test strip was developed and field tested, and in Work Package Two, the test strip and system was modified and optimised to produce two [pre-prototype-02] test strips with improved sensitivity.

2) AIMS AND OBJECTIVES

The aim of the project was to develop a low-cost lateral flow test strip for rapid on-farm detection of *Campylobacter* in poultry in accordance with the guidelines described in the FSA/TSB-SBRI Competition.[1] The purpose of the test was to enable poultry farmers to monitor the *Campylobacter* status of their flocks in order to assess the success/failure of biosecurity procedures. According to the original proposal submitted to the FSA, the four scientific Objectives of the project were:-

Objective 1: Development of *Campylobacter* immunoassay

Expected achievement: Develop a *Campylobacter* detection immunoassay in a microtitre plate and identify the best [immobilised and detection] anti-*Campylobacter* antibody pair. These antibodies will be used to port the assay onto a lateral flow test strip platform.

Objective 2: Production and evaluation of prototype lateral flow test strips

Expected achievement: Port the assay onto a lateral flow platform, create a prototype test strip for lab testing, modify as necessary and then manufacture a batch of test strips packaged in a test kit, i.e., develop a prototype product containing all the necessary accessories for on-farm testing of poultry flocks.

Objective 3: Field testing of *Campylobacter* detection test kit

Expected achievement: Field evaluation of the test kit by farm operators.

Objective 4: Project close-out

Expected achievement: Final report describing key development activities, experimental data, conclusions drawn from the work and recommendations for further development and commercialisation activities.

3) SCOPE OF THIS REPORT

This is the project's Final Report (Deliverable 10). It summarizes nine preceding Interim Reports in which the technical aspects of the development work are described in greater

[1] http://www.innovateuk.org/_assets/pdf/competition-documents/briefs/sbri%20_fsa_45_002%20brief%20campylobacter%20v4%20(2).pdf

detail. It comments on technical hurdles encountered during development and how they were overcome, and appraises progress made to date by highlighting key findings and unique selling points (USPs) of the proposed technology and its potential industrial impact in addressing the rising problem of *Campylobacter* in the human food chain.[2] The Report offers suggestions for further work and concludes with some recommendations.

4) WORK PACKAGE ONE: Development of Prototype-01 Test Strip

4.1 Project Team

The project team and their role in the development work is summarised in Table 4.1.

Table 4.1 – Project Team

Team member	Role
Moredun Scientific	Principal Investigator / Assay development and characterisation
Forsite Diagnostics	LFD prototype development and manufacture
Modha Biomedical	Project management and coordination

In addition to the above project team, field evaluation was conducted on poultry farms by Dr Vivien Allen's group (School of Veterinary Science at the University of Bristol).

4.2 Project Plan

The project commenced in March 2011 and concluded 3 months prematurely in September 2012 (see Table 4.2). Therefore, project duration was 18 months.

Table 4.2 – Project Plan

Work Package	Stage	Duration	Activity
WP1	Phase 1	Mar – Jun 2011	Assay development and identification of suitable anti-*Campylobacter* antibodies
	Phase 2	Jul – Oct 2011	Assay porting and development of Prototype-01 lateral flow test strip
	Phase 3	Nov – Dec 2011	Field evaluation and benchmarking
WP2	Phase 3a	Jan – Jun 2012	Optimisation (modification of Abs) and development of Prototype-02 test strip
	Phase 3b	Jul – Sep 2012	Characterisation of Prototype-02 test strip and optimisation of samples
	Phase 3c	Oct – Nov 2012	Field evaluation of P-02 test strip with optimised samples (boot swabs and faeces)
	Phase 4	Dec 2012	Project close-out and Final Report

[2] "*BBC report highlights rise in campylobacter incidences*"… (World Poultry, 22nd January 2013)

4.3 Project Milestones and Deliverables

The project's objectives, milestones and deliverables (interim reports) are summarised in Table 4.3 below.

Table 4.3 – Project Objectives and Milestones and Interim Reports

Objective	Milestone	Description of work (milestone)	Interim Report	Deliver-able #	Milestone completed?
Set-up	Set-up	Project set-up and identification of anti-*Campylobacter* antibodies	IR-01	D1	Yes
01	01/01	Selection of anti-*Campylobacter* antibodies	IR-02	D2	Yes
02	02/01	Porting a *Campylobacter* assay onto a Lateral Flow Platform	IR-03	D3	Yes
	02/02 02/03	Lab testing, Optimisation and Manufacture of a Prototype *Campylobacter* a Lateral Flow Test Strip (and Kit)	IR-04	D4	Yes
	02/03	Sensitivity of the Test Strip: Benchmarking against conventional microbiological techniques	IR-04 Addendum		
03	03/01	Field Evaluation of a Prototype *Campylobacter* Lateral Flow Test Strip	IR-05	D5	Yes
		Work Package 1: Close-out	IR-06 Draft IR-07	D6 D7	Yes
	03/02	Work Package Two: Set-up, strategy and experimental plan	IR-08	D8	Yes
	03/03	Prototype-02 test strip developed and characterised (basic assay parameters determined)	IR-09	D9	80% completed
	03/04	Sample preparation optimised (optimal run buffer / concentration of boot swabs)			50% completed
	03/05	Prototype-02 test strips manufactured and field evaluation and benchmarking completed			No
04	04/01	Project closed-out	Final Report	D10	Yes

A summary of the development, optimisation and lab- and field evaluation of the test strip is described below with citation of the interim reports where further details of the work is reported.

4.4 Project set-up and identification of anti-*Campylobacter* Abs (Interim Report 01)

Interim Report 01 describes details of the project set-up activities[3] leading up to the experimental assay development work described in Objective 1 above. A key feature for the success of this project was to identify the best anti-*Campylobacter* antibodies to take forward from assay development (Moredun Scientific) to the assay porting work aimed at creating prototype test strip (Forsite Diagnostics). The due diligence performed on identification and selection of antibodies to *Campylobacter* is described in Interim Report 01.

[3] For example, Contract Agreements, designing the experimental strategy, procuring key equipment, staff training, identification of protocols and procurement and preparation of *C. jejuni* and *C. coli* stock cultures.

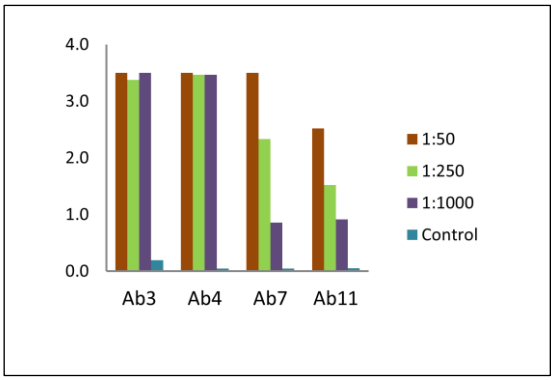
4.5 Selection of Optimal anti-*Campylobacter* antibodies (Interim Report 02)

Interim Report 02 describes experimental evaluation of 12 anti-*Campylobacter* antibodies identified as being potentially valid for use in development of lateral flow test strips at Moredun Scientific and pertains to the milestone of Objective 1.

From a list of 43 available anti-*Campylobacter* antibodies, 12 (8 monoclonals and 4 polyclonals) were procured and tested by direct ELISA using preparations of *C. jejuni* (strain ATCC 29428 and/or 81176) at 10^7 cfu/mL in the detergents 1% NP40, 1% SDS, 2% CHAPS or PBS as antigen to coat the microtitre plates. Since optimally performing Abs were desired, assays were incubated for only 10 minutes prior to addition of conjugate and development with substrate. Four Abs reporting the highest signal (OD_{450nm}) were selected for further study, i.e., Ab3, Ab4, Ab7 and Ab11. These four Abs were further examined to identify which performed best (i.e., reported the greatest signal) at the highest dilution where three dilutions were tested; $^1/_{50}$, $^1/_{250}$ and $^1/_{1000}$. These experiments demonstrated that two antibodies, Ab3 and Ab4, were the best pair for porting onto a lateral flow platform, since they not only reported high signals (OD_{450nm} >3.0) at $^1/_{1000}$ dilution, but also did so without requiring the use of detergent for antigen preparation, i.e., antigen was prepared in PBS alone (see Figure 4.1).

Figure 4.1 – Optimal pair of anti-*Campylobacter* Abs

The Figure shows the OD_{450nm} signal (y-axis) reported by four anti-*Campylobacter* Abs (X-axis) at the dilutions indicated in a 10-minute ELISA using *C. jejuni* at 10^7 cfu/mL in PBS.



4.6 Porting *Campylobacter* assay onto a Lateral Flow Platform (Interim Report 03)

This report describes the early stages of work at Forsite Diagnostics aimed at porting the *Campylobacter* assay onto a platform suitable for development of a prototype lateral flow test strip. As such, the work reported in Interim Report 03 pertains to the first milestone of Objective 2 (i.e., milestone 02/01) and formed a part of the work identified as "Phase 2" of the project (specifically, Phase 2a).

To develop a prototype test strip, the two selected anti-*Campylobacter* antibodies (Ab3 and Ab4) were tested in two possible positions in the test strip (conjugate pad and/or T-line) using one of two possible detection labels (colloidal gold or latex beads) on a nitrocellulose platform comprising either a Pall membrane or a Sartorius membrane. This resulted in experimentally examining 16 possible permutations for test strip configuration. These are

illustrated in Figure 4.2. Permutations highlighted in yellow (nos. 6, 7, 10, 11 and 14) showed promising results when experimental test strips were constructed. However, best results in terms of optimal signal intensity on the T-line were seen with permutation nos. 2 and 3 (highlighted in blue in Figure 4.2). Both of these employed Ab3 on the T-line and on the conjugate pad, the difference being one test strip used colloidal gold as detection label and a membrane from Pall (no. 3) whereas the other used blue-coloured latex beads and a membrane from Sartorius (no. 2). Both membranes are typically categorised as membranes with medium flow rates.

Figure 4.2 – Sixteen possible permutations for specification of prototype test strip

		T-line							
		Goat (Ab3)				**Rabbit (Ab4)**			
Conjugate Pad		**Sartorius**		**Pall**		**Sartorius**		**Pall**	
	Goat (Ab3)	1 Gold	2 Latex	3 Gold	4 Latex	5 Gold	6 Latex	7 Gold	8 Latex
		Sartorius		**Pall**		**Sartorius**		**Pall**	
	Rabbit (Ab4)	9 Gold	10 Latex	11 Gold	12 Latex	13 Gold	14 Latex	15 Gold	16 Latex

When experimental test strips corresponding to permutation nos. 2 and 3 above were tested using *C. jejuni* and *C. coli* antigen at 10^7 cfu/mL in PBS, test strips using colloidal gold demonstrated some non-specific background (nsb, arrowed in Figure 4.3) signal on the T-line whereas those using latex beads did not (note, this situation was to change once faeces was included in the sample).

Therefore, it was decided to produce test strips for experimental purposes based on permutation 2; for example, using Sartorius membrane with Ab3 on the T-line and on the conjugate pad and with latex beads as the detection label.

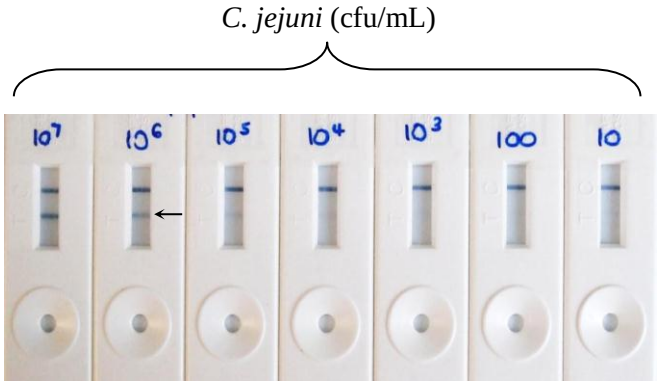
Figure 4.3 – Experimental lateral flow test strips

Experimental test strips using Ab3 on the T-line and on the conjugate pad with colloidal gold or latex beads as detection label. Both types of test strips were probed with antigen prepared in PBS using *C. jejuni* or *C. coli* at 10^7 cfu/mL as indicated.

Sensitivity testing indicated that a test strip with this configuration had a LOD of 10^6 cfu/mL (arrowed, Fig. 4.4) and a strong visible signal at 10^7 cfu/mL using *C. jejuni* or *C. coli* in PBS (see Figure 4.4).[4]

Figure 4.4 – Sensitivity of experimental test strips

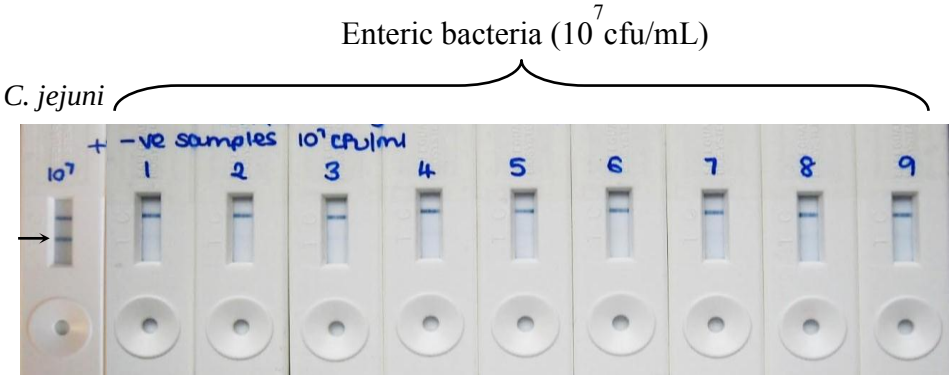
Lateral flow test strips were probed with 10^7 to 10^1 cfu/mL *C. jejuni* in PBS.



The test strips were specific for *C. jejuni* (arrowed, Fig 4.4 and 4.5) and *C. coli* (not shown) but did not recognise nine other enteric bacteria (see Figure 4.5).

Figure 4.5 – Specificity of experimental test strips

Lateral flow test strips were probed with 10^7 cfu/mL of *C. jejuni* or one of nine other species of enteric bacteria as indicated.



1. *Salmonella typhimurium*
2. *Serratia marcescens*
3. *Clostridium perfringens*
4. *Pseudomonas aeruginosa*
5. *Klebsiella pneumoniae*
6. *Bacillus subtilis*
7. *Staphylococcus aureus*
8. *Escherichia coli*
9. *Enterococcus faecalis*

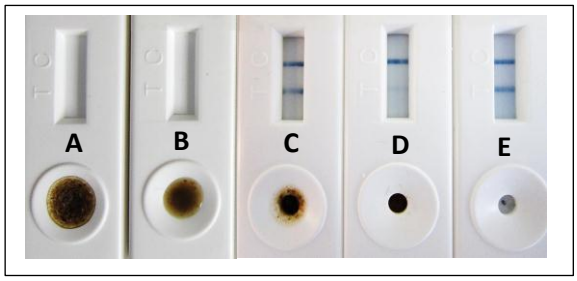
[4] Later, when spiked faecal samples were used for sensitivity testing, the LOD dropped to $3x10^7$ cfu/g.

After initial assay porting in the $Ab3^{latex}$:$Ab3^{T\text{-}line}$ format on Sartorius membrane using latex as the label, putatively negative poultry faeces samples spiked with *Campylobacter* were tested to determine if the ported assay can detect *Campylobacter* in the matrix of poultry faeces. These experiments showed that although a high concentration of faecal debris plugged the aperture of the sample application port and blocked the filter pad underneath (see Figure 4.6, A and B), *C. jejuni* could be detected in poultry faeces if sample was diluted $^1/_4$ and allowed to sediment for 2 minutes (Figure 4.6, C).

Figure 4.6 – Detection of *C. jejuni* in poultry faeces

A) $^1/_2$ dilution of hen faeces spiked with $3x10^7$ cfu/mL of *C. jejuni*.
B) $^1/_3$ dilution of hen faeces spiked with $3x10^7$ cfu/mL of *C. jejuni*.
C) $^1/_4$ dilution of hen faeces spiked with $3x10^7$ cfu/mL of *C. jejuni*.
D) $^1/_4$ dilution of hen faeces spiked with TBST buffer.
E) TBST buffer spiked with 3×10^7 cfu/mL of *C. jejuni*.

In Fig 4.6A the sample application aperture is plugged with faecal debris (sample has dried) and in Fig. 4.6B the sample filter pad is blocked by faecal debris and so the sample still sitting on top of the filter pad at the sample application port. In both cases the sample has failed to pass through the filter pad to be released onto the membrane in order to perform the test. Therefore, as can be seen in test windows A and B (which are clear), the samples failed to run. However, when the dilution of the sample is adjusted, as in Fig. 4.6 C and D, the sample application port and the filter pad underneath are not blocked and so samples flow well in the test strip. Fig 4.6E shows that no sample flow problems are evident when there is no faecal debris in the bacterial sample.



An observation that was noted at this stage was that the LOD of the test strips was reduced by 1.5 log, from $\log_{10}$ 6.0 to $\log_{10}$ 7.5 cfu/mL, when testing was performed using spiked faecal samples in comparison to *C. jejuni* prepared in buffer alone. Therefore, loss of sensitivity due to matrix interference was observed and required circumvention.

At this stage, the objective of Phase 2a (i.e., to port anti-*Campylobacter* antibodies Ab3 and/or Ab4 onto a lateral flow platform and develop an initial working prototype assay) was achieved and milestone 02/01 of the project was satisfied.

Work then proceeded to characterise basic assay parameters using the prototype test strip in the lab (i.e., milestone 02/02), with a view to optimising the strip where necessary and possible prior to on-farm testing using real poultry samples (i.e., milestone 03/01).

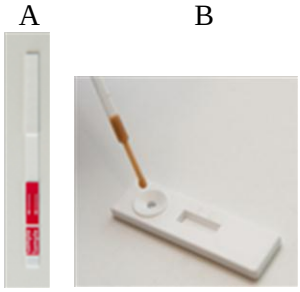
4.7 Lab testing, Optimisation and Manufacture of a Prototype *Campylobacter* a Lateral Flow Test (Interim Report 04)

Interim Report-04 describes laboratory testing of the first prototype *Campylobacter* lateral flow test strip (Prototype-01), subsequent optimisation work and manufacture of the first batch of test strips in kit form for field testing. The reported work pertains to two milestones of Objective 2 (i.e., milestones 02/02 and 02/03) and forms a part of the work identified as 'Phase 2' of the project (specifically, Phase 2b and 2c). This is described briefly below.

4.7.1 Lab testing and characterisation

A batch of 120 prototype strips in two formats, dipsticks and cartridges (see Figure 4.7), was produced for lab testing at Forsite and Moredun using *Campylobacter*-negative faecal samples spiked with various concentrations of *C. jejuni* and *C. coli*.

Figure 4.7 – Lateral flow test strips in two formats; dipstick (A) and cartridge (B)



The purpose of lab testing work was to characterise the performance of the test strips using mock samples and to identify and iron out any anomalies with respect to signal generation and intensity (assay running time/speed), non-specific background on the T-line, discolouration of the test strip due to sample soiling, sample application and flow, and operator issues (e.g., strip handling and/or interpretation).

Results of lab testing and characterisation of basic assay parameters are summarised in Table 4.4 below. The work demonstrated that the experimental test strip could detect *C. jejuni* and *C. coli* if faecal samples comprised $\geq 3 \times 10^7$ cfu/g. Hence, there was scope for improvement in making the test strip more sensitive, and also in removing non-specific background signal (nsb) which was thought to be due to conjugate directly binding to the T-line, most probably by protein-protein interactions.

However, for concentrations of *Campylobacter* that were within the capability of this first pre-prototype test strip (e.g., $\geq 10^7$ cfu/g), the reproducibility of the measurements was good, with a CV of < 20% (see Table 4.4).

Table 4.4 – Summary of Results from Lab Testing

Parameter	**Forsite** (data using LFD meter)	**Moredun** (by visual examination)	**Comments**
Assay time	< 10 minutes	< 10 minutes	Satisfactory
Specificity	*C. jejuni* & *C. coli*	N/A	Test strips did not detect nine other gram negative and gram positive enteric bacteria
Working range	$10^6 – 10^9$ cfu/g	$10^7 – 10^9$ cfu/g	Scope for improvement
Linearity	$10^6 – 4x10^8$ cfu/g	N/A	This parameter is less pertinent in a visually detected signal
LOD	$3x10^6$ cfu/g	$3x10^7$ cfu/g (visual)	Scope for improvement
Repeatability	CV < 20%	N/A	Very satisfactory

Other observations noticed at this stage were that faecal samples occasionally discoloured the test strip membrane (called 'blueing') by causing the blue dye on the latex beads to leach off and bind directly to the membrane.

It was also discovered that the dipsticks would require more sample to be applied to the sample pad (~200µL) than test strips in cartridge format (100µL). Better still, would be to immerse the dipstick in approximately 250µL of sample in an eppendorf tube. The dipstick test strip could not be flooded, whereas the one in cartridge format could. Hence, the sample volume applied to the two types of test strips needed to be controlled accordingly.

Lab testing showed that the sample (chicken faeces) caused assay interference; namely, loss of sensitivity of at least 1 log cfu/mL compared to *Campylobacter* in buffer alone, slightly increased nsb and occasional soiling of the test strip membrane. Hence, although previous problems associated with the sample (such as faecal debris blocking the sample port and clogging the filter) were resolved, further work was necessary to optimise the test strip.

4.7.2 Optimisation of the test strip

In seeking to optimise the performance of the test strip, two modifications were beneficial. Firstly, increasing the assay run time from 10 minutes to 15 minutes reduced discolouration of the membrane. Secondly, replacing the glass fibre filter in the sample pad (which was prone to blockage by faecal debris) with a slightly thicker (12mm) cellulose-based filter (CO83) allowed a better flow of sample through the filter and onto the release pad.

An attempt to increase assay sensitivity by increasing the amount of conjugate on the release pad was unsuccessful as it increased the nsb signal as well. Similarly, attempts to reduce the nsb by increasing the concentration of the [polymer/sugar] blocking solution during nitrocellulose membrane preparation were unsuccessful.

It was noticed that poultry faeces decreased the pH of the diluted faecal sample from pH 7.0 to pH 6.6. This was not thought to have a significant effect on the performance of the test strip but it was clear that in the future, the buffering capacity of the sample buffer will need to be increased. This was not a modification that could have been made in time for field testing

activities because there was no time available, no additional anti-*Campylobacter* antibody (Ab3) available and no budget to assess its impact.

The performance specification of the assay/test strip at this stage is indicated in Table 4.5.

Table 4.5 – Assay specification

Parameter	Specification
Assay type	Sandwich immunoassay, semi-quantitative (low, moderate, high)
Analyte	*C. jejuni* whole organisms (and antigens)
Sample type	Fresh poultry faeces ($^1/_5$ dilution required to prevent blockage of port)
Sample volume	100 µL (cartridge) and ~200 µL (dipstick)
Assay time	< 15 minutes
Specificity	*C. jejuni* and *C. coli*
Sensitivity (LOD)	$3x10^7$ cfu/mL faeces visually … ~ 10^6 cfu/mL using Forsite's reader
Working range	10^7 … $>10^9$ cfu/mL faeces
Detection system	Latex beads (blue-coloured)
Sample buffer	PBS + 0.05% Tween 20-100, pH 7.0, + 0.5% casein
Test strip design	Dipstick and cartridge
Storage (temperature)	Ambient (4 – 40^{o}C)

The assay was rapid, reporting a result in less than 15 minutes (usually within 10 minutes), easy to perform on-site and capable of detecting *C. jejuni* and *C. coli* in faeces from chickens with a moderate or high level of colonisation, i.e., birds depositing ≥ $3x10^7$ cfu/g or mL faeces. The test strip required an inoculum of ~10^7 total cfu in order to produce a *strong* visible signal on the T-line. As such, the test strip demonstrated a similar level of performance to that developed at the Austrian Agency for Health and Food Safety and reported by Wadl *et al*., 2009.[5]

It was noticed that using Forsite's portable reader to quantify (and digitalise) the signal reported by the strip, the strip offered increased sensitivity, adding an extra 1 log cfu/mL to its performance. This would take the LOD down to ~10^6 cfu/mL faeces, which was equivalent to 10^5 cfu total in the 100µL inoculum that is applied to the test strip. However, no further development work could be carried out at this point. A batch of strips awaited manufacture for field testing and we had delayed Forsite's timelines (and overstretched its budget) and the Company needed to complete on our contract and attend to the work of other clients. Moreover, it was felt that the best time to carry out further development work would be after field testing when other modifications to optimise the test strip may also be indicated/required as a result of evaluating the test strip using 'live' or real samples. In fact, looking back at the results of field evaluation, this has been the case.

[5] Wadl, M. et al. (2009) Easy-to-use rapid test for direct detection of *Campylobacter* spp in chicken faeces. J. Food Production **72** (12), 2483-2488.

4.7.3 Manufacture of Prototype-01 Test Strips

After lab testing and optimisation, manufacturing specifications for the first prototype test strip were drawn up and a batch of 300 dipsticks and 300 cartridge test strips was manufactured at Forsite for field testing. This exhausted our supply of anti-*Campylobacter* antibody (Ab3) that was purchased for the project and no further supplies were available within the timeframe of this phase of the work.

4.7.4 Manufacture of Camp-U-Test Kit™ (the product concept)

The test strips were assembled into a "Camp-U-Test™" kit comprising 20 strips in each format and included all the necessary accessories to enable poultry farmers to evaluate the test strips on-site without the need for additional resources, consumables or equipment (see Figure 4.8).[6] These components and accessories were purchased from commercial sources (e.g., sample tube with scoop, small epp tubes, plastic droppers and nitrile gloves), made by Forsite/Modha Biomedical (i-card and kit insert) or manufactured externally by a specialist company (box and pull-out rack for sample tubes).

Figure 4.8 – Camp-U-Test™ Kit

The kit components were packaged into a purpose-built box, which included a rack for sample tubes which comprised plastic universals and 0.5mL 'epp' tubes (see Figure 4.8). The dipsticks were supplied in a plastic tub with desiccant and the cartridge test strips were individually foil wrapped with desiccant. Instructions for performing the test using both types of test strips were developed with illustrations for ease-of-use.[7]

Therefore, Objective 02 was achieved and its milestones, namely, 02/02 and 02/03, were satisfied.

4.8 Field Evaluation (Interim Report 05)

The test kits were dispatched on 25/10/2011 to Moredun Scientific (5 kits) and the School of Veterinary Science at the University of Bristol (SVS-UoB, 6 kits) for field evaluation (milestone 03/01) using faecal samples and boot swabs on poultry farms.[8] One kit was sent to AFBI, Belfast, for assessment and to develop a protocol for testing boot swab samples.

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[6] We were strongly urged to solicit end-user opinion as soon as possible in our remit (reviewed application).

[7] Needless to say, 'productising' or packaging the test strips into a complete product concept for evaluation was a significant undertaking. However, it demonstrated that the test strip technology and all the accessories necessary for testing can be realised by this project consortium into a product concept and it was a response to the request of one of the reviewers of our application and to our remit to obtain end-user opinion of our proposed technology as soon as possible.

[8] It was only possible to manufacture 14 complete test kits with the available antibody. From these, 11 were sent to Moredun and Bristol, one to AFBI, one to the project manager (Jay Modha) and one retained at Forsite as a contingency for future reference with respect to specification.

For field testing, one poultry house per farm was tested and two types of samples were examined; boot swabs and faecal samples. A farm operator carried out some sample collection and testing according to the kit instructions (in order to record end-user opinion) and the lab technician completed the remainder of the testing protocol, as necessary. The samples collected (and tested using the test strips) on the farm were subsequently taken back to the lab as all the test strips were benchmarked against conventional microbiological methods for comparison of their performance.

Dr Vivien Allen's group at SVS-UoB commenced and completed field evaluation in November 2011. Moredun Scientific subsequently delayed its work of field testing pending further improvement of the test strip, based on field results from SVS-UoB. Results from AFBI (Malcolm Taylor) on their experience with the test strip are pending.

A summary of the results of field testing from SVS-UoB is shown in Table 4.6.

Table 4.6 – Summary of results from LF test strips and microbiological methods

Sample	Test	Farm B	Farm C	Farm D
Faeces (2x1g in 10mL)	LF test strip (n=12)	< LOD	< LOD	< LOD
	Campy culture (log_{10} cfu/mL) ~ 0.1mL applied to test strips	5.7 ± 0.6 (range, 4.3 – 6.7)	5.3 ± 1.2 (range, 2 – 7.8)	4.6 ± 0.4 (range, 3.3 – 5)
Boot swabs (pair, 100mL)	LF test strip (n=2)	< LOD	< LOD	< LOD
	Campy culture (log_{10} cfu/mL) ~ 0.1mL applied to test strips	7.4 ± 0.4	6 ± 0.4	5.1 ± 0.4

Field testing demonstrated that although both types of test strips operated correctly, all the test strips reported negative results with faecal samples and with boot swabs in comparison to conventional microbiological methods (see Table 4.6). This was most likely because the extent of *Campylobacter* colonisation of the tested flocks (and therefore, the cfu/mL of the inoculum applied to test strips) was lower than the limit of detection (LOD) of the test, which according to lab data was $3x10^7$ cfu/mL (or log_{10} 7.5). This conclusion is supported by data in Table 4.6 from conventional microbiology which shows that the average number of *Campylobacter* in the [diluted] samples derived ranged from log 4.6 – 5.7 cfu/mL (i.e., $4x10^4$ to $5x10^5$ cfu/mL) which are all below $3x10^7$ cfu/mL, the LOD of the test strip. There was also some deviation from the prescribed sampling and testing protocol and this may have significantly affected the results but its impact (after the event) is difficult to quantify.

Therefore, although the test strips operated correctly, the sensitivity of the prototype-01 strip and sample preparation required optimisation. The sensitivity of the *whole system* (i.e., LOD of the test strip, plus improvements in signal detection and to sample preparation) needed to be improved by 2.5 – 3 logs, in order for the test strip to report a clearly detectable signal from low to moderately infected samples, i.e., those containing 10^5-10^7 cfu/g faeces, as well

as those containing a high number of organisms (e.g., $>10^8$ cfu/g faeces). Table 4.7 describes the modifications that were proposed to increase sensitivity by 3 logs in total.

Table 4.7 – Modifications to the test strip anticipated to benefit assay sensitivity

#	Proposed modification		Expected increase in signal	
			Linear (fold)	**Log_{10}**
1	Test strip	Utilise the biotin-streptavidin signal amplification system to optimise detection reagent	100-1000	2-3
2		Use colloidal gold as label		
3	Signal detection	Detect signal using electronic handheld meter	5-10	0.7-1.0
4	Sample buffer and preparation	Optimise running buffer strength and salt concentration	2	0.3
5a		Faeces: increase the faeces concentration in the sample from 0.2g/mL to 1g/mL by either reducing the volume of sample diluent from 10mL to 2mL, or by increasing the amount of faeces from 2g to 10g in the sample tube containing 10mL kit diluent	5-10	0.7-1.0
5b		Boot swabs: use neat sample (in a smaller prep volume) or concentrate 100mL sample 5-10 fold		
	Cumulative effect of modifications (total)		**5000 – 200,000**	**3.7–5.3 logs**
	Expected improvement		**1000**	**3 logs**

4.9 Close-out of Work Package One (Interim Reports 6 and 7)

These modifications are described in detail in Work Package One Close-Out Report (Interim Reports 6 and 7).

The proposed modifications were not performed previously because the issue of insufficient sensitivity was not known prior to field testing. In that sense, the proposed modifications were remedial. The prototype to date defined the work that needed to be done and was the starting point, not the finishing line, for rapid on-farm detection of *Campylobacter* in poultry, especially in flocks close to their departure date.

5.0 WORK PACKAGE TWO: Development of Prototype-02 Test Strip (Project extension)

The work of this project was extended by additional funds being provided by the FSA to optimise prototype-01 test strip (which was developed in Work Package One) and thereby to create a more sensitive Prototype-02 test strip. Hence, this part of the work was referred to as Work Package Two.

5.1 Set-up, strategy and experimental plan (Interim Report 08)

Interim Report 08 (Deliverable 8) describes the set-up activities and the strategy and experimental plan adopted for optimising the sensitivity of the test strip and system as a whole. The main part of the development work was to improve the performance of the test strip (any improvements made to sample preparation, especially regarding boot swabs, were deemed as a bonus because the existing project partners did not have access to boot swab samples and an additional collaborator would need to be brought on board). The *limit* of detection of prototype-01 strip was ~ $3x10^7$ cfu/mL but the strip required an inoculum of 10^7 cfu of *Campylobacter* in a volume of 0.1mL (i.e., a sample containing 10^8 cfu/mL) to report a strong signal. Results of field testing indicated that the test strip needed to perform well with an inoculum of 10^4 cfu in 0.1mL, i.e., it needed to be able to report a signal detectable using the LFD reader with a sample containing 10^5 cfu/mL of *Campylobacter*.

The strategy for improving the sensitivity of the *Campylobacter* detection *system* as a whole is described in Interim Report 08 (Deliverable 8). Briefly, it involved addressing three aspects of the test system that could, to varying degrees, be modified or improved; namely,

1. the test strip
2. signal detection
3. the sample (i.e., run buffer, concentration of the inoculum applied into the test strip)

The rationale for modifications to each of these is discussed in detail in Interim Report 08 (Deliverable 8). The cumulative effect of the modifications on improvement of signal intensity was anticipated to be approximately 3 logs.

5.2 Optimisation of Test Strip Prototype-01 to create Prototype-02 (Interim Report 09)

Interim Report 09 (Deliverable 9) for milestone 03/03 describes experimental work conducted to optimise the sensitivity of test strip prototype-01 and create prototype-02. By optimisation was meant,

- Increasing the sensitivity of the test strip by 2-3 logs; from a strip that demonstrated a strong visible [by eye] signal at log 7.5-8.0 cfu/mL in faecal samples to one that could detect *Campylobacter* at log 5 cfu/mL (or better) in these samples using Forsite's portable LFD reader.

- Eliminating non-specific binding on the Test (T)-line so that reporting of false positives could be avoided.

A Kick-Off meeting for the work took place on 17 Apr 2012 at Forsite, after which reagents were purchased and detailed protocols developed so that experimental work commenced on 03 May 2012. A total of 42 experiments were conducted in 3.5 months (03 May to 18 Aug 2012)[9] aimed at optimising the sensitivity of the test strip and removing nsb signal on the T-line. Initially (i.e., first 7 weeks, 03 May – 22 June), little progress was made as our key strategy for amplification of signal using the biotin-streptavidin system was unsuccessful. It was difficult to determine whether the problems were due to the inadequacy of the reagents or inexperience of the technical resource deployed by Forsite on the project. However, following discussions with (and technical involvement of) Innova Biosciences and modification of our experimental and operational strategy, progress was made. Two new versions of test strip were developed. The first was an optimised version of Prototype-01 (called "Dev-02") using latex as the label, and the second (called "Dev-03") was a test strip in which colloidal gold nanoparticles were used as a label. Progress in the development of these two test strips is summarised below.

5.2.1 Test strip with Ab3-latex conjugate

Table 5.1 summarises key features of the optimised version of Prototype-01, called "Dev-02" strip, and compares them with its predecessor (referred to as "Prototype 01"), which was developed in Work Package One. Both versions used latex beads as a detection label. The main differences were that with Dev-02 strip, the anti-*Campylobacter* antibodies (Ab3) used to make the detection conjugate Ab3-latex were 'cleaned-up' by chromatography and that a portable LFD reader was used to detect the signal rather than it being detected visually by observing a blue line in the Test zone/line.

Using 'cleaned-up' anti-*Campylobacter* antibodies for the Ab3-latex detection conjugate and for spraying [the membrane] on the Test zone/line significantly reduced the non-specific background (nsb) signal on the T-line (see Table 5.1 and Figure 5.1) and increased the sensitivity of the test strip (see Figure 5.2).

Table 5.1 – Comparison of Prototype-01 and Dev-02 test strips

	Prototype 01	Dev-02
Membrane (flow rate)	Sartorius CN140 (moderate)	MDI-CNPC (fast)
Conjugate pad	Latex	Latex
	Ab3	[cleaned-up] Ab3
T-line	Ab3	[cleaned-up] Ab3
Run buffer	PBST	PBST
Signal detection	Visual (by eye)	LFD reader
LOD	Log 7.3 - 7.7 (visual) Log 6.6 (meter)	Log 5
Nsb (spiked faeces)	350-550	< 150

[9] See Appendix 4.2 in Interim Report 09 (Deliverable 9) for a chronicle of experiments.

Figure 5.1 – Removal of nsb signal on the T-line by chromatography of Ab3

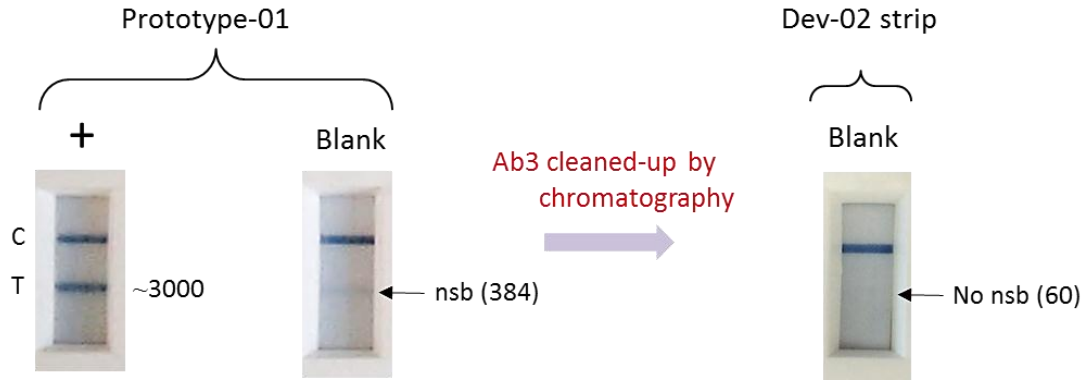


Figure 5.2 compares the performance characteristics of the first prototype test strip (Prototype-01 or P-01) with its successor, Dev-02. Notice, the graph of Dev-02 is shifted to the left, indicating that performance of Dev-02 has improved compared to P-01 in a number of ways. For example,

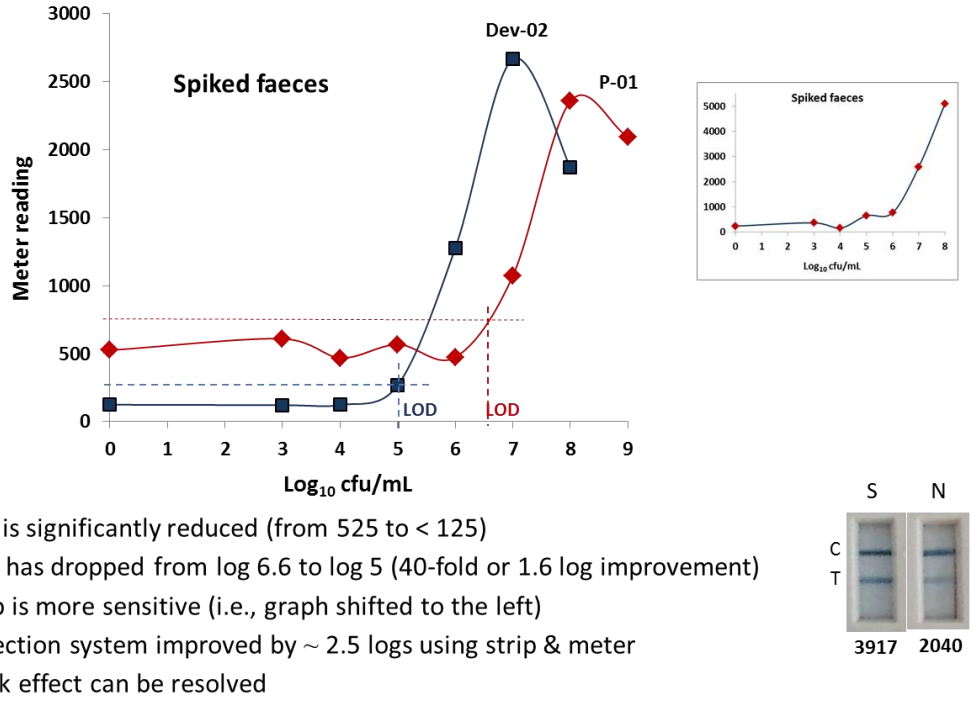
1. The LOD of Dev-02 strip itself is 1.5 log better than its predecessor when both strips are read using the LFD reader (see Table 5.1 and Fig 5.2). Thus, the LOD has improved from log 6.6 in P-01 to log 5 in Dev-02.

2. The detection system as a whole now is 2.5 logs better than it was using Prototype-01 being read by eye, as was the case formerly (Table 5.1).

3. The nsb in Dev-02 was significantly reduced compared to P-01 (see Table 5.1 and Fig 5.1). For example, notice where the lines in the two graphs in Fig 5.2 intersect the Y-axis which is the zero-blank reading, i.e, blue line of Dev-02 hits the Y-axis at a meter reading of ~100, whereas the red line of P-01 hits the same at a meter reading of 525.

4. The strength of a *specific* signal produced by Dev-02 between logs 5 to 8 cfu/mL was higher than that produced by P-01, with the exception of the Hook Effect at log 8 cfu/mL (for which this observation is still true if one looks at the Dev-02 graph in the inset where the log 8 cfu/mL sample is diluted 1 in 2). For example, at log 6 cfu/mL, Dev-02 reported a signal of 1275 whilst P-01 reported 473. Similarly, at log 7 cfu/mL, Dev-02 reported a signal of 2665 whilst P-01 reported 1071. Hence, the overall performance of test strip Dev-02 was better than P-01.

Figure 5.2 – Comparison of Prototype-01 and Dev-02 Test Strips



The Hook Effect, which was observed in Prototype-01, was also seen with Dev-02. This phenomenon occurs when the assay is forced to perform under antigen excess conditions, i.e., too much analyte in the sample. Hence, it is seen at high concentrations of *Campylobacter*, e.g., >log 7 cfu/mL. However, the inset graph in Fig 5.2 shows that dilution of the sample by 1 in 2 at high concentrations of *Campylobacter* (e.g., log 8 cfu/mL) can circumvent the effects of the Hook phenomenon and produce an accurate meter reading for the sample.[10]

In an attempt to identify the most optimal buffer for this test strip, three buffers were examined (see Table 5.2), namely,

[1] Tris-buffered saline with 0.1% Tween 20 and casein (TBSC), pH 8

[2] Buffer D[11]

[3] Extraction buffer C (EBC), pH7.2.

Of these, buffer D enabled the strongest signal to be reported by the test strip (and the lowest nsb signal) in *Campylobacter*-spiked buffer samples; for example, 3 times stronger signal than EBC (see Tables 5.2A and 5.2B). However, it did cause the strip to demonstrate the Hook phenomenon (H) at high concentrations of *Campylobacter*; for example, above log 7 cfu/mL (see Table 5.2A). When spiked faecal samples in buffer D were examined, the results

[10] The meter reading for the ½ diluted sample was doubled.

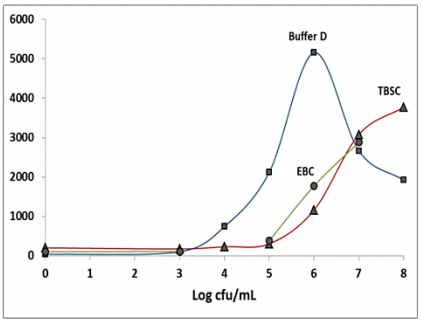
[11] Buffer D was proprietary, hence composition was undisclosed.

were very poor and inconsistent but it is not clear whether this was a genuine result of the incompatibility of buffer D with faecal samples or whether it was due to operator error.

Therefore, extraction buffer C (EBC) was used for evaluation of the Ab3-latex test strip with faecal samples since it performed most consistently with this sample matrix.

Tables 5.2A and 5.2B – Comparison of Run Buffers

Graphical plot of Table 5.2A



- **Buffer D** showed **best performance** (with purified Ab3)
  - ✓ Strongest signal (5154)
  - ✓ Best LOD (log 4 cfu/mL or lower)
  - ✓ Lowest nsb (39)
  - ✓ Best performance at log 5 cfu/mL (554%)
- Hook effect (H) resolves with $^1/_2$ sample dilution, and in faecal matrix

Table 5.2A

Log cfu/mL (buffer)	TBSC	Buffer D	EBC
8	3770	1923 (H)	-
7	3078	2666 (H)	2880
6	1163	5154	1775
5	307	2123	383
4	235	744	-
3	177	106	110
(blank)	204	39	108

Table 5.2B

Run buffer	Meter reading @ 10^5 cfu/mL	Performance (%)
EBC	383	100
TBSC	307	80
D	2123	554

5.2.2 Test strip with Ab3-gold conjugate

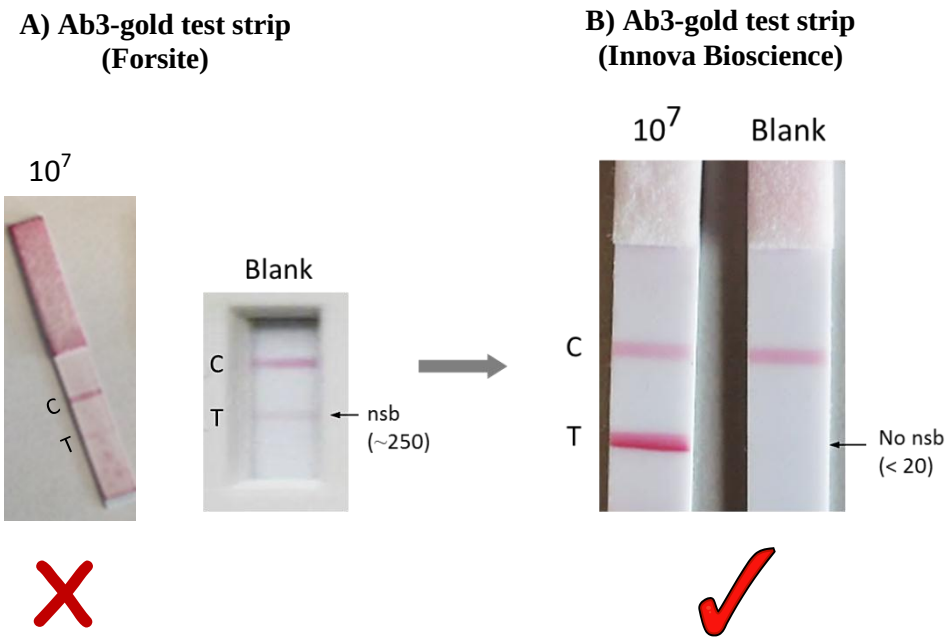
Two types of experimental test strips with Ab3-gold detection conjugate showed promise, although the optimal method for conjugating Ab3 onto the gold particles (e.g., passive adsorption or covalent linkage) remains to be determined. Both types of strips required the anti-*Campylobacter* antibody (Ab3) to be further purified or 'cleaned-up' by chromatography prior to conjugation to 40nm gold particles. Progress in the work undertaken on these two types of strips is described below.

A) Test strip with Ab3-gold conjugate produced by covalent linkage

InnovaCoat Gold™ was used to generate Ab3-gold conjugate but we were not successful in being able to use the reagent productively in the test strip. Signals reported by the strips were very poor and the strips looked ragged, inferior and had many tell-tale signs that the conjugations were not well performed (see the left hand side strip in Fig 5.3A below). Therefore, the manufacturer (Innova Biosciences) kindly offered two free days of consultancy time of its senior development scientist in order to conjugate some of our Ab3 to gold using InnovaCoat reagent, and to use this Ab3-gold conjugate to detect varying concentrations of

Campylobacter spiked into a 'standard' buffer using a 'wet' loading method in experimental strips. Data from this work was very encouraging and is reported below and illustrated in Figures 5.3B and 5.4.

Figure 5.3 – Development test strip using InnovaCoat™ gold to prepare Ab3-gold conjugate



The first observation that was apparent in Innova's test strips was that the signal was much stronger and sharper at 10^7 cfu/mL, the membrane itself was cleaner and nsb on the T-line was eliminated (as can be seen from the arrow in Figure 5.3B above).

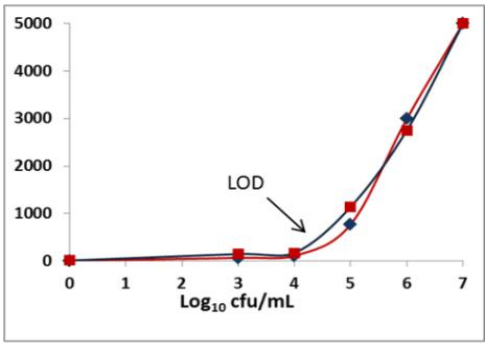
Spiking various concentrations of *Campylobacter* into the standard buffer (Figure 5.4, strips) and buffers B and D (Figure 5.4, table and graph) showed that a visual signal could be detected by eye at log 5 cfu/mL (see Fig 5.4, strips). Using the LFD reader, a signal statistically higher than the blank (zero) could be detected at log 4.3 cfu/mL with both buffers (see Fig 5.4, table and graph). Moreover, the zero-blank was ≤10 units which was the lowest achieved in the project.

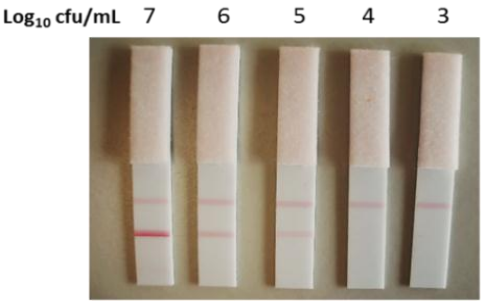
These were the best results achieved with test strips using Ab3-gold as a detection conjugate[12], although it was with spiked buffer and not with spiked faeces. However, the data represents two days' work and expert opinion is that there is much scope for optimisation of the test strip by investigating the optimal concentration of Ab3 for conjugation, evaluating different buffers, membranes (slower membrane might be better), and cleaning-up the Ab3 sprayed on the T-line (note, the present experiments were conducted with non-cleaned-up Ab3 on the T-line; only the Ab3 used for conjugation to gold was cleaned up).

[12] After generating this data, Innova's Technical Lead and Sales & Marketing Manager spent a full day training our operator but it was still not possible to use the InnovaCoat reagent successfully.

Figure 5.4 – Test strips and data from buffer spiking studies using Ab-gold prepared with InnovaCoat Gold™

Log_{10} cfu/mL	Mean of 4 readings	
	Buffer B	Buffer D
7	>5000	> 5000
6	2993	2752
5	769	1139
4	110	171
3	66	145
0	7	10





- Data is with buffer (not spiked faeces)
- LOD ~ log 4.3 cfu/mL (buffer)
- Nsb is low
- Ab3 on T-line was not cleaned-up
- Ab3-gold conjugate is not optimised
- Potential for good linearity
- Ab3-gold conjugate is robust, not pH or salts sensitive)

B) Test strip with Ab3-gold conjugate produced by passive adsorption

In order to advance the work in the absence of developing a successful protocol for InnovaCoat conjugation method (see footnote 12), experimental strips were prepared with 0.5µL of *cleaned-up* Ab3 spotted onto each membrane strip which was then oven dried as usual. These strips were referred to as "T-dot" strips (because a small volume [0.5µL] of Ab3 was spotted onto the membrane as a dot and represented the Test line), and when chromatography purified Ab3 was used, they were referred to as "*pure* T-dot" strips to signify that purified Ab3 was used instead of the commercially supplied Ab3 being used without further chromatographic purification. This principle is similar to dot [immuno]blots, except that with T-dot lateral flow strips, the sample is applied at the bottom end of the test strip (using the conventional wet load method) and allowed to flow up the membrane as with conventional lateral flow test strips. This procedure was followed because sufficient quantity of cleaned-up Ab3 was not available for spraying a membrane to make lateral flow strips with Test *lines* in the conventional way[13] and attempts to generate more cleaned-up Ab3 were unsuccessful.[14]

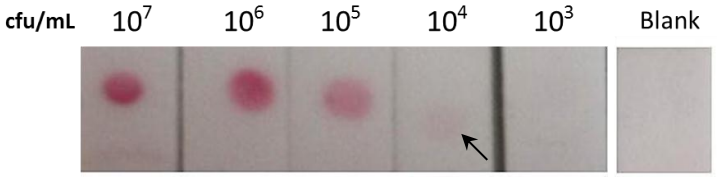
[13] For example, only 80µL at 0.8mg/mL of cleaned-up Ab3 was available, whereas a minimum of 350µL at 1mg/mL was required to spray membranes to make T-lines in the conventional way.

[14] Attempts were made to purify more Ab3 by chromatography but they were not successful. The problem was that either the operator did not collect the correct fraction eluting from the column, or that he failed to gauge correctly the protein concentration of the eluted fraction since an in-house protein assay was not available. Therefore, whatever cleaned-up Ab3 was left from a previous successful purification had to be used.

The Ab3-gold conjugate used on these experimental strips was prepared by a standard conjugation method which involved passive adsorption of the antibody onto gold particles. This method does not always produce a clean preparation of Ab-gold conjugate since conjugation is passively controlled by adjusting the pH of the conjugation; in our case, by performing it at high pH (typically around pH 8.5), which suits adsorption of antibodies (but not most other proteins) to gold particles. Nevertheless, other proteins with isoelectric points in the same region as that of antibody, if they are present in the antibody preparation, could adsorb to the gold particles as well. The passive adsorption reaction, in that sense, is not antibody specific. However, since we were using *cleaned-up* Ab3 for the conjugation, from which contaminant proteins in the antibody preparation would have been removed, we anticipated a cleaner Ab3-gold conjugate. The passive method however does not prevent adsorbed Ab from desorbing (falling) off the gold particles if the pH of the sample matrix and run buffer changes significantly, i.e., by one or more pH units. In this case, the conjugate becomes weaker as a detection reagent. This was a concern for us because our samples contain faeces which is probably the most complex and difficult sample matrix.

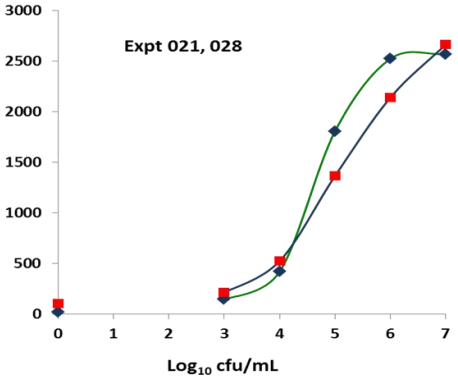
Notwithstanding, titration of *C. jejuni* from log 7 to log 3 cfu/mL in buffer demonstrated encouraging results in two separate experiments (Experiments 021, 028; see Figure 5.5). Firstly, the zero-blank was very low, typically, less than 50 units. Secondly, the detection of a *Campylobacter*-specific signal was good, with a visual signal observed by eye from 10^7- to 10^4 cfu/mL (arrowed in T-dots in Figure 5.5). Using the LFD reader a signal that was statistically higher ($P < 0.0001$) than the zero-blank was detected at 10^3 cfu/mL, indicating that the LOD of this experimental strip was between log 3 to log 4 cfu/mL. This was the best performance observed in an experimental test strip to date. Looking at the plotted data in the graph in Figure 5.5, assay linearity encompassed the range log 3 to log 7 cfu/mL.

Figure 5.5 – Spiked buffer data with Ab3-gold conjugate prepared by passive adsorption method



Mean of 4 readings	2571	2524	1808	420	145	21
(SD)	(341)	(484)	(120)	(52)	(21)	(17)

- [Cleaned-up] Ab3 on T-dots
- [Cleaned-up] Ab3-gold conjugate (passive adsorption)
- 10 minutes run time
- 'Standard' run buffer
- LOD: ~ 10^3 to 10^4 cfu/mL (buffer)
- Nsb: Low, < 50



The run buffer used in these experiments was a 'standard' buffer, which suggests that there is scope for further optimisation of this test strip using other, proprietary, buffers, e.g., buffers B or D, which have enabled up to 3-5 fold stronger signals with gold label regardless of the method of conjugation of the Ab3 to gold (see Table 5.3 below and Tables 5.2A and 5.2B above). Using a slower membrane and increasing the run time to 15 minutes may also increase signal intensity. In addition, [1 in 2] dilution of the sample at concentrations of *Campylobacter* above log 6 cfu/mL, would permit a more accurate [higher] meter reading for the sample by circumventing the Hook phenomenon.

However, a number of caveats should be borne in mind regarding these results. Firstly, this data is using T-dot lateral flow strips and must be repeated using sprayed T-lines on the membrane and dried-down conjugate on the conjugate pad, which would be the case in the final version of the test strip. Secondly, the data was generated using spiked run buffer without faeces in it. The faecal sample matrix usually reduces the performance of the test strip by 1 to 1.5 logs, but perhaps the case may not be so severe using *cleaned-up* Ab3 on the T-line and for the Ab3-gold detection conjugate. Nevertheless, that remains to be investigated.

Table 5.3 – Performance of different run buffers

Ab3-Gold conjugation method	Run buffer	Meter reading @ 10^6 cfu/mL (10^5 cfu/mL)	Performance (%)
Covalent linkage (InnovaCoat)	'Standard'	1113	100
	A	1760	158
	B	3118 (980)	280 (88)
	C	2128	191
	D	3374 (1783)	303 (160)
Passive adsorption (standard)	D	3223 (1261)	290 (113)
	TBSC	522	47

- Run buffers B and D demonstrated optimal performance at 10^6 cfu/mL
- Buffer D enables better sensitivity
- Covalent linkage enables better sensitivity than passive adsorption

6.0 DISCUSSION

An easy-to-use and low-cost test for rapid on-farm detection of *Campylobacter* in poultry has been developed to enable poultry farmers to monitor their flocks and assess the effectiveness of their biosecurity procedures. Suitable capture and detection antibodies were experimentally identified (Interim Report 02) and a sandwich immunoassay for *Campylobacter* was ported onto a lateral flow test strip (Interim Report 03). The test strip was developed and optimised by modifying the nitrocellulose membrane, antibody concentrations (for conjugation and for spraying on the T-line), detection label, sample buffer and assay run time. It is specific for *C. jejuni* and *C. coli* and reports a semi-quantitative result within 15 minutes. The first prototype (Prototype-01 or P-01) test strip demonstrated a LOD of log 7.5 cfu/mL using spiked poultry faeces (Interim Report 04). It was productised into a *Campylobacter* test kit and supplied to poultry farmers for field evaluation and end-user opinion on the product concept and its feasibility (Interim Report 05). Field evaluation indicated that the test strip needed to demonstrate increased sensitivity and end-user and industry opinion indicated a preference for the signal to be reported as a digital readout (rather than a blue-coloured Test line being observed visually by eye, as was the case with Prototype-01), for which a strategy was formulated (Interim Reports 06 and 07).

Therefore, a project extension was granted with an additional budget so that the sensitivity of the test strip could be improved by 2-3 logs cfu/mL and that the test would report a digital result/output using a portable LFD reader (Interim Report 08).

As a result of work conducted under the extension (i.e., Work Package Two), two improved test strips have been developed capable of rapid on-farm detection of *Campylobacter* in poultry (Interim Report 09). The first of these, referred to as test strip "Dev-02", has latex microspheres (blue-coloured) as a detection label and demonstrates a LOD of log 5 cfu/mL using spiked chicken faeces samples. It demonstrates encouraging performance attributes and could potentially identify poultry flocks moderately- to highly colonised with *Campylobacter*. However, it remains to be field tested and benchmarked against conventional microbiology using 'live' samples from poultry houses.

The second test strip, referred to as "Dev-03", is at an earlier, more experimental, stage and has gold nanoparticles as a detection label (pinkish red signal). It demonstrates a LOD of log 3 cfu/mL, which is better than its latex counterpart (Dev-02) in spiked buffer samples. Spiked faeces is yet to be tested, however, this would be best done after further development and optimisation of the test strip (for which there is much scope) has been performed.[15] Notwithstanding, the test strip shows great potential as a rapid on-farm *Campylobacter* detection system, possibly with an LOD of ~log 4 cfu/mL for faecal samples using the portable LFD reader for signal detection. Such sensitivity could potentially identify poultry flocks colonised with low- to moderate and high levels of *Campylobacter*, i.e., ≥log 4 cfu/mL.

A summary of the components, features and performance attributes of Dev-02 (latex) and Dev-03 (gold) test strips is presented in Table 6.1.

[15] After identification of the optimal membrane based on flow rate, test strips with chromatography-purified Ab3 immobilised on the T-lines need to be prepared, and used with chromatography-purified Ab3 conjugated to gold nanoparticles by a method that produces a stable and robust Ab3-gold conjugate.

Table 6.1 – Summary of components, features and attributes of two *Campylobacter* test strips

Component or parameter	*Campylobacter* Test Strips	
	Latex version (Dev-02)	**Gold version (Dev-03)**
Test strip (development stage)	Pre-prototype 	Experimental 
Membrane	MDI-CNPC (fast) (Sartorius CN140 might be better)	MDI-CNPC (fast) (a slower membrane might be better)
Detection label	500nm latex beads (blue)	40nm gold particles (dark pink)
Conjugate pad antibody	[cleaned-up] Ab3	[cleaned-up] Ab3
T-line antibody	[cleaned-up] Ab3	[cleaned-up] Ab3
Run buffer	Extraction buffer C (EBC) (Buffer D might be better)	Extraction buffer C (EBC) (Buffer D might be better)
Run time	10 minutes (but since membrane is fast, assay has run in < 5 mins)	10 minutes (but since the membrane is fast, assay has run in < 5 mins)
Availability of portable LFD reader	Yes	Yes
Sample pad filter	Optimal filter to be determined	Optimal filter to be determined
LOD (*C. jejuni*)	Log 5 cfu/mL (spiked in ¼ diluted faeces)	Log 3-4 cfu/mL (spiked in standard buffer)
Nsb signal on the T-line	50-100 units (faeces)	< 50 units (buffer)
Possibility of silver enhancement?	No	Yes (enhancement could be 2-3 logs)
Additional Comments	1. Test strip is closer to being completed in its development and optimisation. Additional work would seek to optimise the membrane (a slower flowing one might improve sensitivity) and sample/run buffer (buffer D might enhance signal intensity). 2. Test strip is likely to be cheaper to produce than its counterpart. 3. Further improvements to the LOD may not be possible, i.e., the strip is maximally optimised with latex as a label. 4. Finished test strip should be able to identify birds with moderate to high levels of *Campylobacter* (i.e., >log 6 cfu/mL).	1. Test strip requires additional work to complete its development and optimisation but the strip shows greater potential for higher sensitivity compared to its latex counterpart. 2. Sensitivity may be further enhanced by 2-3 logs using silver reagent. 3. A slower membrane and an improved release pad could further optimise sensitivity. 4. Completion of development of this strip is highly recommended. 5. Finished test strip should be able to identify birds with low, moderate and high levels of *Campylobacter* (i.e., >log 5 cfu/mL).

6.1 Key Findings and unique selling points of Camp-U-Test™ strip

Key findings from this project are that an on-farm test for rapid detection of *Campylobacter* in poultry is feasible. The test could be used on all types of samples; faecal, caecal or boot swab. It would be specific for the two major species of *Campylobacter* pathogenic to humans, *C. jejuni* and *C. coli*, and able to detect *Campylobacter* at log 5 cfu/mL in spiked faeces. The Dev-03 test strip developed here could demonstrate sensitivity even better than this if it was fully optimised. The on-farm test would be easy-to-operate, require no complex sample preparation and reports a [digitally displayed] result within 15 minutes of sample application using a low-cost portable LFD reader.[16] It would be low-cost and would not incur a high set-up cost. It would be able to identify moderately- to highly colonised flocks for ~£15 per broiler house plus the initial cost of the reader (currently £350, but this would be considerably less with re-engineering of the reader and economies of scale).

Some unique features of the *Campylobacter* test strip developed here are described below:-

- **Rapid** on-farm test for *Campylobacter*; results within 15 minutes. A poultry house can be [flock] tested within 30 minutes of sampling
- **Easy-to-use**; no complex sample preparation
- **Specific** for *Campylobacter*; detects *C. jejuni* and *C. coli*
- **Sensitive**: LOD of ~log 4-5 cfu/mL
- **Can be used for any sample type**; boot swabs, faeces and caecal droppings
- **Low-cost**; a poultry house could be tested for £15 (using two pooled samples per house)
- **Available in two formats**; dipstick or cartridge device (dipstick not suitable for LFD reader)
- **Semi-quantitative "traffic light" reporting** possible; e.g., as red, amber or green for high, moderate or low levels of *Campylobacter*, respectively
- **Low set-up costs**; portable LFD reader would cost approx. £350 (cost would be lower with economies of scale)

6.2 Industrial Impact

Currently, there are no rapid, easy-to-use and low-cost methods for on-farm detection of *Campylobacter* in poultry. Therefore, poultry farmers cannot assess on-farm in real time the effectiveness of their biosecurity measures or determine the *Campylobacter* status of their flocks prior to departure for the abattoir. Once optimised, the test strip developed here will enable farmers to determine the level of *Campylobacter* in their flocks within 30 minutes of sampling and for less than £15 per broiler house (assuming two pooled samples are tested per poultry house). It will identify moderate- to highly infected houses (i.e., birds with $\geq \log_{10}$ 6 cfu/mL faeces) prior to departure for the abattoir, and could even identify low- to moderately colonised flocks if Dev-03 is optimised to realise its full potential. This will not only enable poultry farmers to evaluate their biosecurity measures, especially post-thinning and prior to departure, but would also allow better-informed scheduling or channelling at the abattoir; for example, to apply a processing intervention to highly contaminated slaughter batches, which otherwise contaminate lesser contaminated birds via the use of shared equipment in the

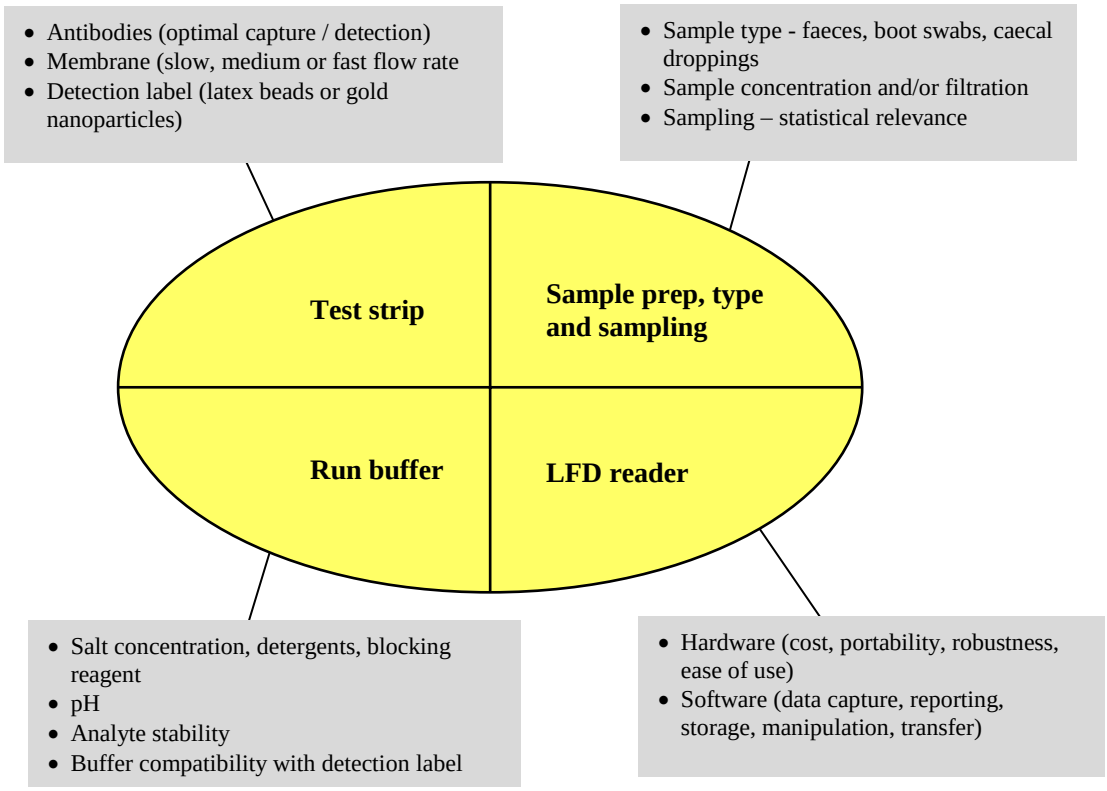
[16] Although Forsite's LFD reader costs ~£350, we could develop one that could retail for less than £175.

abattoir. Both of these capabilities will help to reduce the risk of introducing poultry that is highly colonised with *Campylobacter* into the human food chain. In any food safety strategy, these would be the first slaughter batches that would be prioritised for intervention. It is believed that Camp-U-Test™ could assist poultry companies to achieve the target set by the UK government that no more than 27% retail chicken products in the UK should have ≥1000 cfu *Campylobacter* per gram meat.

6.3 Next Steps

This project is now closed-out. However as indicated above, further development and/or optimisation of the test strip (Dev-03), run buffer and sample preparation methodology (e.g., sample filtration and/or concentration) would maximise the sensitivity of the system as a whole and ensure that each component performs optimally with respect to the others (see Figure 6.1).[17] This will enable the poultry industry fully exploit the capabilities of the test strip and system.

Figure 6.1 – Components of the *Campylobacter* test system



[17] In any lateral flow development project, components are optimised individually by keeping the other variables constant. However, since changing the characteristics of one component can dramatically affect the performance of others, after all the components have been optimised individually, they must be examined collectively to ensure that the whole system is functionally optimally. Failure to do this might result in some components operating sub-optimally to the detriment of the whole system.

Based on our previous experience in assay development and expert opinion from LFD developers, the above data indicates that further work could increase the sensitivity of the test system to a LOD of log 3-4 cfu/mL. Therefore, it is strongly recommended. Subsequent to these improvements, the test strip would need to be evaluated in the lab using spiked faecal samples, after which it could be benchmarked and validated against conventional microbial culture methods using live samples on poultry farms, i.e., field tested.

These improvements were not possible during this project for a number of reasons. Firstly, because the technical resource on the project was on a temporary contract and left Forsite, and the Forsite did not have additional resource with the required technical experience to allocate to the project. The Company was acquired during the term of our project and there was a deployment of key resources to new owner's own projects. Therefore, there was no possibility of further development work being undertaken at Forsite and the work had to be closed-out on 15th August 2012.[18]

Secondly, the budget for the work was spent for progress to date, i.e., having two test strips (Dev-02 and Dev-03) with better performance attributes than the first prototype (P-01).

Hence, additional funds (~ £43,400)[19] are required for,

1. Completion of optimisation work prior to lab evaluation using spiked faecal samples. If funds were available, this work would be conducted by Dr Ahmed Jehanli of IPRO Interactive Ltd who is reputed among industry experts to be one of the most experienced lateral flow test developers in the UK. He has developed a number of rapid lateral flow POC tests, including proprietary buffers (e.g., buffer D, which seems to be the most optimal buffer for our test strip), for the sports market. In fact, IPRO supply LFD tests to around ten Premier League football clubs for the measurement of performance and wellness biomarkers in saliva.

   The project manager (Jay Modha) has discussed the work necessary to complete development, optimisation and basic characterisation of the *Campylobacter* test strip with Dr Jehanli who believes he can undertake this work and bring it to a satisfactory conclusion in around 3 months. We highly recommend this option to the FSA in order to realise a fully developed, maximally optimised and field trialled test strip.[20]

2. Growth and provision of *Campylobacter* and lab evaluation of the test strip by Moredun Scientific across the 6-month project period.

3. Field evaluation and benchmarking by Professor Tom Humphrey (University of Liverpool). Tom has access to some *well-characterised* poultry farms. He is keen to assist development of the test strip and has offered to undertake sample optimisation work (boot swabs and faeces) and field testing of the test strip at minimal cost.

[18] Besides not having the necessary technical resource for our project, Forsite did not have a spectrophotometer or an in-house protein assay, both of which were seen as crucial for making further progress in the work

[19] This is a best estimate cost at the time of writing this Report.

[20] Note, sample optimisation is a separate issue and would be undertaken by Professor Tom Humphrey's group at University of Liverpool.

4. Additional reagents, in particular Ab3 and conjugation kits, which are necessary to complete optimisation of the test strip and produce batches for lab evaluation and field testing (benchmarking).

5. Project management and coordination by Jay Modha (Modha Biomedical) whose input and involvement is crucial to successful completion of the work since he has managed and coordinated the design, development and field evaluation of the test strip from the start.

6.4 Concluding Remarks

Data presented here shows that the overall aim of the project to develop a rapid on-farm test for *Campylobacter* has been largely achieved, in that a rapid on-farm test for detection of Campylobacter in poultry is feasible.

Two test strips have been developed; Dev-02 (see Fig 5.2 and Table 6.1) and Dev-03 (see Figs 5.4 & 5.5 and Table 6.1). Dev-02 uses latex microspheres as a detection label and demonstrates an LOD of log 5 cfu/mL in spiked faeces, and Dev-03 uses gold nanoparticles as a detection label and shows an LOD of log 3-4 cfu/mL in spiked buffer. The former meets the requirement of the [extended] project's objective, which was for a 2.5 log increase on prototype-01 (which demonstrated an LOD of ~ log 7.5), and the latter, if this performance is demonstrable in the field, would exceed expectations and be 'fit-for-purpose' if we define that as being able to identify moderate- to highly colonised flocks (≥ log 6 cfu/mL). These are the flocks that need to be identified first in order to reduce the problem of *Campylobacter* in the human food chain. In flocks with less than log 6 cfu/mL on the farm, *Campylobacter* is reduced to more acceptable levels (i.e., < log 3 cfu/gram neck skin) by the processing process in the abattoir which can reduce the *Campylobacter* concentration by 3-4 log cfu/mL.[21] Hence, our need is for a test strip that will identify flocks carrying ≥log 6 cfu/mL. We think our test strip Dev-03 will be capable of doing that, and perhaps more, if we can complete the work of optimisation and conduct field trials. It is our opinion that the time (6 months) and budget (£43,400) required are modest and easily justify the utility of a rapid and low-cost on-farm test that would help to reduce the rising problem of *Campylobacter* in the human food chain[22] and assist in achieving government targets for its control by 2015.[23]

[21] Confidential data (personal communications)

[22] "*BBC report highlights rise in campylobacter incidences*"… (World Poultry, 22nd January 2013)

[23] http://www.food.gov.uk/multimedia/pdfs/campytarget.pdf