A UK Food Standards Agency final project report

Project title: A new on-farm *Campylobacter* testing provision covering the independent broiler farming sector across the United Kingdom of England, Scotland, Northern Ireland and Wales

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INTRODUCTION AND BACKGROUND

In recent years, infections by campylobacters have become recognised as the most common bacterial cause of food-borne gastrointestinal disease in England and Wales (Anonymous, 2012). Around 250,000 people are infected annually in the UK (FSA, 2014a; 2014b) and poultry meat has been identified as the major source of food-borne campylobacters (Adak et al. 2005). Chicken broiler meat has made the largest contribution to the burden of infection in humans (Anonymous, 2009). As a consequence, a reduction to the numbers and prevalence of campylobacters in poultry was the first 'Priority Evidence Theme' described in the 'Structure and Key Components' section of the UK Food Standards Agency's (FSA) 2010-2015 strategy. It is important to note however, that human infections caused by campylobacters are not an issue that is confined to the UK. An EU-wide survey of *Campylobacter* contamination of poultry meat undertaken by the European Food Safety Authority (EFSA) during 2008 made it apparent that *Campylobacter* contamination of poultry meat at retail was an issue across many EU member states (Anonymous, 2010a). Most recently, retail surveillance of whole broiler carcasses has indicated that more than 70% of raw chickens sampled in UK shops tested positive for campylobacters (FSA, 2015).

As a direct response to the issue of campylobacters in poultry meat, the FSA has stated publically that "tackling campylobacter in chicken is a priority" and that problem was its primary focus towards achieving safer food for UK consumers as part of its 2015-20 strategy (FSA, 2014b; FSA 2014c). As preparation towards that end, in June 2014, the Acting on *Campylobacter* Together (ACT) programme was established as a collaboration between the FSA, farmers, the food processing industry, retailers and consumer representatives. Participants in the ACT programme signed a formal pledge that they would do whatever they could to reduce the prevalence and levels of contaminated chicken meat at retail. The ACT programme broadens a scope that was prepared by an ACT predecessor called the Joint Working Group (JWG) established in 2010 between farmers, the processing/retail industries and the FSA to manage strategies that would reduce *Campylobacter* contamination of chicken. One of the outputs from the JWG is that the FSA have instigated retail surveillance and established other systems (with the participation of the poultry processing industry) to measure progress towards the goal of achieving safer food for UK consumers.

At the European level, the EFSA Baseline B *Campylobacter* in Broilers analyses report (Anonymous 2010b) recommended that member states should undertake "Further national studies to identify more closely, at batch- and slaughterhouse level, the factors that put broiler batches and carcasses at risk of becoming respectively colonised or contaminated with *Campylobacter* in a country are recommended". This proposal goes some way to addressing such a recommendation should the Agency decide that it will adopt the recommended European strategy.

A formal campylobacters reduction target has been declared by the FSA in collaboration with poultry farmers, production and processing industries. In brief, using a test sample of three pooled neck skins excised from different carcasses, post chill, the Agency has grouped the amount of contamination into three bands, which are

<100 cfu/g, 100-<1000 cfu/g and ≥1000 cfu/g. The ultimate goal is that only 10% of samples will have enough contamination to qualify as the highest band. If such a goal were accomplished there would be a reduction of around 50% to the number of food-borne *Campylobacter* infections each year in the UK (FSA, 2014b).

In summary, this study sought to help the Agency deliver its general strategic goal of having consumers eating safer food (FSA, 2014c). This project also sought to further raise awareness of campylobacters as a hazard to food safety amongst bird growers. Previous recent work funded by the FSA as study MO1056 identified that 2/9 of the variance observed in the contamination of whole chickens by campylobacters was explained by slaughterhouse processing factors. The remaining 7/9 of the variance stemmed from on-farm factors. In essence the finding meant having the birds free of campylobacters prior to processing had a greater impact on final carcass campylobacter numbers than attempting to control cross-contamination during processing. Consequently, this study attempted to identify risk factors for bird colonisation on farms with a view to reducing on-farm colonisation. The proposed work will directly address the Agency's current strategy's first 'Priority Activity Theme' of 'identifying and obtaining the information it needs' by providing robust and independent data. Furthermore, the proposed schedule of work was specifically designed to fully comply with the Agency's guidance for ensuring thorough statistical robustness for research work.

MATERIALS AND METHODS

CONSTRUCTION OF THE QUESTIONNAIRE CONTENTS AND THE RATIONALE FOR THE MULTI-FORM QUESTIONNAIRE STRUCTURE

Previous work funded by the FSA as project MO1045 resulted in a set of questionnaires designed to capture information relating to on-farm growing practices for birds and processing conditions. The purpose of the original questionnaire was to objectively assess processing hygiene in plants and there was a strict evidence base of peer-reviewed papers supporting the inclusion of each question. For the current study, the farm section of the MO1045 questionnaire was used as a starting basis. The opinion of eight NFU members that farmed chickens was solicited to determine what they thought might be important influences on the colonisation status of chicken broilers by campylobacters. In addition poultry industry veterinary input was obtained. Since the current study was designed to identify potential risk factors, there was no requirement to justify the inclusion of a question from a science or evidence basis. The overall strategy was to identify what might be important and to test the entries on that list to determine those that played a role in predicting campylobacter numbers in broiler shed litter.

In addition to including questions on the basis of informed opinion, there was variation in how the questionnaires were organised for the current project compared with the historic MO1045 study. A significant number of process hygiene assessment tool users for MO1045 study commented that a number of the same basic questions were asked each time an assessment was undertaken. In some cases, the responses to some questions were likely never to change. In order to address these widely-made industry comments, the farm information questionnaire was split into three smaller ones. Questions with responses that were unlikely ever to change relating to the farm (e.g. farm postcode) and the broiler houses (e.g. frame material used to construct the house(s)) were grouped together so that they could be asked only once. Questions with responses that were likely to change from one batch of birds to another (e.g. antibiotic use) were grouped and asked for every batch.

The three questionnaires used for this study were:

1. A questionnaire describing the farm details that were likely to change infrequently if at all. Examples of the questions asked included: Number of broiler houses on the farm, the farm CPH (county parish holding) identifier and postcode and the farm address. The farm questions were presented to farmers only once.
2. A questionnaire that described the broiler sheds, again that were likely to change infrequently. Examples of the questions asked included: The material covering the ground in-between sheds, the shed base material and whether the shed construction was wood or metal. The shed questions were presented to farmers only once.
3. A questionnaire that captured information likely to change between batches of farmed birds was asked every time a matched litter sample was collected for laboratory testing to determine *Campylobacter* load. Examples of the batch questions included: whether the birds were stressed by fan, feeder or drinker line breakdown during farming, whether there was disease and subsequent antibiotic use for the batch, the source hatchery and the numbers of day-old chicks placed in the shed at the start of the cycle.

A complete listing of the questions and allowed responses used for the study are provided as Appendix 1.

DEVELOPMENT OF THE ON-LINE DATA COLLECTION AND REPORTING FACILITIES

The questionnaire contents and allowed responses are shown as Appendix 2. The questionnaires were converted into web forms and systems were established to allow farmers to securely login to a website located at www.act-nfu.org. Although farmers were actively encouraged to participate, it was made clear to them from the outset that this was a voluntary scheme and the results would not be used for enforcement purposes. There would also be a limited window within which participation was free. The basic sign-up process was that farmers were informed about the study from a variety of NFU sources e.g. emails from the NFU, the fortnightly NFU poultry member newsletter, articles on the NFU website, NFU poultry meetings and directly approached by NFU advisors to raise awareness. Farmers used a web browser to visit the act-nfu website to choose a username and password and provide some basic details about their operations, including a valid email address and a mobile phone number. The email address was verified by sending an activation link for the logon details. The contractor project manager then scrutinised the farm details supplied before deciding whether the farm was suitable for inclusion into the project. The inclusion criteria were chiefly that farms were independently owned, were not already testing as part of an established integrator company testing scheme, and were willing to provide information describing their farms and flocks for risk factor identification. Once approved, farmers were able to login to the site. The site was organised in a manner that automatically guided each approved user through the farm and shed questionnaires. A farm approval triggered automated systems that emailed the laboratory to send testing kits to the farms. Each testing kit included a paper-based flock questionnaire that was returned to the laboratory with each litter-based test sample. Test results were reported by SMS-text to the supplied mobile phone number. The flock forms were electronically scanned to an image file by the testing laboratory and emailed to a data entry clerk for manual entry into the database.

The technical details of the website were that it was built using the Microsoft (MS; Redmond, WA, USA) ASP.NET framework v2.5 on a webserver running the MS Server 2008 operating system. Customised webpages were coded using either the C#.NET or VB.NET programming languages, and the data collected was stored in an instance of MS-SQL (structured query language) database programme version 2008. All data saved and retrieved from the database was as parameterised, HTML-decoded queries that prevented malicious script injection into the database and unintended manipulation of the page script. Technically-advanced coding methodologies such as dynamic page control placement during the page load event (Figure 1) and retrieval of control contents from the page viewstate were used to make the site as easy as possible for the farmers to use.

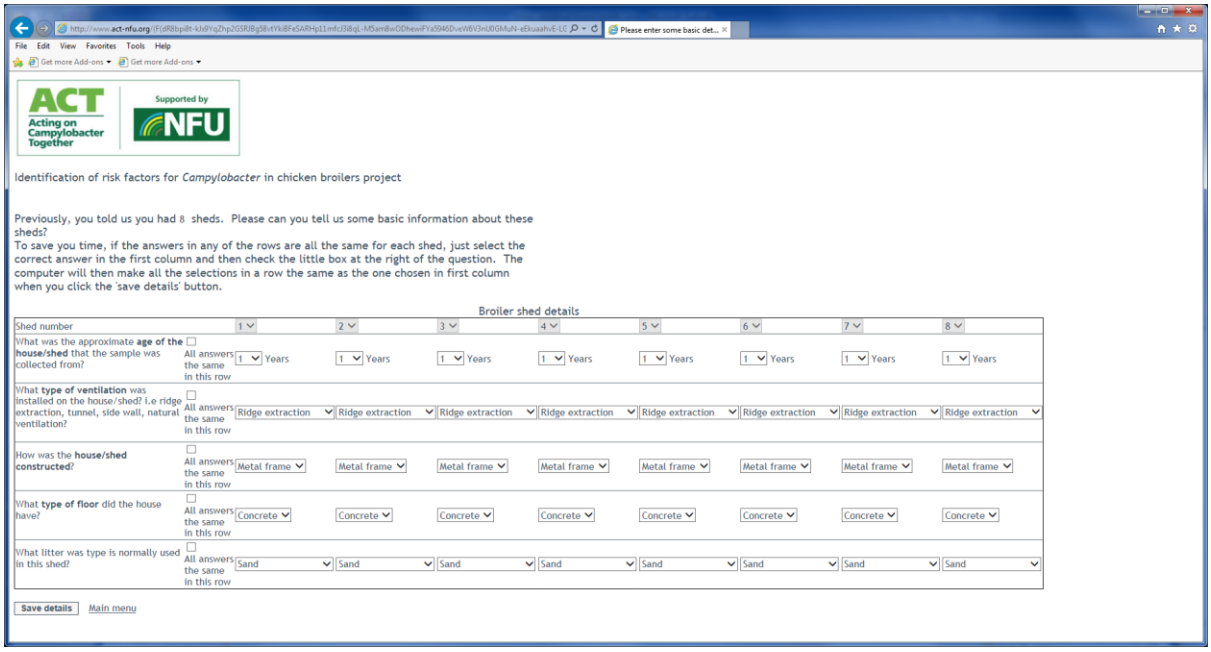


Figure 1 Dynamic control placement was used to create customised data entry forms on a farm-by-farm basis to the site easy-to-use for farmers. In the figure above, the form for the shed detail data was dynamically-created in response to a previous question that asked 'How many sheds are there on the farm?'.

LABORATORY TESTING OF LITTER SAMPLES

SAMPLING KITS

The number of sampling kits required by each farm was determined by consideration of the number of broiler houses on the farm, the number of crops expected to be completed before the end of the project and the total number of kits available for distribution during the study (4200 kits in total). It was anticipated that if a farm was un-colonised at thin (depopulation), the farm would test again at clearance (depletion). This assumption helped to calculate the number of test kits sent to each farm. Two sample collection kits were provided for each house, crop and farm to facilitate sample collection at thin and clearance. Farms registered at the start of the project were supplied with kits sufficient for three crops; subsequent registered farms were supplied with sufficient kits for two crops and in the last month of the study, a single crop.

The sampling kit supplied to all participants consisted of the following components:

- Envelopes: Padded bubble-lined envelopes were used to protect the samples. The envelopes were weather-proof, burst-proof, tear and puncture resistant and had secure peel and seal closures (PostSafe, EPA7 (230x345) http://www.postsafe.co.uk).
- Postage: An AFBI address label and prepaid first class Royal Mail delivery were provided to farmers as part of the kit.
- Sample bags: A grip seal polythene plastic clear food use freezer storage bag was provided for each set of swabs.
- Boot swab: A pair of white tunika overboots (Bowden and Knights , Shadwell, Norfolk, UK) was provided for litter sampling.

- Sample protocol: A detailed description of how to collect samples was also provided (Appendix 3)
- Contact details for the project team in case of queries/clarification were included in the sampling protocol.

- Flock (House/shed) Information Form: A crop information form that provided supplementary information on each batch of birds was also included in the kit.
- Sample labels: An adhesive label, with a printed sample ID barcode, farm name, ACT-NFU farm number, CPH number, house number, date sampled was supplied for application onto the bag containing the swabs.

In addition, a farm-specific identification label displaying the NFU farm identifier was applied to the postage label of each ACT-NFU sample kit to enable sample identification and sorting on arrival at the laboratory.

The sampling protocol is familiar to poultry farmers as the UK National Control Programme (NCP) for Salmonella samples are obtained in the same way.

QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

The testing methodology is an output from the FSA funded project reference M01060 entitled:

The development of a rapid on-farm test for the detection of *Campylobacter*.

SAMPLE RECEPTION AND PROCESSING

Mail was collected by AFBI laboratory staff from the local regional royal mail sorting office before 9.00 am each week day (Monday-Friday) to facilitate the target eight hour sample receipt to reporting turnaround time. Sample envelopes are sorted into customer batches on arrival at the laboratory using the customer specific identification label on the sample envelop address label. In the very rare event samples could not be processed on the arrival date, unopened envelopes were stored at 4^{o}C overnight.

Each customer batch of envelopes was processed separately. Sample bags were removed from each envelope. Each labelled sample bag was grouped with other samples from identified farms (Farm codes) then placed in order, depending on their farm name (Farm code) , sample date and house number.

When the samples had been sorted and the details logged, a barcode that corresponded to a unique lab number was assigned to each sample. After bar-coding was completed, 50mls of MRD (maximum recovery diluent) was poured into each bar-coded sample bag before stomaching (Interscience Bag Mixer 400W) for 60s.

The DNA from the stomached sample was extracted using an automated QIAxtractor robot. Positive and negative extraction controls were included.

QIAGILITY LOADING OF DEEP WELL DNA EXTRACTION PLATE

A QIAgility robot loaded the extracted sample into deep well 96 microtitre plates and added the additional components required for the PCR reactions. The *Campylobacter* qPCR assay used was the commercially available mericon *Campylobacter* spp Kit which was used in conjunction with the mericon pathogen detection kit. The amplified product was detected using target-specific fluorescent probes and monitoring the fluorescence intensity increase during the PCR run. All reactions included an internal positive control to identify reaction inhibition.

QPCR ASSAY SETUP

Each reaction was supplied from the manufacturer as a lyophilised mix of primers and labelled probe, which was reconstituted as the master mix supplied to the robot. 5ul of Mastermix, was aliquoted into each test well of a 96 well RTPCR plate (Life Technologies Cat no 4346906). qPCR plates were sealed with adhesive film (MicroAmp Optical Adhesive Film, Life technologies; Cat No – 4311971) and briefly centrifuged (5000g, 30s) before thermal cycling. Reactions were undertaken on an Applied Biosystems ABI7500 instrument running 7500 fast systems sequence detection software (v1.4.0.27). The instrument cycling conditions were an initial heat to 95°C for 5 min to activate the HotStarTaq Plus DNA Polymerase. Followed by 40 cycles of a three-step amplification cycling:

- Denaturation 15s at 95°C
- Annealing 23s at 60°C, with data collection at 60°C
- Extension at 10s 72°C

The detection reporter excitation and emission channels for *Campylobacter* DNA were 495 and 520 nm respectively. The internal controls used excitation at 524nm and detection at 557 nm.

DATA INTERPRETATION

Applied Biosystems sequence detection software (SDS) Version 1.4.0.27 was used to analyse and interpret the mericon *Campylobacter* qPCR assays. The DNA profiles for the *Campylobacter* standards, together with the positive and negative sample extraction controls were viewed in the amplification plot window of the software to identify abnormal amplifications. The specific checks included:

- Increased fluorescence in negative control wells
- Absence of positive control detectable fluorescence at an expected cycle
- A profile considered at significant variance from the DNA standards and controls

As part of FSA study MO1060, a relative standard curve was constructed for the qPCR assay using the detection cycle values of the *Campylobacter* DNA standards from known numbers of cells. The range spanned six decimal dilutions of a *Campylobacter* type culture ($1x10^7$ to $1x10^2$ cfu/ml). At least five separate amplifications were used for each point on the standard curve. The standard curve was used to convert the detection cycle to numbers of campylobacters.

TEST RESULT REPORTING

An SDS (software diversified services) report export file for each qPCR analysis containing both mericon *Campylobacters* and IPC qPCR, CT and enumeration quantities (mericon only) for each sample was transferred onto a reporting computer. The SDS report export file was opened in Microsoft Excel and the qPCR data was manipulated by removing any superfluous information (e.g. the control results). The spreadsheet was saved and used to update the results database on the ACT-NFU.org website. Provision was made for the direct import of spreadsheets, extraction of the required results fields and the automatic update of the SQL-server database by the website.

In addition, participating NFU farmers were sent an SMS (short message service) text message using a PC based texting service (BulkSMS messenger). Typically, the content of the message was as outlined below:

To: John Doe farm NFU identifier 567.

Campylobacter testing on house 4 taken on the 31/01/2015 has tested positive.

Campylobacter testing on house 6 taken on the 31/01/2015 has tested negative.

MODELLING TO IDENTIFY FACTORS THAT PREDICTED THE NUMBERS OF CAMPYLOBACTERS IN BROILER SHED LITTER

Information was held in the database tables as standard English language ASCII (American standard code for information interchange) text. The first stage of modelling was to convert the text into numeric information. Two types of variable were defined as nominal or categorical. Nominal information bore some relation to the encoded number. For example, the first clearance of birds from a house was encoded as '1', the second removal of birds as '2'. For categorical variables, there was no relationship between the value of the number and the information encoded. Numerical information such as bird age in days or house age in years was used without further conversion.

The software package MLwiN (Rasbash et al 2009) was used to construct a hierarchical linear model to account properly for the correlation structure within the collected data. In the model, a three-level hierarchy was specified as: livestock batch, house identifier and farm identifier. In the model developed, the assumptions necessary for fitting models of this type (e.g. normally distributed residuals and homogeneity of their variance) were verified as satisfactory. The modelling process proceeded by alternately fitting predictor variables to a model that attempted to predict the log numbers of campylobacters and removing those that were not significant at $\alpha \leq 0.05$, using a Chi-square test of the change in likelihood. Variables that had been removed were then retested in later iterations as the model was developed until only statistically significant predictor variables remained.

The model was developed starting from a base model which included a 'constant' term and a categorical variable which specified an additive effect for each sample collected. A key to describe the shortened variable names presented in the analysis is included in Appendix 1.

RESULTS

220 broiler farmers representing in excess of 1,200 broiler sheds participated in the study.

In total 4525 sampling kits were dispatched and 3223 tests were undertaken. However, a number of fields in the supplementary information describing flock growing conditions were not provided. Complete datasets from 1,844 tested batches of birds were available for the final analysis, although a preliminary inspection showed there to be a small number of flocks from which data had been collected at less than 26 days or at greater than 50 days. The general feeling of the project team was that these flocks were not typical of the majority of birds farmed in the UK. Therefore, since these flocks were not representative of normal commercial practices, they were dropped from the analysis leaving data from 1,780 flocks with birds aged from 26 to 50 days at the time of testing.

COLLATION AND GROUPING OF INFORMATION.

As part of this study, information was collated and grouped. There were no specific criteria for the grouping; generally, it was an *ad hoc* response to a previous iteration of the evolving model. In overview, the following groupings were attempted.

1. In an early model iteration, there was evidence that some source hatcheries were predictors of higher numbers of campylobacters. Some of the hatcheries supplied the same farms and sometimes the same sheds were filled from more than one hatchery. Although different combinations of hatchery were attempted on the basis of grouping by the farms supplied, the parent company for subsidiary hatcheries owned by the same entity and hatcheries supplying farms that supplied specific slaughterhouses. However, the initially-observed significance was not restored.
2. Farms were categorised according to specific criteria that would identify the contributing businesses. In order to preserve anonymity of the participating farms, the finer details of these categories are not provided as part of this report. However, there was significance for some categories, which was preserved when (for example) two separate categories of independent farms that supplied a different integrator were combined. Farm categories and grouped categories both contained farm collations that could be protective or risk factors (Figure 2).
3. A number of variables were assigned to summarise some of the collected information. For example, one question was whether supplements had been supplied to the birds. If the answer was yes, the name of the supplement was requested. Supplements were grouped by product name, general class of compound and manufacturer name to investigate any significance for specific supplement use. A similar approach was used for an unusually high CDMR, the reasons for that an elevated CDMR and stress events and the nature of the stress.

THE FINAL MODEL

The final model developed using MLwinN is shown as Figure 2.

$$\text{LogLoading}_{ijk} \sim N(XB,\ \Omega)$$

$$\begin{aligned}\text{LogLoading}_{ijk} = {} & \beta_{0ijk}\text{Constant} + 0.331(0.013)\text{BirdAge}_{ijk} + \text{-}1.107(0.277)\text{BirdGender\_2}_{ijk} + \\ & \text{-}0.785(0.339)\text{BirdGender\_3}_{ijk} + 1.400(0.360)\text{chkPrebiotic\_1}_{ijk} + \text{-}1.091(0.422)\text{NewFmCat\_2}_{k} + \\ & \text{-}0.477(0.986)\text{NewFmCat\_5}_{k} + 2.459(0.448)\text{NewFmCat\_6}_{k} + \text{-}0.263(1.036)\text{NewFmCat\_7}_{k} + \\ & 0.807(1.646)\text{NewFmCat\_10}_{k} + \text{-}2.249(1.519)\text{NewFmCat\_11}_{k} + 1.789(0.962)\text{NewFmCat\_13}_{k} + \\ & \text{-}1.237(0.433)\text{NewFmCat\_14}_{k} + 0.462(0.175)\text{HouseConstruction\_2}_{jk} + \\ & 1.438(0.843)\text{HouseConstruction\_3}_{jk}\end{aligned}$$

$$\beta_{0ijk} = \text{-}8.561(0.521) + v_{0k} + u_{0jk} + e_{0ijk}$$

*-2*loglikelihood(IGLS Deviance)* = 7616.578(1780 of 1780 cases in use)

Figure 2 The final model produced from the MLwiN multilevel analysis. Parameter estimates are in green with their standard errors shown within the brackets. The statistical significance of the individual terms can be calculated by dividing the parameter estimate by its standard error and referring the result to a normal distribution. Suffixes i, j and k refer to farms, broiler houses and farm batches respectively and depict the respective impact of each term to the observed variance.

The model calculated the terms exerting significant influence to the $\log_{10}$ campylobacter count. The constant in the equation is tied to the first category of each categorical variable and to a bird age of zero. Thus, for example, to calculate the predicted (mean) count for a flock of age 42 days of bird gender category 3 (mixed gender birds), in a house with house construction category 2 (metal framework) but otherwise within the first group of the remaining categorical variables, the equation shown in Figure 3 would be used:

$$\text{Log}_{10}\ \text{Count} = -8.561 + (0.331 \times 42\ (\text{days})) - 0.785 + 0.462$$

Figure 3 An equation to calculate the predicted (mean) $\log_{10}$ *Campylobacter* count for a flock of age 40 days for mixed gender birds in a metal framed broiler house.

MAIN FINDINGS

Appendices 1 and 2 show the questions asked to farmers, the table column names used to hold the collected responses and the commands entered into the computer to create the tables to hold the collected data. In combination, these are a listing of the 48 unique on-farm factors investigated by this study. The level of significance for each factor identified as exerting an influence on the numbers of campylobacters in the shed litter is provided as a p value within the explanatory text below.

Gender and Flock age

Overall the analysis showed that, between an age of 26 to 50 days, for every one day increase in the age of a flock there was a mean increase in $\log_{10}$ campylobacters counts of 0.331 cfu/g ($p < 0.001$). There was also an overall protective effect for some bird genders. Female birds had a geometric mean that was 1.107 $\log_{10}$ cfu/g lower compared with male gender birds ($p < 0.001$). Sheds containing mixed gender birds tended to have significantly lower counts of 0.785 $\log_{10}$ cfu/g compared with sheds containing only male birds ($p = 0.020$). Whether the gender and age predictors were correlated was also investigated. It was determined that there was no relationship between gender and age because the outcome for age was not significantly different between each gender and those cases where genders were mixed. In essence the finding is strongly indicative that there was no significant effect on the log numbers of campylobacters as a consequence of different gender birds being farmed differently e.g. were placed into sheds or harvested at different ages.

Processor supplied

Farm category also exerted an influence on $\log_{10}$ *Campylobacter* numbers. More specifically, compared with independent farms supplying independent processors, independent farms supplying integrated processors and not testing before the commencement of the study had lower counts in their litter by around 1.091 $\log_{10}$ cfu/g ($p = 0.001$). Furthermore, there were two categories of farms supplying two different independent processors that had counts which were 2.459 $\log_{10}$ cfu/g lower ($p =< 0.001$) and 1.237 $\log_{10}$ cfu/g lower ($p <0.001$) than a general group of independent farms supplying independent processors. The remaining categories of farm were not significantly different from category 1 (independent farms supplying independent processors), although an elevated $\log_{10}$ count for the category 13 farms (a mix of farms supplying a specific independent processor) that was 1.789 higher only just failed to reach statistical significance ($p = 0.062$).

House construction

There was also an overall effect of the type of house construction on campylobacter numbers with those broiler houses constructed from metal frames having 0.462 $\log_{10}$ counts greater than those with wooden frames ($p < 0.001$). Although there was a mean increase of 1.438 $\log_{10}$ numbers in type 3 (other frame type) construction houses compared with wooden framed houses, this was not statistically, significantly different ($p = 0.088$).

Prebiotics

If prebiotics were fed to birds, the $\log_{10}$ count was increased by 1.400 ($p < 0.001$). However, there were too few specific products listed for robust analyses. Consequently, further investigation of the nature of the risk for prebiotic use was not possible.

Prebiotics are used to promote healthy gut flora rather than counter Campylobacter colonisation. Given their beneficial impact on gut health – which is essential for modern poultry production – they are often used proactively to reduce or even eliminate the use of antibiotics or in response to treatment with antibiotics to re-establish a healthy gut flora. Prebiotics are compounds that function in a wide-ranging manner. Generally, their mechanism of action is to influence gut microbiota either by providing a nutrient preferentially to a specific group of bacteria or specific inhibition of the growth of some bacterial groups. However, prebiotics can also influence immunological targets and adhesion to gut columnar epithelia and thereby indirectly promote or inhibit bacterial populations (Pourabedin and Zhao, 2015).

Although a number of farmers responded that prebiotics had been used, there were too few specific products listed for robust analyses. Consequently the use of prebiotics is an area that could perhaps be investigated as further work and an extension to this project.

SURVEY

Survey of broiler farmers

Responses were obtained from 50 famers that took part in an NFU survey in October 2014. The summary findings are as follows:

- The ACT-NFU Project has provided free on farm campylobacter testing over the last 12 months. Were you aware of the project? Yes 84% No 16%.

- Did you participate in the ACT-NFU project? Yes 72%, No 28%.

- Were there any particular reasons why you didn't participate in the ACT-NFU project?
  Already testing 55%; Concerns about use of the data 9%; Production system not suitable 18%; Other 18%.

- For those that participated v those that did not, how would you rate your level of knowledge of campylobacter in broilers?:

	Participated	v Not participated
Very knowledgeable	24%	9%
Fairly knowledgeable	76%	82%
Limited or no knowledge	0%	9%

- Effectiveness of the project:

	Very effective	Fairly effective	Not effective
Providing you with more information about campylobacter in broilers	45%	45%	10%
Highlighting the importance of on-farm bio-security in keeping flocks free from campylobacter	45%	38%	10%

DISCUSSION

RESTRICTIONS OF THE STATISTICAL PROCESS

The approach of the current study was to record information about already-operating farms and to analyse that information to determine if there were factors that influenced the numbers of campylobacters. In recent years, the collection of data, and in some cases, big data, and statistical analyses has become an accepted scientific method for the identification of factors influencing a target of interest. The range of applications includes effective disease treatment identifications, better strategies for educating children and risk factor analyses for the insurance industries. Although it is now firmly established, the approach is not perfect. In the current study, our role was to observe systems already operating to identify risk factors. Specifically, no attempt was made to ensure there were broadly equal numbers of different responses across the datasets. Some of the responses provided were unevenly balanced e.g. there were only around 150/1780 sheds that contained beetles at harvest. In order for the statistics to be able to make credible comparisons, a larger number of sheds containing beetles would be required.

In addition, for those factors where there was sufficient representation across all responses our identification of specific risk factors involved an original assessment to identify a broad area of risk, followed by more detailed investigations. Some attempt was made for this study to investigate the basis for the observed elevated and protective risk factors; however, little further detail that was concrete was identified. Although the experience of the project team was that the participating farmers were enthusiastic, response rates for the detailed, follow-on, investigative questions were quite low. For example, there were around 400 positive responses made that probiotic supplements had been used when growing birds, but less than 200 further responses explaining the nature of the prebiotic used.

Finally, a general drawback of the statistical process is that it identifies factors that influence campylobacter numbers, but does not provide much explanation on the reasons for the influence. As a general strategy, modelling is useful to identify promising factors as a precursor to an experimental investigation that is balanced and appropriately replicated. Consequently, any discussion of the current study findings might be open to a criticism of being speculative. However, bearing that in mind, there was considerable discussions with farmers and knowledgeable industry representatives regarding the study findings. There is much that seems sensible and so should be recorded and discussed because it may be helpful to others working to tackle the same issues.

INTERPRETATION OF THE FACTORS IDENTIFIED BY THE MODEL

Gender

One result of the statistical process was that female gender was protective for *Campylobacter* numbers in litter. From a practical viewpoint on farm, both male and females will be placed into houses on the same day and will generally be sourced from the same breeding stock (i.e. the famer will get males and females from the same parent flock). In around 50% of cases, the day old chicks are delivered with the males and females mixed together i.e ‘as hatched’.

Bird age/weight

Although the model did not find a significantly-different correlation for bird ages and *Campylobacter* numbers when different genders were compared, it is common in the UK independent sector for the lighter female birds to be cleared from houses first, with the males allowed to grow on to a greater weight. The underlying reason for that practice is because males have the capacity to grow to a heavier weight and also because males achieve a better feed conversion ratio (FCR, an index of how effectively the energy content of feed is converted to bird muscle) compared with females of the same age. In the current statistical model, it was determined that although females were cleared in preference to males for approximately half of the time, there were some processors that would harvest males first if they reached a set target weight before the females, thereby potentially masking any effect for age by gender.

In contrast to integrated processors, independent slaughterhouses tended to favour heavier weight birds because a higher percentage of carcasses are boned out rather than sold whole. One further contributing factor might be that final clearance male birds may experience catching in their house as many as five times during their life particularly in larger sheds. Initial thinnings will most likely involve the females before commencing onto lighter males before clearing heavier males in the shed. The employees undertaking catching are a risk factor for *Campylobacter* colonisation by birds in a house (Allen et al., 2008; Hue et al., 2010). Thus, if catching occurs in large sheds many times before some birds are caught, then there is an increasing likelihood the remaining, mostly male birds, will become colonised with *Campylobacter*.

As was noted in the results section, for each day a bird was farmed, there was a mean increase in $\log_{10}$ campylobacter numbers of 0.331 cfu/g and investigations were undertaken to make sense of that finding. As a result of discussions between members of the project team and integrated and independent processors, it became clear that most integrators will thin only once i.e. one thin, and then houses are fully cleared. Independent processors however, will practice multiple thinnings before a shed is cleared. In particular, farms with very large sheds, such as those containing more than 50,000 birds, might thin as many as 6-8 times before final clearance - typically starting at 28 days and concluding at 50+ days of age. The long clearance times are a consequence of independent processors servicing customers that want a range of weights between 1.35 kg live weight and 3.5 kg live weight and most independent processors having a relatively low throughput capacity compared with integrated slaughterhouses.

Shed size and relevance of processor

An attempt was made to reclassify the originally-assigned farm categories to take account of farms with larger houses and determine any influence on $\log_{10}$ *Campylobacter* numbers. The attempt was partly successful, although it was not possible to identify house areas (only the bird numbers typically placed) for all farms. Independent farms supplying integrated processors would be expected to thin fewer times and these types of farm did have lower counts in their litter compared with farms supplying independent processors. We also observed that two categories of farms supplying two separate independent processors had counts which were significantly lower than the general group of independent farms supplying independent processors. The houses on these two farm groups were predominantly wood-framed and it was these two farm groups that were mainly responsible for a study finding of a protective effect for wood framing as the house material.

Shed construction

We speculate that steel frames are generally stronger than the equivalent timber ones. Consequently, steel frames can be used to construct larger sheds than timber framed ones. Larger sheds can hold larger numbers of birds, and so the protective effect of wood framing may simply be a proxy of numbers of birds placed and the number of thins, stress events and exposure to catchers required to clear the shed. We also noted that in contrast to metal, unpainted and pressure stained wood is porous. Thus, it might generally be expected that an exposed porous wooden surface would provide a niche for campylobacters. An alternative possible explanation for wood being beneficial is that there are natural antimicrobial resins in wood, and pressure impregnated preservatives have at least the potential to be antimicrobial (Willfor et al. 2004). The species of wood used and it's structure in terms of knots influence the distribution and concentration of antimicrobials contained within the structure (Willfor et al. 2004).

Feed withdrawal

In the early stages of the study we observed significance for short feed withdrawal time and lower campylobacter numbers. The significance did not extend to the end of the project or survive multivariate analyses. However we noted during the study duration that every time birds were thinned, feed was withdrawn from all the birds in the house, not just those being thinned. Feed withdrawal stresses birds and so larger sheds with multiple birds will have birds that were stressed multiple times. As was stated previously modelling can provide clues on important factors and follow up work can be properly designed to investigate such clues. The early significance of feed withdrawal time may be a clue it is important and that the influence was masked because the sample representing higher risk, longer feed times was under-represented as a coincidental consequence of the types of farms that participated in the study.

GENERAL CONCLUSIONS AND FURTHER WORK

This study has identified a number of factors that influence the numbers of campylobacters in broiler house litter. These include bird gender, with a protective effect for female birds; the material used to construct sheds, with a protective effect for wooden frames and some groups of farms supplying specific processors. The study has highlighted that there is merit in further investigation in the use of prebiotics as a factor for colonisation.

As was previously discussed, industry has provided some credible explanations as to why some of the identified factors influence campylobacter numbers in litter. However, in order to confirm (or further investigate) the mechanisms that are operating, experimental work should be commenced. Some of the required studies could be undertaken at small expense, by making use of existing standard operations for some companies. For example, there is at least one integrated processor in the UK that routinely sexes and grows birds in single sex houses on the same farm. The establishment of sample collections and testing on a series of suitable farms could form the foundation of further investigations to determine the nature of the protective effect for the female gender. Similarly, there are farms contained within the current study that have a mixture of metal and wooden framed houses. Extended study of these farms and an appropriate balanced mixture of farms with exclusive different house frames might provide further clues regarding the nature of the protective effect of wooden framed houses.

In addition to the factors that remained significant through the multi-level, multi-variate modelling process, there were some factors that were significant for most of the duration of the study but did not survive the final analyses. The most interesting of these was the length of time that feed was withdrawn from birds during thinning, with shorter withdrawal times having a protective effect. As was previously stated, the current study was observational and no attempt was made to recruit a balance of farms with broadly equal numbers for each of the different answer options on the questionnaires. Consequently, feed withdrawal time may be an important consideration that was masked by answer bias in the participating farms. Given feed withdrawal is likely to stress birds, and feed is withdrawn from all of the birds in a shed (not just the ones being thinned), there may be merit in further investigation using a balanced range of withdrawal times. Possibly, the role of feed withdrawal could be investigated in isolation from the other stresses associated with thinning i.e. investigation of feed withdrawal without exposure to potentially-contaminated catchers or the stress of the catching process.

The final risk factor that could be investigated further is the use of prebiotics which saw an effect in this project but there were too few specific products listed for robust analyses. Prebiotics are often used to promote good gut health which is essential in poultry production particularly in the drive for antibiotic free production. They are also often used as a follow up to antibiotic treatment in order to establish a healthy gut flora. Prebiotics are compounds that manipulate gut microbiota using a diverse range of mechanisms. A general poor response to questions asking the probiotic products used hampered better investigation of the mechanisms promoting bird colonisation by campylobacters for the present study. However, it should be straightforward to design experimental work that compares colonisation in birds grown with and without popular prebiotic products.

Raising awareness: The project was particularly successful in raising awareness of:

a) testing for Campylobacter amongst broiler farmers;
b) a farmer's own Campylobacter status; and
c) the differences between shed and site status and lessons that farmers could extract from this.

As reported, 220 farmers took part. This not only exceeded the expectation of the project team but also showed that there is interest in this area of work.

The methodology of collecting the samples was familiar to broiler farmers as a result of taking Salmonella samples as part of the National Control Programme over a number of years.

The results from the survey conducted by the NFU in October 2014 demonstrated the importance and value of the ACT-NFU on farm testing project.

APPENDIX 1 THE QUESTIONNAIRES USED FOR THIS STUDY

The following questionnaires were used to capture information relating to the farms, broiler houses and different batches of farmed birds. The storage location of the collected information is provided as the table name and column title separated by a full stop.

THE SHED (BROILER HOUSE) QUESTIONNAIRE

What is the shed number?

Allowed options: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.

Storage location: tblShedDetails.HouseNumber

What was the approximate age of the house/shed that the sample was collected from?

Allowed options: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50. Years.

Storage location: tblFlockDetails. HouseAge

What type of ventilation was installed on the house/shed?

Allowed options: Ridge extraction, Wall extraction, Tunnel extraction, Natural ventilation.

Storage location: tblShedDetails.VentilationType

How was the house/shed constructed?

Allowed options: Metal frame, Wood frame, Other frame.

Storage location: tblShedDetails.HouseConstruction

What type of floor did the house have?

Allowed options: Concrete, Soil, Other.

Storage location: tblShedDetails.HouseFloorType

What litter was type is normally used in this shed?

Allowed options: Sand, Shavings, Straw, Mix shavings-straw, Other bedding.

Storage location: tblShedDetails.LitterType

THE FARM QUESTIONNAIRE

The following questions were asked for each farm. Validation messages (to help ensure sensible input) are shown in red.

Farm Name

Storage location: tblFarmDetails.FarmName

Address

Storage location: tblFarmDetails.FarmAddress1

Address please.

Town

Storage location: tblFarmDetails.FarmAddress2

Town please.

County

Storage location: tblFarmDetails.FarmAddress3

County please.

Postcode

Storage location: tblFarmDetails.FarmAddress4

Postcode please.

What is the **mobile phone number** that you want to receive the test results by text?
(Please enter the number without any spaces)

Storage location: tblFarmDetails.MobileNumber

Phone number please.
Eleven numbers, no spaces, starting with a zero.

What is the farm county parish holding (**CPH**) number?
(Please enter in the following format: 12/345/6789)

Storage location: tblFarmDetails.FarmCPH

CPH number please.
Format as nn/nnn/nnnn.

How long before catching is feed usually withdrawn from the birds?
(Please enter a single number of hours. If your farm uses a range such as 3 to 5 hours,
enter the number in the middle of the range. For example,

hours

Feed withdrawal time please.
RangeValidator

for 2 to 5 hours, 3.5 hours would be entered)

Storage location: tblFarmDetails.FeedWithdrawalTime

How many **broiler houses** are there on your farm?

Storage location: tblFarmDetails.NumberOfHouses

Allowed options: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.

What **production method** do you usually use for your birds?

Storage location: tblFarmDetails.ProductionMethod

Allowed options: Standard, Red tractor assurance, Free range, RSPCA freedom food, High welfare, Other.

What **material covers the ground** in the spaces between your broiler houses?

Storage location: tblFarmDetails.GroundMaterial

Allowed options: Grass or vegetation, Bare soil, Concrete or tarmac, Stone or hardcore, Other.

Do you usually **disinfect the drinker water lines** at turnaround?

Storage location: tblFarmDetails.DoesWaterDisinfect

Allowed options: Yes, No.

Do you usually **fog your sheds** with formaldehyde or other similarly effective chemical (e.g. peroxyacetic acid) at turnaround?

Storage location: tblFarmDetails.DoesFogging

Allowed options: Yes, No.

What **cycle length** are you using?

Storage location: tblFarmDetails.CycleLength

[ ] days

The time it takes for a batch of birds to be farmed in days please.

When was the current crop of **birds placed**? (use YYYY-MM-DD as the date format)

This and the above question let us work out how many kits we need to send to you.

Storage location: tblFlockDetails.DatePlaced

[ ]

A rough estimate of placement date please. Please format as yyyy-mm-dd with dashes in-between the numbers.

Does your farm have a **formal written protocol** describing its biosecurity measures? measures?

Storage location: tblFarmDetails.BestPracticeBioSecurity

Allowed options: Yes, No.

If it is known, what **slaughterhouse** does your farm usually send the birds to?
If not known leave blank, or enter (as examples) Frank Bird, Gafoor, 2 Sisters (Eye) or Banham etc.

Storage location: tblFarmDetails.Slaughterhouse

THE FLOCK QUESTIONNAIRE

What was the **sample collection date**?

Storage location: tblFlockDetails.SampleCollectionDate

Date please. Format as yyyy-mm-dd.

What was the **broiler house number** that the sample was collected from?

Storage location: tblFlockDetails.HouseNumber

Allowed options: Selection of a number (up to the maximum number of houses on the farm.)

What **hatchery** were your day-old chicks sourced from?

Storage location: tblFlockDetails.SourceHatchery

How many days were there **between the shed being cleaned of litter from the previous crop and the day old chicks being placed**?

Storage location: tblFlockDetails.TimeHouseEmpty

days

What was the **date that the flock was placed**? (please use yyyy-mm-dd)

Storage location: tblFlockDetails.DatePlaced

Date please. Format as yyyy-mm-dd.

What was the **age of the birds** when the test sample was collected?

Storage location: tblFlockDetails.BirdAge

days

What type of **sample** was collected?

Storage location: tblFlockDetails.SampleType

Allowed options: Pre-thin sample, 2nd thin or later sample, Final clearance sample.

How many **day old chicks were originally placed** in the house?

Storage location: tblFlockDetails.DayOldsPlaced

birds

What was the **cumulative daily mortality rate** (CDMR%) at 14 days?

% dead birds

Storage location: tblFlockDetails.CDMR

If the mortality rate was higher than expected, was there any special reason?

Storage location: tblFlockDetails.CDMRReason

Were the birds subjected to any **stress events** such as no drinking water or shed ventilation failure during rearing?

Allowed options: Yes, No.

Storage location: tblFlockDetails.BirdsStressed

What was the **breed** (e.g. Ross, Cobb or Hubbard) of the flock that was sampled?

Storage location: tblFlockDetails.Breed

What was the **sex of the birds** in the batch that was sampled?e birds in the batch that was sampled?

Allowed options: Male, Female, Mixed gender.

Storage location: tblFlockDetails.BirdGender

Were there any **standard supplements** added to the birds' water or feed during their production?
(please check any that apply, or leave everything blank if no supplements were used)

Storage location: tblFlockDetails.Various; prefixed by 'chk'

- ☐ Drinker line sanitiser
- ☐ Vitamins
- ☐ Vaccinations
- ☐ Probiotics
- ☐ Prebiotics
- ☐ Other supplement not listed

If supplements were used, please give the **name of the product(s)** and the manufacturer(s)

Storage location: tblFlockDetails.SpecificSupplements

Was there any **disease diagnosed** in the flock during their growth?

Allowed options: Yes, No.

Storage location: tblFlockDetails.DiseaseDiagnosed

If yes, what was the **disease**?

No

Were any **antibiotics** given to the birds during production?	Allowed options: Yes, No.
If yes, **what antibiotic(s)** were administered?	
If antibiotics were administered, what was the **bird age** when the treatment finished?	days
How **wet was the litter** when the sample was tested? (use a scale of 1-10, with 10 being the wettest)	Allowed options: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.
Was there any evidence of **litter beetle presence** during the current rearing?	Allowed options: Yes, No.
What type of **birds did the catchers catch** before they caught your birds?	Allowed options: First catch of the night, Poussin (1.5-1.75kg), Small/medium (1.8-1.9kg), Medium (2.0-2.4kg), Large (more than 3.5kg), Not known
Did you allow your **dog into the shed** at any time during the rearing of the flock?	Allowed options: Yes, No.
Did you change or **dip your boots** before you went into the shed?	Yes, most of the time; Yes, almost every time; Yes, every time; Sometimes; Never.
Is there anything else you think might be important about these birds that you'd like to tell us?	
Do you have a **300mm high barrier in your control** room to segregate the clean (bird side) and the dirty (outside) areas?	Allowed options: Yes, No.

APPENDIX 2 INFORMATION ORGANISATION IN THE MS-SQL SERVER DATABASE

Farm, broiler house and bird batch information was stored in a relational database (MS SQL server 2008). Tables were created to hold data describing farms, broiler sheds, batch-specific flock information and laboratory testing results. The tables were created by executing the following statements inside the SQL server programme.

1. CREATE TABLE tblFarmDetails (UserName nvarchar(50), FarmName nvarchar(50), FarmAddress2 nvarchar(50), FarmAddress3 nvarchar(200), FarmAddress4 nvarchar(50), FarmAddress5 nvarchar(50), MobileNumber nvarchar(50), FarmCPH nvarchar(50), FeedWithdrawalTime nvarchar(50), NumberOfHouses nvarchar(50), GroundMaterial nvarchar(50), DoesThinning nvarchar(50), BestPracticeBiosecurity nvarchar(50), AccreditedFeedMill nvarchar(50), SalmonellaTestDone nvarchar(50), OtherSpeciesPresent nvarchar(50), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

2. CREATE TABLE tblShedDetails (UserName nvarchar(50), HouseNumber nvarchar(50), VentilationType nvarchar(50), HouseConstruction nvarchar(50), HouseFloorType nvarchar(50), LitterType nvarchar(50), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

3. CREATE TABLE tblFlockDetails (UserName nvarchar(50), SourceHatchery nvarchar(50), TimeHouseEmpty nvarchar(50), DatePlaced datetime, BirdAge nvarchar(50), SampleCollectionDate datetime, DayOldsPlaced nvarchar(50), LiveWeight nvarchar(50), ThinDate datetime, CDMR nvarchar(50), CDMRReasonHigh nvarchar(500), Breed nvarchar(50), PreviouslyThinned nvarchar(50), HouseNumber nvarchar(50), HouseAge nvarchar(50), BirdGender nvarchar(50), chkDrinkSan int, chkVitamin int, chkVaccination int, chkProbiotics int, chkPrebiotic int, chkOtherSupplement int, SpecificSupplements nvarchar(500), DiseaseDiagnosed nvarchar(50), SpecificDisease nvarchar(50), AntibioticsGiven nvarchar(50), SpecificAntibiotics nvarchar(500), BirdAgeAbGiven nvarchar(50), LitterWetness nvarchar(50), SalmonellaTestResult nvarchar(50), CrateDesign nvarchar(50), Slaughterhouse nvarchar(50), LicenceNumber nvarchar(50), AnythingElse nvarchar(500), WhenSaved datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

4. CREATE TABLE tblResults (NFUidentifier nvarchar(50), DateSampled datetime, HouseNumber nvarchar(50), Results nvarchar(50), Loading decimal (12, 3), ReportingDate datetime, ID INT NOT NULL IDENTITY PRIMARY KEY)

The tables were linked using the NFU identifier (i.e. the farm identifier), the sample collection date and the broiler house number. In combination these three keys were a unique sample identifier. Data were harvested for analyses by executing the SQL statement:

```
SELECT tblResults.NFUidentifier, tblResults.DateSampled as ResultsDateSampled, tblFlockDetails.DateSampled
as FlockDateSampled, tblResults.HouseNumber, tblResults.Results, tblFlockDetails.Username,
tblFlockDetails.DatePlaced, tblFlockDetails.BirdAge, tblFlockDetails.SourceHatchery,
tblFlockDetails.TimeHouseEmpty, tblFlockDetails.DayOldsPlaced, tblFlockDetails.CDMR,
tblFlockDetails.CDMRReasonHigh, tblFlockDetails.Breed, tblFlockDetails.HouseNumber AS Expr2,
tblFlockDetails.BirdGender, tblFlockDetails.chkDrinkSan, tblFlockDetails.chkVitamin,
tblFlockDetails.chkVaccination, tblFlockDetails.chkProbiotics, tblFlockDetails.chkPrebiotic,
tblFlockDetails.chkOtherSupplement, tblFlockDetails.SpecificSupplements, tblFlockDetails.DiseaseDiagnosed,
tblFlockDetails.SpecificDisease, tblFlockDetails.AntibioticsGiven, tblFlockDetails.SpecificAntibiotics,
tblFlockDetails.BirdAgeAbGiven, tblFlockDetails.LitterWetness, tblFlockDetails.AnythingElse,
tblFlockDetails.Barrier, tblFlockDetails.WhenSaved, tblFlockDetails.BootsDipped, tblFlockDetails.DogInShed,
tblFlockDetails.PreviouslyCaughtBirds, tblFlockDetails.SampleType, tblFlockDetails.BeetlePresence,
tblFlockDetails.BirdsStressed, tblResults.Loading, tblResults.ReportingDate, tblFarmDetails.FarmName,
tblFarmDetails.FarmAddress, tblFarmDetails.FarmTown, tblFarmDetails.FarmCounty,
tblFarmDetails.FarmPostCode, tblFarmDetails.MobileNumber, tblFarmDetails.FarmCPH,
tblFarmDetails.FeedWithdrawalTime, tblFarmDetails.DoesWaterDisinfect, tblFarmDetails.NumberOfHouses,
tblFarmDetails.CycleLength, tblFarmDetails.GroundMaterial, tblFarmDetails.DoesFogging,
tblFarmDetails.BestPracticeBiosecurity, tblFarmDetails.ProductionMethod, tblFarmDetails.Slaughterhouse,
tblFarmDetails.LicenceNumber, tblFarmDetails.WhenSaved, tblFarmDetails.chkDog, tblFarmDetails.chkCat,
tblFarmDetails.chkCattle, tblFarmDetails.chkSheep, tblFarmDetails.chkPig, tblFarmDetails.chkTurkey,
tblFarmDetails.chkHorse, tblFarmDetails.chkLlama, tblFarmDetails.chkOther, tblFarmDetails.Accepted,
tblFarmDetails.NFUCode, tblFarmDetails.NFUApprovalTimeStamp, tblFarmDetails.KitsDispatchedDate,
tblFarmDetails.AlreadyCampyTesting, tblFarmDetails.FarmCategory, tblFarmDetails.KitNumberSent,
tblFarmDetails.AdditionalInfo, tblShedDetails.HouseAge, tblShedDetails.VentilationType,
tblShedDetails.HouseConstruction, tblShedDetails.HouseFloorType, tblShedDetails.Littertype FROM tblResults
full JOIN tblFlockDetails ON (tblFlockDetails.DateSampled = tblResults.DateSampled) AND
(tblResults.HouseNumber = tblFlockDetails.HouseNumber) AND (tblResults.NFUidentifier =
tblFlockDetails.UserName) LEFT JOIN tblFarmDetails ON (tblResults.NFUIdentifier = tblFarmDetails.NFUCode)
LEFT JOIN tblShedDetails ON (tblResults.HouseNumber = tblShedDetails.HouseNumber) AND
(tblFarmDetails.Username = tblShedDetails.UserName)
```

APPENDIX 3 SAMPLING PROTOCOL DOCUMENTATION

Included as part of the sample kits, the following instructions were sent to farmers.

ACT-NFU Boot swab sampling protocol Campylobacter detection in poultry houses

Kit contents:

1x addressed postage paid large letter tear proof envelope
1x pair Tunika boot swabs
1x sample bag
1x sample label
1x sampling instructions
1x Flock (House/Shed) sample information form

Equipment required not in the kit

Polythene overshoes
Pen for completing label on sample bags with house number etc

When to sample

1. Sample all houses no earlier then 24hrs prior to the commencement of 1st thin. Results will be text to the farm mobile number within 24hr of laboratory receipt of samples.
2. If the result of the first test is negative sample again immediately before (within 24 hours) the birds are next thinned or cleared (depopulated) whichever comes first.
3. If the first test is positive at first thin there is no need to sample that house again.

Instructions per house

1. Clearly complete the sample label and attach to the sample bag :
   - Check Farm name on labels provided with sampling kits
   - Add house number
   - Add date sampled
2. The sampler should observe the normal bio-security protocols up to the step-over barrier in the control room and prepare to enter the house. **The sampler should NOT use any foot dips in the control room as this will affect the result.**
3. After crossing the step-over barrier into the house, and once footwear for wearing inside the house has been put on, a pair of clean, disposable polythene overshoes should be put on (not included in the kit).
4. The pair of boot swabs present in the sampling pack should then be placed over the polythene overshoes.
5. The sampler will then walk the full length of the house and return. Remove the two boot swabs (one per foot) carefully, place in the labelled sealable bag provided.
6. **As much air as possible should be removed from the bag before sealing (as flat as possible failure to may result in postage delays) and before being placed in the pre-addressed / postage paid envelope. Check the sample bag is labelled and sealed before placing in the return envelope.**

   Envelopes should always be free from faecal contamination.
7. A completed Flock (House/ Shed) sample information form together with the Food Chain Information form (FCI) must be completed and returned together with the boot swab. **A single copy of each of these forms recording information for all houses sampled on that day, must be returned in the pre-paid envelope along with one of the samples.**
8. This large letter should be posted by Royal Mail on the day of sampling (critically to meet that day's postal pickup). This should ensure the sample arrives at the laboratory the following day enabling results to be reported within 24 hours.

Summary - What to send back in the envelope to AFBI

1. The sample
2. The Flock (house/shed) information sheet completed for all the sheds sampled
3. A copy of the FCI (alternatively the FCI can be e mailed to Malcolm.Taylor@afbini.gov.uk, CC. cw@hutchisonscientific.com))

Any questions or queries should be directed to Malcolm Taylor (Food Hygiene Unit, Agri-Food and Biosciences Institute, Newforge Lane Belfast BT9 5PX tel 02890255313, 07533064599, malcolm.taylor@afbini.gov.uk)

APPENDIX 4 DNA EXTRACTION PROTOCOL USED BY THE QIAXTRACTOR ROBOT

The robot was running the QIAxtractor software version 4.12.7.

The following DX reagents were used in this protocol and were prepared as outlined in the QIAxtractor DNA handbooklet 01/2011 (https://www.qiagen.com/resources/download.aspx).

Key to abbreviations:

DXL contains DX- liquid digest and digest enzyme (protease K),

DXB contains DXB- binding agent and DX binding agent,

DXW- wash 1 containing Tris-HCL, NaCl & EtOH,

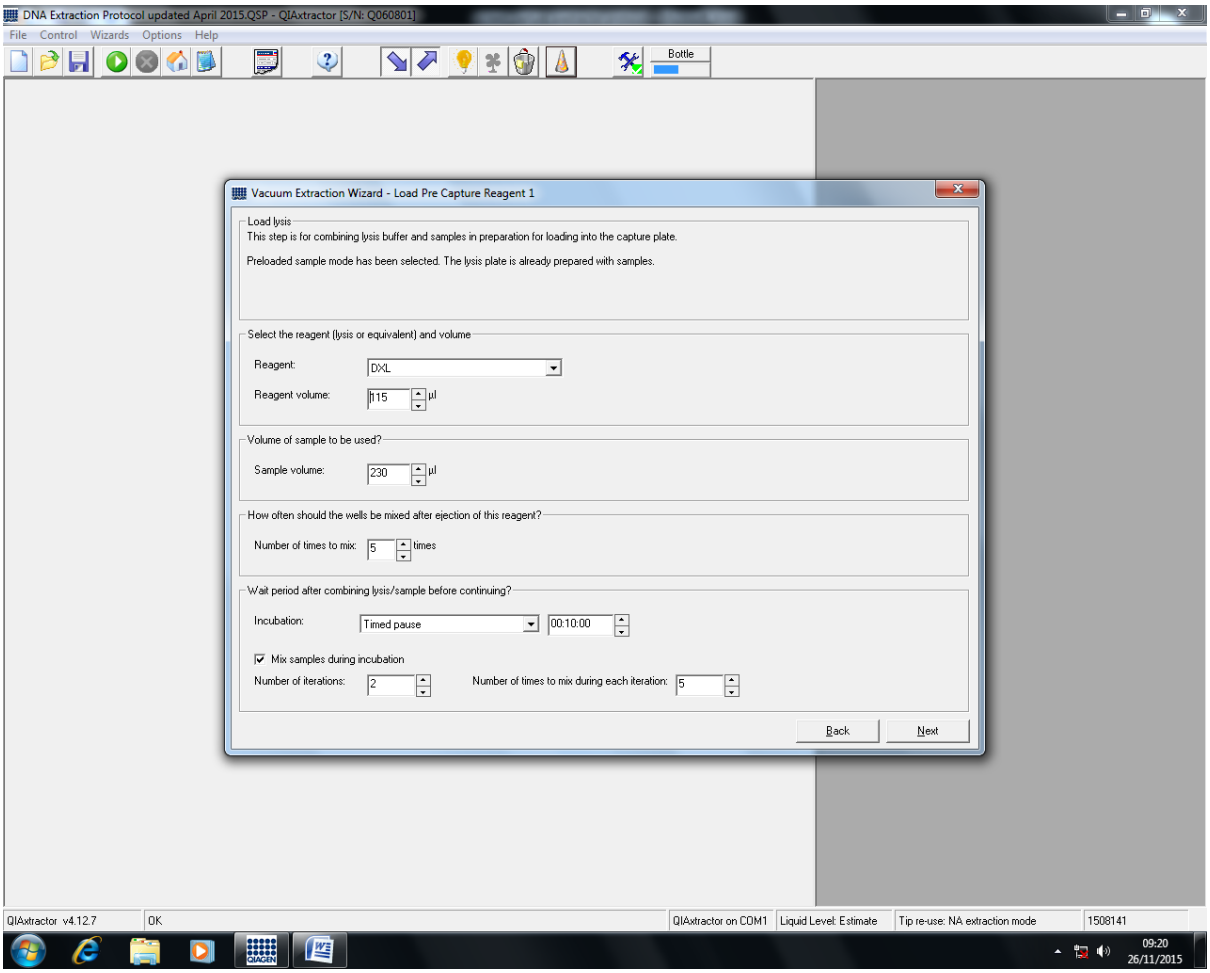
DXF- wash 2 containing EtOH & iPrOH

E- Elution buffer containing Tris HCL(PH 8.5) & 0.5mM EDTA

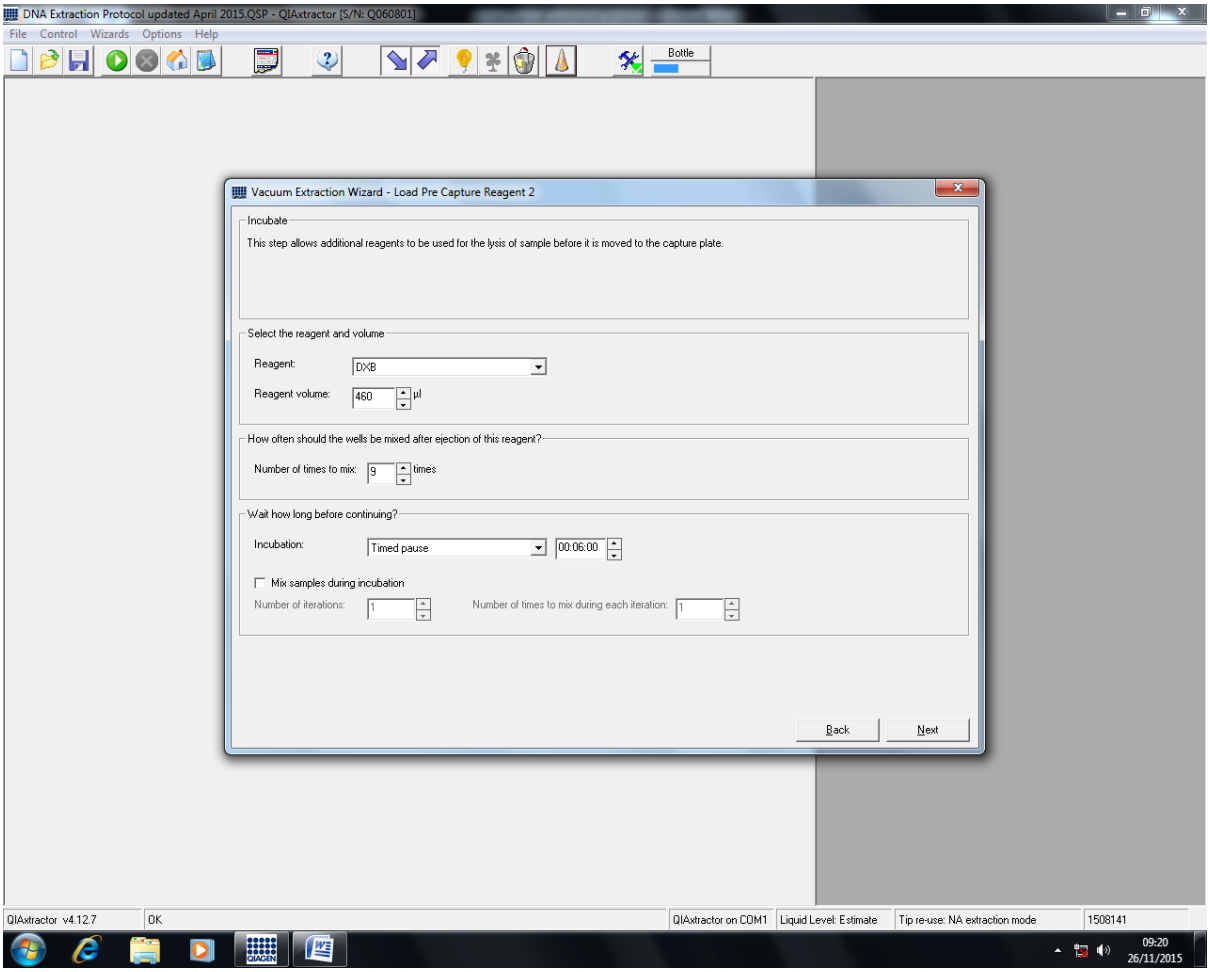
1. 115µl of DXB was added to 230µl of bootswab diluent in a deep well plate. Each sample was mixed five times before a pause of 10 minutes and two further mixes.
2. 460µl DXB was added to each sample. Samples were again mixed for five times before a pause of 10 minutes and further single mix.
3. 600µl of each sample was transferred from deep well plate to a corresponding well on a capture plate. Samples were vacuumed at 50kpa for five minutes (with manual clearing of wells where required).
4. 200µl of DXB per sample was added to each well of the capture plate before the samples were vacuumed at 50kpa for five minutes (again manual clearing of well where required).
5. 600µl of DXW per sample was added to each well in the capture plate and samples were again vacuumed at 40kpa for 1 minute. The step was repeated before proceeding to step 6.
6. 600µl of DXW per sample was added to each well in the capture plate before vacuuming samples at 30kpa for 1 minute.
7. The capture plate was vacuumed at 20kpa for a further five minutes to allow the filters to dry before transfer to the top of an elution plate.
8. 150ul of E per sample was added to the capture plate and incubated with gentle agitation for five minutes. Samples were vacuumed at 20kpa for two minutes to transfer the elution buffer containing any residual DNA to the elution plate.
9. Plates were sealed and stored until mericon *Campylobacter* spp PCR assay assembly was commenced.

A graphic representation of the above protocol in the robot setup is depicted below.

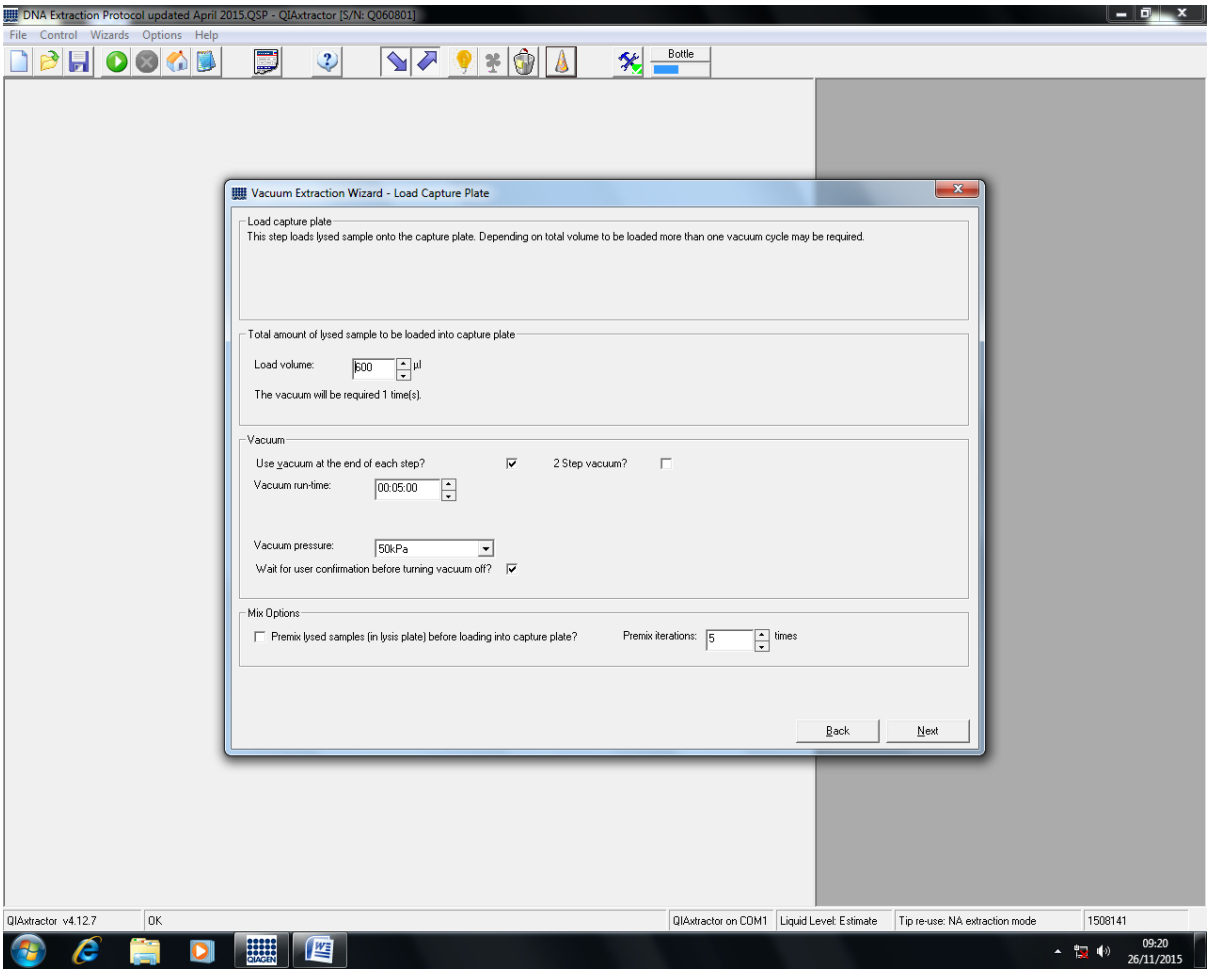
Step 1


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Load Pre Capture Reagent 1
Load lysis
This step is for combining lysis buffer and samples in preparation for loading into the capture plate.
Preloaded sample mode has been selected. The lysis plate is already prepared with samples.
Select the reagent (lysis or equivalent) and volume
Reagent:
DXL
Reagent volume:
115
µl
Volume of sample to be used?
Sample volume:
230
µl
How often should the wells be mixed after ejection of this reagent?
Number of times to mix:
5
times
Wait period after combining lysis/sample before continuing?
Incubation:
Timed pause
00:10:00
Mix samples during incubation
Number of iterations:
2
Number of times to mix during each iteration:
5
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:20
26/11/2015

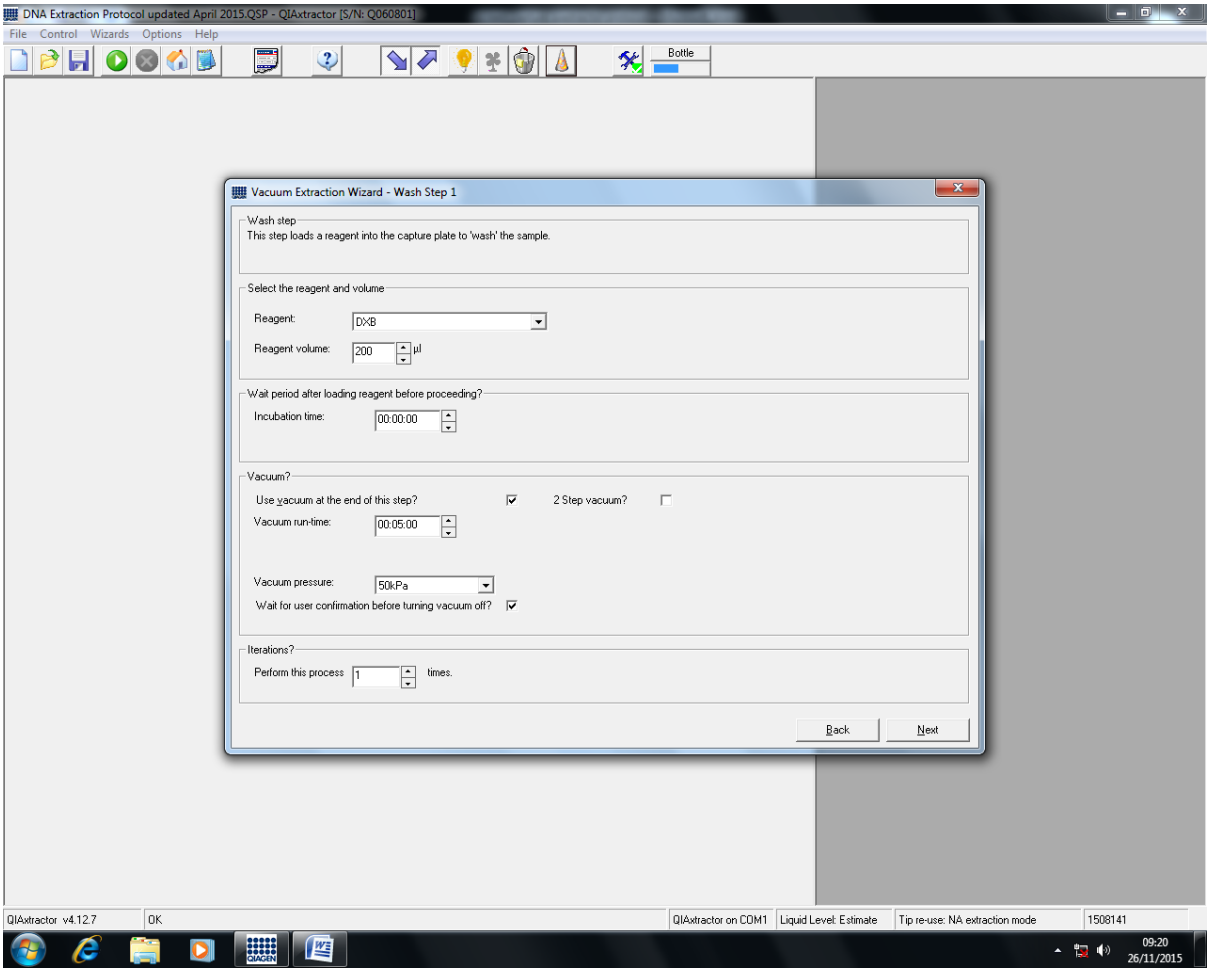
Step 2


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Load Pre Capture Reagent 2
Incubate
This step allows additional reagents to be used for the lysis of sample before it is moved to the capture plate.
Select the reagent and volume
Reagent:
DXB
Reagent volume:
460
µl
How often should the wells be mixed after ejection of this reagent?
Number of times to mix:
9
times
Wait how long before continuing?
Incubation:
Timed pause
00:06:00
Mix samples during incubation
Number of iterations:
1
Number of times to mix during each iteration:
1
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:20
26/11/2015

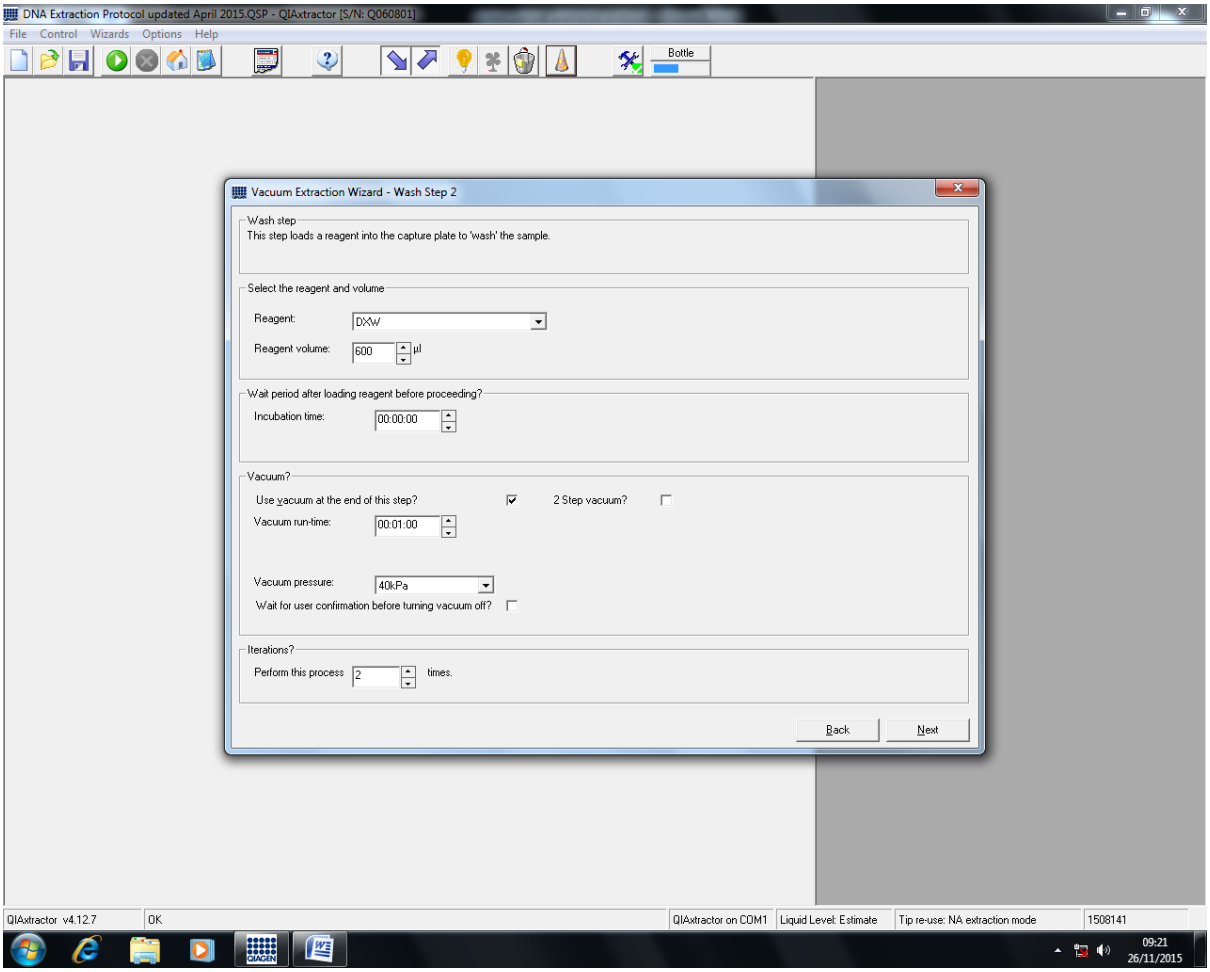
Step 3


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Load Capture Plate
Load capture plate
This step loads lysed sample onto the capture plate. Depending on total volume to be loaded more than one vacuum cycle may be required.
Total amount of lysed sample to be loaded into capture plate
Load volume:
600
µl
The vacuum will be required 1 time(s).
Vacuum
Use vacuum at the end of each step?
2 Step vacuum?
Vacuum run-time:
00:05:00
Vacuum pressure:
50kPa
Wait for user confirmation before turning vacuum off?
Mix Options
Premix lysed samples (in lysis plate) before loading into capture plate?
Premix iterations:
5
times
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:20
26/11/2015

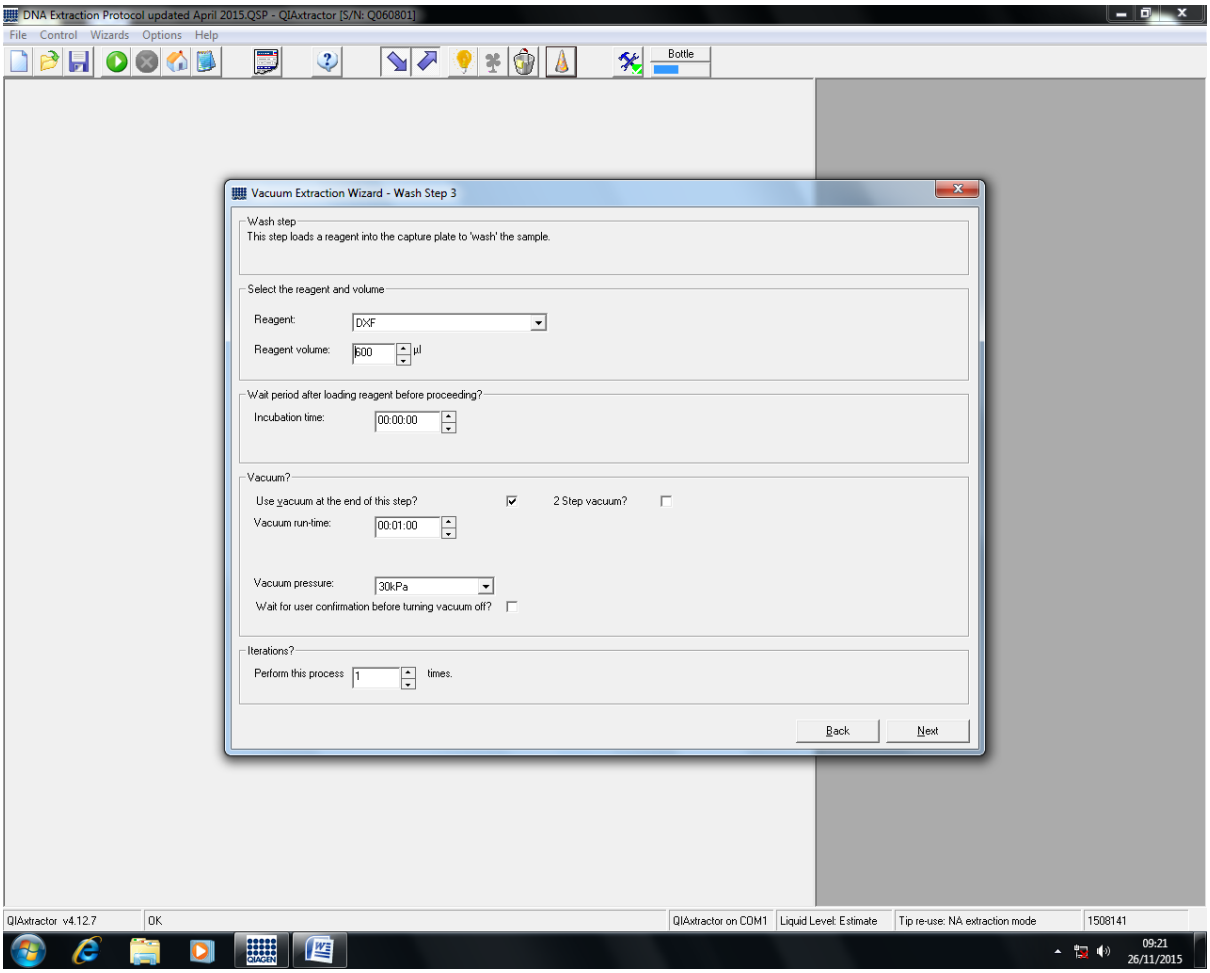
Step 4


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Wash Step 1
Wash step
This step loads a reagent into the capture plate to 'wash' the sample.
Select the reagent and volume
Reagent: DXB
Reagent volume: 200 µl
Wait period after loading reagent before proceeding?
Incubation time: 00:00:00
Vacuum?
Use vacuum at the end of this step?
2 Step vacuum?
Vacuum run-time: 00:05:00
Vacuum pressure: 50kPa
Wait for user confirmation before turning vacuum off?
Iterations?
Perform this process 1 times.
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:20
26/11/2015

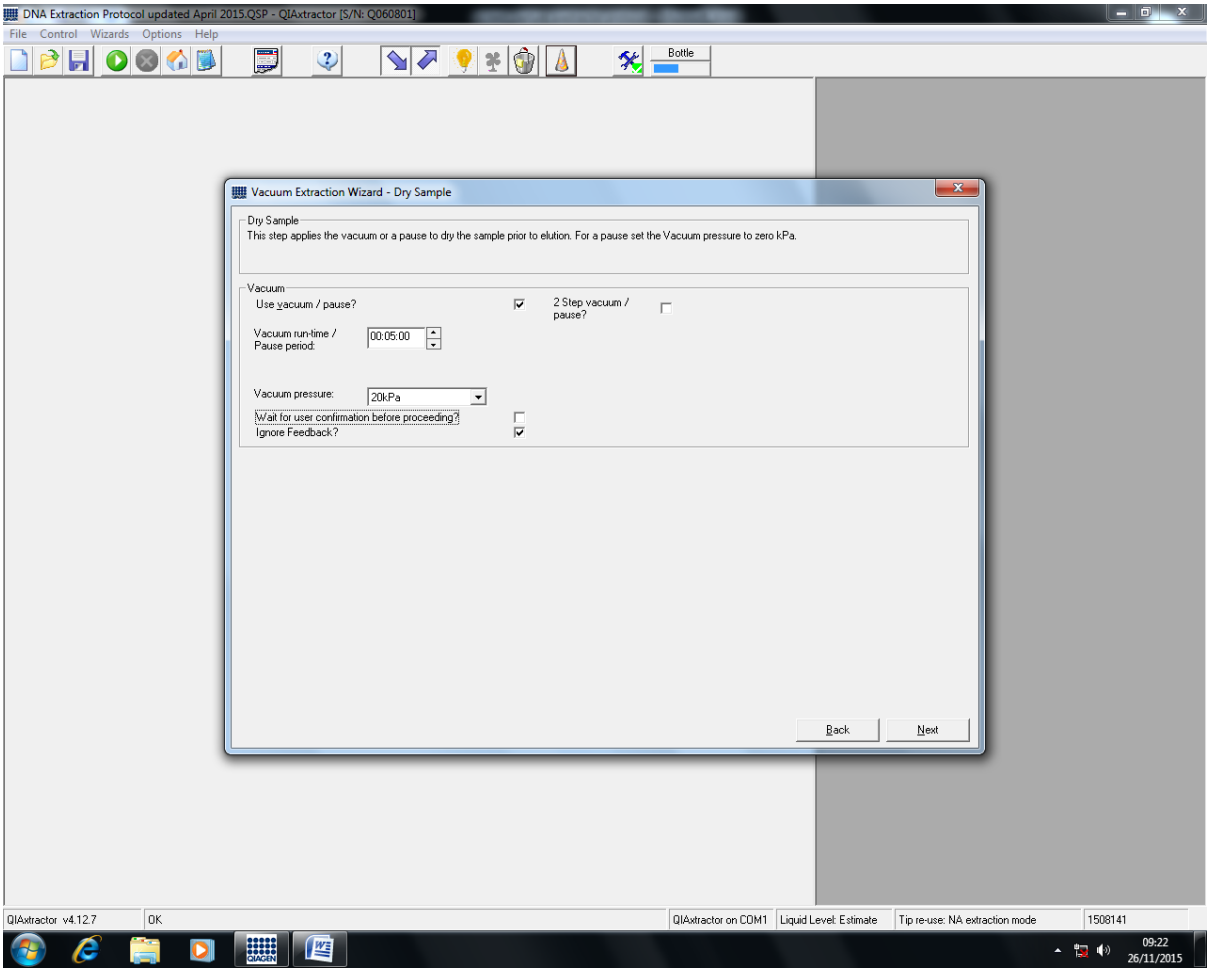
Step 5


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Wash Step 2
Wash step
This step loads a reagent into the capture plate to 'wash' the sample.
Select the reagent and volume
Reagent: DXW
Reagent volume: 600 µl
Wait period after loading reagent before proceeding?
Incubation time: 00:00:00
Vacuum?
Use vacuum at the end of this step?
2 Step vacuum?
Vacuum run-time: 00:01:00
Vacuum pressure: 40kPa
Wait for user confirmation before turning vacuum off?
Iterations?
Perform this process 2 times.
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:21
26/11/2015

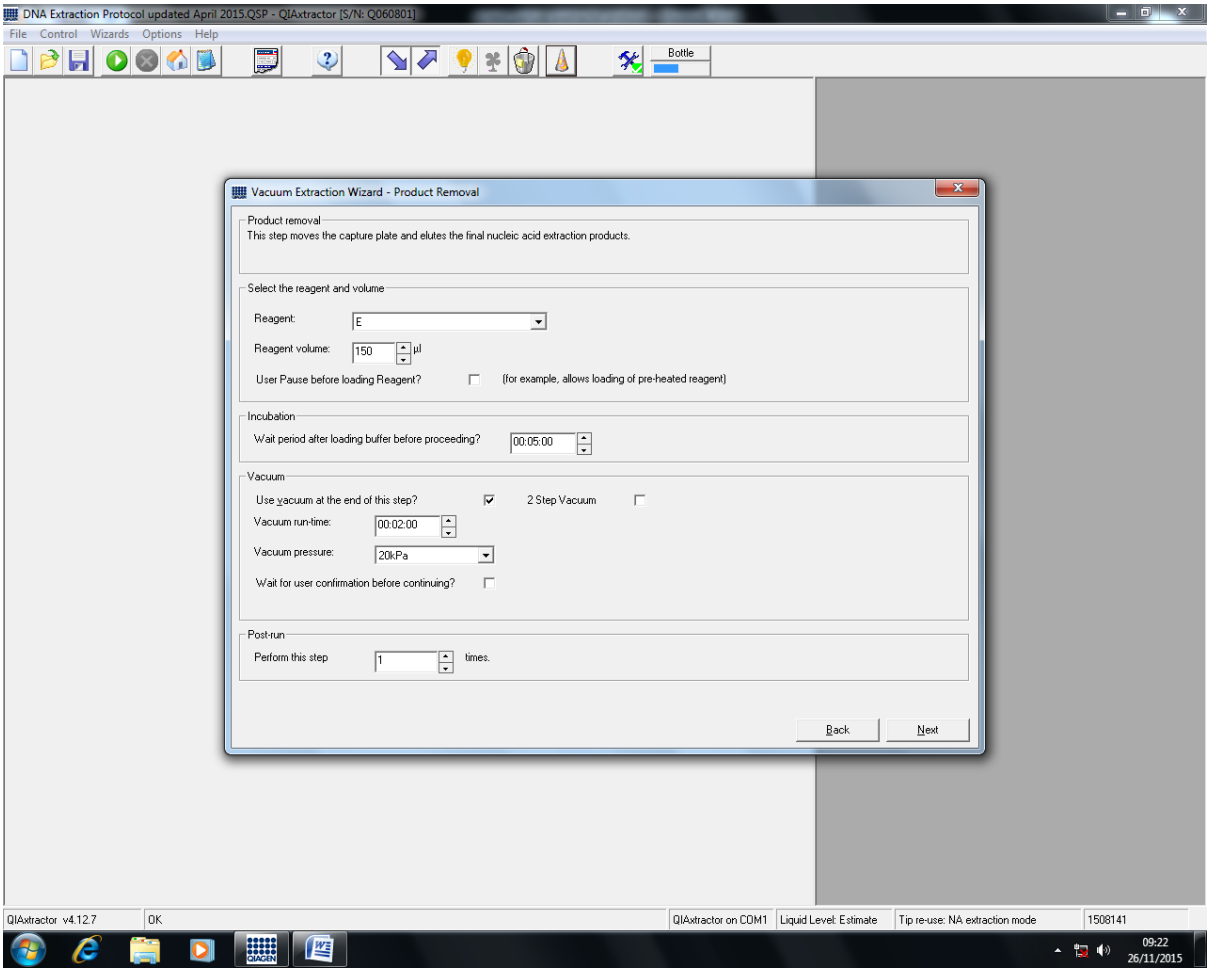
Step 6


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Wash Step 3
Wash step
This step loads a reagent into the capture plate to 'wash' the sample.
Select the reagent and volume
Reagent:
DXF
Reagent volume:
600
µl
Wait period after loading reagent before proceeding?
Incubation time:
00:00:00
Vacuum?
Use vacuum at the end of this step?
2 Step vacuum?
Vacuum run-time:
00:01:00
Vacuum pressure:
30kPa
Wait for user confirmation before turning vacuum off?
Iterations?
Perform this process
1
times.
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:21
26/11/2015

Step 7


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Dry Sample
Dry Sample
This step applies the vacuum or a pause to dry the sample prior to elution. For a pause set the Vacuum pressure to zero kPa.
Vacuum
Use vacuum / pause?
2 Step vacuum / pause?
Vacuum run-time / Pause period:
00:05:00
Vacuum pressure:
20kPa
Wait for user confirmation before proceeding?
Ignore Feedback?
Back
Next
QIAxtractor v4.12.7
OK
QIAxtractor on COM1
Liquid Level: Estimate
Tip re-use: NA extraction mode
1508141
09:22
26/11/2015

Step 8


DNA Extraction Protocol updated April 2015.QSP - QIAxtractor [S/N: Q060801]
File Control Wizards Options Help
Bottle
Vacuum Extraction Wizard - Product Removal
Product removal
This step moves the capture plate and elutes the final nucleic acid extraction products.
Select the reagent and volume
Reagent: E
Reagent volume: 150 µl
User Pause before loading Reagent? (for example, allows loading of pre-heated reagent)
Incubation
Wait period after loading buffer before proceeding? 00:05:00
Vacuum
Use vacuum at the end of this step? 2 Step Vacuum
Vacuum run-time: 00:02:00
Vacuum pressure: 20kPa
Wait for user confirmation before continuing?
Post-run
Perform this step 1 times.
Back Next
QIAxtractor v4.12.7 OK QIAxtractor on COM1 Liquid Level: Estimate Tip re-use: NA extraction mode 1508141
09:22
26/11/2015

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