

Rapid Sample Preparation and Real-Time PCR Analysis of *Listeria monocytogenes* from Fresh Poultry and Poultry-Related Surfaces

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Abstract

A rapid DNA sample preparation method was developed and optimized for real-time Polymerase Chain Reaction (PCR) analysis of *Listeria monocytogenes* from poultry, poultry fluids and poultry packaging materials. Sample preparation was based on a rapid, three-step extraction procedure. Two types of samples were tested in this project; 1) poultry fluids and 2) swabs taken directly from meat, package materials, and working surfaces. Control samples of fluids and swabs were collected. Samples of store-bought chicken were placed in plastic packaging material and inoculated with serial dilutions of *L. monocytogenes* from a 24-hour stock culture (10^5 to 10^6). All samples were placed in a 37°C incubator for 6 hours. Following the incubation period, fluid and duplicate swab samples of the meat and package material were collected. One half of the swab samples were placed in Listeria enrichment media and incubated for an additional 6 hours. Swabs collected from the working surfaces after 6-hours were processed immediately by placing the swabs into sterile bead tubes and disrupting for 5 minutes. DNA was extracted from all samples using the R.A.P.I.D. 1-2-3 DNA extraction kit. Chicken fluids (400 µl) were placed into the bead tubes with 50 µl of protease and disrupting for 5 minutes. Samples were then incubated at 70°C for 10 minutes. All samples were then loaded into spin columns and the DNA sample was captured on the column resin. Samples were washed and eluted from the resin in a two-step process. Real-time PCR was performed using the *Listeria monocytogenes* assay, which is designed to detect all strains of *L. monocytogenes*. Amplification results demonstrated that *L. monocytogenes* could be detected directly from chicken fluids, packaging, and processing surfaces without enrichment. Swab samples of meat required the initial enrichment period. Sample preparation of up to 64 fluid and/or swab samples can be completed within 60 minutes. The limit of detection of direct samples, as confirmed by plate counts, was between 550 and 600 cfu/ml.

Introduction

L. monocytogenes is a ubiquitous gram-positive bacterium commonly associated with food processing environments, raw dairy products, and various meat and poultry products. *L. monocytogenes* is a well-documented food pathogen and has recently been the focus of a number of food product recalls. One of the largest recalls was the October 12, 2002 recall of 27.4 million pounds of turkey and chicken products by the Pilgrim's Pride Corporation. Previous reports show that the distribution of *L. monocytogenes* in poultry products ranges from 90% in turkey frankfurters (1), 76% in ground turkey (2), and 35% in processed poultry parts such as wings and legs (3).

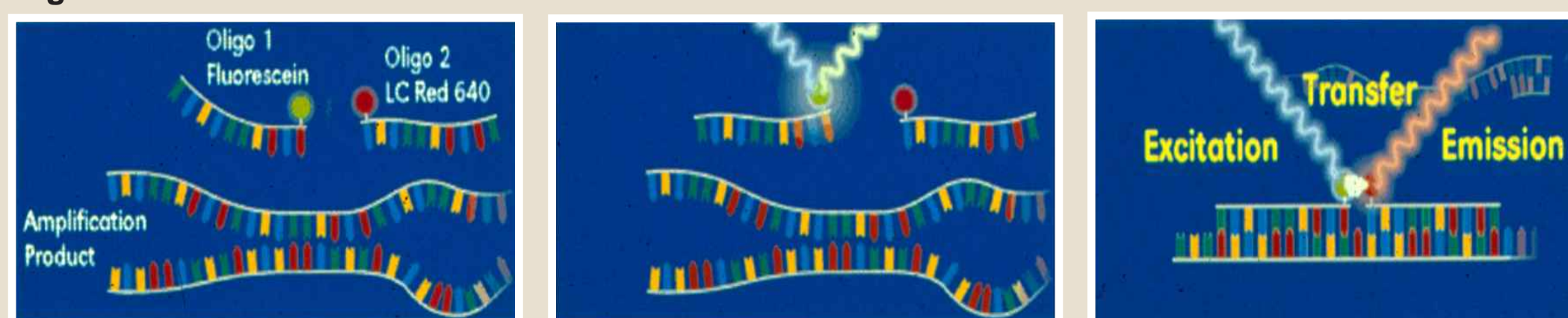
Listeriosis is an atypical food-borne illness of major public health concern because of the severity of the disease, its high rate of fatalities (35%), long incubation periods, and a significant incidence among young children, pregnant women, and/or immuno-compromised individuals. The epidemiology of Listeriosis requires appropriate preventative measures, but the characteristics of *L. monocytogenes* suggest that it is unrealistic to expect all foods to be Listeria-free. Therefore, there exists a requirement for rapid sample processing and detection techniques.

Real-time PCR is widely employed as a rapid, sensitive, and specific molecular diagnostic technique for the testing and identification of bio-threat agents and food pathogens from numerous biological and environmental samples. However, the full potential of diagnostic PCR has been limited in the past by the presence of PCR inhibitory substances in microbial culture media and biological samples. Ramos et al. (4) have demonstrated that in some cases, *L. monocytogenes* could not be detected by PCR due to unknown inhibitors in the enrichment media and/or the sample. The R.A.P.I.D. 1-2-3 DNA Purification kit provides DNA sample purification specifically designed to effectively remove PCR inhibitors such as poultry fat, myoglobin, and enrichment media components, while meeting the stringent requirements of real-time PCR. In addition, the bind, wash, and elute format of this kit is simple and easy to use.

The assay is designed for real-time PCR using the R.A.P.I.D. instrument and probes for DNA sequence specific detection of *L. monocytogenes*. A unique DNA sequence of *L. monocytogenes* genome is amplified using specific primers. The amplified product is then detected by fluorescence using a specific set of probes. The probes consist of two oligonucleotides that hybridize to a specific internal sequence of the amplification product during the annealing phase of the PCR cycle. One probe is labeled at the 5' end with LC-Red 640 and has a phosphorylated 3' end (to prevent extension of the probe sequence). The second probe is labeled at the 3' end with fluorescein. Only after hybridization to the amplified DNA do the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores and the emission of a signal at 640 nm (Figure 1). The intensity of the signal is proportional to the amount of amplification product generated during PCR. The *Listeria monocytogenes* assay has demonstrated 100% specificity to *L. monocytogenes* against a panel of 73 related and non-related species with a sensitivity of 100 fg of amplifiable DNA per reaction capillary.

In this study, methods for the rapid sample preparation and detection of *Listeria monocytogenes* from fresh poultry and poultry related surfaces are presented.

Figure 1.



Materials and Methods

L. monocytogenes was cultured in Listeria Enrichment Broth for 24 hours at 37°C prior to use. The cell numbers of serial dilutions (10^5 to 10^8) were determined by plate counting. Control samples of the liquid fraction from each sample package ($n = 3$) of fresh, raw chicken breast were collected into a sterile 15 ml tube. The sample (400 µl) was mixed with 50 µl of protease (Sigma, St. Louis, MO) and incubated for 10 minutes at 70°C. Following the incubation, the sample was processed using the standard 1-2-3 kit protocol for liquid samples. Prior to inoculation with *L. monocytogenes*, poultry samples were manipulated upon a plastic resin kitchen board. The working surface of the board remained static at room temperature for 6 hours. The surface was divided into 6 sections (8" x 6") and each section was sampled with a rayon swab. Swabs were then processed using the standard kit protocol for environmental swab samples.

Following collection of the control samples, chicken breasts were inoculated with 10 ml volumes of serial dilutions of the 24-hour culture. Control ($n = 3$) and inoculated samples ($n = 15$) were placed in a 37°C incubator for the duration of the study. Liquid samples were collected at 6 and 12 hours. The samples were processed using the standard 1-2-3 kit protocol for liquid samples. Swab samples of the chicken breasts and the plastic packaging were collected at 6 hours. Swabs were processed using the standard kit protocol.



R.A.P.I.D. IT 1-2-3 DNA Purification Kit™

Equipment Needed

- Biospec Bead Beater or Scientific Industries Turbomix 2.0 ml adapter for Vortex-Genie 2.
- Low Speed Desktop Microcentrifuge
- Pipettes and Tips
- Gloves and other Protective Gear

Kit Components (store at room temperature)

- 20 ml Buffer 1
- 30 ml Buffer 2
- 25 ml Buffer 3
- 50 Bead tubes with lysis buffer
- 50 Spin filters (filter-receiver assembly)
- 200 Receiver Tubes
- 50 Swabs

DNA Purification Instructions

Wear gloves and protective gear throughout this procedure. Discard all used disposables into a biosafety waste container. We recommend that you divide the buffers into small containers to control possible contamination.

Protease Treatment for Liquid Sample.

- Add 50 µl of protease to sample.
- Incubate at 70°C for 10 minutes.
- Add to a bead tube containing 400 mg of zirconium-silica beads.
- Screw cap on tight.
- Place bead tube in the bead beater for 5 minutes.

Gathering and Disruption of Micro-Organisms in Sample.

- Dip swab in a bead tube and make sure the swab is wet with lysis buffer.
- Swab the affected area with the damp swab (i.e. swab surface).
- Place the swab into the bead tube and snap off the swab stick end, leaving the swab in the tube. (alternatively, if you are using cultured samples, add 400 µl to a bead tube containing 400 mg of zirconium-silica beads).
- Screw cap on tight.
- Place bead tube in the bead beater for the specified amount of time.

Bead Beating Settings

| | |
|-----------------|---------------|
| Vortex Genie 2: | Maximum Speed |
| BioSpec: | 5 Minutes |

Part 1: Binding DNA to Filter

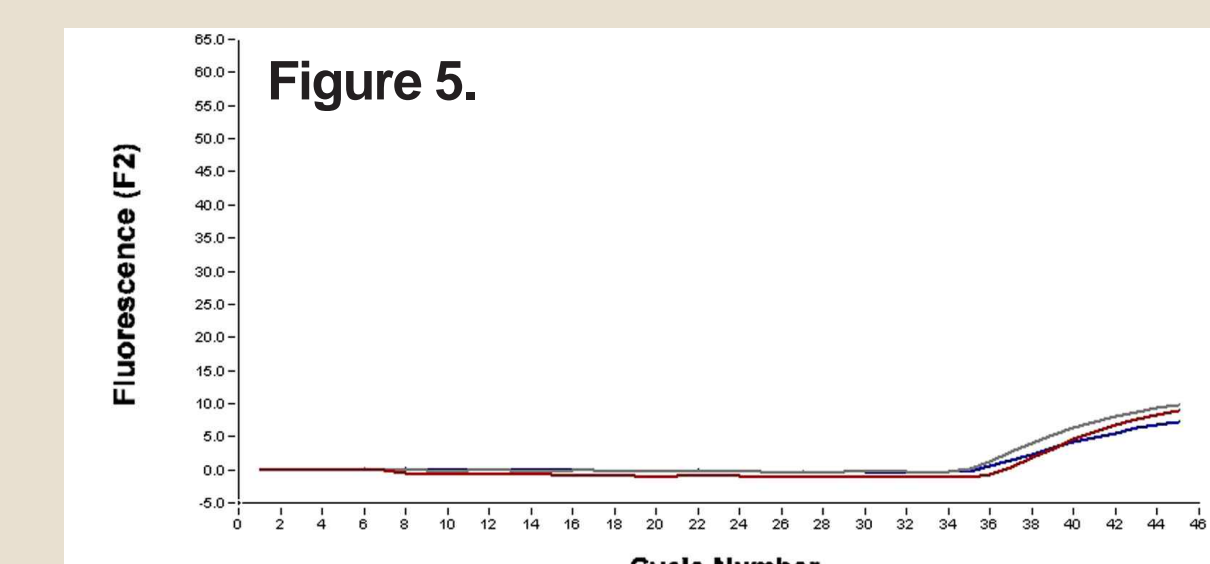
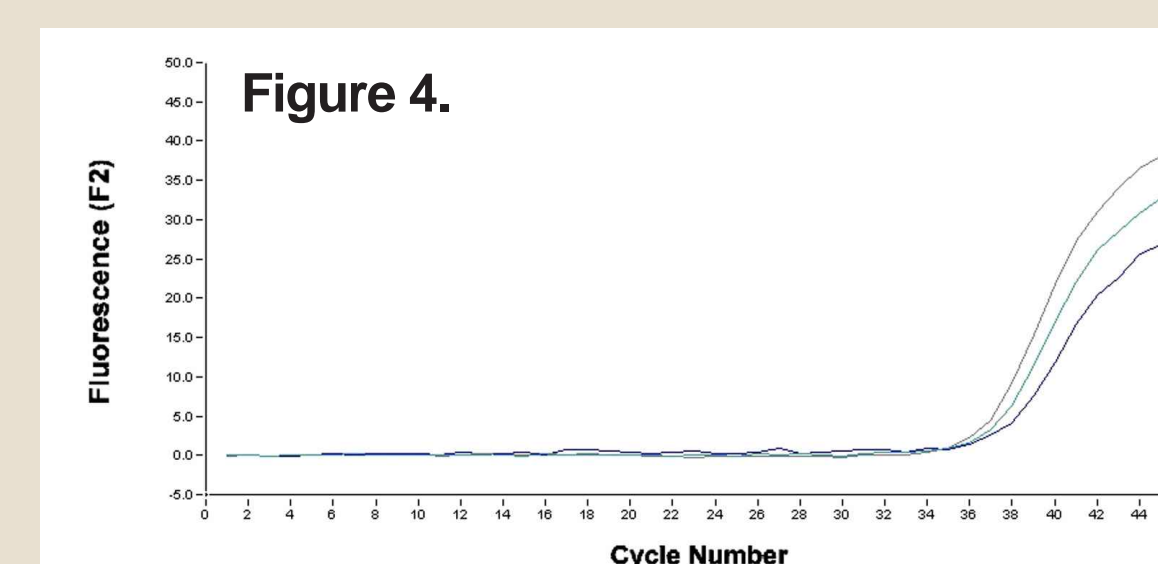
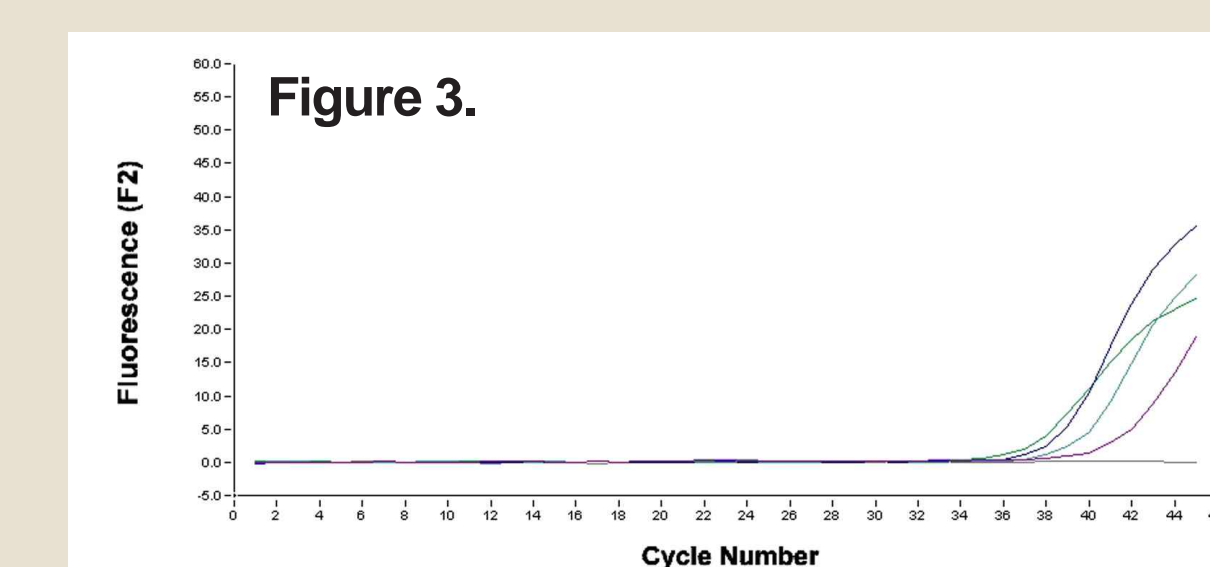
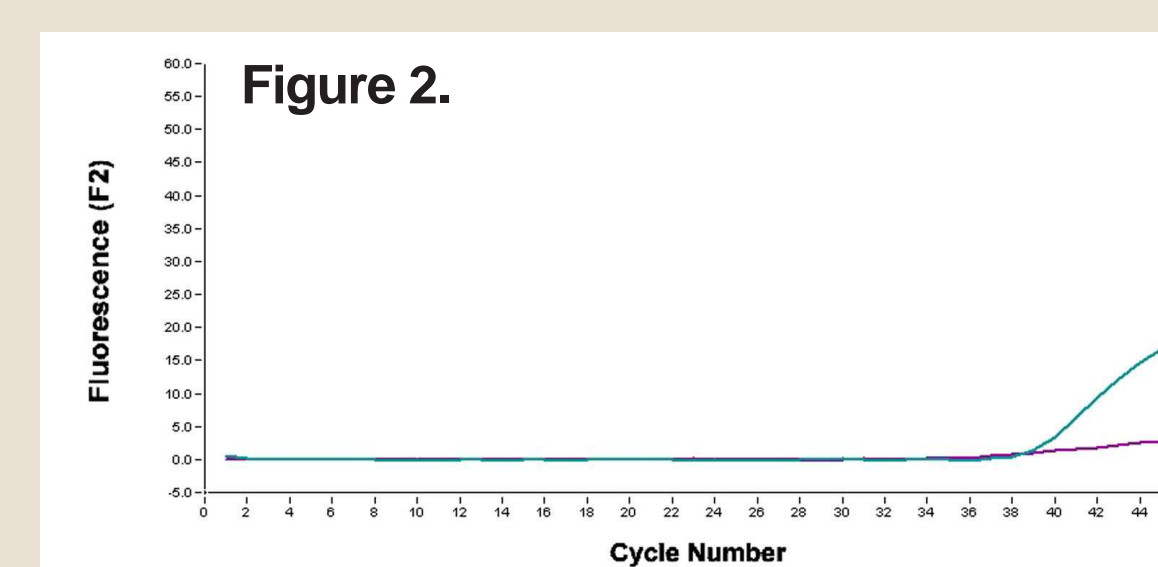
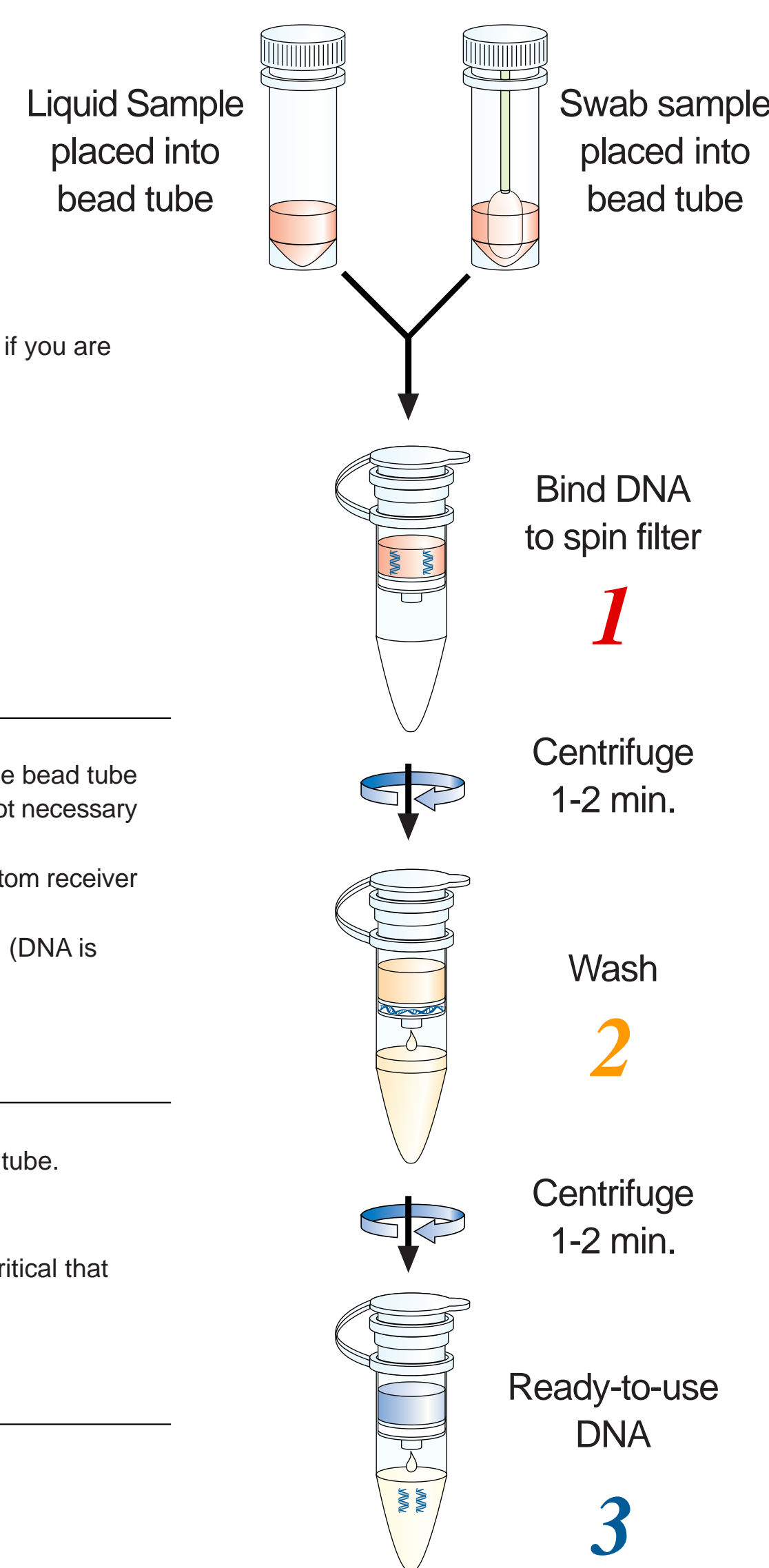
- Pipette 350µl of Buffer 1 into the bead tube containing the swab stick end. Centrifuge briefly.
- Using the P-1000 pipette set at 350µl and a clean tip, draw out as much of the liquid sample as you can from the bead tube without drawing out any of the beads and place the liquid sample into the top of the filter-receiver assembly (it is not necessary to remove all the liquid sample from the tube). Repeat if necessary.
- Close the cap on the filter-receiver assembly. Centrifuge 1-2 minutes or until all liquid has been pulled into the bottom receiver tube.
- Open the lid and remove the filter. Close the lid of the receiver tube. Discard the receiver tube with its contents. (DNA is captured in the filter).
- Place the filter into a new receiver tube.

Part 2: Wash Filter – DNA Clean-Up

- Pipette 550µl of Buffer 2 into the filter-receiver assembly.
- Close assembly lid and spin in centrifuge for 1-2 minutes or until all of the liquid is in the bottom of the receiver tube.
- Open the lid and remove the filter. Close the lid of the receiver tube. Discard the receiver tube with its contents.
- Place the filter into a new receiver tube and centrifuge for 3 minutes.
- Note: This step is very important because the residual ethanol in Buffer 2 can hinder your PCR reaction. It is critical that all of Buffer 2 be removed from the filter.
- Open the lid and remove the filter. Close the lid of the receiver tube. Discard the receiver tube with its contents.

Part 3: DNA Elution

- Place the filter into a new receiver tube.
- Pipette 400µl of Buffer 3 into the filter-receiver assembly.
- Close the lid of the assembly and let it sit at room temperature for 2 minutes.
- Centrifuge for 1-2 minutes or until all of the liquid is in the bottom of the receiver tube.
- Keep the receiver tube containing the DNA. Discard the filter.
- Close the lid of the receiver tube. Label and date the tube containing the sample DNA. Carefully store DNA after use at 4°C to -20°C. This is the DNA that will be used for PCR analysis.



Results

Due to the high incidence of *L. monocytogenes* in poultry, the samples of chicken breasts were screened for the presence of the bacteria prior to testing. Poultry fluids recovered from bags of raw processed chicken contain a high concentration of fats, myoglobin, proteins, and a plethora of unidentified constituents. The addition of a protease step with these types of samples significantly reduces their inherent viscosity and in some cases clarifies the sample. Two poultry fluid samples, collected prior to swabs of the chicken breasts, were positive for the presence of the bacteria (figure 2). Control swabs taken of the chicken breasts and packaging material were negative.

Testing of the environmental swabs, taken from the kitchen board at 6 hours, resulted in four positive results (figure 3.). Swabs collected from the control and inoculated packages of chicken breasts at six hours were all positive (figure 4).

Liquid samples (400 µl) taken from the control and inoculated packages of chicken at 6 hours tested positive. Samples inoculated with a 10^6 dilution of *L. monocytogenes* (105 cfu/ml) all tested positive at 6 hours (figure 5). Only one sample inoculated at 10^7 (10 cfu/ml) resulted in a positive test at 6 hours.

Discussion

PCR has revolutionized scientists' ability to detect and quantify nucleic acid sequences for research and diagnostic purposes. The success of any real-time PCR analysis is based on the purity and recovery of the nucleic acid target and the design and quality of the PCR reagents. PCR methods usually require a purification step prior to template amplification. Typically, this involves lysis of the cell and nuclear membranes (if present), followed by the extraction and purification of the nucleic acids. The traditional approach to isolating DNA has taken advantage of its solubility characteristics. Cells are lysed and the protein digested into smaller fragments through the use of a powerful protease that spares the DNA. When mixed in a solution of phenol/chloroform, the DNA remains in the aqueous phase and the proteins dissolve into the organic phase. Once the organic phase (chloroform) has been removed, the DNA can be precipitated out of solution using pure alcohol or high salt concentrations. Phenol-chloroform extraction has been shown to be highly effective, but uses toxic substances and is labor intensive.

PCR inhibitors in complex biological samples and/or culture media have been shown to interfere with the cell lysis step, inactivate the thermostable DNA polymerase, and/or interfere with the amplification of nucleic acids (Burkhardt, 2000). Various attempts have been made to reduce PCR inhibition in microbial samples, particularly with regards to a specific matrix. Inexpensive methods, such as boiling, have been effective with various biological fluids. However, boiling can also cause inhibition. Sample dilution has worked for the amplification of microbes from urine samples, but was inadequate for more viscous matrices. The addition of bovine serum albumin protects PCR from the effects of blood, but this procedure has not been tested on a wide variety of raw meat samples. Previous methods for sample preparations have proven to be complicated, require significant experience, are difficult to apply to large numbers of samples, and are labor intensive and time consuming.

The binding of specimen DNA to silica spin columns represents a strategy for eliminating a variety of inhibitors in a single step. The cell sample is lysed using zirconium-silica beads. The homogenization with zirconium beads effectively destroys the cell membrane and nucleus (if present), fragmenting the chromosomes in the process. These smaller DNA molecules are more readily amplified over the larger native molecules. Using this procedure, a DNA sequence from as few as 10 cells has been successfully detected. The sample is applied to a silica resin spin column that binds the DNA based on net charge of the molecule. Unwanted proteins and other potential PCR inhibitors are washed from the column. The purified DNA is eluted from the column and collected in the bottom of a receiver tube. The bind, wash, and elute format provides a simple yet effective approach for DNA isolation from a variety of cell types and sample matrices.

The bind, wash, and elute silica membranes used in this study were effective in removing PCR inhibitors from poultry and poultry related samples. The binding of DNA to silica membranes is rapidly performed and easy to use. Given the sensitivity of our PCR assay, we believe that the use of the R.A.P.I.D. 1-2-3 DNA purification kit represents a significant step in improving the quality of real-time PCR analysis of poultry samples.

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