Ozarks Environmental and Water Resources Institute (OEWRI)
Missouri State University (MSU)

# Bacteria Source Tracking to Support Watershed Planning, Little Sac River, Southwest Missouri.

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**June 7, 2018**

OEWRI EDR-18-004

## Introduction

The Little Sac Watershed in Greene and Polk Counties of southwest Missouri was placed on the 303d list for bacteria impairment in 1998 (WCO 2016). In 2006, a Total Maximum Daily Load (TMDL) was developed for the watershed to address bacteria impairments within the Little Sac River and an initial watershed management plan was finalized in 2010 (Baffaut 2006, WCO 2009). The Watershed Committee of the Ozarks (WCO) is presently updating that plan with the most recent information on bacteria within the watershed. As part of that process, the WCO has contracted the Ozarks Environmental and Water Resources Institute (OEWRI) at Missouri State University (MSU) to complete a bacteria source tracking study within the watershed to identify potential bacteria pollution sources. The purpose of this study is to collect water samples throughout the watershed and evaluate bacteria DNA using real-time PCR for specific marker genes that can help identify specific bacteria sources from different locations in the Little Sac River watershed.

## Water Sampling

Water samples were collected from five different location of Little Sac River in Greene and Polk Counties (Figure 1, Table 1). Three sites were located along the main stem of the Little Sac River at Farm Road 68 and Farm Road 129 in Greene County and State Highway 215 in Polk County. Another site was located along Asher Creek on E. 560$^{th}$ in Polk County and the final site was located along Pea Ridge Creek at Farm Road 102 in Greene County. Drainage areas ranged from 10.7 km$^2$ at PR_102 to 609 km$^2$ at LS_215 (Table 2). The watershed above PR_102 is mostly urban (83.3%), with LS_68, AC_560, and LS_215 being more rural with greater than 80% of the land use agricultural and forested. Site LS_129 is more mixed with 22.1% urban, 43.4% agriculture, and 30.1% forested.

Samples were collected two weeks apart on September 22$^{nd}$ and October 6$^{th}$, 2017. During each sampling day a duplicate sample was collected from a randomly selected site. Water samples were collected in 8 L sterilized polypropylene carboy containers. All samples were placed on ice after collection and transported to the Microbiology Laboratory at MSU within two hours of sampling. During the bacteria source tracking sampling, additional samples were collected for quantifying bacteria using the IDEXX method to compare with the source tracking results. These samples were collected in sterile 125 mL plastic bottles and processed in the OEWRI laboratory at MSU within two hours of sampling. Duplicate samples were also collected at the same locations as the bacteria source tracking duplicates. Sample conditions on both days were similar with discharge at the USGS gaging station at Site LS_215 between 25-30 ft$^3$/s (Figure 2).

**Laboratory Methods**

IDEXX

The IDEXX Quanti-Tray/2000 system is used to analyze water samples for the presence of total coliform and *E. coli* flowing manufactures recommendations and laboratory SOPs (OEWRI 2013).

DNA Extraction

From all water samples, one liter of water per sample was filtered through 0.22 μm Sterivex filters (Millipore Corporation, MA) using a peristaltic pump (Masterflex, Cole–Pamer Co, Vernon Hills, IL, USA). Filters were broken and membranes were removed and cut into small pieces using sterile scissors then placed in 1.5 mL micro-centrifuge tubes that were used for DNA extraction. Genomic DNA from the 12 water samples was extracted using the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA). All extraction steps were followed according to the manufacturer's instructions, and DNA samples were stored at –20°C until analyzed.

Real-time PCR for specific marker genes

*Bacteroidetes* specific to human, bovine, goose, and dog fecal bacteria were determined using qPCR. Detection of bacterial contamination of human, bovine, goose, chicken and dog fecal material was performed using the group-specific primers (Table 3). These assays were carried out using the same master mix concentrations and qPCR-cycling conditions as described previously (Mirza et al., 2017). Briefly, qPCR was carried out in 25 μL volumes containing 12.50 μL of iTaq Fast SYBR green supermix with ROX (Bio–Rad, Inc., Hercules, CA), 100 nM primers, and 10 ng of template DNA. PCR conditions were as follows: 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 30 s. PCR grade water was used as a negative control. The specificity of the qPCR products was confirmed by melting curve analysis. A standard curve was generated from serial dilutions ($10^0$ to $10^{-9}$) of plasmid DNA or serially diluted PCR product of the specific marker gene. The qPCR efficiency ($E$) was calculated according to the equation $E = 10^{[-1/\text{slope}]}$.

QA/QC

The PCR primer combination used in this study has been previously well tested and optimized for the specific amplification of bacterial marker genes from human (Green et al., 2014a; Ahmed et al., 2015), bovine (Ravaliya et al 2014; Shanks et al., 2010), goose (Green et al., 2012; Lu et al., 2009), chicken (Weidhass et al. 2010), and dog (Green et al., 2014b) fecal materials. The positive standard DNA material (plasmid or gene amplicon) that was amplified from the fecal material of different source animals (human, dog, cow, chicken and goose) showed consistent PCR amplification. This was used as a reference material for our unknown water samples. The negative samples (sterile water) did not show any amplification. The regression line of the standard curve generated through serial dilutions of specific marker genes showed a coefficient of determination of 0.996 to 0.997 and a PCR amplification efficiency of 91 to 104%. The specificity of the amplicon was confirmed by the

melting curve analysis, which indicated the presence of a single peak for each marker gene (Figure 3).

**Conclusions and Interpretation**

Results of the IDEXX sampling shows some variability in concentrations of *E. Coli* between the two sampling periods while total coliform concentrations exceeded the upper limit of the procedure. On September 22nd, *E. Coli* concentrations ranged from 60.2 MPN/100 mL at LS_215 to 118.7 MPN/100 mL at AC_560 (Table 4). These concentrations are all lower than the Missouri Department of Health whole body contact limit of 126 cfu/100 mL for class A streams (MEC 2007). On October 6th *E. Coli* concentrations were more variable ranging from 30.0 MPN/100 mL at LS_215 to 517.2 MPN/100 mL at PR_102 (Table 5). Site LS_068 exceeded the whole body contact limit of 126 cfu/100 mL for class A streams and LS_129 and PR_102 exceeded the Whole Body Contact Recreation (WBCR) Class-B designation of 206 cfu/100 mL (MEC 2007). The lowest *E. Coli* concentrations were found at LS_215 at the USGS gaging station located near Morrisville above Stockton Lake. Field duplicates varied less than 15% for both sample dates (Table 6). All total coliform concentrations exceeded 2,419.6 MPN/100 mL.

Four out of the five sites had positive results for at least one of the markers examined for the bacteria source tracking portion of this project and the variability of field duplicates and repeat sampling at each site was low. Site PR_102, which is mostly urban, was the only positive for human bacteria (Table 7). The two sites with the most urban land use, PR_102 and LS_129, were positive for the goose bacteria marker (Photo 2). The more agricultural sites LS_068 and AC_560 were positive for the bovine bacteria marker. The furthest downstream site LS_215 did not have positive markers for any of the sources accessed for this project. There were no positive samples for the chicken or dog marker at any of the sites sampled. Field duplicates for LS_215 in September both were negative for all markers. Field duplicates on LS_068 in October were similar for the bovine marker with 6,577 copies per liter and 6,769 copies per liter. All sites had the same positive markers for both the September and October sample dates.

Bacteria source analysis generally indicated two source signatures. First, urban areas are linked to both human and goose sources. Human bacteria probably indicate diffuse nonpoint contributions from the suburban and urban areas of the watershed. However, it is possible that the presence of human bacteria may also reflect releases from leaking sewer infrastructure or septic waste migration through karst conduits. Goose bacteria is expected given the use of the local area by residential or migrating geese (Photo 2). The goose contribution is probably localized and not related to broader watershed trends. Second, agricultural source signatures are linked to bovine sources probably where streams flow through cattle pen or grazing areas. Bovine concentrations in the stream water are 3.6-8 times those from human sources measured in urban areas. Bacteria sources from rural areas, as distinct (or distant) from agricultural areas,

tend to be low as indicated by low *E. Coli* concentrations and no positive tests for specific markers measured at Morrisville.

Dog and chicken bacteria were not detected at any site. However, this study sampled during fair-weather, base flow conditions when runoff sources from watershed surfaces would be diminished. Therefore, pet or chicken house waste source contributions to the stream were expected to be negligible. To address our lack of understanding of rainfall-related inputs, bacteria source analysis of storm water samples at these sites is needed to fully evaluate nonpoint sources of bacteria to the Little Sac River. The results of this study support management efforts to reduce bacteria from human sources in urban areas and from cattle operations, and possibly manure spreading areas, in rural areas of the watershed.

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## Tables

### Table 1. Sample site locations

| Site | North_m | East_m | Stream | Location |
|---|---|---|---|---|
| PR_102 | 4,124,106.295 | 472,519.282 | Pea Ridge Creek | FR 102 bridge in Greene County |
| LS_068 | 4,130,301.675 | 475,487.948 | Little Sac River | FR 68 bridge in Greene County between McDaniel and Fellows Lake |
| LS_129 | 4,127,392.990 | 468,898.117 | Little Sac River | FR 129 bridge in Greene County below NW WTP |
| AC_560 | 4,143,465.108 | 458,857.212 | Asher Creek | E. 560th bridge in Polk County |
| LS_215 | 4,148,542.318 | 457,081.672 | Little Sac River | SH 215 bridge in Polk County |

### Table 2. Drainage area characteristics

| Site | Ad (km2) | % Urban | % Agriculture | % Forest | % Water | % Other |
|---|---|---|---|---|---|---|
| PR_102 | 10.7 | 83.3 | 6.5 | 9.8 | 0.1 | 0.2 |
| LS_068 | 78.4 | 7.2 | 51.1 | 34.9 | 4.5 | 2.3 |
| LS_129 | 204.8 | 22.1 | 43.4 | 30.1 | 2.6 | 1.9 |
| AC_560 | 91.9 | 5.1 | 64.7 | 27.7 | 0.8 | 1.7 |
| LS_215 | 609.2 | 10.9 | 45.8 | 39.9 | 1.3 | 2.1 |

### Table 3. PCR Primers used in qPCR

| Primer | Sequence | Marker | Reference |
|---|---|---|---|
| F Primer HF183 | 5'- ATCATGAGTTCACATGTCCG | Human | Layton et al., 2006 |
| R Primer SSHBacR | 5'- TACCCCGCCTACTATCTAATG | | |
| BoBac367f | 5′- GAAG(G/A)CTGAACCAGCCAAGTA | Bovine | Layton et al., 2006 |
| BoBac467r | 5′- GCTTATTCATACGGTACATACAAG | | |
| DG72F | 5′- GCAACTTGGTGAGGAAAAGG | Dog | Green et al., 2014b |
| DG72R | 5′- TCCAGTATTTCCCGTCGTGT | | |
| CGPrevf5-F | 5′- CCC ACC AAG CCG TCG AT | Goose | Lu et al., 2009 |
| CGPrevf5-R | 5′- GCT TAA CCT GCG GCC TG | | |
| LA35F | 5'- ACCGGATACGACCATCTGC | Chicken | Weidhaas et al., 2010 |
| LA35R | 5'- TCCCCAGTGTCAGTCACAGC | | |

**Table 4. IDEXX sample results from September 22nd**

| Site | Time | Total Coliform (MPN/100 mL) | *E. coli* (MPN/100 mL) |
|---|---|---|---|
| PR_102 | 9:40:00 AM | >2,419.6 | 96 |
| LS_068 | 10:00:00 AM | >2,419.6 | 104.3 |
| LS_129 | 10:25:00 AM | >2,419.6 | 88.8 |
| AC_560 | 10:55:00 AM | >2,419.6 | 118.7 |
| LS_215 | 11:05:00 AM | >2,419.6 | 60.2 |

**Table 5. IDEXX sample results from October 6th**

| Site | Time | Total Coliform (MPN/100 mL) | *E. coli* (MPN/100 mL) |
|---|---|---|---|
| PR_102 | 9:36:00 AM | >2,419.6 | 517.2 |
| LS_068 | 9:50:00 AM | >2,419.6 | 161.6 |
| LS_129 | 10:09:00 AM | >2,419.6 | 285.1 |
| AC_560 | 11:09:00 AM | >2,419.6 | 93.3 |
| LS_215 | 11:21:00 AM | >2,419.6 | 30.0 |

**Table 6. Duplicate analysis of IDEXX samples**

| Site | Date | Total Coliform (MPN/100mL) | *E. coli* (MPN/100mL |
|---|---|---|---|
| LS_215 | 9/22/2017 | >2,419.6 | 60.2 |
| LS_215 | 9/22/2017 | >2,419.6 | 69.7 |
| | RPD | | -14.6 |
| LS_068 | 10/6/2017 | >2,419.6 | 161.6 |
| LS_068 | 10/6/2017 | >2,419.6 | 167.0 |
| | RPD | | -3.3 |
| | **Avg. RPD** | | **-9.0** |

**Table 7. Group Specific Bacterial Contamination**
Copies per 1,000 mL of water. Non-detect (-). Duplicate samples in yellow.

| Sample | Human | Bovine | Goose | Chicken | Dog |
|---|---|---|---|---|---|
| September 22nd | | | | | |
| PR_102 | 1,149 | – | 2,561 | – | – |
| LS_068 | – | 4,122 | – | – | – |
| LS_129 | – | – | 3,296 | – | – |
| AC_560 | – | 5,187 | – | – | – |
| LS_215-A | – | – | – | – | – |
| LS215-B | – | – | – | – | – |
| October 6th | | | | | |
| PR_102 | 855 | – | 1,392 | – | – |
| LS_068-A | – | 6,577 | – | – | – |
| LS_068-B | – | 6,769 | – | – | – |
| LS_129 | – | – | 1,428 | – | – |
| AC_560 | – | 2,650 | – | – | – |
| LS_215 | – | – | – | – | – |

## Figures

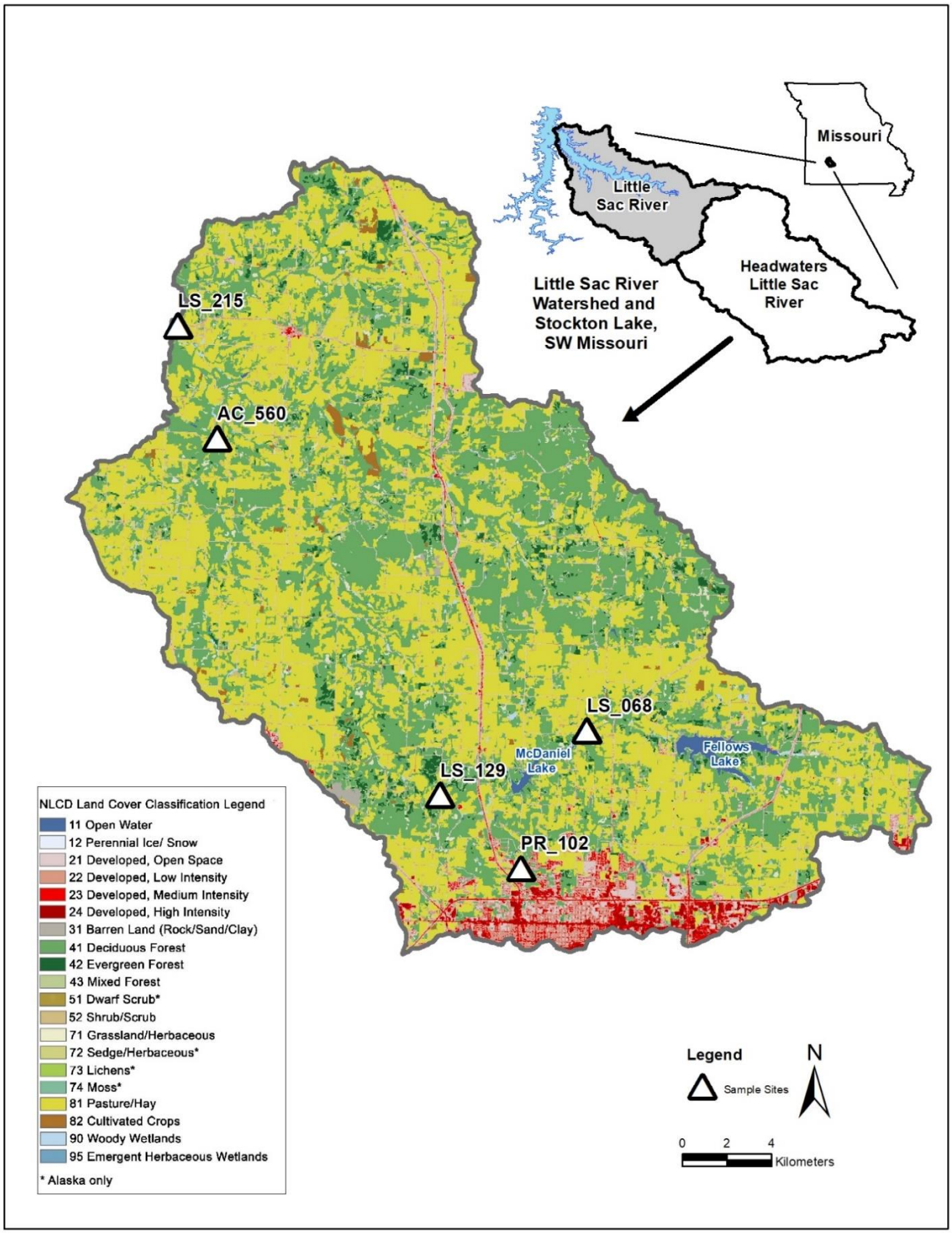


**Figure 1. Land use map and sample locations.**

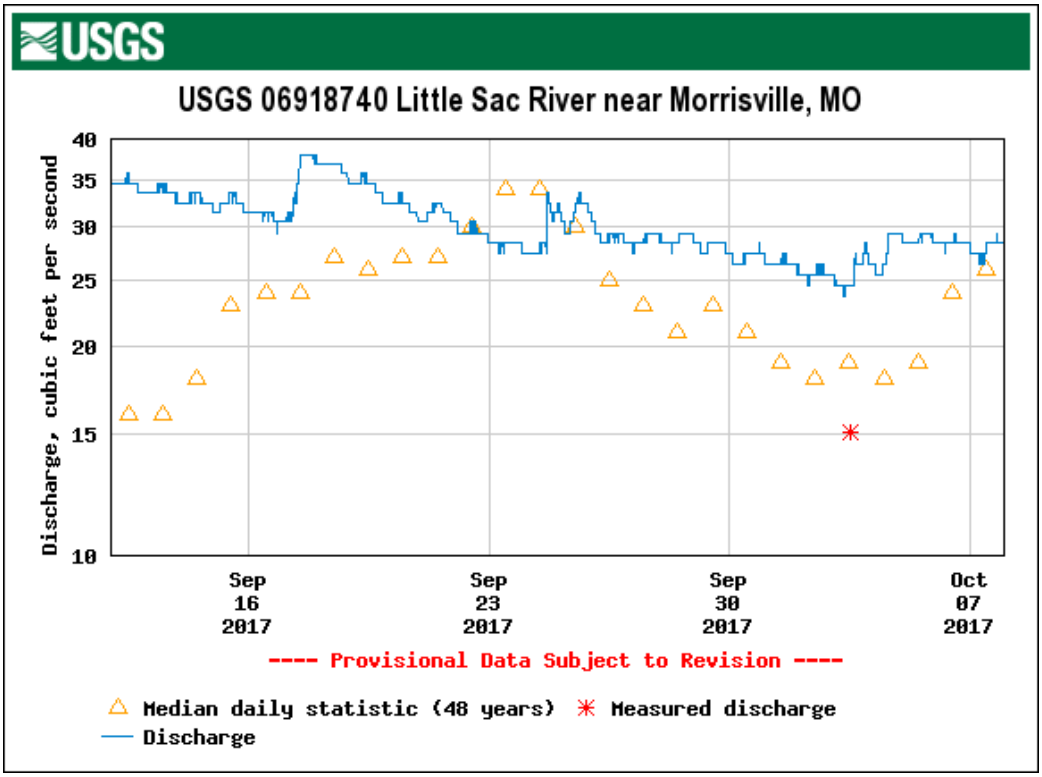


**Figure 2. Hydrograph from the USGS gaging station on the Little Sac River near Morrisville over the study period.**

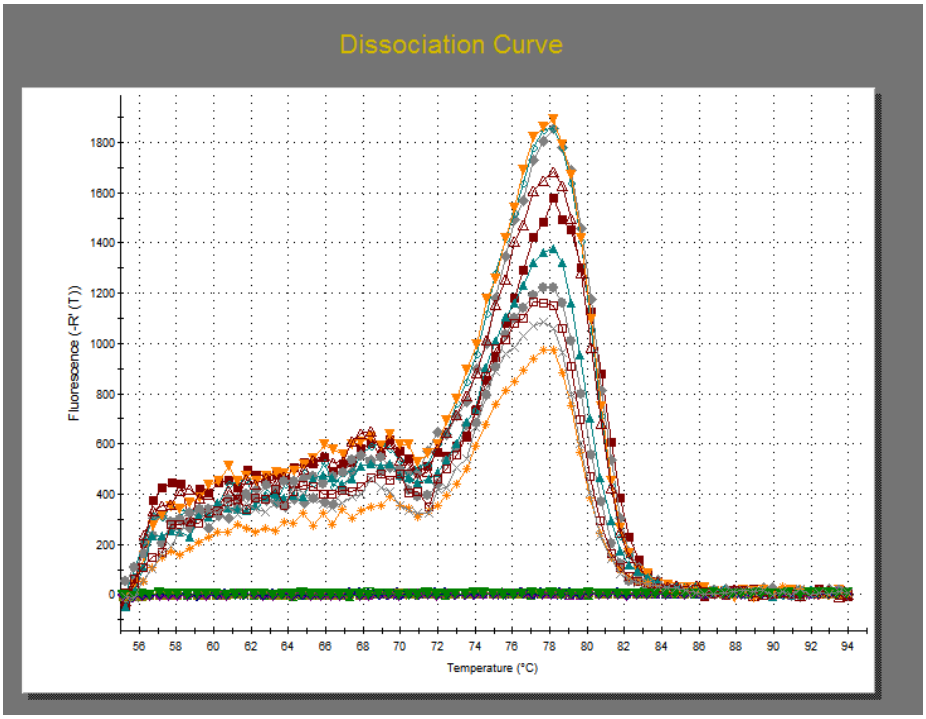


**Figure 3. A dissociation curve of bovine specific primers indicating a specific amplification of single DNA fragment.**

**Photographs**

**Photo 1. PR_102 (Oct. 6, 2017).**

**Photo 2. Above site PR_102 (Oct. 6, 2017).**

**Photo 3. LS_68 (Oct. 6, 2017).**

**Photo 4. LS_129 (Oct. 6, 2017).**

**Photo 5. AC_560 (Oct. 6, 2017).**

**Photo 6. LS_215 (Oct. 6, 2017).**