Standard Operation Procedure-Molecular Verification of *Listeria monocytogenes (Lm)* and *Listeria* spp. isolates and Molecular Identification of *Lm* Serogroups Using Real-Time PCR (qPCR)

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Introduction

This method is intended to verify presumptive *Lm* isolates as *Lm* while also identifying other isolates as either *Listeria* sp. or non-*Listeria*, using the G/S reaction. The Sero reaction is intended to subtype *Lm* isolates into molecular serogroups that generally correspond to phylogenetic lineages and serotypes. This method was based on the method originally proposed by Doumith et al. (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004; Doumith et al., 2005) but has been adapted to a real-time PCR format that has been evaluated by single laboratory validation(Burall et al., 2021) and multi-laboratory validation. For any questions regarding the method, please contact Laurel Burall (Laurel.Burall@fda.hhs.gov).

A. Equipment and Supplies

1. -20°C manual defrost freezer
2. 4°C refrigerator (optional)
3. 37°C incubator
4. Micropipettors
5. Aerosol resistant micropipette tips
6. Benchtop microcentrifuge capable of spinning 1.5mL tubes
7. Benchtop microcentrifuge capable of spinning 96-well plates
8. ABI 7500 FAST System calibrated for Cy5, FAM, TAMRA, TEXAS RED, and VIC with 7500 Software v2.3

9. MicroAmp Fast Optical 96-well Plates (Fisher Scientific, Catalog #43-469-07)
10. MicroAmp Optical Adhesive Films (Fisher Scientific, Catalog #43-119-71)
11. 1.5mL microcentrifuge tubes, sterile
12. 1.5mL opaque microcentrifuge tubes
13. Inoculating needles
14. Aluminum foil
15. Vortex Mixer
16. NanoDrop (ThermoFisher)

B. Media and Reagents

1. Crude lysis buffer (0.05N NaOH, 0.25% SDS)
2. 1N NaOH
3. QuantiNova Multiplex PCR Kit, Multiplex Probe (Qiagen, Catalog # or #208456)
4. Molecular grade water
5. Tris-EDTA, pH 8.0 (TE)
6. BHI agar plates
7. Invitrogen™ Platinum™ Green Hot Start (Thermo Fisher, Catalog #13001012)
8. QiaQuick PCR Purification (Qiagen, Catalog #28104)
9. Primers (See Tables 1)
    i. Stock solutions can be prepared from commercially synthesized probes (Fisher/Biosearch Technologies or equivalent) by rehydrating with sterile molecular grade water to 100uM. Working solutions can be prepared from the stock solution by dilution with molecular grade water to the concentration indicated in Tables 5 and 6.
10. Probes (See Tables 2, 1-3)
    i. Stock solutions can be prepared from commercially synthesized probes (Fisher/Biosearch Technologies or equivalent) by rehydrating with sterile TE to 100uM. Working stocks can be prepared from the stock solution by dilution with molecular grade water to the concentration indicated in Table 4.
11. IAC Target (See Section C)
12. Controls (see Section D)

Table 1. Primer Names and Sequences.

Purpose	Item Name	Sequence
Forward primer for amplification of the IAC target	IAC_F	5'-GGCGCGCCTAACACATCT-3'
Reverse primer for amplification of the IAC target	IAC_R	5'-TGGAAGCAATGCCAAATGTGTA-3'
Forward primer for detection of the *Lm* target	q*hly*3_F	5'-GCTCATTTCACATCGTCCATCTA-3'
Reverse primer for detection of the *Lm* target	q*hly*3_R	5'-CCGGTCATCAATTACCGTTCTC-3'
Forward primer for detection of the *Listeria* target	q*iap*_F	5'-GTTAAAAGCGGYGAYACWATTTGG-3'
Reverse primer for detection of the *Listeria* target	q*iap*_R	5'-TTTGACCYACATAAATAGAAGAAGAAGATAA-3'
Forward primer for detection of the Lineage 2 target	qlmo0737_F	5'-AGATGAACGGCAGAGACTTAAA-3'
Reverse primer for detection of the Lineage 2 target	qlmo0737_R	5'-CCGATCCGAATGCTGCTAATA-3'
Forward primer for detection of the Serogroup 2C target	qlmo1118_F	5'-TGCTTAATAACAGATGAAGAGGATG-3'
Reverse primer for detection of the Serogroup 2C target	qlmo1118_R	5'-CTTGTTCCTTAGTATTCCAGGATTT-3'
Forward primer for detection of the Serogroup 4B target	qORF2110_F	5'-CAGAATACGGCATCCCTGATAA-3'
Reverse primer for detection of the Serogroup 4B target	qORF2110_R	5'-AGCTCCACGTCCAAAGTAAG-3'
Forward primer for detection of the Lineage 1 target	qORF2819_F	5'-CATCACTAAAGCCTCCCATTGA-3'
Reverse primer for detection of the Lineage 1 target	qORF2819_R	5'-CCCTCCAACATATACGGAAAGAG-3'

Table 2. Probe information for ordering, including supplier and sequence.

Name	5' ⟶ 3' Sequence
IAC-PCF610	CAL Fluor Red 610-TTACAACGGGAGAAGACAATGCCACCA-BHQ-2
Lall-PFAM (*iap*)	FAM-ATGTCATGGAATAAT-MGB-NFQ
*hly*3-PTET	TET-TGCACTGGTTTAGCTTGGGAATGGT- BHQ-1
lmo0737-Q670	Quasar 670-TTTGCAAGTCAGGGTCTTGTCCGA- BHQ-2
lmo1118-TET	TET-AGGCGTATACACTCAGGAGAAGATAAAGGT- BHQ-1
ORF2110-TAM	TAMRA-AGTATGACTTCGGGCACAGTTGGC- BHQ-2
ORF2819-FAM	FAM-TGGCAGTTTCCAGGACTTCACTTGT- BHQ-1

C. IAC Target

The IAC reaction target is a 79 bp oligomer obtained by PCR amplification of the template using the same primers, IAC-F and IAC-R, indicated in Table 1 (Suo, He, Tu, & Shi, 2010). Amplification is performed using Platinum Green Hot Start (Invitrogen) according to the manufacturer's protocol. Cycling is as follows: 95°C for 5 minutes for 1 cycle, then 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a single final extension at 72°C for 7 minutes. The product should be checked to verify a single amplicon and the PCR should be cleaned using a kit, such as Qiagen's QiaQuick PCR clean-up kit, according to the manufacturer's protocol and eluting in 15µL of Buffer EB. The concentration is determined by measuring with a NanoDrop (ThermoFisher or similar approach). The IAC template is stored as a freezer stock solution at -20°C and diluted to a 0.05ng/µL working stock. This working stock is also stored at -20°C and used in both the G/S and Sero primer mixes (Tables 5 & 6).

D. Controls

Three positive control strains (Table 3) are used to generate positive controls, as described in Section E. A negative control, also referred to as a mock lysate, is generated in parallel with the sample templates, as described in Section E, to verify the absence of contamination of the reagents.

Table 3. Control strains used for both the PR and MLV portions of the study. Each strain serves as a specific positive control to verify reaction amplification and specificity. All three control strains have publicly available whole genome sequence data. The control column indicates the G/S and Sero results expected for each. n/a, not applicable.

Strain ID (Biosample)	Strain	Control (G/S, Sero)
LS32 (SAMN02769662)	*L. monocytogenes BS1*	Lm, 2C
LS411 (SAMN06216090)	*L. monocytogenes FSL-J1-110*	Lm, 4B
LS168 (SAMN14414661)	*L. innocua N7196*	LS, n/a

E. Generation of Sample Templates

1. Template samples are prepared from overnight, single isolate BHI agar cultures incubated at 37°C for 16-20 hr.
2. Aliquot 50µL of lysis buffer into sterile 1.5 mL microcentrifuge tubes. Label tubes with the corresponding ID number as noted on the BHI agar plate.
3. Using a sterile inoculating needle, pick 1-3 colonies from a plate, depending on colony size, and resuspend in 50µL of lysis buffer.
4. Prepare one extra tube that receives no isolate sample and will be a mock lysate, serving as the negative control.
5. Firmly close all tubes and heat tubes on a dry hot plate set for 99°C for 15 minutes.

6. Remove tubes from the hot plate and allow to cool for 2 minutes.
7. Add 100µL of sterile distilled water to the lysis buffer.
8. Centrifuge for 5 minutes at 16,000xg in a tabletop microcentrifuge and remove for use in the next section. Samples may be used immediately, stored at 4°C for <8 hr, or stored at -20°C for later use.

F. Reaction Setup and Execution

I. Reaction Setup

1. Prepare probes as indicated in Table 4.

Table 1. Working concentrations for probe stocks.

Reagent	Working Stock Concentration
IAC Probe	15µM
iap probe	10µM
*hly*3 probe	15µM
lmo0737 probe	15µM
lmo1118 probe	15µM
ORF2110 Probe	15µM
ORF2819 Probe	15µM

2. Prepare primer mixes as indicated in Table 5, for G/S reactions, and Table 6, for Sero reactions. Volumes should be scaled up as needed while maintaining the proportions.

Table 5. Primer and IAC working stock concentrations and the volume of said working stock added to the final G/S primer mix.

Reagent	Working Stock Concentration	Volume (µL)
IAC Working Solution	0.05ng/µL	25
IAC_F primer	10µM	25
IAC_R primer	10µM	25
*iap*_F primer	12.5µM	100
*iap*_R primer	12.5µM	100
*hly*_F primer	15µM	50
*hly*_R primer	15µM	50
Molecular grade water	n/a	100

Table 6. Primer and IAC working stock concentrations and the volume of working stock added to the final Sero primer mix.

Reagent	Working Stock Concentration	Volume (µL)
IAC template	0.05ng/µL	25
IAC_F primer	12.5µM	25
IAC_R primer	12.5µM	25
lmo0737_F primer	12.5µM	50
lmo0737_R primer	12.5µM	50
lmo1118_F primer	12.5µM	50
lmo1118_R primer	12.5µM	50
ORF2110_F primer	12.5µM	50
ORF2110_R primer	12.5µM	50
ORF2819_F primer	12.5µM	50
ORF2819_R primer	12.5µM	50

3. Add reaction components to an opaque microcentrifuge tube as indicated in Table 7 and 8, for G/S and Sero reactions, respectively. Volumes should be scaled up as needed while maintaining the proportions.

Table 7. The components added to G/S reaction mix (GS-qRM) as well as the volume added.

Reagent	**Working Concentration**	**Volume**
G/S Primer Mix	n/a	125 µL
QuantiNova	n/a	250 µL
IAC Probe	15µM	5 µL
iap probe/Lall-PFAM	10µM	5 µL
*hly*3 probe/*hly*3-PTET	15µM	5 µL
Molecular Grade Water	n/a	560 µL

Table 8. The components added to Sero reaction mix (Sero-qRM) as well as the volume added.

Reagent	**Working Concentration**	**Volume**
Sero Primer Mix	n/a	125 µL
QuantiNova	n/a	250 µL
IAC Probe	15µM	5 µL
lmo0737 probe	15µM	5 µL
lmo1118 probe	15µM	5 µL
ORF2110 Probe	15µM	5 µL
ORF2819 Probe	15µM	5 µL
Molecular Grade Water	n/a	

4. Load 9.5uL of either GS-qRM or Sero-qRM to wells, as appropriate, in a MicroAmp Fast Optical plate (ThermoFisher). Shield the plate from light.
5. Add 0.5 µL of control or sample lysate to the appropriate well, collecting sample from the top of the lysate mix, just under the meniscus.
6. Seal the plate with MicroAmp Optical Adhesive Film (ThermoFisher) or comparable optical film.

II. ABI 7500 Initiation

1. Load the reaction plate into the ABI7500, after briefly centrifuging in a tabletop centrifuge.
2. In the ABI 7500 Software Package, select “Advanced Setup”.
3. Select setup parameters as indicated in Figure 1.

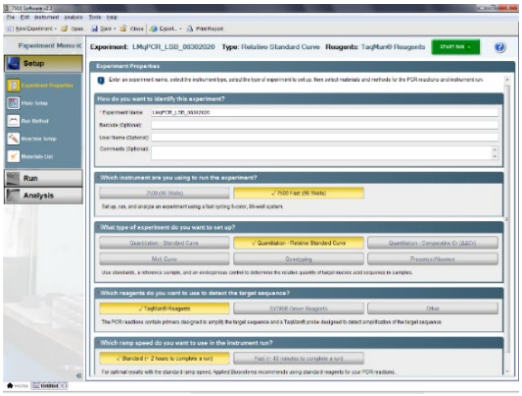

Figure 1. Sample of the “Experiment Properties” section for Setup of the ABI7500 Real-Time PCR run. Yellow buttons are the selected options for this panel.

4. In “Plate Setup” input target and probe information as shown in Figure 2.

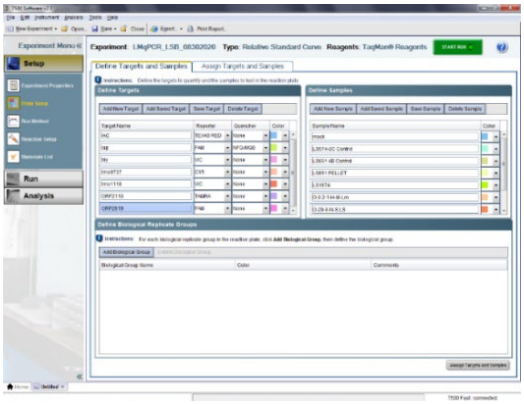

Figure 2. Sample of how to fill out the “Target and Template” Section. Add a sample for each sample analyzed under the “Define Sample” section.

5. Under the “Select the dye to use as the passive reference” click the drop down and select “None”. **<u>Failure to do this will result in no signal detection during the run.</u>**

6. Assign samples and controls, along with their paired targets, to reaction wells as appropriate. Input the run method with a reaction volume of 10µL and 40 cycles. The cycle steps are: Holding stage, 95°C for 5 minutes; Cycling Stage, 95°C for 30 seconds followed by 60°C for 30 seconds, with fluorescence measurement after the 60°C step.

III. Results Analysis

1. In the analysis section of the ABI software, manually set thresholds for each target to be within the exponential phase of amplification, while ensuring that non-sigmoidal amplification curves do not cross the threshold before cycle 30.
2. Export the data to a Microsoft Excel file and review the results to determine whether targets are positive (<30) or negative (>30 or undetermined) for each sample.
3. Evaluate any amplifications with a high C_T value compared to others for the possibility of cross-contamination or inhibition. The latter can be assessed by comparing IAC C_T values.
4. Collate target results by sample and use Tables 9 and 10 to determine the G/S and Sero result, respectively.

Table 9. Determination of G/S Result. +, indicates that target is positive. -, indicates that target is negative. Failed reactions must be repeated. IAC values greater than 3 standard deviations from the average should be considered negative as they are indicative of inhibition.

Result Call	***iap***	***hly***
Non-*Lm*	-	-
Listeria spp.	+	-
Lm	+	+

Table 10. Serogroup Result Determination. Results are split based on lineage with the two markers typically associated with each lineage indicated. The negative (-) and positive (+) pattern identifies the serogroup.

Sero Result	**lmo0737/lmo1118 (Lineage 2)**	**ORF2110/ORF2819 (Lineage 1)**
NT	-/-	-/-
2A	+/-	-/-
2C	+/+	-/-
2B	-/-	-/+
4B	-/-	+/+
4bV	+/-	+/+

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Suo, B., He, Y., Tu, S. I., & Shi, X. (2010). A multiplex real-time polymerase chain reaction for simultaneous detection of Salmonella spp., Escherichia coli O157, and Listeria monocytogenes in meat products. *Foodborne Pathog Dis, 7*(6), 619-628. doi:10.1089/fpd.2009.0430