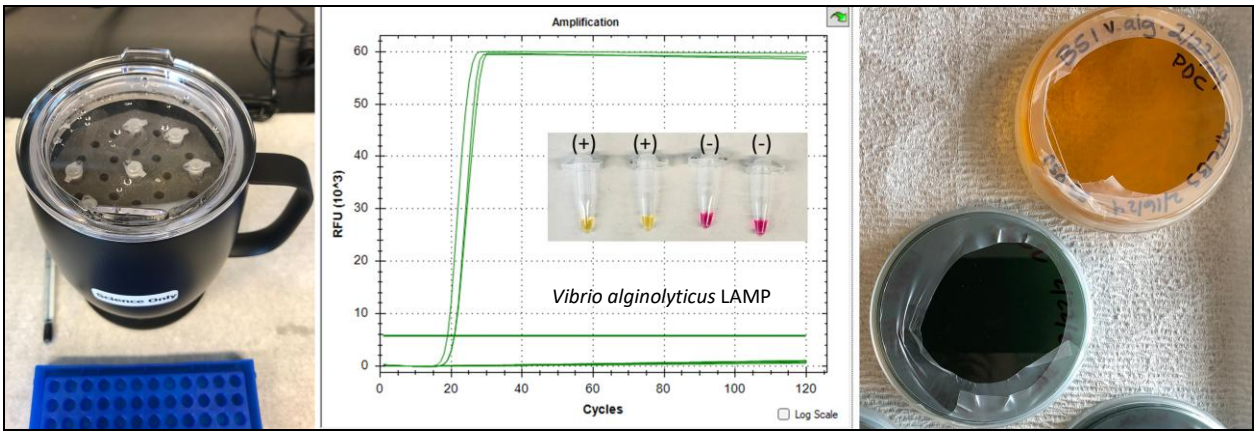

Loop-mediated isothermal amplification (LAMP) for farm-based detection of pathogens linked to shellfish hatchery failures

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Protocol Overview: LAMP is a rapid molecular method that can be used to detect possible bacterial pathogens in a range of environmental sample types. This protocol describes the use of newly-developed LAMP assays for the detection of 5 species of *Vibrio* including (*V. alginolyticus*, *V. coralliilyticus*, *V. harveyi*, *V. splendidus*, *V. tubiashii*) plus the causative agent of Juvenile Oyster Disease (*Aliiroseovarius crassostreae)*, all of which pose threats to the aquaculture industry. The setup for each of the different tests is identical, with the exception that a different set of six LAMP primers are used for targeting each bacterial species. Advantages of LAMP over PCR-based methods include its generally higher tolerance to sample impurities – thus reducing the number of steps needed for DNA extraction, its capacity for rapid detection of target DNA – most positive samples amplify in < 30 min., and its minimal requirements for specialized lab equipment. The LAMP reagents are relatively inexpensive and are available in a wide variety of formats, which include options for either a colorimetric (end-point) or fluorescence (real-time) read-out of amplification results. Newer options include lyophilized versions of the LAMP reagents, which offer reagent stability at ambient storage temperatures for up to two years. In its simplest form, LAMP is performed in a heated container

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(e.g., a self-heating coffee mug) resulting in a visual change in the color of an indicator reagent when the targeted DNA is amplified.

Testing Procedures

I. DNA extraction

Loop-mediated isothermal amplification (LAMP) will work on a variety of different sample types. However, each sample type may require different levels of processing to ensure that target DNA is available for amplification. In general, a crude cell lysate (CCL) approach should work for most sample types, but note that the methods to generate CCLs may require further optimization.

For best results, use a commercial DNA extraction kit that matches your sample type. In general, these kits require basic lab equipment (e.g., a vortex mixer, dry heat block, benchtop centrifuge capable of 10,000 – 15,000 xg, and a set of pipettors). Many of these kits incorporate chemicals that require safe handling protocols and proper disposal of small amounts of waste. It is best to consult with your environmental safety officer before using a commercial kit to ensure that you are equipped to handle and dispose of wastes properly. In the lab, we have used the following kits to extract DNA from various types of environmental samples:

- For Water Samples, see the Omega Bio-Tek Water kit, the Qiagen PowerWater kit or Qiagen PowerSoil kit
- For Shellfish Tissue Samples, see the Omega Bio-Tek Mollusc kit or the Qiagen Blood and Tissue kit

There are many alternative methods for preparing crude DNA extracts, but note, that minimally-extracted samples may not amplify due to incomplete release of DNA from shellfish tissue or free cells (e.g., filtered water samples), Additionally, the presence of contaminants in minimally-extracted samples can inhibit PCR. One low-tech method for preparing crude cell lysates (CCL) from oyster seed, a filtered water sample, or pelleted cells, is described in the procedure below. The protocol assumes that lysis will be conducted with DNA/Nuclease-free water only, to achieve a 'chemical-free' CCL. A lysis buffer (such as the one used to prepare samples for qPCR in Countway and Caron, 2006) can be substituted for the DNA/Nuclease-free water, if the water-based method is insufficient for lysing cells. This lysis buffer contains 100 mM Tris [pH 8], 40 mM EDTA [pH 8], 100 mM NaCl, and 1% Sodium Dodecyl Sulfate (SDS) and may be combined with bead-beating and heating (70 °C) to more-completely lyse the sample and release DNA into solution. A 1:100 dilution of the CCL is generally required to overcome inhibition of amplification caused by the lysis buffer components or inhibitors in the sample.

a. Add deionized water to the self-heating 'NextMug' coffee cup, and set the mug to 'piping hot', which also happens to be the required temperature for LAMP. Allow the mug to reach temperature (65 °C), checking with a thermometer.

b. While the mug is preheating, clean the working area. Spray and wipe down countertops with 10% bleach. Next, spray and wipe the working area with tap water, and then spray and wipe with 70% isopropanol.
c. Add oyster seed to a sterile 1.5 – 2.0 mL microcentrifuge tube. The exact size and amount of seed is up to user discretion, but keep the volume to less than 0.5 mL. If extracting from a filtered sample, a 5 mL tube may be necessary.
d. Add 0.5 mL of DNA/Nuclease-free water to the tube (or up to 1 mL if extracting from a filter in a 5 mL tube), making sure to leave enough space for a plastic pestle to be inserted into the tube without overflowing with water.
e. Grind the sample using a 10% bleach-cleaned (or 10% HCL) plastic pestle and place the microcentrifuge tube into the 'piping hot' (65°C) mug for 3 minutes. Note: make sure that no traces of bleach (or acid) remain on the pestle by rinsing well with lab-grade water. Residual bleach or acid will destroy and DNA in the sample.
f. Repeat the grinding and heating process a total of 3 times.
g. In a separate microcentrifuge tube, add 900 µL of DNA/Nuclease-free water.
h. Pipette 100 µL of the crude cell lysate into the 900 µL of water to create a 1:10 sample dilution. Note: The dilution is required to reduce LAMP inhibitors in the sample
i. Vortex to mix. Alternatively (if a vortex is not available), flick tube and invert a minimum of 10x to adequately mix.
j. This 1:10 dilution is the sample that will be used for the LAMP testing. It is generally good practice to perform a second dilution, using 100 µL of the 1:10 dilution plus 900 µL of DNA free water. Mix thoroughly. If possible, test both the 1:10 and 1:100 dilutions.
k. If not using the sample for LAMP immediately, put the crude cell lysate and the CCL dilutions into the freezer (-20 °C). Note: If not run or frozen immediately, DNAses in the CCL could begin to break down free DNA in solution when extracting with water.

Note: DNA/Nuclease-free water can be obtained from Integrated DNA Technologies (IDT).

II. Colorimetric LAMP Sample Testing

a. Fill the NextMug with deionized water and set mug to 'piping hot' if it is not already at temperature. Allow the mug to reach the correct temperature (65 °C). This can take 20 - 30 minutes. Alternatively, pre-heat water in a lab microwave or tea-kettle and titrate with cold water to achieve the required temperature. Transfer to the mug to maintain the correct working temperature. **Note:** The mug will shut off automatically after 2 hours of non-movement. Make sure to lift the mug off of its charging base just prior to the start of your run to ensure that it will remain on for the duration of the test.
b. Remove the 10X Primer Mix(es), WarmStart Colorimetric LAMP 2X Master Mix with UDG (referred to hereafter as NEB Master Mix)[1], and samples (if frozen) from the freezer. **Note:** The 10X Primer Mix contains all 6 LAMP primers at recommended concentrations to facilitate reaction setup (**Table 1**).

[1] Note: There is an individual reagent called 'Warmstart Colorimetric LAMP 2X Master Mix' produced by New England Biolabs (NEB). The solution of all three reagents is also referred to as 'Master Mix'. For clarity, the reagent will be referred to as 'NEB Master Mix' and the combined solution as 'Master Mix'.

Primer	Concentration of primers in 10X mix	Volume of 100 µM stock primers (µL)
FIP	16	80
BIP	16	80
F3	2	10
B3	2	10
LOOP F	4	20
LOOP B	4	20
sum of mixed primers (µL) -->		220
add this much water (µL) -->		280
Total Volume of 10X mix (µL) -->		500

Table 1. Chart to prepare the 10X primer mix for LAMP. Water is added to the mix to make a final volume of 500 µL. This chart assumes that all stock primers have been resuspended to 100 µM before mixing. Final concentrations of the primers in the LAMP reactions are 1.6 (FIP & BIP), 0.2 (F3 & B3), and 0.4 (Loop F & B) µM.

c. Prepare Master Mix(es) and set up reactions.
   i. Open the 'LAMP_MasterMix' MS Excel file, and fill in the missing fields.
      1. The missing fields include: Name of Run, Date, Target, Sample Number, Sample Type, Sample ID, Purpose of Run.
   ii. Print out the Excel file (preferable), or record the quantity of each reagent necessary to prepare the Master Mix.
   iii. After all reagents have thawed completely, add the appropriate amounts of each reagent to a sterile 1.5 – 2.0 mL microcentrifuge tube.
      1. Thoroughly mix the 10X Primer Mix and the NEB Master Mix by flicking each tube a couple of times before using.
      2. **Tip:** Add the reagents in order of least expensive to most expensive (first water, then 10X Primer Mix, and lastly the NEB Master Mix).
   iv. After all the reagents have been added to the Master Mix, thoroughly mix the solution by flicking the tube. Briefly centrifuge to remove bubbles.

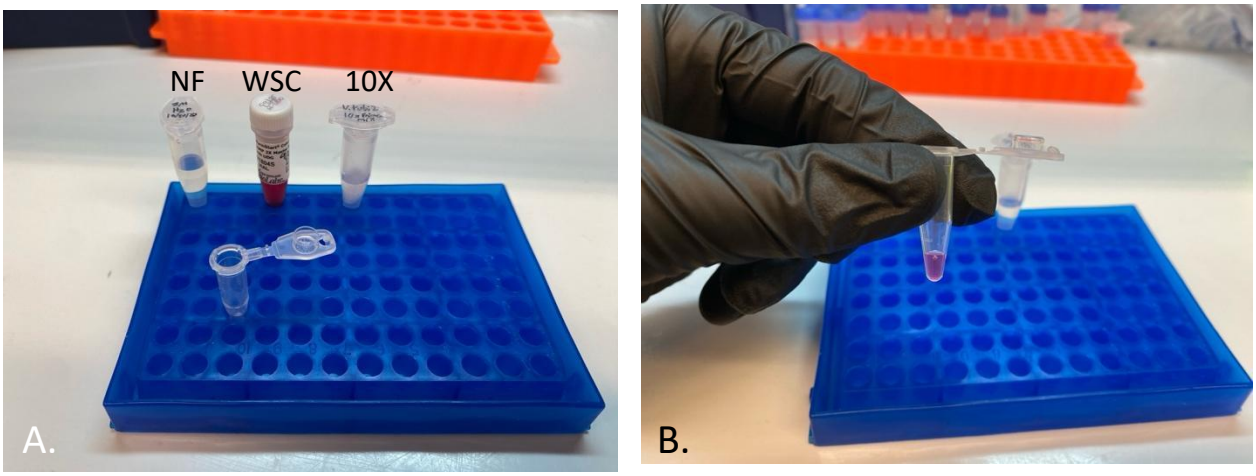

Figure 1. Preparation of the LAMP Master Mix. (A) Nuclease-free H_2O (NF), 2X WarmStart Colorimetric (WSC) master mix and 10X Primer Mix (10X) should be allowed to thaw at room temperature. (B) Anticipated color and volume of LAMP Master Mix for 2 reactions in a 0.5mL tube before the run.

v. Remove PCR tubes from the storage container/bag and arrange in a PCR tube rack (**Fig. 2**). Label each tube. Note: PCR tubes are very small and difficult to label. It may be easiest to write the full sample information (Master Mix Type and Sample ID) in the LAMP MasterMix Excel sheet and label each tube with a sequential number that corresponds to the sample information.

vi. Pipette 22 µL of Master Mix into each of the appropriate PCR tubes.

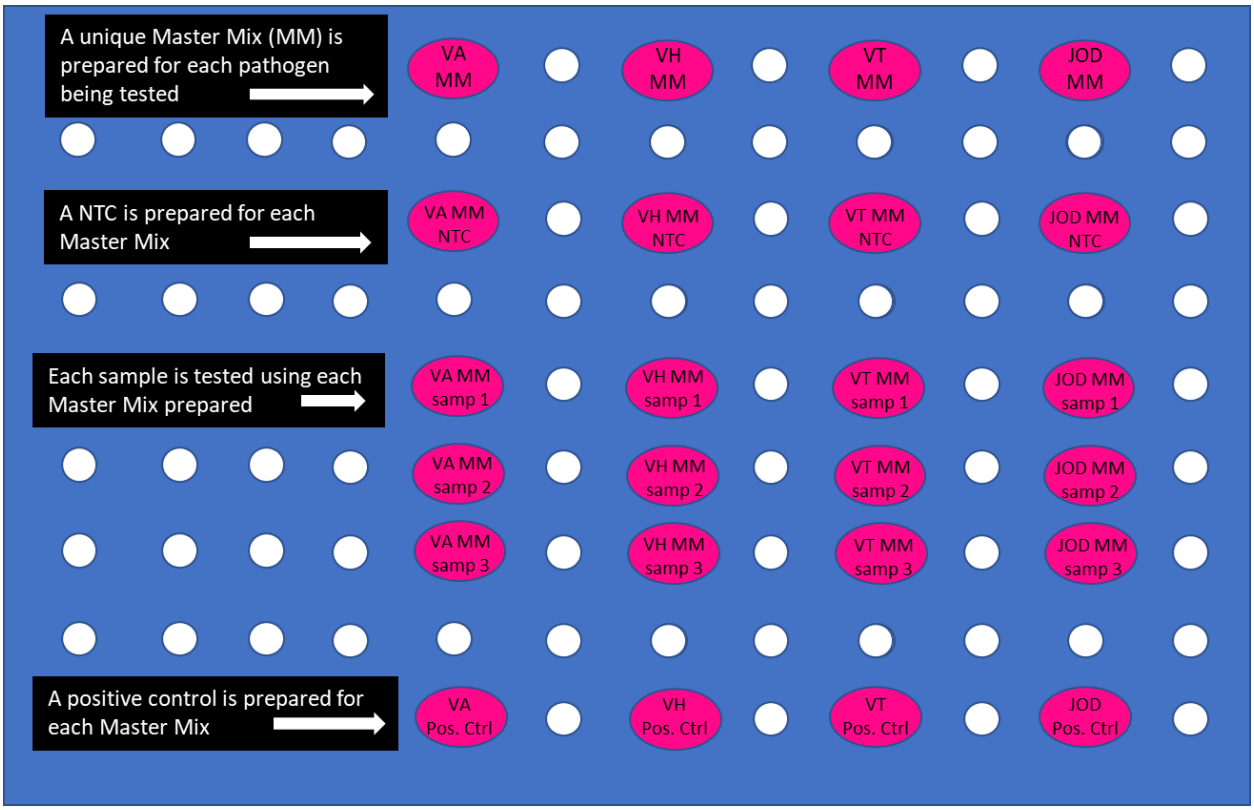

Figure 2. Visual representation of a potential testing matrix for 4 different LAMP assays, testing for *Vibrio alginolyticus* (VA), *Vibrio harveyi* (VH), *Vibrio tubiashii* (VT) and *Aliiroseovarius crassostreae* (JOD). The blue rectangle represents the PCR test tube rack. Pink ovals represent PCR test tubes holding LAMP Master Mix, primers, and water.

vii. Add 3 µL of sample to the appropriate tube.
   1. If the sample was frozen ahead of time, ensure the sample has completely thawed and is well-mix before using.
   2. Thoroughly mix each sample before adding to the reaction tube.

viii. Add 3 µL of positive control to the appropriate tube.
   1. Make sure the positive control has completely thawed before using.
   2. Thoroughly mix the positive control before adding to the reaction tube.

Note 1: The Positive Control is provided at a million-fold dilution from its stock concentration, to minimize chances for contamination.

Note 2: The Positive Control should always react (e.g., color change from pink to yellow before 30 minutes) or the results of the test cannot be trusted.

ix. Add 3 µL of nuclease-free/deionized water to the appropriate No-Template Control (NTC) tube.
Note 1: The NTC serves as a check on contamination and should remain pink for at least 30 minutes. A positive reaction in the NTC (color change from pink to yellow) means that any positive reaction in your samples cannot be trusted.
Note 2: We have seen one case for the *Vibrio alginolyticus* LAMP assay, in which the NTC reacted without obvious sources of contamination, while other NTCs (from other *Vibrio* assays run at the same time) remained yellow. Given that the color change is dependent on a drop in pH over time (normally due to DNA synthesis) there is a possibility that diffusion of CO_2 from air into the headspace of the reaction tube can cause a shift in the pH (NEB Tech Support). Although we remain unsure of why this happened for only one of our assays, we were able to obtain a 'clean' NTC reaction by capping the reactions with Vapor Lock (Qiagen) to prevent diffusion of CO_2 into the reaction.
Note 3: There are other non-pH dependent colorimetric reagents that can be used for LAMP, however these require customized reaction mixtures – See note on hydroxynaphthol blue (HNB) below. We will be exploring these options, but it was beyond the scope of the current project.

x. Double check that all PCR tubes are tightly closed.

xi. Vortex the PCR tubes for at least 30 seconds to ensure the samples are thoroughly mixed (LAMP reactions need to be mixed well prior to the start of the reaction)
    1. If necessary, rearrange the PCR test tubes in the PCR test tube rack so all the samples will fit onto the vortex.
    2. Gently hold the PCR tubes down so the test tube will not fly out of the plate.
    3. Make sure each sample is thoroughly mixed for at least 30 seconds. **This step is very important. Results may be inconsistent if samples are not well-mixed.**

xii. Optional: Add 10-20 of a hydrophobic solution (e.g., Vapor-Lock, Qiagen) – this is sometimes needed to prevent false positive reactions which can occur if the LAMP mixture absorbs CO_2 from air in the head-space of the PCR tube.

xiii. Briefly centrifuge each sample – or (if no centrifuge is available) force the reaction mixture to the bottom of the tube by rapidly 'snapping' the PCR tube from above your head to waist level with a single arm motion. Repeat as needed.

xiv. Load test tubes into a foam floating tube holder.

xv. Check the water temperature in the NextMug to ensure it is at 65 °C.

xvi. Float the foam holder and test tubes in the NextMug and incubate for 30 minutes with the NextMug lid on.

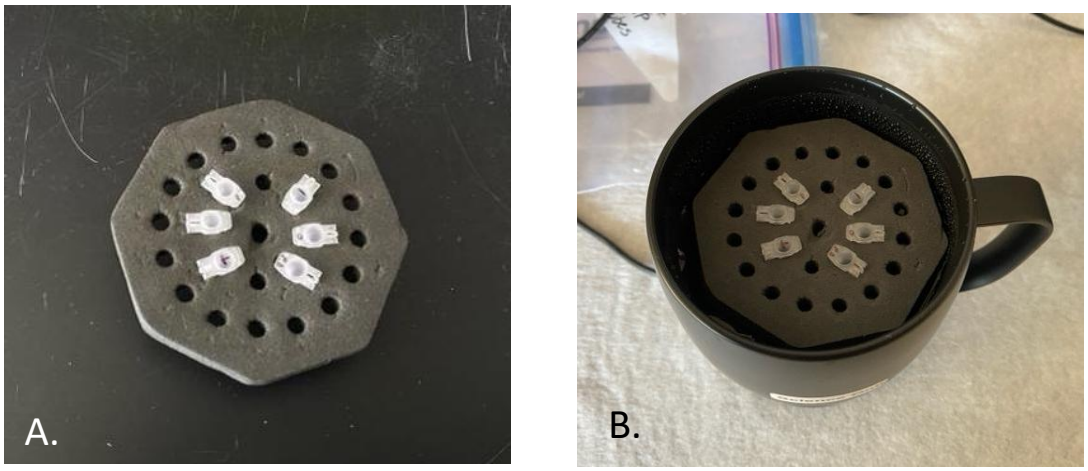

Figure 3. Preparation of sample PCR tubes for incubation. (A) Tubes loaded securely into a foam holder. (B) Float the foam holder in the NextMug so the samples are submerged but the tops of the tubes are not.

xvii. After 30 min., remove the test tubes from the NextMug and foam holder, dry off the tubes, and observe against a white background

1. Ensure all positive control tubes are yellow and all NTC tubes are pink.
    a. If the NTC turns pink, there is contamination in the Master Mix and the results are not reliable.
2. Samples with a specific bacterial pathogen will be yellow. Note: Varying degrees of yellow/pink are possible depending on how 'complete' the reaction is and how much target DNA was in the original sample. The pink to yellow color change is due to a Phenol Red pH indicator solution.

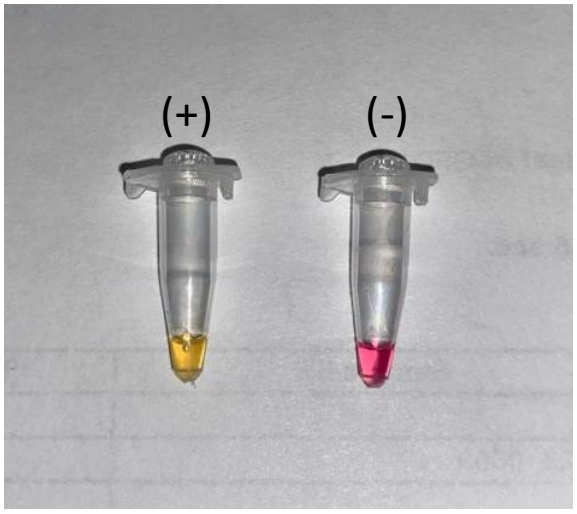

Figure 4. Expected positive and no template control (NTC) results. Positive control (left) should be yellow and NTC (right) should be pink. Photographed with flash for clearest results.

xviii. After running the LAMP and recording results, take tubes to a remote location where you will not be setting up reactions in the future. Open up each PCR tube and submerge contents in a 10% bleach bath to destroy amplification products and prevent contamination of lab space. Soak tubes for a few minutes and then dispose in regular garbage. The water inside the coffee mug can be poured down the drain. **Note:** The NEB WarmStart mastermixes that we recommend contain Uracil-DNA Glycosylase (UDG), which prevents LAMP products from amplifying after the 65 °C incubation step. The bleach treatment is an added contamination control – but likely not needed, if mastermix with UDG is used.

Additional Notes:

Storage:

- The 10X Primer Mixes, NEB Master Mix, and positive control must be stored in a freezer (-20 °C) when not in use. The reagents and positive control will degrade if stored under different conditions.
- Nuclease-free or deionized water can be stored at room temperature.
- Keep the 10X Primer Mixes and Master Mixes as separate from positive controls and samples as much as possible. Store the 10X Primer Mixes and Master Mixes in one box and positive controls and frozen samples together in a different box to avoid contamination – ideally in different freezers.
- Anytime something (reagent, sample, positive control, etc.) is frozen and then thawed, thoroughly mix the solution before using.

Master Mix:

- For this LAMP, the Master Mix consists of 3 reagents (water, 10X primer mix, and NEB Master Mix).
- The 10X primer mix is highly specific to the pathogen being tested. For example, the "VA 10X primer mix" will only detect the *clg* gene in *Vibrio alginolyticus* (a gene that indicates if potentially pathogenic *V. alginolyticus* is present).
- Each Master Mix should contain only one 10X Primer Mix. Therefore, each Master Mix can only test for one type of pathogen at a time.
- If testing for multiple pathogens (e.x. testing for all 5 *Vibrio species*), a Master Mix will need to be prepared for each pathogen being tested.
- A new lyophilized version of the NEB Master Mix for LAMP (LyoPrime WarmStart) has become available recently and can be used for colorimetric LAMP detection with the color indicator hydroxynaphthol blue (HNB) and a mixture of calcein dye and manganese II chloride tetrahydrate (Pang et al., 2019). We did not have access to this

Disclaimer:

The procedures described herein are to be used for research purposes only – to screen for potential bacterial pathogens of shellfish. Positive results should be investigated further by alternative methods, where possible. LAMP is an incredibly sensitive tool and can be subject to false positive (due to contaminants) or false negative (due to inhibition) results, particularly if the protocol is conducted in a non-traditional laboratory setting. As with any chemical test, the possibility exists for human error, equipment error, or reagent degradation that will affect experimental outcomes. Although all LAMP methods herein were designed and tested to be as accurate as possible for the targeted species, no warranties are provided or implied regarding the performance of these tests.

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Pang B, Yao S, Xu K, Wang J, Song X, Mu Y, Zhao C, Li J (2019) A novel visual-mixed-dye for LAMP and its application in the detection of foodborne pathogens, Analytical Biochemistry, Vol. 574:1-6, doi.org/10.1016/j.ab.2019.03.002.

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Complete Supplies and Packing List:

Sample Collection

- o Vacuum pump*
- o Vacuum tubing*
- o Vacuum vapor trap*
- o Filter tower (47 mm)*
- o 47 mm filters*
- o Filter tower (25 mm)
- o 25 mm filters
- o Forceps x2
- o Plastic 1-L sidearm flask

Extraction

- o Clean pestles
- o DNA/Nuclease-free water
- o 2x lysis buffer - custom
- o Tea kettle (pre-warm water)
- o Thermos
- o LoBind tubes 1.5, 2, 5 mL
- o Tube racks
- ○ Sample storage boxes
- o Vortex and tube adapter(s)*
- o Mini-centrifuge & adapters*
- o Distilled or deionized water to fill NextMug
- o Mini dry bath & block*
- ○ Mini high-speed centrifuge*

LAMP set-up

- o PCR tube racks
- o 10x LAMP Primers - custom
- o WarmStart color Master Mix
- o WarmStart fluorescent Master Mix*
- o Fluorescent dye*
- o DNA-free water
- o DNA positive controls - custom
- o Genomic DNA or CCLs - custom
- ○ Vapor-Lock*
- o Pipettors (2 - 1000 µL)
- o Filter tips (2 - 1000 µL)
- o PCR tubes - individual
- o PCR tubes - clear strips
- o Scissors (small – to cut PCR strip tubes)

Amplification

- o Water Bath (65 °C)
- o Thermometer
- o Foam ring
- o Biomeme qPCR device*

Personal Protective Equipment

- o Gloves (S, M, L)
- o Bench paper
- o Lab glasses
- o Lab coat

Miscellaneous

- o LAMP setup sheet
- o LAMP lab manual
- o Ice packs
- o Sharpies/pens
- o Scissors (large)

* = Not a strict requirement

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