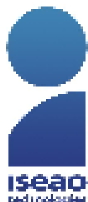

LiMA Bacterial Detection Assay

LiMA100-01 Instructions for Use

A. Intended use. For the detection of bacterial ligase by nucleic acid amplification. This kit can detect bacterial ligase in prepared samples. For example, platelet samples prepared by the recommended protocol (see Appendix) for red cell and white cell lysis can be tested by this kit.

B. Warning and precautions. To be used by trained personnel only. If there is an infections risk for the sample being used, please use within an appropriate bio-safety cabinet. Wear appropriate protective clothing including gloves and laboratory coats. Dispose of all potentially infectious waste in a safe and responsible manner. When performing molecular analysis, please use appropriate precautions for preventing PCR contamination.

C. Kit contents:

For bacterial lysis

6 ml	LigA
1.5ml	LigB
100	2 ml microtubes containing lysis beads

For amplification

Ligase inhibitor	500µl
PCR primers	400µl

Do not use reagents beyond stated expiration date (see outer packaging).

D. Reagents required but not supplied.
Hot-start PCR mix with SYBR Green detection such as Stratagene hot-start PCR mix, catalogue number 600830. UDGase such as Bioline UDGase, catalogue number BIO-27044. Other Hot-start PCR mixes with SYBR Green and UDGase may also work.

E. Equipment required but not supplied.
Real time thermal cycler such as Stratagene Mx3005P but other thermal cyclers may be used. Vortex genie VWR, catalogue number 444-0486 with microtube adaptor VWR, catalogue number SI0-0562. A heating block capable of holding 2 ml tubes at 75ºC

F. Protocol. Samples must first be prepared in a manner appropriate for analysis by LiMA. This protocol varies with type of sample to be investigated (see Appendix for appropriate protocols).

Preparation of the lysis beads

1. One labelled tube will be required for each sample and any controls.
2. Collect the bacterial lysis beads to the bottom of the tube by pulsing briefly in a microfuge.
3. Add 60 µl of LigA to each tube and heat at 75ºC for 15 min and then cool to room temperature.

Bacterial lysis

4. To the prepared sample add 15µl of LigB and mix thoroughly.
5. Add this suspension to the heated and cooled LigA and lysis beads (see above).
6. Collect the bacterial lysis beads and solutions to the bottom of the tube by pulsing briefly in a microfuge.
7. Lyse the bacteria by placing the tubes in the vortex genie and vortex for 5min at 2800rpm.

Bacterila ligase assay

8. Collect the bacterial lysis beads and solutions to the bottom of the tube by pulsing briefly in a microfuge and then place the tubes at 37 °C for at least 30 min (leaving the reaction for longer will give proportionally more signal).
9. After ligation add 7.5µl ligase inhibitor and mix (Note; this will inhibit any ligase contamination that may have co-purified with the Taq enzyme in the PCR mix).
10. Heat the samples at 80ºC for 5 min.
11. Allow to cool prior to PCR analysis. 2µl of each ligated sample can be analysed in a 20µl PCR (see below).

Real Time PCR Analysis
Typical PCR conditions: 20µl Hot-start PCR should be performed containing 4µl primers (supplied) and 0.4µl UDGase. Note: a master mix can be prepared and aliquotted prior to use. Each 20µl PCR should contain 2µl of ligated sample. If the signal during PCR rises slowly or is weak, double the quantity of SYBR Green in the assay.

Appropriate PCR parameters are:
1cycle each of
50ºC 10 min (UDGase step)
95ºC 10 min (Taq activation)

30-40 cycles of
95ºC 10 sec (denaturation)
65ºC 10 sec (aneal)
72ºC 10 sec (extension and read)

G. Controls
Commercial NAD ligase control. Commercial NAD ligase such as New England Biolabs M0205-S can be used. We recommend preparing mock reactions that can be spiked with commercial ligase. The commercial ligase should be diluted10^{-5} before use and 5µl used in a ligase reaction (added at step 8 of the protocol, see section F above). The PCR signal with this dilution should be detected before cycle 20.

Bacterial control. Mock reactions can be spiked with known numbers of bacteria (confirmed by plating out on nutrient agar and counting colonies). The bacteria should be added at step 4 of the protocol, see section F above). For example 2µl of a 10^{-4} dilution of an overnight bacterial culture can be used.

Negative controls. Mock reactions can be prepared without sample processing.

H. Determining positive and negative samples
A cut-off should be established for each combination of sample type, PCR machine and PCR mix used. This cut-off can be established by testing samples confirmed negative (for example by culturing the sample in parallel to analysis by LiMA). Ideally the cut-off for a positive sample should be between 30-34 cycles i.e. all negative samples give PCR signals beyond cycles 30-34 thus any sample giving a signal prior to this cycle number is deemed to be positive). Note: once the cut-off is established it may be appropriate to set the number of PCR cycles performed to this cut-off.

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Appendix - Protocol for Platelet Preparation

A. Solutions required for 100 tests
All solution should be prepared with autoclaved water.

50 ml 10% (w/v) Zwittergent (Sigma 40772). Add sodium azide to 0.05% (w/v) and store at room temperature.
10ml 50mM NaOH (Molekula M80406338). Store at room temperature.
100ml 50mM Tris.HCl pH 7.5 (Sigma 93372 – supplied as a powder). Dissolve, autoclave and store at room temperature.

Alternatively, reagents can be supplied if required.

B. Reagents required but not supplied
Autoclaved, ligase and bacterial-free distilled water. 10 ml per test is required
100 1.5 ml sterile microfuge tubes
100 15 ml sterile tubes

C. Equipment required
Swing-out centrifuge capable of handling 15 ml tubes at speeds up to 4000 x g.

D. Preparation Protocol
1. Add 10 ml platelets to a labeled, sterile 15 ml tube.
2. Add 500µl 10% (w/v) Zwittergent and mix. (Note: If spiking with bacteria they can be added at this step).
3. Incubate 6 min room temperature then centrifuge 4,000g for 20min.
4. Pour off supernatant and tap the tube upside down onto a clean tissue.
5. Resuspend the pellet in 900µl sterile distilled water.
6. Add 100µl 50mM NaOH. Mix and incubate 3 min room temperature.
7. Add 9 ml sterile distilled water and mix.
8. Centrifuge 4,000g for 20min.
9. Pour off supernatant and tap the tube upside down onto a clean tissue.
10. Resuspend the pellet (Note: the pellet may not be visible by eye) in 1ml 50mM Tris. HCl pH 7.5 and transfer the resuspended pellet to a 1.5ml microfuge tube.
11. Centrifuge 8,000 rpm for 5 min and then carefully remove the supernatant.

The prepared pellet is now ready for bacterial ligase analysis by LiMA (see section F, Bacterial lysis, step 4).