Bacterial endotoxin by kinetic chromogenic LAL

Onset time against endotoxin concentration on a standard curve, with the positive product control, the negative control and the validity rules of the chapter, reported in EU per mg of powder.

Not yet approved
No approval of this revision is on file. This revision states what the laboratory's results system enforces today; it takes effect when a named reviewer approves it.

Document		Laboratory	
Number	SOP-04	Name	Cupel Laboratories
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1 Purpose

To determine the bacterial endotoxin content of a lyophilised peptide by the kinetic chromogenic Limulus amoebocyte lysate method, reported as EU per vial and EU per mg of powder, gross fill by weighing, EU per mg of nominal fill and EU per mg of peptide, free peptide by assay.

2 Scope

An add-on (code LAL, "Bacterial endotoxin") to ID, PUR, IDP; the full panel includes it. Minimum sample, as the intake conditions state it: "one sealed vial as sold".

An endotoxin result within limits does not establish sterility, and a sterile preparation can still carry endotoxin. Ordering one does not answer the other.

3 Responsibilities

Role	Responsibility
Analyst	Prepares the standards, the sample, the positive product control and the negative control with pyrogen-free technique, runs the plate, enters every onset and signs as analyst.
Reviewer	Checks the validity block, the curve and the warnings; an invalid assay is repeated, never reported as a value; signs the content as reviewed (SOP-07).

Named: Timothy Ian Benedicto, Medical Laboratory Technician; Roxanne Manlapaz, Laboratory Technician.

4 References and controlled sources

Standards and other documents

- USP <85>; Ph. Eur. 2.6.14 Method D: the kinetic chromogenic technique, the validity of the standard curve, the positive product control and the negative control.
- The lysate manufacturer's insert: the replicate CV criterion, which is the insert's and not the chapter's.
- JCGM 100:2008 (GUM) 6.2 and Annex G; ISO/IEC 17025:2017 7.6 and 7.8.6.

References and controlled sources, continued

– SOP-01 (the vial as received), SOP-07 (review and issue), SOP-09 (the reader).

Controlled sources, read at every build

– lab/results.py: the regression, the validity checks, the reporting limits and the two-significant-figure rule; lab/CONTRACT.md section 1 (lal).
– data.json pricing (the code and what it rides on), intake (minimum sample), limitations.

5 Safety

– Read the current safety data sheet of every reagent before first use and keep it where the work is done. Wear a laboratory coat, safety glasses and chemical-resistant gloves for every step below.
– Treat every sample as a potent, biologically active substance of unconfirmed identity until its report says otherwise: open and weigh lyophilised powder so that it is not inhaled, and keep it off bare skin.
– Control standard endotoxin is purified lipopolysaccharide: pyrogenic through broken skin or a mucous membrane, not a hazard on intact gloved skin; cover cuts, never aspirate by mouth, wipe a spill with water and then 70% ethanol. Work with pyrogen-free tips and tubes, which also protects the assay.
– The depyrogenation oven runs at 250 °C: loads in metal racks or foil, never plastics; heat-resistant gauntlets; the door opened only below 60 °C; the 70% ethanol wipe bottle, which is flammable, kept away from it. The 37 °C reader is not a hazard. Used plates and tips are sealed in a bag and disposed of as the waste plan says.
– Collect solvent and reagent waste in closed, labelled containers for disposal as hazardous chemical waste; never pour it to drain.
– SAF-01, the laboratory safety document (the chemical hygiene plan, the waste plan and the exposure and spill response), is published beside this procedure and governs beyond the notes here: its hazard table, the protective equipment by task and the facts it marks as not yet supplied by the owner are the controlling text; the notes here are the bench reminders of it.

6 Equipment and materials

– A 96-well kinetic incubating microplate reader reading at 405 nm under assay software that records the onset time of every well. No instrument is listed yet: make, model and serial number are published on the methods page and on each certificate once the instrument is installed and its serial plate can be photographed, and never for an instrument the laboratory does not own.
– Everything that touches the stock before the aliquot, and the aliquot itself, is certified pyrogen-free single use (the medium, serological pipettes, tips, the receiving tube: certificate and lot on WS-07 (endotoxin)) or borosilicate depyrogenated in this laboratory at 250 °C for at least 30 min in a cycle validated with endotoxin indicators to at least a 3 log10 reduction (USP <1228.1>), the cycle number on the worksheet. A new lot of tubes is verified once: one tube extracted with LAL reagent water for 1 h and the extract run as a negative control, below lambda. The vial's neck and stopper top are wiped with filtered 70% ethanol; the stopper is lifted with clean forceps and never laid down. Powder-free gloves; no talking over open tubes or the plate.
– The reader's block temperature is read against a calibrated thermometer monthly (SOP-09); the set-point and the displayed temperature at plate start are on the worksheet.
– The instrument identifier, the data file and the acquisition time are recorded and required to issue.

7 Reagents and reference standards

Lysate	Kinetic chromogenic LAL reagent; its lot and the insert's lambda are recorded (the curve is not reproducible without them); reconstituted with the insert's volume of LAL reagent water, swirled, never vortexed, and used within the insert's time, which is recorded.

Reagents and reference standards, continued

CSE	The control standard endotoxin, named, with its lot, the reference of the certificate for this lysate lot and the potency it states in EU/ng, which is what the standards are traced through; the EU-per-vial figure on the vial label is not the potency; a certificate that states no uncertainty is given 7.0% relative (lab/results.py) and the budget says so.
LAL water	LAL reagent water, its lot and certificate recorded; the diluent, the negative control and the medium of the stock. It is poured, never pipetted from the bottle; an opened bottle is used the same day or decanted into depyrogenated tubes, the date opened on the bottle.
Medium	10 mM Tris, pH 7.8, LAL grade, with an endotoxin certificate below lambda, for Semaglutide, tirzepatide and retatrutide whatever their salt form (SOP-06); its pH is set and recorded at the temperature the procedure states (the buffer drifts about 0.03 pH per °C), lot and expiry recorded; a medium-only control runs beside the negative control whenever the stock is made in it.
Assay date	Recorded (UTC).

8 Sample preparation

The preparation, as the methods page states it:

> Endotoxin: an aliquot of that stock first, 1:40 in LAL reagent water, 0.05 mg/mL in the well.
>
> The methods page, “Sample preparation” (data.json method.rows)

8.1 The aliquot is drawn from preparation 1’s stock (2 mg/mL nominal in the medium, SOP-06) before any mobile phase A is opened at the bench and within 15 min of the clarity record on WS-03 (dissolution): 100 µL of stock into 3.90 mL LAL reagent water (1 + 39, D = 40), vortexed 30 s; 0.05 mg/mL of nominal powder in the well. The dilution is used within 1 h.

8.2 The diluted sample in the reaction well is called “in the well” everywhere on the certificate and in this procedure; the figure quoted is the concentration of the test solution added to the well, before the lysate halves it, the convention lambda and the standards share.

8.3 The dilution used is one VR-03 covers for the analyte in this medium (recovery 50–200% there and at every greater dilution). A tighter supplied limit may need 1:20 (0.1 mg/mL in the well) where VR-03 shows no interference there; an analyte whose positive product control fails at 1:40 (a copper complex, for one) moves to 1:80, and its reporting limit doubles. The pH of the test dilution mixed 1 + 1 with lysate lies in the insert’s range (6.0–8.0 unless the insert says otherwise), shown once per analyte and medium in VR-03 and recorded on the worksheet whenever a new salt form or medium appears.

8.4 Maximum valid dilution. When an endotoxin limit is supplied with the order, MVD = limit × c / lambda with c the stock concentration on the same mass basis as the limit (the nominal basis unless the order names another), and the test dilution must not exceed it; the certificate prints the MVD and says when the test dilution exceeds it. A limit stated per vial is compared with EU per vial directly. Without a limit no MVD is defined, and the certificate says so.

8.5 Hold of the stock before the aliquot, for Semaglutide, tirzepatide and retatrutide: the positive product control spiked at assay time shows inhibition or enhancement, not endotoxin lost into the peptide’s aggregates during a hold, so the 15 min rule above is bounded by VP-03’s hold-time spike arm (the stock spiked with CSE at the moment of dissolution and assayed at 15 min and 2 h, recovery 50–200% at both; USP <85>, interfering factors under the conditions of use; USP <1085> by analogy). A repeat from a held stock (Procedure) relies on that arm’s 2 h point, and the open point says so until VR-03 has run it for the analyte.

In a full panel the method as written consumes 5 vials (mass per vial 3; water content 2; HPLC from the mass per vial preparation; LC–MS from the HPLC preparation; endotoxin from the mass per vial preparation) (results.vials_required). This test takes no vial of its own there: its solution comes from the mass per vial preparation, 0 vials are recorded as consumed by it, and the certificate says “endotoxin from the mass per vial preparation”. The endotoxin aliquot is drawn first, from each whole-vial solution while it is still in LAL reagent water and before any dilution in mobile phase A for the other tests; the concentration recorded is that solution’s.

9 Standards and controls

- Standard curve: at least three levels of CSE, distinct, spanning at least two decades, each level in duplicate wells: the levels VP-03 validates, 5, 0.5, 0.05, 0.005 EU/mL (lab/validation.py). Lambda, the labelled sensitivity, is by definition the lowest standard on the curve and must equal it.
- CSE: reconstitute with the volume of LAL reagent water its certificate gives for this lysate lot; vortex 15 min (or as the insert says) after reconstitution and 1 min before every use; record the date, the time and the potency (EU/ng, and the EU/mL it gives) on WS-07 (endotoxin); the stability after reconstitution is the certificate's, the expiry written on the vial. Working series: from the reconstituted CSE, 1 + 9 steps in LAL reagent water in depyrogenated tubes, 1 min vortex after each step, a new tip each step, made fresh each assay day; the volumes on the worksheet.
- Positive product control: the final sample dilution made twice from the same previous step, once with LAL reagent water and once with a CSE working solution at the spike concentration in place of the water (so the spike dilutes nothing; USP <85> solution B), the spike at or near the log midpoint of the curve (within 0.5 decade of it and not equal to an end standard; otherwise a warning); both recipes on the worksheet.
- Negative control: LAL reagent water. It shows no onset within the run, or an onset longer than every standard's. Medium control: the medium alone, run the same way whenever the stock is not made in LAL reagent water.
- Run time: recorded; it must be longer than the longest standard onset, or the curve was not read to completion. Onset threshold 0.2 OD at 405 nm by default; both are recorded.

10 Validity (system suitability)

Check	Criterion	Basis
Standard curve	|r| at or above 0.980 over the standards	USP <85>; Ph. Eur. 2.6.14 Method D
Positive product control	Recovery 50–200% of the spike, net of the sample	USP <85>; Ph. Eur. 2.6.14 Method D
Negative control	Below the lowest standard: no onset within the run time, or an onset longer than every standard onset	USP <85>; Ph. Eur. 2.6.14 Method D
Replicate onsets	CV at or below 10% in every well and standard	Lysate manufacturer's insert criterion, not USP <85>

Replicate onset CV at or below 10% in every well (lysate manufacturer's insert criterion, not USP <85>).

An assay that fails the curve, the positive product control or the negative control is invalid: the result is "Not evaluated" and the assay is repeated. A CV above the insert's criterion is a warning for the reviewer.

11 Procedure

11.1 Prepare the standards from the CSE at the recorded levels, the sample at the test dilution, the positive product control and the negative control (and the medium control), in duplicate wells, 100 µL per well, on the fixed plate map printed on WS-07 (endotoxin): standards in columns 1 and 2, the negative control, the sample, the positive product control and the medium control in column 3, each in two wells.

Procedure, continued

11.2 The one-sample plate above is the method in force. A shared plate is written here as an alternative and takes effect only when the owner's decision is recorded in data.json (method.lal_plate: samples_per_plate 3 or 6, decided_by, decided_on; the saving is ECONOMICS.md's to state, not this procedure's): on a shared plate the standards, the negative control and the medium control are run once per plate, and each sample brings its own duplicate wells and its own positive product control in duplicate (2 × (4 standards + 2 controls) + 4 wells per sample: 16 wells for one sample, 24 for three, 36 for six); every sample is judged by the plate's one curve and negative control and by its own positive product control recovery and replicate CV, so a failed positive product control invalidates that sample alone and a failed curve or negative control the whole plate; the lysate still goes into every well within 2 min, in column order, and the plate map on WS-07 (endotoxin) gains a column per sample. Samples are batched in threes: three samples use the whole of one reconstituted lysate vial (the wells a vial serves are the kit insert's figure; ECONOMICS.md 3.1 gives it for the kit considered), six take two, and the fourth sample on a plate opens a second vial, so a plate carries 3 or 6 samples and never 4 or 5. Until the decision is recorded nothing in this procedure changes.

11.3 Pre-incubate the loaded plate 10 min at 37 ± 1 °C in the reader, its block temperature verified and recorded. Add 100 µL of lysate to every well with an 8-channel pipette, in column order, within 2 min; start the read at once and record its start time (the acquisition time); let it run for the recorded run time. Record the onset time of every well.

11.4 Enter the standards with their duplicate onsets, the sample's stock concentration and dilution with its onsets, the spike with its onsets, and the negative control (no onset, or its onsets).

11.5 The model fits the curve, checks validity, reads the sample and the positive product control, and writes the result sentence; the certificate prints the curve, the standards table with back-calculated recovery and CV per level, the sample and control wells, and the validity block.

11.6 A repeat of an invalid assay uses a fresh 1 + 39 dilution from the stock held at 2–8 °C within 24 h of dissolution; past that, a retained vial, recorded as consumed. The invalid plate's data file is named in the notes with the failed check; no well is excluded by hand.

12 Calculations

$\log_{10}(t_{onset}) = a + b \times \log_{10}(C)$ (least squares over the standards, C in EU/mL)

$C_{well} = 10^{x}$, where $x = (\log_{10}(\text{mean } t_{onset}) - a) / b$

EU per vial = $C_{well} \times D \times V$ (D the dilution from stock to well, V the stock volume of the preparation the aliquot came from)

EU per mg of powder = EU per vial / G (G the gross fill of the vial dissolved, by weighing: the headline when G exists)

EU per mg of nominal fill = EU per vial / N (N the nominal fill; the basis of the MVD and of a supplied EU/mg limit unless the order names another)

EU per mg of peptide = EU per vial / P (P the peptide content from SOP-06, for information)

PPC recovery (%) = $100 \times (C_{PPC} - C_{sample}) /$ spike

CV (%) = 100 × s / mean of the replicate onsets

reporting limit (EU/mg of nominal fill) = lambda × D / c, c = N / V; upper limit = top standard × D / c

– A sample reading below the lowest standard is reported as less than the reporting limit, and above the top standard as more than the upper limit; never extrapolated. The reviewer sees a warning to retest at another dilution.

– Results are reported to 2 significant figures everywhere, each figure from the unrounded EU per vial: page 1, the prose and the headline. r is printed to four decimals and the recovery to one.

– The headline is EU per vial and EU per mg of nominal fill; the three denominators are printed with their names ("EU per mg of powder, gross fill by weighing"; "EU per mg of nominal fill"; "EU per mg of peptide, free peptide by assay"), and the powder basis is flagged when the gross fill differs from the nominal fill by more than twice its own uncertainty, so a reader is never shown only the smaller figure.

13 Measurement uncertainty and decision rule

The endotoxin result is log-normal: the natural log of each duplicate reading through the curve gives a standard error in ln EU, which is combined in quadrature with the method floor of ×/÷ 1.5 taken as an expanded (k = 2) figure and with the Type B terms below, multiplied by 2 and exponentiated. The result is reported as a ×/÷ factor with its interval, each term printed with its value and source; the floor's status for this method is "provisional" (the floor is a provisional pre-validation constant, the laboratory's prior estimate, until the method's validation has measured intermediate precision).

$$u_{floor} = \ln(1.5) / 2$$

$$U\ factor = \exp(2 \times \mathrm{sqrt}(u_{rep,ln}^2 + u_{floor}^2 + u_{CSE}^2 + u_{curve}^2 + u_{dil}^2 + u_V^2 + u_{PPC}^2))$$

- Terms (lab/results.py): the CSE potency from its certificate (7.0% relative when the certificate states none, a design estimate); the curve interpolation, the standard error of log10 C at the sample's onset from the regression, in ln; the aliquot and the dilution (1.0%); the stock volume by mass (0.1%); and the sample's own matrix effect, u_PPC = |ln R| / sqrt(3) with R the positive product control's recovery (zero at full recovery), because USP <85> does not correct for recovery and a valid assay at 60% recovery is biased by what the budget must then carry.
- Once VR-03 measures the run-to-run spread from the positive product controls (its s_IP), that spread already holds the curve and pipetting variance, and those two terms are dropped for the validated state while the CSE potency, a bias shared by every run, stays; the certificate prints the rule it applied.

Decision rule (ISO/IEC 17025:2017 7.8.6; ILAC-G8:09/2019): the conclusion is reached by simple acceptance (guard band w = 0) on the value rounded to its reported precision; the statement beside it says whether the conclusion still holds with the expanded uncertainty (k = 2) included, the guard band w = U. Where a limit is stated more coarsely than the result, comparing at the reported precision is stricter than USP General Notices 7.20 and Ph. Eur. General Notices 1.2, which round to the limit's own digits.

Point comparisons with a limit are made on the value at its 2 significant figures; a limit below the reporting limit cannot be evaluated and the conclusion is "Not evaluated".

14 Acceptance and reporting

- Against a supplied limit in EU/mg or EU/vial: Conforms or Does not conform, with the decision-rule sentence.
- Without a limit: "None supplied; for information"; the result sentence ends by saying that no endotoxin limit was supplied for this material and the value is reported for information.
- The certificate prints the design (levels, replicates, spike, run time), the lysate and CSE lots, the water lot, the reader and the assay date, and every onset time.

15 Deviations and open points

- A departure from this procedure, planned or found afterwards, is nonconforming work and is handled under SOP-08. One that bears on a result is stated on the certificate, in the open point or the notes of the section concerned, never left out.
- An endotoxin result within limits does not establish sterility; the test does not answer the sterility question, and the certificate says so.
- Research peptides have no registered endotoxin limit; the laboratory applies one only when the order supplies it.
- The method is not yet verified for each peptide matrix (the interference and recovery work a validation would include); until it is, the positive product control of every run is the only evidence that the sample does not inhibit or enhance the reaction.

16 Records

- The result set lab/results/<set>.json, its audit trail and its emails; the register row; the certificate and record.json as issued (SOP-07).
- WS-07 (bacterial endotoxin): the lysate, CSE and water lots with their certificates, the consumable lots and the depyrogenation cycle, the standard series and the control recipes, the plate map, the incubation and addition times, the pH where recorded (SOP-07, Records and retention).
- Every standard, well and onset time, the lysate, CSE and water lots, in the result set.
- The reader's data file, named on the certificate with its acquisition time.

17 Revision history

Revision	Change	Approval
1	First issue.	Not yet approved

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Current revisions

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