Validation of bacterial endotoxin by kinetic chromogenic LAL

The preparatory testing USP <85> asks of a kinetic chromogenic method, analyte by analyte: the standard curve and lambda in independent runs, inhibition and enhancement across dilutions, and the maximum valid dilution.

Not yet approved
No approval of this revision is on file. This protocol states the validation the laboratory will perform; no run under it counts until a named reviewer approves it.

Document		Laboratory	
Number	VP-03	Name	Cupel Laboratories
Revision	1, first issue	Address	2599 Speakman Dr Mississauga, ON L5K 1B1
Status	Not yet approved		
Effective	Not yet in effect; takes effect when approved	Country	Canada
		Not on file	Not yet published: legal entity, postal address, telephone and country
Applies to	Test codes LAL, FULL; every analyte of the scope table, one report each	Web	cupel.ca
Supersedes	Nothing; first issue	Email	help@cupel.ca
Fingerprint	d343 227d 0ea7 89b6		

1 Purpose

To establish, for one analyte at a time, the conditions under which SOP-04 measures endotoxin in that peptide: that the standard curve meets the chapter's criteria in independent runs and the lysate sensitivity (lambda, the lowest standard) is confirmed, the dilutions at which the peptide neither inhibits nor enhances the reaction, whether the routine dilution (1:40) is one of them, the maximum valid dilution when a limit is stated, and how far a reading moves between runs against the provisional floor.

2 Scope and status

The method is Bacterial endotoxin (SOP-04). It is validated one analyte at a time: each analyte of the scope table gets its own data file and its own report, VR-03, against this protocol. Test codes LAL, FULL.

Category: USP <85>, photometric techniques: the preparatory testing the chapter prescribes (assurance of the criteria for the standard curve; the test for interfering factors). USP <1225> does not set the characteristics of a compendial endotoxin test; the chapter does.

Status: 0 of 14 analytes have a data file under this protocol and 0 are validated by it. No validation has been performed. This protocol takes effect when it is approved; no validation run counts before then.

3 What the category requires

Characteristic	ICH Q2(R2)	USP <1225>	This protocol
Standard curve and lysate sensitivity	USP <85>	USP <85>	section 7
Interfering factors (inhibition, enhancement)	USP <85>	USP <85>	section 8
Maximum valid dilution	USP <85>	USP <85>	section 8
Hold-time spike (lipidated peptides)	not in the table	not in the table	section 9
Intermediate precision	not in the table	not in the table	section 10

What the category requires, continued

Required and not required as ICH Q2(R2) Table 1 and USP <1225> Table 2 set them for this category; a characteristic outside both tables is studied because the method's use needs it.

4 Responsibilities

Role	Responsibility
Analyst	Runs the experiments as written here, records every value in the data file as it is produced, and names the raw data files they came from.
Reviewer	Checks the data file against the raw data, composes the report, and signs it as reviewed; does not review their own runs.
Approver	Approves this protocol before any run, and the report after; a person named in the laboratory's people.

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

5 Prerequisites

5.1 SOP-04 is approved: its parameters are the ones validated, and a change to them after validation is a change to the method.

5.2 The instrument is installed, qualified and calibrated under SOP-09, and named in the data file. 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.

5.3 Reference materials are on file with their certificates: the analyte's reference standard with its assigned content and the basis of it, and for each experiment the materials it names.

5.4 The people who run and review the work are trained on SOP-04 and on this protocol, and the training is recorded.

5.5 The data file is opened before the first run and filled as the work proceeds; a value is never entered from memory.

6 Decisions and the verdict

- Every criterion is evaluated on the value rounded to its reported precision, the decision rule the certificates apply: a limit of 102.0% is met by 102.04% (reported 102.0) and not by 102.05% (reported 102.1).
- Each criterion is decided Pass, Fail, Not evaluated (its data are missing or short of the design) or Not applicable (its condition is absent, with the reason stated); a statistic with no criterion is reported for information.
- A characteristic passes when none of its criteria fails or is missing. The method is validated for the analyte when every criterion of every characteristic passes, and the report is approved by a person named in the laboratory's people; the scope row then reads Validated, with the date.
- Criteria marked laboratory default are the laboratory's own and are not regulatory limits; a criterion from a cited text cites it. A criterion may be changed before data are taken, as an amendment with its reason, recorded in the data file and printed in the report beside the protocol's own; never after.
- A departure from the design is recorded as a deviation with its impact and printed in the report. A failed criterion is not repeated until a different answer comes: a repeat needs a cause found and recorded (SOP-08).

7 Standard curve and lysate sensitivity

USP <85> assurance of criteria for the standard curve

In every run the curve meets the chapter's criterion over its range, the lowest standard reacts in every replicate and the negative control does not.

Design

7.1 In each of three independent runs on different days, with at least two lysate lots across them (the lot of each run recorded), prepare the control standard endotoxin at 5, 0.5, 0.05, 0.005 EU/mL in LAL reagent water and run each in triplicate, with the negative control (LAL reagent water) in duplicate. Every criterion below holds per lot as it holds per run.

7.2 Record every onset time; a well that does not reach the onset threshold within the run time is recorded as no onset.

Statistics

$\log_{10} t_{onset} = a + b \log_{10} c$, least squares over every well

$r = S_{xy} / \mathrm{sqrt}(S_{xx} S_{yy})$; CV (%) = 100 s / mean of the replicate onsets

back-calculated $c = 10^{y}$, $y = (\log_{10} t_{mean} - a) / b$

Acceptance criteria

Id	Criterion	Source
SC1	|r| over every well of each run's curve at least 0.980	USP <85> (results.LAL_R_MIN)
SC2	Replicate onset CV of every standard at most 10%	the lysate insert, not USP <85> (results.LAL_CV_MAX)
SC3	Every curve has at least three standards in at least triplicate spanning at least two decades, no step wider than one decade	USP <85>
SC4	Every replicate of lambda reacts within the run time and the negative control does not	USP <85>
SC5	At least two lysate lots across the runs, each lot's curves meeting SC1 to SC4	laboratory default (CRITIQUE_17025 F18)

Recorded in the data file as runs (each: lysate_lot; standards with eu_ml and onset_s; negative_control).

8 Interfering factors and the maximum valid dilution

USP <85> test for interfering factors

The dilutions at which a spike is recovered from the peptide within the chapter's window, so the routine dilution is known to be free of inhibition and enhancement.

Design

8.1 Dissolve the peptide as the routine method does: the vial whole, by mass, to 2.0 mg/mL nominal in LAL reagent water (10 mM Tris of LAL grade for semaglutide, tirzepatide and retatrutide), the stock at 2.0 mg/mL nominal (SOP-06). In each run dilute the stock 1:10, 1:20, 1:40, 1:80, 1:160 in LAL reagent water and test each dilution in duplicate unspiked (solution A) and spiked with the positive product control at 0.5 EU/mL (solution B), against that run's curve (solution C) and negative control (solution D).

8.2 When the stock is made in 10 mM Tris, pH 7.8, LAL grade, run a medium-only control (the medium diluted as the routine dilution, no peptide) in duplicate beside the negative control: the medium itself must read below lambda.

8.3 Record the pH of each dilution and of the routine dilution mixed 1 + 1 with the reconstituted lysate, against the range the lysate insert states; once per analyte and medium, and again whenever a new salt form or medium appears.

8.4 One lot of the peptide per run, three lots in all where that many have been received; where the analyte is received as more than one salt form (trifluoroacetate, acetate), each form is one of the lots. For GHK-Cu the copper of the complex is noted in the record: a metal ion can inhibit the reaction, and the dilution series is the evidence that the routine dilution does not.

Interfering factors and the maximum valid dilution, continued

8.5 The endotoxin limit, when one is stated for the material, is recorded with its source; none is set by the laboratory.

Statistics

c_{well} from the run's curve; a well with no onset within the run time is taken as 0

recovery (%) = (c_{PPC} − c_{sample}) / c_{spike} × 100 (USP <85>)

NID = the smallest dilution from which every dilution tested recovers 50–200%

MVD = L c / lambda (USP <85>); reporting limit = lambda × dilution / c

Acceptance criteria

Id	Criterion	Source
IE1	Every lot has a non-interfering dilution in the series (recovery 50–200% there and at every greater dilution)	USP <85> (results.PPC_LO, PPC_HI)
IE2	The routine dilution (1:40) is at or beyond the NID in every lot	laboratory default
IE3	The NID is within the MVD	USP <85>
IE4	Replicate onset CV of every sample and PPC well pair from the NID at most 10%	the lysate insert (results.LAL_CV_MAX)
IE5	pH of the routine dilution mixed 1 + 1 with the lysate inside the insert's range, in every lot	the lysate insert (METHOD_REVIEW SOP-04 §8)
IE6	The medium-only control reads below lambda (no onset before the lowest standard's) in every run where the stock is made in a medium other than LAL reagent water	USP <85> negative control, applied to the medium

Recorded in the data file as runs (each: lot, salt_form; dilutions with dilution, sample_onset_s, ppc_onset_s and ph; lysate_ph of the routine dilution; medium_control with its onsets or no_onset); ph_range of the insert.

9 Hold-time spike (the lipidated peptides)

USP <85> interfering factors under the conditions of use; USP <1085> by analogy

The positive product control spiked at assay time shows inhibition and enhancement, not endotoxin lost into the peptide's self-associated aggregates during the hold between dissolution and assay. One arm proves the hold harmless for semaglutide, tirzepatide and retatrutide, whose stocks are made in 10 mM Tris, pH 7.8, LAL grade.

Design

9.1 Spike the stock with the control standard endotoxin at time zero (dissolution), so that the routine dilution carries 0.5 EU/mL, and assay the routine dilution at 0.25 and 2 h after the spike against the run's curve; one plate per analyte. Recovery at each time against the unspiked stock's own reading.

9.2 Not applicable to an analyte outside semaglutide, tirzepatide and retatrutide: the record says so and the characteristic is not evaluated.

Statistics

recovery(t) (%) = (c_{spiked}(t) − c_{sample}) / c_{spike} × 100

Acceptance criteria

Id	Criterion	Source
HT1	Recovery of the held spike at every time point from 50 to 200%	USP <85> (results.PPC_LO, PPC_HI); METHOD_REVIEW S14

Recorded in the data file as hold_time (run; points, each with hours, sample_onset_s and spiked_onset_s), or null for an analyte it does not apply to.

10 Intermediate precision

ICH Q2(R2) intermediate precision; results.py budget

The run-to-run spread of a reading, on the log scale the uncertainty is stated on. s_IP from the PPC spreads already contains the curve-interpolation and pipetting variance, so PF1 is a sanity criterion against the provisional factor of ×/÷ 1.5, not the floor's replacement: "validated" means this report's s_IP replaces the provisional run-to-run term in the budget of lab/results.py, with the CSE potency term kept and the curve term dropped as already inside s_IP.

Design

10.1 The recovered spike of every PPC well at the routine dilution, in every run, from the interference test.

10.2 The report tabulates, beside s_IP, the other terms the certificate's budget carries so that PF1 is compared on one basis: the CSE potency term (7% relative when the certificate states no uncertainty, results.LAL_CSE_U_REL_PCT_DEFAULT), the dilution term (1.0%, results.LAL_DILUTION_U_REL_PCT) and the curve interpolation, which s_IP already contains.

Statistics

$y = \ln(c_{PPC,\,well} - c_{sample,\,mean})$

one-way ANOVA, runs as groups: $MS_w = \text{sum}\,(y_{ij} - y_j)^2 / (N - k)$; $MS_b = \text{sum}\, n_j\,(y_j - y_m)^2 / (k - 1)$

$s_r^2 = MS_w$; $s_{run}^2 = \max(0, (MS_b - MS_w) / n_0)$; $n_0 = (N - \text{sum}\, n_j^2 / N) / (k - 1)$

$s_{IP} = \text{sqrt}(s_r^2 + s_{run}^2)$ (ISO 5725-2; USP <1210>)

expanded factor = $\exp(2\, s_{IP})$

Acceptance criteria

Id	Criterion	Source
PF1	Expanded run-to-run factor exp(2 s_IP), a sanity criterion, at most 1.5	the provisional factor (results.U_FLOOR_ENDOTOXIN_FACTOR); METHOD_REVIEW M3, S2

Recorded in the data file as derived from the dilutions of every run.

11 Data and report

- The data file is lab/validation/lal-<analyte>.json, one per analyte (the annex lists every file name), with kind "plumbline-validation" and schema 1. Every value is entered as measured, with the raw data file it came from in the run's record.
- The report VR-03 is composed from that file by python lab/validation.py report <file> <out.pdf>: every statistic computed from the file and printed with its formula and this data's numbers, every criterion with its decision.
- lab/validation_examples/lal-semaglutide.json shows the format; it is illustrative data, marked as such in the file and on every page of its report, and no scope row ever reads from it.
- The scope table reads each analyte's file: Not yet validated without one, In validation with one, Validated when every criterion passes and the approval is signed.

12 Annex A: analytes and their data files

Analyte	Data file	Status
Semaglutide	lab/validation/lal-semaglutide.json	Not yet validated
Tirzepatide	lab/validation/lal-tirzepatide.json	Not yet validated
Retatrutide	lab/validation/lal-retatrutide.json	Not yet validated
BPC-157	lab/validation/lal-bpc-157.json	Not yet validated
TB-500 (Tbeta-4 17–23)	lab/validation/lal-tb-500.json	Not yet validated
Ipamorelin	lab/validation/lal-ipamorelin.json	Not yet validated
CJC-1295 (no DAC)	lab/validation/lal-cjc-1295-no-dac.json	Not yet validated

Annex A: analytes and their data files, continued

Tesamorelin	lab/validation/lal-tesamorelin.json	Not yet validated
Bremelanotide (PT-141)	lab/validation/lal-bremelanotide.json	Not yet validated
Melanotan II	lab/validation/lal-melanotan-ii.json	Not yet validated
AOD-9604	lab/validation/lal-aod-9604.json	Not yet validated
Thymosin alpha-1 (thymalfasin)	lab/validation/lal-thymosin-alpha-1.json	Not yet validated
Epitalon (AEDG)	lab/validation/lal-epitalon.json	Not yet validated
GHK (sold as GHK-Cu)	lab/validation/lal-ghk-cu.json	Not yet validated

13 References and controlled sources

Standards and other documents

- USP <85> Bacterial endotoxins test, photometric techniques; Ph. Eur. 2.6.14 Method D.
- ISO 5725-2 and USP <1210> for the variance components.
- Cited for what they ask; this protocol claims no conformity with them, and a number they do not set is marked as the laboratory's.

Controlled sources, read at every build

- lab/validation.py: this protocol's characteristics, design and criteria, and the arithmetic of the report.
- data.json method rows (the routine conditions); lab/results.py (the routine criteria and the provisional uncertainty floors the validation tests).

14 Deviations and open points

- A departure from this protocol's design is recorded in the data file as a deviation with its impact and printed in the report; one found afterwards is nonconforming work, handled under SOP-08.
- 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.
- No endotoxin limit is set by the laboratory, so no MVD is defined unless the material's limit is stated; the non-interfering dilution is established instead.
- No analyte has been validated under this protocol.

15 Records

- The data file and the raw data files it names; the report as composed and as approved; the approvals.
- Kept for the life of the method and never deleted while a report relies on the validation.

16 Revision history

Revision	Change	Approval
1	First issue.	Not yet approved

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Current revisions

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