European Union Reference laboratory for monitoring bacteriological and contamination of bivalve molluscs

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WORK PROGRAMME FOR THE EURL FOR BACTERIOLOGICAL AND VIRAL CONTAMINATION OF BIVALVE MOLLUSCS, 2013

LEGAL FUNCTIONS AND DUTIES

The functions and duties of the EURL are specified in Article 32 of Regulation (EC) No 882/2004 (Official Journal of the European Communities No L 165 of 30.4.2004).

In the 2013 work programme year 27 Member States, and 1 acceding country (Croatia) are considered eligible for EURL assistance and invited to participate in EURL organised training programmes, comparative testing etc. Candidate countries are also invited to participate in comparative testing and training workshops. The full integration into the European Union of Member States continues to be a priority area, and is facilitated via the provision of additional advice, training and assistance where required.

WORK PROGRAMME, 2013

1. **Scientific advice and support** – (up to 100 days) **Expected outputs**

1.1. The EURL will provide scientific assistance to DG SANCO in operation and implementation of European Union food hygiene legislation, and in particular in 2013 the following activities have been identified:

1.1.1. Provide scientific and statistical assistance with the ongoing equivalency negotiations between EU and US for live bivalve molluscs (LBM), especially following on from the 2nd International Workshop on Molluscan Shellfish Area Classification co-organised by the EURL and US FDA[a]. [a]Progress towards technical equivalence between EU and US sanitation systems

1.1.2. Provide scientific assistance, specifically through the Commission expert working group on live bivalve molluscs[b]. [b]Scientific support (Codex, class A, viruses etc)

NOTE. The EURL will provide any other additional advice within its area of expertise as required, and undertake supporting expert missions on request of the European Union.

1.2 Participate in relevant EU and International scientific committees (EFSA, ISO/CEN, WHO/FAO, ICMSS etc). In 2013 the EURL will:

1.2.1 Participate as a member of the international steering committee towards the organisation of the 9th International Conference on Molluscan Shellfish Safety, Sydney, Australia 17-21st March 2013 http://www.icmss2013.com/[c] [c]Successful conference

1.2.2 Further to the above EURL staff (maximum 2) to present keynote speeches, chair sessions, deliver oral scientific presentations and poster presentations on application of sanitary surveys, control options and surveillance of norovirus in live bivalve shellfish at the 9th International

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Conference on Molluscan Shellfish Safety, Sydney, Australia 17-21st March 2013[d].

[d]As above, with strong EU representation

1.2.3 Oversee the publication of ISO TS 15216-1, Microbiology of food and animal feed — Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR — Part 1: Method for quantitative determination and ISO TS 15216-2, Microbiology of food and animal feed — Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR — Part 2: Method for qualitative determination). To include responding to official technical and editorial comments from voting members at ISO SC9 level[e].

[e]Publication of reference method for viruses in LBM

1.2.2 Lead and co-ordinate the activities of CEN/TC 275/WG6/TAG3 in the elaboration of molecular based enumeration methods for pathogenic marine vibrios in bivalve shellfish, particularly for *V. parahaemolyticus*. Two missions associated with this activity are anticipated in 2013[f].

[f]Progress toward quantitative method for determination of total and toxigenic *V. parahaemolyticus* in LBM

1.2.3 Complete the revision of the EU reference method for enumeration of *E. coli* in LBM for official control (ISO TS 16649-3) to establish the method as a full standard. Including responding to official technical and editorial comments from voting members at ISO SC9 level[g].

[g]Publication of *E. coli* reference method as a full standard

1.2.4 Project leader for the revision of the ISO 6887 series part 3 initial preparation and dilutions for aspects of microbiology associated with LBM (incl. in fish and fisheries products). One mission is envisaged in 2013, to include completion of the document and responding to official technical and editorial comments from voting members at ISO SC9 level[h].

[h]Completion of revision of ISO 6887-3 to bring in line (*inter alia*) with Reg (EC) No. 2073/2005

1.2.5 Lead the revision of ISO TS 21872-1 and 2 detection of *Vibrio* spp. in seafood. To inform the Commission of progress under the CEN mandate M/381[i.]

[i] provide updates on revision of vibrio ISO to include provision for detection of toxigenic *V. parahemolyticus*

1.2.6 To continue to contribute to relevant EFSA expert working groups as required[j.]

[j]provide feedback on progress at EFSA with respect to developments regarding control options for norovirus and hepatitis A virus in LBM

1.2.7 To contribute towards the FAO/WHO initiatives in the development of a broader application of the current international risk assessments for *V. parahaemoloyticus* and *V. vulnificus*, in terms of both geographical relevance and bivalve species[k].

[k]provide feedback

1.2.8 Assist DG SANCO with specialist advice in relation to food and veterinary inspections of Member States, Accession Countries and Third Countries as they arise.

1.2.9 Represent the EURL at the annual plenary meeting of the ISO SC9 and CEN WG6 Microbiology working group meeting. One mission in 2013[l].

[l]provide through collaborative report summary of plenary meeting

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2 **Co-ordination of activities of NRL network –** (up to 170 days)

2.2 Participate in annual EURL Director's co-ordination meeting and other EURL co-ordination meetings/workshops as appropriate.

2.3 Organise, host, and participate in the twelfth annual EURL workshop, produce resolutions and other workshop outputs (May 7th -9th 2013, Rome, Italy). To include administrative assistance[m].

[m]successful workshop provide feedback as required through report and briefing

2.4 Further to the above, undertake EURL activities and commitments agreed in resolutions at annual workshop above (as posted on www.eurlcefas.org).

2.5 Continue to improve the EURL website (www.eurlcefas.org) to improve relevance and accessibility of contents and development strategies to increase usage of the website services by NRLs and other stakeholders[n].

[n]increased use of website for dissemination of information to stakeholders and maximum efficiency

3 Provision of technical advice and training - (up to 70 days)

3.1 Provide specialist training and/or training courses to NRLs, accession country NRLs and others in relation to official control analyses (*E. coli*, *Salmonella* spp.,) and non-statutory analyses (*Vibrio* spp., FRNA bacteriophage, Norovirus, hepatitis A virus) and other aspects of bivalve shellfish hygiene as required[o].

[o]improvements in performance and increased harmonisation across laboratories

3.2 Further to 3.1, organise a limited training course at the EURL to provide specialist training in quantitative analysis of noroviruses in bivalve shellfish for NRLs[p].

[p]as 3.1

3.3 Further to 3.1, host a training visit from a member of staff at NRL Italy (Ancona) to provide targeted training in application of EU official controls related to bivalve molluscs (sanitary surveys, laboratory analyses and quality assurance, depuration and traceability)[q].

[q]as 3.1

3.4 Continue to build scientific expertise and capacity across the network in the area of application of methods to detect human pathogenic *Vibrio* spp. associated with LBM (particularly raw oysters) developing on the outputs of the EURL expert *Vibrio* expert working group of 2012[r].

[r]as 3.1

3.5 Supply technical advice on bacteriological and viral methods to NRLs, Official Control testing laboratories, and third county laboratories. In the form of EURL harmonised protocols, standard operating procedures etc, to include approved alternative methods for official control analysis[s].

[s]as 3.1

3.6 To include assistance on implementation of methods, accreditation to IEC ISO17025 and quality control requirements (see above).

3.7 To provide guidance and review of procedures/data to laboratories wishing to undertake studies to validate of alternative methods according to ISO 16140[t].

[t]use of approved of methods across the

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3.8	Provide specialist training and/or training courses to NRLs, accession country NRLs and others in relation to analyses of LBM for microbiological contaminants as required[u].	community [u]harminsation of approaches
4	**Comparative testing and ring trials -** (up to 270 days)	
4.2	Organise comparative testing for NRLs for *E. coli* and *Salmonella* spp. in bivalve molluscs via the EURL/HPA shellfish EQA scheme. Analyse results, produce report, advice and recommendations (by May 2013)[v].	[v]comparative testing report published on the EURL website, presented at workshop
4.3	Organise norovirus and hepatitis A virus comparative testing distribution for quantitative and qualitative analyses. Analyse results, produce report and recommendations (by May 2013)[w].	[w]comparative testing report published on the EURL website, presented at workshop
4.4	Undertake collaborative trials to test aspects of developmental *Vibrio* spp. methods in matrix and laboratory constructed samples. Analyse results, produce report (by December 2013)[x].	[x]comparative testing report published on the EURL website, presented at workshop
4.5	Organise comparative testing amongst NRLs for *E. coli* and *Salmonella* spp. in live bivalve molluscs samples to test aspects of official methods no examined in standard EQA, i.e. initial dilutions, homogenisation. This item is specifically at the request of the NRL network to assist in the requirements of accreditation bodies. Analyse results, produce report, advice and recommendations (by May 2013)[y].	[y]comparative testing report published on the EURL website, presented at workshop
4.6	Distribution of reference materials for all relevant microbiological determinants on request of NRLs[z].	[z]improvements in performance and increased harmonisation across laboratories
5	**Confirmatory testing and quality assurance –** (up to 100 days)	
5.1	Maintenance of EURL laboratory competence and expertise in analytical methods for monitoring virological contaminants of bivalve molluscs (norovirus and hepatitis A virus). To include maintenance of requirements for ISO/IEC 17025 accreditation for quantitative determination of norovirus in LBM[aa].	[aa]retention of ISO 17025 accreditation where relevant at annual external audit
5.2	Maintenance of EURL laboratory competence and expertise in analytical methods for monitoring bacteriological contaminants of bivalve molluscs (*E. coli*, *Salmonella* spp., marine vibrios). To include maintenance of ISO/IEC 17025 accreditation of enumeration of *E. coli*, and the detection of *Salmonella* spp. and *Vibrio parahaemolyticus*[bb].	[bb]retention of ISO 17025 accreditation at annual external audit
5.3	Progress towards accreditation to ISO/IEC 17025 of approved, validated alternative methods for enumeration of *E. coli* in LBM, to include[cc]	
5.3.1	Enumeration of *E. coli* in live bivalve molluscs by the direct impedance technique using BacTrac 4200 analyser (see annex I for experimental detail)	[cc]assessment at annual external audit

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5.3.2 Enumeration of *E. coli* in live bivalve molluscs by the colony count technique (based on ISO 16649-2).

5.4 Contribution to costs of the maintenance of EURL capability to perform analysis for human pathogenic strains of marine vibrios associated with LBM (e.g. serotyping *V. parahaemolyticus*, molecular characterisation of pathogenic strains of *V. parahaemolyticus*, *V. vulnificus* and) non01/0139*V. cholerae*)[dd].

[dd]retention of demonstrable capacity at EURL to performance analysis

5.5 Performance of above tests on outbreak material or on occasion of disputed test results (on request of DG SANCO).

6 Development of analytical methods – (up to 100 days)

6.1 Practical developmental to support elaboration of standard molecular methods to detect pathogenic vibrios in foodstuff; including bivalve shellfish (see section 1.2.2)[ee].

[ee]draft procedure presented to TAG3 expert working group

6.2 Undertake practical laboratory based studies to support the accreditation to ISO/IEC 17025 of approved, validated alternative methods for enumeration of *E. coli* in LBM, to include in house verification/secondary validation of:

- Enumeration of *E. coli* in live bivalve molluscs by the direct impedance technique using BacTrac 4200 analyser (see Annex I for detail of experimental work)[ff]

[ff]completion of work outlined in Annex I, and report- submitted for accreditation audit

- Enumeration of *E. coli* in live bivalve molluscs by the colony count technique (based on ISO 16649-2)[gg].

[gg]submission of data for annual audit

6.3 Undertake preliminary work to investigate methods to examine the efficacy of technical adaptations to ISO TS 15216-1, Microbiology of food and animal feed — Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR — Part 1: Method for quantitative determination, to enable determination of viable viruses[hh].

[hh]EURL method for quantitation of noroviruses and HAV as a generic protocol

6.4 Undertake preliminary work to investigate the efficacy of depuration to remove norovirus in oysters[ii.]

[ii]generation of data on the efficacy of depuration in the removal of noroviruses from LBM

NOTE. In 2013 it is recommended that a studentship is partially supported by the EURL up to a maximum value of £8,000 to assist with development of analytical methods under 6

Rachel Hartnell
EURL Co-ordinator, August 2012 as amended October 2012

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Annex I

In house verification of an impedance method for enumeration of *E. coli* in shellfish using the Bactrac 4300 series analyser or µ-Trac 4200.

1. Introduction

Bacterial impedance using Bactrac 4300 series analyser for the enumeration of *E. coli* in bivalve shellfish has been validated according to the requirements of ISO 16140 by the French NRL (IFREMER)[1]. The validation has been accepted by the EURL for monitoring bacterial and viral contamination of bivalve molluscs and was presented to the European Commission in June 2011. This method as specified in IFREMER/EURL generic protocol [08_06_11.issue 01] is considered acceptable for official control testing for *E. coli* concentrations in bivalve molluscs.

This document sets out a procedure for demonstration of satisfactory performance of impedance (using Bactrac 4300 series analyser or µ-Trac 4200) for enumeration of *E. coli* in bivalve molluscs. This procedure will generate data in support of ISO/IEC 17025 accreditation for impedance techniques.

2. Scope

This procedure specifies a protocol for in house verification of bacterial impedance using Bactrac 4300 series analyser or µ-Trac for enumeration in live bivalve molluscs. This procedure is appropriate for the following bivalve shellfish species *Crassostrea gigas, Mytilus edulis, Mytilus galloprovincialis, Cerastoderma edule, Ruditapes deciussatus, Ruditapes philippinarum* and *Donax trunculus*

Note. This procedure is not appropriate for echinoderms, tunicates and marine gastropods.

3. Principle

A doubling dilution series of shellfish homogenate within the range <140 - ≈18,000 *E. coli* MPN per 100g is analysed in duplicate on two separate occasions using impedance and MPN (UK NRL SOP). Regression analysis of detection times (DT) and *E. coli* MPN/100g is performed on the dataset. The curve coefficients are tested against the calibration coefficients determined by IFREMER using the *t*-statistic. The slope and intercept values obtained from the IFREMER validation study (all species) are considered the true values (http://www.crlcefas.org/InformationCentre/docs/E_coli_enumeration_BacTrac_impedance_technique_v1_08_06_11_issue_01.pdf)

4. Safety precautions

Standard microbiological safety precautions should be applied throughout.

5. Equipment

[1] Validation reports available to download from www.crlcefas.org

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- Safety gloves
- Shucking knife, Florida cracker
- Sterile glass containers of varying size and capacity
- Pipettes with the capacity to deliver volumes of 7.5 ml (with 0.1 ml divisions)
- Electric top pan balance
- Waring blender
- BacTrac 4300 series microbiological analyser or µ-Trac (Sy-Lab, Neupurkerdorf, Austria). The BacMonitor program should be set as the following:
    - BacTrac settings
        - Set temperature 44.0ºC
        - Measuring cycle 10 min
    - Evaluation and BacTrac settings
        - Type M/E M2: cells with 2 pins (no E-value)
        - Threshold M 3.00%
        - Time limits M 0.00h
        - Warm-up time 1.00h
        - Measurement duration 20.00h
        - Drift compensation M 0.00h
        - Do not check DropStop checkboxes for M and E
- Incubator at 37±1°C
- Incubator at 44±1°C

6. Media

- 0.1% peptone
- Minerals modified glutamate broth (MMGB x 1, MMGB x 2)
- Tryptone bile glucoronide agar (TBGA)

7. Procedure

7.1. Preparation of dilution series

7.1.1 Weigh approximately 150 g of flesh and intravalular fluid from the species under test.
7.1.2 Prepare a 1 in 3 homogenate by adding 2ml of 0.1% peptone water for each 1 g flesh and blend in a Waring blender for 60 secs (3 bursts of 15 secs).
7.1.3 Add an inoculum to give an estimated *E. coli* concentration of ≈18,000 *E. coli* MPN per 100g flesh and intravalular fluid.
7.1.4 Blend for a further 15 secs, to thoroughly distribute the inoculum.
7.1.5 Allow the homogenate to settle for 15 min at room temperature.
7.1.6 Prepare serial doubling dilutions (1/2, 1/4, 1/8, 1/16 etc to 1/2048) using uncontaminated 1 in 3 homogenate (1 part FIL to 2 parts 0.1% peptone water) as the diluent, prepared as in 7.1.2. The volume at each dilution point should be at least 45 ml.

Note. Uncontaminated homogenates should be tested in triplicate using the UK NRL SOP (derived from ISO TS 16649-3) prior to use, all results must be <20 MPN per 100g FIL.
Note. Typically a doubling dilution series from ≈18,000 E. coli MPN/100g will require 12 points (i.e. to 1/2048) to reach <20 *E. coli* MPN/100g.

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7.1.7 Allow the dilution series to settle for a further 15 min at room temperature.
7.1.8 Transfer 30 ml of the liquid part of the homogenate from each dilution point to a flask containing 70 ml of 0.1% peptone water to produce a series of 10^{-1} FIL suspensions. Mix thoroughly by shaking.

8 Impedance - Inoculation of measurement cells

8.1 Take two ready to use disposable measurement cells for each dilution point and remove the membrane seal covering the cells. Transfer 7.5 ml of the 10^{-1} FIL suspension from each dilution into each measurement cell using a sterile pipette. Close the cells with sterile caps. Do not shake.
8.2 Insert the measurement cells into the instrument and set to 44ºC.
The calibration line for the determination of *E. coli* per 100g FIL (N) form detection times (DT) takes the form of $\log_{10}N = k_0 + k_1 \times DT$. The k_0 and k_1 values to be entered in the BacMonitor program are:
$k_0 = 9.7174$
$k_1 = -0.8964$
Continue to measure impedance for at least 2 hours after the beginning of the plateau phase for all cells to obtain complete curves for each sample.
Check the validity of the results and the curve shape according to the IFREMER/EURL generic protocol. Record the detection times (DT) for each dilution.

9 MPN – inoculation and incubation of primary broth

9.1 Make further decimal dilutions in 0.1% to 10^{-2} by adding 1 ml of the 10^{-1} dilution (prepared in 7.1.8) to 9ml of 0.1% peptone. For the more heavily contaminated dilutions (1/2, 1/4, 1/8, 1/16) prepare further decimal dilutions to 10^{-3}.
9.2 Inoculate five bottles containing double strength MMGB with 10±0.2ml of the 10^{-1} diluted homogenate. Inoculate five bottles of single strength MMGB with 1±0.1ml of the 10^{-1} diluted homogenate. Inoculate five bottles of single strength MMGB with 1±0.1ml of the 10^{-2} diluted homogenate. And repeat for any further dilutions.
Incubate inoculated bottles of MMGB at 37±°1C for 24±2 hours.
9.3 Confirmation of *E. coli*
Examine MMGB for the presence of acid. Confirm the presence of *E. coli* by subculturing onto TBGA within 4 hours, streaking to obtain single colonies. Incubate TBGA at 44±°1C for 22±2 hours.
After incubation examine TGBA for the presence of blue-green colonies. Record the positive results, and use to derive the 3 figure tube combination (for details of how to derive the MPN see UK NRL SOP issue 9, 28.10.10).

REPEAT STEPS 7 TO 9 ON THREE SEPARATE OCCASSIONS.

10 Interpretation of results

Plot a scatter graph of the results obtained in 9 ($\log_{10}$ transformed MPN values) against the detection times (DT) for each dilution for each occasion obtained in 8 using excel or equivalent software. Calculate the slope and intercept for the regression line. Calculate the standard error for the slope and the intercept.

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Estimate the difference between the curve coefficients (slope and intercept) of the data generated in this study and the curve coefficients produced in the original IFREMER validation; the true values (slope = -0.8964 and intercept = 9.7174) according to the following:

$$t_b = \frac{(\text{slope}_{\text{testing lab}} - \text{slope}_{\text{validation study}})}{\text{standard error}_{\text{testing lab slope}}}$$

$$t_a = \frac{(\text{intercept}_{\text{testing lab}} - \text{intercept}_{\text{validation study}})}{\text{standard error}_{\text{testing lab intercept}}}$$

Calculate t_b (slope) and t_a (intercept). Using the Student *t*- Statistical table with n=2 degrees of freedom (where n = the total number of tests) look up the tabulated *t*-statistic. If the calculated *t* values for both slope and intercept are less than the tabulated *t*-statistic with the appropriate degrees of freedom at the 0.05 level (5%) the curve coefficients can be considered to be not significantly different from the true values and the method is verified.
An example calculation is given in Appendix 1.

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Appendix 1. Example interpretation of results of verification study

Dataset – occasion 1

Dilution series	MPN value	log MPN value	DT
1	18000	4.2553	6.25
2	9200	3.9638	6.4
4	3500	3.5441	6.6
8	1700	3.2304	6.8
16	940	2.9731	7
32	490	2.6902	7.6
64	260	2.4150	7.6
128	130	2.1139	7.9
256	70	1.8451	8.4
512	40	1.6021	8.7
1024	20	1.3010	9.55
2048	10	1	9.8

Dataset – occasion 2

Dilution series	MPN value	log MPN value	DT
1	18000	4.2553	6.3
2	9200	3.9638	6.45
4	5400	3.7324	6.64
8	2800	3.4472	6.86
16	1400	3.1461	7.1
32	700	2.8451	7.65
64	340	2.5315	7.6
128	170	2.2304	7.95
256	90	1.9542	8.5
512	50	1.6990	8.75
1024	10	1	9.6
2048	10	1	9.85

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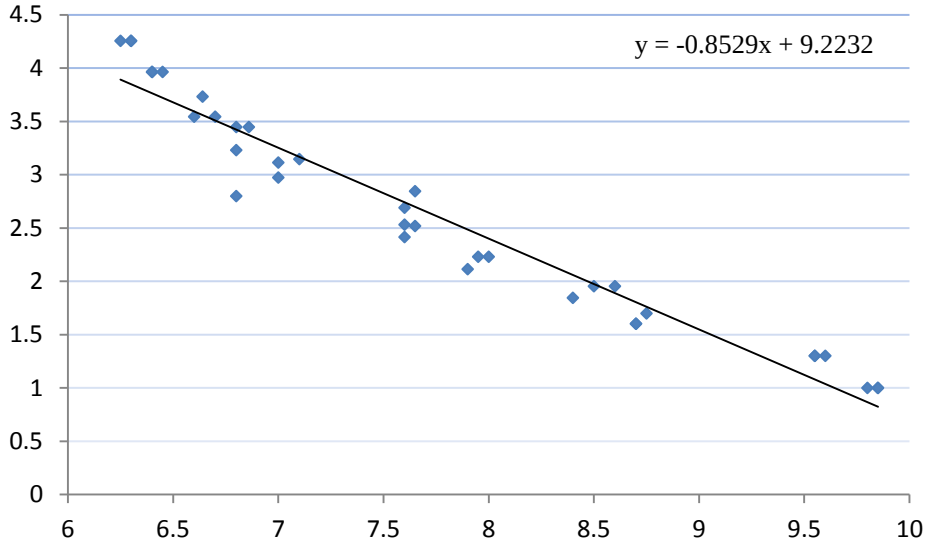
Dataset – occasion 3

Dilution series	MPN value	log MPN value	DT
1	18000	4.2553	6.3
2	9200	3.9638	6.4
4	3500	3.5441	6.7
8	2800	3.4472	6.8
16	1300	3.1139	7
32	630	2.7993	6.8
64	330	2.5185	7.65
128	170	2.2304	8
256	90	1.9542	8.6
512	40	1.6021	8.7
1024	20	1.3010	9.55
2048	10	1	9.85

Using Excel or similar software plot a regression analysis of the entire dataset. Display the equation on the graph



Equation of the line is y = -0.8529x + 9.2232
Slope (*b*) = -0.8529
Intercept (*a*) = 9.2232

Equation of the line form the validation study y = -0.8964x +9.7174
Therefore the **true values** are:
Slope = -0.8964
Intercept = 9.7174
Calculate the standard error (se) of the slope (*b*) and the intercept (*a*)

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Note. The standard error can be calculated manually or using the regression function on excel or similar software.
se (*b*) = 0.03467
se (*a*) = 0.2709

$$t_b = \frac{-0.8529 - (-0.8964)}{0.03467} = \frac{0.0435}{0.03457} = 1.258$$

$$t_a = \frac{9.2232 - 9.7174}{0.2709} = \frac{-0.4942}{0.2709} = -1.824$$

The tabulated *t*-statistic at the 0.05 level with 34 degrees of freedom (*df* = 36-2) is 2.032, as both t_b and t_a are less than the tabulated value the method can be considered verified.