

**PIPETTE CALIBRATION: COLORMETRIC METHOD FOR
VERIFYING PIPETTE DELIVERY VOLUME FOR USE WITH
PIPETTES DISPENSING VOLUMES OF UP TO 250
MICROLITRES.**

**AUSTRALIAN ANIMAL HEALTH LABORATORY
DISEASE DIAGNOSIS PROJECT**

Colormetric Verification of Pipette Delivery Volume

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1. 1 INTRODUCTION

1.1 Applicability

This is an AAHL “in-house” method to be used to check the delivery volume of piston operated micro-volumetric pipettes having delivery volumes of no more than 250 microlitres (μl). All pipettes for use in accredited work must be checked for satisfactory operation at intervals not exceeding 3 months. This method has been designed for testing pipettes used in immunoassay procedures, in particular ELISA. The approach may not be applicable for the use of pipettes in all other methods (see [1.4](#) for specifications).

1.2 The Verification Process

The sequence for the process of verifying pipette delivery volume in summary is:

- Colormetric assessment of pipettes at a selected delivery volume (see [5.](#) and [6.](#))
- Comparison of pipette delivery volumes to a set range (see [6.3.3](#))
- Gravimetric checking of a selected pipettes (see [5.2](#))
- Identification of conforming pipettes by a scheme of colour-codes (see [7.2](#), [10.8](#)).
- Filing of pipette verification test result sheets in specified suite filing cabinets (QC0110 for south suite).
- Storing results on O drive in (O:\General\ISO900\Scientific\Pipette Testing\).
- Retesting on a quarter yearly testing schedule

The colormetric method for verifying pipette delivery volume has been designed to facilitate laboratory staff checking the accurate delivery volume of micropipettes

The method complements the gravimetric method for calibration but cannot achieve the narrow error limits of the gravimetric method. This broadening of error limits occurs because the assessment combines the errors of three rather than the two procedures for gravimetric assessment (ie. colormetric method errors: test pipette, reference pipette, plate reader; gravimetric method errors: test pipette, balance). Hence a result by the gravimetric method takes precedence over a result by the colormetric method.

The principle features of the method are:

- assessment of delivery volumes of 1 to 250 μl
- delivery of a volume of coloured dye into a 96 well microplate
- colormetric assessment of the coloured dye
- assessment relative to targets based on the mean colormetric determinations of at least 10 different pipettes
- the target is related to a gravimetric standard by demonstrating that one pipette at each target value conforms for gravimetric calibration

Advantages of the method are:

- rapid screening of large numbers of pipettes
- assessment delivery under real operating conditions
- assessment of delivery by staff who use the instruments
- assessment of all channels of multi-channel pipettes
- electronic transfer of data
- spread sheet for analysis of data.

Disadvantages of the method are:

- less direct than gravimetric and therefore greater associated error
- cumbersome for small numbers of pipette

1.3 Results

Data are recorded as O (O:\General\ISO900\Scientific\Pipette Testing\).
drive computer files.

The results are evaluated by the pipette calibration supervisor (currently Ross Lunt or Lynda Wright) or nominated trained staff.

Pipettes passing performance verification will be marked with a colour code (see Appendix [10.8](#)).

Pipettes failing performance verification are removed from use, decontaminated, and passed together with a copy of the results to the Electronics Technician (currently David Reid for the secure laboratories).

1.4 Definitions

Verification: This confirms that pipettes are operating within the allowable in-house limit defined by:

- $\pm 5\%$ of the target OD
- a co-efficient of variation of less than 5%.

Calibration: This establishes the performance of a pipette relative to manufacturer's specifications and in accordance with methods described in ETG-003 Gravimetric Calibration Procedure For Pipettes.

Test Pipettes: All pipettes on the equipment list (other than reference pipettes) for verification of delivery volumes of not more than 250 μ l.

1.5 References

There is no direct reference for this procedure.

2. 2 EQUIPMENT

2.1 Test pipettes

Test pipettes are any pipettes listed in the Equipment List which have not been shown to be currently out of calibration by colorimetric or gravimetric methods.

2.2 Tips which are standard for test pipette

2.4 Tips which are standard for reference pipette

2.5 Flat bottom 96 well microtitre plate with lid

2.6 ELISA plate readers, GA02756H located in 3L3137 or GA00331 in 3L3139.

2.7 Filing Cabinets

QC0110 in 3L3139 – South suite

3. 3 REAGENTS

3.1 Chemicals

3.1.1 Borate buffered saline (BBS) pH 9.0 (see [10.3 Borate buffer](#))

3.1.2 Phenol red standard solutions (see [10.4 Phenol Red Standard Solutions](#)).

The method uses a set of standardised dye solutions. These are prepared and obtainable from the pipette calibration manager for each round of testing.

4. 4 PREPARATION

4.1 Preparation of Staff

4.1.1 Staff should be:

- familiar with the correct technique for pipetting as described in the manual for operation of that pipette (located in the South Suite Reading Room)
- familiar with the correct operation of the ELISA plate reader as described in the ELISA plate protocol “Operation And Calibration Of ELISA Plate Readers”
- trained in the use of this technique and be familiar with this protocol.

4.2 Preparation of Pipettes

4.2.1 Pipettes should be clean and surface decontaminated.

4.2.2 Pipettes for use in isolation rooms can be assessed in the isolation room, so no internal decontamination is necessary.

4.2.3 Pipettes which are known to have a delivery problem need not be assessed, but should be immediately taken out of service, decontaminated, labelled and sent to the calibration laboratory for repair.

5. 5. PERFORMING THE TEST

5.1 Assay Procedure

5.1.1 The plating out of dye reagent may be carried out the laboratory in which the pipette is used by trained staff.

5.1.2 Reading of plates is carried out using the ELISA plate reader in 3L3137. Plates can be transported from the Histology laboratory or from the non secure laboratories if care is taken to prevent evaporation and spillage.

5.1.3 Commence Test Cover Sheet (see [10.1](#)).

5.1.4 Sort test pipettes based on the volume at which they will be compared to a target volume delivered by a reference pipette, for example:
10 25 40 50 100 200 250
(Strict adherence to this order is not essential, but aids orderly use of dye solutions).

For variable volume pipettes, select a single testing volume.

For example:

0.5 - 10ul	10
5 - 40ul	40
10 - 100ul	100
40 - 200ul	200

Set pipettes to deliver the selected volume.

5.1.5 Using copies of the format in [10.2](#) Pipette Record Sheet prepare a list of pipettes for testing based on the pipette identification number and delivery volume. Allow for one column per single channel pipette and one column per channel per multichannel pipette. Twelve channel pipettes should be tested on a single plate. Copies of the current pipette lists is maintained by Lynda Wright and a current list is stored on the O drive with the last pipettes tested .

5.1.6 Obtain sufficient flat-bottomed microtitre plates to complete testing.

5.1.7 Uptake and Dispensing of Phenol Red

Ensure the correct dye for the required delivery volume is selected.

Uniform and correct technique is critical to the accurate assessment of pipette performance:

- ensure the correct tip for the pipette is used. Tips should be those recommended for use with a particular pipette, or if unavailable, those normally used for operation of the pipette
- fill and dispense dye from tip
- use the “forward delivery technique”:
 - depress plunger to first stop,
 - dip the tip to a minimal extent,
 - slowly release the plunger,
 - withdraw the tip from the liquid and touch to the side of the container,
 - deliver the dye to the appropriate well by depressing the plunger past the first stop, touch the side of the well
- for single channel pipettes, pipette from a small container eg. Micronic tube, Eppendorf tube or bijou bottle
- Allow tip to enter the minimal extent into the dye to avoid excessive external carry-over
- Avoid allowing bubbles to enter pipette
- If the operator is not satisfied with the pick-up, discard the uptake from that tip
- Drain any excess by touching side of pipette to rim of dye container
- Pipette the volume to plate.
- Repeat delivery over eight wells.

5.1.8 For each pipette, according to the Pipette Record Sheet, add dye to wells of the plate as described in 5.1.7.

5.1.9 Cover plate with lid.

5.1.10 Take plates to Calibration Supervisor.

5.1.11 Add 50µL of BB + 0.5% Tween 20 to all wells for delivery volumes of 50µl or less, using a multidropper **784 (QC0441)** supplied by the Calibration Supervisor.

5.1.12 Use plate shaker to ensure mixing of dye and buffer and regular meniscus formation.

5.2 We do not do this

6. 6. READING AND INTERPRETATION OF RESULTS.

6.1 Reading

6.1.1 Plates should be read within 2 hours of setup. (Note: If sealed in bags, plates will give similar readings after 24 hours).

6.1.2 If the plate reader has been turned off, turn the instrument on, reset measurement parameters and allow 10 minutes before reading the plate.

6.1.3 Check plates for obvious discrepant wells, eg marked differences between duplicates or colour in blanking wells. Check also for plate well deformities. Record any discrepancies on work sheet.

6.1.4 Plates are read at 540 nm (eg filter #5 on the Flow Multiskan).

6.1.5 Run ELISA program (see Plate Reader Protocol)

6.1.6 Set the plate reader to retain blank, then blank on air.

6.1.7 Proceed to read plates. Remove lid and read plate to file 3 times. There is no need for a hard copy at this stage.

6.1.8 If there is more than one plate, read plates in numbered order.

6.2 Acceptance of assay

6.2.1 The test is valid if the reference target OD values (see [6.3.3](#)) are in the range to 1.90 to 2.10.

6.2.2 If the assay run does not pass control checks, results should be scrutinised by the laboratory supervisor and the test re-run in part or in full.

6.2.3 Record if assay has passed or failed control checks and any associated comments.

6.3 Data Analysis and Interpretation

6.3.1 Data analysis will be performed by Ross Lunt or nominated trained staff. Results are imported into a sheet (O:\general\iso9000\calibration procedures\scientific procedures\pipette colormetric calculations.xls) which calculates:

- the mean of the three OD readings made for each well
- the mean OD for the set of 8 deliveries by any one pipette or pipette channel
- the coefficient of variation for the 8 readings
- a listing of results relative to acceptable range values.

6.3.2 Importing results

Run Microsoft Excel.

Open the plate reading files as spread sheets:

- click on open
- add the file name as "filename".dat and click on OK
- use the text import wizard and "fixed width files".
- click on next and ensure that the columns of data are correctly delimited (move all separating lines to the left digit in each column) and add a separating line between the first column of text (the A to H plate labels)
- click on finish

- press Ctrl A
- press Ctrl C
- go to the "Results" sheet of the "Calculations" work book
- Go to cell A1
- press Shift Insert
- save the calculations sheet on a H drive file for that round of testing.

6.3.3 Determination of reference targets.

Determine the mean OD for each dye solution using the results of **at least 10 different pipettes** set to deliver at that volume. Do not include data from pipettes that have co-efficients of variation > 5%. Use these values as the target means for the evaluation of test pipettes.

6.3.4 Printing result sheets

Print result sheets

- Determine if any test pipettes are associated with OD values outside the target range.
- Determine if any test pipettes are associated with Coefficient of Variation values of more than 5%

6.4 Assessment

Delete as appropriate "Pass" or "Fail" in the Assessment box on the completed pipette record sheet (see [5.1.5](#)) for each pipette or pipette channel.

6.5 Confirmation of Gravimetric standard.

All pipettes used as gravimetric standards must pass gravimetric calibration.

7. 7. THE PIPETTE VERIFICATION PROCEDURE

7.1 Responsibilities of Laboratory Staff

7.1.1 Always use pipettes with a current sticker.

7.1.2 Remove from use any pipette identified as having defective performance, remove any current sticker, label the pipette "defective" and contact the Calibration Manager.

7.1.3 Ensure that any pipettes for servicing have been appropriately decontaminated.

7.1.4 At the end of the accredited period (every three months), check the delivery volume by the colorimetric of all pipettes for which responsibility is held.

7.1.5 Remove and replace stickers for pipettes with verified performance according to a list of pipettes that have been certified.

7.2 The Schedule for Testing Pipettes

7.2.1 The verification of pipettes remains current for three months, only if the pipette has been verified at the set time (see [7.3.1](#)). There is a maximum extension period of two weeks.

7.2.2 Certification expires on the following dates: 30-Jan, 30-April, 30-July and 30 October.

7.2.3 The verification testing will be conducted in the last two weeks of the current verification period.

7.3 Colour Coding

7.3.1 Pipettes are recognised as currently verified for delivery volume based on the following cycle of colour sticker replacement:

Sticker Colour	Current for
RED	1 st of February to 30 th of April
BLUE	1 st of May to 31 st July
WHITE	1 st of August to 31 st of October
YELLOW	1 st of November to 31 st of January

7.3.2 This schedule is to be posted in each laboratory with registered pipettes (see Appendix [10.8](#)).

7.4 Recognition of Pipettes with Expired Certification.

7.4.1 A pipette for which the period of certification has expired (without extension) will be recognised by having a colour sticker that is not current.

7.4.2 A pipette for which the period of certification has expired will not have a current certificate on file in the South Suite Reading Room.

7.4.3 The pipette will also be identified in the spreadsheet “pipette register.doc” in the column “Status” as “DUE”.

7.4.4 A “Defective Performance” tag may be attached to a pipette at any time and supersedes any current certification.

7.5 Certification of Pipettes Outside Normal Schedule of Colormetric Testing.

- 7.5.1 Testing to certify pipettes outside of the normal schedule for colormetric testing may be undertaken by gravimetric methods at any time.
- 7.5.2 Gravimetric Calibration and Certification may be undertaken at any time by Calibration Laboratory staff. After calibration:
- the pipette is given a current colour code which will expire in the normal schedule.
 - a certificate of calibration will accompany the pipette on return to the laboratory. This certificate will be filed appropriately in the South Suite Reading Room.
- 7.5.3 Gravimetric testing for verification of performance may be undertaken at any time by trained laboratory staff. After testing:
- results are communicated to the Pipette Manager
 - if the pipette conforms, it is given a current colour code which will expire in the normal schedule.
 - the spreadsheet "pipette register.doc" pipette must be updated by Pipette Manager
 - a certificate of performance verification will be generated and filed appropriately in the South Suite Reading Room.

7.6 Action for conformities

- 7.6.1 The performance of pipettes which conforms to set standards is recognised by the presence of a colour coded sticker applied to all pipettes which have passed a gravimetric or colormetric verification process and the absence of a tag indicating defective performance. There should also be documented confirmation that the pipette has been certified.
- 7.6.2 A currently certified pipette may be used appropriately and in accordance with any test protocol.

7.7 Action for non-conformities

- 7.7.1 When a pipette fails either accuracy or reproducibility, examine data for apparent correctness and any out-lying results that may have caused the failure.
- 7.7.2 If it appears that one or two out-lying results have caused failure, retest with careful operation. Another delivery volume may be selected. Training of operator should be considered.
- 7.7.3 When a pipette passes reproducibility but fails accuracy there may be a significant error in delivery volume that would require the pipette to be re-calibrated. The pipette should be re-tested using the gravimetric or colormetric procedure to confirm the intial results. If the pipette fails

on colormetric recheck, a gravimetric check may additionally be carried out by laboratory staff (see <\\VADER\CORPDIR\GENERAL\iso9000\Calibration Procedures\ETG Procedures\ETG-003 Gravimetric Pipette Calibration.doc>). An acceptable gravimetric result should take precedence over the colormetric results (as explained in [1.2](#)).

- 7.7.4 When a pipette fails both reproducibility testing independently of operator action, there may be a fault in regular volume delivery and pipette will need servicing and recalibration.
- 7.7.5 Any pipettes failing the accuracy and /or the reproducibility test should be removed from use by labeling “Defective Performance”. Notify the Calibration Manager.
- 7.7.6 If it is possible to service the pipette in the Suite, it is the responsibility of the reporting staff to ensure that the pipette has been appropriately decontaminated before servicing is undertaken.
- 7.7.6 If a “Defective Performance” pipette must be removed from an isolation room or laboratory suite, the pipette must be decontaminated in accordance with Microbiological Security Manual specifications (see Section 3 Appendix 3(ii) page 3.21).
- 7.7.7 Each “Defective Performance” pipette will be identified by type and number on Form [10.7](#) which will be signed by the person who has decontaminated the pipette.
- 7.7.8 The pipette, together with Form 10.7 and a copy of the relevant testing details and should be passed to the Calibration Manager.

8. 8. MAINTENANCE OF RECORDS

8.1 Test data and verification records

8.1.1 A record will be held for each round of testing.

This will consist of

- listings of pipettes and summary colormetric test results
- a record of establishment of target values at each level
- a record of any gravimetric calibrations performed,

Colormetric test data and analysis records are maintained by the Suite Calibration Supervisor on the O Drive (O:\General\ISO900\Scientific\Pipette Testing\).

9. 9. QUALITY ASSURANCE

- 9.1 The colormetric method for confirming pipette delivery volume can only be carried out on an appropriately certified ELISA plate reader.

10. 10. APPENDIX

Pipette Verification: Colormetric Method

Plate Number

Date

LAB #

Operator

Plate Column	Delivery Volume	Pipette Number and Identification								Assessment (Delete as appropriate)	Comment
		A	B	C	D	E	F	G	H		
1										Pass / Fail	
2										Pass / Fail	
3										Pass / Fail	
4										Pass / Fail	
5										Pass / Fail	
6										Pass / Fail	
7										Pass / Fail	
8										Pass / Fail	
9										Pass / Fail	
10										Pass / Fail	
11										Pass / Fail	
12										Pass / Fail	

Plate Number

Plate Column	Delivery Volume	Pipette Number and Identification								Assessment (Delete as appropriate)	Comment
		A	B	C	D	E	F	G	H		
1										Pass / Fail	
2										Pass / Fail	
3										Pass / Fail	
4										Pass / Fail	
5										Pass / Fail	
6										Pass / Fail	
7										Pass / Fail	
8										Pass / Fail	
9										Pass / Fail	
10										Pass / Fail	

10.3 Borate buffer (BB)

Boric acid	9.0 g
Sodium hydroxide	2.0 g

Dissolve in DD water to 1 litre.
Check pH which should be 8.5-8.8. Adjust if necessary.

Borate buffer + 0.05% Tween 20

Add 0.5ml Tween 20 per liter of borate buffer.
Mix to disperse,

10.4 Phenol Red Standard Solutions

Phenol red has been selected as a useful dye because of stability, low toxicity, solubility and high absorbance levels. However a high pH buffer, is required to stabilise against pH induced colour change.

Diluent

Borate buffer

Stock phenol red solution:

0.1 g/mL phenol red (water soluble eg. Sigma cat# P2417) in BBS.

The following dilutions have been selected to achieve a target OD of around 2.00 ± 0.05 . (Note: 50µl BBS + 0.5% Tween 20 is added per well to the plate where the delivery volume is 50µl or less).

Pipette Volume	Parts of stock	Parts BBS
1 µL	1	
10 µL	1	88
25 µL	1	250
40 µL	1	374
50µL	1	508
100 µL	1	1040
200 µL	1	2200
250 µL	1	3000

BBS or dye is added to stock solutions if the dilution suggested above does not yield OD values within the specified range.

10.5 The Spreadsheet Calculations

The Excel workbooks have been written to calculate the following values:

$$\overline{OD} = \frac{\sum OD_i}{n}$$

10.5.1 The sample mean OD based on n delivery volumes:

Where the OD represents a blank adjusted value, i is the individual observation and n is the number of observations. Calculated for the reference pipette based on four observations, and for the test pipette based on 10 observations.

$$s = \sqrt{\sum_{i=1}^n \frac{(OD_i - \overline{OD})^2}{(n-1)}}$$

10.5.2 Sample standard deviation (s)

Calculated for the reference pipette based on four observations, and for the test pipette based on 10 observations.

10.5.3 Coefficient of Variation (CV)

$$CV = \frac{s \times 100}{\overline{OD}}$$

Calculated for the reference and test pipettes and is used as a measure of the reproducibility of the set of observations, independent of the target or reference OD. CV levels of 5% are taken as the maximum acceptable value and the report indicates PASS or FAIL dependent on this level.

REQUEST FOR RECALIBRATION OF PIPETTES AND RECORD OF DECONTAMINATION

*This form is for pipettes that have failed performance verification testing.
 The form is to confirm that pipettes have been decontaminated according to the
 Microbiological security Guidelines (See Section 3 Appendix 3(ii) page 3.21).*

*The completed form must accompany all pipettes sent to the Calibration Laboratory, or
 pipettes will not be repaired.*

Item	Pipette	Number	Comments	Decontamination		Cost Code
				Date	Signed	

Delivered to Calibration Laboratory

By

Date

10.8 Recognition of Pipettes Currently Verified For Use.

To Laboratory Supervisors and Staff: Place the schedule for pipette testing in a prominent position in the area(s) in which pipettes are used. Verification testing of all pipettes registered for use should commence in the final two weeks of the accredited period.

Recognition of Pipettes Currently Verified For Use.

Sticker Colour	Current for
Red	1 st of February to 30 th of April
Blue	1 st of May to 31 st July
White	1 st of August to 31 st of October
Yellow	1 st of November to 31 st of January

Note: Pipettes with a "G" on the current sticker have been verified by gravimetric assessment of delivery volume. All others with a current sticker have been verified colorimetrically.

11.

12. CHECKING YOUR PIPETTES

1. Obtain phenol red solutions and borate buffer from Ross/Lynda.
2. Sort pipettes according to delivery volume (useful but not essential).

For variable volume pipettes, select a single testing volume.

For example:

Pipette	Test at:
0.5 – 10 μ l	10
5 – 40 or 50 μ l	40
10 – 100 μ l	100
40 – 200 μ l	200

Set pipettes to deliver the selected volume.

3. Complete “Pipette Record Sheets”.

Each single channel pipette is tested in eight wells. Multi-channel pipettes have one channel per plate column. A 12-channel pipette will take up a whole plate.

4. Pipette dye into plate.

NB:

A) Check pipette operation. If the pipette is leaking or otherwise requires servicing do not attempt to validate the pipette delivery volume.

B) Pipette with care, ensuring complete delivery of the dye.

C) Ensure that the correct dye is used for the selected delivery volume.

5. Take plates and sheets to pipette testing laboratory.

6. For delivered dye volumes of 50 μ l or less, add 50 μ L of BB + 0.05% Tween 20 to all wells using multidropper supplied by the Calibration Supervisor.

7. Give plates and sheets to Ross/Lynda to read.

Or read plates at 540 nm using retained blank (mean column blank on empty wells). Save results on your H:\ drive and then forward file to Ross/Lynda. Select the no print option. Read each plate three times. Give sheets to Ross/Lynda to assess results.

RESULTS

13. RULES FOR ACCEPTANCE.

1. Mean OD falls within 5% low and high range set by target.
2. Coefficient of Variation is less than 5%.

Checking Results

Check the print out of your results and mark "Pipette Record Sheet" as "Pass" or "Fail".

Retest any failures. A gravimetric or colormetric check may be performed. See Ross for details.

Replace sticker on "Pass" pipettes.

Failed pipettes should be decontaminated, labelled and passed to David Reid.

14. END