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Application Note

Application topics:

- (1) Nucleic acid extraction and enrichment
- (2) Standard, *rapid* and real-time PCR
- (3) Kits and reagents
- (4) Automated liquid handling
- (5) Molecular, cellular and in-vitro assays
- (6) Sample preparation and BioBanking
- (7) Protein research
- (8) Homogenization
- (9) Spectrophotometry
- (10) BioImaging
- (11) Electrophoresis and blotting
- (12) Others

CyBio PRODUCT LINE

Photometric and Gravimetric Liquid Handling Check Procedure to determine the Random Error (Precision) and the Systematic Error (Accuracy) of Automated Liquid Handling Systems (ALHS)

Content

1. Summary of the Method Principle

The Method describes a reliable liquid handling check procedure for automated liquid handling systems (ALHS). In a first step the random error (precision) is determined by an absorption measurement in microplates using p-Nitrophenol (synonym = 4-Nitrophenol, abbr. = p-NP). This dye is stable at room temperature and soluble in water (11.6 mg/ml at 20 °C), chloroform, methanol, ethanol and DMSO (100 mg/ml at 20 °C). It has the absorption maximum at 405 nm and a pH > 9.2 which is realized by using 0.1 N NaOH as well as standard solvent and diluent.
The coefficient of variation (CV in %) is calculated from the absorption measurement signals of individual microplate wells. Smaller test volumes are transferred in wells forwarded with 0.1 N NaOH, where they have to be dispersed homogeneously before measurement. The dye concentrations of the different test solutions are specifically adapted to the test volumes to give always a microplate type specific constant final volume and a constant final dye concentration of 120 µM in all wells of the measurement plate, which is within the optimal dynamic range of the absorption reader. The random error (precision) is determined always at first in the evaluation of an automated multichannel pipetting system followed by a gravimetric determination of the systematic error (accuracy). The microplate absorption reader and the analytical balance have to be calibrated at regular intervals and the test conditions have to be considered strictly.

2. Method details

2.1 Test Equipment

- Photometer for 96- and 384-well microplates with 405 nm and 620 nm option, e. g. Tecan GENios Plus (resolution 0,0001 OD, linearity 0 – 3 OD ± 1,5% and 0.005 OD, precision 0 – 3.0 OD ± 1,0% and 0.005 OD, accuracy 0 – 2.0 OD ± 1,0% and 0.01 OD), scheduled service and manufacturer calibration is needed
- Calibrated analytical balance, e. g. Mettler Toledo AG245 (Capacity 41g – 210g, readability 0.1 – 0.01 mg, repeatability 0.1 – 0.02 mg, linearity ± 0.2 mg), scheduled service and authorized certified calibration is needed
- Calibrated manual pipettes to prepare the test solutions
- High quality transparent flat bottom polystyrol 96- and 384-well microplates e. g. Greiner Bio-one # 655 010 and # 781 101, respectively, vacuum packed microplates have to be unpacked 1 week before the measurement
- High quality microplate sealing tape, e. g. Nunc # 236 269
- Microplate shaker, e. g. BioShake iQ
- Microplate centrifuge, e. g. SIGMA 6K 15

2.2 Test Reagents

- p-Nitrophenol, MW 139.11 g/mol, spectrophotometric grade,
- Sodium hydroxide (NaOH) pellets, MW 40.00 g/mol, p.a., for preparation of 0.1 N NaOH as solvent and diluent, density = 1.004 g/cm^3 at 20 °C
- DI water (purity type II, conductivity < 1 µS/cm), density = 0.998 g/cm^3 at 20 °C
- Optional DMSO dried, purity ≥ 99.9 %, density = 1.10 g/cm^3 at 20 °C

The final p-NP concentration in the wells of the measurement plate should be 120 µM to result in an OD value of about 1, which is within the optimal dynamic range. In Tab. 1 the corresponding optimal p-NP concentrations for the different test volumes in both microplate formats 96 and 384 are summarized.

tests in **96-well** MTPs with 200 µl final volume/well		
test volume [µl]	forward volume [0.1 N NaOH in µl/well]	test concentration [p-NP in mM]
200.00		0.12
100.00	100.00	0.24
50.00	150.00	0.48
25.00	175.00	0.96
10.00	190.00	2.40
5.00	195.00	4.80
2.00	198.00	12.00
1.00	199.00	24.00
0.50	199.50	48.00
0.25	199.75	96.00
0.20	199.80	120.00
0.10	199.90	240.00

tests in **384-well** MTPs with 50 µl final volume/well		
test volume [µl]	forward volume [0.1 N NaOH in µl/well]	test concentration [p-NP in mM]
50.00	0.00	0.12
25.00	25.00	0.24
10.00	40.00	0.60
5.00	45.00	1.20
2.00	48.00	3.00
1.00	49.00	6.00
0.50	49.50	12.00
0.25	49.75	24.00
0.20	49.80	30.00
0.10	49.90	60.00

Tab. 1: Optimal p-NP test concentrations for different test volumes in the microplate formats 96 and 384.

The different p-NP test concentrations are prepared from a stock solution. All solutions have to be filtrated before use. 0.1 N NaOH is used as diluent as well as standard solvent to prepare the test solutions for the liquid handling check procedure, other solvents to prepare the test solutions are possible. According all our experiences the liquid handling performance for higher test volumes is comparable or better.

2.3 Test Conditions

The tests have to be performed in an air conditioned room or at room temperature at constant environmental conditions (temperature variation < ± 2°C, air humidity variation < ± 10%). All test equipment, disposables and all reagents have to be in equilibrium with these environmental conditions for at least 1 hour. Temperature and air humidity at the beginning and the end of the tests have to be documented in the test report. The analytical balance has to be placed nearby the liquid handling device under evaluation to limit evaporation.

2.4 Test Execution

The test results have to be calculated from at least 3 parallel measurements per test volume.
The tests have to be performed at least with the smallest specified volume and one or even more higher volumes.

2.4.1 Photometric Determination of the Random Error (Precision)

The precision as described here defines the variation of the transferred liquid volume between the different wells of a microplate. The measurement plates have to be prefilled with the forward volume (see Tab. 1) considering the test volume and the microplate format. The pipetting methods have to be set-up in the software of the liquid handling device with the following rules and parameter settings:

- One set of new tips per volume and pipetting mode,
- Reduce piston speed to default speed/3 *
- Reduce vertical speed for moving tips out of the liquid to default speed/3 **
- Prime tips at least 5 x with the maximum tip volume
- Implement a break of 1s after every aspiration and dispensing step
- Immersion depth of the tips in the liquid 1 mm – 2 mm
- Reverse pipetting *** of the test volume with an additional aspiration volume, the remaining volume after the test volume transfer should be > 10 µl
- For test volumes < 10% of the maximum pipetting volume the first test volume should be dispensed back in the test solution reservoir followed by transfer of the test volume in the measurement plate
- Ejecting the residual volume with maximum blow-out volume back into the source reservoir or waste, move the tips out of the liquid and set pistons back to start position (zero)
- Immediate sealing of the measurement plate
- Shaking of the plates for at least 10 min (careful acceleration and microplate format adapted final speed to avoid splashing)
- Centrifugation of the measurement plates to remove bubbles and to align meniscus (e. g. 2 min at 2000 rpm)
- Read-out not earlier than 1 h after finishing the pipetting procedure
- Daily quick cross-check of the absorption reader performance by turning a test plate through 180° and comparison of the results (additionally to scheduled service and manufacturer calibration) to exclude trending or patterns caused by the reader

* default piston speed = fill or empty a tip completely in 2 s

** default vertical speed = 140 mm/s

*** revers pipetting = pipetting mode in which excess volume is aspirated and remains in tip after delivery, piston cycle has to be finished with blowout and setting pistons back to zero position

2.4.2 Gravimetric Determination of the Systematic Error (Accuracy)

The accuracy as described here defines the agreement between the mean transferred volume in the corresponding destination wells of a microplate and the volume setting per well in the pipetting method. The variation of the accuracy from well to well is defined by the random error of the pipetting device, which should be checked at first and has to be in the specified range.

Immediately after the prefilling of the measurement plates with the forward volume (see Tab. 1) the tare weight has to be read after the stabilization of the balance. Then the test solution has to be transferred as described in 2.4.1 and the microplate weight has to be read again immediately. The time interval from weighing before and after the test solution transfer should not be greater than 15s to limit the evaporation to be less than 1‰ of the test volume, otherwise the evaporation has to be measured at the specific environmental conditions and the calculation has to be corrected respectively.

It is possible to combine the determination of the systematic error with the determination of the random error via weighing the measurement plates before and after the transfer of the test solution. Here the specific density of the test solution (see point 2.2) has to be considered in the calculation of the accuracy (see point 2.5) and the results are only acceptable if the random error is in the
specified range.

2.5 Test Evaluation

The random error (precision) is calculated as relative coefficient of variation (CV in %) according to:

$CV = \frac{\sigma}{S_m} 100\,(\%)$ CV = coefficient of variation, σ = standard deviation, $\sigma = \sqrt{\frac{\sum_{n=1}^{n=N} (S_n - S_m)^2}{N-1}}$

S_m = mean OD signal, $S_m = \frac{\sum_{n=1}^{n=N} S_n}{N}$, S_n = OD signal of a single well,

N = number of wells

The systematic error (accuracy) is calculated as follows:

$$Accuracy = \frac{V_m - V_{adj.}}{V_{adj.}} 100\,\%$$

V_{adj} = volume per well adjusted in the method

V_m = volume per well determined by weighing the microplate and calculated according to $V_m = \frac{m_{plate}^{disp.} - m_{plate}^{0}}{N \rho_{liquid}(T)}$

m_{plate}^{0} = weight of the microplate before the test solution transfer

$m_{plate}^{disp.}$ = weight of the microplate after the test solution transfer

$\rho_{liquid}(T)$ = density at 20°C, N = number of wells,

2.6 Test Report

The test report has to include at least the following details:

- Title
- Name and serial number of the liquid handling device under evaluation
- Software and Firmware version of the tested liquid handling device (optional)
- Tip type, order number
- Test environment (laboratory, temperature and humidity before and after the test)
- Reader / Balance name, serial number and settings, date of the last calibration
- Test volume and test solution (p-NP concentration solved in 0,1 N NaOH or another solvent)
- Test method with liquid handling parameters (e.g. pre-wetting of tips, forward pipetting or reverse pipetting, single or multi-dispensing, forward volume, additional aspiration volume, piston speed, breaks, vertical speed, method references…)
- Test results, test criteria and evaluation of the results
- Comments (optional)
- Test date, name of test operator
- Name, function and signature of authorized person

2.7 Possible Error sources

- Bubbles or uneven liquid surfaces in the wells
- Splashing due to heavy shaking or inattentive removal of the sealing tape
- Inhomogeneous distribution of the test solution in the buffer, e. g. because of too short shaking or waiting time
- Incomplete or incorrect liquid handling parameter settings
- Irregularities in the measurement plates
- Evaporation
- Incorrect or instable environmental conditions

2.8 Method Traceability

The traceability of the gravimetric method to SI units is achieved through frequent calibration of the balance with certified standards by authorized institutions. The date of the last calibration has to be documented in the test report.

2.9 Method Uncertainty

The uncertainty of the photometric and gravimetric method was determined according to EN ISO 8655-6:2002.
To determine the uncertainty of the photometric method a 96-well microplate was filled homogeneously with 200 µl 120 µM p-NP solution in 0.1 N NaOH per well. The microplate was centrifuged to align the meniscus and then the absorbance at 405 nm was read 10 times immediately under defined environmental conditions (see point 2.3). To evaluate the uncertainty of the method the standard deviation of the 10 OD values per well was calculated well-specific and resulted in an average variation of ± 0.0014 OD, which is within the manufacturer´s specification (precision 0 – 3.0 OD ± 1.0% and 0.005 OD, see point 2.1).

The same test was repeated with a 384-well microplate filled homogeneously with 50 µl 120 µM p-NP solution in 0.1 N NaOH per well. The average variation here was ± 0,002 OD and thus also within the manufacturer´s specification.

The uncertainty of the photometric method is independent from the test volume because all volumes are tested with a microplate type specific constant final measurement volume and a constant p-NP concentration of 120 µM.

The uncertainty of the gravimetric method was determined by 10 x weighing of a sealed test microplate and calculating the standard variation of the different results. The repeatability was within the manufacturer specification (0.1 – 0.02 mg, see point 2.1) resulting in an uncertainty of the gravimetric method of < 1 % for the volume range 0.1 µl – 2 µl and < 0.1 % for > 2 µl – 200 µl.

2.10 References

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