Turbo-Mixing in Microplates

H. Rhode[1], M. Schulze[1], E. Mitre[1], G.A. Cumme[1], T. Rausch[2], D. Philipp[2], A. Horn[1†]
[1] Institute of Biochemistry, Klinikum, FSU, Jena [2] CyBio-AG, Jena

INTRODUCTION

Currently microplate technology has occupied high-throughput analysis including rapid reactions. However, sufficiently rapid mixing still represents an unsolved and/or neglected problem. **Actual mixing methods in microplates***: Ultasound*: Mixes quickly, contamination by coupling source to fluid; *Repeated aspiration*: Hampered by adhesive and cohesive forces, causes contamination; *Shaking*: Hampered by adhesive and cohesive forces; *Diffusion*: Ineffective with 0.1 to 1 cm distances. **Method introduced here: mixing by Marangoni convection** (Walters D.A. Langmuir 6 (1990) 991-4) :
Principle: A solvent drop placed onto a watery surface causes violent mixing due to the strongly different surface tensions; *Implementation*: Pipetting the solutions to be mixed, thereafter spotting organic solvent drops to the liquid surfaces.

SUMMARY

- Mixing by microplate shaker: ineffective with 384- and 1536-well microplates, mixing in 96-well plates takes at least 5 min
- **Marangoni convection** caused by solvent spotting reduces mixing time to about **1 sec** in 96- and 384-well microplates
- Amount of organic solvent needed: small enough not to alter most bioactive molecules
- Temperature increase: small, negligible for enzyme activity activity determination
- Rapid mixing increases performance speed, monitoring of initial events and determination of initial velocities with microplates

RESULTS

Mixing times :

Microplate format	96	384	1536
spotted volume	5 µl	3 µl	0.5 µl
layered liquid volume	170 µl	55 µl	8.0 µl
Monitored:	O	O	FI

Fluid spotted	Mixing time (min)		
buffer (♥ diffusion)	51.6 ∂9.7	43.0 ∂ 3.3	18.5 ∂3.4
ethanol	< 0.5	< 0.5	11.9 ∂ 3.0
methanol	< 0.5	< 0.5	11.9 ∂ 1.6
propanol	< 0.5	< 0.5	11.9 ∂ 3.0
acetonitril	< 0.5	< 0.5	12.6 ∂ 2.4
acetone	< 0.5	< 0.5	12.5 ∂ 3.3
Shaking only	5-15	no acceleration effect	

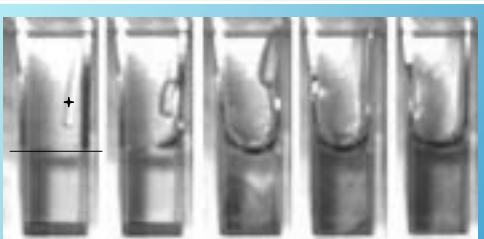
Mixing time: time elapsed until CV(Q) or CV(FI) fell below $1.05\Delta CV_{mix}$ for the first time (cf. Photometric monitoring of mixing).
Mixing time entry: mean value ∂ SD of four experiments. < 0.5 min, mixing was complete with first measurement. Mixing times below one minute cannot be determined exactly due to the time required for measurement at all wells.

Minimum spotting volumes for mixing within < 0.5 min:

Microplate format	96	384	
Fluid spotted			
ethanol	0.70 ∂ 0.0	0.50 ∂ 0	µl
methanol	0.90 ∂ 0.14	0.83 ∂ 0.24	
propanol	0.48 ∂ 0.18	0.25 ∂ 0	
acetonitril	1.00 ∂ 0	0.83 ∂ 0.24	
acetone	0.63 ∂ 0.09	0.67 ∂ 0.24	

Mixing is complete before first reading, only Ω1 µl of solvent is needed.

Camera-monitoring of mixing



sec 0.15 0.25 0.55 1.16

Mixing is complete within about 1 sec!

4 µl of cresol red (0.15 M Tris/HCl, pH 7.6), was placed beneath 50 µl of deionized water. Numbers: time elapsed since spotting 3 µl of ethanol to the liquid surface. Horizontal line: meniscus position prior to solvent addition.
+ , pipette tip

Photometric monitoring of mixing

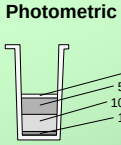
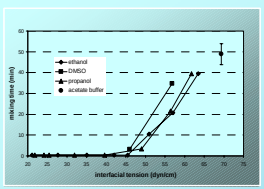


Plate format 96	384	1536	pH	buffer	dyes
5µl	5µl	2µl	4.0	acetate 10mM	p-NP(96, 384)/MU(1536) + F
50µl	20µl	5µl	4.0	acetate 10mM	F
100µl	25µl	0µl	7.0	deionized water	F
15µl	5µl	1µl	11.0	Na-Pi 0.5M , sucrose	F

p-NP, p-nitrophenol; MU, methylumbelliferone; F, fluorescein

Principle: p-NP light absorbance (400 nm) and MU fluorescence intensity (460 nm, ex 365 nm) increase with pH upon mixing + Calculating quotients, Q, of absorbances of p-NP and fluorescein (492 nm) eliminates light path differences between wells + Change of Q or fluorescence intensity, FI, (mean or CV over the plate) indicates progress of mixing + Constant maximum mean, M_{mix}, and minimum CV, CV_{mix}, of Q or FI as observed after 1h are taken as indicative of complete mixing

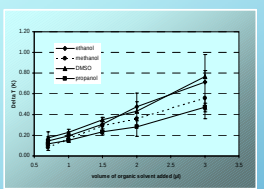
Dependence of mixing time on surface tension of fluid spotted



Fluid mixtures differing in surface tension were prepared from solvents and acetate buffer, surface tensions were examined by a Drop Shape Analysis System (KRÜSS, Hamburg, Germany). 5 µl of the mixtures were spotted onto the surfaces of 50 µl of layered liquids (see photometric monitoring) in 384-well microplates.

All mixtures exhibiting surface tensions below 40 dyn/cm caused immediate mixing (surface tension of upmost liquid layer, 67.2 dyn/cm). Concentrations of propanol, ethanol, and DMSO required for immediate mixing are 20%, 50%, and 100% v/v, respectively.

Temperature effects of mixing by solvent spotting



96-well microplate:

Transient temperature increase after spotting solvents onto 150 µl of deionized water. With 386-well microplates, 1.5- to 3- fold increase was caused by spotting the same volumina (not shown).

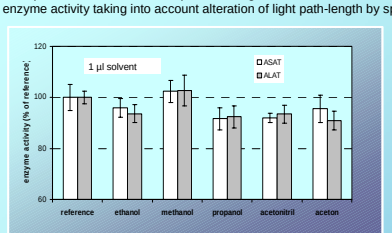
Temperatures were measured by chromel/alumel thermocouple (0.04 mm wires).

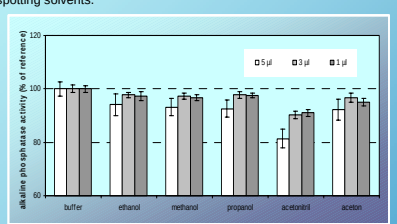
Effect of transient temperature increase on enzyme activity:
Spotting minimum solvent volumes as required for rapid mixing caused 0.2 and 0.5 K increase, ÷T, in 96- and 384-well plates, respectively. Temperature restoration took no more than 1 min. Assuming exponential decay of ÷T to 5% of maximum within 1 min and double enzyme activity at 10 K temperature increase, 1.1% and 0.6% increase of turnover would result after 1 and 2 min, respectively, with 384-well plates; and 0.4% and 0.2% with 96-well plates.

APPLICATIONS

1) Enzyme activity determination

After pipetting enzyme solution and assay mixture into the wells of a 96-well microplate, solvents or reference (buffer) was spotted onto the liquid surfaces. After subsequent shaking for 5min, absorbance was recorded. Increase of absorbance with time was converted into enzyme activity taking into account alteration of light path-length by spotting solvents.





ASAT / ALAT, aspartate / alanine aminotransferase
30 µl of 0.045 IU/ml enzyme solution (Peripath U Serum containing both enzymes, Roche Diagnostics GmbH)
+ 150 µl of respective standard assay (Human GmbH); 25 °C; NADH absorbance at 340 nm

AP, alkaline phosphatase
10 µl of 0.5 IU/ml enzyme solution (purified according to B. Schwenzer et al., Biomed. Biochim. Acta 46 (1987), 15-21)
+ 130 µl of 5 mM p-NP phosphate; pH 9.8; 22 °C;
p-NP absorbance at 400nm

Activities of ASAT and ALAT were not affected significantly by spotting 1µl of methanol, AP not by up to 3 µl of methanol, ethanol, propanol, or acetone.

2 Liquid handler evaluation

Principle: + Artificial samples containing an indicator dye are pipetted into wells pre-filled with dilution fluid + Mixing by shaking or by spotting ethanol + Absorbance, A1, of indicator dye reflects volume pipetted + Absorbance, A2, of second dye contained in both sample and dilution fluid is used to calculate A1/A2 which is free from path length variability + From CV(A1/A2) over all microplate wells, CV of sample volumes is estimated (H. Rhode et al., J. Biomol. Sceening, in press)

Identical results obtained with mixing by solvent spotting and by shaking:

6 groups of 384-well microplates loaded with 48 µl dilution fluid per well and 2 µl of indicator solution, mixing by spotting 2 µl of ethanol or by shaking for 60 min, Mean CV(A1/A2) determined for shaking and for spotting.
CV(spotting)/CV(shaking) = 0.92 ± 0.12. Plate means of A1/A2 determined for checking possible disturbing effects of ethanol: **Mean(spotting)/Mean(shaking) = 1.009 ± 0.011.**

Materials and Devices
Microplates were from Greiner bio-one (Frickenhausen, Germany). Fluorescence in 1536-well microplates was measured with SpectraFluor Plus reader from Tecan (Maennedorf, Switzerland). Absorbance reader Spectramax Plus[384] and fluorescence reader Spectramax Gemini from Molecular Devices (Sunnvale, CA, USA) were used for measurements in 96-well and 384-well microplates. Microplate shaker Titramax 100 was from Heidolph Instruments (Schwabach, Germany).
Liquid-handling systems CyBi™-Well (96 channels, maximum 250 µl each; or 384 channels, maximum 25 µl each) were from CyBio AG (Jena, Germany). The fast camera HCC-1000 from VDS Vosskühler GmbH (Osnabrück, Germany) was applied for vizualization of the mixing process.