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With 1 *plate and* 2 *text-figures*
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A semi-automatic technique for conducting many bacterial matings concurrently*

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SUMMARY

A technique using microserological equipment is described whereby up to 144 quantitative interrupted bacterial matings may be performed concurrently. The technique is easy to use, accurate and reproducible, and eliminates the need for agar plates. However, it is only suitable for 40–42 °C matings and mating efficiency is approximately threefold lower than in test tubes.

The replica-plating technique (Lederberg & Lederberg, 1952) may be used to estimate the efficiencies of large numbers of matings but will only give semi-quantitative results. Furthermore it does not allow matings to be efficiently interrupted. Matings in liquid media (liquid matings) yield quantitative results and may be interrupted by blending (Wollman & Jacob, 1955; Low & Wood, 1965) or by treatment with T6 phage (Hayes, 1957). However, it is inconvenient to perform large numbers of such matings as they are laborious, time-consuming, and expensive. A genetic analysis described elsewhere (Achtman, Willetts & Clark, manuscripts submitted to *J. Bacteriol.*, and in preparation) necessitated thousands of quantitative interrupted matings. For that analysis I developed a semi-automatic version of the liquid mating technique. It should prove useful in other situations as it enables the concurrent performance of up to 144 quantitative interrupted matings. In place of test tubes, pipettes, and agar plates, this semi-automatic version uses 'Microtiter' microserological equipment, which is commercially available.‡ Mating, T6 interruption, dilution, and plating for recombinant colonies are all performed in small volumes in the rows of wells of a Microtiter plate (Plate 1) (see below). Dilutions are made by means of microdiluters fitted into a Microtiter handle (here called a microdiluter set and shown in Text-fig. 2). These dilutions are performed 8 or 12 at a time between one row of wells and the next (e.g. between row 2 and row 3 or between row G and row F of the plate shown in Plate 1). Fluid is added to the wells with conventional pipettes or with Microtiter micropipettes which deliver drops of a constant volume. As shown below, the efficiency of mating with this technique is almost as high as that in

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‡ Obtained from Microbiological Associates, Bethesda, Md., U.S.A., and Flow Laboratories, Irvine, Scotland. The catalogue numbers are: 24A for disposable Microtiter plates; 33-1 for 0·025 ml microdiluters; 27 for handles, each of which can hold up to 12 microdiluters; 5 for micropipettes (0·025 ml per drop); and 30 for plate sealers.

tubes and the accuracy of dilution and recovery of recombinant colonies is at least as good as with conventional techniques.

Eight parallel matings were performed between exponential broth cultures of a $T6^s$ Str^s Hfr* strain transferring *his*$^+$ early and of a $T6^r$ Str^r His^- F^- strain. Plate 1 shows the resulting Microtiter plate. The donor culture was added to the wells of row 1 and the recipient culture to the wells of row 2. The mating was started by making a 1:5 (0·025 + 0·10 ml) dilution from the wells of row 1 into the corresponding wells of row 2. After 45 min the matings were interrupted by a further 1:5 dilution from the wells of row 2 into T6 lysate in the wells of row 3. Twenty minutes later serial 1:6 (0·025 + 0·125 ml) dilutions were made into molten minimal agar selective for His^+ [Str^r] colonies in the remaining rows. All these operations were performed at 42 °C so as to ensure that the molten agar did not set. In fact, mating is more efficient at 42 °C than at 37 °C (Wood, 1968; Achtman, Willetts & Clark, manuscript submitted to *J. Bacteriol.*).

The Microtiter plate was removed from the temperature-control device (see below) after the dilutions had been made and was chilled to set the agar. The plate was then incubated for 2 days at 37° to allow the colonies to develop. Plate 1 shows that recombinant colonies large enough to count had grown by that time.

1. STERILIZATION

Microtiter plates were sterilized by exposure to 10^5 ergs/mm^2 of ultraviolet light and were then covered with a second sterilized Microtiter plate (here called cover plate; the plates may easily be stacked one upon another). The cover plate was only removed during the various additions and dilutions and was immediately replaced afterwards. The cover plate also helped reduce evaporation.

The top of the cover plate was sealed with Microtiter plate sealers. Round sticky labels (1·3 cm diameter) were stuck on the plate sealers above each mating and pertinent information identifying each mating was written on them. The Microtiter test plates and plate sealers were used only once and then discarded but the cover plates were resterilized and used again.

The microdiluters were sterilized shortly before and after each dilution step by swirling for 3–5 sec in boiling water. This pre-wets the microdiluters (for greater accuracy) and removes any molten agar in them. Just before use, the microdiluters were drained on autoclaved and dried Whatman No. 1 filter paper. For occasional use, microdiluters treated in this way are cool enough that bacteria are not killed. When several consecutive dilutions were performed, two microdiluter sets were used alternately to allow sufficient cooling before use. Approximately 15–20 sec cooling time elapsed between immersion in boiling water and use for dilution.

Microtiter micropipettes were used for delivering molten agar (see below). They were stored in water after use, boiled to remove any solidified agar, and sterilized individually by autoclaving.

2. DETAILS OF MATING PROCEDURE

Bacterial cultures and T6 lysate were added to the wells with sterile 1 ml pipettes. Molten agar was added with sterile Microtiter micropipettes (0·025 ml per drop; 5 drops per well; reservoir of approximately 5 ml). Fluid was added to each row no more than 5 min before diluting into that row in order to minimize volume changes due to evaporation. The minimal selective agar used was as used for minimal selective agar plates except that the agar concentration was 0·7 %.

* The nomenclature is that recommended by Demerec *et al.* (1966) and Taylor & Trotter (1967).

At each dilution the microdiluters remove 0·025 ml from the wells in one row, mix it (turning a knob on the handle turns all the microdiluters and allows mixing) with the diluent in another row, and retain 0·025 ml of the mixture from the second row. Thus all but the wells in the last row have 0·025 ml removed from them for the next dilution. To allow for this, after all dilutions had been made, 0·025 ml was removed from the last row of wells and discarded. The 1:6 dilutions in the agar wells (0·025 + 0·125 ml) thus result in a final volume of 0·10 ml in each well.

The plates were then removed from the temperature-control device, chilled in a refrigerator for 15–30 min and incubated for 2 days at 37°. Plates, still covered with their individual cover plates, were stored three or four pairs deep to reduce drying and cracking of the agar.

The colonies are large enough to be easily counted with a bacterial colony counter. Between 5 and 50 colonies (usually between 5 and 30) were counted per well. The 1:6 dilutions ensure that at least one well in a dilution series will have a countable number of colonies.

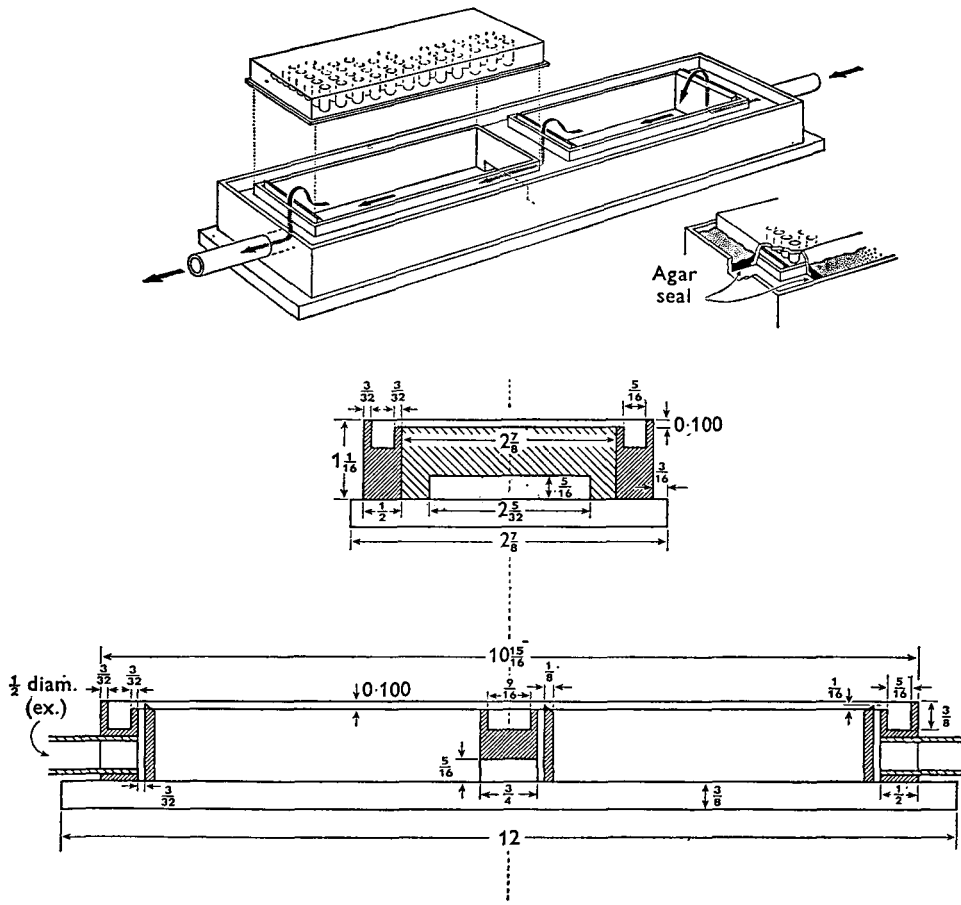

Fig. 1. Temperature-control device. TCD's were constructed of perspex (polymethylmethacrylate or Lucite). The top surface of the TCD is open; during use, 2 Microtiter plates are sealed into position with molten agar and thus form the top surfaces of the two chambers. Measurements in inches.

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3. TEMPERATURE CONTROL

A temperature-control device (TCD) was devised in order to maintain a constant temperature in the Microtiter wells. Temperature is regulated by warm water flowing under the bottom of the plates which sit within the TCD's. The plates are sealed into the TCD's with molten agar, which sets to form an air- and water-tight seal. The Microtiter plates now form the top surface of the chambers through which water flows.

With the cover plate in place (see above) and a water flow rate of 1 l. per min through the TCD's the temperature of fluid within the Microtiter wells is $2{\cdot}0 \pm 0{\cdot}5$ °C lower than the water temperature. The temperature drops a further 2 °C when the cover plates are removed and takes approximately 3 min to reach the original value when the cover plates are replaced.

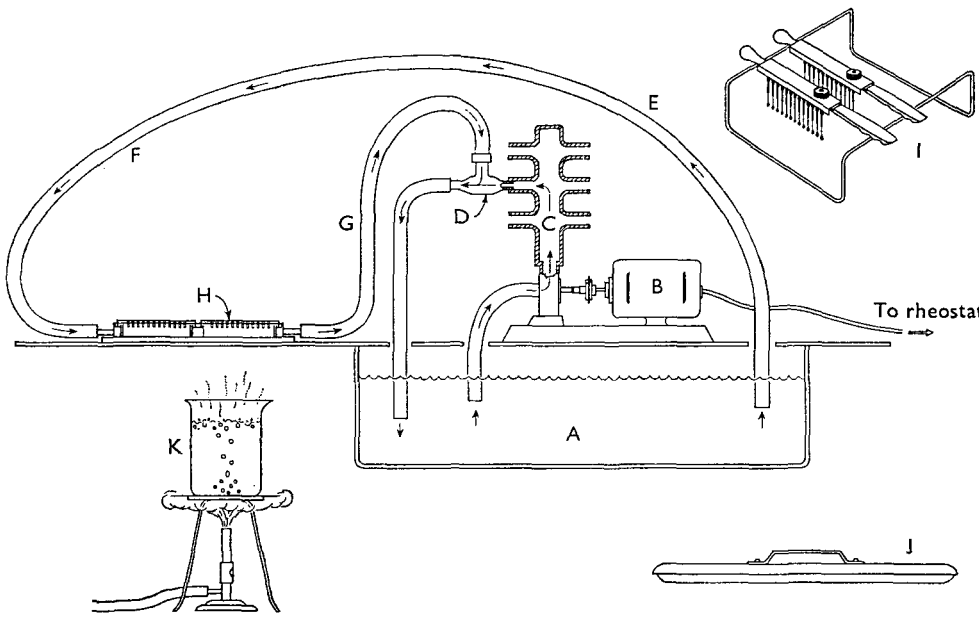

Fig. 2. Equipment used with Microtiter plates. Water is drawn from a constant-temperature water bath (A) by a centrifugal water pump (B) of 950 gal/h capacity connected to a rheostat. The water flows through a plastic fitting (C), through venturi filter pumps (D), and then back to the water bath. The venturi filter pumps suck water via the route EFG from the water bath and through TCD's (H) in which Microtiter plates have been sealed with agar. Only one TCD is shown although six were used together routinely. The length of hosing from the water bath to each TCD was the same to ensure that the temperature of water entering the TCD's was the same. Also shown are two microdiluter sets (I) with stand (not commercially available), a covered glass dish (J) containing sterile dry filter paper for draining microdiluters, and a beaker (K) (5 l. volume) with boiling water for sterilizing microdiluters. Not shown are pipettes, Microtiter micropipettes, ice bucket with bacterial cultures, or cover plates for the Microtiter plates.

Text-Fig. 1 gives a blueprint of a TCD. The TCD's have been constructed to hold two plates. It was felt that single-plate TCD's would be less convenient (routinely, 12 plates and 6 TCD's were used simultaneously) and that TCD's holding more than two plates each would be less flexible and might give less accurate temperature control.

Text-fig. 1 also shows the principle of sealing Microtiter plates into position. Fifteen

ml of hot molten 3 % agar were added to the outer groove of the TCD and the plates were placed in position in the agar. When the agar had set, a further 15–20 ml were added. When this in turn had set, the agar was covered with water (replenished every 60 min during the experiment) to prevent it from drying and cracking. At the end of the experiment, the plates were manually pried out of the agar seal.

The depth of water flowing through the TCD's is determined by the height of the weirs at the entrance and exit of the first chamber and at the exit of the second chamber. The entrance to the second chamber deliberately does not have a weir so that any agar which is accidentially spilt into the space between chambers can be removed. The weirs are high enough to ensure that water covers the under surface of the Microtiter wells. Air bubbles are usually sucked through the TCD but any that accumulate can be dislodged by tilting the TCD and tapping it sharply.

Water flows through the TCD because of suction from a venturi filter pump driven by a centrifugal water pump. Text-fig. 2 shows a schematic representation of the equipment used. The operation is described in the figure legend.

4. ACCURACY AND REPRODUCIBILITY

The manufacturers specify an accuracy of ± 2 % for microdiluters and micropipettes. To determine the accuracy under the conditions described here, 22 matings were performed in Microtiter plates between an (F*lac*)$^+$ T6^s Str^s donor and an F$^-$ Lac$^-$ T6^r Str^r recipient (both exponential cultures grown in broth at 42 °C to 3×10^8/ml). The procedure was as outlined above except that the minimal agar was selective for Lac$^+$ [Str^r] colonies. These colonies appeared in countable numbers in the fifth agar row (a $1:3{\cdot}9 \times 10^4$ dilution of the mating mixture; final volume 0·1 ml). The number of colonies resulting from the 22 matings ranged from 23 to 59 with a mean of 37·2 and a stranded deviation of 8·6. The theoretical standard deviation, assuming Poisson variability, is $\sqrt{37{\cdot}2} = 6{\cdot}1$. Thus over a series of seven dilutions (two dilutions to form a mating mixture and treat it with T 6 and five dilutions in agar) the variation between replicates can almost be attributed to chance alone. However, because of the small number of colonies that can be conveniently counted (< 50), this semi-automatic technique cannot be recommended for bacterial matings where differences of 2- to 3-fold or less from one mating to another are to be measured.

The numbers of recombinant colonies in the mating described above correspond to a mating efficiency of 26 % per donor cell. A parallel liquid mating performed in 2 ml volumes in test tubes yielded a value of 96 % per donor. In a separate experiment, parallel tube and Microtiter matings were performed as above. In addition, 0·04 ml were withdrawn from the Microtiter plate immediately after T6 treatment, diluted with conventional pipettes and assayed on agar plates. The number of Lac$^+$ [Str^r] recombinant colonies in the Microtiter well was $3{\cdot}9 \times 10^6$/ml as assayed with pipettes and agar plates and $3{\cdot}5 \times 10^6$/ml as assayed in the Microtiter system. This corresponds to an efficiency of 28 % per donor cell, while the efficiency in the parallel tube matings was 93 %. Thus there is a consistent lowered mating efficiency of about 3- to 4-fold in the Microtiter plates, but the accuracy of dilution is very high. The reason for the lowered efficiency is not known. On different occasions, when the same matings were performed in Microtiter plates, the mating efficiency showed less than a 2- to 3-fold variation. Thus the lowered efficiency of mating is quite consistent.

I am grateful to Dr A. J. Clark for allowing me to buy the appropriate equipment and experiment with it when the need for a semi-automatic technique became apparent and for suggesting to me that dilutions in molten agar might prove more usable than other techniques tried. I am also very grateful to Mr A. F. Milligan for designing and constructing the TCD's.

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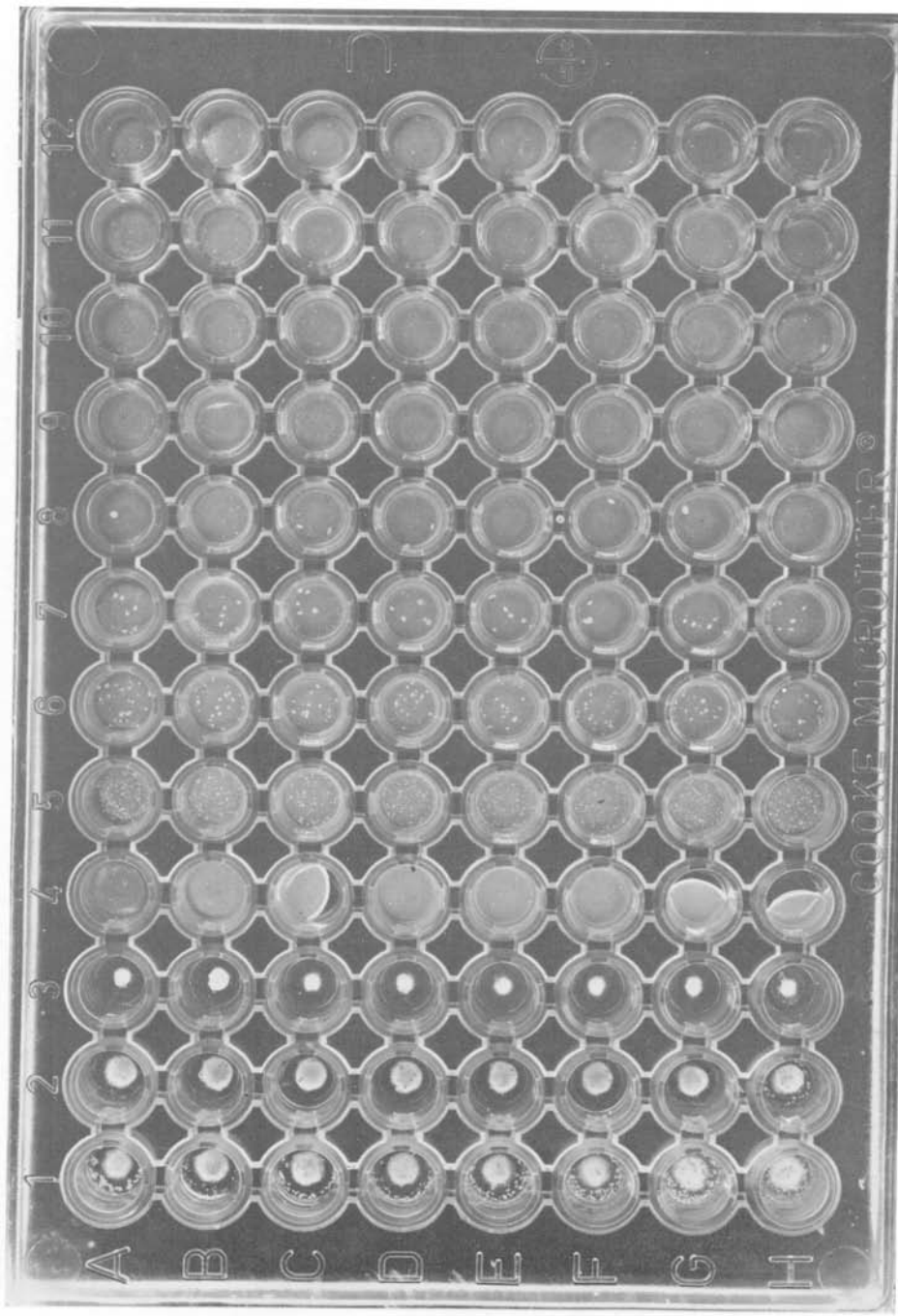
EXPLANATION OF PLATE

Microtiter plate after use. Row 1 (now dry) contained a $T6^s$ Str^s Hfr culture while row 2 (now dry) originally contained a $T6^r$ Str^r F^- culture. Row 3 (now dry) contained T6 lysate and the remaining rows contained agar selective for His^+ [Str^r] recombinants. Mating, T6 interruption, and dilutions were performed as described in the text. The final volume of agar in rows 4–12 was 0·10 ml. The Microtiter plate was chilled to set the agar and incubated for 2 days at 37 °C. It was then stored at 4 °C until it could be photographed. During this storage the agar in wells C 4, G 4, and H 4 has cracked and shrunk. The wells in row 7 ($1:6{\cdot}5 \times 10^3$ dilution of the mating mixture) contain the following numbers of recombinant colonies. 6 (A 7), 6 (B 7), 5 (C 7), 5 (D 7), 7 (E 7), 3 (F 7), 8 (G 7), 6 (H 7). Growth is confluent in the wells in row 4. The Microtiter plate could as easily have been used in a different orientation with 12 simultaneous matings in row G, T6 treatment in row F, and dilutions in agar in rows E, D, C, B and A. Countable numbers of colonies would then have appeared in row B.

NOTE ADDED IN PROOF

Flow Laboratories, Scotland, will now only supply a British imitation of Microtiter plates which have supporting struts underneath. These can be fitted into the TCD's by breaking or cutting away the struts.



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