Minipette System: A Simple and Convenient Ultramicro Technique for Routine Clinical Chemistry (Part II)

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EQUIPMENTS NECESSARY FOR ULTRAMICRO PROCEDURE IN ADDITION TO MINIPETTE

Minipette system for ultramicro analysis demands several equipments which are employed in the ordinary micro or ultramicro procedures, such as mixing devices, heating bath, centrifuge, reagent bottles, test tube racks, colorimeters, incubators, etc. Several inventions were made to modify or improve them so that they may be applied conveniently to our minipette system. The following is the description of those equipments.

(1) Minimixer*, a device for mixing small amounts of sample and reagent solution

In ultramicro procedures, the sample and reagent are taken in small test tubes. This makes it difficult to mix by ordinary stirring and shaking which are employed in micro methods. Therefore, a special convenient mixing device called “minimixer” was invented.

Principle and manipulation of Minimixer

A rubber cap is fitted eccentrically on an end of the axis (rotor) of an electric motor (the axis is fixed perpendicularly) so that it may be rotated. When the motor is switched on, the rubber cap will rotate. The bottom of a small test tube which contains a mixture of the sample and reagent is touched lightly upon the rubber cap. The solution in the test tube will go into centrifugal motion to create a whirlpool circling up along the test tube wall, thus mixing together. Therefore, even when some portion of liquid which was transferred to the test tube by Sanz pipet may be adhering to the midsection of the tube wall (without having reached the bottom), it will be washed down by the whirlpool and become completely mixed together.

Construction of Minimixer

The construction of the Minimixer is shown in Figure 1. Such a device can be made easily from a motor of an electric record player and a rubber cap of

*Such devices known as Thermomixer and Vortex Mixer are available on the market, but these are all made for the purpose of micro-analysis work.
test tubes used for bacteriological culturing.

\[\text{Fig. I. Minimaxer}\]

\[E: \text{Electric power cord}\]
\[l: \text{Signal lamp}\]
\[t: \text{Test tube}\]
\[ca: \text{Rubber cap}\]
\[s: \text{Switch}\]

(2) Heating—Double-Walled (Jacketed) heating bath

There are several procedures of routine clinico-biochemical analysis in which heating (100 °C) is needed. For such purpose, many laboratories are using a boiling water bath. However, when test tubes are put directly into such a bath for heating, droplets of boiling water sometimes get into them. Therefore, rubber plugs must be inserted to or aluminum foil must be applied over the mouth of the tubes in order to prevent such accidents. As ultramicro methods require the use of small test tubes, they are even more greatly exposed to the danger mentioned above than the larger test tubes under the micro method. Therefore, the conventional boiling water bath cannot be used for heating. Furthermore, in the ordinary boiling water bath there is uneven heating causing a temperature difference of (2–3 °C) between the center and the periphery, making uniform heating of the contents impossible. The temperature also fluctuates between 88–99 °C. The double-walled heating bath was devised so as to be free of such defects and provide uniform heating. (Photo 1)

Construction of the Double-walled heating bath

The construction is shown in Figure 2.

A: Inner kettle (ik). A large empty drug can (about 20 cm in diameter) is used. It should be the one which has been coated with anticorrosive agent inside.

B: Outer kettle (ok). A container available on the market under the brand name of Million Pot (garbage can) with a diameter of 30 cm is used. The lid of a one-size smaller Million Pot is used so that the dew (condensed water of the steam) that collects on the lid does not leak outside, but will drip into the outer bath.

*Manufactured and distributed by Fuji Riken (2-6-4 Yushima, Bunkyo-ku, Tokyo) and Shinoda Yoshimasa Shoten (Kami Ube, Higashi-ku, Ube City).
Photo 1. Double-Walled Heating Bath
(Shows wire basket with test tubes being introduced to it.)

Fig. 2. Construction of Double-Walled Heating Bath

L: Lid.  hk: Fixtures to hold inner and outer kettle.  ik: Inner kettle.  ok: Outer kettle. The side wall of the inner kettle and the lid fit tightly against one another at c point. Margin of the lid must be opened as shown(**).

C: Wire basket to stand test tubes. It can hold 7 × 7 test tubes. A long handle is fitted to the center so that it can be used for putting in and taking out the basket (Fig. 3).

The inner kettle (empty drug can) is suspended about 5–6 cm above the bottom of the outer kettle (Million Pot) and held in its place to the side wall of the outer kettle by 4 bolts and screws. The space between the inner and outer kettles is called “the outer bath” and that within the inner kettle “the inner bath”.
Handling of the Double-walled heating bath

The outer bath is filled up to 7/10 with water and up to 4–5 cm from the bottom of the inner bath. Polyethylene glycol or glycerine, in amount of 5–6 ml, is added to the inner bath (To raise the boiling point and prevent boiling).

The whole unit is placed upon a heat source (a gas stove or 2 KW electric hot plate) and heated. Ten minutes after the water in the outer bath has begun to boil, the lid is removed and the wire basket with test tubes inserted is put into the inner bath. The lid is gently replaced* and heating is continued. When the specified time has come, the basket is removed and put in a container for cooling with cold water**.

(3) Centrifuge

In micro-analysis filtration through a filter paper is frequently preferred to centrifugation to obtain a clear sample. However, in ultramicro methods, as comparatively small volumes of solutions are handled, the amount absorbed and lost by use of dry filter paper cannot be ignored. It is also difficult to use the usual filtering apparatuses for small volumes of solution. Therefore, in ultramicro-analysis, centrifugal methods are favored more often than filtering procedures.

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*The lid must be handled gently lest any of the dew should drop directly into the test tubes.

**The depth of water in the cooling bath should be 4–5 cm, and the flow of water should be maintained so that it always enters from the bottom, moves upward and overflows.
a) Micro-hematocrit centrifuge (Fig. 4)

This is a centrifuge used when a small amount of blood is collected in a glass capillary tube to determine the hematocrit value. It is used in a way described below.

![Diagram of Micro-hematocrit Centrifuge](image)

**Fig. 4. Micro-hematocrit Centrifuge**

The glass capillary tubes are placed in the radial grooves (G) of rotor (R) with the bottom of the tubes fitted closely in contact with rubber frame (F). Cover lid (L) is placed in position tightly so that the tubes will not move during centrifugation and lid (L) is applied. Time (T) is set and the circuit is connected for centrifugation.

**Collection of blood:** The tip of the finger is wiped with a pledget of cotton which has been soaked in alcohol (70%), wiped again with a dry piece of gauze and the tip of the heparin-coated tube (diameter 1.2–1.4 mm, length 75 mm) is touched to the second drop. The other end is lowered slightly so that the blood may readily flow into the capillary tube, and when the blood reaches 2/3 of the length of the tube, it is removed from the drop of blood. The part near the end that has not been filled with blood is held between the thumb and index finger, placed over the flame of an alcohol lamp and heated to be sealed by gentle rotating motions*.

**Centrifugation:** The capillary tubes are inserted in the radial grooves of the microhematocrit centrifuge so that the heat-sealed end is in close contact with the frame. The lid is fitted securely and centrifugation is commenced (12,000 rpm, 5 minutes).

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*If a padding of rubber is glued to the frame of the centrifuge (where the lower end of the capillary tube is placed), it will not be necessary to heat seal the tube, and a mere plugging of the lower end of the tube with oil clay or crit seal is sufficient for centrifugation. Blood will not leak out from the lower end.
**Reading:** The capillary tube is placed in the micro-hematocrit reader (Fig. 5). The lower level of the blood cell layer at the heat-sealed end is matched with index line B and graduation mark 100 on outer disk S is fixed in a position aligned with marker line S. The inner disk C' is rotated so that upper surface of the plasma in the capillary tube is aligned with logarithmic spiral curve A and then outer disk C is rotated (C' moves together with C) so that logarithmic spiral curve A becomes in alignment with the top of the red blood cell layer. The graduation on the outer disk to which marker line S points is read. This reading is the hematocrit value sought.

![Fig. 5. Hematocrit Reader](image)

First, the bottom of the erythrocyte layer in the glass tube is aligned with index line B, next marker line S is aligned with 100%, a finger is stuck into the hole in disk C which is rotated so that spiral curve A may become aligned with the top of the plasma in the glass capillary tube in (1). Next, spiral curve A is aligned with the top of the erythrocyte layer as shown by A' in (2). The position of marker line S is read from disk C. This is the hematocrit value.

b) **Centrifugal sedimentation using a ordinary centrifuge**

The ordinary centrifuge (24 racks, 3000 rpm) can be used to separate serum from the blood sample. The method is the same as that for the micro method. The blood collected in capillary tubes whose one end has been sealed in the manner described above (Part I), is placed in an ordinary round bottomed centrifugal tube* and spun for 10 minutes (1500 rpm).

*When the bottom of the sedimentation tube is made flat by placing on it a piece of rubber or a portion of a rubber plug, the capillary tube simply sealed with crit-seal may be safely subjected to centrifugation (2500 rpm, 5 minutes) without any additional protection.
When blood and serum are to be deproteinized, the round bottomed tube is used as the container, to which deproteinizing reagent is added and mixed. After centrifugation (2500 rpm, 10 minutes), a clear supernatant can be obtained*. Furthermore, the bottom of a case (3.8 × 11.0 cm) for large centrifuge tubes (volume 50 ml) may be beaten flat, the upper 1 cm portion cut off with a hacksaw and a piece of rubber of appropriate thickness made from a rubber stopper (No. 12) fitted into the bottom. This can be used as a holder for capillary tubes. If 2 such holders are made, adjusted so that their weight is the same**, and placed diagonally across the axis of rotation each other, it would be possible to process simultaneously, the same number of the same sized tubes filled with the same volume of sample (about 8 small No. 1 or No. 2 test tubes described in this paper) (Fig. 6).

![Fig. 6. Holders for Centrifuge Tubes](image)

A: Regular case  B: Case with bottom flattened.  C: Cut section of B. Bottom is made flat with padding or plug.  g: Rubber padding or rubber plug.

c) Microcentrifuge (Coleman)*** (Fig. 7)

The Coleman ultramicro centrifuge (Catalog No. 6–810) has the capacity to process 24 (400 μl) polyethylene microcentrifuge tubes at 13,000 rpm (10,800 g) at a time. This apparatus has a cycle timer which can be set for the period of centrifugation (e.g. 5 minutes) and when the time comes it will stop automatically. It is not necessary to balance the centrifuge tubes (roughly balanced centrifuge tubes may be inserted diagonally across the axis of rotation from one another). This apparatus is usually used to obtain clear centrifuged supernatant (protein-free

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*Recently, practical and convenient small test tubes (No. 1 and No. 2) suited for this method are being manufactured (Mitsuwa Rika; Kogushi, Nishi-ku, Ube City—in front of Yamaguchi Medical College).

**The bottom of the test tubes are protected and other damage is prevented by the fitting rubber plugs into the bottom. The weight of the pair of holders to be used can be made to be approximately the same by adjusting the thickness of the rubber plugs. Of course, the surface of the rubber must be flat. The thickness should be more than 1 cm.

***Similar centrifuge is obtainable from Japanese manufacturers (Kokusan Enshinki Co., Ltd; 3–9 Daito 2-chome, Daito-ku, Tokyo, and Tominaga Seisakusho, Tokyo).
Axis A has 4 grooves (G). As shown in the figure, 6 microcentrifuge tubes with polyethylene caps (mct) may be inserted into holder (H). The holders are to hold the same number of tubes and be fitted diagonally across from one another into grooves (G). The lid is lowered and the timer is set so as to connect the electric circuit for centrifugation.

filtrate) by removing protein from a minute amount of serum using a small amount of deproteinizing reagent. However, this is used also to separate serum from a minute amount of blood. The procedure is to collect blood directly from the finger tip into a polyethylene microcentrifuge tube instead of using a glass capillary tube (Blood drawing capillary). Refer to section on direct collection of blood into a micropolyethylene centrifuge tube (Coleman) (Part I, Fig. 6)

(4) Test tubes (Fig. 8)

a) Small test tube No. 1: 10 × 75 mm. These are made from the pilot tubes used in the blood banks*. A file mark is made on the glass wall 75 mm from the bottom and the tip of a red hot glass rod is touched to this site causing a crack which makes the starting line to cut off the upper portion. The pilot tubes thus shortened can be used for preserving serum and for carrying out reactions taking place in less than 1.0 ml of medium.

b) Small test tube No. 2: 12 × 75 mm. Pilot tubes of exactly this size used in the blood banks* are employed. These round bottomed tubes may be used as centrifuge tubes and as the test tubes for carrying out reactions in less than 2.0 ml of medium.

*Available from Hiroshima Block Bank (1-23 Ote-machi 5-chome, Hiroshima City) or Toyo Denshi Co., Ltd. (Kotoshibadai, Ube city).
c) **Small test tube No. 3**: 15 × 75 mm. Thick test tubes (15 mm in diameter) are cut to 70 mm length for use. This may be used for carrying out the reactions in a medium of less than 3.0 ml.

d) **Small test tube No. 4**: 18 × 75 mm. Thick test tubes (18 mm in diameter) are cut to 70 mm length for use. These may be employed for carrying out reactions in a medium of less than 5 ml.

(5) **Capillary Tubes**

Glass capillary tubes 2 × 110 mm (manufactured by Futaba Kikai Co., Ltd.; 4-3-6 Kogo Naka-machi, Hiroshima City). These may be used for collection of blood from the finger tip and also after coating the bore with heparin, EDTA, sodium fluoride, etc.*

**Washing of capillary tubes**: Capillary tubes are bundled and placed sideways in a small polyethylene bucket (15 cm in diameter and 15 cm in height) and washed in running water for 24 hours. These are then put into a dryer or a centrifuge.

*Capillary tubes which have been coated inside with heparin and sodium fluoride should be cut slightly shorter than the capillary tubes for blood collection (2 × 90 mm) so that it may be possible to distinguish the two kinds of capillaries.

a) **Method for coating with heparin**: Heparin solution (500U/5ml) is diluted with 2 parts of water. Well washed and dried glass capillary tubes are completely submerged and quickly removed from the solution and stood up in a glass container in which gauze has been lined on the bottom and then placed in a dryer (about 100°C) to dry.

b) **Method for coating with fluoride**: The capillary tubes are treated with 2.5g/dl sodium fluoride (aqueous) solution in the same way as in a).
to remove the water. Tubes which have been thus washed and dried are placed lengthwise in a bottle (10 cm in diameter and 20 cm in height)—a 1 ℓ measuring cylinder whose oral portion has been cut off to adequate length may be used as well—, dichromate sulfuric acid is introduced into the bottle until the tubes are completely submerged, and are left to stand for 4–5 hours. Then the dichromate sulfuric acid is discharged by tilting the bottle and the tubes are washed briefly with water. These are returned to the polyethylene bucket and washed again for 24 hours with running water. Finally, the tubes are transferred to a container of H₂O (distilled water), washed and either put into a dryer or centrifuge to remove water in the tubes. Thus, the tubes are ready for drawing blood.

(6) A Kit of Small Bottle for Standard Solutions

The glass bottles which contained sensitivity disks for bacteriological tests (Eiken) may be adapted for use. These bottles have a polyethylene cap which can be used to make it air tight, which is convenient. The bottles are of a size which make measurements with the Sanz pipet easy. After the bottles are thoroughly washed out, standard solutions are put into them and capped (Fig. 9).

![Fig. 9. A set of Small Bottles used as Containers for Storing Calibration Standards](image)

(7) Other Glassware

Other glassware consists of volumetric flask, measuring cylinder, flask, beaker, glass rod, measuring pipet, hole pipet, etc., which are used for conventional micro-analyses. (Colored polyethylene bottles are preferred to glass bottles).

(8) Test Tube Rack*

A stand that can hold 3 rows** of 12 test tubes (No. 1–4) of an identical type

*Manufactured and distributed by Kayagaki Irika Co., Ltd. (Tokyo) and Shinoda Masayoshi Shoten (Numa, Kami Ube, Higashi-ku, Ube city).
**All of the 3 rows of the holes of a rack are not filled with test tubes. The test tubes are lined 2×10 from the left end, and as sampling (taking 20 μl aliquot of each specimen) has been completed, the tubes are moved to the row in front of that of the untreated tubes (see Fig. 10).
should be used. The diameter of the holes should be 1 mm larger than those of the test tubes. It should be of stainless steel make so that it can be immersed in the watered incubator together with the test tubes. (Fig. 10)

A stand with a capacity for 3×12 is best. A and B show stand with tubes containing separated serum. Using the section of the stand to the left of the vacant hole line, there is room for 50 tubes. C shows tubes containing an aliquot of 20 μl sample each. Tubes which have been treated for sampling (S1, S2, S3, S4) are moved to the front row from that of the untreated ones. This will make it possible to distinguish between the test tubes which have been treated and those not yet treated, and thus mistakes can be avoided. B, 1, 2, 3 and 4 are for calibration standards.

(9) Test Tube Rack for CCFT*

CCFT is performed in a dark place at 37°C. Therefore, the test tubes should be placed in a stand which is encased in a stainless steel container so that the test tubes are not exposed to light (Fig. 11).

The stand is of the shape of a box so that the tubes can be protected from light. The bottom is of wire mesh which allows the tubes to be surrounded by warm water when immersed in a warm water bath.

*Commercially available from Kayagaki Irika Co., Ltd. (Tokyo) and Shinoda Masayoshi Shoten (Numa, Kami Ube, Higashi-ku, Ube City).
(10) Test Tube Rack for Heating the Test Tubes

This consists of a wire basket (See section on Double-Walled Heating Bath).

(11) Incubator with Thermostat

This water bath is the same as that used for micro analysis. The only difference is that a shelf is placed inside of the bath and water is added until about 5 cm above the shelf. Recently, a type suitable for ultramicro methods has been put on sale at a reasonable price*.

(12) Washing of Glassware

As a large number of small test tubes are used in the ultramicro method, it is very troublesome and time consuming to wash each one by one brushing, with detergents as done in the micro method. Therefore, an efficient means of washing glassware are desired. A simple method which does not require any special apparatus will be introduced in the following.

The method, in brief, is to line test tubes of the same size in one direction in a stainless steel (SUS27) wire basket. Cover it with its lid and first dry the tubes. Next, immerse the basket in a dichromate sulfuric acid bath for 3–4 hours and then drain the acid. Next, wash with running tap water and dry in a heating dryer box.

By means of this method, once the tubes have been put into the basket, they can be immersed in dichromate sulfuric acid, washed in water and dried, and each step will be perfectly processed without taking the test tubes individually out of the basket.

i) Wire Basket for Washing**: The basket is $15 \times 15 \times 10$ cm and woven with

![Diagram of Wire Basket for Washing](image)

Fig. 12. Wire Basket for Washing (made of stainless steel)

- $c$: Wire hooks to fasten the lid to the basket.
- $h$: Handle.
- $l$: Lid.

*Commercially available from Fuji Riken (2–6–4 Yushima, Bunkyo-ku, Tokyo).

**Commercially available from Fuji Riken (2–6–4 Yushima, Bunkyo-ku, Tokyo) and Shinoda Masayoshi Shoten (Numa, Kami Ube, Higashi-ku, Ube City).
stainless steel wire. It is divided in the middle into 2 compartments. A cover is made to serve as a lid. Stainless steel handles are fitted to one edge along the top and to another edge diagonally opposite to it. The anchoring edges of the handles and the divider should be parallel to one another (Fig. 12).

When making the basket, soldering should not be used. Weave the wires or weld them together*.

ii) Dichromate Sulfuric Acid Bath**: A box $50 \times 50 \times 50$ cm is made by putting together vinyl chloride boards (15 mm thick), and a vinyl chloride cock (inner diameter, 3 cm) is fitted to the lower portion of one side***. A vinyl chloride lid (5 cm deep) is placed upon the box. A handle is attached to the center of the lid. A board of vinyl chloride, longer than the height of the box, is placed upright against one of the sides of the box. Fitted to this board are stainless steel hooks+ (Fig. 13).

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*If the basket is made of stainless steel SUS27 wire, it will not be corroded by dichromate sulfuric acid. When solder comes into contact with dichromate sulfuric acid, it will dissolve and give off hydrogen gas. Therefore, solder should not be used.

**Commercially available from Fuji Riken (2-6-4 Yushima, Bunkyo-ku, Tokyo).

***Potassium dichromate will settle on the bottom. Therefore, fit the box with as large a drainage pipe as possible and attach the cock, or else the drainage pipe will become plugged up.

+The hooks can be made of thick wire (No. 10) of stainless steel SUS27. These hooks serve the purpose of suspending the basket and draining the tubes after the basket has been removed from the bath.
Concentrated sulfuric acid is pumped into the box until it fills 2/5 of the box* and powdered potassium bichromate crystals are added in proportion of about 10%. This is mixed thoroughly with a stainless steel rod. When not used, the lid is kept closed**. When dichromate sulfuric acid becomes old and loses its activity, the cock of the box is opened and the contents are allowed to flow out slowly together with running water from a tap***. New dichromate sulfuric acid is put into the box.

A hook (stainless steel) fitted to a wooden grip to catch the handle of the wire basket when immersed in dichromate sulfuric acid should be made available (Fig. 13)⁺.

iii) Dryer: An ordinary electric heating dryer box with 2 shelves is recommended.

How to wash: Fill a polyethylene bucket which is available commercially, with water (if neutral detergents are added in adequate amounts, the efficiency of washing will be enhanced). The used small test tubes are put into this bucket, rinsed rather briefly, and arranged in a washing basket with the mouths of the tubes at right angles to its septal wall++. After the basket becomes full (about 200 tubes), the lid is fastened and the test tubes are dried in a dryer+++.

The basket is removed from the bucket and left to cool at room temperature×. Then it is put into the dichromate sulfuric acid bath. Care should be taken so that dichromate sulfuric acid fills the lumen of every tube̶. At the end of 3–4 hours, the bottom of the basket is caught with the hook and the basket is pulled up with its bottom upside, and then one of the handles of the basket is hanged on the hooks of the vinyl chloride board which is standing vertically to drain the acid.

*Care should be exercised so as not to put too great an amount of dichromate sulfuric acid into the box, because drops arising during operations may cause splashes which may burn the body of the operator.

**When dichromate sulfuric acid absorbs water, it loses its activity (cleaning power). Therefore, the bath is covered lest the activity of dichromate sulfuric acid should be lost.

***When discarding the dichromate sulfuric acid, it should be mixed at a rate of 1 to 10 parts of water so that it will not cause damage to the sink.

⁺Made of stainless steel SUS27 wire (No. 10). The hook should not be soldered, for the solder will dissolve.

++For the purpose of subsequent operations, the mouth of the tubes should be arranged so that they all face one direction.

+++If the basket which is wet with water is immersed in the dichromate sulfuric acid bath heat will be produced in the bath causing a spray of acid which is dangerous to the operation. Furthermore, if water mixes with the acid, the cleaning effect of dichromate sulfuric acid is reduced. Therefore, the wire basket containing the tubes should be dry before placing in the acid bath.

×The tubes should be cooled before placing in the acid bath, or the heated tubes will be suddenly cooled by the acid and will result in cracks and breakage.

××The basket should be immersed so that the bottom of the tubes may come downward. This will allow all tubes to become filled with dichromate sulfuric acid.
After there is no more dripping of the acid, the basket is removed from the hooks and immersed in a running water bath (this is arranged so that the unfastened end of the hose is placed on the bottom of the bath and water is allowed to flow forcefully and overflow) until water loses dichromate sulfuric acid discoloration*. Then, the basket is lifted from the water with its handles taken by the hands of the operator, one with the right and other with the left hand, and inclined in order to drain water from the test tubes (Fig. 14). The basket is re-immersed in the water bath and all of the tubes are filled. During this time the water is introduced into the water bath from the bottom and made to overflow. This is repeated 15–20 times to clean test tubes thoroughly (It would be ideal if the tubes were washed with distilled water after this washing with tap water).

![Fig. 14. Washing the Test Tubes in a Basket with Water](image)

Sc: Stainless steel wire basket.
T: Tank with running water (water bath).
W: Water. Look at all the water contained in the test tubes packed in the basket is drained and emptied when the basket is inclined.

The basket is suspended from the hooks on the board to drain and placed in a dryer (120°C) with the bottom ends of the test tubes contained in it to the

*Efficiency will be increased if there are 2 washing baths available, one for the first bath and the other for the finish bath.
top (inverted posture) for drying*. After 30 minutes, the basket is taken out of
the dryer to be cooled to room temperature.

(13) **Photoelectric Colorimeter**

A device equipped with a cuvette which is the same in type as that used in
the photoelectric colorimeter or spectrophotometer but much smaller in volume is
now commercially available for ultramicro-analysis. This will enable us to measure
the extinction of colored solution in volumes less than 0.5 ml. However, our
experience has shown that, attempt to perform colorimetry with a very small
volume of colored solution, is disturbed by the fluctuation of reading, on account
of air bubbles that got mixed into the solution by chance. In the case of
colorimetry in succession of many colored solutions of various concentration, it is
possible that the solution which has already measured for its optical density may
remain in the cuvette and contaminate the next colored solution which is introduced
into the same cuvette, thus resulting in the error in reading its true extinction.
In order to prevent such accidents one has to be constantly alert and careful during
colorimetry. From this view point it is thought to be unwise to reduce the volume
of cuvette of the colored solution which is to be measured for optical density
below the level of 1.0 ml. However, whatever the case may be, ultramicro-analysis
will necessitate a smaller amount of colored solution for colorimetry than the micro
method (3–5 ml). Special note should be made that in colorimetry with about
1 ml of colored solution, it is not always possible to insert the cuvette into the
optical path in identical way for every measurement if it must be removed and
re-inserted for each measurement of optical density, thus resulting in technical
error in reading. Therefore, the cuvette should be fixed to the colorimeter.
Fluctuations in readings due to inaccurate positioning of the cuvette can be avoided
if the fixed cuvette is of the drain type whereby a colored solution which have
been measured for optical density can be removed by suction to be replaced by
another colored solution.

When the solution to be subjected to colorimetry is too small in volume and
the cuvette is reduced in size by shortening the optical path in accordance with
it, the sensitivity of colorimetry will be decreased. Therefore, the optical path
must be maintained at a level at least equivalent to that of the colorimetry
employed in micro method. Otherwise, the ultramicro method will become
inferior in precision to the micro method. One of the measures which enable us
to solve this problem would be to cut away as much dead space as possible on
both sides of the cuvette which does not contribute to the passage of light directly,
thus reducing the capacity of the cuvette without shortening the optical path.

*The test tubes are dried with their bottom ends up in inverted posture. If the bottoms
are down (erect posture), the drying process is poor and any foreign substances which
were in the water by chance will adhere to the bottom.
In the ordinary drain type colorimeter where the cuvette is fixed for reading optical density, it is impossible to recheck reading about the colored solution which has once been read. However, as there are instances where recheck is desired, the authors attached a trap to a section of the suction pipe for the purpose of collecting the solution which had been determined for extinction in a small tube. The trap invented by us proved to be very effective; all the solution in the cuvette could be removed in 5 seconds (at a negative pressure of 22 mmHg). The residual rate referring to the ratio of the amount of solution remaining in the cuvette to the total volume of the solution introduced in the cuvette was less than 1%.

In order that the ultramicro method may become a practical procedure, the first problem to be solved is the invention of a colorimeter which can satisfy the various requirement inherent in this method. Introduced hereunder is a colorimeter devised by us which is currently in use. It is inexpensive and simple to operate. It is a special modification of the compensation circuit type ADS photoelectric colorimeter.\(^{(1,2)}\)

a) Construction of the colorimeter

The improvements made to the ADS colorimeter for the purpose of ultramicro analysis are as follows. (Photo 2).

![Photo 2. Photoelectric Colorimeter for Ultramicro analysis](image)

\(A: \text{Cuvette. } B: \text{Recovery Unit. } C: \text{Zero Adjustors.}\)

(i) Cuvette (A) (Photo 2, Fig. 15)

In the conventional photoelectric colorimeter the cuvettes had been either round or square in shape, but in this colorimeter the cuvette was made oblong with transverse cut surface of 10 mm (length) \(\times\) 3 mm (width). This cuvette enabled us to determine extinction of a colored solution with only 1.0 ml. The lower portion of the cuvette is tapered down so that, after the extinction has been measured, the solution may be drained from the bottom of the cuvette by suction
created by negative pressure using a polyethylene tube connected to a water stream
pump. The upper aperture of the cuvette is expanded to form a funnel for the
easiness in pouring the colored solution into the cuvette.

(ii) Recovery Unit (B) (Photo 2, and Fig. 15)

In conventional colorimeters the solution was discarded through the draining
device after determination of extinction. In our apparatus, a trap is connected to
the draining device to recover the solution after colorimetry. As shown in Figure
15, a piece of sponge (F) is attached so that the mouth of the test tube fits tightly
against the wooden frame (D), and 2 stainless steel pipes are inserted in the middle.
Polyethylene tubes are connected to each pipe, and one pipe (J) is lead to suction
pipe (H), while the other (I) is connected via an introduction pipe (E) to the lower
end of cuvette (A) located within the colorimeter case (B).

![Fig. 15. Construction of photoelectric colorimeter (left) and conventional
drain type cuvette (A), and cuvette (A), whose capacity has
been reduced by elimination of dead space, but retaining the
same optical path length. Dimensions are in mm.
A: Cuvette. B: Colorimeter cover case.
C: Wooden frame fixing nuts. D: Wooden frame for recovery unit.
E: Polyethylene tube for connecting recovery unit and cuvette.
F: Sponge rubber sheet for recovery unit which is attached to wooden frame.
G: Test tube (the recovered solution is collected in this).
H: Tube to connect aspirator with recovery unit.
I, J: Stainless steel pipes. K: Screw pressing polyethylene tube E]

When solution is poured into the cuvette, the solution will automatically settle
at a level proportional to the volume. If the volume is 1.0 ml, the main section
(optical path) of the cuvette will become completely full. After extinction has
been determined, the mouth of the test tube (G) is pushed tightly against the
rubber sheet (F), and as the aspirator is functioning a satisfactory negative pressure
will develop within the test tube. Thus, the solution in the cuvette will be aspirated
into the test tube via the pipe (E). In other words, the solution that has been
determined will be recovered in the test tube (G). Test tube (G) will stick to rubber sheet (F) while the solution is being drawn into it. After all of the solution has been drawn into the test tube and only air is being suctioned, the test tube is picked and pulled downward by fingers to be released from the rubber sheet and to stop the suction.

The smaller the diameter of the introduction pipe (E), the stronger will be the negative pressure created in the test tube, thus causing the tube to stick stronger to the rubber sheet. The bore of the introduction pipe may be made small or large by increasing or decreasing the pressure on the bore, using screw (K), thus making it possible to control the suction power of the tube at will.

(iii) Fine Zero Adjuster (C)

As the width of the cut surface of the cuvette is made small, it is difficult to perform zero adjustment by the ADS photoelectric colorimeter’s zero adjustors alone (2K Ω). Therefore, a separate variable resistor (100 Ω) is connected in a series circuit and used as the zero fine adjustor. The original zero adjustor is used for coarse adjusting while the new one is used for fine adjusting.

(iv) Change in position of cuvette

The ADS photoelectric colorimeter is designed for right hand operation. In the colorimeter for ultramicro procedures, it is arranged so that the left hand is also put to use. The cuvette is placed on the left side of the colorimeter case and the solution to be determined is put into the cuvette. The left hand is used to recover the solution after colorimetry while the right hand is used to rotate the potentiometer (dial)*.

(b) Colorimetry Procedures

1. **Zero adjustment**: Hold test tube which contains a blank solution in the left hand and introduce the solution into the cuvette (Photo 3). Set potentiometer (dial) at 0 and align the needle to 0 using the Zero Adjustors (coarse and fine).

*The modified photoelectric colorimeter is called the S-ADS photoelectric colorimeter and is sold by Fuji Kogyo Co., Ltd. (Front of Haruki-cho streetcar station, Yushima 4-chome, Bunkyo-ku, Tokyo).
2. **Discharge the blank solution**: Push the mouth of an vacant test tube against the rubber sheet of the recovery unit and aspirate the blank solution into the test tube. Remove the test tube.

3. **Colorimetry**: Hold the colored solution in the left hand and pour it into the cuvette. The needle of the meter will leave 0. Turn potentiometer with the right hand until the needle is reset to 0. (Photo 4).

![Photo 4.](image)

**Operation 3**: Colorimetry-Potentiometer is being turned so that the needle is aligned with zero.

4. **Discharge or recover the colored solution**: The procedure is the same as described in 2, but the solution is collected in another test tube (Photo 5). If it is not necessary to collect the colored solution already read for optical density the same tube as that used for blank solution may be pushed up against the rubber sheet. The colored solution will drain into it. The test tube is left attached to the rubber sheet.

![Photo 5.](image)

**Operation 4**: Discharge and recovery of colored solution.

5. **Taking readings of extinction**: Check the reading of the potentiometer (dial) and record in notebook (Photo 6). This is the extinction.

6. The test tube is removed from the rubber sheet. This constitutes the final step of one cycle of the colorimetry procedure.

7. Procedures 3–6 are repeated and extinction of the new colored solution is determined.
8. **Washing of cuvettes**: As soon as colorimetry is completed, H₂O is poured from a washing bottle into the cuvette until full. Then, suction is applied by pushing the test tube which serves as a container of used water tightly against the rubber sheet. This is repeated 3 times. After washing in this way, a lid (polyethylene) is placed over the mouth (funnel) of the cuvette*

c) **Preservation of Colorimeter**

Once a week, a rubber plug is put to the tip of the stainless steel pipe (I) of the recovery unit to stopper it and fresh dichromate sulfuric acid is poured into the cuvette (Photo 8) through the funnel to fill up to eight tenths its volume. It is allowed to stand for 2-3 hours. The rubber plug is removed and test tube *Do not forget to cover the cuvette to prevent foreign particles etc. from entering into it. If anything should get into it by accident or the cuvette becomes clogged, do not poke the inside of the cuvette. The free end of the rubber hose from the aspirator should be brought directly to the funnel of the cuvette and the solution inside the cuvette should be aspirated from the opposite end (Photo 7). If a test tube filled with water is applied to the tip of stainless steel pipe (I) of the recovery unit at this time, the water in the test tube will flow in the reverse direction from introducing pipe to the cuvette and will be drawn into the rubber pipe, washing out the foreign particle along with it.*
is pushed tightly against the rubber sheet and the aspirator is operated to discharge the dichromate sulfuric acid contained in the cuvette.

H₂O is poured into the cuvette after the dichromate sulfuric acid has been completely discharged. If there is any acid left, introducing water into the cuvette will cause generation of heat and may result in its breakage.

Photo 8.
A plug is placed on the tip of the stainless steel pipe of the recovery unit (←) and fresh dichromate sulfuric acid is introduced into the cuvette.

(14) Titrimetric Apparatus—Piston Buret (Metrohm Co.)*

As a rule, ultramicro-analysis is carried out chiefly resorting to the use of colorimetric methods. However, at present there are some instances for which titrimetric procedures are more convenient than colorimetry. Therefore, some account of titrimetric procedures will be given with respect to ultramicro analysis.

The use of a titrimetric apparatus in ultramicro-analysis will naturally involve the treatment of a very small volumes of sample material. In microanalysis ordinary microburets (2 ml) are used, but they are not helpful to us in ultramicro procedures, because titration must be made to minute volume of sample with a buret of less than 1/10 of this size. Beckman ultramicro titrimetric apparatus, micropipette, buret and Lehberg titrimetric apparatus are used for this purpose.

However, as the volume of solution used in these equipments is so minute, they burden a great mental strain on the operator. Therefore they are not always convenient. The authors prefer the Piston Buret (Metrohm Co.) Model E247 (5 ml) which can be used with little strain for ultramicro titrimetry. The apparatus is shown in Figure 16.

Preparation of piston buret (Figure 16)

The vernier scale is turned to the left and the piston is pushed all the way up the cylinder. Glass tube (gt) is connected to cock (k) by connection tube (ct).

The tip of the glass tube is inserted into the reagent in the reagent bottle and when the handle is lowered, the reagent will be drawn into buret cylinder (c) through the glass and teflon tubes by the negative pressure. After the reagent

*Imported by Riko Shoji (3-11 Minami-dori, Nishinaga-dori, dori, Nishi-ku, Osaka).
has filled in the buret cylinder, connect cock (k) to air and upraise handle (h) as far as air bubbles in the buret cylinder can be forced out. Then, turn cock (k) and again connect the cylinder and connecting tube (ct).

The vernier scale is turned and aligned to zero, after which the reagent bottle is removed.

**Method for titrimetry using the piston buret**

Hold the test tube containing the sample to be titrated in the left hand, insert the small glass tube (gt) to the test tube and shake them incessantly while the vernier scale is turned slowly to the left with the thumb of the right hand. This is continued until the end point of titration is reached*. (The vernier scale is to be turned to the left only. Do not push it too far nor make it recede it by turning it to the right.) The amount of reagent consumed is read from the macro-scale and vernier scale of the buret.

**Precision of piston buret**

It is reported that a buret with a capacity of 10 ml has a precision of ± 0.001 ml in titrimetry. One calibration of the vernier scale is 0.005 ml. Therefore, it is readily possible to read up to about one-half of this or 0.0025 ml. Thus, in

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*In order to determine the end point accurately, a control test tube containing a blank solution should be shaken and titrated for the sake of comparison.
titrimetry of 0.1 ml, a precision of ± 1 %, at worse, can be attained. It is felt that using a buret with a capacity of 5 ml, a precision of ± 0.5 % can be obtained.

An ultramicro buret* (Fig. 17) which can be used for titrimetry of even more minute volumes is commercially available.

![Fig. 17. Ultramicro Buret (Micrometric Instrument Co., Cleveland)](image)

Small syringe cylinder (C) is fitted with piston (P) and a beak-shaped tube (q) for titrimetry is attached to the tip. The base of the handle is a screw (S) which when rotated turns meter needle (M) and advancing device (circular plate) (D). The handle pushes the end (d) of piston (P) to the left. The solution in the cylinder is forced out into the test tube (T), and thus titrimetry is performed. The amount of solution forced out can be read from the scale of the meter (M). In addition, a stirrer and a cup which substitutes for the test tube (T) are available as an accessory equipment. They are subjected to an automatic vibrator for mixing the fluid in the cup. This apparatus is more suitable for titrimetry of smaller volumes than the Metrohm’s Piston Buret.

(15) Titrimetric Apparatus—Measuring pipet with screw cap (0.35 ml)**

When a Piston Buret is not available, a measuring pipet fitted with a screw cap may be substituted (Fig. 18). The tip of a 0.5 ml measuring pipet (0.1 ml is divided into 20 calibration) is fused in the flame of a gas burner and tapered to make the tip into a capillary tube (about 3 cm long) with which a capacity up to 0.35 ml can be accurately read. The other end (base) is also heated and slightly extended so that it will fit into a screw cap.

The screw cap consists of a metal cylinder with internal screw threads upon which vaseline is applied and into which a metal screw is tightly fitted. Fitted to the end is a thick rubber tube into which the base of the pipet is inserted.

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*Titrimetric apparatuses of this type include products of Beckman and Coleman. The units are imported by Abe Shoji Co., Ltd. (c/o Osaka Building, 1 Munedore-cho, Kita-ku, Osaka) and Fuji Kogyo (1-14 Yushima 4-chome, Bunkyo-ku, Tokyo). Recently, Toshiba-Beckman are reported to be manufacturing sets for ultramicro purposes in Japan.

**Screw cap type are commercially available from Kayagaki Irika Co., Ltd., (Tokyo).
Fig. 18. Screw Cap Measuring Pipet

pp': Measuring pipet (the one shown has a volume capacity of approximately 0.35 ml).
S: Screw cap. The p' end of the measuring pipet is inserted into the rubber tube (r).

When the screw is loosened and retreated, negative pressure is created drawing solution into the pipet. Blowing-out and aspiration of a minute amount of solution can be readily controlled. It is an instrument suitable for expelling and aspiration very small volumes of solution. The maximum volume that can be taken is 0.5 ml. The pipet with capillary-ended-tube mentioned above is connected with the screw cap on use.

The precision of titrimetry with the screw cap measuring pipet is comparable with the Piston Buret. However, it has the disadvantages that its operation cannot enjoy the easiness and speed of the Piston Buret.

(16) Method for dilution of standard solution to construct calibration curve

In ultramicro colorimetric methods, the best way for the construction of a calibration curve is to use the standard solutions of known concentrations of a particular substance to be determined which are equal in composition with respect to other chemical ingredients to the sample (or serum) as much as possible. Commercial control serum may be employed as such standard solutions. When they are not available, aqueous solutions of the particular substance in known concentration are used. On such occasions, the following method of dilution is recommended for the preparation of a series of gradedly diluted standard solutions.

Test tube (4) which contains the standard solution with concentration "C" (maximum concentration for a particular substance), blank test tube (B) which
contains solvent (generally $\text{H}_2\text{O}$), three test tubes ($18 \times 180$ mm) (1), (2) and (3) or a 50 ml beakers, a 10 ml pipet, and a 5 ml pipet are made available.

With the 10 ml pipet, 10.0 ml of solvent is accurately measured from test tube (B) and transferred to test tube (2). Using the same 10 ml pipet, 10.0 ml of standard solution (concentration "C") is transferred to test tube (2) and the contents are thoroughly mixed (the concentration in (2) will have become 1/2 "C").

Using the 5 ml pipet, 5.0 ml of the solvent is accurately measured from test tube (B) and transferred to test tube (1). With the same 5.0 ml pipet, 5.0 ml of the solution in test tube (2) (concentration 1/2 "C") is transferred to test tube (1) and mixed thoroughly*. Thus, in this test tube a solution with a concentration of 1/4 "C" has been obtained.

Using the same 5 ml pipet, 5.0 ml of the solution in test tube (2) is transferred to test tube (3). With the same 5 ml pipet, 5.0 ml of the solution in test tube (4) is transferred to test tube (3) and thoroughly mixed. The solution in test tube (3) will have a concentration of 3/4 "C" (Fig. 19).

![Diagram of test tubes and pipets](image)

Fig. 19. Method for preparing standard solution for calibration curves

(1), (2), .... are the various test tubes. (1), (2), .... refer to the order of the procedures.
The concentration of the standard solutions prepared are: B ...... 0, 1 ...... 1/4 C, 2 ...... 1/2 C, 3 ...... 3/4 C, 4 ...... 1 C.

SUPERVISION OF ROUTINE TESTS

The results of chemical analysis must be examined from the following 3 view points.

1. **Accuracy.** Is the substance to be analyzed determined specifically?
2. **Precision.** Is the variation of the values obtained by successive analysis of

*As this method is different from the conventional method, one will tend to be opposed to adopting it. However, once he becomes accustomed to it, he will realize that it is efficient and much more accurate.
the same sample reasonably small?

3. Reproducibility. Can the same value always be obtained when determinations using the same sample are repeated on separate days?

Needless to say that accurate methods of analysis with good precision and reproducibility are desirable for routine clinical chemistry, but speed and simplicity cannot be sacrificed even for the purpose of satisfying these 3 requirements. No matter how high the reliability of a particular analyzed value may be, if the procedure required for it is time-consuming, it will hardly be evaluated as a diagnostic index in routine clinical chemistry. Further, if the technique involved is highly complicated, its practical use will be hampered. Therefore, quantitative methods for clinical biochemistry should be simple and rapid in performance. The reproducibility must, of course, be good. As to the precision the coefficient of variation of the analysis of less than ± 5 % will be approved as allowable.

Accuracy

If analysis is performed N times on the same sample and the estimations obtained were \( X_1, X_2, X_3, \ldots, X_{N-1}, X_N \), the range of variation of the estimated values would be represented by \( \bar{X} \pm 2\sigma \), where \( \sigma \) and \( \bar{X} \) are expressed by the following equations.

Standard deviation:

\[
\sigma = \sqrt{\frac{\Sigma X^2 - (\Sigma X)^2}{N - 1}} \tag{1}
\]

Where \( \Sigma X = X_1 + X_2 + X_3 + \ldots + X_{N-1} + X_N \)

\( \Sigma X^2 = X_1^2 + X_2^2 + X_3^2 + \ldots + X_{N-1}^2 + X_N^2 \)

Mean value:

\[
\bar{X} = \frac{X_1 + X_2 + \ldots + X_N}{N} \tag{2}
\]

When the \( N \)-plicate estimations obtained follow Gauss' normal distribution (symmetrical bell-shaped curve), 68.3 % are located within the range from \( (\bar{X} - \sigma) \) to \( (\bar{X} + \sigma) \), that is \( \bar{X} \pm \sigma \), and 95.5 % are between \( \bar{X} \pm 2\sigma \), 99.7 % being within \( \bar{X} \pm 3\sigma \). In other words, the smaller the variation of the estimations, the smaller is the value of \( \sigma \). In other words, the smaller the \( \sigma \) value is, the greater is the precision. Therefore, precision can be expressed by \( \sigma \). However, if the absolute values of the respective estimations \( X_1, X_2, \ldots \) are large, \( \sigma \) will also become large. Thus, it will be germane to evaluate the precision as the \( \sigma \) expressed in terms of the percentage of the mean value \( \bar{X} \) rather than as the absolute value. This ratio is called coefficient of variation (CV).

\[
CV = \frac{\sigma}{\bar{X}} \times 100 \% \tag{3}
\]

It would be ideal to calculate CV from the result obtained by a series of multiple
analysis on the same samples \((N>30)\), but this is troublesome and even if one wished to do so, there might not be enough sample material available. Therefore, in many instances one will be forced to make duplicate determinations, that is, determinations of \(X_1\) and \(X_2\), and be satisfied with mere calculation of the difference between the two, namely \(|X_1 - X_2|\).

However, it is fortunate that a rough estimate of \(\sigma\) is obtained from \(|X_1 - X_2|\) by the following equation.

\[
\text{Difference} = |X_1 - X_2| = \frac{2\sigma}{\sqrt{\pi}} = 1.128\sigma \quad \text{………(4)}
\]

**Tonks' Allowable Error**

Tonks claims that the allowable error in clinical chemistry depends on the normal range of the relevant substances to be analyzed. According to his opinion, the value obtained by analysis is significant for clinical interpretation, only when the quantititative error (analyzed value \(v\) minus true value \(v_o\)) of the analytical method employed for the determination of the relevant substance is smaller than \(1/4\) of the normal range of the same substance in serum or blood. If this requirement is satisfied, the result of analysis is regarded to be reliable and useful for clinical observation even though \(v\) and \(v_o\) are not equal. His idea will be explained, taking serum sodium as an example.

The normal range of serum Na is \(131 - 143\) mEq/\(\ell\). Assume that a serum with known concentration of \(120\) mEq/\(\ell\) Na was analyzed by a particular method and \(118\) mEq/\(\ell\) was obtained for it. Then, according to Tonks this estimation would be considered sufficiently accurate and precise for clinical use. The reason is as follows.

**Allowable limit of error for serum Na**

\[
\begin{align*}
\text{Allowable limit} & = \pm \frac{1/4 \times \text{normal range}}{\text{mean normal value}} \times 100 \\
& = \pm \frac{1/4 \times (143 - 131)}{137} \times 100 \\
& = \pm \frac{1/4 \times 12}{137} \times 100 \\
& = \pm \frac{3}{137} \times 100 = \pm 2.2\% \quad \text{………(5)}
\end{align*}
\]

The allowable limit for the true serum Na concentration of \(120\) mEq/\(\ell\) will, therefore, be \(\pm 2.2\%\) of \(120\) mEq/\(\ell\) or \(\pm 2.64 \div 2.6\) mEq/\(\ell\). So, if the estimated value obtained is within \(120 \pm 2.6\) mEq/\(\ell\) or in the range \(117.4 - 122.6\) mEq/\(\ell\), it will be accepted as satisfactory. The value \(118\) mEq/\(\ell\) is within this range.

Tonks' method will be employed when a particular procedure for analysis is appraised with respect to its usefulness in clinical chemistry by using commercially available control sera (Versatol, Hyland's control serum, Chemtrol, etc.) which have
the record of the true value of analysis on their labels.

**Recovery test**

A particular substance is added to the sample possessing this substance in concentration $c$ until concentration $(c+a)$ is reached and both the original sample and the sample to which the substance has been added are analyzed simultaneously to see if the difference $d$ of the estimations between the two is equal to $(c+a) - c = a$. This is called recovery test. If $d$ is entirely equal to $a$, the recovery test is said to be 100%. If $d$ is smaller than $a$ or greater than $a$, it is said that "recovery is poor" or "excessive", respectively. The ratio of $d$ to $a$ expressed in percentage is called recovery rate. A quantitative method which gives a recovery rate of 100% is, of course, satisfactory for clinical use. If the rate is outside of the range from 85% to 115%, the method may be considered unsatisfactory.

Hence, a "recovery rate of 100%" has been interpreted as an evidence for that a particular substance is estimated specifically by the relevant method of analysis, namely, the method is accurate. Therefore, it has become our habit that every newly invented quantitative method is examined for its usefulness by recovery test.

However, unfortunately recovery test does not always assure the accuracy of the estimation, because it fails to reveal the concomitant presence of a substance which gives a reaction similar to that of that of the substance contained in the sample. The concentration $c$ which is obtained by analysis of the sample includes the false substance simulating true substance by reaction as well as the particular substance to be determined as a whole, and this false substance can not be discriminated from the true by recovery test. A recovery rate of 100% means merely that there is a linear relationship between concentration and optical density (or titration value) within the range of concentration from $c$ to $(c+a)$. In other words, recovery test is no more than a check-up for the limits in which lineality of calibration curve is insured.

**Control of Routine Tests**

The greatest interest taken in clinical biochemistry will be the reliability or trustworthiness of the estimation values obtained in the laboratories. Administration or control of routine test, particularly with respect to reproducibility is, therefore, an important problem for clinical biochemists. Daily use of control sera of known estimated value for particular substances are recommended for this purpose. These sera are obtainable from the market, but they can be prepared in the ordinary laboratory for clinical chemistry.

In ultramicro analysis, a Sanz pipet, the same one as that used for taking samples, is used for pipetting the standard solutions for the construction of a calibration curve (that is, the pipet is not changed for samples and standard solutions). At the same time, control serum of known concentration is pipetted by the same Sanz pipet, in duplicate, into two control test tubes. These are
inserted in a row of sample test tubes separately, being set one from another by intervention of a considerable number of other tubes. The operators or the technologists responsible for the performance of determination should not be notified of the location of the control test tubes. Therefore it is recommended that one of the technologists is assigned to pipet the samples and standard solutions into the test tubes, and these are handed over to other technologists who are charged with actual performance of determinations by means of minipettes and colorimeters.

**Control Chart**

The results of the duplicate determinations of the control serums which were inserted between the sample tubes should be plotted on a graph for each of routine test every day. The daily mean value of the two estimations should be connected by lines (Fig. 20). This will show day-to-day variation (that is, reproducibility) and the daily differences between the 2 values will indicate the precision of analysis from day to day.

![Control Chart](image)

**Fig. 20. One example of a Control Chart**

The daily duplicate determinations are entered onto the graph, and when conditions have changed, such as by the preparation of new agents or by change of the examiners, a notation as to such effect should be made.

The control would be ideal if the fluctuations of the mean values remain within ± 5% of the true concentration of a particular chemical component in the control serum, or it is thought to be acceptable if the difference from the true value is within Tonks' range of allowable error.

It would be desirable that the difference between the duplicate determinations were 0, but this is quite difficult to achieve for some types of analysis. If the degree of difference is within the range of the standard deviation as estimated by Equation (4), the control of determinations is considered acceptable.
Control Serum\textsuperscript{7–10)}

The following types of control serum are commercially available.

VERSATOL (WARNER-CHILCOTT)*

A: Pathologic serum
Alternate: pathologic serum

E: For enzyme
Normal: Normal serum
P: For pediatric cases

HYLAND**

Normal clinical chemistry control serum

DADE*** control serum

Since the commercial control sera are expensive, it is hardly be possible financially to use them every day. Thus, it would be wise to use self-prepared control serum for daily requirements.

How to Prepare Control Serum

Obtain 300–400 ml of human blood serum from a blood bank, and filter it through a Seitz filter or centrifuge it to get clear supernate. Dissolve crystallized glucose (Merck) to the supernate so that glucose concentration may be adjusted to 100–200 mg/dl (roughly addition of 150 mg glucose to 100 ml serum will be sufficient). The glucose concentration of purchased serum is usually 40–50 mg/dl.

A large number of empty, washed and dried penicillin vials are arranged and 5.0 ml (minipette No. 5) aliquots of serum are distributed into each vial to be frozen in the freezing chamber of a refrigerator. Next, they should be lyophilized in a refrigerating vaccum dessicator (lyophilizer). If all of the bottles cannot be put in the lyophilizer at a time, they may be divided into 2 or 3 for lyophilization (the vials should be kept in the freezing chamber until they are placed in the lyophilizer). It is possible to process 400 ml of serum in about 48 hours.

A rubber stopper followed by an aluminum cap should be applied to the serum vials which have been lyophilized. (The serum in the vial will be seen as light yellow powder). These are kept in a refrigerator (2–10°C). They will keep for 2 months or more over.

On use, the commercial product should be dissolved in its vial by adding the amount of \( H_2O \) (25°C) specified on its label, while the self-prepared product should be dissolved similarly by adding a fixed adequate amount of \( H_2O \) (25°C) from 3 to 4 ml using a measuring pipet. For the dissolved self-prepared control serum, its chemical components should be analyzed individually by repeated determinations (several times) to establish their true estimation values. It is desirable

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*Ono Yakuhin (Pharmaceutical Co.), (2-14 Doshu-machi, Higashi-ku, Osaka).
**Sanko Junyaku Co., (1-2 Koji-machi, Kanda, Chiyoda-ku, Tokyo).
***Midori Juji (3-1 Gamo-cho, Higashi-ku, Osaka).
that estimations of chemical ingredients except those of sugar and phenol turbidity are maintained within range of the normal human serum by adjusting the amount of water to be added to the lyophilized powder adequately.

REFERENCE