PART 4 Laboratory Investigation Using Only Manual Tests

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TOPICS COVERED

- ✓ When to Use Manual Tilt-Tube Technique
- ✓ How to Perform Manual Tilt-Tube Technique
- ✓ PT by Manual Tilt-Tube Technique
- ✓ APTT by Manual Tilt-Tube Technique
- ✓ Thrombin Time and Fibrinogen by Manual Tilt-Tube Technique
- ✓ PT-Derived Fibrinogen Test

There are advantages to using coagulometers for coagulation testing, including speed of analysis/throughput and consistency of analysis, which deliver precise and accurate results in a timely fashion. Although many different instruments for coagulation tests are available and in use throughout the world, the manual tilt-tube technique can still be employed successfully for determination of clotting time. This can be done for all samples if no suitable automated method is available, or for a subgroup of samples, either because automated analysis does not generate results on samples with specific features, causing incompatibility of sample with the instrument in use, or because the coagulometer method is temporarily unavailable. Tilt-tube methods are suitable as alternatives for clot-based methods and even comprehensive hemophilia center laboratories should have the manual tilt-tube method available for those very few samples where automated analysis fails, but where results are really needed for safe patient management. This may be the case in the presence of grossly elevated plasma lipid concentrations, or where the clot formation pattern in the sample differs markedly from normal samples, particularly when the fibrinogen to fibrin polymerization is markedly abnormal. Because of the many variables and possible sources of contamination associated with manual techniques, these may require duplicate tests. If the between-day CV of IQC results is >5%, then duplicate testing should be considered. Where tests are performed in duplicate, the two results should be within +/- 5% of the mean with practice.

When to Use Manual Tilt-Tube Technique: Then manual tilt-tube method can be successfully used for determination of PT, APTT, thrombin time, and fibrinogen, as well as clotting factor assays based on PT and APTT.

How to Perform Manual Tilt-Tube Technique: The method of performing the tilt-tube technique for coagulation testing has been recently harmonized in relation to PT testing as part of the calibration of reference thromboplastins for the international normalized ratio (INR) system used for monitoring vitamin K antagonist drugs (Van den Bessellaar et al, 2020). This harmonized method has improved agreement between tilt-tube PT results when testing is done by different operators and in different centers. This method can be used for tilt-tube testing for APTT, thrombin time, and fibrinogen analysis in addition to PT testing.

Materials required:

1) Water bath for keeping the test tubes at a constant temperature of 37°C. Dimensions close to 40 x 30 x 20 cm are convenient. The water in the bath should be circulated continuously by a pump if possible. The temperature should be 37°C (tolerance limits: 37 ± 0.5°C). The temperature should be controlled with a calibrated thermometer.
2) A light source, such as an angle poise lamp, mounted 20 cm above the water level can be used to illuminate the test tube during tilting which facilitates the endpoint clot detection by the operator. LED as a light source is preferred over bulbs which generate heat since that can elevate the temperature of test tubes held close to the light source.

3) Test tubes should be non-siliconized, previously unused glass tubes. Disposable culture tubes (catalogue number 73500-1275, Kimble Chase Life Science and Research Products LLC, Vineland, New Jersey) with dimensions of 75 x 12 mm and wall thickness 0.8 mm were used in the work to harmonize PT tilt-tube testing (van den Besselaar et al, 2020) but 75 x 10 mm tubes can also be used. The test tubes should be made of borosilicate glass. Test tubes should be discarded after use and not washed for re-use. Different sources of glass test tubes can be used successfully, but they may influence the clotting times obtained, particularly in screening tests such as APTT. If the source (manufacturer or composition) of test tubes is changed, the possibility that results have been influenced should be considered. This could be assessed by comparing a small number of tests, such as APTT, with the two types of tube. If systematic differences are present, a new normal range should be established.

Technique:

- The temperature of the room in which the equipment is installed and where the technique is to be performed should be maintained at 20–22°C.
- Prior to commencing PT, APTT, TT, or fibrinogen manual tests in the water bath, the temperature should be checked and subsequently recorded.
- Empty test tubes should be kept in a vertical position in a rack in the water bath at 37°C for at least 4 minutes at a depth of 3.5 cm before addition of reagents and plasma.

PT by Manual Tilt-Tube Technique:

- For PT testing, add 200 µl of the thromboplastin/calcium reagent and incubate for 2 min.
- Then pipette 100 µl of not pre-warmed plasma, dispensing from a height of 1 cm above the level of thromboplastin, with the tip resting against the wall of the test tube, and immediately start the stop watch with the other hand.
- Shake the tube gently to mix the contents, with the tube immersed in the water.
- Put the tube in the rack in the water bath.
- Lay down the pipette.
- The test tube should manually be kept in the water, with the water covering the bottom 5 cm of the tube (Figure 1).
- Manual titling of the tube should commence 7 seconds after starting the stopwatch.
- The tube should be tilted at an angle of nearly 90° by taking the tube out of the water for 2 seconds and putting it back in the water for 1 second (Figure 1). Note that tilting the tube by 90° or more will usually lead to the reaction mixture spilling out from the tube.
- The tube should not be stationary during this cycle but continuously tilted with the operator's hand resting on the side of the water bath.
- This cycle is repeated until the clot forms.
- In the horizontal position, the tube is kept not more than 10 cm and not less than 2 cm above the water level (Figure 1).
- Before the mixture clots, the operator observes the mixture flowing from the bottom to three quarters of the length of the tube in the nearly horizontal position and back to the bottom.
- When clotting commences, the speed of flowing is reduced.
- When flow is stopped, the operator stops the timer and records the clotting time in seconds to one decimal place.

The operator performing the manual tilt-tube technique lifts the test tube regularly out of the water. Taking the test tube out of the water will result in a temperature drop of the reaction mixture. Immersing the tube back into the water will result in a temperature increase. The average temperature drop observed in the manual tilt-tube technique using the method described above is limited to 0.4°C (van den Bessellar et al, 2020).

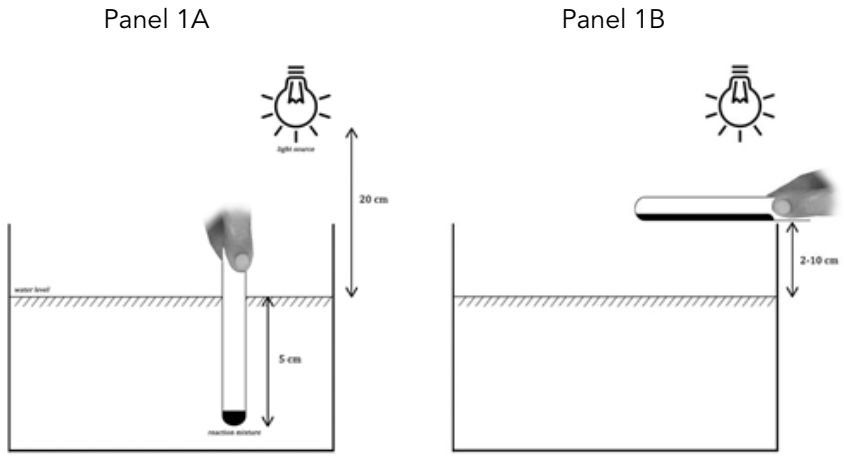

Figure 1. Schematic representation of the manual tilt-tube technique. Panel 1A: the test tube is in the vertical position in the water bath. Panel 1B: the test tube is in the horizontal position out of the water bath. The hand of the operator is resting on the edge of the water bath. Due to variation in the size of the operators' hands, the distance of the tube in the horizontal position to the water surface varies between 2 and 10 cm. The dimensions of the picture are not to scale. (Reproduced by kind permission of Elsevier Publishing, Amsterdam, Netherlands, from: Van den Besselaar et al. J Thromb Haemost. 2020; 18: 1986-1994.)

The above harmonized method was developed over two wet workshops assessing PT testing by up to seven operators from three centers, which identified a number of variables in technique (van den Besselaar et al, 2020). In particular, the workshops confirmed that dispensing the final component of the reaction mixture to initiate coagulation high in the upper part of the test tube was associated with longer clotting times then dispensing close to the surface of the reaction mixture lower down in the tube. Using the harmonized method described, the between-operator CV of Ts on the same test plasmas was 3% for a normal PT and 1.4% for a prolonged PT.

APTT by Manual Tilt-Tube Technique:

- Add 100 µl of APTT reagent to tube and incubate for 2 min.
- Then pipette 100 µl of not pre-warmed plasma, dispensing from a height of 1 cm above the level of APTT reagent, with the tip resting against the wall of the test tube and immediately start a stop watch with the other hand.
- Shake the tube gently to mix the contents, with the tube immersed in the water.
- Put the tube in the rack in the water bath.
- After the activation time recommended by the APTT reagent manufacturer (usually this is 3 mins, but can be 5 mins for others), add 100 µl calcium chloride which has been pre-warmed to 37°C in a separate tube in the water bath, dispensing from a height of 1 cm above the level of the reaction mixture, with the tip resting against the wall of the test tube, and immediately start a new stop watch with the other hand.
- Shake the tube gently to mix the contents, with the tube immersed in the water.
- Put the tube in the rack in the water bath.
- Lay down the pipette.
- The test tube should be kept manually in the water, with the water covering the bottom 5 cm of the tube (Figure 1).
- Manual titling of the tube should commence 15 seconds after starting the stopwatch.
- The tube should be tilted to an angle of nearly 90° by taking the tube out of the water for 2 seconds and putting it back in the water for 1 second (Figure 1). Note that tilting to 90° or more will usually lead to the reaction mixture spilling out from the tube.
- The tube should not be stationary during this cycle but continuously tilted with the operator's hand resting on the side of the water bath.

- This cycle is repeated until the clot forms.
- In the horizontal position, the tube is kept not more than 10 cm and not less than 2 cm above the water level (Figure 1).
- Before the mixture clots, the operator observes the mixture flowing from the bottom to three quarters of the length of the tube in the nearly horizontal position and back to the bottom.
- When clotting commences, the speed of flowing is reduced.
- When the flow is stopped, the operator stops the timer and records the clotting time in seconds to one decimal place.

Thrombin Time and Fibrinogen by Manual Tilt-Tube Technique: The manual tilt-tube methods should use the proportions of reagents and plasma or plasma dilutions recommend by the reagent manufacturer and follow the principles of the methods described for PT/APTT above.

Samples with lipidemia: Many coagulometers in current use that utilize photo-optical endpoints for analysis, are very tolerant of high lipid levels in the samples. However, samples may occasionally have such a high level of lipids that the analyzer cannot detect clot formation. Such samples can be analyzed manually and usually produce a solid clot that can be observed visually for PT, APTT, and thrombin time. It can be difficult to observe clot formation during Clauss fibrinogen analysis of such samples. If that is the case, another option is to subject the samples to centrifugation at 10,000g for 10 min at ambient temperature if available (ICSH guidance; Kitchen et al, 2021). After this ultracentrifugation, the lipid is sedimented and the sample can be analyzed on an automated coagulometer if available, or by the manual tilt-tube technique for PT, APTT, thrombin time, or fibrinogen.

Samples with fibrinogen abnormalities: Clot formation can be deranged in the presence of some fibrinogen abnormalities. For example, fibrinogen Longmont is associated with a weak and translucent clot during the Clauss fibrinogen assay. Photo-optical systems that monitor scattered light as the clot forms (as opposed to transmitted light monitoring) may be unable to detect the endpoint. Such samples can be analyzed using the tilt-tube method, but the operator must be aware that the clot formation may be difficult to discern visually. For some dysfibrinogenemia samples where automated analysis fails, the manual technique should be done with very careful scrutiny of the clotting process, since clots may be fragile and easily disrupted by additional mixing/tilting after the initial clot has formed.

Quality control: Quality control material, as described elsewhere in this manual for PT, APTT, thrombin time, and fibrinogen, is suitable for use with manual test methods. It is acceptable to test a single level of IQC, where the manual technique is reserved for occasional samples that fail automated analysis. Two levels should be available and tested according to the criteria described elsewhere in this manual, where manual testing is the primary analytical procedure in the laboratory.

The range of IQC results obtained by a small group of different operators experienced in the manual tilt-tube technique should typically be as follows:

Mean +/- 1 second for a QC with a mean PT in the range of 10 to 12 seconds,

Mean +/- 2.5 second for a QC with a mean APTT in the range 25 to 30 seconds,

Mean +/- 2.5 second for a QC with a mean thrombin time in the range 12 to 20 seconds,

Mean +/- 0.5 g/l for a QC with a mean fibrinogen of 2.5 to 3 g/l

Normal ranges: Manual test results are usually different from those generated by coagulometers. Typically, clotting times for PT and APTT are shorter for analyzers using photo-optics to monitor clotting compared with results that are obtained manually. The degree of difference is not consistent across different analyzers which may use transmitted or scattered light. Analyzers monitor change in light scatter/transmission over time after clotting is initiated and record clotting time as the time taken to exceed a particular threshold

of change. This may be a threshold as low as 3% change over baseline or a high as 50% change. The lower the % change used in clotting curve analysis, the shorter the clotting time reported. This means that results of manual tests should not be reported alongside a reference range established for an automated technique, even when the same reagents are being used. There are two options for dealing with this issue. One is to establish a reference range for the manual technique using the process described elsewhere in this manual. This should be done if all testing in the lab is carried out using manual techniques. More often, manual testing is restricted to a small subgroup of samples where automated analysis has failed. In this case, most results being released by the lab will be issued with the relevant automated reference range. It is unhelpful to users of the service to see occasional PT or APTT results issued with a different reference range. In this case, the lab can take a pragmatic approach and use a conversion chart as described below, so that testing is done by manual technique, but the test result is converted to the result that would have been obtained if the sample had been analyzed using the automated method.

Converting Manual PT, APTT, and Thrombin Time Results into Automated Equivalent Numbers: A series of 20 to 30 samples covering a range of normal and abnormal results should be tested using both manual and automated methods. The data should be analyzed using regression analysis to establish the relationship between results obtained using the two methods. There should be a significant correlation between results, with a correlation coefficient >0.8. If so, the regression relationship can then be used to create a table which shows the manual result and the equivalent result that would have been obtained on the automated system. When a manual test is performed, the manual result is converted into the automated result, which is then reported alongside the usual automated method reference range. This means the users of the service will only see a single reference range for PT, APTT, or thrombin time. This is important, since such reference ranges are likely to be incorporated into clinical protocols used for patient management. Table 5 shows the manual and automated results obtained by testing the same 21 samples using both methods.

Table 5. Manual and automated APTTs on the same samples

Sample number	Manual APTT (sec)	Automated APTT (sec)	Sample number	Manual APTT (sec)	Automated APTT (sec)
1	31.7	27.0	12	31.7	30.1
2	51.1	46.9	13	31.9	28.9
3	27.2	26.5	14	36.2	33.2
4	42.2	39.4	15	33.1	28.5
5	34.5	30.4	16	40.0	36.4
6	44.2	43.2	17	37.1	30.7
7	33.0	30.5	18	29.2	25.6
8	31.9	30.0	19	35.2	28.1
9	22.2	19.6	20	37.1	34.7
10	34.0	27.8	21	36.5	31.4
11	32.5	31.3			

The linear regression relationship between the two data sets is calculated using a statistics package. In this example, the correlation coefficient (r) is 0.96 and the regression relationship is calculated as:

$$y = 0.9551x - 1.887$$

where

y is the automated APTT,
x is the manual APTT
0.9551 is the slope of the linear regression line
– 1.887 is the y intercept.

This equation is used to derive the automated APTT from the manually determined APTT of any test sample. It is convenient to prepare a table relating manual APTT to the automated equivalent result. Table 6 was derived using the above regression equation.

Table 6. Conversion chart: Manual APTT to Automated equivalent APTT

Manual APTT (sec)	Automated APTT (sec)	Manual APTT (sec)	Automated APTT (sec)	Manual APTT (sec)	Automated APTT (sec)
19	16.3	40	36.3	61	56.4
20	17.2	41	37.3	62	57.3
21	18.2	42	38.2	63	58.3
22	19.1	43	39.2	64	29.2
23	20.1	44	40.1	65	60.2
24	21.0	45	41.1	66	61.2
25	22.0	46	42.0	67	52.1
26	22.9	47	43.0	68	63.1
27	23.9	48	44.0	69	64.0
28	24.9	49	44.9	70	65.0
29	25.8	50	45.9	71	65.9
30	26.8	51	46.8	72	66.9
31	27.8	52	47.8	73	67.8
32	28.7	53	48.7	74	68.8
33	29.6	54	49.7	75	69.8
34	30.6	55	50.7	76	70.7
35	31.5	56	51.6	77	81.7
36	32.5	57	52.6	78	82.6
37	33.4	58	53.5	79	73.6
38	34.4	59	54.5	80	74.5
39	35.4	60	55.4		

Manual Clauss fibrinogen assay: The regents and methods used for the manual Clauss fibrinogen assay are the same as for the automated version (i.e. same buffer, thrombin, and test sample dilution). The clotting time of the manual tests is converted into fibrinogen concentration using a calibration curve. The calibration curve should be constructed using the same calibrator and calibrator dilutions as would be used for automated testing (see Clauss assay section elsewhere in this manual), however, the clotting times of each calibrator dilution used to construct the calibration curve is determined manually. The results are thereby converted into fibrinogen concentration and the result is reported in the same format as the automated version (g/l or mg/dl) using the same reference range as for the automated Clauss method in the same center.

References

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