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REVIEW

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International Council for Standardization in Haematology (ICSH) recommendations for the performance and interpretation of activated partial thromboplastin time and prothrombin time mixing tests

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Abstract

This guidance document has been prepared on behalf of the International Council for Standardization in Haematology (ICSH). The aim of the document is to provide guidance and recommendations for the performance and interpretation of activated partial thromboplastin time (APTT) and prothrombin time (PT) plasma mixing tests in clinical laboratories in all regions of the world. The following areas are included in this document: preanalytical, analytical, postanalytical, and quality assurance considerations as they relate to the proper performance and interpretation of plasma mixing tests. The recommendations are based on good laboratory practice, published data in peer-reviewed literature, and expert opinion.

KEYWORDS
APTT, coagulation, ICSH, mixing test, PT

1 | INTRODUCTION

Plasma mixing tests (also known as plasma mixing studies or inhibitor screen) are often performed in the laboratory evaluation of an unexpected prolongation of an activated partial thromboplastin (APTT), prothrombin time (PT), or both, to aid in the determination of the cause of the prolongation, whether it is due to factor deficiency(ies) or an inhibitor.[1] This distinction is clinically important and likely will direct further evaluation and treatment. Plasma mixing tests are often also performed in the screening for the presence of a specific factor inhibitor in a patient under treatment for factor deficiency and are a part of the diagnostic criteria for detecting a lupus anticoagulant (LA).[2,3] Plasma mixing tests are typically performed by combining equal portions of patient plasma with normal pooled plasma (NPP) followed by immediate performance of the APTT and/or PT. Variations on this 1:1 ratio, as well as the addition of an incubated step at 37°C, are sometimes performed.[4,5] In most cases, correction of the prolonged clotting time by the addition of NPP is indicative of a factor deficiency while lack of correction or incomplete correction suggests the presence of an inhibitor.[5,6] Incorrect performance or interpretation of plasma mixing tests can lead to patient mismanagement.[7] While this document describes APTT and PT plasma mixing tests, the mixing test can also be applied to other activity-based hemostasis assays, such as the dilute Russel's viper venom and von Willebrand activity, to name a few.

The majority of peer reviewed literature on PT and APTT plasma mixing tests is limited to a single instrument or reagent platform,

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limited sample selection (i.e., investigation of LA), limited methods of result interpretation, and rarely include PT mixing studies.[8–11] There is in addition, no published guidance document on the recommended performance and interpretation of mixing tests, other than for LA.[12,13]

The recommendations in this guidance document are based on good laboratory practice, published data in peer-reviewed literature, and expert opinion. Plasma mixing tests are considered laboratory-developed tests and the validation and performance of such should follow local regulatory authorities.

2 | PREANALYTICAL

Preanalytical considerations as they pertain to plasma mixing tests, include patient history, medications the patient is taking, specimen collection, handling, transport, processing, storage, and NPP source.[14,15] In the ideal scenario, patient history is available and can be reviewed prior to the determination as to whether a plasma mixing test should be performed. See Table 1 for a list of potential preanalytical interferents or conditions that may yield factitious mixing test results.

2.1 | Appropriate use of the mixing test

Mixing tests are often indicated in the evaluation of an unexpected prolongation of an APTT and/or PT. They are also indicated in algorithms to detect the presence of a LA. In patients with hereditary or acquired factor deficiency(ies), mixing studies can be performed to screen for the presence of an inhibitor. There is no clinical value in performing mixing studies when baseline APTT and/or PT values fall within the reference interval (RI).[5] Most RIs are normalcy for 95% of the population. When a ±2SD of the mean is used to determine the RI it may be of value to use +3SD of the mean as the indication for performing mixing studies, if statistically appropriate based on sample size, as it reflects normalcy for 99.87% of the population, thereby reducing performance of unnecessary mixing studies and partly accounting for reagent variation.[16,17] Best practices in the evaluation of a prolonged APTT and/or PT are beyond the scope of this guidance document. It should be noted however, that the initial evaluation of samples with minor prolongations of clotting times is often to repeat testing on a new plasma sample. This will potentially exclude preanalytical errors that could cause a spurious result. Furthermore, many isolated slightly prolonged APTT results in an acute setting are not associated with a clinically significant underlying abnormality.

1. Recommendation 1.1.1: Plasma mixing studies should not be performed when baseline APTT and/or PT are within the normal RI.

TABLE 1 Provisional list of mixing study interferants or conditions that may yield factitious mixing study results.

False negative mixing study (i.e., missing the presence of a deficiency or inhibitor in the sample)	False positive mixing study (i.e., suggesting a deficiency or inhibitor is presence when it is not)
1. Poorly processed samples, excess platelets in plasma before freezing 2. Incubation step not performed 3. Water bath temperature not at 37 ± 2°C 4. Factor replacement therapies administered before sample collection. 5. Patients receiving bypassing agents such as FEIBA, emicizumab 6. Non-neutralizing antibodies 7. Weak or low titer inhibitors 8. Incorrect plasma: NPP ratio 9. Acute phase reaction i.e., elevated FVIII, fibrinogen, and C-reactive protein (APTT only) 10. Unsuitable Normal pooled plasma 11. Poorly assigned cut-offs 12. Upper end of normal reference interval set too high	1. Blood collected in tubes containing anticoagulants other than sodium citrate 2. Poorly assigned cut-offs 3. Normal pooled plasma sources with factor levels <80% 4. Upper end of normal reference interval set too low 5. Heparins and heparinoids 6. Parenteral and oral thrombin inhibitors 7. Oral factor Xa inhibitors 8. Thrombolytic therapy 9. Lipoglycopeptide class antibiotics

2.2 | Patient history

Patient history should include personal or family history of any coagulation abnormality such as a factor deficiency or the presence of an inhibitor. It should be determined if the patient has recently been treated for a coagulation disorder, specifically, have there been recent therapies that may interfere with the proper interpretation of plasma mixing tests, such as the recent infusion of fresh frozen plasma, cryoprecipitate, or single factor replacement therapy. The recent administration of bypassing agents such as Factor Eight Inhibitor Bypass Activity (FEIBA, Takeda Pharmaceuticals) or emicizumab (Hemlibra, Genentech USA) may mask the presence of an inhibitor and are likely to normalize the clotting time.[18–20] Medications should be reviewed for those that may impact the APTT and/or PT, especially anticoagulant agents including direct oral anticoagulants (DOACS), heparins, and oral anti-vitamin K antagonists (OVKA).[21,22] Heparins, heparinoids, direct thrombin inhibitors (DTIs), and direct activated X (Xa) inhibitors, function as inhibitors generally resulting in failure to correct the mixing test. Performance of a mixing study in the presence of recent anticoagulant therapies, including inhibitory anticoagulants without knowledge of their presence, can lead to misinterpretation of test results with potential patient mismanagement. Performing mixing studies when the patient's plasma contains parenteral or orally administered DTIs, as an example, can mimic a factor inhibitor or the presence of a LA.[21,23] When patient clinical history and/or medication list is not available to the laboratory, the performance of additional tests on the patient sample such as thrombin time or anti-Xa activity may be useful to exclude anticoagulant drug exposure. Certain lipoglycopeptide antibiotics such as those effective against methicillin resistant *Staphylococcus aureus*, such as Telavancin (Vibativ; Cumberland Pharmaceuticals, USA) and Daptomycin (Cubicin; Merck & Co, USA), may

cause factitious prolongation of the APTT, and/or PT in a dose-dependent manner, due to an in vitro effect and leads to noncorrection in a plasma mixing study.[24,25] Select drugs or biologics that are pegylated may lead to clinically insignificant (i.e., factitious) prolongation of the APTT depending on the specific polyethylene glycol and the APTT reagent used.[26] C-reactive protein, an acute phase protein elevated in inflammatory conditions, may factitiously prolong the APTT in the presence of certain APTT reagents.[27] Patients on thrombolytic therapy may also yield results in mixing tests that suggest the presence of a deficiency.[28] Plasma mixing testing should be avoided in patients currently on these described therapies, but even "recent" exposure may affect coagulation testing due to variable drug half-lives. For assessing the potential impact of recent exposure, the time for drug clearance can be estimated by using 5–6 times the cited drug half-life.[29]

Mixing studies should be performed on the same sample as the prolonged APTT and/or PT.[30] It is conceivable that the remaining volume will be insufficient to perform mixing studies, especially if other testing was performed. If the original sample has insufficient volume to perform the mixing test, it is necessary that the newly collected sample be repeated for the test that was previously noted to be prolonged.

1. Recommendation 1.2.1: Patient history should be obtained and reviewed whenever possible to evaluate for potential personal or family history of a coagulation disorder.
2. Recommendation 1.2.2: Patient history should include recent treatments that may interfere with the interpretation of plasma mixing studies.
3. Recommendation 1.2.3: Patient medications should be reviewed for those that may alter the results of the APTT and/or PT such as anticoagulants, factor replacement or bypassing agents, thrombolytic therapies, certain antibiotics, and select pegylated drugs or biologics.
4. Recommendation 1.2.4: Mixing tests should not be performed when patients are known to be currently (or recently) on therapies described in recommendations 1.2.3.
5. Recommendation 1.2.5: Mixing studies should be performed on the same sample as the prolonged APTT and/or PT. If a repeat sample is collected, the prolongation of the clotting time(s) should be confirmed prior to performing mixing study testing.

2.3 | Patient sample: sample collection

Plasma mixing tests should be performed only on citrated plasma that has been collected, handled, and processed according to international guidance such as those developed by International Council for Standardization in Haematology (ICSH) or Clinical Laboratory Standards Institute (CLSI).[14,15,31] Samples should be collected from a peripheral vein, when possible, due to the potential for contamination of intravenous (IV) fluids. Blood samples may be acquired from indwelling catheters or lines once a sufficient volume of discard blood has been removed. Blood samples should not be collected above IV lines or other indwelling catheters.

Samples collected into additive tubes other than sodium citrate such ethylenediaminetetraacetic acid (EDTA) tubes (purple top), lithium or sodium heparin tubes (green tops) or a nonadditive tube (red or tiger top) will yield erroneous results in a mixing test and are unacceptable sample types that must be rejected.[14,31,32] EDTA samples, furthermore, yield results mimicking the presence of factor (F) VIII inhibitor, specifically demonstrating a decreased FVIII activity, incomplete correction upon mixing with NPP, and can demonstrate further prolongation with 37°C incubation.[32] Samples collected into lithium heparin collection tubes will also mimic the presence of an inhibitor. Serum samples are not acceptable due to in vitro consumption of clotting factors leading to prolongation of the APTT and PT.

When collecting blood into sodium citrate, it is imperative that the ratio of blood to anticoagulant is standardized to a ratio of 9:1 (blood: anticoagulant).[14,32] Tubes that are underfilled have a relative excess of citrate, which will subsequently bind reagent provided calcium resulting in artefactual prolongation of the APTT and/or PT. This effect may be minor in those tubes filled to between 80% and 90% of the target fill volume but are more likely to be clinically significant if the fill volume falls below 80% of the target volume.[33] Fill volumes below 90% may be acceptable if local validation confirms that the impact on results would not affect patient management. Tubes that are overfilled may have an inadequate volume of citrate and a tendency to develop clots. Samples that are clotted, even those that are partially clotted, or those that contain small clots in an otherwise liquid sample must be rejected as a clot in the sample may impact APTT and/or PT results. Even if no clotting occurred in an overfilled tube, the reduced amount of citrate relative to plasma volume requires less $Ca2^{+}$ to counteract the anticoagulant effect and can lead to shortened clotting times.

Another issue that may alter the ratio of blood to anticoagulant, other than filling, is the level of the patient's hematocrit. This is because the greater the hematocrit, the smaller the amount of plasma in a given volume. When the value of the hematocrit exceeds 0.55 (i.e., 55%), the ratio of plasma to anticoagulant is altered and mimics that of an underfilled tube, potentially causing spurious prolongation of the APTT and/or PT. This can be avoided by altering the volume of citrate in the collection tube in accordance with recommendations from CLSI or the World Federation of Haemophilia (WFH).[31,34] Prolonged baseline APTT and/or PT samples from patients with high hematocrits should have repeat testing performed on blood collection tubes with adjusted citrate volume. If the repeat APTT and/or PT testing is within normal limits using the adjusted citrate volume blood collection tube, then a mixing test is not indicated.

1. Recommendation 1.3.1: Samples should be collected into 3.2% sodium citrate at a 9:1 ratio of blood to anticoagulant.
   a. Samples collected into EDTA or heparin (sodium or lithium) as well as serum samples must be rejected.
   b. Collecting samples into 3.8% sodium citrate for APTT and/or PT analysis is not recommended.

2. Recommendation 1.3.2: Blood tubes with < 90% of normal filling volume should be rejected by the laboratory and should not be analyzed.
   a. Tubes with 80%–90% of nominal filling may be acceptable if local validation confirms that the impact on results would not affect patient management.
3. Recommendation 1.3.3: Samples that are clotted or that contain clots, even those with small clots in an otherwise liquid sample must be rejected.
4. Recommendation 1.3.4: The ratio of plasma to citrate anticoagulant should be adjusted when patient's hematocrit results exceed >55% (0.55).
   a. If the baseline clotting times were performed using uncorrected citrate volume on high hematocrit samples, the testing should be repeated with corrected citrated samples.
   b. If the repeat testing from citrate corrected plasma is within normal limits, mixing tests are not indicated.

2.4 | Patient sample: handling

Freshly collected citrated blood samples should be maintained at ambient (18–24°C) temperature until delivery to the laboratory.[15,31] Samples that are chilled (i.e., refrigerated or transported on ice) prior to centrifugation may demonstrate precipitation of certain factors including fibrinogen, FVIII, and Von Willebrand factor leading to prolongation of clotting times, as well as cold activation of FVII, resulting in shortening of the PT.[35,36] In cases of extreme weather conditions, the use of storage containers (i.e., styrofoam) that can maintain ambient temperature of the blood sample should be used to transport samples to the laboratory.

1. Recommendation 1.4.1: Whole blood samples for coagulation tests should be maintained at ambient temperature of 18–24°C prior to processing.

2.5 | Patient sample: centrifugation

Samples should be centrifuged at ambient temperature to yield platelet poor plasma (PPP), defined as having platelet counts <10 000/mm^3, to avoid the potential for platelet neutralization of an LA (this can occur when testing is performed on a frozen–thawed sample) as well as acceleration of the enzymatic reactions of the APTT and PT.[15,31] If samples will be processed for later testing, the samples should be prepared to ensure they are platelet poor, which can be accomplished by double centrifugation or centrifuging at a higher speed for a longer period of time prior to freezing. In the process of double centrifugation, following the initial spinning of the specimen, the plasma is carefully removed from the cells, avoiding the platelet/buffy coat using a plastic transfer pipette. The plasma should not be poured off. The aliquoted plasma is centrifuged again, and the top portion of the plasma removed, leaving about 0.25 mL in the bottom to discard. The double-centrifuged plasma should be aliquoted, labelled with the appropriate patient identification and as citrate plasma and frozen. Post-centrifugation platelet counts on citrated plasma should be checked before introducing new centrifuges into routine use, following any significant centrifuge repair or recalibration, at least annually, or at a frequency as required by the manufacturer. If each individual centrifuge cannot be checked locally, a process should be established to ensure centrifuges of like make and model yield PPP.

1. Recommendation 1.5.1: Prior to testing, samples should be centrifuged to yield post-centrifugation plasma platelet counts of <10 000/mm^3.
2. Recommendation 1.5.2: Prior to sample freezing, citrated samples should be double centrifuged.
3. Recommendation 1.5.3: Processing to generate PPP should be checked upon implementation of a new centrifuge, after major maintenance, at least annually, or per any frequency defined by the instrument IFU.

2.6 | Patient sample: sample stability

Plasma clotting factors are potentially unstable depending on the conditions and duration of storage that occurs prior to testing. In general, whole blood samples should be capped and maintained at ambient temperatures prior to processing.[15,31] Once separated (i.e., centrifuged but the plasma does not have to be removed from the cells), plasma can generally be maintained at room temperature for up to 4 h.

For those samples that cannot be tested within 4 h of collection, the PPP should be carefully transferred to an appropriately labelled polypropylene vial with suitable cap. It has been demonstrated that storage vial type (snap top cap vs. screw cap) and storage conditions (i.e. dry ice vs. ultralow freezer) may alter the performance of PT and APTT testing.[37,38] To thaw previously frozen samples, use a monitored 37 + 2°C water bath, not dry heat block or incubator and sufficiently submerge the vial to ensure the water line is higher than plasma level. Thaw at least 5 min/mL of sample and if not completely thawed, maintain vial in water bath another 3 min. The sample should be mixed before testing. At a minimum the reagent instructions for use (IFU) and international guidance documents should be followed.[14,15,31] Sample stability should be validated or verified for the coagulation tests and methods in use as well as the blood collection system used. If the original patient sample has been consumed or exceeds stability limits, performance of a mixing test on a new plasma sample should be performed only once the abnormal baseline clotting time has been confirmed on the new sample.

1. Recommendation 1.6.1: The allowed time between sample collection and testing should be predicated on the APTT and/or PT reagent IFU or international guidance documents.
2. Recommendation 1.6.2: Use of plasma samples that exceed the reagent manufacturer IFU or international guidance recommendations should be locally validated prior to implementation.

2.7 | Normal pooled plasma

NPP can be purchased as a frozen or lyophilized product, or made locally, but regardless, should be well characterized, have sufficient factor activity, and known to be absent of any inhibitor.[30] The NPP source should be of the same citrate concentration as used in collection of the patient samples. It should be determined prior to use that purchased NPP is approved by appropriate regulatory agencies or local standards for use in mixing tests. Commercially prepared NPP may have the advantage that it has been evaluated for individual factor activity levels. At a minimum, FVIII activity should be determined. Factor levels, including FXII, prekallikrein, and high molecular weight kininogen should be measured when possible. NPP should contain about 100% (80%–120%) of each factor and should have a fibrinogen value above 2.0 g/L. Pools should contain a minimum of 20 individual, ostensibly healthy donors in order to likely obtain 100% activity for all factors. The greater the number of donors in the pool, the more likely each factor activity of the pool will approximate 100%. NPP that contains close to 100% factor activity will provide at least about 50% factor activity in a 1:1 mix which should yield a normal clotting time (providing the reagent is responsive at that level) in the presence of a significant factor deficiency. This further emphasizes the need to characterize NPPs before use. Commercially prepared NPPs, particularly those that are lyophilized, may also be buffered. There is no consensus amongst experts as to the impact, positive or negative, of using buffered NPP in mixing tests. NPP, whether commercially prepared or made locally, must be prepared in a timely fashion and in accordance with international preanalytic guidance documents.[14,15,31] Caution is needed if patient plasma samples with normal clotting times are used in the creation of the NPP as they likely contain acute phase reactants including elevated fibrinogen and FVIII levels, which can cause false negative mixing studies.[30] As such, use of individual patient samples with normal APTT and PT values must not be substituted for NPP in a plasma mixing study. It is possible that a NPP with a PT or APTT clotting time too close to the lower limit of local RI as well as NPPs with a PT or APTT too close to the upper limit of RI may yield factitious mixing study results. For this reason, the PT and APTT of the NPP should fall near the mean value of the local laboratory's RIs.

The stability of NPP depends on a number of variables, including the temperature at which it is stored, that is, room temperature, on instrument, or refrigerated. In general, NPP should be used within 2 h of thawing or reconstitution although manufacturer recommendations should be followed unless local validation is performed to determine the stability for a given temperature.

1. Recommendation 1.7.1: Individual patient samples with normal APTT and PT values must not be substituted for NPP in a plasma mixing study.
2. Recommendation 1.7.2: NPP should contain about 100% factor activity (optimal range of 80%–120% activity) of each factor and must be free of inhibitors.
3. Recommendation 1.7.3: NPP should be used within 2 h of thawing or reconstitution, as defined by manufacturer, or according to local validation.

3 | ANAYLTICAL

Analytical considerations as they pertain to plasma mixing studies include the characteristics of the APTT and PT reagents used, the instrument platform, the NPP source, the procedure for performing the mixing study, whether this test is performed manually or on the coagulation analyzer, the ratio of patient plasma to NPP, the inclusion of an incubation step, as well as APTT and PT RI establishment.

3.1 | APTT, PT reagents, and instrument platform

APTT and PT reagents vary in their responsiveness (also referred to as sensitivity) to factor deficiencies as well as inhibitors, particularly LA, heparin, and DOAC anticoagulants.[21,39,40] APTT and PT reagents should have adequate responsiveness to factor deficiency such that there is prolongation of the clotting time when clinically significant factor levels (FII, FV, FVII, FVIII, FIX, FX, and FXI) fall below 35%–40%.[6,40] Reagent responsiveness varies between reagents from different manufacturers, can be different for each individual factor, and may vary between reagent lots of a single manufacturer. Reagents that are insensitive, that is, will not prolong when clotting factor activity falls below about 40%, may not be able to identify the presence of a deficiency, while reagents that are too sensitive, that is, cause prolongation when factor activities are >50%, may falsely suggest the presence of a deficiency. Information regarding reagent responsiveness to factor deficiencies, various anticoagulants, or LA should be available from reagent manufacturers or may be included in the reagent IFU. For a multitude of reasons, the individual factor responsiveness of each lot of APTT and PT reagents is difficult, if not impossible to determine in a standardized fashion, although manufacturers may be able to provide an estimate of relative responsiveness of a given reagent. There are published methods that can be used in local laboratories to determine reagent responsiveness.[40,41] Rather than using commercial factor deficient plasmas, well-characterized samples from patients with inherited coagulation deficiencies should be used to determine reagent responsiveness, although it is typically difficult for individual laboratories to obtain such samples in the necessary volumes for responsiveness testing.

Reagent stability varies as to the condition of storage, that is, room temperature, on instrument, or refrigerated. Stability must be followed in accordance with manufacturer insert or validated locally.

1. Recommendation 2.1.1: Laboratories should be aware of the relative responsiveness of the APTT and/or PT in use to the presence of LA, clinically significant factor activities, and to DOACS (i.e., dabigatran, apixaban, rivaroxaban, and edoxaban).

2. Recommendation 2.1.2: APTT and PT reagents should be used within stability limits as defined by the manufacturer for a given temperature or in accordance with local validation.

3.2 | PT and APTT RI determination

Establishment of an appropriate RI is an important variable as this will likely determine when a mixing test is indicated. RIs should be checked with each change in APTT and/or PT reagent lot. When the upper limit of the RI is set too high, this may cause a deficiency or inhibitor to be missed, while an upper limit of the RI that is set too low may falsely suggest a deficiency or inhibitor when not present. RIs should be established in accordance with international guidance documents such as CLSI or the International Federation of Clinical Chemistry Expert Panel on Theory of Reference Values.[40,41] Historically, RI is determined using a parametric or nonparametric calculation based on values from an appropriate population of at least 40 individuals providing the distribution of results is Gaussian.[17,42,43]

More recently, other methods of RI determination such as transference or an indirect method of RI determination have been applied.[43,44] The transference method requires less effort and less data than that required for the local establishment of a RI while the indirect method requires relatively large amount of data generated locally (or remotely if using the same population and methods).

When the transference method of RI determination is used, the local laboratory must always use RIs that are appropriate for their patient population and methodologies, and it is assumed that the original RI study from the external source was done with robust methodology, and statistical procedures.[44] If the RI originates from a different population and different laboratory method than the local laboratory, a method comparison must first be performed with acceptable performance. RIs can also be transferred from one laboratory to another as long as they each share common reagents and instruments and test similar populations. With each option, the RI to be transferred must be verified before implemented into use.

The indirect approach of RI determination uses local results from specimens collected for routine purposes, including screening, diagnosis or monitoring. This generally requires sample sizes in the thousands with proper statistical analysis, although in poorly represented populations (e.g., extremes of age), smaller numbers may still provide useful RI information.[43] Age adjustment of an RI may be required for laboratories that perform testing on pediatric patients.

1. Recommendation 2.2.1: Normal PT and APTT RI (including age adjusted RI) should be established in accordance with international guidance documents.

3.3 | Mixing study procedure

The classical mixing study is performed using a 1:1 ratio of patient to NPP and the mix tested immediately in the test system that was originally prolonged.[1,5,30] (Figure 1) Certain methods of mixing study

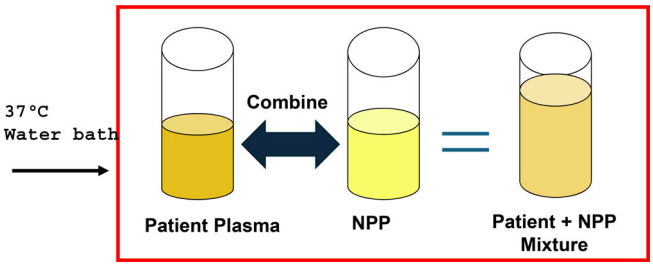

FIGURE 1 Incubated activated partial thromboplastin (APTT) mix procedure. Incubate normal pooled plasma (NPP) and patient/NPP mixture, each in a capped tube, in a controlled 37°C water bath for 60–120 min, then perform APTT on NPP and patient/NPP mixture.

interpretation require the NPP to be tested concurrently (see Section 4 below). A 4-part patient to 1-part NPP ratio is sometimes advocated to aid in the detection of a weak inhibitor. A 4:1 mixing test ratio has the disadvantage in that it can lead to a false positive lack of correction result in a patient with a severe factor deficiency when the NPP contains less than 50% of that factor and may be less effective in the presence of multiple factor deficiencies (i.e., high dose VKA anticoagulation).

The mixing of patient plasma with NPP can be performed using coagulation instruments that have been regulatory approved or locally validated for that purpose, but incubated mixing study samples will likely require manual preparation. Mixing studies should be performed within the stability limits of the patient sample and the thawed NPP. Mixing studies should be tested using the same reagent platform that yielded prolonged clotting times for the baseline APTT or PT. If a different reagent platform is introduced for mixing studies, then the APTT and/or PT should be retested using the new reagent system to verify the prolongation of the clotting time.[30] When setting up mixing studies, only polypropylene tubes (with accompanying caps if an incubation step is required) should be used, and of sufficient size to contain plasma mixtures.[31]

Incubated mixing tests can be of value for the identification of inhibitors that are time and temperature dependent.[1,5,30,32] Time and temperature dependence means that the inhibitor effect may not be evident in the immediate mix and may require incubation for a period of time at 37°C to become apparent. If the immediate mix indicates the presence of an inhibitor, an incubated mixing step is not mandatory, but could be performed if more specific tests are not available. In this situation, care must be taken to ensure that the plasma is not EDTA plasma, as clotting times in EDTA plasma may prolong with incubation. Typically, the presence of EDTA plasma can be indicated by a very elevated potassium level.[45] In the incubation step, a 37°C water bath should be used rather than a 37°C dry incubator, and the temperature monitored each day of use, with acceptability limits of 37 ± 2°C. (Figure 1) Samples should be capped and sufficiently submerged in the water bath such that the plasma-containing portion of the tube is submerged for the duration of the incubation. The incubated mixing test can theoretically be performed based on the APTT or PT although a recent study indicates that incubation of a PT mix leads to a high misprediction (false negative) rate for inhibitors.[46] For PT-only unexpected prolongations, time and temperature dependent inhibitors have not

been described, so routine use of incubated PT mixing studies is likely unnecessary. The only well characterized time and temperature dependent inhibitor is a factor VIII inhibitor. Incubation of the patient and NPP mix should occur for at least 1 h but longer incubations (i.e. 2 h) may be necessary to detect low titer inhibitors. Longer periods of incubation at 37°C (i.e., >2–3 h) may lead to loss of labile clotting factors to a degree that interferes with result interpretation.

The use of a control mixture for the incubation phase of a mixing study is a well-established method used to ascertain any factor lability in the NPP during to the 37°C incubation that may lead to a factitious incubated mixture study interpretation.[6] However, the control mixture is not required when using calculation indices (percent correction or Rosner Index) or the subtraction method as described in this recommendation. The recommended APTT based mixing study procedure (see Figure 1) and interpretation methods (see Figures 2 and 3) include the analysis of neat NPP at both phases (immediate and incubated) of mixing study testing, thus will account for any factor lability associated with the incubated phase testing.

1. Recommendation 2.3.1: Only polypropylene tubes with accompanying caps (if incubation is required) should be used for mixing studies. The tube size should be sufficient to handle the mixture volume, but not too large as to create potential pH changes in the plasma.
2. Recommendation 2.3.2: Mixing studies should be performed on plasma samples using a 1:1 ratio of patient to NPP.
3. Recommendation 2.3.3: APTT and/or PT of the NPP must be analyzed for each phase (immediate and incubated) in each batch of mixing tests.
4. Recommendation 2.3.4: The same reagent system used for baseline prolonged APTT or PT should be used for mixing studies.
   a. Baseline APTT or PT must be repeated if using a new reagent system to confirm prolongation prior to performing mixing studies.
5. Recommendation 2.3.5: Routine incubated PT mixing tests are not recommended.
6. Recommendation 2.3.6: Immediate and incubated mixing tests must be completed within the stability parameters of the patient sample and the NPP.
7. Recommendation 2.3.7: A monitored 37°C (±2°C) water bath should be used when performing an incubated mixing study.

4 | POSTANALYTICAL

Postanalytical considerations as they pertain to plasma mixing studies include the method to determine correction or noncorrection as well as result reporting. Typically, "correction" suggests a factor deficiency (ies), whereas "noncorrection" typically suggests an inhibitor.

4.1 | Methods to determine correction

A number of different methods and schemes are available to determine mixing study correction or lack thereof.[8,9,46] These methods vary in complexity as to how much information is required for mixing test result interpretation, including the use of different cut-off values or

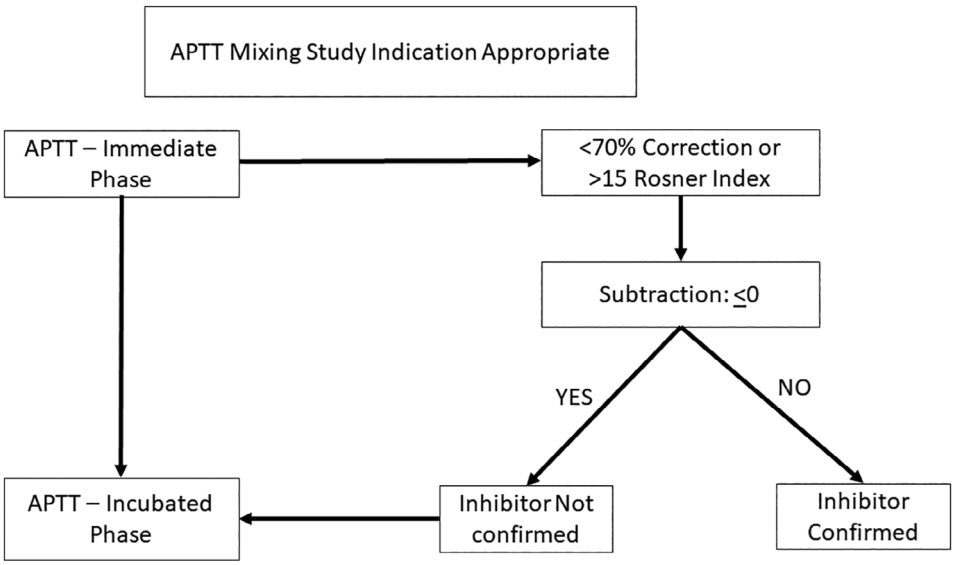

FIGURE 2 The immediate activated partial thromboplastin (APTT) mix should be performed when appropriately indicated. Equal volumes of patient and normal pooled plasma are mixed and an APTT is performed promptly. Either the percent correction calculation or the Rosner index can be used to interpret the result of the mix. Cut-off values for each of these calculations should be locally validated. In the published field study, a precent correction result ≥70% or a Rosner index result of ≤15 suggests correction indicating a likely factor deficiency. To determine if a time and temperature dependent inhibitor is present, an incubated mixing study is indicated. If the percent correction result is <70% or the Rosner index is >15, the subtraction method (result of the mix − NPP result) should be performed and if the result is ≥0, an inhibitor is present. If the result of the subtraction method is ≤0, proceed to the incubated APTT mix.

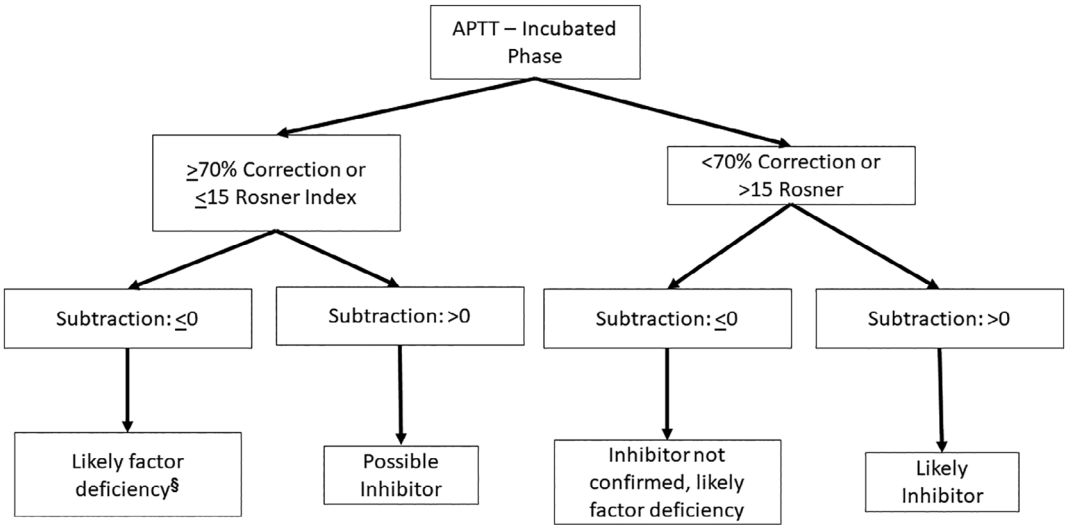

FIGURE 3 The incubated activated partial thromboplastin mix should be performed by combining equal volumes of patient and NPP and incubating in a capped tube in a controlled 37°C water bath for 60 to 120 min. Either the percent correction or the Rosner index can be used to interpret the result. Locally validated cut-offs for these equations should be used. It is important to note that when the algorithm suggests a factor deficiency that a non-neutralizing antibody cannot be excluded.

TABLE 2 Recommended Calculations for interpreting prothrombin time and activated partial thromboplastin time mixing studies.

Method ID	Formula	Explanation	Threshold for correction
Percent (%) correction	$\left(\frac{\text{Patient baseline result} - \text{1:1 mixture result}}{\text{Patient baseline result} - \text{NPP result}}\right) \times 100$	See calculation	≥70%
Rosner index	$\left(\frac{\text{1:1 mixture result} - \text{NPP result}}{\text{Patient baseline result}}\right) \times 100$	See calculation	≤15%
Subtraction method	1 : 1 mixture result − NPP result	NPP result (run concurrently) subtracted from mixed test result	≤0

Abbreviation: NPP, Normal Pooled Plasma.

thresholds. The mixing test calculation methods recommended in this guidance are provided in Table 2.

There is no one mixing test interpretation method that can consistently differentiate factor deficiency from inhibitor with 100% certainty.[5,46] Some overlap within interpretation methods between factor deficiency and inhibitor sample types is inevitable. This was confirmed in a recently published international APTT and PT mixing test field study evaluating multiple reagent platforms and 11 different methods of correction.[46] In this study, subtraction and ratio methods, while relatively easy to perform, tended to have high degrees of mispredictions. These methods tended to perform well for either factor deficiency(ies) or inhibitor samples, but not both. Overall, the percent correction method (using a >70% threshold) performed best for the PT mixing test (see Figure 4). The percent correction (using a 70% threshold) and the Rosner index (using a value <15), performed better for APTT mixing results, for all reagents tested, than any subtraction or ratio methods alone. While for APTT mixing studies, the percent correction method was slightly superior in accurate distinction than the Rosner index, neither demonstrated 100% specificity. Given this, an algorithmic approach for APTT mixing study result interpretation is recommended, employing either the percent correction or the Rosner index, followed by a NPP subtraction method (test result − NPP result) (see Figures 2 and 3). Supplemental figures are included that provide immediate and incubated APTT (Figures S1 and S2) and immediate PT (Figures S3 and S4) mixing test example calculations for both factor deficient and inhibitor samples. While a number of mixing test interpretation methods in the published field study had high rates of misprediction, these methods may be employed if successfully validated locally.

It is important to note that not all inhibitors result in noncorrection of an immediate or incubated mixing test.[47–49] Most factor inhibitors are neutralizing antibodies, and these are often evident in a mixing study as the antibody will interfere with the factor present in the NPP in vitro as well as the patient's factor. Some neutralizing antibodies that have type 2 kinetics, which occur more commonly with acquired inhibitors, demonstrate correction with a mixing test. Few inhibitors are non-neutralizing.[48,49] With non-neutralizing inhibitors, the antibody binds the factor, and the resultant antibody/antigen complex is cleared in vivo by the reticuloendothelial system. These non-neutralizing (or clearing) antibodies cannot be detected in a

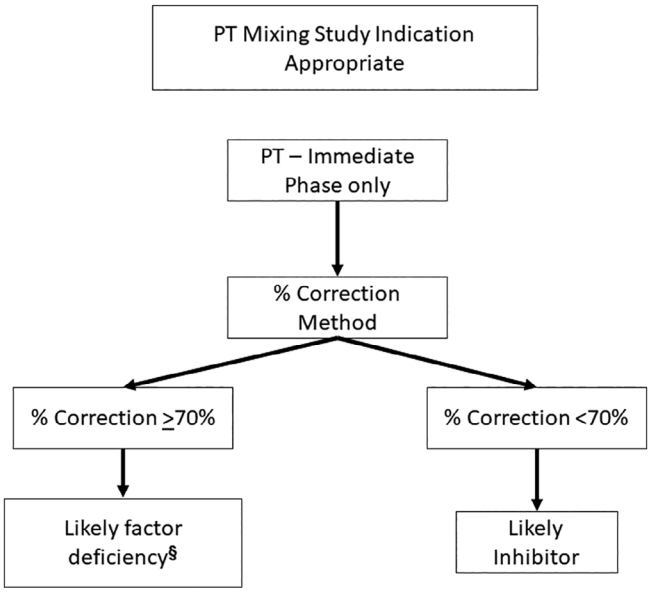

FIGURE 4 The immediate prothrombin time (PT) mixing test is performed by combining equal volumes of patient sample and normal pooled plasma and the PT performed promptly after mixing. The percent correction method should be used for result interpretation and the cut-off should be validated locally.

mixing test as the mix typically results in correction (since that antibody/antigen complex was cleared in vivo). The most common example of a non-neutralizing factor inhibitor is a prothrombin (factor II) antibody that develops in some patients with LA. These patients have very decreased levels of prothrombin when the activity is measured. The presence of non-neutralizing antibodies can usually be determined by pharmacokinetic studies that compare expected in vivo factor stability to actual factor activity levels after factor replacement. To perform pharmacokinetic studies, blood samples are collected, and factor activity measured over defined time periods and compared to the expected in vivo half-life of the factor activity. In the case of antibodies to prothrombin, an ELISA assay can be performed to confirm their presence. Not all patients with antiprothrombin antibodies as determined by ELISA have antibodies that bind and clear prothrombin.

1. Recommendation 3.1.1: The use of a single method of mixing test interpretation is not recommended to determine correction of an APTT mixing test, unless it is validated locally.
2. Recommendation 3.1.2: An algorithmic approach to determining correction in an APTT mixing study is recommended, specifically using the percent correction or Rosner index followed by a subtraction method (test result − NPP result) to determine if the mixing test result falls at or below zero (to indicate a likely factor deficiency) or greater than zero (to indicate a likely inhibitor).
3. Recommendation 3.1.3: For the PT mixing test, the percent correction should be used as the mixing test method of result interpretation (unless a different method is validated locally) and incubation of the mixing test should not be performed.
4. Recommendation 3.1.4: Non-neutralizing antibodies will likely yield correction in the mixing studies suggesting a factor deficiency. If non-neutralizing antibodies are suspected, pharmacokinetic studies are recommended to compare expected in-vivo factor stability to measured factor activity levels.

4.2 | Result reporting

Results should be reported with an indication that there is likely a factor deficiency or factor inhibitor present. Additional information can be provided to suggest, which factor deficiency(ies) or inhibitor(s) are most likely present based on the prolonged screening test results and available clinical data. For laboratories that perform manual calculations or manual data entry into an electronic medical record or laboratory information system, a verification process should be implemented for ensuring no mathematical or clerical entry errors have been made prior to verifying and reporting the results.

1. Recommendation 3.2.1: Mixing study results should include an interpretation stating that the prolongation is most likely due to a factor deficiency or factor inhibitor.
2. Recommendation 3.2.2: If values for each mixing study result or calculation method used are reported, they must be accompanied by a RI.
3. Recommendation 3.2.3: For sites with manual calculations or data entry, a verification process for ensuring mathematical or clerical entry accuracy should be implemented prior to verifying and reporting the results.

5 | QUALITY ASSURANCE CONSIDERATIONS

The mixing test procedure, and interpretation method used (including any interpretation algorithm) should be validated prior to clinical use. Quality controls must be performed in accordance with local regulatory requirements using well-characterized patient or purchased samples. One control should be a known factor deficient sample and a second control a known factor inhibitor sample, which would represent a corrected and noncorrected interpretation, respectively. It is also imperative that laboratories performing mixing studies subscribe to external proficiency testing mixing study programs and ideally ones that also include and evaluate result interpretation.

5.1 | Validation studies

The mixing test validation should be based on the intended use for the population tested. If mixing studies are used for evaluating unexpected prolongations of a PT or APTT, then samples with various coagulopathies associated with prolonged PT and APTT should be used. If the population requiring mixing tests are patients with known or

suspected factor deficiency, then samples with specific factor deficiencies and inhibitors should be considered. If the intended population is primarily for assessing LA patients, the validation should encompass LA negative and positive samples. At a minimum, 10 factor deficient samples and 10 inhibitor samples should be evaluated for the interpretation method, although optimally, 25 samples from each group are preferred.

Factor deficiency samples should include, when possible, congenital and/or acquired single or multiple factor deficiencies. Inhibitor samples should include LA, specific factor inhibitors, or drugs that mimic inhibitors to verify the interpretation method. Combined and severe single factor deficiency(ies) as well as weak factor-specific or nonspecific inhibitors should be included in the validation process. While every effort should be made to validate mixing studies using native, real world patient samples, in laboratories where abnormal samples are not readily available, the use of surrogate (contrived) samples may also be considered such as drug enrichment of normal plasma to demonstrate inhibitor effect or saline diluted plasma to represent factor deficiencies. Surrogate samples are not suitable for mimicking antibodies that demonstrate an inhibitor effect such as LA. A 90% agreement should be the lowest threshold of acceptability for a given interpretation method. Verification of continuous performance should be assessed using quality control material that would generate the expected calculation for correction and noncorrection, representing a factor deficiency or inhibitor, respectively.

New lots of coagulation reagents and NPP should be verified prior to being put into use, using QC materials or previously tested samples to verify expected mixing test interpretations.[50]

A written protocol for the performance of mixing studies, in conformance with local regulatory requirements, should be available for laboratory staff. At a minimum, the protocol should include appropriate patient selection, acceptable sample requirements, stepwise procedure for performing a mixing study, test interpretation, and limitations of testing.

1. Recommendation 4.1.1: Before the results of mixing tests are reported, the assay procedure and methods used for mixing test results interpretations, including thresholds, should be validated.
2. Recommendation 4.1.2: New lots of coagulation reagents and NPP should be verified for mixing study test performance prior to clinical use.
3. Recommendation 4.1.3: A written protocol for performing mixing studies should be available for laboratory staff.

5.2 | Quality control

A negative (mixing study correction interpretation) and positive (mixing study noncorrection interpretation) control sample should be run with each mixing study batch or as minimally required by regional regulatory authorities. Optimally, the mixing study QC would include a severe factor deficiency(ies) for correction/negative control, and a weak positive inhibitor for noncorrection/positive control. It is recommended that the conditions to consider a control as a weak positive would be one that has:

1. Percent (%) correction between approximately 50%–65%, or
2. Rosner Index of approximately 17–20, or
3. Subtraction method that is approximately 3–5 s higher than the reported NPP value.

For resource limited laboratories, or if allowed by local regulatory authorities, an alternative, individualized QC plan (IQCP) could be adopted to determine the acceptable frequency of QC testing for mixing studies.[51] The IQCP should be based on QC test performance, including those materials used for mixing studies and routine monitoring of PT and APTT testing. A risk assessment should be evaluated for any IQCP prior to implementation. At a minimum, QC should be performed once monthly and before each new lot of reagents or NPP is introduced. Each representative QC sample should reflect the intended patient population to be assessed (i.e., weak factor VIII or IX inhibitor QC for mixing studies used to screen for these conditions).

1. Recommendation 4.2.1: Control samples of well-characterized factor deficient and inhibitor samples should be tested and expected results obtained before patient results are reported.
2. Recommendation 4.2.2: An IQCP should be considered if allowed by regional regulatory authorities to reduce QC testing frequency.

5.3 | External proficiency program

Laboratories that perform and report mixing studies should enroll in an external proficiency program, ideally one that evaluates the results of mixing tests. If external programs are not available, proficiency should be determined at least twice per year using well-characterized samples that are resubmitted for testing after being blinded. These well-characterized samples can be shared between institutions. Proficiency samples should be tested in the same manner as routine samples.

1. Recommendation 4.3.1: Laboratories performing mixing tests should enroll in external proficiency programs and ideally ones that evaluate result interpretation of mixing tests or should develop their own proficiency samples as long as these samples meet the requirements of local authorities.

6 | CONCLUSION

Proper performance and interpretation of APTT and PT mixing studies is an important step in the evaluation of an unexpectedly prolonged clotting time or the evaluation for the presence of an inhibitor. This ISCH publication provides recommendations based on published studies and expert opinion. When performing mixing studies, good laboratory practice should be followed such that proper attention is given to

preanalytical, analytical, and postanalytical considerations. The mixing study test method used should be locally validated prior to clinical use, and include considerations for quality controls, result interpretations and enrollment in a suitable external proficiency program.

AUTHOR CONTRIBUTIONS

DMA, RCG, and GWM wrote the guidance and it was reviewed by SALM and GWK.

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The document benefited from careful review by ICSH Board, and ICSH Haemostasis subgroup.

CONFLICT OF INTEREST STATEMENT

RG has received consulting fees from Sysmex America, Inc; and the University of California, Berkeley, speaker honoraria from Mindray and the University of Wisconsin-Madison School of Pharmacy, is a current member of the ICSH coagulation committee, and former ICSH board member. GM reports consultancy fees from Technoclone. DA, GK, SM have no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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