Preanalytical note's for Blood coagulation tests
importance

- The use of modern laboratory instrumentation with high levels of test reliability and appropriate quality assurance measures will lead to very few analytical errors within hemostasis testing. Nevertheless incorrect or inappropriate test results are still reported, often due to events outside the control of the laboratories performing the tests. This is due primarily to pre-analytical events associated with sample collection and processing, as well as post-analytical events related to the reporting and interpretation of test results. This
Sampling time and patient preparation

- **Time:**
  - Samples should be taken before **10:00 am** with the patient sitting down.

- **Fasting:**
  - The patient should have fat-fasted since midnight, or for at least **4 hours**.
  - calm and relaxed,
  - sit there for a little while (**15–20 min.**.) before samples are taken.
Sampling time and patient preparation

- **DRUG:**
- ASA or clopidogrel for the last 7–10 days
- Nonsteroidal anti-inflammatory drugs (NSAID) for the last 1–3 days.
- P-pills and other hormone drugs should have been withdrawn, if possible, for at least 1 month
Points to note prior to sampling

- **DRUG**
  - Estrogen, high-dose p-pills (and other hormone drugs) also affect coagulation and fibrinolysis
  - Increased: FVIII, VWF and fibrinogen
  - **lowered**: antithrombin, protein C and FVII are low at high levels of estradiol.
Points to note prior to sampling

- **Patients on Anticoagulant Therapy**
- Heparin therapy may influence antithrombin detection,
- warfarin therapy may influence protein C and protein S levels,
- Heparin and warfarin therapy may also influence the appropriate identification of LA
Points to note prior to sampling

- **variations:**
- **Physical Activity, Illness, and Stress**
  - FVIII, von Willebrand PAI-1 and fibrinogen, are *acute phase* reactants (i.e. increased by inflammation, infection, surgery, etc.).
- misdiagnosis of (mild) hemophilia A or VWD Type 1
- **mental stress** and physical activity increase the concentrations of FVIII and VWF many times over.
- **Smoking and age** affect the levels of several coagulation factors (e.g. VWF and fibrinogen are increased).
Points to note prior to sampling

- **Circadian and Diurnal Rhythms**
  - Levels of some hemostasis components follow a circadian or diurnal rhythm, with differential levels detectable at different times of the day.4,44
  - For example, fibrinogen and plasminogen activator inhibitor-1 levels tend to be higher in the early morning hours.
Clinical Ordering and Inappropriate Requests as a Pre-analytical Issue

- Following a thrombotic event, some loss (consumption) of the natural anticoagulants might arise; hence, testing too soon after a thrombosis might lead to false conclusion of a deficiency.

  **Example:**

  - FVIII may also be elevated post thrombosis, leading to a missed LA diagnosis if only APTT-based screening tests are used.
Clinical Ordering and Inappropriate Requests as a Pre-analytical Issue

- Considered another way, upwards of 80% of abnormal thrombophilia test results may be a reflection of inappropriate testing while on anticoagulant therapy.42
Points to note prior to sampling

- **menstrual days**: In an investigation of bleeding tendency, fertile women should preferably be sampled during menstrual days 1–4. It is then easier to diagnose a suspected mild VWD.

- **Pregnancy**: In women who have been pregnant, a deficiency cannot be determined exactly until breastfeeding.
Sample Collection

**anticoagulant**

- **citrate-based anticoagulant tubes**
- (generally 105-109 mM or 129 mM sodium citrate, also referred to as 3.2% or 3.8%, respectively).
- **Clinical and Laboratory Standards Institute (CLSI) guidelines** favor the use of the lower citrate concentration, except for specific applications.\(^4\)\(^,\)\(^16\)
- Specimens collected in 129 mM (3.8%) buffered sodium citrate may **overestimate** the PT and APTT and **underestimate** fibrinogen if the normal range is based on 3.2% citrated samples.\(^18\)
Sample Collection

- Conversely, samples collected into 129 mM (3.8%) citrate may provide a more stable sample for assessing antiplatelet (eg, aspirin) therapy response using the PFA-100.

- Sometimes there is no apparent difference in relation to testing (eg, anti-Xa [heparin] testing) based on citrate concentration.
Sample Collection
anticoagulant

- conclusion
- The major recommendation therefore is that laboratories standardize to 1 citrate concentration and develop normal ranges appropriate for that concentration.
- This standardization should include all components of the assay (eg, including determination of patient PT, mean normal PT [NMPT], and international sensitivity index [ISI] for the INR).
Coagulation samples should preferably be collected before other test samples are drawn, if these contain stronger anticoagulant agents such as ethylenediaminetetraacetic acid (EDTA) (for a complete blood count), lithium-heparin (for clinical chemistry testing), as well as clot activators (ie, thrombin), since these materials may contaminate a subsequent coagulation test sample.
Sample Collection

“order of draw”

- The old dogma that the first collection tube should be discarded may not generally be required, as evidence for differential effects on coagulation assays are lacking.\textsuperscript{20}
- Tubes should be adequately filled (to the mark noted on the tube if provided) or to no less than 90\% of this total volume
Sample Collection

mixing

- Samples should be mixed thoroughly (but gently) by 3 to 6 end-over-end tube inversions to ensure adequate mixing of test sample with anticoagulant\(^\text{19}\).
- Conversely, too vigorous mixing (eg, by shaking of tubes) might lead to *in vitro* hemolysis or spurious factor activation resulting in false shortening of test clotting times and even possible false elevation of clotting factor activity (eg, FVII).
Sample Collection

**site selecting**

- blood collections from central venous lines, leading to
- contaminated with heparin
- partially clotted,
- hemolyzed,
- or activated samples,
- or samples diluted by saline or
Sample Collection

**site selecting**

- Collections from venous lines should include a process for
- **flushing and/or discarding the initial collection volumes.**
Sample Collection

needle size

- Size: 16-25

- and heparinized needles (sometimes used for blood gas collection) not used.²⁴
Sample Transport

- Samples should be transported non-refrigerated at ambient temperature (15–22°C) in as short a time as possible.
- Ideally, testing for routine coagulation tests like the PT and the APTT should be accomplished within 4 hours of collection, although allowable tolerances may be greater than this.\textsuperscript{25,26}
- However, APTT testing for unfractionated heparin monitoring should preferably be processed within 1 hour due to the potential for heparin neutralization by platelet releasates.\textsuperscript{16,27,28}
Sample Transport

- Labile factors (FV, FVIII)
- Extremes of temperature (ie, both refrigerated or high) should be avoided.
- **Delays** in transport may affect in particular the leading to prolonged clotting times and *in vitro* loss of factor activity. In such cases, local centrifugation and separation of plasma followed by freezing and frozen transport of the plasma should be considered.
Sample Processing and Storage

Centrifugation

- Once or more?
- Most coagulation-based tests, including PT, APTT, and clotting factor assays, are performed on plasma derived from once-centrifuged samples.
- Some samples, such as those for LA and heparin (UFH/LMH) testing, should be double centrifuged to ensure platelet-free (<10 × 10⁹/L) preparations prior to freezing.\(^{30}\)
- Temperature
  - Centrifugation should essentially be at an ambient Temperature (15°C–22°C),
  - low temperatures, which can lead to platelet activation and adverse effects.
Sample Processing and Storage

Centrifugation

- **TIME and g**: 
- Centrifugation should ideally be at **1500 g** for a minimum of **10–15 minutes**.\(^\text{16}\)
- **Shorter centrifuge times** might be acceptable for **routine coagulation** tests performed immediately post-centrifugation when there are no subsequent test requirements (ie, plasma not to be frozen or processed for additional assays)
- Using centrifugal forces **greater than 1500 g** are not recommended as this may induce **platelet activation and lysis of RBCs**
Sample Processing and Storage

Centrifugation

- The use of centrifuge breaks should also be avoided or monitored to avoid remixing of test and platelet contamination, which may subsequently affect most hemostasis assays.
Sample Processing and Storage

- **Filtered Plasma**: yes or not?
- Not recommended?
- because the process leads to loss of other plasma components, including FVIII and VWF
- a false conclusion of an elevated APTT
- In extreme cases, microfiltration may even generate false (weak) positive LA findings.
the double centrifugation approach for preparation of hemostasis samples prior to freezing is now strongly favored.
Moreover, storage of refrigerated whole blood is now actively discouraged and leads to activation events affecting FVII, FVIII, VWF, and possibly others.\textsuperscript{16,33}
Sample Processing and Storage process

- ideally **within 1 hour** of collection
- and **testing** performed within **4 hours** of procurement (or else be processed by centrifugation and plasma frozen).
- During this short-term storage, whole blood samples should be **kept** capped and maintained at **room temperature**
- Frost-free freezers with automatic defrost cycles are generally unsuitable,
Sample Processing and Storage

- freezer
- When storing plasma, the lower the freezer temperature, the longer the specimens can be maintained for future testing
  - \(-20^\circ C\) 2–4 weeks of storage,
  - \(-80^\circ C\) several months and sometimes years later (useful for research studies and prospective trials).\(^{34}\)
Controlled Thawing of Frozen Plasma Samples

- **Thawing**
  - rapidly thawed in a 37°C water bath for 5–10 minutes or until completely thawed.\(^\text{4,16}\)
  - Once samples are thawed, it is imperative they are thoroughly and adequately mixed prior to testing.
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Serum**
  - Serum will lead to loss of fibrinogen and many other coagulation factors (notably FII, FV, and FVIII) as well as differential loss of high molecular weight VWF.
  - Serum may also yield high values for some factors (e.g., FVII) due to activation.
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **EDTA Plasma**
  - This will raise coagulation test times such as PT and APTT and reduce FV and FVIII
  - Assessment of potassium (extremely increased) or calcium (very low to absent) will usually identify the presence of an inappropriate EDTA collection.  

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Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Heparin**
  - much more common
  - clotting times (APTT and especially the TT) are prolonged, and fibrinogen and clotting factors (especially APTT based, viz FVIII, FIX, FXI, FXII) reduced.
  - Sometimes, test reagents (eg, for PT or LA detection) include heparin neutralizers, at levels sufficient to neutralize about 1 U/mL unfractionated heparin
  - Heparin contamination can be provisionally identified by testing of select clot-based assays (especially APTT and TT), and then by mixing studies (see end of serum section above), and confirmed by using an anti-FXa assay or by repeat APTT or TT testing after addition of a heparin neutralizer.37
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Hemolysis**
  - *in vitro*
  - *in vivo* (autoimmune hemolytic anemia, severe infections,...)
  - Hemolysis increases the spectrometric absorbance of the plasma sample and leads to high background absorbance readings
  - Instruments utilizing mechanical means of clot detection are not affected by this interference
  - the test result may still be compromised since cell lysis products include tissue factors that may activate coagulation.
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Hemolysis**

- PT: decrease
- whereas APTTs may increase or decrease depending on the net effect of activation vs the loss of fibrinogen
- fibrinogen levels may fall
- D-dimer levels may increase
- decrease antithrombin levels
- If possible, grossly hemolyzed specimens should be rejected
- If testing must be pursued (eg, if in vivo hemolysis is present), testing using a mechanical end point detection system is recommended, although the potential effect of activation should also be noted
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

Hemolysis

PT

FIB?

APTT

D-Dimer?

ATIII
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Hematocrit**
  - The presence of significant anemia has not been shown to influence test results.\(^{39}\)
  - hematocrit values are above 55%
1:9 for all??

\[ X = \left( \frac{100 - \text{Hct}}{595 - \text{Hct}} \right) \times \text{vol} \]

- **X**: volume of sodium citrate
- **Vol**: volume of whole blood

Example:

- Hct: 60 %
- Volume of whole blood: ?
- \( X = 0.25 \text{ ml} \)
- \( 0.25 = \left( \frac{100 - 60}{595 - 60} \right) \times \text{vol} \)
- \( \text{Vol} = 0.25 / (40/535) \)
- \( \text{vol} = 4.2 \text{ml} \)
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Lipemia**
- It is not easy to dichotomize the biological and analytical effect of lipemia on coagulation tests.\(^4\text{16}\)
- biological
- Acute elevation of the coagulant activity of FVII is observed after consumption of **high-fat meals**, mostly due to an increase in the concentration of activated FVII (FVIIa)
- Analytical interferences in some laboratory assays (especially those based on optical clot detection) also occur but are minimized using mechanical or electromechanical-based procedures or using analyzers comparing the absorption of samples at **2 wavelengths** or performing coagulation assays at alternative wavelengths.\(^3\text{4}\)
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Lipemia**
- **Analytical** interferences in some laboratory assays (especially those based on optical clot detection) also occur but are minimized using mechanical or electromechanical-based procedures or using analyzers comparing the absorption of samples at 2 wavelengths or performing coagulation assays at alternative wavelengths.\(^3\)\(^4\)
- the **best approach** might be recollection of blood samples at fasting, provided that metabolic problems (ie, dyslipidemia) are absent.
Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- **Freeze-thawing Events**
- These result in the loss of some labile factors, notably FV and FVIII
Sampling

- Technique
  1. The patient should be sitting up
  2. The blood should flow fast.
  0.109 mol/L
  trisodium citrate (9 parts of blood + 1 part of trisodium citrate), pH 7.4.
  (If blood is taken in open tubes, the proportions of blood/citrate should be the same.)
  Note that only filled tubes (±10% deviation) are accepted for further handling.
  3. Reverse the tubes immediately 5–10 times.
Centrifuge

**Time?**
citrate blood samples as soon as possible (preferably within 30 min)
at +15°C, alternatively at room temperature for 15 minutes at 2000 x g.

- Samples for determination of heparin (UFH/LMH) of lupus anticoagulant must be centrifuged **twice** in order to obtain platelet-free plasma.
- **Deep freeze** the plasma at −70°C within 1 hour after sampling.
Reference

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Typical Issues Related to Inappropriate Sample Collection, Processing, and Storage

- Incorrect Patient Collected or Wrong Label Attached
- Incorrect Anticoagulant Matrix Collected or Provided to the Laboratory
- Serum or Clotted Samples
  - prolonged use of a tourniquet,
  - or considerable manipulation of the vein by the needle may be prone to develop a clot \textit{in vitro}.
  - incompletely mixed immediately following collection or in under-filled tubes
For analysis of homocysteine (EDTA tube)

- centrifuged within 1 hour (for clinical use).
- For research : empty stomach, level 10–15%.
- If this is not possible, the sample must be taken on ice.
- After centrifugation, homocysteine is stable in the plasma at room temperature for several days,

- In the frozen state (−20°, −70°) the sample is stable for several months.
INR

- ISI : International Sensitivity Index
- INR : International Normalizing Ratio
- INR = \((\text{PT patient/PT control})^{\text{ISI}}\)
Influence of blood group: blood group O+(positive)

- The level of FVIII is about 30% lower
Essential Guide to Blood Coagulation

Edited by

Jovan P. Antovic
Department of Molecular Medicine and Surgery, Coagulation Research, Karolinska Institute;
Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden

Margareta Blombäck
Department of Molecular Medicine and Surgery, Coagulation Research, Karolinska Institute;
Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden

A John
http://www.practical-haemostasis.com/Miscellaneous/Miscellaneous%20Tests/pre_analytical_variables.html
Points to note prior to sampling

- LMH:
- “low-dose” (1 injection/day): 3 hours after an injection
- “high-dose” treatment (2 injections/day): prior to injection
Sample Collection and Pre-Analytical Variables

- **Tube:**
  - The sample container should not induce contact activation (i.e. use plastic or siliconized glass).
  - The **tourniquet** should be applied just before sample collection.
  - The needle should not be more than 21-gauge for adults, rapid collection.
  - The blood should be drawn **gently** into the syringe.
  - For infants, a 22- or 23-gauge needle may be necessary.
  - mixed by gentle inversion five times. Avoid vigorous shaking.
Sample Collection and Pre-Analytical Variables

- **rapid** collection of the blood
- sample. The blood should be **drawn gently** into the syringe.
- The blood should not be **passed back** through the needle after
- mixed by gentle inversion five times. Avoid vigorous shaking.
- The blood should be mixed with sodium citrate anticoagulant in the
- **proportion 9 parts blood: 1 part anticoagulant.**
Anticoagulant solution

- This should be **0.109M**
- can be stored **at 4°C for up to three months**, but it should be inspected
- prior to use and discarded if particulate material is present