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Determination of Factor VIII potency

Testing of in-process and final product FVIII concentrates requires robust methods which provide a high precision and a reproducible performance. Regulatory bodies prescribe different FVIII reference methods in Europe (chromogenic method) and in USA (one-stage clotting method) for product release testing and there is not yet a global harmonization of FVIII reference methods.

Most clinical laboratories are still using a one-stage method, much due to that it is technically easy to perform. However, it should be recognized that the one-stage method is biochemically quite complex and, in contrast to what is often claimed, do not mimic physiological conditions. This is illustrated by the use of artificial activators and fast coagulation activation with clotting times considerably shorter than in vivo.

Chromogenic methods, on the other hand, rely upon activation of FX by FIXa and the activation times prescribed by manufacturers of chromogenic kits are in the order of 2-5 min, which range agrees well with in vivo clotting times. Still, it should be recognized that also chromogenic methods deviate from physiological conditions.

An inherent advantage of chromogenic methods is their insensitivity towards any presence of preactivated FVIII in a sample whereas one-stage methods will overestimate the FVIII activity considerably under such conditions.

Chromogenic methods have been available for more than 25 years and are e.g. used since long in multicenter calibrations of primary and secondary FVIII reference plasma and concentrate standards. By strict adherence to assay recommendations by SSC/ISTH, chromogenic methods have repeatedly demonstrated a close agreement with one-stage methods and a higher precision on analysis of native FVIII preparations. Analyses of recombinant FVIII (rFVIII) preparations may often give discrepant results between these methods and it is interesting to note that the use of higher phospholipid dilutions and/or use of phospholipids with a composition which approach that of the platelet membrane seem to give one-stage method results which are in closer agreement with chromogenic methods.

Field studies involving clinical hospital laboratories generally result in a larger variation between laboratories and, probably influenced by use of different APTT reagents and different FVIII deficiency plasmas, one-stage methods generally show a considerably larger inter laboratory CV than chromogenic methods.

The following study by D Viuff et al is a representative illustration:

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Table 2.	Intra- and interlaboratory CV, %								
	Advate plasma sample, IU/mL				N8 plasma sample, IU/mL				Plasma std
	0.03	0.2	0.6	0.9	0.03	0.2	0.6	0.9	0.8
One-stage									
Interlab-CV, %	**32.5**	**20.9**	**14.2**	**12.4**	**35.1**	**21.0**	**14.0**	**13.1**	**12.6**
Intralab-CV, %	11.6	9.0	5.8	6.6	11.4	7.6	5.0	5.6	6.5
Chromogenic									
Interlab-CV, %	**14.4**	**11.6**	**8.3**	**5.4**	**13.9**	**10.4**	**8.2**	**7.4**	**3.1**
Intralab-CV, %	11.9	5.2	7.1	5.2	13.7	5.4	4.2	5.1	6.1

From Viuff D et al. Haemophilia 17, 695-702 (2011)

Another outcome in the above study by Viuff et al. was that one-stage methods over-assigned a plasma sample with a low FVIII activity whereas the target level was reached by chromogenic methods. This finding for one-stage methods is supported by earlier data demonstrating improved accuracy in such methods when a constant level of FVIII deficiency plasma is used for all standards in the low range.

With the advent of a new generation of rFVIII variants, which have a considerably increased half- life, the accuracy of FVIII methods is a key matter. Different one-stage methods may show quite variable FVIII potencies on analysis of e.g. PEGylated rFVIII, whereas chromogenic methods appear to be suitable.

Notwithstanding the inherent advantages of chromogenic FVIII methods, there are several aspects which require attention and, if not, may compromise the performance.

- On analysis of in-process samples, it is important to consider whether the sample matrix may result in a more vulnerable and less stable FVIII protein, which may require a minimal delay between sampling and assay start. Final formulations of FVIII mostly display a stability of several hours after reconstitution.
- Predilution of FVIII concentrate to about 1 IU/mL should be made with FVIII deficiency plasma containing a normal level of vWF as recommended by the SSC/ISTH.
- BSA is usually included in buffers used as diluent and may also be included in reagents. The BSA concentration should be 1% as recommended by SSC/ISTH. The quality of each candidate BSA lot should be evaluated carefully before it is approved as a bulking agent. Absence of proteolytic activity assessed from hydrolysis of a chromogenic substrate is a necessary but not sufficient condition. Thus, extensive testing should be performed on stability and activity of various coagulation factors.
- Temperature homogeneity is crucial as for any activity based assay. In case of microplate assays, the temperature difference between wells should be within 0.5^{o}C.
- When using end-point (acid-stopped) assays, the hydrolysis time may be adjusted to allow for an optimal color development. However, in the case of microplate assays with reading at 405-490 nm, the absorbance should preferably not exceed 1.3 absorbance units in order to avoid substrate depletion.
- One approach to ensure a consistent performance, which is utilized since years by some users, is to pool a large number of reconstituted factor reagent vials and aliquot suitable volumes into plastic tubes followed by freezing and storage at -70^{o}C or below. By adhering to well standardized procedures for freezing and thawing as well as for use of the thawed reagent within a stated time frame, the inter assay variability may be kept to a minimum.