European Medicines Agency
Pre-Authorisation Evaluation of Medicines for Human Use

London, 21 September 2006
Doc. Ref. EMEA/CHMP/BWP/94182/2006

OVERVIEW OF COMMENTS RECEIVED ON DRAFT GUIDELINE ON VALIDATION OF IMMUNOASSAY FOR THE DETECTION OF ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS (ANTI-HIV) IN PLASMA POOLS

Table 1: Organisations that commented on the draft Guideline as released for consultation

	Name of Organisation or individual	Country
1	Amgen – Dated 21.12.05	UK
2	BAXTER – Dated 21.01.06	AU
3	International Plasma Fractionation Association (IPFA) – Dated 19.12.05	NL
4	International Plasma Fractionation Association (IPFA) – HbsAg – Dated 22.12.05	NL
5	Plasma Protein Therapeutics Association (PPTA) – Dated 9.02.06	BE
6	A. Portela - Agencia Española de Medicamentos y Productos Sanitarios – Dated 19.03.2006	ES
7	Dr. E. Rusvai, rusvaie@oek.antsz.hu Dr. M. Takács, Head of Hepatitis and Molecular Virology Department, takacsm@oek.antsz.hu – Dated 13.12.06	HU
8	Dr. B. de Vries - National Institute for Public Health and the Environment - RIVM-BMT – Dated 28.11.06	NL

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Table 2:Discussion of comments

GENERAL COMMENTS - OVERVIEW	
The guidance is felt to be very general. Specific examples where further detail would be advantageous have been provided below. It is felt that there is not enough specific information around the very important topics of sensitivity, robustness and specificity. For example, the assay criteria do not specify that all classes of antibodies should be detected. There is no mention of discriminating IgM from IgG antibodies. Greater clarity on the kind of examples which should be evaluated as negative controls would be valuable. Further information regarding the required level of sensitivity would be useful.	As specified by the NfG on plasma derived medicinal products, pool serology was introduced as a measure to detect errors in testing or pooling. In the early phase of infection, the technical limitations of tests available exclude 100% sensitivity, as low affinity antibodies (and IgM) are rapidly diluted below the detection limit. There are no kits specifically for the detection of anti-HIV IgM. Due to the nature of pool serology, pools contaminated with HBsAg or anti-HIV should be a rather rare event. For the purposes of this guideline, a selection of representative pool samples found negative by the manufacturer and by an OMCL is considered adequate. The requirement for sensitivity has to be deduced from the size of the pools to be tested, see NfG on plasma derived medicinal products: "... in relation to pool size ...". Detection limit is addressed in the document.
Both documents describe the potential use of a 'user-defined' grey-zone below the cut-off for reactive/non reactive as presented in the 'Instruction for Use'. The argument for the lower value is based on the dilution of non-specific factors potentially present in the single donations and diluted in the pool. We observe in practice also the opposite, mixing of different plasmas can induce non-specific factors in the pooled material, leading to a higher cut-off value in comparison to negative samples of blood donors. We prefer that the diagnostic kits used for 'in process' control should be used according to the 'Instruction for Use' for the diagnostic device, except in cases where this is not feasible because of the type of material tested or other typical aspects of pooled plasma for manufacturing. Compliance with the 'Instruction for Use' is necessary because the manufacturer can only be made accountable for the claims in these instructions. The S/CO ratio for non reactive/reactive is controlled by the diagnostic manufacturer as part of the batch release for the qualitative test. It is possible that the 'user-defined' grey zone S/CO level is not controlled in the batch release of the diagnostic device. The grey-zone value can fluctuate between lots of kits that are released within specification. The Notified Body responsible for the batch release of these kits under the CE-market authorization will only control the claims presented in the 'Instruction for Use'. When the manufacturer of the diagnostic device claims a grey zone in the 'Instruction for Use', this cut-off value can be included in the procedure for plasma pool testing, when further guidance for the resolution to a final result by repeat testing is given for the diagnostic device.	The evaluation criteria (cut-off, grey-zone) established for single donations must not be applied to pool samples without critical (re)evaluation/validation. The cut-off calculation laid down in the Instructions for Use (IFU) is validated for the intended use (testing of single donations) only. When the test kits are used for plasma pool samples, the user is fully accountable for the evaluation criteria (including cut-off calculation and retesting strategy) applied and the final results obtained, as the use for pool samples is not covered by the Common Technical Specifications (CTS), the validation of the IVD manufacturer, or the evaluation by notified bodies in the CE marking process. If validation of sensitivity and specificity according to this guideline demonstrates adequate performance on negative and spiked pool samples, the interpretation criteria proposed by the IFU may of course be used, but experimental results demonstrate that significant (and in some cases crucial) increase of dilutional sensitivity without loss of specificity may be achieved by definition of a lower cut-off. The argument of unspecific results after pooling might hold true for some assays or pools although the experts involved in the drafting of the guideline do not really experience this phenomenon. The definition of a new (lower) cut-off is just a potential decision one might take, of course based upon respective results and experience. Most important is the identification of an assay with high dilutional sensitivity.

If it comes to that a 'user-defined' grey-zone is allowed in the guidelines EMEA/CHMP/298388/2005 and EMEA/CHMP/298390/2005, the documents should contain guidance how to solve a sample after an initial grey-zone result. This repeat testing (e.g. testing in duplicate) should use the cut-off ratio for reactive/non-reactive as presented in the 'Instruction for Use'. The concept guideline indicates insufficiently the status of a grey-zone result. Should grey-zones be considered as reactive samples or as potential reactive sample for which the non-reactivity should be proven? We propose that a grey-zone value will never be used to formulate a final result.	For all practical applications in the context of this Guideline, if a grey-zone limit is used as the cut-off value for pool samples, this limit is used to identify initially and repeatedly reactive plasma pools. A negative result on repeat testing in duplicate is accepted as a negative. Repeatedly reactive samples should be considered positive unless proven otherwise by adequately validated assays. HIV: A repeat reactive on repeat testing should be confirmed through the use of alternative assays. If immunoblots are used as HIV confirmation tests, great care in the formulation of interpretation criteria is advised, as the highly specific ENV bands are hardly detectable in high dilutions, and some pool samples show unspecific bands at 24 and 40 kDa.
What is the status of the guidelines EMEA/CHMP/298388/2005 and EMEA/CHMP/298390/2005 with regard to the tests in place in the documentation of products with market authorization at the date for coming into effect of the guidelines? Should the validation be repeated when the validation report or SOP in place does not comply with the new guidelines or are the guidelines applicable to validation reports and/or SOPs to be filed for a new market authorization or a renewal procedure.	This guideline has been developed to respond to inadequacies in the validation of plasma pool testing for anti-HIV and HBsAg observed during evaluation of dossiers. Marketing Authorisation Holders and Plasma Master File Holders should review the validation of their pool testing methods in the light of this guidance. If the key aspects described in the guideline have already been covered by existing validation, no further validation is needed. If this is not the case, pool testing should be validated in accordance with this guideline and reported in the next annual update of the documentation on the plasma starting material.
Both documents do not clearly indicate in the § 5 Confirmation Strategies' whether a reactive results of manufacturing pool with the initially used serological assay can be overruled by the confirmation assay based on another test principle. Can NAT-tests or immunoblot assays be used to demonstrate that the EIA result is 'false'-positive?	The initially reactive test result may be overruled by retesting in duplicate with the same assay (note!: for HBsAg: on a fresh sample). Repeat reactive samples have to be confirmed with a properly validated serological method (HBsAg: alternative assay, neutralization with neutralization reagent, "self-neutralization" by signal loss kinetics over time in comparison to appropriate controls; anti-HIV: alternative assay, immunoblot (see comment above)). For HBsAg testing a neutralization test should always be used on repeat reactive samples. NAT should be used in addition to but not instead of a neutralization test. Negative NAT results do not invalidate positive serological results, as HBsAg and anti-HIV may be present in patients with low or undetectable nucleic acid plasma levels. On the other hand, positive NAT results do confirm serological results.

The use and status of the grey-zone is not clear: The difference between the cut-off for pool samples and the grey-zone should be explained. The grey-zone is introduced as an example in par. 3.1. However, the title of par. 3.1 and the wording in par. 5 suggest that the grey-zone is more than just an example. The status of the grey-zone should be clarified. The robustness study described in par. 3.2.1 seems to cover precision instead of robustness. In this context, the use of the word 'robustness' is confusing. Furthermore, for a reliable calculation of the %CV of the inter- and intra-assay variability at least 6 determinations are needed.	See comment above. Grey-zone is an option and it is up to the test lab, which has to validate its approach and this approach has to be scientifically defendable. In any case, the interpretation criteria applied have to be defined on the basis of relevant data (produced with negative and spiked pool samples). A clarification on the status of grey-zone reactive samples was introduced in both guidelines. 3.2.1. covers robustness and precision. Text changed to recommend 6 determinations.

The guidelines summarize requirements in order to validate commercially available serological test kits for the testing of plasma pools to ensure that single donations being positive for HIV-Ab do not escape testing or might reach plasma pools for production otherwise. Commercially available serological test kits are validated for single donation testing only. No test is commercially available that has been released by kit manufacturers for plasma pool testing. Test kits validated for single donation screening might not detect all HIV-Ab positive donations in plasma pools. This depends mainly on the concentration of HIV-Ab in the donation, the size of the pool (i.e. dilution factor) and may partly be influenced by the necessary compromise between specificity and sensitivity in single donation screening assays. The drafted guideline proposes validation procedures for commercially available single donation test kits including improvement of sensitivity and to use these altered tests for screening plasma pools. In presence of a regulatory requirement for plasma pool testing and in absence of suitable test systems, the fractionators are complying by using test kits designed for single donation testing. Such test kits are validated by the fractionators for use in pool testing within justifiable limits. The drafted guideline suggests, that each fractionator qualifies commercially available single donation kits for the use in pools by adjusting (i.e. lowering) their sensitivity threshold, following certain procedures as outlined in the drafted guideline. This, however, will result in the fact that each fractionator will validate a given commercially available single donation screening assay with individually characterized positive samples. In addition, each fractionator will establish individual evaluation criteria based on many different approaches. This redundant approach (various assays, different positive samples, different plasma pool compositions) will lead to highly divers results. To avoid this situation, it would generally be highly preferable if the test kit manufacturer validate their single donation kits for use in plasma pool testing or produced test kits specifically designed for this purpose. The kit producers know their assays best and, by following the outline of the new validation guideline, could produce generally available assays for plasma pool testing. Those assays would have the same characteristics for all users. This approach not only would lead to a controlled improvement of the kits but also support a more harmonized and uniform approach to this testing issue.	Kit manufacturers cannot be required to develop kits for pool testing. Until now, kit manufacturers have not validated their assay kits for use on pooled plasma presumably because there is not a large enough market to make this worthwhile.

In conclusion A series of measures are implemented to avoid positive donors from donating and to intercept positive donations once received. In addition, state-of-the-art data management and sample/donation logistics make the escape of a positive donation (once identified) highly unlikely. Should a donation not be detectable at single unit level by a commercially available test kit, no other assay will react positive on pool level, regardless of additional measures taken to improve its sensitivity. Already 10 years ago Dr. Rabenau (Institute of Medical Virology, J.W.Goethe University, Frankfurt) concluded in his work on serological testing of plasma pools (1): "If a contamination with donations originating from individuals with acute HBV, HIV, or HCV infection or with a poor humoral immune response exists, serologic testing for HBsAg, anti-HIV, and anti-HCV may fail to detect potentially infectious plasma pools. Serologic testing of plasma pools in addition to single blood donation screening may not necessarily increase the security of blood products. However, when screening of blood donations or inactivation procedures are not adequate, it might be of (limited) value for the detection of potentially infectious plasma pools." In light of this statement, dated from 1996, when wide-spread NAT testing was not yet established, and taking into consideration the improvements in screening assays and inactivation methodology that were made since then - it might be questioned whether ten years later serology-based virus marker testing of plasma pools has retained any value at all for the viral safety of plasma derived products. This view is corroborated by the recent changes in the European Pharmacopoeia (2), concerning HCV viral marker screening. From 2006 on, serological screening for anti-HCV antibodies in production pools is no longer required. References 1. Rabenau, H., R. Schutz, A. Berger, H. W. Doerr, and B. Weber. 1996. How accurate is serologic testing of plasma pools for hepatitis B virus surface antigen, anti-human immunodeficiency virus 1 and 2, and anti-hepatitis C virus? Infusionsther. Transfusionsmed. 23:124-130 European Pharmacopoeia, Supplement 5.3, valid from 1.1.2006.	The purpose of pool testing for anti-HIV and HBsAg is a safeguard against errors in testing of individual donations or errors in pooling of donations. It is a measure to detect GMP failures. As anti-HIV and HBsAg may be present in donations with low or undetectable viral nucleic acid levels, a pool positive for anti-HIV or HBsAg because of a GMP failure may not have detectable levels of nucleic acid. 3 confirmed HBsAg positive pools have been detected during OCABR in the last 4 years and communicated as rapid information. Also see: Ferguson M, Minor PD, Garrett PD, Page M, Thorpe R, Barrowcliffe T: Testing Plasma Pools for markers of viral infection: the UK experience, Vox Sang 1996;71:21-26. Linden JV: Error contributes to the risk of transmittable disease, Transfusion 1994;34:1016. This topic was also addressed in the report of the EMEA Workshop on the Plasma Master File 2001 (EMEA/CPMP/BWP/1737/02, December 2002) published on the EMEA website. In contrast to state-of-the-art HBsAg and anti-HIV test kits, dilutional sensitivity of currently available anti-HCV kits is insufficient to detect single donation contaminations in manufacturing pools. This is the reason why pool testing for anti-HCV is no longer required. It should be noted that it is possible to detect anti-HCV in pools from unscreened donations (multiple positive donations in a pool, as could be demonstrated with samples from 1994 and 1995 evaluated retrospectively).

2 SELECTION OF THE TEST KIT(S)		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
Par. 1	"Selection of test kits should be based on a high analytical sensitivity." This requirement results in a very large evaluation study in order to determine the most sensitive kit on the market. Authorities could be of much help in this respect. Since all assays are either CE marked or otherwise controlled it would be easy for the authorities to recommend/approve certain assays that fulfill this requirement. Alternatively, the authorities could define a lower limit of detection as done e.g. for the HCV NAT test (100 IU/ml), which automatically would narrow the selection of suitable tests. The guideline suggests selecting only kits that show high dilutional sensitivity. As a selection criterion, it is suggested that "relative endpoint dilution titers of well-characterized positive samples" should be compared. However, such "well-characterized positive samples" with a few exceptions do not exist and the guideline lacks further detail on this topic.	Authorities cannot recommend the use of specific assays. Analytical sensitivity may change when assay design changes and the users must be aware of this eg several of the HIV antibody/antigen combination assays have a lower analytical sensitivity than the antibody only version. CE marking of anti-HIV assays does not include an evaluation of analytical sensitivity although there is a limit given for HBsAg. Working standards, which are dilutions of positive donations or pools of positive donations, which can be used to monitor the analytical sensitivity of assays are available.
3. Validation **3.1 Specificity and determination of a grey-zone for pool samples**		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
Par. 1-3	In theory, it appears feasible to increase sensitivity of an assay by lowering specificity provided that non-specific factors are sufficiently diluted in pools as assumed by the authors. However, the introduction of such a "grey-zone" leaves important questions unanswered. By definition, the grey-zone would be positioned between the cut-off value validated by the kit manufacturer for single donation testing and the average result of the plasma manufacturer obtained from pool testing with the suggested mean cut-off of negative pools plus 3x standard deviation. The draft does however not state what the required action would be, if a plasma pool result fell into this grey-zone. Would a pool with a result within the grey-zone have to be destroyed? If no, the question arises why a grey-zone should be determined in the first place. If yes, this would be no grey-zone but a lowered cut-off. Lowering the cut-off value to a limit below the one validated for single donation testing based on a small set of pool test data is not without danger, because this	Section 3, paragraph 2: The use of the grey zone was clarified. Experience of OMCLs who test a wide range of manufacturers' plasma pools has

	measure may lead to an increased rate of false-positive results. It is difficult to predict the response of a given commercial test to changes in the plasma pool composition. Fractionating plasma from many different suppliers with a high degree of variability in the composition of plasma pools it will be very difficult to come up with a reliable cut-off applicable for all pools testing. If new plasma suppliers are added, the influence of their plasma on the previously established system will be an important unknown. An additional factor to be considered is pool size. Since pool sizes vary between manufacturers (and often within manufacturers for different pathways) it is important to keep in mind that such a suggested grey zone would have to be validated for each pool size individually, since non-specific factors present in single donations would be diluted to a different degree in fractionation pools of different size. In addition, how could such a grey-zone result be verified? Using a different standard kit for single donation screening would not be sufficient, thus, a second validated assay system with an identical grey-zone needed to be kept available. NAT would not be helpful because the presence of HIV-Ab is not necessarily accompanied by presence of virus.	not shown this theoretical concern about the influence of plasma pool composition to be a practical problem.
3.2 Robustness		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
	Neither certified "representative negative pool samples" nor "low positive samples" exist and their definition is missing in the document If the inter-assay variability is investigated in 3 independent assays, the term “if available” should be added after “equipment”.	3.2: Manufacturers can use a panel of their own negative pools. These will have been ‘validated’ through their own assays and those of the OMCL undertaking plasma pool testing on their behalf. Alternatively, the panels of pools set up by EDQM for the validation of HCV NAT assays (H1005000) could be used if available. However, it is recommended to use the former. The definition of a low positive control is given in Section 4.3. Editorial change to add “if available” has been made..

3.3 Detection limit HIV-Ab		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
Par. 1	"… the detection limit has to be determined with a representative panel of positive samples reflecting different subtypes and groups taking into consideration the epidemiological situation in the respective regions where plasma is sourced". This requirement loads a fairly high burden on the presumptive user of an assay. First, samples of all respective subtypes have to be obtained and skillfully manipulated. Subsequently, all available assays for single donation screening have to be assessed for the given composition of subtypes in order to evaluate the one, which gives the highest dilutional sensitivity under the given conditions (Selection of the test kit(s), Section 2). From this assessment procedure the most suitable kit will be chosen and validation will be conducted.	3.3, paragraph 1: Selection of the test kit with the relatively best dilutional sensitivity may be performed with a single spiking sample (see Section 2 of the guideline). The panel is needed for assessment of sensitivity of the optimized assay (after evaluation of the cut-off) Panels with sub-typed samples are available from commercial sources. Positive single donations detected in the routine donor screening are considered representative without further characterization (if the number of samples in the panel ensures representativity). Alternatively/additionally, abundant information on geographic distribution of subtypes is publicly available. What should be avoided, for example, is to validate a pool test with US spiking samples only (almost exclusively HIV-1 subtype B) and use it for European plasma (approx. 30% of subtypes A, C and E) without assessment of sensitivity of other subtypes.
Par. 2	In the absence of independent reference material the data created in such a sensitivity determination will not allow any direct evaluation of assay sensitivity as the content of HIV-Ab in the samples used is not known. This fact drastically limits the value of a validation based on such sample material.	A WHO panel will be available in the future.
Par. 3	Consideration of the number of donations in a typical pool may be difficult since pools sizes may vary even for the same manufacturer for different production pathways. Testing samples at pool dilution will only demonstrate what is already known: Some samples may be detected at pool dilution and others not. However, would non-detection of a positive sample at a certain pool dilution require to reduce pool size in fractionation? If yes, this would have a huge impact on the established, validated and approved production processes that needs to be considered. If not, why would pool size be a matter of concern?	The worst case (maximum number of donations that are theoretically possible) should be taken into account. Validation should demonstrate the limits of a method and show which samples are detected and where improvement is necessary, if technically feasible. Non-detection of a positive sample at the maximum pool dilution would not result in a requirement to reduce the pool size in fractionation.
Section 3.3.	"We do not believe it is possible to quantify the limit of detection by testing "a representative panel". It is suggested that the true measure of sensitivity is to look at published data from seroconversion panels etc. and then confirming that the presence of plasma does not inhibit the test when selected samples are diluted in plasma versus kit diluent. We suggest that this paragraph should be reworded, otherwise there is a danger that all kits would have to be completely re-validated for sensitivity and replicate the data used for CE marking. In for example the UK, NIBSC produces a control. We suggest that this could be used "to facilitate comparability of detection limit data" without the sensitivity validation suggested	It is relevant to have information about the sensitivity and ability of the assay to detect different subtypes and groups when used on individual donations (e.g. from CE marking). It is likely that each anti-HIV assay will detect antibodies of different subtypes to varying extents eg some detect subtype O very poorly and it is of interest that people testing plasma pools are aware of the differences in analytical sensitivity of different subtypes. Then dilutional sensitivity needs to be investigated as stated in this comment. The text has been modified to make this clearer.

	by the first paragraph of Section 3.3."	
4.1 Standard operation procedures for plasma pool testing		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
	Pertaining to bullet point four we would like to point out that for some automatic instruments the operative parameters of the test are not disclosed in detail by the manufacturer	Bullet point 4 was adapted: Incubation procedures (including tolerance limits for time and temperature, e.g. according to the test kit manufacturer's specifications**/instrument settings**)
4.3 Test kit independent controls		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
Par. 1	The "low level of reactivity likely to be found in contaminated pool samples" is not specified. Also, the requested "low-level positive control" remains undefined. Qualification of each new lot of test kits, using an "off-the-shelf" qualification panel, is suggested as an alternative to inclusion of kit independent controls on every test run.	The low level of reactivity depends on the dilution factor of an individual positive donation in the plasma pool. For preparation of the control, any confirmed anti-HIV positive sample may be used as spiking material, pooled plasma may be used as diluent. The signal of the low-level positive control should be near the cut-off and in the dynamic range of the assay, e.g. 2-3 times the single donation cut-off. The qualification of every test kit lot with an "off-the-shelf" qualification panel is not considered an adequate alternative to low level controls in every test run, as day-to-day performance is not monitored.
4.4 Proficiency testing		
Line no. + paragr. no.	**Comment and Rationale**	**Outcome**
Par. 1	Proficiency testing schemes that incorporate the considerations laid out in this document are currently not offered.	Proficiency schemes aimed at diagnostic laboratories are not necessarily appropriate for laboratories testing only plasma pools as labs testing pools are interested in analytical and not clinical sensitivity.
5. Confirmation strategies		
Line no. + paragr. no.	**Comment and Rationale**	Outcome
Par. 1	Alternative tests are suggested in the document. Since the standard assay has been diligently and individually optimized for the given purpose it is highly unlikely to find positivity in a low level positive pool with non-optimized assays or with assays optimized for another system. Therefore, two independent assays	NAT is not confirmation of a serological assay. A strategy for confirmation based on alternative serological assays and immunoblots should be documented for anti-HIV tests.

	need to be maintained one for routine screening and one for verification. NAT is of little support because presence of antibodies is not necessarily accompanied by presence of virus.	