Figure IIIA-8. Treatment of Factor VIII Solution with 0.3% TNBP and Detergent.

Virus	Extent of Inactivation (log ID_{50})	Inactivation Time (hours required)
VSV	$\geq$4.5	2
Sindbis virus	$\geq$5.5	1
Sendai virus	$\geq$6.0	1
HBV	$\geq$6.0	6*
HCV	$\geq$5.0	6*
HDV	$\geq$4.0	6*
HIV-1	$\geq$6.0	0.25

From Horowitz B. Curr Stud Hematol Blood Transfus. 1989; 56: 83-96; and Horowitz B., et al.. Dev Biol Stand. 1993; 81: 147-61.

*These studies were conducted in the chimpanzee model; six hours was the only time point tested.

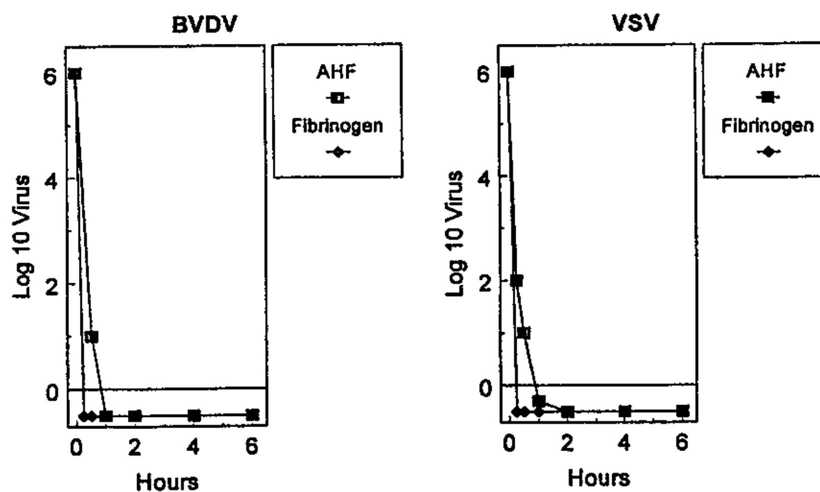


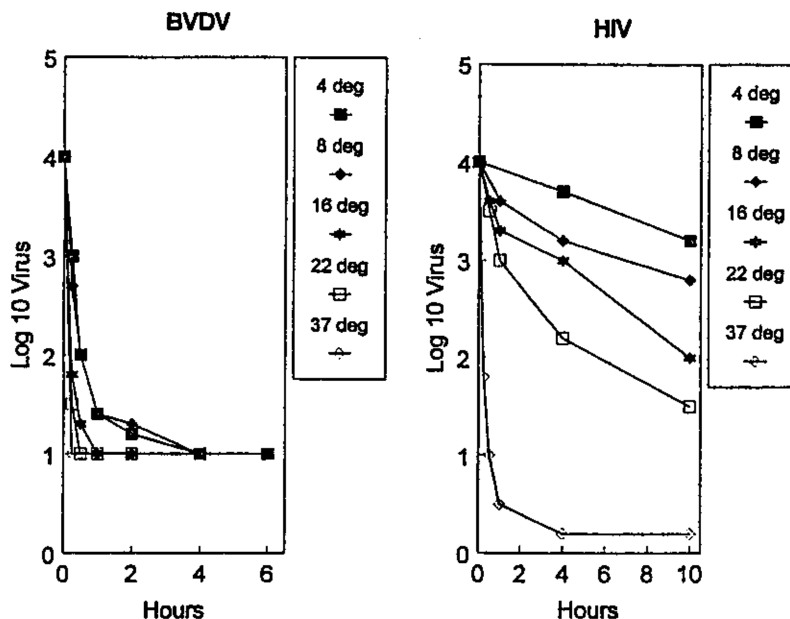
Figure IIIA-9. Treatment of AHF and fibrinogen by solvent/detergent.

AHF was treated with 0.3% TNBP and 1% Tween 80 at 24°C and fibrinogen was treated with 0.3% TNBP and 1% Triton X-100 at 24°C. At the indicated time points, BVDV and VSV infectivity was measured. Data provided courtesy of V.I. Technologies.

6. Low pH

Most proteins are damaged on exposure to the acidic conditions needed to kill virus. For example, little virus is killed at pH 5.0 - 5.5, a condition known to inactivate factor VIII. Immune globulin solutions are an exception. Various studies have shown that low pH such as pH 4 treatment used in the preparation of immune globulins, sometimes in the presence of pepsin, inactivates several enveloped viruses.[11] However, since this treatment was originally designed to reduce IgG aggregation and anticomplementary activity, a number of variants of this procedure have been developed; hence, the conditions being used may or may not inactivate virus efficiently. Each manufacturer's process needs to be validated since virus inactivation is influenced by pH, time, temperature, pepsin content, protein content and solute content. As an example, the effects of time and temperature on the inactivation of BVDV and HIV in one preparation are given in Figure IIIA-10.

Figure IIIA-10. Inactivation of viruses in IgG with pH 4/pepsin.



Results taken from Omar et al., Transfusion 1996; 36: 866-872.

B. Methods of Removal

1. Precipitation

Precipitation with ethanol is the single most widely used fractionation tool worldwide, although other reagents have been used. In addition to its use as a precipitant, ethanol is also a disinfectant. Unfortunately, it acts as a disinfectant mostly at room or even elevated temperatures, while plasma fractionation is carried out at low temperature in order to avoid protein denaturation. Contribution of ethanol to viral safety through inactivation is, therefore, marginal at best. Nonetheless, ethanol can also partially separate virus from protein. Viruses, as large structures, tend to precipitate at the beginning of the fractionation process, when the ethanol concentration is still relatively low. As with any other precipitation, the distribution of viruses between precipitate and supernatant is never absolute.

The following log reduction factors (LRFs) were reported for three distinct steps of albumin production by cold-ethanol precipitation (Figure IIIB-1; the designations of the steps correspond to the Kistler/Nitschmann fractionation scheme) and for the production of immunoglobulin (Figure IIIB-2). [Cautionary note: LRFs should not be summed across steps unless the mechanism of action is shown to be independent or other data demonstrates the that summing is legitimate.]

Figure IIIB-1: LRFs for 4 different viruses and for 3 precipitation steps used during manufacture of albumin.

Step	Ethanol (%)	pH	Log Reduction Factor			
			HIV	PRV	Sindbis	BEV
Step A	19%	5.85	3.3	3.7	4.2	4.2
Step IV	40%	5.85	4.4	5.7	5.4	3.6
Step D	10%	4.6	0.9	1.7	3.1	1.2

From C. Kempf. Haemo April 1997.

Figure IIIB-2: LRFs for 5 different viruses and for 4 precipitation steps used during manufacture of intravenous immunoglobulin.

Step	Ethanol (%)	pH	Log Reduction Factor				
			HIV	PRV	Sindbis	SFV	BEV
Step A	19%	5.85	4.0	3.6	3.2	3.6	3.4
Step B	12%	5.1	5.3	4.7	4.6	2.2	4.1
Step C	25%	7.0	4.0	4.7	2.9	3.5	3.8
Step D			2.2	3.0	1.7	-	2.8

From Kempf C. Haemo April 1996.

Since the result of any precipitation step is a partitioning of components between a solid and a liquid phase it should be kept in mind that, in the absence of inactivation, fractionation results in distribution of viruses between these phases; in other words, if viruses are indeed removed from one fraction the bulk of virus will be found in another fraction, which may or may not be used for making final product. Many manufacturers separate the precipitated proteins by centrifugation while others have introduced filtration as an alternative. To prevent clogging of the filters, filtration uses filter aids. Since these substances (diatomaceous earth or similar products) may also adsorb virus, it is often possible to remove more of the viral infectivity from the supernatant than would be expected based on precipitation alone. This may also explain some of the discrepancies found in the literature. Some authors came to the conclusion that BVDV as a model for HCV was not removed to any significant extent by Cohn-Oncley fractionation[12] while others found substantial partitioning in several steps of cold ethanol fractionation when separation was carried out in the presence of filter aids, as shown for one step in Figure IIIB-3.

Figure IIIB-3: Removal of various viruses from an immunoglobulin solution by filtration in the presence of Celite.

Virus	Log Reduction Factor
Semliki forest virus	3.4
Vesicular stomatitis virus	2.5
Bovine viral diarrhea virus	3.1
Pseudorabies virus	3.4
Sindbis virus	4.1
Human immunodeficiency virus	5.4
Coxsackie virus	>6
Bovine parvovirus	3.4
Bovine enterovirus	4.1

From Omar and Morgenthaler; US patent 5,696,236.

When virus inactivation steps are implemented it is usually relatively easy to ensure that every single drop of a large batch is treated exactly the same way, e.g. by thorough mixing or by transfer of the whole volume from one tank to another (see above). This is much more difficult to achieve for precipitation; the first volumes that contact a filter press encounter an environment which is quite different from the last volumes of the same batch. Although it is probable that these changes occur in a reproducible way in each batch, this might be quite difficult to prove. Similarly, model experiments are relatively easy to perform in a homogenous system, as may be the case during chemical or physical inactivation. However, large-scale centrifugation is usually done in continuous-flow machines; although they could be reduced in size to laboratory scale, parameters like path lengths and residence times are unlikely to be the same. Filtration is not any easier to model on a small scale. In either case, manufacturers need to show with carefully selected parameters (e.g., protein composition; enzyme activity) that both large and small scale processes achieve the same level of phase separation. Demonstration that the downscaled method provides a similar product to that achieved at full scale is at least as important as the demonstration of virus inactivation.

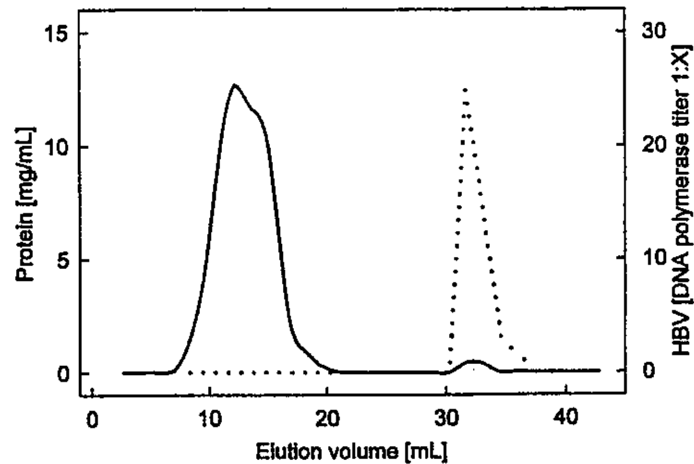
In spite of all the problems associated with precipitation as a means of removing viruses, ethanolic precipitation has proven its value over the years. There can be little doubt that partitioning through precipitation has contributed substantially to the safety of some plasma-based products, e.g. intravenous and intramuscular immunoglobulins, which very rarely transmitted viral diseases in spite of the fact that until very recently the manufacturing processes for these products did not include a dedicated virus inactivation step.

2. Chromatography

Chromatography has been designed to separate closely related molecules; some variants of chromatography, e.g., affinity chromatography, have an exquisite specificity for only one molecular species. The logical expectation would therefore be that chromatography is a good way to physically separate viruses from therapeutic proteins. Both enveloped and non-enveloped viruses can be removed. The log reduction factors are usually in the order of 2 to 3 for ion exchange chromatography and may reach 5 for very specific steps, e.g., affinity chromatography. However, because viruses can bind to protein or the resin backbone, success in removing virus by chromatography is influenced by a number of factors, including column geometry, the composition and flow rate of the buffers used, intermediate wash steps, and the protein composition of the preparation, all of which need to be defined and controlled.

For example, viruses may bind more tightly to a hydrophobic resin than the protein of interest, resulting in substantial virus removal (Figure IIIB-4). From similar experiments carried out with increasing amounts of HbsAg as spike the authors estimated the LRF to be in the order of 4 to 5. Although inadequate by itself to provide safety during routine human use, a subsequent study in chimpanzees confirmed the elimination of 2.5 $\log_{10}$ CID_{50} of HBV by this method.[13]

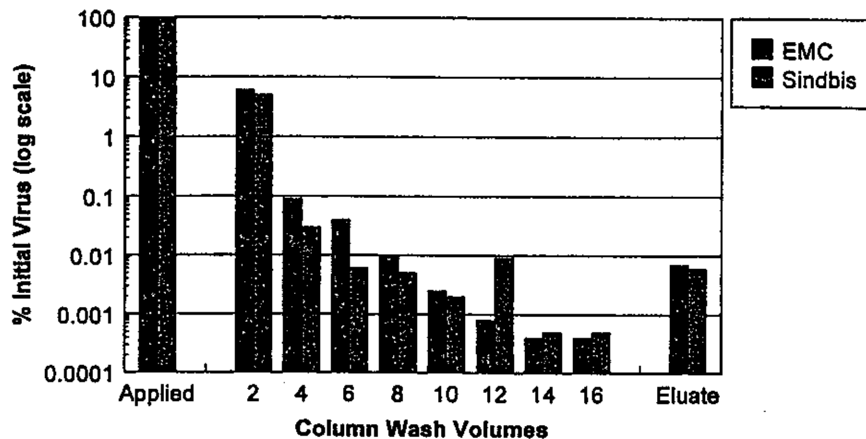
Figure IIIB-4 Chromatography of Factor IX Concentrate Spiked with HBsAg on an Octanohydrazide-Sepharose 4B column.



Solid line, protein; dotted line, HBsAg. From M. Einarsson, et al. J Virol Meth 1981; 3:213-228.

A second and more commonly applied approach is the use of affinity chromatography, frequently antibody mediated, of the protein of interest. In the preparation of monoclonal antibody purified factor VIII, approximately 4 $\log_{10}$ of EMCV and Sindbis virus were removed. Extensive washing of the column prior to eluting factor VIII contributed to the overall removal factor (Figure IIIB-5).

Figure IIIB-5. Method M immunoaffinity step reduction in model viruses.



For EMCV, TNBP and Triton X-100 were present; for Sindbis virus, they were omitted due to the rapid inactivation that otherwise would occur. From Griffith MJ in Roberts HH (Ed) Biotechnology and the Promise of Pure Factor VIII. Baxter Healthcare Publications. Brussels, 1989.

More modest reduction factors were reported for three consecutive chromatographic purification steps used in an albumin isolation scheme. LRFs of <0.3, 0.3, and 1.5 were observed for HbsAg removal during chromatography on DEAE-Sepharose FF, CM-Sepharose FF, and Sephacryl S200 HR, respectively.[14] The same group found LRFs of 5.3, 1.5, and 4.2 for HAV for the same three steps.[15] In another study, the first two chromatographic steps of the same process were investigated for their potential to remove poliovirus and canine parvovirus from albumin. When the two steps were conducted in sequence, overall LRFs of 5.3 and 1.8 were obtained for poliovirus type 1 and canine parvovirus, respectively.[16]

Sanitization of resins and associated chromatography equipment between runs is essential since viruses tend to stick to resins and a complete wash-out is often impossible. Discarding resin is, for financial reasons, normally not an option. Many resins withstand chemical or physical treatments which inactivate viruses. Typical treatments include overnight incubation with 0.1–1 N NaOH or HCl, oxidizing conditions such as provided by sodium hypochlorite, and very high temperatures or autoclaving. The selection of sanitization procedure depends on the column matrix in use. As examples, silica backbones are degraded on exposure to alkali, and immobilized antibody used in affinity chromatography can be degraded by harsh chemical treatments and by enzymes present in the material being purified.

Since sanitization is an essential part of the production process, it must be validated to the same extent as virus inactivation/elimination steps are. The aim of the validation is to prove that there is no cross-contamination from one batch to the next. If it can be shown convincingly that at least one of the solutions used during the regeneration cycle completely inactivates all relevant viruses under the conditions used during cleaning, validation will be relatively simple and can be limited to demonstrating that the column material and all associated equipment has been exposed to the cleaning solution. However, in most cases, inactivation of certain viruses will be incomplete. In these cases, wash-out of viruses during the sanitization cycle needs to be monitored. If necessary, washing may be prolonged until no more virus is removed from the column. Finally, an attempt should be made to demonstrate that no infectious virus remains on the resin, usually by subjecting it to the next purification cycle. These validation experiments need to be done with fresh resin as well as with resin that has been used for the specified maximum number of cycles.

3. Nanofiltration

Nanofiltration can remove viruses based on size while permitting flow-through of the desired protein. However, large proteins – particularly those that tend to form aggregates – are as large or larger than small viruses so nanofiltration cannot be used with all products. Effective removal requires that the pore size of the filter be smaller than the effective diameter of the virus. With filters whose pore size exceeds the virus diameter, some removal may still occur if the virus is aggregated such as by inclusion in antibody/antigen or lipid complexes. Adsorption of the virus to the filter surface may also contribute to virus removal, though this will be affected greatly by the general milieu of the solution being filtered. Only a careful validation of the down-scaled process with several virus species will reveal the potential of the method for specific applications.

Nanofilters are usually available in many different sizes (surface areas), which makes it easy to increase to production scale and to decrease to laboratory scale for validation experiments. Careful monitoring of the performance of the nanofilters in every run is mandatory. Filter integrity should be ascertained before and after use, and every filter manufacturer offers test methods that have been developed specifically for this purpose. If a filter fails the integrity test after its use the filtration step has to be repeated. So far, nanofilters may be used only once.

Although nanofiltration is a gentle method, proteins are subjected to shear forces which may damage their integrity and functionality. Appropriate tests should be conducted during the development phase to rule out this possibility, keeping in mind that several filters may be used in series.

Membranes with 15 and 35nm pore size were able to remove 6-7 $\log_{10}$ of murine xenotropic retrovirus, SV40, and pseudorabies virus from IgG and IgM solutions.[17] Troccoli and coworkers found that all viruses larger than 35nm spiked into an IVIG-solution were completely removed by cascade filtration through one 75nm pre-filter, followed by two 35nm virus removal filters; the pre-filter was used to increase the

capacity of the small pore filters. Even smaller viruses like EMC, HAV, and PPV were removed to a significant extent (LRFs of 4.3, >4.7, and 2.6, respectively). Some small viruses (BPV; Sindbis; SV40) could not be evaluated due to neutralization by cross-reacting antibodies.[18] A single dead-end filtration was able to remove HIV, BVDV, PPRV (porcine pseudorabies virus), RT3 (reovirus type 3), and SV40 with LRFs of >5.7 to >7.8, when these viruses were added to high-purity factor IX and factor XI concentrates.[19] Numerous other studies also demonstrate the efficiency of virus removal with appropriate membranes, either with model solutions or in the presence of (purified) plasma proteins. Protein recovery has almost always been reported to be excellent.

IV. VALIDATION AND CONTROL

A. Virus Specific Issues

A broad range of viruses may contaminate blood and blood products, representing many of the types known. They include viruses with a DNA or RNA genome, with or without a lipid membrane and ranging in size from the smallest such as parvovirus B19 to the middle range such as hepatitis B. The cellular but not the protein components of blood may also be contaminated with herpes viruses including cytomegalovirus (CMV) and Epstein Barr Virus (EBV) which are among the largest viruses known. Processes must be shown to be able to remove or inactivate a wide range of viruses if they are to be considered satisfactory, and typically studies have involved at least four viruses, chosen to represent different kinds of agent.

Viruses have been selected to resemble those which may be present in the starting material, but all are laboratory strains which may be grown to high titer and assayed readily (Figure IVA-1). They include bovine viral diarrhea virus (BVDV), Sindbis virus, Semliki Forest virus or yellow fever virus as models for hepatitis C virus as they share many properties, including a lipid membrane, an RNA genome and a particle size of 40 to 50nm. Laboratory strains of HIV or hepatitis A virus are used, and canine or porcine parvovirus have been used as models for parvovirus B19. Models for hepatitis B virus have been more difficult to identify because few viruses of this family can be grown in culture. Duck hepatitis virus has been used but the herpes virus pseudorabies virus has also been employed as a large DNA virus. The safe handling of the viruses for both human and animal contacts should be taken into account in the design and execution of the studies. The main viruses of concern are HIV, HBV and HCV, and laboratory viruses to represent them should be considered. Readers are directed to existing guidance documents for additional details on the selection and assay of model viruses[20].

Figure IVA-1. Examples of viruses found in blood

Virus	Genome	Envelope	Size	Models which have been used
Human Immunodeficiency Virus (HIV)	RNA	Enveloped	80-100 nm	HIV
Hepatitis B Virus (HBV)	DNA	Enveloped	45 nm	Duck hepatitis virus, pseudorabies virus
Hepatitis C Virus (HCV)	RNA	Enveloped	40 nm	Bovine Viral Diarrhea Virus, Sindbis virus, Semliki Forest virus, Yellow fever virus
Hepatitis A Virus (HAV)	RNA	Non-enveloped	27 nm	HAV, polio, encephalomyocarditis (EMC) virus
Parvovirus B19	DNA	Non-enveloped	20 nm	Canine parvovirus, porcine parvovirus

The production process can be viewed as a series of steps, and it is the obligation of the manufacturer to identify those steps likely to remove or inactivate virus and to demonstrate the degree of virus reduction achieved with those steps. Not all steps need to be evaluated. The ability of a particular step to remove or inactivate virus can be assessed on a laboratory scale by modeling it, introducing virus (spiking) into material derived from the fractionation process just prior to the step being evaluated and measuring the amount remaining after the modeled process step. The accuracy of the resulting clearance figure and its relevance to the production scale process depends on how representative the virus spike and the conditions used are of the full scale process. Models of ethanol fractionation processes have proved particularly variable, in part because of difficulties in scaling down centrifugational processes and in controlling subzero temperatures at small scale. Models of inactivation by beta-propiolactone are difficult to evaluate because of the very rapid reaction of the chemical with water which makes mixing an essential part of the model. It is necessary to evaluate the effect of changes in conditions on the clearance observed; examples might include changes in temperature or composition. A robust reliable process step will be able to remove or inactivate substantial amounts of virus, be easy to model convincingly and be relatively insensitive to changes in process conditions. A production process with several steps of this kind is likely to give a very safe product.

Assuming that the laboratory model can be shown to be representative of the production step (for example in terms of flow rates and the composition of the effluent for a chromatographic column), data need to be collected that demonstrate the degree of virus removal or inactivation and the robustness of the process. For viral inactivation procedures, both the rate and extent of virus kill needs to be demonstrated. For viral removal systems, the attempt should be made to show mass balance, i.e., account for all of the virus added. Additionally, the reliability of the viral assays employed need to be demonstrated. This may include repeat runs of the experiment with or without slight changes in conditions to evaluate the robustness of the procedure, and use of viral assay systems of appropriate statistical reliability. A well controlled in vitro virus assay should have 95 percent confidence limits of plus or minus 0.5 $\log_{10}$.

The subdivision of the process into individual steps which are separately assessed assumes that the effects of different procedures can be added up in some way. This is only true if the fraction of virus surviving one step is not resistant to the other, which is not always the case. If virus is resistant to a chemical treatment because it is present as an aggregate which the chemical cannot penetrate, it may also be resistant to a second, different, chemical treatment. Care must be taken to not count the same treatment twice, for example if ethanol has a direct inactivating effect on a virus, steps in fractionation involving increasing concentrations may all inactivate the virus in the same way, and therefore not have additive effects. In contrast if the reduction in viral infectivity results from the removal of virus particles at one ethanol concentration and the inactivation of virus at a higher concentration, the effects may be summed. Care must therefore be taken to justify summing the effects of different steps which, ultimately, is dependent on the steps removing or inactivating viruses by different mechanisms.

As part of the overall evaluation of product safety, in addition to laboratory scale process evaluation, it is sometimes possible to evaluate plasma pools or final products for viral contamination. As an additional measurement of the effectiveness of donor screening, the pooled plasma entering the production process can be screened for viral antibody or antigen with immunologic methods or for the quantity of viral genomic nucleic acid by NAT. The latter, even if only performed intermittently, provides a basis for assessing product safety when coupled with the data quantifying virus removal or inactivation. On the other hand, the testing of final product has been found to contribute little to safety. The purification process is likely to remove viral antibody or antigen to levels below the test's sensitivity limit, testing of immune globulin preparations by ELISA typically yields false positive results because of their high immune globulin content, and NAT cannot distinguish virus surviving an inactivation step from that which was inactivated. Additionally, if infectious virus was present, it is likely to be at very low concentration, thereby escaping detection. Recently in Europe, post marketing surveillance was concluded to be the best way of monitoring the safety of the product.

B. Protein Issues

To assure efficacy as well as consistency of manufacture, the integrity of the final product with respect to protein function and structure must be demonstrated. Several analytical approaches are typically employed in tandem and almost always include a functional assay for the protein of interest and an assessment of its aggregation/fragmentation. More sophisticated assays are occasionally employed, dependent on the product being manufactured. As examples, anticomplementary activity of every lot of IVIG is usually measured, while the thrombogenic potential of prothrombin complex concentrates, which is usually assessed during process development, may or may not be assessed on every lot. For newer methods of viral inactivation or for unusual methods of purification, a more thorough evaluation may be required to help discern whether neoimmunogens have formed. Unfortunately, laboratory and animal tests of neoimmunogenicity are not definitive.

1. Analysis of widely distributed products

The specifications for many plasma products are provided in several pharmacopeias, including those from Europe, the United States and Japan. As examples, Figures IVB-1 and IVB-2 provide specifications for albumin and IMIG products taken from the US Code of Federal Regulations. However, codified specifications are not necessarily comprehensive or complete, and many specialty assays are also frequently required (Figure IVB-3), the selection of which is at the discretion of the appropriate regulatory authority and may depend on whether the manufacturing protocols in use are well established or relatively new.

Figure IVB-1. Tests commonly employed when assessing widely distributed plasma products -- ALBUMIN

Test*	Result
Total protein	One of: 4.0 ± 0.25% 5.0 ± 0.30% 20.0 ± 1.2% 25.0 ± 1.5%
Purity	> 96%
Heat stability; 57°C, 50 hrs	Unchanged on visual inspection
Na^+	130-160 mEq/L
K^+	<2 mEq/L
pH	6.9 ± 0.5

* Taken from the US Code of Federal Regulations 21 CFR 640.80. The requirements of other national bodies may differ.

Figure IVB-2. Tests commonly employed when assessing widely distributed plasma products – IMIG

Test*	Result
Total protein	16.5 ± 1.5%
Purity	≥ 96% IgG
Diptheria antitoxin	≥ 2 units/mL
Anti-measles antibody	matches or exceeds standard
anti-polio antibody	matches or exceeds standard
Heat stability; 57°C, 4 hrs	No gelation on visual inspection
pH	6.8 ± 0.4

Taken from the US Code of Federal Regulations 21 CFR 640.1. The requirements of other national bodies may differ.

Figure IVB-3. Specialized assays commonly employed when assessing the activity, molecular integrity and safety of widely distributed plasma products

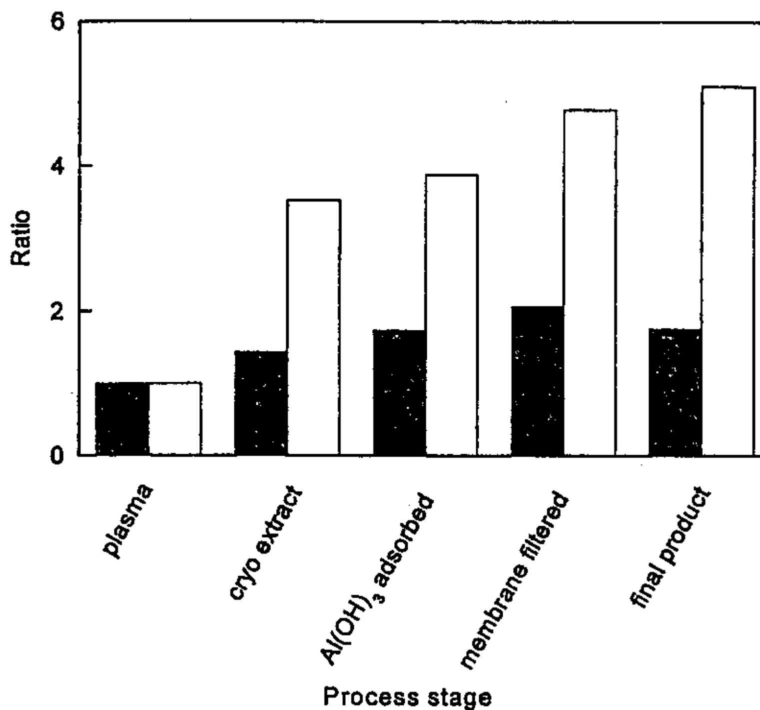
Product	Test	Common Results
AHF	Factor VIII procoagulant activity	30-100 IU/mL
	vWF antigen	Depends on purification method. May be absent or present at a concentration 2- to 3-times that of FVIII procoagulant activity
	Rabbit pyrogenicity	Passes. Endotoxin determination may be an acceptable alternative.
	Moisture content	≤2%
	Sterility	passes
PCC/ Factor IX	Factor IX coagulant activity	30-100 IU/mL
	Factors II, VII, and X	Depends on the purification method.
	Measure of activated clotting factors, e.g., NAPTT, thrombin generation time, fibrinogen clot test, etc.	"Prolonged" (typically > 100 seconds) clot times.
	Rabbit pyrogenicity	Passes. Endotoxin determination may be an acceptable alternative.
	In vivo thrombogenicity	Not typically a lot release test. Useful in validating manufacturing method
	Residual moisture	≤2%
	Sterility	Passes
IVIG	IgG content	≥95%
	Potency via specific assays for viral and bacterial antigens	matches or exceeds standard
	Monomer + dimer content	≥90%
	IgG aggregates	≤10%
	IgG fragments	≤10%
	IgG subclass distribution	Reflects plasma
	Anticomplementary activity	Passes
	Prekallikrein activator	≤ Standard
	Rabbit pyrogenicity	Passes
	Residual moisture (if lyophilized)	≤2%
	Sterility	Passes

2. Other integrity measurements

Depending on the experience with the process methods being employed, manufacturers and/or regulatory authorities may wish to see the results from additional laboratory and animal studies, some of which are described below.

Antigen/activity ratio: During process qualification, it may be useful to simultaneously measure protein activity as well as antigen concentration in an immunological assay. A constant ratio of activity to antigen during the isolation process provides evidence that fractionation did not affect protein structure while a decline in this ratio is indicative of detrimental effects. As an example, a clear increase in the antigen to functionality ratio has been shown during the isolation of factor VIII (Figure IVB-4); nonetheless, this preparation functioned normally in man.

Figure IVB-4. Changes in Ratio of Antigen/Functionality Ratio during Isolation of Factor VIII



Solid bars: VIIIC:Ag/VIII:C; open bars: VIII:RAg/VIII:C. From C.V. Prowse, B.Griffin, D.S. Pepper et al. Thromb Haemost 1981; 46: 597-601.

Protein size and shape: Gel chromatography detects overall changes in size and shape of proteins and has been used, e.g., to assess the molecular weight distribution of a range of IVIG products that were subjected to various treatments (Figure IVB-5). The same information may be obtained from HPLC. Other physico-chemical methods like diffusion coefficients, sedimentation constants and viscosity are also sensitive to the general shape of the protein molecule, but they normally will not detect small changes and cannot be used with protein mixtures.

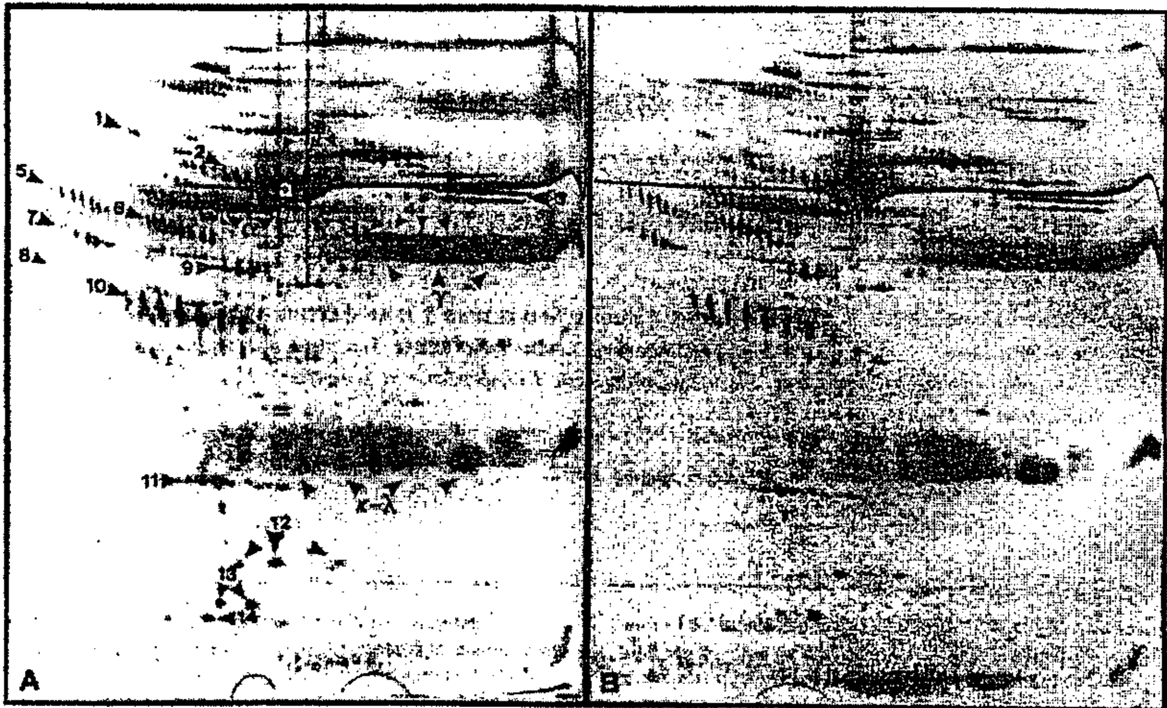
Figure IVB-5. Gel filtration of immunoglobulin preparations that underwent treatment to reduce their anticomplementary activity

Procedure	Oligomers [%]	Dimers [%]	Monomers [%]	splits [%]
Untreated	12	17	70	0
Plasmin treated	1	3	39	57
β-Propiolactone treated	2	12	85	0
Sulfonated	7	8	85	0
Reduced and alkylated	3	7	89	1
PH4/pepsin treated	0	4	90	6
PEG treated	0	4	96	1

From J. Römer, J.-J. Morgenthaler, R. Scherz, F. Skvaril. Vox Sang 1982; 42:2-73.

Enhanced SDS-PAGE: When a viral inactivation step is being first qualified, SDS-PAGE is usually applied to the protein solution prior to and following the viral inactivation step. If the protein is made up of several chains linked by disulfide bonds, SDS-PAGE under reducing and non-reducing conditions is used. When a more thorough evaluation is desired, the SDS-PAGE procedure can be refined by first subjecting the protein to enzymatic or chemical digestion. The combination of isoelectric focusing with PAGE is a very powerful tool for the detection of even small differences in protein structures and properties. In a study of the effects of methylene blue and light treatment on fresh frozen plasma no differences could be detected when the patterns of treated and untreated samples were compared (Figure IVB-6).

Figure IVB-6. Separation of Plasma Proteins by Isoelectric Focusing and PAGE: Comparison of Methylene Blue Treated Plasma (A) and Untreated Plasma (B)



Acidic side is on the left; high molecular weight proteins are on top. From J.-D. Tissot, D.F. Hochstrasser, B. Schneider, J.-J. Morgenthaler, P. Schneider: Brit J Haematol 1994; 86: 143-146.

Circulatory survival: Another possibility – albeit difficult, time-consuming, and expensive – consists in injecting the protein intravenously into a suitable animal (e.g., rat or rabbit) and comparing the half-life with a reference preparation of the same protein, possibly with the protein in its native state, i.e. in plasma. The kinetics of removal of a foreign protein from circulation is quite sensitive to minor changes in protein structure. Half-life times of albumin preparations were shown to depend on previous treatments (Figure IVB-7).

Figure IVB-7. Circulatory Half-life of Various Human Albumin Preparations in Rabbits

Albumin treatment	Average Half-life (hours ± SEM)
Unfractionated	143.0 ± 5.15
Pasteurized, cold-ethanol fractionated	139.0 ± 14.75
Reprocessed	125.1 ± 5.07
Heat-ethanol fractionated	124.1 ± 5.25
Chemically modified	79.9 ± 4.08

From C. Anhorn, S. Sheldon, C. Laschinger, D.H. Naylor: Vox Sang 1982; 42: 233-242.

Analogous experiments were also done with immunoglobulins and coagulation factors. A study demonstrated that recovery and half-life time of solvent-detergent treated factors VIII and IX in hemophilia A and B patients were not significantly different from historic controls (Figure IVB-8).

Figure IVB-8. *In Vivo* Circulatory Recovery and Half-life of Solvent-Detergent Treated Factor VIII and Factor IX in Man

	FVIII	FIX
Recovery	83.5 ± 20.1%	53.3 ± 17.4%
Half-life	12.5 ± 2.8h	19.4 ±4.2h

From C. Baumgartner, B.A. Perret, E. Meili, M. Furlan, H. Friedli, J.-J. Morgenthaler. Thromb Haemost 1987; 58: 350.

Neoimmunogenicity: Neoimmunogenicity may be looked at as a special case of changes to the higher order structure of proteins, which does not necessarily impair the protein's functionality, but which results in an immune response in the recipient. Some products had to be withdrawn from the market because clinical practice showed an increased number of inhibitors arising in patients treated with a new preparation as compared to older products. Detection of neoimmunogenicity preclinically is very difficult. One approach is to immunize one group of laboratory animals (rabbits) with a new preparation and another group with the same preparation with the viral inactivation step omitted or a similar preparation with proven lack of neoimmunogenicity. The resulting antibodies are compared with each other in a cross-over experiment; if the antibodies raised against the new preparation are completely adsorbed by the old preparation, the new one most likely does not contain neo-antigens. However, these

experiments have to be conducted in a heterologous system and there is no guarantee that the human immune system recognizes the same epitopes as the immune system of laboratory animals does. The ongoing use of these models with any of the well recognized viral inactivation procedures generally is considered to be optional.

The best proof for absence of neo-antigens is derived from careful clinical studies. The determination of circulatory recovery and half-life in repeatedly infused subjects can be very useful and are typically performed prior to licensure. A full assessment of immunogenicity is best monitored over the long term and, therefore, is typically monitored following licensure in human users. If there is no increase in the appearance of clinically relevant antibodies or other adverse immunological reactions in patients when the product is compared to older variants, it is reasonable to assume that the newly developed product does not exhibit any neoantigens.

C. Pre-Market Clinical Trials

Historically, the role of clinical trials is to assess efficacy, general as well as viral safety, and immunogenicity. Trial design for established products is a subject of considerable discussion within the EU and USA with an overall trend toward simplifying trial design and reducing the number of patients that are required. Viral safety is assessed principally by review of donor demographics, test procedures, and process validation. Within the EU, there is a trend to assess viral safety in man after rather than before receipt of marketing authorization. This trend takes into account the safety that current products have exhibited, recent reductions in viral loads, the universal use of well validated methods of virus removal and inactivation, and the relative insensitivity of small clinical trials.

Special circumstances in individual countries and the divergent medical uses of the established products makes the formation of guidelines a daunting task. Generally, all products made by new processes or new manufacturers should undergo general safety testing in a minimum of 5-10 volunteers, and in many cases, clinical testing will involve 25 or more patients.

D. Implementation in a Manufacturing Setting

1. Facility layout and product flow

The benefit of virus inactivation/removal will be negated if materials that precede such steps are permitted to recontaminate the intermediates or product that follow. The simplest and best solution from a facility management perspective is to transfer product from one room to the next in the course of the inactivation/removal procedure. This serves to create different safety zones which, when arranged in a clear and logical way, helps avoid cross-contamination. In the best implementations, every zone has its own dedicated staffing, equipment, entrance, and air handling and other services. Product flow is unequivocal, from less safe from the point of view of viral contamination through the various decontamination steps to the safest product. When this is not practical, the same effect can be achieved through management practices, which at the extreme, become very difficult to enforce. For example, some facilities utilize the same staff in