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Annex 4
Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products

List of abbreviations and definitions used in this Annex

The definitions given below apply to the terms used in these guidelines. They may have different meanings in other contexts.

AHF	Antihaemophilic factor. Blood coagulation factor VIII, missing in patients with classic haemophilia.
Blood components	These typically refer to red blood cell concentrates, platelet concentrates and plasma.
BEV	Bovine enterovirus. A non-enveloped, single-stranded RNA virus used as a model for hepatitis A virus.
BVDV	Bovine viral diarrhoea virus. An enveloped, single-stranded RNA virus used as a model for hepatitis C virus.
CMV	Cytomegalovirus. An enveloped, double-stranded DNA virus, typically cell-associated.
Coxsackie virus	A non-enveloped, single-stranded RNA virus.
CPV	Canine parvovirus. A non-enveloped, single-stranded DNA virus.
Donor retested plasma	A process for reducing window period transmissions whereby fresh frozen plasma is held in the inventory for a designated period of time until the donor returns and tests negative for virus exposure. The initial unit is then released for use. Also called *quarantine plasma*.
Dry heat	A process of heating protein following lyophilization, typically at 80 °C or higher.
EBV	Epstein–Barr virus. An enveloped, double-stranded DNA virus, typically cell-associated.
EMCV	Encephalomyocarditis virus. A non-enveloped, single-stranded, RNA virus.
Factor IX	Blood coagulation factor IX, missing in patients with haemophilia B.
Factor VIII	Blood coagulation factor VIII, missing in patients with haemophilia A. Also called antihaemophilic factor.
FFP	Fresh frozen plasma.
Fluence	The total quantity of light delivered. Expressed in J/cm^2.
Gamma-irradiation	A process of virus inactivation or bacterial sterilization using gamma-irradiation of liquid, frozen or lyophilized product.

GE	Genome equivalents. The amount of nucleic acid of a particular virus assessed using nucleic acid testing.
GMPs	Good manufacturing practices. Sometimes referred to as current good manufacturing practices.
HAV	Hepatitis A virus. A non-enveloped, single-stranded RNA virus.
HBsAg	Hepatitis B surface antigen. The antigen on the periphery of hepatitis B virus.
HBV	Hepatitis B virus. An enveloped, double-stranded DNA virus.
HCV	Hepatitis C virus. An enveloped, single-stranded, RNA virus.
HDV	Hepatitis delta virus. A defective virus which requires co-infection by hepatitis B virus.
High purity factor VIII	Factor VIII concentrate with a specific activity typically greater than 100 IU/mg.
HIV	Human immunodeficiency virus. An enveloped, single-stranded RNA virus.
HSV	Herpes simplex virus. An enveloped, double-stranded DNA virus, typically cell-associated.
HTLV 1 and 2	Human T-cell lymphotropic virus, types 1 and 2. Enveloped, single-stranded RNA viruses, typically cell-associated.
ID_{50}	The quantity of virus or other infectious agent that will infect 50% of subjects or tissue cultures. Frequently expressed on a log scale; thus, 6 $\log_{10}$ ID_{50} represents 1 million infectious units.
Immunogenic	Causing the formation of antibody. Harsh processing conditions may modify the structure of a protein so as to make it immunogenic.
Intermediate purity factor VIII	Factor VIII concentrate with a specific activity between 1 and 50 IU/mg.
IVIG	Intravenous immunoglobulin.
Limiting dilution	A way of determining titre by diluting the sample continually until the positive signal is lost.
LRF	Log reduction factor. The quantity of virus, expressed on a log 10 scale, inactivated or removed.

MB-plasma	Methylene blue-treated plasma intended as a substitute for fresh frozen plasma.
Nanofilters	Filters that usually have effective pore sizes of 50 nm or less, designed to remove viruses from protein solutions.
NAT	Nucleic acid testing, using amplification techniques such as polymerase chain reaction.
Pasteurization	A process of heating protein in solution, typically at 60 °C.
Polio virus	A non-enveloped, single-stranded, RNA virus.
PPRV	Porcine pseudorabies virus. An enveloped, double-stranded DNA virus.
PPV	Porcine parvovirus. A non-enveloped, single-stranded DNA virus.
Prion	The infectious particle associated with transmissible spongiform encephalopathies. It is believed to consist only of protein and to contain no nucleic acid.
PRV	Pseudorabies virus. An enveloped, double-stranded DNA virus.
Psoralen	A furocoumarin ring structure, which when exposed to light, cross-links nucleic acid.
Quarantine plasma	*See donor retested plasma*.
RT3	Reovirus type 3. A non-enveloped, double-stranded RNA virus.
Rutin	A flavonoid used as an antioxidant that reduces the action of reactive oxygen species.
Solvent/detergent treatment	A process of treating protein in solution, usually with the organic solvent, tri(*n*-butyl)phosphate, and a detergent such as Tween 80 or Triton X-100.
SD-Plasma	Solvent/detergent-treated plasma intended as a substitute for FFP.
Sindbis virus	An enveloped, single-stranded RNA virus.
SLFV	Semliki forest virus. An enveloped, single-stranded, RNA virus.
Titre	The quantity of virus, typically expressed on a $\log_{10}$ scale. Six logs of virus are equal to 1 million infectious units.
TNBP	Tri(*n*-butyl)phosphate. The organic solvent used with *solvent/detergent treatment*.
Triton X-100	A non-ionic detergent frequently used as part of *solvent/detergent treatment*.

Tween 80	A non-ionic detergent frequently used as part of *solvent/detergent treatment*.
UVC	Ultraviolet irradiation, usually at a wavelength of 254 nm.
Vaccinia virus	An enveloped, double-stranded DNA virus.
Vapour heating	A process of heating protein following lyophilization and then reintroducing moisture normally at 60 °C and in some cases at 80 °C.
Viral inactivation	A process of enhancing viral safety in which virus is intentionally "killed".
Viral removal	A process of enhancing viral safety by removing or separating the virus from the protein(s) of interest.
VSV	Vesicular stomatitis virus. An enveloped, single-stranded RNA virus.
West Nile virus	An enveloped, single-stranded RNA virus.

1. Introduction and scope

Human blood is the source of a wide range of medicinal products used for the prevention and treatment of a variety of often life-threatening injuries and diseases. Despite measures such as donor selection, testing of donations and of plasma pools, the transmission of blood-borne viruses by plasma and purified plasma products is still considered to constitute a risk to patients. Over the past 15–20 years, the transmission of the principal viral threats historically associated with these products — hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) — has been greatly reduced or eliminated in many areas of the world. This is a consequence of the more sensitive methods being used to screen donated blood and plasma pools, and of the establishment of manufacturing practices that lead to significant virus inactivation and removal. Several procedures for virus inactivation and removal have proven to be robust and to contribute substantially to blood product safety. Viral inactivation methods should be applied to all blood plasma-derived protein solutions.

Continuing concerns about the quality and safety of plasma-derived medicinal products have resulted in a number of urgent requests from Member States for support and advice from WHO. Moreover, the World Health Assembly Resolution No 50.20, of 13 May 1997 on the "Quality of biological products moving in international commerce", requested WHO to extend the assistance offered to Member States to

develop and to strengthen their national regulatory authorities and control laboratories to increase competence in the area, and to extend efforts to upgrade the quality and safety of all biological products worldwide.

The present WHO Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products were developed to complement the WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives"(*1*), in response to the above requests. These Guidelines pertain to the validation and assessment of the steps for viral inactivation and removal employed in the manufacture of human blood plasma derivatives and virally inactivated plasma for transfusion, prepared either from plasma pools or from individual donations. It is hoped that this document, by summarizing current experience with well recognized methods, will help set expectations, serve as a guide to speed implementation, and ensure that implementation is appropriate.

Inevitably, individual countries may formulate different policies, not only in relation to procedures for validation and control, but also regarding donor selection and methods of blood screening. These Guidelines do not replace the requirements of regulatory authorities in various parts of the world (*2–4*); rather, they are primarily intended to assist those national regulatory authorities and manufacturers that are less familiar with viral decontamination processes.

The document does not address products of animal origin or those manufactured by recombinant techniques.

2. General considerations

Viral safety derives from three complementary approaches during manufacture, i.e. donor selection, testing of donations and plasma pools, and the introduction of viral inactivation and removal procedures in the course of manufacture, each of which requires strict adherence to good manufacturing practices (GMPs). Although these Guidelines address only viral inactivation and removal, no individual approach provides a sufficient level of assurance, and safety will only be achieved by using a combination of the three.

Some of the principles that relate to viral inactivation and removal procedures as applied to purified blood plasma products and to plasma intended for transfusion are listed below.

- Viral contamination can arise from the donor, or, less commonly, from other sources introduced during manufacture (e.g. from the reagents employed).
- Viral validation studies are intended to assess the degree to which virus infectivity is eliminated during manufacture. These studies can only approximate the inactivation and removal that occur during routine manufacture because the model viruses employed in the studies may differ from those present in blood, and it may be difficult or impossible to truly model the conditions employed during manufacture. Thus, the appropriateness of the studies needs to be reviewed on a case-by-case basis, and the manufacturer should justify the choice of viruses and the validation conditions employed.
- Viruses to be studied, where required, include: HIV; a model for hepatitis C such as Sindbis virus or bovine viral diarrhoea virus (BVDV); one or more non-enveloped viruses such as hepatitis A virus, encephalomyocarditis virus (EMCV), or porcine parvovirus; and an enveloped DNA virus such as pseudorabies virus or duck hepatitis B virus.
- The ability of a process to inactivate or remove viruses should take into account:
  — the reduction in virus titre achieved;
  — for inactivation processes, the rate of inactivation and the shape of the inactivation curves; for removal, mass balance;
  — how robust the step is in response to changes in process conditions; and
  — the selectivity of the process for viruses of different classes.

  Data should be analysed using appropriate statistical procedures.
- Virus removal should be distinguished from virus inactivation. This is important in ensuring the accurate modelling of a process step and identifying the parameters that are most effective in reducing infectivity in that process. For example, if a chromatography step removes viruses, flow rates and column dimensions are important process variables, whereas if the buffer used inactivates viruses, temperature and pH are likely to be more significant.
- Purification procedures such as precipitation or chromatography can contribute to virus removal; however, removal depends critically on the protein composition and the separation conditions used, and it is difficult to scale down partition processes for validation purposes. Therefore, all appropriate specifications and accepted tolerances should be stated, and control data provided. For chromatographic columns and media, the conditions of storage, preservation and regeneration should be described.

- Validation studies need to be well documented to ensure proper execution of the procedure. The highest titre of virus that can reasonably be employed should be added (spiked) into the solution to be tested at a ratio not exceeding one part virus to nine parts sample. Virus infectivity starting titre should be measured, ideally after addition to the sample, and then with time during the virus inactivation and removal procedure. Worst case conditions must be studied. Appropriate controls should be run to demonstrate the validity and sensitivity of the assay.
- All viral infectivity tests suffer from the limitation that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Consequently, the largest sample size that can be practically assayed should be chosen if the study indicates that all viruses are inactivated or removed.
- Appropriate procedures should be employed throughout the manufacturing process to prevent recontamination following use of a virus inactivation or removal method.
- Priority for validating the viral inactivation steps used in the manufacture of plasma protein solutions should be given to those products with the highest risk potential, such as coagulation factors, proteolytic inhibitors and intravenous immunoglobulins.

3. Infectious agents

3.1 Viruses, viral burden and screening methods

Medicinal products made from human blood include clotting factors, immunoglobulins and albumin among others, have all at some time transmitted serious virus infections to recipients. The object of viral inactivation and removal procedures is to improve viral safety so that such transmissions no longer occur. The viruses of particular concern, HBV, HCV and HIV, have all been transmitted by some plasma products, and all cause life-threatening diseases. Other viruses of concern include hepatitis A virus and parvovirus B19, both of which have been transmitted by clotting factor concentrates. Some of the properties of these viruses are listed in Table 1.

The pathogenicity of a virus may depend on the patient group and on the product being administered. For example parvovirus B19 infects the red blood cell precursors and effectively eliminates them for a period. Parvovirus infections are usually relatively mild in the general population because most people have a substantial buffer of mature red cells. However, in patients with haemolytic anaemias (such as sickle-cell anaemia), parvovirus infections can be fatal because the

Table 1
Selected properties of some plasma-borne viruses

Virus	Genome	Envelope	Size (nm)
Hepatitis B virus	dsDNA	yes	40–45
Hepatitis C virus	ssRNA	yes	40–50
Human immunodeficiency virus	ssRNA	yes	80–130
Hepatitis A virus	ssRNA	no	28–30
Parvovirus B19	ssDNA	no	18–26

ds, double-strand; ss, single-strand

lifespan of mature red cells is shorter. Parvovirus B19 may be of greater concern in Africa where sickle-cell anaemia is relatively more common than in Europe, and it is possible that other agents (e.g. hepatitis E virus) would be significant in other geographical settings depending on their prevalence in the donor population. Other examples include cytomegalovirus and human T lymphotropic virus I and II (HTLV I + II) which are strongly cell-associated and are therefore not considered to pose a significant risk in therapeutic proteins derived from human plasma, although they have been transmitted by cellular components in blood transfusions, and HAV, which can be transmitted by purified coagulation factor concentrates, but is not usually a problem with products such as intravenous immunoglobulin (IVIG) that contain anti-HAV antibodies.

For the product to be safe, the production process must inactivate and/or remove all the virus present. The quantity of virus depends on the number of infected donors contributing to the pooled starting material and the titre (concentration) of infectious virus in those donations. Estimates of the frequency of occurrence of hepatitis viruses, HIV and parvovirus and their titres prior to the implementation of screening tests, in European and US donor populations are given in Table 2. For example, before tests for HCV antibody were developed, approximately 1–2% of donors were unknowingly infected with HCV. Parvovirus is now known to be present in 1/1000–1/7000 blood donors, largely because it is a common infection in the general population, and tests for it are not routinely employed. Most pools of 10 000 or more unscreened donor units would therefore be expected to be contaminated with HCV and parvovirus. When this information is combined with the titre of virus in contaminated units and the number of donors contributing to the plasma pool, the titre in the plasma pool can be calculated (Table 2). Because the titres of HCV RNA in an infected individual may range from 10^4 to 10^6 ge-

Table 2
Viruses in plasma from unscreened donor blood

Virus	Prevalence in donor blood	Viral titre (GE/ml)	Calculated titre in plasma pool (GE/ml)[a]
Hepatitis B virus	1/10000	10^3–10^8	0–10^4
Hepatitis C virus	1/50–1/100	10^4–10^6	10^2–10^4
Human immunodeficiency virus	1/1000–1/10000	10^3–10^7	0–10^4
Hepatitis A virus	1/500000	10^3–10^5	0–10^1
Parvovirus B19	1/1000–1/7000	10^2–10^{12}	0–10^9

[a] Assumes the pooling of 10000 units.

Table 3
Frequency of HCV RNA-positive plasma pools following testing of single donations for anti-HCV antibody

Screening test on individual unit	Number of pools (positive/total)	Percentage hepatitis C virus PCR positive
None	8/8	100
First-generation antibody test	65/85	76
Second-generation antibody test	49/123	39

Source: Nübling, Willkommen & Löwer (*5*).
PCR, polymerase chain reaction.

nome equivalents (GE)/ml and those of parvovirus B19 DNA from 10^2 to 10^{12} GE/ml, plasma pools would be expected to contain 10^2–10^4 GE/ml of HCV and 0–10^9 GE/ml of parvovirus. Put more simply, most pools of 10000 or more unscreened donor units would be expected to be contaminated with HCV and parvovirus, whereas contamination with HBV, HIV and HAV would occur at a lower frequency. Viral titres of HBV, HCV and HIV in the plasma pool can reach 10^4 GE/ml. It should be noted that the incidence of virally infected units depends on several factors including the population from which the donors are drawn and, for parvovirus, on seasonal variations.

A study conducted at the Paul Ehrlich Institute, Germany, determined the frequency of HCV RNA-positive pools before and after screening of donors was introduced, using first- or second-generation tests for HCV antibody (Table 3) (*5*). Although screening reduced the number of antibody-positive pools, it is important to note that the viral titre in those pools that were contaminated was not reduced. This is a consequence of using a test for the antibody rather than for

Table 4
Average window period estimates for HIV, HCV and HBV

Virus	Window period without nucleic acid amplification technology (days)	Window period with minipool nucleic acid amplification technology (days)
Human immunodeficiency virus	22	10
Hepatitis C virus	82	9
Hepatitis B virus	59	49

Sources: Schreiber et al. (*8*); Kleinman et al. (*9*).

the virus and because in the case of HCV, and many other viruses, peak titres occur before the appearance of the antibodies in the circulation (i.e. the so-called window period). Nonetheless, because the screening of donors for markers of infection such as hepatitis B surface antigen or antibodies to HIV or HCV can reduce the number of positive pools and, in certain circumstances, the virus load in the starting material, screening is an important element in assuring viral safety.

Nucleic acid amplification technology (NAT) has been introduced in some instances to detect viral nucleic acid. As nucleic acid is associated with the virus itself rather than the host response to infection, NAT minimizes the window period and reduces the total quantity of virus in the plasma pool (*6*, *7*). Window period estimates are given in Table 4. As an additional measurement of the effectiveness of donor screening, the quantity of viral genomic nucleic acid present in the plasma pool can be assessed by NAT. Even if only carried out intermittently, performing NAT on plasma pools provides a basis for assessing product safety when coupled with the data quantifying virus removal or inactivation.

Finally, it should be recognized that all screening methods are subject to the criticisms that they are unable to detect virus infection below a certain level, and that errors in the screening process may occur, particularly where large numbers of donations are used. Additionally, screening is limited to the viruses being tested for. Thus, while screening helps to ensure that the virus load is kept to a minimum, it is not sufficient to ensure safety in itself, and the ability of the production process to remove or inactivate viruses is a crucial second element. The proportion of potential donors who are infected with viruses will depend on the particular geographical region. In donors from certain areas, HBV or HIV infections may be far more common than in those

from countries where the strategies for ensuring viral safety have evolved. Where this is the case, the ability of the production process to inactivate or remove viruses will be even more important.

3.2 Other infectious agents

Bacteria and parasitic infections including malaria and trypanosomes do not pose a risk in plasma products that have been sterile filtered with a 0.2 µm filter.

Prions, the putative causative agent of the transmissible spongiform encephalopathies including Creutzfeld Jakob Disease (CJD) of humans, are a matter of concern, especially as a result of the occurrence of variant CJD (vCJD) in the United Kingdom following the epidemic of bovine spongiform encephalopathy. The continuing concern stems, in part, from experimental evidence in animal models that infectivity could be present in blood, albeit late in infection and at low levels. However, there has been no increase in the incidence of classic CJD (currently one death per million head of population per year wherever it has been measured), despite the increased transfusion of blood and the extremely hardy nature of the agent. As with CJD, there is no evidence that vCJD has been transmitted by blood, blood components or plasma-derived products in clinical practice. However, since vCJD is a newly emerging disease, it is too early to conclude that there is no risk. Measures to minimize the risks to humans from human- and bovine-derived materials are summarized in the report of a WHO consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies (*10*).

3.3 Validation of viral inactivation and removal procedures

3.3.1 *Selection of relevant and model viruses*

The viruses that may contaminate blood and blood products encompass all of the viral types, including viruses with a DNA or RNA genome, with and without a lipid membrane, and ranging in size from the smallest, such as parvovirus, to the middle range, such as HBV. The processes employed should therefore be shown to be able to remove or inactivate a wide range of viruses if they are to be considered satisfactory; typically, validation studies have involved at least three viruses, chosen to represent different kinds of agent.

Viruses have been selected to resemble those that may be present in the starting material (Table 5). All are laboratory strains that may be grown to high titre and assayed readily. The models for hepatitis C virus include BVDV, Sindbis virus, Semliki forest virus and yellow

Table 5
Plasma-borne viruses and their models

Virus	Examples of viruses used to model inactivation/ removal studies
Hepatitis B virus	Duck hepatitis B virus, pseudorabies virus[a]
Hepatitis C virus	Bovine viral diarrhoea virus, Sindbis virus, Semliki forest virus, Yellow fever virus
Human immunodeficiency virus	Human immunodeficiency virus
Hepatitis A virus	Hepatitis A virus, encephalomyocarditis virus
Parvovirus B19	Canine parvovirus, porcine parvovirus

[a] Because there are no convenient models for hepatitis B virus, pseudorabies virus is frequently used: both pseudorabies virus and hepatitis B virus are enveloped, double-stranded DNA viruses.

fever virus as they share many properties, including a lipid membrane, an RNA genome and a particle size of 40–50 nm. Laboratory strains of HIV or hepatitis A virus are used, and canine and porcine parvovirus have been used as models for parvovirus B19. Suitable 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 pseudorabies virus has also been employed as a large DNA virus. This list is not exhaustive and other appropriate viruses are acceptable. The main viruses of concern are HIV, HBV and HCV, and laboratory viruses are almost always used to represent them. During the developmental phase, viruses that are particularly resistant to the approach taken often serve as useful surrogates. As an example, the use of vesicular stomatitis virus (VSV) has proven useful when first qualifying a viral inactivation step based on low pH or solvent/detergent treatments. Nonetheless, for product registration, the use of viruses that better resemble those present in the starting material should be used. Precautions needed for 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. Readers are directed to existing guidance documents for additional details on the selection and assay of model viruses (*2*, *3*).

3.3.2 ***Modelling (downscaling) the production process***

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 by following them. Not every step needs to be evaluated. The ability of the steps in a process to remove or inactivate viruses is measured on a laboratory scale and not in the production facility

where it would be inappropriate to introduce infectious virus deliberately. The accuracy of the model is crucial, and should be assessed by comparing the characteristics of the starting material and the product for that step for both laboratory and full-scale processes. In the model of the process, physical factors (e.g. temperature, stirring, column heights and linear flow rates, and sedimentation or filtration conditions) and chemical factors (e.g. pH, ionic strength, moisture and the concentration of inactivating agents) should be equivalent to the real process) where possible. It should be noted that whereas many process steps can be modelled readily, models of ethanol fractionation processes have proved particularly variable, in part because of difficulties in scaling down centrifugational processes and in controlling subzero temperatures on a small scale.

Once the step is accurately modelled, virus is introduced into material derived from the fractionation process just prior to the step being evaluated, and the amount remaining after the modelled process step is measured. The results are conventionally expressed in terms of the logarithm of the reduction in infectivity reported. Total infectivity or viral load is calculated as the infectious titre (infectious units per ml) multiplied by the volume. Viral clearance compares the viral load at the beginning with that at the conclusion of the step being evaluated.[a]

For viral inactivation procedures, both the kinetics and extent of virus inactivation need to be demonstrated. The kinetics of inactivation are important since the rapid kill of large amounts of virus is a further indication of the virucidal potential of the step and, for well-characterized procedures for viral inactivation, enables comparison of a process with similar processes executed by others (see section 4). For viral removal systems, an attempt should be made to show mass balance, i.e. to account for all of the virus added. If the buffers used are virucidal, it is important to distinguish virus inactivation from virus removal.

It is necessary to evaluate the effect of possible variations in the process conditions on the virus clearance observed, for example the effects of changes in temperature or composition of the starting material for the particular step. A robust, effective and reliable process step will be able to remove or inactivate substantial amounts of virus, typically 4 logs or more, be easy to model convincingly and be rela-

[a] For example, if at the start of a step the viral titre is 10^5/ml and the volume is 20 ml and at the conclusion of the step the viral titre is 10^1/ml and the volume is 60 ml, then the viral load at the start is 6.3 logs and at the end is 2.8 logs, and the viral clearance is 3.5 logs.

tively insensitive to changes in process conditions. Steps removing 1 log of virus or less cannot be regarded as significant. A production process that includes two robust steps able to remove or inactivate enveloped viruses is likely to give a safe product, particularly if the steps act by different mechanisms (e.g. inactivation by a chemical treatment followed by a robust physical removal step). Non-enveloped viruses are more difficult to remove or inactivate. A process that includes one robust step effective against non-enveloped viruses may give a safe product; failing this, other approaches including implementing screening procedures, e.g. NAT, may prove helpful in excluding infectious material.

Virus validation studies are subject to a number of limitations. 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 true only if the fraction of virus surviving one step is not resistant to another, which is not always the case. If virus is resistant to a chemical treatment because it is present as an aggregate that 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 of ethanol may all inactivate the virus in the same way, and will therefore have no additive effects. In contrast, if the reduction in viral infectivity results from the removal of virus particles at one ethanol concentration, followed by the inactivation of virus at a higher concentration, the effects may be summed. Care must therefore be taken to provide justification for summing the effects of different steps which, ultimately, is dependent on the steps removing or inactivating viruses by different mechanisms. Other limitations are that the properties of the virus used in the laboratory studies may differ in from that occurring in nature, the plasma may contain antibodies to the virus of interest that may affect virus inactivation or removal in unpredictable ways, there may be fractions of virus that are resistant to a number of steps, and the modelling of the process may be imperfect. The clearance figures obtained are therefore approximate.

The difficulties of establishing an adequate laboratory model for virus inactivation and removal mean that the figures produced are unlikely to fully reflect manufacturing operations. In general, for a product to be safe, the process must remove or inactivate virus infectivity to a much greater extent than the level of virus in the starting materials. The use of two complementary steps for virus inactivation and removal may be especially important if the population of donors con-

tributing to the plasma pool has a high incidence of bloodborne viruses, leading to a high viral load in the material being processed. A second advantage in employing two complementary methods of virus inactivation and removal is the potential to increase the spectrum of viruses covered.

3.3.3 *Other considerations*

In practice many inactivation and removal processes result in a product that is safe. For bacteria, a sterile product is conventionally defined as one having fewer than one infectious organism in one million doses. No comparable figure has been agreed upon for viral sterility because viruses are more difficult to assay in the final product, the titre of virus in the stocks used to spike product is limited, and assessing the ability of a process to remove or inactivate viruses is subject to significant sources of error.

The testing of a final product for viral markers, as part of the routine batch release, has generally been found to contribute little to safety. Commercially available serological tests are generally not designed or validated for use with purified fractions. For most products, the purification process is likely to remove viral antibody or antigen to levels below the limit of sensitivity of the test, and for immune globulin preparations testing by ELISA typically yields a very high rate of false-positive results because of their high immune globulin content. With respect to genomic tests, NAT testing of plasma pools has proven useful; however, NAT cannot distinguish virus that has survived an inactivation step from inactivated virus, and if infectious virus is present, it is likely to be at very low concentration. Therefore NAT testing of final product is not recommended. Should final product testing be performed, the tests used must be shown to be suitable for their intended purpose.

3.3.4 *Measurement of infectivity*

The provision of details on the methods used to measure viral infectivity is beyond the scope of this document, and readers are referred to other available guidelines (see references *2–4*). A sample final report of a viral inactivation study is given in Appendix 1. A few points to consider are given below.

- Care should be taken when preparing virus stocks with high titres to avoid aggregation which may enhance physical removal and decrease inactivation thus distorting the correlation with actual production.

- The virus spike should be added to the product in a small volume so as to not dilute or alter the characteristics of the product. Typically, a spike of 5–10% of the total volume is employed.
- Buffers and product should be evaluated independently for toxicity or interference in viral infectivity assays used to determine viral titres, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, dialysis of the buffer, or other steps to eliminate toxicity or interference will be necessary. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g. dialysis and storage) on the removal or inactivation of the spiking virus should be included.
- If samples are frozen prior to assay, sufficient controls need to be run to show that the freeze/thaw cycle does not affect virus infectivity. Inactivating agents should be removed prior to freezing.
- The reliability of the viral assays employed needs to be demonstrated. This may necessitate 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 a within-assay 95% confidence interval of plus or minus $0.5 \log_{10}$.

4. Review of well-recognized methods for viral inactivation and removal

The methods described in this section are generally recognized as contributing substantially to viral safety based on the following factors:

— their application to a variety of products;
— use by several manufacturers;
— the availability of a substantial body of preclinical and clinical information; and
— their robust nature.

Well recognized methods of inactivation (pasteurization, dry heat, vapour heat, solvent/detergent and low pH) are described in section 4.1, and well recognized methods of removal (precipitation, chromatography and nanofiltration) are described in section 4.2. The selection of the methods to be employed for viral inactivation and removal depends on the size and lability of the protein being prepared, the method(s) of purification the manufacturer wishes to use, and the nature and titre of the viruses of concern. Each method of inactivation and removal has special characteristics that need to be taken into

account. For example, solvent/detergent is very effective against enveloped viruses, but does not inactivate non-enveloped viruses. If HBV is a principal concern, solvent/detergent may have an advantage over methods that employ heating because HBV is known to be relatively heat stable. On the other hand, several methods of heating have been shown to inactivate 4 logs or more of HAV; therefore if HAV is the virus of concern, heat has an advantage over solvent/detergent. As mentioned above, from a virus safety perspective, the best procedures will use a combination of complementary methods because combinations have the advantage of increasing the spectrum of viruses covered as well as of increasing the total quantity of virus that is eliminated. Whether one or more methods of inactivation and removal are used, the maintenance of protein structure and function is equally as important as viral safety and must be evaluated thoroughly. The general characteristics of well recognized methods of inactivation and removal are listed in Tables 6a and 6b, and examples of the successful application of individual, dedicated viral inactivation and removal procedures to commercialized products are provided in Table 6c. Subsequent sections provide representative data applicable to a variety of products; nonetheless, manufacturers are obligated to evaluate virus inactivation and removal in each of their products.

4.1 Methods of inactivation

4.1.1 *Pasteurization of albumin*

Albumin solutions are heated as a liquid at 60 ± 0.5 °C for 10–11 hours continuously, usually following sterile filtration and dispensing into final containers (glass vials). If pasteurization is conducted before filling, care must be taken to prevent post-treatment contamination, and bacterial sterility may be compromised. To prevent denaturation of albumin, low concentrations of sodium caprylate alone or with *N*-acetyl tryptophan are added prior to sterile filtration. Safety with respect to hepatitis viruses and HIV has been demonstrated for decades, with few exceptions (*11*). Much of this history derives from albumin manufactured using cold ethanol fractionation, which also contributes to safety. The inactivation of model viruses added to 5% albumin solution on heating at 60 °C is shown in Figure 1. Infectious virus can no longer be detected after 10 minutes of treatment. Because the conditions of treatment are well established and, in some countries, specified by regulation, manufacturers are not required to validate the effectiveness of the treatment itself; however, they need to demonstrate that the process parameters of temperature and time are met. Homogeneity of temperature is typically achieved by total immersion of the vials in a water-bath or by placing them in a forced-

Table 6a
Characteristics of well recognized virus inactivation procedures

Treatment	Advantages	Points to consider	Most relevant properties to be recorded
Pasteurization	• Inactivates both enveloped and some non-enveloped viruses including HAV • Relatively simple equipment	• Protein stabilizers may also protect viruses • HBV is relatively heat stable • Does not inactivate parvovirus B19 • Process validation required	• Temperature • Temperature homogeneity • Duration • Stabilizer concentration
Terminal dry heat	• Inactivates both enveloped and some non-enveloped viruses including HAV • Treatment applied on the final container	• At least 80 °C usually required for elimination of hepatitis viruses • Does not inactivate parvovirus B19 • Requires strict control of moisture content • Freezing and lyophilization conditions require extensive validation	• Freeze cycle • Lyophilization cycle • Temperature homogeneity • Residual moisture
Vapour heat	• Inactivates both enveloped and some non-enveloped viruses including HAV	• Does not inactivate parvovirus B19 • Freezing and lyophilization conditions require extensive validation • Relatively complex to implement	• Freeze cycle • Initial lyophilization cycle • Temperature homogeneity • Moisture before and after heating
Solvent/detergent	• Very efficient against enveloped viruses • Does not denature proteins • High process recovery • Relatively simple equipment	• Non-enveloped viruses unaffected • Not generally affected by buffers used • Solvent/detergent reagents must be removed	• Temperature • Duration • Reagent concentration
Acid pH	• Effective against enveloped viruses • Relatively simple equipment	• Limited efficacy against non-enveloped viruses • Use largely restricted to IgG • At pH 4, effective virus kill requires elevated temperatures • Process validation required	• pH • Temperature • Duration

HAV, hepatitis A virus; HBV, hepatitis B virus.

Table 6b
Characteristics of well recognized virus removal procedures

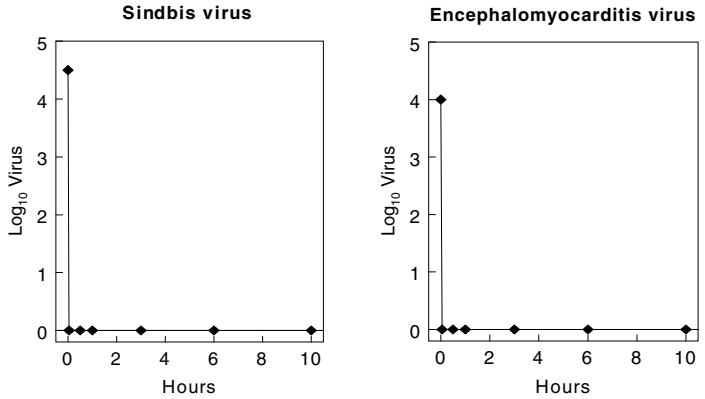
Treatment	Advantages	Points to consider	Most relevant properties to be recorded
Precipitation	• Purifies protein • Can be effective against both enveloped and non-enveloped viruses including HAV and parvovirus B19	• Virus removal usually modest • Difficult to model	• Concentration of precipitation agent(s) • Protein concentration, pH, and possibly ionic strength . • Temperature • Timing for the addition of precipitation agent and for precipitate ageing • Degree of contamination of precipitate with supernatant (or vice versa)
Chromatography	• Purifies protein • Can be effective against both enveloped and non-enveloped viruses including HAV and parvovirus B19	• Virus removal highly dependent on choice of resin, protein solution and buffers • May be highly variable from one virus to another • Degree of virus removal may change as resin ages • Resin must be sanitized between lots	• Resin packing by e.g. HETP measurements • Protein elution profile • Flow rate and buffer volumes • Number of cycles of resin use
Nanofiltration	• Effective against enveloped viruses • Can be effective against non-enveloped viruses including HAV and parvovirus B19 • Does not denature proteins • High recovery of “smaller” proteins such as coagulation factor IX • Risk of downstream contamination limited when performed just prior to aseptic filling	• Degree of virus removal depends on the pore size of filter used • Elimination of small viruses may be incomplete • Filter defects may not be detected by integrity testing	• Pressure • Flow-rate • Filter integrity • Protein concentration • Ratio of product volume to filter surface area

HAV, hepatitis A virus; HETP, height-equivalent theoretical plates.

Table 6c
Established applications of dedicated viral inactivation and removal procedures to marketed plasma products

Treatment	Product type
In-process	
Solvent/detergent treatment	• IgG • Coagulation factors (e.g. factor VIII, factor IX, prothrombin complex, fibrin sealant) • Protease inhibitors (e.g. antithrombin III) • Plasma
Pasteurization	• IgG • Coagulation factors (e.g. factor VIII, factor IX, von Willebrand factor, prothrombin complex, fibrin sealant) • Protease inhibitors (e.g. antithrombin III and alpha-1-proteinase inhibitor)
Steam-treatment	• Coagulation factors (e.g. factor VIII, factor IX, fibrin sealant) • Protease inhibitors (e.g. C1-inhibitor)
Incubation at pH 4	• IgG
Nanofiltration (35nm or less)	• IgG • Coagulation factors (e.g. factor VIII, factor IX, von Willebrand factor, prothrombin complex) • Protease inhibitors (e.g. antithrombin III)
Terminal (final container)	
Terminal pasteurization	• Albumin
Terminal dry-heat treatment	• Coagulation factors (e.g. factor VIII, factor IX and factor XI)

Figure 1
Rate of virus inactivation on pasteurization of 5% albumin at 60 °C[1]



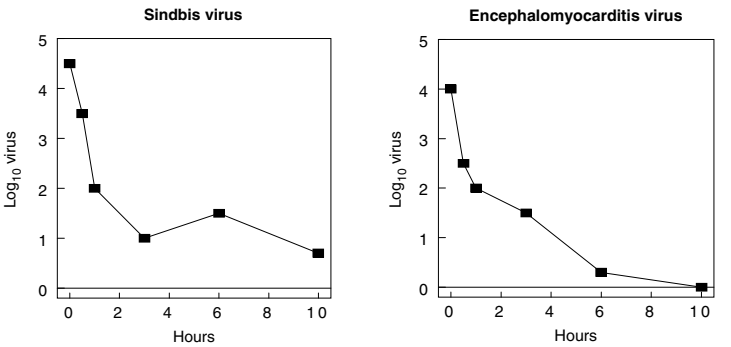
[1] On this and other similar graphs, "0" indicates that no infectious virus was detected.
Source: Horowitz et al. (*12*).

air oven. In both cases, temperature-mapping studies are required to demonstrate homogeneity, including measurements of both the temperature of the water or air and of the product itself. These studies must be performed with representative loads. Once validated, temperature probes are placed at strategic points in the water-bath or oven during each pasteurization run. Albumin used to stabilize other parenteral drugs should conform to the same requirements as albumin for therapeutic use.

4.1.2 *Pasteurization of other protein solutions*

Most proteins denature when heated in solution at 60 °C. To maintain the biological function of the more labile proteins, general stabilizers such as amino acids, sugars or citrate are added. Because these may also stabilize viruses, virus inactivation procedures need to be validated in model studies for each product under the conditions of treatment specified by the manufacturer. Following pasteurization, the stabilizers usually need to be removed. This is typically accomplished by diafiltration, size exclusion chromatography, or positive adsorption chromatography where the protein of interest binds to a chromatographic resin. Pasteurization has been used successfully with a variety of plasma protein products including coagulation factors and immune globulin solutions, although in rare instances transmission of HBV has been reported (*13*). A common method of preparing factor VIII is to heat it at 60 °C for 10 hours in the presence of high concentrations of glycine and sucrose or selected salts. Published results

Figure 2
Rate of virus inactivation on pasteurization of blood coagulation factor VIII at 60 °C in the presence of 50% sucrose and 2.75 M glycine



Source: Horowitz et al. (*12*).

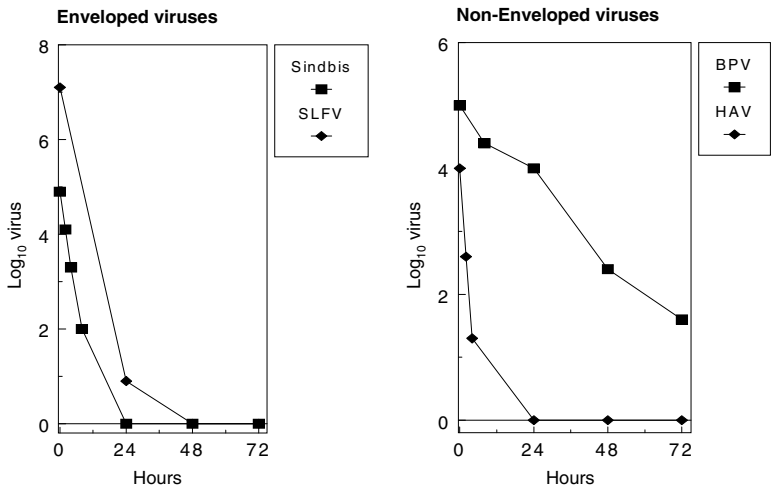
showing the extent and rate of virus inactivation of blood coagulation factor VIII are illustrated in Figures 2 and 3.

Prior to heating, the solution is typically filtered through a 1 µm or finer filter to eliminate particles that might entrap and further stabilize viruses. Heating is conducted in a jacketed tank and the solution is usually stirred throughout the heating cycle. Temperature-mapping studies are conducted to ensure that the temperatures at all points in the tank are within the range specified by the process record. Care must be taken to ensure that all parts of the tank, including the lid, where solution might splash, are heated. Viral inactivation studies, conducted under worst-case conditions, are performed at the lowest temperature that might be encountered in an acceptable production run. Protein recovery should be monitored during virus inactivation studies and should be comparable to that achieved at scale.

4.1.3 *Heating of dry (lyophilized) products*

Proteins can withstand being heated at temperatures of 60–80 °C or higher when they are first lyophilized to remove water. Heating at 60–68 °C for up to 72 hours has generally not been found to eliminate hepatitis transmission (*15*), whereas heating at 80 °C has produced favourable results with respect to transmission of HBV, HCV, HIV and HAV. (*16*) Recently, at least one manufacturer has been treating its coagulation factor VIII with solvent/detergent and also heats final product for 30 minutes at 100 °C. All HAV (≥5 logs) was inactivated within 4 minutes (*17*). Since viruses may be more stable following lyophilization, virus inactivation needs to be validated for each

Figure 3
Rate of inactivation of enveloped and non-enveloped viruses during dry-heat treatment of blood coagulation factor VIII at 80°C



BPV, bovine parvovirus; HAV, hepatitis A virus; SLFV, Semliki forest virus.
Results generously provided by the Scottish National Blood Transfusion Service.

product under the conditions of treatment specified by the manufacturer. Viral inactivation is influenced by residual moisture, the formulation (e.g. content of protein, sugars, salts and amino acids), and by the freezing and lyophilization cycles. Residual moisture is influenced by the lyophilization cycle and may be introduced inadvertently by the rubber stoppers.

Since virus inactivation is very sensitive to residual moisture content, the setting of upper and lower limits for moisture should be based on viral validation studies, and the variation of moisture content between vials should be within the limits set. To ensure reproducibility, one manufacturer has stipulated that, during the freeze-drying process, the temperature in three or more product vials, the shelf coolant temperature and the chamber pressure must remain within defined limits for each timed phase of the lyophilization cycle for every batch manufactured. Following freeze-drying, vials are stoppered under sterile, dry nitrogen at atmospheric pressure to ensure a constant atmosphere from vial-to-vial during dry-heat treatment. In addition, from every lyophilization run, the residual moisture content of five vials out of a lot of 1500 is measured following heat treatment. The moisture contents of these vials are used to calculate the 95% confi-

Table 7
Treatment of a solution of blood coagulation factor VIII by pasteurization

Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Human immunodeficiency virus	≥5.0	1.0
Cytomegalovirus	≥6.0	8
Epstein–Barr virus	≥3.3	0.5
Herpes simplex virus	≥5.9	4
Poliovirus	≥7.1	10
Vaccinia virus	6.2	10

Source: Hilfenhaus, et al. (*14*).

dence interval for the batch, and this interval must be within the upper and lower limits of moisture defined for the product.

Again using the specifications of one manufacturer, the dry-heat treatment, itself, is performed at 80.25 ± 0.75 °C for 72 hours. Process monitoring during heat treatment is carried out by means of temperature sensors located in 10 vials distributed throughout the load and two "air" probes located at the previously determined warmest and coldest points in the oven. All temperature sensors (both those in the vials and those measuring air temperature) must reach 79.5 °C before the cycle timer starts. Temperatures recorded by all sensors should remain stable between 79.5 °C and 81 °C for a continuous period of 72 hours. In addition, the dry-heat ovens are validated every 6 months, when a further 12 independent probes (10 in vials and two "air" probes) linked to a separate chart recorder are included to increase the temperature coverage to 24 points. In this way the temperature control is tested and the temperature spread within the cabinets established. The cycle time on the automatic control is also checked for accuracy.

Typical results achieved by heating factor VIII at 80 °C are given in Table 8 and Figure 3.

4.1.4 *Heating of lyophilized products under humidified conditions (vapour heating)*

At equivalent temperatures, a higher level of virus inactivation can be achieved by the addition of water vapour before initiating the heat cycle. To assure proper application of this approach, the material to be heated, the addition of moisture and the heat cycle need to be tightly controlled. In one case, freeze-dried intermediate bulk product is homogenized by a combination of sieving and milling. After deter-

Table 8
Treatment of lyophilized blood coagulation factor VIII at 80°C for 72 hours

Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Sindbis virus	8	72
Human immunodeficiency virus	≥6.4	72
Vaccinia virus	2.6–3.3	72
Herpes simplex virus	2.2	48
Semliki forest virus	≥6.9	24
Hepatitis A virus	≥4.3	24
Canine parvovirus	≥2.1	48

Sources: Knevelman et al. (*18*) Winkelman et al. (*19*) and Hart et al. (*20*).

mination of the residual water content, the freeze-dried intermediate is transferred into a stainless steel tank where an amount of water vapour, that has been predetermined based on the weight and the residual water content of the lyophilized product, is slowly added to adjust the water content to 7–8% (w/w). After an equilibration period, the water content is measured again before the product is ready for vapour heating. The intermediate product is transferred to a stainless steel cylinder. The cylinder is flushed with dry nitrogen to remove oxygen, and a pressure test is performed to ensure that the cylinder is airtight. This cylinder is then transferred to a heating cabinet equipped with an electric heater and a fan to ensure even temperature distribution. The intermediate product within the cylinder is heated according to the temperature regimen specified for the particular product. The cylinder is subjected to an oscillating rotation, changing direction every half-turn, until the end of vapour heating. During the heating process the pressure inside the vessel rises due to heating of the enclosed nitrogen, which cannot expand in the closed cylinder, and also due to evaporating water vapour from the moist intermediate product. After vapour heating, the heating cabinet is opened from the other side, and the product is further processed in a different and isolated manufacturing zone to prevent cross-contamination from non-inactivated product.

To assure consistency from lot-to-lot, the ranges for protein, salt and water content are set on the basis of the results of preliminary viral infectivity and protein functional studies. Additionally, the ratio of product weight to cylinder volume is specified for each product. A pressure test is performed before the start of vapour heating to ensure that the cylinder is airtight. During heating, product temperature and air temperature (one temperature sensor each) and pressure within

Table 9
Virus inactivation by Vapour heating at 60°C for 10 hours

Product	Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Factor VIII: intermediate purity	HAV	>3.3	8
	HIV	>6.8	10
	PRV	5.9	10
Factor VIII: high purity	HAV	3.9	10
	HIV	6.7	10
	PRV	5.6	10
Factor IX: intermediate purity	HAV	>5.7	6
	HIV	>6.5	6
	PRV	>7.1	8
Factor IX: high purity	HAV	>6.7	3
	HIV	>7.9	8
	PRV	>6.8	8

Data and process information provided courtesy of Baxter/Immuno. See also Barrett et al. (*24*) and Dorner and Barrett (*25*)
HAV, Hepatitis virus A; HIV, Human immunodeficiency virus; PRV, pseudorabies virus.

the cylinder are measured continuously and must conform to the specifications set for each. Following vapour heating, the water content of the intermediate is measured again.

Although historical reports indicate some cases of transmission of enveloped virus (*21*, *22*), the preponderance of clinical data indicate safety with respect to to transmission hepatitis viruses and HIV. (*23*) It should be noted that some products are heated at 60°C for 10 hours and others are additionally heated at 80°C for 1 hour; however, this cannot be considered as the use of two independent steps and the viral kill observed cannot be summed. Typical results achieved by vapour heating are given for several products in Table 9.

4.1.5 ***Solvent/detergent treatment***

Organic solvent/detergent mixtures disrupt the lipid membrane of enveloped viruses. Once disrupted, the virus can no longer bind to and infect cells. Non-enveloped viruses are not inactivated. The conditions typically used are 0.3% tri(*n*-butyl) phosphate (TNBP) and 1% nonionic detergent, either Tween 80 or Triton X-100, at 24°C for a minimum of 4 hours with Triton X-100, or 6 hours with Tween 80. When using TNBP and Triton X-100, some preparations can be treated successfully at 4°C. Since high lipid content can adversely affect virus inactivation, the final selection of treatment conditions must be based on studies demonstrating virus inactivation under

worst-case conditions; i.e. lowest permitted temperature and reagent concentration and the highest permitted product concentration. Prior to treatment, solutions are filtered through a 1-μm filter to eliminate virus entrapped in particles. Alternatively, if filtration is performed after addition of the reagents, the process should be demonstrated to not alter the levels of solvent and detergent added. The solution is stirred gently throughout the incubation period. When implementing the process in a manufacturing environment, physical validation should be used to confirm that mixing achieves a homogeneous solution and that the target temperature is maintained throughout the designated incubation period. Mixture homogeneity is best verified by measuring the concentrations of TNBP or detergent at different locations within the tank, although measuring dye distribution might be an acceptable substitute. To ensure that every droplet containing virus comes into contact with the reagents, an initial incubation for 30–60 minutes is typically conducted in one tank after which the solution is transferred into a second tank where the remainder of the incubation takes place. In this manner, any droplet on the lid or a surface of the first tank that might not have come into contact with the solvent/detergent reagents is excluded. The use of a static mixer where reagents and plasma product are mixed before being added to the tank is an acceptable alternative. The tank in which viral inactivation is completed is located in a separate room in order to limit the opportunity for post-treatment contamination. This room typically has its own dedicated equipment and may have its own air supply. When the treatment is complete, the solvent/detergent reagents must be removed. This is usually accomplished by extraction with 5% vegetable oil, positive adsorption chromatography (where the protein of interest binds to a chromatographic resin), or adsorption of the reagents on a C-18 hydrophobic resin. Depending on the volume of product infused and the frequency of infusion, the permitted residual levels of TNBP, Tween 80 and Triton X-100 are generally, 3–25, 10–100 and 3–25 ppm, respectively.

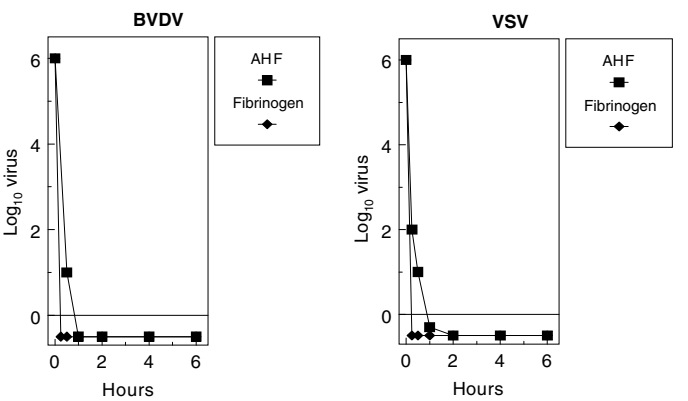
When performing viral validation studies, the reaction is stopped either by dilution or, in some cases, adsorption of the TNBP and Triton X-100 by a C18 hydrophobic resin. An appropriate control needs to be run to establish that virus inactivation does not continue following the use of the stop procedure. Safety with respect to HBV, HCV and HIV has been demonstrated in numerous clinical studies that reflect the high level of virus inactivation demonstrated in both laboratory and chimpanzee studies. Typical results achieved on treating a coagulation factor VIII concentrate and fibrinogen at 24 °C are given in Table 10 and Figure 4.

Table 10
Treatment of blood coagulation factor VIII solution with 0.3% TNBP and Tween 80

Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Vesicular stomatitis virus	≥4.5	2
Sindbis virus	≥5.5	1
Sendai virus	≥6.0	1
Hepatitis B virus	≥6.0	6[a]
Hepatitis C virus	≥5.0	6[a]
Hepatitis D virus	≥4.0	6[a]
Human immunodeficiency virus-1	≥6.0	0.25

Sources: Horowitz (*26*) and Horowitz et al. (*27*).
[a] These studies were conducted in the chimpanzee model; 6 hours was the only time-point tested.

Figure 4
Treatment of AHF and fibrinogen by solvent/detergent[a]

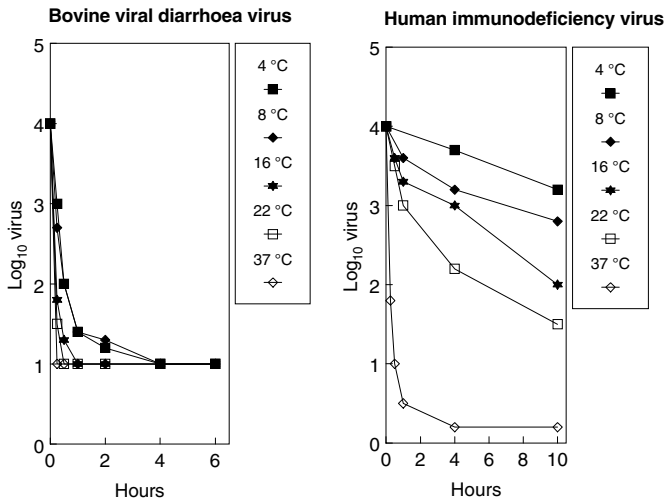


AHF, blood coagulation factor VIII; BVDV, bovine viral diarrhoea virus; TNBP, tri(*n*-butyl) phosphate; VSV, vesicular stomatitis virus.
[a] AHF was treated with 0.3% TNBP and 1% Tween 80 at 24 °C and fibrinogen was treated with 0.3% TNBP and 1% Triton X-100 at 24 °C. At the time-points indicated, BVDV and VSV infectivity were measured.
Data provided courtesy of V.I. Technologies.

4.1.6 ***Low pH***

Most proteins are damaged by exposure to the acidic conditions needed to kill viruses. For example, few viruses are 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 in the pH 4-treatment used in the preparation of

Figure 5
Inactivation of viruses in IgG at pH 4 in the presence of pepsin



Source: Omar et al. (*28*).

immunoglobulins, inactivates several enveloped viruses. (*28*) The presence of trace concentrations of pepsin added to reduce anticomplementary activity during this procedure has been shown to contribute little to virus inactivation. Since acid 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 separately because 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 5. On the basis of these and other results, one manufacturer incubates its immunoglobulin preparation at pH 4.0 for at least 6 hours at 37 °C whereas another follows solvent/detergent treatment by incubating in the container at pH 4.25 for a minimum of 21 days at 20 °C.

4.2 Methods of virus removal

Before the 1980s, conditions for the fractionation of plasma were selected largely on the basis of considerations of protein purification and less on the capacity of the process to remove virus. Modern purification procedures frequently consider both protein purification and virus removal. For example, an ion-exchange or monoclonal

antibody column may be selected for the degree of protein purification provided, but also be characterized fully with respect to virus removal. Based on this characterization, additional wash buffers or greater volumes of wash buffer may be used to increase the degree of virus removal. Additionally, in the last few years, specific removal methods such as nanofiltration have been developed, and others, such as viral affinity adsorbents, are under development. Such methods are intended to remove viruses. Where virus removal is believed or claimed to be an important consideration for a particular purification step, whether intended or not, the same discipline in validating and implementing that step should be used as is applied to a virus inactivation step.

4.2.1 *Precipitation*

Precipitation with ethanol is the single most widely used plasma 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 temperature or above, whereas plasma fractionation is carried out at a low temperature to avoid protein denaturation. The 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 reaction, the distribution of viruses between precipitate and supernatant is never absolute.

The following log reduction factors (LRFs) were reported for three distinct steps in albumin production by cold-ethanol precipitation (Table 11; the designations of the steps correspond to the Kistler/Nitschmann fractionation scheme) and for the production of immunoglobulin (Table 12). (Note that LRFs should not be summed across

Table 11
Log reduction factors for four different viruses and for three precipitation steps used during the 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	4.60	0.9	1.7	3.1	1.2

BEV, bovine enterovirus; HIV, human immunodeficiency virus; PRV, pseudorabies virus.
Source: reference *29*.

Table 12
Log reduction factors for five different viruses and for four precipitation steps used during the 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	5.10	5.3	4.7	4.6	2.2	4.1
Step C	25	7.00	4.0	4.7	2.9	3.5	3.8
Step D	—	—	2.2	3.0	1.7	—	2.8

Source: reference *30*.
BEV, bovine enterovirus; HIV, human immunodeficiency virus; PRV, pseudorabies virus; SFV, semliki forest virus.

steps unless the mechanism of action has been shown to be independent, or other data demonstrate that the summing is legitimate.)

Because the result of any precipitation step is a partitioning of components between a solid and a liquid phase it should be borne in mind that, in the absence of inactivation, fractionation results in distribution of viruses between these phases. Therefore, if viruses are indeed removed from one fraction, the bulk of virus will be found in another fraction, which may or may not be that used for making the final product. Many manufacturers separate the precipitated proteins by centrifugation whereas others have introduced filtration as an alternative. To prevent clogging of the filters, filtration is carried out using filter aids. Because 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 concluded that BVDV, as a model for HCV, was not removed to any significant extent by Cohn–Oncley fractionation, (*31*) whereas 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 Table 13.

When virus inactivation steps are implemented, it is usually relatively easy to ensure that every drop of a large batch is treated in 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 come into contact with a filter press encounter an environment that is quite different from that encountered by the last volumes of the same batch. Although it is probable that these changes occur in a reproducible way in each

Table 13
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 diarrhoea 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

Source: Omar & Morgenthaler, (*32*).

batch, this could be difficult to prove. Similarly, model experiments are relatively easy to perform in a homogeneous system, as may be the case during chemical or physical inactivation. However, large-scale centrifugation is usually done in continuous-flow machines and although the could be reduced in size to laboratory scale, parameters such as path lengths and residence times are unlikely to 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 and enzyme activity) that both large-scale 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 removal.

In spite of all the problems associated with precipitation as a means of removing viruses, ethanol precipitation has proven its value over the years. There can be little doubt that partitioning though precipitation has contributed substantially to the safety of some plasma-based products, e.g. intravenous and intramuscular immunoglobulins, which have very rarely transmitted viral diseases although until very recently the manufacturing processes for these products did not include a dedicated virus inactivation step. Nonetheless, reliance on virus removal alone is not recommended because small changes in process conditions can affect virus partitioning and safety. As an example, HCV partitioning was modified on introduction of anti-HCV screening, with the result that an IVIG became infectious (*33*).

Chromatography

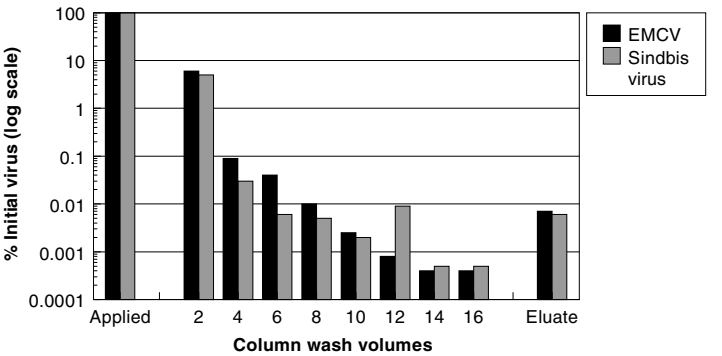
Chromatography has been designed to separate closely related molecules; some variants of chromatography, e.g. affinity chromatography, are specific for a single molecular species. The logical expectation would therefore be the chromatography is a good way to physically separate viruses from therapeutic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

Relatively modest reduction factors were reported for three consecutive chromatographic purfication steps used in an albumin isolation scheme. LRFs of <0.3, 0.3 and 1.5 were reported for removal of HBsAg during chromatography on DEAE-Sepharose FF, CM-Sepharose FF and Sephacryl S200 HR, respectively (*34*). The same group recorded LRFs of 5.3, 1.5 and 4.2 for HAV for the same three steps (*35*). 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 (*36*).

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 6).

Sanitization of resins and associated chromatography equipment between runs is essential because viruses tend to stick to resins and a complete wash-out is often impossible. Discarding used resin is, for financial reasons, normally not a practical option. Many resins withstand chemical or physical treatments that inactivate viruses. Typical treatments include overnight incubation with 0.1–1 N sodium hydroxide or hydrochloric acid, oxidizing conditions such as provided by sodium hypochlorite; very high temperatures, or autoclaving. The selection of a sanitization procedure depends on the column matrix in use. For example, silica backbones are degraded on exposure to

Figure 6
The reduction of model viruses during method M immunoaffinity purification of factor VIII[a]



EMCV, encephalomyocarditis virus; TNBP, tri (*n*-butyl) phosphate.
[a] For EMCV, TNBP and Triton X-100 were present; for Sindbis virus, they were omitted due to the rapid inactivation that would otherwise occur.
Source: Griffith (*37*).

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 or elimination steps. 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 such 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.

4.2.2 *Nanofiltration*

Nanofiltration is a technique that is specifically designed to remove viruses. Simplistically, nanofiltration removes viruses according to

their size while permitting flow-through of the desired protein. However, large proteins — particularly those that tend to form aggregates — are as large as 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. Filters with a pore size that exceeds the virus diameter may still remove some virus if it is aggregated such as by inclusion in antibody/antigen or lipid complexes. In reality, nanofiltration is a more complex process. Apart from sieving effects, adsorption of the virus to the filter surface may also contribute to virus removal, though this will be strongly influenced by the intrinsic characteristics 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 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 that 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 35 nm pore size were reported to remove 6–7 $\log_{10}$ of murine xenotropic retrovirus, SV40 and pseudorabies virus from IgG and IgM solutions (*38*). Troccoli and coworkers found that all viruses larger than 35 nm spiked into an IVIG-solution were completely removed by cascade filtration through one 75 nm pre-filter, followed by two 35 nm virus removal filters; the pre-filter was used to increase the capacity of the small-pore filters. Even smaller viruses like EMCV, HAV and PPV were removed to a significant extent (LRFs were 4.3, >4.7 and 2.6, respectively). The removal of some small viruses (BPV, Sindbis and SV40) could not be evaluated due to neutralization by cross-reacting antibodies (*39*). A single dead-end filtration was able to remove HIV, BVDV, PPRV, RT3 and SV40 with LRFs of >5.7 to >7.8, when these viruses were added to high-purity factor IX and factor XI concentrates (*40*). Numerous other

studies have also demonstrated 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. It should however be borne in mind that the virus stocks used in validation studies may be artifactually aggregated as a consequence of achieving high titres in culture systems or of the concentration methods used.

4.3 Protein issues

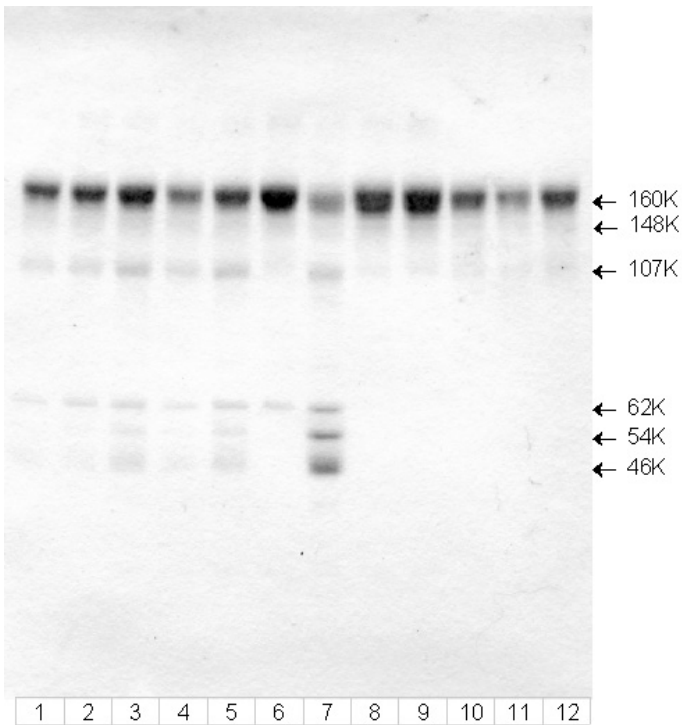
When considering processes that inactivate or remove viruses, just as with other manufacturing procedures, manufacturing consistency and the integrity of the final product with respect to protein function and structure must be demonstrated. Several analytical tests are typically applied to in-process samples and to the final product. These almost always include total protein, one or more functional assays for the protein of interest, and an assessment of its aggregation/fragmentation. Additional final product protein assays are occasionally employed depending on the product being manufactured. For example, anticomplementary activity in IVIG and activated coagulation factor activity in prothrombin complex concentrate are usually measured in every production lot, while the in vivo measurement of the thrombogenic potential of prothrombin complex concentrates is usually assessed during process development.

4.3.1 *Measurements of protein structure*

Depending on the experience with the process methods being employed, laboratory and animal studies such as those described below can be of value for characterizing products under development.

Electrophoresis is a fast and easy method for evaluating the overall integrity of a protein. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) is particularly useful for analysing protein composition, aggregation and fragmentation. The procedure separates proteins approximately according to their relative molecular mass, although protein shape and glycosylation can affect migration. Under nonreducing conditions, disulfide-linked protein chains usually remain together. For instance, under nonreducing conditions, immunoglobulins migrate as a single molecule with a relative molecular mass of approximately 160 kD while under reducing conditions, the chains that were linked by disulfide bridges fall apart, producing two bands with approximate relative molecular masses of 50 kD (heavy chain) and 25 kD (light chain). Cleavage in the primary sequence of proteins is usually easily detected (Figure 7). SDS-PAGE

Figure 7
SDS-PAGE of intramuscular and intravenous immunoglobulin products[a]



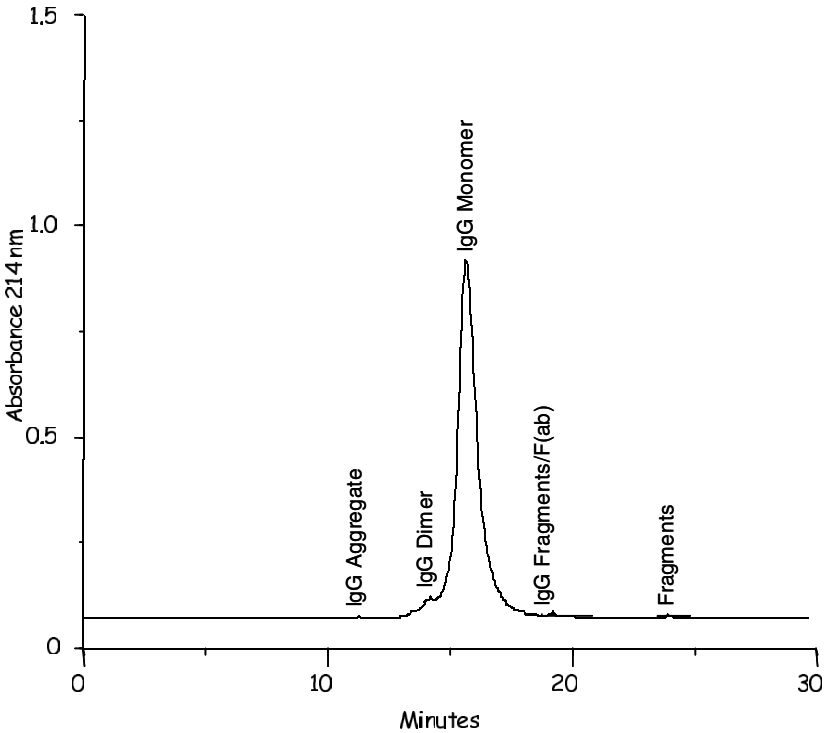
SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.
[a] The products analysed in lanes 1–7 show variable amounts of IgG fragments, whereas those in lanes 8–12 show little, if any evidence of fragmentation.
Figure reproduced with the kind permission of R. Thorpe, National Institute of Biological Standards and Control, England.

will not normally reveal changes in higher-order structures or covalent modifications of amino acids.

Capillary electrophoresis has recently been introduced as an adjunct method to PAGE. Although it is more amenable to automation, and therefore useful for high-throughput analyses, it does not yield substantially more information than PAGE and requires sophisticated equipment.

Size-exclusion gel chromatography separates proteins according to their overall size and shape. The use of size-exclusion high performance liquid chromatography (SE-HPLC) allows rapid analysis and high resolution of protein components and also better reproducibility than that obtained using conventional gel permeation chromatography. Fragmentation and/or aggregation of plasma proteins are usually

Figure 8
SE-HPLC of an intravenous immunoglobulin product showing the expected distribution of molecular species[a]



[a] The product consists primarily of IgG monomers with small amounts of dimer and trace amounts of fragments and aggregates.
Figure reproduced with the kind permission of R. Thorpe, National Institute of Biological Standards and Control, England.

easily demonstrated and quantifiable and gross modifications to the molecular shape of the protein may also be detectable. More subtle changes may not be detected, and the method is insensitive to chemical modifications of amino acids. An example of SE–HPLC analysis of IVIG is given in Figure 8.

Isoelectric focusing separates proteins according to their isoelectric point. Separation is normally performed in the presence of a supporting matrix (e.g. polyacrylamide), and the proteins may be subjected to this method either in a native or denatured state. As covalent modification of amino acids usually changes their electric charge, it also affects their isoelectric point and therefore the protein's position in an isoelectric focusing gel. Isoelectric focusing combined with PAGE is a very powerful tool for the detection of even small differences in

protein structures and properties, although the gel patterns can be very complex depending on the purity of the sample.

Antigen/activity ratio. During process qualification, it may be useful to measure protein functional activity and antigen concentration simultaneously in an immunoassay. A constant ratio of activity to antigen during the isolation process and before and after virus inactivation provides evidence that protein structure was not affected while a decline in this ratio is indicative of detrimental effects.

Neoimmunogenicity may be regarded as a special case of changes to the higher-order structure of proteins, which do not necessarily impair the protein's functionality, but result an immune response in the recipient. Products made with current methods of viral inactivation and removal do not generally stimulate an antibody response in humans. There are, however, two documented instances (one involving a pasteurized product and the other a product treated with solvent/detergent combined with pasteurization) where treated products had unexpected immunogenicity and were therefore withdrawn from the market (*41–43*).

The detection of neoimmunogenicity preclinically is very difficult, and several animal models have been developed. One approach is to immunize one group of laboratory animals (e.g. rabbits) with the preparation to be tested and another group with the same preparation in which the viral inactivation step has been omitted, or a similar preparation with proven lack of neoimmunogenicity. The resulting antisera are compared with one another in a cross-over experiment; if the antibodies raised against the new preparation are completely adsorbed by the old preparation, the preparation under test is not likely to contain neoantigens. 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 those recognized by the immune systems of laboratory animals.

Because the models are not believed to adequately predict human response, animal neoimmunogenicity studies are not generally required for products manufactured using well recognized techniques for viral inactivation and removal. If the manufacturing conditions differ substantially from well recognized treatment conditions, such as the use of a higher temperature than that normally employed during solvent/detergent or heat treatments, new combinations of treatments, or the use of a new method of virus inactivation and removal, then animal neoimmunogenicity studies using one of the available models should precede first use of the product in humans.

The best proof of absence of neoantigens is derived from careful clinical studies involving a number of patients determined on a case-by-case basis. The determination of circulatory recovery and half-life in repeatedly infused subjects can be very useful and such measurements are typically made prior to licensure. A full assessment of immunogenicity is best monitored over the long term and is, therefore, is typically monitored in humans following licensure of the product. This has proven to be especially important in the care of patients with haemophilia, and recommendations for the conduct of such studies have been published (*44*). If there is no increase in the appearance of clinically relevant antibodies or of other adverse immunological reactions in patients (as compared with the incidence expected from earlier studies, when available), it is reasonable to assume that the newly developed product does not exhibit neoantigens.

The following are not usually applied to well-established procedures, products and processes, but may prove valuable with new viral inactivation and removal procedures.

- ***Amino acid analysis*** determines the overall quantitative composition of a protein. It may help to detect changes that were inadvertently introduced, e.g. covalent modifications of amino acids.
- ***Amino acid terminus analysis*** may identify changes in the covalent structure of proteins because cleavage of protein chains may produce new terminal sequences that can be identified and located by alignment with the native sequence (if it is known) or when comparing pretreatment to post-treatment samples.
- ***Cleavage with proteolytic enzymes*** can be used to assess protein integrity because denatured proteins or proteins with altered conformation often contain new sites that are now recognized by sequence-specific proteases. Comparing the fragmentation patterns produced by addition of proteolytic enzyme(s) before and after virus inactivation and removal may give clues to subtle changes that have occurred during treatment. The degradation patterns may be analysed by several of the methods already mentioned such as SDS-PAGE and size-exclusion gel chromatography (*45*).
- ***Circulatory survival*** may be considered as an in vivo variant of using proteolytic enzymes, albeit a difficult, time-consuming and expensive variant. It is carried out by injecting the protein intravenously into a suitable animal (e.g. rat or rabbit) and comparing the half-life with that of 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 are quite sensitive to minor changes in protein structure. As a demonstration of the utility of this method, the circulatory half-life of human

albumin prepared by standard procedures was shown to be unaltered whereas that of chemically modified albumin was halved (*46*). Analogous experiments performed with virally inactivated (by solvent/detergent) immunoglobulins and coagulation factors demonstrated no change from historical controls (*47*).

- ***Other methods that could be indicative of overall changes in shape*** include measurements of sedimentation and diffusion coefficients, viscosity, circular dichroism and optical rotatory dispersion. Most of these methods are difficult and slow to perform. They are of limited value because their results are hard to interpret and can only be evaluated by comparison with a difficult to establish standard preparation of the same protein.

4.3.2 *Final product characterization*

The specifications for many plasma products are provided in several pharmacopoeias, national regulations and in the WHO requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (*48*). Common tests that are generally considered in the characterization of plasma derivatives in final product are listed in Figure 9. Tests to be conducted on each final bulk solution or filling lot should comply with methods and specifications approved by the national regulatory authority.

4.3.3 *Stability assessment*

The purpose of stability studies is to prove that a product remains stable, safe and efficacious during the shelf-life that is claimed for it by the manufacturer. A set of relevant parameters has to be chosen and measured at regular, predefined intervals. These parameters will include measures of potency as well as indicators of protein integrity. Limits for these parameters that may not be exceeded are generally predefined.

True stability tests can only be conducted in real time. Since most plasma-protein products have a shelf-life of 2–3 years, stability tests are usually incomplete at the time of licensure. Real-time stability studies need to be done under worst-case conditions. For example, if the storage conditions for a particular product are specified to be within a temperature range of 2–8 °C, the minimum testing would be carried out within the specified temperature range and at some other higher temperature.

To obtain an indication of product stability before the data from real-time stability studies are available, it is possible to conduct accelerated stability studies (*49*, *50*). For these, the product is exposed to

Figure 9
Tests commonly applied to final product characterization

Albumin

- Protein composition (albumin content)
- Molecular size distribution (polymers, aggregates)

Normal and specific immunoglobulins

Intramuscular administration

- Protein composition (IgG content)
- Molecular size distribution (aggregates, dimers, monomers, fragments)
- Potency tests of antibody reactivity against selected antigens

Intravenous administration

- Protein composition (IgG content)
- Molecular size distribution (aggregates, dimers, monomers, fragments)
- Anti-complementary activity
- Potency tests of antibody reactivity against selected antigens

Coagulation factor concentrates

Anti-haemophilic factor

- Factor VIII coagulant activity
- von Willebrand factor activity (if required)

Prothrombin complex / factor IX

- Factor IX coagulant activity
- Factor II, VII, X coagulant activities
- Measurement of activated clotting factors

Tests common to all products

- Total protein
- Moisture and solubility (if lyophilized)
- pH

more severe conditions than are normally expected to be encountered during routine storage and shipping, e.g. higher temperatures, and stability is assessed over a shorter period than that used for the real-time study. The data can be used to predict stability under the prescribed storage conditions, but cannot replace real-time studies because predictions from accelerated studies do not always correlate with what occurs during real time. Other stress factors that are often incorporated into an accelerated stability test include humidity, light,

mechanical stress (shaking) and combinations of these. Parameters that are identified as critical during accelerated testing will receive particular attention during real-time testing.

In both accelerated and real-time studies, time points need to be chosen such that both transient and permanent deviations from the initial value will be recognized. Should the predefined limits for any parameter be exceeded, a reconsideration of the storage conditions will be unavoidable.

4.4 Clinical trials to assess safety

Historically, the role of clinical trials has been to assess efficacy, both general and viral safety, and immunogenicity. Trial design for established products is a subject of considerable discussion with an overall trend towards simplifying design and reducing the number of patients required. Viral safety is assessed principally by the review of donor demographics, test procedures and process validation. Within the EU, there is a trend towards assessment of viral safety in humans after, rather than before, receipt of marketing authorization (postmarketing surveillance). This trend takes into account the safety exhibited by current products, 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 diverse medical uses of the established products makes the setting of generally applicable guidelines a daunting task. Prior to licensure, all products typically undergo safety evaluation in a minimum of 5–10 volunteers, and in many cases, 25 or more. More patients are included in testing of products made by new processes.

4.5 Implementation in a manufacturing setting

A set of measures should be implemented to ensure that virus inactivation and removal procedures are correctly carried out in a manufacturing process and that cross-contamination following these procedures is avoided. The examples of viral reduction treatment practices given below should not be understood as requirements, but rather as general points for consideration. They are not the only acceptable way of conducting viral reduction treatments but provide examples of the solutions employed by some manufacturers when addressing this issue.

4.5.1 *Overall process design*

When planning to implement a new viral inactivation and removal treatment, the following conditions should be set ahead of time to facilitate their implementation:

— batch-size or volume at the stage of the viral reduction step, and potential up-scaling in the future;
— floor area in the manufacturing facility required for the viral reduction step itself, and for downstream processing (e.g. for removal of stabilizers or chemicals);
— the possibility of creating a "safety area" where successive production steps are arranged in a clear and logical way so as to avoid cross-contamination from a consideration of how the various flows (operators, product, equipment, wastes) will be organized during and after the viral reduction step.
— Whether cleaning or sanitation procedures will be in that place or executed in another location.

4.5.2 *Equipment specifications*

Because viral reduction treatments are critical to product safety, the specifications of the equipment employed in these steps are of particular importance. The following examples are illustrative:

In-process/bulk virus inactivation (e.g. solvent/detergent, low pH, pasteurization)

- Ideally, incubation vessels should be fully enclosed fitted with an appropriate mixing device. Usually, these are temperature-controlled vessels in which the source of heat is a jacket or a heating coil. They often have hygienic, polished internal finishes, flush-fitting valves, hygienic entry ports for the addition of reagents and sampling (e.g. to control pH and osmolality), and probe ports for relevant in-process monitoring (such as measurement of temperature).
- There should be no "dead points" i.e. areas where the temperature defined in the specification or where homogeneity of the mixture cannot be ensured.
- For heat inactivation processes, temperature monitoring equipment should provide a continuous, accurate and permanent record of temperature during the treatment cycle.

Terminal virus inactivation (e.g. pasteurization or dry heat in the final container)

- The heating device (such as a water-bath, steam autoclave, or forced-air oven) should provide even temperature distribution

across the range of batch sizes encountered. This should be demonstrated as part of the equipment qualification.

- The temperature-monitoring equipment should be capable of providing a continuous, accurate and permanent record of the heat-treatment cycle.

4.5.3 *Pre-qualification and validation*

Once the equipment for the virus inactivation or removal step has been received, the following steps are usually followed prior to routine use.

- The installation qualification verifies that the viral inactivation and removal equipment conforms to the predefined technical specifications and relevant good manufacturing practices regulations applicable at the time of installation in the manufacturing environment. This includes confirmation that the required services (e.g. voltage, cooling/heating fluid and steam) are available and appropriate.
- The operational qualification demonstrates experimentally, typically without product, that the equipment for the inactivation and removal of viruses functions within the specified limits and under the requirements for good manufacturing practices in the manufacturing environment.
- The performance qualification establishes that the equipment for the inactivation and removal of viruses operates to the predetermined performance requirements in the presence of product under routine manufacturing conditions.
- Product validation provides evidence that intermediate and/or final product prepared with the newly installed equipment reproducibly meets its specifications.

4.5.4 *Process design and layout*

The benefit of viral inactivation and removal will be negated if the plasma fractions from preceding steps are permitted to recontaminate the intermediates or products that follow; thus, the manufacturer must describe how the operating procedures reduce the likelihood of cross-contamination. Usually, decisions are made after a multidisciplinary team consisting of responsible staff from manufacturing, engineering, quality assurance and microbiology has made its recommendations.

The simplest and best solution to the problem of cross-contamination, from a facility management perspective, is to transfer product from one room to the next in the course of the specific inactivation and

removal procedure. This serves to create different safety zones, which, when arranged in a clear and logical way, help avoid cross-contamination. In the best implementations, every zone has its own dedicated staff, equipment, entrance, air-handling and other services. When this arrangement is not practical, the same effect can be achieved through appropriate management practices. For example, some facilities utilize the same staff in both downstream and upstream areas, and personnel moving into a safer zone must change their outer overalls, shoes or shoe covers, gloves, etc before entering. Equipment must also be decontaminated when moving it into a safer zone. Preferably, the equipment in one safety zone should not be shared with a second zone. Strict segregation has generally been adopted for continuous flow centrifuges, column chromatography matrices and ultrafiltration membranes which are notoriously difficult to decontaminate with the methods that are currently available.

The following points illustrate how some manufacturers have addressed these cross-contamination issues.

In-process/bulk virus inactivation (e.g. solvent/detergent, low pH, pasteurization)

- Inactivation procedures are usually carried out in two stages. For example, the first stage may be a treatment at acid pH 4 which takes place in a normal production room, followed by a second incubation in another tank located in a segregated, contained area.
- For solvent/detergent treatment, most of the inactivation is usually during the first 30–60 minutes of the 4–6 hour total treatment time.
- If bacterial growth during virus inactivation is a consideration, the solution is sterile-filtered (pore size 0.45 µm or less) before treatment.
- Samples are usually taken to confirm that the process conditions for inactivation meet the specified limits (e.g. for pH, stabilizer concentrations and concentration of virus inactivating agent).
- On completion of the first stage of inactivation, the product is aseptically transferred (sterile coupling) into the second vessel, which is located in a safety zone, for completion of the second stage of viral inactivation.
- Ideally, the "safety area" has an independent air-handling system, designated controlled clothing for personnel, and defined routes of entry for all equipment, reagents (including process buffers) and consumables.
- The process water and the reagents supplied to the safety area are of water for injection (WFI) standard or demonstrated to be free of infectious agents.

- All processing after virus inactivation and prior to sterile filtration and dispensing (e.g. removal of solvent/detergent or stabilizers and further purification steps) are carried out in the safety area.
- All the equipment used in the safety area that is in contact with the product is dedicated, or decontaminated in a manner that can be shown to inactivate any remaining virus.
- In some cases, a dedicated aseptic filling area is used for virus-inactivated products while a separate dispensing area is used for products that have not been virally inactivated during the purification process and are treated at the end of the process. Alternatively, products that will be inactivated in the final container can also undergo a preliminary virus inactivation in bulk, or the filling line is cleaned by procedures that can be shown to inactivate virus.

In-process virus removal (e.g. nanofiltration, specified purification steps)

The principles relating to product segregation described above also apply to procedures for virus removal.

Terminal virus inactivation (e.g. pasteurization or dry heat in the final container)

Inherently, terminal virus inactivation procedures greatly reduce the likelihood of recontamination.

- Temperature is monitored at several locations throughout the load including the previously determined locations at which the highest and lowest temperatures occur.
- The temperature control probe is independent from the probes used to monitor product temperature during the heat treatment.
- A maximum time is specified for the temperature to reach its set point.
- The specified temperature is maintained by all probes for the required period.

4.5.5 ***Process control***

Quality assurance is a critical part of the manufacturing process because completeness of virus inactivation and removal cannot be guaranteed by testing the final product. It is the responsibility of quality assurance to ensure that the execution of virus inactivation and removal methods in a production setting conforms to the conditions that were validated in the virus spiking studies. Additionally, it is their responsibility to ensure that the procedures that are designed to avoid cross-contamination are strictly followed. In the case of any departure

from the standard, specified manufacturing processes or in environmental conditions, the independent quality assurance team, typically with the assistance of a select committee, will conduct a deviation investigation to determine whether or not the product can be released. Generally, the quality assurance team has final authority to release or reject product.

The following points should be taken into consideration.

- As with all other procedures, viral inactivation and removal procedures should be described in approved standard operating procedures.
- The standard operating procedures should contain critical process limits for the viral inactivation and removal methods.

Because of the critical importance of the viral inactivation and removal step, quality assurance personnel may review and approve the recorded conditions for viral inactivation and removal while the batch is being processed; i.e. not just as part of the final overall review of the batch file.

5. Virally inactivated plasma for transfusion

In the past, plasma has been used to treat a variety of haemostatic disturbances and immune deficiencies and even to provide a source of nutrients. This has led to a significant increase in the often inappropriate use of fresh frozen plasma (FFP). For many of those applications, alternative, safer and more economical treatments are now a better choice than FFP. According to the recommendations of consensus development conferences in various countries, there are a limited number of indications for the use of FFP (*51–53*). These include patients who require massive transfusions, patients with multiple coagulation factor deficits who are bleeding or who need an invasive procedure, patients with thrombotic thrombocytopenic purpura and patients with protein-losing enteropathy. In addition, FFP is indicated where there are no concentrates or purified preparations available such as for congenital coagulation factor deficiencies and immune deficiencies.

Regulatory approvals have been granted to three approaches designed to enhance the viral safety of transfusion plasma, namely:

— quarantine or donor-retested plasma;
— solvent/detergent-treated plasma; and
— methylene blue-treated plasma.

Each of the three approaches is described below, and all have been recently reviewed (*54*). All transfusion plasma options, including the continued use of FFP from well-screened donors, have advantages and disadvantages, and it is up to the local medical community and relevant regulatory bodies to determine which option is preferable and most suitable for the particular setting. Implementation should adhere to the applicable measures described in section 4.5.

5.1 Quarantine or donor-retested plasma

One approach to reducing window-period transmissions is to hold donor units in quarantine for a suitable period of time until the donor returns and can be retested. This method is useful only for the viruses being tested for, although interviewing the donor at the time of the second test may help to identify any transient illnesses that occurred between the two donations. The length of the quarantine period is related to estimates of the window period, which differs for each virus. To reduce transmission of HIV, HBV and HCV, a sufficient hold period should be chosen to give a 95% confidence level of not releasing a product during a window period. Periods of 3–4 months have typically been considered to prevent almost all window-period transmissions. The option to quarantine is made possible by the relatively long outdating (shelf-life) period of FFP, typically 1 year.

Although the transmission of HBV, HCV and HIV will be greatly reduced by use of quarantine plasma, it will not have been eliminated. For example, HCV has been reported to have been transmitted by quarantine plasma (*55*), blood donations that are not screened by genomic techniques continue to harbour HIV and other viruses of concern (*56*), and quarantine has little or no impact on viruses that are not tested for. However, the advantages of this method are that the plasma itself is unchanged and thus has the same properties and indications as FFP, and no sophisticated equipment, other than that used for donor tracking, is required. On the other hand, supply logistics may prove difficult in some circumstances where a large number of donors need to re-donate well before the expiry date of the initial FFP unit. This is of special concern in an environment based on blood donations volunteers, where many donors give blood infrequently, with consequent losses of plasma units.

Implementation requires systems that correctly match donated units with the returning donors and that prevent premature release of units labelled as being either "quarantined" or "donor retested". Although manual systems may be used, computerization greatly facilitates this process and provides improved security.

Table 14
Inactivation of viruses on treatment of plasma with 1% tri(*n*-butyl) phosphate and 1% Triton X-100 at 30 °C for 4 hours

Virus	Inactivation (log_{10})	Inactivation time (hours required)
Vesicular stomatitis virus	≥7.5	0.25
Sindbis virus	≥6.9	0.25
Duck hepatitis B virus	≥7.3	2.5
Bovine viral diarrhoea virus	≥6.1	0.25
Human immunodeficiency virus	≥7.2	0.25
Hepatitis B virus	≥6.0	4[a]
Hepatitis C virus	≥5.0	4[a]

[a] Only one time point tested.

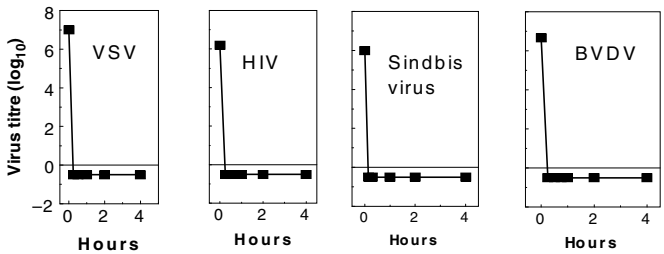
5.2 Solvent/detergent-treated plasma

Routinely collected source, recovered, or FFP is pooled and treated with 1% TNBP and 1% Triton X-100 at 30 °C for a minimum of 4 hours to inactivate enveloped viruses. The reagents are removed by hydrophobic chromatography to near undetectable levels (*57*). The compounds used are non-mutagenic and have an overall benign toxicology profile. Leukocytes, bacteria and parasites are removed by sterile filtration. The final product is available frozen and, in some countries, also in a lyophilized form. Inactivation of HIV, HBV and HCV and of many other enveloped viruses has been demonstrated (Table 14, Figure 10). To reduce the risk from non-enveloped viruses, the application of NAT can eliminate positive pools. Little change is observed in the level of most procoagulant factors, and bag-to-bag consistency is ensured through the pooling process.

Clinical trials conducted in both Europe and the USA have shown that solvent/detergent (SD)-treated-plasma can replace FFP in all of its indications, including the replacement of coagulation factors and the treatment of thrombotic thrombocytopenic purpura (*58–61*). More recently, several deaths were reported in liver transplant patients who received a product provided by one manufacturer in the USA.[1] Although the link with this product or with reduced levels of some anticoagulant proteins in SD-plasma is uncertain, the US manufacturer's product label has been amended to indicate that this product should not be used in patients undergoing liver transplant or in patients with severe liver disease and known coagulopathies.[1]

[1] Information available from the FDA/CBER web site (http://www.fda.gov/medwatch/safety2002) became available after the fifty-second meeting of the WHO Expert Committee on Biological Standardization. This information was added during editing of the Guidelines.

Figure 10
Rate of virus inactivation on solvent/detergent treatment of plasma



BVDV, bovine viral diarrhoeavirus; HIV, human immunodeficiency virus; VSV, vesicular stomatitis virus. Data generously provided by V.I. Technologies, Inc.

Additionally, the coagulation status of patients receiving large volumes of SD-plasma should be monitored for evidence of thrombosis, excessive bleeding or exacerbation of disseminated vascular coagulation.

The same parameters need to be defined and controlled as for other solvent/detergent-treated products. In addition, some regulatory bodies have instituted a maximum for the number of donors that can contribute to an individual lot; the maximum number permitted ranges from 100 to 2500.

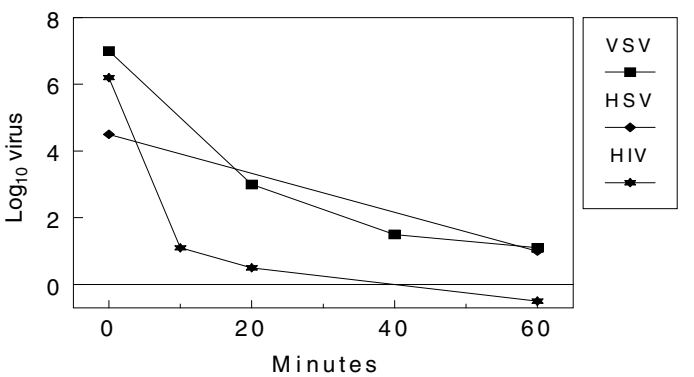
5.3 Methylene blue and visible light

Methylene blue is a photosensitizer, and in conjunction with light and in the presence of oxygen it can inactivate biological systems. The virucidal action of methylene blue is well known (*62*) but the mechanism of action is not entirely clear. Nucleic acid damage usually results from photosensitization with methylene blue. This was ruled out as the cause of virus kill in one case (*63*), but not in others (*64*). In the current procedure, individual plasma units are treated with 1 µM methylene blue and white fluorescent light for 1 h at 45 000 lux (*65*) or with low-pressure sodium lamps at 200 Joules/cm^2 for 20 minutes. The individual units are re-frozen and stored for later use. Added methylene blue is not usually removed although special filters for its removal are being developed (*66*). Model enveloped viruses and cell-free HIV are inactivated effectively, but non-enveloped viruses, (Table 15 and Figure 11) (*67–68*) cell-associated HIV and other cell-associated viruses are less affected. The latter must be removed completely by filtration or other means. A recent study has suggested that parvovirus may be inactivated (*69*). The in vitro coagulation capacity of plasma treated with methylene blue is well maintained, but the

Table 15
Inactivation of viruses on treatment of plasma with 1 μM methylene blue and white light for 1 hour

Virus	Inactivation (log_{10})	Inactivation time (minutes required)
Vesicular stomatitis virus	5.0	60
Simian immunodeficiency virus	≥6.3	—
Semliki forest virus	≥7.0	10
Herpes simplex virus	≥5.5	60
West Nile virus	≥6.5	—
Sindbis virus	≥9.7	—
Bovine viral diarrhoea virus	≥5.9	2
Human immunodeficiency virus (extracellular)	≥6.3	10–30
Human immunodeficiency virus (cellular)	0	—
Duck Hepatitis B virus	3.9	60
Hepatitis A virus	0	60
Porcine parvovirus	0	60

Figure 11
Rate of virus inactivation on methylene blue treatment of plasma



HIV, human immunodeficiency virus; HSV, herpes simplex virus; VSV, vesicular stomatitis virus.
Results generously provided by H Mohr, DRK Blutspenddienst, Springe.

activities of fibrinogen and factor VIII are reduced (*70*). Photodynamic methylene blue treatment of plasma resulted in no adverse reactions in a controlled clinical study (*71*) and there is no evidence of neoantigen formation (*72*). The advantage of this approach compared with solvent/detergent-treatment (see above) is the absence of pooling, i.e. recipients would receive plasma from individual donations,

rather than from a plasma pool made from hundreds or thousands of donations. Because it is well known that methylene blue and its reaction products are mutagenic (genotoxic) in bacteria, some regulatory authorities in Europe have requested additional data on the mutagenic potential of these substances in mammals and/or on the validated use of filters to efficiently remove them from treated plasma units.

On the basis of the above considerations, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— the volume of plasma being treated;
— the geometry of the sample;
— the light intensity and duration of exposure;
— the effect of residual cells;
— the transparency of the bag;
— mixing efficiency; and
— residual levels of reagent and its photoproducts.

Some of this information may be available from the manufacturer of the specialty equipment employed during this procedure.

6. Review of newer viral inactivation methods under development

Several new viral inactivation procedures are being investigated, with the principal objectives of providing broader viral coverage, complementing existing methods, reducing cost and/or improved applicability to FFP. Several of these newer approaches are reviewed here, but it should be noted that in many cases, there is little or no clinical experience with these methods.

6.1 Psoralen-treated fresh frozen plasma

The use of the psoralen, S-59, together with ultraviolet (UVA) irradiation is being investigated with both FFP and platelet concentrates. Published data on viral kill are provided in Table 16. The amount of virus killed by S-59 treatment of platelet concentrates is somewhat greater than that in plasma because of its lower protein content. In phase 1 studies involving six healthy volunteers, infusion of up to 1 l of plasma resulted in no adverse events and no significant clinical changes in blood chemistries or haematological measurements (*73*). Three phase 3 trials are under way. In an open-label trial in patients (to date, $n = 34$) with congenital deficiencies in blood clotting factors,

Table 16
Inactivation of viruses on treatment of plasma with 150 μM psoralen S-59 and 3 J/cm2 UVA

Virus	Inactivation (log_{10})
Duck hepatitis B virus	5.4
Hepatitis B virus	≥4.5
Hepatitis C virus	≥4.5
Bovine viral diarrhoea virus	≥6.7
Human immunodeficiency virus	≥5.9
Human immunodeficiency virus (cellular)	6.4

infusion of S-59-treated plasma resulted in a similar increase in coagulation factor levels to those reported with untreated plasma (*74*) in historical data.

Based on the above considerations, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— the volume of plasma being treated;
— the geometry of the sample;
— the light intensity and duration of exposure;
— the effect of residual cells;
— the transparency of the bag;
— mixing efficiency; and
— residual levels of the reagent and its photoproducts.

Some of this information may be available from the manufacturer of the specialty equipment employed during this procedure.

6.2 Irradiation with ultraviolet light (UVC)

Ultraviolet irradiation, typically at a wavelength of 254 nm (UVC) targets nucleic acid, thus a wide variety of viruses are inactivated irrespective of the nature of their envelope. Viruses containing single-stranded nucleic acids are more sensitive, because they are unable to repair damage in the absence of a complementary strand, and sensitivity increases with genome size (*75*), because a larger target is hit more often. Attempts to use UVC in the 1950s failed to prevent hepatitis transmission by whole plasma, but this probably reflects the relatively high titre of HBV present in donor plasma at that time and the fact that HBV is a double-stranded DNA virus. Based on these principles, HAV and parvovirus should be relatively sensitive to UVC. Following the early efforts, considerable thought was given to the factors that affect UVC efficacy, particularly to the various ways

in which a uniform thin film can be formed in continuous flow. For most protein solutions thin films are necessary to ensure complete penetration of the UVC light because the protein solutions at least partially absorb UVC energy. The difficulty in assuring maintenance of an appropriate thin film may be the reason that that a prothrombin complex concentrate treated with UVC was reported to transmit HIV (*76*). UVC has also been shown to damage protein. For example, albumin prepared from whole plasma irradiated with UVC was reported to be appreciably less stable during storage than albumin prepared from unirradiated plasma (*77*, *78*).

The most practical applications use a light source that emits at 254 nm. With such a source, Hart et al. (*79*) have shown that both albumin and IVIG solutions could be treated with 5000 Joules/m^2 UVC before an unacceptable level of IgG aggregates was observed. Non-enveloped and heat and/or acid-resistant viruses (e.g. polio 2, T4 phage and vaccinia) were effectively inactivated. The results of validation studies performed with albumin appear encouraging (*80*). Horowitz et al. have shown that the addition of quenchers of reactive oxygen species enhances the specificity of virus inactivation by UVC in protein solutions. By adding the plant flavonoid rutin to the protein solution prior to treatment with UVC, these investigators found that the inactivation of several viruses was largely unaffected (Figure 12), but that several coagulation factors were protected against UVC-induced damage (*81*). The beneficial effect of including rutin during UVC treatment was also observed with fibrinogen incorporated into a fibrin sealant, albumin and IVIG (*82*).

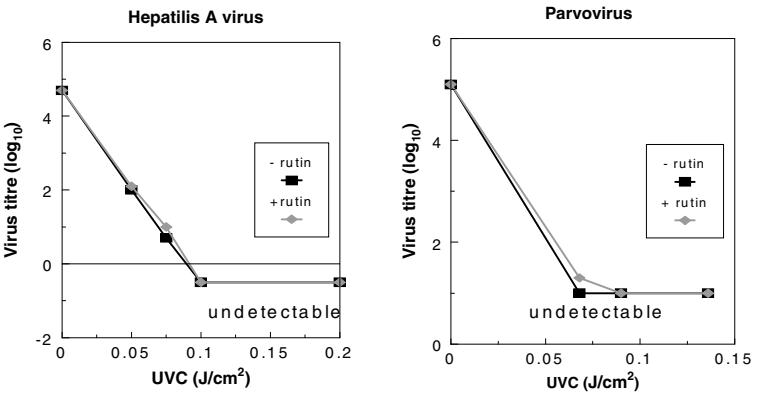
The above mechanistic considerations and experimental findings indicate that the following factors are likely to affect outcome and therefore need to be defined and controlled:

— UVC dose;
— uniformity of dose over time;
— flow rate; and
— optical density of the material being treated.

6.3 Gamma-irradiation

The use of gamma irradiation has been studied extensively for a range of applications from sterilizing hospital supplies to reducing bacterial and viral contamination of meats, other foods and sewage sludge. In most installations, ^{60}cobalt serves as the source. Gamma irradiation can act by two different mechanisms. The first is the direct rupture of covalent bonds in target molecules including both proteins and nucleic acids. The second is an indirect, mechanism, such as with

Figure 12
Inactivation of non-enveloped viruses added to a concentrate of coagulation factor VIII with UVC

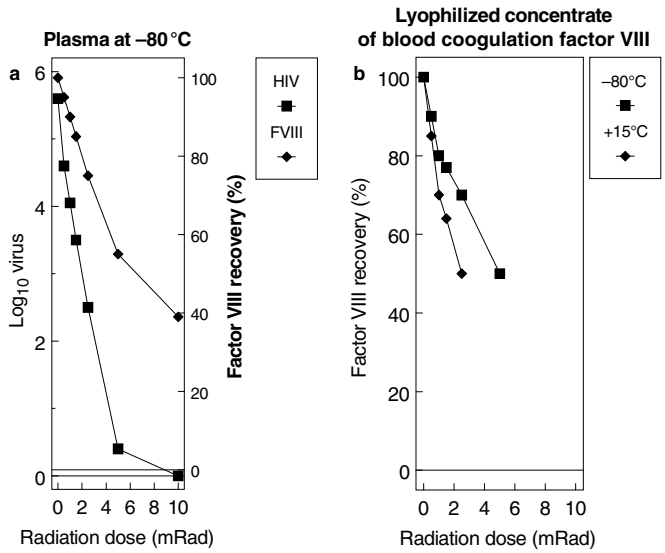


0.5 mM rutin was either present or absent
Source: Chin et al. (*81*).

water, producing reactive free radicals and other active, radiolytic products, which in turn can react with a variety of macromolecules including both proteins and nucleic acids. Indirect reactions can be reduced by adding radical scavengers, removing water by lyophilization, and/or working at cold temperatures. More recently, for the same total dose of radiation, reducing the dose rate has been reported to improve the balance between protein recovery and virus inactivation. The kinetics of viral kill are typically linear in a semi-logarithmic plot of virus titre versus radiation dose, suggesting that inactivation occurs with a single hit of radiation that is absored of directly by the nucleic acid is the likely basis of the inactivation.

The principal challenge in using gamma irradiation is the inactivation of the desired quantity of virus while maintaining the structural and functional integrity of protein. For example, Hiemstra et al. showed that on treating plasma, the inactivation of 5–6 logs of HIV required 5–10 mRad, whereas recovery of at least 85% of factor VIII demanded that the dose not exceed 1.5 mRad (Figure 13). Coagulation activity present in a lyophilized blood coagulation factor VIII, concentrate was even more sensitive whether the treatment was at –80 °C or +15 °C. Moreover, following irradiation of either lyophilized antihaemophilic factor or lyophilized prothrombin concentrates, high-pressure size-exclusion chromatography revealed protein changes at doses as low as 0.5–1 mRad.

Figure 13
Gamma irradiation of plasma and of blood coagulation factor VIII



FVIII, factor VIII; HIV, human immunodeficiency virus.
a Effect on HIV (squares) and factor VIII activity (diamonds) on treatment of frozen plasma. **b** Effect on factor VIII activity in a lyophilized concentrate of blood coagulation factor VIII.
Source: Hiemstra et al. (*83*).

These results contrast with those of Kitchen et al. (*84*), who reported a recovery of 85% for factor VIII and of 77% for factor IX on treatment of frozen plasma with 4 mRad gamma irradiation. This dose of radiation resulted in the inactivation of 4.3 logs of HIV and more than 4 logs of several other viruses including polio and measles. It has not yet been possible to explain the different findings in these two studies.

More recently, Miekka et al. (*85*) reported that treatment of lyophilized preparations of blood coagulation factor VIII with 2–3 mRad of gamma irradiation resulted in the inactivation of 4 logs of porcine parvovirus while retaining 93% of fibrinogen solubility, 67% of factor VIII activity and over 80% of α-1-proteinase inhibitor activity. The dose rate may have been an important variable in these studies. Since then, Drohan et al. have reported that treatment of a monoclonal antibody preparation in the presence of an antioxidant protein protection cocktail resulted in the inactivation of ≥4.8 $\log_{10}$ of PPV. The retention of antigen-binding activity was improved 3- to 4-fold by the presence of the protectant cocktail.

On the basis of the above mechanistic considerations and experimental findings, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— total dose;
— dose rate and dose uniformity;
— composition;
— oxygen content;
— temperature; and
— (for lyophilized products) residual moisture.

6.4 Iodine

Iodine is a strong oxidizing agent and, as a result, is a powerful microbicidal agent. However, in its free form iodine is not sufficiently selective. When bound to polymers, such as polyvinylpyrrolidone (*86*), cross-linked starch (*87*), or dextran chromatographic medium such as Sephadex, the virucidal action of iodine is more controlled. The iodine in these bound forms is slowly released into the protein solution, and virus inactivation occurs over the course of hours. For example, starch-bound iodine at a concentration of 1.05 mg/ml resulted in more than 7 $\log_{10}$ inactivation of model lipid enveloped and non-enveloped viruses while more than 70% of the activity of the clotting factors in plasma was retained. In another implementation, protein was passed through a bed of iodine–Sephadex followed immediately by a bed of Sephadex used to trap and remove free iodine.

Based on the above mechanistic considerations and experimental findings, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— iodine concentration;
— age of iodine–Sepharose;
— temperature;
— contact and incubation times; and
— composition of protein solution being treated.

In addition, careful studies evaluating the covalent incorporation of iodine into macromolecules are required.

6.5 Pasteurized fresh frozen plasma

A system for pasteurizing pooled plasma in bulk at 60 °C for 10 hours with 80–90% retention of coagulation factor activity has been described (*88*). Added stabilizers are removed by diafiltration. Data on viral kill are provided in Table 17. No changes in blood pressure or

Table 17
Inactivation of viruses on pasteurization of plasma at 60 °C for 10 hours[a]

Virus	Inactivation (log_{10})
Human immunodeficiency virus-1	≥5.0
Vaccinia	≥4.3
Pseudorabies	≥4.1
Parainfluenza type 3	≥6.3
Sindbis	≥5.7
Polio Sabin 1	≥6.2
Reovirus	3.2

[a] Stabilized with 1300 g/l sorbitol, 514 g/l sucrose, 4 mM calcium gluconate, 15 mM trisodium citrate, 5 g/l L-lysine and 5 g/L L-arginine.
Source (*83*)

heart rate were observed when the treated plasma was infused in rat at 4 ml/kg body weight, and there was no sign of toxicity on infusion of a single dose of 25 mL/kg body weight of treated plasma into mice. Clinical studies have not been initiated. One alternative that does not require a manufacturing plant, described in a preliminary report, is to heat plasma from a single donor at 50 °C for 3 hours in the presence of lower concentrations of stabilizers, thus avoiding the need for diafiltration (*89*). Although this approach results in lower levels of virus inactivation with some viruses, complete inactivation of HIV (≥6.6 logs) was achieved.

The same factors need to be defined and controlled as for other pasteurized products. In addition, if single units of plasma are treated, the effect of varying the ratio of plasma volume to stabilizer mixture needs to be evaluated.

7. Summary

A number procedures for the inactivation and removal of viruses are now in common use and are well recognized as contributing substantially to the virus safety of plasma products and plasma for transfusion. Adoption of these or equivalent methods is encouraged. For the virus inactivation and removal procedures commonly employed, the information above should help define criteria for acceptance often based on a decade or more of experience. For new products or products from new manufacturers, the rate of virus kill and the extent of virus kill or removal should match those shown for products with good safety records. Assuming this requirement is met for selected viruses, the details of how a process is installed in the production

facility, including staff training, equipment selection, steps taken to monitor the process and process controls, and measures taken to prevent recontamination, probably deserve more emphasis than increasing the number of different viruses studied or the number of slight variations explored.

Which method is most appropriate depends on a variety of factors such as the type of virus, the nature of the product and the characteristics of the production process. The method selected needs to guarantee both viral and general safety without affecting clinical effectiveness, and full safety may require the use of more than one method. The use of more than one robust virus inactivation and removal procedure may be especially important if the viral load present in plasma is substantially higher than that encountered in the countries where the strategies for ensuring viral safety have evolved.

National regulatory authorities frequently need to address the question of how much viral and protein data should be required prior to initiation of clinical trials or routine clinical use. No definitive answer to this question is yet available. Decisions of this nature need to take local circumstances into consideration. For example, to initiate clinical trials, the US Food and Drug Administration usually limits its virus requirements to studies demonstrating the adequate inactivation and removal of HIV, a model for HCV such as BVDV, and a single non-enveloped virus such as parvovirus or HAV.

This guidance document is intended to define the scientific principles that should be taken into consideration as a common basis in the evaluation of the safety of a plasma-derived product, both by the regulatory authorities and the manufacturer. The following principles should be applied.

- Viral inactivation and removal are part of an integrated process designed to guarantee product safety; they cannot replace other safety measures such as donor selection, donation screening or overall compliance with current good manufacturing practices.
- The preparation of all purified plasma products should incorporate two independent and complementary methods able to eliminate enveloped viruses, at least one of which is a viral inactivation step.
- The inactivation and removal of nonenveloped viruses with current methods is frequently incomplete. Manufacturers are therefore encouraged to develop procedures to deal with such viruses.
- Studies that assess viral clearance are required for all products. An exception can be made for albumin produced by the established methods using ethanol fractionation followed by pasteurization. This means that even if the manufacturing process, including virus

inactivation and removal, has been validated by other manufacturers and has a history of use, additional viral validation by the new manufacturer is still required.

- When validating virus inactivation and removal, viruses should not be brought into the production facility.
- When applying established methods of viral inactivation to a particular product, the kinetics and extent of viral inactivation should be assessed with reference to existing data derived from products with a history of safety in which viral inactivation has been carried out by the same or similar procedures.
- When applying established methods of virus removal to a particular product, the extent of removal should be assessed with reference to existing data derived from products with a history of safety that have been manufactured by the same or similar procedures. Studies should include an attempt to show mass balance, i.e. to account for the entire quantity of virus added.
- A robust, effective, reliable process step will be able to remove or inactivate substantial amounts of virus, typically 4 logs or more, be easy to model convincingly and be relatively insensitive to changes in process conditions.
- Final product testing for viral markers, as part of the routine batch release, is not recommended as the outcome is generally of very limited value in determining viral safety. The results of such tests (both serological and NAT) can often be misleading and difficult to interpret.
- The manufacturer should demonstrate, using appropriate methodologies, that the viral inactivation step(s) has (have) not adversely affected the required characteristics of the product.
- Manufacturing aspects such as facility layout, equipment, product flow, staff training and standard operating procedures need to comply with current good manufacturing practices, including measures to prevent the recontamination of product or intermediates.
- Regulations can be established only by the national regulatory authority. Products imported into a country should comply with both the requirements in the country of origin and in the country where the product will be used. Batches of plasma derivatives recalled or withdrawn in one country should under no circumstances be exported to another country.

8. Authors

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Appendix

Example of a study on the inactivation of human immunodeficiency virus-1 by treating a therapeutic plasma protein preparation with tri(n-butyl)phosphate and Tween 80

A solvent/detergent procedure was evaluated for its ability to inactivate human immunodeficiency virus type 1 (HIV-1) added to a therapeutic plasma protein preparation. The study evaluated the rate and extent of HIV-1 inactivation under "worst-case" conditions in that the concentrations of tri(n-butyl)phosphate (TNBP) and Tween 80 were 85% of that specified and the temperature was at the minimum specified under routine manufacturing conditions. Samples were titrated by 50% tissue culture infectious dose ($TCID_{50}$) end-point assay using C8166 cells.

The calculated log reduction factor for the solvent/detergent procedure was:

$\geq 6.00 \pm 0.31 \log_{10} TCID_{50}$.

Validation study report

Objective

The objective of this viral validation study is to provide information concerning the inactivation of HIV-1 on treatment of a therapeutic plasma protein (hereinafter "Test Article") with a solvent/detergent procedure.

Testing facility

Responsibilities for preparing the spiking virus, performing the scale-down process, performing the virus titration, writing the final report and maintaining an archive with the raw data were defined. The validation studies were reviewed by the quality assurance unit.

The following records were stored in the archives: virus spiking records, sample records, cell culture records, culture treatment records, virus titration records, dilution records, inoculation records and records of examination of cells.

Selection criteria for viruses

Validation of virus removal and inactivation should include relevant viruses that are known to, or likely to, contaminate the source mate-

Table 1
Virus used in this viral clearance study

Virus	Genome	Envelope	Family	Size (nm)	Resistance to physicochemical reagents
HIV-1	RNA	Yes	Retro	80–110	Low

rial. The virus proposed for this study is HIV-1, a potential contaminant of human blood products. The characteristics of HIV-1 are given in Table 1.

Equipment and supplies

All equipment and supplies required for this study, including pipettes, pH meters, water-bath, biohazard hoods and incubators were provided. All had been calibrated and certified within the past 6 months.

Test article

Responsibilities for the preparation, stability, purity and concentration of the Test Article were defined. The Test article was sampled from the point in manufacture just prior to virus inactivation, frozen at –70 °C or below, and shipped to the testing facility on dry ice. Once received, the test Article was stored at –70 °C or below.

Virus preparation

Stock virus was prepared at the testing facility. Its titre was determined with three independent assays of its $TCID_{50}$ using 5-fold dilutions and eight replicates per dilution. The certified titre was the average of these three determinations.

Cytotoxicity and viral interference

A previous study had been conducted to determine whether the test article, in the presence or absence of the solvent/detergent reagents, was cytotoxic to the indicator cells used in assessing infectivity of the virus, or interfered with its detection. The results indicated that cytotoxicity could be overcome by diluting the Test Article 81-fold (3^4) with RPMI-1640 + 10% FBS (culture medium) and that, at this dilution, the Test Article did not interfere with the detection of 100 $TCID_{50}$ of HIV-1.

Protocol

1. On the day of testing, the Test Article was thawed in a water bath at 37 °C for approximately 1 hour and clarified by centrifugation at

5000×*g* for 10 minutes and the precipitate discarded. The pH following centrifugation was 7.2, and the A_{280} was 25.6.

2. (a) HIV-1 stock (1 ml) was added to Test Article (19 ml) at a dilution of 1:20 and mixed thoroughly. This was divided into two aliquots, one of 15 ml (to receive solvent/detergent) and one of 5 ml (to receive water). Both were brought to 21 ± 1 °C in a shaking water-bath.
   (b) An additional aliquot (50 µl) of HIV-1 stock was diluted 1000-fold into culture medium containing 10% FBS to serve as the positive control. This was placed on ice during the remainder of the experiment.
3. (a) To the 15 ml aliquot was added 667 µl of 20% Tween 80 followed immediately (after mixing) by 40 µl of TNBP.
   (b) To the 5 ml aliquot was added 222 µl water for injection.
   (c) Both were returned to the shaking water-bath set at 21 ± 1 °C[(1)].
4. (a) From the vessel to which solvent/detergent had been added (the +SD vessel), 0.5 ml was removed after 0, 15, 30, 60, 120 and 240 minutes and diluted immediately 81-fold with culture medium containing 10% FBS. Following dilution, the samples were placed on ice.
   (b) From the –SD vessel, 0.5 ml was removed after 0 and 240 minutes and diluted immediately 81-fold with culture medium containing 10% FBS. Following dilution, the samples were placed on ice.

Assay of infectivity

1. Samples were assayed for HIV infectivity on the day of sampling using C8166 as indicator cells, 3-fold serial dilutions with eight replicates per dilution and 50 µl/well. In addition, to increase the sensitivity of the assay, the +SD, 240-minute time point was also assayed in "large volume" using 800 replicates without further dilution and 50 µl/well. Excess samples of the original dilution (approximately 10 ml) were placed on ice and stored at –70 °C or below until completion of the study in case additional assays were required.
2. For the test to be valid, the titre of the positive control must be within ±1 log of the certified titre.
3. Calculation of titre

(1) The final concentrations of TNBP and Tween 80 were 0.255% and 0.85%, respectively. These are intentionally 85% of that specified for use during manufacture to test the worst case likely to be encountered. Similarly, the temperature specified during manufacture is 24 ± 2 °C; thus, the use of 21 ± 1 °C should also represent worst case conditions.

The following formula for the calculation of $TCID_{50}$ is based on the Karber method:

$$LT = LT_{min} + (\log SDF)/2 + \log SDF \, \Sigma P_i$$

where:

LT = log titre for the sample volume tested
LT_{min} = log of smallest dosage causing infection in all cultures
SDF = serial dilution factor (usually 3, 5 or 10)
ΣP_i = the sum of the proportion of positive results observed at all dilutions greater than that causing infection in all cultures.

4. Calculation of 95% confidence interval
   The 95% confidence interval was calculated using the following formula:

$$SE^2 = (\log SDF)^2 \times \Sigma\{(P_i(1 - P_i))/(n_i - 1)\};$$

and the 95% confidence interval is: $\pm 1.96 \times SE$

where:

SE = the standard error
SDF = serial dilution factor (usually 3, 5 or 10)
P_i = proportion of positive results at dilution i
n_i = the number of replicates at dilution i
Σ = the summation over all dilutions

5. Calculation of viral reduction factor (RF)

$$RF = \log_{10} \frac{\text{input virus titre (per unit volume)} \times \text{input volume}}{\text{output virus titre (per unit volume)} \times \text{output volume}}$$

For example:

$$RF = \log_{10} \frac{10^8 \text{ IU/ml} \times 10 \text{ ml}}{10^2 \text{ IU/ml} \times 20 \text{ ml}}$$

Results

The controls met the criteria for a valid test. The positive control was within ±1 log of the certified titre of the stock virus, and the negative control did not elicit any cytopathology during the test period. The raw data recorded are given in Table 2.

The $TCID_{50}$ titres of the samples tested were as shown in Tables 3 and 4.

Table 2
Raw data

Sample	No. of wells positive for HIV-1/total[a]																
Serial dilution factor (3^x)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Positive control	8/8	8/8	8/8	8/8	8/8	8/8	8/8	7/8	1/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Negative control	0/8	0/8	0/8	0/8	0/8	0/8												
+SD T = 0 min	8/8	8/8	8/8	8/8	8/8	3/8	1/8	0/8	0/8	0/8	0/8							
+SD T = 15 min	8/8	4/8	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8							
+SD T = 30 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 60 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 120 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 240 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 240 min (large volume)	0/800																	
–SD T = 0 min				8/8	8/8	8/8	7/8	5/8	2/8	0/8	1/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8
–SD T = 240 min				8/8	8/8	8/8	8/8	7/8	3/8	3/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

+SD, solvent/detergent present; –SD, solvent/detergent absent.
[a] 50 µl were tested per well.

Table 3
Calculation of $TCID_{50}$ values

Sample	Titre ± 95% CI (log_{10} $TCID_{50}$/ml)	Volume (ml)	Volume correction (dilution prior to titration)	Viral load (log_{10} $TCID_{50}$)
Certified titre of spiking virus	8.45 ± 0.24			
Positive control	4.94 ± 0.20		1000	7.94 ± 0.20
Negative control	no virus detected			
Solvent/detergent treatment				
+SD; T = 0 min	3.69 ± 0.21	15.67	81	6.79 ± 0.21
+SD; T = 15 min	1.96 ± 0.25	15.67	81	5.06 ± 0.25
+SD; T = 30 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 60 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 120 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 240 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 240 min (large volume)	≤–1.12[a]	15.67	81	≤1.98
–SD; T = 0 min	4.88 ± 0.31	15.67	81	7.98 ± 0.31
–SD; T = 240 min	5.24 ± 0.29	15.67	81	8.34 ± 0.29

+SD, solvent/detergent present; –SD, solvent/detergent absent; $TCID_{50}$, 50% tissue culture infectious dose.
[a] No virus detected. The theoretical titre was based on the Poisson distribution.

Table 4
Reduction factors (viral clearance)

Process step	Initial load (log_{10} $TCID_{50}$)	Output load (log_{10} $TCID_{50}$)	Log_{10} reduction
Solvent/detergent treatment (240 min)	7.98 ± 0.31	≤1.98	≥6.00 ± 0.31

$TCID_{50}$, 50% tissue culture infectious dose.