both downstream and upstream areas. If facility layout permits it, the movement of production personnel is unidirectional, starting from the area which handles the more highly processed product. The same holds true for production materials, e.g., chemicals and filters. In another implementation, personnel moving into a safer zone must change their outer coveralls, shoes or shoe covers, gloves, etc., and equipment such as a pH meter moving into a safer zone is surface decontaminated with sodium hypochlorite. Most preferably, 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 since these are notoriously difficult to decontaminate with present methods.

Ultimately, it is the manufacturer's responsibility to describe how facility systems and operating procedures reduce the likelihood of cross-contamination. Usually, decisions are made after a multidisciplinary team consisting of manufacturing, engineering, QA, and microbiology meets and makes recommendations.

2. Quality assurance's role in viral safety

Quality assurance (QA) is a critical part of the manufacturing process since quality cannot be guaranteed simply by checking the final products for conformity to a set of parameters. It is QA's responsibility to ensure that the execution of virus inactivation/removal methods in a production setting conforms to the conditions which were validated in the virus spiking studies. Additionally, it is their responsibility to ensure that the procedures which are designed to avoid cross-contamination are strictly followed. In case there are any departures from the standard, specified manufacturing processes or in environmental conditions, QA, typically with the assistance of a select committee, will conduct a deviation investigation to determine whether or not product can be released. Generally, QA/QC has final authority to release or reject product.

E. Key Predictive Studies

National regulatory authorities frequently need to address the question as to how much viral and protein data should be required prior to initiation of clinical trials or routine clinical use. At this time, a strict answer is unavailable. Clearly, decisions of this nature need to take local circumstances into consideration. As an example, to initiate clinical trials, the US FDA usually limits its virus requirements to studies demonstrating the adequate inactivation/removal of HIV, a model for HCV such as BVDV, and a single non-enveloped virus such as parvovirus or HAV. Regulatory authorities need to determine whether a similarly restricted data set is sufficient when availability of virally inactivated plasma proteins needs to be accelerated. For the commonly employed virus inactivation/removal procedures, the information detailed above should help define expectations based on, in many cases, a decade or more of experience. At the risk of simplicity, for new products or products from new manufacturers, the rate of virus kill and the extent of virus kill/removal should match those shown for products with excellent safety records. Assuming this is met for selected viruses, the details of how a process is installed in a factory may deserve more emphasis than expanding the

number of different viruses which are studied or the number of slight variations which are explored. Fortunately, protein recovery and maintenance of integrity can be shown typically using relatively simple assays.

Inevitably, countries formulate different policies not only in relation to validation and control procedures but also regarding donor selection and methods of blood screening. Different stances can currently be applied by regulatory authorities in different countries. This guidance document intends 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. Regulations can only be established by the National Regulatory Authorities. For products imported into a country, the requirements should comply at least with the requirements approved by the regulatory authorities in the country of origin and batches of plasma derivatives withdrawn in one country should not be exported to another country.

V. VIRALLY INACTIVATED TRANSFUSION PLASMA

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 is described below.

A. 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 only useful for viruses being tested for, although interviewing the donor at the time of the second test may help identify any transient illnesses which occurred between the two donations. To reduce HIV, HBV and HCV transmissions, in the U.S. the regulated hold period is 110 days, estimated to reduce 95% of window period transmissions. The option to quarantine is made possible by the relatively long outdating period of FFP, typically one year.

One advantage of this method is that the plasma itself is unchanged and thus has the same properties and indications as FFP. Implementation requires systems that match donated unit with returning donors and that prevent premature release of units which are labeled as being "Quarantined" or "Donor Retested." Although manual systems are possible, computerization greatly facilitates this effort and provides improved security.

B. SD-Plasma

SD-Plasma was the **first** preparation intended as a virally inactivated substitute for Fresh Frozen Plasma. Plasma is pooled from up to 2,500 donors and treated to inactivate enveloped viruses by using 1.0% TNBP and 1% Triton X-100 at 30 deg C for a minimum of four hours. To reduce risk from non-enveloped viruses, in the USA, incoming plasma is screened for parvovirus DNA by NAT using a minipool strategy, and final product is screened for HAV RNA by NAT. Leukocytes, bacteria, and parasites are removed by sterile filtration. The reagents are removed by hydrophobic chromatography to near undetectable levels[21]. The compounds used are non-mutagenic and have an overall benign toxicology profile. Kill of HIV, HBV, and HCV as well as many other enveloped viruses has been demonstrated (Figures VB-1 and VB-2). Little change in the level of coagulation factors is observed, and bag-to-bag consistency is ensured through the pooling process. **Clinical trials conducted in both Europe and the USA have shown that SD-Plasma can replace FFP in all of its indications, including the replacement of coagulation factors and in the treatment of thrombotic thrombocytopenic purpura (TTP).**

The same factors need to be defined and controlled as with 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.

Figure VB-1. Inactivation of viruses on treatment of plasma with 1% TNBP and 1% Triton X-100 at 30°C for 4 hours

Virus	Inactivation (log 10)	Inactivation Time (hours required)
VSV	≥7.5	0.25
Sindbis virus	≥6.9	0.25
Duck hepatitis B virus	≥7.3	2.5
BVDV	≥6.1	0.25
HIV	≥7.2	0.25
HBV	≥6.0	4*
HCV	≥5.0	4*

* Only time point tested.

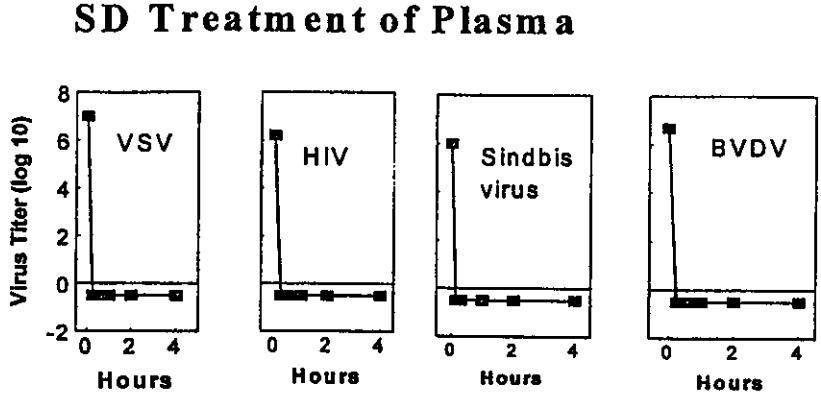


Figure VB-2. Rate of virus inactivation on SD treatment of plasma.

Data graciously provided by V.I. Technologies, Inc.

C. *Methylene Blue and Visible Light*

Methylene blue (MB) is a photosensitizer, i.e. in conjunction with light it can inactivate biological systems. Oxygen is required for action. The virucidal action of MB is well known[22] but the mechanism of action is not entirely clear. Nucleic acid damage is usually produced as a result of MB photosensitization but was ruled out as the cause of virus kill in one case[23] but not in others[24]. In the current procedure individual plasma units are treated with 1µM MB and white fluorescent light for 1 h at 60000 lux[25]. The individual units are refrozen and stored for later use. Added MB is not typically removed although special filters, designed for its removal, are being developed. Model enveloped viruses and cell-free HIV are inactivated effectively, but non-enveloped viruses are less affected (Figures VC-1 and VC-2)[26, 27] as are cell-associated HIV or other cell-associated viruses. The latter must be removed by filtration. Studies on the inactivation of human HBV and HCV have not been performed. The *in vitro* coagulation capacity of MB-treated plasma is well maintained although fibrinogen and factor VIII activities are reduced[28]. MB photodynamic treatment of plasma resulted in no adverse reactions in a controlled clinical study[29] and there is no evidence of neoantigen formation[30]. The advantage of this approach compared with SD-treatment (see above) is the lack of pooling, i.e., recipients would receive plasma from individual donations, rather than from a plasma pool made from hundreds or thousands of donations. Recent reports that MB is mutagenic[31] have caused some regulatory agencies in Europe to demand additional studies prior to continued distribution of this product.

Based on the above considerations, the following factors are deemed important: the impact of varying the concentration of methylene blue with changes in donor plasma volume, lipemia in the donor unit, the degree of cell removal prior to treatment, the total quantity (fluence) of light as well as its intensity (referred to as irradiance or lux) and wavelength, plastic bag transparency, sample depth, mixing efficiency, and residual levels of methylene blue and its breakdown products.

Figure VC-1. Inactivation of Viruses on Treatment of Plasma with 1 µM Methylene Blue and Light for 1 Hour

Virus	Inactivation (log 10)
VSV	5.0
SIV	≥6.3
Semliki forest virus	≥7.0
HSV	≥5.5
West Nile virus	≥6.5
Sindbis virus	≥9.7
BVDV	≥5.0
HIV (extra-cellular)	≥6.3
HIV (cellular)	0
HAV	0
PPV	0

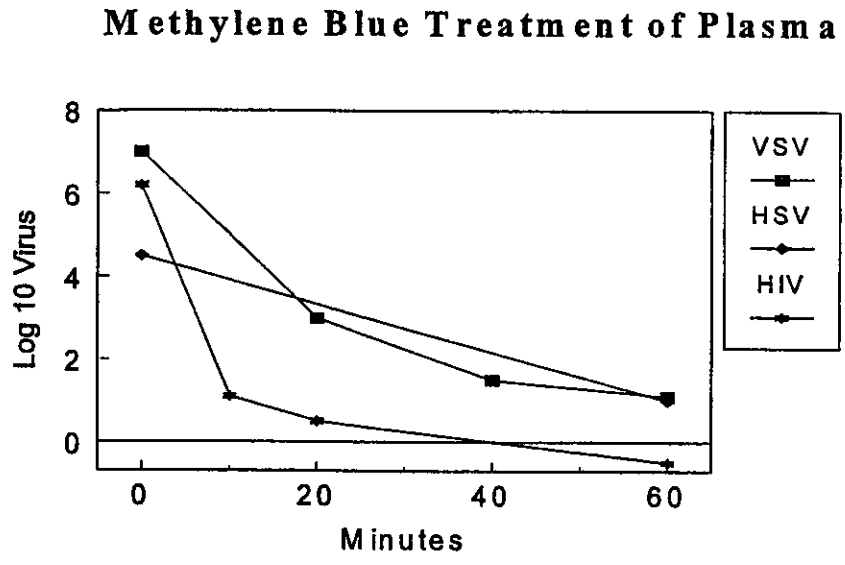


Figure VC-2. Rate of virus inactivation on methylene blue treatment of plasma.
Results graciously provided by H. Mohr, DRK Blutspendidienst, Springe.

VI. REVIEW OF NEWER VIRAL INACTIVATION METHODS APPLIED TO PLASMA PROTEINS

Several new viral inactivation procedures are being pursued, with the principal objectives of broader viral coverage, improved complementation to existing methods, reduced cost, and/or applicability to Fresh Frozen Plasma. Several of these newer approaches will be reviewed here, but it should be recognized that in many cases, there is either no clinical experience with these methods or such experience is very limited.

A. UVC Light Irradiation

UVC light 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, and sensitivity increases with genome size[32]. The former results from the inability to repair damage in the absence of a complementary strand while the latter reflects the fact that a larger target is hit more often. Attempts to use UVC in the 1950's failed to prevent hepatitis transmission by whole plasma, but this likely reflects the relatively high titer 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. Because of that early effort, considerable thought was given to the factors which affect UVC efficacy, the most important of which relates to various ways in which a uniform thin film can be formed in continuous flow. Thin films

are necessary for most protein solutions to ensure complete penetration of the UVC light since 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[33]. Additionally, UVC has been shown to damage protein. For example, albumin prepared from UVC irradiated whole plasma was appreciably less stable during storage than albumin prepared from unirradiated plasma[34].

The most practical applications use a light source which emits at 254nm. With such a source, Hart et al.[35] have shown that both albumin and IVIG solutions could be treated with 5,000 J/m^2 UVC before an unacceptable level of IgG aggregates was observed. Non-enveloped and heat and/or acid resistant viruses (polio 2, T4 phage, vaccinia and T4 phage) were effectively inactivated. Validation studies performed with albumin appear encouraging[36]. Horowitz et al. have shown that the addition of quenchers of reactive oxygen species (ROS) enhances the specificity of virus inactivation by UVC in protein solutions. By adding the plant flavonoid rutin to the protein solution prior to treatment, this group has shown that the inactivation of several viruses was largely unaffected (Figure VIA-1) but several coagulation factors were protected against UVC-induced damage[37]. Rutin also protects fibrinogen, albumin and IVIG against UVC irradiation of fibrin sealant for viral inactivation[38].

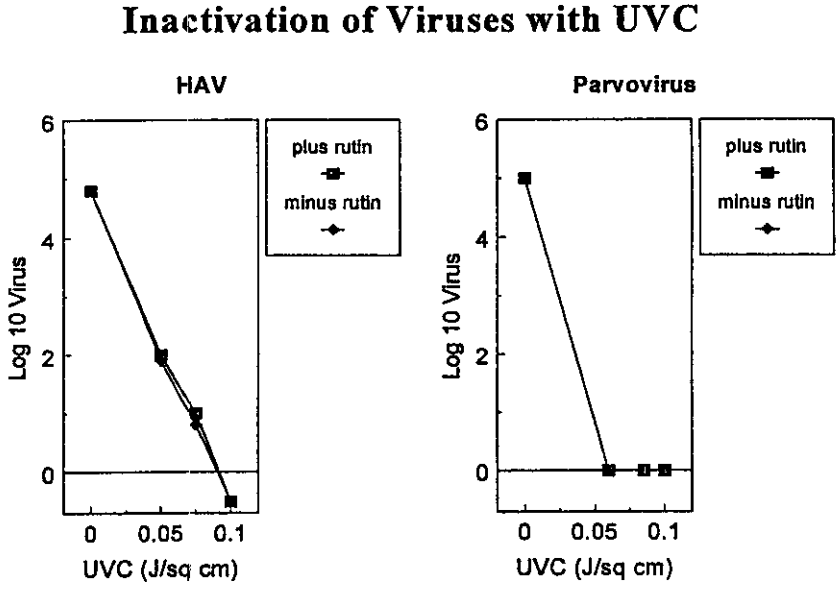


Figure VIA-1. Inactivation of nonenveloped viruses added to an AHF concentrate with UVC.

0.5mM rutin was either present (squares) or absent (diamonds)

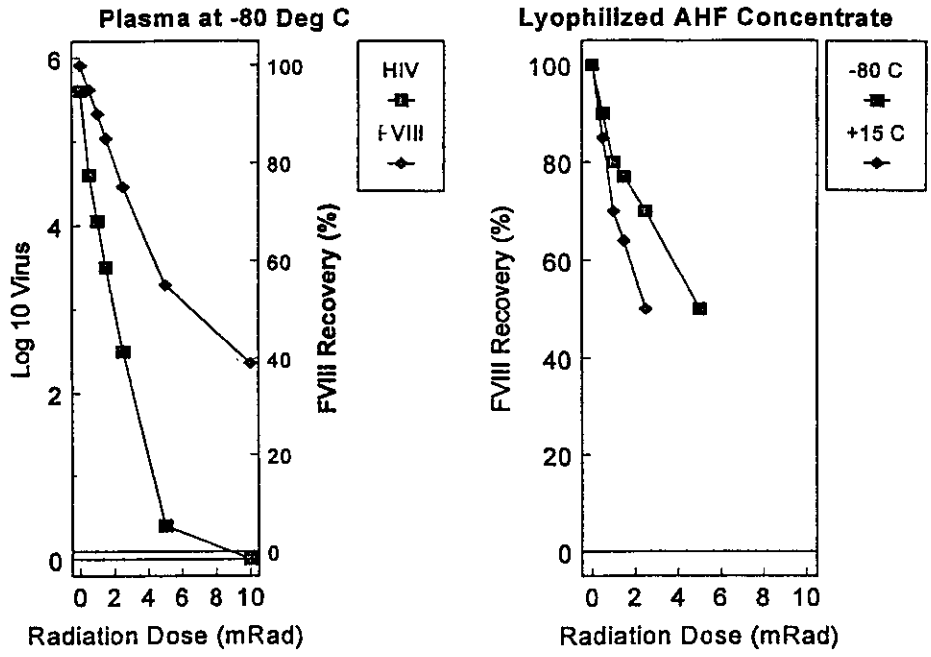
From Chin et al., Blood 1995; 86: 4331-4336.

B. *Gamma-Irradiation*

Gamma irradiation has been studied extensively for the treatment of a variety of materials ranging 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. First, it can directly rupture covalent bonds in target molecules including both proteins and nucleic acids. Second, it can act indirectly, such as with 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. Viral kill kinetics typically are linear in a semi-logarithmic plot of virus titer versus radiation dose, suggesting that inactivation occurs with a single hit on direct absorption of radiation energy by, most likely, nucleic acid.

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

Figure VIB-1. Gamma radiation of plasma and of AHF



Left figure: Effect on HIV (squares) and factor VIII activity (diamonds) on treatment of frozen plasma. Right figure: Effect on factor VIII activity in a lyophilized AHF concentrate. Results taken from Hiemstra et al., Transfusion 1991; 31: 32-39.

These results contrast with those reported by Kitchen et al., 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 viruses. Thus far, it is not possible to resolve the difference in findings between these two studies.

More recently, Miekka et al. reported that treatment of lyophilized preparations with 2 to 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. Dose rate may have been an important variable in these studies. Since then, Drohan et al. reported that treatment of a monoclonal antibody preparation in the presence of an anti-oxidant protein protection cocktail resulted in the inactivation of $\geq$4.8 $\log_{10}$ of PPV. The retention of antigen binding activity was improved by 3- to 4-fold by the presence of the protectant cocktail.

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: total dose, dose rate, and dose uniformity; composition; oxygen content; temperature; and for lyophilized products, residual moisture.

C. *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[39], crosslinked starch[40], or dextran chromatographic medium such as Sephadex, the virucidal action of iodine is more controlled. With iodine in these bound forms, there is a slow release of iodine into the protein solution, with virus inactivation occurring 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 clotting factors activity 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.

D. *Psoralen-Treated Fresh Frozen Plasma*

The psoralen, S-59, together with UVA irradiation is being investigated with both Fresh Frozen Plasma and Platelet Concentrates. Published viral kill data are provided in Figure VID-1. As compared to S-59 treatment of platelet concentrates, virus kill on S-59 treatment of plasma is somewhat reduced because of its higher protein content. In Phase 1 studies involving 6 healthy volunteers, infusion of up to one liter of plasma resulted in no adverse events and no significant clinical changes in blood chemistries or other hematological measurements[41]. In a subsequent open-label Phase IIIa trial in patients (to date, n=9) with congenital deficiencies in blood clotting factors, infusion of S-59 treated plasma resulted in a similar increase in coagulation factor levels when compared with historical data with untreated plasma[42]. Similar factors need to be controlled as outlined for methylene blue treatment of plasma.

Figure VID-1. Inactivation of Viruses on Treatment of Plasma with 150 µM Psoralen S-59 and 3 J/cm^2 UVA

Virus	Inactivation (log 10)
DHBV	5.4
HBV	≥4.5
HCV	≥4.5
BVDV	≥6.7
HIV	≥5.9
HIV (cellular)	6.4

VII. GLOSSARY

AHF. Antihemophilic factor. Coagulation factor VIII, missing in classic hemophilia.

BEV. Bovine enterovirus. A non-enveloped, single stranded RNA virus used as a model for HAV.

BVDV. Bovine viral diarrhea virus. An enveloped, single stranded RNA virus used as a model for HCV.

Cellular components of blood. One of red blood cell concentrates and platelet concentrates.

GMP's. Good manufacturing practices.

CMV. Cytomegalovirus. Enveloped, double stranded DNA virus, typically cell-associated.

Components of blood. One of red blood cell concentrates, platelet concentrates, and fresh frozen plasma

Coxsacki virus. A non-enveloped, single stranded RNA virus.

CPV. Canine parvovirus. A non-enveloped, single stranded DNA virus.

Donor retested plasma. A process of reducing window period transmissions whereby FFP is held in inventory for a designated period of time (approximately 110 days) 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 deg C or higher.

EBV. Epstein Barr virus. Enveloped, double stranded DNA virus, typically cell-associated.

EMCV. A non-enveloped, single stranded, RNA virus.

Factor IX. Coagulation factor IX, missing in hemophilia B.

Factor VIII. Coagulation factor VIII, missing in classic hemophilia. Also called antihemophilic factor.

FFP. Fresh frozen plasma.

Fluence. The total quantity of light delivered. Expressed in J/sq cm.

Gamma-irradiation. A process of virus inactivation or bacterial sterilization which uses gamma-irradiation of liquid, frozen, or lyophilized product.

GE. Genome equivalents. The amount of nucleic acid of a particular virus assessed using NAT.

HAV. A non-enveloped, single stranded RNA virus.

HBsAg. Hepatitis B surface antigen. The antigen on the periphery of HBV.

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 HBV.

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. 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.

Immunogenic. Causing the formation of antibody. Harsh processing conditions may modify a protein's structure so as to make it immunogenic.

Inactine. A family of compounds which bind to and covalently modifies nucleic acid. The exact structure has not been disclosed.

Intermediate purity factor VIII. Factor VIII concentrate with a specific activity between 1 and 50 IU/mg.

Limiting dilution. A way of determining titer 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/removed.

MB-Plasma. Methylene blue-treated plasma intended as a substitute for FFP.

Nanofilter. Small pore filters designed to remove viruses from protein solutions.

NAT. Nucleic acid testing, using amplification techniques.

Pasteurization. A process of heating protein in solution, typically at 60 deg C.

Polio virus. A non-enveloped, single stranded, RNA virus.

PPV. Porcine parvovirus. A non-enveloped, single stranded DNA virus.

Prion. The infectious particle associated with transmissible spongiform encephalopathies. 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, crosslinks nucleic acid.

Quarantine plasma. A process of reducing window period transmissions whereby FFP is held in inventory for a designated period of time (approximately 112 days) until the donor returns and tests negative for virus exposure. The initial unit is then released for use. Also called Donor retested plasma.

ROS. Reactive oxygen species, such as hydroxyl radicals, singlet oxygen.

Rutin. A flavonoid, used as an antioxidant, reducing the action of ROS.

SD. Solvent/detergent treatment. A process of treating protein in solution, typically 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.

Titer. The quantity of virus, typically expressed on a log 10 scale. Six logs of virus equates to 1 million infectious units.

Tri(n-butyl)phosphate. The organic solvent used with so-called solvent/detergent treatment.

Triton X-100. A non-ionic detergent frequently used as part of SD treatment.

Tween 80. A non-ionic detergent frequently used as part of SD treatment.

UVC. Ultraviolet irradiation, typically at a 254 nm wavelength.

Vaccinia virus. An enveloped, double stranded DNA virus.

Vapor heated. A process of heating protein following lyophilization and then reintroducing moisture. Typically at 60 deg C and also at 80 deg C in some cases.

Viral inactivation. A process of enhancing viral safety where virus is intentionally "killed"

Viral removal. A process of enhancing viral safety where virus is removed or separated 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.

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