The pathogen safety of plasma derivative and labile blood components has been dramatically enhanced over the last 25 years through the implementation of nucleic acid testing (NAT) technologies that effectively screen out units with high virus titers. Additionally, the incorporation of pathogen inactivation (PI) and removal steps into the manufacturing processes of plasma-derived medicinal products has greatly enhanced the safety of the final products against both known and emerging/reemerging viruses. The development of pathogen reduction technologies for labile blood components has proven to be more challenging, but significant advancements have been made over the last decade in the development, commercialization, and routine implementation of pathogen reduction technology (PRT) systems for fresh frozen plasma (FFP) and for platelets. The development of a PRT suitable for whole blood or red cells remains an ongoing challenge.

This chapter discusses the PRTs that are commonly used or under development for the various product classes: (1) labile blood components (whole blood [RBCs], platelets, or FFP); (2) manufactured pooled, solvent–detergent (S/D)-treated plasma; and (3) manufactured plasma-derived medicinal products (PDMPs) such as immunoglobulins, purified clotting factors, and albumin. Each class of product faces unique challenges and incorporates different safety measures in order to prevent transfusion-transmitted infections (TTIs).

**PRTs for blood components**

Blood component transfusions represent a portal of entry for infectious agents and may contain trace amounts of endogenous bacteria (mostly from the gut or from presymptomatic infections) and/or exogenous bacteria (from the skin). With the volumes generally collected during a donation, trace contaminations are a problem. This is particularly true for platelet products (stored at 22°C) and in the transfusion of immunocompromised patients. Important interventions to reduce the risk for trace contaminations are careful donor selection, enhanced cleansing of the venipuncture site, use of diversion pouches for the first 15 to 20 mL of a blood collection,¹ and the testing of platelet preparations for the presence of bacteria. Risks for TTIs are escalated by emerging infections combined with increased international air travel.² Programs for careful donor selection and extensive laboratory testing pose both organizational and economic challenges in most developing countries. In addition, the epidemiology of the donor base is less favorable than in most developed countries, the use of whole blood is still dominating, and safe fractionated plasma proteins are mostly unavailable. Therefore, TTIs are a serious problem, causing thousands of infections a year, particularly with hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and malaria. Robust and affordable PRTs for whole blood and blood components would be of great value in these countries. In developed countries, the risk for bacterial and protozoan infections and emerging infectious agents are the main driver for PRTs.

PRTs have been developed and licensed for plasma and platelet components, but the implementation of these systems, although increasing, still remains sporadic and varies widely by country.³ The development of a suitable PRT for whole blood or RBC components is much more challenging, and although development activities are continuing, to date, no technology is commercially available. The lack of approved PRT systems for whole blood (or for RBCs) may be a significant barrier for the adoption and implementation of PRTs for platelets and plasma.

The goal of PRTs is to provide broad-spectrum inactivation of viral and/or bacterial pathogens that are not routinely tested for by NAT or cultivation methodologies. PI of blood components should inactivate or remove all types of infectious agents, without inducing neo-antigens or reducing the function or lifespan of a blood component. PI should not result in residual toxic substances or involve a risk greater than any TTI associated with the original blood component.⁴ Because toxicity may first be revealed after large-scale clinical use, Phase IV postmarketing studies and hemovigilance programs are important.

In general, PRT systems utilize a single type of technology that has a relatively broad-spectrum efficacy against enveloped viruses (including emerging viruses such as West Nile virus [WNV], dengue virus, and chikungunya virus), vegetative bacteria, and parasites. Arguably, the prophylactic use of these technologies on platelet and plasma components will further improve the safety of the blood supply by preventing window period virus transmission, preventing transfusion transmission of infections during emerging virus outbreaks, and reducing transfusion transmission of cell-associated cytomegalovirus (CMV) and bacterially contaminated components, especially platelets.⁵ PRTs that target nucleic acids have the advantage of inactivating residual white blood cells, thereby potentially preventing transfusion-related acute lung injury.

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(TRALI) and graft-versus-host disease (GVHD). Unlike plasma-derived medicinal products that are produced using manufacturing processes that contain multiple complementary measures with orthogonal modes of action to assure the safety of the final product against cellular pathogens, viruses, and prions (see later in this chapter), PRT systems for labile blood components generally rely on a single type of inactivation technology (which is not universally effective), and therefore each system has inherent limitations that may allow specific pathogens to escape inactivation and removal and transmit disease. Nonetheless, even while recognizing these inherent limitations, PRTs have the potential to significantly reduce the safety gap of untreated components and provide benefit to patients.

**PRTs for single-donor plasma and platelets**

**Methylene blue (MB)-light treatment of FFP**

MB, coupled with visible-light treatment, has been used clinically in Europe as a pathogen reduction technology for single-unit FFP for nearly two decades. Over 4.4 million units of FFP have been treated and transfused. MB light treatment has been used clinically in Europe (CE) marked and is intended to be employed at regional blood centers.

MB-treated FFP has not been approved by the US Food and Drug Administration (FDA) for use in the United States, but variations of the technology have been commercially developed for clinical use in Europe by Grifols SA and by Macopharma (Thera) centrifally processes MB-treated plasma at an industrial facility near Barcelona, whereas the Theraplex MB-Plasma system is Council of Europe (CE) marked and is intended to be employed at regional blood centers.

MB treatment reduces the potency of some plasma proteins; fibrinogen, factor V, and factor VIII activity are decreased by approximately 10–30%, and the effect on other coagulation factors and inhibitors is smaller. No neo-antigenicity after MB treatment has been reported. However, hemovigilance data have raised debate as to whether MB treatment increases, decreases, or has no effect on the rate of adverse events versus quarantined FFP. Satisfactory clinical results and efficacy have been reported with MB-treated plasma except for observational studies from Spain that suggest that MB-treated plasma may be less effective in the treatment of thrombotic thrombocytopenic purpura (TTP) than quarantined FFP.

**Psoralen-ultraviolet light treatment of FFP and platelets (INTERCEPT System)**

Cerus (Concord, CA) has developed a psoralen/ultraviolet-A (UV-A)-based PI method (INTERCEPT Plasma or Platelet System)

### Table 56.1 Inactivation agents, susceptible pathogens, and modified targets

<table>
<thead>
<tr>
<th>Inactivation Method</th>
<th>Susceptible Organism</th>
<th>Target/Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue plus light</td>
<td>Enveloped viruses</td>
<td>Free radical damage of negatively charged nucleic acids and lipid bilayers</td>
</tr>
<tr>
<td>-Theraflex-MB</td>
<td>Some non-enveloped viruses</td>
<td></td>
</tr>
<tr>
<td>-Grifols/Biomat MB treated plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-59 Psoralen plus UVA light</td>
<td>Enveloped viruses</td>
<td>Pyrimidine adducts and cross-links</td>
</tr>
<tr>
<td>-INTERCEPT Plasma</td>
<td>Intercellular viruses</td>
<td></td>
</tr>
<tr>
<td>-INTERCEPT Platelets</td>
<td>Some non-enveloped viruses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetative bacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protozoa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leukocytes</td>
<td></td>
</tr>
<tr>
<td>Riboflavin plus light</td>
<td>Most enveloped viruses</td>
<td>Nucleic acid (guanidine) oxidation</td>
</tr>
<tr>
<td>-MIRASOL Plasma</td>
<td>Intercellular viruses</td>
<td></td>
</tr>
<tr>
<td>-MIRASOL Platelets</td>
<td>Some non-enveloped viruses</td>
<td></td>
</tr>
<tr>
<td>-MIRASOL Whole Blood</td>
<td>Vegetative bacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protozoa</td>
<td></td>
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<tr>
<td></td>
<td>Leukocytes</td>
<td></td>
</tr>
<tr>
<td>Short UV Light Treatment</td>
<td>Enveloped viruses</td>
<td>Dimerization of nucleic acid pyrimidines</td>
</tr>
<tr>
<td>-THERAFLEX UV-Platelets</td>
<td>Intercellular viruses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetative bacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leukocytes</td>
<td></td>
</tr>
<tr>
<td>Solvent Detergent + Filtration</td>
<td>Enveloped viruses</td>
<td>[4,0]Phospho-lipid bilayer of membranes</td>
</tr>
<tr>
<td>-VIPS S/D-F Plasma/Cryo</td>
<td>Intracellular viruses*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetative bacteria*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protozoa*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leukocytes*</td>
<td></td>
</tr>
<tr>
<td>S-303 + GSH</td>
<td>Enveloped viruses</td>
<td>Nucleic acid adducts</td>
</tr>
<tr>
<td>-INTERCEPT Red Blood Cells</td>
<td>Intercellular viruses</td>
<td>Some nonspecific modification of membrane/protein nucleophiles</td>
</tr>
<tr>
<td>(second generation)</td>
<td>Some non-enveloped viruses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetative bacteria</td>
<td></td>
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<td></td>
<td>Protozoa</td>
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<td></td>
<td>Leukocytes</td>
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</table>

* In preclinical or clinical development.
suitable for the treatment of plasma (recovered or apheresis) and platelets. Because hemoglobin absorbs UVA light, the method is not appropriate for use with red cells. The INTERCEPT treatment system utilizes a synthetic photoreactive psoralen-based derivative (amotosalen, or S59) in conjunction with long-wavelength UV irradiation (UVA). S59 crosses plasma membranes efficiently and intercalates with the RNA and DNA of viruses, bacteria, parasites, and leukocytes. Upon illumination with UVA light, S59 is activated and covalently crosslinks pyrimidine bases, preventing subsequent replication of the nucleic acid. The treatment system is designed to control the concentration of amotosalen and the UV dose to ensure robust PI while minimizing damage to protein and platelet function. After treatment, the residual amotosalen and by-products are removed to below pharmacologically and toxicologically safe levels using an adsorption device integrated as part of the treatment system.20

The INTERCEPT system is intended to be implemented under good manufacturing practice (GMP) conditions in blood centers. The systems were granted CE mark for PI of platelets in 2002 and plasma in 2006, and have received regulatory approval for use from a variety of international regulatory bodies, including the PEI (Germany), Swissmedic (Switzerland), and AFSSAPS (France); in 2014, it was approved by the FDA for use in the United States.

The INTERCEPT System is broadly effective against enveloped viruses (including emerging viruses such as chikungunya virus [CHIKV] and dengue virus) and cell-associated viruses (like CMV and HTLV).20,24 The efficiency with which non-enveloped viruses can be inactivated is variable and virus specific; bluetongue virus and human adenovirus are effectively inactivated, B19 V parvovirus and calicivirus are only moderately inactivated, and HAV and hepatitis E virus (HEV) are resistant.20,6

The treatment method is also effective against a broad variety of both aerobic and non-aerobic Gram-positive and negative bacteria as well as parasites, including *Plasmodium*, babesia, and *T. cruzi*.23,24

The INTERCEPT treatment was proactively employed and may have prevented transfusion transmission of CHKV in the Ile de Reunion epidemic of chikungunya virus (an enveloped virus spread by mosquitoes) in 2006.25 However, the INTERCEPT treatment system was ineffective in preventing transfusion-associated transmission of HEV (a reemerging non-enveloped virus).6

Although INTERCEPT treatment of plasma can moderately affect certain coagulation factors,26 INTERCEPT-plasma remains clinically efficacious as demonstrated in clinical trials on patients with congenital coagulopathies,26 those with acquired coagulopathies,27 and TTP patients requiring therapeutic plasma exchange.28

An active hemovigilance program of over 7000 transfusions demonstrated high levels of safety and tolerability and a decreased frequency of adverse transfusion reactions compared to untreated plasma.29

INTERCEPT treatment of platelets results in some changes in biochemical qualities (e.g., increased P-selectin and CD42b).30 However, at least 11 clinical trials enrolling more than 1000 patients have been conducted with INTERCEPT platelets that demonstrate efficacy and safety for clinical use.21–35 In addition, the clinical efficacy has been indirectly confirmed by a retrospective analysis of over 13,000 platelet transfusions from a regional blood center that showed no increased usage of platelet concentrates.36 No neo-antigenicity, either biochemically or clinically, was observed in clinical trials as a result of INTERCEPT treatment of platelets.

Riboflavin light treatment: Mirasol® PRT System

TerumoBCT (formerly CaridianBCT, Lakewood, CO) has developed the Mirasol Pathogen Reduction Technology (PRT) System for pathogen and leukocyte inactivation in platelet and plasma components. The system utilizes riboflavin (vitamin B2) plus UV light to induce damage to nucleic acids. Riboflavin preferentially associates with nucleic acids and acts as a photosensitizer by mediating an oxygen-independent electron transfer to, primarily, guanine residues upon exposure to light.37 Damage induced by riboflavin is irreversible because replication and repair processes are impaired due to the guanine base modification.38 In addition, inactivation can be mediated by direct damage to nucleic acids by UV light or indirectly by damage of membranes, proteins, or nucleic acids by reactive oxygen species created by dissolved oxygen when riboflavin absorbs light.39

Riboflavin is a naturally occurring vitamin with a well-known and well-characterized safety profile.40 Preclinical studies with Mirasol-treated plasma and platelets support a minimal in vitro and in vivo toxicity profile (including chronic administration), and no neo-antigenicity has been reported.39,41 In vitro studies have shown that Mirasol-treated platelets remain viable and functional throughout storage whether stored in plasma or in platelet additive solutions.2,43

The Mirasol PRT System inactivates a wide range of clinically relevant pathogens in both plasma and platelet components. A broad panel of enveloped viruses, including free HIV, intracellular HIV, WNV, and CHIKV, is effectively (4 log10), but often not completely, inactivated.21,40,44 However, certain enveloped viruses such as dengue virus and the model viruses bovine viral diarrhea virus (BVDV) and pseudorabies virus (PRV) appear to be only partially susceptible (∼1.5 to 3.5 log10 inactivation)45 to riboflavin–UV treatment and, in side-by-side studies, are inactivated much less efficiently than by INTERCEPT treatment.21,45 Inactivation of non-enveloped viruses is virus specific; the model parovirus (PPV) is inactivated effectively, but the relevant human viruses parovirus B19 V and picornavirus HAV are resistant to treatment (0 to <2 log10 inactivation)45,44 (Table 56.1). Parasites are effectively inactivated by Mirasol treatment.55–48 A broad range of Gram-positive and Gram-negative bacteria are also susceptible to Mirasol riboflavin treatment.46,44 In side-by-side laboratory studies conducted using high bacterial spike challenges, Mirasol PRT failed to produce complete inactivation—whereas INTERCEPT treatment inactivated the bacterial challenge to the limit of detection.49 It can be argued that high bacterial spike challenges may not be as relevant for blood components where bacterial levels are low,50 but the data suggest a diminished robustness of pathogen inactivation efficacy of Mirasol PRT versus INTERCEPT treatment. Leukocytes are inactivated by the Mirasol PRT system, and therefore, Mirasol treatment should reduce transfusion-associated GVHD and TRALI.51,52 In Mirasol PRT plasma units, coagulation factor activity is decreased by between ∼15% and 35%, whereas coagulation inhibitor functionality is well preserved; a loss in high multimeric vWF has also been observed.53,54

The Mirasol PRT system for platelets and plasma received CE mark approval in 2007 and 2008, respectively. The systems are used clinically in various countries in Europe, South America, Africa, and the Middle East.5 A small, prospective, randomized controlled study of 30 patients who received transfusion of FFP for therapy of congenital or acquired coagulopathies showed that hemostatic effectiveness was retained but larger volumes versus FFP were required.55 A small randomized controlled clinical trial evaluating
the performance and efficacy of platelets treated with the Mirasol PRT (MIRACLE trial) confirmed that there was no increased usage of platelet or red blood cell transfusions in patients receiving Mirasol-treated platelets. In addition, the trial showed that platelet corrected count increments (CCIs) remained stable throughout multiple transfusions of Mirasol-treated platelets.56

**Theraflex UV light treatment of platelets**

Macopharma is developing a UV light PRT system for platelets (the THERAFLEX UV-Platelet system). The system uses low-dose short-wave UV light combined with an efficient agitation system without the need for additional photoreactive compounds.57,58 UV light is directly absorbed by the nucleic acids of pathogens and leukocytes, resulting in the formation of intra- and inter-pyrimidine dimers that block transcription.

Preclinical studies demonstrate that the Theraflex UV-Platelet system produces a dose-dependent inactivation of bacteria and viruses. Effective inactivation of most enveloped and non-enveloped viruses was achieved at doses of 0.4 J/cm². At the proposed treatment dose of 0.2 to 0.3 J/cm², small non-enveloped viruses were still effectively inactivated, but larger non-enveloped viruses were inactivated to a lesser degree; HIV was essentially resistant to UV treatment.59 A broad panel of bacterial species were inactivated by at least 4 logs at the proposed treatment dose of 0.3 J/cm²; inactivation studies conducted using low-level challenges of bacteria resulted in sterile units followed by treatment and six-day storage.60 Leukocytes are also inactivated by the treatment procedure.58

In vitro studies have shown Theraflex UV-treated platelets show marginal changes in metabolism and activation throughout five-day storage. In an autologous radiolabeled clinical study, recovery and survival were reduced versus untreated platelet concentrates, which is similar to what is observed for other PRT-treated platelets.54 Additional studies and clinical trials are ongoing.

**Red cell pathogen inactivation**

To date, no PRT for red cells or whole blood has been licensed for routine use. VI Technologies (VITEX, Watertown, MA) pursued development of INACTINE (e.g., ethylenimine) treated packed RBCs into Phase III clinical trials. Although INACTINE was highly effective at inactivating a broad range of enveloped and non-enveloped viruses, bacteria, protozoa, and leukocytes,72,73 the Phase III trials were stopped because the treatment caused neo-antigen formation and antibody responses to PEN110-treated red cells.74

**INTERCEPT Red Blood Cell System**

Cerus Corp. (Concord, CA) is developing a second-generation pathogen and leukocyte reduction system for erythrocytes (INTERCEPT for Red Blood Cells) using S303 (an alkylating agent) and glutathione (GSH). S303 is an alkylating agent with three functional components: (1) an acridine anchor that intercalates into nucleic acids, (2) a bis-alkylator group that reacts with nucleophiles, and (3) a small flexible carbon linker that hydrolyzes at neutral pH to yield a nonreactive breakdown product (S300).65 In the current prototype, after the inactivation step, reagents, and S303 byproducts are removed to toxicologically acceptable levels by centrifugation and removal of the supernatant. S303 readily crosses the membranes of cells and enveloped viruses, allowing it to intercalate with nucleic acids and crosslink them. However, S303 can potentially react nonspecifically with other nucleophiles, including small molecules and proteins. Phase III clinical trials with the first-generation system were suspended after two patients in a chronic transfusion trial developed antibodies to the acridine moiety of S303 on the RBC surface after exposure to several transfusions.66-68 The second-generation system has been modified to include GSH to minimize the potential for natural or induced immune responses to S303-treated RBCs.65 Clinical trials with the second-generation system INTERCEPT RBCs are ongoing.

The second-generation INTERCEPT RBC system is effective at inactivating both enveloped and non-enveloped viruses, although the virus panel studied to date is limited (e.g., B19V, HAV, and HEV were not included).69 Inactivation of between 4 and 6 log₁₀ of a wide variety of relevant Gram-positive and Gram-negative bacteria was also demonstrated. Residual leukocytes are also inactivated by the INTERCEPT RBC system.65

**Riboflavin light treatment (Mirasol RBC)**

Preliminary studies show promising results with PI of red cells using the Mirasol system adapted for red cells. Pathogen inactivation of viruses and virus susceptibility was similar to that observed for viruses.70 Pathogen inactivation of bacteria was tested in units spiked with low levels of contamination followed by treatment and storage for seven days. At higher doses, the units tested negative for bacterial outgrowth, but at the proposed dose, some bacterial breakthrough was observed. In vitro red cell properties (hemolysis and adenosine diphosphate [ADP] level) were minimally impacted versus untreated controls, and hemolysis remained under 1% over 25 days of storage.60,70 In vivo viability of RBCs from Mirasol-treated whole blood treated at different dose levels was studied in a prospective, single-center, open-label design in normal volunteers,71 with no serious adverse events reported. Correlative relationships between 24-hour survival/half-life and ADP levels and hemolysis were observed.72 The data are being used to optimize the Mirasol system for RBCs during further development.

**Small-pool S/D treatment and filtration for plasma and cryoprecipitate (VIPS S/D-F)**

VIPS SA (Viral Inactivated Plasma Systems, Columbier, Switzerland) has recently developed an S/D-based, easy-to-use system for viral inactivation and sterile filtration of small pools of plasma units (recovered or apheresis) and for small pools of cryoprecipitate. The VIPS Plasma and VIPS Cryo systems are designed for implementation in blood collection establishments. The system is CE marked and is in clinical use in several countries, including Egypt, Thailand, and Indonesia.

The process employs a two-stage viral inactivation step using 1% TnBP and 1% Triton X445. After S/D treatment, the added reagents and lipids are extracted with oil and removed to low levels by passage through a S/D and phthalate adsorption device, followed by passage through a 0.2 sterile filter.73,74 The methodology has been validated; >90% of plasma coagulation factors, coagulation inhibitors, as well as albumin and immunoglobulin are recovered. S/D treatment (see the “Pooled plasma, S/D treated” subsection) is highly effective at inactivating all extracellular enveloped viruses, but non-enveloped viruses are not inactivated; the process system has been validated to inactivate a wide variety of model and relevant enveloped viruses.73-76 The sterile filtration step removes bacteria, parasites, leukocytes, cell debris, and plasma microparticles, and it reduces pyrogen.74 A clinical study using VIPS S/D-F cryoprecipitate FVIII showed a normal pharmacokinetics profile and control of acute and chronic bleeding episodes in hemophilia A patients.77
Prion removal

None of the mentioned PRT technologies for blood components eliminate prions. Several companies are developing prion reduction filters for red cell components. A filter employing ligand affinity chromatography has shown significant reduction capacity for exogenously spiked prion spikes, and protection against transmission of vCJD has been verified in ongoing primate transfusion studies.

Pooled plasma, S/D treated

Pooled, S/D-treated plasma (SDPP) is produced in accordance with the EU Pharmacopeia. S/D-treated plasma is prepared using an industrial-scale process in which small manufacturing pools of plasma are created (~60–650L) by pooling multiple units of recovered or source plasma. Prior to manufacture, the plasma pools are sampled and must test nonreactive for certain virological markers—anti HIV-1 and -2, HBsAg, HCV RNA, HAV RNA, and HEV RNA—by NAT. The plasma pool cannot contain more than 10^{4} IU/mL of non-enveloped B19 virus DNA. During the manufacturing process, the plasma pool is incubated in the presence of an S/D mixture (e.g., tributyl phosphate and octoxinol 10 [Triton X-100]) under tightly controlled conditions in order to inactivate enveloped viruses that may be present. The S/D chemicals are subsequently removed to clinically safe levels by either oil or solid phase extraction. The treated product is subsequently sterile filtered into individual doses. The manufacturing process produces standardized biopharmaceutical product with extensive in-process control and without the significant normal variation in plasma protein concentrations observed in single plasma units.

S/D treatment inactivates enveloped viruses by disrupting and destroying the lipid bilayer membranes required for cell adhesion and receptor binding to initiate an infection. Virus validation studies are completed on the S/D manufacturing step to demonstrate that the S/D treatment conditions are robust and effective against a broad variety of enveloped viruses. However, the method does not inactivate non-enveloped viruses and therefore there remains a potential risk for transmission of non-enveloped viruses, especially those that are newly emerging within the population. Therefore, S/D plasma pools (60–650 L) are generally much smaller than those used in the production of fractionated plasma proteins. It is assumed that neutralizing immune antibodies in a plasma pool (i.e., HAV and parvovirus B19) considerably lowers the risk of transmission of non-enveloped viruses already circulating in the population. In addition, as an essential regulatory requirement, NAT screening for the non-enveloped viruses HAV, HEV, and parvovirus B19 V assures the safety of S/D-treated plasma. Manufacturers also carefully follow and assess the epidemiology of known and emerging viruses in their donor population.

Prions (transmissible spongiform encephalopathies [TSEs]) are not inactivated by S/D treatment. Octapharma has introduced a prion affinity chromatography step into their S/D manufacturing process without impacting hemostatic quality. The ability of the prion removal step to remove 2.5 to 3 log_{10} of a surrogate marker of the prion agent has been demonstrated in in vitro studies.

In Europe, an ABO-independent universal SDPP has been developed by Octapharma by proportionally pooling A, B, and AB plasma. The product (tentatively named Uniplas) is not yet licensed, but has undergone clinical trials in cardiac surgery and liver resection in Europe. Adverse events are less common with SDPP than with ordinary standard plasma components. Particularly important is that there have been no reports of TRALI after transfusion of over five million units of SDP in Europe. Hemovigilance data indicate that febrile, allergic, or anaphylactic reactions are reduced by 70% to 80% with SDPP. These observations are best explained by dilution/neutralization that results from pooling and removal of all cellular components during the S/D treatment process.

Plasma-derived medicinal products

Plasma-derived medicinal products are manufactured from relatively large pools (~2000–4000 L) of plasma. Intermediates from multiple pools of plasma may be combined such that a final container may contain proteins from up to 50,000 individual donations. The pooling of many donations inherently increases the risk that the starting material may be contaminated by an unknown or untested-for virus as compared to individual blood components. However, the pooling process also provides a dilution factor that contributes to safety, and, most importantly, the manufacturing processes of all plasma-derived medicinal products contain steps specifically designed to have a high capacity to inactivate or remove virus. The overall virus safety of plasma-derived medicinal products is achieved by the complementary combination of measures of donor selection, testing of donations and plasma pools, and virus inactivation and removal in the manufacturing process, as demonstrated in virus validation studies. These measures have been highly successful in assuring virus-safe products.

In order to achieve virus-safe plasma-derived products, effective virus reduction steps inherent in the manufacturing process are essential. Usually, a minimum of two distinct effective virus reduction methods are implemented that complement each other in their mode of action. If, for one manufacturing step, a gap is demonstrated in the virus reduction capacity for a range of viruses, a second step covering that gap has to be introduced into the manufacturing process (e.g., dry heat treatment or virus filtration for the reduction of non-enveloped viruses together with S/D treatment for inactivation of enveloped viruses).

Virus inactivation and removal

Following the appearance of HIV transmission by transfusion of blood components and plasma derivatives, a major portion of scientific activity in the plasma industry has been directed at either inactivation (such as pasteurization developed by Norbert Heimburger or S/D treatment developed by Bernard Horowitz) or removal of viruses to ensure product safety. By 1994, the plasma fractionation industry had largely achieved this goal, as indicated by the absence of disease transmission of hepatitis or HIV by any product manufactured by American or European biological producers. After the introduction of second-generation HCV antibody testing, the resulting loss of protection of the antibody in immune globulin products led to transmission of HCV. The introduction of dedicated virus inactivation methods into the manufacturing process of IVIG has prevented such transmission from occurring again.

The success of virus inactivation/removal requires not only validation of the reduction in viral load but also maintenance of the intact therapeutic protein. Techniques such as electrophoresis (especially capillary electrophoresis), size exclusion gel chromatography, isoelectric focusing, or antigen–activity ratio are needed. In addition, nonimmunogenicity must be demonstrated to ensure that neo-antigens have not been introduced. Amino acid analysis, cleavage with proteolytic enzymes, circulatory survival in animal
models, and measurement of sedimentation and diffusion coefficients, viscosity, circular dichroism, and optical rotary dispersion are used.98

The virus validation studies must be performed in dedicated laboratories separate from manufacturing facilities so as not to contaminate the production facility. A process step that removes four logs or more of virus is considered effective when the process step can be reliably performed and is insensitive to modifications within the specifications of the manufacturing process.99

Viruses chosen for validation studies should closely resemble the viruses that may potentially be present in contaminated plasma (i.e., transfusion-relevant viruses). To test the ability of the production process to remove viruses in general, they should also represent a wide range of physicochemical properties. For these studies, strains of viruses should be chosen that replicate to high titers in cell culture and can be assayed in an effective, sensitive, and reliable manner in in vitro infectivity assays. Routine infectivity assays are currently not available for HBV, HCV, or parvovirus B19. Research infectivity assays for parvovirus B19 have been established in different laboratories.100,101 and parvovirus neutralization during manufacturing has recently been reported.102 Consequently, these actual viruses cannot be used for virus validation studies, and, where appropriate, model viruses are used instead.

Viruses chosen for validation studies should be performed with HIV and HAV as relevant viruses. For those blood-borne viruses that cannot be propagated in cell culture systems, model viruses must be used. Model viruses for HCV include, for example, bovine viral diarrhea virus (a flavivirus) or Sindbis virus (a togavirus). A specific model virus for HBV is not available; therefore, nonspecific model viruses must be used or, potentially, duck HBV but only under situations where a novel inactivation method is employed. A wide range of viruses must be used in virus validation studies. For parvovirus B19, currently only experimental cell culture systems are available; therefore, animal parvoviruses (canine or porcine parvoviruses) are used as model viruses. In case of interaction of human immunoglobulins with viruses used in virus validation studies, the use of model viruses may be essential: HAV can be replaced by porcine or bovine enteroviruses or encephalomyocarditis virus.99,103

The virus reduction factor is determined as the difference of the virus load (virus titer × volume) in the spiked starting material and in the final sample. In order to demonstrate a high virus reduction factor, it is often appropriate to detect low virus concentration (i.e., the largest practical sample size should be evaluated for residual virus infectivity). However, the impact of potential cytotoxicity or interference of test matrix (composition of product intermediate) on the accurate determination of a virus titer has to be evaluated and taken into account in the calculation of the virus reduction factor.

In virus validation studies, selected steps (stages) of the manufacturing process are studied to assess and quantify how effectively they can inactivate or remove a broad-range variety of viruses. These experiments are carried out using a bench-scale version of the commercial manufacturing process with aliquots of starting materials (production intermediate) obtained from the commercial-scale process. The production-scale manufacturing process is closely mimicked in the bench-scale model by tightly controlling key manufacturing parameters (pH, conductivity, flow rates, filter load ratios, etc.). The performance of the bench-scale model is confirmed by comparing key performance characteristics (e.g., filtration time and chromatography profiles) to the full-scale process and by demonstration that the biochemical characteristics (e.g., activity, protein concentration, monomer and aggregate, purity, and yield) of the resultant output material from the bench-scale model are comparable to those of the production-scale model. The virus validation studies are then performed by deliberately spiking small volumes of high-titer virus into aliquots of product intermediates derived from actual production lots. The virus-spiked product intermediate is then run through the validated bench-scale model of the commercial manufacturing process, and samples are removed for subsequent assay. The amount of virus infectivity removed or inactivated by the manufacturing step is quantified using infectivity assays. The extent of virus removal and inactivation at each individual stage of the production process should be tested at least twice, independently. The results of the virus validation studies (in scaled-down procedures) are predictive for production scale, as the laboratory scale was validated with regard to the comparability of the laboratory scale (model) and full scale (manufacturing), demonstrated in scaled-down validation studies. The demonstration that a step is broadly effective against the wide range of viruses employed in the virus validation studies also provides indirect evidence that the production process can effectively inactivate or remove any novel or emerging virus that could potentially be present.

In addition to providing an accurate reflection of the full-scale manufacturing procedure, scaled-down versions of the production process are also used in so-called robustness studies. These robustness studies evaluate the production parameters that may have an influence on virus inactivation and/or removal. These include parameters such as concentration of protein and other components such as the precipitating agents, temperature, pH, reaction time, amount of stabilizer, temperature for the pasteurization step, and amount of solvent or detergent in S/D treatments. Furthermore, for chromatographic steps, protein concentration, flow rate, washing and elution volume, and resin reuse should be assessed. These robustness studies are performed in the laboratory using parameters in the range of, or even beyond the specifications for, routine production, and assess the impact of these extreme production parameters on the effectiveness of virus reduction steps. The goal of these studies is to demonstrate that the process is robust and effective at removing viruses across the entire potential range of parameters under which the manufacturing process is licensed to be run.

When inactivation processes are validated, the rate of inactivation and the shape of the inactivation curves are assessed. For virus removal, the mass balance of virus infectivity in the starting material versus virus infectivity in the final sample and the final fractions has to be established.

Virus reduction or clearance is achieved during the manufacturing process by removal of viruses. This is accomplished by manufacturing steps designed to purify and concentrate the desired protein, dedicated virus filtration steps, and inactivation of viruses. The inactivation of viruses uses dedicated steps introduced into the manufacturing process.

**Virus inactivation**

**Heat treatment in aqueous solution (pasteurization)**

Heating in aqueous solution at 60°C for 10 hours or greater can be a highly effective method for the inactivation of both enveloped and non-enveloped viruses. The effectiveness of inactivation is dependent upon the composition of the solution and the concentration of
stabilizers that are used to protect proteins and minimize neo-
antigen formation; stabilizers can also protect viruses (especially non-enveloped virus). Stabilizer concentrations are carefully chosen to find conditions that maximize retention of biological activity of the therapeutic molecule while maximizing virus inactivation. All parts of the tank must be heated equally to provide constant temperature in the aqueous solution. The impact of stabilizers and temperature on the virus inactivation capacity for a wide range of viruses must be carefully validated.  

The pharmacopoeia method for albumin requires formulation with low concentrations of sodium caprylate alone or with N-acetyl tryptophan as a stabilizer prior to sterile filtration and filling and heat treatment for 60 °C for 10 to 11 hours in the final containers. The safety of the albumin pasteurization method has been demonstrated for decades. An additional contribution to the virus safety of albumin also comes from virus partitioning during ethanol fractionation that contributes to the removal of viruses.  

It is possible to pasteurize relatively labile molecules such as factor VIII concentrates in the presence of high concentrations of glycine and sucrose or selected salts. This approach was developed by Heimburger in the late 1970s, resulting in a plasma-derived factor VIII/vWF product with no proven cases of virus transmissions in more than 25 years.

**Dry heat**

After lyophilization of proteins (in the final container) to remove water, heating at 80 °C for 72 hours or 100 °C for 30 minutes is also an effective virus inactivation step. Higher residual moisture in the lyophilized product can enhance the inactivation of some viruses, but under such conditions protein aggregates increased and the stability of the product was reduced. However, even at residual moisture levels as low as 0.3%, effective inactivation of most viruses can be obtained. In the commercial manufacturing process, the residual moisture is controlled and the process is validated to ensure that each vial remains between predefined limits demonstrated in viral validation studies.  

Upper and lower limits of residual moisture should be set based on virus validation studies as well as protein integrity studies and aggregate formation studies. Where such a treatment is applied to the product in its final containers, the variation in residual moisture between vials of product should be within the limits set. Temperature and duration of heating should be monitored throughout the process step.

**Vapor heat**

Lyophilized (intermediate) products are heated at defined (relatively high) residual moisture (for, e.g., 60 °C for 10 hours followed by 80 °C for 1 hour). Low residual moisture levels of dry heat treatment do not interfere with the success of this method because the product intermediate is further processed to the final product.

**S/D treatment**

Organic S/D treatment works by disrupting the lipid membrane of enveloped viruses, rendering them unable to bind to and infect cells. This method has been shown to be extremely robust and effective in inactivating a broad range of enveloped viruses, including WNV, HIV, HBV, and HCV. This method does not inactivate non-enveloped viruses. An example of conditions used for virus inactivation is: 0.3% tr(n-butyl) phosphate (TNBP) and 1% ionic detergent (usually either Tween 80 or Triton X-100) at approximately 24 °C for 4 to 6 hours. It is even possible to treat some preparations at 4 °C. Product intermediate has to be filtered to eliminate virus trapped in gross aggregates. Mixing throughout the process is generally used, and homogeneity of distribution of the S/D reagents throughout the S/D treatment process step is assured at the commercial scale through careful validation studies. Every droplet must be treated; solutions are often transferred from one tank to another to ensure that material on the ports, lid, or surfaces of the first tank is subject to the treatment. At the end of the process, the S/D reagents are removed by downstream process steps. In virus validation studies, the key process variables such as the concentration of the S/D compounds, the temperature, and the incubation time as well as the impact of potential lipid content in the product intermediate (because this interferes with the availability of the S/D compounds for virus inactivation) have to be covered.

**Octanoic acid (caprylate) treatment**

Octanoic acid treatment works by the non-ionic form of octanoic acid disrupting the lipid membrane of enveloped viruses, rendering them unable to bind to and infect cells. Similar to S/D treatment, this method is not effective against non-enveloped viruses. The rapidity and effectiveness of inactivation are dependent upon the amount of free non-ionized octanate present, and therefore octanoic acid concentration, pH, temperature, and protein concentration are all critical variables that need to be controlled during treatment.

**Low pH**

Low pH (approximately 4) has been shown to be an effective method for some immune globulin preparations for the inactivation of enveloped viruses and certain non-enveloped viruses such as human parvovirus B19 V. The effectiveness and robustness of a low-pH inactivation treatment are highly dependent upon the exact conditions used (pH, time, temperature, and excipients).

**Virus removal by methods for protein purification**

Precipitation of proteins can also contribute to the safety of the final product by partitioning away pathogens from the protein of interest. Most often, the precipitate is separated by depth filtration or centrifugation. Because the precipitation conditions are designed to purify or concentrate the protein of interest, these partitioning steps often remove only a small range of different viruses. Careful virus validation studies are required to assess the reliable contribution of such precipitation steps to the overall virus reduction for a final product.

**Ethanol, polyethylene glycol (PEG), and caprylate precipitation**

At the low temperatures and pH typically used in plasma fractionation processes, the inactivation property of ethanol against enveloped viruses is minimal. However, cold ethanol fractionation of plasma can contribute to virus safety by partitioning viruses away from the therapeutic protein of interest (Figure 56.1). Prior to the incorporation of dedicated virus reduction steps into manufacturing processes, partitioning during fractionation and purification, along with antibody neutralization in plasma pools, was largely responsible for ensuring the safety of immune globulin products. Comparable principles related to ethanol precipitation steps apply to other precipitation principles such as octanoic acid (caprylate) precipitation, PEG precipitation, glycine and NaCl precipitation, or ammonium sulfate precipitation. Although octanoic acid can
rapidly inactivate enveloped viruses, under some process conditions non-enveloped viruses and certain enveloped viruses can be shown to be precipitated and partitioned (rather than inactivated) by octanoic acid.\textsuperscript{114–116} Virus removal by precipitation and depth filtration is virus specific but can contribute significantly to the overall safety of the final product.

**Chromatography**

Ion exchange, hydrophobic interaction, or affinity chromatography can remove viruses (Figure 56.2).\textsuperscript{117,118} When the resin of the chromatography column is reused, efficient sanitization of resins (and column parts) is a necessary step to avoid batch-to-batch contamination.

**Virus removal by dedicated manufacturing steps for virus filtration**

*Virus filtration*, also called *nanofiltration* (Figure 56.3), is specifically designed to remove virus. It does so by removing virus based on size while allowing flow-through of the desired protein. Large protein molecules or easily aggregated proteins pose a challenge for this method. Virus filtration using small-pore (~20 nm) or parvo-virus-retentive filters is generally a robust and effective means for removing viruses as small as 18–24 nm from process streams, but virus filtration is a complex process. The success depends potentially not only on pore size but also on hydrodynamic forces, adsorption of the virus to the filter surface, and removal of virus aggregated by inclusion in antigen–antibody or lipid complexes.\textsuperscript{119} Virus filtration is gentle, but appropriate testing must ensure that shear forces are not damaging the proteins.

**Other methods**

Besides the established virus reduction factors discussed throughout this section, research into methods such as irradiation, bifunctional hyperoxides, and nucleic acid intercalating substances is ongoing.\textsuperscript{120} The suitability of these inactivation methods with respect to modification of proteins has still to be demonstrated. Although UVC irradiation is not used in the manufacturing process of therapeutic proteins (due to the potential for protein modification), utility may be found as a preventative safety step in the treatment of cell culture media entering the production bioreactors for the manufacture of recombinant-derived proteins in mammalian cell lines.\textsuperscript{121}

**Prions**

Prions, the causative agents of human neurodegenerative diseases such as Creutzfeldt–Jakob disease (CJD) and vCJD, cannot be inactivated under conditions used for the manufacture of plasma-derived products without negatively impacting the qualities of the therapeutic protein. However, the manufacturing processes have been shown to contain process steps that have the potential to significantly reduce prion agent infectivity.\textsuperscript{122} The prion removal capacity of the manufacturing process can be measured in investigative studies using scale-down models and model infectious agents of TSE using principles consistent with virus validation studies. In such investigative studies, a variety of commonly used manufacturing steps (virus filtration [nanofiltration], octanoic acid and caprylate precipitation, PEG precipitation, and affinity chromatography) were shown to be capable of significantly reducing TSE
infectivity. These studies provide reasonable assurance that low levels of TSE infectivity, if present, would be removed by the manufacturing process. There has been one putative report of vCJD (not CJD) transmission of a medium-purity factor VIII concentrate to a hemophiliac patient who did not develop clinical vCJD.123 The hemophiliac had multiple TSE risk exposures, including exposure to 8Y (BPL) factor VIII concentrate manufactured from UK-sourced plasma, which in at least one lot contained plasma from a preclinical vCJD donor. It should also be noted that the pri...
Key references

A full reference list for this chapter is available at: http://www.wiley.com/go/simon/transfusion