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The safety of donor skin preserved with glycerol
Evaluating the Euro Skin Bank preservation procedures of human donor skin against the prEN 12442 standard

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Abstract

The procedures for preservation of human donor skin with glycerol, as applied by the Euro Skin Bank (ESB), were evaluated against the prEN 12442 standard: Animal tissues and their derivatives utilized in the manufacture of medical devices. The focus chosen for this review is on risks related to the transmission of diseases. Product-related hazards were identified. Subsequently, the associated risks and the ESB measures to reduce these risks were discussed. The acceptability of the residual risks was also evaluated by comparing the risk-reducing measures with the prEN 12442, EN 1441, EN 1174 and ISO 14160 standards. Conclusions drawn indicate that ESB procedures as they now stand do not meet all the requirements of these standards. Nonconformities were used to formulate a validation plan containing considerations and suggestions on process definition and control, as well as assessment of bacteriological and virological inactivation and/or elimination. The implementation of this plan should lead to full conformance to the standards.

Preface

For an effective treatment of burns, whether they are superficial or deep, partial or full thickness, it is required that the burned area is covered quickly. This mechanical and biological barrier is necessary to prevent the loss of fluid, protein, electrolyte and heat and also to inhibit infections.
An autograft is commonly regarded as a nearly ideal covering and consists of an epidermal layer with part of the dermal layer which is removed from an unaffected area and placed over the wound. However, a lot of patients do not have sufficient donor sites and/or their physical condition does not permit the procedure, which introduces additional wounds. Human skin from cadaveric sources has proved to be a very effective alternative in these cases.
Both the demand for and the donation of this allograft skin, however, are unpredictable and therefore the availability could be a problem. The creation of skin banks supported a (more) steady supply of human allograft skin.
Crucial for tissue banking is the availability of adequate preservation techniques. Simple preservation techniques like refrigeration have disadvantages, such as high cost, risk of contamination and a limited shelf-life. Therefore other methods had to be developed, such as cryopreservation, which is known to yield excellent results. One of the more recently developed methods for skin banking is the use of glycerol as the preservation medium at moderately low storage temperatures (around 4°C).
In 1982 Basile , who was inspired by successes in experiments with human dura mater, found that glycerolised porcine skin had very good properties for the use as a biological dressing.
Some years later, the Dutch National Skin Bank (now the Euro Skin Bank) developed the glycerol preservation method for human donor skin, which is currently applied in several European burn centres.
Many successful applications of glycerol-preserved donor skin have been reported today, in which the allograft is either used alone, or in combination with widely meshed autografts or cultured keratinocyte sheets. However, a quality system requires more than reports of successful applications. All procedures need to be described adequately and processing methods need to be validated.

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Samenvatting

De procedures voor de preservatie van humane donorhuid, zoals toegepast door de Euro Skin Bank (ESB) werden geëvalueerd aan de hand van de voorlopige Europese norm prEN 12442 "Dierlijke weefsels en daarvan afgeleide producten die voor de vervaardiging van medische hulpmiddelen worden gebruikt"[1]. Hoewel deze norm niet ontworpen is voor het beoordelen van de veiligheid van producten die uit humane weefsels zijn vervaardigd, werd hij toch beschouwd als toepasbaar gezien de mate van vergelijkbaarheid van humane donorhuid met dierlijke weefsels. Betreffende kwesties die specifiek zijn voor humane weefsels is getracht aanvullende of aangepaste eisen te stellen.
De evaluatie richtte zich op risico's inzake de overdracht van ziekten. De gevaren van het product werden geïdentificeerd, waarna de daarmee verbonden risico's alsmede de maatregelen van de ESB om de risico's te reduceren werden bediscussieerd. Bovendien werd de aanvaardbaarheid van de resterende risico's geëvalueerd door ze te vergelijken met de eisen in prEN 12442 en gerelateerde normen (EN 1441, EN 1174, ISO 14160). Belangrijke onderdelen van deze evaluatie waren de literatuuronderzoeken naar de risico's van overdracht van virussen en "transmissible spongiform encephalopathy" (TSE) agentia.
De conclusie van de evaluatie was dat de ESB-procedures op dit moment niet aan alle eisen van de gebruikte normen voldoen. De belangrijkste geconstateerde tekortkomingen zijn de volgende: 1) Er was geen volledig en gecertificeerd kwaliteitssysteem; 2) Het preserveringsproces was niet voldoende omschreven in termen van tijd, temperatuur, en glycerolconcentratie; 3) De inactivatie en/of eliminatie van bacteriën, schimmels, gisten en virussen is niet voldoende gevalideerd.
Betreffende de eliminatie en/of inactivatie van TSE's werden volgens de huidige stand van de wetenschap verdere studies niet noodzakelijk geacht.
Op basis van de geconstateerde tekortkomingen zijn in dit rapport in de vorm van een validatieplan overwegingen en aanbevelingen beschreven die zouden moeten leiden tot conformiteit van het ESB-proces met de prEN 12442 en gerelateerde normen.
Het risico van virusoverdracht wordt op dit moment door de ESB beheerst door de toepassing van donorselectiecriteria en het uitvoeren van serologietesten. Als risico-beheersingsmaatregel ten aanzien van overdracht van bacteriën, schimmels en gisten hanteert de ESB een vrijgifteprocedure waarin resultaten van kweken worden betrokken. Deze maatregelen wijken niet af van hetgeen gebruikelijk is bij huidbanken en bieden een hoge mate van veiligheid. De ESB werkt daarnaast aan ISO certificering.

[1] Inmiddels is deze norm zonder ingrijpende wijzigingen definitief geworden

Summary

The procedures for preservation of human donor skin with glycerol as applied by the Euro Skin Bank (ESB) were evaluated against the draft prEN 12442 standard: Animal tissues and their derivatives utilized in the manufacture of medical devices[2]. Although not specifically designed to assess the safety of products prepared from human tissues, the standard was thought to be applicable because of the similarity of the products. In order to address issues that are specific for human tissues additional or altered requirements were applied.
The review focused on the risks related to transmission of diseases. The hazards that are related to the product were identified and their risks and the ESB measures to reduce these risks were discussed. Furthermore, the acceptability of the residual risks was evaluated by comparison of the risk reducing measures with the requirements in prEN 12442 and related standards (EN 1441, EN 1174, ISO 14160). Important parts of this evaluation included literature searches on the risks regarding transmission of viruses and transmissible spongiform encephalopathy agents.
It was concluded that at this moment the ESB procedures do not meet all of the requirements of the applied standards. The most important nonconformities are the following: 1) There was no complete and certificated quality system; 2) The preservation process was not sufficiently characterised in terms of time, temperature and glycerol concentration; 3) The elimination and/or inactivation of bacteria, moulds, yeasts and viruses was not sufficiently validated.
Regarding the elimination and/or inactivation of TSE agents and according to the current state of the art further studies were not considered necessary.
Based on the nonconformities considerations and suggestions are described in this report in the form of a validation plan. Implementation of this plan should lead to conformance to the prEN 12442 and related standards.
At the moment the risk of virus transmission is managed by the ESB by the application of donor selection criteria and serology testing on several viruses which are judged to represent the greatest risks. In order to control the risk of transmission of bacteria, moulds and yeasts the ESB applies a batch release procedure which consists of testing samples of each batch for bacterial contamination. These measures do not deviate from commonly applied procedures in skin banks and offer a high level of safety. The ESB is in the process of acquiring ISO9002 certification.

[2] By now the standard has been finalised with no major changes

1. Introduction

In this review, the procedures for glycerol preservation of donor skin as applied by the Euro Skin Bank (ESB) were evaluated against the prEN 12442 standard: Animal tissues and their derivatives utilized in the manufacture of medical devices (Feb. 1998). Also CPMP/BWP/268/1995 "Note for Guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses", as issued by the Committee for Proprietary Medicinal Products (CPMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) was taken into account. This analysis was initiated by the ambition of the ESB to validate their preservation procedures with respect to elimination and/or inactivation of micro-organisms, as validation of procedures contributes to the safety of a product. In the absence of specific standards for the manufacture/processing of human tissues, the ESB procedures have been reviewed against this standard for animal tissues. This prEN 12442 standard specifies a procedure to assess the safety of the device (*skin*) by identifying the hazards associated with the device (*skin*) and estimating their risk. This standard is used in close conjunction with EN 1441. Although not specifically designed to assess the safety of products prepared from human tissues, the standard was thought to be applicable because of the similarity of the products. Whenever this was deemed necessary additional or altered requirements were used to address issues that are specific for human tissues.

The 12442-1 standard introduces a flow diagram for the evaluation of the risks related to the device. Such an evaluation is meant to be a dynamic and iterative process until an acceptable state has been reached. That approach, however, was considered unsuitable for this report, which evaluates a steady state, i.e. the current procedures of the ESB. In order to reflect a more realistic approach for this specific study, the sequence of the paragraphs in this review differs slightly from the sequence of clauses as defined in the 12442-1 standard.

This evaluation was based on a selection of the ESB Kwaliteitshandboek and published literature studies. Peer review was carried out by representatives of the ESB, a bacteriologist, a virologist, and a chemical pharmaceutical assessor of biologicals.

A considerable part of the standards is directed at the implementation of a Quality System in the organisation of the manufacturer. Although considered as highly relevant for the reproducibility and control of the total process, this topic and its ramifications, e.g. training of personnel, was excluded from the present review. It was judged to be lying outside its scope. Conducting an audit at the ESB to evaluate the implementation and effectiveness of procedures is considered essential.

The present review emphasises the evaluation of the risk of disease transmission related to donor skin as processed by the ESB. Other risks, although important to the quality and safety of the product, are excluded.

2. A complete description and identification of the product under consideration

The product is human cadaveric donor skin for transplantation purposes and is processed by ESB (Appendix 1: Summary of the procedures of the ESB). The final product is packed in containers with glycerol 85%, each containing a roll of skin with a maximal area of 450 cm^2. The skin has a thickness of about 0.2-0.4 mm and consists of epidermis and part of the dermis. The product is intended as a temporary wound dressing for burn patients. A far more infrequent application is the treatment of chronic skin defects, e.g. ulcers. As an alternative to direct application, it can also be used in a sandwich technique covering meshed autologous skin.
Glycerol is claimed to act as an anti-bacterial and anti-viral agent and preservative of the fine structure of the tissue [1]. It has been shown that the glycerol renders the skin nonviable and attenuates its immunogenicity [2,3]. Notwithstanding the debate on whether transplanted skin should be viable or nonviable for optimal clinical results, adequate results have been achieved with transplantation of glycerolised donor skin [4]. It is beyond the scope of this review to evaluate the fitness for use of the skin as processed by ESB.
Before use, the skin should be washed with lukewarm 0.9% saline for 10 minutes to remove the glycerol. If the transplantation is successful, the donor skin will eventually be replaced by skin of the recipient and is sloughed.

3. Analysis of qualitative and quantitative characteristics related to medical devices

Although it is acknowledged that these aspects bear significance to virus transmission, a detailed assessment of the relationship between cell viability and virus transmission in the case of donor skin was not made. Based on publications by ESB [2,3] it was assumed that the skin contains only non-viable cells. The scope of the EN12442 series, which was used for this evaluation, is also restricted to non-viable tissues.

3.1	What is the intended use and how is the device to be used?	Glycerol-preserved donor skin is to be used by surgeons in burn patients as a wound dressing. After preparation of the skin according to the enclosed instructions, it is applied on the wound, according to the technique used by the surgeon, e.g. in a meshed fashion. This medical procedure is part of standard surgical practice and is performed in an aseptic environment.
3.2	Is the device intended to contact the patient or other persons?	Yes, skin is in contact with burn wounds of the patient; the quantity of material applied is theoretically unlimited. The duration of contact depends on the speed of the repair process of the recipient.
3.3	What materials and/or components are incorporated in the device or are used?	a) Human skin, composed of epidermis and part of the dermis of the arms, legs and posterior thoracic and lumbal regions. Skin derived from a single donor is processed in one batch. b) Glycerol. The concentration in the donor skin during application depends on the efficacy of the washing procedure. The concentration will vary between 85% and 0% (v/v). According to unpublished results the concentration will be about 1% if the prescribed 10 minutes washing time is used [pers. commun. JdP].
3.4	Is energy delivered to and/or extracted from the patient?	No.
3.5	Are substances delivered to and/or extracted from the patient?	Not in an active manner. Glycerol will be resorbed by the wound of the recipient if still present after washing. By application of donor skin on a wound, donor antigens are presented to the recipient. This could induce an immune response.
3.6	Are biological materials processed by the device for subsequent re-use?	No.

3.7	Is the device supplied sterile or intended to be sterilized by the user or are other microbiological controls applicable?	It is not labeled sterile, nor intended to be sterilised by the user. Microbiological controls include serological screening of donors (see Appendix 2: prEN 12442-2,) and bacteriological controls of tissue samples. These controls must show negative results before the skin is released by ESB for transplantation.
3.8	Is the device intended to modify the patient environment?	No.
3.9	Are measurements made?	No.
3.10	Is the device interpretative?	No.
3.11	Is the device intended to control or to interact with other devices or drugs?	No.
3.12	Are there unwanted outputs of energy or substances?	No. An unknown amount of glycerol may be resorbed through the wound.
3.13	Is the device susceptible to environmental influences?	As it is applied on damaged skin and covered by bandages, wound healing is susceptible to mechanical influences.
3.14	Are there essential consumables or accessories associated with the device?	No.
3.15	Is maintenance and/or calibration necessary?	No.
3.16	Does the device contain software?	No.
3.17	Does the device have a restricted shelf life?	Yes, five years.
3.18	Possible delayed and/or long term effects?	Possible sensitisation to donor antigens.
3.19	To what mechanical forces will the device be subjected?	Slight compression.
3.20	What determines the shelf life of the device?	The structure of the skin and bacteriological contamination determine the shelf life.
3.21	Is the device intended for single use or reuse?	Single use only.

4. Identification of possible hazards

4.1 Bio-contamination

Transmission of micro-organisms has been proven after transplantation of allograft skin [5,6] and the recipient of donor skin is often in a weak clinical condition and is sometimes immunologically compromised. For these reasons it is generally accepted that donor skin should be free from contaminants.
For a critical appraisal of the full process, it is relevant to be informed about the number and type of contaminants at the onset of the procedures (i.e. after selection and screening of donors, harvesting of donor skin, before and after decontamination) and the decontaminating capabilities of the subsequent preservation procedures. Also the risk factors for contamination of donor skin during harvesting will contribute to the insight of the process.
In this review bio-contamination by bacteria, moulds, yeast and fungi have been addressed separately from viruses and unconventional transmissible agents.

4.2 Other hazards

Transplantation of glycerolised nonviable donor skin can induce an immunological response, although attenuated when compared with viable donor skin [3]. Additionally, the potential toxic effects of glycerol in concentrations up to 85% on damaged skin could be considered a hazard if the user does not apply the prescribed washing procedure. These and other biocompatibility aspects could be evaluated by using the harmonised standard EN/ISO 10993 “Biological evaluation of medical devices”. However, biological evaluation was excluded from this review.
Inadequate packaging can be identified as a potential hazard, but it is considered beyond the scope of this review. Similar reasoning applies to inadequate labeling of containers/packaging.

5. Estimation of the risk for each hazard

5.1 Bio-contamination

5.1.1 Bacteria, moulds, yeast, fungi

Healthy human skin is colonised by multiple strains of bacteria. They live on the outer surface of the skin as well as between the layers of the stratum corneum, in the follicles of the sebaceous glands and in the hair [7]. The number of micro-organisms is estimated at approximately $10^{3.7}/cm^2$ [8]. The skin harbours predominantly Gram-positive bacteria, such as micrococci, coagulase-negative staphylococci, Propionibacterium and Corynebacteria [7,8]. These bacteria are considered not to be pathogenic in normal circumstances. Although other organisms, such as Gram-negative bacilli, aerobic spore formers and yeast and moulds may be present in only small amounts, these micro-organisms can lead to serious infections.
The effects of measures that are applied by the ESB to reduce these initial risks are reviewed in paragraph 6.

5.1.2 Viruses and transmissible agents

As opposed to bacteria, viruses are normally not present in human skin. As discussed in full detail in the literature review (Appendix 5: Literature review, Inactivation and/or elimination of viruses from donor skin), viruses can be present in human skin, or in skin associated cells during certain stages of infections. This poses a risk for transmission of infections when infected tissues are used for transplantation. Actual transmission has been documented for HIV-1 and cytomegalovirus. An estimation of the risk for virus transmission in a donor population could be approximated by the chance on a positive result on serologic testing of potential donors.
For TSE-agents, infectivity of skin is less clear. Although the “normal”, cellular form of the agent can be present in skin, the infectious form has not been detected. On the other hand, in the animal model infection through breached skin is possible. Full details are discussed in Appendix 6: Literature review, Inactivation and/or elimination of TSE agents from donor skin.

6. Risk reduction

At this moment, the ESB has implemented several procedures to reduce the risk of bio-contamination. Firstly, the ESB is in the process of introducing a Quality System that is based on the ISO system and defines a general structure and relations between processing steps. Furthermore, with respect to the risks as estimated in section 5, the ESB has defined several procedures to reduce these risks. These procedures describe measures like selection of donors, aseptic harvesting and bacteriological controls after processing.

6.1 Selection of donors

Selection of donors is based on a defined list of contraindications referring to the social and medical history. This initial selection is combined with subsequent serological testing of blood samples of the potential donor. The ESB selection criteria are stricter than those mentioned in the common standards of the European Association of Tissue banks (EATB). A statement in the ESB procedures about the inclusion of findings of an autopsy (when performed) in the decision of acceptance of a donor would be appropriate. As stated in paragraph 7.1, a procedure should describe the revision of this list of selection criteria on a regular basis.
The serological tests of the donor blood conform to those listed in the recommendations of the EATB. The quality of serological testing is relevant to the selection of donors. The serology testing is performed by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), where a certified quality system is in place.

6.2 Disinfection of skin

The harvesting area on the donor is cleaned and disinfected prior to harvesting and sterile instruments are being used. The disinfection aims at reducing the total number of micro-organisms. The efficacy of this reduction varies around 1.4 - 1.9 $\log^{10}$, although the effect on different species can vary between various agents [8]. This leads to a theoretical residual contamination after disinfection of approx. $10^{1.8}$ - $10^{2.3}/cm^2$. Although the bacterial load of these skin segments may be reduced and certain species may have been eliminated, Gram-negative species may persist after disinfection [9]. These disinfection procedures are not effective against intracellular viruses.
The efficacy of the disinfection procedure as followed by the ESB (povidone iodine scrub, followed by chlorhexidine digluconate 0.5% in isopropanolol 70%) has been evaluated in daily practice by an other skin bank and shows 81.6% donors free from contamination, or 94.4% of all harvested skin segments [9]. Other studies, analysing different disinfection protocols, report 46% of all donors free from contamination, or 92% of all donor sites (multiple sites per donor) [10].

6.3 Antibiotic treatment

Antibiotics are added during the glycerolisation procedure (see also 6.4 and Appendix 2). This procedure has been generally accepted to reduce the risk, although the effect of the ESB procedure has not been quantified.

6.4 Glycerol treatment

The antibacterial capacities of glycerol [11] are monitored by bacteriological culturing of samples of the final product. In case of a positive culture the storage in glycerol is extended with 5 weeks followed by re-testing until results show only negative cultures.
The effect of these measures can be partly deduced from the analysis of the results of bacteriological culturing of skin samples after processing and storage in glycerol. Review of the results of almost 2000 donors of the ESB from 1987 to 1995 revealed an average 10.1% contamination of the first series of cultures (three weeks after processing and storage in glycerol) [11]. For a subset of 25 donors of which the skin was not treated with antibiotics during the glycerolisation procedure a contamination rate of about 80% was shown after first culture. S. epidermidis was the most frequently found (76.7%) contaminant. Subsequent cultures were positive in 36% after 60 days, 25% after 90 days and 0% after 130 days. In contaminated skin that was treated with antibiotics during the glycerolisation procedure, these results were 2.8% after 60 days and 0% after 90 days. It appears therefore that antibiotics have an additional effect, even after an average treatment duration of 4.2 hours (minimum 2 hours) at room temperature. The authors claim an LD_{50} (time needed to reduce the number of skin samples positive for certain bacteria species by 50%) of about 25 days for S. epidermidis and 110 days for Bacillus species.
Determination of LD_{50} values as described in this study is not optimal. The initial contamination was not known, and the results were recorded as positive/negative only. Furthermore, only two of the 6 frequently found micro-organisms were tested. Determination of LD_{50} values requires measurements in media that are spiked with known numbers of micro-organisms and culturing of subsequent samples taken after fixed time intervals or after certain processing steps (see 9.4.2). From the above mentioned study one can conclude that glycerol has certain antibacterial capacities, of which the efficacy is not exactly known, however.
Recently the glycerolisation procedure has been changed slightly. The antibiotics are no longer added during the first step (the 50% glycerol transport medium), but during the second step (3 h 33ºC in 70% glycerol). The effect of this change on the microbiological quality of the product is unclear.
A vital aspect in the strategy of the ESB is the quality of the final bacteriological test. The sensitivity and specificity of the test system contribute to the safety of the final product.
The glycerolisation procedure has also been shown to have a virucidal effect. The ESB has performed research in order to characterise and quantify this effect. Full details are discussed in Appendix 4 and Appendix 5

7. Acceptability of risk

Acceptability of risk is reviewed by comparison of the ESB procedures with prEN 12442-1 (in close conjunction with EN 1441). This standard in turn refers to prEN 12442-2 (Appendix 3), prEN 12442-3 (Appendix 4), EN 1174-1 (Appendix 7) and ISO 14160 (Appendix 8). These standards rely heavily on the establishment and maintenance of a Quality System, such as the ISO 9000 series. The ESB has started a program to acquire ISO 9000 certification.
Furthermore, the standards introduce specific requirements within this Quality framework and require sound validation of procedures, a strategy that is pursued by the ESB, but which has not yet been completed. Consequently, at this moment the ESB quality System can be expected not to conform to the referred standards. Also because of this situation, this review focuses on the preservation procedures proper and no attempts have been made to check whether every element relating to e.g. training of personnel of each standard has been adequately addressed in the sections of the ESB Quality Manual. Whether the ESB has adequately implemented their procedures has also not been evaluated.

7.1 prEN 12442-2

Review of the ESB procedures against this standard is described in detail in Appendix 2. Procedures that are relevant for the risk of transmission of micro-organisms relate to the Clauses General 4.1/a-c, 5 Sourcing, 6 Collection, 8 Handling and 9 Storage and transport. The ESB procedures conform to these clauses. The clauses 4.2 Procedures and 4.3 Personnel have not been reviewed because they refer to general requirements of a Quality System. Clause 4.1 Quality System Elements identifies the acceptance criteria for skin donation as relevant for the quality of the product. As a consequence, it is relevant to define procedures which describe the review of these criteria on a regular basis, or when new knowledge has emerged. This may have been included in a general standard procedure for regular total review of the Quality Manual.
Not referred to by prEN 12442-2 are the standards of the EATB, representing the state of the art opinion of a selection of experts in the field of human tissue transplantation. The selection criteria for donors for skin (KHB 13v01) comply with the standards of the EATB (see also 6.1).

7.2 prEN 12442-3

Review of the ESB procedures against this standard is described in detail in Appendix 4. Apart from clauses that apply to general aspects of a quality system (e.g. documentation of procedures), the major part of this standard focuses on the literature search and conduction of elimination and/or inactivation studies with respect to viruses. Obviously, the ESB does not comply with this standard, as this notion was the incentive to start this review.
The literature review on the inactivation of viruses (Appendix 5) concludes that additional validation is necessary. The requirements as defined in prEN 12442-3 have been included in the validation plan (paragraph 9). For TSE-agents (Appendix 6), the literature review indicates that with the current knowledge of these agents, it is acceptable not to perform inactivation studies.

7.3 EN 1174-1

Review of the ESB procedures against this standard is described in detail in Appendix 7. Not included in the review are clauses 4.1 Documentation, 4.2 Personnel, 4.3 Equipment, 4.4 Media and materials, 8 Revalidation.
After review of this standard, it can be concluded that the ESB has not met the requirements of the EN 1174-1. Several shortcomings can be mentioned:
Not only is the ESB in the process of implementing a Quality System, but also one of their subcontractors: the Streeklab Haarlem. This means that full documentation of the testing techniques has not been completed. The Streeklab Haarlem works towards a Quality System that is supported by their branch organisation. Review of this Quality System was not included in this review. The ESB should assess the suitability of this system for their situation as the requirements for microbiological evaluation of their samples may differ from those applying to clinical samples, being a type of samples that is frequently processed by the Streeklab Haarlem.
As the product for testing releases glycerol, clause 6.2 applies and requires the use of a technique that neutralises/removes or minimizes the effect of glycerol during culturing. The ESB has overcome this requirement by culturing the sample directly in broth. The ESB skin trimmings are cultured in broth on 50/50 (v/v) basis. However, the requirement of documentation of the effectiveness of this system is not met by the ESB.
Clause 6.3: Culture conditions shall be selected after consideration (to be documented) of the types of micro-organisms expected to be present. Instead, the ESB has selected culture conditions on a subset of micro-organisms, namely those that have been shown to cause wound infection in burn patients. This approach is practical, but not sufficient when aiming at sterility. With the current protocol several types of bacteria, like leprosy and skin tuberculosis bacteria as well as anaerobic bacteria, cannot be detected. However, the risk of these bacteria being present on the product is probably covered by other control measures.
Clause 6.4: Validation of the technique is only partly performed. The Streeklab Haarlem evaluates the quality of culture media on a routine basis. The ESB shall validate (including periodical revalidation) the full technique for bioburden estimation.
Furthermore, (clause 9.3) pre-sterilisation counts shall be assessed according to a plan, which describes frequency and size of sampling.

7.4 ISO 14160

Review of the ESB procedures against this standard is described in detail in Appendix 8. The clauses that relate directly to the ESB preservation process are 5.3 Performance qualification, 5.4 Certification of validation, 6 Process control and monitoring and 7 Product release from sterilisation. The ESB procedures have not met the ISO 14160.
Non-conformities relate e.g. to:
Clause 4.1: pre-sterilisation counts are not determined (and limits have not been set).
Clause 4.5 and 5.1: the process is defined and documented, but shall also be validated.
Clause 5.3: the performance qualification has not been performed. This includes e.g. assessment of appropriate lethal activity against a representative range of micro-organisms (inactivation curves) and validation of the neutralizing capacity of the testing method.
Clause 6: Process control and monitoring requires e.g. temperature recordings, which are not routinely performed by the ESB.

8. Generation of other hazards

If donor selection criteria are too stringent, they may cause an availability problem. This potential problem was not evaluated in the present review. Possible adverse effects of glycerol residues on the product when it is applied have already been discussed in 4.2. Other potential hazards introduced by risk reduction measures have not been identified.

9. Validation plan

9.1 Introduction

After the literature search (see Appendix 5 and 6) and the evaluation of the relevant standards (see Appendix 3, 4, 7 and 8) and the draft ESB Quality Manual (version 01-01-1999) several conclusions can be drawn regarding the need for further actions/studies to validate the glycerol preservation method:

- A complete and certificated quality system is required (ESB is in the process of acquiring ISO9002).
- The process should be characterised in terms of time, temperature and glycerol concentration (see 9.2). In 9.3 some considerations are presented regarding the current glycerolisation process, which should perhaps be adapted.
- Regarding the elimination and/or inactivation of bacteria, moulds and yeasts further studies are required (see 9.4).
- Regarding the elimination and/or inactivation of viruses, additional studies are required (see 9.5).
- Regarding the elimination and/or inactivation of TSE agents, further studies are not required (provided that the donor selection criteria are strictly applied under a quality system and according to the current state of the art).

9.2 Characterisation of the process to be applied

Before the validation studies on bacteria and viruses can be designed in detail, the process needs to be characterised on physical and chemical parameters (time, temperature and glycerol concentrations in the different steps). Upper and lower limits for the different parameters should be set. The worst case conditions should then be applied in the validation studies.

The reasons for choosing the sequence of the different steps in the protocol should also be identified: is this sequence critical for e.g. product quality, or are other time/temperature/concentration combinations possible without yielding a final product of poorer quality?

Judging from the description of the process in the “Kwaliteitshandboek” of the ESB, it is in particular questionable whether the claimed temperatures are actually reached during the claimed periods of time. The skin is put in a plastic bag with the preservation medium, which is subsequently placed on a rotating device in an incubator. The temperature in the plastic bag will probably adapt slowly to the temperature of the incubator. Currently, this is not monitored by ESB, nor has it been determined in the past.

The characterisation of the process can be relatively simple. It can be carried out at the premises of ESB. The temperature profile during the course of the complete glycerolisation process shall be measured by inserting a thermocouple in the plastic bag that contains the skin and the preservation medium. The glycerolisation process shall be carried out by one of the employees of ESB who routinely performs this procedure. Worst-case conditions shall be taken into account (e.g. glycerol from the coldest of the possible storage rooms).

9.3 Considerations for adaptation of the process

Before starting the validation studies, the process conditions should be critically evaluated. Two approaches are possible: 1) Validation studies can be performed using the conditions used in the current process. Based on the results obtained, adaptations of the process conditions may then be necessary. 2) Adjustment of the process conditions before the validation studies are designed. In view of the results of the literature search on the capacity of the glycerolisation process, the latter approach is recommended. Parameters that should be considered are temperature and glycerol concentration. However, the impact of the changes in process conditions on the quality of the donor skin should be carefully evaluated before changes are introduced.

- The inactivation studies with HSV, PV and HIV that were carried out earlier were all performed at temperatures of 4°C, 20°C, 37°C and did not test at 33°C. HIV was shown to be inactivated in the glycerolisation process, if the higher temperature (37°C) is used. If the ESB-process would be changed to these conditions, this would mean that a virus inactivation study with HIV could be omitted. If the temperature of the process will be kept at 33°C, this means that additional studies with the HIV virus are required. Also other viruses can be expected to be more effectively inactivated at 37°C.

Consideration 1: **Investigate whether the processing temperature can be changed from 33°C to 37°C without unacceptable adverse effects to the product.**

- The studies with HSV and PV a definite risk that studies on the current protocol - even if the temperature is changed from 33°C to 37°C - will not provide the decisive evidence that the glycerolisation process yields a product that is virologically safe. It can be argued on the basis of the same studies that processing at high temperature (33 / 37°C) in 98% glycerol would probably yield a product that is virologically safe (see Appendix 5). Introducing a processing step with 98% glycerol would thus be favourable. On the other hand, it has also been suggested that this might lead to clusters of micro-organisms that are hard to eliminate [Du Pont, pers. commun.]. If the introduction of a 98% glycerol processing step is not deemed feasible, the virus validation studies will be carried out with 85% glycerol, with the risk that the obtained results will not be acceptable. Another possibility is to test both protocols (85% and 98% glycerol), however, this would mean that double the amount of experiments has to be performed. (NB: This does not necessarily mean the total amount of work is doubled: a lot of the groundwork can be combined)

Consideration 2: **Investigate whether a processing step with 98% glycerol can be introduced without unacceptable adverse effects to the product.**

9.4 Bacteria, moulds and yeasts - proposed additional studies

As guided by the conclusions of paragraphs 7.1, 7.3 and 7.4 several considerations with respect to the design of a study for validation of the inactivation of bacteria, moulds and

yeasts can be made. It appears that considerations regarding virus inactivation (paragraph 9.3) are more strictly defined than those for inactivation of bacteria and therefore the former will determine the exact process in terms of the time/temperature/concentration combination.
The validation of the process in terms of the physical and chemical parameters (see 9.2) needs to be performed before inactivation studies can be successfully executed.
The main elements of validation are the testing technique (including sampling), performance qualification and the selection (and implementation) of controls during routine processing. These elements are described in more detail in the following paragraphs. However, a detailed validation plan should be based on EN 1174 (including 1174-2 and 1174-3) and ISO 14160 and requires the input of a bacteriologist.

9.4.1 The test system

1. It should be assessed that the product for testing (sampling) represents the whole product, e.g. by testing multiple samples from the whole product directly after harvesting. The whole product can be appreciated as the contents of the final container.
2. It should be assessed that the inactivation of glycerol by immersion of the skin sample in broth (at a 50/50 volume ratio of skin/broth) is effective. This may be evaluated by comparison of culture results of contaminated skin with and without adherent glycerol.
3. The culturing media should be selected after consideration of the expected micro-organisms. This requires the assessment of the bioburden directly after harvesting, before the start of the preservation procedure.
4. The adequacy of the technique to enumerate micro-organisms should be determined.

9.4.2 Performance qualification

Performance qualification is described in detail in Clause 5.3 of ISO 14160, main elements are:

1. The lethal activity of the process should be demonstrated against a representative range of micro-organisms, on skin, by construction of log-survival curves (minimum 5 points), covering at least a thousandfold reduction in numbers. The “normal” microbial flora on skin may pose problems in this experiment. Skin may be sufficiently decontaminated by e.g. immersion in chlorhexidine.
2. The selection of micro-organisms is based on screening for micro-organisms that are resistant to the process (see also table A2 of ISO 14160), e.g. by sampling before the process has ended.
3. A minimum requirement is the evaluation of the full process, beginning at harvesting and ending at final packaging, or at a defined period following packaging. This strategy will evaluate the combined effects of disinfection, antibiotic incubation and stepwise glycerol incubation. If more time and resources are available, the effect of each step (exposure to disinfectants, antibiotics, glycerol) can be evaluated, yielding more information to choose a time/temperature/concentration combination.
4. The inactivation of micro-organisms from the pre-processing count as they are induced to grow onto the tissue shall be assessed.
5. A validation report shall be written, including full description of physical and chemical characterisation (values and tolerances, e.g. volume/surface area of skin pieces per volume of glycerol) of critical variables of the process.

9.4.3 Routine controls

The ESB Standard operating procedures should be revised / evaluated against, or newly established conforming to clause 6 and 7 of ISO 14160. They should include:

1. Bioburden estimation at regular intervals, in order to detect changes in the population of micro-organisms on donor skin.
2. Recording of critical process variables for each batch.
3. For each batch subjected to the process, the absence of micro-organisms should be assessed in the glycerol solution and in representative pieces of skin.
4. For each batch, a portion of the glycerol should be challenged under the same conditions as the batch by processing a piece of skin, which is inoculated with at least 10^6 organisms with a known high resistance to the process. This would require the introduction of test organisms into the manufacturing environment. This may not be acceptable in view of GMP/ISO9002 quality system requirements.
   An alternative approach is the determination of the concentration of glycerol of a sample taken at the end of the process for each batch. However, it is then required to 1) assess the relation between the chemical composition of the glycerol solution) and its microbicidal activity, and 2) to validate the decontaminating effect (assessed by the above mentioned method) to be successful for at least 30 batches.
5. The documented results from the above mentioned tests in the above mentioned points 2 to 4 should form part of the evidence permitting release of skin as bacteriologically safe.

9.5 Viruses - proposed additional studies

9.5.1 Process definition

As a minimum requirement samples have to be spiked with the selected viruses (see 9.5.2) before the start of the overall glycerolisation process (not before each step of the process). This means the validation of the actual process with the risk that the conclusion at the end will be that viral inactivation is not sufficient for the process as it is, while there are no additional data available that could indicate which changes should be made to one of the process steps in order to meet the demands for virus inactivation. In this case the process to which samples are subjected should follow the exact ESB protocol (using worst case assumptions) with regard to the different time/temperature/concentration combinations. This can be either the current protocol or the protocol after adaptation according to the considerations in 9.3. This means there are at least four possibilities A, B, C, D:

A	step 1:	2 hours in 50% glycerol at room temperature
	step 2:	3 hours in 70% glycerol at 33°C
	step 3:	3 hours in 85% glycerol at 33°C
B	step 1:	2 hours in 50% glycerol at room temperature
	step 2:	3 hours in 70% glycerol at 37°C
	step 3:	3 hours in 85% glycerol at 37°C
C[1]	step 1:	2 hours in 50% glycerol at room temperature
	step 2:	x hours in 70% glycerol at 33°C
	step 3:	y hours in 85% glycerol at 33°C
	step 4:	z hours in 98% glycerol at 33°C
D[1]	step 1:	2 hours in 50% glycerol at room temperature
	step 2:	x hours in 70% glycerol at 37°C
	step 3:	y hours in 85% glycerol at 37°C
	step 4:	z hours in 98% glycerol at 37°C

[1] x,y,z to be determined by ESB

After each of the steps the residual infectivity shall be assayed. Since the possible inactivation obtained in the different steps is based on the same mechanism, the reduction factors for the three steps cannot be added. However, evaluation of the individual steps separately and during an extended period of time gives the advantage that it is possible to make a reliable estimate of which conditions are required for an optimal virus inactivation.

9.5.2 Test system

It is proposed to perform additional inactivation studies following the methodology as used in the HIV study that was performed in Australia. Their choice to assess the inactivation of both extracellular and intracellular virus, as well as of virus inside infected skin tissue yields complete information and can be considered thorough and elegant. This is therefore also the most favourable option for future studies. In the standard, however, this is not required: a permissive cell model is acceptable. If this approach is chosen, the number of experiments to be conducted will obviously be reduced significantly.

NB: The HIV study has shown that virus inside skin tissue is more difficult to inactivate; for optimum security, and if practically feasible, it is recommended to include this option. At this moment it is not possible to predict requirements on this aspect in future legislation for human tissues.

The additional studies should be performed with a defined set of viruses. For validation purposes the following guidance should be taken in selecting this set of viruses:

1 whenever possible, a relevant virus should be chosen
2 if model viruses are chosen, they should:
- represent the relevant viruses as much as possible
- span a wide range of physico-chemical characteristics

Obviously, the most relevant virus is HIV. It has already been shown that studies with HIV viruses are possible. If the ESB process is not adapted according to Consideration 1 (see 10.2), HIV should be included in the set of viruses. The question whether a model virus could be chosen for HIV is controversial. For medical devices manufactured utilizing animal tissues this is allowed and for human plasma-derived pharmaceutical products it is not allowed. For human tissues no specific regulation exists at the moment.

The second relevant virus is CMV, which has been reported to be transmissible by skin transplantation. It is also estimated that the majority of the donors may be infected with this virus. CMV is one of the herpes viruses. It is thus advisable to include one of the herpes viruses in the inactivation studies.

Other potentially relevant viruses include HBV and HCV. These viruses are known to be transmissible by e.g. puncture accidents, and also by transplantation of organs (transmission by skin transplantation not yet reported). Since HBV (which is more resistant than HIV) cannot be cultured, another enveloped DNA virus should be used. No specific model virus for HBV is known.

Additionally, a set of model viruses should be selected, e.g. using Table C from Annex C of EN12442-3 (see Appendix 4). An example of a possible set of viruses is the following:

Agent	genome	Enveloped	resistance (phys-chem)	comments
Human immuno-deficiency virus (HIV)	RNA	yes	low	
Poliovirus	RNA	No	medium	
Bovine viral diarrhoea virus (BVDV)	RNA	Yes	low	model for HCV
Parvovirus	DNA	No	v. high	
Pseudorabies virus	DNA	Yes	medium	model for HSV/CMV

As is obvious from the above table, this set of model viruses has been selected in such a way, that it contains an enveloped and a non-enveloped RNA virus, as well as an enveloped and a non-enveloped DNA virus. Further, they span a range of physico-chemical properties from low to very high. Finally, the set contains model viruses for CMV and HCV, which might be considered relevant viruses, judging from the fact that serology screening for these viruses is performed on prospective skin donors. Other viruses can be used instead of the examples above, provided that the combination of viruses still meets the indicated requirements.

The detailed design of virological testing, including e.g. the identification of permissive cell models and infectivity assays, should be carried out in close cooperation with a virologist. Specific virological expertise is absolutely required for the design of that part of the study. It is advised to start the validation studies with a pilot during which all the experimental details of the studies are evaluated and designed. After this pilot it would also be possible to make an accurate estimate of the required budget for such a study. At that point a decision point (go/no go) can be set.

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List of reviewed documents:

ESB Quality Manual 01 jan 1999:
PRO 06v01 - 19v01
KHB 13v01 - 17v01
WERK 01v01 - 04v01

Streeklab voor de Volksgezondheid, Haarlem
Protocol Bacteriologische screening Huidbank, 24 mei 1998.

Appendix 1 Mailing list

1 Inspecteur-Generaal voor de Gezondheidszorg, Inspectie voor de Gezondheidszorg (IGZ), VWS
2 Directeur Genees- en Hulpmiddelenvoorziening (GMV), VWS
3 Directeur-Generaal Volksgezondheid, VWS
4 Directeur Curatieve Somatische Zorg, VWS
5 Hoofdinspecteur voor de Farmacie en Medische Technologie, IGZ, VWS
6 Hoofdinspecteur Preventieve en Curatieve Gezondheidszorg, IGZ, VWS
7 Coördinator Inspectie voor de Gezondheidszorg, IGZ, VWS
8 Mw. dr. A. van Sliedregt, IGZ, VWS
9 Dhr. J. Kraus, IGZ, VWS
10 Coördinator VWS
11 Dhr. A. de Vries, GMV, VWS
12 Mw. dr. I. Steneker, GMV, VWS
13 Dr. M.J. van der Velde, GMV, VWS
14 Mw. ir. G.J. Huisman, GMV, VWS
15 Voorzitter van de Gezondheidsraad
16 Dhr. P.J. Baart, Nederlandse Brandwondenstichting, Beverwijk
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18 Dr. J.S. du Pont, Nederlandse Brandwondenstichting, Beverwijk
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22 Depot Nederlandse Publikaties en Nederlandse Bibliografie, Den Haag
23 Directie RIVM
24 Dr. ir. G. de Mik, directeur sector Risico’s, Milieu en Gezondheid, RIVM
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26 Dr. ir. H.J.G.M. Derks, hLGO/clustercoördinator, RIVM
27 Dr. T.G. Kimman, hLIO, RIVM
28 Dr. B. van Klingeren, SB2, RIVM
29 Mw. dr. M. Lambert, LGM, RIVM
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31 Hoofd van de afdeling Geneesmiddelenbeoordeling, LGM, RIVM
32 Dr. W.H. de Jong, LPI, RIVM
33 Secretariaat LGM, RIVM
34 Ir. R.E. Geertsma, LGM, RIVM
35 Dr. C. Wassenaar, LGM, RIVM
36 SBD/Voorlichting & Public Relations
37 Bureau Rapportenregistratie

38 Bibliotheek RIVM
39-49 Bureau Rapportenbeheer
50-60 Reserve-exemplaren

Appendix 2 Summary of Procedures of the Euro Skin Bank

Sourcing:
The skin that will be processed is donated by deceased human subjects (donors). The procedure starts with the identification of a potential donor by a medical doctor, who informs the central registry in the Netherlands, i.e. the Nederlandse Transplantatie Stichting (NTS, or Dutch Transplantation Foundation).
The NTS checks the presence in their registry of a previously formulated consent of this potential donor. If this is not available from the registry, the MD shall ask permission for donation from the next of kin. When permission is granted, review of the suitability of the potential donor is guided by a preformulated questionnaire. The items on the questionnaire (ESB KHB 13v01) relate to the general status of health, medical history and physical examination. Serological screening is part of the selection procedure and consists of TPHA, VDRL, HBsAg, HCV-ab, HIV-1/2-ab, HTLV-1-ab.

Collection:
When the donor has passed the health status review, a team of the bank will go to the donor (in hospital or at home), re-examines the donor and decides where to remove skin. The removal procedures have been standardised. By using aseptic procedures (disinfection, covering of donor, sterile instruments, surgical dress) skin is removed. Donor preparation includes disinfection with povidone iodine scrub, followed by chlorhexidine digluconate 0.5% in isopropanolol 70%. The donated skin is acceptable if the thickness is 0.3 - 0.5 mm. The minimal size that is acceptable is about 4x12.5 cm, an average piece of skin measures 7x60 cm. Harvesting is performed within 24 hours after death if the donor is cooled within 3 hours after death. If these cooling requirements have not been met, harvesting is performed within 12 hours after death.

Transport:
Step 1: Skin is immersed in glycerol 49% with NaCl 0.45%. The containers are transported at room temperature to the bank. The time between harvesting and the next step in preparation is referred to as the first glycerolisation time and has an average duration of 4.2 h (range 1-12) [deBa94]. Since 1998 this interval has been stated as at least 2 hours [vBa98], while in daily practice it is within 12 hours [pers. comm.].

Processing at the Bank:
Step 2: The quality of the donated skin is reviewed (mechanical damage, thickness and dimensions) and accepted samples are transferred to a sterile plastic bag containing glycerol 70% in NaCl 0.26% containing 1 g streptomycin sulphate and 10^6 IU of sodium penicillin G in a total volume of 1 litre. The bag is placed on a revolving shaker for 3 hours at 33°C. This stage is called the second glycerolisation time and equals an average 4.2 h (range 4-18). At the end of this stage, either the skin is left in the 70% glycerol until further processing at room temperature (resulting in a slow reduction of the temperature), or it is directly taken to the next processing step. This decision depends on the time of day.
Step 3: Subsequently, the skin is placed in a plastic bag containing glycerol 85% for 3 hours at 33°C on a revolving shaker. Thereafter, the bag is put in quarantine at 4°C and stored for about 3 weeks. In daily practice, the skin has reached this quarantine phase within 24 hours after collection.

Storage:
Step 4: Finally, from those donors that have passed the serological screening, the skin strips are trimmed, measured and transferred to packing containers with 85% glycerol, which are stored at a temperature of 4°C awaiting the results of bacteriological screening. A selection of the remnants of trimmed skin from a single donor is put in 5 separate containers with 85% glycerol for bacteriological testing. The final package contains maximal 450 cm^2 skin.

Transport:
The packed skin is transported at room temperature to the customer.

Appendix 3 prEN12442-2 and the Euro Skin Bank preservation procedures

prEN 12442 (1998): Animal tissues and their derivatives utilized in the manufacture of medical devices - Part 2: Sourcing, controls, collection and handling.
Clauses 4 General requirements, 5 Sourcing of animals: General, 6 Sourcing of animal materials: Inspection, certification and traceability, 7 Collection, 8 Handling, 9 Storage and transport, 10 Derivatives and other specialized cases.

4 General requirements
4.1 Quality system elements

A documented system shall be established and maintained to control the quality of materials of *animal* / *human* origin. This system shall include at least the following:	see ESB PRO 06v.01.
a) specification of the geographical origin (such as country or region) of the *animal* / *human* material, state of health of the *animals* / *humans*, and acceptance criteria for *animals* / *humans* taking into account the source species, perceived risk from pathogens and ability to obtain appropriate assurances;	Donor data are documented by Bio Implant Services (BIS). A copy of the Tissue Donor Information Form is sent to ESB. The state of health is reviewed according to BIS procedures, acceptance criteria have been established, see ESB KHB 13v01.
b) hygiene and quality assurance requirements to be met by the slaughterer / harvesting team;	Ok, see ESB WERK 01v01.
c) procedures for the collection, preservation, handling, storage and transport of materials of animal / human origin;	Ok, see ESB PRO 07v01, PRO 08v01, WERK 01v01.
d) records to be maintained (including as a minimum items a), b) and c) above; see also 6.2);	Excluded from this review (ESB claims to maintain records of donors and processing for an indefinite period).
e) audit of the effectiveness of controls defined in a), b) and c) above.	Audits have not been included in the Kwaliteitshandboek ESB.

4.2 Procedures

The documented procedures and instructions required by this standard shall be established, implemented and maintained. These procedures and instructions shall be approved on issue and shall be controlled as follows.	Excluded from this review.
The manufacturer shall establish and maintain procedures to control all documents and data that relate to the requirements of this Part of EN 12442. These documents shall be reviewed and approved for adequacy by authorised personnel prior to issue.	Excluded from this review.

This control shall ensure that: a) the pertinent issues of appropriate documents are available at all locations where operations essential to the effective functioning of the quality system are performed; b) obsolete documents are promptly removed from all points of issue or use.	Excluded from this review.
Changes to documents shall be reviewed and approved by the same functions/organisations that performed the original review and approval unless specifically designated otherwise. The designated organisations shall have access to pertinent background information upon which to base their review and approval.	Excluded from this review.
Where practicable, the nature of each change shall be identified in the document or the appropriate attachments.	Excluded from this review.
A master list or equivalent document control procedure shall be established to identify the current revision of documents in order to preclude the use of non-applicable documents.	Excluded from this review.
4.3 Personnel	
Responsibility for the collection, handling and storage of materials shall be assigned to qualified personnel as follows: The manufacturer shall establish and maintain procedures for identifying the training needs and provide for the training of all personnel performing activities affecting quality. Personnel performing specific assigned tasks shall be qualified on the basis of appropriate education, training and/or experience as required. Appropriate records of training shall be maintained.	Excluded from this review.
Personnel directly involved in the collection and handling of material of *animal / human* origin shall be personnel employed by the device manufacturer or designated and adequately trained *abattoir / hospital* employees or the equivalent. The same requirements apply to personnel of all subcontractors. The manufacturer shall identify the in-house verification requirements, and shall provide adequate resources and assign trained personnel for	Excluded from this review.

verification activities. Audits shall be carried out by personnel independent of those having direct responsibility for the work being performed.	
5 Sourcing of *animals/humans*: General	
NOTE: Clauses 5, 6, 7, 8 and 9 should be applied by suppliers of *animal / human* materials, intermediaries and medical device manufacturers as relevant.	-
The procedures adopted before, during and after *slaughter / harvesting* shall not prejudice the risk reduction provided by sourcing.	Ok.
6 Sourcing of *animal / human* materials: Inspection, certification and traceability	
6.1 Sourcing of animal / human material shall where technically practicable be subject to control and individual inspection by a veterinarian/physician. (There will however be some source species where this is not possible (e.g. fish)).	BIS has defined procedures for donor selection. ESB KHB 13v01, ESB PRO 08v01, WERK 01v01.
If individual animal / humans cannot be inspected, the justification for this shall be documented and a relevant sampling plan provided.	Not applicable.
To minimise the potential risk of the causative agents of spongiform encephalopathies in medical devices the requirements of normative Annex A shall be applied to relevant animal / human species.	ESB KHB 13v01.
6.2 Material of animal / human origin intended for utilisation in medical devices shall have originated from animals / humans confirmed by a veterinarian/physician as being fit (for human consumption. For species not usually consumed by humans a status equivalent to "fit for human consumption' is required.) Records to demonstrate conformance with veterinary/ medical inspection criteria at the abattoir/hospital, certificate details and source shall be available.	see Clause 6.1.
6.3 A certificate relating to the animal / human material utilised in medical devices (see for example Annex B. 1) signed by a person having personal knowledge of the facts being declared and a health attestation signed by a veterinarian/physician (see for example Annex B.2) shall be retained by the	Donor data are reviewed by BIS doctor who signs the TDIF form.

manufacturer. In countries where local regulations ensure that the relevant requirements of this standard are met, the health attestation (see Annex B.2) may be omitted provided that adequate documentary evidence of compliance is available.	
6.4 Depending on the source species of the tissues used, the perceived risk from pathogens, and the ability to obtain appropriate assurances, it may be necessary to specify the origin of the animals / humans (such as country, region or farm) and to obtain additional assurances on their state of health and system of management (see Part 1 of EN 12442).	Not applicable.
7 Collection	
7.1 Between the manufacturer and the supplier of material of animal / human origin there shall be a technical agreement defining the limits of responsibilities, a description of the material, documentation, inspection criteria, procedures and audits (see Annex C). The medical device manufacturer shall document the arrangements for any intermediate stages after collection.	A technical agreement between ESB and BIS has been formulated and approved by both parties.
7.2 The manufacturer shall be responsible for ensuring that the collection of the material is conducted in accordance with the documented procedures.	Ok.
7.3 The manufacturer shall give special consideration to the certification and traceability relating to materials of animal / human origin if pooled at the abattoir/hospital or subsequently. The limits of pooling permitted shall be documented.	Not applicable.
8 Handling	
8.1 If any material of animal / human origin requires further dissection or summing, it shall be removed as soon as possible to an area separate from that used for slaughtering / harvesting and collection. This separate area shall be suitably equipped and maintained at an appropriate level of cleanliness and environmental protection. Implements for dissection and trimming shall be kept clean to minimize risk of cross-contamination.	Ok, ESB PRO 09v01, WERK 02v01, PRO 10v1, WERK 03v01, PRO 17v01, WERK 04v01.
8.2 Source materials to be utilised in medical devices shall be segregated into	Source materials are segregated per donor, see ESB PRO 08v01, WERK 01v01.

consignments according to a documented procedure.	
8.3 The manufacturer shall be responsible for ensuring that the handling of the material is conducted in accordance with documented procedures.	Ok.
9 Storage and transport	
9.1 Consignments of collected material shall be stored and transported in closed containers.	Ok, see Kwaliteitshandboek ESB PRO 08v01, WERK 01v01.
9.2 The conditions for storage and transport shall not compromise compliance with the relevant qualities of the animal / human material, in particular by environmental or enzymatic degradation or microbial proliferation.	Ok, see Kwaliteitshandboek ESB PRO 08v01, WERK 01v01.
9.3 The manufacturer shall be responsible for ensuring that the storage and transport of the material is conducted in accordance with documented procedures.	Ok.
10 Derivatives and other specialized cases	
10.1 The derivative specification shall be documented by the medical device manufacturer and shall ensure that the derivative is suitable for its intended purpose.	Not applicable.
10.2 The manufacturer shall encourage specialised industries to apply specified clauses of EN 12442 but it may be necessary to rely on other procedures which have shown to be effective for risk reduction (see Part 1 of EN 12442).	Not applicable.

Appendix 4 prEN12442-3 and the Euro Skin Bank preservation procedures

prEN 12442: Animal tissues and their derivatives utilized in the manufacture of medical devices - Part 3: Validation of the elimination and/or inactivation of viruses and other transmissible agents.

This part of prEN 12442 specifies requirements for the validation of elimination and/or inactivation of viruses and/or transmissible agents during the manufacture of medical devices utilizing materials of animal origin. In this report it is applied, by analogy, to (non-viable) human donor skin. Given the supposed great similarity between human and animal tissues this was deemed appropriate. However, caution is taken to identify not-applicable and/or missing requirements. For this purpose also the provisions of the "Note for Guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses", as issued by the Committee for Proprietary Medicinal Products (CPMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) were taken into account.

1. General aspects

1.1 Sourcing and manufacturing process

A documented system shall be established and maintained to control the source of raw materials of *animal / human* origin.	See prEN 12442-2
The manufacturing process shall be established and controlled to minimise the load of viruses and transmissible agents in starting materials, intermediate products and finished products.	Starting materials: see prEN 12442-1,2 (donor selection and serology testing) Intermediate products: not applicable Finished products: adequate virucidal effects of glycerol are claimed, but not established, yet.
Appropriate documented procedures shall be applied to ensure that the validated processing parameters are applied during routine manufacture.	See prEN12442-1 (ESB is in the process of obtaining ISO9000 certification - for this aspect EN ISO 9001 + EN 46001 or EN ISO 9002 + EN 46002 is applicable)

1.2 General aspects related to validation

1.2.1 Documented procedures

The documented procedures and requirements of this standard shall be implemented. Documentation and records shall be reviewed and approved by designated personnel (see clause 1.2.2).	Excluded
Procedures for any literature search and/or any inactivation study shall be documented and records shall be retained for a period defined by the manufacturer.	Excluded

1.2.2 Personnel

Responsibility for the implementation of this part of EN 12442 shall be assigned to qualified personnel.	Excluded

The requirements for the qualification, training or experience of personnel shall be documented and suited for the individual's work, responsibility and authority.	Excluded
1.2.3 Calibration	
An effective system shall be established, documented and maintained for the calibration of all controlling, indicating and recording instruments used for validation.	Excluded
1.2.4 Equipment	
Appropriate equipment as specified in the protocol shall be used. All equipment requiring planned maintenance shall be maintained in accordance with documented procedures. Records of maintenance shall be retained. In particular, it shall be capable of delivering the intended process within defined limits. In addition, if the equipment is not identical with that used in normal production cycles, adequate documentation shall be available to demonstrate that the performance parameters are equivalent to those used in the production cycle.	Excluded
1.2.5 Ancillaries	
Ancillaries used for validation studies such as chemicals, cell systems and laboratory animals shall be adequately justified, controlled and documented.	Excluded
2 Literature search	
2.1 Conduct of the literature search	
A literature search shall be performed as specified in Annex A, to identify and analyse data on the elimination and/or inactivation of viruses and transmissible agents (see C.2).	See Annex A
2.2 Application of literature search output	
Technical information from the literature search shall be used in optimising the design of an inactivation and/or elimination study.	See clause 3
Any extrapolation based on the inactivation of viruses and transmissible agents shall be justified and documented.	See clause 3
Intrinsic variability of materials of *animal / human* origin utilised in medical devices and of manufacturing processes can lead to misinterpretation of the validity of published data and shall be taken into account.	See clause 3

2.3 Viruses	
The manufacturer shall demonstrate whether the literature search provides an indication of which inactivation and/or elimination steps are likely to be effective and is a prerequisite to performing a viral inactivation study. In exceptional cases, if a manufacturer chooses not to perform a study this shall be justified and documented.	The glycerol preservation process in total is likely to be effective for the inactivation of viruses to an as yet unknown extent. (see Appendix 5) The different steps in the process, although likely to be of different efficacy, are all based on the same mechanism and therefore their respective inactivation effects cannot be added if assessed individually. This means that at least the effect of the total process should be evaluated. Any studies regarding the individual steps may yield useful extra information.
2.4 Transmissible agents	
The manufacturer shall demonstrate whether the literature search provides an indication of which methods are likely to be effective in the elimination and/or inactivation of transmissible agents. In particular it shall be demonstrated that the specific materials of *animal/human* origin and the specific processes referred to in the literature are comparable to those used for the medical devices concerned. Where the materials or processes are not comparable, an inactivation study shall be performed.	No indication is found in the literature on the effectiveness of the glycerol process regarding the elimination and/or inactivation of transmissible agents. (see Appendix 6) Methods known to be effective in the elimination and/or inactivation of transmissible agents cannot be applied, because they would destroy the product.
If the available information does not support the elimination and/or inactivation of transmissible agents, then an alternative risk management strategy shall be implemented (see EN 12442-1).	An alternative risk management strategy is applicable (see EN 12442-1). This consists of: - Sourcing/selection of donors (see EN 12442-2) - Literature data on the infectivity of skin tissue (see Appendix 6)
3 Elimination and/or inactivation study of viruses and transmissible agents	
If the need for an elimination and/or inactivation study is identified (see 2.3 and 2.4) this shall be performed so that it substantiates the effectiveness for the selected steps of manufacture against selected agents (see Annex C).	For viruses a study should be performed (see 2.3, Annex C). For transmissible agents an alternative risk management strategy is applicable (see 2.4).
If the manufacturer uses sterilization processes which have been validated for bacteria, moulds and yeasts, these processes shall still be supported by relevant validation data for the elimination and/or inactivation of viruses and transmissible agents.	OK

3.1 Protocol	
The protocol for the study to demonstrate the elimination and/or inactivation of viruses and transmissible agents during manufacture shall detail the following, including, where appropriate, values and tolerances:	see Validation Plan (Chapter 9).
a) the identified risks associated with the tissue concerned (see EN 12442-1);	microbiological contamination (i.c. viruses).
b) identification of the relevant agent(s);	From literature search (see Appendix 5).
c) the rationale for the choice of the particular combinations of model agents. The models for the elimination and/or inactivation study shall be chosen by the manufacturer. The justification for the choice of model(s) shall be documented;	See Validation Plan (Chapter 9).
d) identification and definition of the manufacturing step(s) chosen to eliminate and/or inactivate the relevant viruses and transmissible agents;	The total glycerol (preservation) process is chosen (see 2.3). Treatment with antibiotics/disinfectants is considered not (significantly) effective against viruses/transmissible agents
e) if scaling down is performed, it shall be documented. The validity of the scaled down version of the manufacturing process shall be demonstrated.	See Validation Plan (Chapter 9).
f) the methods of calculation for the reduction factors shall be stated; in addition the method of the estimation of reduction kinetics shall be stated where applicable (see Annex E and F).	See Validation Plan (Chapter 9).
3.2 Conduct of the study	
The study shall be conducted in accordance with the protocol.	Obviously
3.3 Interpretation of data	
The reduction factor shall be determined (see C.3.4 and Annex E). The efficacy of the identified manufacturing steps for the elimination and/or inactivation of viruses and transmissible agents shall be reviewed. Particular attention shall be given to the validity of the down scaling and other variables which may influence the results.	Report to be written after conduct of the study.
4 Final report	
A final report shall be compiled containing a critical evaluation of the literature search (see clause 5 and Annex A) and a critical evaluation of the data obtained during any elimination and/or inactivation study undertaken (see clause 6) together with an overall conclusion.	Report to be written after conduct of the study

The report shall identify manufacturing parameters that are critical to the effectiveness of the inactivation or elimination process. Acceptable limits shall be determined and specified for such parameters.	Report to be written after conduct of the study.
An overview shall be given showing all relevant processing steps with the statement of agent reduction factor (see C.3.2.8).	Report to be written after conduct of the study.
This report shall be signed by persons designated as responsible for its preparation, review and approval. This report shall be retained (e.g. for re-validation(s), see clause 8).	ESB

5 Review of final report

Procedures for the review of the final report by persons designated as responsible shall be documented.	ESB
The review of the final report shall be conducted when significant changes in the manufacturing process(es) occur and/or when relevant information, e.g. in the literature, not previously considered in the final report becomes available. Where necessary, corrective actions and/or additional studies shall be undertaken and reported to revalidate the manufacturing process.	ESB
Records of the review of the final report shall be retained.	ESB

6 Routine monitoring and control of critical process parameters

The manufacturer shall assure that all critical parameters identified in the final report are monitored and controlled during manufacture.	ESB

Annex A (normative)
Requirements related to literature search

The literature search was performed and reported in two separate reviews (Annex 4 Literature review, viruses; Annex 5 Literature review, transmissible agents). This annex was subsequently used as a checklist to control if the reviews contained all the required elements.

A.1 The literature search shall identify investigations into the elimination and/or inactivation capacity of the manufacturing process for viruses and transmissible agents.	OK
A.2 The manufacturer shall justify the selection of the elimination and/or inactivation studies from the literature.	OK
The literature search shall include the following steps:	
1) establish a precise definition of the questions to be answered relating to the elimination and/or inactivation of viruses and transmissible agents;	OK
2) conduct a thorough search of the literature to reduce the risk of introducing bias;	OK
3) draw conclusions.	OK
A.3 The definition of the questions, as specified in A.2.1 shall include at least:	
1) identification of the relevant viruses and transmissible agents that will be the basis for the search (see Annex C);	OK
2) identification of the manufacturing steps which may inactivate and/or eliminate the relevant viruses and transmissible agents;	OK
3) identification of the relevant parameters of these manufacturing steps (see C.3);	OK
4) review of the potential efficacy of the process for the elimination and/or inactivation of viruses and transmissible agents.	OK
5) evaluation of the effect of the tissue on the efficacy of the process	OK
A.4 The conduct of the search as specified in A.2.2 shall include:	
1) identification of appropriate inclusion criteria for selecting publications (i.e. peer review or safeguards for non public information, non peer reviewed publications, methodology, scientific quality, relevance of the publications (see A.3), representativeness of the literature as a whole and reflecting the current state of the art, including favourable and unfavourable information as relevant);	OK
2) selection of the studies using the criteria mentioned under A.4 1;	OK
3) analysis and correlation of the conclusions with the data, and noting whether the results are sensitive to changes in the way the analysis is performed.	OK
A.5 The results of the literature search shall be compiled in a report. This shall include a critical evaluation of the literature and conclusions (see clause 5).	OK

Annex C (informative)
Guidance on the elimination and/or inactivation study for viruses and transmissible agents ('agents')

The guidance in this (informative) annex is used to draw up the Validation Plan (Chapter 9). As explained in the Validation Plan, only part of the elements described in this annex can be found in the Validation Plan. The experimental details will be described in a separate protocol, with the help of a virologist.

C.1 General
Elimination and/or inactivation of viruses and transmissible agents is assumed to follow probabilistic concepts and it is therefore not possible to guarantee absolute freedom of contamination in products.
All testing suffers from the inherent limitation of quantitative agent assays, where the ability to detect low agents concentrations, for statistical reasons depends on the size of the sample (see also Annex F).
Establishing the freedom from relevant agents in or on tissue, will not derive solely from direct testing for their presence but also from a demonstration that the manufacturing steps are capable of inactivating or removing them.

C.2 Selection of agents
C.2.1 The selection of agents to be used when performing the elimination and/or inactivation study is critical. Whenever possible the relevant agent should be included. If the use of relevant agents does not demonstrate a wide range of properties where required, then validation should be performed with model agents.
C.2.2 Model agents for elimination and/or inactivation studies should be chosen to represent as closely as possible the relevant agents which may contaminate the product and to represent as wide a range of physico-chemical properties as possible in order to test the ability of the process to inactivate agents. The choice of the model agents should also be influenced by the quality and characteristics of the source material and the manufacturing process.
C.2.3 Unless otherwise justified, where two possible agents could be used for an elimination and/or inactivation study of a particular step either because of their equal resemblance to possible contaminants or similarities in their properties, the more resistant should be chosen.
C.2.4 Consideration should be given to the continued recoverability of the model agent during the inactivation study.
C.2.5 Where possible the choice of the agents should be such that the agents can be grown to high titre.
C.2.6 There should be an efficient, sensitive and reliable assay for the detection of the chosen agents, before and after processing through a manufacturing step.
C.2.7 Consideration should be given to health hazards which certain may pose to the personnel performing the validation studies, and to the use of suitable protective measures.
C.2.8 Examples of viral models which have been used or have been recommended for use in inactivation validation studies are given in table C.
C.2.9 Examples of transmissible agent models which have been used or have been recommended for use in inactivation validation studies are scrapie strains (263K, 1 39A, 22C, ME7, 87A).

C.3 Design and implications of elimination and/or inactivation studies

C.3.1 General

Elimination and/or inactivation studies involve the deliberate addition (spiking) of an agent at various production manufacturing steps and measuring the extent of the inactivation during the subsequent individual manufacturing step or manufacturing steps. It is not necessary to validate each individual manufacturing step of a manufacturing process. Only those manufacturing steps which are likely to or could conceivably contribute to elimination and/or inactivation of agents need to be subject to an elimination and/or inactivation study. In the case where a process has a number of small stage reductions the validation of the overall process may be necessary (see C.3.5).

Careful consideration should be given to the exact definition of an individual manufacturing step.

The deliberate introduction of any agent into the production facility should be avoided. The validation should be conducted in a separate laboratory equipped for virological work usually on a scaled down version of the production process and performed by suitable qualified and experienced staff.

C.3.2 Design of the study

C.3.2.1 Consideration should be given to the minimum detectable quantity of agent which can be assayed reliably (see Annex F).

C.3.2.2 The effectiveness of inactivation/elimination of agents will depend on the structure, size and shape of the material and on their distribution in it. The study should be designed to take this into account.

C.3.2.3 The amount of agent added to the starting material for the production step which is to be studied should be as high as possible in order to determine the capacity of the production step adequately. However, the volume of the added suspension containing the agent should not exceed 10 % of the total product to be spiked so that the test sample remains similar in composition to the production material. Calculated reduction factors should be based on the agent which can be detected in the spiked starting material and not on the amount of agent added.

C.3.2.4 If possible, agent in samples from model experiments should be titrated without further manipulations such as ultra-centrifugation, dialysis or storage. Where further treatments are unavoidable, e.g. to remove or neutralize inhibitors or toxic substances, or storage for a period to ensure that all samples are titrated together, appropriate controls should be included to determine what effect the procedures have on the result of the study e.g. dilution effects. Effects of the sample on the detection system, including toxic effects, should be recorded as they influence the limits of detection.

C.3.2.5 Whenever possible kinetics of inactivation of agents should be obtained in order to measure the slope of the curve and to determine the theoretical time necessary to inactivate the total agent population. Inactivation studies should be planned in such a way that samples are taken at different times allowing an inactivation curve to be constructed. Often virus inactivation has a fast initial phase followed by a slower phase. For the characterisation of the inactivation process in particular the study of the second phase is important (see C.4.1c).

C.3.2.6 In case of elimination, e.g. reduction of agent infectivity by partition into precipitates or removal of certain fractionates, the sample which is removed should always be studied as well. A balance of the distribution of agent in the different fractions should be drawn up wherever possible.

C.3.2.7 The method of quantitative infectivity assay of agents should have adequate sensitivity and reproducibility and should be performed with sufficient replicates and controls to ensure adequate statistical accuracy of the result (see Annex E).

C.3.2.8 The validity of the log reduction achieved should be established from investigation of the effects of variation in critical process parameters used to set in-process limits.

C.3.3 Culturing model agents
Viral model agents used to validate the inactivation process are preferably produced in cell cultures where these are available. The cell culture system chosen should not alter the properties of the model agent.

C.3.4 Conduct of cell culture tests
C.3.4.1 Model viruses used to validate the inactivation processes are preferentially tested in cell cultures. A permissive cell model for testing the inactivation potential of the different manufacturing step(s) should be used.
C.3.4.2 Intracellular viruses are usually more difficult to inactivate than extracellular viruses. The permissive cell approach allows testing the effectiveness of the processing parameters in inactivating both intracellular and extracellular viruses.
C.3.4.3 The testing should be extended to the point where no infective agents can be recovered from the cell cultures infected with the selected virus model agents.

C.3.5 Reduction factors
C.3.5. The objective of the elimination and/or inactivation study is to identify steps effective in the inactivation and/or elimination of agents and to obtain a measure of the overall capacity of the manufacturing process to inactivate/remove them. An overall reduction factor is generally expressed as the sum of the individual factors. However, a simple summing of the individual reduction factors may not be justifiable, especially in the case of agents for which the resistance characteristics are not fully known. Reductions in agent titre during a process step of the order of 1 log or less are considered to be negligible and should be ignored. Manufacturers should differentiate effective steps from process steps which may contribute to removal but upon which less reliance can be placed. Consideration should also be given to whether agents surviving one -step would be resistant to a subsequent step or alternatively have increased susceptibility. For example, it has been shown that formaldehyde treatment of transmissible agents enhanced their resistance to heat. In general, a single step having a large effect gives more assurance of safety than several steps having the same overall effect.
C.3.5.2 For all agents, manufacturers should justify the acceptability of the reduction factors obtained. An overall testing of the manufacturing process should be taken into consideration, where appropriate, to substantiate the overall capacity of the process as regards inactivation and/or elimination of TSE agents (test of the product after the process run following addition of a spike with maximum contamination to the starting material).

C.4 Elimination and/or inactivation study limitations
C.4.1 Elimination and/or inactivation studies are useful in contributing to the risk analysis and risk management to assure that an acceptable level of safety is established for the medical device. However, a number of factors in the design and execution of elimination and/or inactivation studies may lead to an incorrect estimate of the ability of the process to eliminate and/or inactivate agents. These factors include the following:
a) different laboratory strains of virus may differ in their sensitivity of the same treatment;
b) if agent preparations used to validate an elimination and/or inactivation step are produced in tissue culture, the behaviour of tissue culture derived agent in the elimination and/or inactivation study may be different from that of the native agent (for example if native and culture agents differ in purity or degree of aggregation);

c) the ability of the overall process to reduce infectivity is often expressed as the sum of the logarithm of the reductions of each step. This is a useful way of calculating the overall reduction factor, though there are some situations in which it may not be valid to add logarithmic reductions, {e.g. where reduction depends on agent adsorption by a matrix),
d) inactivation of agent infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that agents surviving a particular inactivation step may be more resistant to subsequent steps. As a consequence, the overall reduction factor is not necessarily the sum of reduction factors calculated from each individual step, using each time a fresh agent inoculum. For example, if the resistant fraction takes the form of agent aggregates, infectivity may be resistant to a range of different treatments like fixation and sterilization;
e) If the inactivation curve is atypical by comparison to historic data this should be given particular consideration.
f) the expression of reduction factors as logarithmic reductions in titre implies that, while residual agent infectivity may be greatly reduced, it will never be reduced to zero.
C.4.2 Model scaled down processing is likely to differ from full scale processing despite care taken to design the scaled down process (see Annex B).
C.4.3 In certain circumstances antibodies to a native agent may be present in the material. This may affect partition of the agent or its susceptibility to inactivation; but it may also complicate the design of the study by neutralizing the infectivity. The appropriateness of the study design may be difficult to judge. The level of antibody present may be considered as a significant process variable.
C.4.4 Small differences in production parameters such as protein content or temperature can produce large differences in the reduction of agent infectivity by whatever mechanism. Worst case conditions should be applied during the validation studies.

Appendix 5 Literature review: Inactivation and/or elimination of viruses from donor skin

INTRODUCTION

In order to enable an assessment of the risk of virus transmission by glycerol-preserved skin allografts, several questions need to be asked and answered:

1 Which viruses can be present in skin tissue?
2 Is there a risk of transmitting these viruses by skin transplantation?
3 Are there any data on the virucidal effect of glycerol?
4 To what extent is glycerol able to penetrate the skin cells and after how much time is an equilibrium concentration reached?
5 Is the available information sufficient to assess the potential of the actually applied protocol to prevent the transmission of viruses?
6 If so, is the (residual) risk of virus transmission acceptable?
7 If not, what additional research is needed to fully evaluate the potential of the actually applied protocol to prevent the transmission of viruses?

The literature search was based primarily on searches in the Medline database supplemented with cross-references from the publications that were identified in these searches. Publications were selected from peer-reviewed journals and books, thus accounting for their scientific quality. Relevance of the publications was determined by checking against the above mentioned questions. In view of the subject it was assumed that there was no need to search other databases besides the Medline.

1 THE PRESENCE OF VIRUSES IN SKIN TISSUE

A search in the Medline database revealed that a broad range of viruses can be present in skin tissue and/or in skin-associated cells. Therefore it was deemed not necessary and beyond the scope of this report to try and draw up an exhaustive list. If the need for additional virus inactivation studies is identified, it can be anticipated that a number of model viruses will be chosen, since it is not feasible to do the studies for any virus that might be expected. The most relevant viruses regarding the risk of disease transmission will be identified in Chapter 2. In order to provide some insight in the variety of viruses that can be present in the skin, Table 1 lists a number of randomly chosen examples.

Table 1: Viruses reported to be present in skin tissue (non-exhaustive list)

virus	comments	ref.
HIV		[1,2]
cytomegalovirus		[3]
HTLV-1		[4,5]
Human papillomavirus		[6,7]
Epstein-Barr virus	associated with T-cell lymphomas / amyloidosis	[8,9]
Human Herpesvirus-8	associated with AIDS-related Kaposi's sarcoma	[10,11]
Leishmaniavirus	diagnostic marker of Leishmaniasis	[12]
Herpes Simplex virus		[13,14]
Parvovirus B19		[15]
rabies		[16]

2 RISK OF VIRUS TRANSMISSION BY SKIN TRANSPLANTATION

2.1 General

As was reported in Chapter 1, a variety of viruses can be present in skin tissue. Therefore transmission of viral disease by skin transplantation is certainly a possibility. Infectious disease transmission through transplantation has been confirmed for a number of organs and tissues [17,18]. Organs can transmit HIV, Hepatitis B and C, cytomegalovirus, Epstein-Barr virus, Herpes simplex virus and Parvovirus. From several tissues like bone, cornea and heart valves infections by HIV, Hepatitis B and C, cytomegalovirus, Herpes simplex virus and rabies were reported. For skin, however, the only viruses that were reported to be transmitted through transplantation, are HIV-1 [19] and cytomegalovirus [3]. This shows that a great part of the risk is dealt with by the application of stringent donor selection criteria and serology testing [EATB]. Nevertheless, as stated by Kearney [ski-84], even this combination may not be sufficient, and therefore, where appropriate, inactivation and/or elimination processes for viruses should be applied. This statement is supported - at least regarding the risk of HIV - by several reports on HIV transmission from seronegative donors [21-23]. Most of these cases are attributed to the existence of a 'window phase' during which a person is infectious, but cannot be identified, yet, with the current detection methods. The risk of HIV transmission is further discussed in paragraph 2.2.

For other viruses that are tested for by serology (HBV, HCV, HTLV) 'window phases' exist as well. Even though transmission of these viruses has not been reported yet, the risk of their transmission should also be taken into account if virus inactivation studies are set up for validation of the method. Cytomegalovirus (CMV) is not tested for routinely, despite the fact that transmission by skin transplantation has occurred. The reason for this situation is that firstly CMV is not recognised as a virus that significantly affects patient clinical outcomes, and secondly the exclusion of CMV-positive donors would exacerbate the already existing shortage of skin allografts since the majority of healthy adult prospective donors have CMV antibodies [18,24]. For immunesuppressed individuals like organ donors a high mortality and morbidity

rate from transplant transmitted CMV is known to exist. However, the natural immunosuppression associated with burns does not appear to lead to CMV related problems in skin allograft recipients.

2.2 Risk of HIV transmission

HIV transmission by organ and tissue transplantation is well documented [17,18,22,25-29]]. A review by Simonds [29] identifies infection following transplantation of kidney (n=50), liver (n=13), heart (n=6), pancreas (n=1), bone (n=4) and skin (n=1). In 14 cases transplantation occurred after routine donor screening for HIV antibody was introduced. In the case of transmission by skin the transplantation was performed before the serology results were available [19]. Further, the skin was unprocessed. Simonds [29] and Asselmeier et al. [30] report that the transmission risk appears lower for processed (e.g. irradiated, lyophilised or treated with ethanol) or avascular tissues.

As far as the specific situation of skin is concerned, there is certainly a risk for HIV transmission if no inactivation procedure is carried out. A review by Pirnay et al. [1] describes a series of sometimes conflicting reports on the possibility of in vivo infection of skin cells by HIV. It has been confirmed that HIV is very frequently present within the skin during the course of HIV infection [31]. Kalter et al. [32] demonstrated viral DNA in the dermis of 26 out of 28 patients, but found that epidermal Langerhans cells are not principal reservoirs of HIV. Blauvelt and Katz [2] acknowledge that Langerhans cells and possibly other skin cells are targets for infection with HIV as claimed for instance by Näher et al. [33], but they also conclude that the skin is not a major viral reservoir in HIV-infected individuals. Although they may be right, a recent study by Gala et al. [34] demonstrated the potential viral infectivity of fresh or stored post-mortem samples skin in HIV infected patients.

In summary, it may be concluded that transplantation of unprocessed skin bears a risk of transmission of HIV, even after application of donor selection criteria and serology testing. The risk reduction achieved by processing the skin in glycerol will be discussed in Chapter 5.

3. VIRUCIDAL EFFECT OF GLYCEROL

3.1 General

For ten years now, the Euro Skin Bank in the Netherlands has investigated the antiviral effect of glycerol as used in donor skin preservation. In their first studies [35-37], two model viruses were used: Herpes simplex virus (HSV), which has an envelope comparable to HIV, and Poliovirus (PV), as an example of a small, hard-to-inactivate virus without an envelope. Experiments were carried out at three different temperatures (4°C, 20°C, and 37°C) with two different concentrations of glycerol (85% and 98%).

The results showed that with 98% glycerol both HSV and PV were immediately (samples taken on day 0 directly after start of incubation) completely inactivated at both 4°C and 20°C. With 85% glycerol HSV was inactivated after 50 days, 8 days and 2 hours at resp. 4°C, 20°C, and 37°C; PV was not inactivated at 4°C, but at 20°C and 37°C it was inactivated after 22 days, resp. 24 hours.

On the basis of these results the protocol used by the Euro Skin Bank was determined. It is described by De Backere [38] as follows: transport in 50% glycerol to the Euro Skin Bank, where it is incubated in 70% glycerol for 3 hours at 33°C, followed by incubation in 85% glycerol for 3 hours at 33°C.
According to Van Baare et al. [37] skin preserved in 98% glycerol can be difficult to rehydrate and is inferior for transplantation purposes. This is probably the reason why 85% glycerol is used instead of the much stronger virucidal 98% glycerol. An earlier report by the same group, however, states that allograft skin lyophilised in 98% glycerol is an effective overlay for widely expanded autografts [39]. Also Basile reports no problems in rehydrating porcine skin that had been preserved in 98% glycerol [40]. A study by Ghosh et al. [41] compared methods of sterilisation of allograft skin. They concluded that a treatment with 98% glycerol routinely gave the most consistently normal-appearing dermal matrix as compared with ethylene oxide sterilisation or irradiation (treatment with 85% glycerol was not investigated).

A comment to the above described research was made by Ghosh and Freedlander [42]. They conclude that on the basis of the data as shown in the study by Van Baare et al. [37] the protocol that is applied by the Euro Skin Bank will not be effective (see above reported time/temperature combinations to inactivate PV). Furthermore they state correctly that the study only investigated the effect of glycerol on extracellular viruses, which may not parallel the situation concerning intracellular viruses.
The authors from the Euro Skin Bank respond to this that, firstly, Ghosh and Freedlander misinterpreted the protocol (twice for 3 h. at 33°C instead of once). Secondly, they estimate their procedure to give a reduction of 7 ^{10}log-units, which they claim should be adequate. Thirdly, they point out that the extracellular situation was chosen in order to be able to experiment with titres that are very much higher than in any possible situation with donor skin.

In 1995 Marshall et al. (including Ghosh and Freedlander) reported the results of investigation of a model studying the effect of glycerol on the inactivation of intracellular viruses [43]. They used the same two model viruses as in the above described research on extracellular viruses: HSV and PV.
Their results show that with 85% glycerol neither HSV nor PV could be fully inactivated at 4°C within 4 weeks; at 20°C HSV was inactivated within 1 week, but PV could not be fully inactivated within 4 weeks. With 98% glycerol at 4°C HSV was inactivated, but PV could not be fully inactivated within 4 weeks; at 20°C both HSV and PV were inactivated within 1, resp. 2 weeks. Unfortunately they did not perform experiments at higher temperatures.
On the basis of these results they recommend that allograft skin be exposed to 98% glycerol for a minimum of at least 4 weeks at a minimum temperature of 20°C before clinical use.

3.2 Effect of glycerol on HIV

Several reports show that HIV can be remarkably resistant under certain circumstances. This is contrary to what is generally thought of viruses with a lipid envelope like HIV [44]. Especially when attached to surfaces (either organic/cellular or metal/plastic/glass/etc.) the virus is hard to

inactivate, even when completely dried out [44-47]. Reports on the effectiveness of various chemical disinfectants, heat, gamma rays and UV-light were made by Spire et al. [48,49] and the procedures used in the preparation of factor VIII in relation to their inactivation effect on AIDS-associated retroviruses were discussed by Levy et al. [50].
In 1995 a study was performed by Bujia et al. in order to determine the influence of different chemical preservation procedures on the presence of HIV DNA in stored allograft tissues [51]. HIV-infected tissues were obtained from several patients and examined using the RT-PCR (reverse transcriptase polymerase chain reaction) technique. After chemical treatment with Cialit, Merthiolate and formaldehyde the presence of HIV was still observed in all examined tissues. Unfortunately, neither skin as a tissue nor glycerol as a preservation medium were included in the research. The results do, however, indicate that HIV in allograft tissues is not easily inactivated.

Still, the question whether HIV is inactivated in the ESB process had not been addressed directly. Therefore a study was set up by ESB in cooperation with the AIDS Pathogenesis Research Unit of the Macfarlane Burnet Centre for Medical Research in Victoria, Australia. Preliminary results were reported earlier [52,53], and the full studies are now available [54,55].
Both the effects of glycerol preservation and cryopreservation on inactivation of HIV-1 were investigated. The studies comprised experiments on both extracellular and intracellular virus, as well as infected split skin grafts. The extracellular virus was readily inactivated within 30-60 minutes in glycerol concentrations of 70% and 85% even at 4°C. Intracellular virus was more difficult to inactivate. Treatment with 70% or 85% glycerol at 37°C for up to 4 hours markedly reduced but did not totally eliminate infectious cells. For both intracellular and extracellular virus the authors have assessed the individual steps of the glycerolisation procedure and then claim a cumulative reduction in titre of the sum of these effects. This is not allowed in virus inactivation studies. Titre reductions of different steps in a procedure may only be added if the inactivation in the individual steps occurs by different mechanisms. Therefore the claimed cumulative titre reductions of $10^{10.76}$ for extracellular virus and $10^{5.9}$ for intracellular virus may give a false sense of security. According to the guidelines for virus inactivation studies [56] this should be changed in $10^{4.5}$ and $10^{2.3}$ respectively.
Experimentally infected skin was tested after storage in 85% glycerol at 4°C without being subjected to elevated temperatures. HIV-1 could consistently be recovered for up to 5 days. Experiments at elevated temperatures showed that the influence of the temperature is very important. If the glycerolisation procedure was carried out at room temperature, residual infectivity after the complete procedure was found. Only if a temperature of 37°C was used, complete inactivation of the virus was shown after the second stage (3 hours in 70% glycerol).
The cryopreservation procedure did not result in any inactivation of extracellular or intracellular HIV-1 virus, nor of the infected skin samples.

4 INTRACELLULAR PENETRATION OF GLYCEROL

As was already recognised by Marshall et al., [43] viruses can be found intracellularly. Therefore it is not enough to establish the inactivation effect of glycerol on extracellular viruses. It is also very important to know if the inactivating agent actually reaches the virus inside the cell in an effective concentration inside the cell.
Actual measurements of the intracellular glycerol concentration in glycerolised skin have not been reported yet. Therefore the question cannot be answered directly, but there are several reports that are noteworthy in this respect.

A lot of data on the influence of glycerol on skin were gathered from literature by Mast [57]. For example, the in vitro epidermal water permeation was determined in a study comparing rat, pigeon and lizard skin. It was found that glycerol treated skin shows negligible water permeation. [58].
Very interesting are the observations of Ackermann and Flynn [59]. They determined the permeation coefficient of glycerol for full thickness nude mouse skin and the dermis of nude mice. For full thickness skin a very low coefficient was found: 1.4×10^{-4} cm/h. For the dermis alone, however, a coefficients 0.4 cm/h was found. With a thickness of 0.3 mm of the human donor skin, and assuming that these permeation coefficients are applicable to the situation during the ESB glycerolisation process, this would mean that the dermis is permeated (not necessarily saturated) after 4.5 minutes, but for the epidermis 214 hours are needed.
Glycerol, although in a low concentration (10-15%), is also used as a cryoprotectant in cryopreservation of skin [60,61]. Mast states that, given the fact that glycerol needs to enter the cell in order to be effective as a cryoprotectant, sufficient pre-treatment time must be allowed, as it does penetrate slowly [57]. Probably this is also valid when high concentrations of glycerol are used.

Interestingly, Basile claims that glycerol dehydrates tissue by replacing most of the intracellular water [40]. This statement, however, is not theoretically or experimentally supported, and also the time this process takes or the intracellular glycerol concentration that is reached are not specified.

5 DISCUSSION AND CONCLUSIONS

In the introduction several questions were formulated that need to be answered in order to be able to assess the risk of virus transmission by glycerol preserved skin allografts. In Chapters 1-4 the first four questions were answered. The remaining questions will be answered here.
It has been established that a range of viruses can be present in skin tissue. In theory all of these viruses can be transmitted by transplantation. Documented transmissions by organ and tissue transplantation led to the conclusion that in practice the risk of virus transmission by processed (e.g. irradiated, lyophilised or treated with ethanol) tissues is lower than for organs and unprocessed tissues. Case reports of virus transmission by skin allografts exist solely for CMV and HIV. In the case of HIV transmission unprocessed skin was used, the study on CMV

transmission did not report skin processing, but it was not explicitly stated that the skin was used unprocessed. CMV transmission is judged an acceptable risk, given the fact that most of the prospective donors are CMV-positive and the consideration that CMV is not recognised as a virus that significantly affects patient clinical outcomes. Only immunesuppressed patients are at considerable risk to experience adverse effects from a CMV infection. HIV transmission is obviously not an acceptable risk. This risk is managed partly by selection of donors on medical and social history and the performance of serology testing of prospective donors. In view of the existence of a window phase additional risk management by processing of the skin is desirable. Although other viruses have hitherto not been reported to be transmissible by skin transplantation, the risk of their transmission cannot be excluded. Moreover, emerging infectious diseases, which are currently seen as a public health problem, could introduce new viruses. In case virus validation studies will be carried out, this should therefore be done with viruses that span a wide range of (physicochemical) properties.

Regarding the virucidal effect of glycerol, two studies have now been performed with Herpes simplex virus (HSV) and polio virus (PV), and one study with HIV-1. From the resulting data of the studies by Van Baare et al. [37] and Marshall et al. [43] with HSV and PV as model viruses, it is difficult to determine the effectiveness of the actually applied protocol at the Euro Skin Bank. The exact protocol (combinations of time/temperature/glycerol concentration) was not tested as such. The results show that none of the individual steps is sufficient for the complete inactivation of HSV and PV. In any case, it can be concluded that no decisive evidence exists for total inactivation of even the model viruses by the applied protocol.

Unfortunately Marshall et al. did not test the combination of 98% glycerol and 37°C. In view of the other results in both investigations with HSV and PV, it is quite possible that a short processing time (hours) under these circumstances is sufficient to inactivate the model viruses both extracellularly and intracellularly. On the other hand, this would still not answer the question of inactivation of the viruses in infected skin. The third study (with HIV-1) [54,55] demonstrated that extracellular virus is more readily inactivated than intracellular virus, which in turn is more effectively inactivated than the virus in infected skin.

This observation is consistent with the data in Chapter 4. Actual measurements of the penetration of glycerol into the skin cells have not been reported. It is, however, known that glycerol negatively affects the permeation of other substances through the skin. Also the permeation coefficient of glycerol has been measured: only the dermis is permeated with reasonable velocity. From these data, it can be concluded that glycerol does penetrate the skin cells, but only at a slow speed and until an unknown equilibrium concentration. Sufficient pre-treatment and processing time is therefore required. A comparison between the experimental conditions used during the laboratory study and the actual processing at the ESB should be performed, in order to identify factors that would influence glycerol penetration, e.g. characteristics of the sample, process scale, etc.

From the results in the study with HIV it seems likely that this virus is effectively eliminated and/or inactivated by the glycerolisation process as it was tested. Unfortunately, however, there is a discrepancy between the temperature used in the ESB protocol and the temperatures included in the investigations: 33°C versus 37°C (“for laboratory convenience”). The same problem is also encountered in assessing the results of the studies with the model viruses HSV

and PV. This means that interpolation between the results found at 20°C and 37°C is necessary. At room temperature residual infectivity of HIV was determined, indicating that the influence of the temperature is critical. If further experiments are conducted this problem should be avoided. Another solution to this problem, which seems preferable, would be to adapt the ESB protocol and process the skin at 37°C. The critical influence of temperature also leads to the strong recommendation that the temperature profile of the skin samples during actual processing at the ESB should be determined. Judging from the oral description of the procedure, there is a definite possibility that the desired temperature of 33°C is not reached in the sample.
In conclusion, the studies that have been performed do not yield conclusive evidence that the ESB glycerolisation procedure yields virologically safe donor skin. The relevant (i.e. used by ESB) time/temperature/concentration combinations have not been shown to be effective against HIV or other viruses. Additional virus validation studies are required to achieve this.

The ESB states that 98% glycerol, which has been demonstrated to be much more effective in virus inactivation than 85% glycerol, yields skin that is inferior for transplantation. In order to obtain optimally preserved skin, while still profiting from the stronger virucidal action of the highest concentration, it might be possible to introduce a processing step with 98% glycerol, and subsequently store the skin in 85% glycerol. Experiments using this procedure have not been reported yet. Furthermore, their statement about clinical inferiority might need to be re-evaluated, given the reports of other groups on the applicability of 98% glycerolised skin.

As long as it is not validated that viruses that are present in the skin are inactivated during the preservation procedure, the rules of the Berlin Burn Centre are certainly worth considering [62,63]. They quarantine the skin until recipients of other organs from the skin donor have been shown to be seronegative three months after their transplantation. Perhaps the three months period should even be extended in order to be sure. Since glycerolised skin can be stored for at least two years this should be possible, although all sorts of practical and legal problems are obviously connected to this protocol.

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Appendix 6 Literature review: Inactivation and/or elimination of Transmissible Spongiform Encephalopathy (TSE) agents from donor skin

INTRODUCTION

In order to enable an assessment of the risk of TSE agent transmission by glycerol-preserved skin allografts, several questions need to be asked and answered:

1 Can TSE agents be present in skin tissue?
2 Is there a risk of transmitting TSE agents by skin transplantation?
3 Are there any data on the effect of glycerol on TSE agents?
4 To what extent is glycerol able to penetrate the skin cells and after how much time is an equilibrium concentration reached?
5 Is the available information sufficient to assess the potential of the actually applied protocol to prevent the transmission of TSE agents?
6 If so, is the (residual) risk of TSE agents transmission acceptable?
7 If not, what additional research is needed to fully evaluate the potential of the actually applied protocol to prevent the transmission of TSE agents?

The literature search was conducted based primarily on searches in the Medline database supplement with cross-references from the publications that were identified in these searches. Publications were selected from peer-reviewed journals and books, thus accounting for their scientific quality. Relevance of the publications was determined by checking against the above mentioned questions. In view of the subject it was assumed that there was no need to search other databases besides the Medline.

1 THE PRESENCE OF TSE AGENTS IN SKIN TISSUE

In the tissue classification that is generally used in risk assessments regarding the transmission of TSE agents (or prions), skin is listed in Category IV - no detectable infectivity [1]. This classification is based on titration of (scrapie) infectivity in mice by the intracerebral route, which is the most efficient route of transmission. It is generally accepted that this classification is also applicable to human tissues. The fact that skin is listed in Category IV means that the infective form of the prion PrP^{Sc} has not been found in skin tissue.
On the contrary, the normal cellular prion-related protein PrP^{C} has been demonstrated to be present in squamous epithelia of normal and diseased skin [2]. In inflammatory skin diseases like psoriasis and contact dermatitis, as well as in squamous cell carcinomas and viral warts, PrP^{C} was strongly present in both keratinocytes and infiltrating mononuclear cells. In normal skin only little expression of PrP^{C} was detected.

In principle, the presence of PrP^{C} is not a problem. It is a protein that, like any other protein in the human body, is constantly being formed and then decomposed again. However, if it is transformed into a PrP^{Sc}, enzyme digestion is no longer possible. This transformation can occur spontaneously (in very rare cases) or it can be induced by the presence of another PrP^{Sc} molecule. Because of the latter mechanism prions are considered infectious. Therefore the presence of PrP^{C} in skin tissue must be considered relevant information. If sufficient accumulation of PrP^{Sc} takes place, morbidity of the tissue will result. In people (or animals) suffering from prion diseases, this eventually fatal phenomenon is predominantly seen in brain tissue.

2 RISK OF TRANSMISSION OF TSE AGENTS BY SKIN TRANSPLANTATION

It has been established that TSE's can be transmitted by transplantation of tissues. From the known cases of iatrogenic CJD, 80 cases have been attributed to dura mater grafts and 3 to a corneal transplant [3]. These tissues are closely related to the central nervous system, in which high levels of infectious agent can be present.
Iatrogenic CJD through skin transplantation has never been reported. However, in view of the fact that PrPC can be present in the skin (see Chapter 1), there is a theoretical possibility that infection might take place via the skin. In fact, Taylor has reported the effective transmission of prion diseases via inoculation in the skin in rodents [4]. It has also been hypothesised that the prion disease Kuru in New Guinea was not only transmitted by oral exposure to the brains of deceased relatives (a cannibalistic ritual), but that exposure via skin lesions during the preparation of the "food" may also have played a role [5-6].
In theory, if prions from contaminated material would penetrate into the epidermis, which is possible via the broken skin barrier in burn wounds, they would be able to start the conversion of PrPC in the skin. Skin-associated lymphoid tissue could then play a role in the further propagation of prions to lymphoid organs and to the CNS.
Through the inverse route, infected individuals may also have prion infectivity in the skin, albeit probably in very low concentrations. An important difference with other organs and tissues, where PrPSc accumulates in cells and intracellular spaces, is that the epidermis is constantly self-renewing and sheds terminally differentiated cells. Therefore a significant accumulation of infectivity will probably not take place, especially if the fact is taken into account that in normal skin little expression of PrPC is expected [2], whereas people with skin diseases (and thus possibly a high expression of PrPC) are excluded as skin donors. This means that even in case PrPSc reaches the skin, only very small doses of infectivity will presumably be present.

In theory glycerol itself also represents a risk factor regarding the possible transmission of TSE agents through skin transplantation. This product is manufactured from tallow, which in turn has been prepared from animal materials, mostly from bovine/ovine origin. This means that glycerol could theoretically be contaminated with TSE agents. Since it is almost certain that the new variant of CJD (nvCJD) has originated from Bovine Spongiform Encephalopathy (BSE) [7-11], this would represent a risk. However, tallow derivatives like glycerol have been the subject of

specific consideration for the Committee of Proprietary Medicinal Products (CPMP) and are thought unlikely to be infectious because of their extremely rigorous manufacturing processes [1]. The CPMP Note for Guidance has recently been adopted as a general chapter on minimising TSE risk in the European Pharmacopoeia (Ph.Eur.) [12]. Furthermore a general monograph has been adopted which implements this general chapter for all products with TSE risk, applying to all substances used in the production of medicinal products (raw materials, starting materials, ingredients), irrespective of whether a monograph for the substance is included in the Ph.Eur. [13].

Although not applicable to the glycerolised donor skin, there is another risk that should be mentioned here, because it is a closely related subject. In some cases skin defects are treated utilising cultured keratinocytes. Growth of these cells is mostly carried out in media containing bovine constituents. Since it is almost certain that the new variant of CJD (nvCJD) has originated from BSE [7-11], it should be made absolutely sure that the culture media are free from TSE agents contamination in order to prevent the possibility of accidental transmission of BSE to humans.

3 EFFECT OF GLYCEROL ON TSE AGENTS

TSE agents are more difficult to inactivate than conventional micro-organisms. A lot of studies have been carried out to assess the effectiveness of all kinds of inactivation and/or elimination processes [14-15]. Alcohols in general are considered not to be effective [16]. The effectiveness of glycerol itself has not been evaluated experimentally, but given the available information on other alcohols, it is most likely that also glycerol will not be effective against TSE agents.
Chemicals and processes which have been found effective against TSE agents include the use of hypochlorite (20,000 ppm av. Cl_2), 2 M NaOH and porous load autoclaving at 134°C for 18 minutes [16]. Such procedures would damage or even destroy the skin tissue, and therefore cannot be applied.

4 INTRACELLULAR PENETRATION OF GLYCEROL

As was already mentioned in Chapter 1 and 2, TSE agents can be found intracellularly. If glycerol had been found to be effective against TSE agents, it would therefore also be important to know if the inactivating agent actually reaches the TSE agent in an effective concentration. However, since glycerol is not likely to be effective in the inactivation of TSE agents, this question is no longer relevant in this context.
Since it is relevant in the context of the inactivation and/or elimination of viruses, however, the subject will be elaborated in the review on viruses.

5 DISCUSSION AND CONCLUSIONS

In the introduction several questions were formulated that need to be answered in order to be able to assess the risk of TSE agent transmission by glycerol preserved skin allografts. In Chapters 1-4 the first four questions were answered. The remaining questions will be answered here.

It has been established that the cellular form of the TSE agent, PrP^{C} can be present in skin tissue. However, until now the infective form, PrP^{Sc}, has not been detected in skin tissue.

Theoretically, there is a risk of transmitting TSE agents by skin transplantation. Given the presence of PrP^{C} in the skin, and the fact that it has been proven in animal models that infection through a breached skin is possible, the possibility that transmission occurs cannot be excluded completely.

There are no data on the resistance of TSE agents to glycerol. However, in view of available data about the resistance of TSE agents to alcohols, it may be assumed that glycerol is not effective in the elimination and/or inactivation of TSE agents. This means that the question whether glycerol is able to penetrate skin cells, and at which rate, is no longer relevant.

It could be argued that the lack of data on the effectiveness of the glycerol process regarding the elimination and/or inactivation of TSE agents warrants specific research into this matter. However, considering the following arguments, we think that this is neither useful nor necessary:

- It is to be expected that glycerol is not effective against TSE agents, while the studies are lengthy and expensive and may not even yield conclusive evidence.
- The risk of TSE transmission by skin transplantation is probably very small, since presumably no accumulation of infectivity takes place in the skin and the infectious dose - although not known - may thus not even be reached. In any case, currently available detection methods are not able to measure infectivity in skin tissue. Only in case an epidemic of nvCJD emerges, the risk should be re-evaluated, since there are indications that the tissue distribution of nvCJD-infectivity is different [17].
- The risk of TSE transmission is reduced significantly by the donor selection criteria. By applying these criteria people from CJD risk groups are excluded as a donor. The criteria were checked and found to be in accordance with the state of the art [16].

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Appendix 7 EN 1174-1 and the Euro Skin Bank preservation procedures

EN 1174 (1996): Sterilisation of medical devices. Estimation of the population of micro-organisms on product. Part 1: Requirements.
Clauses 4 General, 5 Selection of product, 6 Selection of technique, 7 Validation of technique, 8 Revalidation, 9 Use of technique (italics copied from original text).

4 General

4.1 Documentation

4.1.1 Documented procedures and instructions on the testing techniques to be employed and on the use and operation of all relevant equipment shall be available. These procedures and instructions shall be approved on issue and shall be controlled as specified in 4.5 of EN ISO 9001 : 1994.	See ESB Quality Manual (01/01/99) PRO 19v.01, KHB 17v.01 and Streeklab Haarlem Protocol MML 12.3 Donorhuid (12/05/95). Furthermore, the Streeklab has started implementation of a Quality System, which is defined by the branch organisation.
4.1.2 The procedures and instructions required by this Part of prEN 1174 shall be implemented effectively.	Not included in this review.
4.1.3 Calculations and data transfers shall be subject to appropriate checks.	Not included in this review.
4.1.4 Records of all original observations, calculations, derived data and final reports shall be retained as specified in 4.16 of EN ISO 9001: 1994. The records shall include the identity of all personnel involved in sampling, preparation and testing.	Not included in this review.

4.2 Personnel

4.2.1 Responsibility for bioburden estimation shall be assigned to specific personnel as specified in 4.1.2.2 and 4.18 of EN ISO 9001: 1994.	Not included in this review.
4.2.2 Training shall be performed in accordance with documented procedures. Records of the relevant qualifications, training and experience of technical personnel shall be maintained.	Not included in this review.

4.3 Equipment

4.3.1 All items of equipment required for correct performance of the specified tests and measurements shall be available.	Not included in this review.
4.3.2 All equipment requiring planned maintenance shall be maintained in accordance with documented procedures.	Not included in this review.

Records of maintenance shall be retained.	
4.3.3 An effective system shall be established, documented and maintained for the calibration of all equipment with measurement or control functions. This system shall comply with 4.11 of EN ISO 9001: 1994.	Not included in this review.
4.4 Media and materials	
Methods shall be established and documented for the preparation and sterilization of materials used in *bioburden* estimation, including appropriate quality tests.	Not included in this review.
5 Selection of product	
5.1 The procedures for selection and procurement of product for testing shall be established to ensure that the product is representative of routine production.	Ok, see ESB PRO 18v.01, WERK 04v.01.
5.2 If a specified portion of product is to be used, it shall be selected to possess micro-organisms representative of the whole product. If it has been demonstrated that the micro-organisms are evenly distributed on product, the portion shall be selected from a random location. In the absence of such a demonstration, the portion shall be made up of pieces of product from a number of random locations.	Ok, it is assumed that the bioburden is evenly distributed on a batch of harvested donor skin. Trimmings of skin pieces are selected from a number of random locations for bacteriological culturing.
6 Selection of technique	
6.1 For an identified product, factors relevant to the efficiency of removal of viable micro-organisms from product shall be considered and recorded if such removal is part of the technique. Such factors shall include: a) ability to remove microbiological contamination; b) possible type(s) of contaminating micro-organisms and their locations on product; c) effect(s) of the removal method on the viability of microbiological contamination; d) the physical or chemical nature of product to be tested.	Not applicable.
6.2 If the physical or chemical nature of *product* to be tested (see d) of 6.1) is such that substances can be released which would	Not ok. Half the volume of a container is filled with skin samples; the glycerol is replaced by broth. Whether this volume

adversely affect either the number or the types of micro-organisms detected, then a system to neutralize, remove or, if this is not possible, minimize the effect of any such released substance shall be used. The effectiveness of each system shall be demonstrated.	50/50% ratio skin/broth is adequate to establish inhibition of the sterilizing effect of glycerol has not been determined.
6.3 Culture conditions shall be selected after consideration of the types of micro-organisms expected to be present. The results of this consideration and the rationale for the decisions reached shall be documented.	Not ok. The selection of culturing conditions was based on the capability to detect micro-organisms that caused wound infection in burn patients. These considerations have not been documented.
6.4 The selected technique shall be validated as specified in clause 7.	Has not been performed.
7 Validation of technique	
7.1 Each procedure for the validation of bioburden estimations shall be documented.	Has not been performed.
7.2 The validation procedures shall consist of the following steps:	
a) assessment of the adequacy of the technique used to remove micro-organisms from the product, if such removal is part of the technique;	Not applicable.
b) assessment of the adequacy of the technique used to enumerate removed micro-organisms, including microbiological counting techniques and culture conditions; and	Has not been performed.
c) establishment of the recovery efficiency of the method used in order that the correction factor can be calculated.	Not applicable.
7.3 Any change in a routine method shall be assessed. This assessment shall include:	Not included in this review.
a) evaluation of the change;	
b) establishment of the recovery efficiency of the revised method.	
8 Revalidation	
8.1 The validation data and any subsequent revalidation data shall be reviewed periodically and the extent of revalidation determined and documented. Procedures for the review of validation and revalidation shall be documented and records of the revalidation shall be retained.	Not included in this review.
8.2 A revalidation report shall be documented. The report shall be signed by the persons designated by the same	Not included in this review.

functions/organizations that prepared, reviewed and accepted the original validation report.	
9 Use of technique	
9.1 Pre-sterilization counts shall be performed in accordance with documented sampling plan(s) with defined sampling frequency and sample size.	Pre-sterilisation counts have not been performed.
9.2 If contaminants that are not normally encountered are isolated while performing pre-sterilization counts, they shall be characterized. The potential effect of such contaminants on the manufacturing process, including the sterilization process, shall be considered and documented	Pre-sterilisation counts have not been performed.
9.3 Acceptable limits for either pre-sterilization counts or bioburden estimates shall be established on the basis of previous data and documented. If these limits are exceeded, corrective action shall be undertaken as specified in 4.14 of EN ISO 9001: 1994. Established limits shall be reviewed formally at defined intervals and revised if necessary.	Limits for pre-sterilization counts or bioburden estimates have not been defined.
9.4 The use of statistical methods to define sample size, sampling frequency and acceptable limits shall conform to 4.20 of EN ISO 9001: 1994.	Not included in this review.
9.5 If pre-sterilization counts are to be used to determine the extent of treatment of a sterilization process (unless a requirement in a standard for the validation of the particular sterilization process specifies otherwise), then: a) a correction factor, based on recovery efficiency as determined during validation (see 7.2), shall be applied to the pre-sterilization count to calculate the bioburden estimate before the extent of treatment is determined; and b) the resistance of the micro-organisms comprising the population present on product shall be considered in determining the extent of treatment.	Not applicable.
9.6 If bioburden estimates have been used to determine the extent of treatment of the sterilization process: a) consideration shall be given to the effect on the assurance of sterility if the	Not applicable.

acceptable limits are exceeded; and

b) the characterization of contaminants that are not normally encountered shall include an estimation of the resistance of those contaminants to the sterilization process.

The consequences in b) of the presence on product of contaminants with high resistance to the sterilization process on the assurance of sterility shall be considered.

All these considerations shall be documented and included in the determination of corrective action. This corrective action shall be in accordance with 4.14 of EN 9001: 1994.

9.7 Changes to product and/or processes shall be reviewed formally to determine whether they are likely to result in a change in the bioburden (see also 9.3). The results of the review shall be documented. If a potential change in the bioburden is determined, specific bioburden estimations shall be performed to evaluate the effects of the change.

Not included in this review.

Appendix 8 ISO 14160 and the Euro Skin Bank preservation procedures

ISO 14160 (1998-03-15): Sterilisation of single-use medical devices incorporating materials of animal origin - Validation and routine control of sterilisation by liquid sterilants.
Clauses 4 General, 5 Validation, 6 Process control and monitoring and 7 Product release from sterilisation.

4 General

4.1 Control of manufacturing

1. The manufacturing process shall be established and controlled to maintain the presterilization count below a specified limit.	Not ok. The presterilisation count is reduced by disinfection of the harvesting site on the donor with povidone iodine scrub, followed by chlorhexidine. Limits have not been specified.
2. A documented system shall be established and maintained to control the sourcing of raw materials of animal / human origin.	Donors are registered by BIS according to written procedures. BIS has been ISO 9000 certified for their activities.
3. The documented procedures and instructions required by this International Standard shall be implemented effectively. Documentation and records shall be reviewed and approved by designated personnel (see 4.2).	Excluded from this review. Effective implementation of procedures and instructions in ESB will be evaluated in the near future by a Certifying Agent in the process of obtaining ISO certification.

4.2 Personnel

Responsibility for the maintenance of equipment (see 4.4), for the validation (see clause 5) and routine control (see clause 6) of sterilization by exposure to liquid chemical sterilants and for the release of product shall be assigned to qualified personnel as specified in ISO 9001 or in ISO 9002.	Excluded from this review.

4.3 Calibration

An effective system shall be established, documented and maintained for the calibration of all controlling, indicating and recording instruments used for validation and routine control of the sterilization process. This system shall comply with the requirements of either ISO 9001 or ISO 9002.	Excluded from this review.

4.4 Maintenance of equipment

1. Preventative maintenance shall be planned and performed in accordance with documented procedures. The procedure for	Excluded from this review.

each planned maintenance task and the frequency at which it is to be carried out shall be specified and documented.	
2. Equipment shall not be used to process medical devices unless all maintenance tasks have been satisfactorily completed and recorded.	Excluded from this review.
3. Records of maintenance shall be retained as specified in ISO 9001 or ISO 9002.	Excluded from this review.
4. The maintenance scheme, maintenance procedures and maintenance records shall be reviewed periodically by a designated person (see 4.2).	Excluded from this review.
4.5 Process development and product compatibility	
1a Prior to the introduction of a new or altered product, package, loading pattern or sterilization process, the sterilization process to be validated shall be defined and documented.	Ok. The process is defined and documented in PRO 09v01, WERK 02v01, PRO 10v01, WERK 03v01.
1b A demonstration of equivalence to previously validated product, package or loading pattern shall be considered to meet this requirement. Any demonstration of equivalence shall be documented.	Not applicable.
2 Product and packaging shall be designed to allow contact with liquid chemical sterilant and so that residues of the liquid chemical sterilant are below levels as specified by the manufacturer. The location within the product at which sterilization is most difficult to achieve shall be identified.	Ok. Packaging in flexible, sealed bags or in containers allows contact between sterilant and product. Relevance needs to be assessed: The final product is shipped in glycerol 85%. Residues of sterilant remaining in the product after washing, before use, are not specified. Given the shape of the product (sheets of 0.2-0.4 mm thickness), sterilisation depends on diffusion speed of the sterilant.
3. It shall have been demonstrated and documented that the sterilization process does not affect adversely the fitness for use of the product or its packaging. If resterilization is to be permitted, the effects of such processing shall be evaluated and documented.	Excluded from this review. Resterilisation not permitted, single use only.
5 Validation	
5.1 General	
Procedures for validation shall be documented and records of each validation shall be retained (see 5.4.1).	Not performed.

5.2 Commissioning

Commissioning shall demonstrate that the specifications for equipment used for the sterilization process are met.	Excluded from this review.

5.3 Performance qualification

1. The performance qualification shall demonstrate that the sterilization process has: a) appropriate lethal activity against a representative range of microorganisms; b) defined processing parameters (e.g., time, temperature, liquid chemical sterilant concentration, pH) which are capable of control throughout the process.	Not performed.
2. For the performance qualification, the part of the product which is most difficult to sterilize, as defined according to 4.5.2, shall be taken into consideration during the performance qualification.	Not performed.
3. The presterilization count of the product shall be established as described in ISO 11737-1 (tech. equivalent EN 1174-1).	See review of ESB procedures against EN 1174-1 (Annex 6).
4. Before performance qualification is undertaken, a method shall be validated for the neutralization of the liquid chemical sterilant prior to culturing survivors. The method shall not in itself adversely influence the ability to interpret the results.	Not ok. Samples of product for culturing for bacteria are fully immersed in a volume of culturing broth. Whether the adhering sterilant is inactivated by this dilution has not been assessed (ISO 1174-2, 5.2.6.1).
5. The combination of conditions with the lowest microbicidal activity within the process specification shall be identified and this combination of conditions shall be used in the performance qualification.	Not performed.
6. Microbiological performance qualification shall include the following three stages:	
a) A screening test to identify micro-organisms with a high resistance to the process.	(A.4.2.3.) Not performed.
b) Determination of inactivation kinetics. This consists of the construction of log survival curves for the microorganisms identified as having a high resistance to the process. The inactivation curve shall include a minimum of 5 points covering at least a thousandfold reduction in numbers.	(A.4.2.4.1. and A.4.2.5.) Not performed.
If the product does not allow the above-mentioned procedure, the MPN method as specified in A.4.2.4.2 may be used. This shall be rationalized and documented.	Not performed.

Microorganisms shall be presented to the process on carrier material(s) representative of the medical device.	Not performed.
c) Assessment of inactivation of the microorganisms from the presterilization count as they are induced to grow onto carriers of tissue.	Not performed.
The range of microorganisms employed, in addition to isolates from the bioburden, shall include microorganisms with a known high resistance to the sterilization process and, in any event, resistance equivalent to spores of Bacillus subtilis complying with ISO 11138-1 (related to EN 866).	Not performed.
7. Within the sterilization process, the exposure time shall not be less than D[6 + log10(100 + B)] where D is the D-value of the most resistant microorganism identified during performance qualification and B is the value of the bioburden estimated as described in ISO 11737-1 (tech. equiv. EN 1174-1).	Not performed.
8. If the medical device is subjected to an aseptic transfer following the completion of sterilization process:	Not applicable.
a) processes used for the sterilization of components for manufacture (e.g. containers, storage solutions) shall be validated and routinely controlled in accordance with the appropriate International Standard;	Excluded from this review.
b) transfer procedures after exposure to the liquid chemical sterilant shall be validated in accordance with ISO 13408.	Relevance needs to assessed. Handling of skin is performed in LAF cabinets, but is not validated against ISO 13408.
5.4 Certification of validation	
1. A validation report containing the results of all validation exercises shall be documented. The report shall be signed by persons designated as responsible for preparing, reviewing and accepting this report. The validation report shall be retained as specified in ISO 9001 or in ISO 9002.	Not performed.
2. The validation report shall contain or reference the documented process specification for liquid chemical sterilization.	Not ok. A validation report has not been written, but the procedures have been defined.
The process specification shall specify the medical device for which the validation has been performed and shall detail, including	Not performed.

values and tolerances where appropriate, the following:	
a) frequency and method(s) for bioburden estimations, together with action limits;	Not performed.
b) specification for the environment in which the liquid chemical sterilant and containers are prepared, and aseptic transfers (see 5.3.8) are undertaken;	Ok. Handling of skin is performed in LAF cabinets.
c) training and certification criteria for approval of personnel to be authorized to undertake aseptic transfers;	(5.3.8.) Excluded from this review.
d) method of ensuring the absence of viable microorganisms from the liquid chemical sterilant solution(s) (seeA.6);	Not performed.
e) formulation of the liquid chemical sterilant, including the specification of its constituents;	Ok. The sterilant consists of glycerol in concentrations of 50%, 70% and 85% in saline.
f) pH of the liquid chemical sterilant;	Not performed.
g) residual activity required for the liquid chemical sterilant after the sterilization process in terms of chemical concentration and/or microbicidal activity;	Not performed.
h) specification of the exposure vessel in which products come into contact with the liquid chemical sterilant, including materials of construction, size, and details of any pretreatment to be applied;	Sterile (γ-sterilized) flexible PAPA bag, containing 1 litre of glycerol and skin, can be heat-sealed. For final packing polystyrene/polyethylene 25 ml containers are used, containing maximal 450 cm^2 skin.
i) number of products to be sterilized per unit volume of liquid chemical sterilant;	Up to approximately 8.000 cm^2 skin / liter glycerol.
j) exposure time;	Not ok. Exposure times have been defined, not validated.
k) temperature to be used for sterilization;	Not ok. Exposure temperatures (settings of incubator) have been defined, not validated.
l) any other critical process variable(s) determined during process development;	No.
m) method of sterilizing any storage solution in which the product is presented after sterilization (see 5.3.8).	Not applicable.
3. In cases where a validation for a specific device is also judged valid for other devices, the justification for this shall be documented.	Not applicable.

5.5 Revalidation

1. The validation and any subsequent revalidation data shall be reviewed at least annually and a rationale shall be prepared and documented whether or not revalidation is required.	Excluded from this review.
A revalidation exercise shall be undertaken unless sufficient data have been generated to	

demonstrate the continued appropriateness of the sterilization process. Procedures for the review of validation and revalidation data shall be documented and records of revalidation shall be retained.	
2. A revalidation report shall be documented. The report shall be signed by the persons designated by the same functions / organizations that prepared, reviewed and accepted the original validation report.	Not performed.
6 Process control and monitoring	
1. At stipulated intervals, the bioburden shall be estimated as described in ISO 11737-1 (EN 1174-1). If a microorganism that has not been studied in the original performance qualification is isolated during routine estimation of the presterilization count, the exercise in 5.3.5 shall be performed with this microorganism.	Not performed.
2. Data shall be recorded and retained for each batch of sterilized product to demonstrate that the sterilization process specification has been met. These data shall include at least the following:	Culturing data are recorded from each donor and retained for an indeterminate period.
a) variables monitored during sterilization of final container(s), if appropriate	Not applicable.
b) variables monitored during sterilization of storage solution, if appropriate	Not applicable. Non-sterile glycerol of Ph.Eur. quality is used.
c) initial chemical concentration(s) and pH of liquid chemical sterilant	Glycerol of Ph.Eur. quality and known concentration is used. No pH control.
d) parameters monitored during preparation of liquid chemical sterilant	None.
e) results of integrity tests on any filters used to sterilize solutions, if appropriate	Not applicable.
f) exposure time	Is recorded.
g) temperature during the exposure time	Not monitored.
h) results of environmental monitoring during aseptic transfer, if appropriate	Performance evaluation of the LAF cabinets is claimed to be part of the maintenance plan.
i) identities of personnel who are	
1) preparing storage solutions and liquid chemical sterilant solutions;	At the moment by ESB personnel.
2) controlling the sterilization process	Not applicable.
3) performing aseptic transfer, if appropriate.	
j) numbers (or other unique identification) of products processed	Yes, Donor Identification Number.
3. For each batch of medical devices subjected to liquid chemical sterilization, the following shall be examined for the presence	

of viable microorganisms:	
a) chemical sterilant solutions;	Not performed.
b) storage solutions, if applicable;	Not performed.
c) at least one of the following:	
1) finished product;	Not applicable.
2) product which has been rejected but subjected to the complete manufacturing process; or	Not applicable.
3) isolated pieces of animal / human tissue, justified as being representative of the medical device, which have been subjected to the complete manufacturing process.	Ok.
4. For each batch of medical devices, a portion of either:	To be discussed with the ESB. When the ESB concludes that "it is not appropriate to introduce test organisms into the sterilization vessel or manufacturing environment" (see Clause 6.5 b), then this Clause 6.4 is not applicable and Clause 6.5 applies.
a) liquid chemical sterilant solution, or	
b) liquid chemical sterilant solution remaining following the sterilization process shall be challenged under the same conditions as the batch of medical devices by a carrier of the animal / human tissue inoculated with a microorganism complying with ISO 11138-1 and containing at least 106 organisms with a known high resistance to the sterilization process, as identified during performance qualification.	
5. If a bulk liquid chemical sterilization process is used for which it is not appropriate to introduce test organisms into the sterilization vessel or manufacturing environment; and	Not performed.
a) the relationship between the complete chemical composition of the liquid chemical sterilant and microbicidal activity of the liquid chemical sterilant has been demonstrated; and	Not performed.
b) the sterilization process has been applied for a significant period for at least 30 batches of medical devices during which time no failures have been detected in accordance with 6.4, then testing specified in 6.4 may be discontinued and complete chemical analysis capable of demonstrating compliance with the limits of the process specification shall be performed on the liquid chemical sterilant remaining at the end of the sterilization process.	Not performed. Complete chemical analysis of the liquid chemical sterilant remaining at the end of the sterilization process is not performed.

6. The results from 6.2, 6.3 and either 6.4 or 6.5 shall form part of the evidence permitting release of product as sterile and shall be retained as part of the sterilization records.	Not completely performed.
7. All records shall be retained as specified in ISO 9001 or in ISO 9002.	ESB retains all records.
7 Product release from sterilization	
1. The criteria for designating a given sterilization process as conforming shall be documented. These criteria shall include:	
a) conformance to the process specification(s); and	Excluded from this review.
b) no growth in microbiological testing (see 6.3 and 6.4) following incubation.	Ok. PRO 19v01 (Bacteria).
2. A given sterilization process shall be considered as non-conforming and non-conforming product shall be handled as specified in ISO 9001 or in ISO 9002 if:	
a) a process variable is outside the documented tolerances; or,	Ok. PRO 17v01.
b) any microbiological test (see 6.3 and 6.4) shows growth following incubation.	PRO19v01. However, the procedure has not been defined in case all bacteriological samples have been used.