(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 March 2006 (23.03.2006)

PCT

(10) International Publication Number
WO 2006/029467 A1

(51) International Patent Classification[7]: F26B 5/06, 25/22

(21) International Application Number:
PCT/AU2005/001408

(22) International Filing Date:
15 September 2005 (15.09.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2004905354 16 September 2004 (16.09.2004) AU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/029467 A1

(54) Title: RAPID FREEZE DRYING PROCESS

(57) Abstract: A process for rapid freeze drying a sample including freezing the sample followed by freeze drying the sample under temperature and vacuum conditions such that the sample is dried close to the collapse temperature of the sample. T collapse temperature of the sample has been defined as the temperature during freezing that results in the collapse of the structural integrity of the product. Further, 'close to the collapse temperature' means a temperature about 0.1 to 1O°C below the collapse temperature.

RAPID FREEZE DRYING PROCESS

Technical Field

The invention relates to a process for freeze drying, particularly freeze drying samples containing biological material.

Background Art

The process of lyophilization, or freeze-drying involves removing water from a material by sublimation and desorption. Freeze drying is used widely in the food and pharmaceutical industries to preserve biological molecules as well as whole cells.

Freeze drying involves firstly freezing the material that is to be freeze dried and then placing the frozen material into a sealed vessel and applying a vacuum. The sealed vessel is normally comprised of two compartments, one which holds the material and a second that acts as a condenser. The condenser is usually a cylindrical chamber that can be cooled to below 0°C. When a vacuum is applied to the vessel the frozen water molecules within the material undergo sublimation and are collected on the cooled surface of the condenser. This process is continued until sufficient water has been removed from the material.

In many freeze dryers the material that is being freeze dried is held on shelves that can be heated. The application of heat to the material speeds up the drying process and provides control over the rate of drying. The application of heat through a shelf or tray that is in contact with a large surface area of the material that is being freeze dried, has the added advantage that it reduces variation in the rate of drying across the material.

Sensitive biological material is normally freeze dried in glass vials. The vials are loaded into a tray that is placed onto a heat controlled shelf within the freeze dryer. The material may be frozen prior to loading into the freeze dryer or the materials loaded in a liquid form and then frozen by lowering the temperature of the shelf. The pressure in the chamber is then lowered and the drying process begins. The temperature of the shelves is gradually increased to help drive the water molecules from the frozen material. The drying process is normally complete within 24 to 72 hours depending on the amount of material loaded into the freeze dryer and the capabilities of the freeze dryer.

It is important to control the drying rate and the heating rate during the freeze drying process. If the product is heated too rapidly, it will melt or collapse. This may

cause degradation of the product, and will certainly change the physical characteristics of the dried material, making it visually unappealing and harder to reconstitute. While frozen mobile water is present, the product must be held below the collapse temperature. It is difficult to accurately determine the collapse temperature for a particular material, therefore, heat is normally applied very gradually to ensure that the collapse temperature is not reached.

If heat is applied to the product at a rate that brings the product close to the collapse temperature, then variation between the vials occurs depending on the position of the vial on the shelf. Material in some vials will collapse whilst others will not. This is because there is variation in the temperature of the vials depending on the position of the vial. For example, vials on the outside of the shelf may warm up quicker than vials in the middle of the shelf because the ones in the middle are surrounded by a large mass of cold material of the neighbouring vials, whereas vials on the outside of the shelf are not surrounded by such a large mass of cold material.

Sensitive biological materials such as cells are often irrevocably damaged during freeze drying. It is thought that changes occur in the physical state of lipids and structure changes occur in some proteins. These changes can cause lethal damage to biological membranes. It is well known that crystal growth during freezing causes damage to biological material. Furthermore, it is likely that crystal growth during the freeze drying process contributes to cell damage and cell death.

Additives used to exert a mechanism of protection to biological materials are termed cryoprotectants, if the material is frozen, and lyoprotectants if the material undergoes freeze drying. There are a wide range of protectants used in the field and include sulfoxides, alcohols and derivatives, saccharides, polysaccharides, amides, peptides, proteins, glycoproteins, complex compounds and cations.

There are three mechanisms of protection afforded by protectants according to their permeability. Additives such as glycerol, dimethylsulfoxide (Me_2SO) and glucose are cell permeant and can therefore exert intracellular protection. Larger molecules such as mono- and disaccharides, amino acids and low molecular weight polymers that cannot pass through the cell membrane, but are small enough to cross the cell wall are termed semi-permeable protectants. The large molecules, for example polymers with high molecular weights, proteins, polysaccharides and dextran, that cannot pass either the cell wall or the cell membrane can only exert extracellular protection mechanisms.

Permeant protectants are generally hydrophilic (water – loving), and possess chemical groups that enable strong hydrogen bonds with water molecules. This ability to bind intracellular water prevents excessive dehydration, reduces the build up of high salt concentrations and stops the formation of large ice crystals. Permeable protectants stimulate a fine crystalline ice structure and form a gel-type glass phase, helping cells to avoid hyperosmotic injury, as the salt is bound in the gel phase and not allowed to concentrate in solution as water crystals remove the water solvent.

Semi-permeable protectants can induce partial dehydration of cells prior to freezing, by concentrating as a buffer layer between the cell wall and the cell membrane and drawing water osmotically from the cell. They can also exert a mechanical support to the cell wall.

Non-penetrating protectants generally protect against extracellular ice crystal formation, they do not interact directly with the cells, but they form a viscous protective layer around cells. Like the semi-permeable protectants, they can cause an osmotic efflux of water from the cell, but more importantly they inhibit the growth of ice crystals by forming amorphous glass structures during drying, due to gradually increasing viscosity of the protectant solution.

The present inventors have now developed a process for freeze-drying which results in minimal loss of activity or viability of biological materials.

Summary of Invention

The present invention provides a process for rapid freeze drying samples which results in improved recovery of activity or viability of the freeze-dried samples compared with conventional freeze-drying processes for equivalent samples.

In a first aspect, the present invention provides a process for freeze drying a sample comprising:

freezing the sample; and

freeze drying the sample under temperature and vacuum conditions such that the sample is dried close to the collapse temperature of the sample.

In a preferred form, the process further includes:

adding a penetrating cryoprotectant to the sample.

Preferably, the penetrating cryoprotectant is selected from glycerol, 2-methyl-2,4-pentanediol (MPD), polyethylene glycols (PEGs) of various molecular weights, dimethyl sulfoxide (DMSO), methanol, 1,2-propanediol, proline, and ethylene glycol.

Preferably, the cryoprotectant is glycerol.

Preferably, one or more cryoprotectants are included in the sample medium, usually prior to freezing. In a preferred embodiment, at least about 0.1% cryoprotectant (v/v) is included, preferably at least about 0.2%. Typically, not more than about 10% cryoprotectant is added, preferably not more than about 5% cryoprotectant, more preferably not more than about 1% cryoprotectant.

In a particularly preferred embodiment, at least 0.1% glycerol (v/v) is included, preferably at least 0.2%. Typically, not more than about 10% glycerol is added, preferably not more than about 5%, more preferably not more than about 1% glycerol. It will be appreciated that other suitable cryoprotectants can also be used.

In a second aspect, the present invention provides a process for freeze drying a plurality of samples comprising:

freezing a plurality of samples;

placing the plurality of frozen samples into a plurality of containers; and

freeze drying the plurality of samples under temperature and vacuum conditions such that the samples are dried close to the collapse temperature of the sample.

In a preferred form, the process further includes:

adding a penetrating cryoprotectant to the samples.

The present inventors have found that activity or viability of biological materials can be maintained in samples when freeze dried according to the present invention.

Biological materials include, but not limited to, microorganisms such as bacteria, yeasts, fungi, viruses; proteins such as antibodies, growth factors, hormones and the like; nucleic acids such as DNA and RNA. It will be appreciated, however, that the process of the present invention is suitable for freeze drying any suitable material.

The collapse temperature is the temperature during freeze drying that results in collapse of the structural integrity of a product. It will be appreciated therefore that the collapse temperature will vary between different sample types. By close to the collapse temperature, it is meant to be about 0.1 to 10°C below the collapse temperature. It will be appreciated that a collapse temperature may be calculated by experiment or measured for each sample type.

When a sample is freeze dried close to its collapse temperature, the present inventors have found that the product looks shrivelled and / or shrunken. Freeze drying further away from the collapse temperature produces a more smooth and well formed product. It is therefore preferred to form a shrivelled and shrunken product using the present invention. This is quite different from what has been done prior to the present invention as a smooth and well formed product was considered desirable.

Prior art freeze drying methods are carried out far from the collapse temperature of the sample to produce smooth and evenly formed products.

In a preferred form, the plurality of containers are arranged in the freeze dryer such that the containers are isolated from each other.

In a further preferred form, heat is applied to the plurality of containers such that each container is exposed to substantially an equivalent temperature at any given point during the freeze drying process.

Preferably, during the freeze drying the rate of heating and rate of vacuum pressure are controlled such that the drying process occurs as rapidly as possible without the sample collapsing.

Preferably, there is substantially no inactivation or loss of activity or viability of the samples during the process. The present inventors have surprisingly found that by freeze-drying samples of labile or living material close to the collapse temperature not only results in rapid freeze drying but retains the activity or viability of the sample content.

Preferably, there is no adverse affect to the appearance of the freeze-dried samples.

Preferably, the heating is rapid. Such rapid heating is defined as increasing the temperature by about 1°C per minute. A starting temperature of around -40°C and a finish temperature of around 25°C are typically used for the present invention. It will be appreciated that the process may be started at other temperatures such as about -35°C, -30°C, -25°C, or -20°C, for example. The finishing temperature may be about 10°C, 15°C, 20°C, or 30°C, for example.

A vacuum is typically applied during the heating process.

Preferably, the process takes about 3 to 5 hours depending on the volume of material within the freeze dryer.

The process may further include sealing the containers.

The sample can be a compound, composition, or biological material. Preferably, the compound or composition is a pharmaceutical agent, an antibody, an enzyme, a protein, a peptide or a nucleic acid. Preferably, the biological sample is a microorganism, cell, cell component or a virus. The present invention is particularly suitable for freeze-drying bacteria in relatively small numbers without significant loss of viability.

The sample may further contain bulking agents, cryoprotectants, lyoprotectants, surfactants, buffering agents, buffers or excipients. It will be appreciated, however, that other components as required may also be added to the sample prior to processing.

Preferably, the plurality of containers are placed in an rack made of an insulating material to isolate the containers from each other. Preferably, the rack is made of polystyrene, or other suitable insulating material, having holes adapted to receive the containers. The rack preferably does not provide insulation to the bottom of the containers such that the heating is provided from above and below the containers in a freeze drying apparatus.

The present inventors have found that if the temperature applied to each container during freeze-drying is relatively consistent, then it is possible to rapidly freeze dry samples with significantly less adverse affects on the characteristics of the sample. Instead of requiring several days to prepare samples by conventional freeze-drying, the present invention can be carried out over several hours. Not only does this result in an improved product, it also reduces processing time and thus production costs.

In a third aspect, the present invention provides a freeze-dried product produced by the process according to the first and second aspects of the present invention.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this specification.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 shows vacuum pressure and temperature profile of a preferred freeze drying cycle according to the present invention.

Figure 2 shows results of the lowest freeze dried count of 5 replicates produced after *E. coli* 0157 BioBall™ had been stored for 1 week at 22°C.

Mode(s) for Carrying Out the Invention

Definitions

Protein stability can be affected inter alia by such factors as ionic strength, pH, temperature, repeated cycles of freeze/thaw and exposures to shear forces. Active protein may be lost as a result of physical instabilities, including denaturation and aggregation (both soluble and insoluble aggregate formation), as well as chemical instabilities, including, for example, hydrolysis, deamidation, and oxidation. For a general review of stability of protein pharmaceuticals, see, for example, Manning, et al., Pharmaceutical Research 6:903-918 (1989).

"Collapse temperature" is the temperature during freeze drying that results in collapse of the structural integrity of a product. It will be appreciated therefore that the collapse temperature will vary between different sample types.

"Close to the collapse temperature" is meant to be about 0.1 to 10°C below the collapse temperature. It will be appreciated that a collapse temperature may be calculated by experiment or measured for each sample type.

"Storage-stable" means the product is stabilised for storage at temperatures between -20°C to 25°C and remains within product specifications for a suitable time period, often several months.

"Bulking agent" generally includes agents which provide good lyophilised cake properties, which form a pharmaceutically elegant product, which help the material overcome various stresses (shear/freezing for example) associated with the lyophilization process, and which help to maintain protein activity levels during the freeze-drying process and subsequent storage. Examples of often-used bulking agents

include mannitol, glycine, sucrose, lactose, etc. The agents can also contribute to the tonicity of the formulations.

"Cryoprotectants" generally includes agents which provide stability to the material to be freeze-dried from freezing-induced stresses. The term also includes agents that provide stability, e.g. to bulk drug formulations during storage from non-freezing-induced stresses. Examples of cryoprotectants include solvents such as glycerol and dimethyl sulfoxide (DMSO), polyols such as, for example, mannitol, and include saccharides such as, for example, sucrose, as well as including surfactants such as, for example, polysorbate, poloxamer or polyethylene glycol, and the like. Cryoprotectants can also contribute to the tonicity of the formulations.

The term "lyoprotectant" includes agents that provide stability to the material during water removal from the system during the drying process, presumably by maintaining the proper conformation of the protein through hydrogen bonding. Examples of lyoprotectants include saccharides and di- or trisaccharides and complex mixtures such as serum. Cryoprotectants may also have lyoprotectant effects. While preferred concentrations range from about 0.5 to 2%, relatively higher concentrations (for example 75%) of serum is often used.

The term "surfactants" generally include those agents, which protect the material from air/solution interface-induced stresses and solution/surface induced-stresses (e.g. resulting in protein aggregation), and may include detergents such as polysorbate, poloxamer or polyethylene glycol, and the like. Optionally, concentrations from about 0.01% to about 1% (w/w) are suitable for maintaining protein stability, however, the levels used in actual practice are customarily limited by clinical practice.

The term "buffering agent" or "buffer" encompasses those agents which maintain the solution pH in an acceptable range prior to and during lyophilization and may include histidine, phosphate, citrate, tris, diethanolamine, and the like. The upper concentration limits are generally higher for "bulk" materials than for "dosage" material forms as is readily appreciated by one skilled in the art. For example, while buffer concentrations can range from several millimolar up to the upper limit of their solubility (e.g., histidine could be as high as 200 mM), one skilled in the art would also take into consideration achieving/maintaining an appropriate physiologically suitable concentration. Percentages are weight/weight when referring to solids and weight/volume when referring to liquids.

The term "isotonic", 300+-.50 mOsM, is meant to be a measure of osmolality of the material solution prior to lyophilization; reconstitution is typically with water for

injection (WFI). Maintaining physiological osmolality can be important for the dosage formulations. However for bulk formulations, much higher concentrations can be effectively utilised as long as the solution is made isotonic prior to use.

The term "excipients" includes pharmaceutical acceptable reagents to provide good lyophilised cake properties (bulking agents) as well as provide lyoprotection and cryoprotection of the protein, maintenance of pH, and proper conformation of the material during storage so that substantial retention of biological activity and protein stability is maintained.

The term "viability" refers to the ability of a culture of microorganisms to grow. For example, a "viable" sample is comprised of live organisms that are capable of metabolism and growth. Consequently, a freeze-dried sample is viable if it can be revived, such as by rehydration, and grown in a suitable median. Preferably, the sample remains viable for at least one month, preferably at least two, three or six months, more preferably at least a year.

The term "activity" refers to the ability of a biological material to react in a specific manner. For example, an "active" antibody is capable of binding to an antigen. Consequently, a freeze-dried sample is active if it maintains activity after rehydration. Preferably, the sample remains active for at least one month, preferably at least two, three or six months, more preferably at least a year.

Microorganisms

The term "microorganism" includes prokaryotes and eucaryotes such as yeast, fungi and animal and plant cells capable of being propagated in culture, such as immortalised cell lines. Examples of prokaryotes include eubacteria and archaebacteria. Eubacteria include gram negative bacteria such as Escherichia sp. (e.g. *E. coli*), Neisseria sp, Campylobacter sp, Haemophilus sp, Streptococcus sp and Staphylococcus sp and gram positive bacteria such as Bacillus sp (e.g. *B. subtilis*).

Table 1 contains a list of bacteria that can be preserved or freeze-dried according to the present invention. It will be appreciated, however, that this list is not exhaustive.

Table 1

Acinetobacter baumannii ATCC19606	*Mycobacterium smegmatis* CIP 7326
Aeromonas hydrophila ATCC7966	*Mycobacterium terrae* ATCC15755
Aspergillus niger ATCC16404	*Neisseria gonorrhoeae* NCTC 8375
Bacillus cereus NCTC 10320	*Neisseria gonorrhoeae* ATCC49226
Bacillus cereus NCTC 7464	*Neisseria lactamica* ATCC23970
Bacillus subtilis ATCC6633	*Neisseria meningitidis* ATCC3090
Bacteroides fragilis ATCC285	*Neisseria sicca* ATCC9913
Campylobacter jejuni NCTC 11322	*Proteus mirabilis* ATCC14153
Candida albicans ATCC2091	*Proteus vulgaris* ATCC13315
Candida albicans ATCC10231	*Pseudomonas aeruginosa* ATCC27853
Citrobacter freundii ATCC8090	*Pseudomonas aeruginosa* CIP A22
Clostridium sporogenes ATCC19404	*Pseudomonas aeruginosa* ATCC9027
Clostridium perfringens ATCC3124	*Pseudomonas aeruginosa* ATCC15442
Enterobacter aerogenes ATCC13048	*Rhodococcus equi* NCTC 1621
Enterobacter cloacae ATCC23355	*Salmonella poona* NCTC 4840
Enterococcus faecalis ATCC19433	*Salmonella niarembe* NCTC 8279
Enterococcus faecalis ATCC29212	*Salmonella salford* IMVS 1710
Enterococcus faecalis ATCC33186	*Salmonella typhimurium* ATCC14028
Enterococcus hirae ATCC10541	*Serratia marcescens* ATCC8100
Escherichia coli ATCC10536	*Shigella flexneri* ATCC12022
Escherichia coli ATCC922	*Shigella sonnei* ATCC931
Escherichia coli ATCC35218	*Staphylococcus aureus* ATCC6538P
Escherichia coli ATCC8739	*Staphylococcus aureus* ATCC9144
Escherichia coli ATCC11229	*Staphylococcus aureus* ATCC923
Pseudomonas fluorescens	*Salmonella abaetetuba*
Escherichia coli NCTC 9001	*Staphylococcus aureus* ATCC29213
Escherichia coli 0157 NCTC 12900	*Staphylococcus aureus* NCTC 10442
Haemophilus influenzae NCTC 11931	*Staphylococcus aureus* ATCC6538
Haemophilus influenzae ATCC35056	*Staphylococcus aureus* NCTC 6571

Table 1 continued

Haemophilus influenzae ATCC49247	*Staphylococcus epidermidis* ATCC12228
Klebsiella aerogenes NCTC 9528	*Streptococcus agalactiae* ATCC13813
Klebsiella pneumoniae ATCC13883	*Streptococcus pneumoniae* ATCC6303
Lactobacillus brevis ATCC8287	*Streptococcus pneumoniae* ATCC49619
Listeria monocytogenes ATCC7644	*Streptococcus pyogenes* ATCC19615
Listeria monocytogenes NCTC7973	*Yersinia enterocolitica* ATCC9610
Listeria monocytogenes NCTC11994	*Zygosaccharomyces bailii* NCYC 417

Microorganisms are typically grown prior to freeze-drying in standard culture medium appropriate to that specific type of microorganism, such as nutrient broth for *E. coli*. In a preferred embodiment of the present invention, the microorganisms are grown in the presence of a high concentration of a carbohydrate at a desired pH for sufficient time so as to cause an accumulation of the carbohydrate in the cell.

Prior to freeze drying the microorganisms are mixed with a lyoprotectant to provide a sample with a suitable glass transition point (Tg), as discussed below.

Freeze-drying

Freeze-drying involves the removal of water or other solvent from a frozen product by a process called sublimation. Sublimation occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase. Freeze-drying is a routine technique used in the art and suitable equipment is available from commercial sources. However, for completeness, a brief description of the freeze-drying process is set out below, some of which is based on information provided in "A Guide to Freeze-drying for the Laboratory"--an industry service publication by Labconco, 1998. See also Franks, F. (1994), Effective freeze-drying: a combination of physics, chemistry, engineering and economics. Proc. Inst. Refrigeration 91: 32-39.

The freeze-drying process consists of three stages: pre-freezing, primary drying and secondary drying.

Pre-freezing: the material to be freeze-dried is adequately prefrozen. In traditional freeze-drying it is usual to pre-freeze the product below the glass transition point (Tg). As the temperature of the sample is lowered, the water in the sample freezes, increasing the concentration of solutes in the sample and increasing the viscosity of the

mixture. As the viscosity increases steeply, the rate of freezing reduces, eventually stopping altogether. The residual mixture, including the ice, undergoes a glass transition, where the viscosity increases by many orders of magnitude over a narrow temperature interval. Structurally, the sample is then in the form of an amorphous solid and is described as a glass. This transition from a super-saturated, frozen aqueous solution of very high viscosity to a brittle solid is operationally characterized by a glass transition temperature (Tg). The Tg for a sample can be determined by methods known in the art, such as differential scanning calorimetry or thermomechanical analysis.

However the Tg for samples of microorganisms in standard culture medium is often too low for practical purposes and must be raised by the addition of excipients. Suitable excipients include skimmed milk powder, glucose, sucrose, maltose, lactose, trehalose, raffinose, maltotriose, stachyose and dextran. Other suitable excipients are known in the art. Preferably the excipient is added in such an amount as to raise the Tg to about from –40°C to –20°C. The amounts may vary between types of sample but may readily be determined by the person skilled in the art. A suitable amount is typically from 5 to 15% w/v, such as from 7.5 to 12.5% w/v.

Primary drying: after pre-freezing the sample, conditions are established in which ice can be removed from the frozen product by sublimation. Samples are typically pre-frozen to well below their Tg and then the temperature is raised to just below the Tg. The samples are then subjected to reduced pressure in a vacuum chamber. At this point the freeze-drying process begins.

Secondary drying: after primary freezing is completed, all the frozen water has sublimed. However, non-frozen bound moisture is still present in the product. Continued drying at a higher temperature is often required to drive off the residual moisture by evaporation.

Several types of freeze-drying methods are currently in use including the manifold method, the batch method and the bulk method. It is preferred according to the present invention to use an apparatus suited to the batch method, such as a tray dryer.

Samples of microorganisms may be grown in the container prior to freeze-drying or grown in a separate vessel and dispensed into containers.

If a suitable excipient has not yet been added then it is added to the material prior to freezing. Once the excipient has been added, if not already added, the container may be frozen at a temperature below the Tg (e.g. in a -80°C freezer or by a cryogenic liquid such as liquid nitrogen) and maintained at that temperature until it is freeze-dried, if it

cannot be freeze-dried immediately. The container is then placed in the freeze-drying apparatus and freeze-dried according to the present invention. Once freeze-drying is complete, the container is typically sealed. The container may then be kept at room temperature, or below room temperature, as required, such that the viability of the microorganisms is maintained.

In general, cryoprotectants such as DMSO and glycerol, which are often used to protect the cells from damage during the freezing process, are not required in the method of the invention, but may be added if necessary. Preferably cryoprotectants are included in the sample medium, more preferably glycerol is included in the sample medium, prior to freezing. In a particularly preferred embodiment, at least 0.1% glycerol v/v is included, preferably at least 0.2%. Typically, not more than 10% glycerol is added, preferably not more than 5 or 1% glycerol.

Conventionally employed freeze-drying apparatus have a construction in which a plurality of heating shelves in a form of a multistage unit are positioned in a sealable vessel provided with an exhaust manifold. The frozen material to be freeze-dried is placed on each shelf and the shelves holding the frozen material are placed in the vessel. The vessel is closed, sealed and evacuated to generate a vacuum. The shelves are then heated to effect vacuum drying of the frozen material on the shelves, while the gases and moisture given off under sublimation from the frozen material are removed.

By such a conventional freeze-drying apparatus, a large scale processing of materials can be difficult due to restrictions in the amount and the size of the material to be freeze-dried. It can often take several days to ensure complete freeze drying of the samples therefore increasing the likelihood of inconsistency between the products. A further problem is that irregular heat transfer on heating the material to be freeze-dried may occur due to confinement of the material within a narrow interstitial spaces between the shelves, resulting in potential denaturation or inactivation of the material caused by local heat accumulation in different parts of the freeze-dryer.

Figure 1 shows vacuum pressure and temperature profile of a preferred freeze drying cycle according to the present invention.

EXAMPLE 1

This example describes a process for freeze drying droplets containing *Escherichia coli* cells.

Culture conditions

E. coli ATCC 11775 was grown in 2xYT media (tryptone-yeast extract rich medium) (Sun, 1991) supplemented with 250 mM HEPES buffer pH 7.2 and 20% w/v sucrose in 250 ml erlenmeyer flasks. A standard aliquot containing 10^5 cells was used to inoculate the media before incubating for 24 hrs in a 37°C shaker at 300 rpm.

Preparation of droplets containing *E. coli* cells

Diluted culture was then mixed 1:1 with filter sterilized sheep serum. A Pasteur pipette was used to dispense drops from this mixture into liquid nitrogen. The sample was then formed into frozen spherical pellets after freezing in liquid nitrogen for 20 seconds. The sample pellets were then transferred individually into labelled 2 ml freeze drying vials and partially capped with a freeze drying bung. The capped vials were held in liquid nitrogen until loaded into the freeze dryer.

To obtain a control plate to represent the number of cells contained in a drop prior to freeze-drying, drops were spread plated onto nutrient agar plates for each sample. The process of pre-freezing and control plating for each sample tested was repeated at least three times. The control plates were then incubated at 37°C overnight until growth was achieved.

Freeze drying

Two different freeze drying cycles were tested. The cycles differed in the temperature that the shelves were set during various stages of the drying cycle. The first cycle involved freeze drying slowly and maintaining the shelves at a temperature well below the collapse temperature. The second cycle involved starting the shelves at a higher temperature and rapid increasing the shelf temperature to 25°C so that the product was freeze dried close to the collapse temperature. Details of the freeze drying cycles are given in Table 2.

Table 2.

Freeze drying cycle	Shelf start temperature	Shelf temp set to reach 25°C over this many hours	Cycle completed
1	-50°C	8 hours	15 hours
2	-10°C	0.5 hours	3 hours

A Telstar Lyobeta freeze dryer (Telstar, Spain) was prepared by setting the shelf temperature to the start temperature and setting the condenser temperature to -60°C.

The vials containing the frozen droplets were placed directly onto the shelves of the freeze dryer. Alternatively, the vials were loaded into a polystyrene test tube rack that had had the bottom cut off so that the holes passed completely through the rack. The polystyrene rack was held on a stainless steel tray. The tray and rack were placed onto a shelf within the freeze dryer. The tray was slid out from the rack leaving the vials in contact with the shelf.

The vacuum pump was started and the heater for the shelf was adjusted as detailed in Table 2. At the end of the cycle the vials were capped and the freeze dryer turned off. The vials were removed from the freeze dryer and analysed immediately. Vials were examined to determine if the freeze dried product had formed a uniform ball shape or if the product had melted.

Determination of cell viability

To rehydrate the freeze-dried sample pellets, the vials containing the sample were opened and the freeze-dried pellet was tipped onto a nutrient agar plate. Some 100 µl of sterile 0.9% saline was then dispensed over the pellet on the plate and a spreader was then used to dispense the sample. The plates were then incubated at 37°C over night.

Control counts from samples plated prior to freeze-drying experiments were compared to counts obtained from samples re-hydrated after freeze-drying. From the data a mean with its corresponding standard deviation was obtained for every control and freeze-dried sample. Percentage survival was obtained from this data by dividing

the mean of the freeze-dried sample by the mean of the control sample, then multiplying by one hundred.

RESULTS

Freeze drying cycle number one resulted in uniform and well shaped freeze dried balls, however, the recovery of viable *E. coli* was low (Table 3). Freeze dryer cycle number 2 resulted in a collapsed product in approximately half of the vials and a variably shaped product in all the vials. Many vials contained product that stuck to the side of the vial.

The recovery of viable *E. coli* was high for freeze drying cycle 2 (Table 3). The use of freeze drying cycle number 2 with the vials insulated by placing in polystyrene racks resulted in uniform and well shaped freeze dried balls and high recovery of *E. coli* cells (Table 3).

Table 3.

Freeze drying cycle	**Appearance of product**	**Recovery of *E. coli***
1	Uniform and well shaped	60%
2 (uninsulated)	Variable and approximately 50% collapsed	100%
2 (insulated)	Uniform and well shaped	100%

In the present inventors' experience with bacteria, over 90% of the cells die during the freezing and freeze drying process that are normally employed to preserve bacteria. However, the present inventors have found that when biological material is freeze dried at a rapid rate so that the freeze dried material is close to its collapse temperature then less damage to the material occurs. In fact the present inventors have successfully freeze dried cells in this manner without causing death to any of the cells. This has been performed by freeze drying frozen droplets of fluid that contain bacteria.

The frozen droplets are placed into vials and then freeze dried rapidly just below the collapse temperature of the droplets. Whilst the viability of the bacteria was maintained, the physical appearance of the freeze dried droplets varied between the vials depending on the position of the vial within the freeze dryer. Some of the droplets collapsed resulting in a freeze dried product with a poor appearance and which was often stuck to the bottom of the vial.

This effect was overcome by insulating each vial in a polystyrene foam rack that consisted of a polystyrene tray with holes, slightly larger than the vials. The holes pass all the way through the rack so that the vials are insulated from each other but the vials touch the heated shelf.

EXAMPLE 2

This example describes a process for freeze drying droplets containing a fluorescent monoclonal antibody.

Monoclonal antibody

A monoclonal antibody specific to the surface of *Cryptosporidium* oocysts was conjugated with fluorescein isothiocyanate (FITC) as previously described (Weir, C, Vesey, G, Slade, M, Ferrari, B, Veal, DA, and Williams, K. 2000. An immunoglobulin G1 monoclonal antibody highly specific to the wall of *Cryptosporidium* oocysts. *Clinical & Diagnostic Laboratory Immunology.* 7(5):745-750). The conjugated antibody was diluted in sterile sheep serum to a concentration of 20 µg per ml.

Freezing and freeze drying

A Pastuer pipette was used to dispense drops of the antibody solution into liquid nitrogen. The frozen spherical pellets were then transferred individually into labelled 2 ml freeze drying vials and partially capped with a freeze drying bung. The capped vials were held in liquid nitrogen until loaded into the freeze dryer.

Additional samples were prepared by pipetting 750 µl of antibody solution directly into freeze drying vials and then freezing the samples by placing the vials into liquid nitrogen. A frozen cake of antibody was obtained.

The two different freeze drying cycles described in Example 1 were used. Vials containing frozen spheres were freeze dried using cycle number 2. Vials containing 750 µl of frozen antibody were freeze dried using cycle 1.

After completion of the freeze drying cycle the vials were removed from the freeze dryer, crimped and stored at -20°C for 8 weeks prior to analysis.

Flow cytometric analysis of antibodies

The vials of antibody were reconstituted by adding a volume of water equivalent to the volume of antibody prior to freeze drying. The vials were then vortexed for 2 minutes.

A 20 µl aliquot of antibody was mixed in a flow cytometry test tube with 200 µl of saline that contained approximately 50,000 *Cryptosporidium* oocysts. The samples were vortexed for 10 seconds and then incubated at room temperature for 30 minutes.

The samples were then analysed using a FACScalibur flow cytometer (Becton Dickinson, CA, USA) and the intensity of the green fluorescence (525 nm) of 2000 oocysts was recorded.

RESULTS

The fluorescence intensities of oocysts stained with the freeze dried fluorescent antibodies are presented in Table 4.

Table 4

Freeze drying cycle	**Format of frozen antibody**	**Mean fluorescence of three replicate samples (standard deviation)**
1	750 µl frozen cake	94 (4)
2 (uninsulated)	Frozen sphere	125 (3)

The antibody that was freeze dried as a frozen sphere using the rapid freeze drying cycle produced significantly brighter oocysts than the antibody freeze dried as a cake using the slower freeze drying cycle.

Freeze drying sensitive biological material in this manner resulted in preservation of the material without damage and results in a freeze dried material that is not stuck to the vial and has an acceptable visual appearance.

EXAMPLE 3

This example describes the difference when glycerol is used in protectant for freeze drying *E. coli* cells.

Culture Conditions

The same culture conditions as described in Example 1 above were used.

Preparation of *E. coli* cells for freeze drying

An aliquot of 1 µl of *E. coli* cell culture was diluted into 1 ml PBS. This cell dilution was loaded onto a FACScalibur flow cytometer (Becton Dickinson). The cells were sorted by the cytometer and released as a single droplet from the outlet nozzle. An equal flow rate of a lyoprotectant was pumped into the nozzle, so that the resulting droplet consisted of 50% cytometer fluids (PBS) and sorted cells and 50% lyoprotectant.

Two lyoprotectants were tested:

A. Lyoprotectant + 4% glycerol;

B. Lyoprotectant

Freeze drying

The droplets dispensed by the cytometer were collected in cups of liquid nitrogen and allowed to freeze. Once frozen, the droplets were placed into vials and loaded onto the freeze dryer, with the shelves chilled to -30°C.

The freeze drying cycle, ramped the shelf temperature from -30°C to 25°C in 45 minutes, and then remained at 25°C for a further 2.25 hours, until the chamber pressure level reached 0.06 mbar.

Table 5

Lyoprotectant	**Recovery of *E. coli* directly after freeze drying**	**Recovery of *E. coli* after 1 week storage at 22°C**
With glycerol	97.5%	97.5%
Without glycerol	87.5%	73.1%

There was no significant difference observed between the two different lyoprotectants immediately after freeze drying. However, there was a significant difference between the *E. coli* cell recoveries after a 1 week period of storage at 22°C. The addition of glycerol to the lyoprotectant enables *E. coli* to survive for longer periods of time at elevated temperatures.

EXAMPLE 4

***E. coli* 0157**

Figure 2 shows results of the lowest freeze dried count of 5 replicates produced after *E. coli* 0157 BioBall™ had been stored for 1 week at 22°C and then plated onto non-selective agar. The limit pass level for these batches was all 5 counts to be above 24 cfu. The light shaded bars show batches that were freeze dried using a slow cycle and failed to meet the pass specification of 24 cfu whereas, the black shaded bars are batches that used a rapid freeze drying cycle and passed the limit of 24 cfu.

The rapid freeze drying cycle had a shelf temperature ramp to 25°C of 20 minutes, whereas the slow freeze drying cycle had a shelf temperature ramp to 25°C of 45 minutes. The shelf temperature ramp rate was the controlling force behind the speed of the drying process. Both the mentioned rates were significantly faster than traditional freeze drying methodologies. However, the 25 minute increase in rate resulted in differences between a freeze dried *E. coli* 0157 being stable at 22°C for 1 week and producing significantly lower counts after 1 week storage.

Table 6

Batch No	Mean cfu count (SD) post freeze drying n=50	Mean cfu count (SD) post 1 week storage at 22°C n=5	Lowest count after 1 week storage at 22°C	Pass/Fail storage stability test (above 24 cfu)
B337	31.06 (2.98)	25.5 (4.09)	19	FAIL
B342	33.2 (3.06)	24.4 (3.51)	20	FAIL
B352	29.55 (3.43)	23.4 (4.16)	19	FAIL
B357	31.77 (2.94)	26 (3.32)	23	FAIL
B366	30.68 (2.37)	27.9 (2.45)	24	PASS
B376	30.34 (2.26)	29.6 (2.07)	26	PASS
B377	31.24 (2.39)	27.8 (2.77)	25	PASS
B378	31.68 (1.89)	31.2 (2.77)	29	PASS
B379	29.54 (2.96)	26.6 (2.3)	24	PASS
B383	31.36 (2.62)	28.2 (1.79)	26	PASS

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A process for rapid freeze drying a sample comprising:

freezing the sample; and

freeze drying the sample under temperature and vacuum conditions such that the sample is dried close to the collapse temperature of the sample.

2. The process of claim 1 wherein close to the collapse temperature is about 0.1°C to 10°C below the collapse temperature of the sample.

3. The process of claim 1 or 2 wherein the sample is in a plurality of containers.

4. The process of claim 3 wherein the plurality of containers are arranged in a freeze dryer such that the containers are isolated from each other.

5. The process of claim 4 wherein the plurality of containers are isolated from each other in a rack having holes adapted to receive the containers.

6. The process of claim 5 wherein the rack is made of an insulating material.

7. The process of claim 6 wherein the insulating material is polystyrene.

8. The process of any one of claims 3 to 7 wherein the sample is heated such that each container is exposed to substantially an equivalent temperature at any given point during the freeze drying process.

9. The process of any one of claims 1 to 8 wherein the freeze drying is started at a temperature of about -40°C and finished at a temperature of about 25°C.

10. The process of claim 10 wherein the sample is heated at a rate of about 1°C per minute.

11. The process of any one of claims 1 to 10 further comprising adding a penetrating cryoprotectant to the sample.

12. The process of any one of claims 1 to 11 wherein the penetrating cryoprotectant is selected from the group consisting of glycerol, 2-methyl-2,4-pentanediol (MPD), polyethylene glycols (PEGs) of various molecular weights, dimethyl sulfoxide (DMSO), methanol, 1,2-propanediol, proline, and ethylene glycol.

13. The process of claim 12 wherein the cryoprotectant is glycerol.

14. The process of any one of claims 11 to 13 wherein at least about 0.1% (v/v) cryoprotectant is added.

15. The process of claim 14 wherein at least about 0.2% (v/v) cryoprotectant is added.

16. The process of claim 15 wherein not more than about 10% cryoprotectant is added.

17. The process of claim 16 wherein not more than about 5% cryoprotectant is added.

18. The process of claim 17 wherein not more than about 1% cryoprotectant is added.

19. The process of any one of claims 1 to 18 further comprising adding bulking agents, cryoprotectants, lyoprotectants, surfactants, buffering agents, buffers or excipients to the sample.

20. The process of any one of claims 1 to 19 wherein the sample is a compound, composition, or biological material.

21. The process of claim 20 wherein the compound or composition is a pharmaceutical agent, an antibody, an enzyme, a protein, a peptide or a nucleic acid.

22. The process of claim 20 wherein the biological material is a microorganism, cell, cell component or a virus.

23. A freeze-dried product produced by the process of any one of claims 1 to 22.

24. The product according to claim 23 having shrivelled or shrunken appearance.

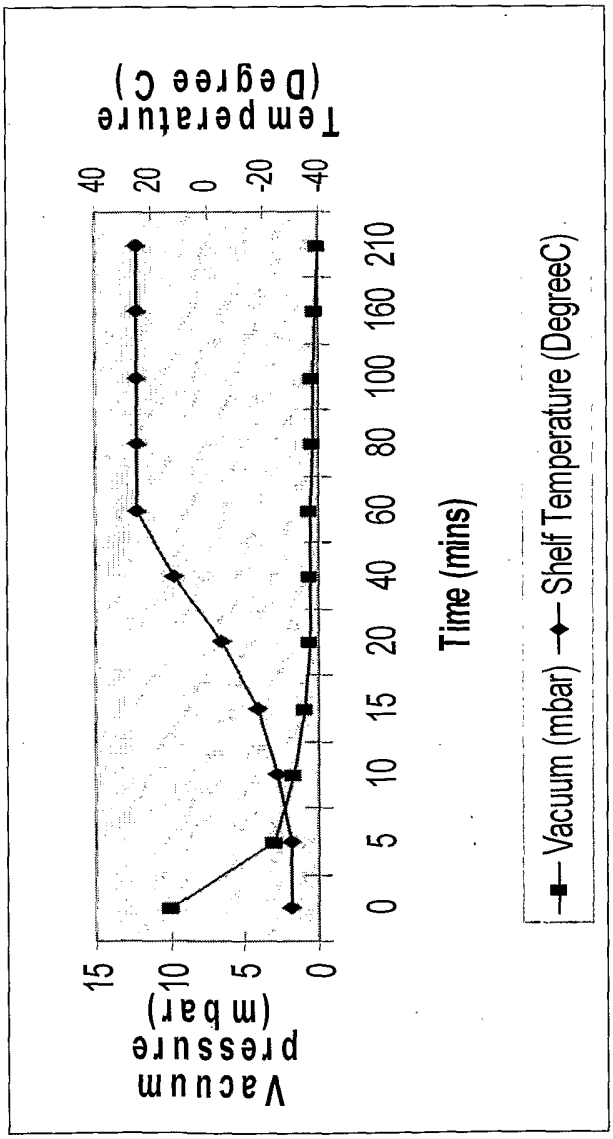
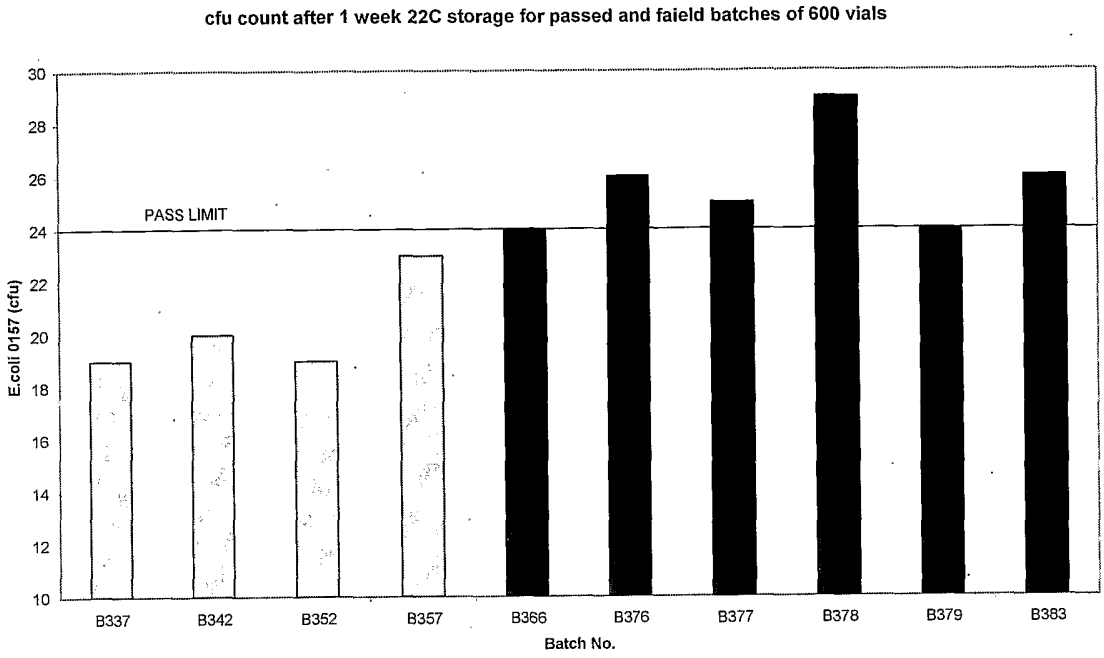

Vacuum pressure (m bar)
Temperature (Degree C)
Time (mins)
0 5 10 15 20 40 60 80 100 160 210
0 5 10 15
40 20 0 -20 -40
Vacuum (mbar)
Shelf Temperature (DegreeC)

Figure 1

Figure 2


cfu count after 1 week 22C storage for passed and faield batches of 600 vials
30
28
26
24
22
20
18
16
14
12
10
PASS LIMIT
E.coli 0157 (cfu)
B337
B342
B352
B357
B366
B376
B377
B378
B379
B383
Batch No.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001408

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: F26B 5/06, F26B 25/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI: (IPC) A01N 1/+, 3/+; C12N 1/04, 5/+, 7/+, 9/+; F26B 5/06, 25/22; A23C/IC, C12M/IC, A61K 9/19; & Keywords - (freeze dry), lyophili+, sublim+, (collapse temperature), collaps+

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2003/053463 A (BAVARIAN NORDIC A/S) 3 July 2003 See especially Example 1 and the claims	1-24
X	Derwent Abstract Accession No. 86-167668/26, Class Q76 JP 86022943 B (SHOWA DENKO KK) 3 June 1986 Abstract	1-24
X	Fonseca et al. "Collapse Temperature of Freeze-Dried Lactobacillus bulgaricus Suspensions and Protective Media". *Biotechnol. Prog.* 2004, 20(1). Pages 229-238. Whole document	1-24
X	Gatlin, L.A and Nail, S.L. "Freeze Drying: A practical Overview". *Bioprocess Technol.* 1994, vol 18, pages 317-67. See especially pages 361-365	1-24

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
21 October 2005

Date of mailing of the international search report - 9 NOV 2005

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001408

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Derwent Abstract Accession No. 98-607906/51, Class B04 D16 RU 2111426 C (VEKTOR RES CENTRE) 20 May 1998 Abstract	1-24
A	US 6060223 A (WIGGINS) 9 May 2000 Whole document	1-24
A	US 6098410 A (HORIGANE) 8 August 2000 Whole document	1-24
P, A	US 2005/0084576 A (SAKUMA ET AL) 21 April 2005 Whole document	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2005/001408

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	03053463	AU	2002361962	BR	0214822	CA	2467365
		CN	1602205	EP	1418942	HU	0402179
		NO	20042958	US	2005019349		
JP	86022943	NONE					
RU	2111426	NONE					
US	6060223	AU	42892/96	AU	42924/96	AU	47020/96
		AU	55352/96	AU	59707/96	AU	59725/96
		AU	75173/94	BR	9407181	BR	9510258
		BR	9510279	BR	9607572	BR	9608387
		BR	9608388	BR	9609295	CA	2120838
		CA	2123281	CA	2168727	CA	2207571
		CA	2207607	CA	2209480	CA	2219459
		CA	2219463	CA	2221565	CN	1131468
		CZ	9600275	EP	0712506	EP	0799246
		EP	0804511	EP	0807294	EP	0830256
		EP	0830676	EP	0846146	EP	1020478
		EP	1116708	EP	1123811	EP	1123812
		EP	1123813	EP	1123814	EP	1132119
		FI	960483	HU	73681	NO	960455
		PL	312835	PL	321370	SK	15296
		SK	94597	US	5616443	US	5643356
		US	5643701	US	5645964	US	5681380
		US	5683843	US	5700850	US	5721287
		US	5733693	US	5773182	US	5858586
		US	5865471	US	5908495	US	6017471
		US	6017661	US	6033465	US	6054256
		US	6060200	US	6066439	US	6120949
		US	6127073	US	6211383	US	6235095
		US	6342305	WO	9504955	WO	9619502
		WO	9619776	WO	9622335	WO	9639302
		WO	9639693	WO	9701605	ZA	9510848
		ZA	9510849	ZA	9600360	ZA	9604549

		ZA	9604553	ZA	9605031
US	6098410	EP	0917825	JP	11151080
US	2005084576	JP	2005218442	WO	2005036974

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX