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(71) Demandeur/Applicant:
NANOGEN PHARMACEUTICAL BIOTECHNOLOGY,
VN
(72) Inventeur/Inventor:
HO, NHAN, VN
(74) Agent: DENNISON ASSOCIATES

(54) Titre : CONJUGUES LAMBDA 1 D'INTERFERON PEG
(54) Title: PEG-INTERFERON LAMBDA 1 CONJUGATES

(57) **Abrégé/Abstract:**

The present application discloses new PEG-interferon lambda 1 conjugates (PEG-IEN λ 1), processes for their preparation, pharmaceutical compositions containing these conjugates and processes for making the same. These conjugates have increased blood half-lives and persistence time compared to IEN λ 1 and are effective in the treatment of hepatitis B and hepatitis C.



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- (71) Applicant (for all designated States except US): **NANO-GEN PHARMACEUTICAL BIOTECHNOLOGY** [VN/VN]; Lot I-5C Saigon Hitech Park, Tang Nhon Phu A Ward, District 9, Ho Chi Minh City (VN).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): **HO, Nhan** [VN/VN]; Nanogen Pharmaceutical Biotechnology, Lot I-5C Saigon Hitech Park, Tang Nhon Phu A Ward, District 9, Ho Chi Minh City (VN).
- (74) Agent: **NGUYEN, Sam, L.**; Hamilton, DeSanctis & Cha LLP, 3239 El Camino Real, Suite 220, Palo Alto, CA 94306 (US).

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(54) Title: PEG-INTERFERON LAMBDA 1 CONJUGATES

(57) Abstract: The present application discloses new PEG-interferon lambda 1 conjugates (PEG-IENλ1), processes for their preparation, pharmaceutical compositions containing these conjugates and processes for making the same. These conjugates have increased blood half-lives and persistence time compared to IENλ1 and are effective in the treatment of hepatitis B and hepatitis C.

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PEG-INTERFERON LAMBDA 1 CONJUGATES

Related Application:

[0001] The present application claims the benefit of Vietnamese Patent application serial No. VN1-2011-02222, filed August 25, 2011, which is incorporated herein by reference.

Field of the Invention:

[0002] In one embodiment, the present application discloses pegylated derivatives of recombinant human interferon lambda 1 (PEG-interferon lambda 1 conjugates or PEG-IFN λ 1), processes for their preparation, pharmaceutical compositions containing these conjugates and processes for making the same.

Background of the Invention:

[0003] Hepatitis C virus (HCV) is a major health problem and the leading cause of chronic liver disease throughout the world. It is estimated that at least 180 million people worldwide are chronically infected with HCV. In Vietnam, the proportion of infected HCV individuals in the population is 4-9%. Approximately 55-85% of acutely infected HCV individuals will convert to chronic infection, 5-25% of these chronic carriers are at risk of developing cirrhosis after 25-30 years and of those with cirrhosis, 30% are at risk of liver decompensation over 10 years, and 1-3% will develop liver cancer each year. According to epidemiological research, HCV is the cause of 40% of individuals in final stage cirrhosis and 60% in hepatoma.

[0004] Currently, α -interferons (AI) are the therapies of choice for the treatment of chronic HCV infection. AI can give a persistent response to HCV in approximately 70% of cases, however these interferons cause many side-effects, even in the case of PEG-interferon alpha. These side-effects can sometimes limit treatment, leaving the treatment of patients incomplete. Side-effects include influenza-like symptoms and hematologic effects such as thalassemia and anemia.

[0005] Interferons are currently used for the treatment of many viral diseases such as hepatitis B, hepatitis C, hepatitis D, condyloma acuminata, lepromatous leprosy, chronic leukaemia and AIDS. AI are also effective in reducing malignant tumors and treating Kaposi's sarcoma, melanoma, and renal cell carcinoma. Moreover, AI are applicable in the prevention and treatment of diseases in cattle and other livestock. For example, AI enhance the activity of vaccines used in prophylaxis and treatment of foot and mouth disease and porcine reproductive and respiratory syndrome.

[0006] AI have been produced from human cell lines incubated in tissue culture media or leukocytes derived from donors. However, these methods are time consuming, labor intensive, expensive, and not amenable to large scale manufacturing. Furthermore, there is the risk of septicaemia caused by infectious agents from the cell lines.

[0007] With the development of recombinant DNA technology, we can now introduce AI genes into microorganisms that enable production of large amounts of interferons. However, these methods also present certain advantages and difficulties, mainly in the steps of expression and large-scale protein production.

[0008] IL-29 is a member of the helical cytokine family and is a type III interferon. It is also known as interferon lambda 1 (IFN λ 1) and is highly similar in amino acid sequence to IL-28, the other type III interferon. IL-28 and IL-29 (IFN λ 1) were recently described as members of a new cytokine family that shares with type I interferon (IFN), the same Jak/Stat signaling pathway driving expression of a common set of genes. Accordingly, they have been named IFN λ . IFNs λ exhibit several common features with type I IFNs: antiviral activity, antiproliferative activity and *in vivo* antitumor activity. Importantly, however, IFNs λ bind to a distinct membrane receptor, composed of IFNLR1 and IL10R2.

[0009] The major disadvantage with the therapeutic use of most biologicals is that they are administered parenterally, e.g. intravenously (i.v.), subcutaneously (s.c.), intramuscularly (i.m.) etc. This means that delivery to the patient is associated with pain and discomfort. Furthermore, because of their usually very short half-lives, biologicals require frequent administration to the patient in order to maintain therapeutic blood serum or plasma levels of the drug. Injections that cannot be self-administered require frequent trips to the clinic and trained medical personnel, making such therapy inconvenient and expensive. Interferon alpha-2a (Roferon, Roche) and interferon alpha-2b (Intron A, Schering A G), the two recombinant forms of human interferon alpha used in the treatment of chronic hepatitis B and C, have a serum half-life of less than 12h (McHutchison, et al., Engl. J. Med. 1998, 339, 1485-1492; Glue, et al., Clin. Pharmacol. Ther. 2000, 68, 556-567) and therefore require administration 3 times a week. Repeated injections with interferon beta-1b (Betaseron) are also required to treat the patients of multiple sclerosis (MS).

[0010] One very successful and well accepted method of overcoming the above requirement of frequent high dose injections to maintain threshold levels of the drug in the body is to increase the *in vivo* half-life of the therapeutic protein by conjugating it with a polymer, such as polyethylene glycol (PEG or Peg). PEG molecules with their long chains not only create a protective shield

around the pegylated drug molecule in aqueous solution, thereby reducing the immunogenicity of protein drugs while also protecting them from the action of proteases, but they further help increase the circulation half-life of the drug by increasing its hydrodynamic volume which reduces its loss from the filtration mechanisms of the kidney glomeruli network. After their separation from the protein molecule, the PEG moieties are cleared without any structural changes and their clearance is proportional to their molecular weight.

[0011] Usually PEG moieties are attached to the protein by first activating the PEG moiety and then reacting the activated PEG agent with the side chains of an amino acid of a protein, such as the lysine residue and/or the N-terminal amino group on the protein. The most frequently used PEG is monofunctional PEG because this moiety resists cross-linking and aggregation. One such example has been disclosed by Davis et al. in U.S. Pat. No. 4,179,337.

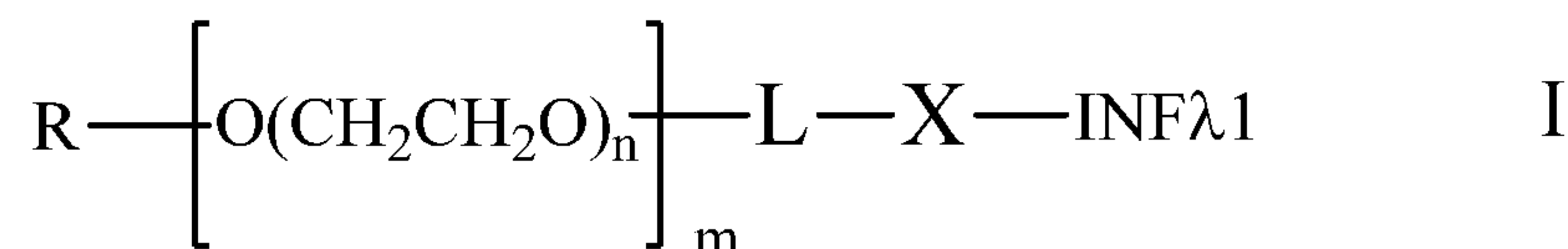
Summary of the Invention:

[0012] PEG-interferon lambda 1 (PEG-IFN λ 1) is a pegylated derivative of human recombinant IFN λ 1 (wherein polyethylene glycol is conjugated to IFN λ 1, also referred to as the “conjugate”) that is useful in the treatment of chronic hepatitis C in adult patients. PEG-IFN λ 1 bypasses the action of extracellular enzymes and resists filtration in the kidney after injection into the patient’s body; therefore its half-life in circulation is extended. That is, the conjugate has significantly improved stability, better solubility, and enhanced circulating half-life and plasma residence times when compared to the corresponding non-PEG-conjugated IFN λ 1.

[0013] Interferon lambda 1 (IFN λ 1, Zcyto21 or IL-28A) is known in the art, for example, from U.S. Pat. Nos. 7,038,032, 6,927,040, 7,135,170, 7,157,559 and 7,351,689; and PCT publication Nos. WO 05/097165, WO 07/012,033, WO 07/013,944 and WO 07/041,713; all of which are herein incorporated by reference in their entirety.

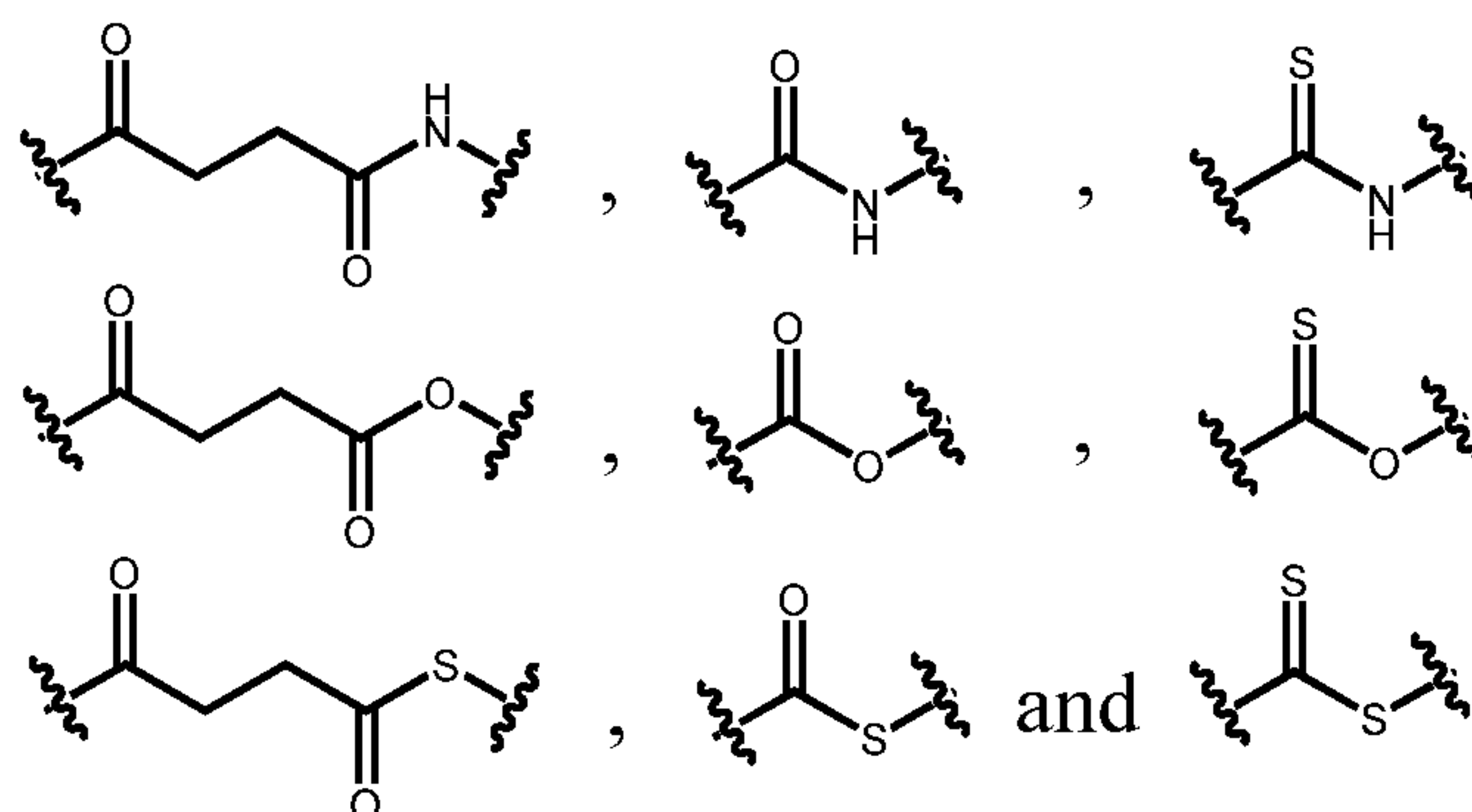
[0014] In one embodiment, the present application discloses novel PEG-IFN λ 1 conjugates. In one aspect, conjugates of the present application have a linear PEG chain structure. As compared to unmodified IFN λ 1 (that is, the IFN λ 1 that is not conjugated with PEG or mPEG), these conjugates have increased circulating half-life and persistence in plasma. Water soluble PEGs include polyethylene glycol (PEG), monomethoxy-PEG (mPEG), mono-C₁₋₁₀ alkoxy-PEG and mono-C₁₋₃ alkoxy-PEG. These PEGs that may be employed may have a molecular weight of about 600 to 60,000 and include those, for example with about 10 kDa, 20 kDa, 30 kDa, 40 kDa, 50 kDa and 60 kDa. In one aspect, the PEG employed in the present conjugates are mPEG with a molecular weight of 40 kDa.

[0015] In one embodiment, there is provided a physiologically active PEG-IFN λ 1 conjugate comprising the formula I:

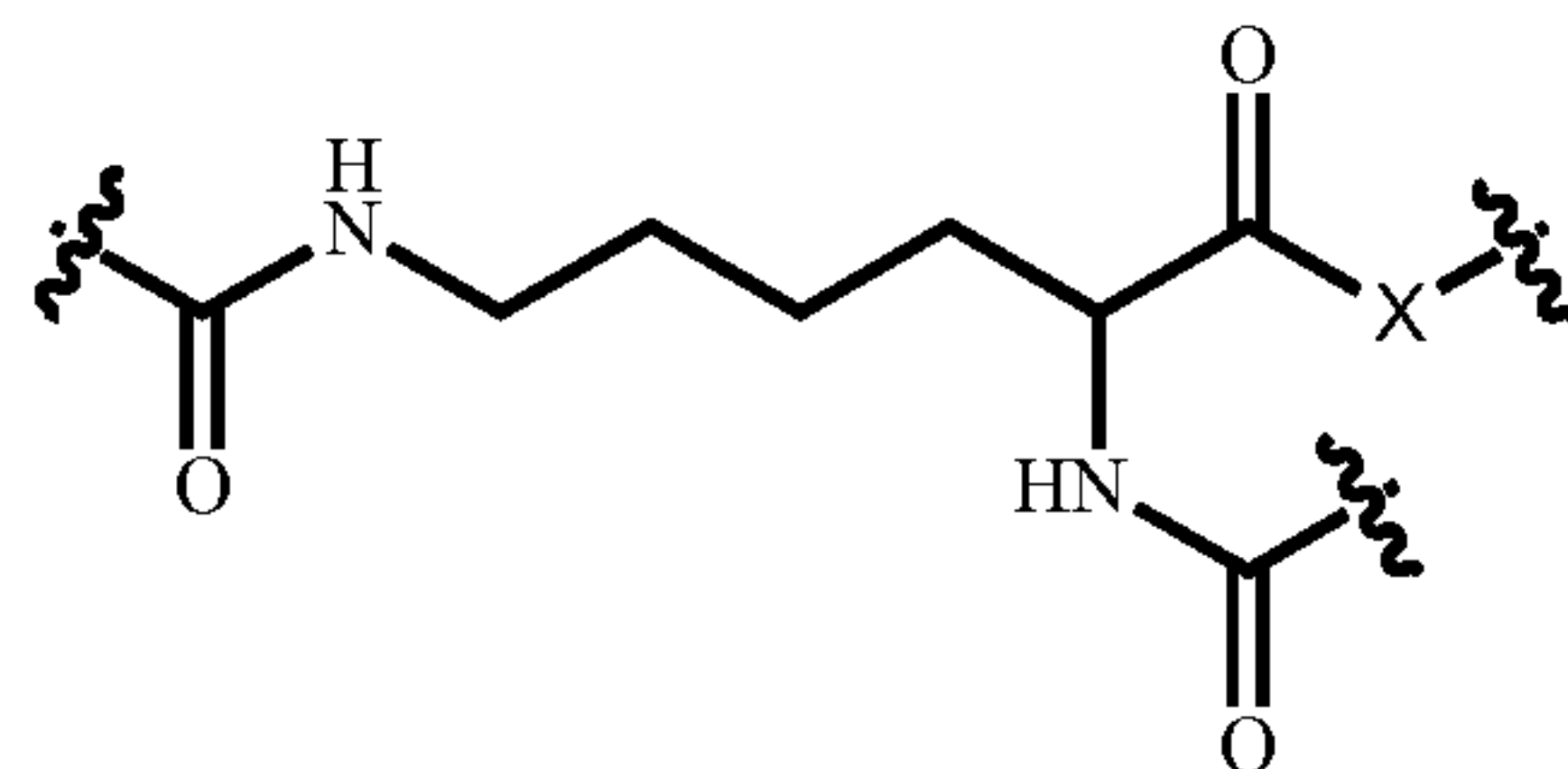


wherein: R is H or C₁₋₃ alkyl; m is 1, 2, 3 or 4; n is a positive integer selected in the range from 400 to 550; L is a C₁₋₁₀ alkyl or heteroalkyl linker; X is -O-, -NH- or -S-; and IFN λ 1 is interferon lambda 1; or a pharmaceutically acceptable salt thereof. In one aspect, interferon lambda 1 is a human recombinant interferon. In another aspect, the IFN λ 1 may be a natural or recombinant protein. In another aspect, the IFN λ 1 is a human protein derived from a source such as tissues, protein synthesis, or cell culture using natural cells or recombinant cells. In a still another aspect, IFN λ 1 is a human recombinant protein. In another aspect, the conjugate interferon lambda 1 of the formula I is the SEQ ID 2. In one embodiment, the PEG chain is coupled to the IFN λ 1 via an amide bond on a primary amino group of, for example, lysine, or the N-terminal of IFN λ 1.

[0016] Heteroalkyl is defined as a C₁₋₁₀ alkyl wherein at least one of the carbon of the C₁₋₁₀ alkyl is replaced by an -O-, -C(=O)-, -NH- or combinations thereof. Such combinations include, for example, -OC(=O)-, -C(=O)O-, -NHC(=O)-, -C(=O)NH- and the like. In one aspect, n is about 500 to 550. In another aspect, n is about 420, 520 or 455. In another aspect, the molecular weight of the Peg group is about 35 kDa to 45 kDa, or about 40 kDa. In another aspect of the above, m is 1 or 2. A C₁₋₁₀ alkyl or heteroalkyl may be a linear or a branched alkyl or heteroalkyl group. In one aspect, the C₁₋₁₀ alkyl group is a -C(O)- group. In one embodiment, m is 1 and L-X- is selected from the group consisting of the formulae:

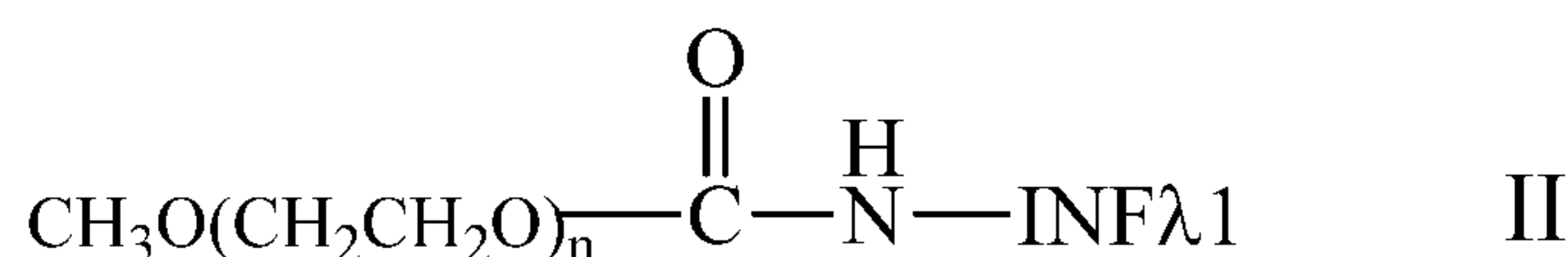


[0017] In one aspect of the above conjugate, m is 2 and L-X- is of the formula:



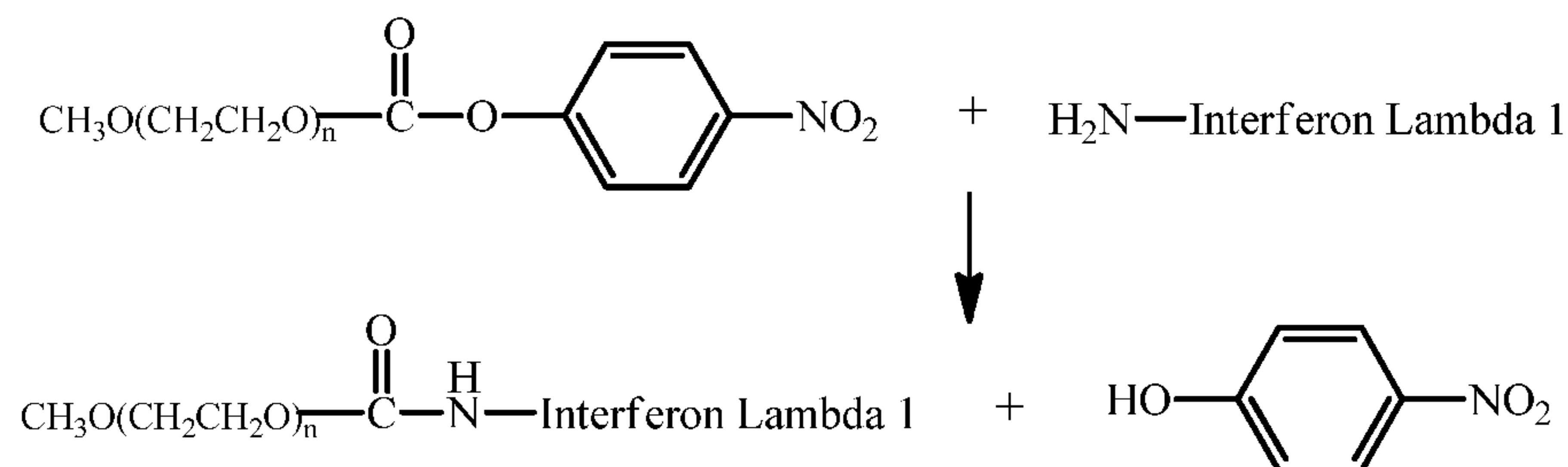
[0018] In another aspect of the above conjugate, the two PEG groups, PEG, alkyl-PEG or m-PEGs, are attached to the two formamide groups (i.e. $-\text{C}(\text{O})\text{NH}-$) of the above formula. In another aspect, X is $-\text{NH}-$ or $-\text{O}-$ and m is 2. In another aspect, the linker is attached to two PEG groups. In another aspect, X is $-\text{NH}-$, and the group attached to the linker is the residue of a lysine on IFN λ 1. In one aspect, the $-\text{NH}-$ group (i.e., the amino group) attached to the linker is the residue of a histidine. In another aspect, X is $-\text{O}-$, and the group attached to the linker may be derived from the residue of a serine on IFN λ 1. In one variation of the above formula, R is $-\text{CH}_3$. In another aspect, the linker is attached to the residue of a lysine, a serine, a histidine or mixtures thereof on the IFN λ 1. In another aspect, the linker is attached to a positional isomer of the residue of a lysine, a serine, a histidine or mixtures thereof on the IFN λ 1. In another aspect of the conjugate, R is H or $-\text{CH}_3$, m is 1, L is $-\text{C}(\text{O})-$ and X is $-\text{NH}-$. In another aspect, n is 500 to 550.

[0019] In another aspect, the conjugate (Nanogen PEG-IFN λ 1) comprises the formula II:



wherein IFN λ 1 is interferon lambda 1; and n is 500 to 550. In one aspect, n is a number of units of ethylene glycol in the PEG structure and it is a positive integer selected from any numbers such that the molecular weight of PEG moiety is about 40 kDa, and IFN λ 1 is interferon lambda 1. In another embodiment of the above, the conjugate has a blood serum half-life and persistence time that are prolonged or extended when compared to IFN λ 1. In another aspect of the conjugate, the PEG is attached to a methionine at the N-terminal of the IFN λ 1. In yet another aspect, the conjugate is effective in the treatment of hepatitis B and hepatitis C.

[0020] In another embodiment, there is provided a process for the preparation of a human recombinant conjugate as disclosed above, the process comprises the step of covalently binding (α -methoxy- ω -(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC) 40 kDa with IFN λ 1 through a conjugation reaction as follows:

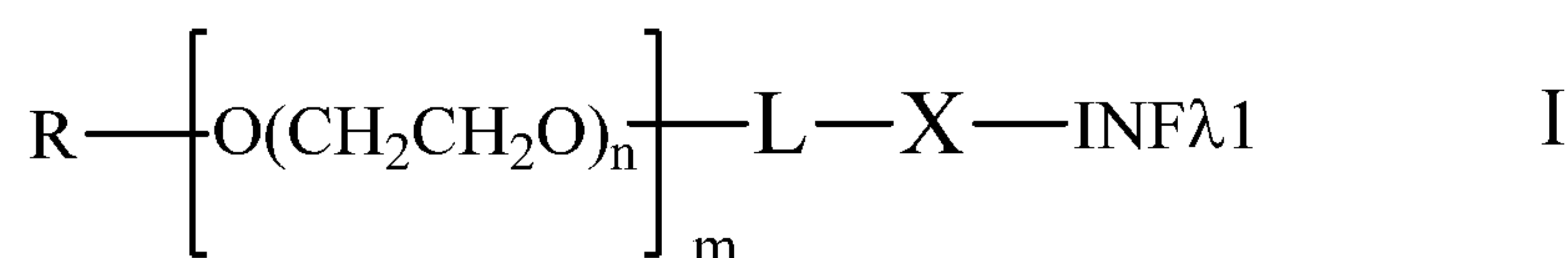


wherein, n is a positive integer selected such that the molecular weight of PEG moiety is about 40 kDa; and isolating the conjugate. In one aspect, n is from about 500 to 550.

[0021] In another embodiment, there is provided a pharmaceutical composition containing a conjugate as disclosed above and pharmaceutically acceptable carriers and excipients.

Conventional pharmaceutical formulations can be also prepared using the compositions comprising the conjugate of the present application. The formulations may comprise a therapeutically effective amount of the composition comprising the conjugate together with pharmaceutically acceptable carriers as known in the art. For example, adjuvants, diluents, preservatives and/or solubilizers, if needed, may be used. Pharmaceutical compositions comprising the conjugate may include diluents of various buffers (e.g., Tris-HCl, acetate, phosphate) having a range of pH and ionic strength, carriers (e.g., human serum albumin), solubilizers (e.g., polyoxyethylene sorbitan or TWEEN®, polysorbate), and preservatives (e.g., thimerosal, benzyl alcohol), as disclosed, for example, in U.S. Patent No. 4,496,537. In one aspect, the pharmaceutical composition is formulated as a sterile lyophilized powder for injection. In another aspect, the composition comprises a combination of pharmaceutically acceptable vehicles, including saline, buffered saline and 5% dextrose in water. In another aspect, the pharmaceutical composition is formulated as a solution for injection in vials or pre-filled syringes. In one aspect of the above, the pharmaceutical composition is used in the treatment of hepatitis B and hepatitis C. Pharmaceutical formulations and methods for preparing such formulations are well known in the art and are disclosed, for example, in *Remington, The Science and Practice of Pharmacy*, Gennaro, ed., Mack Publishing Co., Easton, Pa. 19th ed. 1995.

[0022] In another embodiment, there is provided a process for preparing a Peg-IFNλ1 conjugate comprising the formula I:



wherein: R is H or C₁₋₃ alkyl; m is 1, 2, 3 or 4; n is a positive integer selected in the range from 400 to 550; L is a C₁₋₁₀ alkyl or heteroalkyl linker; X is -O-, -NH- or -S-; and IFNλ1 is interferon lambda 1; or a pharmaceutically acceptable salt thereof; the process comprising: contacting the IFNλ1 with a pre-activated Peg under conditions that are sufficient to facilitate covalent conjugation with an amino acid residue of the IFNλ1. In one embodiment, there is provided a PEG-IFNλ1 conjugate prepared by the process as described herein. In one aspect, there is disclosed a method of preparing the above conjugate comprising contacting the IFNλ1 with a sufficient amount of an activated PEG or mPEG under conditions that are sufficient to facilitate covalent attachment of the PEG or mPEG on the IFNλ1. In another aspect, the activated mPEG is mPEG-pNC. In another aspect, the attachment of the activated mPEG is on a methionine at the N-terminal of the IFNλ1. In another aspect, the mPEG has a molecular weight of about 40 kDa. In another aspect, the activated oxycarbonyl agent is a mono- or di-activated agent.

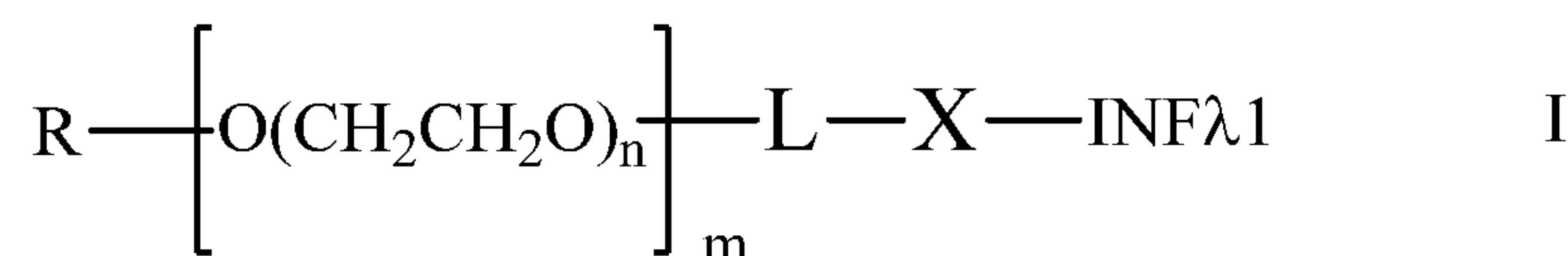
[0023] In another embodiment, there is provided a method for inhibiting the proliferation of a cancer cell in a patient comprising contacting the cancer cell with the conjugate described above, wherein the conjugate has a blood serum half-life and persistence time that are prolonged or extended when compared to IFNλ1. In one aspect, the conjugate of the present application has a blood serum half-life that is extended by more than twice, three times, five times, eight times or more than 10 times the serum half life of the corresponding unconjugated IFNλ1.

[0024] In another embodiment, there is provided a method for treating a proliferative disorder in a mammal comprising administering to the mammal a therapeutically effective amount of the above conjugate. In one embodiment, the conjugate may be used for treating interferon-susceptible conditions or conditions which would respond positively or favorably to interferon based therapy. In one aspect, the treatment using the conjugate results in substantially reduced or elimination of side effects when compared to conventional treatment with interferons.

[0025] In one aspect, exemplary conditions which can be treated with the conjugates of the present application include, but are not limited to, cell proliferation disorders, in particular cancer (e.g., hairy cell leukemia, Kaposi's sarcoma, chronic myelogenous leukemia, multiple myeloma, basal cell carcinoma and malignant melanoma, ovarian cancer and cutaneous T cell lymphoma), and viral infections. In another aspect, the conjugates may be used to treat conditions which would benefit from inhibiting the replication of interferon-sensitive viruses. Viral infections which may be treated with the conjugate of the present application include hepatitis A, hepatitis B, hepatitis C, other non-A/non-B hepatitis, herpes virus, Epstein-Barr virus (EBV), cytomegalovirus (CMV),

herpes simplex, human herpes virus type 6 (HHV-6)), papilloma, poxvirus, picomavirus, adenovirus, rhinovirus, human T lymphotropic virus-type 1 and 2 (HTLV-1/-2), human rotavirus, rabies, retroviruses including human immunodeficiency virus (HIV), encephalitis and respiratory viral infections.

[0026] In another embodiment, there is provided a method of treating a patient infected or at risk of infection with a viral infection, comprising administering to a patient in need thereof, a therapeutically effective amount of a conjugate of the formula I:



wherein: R is H or C₁₋₃ alkyl; m is 1, 2, 3 or 4; n is a positive integer selected in the range from 500 to 550; L is a C₁₋₁₀ alkyl or heteroalkyl linker; X is -O-, -NH- or -S-; and IFNλ1 is interferon lambda 1; or a pharmaceutically acceptable salt thereof; or a pharmaceutical formulation comprising the conjugate of the formula I. In one aspect, the conjugate interferon lambda 1 of the formula I is the SEQ ID 2. In one aspect, the mammal is a human. In another aspect of the method, the viral infection is caused by a hepatitis C virus, or the viral infection results in advance liver cirrhosis. In a particular aspect, the patient is an HCV resistant or refractory patient. In another aspect of the above method, the PEG-IFNλ1 is administered in a dose of about 0.5 μg/kg to 10.0 μg/kg weekly. In one aspect of the method, the PEG-IFNλ1 is administered in a dose of about 2.5 μg/kg weekly. In another aspect, the PEG-IFNλ1 is administered for about 8 weeks to about 52 weeks. In another aspect, the PEG-IFNλ1 is administered for about 12 weeks, about 16 weeks, about 20 weeks or about 24 weeks. In another aspect, the PEG-IFNλ1 is administered until the patient is determined to be free of HCV RNA in blood serum. In another aspect, the administration of the conjugate provides significant improvement over the standard PEG-INF-α therapy because the method does not result in the significant reductions in neutrophil counts, platelet counts or hemoglobin levels. In yet another aspect of the above method, the method further comprises the administration of a nucleoside analogue selected from ribavirin and viremagine. In another aspect of the method, the ribavirin is administered orally in a dose of 5 mg/kg to 25 mg/kg daily; or 15 mg/kg to 25 mg/kg daily. In one aspect, the ribavirin is administered in a dose of about 10 mg/kg to 30 mg/kg once or twice daily, or about 15 mg/kg daily once or twice daily. In one aspect of the above, the conjugate is administered parenterally.

[0027] In one embodiment, HBV may be treated using a dose of about 200 µg of the PEG-IFNλ1 conjugate per week, combined with tenofovir (tenofovir disoproxil fumarate) at about 300 mg per day. Under this treatment, it is determined that most patients are clear of HBV after about four injections over about 30 days. Under these conditions, the HBV is found to be suppressed, and the HBsAg (virus surface antigen) is released, which triggers the immune system to make the antibody against this antigen, resulting in the optimal endpoint in the particular treatment. The treatment may be continued as disclosed herein, for about 12 weeks to 24 weeks, depending on the patient's initial viral load.

Definitions:

[0028] "Alkyl" means a straight or branched, saturated or unsaturated, aliphatic radical having a chain of carbon atoms, optionally substituted with oxygen (e.g., a C₁ alkyl may be -C(O)-), nitrogen atoms (e.g., a C₁ alkyl may be -C(NH)-) or sulfur atoms (e.g., a C₂ alkyl may be -CH₂C(S)-). A C_X alkyl and C_{X-Y} alkyl, such as a C₁₋₁₀ alkyl or a C₁₋₆ alkyl, are typically used where X and Y indicate the number of carbon atoms in the chain. For example, C₁₋₆ alkyl includes alkyls having between 1 and 6 carbons (e.g., methyl, ethyl, propyl, isopropyl, butyl, isobutyl, vinyl, isopropenyl, 1-butenyl, ethynyl, 1-propynyl and the like).

[0029] A "heteroalkyl" or "heteroalkylene" is an alkyl that may have an oxygen, nitrogen or sulfur between the carbon atoms. Examples of such heteroalkyl groups include -C(O)NH-, -OC(O)-, -CH₂CH₂C(O)-NH-, -CH₂-O-CH₂-CH₂-, -CH₂-NH-CH₂-CH₂-, -CH₂-S-CH₂-CH₂- and -CH₂O-CH₂-CH₃ and the like.

[0030] The term "PEG" as in "PEG-IFNλ1" means polyethylene glycol as used in the art, and generally includes both alkyl-PEG such as mPEG (methoxy-polyethylene glycol) and PEG, unless specified otherwise.

[0031] A "therapeutically effective amount" is an amount of the PEG-IFNλ1 conjugate that is sufficient to produce a clinically significant change in the treated condition, such as a clinically significant change in the viral load or immune function, a significant reduction in morbidity or a significantly increased histological score, or combinations thereof.

[0032] As used herein, "treatment" or "treating" refers to a therapeutic treatment and prophylactic or preventive measures. Patients who are in need of treatment include patients already infected with hepatitis C virus as well as those in which the hepatitis C disease is to be prevented.

[0033] In one aspect of the above method, the conjugate is administered by injection or infusion. In another aspect, the conjugate is administered intravenously, intramuscularly, subcutaneously,

intradermally or intraperitoneally. In another aspect, the conjugate is administered to the patient in a dose amount selected from less than 0.5 µg/kg, 0.5 to 1.0 µg/kg, 1.0 to 1.5 µg/kg, 1.5 to 2.0 µg/kg, 2.0 to 2.5 µg/kg, 2.5 to 3.0 µg/kg, 3.0 to 3.5 µg/kg, 3.5 to 4.0 µg/kg, 4.0 to 4.5 µg/kg, 4.5 to 5.0 µg/kg, 5.0 to 5.5 µg/kg, 5.5 to 6.0 µg/kg, 6.0 to 6.5 µg/kg, 6.5 to 7.0 µg/kg, 7.0 to 7.5 µg/kg, 7.5 to 8.0 µg/kg, 8.0 to 8.5 µg/kg, 8.5 to 9.0 µg/kg, 9.0 to 9.5 µg/kg, 9.5 to 10.0 µg/kg, or greater than 10.0 µg/kg. In another aspect, the conjugate is administered in a fixed dose of about 60-80 µg, 80-100 µg, 100-120 µg, 120-140 µg, 140-160 µg, 160-180 µg, 180-200 µg, 200-220 µg, 220-240 µg, 240-260 µg, 260-280 µg, or about 280-300 µg. In one embodiment, the conjugate is administered subcutaneously at 200 µg for 12 consecutive weeks.

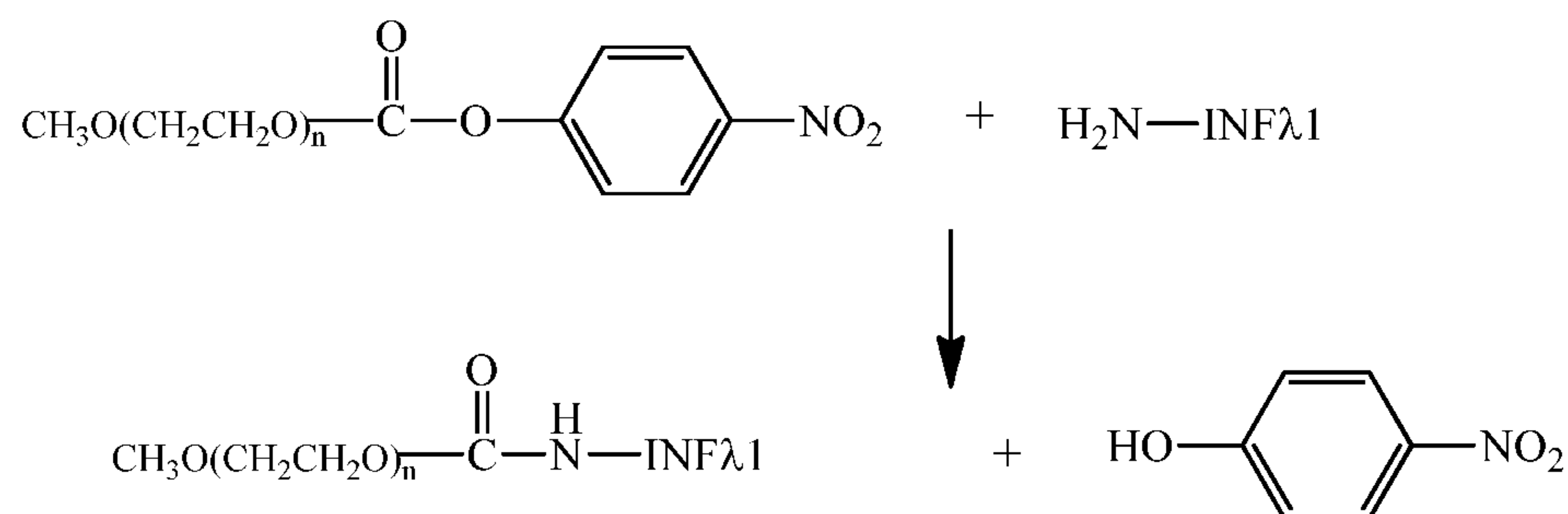
[0034] In another embodiment, there is provided a pharmaceutical composition containing the above conjugate and pharmaceutically acceptable carriers and excipients. In another aspect, the pharmaceutical composition is used in treatment of hepatitis B and hepatitis C. In another embodiment, there is provided a process for the preparation of a pharmaceutical composition containing the above conjugate comprising mixing the conjugate with pharmaceutically acceptable carriers and excipients.

[0035] The conjugates of the application have similar effects or activities as those of IFNλ1. For example, the conjugates may be used as anti-proliferative agents, antiviral agents, or antitumor agents. Specifically, the conjugates of the present application are effective in treatment of hepatitis B and hepatitis C, and they have a longer persistence time in blood than IFNλ1. In one embodiment, pharmaceutical compositions containing the conjugates of the present application are prepared as sterile lyophilized powders for injection, or as solutions for injection in vials or pre-filled syringes. These pharmaceutical compositions may be formulated by mixing the conjugates with relevant pharmaceutically acceptable carriers and excipients.

[0036] In another embodiment, the present application provides processes for the preparation of human recombinant PEG-IFNλ1 conjugates. First, human recombinant IFNλ1 is produced by recombinant DNA technology in *E.coli*, then reacted with a pegylating agent (such as α-methoxy-ω-(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC) to produce the PEG-IFNλ1. In one aspect, the PEG-IFNλ1 is a linear chain PEG 40 kDa that is conjugated to IFNλ1. This product bypasses the action of extracellular enzymes and kidney filtration when injected into the patient's body, therefore its blood serum half-life is extended.

[0037] The pegylation reaction between (α-methoxy-ω-(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC) 40 kDa and IFNλ1 to form the conjugate, is shown below. In one

aspect, the -NH₂ group is a methionine residue at the N-terminal on a site of the interferon lambda 1 molecule. In another aspect, the -NH₂ group is the amine of a lysine residue on a site of the interferon lambda 1 molecule.



[0038] In one embodiment, the conjugates of the present application may be prepared by covalently binding Interferon lambda 1 with pre-activated PEG. In one embodiment, PEG may be activated by substituting the PEG hydroxyl group with a linking group to form the coupling agent, or an activated PEG agent that is (α-methoxy-ω-(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC). In one embodiment, the PEG-INFλ1 conjugates may be prepared by the preparation of INFλ1 and the pegylation of the INFλ1. Also disclosed are processes for purifying and assaying the conjugated products.

Brief description of the drawings:

[0039] Figure 1 exemplifies a nucleic acid sequence (SEQ ID 1) used to produce human recombinant INFλ1 after the sequence was synthesized and introduced into the expression vector pNanogen 1-IL29.

[0040] Figure 2 is a representative amino acid sequence (SEQ ID 2) of human recombinant INFλ1 produced by Nanogen Pharmaceutical Biotechnology Co., Ltd.

[0041] Figure 3 exemplifies a plasmid pNanogen 1-IL29 containing the gene encoding human INFλ1 (interleukin-29).

[0042] Figure 4 depicts a result of analyzing plasmid pNanogen 1-IL29.

[0043] Figure 5 exemplifies a result of an electrophoresis process for examining the ability of *E. coli* containing pNanogen 1-IL29 used to produce INFλ1.

[0044] Figure 6 is a representative spectrum of the salt phase and SDS-PAGE electrophoresis after refolding protein. The spectrum of Figures 6, 7, 8 and 9 are all coomassi blue stained.

[0045] Figure 7 exemplifies a spectrum and SDS-PAGE electrophoresis after cation 1 phase.

[0046] Figure 8 exemplifies a spectrum and SDS-PAGE electrophoresis after cation 2 phase.

[0047] Figure 9 exemplifies a spectrum and SDS-PAGE electrophoresis after a gel filtration phase.

[0048] Figure 10 exemplifies a spectrum of the purification process and SDS-PAGE electrophoresis of PEG-interferon lambda 1.

[0049] Figure 11 exemplifies an identification results of IFN λ 1 and PEG-IFN λ 1.

[0050] Figure 12 exemplifies a Maldi-Tof mass-spectrum of PEG-IFN λ 1 produced by Nanogen Pharmaceutical Co., Ltd.

Detailed Description of the Invention

[0051] In one embodiment, the present application discloses processes for preparing a recombinant bacterial strain containing the gene encoding IFN λ 1, large scale or industrial manufacture of IFN λ 1, pegylation reaction of IFN λ 1, purification of the produced the PEG-IFN λ 1, and assays of PEG-IFN λ 1.

[0052] In one embodiment, the present application discloses an artificial synthesis of the gene encoding IFN λ 1 based on the published sequence available from the National Center for Biotechnology Information (the encoding gene was modified to conform to the industrial production process on *E.coli*), creating of the gene transfer vectors, introducing these vectors into the bacteria, and selecting the bacterial strain that best produced IFN λ 1.

[0053] In one embodiment, the industrial manufacturing process for IFN λ 1 includes the steps of: fermenting the initial material, collecting the solution of crude proteins and purifying the IFN λ 1 protein. In a representative process, the fermentation process may be carried out in a 10 liter fermenting tank containing a nutrient medium and production of IFN λ 1 was induced by lactose. The biomass obtained was separated and purified. IFN λ 1 was collected and refined through a number of steps including: refolding the protein, separating the protein, for example by ion exchange chromatography (cation 1 and cation 2), and refining the protein on a gel.

[0054] In one embodiment, the pegylation process comprises a reaction between the linear chain (α -methoxy- ω -(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC- with a molecular weight of 40 kDa) and IFN λ 1. The resulting conjugate product may be purified by chromatography, such as using an HPLC system, and tested for quality and purity.

[0055] The present application will be more fully appreciated by reference to the following examples, which are to be considered merely illustrative and not limiting to the scope of the invention as claimed.

Examples:

[0056] Example 1: Process for preparing *E. coli* strain containing the gene encoding human recombinant interferon lambda 1 (IFN λ 1)

[0057] The gene encoding IFN λ 1 was artificially synthesized based on the protein sequence data available from NCBI or other databases. The novel method provided herein reduces the time required to isolate the gene but still provides a result as accurate as the conventional method. The nucleic acid sequence used to produce IFN λ 1 in Nanogen Pharmaceutical Biotechnology Co., Ltd. is shown in Figure 1 and the amino acid sequence of this protein is shown in Figure 2.

[0058] The expression vector pNanogen-IL29 (comprising the T7 transcription promoter region, the IFN λ 1 transgene, the T7 reverse priming site, the T7 transcription terminator, the f1 origin, the kanamycin resistance gene, and the pUC origin of replication) was specifically designed to enable high expression of the protein and facilitate fermentation for industrial production of a large quantity of IFN λ 1. Figures 3, 4 show the process for creation of vector pNanogen 1-IL29.

[0059] Vector pNanogen 1-IL29 was then transferred into an *E. coli* strain suitable for expression of promoter T7. This strain has a genotype *F⁻ ompT hsdS_B (rB⁻mB⁻)gal dcm (DE3)*. The strain containing the IFN λ 1 gene is termed *E. Coli*-pNanogen1-IL29. It has the ability to produce higher than 100 mg of IFN λ 1 per litre by fermentation (see Figure 5) and was introduced into the original strain bank.

[0060] Example 2: Process for fermentation of *E. coli* to produce human recombinant IFN λ 1

[0061] The fermentation process was carried out in a 140 liter fermentation tank with nutrient medium at a temperature of 37 $\pm$ 0.5 °C, air pressure 0.5 m³/h, pH 7.0 $\pm$ 0.2, stirring rate of 300 rpm and the pH was maintained at between 6.8-7.2 by adding H₃PO₄ or NH₄OH. After 8 hours (when *E. coli* grew in log phase is the time that cells develop most efficiently), the temperature was cooled to 30 $\pm$ 0.5 °C and the stirring rate was reduced to 200 rpm to start the process for the generation of IFN λ 1. The fermentation process was stopped after 4 hours and the cold product was centrifuged at 6000 rpm to obtain biomass.

[0062] The biomass was disrupted in a cell lysis solution (12 ml solution per 1 g wet biomass) by homogenizing in a homogenizing device. The temperature was maintained at 4 °C for 1 hour, then the cells were disrupted 2 times by an ultrasonic device. The resulting suspension was centrifuged at 6000 rpm for 30 minutes to give a pellet. The pellet was then washed with an inclusion body wash buffer (12 ml buffer per 1g wet biomass), the resulting suspension was kept at 4 °C for 1 hour, then centrifuged twice at 13,000 rpm for 30 minutes to obtain a pellet. The pellet was

dissolved in 2M urea solution and incubated ice-cold for 1 hour, the suspension was then centrifuged at 13,000 rpm for 30 minutes to give the pellet. The pellet was dissolved in a wash solution and centrifuged at 13,000 rpm for 30 minutes to give a resulting pellet. The pellet was then dissolved in 6M guanidine solution, the suspension was kept ice-cold for 12-16 hours, and centrifuged at 13,000 rpm for 30 minutes. The solution containing protein was recovered and purified in next step.

[0063] The components of culture medium and solutions used to separate IFN λ 1 are shown in Table 1.

Table 1

Culture medium of <i>E. coli</i> - pNanogen1 – IL29 bacteria	- 70 μg/ml Kanamycin - 2 mM MgSO₄ - 0.1% aspartate - 25 mM Na₂HPO₄ - 25 mM KH₂PO₄ - 50 mM NH₄Cl - 5 mM Na₂SO₄ - 0.5% Glycerol - 0.05% Glucose - 0.2% α-lactose - 200 μg/ml each amino acid (18)
Cell lysis solution	- NaCl 50 mM - EDTA 1 mM - Tris base 20 mM
Inclusion body wash solution	- EDTA 1 mM - Tris base, pH 8, 20 mM - Triton X100 1%
Wash solution	- EDTA 1 mM - Tris base 20 mM
6M guanidine solution	- EDTA 2 mM - Tris base 50 mM - Guanidine 6M - Cysteine HCl 75 mM

[0064] Example 3: Process for purification of human recombinant IFN λ 1

[0065] IFN λ 1 was refolded by dissolving the inclusion bodies in refolding solution (25mM Tris buffer, 1mM EDTA, 1.2M guanidine, pH 8.2) such that the final concentration of the inclusion bodies were 500 μ g/ml. The mixture was then kept at 2-8 °C for 16-24 hours. The resulting mixture was desalted before being subjected to a purification step on a Sephadex G25 column. The salt exchange buffer was a phosphate buffer (10mM, pH 8.0). In the step “cation 1”, the

desalted mixture was loaded onto a Sephadex G25 column (this column was prefilled with CM-Sepharose FF gel and equilibrated in 10mM phosphate buffer pH 8.0), the product was eluted using 10mM sodium phosphate + 0.5M NaCl pH 8.0. The resulting protein solution was desalted and chromatographed as above (step "cation 2"). The protein solution was then filtered through a gel column to give the product human recombinant IFN λ 1 with purity greater than 95% (see the spectrum and electrophoresis results in Figures 5, 6, 7, 8 and 9).

[0066] Example 4: Pegylation Process for Preparing the Conjugate

[0067] The solution of 5 mg/ml human recombinant IFN λ 1 (MW~20.1 kDa) in 50mM sodium borate-phosphate pH 8.0 was added (α -methoxy- ω -(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC) (MW~40 kDa) at a molar ratio of PEG-pNC:IFN λ 1 about 3:1. The reaction mixture was kept at 2-4 °C for 20 hours. The reaction was stopped by adjusting the pH to 4.0 using 30%w/w acetic acid solution. The resulting mixture was then diluted 5-fold with water.

[0068] In a general exemplary process, the reaction conditions for the conjugation reaction of the activated PEG or m-PEG reagent to the IFN λ 1 further include conducting the reaction using about equi-molar to a relatively small molar excess of the activated PEG or m-PEG with respect to IFN λ 1. In one variation, the conjugation may be carried out with about 1-10 fold molar excess; or about 1.5 to 7 fold molar excess; or about 1.75 to 5 fold molar excesses. In one variation, the conjugation reaction can be carried out at about room temperature, or about 20-25 °C. The conjugation reaction may be allowed to proceed for about 1 to 10 hrs, 1 to 5 hrs, 1 to 3 hrs or about 1 to 2 hrs, before the reaction is terminated by quenching. In some cases, the reaction conditions provide a mixture of the PEG-IFN λ 1 positional isomers. In one aspect, each isomer contains a single PEG-linker unit attached to the IFN λ 1 via an amino acid residue as disclosed herein. In certain cases where more than one PEG-linker unit is attached to an IFN λ 1, if desired, the resulting composition containing these conjugates may be used or may be separated by chromatography using standard purification methods, including ultrafiltration, ion exchange chromatography, affinity chromatography and size exclusion chromatography. In one aspect, the purification method used for the separation and purification of the conjugates is cation exchange chromatography as described herein.

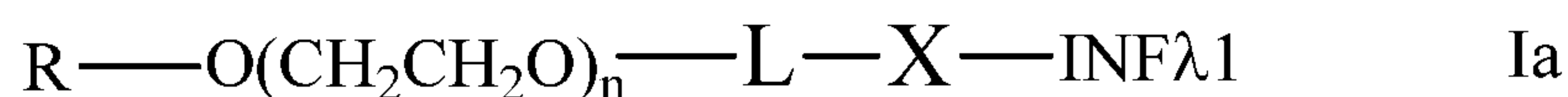
[0069] In certain conditions, the site of conjugation on the IFN λ 1 may be influenced by the pH of the reaction medium. Modification of the particular pH of the conjugation process will result in certain preferred sites of conjugation. For example, under certain conditions, the conjugation at

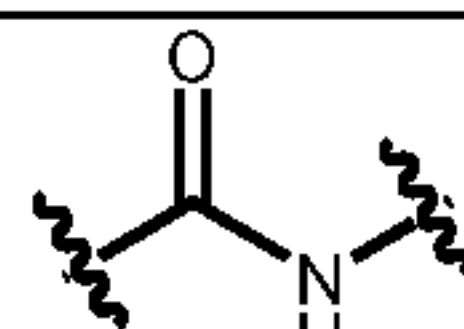
basic pH values, such as pH of 7.5 or higher, 8.0 or higher, 8.5 or higher or 9.0 or higher, favors the conjugation to a lysine group of the IFN λ 1.

[0070] In the above method, the pegylation reagent, such as PEG-pNC, forms a carbamate linker between the PEG and IFN λ 1. Additional pegylation reagents that may be employed in the above process include oxycarbonyl-oxy-N-dicarboximide (such as succinimidyl carbonate, succinimidyl succinate), para-nitroaryl carbonates, para-nitrophenyl carbonates, carbonyl di-imidazole, benzotriazole carbonates, pyridyl carbonates, N-succinimide, N-phthalimide, N-glutarimide, and N-tetrahydrophthalimide as disclosed in U.S. Patent No. 5,122,614. Representative activated PEG or mPEG compounds that may be used to form the conjugate include PEG-2,4,6-trichloro-S-triazine, mPEG-2,4,6-trichloro-S-triazine, PEG-N-succinimidyl glutarate, mPEG-N-succinimidyl glutarate, PEG-N-succinimidyl succinate and mPEG-N-succinimidyl succinate.

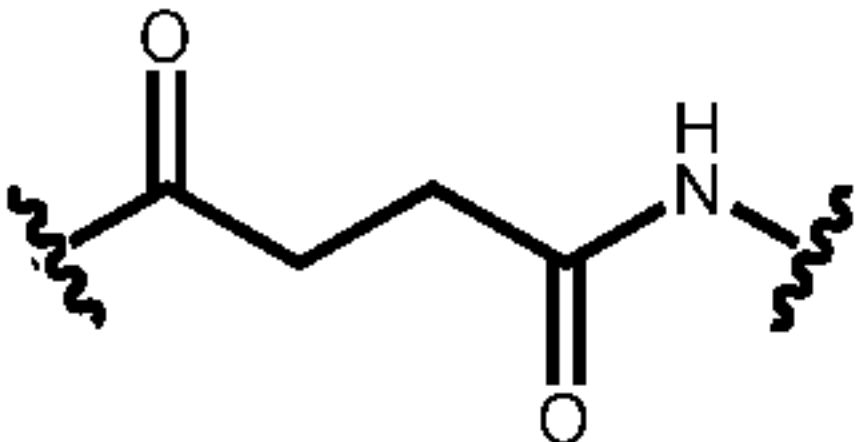
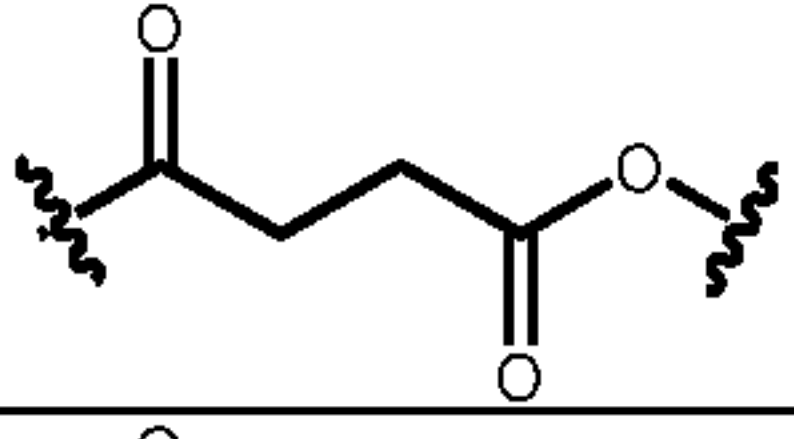
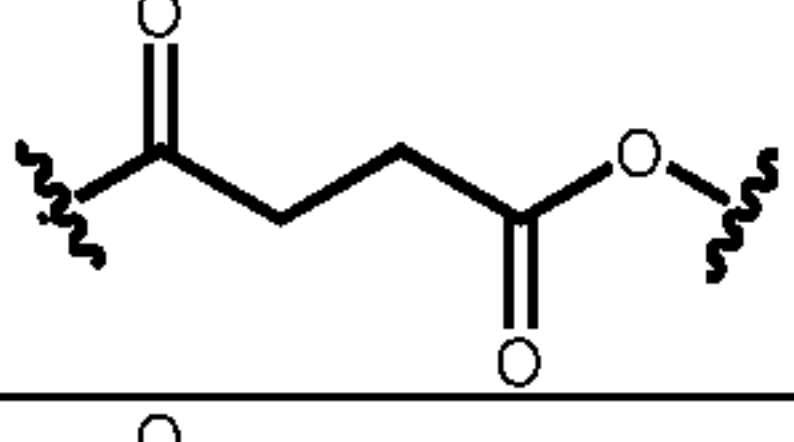
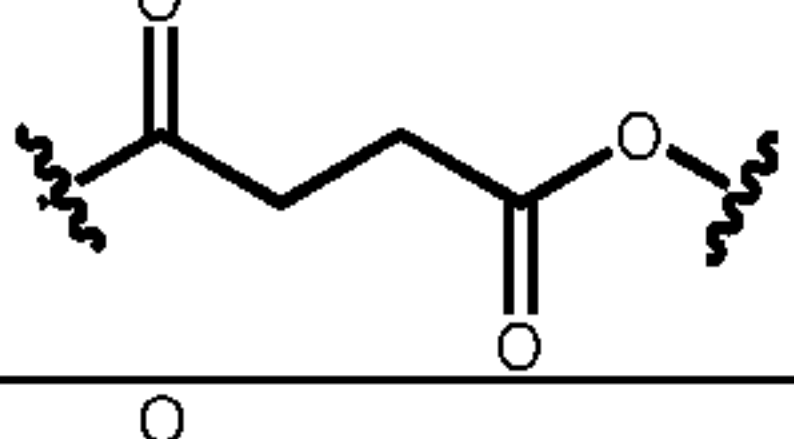
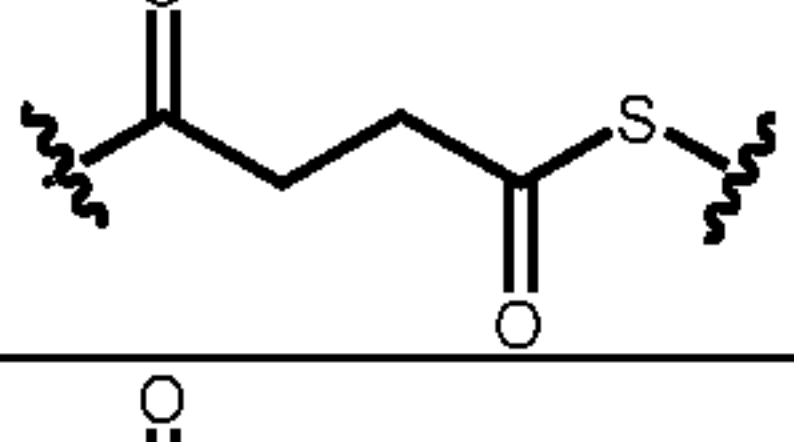
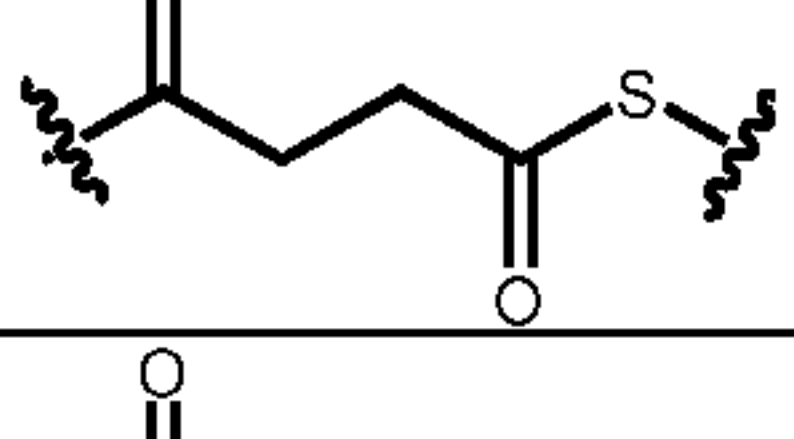
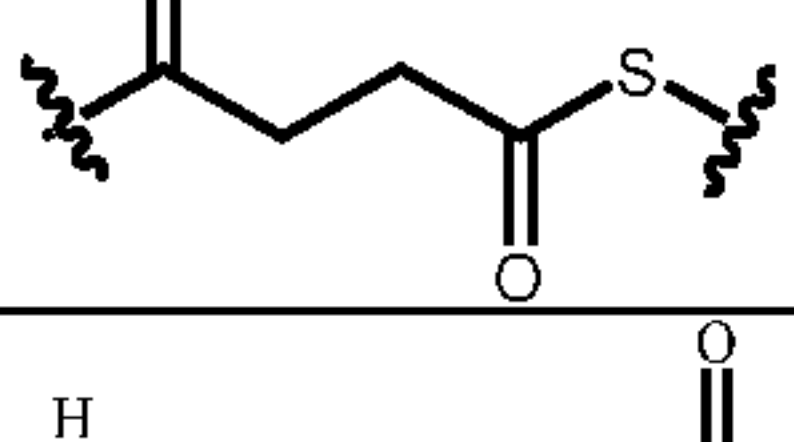
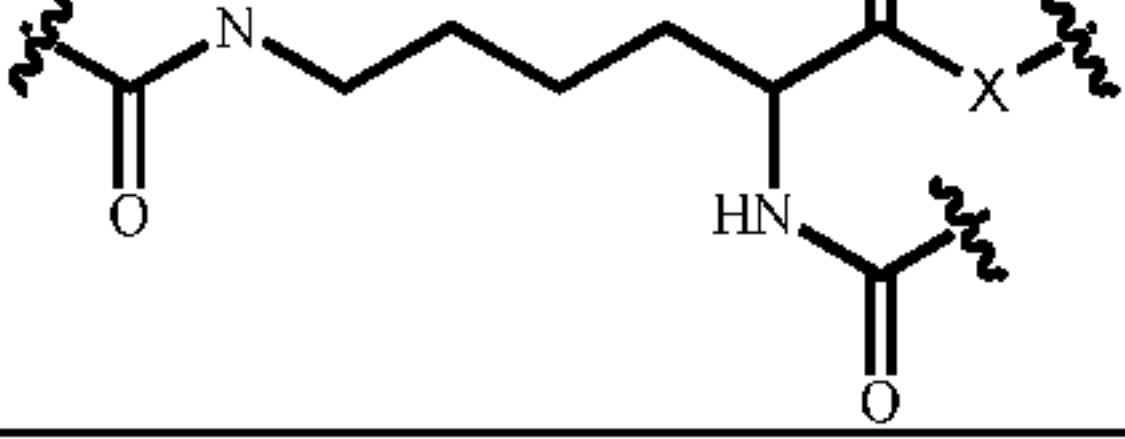
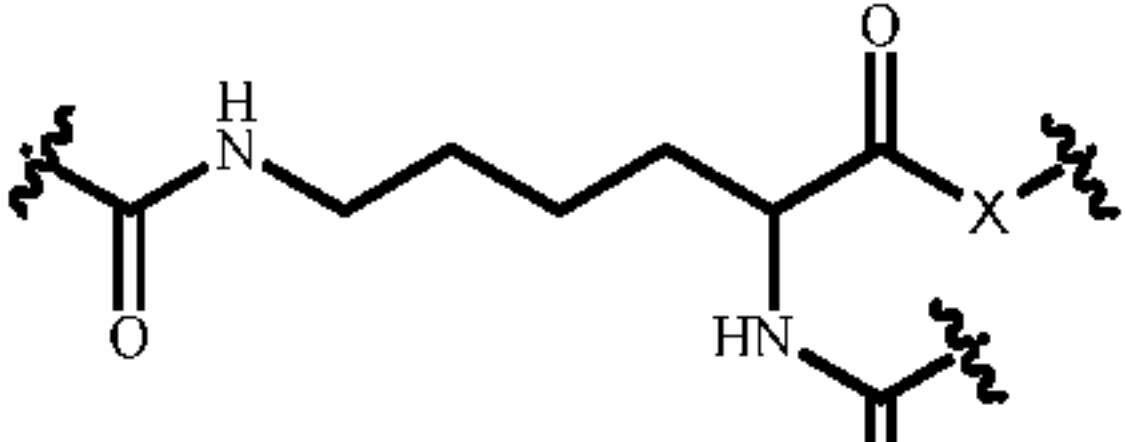
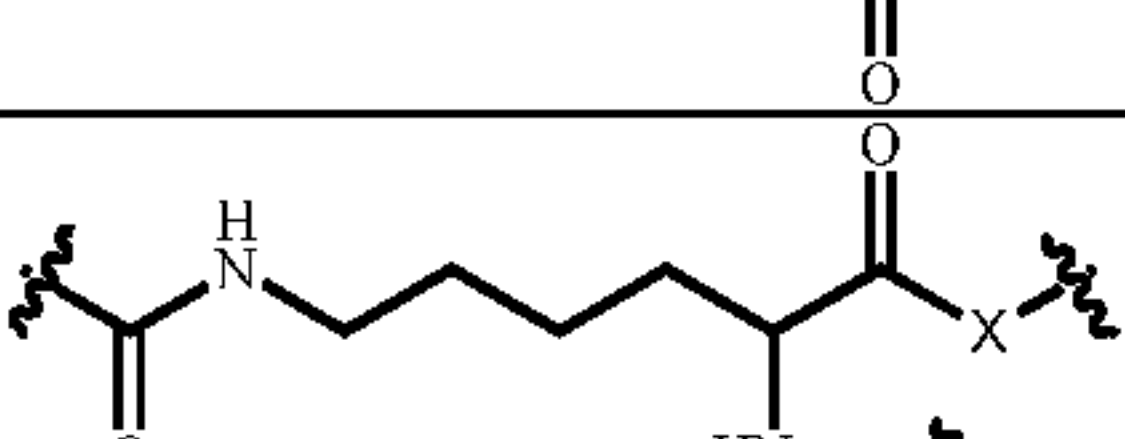
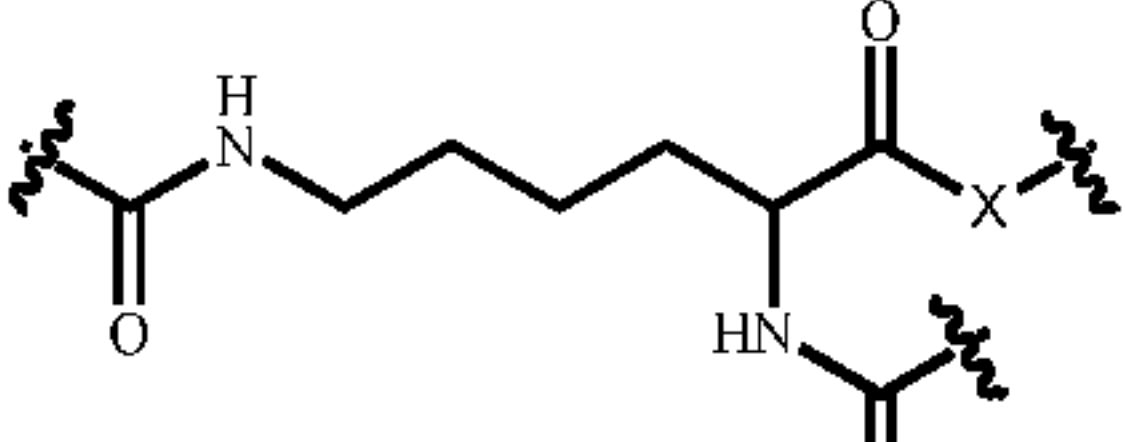
[0071] Representative Compounds of the Examples:

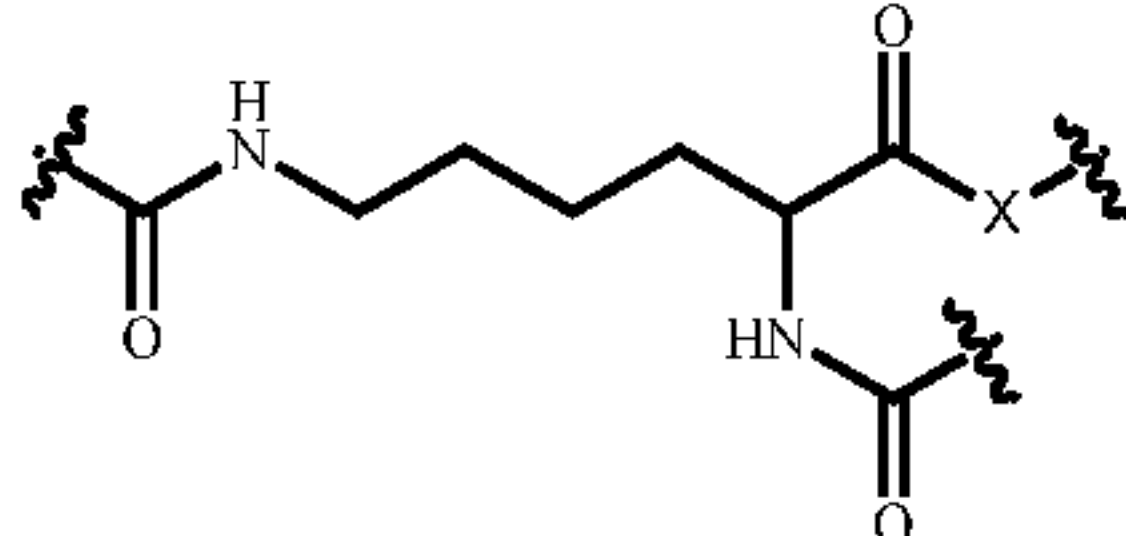
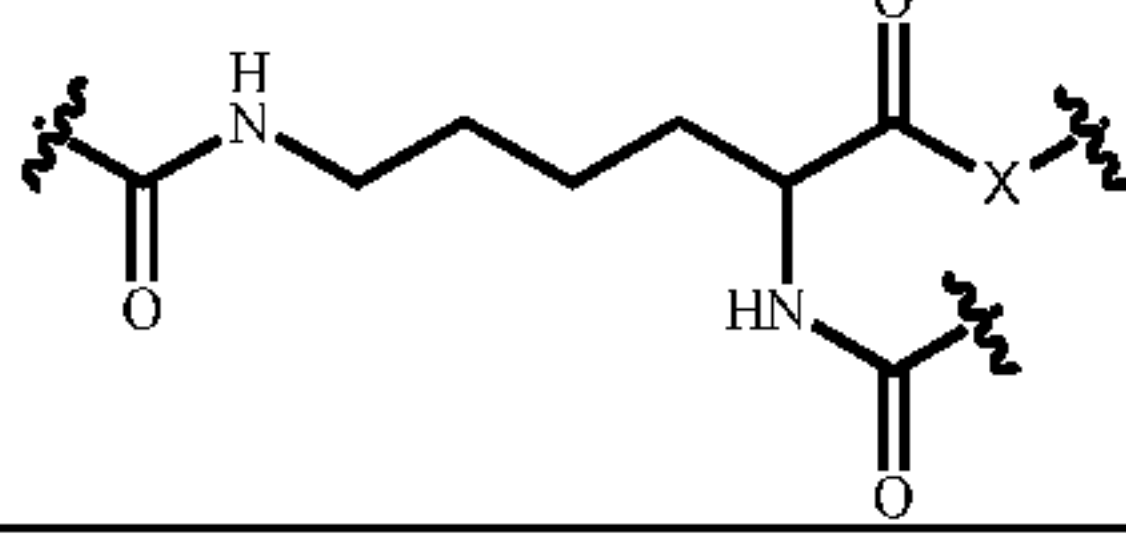
[0072] The following Table provides a summary of selected compounds of the Examples as described herein:



Conjugates	Variables			
	R	n (kDa)	-L-	-X-
1	CH ₃ -	900-945 (40)	-C(=O)-	-NH-
2	CH ₃ -	900-945 (40)	-C(=O)-	-O-
3	CH ₃ -	900-945 (40)	-C(=O)-	-S-
4	CH ₃ -	790-830 (35)	-C(=O)-	-NH-
5	CH ₃ -	790-830 (35)	-C(=O)-	-O-
6	CH ₃ -	790-830 (35)	-C(=O)-	-S-
7	CH ₃ -	1,010-1,060 (45)	-C(=O)-	-NH-
8	CH ₃ -	1,010-1,060 (45)	-C(=O)-	-O-
9	CH ₃ -	1,010-1,060 (45)	-C(=O)-	-S-
10	CH ₃ -	900-945 (40)		-NH-

Conjugates	Variables			
	R	n (kDa)	-L-	-X-
11	CH ₃ -	900-945 (40)		-O-
12	CH ₃ -	900-945 (40)		-S-
13	CH ₃ -	900-945 (40)		-NH-
14	CH ₃ -	900-945 (40)		-O-
15	CH ₃ -	900-945 (40)		-S-
16	CH ₃ -	900-945 (40)		-NH-
17	CH ₃ -	900-945 (40)		-O-
18	CH ₃ -	900-945 (40)		-S-
19	CH ₃ -	900-945 (40)		-NH-
20	CH ₃ -	900-945 (40)		-O-
21	CH ₃ -	900-945 (40)		-S-
22	CH ₃ -	900-945 (40)		-NH-
23	CH ₃ -	900-945 (40)		-O-
24	CH ₃ -	900-945 (40)		-S-
25	CH ₃ -	900-945 (40)		-NH-
26	CH ₃ -	900-945 (40)		-O-
27	CH ₃ -	900-945 (40)		-S-
28	CH ₃ -	900-945 (40)		-NH-
29	CH ₃ -	900-945 (40)		-O-

Conjugates	Variables			
	R	n (kDa)	-L-	-X-
30	CH ₃ -	900-945 (40)		-S-
31	CH ₃ -	900-945 (40)		-NH-
32	CH ₃ -	900-945 (40)		-O-
33	CH ₃ -	900-945 (40)		-S-
34	CH ₃ -	900-945 (40)		-NH-
35	CH ₃ -	900-945 (40)		-O-
36	CH ₃ -	900-945 (40)		-S-
37	CH ₃ -	900-945 (40)		-NH-
38	CH ₃ -	900-945 (40)		-O-
39	CH ₃ -	900-945 (40)		-S-
40	H-	900-945 (40)	-C(=O)-	-NH-
41	H-	900-945 (40)	-C(=O)-	-O-
42	H-	900-945 (40)	-C(=O)-	-S-
43	H-	900-945 (40)		-NH-

Conjugates	Variables			
	R	n (kDa)	-L-	-X-
44	H-	900-945 (40)		-O-
45	H-	900-945 (40)		-S-

[0073] Processes for the purification of the conjugates of the formula Ia in the above table are performed using the methods described herein. The resulting PEG-IFN λ 1 has a purity that is higher than about 95%. A spectrum and SDS-PAGE electrophoresis of the conjugate after a gel filtration phase is exemplified in Figure 9. A spectrum of the purification process and SDS-PAGE electrophoresis of the conjugate is exemplified in Figure 10. The conjugates have antiviral EMC activity on Hep-2C cell with ED₅₀ in a range of about 10-50 ng/ml. Antiviral activities of the conjugates of the formula Ia in the above table at ED₅₀ (ng/ml) are about 25.00 to 28.00; with a Mean (ng/ml) of about 1.0 to about 30.0; SD of about 0.1 to about 1.0 and RSD of about 3.0 to 7.0.

[0074] The conjugates of the formula Ia in the above table are administered to patients at 200 μ g (weekly subcutaneous injection) + ribavirin 15 mg/kg (daily). In the first 4 weeks, all patients are determined to be free of HCV RNA (free virus in serum). The treatment protocols are continued for 12 weeks. All patients achieve primary endpoint of total viral suppression after 12 weeks treatment and 12 weeks follow up.

[0075] Example 5: Purification of PEG-IFN λ 1

[0076] The solution containing PEG-IFN λ 1, quenched reagent and unmodified IFN λ 1 was purified on a cation column (this column was prefilled with Sepharose CM gel and equilibrated in 10mM sodium phosphate pH 6.0), eluted with a solution of 10mM sodium phosphate, 0.5M NaCl pH 6.0. The eluted fractions containing protein were transferred into preservative buffer using a solution of 10mM sodium phosphate pH 6.0. This product was then subjected to a sterile filtration process and stored at -20 °C.

[0077] Figure 10 shows the spectrum of the purification process and SDS-PAGE electrophoresis of IFN λ 1. The resulting PEG-IFN λ 1 had a purity that is higher than 95% and antiviral EMC activity on Hep-2C cell with ED₅₀ about 10-50 ng/ml (see example 6).

[0078] Example 6: Examination of antiviral activity of IFN λ 1 and PEG-IFN λ 1

[0079] The examination was based on the antiviral activity in EMC viral model and Hep-2C cell according to the study of Ank et al. (J Virol. 2006 May; 80(9):4501-9). The experiment was carried out with 3 lots (IL290010111, IL290020311 and IL290030411). The results indicated that the antiviral activity of Nanogen's interferon lambda 1 (ED₅₀ about 1-5 ng/ml) is equivalent to the study results of Sheppard et al. (Nat Immunol. 2003 Jan; 4(1):63-8.) (see Table 2).

[0080] The antiviral activity of PEG-IFN λ 1 was compared to IFN λ 1. The experiments were carried out with 5 lots (PIL290010111, PIL290020211, PIL290030311, PIL290040411, PIL290050511). Similar results were obtained in all lots, with an ED₅₀ about 10-50 ng/ml (see Table 3).

[0081] Patients' clinical information prior to treatment:

[0082] All patients in this treatment group were diagnosed with HCV chronic have been previously treated with Pegasys (PegInterferon alfa 2a) and PegIntron (PegInterfeon alfa 2b) combined with ribavirin (15 mg/kg) for over six months with no HCV RNA reduction of more than one log. HCV RNA >500,000 IU/ml serum; HCV Genotype 1-6; quantity 150; Age 26-78 years old; median age 52 yr; some patient with high Ferritin, low Platelet (<50,000/ml), low Hb. Most patients have high level of fibrosis on the Fibro scale of F4 due to being chronically infected with HCV, where high AST/ALT ratio over 1 indication of liver fibrosis. Some of the patients were under insulin treatment for diabetes. All patients in the treatment group that are over 50 years old have high blood pressure.

[0083] Treatment regimen:

[0084] Peglamda (PEG-IFN λ 1) 200 μ g (weekly subcutaneous injection) + ribavirin 15 mg/kg (daily). First 4 weeks, all patients were determined to be free of HCV RNA (free virus in serum). The treatment was continued for 12 weeks.

[0085] Results: All patients achieved primary endpoint of total viral suppression after 12 weeks treatment and 12 weeks follow up.

Table 2: Antiviral activity of Nanogen's interferon lambda 1 (PEG-IFN λ 1)

	Lot IL290010111	Lot IL290020311	Lot IL290030411
ED ₅₀ (ng/ml)	1.23	2.10	1.99
Mean (ng/ml)	1.97		
SD	0.13		
RSD	6.78		

Table 3: Antiviral activity of Nanogen's PEG-IFN λ 1

	Lot PIL 290010111	Lot PIL 290020211	Lot PIL 290030311	Lot PIL 290040411	Lot PIL 290050511
ED ₅₀ (ng/ml)	26.94	28.70	26.27	26.00	27.31
Mean (ng/ml)	27.04				
SD	1.06				
RSD	3.92				

[0086] Therapy for HCV Resistant Patients:

[0087] More than 50 patients who have been previously treated using standard therapies, such as a combination of PEGASYS® (peginterferon alfa-2a) with ribavirin, were found to be ineffective.

The non-responder patients, defined by the guidelines for HCV treatment from AASLD as patients who do not show a clearance of HCV RNA from serum from 24 weeks of therapy, and the null-responder patients, defined as those who show a failure to decrease HCV RNA at the 12th week by > 2 log, were enrolled in the treatment regiment using the PEG-IFN λ 1 of the present application.

[0088] After 4-12 weeks or 4-24 weeks of treatment with PEG-IFN λ 1 using the disclosed treatment protocol, substantially all patients were tested and determined to be HCV RNA negative; or all patients had a sustained virological response (SVR), defined as having no detectable virus 24 weeks after the final treatment dose.

[0089] In other studies using HCV resistant patients, the presently disclosed treatment protocol was found to be effective for greater than 80%, 85%, 90 % or greater than 95% of the HCV resistant patient population. Accordingly, the treatment methods using the PEG-IFN λ 1 demonstrate efficacy in HCV including cases of resistance to current standard therapy of peginterferon alfa-2a with ribavirin. No significant side effects that are typically associated with the combination therapy of PEGASYS® with ribavirin were observed.

[0090] Example 7: Identification of IFN λ 1 and PEG-IFN λ 1

[0091] Western blotting method was used to identify IFN λ 1 and PEG-IFN λ 1 using anti-IFN λ 1 antibody. The protein solution after being analyzed on an SDS-PAGE gel was transferred to a nitrocellulose membrane and probed with the anti-IFN λ 1 antibody. Antibody was detected with peroxidase coupled protein A and TMB substrate (see Figure 11).

[0092] Example 8: Molecular weight of PEG-IFN λ 1

[0093] The MALDI-TOF assay was applied to determine the molecular weight of PEG-IFN λ 1. The result is provided in Figure 12. In the present example, the Nanogen's PEG-IFN λ 1 has a molecular weight of approximately 62 kDa.

[0094] Example 9: Purity of PEG-IFN λ 1

[0095] Five lots (PIL290010111, PIL290020211, PIL290030311, PIL290040411, PIL290050511) were used to determine the purity of PEG-IFN λ 1 by SDS-PAGE electrophoresis. The electrophoresis gel was stained with coomassie blue, destained and then analyzed using Phoretix software (TotalLab, England). All tested lots showed purity higher than 95%.

[0096] The following tests employ the PEG-IFN λ 1 as prepared above:

[0097] Example 10: Toxicity of PEG-IFN λ 1

[0098] Acute toxicity of PEG-IFN λ 1: The acute toxicity of PEG-IFN λ 1 was assessed in Swiss mice and rats. Healthy ICR mice and Sprague-Dawley rats, at 5 week old, were chosen for the study. The animals were inspected for two weeks. PEG-IFN λ 1 was administered at three different dosages (high dose 3 mg/kg, medium dose 0.3mg/kg, low dose 0.03 mg/kg and the vehicle treatment (phosphate buffer saline, pH 7.2)) by subcutaneous or intraperitoneal injection. Animals were observed for clinical signs, body weight changes, and mortality 14 days after treatment. At the end of the study, all animals were sacrificed, and their tissues and organs were examined for abnormalities. The results are summarized in table 4.

Table 4

Experiment						Results		
Animal	Number of animals	Administration route	Administration period	Dose (mg/kg)	Volume (ml)	Body weight	Clinical signs	Autopsy result
Swiss mice	5 males, 5 females	Intraperitoneal injection	Once a week	3 0.3 0.03	0.5	No significant difference (*)	None	No abs. ¹
Rats	5 males, 5 females	Intraperitoneal injection	Once a week	3 0.3 0.03	1.0	No significant difference (*)	None	No abs. ¹
Swiss mice	5 males, 5 females	Subcutaneous injection	Once a week	3 0.3 0.03	0.5	No significant difference (*)	None	No abs. ¹

Experiment						Results		
Animal	Number of animals	Administration route	Administration period	Dose (mg/kg)	Volume (ml)	Body weight	Clinical signs	Autopsy result
Rats	5 males, 5 females	Subcutaneous injection	Once a week	3 0.3 0.03	1.0	No significant difference (*)	None	No abs. ¹

(*): ANOVA, single factor, compare to the vehicle treatment, ($p > 0.05$)

¹ means no abnormalities

[0099] All animals survived for the test period even at the highest dosage. The body weight did not significantly change in the treated animals compared to the control. There were no clinical signs or organ abnormalities observed in either group of the tested animals.

[00100] Based on these results, the lethal dose (LD50) of Nanogen's PEG-IFN λ 1 in mouse and rat was greater than 3 mg/kg. Subacute toxicity of PEG-IFN λ 1: Animals (5 weeks old rats) were administered PEG-IFN λ 1 at three different dosages (high dose 3 mg/kg, medium dose 0.3 mg/kg, low dose 0.03 mg/kg) by subcutaneous or intraperitoneal injection once a day for 4 weeks.

[00101] The rats were examined throughout the study for any clinical and behavioral adverse effects caused by Nanogen's PEG-IFN λ 1 administration. After the test period, the survived rats were sacrificed for autopsy and biochemical analyses. Blood samples were also collected from abdominal artery to conduct hematologic tests.

[00102] The test method and results are summarized in table 5.

Table 5

Animals	Administration route	Administration period	Dose (mg/kg)	Volume (ml)
Rats 5 males, 5 females	Subcutaneous injection	Once a day for 4 weeks	3 0.3 0.03	1.0
Rats 5 males, 5 females	Intraperitoneal injection	Once a day for 4 weeks	3 0.3 0.03	1.0
Examination/Analysis		Male rats	Female rats	
Clinical signs		none	none	
Body weight		Normal	Normal	
Food consumption		Normal	Normal	
Water consumption		Normal	Normal	
Urinalysis		Normal	Normal	
Hematology		Normal	Normal	

Urinalysis	Normal	Normal
Hematology	Normal	Normal
Serum biochemistry	Normal	Normal
Absolute and relative organ weight	Normal	Normal
Autopsy result	Normal	Normal
Histopathological examination	Normal	Normal

[00103] There was no death in any groups during the entire study and no clinical signs were detected from the tested rats. The tested rats were normal in other examination categories and analyses even in the high dosage group. Therefore, the study shows that Nanogen's PEG-IFN λ 1 does not have toxic effects in rats when it is administered repeatedly at the dosage of 3 mg/kg.

[00104] Immunological toxicity of PEG-IFN λ 1: A study was carried out to investigate immunologic potential of Nanogen's PEG-IFN λ 1 in guinea pigs. Healthy male Hartley guinea pigs with body weight of 300-500 gram were injected with PEG-IFN λ 1 twice a week for 3 weeks either at a high dose (3 mg/kg) or low dose (0.03 mg/kg) and ovalbumin as control. Fourteen days after the final sensitization, the anaphylaxis test was performed by intravenously injecting a high dose of PEG-IFN λ 1. The study included PEG-IFN λ 1 incorporated in Freund's complete adjuvant

(FCA). The sensitized guinea pigs were observed for active systemic anaphylaxis reactions after injection of a high dose PEG-IFN λ 1. A list of indications was used as a sign of anaphylactic reaction and their occurrence was monitored in each tested animal.

[00105] Table 6 shows the study method and results.

Table 6

Test groups	Dose	Administration route	Anaphylaxis challenge	No. of animals					
Negative control (PBS)	-	Subcutaneous	Intravenous injection of PBS	5 males					
Positive control (ovalbumin)	2 mg/kg	Subcutaneous	Intravenous injection of ovalbumin	5 males					
Low dose	0.03 mg/kg	Subcutaneous	Intravenous injection of 3 mg/kg PEG-IFNλ1	5 males					
High dose	3 mg/kg	Subcutaneous	Intravenous injection of 3 mg/kg PEG-IFNλ1	5 males					
High dose + FCA	3 mg/kg+ FCA	Subcutaneous	Intravenous injection of 3 mg/kg PEG-IFNλ1	5 males					
Anaphylaxis challenge	Animal	Physical observation ⁽¹⁾							
		1	2	3	4	5	6	7	8
PBS	1	-	-	-	-	+	-	-	-
	2	-	-	-	-	+	-	-	-
	3	-	-	-	-	+	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	+	-	-	-
OVALBUMIN (2mg/kg)	1	-	+	+	-	+	+	+	+
	2	-	+	+	-	-	-	-	-
	3	-	+	+	+	+	+	+	+
	4	-	+	+	+	+	+	+	+
	5	-	+	+	+	-	-	-	-

Peg-IFN λ 1 (0.03mg/kg)	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	+	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	+	-	-	-
	5	-	-	-	-	+	-	-	-
Peg-IFN λ 1 (3mg/kg)	1	-	-	-	-	+	-	-	-
	2	-	-	-	-	+	-	-	-
	3	-	-	-	-	+	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	+	-	-	-
Peg-IFN λ 1 (3mg/kg) + FCA	1	-	+	+	+	-	-	-	-
	2	-	+++	+++	-	+	-	-	-
	3	-	-	+	+	+	-	-	-
	4	-	+	+	-	+	-	-	-
	5	-			+	+	-	-	-

⁽¹⁾ 1. Licking nose, rubbing nose; 2. Ruffling fur;
3. Labored breathing; 4. Sneezing, coughing;
5. Evacuation of feces, micturition; 6. Convulsion; 7. Prostration;
8. Death. -, negative ; +, positive

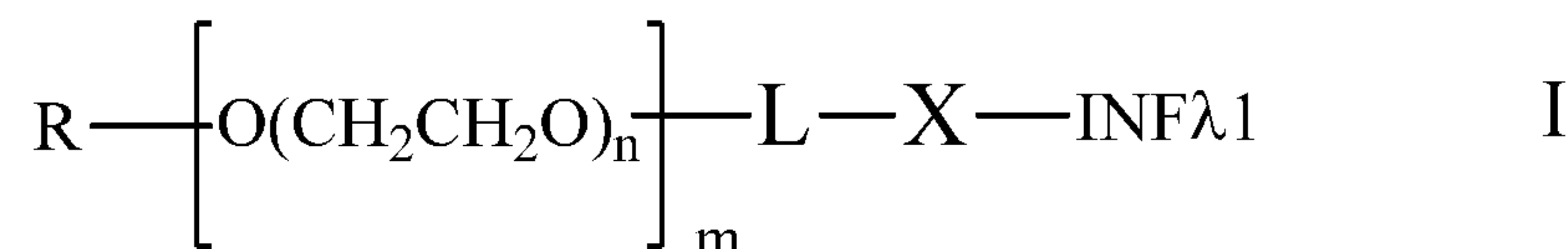
[00106] In the active systemic anaphylactic test, the guinea pigs slightly sensitized with high dose of PEG-IFN λ 1 (3 mg/kg) incorporated in Freund's complete adjuvant (FCA) showed some indications of anaphylactic reaction. On the other hand, no guinea pigs sensitized with low dose and high dose of PEG-IFN λ 1 (0.03 and 3 mg/kg) alone showed any anaphylactic reaction. No guinea pigs were dead after administration with Nanogen's PEG-IFN λ 1 and negative treatment (PBS), but 3 pigs were dead after administration with ovalbumin (positive treatment). Therefore, it can be concluded that Nanogen's PEG-IFN λ 1 does not induce systemic allergic reaction when administered alone in its clinical use.

[00107] It will be apparent to those skilled in the art that various modifications and variations can be made to the compounds, compositions, and methods of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

What is claimed:

Claims:

1. A physiologically active PEG-IFN λ 1 conjugate comprising the formula I:



wherein:

R is H or C₁₋₃ alkyl;

m is 1, 2, 3 or 4;

n is a positive integer selected in the range from 400 to 550;

L is a C₁₋₁₀ alkyl or heteroalkyl linker;

X is -O-, -NH- or -S-; and

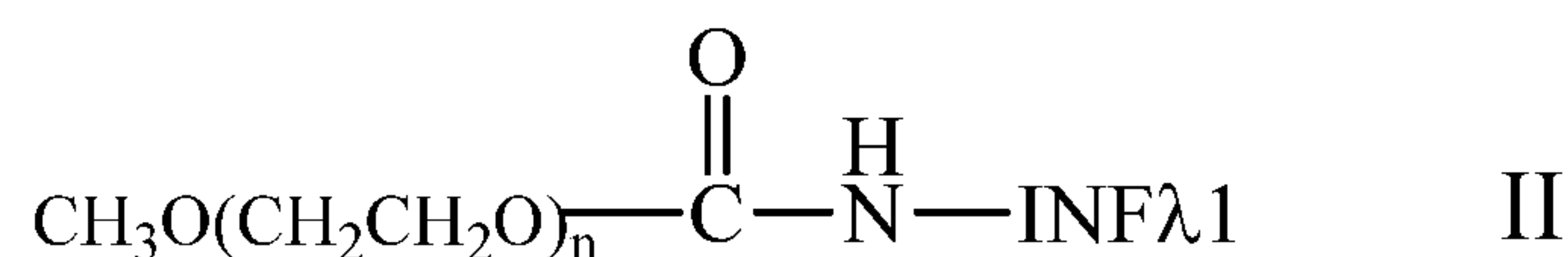
INF λ 1 is interferon lambda 1;

or a pharmaceutically acceptable salt thereof.

2. The conjugate of Claim 1, wherein R is H or -CH₃, m is 1, L is -C(O)- and X is -NH-.

3. The conjugate of Claim 1 or 2, wherein n is 500 to 550.

4. The conjugate of Claim 1 comprising the formula:



wherein INF λ 1 is interferon lambda 1 of SEQ ID2; and

n is 500 to 550.

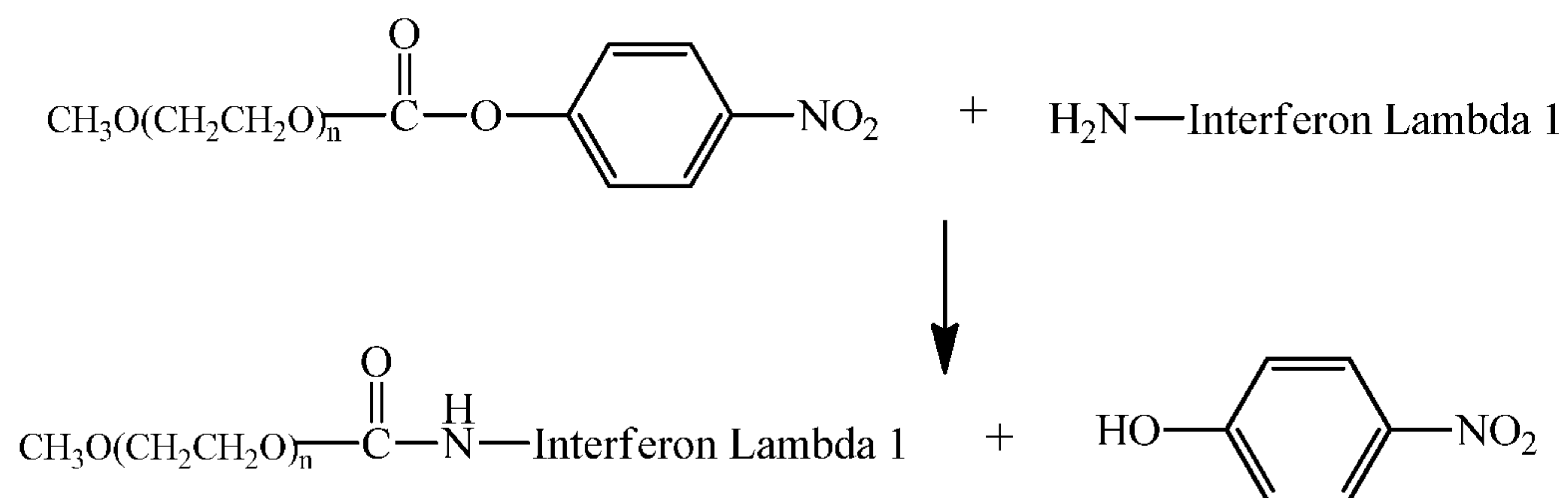
5. The conjugate of any one of Claims 1 to 4, wherein the conjugate has a blood serum half-life and persistence time that are prolonged or extended when compared to INF λ 1.

6. The conjugate of any one of Claims 1 to 5, wherein the PEG is attached to the methionine at the N-terminal of the INF λ 1.

7. A pharmaceutical composition containing a conjugate according to any one of Claims 1 to 6 and pharmaceutically acceptable carriers and excipients.

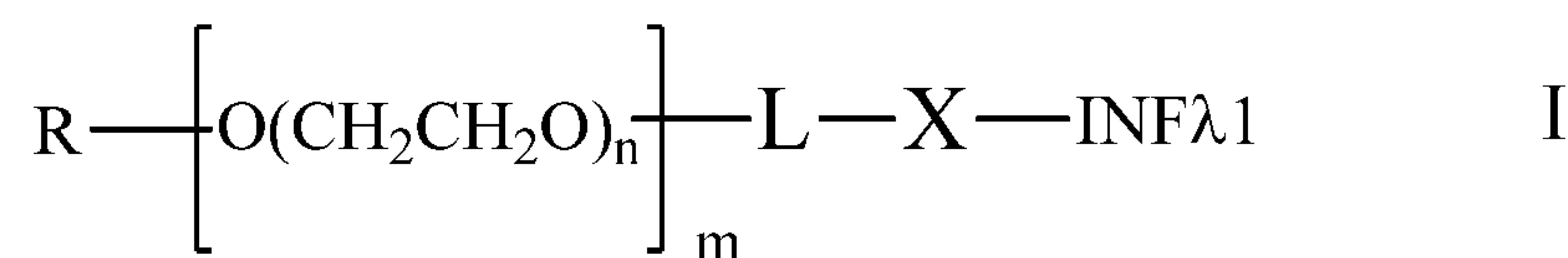
8. The pharmaceutical composition of Claim 7, wherein the pharmaceutical composition is used in the treatment of hepatitis B and hepatitis C.

9. A process for preparation of a human recombinant conjugate according to Claim 1, comprising the step of covalently binding (α -methoxy- ω -(4-nitrophenoxy carbonyl)) polyoxyethylene (PEG-pNC) 40 kDa with IFN λ 1 through a conjugation reaction as follows:



wherein, n is a positive integer selected in the range from 500 to 550 such that the molecular weight of PEG moiety is about 40 kDa; and isolating the conjugate.

10. A process for preparing a Peg-IFN λ 1 conjugate comprising the formula I:



wherein:

R is H or C₁₋₃ alkyl;

m is 1, 2, 3 or 4;

n is a positive integer selected in the range from 400 to 550;

L is a C₁₋₁₀ alkyl or heteroalkyl linker;

X is —O—, —NH— or —S—; and

IFN λ 1 is interferon lambda 1;

or a pharmaceutically acceptable salt thereof;

the process comprising:

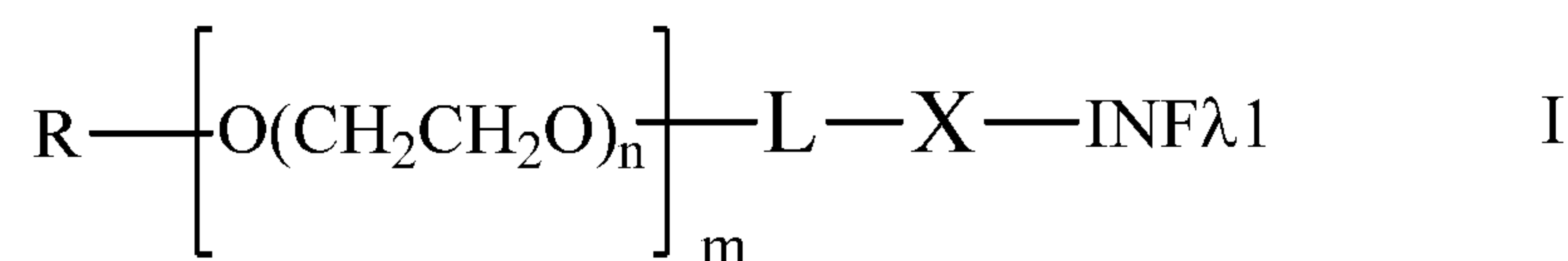
contacting the IFN λ 1 with a pre-activated Peg under conditions which are sufficient to facilitate covalent conjugation with an amino acid residue of the IFN λ 1.

11. The PEG-IFN λ 1 conjugate prepared by the process of Claim 10.

12. A method for inhibiting the proliferation of a cancer cell in a patient comprising contacting the cancer cell with the conjugate of any one of Claims 1 to 4, wherein the conjugate has a blood serum half-life and persistence time that are prolonged or extended when compared to IFN λ 1.

13. A method for treating a proliferative disorder in a mammal comprising administering to the mammal a therapeutically effective amount of the conjugate of Claim 1.

14. A method of treating a patient infected or at risk of infection with a viral infection, comprising administering to a patient in need thereof, a therapeutically effective amount of a conjugate of the formula I:



wherein:

R is H or C₁₋₃ alkyl;

m is 1, 2, 3 or 4;

n is a positive integer selected in the range from 500 to 550;

L is a C₁₋₁₀ alkyl or heteroalkyl linker;

X is -O-, -NH- or -S-; and

IFN λ 1 is interferon lambda 1;

or a pharmaceutically acceptable salt thereof; or a pharmaceutical formulation comprising the conjugate of the formula I.

15. The method of Claim 14, wherein R is -CH₃, m is 1, L is -C(O)-, X is -NH- and IFN λ 1 is of SEQ ID 2.

16. The method of Claim 14 or 15, wherein the viral infection is caused by a hepatitis C virus or the viral infection results in advance liver cirrhosis.
17. The method of any one of Claim 14, 15 or 16, wherein the PEG-IFN λ 1 is administered in a dose of about 0.5 μ g/kg to 10.0 μ g/kg weekly.
18. The method of any one of Claims 14 to 17, further comprising the administration of a nucleoside analogue selected from ribavirin and viremagine.
19. The method of Claim 18, wherein the ribavirin is administered in a dose of 5 mg/kg to 25 mg/kg daily.
20. The method of any one of Claims 14 to 17, wherein the patient is an HCV resistant or refractory patient.

Drawings

SEQ ID 1:

ATGGGCCCTGTTCCGACCTCTAAACCTACCACCACGGGTAAGGGTTGTCATATTGGTC
GTTTCAAGTCTCTGTCCCCGCAGGAAGTGGCCTCTTTCAAGAAAGCTCGTGATGCCCT
GGAAGAGTCTCTGAAGCTGAAAAAAGTGGAGCTGTTCTTCCCCGGTGTTCCTCCGGGCAAT
TGGGACCTGCGCCTGCTGCAGGTTTCGCGAACGCCCCGGTGGCTCTGGAAGCTGAGCTG
GCTCTGACCCTGAAAGTACTGGAAGCGGCGGCTGGTCCGGGCCCTGGAAGACGTTCTG
GATCAGCCGCTGCACACCCTGCATCATATTCTGTCCCAGCTGCAGGCTTGCATCCAGC
CTCAGCCAACCGCCGGTCCGCGTCCACGTGGCCGTCTGCACCACTGGCTGCATCGCCT
GCAGGAAGCGCCGAAAAAGGAGAGCGCTGGCTGCCTGGAAGCAAGCGTAACCTTTA
ACCTGTTCCGTCTGCTGACTCGTGACCTGAAATATGTTGCAGATGGCAATCTGTGCCT
GCGTACCTCCACCCACCCGGAATCCACCTGA

Figure 1

SEQ ID 2:

MetGPVPTSKPTTTGKGCHIGRFKSLSPQELASFKKARDALEESL
KLKNWSCSSPVFPGNWDLRLLQVRERPVALEAELATLKVLEA
AAGPALEDVLDQPLHTLHHILSQLQACIQPQPTAGPRPRGRLHH
WLHRLQEAPKKESAGCLEASVTFNLFRLLTRDLKYVADGNLCL
RTSTHPEST

Figure 2

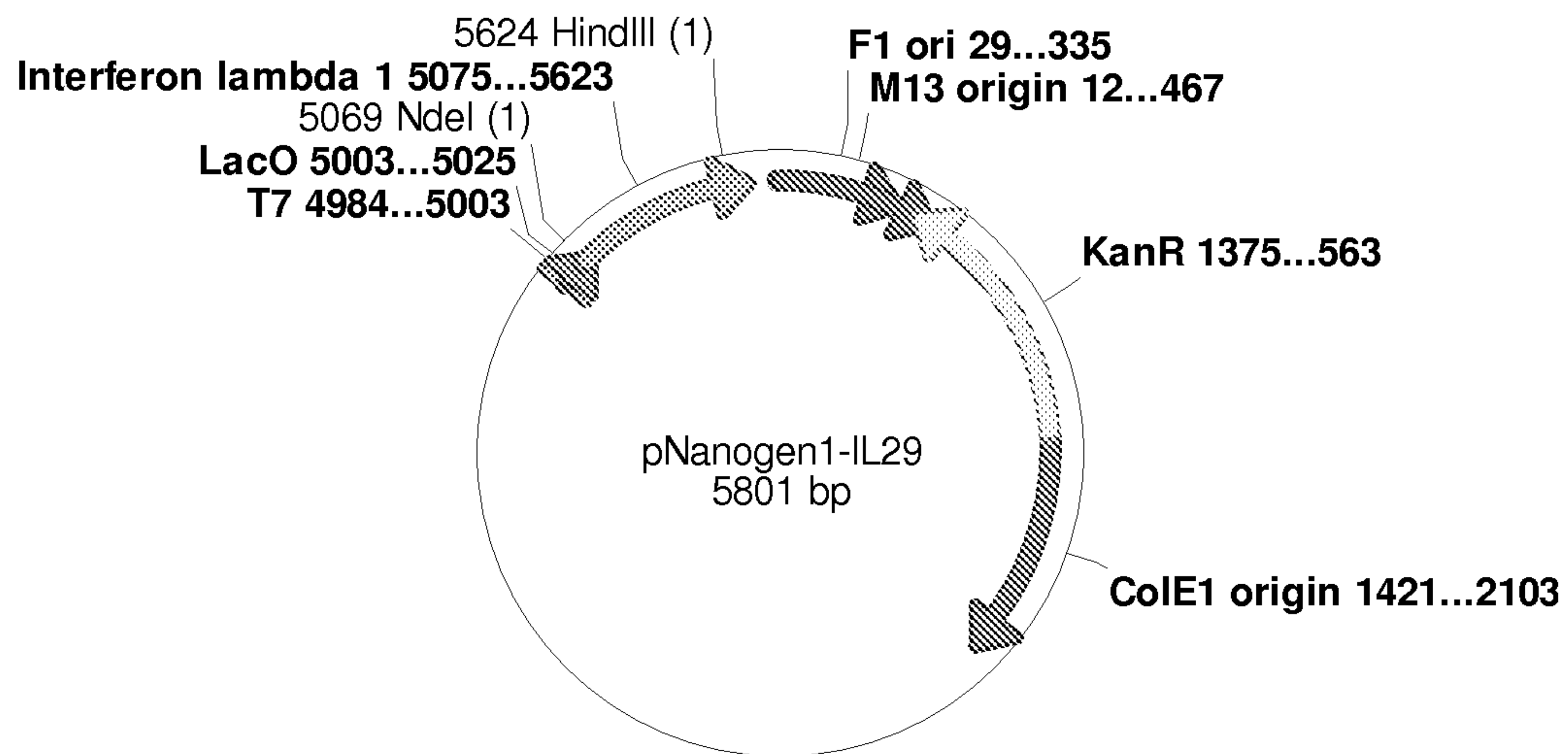
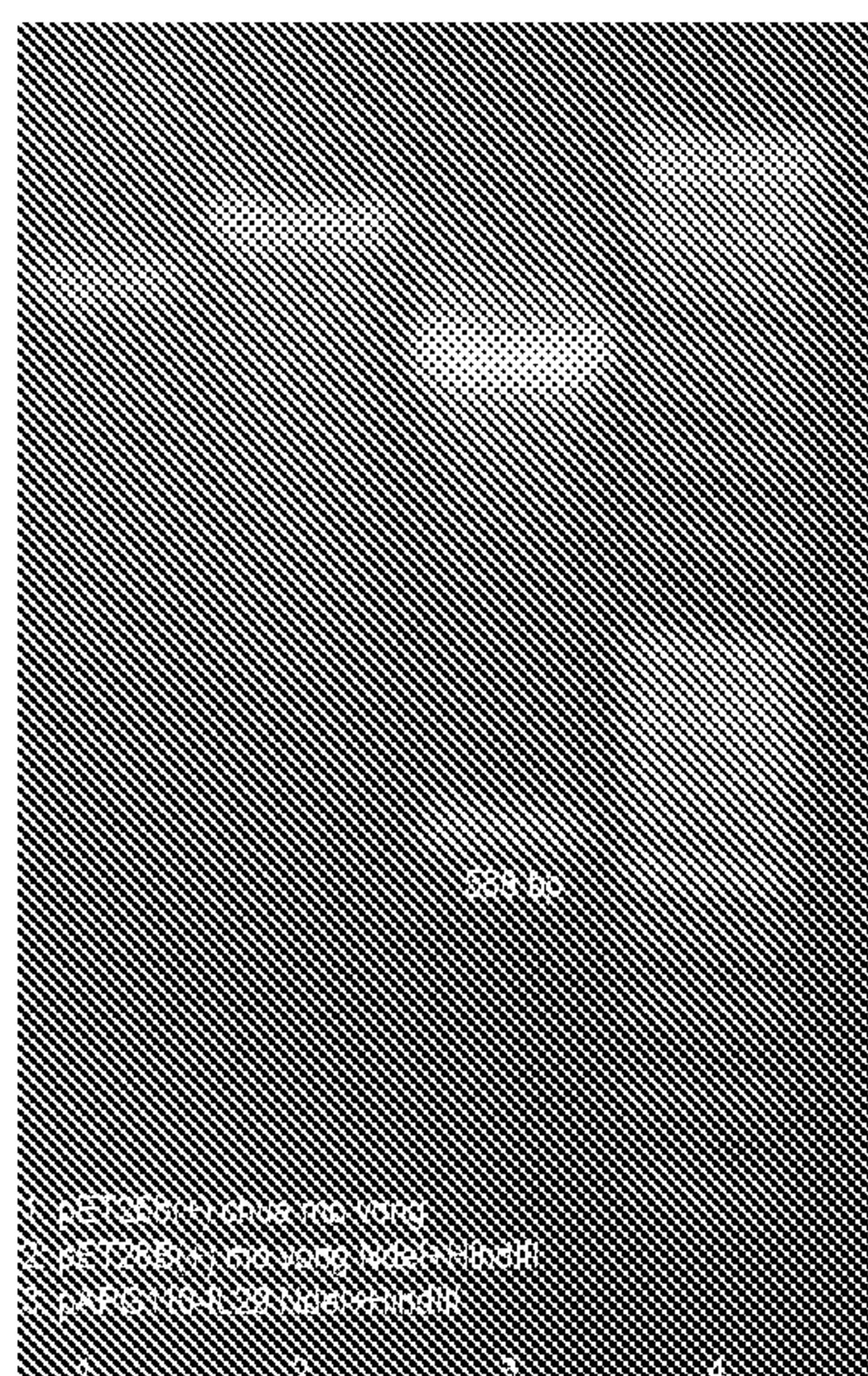
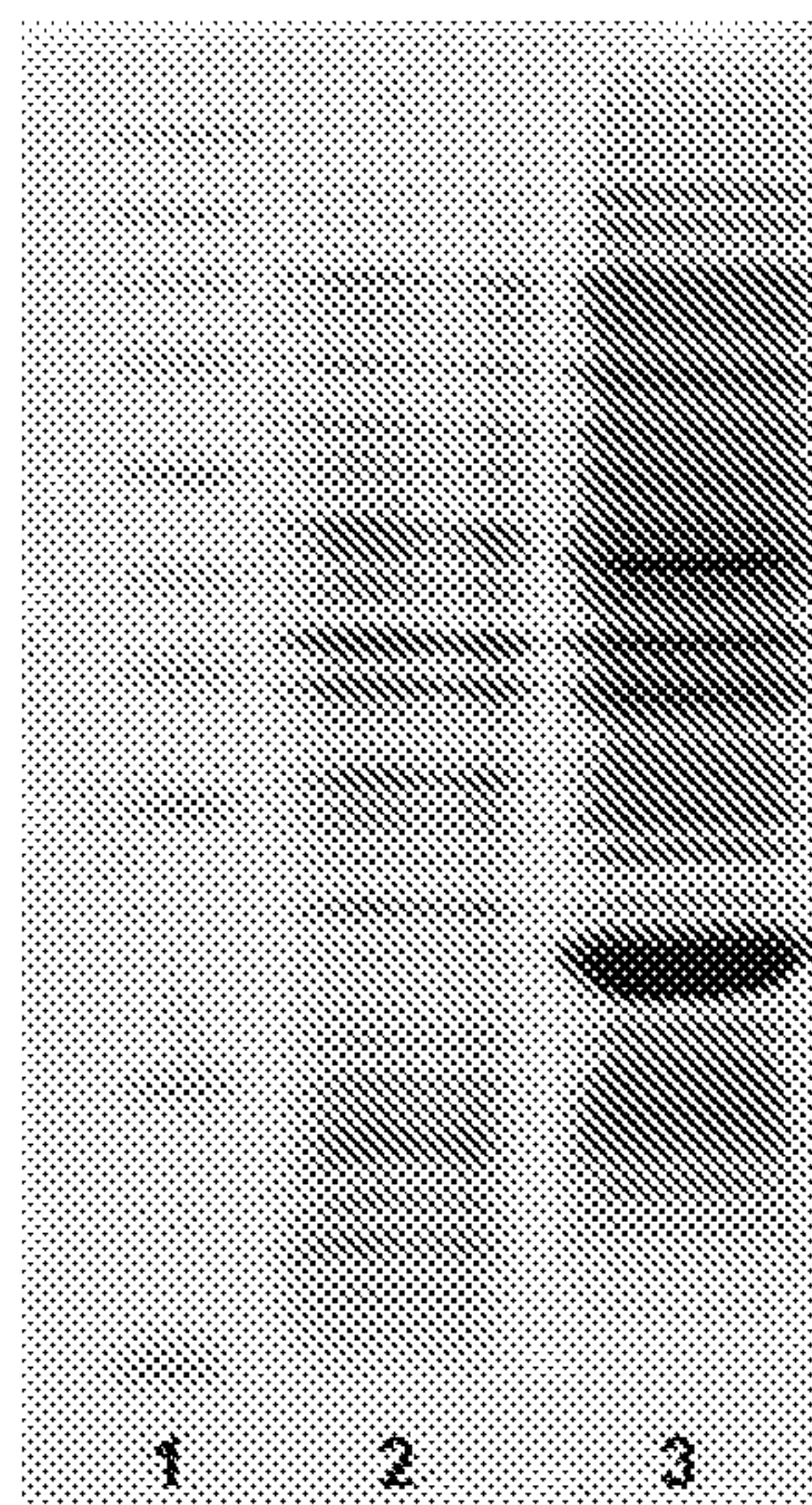


Figure 3



- 1: Vector pET26b(+)
- 2: Vector pET26b(+) digested by NdeI and HindIII
- 3: Vector PAPG110-IFN lambda digested by NdeI and HindIII
- 4: DNA massRuler pattern of Fermentas

Figure 4



1: Protein pattern of Fermentas
2: *E.coli*-pNanogen1-IL29 uninduced
3: *E.coli*-pNanogen1-LI29 induced by IPTG

Figure 5

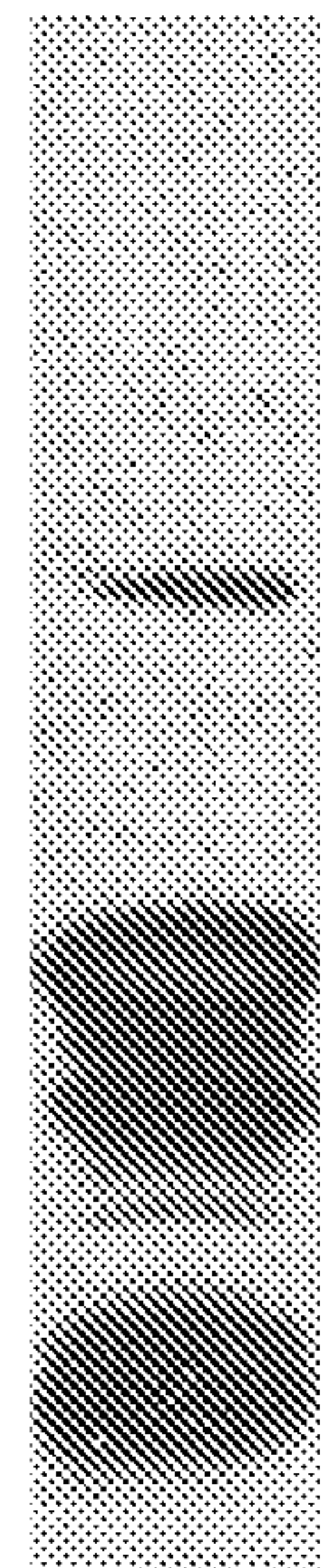
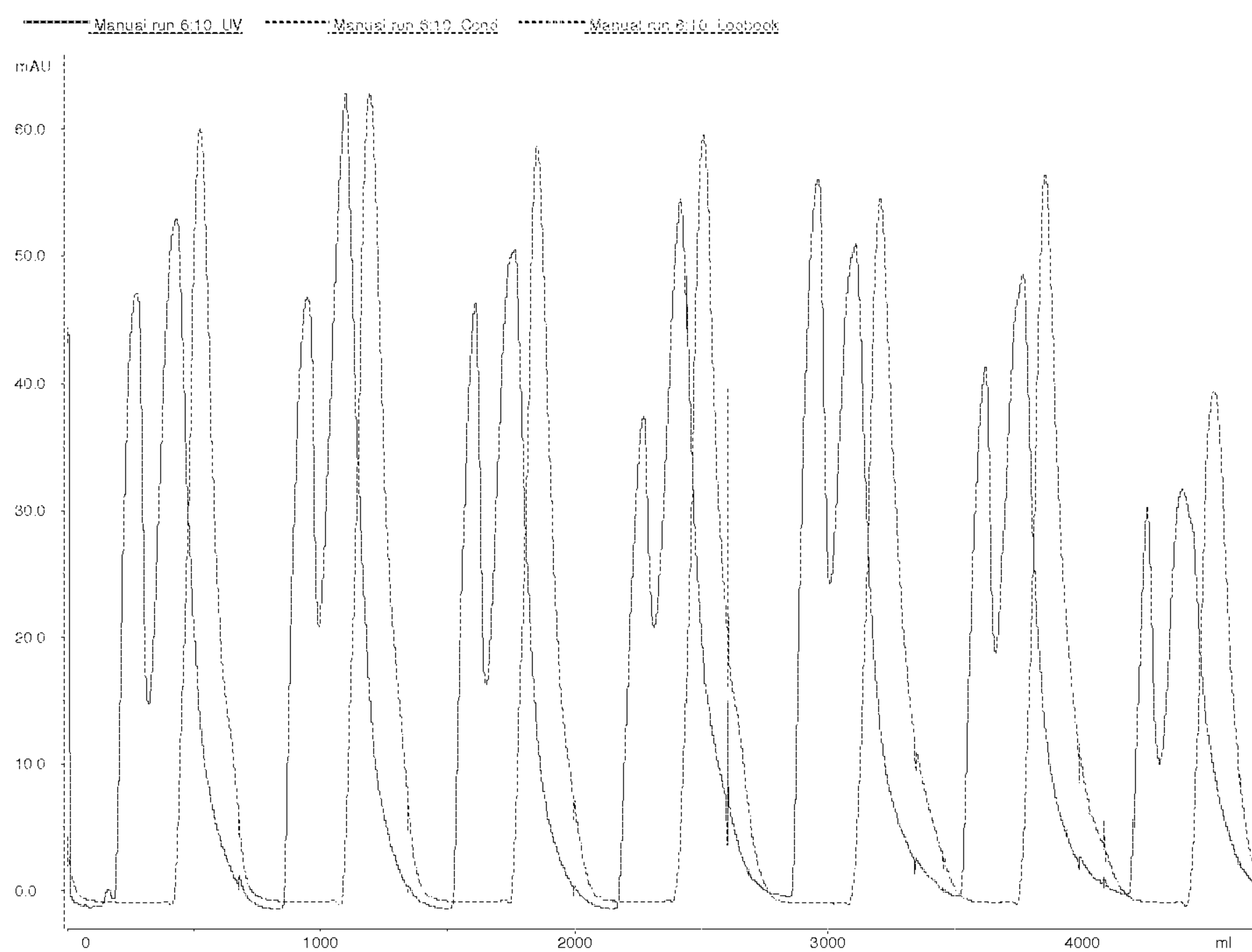


Figure 6

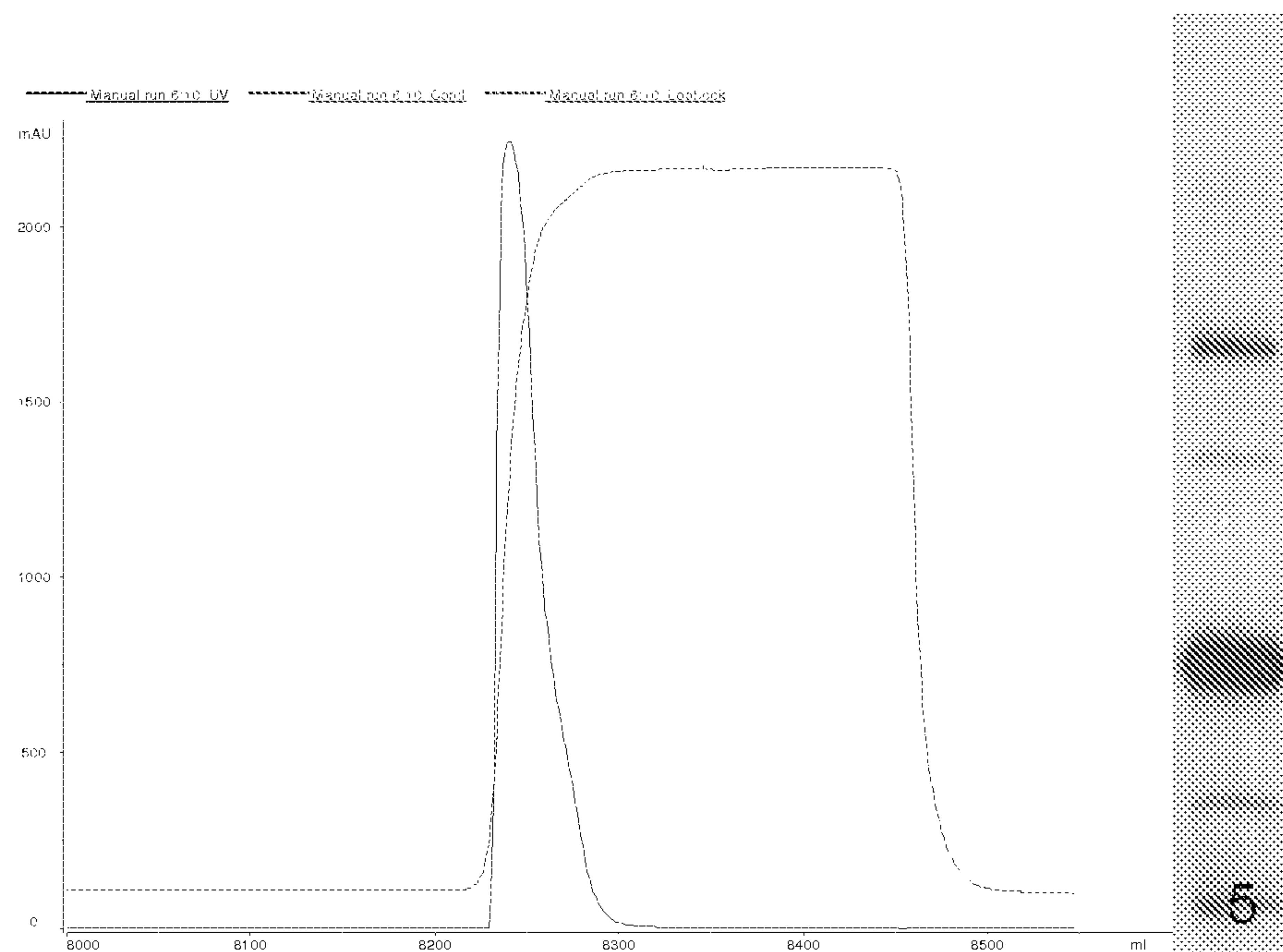


Figure 7

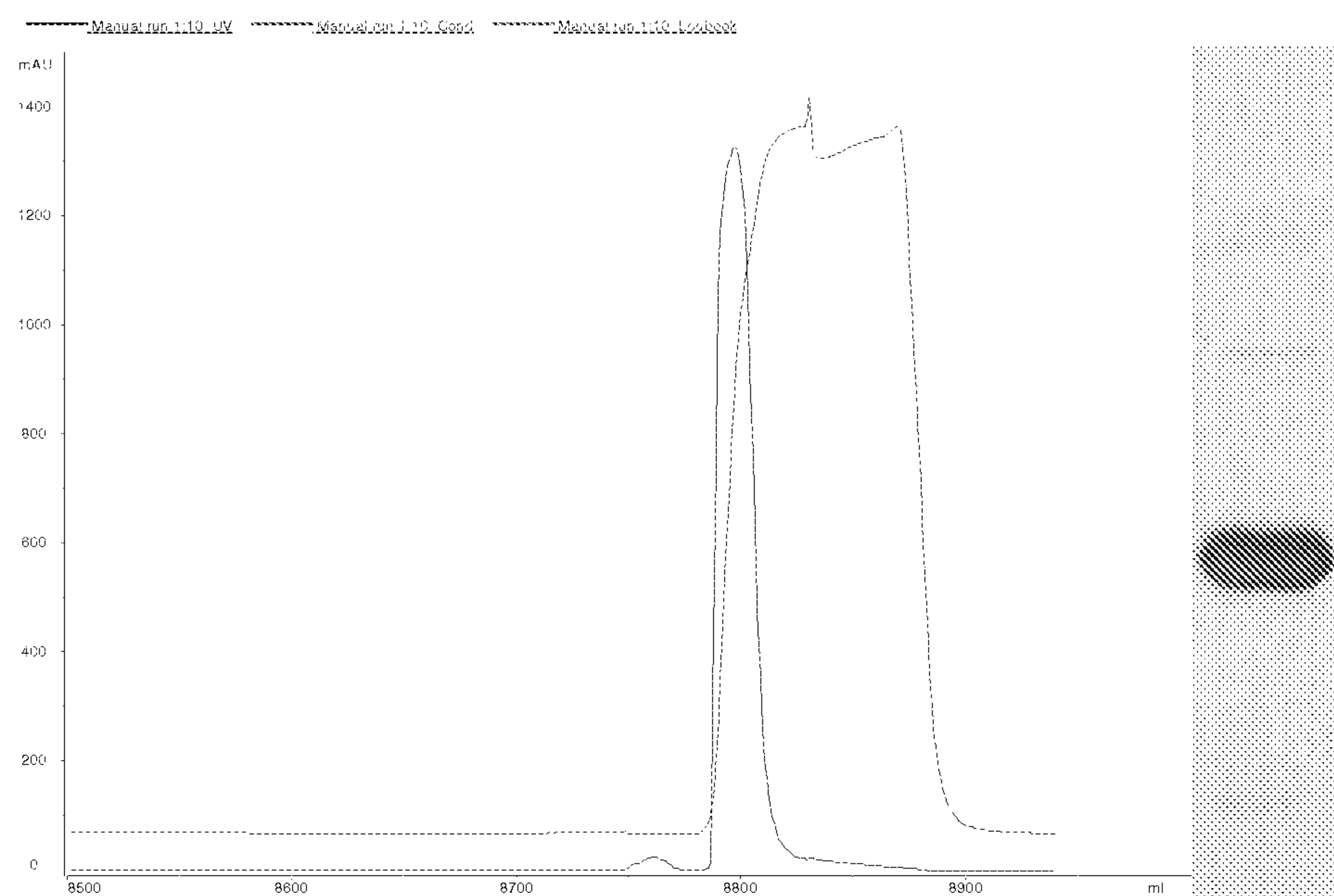


Figure 8

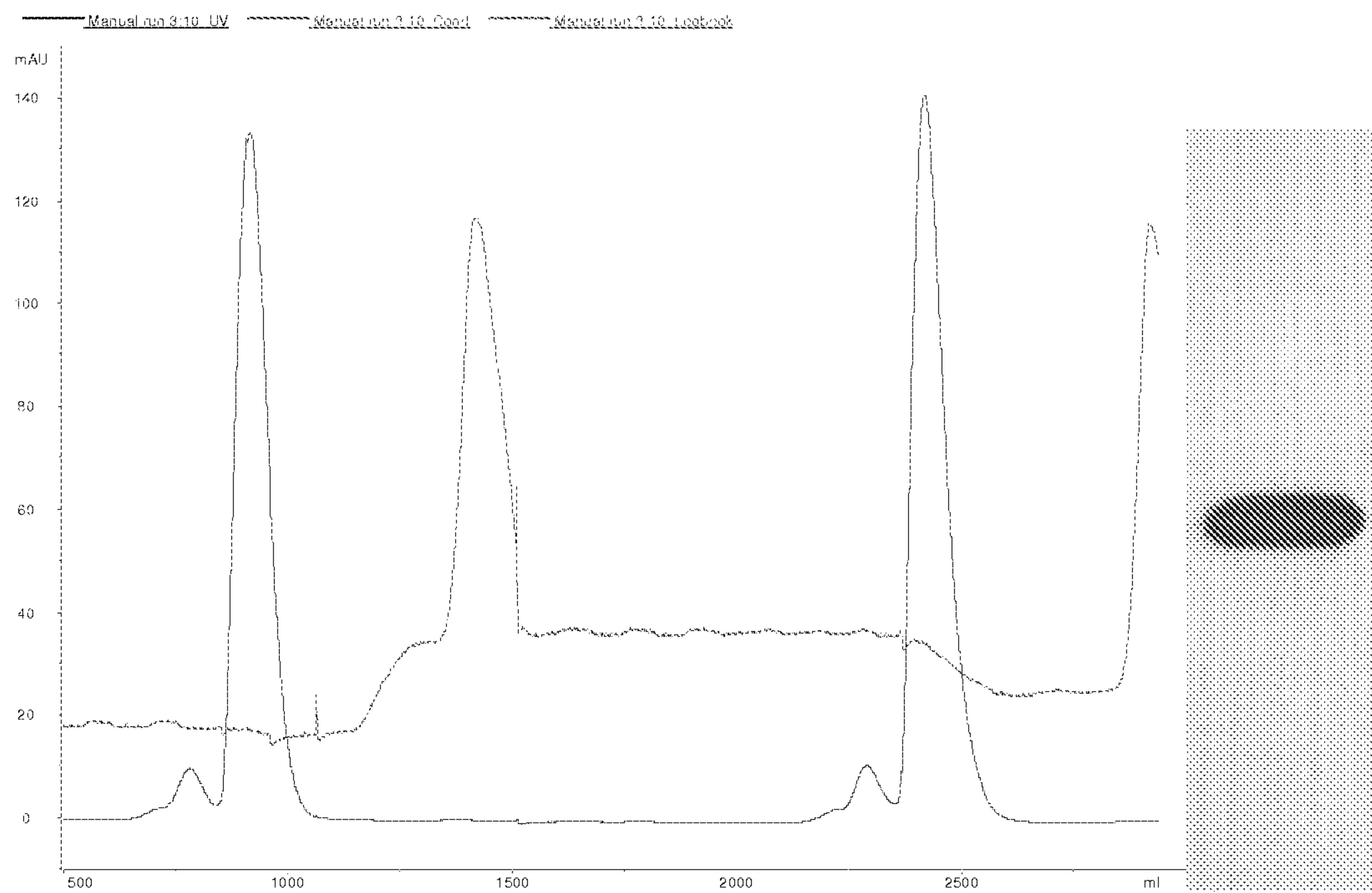


Figure 9

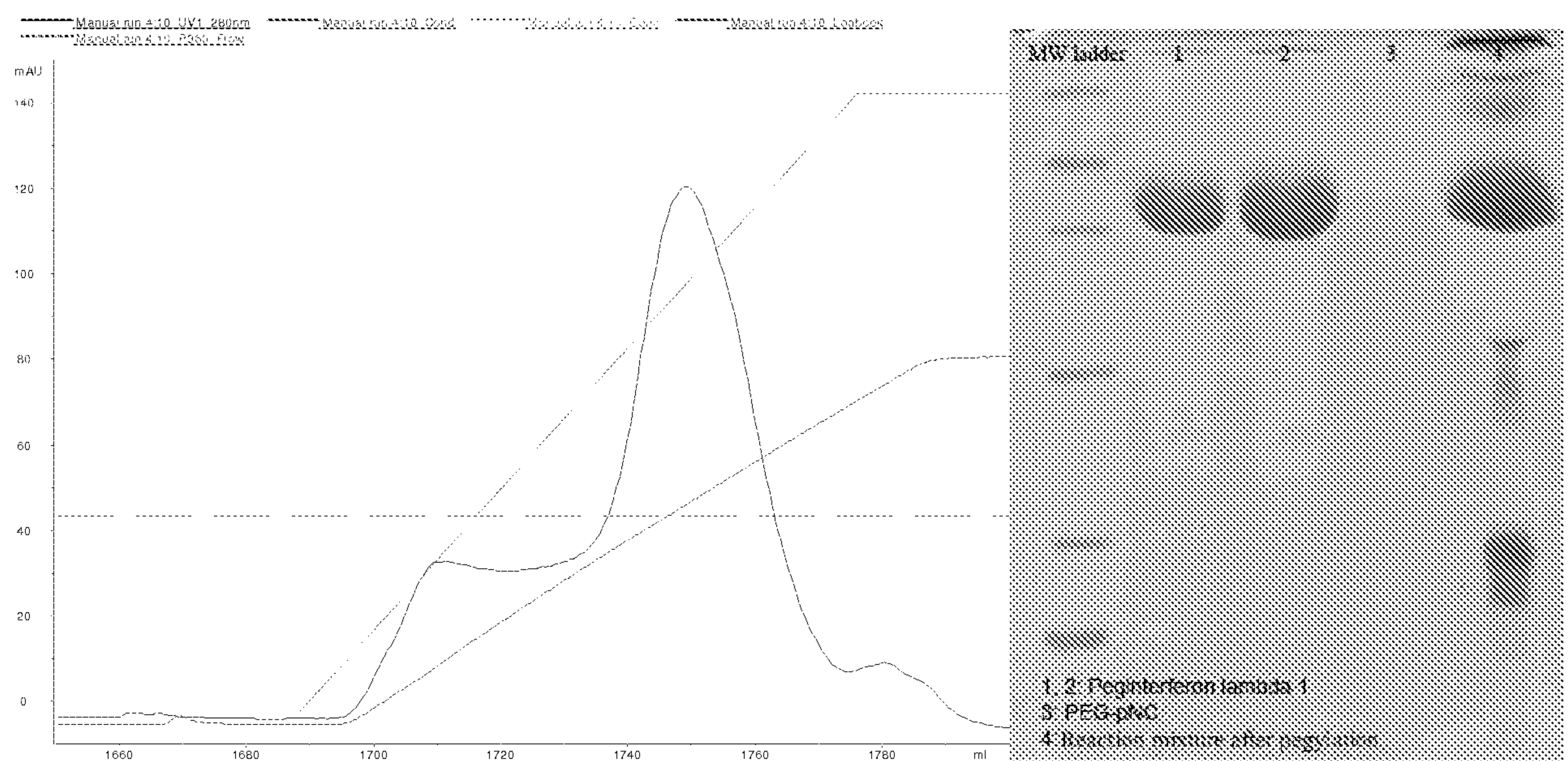
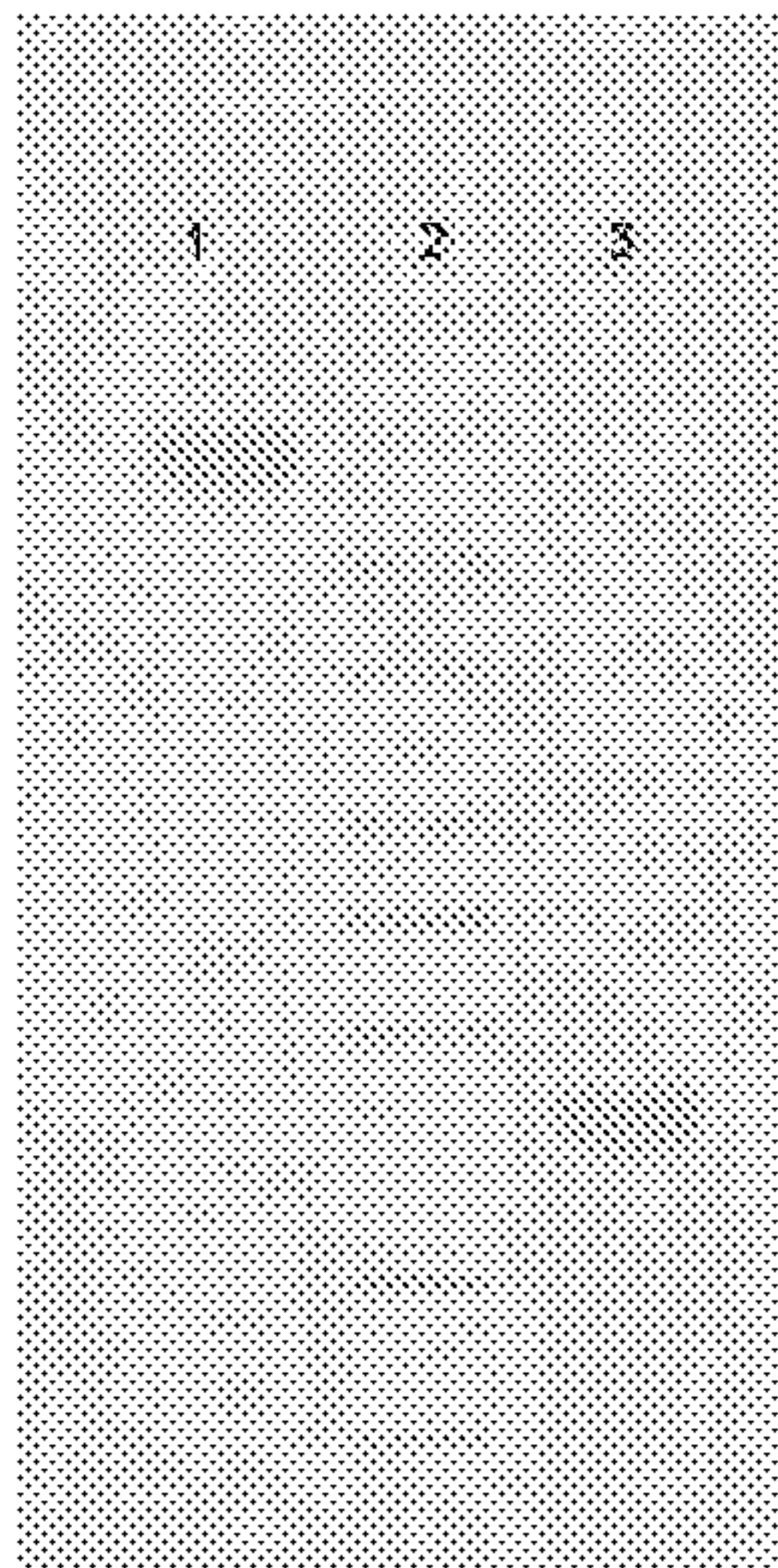


Figure 10



1: Peginterferon lambda 1, lot PIL290050511

2: Protein pattern of MBI Fermentas

3: Interferon lambda 1, lot IL290030411

Figure 11

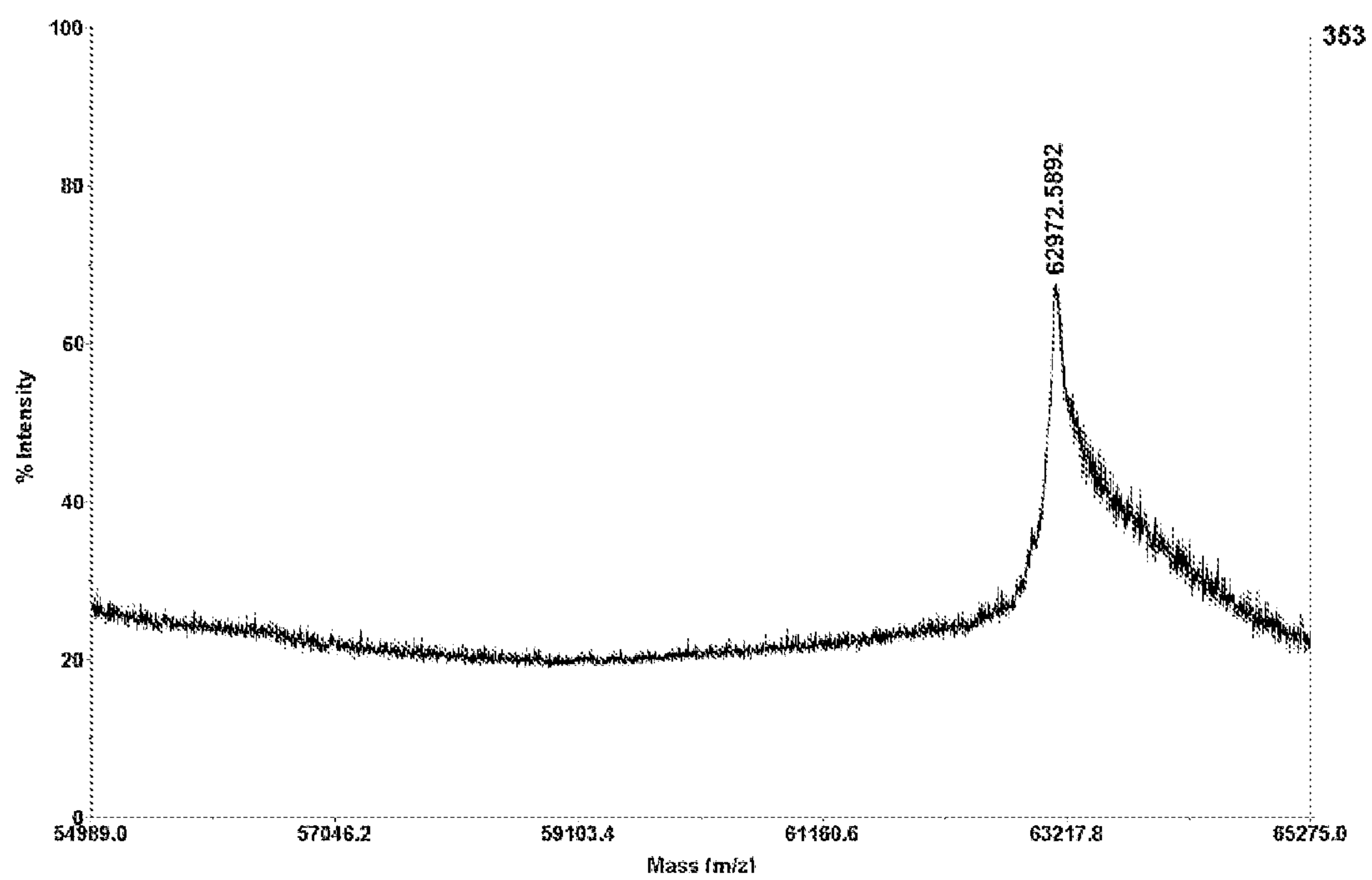


Figure 12